

JANUARY/FEBRUARY 1998
Volume 9, Number 1

© Copyright 1998 by the American Chemical Society

Bioconjugate Chemistry

EDITORIAL: What's New for 1998

Professor Mitsuru Hashida of the University of Kyoto has joined the editorial staff of the journal as Associate Editor. Professor Hashida has an outstanding record of achievements in many areas of science and technology related to bioconjugate chemistry, and we are delighted to have his advice and help as the manuscript flow continues to increase.

There is good news for authors who use color illustrations: the ACS will now charge \$300 per color figure. The 1998 Instructions to Authors for *Bioconjugate Chemistry*, which may be downloaded from the worldwide web address <http://pubs.acs.org/journals/bcches/index.html>, informs authors about practical aspects of electronic manuscript submission and the preparation of illustrations.

Finally, we note the passing of Professor Edgar Haber, who made fundamental contributions to our field, and who will be remembered with friendship and admiration.

Thanks to Our Reviewers

It is a pleasure to acknowledge the contributions of the reviewers of manuscripts submitted in 1997 for publication in *Bioconjugate Chemistry*. Their unselfish service has been essential in the shaping of the eighth volume of the journal. The scientific community is fortunate that its members are willing to share their time and specialized knowledge in this way. The list of reviewers is on the following page.

Claude F. Meares
Editor-in-Chief

List of Reviewers

Abe Abuchowski	A. T. Florence	Robert L. Letsinger	Hartmut Seliger
Ralph Abraham	Joseph Francisco	Susan Z. Lever	Peter Senter
Michael J. Abrams	Art Frankel	Shuang Liu	Robert Shapiro
Maciej Adamczyk	Philip M. Friden	E. L. Loechler	Steven Sheriff
Stephen Adams	Alan R. Fritzberg	Harri Lönnberg	Clay Siegall
Thomas E. Adams	James Frost	Joseph Loo	David S. Sigman
Sudhir Agrawal	Hubert F. Gaertner	Helmut R. Mäcke	Hans Sigrist
Vernon L. Alvarez	Jean Gariépy	Jeffry S. Mann	Mark Smit
Carl Alving	Kyle Gee	Stephen Marburg	Lloyd M. Smith
James R. Baker	Kieran F. Geoghegan	Gary R. Matsueda	Sandra Smith-Gill
John D. Baldeschwieler	Rosina M. Georgiadis	Mark Matteucci	E. M. Southern
Robert W. Baldwin	William F. Goeckler	Phillip G. Mattingly	Caroline J. Springer
Marcel B. Bally	Alexander N. Glazer	Richard May	Mark Stebbins
Carlos F. Barbas, III	Victor A. Goldmacher	Richard Mazurchak	Cy A. Stein
George Barisas	Wayne Gombotz	Claude F. Meares	Valentina Stella
Jacqueline K. Barton	David Goodlet	R. G. Melton	Mark Stolzowicz
Hagan Bayley	David A. Goodwin	Bernard Meunier	Lucjan Strekowski
Jean-Paul Behr	Marc Greenberg	Damon L. Meyer	H. W. Strobel
Laurent Bellon	Gary L. Griffiths	Karen L. Meyer	Ramaswamy Subramanian
G. V. Betageri	Angelo Gunasekera	Rich B. Meyer	Håkan P. Svensson
Purshotam Bhan	Jon Hangeland	Patrick Midoux	Christopher Switzer
Gary Bignami	Milton J. Harris	Michel Monsigny	Francis C. Szoka, Jr.
David Blakey	Mitsuru Hashida	Akira Murakami	Sho Takahashi
Forrest Blocker	Richard Haugland	Vasu Nair	G. I. Tesser
Victor A. Bloomfield	Sidney M. Hecht	Narasimhachari Narayan	Matthew L. Thakur
Albert M. Bobst	Stanley Hefta	Francois Nepveu	Lou Theodore
Nicholas Bodor	Ned D. Heindel	Peter Nielsen	H. M. Therien
Alexei A. Bogdanov	Claude Hélène	Teruo Okano	David H. Thompson
Ronald T. Borchardt	Donald Hilvert	Leslie E. Orgel	Louis X. Tiefenauer
Robert C. Brasch	Richard G. Hiskey	Giovanni Paganelli	Vladimir P. Torchilin
Michael Brinkley	Donald Hnatowich	Chang C. Paik	Michael F. Tweedle
Thomas C. Bruice	Thomas Horn	William M. Pardridge	Olke C. Uhlenbeck
Cynthia J. Burrows	Leaf Huang	Kinam Park	Nassim Usman
Charles R. Cantor	Brian E. Huber	Ira Pastan	Daniel A. Vallera
Jorge A. Carrasquillo	Ferenc Hudecz	Jose C. Perales	Jacques Van Boom
Paul Carter	James S. Huston	Dean Pettit	Jeffrey Van Ness
Elliott L. Chaikoff	Yoshitaka Ichikawa	Geoffrey A. Pietersz	Wynn A. Volkert
C. Allen Chang	Yoshihiro Ito	Michael C. Pirrung	Murthy Vrudhula
Chien-Hsing Ken Chang	Sumedha D. Jayasena	Robert Plourde	Ernst Wagner
Joon S. Cho-Chung	Tae H. Ji	Richard T. Pon	John Walker
Jayanta Roy Chowdhury	David K. Johnson	Glenn D. Prestwich	Andrew H-J. Wang
Daniel J. Coughlin	Gary M. Johnson	Michael Przybylski	Daguang Wang
John H. Crowe	William T. Jones	Thomas P. Quinn	E. Sally Ward
Clark H. Cummins	Alexander V. Kabanov	J. D. Readio	Andy Watson
David Curiel	Daniel Kahne	M. P. Reddy	David S. Watt
Massad Damha	Kazunori Kataoka	Michael W. Reed	Jen Wigren
Gary S. David	John A. Katzenellenbogen	Steven L. Regen	John N. Weinstein
William Dick	John F. W. Keana	Mark A. Reynolds	Michael J. Welsh
Mark Distefano	George L. Kenyon	Kevin G. Rice	Joel Weltman
Richard A. Dluhy	David E. Kerr	David E. Richardson	David E. Wemmer
Ruth Duncan	Ban An Khaw	P. Riesz	Erick Wickstrom
Bruce Eaton	Eiichi Kimura	Annie C. Roche	D. Scott Wilbur
William Eckelman	Irving M. Klotz	Steven E. Rokita	Meir Wilchek
Fritz Eckstein	Junji Konishi	Keith Rose	C. H. Williams
Andrew Ellington	Eric T. Kool	Scott Rosebrough	Francine Wincott
John E. Emswieler	Jindrich Kopecek	Rene Roy	Martin Yarmush
Volker A. Erdman	Ken Krohn	Tomikazu Sasaki	Samuel Zalipsky
Arnold Falick	Hank Kung	Andrew V. Schally	Michael Zalutsky
Gary Felsenfeld	Bachir Latli	Gary B. Schuster	R. N. Zuckerman
Mark A. Findeis	John M. Lambert	William H. Scouten	
Raymond A. Firestone	Robert Langer	Nadrian Seeman	
A. J. Fischman	Bernard Lebleu		BC9702112

In Memoriam

Friends, colleagues, former fellows, and residents mourn the death of a preeminent scientist, cardiologist, immunologist, mentor, an ultimate renaissance gentleman and a friend: Edgar Haber, MD. Dr. Haber, the Elkan R. Blout professor of biological science at the Harvard School of Public Health and professor of medicine at the Harvard Medical School, died on October 13, 1997, at the age of 65. Dr. Haber is survived by his wife Carol Avery Haber, three sons, Justin, Graham, and Eben, and his mother and sister.

Edgar Huber was born in Berlin, Germany, in 1932 to Fred Siegfried and Dorothy Judith Haber. The family immigrated to America, and in 1944 Edgar Haber, became a naturalized U.S. citizen. He received his undergraduate degree in philosophy from Columbia College at New York in 1952 and an M.D. from Columbia College of Physicians and Surgeons in 1956. An illustrious career took a maiden flight at Massachusetts General Hospital in 1957. From 1957 to 1962, he served as a resident in internal medicine, with a 3-year sabbatical postdoctoral fellowship in the Laboratory of Cellular Physiology of Christian Anfinsen, at the National Institutes of Health. Dr. Haber's penchant for excellence in science was evident even then. While at the laboratory of Dr. Anfinsen, Ed Haber's work led to the demonstration that the amino acid sequence dictated the three-dimensional structure of ribonuclease. The importance of this finding was manifested by the awarding of the Nobel Prize for Chemistry to Dr. Anfinsen in 1972 for the establishment of the concept that the information necessary for the three-dimensional conformation of a protein is inherent in the linear sequence of the nucleotide bases of the gene. After an additional year of training in the Cardiac Department of St. George's Hospital in London, Dr. Haber was appointed the (young-

est ever) chief of the Cardiac Unit of the Massachusetts General Hospital in 1964, a full professor of Medicine at Harvard Medical School in 1971 and an endowed Higgins professor of medicine in 1982.

Unparalleled success in clinical cardiology and immunology was the hallmark of Dr. Haber's 24-year tenure as the chief of the cardiac unit at the Massachusetts General Hospital. In 1988, Dr. Haber became president of the Squibb Institute for Medical Research. After the merger of Bristol Myers and Squibb, Dr. Haber, as president of the BMS Pharmaceutical Research Institute, was instrumental in creating one of the largest pharmaceutical research organizations in the world. In 1991, Dr. Haber returned to Boston to head the Division of Biological Sciences and Center for the Prevention of Cardiovascular Disease, as Director at the Harvard School of Public Health. He was appointed Elkan R. Blout professor of biological sciences.

Dr. Haber was a fellow of the American Academy of Arts and Sciences, and a member of the Institute of Medicine of the National Academy of Sciences. He had also been showered with innumerable prestigious awards and honors. He published more than 550 manuscripts in cardiology, immunology, and molecular biology. He mentioned more than 100 postdoctoral fellows and graduate students. Ten are now department chairs, 35 division directors, and 48 full professors. "It was his pride and joy to follow the success of his charges", said Carol.

Those of us who benefited directly will deeply miss the gentle smile, inordinate patience and immense wisdom of a scientific giant and patriarch. We are indeed fortunate to be touched by the life of this illustrious mentor and friend.

BC970210+

REVIEWS

Gene-Directed Enzyme Prodrug Therapy

Ion Niculescu-Duvaz, Robert Spooner, Richard Marais,[†] and Caroline J. Springer*

CRC Centre for Cancer Therapeutics at the Institute of Cancer Research, 15 Cotswold Road, Sutton, Surrey SM2 5NG, United Kingdom, and CRC Centre for Cells Molecular Biology at the Institute of Cancer Research, 237 Fulham Road, London SW3 6JB, United Kingdom. Received June 21, 1997

TABLE OF CONTENTS

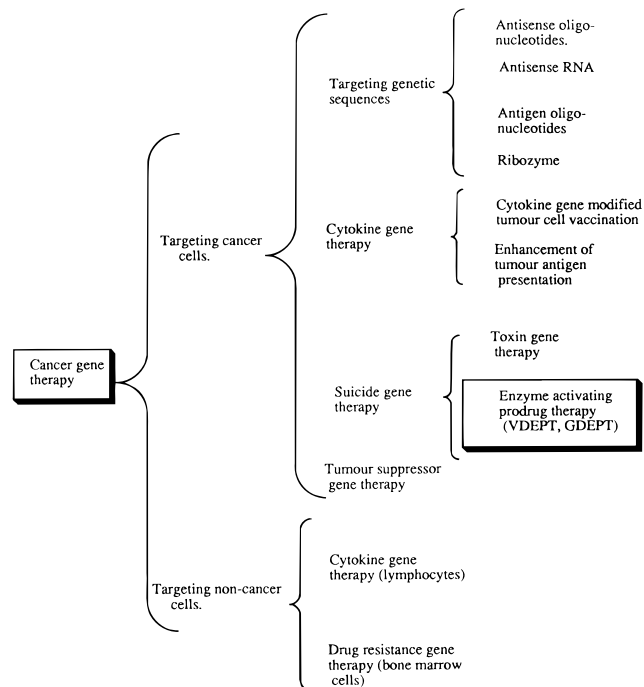
1. Introduction	1
2. Vectors Used in GDEPT	2
3. Enzyme/Prodrug Systems Used in GDEPT	4
4. Bystander Effect and Acquired Immunity	12
5. Future Perspectives	15

1. INTRODUCTION

Gene therapy may be broadly defined as a genetic technology aimed at modifying cells for therapeutic gain. There have been many proposals to modify genetically, or to suppress gene expression in, tumor cells (1). An early suggestion was antisense oligonucleotide strategies that target specific mRNA sequences. Another approach is the "antigen oligonucleotide" approach, which aims to target DNA sequences. Other strategies involve antisense RNA (which interferes with transcription and translation, blocks RNA splicing, or induces double-stranded DNA cleavage) and antioncogene ribozymes (1, 2). Cytokine gene therapy has also been proposed in an attempt to elicit an immune response against tumor cells and thus enhance the patient's immune system to destroy the tumor. The introduction of tumor suppressor genes is also being investigated (1).

Other approaches include attempts to stabilize normal tissues, especially those that are rapidly dividing (e.g., bone marrow, duodenum) by expressing enzymes that regulate DNA repair in these tissues but not in the tumor cells. In this approach, it is hoped that the normal tissues which are most sensitive to chemotherapeutic agents can be modified to overcome drug toxicity and thus reduce the side effects of chemotherapy (3–6). Alternatively, cancer cells may be rendered more sensitive to chemotherapy or toxins, either by suppressing the expression of resistance genes [e.g., multidrug resistance (MDR)] or by introducing "suicide genes", which increase sensitivity to chemotherapeutics (1). Hence, it can be seen that it is possible to modify genetically either the malignant or nonmalignant cells for therapeutic gain. There are two suicide gene approaches. The first is a toxin gene therapy, whereby genes are introduced into malignant cells and the protein produced directs the killing of the tumor cells. The second is termed gene-directed enzyme prodrug therapy (GDEPT) (7, 8) or

Scheme 1. Cancer Gene Therapy



virally directed enzyme prodrug therapy (VDEPT) (9). This review will discuss the GDEPT system of making tumor cells more sensitive to chemotherapeutic agents. (See Scheme 1.)

In GDEPT (or VDEPT), foreign enzymes are expressed in target cells, where they can activate subsequently administered nontoxic prodrugs in to active drugs.

To achieve success in GDEPT, a number of factors must be accomplished. GDEPT requires (1) a gene expressing a nonendogenous enzyme or an endogenous enzyme that is poorly expressed in tumors, which is able to activate a prodrug; (2) a means of delivering the gene to the target cells; and (3) a prodrug.

The gene must be expressed exclusively or with a relatively high ratio in tumor cells compared to normal tissues and blood. The catalytic activity of the foreign or endogenous enzymes, which are only expressed at low concentrations, should be high enough to achieve therapeutic benefit, which may require a specific threshold to be reached. A wide range of enzyme/prodrug systems have been considered. (See section 3.)

The gene should be expressed exclusively or with relatively high ratio in tumor cells compared to normal tissues and blood and must occur in sufficient number

* Author to whom correspondence should be addressed (telephone 44-181-643 8901; fax 44-181-770 7899).

[†] CRC Centre for Cells Molecular Biology at the Institute of Cancer Research.

of cells that there be a clinical benefit (see section 4 for bystander effect). After gene delivery, prodrug administration must be delayed to permit protein expression and stable accumulation in the targeted cells, and the catalytic activity of the protein should be high enough to achieve a therapeutic benefit at these expression levels, which may require a specific threshold to be reached.

2. VECTORS USED IN GDEPT

A number of vector systems have been proposed for gene therapy. These include the RNA viruses: retroviruses (10); the DNA viruses: adenovirus, parvovirus (10, 11), and herpes simplex virus (12); cationic lipids (13, 14), peptides (15), and naked DNA (16). In addition, antibody-directed and ligand-directed (17) delivery of DNA have been proposed. There has also been a suggestion that the anaerobic bacterium *Clostridium* could be used for targeting to the hypoxic regions of tumors (18).

The majority of the proposals have been for viral vectors that have been modified to be deficient in replication functions, although the use of replication-competent viruses has also been considered (19). For applications such as *ex vivo* infections, direct administration of vectors to target tissues *in vivo* or for locoregional delivery, the ability to target malignant cells specifically may not be necessary. However, if systemic delivery is used, targeting to malignant cells is an absolute necessity. Some viruses have restricted tropisms that may be of value. For example, the minute virus of mice is oncotropic and can deliver DNA to transformed cells (20); similarly, the human parvovirus B19 targets primary hematopoietic progenitor cells. The B19 tropism can be transferred to adeno-associated virus by recombinant means (21). The two groups of viruses that have been most extensively studied, retroviruses and adenoviruses, have much less restricted tropisms, and efficient targeting in the clinic is, as yet, unaccomplished. The manipulation of these viruses to modify their tropism by altering their coat proteins is described below. Furthermore, the possibility of ensuring expression of therapeutic genes in particular subsets of cells, by modifying the regions that control gene expression and by adding promoter sequences that will only be active in malignant cells, is discussed.

The issue of transfer efficiency is of paramount importance in GDEPT. *Ex vivo* transfer efficiencies of ~100% have been achieved with viral carriers. However, *in vivo* transfer efficiencies are much lower, and conflicting data are reported.

Retroviruses mediate stable gene transfer with low potential immunogenicity. Difficulties in producing high-titer virus (maximum 10^7 cfu mL⁻¹), the fact that only dividing cells are infected, and the possibility of insertional mutations are nevertheless serious drawbacks. Direct injection of retrovirus in brain tumors resulted in low efficiency (1–3%) (22). However, 10–55% transfer efficiencies were reported in the case of direct implantation of retrovirus producer cells in such systems (22).

Adenoviruses as alternative vectors, have the advantage of producing higher titers (maximum 10^{11} – 10^{12} pfu mL⁻¹) capable of generating infections regardless of the proliferative status of the cells (23). Efficiency ≈50% (at virus titer, 10^{10} pfu mL⁻¹) was observed in mouse hepatocytes after an intravenous injection of adenovirus carrying the lac Z gene (24).

2.1. Retroviruses. Most of the retroviral vectors currently being examined for gene therapy have been derived from the Moloney murine leukaemia virus (MoMuLV). There are of two types, defined by their infection

spectrum: ecotropic, which infect murine cells only; or amphotropic, which can infect a wide range of mammalian cells, including human and murine cells. Neither ecotropic nor amphotropic viruses display tissue specificity, although different cell types within the host organism differ in their sensitivity to infection and some cells are very resistant (e.g., hepatocytes and early hematopoietic progenitor cells). Therefore, for targeting specific populations of cells *in vivo*, a means of altering retrovirus specificity would be of great value.

Mammalian type C retroviruses can infect human cells *in vitro*, but since they are inactivated by human serum, transmission of these viruses to humans is restricted. Inactivation correlates in part with the sensitivity of producer cells to lysis by human serum, so that, for example, retroviruses produced from murine or dog cells are all sensitive to human serum while viral particles from human cells are more resistant (25). Exposure of sensitive producer cells and sensitive retrovirus to human serum would presumably decrease gene transfer *in vivo*, a serious barrier for gene therapy. It had been thought that antibody-independent activation of the classical complement pathway was responsible, due to direct binding of human C1q (the first component of this pathway) to retroviral envelope proteins. However, recently, this view has been challenged, since anti- α -galactosyl antibodies in human serum inactivate retroviruses whose envelopes contain the Gal α 1–3Gal β 1–4GlcNAc-R carbohydrate epitope (26, 27). These experiments have implications for the production of retroviruses for gene therapy: soluble Gal α 1–3Gal can protect retrovirus from human serum and might be administered with the retrovirus. Alternatively, production of retrovirus from producer cell lines that do not express α -galactosyl modifications should permit production of retroviral particles with prolonged survival in human serum.

Another recent advance has been the discovery of a number of packaging cell lines that produce high-titer recombinant retroviruses, and these have been engineered to be resistant to human serum (28, 29). These cell lines will overcome the technical barriers that prevent production of the large number of viral particles required for systemic administration of retrovirus into patients. It should be remembered that, although human serum inactivates many retroviruses, both retroviruses and producer cells survive exposure to cerebrospinal fluid and fluid from the tumor bed in patients with malignant glioma (30, 31). Thus, gene transfer from nonmodified retroviruses to tissues within the central nervous system may be more successful than gene transfer to other tissues.

Retroviral host range is determined by the interaction of the retrovirus envelope (env) glycoprotein with host cell surface proteins (32). Glycoprotein env is synthesised as a precursor, which is proteolytically cleaved into two disulfide-linked proteins. In MoMuLV, one is the hydrophobic p15(E) transmembrane protein (TM) responsible for tethering env to the viral particle, and the other is the hydrophilic glycosylated surface gp70 protein that is responsible for interacting with host cell receptors.

These env host cell receptor interactions can be overcome by biochemical means to retarget virus particles. Bispecific antibody complexes have been prepared by using streptavidin to link biotinylated anti-major histocompatibility complexes (MHC) class I antibodies with biotinylated anti-ecotropic receptor antibodies and to link biotinylated anti-MHC class II antibodies with biotinylated anti-ecotropic receptor antibodies. These complexes

can act as bridges permitting infection of human cells (33). Similar bispecific complexes have permitted targeting via the epidermal growth factor receptor and the insulin receptor, but this system is not universal, since it does not work via the transferrin, high-density lipoprotein, or galactose receptors (34). However, chemical conjugation of lactose to ecotropic virus particles allows infection of human hepatic cells, presumably via the galactose receptor (35).

Some proteins, such as Thy-1 (36), CD4 (37), β 2-microglobulin, HLA DR, actin, and ubiquitin (38), can be incorporated naturally into retroviral particles and so have the potential to influence retroviral tropism. Many approaches have followed a more directed route, specifically altering env to attempt retroviral retargeting. Since structural variations of gp70 determine whether the virus has ecotropic or amphotropic host range, one approach to targeting might be to engineer the env gene to confer new tissue specificity to the env protein. Early attempts fused DNA encoding a single-chain Fv antibody fragment (scFv) specific for a hapten with DNA encoding the env glycoprotein of MoMuLV (39) and spleen necrosis virus (40). When coexpressed with a retrovirus expressing wild-type env, retrovirus particles that could bind haptens were recovered. The hapten specificity had been overlaid upon the normal cell tropism, but the presence of wild-type env meant that normal tropism was not ablated.

Similar approaches fused a scFv specific for the low-density lipoprotein receptor to MoMuLV env (41) and a scFv specific for a cell-surface antigen expressed on human colon carcinoma cells to spleen necrosis virus env (42). Other targeting motifs fused to env include a 16 amino acid peptide containing the integrin-binding motif RGD fused to avian leukosis virus env (43); erythropoietin fused to MoMuLV env (44); heregulin, a ligand for human epidermal growth factor receptors 2 and 4, fused to MoMuLV env (45); CD4, for targeting HIV-infected cells fused to MoMuLV env (46); epidermal growth factor (47); and the first 208 amino acids of amphotropic virus env fused to murine leukemia virus ecotropic env (47). In all cases, expression was in packaging cell lines that also expressed the appropriate wild-type env, resulting in viral particles with an overlaid specificity that permitting redirected cell binding, although not always productive infection (for example, the EGF-env fusion was blocked at a postbinding stage) (47).

A substantial reduction in postbinding block and up to a 100-fold increase in gene transfer efficiency were achieved by introducing spacer peptides separating the amphotropic and ecotropic moieties of an amphi/ecotropic fusion env (48). If this proves to be a general phenomenon, it might be of great value for gene therapy.

The requirement for wild-type env glycoprotein has led to the idea that it may act as a helper function (42), but it may depend upon the nature of the fused ligand. A scFv (anti-MHC class I)-ecotropic env fusion permitted retroviral binding to, and infection of, human cells, even when produced from cells that did not coexpress wild-type env (49). A strategy that may also target cells efficiently relies upon the fusion of the targeting ligand to env via a protease-sensitive linker (50). Here, cell-specific targeting was determined by the fused EGF ligand, but the ligand blocked the env-receptor interaction and gene delivery did not proceed until the linker was cleaved.

Despite the generally low efficiencies of gene transfer, the principle of retargeted retroviruses is now well established, although the criticism that the important

event may simply be to concentrate the modified retrovirus to a particular cell surface cannot be ignored (51).

2.2. Adenoviruses. The adenoviral capsid is icosahedral, each vertex terminating in a penton base structure that interacts with cellular integrins. A trimeric fiber that terminates in a trimeric knob is inserted into each penton base. The knob is the primary ligand for the cellular adenoviral receptor. Serotypes differ, in part, in the length of the fiber shaft. A chimeric fiber, with the shaft of adenovirus serotype 5 (Ad5) fused to the knob of Ad3, confers Ad3-type specificity (52). Thus it is possible to alter adenoviral specificity by manipulating the knob domain. A refinement was the introduction of a decapeptide encoding part of the gastrin releasing peptide (GRP) to the carboxy terminus (knob) of an adenoviral fiber (53). The resulting fiber could still trimerize, and the peptide was accessible to an anti-GRP antibody, raising hopes that incorporation of such a fiber into an adenovirus might permit targeting to cells expressing GRP receptors. Whether such manipulations ablate normal targeting is not clear, although it is known that there are considerable constraints in knob structure. While the shaft can tolerate large deletions, removal of just the two carboxy-terminal amino acids of the Ad2 knob results in monomeric fibers, as does addition of a 27-mer peptide (54). To date, none of these targeting strategies has been attempted for a GDEPT approach to cancer.

2.3. Transcriptional Targeting. Another area in which malignant cell targeting progress has been made is at the transcriptional level. If a tumor cell overexpresses a particular protein due to increased specific transcriptional activity of its promoter (rather than through gene duplication), this characteristic can be exploited for the GDEPT approach. Foreign genes can be cloned downstream of the promoter and introduced into appropriate tumor cells to allow specific high-level expression of the protein. Here, normal tissue that is also transduced would express much lower levels of the gene product and in an ideal system, none.

High-level expression of hepatoma-associated α -feto-protein (AFP) makes hepatocellular carcinoma (HCC) a prime target for this approach (55). Varicella zoster virus (VSV) thymidine kinase (TK) was cloned under the transcriptional control of specific promoters, the AFP tissue regulatory sequence (TRS) and the albumin (ALB) TRS, in replication defective retroviruses. Infection of Hep G2 hepatoma cells and HuH7 non-hepatoma cells, both of which express AFP and ALB, resulted in greatly increased sensitivity to the prodrug 6-methoxypurine arabinonucleoside (ara-M) (a good substrate for VZV-TK, see section 3.1). If these systems were used *in vivo*, further levels of selectivity might be expected, due to failure of retrovirus to infect nondividing hepatocytes and the lack of toxicity of ara-ATP to nondividing cells. A retrovirus expressing HSV-TK under AFP regulation control conferred ganciclovir (GCV) sensitivity to infected AFP-producing cells, but not to cells that did not express AFP (56). In addition, the presence of a glucocorticoid-responsive element in the AFP promoter permitted enhancement of acyclovir (ACV)-activated cytotoxicity by administration of dexamethasone, raising the possibility of pharmacological modulation of therapy.

Similarly, replication defective adenovirus expressing herpes simplex virus HSV-TK from AFP promoter/enhancer elements increased sensitivity of Hep G2 and HuH7 cells to GCV, but did not increase sensitivity of cells that did not express AFP (24). In murine tumor models, gene transfer by adenovirus delivery of HSV-TK

under AFP promoter/enhancer control and subsequent GCV administration led to inhibition of HCC growth (57). In nude mice, adenoviral transduction of HSV-TK expressed from a Rous sarcoma virus promoter led to nonspecific killing of human tumor cells upon subsequent administration of GCV, but using the AFP promoter restricted cell death to only those cells expressing AFP, leading to complete regression of HuH7 tumors (58). In transgenic mice, expression of HSV-TK was found to be predominantly in the liver when under the transcriptional control of an AFP/enhancer/ALB promoter fusion, demonstrating the high degree of tissue specificity of this promoter system (59). Adenovirus-associated virus delivery of this construct followed by GCV treatment was shown to kill cells expressing both AFP and ALB, but not cells expressing neither.

A range of tissue-specific promoters has been suggested for use in the GDEPT approach. Secretory leukoprotease inhibitor is expressed in a wide range of carcinomas, including lung, breast, ovarian, bladder, oropharyngeal, and colorectal, and its promoter has been used to express HSV-TK (60). Osteocalcin is a noncollagenous bone matrix protein expressed at high level in osteoblasts. Its promoter has also been used to express HSV-TK and, when delivered to cells in an adenovirus vector, results in TK activity in both murine and human osteoblast cell lines. There was no expression in a panel of murine and human nonosteoblast cell lines, and subsequent treatment with ACV led to cell death only in the targeted cells (61). Intratumoral (i.t.) injection of this adenovirus into murine osteosarcoma followed by intraperitoneal (ip) injection of ACV abolished tumor growth. For small-cell lung cancers that overexpress Myc, transcriptional control of HSV-TK by the Myc-Max response elements appears to be of value (62).

Carcinoembryonic antigen (CEA) is overexpressed by a number of carcinomas. Transfection of HSV-TK under the transcriptional control of a truncated (445 bp) CEA promoter into a CEA-producing human lung cancer cell line increased its sensitivity to GCV, while transfection into two CEA-nonproducing lines, a lung cancer cell line and HeLa cells, did not. In nude mice, significant regressions of CEA-producing lung cancer tumors expressing this CEA/HSV-TK construct were obtained after GCV treatment (63). Tumors were derived by subcutaneous (sc) implantation of CEA-expressing pancreatic carcinoma cells transduced with a retrovirus expressing HSV-TK downstream from a CEA promoter into severe combined immunodeficient (SCID) mice (64). After GCV treatment, reductions in tumor size were noted. An adenovirus expressing HSV-TK from the CEA promoter conferred GCV sensitivity to CEA-producing gastric carcinoma cell lines, but not to CEA-nonproducing cell lines or to HeLa cells (65). Optimization of expression by recombinant techniques has resulted in a small CEA promoter (important because of virus packaging limitations) that can direct high-level expression of cytosine deaminase (CD) in CEA-positive cells (66).

The c-erb B2 proto-oncogene is overexpressed in 25–30% of breast and pancreatic carcinomas, ~40% of non-small-cell lung cancers (NSCLC), 20–30% of ovarian carcinomas, and 20% of stomach and bladder carcinomas. In breast cancer, overexpression correlates with poor prognosis and resistance to chemotherapy, so a new treatment modality is very important. Overexpression of c-erb B2 occurs in part at the level of gene transcription, owing to elevated levels of a developmentally regulated transcription factor (67, 68). Expression of CD from a truncated c-erb B2 promoter has been proposed

for VDEPT in cells overexpressing c-erb B2 (69). After treatment of retrovirally transduced cells with 5-fluorocytosine (5-FC), significant cell death was noted in c-erb B2-positive cell lines but not in c-erb B2-negative cell lines. The enhancer sequence of the DF3/MUC1 gene that encodes a polymorphic high molecular weight glycoprotein can also regulate TK expression, limiting it to breast cancer cells (70). Similarly, the DF3 promoter has been used in adenovirus to confer selective expression of HSV-TK in DF3 positive breast carcinoma cell lines (71).

A problem frequently encountered using retroviral vectors containing tissue-specific promoters is interference with specificity from the viral enhancer in the 3' long terminal repeat (LTR). For example, the SV40 enhancer and the immunoglobulin heavy chain enhancer both promote gene expression in lymphoid cells. However, introduction of these promoter regions into retroviral vectors that express HSV-TK fails to enhance expression in a murine lymphoma cell line (72).

Melanin biosynthesis is restricted to melanocytes due to transcriptional regulation. The tyrosinase promoter has been used successfully for specific expression of genes in melanoma cells (73–75), as has the promoter for tyrosinase-related protein 1 (76). Recently, a truncated (769 bp) tyrosinase promoter/enhancer has been used to replace the enhancer in the 3' LTR of MoMuLV (10). The result was abolition of promoter interference, with very low expression levels of the downstream gene, interleukin 2, in nonmelanoma cell lines, but high-level expression in melanoma cell lines infected with the retrovirus. Increasing the length of the tyrosinase promoter/enhancer to 2.5 kb improved levels of gene expression further. If such an approach can be extended to other tissue-specific or cancer-specific promoters, then powerful tools for transcriptionally regulated gene transfer should become available.

3. ENZYMES/PRODRUG SYSTEMS USED IN GDEPT

Enzymes used in GDEPT should meet some specific requirements. Ideally they should be: able to catalyze scission reactions or other types of reactions (e.g., redox reactions); different from any circulating endogenous enzyme accessible to prodrug; expressed in sufficient amounts in the tumor target cells; and able to effect high catalytic activity. It may be possible to improve on the enzymes that are currently available. For example, mutants have been created in viral TK that improved the kinetics of the enzyme for the prodrugs GCV and ACV (see section 3.2) (77). Alternatively, the prodrug may be modified to make it a better substrate for the enzyme to overcome enzyme inadequacies.

The enzymes proposed for GDEPT are summarized in Table 1 and can be characterized into two classes: (a) One class consists of enzymes of nonhuman origin, e.g., viral TK, *Escherichia coli* CD, *Pseudomonas* sp. carboxypeptidase, *E. coli* purine nucleotide phosphorylase, *E. coli* xanthine-guanine phosphoribosyltransferase, and *E. coli* nitroreductase. They may elicit cell-mediated immune responses in humans, which may be an advantage, producing more extensive bystander cytotoxicity (see section 4). In experiments with cytosolically expressed herpes simplex virus thymidine kinase in mouse of malignant melanoma (78) and colon carcinoma (79), the bystander effect was greater in immunocompetent mice than in nude mice and was diminished after sublethal irradiation, implying a strong immune component. After treatment with GCV, tumor deposits had greater leukocyte infiltration than those of control animals (80) and

Table 1. Enzymes Used in GDEPT

no.	enzyme	code	source	reaction catalyzed by the enzyme	comments	refs
1	thymidine kinase	EC 2.7.1.21	herpes simplex virus	phosphorylation of GCV, ACV, and FIAU	exogenous, viral origin	82
2	thymidine kinase	EC 2.7.1.21	varicella zoster virus	phosphorylation of ara-M (6-methoxypurine); poor activity against GCV, ACV, and FIAU	exogenous, viral origin	52, 81
3	cytosine deaminase cytochrome P450	EC 3.5.4.1	<i>E. coli</i> rat	conversion of cytosine to uracil (or 5FC to 5FU) activation of oxazaphosphorines (CP, IF, etc.) by hydroxylation of the oxazaphosphorine cycle	exogenous, bacterial origin exogenous, CYP2B1, mammalian origin; its human counterpart, CYP2B6 is expressed in liver but not in tumor cells	106–108 116
4	purine nucleoside phosphorylase	EC 2.4.2.1	<i>E. coli</i>	reversible phosphorylation of purine nucleosides	exogenous; human counterpart expressed in erythrocytes but presents important differences in specificity	122
5	thymidine phosphorylase	EC 2.4.2.4	human	reversible phosphorolytic cleavage of thymidine, deoxyuridine, and their analogues	endogenous, low level in tumor cells	124
6	deoxycytidine kinase	EC 2.7.1.74	human	phosphorylation of deoxycytidine (but as well some purine deoxynucleosides)	endogenous, low level in tumor cells	125
7	carboxypeptidase G2	EC 3.4.22.12	<i>Pseudomonas</i> sp.	cleavage of the amidic bond between an aromatic nucleus and glutamic acid	exogenous, bacterial origin	127
8	xanthine-guanine phosphoribosyltransferase	EC 2.4.2.22	<i>E. coli</i>	catalyzes the conversion of hypoxanthine, xanthine, and guanine to IMP, XMP, and GMP	bacterial origin with human counterpart (HPRT)	135
9	nitroreductase		<i>E. coli</i>	reduction of aromatic nitro groups	exogenous, bacterial origin having a human counterpart with different substrate specificity	134

cytokine release from these was implicated in up-regulation of the costimulatory molecules B7 and ICAM and with enhanced host T-cell proliferative response.

(b) The other class of enzymes are of human origin and are not present or are expressed only at low concentrations in tumor cells, e.g., deoxycytidine kinase, thymidine phosphorylase, and cytochrome P450. Their main advantage resides in the reduction of the potential for inducing an immune response. However, their presence in normal tissues is likely to preclude specific activation of the prodrugs only in tumors.

The prodrug/drug system for GDEPT should also meet a number of requirements: the prodrug must be able to cross the tumor cell membrane for intracellular activation; the cytotoxicity differential between the prodrug and its corresponding drug should be as high as possible; the prodrug should be a good substrate for the expressed enzyme but not activated by endogenous enzymes in other tissues; and the active drug should be highly diffusible or be actively taken up by neighboring cells to achieve the bystander effect (see section 4). The differential between the prodrug and the active drug should be as large as possible.

The design of a prodrug with low cytotoxicity which can release highly toxic active drug requires effective knowledge of the structure–activity relationship (SAR) or preferably of the quantitative structure–activity relationship (QSAR) for the particular type of compound. For this reason, most of the prodrugs used in GDEPT to date have been compounds that have already been used clinically as anticancer agents. An additional advantage of this approach is that the pharmacological, pharmacokinetic, dosage, and safety parameters of these compounds are known.

To date there have been many different prodrugs/enzyme combinations that have been tested in various GDEPT systems. They are summarized in Table 2.

The rationale for their use and the results obtained with each of them are discussed below.

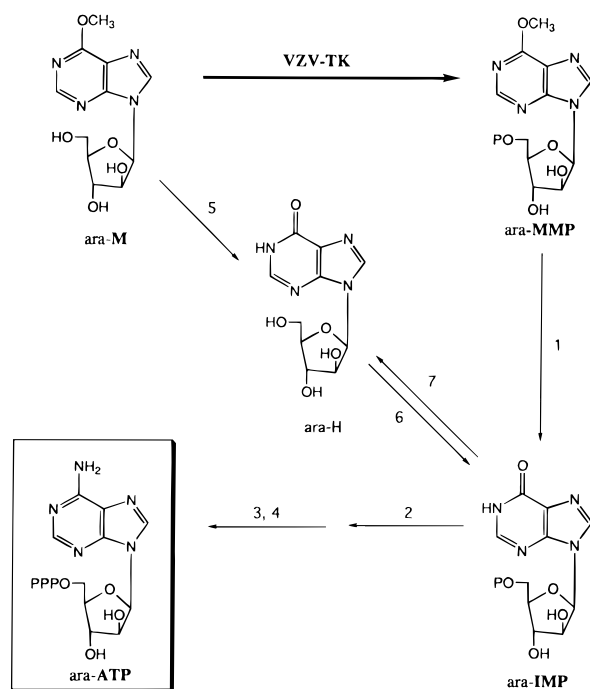
3.1. 6-Methoxypurine Arabinonucleoside (Ara-M)/Varicella Zoster Virus Thymidine Kinase (VZV-TK). The rationale for this prodrug choice was that ara-M is a good substrate for VZV-TK but a poor substrate for the three major mammalian nucleotide kinases (55, 81). The prodrug is activated by the viral enzyme to ara-M-monophosphate (ara-MMP), which is then further converted by the mammalian AMP-deaminase to inosine arabinonucleoside monophosphate (ara-IMP). This metabolite is further catalyzed by mammalian adenylosuccinate synthetase lyase into adenine arabinonucleoside monophosphate (ara-AMP), which is then phosphorylated by the AMP-kinase and subsequently by the cellular nucleoside diphosphate kinases to the anabolite adenine arabinonucleoside triphosphate (ara-ATP) (see Scheme 2). An alternate possible pathway is through hypoxanthine arabinonucleoside (ara-H), which involves the adenosine deaminase-catalyzed demethoxylation of ara-M. Ara-ATP exerts its cytotoxic effects on dividing cells, by inhibiting DNA synthesis.

In the absence of VZV-TK, traces of ara-ATP are formed presumably through the intermediate ara-H. Ara-M is converted to ara-H by adenosine deaminase and undergoes further catalysis to ara-IMP by cellular kinases.

Activation of ara-M was used to develop a therapeutic model for the treatment of HCC (55) (see also section 2.3). This model takes advantage of the transcriptional differences between normal and malignant cells, by cloning the VZV-TK gene under the transcriptional control of the

Table 2. Prodrugs and Enzymes Used in GDEPT

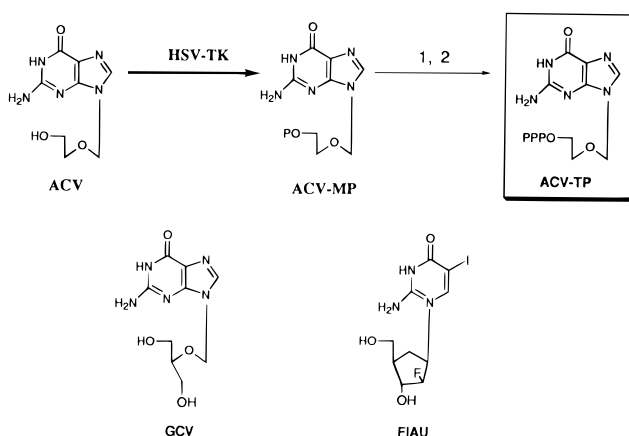
no.	prodrug	enzyme	refs
1	6-methoxypurine arabinonucleoside	thymidine kinase (varicella zoster virus)	55, 81
2	ganciclovir, acyclovir, 1-(2'-deoxy-2'-fluoro- β -D-arabinofuranosyl)-5-iodouracyl	thymidine kinase (herpes simplex virus)	72–74, 77, 84–105
3	5-fluorocytosine	cytosine deaminase	66, 69, 109–112
4	cyclophosphamide, ifosfamide	cytochrome P450	116–121
5	9-(β -D-2'-deoxy-erythro-pentafuranosyl)-6-methylpurine	purine nucleoside phosphorylase	75, 123
6	5'-deoxy-5-fluorouridine	thymidine phosphorylase	124
7	arabinosyl cytosine, 2-chloro-2'-deoxyadenine, 2-fluoro-9-(β -D-arabinofuranosyl)cytosine, 2',2'-difluorodeoxycytidine	deoxycytidine kinase	125, 126
8	(2-chloroethyl)(2-mesyloxyethyl)aminobenzoyl-L-glutamic acid	carboxypeptidase G2	8
9	5-aziridinyl-2,4-dinitrobenzamide	nitroreductase	7
10	6-thioxanthine, 6-thioguanine	xanthine-guanine phosphoribosyltransferase	136

Scheme 2. Metabolism of Ara-M^a

^a 1, AMP-deaminase; 2, adenylosuccinate synthetase; 3, AMP-kinase; 4, nucleoside diphosphate kinase; 5, adenosine deaminase; 6, cellular kinases; 7, cellular phosphatases.

hepatoma-associated AFP, TRS, or the liver-associated (ALB) TRS. These chimeric tumor and liver specific genes were cloned into replication defective retroviral vectors and used to infect human hepatoma Hep G2 cells (expressing high levels of both ALB and AFP) and HuH7 nonhepatoma cells (expressing high levels of AFP and moderate levels of ALB). In control Hep G2 parental cells, the IC₅₀ ratio of prodrug to drug was >2000 (ara-M, IC₅₀ > 2000 μ M; araATP < 1 μ M). After infection, the IC₅₀ of araM was greatly decreased in Hep G2 cells (IC₅₀ = 3.1–6.5 μ M) as well as in HuH7 cells (IC₅₀ = 11–36 μ M). The cytotoxicity of ara-M in infected cells paralleled the level of TK activity, and metabolic studies demonstrated an increase in the levels of ara-A of 2000- and 7000-fold in the VZV-TK expressing cells (55).

3.2. Purine Nucleosides: ACV, GCV, and 1-(2'-Deoxy-2'-fluoro- β -D-arabinofuranosyl)-5-iodouracyl (FIAU)/Herpes Simplex Virus TK (HSV-TK). This system is based on the metabolic activation by the HSV-TK of certain purine nucleosides which have previously demonstrated clinical efficacy for the treatment of herpes in humans. HSV-TK catalyzes the phosphorylation of the purine nucleoside analogues ACV, GCV, and FIAU, which are poor substrates for VZV-TK (81). The conver-

Scheme 3. Metabolism of Acyclovir^a

^a 1, human GMP-kinase; 2, nucleoside diphosphate kinases; MP, monophosphate; TP, triphosphate. The same metabolic pathway occurs for GCV and FIAU.

sion is to the corresponding nucleoside monophosphates, which are catalyzed to nucleoside triphosphates by mammalian nucleoside monophosphate kinases. These latter nucleotides are able to inhibit DNA synthesis and thus kill dividing cells (82) (see Scheme 3). Evidence has been obtained for the inhibition of DNA polymerase by ACV-triphosphate, resulting in inhibition of the elongation of newly synthesized DNA. Incorporation of ACV-triphosphate into DNA is also a chain-terminating event, and GCV-triphosphate also acts as a chain terminator (82, 83).

Cells transfected with the HSV-TK gene and treated with GCV have been shown to die by apoptosis. This process is probably mediated by induction of DNA damage and occurs via a pathway that is independent of p53 (84).

The GCV/HSV-TK system is the most intensively studied and reported system despite some limitations. These include the fact that the bystander effect requires cell to cell contact and that deoxythymidine (dT) is a better substrate than GCV for HSV-TK. However, it represents the best example of the evolution of a GDEPT system from studies *in vitro* to the clinic.

This system is also interesting as a method to monitor therapy for the noninvasive imaging, using a gamma camera and single-photon emission tomography (SPET) with ¹³¹I-FIAU, of the HSV-TK enzyme (85). Furthermore, it may not be necessary to administer GCV systemically. In a mouse transgenic system for HSV-TK, regressions of breast tumours were obtained by direct i.t. administration of GCV (86).

The following steps, which are essential for all GDEPT systems, have been demonstrated for the HSV-TK sys-

tems: (1) demonstration *in vitro* of the stability of the transfected/infected tumor cells and their ability to express the encoded enzyme at concentrations high enough to activate the prodrug of choice; (2) demonstration *in vivo* of the viability of the transfected/infected cells, their propensity to generate tumors, and their sensitivity to the selected prodrug; (3) demonstration of a bystander effect; (4) feasibility of the delivery *in vivo* of the suicide gene.

The *in vivo* experimental studies have shown cures for GCV/HSV-TK and have led to at least 15 ongoing clinical trials involving this gene (87).

Early studies were undertaken with the GCV/HSV-TK system to substantiate the "tissue mosaicism" strategy, which represents a preventative approach (72, 88). This strategy assumes that genes which modify cellular sensitivity to various anticancer agents could be introduced prophylactically into a tissue so that some cells acquire a given gene while others do not. The result is a mosaic of cells that differ in sensitivity to the anticancer agent under consideration. If a tumor of clonal origin develops later from this tissue, therapy could be conducted at whatever sensitivity the tumor displays, while the host would be protected by those cells in the mosaic normal population that do not share this gene (88). This strategy was tested using BALB/c murine cancer cells bearing the HSV-TK gene. Treatment with GCV in clonogenic assays showed that the HSV-TK negative lines were resistant to doses 200- to >1000-fold higher than the HSV-TK positive lines. A bystander effect, called "metabolic cooperation", was shown. Treatment of TK⁺-tumor-bearing animals with GCV (150 mg/kg/injection, twice daily, for 5 days), after tumors became visible, induced complete regressions, and long-term survivors were obtained. In contrast, there were no survivors in the control animals with TK⁺ tumors that did not receive GCV or in TK⁻-tumor-bearing animals treated with GCV. Furthermore, animals treated with CP alone, in another chemotherapeutic control, also have no survivors (88). These studies were further extended using retrovirus delivery of the HSV-TK gene to murine sarcoma and lymphosarcoma cell lines. Tumors derived from these were sensitive to GCV treatment, and long-term regressions were obtained in 12 of 19 animals for sarcoma cell lines. Although regressions were seen for sarcoma cells, the tumors regrew and were found to have become TK-negative, a failure attributed to genomic instability (72).

More recent studies used a retroviral vector to transduce the HSV-TK gene into preneoplastic murine mammary epithelial cells, to yield a clonal subline sensitized to GCV. GCV treatment of tumors that arose from the transduced cells retarded tumor growth and induced durable regressions in 7 of 20 mice. GCV was ineffective in the controls (89). Unfortunately, the mosaicism strategy suffers from the drawback that the gene transfection cannot be confined to a single tissue and is therefore only justified for people with exceptionally high risks of malignancies (e.g., myelodysplastic syndrome) (72).

Further attempts focused on generating curative tumor models. Plasmid vectors have been engineered, with the TK gene under the transcriptional control of the mouse mammary tumor virus long terminal repeat (MTV-LTR). These were used to express TK in rat fibroblasts that subsequently became sensitive to ACV (60% cell death at 10 μ M and 98% at 100 μ M) or to FIAU (>60% cell death at 0.1 μ M and 98% at 3.0 μ M) (90). The cytotoxicity was proportional to the level of HSV-TK expression and dose dependent for ACV and FIAU.

The GCV/HSV-TK system was not as effective in inhibiting human pulmonary adenocarcinoma cells. No bystander effect was observed with TK-positive cells, but confluence was not achieved in these assays (91). A number of strategies involve tissue specific expression of HSV-TK, using promoter and enhancer sequences of a variety of genes (see also section 2.3).

The choice of prodrug for a given HSV-TK system is an important one. In murine and rat HCC retrovirally transduced with the HSV-TK gene, GCV treatment killed the cells at concentrations lower than the plasma level attainable in clinical trials. By contrast, ACV was less effective in GDEPT (92).

Recently, random sequence mutagenesis was used to create mutant genes of HSV-TK that render cells more sensitive to specific nucleotide analogues than cells expressing wild-type HSV-TK (77). Twenty-six HSV-TK mutants were obtained that made cells more sensitive to GCV, and 56 made cells more sensitive to ACV. Only 6 of the mutants gave increased sensitivity to both compounds. Using these genes, stable mammalian cell transfectants were obtained that were 43-fold more sensitive to GCV and 20-fold more sensitive to ACV than cells expressing wild-type HSV-TK (77).

The HSV-TK system has been tested in numerous tumor models to date. One of the earliest of these was in brain tumours, which are an interesting model for GDEPT for the following reasons: the brain represents an immunologically privileged site; the tumors are localized to the CNS; retroviral vectors integrate only into dividing cells, therefore the majority of the cells in the brain will not be infected; and GCV is able to cross the blood-brain barrier and tumor destruction is not immune-mediated (22).

In these *in vivo* studies, numerous long-term survivors have been described and tumor regressions have also been documented (93–102) (see Table 3).

HSV-TK has also been used successfully in animal model HCCs, using the AFP promoter to express HSV-TK (56–58); it has been employed for treatment of metastatic liver disease in conjunction with retroviral delivery, which does not infect normal hepatocytes (80), for the treatment of melanoma, where the tyrosinase promoter directed the expression of HSV-TK in the melanocytes (73, 74), and in a wide variety of other models (103–105).

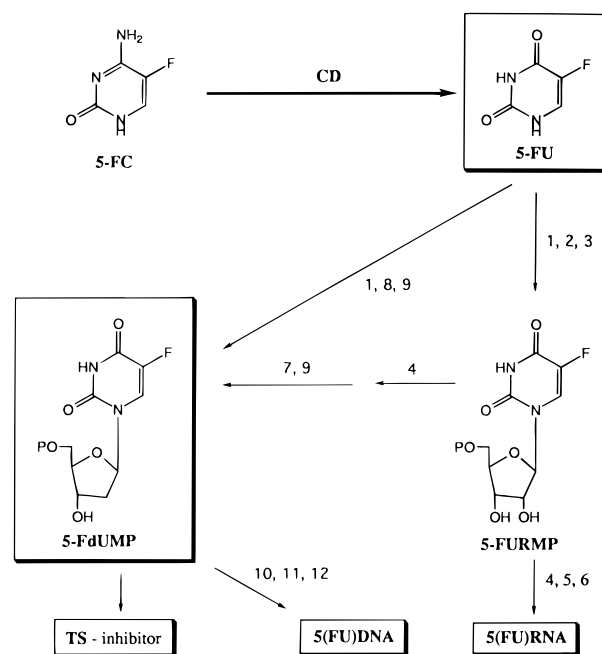
GCV/HSV-TK is perceived to be safe for patient studies since retroviral producer cells are likely to be destroyed by the GCV treatment, and coupled with the success of the *in vitro* and animal studies, clinical studies are currently proceeding in brain tumors (nine studies), melanoma (one study), head and neck tumors (one study), and ovarian carcinomas (two studies) (87).

3.3. 5-Fluorocytosine (5-FC)/*E. coli* Cytosine Deaminase (CD). CD is an *E. coli* enzyme, which has homology to human cytidine deaminase but different substrate specificity. CD converts the nontoxic 5-FC to 5-fluorouracil (5-FU), which is then transformed by cellular enzymes to a potent pyrimidine antimetabolite. Its cytotoxicity depends upon cellular anabolism to 5-fluorodeoxyuridine-5'-monophosphate (5-FdUMP) and then to 5-fluorouridine-5'-monophosphate (5-FUMP) by endogenous enzymes (106, 107). 5-FdUMP is a powerful inhibitor of thymidylate synthase (TS) through the formation of the covalently linked ternary enzyme-inhibitor-cofactor complex, resulting in an irreversible inhibition of TS, a key enzyme for DNA biosynthesis and leading to cell death (see Scheme 4). 5-FUMP is phosphorylated to 5-fluorouridine-5'-triphosphate (5-FUTP),

Table 3. Studies on HSV-TK/GCV Gene Therapy in Animal Brain Tumor Models

no.	ref	type of tumor	no. of tumor cells	type of viral vector	no. of packaging cells	virus titer (cfu/mL)	% of tumor cells expressing transgene	treatment schedule: GCV (mg/kg/day × days)	50% survival with GCV (days)	50% survival untreated (days)	% long time survivors ^a
1	93	9L	4 × 10 ⁴	retrovirus	3 × 10 ⁶			300 × 5			several animals survive > 100 days; *
2	97	9L	4 × 10 ⁴		1.8 × 10 ⁶	5 × 10 ⁵	30–70 (estimated by LacZ transfer)	30 × 14			38 ⁺
		9L	4 × 10 ⁴	retrovirus	3 × 10 ⁶	5 × 10 ⁵		30 × 14			72 ⁺
		9L	4 × 10 ⁴		5 × 10 ⁶	5 × 10 ⁵		30 × 14			83 ⁺
3	95	9L	2 × 10 ⁵	retrovirus	2 × 10 ⁵		—	50 × 7	30	22	22** (7/32)
					(2 × 10 ⁶)		—	50 × 14			
4	98	9L	2 × 10 ⁵	retrovirus	3 × 10 ⁶	1 × 10 ⁶	—	90 × 5			0
5	99	9L	1 × 10 ⁵	retrovirus	5 × 10 ⁵	7.5 × 10 ⁵	45	30 × 10	35	20	0
6	100	C6	1 × 10 ⁴	adenovirus		2.5 × 10 ⁸ (pfu)	—	50 × 14	101 ± 26	25.4 ± 1.8	
7	101	9L	4 × 10 ⁴	retrovirus	1.5 × 10 ⁶		0.1–10.5	30 × 10	25.9 ± 0.9	24.0 ± 0.4	0
					6 × 10 ⁶			30 × 10			
		9L	4 × 10 ⁴	retrovirus			100 ⁺	30 × 10	60	24.0 ± 0.4	26 (> 150 days)
		9L	4 × 10 ⁴ (++)	retrovirus			100 ⁺	30 × 10		24.0 ± 0.4	67 (> 270 days)
8	102	9L	4 × 10 ⁴	adenovirus		1 × 10 ⁸ (pfu)	—	(2 × 15)		20	10 (at day 80)
		9L	4 × 10 ⁴				—	(2 × 15)		20	30 (at day 80)
		9L	4 × 10 ⁴	adenovirus		5 × 10 ⁸ (pfu)	—	(2 × 15)		20	20 (at day 80)
		9L	4 × 10 ⁴	retrovirus	5 × 10 ⁵		—	(2 × 15)	?	20	20 (at day 80)

^a *, 11 of 14 animals were free of tumors after treatment; +, % animals with no tumors at the end of the treatment; **, >90 days; +, ex vivo transfection; ++, the transfected cells were implanted sc.

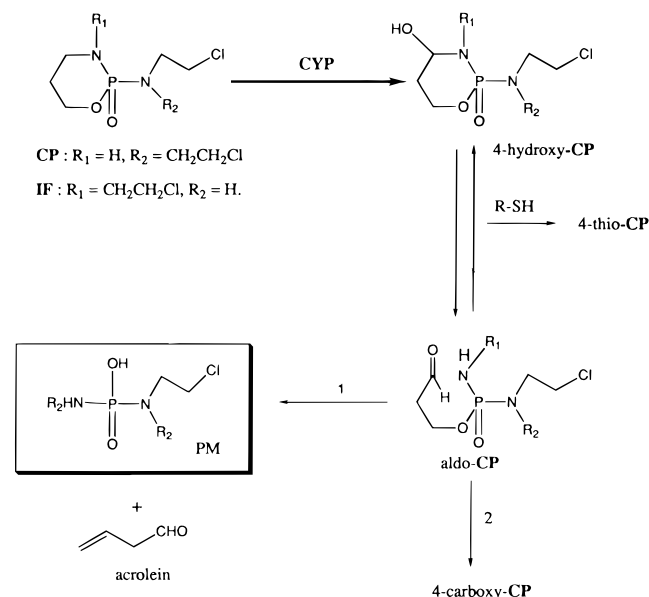
Scheme 4. Metabolism of 5-FC^a

^a 1, nucleoside phosphorylases; 2, uridine kinase; 3, phosphoribosylpyrophosphate-dependent orotate phosphoribosyltransferase; 4, UMP-kinase; 5, nucleotide diphosphate kinases; 6, DNA polymerase; 7, ribonucleoside reductase; 8, thymidine and/or deoxyuridine phosphorylases; 9, deoxyuridine kinase; 10, dUMP-kinase; 11, (deoxy)nucleoside kinases; 12, DNA polymerase; R-1-P, ribose-1-pyrophosphate; dR-1-P, deoxyribose-1-pyrophosphate.

resulting in the formation of “fraudulent” (5-FU)RNA. This affects RNA maturation and processing and may sometimes be an essential event for cytotoxicity. 5-FUMP is also converted into the corresponding triphosphate (5-FdUTP), by diphosphate kinase, which is incorporated into DNA, interfering with its biosynthesis.

The CD gene has an unusual protein synthesis initiation codon, GTG, and when this is mutated into an ATG, its expression in eukaryotic cells was considerably enhanced (108). Murine cells expressing CD with the altered initiation codon showed increased sensitivity to 5-FC, although in this study no bystander effect was seen. In human glioblastoma cells expressing CD, by contrast, a bystander effect was observed with 5-FC, and 5-FU could be detected in tissue culture medium of these cells. Uptake studies revealed a moderate and nonsaturable accumulation of 5-FC in tumor cells supporting its entry by passive diffusion, but a rapid efflux has also been demonstrated, suggesting a possible mechanism for the bystander effect and the reason for the accumulation of 5-FU in tissue culture medium (109).

Colorectal carcinoma models have been developed *in vivo* to test 5-FC/CD as a possible novel therapy for metastatic disease in the liver. This is suggested as an innovative alternative therapy since 5-FU is used as chemotherapeutic agent for colorectal carcinoma. Some of these models have recently been reviewed (9). In one such system both nonmetastatic colon carcinoma cells and fibrosarcoma cells expressing CD demonstrated dose-dependent sensitivity to 5-FC. In immune competent mice, these cell lines all produced tumors, but treatment with 5-FC was able to give significant tumor regressions. However, different rates of regression were observed (6–47%), depending on the parental tumor line used, indicating that tumor cells differ greatly in their sensitivity to the 5-FC/CD system. Intriguingly, the animals that

Scheme 5. Metabolism of Cyclophosphamide and Ifosfamide^a

survived the experiment were found to be resistant to subsequent rechallenge with the unmodified parental cell line used, indicating that long-term immune responses had been induced.

Another study with 5-FC/CD has indicated the efficacy of this treatment for colorectal carcinoma. Expression of CD increased the sensitivity of WiDr cells to 5-FC *in vitro*, by ~1000 fold (from $IC_{50} = 26$ mM to $27 \mu M$), while the expression of CD in these cells did not affect their sensitivity to 5-FU ($IC_{50} = 5 \mu M$) (110).

In mice bearing xenografts expressing CD, 5-FU concentration in the tumors, of $>400 \mu M$, could be generated following 5-FC administration and 5-FU could be detected in the environment surrounding the tumor (111). The liberated 5-FU may account for the bystander effect seen in this studies, where it was found that only 2% of tumor cells were needed to express CD to obtain significant regressions following 5-FC administration. These studies have also addressed dose scheduling and found that appropriate dosing could give cure rates of up to 75% in tumor-bearing mice.

Further studies were conducted in attempts to target CD expression to human models, using promoters [c-erb B2 and CEA (66, 69)], adenoviral vectors (112), and recently *Clostridium* bacteria, which grow specifically in hypoxic regions of tumors (18). Although these studies have met with encouraging success, they have also shown that whereas 5-FC/CD is effective against colorectal cancers (110) and lung adenocarcinoma (91), disappointing results were obtained with breast and pancreatic cancer models.

3.4. Oxazaphosphorines [Cyclophosphamide (CP), Ifosfamide (IF)]/Rat Liver Cytochrome P450 Isoenzyme (CYP2B1) or Human Isoenzyme (CYP2B6). CP and IF are good candidates for GDEPT since they are prodrugs and their biochemical and pharmacological properties are known. CP is activated in rat liver by microsomal CYP to 4-hydroxy-CP, which exists in equilibrium with its ring-opened tautomer aldophosphamide (113) as shown in Scheme 5.

This reaction requires NADH and O_2 , and its $K_M = 0.5$ – 1.5 mM (113). There is evidence that the significant

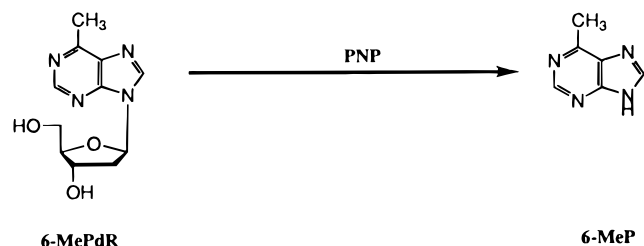
circulating and pharmacologically important metabolite is 4-hydroxy-CP/aldo-CP. The cytotoxic metabolite is the corresponding phosphoramidate mustard, which is generated together with acrolein from the decomposition of aldo-CP (see Scheme 5). The spontaneous β -elimination of acrolein from aldo-CP is a base-catalyzed process (involving hydroxide ions, carbonate anion, and organic or inorganic phosphate) (113). There are, however, data suggesting that 3',5'-exonucleases (and especially those related to DNA polymerase) catalyze the release of the phosphoramidate mustard, and this could be the basis of its relative specificity to the cancer cells (114). The activation of IF occurs in a similar way (115). These bifunctional alkylating agents kill both cycling and noncycling cells by cross-linking DNA.

CYP is a complex enzyme of which there are many isoenzymes with different substrate specificities. Not all of the isoenzymes are equally effective in activating CP or IF. Isoenzymes 2B1 (CYP2B1, phenobarbital inducible), 2C6 (CYP2C6), and 2C11 (CYP2C11) (the latter two are constitutively expressed) are the enzymes involved in activating oxazaphosphorines in rats. Isoenzymes 2B6 (CYP2B6) and 3A4 (CYP3A4, expression restricted to liver) are responsible for this process in humans (116). CYP2B6 and CYP3A4 are not found in tumor cells. Glioma cell lines expressing CYP2B1 were found to be sensitive to both CP ($IC_{50} = 70 \mu M$) and IF ($IC_{50} = 145 \mu M$), whereas the parental lines were resistant to millimolar concentrations of these prodrugs. The difference in the IC_{50} figures of CP and IF is in good agreement with the kinetic data, which showed that the CYP2B1 enzyme cleaves IF with an efficiency (V_{max}/K_M) 3–4-fold lower than that of CP. In xenograft models in rats tumors expressing CYP2B1 were found to be sensitive to subsequent CP administration. After 7–8 weeks, none of the rats bearing non-CYP2B1-expressing tumors had survived, whereas 19 of 20 of the animals carrying CYP2B1-expressing tumors were still alive. In these experiments a bystander effect was seen.

The CP/CYP2B1 system has also been shown to be effective in murine glioma models (117). In this system, an important observation was that the CP treatment was effective against the tumors, and there was no apparent host toxicity, despite significant levels of isoenzyme capable of activating CP present in normal liver. It should be noted, however, that since the activating enzyme in liver, CYP2C6, is distinct from CYP2B1 used in the therapy, potential problems of activation of CP by liver enzymes could be overcome by the use of CYP-isoform specific inhibitors such as 21,21-dichloroprogesterone or 3,5-dimethoxy-2,6-dimethyl-4-ethyl-1,4-dihydropyridine (118, 119).

Retroviral and adenoviral vectors have been used to transduce CYP2B1 into established intracerebral gliomas in rats resulting in significantly prolonged survival of the animals after treatment with prodrugs (120). When compared to GCV/TK, the CYP/CP system was found to be less efficient, possibly due to poor penetration of CP across the blood–brain barrier or low viral titers (120).

In addition to glioma models, expression of CYP2B1 in other tumor cell types (breast and prostate cancer lines) has been shown to increase the sensitivities of these cells to CP and IF, *in vitro* (121). These sensitivities to CP and IF can be reversed by metyrapone, a CYP2B1-selective enzyme inhibitor. Intratumoral CYP2B1 expression conferred a distinct therapeutic advantage in the treatment of MCF-7 (human breast) xenografts in nude mice. CYP2B1-expressing tumors were found to be 15–20-fold more sensitive to appropriate prodrugs than

Scheme 6. Metabolism of 6-MePdR^a

^a PNP, purine nucleotidophosphorylase.

the nonexpressing controls (121). Encouragingly, the therapeutic benefit was obtained without apparent increases in host toxicity.

3.5. 6-Methylpurine-2'-deoxyribonucleoside (6-MePdR)/*E. coli* Purine Nucleoside Phosphorylase (PNP). PNP (or purine nucleoside orthophosphate transferase) catalyzes the reversible phosphorolysis of purine ribonucleotides (122). These enzymes are found in bacteria and in eukaryotes, but there are important differences in substrate specificity between the bacterial and the human enzymes. For instance, adenine is a good substrate for bacterial PNP but not for the human enzyme. Purine arabinosides have at least 10-fold greater activity with *E. coli* PNP compared to the human enzyme. On the other hand, mammalian PNP is more efficient in the synthesis of nucleosides from 2-oxo-6-substituted purines and their analogues than is the bacterial PNP.

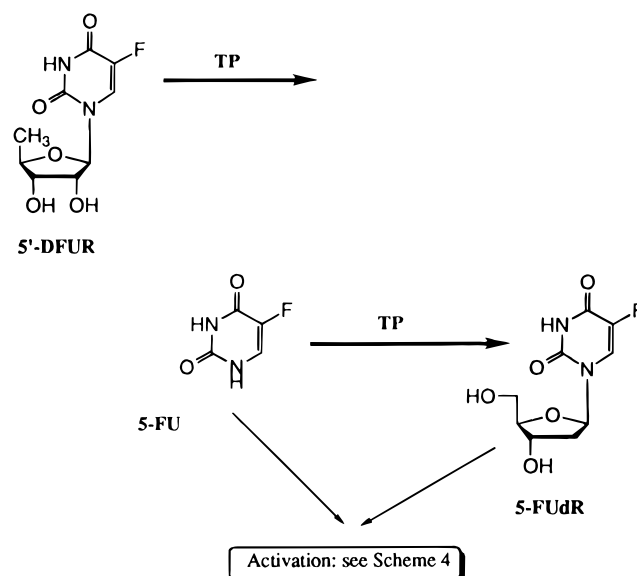
In GDEPT, the bacterial PNP was used to activate the relatively nontoxic 6-MePdR to the toxic 6-methylpurine (6-MeP) (see Scheme 6). In human colonic carcinoma cells, 6-MeP was ~100-fold more toxic than 6-MePdR (123). When these cells were engineered to express *E. coli* PNP, their sensitivity to 6-MePdR was found to increase 60-fold. Furthermore, a significant bystander effect has been shown, with <1% of cells expressing *E. coli* PNP resulting in the death of virtually all of the cells in the population following prolonged 6-MePdR treatment (123).

This system has been tested in human melanoma cell lines, using the tyrosinase promoter to direct melanocyte expression (75). Human colonic carcinoma cells were used as a control for the promoter. The melanoma cells were sensitized to 6-MePdR, whereas the control cells were not. Furthermore, using murine melanoma cell lines, *in vitro*, complete cell kill could be achieved with only 2% of cells expressing *E. coli* PNP under the control of the SV40 promoter, following long incubation with prodrug.

Other adenine nucleosides such as 2-fluoroadenine, 2-azaadenine, and 4-aminopyrazolo[3,4-*d*]pyrimidine are also potential prodrugs for bacterial PNP and, indeed, tumor cell sensitization to arabinofuranosyl-2-fluoroadenine has been demonstrated using this enzyme (75).

3.6. 5'-Deoxy-5-fluorouridine (5'-DFUR)/Thymidine Phosphorylase (TP). Human TP has been proposed for GDEPT in connection with the activation of 5'-DFUR (124). TP catalyzes the reversible phosphorolytic cleavage of deoxythymidine, deoxyuridine, and some of their analogues to the corresponding bases and deoxyribose-1-phosphate. (See Scheme 7.) Although TP is implicated in the activation of 5'-DFUR, there has been no clear demonstration that TP of human origin can cleave the glycosidic bond of 5'-DFUR to yield 5-FU.

Nevertheless, it was shown that overexpression of human TP in human breast cancer cells led to a ~90-fold increase in enzyme levels and a concomitant 165-

Scheme 7. Metabolism of 5'-DFUR^a

^a TP, thymidine phosphorylase.

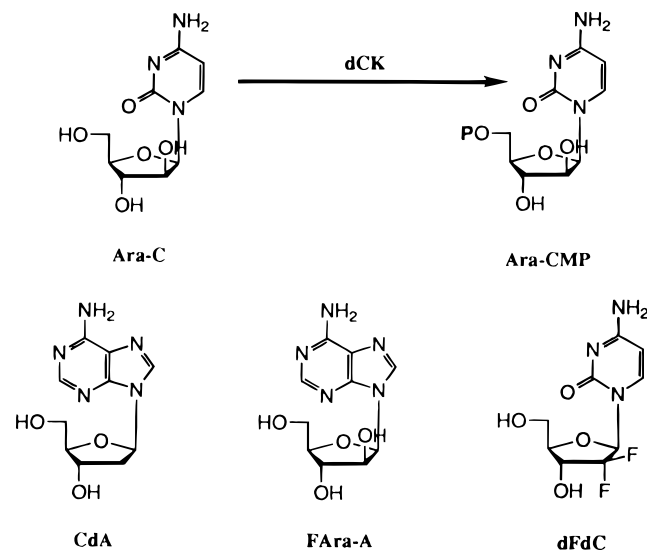
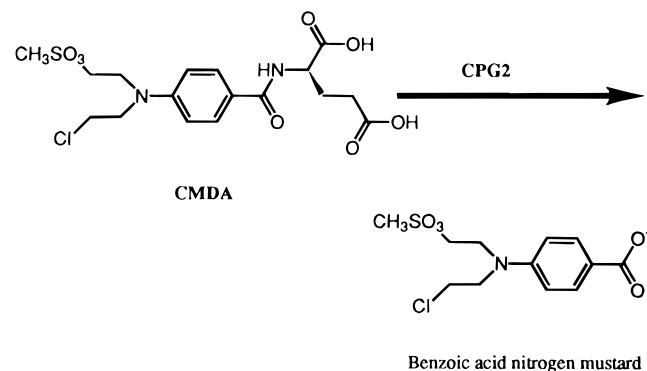
fold increase in sensitivity to 5'-DFUR. The sensitivity of the cells to 5-FU and 5-FUdR remained unchanged (124).

3.7. Arabinosylcytosine [1-(β-D-Arabinofuranosyl)cytosine (Ara-C), 2-Chloro-2'-deoxyadenosine (CdA), 2-Fluoro-9-(β-D-arabinofuranosyl)cytosine (Fara-C), 2-Fluoro-9-(β-D-arabinofuranosyl)adenine (Fara-A), and 2',2'-Difluorodeoxycytidine (dFdC)/Deoxycytidine Kinase (dCK)]. Human dCK is an enzyme involved in the salvage pathway of deoxyribonucleotides biosynthesis. The phosphorylation reaction is the limiting step in this pathway (125). The prodrugs ara-C, CdA, Fara-C, Fara-A, and dFdC can all be activated by a phosphorylation reaction to the corresponding deoxynucleotide monophosphates which, after further phosphorylation to the corresponding triphosphates, are biosynthetically incorporated into RNA and DNA, leading to DNA fragmentation and consequent cell death. (See Scheme 8.)

There have been suggestions that tumor sensitivity to these drugs correlates with dCK activity, but since tumors have relatively low levels of dCK, it has been proposed that dCK-gene transfer will be useful for chemotherapy.

To investigate this proposition, human colon carcinoma and small-cell lung adenocarcinoma cell lines have been engineered to express the human dCK-protein. MCF-7 cells expressing dCK showed a 2.5-fold increased sensitivity to ara-C, whereas H1437 cells expressing dCK displayed 106-fold increase to ara-C. In these studies a linear correlation between IC₅₀ and dCK activity was suggested (125).

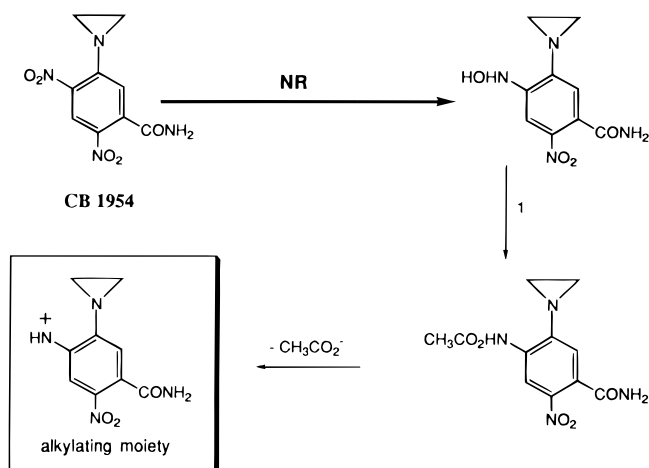
The ara-C/dCK system represents a good model for brain tumors, since ara-C can cross the blood-brain barrier and the activated drug is not toxic to postmitotic cells. Glioma cells expressing dCK were ~10-fold more sensitive to ara-C than parental cells, although this was observed following 4 days of continuous exposure (126). The bystander effect, which requires cell to cell contact, was disappointing, with only 50% cell kill occurring when 10% of the cells expressed dCK. In spite of these results, six of seven rats with intracerebral gliomas that expressed dCK were cured (survival >120 days, in comparison with control animals, which were dead after 19-

Scheme 8. Activation of Ara-C by dCK and Other Nucleosides That Could Be Activated by the Same Enzyme^a^a dCK, deoxycytidine kinase.**Scheme 9. Activation of CMDA by the CPG2 Enzyme^a**^a CPG2, carboxypeptidase G2.

29 days). Furthermore, the dCK gene was transduced into established intracerebral glioma tumors using adenoviral vectors which, following ara-C treatment, resulted in significantly longer survival than found for animals treated with the control adenoviral vector plus ara-C.

3.8. (2-Chloroethyl)(2-mesyloxyethyl)aminobenzoate-L-glutamic Acid (CMDA)/*Pseudomonas* Sp. Carboxypeptidase G2 (CPG2). CPG2 is a bacterial enzyme from a *Pseudomonas* species (127) for which there are no known human homologues, and it is therefore a good candidate for GDEPT. CPG2 catalyzes the scission of amidic, urethanic, and ureidic bonds between an aromatic nucleus and L-glutamic acid (see Scheme 9) (128–130). It can therefore be used to activate prodrugs such as CMDA to aromatic bifunctional alkylating agents.

CMDA, a benzoylglutamate mustard, is cleaved by CPG2 to a benzoyl mustard which can cross-link DNA. Since no endogenous enzymes are required for this activation, the chance of developing resistance is greatly reduced. The released drug is also active against cycling and noncycling cells and therefore has an advantage over antimetabolite toxins (e.g., GCV or 5-FC). CPG2 has been expressed stably in a variety of human tumor cell lines (breast, ovarian, and colon carcinomas) which rendered the cells sensitive to the prodrug CMDA. The cell lines expressing CPG2 were found to be 10–100

Scheme 10. Metabolism of CB 1954^a^a NR, nitroreductase; 1, acetyl-coenzyme A.

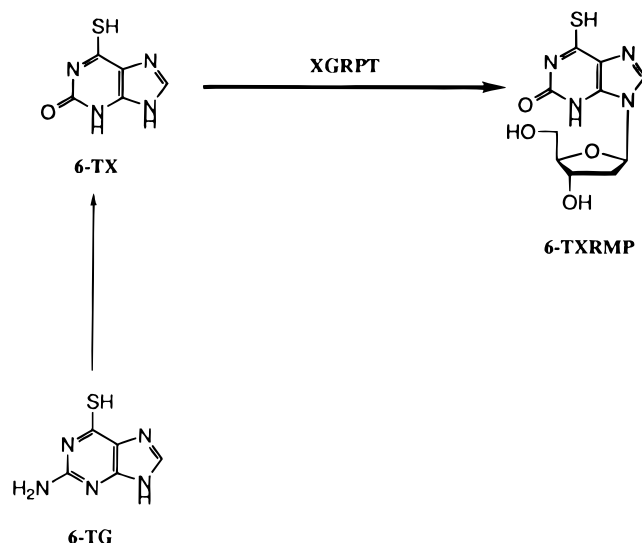
times more sensitive to CMDA than appropriate controls *in vitro*. Total cell kill could be achieved when 4–12% of the cells expressed CPG2, depending on the cell line tested (8).

3.9. 5-Aziridinyl-2,4-dinitrobenzamide (CB 1954)/*E. coli* Nitroreductase (NR). The prodrug CB 1954, a monofunctional alkylating agent, was found to be extremely potent against the Walker carcinosarcoma in rats but inactive against a panel of tumors used in screening anticancer agents (131). It has subsequently been shown that the sensitivity of the Walker cell line was due to DT-diaphorase present in these cells, which activates CB1954 into the bifunctional alkylator 5-aziridinyl-4-(hydroxylamino)-2-nitrobenzamide (132, 133). This is probably converted by acetyl-CoA or other endogenous enzymes to a more potent electrophile, for example, the corresponding acetate, which then reacts with DNA. The metabolism of CB 1954 is shown in Scheme 10. Since the active drug is an alkylating agent, it is able to kill both cycling and noncycling cells, an advantage compared to antimetabolite toxins. Human DT-diaphorase is much less able to activate CB 1954 than is the rat enzyme and therefore should not pose a problem for the use of CB1954 in patients.

Bacterial NR is also able to activate CB 1954, and NR is much more effective than human DT-diaphorase in activating the prodrug (134). A recent study found that expression of *E. coli* NR in NIH3T3 cells increased their sensitivity to NR. Some co-operative killing was observed when cells expressing both NR and TK were treated with a combination of CB 1954 and GCV, supporting their different mechanisms of action (7).

3.10. 6-Thioguanine (6-TG) and 6-Thioxanthine (6-TX)/*E. coli* Xanthine-Guanine Phosphoribosyl-transferase (XGPRT). Bacterial XGPRT and its human counterpart (HPRT) catalyze the conversion of hypoxanthine and guanine to inosine monophosphate (IMP) and guanosine monophosphate (GMP), respectively. XGPRT also efficiently converts xanthine to XMP, a reaction that is poorly catalyzed by the human enzyme (135). These nucleoside monophosphates serve as substrates for pathways involved in the biosynthesis of DNA and RNA. The bacterial enzyme is also able to transfer the ribose phosphate moiety to 6-TX and 6-TG. (See Scheme 11.)

XGPRT is encoded by the *E. coli* gpt gene (135, 136). XGPRT expression in mammalian cells can be used either as a positive or negative selection depending on the

Scheme 11. Activation of 6-TX and 6-TG by XGPRT^a

^a XGPRT, xanthine-guanine phosphoribosyltransferase.

prodrug used (e.g., hypoxanthine and xanthine positively select cells that express XGPRT, whereas 6-TX kills these cells) (136). 6-TG can also be used, despite the fact that it is not a XGPRT substrate, since 6-TG is metabolized to 6-TX by endogenous enzymes, which are subsequently activated by XGPRT.

Clonal glioma lines expressing XGPRT showed significantly increased sensitivity to 6-TX ($IC_{50} = 2.5 \mu\text{M/L}$; parental line $IC_{50} > 50 \mu\text{M/L}$). The XGPRT expressing cell line was also sensitive to 6-TG ($IC_{50} = 0.05 \mu\text{M/L}$, parental line $IC_{50} = 0.5 \mu\text{M/L}$). Cell to cell contact was shown to be required for bystander effects, and under these conditions, significant bystander effects were seen when $\sim 11\%$ of cells expressed XGPRT. Both 6-TG and 6-TX significantly inhibited the growth of tumors in nude mice formed from the sc or intracerebral (ic) transplantation of XGPRT expressing cells.

3.11. Design of Prodrugs for GDEPT. The design of prodrugs for GDEPT should address several issues related to clinical situations. These include the following: penetration of prodrugs into cancer cells for intracellular activation; low toxicity of prodrugs; effective activation of prodrug by the expressed enzyme with favorable activation kinetics; high cytotoxicity of the active drug; ideally, the released drugs should be effective in both cycling and noncycling cells; and the released drugs should induce a bystander effect.

Lipophilic prodrugs are required to penetrate across cell membranes, or prodrugs could be synthesized that are taken up by active transport. It appears that the prodrugs so far in use (nucleoside analogues, 5-FC, CP, CMDA, and CB 1954) penetrate cells by passive diffusion as opposed to active uptake. Low cytotoxicity for the prodrugs is essential since they will also be taken up by normal cells. Activation of the prodrugs is one of the critical steps in GDEPT. It is an advantage if prodrug activation proceeds directly by the expressed enzyme, without the requirement for further catalysis by endogenous enzymes. The involvement of host enzymes in prodrug activation may lead to resistance of tumor cells if the endogenous enzymes are deficient or defective. Furthermore, cells may differ in the levels of the endogenous enzymes they express and thus individual tumors may differ in their sensitivity to prodrugs, even if they express equivalent amounts of exogenous protein. With ara-M, GCV, and ACV the toxic metabolites are gener-

ated by mammalian adenosine monophosphate deaminase, AMP- and/or GMP-kinases following initial activation by TK (see Schemes 2 and 3). With 5-FC, which is converted to 5-FU by CD, the active metabolite is 5-FdUMP or 5-FUTP, which results from 5-FU conversion by a number of mammalian enzymes involving a complicated activation pattern (see Scheme 4). The complex activation pathway of these antimetabolites is partially responsible for their propensity to induce resistance (137).

The prodrugs CP, IF, and CB 1954 are dependent on fewer endogenous enzymes for activation than those cited above. CP, after the primary activation by CYP, requires a second step by 3',5'-exonuclease to generate the active metabolite. For CB 1954, the 5-aziridinyl-4-hydroxylamino-2-nitrobenzamide intermediate resulting from the prodrug reduction by NR needs a further enzymatic step to be converted to the electrophile which alkylates DNA. With the alkylating agent prodrug CMDA, catalysis by CPG2 leads directly from the prodrug to the activated drug (see Scheme 9). These prodrugs, theoretically, have less opportunity to generate resistance.

It is an advantage if the prodrugs are activated to drugs with high cytotoxicity and optimum half-lives to maximize the benefits for GDEPT. The half-lives should be short enough not to allow the drug to leak out from the tumor but long enough to induce a bystander effect. Good kinetic parameters (higher k_{cat}) seem favorable. This effect was noticed when the activity of CP (higher k_{cat} , lower IC_{50}) was compared with that of IF (lower k_{cat} , higher IC_{50}) (138). Also, as described above, improving the kinetic parameters of TK by mutagenesis has resulted in enzymes that make cells more sensitive to prodrugs than wild-type enzymes (77).

Ideally the released drug should be effective against both cycling and noncycling cells. Unfortunately, the majority of prodrugs used in GDEPT release active drugs that are antimetabolites, which require cycling cells (S phase) for cytotoxicity. It has been speculated that the resistance to GCV in GDEPT is not an acquired one but resulted from tumor cells being in G_0 during the time of GCV administration (139). This argument is supported by the fact that the tumors which grew out remained sensitive to GCV. The use of prodrugs that are activated to alkylating agents should have an advantage over purine nucleosides or 5-FC, since they are cytotoxic to both cycling and noncycling cells and this has been shown to be true for the alkylating agent CB 1954 (7). Alkylating agents also have the advantage that they are less prone than other anticancer compounds to induce resistance (140, 141).

4. BYSTANDER EFFECT AND ACQUIRED IMMUNITY

Since expression of the foreign enzymes will not occur in all cells of a targeted tumor *in vivo*, a bystander cytotoxic effect is required whereby the prodrug is cleaved to an active drug that kills not only tumor cells but also neighboring nonexpressing tumor cells (111). This means that expression in $<100\%$ of tumor cells can still give total tumor cell kill. It was demonstrated, *in vivo*, that when as few as 2% of the tumor cells expressed the foreign enzyme, subsequent treatment with suitable prodrug gave long-term animal survivors (111). Therefore, an expression efficiency of 10–20% should be enough to achieve 100% cell kill in tumors, and efficiencies of 1–5% are considered sufficient for a therapeutic benefit (111). Even with lower transfer efficiencies,

beneficial results were obtained if the transduced cell population was allowed to expand before prodrug administration.

The systems reported have exhibited a significant bystander effect *in vivo*. However, the mechanisms may be different for individual drugs. For instance, for the toxic nucleoside phosphates resulting from the activation of purine (GCV, ACV, etc.) or pyrimidine (ara-C, 5-FUDR, etc.) nucleosides, which do not diffuse across cell membranes, cell to cell contact is required to obtain the bystander effect (142). By contrast, for diffusible drugs such as 5-FU or aldophosphamide (or phosphoramidate mustard) no such requirement is necessary (see below) (111, 138).

The responses described in GDEPT would surely not be possible without the existence of the bystander effect. It is very difficult to compare bystander effects obtained in different experimental models due to the different conditions employed by the various investigators. However, we have summarized the data on the bystander effects in different systems in Table 4.

The bystander effects vary depending not only on which prodrug is combined with which enzyme but also on whether the effects are monitored with *in vitro* or *in vivo* systems. It is also clear that different tumor cell types give different results even within the same prodrug/enzyme system. For example, using CMDA with CPG2 *in vitro* in the colorectal carcinoma cell line WiDr, 12% transfected cells were required for total cell kill, whereas with the colorectal carcinoma cell line LS174T only 4% were needed to give 100% cytotoxicity (8). With the GCV-TK combination, 25% of Ov-Ca-1225 TK expressing cells were required for total cell kill *in vitro*. In a liver carcinoma cell line, 10–20% of cells expressing TK were needed for total regression of tumors (80).

The mechanisms responsible for the bystander effects are not completely understood. Several explanations are plausible, and there is the possibility that more than one mechanism is involved. It is also plausible that the mechanisms are different when different types of prodrugs are used. In addition, mechanisms *in vivo* are different from those *in vitro*.

Toxic metabolites are formed during prodrug activation. These are released by efflux from dead and dying genetically modified cells. This is postulated for 5-FU formed from 5-FC, for the metabolites of CP or IF, aldophosphamide, phosphoramidate mustards, or acrolein (7, 111, 138), and for 6-MeP, formed from the corresponding deoxynucleoside (123). This assumption is supported by the fact that no cell to cell contact is required to obtain a bystander effect in these systems. In WiDr cells, this effect was observed both *in vitro* and *in vivo*. *In vitro*, 30% of cells expressing CD were required to eradicate the whole cell population (111). However, *in vivo* the bystander effect was more dramatic and 2% WiDr-CD cells were enough to obtain 100% tumor regression in nude mice, while 4% of cells expressing CD resulted in cures in 66% of the animals (91, 111, 143). When similar experiments were performed in immunocompetent animals, the results were better, indicating that there is a significant immune component to GDEPT.

For purine or pyrimidine nucleosides, where the toxic metabolites, being phosphorylated, are not diffusible across cell membranes, direct cell to cell contact is required to achieve a bystander effect (142). When TK⁺ cells were separated by a 0.4 μ m filter membrane from TK[−] cells, the bystander effect was abrogated. A similar mechanism could also be postulated for other systems involving different purine or pyrimidine nucleotides as

active drugs, for instance ara-C/dCK (126), 6-TA, 6-TX/XGPRT (136), or 5'-DFUR/TPP (124). Since purine and pyrimidine nucleotides formed during activation cannot cross cell membranes, it was assumed that gap junctions are involved in the transfer of toxic metabolites from cell to cell (142, 144). Another suggestion is that the TK-enzyme itself is transported by apoptotic vesicles or through gap junctions (142, 145, 146). Using a flow cytometry assay, it has been shown (in a coculture of HSV-TK expressing murine fibroblasts with rat glioma cells or human hepatoma cells) that bystander tumor cytotoxicity during GCV treatment was highly correlated ($p < 0.001$) with the extent of gap junction mediated coupling, supporting their role in mediating the bystander effect (147).

Phagocytosis by the nonenzyme-expressing cells of materials other than the active drug or the activating enzyme from the dying cells (e.g., hydrolases or other lytic enzymes) has been suggested as a mediator of the bystander effect (142, 148). However, in contrast with this theory, during the period of apoptosis, in bystander cells cocultured with HSV-TK-expressing cells, no phagocytosis could be observed (149). Apoptosis has been shown in bystander cells and was found to be inhibited by bcl2 expression supporting a role for apoptosis in bystander events (149). Furthermore, it has also been suggested that killing of tumor cells by apoptosis could heighten the immune response to wild-type tumor cells (142).

There have been suggestions that the immune response is a favorable event in GDEPT. Although data are available which show that the bystander effect also occurs in immunoincompetent animals, more recent findings suggest that the bystander effect *in vivo* can be mediated through the release of cytokines (148, 150). Co-injection of mouse colon carcinoma cells with HSV-TK-expressing retroviral packaging cells followed by GCV treatment resulted in almost total tumor ablation in immunocompetent BALB/c mice but not in immunocompromised athymic BALB/c animals (79). In another study, HSV-TK-expressing 3T3, which were transformed by Ki-Ras V12G, were shown to be sensitive to GCV. Tumor growth with these cells was inhibited for up to 50 days following GCV treatment in immunocompromised nude mice, but this was not sufficient to eliminate all of the tumor cells in these animals (tumors regrew 40–50 days after cell implantation). By contrast, in immunocompetent BALB/c mice, this treatment led to the development of a long-lasting immunity (151). Similarly, in experimental metastasis of murine melanoma models, a greater reduction in the number of metastatic sites was seen in immunocompetent mice than in nude mice, indicating an immune component to the bystander effect (152).

The role of IL-2 in these systems has also been investigated. Syngeneic colon carcinoma cells grown in BALB/c mice were injected with an adenoviral vector containing the HSV-TK gene or the IL-2 gene, followed by treatment with GCV. While the tumors continued to grow in the animals injected with a control vector or the vector carrying the IL-2 gene, those treated with HSV-TK, with or without coadministration of IL-2, exhibited tumor necrosis and regressions. However, only animals treated with HSV-TK together with the IL-2 genes developed effective systemic antitumoral immunity against tumorigenic rechallenges. The antitumoral immunity was associated with the presence of tumor cell specific cytolytic CD8⁺ T-lymphocytes (153). To enhance and prolong the antitumoral immunity, a third vector con-

Table 4. Bystander Effect

no.	system	bystander effect (transgenes needed to obtain a certain response)			refs
		<i>in vitro</i>	<i>in vivo</i>	comments	
1	herpes simplex virus thymidine kinase/ganciclovir	1% 9L/TK1.3 cells required for 60% cell kill; 25% Ov-Ca-1225 TK ⁺ cells required for 100% cell kill	50% WiDrTK cells are required for 60% tumor-free animals, day 70; 10–20% cells transfection efficiency is needed in liver tumors for regressions	cell to cell contact required	80, 96, 143
2	cytosine deaminase/5-fluorocytosine	significant bystander effect in H1347 and H2122 cells	4% WiDrCD cells are needed for xenografts cures; 2% WiDrCD cells are needed for tumor regressions and 4% for 66% cures	diffusible active drug	111, 143
3	cytochrome P450/cyclophosphamide, ifosfamide	25% transgenes required for 75% cell kill	significant (no quantitative data)	diffusible active drug	117, 121
4	purine nucleoside phosphorylase/9-(β -D-2'-deoxy-erythro-pentafuranosyl)-6-methylpurine	>1% T84-PNP cells (colon cancer) required for 100% cell kill; 1–2% MeI-PNP (human melanoma) required for 100% cell kill		diffusible active drug; prolonged produg exposure	75, 123
5	thymidine phosphorylase/5'-deoxy-5-fluorouridine	MCF-7 cell population reduced 10-fold in a mixture containing 20% MCF7-TP cells (estimated on IC ₅₀ basis)		diffusible metabolite	124
6	deoxycytidine kinase/arabinosylcytosine	10% 9LdCK cells required for 50% cell kill			
7	carboxypeptidase G2/(2-chloroethyl)-(2-mesyloxyethyl)aminobenzoyle-L-glutamic acid	~12% WiDr-CPG2 or SK-Ov3-CPG2 cells required for 100% cell kill; 4–5% LS174T-CPG2 or A2780-CPG2 cells required for 100% cell kill		cell to cell contact required diffusible metabolite	126 8
	nitroreductase/5-aziridinyl-2,4-dinitrobenzamide	30–50% transgenes needed for 90% cell kill		diffusible metabolite	7
8	xanthine-guanine phosphoribosyl-transferase/6-thioxanthine, 6-thioguanine	complete inhibition of growth at C6GPT:C6 glioma cells ratio of 1:1		cell to cell contact required	136

taining the mouse granulocyte macrophage colony stimulating factor (mGM-CSF) gene was also employed. The animals treated simultaneously with HSV-TK + IL-2 + mGM-CSF vectors, followed by administration of GCV, developed long-term antitumor immunity and survived for >4 months without recurrence (154).

The synergism between "suicide gene" and "cytokine gene" therapies has also been tested in head and neck squamous carcinoma in mice. Animals receiving both HSV-TK and IL-2 genes, followed by GCV treatment demonstrated a significantly greater regression in tumours compared to those treated with the components without the cytokine gene (155). Taken together, these studies suggest strongly that an intact immune system is important for long term tumour suppression *in vivo*.

5. FUTURE PERSPECTIVES

As described above there are a large number of prodrug/enzyme combinations that have shown efficiency *in vitro* and *in vivo*. There remain some hurdles to overcome before GDEPT will become a clinically efficient treatment of solid tumors. These include the development of better and more efficient delivery systems for the exogenous genes together with restricted expression in the tumour alone; the synthesis of prodrugs that are better substrates for the transfected genes, which are not substrates for endogenous enzymes and that exhibit good bystander effects; and the use of mutated genes that express enzymes with higher specificity for their substrates.

The following steps have already been achieved in GDEPT: transfection of tumor cells with the gene for an enzyme *in vitro* have been able to activate specific prodrugs, converting them to cytotoxic drugs; transfected tumor cells, when injected into animals, are able to grow and to generate tumors, which have subsequently undergone regressions or been cured by administration of appropriate prodrugs; the "bystander effect" has been measured *in vitro* and *in vivo* (in the presence of an immune system, this bystander effect may become long-term); gene transfection has been achieved *in vivo* using direct DNA injection (naked DNA) liposomes, cationic lipids, or gene transduction with adenoviral or retroviral vectors; and gene infection has been achieved *in vivo* using packaging cells that continuously produce the desired retroviral vector.

The demonstration of these steps in combination with the prodrugs described herein illustrates not only the feasibility of the current systems but also the future promise of GDEPT.

ACKNOWLEDGMENT

Work in this area is funded by the Cancer Research Campaign. We thank Professors Ken Harrap and Chris Marshall for their support.

LITERATURE CITED

- (1) W. W. Zhang, T. Fujiwara, E. A. Grimm, and J. A. Roth (1995) Advances in cancer gene therapy. *Adv. Pharmacol.* 12, 289–341.
- (2) R. E. Christoffersen and J. J. Marr (1995) Ribozymes as human therapeutic agents. *J. Med. Chem.* 38, 2023–2037.
- (3) B. P. Sorrentino, C. J. Brandt, D. Bodine, M. Gottesman, I. Pastan, A. Cline, and A. W. Nienhuis. (1992) Selection of drug-resistant bone marrow cells *in vivo* after retroviral transfer of human MDR1. *Science* 257, 99–103.
- (4) G. Wang, C. Weiss, P. Sheng, and E. Bresnick. (1996) Retrovirus mediated transfer of the human O6-methylguanine-DNA methyl transferase gene into a murine hematopoietic stem cell line and resistance to the toxic effects of certain alkylating agents. *Biochem. Pharmacol.* 51, 1221–1228.
- (5) J. E. Reese, O. N. Koc, K. M. Lee, L. Liu, J. A. Allay, W. P. Phillips, and S. L. Gerson (1996) Retroviral transduction of a mutant methylguanine DNA methyltransferase gene into human CD34 cells confers resistance to O6-benzylguanine plus 1,3-bis(2-chloroethyl)-1-nitrosourea. *Proc. Natl. Acad. Sci. U.S.A.* 93, 14088–14093.
- (6) S. Letourneau, M. Greenbaum, and D. Cournoyer. (1996) Retrovirus-mediated gene transfer of rat glutathione-S-transferase Yc confers *in vitro* resistance to alkylating agents in human leukemia cells and in clonogenic mouse hematopoietic progenitor cells. *Hum. Gene Ther.* 7, 831–840.
- (7) G. Bridgewater, C. J. Springer, R. Knox, N. Minton, P. Michael, and M. Collins. (1995) Expression of the bacterial nitroreductase enzyme in mammalian cells renders them selectively sensitive to killing by the prodrug CB1954. *Eur. J. Cancer* 31A, 2362–2370.
- (8) R. Marais, R. A. Spooner, Y. Light, J. Martin, and C. J. Springer (1996) Gene-directed enzyme prodrug therapy with a mustard prodrug/carboxypeptidase G2 combination. *Cancer Res.* 56, 4735–4742.
- (9) B. E. Huber, C. A. Richards, and E. A. Austin (1995) VDEPT: An enzyme/prodrug gene therapy approach for the treatment of metastatic colorectal cancer. *Adv. Drug Delivery Rev.* 17, 279–292.
- (10) R. G. Vile, R. M. Diaz, N. Miller, A. Tuszyanski, and S. G. Russell (1995) Tissue-specific gene expression from Mo-MLV retroviral vectors with hybrid LTRs containing the murine tyrosinase enhancer/promoter. *Virology* 214, 307–313.
- (11) E. J. Kremer and M. Perricaudet (1995) Adenovirus and AAV mediated gene transfer. *Br. Med. Bull.* 51, 31–44.
- (12) J. C. Glorioso, N. A. DeLuca, and D. J. Fink (1995) Development and application of herpes simplex virus vectors for human gene therapy. *Annu. Rev. Microbiol.* 49, 675–710.
- (13) F. Ledley (1995) Nonviral gene therapy: the promise of genes as pharmaceutical products. *Hum. Gene Ther.* 6, 1129–1144.
- (14) G. P. Behr (1994) Gene transfer with synthetic cationic amphiphiles: prospects for gene therapy. *Bioconjugate Chem.* 5, 382–389.
- (15) M. S. Wadha, W. T. Collard, R. C. Adami, D. L. McKenzie, and K. G. Rice (1997) Peptide-mediated gene delivery: influence of peptide structure on gene expression. *Bioconjugate Chem.* 8, 81–88.
- (16) R. A. Spooner, M. P. Deonarain, and A. A. Epenetos (1995) DNA vaccination for cancer treatment. *Gene Ther.* 2, 173–180.
- (17) S. W. Ebbinghaus, N. Vigneswaran, C. R. Miller, R. A. Chee-Awai, C. A. Mayfield, D. T. Curiel, and D. M. Miller (1996) Efficient delivery of triplex forming oligonucleotides to tumour cells by adenovirus-polylysine complexes. *Gene Ther.* 3, 287–297.
- (18) M. E. Fox, M. J. Lemmon, M. L. Mauchline, T. O. Davis, A. J. Giaccia, N. P. Minton, and J. M. Brown (1996) Anaerobic bacteria as a delivery system for cancer gene therapy: *in vitro* activation of 5-fluorocytosine by genetically engineered clostridia. *Gene Ther.* 3, 173–178.
- (19) S. J. Russell (1994) Replicating vectors for gene therapy of cancers: risks, limitations and prospects. *Eur. J. Cancer* 30A, 1165–1171.
- (20) S. J. Russell, A. Brandenburger, C. L. Flemming, M. K. Collins, and J. Rommelaere (1992) Transformation-dependent expression of interleukin genes delivered by a recombinant parvovirus. *J. Virol.* 66, 2821–2828.
- (21) X. S. Wang, M. C. Yoder, S. Z. Zhou, and A. Srivastava (1995) Parvovirus B19 promoter at map unit 6 confers autonomous replication competence and erythroid specificity to adeno-associated virus 2 in primary human hematopoietic progenitor cells. *Proc. Natl. Acad. Sci. U.S.A.* 92, 12416–12420.
- (22) K. W. Culver (1996) The role of herpes simplex thymidine kinase gene transfer in the drug treatment of brain tumors. *CNS Drugs* 6, 1–11.

- (23) E. Benaïm and B. P. Sorrentino (1996) Gene therapy in pediatric oncology. *Invest. New Drugs* 14, 87–99.
- (24) F. Kanai, Y. Shiratori, H. Wakimoto, Y. Kanegae, I. Saito, H. Nakabayashi, T. Tamaoki, T. Tanka, K. H. Lan, N. Kato, S. Shiina, and M. Omata (1996) Gene therapy for α -fetoprotein producing human hepatoma cells by adenovirus mediated transfer of the herpes simplex virus thymidine kinase gene. *Hepatology* 23, 1359–1368.
- (25) Y. Takeuchi, F. L. Cosset, P. J. Lachmann, H. Okada, R. A. Weiss, and M. K. Collins (1994) Type-C retrovirus inactivation by human complement is determined by both the viral genome and the producer cell. *J. Virol.* 68, 8001–8007.
- (26) R. P. Rother, W. L. Fodor, J. P. Springhorn, C. W. Birks, E. Setter, M. S. Sandrin, S. P. Squinto, and S. A. Rollins (1995) A novel mechanism of retrovirus inactivation in human serum mediated by anti- α -galactosyl natural antibody. *J. Exp. Med.* 182, 1345–1355.
- (27) Y. Takeuchi, C. D. Porter, K. M. Strahan, A. F. Preece, K. Gustafsson, F. L. Cosset, R. A. Weiss, and M. K. Collins (1996) Sensitization of cells and retroviruses to human serum by (α 1–3) galactosyl transferase. *Nature* 379, 85–88.
- (28) F.-L. Cosset, Y. Takeuchi, J. L. Battini, R. A. Weiss, M. K. Collins, and S. J. Russell (1995a) High-titer packaging cells producing recombinant retroviruses. *J. Virol.* 69, 7430–7436.
- (29) R. J. Rigg, J. Chen, J. S. Dando, S. P. Forestell, I. Plavec, and E. Bohnlein (1996) A novel human amphotropic packaging cell line: high titer, complement resistance, and improved safety. *Virology* 218, 290–295.
- (30) D. W. Russell, S. M. Berger, and A. D. Miller (1995) The effects of human serum and cerebrospinal fluid on retroviral vectors and packaging cell lines. *Hum. Gene Ther.* 5, 635–641.
- (31) K. Shimizu, Y. Miyao, M. Tamura, H. Kishima, M. Okhawa, E. Mabuchi, M. Yamada, T. Hayakawa, and K. Ikenaka (1995) Infectious retrovirus is inactivated by serum but not by cerebrospinal fluid or fluid from the tumor bed in patients with malignant glioma. *Jpn. J. Cancer Res.* 86, 1010–1013.
- (32) R. Weiss (1993) In *The Retroviridae* (J. Levy, Ed.) Vol. 2, pp 1–108, Plenum Press, New York.
- (33) P. Roux, P. Jeanteur, and M. Piechaczyk (1989) A versatile and potentially general approach to the targeting of specific cell types by recombinant retroviruses. *Proc. Natl. Acad. Sci. U.S.A.* 86, 9079–9083.
- (34) M. Etienne-Julan, P. Roux, S. Carillo, P. Jeanteur, and M. Piechaczyk (1992) The efficiency of cell targeting by recombinant retroviruses depends on the nature of the receptor and the composition of the artificial cell-virus linker. *J. Gen. Virol.* 73, 3251–3255.
- (35) H. Neda, C. Wu, and G. Y. Wu (1991) Chemical modification on a ecotropic murine leukemia virus results in redirection of its target specificity. *J. Biol. Chem.* 266, 14143–14146.
- (36) J. Calafat, H. Janssen, P. Demant, J. Hilgers, and J. Zavada (1983) Specific selection of host cell glycoproteins during assembly of murine leukemia virus and vesicular stomatitis virus: presence of H-2, Pgp-1 and t-200 glycoproteins on the envelopes of these virus particles. *J. Gen. Virol.* 64, 1241–1253.
- (37) J. A. T. Young, P. Bates, K. Willert, and H. E. Varmus (1990) Efficient incorporation of human CD4 protein into avian leukosis virus particle. *Science* 250, 1421–1423.
- (38) L. O. Arthur, J. V. J. Bess, R. R. Sowder II, R. E. Benveniste, D. L. Mann, J. C. Chermann, and L. E. Henderson (1992) Cellular proteins bound to immunodeficiency viruses: implications for pathogenesis and vaccines. *Science* 258, 1935–1938.
- (39) D. W. Russell, R. E. Hawkins, and G. Winter (1993) Retroviral vectors displaying functional antibody fragments. *Nucleic Acids Res.* 21, 1081–1085.
- (40) T.-H. T. Chu, I. Martinez, W. Sheay, and R. Dornburg (1994) Cell targeting with retroviral particles containing antibody-envelope fusion proteins. *Gene Ther.* 1, 292–299.
- (41) N. V. Somia, M. Zoppe, and I. M. Verma (1995) Generation of targeted retroviral vectors by using single-chain variable fragment: an approach to in vivo gene therapy. *Proc. Natl. Acad. Sci. U.S.A.* 92, 7570–7574.
- (42) T.-H. T. Chu and R. Dornburg (1995) Retroviral vector particles displaying the antigen-binding site of an antibody enable cell-type-specific gene transfer. *J. Virol.* 69, 2659–2663.
- (43) S. Valsesia-Wittman, A. Drynda, G. Deleage, M. Aumailley, J.-M. Heard, O. Danos, G. Verdier, and F. L. Cosset (1994) Modifications in the binding domain of avian retrovirus envelope protein to redirect the host range of retroviral vectors. *J. Virol.* 68, 4609–4619.
- (44) N. Kasahara, A. M. Dozy, and Y. W. Kan (1994) Tissue-specific targeting of retroviral vectors through ligand-receptor interactions. *Science* 266, 1373–1376.
- (45) K. Han, N. Kasahara, and Y. W. Kan (1995) Ligand-directed retroviral targeting of human breast cancer cells. *Proc. Natl. Acad. Sci. U.S.A.* 92, 9747–9751.
- (46) T. Matano, T. Odawara, A. Iwamoto, and H. Yoshikura (1995) Targeted infection of a retrovirus bearing a CD4-Env chimera into human cells expressing human immunodeficiency virus type 1. *J. Gen. Virol.* 76, 3165–3169.
- (47) F.-L. Cosset, F. J. Morling, Y. Takeuchi, J. L. Battini, R. A. Weiss, M. K. Collins, and S. J. Russell (1995b) Retroviral retargeting by envelopes expressing an N-terminal binding domain. *J. Virol.* 69, 6314–6322.
- (48) S. Valsesia-Wittman, F. J. Morling, B. H. K. Nilson, Y. Takeuchi, S. J. Russell, and F.-L. Cosset (1996) Improvement of retroviral retargeting by using amino acid spacers between an additional binding domain and the N-terminus of Moloney leukemia virus SU. *J. Virol.* 70, 2059–2064.
- (49) M. Marin, D. Noel, S. Valsesia-Wittman, F. Brockly, M. Etienne-Julan, S. Russell, F.-L. Cosset, and M. Piechaczyk (1996) Targeted infection of human cells via Major Histocompatibility Complex Class I molecules by Moloney murine leukemia virus-derived viruses displaying single-chain antibody fragment-envelope fusion proteins. *J. Virol.* 70, 2957–2962.
- (50) B. H. Nilson, F. J. Morling, F.-L. Cosset, and S. J. Russell (1996) Targeting of retroviral vectors through protease-substrate interactions. *Gene Ther.* 3, 280–286.
- (51) D. Kabat (1995) Targeting retroviral vectors to specific cells. *Science* 269, 417.
- (52) V. N. Krasnykh, G. V. Mikheeva, J. T. Douglas, and D. T. Curiel (1996) Generation of recombinant adenovirus vectors with modified fibers for altering viral tropism. *J. Virol.* 70, 6839–6846.
- (53) S. I. Michael, J. S. Hong, D. T. Curiel, and J. A. Engler (1995) Addition of a short peptide to the adenovirus fiber protein. *Gene Ther.* 2, 660–668.
- (54) J. S. Hong and J. A. Engler (1996) Domains required for assembly of adenovirus type 2 fiber trimers. *J. Virol.* 70, 7071–7078.
- (55) B. A. Huber, C. A. Richards, and T. A. Krenitsky (1991) Retroviral-mediated gene therapy for the treatment of hepatocellular carcinoma: An innovative approach for cancer therapy. *Proc. Natl. Acad. Sci. U.S.A.* 88, 8039–8043.
- (56) A. Ido, K. Nakata, Y. Kato, K. Nakao, K. Murata, M. Fujita, N. Ishii, T. Tamaoki, H. Shiku, and S. Nagataki (1995) Gene therapy for hepatoma cells using a retrovirus vector carrying herpes simplex virus thymidine kinase under the control of human α -fetoprotein gene promoter. *Cancer Res.* 55, 3105–3109.
- (57) N. K. Wills, W. M. Huang, M. P. Harris, T. Machemer, D. C. Maneval, and R. J. Gregory (1995) Gene-therapy for hepatocellular carcinoma; chemosensitivity conferred by adenovirus mediated transfer of the HSV-1 thymidine kinase gene. *Cancer Gene Ther.* 2, 191–197.
- (58) S. Kaneko, P. Hallenbeck, T. Kotani, H. Nabayashi, G. McGarrity, T. Tamaoki, W. F. Anderson, and Y. L. Chiang (1995) Adenovirus-mediated gene therapy of hepatocellular carcinoma using cancer-specific gene expression. *Cancer Res.* 55, 5283–5287.
- (59) H. Su, J. C. Chiang, S. M. Xu, and Y. W. Kan (1996) Selective killing of AFP-positive cells by adeno-associated virus transfer of the herpes simplex virus thymidine kinase gene. *Hum. Gene Ther.* 7, 463–470.

- (60) R. I. Garver Jr., K. T. Goldsmith, B. Rodu, P. C. Hu, and D. T. Curiel (1994) Strategy for achieving selective killing of carcinomas. *Gene Ther.* 1, 46–50.
- (61) S. C. Ko, J. Cheong, C. Kao, A. Gotoh, T. Shirakawa, R. A. Sikes, G. Karsenty, and L. K. W. Chung (1996) Osteocalcin promoter-based toxic gene therapy for the treatment of osteosarcoma in experimental models. *Cancer Res.* 56, 4614–4619.
- (62) T. Kumagai, Y. Tanio, T. Osaki, S. Hosoe, Y. Tachibana, K. Veno, T. Kijima, T. Horai, and T. Kishimoto (1996) Eradication of Myc-overexpressing small cell lung cancer cells transfected with herpes simplex virus thymidine kinase gene containing Myc-Max response elements. *Cancer Res.* 56, 354–358.
- (63) T. Osaki, Y. Tanio, I. Tachibana, S. Hosoe, T. Kumagai, I. Kawase, S. Oikawa, and T. Kishimoto (1994) Gene therapy for carcinoembryonic antigen-producing cancer cells by cell type-specific expression of herpes simplex virus thymidine kinase gene. *Cancer Res.* 54, 5258–5261.
- (64) J. M. DiMaio, B. M. Clary, D. F. Via, E. Coveney, T. N. Pappas, and H. K. Lyerly (1994) Directed enzyme pro-drug gene therapy for pancreatic cancer in vivo. *Surgery* 116, 205–213.
- (65) T. Tanaka, F. Kanai, S. Okabe, Y. Yoshida, H. Wakimoto, H. Hamada, Y. Shiratori, K. Lan, M. Ishitobi, and M. Omata (1996) Adenovirus-mediated prodrug gene therapy for carcinoembryonic antigen producing human gastric carcinoma cells in vitro. *Cancer Res.* 56, 1341–1345.
- (66) C. A. Richards, E. A. Austin, and B. E. Huber (1995) Transcriptional regulatory sequences of carcinoembryonic antigen: identification and use with cytosine deaminase for tumor-specific gene therapy. *Hum. Gene Ther.* 6, 881–893.
- (67) J. M. Bosher, T. Williams, and H. C. Hurst (1995) The developmentally regulated transcription factor AP-2 is involved in c-erbB2 overexpression in human mammary carcinoma. *Proc. Natl. Acad. Sci. U.S.A.* 92, 744–747.
- (68) D. P. Hollywood and H. C. Hurst (1993) A novel transcription factor, OB2-1, is required for overexpression of the proto-oncogene c-erbB-2 in mammary tumour lines. *EMBO J.* 12, 2369–2375.
- (69) J. D. Harris, A. A. Gutierrez, H. C. Hurst, K. Sikora, and N. R. Lemoine (1994) Gene therapy for cancer using tumor-specific prodrug activation. *Gene Ther.* 1, 170–175.
- (70) Y. Manome, M. Abe, M. F. Hagen, H. A. Fine, and D. W. Kufe (1994) Enhancer sequences of the DF3 gene regulate expression of the herpes simplex virus thymidine kinase gene and confer sensitivity of human breast cancer. *Cancer Res.* 54, 5408–5413.
- (71) L. Chen, D. Chen, Y. Manome, Y. Dong, H. A. Fine, and D. W. Kufe (1995) Breast cancer selective gene expression and therapy mediated by recombinant adenoviruses controlling the DF3/MUC1 promoter. *J. Clin. Invest.* 96, 2775–2782.
- (72) F. L. Moolten and J. M. Wells (1990) Curability of tumors bearing Herpes virus thymidine kinase genes transferred by retroviral vectors. *J. Natl. Cancer Inst.* 82, 297–300.
- (73) R. G. Vile and I. R. Hart (1993a) In vitro and in vivo targeting of gene expression to melanoma cells. *Cancer Res.* 53, 962–967.
- (74) R. G. Vile and I. R. Hart (1993b) Use of tissue-specific expression of the herpes simplex virus thymidine kinase gene to inhibit growth of established murine melanomas following direct intratumoral injection of DNA. *Cancer Res.* 53, 3860–3864.
- (75) B. W. Hughes, A. H. Wells, Z. Bebok, V. K. Gadi, R. I. Garver Jr., W. B. Parker, and E. J. Sorscher (1995) Bystander killing of melanoma cells using the human tyrosinase promoter to express the E. Coli purine nucleoside phosphorylase gene. *Cancer Res.* 55, 3339–3345.
- (76) I. R. Hart (1996) Tissue-specific promoters in targeting systemically delivered gene therapy. *Semin. Oncol.* 23, 154–158.
- (77) M. E. Black, T. G. Newcomb, H. M. Wilson, and L. A. Loeb (1996) Creation of drug-specific herpes simplex virus type 1 thymidine kinase mutants for gene therapy. *Proc. Natl. Acad. Sci. U.S.A.* 93, 3525–3529.
- (78) R. Vile, J. A. Nelson, S. Castledon, H. Chang, and I. Hart (1994) Systemic gene therapy of murine melanoma using tissue-specific expression of HSV-TK gene involves an immune response. *Cancer Res.* 54, 6228–6234.
- (79) S. Gagandeep, R. Brew, B. Green, S. E. Christmas, D. Klatzmann, G. J. Poston, and A. R. Kinsella (1996) Prodrug-activated gene therapy: Involvement of an immunological component in the “bystander effect”. *Gene Ther.* 3, 83–88.
- (80) M. Caruso, Y. Panis, S. Gagandeep, D. Houssin, J. L. Salzmann, and D. Klatzmann (1993) Regression of established macroscopic liver metastases after *in situ* transduction of a suicide gene. *Proc. Natl. Acad. Sci. U.S.A.* 90, 7024–7028.
- (81) D. R. Averett, G. A. Kozalka, J. A. Fyfe, G. B. Roberts, D. J. M. Purifoy, and T. A. Krenitsky (1991) 6-methoxypurine arabinoside as a selective and potent inhibitor of varicella-zoster virus. *Antimicrob. Agents Chemother.* 35, 851–857.
- (82) G. B. Elion (1980) The chemotherapeutic exploitation of virus-specified enzymes. *Adv. Enzymes Regul.* 18, 53–66.
- (83) H. S. Allaudeen, J. Descamps, R. K. Sehgal, and J. J. Fox (1982) Selective inhibition of DNA replication in herpes simplex virus infected cells by 1-(2'-deoxy-2'-fluoro- β -D-arabinofuranosyl)-5-iodocytosine. *J. Biol. Chem.* 257, 11879–11882.
- (84) H. Wallace, A. R. Clarke, D. J. Harrison, M. L. Hooper, and J. O. Bishop (1996) Ganciclovir-induced ablation of non-proliferating thyrocytes expressing herpes virus thymidine kinase occurs by p53-independent apoptosis. *Oncogene* 13, 55–61.
- (85) J. G. Tjuvajev, R. Finn, K. Watanabe, R. Joshi, T. Oku, J. Kennedy, B. Beattie, J. Koutcher, S. Larson, and R. G. Blasberg (1996) Noninvasive imaging of herpes simplex virus thymidine kinase gene transfer and expression: A potential method for monitoring clinical gene therapy. *Cancer Res.* 56, 4087–4095.
- (86) M. G. Sacco, L. Mangiovini, A. Villa, P. Macchi, O. Barbieri, M. C. Sacchi, E. Monteggia, V. Fasolo, P. Vezzoni, and L. Clerici (1995) Local regression of breast tumors following intramammary ganciclovir administration in double transgenic mice expressing neu oncogene and herpes simplex virus thymidine kinase. *Gene Ther.* 2, 493–497.
- (87) (1995) Clinical protocol list. *Cancer Gene Ther.* 2, 225–234.
- (88) F. L. Moolten (1986) Tumor chemosensitivity conferred by inserted Herpes thymidine kinase genes: Paradigm for a prospective cancer control strategy. *Cancer Res.* 46, 5276–5281.
- (89) F. L. Moolten, B. K. Vonderhaar, and P. J. Mroz (1996) Transduction of the herpes thymidine kinase gene into premalignant murine mammary epithelial cells renders subsequent breast cancers responsive to ganciclovir therapy. *Hum. Gene Ther.* 7, 1197–1204.
- (90) E. Borelli, R. Heyman, M. Hsi, and R. M. Evans (1988) Targeting of an inducible toxic phenotype in animal cell. *Proc. Natl. Acad. Sci. U.S.A.* 85, 7572–7576.
- (91) D. K. Hoganson, R. K. Batra, J. C. Olsen, and R. C. Boucher (1996) Comparison of the effect of three different toxin genes and their levels of expression on cell growth and bystander effect in lung adenocarcinoma. *Cancer Res.* 56, 1315–1323.
- (92) S. Kuriyama, T. Nakatani, K. Masui, T. Sakamoto, K. Tominaga, M. Yoshikawa, H. Fukui, K. Ikenaka, and T. Tsujii (1996) Evaluation of prodrugs ability to induce effective ablation of cells transduced with viral thymidine kinase gene. *Anticancer Res.* 16, 2623–2628.
- (93) K. W. Culver, Z. Ram, S. Wallbridge, H. E. H. Oldfield, and M. R. Blaese (1992) In vivo gene transfer with retroviral vector-producer cells for treatment of experimental brain tumors. *Science* 256, 1550–1552.
- (94) D. Barba, J. Hardin, J. Ray, and F. H. Gage (1993) Thymidine kinase-mediated killing of rat brain tumors. *J. Neurosurg.* 79, 729–735.
- (95) D. Barba, J. Hardin, M. Sadelain, and F. H. Gage (1994) Development of anti-tumor immunity following thymidine kinase-mediated killing of experimental brain tumors. *Proc. Natl. Acad. Sci. U.S.A.* 91, 4348–4352.

- (96) S. H. Chen, H. D. Shine, J. C. Goodman, R. G. Grossman, and S. L. C. Woo (1994) Gene therapy for brain tumors: Regression of experimental gliomas by adenovirus-mediated gene transfer *in vivo*. *Proc. Natl. Acad. Sci. U.S.A.* 91, 3054–3057.
- (97) Z. Ram, K. W. Culver, S. Walbridge, R. M. Blaese, and E. H. Oldfield (1993) In situ retroviral-mediated gene transfer for the treatment of brain tumors in rats. *Cancer Res.* 53, 83–88.
- (98) S. J. Tapscott, A. D. Miller, J. M. Olson et al. (1994) Gene therapy of 9L rat gliosarcoma tumors by transduction with selectable genes does not require drug selection. *Proc. Natl. Acad. Sci. U.S.A.* 91, 8185–8189.
- (99) N. G. Rainov, C. M. Kramm, K. Aboody-Guterman, M. Chase, K. Ueki, D. N. Louie, G. R. Harsh IV, E. A. Chiocca, and X. O. Breakefield (1996) Retrovirus-mediated gene therapy of experimental brain neoplasms using the herpes simplex virus-thymidine kinase/ganciclovir paradigm. *Cancer Gene Ther.* 3, 99–106.
- (100) A. Maron, T. Gustin, A. Le Roux, I. Mottet, J. F. Dedieu, J. P. Brion, R. Demeure, M. Perricaudet, and J. N. Octave (1996) Gene therapy of rat C6 glioma using adenovirus-mediated transfer of the herpes simplex virus thymidine kinase gene: long time follow-up by magnetic resonance imaging. *Gene Ther.* 3, 315–322.
- (101) V. Cool, B. Pirotte, C. Gerard, J. L. Dargent, N. Baudson, M. Levivier, S. Goldman, J. Hildebrandt, J. Brotchi, and T. Velu (1996) Curative potential of herpes simplex virus thymidine kinase gene transfer in rats with 9L gliosarcoma. *Hum. Gene Ther.* 7, 627–635.
- (102) A. J. P. E. Vincent, R. Vogels, G. V. Someren, M. C. Esandi, J. L. Noteboom, C. J. J. Avezaat, C. Vecht, D. W. V. Bekkum, D. Valerio, A. Bout, and P. M. Hoogerbrugge (1996) Herpes simplex virus thymidine kinase gene therapy for rat malignant brain tumors. *Hum. Gene Ther.* 7, 197–205.
- (103) M. E. Rosenfeld, M. Wang, G. P. Siegal, R. D. Alvarez, G. Mikheeva, V. Krasnykh, and D. T. Curiel (1996) Adenoviral-mediated delivery of herpes simplex virus thymidine kinase results in tumor reduction and prolonged survival in SCID mouse model of human ovarian carcinoma. *J. Mol. Med.* 74, 455–462.
- (104) D. Yee, S. E. McGuire, N. Brunner, T. W. Kozelsky, D. C. Allred, S. H. Chen, and S. L. C. Woo (1996) Adenovirus-mediated gene transfer of herpes simplex virus thymidine kinase in an ascites model of human breast cancer. *Hum. Gene Ther.* 7, 1251–1257.
- (105) J. A. Eastham, S. H. Chen, I. Sehgal, G. Yang, T. L. Timme, S. J. Hall, S. L. C. Woo, and T. C. Thompson (1996) Prostate cancer gene therapy: Herpes simplex virus thymidine kinase gene transduction followed by ganciclovir in mouse and human prostate cancer models. *Hum. Gene Ther.* 7, 515–523.
- (106) M. MacCoss and M. J. Robins (1990) In *Chemistry of Antitumor Agents* (D. E. V. Wilman, Ed.) pp 261–298, Blackie & Son Ltd., Chapman & Hall, London, New York.
- (107) D. S. Martin (1987) In *Metabolism and Action of Anti-cancer Drugs* (G. Powis and R. A. Prough, Eds.) pp 91–140, Taylor & Francis, London.
- (108) C. A. Mullen, M. Kilstrup, and R. M. Blaese (1992) Transfer of the bacterial gene for cytosine deaminase to a mammalian cells confers lethal sensitivity to 5-fluorocytosine: A negative selection system. *Proc. Natl. Acad. Sci. U.S.A.* 89, 33–37.
- (109) U. Haberkorn, F. Oberdorfer, J. Gebert, I. Morr, K. Haack, K. Weber, M. Lindauer, G. Van Kaik, and H. K. Schackert (1996) Monitoring gene therapy with cytosine deaminase: In vitro studies using tritiated-5-fluorocytosine. *J. Nucl. Med.* 37, 87–94.
- (110) B. E. Huber, E. A. Austin, V. C. Good, V. C. Knick, S. Tibbels, and C. A. Richards (1993) In vivo antitumor activity of 5-fluorocytosine on human colorectal carcinoma cells genetically modified to express cytosine deaminase. *Cancer Res.* 53, 4619–4626.
- (111) B. E. Huber, E. A. Austin, C. A. Richards, S. T. Davis, and S. S. Good (1994) Metabolism of 5-fluorocytidine to 5-fluorouracil in human colorectal tumor cells transduced with the cytosine deaminase gene: Significant antitumor effects when only a small percentage of tumor cells express cytosine deaminase. *Proc. Natl. Acad. Sci. U.S.A.* 91, 8302–8306.
- (112) A. Ohwada, E. A. Hirschowitz, and R. G. Crystal (1996) Regional delivery of an adenovirus vector containing the *Escherichia coli* cytosine deaminase gene to provide local activation of 5-fluorocytosine to suppress the growth of colon carcinoma metastatic to liver. *Hum. Gene Ther.* 7, 1567–1576.
- (113) N. E. Sladek (1987) In *Metabolism and Action of Anti-cancer drugs* (G. Powis and R. A. Prough, Eds.) pp 48–89, Taylor & Francis, London.
- (114) L. Bielicki, G. Voelcker, and H. J. Hohorst (1984) Activated cyclophosphamide: an enzyme-mechanism-based suicide inactivator of DNA polymerase/3',5' exonuclease. *J. Cancer Res. Clin. Oncol.* 107, 195–198.
- (115) T. A. Connors, P. J. Cox, P. B. Farmer, A. B. Foster, A. M. Gilsenan, and M. Jarman (1974) Some studies of the active intermediates formed in the microsomal metabolism of cyclophosphamide and isophosphamide. *Biochem. Pharmacol.* 23, 115–129.
- (116) T. K. H. Chang, G. F. Weber, C. L. Crespi, and D. J. Waxman (1993) Differential activation of cyclophosphamide and ifosfamide by cytochromes P450 2B and 3A in human liver microsomes. *Cancer Res.* 53, 5629–5637.
- (117) M. X. Wei, T. Tamiya, M. Chase, E. J. Boviatsis, T. K. Chang, N. W. Kowall, F. H. Hochberg, D. J. Waxman, X. O. Breakefield, and E. A. Chiocca (1994) Experimental tumor therapy in mice using the cyclophosphamide-activating cytochrome P450–2B1 gene. *Hum. Gene Ther.* 5, 969–978.
- (118) J. Halpern, J. Y. Jaw, L. J. Cornfield, C. Balfour, and E. Mash (1989) Selective inactivation of rat liver cytochromes P450 by 21-chlorinated steroids. *Drug Metab. Disp.* 17, 26–31.
- (119) M. A. Correia, K. Yao, S. A. Wrighton, D. J. Waxman, and A. E. Rettie (1992) Differential apoprotein loss of rat liver cytochromes P450 after their inactivation by 3,5-dicarbethoxy-2,6-dimethyl-4-ethyl-1,4-dihydropyridine: a case for distinct proteolytic mechanism. *Arch. Biochem. Biophys.* 294, 493–503.
- (120) Y. Manome, P. Y. Wen, L. Chen, T. Tanaka, Y. Dong, M. Yamazoe, A. Hirshowitz, D. W. Kufe, and H. A. Fine (1996) Gene therapy for malignant gliomas using replication incompetent retroviral and adenoviral vectors encoding the cytochrome P-450 2B1 gene together with cyclophosphamide. *Gene Ther.* 3, 513–520.
- (121) L. Chen, D. J. Waxman, D. Chen, and D. C. Kufe (1996) Sensitization of human breast cancer cells to cyclophosphamide and ifosfamide by transfer of a liver cytochrome P-450 gene. *Cancer Res.* 56, 1331–1340.
- (122) T. P. Zimmerman, N. B. Gersten, A. F. Ross, and R. P. Miech (1971) Adenine as substrate for purine nucleoside phosphorylase. *Can. J. Biochem.* 49, 1050–1054.
- (123) E. J. Sorscher, S. Peng, Z. Bebok, P. W. Allan, L. L. Bennett Jr., and W. B. Parker (1994) Tumor cell bystander killing in colonic carcinoma utilizing the *Escherichia coli* deo-D gene to generate toxic purines. *Gene Ther.* 1, 233–238.
- (124) A. V. Patterson, H. Zhang, A. Moghaddam, R. Bicknell, D. C. Talbot, I. J. Stratford, and A. L. Harris (1995) Increased sensitivity to the prodrug 5'-deoxy-5-fluorouridine and modulation of 5-fluoro-2'-deoxyuridine sensitivity in MCF-7 cells transfected with thymidine phosphorylase. *Br. J. Cancer* 72, 669–675.
- (125) D. M. Hapke, A. P. A. Stegmann, and B. S. Mitchell (1996) Retroviral transfer of deoxycytidine kinase into tumor cell lines enhances nucleoside toxicity. *Cancer Res.* 56, 2343–2347.
- (126) Y. Manome, P. Y. Wen, Y. Dong, T. Tanaka, B. S. Mitchell, D. W. Kufe, and H. A. Fine (1996) Viral vector transduction of the human deoxycytidine kinase cDNA sensitizes glioma cells to the cytotoxic effects of cytosine arabinoside in vitro and in vivo. *Nat. Med.* 2, 567–573.
- (127) R. F. Sherwood, R. G. Melton, S. M. Alwan, and P. Hughes (1985) Purification and properties of carboxypeptidase G2 *Pseudomonas* sp strain RS-16. *Eur. J. Biochem.* 148, 447–453.

- (128) C. J. Springer, P. Antoniow, K. D. Bagshawe, F. Searle, G. M. F. Bisset, and M. Jarman (1990) Novel prodrugs which are activated to cytotoxic alkylating agents by carboxypeptidase G2. *J. Med. Chem.* 33, 677–681.
- (129) R. Dowell, C. J. Springer, D. H. Davies, E. M. Hadley, P. J. Burke, F. T. Boyle, R. G. Melton, T. A. Connors, D. C. Blakey, and A. B. Mauger (1996) New mustard prodrugs for antibody-directed enzyme prodrug therapy: Alternative for the amide link. *J. Med. Chem.* 39, 1100–1105.
- (130) C. J. Springer, R. Dowell, P. J. Burke, E. Hadley, D. H. Davies, D. C. Blakey, R. G. Melton, and I. Niculescu-Duvaz (1995) Optimization of alkylating agent prodrugs derived from phenol and aniline mustards: A new clinical candidate prodrug (ZD2767) for antibody-directed enzyme prodrug therapy (ADEPT). *J. Med. Chem.* 38, 5051–5065.
- (131) A. C. Khan and W. C. J. Ross (1967) Tumour growth inhibitory nitrophenylaziridines and related compounds: structure activity relationships. *Chem. Biol. Interact.* 1, 27–47.
- (132) R. J. Knox, M. P. Boland, F. Friedlos, B. Coles, C. Southan, and J. J. Roberts (1988) The nitroreductase enzyme in Walker cells that activates 5-(aziridin-1-yl)-2,4-dinitrobenzamide (CB 1954) to 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide is a form of NAD(P)H dehydrogenase (quinone) (EC 1.6.99.2). *Biochem. Pharmacol.* 44, 2297–2301.
- (133) R. J. Knox, F. Friedlos, R. F. Sherwood, R. G. Melton, and G. M. Anlezark (1992) The bioactivation of 5-(aziridin-1-yl)-2,4-dinitrobenzamide (CB 1954). II. A comparison of an *Escherichia coli* nitroreductase and Walker DT-diaphorase. *Biochem. Pharmacol.* 44, 2297–2301.
- (134) M. P. Boland, R. J. Knox, and J. J. Roberts (1991) The differences in kinetics of rat and human DT-diaphorase result in a differential sensitivity of cells lines to CB 1954 [5-(aziridin-1-yl)-2,4-dinitrobenzamide]. *Biochem. Pharmacol.* 41, 867–875.
- (135) R. C. Mulligan and P. Berg (1981) Selection for animal cells that express the *Escherichia coli* gene coding for xanthine-guanine phosphoribosyltransferase. *Proc. Natl. Acad. Sci. U.S.A.* 78, 22072–22076.
- (136) T. Tamiya, Y. Ono, M. X. Wei, P. J. Mroz, F. L. Moolten, and E. A. Chiocca (1996) *Escherichia coli* gpt gene sensitizes rat glioma cells to killing by 6-thioxanthine or 6-thioguanine. *Cancer Gene Ther.* 3, 155–162.
- (137) A. R. Kinsella, D. Smith, and M. Pickard (1997) Resistance to chemotherapeutic antimetabolites: a function of salvage pathway involvement and cellular response to DNA damage. *Br. J. Cancer* 75, 935–945.
- (138) L. Chen and D. J. Waxman (1995) Intratumoral activation and enhanced chemotherapeutic effect of oxazaphosphorines following cytochrome P-450 gene transfer: Development of a combined chemotherapy/cancer gene therapy strategy. *Cancer Res.* 55, 581–589.
- (139) P. T. Golumbek, F. M. Hamzeh, E. M. Jaffee, H. Levitsky, P. S. Lietman, and D. M. Pardoll (1992) Herpes simplex-1 virus thymidine kinase gene is unable to completely eliminate live, nonimmunogenic tumour cell vaccines. *J. Immunother.* 12, 224–230.
- (140) B. A. Teicher and E. Frei III (1988) Development of alkylating agent resistant human tumor cell lines. *Cancer Chemother. Pharmacol.* 21, 292–298.
- (141) E. Frei, B. A. Teicher, and S. A. Holden (1988) Preclinical studies and clinical correlation of the effect of alkylating drugs. *Cancer Res.* 48, 6417.
- (142) S. M. Freeman, C. N. Abboud, K. A. Whartenby, C. H. Packman, D. S. Koeplin, F. S. Moolten, and G. N. Abraham (1993) The “bystander effect”: Tumor regression when a fraction of the tumor mass is genetically modified. *Cancer Res.* 53, 5274–5283.
- (143) Q. T. Trinh, E. A. Austin, D. M. Murray, V. C. Knick, and B. E. Huber (1995) Enzyme/prodrug gene therapy: Comparison of cytosine deaminase/5-fluorocytosine versus thymidine kinase/ganciclovir enzyme/prodrug system in a human colorectal carcinoma cell line. *Cancer Res.* 55, 4808–4812.
- (144) G. Goldberg and J. S. Bertram (1994) Correspondence re: Z. Ram et al. In situ retroviral-mediated gene transfer for the treatment of brain tumors in rats. *Cancer Res.* 53, 83–88; (1993) *Cancer Res.* 54, 3947–3948.
- (145) S. F. Dagher, S. E. Conrad, E. A. Werner, and R. J. Patterson (1992) Phenotypic conversion of TK-deficient cells following electroporation of functional TK enzyme. *Exp. Cell Res.* 198, 36–42.
- (146) W. L. Bi, L. M. Parysek, R. Warnick, and P. J. Stambrook (1993) *In vitro* evidence that metabolic cooperation is responsible for the bystander effect observed with HSV tk retroviral gene therapy. *Hum. Gene Ther.* 4, 725–731.
- (147) J. Fick, F. G. Barker, P. Dazin, E. M. Westphale, E. C. Beyer, and M. A. Israel (1995) The extent of heterocellular communication mediated gap junctions is predictive of bystander tumor cytotoxicity *in vitro*. *Proc. Natl. Acad. Sci. U.S.A.* 92, 11071–11075.
- (148) S. M. Freeman, R. Ramesh, M. Sastri, A. Munshi, A. K. Jensen, and A. J. Marrogi (1995) The role of cytokines in mediating the bystander effect using HSV-TK xenogeneic cells. *Cancer Lett.* 92, 167–174.
- (149) W. Hamel, L. Magnelli, V. P. Chiarugi, and M. A. Israel (1996) Herpes simplex virus thymidine kinase/ganciclovir-mediated apoptotic death of bystander cells. *Cancer Res.* 56, 2697–2702.
- (150) R. Ramesh, A. J. Marrogi, A. Munshi, C. N. Abboud, and S. M. Freeman (1996) *In vivo* analysis of the “bystander effect”: A cytokine cascade. *Exp. Hematol.* 24, 829–838.
- (151) J. Pavlovic, M. Nawrath, R. Tu, T. Heinicke, and K. Moelling (1996) Anti-tumor immunity is involved in the thymidine kinase-mediated killing of tumors induced by activated Ki-ras (G12V). *Gene Ther.* 3, 635–643.
- (152) R. Vile, N. Miller, Y. Chernayovsky, and I. Hart (1994) A comparison of the properties of different retroviral vectors containing the murine tyrosinase promoter to achieve transcriptionally targeted expression of the HSVtk or IL-2 genes. *Gene Ther.* 1, 307–316.
- (153) S. H. Chen, X. L. Li Chen, Y. Wang, K. Kosai, M. J. Finegold, S. S. Rich, and S. L. C. Woo (1995) Combination gene therapy for liver metastasis of colon carcinoma *in vivo*. *Proc. Natl. Acad. Sci. U.S.A.* 92, 2577–2581.
- (154) S. H. Chen, K. Kosai, B. Xu, K. Pham-Nguyen, C. Contant, M. J. Finegold, and S. L. C. Woo (1996) Combination suicide and cytokine gene therapy for hepatic metastases of colon carcinoma: Sustained antitumor immunity prolongs animal survival. *Cancer Res.* 56, 3758–3762.
- (155) B. W. O'Malley, K. A. Cope, S. H. Chen, D. Li, M. R. Schwartz, and S. L. C. Woo (1996) Combination gene therapy for oral cancer in a murine model. *Cancer Res.* 56, 1737–1741.

BC970116T

ARTICLES

Immunoassay Reagents for Thyroid Testing. 3. Determination of the Solution Binding Affinities of a T₄ Monoclonal Antibody Fab Fragment for a Library of Thyroxine Analogs Using Surface Plasmon Resonance

Maciej Adamczyk,* Donald D. Johnson, Phillip G. Mattingly, Jeffrey A. Moore, and You Pan

Diagnostics Division Organic Chemistry (9-NM), Abbott Laboratories, Building AP 20, 100 Abbott Park Road, Abbott Park, Illinois 60064. Received July 23, 1997; Revised Manuscript Received October 1, 1997[®]

A library of thyroxine analogs and tracers was prepared, and their solution binding affinities for an anti-T₄ Fab fragment were determined using a single high-density L-T₄ biosensor surface in a BIAcore surface plasmon resonance instrument. The high-density L-T₄ analog biosensor was calibrated by determination of the initial binding rate was of known concentrations of free anti-T₄ Fab fragment in solution to the biosensor surface. A range of individual thyroxine analog and tracer concentrations was subsequently mixed with a fixed concentration of anti-T₄ Fab fragment. The concentration of free anti-T₄ Fab fragment in each solution at equilibrium was determined, and the equilibrium dissociation constant (K_D) for each case was derived. The K_D values determined in solution are compared to values determined by a direct kinetic analysis on the BIAcore instrument using individual biosensor surfaces.

INTRODUCTION

The need for well-characterized immunoreagents utilized in immunoassays is widely recognized (1–6). While haptens, tracers, and antibodies are routinely characterized chemically to determine their identity and purity, the kinetic and thermodynamic properties of their binding interactions are generally only inferred from their performance in a particular assay format. These binding interactions are critical to all stages of assay development from selection of antibody through determination of the structural features important for tracer design. Unfortunately, determination of these binding parameters traditionally has been a laborious endeavor (7). The recent development of automated BIAcore (BIAcore, Inc.) surface plasmon resonance technology (SPR) has greatly simplified this task.

We recently reported the utility of the automated BIAcore SPR technology in assessing the binding kinetics of an anti-T₄ mAb and its Fab fragment to a library of surface-bound thyroxine analogs (2). In that study, individual thyroxine analogs were conjugated to a solid-supported hydrophilic dextran matrix of an SPR chip to form a biosensor, mimicking a heterogeneous assay format. A solution of the mAb or Fab fragment was then introduced at a constant rate to the surface of each biosensor, and binding was monitored continuously, providing real-time kinetic information. Dissociation of

bound mAb or Fab fragment was subsequently monitored after the sample had traversed the biosensor surface. By using a range of binding protein concentrations, a series of sensorgrams was produced for each thyroxine analog biosensor. Nonlinear regression analysis of the data provided the association and dissociation rate constants (k_a and k_d) from which the equilibrium association constant (K_A) and the change in free energy (ΔG) of binding were calculated. These values helped to define the structural features of the thyroxine analogs that were required for the binding interaction, but necessitated production of a different biosensor for each analog studied. Additionally, this format neither allowed for the direct measurement of antibody interaction with a native analyte (underivatized T₄) or tracer nor served as a good model for homogeneous assay formats. These issues are addressed in this study.

The BIAcore instrument was used to study the binding interactions between an anti-T₄ Fab fragment and a library of thyroxine analogs and tracers in solution using a single thyroxine analog biosensor. Thus, anti-T₄ Fab fragment is known to bind to a biosensor surface of thyroxine immobilized through an aminoalkyl linker (2). With high-density T₄ biosensor surfaces, the binding observed is limited by the diffusion of the protein to the surface. Under such conditions, initial binding rates are proportional to the free anti-T₄ Fab fragment concentration in solution and independent of the interaction kinetics or analyte–ligand affinity (8, 9). High-density T₄ biosensor surfaces were prepared, and the initial binding rates were measured with known concentrations of anti-T₄ Fab fragment to construct a calibration curve. A range of individual thyroxine analog and tracer con-

* To whom correspondence should be addressed. Telephone: (847) 937-0225. Fax: (847) 938-8927. E-mail: maciej.adamczyk@abbott.com.

[®] Abstract published in *Advance ACS Abstracts*, November 15, 1997.

centrations were subsequently mixed with a fixed concentration of antibody fragment in solution and allowed to reach equilibrium. The concentration of free anti-T₄ Fab fragment in each solution was subsequently determined, and the equilibrium dissociation constant (K_D) for each case was derived.

MATERIALS AND METHODS

Sodium L-thyroxine pentahydrate (**L-1**) and all other reagents were obtained from Aldrich Chemical Co. (Milwaukee, WI) unless otherwise noted. Sodium D-thyroxine pentahydrate (**D-1**), sodium L-3,3',5-triiodothyronine (**L-2**), and L-3,5-diiodothyronine (**3**) were obtained from Sigma Chemical Co. (St. Louis, MO). D-3,3',5-Triiodothyronine (**D-2**) was obtained from ICN Biochemicals (Cleveland, OH). Compounds **4–16** were prepared as reported earlier (2). 5-Carboxyfluorescein was obtained from Calbiochem (La Jolla, CA). Acridinium chemiluminescent label **39** was prepared following the general procedure of Mattingly *et al.* (10, 11). Synthesized compounds were purified by HPLC [Waters (Millford, MA) system consisting of a model 590 pump, a Lambda-Max 481 UV detector, a model 745B data module, and a 40 × 100 mm μ Bondapak C18 column; flow rate, 45 mL/min]. Analytical HPLC was performed on a Beckman Gold system with UV detection at 254 nm using a Waters 8 × 100 mm μ Bondapak C18 column eluting with aqueous acetonitrile (v:v, CH₃CN/H₂O) containing 0.05% trifluoroacetic acid at a flow rate of 2 mL/min. ¹H NMR spectra were recorded at 300 MHz on a Varian Gemini spectrometer (Palo Alto, CA). Chemical shifts are reported in parts per million (δ) using tetramethylsilane (TMS) as the internal reference; coupling constants (J) are in hertz. Electrospray ionization mass spectrometry (ESI/MS) was carried out on a Perkin-Elmer (Norwalk, CT) Sciex API 100 Benchtop system employing the Turbo IonSpray ion source. Surface plasmon resonance measurements were carried out on a BIAcore 2000 (BIAcore, Inc., Piscataway, NJ) automated system using CM-5 four-channel sensor chips. Reagents for the BIAcore instrument consisted of HBS buffer [10 mM Hepes (pH 7.4), 150 mM NaCl, 3.4 mM EDTA, and 0.05% surfactant P-20] and a coupling kit containing *N*-hydroxysuccinimide (NHS), *N*-ethyl-*N*-(3-(diethylamino)propyl)carbodiimide (EDAC), and 1 M ethanolamine hydrochloride (pH 8.5), all from BIAcore, Inc. Anti-thyroxine Fab fragment was prepared and purified as previously described (2).

General Procedure for Preparation of 5-Carboxyfluorescein Tracers. (A) *In Situ Activation of 5-Carboxyfluorescein.* A solution of 5-carboxyfluorescein (250 mg, 0.681 mmol), EDAC (196 mg, 1.02 mmol), and *N*-hydroxysuccinimide (117 mg, 1.02 mmol) in DMF (6.8 mL) was stirred overnight. The reaction solution (0.1 M) was used without further purification.

(B) *Coupling of Succinimidyl 5-Carboxyfluorescein to Thyroxine Analogs.* To a solution of the analog compound (**9–16**) (0.03 mmol) in DMF (3 mL) were added a 5-carboxyfluorescein-activated ester stock solution (0.34 mL, 0.1 M, 0.34 mmol) and *N,N*-diisopropylethylamine (100 μ L). After stirring for 4 h, the reaction mixture was acidified with TFA and purified by preparative HPLC.

Compound **L-17** was obtained from compound **L-9** (79%). HPLC (55:45): retention time, 5.8 min, 98%. ¹H NMR (methanol-*d*₄): 8.45 (1 H, s), 8.20 (1 H, d, J = 8), 7.89 (2 H, s), 7.33 (1 H, d, J = 8), 7.05 (2 H, s), 6.74 (2 H, s), 6.65 (2 H, d, J = 8), 6.60 (2 H, d, J = 8), 4.70 (1 H, m), 3.43 (2 H, m), 3.22 (1 H, m), 2.93 (1 H, m), 2.24 (2 H, m), 1.65 (4 H, m), 1.40 (2 H, m). ESI/MS m/z : 1247 ($M - H$)⁻.

Compound **D-17** was obtained from compound **D-9** (59%). HPLC (55:45): retention time, 5.8 min, 96%. ¹H NMR (methanol-*d*₄): 8.48 (1 H, s), 8.19 (1 H, d, J = 8), 7.80 (2 H, s), 7.34 (1 H, d, J = 9), 7.05 (2 H, s), 6.78 (2 H, s), 6.71 (2 H, d, J = 8), 6.65 (2 H, d, J = 8), 4.72 (1 H, m), 3.42 (2 H, m), 3.24 (1 H, m), 2.90 (1 H, m), 2.24 (2 H, m), 1.67 (4 H, m), 1.38 (2 H, m). ESI/MS m/z : 1247 ($M - H$)⁻.

Compound **L-18** was obtained from compound **L-10** (61%). HPLC (55:45): retention time, 4.4 min, 98%. ¹H NMR (methanol-*d*₄): 8.45 (1 H, s), 8.20 (1 H, d, J = 8), 7.79 (2 H, s), 7.33 (1 H, d, J = 8), 6.98 (1 H, d, J = 3), 6.74 (2 H, s), 6.71 (3 H, m), 6.65 (2 H, d, J = 8), 6.57 (1 H, dd, J = 8, 3), 4.71 (1 H, m), 3.42 (2 H, m), 3.25 (1 H, m), 2.91 (1 H, m), 2.24 (2 H, m), 1.66 (4 H, m), 1.41 (2 H, m). ESI/MS m/z : 1123 ($M + H$)⁺.

Compound **D-18** was obtained from compound **D-10** (42%). HPLC (55:45): retention time, 4.4 min, 99%. ¹H NMR (methanol-*d*₄): 8.48 (1 H, s), 8.19 (1 H, d, J = 8), 7.79 (2 H, s), 7.35 (1 H, d, J = 8), 6.98 (1 H, d, J = 3), 6.80 (2 H, s), 6.76 (3 H, m), 6.64 (2 H, d, J = 8), 6.56 (1 H, dd, J = 8, 3), 4.68 (1 H, m), 3.42 (2 H, m), 3.25 (1 H, m), 2.88 (1 H, m), 2.24 (2 H, m), 1.64 (4 H, m), 1.38 (2 H, m). ESI/MS m/z : 1123 ($M + H$)⁺.

Compound **19** was obtained from compound **11** (49%). HPLC (50:50): retention time, 4.6 min, >99%. ¹H NMR (methanol-*d*₄): 8.45 (1 H, s), 8.20 (1 H, d, J = 9), 7.77 (2 H, s), 7.32 (1 H, d, J = 9), 6.75 (2 H, s), 6.65 (4 H, m), 6.61 (2 H, d, J = 9), 6.49 (2 H, d, J = 9), 4.67 (1 H, m), 3.42 (2 H, m), 3.23 (1 H, m), 2.87 (1 H, m), 2.21 (2 H, m), 1.64 (4 H, m), 1.23 (2 H, m). ESI/MS m/z : 995 ($M - H$)⁻.

Compound **20** was obtained from compound **12** (59%). HPLC (55:45): retention time, 5.5 min, >99%. ¹H NMR (methanol-*d*₄): 8.46 (1 H, s), 8.21 (1 H, d, J = 8), 7.81 (2 H, s), 7.33 (1 H, d, J = 8), 7.03 (1 H, d, J = 3), 6.83 (1 H, d, J = 3), 6.76 (2 H, s), 6.68 (2 H, d, J = 9), 6.62 (2 H, d, J = 9), 4.72 (1 H, dd, J = 10, 4), 3.42 (2 H, m), 3.23 (1 H, m), 2.85 (1 H, m), 2.23 (2 H, m), 1.66 (4 H, m), 1.38 (2 H, m). ESI/MS m/z : 1201, 1203 ($M + H$)⁺.

Compound **21** was obtained from compound **13** (47%). HPLC (55:45): retention time, 4.1 min, >99%. ¹H NMR (methanol-*d*₄): 8.46 (1 H, s), 8.21 (1 H, d, J = 8), 7.79 (2 H, s), 7.34 (1 H, d, J = 8), 6.77 (4 H, m), 6.72 (2 H, d, J = 8), 6.63 (2 H, d, J = 9), 6.54 (1 H, m), 4.69 (1 H, m), 3.44 (2 H, m), 3.22 (1 H, m), 2.88 (1 H, m), 2.24 (2 H, m), 1.66 (4 H, m), 1.57 (2 H, m). ESI/MS m/z : 1075, 1077 ($M + H$)⁺.

Compound **22** was obtained from compound **14** (44%). HPLC (55:45): retention time, 5.1 min, 98%. ¹H NMR (methanol-*d*₄): 8.43 (1 H, s), 8.20 (1 H, d, J = 8), 7.81 (2 H, s), 7.31 (1 H, d, J = 8), 6.83 (2 H, s), 6.72 (2 H, s), 6.62 (2 H, d, J = 9), 6.57 (2 H, d, J = 9), 4.72 (1 H, m), 3.41 (2 H, m), 3.28 (1 H, m), 2.88 (1 H, m), 2.23 (2 H, m), 1.63 (4 H, m), 1.38 (2 H, m). ESI/MS m/z : 1151, 1153, 1155 ($M - H$)⁻.

Compound **23** was obtained from compound **15** (58%). HPLC (55:45): retention time, 7.6 min, 98%. ¹H NMR (methanol-*d*₄): 8.44 (1 H, s), 8.21 (1 H, d, J = 8), 7.83 (2 H, s), 7.37 (1 H, d, J = 8), 7.05 (2 H, s), 6.71 (2 H, s), 6.62 (2 H, d, J = 8), 6.58 (2 H, d, J = 8), 5.07 (1 H, m), 3.42 (2 H, m), 3.02 (3 H, s), 2.96 (1 H, m), 2.91 (3 H, s), 2.85 (1 H, m), 2.23 (2 H, m), 1.66 (4 H, m), 1.35 (2 H, m). ESI/MS m/z : 1276 ($M + H$)⁺.

Compound **24** was obtained from compound **16** (60%). HPLC (55:45): retention time, 5.3 min, >99%. ¹H NMR (methanol-*d*₄): 8.46 (1 H, s), 8.22 (1 H, d, J = 8), 7.81 (2 H, s), 7.31 (1 H, d, J = 8), 6.94 (1 H, d, J = 3), 6.72 (2 H, s), 6.61 (3 H, m), 6.58 (3 H, m), 5.07 (1 H, m), 3.42 (2 H, m), 3.03 (3 H, s), 2.98 (1 H, m), 2.92 (3 H, s), 2.85 (1 H,

m), 2.23 (2 H, m), 1.64 (4 H, m), 1.38 (2 H, m). ESI/MS m/z : 1150 ($M + H$)⁺.

Compound **29** was obtained from compound **D-1** (80%). HPLC (55:45): retention time, 6.9 min, >99%. ¹H NMR (DMSO-*d*₆): 9.39 (1 H, d, $J = 7$), 8.44 (1 H, s), 8.15 (1 H, d, $J = 8$), 7.89 (2 H, s), 7.36 (1 H, d, $J = 8$), 6.99 (2 H, s), 6.66 (2 H, d, $J = 2$), 6.54 (4 H, m), 4.67 (1 H, m), 3.03 (1 H, m), 2.95 (1 H, m). ESI/MS m/z : 1136 ($M + H$)⁺.

L-Thyroxine 6-carboxyfluorescein tracer (**25**) was prepared from compound **L-9** and 6-carboxyfluorescein according to the general procedure used for the 5-carboxyfluorescein tracers above (70%). HPLC (55:45): retention time, 5.4 min, >99%. ¹H NMR (methanol-*d*₄): 8.11 (2 H, d, $J = 3$), 7.77 (2 H, s), 7.67 (1 H, s), 7.03 (2 H, s), 6.72 (2 H, s), 6.65 (2 H, d, $J = 9$), 6.58 (2 H, d, $J = 9$), 4.66 (1 H, m), 3.21 (3 H, m), 2.85 (1 H, m), 2.16 (2 H, m), 1.54 (4 H, m), 1.27 (2 H, m). ESI/MS m/z : 1247 ($M - H$)⁻.

N-(Carboxymethyl)-L-thyroxine 4'-Aminomethylfluorescein Tracer (26). (A) *N*-(Carboxymethyl)-L-thyroxine *tert*-butyl ester (**4**) (38 mg, 0.04 mmol) and *N*-hydroxysuccinimide (5 mg, 0.04 mmol) were dissolved in DMF (500 μ L) and stirred with DCC for 14 h under nitrogen. The crude active ester solution was filtered and then added to a solution of 4'-aminomethylfluorescein hydrochloride (17 mg, 0.04 mmol) and triethylamine (3 drops) in DMF (500 μ L). The reaction mixture was stirred under nitrogen in the dark. After 14 h, the reaction solvent was removed *in vacuo*, and the residue was purified by preparative HPLC (78:22:0.4, CH₃OH/H₂O/HOAc). Product fractions were pooled and evaporated *in vacuo* to give the *tert*-butyl ester-protected tracer (30 mg, 57%) as an orange powder. Analytical HPLC (78:22:0.4, CH₃OH/H₂O/HOAc; 1 mL/min, 240 nm): retention time, 12.97 min, 99.7%. FAB/MS m/z : 1235 ($M + H$)⁺. (B) The *tert*-butyl ester-protected tracer (13 mg) was dissolved in 1:1 TFA/CH₂Cl₂ (1 mL). After the mixture was stirred for 4 h, the reaction solvent was removed *in vacuo*. The residue was dissolved in methanol (0.6 mL) and purified by preparative HPLC as above to give **26** (9 mg, 69%). Analytical HPLC (75:25:0.4, CH₃OH/H₂O/HOAc; 1 mL/min, 240 nm): retention time, 11.2 min, 99%. FAB/MS m/z : 1179 ($M + H$)⁺.

N-(Carboxymethyl)-L-thyroxine 5-aminomethylfluorescein tracer (**27**) was prepared from *N*-(carboxymethyl)-L-thyroxine *tert*-butyl ester (**4**) and 5-aminomethylfluorescein hydrobromide (**12**) according to the procedure used for compound **26** (61%). Analytical HPLC (80:20:0.4, CH₃OH/H₂O/HOAc; 1 mL/min, 240 nm): retention time, 6.1 min, 99%. FAB/MS m/z : 1179 ($M + H$)⁺.

L-Thyroxine 4'-N-glycylaminomethylfluorescein tracer (**28**) was prepared from *N-tert*-(butoxycarbonyl)-L-thyroxine (**2**) and 4'-N-glycylaminomethylfluorescein (Molecular Probes, Eugene, OR) according to the procedure outlined for compound **26** (61%). Analytical HPLC (75:25:0.4, CH₃OH/H₂O/HOAc; 1 mL/min, 240 nm): retention time, 10.3 min, 99%. ESI/MS m/z : 1178 ($M + H$)⁺.

General Procedure for Preparation of 10-(3-Sulfofpropyl)-*N*-tosyl-*N*-(3-carboxypropyl)acridinium-9-carboxamide Tracers. To a solution of the analog compound (**9-16**) (0.03 mmol) in DMF (3 mL) were added the acridinium active ester (**39**) (0.03 mmol) and *N,N*-diisopropylethylamine (100 μ L). After being stirred for 6 h, the reaction mixture was acidified with TFA and purified by preparative HPLC.

Compound **L-31** was obtained from compound **L-9** (52%). HPLC (50:50): retention time, 6.5 min, >99%. ¹H NMR (methanol-*d*₄): 8.92 (2 H, d, $J = 10$), 8.43 (2 H, m), 8.05 (2 H, d, $J = 10$), 7.85 (2 H, m), 7.77 (2 H, s), 7.17 (4 H, bs), 7.03 (2 H, s), 5.73 (2 H, b), 4.67 (1 H, m),

4.24 (2 H, m), 3.24 (5 H, m), 2.90 (1 H, m), 2.65 (2 H, m), 2.44 (2 H, m), 2.36 (3 H, s), 2.32 (2 H, m), 2.20 (2 H, m), 1.55 (4 H, m), 1.34 (2 H, m). ESI/MS m/z : 1457 ($M + H$)⁺.

Compound **D-31** was obtained from compound **D-9** (51%). HPLC (50:50): retention time, 6.5 min, >99%. ¹H NMR (DMSO-*d*₆/TFA-*d*): 9.01 (2 H, dd, $J = 12, 9$), 8.42 (2 H, bt), 8.16 (1 H, d, $J = 7$), 8.01 (2 H, m), 7.85 (2 H, m), 7.85 (1 H, t, $J = 8$), 7.80 (2 H, d, $J = 6$), 7.69 (1 H, d, $J = 9$), 7.63 (1 H, d, $J = 9$), 7.16 (1 H, d, $J = 8$), 7.11 (1 H, d, $J = 8$), 7.03 (2 H, d, $J = 2$), 5.66 (2 H, b), 4.44 (1 H, b), 4.20 (2 H, b), 3.42 (1 H, m), 3.08 (2 H, m), 2.92 (2 H, m), 2.81 (1 H, m), 2.47 (2 H, m), 2.37 (1 H, m), 2.30 (3 H, s), 2.27 (1 H, m), 2.06 (2 H, m), 1.65 (1 H, b), 1.43 (4 H, m), 1.23 (2 H, m). ESI/MS m/z : 1457 ($M + H$)⁺.

Compound **L-32** was obtained from compound **L-10** (63%). HPLC (50:50): retention time, 4.8 min, >99%. ¹H NMR (DMSO-*d*₆/TFA-*d*): 8.96 (2 H, dd, $J = 14, 9$), 8.40 (2 H, bt), 8.13 (1 H, d, $J = 9$), 7.97 (2 H, m), 7.82 (1 H, t, $J = 7$), 7.76 (2 H, d, $J = 6$), 7.68 (1 H, d, $J = 9$), 7.60 (1 H, d, $J = 9$), 7.16 (1 H, d, $J = 8$), 7.08 (1 H, d, $J = 8$), 6.94 (1 H, t, $J = 3$), 6.80 (1 H, dd, $J = 9, 3$), 6.53 (1 H, m), 5.65 (2 H, b), 4.46 (1 H, m), 4.18 (2 H, b), 3.42 (2 H, m), 3.06 (4 H, m), 2.73 (1 H, m), 2.43 (2 H, m), 2.30 (1 H, m), 2.27 (3 H, s), 2.14 (1 H, m), 2.05 (2 H, m), 1.64 (1 H, m), 1.40 (4 H, m), 1.22 (2 H, m). ESI/MS m/z : 1331 ($M + H$)⁺.

Compound **D-32** was obtained from compound **D-10** (55%). HPLC (50:50): retention time, 4.8 min, >99%. ¹H NMR (DMSO-*d*₆/TFA-*d*): 9.01 (2 H, dd, $J = 13, 10$), 8.41 (2 H, bt), 8.15 (1 H, d, $J = 8$), 7.98 (2 H, m), 7.85 (1 H, bt), 7.78 (2 H, d, $J = 5$), 7.66 (1 H, d, $J = 9$), 7.62 (1 H, d, $J = 9$), 7.16 (1 H, d, $J = 8$), 7.12 (1 H, d, $J = 8$), 6.95 (1 H, t, $J = 3$), 6.78 (1 H, dd, $J = 9, 3$), 5.66 (2 H, b), 4.45 (1 H, m), 4.20 (2 H, b), 3.45 (2 H, b), 3.07 (2 H, m), 2.94 (2 H, m), 2.86 (1 H, m), 2.47 (2 H, m), 2.31 (1 H, m), 2.29 (3 H, s), 2.15 (1 H, m), 2.03 (2 H, m), 1.65 (1 H, b), 1.42 (4 H, m), 1.27 (2 H, m). ESI/MS m/z : 1331 ($M + H$)⁺.

Compound **33** was obtained from compound **11** (67%). HPLC (45:55): retention time, 9.2 min, >99%. ¹H NMR (DMSO-*d*₆/TFA-*d*): 9.87 (2 H, dd, $J = 13, 10$), 8.42 (2 H, bt), 8.17 (1 H, d, $J = 8$), 8.01 (2 H, m), 7.86 (1 H, t, $J = 8$), 7.76 (2 H, d, $J = 5$), 7.67 (1 H, d, $J = 9$), 7.63 (1 H, d, $J = 9$), 7.18 (1 H, d, $J = 8$), 7.11 (1 H, d, $J = 8$), 6.64 (2 H, d, $J = 9$), 6.49 (2 H, d, $J = 9$), 5.65 (2 H, b), 4.43 (1 H, m), 4.20 (2 H, b), 3.44 (2 H, b), 3.06 (2 H, m), 2.95 (2 H, m), 2.85 (1 H, m), 2.48 (2 H, m), 2.31 (1 H, m), 2.29 (3 H, s), 2.14 (1 H, m), 2.06 (2 H, m), 1.65 (1 H, b), 1.43 (4 H, m), 1.23 (2 H, m). ESI/MS m/z : 1205 ($M + H$)⁺.

Compound **34** was obtained from compound **12** (57%). HPLC (50:50): retention time, 4.8 min, >99%. ¹H NMR (DMSO-*d*₆/TFA-*d*): 9.00 (2 H, dd, $J = 14, 10$), 8.42 (2 H, bt), 8.14 (1 H, d, $J = 7$), 7.97 (2 H, m), 7.83 (1 H, m), 7.78 (2 H, d, $J = 6$), 7.68 (1 H, d, $J = 9$), 7.62 (1 H, d, $J = 9$), 7.15 (1 H, d, $J = 9$), 7.10 (1 H, d, $J = 9$), 7.03 (1 H, t, $J = 3$), 6.85 (1 H, t, $J = 3$), 5.65 (2 H, m), 4.48 (1 H, dd, $J = 10, 4$), 4.19 (2 H, m), 3.44 (2 H, m), 3.05 (2 H, m), 2.93 (2 H, m), 2.81 (1 H, m), 2.46 (2 H, m), 2.30 (1 H, m), 2.28 (3 H, s), 2.15 (1 H, m), 2.01 (2 H, m), 1.64 (1 H, b), 1.38–1.23 (4 H, m). ESI/MS m/z : 1407, 1409 ($M - H$)⁻.

Compound **35** was obtained from compound **13** (35%). HPLC (50:50): retention time, 4.5 min, >99%. ¹H NMR (methanol-*d*₄): 8.93 (2 H, d, $J = 9$), 8.43 (2 H, dd, $J = 11, 9$), 8.05 (2 H, d, $J = 9$), 7.83 (2 H, m), 7.76 (2 H, s), 7.16 (4 H, s), 6.77 (2 H, m), 6.54 (1 H, m), 5.73 (2 H, b), 4.66 (1 H, m), 4.24 (2 H, b), 3.26 (5 H, m), 2.84 (1 H, m), 2.61 (2 H, m), 2.42 (2 H, m), 2.37 (3 H, s), 2.37 (2 H, m), 2.20 (2 H, m), 1.56 (4 H, m), 1.38 (2 H, m). ESI/MS m/z : 1283, 1285 ($M + H$)⁺.

Compound **36** was obtained from compound **14** (51%). HPLC (50:50): retention time, 5.7 min, 97%. ^1H NMR (methanol- d_4): 8.93 (2 H, d, $J = 9$), 8.44 (2 H, dd, $J = 11, 9$), 8.07 (2 H, d, $J = 9$), 7.87 (2 H, m), 7.81 (2 H, s), 7.17 (4 H, s), 6.83 (2 H, s), 5.76 (2 H, b), 4.67 (1 H, m), 4.25 (2 H, m), 3.22 (5 H, m), 2.87 (1 H, m), 2.61 (2 H, m), 2.44 (2 H, m), 2.37 (3 H, s), 2.29 (2 H, m), 2.18 (2 H, m), 1.60 (4 H, m), 1.32 (2 H, m). ESI/MS m/z : 1361, 1363, 1365 ($M + H$) $^+$.

Compound **37** was obtained from compound **15** (67%). HPLC (50:50): retention time, 8.6 min, 96%. ^1H NMR (DMSO- d_6 /TFA- d): 9.04 (2 H, dd, $J = 12, 9$), 8.41 (2 H, bt), 8.17 (1 H, d, $J = 7$), 7.84 (1 H, m), 7.80 (2 H, d, $J = 3$), 7.69 (1 H, d, $J = 9$), 7.66 (1 H, m), 7.15 (1 H, d, $J = 9$), 7.11 (1 H, d, $J = 9$), 7.04 (2 H, s), 5.66 (2 H, b), 4.87 (1 H, m), 4.20 (2 H, m), 3.44 (2 H, m), 3.05 (2 H, m), 2.94 (3 H, s), 2.85 (2 H, m), 2.80 (3 H, s), 2.72 (2 H, m), 2.41 (2 H, m), 2.30 (3 H, s), 2.14 (2 H, m), 2.05 (2 H, m), 1.66 (1 H, m), 1.43 (4 H, m), 1.22 (2 H, m). ESI/MS m/z : 1484 ($M + H$) $^+$.

Compound **38** was obtained from compound **16** (41%). HPLC (50:50): retention time, 5.9 min, >99%. ^1H NMR (DMSO- d_6 /TFA- d): 8.99 (2 H, dd, $J = 12, 9$), 8.41 (2 H, bt), 8.18 (1 H, d, $J = 9$), 8.01 (2 H, d, $J = 9$), 7.87 (1 H, bt), 7.80 (2 H, d, $J = 3$), 7.69 (1 H, d, $J = 8$), 7.66 (1 H, b), 7.18 (1 H, d, $J = 8$), 7.11 (1 H, d, $J = 8$), 6.91 (1 H, t, $J = 3$), 6.82 (1 H, dd, $J = 9, 4$), 6.56 (1 H, m), 5.65 (2 H, b), 4.88 (1 H, m), 4.20 (2 H, b), 3.44 (2 H, m), 3.06 (2 H, m), 2.94 (3 H, s), 2.89 (2 H, m), 2.80 (3 H, s), 2.71 (2 H, m), 2.46 (2 H, m), 2.29 (3 H, s), 2.15 (2 H, m), 2.04 (2 H, m), 1.65 (1 H, m), 1.42 (4 H, m), 1.22 (2 H, m). ESI/MS m/z : 1358 ($M + H$) $^+$.

Preparation of a Biosensor Surface. Immobilization of L-T₄ analog L-**9** through the free amine to the CM-5 sensor chip was performed by a modification of the method previously described (2). Briefly, a continuous flow of HBS buffer at 20 $\mu\text{L}/\text{min}$ was initiated over the biosensor surface. The carboxymethylated dextran matrix on the sensor surface was activated by a 6 min injection of a solution of 0.2 M EDAC and 0.05 M NHS. L-T₄ analog L-**9** (25 $\mu\text{g}/\text{mL}$ in 10% EtOH, 50 mM NaHCO₃, and 1 M NaCl at pH 8.5) was then immobilized (5 min injection), followed by a 6 min injection of 1 M ethanolamine hydrochloride to block remaining unreacted active ester groups. Typical immobilizations yielded 350–450 RU of L-**9** bound to the biosensor surface.

Solution Competition Analysis. (A) *Construction of a Standard Curve.* All BIAcore studies were carried out at 25 $^{\circ}\text{C}$ using the Fab fragment of an anti-T₄ mAb which eliminated the complexity associated with the bivalency of the monoclonal antibody. Seven concentrations (0, 5, 10, 15, 20, 25, and 30 nM) of anti-T₄ Fab fragment were serially diluted into HBS buffer and allowed to equilibrate for 1 h. Each sample was then injected over the L-T₄ biosensor surface at 5 $\mu\text{L}/\text{min}$ for 2 min. The biosensor surface was regenerated after each run with a 1 M formic acid wash for 1 min. Initial rates of binding of anti-T₄ Fab fragment to the biosensor surface were measured over a 15 s window beginning 15 s postinjection using homogeneous association model type 3 (13). A standard curve was obtained from the linear fit of a plot of initial binding rates versus concentration of added anti-T₄ Fab fragment.

(B) *Determination of Solution Affinities.* Ten concentrations of each soluble T₄ analog, determined from preliminary studies to completely define the competition curve, were serially diluted into HBS buffer and mixed with 20 nM anti-T₄ Fab fragment. Samples were allowed to equilibrate (≥ 2 h) and then injected over the calibrated

T₄ biosensor surface at 5 $\mu\text{L}/\text{min}$ for 2 min. The biosensor surface was regenerated after each run with a 1 M formic acid wash for 1 min. Sensorgrams for each solution were obtained in duplicate, and initial binding rates of free anti-T₄ Fab fragment to the biosensor surface were measured as described above. The resulting free anti-T₄ Fab fragment concentrations in solution, measured as initial binding rates to the biosensor surface, were plotted against the concentration of added soluble T₄ analog to generate competition curves. Equilibrium dissociation constants (K_D) were obtained by nonlinear regression analysis of the data using the solution affinity model built into BIAevaluation 2.1 software (BIAcore, Inc.) with the slope and intercept obtained from the standard curve.

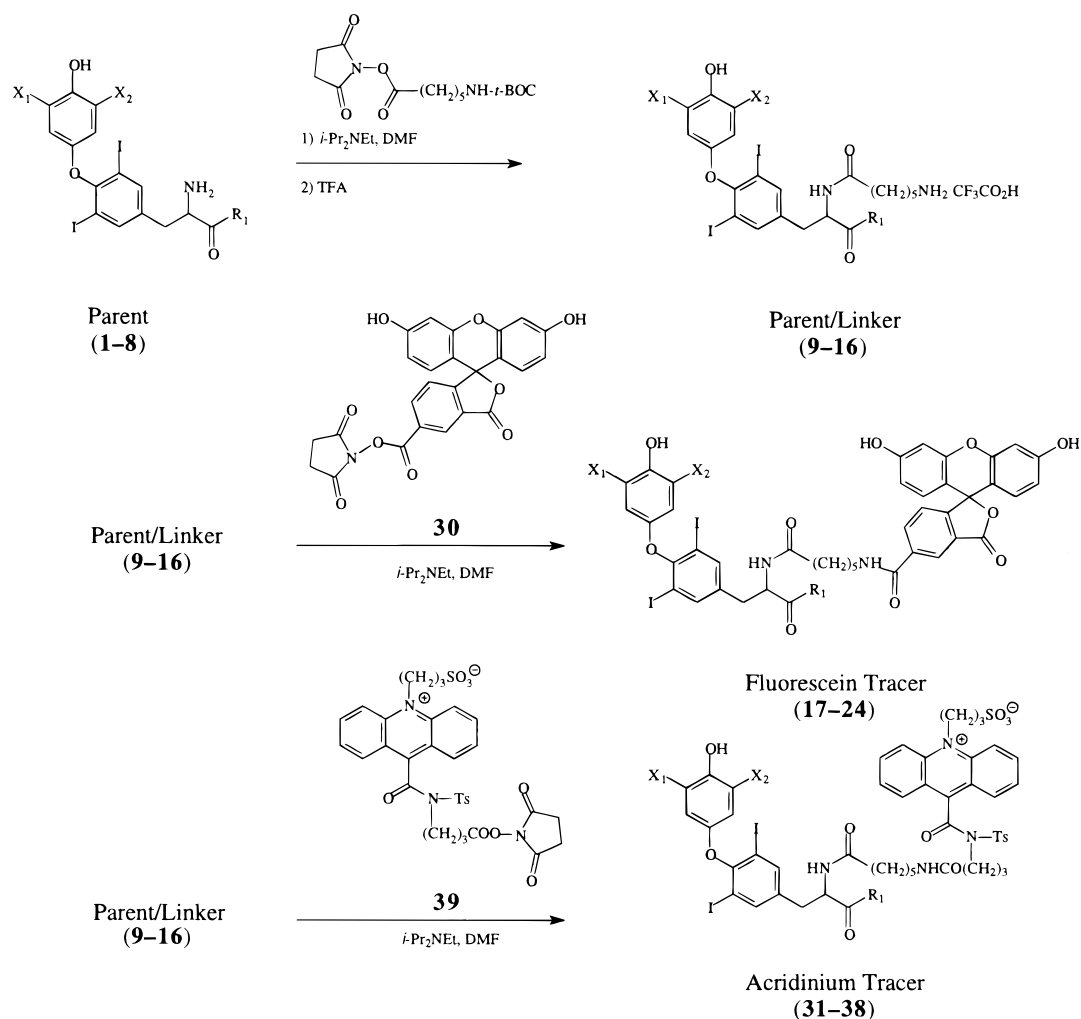
RESULTS

Preparation of Soluble T₄ Analogs. Thyroxine analogs with and without aminoalkyl linkers (**1–16**) were obtained from commercial sources or synthesized as previously described (2). Analogs **9–16** bearing an aminoalkyl linker were coupled with the NHS active ester of 5-carboxyfluorescein to give tracers **17–24** in 42–79% yield after preparative HPLC (Scheme 1). Analog L-**9** was coupled in an identical manner to the NHS active ester of 6-carboxyfluorescein to yield compound **25** in 70% yield. The fluorescein tracers **26** and **27** were prepared from the NHS active ester of *N*-(carboxymethyl)-L-thyroxine *tert*-butyl ester and 4'-aminomethylfluorescein or 5-aminomethylfluorescein in 39 and 61% overall yields, respectively, after deprotection with TFA. *N-tert*-(Butoxycarbonyl)-L-thyroxine NHS active ester and 4'-*N*-glycylaminomethylfluorescein were reacted to give the carboxy-substituted thyroxine tracer **28** after deprotection with TFA in 61% overall yield. Fluoresceinated compound **29** was prepared by the direct conjugation of D-thyroxine and 5-carboxyfluorescein NHS ester in 80% yield. Analogs **9–16** were also coupled with the NHS active ester of 10-(3-sulfopropyl)-*N*-tosyl-*N*-(3-carboxypropyl)acridinium-9-carboxamide (**39**) to give chemiluminescent tracers **31–38** in 35–67% yield after preparative HPLC (Scheme 1).

Preparation of an L-T₄ Biosensor. L-T₄ containing an aminoalkyl linker (L-**9**, Figure 1) was immobilized on the activated carboxymethylated dextran surface of a CM-5 sensor chip through amine coupling and used for all experiments to determine the concentration of free anti-T₄ Fab fragment in solution. To ensure that initial rates of binding of anti-T₄ Fab fragment to the immobilized L-T₄ biosensor surface were mass transport limited, a relatively high-density surface was prepared via a modification of the method utilized for the preparation of low-density thyroxine analog biosensors previously described (2). Thus, high-density L-T₄ surfaces were obtained by increasing the flow rate across the biosensor surface from 5 to 20 $\mu\text{L}/\text{min}$ for the activation (NHS/EDAC), coupling (L-**9**), and blocking (ethanolamine) solutions. Additionally, the concentration of L-**9** in the coupling solution was increased from 5 to 25 $\mu\text{g}/\text{mL}$. Typical immobilizations yielded 350–450 RU due to L-**9** covalently bound to the biosensor surface. Initial binding rates obtained for a given concentration of anti-T₄ Fab fragment (≤ 30 nM, *vide infra*) to all biosensor surfaces generated during these studies were identical within experimental error, indicating that mass transport limiting binding conditions were met. Biosensors prepared in this manner were suitable for use in the solution affinity analyses described below.

Construction of a Standard Curve. Typical sensorgrams obtained for different concentrations of anti-

Scheme 1



	X ₁	X ₂	R ₁	Parent	Parent with Linker	Fluorescein Tracer	Acridinium Tracer
L-T ₄	I	I	OH	L-1	L-9	L-17	L-31
D-T ₄	I	I	OH	D-1	D-9	D-17	D-31
L-T ₃	H	I	OH	L-2	L-10	L-18	L-32
D-T ₃	H	I	OH	D-2	D-10	D-18	D-32
L-T ₂	H	H	OH	3	11	19	33
L-T ₃ Br	I	Br	OH	4	12	20	34
L-T ₂ Br	H	Br	OH	5	13	21	35
L-T ₂ Br ₂	Br	Br	OH	6	14	22	36
L-T ₄ NMe ₂	I	I	N(CH ₃) ₂	7	15	23	37
L-T ₃ NMe ₂	H	I	N(CH ₃) ₂	8	16	24	38

T₄ Fab fragment binding to a high-density L-T₄ biosensor surface under mass transport limiting conditions are shown in Figure 2. Both the initial binding rates and the total response units obtained during the binding phase of the sensorgrams increase proportionately with the increase in binding protein concentration within the range examined (0–30 nM). The initial binding rate for each anti-T₄ Fab fragment concentration was obtained

by averaging the binding rate observed over a 15 s window beginning 15 s postinjection. Data from the first 15 s of each sensorgram were omitted due to sample dispersion effects at the start of injections. A plot of initial binding rate versus concentration of anti-T₄ Fab fragment yields a standard curve which can be used to determine the concentration of free Fab fragment in equilibrium with various T₄ analogs from which K_D

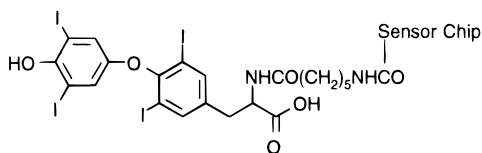


Figure 1. Biosensor utilized for solution affinity analysis of T_4 analogs for anti- T_4 Fab fragment.

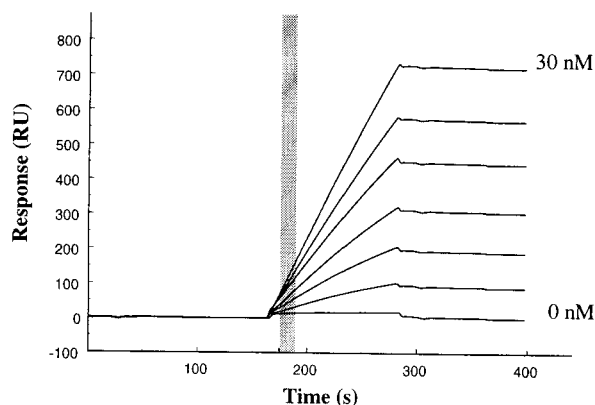


Figure 2. Overlay plot of sensorgrams generated with 0, 5, 10, 15, 20, 25, and 30 nM anti- T_4 Fab fragment binding to the immobilized T_4 biosensor surface. Initial binding rates were obtained from the shaded portion of each sensorgram.

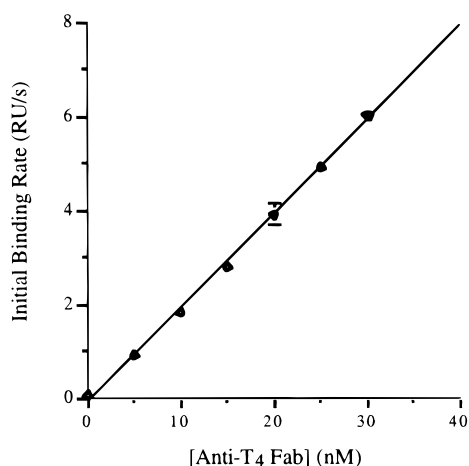


Figure 3. Average standard curve obtained for binding of anti- T_4 Fab fragment to the immobilized T_4 biosensor surface.

values in solution can be determined. Thirteen identical standard curves were generated during the course of these studies. The initial binding rates measured for each concentration of anti- T_4 Fab fragment were averaged and plotted against the Fab fragment concentration, providing an average standard curve depicted in Figure 3. The standard curve is linear ($r^2 = 0.998$) over the Fab fragment concentration range of 0–30 nM with measured binding rates from 0 to 6 RU s^{-1} . The slope and intercept for the average standard curve are $2.0 \times 10^8 \text{ RU M}^{-1} \text{ s}^{-1}$ and -0.099 RU s^{-1} , respectively.

Determination of Solution Affinities of Soluble T_4 Analogs for Anti- T_4 Fab Fragment. Ten concentrations of each T_4 analog tested were mixed with a fixed concentration of anti- T_4 Fab fragment (20 nM) in HBS buffer. After the solutions had reached equilibrium, the samples were individually injected over the calibrated high-density T_4 biosensor surface to generate a series of sensorgrams. A typical result is illustrated in Figure 4A for the solution affinity analysis of L- T_3 Br fluorescein tracer (**20**) with anti- T_4 Fab fragment. As the concentration of T_4 analog increases, the amount of free anti- T_4

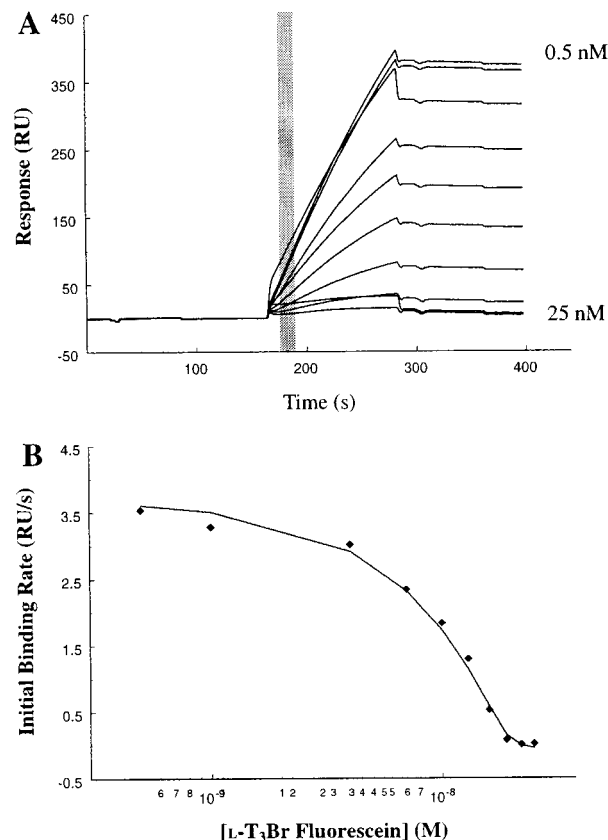


Figure 4. Analysis of anti- T_4 Fab fragment binding to L- T_3 Br fluorescein tracer in solution. (A) Overlay plot of sensorgrams generated from solutions of 0.5, 1, 4, 7, 10, 13, 16, 19, 22, and 25 nM L- T_3 Br fluorescein tracer with 20 nM anti- T_4 Fab fragment. Initial binding rates were obtained from the shaded portion of each sensorgram. (B) Nonlinear regression plot of initial binding rate versus concentration of added L- T_3 Br fluorescein tracer.

Fab fragment in solution available for binding to the biosensor surface decreases. Since the concentration of free binding protein is proportional to the binding rate of anti- T_4 Fab fragment to the biosensor surface, the initial binding rate also decreases. A plot of initial binding rate, determined as above, versus total concentration of added L- T_3 Br fluorescein tracer (**20**) provides the competition curve depicted in Figure 4B. The data points represent the experimentally determined initial rates of binding of free Fab fragment to the biosensor surface at a given concentration of soluble T_4 analog in solution. The curve represents the best nonlinear fit of the data using eq 1 (solution affinity model in BIAevaluation 2.1 software)

$$r_0 = r_m - \frac{k \left(x + \frac{r_m - I}{k} + K_D \right)}{k \sqrt{\frac{1}{4} \left(x + \frac{r_m - I}{k} + K_D \right)^2 - x \left(\frac{r_m - I}{k} \right)}} \quad (1)$$

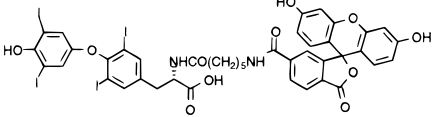
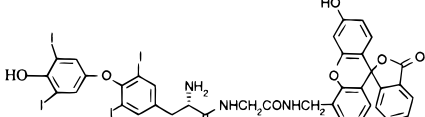
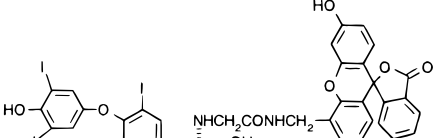
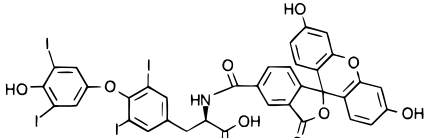
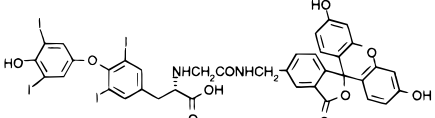
where r_0 is the initial binding rate, r_m is the maximum initial binding rate where $[T_4 \text{ analog}] = 0$, x is the total concentration of T_4 analog or tracer, and k and I are the slope and intercept, respectively, determined from the standard curve above. K_D is the equilibrium dissociation constant for the binding of the T_4 analog to anti- T_4 Fab fragment in solution. In the example depicted, values for K_D of $122 \pm 50 \text{ pM}$ and an r_m of 3.76 RU s^{-1} were obtained. Using the standard curve slope and intercept

Table 1. Effect of T₄ Analog Structure on the Binding Properties of T₄ Analogs and Tracers with an Anti-T₄ Fab Fragment

	X ₁	X ₂	R ₁	equilibrium dissociation constant (K _D)				
				parent	parent with linker	fluorescein tracer	acridinium tracer	solid phase ^a
L-T ₄	I ^b	I	OH	≤100 pM ^c	≤100 pM	≤100 pM	≤100 pM	75 ± 10.2 pM
D-T ₄	I ^d	I	OH	≤100 pM	≤100 pM	924 ± 240 pM	≤100 pM	225 ± 42 pM
L-T ₃	H ^b	I	OH	194 ± 12 nM	44 ± 6.2 nM	28 ± 4.0 nM	18 ± 3.0 nM	0.82 ± 0.19 nM
D-T ₃	H ^d	I	OH	282 ± 35 nM	31 ± 3.5 nM	18 ± 3.1 nM	19 ± 3.4 nM	1.3 ± 0.17 nM
L-T ₂	H	H	OH	16 ± 3.7 μM	12 ± 1.5 μM	6 ± 0.83 μM	22 ± 3.7 μM	nd ^e
L-T ₃ Br	I	Br	OH	417 ± 96 pM	≤100 pM	122 ± 50 pM	≤100 pM	225 ± 17 pM
L-T ₂ Br	H	Br	OH	3.6 ± 0.3 μM	585 ± 57 nM	298 ± 45 nM	214 ± 41 nM	0.89 ± 0.3 nM
L-T ₂ Br ₂	Br	Br	OH	831 ± 150 pM	283 ± 55 pM	336 ± 60 pM	329 ± 50 pM	367 ± 49 pM
L-T ₄ NMe ₂	I	I	N(CH ₃) ₂	≤100 pM	≤100 pM	145 ± 87 pM	241 ± 117 pM	60 ± 19 pM
L-T ₃ NMe ₂	H	I	N(CH ₃) ₂	41 ± 12 nM	20 ± 3.2 nM	55 ± 12 nM	142 ± 12 nM	7.0 ± 1.2 nM

^a These data were obtained from an earlier study (2) in which the Fab interacted with each parent/linker structure conjugated to a separate sensor surface. Data were reported as equilibrium association constants (K_A) in the original reference. ^b L-Enantiomer. ^c K_D was too small to be reliably measured by solution affinity experiments. ^d D-Enantiomer. ^e Not determined.

Table 2. Effect of the Linker and Fluorescein Regioisomer on the Binding Properties of T₄ Fluorescein Tracers with Anti-T₄ Fab Fragment

Tracer	K _D	Tracer	K _D
 25	227 ± 47 pM	 28	2.5 ± 0.72 nM
 26	166 ± 60 pM	 29	≤ 100 pM
 27	≤ 100 pM		

determined above and the equation for a straight line ($y = mx + b$), an r_m value of 3.76 RU s⁻¹ corresponds to 19.3 nM free anti-T₄ Fab fragment without any L-T₃Br fluorescein tracer added which is very close to the predicted value of 20 nM. Solution affinity studies with all T₄ analogs were conducted in a similar manner.

Equilibrium dissociation constants obtained for several T₄ analogs and tracers with the anti-T₄ Fab fragment in solution are summarized in Table 1. For comparison, the table also contains K_D values obtained previously for the same T₄ analogs bound to the biosensor chip surface through aminoalkyl linkers (compounds 9–16) using the direct kinetic analysis protocol on the BIAcore instrument (2). In general, the K_D values obtained in solution were comparable to those determined by direct kinetic analysis. The largest differences are seen in the T₃ analog series (L- and D-T₃, L-T₂Br, and L-T₃NMe₂) with K_D values obtained in solution being higher. As seen previously, the T₄ analogs (L- and D-T₄, L-T₃Br, and L-T₄NMe₂ series) bind exceedingly tight with several K_D values being outside the range for which the BIAcore instrument can accurately provide values from solution competition studies (≤100 pM) (14). Conversely, the L-T₂ analogs

bind much weaker with K_D values in the micromolar range. There is no preference for the stereochemistry of the α-amino group. Significantly, incorporation of fluorescein or acridinium moieties into any of the T₄ analogs for generation of tracers had little effect on the observed K_D.

To determine the effect of the linker position and the fluorescein regioisomer utilized for a tracer on the K_D values, five additional fluorescein tracers were studied. The results are summarized in Table 2. 5-Carboxyfluorescein tracers (27 and 29) bind the anti-T₄ Fab fragment the tightest with K_D values being outside the range for which the BIAcore instrument can accurately provide values from solution affinity studies (≤100 pM). For the tracers linked through the 5-position of fluorescein studied here, there is no apparent preference for the stereochemistry of the α-amino group or the length of the linker arm. Similar K_D values were obtained for analogs containing aminoalkyl linkers attached to fluorescein through the 4'- and 6-positions (25 and 26). However, the tracer bearing a 4'-N-glycylaminomethylfluorescein moiety linked through the carboxyl group of L-T₄ (28)

showed a considerable decrease in affinity for the anti-T₄ Fab fragment.

DISCUSSION

Surface plasmon resonance technology allows for the direct real-time observation of molecular interactions between an analyte in solution and a binding partner immobilized on a biosensor surface. The SPR phenomenon measures small changes in the refractive index at the biosensor surface which is directly proportional to the mass of the bound analyte (8, 15). The larger the mass of the analyte used, the larger the instrumental response. Thus, the majority of studies which have utilized BIAcore SPR technology have involved binding interactions between two macromolecules. In such cases, the choice of which macromolecule to conjugate to the biosensor surface is largely arbitrary since binding of either macromolecule to the immobilized partner will result in an easily measurable instrument response. However, the observed kinetics of the interaction may be different for each approach because the process of immobilization of a macromolecule is generally nonspecific and can effect the conformation and reactivity of the species involved (16).

Relatively few studies have been reported in which one of the binding partners is a low-molecular weight ligand or analyte. In this case, the design of the experiment is critical to the reliability of the information obtained. Conjugation of the macromolecule member of the binding pair to the biosensor surface is again nonspecific, and a subsequent binding interaction with the low-molecular weight analyte would generate a minimal instrument response. The complementary experiment in which the low-molecular weight species is conjugated to the biosensor surface is more advantageous with regard to generation of an instrument response upon binding of the macromolecular analyte. However, there are limitations in the conjugation of low-molecular weight ligands to a biosensor surface. Small molecules generally have fewer functional groups available for coupling to a biosensor surface and in many cases require chemical modification for incorporation of a linker functionality (2, 14, 17). In fact, the acridinium and fluorescein tracers described here contain no functionalities which would allow them to be coupled to a biosensor surface. Low-molecular weight ligand immobilizations also cannot be followed directly, since the instrument response obtained for small molecules is too low. A subsequent binding experiment with saturating amounts of the macromolecular analyte is required to obtain a large and easily quantitated instrument response. Moreover, the amount of a low-molecular weight ligand immobilized on the biosensor surface is difficult to control and often represents an excessively high binding capacity for the macromolecular analyte.

The need to study binding interactions involving low-molecular weight ligands or analytes using SPR technology is still of considerable importance since many ligand–receptor interactions of biological significance involve at least one low-molecular weight interactant. Several approaches to the study of binding interactions involving a low-molecular weight ligand or analyte have been described. One approach involves the direct, simultaneous analysis of a low-molecular weight analyte binding to multiple sensing surfaces with varying amounts of an immobilized ligand (multispot sensing) (18). The methodology was shown to be applicable to analytes as small as 180 Da and particularly useful for low-affinity interac-

tions. Drawbacks to this approach include a requirement for the most sensitive BIAcore instrument, BIAcore 2000, and the need to use several sensor surfaces for a single binding study. Furthermore, the kinetic information obtained from this method is more qualitative than quantitative. A more quantitative analysis of binding interactions involving a low-molecular weight analyte can be obtained from a competitive study in which the low-molecular weight analyte competes with a larger macromolecule for the same immobilized ligand (surface competition) (8). The method provides both the rate and affinity constants in a one-step procedure that monitors binding directly. In a qualitative manner, the method allows for a rapid affinity ranking of different low-molecular weight analytes interacting with the same immobilized ligand. However, the mathematics used to characterize the surface competition experiment are much more complex than that required for a direct binding experiment. The instrument response for a given low-molecular weight/high-molecular weight analyte combination binding to an immobilized ligand is dependent upon the association and dissociation rate constants of both the low- and high-molecular weight analytes and their respective molecular weights. The approach also requires the additional preparation and characterization of the high-molecular weight analyte to be used in the competition studies. An alternative and simpler competitive experiment involves an immobilized ligand and a soluble ligand free in solution competing for binding sites on a single analyte (solution competition) (8, 19, 20). For the solution competition experiment, if high-density immobilized ligand surfaces are employed and the association rate between the analyte and immobilized ligand is $\geq 1 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$, the observed binding will be limited by mass transfer. Under such conditions, initial binding rates are directly proportional to the amount of free analyte (not bound to soluble ligand) in solution and independent of the interaction kinetics or analyte–ligand affinity. Measuring concentrations of free analyte in equilibrium solutions with different concentrations of soluble ligand allows one to determine equilibrium dissociation constants. This was the method utilized in these studies. The advantage to the solution competition experiment is that a single biosensor surface can be utilized for determination of binding affinities for a series of related analogs, making direct comparison of results more reliable. Unfortunately, the method is indirect and does not provide individual association and dissociation rate constants.

Solution competition experiments require the generation of a standard curve from which the concentration of free analyte in equilibrium solutions with a soluble ligand can be determined. Several high-density L-T₄ surfaces, ranging from 350 to 450 RU of immobilized L-9, were prepared during the course of these studies, and a standard curve was generated at the start of each experiment. As depicted in the average standard curve (Figure 3), the measured initial binding rate for a given concentration of anti-T₄ Fab fragment binding to the different immobilized L-T₄ surface densities was quite reproducible, indicating a mass transport limiting situation. The high reproducibility of initial binding rates also supports the accuracy of the slope and intercept obtained from the average standard curve which are critical to evaluation of solution competition data by the solution affinity model (cf. eq 1), particularly for high-affinity interactions. For low-affinity interactions (micromolar concentrations), K_D values obtained are largely independent of the slope and intercept values from the

standard curve (8). The standard curves and solution competition studies described here were all carried out with the Fab fragment of an anti-T₄ mAb. Anti-T₄ mAb contains two identical binding sites and under the conditions described for solution competition experiments can bind zero, one, or two T₄ antigens (immobilized and/or soluble). Under such conditions, the observed binding rate is no longer proportional to free antibody binding sites in solution. Equations accounting for mAb bivalency have been derived, but they do not necessarily correct the problem (8, 14). The Fab fragment contains a single binding site and eliminates the problems associated with mAb bivalency.

In general, structural requirements important for the anti-T₄ Fab fragment-T₄ analog binding interaction in solution were the same as those identified from the direct analysis of Fab fragment interaction with individual T₄ analog biosensors (2). Specifically, the diphenyl ether ring system, including the degree and type of halogenation, was the major determinant in binding affinity. The binding interaction became less favorable with the loss of each halogen from the terminal aryl ring (K_D for a series, L,D-T₄ $\approx$ L-T₄NMe₂ < L-T₃Br < L-T₂Br₂ < L,D-T₃ $\approx$ L-T₃NMe₂ < L-T₂Br < L-T₂). Iodo-bromo exchanges in the terminal aryl ring system also resulted in slight decreases in binding affinity. These observations have been attributed to the acidity of the phenolic hydroxyl group and the effect the different halogenation patterns have on the pK_a of this moiety (2). As previously observed in the direct kinetic analysis, the binding interaction in solution is relatively insensitive to modifications to the amino acid portion of the T₄ analogs. Specifically, there is no preference for the stereochemistry of the α -amino chiral center (L-T₄ series $\approx$ D-T₄ series) or a requirement for an ionizable carboxyl group (L-T₄ series $\approx$ L-T₄NMe₂ series). Binding interactions were generally more favorable for the analogs bearing an aminoalkyl linker relative to those of the native analytes. This was not unexpected since the immunogen used to elicit the antibody was prepared by conjugating *N*-acetyl-L-T₄ to the carrier protein. With an immunogen thus prepared, the animal's immune system was presented a hapten in which both the α -amino and carboxylic acid groups had been converted into amides, unlike the native analyte. Incorporation of the acridinium or 5-carboxyfluorescein labels via an aminohexyl linker also had little effect on the observed binding affinity in solution (Table 1). However, the linker, linker position, and regioisomer of the label incorporated can effect the binding interaction as revealed by the particular combinations that were tested (Table 2).

In absolute terms, some significant differences were observed between K_D values determined by the solution competition method and those determined by direct kinetic analysis (solid phase) on the BIAcore instrument (Table 1), with the disparity being more pronounced for the weaker binding interactions. In these cases, the K_D value determined by the solution competition method was always higher than the K_D value determined by direct kinetic analysis. Previous literature reports applying BIAcore technology to the study of binding interactions have noted similar differences between solution competition studies and direct kinetic analysis studies (14, 21, 22). The disparity has been primarily attributed to the different experimental designs of the two methods. A direct kinetic analysis of binding interactions involves the determination of association and dissociation rate constants (k_a and k_d) for an analyte free in solution binding to individual immobilized ligands. Equilibrium

dissociation constants (K_D) can then be calculated from the measured rate constants. However, the density of the immobilized ligand can greatly effect the measured rate constants due to mass transport effects and rebinding of analyte to the immobilized ligand. Rebinding can become particularly problematic with rapid association rates which will favor reassociation of analyte to the immobilized ligand during the dissociation phase of the study. Rebinding of analyte to the immobilized ligand will artificially slow the observed dissociation rate constant. This phenomenon will become more pronounced as the true dissociation rate becomes faster and can result in an artificially low equilibrium dissociation constant ($K_D = k_d/k_a$) for weaker binding interactions. Association rates measured by direct kinetic analysis of the anti-T₄ Fab fragment-T₄ analog binding interaction were all rapid, ranging from 1 to $7 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ (2). Dissociation rates measured by the direct kinetic analysis method were much more varied, ranging from 6 to $920 \times 10^{-5} \text{ s}^{-1}$. It would not be surprising if some of the anti-T₄ Fab fragment-T₄ analog binding affinities previously determined by direct kinetic analysis were artificially low. Thus, due to the solution-surface reaction format, the values can only be regarded as apparent rate and equilibrium constants. In contrast, the K_D values determined here by the solution competition method involve equilibrium mixtures in solution which are independent of the solution-surface interactions described above for the direct kinetic analysis method. Equilibrium dissociation constants measured in solution can be regarded as true affinity constants.

CONCLUSION

Direct kinetic analysis of binding interactions using BIAcore SPR technology provides individual association and dissociation rate constants from which equilibrium constants can be calculated. However, the biosensor optimization process for minimizing mass transport effects and analyte rebinding during the dissociation phase of the sensorgram can be time-consuming and subjective. Furthermore, direct comparison of binding affinities determined for a series of related analogs with a single binding protein is difficult since a different biosensor must be generated for each analog studied. In contrast, the solution competition format utilized in these studies provides only equilibrium constants but allows for a reliable, direct comparison of binding affinities using a single biosensor surface.

The binding affinities of an anti-T₄ Fab fragment for a series of T₄ analogs and tracers were determined using the solution competition format and compared to results obtained by the direct kinetic analysis method on the BIAcore instrument. Qualitatively, results obtained from solution competition studies of the anti-T₄ Fab fragment-T₄ analog binding interaction followed the same trend as that observed in the direct kinetic analysis. Primary T₄ analog structural features important for the binding interaction include the diphenyl ether ring system and the ionizable phenolic hydroxyl moiety. Incorporation of fluorescein or acridinium labels to generate tracers had little effect on the observed binding affinities. Quantitatively, some significant differences were observed between results determined by the solution competition method and those determined by direct kinetic analysis. We attribute the differences to the different experimental designs (solution-surface versus solution phase) and the considerations affecting these interactions.

LITERATURE CITED

- (1) Henry, C. (1996) FDA, reform, and the well-characterized biologic. *Anal. Chem.* 68, 674A–677A.
- (2) Adamczyk, M., Gebler, J. C., Gunasekera, A. H., Mattingly, P. G., and Pan, Y. (1997) Immunoassay reagents for thyroid testing. 2. Binding properties and energetic parameters of a T₄ monoclonal antibody and its Fab fragment with a library of thyroxine analog biosensors using surface plasmon resonance. *Bioconjugate Chem.* 8, 133–145.
- (3) Adamczyk, M., Johnson, D. D., Mattingly, P. G., Clarisse, D. E., Tyner, J. D., and Perkowitz, M. M. (1994) Reagents and methods for the detection and quantitation of thyroxine in fluid samples. U.S. Patent 5,359,093; *Chem. Abstr.* 122, 124579.
- (4) Adamczyk, M., Fino, L., Fishpaugh, J. R., Johnson, D. D., and Mattingly, P. G. (1994) Immunoassay reagents for thyroid testing. 1. Synthesis of thyroxine conjugates. *Bioconjugate Chem.* 5, 459–462.
- (5) Adamczyk, M., Fishpaugh, J. R., Harrington, C., Johnson, D. D., and Vanderbilt, A. (1993) Immunoassay reagents for psychoactive drugs. II. The method for the development of antibodies specific to imipramine and desipramine. *J. Immunol. Methods* 163, 187–197.
- (6) Adamczyk, M., Fishpaugh, J. R., Harrington, C., Hartter, D., Johnson, D. D., and Vanderbilt, A. (1993) Immunoassay reagents for psychoactive drugs. I. The method for the development of antibodies specific to amitriptyline and nortriptyline. *J. Immunol. Methods* 162, 47–58.
- (7) Van Regenmortel, M. H. V., and Azimzadeh, A. (1994) Determination of antibody affinity. In *Immunochemistry* (C. J. Van Oss and M. H. V. Van Regenmortel, Eds.) pp 805–824, Marcel Dekker, Inc., New York.
- (8) Karlsson, R. (1994) Real-time competitive kinetic analysis of interactions between low-molecular-weight ligands in solution and surface-immobilized receptors. *Anal. Biochem.* 221, 142–151.
- (9) Karlsson, R., Fägerstam, L., Nilshans, H., and Persson, B. (1993) Analysis of active antibody concentration. Separation of affinity and concentration parameters. *J. Immunol. Methods* 166, 75–84.
- (10) Mattingly, P. G. (1991) Chemiluminescent 10-methyl-acridinium-9-(N-sulfonylcarboxamide) salts. Synthesis and kinetics of light emission. *J. Biolumin. Chemilumin.* 6, 107–114.
- (11) Mattingly, P. G., and Bennett, L. (1995) Chemiluminescent acridinium salts. U.S. Patent 5,468,646.
- (12) Mattingly, P. G. (1992) Preparation of 5- and 6-(amino-methyl)fluorescein. *Bioconjugate Chem.* 3, 430–431.
- (13) *BIAevaluation Software Handbook* (1995) pp A1–A16, BIAcore, Inc., Piscataway, NJ.
- (14) Stenberg, E., Persson, B., Roos, H., and Urbaniczky, C. (1991) Quantitative determination of surface concentration of protein with surface plasmon resonance by using radiolabeled proteins. *J. Colloid Interface Sci.* 143, 513–526.
- (15) Karlsson, R., Mo, J. A., and Holmdahl, R. (1995) Binding of autoreactive mouse anti-type II collagen antibodies derived from the primary and the secondary immune response investigated with the biosensor technique. *J. Immunol. Methods* 188, 63–71.
- (16) Nieba, L., Krebber, A., and Plückthun, A. (1996) Competition BIAcore for measuring true affinities: large differences from values determined from binding kinetics. *Anal. Biochem.* 234, 155–165.
- (17) Minunni, M., and Mascini, M. (1993) Detection of pesticide in drinking water using real-time biospecific interaction analysis (BIA). *Anal. Lett.* 26, 1441–1460.
- (18) Karlsson, R., and Stahlberg, R. (1995) Surface plasmon resonance detection and multispot sensing for direct monitoring of interactions involving low-molecular-weight analytes and for determination of low affinities. *Anal. Biochem.* 228, 274–280.
- (19) Sternesjö, A., Mellgren, C., and Björck, L. (1995) Determination of sulfamethazine residues in milk by a surface plasmon resonance-based biosensor assay. *Anal. Biochem.* 226, 175–181.
- (20) Zeder-Lutz, G., Van Regenmortel, M. H. V., Wenger, R., and Altschuh, D. (1994) Interaction of cyclosporin A and two cyclosporin analogs with cyclophilin: relationship between structure and binding. *J. Chromatog. B* 662, 301–306.
- (21) Morelock, M. M., Ingraham, R. H., Betageri, R., and Jakes, S. (1995) Determination of receptor–ligand kinetic and equilibrium binding constants using surface plasmon resonance: application to the *lck* SH2 domain and phosphotyrosyl peptides. *J. Med. Chem.* 38, 1309–1318.
- (22) Payne, G., Shoelson, S. E., Gish, G. D., Pawson, T., and Walsh, C. T. (1993) Kinetics of p56^{lck} and p60^{src} Src homology 2 domain binding to tyrosine-phosphorylated peptides determined by a competition assay or surface plasmon resonance. *Proc. Natl. Acad. Sci. U.S.A.* 90, 4902–4906.

BC9701353

Nucleosides and Nucleotides. 170. Synthesis and Properties of Oligodeoxynucleotides Containing 5-[N-[2-[N,N-Bis(2-aminoethyl)-amino]ethyl]carbamoyl]-2'-deoxyuridine and 5-[N-[3-[N,N-Bis(3-aminopropyl)amino]propyl]carbamoyl]-2'-deoxyuridine[†]

Yoshihito Ueno, Mai Mikawa, and Akira Matsuda*

Faculty of Pharmaceutical Sciences, Hokkaido University, Kita-12, Nishi-6, Kita-ku, Sapporo 060, Japan.
Received August 11, 1997; Revised Manuscript Received October 7, 1997[®]

The synthesis of oligodeoxynucleotides (ODNs) containing 5-[N-[2-[N,N-bis(2-aminoethyl)amino]ethyl]carbamoyl]-2'-deoxyuridine (**BAE**) and 5-[N-[3-[N,N-bis(3-aminopropyl)amino]propyl]carbamoyl]-2'-deoxyuridine (**BAP**) is described. The thermal stabilities of duplexes containing these ODNs and either the complementary DNA or RNA strand and of triplexes consisting of these ODNs and the target duplex were studied by thermal denaturation. ODNs containing **BAE** or **BAP** stabilize duplex formation with either the complementary DNA or RNA strands but destabilize triplex formation with the target duplex. Furthermore, the resistance of these ODNs to nuclease hydrolysis was studied by using snake venom phosphodiesterase (a 3'-exonuclease) and nuclease S1 (an endonuclease). It was found that ODNs containing either **BAE** or **BAP** were more resistant to nucleolytic hydrolysis by either of the nucleases than the unmodified ODN.

INTRODUCTION

Oligodeoxynucleotide (ODN)¹ analogues with a variety of functional groups (i.e. intercalating, alkylating, DNA-degrading, and fluorescent groups) have been synthesized and used for biological and biochemical studies, including antisense and antigen studies (1–6). To be able to apply these ODN analogues to antisense and antigen studies, the ODNs must form stable Watson–Crick hybrids with the complementary target RNAs and form stable triple-stranded DNA with target DNA duplexes (1–4).

It is well-known that naturally occurring polyamines, such as spermidine and spermine, bind strongly to DNA (7–9) and stabilize duplex (10, 11) and triplex formations (12–14), although the precise mode of binding is not clear. Their enhanced thermal stability is explained by the reduction of the anionic electrostatic repulsion between the phosphate moieties by the cationic polyamines. Therefore, ODN analogues carrying various polyamines have been synthesized to improve the thermal stability of duplexes and triplexes (15–24).

We recently developed a new and convenient postsynthetic modification method for the synthesis of ODNs containing various amino linkers using either 5-(methoxycarbonyl)-2'-deoxyuridine (**1**) or 5-(trifluoroethoxycarbonyl)-2'-deoxyuridine (**2**) as a convertible nucleoside

(25–30). If ODNs containing **1** or **2** at specific positions are treated with one of several diaminoalkanes, the desired ODNs containing 5-(N-aminoalkyl)carbamoyl-2'-deoxyuridines are readily obtained. Using this method, we found that ODNs containing 5-(N-aminoethyl)carbamoyl-2'-deoxyuridines (**Hs**) efficiently stabilize duplex formation with either the complementary DNA or RNA strands (25, 26, 30). The terminal amino groups of the amino linkers can be further modified by the addition of a variety of functional groups (27). By converting the amino linker to a mercapto linker, we successfully synthesized 5'-5'-linked ODNs with the potential for triplex formation (28) and succeeded in the chemical ligation of ODNs via a disulfide bond in the presence of a template (29).

The enhanced thermal stability of duplexes containing **Hs** has been explained in the following manner. Reduction of the electrostatic repulsion between the phosphate moieties by the ammonium ion of the aminoethylcarbamoyl linker and the enhanced hydrogen-donor ability at N3-H combine to give duplex stabilization, due to incorporation of an electron-withdrawing carbamoyl group at the 5-position of the uracil (26). Tris(2-aminoethyl)amine and tris(3-aminopropyl)amine have a greater positive net charge than diaminoalkanes such as diaminoethane and 1,6-diaminohexane. Therefore, we envisioned that ODNs containing either tris(2-aminoethyl)amine or tris(3-aminopropyl)amine would stabilize duplex formation with the complementary DNA or RNA strand more efficiently than the ODNs carrying the diaminoalkanes.

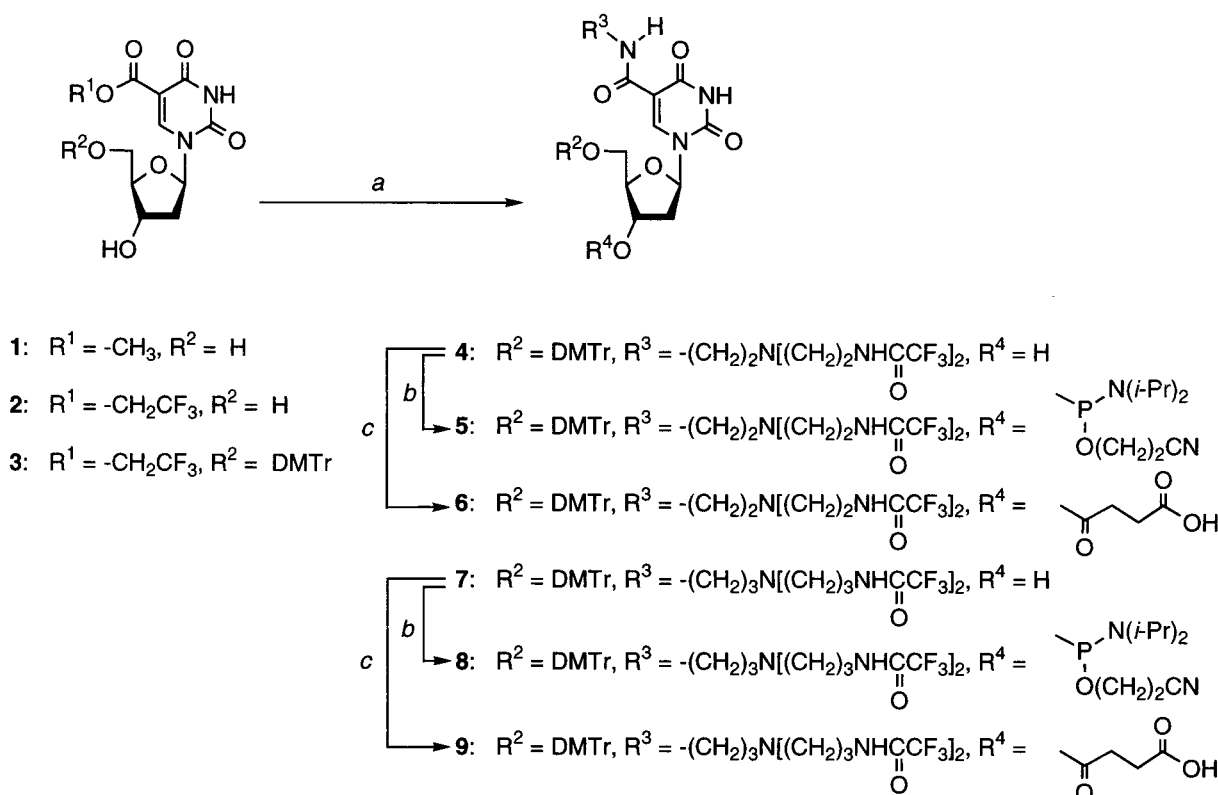
In this paper, we report the synthesis of ODNs containing 5-[N-[2-[N,N-bis(2-aminoethyl)amino]ethyl]carbamoyl]-2'-deoxyuridine (**BAE**) and 5-[N-[3-[N,N-bis(3-aminopropyl)amino]propyl]carbamoyl]-2'-deoxyuridine (**BAP**). The thermal stability of duplexes containing these ODNs and either the complementary DNA or RNA strand, the thermal stability of triplexes consisting of these ODNs and the target duplex, and the resistance of

[†] For part 169 in this series, see the following: Shuto, S., Niizuma, S., and Matsuda, A. (1997) *J. Org. Chem.* (submitted for publication).

* To whom reprint requests should be addressed. Phone: +81-11-706-3228. Fax: +81-11-706-4980. E-mail: matuda@pharm.hokudai.ac.jp.

[®] Abstract published in *Advance ACS Abstracts*, November 15, 1997.

¹ Abbreviations: ODN, oligodeoxynucleotide; CPG, controlled pore glass; HPLC, high-performance liquid chromatography; *T*_m, melting temperature; WSCI, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride; DMAP, 4-(dimethylamino)pyridine; TEAA, triethylammonium acetate.

Scheme 1^a

^a (a) (1) $(NH_2CH_2CH_2)_3N$ or $(NH_2CH_2CH_2CH_2)_3N$, pyridine, room temperature; (2) CF_3COOEt , Et_3N , pyridine, room temperature; (b) 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite, *N,N*-diisopropylethylamine, CH_2Cl_2 , room temperature; and (c) succinic anhydride, DMAP, pyridine, room temperature.

the ODNs to nucleolytic hydrolysis by snake venom phosphodiesterase (a 3'-exonuclease) and nuclease S1 (an endonuclease) were also studied.

RESULTS AND DISCUSSION

Synthesis. The synthesis of ODNs containing **BAE** or **BAP** was accomplished in a DNA synthesizer by using a suitably protected 5-[*N*-[2-[*N,N*-bis(2-aminoethyl)amino]ethyl]carbamoyl]-2'-deoxyuridine (**5**) or 5-[*N*-[3-[*N,N*-bis(3-aminopropyl)amino]propyl]carbamoyl]-2'-deoxyuridine phosphoramidite (**8**). The synthesis of the 3'-phosphoramidite derivatives **5** and **8** is shown in Scheme 1. 5'-*O*-(Dimethoxytrityl)-5-(trifluoroethoxycarbonyl)-2'-deoxyuridine (**3**), prepared by a previously described method (27), reacted readily with either tris(2-aminoethyl)amine or tris(3-aminopropyl)amine. Without purifying the product, we protected the amino group with a trifluoroacetyl group to give **4** or **7**. The modified nucleoside (**4** or **7**) was converted to the protected nucleoside phosphoramidite, **5** or **8**, by the usual method of ODN synthesis (31). To incorporate **BAE** or **BAP** into the 3'-end of the ODNs, **4** or **7** was further modified to produce the corresponding 3'-succinate, **6** or **9**, which was then reacted with controlled pore glass (CPG) to give a solid support containing **4** (35.0 $\mu\text{mol/g}$) or **7** (34.7 $\mu\text{mol/g}$).

An unmodified DNA heptadecamer (control) and heptadecamers containing **BAEs** or **BAPs** were synthesized in a DNA synthesizer (31). The average coupling yields of **5** and **8** were 96 and 95%, respectively, using a 0.12 M solution of the amidite derivatives in CH_3CN and 360 s for the coupling time. The fully protected ODNs (each 1 μmol scale) linked to the solid supports were treated with concentrated NH_4OH at 55 $^\circ\text{C}$ for 16 h. This was followed by C-18 column chromatography. Detritylation gave

ODNs **11–20**. Each ODN in this preparation showed a single peak by reversed phase HPLC analysis. ODNs **13** and **17** were analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS), and the observed molecular weight supported its structure.

UV Melting Studies of Duplex and Triplex Formation. Duplex formations of the heptadecamers **10–20** with the complementary DNA, $d[TGG(AG)_9GT]-3'$ (**21**), was studied by thermal denaturation in a buffer of 0.01 M sodium cacodylate (pH 7) containing 0.01 M NaCl. One transition was observed in a melting profile of each duplex. Melting temperatures (T_m s) are listed in Table 1. All of the T_m values for the modified ODNs containing **BAEs** or **BAPs** were greater than that of the corresponding unmodified ODN **10**. Therefore, the modified nucleosides, **BAE** and **BAP**, were found to thermally stabilize duplex formation. The stability of the duplexes was dependent on the position and number of the modified nucleosides. Duplexes with ODN **17** or **18** containing one molecule of **BAP** at either the center or 5'-end ($T_m = 56.1$ and 56.2 $^\circ\text{C}$, respectively) were more stable than the duplex with ODN **16** containing **BAP** at its 3'-end ($T_m = 54.3$ $^\circ\text{C}$). On the other hand, the T_m values for the duplexes containing ODNs **11–13** which had one molecule of **BAE** at either the 3'-end, center, or 5'-end were almost same. The duplexes became more stable as the number of **BAEs** and **BAPs** increased. ODNs containing **BAPs** stabilized duplex formation more efficiently than ODNs containing **BAEs**. A 9.8 $^\circ\text{C}$ increase in T_m was observed for ODN **20**.

In a previous paper, we reported the thermal stability of duplexes constructed from ODNs containing **Es** or 5-(*N*-aminoethyl)carbamoyl-2'-deoxyuridines (**Es**) using

Table 1. Thermal Denaturation^a

ODNs	ODN–DNA ^b				ODN–RNA ^c		triplex ^d	
	0.01 M NaCl		0.1 M NaCl		0.1 M NaCl		0.5 M NaCl	
	<i>T_m</i> (°C)	ΔT_m (°C)	<i>T_m</i> (°C)	ΔT_m (°C)	<i>T_m</i> (°C)	ΔT_m (°C)	<i>T_m</i> (°C)	ΔT_m (°C)
10	53.4	—	62.7	—	70.7	—	40.8	—
11	54.9	1.5	nd ^e	nd	72.4	1.7	39.8	–1.0
12	54.5	1.1	64.1	1.4	72.2	1.5	35.5	–5.3
13	54.7	1.3	nd	nd	72.9	2.2	40.0	–0.8
14	56.1	2.7	66.2	3.5	72.2	1.5	27.2	–13.6
15	59.7	6.3	68.8	6.1	74.1	3.4	35.5	–5.3
16	54.3	0.9	nd	nd	71.9	1.2	39.7	–1.1
17	56.1	2.7	65.8	3.1	73.8	3.1	36.3	–4.5
18	56.2	2.8	nd	nd	73.8	3.1	40.6	–0.2
19	61.2	7.8	68.1	5.4	76.0	5.3	31.0	–9.8
20	63.2	9.8	70.7	8.0	77.7	7.0	35.3	–5.5

^a Experimental conditions are described in Experimental Procedures. ^b The complementary DNA 5'-d[TGG(AG)₉GT]-3' (**21**). ^c The complementary RNA 5'-r[UGG(AG)₉GU]-3' (**22**). ^d The target sequence 5'-d[AG(TC)₉C(T)₅AG(GA)₉CT]-3' (**23**). ^e nd, not determined.

the same sequences and conditions described here (25, 26). The length of the amino linkers was found to affect the thermal stability of the duplexes. The ODNs containing **H** stabilized duplex formation with either the complementary DNA or RNA strands more efficiently than ODNs containing **Es** (25). If ODNs containing **Hs** and **Es** are considered, the order of ΔT_m values for ODN–DNA duplexes containing three molecules of the modified nucleosides in a buffer containing 0.01 M NaCl is as follows: ODN **20** containing **BAEs** ($\Delta T_m = 9.8$ °C) > ODN containing **Hs** ($\Delta T_m = 8.0$ °C) > ODN **15** containing **BAEs** ($\Delta T_m = 6.3$ °C) > ODN containing **Es** ($\Delta T_m = 6.0$ °C).

The enhanced thermal stability of duplexes containing **Hs** and **Es** is thought to be due to both a reduction of the electrostatic repulsion between phosphate moieties by the ammonium ion of the aminoalkylcarbamoyl linker and the enhanced hydrogen-donor ability at N3-H caused by incorporation of an electron-withdrawing carbamoyl group at the 5-position of the uracil (26). Therefore, to confirm the importance of the terminal ammonium ion of the *N,N*-bis(2-aminoethyl)aminoethyl and *N,N*-bis(3-aminopropyl)aminopropyl linkers in stabilizing duplexes, T_m s were measured under conditions with a higher ionic strength (0.1 M NaCl) and the ΔT_m values were compared with those measured under conditions with a low ionic strength (Table 1). The ΔT_m values with the high ionic strength for the duplexes containing **BAEs** were similar to that with the low ionic strength, whereas the ΔT_m values with the high ionic strength for the duplexes containing **BAP** became smaller than that with the low ionic strength as the number of **BAP** increased. These results indicate that the terminal ammonium ion of the *N,N*-bis(3-aminopropyl)aminopropyl linker more effectively neutralized the phosphate anions than that of the *N,N*-bis(2-aminoethyl)aminoethyl linker. The *N,N*-bis(3-aminopropyl)aminopropyl linker is longer than the *N,N*-bis(2-aminoethyl)aminoethyl linker and has a more positive net charge than either the aminoethyl or aminoethyl linkers. Therefore, ODNs containing **BAPs** should stabilize duplex formation with the complementary DNA more efficiently than ODNs containing **Hs**, **Es**, or **BAEs**.

Stable duplex formation with mRNA is one of the most important factors in antisense research. Therefore, duplex formation by heptadecamers with the complementary RNA strand, 5'-[UGG(AG)₉GU]-3' (**22**), was next studied by thermal denaturation in a buffer containing 0.1 M NaCl. As shown in Table 1, the trend shown by the T_m s of the modified ODNs containing **BAEs** or **BAPs** was similar to that of the corresponding ODN–DNA duplexes. However, the ΔT_m values were evidently different. The ΔT_m values for ODNs **11–13** and **16–18**,

containing one molecule of **BAE** or **BAP**, were slightly greater than those of the corresponding ODN–DNA duplexes. On the other hand, those for ODNs **14**, **15**, **19**, and **20**, containing either two or three molecules of **BAE** or **BAP**, were smaller than those of the ODN–DNA duplexes. The duplexes were more stable as the number of **BAE** and **BAP** molecules increased. A 7.0 °C increase in T_m was observed for ODN **20**. If ODNs containing **Hs** and **Es** are considered, the order of ΔT_m values for ODN–RNA duplexes containing three molecules of the modified nucleosides in a buffer containing 0.1 M NaCl is as follows: ODN **20** containing **BAEs** ($\Delta T_m = 7.0$ °C) > ODN **15** containing **BAEs** ($\Delta T_m = 3.4$ °C) > ODN containing **Hs** ($\Delta T_m = 3.0$ °C) > ODN containing **Es** ($\Delta T_m = 2.0$ °C).

When ODN analogues containing **BAEs** and **BAPs** form duplexes with the complementary DNA or RNA strand, the amino linkers should be accommodated in the major groove, since the 5-position of 2'-deoxyuridine is in the major groove. The major groove of a DNA–RNA duplex is narrower than that of a DNA–DNA duplex (32). So bulky functional groups may not fit into the narrower major groove of DNA–RNA duplexes as well as they do in DNA–DNA duplexes. The difference in ΔT_m values between DNA–RNA and DNA–DNA duplexes may reflect this conformational difference.

Triplex formation of heptadecamers with the target duplex, d[AG(TC)₉C(T)₅AG(GA)₉CT]-3' (**23**), was also studied by thermal denaturation in a buffer containing 0.5 M NaCl (pH 7.0). Two transitions were observed in the melting profile of each triplex; the transition with the higher T_m was due to melting of the target duplex **23** (88 °C), and the transition with the lower T_m corresponded to dissociation of the third strand from the triplex. The T_m s are also listed in Table 1.

While ODNs containing one molecule of **BAE** at either the 5'- or 3'-end have slightly destabilized triplex formation ($T_m = 39.8$ and 40.0 °C, respectively), the ODN with one molecule of **BAE** in its center showed markedly destabilized triplex formation ($T_m = 35.5$ °C). Furthermore, a 13.6 °C decrease in T_m was observed in ODN **14**, which contains two molecules of **BAE** in the middle of it. ODN **15**, containing three molecules of **BAE**, one in the middle and one at either end, had a T_m similar to that of ODN **13**. Furthermore, similar results were observed in the case of ODNs containing **BAPs**.

When the polypyrimidine ODNs form triplexes with target duplexes, the ODNs are accommodated in the major groove of the target duplexes and Hoogsteen-type base triplets (T·T and C⁺·GC) are formed (4). When ODN analogues containing **BAEs** and **BAPs** form triplexes with the target duplexes, the amino linkers should lie in the

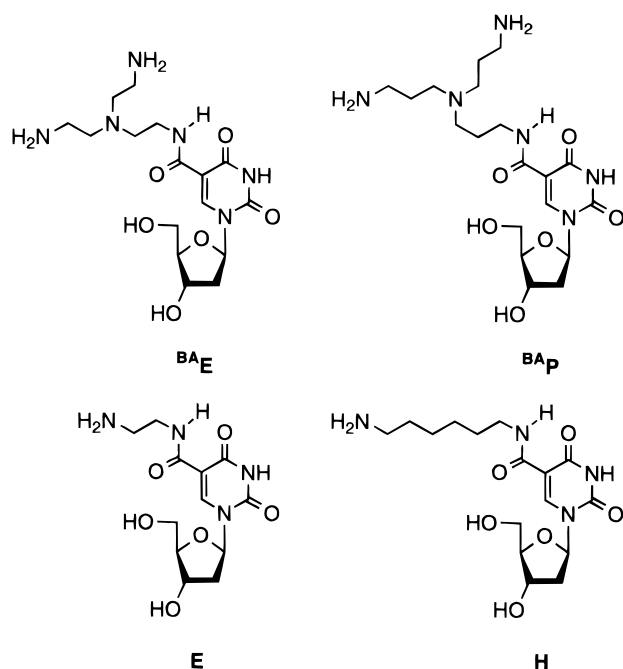


Figure 1. Structures of the 2'-deoxyuridine analogues.

10	5'-TMTMTMTMTMTMTMTMT-3'
11	5'-TMTMTMTMTMTMTMTMT ^{BA_E} -3'
12	5'-TMTMTMTMT ^{BA_E} EMTMTMTMT-3'
13	5'- ^{BA_E} EMTMTMTMTMTMTMTMT-3'
14	5'-TMTMT ^{BA_E} EMTMTMTMT ^{BA_E} EMTMT-3'
15	5'- ^{BA_E} EMTMTMTMT ^{BA_E} EMTMTMTMT ^{BA_E} -3'
16	5'-TMTMTMTMTMTMTMTMT ^{BA_P} -3'
17	5'-TMTMTMTMT ^{BA_P} PMTMTMTMT-3'
18	5'- ^{BA_P} PMTMTMTMTMTMTMTMT-3'
19	5'-TMTMT ^{BA_P} PMTMTMTMT ^{BA_P} PMTMT-3'
20	5'- ^{BA_P} PMTMTMTMT ^{BA_P} PMTMTMTMT ^{BA_P} -3'
target DNA 21	5'-TGGAGAGAGAGAGAGAGAGGT-3'
target RNA 22	5'-UGGAGAGAGAGAGAGAGAGGU-3'
target duplex 23	5'-AGTCTCTCTCTCTCTCTCCT ^T 3'-TCAGAGAGAGAGAGAGAGGA ^T

Figure 2. Sequences of ODNs synthesized. ^{BA_E} = 5-[N-[2-[N,N-bis(2-aminoethyl)amino]ethyl]carbamoyl]-2'-deoxyuridine. ^{BA_P} = 5-[N-[3-[N,N-bis(3-aminopropyl)amino]propyl]carbamoyl]-2'-deoxyuridine. M = 5-methyl-2'-deoxycytidine.

major groove of the target duplexes, since the 5-position of the 2'-deoxyuridine residue of the third strand is located in the major groove of the target duplex in the triplex. The vicinity of the 5-position of the 2'-deoxyuridine residue of the third strand in the triplex is sterically crowded. Therefore, bulky amino linkers, such as the *N,N*-bis(3-aminopropyl)aminopropyl linker and the *N,N*-bis(2-aminoethyl)aminoethyl linker, may not fit into the major groove of target duplexes, especially when they are located in the middle of the third strands.

Partial Digestion of ODNs with Snake Venom Phosphodiesterase and Nuclease S1. Since resistance to nucleolytic hydrolysis by nucleases is an important factor in antisense and antigene studies, the stability of ODNs containing ^{BA_E} or ^{BA_P} to nucleolytic digestion was examined next (1-4). Two nucleases, snake venom phosphodiesterase (a 3'-exonuclease) and nuclease S1 (an endonuclease), were used in this study. ODNs **11**, **14**, **16**, and **19** were labeled at the 5'-end with ³²P and

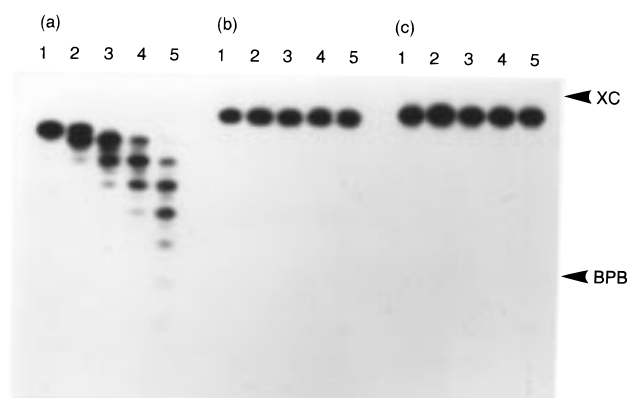


Figure 3. Polyacrylamide gel electrophoresis of 5'-³²P-labeled ODNs hydrolyzed by snake venom phosphodiesterase: (a) **10**, (b) **11**, and (c) **16**. ODNs were incubated with snake venom phosphodiesterase for 0 min (lane 1), 10 min (lane 2), 30 min (lane 3), 60 min (lane 4), and 120 min (lane 5). Experimental conditions are described in Experimental Procedures.

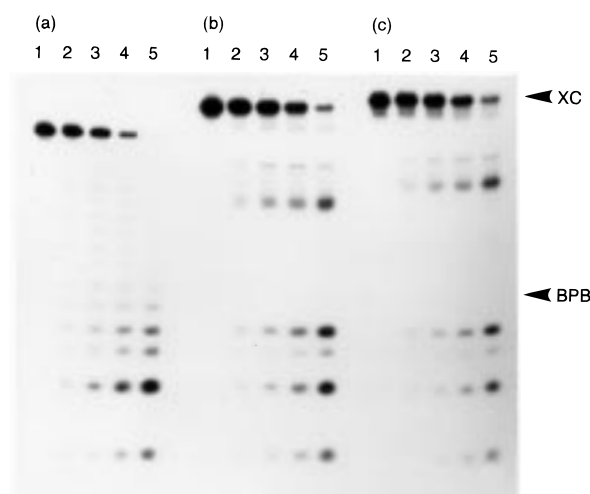


Figure 4. Polyacrylamide gel electrophoresis of 5'-³²P-labeled ODNs hydrolyzed by nuclease S1: (a) **10**, (b) **14**, and (c) **19**. ODNs were incubated with nuclease S1 for 0 min (lane 1), 10 min (lane 2), 30 min (lane 3), 60 min (lane 4), and 120 min (lane 5). Experimental conditions are described in Experimental Procedures.

incubated separately with these nucleases. The reactions were then analyzed by denaturing polyacrylamide gel electrophoresis (31). While the control, ODN **10**, was randomly hydrolyzed by snake venom phosphodiesterase after 120 min of incubation (Figure 3a, lane 5), ODNs containing ^{BA_P} or ^{BA_E} at their 3'-ends were completely resistant to hydrolysis by this nuclease (Figure 3b,c, lane 5). ODNs **14** and **19**, which contained two molecules of the modified nucleoside ^{BA_E} and ^{BA_P} in their middles, were also more resistant to nuclease S1 than the control, ODN **10** (Figure 4). The half-lives of ODNs **10**, **14**, and **19** were 23, 39, and 54 min, respectively. Therefore, ODNs containing ^{BA_E} or ^{BA_P} were more resistant to nucleolytic hydrolysis by both snake venom phosphodiesterase and nuclease S1 than the unmodified ODN.

CONCLUSION

In this paper, we report the synthesis of ODNs containing 2'-deoxyuridine analogues carrying either an *N,N*-bis(2-aminoethyl)aminoethylcarbamoyl or *N,N*-bis(3-aminopropyl)aminopropylcarbamoyl linker. The thermal stabilities of duplexes containing these ODNs and either the complementary DNA or RNA strand and of

triplexes of these ODNs and the target duplex were studied by thermal denaturation. We found that ODNs containing ^{BAE} or ^{BAP} stabilize duplex formation with either the complementary DNA or RNA strand but destabilize triplex formation with the target duplex. Furthermore, resistance of these ODNs to nuclease hydrolysis was examined by using snake venom phosphodiesterase (a 3'-exonuclease) and nuclease S1 (an endonuclease). The ODNs containing ^{BAE} or ^{BAP} were more resistant to nucleolytic hydrolysis by either of these nucleases than the unmodified ODN.

EXPERIMENTAL PROCEDURES

General Remarks. Thin-layer chromatography was done on Merck 60F₂₅₄ coated plates. The silica gel or the neutralized silica gel used for column chromatography was Merck silica gel 5715 or ICN silica 60A, respectively. Melting points were measured on a Yanagimoto MP-3 micromelting point apparatus (Yanagimoto) and are uncorrected. Fast atom bombardment mass spectrometry (FAB-MS) was measured on a JEOL JMS-HX110 instrument (JEOL) at an ionizing voltage of 70 eV. The ¹H-NMR spectra were recorded with a JEOL EX-270 instrument with tetramethylsilane as an internal standard. Chemical shifts are reported in parts per million (δ), and signals are expressed as an s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), or br (broad). All exchangeable protons were detected by the addition of D₂O. UV absorption spectra were recorded with a Shimadzu UV-240 spectrophotometer (Shimadzu Co.).

5-[N-[2-[N,N-Bis[2-(trifluoroacetyl-amino)ethyl]-amino]ethyl]carbamo-yl]-5'-O-(dimethoxytrityl)-2'-deoxyuridine (4). Tris(2-aminoethyl)amine (900 μ L, 6.00 mmol) was added to a solution of **3** (**27**) (1.31 g, 2.00 mmol) in pyridine (20 mL), and the mixture was stirred at room temperature. After 1 h, pyridine (20 mL), triethylamine (5.60 mL, 40.0 mmol), and ethyl trifluoroacetate (4.80 mL, 40.0 mmol) were added to the solution, and the mixture was stirred at room temperature for 5 h. The solvent was concentrated, and then the residue was dissolved in CHCl₃. The whole was washed with aqueous saturated NaHCO₃, dried over Na₂SO₄, and concentrated. The residue was chromatographed over a silica gel column (3.2 $\times$ 10 cm) with 75–100% EtOAc in hexane to give **4** (1.41 g, 79% as a white foam): FAB-MS m/z 895 ($M^+ + 1$); ¹H-NMR (CDCl₃) δ 8.89 (t, 1H, CONH, $J = 5.3$ Hz), 8.70 (s, 1H, 3-NH), 8.64 (s, 1H, H-6), 7.52 (t, 2H, CF₃CONH, $J = 4.6$ Hz), 7.41–7.20 (m, 9H, DMTr-phenyl), 6.84–6.81 (m, 4H, DMTr-phenyl), 6.15 (t, 1H, H-1', $J_{1',2'} = 6.4$ Hz), 4.35–4.31 (m, 1H, H-3'), 3.99–3.94 (m, 1H, H-4'), 3.78 (s, 6H, DMTr-OMe), 3.52–3.34 (m, 8H, H-5'ab, CF₃CONHCH₂, CONHCH₂), 2.69–2.61 (m, 6H, NCH₂), 2.50 (ddd, 1H, H-2'a, $J_{2'a,3'} = 4.3$ Hz, $J_{2'a,1'} = 6.4$ Hz, $J_{2'b,2'a} = 14.0$ Hz), 2.20 (ddd, 1H, H-2'b, $J_{2'b,3'} = 6.4$ Hz, $J_{2'b,1'} = 6.7$ Hz, $J_{2'b,2'a} = 14.0$ Hz), 2.06 (d, 1H, 3'-OH, $J = 3.1$ Hz); FAB exact MS calcd for C₄₁H₄₅F₆N₆O₁₀ ($M^+ + 1$) 895.3099, found 895.3077.

5-[N-[2-[N,N-Bis[2-(trifluoroacetyl-amino)ethyl]-amino]ethyl]carbamo-yl]-5'-O-(dimethoxytrityl)-3'-O-[(2-cyanoethyl)(N,N-diisopropylamino)phosphinyl]-2'-deoxyuridine (5). After successive coevaporation with pyridine, **4** (537 mg, 600 μ mol) was dissolved in CH₂-Cl₂ (6 mL) containing N,N-diisopropylethylamine (310 μ L, 1.80 mmol). 2-Cyanoethyl N,N-diisopropylchlorophosphoramidite (270 μ L, 1.20 mmol) was added to the solution, and the reaction mixture was stirred for 20 min at room temperature. The mixture was diluted with

CHCl₃ and washed with aqueous saturated NaHCO₃ and brine. The separated organic phase was dried over Na₂SO₄ and concentrated. The residue was chromatographed over a neutral silica gel column (3.3 $\times$ 9 cm) with 50–80% EtOAc in hexane to give **5** (488 mg, 74% as a white foam): FAB-MS m/z 1095 ($M^+ + 1$); ³¹P-NMR (CDCl₃) δ 149.90, 149.64 (85% H₃PO₄ as an internal standard); FAB exact MS calcd for C₅₀H₆₂F₆N₆O₁₁P ($M^+ + 1$) 1095.418, found 1095.4240.

5-[N-[2-[N,N-Bis[2-(trifluoroacetyl-amino)ethyl]-amino]ethyl]carbamo-yl]-5'-O-(dimethoxytrityl)-3'-O-succinyl-2'-deoxyuridine (6). After successive coevaporation with pyridine, **4** (179 mg, 200 μ mol) was dissolved in pyridine (2 mL). Succinic anhydride (40 mg, 400 μ mol) and DMAP (12 mg, 100 μ mol) were added to the solution, and the mixture was stirred at room temperature for 3 days. The mixture was diluted with CHCl₃ and washed with H₂O, aqueous saturated NaHCO₃, and then brine. The separated organic phase was dried over Na₂SO₄ and evaporated. The residue was chromatographed on a silica gel column (3.0 $\times$ 9 cm) with 80–100% EtOAc in hexane and then 0–30% MeOH in EtOAc to give **6** (117 mg, 59% as a white foam): ¹H-NMR (CDCl₃) δ 8.73 (s, 1H, H-6), 7.61 (s, 2H, CF₃CONH), 7.41–7.11 (m, 9H, DMTr-phenyl), 6.88–6.78 (m, 4H, DMTr-phenyl), 6.02–5.95 (m, 1H, H-1', $J_{1',2'} = 6.4$ Hz), 5.12–5.10 (m, 1H, H-3'), 4.23–4.15 (m, 1H, H-4'), 3.73 (s, 6H, DMTr-OMe), 3.48–3.24 (m, 8H, H-5'ab, CF₃CONHCH₂, CONHCH₂), 2.65–2.44 (m, 12H, NCH₂, H-2'ab, OCOC-H₂CH₂COOH).

5-[N-[3-[N,N-Bis[3-(trifluoroacetyl-amino)propyl]-aminol]propyl]carbamo-yl]-5'-O-(dimethoxytrityl)-2'-deoxyuridine (7). Tris(3-aminopropyl)amine (90 μ L, 450 μ mol) was added to a solution of **3** (**27**) (98 mg, 150 μ mol) in pyridine (2 mL), and the mixture was stirred at room temperature. After 4 h, pyridine (2 mL), triethylamine (420 μ L, 3.00 mmol), and ethyl trifluoroacetate (360 μ L, 3.00 mmol) were added to the solution, and the mixture was stirred at room temperature for 5 h. The mixture was diluted with CHCl₃, which was washed with aqueous saturated NaHCO₃. The separated organic phase was dried over Na₂SO₄ and concentrated. The residue was chromatographed over a silica gel column (2.5 $\times$ 8 cm) with 0–20% MeOH in EtOAc to give **7** (105 mg, 75% as a white foam): FAB-MS m/z 937 ($M^+ + 1$); ¹H-NMR (CDCl₃) δ 8.66–8.59 (m, 3H, CONH, 3-NH, H-6), 7.76–7.74 (m, 2H, CF₃CONH), 7.50–7.16 (m, 9H, DMTr-phenyl), 6.87–6.80 (m, 4H, DMTr-phenyl), 6.15 (t, 1H, H-1', $J_{1',2'} = 6.5$ Hz), 4.31 (m, 1H, H-3'), 3.97 (m, 1H, H-4'), 3.78 (s, 6H, DMTr-OMe), 3.48–3.32 (m, 8H, H-5'ab, CF₃CONHCH₂, CONHCH₂), 2.51–2.42 (m, 7H, NCH₂, H-2'a), 2.23 (m, 1H, H-2'b), 1.77–1.66 (m, 6H, NCH₂CH₂CH₂-NH); FAB exact MS calcd for C₄₄H₅₁F₆N₆O₁₀ ($M^+ + 1$) 937.3568, found 937.3620.

5-[N-[3-[N,N-Bis[3-(trifluoroacetyl-amino)propyl]-aminol]propyl]carbamo-yl]-5'-O-(dimethoxytrityl)-3'-O-[(2-cyanoethyl)(N,N-diisopropylamino)phosphinyl]-2'-deoxyuridine (8). Compound **7** (562 mg, 600 μ mol) was phosphitylated as described in the preparation of **5** to give **8** (469 mg, 69% as a white foam): FAB-MS m/z 1137 ($M^+ + 1$); ³¹P-NMR (CDCl₃) δ 149.78, 149.57 (85% H₃PO₄ as an internal standard); FAB exact MS calcd for C₅₃H₆₈F₆N₈O₁₁P ($M^+ + 1$) 1137.464, found 1137.4710.

5-[N-[3-[N,N-Bis[3-(trifluoroacetyl-amino)propyl]-aminol]propyl]carbamo-yl]-5'-O-(dimethoxytrityl)-3'-O-succinyl-2'-deoxyuridine (9). Compound **7** (281 mg, 300 μ mol) was succinylated as described in the preparation of **6** to give **9** (145 mg, 47% as a white foam): ¹H-

NMR (CDCl₃) δ 8.85 (m, 2H, H-6, 3-NH), 8.14 (m, 2H, CF₃CONH), 7.40–7.14 (m, 9H, DMTr-phenyl), 6.83–6.80 (m, 4H, DMTr-phenyl), 6.02 (t, 1H, H-1', $J_{1',2'} = 6.2$ Hz), 4.99 (m, 1H, H-3'), 4.24 (m, 1H, H-4') 3.75 (s, 6H, DMTr-OMe), 3.55–3.14 (m, 8H, H-5'ab, CF₃CONHCH₂, CONHCH₂), 2.97–2.21 (m, 12H, NCH₂, H-2'ab, OCOCH₂CH₂-COOH), 1.86–1.59 (m, 6H, NCH₂CH₂CH₂NH).

Synthesis of the Controlled Pore Glass Support Containing **4 and **7**.** Aminopropyl controlled pore glass (323 mg, 29.0 μ mol, 89.8 μ mol/g, CPG Inc.) was added to a solution of **6** (115 mg, 0.116 mmol) and 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (WSCl) (22 mg, 0.116 mmol) in anhydrous DMF (3 mL), and the mixture was kept for 4 days at room temperature. After the resin was washed with anhydrous pyridine, 3 mL of a capping solution (0.1 M DMAP in 9:1 pyridine/Ac₂O) was added, and the whole was kept for 1 days at room temperature. The resin was washed with EtOH and acetone and was dried under vacuum. The amount of nucleoside **4** loaded onto the solid support is 35.0 μ mol/g from the calculation of released dimethoxytrityl cation by a solution of 70% HClO₄/EtOH (3:2, v:v). In a similar manner, the solid support containing **7** was obtained in 34.7 μ mol/g of loading amounts.

Synthesis of ODNs. ODNs were synthesized on a DNA/RNA synthesizer (Applied Biosystem model 392) by the phosphoramidite method (29). Each ODN (1 μ mol) linked to the resin was treated with concentrated NH₄-OH (2 mL) for 16 h at 55 °C. After filtration of the resin, the filtrate was concentrated under reduced pressure. The residue obtained from the reaction with concentrated NH₄OH was purified by C-18 silica gel column chromatography (1 $\times$ 10 cm) with a linear gradient of CH₃CN in 0.1 M TEAA buffer (pH 7.0). Fractions were concentrated, and the residue was treated with 80% AcOH for 20 min at room temperature. The solution was concentrated, and the residue was coevaporated with H₂O. The residue was dissolved in H₂O, and the solution was washed with Et₂O; then, the H₂O layer was concentrated to give deprotected ODN **11** (14.4), **12** (11.1), **13** (27.7), **14** (18.0), **15** (19.7), **16** (16.8), **17** (24.2), **18** (17.5), **19** (10.6), and **20** (8.2). The yields are indicated in parentheses as OD units at 260 nm starting from a 1 μ mol scale.

Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry. Spectra were obtained on a Voyager Elite reflection time-of-flight mass spectrometry system (PerSeptive Biosystems, Inc., Framingham, MA) equipped with a nitrogen laser (337 nm, 3 ns pulse) in the negative ion mode. 3-Hydroxypicolinic acid (HPA), dissolved in H₂O to give a saturated solution at room temperature, was used as the matrix. Time-to-mass conversion was achieved by calibration by using the peak representing the C⁺ cation of the charged derivative to be analyzed. The observed average molecular masses of **13** and **17** were 5258.33 and 5299.89, respectively, and fit the calculated molecular weights (theoretical average molecular masses) for these compounds, i.e. 5258.69 (for **13**, C₁₇₆H₂₄₄N₄₆O₁₁₀P₁₆) and 5300.77 (for **17**, C₁₇₉H₂₅₀N₄₆O₁₁₀P₁₆), within a commonly accepted error range of MALDI-TOF/MS.

Thermal Denaturation. Each solution contains each ODN (3 μ M) and the complementary DNA **21** (3 μ M), RNA **22** (3 μ M), or the target duplex **23** (3 μ M) in an appropriate buffer. The solution containing each ODN was heated at 90 °C for 10 min, then cooled gradually to an appropriate temperature, and used for the thermal denaturation studies. Thermal-induced transitions of each mixture were monitored at 260 nm on a Perkin-

Elmer Lambda2S instrument. The sample temperature was increased 0.5 °C/min.

Partial Hydrolysis of ODNs with Snake Venom Phosphodiesterase. Each ODN labeled with ³²P at the 5'-end (10 pmol) was incubated with snake venom phosphodiesterase (0.4 μ g) in the presence of Torula RNA (0.15 OD₂₆₀ unit) in a buffer containing 37.5 mM Tris-HCl (pH 8.0) and 7.5 mM MgCl₂ (total of 20 μ L) at 37 °C. At appropriate periods, aliquots of the reaction mixture were separated and added to a solution of EDTA (5 mM, 10 μ L), and then the mixtures were heated at 100 °C for 5 min. The solutions were analyzed by electrophoresis on a 20% polyacrylamide gel containing 8 M urea (31).

Partial Hydrolysis of ODNs with Nuclease S1. Each ODN labeled with ³²P at the 5'-end (10 pmol) was incubated with nuclease S1 (0.2 unit) in the presence of Torula RNA (0.54 OD₂₆₀ unit) in a buffer containing 30 mM sodium acetate (pH 4.6), 1 mM ZnSO₄, and 280 mM NaCl (total of 20 μ L) at 37 °C. At appropriate periods, aliquots of the reaction mixture were separated and added to a solution of EDTA (5 mM, 10 μ L), and then the solutions were heated for 5 min at 100 °C. The solutions were analyzed by gel electrophoresis as described above. Densities of radioactivity of the gel were visualized by a Bio-imaging Analyzer (Bas 2000, Fuji, Co. Ltd.).

ACKNOWLEDGMENT

This investigation was supported in part by a Grant-in-Aid for Encouragement of Young Scientists from the Ministry of Education, Science, Sports, and Culture of Japan and a Grant from the "Research for the Future" Program of the Japan Society for the Promotion of Science (JSPS-RFTF97I00301).

LITERATURE CITED

- (1) Uhlmann, E., and Peyman, A. (1990) Antisense oligonucleotides: a new therapeutic principle. *Chem. Rev.* 90, 544–584.
- (2) Milligan, J. F., Matteucci, M. D., and Martin, J. C. (1993) Current concepts in antisense drug design. *J. Med. Chem.* 36, 1923–1937.
- (3) Crooke, S. T., and Lebleu, B., Eds. (1993) *Antisense research and applications*, CRC Press, Boca Raton, FL.
- (4) Thuong, N. T., and Helene, C. (1993) Sequence-specific recognition and modification of double-helical DNA by oligonucleotides. *Angew. Chem., Int. Ed. Engl.* 32, 666–690.
- (5) Beaucage, S. L., and Iyer, R. P. (1993) The functionalization of oligonucleotides via phosphoramidite derivatives. *Tetrahedron* 49, 1925–1963.
- (6) Beaucage, S. L., and Iyer, R. P. (1993) The synthesis of modified oligonucleotides by the phosphoramidite approach and their applications. *Tetrahedron* 49, 6123–6194.
- (7) Tabor, C. W., and Tabor, H. (1976) 1,4-Diaminobutane (putrescine), spermidine, and spermine. *Annu. Rev. Biochem.* 45, 285–306.
- (8) Tabor, C. W., and Tabor, H. (1984) Polyamines. *Annu. Rev. Biochem.* 53, 749–790.
- (9) Etter, M. C. (1990) Encoding and decoding hydrogen-bond patterns of organic compounds. *Acc. Chem. Res.* 23, 120–126.
- (10) Tabor, H. (1962) Protective effect of spermine and other polyamines against heat denaturation of deoxyribonucleic acid. *Biochemistry* 1, 496–501.
- (11) Thomas, T. J., and Bloomfield, V. A. (1984) Ionic and structural effects on the thermal helix-coil transition of DNA complexed with natural and synthetic polyamines. *Biopolymers* 23, 1295–1306.
- (12) Hampel, K. J., Crosson, P., and Lee, J. S. (1991) Polyamines favor DNA triplex formation at neutral pH. *Biochemistry* 30, 4455–4459.

- (13) Thomas, T., and Thomas, T. J. (1993) Selectivity of polyamines in triplex DNA stabilization. *Biochemistry* 32, 14068–14074.
- (14) Musso, M., and Van Dyke, M. W. (1995) Polyamine effects on purine-purine-pyrimidine triple helix formation by phosphodiester and phosphorothioate oligodeoxyribonucleotides. *Nucleic Acids Res.* 23, 2320–2327.
- (15) Tung, C.-H., Breslauer, K. J., and Stein, S. (1993) Polyamine-linked oligonucleotides for DNA triple helix formation. *Nucleic Acids Res.* 21, 5489–5494.
- (16) Prakash, T. P., Barawkar, D. A., Kumar, V. A., and Ganesh, K. N. (1994) Synthesis of site-specific oligonucleotide-polyamine conjugates. *Bioorg. Med. Chem. Lett.* 4, 1733–1738.
- (17) Barawkar, D. A., Kumar, V. A., and Ganesh, K. N. (1994) Triplex formation at physiological pH by oligonucleotides incorporating 5-Me-dC-(N⁴-spermine). *Biochem. Biophys. Res. Commun.* 205, 1665–1670.
- (18) Barawkar, D. A., Rajeev, K. G., Kumar, V. A., and Ganesh, K. N. (1996) Triplex formation at physiological pH by 5-Me-dC-(N⁴-spermine)[X] oligodeoxynucleotides: non protonation of N3 in X of X*G:C triad and effect of base mismatch/ionic strength on triplex stabilities. *Nucleic Acids Res.* 24, 1229–1237.
- (19) Schmid, N., and Behr, J.-P. (1995) Recognition of DNA sequences by strand replacement with polyamino-oligonucleotides. *Tetrahedron Lett.* 36, 1447–1450.
- (20) Sund, C., Puri, N., and Chattopadhyaya, J. (1996) Synthesis of C-branched spermine tethered oligo-DNA and the thermal stability of the duplexes and triplexes. *Tetrahedron* 52, 12275–12290.
- (21) Hashimoto, H., Nelson, M. G., and Switzer, C. (1993) Formation of chimeric duplexes between zwitterionic and natural DNA. *J. Org. Chem.* 58, 4194–4195.
- (22) Hashimoto, H., Nelson, M. G., and Switzer, C. (1993) Zwitterionic DNA. *J. Am. Chem. Soc.* 115, 7128–7134.
- (23) Ozaki, H., Nakamura, A., Arai, M., Endo, M., and Sawai, H. (1995) Novel C5-substituted 2'-deoxyuridine derivatives bearing amino-linker arms: synthesis, incorporation into oligodeoxyribonucleotides, and their hybridization properties. *Bull. Chem. Soc. Jpn.* 68, 1981–1987.
- (24) Ono, A., Dan, A., and Matsuda, A. (1993) Synthesis of oligonucleotides carrying linker groups at the 1'-position of sugar residues. *Bioconjugate Chem.* 4, 499–508.
- (25) Ono, A., Haginoya, N., Kiyokawa, M., Minakawa, N., and Matsuda, A. (1994) A novel and convenient postsynthetic modification method for the synthesis of oligodeoxyribonucleotides carrying amino linkers at the 5-position of 2'-deoxyuridine. *Bioorg. Med. Chem. Lett.* 4, 361–366.
- (26) Haginoya, N., Ono, A., Nomura, Y., Ueno, Y., and Matsuda, A. (1997) Synthesis of oligodeoxyribonucleotides containing 5-(N-aminoalkyl)carbamoyl-2'-deoxyuridines by a new postsynthetic modification method and their thermal stability and nuclease-resistance properties. *Bioconjugate Chem.* 8, 271–280.
- (27) Nomura, Y., Ueno, Y., and Matsuda, A. (1997) Site-specific introduction of functional groups into phosphodiester oligodeoxynucleotides and their thermal stability and nuclease-resistance properties. *Nucleic Acids Res.* 25, 2784–2791.
- (28) Ueno, Y., Ogawa, A., Nakagawa, A., and Matsuda, A. (1996) Facile synthesis of 5'-5'-linked oligodeoxyribonucleotides with the potential for triple-helix formation. *Bioorg. Med. Chem. Lett.* 6, 2817–2822.
- (29) Ueno, Y., Nakagawa, A., and Matsuda, A. (1997) Chemical ligation of oligodeoxynucleotides having a mercapto group at the 5-position of 2'-deoxyuridine via a disulfide bond. *Nucleosides Nucleotides* (in press).
- (30) Ueno, Y., Kumagai, I., Haginoya, N., and Matsuda, A. (1997) Effects of 5-(N-aminohexyl)carbamoyl-2'-deoxyuridine on endonuclease stability and an ability of oligodeoxynucleotide to activate RNase H. *Nucleic Acids Res.* 25, 3777–3782.
- (31) Gait, M. J., Ed. (1984) *Oligonucleotides synthesis: a practical approach*. IRL Press, Oxford, U.K.
- (32) Saenger, W. (1984) *Principle of nucleic acid structure*, Springer-Verlag, Inc., New York.
- (33) Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) *Molecular cloning: a laboratory manual*, Cold Spring Harbor Laboratory Press, Plainview, NY.

BC9701508

Poly(amino acid)-*b*-Poly(*N,N*-diethylacrylamide)-*b*-Poly(amino acid) Conjugates of Well-Defined Structure

Lev Bromberg^{*,†} and Gideon Levin

Department of Materials and Interfaces, The Weizmann Institute of Science, Rehovot 76100, Israel.
Received June 4, 1997; Revised Manuscript Received September 11, 1997[®]

Carboxy-terminated oligomers of *N,N*-diethylacrylamide (DEAAm) with low polydispersity were synthesized by group-transfer polymerization. The oligomers were conjugated with the N terminus of ϵ -*N*-CBZ-protected poly(lysine) and *s*-CBZ-protected poly(L-cysteine). The resulting conjugates precipitated in a narrow region of lower critical solution temperatures (LCST) around 29 °C. The LCST of poly(lysine)–polyDEAAm–poly(lysine) conjugates was pH-independent and independent of the lengths of poly(lysine) segments. Water-soluble nonstoichiometric conjugate–DNA complexes precipitated above the conjugate's LCST. Covalent complexes of human albumin and poly(cysteine)–polyDEAAm–poly(cysteine) conjugates showed temperature-sensitive behavior in aqueous solutions.

INTRODUCTION

Binding of biologically active compounds to polymers that possess a lower critical solution temperature (LCST) leads to conjugates with temperature-dependent solubility in aqueous solutions, a feature useful to control bioaffinity, enzyme separations, bioassays, and drug release (1–9). The field of temperature-sensitive polymers has been dominated by poly(*N*-isopropylacrylamide) (NIPA), because of its sharp, almost discontinuous volume phase transition corresponding to a LCST of about 32 °C (1–8, 10). Poly(*N*-isopropylacrylamides) are synthesized by free-radical polymerization (FRP) that usually results in high molecular weight polymers with a broad distribution of molecular weights and thus undefined molecular architecture. Okano and co-workers (6–8) used thiols as chain-transfer agents in FRP to produce NIPA oligomers with reactive end groups. The resulting oligomers had polydispersities of 1.2–2.0 and contained one carboxyl end group per molecule (6). Limitations of chain-transfer telomerization lie in complications associated with numerous side reactions and difficulties of finding a proper chain-transfer agent for a particular FRP system. Another means of controlling the molecular weight, end groups, pendent functionality, and chain configuration of a polymer is the group-transfer polymerization (GTP) involving transfer of trialkylsilyl groups (12). Since active hydrogen compounds interfere with GTP and stop chain growth, application of GTP to (unprotected) *N*-isopropylacrylamide presents a hurdle. *N,N*-Diethylacrylamide (DEAAm), a monomer that possesses a well-defined LCST in water of around 30 °C (13–15), can be used instead (16–18). DEAAm is devoid of active hydrogens and has been shown to be effectively polymerized by GTP, resulting in isotactic, highly uniform oligomers (16–18).

In this work, we have taken interest in the synthesis of carboxy-terminated polyDEAAm with the goal of

obtaining temperature-sensitive poly(amino acid)–poly-DEAAm triblock copolymers of well-defined architecture. Such conjugates can employ multiple amino acid functionality for bonding with specific drugs (19) via electrostatic interactions or can be used as a carrier molecule to bind and condense DNA into small particles (20, 21). PolyDEAAm was polymerized by GTP and end-capped by addition of methacrylic acid to the living macromonomer. Hydrolysis of the initiator, dimethylketene bis-(trimethylsilyl) acetal, produced a COOH group at the starting end of the polyDEAAm chain. The carboxy-terminated polyDEAAm was conjugated with several poly(amino acids) to result in conjugates with pH-independent LCSTs capable of complexing with DNA or proteins.

EXPERIMENTAL MATERIALS

Tetrahydrofuran (THF, >99%) was distilled under nitrogen from the purple sodium/benzophenone ketyl. Trimethylsilyl methacrylate (97%), chlorotrimethylsilane (>99%, redistilled), lithium diisopropylamide (97%), and isobutyric acid (>99%) were vacuum-distilled prior to use and found to be pure by GC. Tetrabutylammonium acetate (97%), *N*-hydroxysuccinimide (NHSI, 97%), 1,3-dicyclohexylcarbodiimide (DCCD, 99%), anhydrous ammonia (>99.99%), 2,2'-dipyridil disulfide (AldrichiolTM-2, 98%), dithiothreitol (DTT), and sodium spheres (2–5 mm) under nitrogen were used as received. Palladium black was freed from water by repeated washing with anhydrous ethanol. The monomer *N,N*-diethylacrylamide was stirred over CaH₂ for 24 h, vacuum-distilled, and found to be pure by GC. The monomer was kept at –20 °C in a septum-sealed flask prior to use. All above chemicals except for *N,N*-diethylacrylamide (Polysciences, Inc., Warrington, PA) were obtained from Aldrich Chemical Co. (Milwaukee, WI). 2,2'-Azobis(2-methylpropionitrile) (Eastman Kodak, Rochester, NY) was repeatedly crystallized from acetone. Poly(ϵ -CBZ-DL-lysine), poly(ϵ -CBZ-L-lysine), and poly(*s*-CBZ-L-cysteine) with average chain lengths of 90, 10, and 50 amino acid residues, respectively, as well as bacteriophage T7 DNA (lyophilized, from *Escherichia coli* B host, strain ATCC 11303, molecular weight of 25×10^6), human albumin

[†] Present address: MediSense, Inc., An Abbott Laboratories Company, 4A Crosby Drive, Bedford, MA 01730. Phone: (617) 276-4745. Fax: (617) 276-6266. E-mail: cpbrolev@aol.com.

[®] Abstract published in *Advance ACS Abstracts*, December 1, 1997.

(99%, globulin-free) (HA), and 2,4,6-trinitrobenzenesulfonic acid were all obtained from Sigma Chemical Co. (St. Louis, MO) and used as received. 1-Benzotriazolyl-oxy-tris-pyrrolidinophosphonium hexafluorophosphate (Py-BOP) was obtained from Calbiochem-Novabiochem Co. (La Jolla, CA) and was dissolved in *N,N*-dimethylformamide (DMF) immediately prior to use.

All other chemicals, gases, and dry organic solvents used were obtained from commercial sources and were of highest purity available.

PROCEDURES

Glassware was heated at 300 °C until dry. Glassware, syringes, reagents, and solutions used in synthetic procedures involving group-transfer polymerization were stored in a glovebox under a moisture-free argon atmosphere.

Chromatography was carried out at 20 °C on a Hewlett-Packard series 1050 system, including a variable wavelength detector, 1050 pumping system, 3396 series II integrator, and 19395A headspace sampler, as well as a Waters 410 differential refractometer, Viscotek differential viscometer, and right angle laser light scattering detector (Viscotek, Houston, TX). Columns and run conditions are given throughout.

NMR spectra of 10 wt % solutions in D₂O or DMSO-*d*₆ were acquired on a Bruker AM300 or a Bruker AMX400 spectrometer. Tetramethylsilane and dioxane were used as external frequency locks for ¹H and ¹³C NMR, respectively. Infrared spectra of dry polymer samples dispersed in KBr at a concentration of 1 wt % were recorded in a water-free atmosphere on a Nicolet Magna-IR model 560 spectrometer. Resolution was 2 cm⁻¹, and 256 scans of each sample were collected.

Dynamic light scattering experiments were performed with a Brookhaven Instruments BI-200SM goniometer, a BI-9000 correlator, and a Spectra Physics He-Ne model 127 laser operating at a scattering angle θ of 90° and a wavelength of incident light of 633 nm at a power of 50 mW. A 10 μ L quartz scattering cell was thermostated using refractive index matching silicone oil, and the temperature was controlled to within 0.02 $^\circ$ C. The intensity autocorrelation function was fitted to a triple-exponential form as described elsewhere (22). Correlation lengths ξ were calculated from the characteristic decay times of various scatterers using the Stokes-Einstein equation (22).

Lower critical solution temperatures (LCSTs) of polymer suspensions were determined as an onset of the transmittance decrease with the increasing temperature (8). Transmittance at 500 nm was continuously monitored in a flow-through quartz cell (path length of 0.2 cm) under controlled temperature (± 0.1 °C) conditions. The rate of temperature increase was 0.05 °C/min. Electronic absorbance spectra were measured on a Shimadzu UV-1601 spectrophotometer.

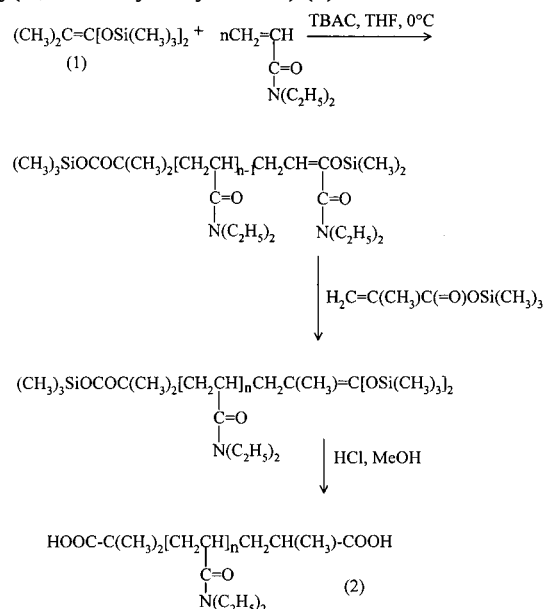
Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) was carried out on a Comstock model LTOF-160 spectrometer (Oak Ridge, TN). The 337.1 nm nitrogen laser parameters were as follows: pulse width, 3 ns; and pulse energy, 120 μ J. The matrix used was 2,5-dihydroxybenzoic acid, dissolved in 60:40 acetonitrile/0.1% TFA at a concentration of about 10 mg/mL (23). Insulin was used as an internal standard.

SYNTHESES

Dimethylketene Bis(trimethylsilyl) Acetal (1) (24).

A solution of isobutyric acid in THF (2 M, 25 mL) chilled

Scheme 1. Synthesis of Carboxy-Terminated Poly(*N,N*-diethylacrylamide) (2)



to 0 °C was added dropwise to 0.1 mol of lithium diisopropylamide. The mixture was stirred at 0 °C for 0.5 h, and 25 mL of chlorotrimethylsilane was added cautiously. After being stirred for 0.5 h at 20 °C, the mixture was filtered and concentrated using a rotary evaporator. The mixture was repeatedly washed with dry diethyl ether and refiltered. Ether was removed by evaporation, and the residue was distilled under vacuum (10 Torr). Calcd for $C_{10}H_{24}O_2Si_2$: C, 51.66%; H, 10.41%. Found: C, 51.49%; H, 10.59%. ^1H NMR (300 MHz, CCl_4): δ 1.48 (s, CH_3), 0.16 [s, $Si(CH_3)_3$].

Carboxy-Terminated Poly(*N,N*-diethylacrylamide)
(2) (Scheme 1). Dry THF (30 mL) was charged to a rigorously cleaned and dried one-necked round-bottom flask equipped with a magnetic stirrer and a rubber septum under a prepurified nitrogen atmosphere. A calculated amount of **1** was added *via* syringe to the reaction flask to yield a polymer with the desired molecular weight. Then calculated amount of *N,N*-diethylacrylamide was slowly added to produce the desired monomer-to-initiator molar ratio. The reaction mixture was cooled to 0 °C, and an aliquot of the 0.04 M solution of tertbutylammonium acetate catalyst (TBAC) solution in dry THF (250 μ L) was charged to the flask by rapid injection under stirring. An aliquot of trimethylsilyl methacrylate in THF was injected after 4 h, and the reaction was allowed to proceed for another 24 h at 0 °C under stirring. An amount of trimethylsilyl methacrylate was chosen which resulted in 1:1 molar ratio of trimethylsilyl methacrylate to **1**. The reaction was then stopped by excess methanol following addition of an aliquot of 1% HCl to remove the trimethylsilyl protecting groups. The solution was evaporated under vacuum (5 Torr), dissolved in dry THF, precipitated from hexane, and dried under vacuum (0.5 Torr) until a constant weight was reached. For comparison, random poly(*N,N*-diethylacrylamide-co-methacrylic acid) was synthesized by free-radical polymerization as follows. *N,N*-Diethylacrylamide (0.1 mol) and various amounts of methacrylic acid ranging from 1 μ mol to 10 mmol were dissolved in 200 mL of THF and deaerated by nitrogen flow overnight and following addition of 1 mmol of 2,2'-azobis(2-methylpropanitrile) in THF were kept at 50 °C for 48 h. The polymer was recovered as described above.

Table 1. Composition of Reaction Mixtures and Molecular Weights of Carboxy-Terminated Poly(*N,N*-diethylacrylamide)

no.	[DEAAM]/[initiator] (mol/mol)	M_n	M_w/M_n	n^a	COOH per molecule ^b
1	9	1060	1.17	7	2.01 ± 0.08
2	12	1570	1.19	11	2.04 ± 0.07
3	17	2100	1.12	15	1.98 ± 0.03
4	20	2800	1.21	20	1.93 ± 0.09
5	30	4000	1.09	30	2.09 ± 0.07
6	free-radical	390000	2.7	nd	nd

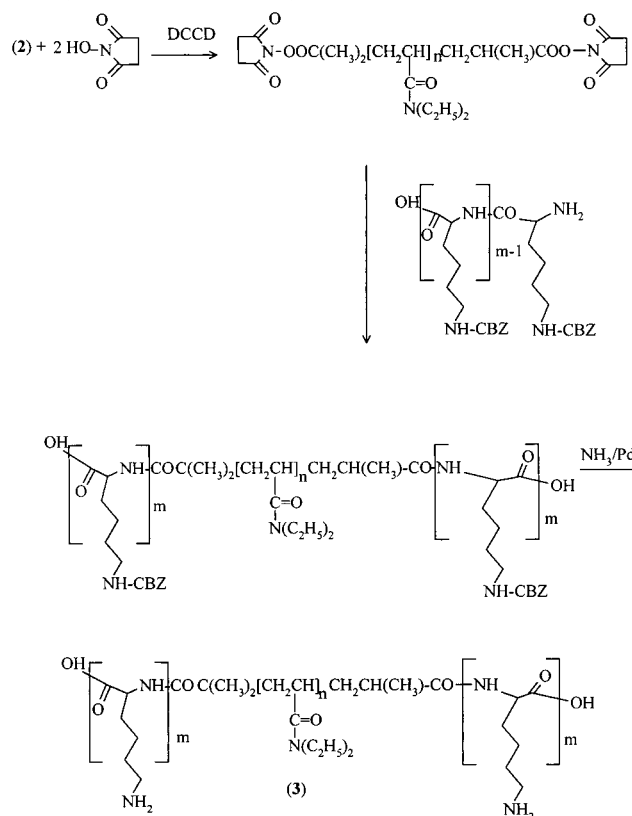
^a n is calculated from the measured number-average molecular weight from formula 2 in Scheme 1. ^b The number of carboxyl groups per molecule was obtained using M_n by titration of the corresponding polymer fractions. Three titrations of each fractions were averaged.

Polymer fractions dissolved in THF at 1 wt % were characterized by gel-permeation chromatography with PLgel analytical GPC columns (porosities, 50, 100, 500, 10^3 , 10^4 , and 10^6 Å; particle sizes, 5 or 10 µm; dimensions, 300 × 7.5 mm, Polymer Laboratories, Inc., Amherst, MA) using the refractive index and differential viscometer detectors. The flow rate, concentration, and injection volume were 1 mg/mL, 0.2%, and 30 µL, respectively. Initial DEAAM-to-initiator (1) mole ratios set in reaction mixtures and molecular weights of the resulting copolymers are collected in Table 1. Oligomer that resulted from run 1 was passed through a PLgel preparative mixed-bed column (10 µm, 300 × 7.5 mm) at a flow rate of 0.05 mL/min with THF as an eluent. The central part of the peak was collected, and the polymer was dried under vacuum until a constant weight was reached and subjected to MALDI-TOF-MS analysis.

Run 5 (Table 1). ¹³C NMR (D_2O , 100.6 MHz): δ 177.5 (O=CN), 173.2 (O=COH), 50.5, 45.1, 43.8 (CN, CO), 38.9, 37.4, 29.7 (secondary CN, CC), 16.2, 15.7, 15.2, 14.9, 11.0 (CC). ¹H NMR (DMSO- d_6 , 400.1 MHz, 343 K): δ 7.10 (COOH), 3.35, 3.20 [m, 4H, (CH₂)₂NCO], 2.50 (m, 1H, CH), 1.62 (m, 2H, CH₂), 1.07 (m, 6H, CH₃). The ratio of integrations of resonances corresponding to CH₂NCO, CH, CH₂, and CH₃ groups, respectively, was found to be 4.0:1.0:1.8:6.9; the calculated ratio was 4.0:1.0:2.0:6.0. FTIR (KBr): 2973, 2934 (asymmetrical and symmetrical CH₂CH₃ stretch), 1724 (COOH), 1634 (CON), 1451 (CH₂-CH₃ deform.), 1381, 1362 (CON), 1309 (COOH), 1276, 1218 (CH₂ wag., C₂H₅), 951 cm⁻¹.

Poly(lysine)-*b*-Poly(*N,N*-diethylacrylamide)-*b*-Poly(lysine) (3). Two methods of synthesis of this triblock copolymer were explored as follows.

Method 1 (Scheme 2). A calculated amount of NHSI was added to the 20 mg/mL solution of 2 in DMF at 4 °C while stirring. After 1 h, a calculated amount of DCCD in DMF was added, and the reaction was allowed to proceed for another 24 h at 4 °C under stirring. The molar ratio DCCD:NHSI:(COOH in polymer) was set at 10:10:1, and the total volume of the mixture was 100 mL. Formed dicyclohexylurea was removed by filtration, and the reaction mixture was reprecipitated into excess diethyl ether four times and dried under vacuum (10 Torr) until a constant weight was reached. Activated ester group was assayed as described in ref 25. Activated polymer in DMF was slowly added to a 100 mM solution of poly(ε-CBZ-DL-lysine) or poly(ε-CBZ-L-lysine) in 10 mM phosphate buffer (pH 8.0) [concentration of poly(CBZ-lysine)s is given on a monomer basis] at 4 °C while stirring. The molar ratio [activated ester group in poly-(DEAAM)]:[α-NH₂ groups in poly(CBZ-lysine)] was set at 1:1, and the total volume of the mixture was 30 mL. α-Amino groups in poly(CBZ-lysine)s were assayed using

Scheme 2. Synthesis of Poly(lysine)-polyDEAAM-Poly(lysine) Conjugate (3) by Method 1

2,4,6-trinitrobenzenesulfonic acid as described elsewhere (26). The coupling reaction was carried out for 24 h at 4 °C under stirring. The reaction mixture was then dialyzed against excess deionized water at 4 °C for 48 h using a Spectra/Por cellulose ester membrane (molecular weight cutoff of 500, Spectrum, Laguna Hills, CA) and passed through a PL-SCX semipreparative cation-exchange column (1000 Å, 8 µm, 100 × 10 mm, Polymer Laboratories, Inc., Amherst, MA) at flow rate of 0.5 mL/min with 10 mM phosphate buffer as an eluent. The fraction absorbance was monitored at 214 nm. The conjugate fractions were collected, dialyzed against deionized water, and lyophilized. Deprotection of ε-amino groups of lysine residues from benzyloxycarbonyl group was carried out by catalytic hydrogenolysis as follows (27). Anhydrous ammonia was passed over KOH pellets and placed in a three-necked round-bottom flask immersed in dry ice and fitted with dry ice reflux condenser and drying tube. Protected conjugate (1 mmol) was dissolved in 50 mL of liquid ammonia with magnetic stirring. Palladium black (0.6 g) wet with methanol was added under a nitrogen blanket. A stream of dried nitrogen was passed through the stirred refluxing solution for 8 h. The solution was then filtered and the solvent evaporated at room temperature under the stream of nitrogen. The conjugates were dissolved in degassed 0.1% TFA and purified on a PLRP-S 300 Å (250 × 4.6 mm) column (Polymer Laboratories, Inc.) which was eluted at 10 mL/min with 0.1% TFA and acetonitrile (5–20% over 0.5 h) while measuring absorbance at 214 nm. Fractionation of the conjugate samples was performed at 15 °C on a Hewlett-Packard 1050 HPLC system with the refractive index detector. A 1.0 mg/mL polymer solution in 0.05 M NaNO₃ was loaded onto a PLgel preparative three-column system (porosities, 50, 100, 500, 10^3 , and 10^4 Å; particle sizes, 10 µm; dimensions, 300 × 25 mm, Polymer Laboratories, Inc.). Frac-

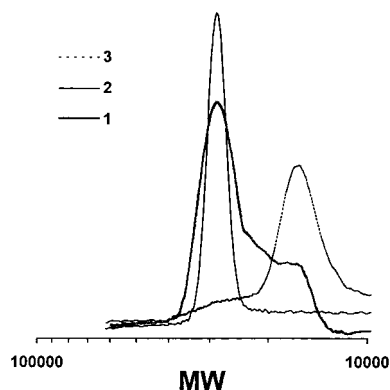


Figure 1. Gel-permeation chromatograms illustrating the process of fractionation of poly(lysine)-poly(*N,N*-diethylacrylamide) conjugates (**3**): (1) a mixture of conjugates obtained after purification of the synthesis products (method 1), (2) conjugate Lys₉₀DEAAm₁₅Lys₉₀ (molecular weight of 29 000, lysine content of 90 wt %) obtained by fractionation of the mixture in chromatogram 1, and (3) conjugate Lys₉₀DEAAm₁₅ (molecular weight of 17 000, lysine content of 80 wt %) obtained by fractionation of the mixture in chromatogram 1.

tions around the peaks were collected and precipitated by heating at 37–45 °C. The precipitates were filtered off and redissolved in cold deionized water. The resulting polymer fractions were subjected to GPC. Fractions were collected and analyzed for lysine content by titration (28). Typical gel-permeation chromatograms illustrating the process of fractionation are shown in Figure 1. The yield of poly(lysine)-*b*-polyDEAAm-*b*-poly(lysine) conjugates obtained by method 1 per initial amount of carboxy-terminated polyDEAAm varied from 9.4 to 14.8%.

Lys₁₀DEAAm₂₀Lys₁₀. ¹³C NMR (D₂O, 100.6 MHz): δ 177.9 (O=CN), 176.5 (O=COH), 56.2 (α-CH of Lys), 50.5, 45.4, 43.7 (CN, CO of DEAAm), 42.0 (ε-CH₂ of Lys), 38.8, 33.5, 37.4 (CN, CC of DEAAm), 33.5 (β-CH₂ of Lys), 29.2 (δ-CH₂ of Lys), 25.0 (γ-CH₂ of Lys), 16.7, 15.7, 15.1, 15.1, 14.8, 11.0 (CC). ¹H NMR (DMSO-*d*₆, 400.1 MHz, 343 K): δ 7.9 (COOH), 4.21 (t, 2H, α-CH of Lys), 3.31, 3.20 (m, CH₂NCO), 3.06 (t, 4H, ε-CH₂ of Lys), 2.77 (m), 2.50 (m, 1H, CH), 1.90 (m, β-CH₂ of Lys), 1.65 (m, δ-CH₂ of Lys, CH₂ of DEAAm), 1.43 (m, γ-CH₂ of Lys), 1.07 (m, CH₃). The ratio of integrations of resonances corresponding to α-CH of Lys, ε-CH₂ of Lys, CH of DEAAm, and CH₃ of DEAAm, respectively, was found to be 2.0:4.2:1.9:14; the calculated ratio was 2.0:4.0:2.0:12. FTIR (KBr): 3671, 3425 (NH₂), 2971, 2935 (asymmetrical and symmetrical CH₂CH₃ stretch), 1639 (CON), 1543, 1455 (CH₂CH₃ deform.), 1381, 1362 (CON), 1309 (COOH), 1275, 1219 (CH₂, C₂H₅), 1136, 1098 (CH₂NH₂), 950 cm⁻¹.

Method 2 (Scheme 3). Solid phase peptide synthesis was conducted by the Fmoc continuous flow methodology in DMF on a PC-interfaced synthesizer (Advanced ChemTech, Lexington, KY) using *N*-α-Fmoc-protected lysine (Boc) attached to NovaSyn PA 500 resin [poly(styrene)-poly(*N,N*-dimethylacrylamide) composite, Novabiochem Co.]. First, a peptide containing five or seven lysine residues with Boc-protected side chains and an Fmoc-protected N terminus was synthesized at a resin concentration of 0.48 mmol/g. Fmoc deblocking was performed by flashing the column with 25% piperidine for 30 min while continuously monitoring the absorbance at 320 nm. A 3-fold molar excess of *N,N*-diethylacrylamide oligomer of the structure HOCC(CH₃)₂-DEAAm₇-CH₂CH(CH₃)COOH in DMF was activated *in situ* with 14 mL of 0.2 M PyBOP and 0.4 M diisopropylethylamine mixture in DMF for 30 min. The activated oligomer was then added at a 1:1 molar ratio of activated

Scheme 3. Synthesis of Poly(lysine)-PolyDEAAm-Poly(lysine) Conjugate (**3**) by Method 2

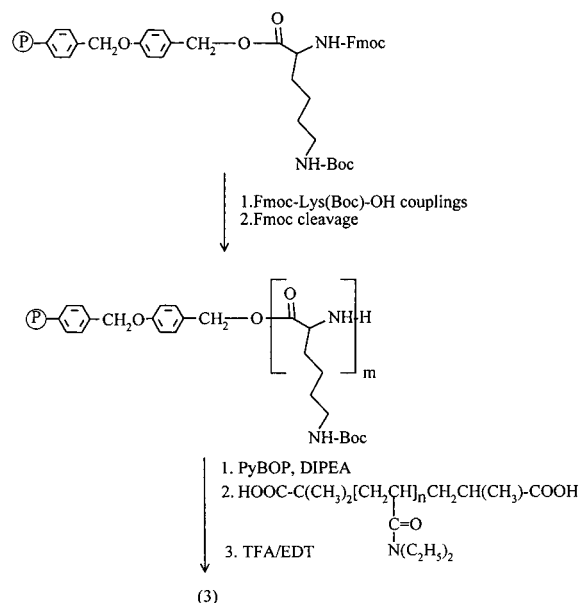


Table 2. Initial Carboxy-Terminated PolyDEAAm (2) and Poly(lysine)-*b*-PolyDEAAm-*b*-Poly(lysine) Conjugates Prepared by Method 2

formula	calcd ^a /obs
HOCC(CH ₃) ₂ -DEAAm ₇ -CH ₂ CH(CH ₃)COOH	1064.5/1065
Lys ₅ OCC(CH ₃) ₂ -DEAAm ₇ -CH ₂ CH(CH ₃)COLys ₅	2362.2/2364
Lys ₇ OCC(CH ₃) ₂ -DEAAm ₇ -CH ₂ CH(CH ₃)COLys ₇	2874.9/2876

^a Masses are calculated on the basis of the formula for a non-ionized compound.

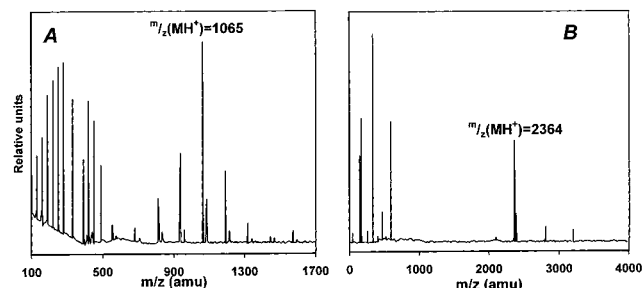


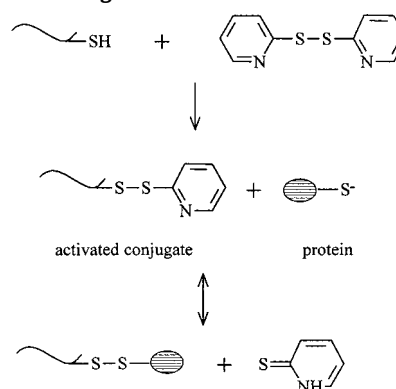
Figure 2. MALDI-TOF-MS spectra of carboxy-terminated poly(*N,N*-diethylacrylamide) (run 1 in Table 1) (A) and Lys₅-DEAAm₇Lys₅ (B).

carboxy groups to amino groups, and the coupling reaction was carried out for 8 h by recirculation through the column. At completion, the resin with the attached conjugate was washed with 1% acetic acid, dichloromethane, and diethyl ether and dried. Cleavage and removal of Boc protective groups were carried out with the TFA/ethanedithiol/water (95:2.5:2.5 v:v) solution for 30 min at room temperature. The resulting conjugate was washed with diethyl ether and lyophilized. The conjugates were dissolved in degassed 0.1% TFA and purified on a PLRP-S 300 Å (250 × 4.6 mm) column (Polymer Laboratories, Inc.) which was eluted at 10 mL/min with 0.1% TFA and acetonitrile (5 to 20% over 0.5 h) while measuring absorbance at 214 nm. Peptides were subjected to MALDI-TOF-MS analysis (Table 2). While mass spectra showed the presence of oligomers ranging from DEAAm₄ to DEAAm₁₁ (Figure 2A) and several peptide-containing species (Figure 2B), fractions corresponding to HOCC(CH₃)₂-DEAAm₇-CH₂CH(CH₃)-COOH and Lys₅OCC(CH₃)₂-DEAAm₇-CH₂CH(CH₃)-

COLys₅ (A and B in Figure 2, respectively) dominated the spectra. The molecular weights of the main fractions in MALDI spectra are listed in Table 2. The overall yield of poly(lysine)-*b*-polyDEAAm-*b*-poly(lysine) conjugates obtained by method 2 per initial amount of carboxy-terminated polyDEAAm was estimated to be 25–33%.

Poly(L-cysteine)-*b*-Poly(*N,N*-diethylacrylamide)-*b*-Poly(L-cysteine). NHSI (0.58 g) was added to the 5 mM solution of **2** (run 5 in Table 1) in 100 mL of DMF at 4 °C while stirring. After 1 h, 1.03 g of DCCD in DMF was added, and the reaction was allowed to proceed for another 24 h at 4 °C under stirring. Formed dicyclohexylurea was removed by filtration, and the reaction mixture was reprecipitated into excess diethyl ether four times and dried under vacuum until a constant weight was reached. Activated polymer in DMF was slowly added to a 10 mM solution of poly(s-CBZ-cysteine) in 10 mM phosphate buffer (pH 8.0) at 4 °C while stirring. The molar ratio [activated ester group in polyDEAAm]:[α-NH₂ groups in poly(s-CBZ-cysteine)] was set at 1:1, and the total volume of the mixture was 100 mL. The coupling reaction was carried out for 48 h at 4 °C under stirring. The reaction mixture was then dialyzed against excess deionized water at 4 °C for 48 h using a Spectra/Por cellulose ester membrane (molecular weight cutoff 1000) and passed through a MetaPore 10 μm C₈ column (250 × 4.6 mm) (MetaChem Technologies, Inc., Torrance, CA) at a flow rate of 0.5 mL/min. Buffer A was 0.1% TFA in water, and buffer B was acetonitrile/water/TFA (90:10:0.1 v:v). The elution profile was set at 5 min to 5% B and then to 100% B over 60 min. The fraction absorbance was monitored at 214 nm. The conjugate fractions were collected, dialyzed against deionized water, and lyophilized. Fractionation of the conjugate samples was performed at 15 °C on a Hewlett-Packard 1050 HPLC system with the refractive index detector. A 1.0 mg/mL polymer solution in 0.05 M NaNO₃ was loaded onto a PLgel preparative three-column system. Fractions around the peaks were collected and precipitated by heating at 37–45 °C. The precipitates were filtered off and redissolved in cold deionized water. The resulting polymer fractions were subjected to GPC. Fractions were collected and analyzed for S content by elemental analysis. Deprotection of thiol groups of cysteine residues from the benzyloxycarbonyl group was carried out by sodium in liquid ammonia as follows (19). Anhydrous ammonia was passed over KOH pellets and immersed in a three-necked round-bottom flask immersed in dry ice and fitted with a dry ice reflux condenser and a drying tube. To a 10 mg/mL solution of protected conjugate in anhydrous liquid ammonia were incrementally added sodium spheres with magnetic stirring which resulted in a 10 mg/mL effective sodium concentration. The suspension was stirred under reflux for 16 h, until a permanent blue color persisted (29). Then, the ammonia was allowed to evaporate, and the residue was dissolved in oxygen-free water. The solution was repeatedly washed with ether and acidified with concentrated HCl. The solution was then filtered on a sintered glass filter and the solvent evaporated over phosphorus pentoxide under the stream of nitrogen. The conjugates were dissolved in degassed 0.1% TFA and purified on a PLRP-S 300 Å (250 × 4.6 mm) column (Polymer Laboratories, Inc.) and eluted at 2 mL/min with 0.1% TFA and acetonitrile (5 to 20% over 1 h) while measuring absorbance at 214 and 280 nm. The solutions of the conjugate in water above pH 8 contained free sulfhydryl groups as detected upon addition of nitroprusside, which gave a faint violet color (30). The yield of poly(cysteine)-*b*-polyDEAAm-*b*-poly(cysteine) con-

Scheme 4. Activation of the Poly(cysteine)–PolyDEAAm–Poly(cysteine) Conjugate by the 2-Pyridyldisulfanyl Group Followed by Reaction with a Thiol-Containing Protein



jugates was 11% per weight of initial carboxy-terminated polyDEAAm.

Cys₅₀DEAAm₂₀Cys₅₀. ¹H NMR (DMSO-*d*₆, 400.1 MHz, 343 K): δ 8.1 (COOH), 3.74 (m, NCHCO), 3.35 (m), 2.94 (m, HCNCO), 2.35 (m, CH₂S), 1.64 (m, CH₂ of DEAAm), 1.1 (m, CH₃). The ratio of integrations corresponding to CH₂S of Cys to CH₂N of DEAAm was found to be 1.0:1.3; the calculated ratio was 1.0:1.2. FTIR (KBr): 2975, 2930 (asymmetrical and symmetrical CH₂CH₃ stretch), 2877 (CH₂S), 1621 (CON), 1553, 1463 (CH₂CH₃ deform.), 1384, 1362 (CON), 1308 (COOH), 1278, 1254 (CH₂S), 1217, 1114, 960 cm⁻¹.

Conjugate–DNA Condensates. Temperature-dependent interaction of DNA with poly(lysine)–polyDEAAm conjugates was assessed with the bacteriophage T7 DNA that has been used by Wilson and Bloomfield in their light scattering study of DNA condensation (31). A 20 μg/mL DNA solution was prepared in a 1.0 mM NaCl, 1.0 mM sodium cacodylate buffer (pH 7.2) as described in ref 31. The DNA solutions were centrifuged at 7000g for 0.5 h to eliminate dust. To prepare condensates, 100 μL of a DNA solution was mixed with 100 μL of a conjugate solution of a known concentration, and the mixture was vortexed for about 10 min and transferred to a scattering cell. The degree of binding of DNA to the conjugates was estimated by a method similar to the one used by Wadhwa et al. (20, 32). Namely, an aliquot of DNA solution was assayed spectrophotometrically (absorbance at 260 nm, Abs₂₆₀) at a given temperature below the conjugate's LCST. At a temperature above the LCST, the ensuing precipitates were removed by centrifugation at 7000g for 0.5 h which was carried out at the same temperature following measurement of Abs₂₆₀ in the resulting supernatant solution. The degree of DNA removal was expressed as $R(\%) = 100[1 - (\text{Abs}_{260} \text{ after precipitation})/(\text{Abs}_{260} \text{ before precipitation})]$.

Pyridine-2-disulfide-Derivatized Poly(cysteine)–PolyDEAAm–Poly(cysteine). The poly(cysteine)–polyDEAAm–poly(cysteine) conjugate was activated by derivatization of the free thiol groups with pyridine-2-disulfide by a procedure analogous to the one developed for activation of glutathione–agarose (33) (Scheme 4).

Namely, the conjugate was dissolved in excess 0.1 M Tris-HCl, 1 mM EDTA, and 30 mM DTT buffer (pH 8.0), and the solution was incubated at 4 °C overnight to reduce all available sulfhydryl residues. The conjugate was then precipitated from the solution at 60 °C, filtered off, and redissolved in excess 2 mM 2,2'-dipyridyl disulfide at room temperature, shaking gently. The activated

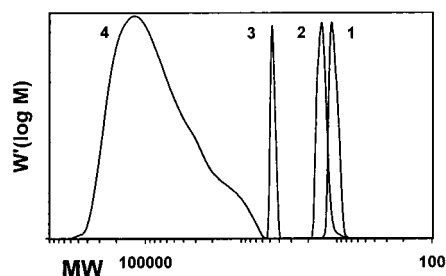


Figure 3. Typical molecular weight distribution of carboxy-terminated poly(*N,N*-diethylacrylamide) (polyDEAAm) polymers prepared by group-transfer polymerizations (GTPs) (1–3) and poly(*N,N*-diethylacrylamide-co-methacrylic acid) synthesized by free-radical polymerization (FRP) (4). In curve 4, the ratio DEAAm:methacrylic acid is 1000:1 on a monomer basis.

conjugate was precipitated at 60 °C, filtered off, and gently shaken at 60 °C in several portions of 10 mM Tris-HCl buffer (pH 8.0) until no further absorbance at 280 nm was detectable in the wash solutions. After washing, the activated conjugate was snap-frozen and lyophilized.

In conjugate–protein interaction studies, HA and activated conjugate solutions of known concentrations in a 10 mM Tris-HCl buffer (pH 8.0) prepared at 4 °C were incubated at a given temperature for 16 h and centrifuged at 16000*g* for 5 min. Precipitate (if any) and supernatant were separated, and the supernatant was evaporated under vacuum, redissolved in fresh 10 mM Tris-HCl buffer (1.0 mL), and assayed for protein content using bicinchoninic acid (34). The degree of albumin removal was expressed as $R_{HA}(\%) = 100[1 - (\text{HA concentration at a given temperature})/(\text{HA concentration at 4 °C})]$.

RESULTS AND DISCUSSION

Temperature Sensitivity of Conjugates. Application of the group-transfer polymerization technique yielded carboxy-terminated polyDEAAm with a narrow molecular weight distribution, when compared to the polymers which resulted from free-radical polymerization (see Table 1 and Figure 3). Average molecular weights calculated from GPC demonstrated good consistency with the initial molar [DEAAm]/[initiator] ratio, with somewhat smaller numbers at higher initiator concentrations. The possibility of minor spontaneous termination reaction in GTP synthesis of polyDEAAm has been shown by Freitag et al. (16–18).

The highly uniform structure of carboxy-terminated polyDEAAm allowed synthesis of well-defined sequences of triblock copolymers and oligomers of polyDEAAm and poly(lysine) (Table 2) as well as poly(cysteine).

Potentiometric titration of carboxy-terminated polyDEAAm and poly(lysine)– and poly(cysteine)–polyDEAAm conjugates yielded pK_a values for the terminal COOH group in the starting copolymer and the ϵ -NH₂ and SH groups in the corresponding poly(lysine) and poly(cysteine) segments of 4.41, 10.25, and 9.25, respectively (Figure 4). These values agree well with the reported pK_a values of methacrylic acid, lysyllysine, and cysteinylcysteine (35). Next, we examined whether ionization of carboxy-terminated polyDEAAm and its conjugates would impact their thermoprecipitation properties.

In contrast to the LCST of carboxy-terminated polyDEAAm synthesized by GTP which was pH-independent, ionization of poly(*N,N*-diethylacrylamide-co-methacrylic acid) prepared by free-radical polymerization (DEAAm:methacrylic acid ratio of 12:1 on a monomer basis) caused a dramatic shift of its LCST to higher

temperatures (Figure 5A). Interestingly, thermoprecipitation curves of poly(lysine)–polyDEAAm–poly(lysine) conjugates showed a slight shift of the LCST to lower temperatures with the increase of the length of the polyDEAAm segments (Figure 5B). Virtually no dependence of the LCST on pH (Figure 5C) or the length of the poly(lysine) chains at fixed lengths of the polyDEAAm segments (Figure 5D) was observed. Similarly, ionization of sulfhydryl groups of poly(cysteine) segments in the corresponding conjugates had no substantial effect on the conjugate thermoprecipitation (Figure 6).

The sharpness of (almost discontinuous) transition in the conjugate solutions can be explained by the low polydispersity of the polyDEAAm segments which resulted from GTP. Random copolymerization of the temperature-sensitive monomer (DEAAm) with the ionizable monomer (methacrylic acid) leads to a drastic shift of the volume transition of the resulting copolymer to higher temperatures, when the pH is higher than the pK_a of the ionizable component (Figure 5A). This is because of the solubility that the ionized component conveys to the incipient formation of insoluble, temperature-sensitive aggregates of polyDEAAm (36). It has been shown that in order to retain reversible temperature-induced volume phase transition of an ionizable copolymer over a broad range of pH the latter should contain long enough segments of a hydrophobic component. The hydrophobic segments will provide entropy-driven aggregation of the entire copolymer at temperatures not significantly different from those of the hydrophobic component itself, whereas ionizable segments will render the copolymer ion-sensitive. The block or graft copolymers where pH- and temperature-sensitive segments either alternate along the chain or are grafted onto each other are therefore the only type of copolymers where volume phase transitions can be due to either pH or temperature or both simultaneously (36, 37).

The observed insensitivity of the conjugates to ionization is an important feature that may allow for their application as switching agents in controlling bioactivity and affinity of the conjugate–DNA or conjugate–protein complexes by means of finely tuned temperature changes. Experiments that support this hypothesis are described below.

Poly(lysine)–PolyDEAAm–Poly(lysine) Conjugates as Temperature-Controlled DNA-Condensing Agents. To verify formation of DNA–conjugate condensates, total light scattering at 90° was measured (Figure 7). When increasing amounts of conjugate were added to T7 bacteriophage DNA, an abrupt increase in the relative intensity was observed at the nanomoles per microgram conjugate-to-DNA ratios of 7.0, 5.0, and 3.5 for Lys₅DEAAm₇Lys₅, Lys₇DEAAm₇Lys₇, and Lys₁₀DEAAm₁₅Lys₁₀, respectively. These ratios indicate, with a good consistency, that about 70 kmol of lysine residues is required for the condensation of 1 mol (about 35 kbp) of DNA under the conditions studied. This corresponds to a 1:1 stoichiometry on the DNA bases per mole of lysine basis. Thus, aggregates form when the DNA–conjugate complex is neutral overall. A further increase in the conjugate:DNA ratio leads to recharging of the complexes and therefore to their redissolution (Figure 7).

Similarly, Wadhwa et al. (20) observed maximum sedimentation of dipeptides such as Lys₈TrpCys-S-S-CysTrpLys₈, Lys₁₃TrpCys-S-S-CysTrpLys₁₃, and Lys₁₈TrpCys-S-S-CysTrpLys₁₈ complexed with a 5.6 kbp DNA at a 1:1 moles of peptide per DNA base ratio. At

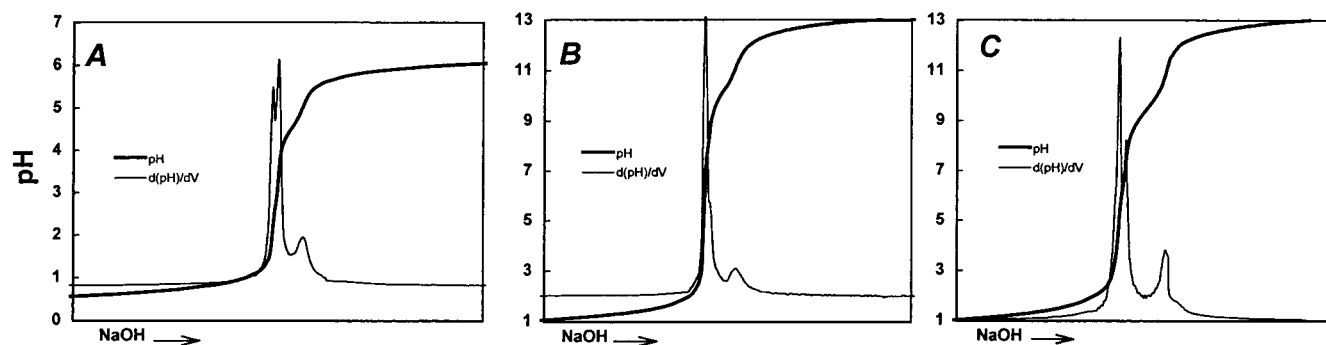


Figure 4. Potentiometric titration curves for 1% w:w aqueous solutions of Lys₁₀DEAAm₁₅Lys₁₀ (A), HOCC(CH₃)₂-DEAAm₂₀-CH₂CH(CH₃)COOH (B), and Cys₅₀DEAAm₃₀Cys₅₀ (C).

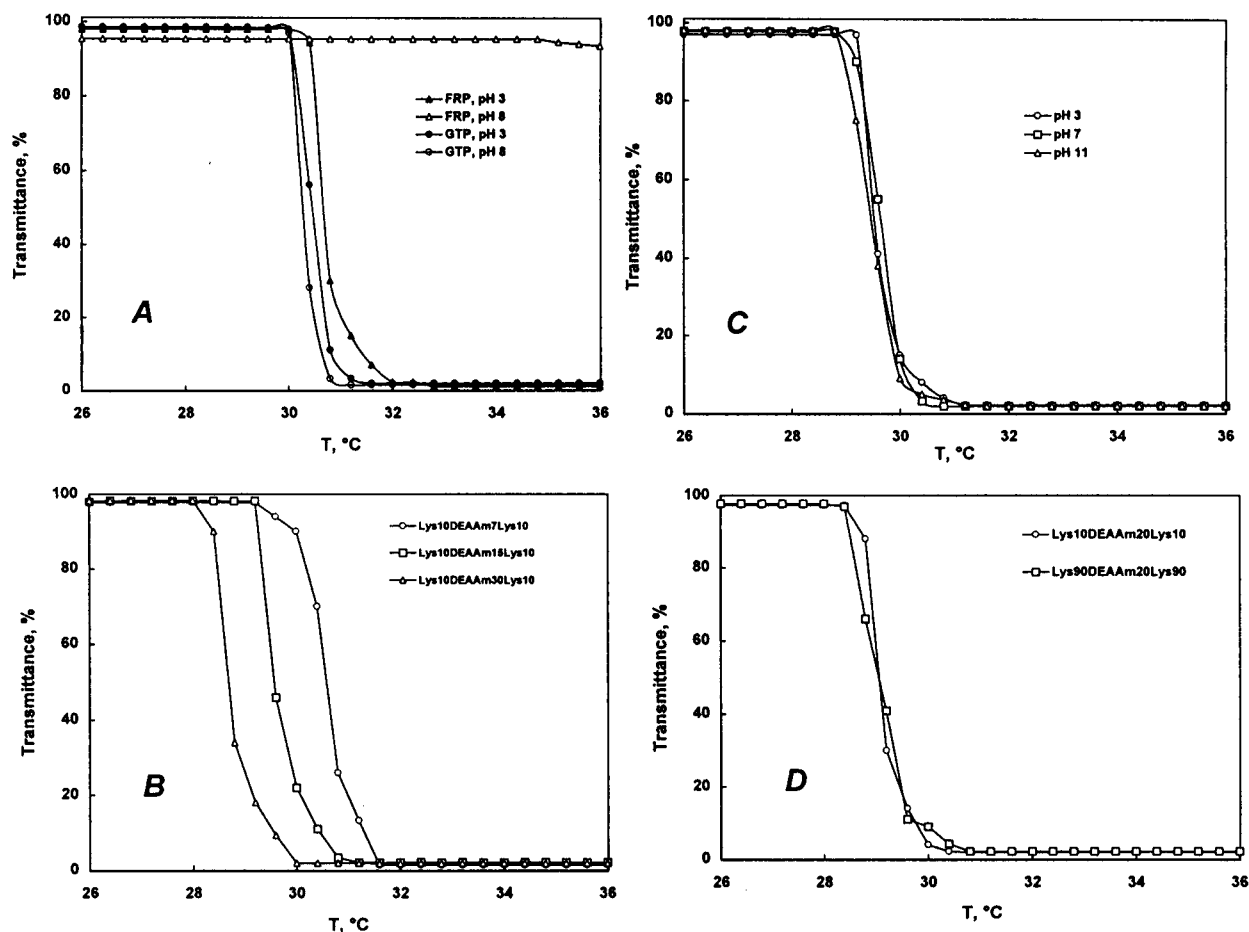


Figure 5. (A) Transmission vs temperature curves for 0.05% w:w aqueous solutions of poly(*N,N*-diethylacrylamide-*co*-methacrylic acid) obtained by FRP and carboxy-terminated polyDEAAm₃₀ obtained by GTP as a function of pH. (B) Transmission vs temperature curves for 0.05% w:w aqueous solutions of poly(lysine)-polyDEAAm-poly(lysine) conjugates as a function of the length of polyDEAAm segments (pH 7.0). (C) Transmission vs temperature curves for 0.05% w:w aqueous solutions of Lys₉₀DEAAm₁₅Lys₉₀ conjugates as a function of pH. (D) Transmission vs temperature curves for 0.05% w:w aqueous solutions of poly(lysine)-polyDEAAm-poly(lysine) conjugates as a function of the length of poly(lysine) segments (pH 7.0).

stoichiometries larger than the charge neutral, the peptide-DNA complexes sedimented to a much lesser extent (20).

Temperature-induced aggregation of the DNA-conjugate complexes was studied by dynamic light scattering at the conjugate-to-DNA nanomoles per microgram ratio of 3.0 when neither conjugate-DNA solution demonstrated an increase of light scattering intensity at 15 °C compared to that of the DNA solution (Figure 7). The effect of temperature on correlation lengths (ξ) and relative intensity of the DNA-conjugate solutions is depicted in Figure 8. Below conjugates' LCST, the scattering intensity was that of the background, whereas above the LCST, the scattering intensity and correlation

lengths drastically increased, indicating formation of larger aggregates.

The ξ values exceeding 1000 nm that are 20-fold larger than the sizes of condensed bacteriophage T7 DNA particles reported by Wilson and Bloomfield (31) indicate formation of aggregates among the DNA-conjugate complexes due to hydrophobic interactions between polyDEAAm segments. The mechanism of such aggregation in poly(alkylacrylamide) solutions is reasonably well understood (9, 10).

To ascertain binding of the conjugates to the DNA throughout the temperature range, amounts of DNA remained in the conjugate-DNA solutions after the temperature-induced aggregate precipitation was mea-

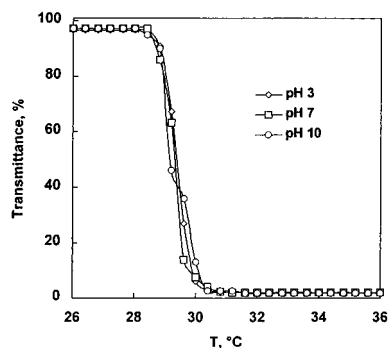


Figure 6. Transmission vs temperature curves for 0.05% w/w aqueous solutions of Cys₅₀DEAAM₃₀Cys₅₀ conjugates as a function of pH.

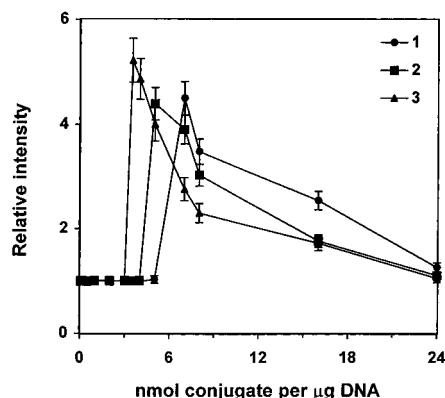


Figure 7. Light scattering titration of DNA with poly(lysine)-polyDEAAM-poly(lysine) conjugates. Slow modes combined (scatterers with ξ values of >10 nm) were normalized to the total intensity without conjugate added, resulting in relative intensity units. Experiments were conducted in a 1.0 mM NaCl, 1.0 mM sodium cacodylate buffer (pH 7.2); $T = 15^\circ\text{C}$: (1) Lys₅-DEAAM₇-Lys₅, (2) Lys₇-DEAAM₇-Lys₇, and (3) Lys₁₀-DEAAM₁₅-Lys₁₀.

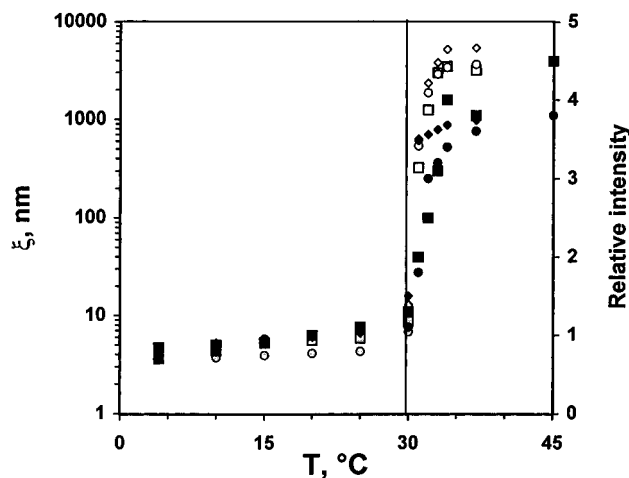


Figure 8. Correlation lengths (ξ) (open points) and relative intensity ($I_{T=20^\circ\text{C}} = 1$) (filled points) of DNA-conjugate solutions versus temperature. Squares- Lys₅DEAAM₇-Lys₅; circles- Lys₇-DEAAM₇-Lys₇; diamonds- Lys₁₀DEAAM₁₅-Lys₁₀. Open and lightly filled points represent ξ , whereas black filled points show relative intensity. Lightly filled points represent background (fast modes with $\xi < 10$ nm). Vertical line corresponds to the conjugates' LCST. Conjugate-to-DNA nmol per μg ratio was 3.0 throughout. For other conditions see the legend to Figure 7.

sured (Figure 9). To ensure the absence of precipitation when $T < \text{LCST}$, the conjugate:DNA nanomoles per microgram ratio was 3.0 throughout.

While the amount of DNA removed did not exceed 11%

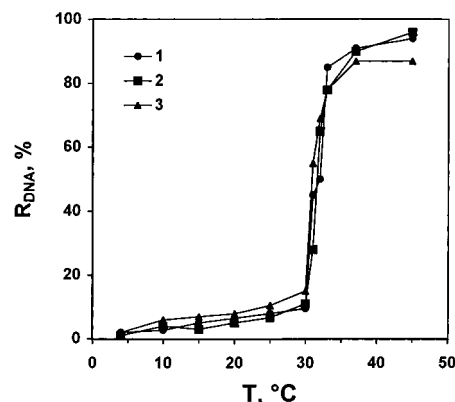
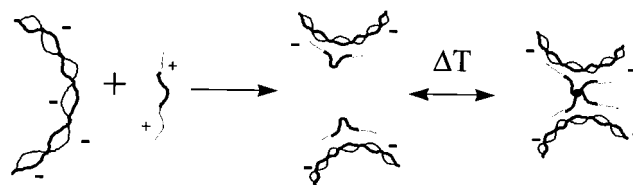


Figure 9. Percent of DNA removal [$R_{\text{DNA}}(\%)$] from DNA-conjugate solutions following centrifugation as a function of temperature: (1) Lys₅DEAAM₇-Lys₅, (2) Lys₇DEAAM₇-Lys₇, and (3) Lys₁₀DEAAM₁₅-Lys₁₀. The conjugate-to-DNA molar ratio was 3.0 throughout. For other conditions, see the legend to Figure 7.

Scheme 5. DNA forms a Nonstoichiometric Complex with the Poly(lysine)-PolyDEAAM-Poly(lysine) Conjugate^a



^a The complex reversibly aggregates in response to changes in the solution temperature.

below the conjugate's LCST, almost complete DNA removal was observed above the LCST. This is a strong indication that the DNA remained complexed to the precipitated conjugates.

The phenomenon of formation and redissolution of interpolyelectrolyte complexes between nucleic acids and synthetic polyelectrolytes is well-known and has been applied to the delivery of genetic material into cells (20, 38, 39). It seems that we hereby introduced a novel, temperature-regulated mode of behavior of interpolyelectrolyte complexes of DNA with a polycation modified with a segment that has a LCST (Scheme 5).

The next step would be to test functioning of poly(lysine)-polyDEAAM-poly(lysine) conjugates in cell transfection.

Poly(cysteine)-PolyDEAAM-Poly(cysteine) Conjugates in Temperature-Controlled Protein Precipitation. A sorption-desorption process involving thiol-disulfide exchange has been utilized in "covalent chromatography", a type of affinity chromatography whereby disulfide-modified gel adsorbents are capable of removing thiol-containing proteins from their mixtures with non-thiol-containing proteins (33, 40, 41). Drawing an analogy with covalent chromatography, we hypothesized that the poly(cysteine)-containing conjugate would be a powerful binding agent toward a thiol-containing protein, due to its numerous thiol groups. Yet, the poly-DEAAM segment of the conjugate may impart thermoreversible solubility to the protein-conjugate complex. To test this concept, we have chosen human albumin, a thiol-containing protein prone to forming covalent attachments to polymer supports modified with reactive disulfide groups (40, 41). The thiol groups in the poly(cysteine)-polyDEAAM-poly(cysteine) conjugate were converted into 2-pyridyldisulfanyl groups (Scheme 4). The resulting

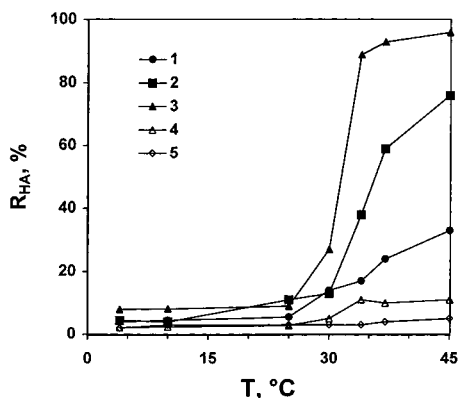


Figure 10. Removal of human albumin [R_{HA} (%)] by the 2-pyridyldisulfanyl-activated $Cys_{50}DEAAM_{30}Cys_{50}$ conjugate as a function of temperature. The initial solution contained 0.1% w/w HA in 10 mM Tris-HCl (pH 8.0). The conjugate was added which resulted in 0.05% w/w (1), 0.1% w/w (2 and 4), and 0.2% w/w (3). Curve 5 represents a control without conjugate added. In curve 4, the solution contained 10 mM dithiothreitol.

protein–conjugate complex precipitated at temperatures above the conjugate's LCST, causing HA removal from the solution (Figure 10). At the conjugate-to-protein weight ratio of 2:1, the protein was removed almost quantitatively at 37–45 °C (curve 3 in Figure 10). Addition of DTT that destroys disulfide bonds in the protein–conjugate complex lowered the HA removal at least 5-fold (compare curves 4 and 2 in Figure 10). These data indicate the possibility of reversible protein–conjugate complexation that, in turn, may provide a thermoreversible precipitation of a thiol-containing protein. We believe this may conceptually add another dimension to the area of protein separation by thermally induced precipitation utilizing polymer–protein conjugates (2–5, 42).

CONCLUSIONS

Carboxy-terminated oligomers of *N,N*-diethylacrylamide (DEAAM) with low polydispersity were synthesized by group-transfer polymerization. Carboxyl functionality allowed conjugation of the DEAAM oligomers with the N terminus of ϵ -*N*-CBZ-protected poly(lysine) in both batch and continuous solid phase synthesis modes, resulting in poly(lysine)–polyDEAAM–poly(lysine) triblock copolymers with well-defined structure. Similarly, *s*-CBZ-protected poly(L-cysteine) was conjugated with polyDEAAM, producing triblock copolymers. The LCST of the conjugates was observed in a narrow region of temperatures around 29 °C and was pH-independent. The LCST was shown to be independent of the lengths of poly(lysine) segments, given constant length of the polyDEAAM segments. A shift of about 2 °C to a lower LCST was observed with increasing lengths of the polyDEAAM segments. Stoichiometric poly(lysine)–polyDEAAM–poly(lysine) conjugates formed condensed complexes with bacteriophage T7 DNA. Nonstoichiometric conjugate–DNA complexes precipitated above the conjugate's LCST due to hydrophobic interactions between the polyDEAAM segments in the conjugates. Poly(cysteine)–polyDEAAM–poly(cysteine) conjugates complexed with human albumin via the thiol–disulfide exchange reaction imparted temperature sensitivity to the conjugate–protein complex. The newly obtained conjugates may appear to be a means of regulating cell transfection with DNA and affinity precipitation of enzymes by regulating the temperature of the aqueous environment.

LITERATURE CITED

- (1) Hoffman, A. S. (1987) Applications of thermally reversible polymers and hydrogels in therapeutics and diagnostics. *J. Controlled Release* 6, 297–305.
- (2) Chen, J. P., Yang, H. J., and Hoffman, A. S. (1990) Polymer–protein conjugates I. Effect of protein conjugation on the cloud point of poly(*N*-isopropylacrylamide). *Biomaterials* 11, 625–630.
- (3) Chen, J. P., and Hoffman, A. S. (1990) Polymer–protein conjugates II. Affinity precipitation separation of immunoglobulin by a poly(*N*-isopropylacrylamide)–protein A conjugate. *Biomaterials* 11, 631–634.
- (4) Chen, G. H., and Hoffman, A. S. (1993) Preparation and properties of thermoreversible, phase-separating enzyme–oligo(isopropylacrylamide) conjugates. *Bioconjugate Chem.* 4, 509–514.
- (5) Chen, G. H., and Hoffman, A. S. (1994) Synthesis of carboxylated poly(NIPAAM) oligomers and their application to form thermo-reversible polymer–enzyme conjugates. *J. Biomater. Sci., Polym. Ed.* 5, 371–377.
- (6) Takei, Y. G., Aoki, T., Sanui, K., Ogata, N., Okano, T., and Sakurai, Y. (1993) Temperature-responsive bioconjugates. 1. Synthesis of temperature-responsive oligomers with reactive end groups and their coupling to biomolecules. *Bioconjugate Chem.* 4, 42–46.
- (7) Takei, Y. G., Aoki, T., Sanui, K., Ogata, N., Okano, T., and Sakurai, Y. (1993) Temperature-responsive bioconjugates. 2. Molecular design for temperature-modulated bioseparations. *Bioconjugate Chem.* 4, 341–346.
- (8) Takei, Y. G., Matsukata, M., Aoki, T., Sanui, K., Ogata, N., Kikuchi, A., Sakurai, Y., and Okano, T. (1994) Temperature-responsive bioconjugates. 3. Antibody–poly(*N*-isopropylacrylamide) conjugates for temperature-modulated precipitations and affinity bioseparations. *Bioconjugate Chem.* 5, 577–582.
- (9) Bromberg, L. E., and Ron, E. S. (1997) Protein and peptide release from temperature-responsive gels and thermogelling polymer matrices. *Adv. Drug Delivery Rev.* (in press).
- (10) Schild, H. G. (1992) Poly(*N*-isopropylacrylamide): experiment, theory and application. *Prog. Polym. Sci.* 17, 163–249.
- (11) Webster, O. W., and Sogah, D. Y. (1989) Group transfer and aldol group transfer polymerization. In *Comprehensive Polymer Science* (G. C. Eastmond, A. Ledwith, S. Russo, and P. Sigwalt, Eds.) Vol. 4, pp 163–169, Pergamon Press, London.
- (12) Webster, O. W. (1987) Group-transfer polymerization. *Encyclopedia of Polymer Science and Engineering*, Vol. 7, pp 580–588, Wiley, New York.
- (13) Ilavsky, M., Hrouz, J., and Havlicek, I. (1985) Phase transition in swollen gels: 7. Effect of charge concentration on the temperature collapse of poly(*N,N*-diethylacrylamide) networks in water. *Polymer* 26, 1514–1518.
- (14) Bromberg, L., and Levin, G. (1996) Ion-selective gel that is sensitive to temperature, pH, and redox reactions. *Macromol. Rapid Commun.* 17, 169–172.
- (15) Bromberg, L., and Levin, G. (1996) Transesterification of poly(*N,N*-diethylacrylamide-co-glycol methacrylate) with diethyldithiophosphate. Facts and implications. *J. Polym. Sci., Part A: Polym. Chem.* 34, 2595–2602.
- (16) Freitag, R., Baltes, T., Eggert, M., Schügerl, K., and Bahr, U. (1994) Synthesis and characterization of a highly uniform, thermosensitive affinity macroligand precursor suitable for carbodiimid coupling. *Bioseparation* 4, 353–367.
- (17) Eggert, M., and Freitag, R. (1994) Poly-*N,N*-diethylacrylamide prepared by group transfer polymerization: synthesis, characterization, and solution properties. *J. Polym. Sci., Part A: Polym. Chem.* 32, 803–814.
- (18) Freitag, R., Baltes, T., and Eggert, M. (1994) Comparison of thermoreactive water-soluble poly-*N,N*-diethylacrylamide prepared by anionic and by group transfer polymerization. *J. Polym. Sci., Part A: Polym. Chem.* 32, 3019–3030.
- (19) Yang, F., and Zhuo, R. (1990) Synthesis and antitumor activity of poly(L-cysteine) bonded covalently 5-fluorouracil. *Polym. J.* 22, 572–577.
- (20) Wadhwa, M. S., Collard, W. T., Adami, R. C., McKenzie, D. L., and Rice, K. G. (1997) Peptide-mediated gene deliv-

- ery: influence of peptide structure on gene expression. *Bioconjugate Chem.* 8, 81–88.
- (21) Kabanov, A. V., and Alakhov, V. Yu. (1997) Micelles of amphiphilic block copolymers as vehicles for drug delivery. In *Amphiphilic Block Copolymers: Self-Assembly and Applications* (P. Alexandridis and B. Lindman, Eds.) Elsevier, Amsterdam (in press).
- (22) Bromberg, L., Orkisz, M., Roos, E., Ron, E. S., and Schiller, M. (1996) Interpenetrating networks of Poloxamer copolymers and poly(acrylic acid) as vehicles in controlled drug delivery. *Abstracts of the 4th European Symposium on Controlled Drug Delivery*, pp 126–131, Noordwijk aan Zee.
- (23) Chilkoti, A., Chen, G., Stayton, P. S., and Hoffman, A. S. (1994) Site-specific conjugation of a temperature-sensitive polymer to a genetically-engineered protein. *Bioconjugate Chem.* 5, 504–507.
- (24) Ainsworth, C., and Kuo, Y.-N. (1972) Ketene bis(tri-alkylsilyl)acetals: synthesis, pyrolysis and spectral studies. *J. Organomet. Chem.* 46, 73–87.
- (25) Miron, T., and Wilchek, M. (1982) A spectrophotometric assay for soluble and immobilized N-hydroxysuccinimide. *Anal. Biochem.* 126, 433–435.
- (26) Fields, R. (1971) The measurement of amino groups in proteins and peptides. *Biochem. J.* 124, 581–590.
- (27) Meienhofer, J., and Kuromizu, K. (1974) Catalytic hydrolysis in liquid ammonia: stability and cleavage of some protecting groups used in peptide synthesis. *Tetrahedron Lett.* 37, 3259–3262.
- (28) Terayama, H. (1952) Method of colloid titration (A new titration between polymer ions). *J. Polym. Sci.* 8, 243.
- (29) Oh, Y.-I., and Cushman, M. (1994) Synthesis and anti-HIV activity of poly(cysteic acid). *Bioorg. Med. Chem. Lett.* 4, 2245–2248.
- (30) Shibata, S. (1972) 2-Pyridyl compounds in analytical chemistry. In *Chelates in Analytical Chemistry* (H. A. Flaschka and A. J. Barnard, Eds.) Vol. 4, pp 1–232, Marcel Dekker, New York.
- (31) Wilson, R. W., and Bloomfield, V. A. (1979) Counterion-induced condensation of deoxyribonucleic acid. A light-scattering study. *Biochemistry* 18, 2192–2196.
- (32) Wadhwa, M., Knoell, D. L., Young, A. P., and Rice, K. G. (1995) Targeted gene delivery with a low molecular weight glycopeptide. *Bioconjugate Chem.* 6, 283–291.
- (33) Oscarsson, S., and Porath, J. (1993) Covalent chromatography. In *Molecular Interactions in Bioseparations* (T. T. Ngo, Ed.) pp 403–413, Plenum Press, New York.
- (34) Smith, P. K., Krohn, R. I., Hermanson, G. T., Malia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., and Klenk, D. C. (1985) Measurement of protein using bicinchoninic acid. *Anal. Biochem.* 150, 76–85.
- (35) Edsall, J. T. (1965) Dipolar ions and acid–base equilibria. In *Proteins, Amino Acids and Peptides as Ions and Dipolar Ions* (E. J. Cohn and J. T. Edsall, Eds.) pp 75–115, Hafner Publishing Co., New York.
- (36) Chen, G., and Hoffman, A. S. (1995) Graft copolymers that exhibit temperature-induced phase transition over a wide range of pH. *Nature* 373, 49–52.
- (37) Bromberg, L. (1997) Zein-poly(N-isopropylacrylamide) conjugates. *J. Phys. Chem. B* 101, 504–507.
- (38) Kabanov, A. V., and Kabanov, V. A. (1995) DNA complexes with polycations for the delivery of genetic material into cells. *Bioconjugate Chem.* 6, 7–20.
- (39) Kabanov, A. V., Astafieva, I. V., Maksimova, I. V., Lukandin, E. M., Georgiev, G. P., and Kabanov, V. A. (1993) Efficient transformation of mammalian cells using DNA interpolyelectrolyte complexes with carbon chain polycations. *Bioconjugate Chem.* 4, 448–454.
- (40) Oscarsson, S., and Porath, J. (1989) Covalent chromatography and salt-promoted thiophilic adsorption. *Anal. Biochem.* 176, 330–337.
- (41) Oscarsson, S., Medin, A., and Porath, J. (1992) Kinetic and conformational factors involved in chemisorption and adsorption of proteins on mercaptopyridine-derivatized agarose. *J. Colloid Interface Sci.* 152, 114–124.
- (42) Ding, Z., Chen, G., and Hoffman, A. S. (1996) Synthesis and purification of thermally sensitive oligomer-enzyme conjugates of poly(N-isopropylacrylamide)-trypsin. *Bioconjugate Chem.* 7, 121–125.

BC9701104

Capture of HIV-1 gp120 and Virions by Lectin-Immobilized Polystyrene Nanospheres[†]

Mitsuru Akashi,^{*,‡} Takashi Niikawa,[‡] Takeshi Serizawa,[‡] Tadao Hayakawa,[§] and Masanori Baba^{*,§}

Department of Applied Chemistry and Chemical Engineering, Faculty of Engineering, Kagoshima University, 1-21-40 Korimoto, Kagoshima 890, Japan, and Division of Human Retroviruses, Center for Chronic Viral Diseases, Faculty of Medicine, Kagoshima University, 8-35-1 Sakuragaoka, Kagoshima 890, Japan. Received March 28, 1997; Revised Manuscript Received October 7, 1997[®]

A lectin such as concanavalin A (Con A) was immobilized on the surfaces of poly(methacrylic acid) branches covered with polystyrene nanospheres with a diameter of 360 nm, which were obtained by the free radical copolymerization of styrene with the poly(*tert*-butylmethacrylate) macromonomer, followed by hydrolysis. Using Con A-immobilized (0.3 $\mu\text{g}/\text{cm}^2$) nanospheres, the interaction of the nanospheres with HIV-1 was determined by the reduction of the gp120 level and the viral infectivity of the HIV-1 suspensions after a 60 min incubation at room temperatures. Con A-immobilized nanospheres achieved a 95 and a 77% reduction of the gp120 level and the infectivity at a concentration of 0.5 mg/mL, respectively, indicating the effective capture of the gp120 and the virions.

INTRODUCTION

Human immunodeficiency virus-1 (HIV-1) is a spherical virus with a diameter of 100 nm and possesses an envelope with glycoprotein gp120 and gp41 (1). Gp120 is a mannose-rich glycoprotein, so it strongly interacts with some kinds of lectins (2, 3). In particular, concanavalin A (Con A) is reported to have a high affinity for gp120. Thus, there is a strong possibility that gp120 and HIV-1 virions will be effectively captured by lectins, if they are ideally immobilized to certain materials. Actually, purification of the HIV envelope glycoprotein by means of Con A–agarose affinity chromatography was reported (4).

Polymeric materials such as gels, films, beads, and particles are frequently used for the immobilization of biomolecules such as antibodies and enzymes in order to realize immuno assays (5, 6) and catalytic systems (7). Among these, polystyrene beads and particles are quite useful because of the ease of their preparation. Since there is no functional group on their surfaces, the immobilization of biomolecules by means of covalent bonds is inadequate. In addition, as the surfaces of the polystyrene beads and the particles are highly hydrophobic, nonspecific adsorption of other biomolecules seems to be unavoidable. To circumvent these problems, we recently developed technology for hydrophilic polymer chains with functional group-coated polystyrene nanospheres (8, 9), which is dispersed well in water and very useful for biomolecule immobilization. Consequently, these nanospheres are quite useful as immunolates, when antibodies, for example, are introduced into surface-

modified nanospheres. Moreover, the peptide drug calcitonin shows a pharmaceutical activity in oral administration when it has been physically immobilized onto polystyrene nanospheres coated with water-soluble polymer chains (10). In this paper, we will discuss the immobilization of lectins onto surface-modified polystyrene nanospheres and their capture of HIV-1 particles through affinity interaction between gp120 mannose and the immobilized lectins.

RESULTS AND DISCUSSION

First, poly(methacrylic acid)-covered polystyrene nanospheres were prepared by the copolymerization of styrene with the poly(*tert*-butyl methacrylate) macromonomer and the following acid hydrolysis, and analyzed according to the method described previously (8). Because methacrylic acid oligomers contain many active carboxyl groups that are inadequate for macromonomer synthesis, we prepared the poly(*tert*-butyl methacrylate) macromonomer by the reaction between poly(*tert*-butyl methacrylate) oligomers and *p*-vinylbenzyl chloride. The resulting nanospheres (mean diameter of 360 nm) were dispersed well in water and were completely collected by centrifugation or freeze-drying after dialysis. Electron spectroscopy for chemical analysis (ESCA) of the surface suggested that poly(methacrylic acid) chains were favorably located on the surfaces of the polystyrene particles. Scheme 1 illustrates the immobilization of Con A onto the surface of the polystyrene nanospheres. Lectins were immobilized on the surface of polystyrene particles by means of their amino group. Using water-soluble carbodiimide (WSC), the condensation between the carboxyl group of poly(methacrylic acid) on the polystyrene surface and the amino group of Con A gave Con A-immobilized polystyrene nanospheres in a 0.01 M HEPES buffer solution (pH 8.0) at 4 °C for 24 h. After reaction, the centrifuged nanospheres were repeatedly washed in order to correct the Con A-immobilized nanospheres. The surfaces of the obtained nanospheres were fully covered with Con A.

To investigate how Con A-immobilized polystyrene nanospheres can capture HIV-1, they were added to an

[†] This paper is part XIV in the series of the study on Graft Copolymers Having Hydrophobic Backbone and Hydrophilic Branches. Part XIII is as follows: Serizawa, T., and Akashi, M. (1997) *Chem. Lett.*, 809.

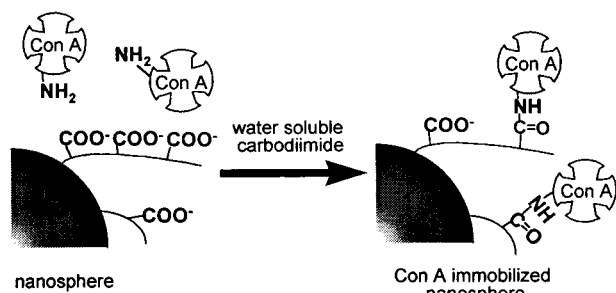
^{*} To whom correspondence should be addressed.

[‡] Department of Applied Chemistry and Chemical Engineering.

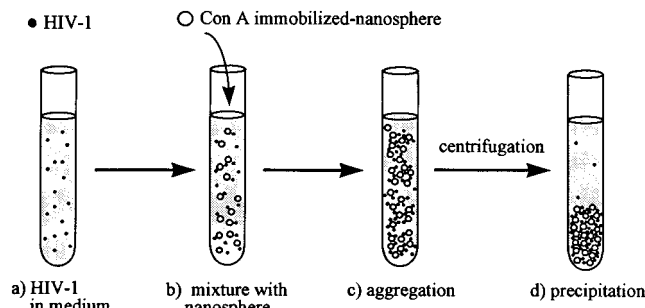
[§] Division of Human Retroviruses.

[®] Abstract published in *Advance ACS Abstracts*, December 15, 1997.

Scheme 1. Immobilization of Lectins onto the Surface of Polystyrene Nanospheres



Scheme 2. Capture of HIV-1 Virions by Lectin-Immobilized Polystyrene Nanospheres



HIV-1 medium, as shown in Scheme 2. The suspension (0.5 mL) of the Con A-immobilized nanospheres (final concentration of 0.5 mg/mL) was added to an equal volume of the medium that contained HIV-1 (III_B strain), and the mixed suspension was incubated at room temperature for 60 min. It was assumed that the Con A-immobilized nanospheres would be aggregated, if the HIV-1 virions or the gp120 could interact with the nanospheres. In fact, the Con A-immobilized nanospheres but not the control nanospheres apparently aggregated. Consequently, the Con A-immobilized nanospheres were easily precipitated by centrifugation (Scheme 2). The amount of captured HIV-1 was determined by the residual gp120 antigen level and the viral infectivity in the supernatants after centrifugation at 8200*g* for 10 min. The gp120 level was measured by using an HIV-1 gp120 antigen capture ELISA kit (Advanced Biotechnologies, Columbia, MD). Viral infectivity was assessed by microscopic observation for HIV-1-induced cytopathicity in MT-4 cells and expressed as a 50% cell culture infectious dose (CCID₅₀).

In these assay systems, Con A-immobilized nanospheres achieved a 95 and a 77% reduction of the gp120 level and the viral infectivity at a concentration of 0.5 mg/mL, respectively (Figure 1). In contrast, there was only a slight reduction of the gp120 level and the viral infectivity was identified for the nanospheres that were not immobilized with Con A (Figure 1). Since a small amount of free Con A was contaminated with the suspension of the Con A-immobilized nanospheres, we also examined the effects of the free Con A on the HIV-1 capture assays. The contamination-free Con A (0.79 µg/mL) did not significantly affect the gp120 level or the viral infectivity (data not shown). In order to know the capture activity using other lectins, the mannose-sepecific lectins such as lens culinaris agglutinin (LCA), galanthus nivalis lectin (GNL), and hippeastrum hybrid lectin (HHL) and also the galactose-specific ricinus communis agglutinin (RCA₁₂₀) were immobilized onto the nanospheres by the same procedure. The amounts of lectins immobilized onto the nanospheres and their capture

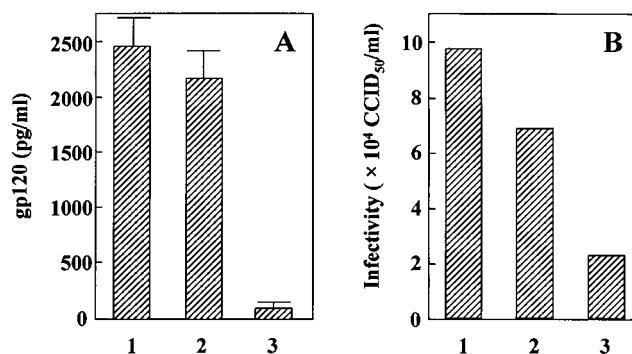


Figure 1. Effect of Con A-immobilized nanospheres on the (A) gp120 and (B) viral infectivity of HIV-1. The medium containing HIV-1 was mixed with an equal volume of (1) control medium, (2) control nanospheres (final concentration of 0.5 mg/mL), or (3) Con A-immobilized nanospheres (final concentration of 0.5 mg/mL). After a 60 min incubation at room temperature, the mixtures were centrifuged, and the supernatants were examined for their gp120 antigen level and viral infectivity. Data represent mean values in triplicate experiments.

Table 1. HIV-1 Capturing Using Various Lectin-Immobilized Nanospheres^a

lectin	immobilized amount (pmol/cm ²)	HIV-1 capturing ^b (%)
Con A	3.18	95
LCA	2.44	89
GNL	2.08	82
NHL	1.10	83
RCA ₁₂₀	7.30	39

^a The concentration of their nanospheres was 0.5 mg/mL.

^b Estimated by the ELISA technique.

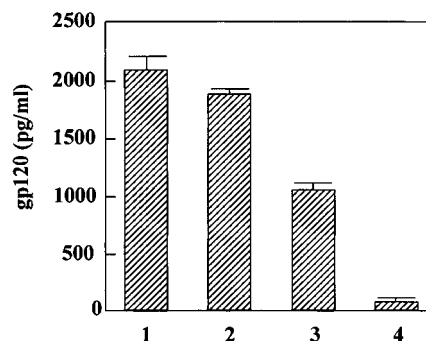


Figure 2. Dose-dependent HIV-1 capture by Con A-immobilized nanospheres. The medium containing HIV-1 was mixed with an equal volume of (1) control medium or Con A-immobilized nanospheres at a final concentration of (2) 0.02, (3) 0.1, and (4) 0.5 mg/mL. After a 60 min incubation at room temperature, the mixtures were centrifuged, and the supernatants were examined for their gp120 antigen level and viral infectivity. Data represent mean values in triplicate experiments.

activities are listed in Table 1. The LCA-, GNL-, and HHL-immobilized nanospheres still performed effective capture activity, even though smaller amounts of their lectins compared to Con A were immobilized. The steric hindrance or direction of binding pockets as well binding specificity of each lectin will be significant for their activity. Also, the RCA₁₂₀-immobilized nanospheres showed little effect on the HIV-1 capturing as expected from its specificity. Figure 2 shows dose-dependent HIV-1 capture activity of Con A-immobilized nanospheres. Even at a concentration of 0.1 mg/mL, the Con A-immobilized nanospheres reduced the gp120 level by 50%. To determine the specificity of the Con A-immobilized nanospheres, we conducted an inhibition assay

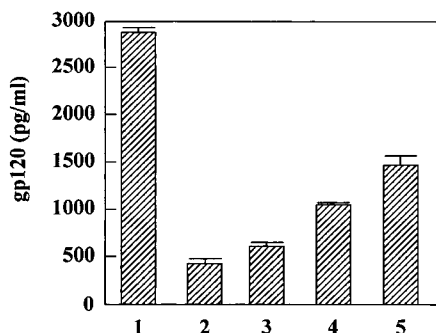


Figure 3. Mannose inhibits HIV-1 capture by Con A-immobilized nanospheres. The medium containing HIV-1 was mixed with an equal volume of (1) control medium or Con A-immobilized nanospheres (final concentration of 0.5 mg/mL) containing mannose at a final concentration of (2) 0, (3) 10, (4) 100, and (5) 250 mg/mL. After a 60 min incubation at room temperature, the mixtures were centrifuged, and the supernatants were examined for their gp120 antigen level and viral infectivity. Data represent mean values in triplicate experiments.

for HIV-1 capture by the addition of mannose. When an excess amount of mannose was added to the Con A-immobilized nanospheres, their capture activity was weakened in a dose-dependent fashion (Figure 3), indicating that the Con A-immobilized nanospheres primarily recognize the mannose molecules in gp120. Furthermore, capture activity was not hampered by the addition of galactose (data not shown).

In this study, we have shown that HIV-1 particles and the gp120 antigen can be effectively captured by lectin-immobilized nanospheres. Thus, the lectin-immobilized nanospheres may be potential chemotherapeutic or prophylactic agents in HIV-1 infection. Apparently, several issues, including efficacy and toxicity *in vivo*, still need to be elucidated. The selection of immobilized lectins and the diameter of nanospheres are important determinants for their capture activity and selectivity. In fact, nanospheres that immobilized mannose-specific lectins other than Con A were found to be equally effective with a smaller amount of immobilization (data not shown). Further research is now in progress that will attempt to obtain more effective and selective nanospheres for the capture of HIV-1.

EXPERIMENTAL PROCEDURES

Materials. Styrene (Wako Pure Chemical Ind., Tokyo, Japan) and *tert*-butyl methacrylate (Wako Pure Chemical Ind.) were used after distillation under reduced pressure. 2-Mercaptoethanol (2-MEtOH) (Wako Pure Chemical Ind.), sodium hydride (NaH) (Nacalai Tesque, Kyoto, Japan), and tetrabutylphosphonium bromide (TBPB) (Wako Pure Chemical Ind.) were used without further purification. 2,2'-Azobis(isobutyronitrile) (AIBN) was recrystallized from methanol and dried under vacuum. Tetrahydrofuran (THF), *N,N*-dimethylformamide (DMF), ethanol, water, and other solvents were distilled by the ordinary ways. *p*-Chloromethylstyrene (CMSt) was a gift from Nippon Oil and Fats Co. (Tokyo, Japan) and used without further purification.

Preparation of *tert*-Butyl Methacrylate Oligomers. *tert*-Butyl methacrylate oligomers that were terminated with a hydroxyl group were prepared by the free radical polymerization of *tert*-butyl methacrylate with AIBN as an initiator in the presence of 2-mercaptoethanol as a chain transfer reagent (8). For instance, into a 200 mL three-necked round-bottomed flask equipped with a mechanical stirrer, a condenser, and a nitrogen

inlet tube were placed 176 mmol of *tert*-butyl methacrylate, 0.176 mmol of AIBN, and 2.18 mmol of 2-mercaptoethanol in 60 mL of THF. After dry nitrogen gas was bubbled into the reaction media at 0 °C for 30 min, it was kept at 60 °C for a further 6 h. The reaction mixture was poured into H₂O/methanol (1/1, v/v). The precipitated polymer was filtered off and dried under reduced pressure. The polymer was resolved again in 2-propanol, and then the solution was poured into H₂O/methanol (1/1, v/v). The purification procedure was repeated several times. The yield was 74%. The M_n and M_w/M_n were 3870 and 2.07, respectively.

Preparation of Poly(*tert*-butyl methacrylate) Macromonomers. The macromonomer was prepared by combination of CMSt with a hydroxyl group of the oligomer under an alkaline condition (8). The 0.78 mmol of the *tert*-butyl methacrylate oligomer was stirred with a 10-fold molar excess of NaH in 100 mL of DMF at 30 °C for 30 min. After that, the 10-fold molar excess (7.8 mmol) of CMSt was added into the flask and the mixture stirred for another 48 h in the presence of TBPB as a phase transfer catalyst. The reaction mixture was poured into H₂O/methanol (1/1, v/v). The precipitated polymer was filtered off and dried under reduced pressure. The polymer was resolved again in 2-propanol, and then the solution was poured into H₂O/methanol (1/1, v/v). The purification procedure was repeated several times. The yield was 84%. The M_n and M_w/M_n were 4100 and 1.97, respectively.

Preparation of Poly(*tert*-butyl methacrylate)-Covered Polystyrene Nanosphere. The poly(*tert*-butyl methacrylate)-covered polystyrene nanosphere was prepared by the free radical polymerization of the corresponding macromonomer and styrene in a polar solvent (8). The 0.115 mmol of the poly(*tert*-butyl methacrylate) macromonomer and the 40-fold molar excess (4.6 mmol) of styrene were weighted into a glass tube with 0.047 mmol of AIBN and 5 mL of ethanol, and then the mixture was degassed by freeze-thaw cycles on a vacuum apparatus, sealed off, and kept at 60 °C for 24 h. The reaction medium was dialyzed in methanol by using a cellulose dialyzer for 5–6 days. The nanosphere was obtained by lyophilization. The yield was 41%. The particle size and its standard deviation of the size were 199 and 34 nm, respectively.

Preparation of Poly(methacrylic acid)-Covered Polystyrene Nanosphere. The poly(methacrylic acid)-covered polystyrene nanosphere was prepared by hydrolysis of the poly(*tert*-butyl methacrylate) that was introduced on the poly(*tert*-butyl methacrylate)-covered polystyrene nanosphere under an acidic condition (8). An adequate amount of the poly(*tert*-butyl methacrylate)-covered polystyrene nanosphere was dispersed in a 12 N HCl water/ethanol (1/5, v/v) solution. The dispersion was heated at 70 °C for 24 h. The reaction mixture was dialyzed in water for several days. The particle size was 360 nm, which was larger than that of the poly(*tert*-butyl methacrylate)-covered polystyrene nanosphere. It seemed that electrostatic repulsion of anionic poly(methacrylic acid) on the nanosphere enlarged its size. The standard deviation was 120 nm, which means its size distribution became slightly larger with the acid hydrolysis of the poly(*tert*-butyl methacrylate).

Immobilization of Con A onto Poly(methacrylic acid)-Covered Polystyrene Nanosphere. A carboxyl group of polystyrene nanospheres (2.5 mg) was first activated by WSC (1.0 mg/mL in 0.05 M KH₂PO₄) for 30 min. The nanospheres that were obtained by centrifugation were mixed with 1.0 mg of lectin in 1.0 mL of 0.01

M HEPES buffer solution (pH 8.0), and the mixture was kept at 4 °C for 24 h. After reaction, the centrifuged nanospheres were repeatedly washed. The amount of immobilized lectin was evaluated by means of the ninhydrin method. The surfaces of the obtained nanospheres were covered with 0.3 μg of Con A/cm².

Characterization Method. The number-average molecular weights (M_n) of the oligomer and macromonomer were determined by GPC (Shimadzu LC-6A system with a Shodex AC-800P column), calibrated with polystyrene standards. The terminal vinylbenzyl group of the macromonomer was confirmed by ¹H-NMR spectra obtained with a JEOL FX-400 (400 MHz) instrument. Particle-size measurements were achieved by direct observation with a scanning electron microscope (SEM) (Hitachi H-7010A) and a submicron particle analyzer (Coulter model N4SD). Surface analysis by ESCA was carried out a Shimadzu ESCA 1000 apparatus.

Viruses. The III_B strain of HIV-1 was used in the experiments. The virus was propagated in MOLT-4 cells. The infected cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum and antibiotics (culture medium), and their culture supernatants were centrifuged at 3000 rpm for 10 min, filtered, and stored at -80 °C until they were used. The infectivity of viral stocks was determined in MT-4 cells, and their gp120 antigen level was measured by a gp120 antigen-capture ELISA kit (Advanced Biotechnologies).

ACKNOWLEDGMENT

This work was financially supported in part by a Grant-in-Aid for Scientific Research in Priority Areas of New Polymers and Their Nano-Organized Systems (277/08246243) and a Grant-in-Aid for Scientific Research (08458291) from the Ministry of Education, Science, Sports and Culture, Japan.

LITERATURE CITED

- (1) Evans, L. A., and Levy, J. A. (1993) The heterogeneity and pathogenicity of HIV. In *HIV Molecular Organization, Pa-*

- thology and Treatment* (W. G. W. Morrow and N. L. Haigwood, Eds.) pp 29-73, Elsevier, Amsterdam.
- (2) Lifson, J., Coutre, S., Huang, E., and Engleman, E. (1986) Role of envelope glycoprotein carbohydrate in human immunodeficiency virus (HIV) infectivity and virus-induced cell fusion. *J. Exp. Med.* 164, 2101-2106.
- (3) Balzarini, J., Schols, D., Neyts, J., Van Damme, E., Peumans, W., and De Clercq, E. (1991) α -(1-3)- and α -(1-6)-D-Mannose-specific plant lectins are markedly inhibitory to human immunodeficiency virus and cytomegalovirus in vitro. *Antimicrob. Agents Chemother.* 35, 410-416.
- (4) Mbemba, E., Carre, V., Atemezem, A., Saffar, L., Gluckman, J. C., Gattegno, and L. (1996) Inhibition of human immunodeficiency virus infection of CD4+ cells by CD4-free glycopeptides from monocytic U937 cell. *AIDS Res. Hum. Retroviruses* 12, 47-53.
- (5) Singer, J. M., and Plotz, C. M. (1956) The latex fixation test: (1) application to the serologic diagnosis of rheumatoid arthritis. *Am. J. Med.* 21, 888-892.
- (6) Kawaguchi, H. (1993) Polymer materials for bioanalysis and bioseparation. In *Biomedical Applications of Polymeric Materials* (T. Tsuruta, T. Hayashi, K. Kataoka, K. Ishihara, and Y. Kimura, Eds.) Chapter 5, pp 299-324, CRC Press, Boca Raton, FL.
- (7) Akashi, M., Maruyama, I., Fukudome, N., and Yashima, E. (1992) Immobilization of human thrombomodulin on glass beads and its anticoagulant activity. *Bioconjugate Chem.* 3, 363-365.
- (8) Riza, M., Tokura, S., Iwasaki, M., Yashima, E., Kishida, A., and Akashi, M. (1995) Graft copolymers having hydrophobic backbone and hydrophilic branches. X. Preparation and properties of water-dispersible microspheres having poly(methacrylic acid) branches on their surface. *J. Polym. Sci., Part A: Polym. Chem.* 33, 1219-1225.
- (9) Chen, M. Q., Kishida, A., and Akashi, M. (1996) Graft copolymers having hydrophobic backbone and hydrophilic branches. XI. Preparation and thermosensitive properties of polystyrene microspheres having poly(*N*-isopropylacrylamide) branches on their surface. *J. Polym. Sci., Part A: Polym. Chem.* 34, 2213-2219.
- (10) Sakuma, S., Suzuki, N., Kikuchi, H., Hiwatari, K., Arikawa, K., Kishida, A., and Akashi, M. (1997) Oral Peptide Delivery Using Nanoparticles Composed of Novel Graft Copolymers Having Hydrophobic Backbone and Hydrophilic Branches. *Int. J. Pharm.* 149, 93-106.

BC970045Y

Terminal Groups in Starburst Dendrimers: Activation and Reactions with Proteins[†]

Pratap Singh*

Dade Behring Inc., P.O. Box 520672, Miami, Florida 33152-0672. Received April 2, 1997;
Revised Manuscript Received August 21, 1997[®]

Starburst dendrimers are novel nanoscopic synthetic polymers of defined molecular mass and geometry. These macromolecules, available in commercial quantities, contain methyl carboxylate and primary amino-terminal groups. The presence of these groups on any macromolecule limits its usefulness especially in cases where, for specific modulation of the properties of biologically active molecules, covalent bond formation is desirable between the biologically active molecules and the macromolecule. This paper describes activation of the surface groups of Starburst dendrimers for incorporation of a number of reactive electrophilic and nucleophilic groups and utilization of these reactive groups in formation of covalent bonds between dendrimers and alkaline phosphatase. The protein–dendrimer complexes have been reacted further with the Fab' fragment of an anti-creatine kinase MB isoenzyme antibody to form multifunctional dendrimer reagents. The enzymatic and immunochemical properties of these protein–dendrimer reagents have been evaluated by an immunoassay system. Nucleophilic thiols and electrophilic phenyliodoacetamido, iodoacetamido, and epoxy groups have been incorporated into amino-terminal dendrimers by their reactions with appropriate heterobifunctional reagents. Two independent sets of reactions have been used to prepare the reactive *N*-hydroxysuccinimidyl esters from dendrimers containing the terminal carboxyl groups. Quantitation of the reactive groups has been carried out by direct titration of these activated dendrimers and the products obtained by reactions of these dendrimers with small molecules and proteins.

INTRODUCTION

In recent years, dendrimers (1–6) have attracted attention as structurally unique synthetic macromolecules with a very broad range of applications in physical, chemical, and biological processes (7–12). Dendrimers are synthesized by either a divergent or a convergent approach (1). The method of choice depends on the desired generation (4) of a specific structure, which in turn dictates the molecular mass, the terminal functional groups, and the physical dimensions of the molecule.

To modulate some specific properties of biologically active molecules, a number of these molecules that show specific activity such as drugs, enzymes, antibodies, and nucleotides have been covalently coupled to macromolecules [e.g. poly(ethylene glycol) or polysaccharides]. Very limited chemical manipulations are generally possible on many of the biologically active molecules, of either natural or synthetic origin, due to the labile nature or specific structural requirements for the maintenance of their biological activity.

Functional groups on the surface of dendrimers show remarkably higher chemical reactivity (13) in comparison to their activity when present in other macromolecules. This unusual reactivity is expected to allow coupling reactions to occur under mild reaction conditions. These characteristics make a dendrimer, with its controlled interior, the macromolecule of choice for covalent coupling

reactions. A biologically active molecule is likely to retain its maximum possible activity when present in a dendrimer–biologically active molecule complex prepared via the surface groups of dendrimers. The feasibility of such a concept has been demonstrated by preparation of dendrimer–peptide (14), dendrimer–antibody (11, 15), dendrimer–fullerene (16), and dendrimer–antibody–porphyrin (17) complexes. However, full exploitation of these molecules for a number of applications is limited due to the nature of the terminal functional groups available in the presently known dendrimer structures. A limited number of dendrimers are known (18) to contain reactive terminal groups. However, these reactive dendrimers have been synthesized by the convergent method, where it is often difficult to prepare large quantities of higher-generation macromolecules, thus limiting the choice of available molecules. Kilogram quantities of highly pure dendrimers, with broad molecular mass ranges, that have been synthesized by the divergent method are available (19, 20). One category

[†] Abbreviations: ALP, calf intestine alkaline phosphatase; ALP–dendrimer (D_n), ALP–dendrimer complex prepared by reaction of ALP with the activated dendrimer of the *n*th generation; ALP–PDH, 6-[3-(2-pyridyldithio)propionamide]hexanoate derivative of ALP; BAHA, [6-(bromoacetamido)hexyl]-amine; CKMB, creatine kinase MB isoenzyme; dendrimer–PDH, 6-[3-(2-pyridyldithio)propionamide]hexanoate derivative of dendrimer; dendrimer–X, derivatized dendrimer containing the surface group X; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]-carbodiimide hydrochloride; NIA, *N*-hydroxysuccinimidyl iodoacetate; sulfo NHS LC SPDP, sulfosuccinimidyl 6-[3-(2-pyridyldithio)propionamide]hexanoate; sulfoSIAB, sulfosuccinimidyl (4-iodoacetyl)aminobenzoate; TSTU, *N,N,N',N'*-tetramethyl-*O*-(*N*-succinimidyl)uronium tetrafluoroborate.

[†] Presented as a poster at the Fourth Pacific Polymer Conference held at Koloa, Kauai, HI, on December 12–16, 1995.

* Author to whom correspondence should be addressed. Telephone: (305) 591-5556. Fax: (305) 597-5176.

[®] Abstract published in *Advance ACS Abstracts*, December 15, 1997.

of dendrimers available in large quantities is the full and half-generations of Starburst dendrimers (1).

This paper describes methods for modifying the terminal amino and carboxyl groups of Starburst dendrimers (1) to prepare dendrimer derivatives containing a defined number of reactive nucleophilic and electrophilic groups. These reactive groups have been utilized to couple two proteins in sequence, namely calf intestine alkaline phosphatase (ALP)¹ and the Fab' fragment of an anti-creatine kinase MB isoenzyme (CKMB) antibody, to form ALP-dendrimer and ALP-dendrimer-Fab' complexes. The performance characteristics of these protein-dendrimer complexes have been studied on Stratus II, an automated enzyme immunoassay system.

MATERIALS AND METHODS

All reagents used were of analytical grade or better and were purchased from either Aldrich Chemical Co. (Milwaukee, WI), Sigma Chemical Co. (St. Louis, MO), or Fluka Chemical Corp. (Ronkonkoma, NY). The heterobifunctional reagents were purchased from BioAffinity Systems Inc. (Rosco, IL). Ultrogels AcA 44 and AcA 34 were obtained from BioSeptra (Marlborough, MA). BCA protein assay reagent was obtained from Pierce (Rockford, IL). Alkaline phosphatase was obtained from Boehringer Mannheim Corp. (Indianapolis, IN). Mass spectra were run by the Mass Spectrometry Laboratory of the University of California at Riverside. The performance of the protein-dendrimer complexes was evaluated on Stratus II, an automated fluorometric enzyme immunoassay system available from Dade International Inc. (Miami, FL). The ALP-Fab' conjugate, sold commercially in the CKMB assay kit for Stratus II, was used to compare the performance of the ALP-dendrimer-Fab' complexes.

Unless otherwise indicated, all reactions were carried out at room temperature. All full and half-generation poly(amidoamine) dendrimers, with ethylenediamine as the initiator core molecule (1), were obtained from Dendritech, Inc. (Midland, MI). The half-generation dendrimers were obtained as the sodium salts prepared by hydrolysis of the corresponding methyl esters with sodium hydroxide. Partially hydroxylated dendrimers were prepared at Dendritech, Inc., by using a mixture of ethylenediamine and ethanolamine in place of ethylenediamine at the final stage of synthesis. This modified synthetic procedure was used to prepare dendrimers containing a mixture of terminal hydroxyl (77%) and primary amino (23%) groups. Partially carboxymethylated dendrimer was prepared by treating a methanol solution of the dendrimer with 0.85 mol of bromoacetic acid at 37 °C for 4 h. The methanol solution was evaporated under reduced pressure, and the number of amino groups remaining in the treated dendrimer was determined by reaction with fluorescamine (21).

Derivatized dendrimers of generations 1–3 were purified by repeated precipitation of a methanol solution on addition of ethyl acetate, benzene, or dioxane. The 4.0-, 4.5-, and 5th generation dendrimers were purified by ultrafiltration in an Amicon cell using a YM 10 membrane (molecular mass cutoff of 10 kDa). The Amicon concentrate, containing the derivatized dendrimer solution, was either used immediately to react with a protein or evaporated under reduced pressure to isolate the derivative. The ALP-dendrimer complexes were separated from the low-molecular mass contaminants, including the derivatized dendrimers by ultrafiltration (Amicon cell with a YM 10 membrane) or by gel filtration. A

column of either Sephadex G-25 or Ultrogel AcA 44 (fractionation range of 10–130 kDa; exclusion limit of 200 kDa) equilibrated with a buffer containing 100 mM phosphate/1.0 mM magnesium chloride (phosphate buffer) at pH 7.0–7.4 was used for gel filtration.

Free thiols in the derivatized dendrimer and the protein-dendrimer-SH complex were determined by measuring the increase in absorption at 324 nm on addition of a solution of 4,4'-dithiodipyridine (22). Titration of electrophilic epoxy, phenyliodoacetamido, and iodoacetamido groups in the derivatized dendrimers and the dendrimer-X and protein-dendrimer-X complex (X = epoxy, phenyliodoacetamido, and iodoacetamido) was carried out by reaction with an excess of dithiothreitol followed by reaction of the remaining dithiothreitol with 4,4'-dithiodipyridine essentially as previously described (23). The *N*-hydroxysuccinimidyl esters were quantitated as described by Miron and Wilchek (24).

The periodate-oxidized ALP was prepared by a procedure similar to that described by Husain and Bieniarz (25). ALP-SH was prepared by the reaction of ALP with sulfosuccinimidyl 6-[3-(2-pyridyldithio)propionamido]hexanoate (sulfo NHS LC SPDP; 26) followed by reaction with dithiothreitol. Excess dithiothreitol was separated from the protein solution by passage over a Sephadex G-25 column equilibrated with the phosphate buffer at pH 7.0. The poly(amidoamine) dendrimers and many of the derivatives prepared in this study do not contribute to absorptions between 260 and 280 nm. Protein concentrations (mg/mL) of the solutions containing ALP and ALP-dendrimer complexes were therefore calculated from absorptions measured at 280 and 260 nm and using the formula $A_{280}/0.689 - A_{260}/1.351$. In this formula, 0.689 and 1.351 are the extinction coefficients (milliliters per milligram per centimeter) at 280 and 260 nm, respectively. BCA protein assay reagent was used to determine protein concentrations for the ALP-dendrimer complexes prepared from a dendrimer derivative containing an aromatic or another chromophoric group, e.g. sulfosuccinimidyl (4-iodoacetyl)aminobenzoate (sulfoS-IAB)-activated dendrimer.

Preparation and Reactions of Dendrimer Derivatives Containing Phenyliodoacetamido 1 and Iodoacetamido Groups 3. The carboxymethylated 5th generation dendrimer (16 mg, 0.5 μ mol) was dissolved in 1 mL of the phosphate buffer at pH 7.4. A 277 μ L solution of sulfoSIAB (10 mg/mL in water, 5.5 μ mol) was then added. After 1 h at 30 °C, the reaction mixture was diluted with 50 mL of the phosphate buffer at pH 7.6 and concentrated to about 2 mL in an Amicon ultrafiltration cell. This dilution-concentration process was repeated two more times. The dendrimer derivative 1 present in about 1 mL of concentrate was combined with a 1 mL solution of the periodate-oxidized ALP (8.1 mg, 58 nmol) in the phosphate buffer at pH 7.4 and the combined mixture concentrated to 1.2 mL. After 16 h at 4 °C, the reaction mixture was adjusted to pH 6.3 by a careful addition of 1 N HCl and then incubated for 1 h with a 200 μ L aqueous solution of sodium cyanoborohydride (30 mg/mL, 95 μ mol). The ALP-dendrimer complex 2 formed by this sequence of reactions was purified by passage over an Ultrogel AcA 44 column equilibrated and eluted with the phosphate buffer at pH 7.4.

A solution of *N*-hydroxysuccinimidyl iodoacetate (NIA, 60.4 mg, 213.4 μ mol) in 1 mL of tetrahydrofuran was added dropwise to a solution of the 1st generation aminoterminal dendrimer (32.7 mg, 20.4 μ mol) in 2 mL of 50% alcohol. After 1 h, excess solvents were removed under reduced pressure, and the residue was washed with

tetrahydrofuran. The tetrahydrofuran-insoluble semi-solid product, the iodoacetamido-dendrimer **3**, was dried. The solid residue dissolved in 1 mL of alcohol and 0.5 mL of the phosphate buffer at pH 7.0 was mixed with a solution of ALP-SH (11.9 mg, 85 nmol) in 12 mL of the phosphate buffer at pH 7.0. ALP-SH, used in these reactions, contained an average of 1.4 free thiols per mole of protein. After incubation for 16 h at 4 °C, the protein-dendrimer complex **4** was purified over a column of Ultrogel AcA 44 in the phosphate buffer at pH 7.0.

Preparation of Dendrimer Derivatives 6 Containing Epoxy Groups. A solution of the 5th generation dendrimer (165.8 mg, 5.7 μ mol), in 1 mL of 50% alcohol, was mixed with sodium carbonate (200 mg, 1.9 mmol) and epibromohydrin (0.96 g, 7.1 mmol). After stirring for 4 h, the reaction mixture was diluted with 10 mL of 50% alcohol. The solution was concentrated to about 1 mL in an Amicon ultrafiltration cell. The concentrate was diluted again to 10 mL with 50% alcohol. This concentration-dilution process was continued till the effluent was neutral (pH paper). The dendrimer derivative **6** present in the concentrate was stored at 4 °C until it was used for reaction with ALP.

A 0.4 mL alcohol solution of the 2nd generation dendrimer (41.5 mg, 13 μ mol) was mixed with epibromohydrin (0.5 g, 3.7 mmol) and triethylamine (36 mg, 0.36 mmol). After 4 h at 37 °C, the reaction mixture was evaporated under vacuum to a semisolid residue. The product **6** was purified by repeated precipitation (alcohol/benzene). A solution of this product in 0.2 mL of alcohol and 0.2 mL of the phosphate buffer at pH 7.2 was mixed with a 1.5 mL solution of ALP-SH (1.9 mg, 13.6 nmol) in the phosphate buffer at pH 7.2. The ALP-SH used for this reaction contained 2.6 free thiols per mole of the protein. After 16 h at 4 °C, the ALP-dendrimer complex **7**, formed in the reaction mixture, was then separated from other contaminants by passage over a Sephadex G-25 column in the phosphate buffer at pH 7.2.

The succinyl-dendrimer-epoxide was prepared by stirring a reaction mixture containing the 5th generation dendrimer (222 mg, 7.7 μ mol) and succinic anhydride (148 mg, 1.5 mmol) in 5 mL of 50% alcohol. After 2 h, epibromohydrin (2 mL, 23.5 mmol) was added and the mixture stirred for an additional 16 h. The derivatized dendrimer was purified by ultrafiltration in an Amicon cell (YM 10 membrane) using 50% alcohol as described above.

Reaction of Dendrimers To Incorporate Thiol Groups. A 3.0 mL methanolic solution of the 5th generation dendrimer (330 mg, 11.4 μ mol) was flushed with nitrogen. The dendrimer solution was then allowed to react for 1 h with a solution of sulfo NHS LC SPDP (50 mg, 95 μ mol) in 0.5 mL of water. This reaction produced the 6-[3-(2-pyridyldithio)propionamide]hexanoate derivative of dendrimer **8**, i.e. NH_2 -dendrimer- $\text{NHCO}(\text{CH}_2)_5\text{NHCO}(\text{CH}_2)_2\text{S-S-Py}$ (dendrimer-PDH). The methanol solution was evaporated under vacuum and the semisolid residue stored at -10 °C.

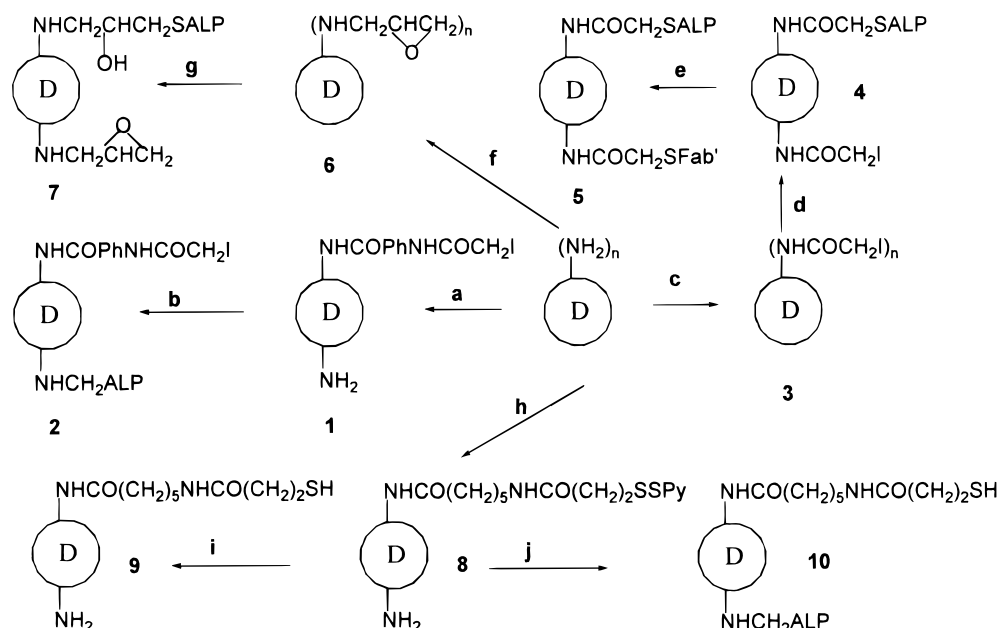
To block the residual amino groups in the dendrimer-PDH derivative prepared above, a 1.8 mL methanol solution of this derivative (148 mg, 5 μ mol) was mixed with succinic anhydride (550 mg, 5.5 mmol) and 2 mL of tetrahydrofuran. The trinitrobenzenesulfonic acid color test (27) was used to monitor reaction of the primary amino groups in the dendrimer-PDH derivative. After 24 h, the organic solvents were removed by evaporation under reduced pressure and the dendrimer derivative was separated from the side products by Amicon ultrafiltration of the mixture using water. The aqueous

solution of the succinyl-dendrimer-PDH, present in the Amicon concentrate, was evaporated under vacuum to provide 173 mg of the succinyl-dendrimer-PDH derivative as an oil.

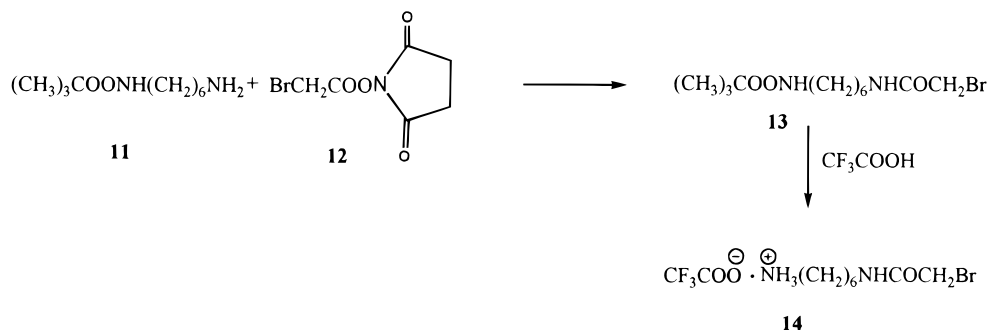
The 2nd generation dendrimer (75 mg, 23.4 μ mol) dissolved in 4 mL of methanol was reacted for 1 h with a solution of sulfo NHS LC SPDP (55 mg, 104.4 μ mol) in 0.5 mL of water. The reaction mixture containing this dendrimer-PDH derivative **8** was concentrated to 1.0 mL by evaporation under reduced pressure and then mixed with a 9 mL solution of the periodate-oxidized ALP (18 mg, 128.6 nmol) in 50 mM sodium phosphate/50 mM sodium carbonate at pH 9.0. After 1 h, the reaction mixture was adjusted to pH 6.5 by a careful addition of 1 N HCl. An aqueous solution (0.7 mL) of sodium cyanoborohydride (27 mg, 0.44 mmol) was then added. After 30 min, the resulting ALP-dendrimer-PDH complex was buffer exchanged with the phosphate buffer at pH 7.0 in an Amicon ultrafiltration cell (YM 10). The dithiothreitol/4,4'-dithiodipyridine titration of this ALP-dendrimer-PDH complex showed the presence of 25 free thiols (as pyridyl disulfide linkage) per mole of the protein. The thiol content was calculated from the increase in absorption at 343 nm resulting from the formation of pyridine-4-thione.

The dendrimer derivative succinyl-dendrimer-PDH (43 mg, 1.5 μ mol) dissolved in 1.0 mL of 100 mM sodium phosphate/5 mM EDTA at pH 6.0 (phosphate/EDTA buffer) was mixed with a 0.2 mL solution of dithiothreitol (3.1 mg, 20 μ mol) in the phosphate/EDTA buffer. After 1 h at 30 °C, the reaction mixture was passed through a Sephadex G-25 column equilibrated and eluted with the phosphate/EDTA buffer. The column fractions, containing the dendrimer derivative with free thiols, were monitored by measuring the increased absorption at 324 nm on addition of a solution of 4,4'-dithiodipyridine (22) to aliquots of the individual fractions. The dendrimer derivatives dendrimer-PDH and ALP-dendrimer-PDH were similarly treated with dithiothreitol to provide the derivatives NH_2 -dendrimer-SH **9** and ALP-dendrimer-SH **10**, respectively. The derivative ALP-dendrimer-SH **10** was separated from excess dithiothreitol either by a Sephadex G-25 column or by an exchange with the phosphate/EDTA buffer in an Amicon ultrafiltration cell till the effluent showed a negative reaction on addition of a solution of 4,4'-dithiodipyridine. A titration of the NH_2 -dendrimer (D5)-SH **9** and succinyl-dendrimer (D5)-SH derivatives with 4,4'-dithiodipyridine (22) showed the presence of 3.8 and 3.7 thiols per mole of the dendrimer, respectively.

Synthesis of [6-(Bromoacetamido)hexyl]amine (BAHA). BAHA **14** was synthesized (Scheme 2) by a procedure similar to that described by Heindel et al. (28). In brief, 2 mL of a methanol solution of *N*-BOC-1,6-diaminohexane **11** (0.5 g, 2.3 mmol) was added to a solution of *N*-hydroxysuccinimidyl bromoacetate **12** (0.5 g, 2.1 mmol) in 5.0 mL of tetrahydrofuran. The ethyl acetate solution (20 mL) of the residue, obtained on evaporation of the organic solvents, was washed with 10 mM HCl (3 $\times$ 5 mL). The dichloromethane solution (5.0 mL) of the dry intermediate **13**, obtained after removal of ethyl acetate, was mixed with trifluoroacetic acid (5 mL, 65 mmol). The trifluoroacetate salt of BAHA **14** was obtained in 50% yield as a white hygroscopic solid on crystallization from 2-propanol/hexane: mp 114-115 °C (corrected). The purity of the material was determined to be >90% by quantitation of the bromoacetamido groups as described before (23). MS *m/z* (relative intensity): 236, 238 (M^+ , due to two isotopes of bromine of

Scheme 1. Activation and Reactions of Dendrimers Containing Terminal Amino Groups^a

^a (a) sulfoSIAB; (b) ALP-CHO, sodium cyanoborohydride; (c) NIA; (d) ALP-SH; (e) Fab'; (f) epibromohydrin; (g) ALP-SH; (h) sulfo NHS LC SPDP; (i) dithiothreitol; (j) ALP-CHO, sodium cyanoborohydride, dithiothreitol.

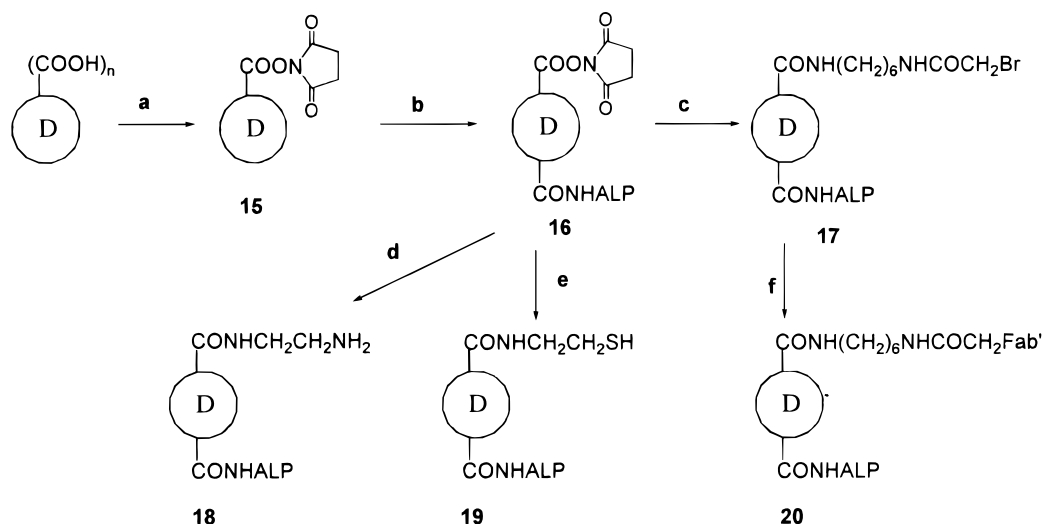
Scheme 2. Synthesis of [6-(Bromoacetamido)hexyl]amine

atomic weights 79 and 81; 28%), 121, 123 (the bromoacetyl residue; 58%), and 116 [the protonated form of the residue $\text{NH}_2(\text{CH}_2)_6\text{NH}$; 100%].

Activation of the Half-Generation Carboxy-Terminal Dendrimers. The sodium salts of the half-generation dendrimers, dissolved in water, were converted to the acid form by acidification with 5 N hydrochloric acid to pH 3.0. The acidified solutions were evaporated to dryness under high vacuum. Activation reaction of the free acid form of these dendrimers was carried out with *N,N,N',N'*-tetramethyl-*O*-(*N*-succinimidyl)uronium tetrafluoroborate (TSTU) essentially as described by Bannwarth and Knorr (29). A solution containing the 2.5th generation dendrimer (30 mg, 7.2 μmol) in 0.2 mL of water and 0.8 mL of DMF was mixed with *N,N*-diisopropylethylamine (90 mg, 0.7 mmol) and TSTU (74 mg, 0.24 mmol). After 1 h, solvents were removed from a red reaction mixture under reduced pressure. The residue was washed with ethyl acetate (3×10 mL), and the red residue obtained on removal of all the solvents was dissolved in 0.4 mL of water. The aqueous solution of the dendrimer-active ester **15** (Scheme 3) was stored in an ice bath while the active ester concentration was being determined by titration (24). A 100 μL aliquot of the aqueous solution, containing 4.4 μmol of the active ester as determined by titration, was mixed with 1 mL of a refrigerated solution of ALP (3 mg, 21 nmol) in the phosphate buffer at pH 7.0. After

incubation for 30 min in an ice bath, the reaction mixture was combined with 0.4 mL of a 2-propanol solution of BAH (20 mg, 62.5 μmol) and stirred gently at 4 $^\circ\text{C}$ for 16 h. The ALP-dendrimer complex **17**, containing reactive bromoacetamido groups, was purified over an Ultrogel AcA 44 column in the phosphate buffer at pH 7.0. To incorporate other functional groups, the intermediate **16**, obtained after reaction of ALP with the activated dendrimer, was combined separately with either a 1 mL solution of 100 mM ethylenediamine or 2-aminoethanethiol in the phosphate buffer to react with a homo- or heterobifunctional nucleophile. These reactions produced the complexes ALP-dendrimer- NH_2 **18** and ALP-dendrimer-SH **19**, respectively.

A solution of the free acid form of the 1.5th generation carboxyl-terminal dendrimer (33.4 mg, 15.2 μmol) in 1 mL of 80% DMF was mixed with *N*-hydroxysuccinimide (115 mg, 1 mmol) and 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDC, 200 mg, 1 mmol). The reaction mixture was stirred for 16 h followed by washing the reaction mixture with ethyl acetate (2×5 mL) and 2-propanol (2×5 mL). The active ester **15** obtained on removal of the combined organic solution was dissolved in 0.5 mL of water. The aqueous solution containing 32 μmol of the active ester was reacted with a 1.8 mL solution of ALP (6.2 mg, 44.3 nmol) after titration of its active ester content as described above.

Scheme 3. Activation and Reactions of Dendrimers Containing Terminal Carboxyl Groups^a

^a (a) TSTU or EDC/NHS; (b) ALP-NH₂; (c) BAHA; (d) ethylenediamine; (e) 2-aminoethanethiol; (f) Fab'.

The nonspecific binding of the ALP-dendrimer complexes was measured by using a solution of the complex in place of the ALP-Fab' conjugate on Stratus II. The complex was diluted to 500 ng of protein/mL of solution in a buffer containing 100 mM Tris/1% BSA/1% Triton X-100/2 mM magnesium chloride/0.1 mM zinc sulfate at pH 7.2.

Coupling of Fab' with the ALP-Dendrimer Complex. The mouse monoclonal IgG₁ was converted to its fragments F(ab')₂ and Fab' as described by Ishikawa et al. (pages 29–30 of ref 23). The number of free thiols per mole of Fab' was found to be 2.5 ± 0.2 .

A typical procedure for the coupling of Fab' and the ALP-dendrimer complex, **4** or **17**, involved mixing of a 5.5 mL solution of Fab' (5.5 mg, 119.6 nmol) in the phosphate/EDTA buffer with 5.4 mL of a solution of the ALP-dendrimer complex (2.9 mg of protein, 20.7 nmol) in the phosphate buffer at pH 7.0. For this specific example, the ALP-dendrimer complex was prepared by a reaction of the NIA-activated 3rd generation dendrimer and ALP-SH. The mixture of Fab' and the ALP-dendrimer complex was concentrated to about 2 mL in an Amicon ultrafiltration cell (YM 10) and then buffer exchanged with the phosphate buffer at pH 7.6 till concentration of EDTA in the concentrate was $\leq 5 \mu\text{M}$ and the pH of the effluent was 7.6. The reaction mixture, 1.6 mL, was incubated at 4 °C for 16 h and then mixed with 32 μL of a 20 mg/mL solution of *N*-ethylmaleimide (0.64 mg, 5.1 μmol) in *N,N*-dimethylformamide. After 2 h, the reaction mixture containing free unconjugated Fab' and the ALP-dendrimer-Fab' complex, **5** or **20**, was applied to an Ultrogel Aca 34 column (fractionation range of 20–350 kDa; exclusion limit of 750 kDa) equilibrated with 10 mM Tris/100 mM NaCl/0.1 mM magnesium chloride/0.01 mM zinc sulfate/0.1% azide at pH 7.0 (Tris buffer). The performance of the fractions was evaluated on Stratus II. The fractions containing the ALP-dendrimer-Fab' complex, eluting as the first peak from the Aca 34 column, were pooled on the basis of their performance being similar to that shown by the ALP-Fab' conjugate. The protein concentration of the ALP-dendrimer-Fab' conjugate was determined using the BCA protein assay with alkaline phosphatase as an internal standard. The column was calibrated with protein standards from Pharmacia Biotech using blue dextran (2000 kDa), thyroglobulin (669 kDa), catalase (232 kDa), aldolase (158 kDa), bovine serum albumin (67

kDa), and ovalbumin (43 kDa). The linear regression analysis of this calibration curve (fraction number vs molecular mass) was used to calculate the apparent molecular mass of the ALP-dendrimer-Fab' complex present in the specific column fraction.

RESULTS

The biochemical and immunochemical evaluations of the protein-dendrimer complexes, ALP-dendrimer and ALP-dendrimer-Fab', were carried out on Stratus II. This heterogeneous immunoassay system utilizes a sandwich format to analyze samples containing an analyte, e.g. CKMB. Glass fiber filter paper, a negatively charged surface, is used in this system to immobilize an anti-CKMB antibody called the capture antibody. The capture antibody provides one component necessary to form the sandwich. The second component of the sandwich is provided by a solution of the conjugate ALP-Fab'. These two sandwich components that utilize antibodies have distinctly different requirements for the solid phase in any heterogeneous immunoassay system. For optimum performance, the capture antibody must be able to bind strongly to the solid phase whereas the antibody-enzyme conjugate should have the least affinity for the solid support. An affinity of the antibody-enzyme conjugate for the solid support would increase nonspecific binding and thus compromise the sensitivity of the system.

During the initial phase of these studies, it was clear that to minimize the solid support (glass fiber) affinity for a protein-dendrimer complex it would be necessary to block all the terminal primary amino groups of the full generation dendrimer used to prepare the complex. Two strategies were applied for this purpose. The first approach involved selection of the activation conditions in such a way so as to functionalize all the terminal groups of the full generation dendrimer. Alternatively, the amino groups that remained free during the activation procedure were blocked by reaction with a reagent so as to introduce terminal carboxyls or neutral hydroxyl groups, the groups that are expected to have the least affinity for a negatively charged solid support such as glass fiber. The reactions used to activate the terminal groups of the full and half-generation dendrimers are shown in Schemes 1 and 3, respectively.

The electrophilic iodoacetamido groups are generally introduced under mild reaction conditions by treatment

Table 1. Incorporation of Electrophilic Iodoacetamido Groups^a in Dendrimers and the ALP–Dendrimer Complex Prepared from the Activated Amino-Terminal Dendrimers

dendrimer generation	activation reagent	number of iodoacetamido groups ^a incorporated			
		dendrimers (1, 3)		ALP–dendrimer complex prepared by	
				Schiff base ^b (2)	thioether ^b (4)
1	NIA	2.1 ^a	26 ^c		1.3
2	NIA	4.2	26		2.5
2	SIAB			1.3	
3	NIA	5.7	18		5.8
4	NIA	8.1	13		10.8
5	NIA	19.2	15		3.7
5 ^d	NIA				6.7
5 ^d	SIAB			14.2	
5 ^e	SIAB			15.3	
5 ^e	NIA				15.2

^a Iodoacetamido groups, as determined by titration, per mole of dendrimer or protein. ^b See Scheme 1 and the text for details.

^c Percent coupling of iodoacetamido groups, calculated from the experimentally determined value of the incorporated groups and the total number of terminal groups expected to be available for reaction. ^d Carboxymethylated dendrimers; see the text for details.

^e Partially hydroxylated dendrimers; see the text for details.

of molecules containing primary amines, such as a full generation poly(amidoamine) dendrimer, with a heterobifunctional reagent (e.g. the commercially available sulfoSIAB and NIA). By activation with a 10–20-fold molar excess of sulfoSIAB, it is possible to incorporate 3–5 phenyl iodoacetamido groups in the 5th generation dendrimer containing a total of 128 surface amino groups to produce **1** (NH₂–dendrimer–NHCOPhNHCOCH₂I). Irrespective of the generation of dendrimer used, all efforts to incorporate the maximum possible number of the reactive phenyliodoacetamido groups by reaction of all the surface amino groups with an excess of the activating reagent produced a white nonreactive precipitate. This precipitate was found to be insoluble in buffers of pH 2.0–12.0 and all common solvents such as alcohol, *N,N*-dimethylformamide, and dimethyl sulfoxide.

A clear solution was obtained when a solution of the full generation dendrimer, in 50% alcohol, was treated with an excess of NIA dissolved in tetrahydrofuran. By this method, it was possible to activate different generations of dendrimers, including partially functionalized dendrimers such as the partially hydroxylated and carboxymethylated dendrimers. Fluorescamine titration of these NIA-activated dendrimers **3** showed the absence of any surface amino groups present originally before the activation reaction. However, the reactive iodoacetamido groups that can be titrated with dithiothreitol in these derivatized dendrimers ranged from 13 to 26% of the values expected (Table 1). To calculate these values, it is assumed that all the terminal amino groups present originally in the dendrimer would react with the activating reagent. The first two generations of dendrimers contained the highest percentage of the incorporated reactive groups. All of these dendrimer derivatives were stable to storage in 50% alcohol or pH 5.0 buffer for at least 3 days at –10 °C.

The dendrimers derivatized by reaction with sulfoSIAB or NIA were reacted with ALP to form the complex ALP–dendrimer–COCH₂I in two different sets of reactions (Scheme 1). A Schiff base was formed between the periodate-oxidized ALP and amino groups of the dendrimer activated with sulfoSIAB to form the intermediate

Table 2. Incorporation of Electrophilic Epoxy Groups^a in Amino Dendrimers and the ALP–Activated Dendrimer Complex on Reaction with Epibromohydrin

dendrimer generation	number of epoxy groups ^a incorporated		
	dendrimer 6	ALP–dendrimer complex (7) prepared by thioether ^b	
2	0.7 ^a	4.4 ^c	5.3
5	2.1	1.6	
5 ^d	2.5	8.5	
5 ^e	2.3	8.0	

^a Epoxy groups incorporated, as determined by titration, per mole of dendrimer or protein. ^b ALP containing 2.6 mol of free SH reacted with an excess of the activated dendrimer. ^c Percent coupling of epoxy groups, calculated from the experimentally determined value of the incorporated groups and the total number of terminal groups expected to be available for reaction. ^d Partially hydroxylated dendrimers; see the text for details. ^e Succinylated dendrimers; see the text for details.

ALP–CH=N–dendrimer–NHCOPhNHCOCH₂I. This intermediate was reduced with sodium cyanoborohydride to form the stable secondary amine derivative **2**. Alternatively, ALP–SH (containing 1–3 free SHs per mole of the protein) was reacted with an excess of the NIA-activated dendrimer derivative to form the complex **4** (ALP–SCH₂CONH–dendrimer–NHCOCH₂I). The reactive iodoacetamido groups present in the complexes prepared by the two methods were quantitated by titration with dithiothreitol (Table 1).

The product formed on reaction of the 1st generation amino-terminal dendrimer with NIA was analyzed with an electrospray ionization mass spectrum. This analysis was carried out on a methanol/water solution of the product under the positive ion mode. The three most intense peaks present in this spectrum show *m/z* (relative intensity) of 903.3 (100%), 780.1 (85%), and 678.1 (50%) corresponding to +3, +2, and +4 charged states of the molecules, respectively. These three peaks represent the molecular species of *m/z* 2708.7, 1558.2, and 2708.4, respectively. The highest peak observed in this spectrum shows *m/z* of 1361.6 (10% relative intensity) corresponding to a +2 charged state. This peak represents a molecular species of *m/z* 2721.2. The 1st generation dendrimer derivative containing eight iodoacetamido groups introduced by reaction of all eight terminal amino groups would be expected to have a molecular mass of 2780 Da.

A reaction of terminal amino groups of the full generation dendrimers with an excess of epibromohydrin resulted in the formation of epoxy–dendrimers **6**. A number of dendrimers were functionalized with the epoxy groups. Fluorescamine titration of the derivatized dendrimers showed that 96–99% of the terminal amino groups had been reacted during this activation procedure. Similar to the NIA activation above, all the epoxy–dendrimers prepared by reaction with epibromohydrin showed complete solubility in 50% aqueous alcohol and these solutions could be stored for several days at –20 °C without loss of the reactive groups. The complex ALP–S–dendrimer–epoxide **7** was prepared by reaction of an excess of the epoxy–dendrimer (prepared from the 2nd generation dendrimer) with ALP–SH containing 2.6 free SHs per mole of protein (Scheme 1). Titration of the electrophilic groups in this complex and epoxy–dendrimer was carried out with dithiothreitol (22), and results are shown in Table 2. The reactive epoxide groups in the dendrimer–epoxide **6**, available for titration, were found to be 1.6–8.5% of the total terminal amino groups present in the specific generation of the dendrimer.

Table 3. Incorporation of Electrophilic *N*-Hydroxysuccinimidyl Ester Groups^a in Carboxyl Dendrimers and the ALP-Coupled Activated Dendrimer

dendrimer generation	activation reagent	number of active ester groups ^a	number of additional groups in the ALP-activated dendrimer complex ^b		
			NH ₂ (18)	SH (19)	COCH ₂ Br (17)
1.5	EDC/NHS			83	42.8
5.5	EDC/NHS		503		
1.5	TSTU	1.2	7.5 ^c		3.9
2.5	TSTU	1.4	4.4		4.8
4.5	TSTU	7.1	5.5		5.2

^a Active groups incorporated, as determined by titration, per mole of dendrimer or protein. ^b See the text for details. ^c Percent coupling of the active ester groups, calculated from the experimentally determined value of the incorporated groups and the total number of terminal groups available for reaction.

Dendrimers containing the nucleophilic sulfhydryls (dendrimer-SH) **9** have been prepared by a reaction of the terminal amino groups in the full generation dendrimers with a 4–10-fold molar excess of sulfo NHS LC SPDP (26) to form the intermediate NH₂-dendrimer-S-S-Py (dendrimer-PDH) **8**, followed by reaction of this intermediate with a reagent such as dithiothreitol. By utilizing a limited amount of the activation reagent, it has been possible to prepare a dendrimer derivative **9** containing two surface nucleophiles with distinctly different reactivity, i.e. NH₂ and SH (Scheme 1). In addition, succinic anhydride/dithiothreitol treatment of the intermediate dendrimer-PDH, containing the surface amino groups that were not involved during reaction with sulfo NHS LC SPDP, resulted in the formation of COOH-dendrimer-SH. This derivative contains the nucleophilic sulfhydryls in addition to the free carboxyl groups which show a low reactivity. A solution of the succinylated dendrimer derivative with free sulfhydryl groups was found to form an insoluble precipitate when stored at –20 °C for 48 h.

Quantitative conversion of the terminal amino groups in the intermediate dendrimer-PDH, during reaction with succinic anhydride, was confirmed by titration with fluorescamine. However, under identical reaction conditions, ≤90% of the amino groups were involved in reaction with reagents such as gluconolactone, butyrolactone, or acetoxyacetyl chloride.

An excess of the intermediate dendrimer-PDH, prepared from the 2nd generation amino-terminal dendrimer, was reacted with the periodate-oxidized ALP. A cyanoborohydride/dithiothreitol treatment of the intermediate Schiff base resulted in the formation of the complex ALP-dendrimer-SH **10** (Scheme 1).

BAHA, a heterobifunctional reagent, was synthesized in three steps by a procedure similar to that described by Heindel et al. (28). The synthetic scheme is shown in Scheme 2.

The electrophilic *N*-hydroxysuccinimidyl esters **15** (Scheme 3) were prepared by activation of the terminal carboxyl groups in the half-generation dendrimers. This activation was carried out by two different methods. An 80% dimethylformamide solution of the free acid form of the dendrimer was treated either with TSTU (29) or with EDC in the presence of NHS. A titration of the active esters incorporated in different generations of the carboxyl dendrimers was found to be 4.4–7.5% of the total carboxyl groups present in the specific generation of the dendrimer (Table 3). The purified active esters **15** were reacted with ALP followed by reaction of the intermediate

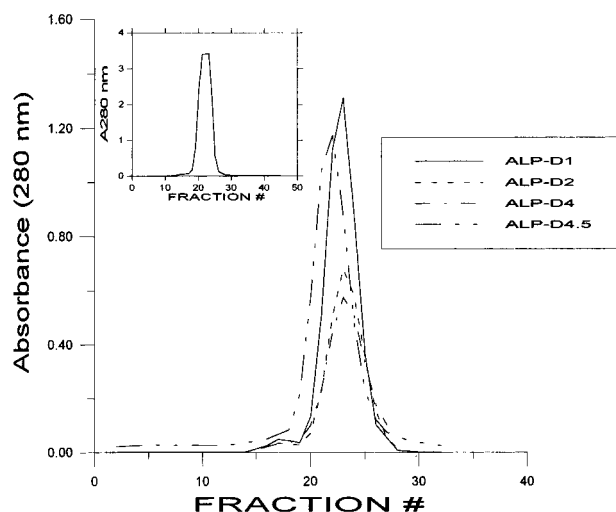


Figure 1. Gel filtration elution profiles of the ALP-dendrimer complexes. The NIA-activated dendrimers were reacted with ALP-SH, and the reaction mixture was applied to an Ultrogel AcA 44 column (1.6 × 70 cm) equilibrated and eluted with the phosphate buffer at pH 7.0. The figure shows the elution profiles of products obtained on reaction of the 1st, 2nd, 4th, and 5th generations of dendrimers (D1, D2, D4, and D5, respectively). The inset shows the elution profile of ALP-PDH obtained by reaction of ALP with sulfo NHS LC SPDP.

ALP-dendrimer-active ester **16** with either BAHA, ethylenediamine, or 2-aminoethanethiol to prepare complexes **17–19**, respectively. Additional amino, sulfhydryl, and bromoacetamido groups present in the complex ALP-dendrimer-X (X = NH₂, SH, and NHCOCH₂Br) were quantitated by titration with fluorescamine, 4,4'-dithiodipyridine, and dithiothreitol/4,4'-dithiodipyridine, respectively (Table 3).

A number of the ALP-dendrimer complexes were purified on a gel filtration column (Ultrogel AcA 44; fractionation range of 10–130 kDa). The elution profiles of a few of these complexes along with that of ALP-6-[3-(2-pyridyldithio)propionamide]hexanoate (ALP-PDH), prepared by activation of ALP with sulfo NHS LC SPDP, are shown in Figure 1. The peak fractions (showing maximum absorption at 280 nm) in these profiles were eluted from this column in the same position, i.e. the fraction number 23 ± 1. Activation of proteins with sulfo NHS LC SPDP does not lead to polymerizations, and since the elution profiles of the ALP-dendrimer complexes are similar to that of ALP-PDH, the predominant component in all these ALP derivatives is the monomeric form of the protein. The polymeric forms of ALP, especially a dimer when present as an impurity in the monomeric form of the protein, elute in front of the main peak containing the monomeric form of the protein when chromatographed on a column prepared with this gel. A small amount of this dimeric form of ALP is evident as a small shoulder (Figure 1) in a few of the ALP-dendrimer complexes as well as in ALP-PDH.

The specific enzyme activity of all of the ALP-dendrimer complexes described above has been found to be very similar to that of ALP used for the coupling reactions. The enzyme activity was determined spectrophotometrically from the rate of hydrolysis of *p*-nitrophenyl phosphate in 1 M Tris buffer at pH 8.0. For example, ALP-dendrimer-COCH₂I, prepared from the 2nd and the hydroxylated 5th generation dendrimers, shows specific activities of 450 and 399 units/mg, respectively. Under identical conditions, ALP, used for these reactions, shows an activity of 500 units/mg.

Kinetic parameters of the ALP–dendrimer complexes were found to be comparable to those of ALP. For example, the ALP–CH₂NH–dendrimer complexes prepared from the 3rd and the 5th generation dendrimers show K_m values of 0.19 and 0.11 mM, respectively, as compared to 0.17 mM for that of ALP. The K_{cat}/K_m ratios for these dendrimer complexes were found to be 8.6×10^6 and $1.20 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$, respectively. This ratio was found to be $9.8 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ for ALP under identical reaction conditions.

Prior to conjugation of Fab' to an ALP–dendrimer complex, the nonspecific binding of these ALP–dendrimer complexes was evaluated on Stratus II. Nonspecific binding is the response generated, at 0 ng/mL of the analyte, by the ALP–dendrimer complex when applied to the glass fiber solid support used in Stratus II. The complexes **4** prepared by a reaction of ALP–SH with the NIA-activated dendrimers showed (15) nonspecific binding of 146–2571 mV/min at a protein concentration of 500 ng/mL. This interaction was found to increase with the generation of the dendrimers containing terminal amino groups. The reagents prepared with the half-generation dendrimers show very low nonspecific binding (35–83 mV/min) regardless of the generation of dendrimer used. The nonspecific binding of complexes prepared by dendrimers activated with the other methods was found to be extremely high. For example, the complexes ALP–dendrimer (D2)–epoxide **7**, ALP–CH₂NH–dendrimer (hydroxylated D5)–NHCOPhNHCOCH₂I **2**, and ALP–CH₂NH–dendrimer (D5)–SH **10** showed responses of 10 668 (20 ng/mL), 2544 (0.94 ng/mL), and 2973 mV/min (100 ng/mL), respectively, as compared to a response of 108 mV/min for the ALP–Fab' conjugate.

The ALP–dendrimer complexes **4** and **17** containing terminal iodoacetamido and bromoacetamido groups were reacted separately with the Fab' fragment of an anti-CKMB antibody to form the multifunctional dendrimer reagents ALP–dendrimer–Fab'. The reaction mixtures containing the ALP–dendrimer–Fab' complexes (**5** and **20**) were purified on an Aca 34 column (fractionation range of 20–350 kDa; exclusion limit of 750 kDa). The column fractions were tested on Stratus II. This testing evaluates both the enzyme activity of ALP and the antigen binding activity of the antibody in the ALP–dendrimer–Fab' complex. The first peak that elutes from the column represents the ALP–dendrimer–Fab' complex followed by elution of the two unreacted components ALP–dendrimer and Fab'. The elution profiles of a few of these representative complexes are shown in Figure 2.

The presence of fractions appearing as a shoulder and representing higher-molecular mass complexes in front of the main ALP–dendrimer–Fab' complex peak is dependent on the generation of the dendrimer used to prepare the ALP–dendrimer complex. For example, in the complex prepared from the 1st generation dendrimer, this front shoulder is almost nonexistent, whereas this set of fractions becomes the major component when the 5th generation dendrimer was used for the coupling reactions. The presence of this shoulder (Figure 2) is quite obvious in cases where the 3rd and the 4.5th generation dendrimers were used for the reactions. The apparent molecular mass of the peak fractions (showing highest absorption at 280 nm) in these ALP–dendrimer–Fab' complexes prepared from the 3rd and the 5th generation dendrimers was calculated to be 194 and 517 kDa, respectively. All complexes, except for the one prepared from the 5th generation dendrimer, showed performances (15) similar to that of the ALP–Fab'

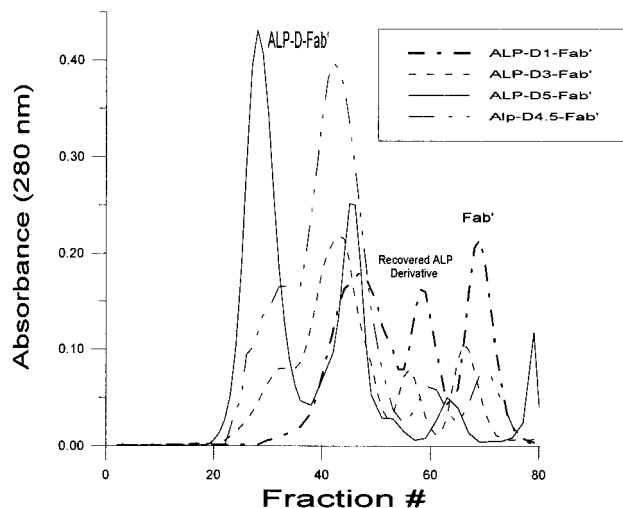


Figure 2. Gel filtration elution profiles for the purification of ALP–dendrimer–Fab' complexes. The reaction mixture obtained after reaction of the ALP–dendrimer complex with an excess of Fab' was applied to an Ultrogel Aca 34 column (1.6 × 100 cm) equilibrated and eluted with the Tris buffer. The figure shows the profiles of products obtained from the 1st, 3rd, 5th, and 4.5th generation dendrimers (D1, D3, D5, and D4.5, respectively).

conjugate. The ALP–dendrimer–Fab' complex, prepared from the 5th generation dendrimer, could not match the performance of the commercial conjugate even at a very high protein concentration of 9.26 µg/mL as compared to other complexes which showed comparable performances at 0.434–0.740 µg/mL protein (15).

DISCUSSION

Reaction conditions normally used to activate functional groups may adversely affect the structural integrity or specific activity of a biologically active molecule. However, a number of reaction conditions affecting the surface groups of Starburst dendrimers do not impact their structural integrity (3, 30, 31). For coupling of biologically active molecules to a natural or a synthetic macromolecule, such as a dendrimer, it is therefore desirable to activate the surface groups of these macromolecules.

For optimum performance, the complex prepared by reacting biologically active material with a dendrimer, e.g. antibody–dendrimer (11) and antibody–dendrimer–enzyme (15), may require either partial or essentially complete reaction of the terminal functional groups present originally in a dendrimer. The results presented here make it possible to achieve these goals by activation of a dendrimer with a specific reagent in defined molar ratios. A number of full and half-generation dendrimers have been used for these derivatizations. Respective reactions with sulfoSIAB and NIA (Scheme 1 and Table 1) allow partial or complete reaction of terminal amino groups of a full generation dendrimer to incorporate the electrophilic iodoacetamido groups **1** and **3**. Similarly, incorporation of the electrophilic epoxy **6** and *N*-hydroxy-succinimidyl groups **15** (Tables 2 and 3) has been achieved by reaction with an excess of the appropriate reagents. By a similar choice of reactions, it has been possible to derivatize dendrimers containing either two nucleophilic groups **9** (i.e. SH and NH₂; Scheme 1) with selective reactivity patterns or a nucleophilic sulfhydryl and a nonreactive carboxyl or hydroxyl. These derivatized dendrimers have been reacted with a limited amount of a model protein (ALP) to form the complex

ALP–dendrimer–active group. This complex can be reacted further with a homo- or heterobifunctional reagent to achieve specific chemical or ionic characteristics. Alternatively, active groups in the intermediate are available for further reaction with other similar or dissimilar biologically active materials for formation of dendrimer-based multifunctional reagents.

The feasibility of preparation of multifunctional reagents has been demonstrated by reaction of the ALP–dendrimer complexes with the Fab' fragment of an anti-CKMB antibody. The resulting bifunctional reagents have been prepared using both full and half-generations of the poly(amidoamine) dendrimers. The performance characteristics of these ALP–dendrimer–Fab' complexes are influenced by the dendrimer generation (15), the nature of the terminal functional groups present, and the method of activation used to prepare the derivatized dendrimer.

The bifunctional reagent ALP–dendrimer–Fab' prepared from the 5th generation dendrimer did not show optimum performance on Stratus II probably due to the very large size of the complex formed. However, it may rather be beneficial to prepare a large sized complex for other applications such as that utilized for a slow and sustained release of a biologically active molecule targeted either at a specific site of action or in circulation. The results presented here clearly show that by an appropriate selection of the dendrimer it is possible to control the size (Figure 2) and thus the performance of these dendrimer-based multifunctional reagents.

The number of reactive functional groups introduced in the derivation reactions described here is lower than that expected on the basis of the number of terminal groups present originally in the specific generation of the Starburst dendrimer. The reasons for this effect are not very clear. Although a standard method for titration of the electrophilic groups when present in proteins, dithiothreitol/4,4'-dithiodipyridine reaction (23) may not be optimum for quantitation of such groups in dendrimers. Furthermore, the terminal functional groups in a dendrimer have been shown (13) to possess enhanced chemical reactivity as compared to their activity when present in other molecules. This higher than usual activity may be responsible for inter- and intramolecular reactions of the incorporated groups and thereby decreasing the number of such groups actually available during titrations and for coupling with biologically active molecules. The gel filtration column profiles of ALP–dendrimer complexes (Figure 1) have shown that the molecular mass of the protein eluted was not dramatically different as compared to that of ALP; i.e. formation of dimers and other polymers of ALP was not evident. This interpretation is supported further by the calculated molecular mass (194 kDa) of the peak fraction (with maximum absorbance at 280 nm) in the complex ALP–D3–Fab'. This number is very close to what would be expected (193.7 kDa) for such a complex containing ALP–D3–Fab' in a 1:1:1 ratio.

The mass spectrum of a product formed from the activation reaction of the 1st generation amino-terminal dendrimer shows the presence of species with a molecular mass of <3 kDa. This observation would rule out the possibility of the multimeric derivatives of the dendrimer being the predominant component of this reaction product. The unusual reactivity of a terminal group in a dendrimer, the reactivity of the introduced iodoacetamido groups, an absence of primary amino terminal groups (fluorescamine), and a small number of the available iodoacetamido groups (dithiothreitol titration) suggest an

intramolecular coupling of the activated derivative. Work is in progress to further characterize the nature of the major component present in this reaction mixture. The intermolecular coupling of appropriately activated dendrimers has been used (2) to prepare multimeric forms of dendrimers but may not be the major reaction path under the conditions utilized in this study.

In conclusion, dendrimer derivatives of different generations have been prepared that contain nucleophilic, electrophilic, and nonreactive groups by an appropriate selection of the specifically defined reaction conditions. The methods described here allow for the preparation of reagents with desirable characteristics such as controlled molecular mass and a defined number of reactive groups. These reactions show that Starburst dendrimers can be subjected to a series of defined chemical reactions. Due to the controlled architecture and geometry of dendrimers and the use of a defined sequence of chemical reactions, the methods developed here allow for reproducible preparation of reagents that have the potential for very broad applications.

ACKNOWLEDGMENT

The author is grateful to Peter Cronin and Tim Goodnow for their support and Ralph Spindler for supplying dendrimers.

LITERATURE CITED

- (1) Tomalia, D. A., and Dvornic, P. R. (1996) Dendritic polymers, divergent synthesis (Starburst polyamidoamine dendrimers). In *The Polymeric Materials Encyclopedia. Synthesis, properties and application* (J. C. Salamone, Ed.) Vol. 3, pp 1814–1830, CRC Press Inc., Boca Raton, FL.
- (2) Tomalia, D. A. (1996) Starburst Dendrimers-Nanoscope supermolecules according to dendritic rules and principles. *Macromol. Symp.* 101, 243–255.
- (3) Tomalia, D. A., and Durst, H. D. (1993) Genealogically directed synthesis: Starburst/cascade dendrimers and hyperbranched structures. *Top. Curr. Chem.* 165, 193–313.
- (4) Tomalia, D. A., Naylor, A. M., and Goddard, W. A., III (1990) Starburst Dendrimers: Molecular-level control of size, shape, surface chemistry, topology, and flexibility from atoms to macroscopic matter. *Angew. Chem., Int. Ed. Engl.* 29, 138–175.
- (5) Newkome, G. R., Yao, Z., Baker, G. R., and Gupta, V. K. (1985) Micelles. Part 1. Cascade molecules: A new approach to micelles. A [27]-arborol. *J. Org. Chem.* 50, 2003–2004.
- (6) Issberner, J., Moors, R., and Vogtle, F. (1994) Dendrimers: From generations and functional groups to functions. *Angew. Chem., Int. Ed. Engl.* 33, 2413–2420.
- (7) Knapen, J. W. J., van der Made, A. W., de Wilde, J. C., van Leeuwen, P. W. N. M., Wijkens, P., Grove, D. M., and van Koten, G. (1994) Homogeneous catalysts based on silane dendrimers functionalized with arylnickel(II) complexes. *Nature* 372, 659–663.
- (8) Jansen, J. F. G. A., and Meijer, E. W. (1995) The dendritic box: Shape-selective liberation of encapsulated guests. *J. Am. Chem. Soc.* 117, 4417–4418.
- (9) Percec, V., Johansson, G., Ungar, G., and Zhou, J. (1996) Fluorophobic effect induces the self-assembly of semifluorinated tapered monodendrons containing crown ethers into supramolecular columnar dendrimers which exhibit a homeotropic hexagonal columnar liquid crystalline phase. *J. Am. Chem. Soc.* 118, 9855–9866.
- (10) Wooley, K. L., Hawker, C. J., and Frechet, J. M. J. (1993) Unsymmetrical three-dimensional macromolecules: Preparation and characterization of strongly dipolar dendritic macromolecules. *J. Am. Chem. Soc.* 115, 11496–11505.
- (11) Singh, P., Moll, F., III, Lin, S. H., Ferzli, C., Kwok, S. Y., Koski, R. K., Saul, R. G., and Cronin, P. (1994) Starburst Dendrimers: Enhanced performance and flexibility for immunoassays. *Clin. Chem.* 40, 1845–1849.

- (12) Tang, M. X., Redemann, C. T., and Szoka, F. C., Jr. (1996) *In vitro* gene delivery by degraded polyamidoamine dendrimers. *Bioconjugate Chem.* 7, 703–714.
- (13) Frechet, J. M. J. (1994) Functional polymers and dendrimers: Reactivity, molecular architecture and interfacial energy. *Science* 263, 1710–1715.
- (14) Haensler, J., and Szoka, F. C., Jr. (1993) Polyamidoamine cascade polymers mediate efficient transfection of cells in culture. *Bioconjugate Chem.* 4, 372–379.
- (15) Singh, P., Moll, F., III, Lin, S. H., and Ferzli, C. (1996) Starburst Dendrimers: A novel matrix for multifunctional reagents in immunoassays. *Clin. Chem.* 42, 1567–1569.
- (16) Wooley, K. L., Hawker, C. J., Frechet, J. M. J., Wudl, F., Srdanov, G., Shi, S., Li, C., and Kao, M. (1993) Fullerene-bound dendrimers: Soluble, isolated carbon clusters. *J. Am. Chem. Soc.* 115, 9836–9837.
- (17) Roberts, J. C., Adams, Y. E., Tomalia, D., Mercer-Smith, J. A., and Lavalley, D. K. (1990) Using Starburst dendrimers as linker molecules to radiolabel antibodies. *Bioconjugate Chem.* 1, 305–308.
- (18) Leon, J. W., Kawa, M., and Frechet, J. M. J. (1996) Isophthalate ester-terminated dendrimers: Versatile nanoscopic building blocks with readily modifiable surface functionalities. *J. Am. Chem. Soc.* 118, 8847–8859.
- (19) Starburst dendrimers, synthesized by Dendritech Inc., are commercially available from Aldrich Chemical Co. (Milwaukee, WI).
- (20) de Brabander-van den Berg, E. M. M., and Meijer, E. W. (1993) Poly(propylene imine) dendrimers: Large-scale synthesis by heterogeneously catalyzed hydrogenations. *Angew. Chem., Int. Ed. Engl.* 32, 1308–1311.
- (21) Lai, C. Y. (1977) Detection of peptides by fluorescence methods. In *Methods In Enzymology* (C. H. W. Hirs and S. N. Timasheff, Eds.) Vol. 47, pp 236–243, Academic Press, New York.
- (22) Grassetti, D. R., and Murray, J. F., Jr. (1967) Determination of sulfhydryl groups with 2,2'- or 4,4'-dithiodipyridine. *Arch. Biochem. Biophys.* 119, 41–45.
- (23) Ishikawa, E., Hashida, S., Kohno, T., and Tanaka, K. (1988) Methods for enzyme-labeling of antigens, antibodies and their fragments. In *Nonisotopic Immunoassay* (T. T. Ngo, Ed.) p 37, Plenum Press, New York.
- (24) Miron, T., and Wilchek, M. (1982) A spectrophotometric assay for soluble and immobilized N-hydroxysuccinimide esters. *Anal. Biochem.* 126, 433–435.
- (25) Husain, M., and Bieniarz, C. (1994) Fc site-specific labeling of immunoglobulins with calf intestine alkaline phosphatase. *Bioconjugate Chem.* 5, 482–490.
- (26) Carlsson, J., Drevin, H., and Axen, R. (1978) Protein thiolation and reversible protein-protein conjugation. N-Succinimidyl 3-(2-pyridyldithio)propionate, a new heterobifunctional reagent. *Biochem. J.* 173, 723–737.
- (27) Obi, I. U. (1982) Application of the 2,4,6-trinitrobenzenesulfonic acid (TNBS) method for the determination of available lysine in maize seed. *Agric. Biol. Chem.* 46, 15–20.
- (28) Heindel, N. D., Zhao, H., Egolf, R. A., Chang, C., Schray, K. J., Emrich, J. G., McLaughlin, J. P., and Woo, D. V. (1991) A novel heterobifunctional linker for formyl to thiol coupling. *Bioconjugate Chem.* 2, 427–430.
- (29) Bannwarth, W., and Knorr, R. (1991) Formation of carboxamides with N,N,N',N'-tetramethyl (succinimido)uronium tetrafluoroborate in aqueous/organic solvent systems. *Tetrahedron Lett.* 32, 1157–1160.
- (30) Aoi, K., Katsuhito, I., and Okada, M. (1995) Globular carbohydrate macromolecule "Sugar Balls". 1. Synthesis of novel sugar-persubstituted poly(amido amine) dendrimers. *Macromolecules* 28, 5391–5393.
- (31) Kim, R. M., Manna, M., Hutchins, S. M., Griffin, P. R., Yates, N. A., Bernick, A. M., and Chapman, K. T. (1996) Dendrimer-supported combinatorial chemistry. *Proc. Natl. Acad. Sci. U.S.A.* 93, 10012–10017.

BC970048A

Synthesis and Reactivity of Aryl Nitrogen Mustard–Oligodeoxyribonucleotide Conjugates

Michael W. Reed,* Eugeny A. Lukhtanov, Vladimir Gorn, Igor Kutyavin, Alexander Gall, Ansel Wald, and Rich B. Meyer

Epoch Pharmaceuticals, Inc., 1725 220th Street, S.E., #104, Bothell, Washington 98021. Received July 23, 1997; Revised Manuscript Received October 6, 1997[®]

A versatile method is described for preparing aryl nitrogen mustard–oligodeoxyribonucleotide (mustard–ODN) conjugates under anhydrous conditions. The chemistry uses DMSO soluble triethylammonium or tributylammonium salts of the ODNs. A G/A motif triplex forming ODN was chosen for study since it had been shown earlier to bind with high affinity and specificity to a duplex DNA target. A 5'-hexylamine derivative of this ODN was reacted with three different 2,3,5,6-tetrafluorophenyl ester derivatives of aryl nitrogen mustards which were designed to have different alkylation rates. An HPLC assay was used to determine reaction rates of these mustard–ODNs under various conditions. The reactivity of the mustard groups depended on chloride concentration and the presence of nucleophiles. Conjugation of mustards to G/A-containing ODNs decreased their aqueous stability. Hydrolysis and alkylation rates of these agents were consistent with reaction via an aziridinium intermediate. Rates of sequence specific alkylation within a triplex were determined by denaturing gel electrophoresis and shown to depend on inherent reactivity of the mustard group. The improved synthesis and chemical characterization of mustard–ODNs should facilitate their use as sequence specific alkylating agents and as probes for nucleic acid structure.

INTRODUCTION

Aryl nitrogen mustards are one of the oldest and most studied classes of cancer chemotherapy agents (1). Their cytotoxic effects are attributed to random alkylation of vital macromolecular targets, especially the nucleophilic N7 position of the purine bases in genomic DNA (ref 2 and references therein). Formation of interstrand cross-links in DNA by these bifunctional alkylating agents is believed to be especially lethal, but monoalkylation, depurination, and strand scission may also contribute to cytotoxic and mutagenic effects (3). Our goal is to deliver these (and other) alkylating agents to specific genetic targets as triplex-forming oligodeoxynucleotide (ODN) conjugates in order to induce sequence specific mutations or gene knockouts. We have recently shown (4) sequence specific targeting and covalent modification of human genomic DNA by aryl nitrogen mustard–ODN conjugates (mustard–ODNs). Mustard–ODNs are also valuable probes for nucleic acid structure. For example, the mechanisms of RecA-mediated synaptic joint formation (5) and RNA polymerase-catalyzed transcription (6) have recently been explored using mustard–ODNs. As shown in Figure 1, the alkylation mechanism of the aryl nitrogen mustards is believed to occur mainly via spontaneous formation of an electrophilic aziridinium cation intermediate (7). Although aromatic aziridinium cations have not been directly detected, the first-order reactions observed for this class of mustards are in accord with the proposed mechanism. Mustards have been attached to ODNs with appropriate length linkers that position the short-lived aziridinium cation in the proximity of a

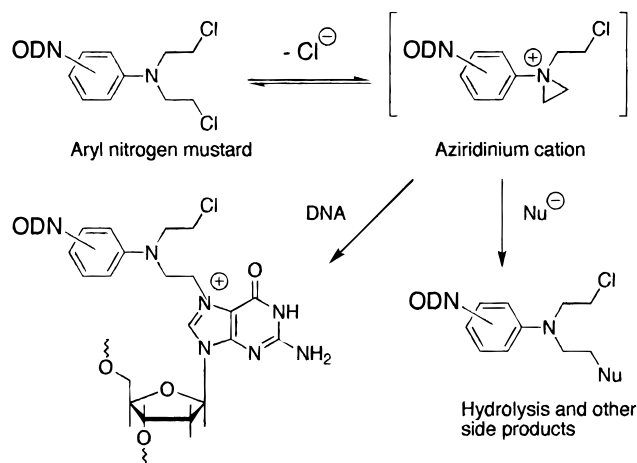


Figure 1. Mechanism for reaction of aryl nitrogen mustard–ODN conjugates. The major pathway is via the aziridinium cation intermediate. Alkylation of N7 in dG is shown, but other nucleophilic centers in DNA can also be alkylated. The second (2-chloroethyl)amine group reacts in a similar manner.

dG residue after hybridization to a single-stranded (ss) or double-stranded (ds) DNA target (ref 8 and references therein). Targeted alkylation of a single DNA strand in dsDNA by a triplex-forming mustard–ODN can occur in high yield (4), and efficient interstrand cross-linking has been accomplished by conjugating a mustard group to each end of the ODN (9). Nonetheless, there are competing side reactions such as hydrolysis and nonsequence specific alkylation that can decrease alkylation efficiency in some triplex systems. To understand effects of sequence-directed DNA alkylation in more complex biological systems, we have explored the aqueous stability and reactivity of mustard–ODNs.

Mustard–ODNs have been used for many years, but

* Corresponding author. Telephone: (425) 485-8566. Fax: (425) 486-8336. E-mail: mreed@epochpharm.com.

[®] Abstract published in *Advance ACS Abstracts*, December 15, 1997.

synthesis and characterization of these reactive ODNs remains a challenging problem (10). ODNs have poor solubility in organic solvents, and the (2-chloroethyl)-amino functionality of biologically relevant nitrogen mustards is rapidly hydrolyzed. In addition, self-reaction of mustard groups with the ODN carrier is possible. In the course of our studies, we have developed a general method for preparation of reactive ODN conjugates in anhydrous organic solvents. In this paper, we describe synthesis of three aryl nitrogen mustard-activated esters and conjugation to a G/A motif triplex-forming ODN. These mustard groups were designed to have different inherent reaction rates. The ability to vary the reactivity of the mustard–ODNs is especially important for biological applications where uptake and degradation rates of the ODN carrier are a concern. Rates of reaction of mustard–ODNs under various conditions were analyzed by reverse phase HPLC, and alkylation of duplex DNA targets was determined by gel electrophoresis.

EXPERIMENTAL PROCEDURES

Nitrogen mustards are potent alkylating agents and were handled with great care. DMSO solutions are especially hazardous, and disposable nitrile gloves were used for these operations. Excess reagents were decomposed in water.

^1H and ^{13}C NMR spectra were recorded on a Varian Gemini 300 MHz spectrometer. Infrared spectra were obtained with a Perkin-Elmer 783 spectrophotometer. Elemental analyses were performed by Quantitative Technologies Inc. (Boundbrook, NJ). Melting points were determined on a Mel-Temp melting point apparatus in open capillary tubes and are uncorrected. All air and water sensitive reactions were carried out under a slight positive pressure of argon. Flash chromatography was performed on 230–400 mesh silica gel. Analytical thin-layer chromatography was carried out on EM Science F_{254} aluminum-backed, fluorescent indicator plates. 4-[Bis(2-chloroethyl)amino]benzenebutanoic acid (chlorambucil) was purchased from Fluka Chemical Co. 4-[Bis(2-chloroethyl)amino]phenylacetic acid and 3-[bis(2-chloroethyl)amino]benzoic acid were made by variations of the literature method (11). 2,3,5,6-Tetrafluorophenyl trifluoroacetate (TFP-TFA) was prepared as described earlier (12). Triethylammonium bicarbonate (TEAB, 0.1 M) and tributylammonium bicarbonate (TBAB, 0.1 M) were prepared by sparging a heterogeneous mixture of the appropriate amine with CO_2 until the organic layer disappeared.

2,3,5,6-Tetrafluorophenyl 4'-[Bis(2-chloroethyl)-amino]benzenebutanoate (1). To a solution of 0.25 g (0.82 mmol) of chlorambucil and 0.3 g (1.1 mmol) of TFP-TFA in 5 mL of dry dichloromethane was added 0.2 mL of dry triethylamine. The mixture was stirred under argon at room temperature for 0.5 h and evaporated. The residual oil was purified by column chromatography on silica gel with hexane/chloroform (2:1) as the eluting solvent to give 0.28 g (75% yield) of **1** as a colorless oil: TLC (CHCl_3) $R_f = 0.6$; IR (CHCl_3) 3010, 1780, 1613, 1521, 1485 cm^{-1} ; ^1H NMR (CDCl_3) δ 7.11 (d, $J = 8.8$ Hz, 2H), 7.06–6.94 (m, 1H), 6.65 (d, $J = 8.8$ Hz, 2H), 3.8–3.6 (m, 8H), 2.7–2.6 (m, 4H), 2.1–2.0 (m, 2H); ^{13}C NMR (CDCl_3) δ 147.69, 144.54 (dt), 144.30 (dt), 142.30 (dd), 138.5 (dd), 129.88, 112.22, 103.17 (t), 53.61, 40.52, 33.63, 32.64, 30.99, 26.64. Anal. ($\text{C}_{20}\text{H}_{19}\text{Cl}_2\text{F}_4\text{NO}_2$) C, H, N, F.

2,3,5,6-Tetrafluorophenyl 4'-[Bis(2-chloroethyl)-aminol]phenylacetate (2). 4-[Bis(2-chloroethyl)amino]phenylacetic acid was converted to TFP ester **2** using a

procedure similar to that described for synthesis of **1** to give 0.45 g (74% yield) of **2** as a white crystalline solid: mp 99–100 $^\circ\text{C}$ (from hexanes/ethyl acetate); TLC (2:1 hexanes/ethyl acetate) $R_f = 0.68$; ^1H NMR (CDCl_3) δ 7.26 (d, $J = 8.8$ Hz, 2H), 6.99 (m, 1H, TFP), 6.71 (d, $J = 8.8$ Hz, 2H), 3.89 (s, 2H), 3.75 (m, 4H), 3.65 (m, 4H); ^{13}C NMR (CDCl_3) δ 167.97, 147.69 (m), 144.40 (m), 142.35 (m), 139.11 (m), 145.61, 130.64, 121.00, 112.17, 103.24 (t), 53.53, 52.06, 40.45, 40.07. Anal. ($\text{C}_{18}\text{H}_{15}\text{NO}_2\text{Cl}_2\text{F}_4$) C, H, N.

2,3,5,6-Tetrafluorophenyl 3'-[Bis(2-chloroethyl)-amino]benzoate (3). To a suspension of 2.62 g (10 mmol) of 3-[bis(2-chloroethyl)amino]benzoic acid in 20 mL of dry dichloromethane was added 3.37 g (13 mmol) of TFP-TFA followed by 2 mL (14.4 mmol) of dry triethylamine. A mild exothermic reaction was observed, and the mixture formed a clear solution within 5 min. The mixture was stirred under argon for an additional 15 min and evaporated. The residual oil was purified by column chromatography on silica gel using dichloromethane as the eluting solvent. The solid was recrystallized from 15 mL of hexanes to give 3.04 g (74% yield) of **3** as a white solid: mp 56–59 $^\circ\text{C}$; TLC (CHCl_3) $R_f = 0.7$; ^1H NMR (CDCl_3) δ 7.65–7.6 (m, 1H), 7.5–7.4 (m, 2H), 7.1–7.0 (m, 2H), 3.9–3.96 (m, 4H); ^{13}C NMR (CDCl_3) δ 147.75 (dt), 146.57, 144.47 (dt), 142.50 (dd), 139.23 (dd), 130.26, 128.50, 119.73, 117.91, 113.41, 103.32 (t), 53.41, 40.30. Anal. ($\text{C}_{17}\text{H}_{13}\text{Cl}_2\text{F}_4\text{NO}_2$) C, H, N, F.

(R/S)-3-[4'-[[Bis(2-chloroethyl)amino]phenyl]-acetamido]-1,2-propanediol (4). To a solution of 241 mg (2.65 mmol) of 3-amino-1,2-propanediol in 5 mL of dry DMF was added 226 mg (0.53 mmol) of TFP ester **2**. After 1 h, the reaction mixture was evaporated and applied onto a silica gel column (2 $\times$ 28 cm). Elution with methylene chloride/methanol (95:5) followed by concentration of the proper fractions gave 174 mg (94% yield) of **4** as a white solid: mp 76–78 $^\circ\text{C}$; TLC (9:1 dichloromethane/methanol) $R_f = 0.42$; ^1H NMR (CDCl_3) δ 7.12 (d, $J = 6.6$ Hz, 2H), 6.66 (d, $J = 6.6$ Hz, 2H), 5.88 (m, 1H), 3.8–3.3 (m, 9H), 1.7 (br s, 2H). Anal. ($\text{C}_{15}\text{H}_{22}\text{N}_2\text{O}_3\text{Cl}_2$) C, H, N.

Synthesis and Purification of Oligodeoxynucleotides. ODNs were prepared on an Applied Biosystems model 394 synthesizer using the 1 μmol protocols supplied by the manufacturer. Protected β -cyanoethyl phosphoramidites, CPG supports, deblocking solutions, cap reagents, oxidizing solutions, and tetrazole solutions were purchased from Glen Research (Sterling, VA). The sequences of the 65-mer dsDNA target and the triplex-forming ODN (ODN-NH₂) are shown in Figure 2. A 3'-hexanol modification was introduced into ODN-NH₂ using 2 μmol of hexanol-modified CPG support (12), and a 5'-aminohexyl linker was introduced using an *N*-MMT-hexanolamine phosphoramidite (Glen Research). Preparative HPLC purification, detritylation, and butanol precipitation of the synthetic ODNs were carried out as previously described (13). Aliquots (0.2 mg) of the 65-mer ODNs were further purified by preparative gel electrophoresis for triplex cross-linking experiments.

Characterization of ODNs. The concentrations of all ODNs were determined from the UV absorbance at 260 nm in phosphate-buffered saline (pH 7.2). An extinction coefficient for each ODN was estimated using a nearest neighbor model (14). For the triplex-forming ODN used in these studies, a value of 29.1 $\mu\text{g/mL}$ per A_{260} unit was used. All modified ODNs were analyzed by reverse phase HPLC (C_{18} HPLC) using a 250 $\times$ 4.6 mm C_{18} column equipped with a guard column (Rainin Dynamax, 10 μm particle size, 300 $\text{\AA}$ pore size). A

gradient of 5 to 65% solvent B over 30 min was used (flow rate = 1 mL/min), where solvent A was 0.1 M triethylammonium acetate (TEAA, pH 7.5) and solvent B was acetonitrile; detection was by UV absorbance at 260 nm. Unless otherwise noted, all modified ODNs were greater than 95% pure by C_{18} HPLC.

Typical Synthesis of Mustard-ODN Conjugates (ODN I). Dried, detritylated ODN-NH₂ from a 2 μ mol scale synthesis was dissolved in 0.5 mL of water and injected onto a Hamilton PRP-1 column (Reno, NV) that was equilibrated with 0.1 M TEAB (pH 7.2). The TEA salt of the ODN was eluted from the column using a gradient of 0 to 60% acetonitrile over 30 min, with a flow rate of 2 mL/min. The desired peak (~15 min) was collected and dried in vacuo on a centrifugal evaporator. The residue was dissolved in 0.5 mL of water, and the concentration was determined to be 2.36 mg/mL. C_{18} HPLC analysis showed one major peak (90% purity). A 1 mg aliquot (424 μ L, 71 nmol) was redried in a 1.7 mL Eppendorf tube, and the residue was dissolved in 0.2 mL of DMSO with 5 μ L of ethyldiisopropylamine. A 20 mg/mL solution of TFP ester **1** in DMSO was prepared, and 31.5 μ L (0.63 mg, 1.4 μ mol) was added to the ODN. The mixture was shaken for 3 h at room temperature, and then the ODN was precipitated by adding 10 mL of 2% NaClO₄/acetone in a 14 mL polypropylene tube. The mixture was centrifuged at 3000 rpm for 5 min, and the pellet was sonicated with 2 mL of acetone and recentrifuged. The pellet was dried in vacuo for 15 min, and the crude product was stored at -20 °C. The crude reaction mixture was analyzed by C_{18} HPLC. Purification by C_{18} HPLC used the same gradient and column specified above. The peak eluting at 19 min was collected in ~1 mL of TEAA/acetonitrile and immediately precipitated by adding 100 μ L of 3 M sodium acetate and 4 mL of ice cold absolute ethanol. The mixture was centrifuged at 3000 rpm for 5 min, and the pellet was sonicated with 2 mL of ethanol and recentrifuged. The pellet was dried in vacuo for 15 min, and the purified product was dissolved in 0.25 mL of water. A 5 μ L aliquot was removed for C_{18} HPLC analysis, and another 5 μ L aliquot was removed for concentration determination. The bulk solution of ODN **I** was immediately frozen and stored at -20 °C for future use. HPLC analysis showed 98% purity. The concentration was 1.76 mg/mL (0.44 mg, 44% yield).

ODN **I** was prepared in similar yield and purity using TBAB for the initial salt exchange. The more lipophilic TBA salt was eluted from the PRP-1 column using a gradient of 0 to 100% acetonitrile over 30 min, with a flow rate of 2 mL/min. The desired peak (~23 min) was collected and dried in vacuo on a centrifugal evaporator as usual. ODN **II** and ODN **III** were prepared in similar yields and purities from the TBA salts.

HPLC Assay for Kinetics Studies. Reverse phase HPLC analysis of mustard-ODNs was carried out using a Rainin Gradient HPLC system (Emeryville, CA) equipped with 10 mL pump heads and a Rainin Dynamax PDA-1 photodiode array detector. For kinetics studies, 10 μ L of each sample was injected onto a 4.6 $\times$ 150 mm Rainin Microsorb C_{18} column and eluted using a gradient of 5 to 65% acetonitrile in 0.1 M triethylammonium acetate (pH 7.5) over 20 min (flow rate of 1 mL/min). ODN products were detected by UV absorbance at 260 nm, and data were integrated and analyzed using Rainin Dynamax software.

Aqueous Stability of Mustard Conjugates. One hundred microliters of a 0.1 mM solution of the conjugate of interest was prepared in 20 mM HEPES buffer (pH

7.2) with 140 mM KCl and 10 mM MgCl₂. For the chloride-free experiments, 20 mM HEPES buffer (pH 7.2) was used with no additional salts. The 0.1 mM stock solution was immediately aliquoted to six Eppendorf tubes that were submerged in a 37 °C bath. For ODN **I**, ODN **II**, and the aminopropanediol conjugate **4**, aliquots were removed at 30, 45, 60, 90, 120, and 240 min. For ODN **III**, aliquots were removed at 2, 4, 6, 8, and 12 h. The aliquots were immediately frozen (-20 °C) and thawed just prior to HPLC analysis. Intact mustard-ODNs eluted at ~10 min, and a mixture of degradation products eluted at 5–8 min. Intact compound **4** eluted at ~14 min. After integration, the percent of intact conjugate was plotted vs time and the half-life for disappearance of starting material was determined from the line of best fit using an equation for exponential decay.

Reaction of Mustard Conjugates with a Model Nucleophile. One hundred microliters of a 0.1 mM solution of ODN **I**, **II**, or **III** was prepared in 20 mM HEPES buffer (pH 7.2) with 140 mM KCl and 10 mM MgCl₂. Sodium thiosulfate (10 mM, 100 equiv) was added to the mixtures. The half-life for disappearance of the starting conjugate was determined as described for the stability studies.

Determination of DNA Alkylation Rates for Triplex-Forming Mustard-ODNs. The duplex used in these studies was 65 bp long and contained a 33 bp homopurine-homopyrimidine run. The purine-rich strand of the duplex was 5'-end labeled by treatment with T4 polynucleotide kinase and [γ -³²P]ATP under standard conditions (15). Labeled ODN was purified using a Nensorb column (NEN Research Products) and had a specific activity of ~6000 cpm/fmol. Duplexes were formed by annealing 20 nM purine-rich strand with 40 nM complementary pyrimidine-rich strand in 20 mM HEPES (pH 7.2) with 140 mM KCl, 10 mM MgCl₂, and 1 mM spermine using an incubation profile of 1 min at 95 °C and 30 min at 37 °C. After annealing, coralyne chloride (Sigma) was added to give a final concentration of 10 μ M. The concentration of the labeled duplex was kept constant (20 nM), while the concentration of TFO was varied (0.2–2 μ M). Triplexes were formed in capped and siliconized polypropylene microcentrifuge tubes (0.65 mL) at 37 °C in a final volume of 25 μ L. The labeled duplex (22.5 μ L) was combined with 2.5 μ L of ODN **I** or ODN **II** (2 or 20 μ M), and the solutions were incubated at 37 °C. Aliquots (2.5 μ L) were removed at 0, 30, 60, 120, 240, 360, and 1320 min and stored frozen in 4 μ L of loading buffer (80% formamide, 0.01% xylene cyanol, and bromphenol blue). The aliquots were thawed, and alkylated products were electrophoretically resolved in a denaturing 8% polyacrylamide gel. The labeled bands were visualized by autoradiography and quantified using a BioRad GS-250 Phosphorimager. The percent of monoalkylated labeled strand was plotted vs time, and the half-life for alkylation was determined from the line of best fit using an equation for exponential growth.

RESULTS AND DISCUSSION

Triplex-Forming Mustard-ODN Model System. The specific ODN chosen for this study (ODN-NH₂) is a 21-mer G/A motif triplex-forming ODN, designed to hybridize to a homopurine run in the human DQB1*0302 allele (16). Inheritance of this allele predisposes individuals to insulin-dependent diabetes mellitus. This endogenous genetic target provides an ideal system for comparison of different triplex-forming motifs. In a

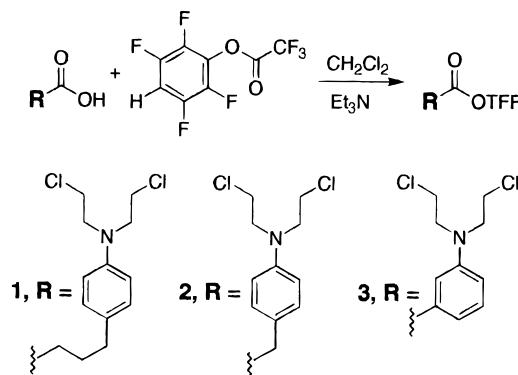


Figure 2. Design of triplex-forming ODN (ODN-NH₂) and the dsDNA target. ODN-NH₂ was reacted with the aryl nitrogen mustard TFP esters **1–3** to give ODN conjugates **I–III** as shown in Scheme 2. The position of the alkylated dG residue in the purine-rich strand is indicated by an arrow.

separate study (17), triplex formation in this model system was examined using a gel retardation assay. In a physiologically relevant buffer (pH 7.2 HEPES, 140 mM KCl, 10 mM MgCl₂, and 1 mM spermine), we found that the G/A motif triplex-forming ODN binds with the highest association constant. This buffer was designed to model salt concentrations expected to be found in the nucleus of cells (18). Although some self-association was observed with this G-rich ODN, it did not appear to affect triplex formation, especially in the presence of the triplex specific intercalating agent coralyne (19). ODN-NH₂ was prepared with a 5'-hexylamine linker in order to provide a site for attachment of the mustards. The linker length was not optimized for this target system but provides a long enough tether to allow each of the mustards studied herein to find a suitable conformation for alkylation of the targeted guanine in the homopurine-containing strand. The sequence of ODN-NH₂, the site of triplex formation, and the position of the targeted dG in the purine-rich strand are shown in Figure 2. Since cell culture experiments were planned, a 3'-hexanol modification was incorporated into the ODN in order to block the 3'-end of the ODN from nuclease degradation (12).

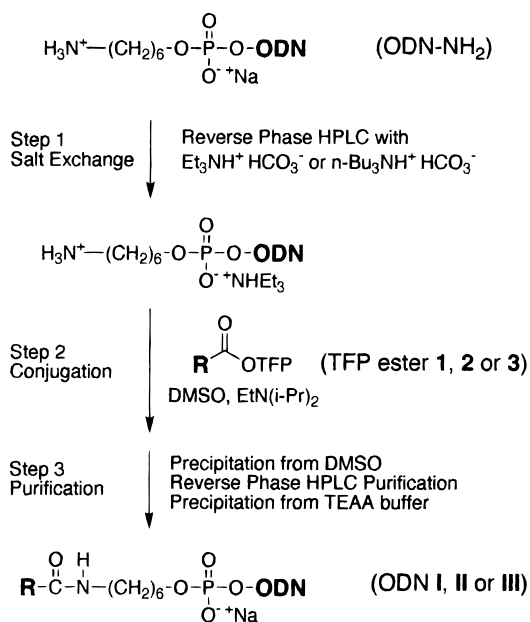
Conjugation Chemistry. Many simple conjugate groups such as amine-containing linkers can be conveniently added to the growing ODN chain during automated synthesis (20). However, the harsh deprotection conditions require postsynthetic addition of more sensitive conjugate groups. This generally involves treatment of an aqueous solution of an ODN containing a nucleophilic linker group with an electrophilic form of the conjugate group. For example, amine-modified ODNs react with "activated esters" to give amide bonds. We have found that 2,3,5,6-tetrafluorophenyl (TFP) esters are particularly convenient to make and use for ODN conjugation reactions. These compounds can be purified by flash chromatography with little decomposition, and they hydrolyze slowly in relation to some other activated esters. In addition, the chemical shift of the H4 proton ($\delta = 7.0$ ppm) can be monitored by ¹H NMR to give an indication of the purity of these compounds. Three different aryl nitrogen mustard TFP esters were prepared as shown in Scheme 1. In our earlier work (9), we used TFP ester **1** prepared from the clinically used anticancer compound chlorambucil. The reactivity of the aniline mustards can be "tuned" by judicious selection of the substituents on the aromatic ring. Increasing the electron density of the aryl group increases the nucleophilicity of the aniline nitrogen, thus increasing the rate of cyclization to the aziridinium cation. Phenyl acetate mustard (**2**) and benzoate mustard (**3**) were prepared as described for **1** and investigated as slower-reacting analogues of **1**. Early work had shown the relative rates of hydrolysis of the carboxylic acid precursors to **1**, **2**, and **3** to be 27, 17, and 4 when measured in 50% acetone (11). Synthesis of mustard-ODNs is problematic due to the rapid hydrolysis of the (chloroethyl)amino functional groups in neutral or basic aqueous conditions. Thus, anhydrous conditions were developed for synthesis of

Scheme 1. Synthesis of Aryl Nitrogen Mustard TFP Esters **1–3**^a



^a TFP is 2,3,5,6-tetrafluorophenyl.

Scheme 2. Synthesis of Mustard-ODN Conjugates^a



^a The structure of ODN-NH₂ is shown in Figure 2. Three different mustard-ODNs (ODNs **I–III**) were prepared using TFP esters **1–3**, respectively. Structures of the different aryl nitrogen mustard groups (R) are shown in Scheme 1.

mustard-ODNs as illustrated in Scheme 2. Others have used organic soluble cetyltrimethylammonium salts of the ODNs (21), but this method can be troublesome since the nonvolatile cetyltrimethylammonium chloride is not easily removed from the reaction mixtures. We recently found that triethylammonium (TEA) or tributylammonium (TBA) salts of ODNs are soluble in polar aprotic solvents such as DMSO. As the first step in mustard-ODN synthesis (Scheme 2, step 1), alkylammonium salts were prepared by loading the sodium form of the ODN onto a reverse phase HPLC column (equilibrated with TEA or TBA bicarbonate) and eluting with a gradient of acetonitrile. The excess volatile TEAB or TBAB is removed in vacuo to give the organic salt form of the

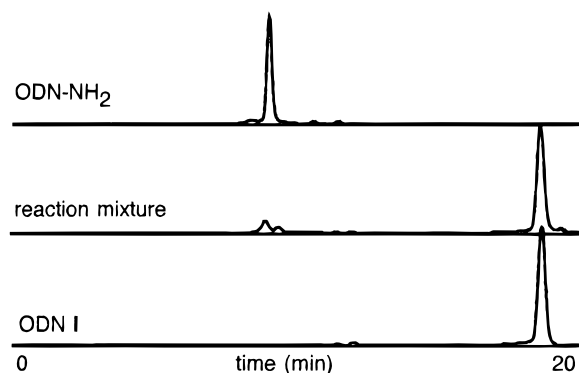


Figure 3. Reaction of ODN-NH₂ with TFP ester **1**. The progress of the reaction was followed by C₁₈ HPLC as described in Experimental Procedures. Unreacted starting ODN was removed by HPLC to give purified ODN **I**.

ODN. Most ODNs are soluble in DMSO as the TEA salts (~5 mg of ODN per milliliter of DMSO), but some modified ODNs require the extra lipophilicity provided by the TBA salts. We used TEA and TBA salts interchangeably for preparation of ODNs **I–III**. Preparation of the mustard-ODNs (Scheme 1, step 2) involved treatment of the DMSO solution of ODN-NH₂ (TEA salt) with 20 equiv of activated ester (**1**, **2**, or **3**) and excess ethyldiisopropylamine. Progress of the reaction from ODN-NH₂ to the more lipophilic mustard-ODN conjugate (**I**, **II**, or **III**) was easily monitored by reverse phase (C₁₈) HPLC as shown in Figure 3. Isolation and purification of the mustard-ODN (Scheme 2, step 3) was accomplished by first precipitating the crude product from DMSO solution by adding sodium perchlorate in acetone. The resulting pellet was dissolved in TEAA buffer and immediately purified by reverse phase HPLC and precipitation from the desired fraction. Although some yield was lost during the final HPLC and precipitation steps, ODNs **I–III** were routinely prepared with >95% purity.

Aqueous Stability of Mustard-ODNs. Reverse phase HPLC was used to study the aqueous reactivity of ODNs **I–III**. Mustard-ODNs were prepared at 100 μ M in an aqueous buffer (pH 7.2) that contained a physiological concentration of chloride ion. Typical chromatograms that illustrate the kinetics for degradation of ODN **II** are shown in Figure 4A. A complex mixture of less lipophilic ODN degradation products was observed. Hydrolyses of ODN **I** and ODN **III** showed similar mixtures of degradation products.

The large separation in retention times between degradation products and starting mustard-ODN allowed disappearance of ODNs **I–III** to be determined as shown in Figure 4B. As expected, the concentration of mustard-ODNs decayed exponentially, consistent with first-order reaction via a reactive aziridinium cation intermediate. Apparent residual ODN **I** at 240 min is due to baseline noise in the HPLC assay. Half-lives for hydrolysis of ODNs **I–III** were approximately 27, 57, and 216 min, respectively.

Aqueous decomposition of mustard-ODNs indicated other degradation products in addition to simple hydrolysis products (Figure 1, Nu⁻ = OH⁻). Observed products for these G-rich ODNs most likely result from intramolecular or intermolecular self-alkylation. G/A-containing ODNs have been shown to self-associate in solution, presumably as parallel strands held together through G–G and A–A hydrogen bonds (22). Analysis of degra-

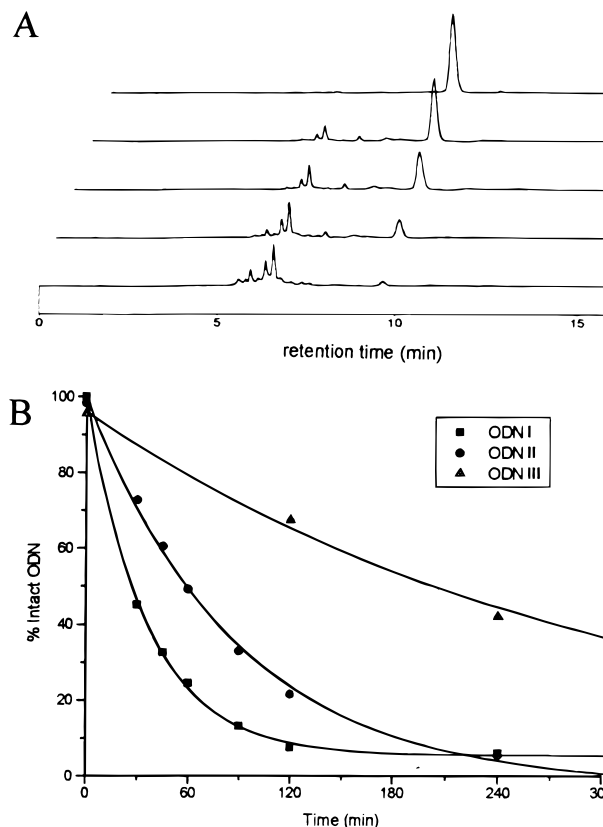


Figure 4. Aqueous stability of ODN **II**. (A) Decomposition of **II** in chloride-containing buffer (pH 7.2) at 37 °C was followed by C₁₈ HPLC at 0, 30, 60, 120, and 240 min. A mixture of ODN degradation products resulted. (B) Plot of the percentage of unreacted ODN vs time for each of the mustard-ODNs. The percentage of intact ODN was calculated from integration of chromatograms.

dation products in a related mustard-ODN by gel electrophoresis provided further evidence of self-alkylation (23).

Reactivity of Mustard-ODNs with a Model Nucleophile. To further characterize the reactivity of mustard ODNs, we used the same HPLC assay and followed reaction of ODNs **I–III** with 100 equiv (10 mM) of sodium thiosulfate. As shown in Figure 5A, only two major reaction products were observed for reaction of ODN **II** with this strong nucleophile. Stepwise formation and disappearance of the 7.8 min peak is indicative of a mono-thiosulfate adduct, and steady appearance of the final reaction product at 6.6 min is indicative of a bis-thiosulfate adduct. The same product profile was observed for ODN **I** and **III**. As shown in Figure 5B, relative rates for reaction of ODNs **I–III** with excess thiosulfate depend on the inherent reactivity of the nitrogen mustard conjugate group. Half-lives for reaction of ODNs **I–III** with thiosulfate were approximately 18, 52, and 192 min, respectively.

Effects of Buffer Composition on Reaction Rate. It appeared from our preliminary studies that adding thiosulfate to the buffer mixture shortened half-lives of mustard-ODNs. To determine the significance of this effect (and accuracy of our HPLC assay), we performed more careful kinetic measurements on ODN **II**. We also examined the reactivity of ODN **II** in a chloride-free buffer. Log[ODN **II**] vs time was plotted, and linear regression analysis gave first-order rate constants as shown in Figure 6. Adding a strong nucleophile (thiosulfate) slightly increased the reaction rate, presumably by trapping the aziridinium intermediate. An increase

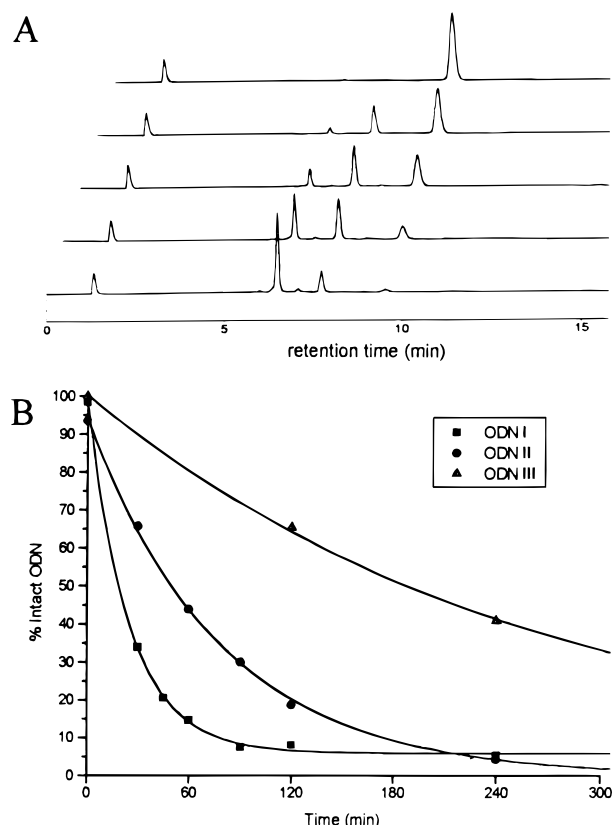


Figure 5. Reaction of ODN II with a model nucleophile. (A) Reaction of II with 100 equiv of sodium thiosulfate at 37 °C was followed by C₁₈ HPLC at 0, 30, 60, 120, and 240 min. Stepwise conversion to mono- and bis-thiosulfate adducts resulted. (B) Plot of the percentage of unreacted ODN vs time for each of the mustard–ODNs. The percentage of intact ODN was calculated from integration of chromatograms.

in the mustard–ODN half-life in the presence of chloride ion was observed, consistent with earlier studies on the reactivity of chlorambucil (24). Chloride ion can react with the aziridinium ion intermediate to regenerate the starting (2-chloroethyl)amine functional group. It should be noted that ionic strength was not held constant in our studies, and Debye–Huckel or specific salt effects may also be affecting reaction rate.

Effects of ODN on Reaction Rate. As described above, mixtures of self-alkylation products result from decomposition of G-rich mustard–ODNs in the absence of a competing nucleophile. The reactive aziridinium cation can be trapped by appropriately positioned nucleophilic DNA residues in a complex. To further explore the effects of the ODN carrier on reactivity of the phenyl acetate mustard, a small molecule analogue (**4**) was prepared by reacting TFP ester **2** with 1-amino-2,3-propanediol. The aqueous stability of **4** was measured using the HPLC assay described above. As expected, two major decomposition products were observed for **4**, consistent with simple hydrolysis of the (chloroethyl)-amine groups. The $t_{1/2}$ for hydrolysis of this derivative was 92 min as compared to 57 min for ODN II in the same buffer. Aminodiol analogue **4** reacted with thiosulfate in the same stepwise fashion with a $t_{1/2}$ of 48 min as compared to 52 min for ODN II in the same buffer. These results show that the ODN carrier can significantly affect the aqueous stability of the mustard conjugate group. The effects may be sequence-dependent, thus making the stability of mustard–ODNs difficult to predict accurately. Relative half-lives of ODNs I–III under various conditions are summarized in Table 1.

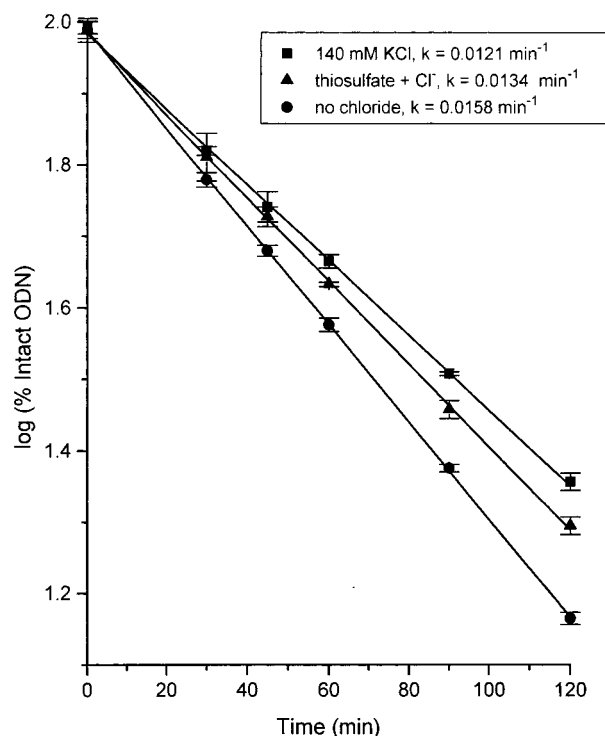


Figure 6. Kinetics plots for reaction of ODN II at 37 °C in different buffers. Buffers are described in Table 1. The HPLC assay described in Figures 5 and 6 was repeated in triplicate for each buffer. Linear regression analysis of log(percentage of intact ODN) vs time gave the rate constants (k) as indicated from the slope.

Table 1. Half-Life (Minutes) for Reaction of Nitrogen Mustard Conjugates^a

buffer ^c	ODN I	ODN II	ODN III	4 ^b
KCl	27	57	216	92
thiosulfate	18	52	192	48
no chloride	—	44	162	—

^a Structures of nitrogen mustards are shown in Scheme 1. $t_{1/2}$ for the disappearance of the starting compound was determined by HPLC assay. ^b Compound **4** is an aminopropanediol derivative of TFP ester **2**. In the reactions, 0.1 mM solutions of the indicated compound were used at 37 °C. ^c Reactions of nitrogen mustards were in pH 7.2 HEPES buffer (20 mM). KCl contains 140 mM KCl and 10 mM MgCl₂. Thiosulfate indicates addition of 10 mM Na₂S₂O₃ to the KCl buffer. No chloride used only HEPES buffer. Except for ODN II, results are from a single representative experiment.

DNA Alkylation Rates for Triplex-Forming Mustard–ODNs. Rates of DNA alkylation by ODNs I and II within a triplex were measured by denaturing gel electrophoresis using a synthetic 65-mer dsDNA target as shown in Figure 7. Alkylation of the labeled purine-rich strand in the duplex by ODN I or II gave a species with two linked strands that was observed as a slower-running band in the gel. This slow-running band is a result of monoalkylation of the dsDNA target. In separate sequencing experiments, the alkylated base was determined to be the dG directly 3' of the triplex region as shown in Figure 2. Trace amounts (<2%) of a very slow-moving band was observed where the mustard–ODN alkylates both strands of the dsDNA target. This level of interstrand cross-linking is low despite the presence of a preferred GNC site in the dsDNA target (25, 26). Hybridization buffers were identical with those used for the aqueous stability studies described in Figure 4 except for the presence of spermine and coralyne in the triplex assays. Initial experiments used duplexes with

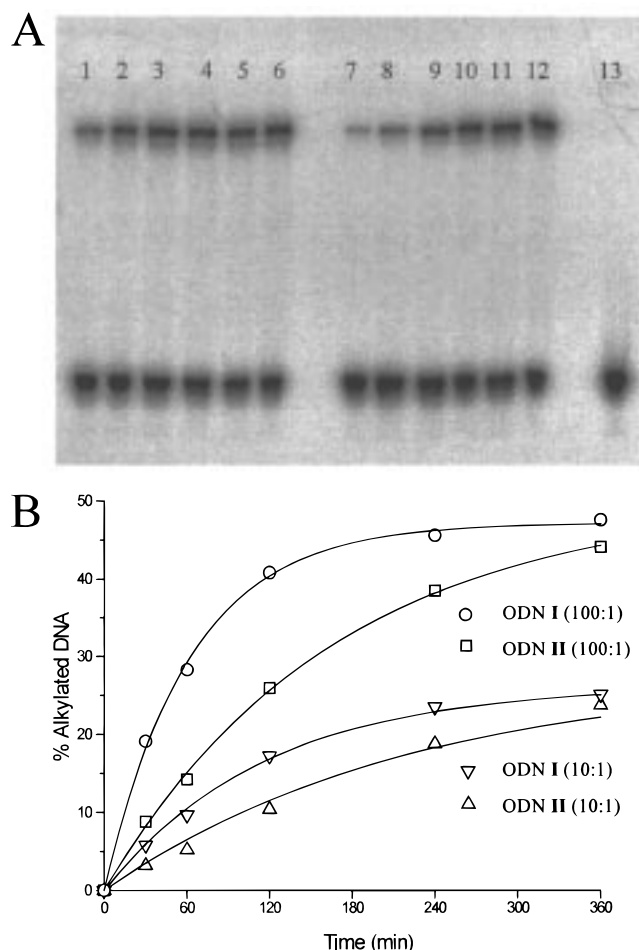


Figure 7. Alkylation of the 65-mer dsDNA target by triplex-forming ODNs **I** and **II**. Hybridization conditions are described in Experimental Procedures. (A) Denaturing gel analysis at an ODN:dsDNA ratio of 100:1. Upper bands correspond to alkylated purine-rich DNA strands. Lower bands are unmodified purine-rich DNA strands: lanes 1–6, ODN **I** at 30, 60, 120, 240, and 360 min; lanes 7–12, ODN **II** at the same time points; and lane 13, no mustard-ODN. (B) Plot of the percentage of DNA alkylation vs time for ODNs **I** and **II** at ODN:dsDNA ratios of 100:1 and 10:1.

either the purine- or pyrimidine-rich strand 5'-labeled with ^{32}P . Since alkylation of the pyrimidine-rich strand was negligible (<2%), we conducted quantitative kinetic studies using duplexes prepared from the 5'- ^{32}P -labeled strand containing the homopurine run.

The extent of alkylation measured at 22 h depended on the ratio of triplex-forming ODN to dsDNA target. At 100:1 ODN:dsDNA ($2\ \mu\text{M}$ ODN), alkylation plateaued at 48% for both ODN **I** and **II**, whereas at 10:1 ODN:dsDNA ($0.2\ \mu\text{M}$ ODN), the extent of alkylation was 27%. We were initially concerned that excess spermine in the buffer would compete for reactive aziridinium ion, but separate experiments showed that DNA alkylation in fact increased (from 42 to 48% for ODN **II**). In the absence of coralyne, alkylation was ~25% lower. Alkylation kinetics were not studied for ODN **III**, but the extent of DNA alkylation after 24 h was approximately the same as that for **I** and **II**.

As expected, the rate of DNA alkylation for ODN **I** was faster than that for ODN **II**. It is also clear that the absolute rate of alkylation depended on the ratio of triplex-forming ODN to dsDNA target. For 100:1 ODN **I**:dsDNA, ~46 min was required for half of the alkylation products to form, whereas at 10:1, ~81 min was required.

Likewise, for 100:1 ODN **II**:dsDNA, ~108 min was required for half of the alkylation products to form, whereas at 10:1, ~160 min was required. DNA alkylation rates for mustard-ODNs in the triplex assay are slower than inherent rates of formation of the aziridinium cation in solution. This may reflect both inherent inefficiency of DNA alkylation and the presence of degraded mustard-ODN that can compete for hybridization to target dsDNA. As expected, the percent of DNA alkylation increases until nitrogen mustard conjugate groups are completely degraded. Since there are two (2-chloroethyl)amine groups, the half-life for complete degradation of mustard-ODN should be 2 times the half-life for reaction of a single alkylating group (Table 1).

CONCLUSION

Versatile methods have been developed for preparation and analysis of mustard-ODNs. The anhydrous conditions are useful for addition of other aqueous sensitive or lipophilic conjugate groups to oligonucleotides. The three aryl nitrogen mustard-ODN conjugates studied herein had different inherent rates of reaction as designed. Although other factors had some effect on reaction rates, kinetic studies support the concept of a reactive aziridinium intermediate which either alkylates neighboring nucleophiles or is hydrolyzed. Electrophoresis studies showed that a large excess of triplex-forming mustard-ODN over dsDNA improved DNA alkylation efficiency and increased alkylation rates. Since genomic DNA targets are present in very low concentrations, large excesses of triplex ODN over DNA are easily achieved. For example, we have used ODN **I** to target a specific sequence in genomic DNA with 90% efficiency at a concentration of $0.5\ \mu\text{M}$ (4). The synthetic and analytical procedures described here will facilitate the use of mustard-ODNs as genetic probes, and allow design of other types of alkylating ODNs for applications in gene therapy.

ACKNOWLEDGMENT

This work was supported in part by the National Institutes of Health through Contract 1-AR-6-2230. The authors thank David Adams and Rene Taffin for technical assistance with HPLC assays and Debbie Lucas for help with conjugation chemistry.

LITERATURE CITED

- (1) Calabresi, P., and Chabner, B. A. (1985) Antineoplastic Agents. In *The Pharmacological Basis of Therapeutics* (A. G. Gilman, T. W. Rall, A. S. Nies, and T. Taylor, Eds.) pp 1208–1263, Pergamon Press, New York.
- (2) Mattes, W. B., Hartley, J. A., and Kohn, K. W. (1986) DNA sequence selectivity of guanine-N7 alkylation by nitrogen mustards. *Nucleic Acids Res.* 14, 2971–2987.
- (3) Povirk, L. F., and Shuker, D. E. (1994) DNA damage and mutagenesis induced by nitrogen mustards. *Mutat. Res.* 318, 205–226.
- (4) Belousov, E. S., Afonina, I. A., Podyminogin, M. A., Gamper, H. B., Reed, M. W., Wydro, R. M., and Meyer, R. B. (1997) Sequence specific targeting and covalent modification of human genomic DNA. *Nucleic Acids Res.* 25, 3440–3444.
- (5) Podyminogin, M. A., Meyer, R. B., and Gamper, H. B. (1995) Sequence-specific covalent modification of DNA by cross-linking oligonucleotides. Catalysis by RecA and implication for the mechanism of synaptic joint formation. *Biochemistry* 34, 13098–13108.
- (6) Nudler, E., Mustaev, A., Lukhtanov, E., and Goldfarb, A. (1997) The RNA-DNA hybrid maintains the register of transcription by preventing backtracking of RNA polymerase. *Cell* 89, 33–41.

- (7) Benn, M. H., Kazmaier, P., and Watanatada, C. (1970) The mechanism of the reaction of aryl nitrogen mustards with nucleophiles. *Chem. Commun.*, 1685–1686.
- (8) Knorre, D. G., and Zarytova, V. F. (1991) in *Prospects for Antisense Nucleic Acid Therapy of Cancer and Aids* (E. Wickstrom, Ed.) p 195, Wiley-Liss, New York.
- (9) Kutyavin, I. V., Gamper, H. B., Gall, A. A., and Meyer, R. B. (1993) Efficient, specific interstrand cross-linking of double-stranded DNA by a chlorambucil-modified, triplex-forming oligonucleotide. *J. Am. Chem. Soc.* 115, 9303–9304.
- (10) Knorre, D. G., and Vlassov, V. V. (1988) *Affinity Modification of Biopolymers*, p 87, CRC Press, Boca Raton, FL.
- (11) Everett, J. L., Roberts, J. J., and Ross, W. C. J. (1953) Aryl-2-halogenoalkylamines. Part XII. Some carboxylic derivatives of *N,N*-di-2-chloroethylaniline. *J. Chem. Soc.*, 2386–2392.
- (12) Gamper, H. B., Reed, M. W., Cox, T., Virosco, J. S., Adams, A. D., Gall, A. A., Scholler, J. K., and Meyer, R. B., Jr. (1993) Facile preparation of nuclease resistant 3' modified oligodeoxynucleotides. *Nucleic Acids Res.* 21, 145–150.
- (13) Reed, M. W., Adams, A. D., Nelson, J. S., and Meyer, R. B., Jr. (1991) Acridine- and cholesterol-derivatized solid supports for improved synthesis of 3'-modified oligonucleotides. *Bioconjugate Chem.* 2, 217–225.
- (14) Cantor, C. R., Warshaw, M. M., and Shapiro, H. (1970) Oligonucleotide interactions. III. Circular dichroism studies of the conformation of deoxyoligonucleotides. *Biopolymers* 9, 1059–1077.
- (15) Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1989) in *Current Protocols in Molecular Biology*, John Wiley, New York.
- (16) Nepom, G. T., and Erlich, H. (1991) MHC class-II molecules and autoimmunity. *Annu. Rev. Immunol.* 9, 493–525.
- (17) Gamper, H. B., Kutyavin, I. V., Rhinehart, R. L., Lokhov, S. G., Reed, M. W., and Meyer, R. B. (1997) Modulation of C^m/T, G/A, and G/T triplex stability by conjugate groups in the presence and absence of KCl. *Biochemistry* 36, 14816–14826.
- (18) Alberts, B. (1989) in *Molecular Biology of the Cell*, p 304, Garland, New York.
- (19) Lee, J. S., Latimer, L. J. P., and Hampel, K. J. (1993) Coralyne binds tightly to both TAT and CGC⁺-containing DNA triplexes. *Biochemistry* 32, 5591–5597.
- (20) Goodchild, J. (1990) Conjugates of oligonucleotides and modified oligonucleotides: A review of their synthesis and properties. *Bioconjugate Chem.* 1, 165–187.
- (21) Boutorine, A. S., Le Doan, T., Battioni, J. P., Mansuy, D., Dupre, D., and Helene, C. (1990) Rapid routes of synthesis of chemically reactive and highly radioactively labeled α - and β -oligonucleotide derivatives for in vivo studies. *Bioconjugate Chem.* 1, 350–356.
- (22) Noonberg, S. B., Francois, J.-C., Garestier, T., and Helene, C. (1995) Effect of competing self-structure on triplex formation with purine-rich oligodeoxynucleotides containing GA repeats. *Nucleic Acids Res.* 23, 1956–1963.
- (23) Lampe, J. N., Kutyavin, I. V., Rhinehart, R., Reed, M. W., Meyer, R. B., and Gamper, H. B. (1997) Factors influencing the extent and selectivity of alkylation within triplexes by reactive G/A motif oligonucleotides. *Nucleic Acids Res.* 25, 4123–4131.
- (24) Chatterji, D. C., Yeager, R. L., and Gallelli, F. (1992) Kinetics of chlorambucil hydrolysis using high-pressure liquid chromatography. *J. Pharm. Sci.* 81, 50–54.
- (25) Millard, J. T., Raucher, S., and Hopkins, P. B. (1990) Mechlorethamine cross-links deoxyguanosine residues at 5'-GNC sequences in duplex DNA fragments. *J. Am. Chem. Soc.* 112, 2459–2460.
- (26) Ojwang, J. O., Grueneberg, D. A., and Loechler, E. L. (1989) Synthesis of a duplex oligonucleotide containing a nitrogen mustard interstrand DNA-DNA cross-link. *Cancer Res.* 49, 6529–6537.

BC970134A

Maleimidocysteineamido-DOTA Derivatives: New Reagents for Radiometal Chelate Conjugation to Antibody Sulfhydryl Groups Undergo pH-Dependent Cleavage Reactions

Michael R. Lewis[†] and John E. Shively^{*,‡}

City of Hope Graduate Program in Biological Sciences, Duarte, California 91010, and Division of Immunology, Beckman Research Institute of the City of Hope, Duarte, California 91010. Received July 18, 1997; Revised Manuscript Received October 15, 1997[®]

We have synthesized two bifunctional derivatives of the macrocyclic chelating agent 1,4,7,10-tetraazacyclododecane-*N,N',N'',N'''*-tetraacetic acid (DOTA) equipped with maleimide groups for conjugation to reduced disulfide bonds of monoclonal antibodies. Using water-soluble carbodiimide chemistry, DOTA was coupled to L-cysteine to incorporate both a "pendant-type" carboxyl group for metal coordination and an orthogonal thiol group for protein attachment. The homobifunctional reagent 1,6-bis(maleimido)hexane was then used to introduce the maleimide functionality via a sulfide linkage to the macrocycle, and alternatively, the sulfide group was converted to a sulfone side chain. Both maleimide derivatives were conjugated to the anticarcinoembryonic antigen chimeric monoclonal antibody cT84.66 after light reduction of the mAb with dithiothreitol. In this manner, antibody conjugates were prepared which afforded near-quantitative labeling with the radiometals ¹¹¹In(III) and ⁹⁰Y(III) as well as quantitative immunoreactivity. Radioimmunoconjugates prepared with the sulfide and sulfone compounds exhibited relatively rapid linker-dependent radiometal loss when incubated in human serum and aqueous solutions at physiological temperature and pH. The unconjugated maleimidocysteineamido-DOTA derivatives and their Y(III) complexes were incubated in aqueous solution at 37 °C, and the resulting decomposition products were analyzed by HPLC and mass spectrometry. These studies revealed that the two bifunctional chelating agents underwent linker-specific cleavage reactions which were considerably faster at pH 7.4 than at pH 5.4. The chemically labile linker systems are expected to release chelated radiometal from mAb conjugates in a pH-dependent manner. This property may impart favorable tumor uptake and normal tissue clearance on radioimmunoconjugates prepared with these reagents, on the basis of the observation that many solid tumors are significantly more acidic than normal tissues.

INTRODUCTION

Bifunctional chelating agents (BCAs¹) have been used widely to derivatize tumor-specific monoclonal antibodies (mAbs) with radiometal ions for cancer imaging and

therapy (1–3). Since most radionuclides are metals, the conjugation of antibodies to radiometal complexes represents one of the most versatile strategies for selective delivery of diagnostic or therapeutic radiation to primary tumors or metastatic disease. This versatility stems not only from the variety of metallic radionuclides available, encompassing a range of coordination chemistries, half-lives, and radioemissions suitable for imaging and therapy, but also from the variety of chemical methods for attachment of radiometal complexes to mAbs. In addition, the versatility of chelate conjugation chemistry introduces the potential of optimizing several important properties in the resulting mAb conjugates, including radiolabeling efficiency, specific activity, immunoreactivity, and physiological stability. Considerable progress has been made in this field, beginning with EDTA derivatives (4) and leading to progressively more stable mAb–radiometal conjugates modified with derivatives of DTPA (5–11) and polyazamacrocyclic chelating agents (12–29).

Each generation of improvements has produced bifunctional radiometal chelates with greater thermodynamic, kinetic, and physiological stability, as well as more versatile chemistry for attachment to antibodies. The costs of these improvements, however, have been progressively elaborate synthetic schemes for generating BCAs and increased difficulty in labeling the resulting mAb conjugates with radiometals. The macrocyclic chelating agents possess relatively rigid, preorganized

* Address correspondence to John E. Shively, Ph.D., Division of Immunology, Beckman Research Institute of the City of Hope, 1450 E. Duarte Rd., Duarte, CA 91010. Phone: (626) 301-8301. Fax: (626) 301-8186. E-mail: jshively@coh.org.

[†] City of Hope Graduate Program in Biological Sciences.

[‡] Beckman Research Institute of the City of Hope.

[®] Abstract published in *Advance ACS Abstracts*, December 15, 1997.

¹ Abbreviations: BCA, bifunctional chelating agent; DOTA, 1,4,7,10-tetraazacyclododecane-*N,N',N'',N'''*-tetraacetic acid; MC-DOTA, maleimidocysteineamido-DOTA; MSC-DOTA, maleimidosulfonylcysteineamido-DOTA; DOTA-OSSu, *N*-hydroxysulfosuccinimide ester of DOTA; sulfo-NHS, *N*-hydroxysulfosuccinimide; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; BMH, 1,6-bis(maleimido)hexane; DTPA, diethylenetriaminepentaacetic acid; EDTA, ethylenediaminetetraacetic acid; TFA, trifluoroacetic acid; PBS, phosphate-buffered saline; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; HSA, human serum albumin; CEA, carcinoembryonic antigen; cT84.66, human/murine chimeric anti-CEA mAb; cT84.66–MC-DOTA, cT84.66 conjugated with MC-DOTA; cT84.66–MSC-DOTA, cT84.66 conjugated with MSC-DOTA; cT84.66–DOTA, cT84.66 conjugated with DOTA-OSSu; ESI MS, electrospray ionization mass spectrometry; FAB MS, fast atom bombardment mass spectrometry.

cavities which enable them to bind a wide variety of metal ions with extremely high stability constants (30–35), but the adverse consequences of this structural property are the slow rate of incorporation of the metal ion into the chelate complex (36) and effective competition for the ligand by trace metals (33). It has been shown that the chelation of metals by the macrocyclic ligand DOTA is often a two-step process in which a reversibly formed initial adduct (a type I complex) slowly transforms to the final chelate (a type II complex) (37, 38).

Given this mechanism, our hypothesis is that low radiochemical yields observed for labeling antibody–macrocycle conjugates, compared to free ligand, may be a result of competition between nonspecific metal-binding sites on the protein surface and formation of the type I complex. Macrocyclic BCAs bearing “pendant-type” carboxyl groups have been synthesized by Takenouchi et al. (39, 40), and the addition of a side chain carboxylate was shown to accelerate the formation of Y(III) complexes. These authors reasoned that the Y(III) ion was captured quickly but loosely by the pendant donor group, forming a more stable type I complex and raising the local metal concentration in the vicinity of the macrocyclic backbone, thus increasing the ultimate rate of complexation. The serum stabilities of the pendant-type bifunctional chelates were found to be identical with those of the corresponding nonpendant-type chelates, indicating that the pendant donor has no effect on type II complex stability. However, the Y(III)-binding properties of only the free chelating agents were investigated, and it was not determined whether the same effects would be borne out at the antibody conjugate level. We believe that structural modifications leading to better kinetics of type I complex formation will lead to improved radiometal labeling efficiency for antibody–macrocycle conjugates, and we set out to test this concept by synthesizing and conjugating to antibody a series of DOTA derivatives containing similarly positioned pendant-type carboxyl groups.

Takenouchi and co-workers employed six- and eight-step synthetic routes to introduce pendant-type carboxyl groups to the side chains of two *C*-substituted DOTA derivatives. They observed a rate enhancement for Y(III) complexation when the carboxylate oxygen of the pendant donor was spaced six, but not seven, chemical bonds from the amino nitrogen of the macrocyclic ring. We have developed a simplified synthetic strategy for introducing a similar pendant-type carboxylate donor, spaced six chemical bonds from the macrocyclic ring nitrogen, via peptide bond formation between one carboxyl group of DOTA and the α -amino group of an amino acid. Previously, we demonstrated that amide bond formation between DOTA and endogenous antibody amino groups allowed for the formation of extremely stable $^{111}\text{In(III)}$ and $^{90}\text{Y(III)}$ chelates on the mAb conjugates (29); that is, the conversion of one carboxyl group of DOTA to an amide did not affect type II complex stability to an appreciable degree. Therefore, we synthesized a monocysteineamide derivative of DOTA to introduce not only the pendant carboxylate donor group but also an orthogonal functionality for attachment to mAbs. The DOTA monocysteineamide compound was subsequently derivatized for antibody conjugation by reaction with 1,6-bis-(maleimido)hexane. Maleimidocysteineamido-DOTA derivatives containing both sulfide and sulfone linkers were prepared and conjugated to cysteine residues of cT84.66 (41), a human/murine chimeric mAb which displays high specificity and high affinity for carcinoembryonic antigen (42). Under the conditions used, it is believed that the

conjugation occurs specifically at the interchain disulfide bonds in the hinge region of the antibody (43–45).

We report here that the cT84.66 conjugate prepared with maleimidocysteineamido-DOTA (MC-DOTA, **2**) exhibits significantly improved rates of radiometal complexation under carrier-free conditions, leading to substantial increases in radiolabeling yields with ^{111}In and ^{90}Y . Furthermore, we have studied the radiometal-labeled antibody conjugates and free chelate complexes of the sulfide compound MC-DOTA and its sulfone derivative (MSC-DOTA, **3**) *in vitro* and found that these molecules undergo pH-dependent cleavage reactions in aqueous media. Our results indicate that both of these BCAs are chemically labile at physiological temperature and pH but significantly more stable under moderately acidic conditions. This attachment of kinetically stable radiometal chelates to monoclonal antibodies using pH-dependent chemically labile linkers offers the possibility of creating radioimmunoconjugates with favorable biodistribution properties.

EXPERIMENTAL PROCEDURES

General. DOTA trisodium salt was purchased from Parish Chemical Co. (Orem, UT). Sulfo-NHS, EDC, and BMH were obtained from Pierce (Rockford, IL). Human serum albumin (99%, fatty acid- and globulin-free) and L-cysteine were purchased from Sigma (St. Louis, MO). DTPA was obtained from Fluka (Milwaukee, WI). EDTA, yttrium chloride hexahydrate (99.999%), cobalt powder (99.995%), and Oxone (potassium hydrogen persulfate) were purchased from Aldrich (Milwaukee, WI). Chelex 100 (Biotechnology Grade, 100–200 mesh, sodium form) was obtained from Bio-Rad (Hercules, CA), and buffers used for radiolabeling reactions were passed over a Chelex 100 column (1 $\times$ 15 cm). Ultrapure water (18 M Ω -cm) was used for all procedures, and all other reagents were of the highest purity available. All laboratory glassware was washed with a mixed acid solution (46) and thoroughly rinsed with ultrapure water. $^{57}\text{CoCl}_2$ and Na^{131}I were obtained from ICN (Costa Mesa, CA). $^{111}\text{InCl}_3$ was obtained from Amersham (Arlington Heights, IL), and $^{90}\text{YCl}_3$ was obtained from Nordion (Kanata, ON) and Hanford (Richland, WA). The mAb cT84.66 was prepared as described previously (41).

HPLC and TLC were performed as described previously (29). Mass spectra were recorded on a Finnigan LCQ ion trap mass spectrometer equipped with a custom nanospray interface built at City of Hope. The mass spectrometer was operated under manual control with the automatic gain control active. In a few cases, manual control of the ion injection time was utilized to improve the quality of weaker product ion spectra. NMR spectra were recorded at pH 2.6 (not corrected for the deuterium isotope effect) on a Varian Unity spectrometer, operating at 300 MHz for ^1H and 75 MHz for ^{13}C , or a Varian Unity Plus spectrometer, operating at 500 MHz for ^1H and 125 MHz for ^{13}C . ^1H chemical shifts are reported relative to HOD (4.80 ppm), and ^{13}C chemical shifts are reported relative to 1,4-dioxane (66.66 ppm) as an internal reference. Concentrations of antibody were determined by absorbance measurements at 280 nm (A_{280} at 1 mg/mL = 1.42, within 5% of the concentration determined by amino acid analysis). UV spectrophotometry was performed on a Pharmacia LKB Ultrospec III instrument, using a 1 cm sample cell. Melting points were recorded on a Thomas Hoover uni-melt capillary melting point apparatus and are uncorrected.

Cysteineamido-DOTA (1). To a solution of 100 mg (213 μmol) of trisodium DOTA and 92.3 mg (425 μmol)

of sulfo-NHS in 1 mL of H₂O was added dropwise with continuous stirring a solution of 81.5 mg (425 μ mol) of EDC in 151 μ L of H₂O. The reaction mixture was stirred at room temperature for 1 h, after which a solution of 51.5 mg (425 μ mol) of L-cysteine in 326 μ L of H₂O was added dropwise with continuous stirring. The pH was adjusted to 7.0 with 50% NaOH, and the reaction mixture was stirred at room temperature for 22 h. The reaction mixture was then cooled to 4 °C, made 1 M in HCl with the addition of 155 μ L of concentrated HCl, and stirred at 4 °C for 17 h. The acidified mixture was applied to a Dowex AG50W-X8 column (H⁺ form, 1 $\times$ 6.5 cm), which was washed with 50 mL of H₂O and then eluted with a 40 mL step gradient from 0.5 to 4 M NH₄OH. A mixture of the desired product, disubstituted cysteine adducts of DOTA, and unreacted DOTA eluted in 2 M NH₄OH and was lyophilized to a pale yellow gum. The crude product was dissolved in 1 mL of H₂O and applied to a Dowex AG1-X4 column (HCO₂⁻ form, 1 $\times$ 11 cm), which was washed with 50 mL of H₂O and eluted with a 60 mL step gradient from 0.05 to 2 M HCO₂H. Unreacted DOTA eluted in 0.2 M HCO₂H, and the desired product eluted in 0.5 M HCO₂H. These fractions were lyophilized to give white powders, which were dissolved in 1 mL of H₂O and concentrated to dryness by lyophilization. This process was repeated four times to remove residual HCO₂H. This procedure afforded 25.2 mg (29.3%) of DOTA, which was recrystallized from H₂O/CH₃OH (1:9) and used for other syntheses, and 23.3 mg (21.6%) of **1** as a white powder: mp 177–178 °C (dec); ¹H NMR (D₂O) δ 2.93–3.06 (m, 2H, CH₂SH), 3.16–3.24 (m, 8H, CH₂NCH₂), 3.42 (m, 8H, CH₂NCH₂), 3.56–3.71 (m, 2H, CH₂CONH), 3.84 (s, 6H, CH₂CO₂H), 4.56 (dd, 1H, CHCH₂SH); ¹³C NMR (D₂O) δ 25.42, 48.61, 50.97, 53.53, 54.41, 55.42, 56.16, 170.50, 173.71, 174.63; FAB MS *m/z* calcd for C₁₉H₃₄N₅O₉S (M + H)⁺ 508.2077, found 508.2098.

Maleimidocysteineamido-DOTA (2). To a solution of 29.2 mg (106 μ mol) of 1,6-bis(maleimido)hexane in 1.0 mL of degassed *N,N*-dimethylformamide, under an argon atmosphere, was added dropwise with continuous stirring a solution of 5.4 mg (10.6 μ mol) of **1** in 1.9 mL of degassed 50 mM K₂HPO₄ at pH 7.5. The reaction mixture was stirred at room temperature for 2 h, after which it was extracted with 3 $\times$ 15 mL of ethyl acetate. The pH of the aqueous layer was adjusted to 1.5 with trifluoroacetic acid, and the desired product was purified by reversed phase HPLC using a 10 $\times$ 250 mm column (Vydac Protein & Peptide C₁₈, 5 μ m, 300 Å), a flow rate of 4.0 mL/min, and a linear gradient from 0 to 60% solvent B (solvent A, 0.1% TFA; solvent B, 0.1% TFA/90% CH₃CN) over 60 min. The major peaks, eluting at retention times of 26.8 and 27.2 min, were collected, pooled, and lyophilized to give 2.0 mg (24.5%) of **2** as a white powder: mp 120–122 °C; ¹H NMR (D₂O) δ 1.25 (m, 4H, CH₂CH₂CH₂N(CO)₂), 1.53 (m, 4H, CH₂CH₂N(CO)₂), 2.69 (dt, 1H, CH₂S), 3.01–3.49 (m, 24H, CH₂S + CH₂NCH₂ + CH₂CHS + CHS + CH₂N(CO)₂), 3.66 (br s, 2H, CH₂CONH), 3.81 (br s, 6H, CH₂CO₂H), 4.08 (m, 1H, CHCH₂S), 6.81 (s, 2H, C=CH); ¹³C NMR (D₂O) δ 25.47, 26.58, 27.55, 30.00, 35.83, 36.27, 37.55, 39.09, 39.87, 48.27, 48.66, 50.77, 51.56, 52.58, 134.34, 173.50, 174.23, 178.69, 179.82, 180.88; FAB MS *m/z* calcd for C₃₃H₅₀N₇O₁₃S (M + H)⁺ 784.3187, found 784.3205. A 5 μ g aliquot of the product was reinjected onto the HPLC instrument, using a 4.6 $\times$ 250 mm column (Vydac Protein & Peptide C₁₈, 5 μ m, 300 Å), a flow rate of 1.0 mL/min, and a linear gradient from 0 to 60% solvent B (solvent A, 0.1% TFA; solvent B, 0.1% TFA/90% CH₃CN) over 60 min. Two peaks were observed at retention times of 26.5 min (peak area of 49.5%) and 26.8

min (peak area of 50.5%). The two peaks gave identical mass spectra and presumably represent diastereomers of the product.

Maleimidosulfonylcysteineamido-DOTA (3). A solution of 2.0 mg (2.6 μ mol) of **2** in 0.4 mL of H₂O was cooled to 0 °C, and 10.2 μ L (2.4 mg, 3.8 μ mol) of a 23% (w/w) solution of Oxone in H₂O was added with continuous stirring. The reaction mixture was then stirred at room temperature for 4 h, after which 1.0 mg (5.1 μ mol) of sodium metabisulfite in 15.2 μ L of H₂O was added. The reaction mixture was stirred for 30 min at room temperature, and the desired product was purified by reversed phase HPLC using a 10 $\times$ 250 mm column (Vydac Protein & Peptide C₁₈, 5 μ m, 300 Å), a flow rate of 4.0 mL/min, and a linear gradient from 0 to 60% solvent B (solvent A, 0.1% TFA; solvent B, 0.1% TFA/90% CH₃CN) over 60 min. The major peak, eluting at a retention time of 25.8 min, was collected and lyophilized to afford 1.0 mg (48.1%) of **3** as a white powder: mp 151–154 °C; ¹H NMR (D₂O) δ 1.26 (m, 4H, CH₂CH₂CH₂N(CO)₂), 1.55 (m, 4H, CH₂CH₂N(CO)₂), 3.12–3.84 (m, 26H, CH₂-CH(SO₂) + CH₂NCH₂ + CH₂CONH + CH₂CO₂H), 3.27 (m, 2H, CH₂(SO₂)), 3.49 (dd, 2H, CH₂N(CO)CH(SO₂)), 3.55 (t, 2H, CH₂N(CO)C=), 4.07–4.11 (m, 1H, CHCH₂(SO₂)), 4.24–4.30 (m, 1H, CH(SO₂)), 6.82 (s, 2H, C=CH); ¹³C NMR (D₂O) δ 25.33, 26.40, 27.58, 30.34, 35.69, 36.54, 37.56, 38.78, 39.43, 47.88, 48.65, 51.52, 52.38, 53.09, 134.35, 170.15, 171.02, 172.47, 173.48, 176.51; FAB MS *m/z* calcd for C₃₃H₅₀N₇O₁₅S (M + H)⁺ 816.3086, found 816.3051. A 5 μ g aliquot of the product was reinjected onto the HPLC instrument, using a 4.6 $\times$ 250 mm column (Vydac Protein & Peptide C₁₈, 5 μ m, 300 Å), a flow rate of 1.0 mL/min, and a linear gradient from 0 to 60% solvent B (solvent A, 0.1% TFA; solvent B, 0.1% TFA/90% CH₃CN) over 60 min. A single peak was observed at a retention time of 25.7 min.

Preparation of cT84.66–MC-DOTA and cT84.66–MSC-DOTA Conjugates. An aliquot of 21.5 mg of cT84.66 in 2 mL of PBS (pH 7.4) was dialyzed against 1 L of PBS/20 mM DTPA (pH 7.4) for 24 h at 4 °C and then against 1 L of PBS (pH 7.4), over Chelex 100, for 43.5 h at 4 °C, with one buffer change. To 17.2 mg (115 nmol) of cT84.66 in 2.05 mL of PBS (pH 7.4) was added 230 μ L (2.30 μ mol) of freshly prepared 10 mM DTT in degassed H₂O. The reaction mixture was incubated at 37 °C for 3 h under an argon atmosphere, after which it was applied in equal volumes to four 1 mL Sephadex G-25–50 spin columns. The mAb was eluted from the spin columns by centrifugation (4) at 1200 rpm for 2 min under argon. The eluents were pooled, and the volume, concentration, and sulfhydryl titer of the reduced mAb solution were measured. Then, to 4.68 mg (31.2 nmol) of reduced cT84.66 was added 245 μ g (312 nmol) of **2** in 49.0 μ L of degassed H₂O, and to 4.38 mg (29.2 nmol) of reduced cT84.66 was added 240 μ g (294 nmol) of **3** in 48.0 μ L of degassed H₂O. The reaction mixtures were incubated on a tube rotator under an argon atmosphere for 1.5 h at room temperature, after which they were dialyzed against 1 L of 0.25 M ammonium acetate/20 mM DTPA (pH 7.0) for 24 h at 4 °C. The conjugates were then dialyzed against 1 L of 0.25 M ammonium acetate, over Chelex 100, for 138 h at 4 °C, with five buffer changes. Typical mAb recoveries ranged from 72 to 79% for the conjugation procedure.

Alternatively, preparations of cT84.66–MC-DOTA and cT84.66–MSC-DOTA were alkylated at unreacted cysteine residues with iodoacetic acid. After reaction with **2** or **3**, 100 μ L (10 μ mol) of freshly prepared 100 mM iodoacetic acid in degassed 0.2 M ammonium bicarbon-

ate/1 mM EDTA (pH 8.4) was added. The reaction mixtures were incubated on a tube rotator in the dark under an argon atmosphere for 1 h at room temperature, and the carboxymethylated conjugates were then dialyzed as described above.

Radiolabeling of cT84.66–MC-DOTA and cT84.66–MSC-DOTA. The cT84.66–MC-DOTA conjugate was labeled with the radiometals $^{111}\text{In}(\text{III})$ and $^{90}\text{Y}(\text{III})$, and the cT84.66–MSC-DOTA conjugate was labeled with $^{111}\text{In}(\text{III})$ only. In a typical $^{111}\text{In}(\text{III})$ labeling reaction, 1.00 mCi of $^{111}\text{InCl}_3$ in 68 μL of 0.04 N HCl was added to 110 μL of 0.25 M ammonium acetate (pH 5.7), followed by an aliquot of 0.2 mg of the mAb conjugate in 44 μL of 0.25 M ammonium acetate (pH 7.0). The reaction mixture was incubated at 43 °C for 45 min, after which the solution was made 1 mM in EDTA with the addition of 24 μL of 10 mM EDTA (pH 6.5). The reaction mixture was incubated at 37 °C for 15 min, and then the ^{111}In -labeled antibody was purified by size exclusion HPLC using a TosoHaas TSKgel G2000 SW column (10 μm , 7.5 $\times$ 300 mm) and an isocratic mobile phase of normal saline at a flow rate of 0.5 mL/min. The mAb peak, eluting at a retention time of 8.3 min, was collected in 0.5 mL fractions containing 1 drop of 25% (w/v) HSA each.

In a typical ^{90}Y labeling reaction, 1.90 mCi of $^{90}\text{YCl}_3$ in 24 μL of 0.05 N HCl was added to 48 μL of 0.25 M ammonium acetate (pH 5.7). Then 0.2 mg of cT84.66–MC-DOTA in 44 μL of 0.25 M ammonium acetate (pH 7.0) was added, and the reaction mixture was incubated at 43 °C for 1 h. The solution was made 1 mM in DTPA with the addition of 13 μL of 10 mM DTPA (pH 6.0) and incubated at 37 °C for 15 min. The ^{90}Y -labeled conjugate was purified by size exclusion HPLC in the same manner as the ^{111}In -labeled antibody.

The cT84.66–MC-DOTA conjugate was labeled with ^{131}I using the Iodogen method (47). An aliquot of 0.4 mg of cT84.66–MC-DOTA in 100 μL of 0.25 M ammonium acetate (pH 7.0) was placed in a polypropylene tube that had been coated with 100 μg of Iodogen. Then, 0.4 mCi of Na^{131}I in 7.3 μL of PBS (pH 7.4) was added, and the reaction mixture was incubated at room temperature for 2 min. The ^{131}I -labeled conjugate was purified by gel filtration HPLC on two Pharmacia Superose 12 HR 10/30 columns (1 $\times$ 30 cm) in series, using an isocratic mobile phase of normal saline and a flow rate of 0.5 mL/min. The mAb peak, eluting at a retention time of 42 min, was collected in 0.5 mL fractions containing 1 drop of 25% (w/v) HSA each.

Immunoreactivity Determination. A dilution of the purified ^{111}In - or ^{90}Y -labeled cT84.66–MC-DOTA conjugate, containing approximately 200 000 cpm of radioactivity, was mixed with a 20-fold molar excess of purified CEA (48, 49) in 150 μL of 1% HSA/PBS (pH 7.4). A control sample was prepared in an identical manner, except that CEA was not added. The reaction mixtures were incubated at 37 °C for 15 min with continuous end-over-end mixing, after which aliquots of 100 μL were analyzed by gel filtration HPLC, using two Pharmacia Superose 6 HR 10/30 columns (1 $\times$ 30 cm) in series and an isocratic mobile phase of 0.05 M Na_2SO_4 /0.02 M NaH_2PO_4 /0.05% NaN_3 (pH 6.8) at a flow rate of 0.5 mL/min. Immunoreactivity was calculated as the percentage of the total radioactivity shifted to complexes with apparent molecular masses higher than that of the mAb.

Serum Stability Studies. An aliquot of radiolabeled cT84.66–MC-DOTA or carboxymethylated cT84.66–MSC-DOTA containing approximately 1.6×10^7 cpm of ^{111}In radioactivity or 4.0×10^7 cpm of ^{90}Y radioactivity was added to 1.1 mL of fresh human serum containing

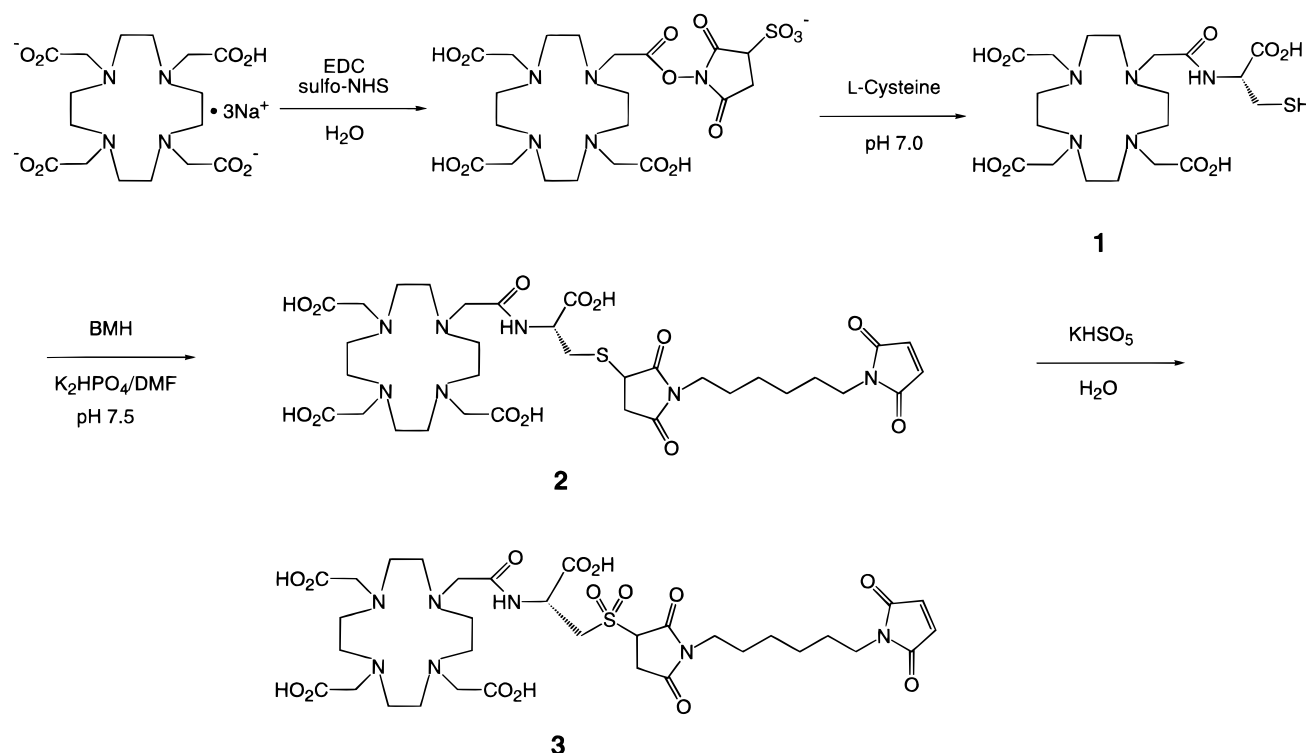
10 μL of 10% NaN_3 . The mixture was incubated at 37 °C throughout the study, during which 100 μL samples were analyzed by gel filtration HPLC on two Pharmacia Superose 12 HR 10/30 columns (1 $\times$ 30 cm) in series, using an isocratic mobile phase of 0.05 M Na_2SO_4 /0.02 M NaH_2PO_4 /0.05% NaN_3 (pH 6.8) and a flow rate of 0.5 mL/min. Serum samples were analyzed at intervals ranging from 6 to 72 h for 7–8 days to determine conjugate stability.

Stability Studies in Aqueous Solutions. The carboxymethylated cT84.66–MC-DOTA conjugate was labeled with ^{111}In and purified by size exclusion HPLC as described above, except that the mAb peak was collected in 0.5 mL fractions without HSA. Aliquots of ^{111}In -labeled carboxymethylated cT84.66–MC-DOTA containing 1.6×10^7 cpm of radioactivity were added to 1.1 mL of PBS/0.1% NaN_3 (pH 7.4), 1.1 mL of 1 mM DTPA/PBS/0.1% NaN_3 (pH 7.2), and 1.1 mL of 1 mM DTT/PBS/0.1% NaN_3 (pH 7.4). The three mixtures were incubated at 37 °C over the course of the study, during which 100 μL samples were analyzed by gel filtration HPLC as described for the serum stability studies. Samples of the aqueous solutions were analyzed at intervals ranging from 12 to 48 h for 7 days to determine conjugate stability.

Bifunctional Chelate Stability Studies. To 100 μg (128 nmol) of **2** in 20.0 μL of H_2O was added 388 μg (1.28 μmol) of freshly prepared $\text{YCl}_3 \cdot 6\text{H}_2\text{O}$ in 77.6 μL of 0.25 M ammonium acetate (pH 5.7). The reaction mixture was incubated at 43 °C for 1 h, after which the Y(III) complex of **2** was purified by reversed phase HPLC, using a 4.6 $\times$ 250 mm column (Vydac Protein & Peptide C₁₈, 5 μm , 300 Å), a flow rate of 1.0 mL/min, and a linear gradient from 0 to 60% solvent B (solvent A, 0.1% TFA; solvent B, 0.1% TFA/90% CH_3CN) over 60 min. The major peak, eluting at a retention time of 24.7 min, was collected, and the correct structure of the Y(III) chelate of **2** was confirmed by ESI MS and MS². This fraction was lyophilized to a white powder and redissolved in 20.0 μL of H_2O , and two 10.0 μL aliquots of the resulting solution were added to 0.5 mL of PBS at pH 7.4 and 0.5 mL of PBS at pH 5.4, respectively. Two control reactions were set up in which **2** was dissolved in PBS at pH 7.4 and PBS at pH 5.4, respectively. The four reaction mixtures were incubated at 37 °C throughout the study, during which 50 μL samples were analyzed by reversed phase HPLC, using a 2 $\times$ 250 mm column (Column Engineering Reliasil C₁₈, 5 μm , 300 Å), a flow rate of 0.2 mL/min, and a linear gradient from 0 to 60% solvent B (solvent A, 0.1% TFA; solvent B, 0.1% TFA/90% CH_3CN) over 60 min. Samples were analyzed at intervals ranging from 3 to 287 h over a period of 23 days to determine the stability of the bifunctional chelate. The peaks corresponding to the major decomposition products were collected and stored at –80 °C prior to analysis by ESI MS, MS², and MS³.

To 100 μg (123 nmol) of **3** in 20.0 μL of H_2O was added 373 μg (1.23 μmol) of freshly prepared $\text{YCl}_3 \cdot 6\text{H}_2\text{O}$ in 74.6 μL of 0.25 M ammonium acetate (pH 5.7). The reaction mixture was incubated at 43 °C for 1 h, after which the Y(III) complex of **3** was purified by reversed phase HPLC in the same manner as the Y(III) complex of **2**. The major peak, eluting at a retention time of 24.3 min, was collected, and the correct structure of the Y(III) chelate of **3** was confirmed by ESI MS and MS². This fraction was lyophilized, redissolved in H_2O , and added to PBS at pH 7.4 and PBS at pH 5.4 in the same manner as the Y(III) complex of **2**. Two control reactions were also set up in which **3** was dissolved in PBS at pH 7.4 and in

Scheme 1



PBS at pH 5.4. The four reaction mixtures were incubated at 37 °C throughout the study, and for 5 days, samples were analyzed by reversed phase HPLC at intervals ranging from 3 to 72 h, using the method employed for **2** and its Y(III) chelate. The peaks corresponding to the major decomposition products were collected and stored at -80 °C prior to analysis by ESI MS, MS², and MS³.

RESULTS

Synthesis. The synthesis of MC-DOTA (**2**) and MSC-DOTA (**3**) is shown in Scheme 1. DOTA *N*-hydroxysulfosuccinimide ester was prepared by a modification of the procedure employed for direct conjugation to proteins (29). Reaction of the active ester with L-cysteine at pH 7.0 results in the formation of an intermediate thioester, which in aqueous solution rearranges spontaneously to the more stable amide derivative (50). After purification by sequential cation- and anion-exchange chromatography, the DOTA cysteineamide adduct **1** was reacted with an excess of the sulfhydryl-specific homobifunctional reagent 1,6-bis(maleimido)hexane. The major product, **2**, could be purified easily by reversed phase HPLC by virtue of its long, hydrophobic side chain. Two byproducts from the preparation of **2** were also collected and identified by mass spectrometry. The side product eluting at a retention time of 22.1 min was determined to be the adduct of BMH and two molecules of DOTA cysteineamide, and the compound eluting at 24.2 min was identified as a product resulting from the addition of one molecule of water to **2**. Compound **3** was prepared by Oxone oxidation of **2**, a reaction which is highly chemoselective for conversion of sulfides to sulfones (51). During the purification of **3** by reversed phase HPLC, a trace byproduct eluting at a retention time of 24.4 min was collected and identified by mass spectrometry as the sulfoxide.

Antibody Conjugation. MC-DOTA and MSC-DOTA were conjugated to the human/murine chimeric anti-CEA

Table 1. Conjugation and ⁹⁰Y Radiolabeling Yields for cT84.66–MC-DOTA Conjugates^a

MC-DOTA:mAb	chelates/mAb	labeling efficiency (%)	specific activity (mCi/mg)
1:1	0.542	0.982	0.0982
2:1	0.655	3.64	0.364
4:1	1.32	25.8	2.58
10:1	3.80	81.2	8.12
20:1	6.28	95.8	9.58
40:1	6.34	97.1	9.71

^a Radiolabeling was performed for 1 h at 43 °C, using 2.00 mCi of ⁹⁰Y and 0.200 mg of mAb conjugate. Immunoreactivity of all ⁹⁰Y-labeled conjugates was 100%.

mAb cT84.66, isotype IgG₁, κ, using a modification of the method of Willner et al. (52). Antibody reduction was monitored using Ellman's reagent (53, 54). Treatment of cT84.66 with 20 molar equiv of DTT (1 mM) resulted in the generation of 10.3 ± 0.7 reactive sulfhydryl groups per antibody molecule, and further addition of DTT did not increase the SH titer (data not shown). In our experience, cT84.66 was fully reduced in a reproducible manner under these conditions, allowing the extent of conjugation to be controlled by the amount of maleimide reagent added.

As shown in Table 1, conjugation of lightly reduced cT84.66 with MC-DOTA at six BCA:mAb molar ratios, ranging from 1:1 to 40:1, resulted in average incorporations of 0.5–6.3 chelates per antibody molecule, as determined by a modification of a ⁵⁷Co(II) binding assay (4, 29). The reduced antibody was maximally modified with 6.3 chelates/mAb after reaction with 20 molar equiv of MC-DOTA. SDS–polyacrylamide gel electrophoresis of the six cT84.66–MC-DOTA conjugates under nonreducing conditions [Figure 1 (top)] showed that the conjugation reaction is relatively specific for reduced interchain disulfide residues. The addition of increasing amounts of MC-DOTA to reduced cT84.66 resulted in progressive conversion of the intact mAb to species which, in the presence of SDS, dissociated into various combina-

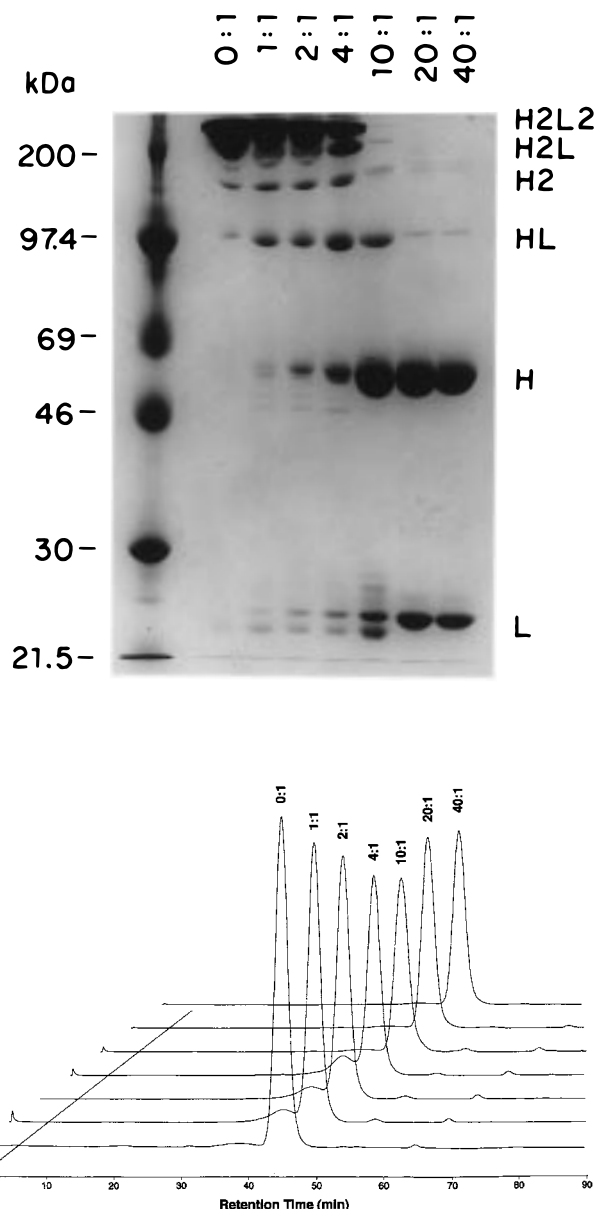


Figure 1. (Top) Nonreducing SDS-polyacrylamide gel electrophoresis of unconjugated cT84.66 and cT84.66-MC-DOTA conjugates prepared with 1–40 molar equiv of MC-DOTA. The MC-DOTA:mAb conjugation ratio is shown above each lane. Molecular mass markers are shown to the left. Aliquots of 8–12 μ g of the conjugates were loaded onto a 10% polyacrylamide gel (8×8 cm) with a 3% stacking layer (2×8 cm). (Bottom) Gel filtration chromatography of unconjugated cT84.66 and cT84.66-MC-DOTA conjugates prepared with 1–40 molar equiv of MC-DOTA. The MC-DOTA:mAb conjugation ratio is shown above each chromatogram. Aliquots of 8–120 μ g of the conjugates were injected onto two Pharmacia Superose 12 HR 10/30 columns (1×30 cm) in series and eluted with an isocratic mobile phase of 0.05 M Na_2SO_4 /0.02 M NaH_2PO_4 /0.05% NaN_3 (pH 6.8) at a flow rate of 0.5 mL/min. Absorbance was monitored at 280 nm. The peak eluting at a retention time of 45 min corresponds to an apparent molecular mass of 150 kDa.

tions of the heavy and light polypeptide chains: H₂L, H₂, HL, and modified and unmodified H and L chains. The same six preparations were also analyzed by gel filtration chromatography [Figure 1 (bottom)], which revealed that, under nondenaturing conditions, all of the conjugates exist as species with an apparent molecular mass of 150 kDa.

Antibody Radiolabeling. Also shown in Table 1 are radiochemical yields and radioimmunoconjugate specific

Table 2. Radiolabeling Yields for cT84.66–DOTA-OSSu and –MC-DOTA Conjugates^a

radiometal	T (°C)	time (min)	cT84.66–DOTA labeling efficiency (%)	cT84.66–MC-DOTA labeling efficiency (%)
¹¹¹ In	43	45	73.5 $\pm$ 12.2	96.6 $\pm$ 2.5
⁹⁰ Y	43	60	56.4 $\pm$ 3.4	78.6 $\pm$ 3.7

^a The average number of chelates per mAb was 3.8 for both conjugates. Immunoreactivity of all radiolabeled conjugates was 100%. Radiochemical yields represent the average of three experiments.

activities for ⁹⁰Y labeling of the six cT84.66–MC-DOTA conjugates. The conjugate prepared at an MC-DOTA:mAb molar ratio of 10:1, modified with an average of 3.8 chelates per antibody molecule, was the least modified product to give high-specific activity labeling with ⁹⁰Y. Using our standard labeling conditions at 43 °C, the average yield for ⁹⁰Y incorporation into this cT84.66–MC-DOTA conjugate was 78.6% after 60 min, and the average ¹¹¹In incorporation was 96.6% after 45 min (Table 2). In contrast, average labeling yields for a cT84.66 conjugate prepared with DOTA *N*-hydroxysulfosuccinimide ester (29), also modified with 3.8 chelates per mAb, were 56.4% for ⁹⁰Y and 73.5% for ¹¹¹In. The efficiency of ¹¹¹In incorporation into the cT84.66–MSC-DOTA conjugate was 72.9% for a single experiment, affording a radioimmunoconjugate with a specific activity of 3.65 mCi/mg.

Immunoreactivity of Radiolabeled cT84.66–MC-DOTA. The immunoreactivity of ¹¹¹In- and ⁹⁰Y-labeled cT84.66–MC-DOTA was determined by a solution phase assay using purified CEA. Gel filtration chromatography on tandem Superose 6 columns routinely allowed separation of the 2:1 CEA–mAb complex, the 1:1 CEA–mAb complex, and free antibody. The binding percentages for a 1:190 dilution of the ¹¹¹In-labeled and a 1:50 dilution of the ⁹⁰Y-labeled mAb were both 100% after a 15 min incubation with 20 molar equiv of CEA at 37 °C. In each case, only the 2:1 CEA–mAb complex was observed by gel filtration chromatography, indicating that both antigen-combining sites remained fully immunoreactive.

Serum Stability of cT84.66–MC-DOTA and cT84.66–MSC-DOTA. The kinetic stabilities of ¹¹¹In- and ⁹⁰Y-labeled cT84.66–MC-DOTA and ¹¹¹In-labeled cT84.66–MSC-DOTA were studied in fresh human serum at 37 °C. The serum samples were analyzed by gel filtration chromatography with radioactivity detection, and the area of each radioactive peak was divided by the total area of all radioactive peaks in the chromatogram to determine the percentage of radiometal associated with that fraction.

The serum stability results for ¹¹¹In-labeled cT84.66–MC-DOTA are shown in Figure 2. Fifty percent of the radiolabel was lost from the antibody fraction of the mixture after approximately 27 h, resulting in the transfer of ¹¹¹In to a fraction with an apparent molecular mass of 70 kDa and to a low-molecular mass fraction (<1350 Da). After 7 days, 24.7% of the ¹¹¹In label remained bound to the mAb, 61.3% was associated with the 70 kDa fraction, and 14.0% of the activity was obtained as a low-molecular mass species. Addition of CEA to the serum and reanalysis by gel filtration chromatography revealed that only the 150 kDa fraction was immunoreactive. Similar results were obtained for ⁹⁰Y-labeled cT84.66–MC-DOTA (data not shown). Carboxymethylation of unconjugated cysteine residues in cT84.66–MC-DOTA with iodoacetic acid resulted in a moderate improvement in serum stability. In that case,

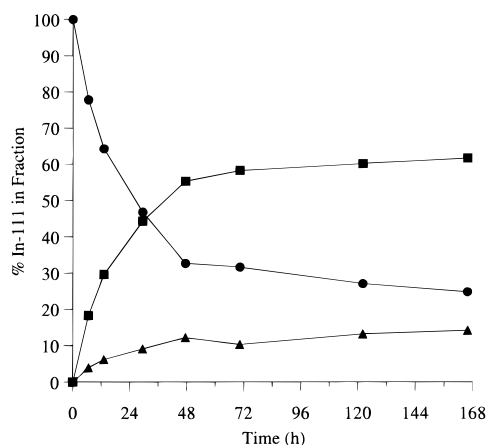


Figure 2. Percentage of ^{111}In associated with cT84.66-MC-DOTA (●), serum albumin (■), and low-molecular mass (▲) fractions as a function of time of incubation in human serum at 37 °C, determined by gel filtration HPLC.

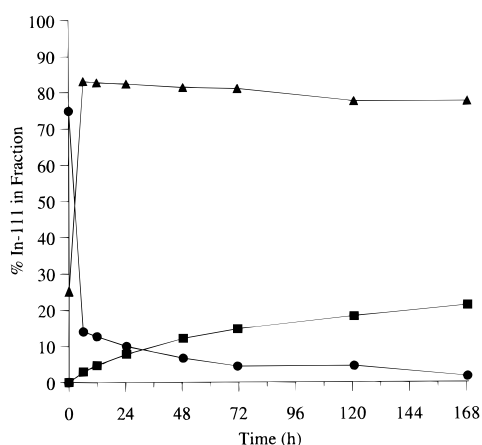


Figure 3. Percentage of ^{111}In associated with cT84.66-MSC-DOTA (●), serum albumin (■), and low-molecular mass (▲) fractions as a function of time of incubation in human serum at 37 °C, determined by gel filtration HPLC.

50% of the ^{111}In label was lost from the mAb in about 47 h, but after 8 days, 21.1% of the ^{111}In activity was bound to the mAb fraction, 59.3% eluted in the 70 kDa fraction, and 19.6% eluted as a low-molecular mass species (data not shown). In contrast, the ^{131}I -labeled cT84.66-MC-DOTA conjugate was completely stable in human serum for a period of 7 days; 100% of the ^{131}I activity remained associated with the mAb fraction (data not shown).

The 70 kDa ^{111}In species was found to coelute with purified, ^{131}I -labeled human serum albumin by gel filtration chromatography. Also, the ^{90}Y activity associated with the 70 kDa peak was collected, analyzed by reducing and nonreducing SDS-polyacrylamide gel electrophoresis with autoradiography detection, and found to migrate with an apparent molecular mass of 66.4 kDa (data not shown). No evidence that either radiometal associated with transferrin was obtained by gel filtration chromatography or gel electrophoresis. When the radiolabeled mAb was incubated with purified HSA at physiological temperature and pH, alkylation of Cys-34 of HSA with iodoacetic acid had no effect on the rate of transfer of ^{111}In to HSA.

The serum stability results for ^{111}In -labeled carboxymethylated cT84.66-MSC-DOTA are shown in Figure 3. After 6 h at 37 °C, only 14.1% of the radiometal label remained with the mAb. However, unlike the cT84.66-MC-DOTA conjugate, most of the ^{111}In activity remained

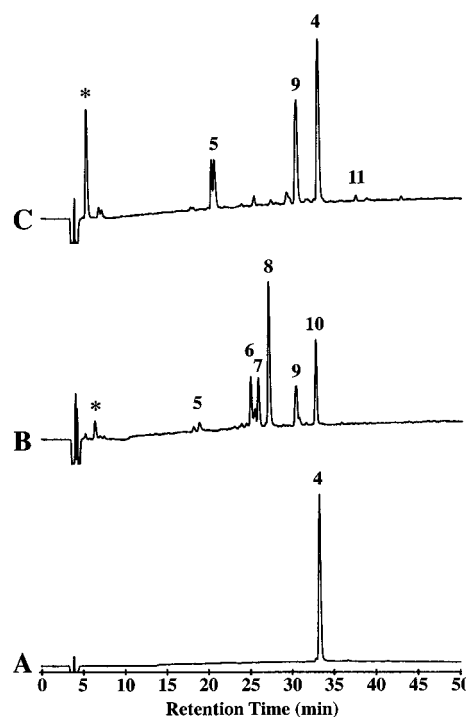


Figure 4. Reversed phase chromatograms of MC-DOTA[Y(III)] before incubation (A) and after incubation in PBS at 37 °C for 555 h at pH 7.4 (B) or for 555 h at pH 5.4 (C). Aliquots of 5 μg were injected onto a C_{18} column (2 $\times$ 250 mm, 5 μm , 300 Å) and eluted with a linear gradient from 0 to 60% solvent B (solvent A, 0.1% TFA; solvent B, 0.1% TFA/90% CH_3CN) over 60 min at a flow rate of 0.2 mL/min. Absorbance was monitored at 214 nm.

in the low-molecular mass fraction (83.1% at 6 h), with very little transfer to HSA (2.90% at 6 h). After 7 days, the amounts of the ^{111}In associated with cT84.66-MSC-DOTA, HSA, and the low-molecular mass species were 1.65, 21.3, and 77.1%, respectively.

Stability of cT84.66-MC-DOTA in Aqueous Solutions. In the absence of other proteins, the kinetic stability of ^{111}In -labeled carboxymethylated cT84.66-MC-DOTA was studied in aqueous media at physiological temperature and pH. At 37 °C in 1 mM DTPA (pH 7.2), 1 mM DTT (pH 7.4), and PBS (pH 7.4), the ^{111}In label was lost from the mAb component and converted to a low-molecular mass species. Formation of the ^{111}In -labeled low-molecular mass fraction was observed to follow slow, pseudo-first-order kinetics in all three mixtures. Therefore, the rates of formation of this species could be expressed in linear plots, where $\exp(-k_i t) \approx 1 - k_i t$ when $k_i t \ll 1$ (28). The observed rate constants for formation of the ^{111}In -labeled low-molecular mass species were $0.336\text{--}0.361\text{ h}^{-1}$ ($r^2 = 0.985\text{--}0.990$) in 1 mM DTPA, 1 mM DTT, and PBS.

Stability of MC-DOTA[Y(III)] and MSC-DOTA[Y(III)] in Aqueous Solution. The kinetic stabilities of the purified yttrium chelates of MC-DOTA and MSC-DOTA were studied in PBS at 37 °C and pH 7.4 or 5.4. Figure 4 shows the reversed phase chromatograms of MC-DOTA[Y(III)] (4) before heating to 37 °C (A) and after incubation at 37 °C for 555 h at pH 7.4 (B) or at pH 5.4 (C). In the absence of chelated yttrium, MC-DOTA exhibited nearly identical reaction rates and produced identical proportions of the analogous decomposition products at the two pH values. Figure 5 shows the rates of formation of decomposition products from MC-DOTA[Y(III)] at pH 7.4 and 5.4. The kinetic profile at pH 7.4 [Figure 5 (top)] indicated that 4 was converted to

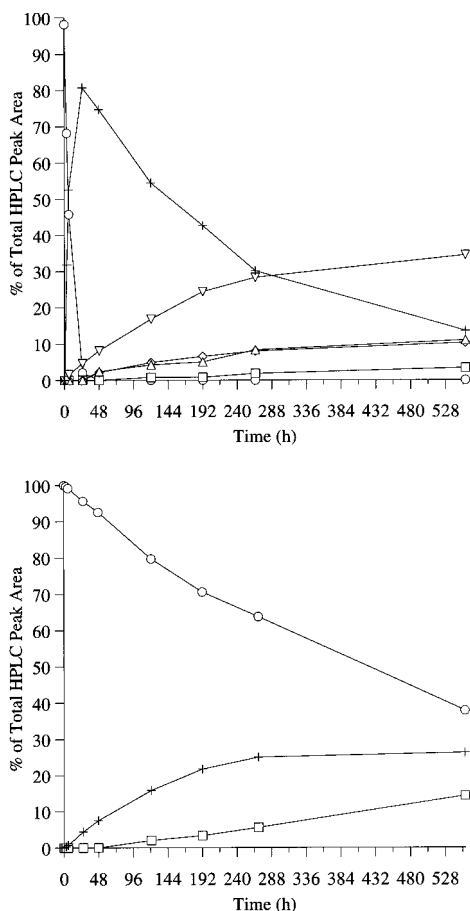


Figure 5. Peak areas, expressed as percentages of the total peak area, of MC-DOTA[Y(III)] (○) and decomposition products **5** (□), **6** (◇), **7** (△), **8** (▽), and **9** (+) as a function of time of incubation in PBS at 37 °C at pH 7.4 (top) or pH 5.4 (bottom). compound **9** within 24 h, followed by the conversion of **9** to compounds **5–8**. In addition, a second decomposition pathway produced compounds **10** and **11** in minor amounts (<15%, data not shown). At pH 5.4 [Figure 5 (bottom)], the reaction was considerably slower, and **4** was converted to compounds **9** and **5**, with small quantities of **11** produced (<2%, data not shown) and negligible formation of products **6–8**. The formation of compound **10** could not be confirmed at pH 5.4, because the comparatively large quantity of unreacted MC-DOTA[Y(III)] eluted at a nearly identical retention time.

Shown in Figure 6 are the reversed phase chromatograms of purified MSC-DOTA[Y(III)] (**12**) before heating to 37 °C (A), after incubation at 37 °C for 6 h at pH 7.4 (B), and after incubation at 37 °C for 50 h at pH 5.4 (C). Figure 7 shows the rates of formation of decomposition products from MSC-DOTA[Y(III)] at pH 7.4 and 5.4. The plot for the decomposition at pH 7.4 [Figure 7 (top)] indicated that **12** was rapidly converted to compounds **13** and **15**, after which **15** was converted to compound **11**, which in turn gave rise to product **10**. The same decomposition products were obtained from MSC-DOTA[Y(III)] at pH 5.4, but at significantly slower rates [Figure 7 (bottom)]. Decomposition of MSC-DOTA produced identical proportions of the analogous decomposition products at both pH values. However, the presence of chelated yttrium resulted in an approximately 3-fold acceleration in the decomposition at pH 7.4 and an approximately 2-fold increase in the reaction rate at pH 5.4.

Mass Spectrometry Analysis of Decomposition Products from MC-DOTA[Y(III)] and MSC-DOTA-

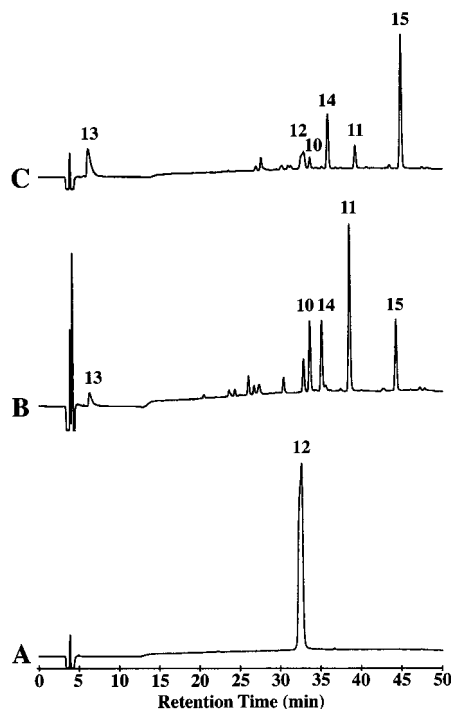


Figure 6. Reversed phase chromatograms of MSC-DOTA[Y(III)] before incubation (A) and after incubation in PBS at 37 °C for 6 h at pH 7.4 (B) or for 50 h at pH 5.4 (C). Aliquots of 5 μ g were injected onto a C₁₈ column (2 $\times$ 250 mm, 5 μ m, 300 Å) and eluted with a linear gradient from 0 to 60% solvent B (solvent A, 0.1% TFA; solvent B, 0.1% TFA/90% CH₃CN) over 60 min at a flow rate of 0.2 mL/min. Absorbance was monitored at 214 nm.

[Y(III)]. After separation by reversed phase HPLC, fractions corresponding to MC-DOTA[Y(III)] (**4**) and its decomposition products **5–11** were collected and analyzed by ESI MS with collision-induced dissociation. Fractions were collected and analyzed from reaction mixtures incubated at pH 7.4 and 5.4, in the presence and absence of chelated yttrium, to determine the identities of products generated at both pH values and of the analogous products generated in the absence of yttrium. Table 3 summarizes the mass spectrometry results obtained for **4** and its decomposition products. Taken together, the results of mass spectrometry and kinetic analysis allowed a mechanism for the decomposition of MC-DOTA[Y(III)], which is depicted in Figure 8, to be proposed.

The mass spectrum of fraction **4** gave a singly charged molecular ion at $m/z = 870.54$, consistent with that expected for MC-DOTA[Y(III)] (calcd for C₃₃H₄₇N₇O₁₃SY, 870.20). MS² of the parent ion produced complete fragmentation of **4** to yield a number of characteristic product ions. The product ions at $m/z = 826.2$, 782.3, 738.3, and 694.5 corresponded to the loss of 1–4 molecules of CO₂, respectively, from the chelate carboxylate groups. The product ion at $m/z = 594.2$ corresponded to the yttrium chelate of DOTA cysteineamide, and the product ions at $m/z = 560.2$ and 516.1 were consistent with progressive loss of H₂S and CO₂ from DOTA-cysteineamide[Y(III)]. The product ion at $m/z = 491.2$ was the smallest fragment obtained and corresponded to the yttrium chelate of DOTA monoamide.

Compound **9** afforded a singly charged molecular ion at $m/z = 888.04$, 18 mass units higher than **4** and consistent with the addition of H₂O to MC-DOTA[Y(III)]. MS² of the $m/z = 888.04$ ion yielded a major product ion at $m/z = 790.2$, which corresponded to the loss of maleic

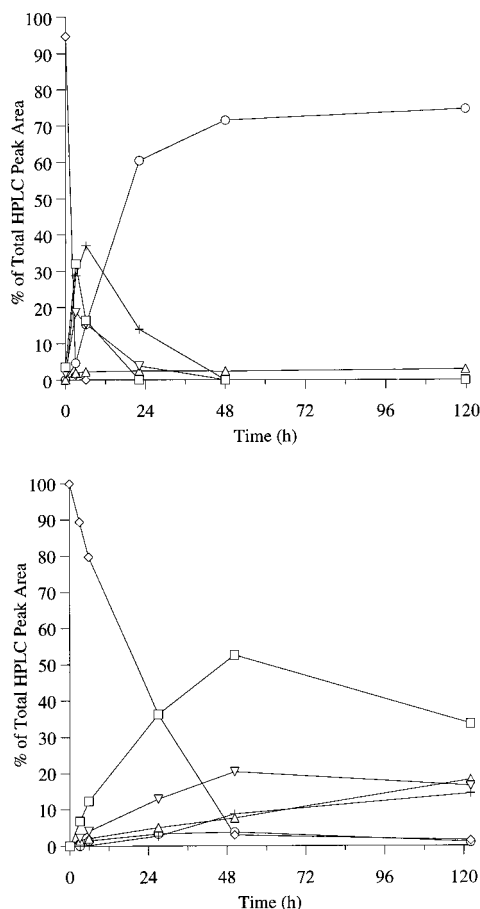


Figure 7. Peak areas, expressed as percentages of the total peak area, of MSC-DOTA[Y(III)] ($\diamond$) and decomposition products **10** ($\circ$), **11** ($+$), **13** (Δ), **14** (∇), and **15** ($\square$) as a function of time of incubation in PBS at 37 °C at pH 7.4 (top) or pH 5.4 (bottom).

anhydride. MS^3 of the ion at $m/z = 790.2$ produced secondary product ions at $m/z = 594.1$, 560.1, 516.0, and 491.1. Compared to **4**, decomposition product **9** appeared to arise from addition of H_2O , contain a maleic acid moiety, and otherwise possess fragmentation patterns similar to those of the starting material. Thus, the structure of **9** was assigned to a hydrolysis product of **4** in which the maleimide ring had been opened.

Fractions **6–8** all gave singly charged molecular ions at a nominal m/z value of 906, an increase of 36 mass units compared to MC-DOTA[Y(III)], and major product ions at $m/z = 808$, corresponding to loss of maleic anhydride. In addition, all three of these decomposition products showed secondary product ions characteristic of the DOTA-cysteineamide[Y(III)] moiety, at $m/z = 594$, 560, 516, and 491. The parent ion was consistent with the addition of 2 equiv of H_2O to **4**, and the only reasonable structure consistent with the mass spectral data was that of a hydrolysis product in which both the maleimide and succinimide rings of MC-DOTA[Y(III)] had been opened. This process would generate two regioisomeric products, each of which would exist as a pair of diastereomers. Compounds **6** and **7** gave nearly identical MS^3 spectra, with the major secondary product ions at $m/z = 790.1$ and 763.0. In contrast, compound **8** yielded major secondary product ions at $m/z = 710.1$ and 692.1. The fact that two HPLC peaks, **6** and **7**, gave identical mass spectra suggested that they represent one pair of diastereomers which are chromatographed with different retention times on the reversed phase column. In the case of compound **8**, only one HPLC peak was

Table 3. Mass Spectrometry Analysis of Decomposition Products from MC-DOTA[Y(III)]

compound	MS m/z	MS^2 m/z	MS^3 m/z
4	870.54	826.2 (43) ^a	nd ^b
		782.3 (15)	
		764.2 (19)	
		746.3 (1)	
		738.3 (3)	
		720.3 (2)	
		711.2 (3)	
		694.5 (1)	
		594.2 (1)	
		560.2 (1)	
		516.1 (1)	
5	790.43	491.2 (100)	nd
		762.1 (50)	
		744.2 (20)	
		719.1 (20)	
		594.1 (1)	
		560.2 (1)	
		516.1 (3)	
		491.1 (100)	
6	906.57	888.1 (5)	790.1 (18), 763.0 (100), 692.0 (13), 607.0 (3), 594.1 (7), 560.1 (2), 516.2 (1), 491.0 (20)
		808.2 (100)	
7	906.30	888.1 (4)	790.1 (26), 763.0 (100), 692.0 (18), 607.1 (2), 594.1 (8), 560.1 (4), 516.1 (2), 491.1 (29)
		808.1 (100)	
8	906.38	888.1 (6)	790.1 (4), 763.1 (10), 737.0 (12), 710.1 (21), 692.1 (100), 676.0 (1), 648.1 (1), 607.2 (9), 594.1 (10), 560.1 (2), 515.9 (1), 491.1 (3)
		808.2 (100)	
9	888.04	870.1 (7)	762.1 (67), 744.1 (27), 702.2 (1), 607.2 (1), 594.1 (2), 560.1 (2), 516.0 (3), 491.1 (100)
		790.2 (100)	

^a Relative intensities are given in parentheses. ^b nd = not done.

obtained, which may represent the coelution of the other diastereomeric pair. We were not able to determine conclusively which regioisomers correspond to fractions **6/7** and **8** on the basis of the MS data. However, opening of the succinimide ring should be favored at the site distal to the sulfide group for steric reasons. Since the peak area for fraction **8** was always greater than the combined peak areas for fractions **6** and **7**, we have tentatively assigned their structures as shown in Figure 8.

Compound **5** yielded a singly charged molecular ion at $m/z = 790.43$, consistent with the mass of a decomposition product arising from hydrolytic loss of maleic acid from **4**. MS^2 of **5** afforded product ions at $m/z = 594.1$, 560.2, 561.1, and 491.1, indicative of the DOTA-cysteineamide[Y(III)] moiety. Hydrolytic loss of maleic acid from MC-DOTA[Y(III)] generated the primary amine derivative of the bifunctional chelate, the only cleavage product we observed in this experiment. The split peak obtained for fraction **5** suggested that this pair of diastereomers was chromatographed with slightly different retention times. When collected separately, the two peaks from fraction **5** gave identical mass spectra.

The peak marked with an asterisk (*) in the chromatograms shown in Figure 4 was also analyzed by ESI MS. This fraction gave a singly charged molecular ion at $m/z = 130.04$. MS^2 of this parent ion generated product ions at $m/z = 74.1$ and 57.1. MS^3 of the product ion at $m/z =$

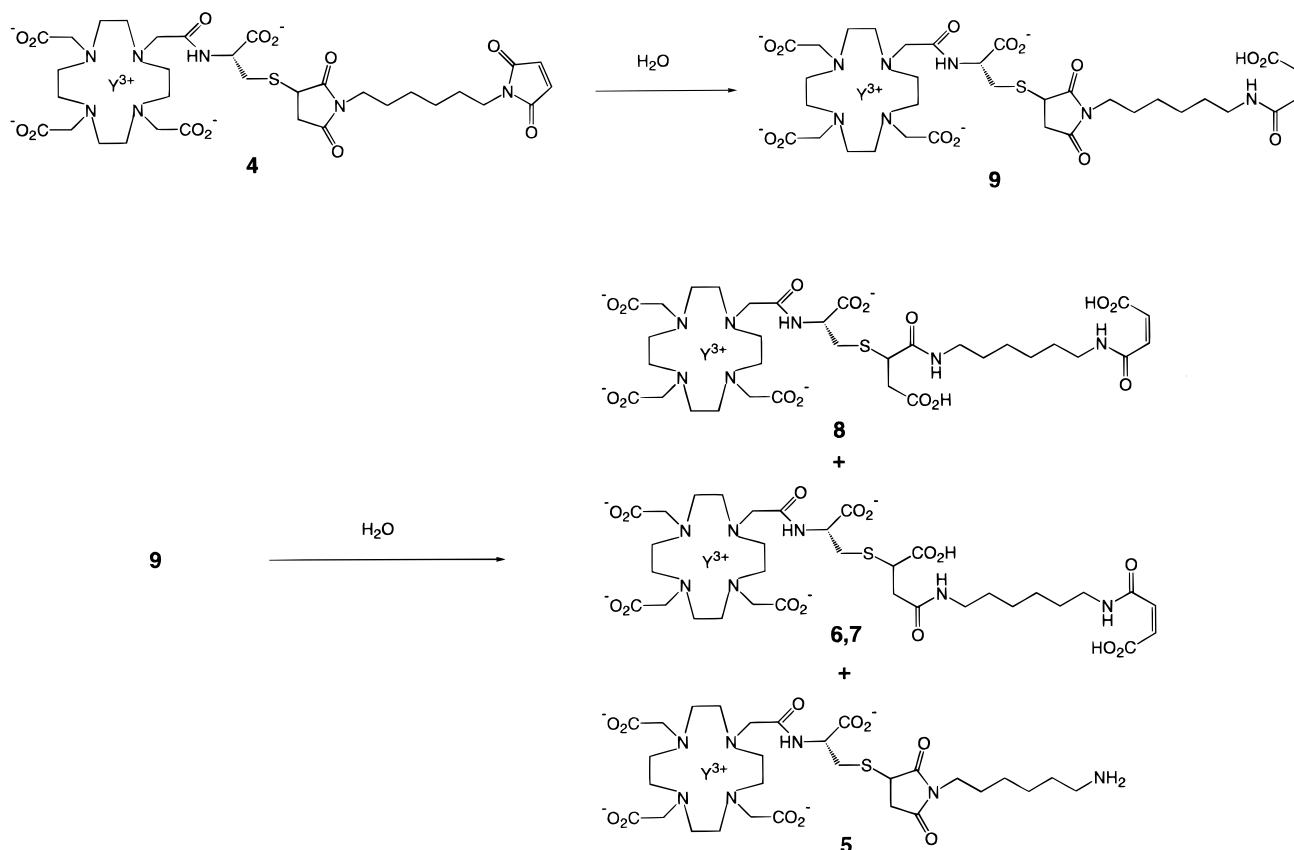


Figure 8. Proposed mechanism for the decomposition of MC-DOTA[Y(III)] in aqueous solution.

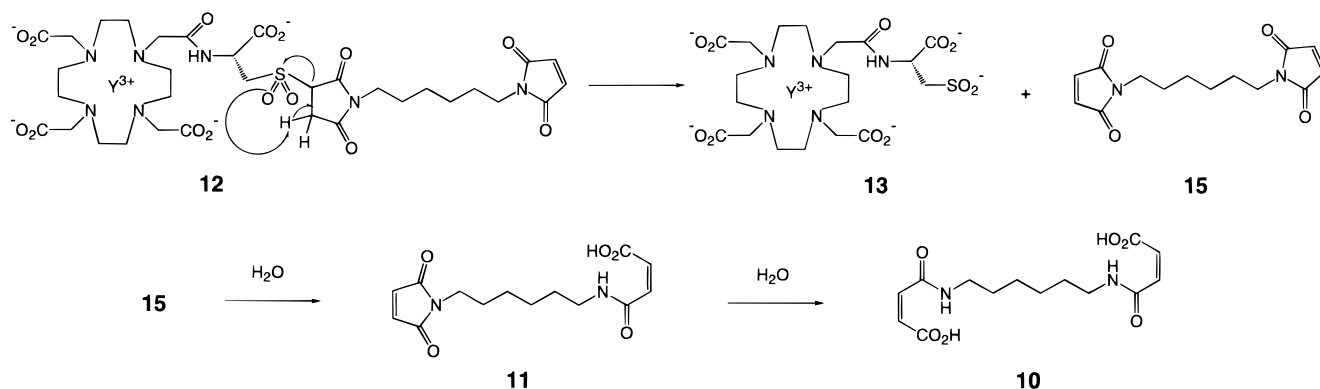


Figure 9. Proposed mechanism for the decomposition of MSC-DOTA[Y(III)] in aqueous solution.

74.1 yielded the ion at $m/z = 57.2$ as the secondary product. Because these studies were performed in an ion trap instrument, the data were consistent with the structure of any isomer of dibutylamine. Dibutylamines are commonly used as stabilizers and vulcanization accelerants in the manufacture of rubber products.² The decomposition reactions were performed in screw-cap polypropylene vials containing rubber O-rings. It is likely that solvent evaporation and condensation was responsible for extraction of dibutylamine from the O-rings and introduction of this contaminant into the reaction mixtures. The observation that considerably more dibutylamine was obtained at pH 5.4 than at pH 7.4 supports this conclusion.

After separation by reversed phase HPLC, MSC-DOTA[Y(III)] (**12**) and its decomposition products, **10**, **11**, and **13–15**, were collected and subjected to the same ESI

MS analysis. Again, fractions were collected from reaction mixtures incubated at pH 7.4 and 5.4, with and without chelated yttrium, to verify that the same products were obtained at both pH values and that the analogous products were obtained in the absence of yttrium. Table 4 summarizes the ESI MS data for compounds **10–15**, and the proposed reaction mechanism derived from the mass spectrometry and kinetic analysis is shown in Figure 9.

MSC-DOTA[Y(III)] (**12**) gave a singly charged molecular ion at $m/z = 902.23$ (calcd for $\text{C}_{33}\text{H}_{47}\text{N}_7\text{O}_{15}\text{SY}$, 902.19). MS² of this parent ion produced complete fragmentation of **12** to afford characteristic product ions at $m/z = 858.2$ and 814.3 , corresponding to loss of 1 and 2 CO_2 molecules, respectively, as well as at $m/z = 626.1$, consistent with the structure of the yttrium chelate of DOTA-cysteinesulfenic acid. The product ions at $m/z = 560.2$ and 516.1 resulted from loss of H_2SO_2 and CO_2 from DOTA-cysteinesulfenic acid[Y(III)]. The product ion cor-

² R. G. Lewis, personal communication.

Table 4. Mass Spectrometry Analysis of Decomposition Products from MSC-DOTA[Y(III)]

compound	MS m/z	MS ² m/z	MS ³ m/z
10	312.99	295.0 (100) ^a	
		277.1 (23)	
		215.1 (36)	197.1 (2), 117.1 (100)
		197.1 (61)	
		117.1 (27)	
11	295.01	277.1 (100)	180.0 (100), 162.1 (3), 138.0 (7), 124.0 (17), 110.1 (15)
		197.1 (12)	
12	902.23	858.2 (17)	nd ^b
		814.3 (1)	
		776.3 (1)	
		732.2 (2)	
		706.4 (1)	
		626.1 (9)	
		608.1 (19)	
		560.2 (65)	
		516.1 (29)	
		491.2 (100)	
		608.2 (100)	590.1 (5), 560.2 (15), 542.2 (100), 516.2 (55), 491.2 (13)
13	626.12	608.2 (100)	
		259.1 (13)	
		249.1 (100)	nd
		224.9 (17)	
		195.0 (91)	
14	277.08	178.0 (23)	
		124.1 (5)	
		180.1 (100)	162.1 (15), 152.3 (8), 138.0 (32), 124.1 (95), 122.1 (21), 110.1 (100), 98.1 (18), 96.2 (8), 83.1 (74)
		162.1 (3)	
		138.1 (5)	
15	277.09	124.1 (17)	
		110.1 (13)	

^a Relative intensities are given in parentheses. ^b nd = not done.

responding to DOTA-monoamide[Y(III)] was also prominent at $m/z = 491.2$.

The initial products formed from the decomposition of MSC-DOTA[Y(III)] were compounds **13** and **15**. Fraction **13** exhibited a singly charged molecular ion at $m/z = 626.12$, one product ion at $m/z = 608.2$, and secondary product ions at $m/z = 560.2$, 516.2 , and 491.2 . These data were consistent with the structure of the yttrium chelate of DOTA-cysteinesulfenic acid. Fraction **15** generated a singly charged molecular ion at $m/z = 277.09$. MS² of **15** gave a product ion at $m/z = 180.1$, corresponding to loss of maleimide, and MS³ afforded a major secondary product ion at $m/z = 83.1$, again consistent with loss of maleimide. These product ions, along with the loss of several 14 mass unit fragments in the MS³ spectrum, led us to assign the structure of **15** as that of 1,6-bis-(maleimido)hexane. Thus, as depicted in Figure 9, the first step in the decomposition mechanism of MSC-DOTA[Y(III)] appeared to be a β -elimination reaction involving the sulfone and succinimide groups, generating **13** and **15** as cleavage products.

Compounds **10** and **11** gave mass spectra which were consistent with the structures of hydrolysis products derived from 1,6-bis-(maleimido)hexane. Fraction **11** had a singly charged molecular ion at $m/z = 295.01$, 18 mass units higher than that of **15**. MS² of **11** yielded a peak at $m/z = 277.1$, corresponding to loss of H₂O in the gas phase to generate BMH as a product ion, and MS³ produced a fragmentation pattern similar to that observed for **15**. The structure of compound **11** was assigned as the BMH hydrolysis product in which one of the maleimide rings had been opened. Fraction **10** gave a singly charged molecular ion at $m/z = 312.99$, 36 mass units higher than that of **15**, and upon MS² analysis

afforded product ions at $m/z = 295.0$, representing loss of H₂O, and $m/z = 215.1$, representing loss of maleic anhydride. MS³ of the ion at $m/z = 215.1$ produced loss of a second molecule of maleic anhydride to give a secondary product ion at $m/z = 117.1$, corresponding to the ion expected for 1,6-hexanediamine. Thus, the structure of **10** was assigned as a BMH hydrolysis product in which both maleimide rings had been opened.

MS of fraction **14**, like that of **15**, showed a singly charged molecular ion at $m/z = 277.08$. However, the MS² fragmentation pattern of **14** was dramatically different from that obtained for compound **15** and was not consistent with any reasonable structure we could derive for a possible MSC-DOTA[Y(III)] decomposition product. At this point, we are not able to assign an unequivocal structure to compound **14**.

DISCUSSION

The macrocyclic chelating agent DOTA is an attractive candidate for labeling monoclonal antibodies with radiometals because it has been shown to bind a large number of main group and transition metal ions with extremely high stability constants (30–35). Unfortunately, for many metals of interest, the rates of incorporation into the DOTA chelate complex are relatively slow (36), especially in the context of an antibody conjugate. Thus, yields for radiometal labeling of these conjugates under ambient conditions can be low or variable. Chelation of a wide variety of metal ions by free DOTA has been shown to be a two-step process, involving the reversible formation of the loosely associated type I complex and a slow transformation to the fully coordinated and kinetically stable type II complex (37, 38).

We hypothesized that for antibody-conjugated DOTA derivatives, especially at carrier-free radiometal concentrations, competition from nonspecific metal-binding sites on the protein surface may cause formation of the type I complex to become the rate-limiting step for radiolabeling. On the basis of the model compounds synthesized by Takenouchi and co-workers (39, 40), we synthesized similar bifunctional DOTA derivatives bearing “pendant-type” carboxylates, to determine whether the presence of such additional metal coordinating groups would have an effect on mAb radiolabeling yields.

The maleimidocysteineamido-DOTA derivatives **2** and **3** were prepared by a simple, straightforward synthetic route, taking advantage of water-soluble chemistry to circumvent the need for lengthy sequences of protection and deprotection reactions. These compounds can be synthesized and purified in a relatively short period of time, on the order of 2 weeks. The key intermediate, the monocysteineamide derivative of DOTA (**1**), was prepared by a reaction that was once commonly employed in peptide synthesis (50) and more recently used to synthesize entire proteins by native chemical ligation (55). The mechanism of coupling active esters to cysteine involves nucleophilic attack of the ionized thiolate to form an intermediate thioester, which in aqueous solution rearranges via a pentacyclic transition state to the more stable amide (50). The thiol group of **1** was then exploited as an orthogonal functionality for antibody conjugation, using the homobifunctional linker 1,6-bis-(maleimido)-hexane. The resulting product, MC-DOTA (**2**), was oxidized with potassium hydrogen persulfate (Oxone), a reagent which is highly chemoselective for conversion of sulfides to sulfones (51), to give MSC-DOTA (**3**).

Compounds **2** and **3** were conjugated to the lightly reduced anticarcinoma embryonic antigen mAb cT84.66,

using a modification of the procedure of Willner et al. (52). According to Ellman's test, treatment of cT84.66 with 20 molar equiv of DTT generated approximately 10 reactive thiol groups per antibody molecule, a titer slightly higher than that anticipated for a human IgG₁ mAb, which contains four interchain disulfide bonds. However, these results are similar to those obtained by Willner et al., who generated a titer of 9.6 sulfhydryl groups per molecule of the isotype-matched mAb BR64 at a DTT:mAb molar ratio of 20:1. Addition of up to 40 molar equiv of maleimide-derivatized bifunctional chelating agent resulted in modification with a maximum of six chelates per antibody molecule. The conjugation results suggested that at least two of the cysteine residues of cT84.66 remained unreactive to modification. Since SDS-polyacrylamide gel electrophoresis of the maximally modified conjugate [Figure 1 (top)] clearly showed 100% modification of the light chain, the heavy chain likely contained the unreactive cysteine residues. Steric hindrance may have played a role in preventing all of the heavy chain cysteines from being modified.

The cT84.66-MC-DOTA conjugate containing an average of 3.8 chelates per antibody molecule was the minimally modified product that gave high specific activity radiolabeling with ¹¹¹In and ⁹⁰Y. Fortunately, the minimally modified cT84.66 conjugate prepared with the *N*-hydroxysulfosuccinimide ester of DOTA (29) which exhibited high-specific activity radiometal labeling was also modified with an average of 3.8 chelates per mAb. Conjugates prepared with both reagents and coupled to an average of 1–2 chelates per antibody displayed an approximate 4-fold reduction in radiometal labeling yield. The "threshold" modification value of 3–4 chelates per mAb, required for the conjugate to carry high radiometal activity, was in good agreement with the findings of other groups (56, 57). However, a direct comparison of the cT84.66 conjugates prepared with MC-DOTA and DOTA-OSSu showed that the MC-DOTA conjugate afforded an average 22–23% increase in radiolabeling yields for both ¹¹¹In and ⁹⁰Y. At present, we cannot conclude unequivocally whether this improvement in radiometal labeling can be attributed to the pendant-type carboxyl group of MC-DOTA, the change in conjugation site to reduced disulfides in the mAb, or the addition of a longer linker to the macrocycle. Using modifications of the water-soluble route for the synthesis of MC-DOTA, we can now test each of these parameters independently for their respective contributions to radiolabeling yields.

Kinetic stability studies of cT84.66-MC-DOTA and cT84.66-MSD-DOTA were performed in human serum and in aqueous solutions containing DTPA, DTT, and buffer alone at physiological temperature and pH. In serum, both conjugates exhibited relatively rapid loss of radiometal, and this process was approximately 10 times faster for the conjugate prepared with the sulfone compound. A dramatic difference in the fate of the radiometal was observed, depending on which type of chelate-antibody linker was used. Radiometal lost from the MC-DOTA conjugate was predominantly associated with serum albumin, while the vast majority of metal lost from the MSD-DOTA conjugate remained as a low-molecular mass species. In these experiments, no ¹¹¹In was associated with transferrin, the usual acceptor protein for this metal. Furthermore, in the absence of serum and other proteins, large quantities of exogenous chelating agent or reducing agent had no effect on the observed rate of radiometal loss. These results were inconsistent with those expected for radiometal ion dissociation or a disulfide exchange reaction, suggesting that a solvent-

mediated or intramolecular cleavage reaction involving the linker portion of the bifunctional chelates might be responsible for radiometal release. The cT84.66-MC-DOTA conjugate, however, has afforded highly reproducible radiolabeling yields over a period of more than 6 months of storage under metal-free conditions at 4 °C and pH 7.0, indicating that the stability of the antibody conjugate is temperature-dependent.

The properties of the unconjugated Y(III) chelates of MC-DOTA and MSD-DOTA at physiological temperature were studied in order to elucidate the mechanisms of their decomposition reactions. When MC-DOTA[Y(III)] (4) and MSD-DOTA[Y(III)] (12) were incubated in PBS at 37 °C, pH-dependent cleavage of the linker moiety occurred in both compounds. HPLC and ESI MS analysis of the reaction products revealed that MC-DOTA (2) and its yttrium chelate underwent imide bond hydrolysis, while MSD-DOTA (3) and its yttrium chelate decomposed by a β -elimination reaction involving the sulfone group. The decomposition of 2–4 and 12 occurred much faster at pH 7.4 than at pH 5.4, and neither yttrium compound showed any evidence of metal ion dissociation during the course of these reactions. Interestingly, MC-DOTA-[Y(III)] showed a much slower rate of decomposition at pH 7.4 than the corresponding radiolabeled antibody conjugate, indicating that the protein also played a role in the decomposition reaction. Further evidence of the mAb's role in the decomposition of the MC-DOTA conjugate was provided by the modest, but significant, improvement in radiolabel stability upon alkylation of the unconjugated cysteines with iodoacetic acid.

In the case of MSD-DOTA and its Y(III) chelate, the same reaction products were generated in the same proportions at both pH values. The presence of chelated yttrium accelerated the elimination reaction 2–3-fold, indicating that the sulfone group is activated by the bound metal ion. In contrast, imide bond hydrolysis of MC-DOTA was not significantly enhanced by chelated yttrium, but the profile of decomposition products depended on the pH of the reaction mixture. At pH 5.4, no products resulting from succinimide ring opening of MC-DOTA or its yttrium chelate were generated, although the cleavage product resulting from maleimide ring hydrolysis (5) was obtained in much greater yield than at pH 7.4. It could be argued that the maleimide ring of MC-DOTA is less stable to hydrolysis than the succinimide ring, causing it to open first to yield intermediate 9, which hydrolyzes further to generate compound 5 as the only observed cleavage product in this system. However, when MC-DOTA is conjugated to an antibody, the maleimide group is converted to a succinimide group. If the hydrolysis mechanism holds for the mAb conjugate, then loss of the radiolabel might occur by cleavage at one of the succinimide rings to release the radiometal chelate. On the basis of the HPLC results, this process would not be expected to occur to an appreciable degree at pH 5.4 but might occur at physiological pH. Maleimide ring opening (58) and succinimide ring opening on protein conjugates (59–64) have been observed or postulated to occur at pH >7.5 in many other systems, but to our knowledge, this is the first case in which succinimide ring cleavage may be responsible for release of a conjugated molecule from a protein.

Conjugation of kinetically stable radiometal chelates to monoclonal antibodies by means of a linker system that is chemically labile at physiological pH offers the possibility of imparting favorable biodistribution properties to the resulting radioimmunoconjugates. The serum studies of cT84.66-MSD-DOTA suggested that this

conjugate would probably exhibit an insufficient *in vivo* stability to be useful for tumor targeting with intact IgG. On the other hand, the *in vitro* serum half-life of cT84.66-MC-DOTA was comparable to the biological half-life of radiolabeled IgGs in animal models and human subjects. Controlled hydrolysis of the linker between chelate and antibody might be expected to improve the clearance of radioactivity from normal organs and tissues, since the radiometal should be released in chelated form, and free chelates have been shown to clear by rapid urinary excretion (65–68).

In contrast, the increase in stability of the MC-DOTA linker and lack of succinimide hydrolysis at moderately acidic pH may have an important effect on the stability of the radiometal-labeled antibody at the tumor site. The interstitial pH of solid tumors in both rodents and humans has been shown to be significantly lower than that of normal tissues (69–77); for reviews, see refs 78 and 79. The extracellular pH of malignant tissue has been measured in the range of 5.8–7.7, while normal tissues fall in the range of 7.0–8.1 (78). In general, tumors with a higher percentage of epithelial tissue are more acidic (72), and adenocarcinomas in particular fall in the lower ranges of extracellular pH, from 5.7 to 7.8 (75). Since the mAb cT84.66 recognizes the adenocarcinoma-associated antigen CEA and is not internalized upon binding, the increased stability of the MC-DOTA linker at moderately acidic pH may allow tumor uptake of the radiometal-labeled mAb to remain high. In the accompanying article (80), we show that ^{111}In - and ^{90}Y -labeled cT84.66-MC-DOTA exhibited high tumor uptake, with improved blood clearance and low uptake in normal organs, in a nude mouse model of human colorectal adenocarcinoma.

The maleimidocysteineamido-DOTA derivatives described here allowed conjugation of macrocyclic radiometal chelates to reduced disulfide bonds of antibodies. Compared to the conjugate prepared with an active ester of DOTA, cT84.66-MC-DOTA showed increases in radiolabeling yields of 22–23% for ^{111}In and ^{90}Y . Using MC-DOTA, we have achieved our goal of near-quantitative antibody labeling with both radiometals. Immunoconjugates prepared with the two maleimidocysteineamido-DOTA derivatives exhibited linker-dependent loss of radiometal at physiological temperature and pH. We attributed this phenomenon to imide bond hydrolysis for the sulfide compound MC-DOTA and to a β -elimination reaction in the case of the sulfone MSC-DOTA. We found that the cleavage reactions were dramatically slower at moderately acidic pH. The use of such pH-dependent chemically labile linkers may be an important determinant of the *in vivo* distribution properties of antibody-radiometal chelate conjugates, on the basis of the observed differences between the extracellular pH of solid tumors and that of normal tissues.

ACKNOWLEDGMENT

The authors thank Jim Kao, Randall Woo, and Mike Davis for excellent technical assistance, Jim Primus and Karen Rickard-Dickson for providing the mAb cT84.66, and Terry Lee and Andrew Raubitschek for helpful discussions. This work was funded by NIH Research Grant CA43904 (J.E.S.) and Department of the Army Grant DAMD17-96-1-6047 (M.R.L.). The content of this article does not necessarily reflect the position or the policy of the United States government, and no official endorsement should be inferred.

LITERATURE CITED

- (1) Meares, C. F. (1986) Chelating Agents for the Binding of Metal Ions to Antibodies. *Int. J. Radiat. Appl. Instrum. Part B Nucl. Med. Biol.* 13, 311–318.
- (2) Gansow, O. A. (1991) Newer Approaches to the Radiolabeling of Monoclonal Antibodies by Use of Metal Chelates. *Int. J. Radiat. Appl. Instrum. Part B Nucl. Med. Biol.* 18, 369–381.
- (3) Liu, Y., and Wu, C. (1991) Radiolabeling of Monoclonal Antibodies with Metal Chelates. *Pure Appl. Chem.* 63, 427–463.
- (4) Meares, C. F., McCall, M. J., Reardan, D. T., Goodwin, D. A., Diamanti, C. I., and McTigue, M. (1984) Conjugation of Antibodies with Bifunctional Chelating Agents: Isothiocyanate and Bromoacetamide Reagents, Methods of Analysis, and Subsequent Addition of Metal Ions. *Anal. Biochem.* 142, 68–78.
- (5) Hnatowich, D. J., Layne, W. W., Childs, R. L., Lantaigne, D., Davis, M. A., Griffin, T. W., and Doherty, P. W. (1983) Radioactive Labeling of Antibody: A Simple and Efficient Method. *Science* 220, 613–615.
- (6) Hnatowich, D. J., Childs, R. L., Lantaigne, D., and Najafi, A. (1983) The Preparation of DTPA-Coupled Antibodies Radiolabeled with Metallic Radionuclides: an Improved Method. *J. Immunol. Methods* 65, 147–157.
- (7) Halpern, S. E., Hagan, P. L., Garver, P. R., Koziol, J. A., Chen, A. W. N., Frincke, J. M., Bartholomew, R. M., David, G. S., and Adams, T. H. (1983) Stability, Characterization, and Kinetics of ^{111}In -labeled Monoclonal Antitumor Antibodies in Normal Animals and Nude Mouse-Human Tumor Models. *Cancer Res.* 43, 5347–5355.
- (8) Buckley, R. G., and Searle, F. (1984) An efficient method for labelling antibodies with ^{111}In . *FEBS Lett.* 166, 202–204.
- (9) Paxton, R. J., Jakowatz, J. G., Beatty, J. D., Beatty, B. G., Vlahos, W. G., Williams, L. E., Clark, B. R., and Shively, J. E. (1985) High-Specific-Activity ^{111}In -labeled Anticarcinoma-embryonic Antigen Monoclonal Antibody: Improved Method for the Synthesis of Diethylenetriaminepentaacetic Acid Conjugates. *Cancer Res.* 45, 5694–5699.
- (10) Brechbiel, M. W., Gansow, O. A., Atcher, R. W., Schlom, J., Esteban, J., Simpson, D. E., and Colcher, D. (1986) Synthesis of 1-(p-Isothiocyanatobenzyl) Derivatives of DTPA and EDTA. Antibody Labeling and Tumor-Imaging Studies. *Inorg. Chem.* 25, 2772–2781.
- (11) Westerberg, D. A., Carney, P. L., Rogers, P. E., Kline, S. J., and Johnson, D. K. (1989) Synthesis of Novel Bifunctional Chelators and Their Use in Preparing Monoclonal Antibody Conjugates for Tumor Targeting. *J. Med. Chem.* 32, 236–243.
- (12) Moi, M. K., Meares, C. F., McCall, M. J., Cole, W. C., and DeNardo, S. J. (1985) Copper Chelates as Probes of Biological Systems: Stable Copper Complexes with a Macrocyclic Bifunctional Chelating Agent. *Anal. Biochem.* 148, 249–253.
- (13) Moi, M. K., Meares, C. F., and DeNardo, S. J. (1988) The Peptide Way to Macrocyclic Bifunctional Chelating Agents: Synthesis of 2-(p-Nitrobenzyl)-1,4,7,10-tetraazacyclododecane- N,N',N'',N''' -tetraacetic Acid and Study of Its Yttrium(III) Complex. *J. Am. Chem. Soc.* 110, 6266–6267.
- (14) Cox, J. P. L., Jankowski, K. J., Katak, R., Parker, D., Beeley, N. R. A., Boyce, B. A., Eaton, M. A. W., Millar, K., Millican, A. T., Harrison, A., and Walker, C. (1989) Synthesis of a Kinetically Stable Yttrium-90 Labelled Macrocyclic-Antibody Conjugate. *J. Chem. Soc., Chem. Commun.*, 797–798.
- (15) Craig, A. S., Helps, I. M., Jankowski, K. J., Parker, D., Beeley, N. R. A., Boyce, B. A., Eaton, M. A. W., Millican, A. T., Millar, K., Phipps, A., Rhind, S. K., Harrison, A., and Walker, C. (1989) Towards Tumour Imaging with Indium-111 Labelled Macrocyclic-Antibody Conjugates. *J. Chem. Soc., Chem. Commun.*, 794–796.
- (16) Parker, D., Morphy, J. R., Jankowski, K., and Cox, J. (1989) Implementation of macrocycle conjugated antibodies for tumour-targeting. *Pure Appl. Chem.* 61, 1637–1641.
- (17) Cox, J. P. L., Craig, A. S., Helps, I. M., Jankowski, K. J., Parker, D., Eaton, M. A. W., Millican, A. T., Millar, K., Beeley,

- N. R. A., and Boyce, B. A. (1990) Synthesis of C- and N-Functionalised Derivatives of 1,4,7-Triazacyclononane-1,4,7-triyltriacetic acid (NOTA), 1,4,7,10-Tetra-azacyclododecane-1,4,7,10-tetra-yltetra-acetic Acid (DOTA), and Diethylenetriaminepenta-acetic Acid (DTPA): Bifunctional Complexing Agents for the Derivatisation of Antibodies. *J. Chem. Soc., Perkin Trans. 1*, 2567–2576.
- (18) Morphy, J. R., Parker, D., Katakly, R., Eaton, M. A. W., Millican, A. T., Alexander, R., Harrison, A., and Walker, C. (1990) Towards Tumour Targeting with Copper-radiolabelled Macrocycle-Antibody Conjugates: Synthesis, Antibody Linkage, and Complexation Behaviour. *J. Chem. Soc., Perkin Trans. 2*, 573–585.
- (19) Deshpande, S. V., DeNardo, S. J., Kukis, D. L., Moi, M. K., McCall, M. J., DeNardo, G. L., and Meares, C. F. (1990) Yttrium-90-Labeled Monoclonal Antibody for Therapy: Labeling by a New Macroyclic Bifunctional Chelating Agent. *J. Nucl. Med.* 31, 473–479.
- (20) Moi, M. K., DeNardo, S. J., and Meares, C. F. (1990) Stable Bifunctional Chelates of Metals Used in Radiotherapy. *Cancer Res.* 50 (Suppl.), 789s–793s.
- (21) Meares, C. F., Moi, M. K., Diril, H., Kukis, D. L., McCall, M. J., Deshpande, S. V., DeNardo, S. J., Snook, D., and Epenetos, A. A. (1990) Macroyclic chelates of radiometals for diagnosis and therapy. *Br. J. Cancer* 62 (Suppl. X), 21–26.
- (22) Ruegg, C. L., Anderson-Berg, W. T., Brechbiel, M. W., Mirzadeh, S., Gansow, O. A., and Strand, M. (1990) Improved *in Vivo* Stability and Tumor Targeting of Bismuth-labeled Antibody. *Cancer Res.* 50, 4221–4226.
- (23) Smith-Jones, P. M., Fridrich, R., Kaden, T. A., Novak-Hofer, I., Siebold, K., Tschudin, D., and Maecke, H. R. (1991) Antibody Labeling with Copper-67 Using the Bifunctional Macrocycle 4-[(1,4,8,11-Tetraazacyclotetradec-1-yl)methyl]-benzoic Acid. *Bioconjugate Chem.* 2, 415–421.
- (24) Kline, S. J., Betebenner, D. A., and Johnson, D. K. (1991) Carboxymethyl-Substituted Bifunctional Chelators: Preparation of Aryl Isothiocyanate Derivatives of 3-(Carboxymethyl)-3-azapentanedioic Acid, 3,12-Bis(carboxymethyl)-6,9-dioxo-3,12-diazatetradecanedioic Acid, and 1,4,7,10-Tetraazacyclododecane-*N,N',N'',N'''*-tetraacetic Acid for Use as Protein Labels. *Bioconjugate Chem.* 2, 26–31.
- (25) McMurry, T. J., Brechbiel, M., Kumar, K., and Gansow, O. A. (1992) Convenient Synthesis of Bifunctional Tetraaza Macrocycles. *Bioconjugate Chem.* 3, 108–117.
- (26) Smith, A., Alberto, R., Blauenstein, P., Novak-Hofer, I., Maecke, H. R., and Schubiger, P. A. (1993) Preclinical Evaluation of ^{67}Cu -labeled Intact and Fragmented Anti-Colon Carcinoma Monoclonal Antibody MAb35. *Cancer Res.* 53, 5727–5733.
- (27) Kruper, W. J., Jr., Rudolf, P. R., and Langhoff, C. A. (1993) Unexpected Selectivity in the Alkylation of Polyazamacrocycles. *J. Org. Chem.* 58, 3869–3876.
- (28) Li, M., and Meares, C. F. (1993) Synthesis, Metal Chelate Stability Studies, and Enzyme Digestion of a Peptide-Linked DOTA Derivative and Its Corresponding Radiolabeled Immunoconjugates. *Bioconjugate Chem.* 4, 275–283.
- (29) Lewis, M. R., Raubitschek, A., and Shively, J. E. (1994) A Facile, Water-Soluble Method for Modification of Proteins with DOTA. Use of Elevated Temperature and Optimized pH To Achieve High Specific Activity and High Chelate Stability in Radiolabeled Immunoconjugates. *Bioconjugate Chem.* 5, 565–576.
- (30) Loncin, M. F., Desreux, J. F., and Merciny, E. (1986) Coordination of Lanthanides by Two Polyamino Polycarboxylic Macrocycles: Formation of Highly Stable Lanthanide Complexes. *Inorg. Chem.* 25, 2646–2648.
- (31) Cacheris, W. P., Nickle, S. K., and Sherry, A. D. (1987) Thermodynamic Study of Lanthanide Complexes of 1,4,7-Triazacyclononane-*N,N',N''*-triacetic Acid and 1,4,7,10-Tetraazacyclododecane-*N,N',N'',N'''*-tetraacetic Acid. *Inorg. Chem.* 26, 958–960.
- (32) Kumar, K., Magerstädt, M., and Gansow, O. A. (1989) Lead(II) and Bismuth(III) Complexes of the Polyazacycloalkane-*N*-acetic Acids nota, dota, and tetra. *J. Chem. Soc., Chem. Commun.*, 145–146.
- (33) Broan, C. J., Cox, J. P. L., Craig, A. S., Katakly, R., Parker, D., Harrison, A., Randall, A. M., and Ferguson, G. (1991) Structure and Solution Stability of Indium and Gallium Complexes of 1,4,7-Triazacyclononanetriacetate and of Yttrium Complexes of 1,4,7,10-Tetraazacyclododecanetetraacetate and Related Ligands: Kinetically Stable Complexes for Use in Imaging and Radioimmunotherapy. X-Ray Molecular Structure of the Indium and Gallium Complexes of 1,4,7-Triazacyclononane-1,4,7-triacetic Acid. *J. Chem. Soc., Perkin Trans. 2*, 87–99.
- (34) Clarke, E. T., and Martell, A. E. (1991) Stabilities of the alkaline earth and divalent transition metal complexes of the tetraazamacrocyclic tetraacetic acid ligands. *Inorg. Chim. Acta* 190, 27–36.
- (35) Clarke, E. T., and Martell, A. E. (1991) Stabilities of trivalent metal ion complexes of the tetraacetate derivatives of 12-, 13-, and 14-membered tetraazamacrocycles. *Inorg. Chim. Acta* 190, 37–46.
- (36) Kodama, M., Koike, T., Mahatma, A. B., and Kimura, E. (1991) Thermodynamic and Kinetic Studies of Lanthanide Complexes of 1,4,7,10,13-Pentaazacyclopentadecane-*N,N',N'',N'''*-pentaacetic Acid and 1,4,7,10,13,16-Hexaazacyclooctadecane-*N,N',N'',N'''*-hexaacetic Acid. *Inorg. Chem.* 30, 1270–1273.
- (37) Kasprzyk, S. P., and Wilkins, R. G. (1982) Kinetics of Interaction of Metal Ions with Two Tetraaza Tetraacetate Macrocycles. *Inorg. Chem.* 21, 3349–3352.
- (38) Wang, X., Jin, T., Comblin, V., Lopez-Mut, A., Merciny, E., and Desreux, J. F. (1992) A Kinetic Investigation of the Lanthanide DOTA Chelates. Stability and Rates of Formation and of Dissociation of a Macroyclic Gadolinium(III) Polyaza Polycarboxylic MRI Contrast Agent. *Inorg. Chem.* 31, 1095–1099.
- (39) Takenouchi, K., Watanabe, K., Kato, Y., Koike, T., and Kimura, E. (1993) Novel Bifunctional Macroyclic Chelating Agents Appended with a Pendant-Type Carboxymethylamino Ligand and Nitrobenzyl Group and Stability of the $^{88}\text{Y}^{\text{III}}$ Complexes. *J. Org. Chem.* 58, 1955–1958.
- (40) Takenouchi, K., Tabe, M., Watanabe, K., Hazato, A., Kato, Y., Shionoya, M., Koike, T., and Kimura, E. (1993) Novel Pendant-Type Macroyclic Bifunctional Chelating Agents: (Carboxymethyl)amino Derivatives of 2-(4-Nitrobenzyl)-1,4,7,10-tetraazacyclododecane-*N,N',N'',N'''*-tetraacetic Acid and Their Complex Formation with Yttrium(III). *J. Org. Chem.* 58, 6895–6899.
- (41) Neumaier, M., Shively, L., Chen, F.-S., Gaida, F.-J., Ilgen, C., Paxton, R. J., Shively, J. E., and Riggs, A. D. (1990) Cloning of the Genes for T84.66, an Antibody That Has a High Specificity and Affinity for Carcinoembryonic Antigen, and Expression of Chimeric Human/Mouse T84.66 Genes in Myeloma and Chinese Hamster Ovary Cells. *Cancer Res.* 50, 2128–2134.
- (42) Gold, P., and Freedman, S. O. (1965) Demonstration of Tumor-Specific Antigens in Human Colonic Carcinomata by Immunological Tolerance and Absorption Techniques. *J. Exp. Med.* 121, 439–462.
- (43) Gunewardena, P., and Cook, K. B. (1966) 1,4-Dithiothreitol as a Reagent for Reductive Degradation of Human γG Globulin. *Biochem. J.* 99, 8P.
- (44) Nisssonof, A. (1984) *Introduction to Molecular Immunology*, p 20, Sinauer Associates, Inc., Sunderland, MA.
- (45) Gorevic, P. D., Prelli, F. C., and Frangione, B. (1985) Immunoglobulin G (IgG). *Methods Enzymol.* 116, 3–25.
- (46) Thiers, R. E. (1957) Contamination in Trace Element Analysis and Its Control. *Methods Biochem. Anal.* 5, 273–335.
- (47) Salacinski, P. R. P., McLean, C., Sykes, J. E. C., Clement-Jones, V. V., and Lowry, P. J. (1981) Iodination of Proteins, Glycoproteins, and Peptides Using a Solid-Phase Oxidizing Agent, 1,3,4,6-Tetrachloro-3 α ,6 α -diphenyl Glycoluril (Iodogen). *Anal. Biochem.* 117, 136–146.
- (48) Coligan, J. E., Lautenschlager, J. T., Egan, M. L., and Todd, C. W. (1972) Isolation and Characterization of Carcinoembryonic Antigen. *Immunochimistry* 9, 377–386.

- (49) Slayter, H. S., and Coligan, J. E. (1976) Characterization of Carcinoembryonic Antigen Fractionated by Concanavalin A Chromatography. *Cancer Res.* 36, 1696–1704.
- (50) Wieland, T., Bokelmann, E., Bauer, L., Lang, H. U., and Lau, H. (1953) Über Peptidsynthesen. 8. Mitteilung Bildung von S-haltigen Peptiden durch intramolekulare Wanderung von Aminoacylresten. *Ann. Chem. Liebigs* 583, 129–149.
- (51) Trost, B. M., and Curran, D. P. (1981) Chemoselective Oxidation of Sulfides to Sulfones with Potassium Hydrogen Persulfate. *Tetrahedron Lett.* 22, 1287–1290.
- (52) Willner, D., Trail, P. A., Hofstead, S. J., King, H. D., Lasch, S. J., Braslawsky, G. R., Greenfield, R. S., Kaneko, T., and Firestone, R. A. (1993) (6-Maleimidocaproyl)hydrazide of Doxorubicin—A New Derivative for the Preparation of Immunoconjugates of Doxorubicin. *Bioconjugate Chem.* 4, 521–527.
- (53) Ellman, G. L. (1958) A Colorimetric Method for Determining Low Concentrations of Mercaptans. *Arch. Biochem. Biophys.* 74, 443–450.
- (54) Riddles, P. W., Blakeley, R. L., and Zerner, B. (1979) Ellman's Reagent: 5,5'-Dithiobis(2-nitrobenzoic Acid)—a Re-examination. *Anal. Biochem.* 94, 75–81.
- (55) Dawson, P. E., Muir, T. W., Clark-Lewis, I., and Kent, S. B. H. (1994) Synthesis of Proteins by Native Chemical Ligation. *Science* 266, 776–779.
- (56) Kukis, D. L., DeNardo, G. L., DeNardo, S. J., Mirick, G. R., Miers, L. A., Greiner, D. P., and Meares, C. F. (1995) Effect of the Extent of Chelate Substitution on the Immunoreactivity and Biodistribution of ZIT-BAT-Lym-1 Immunoconjugates. *Cancer Res.* 55, 878–884.
- (57) Stimmel, J. B., Stockstill, M. E., and Kull, F. C., Jr. (1995) Yttrium-90 Chelation Properties of Tetraazatetraacetic Acid Macrocycles, Diethylenetriaminepentaacetic Acid Analogues, and a Novel Terpyridine Acyclic Chelator. *Bioconjugate Chem.* 6, 219–225.
- (58) Gregory, J. D. (1955) The Stability of *N*-Ethylmaleimide and its Reaction with Sulfhydryl Groups. *J. Am. Chem. Soc.* 77, 3922–3923.
- (59) Wu, C.-W., Yarbrough, L. R., and Wu, F. Y.-H. (1976) *N*-(1-Pyrene)maleimide: A Fluorescent Cross-Linking Reagent. *Biochemistry* 15, 2863–2868.
- (60) Betcher-Lange, S. L., and Lehrer, S. S. (1978) Pyrene Excimer Fluorescence in Rabbit Skeletal α -Tropomyosin Labeled with *N*-(1-Pyrene)maleimide. A Probe of Sulfhydryl Proximity and Local Chain Separation. *J. Biol. Chem.* 253, 3757–3760.
- (61) Lux, B., and Gérard, D. (1981) Reappraisal of the Binding Processes of *N*-(3-Pyrene)maleimide as a Fluorescent Probe of Proteins. *J. Biol. Chem.* 256, 1767–1771.
- (62) Ishii, Y., and Lehrer, S. S. (1986) Effects of the State of the Succinimido-Ring on the Fluorescence and Structural Properties of Pyrene Maleimide-Labeled α -Tropomyosin. *Biophys. J.* 50, 75–80.
- (63) Griep, M. A., and McHenry, C. S. (1990) Dissociation of the DNA Polymerase III Holoenzyme β_2 Subunits Is Accompanied by Conformational Change at Distal Cysteines 333. *J. Biol. Chem.* 265, 20356–20363.
- (64) Griep, M. A., and Mesman, T. N. (1995) Fluorescent Labeling of Cysteine 39 on *Escherichia coli* Primase Places the Dye Near an Active Site. *Bioconjugate Chem.* 6, 673–682.
- (65) Winchell, H. S., Pollycove, M., Loughman, W. D., and Lawrence, J. H. (1964) Autologous Bone Marrow Transplantation Studies in Dogs Irradiated by Y^{90} -DTPA Urine-Recycling Technic. *Blood* 23, 44–52.
- (66) Winchell, H. S., Pollycove, M., Andersen, A. C., and Lawrence, J. H. (1964) Relatively Selective Beta Irradiation of Lymphatic Structures in the Dog Using Y^{90} -DTPA. *Blood* 23, 321–336.
- (67) Le Mignon, M.-M., Chambon, C., Warrington, S., Davies, R., and Bonnemain, B. (1990) Gd-DOTA. Pharmacokinetics and Tolerability After Intravenous Injection into Healthy Volunteers. *Invest. Radiol.* 25, 933–937.
- (68) Katzberg, R. W., Sahler, L. G., Duda, S. W., Morris, T. W., McKenna, B. A., Pabico, R. C., Niedrach, W. L., and Tonetti, F. W. (1991) Renal Handling and Physiologic Effects of the Paramagnetic Contrast Medium, Gadolinium-DOTA. *Invest. Radiol.* 26, S129–S130.
- (69) Ashby, B. S., and Cantab, M. B. (1966) pH Studies in Human Malignant Tumours. *Lancet* 2, 312–315.
- (70) van den Berg, A. P., Wike-Hooley, J. L., van den Berg-Blok, A. E., van der Zee, J., and Reinhold, H. S. (1982) Tumour pH in Human Mammary Carcinoma. *Eur. J. Cancer Clin. Oncol.* 18, 457–462.
- (71) Wike-Hooley, J. L., van den Berg, A. P., van der Zee, J., and Reinhold, H. S. (1985) Human Tumour pH and its Variation. *Eur. J. Cancer Clin. Oncol.* 21, 785–791.
- (72) Kallinowski, F., and Vaupel, P. (1988) pH distributions in spontaneous and isografted rat tumours. *Br. J. Cancer* 58, 314–321.
- (73) Gerweck, L. E., Rhee, J. G., Koutcher, J. A., Song, C. W., and Urano, M. (1991) Regulation of pH in Murine Tumor and Muscle. *Radiat. Res.* 126, 206–209.
- (74) Martin, G. R., and Jain, R. K. (1994) Noninvasive Measurement of Interstitial pH Profiles in Normal and Neoplastic Tissue Using Fluorescence Ratio Imaging Microscopy. *Cancer Res.* 54, 5670–5674.
- (75) Engin, K., Leeper, D. B., Cater, J. R., Thistlethwaite, A. J., Tupchong, L., and McFarlane, J. D. (1995) Extracellular pH distribution in human tumours. *Int. J. Hyperthermia* 11, 211–216.
- (76) McCoy, C. L., Parkins, C. S., Chaplin, D. J., Griffiths, J. R., Rodrigues, L. M., and Stubbs, M. (1995) The effect of blood flow modification on intra- and extracellular pH measured by ^{31}P magnetic resonance spectroscopy in murine tumours. *Br. J. Cancer* 72, 905–911.
- (77) Gerweck, L. E., and Seetharaman, K. (1996) Cellular pH Gradient in Tumor versus Normal Tissue: Potential Exploitation for the Treatment of Cancer. *Cancer Res.* 56, 1194–1198.
- (78) Wike-Hooley, J. L., Haveman, J., and Reinhold, H. S. (1984) The relevance of tumour pH to the treatment of malignant disease. *Radiother. Oncol.* 2, 343–366.
- (79) Tannock, I. F., and Rotin, D. (1989) Acid pH in Tumors and Its Potential for Therapeutic Exploitation. *Cancer Res.* 49, 4373–4384.
- (80) Williams, L. E., Lewis, M. R., Bebb, G. G., Clarke, K. G., Odom-Maryon, T. L., Shively, J. E., and Raubitschek, A. A. (1998) Biodistribution of ^{111}In - and ^{90}Y -Labeled DOTA and Maleimidocysteineamido-DOTA Conjugated to Chimeric Anti-Carcinoembryonic Antigen Antibody in Xenograft-Bearing Nude Mice: Comparison of Stable and Chemically Labile Linker Systems. *Bioconjugate Chem.* 9, 87–93.

BC970136V

Biodistribution of ^{111}In - and ^{90}Y -Labeled DOTA and Maleimidocysteineamido-DOTA Conjugated to Chimeric Anticarcinoembryonic Antigen Antibody in Xenograft-Bearing Nude Mice: Comparison of Stable and Chemically Labile Linker Systems

Lawrence E. Williams,^{*,†} Michael R. Lewis,[‡] Gregory G. Bebb,[§] Kenneth G. Clarke,^{||} Tamara L. Odom-Maryon,^{||} John E. Shively,[⊥] and Andrew A. Raubitschek[#]

Division of Diagnostic Radiology, City of Hope National Medical Center, Duarte, California 91010, City of Hope Graduate Program in Biological Sciences, Duarte, California 91010, Division of Surgery and Department of Biostatistics, City of Hope National Medical Center, Duarte, California 91010, Division of Immunology, Beckman Research Institute of the City of Hope, Duarte, California 91010, and Department of Radioimmunotherapy, City of Hope National Medical Center, Duarte, California 91010. Received July 18, 1997; Revised Manuscript Received October 15, 1997[⊗]

Biodistributions of two radiometal chelate conjugates of the human/murine chimeric anticarcinoembryonic antigen monoclonal antibody cT84.66 were obtained in nude mice bearing LS174T human colorectal carcinoma xenografts. Derivatives of the macrocyclic chelating agent 1,4,7,10-tetraazacyclododecane-*N,N',N'',N'''*-tetraacetic acid (DOTA) were covalently attached to the antibody by a stable amide linkage and by a maleimidocysteineamido side chain (MC-DOTA) that has been shown to be chemically labile at physiological temperature and pH. Biodistributions of both ^{111}In and ^{90}Y labels were obtained in these studies. At common biodistribution time points, it was found that the ^{111}In label had greater uptake in the liver than ^{90}Y for both conjugates. No significant differences were found with respect to bone uptake of ^{90}Y using either chelate. Blood curves were generally lower at comparable time points for MC-DOTA, indicative of faster clearance as compared to DOTA. Tumor uptake was high for both conjugates (57–68% ID/g at 48 h), with a longer tumor residence time in the case of the DOTA conjugate, probably a result of its longer blood circulation times. We conclude that bone uptake of ^{90}Y would be minimal if either DOTA or MC-DOTA were used as the bifunctional chelator. This would imply preference for these macrocyclic ligands if radiation doses to the bone marrow would be considered to be dominated by skeletal uptakes. Alternatively, if bone marrow radiation dose is dominated by circulating antibody, the chemically labile linker system employed by the MC-DOTA conjugate offers the advantage of enhanced blood clearance.

INTRODUCTION

Radiolabeled monoclonal antibodies (mAbs) have applications for both imaging and therapy of malignant tumors (1). A large number of radionuclides, including radioiodines but primarily radiometals, are available for these applications. Generally, pure photon emitters and particulate emitters are favored for imaging and therapy, respectively. In the case of clinical radioimmunotherapy (RIT¹), however, an imageable photon must be emitted along with the particulate radiation so the absorbed dose

can be estimated. If one utilizes only one radiolabel, such as a radioiodine, the associated photon yield may be of the wrong energy and/or too intense for effective quantitation. Human therapies require total injected activities approaching or exceeding 100 mCi (2). An example is the application of ^{131}I -labeled anti-CD20 in the treatment of B-cell lymphomas (3), where injected activities have exceeded 500 mCi. Such levels lead to γ camera count rate limitations at early time points postinjection. This may limit the accuracy of patient iodine uptake measurements and consequently the estimation of the absorbed dose.

Because of this ineffective or hazardous photon production, in many situations two radiolabels are used in RIT. One label must be suitable for the imaging or therapy planning situation, and the second, essentially of a particulate type, provided for the subsequent treatment(s). If the particulate emitter has no associated

* Address correspondence to Lawrence E. Williams, Ph.D., Division of Diagnostic Radiology, City of Hope National Medical Center, 1500 E. Duarte Rd., Duarte, CA 91010. Phone: (626) 301-8252. Fax: (626) 930-5451. E-mail: lwilliams@smtp link.coh.org.

[†] Division of Diagnostic Radiology, City of Hope National Medical Center.

[‡] City of Hope Graduate Program in Biological Sciences.

[§] Division of Surgery, City of Hope National Medical Center.
^{||} Department of Biostatistics, City of Hope National Medical Center.

[⊥] Beckman Research Institute of the City of Hope.

[#] Department of Radioimmunotherapy, City of Hope National Medical Center.

[⊗] Abstract published in *Advance ACS Abstracts*, December 15, 1997.

¹ Abbreviations: RIT, radioimmunotherapy; BCA, bifunctional chelating agent; DOTA, 1,4,7,10-tetraazacyclododecane-*N,N',N'',N'''*-tetraacetic acid; MC-DOTA, maleimidocysteineamido-DOTA; DTPA, diethylenetriaminepentaacetic acid; CEA, carcinoembryonic antigen; cT84.66, human/murine chimeric anti-CEA mAb; cT84.66-DOTA, cT84.66 conjugated with the *N*-hydroxysulfosuccinimide ester of DOTA; cT84.66-MC-DOTA, cT84.66 conjugated with MC-DOTA.

photon suitable for imaging, the provision of an imaging label becomes a necessity. From the earliest discussions of therapeutic efficacy, $^{90}\text{Y}(\text{III})$ has been described as a possible RIT radionuclide (4). Its pure, high-energy β^- (range, 1.1 cm) leads to the clinical advantage of cross-fire capability in treating tumor cells distant from those to which the radioimmunoconjugate has localized. The corresponding γ emitter $^{111}\text{In}(\text{III})$ generally has been used for imaging and dosimetry applications in ^{90}Y therapy. Both trivalent metals can be attached to mAbs using the same bifunctional chelating agent (BCA). ^{111}In emits two γ photons at 171 and 247 keV, energies that are efficiently detected with commercial Anger cameras.

Given that two labels must be used for ^{90}Y -based RIT, it is incumbent upon the investigator to demonstrate that variation of the radiometal has minimal and predictable impact upon biodistributions. If this is so, then an imaging sequence can be used to plan and document patient RIT treatments. Biodistributions in an animal model of human cancer can be used to show the variation of organ uptake with a change in the radiometal or bifunctional chelate coupled to the mAb.

An important consideration for the selection of the BCA is the formation of a physiologically stable radiometal complex, to minimize uptake in and radiation toxicity to normal tissues (5). In the case of ^{90}Y , uptake in mice approaching 20% of the injected dose per gram (% ID/g) of bone has been seen for the ionic form (6). Since bone marrow is an organ at risk in cancer radiotherapy, the dissociation of ^{90}Y in the bloodstream and other tissues as a function of time postinjection of the radiolabeled mAb must be evaluated. The stability of the conjugated radiometal complex will depend upon the type of BCA used.

Macrocyclic ligands have been suggested as one possible methodology for reducing ^{90}Y loss *in vivo* (7–10). Meares and co-workers (7–9) have demonstrated bone uptakes on the order of 2% ID/g in nude mice receiving 1,4,7,10-tetraazacyclododecane-*N,N',N'',N'''*-tetraacetic acid (DOTA) derivatives labeled with ^{90}Y and conjugated to Lym-1 (7) and chimeric L6 (9) antibodies. In these studies, the radiometal was incorporated into the BCA prior to mAb conjugation. Similar bone uptake values have been demonstrated by Harrison et al. (10) using the anti-colon cancer antibody B72.3, which had been covalently linked to DOTA before radiolabeling. In these earlier works, however, little numerical comparison was made between ^{111}In and ^{90}Y biodistributions in animals. Deshpande et al. (7) utilized Lym-1 mAb, which targets to B lymphocytes within the bone and confounds analyses. Harrison and co-workers utilized only ^{90}Y in their B72.3 studies (10). One recent report (9) included both radiometals, but did not analyze their differences statistically *in vivo*. The need to compare ^{111}In - and ^{90}Y -labeled antibody biodistributions for DOTA-based BCAs led to the studies described herein.

The human/murine chimeric anticarcinoembryonic antigen (anti-CEA) mAb cT84.66 (11) has shown minimal cross-reactivity with normal human or murine tissues, and biodistributions of ^{111}In - and ^{90}Y -labeled cT84.66 were evaluated earlier using a bifunctional DTPA derivative (12). Athymic nude mice implanted with CEA-expressing LS174T human colorectal tumor xenografts were used in that study. In this work, we obtained the ^{111}In and ^{90}Y biodistributions of cT84.66 conjugated to two new DOTA derivatives in the LS174T-bearing nude mouse model. When endogenous primary amino groups of the mAb were reacted with the *N*-hydroxysulfosuccinimide ester of DOTA, the radiometal-labeled conjugate

displayed extremely high kinetic stability *in vitro* (13). In contrast, radiometals conjugated to reduced disulfide bonds in the mAb with maleimidocysteineamido-DOTA (MC-DOTA) were chemically labile at physiological temperature and pH. This BCA exhibited pH-dependent linker hydrolysis *in vitro*, which resulted in release of $\text{Y}(\text{III})$ in the chelated form (14).

For both macrocycle–antibody conjugates, we compared the biodistributions obtained with ^{111}In versus those obtained with ^{90}Y . The likelihood of bone uptake of ^{90}Y was evaluated with either macrocycle. In addition, blood clearance curves were analyzed for all four combinations of label and BCA. The latter two studies provided information that can be used to select an optimal BCA for reducing bone marrow absorbed dose during clinical trials.

EXPERIMENTAL PROCEDURES

Conjugation of mAb and Radiolabeling. The preparation, ^{111}In and ^{90}Y labeling, and *in vitro* characterization of cT84.66–DOTA have been described previously (13). Reagents, synthesis of MC-DOTA, antibody conjugation, radiometal labeling, and *in vitro* evaluation of cT84.66–MC-DOTA were detailed in the preceding paper by Lewis and Shively (14). Briefly, the mAb conjugates were labeled with ^{111}In by incubating the conjugate with $^{111}\text{InCl}_3$ in 0.25 M ammonium acetate for 45 min at 43 °C. Then EDTA was added to a final concentration of 1 mM, and the mixture was incubated at 37 °C for 15 min. Labeling with ^{90}Y was performed using a 1 h incubation at 43 °C, after which DTPA was added to a final concentration of 1 mM, and the mixture was incubated for an additional 15 min at 37 °C. The radiometal-labeled mAb conjugates were purified by size exclusion HPLC, using a TosoHaas TSKgel G2000 SW column (10 μm , 7.5 $\times$ 300 mm) and an isocratic mobile phase of normal saline at a flow rate of 0.5 mL/min. In each case, the radiolabeled mAb peak was collected in 0.5 mL fractions containing 1 drop of 25% (w/v) human serum albumin, and the fraction containing the highest activity of radiolabeled mAb was used to prepare biodistribution doses. The cT84.66–MC-DOTA conjugate used for the biodistribution experiments was not treated with iodoacetic acid, a procedure which was shown to afford a moderate improvement in serum stability (14).

cT84.66–DOTA Biodistributions. In the case of cT84.66–DOTA, separate animal experiments were conducted with either the ^{111}In or ^{90}Y label. Female nu/nu BALB/c mice were injected subcutaneously with approximately 10^6 LS174T tumor cells in the hind flank, and tumors were allowed to grow for 10–14 days. Radiolabeled immunoconjugate (8 μCi of ^{111}In per 3.79 μg of mAb or 30 μCi of ^{90}Y per 3.49 μg of mAb) was injected into the tail vein of the animals when single tumors were palpable. Biodistributions were obtained at 0, 4, 18, 48, 72, and 96 h postinjection of the ^{111}In -labeled antibody. Corresponding values for the ^{90}Y label were 0.75, 6, 24, 48, 72, and 96 h. At these times, animals were euthanized and tissues (drained of blood) were taken, weighed, and counted in a well counter. Tissues included blood, liver, spleen, kidney, lung, bowel, bone, tumor, and carcass for the ^{111}In label. ^{90}Y biodistributions included blood, liver, spleen, kidney, bone, tumor, and carcass sampling. For the ^{111}In label, γ radiation was counted directly. ^{90}Y samples were quantitated using a β^- counter detecting Cerenkov radiation produced in a fixed volume (tissue plus saline) of 10 mL. Prior to Cerenkov counting, blood samples were clarified

by treatment with 10% sodium dodecyl sulfate at room temperature, bleach at 56 °C, and 30% hydrogen peroxide at room temperature. Other tissue samples were dissolved in perchloric acid at 56 °C, followed by treatment with 30% hydrogen peroxide at 56 °C and with bleach at room temperature, to make the emitting volume transparent to Cerenkov radiation. By comparison with a standard equal in activity to the injected dose per animal, the decay of either radiolabel was corrected so that normal organ and tumor uptake were calculated as the percent injected dose per gram of tissue.

cT84.66–MC-DOTA Biodistributions. When the cT84.66–MC-DOTA biodistributions were evaluated, we had become aware that tumor sizes could be different in separate groups of animals used for different radiolabels. Thus, in the case of cT84.66–MC-DOTA, the two radiolabeled preparations were mixed (8 μ Ci of ^{111}In per 10 μ Ci of ^{90}Y per 2.22 μ g of mAb) and co-injected into the same animal group. Biodistributions were performed at 0, 4, 12, 18, 48, 96, and 120 h postinjection. ^{111}In and ^{90}Y counting were performed as described above, and count crossover was taken into account in these analyses.

Because of the logistical difficulty of processing a large number of tissues simultaneously for both γ and Cerenkov counting, only blood, liver, bone, and tumor were evaluated for ^{90}Y activity per gram. In addition to those tissues, ^{111}In counts from spleen, lung, kidney, bowel, and carcass were also measured.

Statistical Methods. To compare differences between ^{111}In and ^{90}Y for the DOTA chelate at comparable time points (48, 72, and 96 h) and for each organ separately, the independent two-sample *t*-test was used (15). Similarly, the *t*-test was used to compare differences between the cT84.66–DOTA and cT84.66–MC-DOTA conjugates for a given label at comparable time points (4, 18, 48, and 96 h) and for each organ separately. For the cT84.66–MC-DOTA biodistribution studies, the agent was labeled with both ^{111}In and ^{90}Y . Therefore, to evaluate the agreement between the organ biodistributions for the two labels, Lin's concordance correlation coefficient (16) was estimated for each time point separately and all time points combined (referred to as pooled concordance coefficients). The concordance correlation coefficient evaluates the degree to which the pairs of data fall on the line of identity. All statistical testing was done at the *p* = 0.05 level using the SAS/STAT software package (17).

Blood Clearance. The rapidity of blood clearance was evaluated for all four combinations of BCA and radiometal. In these analyses, first and second biological half-lives were obtained by using the ADAPT II program of D'Argenio and Schumitzky (18). A biexponential function of time (*t*) having the form

$$U_B(t) = A_1 \exp(-k_1 t) + A_2 \exp(-k_2 t) \quad (1)$$

was fit to the blood data that had been corrected for radiodecay. In the equation, $U_B(t)$ represents the blood activity in percent injected dose per gram. Inverting the first and second rate constants allowed us to determine the two corresponding biological half-lives. These were intracompared among the radioimmunoconjugates studied here and also to the values of the antibody–chelate conjugates in the literature (9). It should be kept in mind that a double-exponential function may not be the best description of blood clearance. It is, however, a standard form that has been used in earlier analyses and can be applied, to the lowest order, in these data sets.

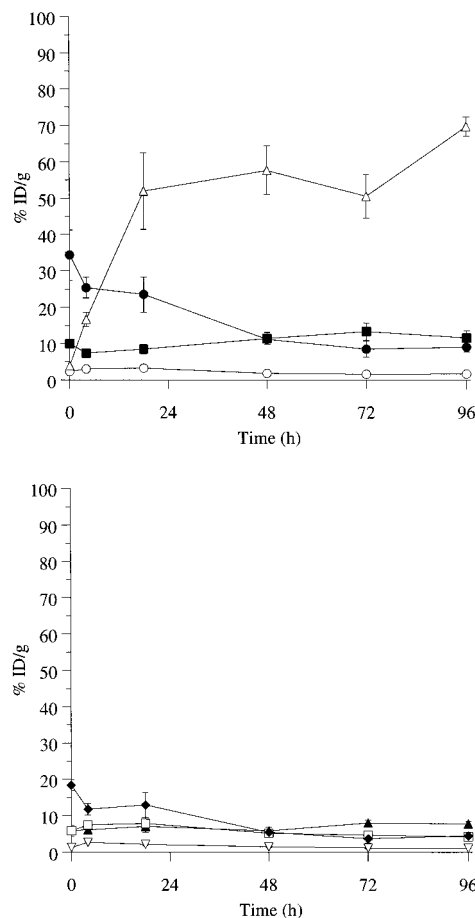


Figure 1. (Top) Percent injected dose per gram (% ID/g) of blood (●), liver (■), bone (○), and tumor (△) for ^{111}In -labeled cT84.66–DOTA in LS174T tumor-bearing nude mice. (Bottom) Percent injected dose per gram (% ID/g) of spleen (▲), kidney (□), lung (◆), and bowel (▽) for ^{111}In -labeled cT84.66–DOTA in LS174T tumor-bearing nude mice. Standard errors of the mean (SE) are indicated; all data were corrected for radiodecay.

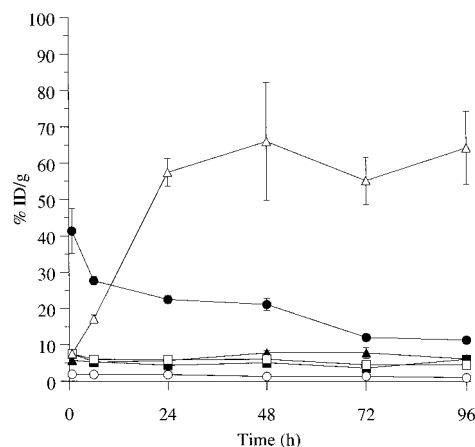


Figure 2. Percent injected dose per gram (% ID/g) of blood (●), liver (■), bone (○), tumor (△), spleen (▲), and kidney (□) for ^{90}Y -labeled cT84.66–DOTA in LS174T tumor-bearing nude mice. Standard errors of the mean (SE) are indicated; all data were corrected for radiodecay.

RESULTS

cT84.66–DOTA Biodistributions. The results of separate biodistribution studies for ^{111}In - and ^{90}Y -labeled cT84.66–DOTA are given in Figures 1 and 2, respectively. For both radiometals, blood was the dominant normal tissue out to 48 h. High uptakes were seen in

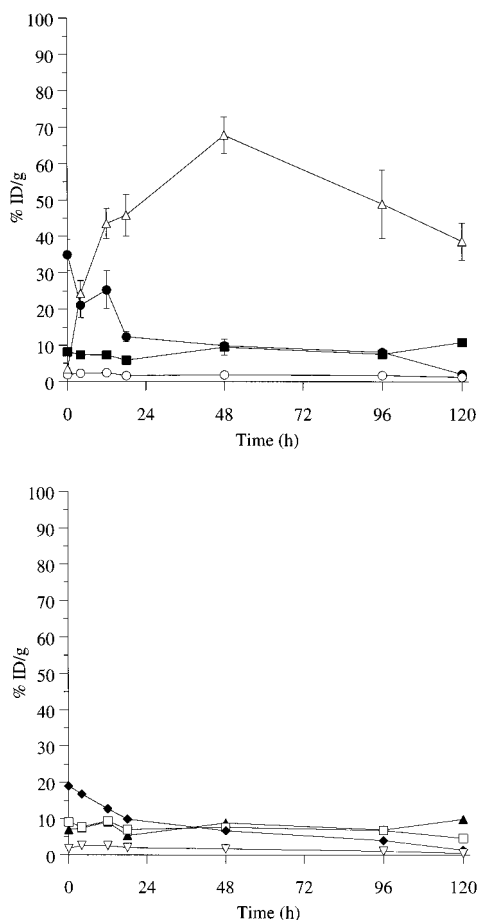


Figure 3. (Top) Percent injected dose per gram (% ID/g) of blood (●), liver (■), bone (○), and tumor (Δ) for ^{111}In -labeled cT84.66-MC-DOTA in LS174T tumor-bearing nude mice. (Bottom) Percent injected dose per gram (% ID/g) of spleen (▲), kidney (□), lung (◆), and bowel (▽) for ^{111}In -labeled cT84.66-MC-DOTA in LS174T tumor-bearing nude mice. Standard errors of the mean (SE) are indicated; all data were corrected for radiodecay.

the lung (18% ID/g) initially and in the liver as time exceeded 18 h. With the ^{111}In label, hepatic accumulation (11–14% ID/g) eventually exceeded that of ^{90}Y (4–7% ID/g). The highest accumulation, however, was ultimately obtained in the tumor, where values approached 60% ID/g at time points as early as 18–24 h. This level of tumor uptake remained relatively constant out to 96 h for both radiometals.

cT84.66-MC-DOTA Biodistributions. Organ and tissue uptakes for the simultaneous biodistribution of ^{111}In - and ^{90}Y -labeled cT84.66-MC-DOTA are shown in Figures 3 and 4, respectively. As in the case of the DOTA conjugate, blood was the dominant normal tissue out to 48 h. At early time points, lung accumulation was the second highest value with the ^{111}In label (9–17% ID/g). No lung data were obtained with ^{90}Y . Hepatic uptakes for both labels approached 10% ID/g by the end of the experimental period. Tumor accumulation exceeded 60% ID/g for both radiometals at 48 h but began to decrease slowly at later time points.

Comparison of Radiometals. Comparison of the ^{111}In and ^{90}Y uptakes resulting from the cT84.66-DOTA agent revealed that only the liver showed consistently different values at the three common time points (48, 72, and 96 h). At these intervals, hepatic uptake of ^{111}In was consistently higher ($p < 0.05$) than that of ^{90}Y . None of the other six tissues showed more than one point at which the uptakes differed significantly ($p > 0.05$).

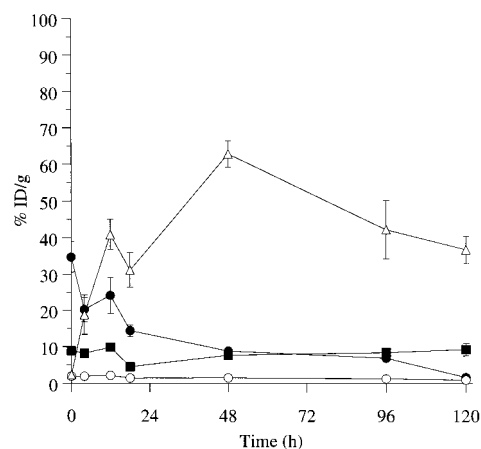


Figure 4. Percent injected dose per gram (% ID/g) of blood (●), liver (■), bone (○), and tumor (Δ) for ^{90}Y -labeled cT84.66-MC-DOTA in LS174T tumor-bearing nude mice. Standard errors of the mean (SE) are indicated; all data were corrected for radiodecay.

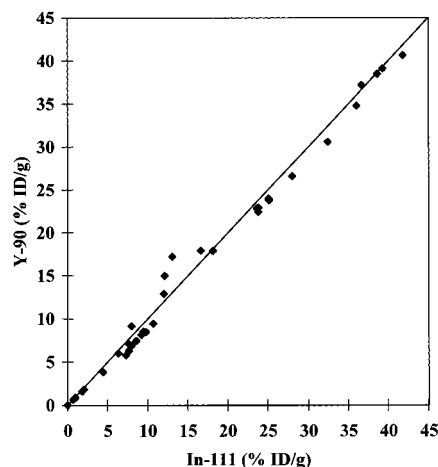


Figure 5. Concordance correlation analysis of blood data for cT84.66-MC-DOTA. Uptakes are plotted along both axes, with the ^{111}In -labeled cT84.66-MC-DOTA along the horizontal and the ^{90}Y -labeled cT84.66-MC-DOTA along the vertical axis. All time points are considered. The reference line shown is the line of identity.

^{111}In and ^{90}Y uptakes from the cT84.66-MC-DOTA biodistributions were evaluated using the Lin concordance coefficient (16). For blood, bone, tumor, and liver, pooled concordance coefficients were 0.9942, 0.8698, 0.9042, and 0.6460, respectively. Graphical representations of the blood and liver data are shown in Figures 5 and 6. It was noteworthy that the reduced concordance of hepatic samples was a result of greater accumulation of ^{111}In from the MC-DOTA conjugate in liver tissues. Reference lines shown on each of the two graphs are the lines of identity.

Bifunctional Chelate Effects. The biodistribution properties of the cT84.66-DOTA and cT84.66-MC-DOTA conjugates were compared for the ^{111}In label. Five time points were common to the two experiments: 0, 4, 18, 48, and 96 h. Blood values of ^{111}In were generally higher for cT84.66-DOTA than for cT84.66-MC-DOTA throughout, but statistically significant differences were found only at 18 and 48 h. The cT84.66-MC-DOTA conjugate showed significantly greater ^{111}In uptake in the kidneys at two of five times (48 and 96 h).

Corresponding tissue uptakes of ^{90}Y from cT84.66-DOTA and cT84.66-MC-DOTA were also compared. Four tissues were available at two common time points.

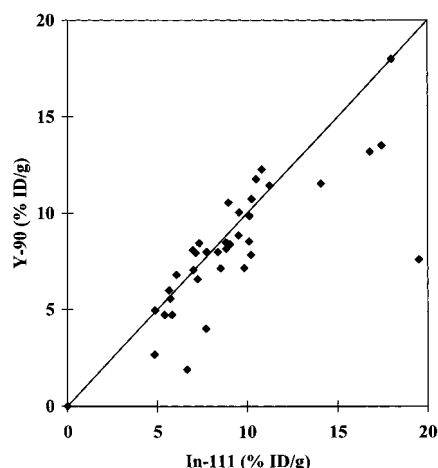


Figure 6. Concordance correlation analysis of liver uptake for cT84.66-MC-DOTA. The ^{111}In -labeled cT84.66-MC-DOTA is plotted along the horizontal and the ^{90}Y -labeled cT84.66-MC-DOTA along the vertical axis. All time points are considered. The reference line shown is the line of identity.

At both times, significantly lower ^{90}Y blood levels were obtained with cT84.66-MC-DOTA. No significant differences were observed for hepatic or bone uptakes.

^{90}Y Loss from Macrocyclic and Bone Uptake. Bone uptake of ^{90}Y was compared for the two macrocyclic BCAs using the *t*-statistic. Using data from Figures 2 and 4, no significant differences were seen in bone uptake at the 5% level of confidence. Initial values ($t = 0$) were on the order of 2% ID/g and decreased to 1.07% ID/g by the end of the experiment ($t = 96$ h) for ^{90}Y -labeled cT84.66-DOTA. In the case of cT84.66-MC-DOTA, the experiments were carried out to 120 h, at which time the bone uptake had been further reduced to 0.95% ID/g.

Blood Curve Analyses. Results from the blood curve analyses are given in Table 1. Half-lives were determined from the rate constants by the relationship $T_{1/2} = \ln(2)/k$. The first exponential (k_1) could be estimated, but its error or that of its associated half-life was indeterminate for either macrocycle-antibody conjugate. The primary reason for this difficulty appeared to be a slight inflection in the blood curves for cT84.66-DOTA and cT84.66-MC-DOTA, making eq 1 a more approximate representation for the early time points (18). For both radiometals, it was found that characteristic blood half-times were comparable for cT84.66-DOTA and cT84.66-MC-DOTA. Initial biological half-times were generally on the order of 0.2–0.4 h, while the second biological half-time was much larger, on the order of 40 h or longer. Figure 7 shows the ^{90}Y blood clearance curves for cT84.66-DOTA and cT84.66-MC-DOTA. The curve for the MC-DOTA conjugate was below that of the corresponding DOTA conjugate throughout the time range; significant differences were observed at both common time points (48 and 96 h). It should be noted that ^{111}In -labeled cT84.66-MC-DOTA exhibited faster blood clearance throughout the experimental period, but this difference was only significant at two time points, as described above.

DISCUSSION

Figures 1–4 show that both the amide-linked cT84.66-DOTA conjugate and the cT84.66-MC-DOTA conjugate, with its cleavable imide linker, demonstrate high tumor targeting in the LS174T nude mouse model. These results were consistent for both radiometals. At the 48 h time point, tumor uptake of the DOTA conjugate was

between 3- and 5-fold greater than that of the normal tissue having the next highest accumulation (typically blood or liver). The MC-DOTA conjugate exhibited an approximate 7-fold difference between tumor and the highest normal tissue uptake at 48 h. The absolute magnitude of tumor uptake was between 50 and 70% ID/g for both conjugates. These values were comparable to those observed previously in the LS174T nude mouse model by our group (12) for cT84.66 conjugated with a benzylisothiocyanate derivative of DTPA. Other xenograft data using the Lym-1 (7) and chimeric L6 (9) antibodies conjugated to DOTA derivatives have indicated maximum uptakes approaching only 17 and 18% ID/g, respectively. These lower values may be a result of differences in antibody-antigen affinity constants and/or xenograft models. Tumor masses were not specified in those earlier analyses; our xenograft sizes ranged from approximately 0.1 to 0.2 g at the 48 h time point.

Table 2 contains a summary of our biodistribution comparisons. It was seen that hepatic accumulation of ^{111}In was significantly greater than that of ^{90}Y when either BCA was coupled to cT84.66. Transchelation of ^{111}In by transferrin is one possible mechanism for transport to the hepatocytes, but we found no evidence of this mechanism with either conjugate *in vitro* or *in vivo*. Kinetic stability studies of radiolabeled cT84.66-DOTA in human serum (13) showed that <1% of ^{111}In or ^{90}Y was converted to a low-molecular mass species, possibly a result of hydrolytic (7, 19) or proteolytic cleavage of the chelate from the mAb. Even though MC-DOTA underwent a linker-dependent cleavage reaction under physiological conditions, both released radiometal chelates were found to associate with serum albumin and not with transferrin upon incubation in human serum at 37 °C (14).

Blood samples drawn from animals in this study were also analyzed by gel filtration HPLC. Radiolabeled mAb was the only radioactive species detected in the circulation of mice injected with cT84.66-DOTA (data not shown). Blood of animals receiving cT84.66-MC-DOTA contained radiolabeled antibody and serum albumin, but the label associated with albumin cleared faster than the mAb (data not shown). It is possible that clearance of serum albumin plays a role in hepatic uptake of radiometal from the MC-DOTA conjugate. However, the differences between ^{111}In and ^{90}Y were not dramatic and amounted to a $\leq 5\%$ ID/g increase with ^{111}In . Figure 6 shows this clearly in the concordance analyses of cT84.66-MC-DOTA. The difference between radiometal uptake in hepatic tissues would have to be considered when estimating the patient-absorbed dose resulting from radioimmunotherapy. In that case, the ^{111}In biodistribution is used to predict the ^{90}Y -induced absorbed dose. No other organ system studied showed such consistent disparity between uptake of the two radiolabels. A similar difference has been observed previously using DTPA benzylisothiocyanate as the BCA for ^{111}In and ^{90}Y (12).

For a given radiolabel, several differences were seen in our analyses with variation of the BCA. With ^{111}In , cT84.66-MC-DOTA demonstrated less uptake in the blood at two of the five common time points. Renal accumulation, however, was found to be greater for the MC-DOTA conjugate in two of five time points. In the case of the ^{90}Y (Figure 7), blood clearance was increased by using the chemically labile MC-DOTA, a result similar to that found with ^{111}In . No significant differences of hepatic uptake were observed comparing ^{90}Y -labeled cT84.66-DOTA and cT84.66-MC-DOTA.

Table 1. Results of Two-Exponential Fits of Blood Curves for cT84.66–DOTA and cT84.66–MC-DOTA^a

BCA-label	A ₁ (% ID/g)	A ₂ (% ID/g)	k ₁ (h ⁻¹)	k ₂ (10 ⁻² h ⁻¹)	T _{1/2,1} (h)	T _{1/2,2} (h)
DOTA- ¹¹¹ In	6.47 (6.1)	27.8 (5.3)	3.26 (–)	1.48 (0.44)	0.212 (–)	46.8 (14.0)
MC-DOTA- ¹¹¹ In	11.4 (7.9)	23.5 (6.1)	3.31 (–)	1.60 (0.82)	0.209 (–)	43.3 (22.1)
DOTA- ⁹⁰ Y	42.4 (–)	29.7 (5.0)	1.69 (–)	1.02 (0.33)	0.410 (–)	68.0 (21.8)
MC-DOTA- ⁹⁰ Y	11.2 (6.3)	23.5 (4.9)	3.49 (–)	1.72 (0.70)	0.199 (–)	40.3 (16.3)

^a Standard deviations are shown in parentheses. The symbol (–) denotes that an error could not be estimated with the two-exponential fit of eq 1.

Table 2. Summary of Biodistribution Comparisons

BCA or radiometal	no. of common time points	organ with significant difference (points/total) ^a	times at which difference was significant with <i>t</i> -test	uptake result
DOTA	3	liver (3/3)	48, 72, 96 h	¹¹¹ In > ⁹⁰ Y
MC-DOTA	7	liver (N/A) ^b	N/A ^b	¹¹¹ In > ⁹⁰ Y
¹¹¹ In	5	blood (2/5)	18, 48 h	MC-DOTA < DOTA
		kidney (2/5)	48, 96 h	DOTA < MC-DOTA
⁹⁰ Y	2	blood (2/2)	48, 96 h	MC-DOTA < DOTA

^a Significant when *p* < 0.05. ^b Significant difference was determined by concordance analysis.

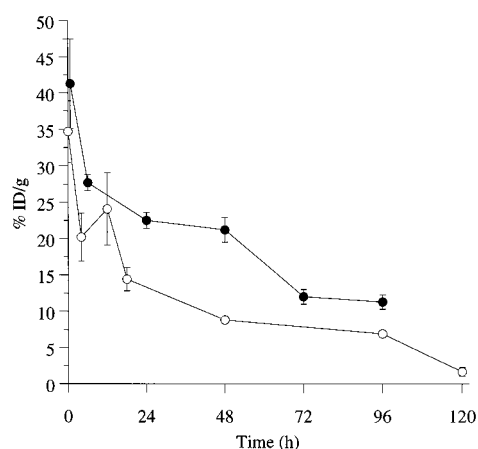


Figure 7. Blood clearance curves (% ID/g) for ⁹⁰Y-labeled cT84.66–DOTA (●) and cT84.66–MC-DOTA (○). All data have been corrected for radiodecay. Measured standard errors of the mean (SE) are indicated.

Blood clearance kinetic analyses were consistent (Table 1) with biphasic curves for all combinations of radiometal and BCA. The difference between the two half-times was almost 2 orders of magnitude, with the larger value being between 40 and 70 h. This *T*_{1/2,2} was somewhat smaller than the comparable quantity observed by DeNardo et al. (9). This difference may not be significant, as no errors were quoted for their *T*_{1/2,2} values. By comparing ⁹⁰Y data for our DOTA and MC-DOTA conjugates, we have been able to show that the latter BCA results in a generally lower blood clearance curve (Figure 7). This difference may have important consequences in the bone marrow-absorbed dose in a clinical situation.

The dominant source organ for the bone marrow-absorbed dose in patients cannot be predicted at this time. If we assume that murine data correspond to the human case, it can be anticipated that blood and bone will be contributors. Both BCAs studied here exhibited substantially reduced bone uptake compared to the benzylisothiocyanato-DTPA chelate studied earlier by our group (12). Thus, the use of a macrocycle can be generally justified for minimization of the marrow dose arising from bone as a source organ. As noted above, skeletal uptake of ⁹⁰Y was comparable for both BCAs.

If the bone marrow radiation dose in human subjects proves to be dominated by circulating ⁹⁰Y-labeled antibody, selection of an optimal agent for RIT applications may be accomplished using a direct numerical compari-

son of blood clearance data. Since β^- -induced tissue radiation doses are proportional to the area under that tissue's curve (AUC), it is appropriate to compare the ratios of such areas for the two BCAs studied here. For blood-associated radioactivity, the relevant ratio is AUC-(tumor)/AUC(blood). In the case of cT84.66–MC-DOTA, this value was 4.06, while the comparable magnitude for cT84.66–DOTA was only 3.22. Thus, assuming that the blood activity curve was of primary toxicity significance as a result of the blood-induced marrow dose, the cT84.66–MC-DOTA conjugate appeared to be superior. This result could provide a reason for selecting this conjugate for use in clinical RIT trials.

ACKNOWLEDGMENT

This work was supported by funds from NIH Research Grant CA43904, Cancer Center Core Grant 33527, and Department of the Army Grant DAMD17-96-1-6047 (predoctoral fellowship to M.R.L.). The content of this article does not necessarily reflect the position or the policy of the United States government, and no official endorsement should be inferred.

Supporting Information Available: Tables of mean percent injected dose per gram (% ID/g) with standard errors (SE) of ¹¹¹In- and ⁹⁰Y-labeled cT84.66–DOTA and cT84.66–MC-DOTA for all tissues and time points evaluated (4 pages). Ordering information is given on any current masthead page.

LITERATURE CITED

- (1) Waldmann, T. A. (1991) Monoclonal Antibodies in Diagnosis and Therapy. *Science* 252, 1657–1662.
- (2) Williams, L. E., Beatty, B. G., Beatty, J. D., Wong, J. Y. C., Paxton, R. J., and Shively, J. E. (1990) Estimation of Monoclonal Antibody-associated Y-90 Activity Needed to Achieve Certain Tumor Radiation Doses in Colorectal Cancer Patients. *Cancer Res.* 50 (Suppl.), 1029s–1030s.
- (3) Kaminski, M. S., Zasadny, K. R., Francis, I. R., Milik, A. W., Ross, C. W., Moon, S. D., Crawford, S. M., Burgess, J. M., Petry, N. A., Butchko, G. M., Glenn, S. D., and Wahl, R. L. (1993) Radioimmunotherapy of B-Cell Lymphoma with [¹³¹I]Anti-B1 (Anti-CD20) Antibody. *N. Engl. J. Med.* 329, 459–465.
- (4) Wessels, B. W., and Rogus, R. D. (1984) Radionuclide selection and model absorbed dose calculations for radiolabeled tumor associated antibodies. *Med. Phys.* 11, 638–645.
- (5) Kozak, R. W., Raubitschek, A., Mirzadeh, S., Brechbiel, M. W., Junghaus, R., Gansow, O. A., and Waldmann, T. A. (1989) Nature of the Bifunctional Chelating Agent Used for Radio-

- immunotherapy with Yttrium-90 Monoclonal Antibodies: Critical Factors in Determining *in Vivo* Survival and Organ Toxicity. *Cancer Res.* 49, 2639–2644.
- (6) Sharkey, R. M., Motta-Hennessy, C., Pawlyk, D., Siegel, J. A., and Goldenberg, D. M. (1990) Biodistribution and Radiation Dose Estimates for Yttrium- and Iodine-labeled Monoclonal Antibody IgG and Fragments in Nude Mice Bearing Human Colonic Tumor Xenografts. *Cancer Res.* 50, 2330–2336.
- (7) Deshpande, S. V., DeNardo, S. J., Kukis, D. L., Moi, M. K., McCall, M. J., DeNardo, G. L., and Meares, C. F. (1990) Yttrium-90-Labeled Monoclonal Antibody for Therapy: Labeling by a New Macrocyclic Bifunctional Chelating Agent. *J. Nucl. Med.* 31, 473–479.
- (8) Meares, C. F., Moi, M. K., Diril, H., Kukis, D. L., McCall, M. J., Deshpande, S. V., DeNardo, S. J., Snook, D., and Epenetos, A. A. (1990) Macrocyclic chelates of radiometals for diagnosis and therapy. *Br. J. Cancer* 62 (Suppl. X), 21–26.
- (9) DeNardo, S. J., Zhong, G.-R., Salako, Q., Li, M., DeNardo, G. L., and Meares, C. F. (1995) Pharmacokinetics of Chimeric L6 Conjugated to Indium-111 and Yttrium-90-DOTA-Peptide in Tumor-Bearing Mice. *J. Nucl. Med.* 36, 829–836.
- (10) Harrison, A., Walker, C. A., Parker, D., Jankowski, K. J., Cox, J. P. L., Craig, A. S., Sansom, J. M., Beeley, N. R. A., Boyce, R. A., Chaplin, L., Eaton, M. A. W., Farnsworth, A. P. H., Millar, K., Millican, A. T., Randall, A. M., Rhind, S. K., Secher, D. S., and Turner, A. (1991) The *In Vivo* Release of ^{90}Y from Cyclic and Acyclic Ligand-Antibody Conjugates. *Int. J. Radiat. Appl. Instrum. Part B Nucl. Med. Biol.* 18, 469–476.
- (11) Neumaier, M., Shively, L., Chen, F.-S., Gaida, F.-J., Ilgen, C., Paxton, R. J., Shively, J. E., and Riggs, A. D. (1990) Cloning of the Genes for T84.66, an Antibody That Has a High Specificity and Affinity for Carcinoembryonic Antigen, and Expression of Chimeric Human/Mouse T84.66 Genes in Myeloma and Chinese Hamster Ovary Cells. *Cancer Res.* 50, 2128–2134.
- (12) Williams, L. E., Primus, F. J., Wong, J. Y. C., Wu, A. M., Odom-Maryon, T. L., Johnson, D. K., Hefta, L. J. F., Shively, J. E., and Raubitschek, A. A. (1996) Biodistribution of an Indium-111 or Yttrium-90-Labelled Chimeric Anti-Carcinoembryonic Antigen Monoclonal Antibody (cT84.66) Following Its Large Scale Production in a Bioreactor. *Tumor Targeting* 2, 116–124.
- (13) Lewis, M. R., Raubitschek, A., and Shively, J. E. (1994) A Facile, Water-Soluble Method for Modification of Proteins with DOTA. Use of Elevated Temperature and Optimized pH To Achieve High Specific Activity and High Chelate Stability in Radiolabeled Immunoconjugates. *Bioconjugate Chem.* 5, 565–576.
- (14) Lewis, M. R., and Shively, J. E. (1998) Maleimidocysteineamido-DOTA Derivatives: New Reagents for Radiometal Chelate Conjugation to Antibody Sulfhydryl Groups Undergo pH-Dependent Cleavage Reactions. *Bioconjugate Chem.* 9, 72–86.
- (15) Zar, J. H. (1984) *Biostatistical Analysis*, 2nd ed., pp 126–131, Prentice-Hall, Englewood Cliffs, NJ.
- (16) Lin, L. I.-K. (1989) A Concordance Correlation Coefficient to Evaluate Reproducibility. *Biometrics* 45, 255–268.
- (17) SAS Institute (1989) *SAS/STAT User's Guide, Version 6*, 4th ed., Vol. 2, SAS Institute, Inc., Cary, NC.
- (18) D'Argenio, D. Z., and Schumitzky, A. (1979) A program package for simulation and parameter estimation in pharmacokinetic systems. *Comput. Prog. Biomed.* 9, 115–134.
- (19) Li, M., and Meares, C. F. (1993) Synthesis, Metal Chelate Stability Studies, and Enzyme Digestion of a Peptide-Linked DOTA Derivative and Its Corresponding Radiolabeled Immunoconjugates. *Bioconjugate Chem.* 4, 275–283.

BC970137N

Table 3. Mean percent injected dose per gram of tissue (% ID/g) for ^{111}In -labeled cT84.66-DOTA in LS174T tumor-bearing nude mice. Standard errors (SE) are given in parentheses; all data were corrected for radiodecay.

Organ	0 h	4 h	18 h	48 h	72 h	96 h
Blood	34.3 (6.9)	25.4 (2.8)	23.5 (4.8)	11.2 (0.2)	8.56 (2.2)	9.09 (1.3)
Liver	10.0 (0.7)	7.40 (0.8)	8.54 (1.3)	11.5 (1.6)	13.4 (2.4)	11.6 (2.0)
Spleen	6.00 (0.8)	6.16 (1.1)	7.11 (1.6)	5.91 (1.0)	8.06 (0.8)	7.82 (0.6)
Kidney	5.93 (1.5)	7.51 (0.2)	7.86 (1.6)	5.30 (0.3)	4.72 (1.1)	4.29 (0.3)
Lung	18.4 (1.5)	11.8 (1.6)	13.0 (3.4)	5.52 (0.4)	3.79 (1.2)	4.59 (0.5)
Bowel	1.30 (0.1)	2.70 (0.3)	2.20 (0.3)	1.50 (0.1)	1.19 (0.2)	1.14 (0.02)
Bone	2.30 (0.2)	3.05 (0.1)	3.32 (0.9)	1.86 (0.1)	1.64 (0.3)	1.66 (0.1)
Tumor	3.89 (0.1)	16.7 (2.0)	51.9 (10.6)	57.6 (6.7)	50.4 (6.0)	69.4 (2.6)
Carcass	2.28 (0.3)	3.00 (0.2)	3.10 (0.3)	2.60 (0.2)	1.60 (0.3)	2.10 (0.2)
Tumor mass (mg)	58 (4)	83 (26)	153 (28)	258 (51)	360 (44)	232 (45)
No. of animals	3	4	4	4	4	4

Table 4. Mean percent injected dose per gram of tissue (% ID/g) for ^{90}Y -labeled cT84.66-DOTA in LS174T tumor-bearing nude mice. Standard errors (SE) are given in parentheses; all data were corrected for radiodecay.

Organ	0.75 h	6 h	24 h	48 h	72 h	96 h
Blood	41.3 (6.2)	27.7 (1.12)	22.5 (1.1)	21.2 (1.7)	12.0 (1.0)	11.3 (1.0)
Liver	7.42 (0.9)	5.37 (0.2)	4.41 (0.3)	5.13 (0.3)	3.71 (0.3)	6.11 (0.9)
Spleen	5.74 (1.0)	5.20 (0.3)	5.63 (0.4)	7.82 (0.6)	7.82 (1.5)	6.12 (1.1)
Kidney	7.50 (1.0)	6.02 (0.3)	5.88 (0.6)	6.10 (0.2)	4.60 (0.2)	4.68 (0.4)
Bone	1.97 (0.4)	1.90 (0.5)	1.86 (0.1)	1.39 (0.3)	1.42 (0.3)	1.07 (0.1)
Tumor	7.57 (0.8)	17.2 (1.1)	57.4 (3.8)	65.9 (16.2)	55.0 (6.5)	64.0 (10.0)
Carcass	2.72 (0.1)	2.70 (0.1)	2.58 (0.1)	2.87 (0.1)	2.28 (0.1)	2.10 (0.1)
Tumor mass (mg)	79 (26)	45 (10)	64 (8)	74 (27)	71 (8)	85 (4)
No. of animals	4	4	4	4	4	4

Table 5. Mean percent injected dose per gram of tissue (% ID/g) for ^{111}In -labeled cT84.66-MCDDOTA in LS174T tumor-bearing nude mice. Standard errors (SE) are given in parentheses; all data have been corrected for radiodecay.

Organ	0 h	4 h	12 h	18 h	48 h	96 h	120 h
Blood	34.9 (4.3)	21.1 (3.4)	25.3 (5.2)	12.4 (1.4)	9.98 (0.3)	8.16 (0.3)	2.00 (0.7)
Liver	8.22 (0.5)	7.44 (0.6)	7.31 (0.8)	5.93 (0.4)	9.54 (2.2)	7.56 (1.2)	10.9 (1.1)
Spleen	7.00 (0.7)	7.41 (0.5)	9.08 (1.1)	5.34 (0.5)	8.87 (1.6)	6.89 (1.0)	9.86 (1.9)
Kidney	9.04 (0.9)	7.74 (0.4)	9.42 (1.2)	7.05 (0.4)	7.63 (0.6)	6.83 (0.2)	4.64 (0.4)
Lung	19.0 (2.0)	16.8 (1.2)	12.7 (1.5)	9.84 (0.8)	6.73 (1.4)	3.98 (0.2)	1.35 (0.3)
Bowel	1.80 (0.3)	2.69 (0.2)	2.61 (0.3)	2.05 (0.1)	1.70 (0.2)	1.05 (0.1)	0.48 (0.1)
Bone	1.90 (0.3)	2.22 (0.1)	2.38 (0.2)	1.64 (0.1)	1.82 (0.1)	1.70 (0.2)	1.20 (0.1)
Tumor	3.44 (0.2)	24.3 (3.5)	43.5 (4.2)	45.8 (5.8)	67.8 (5.0)	48.8 (9.5)	38.5 (5.1)
Carcass	2.42 (0.1)	3.01 (0.1)	3.89 (0.4)	3.33 (0.2)	2.45 (0.2)	1.86 (0.1)	1.00 (0.2)
Tumor mass (mg)	46 (8)	120 (33)	110 (31)	140 (49)	196 (49)	180 (37)	372 (51)
No. of animals	5	5	5	5	5	5	5

Table 6. Mean percent injected dose per gram of tissue (% ID/g) for ^{90}Y -labeled cT84.66-MCDDOTA in LS174T tumor-bearing nude mice. Standard errors (SE) are given in parentheses; all data were corrected for radiodecay.

Organ	0 h	4 h	12 h	18 h	48 h	96 h	120 h
Blood	34.7 (4.2)	20.2 (3.3)	24.1 (5.0)	14.4 (1.6)	8.83 (0.2)	6.93 (0.4)	1.70 (0.6)
Liver	8.86 (0.5)	8.25 (1.2)	9.87 (1.1)	4.53 (1.1)	7.75 (1.0)	8.42 (0.8)	9.30 (1.7)
Bone	1.91 (0.3)	1.97 (0.1)	2.17 (0.2)	1.44 (0.1)	1.51 (0.1)	1.28 (0.2)	0.95 (0.1)
Tumor	2.34 (0.3)	18.8 (5.4)	40.9 (4.2)	31.1 (4.8)	62.8 (3.6)	42.2 (7.9)	36.7 (3.8)
Tumor mass (mg)	46 (8)	120 (33)	110 (31)	140 (49)	196 (49)	180 (37)	372 (58)
No. of animals	5	5	5	5	5	5	5

Polymeric Conjugates of Gd^{3+} –Diethylenetriaminepentaacetic Acid and Dextran. 2. Influence of Spacer Arm Length and Conjugate Molecular Mass on the Paramagnetic Properties and Some Biological Parameters

Richard Rebizak,[†] Michel Schaefer,[‡] and Édith Dellacherie^{*,†}

Laboratoire de Chimie-Physique Macromoléculaire, URA CNRS 494, ENSIC-INPL, BP 451, 54001 Nancy Cedex, France, and Guerbet-GCA, BP 15, 93601 Aulnay-sous-Bois, France. Received August 8, 1997;

Revised Manuscript Received October 5, 1997[©]

Macromolecular conjugates of dextran 40 ($\overline{M}_n = 38$ kg/mol, $\overline{M}_w = 43$ kg/mol) and diethylenetriaminepentaacetic acid (DTPA), capable of complexing Gd^{3+} , were synthesized in order to obtain contrast agents for nuclear magnetic resonance imaging, with long intravascular persistence. As already reported, relaxivity of these macromolecular complexes was greater than that of molecular complexes (2–2.5 times). On the other hand, as relaxivity of paramagnetic agents partially depends on their rotation speed, the influence of the length of the spacer arm inserted between DTPA and dextran was investigated. Various conjugates with spacer arms comprised of methylenic chains $-(\text{CH}_2)_n-$ with varying lengths ($2 \leq n \leq 6$) were prepared. The result was that, whatever the distance between DTPA and dextran, all of the Gd^{3+} -complexed polymeric conjugates with similar DTPA content (0.6–0.8 mmol/g) exhibited, at 20 MHz, about the same relaxivity values. On the other hand, in the same way, no significant effect of the molecular masses ($\overline{M}_n$ between 166 and 224 kg/mol and $\overline{M}_w$ between 332 and 865 kg/mol) of the polymeric conjugates on relaxivity were observed. Toxicity studies carried out on mice, showed very similar LD_{50} values whatever the spacer arm and molecular mass of the macromolecular conjugates. Finally the urinary excretion studied on rats (after 4 h) and on rabbits (after 24 h) was relatively low (15–20% of the administered dose), and, as expected, the lower values were obtained with the higher molecular mass conjugates.

INTRODUCTION

The usefulness of paramagnetic metals to decrease the proton spin relaxation times (T_1 and T_2) of water molecules was discovered in 1948 by Bloch (1). Then, Solomon (2) and Bloembergen et al. (3) contributed to establishing the theoretical relations between the nature of paramagnetic elements (metallic ion or molecular complex) and T_1/T_2 . The most commonly used paramagnetic metals are Mn^{2+} , Fe^{3+} , and Gd^{3+} because of the great number of unpaired electrons on the last electronic orbital, which results in strong dipolar interactions between the electronic and protonic spins.

Because of their high toxicity, these elements have to be used under the form of very stable complexes. In fact, the LD_{50} values of these ions, intravenously injected in rats, are very low, respectively 0.22, 1.6, and 0.5 mmol kg^{-1} for MnCl_2 , FeCl_3 , and GdCl_3 (4). At present, the gadolinium complexes used in NMR imaging in humans are based on two types of structures: gadolinium–diethylenetriaminepentaacetic acid (GdDTPA^{2-}) and gadolinium–tetraazacyclododecanetetraacetic acid (GdDOTA^-). Their thermodynamic stability constants are, respectively, $10^{22.5}$ (5) and 10^{28} L mol^{-1} (6). Studies carried out on healthy volunteers and patients showed

that, when injected, these complexes were well tolerated and that risks of anaphylactic shocks were very low (7). However cation complexation by DTPA or DOTA leads to a decrease in the amount of water molecules in the first hydration shell of the ion and consequently to a decrease in the transversal (r_2) and longitudinal (r_1) relaxivity values. Thus, Gd^{3+} that binds 9 and 1 water molecules in its first hydration shell, when it is respectively free in solution and complexed by DTPA, is characterized by r_1 values of respectively 9.1 and 4.1 $\text{mM}^{-1} \text{s}^{-1}$ at 20 MHz (4).

Besides a relatively low relaxivity, these molecular complexes present another disadvantage that is their rapid removal from the blood vessels through the vascular interstitium, which makes it necessary, in a prolonged clinical examination, to inject several doses. For example, in humans, 50% of GdDTPA^{2-} has been excreted 90 min after the injection, and its blood concentration is only about 30% of the initial administered dose after 5 min (8). To increase the complex *in vivo* half life, many authors have focused their attention on the synthesis of macromolecular paramagnetic compounds, generally obtained by coupling GdDTPA^{2-} or GdDOTA^- to different types of macromolecules or polymers such as polypeptides (9, 10), proteins (8, 11–16) and polysaccharides (17–21).

Besides the expected increase in the intravascular persistence, the use of macromolecules as contrast agent carriers leads to another benefit. In fact, increasing the molecular size of the paramagnetic element improves the transversal and longitudinal relaxivities by reducing the rotation speed of the gadolinium complex (4). Moreover,

* To whom correspondence should be sent. Phone: (33) 383 17 52 21; fax: (33) 383 37 99 77; e-mail: e.dellacherie@kekule.ensic.u-nancy.fr.

[†] ENSIC-INPL.

[‡] Guerbet-GCA.

[©] Abstract published in *Advance ACS Abstracts*, December 15, 1997.

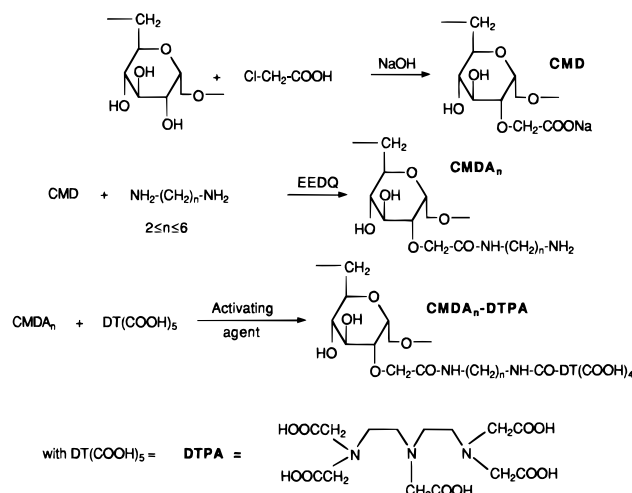


Figure 1. Synthesis of CMDA_n -DTPA samples.

the choice of a particular macromolecule as complex carrier, for example a labeled monoclonal antibody specific to a particular tumor line, can contribute to target the paramagnetic agent (16).

This paper describes the properties of macromolecular conjugates with various molecular masses obtained by coupling DTPA to a polysaccharide dextran with a M_w of about 40 kg/mol, well known for its biocompatibility and its biodegradability. Indeed, it is often used as a plasma expander to prevent blood platelet aggregation (22). DTPA was bound to dextran 40 via spacer arms of various lengths. This paper reports on the influence of the spacer arm length and of the conjugate molecular mass and size on the r_1 and r_2 relaxivity values of the macromolecular Gd^{3+} -complexes. Some results of *in vivo* toxicity and urinary excretion experiments are also commented on with respect to the effect of these parameters.

EXPERIMENTAL PROCEDURES

Syntheses. The polymer synthesis previously described in details (23) was achieved in three steps (Figure 1). The first consisted in the synthesis of carboxymethyl dextran (CMD) by reacting chloroacetic acid with dextran hydroxyl groups in aqueous medium. In the second step, the carboxylate groups of CMD were allowed to react with a linear diamine $\text{NH}_2(\text{CH}_2)_n\text{NH}_2$, with $2 \leq n \leq 6$ by means of a peptide coupling agent, 2-ethoxy-1-(ethoxycarbonyl)-1,2-dihydroquinoline (EEDQ), to give CMDA_n . Finally, three different experimental procedures were used to bind DTPA to CMDA_n in order to obtain CMDA_n -DTPA (23). In the two first ones, DTPA bisanhydride (DTPAba) was used, and in the third, DTPA was activated in the form of succinimidic ester (DTPA-ONSu) by means of dicyclohexylcarbodiimide (DCC) and *N*-hydroxy-succinimide (HONSu). In the first method using DTPAba that is previously prepared according to a method deriving from that of Eckelman and Karesh (24), 1 g of CMDA_n (1.7 mmol of NH_2/g) was dissolved in 30 mL of water. Then DTPAba (11 g, 30.8 mmol) was progressively added to the solution while maintaining the pH between 8 and 10. In the second procedure, 1 g of CMDA_n (1.7 mmol of NH_2/g) was dissolved in 10 mL of water. Then, 1/20 of the CMDA_n solution and 1/20 of the total DTPAba amount (11 g) were successively added to 20 mL of water. During this step, the pH was kept constant at 10 by means of 6 N NaOH. When the pH was stabilized, the operation was repeated until total

consumption of all of the reagents. The third method used DTPA-ONSu: DTPA (8 g, 20.3 mmol) was dissolved in 10 mL of acetonitrile in the presence of triethylamine (11.3 mL, 81.3 mmol). Then, 2.87 g (14.4 mmol) of DCC and 1.66 g (14.4 mmol) of HONSu were added. After 90 min of reaction, the solution was filtered to get rid of the precipitated urea, and the filtrate was dropped into a solution of 1 g of CMDA_n (1.7 mmol of NH_2/g) in 10 mL of distilled water while maintaining a constant pH at 10. The solution was stirred for 24 h, and then acetonitrile was removed by rotative evaporation. All of the final polymeric derivatives were purified by dialysis against water and recovered by freeze-drying (23). Finally, CMDA_n - GdDTPA^- was prepared as already described (23) from CMDA_n -DTPA and $\text{GdCl}_3 \cdot 6\text{H}_2\text{O}$, at pH 6.5 using a $\text{Gd}^{3+}/\text{DTPA}$ molar ratio of 1.

Physicochemical Methods. The DTPA contents of the various conjugates were determined by a reverse complexometric method using EDTA as described elsewhere (23). M_n and M_w were determined by size exclusion chromatography coupled to low angle laser light scattering (SEC-LALLS). The LALLS detector was a Chromatix KMX6 (Milton Roy, Riviera Beach, FL) and the refractometric detector, a Waters R 410 model (Milford, MA). The two SEC columns were a TSK PW G 4 000 and a TSK PW G 6 000 (Touzart et Matignon, Courtaboeuf, France; 7.8 mm $\times$ 30 cm) and were connected in series. The eluent was a 0.15 M NaNO_3 water solution.

The polymer hydrodynamic size distribution profiles were determined by high performance size exclusion chromatography (HP-SEC). The experiments were carried out with a system equipped with a refractometric detector (Waters R 410) and with a Lichrosorb Diol Si 300 column (Merck-Clévenot, Chelles, France; 1 cm $\times$ 30 cm). The eluent was 0.05 M phosphate buffer, pH 7.2.

The values of longitudinal (r_1) and transversal (r_2) relaxivity were measured at 20 MHz and 37 °C with a Minispec PC 20 (Bruker, Karlsruhe, Germany). The NMRD (nuclear magnetic relaxation dispersion) profiles that represent the dependence of longitudinal relaxivity on magnetic field were recorded at 37 °C, between 0.1 and 50 MHz with an IBM Research field cycling relaxometer using an inversion recovery pulse sequence (Mons University, Belgium).

Evaluation of the *in Vivo* Toxicity and of the Urinary Excretion. LD_{50} experiments were carried out on mice (SWISS OF1, Iffa Credo). A water solution of each paramagnetic conjugate was intravenously injected in animals at a rate of 2 mL/min. All of the administered solutions had the same gadolinium concentration (0.1 M). The LD_{50} values were determined by increasing the administered solution volume according a geometric progression and keeping the value smaller than 50 mL/kg. The final value of LD_{50} was calculated by the method of Reed and Muench (25) from the results obtained for five animals per injected dose.

Urinary excretion was determined on anesthetized Wistar rats and New Zealand rabbits after the injection of a 0.1 M gadolinium solution (0.05 mmol Gd/kg). The urine was regularly removed by bladder catheterization, and the gadolinium content of the total volume collected after 24 h and 4 h, respectively, on rats and rabbits, was determined.

RESULTS AND DISCUSSION

Polymer Chemical Structure. The synthesized CMD sample contained 0.6 mol of COONa/mol of glucose

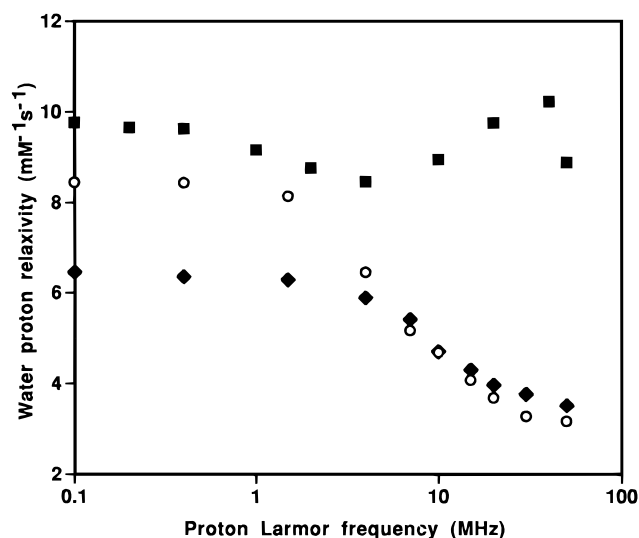


Figure 2. NMRD profiles of water proton molecules for GdDTPA²⁻ (◆), GdDOTA⁻ (○), and CMDA₂-GdDTPA⁻ with 0.60 mmol of GdDTPA/g of CMDA₂-DTPA (■). Profiles obtained with a field cycling relaxometer at 37 °C.

unit. Different CMDA_n derivatives with linear diamines having between 2 and 6 carbons (NH₂(CH₂)_nNH₂, 2 ≤ *n* ≤ 6) were prepared and whatever the *n* value, the different polymers had similar amine and residual carboxylate contents, that is 0.3 mol of NH₂/mol of glucose unit and 0.3 mol of unsubstituted COONa/mol of glucose unit.

The various CMDA_n-DTPA conjugates prepared from DTPAba by the first method (see Experimental Part) had about the same DTPA content, between 0.5 and 0.6 mmol of DTPA/g, whatever the length of the spacer arm. The DTPA content of CMDA_n-DTPA synthesized by the second method using DTPAba was about 0.7 mmol DTPA/g, i.e. slightly higher than those of the derivatives prepared by the first method. The method using DCC and HONSu led to the highly substituted CMDA_n-DTPA sample with about 0.8 mmol DTPA/g. As already noted (23) these values of DTPA contents correspond only to the dextran-monoamidified DTPA molecules since they were determined by a EDTA back titration procedure.

Effect of Polymer on Relaxivity: Comparison between the NMRD Profiles of Molecular and Macromolecular Complexes. Figure 2 shows the NMRD profiles (*r*₁), between 0.1 and 50 MHz, of two molecular complexes GdDTPA²⁻ and GdDOTA⁻ and of one macromolecular complex, CMDA₂-GdDTPA⁻ (0.60 mmol of DTPA/g of CMDA₂-DTPA). For the whole range of frequencies, it is observed that CMDA₂-GdDTPA⁻ *r*₁ relaxivity is greater than that of GdDTPA²⁻. Moreover, while between 1 and 50 MHz, relaxivity of GdDTPA²⁻ regularly decreases from 6.3 to 3.5 mM⁻¹ s⁻¹, that of the polymer decreases from 9.8 to 8.5 mM⁻¹ s⁻¹ between 1 and 6 MHz and then increases up to a maximum of 10.2 mM⁻¹ s⁻¹ for a frequency of 40 MHz. Thus at 20 MHz, an usual frequency of imaging, CMDA₂-GdDTPA⁻ relaxivity is 2.5 times as great as that of GdDTPA²⁻, when at low frequency, below 2 MHz, polymer relaxivity is only 1.5 times as great as that of GdDTPA²⁻. The increase in relaxivity of paramagnetic agents (proton relaxation enhancement, PRE) upon fixation to macromolecular species has already been reported and explained in other works (4, 10, 11) and mainly results from the increase in the rotational correlation time of the paramagnetic metal chelate when bound to a slowly tumbling macromolecule.

Table 1. Relaxivity Values of Various CMDA_n-GdDTPA⁻ Conjugates as a Function of the Spacer Arm Length -(CH₂)_n-

<i>n</i>	DTPA content, mmol/g of CMDA _n -DTPA	<i>r</i> ₁ , ^b mM ⁻¹ s ⁻¹	<i>r</i> ₂ , ^b mM ⁻¹ s ⁻¹
2	0.63	9.4	11.1
3	0.51	9.5	10.9
4	0.59	9.4	10.9
6	0.62	8.9	10.8

^a The CMDA_n-DTPA samples were synthesized according to the first DTPAba method (see Experimental Part). ^b Longitudinal (*r*₁) and transversal (*r*₂) relaxivity values were measured at 20 MHz, 37 °C.

Table 2. Longitudinal (*r*₁) and Transversal (*r*₂) Relaxivity Values of CMDA_n-GdDTPA⁻ Samples of Various Molecular Masses

sample ^a number	DTPA content, mmol/g of CMDA _n -DTPA	<i>M</i> _n , ^b kg/mol	<i>M</i> _w , ^b kg/mol	<i>r</i> ₁ , ^c mM ⁻¹ s ⁻¹	<i>r</i> ₂ , ^c mM ⁻¹ s ⁻¹
1	0.60	224	865	10.4	12.0
2	0.76	166	332	10.1	10.7
3	0.62	183	706	9.3	11.2
4	0.66	154	348	9.9	12.0

^a Samples 1–4 were synthesized as described in the text. Samples 1 and 2 have a spacer arm with *n* = 2 and samples 3 and 4 a spacer arm with *n* = 6. ^b *M*_n and *M*_w were measured by SEC-LALLS. ^c Relaxivity values were measured at 20 MHz, 37 °C.

On the other hand, it is known (26) that between 0.1 and 1 MHz, relaxivity decreases with the paramagnetic element symmetry. *r*₁ relaxivity of GdDOTA⁻ is thus higher than that of GdDTPA²⁻ in this frequency range (Figure 2). As symmetry of GdDTPA²⁻ decreases when it is bound to dextran, relaxivity of CMDA_n-GdDTPA⁻ below 1 MHz should be lower than that of free paramagnetic metal chelate. However, an opposite result is seen in Figure 2. Such a result was also obtained at low frequencies (≤0.4 MHz) by Aime (27) who observed a greater relaxivity for a complex of Gd³⁺ with monoamidified DOTA than for GdDOTA⁻. The conclusion was that the increase in the ligand molecular mass, resulting from the amidification of one of the four carboxylic functions, compensated for the lost of symmetry provoked by DOTA amidification.

Effect of the Spacer Arm Length on Relaxivity. As already mentioned (11), the PRE observed when paramagnetic species are bound to macromolecules depends on how rigidly the chelate is attached to the macromolecule. Thus, as increasing the length of the spacer arm (-HN(CH₂)_nNH-) leads to an increase in the motional freedom of the paramagnetic element, an effect of this increase on *r*₁ and *r*₂ relaxivity values was expected. However, whatever the length of the spacer arm, the various CMDA_n-GdDTPA⁻ samples were characterized by similar *r*₁ and *r*₂ values (Table 1) which means that the motional freedom of the paramagnetic species resulting in the spacer arm motions is not a dominating factor in the relaxation phenomena. As already proposed by Meyer et al. (29), it can thus be concluded that, since it is known that in dextran paramagnetic derivatives the rapid rotation of the glucose units within the polymer plays a significant role in the relaxation phenomena, the chemical modification of the dextran backbone results in a greater effect on relaxivity than the lengthening of the spacer arm.

Influence of the CMDA_n-GdDTPA⁻ Molecular Masses and Sizes on Relaxivity. Table 2 shows the *M*_n and *M*_w molecular masses of several CMDA_n-DTPA

Table 3. Values of LD₅₀ and of Urinary Excretion for Two CMDA_n-GdDTPA⁻ Samples^a

<i>n</i> value	DTPA content mmol/g of CMDA _n -DTPA	$\overline{M}_n$, kg/mol	LD ₅₀ , mmol of Gd/kg	urinary excretion after 4 h (rats), %	urinary excretion after 24 h (rabbits), %
2	0.74	132	2.2	22.7 ± 3.0	20.3 ± 5.4
6	0.68	154	2.4	15.0 ± 1.4	18.6 ± 0.4

^a LD₅₀ experiments were carried out with mice and urinary excretion, with rats and rabbits, under conditions described in the Experimental Part. LD₅₀ of free GdDTPA²⁻ under the same conditions was 5.8 mmol of Gd/kg. The results given for urinary excretion are relative to the total administered dose.

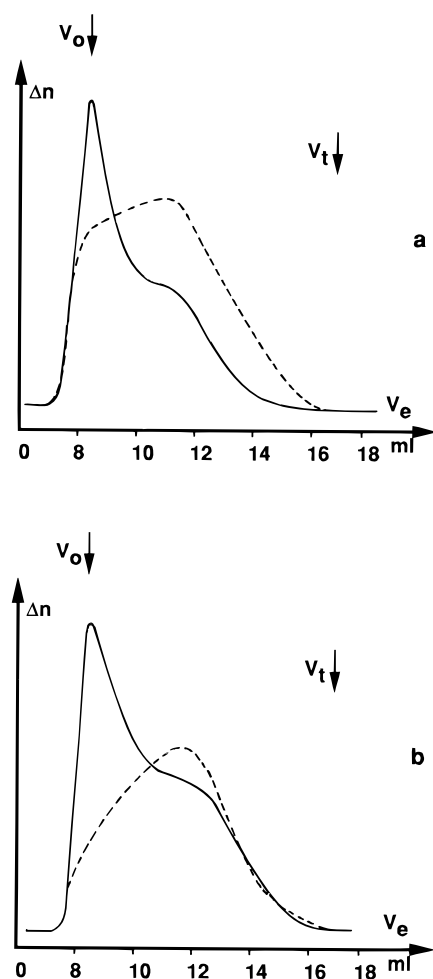


Figure 3. HP-SEC elution profiles of CMDA_n-DTPA samples 1–4 (see Table 2). (a) Samples 1 (—) and 2 (---). (b) Samples 3 (—) and 4 (---) on a Si 300 diol column. Eluent: 0.05 M phosphate buffer, pH 7.2. Refractometric detection. Flow rate: 0.7 mL/min.

conjugates prepared with diaminoethane ($n = 2$, samples 1 and 2) and with diaminoethane ($n = 6$, samples 3 and 4). Sample 2 was synthesized by means of DCC and HONSu, and the three other ones were obtained by using DTPAba. Samples 1 and 3 were prepared following method 1 and sample 4 following method 2 (see Experimental Part). As it is seen in Table 2, sample 1 has $\overline{M}_n$ and $\overline{M}_w$ values greater than sample 2 does, and the $\overline{M}_n$ and $\overline{M}_w$ values of sample 3 are greater than those of sample 4. These results are corroborated by those obtained from the HP-SEC analyses (Figure 3) where it can be seen that the proportions of the elution peaks in the void volume are less large for samples 2 and 4 than for samples 1 and 3. Consequently, samples 2 and 4 have, in average, hydrodynamic sizes smaller than those of samples 1 and 3. The CMDA_n-DTPA molecular sizes and masses ($\overline{M}_n$, $\overline{M}_w$) are too great to only result from

the coupling of DTPA to CMDA_n. In fact, whatever the CMDA_n-DTPA conjugate and the spacer arm length, $\overline{M}_n$ and $\overline{M}_w$ are respectively higher than the theoretical ones which can be calculated for CMDA_n-DTPA polymers with the same chemical composition. These theoretical $\overline{M}_n$ and $\overline{M}_w$ are, respectively, 95 and 142 kg/mol. The difference between the experimental and calculated values thus results from some cross-linking reactions which can occur during CMDA_n-DTPA synthesis to a more or less large extent according to the procedure employed. The cross-linking mechanism results from the reaction between one DTPAba molecule or one DTPA molecule polyactivated by DCC and HONSu, with at least two amine functions carried by different CMDA chains. According to the molecular masses and HP-SEC profiles, the fraction of dextran-diamidified DTPA is smaller for CMDA_n-DTPA samples 2 and 4 than for samples 1 and 3, and taking into account the absolute values of $\overline{M}_n$ and $\overline{M}_w$ for the last ones, it is clear that those samples contain cross-linked fractions, i.e. large molecular size fractions. However, relaxivity of these conjugates is quite similar to that of the smaller molecular size samples (2 and 4) which means that relaxivity of GdDTPA linked to high molecular mass chains must be of the same order as that of GdDTPA bound to smaller molecular size chains.

For molecular complexes with molecular masses between 400 and 900 g/mol, it was shown (28) that longitudinal relaxivity at 20 MHz regularly increased with molecular mass. On the other hand when a paramagnetic element is bound to a macromolecule, its relaxivity is also increased. In all of the cases, the improvement of relaxivity is partially assigned to the slowing down of the paramagnetic agent rotation motion because of the increase in the size of the whole molecule. Nevertheless, a stability of the relaxivity values was observed for the CMDA_n-DTPA samples while their molecular masses increased. Similar observations were done by Spanoghe et al. (10) on polylysines labeled with GdDTPA with molecular masses between 7.7 and 288 kg/mol. The conclusion was that, while binding the molecular paramagnetic agent to a slow tumbling macromolecule results in an increase of its rotational correlation time and consequently of relaxivity, there is no interest in further lengthening the polymer chain because the electron spin relaxation time and the exchange relaxation time are sufficiently short to preclude any additional increase in correlation time (17).

In Vivo Toxicity and Urinary Excretion of Macromolecular Paramagnetic Conjugates. The LD₅₀ values of two conjugates differing by the spacer arm length ($n = 2$ and $n = 6$) are shown in Table 3. These values are very close to each other (2.2–2.4 mmol of Gd/kg) and smaller than that of the molecular complex (5.8 mmol of Gd/kg under the same conditions). On the other hand another CMDA₆-GdDTPA⁻ conjugate with 0.3 mmol of DTPA/g of polymer, prepared from dextran 20

(M_w about 20 kg/mol) instead of dextran 40, exhibited a LD_{50} of 2.9 mmol/kg (29) which means that in this type of conjugate, neither the molecular mass nor the spacer arm length have an effect on the conjugate toxicity. Furthermore, the LD_{50} values shown in Table 3, are higher than those found for other dextran-GdDTPA⁻ samples described by Meyer et al. (29). These samples were prepared by procedures involving dextran periodate oxidation or dextran epichlorohydrin activation, and these methods do not introduce carboxylate functions on the dextran backbone. The difference in toxicity between the conjugates described in this paper and those of Meyer et al. (29) can thus be ascribed to the difference in the chemical structure of the polymeric backbones and probably to the presence of an excess of free carboxylate functions on the conjugates of this paper.

Finally, as shown in Table 3, the urinary excretion of the two conjugates ($n = 2$ and $n = 6$) is relatively low, respectively, 22.7 and 15% of the administered dose for rats after 4 h and 20.3 and 18.6% for rabbits after 24 h. These results have to be compared to those obtained by Bligh et al. [19] who found that 2 h after a GdDTPA²⁻ injection in rats, the urinary excretion was about 90% of the injected dose. This comparison shows that binding GdDTPA²⁻ to dextran leads to a significant decrease in the urinary excretion of gadolinium as a consequence of the increase in the paramagnetic complex size and as expected the lower values were obtained with the higher molecular mass conjugates.

In conclusion, it was shown that dextran-linked DTPA could be complexed with Gd³⁺ and that the r_1 and r_2 relaxivity values of resulting complexes, between 0.1 and 50 MHz, was not influenced by the length of the spacer arm (between two and six methylene groups) inserted between DTPA and the polysaccharidic backbone. On the other hand, it was also shown that coupling GdDTPA²⁻ to a polymer such as dextran 40 had a great influence on relaxivity as for the macromolecular complexes, r_1 and r_2 at 20 MHz were 2.5 times as great as those of free GdDTPA²⁻. However, in the range of the studied high molecular masses (M_n between 166 and 224 kg/mol) no increase in relaxivity was observed when increasing the conjugate molecular mass. Anyway, even these polymeric conjugates carrying some diamidified DTPA ligands (resulting from cross-linking reactions) that, according to Sherry's work (13), have less affinity for Gd³⁺ than monoamidified DTPA, exhibit interesting paramagnetic properties. As the urinary excretion is greatly decreased compared to that of free GdDTPA²⁻, which must lead to a longer intravascular persistence, these conjugates can be regarded as potential contrast agents which could be used in a low dose in magnetic resonance imaging.

ACKNOWLEDGMENT

This work was supported by a CIFRE grant (Société Guerbet-GCA, Aulnay-sous-Bois, France, and ANRT, France). We thank Mrs. T. Geoffroy for her helpful technical assistance in LALLS analyses. We also thank Prof. R. N. Muller (Mons University, Belgium) for his insight concerning the relaxivity values.

LITERATURE CITED

- (1) Bloch, F. (1946) Nuclear introduction. *Phys. Rev.* 70, 460.
- (2) Solomon, I. (1955) Relaxation processes in a system of two spins. *Phys. Rev.* 99, 559.
- (3) Bloembergen, N., Purcell, E. M., and Pound, R. V. (1948) Relaxation effects in nuclear magnetic resonance absorption. *Phys. Rev.* 73, 678.
- (4) Lauffer, R. B. (1987) Paramagnetic metal complexes as water proton relaxation agents for NMR imaging: theory and design. *Chem. Rev.* 87, 901.
- (5) Wang, X., Jin, T., Comblin, V., Lopez-Mut, A., Merciny, E., and Desreux, J. F. (1992) A kinetic investigation of the lanthanide DOTA chelates. Stability and rate of formation and of dissociation of a macrocyclic gadolinium(III) polyazapolycarboxylic MRI contrast agent. *Inorg. Chem.* 31, 1095.
- (6) Desreux, J. F. (1980) Nuclear magnetic resonance spectroscopy of lanthanide complexes with a tetraacetic tetraaza macrocycle. Unusual conformation properties. *Inorg. Chem.* 19, 1319.
- (7) Niendorf, H. P., Alhassan, A., Haustein, J., Clauss, W., and Cornelius, I. (1993) Safety and risk of gadolinium-DTPA: extended clinical experience after more than 5,000,000 applications. *Adv. MRI Contrast* 2, 12.
- (8) Schmiedl, U., Moseley, M. E., Ogan, M. D., Chew, W. D., and Brasch, R. C. (1987) Comparison of initial biodistribution patterns of Gd-DTPA and albumin-(Gd-DTPA) using rapid spin echo MR imaging. *J. Comput. Assist. Tomogr.* 11, 306.
- (9) Schuhmann-Giampieri, G., Schmitt-Willich, H., Frenzel, T., Press, W.-R., and Weinmann, H.-J. (1991) In vivo and in vitro evaluation of Gd-DTPA-polylysine as a macromolecular contrast agent for resonance magnetic imaging. *Invest. Radiol.* 26, 969.
- (10) Spanoghe, M., Lanens, D., Dommissie, R., Van Der Linden, A., and Alderweireldt, F. (1992) Proton relaxation enhancement by means of serum albumin and poly-L-lysine labeled with DTPA-Gd³⁺: relaxivities as a function of molecular weight and conjugation efficiency. *Magn. Reson. Imaging* 10, 913.
- (11) Lauffer, R. B., and Brady, T. J. (1985) Preparation and water relaxation properties of proteins labeled with paramagnetic metal chelates. *Magn. Reson. Imaging* 3, 11.
- (12) Lauffer, R. B., Brady, T. J., Brown III, R. D., Baglin, C., and Koenig, S. H. (1986) 1/T1 NMRD profiles of solutions of Mn²⁺ and Gd³⁺ protein-chelate conjugates. *Magn. Reson. Med.* 3, 541.
- (13) Sherry, A. D., Cacheris, W. P., and Kuan, K.-T. (1988) Stability constants for Gd³⁺ binding to model DTPA-conjugates and DTPA-proteins: implications for their use as magnetic resonance contrast agents. *Magn. Reson. Med.* 8, 180.
- (14) Paaanen, H., Reisto, T., Hemmila, I., Komu, M., Niemi, P., and Kormano, M. (1990) Proton relaxation enhancement of albumin, immunoglobulin G and fibrinogen labeled with Gd-DTPA. *Magn. Reson. Med.* 13, 38.
- (15) Niemi, P., Reisto, T., Hemmila, I., and Kormano, M. (1991) Magnetic field dependence of longitudinal relaxation rates of solutions of various protein-gadolinium³⁺ chelate conjugates. *Invest. Radiol.* 26, 820.
- (16) Unger, E. C., Totty, W. G., Neufeld, D. M., Otsuka, F. L., Murphy, W. A., Welch, M. S., Connett, J. M., and Philpott, G. W. (1985) Magnetic resonance imaging using gadolinium labeled monoclonal antibody. *Invest. Radiol.* 20, 693.
- (17) Armitage, F. E., Richardson, D. E., and Li, K. C. P. (1990) Polymeric contrast agents for Magnetic Resonance Imaging: Synthesis and characterization of gadolinium diethylenetriaminepentaacetic acid conjugated to polysaccharides. *Bioconjugate Chem.* 1, 365.
- (18) Wang, S. C., Wikstroem, M. G., White, D. L., Klaveness, J., Holtz, E., Rongved, P., Moseley, M. E., Michael, E., and Brasch, R. C. (1990) Evaluation of the gadolinium-DTPA-labeled dextran as an intravascular MR contrast agent: imaging characteristics in normal rat tissues. *Radiology* 175, 483.
- (19) Bligh, S. W. A., Harding, C. T., Sadler, P. J., Bulman, R. A., Bydder, G. M., Pennock, J. M., Kelly, J. D., Latham, I. A., and Marriott, J. A. (1991) Use of paramagnetic chelated derivatives of polysaccharides and spin-labeled polysaccharides as contrast agents in magnetic resonance imaging. *Magn. Reson. Med.* 17, 516.
- (20) Rongved, P., and Klaveness, J. (1991) Water soluble polysaccharides as carriers of paramagnetic contrast agents for magnetic resonance imaging: Synthesis and relaxation properties. *Carbohydr. Res.* 214, 315.

- (21) Brash, R. C. (1991) Rationale and applications for macromolecular Gd-based contrast agent. *Magn. Reson. Med.* 22, 282.
- (22) Dellacherie, É. (1996) Polysaccharides in oxygen-carrier blood substitutes. In *Polysaccharides in Medicinal Applications*, (S. Dumitriu, Ed.) Marcel Dekker, Inc., New York, p 525.
- (23) Rebizak, R., Schaefer, M., and Dellacherie, E. *Bioconjugate Chem.*, in press.
- (24) Eckelman, W. C., Karesh, S. M., and Reba, R. C. (1975) New compounds: fatty acid and long chain hydrocarbon derivatives containing a strong chelating agent. *J. Pharm. Sci.* 64, 704.
- (25) Reed, L., Muench, H. (1938) A simple method of estimating fifty per cent endpoints. *Am. J. Hyg.* 27, 493.
- (26) Sherry, A. D., Brown III, R. D., Gerald, C., Koenig, S. H., Kuan, K.-T., and Spiller, M. (1989) Synthesis and characterization of the gadolinium(3+) complex of DOTA-Propylamide: a model DOTA-protein conjugate. *Inorg. Chem.* 28, 620.
- (27) Aime, S., Botta, M., Ermondi, G., Fidelli, F., and Uggeri, F. (1992) Synthesis and NMRD studies of Gd³⁺ complexes of macrocyclic polyaminopolycarboxylic ligands bearing β -benzyloxy- α -propionic residues. *Inorg. Chem.* 31, 1100.
- (28) Aime, S., Anelli, P. L., Botta, M., Fedelli, F., Grandi, M., Paoli, P., and Uggeri, F. (1992) Synthesis, characterization and 1/T1 NMRD profiles of gadolinium(III) complexes of monoamide derivatives of DOTA-like ligands. X-ray structure of the 10-[2-[[2-hydroxy-1-(hydroxymethyl)ethyl]amino]-1-[(phenylmethoxymethyl)-2-oxo-ethyl]-1,4,7,10-tetraazacyclododecane-1,4,7-triacetic acid-gadolinium(III) complex. *Inorg. Chem.* 31, 2422.
- (29) Meyer, D., Schaefer, M., Chambon, C., and Beauté, S. (1994) Paramagnetic dextrans as magnetic blood pool tracers. *Invest. Radiol.* 29, S90.

BC9701499

Streptavidin in Antibody Pretargeting. Comparison of a Recombinant Streptavidin with Two Streptavidin Mutant Proteins and Two Commercially Available Streptavidin Proteins

D. Scott Wilbur,^{*,†} Patrick S. Stayton,[‡] Richard To,[‡] Kent R. Buhler,[§] Lisa A. Klumb,[‡] Donald K. Hamlin,[†] James E. Stray,[§] and Robert L. Vessella[§]

Departments of Radiation Oncology, Urology, and Bioengineering, University of Washington, Seattle, Washington 98195. Received August 11, 1997; Revised Manuscript Received October 10, 1997[®]

In this investigation, a comparison of wild type recombinant streptavidin (r-SAv) with two genetically engineered mutant r-SAv proteins was undertaken. The investigation also included a comparison of the r-SAv with two streptavidin (SAv) proteins from commercial sources. In vitro characterization of the SAv proteins was conducted by HPLC, SDS–PAGE, IEF, and electrospray mass spectral analyses. All SAv proteins studied appeared to be a single species by size exclusion chromatography (HPLC) and SDS–PAGE analyses, but multiple species were noted in the IEF and MS analyses. In vivo comparisons of the SAv proteins were accomplished with dual isotope-labeled SAv in athymic mice. In an initial experiment, tissue localization of r-[¹³¹I]SAv directly radiolabeled using chloramine-T was compared with r-SAv radiolabeled with the *N*-hydroxysuccinimidyl *p*-iodobenzoate conjugate ([¹²⁵I]-PIB), a radioiodination reagent that has been shown to result in iodine-labeled proteins which are stable to in vivo deiodination. The data obtained indicated that there is little difference in the distribution (except kidney localization) when r-SAv labeled by the two methods. Data obtained from comparison of r-[¹³¹I]SAv with a disulfide-stabilized r-SAv mutant (r-SAv-H127C), a C-terminal cysteine-containing r-SAv mutant (r-[¹²⁵I]SAv-S139C), and two ¹²⁵I-labeled SAv proteins obtained from commercial sources indicated that their distributions were quite similar, except the kidney concentrations were generally lower than that of r-[¹³¹I]SAv. On the basis of the similar distributions of the SAv proteins studied, it appears that the r-SAv mutants may be interchanged for the (wild type) r-SAv in pretargeting studies. Further, the similarity of distributions with two commercially available SAv proteins suggests that the results obtained in our studies and those of other groups may be directly compared (with consideration of animal model, sacrifice time, etc.).

INTRODUCTION

A monoclonal antibody-based method of delivering radionuclides to cancer tissue for diagnosis and/or therapy termed tumor "pretargeting" is under investigation by a number of research groups (1–14). In tumor pretargeting, an antibody conjugate is injected into a patient and allowed to localize at the tumor, and then a radiolabeled molecule which binds avidly with the antibody conjugate is administered in a "two-step" or "three-step" procedure (3). Due to the very high affinity of biotin for the proteins streptavidin (SAv¹) and avidin (i.e. 10^{13} – 10^{15} M⁻¹) (15, 16), combinations of these molecules with monoclonal antibodies have formed the basis for a large number of

the investigations conducted in pretargeting research. In addition to the high affinity of biotin for SAv and avidin, the biotin/(strept)avidin system is particularly attractive for application to cancer therapy as SAv and avidin are tetrameric proteins which have the capacity to bind four molecules of biotin (16). This multi-biotin binding nature of SAv and avidin is of particular interest as it provides the potential for increasing the quantity of radioactivity localized on cancer cells. Thus, a major focus of our research effort is directed at developing a method for using the multi-biotin binding capacity of SAv to maximize the amount of radioactivity delivered to cancer cells in vivo. In the approach under investigation, the first step is to pretarget a biotinylated antibody [or antibody–(strept)avidin conjugate] to a tumor, and then alternate administrations of SAv and a radiolabeled cross-linking biotin molecule are given to increase the amount of radioactivity localized at the tumors (14).

A key component of our investigations of antibody-based tumor pretargeting is the biotin binding protein SAv. Although SAv is similar in structure to avidin (16), it has a longer half-life in blood than avidin (17). The longer blood half-life is primarily due to the fact that SAv is not glycosylated (17–19). Interaction of glycosylation sites on proteins with asialoglycoprotein receptors on liver cells results in rapid clearance from blood (20). Importantly, it has been demonstrated that the longer blood half-life of SAv results in higher concentrations in tumor xenografts implanted in athymic mice (14, 18).

* Address correspondence to D. Scott Wilbur, Ph.D., Department of Radiation Oncology, University of Washington, 2121 N. 35th St., Seattle, WA 98103-9103. Phone: (206) 685-3085. Fax: (206) 685-9630. E-mail: dswilbur@u.washington.edu.

[†] Department of Radiation Oncology.

[‡] Department of Bioengineering.

[§] Department of Urology.

[®] Abstract published in *Advance ACS Abstracts*, December 15, 1997.

¹ Abbreviations: PIB, *p*-iodobenzoate; ChT, chloramine-T; cpm, counts per minute; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; ESMS, electrospray mass spectrometry; NEM, *N*-ethylmaleimide; % ID/g, percent injected dose per gram; PMSF, phenylmethanesulfonyl fluoride; r-SAv(s), recombinant streptavidin(s); rt, room temperature; SAv(s), streptavidin(s); T/B, tissue-to-blood.

Deglycosylation of avidin produces a tetrameric biotin binding protein which is similar to SAV in its in vivo clearance (19); however, we chose to use a recombinant SAV (r-SAV) in our studies. The r-SAV employed is obtained from bacterial (*Escherichia coli*) expression and is primarily a single protein with a discrete molecular size (21). SAV obtained through recombinant molecular biology methods was chosen over SAV obtained from commercial sources for use in our studies as production by this method permits specific alterations in the SAV molecule by site-directed mutagenesis (21). In fact, site-directed mutagenesis of r-SAV has allowed for the production of unique r-SAV mutants which may have broad application in biotin/(strept)avidin technology (22–24), as well as in tumor pretargeting. For example, one of the r-SAV mutants produced, r-SAV-H127C,² has two intersubunit disulfide bonds that were designed to provide stabilization of the tetramer from dissociation in vivo. Another r-SAV mutant of interest in our pretargeting studies, r-SAV-S139C,² was obtained by substitution of a cysteine residue for the serine residue at the C-terminal end of the polypeptide subunit. The r-SAV-S139C mutant was designed to allow site-specific conjugations at a location in the molecule that is not adjacent to the biotin binding pockets.

Many other investigators have reported the use of SAV in tumor pretargeting (4, 7, 8, 11–13, 25–28). In reviewing the literature, we were surprised to note the large variation in the biodistribution data of radiolabeled SAV which had been previously reported, with the most striking differences being in kidney localization. Indeed, reported kidney concentrations have ranged from 1% injected dose per gram (% ID/g) to over 100% ID/g. The reported wide variation in the in vivo characteristics, particularly kidney localization of SAV used in tumor pretargeting studies, prompted us to compare the in vitro and in vivo characteristics of two SAV mutants, r-SAV-H127C and r-SAV-S139C, with those of the r-SAV presently used in our pretargeting studies. Since previously reported studies were conducted with SAV obtained from several different commercial sources, we chose to include two commercially available SAV proteins³ in the comparison studies. In vitro comparison of the SAV proteins was obtained by conducting HPLC, SDS–PAGE, IEF, and mass spectrometric analyses. In vivo comparison of radiolabeled SAV was obtained in dual isotope experiments. Direct comparison of sets of biodistribution data was obtained by co-injection of r-[¹³¹I]SAV with each of the other radiolabeled ([¹²⁵I]) SAV proteins in athymic mice.⁴ The results of our comparison of SAV proteins are described herein.

EXPERIMENTAL PROCEDURES

General. All chemicals from commercial sources were analytical grade or better and were used without further

purification. Chloramine-T (ChT) was obtained from Sigma Chemical Co. (St. Louis, MO). SAV proteins were obtained from Boehringer Mannheim (CS1,³ lot 133816721, Indianapolis, IN) and from CALBIOCHEM (CS2,³ lot B12651, La Jolla, CA). Reconstitution of the commercial SAV proteins in water was carried out as recommended by the package inserts.

The radioisotopes, Na¹²⁵I and Na¹³¹I, were purchased from NEN/Dupont (Billerica, MA) as high-concentration/high-specific activity radioiodide in 0.1 N NaOH. Measurement of ¹²⁵I and ¹³¹I was accomplished on a Capintec CRC-15R or a Capintec CRC-6A Radioisotope Calibrator. Dual isotope counting of tissue samples was accomplished in a LKB 1282 γ counter with the following window settings: channels 35–102 (20–90 keV) and channels 165–185 (300–450 keV) when ¹²⁵I and ¹³¹I were counted together. When dual isotope counting was conducted, all cpm measurements of ¹²⁵I were corrected for spillover (13.5%) from the ¹³¹I emissions.

Recombinant Protein Gene Design, Expression, and Purification. The design of the recombinant core SAV gene and the construction of the r-SAV-H127C mutant have been described previously (29). The r-SAV-S139C mutant was created by cassette mutagenesis. The cassette for the r-SAV-S139C gene was constructed from oligonucleotides spanning the 5' *Mlu*I restriction enzyme site and the 3' *Hind*III site. The sequence for the last serine residue, S139, was changed from TCC to TGC, thus encoding a cysteine residue. The S139C cassette was annealed and ligated into a pUC18 construct containing the truncated r-SAV at the *Mlu*I/*Hind*III sites. Colonies containing the *Mlu*I/*Hind*III insert were selected and sequenced to confirm the production of the S139C coding sequence. The mutant r-SAV-S139C sequence was excised from the pUC18 vector by *Nde*I and *Hind*III digestion and ligated into the pET21a T7 expression vector (Novagen, Inc., Madison, WI).

The r-SAV proteins were expressed and purified as previously described (29). Briefly, r-SAV was initially isolated as insoluble inclusion bodies and dissolved in 6 M guanidine·HCl. The guanidine·HCl also contained 10 mM dithiothreitol (DTT) when the cysteine mutants were dissolved. r-SAV was subsequently refolded in 0.05 M Tris·HCl (pH 7.6), 0.1 M NaCl, 0.005 M EDTA, and 0.1 mM phenylmethanesulfonyl fluoride (PMSF). r-SAV-S139C was refolded in the presence of 1 mM DTT to prevent disulfide bond formation. Refolded streptavidin (100 mg or less) was exchanged into binding buffer (pH 11, 50 mM Na₂CO₃ and 500 mM NaCl) and concentrated to a volume of about 30 mL using an ultrafiltration cell (Amicon, Beverly, MA). The concentrated protein solution was applied to a 20–25 mL iminobiotin–agarose column (Pierce, Rockford, IL) that had been pre-equilibrated with at least 125 mL of binding buffer. After washing with an additional 100–150 mL of binding buffer, the protein was eluted with a low-pH aqueous solution (pH 3, 100 mM HOAc). The flow-through was collected in 10 mL fractions, and the streptavidin content was checked by UV. Those fractions containing protein (50–60 mL) were pooled, immediately exchanged into a phosphate buffer (pH 7, 50 mM sodium phosphate and 100 mM NaCl), and concentrated via ultrafiltration. Recovery yields of 70–90% of the initial amount of refolded protein were obtained.

Protein Characterization. Protein concentrations in aqueous solution were estimated using the Beer's law equation, wherein the measured absorbance in water at

² The notations for site-directed streptavidin mutants studied, r-SAV-H127C and r-SAV-S139C, include the amino acid number (127 or 139; based on native streptavidin numbering) and the identity of the amino acid substitution [i.e. cysteine (C) for histidine (H) or serine (S)].

³ To limit the extent of the investigation, two commercial sources were chosen to be representative of commercially available streptavidin. Commercial source 1 (CS1) was Boehringer Mannheim (Indianapolis, IN), and commercial source 2 (CS2) was CALBIOCHEM (La Jolla, CA).

⁴ Athymic mice were used to allow correlation of data from this investigation with data obtained in previous and planned studies employing athymic mice bearing human tumor xenografts.

280 nm was divided by 2.56 L/g.⁵ Characterization of all SAV proteins included IEF, SDS-PAGE, HPLC, and protein electrospray mass spectrometry (ESMS) analyses. IEF and SDS-PAGE analyses were conducted using a PhastSystem electrophoresis apparatus (Pharmacia Biotech, Uppsala, Sweden). Conditions used to conduct the IEF are described in the legend of Figure 1. Reducing denatured and nondenatured SDS-PAGE analyses were conducted on 12.5% homogeneous gels (see Supporting Information). SDS-PAGE analyses were also carried out using precast MiniProtein 10 to 20% gradient gels (BioRad Inc., Hercules, CA) with a discontinuous buffer system. Native PAGE was performed by omitting SDS during the heat denaturation, and in the sample application buffer and the gel running buffer. Mass spectral analyses were obtained for all SAV proteins, except the r-SAV-S139C. ESMS of protein samples was performed on an API-III electrospray mass spectrometer (PE Sciex, Thornhill, ON) or a VG Quattro II tandem quadrupole mass spectrometer (VG BioTech, Cheshire, U.K.). Protein concentrations were 20–60 μ M (subunit) after extensive dialysis against distilled water (dialysis membrane MWCO of 10 000), with 0.1% formic acid added just prior to the ESMS. Mass spectra for r-SAV, SAV-CS1, and SAV-CS2 are provided in the Supporting Information. Mass spectral data have been previously published for r-SAV-H127C (29).

HPLC separations of nonlabeled SAV proteins were conducted on a system consisting of a Hewlett-Packard 1050 Multiple Wavelength Detector (280 nm), an isocratic pump, and a TSK-GEL G3000SW size exclusion column (7.5 mm $\times$ 600 mm, 10 μ m; TosoHaas, Montgomeryville, PA). The mobile phase was an aqueous solution containing 50 mM potassium phosphate (pH 6.8), 300 mM NaCl, 1 mM EDTA, and 1 mM NaN₃. A flow rate of 0.75 mL/min was used. The retention times for SAV proteins were approximately 30 min.

HPLC separations of radioiodinated SAV proteins were conducted on a system consisting of two model 306 Gilson pumps with 10SC titanium pump heads, a Gilson model 118 UV-Visible detector (280 nm), a Beckman model 170 radioisotope detector, a Gilson System Interface Module, and an IBM 486 computer with Gilson Unipoint HPLC Controller software. The proteins were separated by size exclusion chromatography on a 300 SW Protein Pak column (8 mm $\times$ 300 mm glass column; 10 μ m silica particle size), eluting with a solvent mixture of 25 mM Tris-HCl, 75 mM NaCl, and 1 mM EDTA at pH 7.2 with a flow rate of 0.70 mL/min. The retention time for the radiolabeled SAV proteins was approximately 15 min using this system. Chromatograms of the SAV variants are provided in the Supporting Information.

Preparation of r-SAV-S139C-NEM. To 100 μ L of r-SAV-S139C (13 mg/mL) was added 6 μ L of a 1 M aqueous DTT solution. After 1 h at rt, the mixture was passed over a Sephadex G-25 (PD-10) column and eluted with 20 mM sodium phosphate at pH 6.8. To a 2 mL aliquot of DTT-treated r-SAV-S139C (0.25 mg/mL) solution was added 100 μ L (50 equiv, 0.64 mg/mL) of a solution containing *N*-ethylmaleimide (NEM) in 20 mM sodium phosphate at pH 6.8. The reaction was allowed to proceed at rt for 30 min before the mixture was divided

into two equal volume fractions and the fractions were placed in separate Centricon-10 microconcentrators (Amicon) that were prewashed with 1 mL of 20 mM sodium phosphate at pH 6.8. The solutions were centrifuged at 3200 rpm for 15 min to concentrate the solutions to a 100 μ L volume. Each Centricon retentate was rinsed four times with 500 μ L of the phosphate buffer, reducing the volume to 100 μ L by centrifugation during each rinse.

General Procedure for Radioiodination of r-SAV Using ChT. A 500–650 μ g quantity of SAV in 40–100 μ L of a 50 mM Tris/50 mM NaCl solution (pH 8.0) was added to a mixture of 25 μ L of 0.5 M sodium phosphate (pH 7.4) and 1 μ L (0.5–0.7 mCi) of Na^{125/131}I in 0.1 N NaOH. To that solution was added 10–20 μ L of a 1 mg/mL solution of ChT in H₂O (final concentration of 0.15 μ g/mL). After 5 min at rt, 1–2 μ L of a 10 mg/mL solution of sodium metabisulfite in H₂O was added to quench the reaction. The radioiodinated SAV was eluted on a NAP-10 column (Sephadex G-25; Pharmacia Biotech) with 0.9% saline to give a 48–93% radiochemical yield of the radiolabeled SAV. The radiochemical purity of the isolated SAV proteins was >98% (by HPLC) in all reactions conducted.

Radioiodination of r-SAV Using [¹²⁵I]PIB-NHS. Synthesis of *N*-hydroxysuccinimidyl 4-(tri-*n*-butylstannyl)benzoate (PSnB-NHS) was accomplished as previously reported (30). The radioiodination was conducted as follows. A 1.0 mCi quantity of Na¹²⁵I in 3 μ L of 0.1 N NaOH was added to a mixture of 50 μ g of PSnB-NHS in 50 μ L of a 1% HOAc solution in MeOH. To that solution was added 10 μ L of a 1 mg/mL solution of NCS in MeOH. After 5 min at rt, 1 μ L of a 10 mg/mL solution of sodium metabisulfite was added to quench the reaction. The solvent was removed under a stream of argon (through a charcoal filter), and the crude residue containing [¹²⁵I]PIB-NHS was used directly in the radiolabeling reaction.

To the vial containing [¹²⁵I]PIB-NHS (1.04 mCi) was added a mixture containing 35 μ L of a 15 mg/mL solution of r-SAV in PBS and 100 μ L of a 0.5 M sodium borate buffer at pH 9.3. After a 5 min reaction time, the mixture was placed on a NAP-10 column and eluted with 0.9% saline. A total of 496 μ Ci (48%) of PIB-labeled SAV was obtained, with a specific activity of 1.23 μ Ci/ μ g, which had a radiochemical purity of >98% by HPLC analysis.

Biodistribution Studies. All animal use and procedures were approved by the University of Washington's Animal Care Committee. Animal care and use was conducted in accordance with the NIH guidelines.⁶ Male athymic mice (BALB/c *nu/nu*), 4–5 weeks of age, were obtained from Simonsen Laboratories (Gilroy, CA). All mice were housed for 1 week in the animal facility prior to beginning the study.

In the biodistribution studies, mixtures of r-[¹³¹I]SAV and ¹²⁵I-labeled r-SAV mutants, or ¹²⁵I-labeled SAV from commercial sources,³ were administered to mice in a total volume of approximately 100 μ L via the lateral tail vein. The actual amount of injectate administered to each animal was determined by weighing the administering syringe pre- and postinjection. Groups of five mice were sacrificed at 4, 24, and 48 h postinjection to obtain tissue distributions. The tissues excised were muscle, lung, kidney, spleen, liver, intestine, neck (including thyroid), and stomach. Blood samples were obtained by cardiac

⁵ The 2.56 L/g value was obtained by dividing the molar absorptivity of SAV (i.e. 136 000 mol L⁻¹ cm⁻¹) by the quantity per mole of SAV (i.e. 53 084 g/mol). The molar absorptivity was obtained by multiplying the SAV molar absorptivity (ϵ_{280}) of 34 000 mol L⁻¹ cm⁻¹ for one subunit (16) by 4 (subunits). The path length of the cell used in the measurements was 1 cm.

⁶ NIH guidelines are described in NIH Publication 86-23, Guide for the Care and Use of Laboratory Animals.

⁷ Studies with r-[¹²⁵I]SAV-S139C have shown that reduction is needed before conjugations will take place.

Table 1. Distribution of Radioactivity at 4, 24, and 48 h Post-Coadministration of [¹²⁵I]PIB-Labeled r-SAv and ChT-Labeled r-[¹²⁵I]SAv in Athymic Mice^a

tissue	4 h ^{b,c}		24 h		48 h	
	¹²⁵ I (PIB)	¹³¹ I (ChT)	¹²⁵ I (PIB)	¹³¹ I (ChT)	¹²⁵ I (PIB)	¹³¹ I (ChT)
blood	9.05 ± 0.50	8.45 ± 0.46	2.86 ± 0.35	2.42 ± 0.33	0.52 ± 0.08	0.53 ± 0.09
muscle	1.76 ± 0.33	1.57 ± 0.30	0.99 ± 0.16	0.90 ± 0.09	0.61 ± 0.18	0.57 ± 0.17
lung	7.28 ± 0.92	6.46 ± 0.75	3.02 ± 0.26	2.66 ± 0.22	1.53 ± 0.28	1.41 ± 0.27
kidney	16.51 ± 1.58 ^e	23.86 ± 2.17 ^e	16.94 ± 2.56 ^e	24.23 ± 2.13 ^e	18.01 ± 2.98	24.20 ± 3.47
spleen	3.80 ± 0.51	3.31 ± 0.39	2.91 ± 0.39	2.54 ± 0.38	2.47 ± 0.41	2.20 ± 0.40
liver	2.82 ± 0.19	2.52 ± 0.15	2.56 ± 0.26	2.29 ± 0.25	2.40 ± 0.20	2.07 ± 0.17
intestine	2.24 ± 0.37	1.95 ± 0.32	1.15 ± 0.16	0.89 ± 0.13	1.07 ± 0.24	0.65 ± 0.12
neck	5.14 ± 0.65	4.78 ± 0.58	3.48 ± 0.50	3.95 ± 1.33	2.06 ± 0.45	4.48 ± 2.74
stomach	2.10 ± 0.48	2.01 ± 0.45	1.49 ± 0.14	1.65 ± 0.41	0.95 ± 0.21	1.58 ± 0.38
urine ^d	29.71 ± 20.69	29.34 ± 19.96	10.82 ± 1.94	10.01 ± 0.99	8.95 ± 1.85	5.85 ± 0.92

^a Values shown are in % ID/g ± standard deviation. ^b Time of sacrifice from injection of radiolabeled SAv. ^c Data were obtained for five mice at each time point; average animal weight, 23.16 ± 1.17 g. Injectate for each animal had 15 μCi per 15 μg of r-SAv labeled with Na¹³¹I/ChT and 18 μCi per 15 μg of r-SAv labeled with [¹²⁵I]PIB in approximately 100 μL of 0.9% sterile saline. ^d Urine was collected by syringe bladder tap after sacrifice. ^e Statistical significance of difference in paired *t* test; *P* < 0.005.

Table 2. Comparison of Radioactivity Present in Blood and Kidney at 4, 24, and 48 h Post-Coadministration of ¹²⁵I-Labeled SAv Protein and r-[¹³¹I]SAv in Athymic Mice^a

distribution	4 h ^{b,c}		24 h		48 h	
	blood	kidney	blood	kidney	blood	kidney
(A)						
[¹²⁵ I]H127C	11.43 ± 0.40	18.92 ± 1.72	3.07 ± 0.27	17.21 ± 1.31	0.93 ± 0.22	20.17 ± 2.70
r-[¹³¹ I]SAv	10.35 ± 0.35	26.43 ± 2.71	2.12 ± 0.23	22.82 ± 1.47	0.58 ± 0.13	26.50 ± 3.21
(B)						
[¹²⁵ I]S139C	10.67 ± 0.30	19.84 ± 1.12	2.30 ± 0.44	19.53 ± 3.69	0.59 ± 0.05	17.10 ± 3.36
r-[¹³¹ I]SAv	9.15 ± 0.37	22.34 ± 1.14	1.90 ± 0.46	23.30 ± 4.60	0.40 ± 0.03	21.31 ± 3.70
(C)						
[¹²⁵ I]CS1	6.65 ± 0.48	18.69 ± 3.46	1.33 ± 0.19	15.68 ± 1.18	0.31 ± 0.06	15.48 ± 3.97
r-[¹³¹ I]SAv	8.38 ± 0.65	19.31 ± 3.23	1.94 ± 0.24	22.09 ± 1.35	0.48 ± 0.09	23.55 ± 5.41
(D)						
[¹²⁵ I]CS2	6.64 ± 0.23	29.23 ± 1.48	1.35 ± 0.14	24.27 ± 4.43	0.34 ± 0.04	20.33 ± 2.30
r-[¹³¹ I]SAv	9.12 ± 0.40	21.65 ± 1.65	2.16 ± 0.31	27.25 ± 4.64	0.54 ± 0.05	26.56 ± 2.78

^a Values shown are in % ID/g ± standard deviation. ^b Time of sacrifice from injection of radiolabeled SAv. ^c Data were obtained for five mice at each time point.

puncture. Urine samples were obtained by syringe bladder tap at the time the tissues were excised. Excised tissues were blotted free of blood, weighed, and counted. Calculations of the percent injected dose (% ID) and percent injected dose per gram (% ID/g) in the tissues were based on internal standards (quadruplicate) containing 1 μL of the injected dose. The total blood volume was estimated to be 8% of body weight for the calculations (31). A spreadsheet program in Excel (Microsoft Corp., Redmond, WA) was used to perform the calculations, including ratios of isotopes in tissue/fluids for the radiolabeled SAv proteins. Statistical analyses were conducted on each tissue in the set of five mice using Excel's *t* test function for two samples assuming equal variances. The biodistribution data obtained are provided in Tables 1 and 2 in this paper and Tables A–D in the Supporting Information. Information on the number of animals per time point, the specific activity of each preparation, and quantities administered are provided in the footnotes of the tables. The complete biodistribution data obtained, and additional information on average animal weight and pairs of data in which the differences were highly significant (i.e. *P* < 0.005), are provided in Tables A–D in the Supporting Information.

RESULTS

In Vitro Comparison of SAv Proteins. To evaluate the differences in size, charge, and homogeneity of mass of the SAv proteins, each protein studied was evaluated by HPLC, SDS–PAGE, IEF, and electrospray mass spectral analyses. Size exclusion HPLC analyses of the SAv proteins had only one peak present at the expected

molecular mass range (by comparison with MW standards) for the tetrameric protein, indicating that they were not contaminated with higher- or lower-molecular mass proteins. It was interesting to note that the retention times of r-SAv proteins (*t_R* = 29.5 min) were found to be approximately 0.6 min shorter than that of the truncated commercial preparations (*t_R* = 30.1 min).

SDS–PAGE analysis of denatured r-SAv proteins demonstrated the correct molecular mass for the monomer, with the cysteine mutants being indistinguishable from r-SAv under reducing conditions. r-SAv-S139C and r-SAv-H127C also showed the presence of a dimeric species in the absence of reductant due to the formation of disulfide bonds. This dimeric species was not present under reducing conditions (6% β-mercaptoethanol in sample loading buffer). Nondenatured samples demonstrated the expected molecular mass of the tetramer for all r-SAvs.

An IEF gel comparing the five SAv proteins is shown in Figure 1. Although there appears to be one primary protein band for each SAv, all proteins studied had a minimum of three species present (percentages of each species not quantified). The *pI* value of the species present for the r-SAv (wild type) is very nearly the same as that of the site-directed mutant r-SAv-S139C (i.e. pH 6.6–7.4), but the *pI* values of the other mutant, r-SAv-H127C are lower (i.e. pH 5.2–6.6). The SAv protein obtained from one commercial source, SAv-CS2, has a *pI* profile quite similar to that of the r-SAv (wild type), except that it appears that the major species has a slightly higher *pI* (i.e. estimated to be 7.3 vs 7.0). The SAv protein obtained from the other commercial source,

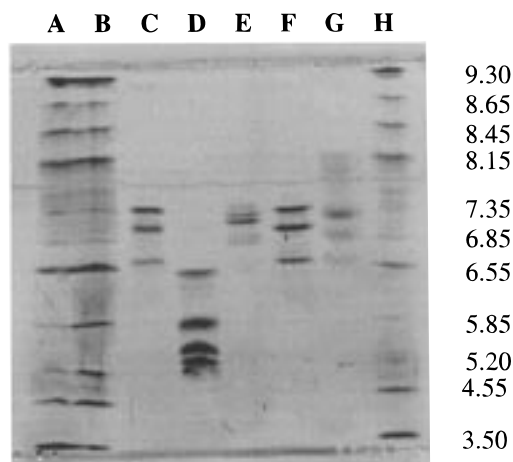


Figure 1. Digitized image of an IEF gel showing SA v protein *pI* values. The gel (PhastGel IEF 3–9) was run on a PhastSystem electrophoresis apparatus using the optimized method for IEF described in “Separation Technique File #100”. The protein staining was done by the silver stain method described in “Development Technique File #210”: lane A, IEF standards (Isoelectric Focusing Calibration Kit, broad *pI*; 3–10) run in the direction of application (bottom to top); lane B, IEF standards run from top to bottom; lane C, r-SA v-S139C (NEM-capped); lane D, r-SA v-H127C; lane E, SA v-CS1 (Boehringer Mannheim); lane F, r-SA v (wild type); lane G, SA v-CS2 (CALBIOCHEM); and lane H, IEF standards run from bottom to top.

SA v-CS1, has a *pI* profile somewhat different from those of the other SA v proteins as the *pI* range for the species present is quite small (i.e. pH 7.0–7.4).

In contrast to r-SA v, electrospray mass spectrometry (ESMS) of the commercially available SA v proteins indicated that they were heterogeneous in nature. Native streptavidin consists of amino acid residues 1–159; however, the recombinant streptavidin used is composed of residues 13–139 (based on native SA v numbering system), and core or “truncated” SA v proteins purchased from commercial sources generally are composed of 125–127 amino acid fragments containing residues beginning as low as amino acid 13 and ending with amino acids 133–139. The major component of the SA v proteins which were analyzed had molecular masses within 1 Da of the expected (calculated) subunit masses. The r-SA v mass spectrum had one major mass peak at the expected mass (13 271 Da). The mass spectrum for the SA v protein from commercial source 1 (Boehringer Mannheim) is a mixture which appears to have the 14–136 residues (12 971 Da) as its major component and the 13–135 residues (12 954 Da) as a minor component. The mass spectrum for the SA v protein from commercial source 2 (CALBIOCHEM) had several species present. It also had the 14–136 residues as the predominant species, but had a significant quantity of a second species with a mass of 13 042 Da, corresponding to either fragment 14–137 or fragment 13–136, and may be a mixture of the two.

Radioiodination of SA v Proteins by the ChT Method. Recombinant “core” (16, 32) SA v has 6 tyrosine residues per subunit (24 per tetramer) and is readily radioiodinated by the ChT method. It should be noted that there is a very important tyrosine residue in both avidin (Tyr-33) and SA v (Tyr-43) which participates in binding with the biotin ureido group (33–35). In avidin, nitration of that tyrosine, which is the only one present in the protein (36), has been shown to decrease biotin binding (37). For this reason, the specific activity of the radioiodinated SA v proteins in this study was kept below

2 $\mu\text{Ci}/\mu\text{g}$ to assure iodination on the more accessible (non-biotin binding) tyrosines in SA v. The SA v proteins were radioiodinated with either ^{125}I or ^{131}I by reaction with chloramine-T at pH 7.4. Generally, the SA v radioiodination yields ranged from 74 to 98%, but radioiodination of SA v from one commercial source (CS2)³ resulted in 48–50% radiochemical yields. It is not known why this protein gave consistently lower labeling yields.

Assessment of Radioiodination Method. As an initial part of the SA v biodistribution study, we were interested in determining if the radioiodine distribution was reflective of the distribution of the ChT-labeled r-SA v. To determine this, r-SA v was radiolabeled directly with $\text{Na}^{131}\text{I}/\text{ChT}$ and separately radiolabeled with *N*-succinimidyl *p*-[^{125}I]iodobenzoate ([^{125}I]PIB). PIB labeling of proteins (e.g. antibodies) has been shown to be resistant to in vivo deiodination (30). After r-SA v was labeled by the two methods, equal quantities of [^{125}I]PIB-r-SA v and r-[^{131}I]SA v were combined and injected into athymic mice. The distribution of radioactivity at 4, 24, and 48 h postinjection is provided in Table 1. The two methods of radiolabeling r-SA v gave similar distributions at all time points, except the amine-conjugated [^{125}I]PIB-labeled r-SA v had slightly reduced kidney concentrations. The data clearly showed that in vivo deiodination of SA v labeled directly with ChT was not a problem. Indeed, very little difference was observed in the neck and stomach concentrations of radioiodine nuclides. If significant deiodination had occurred, large differences between the PIB-labeled protein and the ChT-labeled protein would have been observed. These data indicated that the ChT labeling method was appropriate for labeling the SA v proteins in the comparative investigation.

Comparative Biodistribution of SA v Proteins. The biodistributions of two r-SA v mutant proteins and two SA v proteins available from commercial sources were compared with that of the (wild type) r-SA v. In the comparisons, one of the r-SA v mutants, r-[^{125}I]SA v-H127C or r-[^{125}I]SA v-S139C, or one of the SA v proteins obtained from commercial sources, SA v-CS1 or SA v-CS2, was mixed and co-injected with an equal quantity (i.e. 15 μg) of r-[^{131}I]SA v. By co-injection of the dual isotope-labeled SA v proteins into each animal, a direct comparison of the SA v variants was obtained. With the exception of the blood and kidney concentrations (Table 2), the concentrations in tissues were not appreciably different for the r-SA v mutants or commercially available SA v proteins from those for the r-SA v (wild type) used in our studies. The complete distribution data for the comparisons of SA v variants is provided in the Supporting Information as Tables A–D.

DISCUSSION

Recombinant streptavidin has been used extensively in our pretargeting studies because it is composed of a single protein species and can be prepared readily in large quantities from *E. coli* (21). Another factor in the choice of r-SA v was the fact that, through site-directed mutagenesis of the recombinant gene, it is possible to prepare r-SA v mutants that have unique properties. It is our belief that r-SA v mutants can be prepared which have properties that will improve in vivo characteristics and/or allow the preparation of improved reagents for pretargeting. This study was initiated to compare the in vivo characteristics of two r-SA v mutants, r-SA v-H127C and r-SA v-S139C, with those of the r-SA v being used in our studies (14). In addition to the r-SA v mutants, two commercially available SA v proteins were evaluated for the reasons described below.

In order to determine if the r-SAv mutants constituted "improved" reagents, we needed to understand the *in vivo* characteristics of SAv proteins other than the r-SAv used. As with our studies of r-SAv, inspection of the *in vivo* data previously reported in the literature indicated that SAv does not accumulate to any extent in tissues except kidney. Although localization of SAv in the kidney was consistently reported, the quantity localized varied widely (i.e. from 1 to >100% ID/g) (8, 12, 13, 18, 38–41). Our assumption was that the large reported differences could be caused by heterogeneity in the SAv proteins supplied by commercial sources. In 1990, it was reported that *some* commercial preparations contained unprocessed SAv (16). More recently, it has been reported that many commercially available SAv proteins are treated with proteases to provide the core SAv and maximize the homogeneity of the terminal structure (32).

SAv has 159 amino acid residues in its native state (16); however, native SAv is highly susceptible to enzymatic degradation, and both the C- and N-terminal amino acid sequences are truncated after secretion (16, 42–45). Importantly, truncation occurs which yields a "natural core SAv" which is resistant to further proteolysis. Truncation of SAv does not appear to significantly alter biotin binding properties, but removal of terminal amino acids appears to improve binding with biotinylated macromolecules (32). Further, truncation also improves the aqueous solubility of SAv and decreases the formation of aggregates. Unfortunately, the truncation process can result in heterogeneity within monomer and tetramer (44). The wide variation in the literature reports with regard to the kidney localization of commercially available SAv proteins and the potential for having nonhomogeneous mixtures of SAv proteins from commercial sources prompted us to include two representative SAv proteins from commercial sources in our biodistribution studies.

Before the *in vivo* studies were conducted, the proteins were characterized with regard to their purity, homogeneity, and *pI*. This was done as part of the characterization of r-SAv mutants, and to provide data on the physical differences between SAv variants. Analyses of the SAv variants by HPLC and SDS–PAGE provided data that indicated the proteins were pure. The wild type r-SAv is homogeneous in molecular mass, having 127 amino acid residues and a mass of 13 271 Da per subunit protein (21). The r-SAv mutants were also homogeneous in molecular mass, having subunit masses consistent with the desired amino acid substitution of wild type r-SAv. Contrary to this, the commercial SAv proteins were heterogeneous in molecular mass as judged by ESMS. IEF analyses indicated that multiple charged species exist for all of the SAv and r-SAv preparations. Although the origins of these charged species are not known, the IEF heterogeneity data obtained are consistent with previously reported data from SAv that has been truncated by proteolytic degradation (46) and for commercially available recombinant SAv (Sigma) (47). Indeed, microheterogeneity of proteins observed in IEF analyses, in general, is considered common (48). It is particularly interesting to note the much lower *pI* values found for the r-SAv-H127C mutant. As this protein is different from the r-SAv in only one amino acid (per subunit), the large shift in *pI* was unexpected. The explanation for the change in *pI* is not readily apparent, but the change in *pI* observed could be brought about by a number of factors, such as formation of alternate stable conformations of the subunits and/or various combinations of the subunits in forming the tetramer (48).

The primary goal of this investigation was to compare the *in vivo* characteristics of two r-SAv mutants with those of r-SAv. In one of the r-SAv mutants studied, r-SAv-H127C, the amino acid sequence of r-SAv has been altered by replacing the histidine residue at amino acid 127 with a cysteine. This substitution resulted in formation of two disulfide links between subunits in the r-SAv molecule which can readily be observed when evaluated by nonreducing SDS–PAGE analysis. Formation of disulfide cross-linked subunits in this mutant has been shown to increase the stability of r-SAv (49). This higher stability for subunit dissociation of r-SAv-H127C made it of interest for *in vivo* application as we thought it might alter its degradation and tissue distribution. Unfortunately, little difference in the biodistributions of the r-SAv-H127C and (wild type) r-SAv was noted. The one difference noted was that the kidney localization decreased relative to r-SAv. The relative amount of the decrease in kidney at the three time points is similar (Table 2) to that seen in the PIB-labeled r-SAv (Table 1). The reason for this decrease is not known, but we are investigating whether it might be related to a decrease in the *pI* of the proteins.

In the other site-directed r-SAv mutant studied, r-SAv-S139C, the C-terminal serine was replaced by a cysteine in the subunit polypeptide chain. This mutant is of particular interest as its use should allow conjugations to be carried out at locations which would not be expected to interfere with biotin binding. Although r-SAv which has more than one terminal cysteine has been reported (50), for our studies, it was desirable to have only one terminal cysteine for modification. The sulfhydryl group in the refolded r-SAv-S139C is present as disulfide linked subunits. As our primary interest in this SAv protein is the capacity for site-specific conjugations, prior to the *in vivo* evaluation, the disulfide bridges were reduced with DTT and the free sulfhydryl groups were conjugated with *N*-ethylmaleimide. The biodistribution of the r-SAv-S139C conjugate is quite similar to that of r-SAv. While they may not be applicable for all conjugates, these data suggest that sulfhydryl conjugations (of small molecules) will not affect the *in vivo* distribution appreciably. Additional conjugate studies are planned with r-SAv-S139C.

Aside from the kidney localization, there were only small differences in the tissue distributions of SAv variants studied. A comparison of blood and kidney concentrations of all the SAv variants vs r-SAv is provided in Table 2. It is interesting to note that blood concentrations of commercially available SAv proteins decreased more rapidly than that of the slightly larger r-SAv variants. Importantly, the mass heterogeneity of the commercially available SAv proteins did not appear to alter the kidney localization appreciably. Because all of the SAv variants were heterogeneous with regard to their *pI*, it is not possible to determine with certainty whether such heterogeneity contributes to the kidney localization. Our results indicate that a range of 15–29% ID/g (or 3–6% ID) is accumulated in the kidneys of athymic (BALB/c *nu/nu*) mice. In a three-step pretargeting protocol, and in our proposed method of increasing tumor radioactivity (14), the biotin derivative is the only species which carries radioactivity. Therefore, in these situations, the concentration of SAv in the kidneys may not be important if it is not available to bind with the radiolabeled biotin.

We plan to investigate the localization of r-SAv to kidneys further. If there is a specific receptor uptake, it may be possible to block uptake. Of course, the most

relevant question for our studies is whether the r-SAv localized in kidneys is available for binding with radiolabeled biotin derivatives. Due to concerns about misinterpretation of results, studies of kidney localization of radioiodinated biotin derivatives could not be conducted until a biotinidase-stabilized radioiodinated biotin derivative was obtained. Now that we have synthesized and tested a biotinidase-stabilized biotin derivative (51), its co-localization to SAv localized at kidney will be examined.

Summary. New r-SAv proteins are of interest for development of antibody pretargeting methods. As part of that development, two mutant r-SAv proteins have been compared with the (wild type) r-SAv. In an initial biodistribution study, it was demonstrated that radioiodination of r-SAv with ChT resulted in a (relatively) stable label, permitting the use of that labeling method in the subsequent comparison studies. Data obtained when r-SAv was compared with a disulfide-stabilized r-SAv mutant, r-SAv-H127C, indicated that the distribution was similar to that of r-SAv, except the kidney concentration was lower for the mutant than for r-SAv. Similarly, comparison of the r-SAv with C-terminal cysteine residues, r-SAv-S139C, demonstrated that their distributions were similar. Comparison of r-SAv with two commercially available SAv proteins in mice also indicated that there were only minor differences in their in vivo distributions. The results obtained indicate that truncated or recombinant SAv proteins might be used interchangeably for in vivo applications because they have similar distribution properties.

ACKNOWLEDGMENT

We are grateful for the financial support provided by the Department of Energy, Medical Applications and Biophysical Research Division, Office of Health and Environmental Research, under Grant DE-FG06-95ER62029. The animal studies were also supported in part by funding from the Richard M. Lucas Foundation.

Supporting Information Available: HPLC chromatograms and SDS-PAGE gels (denatured and nondenatured) of SAv proteins studied; mass spectra of r-SAv, SAv-CS1, and SAv-CS2 proteins; and Tables A–D containing biodistribution data for the r-SAv mutants, H127C and S139C, and the two commercially available SAv proteins (9 pages). Ordering information is given on any current masthead page.

LITERATURE CITED

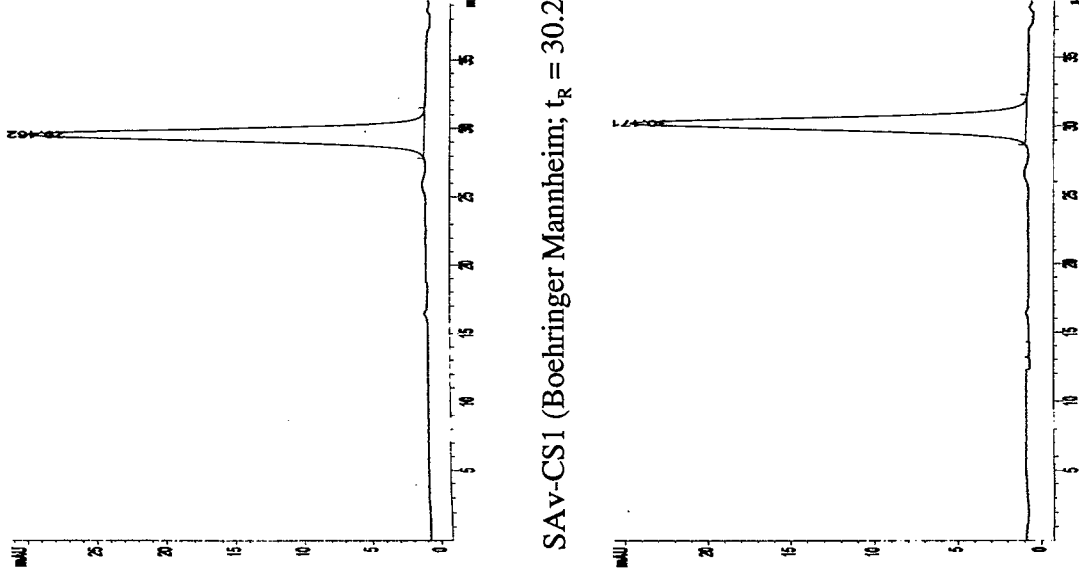
- (1) Hnatowich, D. J., Virzi, F., and Rusckowski, M. (1987) Investigations of Avidin and Biotin for Imaging Applications. *J. Nucl. Med.* 28, 1294–1302.
- (2) Goodwin, D. A. (1995) Tumor Pretargeting: Almost the Bottom Line. *J. Nucl. Med.* 36, 876–879.
- (3) Paganelli, G., Malcovati, M., and Fazio, F. (1991) Monoclonal Antibody pretargeting techniques for tumour localization: the avidin-biotin system. *Nucl. Med. Commun.* 12, 211–234.
- (4) Axworthy, D. B., Fritzberg, A. R., Hylarides, M. D., Mallett, R. W., Theodore, L. J., Gustavson, L. M., Su, F.-M., Beaumier, P. L., and Reno, J. M. (1994) Preclinical Evaluation of an Anti-tumor Monoclonal Antibody/Streptavidin Conjugate For Pretargeted ^{90}Y Radioimmunotherapy in a Mouse Xenograft Model. *J. Immunother.* 16, 158.
- (5) del Rosario, R. B., and Wahl, R. L. (1993) Biotinylated Iodo-Polylysine for Pretargeted Radiation Delivery. *J. Nucl. Med.* 34, 1147–1151.
- (6) Jeong, J. M., Kinuya, S., Paik, C. H., Saga, T., Sood, V. K., Carrasquillo, J. A., Neumann, R. D., and Reynolds, J. C. (1994) Application of High Affinity Binding Concept to Radiolabel Avidin with Tc-99m Labeled Biotin and the Effect of pI on Biodistribution. *Nucl. Med. Biol.* 21, 935–940.
- (7) Oehr, P., Westermann, J., and Biersack, H. J. (1988) Streptavidin and Biotin as Potential Tumor Imaging Agents. *J. Nucl. Med.* 29, 728–729.
- (8) Pimm, M. V., Fells, H. F., Perkins, A. C., and Baldwin, R. W. (1988) Iodine-131 and indium-111 labelled avidin and streptavidin for pre-targeted immunoscintigraphy with biotinylated anti-tumour monoclonal antibody. *Nucl. Med. Commun.* 9, 931–941.
- (9) Hashmi, M., and Rosebrough, S. F. (1995) Synthesis, Pharmacokinetics, and Biodistribution of ^{67}Ga Deferoxamineacetyl-Cysteinylbiotin. *Drug Metab. Dispos.* 23, 1362–1367.
- (10) Shoup, T. M., Fischman, A. J., Jaywook, S., Babich, J. W., Strauss, H. W., and Elmaleh, D. R. (1994) Synthesis of Fluorine-18-Labeled Biotin Derivatives: Biodistribution and Infection Localization. *J. Nucl. Med.* 35, 1685–1690.
- (11) Yao, Z., Zhang, M., Kobayashi, H., Sakahara, H., Nakada, H., Yamashina, I., and Konishi, J. (1995) Improved Targeting of Radiolabeled Streptavidin in Tumors Pretargeted with Biotinylated Monoclonal Antibodies through an Avidin Chase. *J. Nucl. Med.* 36, 837–841.
- (12) Khawli, L. A., Alauddin, M. M., Miller, G. K., and Epstein, A. L. (1993) Improved Immunotargeting of Tumors with Biotinylated Monoclonal Antibodies and Radiolabeled Streptavidin. *Antibody, Immunoconjugates, Radiopharm.* 6, 13–27.
- (13) Kassiss, A. I., Jones, P. L., Matalaka, K. Z., and Adelstein, S. J. (1996) Antibody-Dependent Signal Amplification in Tumor Xenografts after Pretreatment with Biotinylated Monoclonal Antibody and Avidin or Streptavidin. *J. Nucl. Med.* 37, 343–352.
- (14) Wilbur, D. S., Hamlin, D. K., Vessella, R. L., Stray, J., Buhler, K. R., Stayton, P., Klumb, L., Pathare, P. M., and Weerawarna, S. A. (1996) Antibody Fragments in Tumor Pretargeting. Evaluation of Biotinylated Fab' Co-localization with Recombinant Streptavidin and Avidin. *Bioconjugate Chem.* 7, 689–702.
- (15) Green, N. M. (1975) Avidin. *Adv. Protein Chem.* 29, 85–133.
- (16) Green, N. M. (1990) Avidin and Streptavidin. *Methods Enzymol.* 184, 51–67.
- (17) Schechter, B., Silberman, R., Arnon, R., and Wilchek, M. (1990) Tissue distribution of avidin and streptavidin injected to mice. Effect of avidin, carbohydrate, streptavidin truncation and exogenous biotin. *Eur. J. Biochem.* 189, 327–331.
- (18) Rosebrough, S. F. (1993) Pharmacokinetics and Biodistribution of Radiolabeled Avidin, Streptavidin and Biotin. *Nucl. Med. Biol.* 20, 663–668.
- (19) Rosebrough, S. F., and Hartley, D. F. (1996) Biochemical Modification of Streptavidin and Avidin: In Vitro and In Vivo Analysis. *J. Nucl. Med.* 37, 1380–1384.
- (20) Marshall, D., Pedley, R. B., Melton, R. G., Boden, J. A., Boden, R., and Begent, R. H. J. (1995) Galactosylated Streptavidin for Improved Clearance of Biotinylated Intact and F(ab')₂ Fragments of an Anti-Tumour Antibody. *Br. J. Cancer* 71, 18–24.
- (21) Chilkoti, A., Tan, P. H., and Stayton, P. S. (1995) Site-directed mutagenesis studies of the high-affinity streptavidin-biotin complex: contributions of tyrosine residues 79, 108, and 120. *Proc. Natl. Acad. Sci. U.S.A.* 92, 1754–1758.
- (22) Wilchek, M., and Bayer, E. A. (1990) Introduction to Avidin-Biotin Technology. *Methods Enzymol.* 184, 5–45.
- (23) Diamandis, E. P., and Christopoulos, T. K. (1991) The Biotin-(Strept)Avidin System: Principles and Applications in Biotechnology. *Clin. Chem.* 37, 625–636.
- (24) Wilchek, M., and Bayer, E. A. (1988) The Avidin-Biotin Complex in Bioanalytical Applications. *Anal. Biochem.* 171, 1–32.
- (25) del Rosario, R. B., and Wahl, R. L. (1990) Disulfide Bond-targeted Radiolabeling: Tumor Specificity of a Streptavidin-biotinylated Monoclonal Antibody Complex. *Cancer Res.* 50, 804–808.
- (26) Rowlinson-Busza, G., Hnatowich, D. J., Rusckowski, M., Snook, D., and Epenetos, A. A. (1993) Xenograft Localization Using Pretargeted Streptavidin-conjugated Monoclonal An-

- tibody and ^{111}In -Labeled Biotin. *Antibody, Immunoconjugates, Radiopharm.* 6, 97–109.
- (27) Paganelli, G., Pervez, S., Siccardi, A. G., Rowlinson, G., Deleide, G., Chiolerio, F., Malcovati, M., Scassellati, G. A., and Epenetos, A. A. (1990) Intraperitoneal Radio-Localization of Tumors Pre-Targeted by Biotinylated Monoclonal Antibodies. *Int. J. Cancer* 45, 1184–1189.
- (28) Saga, T., Weinstein, J. N., Jeong, J. M., Heya, T., Lee, J. T., Le, N., Paik, C. H., Sung, C., and Neumann, R. D. (1994) Two-Step Targeting of Experimental Lung Metastases with Biotinylated Antibody and Radiolabeled Streptavidin. *Cancer Res.* 54, 2160–2165.
- (29) Chilkoti, A., Schwartz, B. L., Smith, R. D., Long, C. J., and Stayton, P. S. (1995) Engineered Chimeric Streptavidin Tetramers as Novel Tools for Bioseparations and Drug Delivery. *Bio/Technology* 13, 1198–1204.
- (30) Wilbur, D. S., Hadley, S. W., Hylarides, M. D., Abrams, P. G., Beaumier, P. A., Morgan, A. C., Reno, J. M., and Fritzberg, A. R. (1989) Development of a Stable Radioiodinating Reagent to Label Monoclonal Antibodies for Radiotherapy of Cancer. *J. Nucl. Med.* 30, 216–226.
- (31) Fritzberg, A. R., Beaumier, P. L., Bottino, B. J., and Reno, J. M. (1994) Approaches to improved antibody- and peptide-mediated targeting for imaging and therapy of cancer. *J. Controlled Release* 28, 167–173.
- (32) Sano, T., Pandori, M. W., Chen, X., Smith, C. L., and Cantor, C. R. (1995) Recombinant Core Streptavidins. A Minimum-Sized Core Streptavidin Has Enhanced Structural Stability and Higher Accessibility to Biotinylated Macromolecules. *J. Biol. Chem.* 270, 28204–28209.
- (33) Gitlin, G., Bayer, E. A., and Wilchek, M. (1989) Studies on the Biotin-Binding Sites of Avidin and Streptavidin. Tyrosine residues are involved in the binding site. *Biochem. J.* 269, 527–530.
- (34) Gitlin, G., Khait, I., Bayer, E. A., Wilchek, M., and Muszkat, K. A. (1989) Studies on the Biotin-Binding Sites of Avidin and Streptavidin. A Chemically Induced Dynamic Nuclear Polarization Investigation of the Status of Tyrosine Residues. *Biochem. J.* 259, 493–498.
- (35) Hiller, Y., Bayer, E. A., and Wilchek, M. (1991) Studies on the Biotin-Binding Site of Avidin. *Biochem. J.* 278, 573–585.
- (36) Savage, M. D., Mattson, G., Desai, S., Nielander, G. W., Morgensen, S., and Conklin, E. J. (1992) *Avidin-Biotin Chemistry: A Handbook*, Pierce Chemical Co., Rockford, IL.
- (37) Morag, E., Bayer, E. A., and Wilchek, M. (1996) Reversibility of Biotin-Binding by Selective Modification of Tyrosine in Avidin. *Biochem. J.* 316, 193–199.
- (38) Rusckowski, M., Fogarasi, M., Virzi, F., and Hnatowich, D. J. (1995) Influence of endogenous biotin on the biodistribution of labelled biotin derivatives in mice. *Nucl. Med. Commun.* 16, 38–46.
- (39) Ngai, W. M., Reilly, R. M., Polihronis, J., and Shptiz, B. (1995) In Vitro and In Vivo Evaluation of Streptavidin Immunoconjugates of the Second Generation TAG-72 Monoclonal Antibody CC49. *Nucl. Med. Biol.* 22, 77–86.
- (40) Schechter, B., Arnon, R., Colas, C., Burakova, T., and Wilchek, M. (1995) Renal accumulation of streptavidin: Potential use for targeted therapy of the kidney. *Kidney Int.* 47, 1327–1335.
- (41) Zhang, M., Sakahara, H., Yao, Z., Saga, T., Nakamoto, Y., Sato, N., Nakada, H., Yamashina, I., and Konishi, J. (1997) Intravenous Avidin Chase Improved Localization of Radio-labeled Streptavidin in Intraperitoneal Xenograft Pretargeted with Biotinylated Antibody. *Nucl. Med. Biol.* 24, 61–64.
- (42) Bayer, E. A., Ben-Hur, H., Gitlin, G., and Wilchek, M. (1986) An improved method for the single-step purification of streptavidin. *J. Biochem. Biophys. Methods* 13, 103–112.
- (43) Argarana, C. E., Kuntz, I. D., Birken, S., Axel, R., and Cantor, C. R. (1986) Molecular cloning and nucleotide sequence of the streptavidin gene. *Nucleic Acids Res.* 14, 1871–1882.
- (44) Pahler, A., Hendrickson, W. A., Kolks, M. A. G., Argarana, C. E., and Cantor, C. R. (1987) Characterization and Crystallization of Core Streptavidin. *J. Biol. Chem.* 262, 13933–13937.
- (45) Bayer, E. A., Hen-Hur, H., Hiller, Y., and Wilchek, M. (1989) Postsecretory modifications of streptavidin. *Biochem. J.* 259, 369–376.
- (46) Dittmer, J., Dittmer, A., Bruna, R. D., and Kasche, V. (1989) A native, affinity-based protein blot for the analysis of streptavidin heterogeneity: Consequences for the specificity of streptavidin mediated binding assays. *Electrophoresis* 10, 762–765.
- (47) Kang, Y.-S., Saito, Y., and Pardridge, W. M. (1995) Pharmacokinetics of (^3H)Biotin Bound to Different Avidin Analogues. *J. Drug Targeting* 3, 159–165.
- (48) Pharmacia (1982) *Isoelectric Focusing: Principles and Methods*, Ljungföretagen AB, Örebro, Uppsala, Sweden.
- (49) Reznik, G. O., Vajda, S., Smith, C. L., Cantor, C. R., and Sano, T. (1996) Streptavidins with intersubunit crosslinks have enhanced stability. *Nat. Biotechnol.* 14, 1007–1011.
- (50) Sano, T., Smith, C. L., and Cantor, C. R. (1993) A Streptavidin Mutant Containing a Cysteine Stretch That Facilitates Production of a Variety of Specific Streptavidin Conjugates. *Bio/Technology* 11, 201–206.
- (51) Wilbur, D. S., Hamlin, D. K., Pathare, P. M., and Weerawarna, S. A. (1997) Biotin Reagents for Antibody Pretargeting. Synthesis, Radioiodination and In Vitro Evaluation of Water Soluble, Biotinidase Resistant Biotin Derivatives. *Bioconjugate Chem.* 8, 572–584.

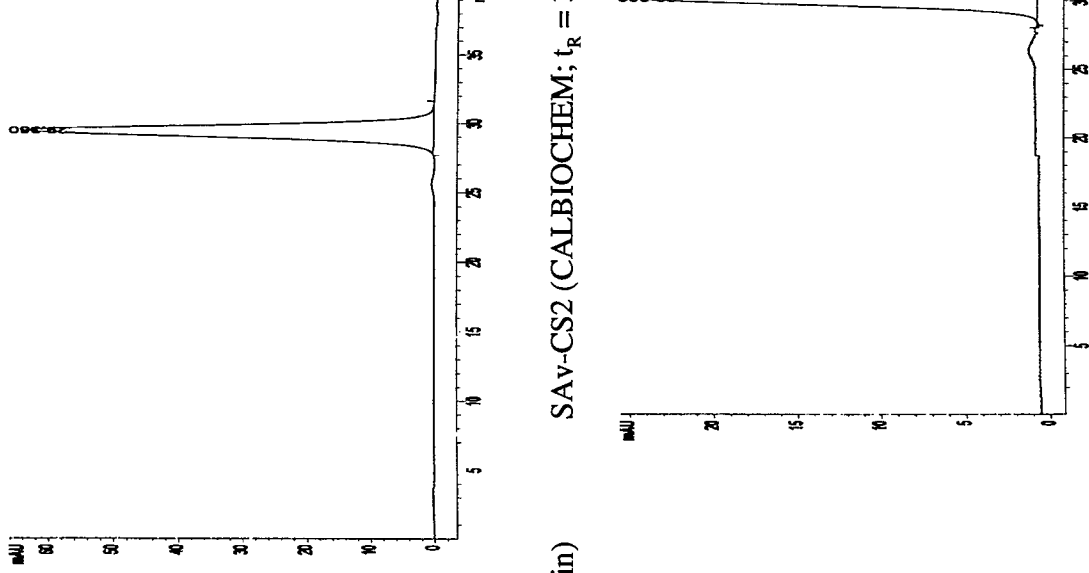
BC970152S

Size exclusion HPLC chromatograms of the streptavidin proteins evaluated. HPLC equipment and conditions were as described for non-labeled proteins in manuscript. All protein evaluations were conducted on the same day under identical HPLC conditions to obtain relative retention times (t_R). Differences in t_R observed for r-SAv proteins and commercial SAv proteins appear to be real as co-injection of r-SAv (wild type) with SAv-CSI had a clearly discernible shoulder at about 30 min on a major peak at 29.5 min.

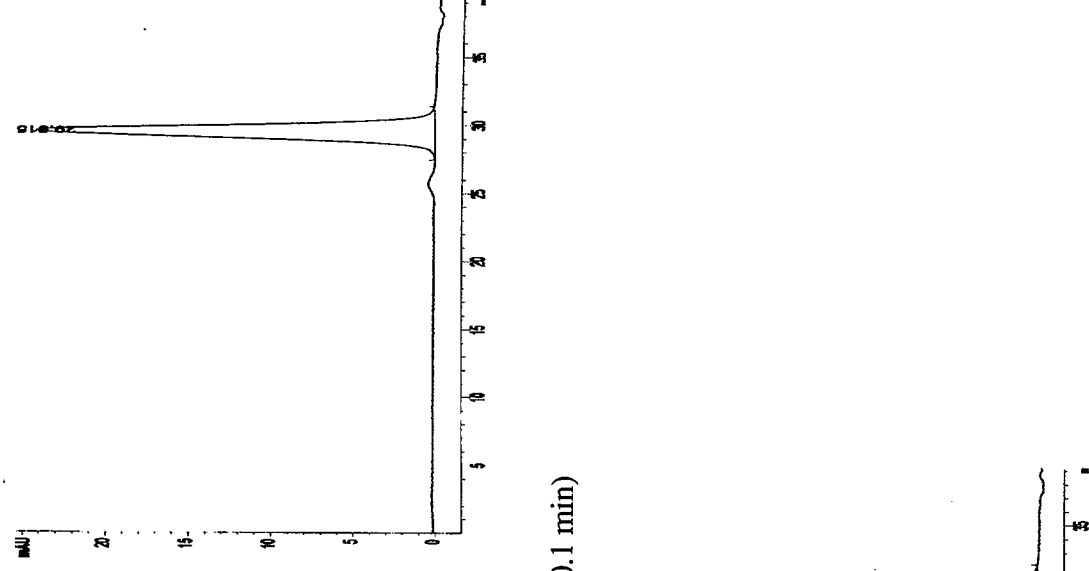
r-Streptavidin (wild type; $t_R = 29.5$ min)



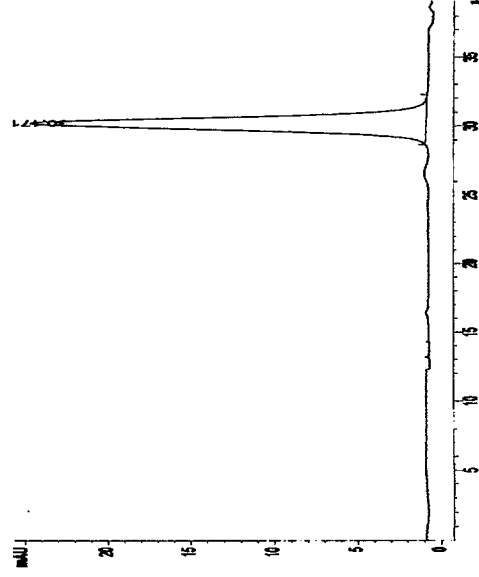
r-SAv-H127C ($t_R = 29.4$ min)



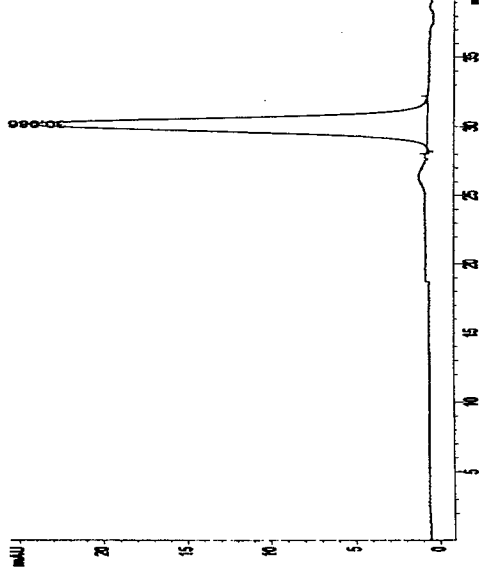
r-SAv-S139C (NEM capped; $t_R = 29.5$ min)



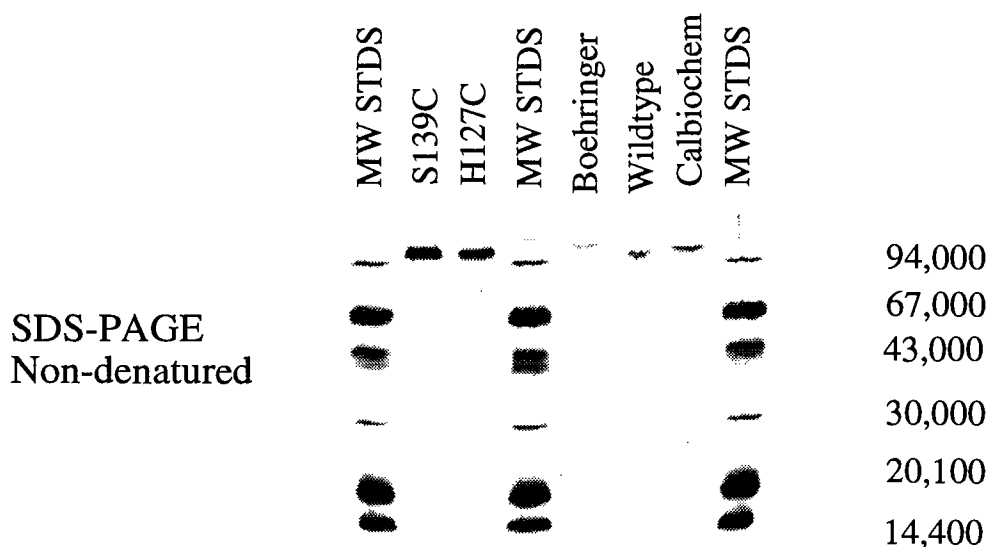
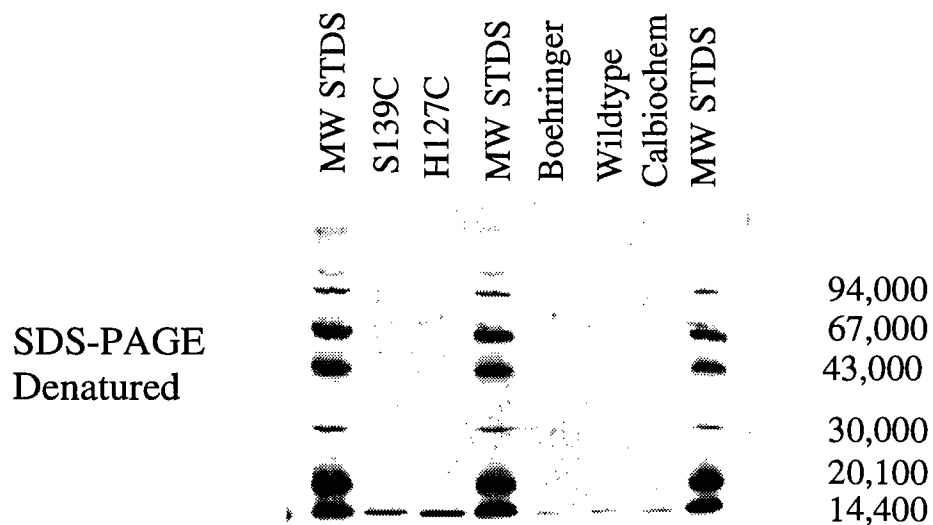
SAv-CSI (Boehringer Mannheim; $t_R = 30.2$ min)



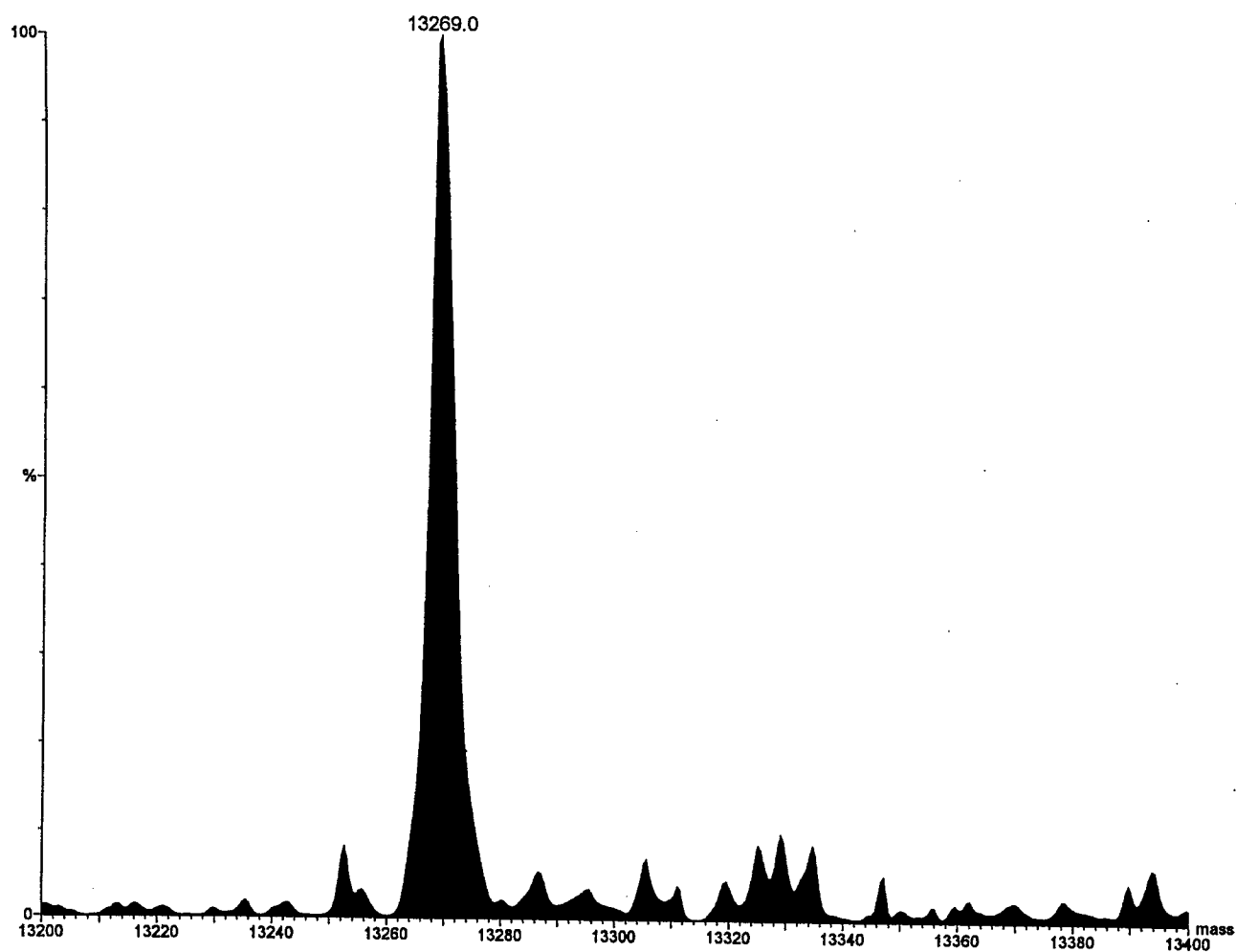
SAv-CS2 (CALBIOCHEM; $t_R = 30.1$ min)



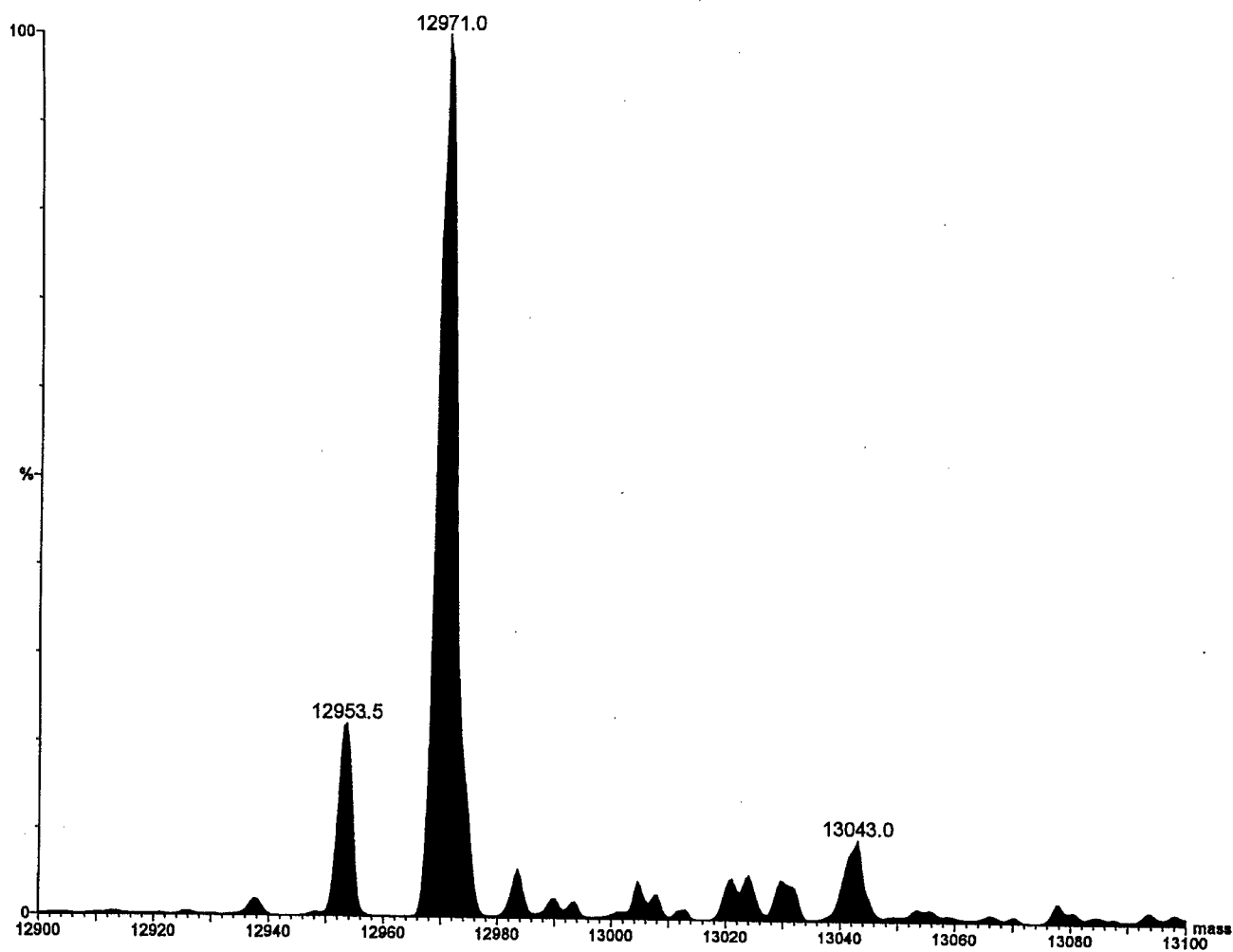
Digitized images of denatured and non-denatured (reducing, β -mercaptoethanol) SDS-PAGE gels of SA_v proteins conducted on a PhastSystem™ electrophoresis apparatus (using PhastGel®, Homogeneous 12.5%). Lanes 1, 4, and 8; molecular weight standards (MW STDS; LMW Electrophoresis Calibration kit (Pharmacia)). Lane 2; r-SA_v-S139C. Lane 3; r-SA_v-H127C. Lane 5; SA_v-CS1 (Boehringer Mannheim). Lane 6; r-SA_v (wild type). Lane 7; SA_v-CS2 (CALBIOCHEM). Gels were run by optimized method for Homogeneous 12.5% gel using "Separation Technique File #111. The protein band was developed by the silver staining method from "Development Technique File #210.



Electrospray Mass Spectrum (ESMS) of the wild type recombinant streptavidin (r-SAv) used in studies. Note that the minor peaks are thought to be Ca ion adducts or other background noise and are not considered to be impurities in the protein.



Electrospray Mass Spectrum (ESMS) of the streptavidin obtained from Boehringer Mannheim (SAv-CS1; lot number 133816721). Three proteins of different masses are readily apparent (with masses marked). Note that the minor peaks are thought to be Ca ion adducts or other background noise and are not considered to be impurities in the protein.



Electrospray Mass Spectrum (ESMS) of the streptavidin obtained from CALBIOCHEM (SAv-CS2; lot number B12651). Two proteins of different masses are readily apparent, with several other peaks likely to be attributed to different SAv proteins. Note that the smallest peaks are thought to be Ca ion adducts or other background noise and are not considered to be impurities in the protein.

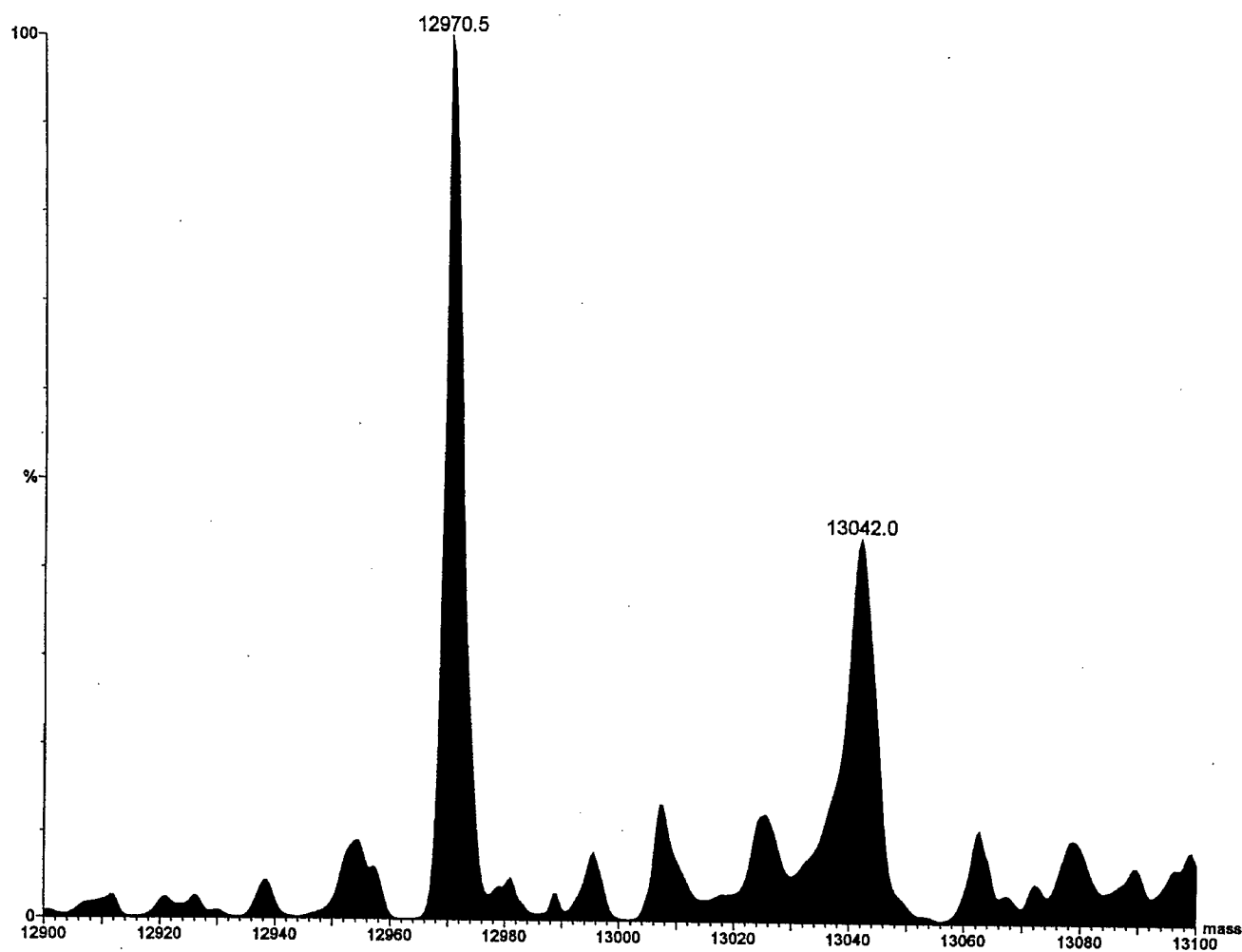


Table A: Distribution of Radioactivity at 4, 24 and 48 h Post Co-administration of r-SAV Mutant, r-[¹²⁵I]SAV-H127C, and r-[¹³¹I]SAV in Athymic Mice^a

Tissues	<u>4 h^{b,c}</u>			<u>24 h</u>			<u>48 h</u>		
	<u>I-125</u>	<u>I-131</u>		<u>I-125</u>	<u>I-131</u>		<u>I-125</u>	<u>I-131</u>	
blood	11.43 ± 0.40 ^e	10.35 ± 0.35 ^e		3.07 ± 0.27 ^e	2.12 ± 0.23 ^e		0.93 ± 0.22	0.58 ± 0.13	
muscle	1.38 ± 0.09	1.24 ± 0.09		0.88 ± 0.08	0.69 ± 0.04		0.59 ± 0.04 ^e	0.47 ± 0.03 ^e	
lung	7.52 ± 0.91	6.60 ± 0.84		2.88 ± 0.16 ^e	2.16 ± 0.13 ^e		2.08 ± 0.53	1.58 ± 0.41	
kidney	18.92 ± 1.72 ^e	26.43 ± 2.71 ^e		17.21 ± 1.31	22.82 ± 1.47		20.17 ± 2.70	26.50 ± 3.21	
spleen	3.96 ± 0.44	3.49 ± 0.39		3.12 ± 0.19	2.53 ± 0.19		2.64 ± 0.76	2.14 ± 0.63	
liver	3.11 ± 0.30	2.77 ± 0.28		2.61 ± 0.07 ^e	2.12 ± 0.07 ^e		2.41 ± 0.31	1.96 ± 0.27	
intestine	2.31 ± 0.26	2.05 ± 0.22		1.07 ± 0.09	0.82 ± 0.08		0.71 ± 0.13	0.55 ± 0.11	
neck	4.51 ± 0.52	4.17 ± 0.48		3.92 ± 0.30	3.06 ± 0.22		6.48 ± 3.31	4.43 ± 1.93	
stomach	2.69 ± 0.30	2.50 ± 0.29		1.78 ± 0.14 ^e	1.24 ± 0.11 ^e		1.44 ± 0.31	1.02 ± 0.19	
urine ^d	15.76 ± 9.32	20.33 ± 8.93		11.90 ± 1.73	8.79 ± 1.44		7.24 ± 1.23	4.96 ± 0.79	

^aValues shown are % ID / g ± standard deviation. ^bTime of sacrifice from injection of radiolabeled r-SAV. ^cData were obtained for *n* = 5 mice at each time point; average animal weight, 27.52 ± 2.04 g; Injectate for each animal had 15 µCi / 15 µg of r-SAV labeled with Na[¹³¹I]I/ChT and 13 µCi / 15 µg of r-SAV mutant, r-SAV-H127C, labeled with Na[¹²⁵I]I/ChT in approximately 100 µL of 0.9% sterile saline. ^dUrine was collected by syringe bladder tap after sacrifice. ^eStatistical significance of difference in paired *t* test; *P* < 0.005.

Table B: Distribution of Radioactivity at 4, 24 and 48 h Post Co-administration of r-^{SAV} Mutant, r-[¹²⁵I]^{SAV}-S139C, and r-[¹³¹I]^{SAV} in Athymic Mice^a

Tissues	<u>4 h^{b,c}</u>		<u>24 h</u>		<u>48 h</u>	
	<u>I-125</u>	<u>I-131</u>	<u>I-125</u>	<u>I-131</u>	<u>I-125</u>	<u>I-131</u>
blood	10.67 ± 0.30 ^e	9.15 ± 0.37 ^e	2.30 ± 0.44	1.90 ± 0.46	0.59 ± 0.05 ^e	0.40 ± 0.03 ^e
muscle	1.65 ± 0.20	1.46 ± 0.20	0.84 ± 0.22	0.74 ± 0.15	0.51 ± 0.03 ^e	0.42 ± 0.03 ^e
lung	6.72 ± 0.27 ^e	5.59 ± 0.29 ^e	2.53 ± 0.34	2.17 ± 0.35	1.44 ± 0.15	1.21 ± 0.13
kidney	19.84 ± 1.12	22.34 ± 1.14	19.53 ± 3.69	23.30 ± 4.60	17.10 ± 3.36	21.31 ± 3.70
spleen	3.98 ± 0.06 ^e	2.99 ± 0.07 ^e	3.69 ± 0.39 ^e	2.59 ± 0.40 ^e	2.96 ± 0.47	2.10 ± 0.27
liver	2.84 ± 0.15 ^e	2.27 ± 0.13 ^e	2.74 ± 0.43	2.22 ± 0.38	2.30 ± 0.23	1.85 ± 0.20
intestine	2.51 ± 0.26	2.14 ± 0.26	1.07 ± 0.15	0.87 ± 0.13	0.68 ± 0.05 ^e	0.54 ± 0.04 ^e
neck	4.91 ± 0.22 ^e	4.01 ± 0.31 ^e	4.75 ± 1.43	3.45 ± 0.91	5.81 ± 1.78	4.05 ± 1.07
stomach	2.46 ± 0.19 ^e	1.96 ± 0.14 ^e	1.81 ± 0.40	1.34 ± 0.32	1.62 ± 0.29	1.24 ± 0.21
urine ^d	25.86 ± 2.25	28.79 ± 3.12	9.54 ± 1.17	8.92 ± 1.21	6.02 ± 1.23	4.73 ± 0.92

^aValues shown are % ID / g ± standard deviation. ^bTime of sacrifice from injection of radiolabeled r-^{SAV}. ^cData were obtained for *n* = 5 mice at each time point; average animal weight, 26.34 ± 1.36 g; Injectate for each animal had 18 µCi / 15 µg of r-^{SAV} labeled with Na[¹³¹I]/ChT and 17 µCi / 15 µg of r-^{SAV} mutant, r-^{SAV}-S139C, labeled with Na[¹²⁵I]/ChT in approximately 100 µL of 0.9% sterile saline. ^dUrine was collected by syringe bladder tap after sacrifice. ^eStatistical significance of difference in paired t test; *P* < 0.005.

Table C: Distribution of Radioactivity at 4, 24 and 48 h Post Co-administration of SAV from a Commercial Source, [¹²⁵I]SAV-CS1^a, and r-[¹³¹I]SAV in Athymic Mice^b

Tissues	<u>4 h^{c,d}</u>			<u>24 h</u>			<u>48 h</u>		
	<u>I-125</u>	<u>I-131</u>		<u>I-125</u>	<u>I-131</u>		<u>I-125</u>	<u>I-131</u>	
blood	6.65 ± 0.48 ^f	8.38 ± 0.65 ^f		1.33 ± 0.19 ^f	1.94 ± 0.24 ^f		0.31 ± 0.06	0.48 ± 0.09	
muscle	1.33 ± 0.15	1.38 ± 0.17		0.55 ± 0.12 ^f	0.77 ± 0.03 ^f		0.43 ± 0.11	0.60 ± 0.14	
lung	5.14 ± 0.41	5.83 ± 0.44		1.79 ± 0.20	2.28 ± 0.25		1.02 ± 0.22	1.34 ± 0.28	
kidney	18.69 ± 3.46	19.31 ± 3.23		15.68 ± 1.18 ^f	22.09 ± 1.35 ^f		15.48 ± 3.97	23.55 ± 5.41	
spleen	1.92 ± 0.26	2.29 ± 0.32		1.68 ± 0.22 ^f	2.29 ± 0.24 ^f		1.45 ± 0.21	2.08 ± 0.37	
liver	1.94 ± 0.21	2.23 ± 0.26		1.97 ± 0.22	2.42 ± 0.25		1.53 ± 0.30	1.96 ± 0.35	
intestine	1.56 ± 0.27	1.73 ± 0.28		0.70 ± 0.12	0.87 ± 0.14		0.43 ± 0.05 ^f	0.57 ± 0.06 ^f	
neck	4.22 ± 0.55	4.67 ± 0.70		3.87 ± 1.46	4.16 ± 1.20		2.84 ± 2.42	3.42 ± 2.48	
stomach	1.87 ± 0.24	1.93 ± 0.26		1.56 ± 0.30	1.65 ± 0.26		1.25 ± 0.11	1.53 ± 0.13	
urine ^e	36.96 ± 17.16	20.33 ± 8.93		10.91 ± 1.44	8.43 ± 0.52		4.62 ± 1.19	5.32 ± 1.19	

^aThe SAV was purchased from Boehringer Mannheim (Indianapolis, IN). ^bValues shown are % ID / g ± standard deviation. ^cTime of sacrifice from injection of radiolabeled SAV.

^dData were obtained for *n* = 5 mice at each time point; average animal weight, 24.54 ± 1.82 g; Injectate for each animal had 15 µCi / 15 µg of r-SAV labeled with Na[¹³¹I]J/ChT and 15 µCi / 15 µg of SAV-CS1 labeled with Na[¹²⁵I]J/ChT in approximately 100 µL of 0.9% sterile saline. ^eUrine was collected by syringe bladder tap after sacrifice. ^fStatistical significance of difference in paired t test; *P* < 0.005.

Table D: Distribution of Radioactivity at 4, 24 and 48 h Post Co-administration of SAV from a Commercial Source, [¹²⁵I]SAV-CS2^a, and r-[¹³¹I]SAV in Athymic Mice^b

Tissues	<u>4 h^{c,d}</u>			<u>24 h</u>			<u>48 h</u>		
	<u>I-125</u>	<u>I-131</u>	<u>I-125</u>	<u>I-131</u>	<u>I-125</u>	<u>I-131</u>	<u>I-125</u>	<u>I-131</u>	<u>I-125</u>
blood	6.64 ± 0.23 ^f	9.12 ± 0.40 ^f	1.35 ± 0.14 ^f	2.16 ± 0.31 ^f	0.34 ± 0.04 ^f	0.54 ± 0.05 ^f			
muscle	1.30 ± 0.07 ^f	1.48 ± 0.07 ^f	0.71 ± 0.16	0.98 ± 0.20	0.38 ± 0.02 ^f	0.57 ± 0.05 ^f			
lung	4.61 ± 0.26 ^f	5.73 ± 0.28 ^f	1.83 ± 0.20 ^f	2.53 ± 0.24 ^f	1.00 ± 0.11 ^f	1.45 ± 0.15 ^f			
kidney	29.23 ± 1.48 ^f	21.65 ± 1.65 ^f	24.27 ± 4.43	27.25 ± 4.64	20.33 ± 2.30 ^f	26.56 ± 2.78 ^f			
spleen	2.17 ± 0.21	2.63 ± 0.23	1.77 ± 0.09 ^f	2.48 ± 0.15 ^f	1.62 ± 0.15 ^f	2.45 ± 0.28 ^f			
liver	2.07 ± 0.13 ^f	2.55 ± 0.18 ^f	1.96 ± 0.20 ^f	2.61 ± 0.27 ^f	1.69 ± 0.15 ^f	2.32 ± 0.22 ^f			
intestine	1.54 ± 0.30	1.86 ± 0.37	0.84 ± 0.31	1.07 ± 0.31	0.46 ± 0.03 ^f	0.65 ± 0.05 ^f			
neck	4.59 ± 0.78	4.67 ± 0.70	2.63 ± 0.48	3.42 ± 0.62	3.64 ± 3.84	3.74 ± 2.37			
stomach	3.49 ± 0.27 ^f	1.93 ± 0.21 ^f	1.79 ± 0.35	1.76 ± 0.31	1.45 ± 0.19	1.78 ± 0.19			
urine ^e	33.77 ± 16.30	20.33 ± 8.93	8.78 ± 5.45	9.11 ± 7.59	4.02 ± 2.51	4.90 ± 2.92			

^aThe SAV was purchased from CALBIOCHEM (La Jolla, CA). ^bValues shown are % ID / g ± standard deviation. ^cTime of sacrifice from injection of radiolabeled SAV. ^dData were obtained for *n* = 5 mice at each time point; average animal weight, 23.38 ± 1.81 g; Injectate for each animal had 15 µCi / 15 µg of r-SAV labeled with NaI [¹³¹I]/ChT and 11 µCi / 15 µg of SAV-CS2 labeled with NaI [¹²⁵I]/ChT in approximately 100 µL of 0.9% sterile saline. ^eUrine was collected by syringe bladder tap after sacrifice. ^fStatistical significance of difference in paired t test; P<0.005.

Simplified Preformed Chelate Protein Radiolabeling with Technetium-99m Mercaptoacetamidoadipoylglycylglycine (N₃S-Adipate)

Sudhakar Kasina,* James A. Sanderson, Jeffrey N. Fitzner, Ananthachari Srinivasan, Tripuraneni N. Rao, Lori J. Hobson, John M. Reno, Donald B. Axworthy, Paul L. Beaumier, and Alan R. Fritzberg

NeoRx Corporation, 410 West Harrison Street, Seattle, Washington 98119. Received April 1, 1997; Revised Manuscript Received October 7, 1997[©]

A simplified kit has been developed for ^{99m}Tc protein radiolabeling using an N₃S triamide mercaptide bifunctional chelating agent and the preformed chelate approach. The process combined N₃S chelating agent, gluconate intermediate transfer agent, stannous reducing agent, and gentisic acid stabilizer into a lyophilized formulation. With sulfur donor atom hemithioacetal protection of the ligand, δ -2,3,5,6-tetrafluorothiophenyl α -S-(1-ethoxyethyl)mercaptoacetamido-L-adipoylglycylglycine, optimum ^{99m}Tc chelation was achieved in a single step. Subsequent reaction with NR-LU-10 antibody Fab fragment followed by purification via QAE Sephadex anion exchange resin filter afforded ^{99m}Tc-N₃S-NR-LU-10 Fab conjugate with retained immunoreactivity and effective tumor targeting properties.

INTRODUCTION

Earlier investigations have shown that amide thiolate (N₂S₂, N₃S)¹ chelating agents form well-defined ^{99m}Tc complexes of high stability (1, 2). Application of N₂S₂ chelating agents for the bifunctional attachment of ^{99m}Tc to proteins such as antibodies was established with 4,5-dimercaptoacetamidopentanoate via chelation, *in situ* esterification, and protein coupling (3). The chelating agent was subsequently modified to a tetrafluorophenyl active ester and incorporated into a kit (4) that has been used clinically with various antibody fragments, including NR-ML-05 Fab fragment for melanoma (5) and NR-LU-10 Fab fragment for small cell lung carcinoma and other solid tumor cancers (6–8). Additionally it has been used with annexin V, a 36 kD human placental anticoagulant protein for thrombus imaging (9).

Although the pentanoate-based N₂S₂ chelating kit served successfully to stably attach ^{99m}Tc without negatively impacting tumor targeting ability of the Fab fragments and annexin V protein, some drawbacks were

experienced. Catabolism of the radiolabeled protein following kidney and liver uptake yielded a lysine adduct of the ^{99m}Tc-chelate that was excreted via the hepatobiliary system (10). This excretion interfered in the detection of disease in the lower abdomen. Furthermore, the kit was cumbersome, involving manipulation of several components prior to the ^{99m}Tc chelation step. Thus, the opportunity existed for further simplification of the radiolabeling process to yield a product of improved biodistribution and ease of use.

Previously (11), we have shown that the addition of a carboxyl group to the N₂S₂ ligand core decreased hepatobiliary excretion of the radioactivity. Additional carboxyl groups added to the ligand further decreased hepatobiliary excretion. We have now extended these results to the N₃S ligand system and herein describe the bifunctional chelate, 2,3,5,6-tetrafluorothiophenyl-S-(1-ethoxyethyl)mercaptoacetamidoadipoylglycylglycine (N₃S-adipate-STFP), and its incorporation into a kit for protein radiolabeling for clinical application. This kit combined N₃S-adipate-STFP, gluconate intermediate transfer agent, and stannous reducing agent into a lyophilized formulation that reduced the number of steps and time required for protein radiolabeling. Comparative mice biodistribution studies of this ^{99m}Tc-N₃S Fab conjugate, noncarboxylate ^{99m}Tc-N₂S₂ Fab conjugate (4), and ¹²⁵I-PIP Fab conjugate (2) are herein described. Comparative rat biodistribution and hepatobiliary excretion studies for the series of carboxylate-substituted amide thiolate bifunctional chelating agents will be described in a separate report.

MATERIALS AND METHODS

Reagents. All reagents were obtained from Aldrich Chemical Co. (Milwaukee, WI) except BSA, transferrin, cysteine, and sodium phosphate, all obtained from Sigma Chemical Co. (St. Louis, MO), and QAE Sephadex obtained from Pharmacia Biotech, Inc. (Piscataway, NJ). All solvents were reagent or HPLC grade. Two intermediate precursor compounds, S-(1-ethoxyethyl)mercap-

* Author to whom correspondence and reprint requests should be addressed. [Phone (206) 281-7001; Fax (206) 286-2537; email skasina@neorx.com].

[©] Abstract published in *Advance ACS Abstracts*, December 15, 1997.

¹ Abbreviations: Ab-NH₂, antibody fragment with lysine ϵ -amino group; BSA, bovine serum albumin; BuOH, butanol; CBZ, carbobenzyloxy; DCC, 1,3-dicyclohexylcarbodiimide; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; DTPA, diethylenetriaminepentaacetic acid; EtOAc, ethyl acetate; HOAc, acetic acid; HPLC, high pressure liquid chromatography; IPA, 2-propanol; ITLC, instant thin layer chromatography; NR-LU-10 Fab, pancarcinoma papain fragmented murine antibody fragment; N₂S₂, diamide disulfur; N₃S-adipate-STFP, δ -2,3,5,6-tetrafluorothiophenyl- α -S-(1-ethoxyethyl)mercaptoacetamido-L-adipoylglycylglycine; N₃S-adipate-OTFP, δ -2,3,5,6-tetrafluorophenyl α -S-(1-ethoxyethyl)mercaptoacetamido-L-adipoylglycylglycine; PBS, phosphate-buffered saline; PIP, P-iodophenylate; QAE, quaternary aminoethyl; TCA, trichloroacetic acid; TLC, thin layer chromatography.

toacetic acid **11** and *N*-hydroxysuccinimidyl *S*-(1-ethoxyethyl)mercaptoacetate **12**, were prepared as described earlier (4). NR-LU-10 Fab antibody fragment was prepared in-house from standard papain digestion of whole antibody isolated from the supernatant of cultured murine hybridoma cells (12, 13). The ^{99m}Tc - N_3S -NR-LU-10 Fab purification column was manufactured to NeoRx specifications by BioProbe, Inc. (Tustin, CA) and Sepratech, Inc. (American Canyon, CA). Lyophilization vials were obtained from Miles, Pharmaceutical Div. (Spokane, WA), and stoppers were purchased from The West Co. (Lionville, PA).

General Instrumentations. Proton NMR spectra were obtained on a Varian Gemini-200 spectrometer with tetramethylsilane as an internal standard. IR data were obtained on a Perkin-Elmer 1310 infrared spectrophotometer. Mass spectral data (both low and high resolution) were obtained on a Micromass 70 SEQ tandem hybrid mass spectrometer (Micromass Ltd., Manchester, U.K.) with associated DEC PDP 11-250J Data System. Mass spectral data were obtained by fast atom bombardment (FAB^+) by electron impact (EI) ionization on the sample introduced by the direct insertion probe (DIP). Melting points were obtained on a Thomas-Hoover apparatus and are uncorrected. Elemental analyses were performed by Desert Microanalytical Laboratories, Tucson, AZ. Moisture analyses were performed on a Photo Volt Moisture Analyzer.

Chromatography. High performance liquid chromatography (HPLC) of small molecule forms of the organic syntheses and radiolabeling of bifunctional chelates was carried out on Beckman-Altex systems using 5 μm ODS (Beckman Ultrasphere) C-18 columns. Beckman Models 153 and 331 spectrophotometers with fixed wavelength or 155–400 variable wavelength were used for optical detection, while a Beckman Model 170 radiometric detection in series with the former was used in identifying the radiolabeled products.

^{99m}Tc - N_3S -adipate-STFP analyses was performed isocratically using a mobile phase of acetonitrile in 0.01 M sodium phosphate (pH 6.8) at a ratio of 24:76 at 1.5 mL/min on a reverse phase column. Additionally the separation was performed on a gradient system going from an acetonitrile:water:glacial acetic acid ratio of 5:94:1 (pump A) to 60:39:1 (pump B) at 1.0 mL/min. The program for the gradient elution involved a 10 min pumping of 100% A followed by a 35 min pumping of 100% B.

Antibody protein conjugate radiochemical purity was assessed by size-exclusion chromatography using Zorbax (DuPont de Nemours) HPLC with 0.2 M phosphate, pH 7.2 as mobile phase. Detection was performed as noted above. Separation of free and protein-bound ^{99m}Tc was also performed using silica gel impregnated glass fiber instant thin layer chromatography (ITLC) supplied by Gelman Sciences, Inc., Ann Arbor, MI, as ITLC SG. The silica gel strips were precut to 2 $\times$ 10 cm, activated at 100 $^\circ\text{C}$ for at least 2 h and stored according to the manufacturer's instructions. The ITLC strips were developed with freshly prepared 12% w/v trichloroacetic acid (TCA). In this system, the ^{99m}Tc -labeled Fab fragment remained at the origin and non-protein bound ^{99m}Tc -labeled species migrated with the solvent front. The TLC strips were cut into two halves and separately counted for final purity on a Packard MINAXI 500 series γ counter.

Syntheses of N_3S -Adipate-STFP (17) and N_3S -Adipate-OTFP (15). Scheme 1 outlines the syntheses of thio and oxo 2,3,5,6-tetrafluorophenyl *S*-(1-ethoxyethyl)mercaptoacetamido-*L*-adipoylglycylglycine bifunctional

chelating moieties, N_3S -adipate-STFP and N_3S -adipate-OTFP. The final compounds were purified by preparative reverse phase HPLC. The detailed procedures were performed as follows.

L- α -*N*-(Carbobenzyloxy)amino adipic Acid (**2**). 4.47 g (27.7 mmol) of *L*-amino adipic acid (**1**) was dissolved in 14 mL of 4 N sodium hydroxide and cooled to 0 $^\circ\text{C}$. To the magnetically stirred solution was added a solution of 4.35 mL (30.5 mmol) of carbobenzyloxy chloride (CBZ-Cl) in 14 mL of dioxane dropwise over a period of 30 min. The pH of the solution was periodically monitored during the addition, maintaining a basic pH by adding portions of 8.2 mL of 4.0 N sodium hydroxide during the addition. After 2 h and 15 min, the progress of the reaction was monitored by TLC in *n*-BuOH:HOAc:H₂O = 3:2:1. Due to the presence of trace amounts of starting amino adipic acid, an additional 1.0 mL (0.25 equiv) of CBZ-Cl in 3.0 mL dioxane was added. Immediately following this addition, 2.0 mL of 4.0 N sodium hydroxide was added to the reaction mixture to maintain basic pH. After incubation at 0 $^\circ\text{C}$ for an additional 3 h, the reaction mixture was allowed to warm up to room temperature. The pH of the reaction mixture was checked, and, as necessary, the pH was adjusted to ~ 10.0 with 4.0 N NaOH during the workup. The reaction mixture was washed two times with ether to remove excess CBZ-Cl, and the ether washes were discarded. The aqueous layer was then acidified with 6.0 N hydrochloric acid to $\sim \text{pH}$ 1.0. The aqueous acidified solution was then extracted with three portions of ethyl acetate. The combined ethyl acetate extracts were then dried over anhydrous sodium sulfate and filtered. The solvent from filtrate was removed under vacuum to concentrate the product to an oil that solidified upon drying. The crude product was recrystallized from hot ethyl acetate:hexane to yield 7.4 g (90.3%) of white crystalline solid, mp 134–135 $^\circ\text{C}$. ^1H NMR (DMSO-*d*₆): δ 1.40–1.80 (m, 4H), 2.10–2.30 (t, 2H), 3.85–4.05 (m, 1H), 5.05 (s, 2H), 7.37 (s, 5H, C₆H₅), 7.64 (d, 1H, NH), 12.36 (s, COOH). IR (KBr, cm^{-1}): 3300, 3000, 2920, 1690, 1520, 1420, 1270, 1240, 1200, 1170, 1050, 900, 750, 700, 670. HRMS: mass calcd for $\text{C}_{14}\text{H}_{17}\text{NO}_6$ ($\text{M} + \text{H}$)⁺, 296.1134; found, 296.1126.

Dibenzyl L- α -*N*-(Carbobenzyloxy)amino adipate (**3**). 6.50 g (22.0 mmol) of *N*-(carbobenzyloxy)amino adipic acid was suspended in 180 mL of benzene containing 4.76 g (44.0 mmol) of benzyl alcohol. To the reaction mixture was added 0.2 g of *p*-toluenesulfonic acid. The reaction mixture was heated overnight under reflux with a Dean-Stark condenser. After 20 h, the reaction mixture turned colorless and homogeneous with about 1.6 mL of water collected. Completion of the reaction was monitored by TLC in 1:1 ethyl acetate/hexane as well as in 96:4 EtOAc/HOAc with spots stained in potassium permanganate. Benzene from the reaction mixture was removed under vacuum. The mixture was taken up into ethyl acetate and washed twice with dilute aqueous sodium bicarbonate followed by brine solution. The organic layer was dried over anhydrous sodium sulfate and filtered. Solvent from the filtrate was removed under reduced pressure to concentrate the product. The crude product was purified by flash chromatography on a silica gel 60 column using 30% ethyl acetate in hexane as an eluting solvent. The fractions containing the product were pooled, and the solvent was removed to yield 9.09 g (87%) desired product as a viscous colorless oil: ^1H NMR (DMSO-*d*₆): δ 1.40–1.90 (m, 4H), 2.30–2.45 (t, 2H), 4.0–4.20 (m, 1H), 5.05 (s, 2H), 5.09 (s, 2H), 5.14 (s, 2H), 7.36 (s, 15H, C₆H₅), 7.84 (d, 1H, NH). IR (NaCl cells, cm^{-1}): 3380, 3060, 3040, 2980, 1740, 1730, 1520, 1470, 1390,

1000, 980, 910, 860, 815, 760, 700, 650. HRMS: mass calcd for $\text{C}_{25}\text{H}_{26}\text{N}_2\text{O}_8$ ($\text{M} + \text{H}$) $^+$, 483.17673; found 483.1767412.

2,2,2-Trichloroethyl N-(Carbobenzyloxy)glycylglycinate (7). 1.0 g (3.76 mmol) of N-(carbobenzyloxy)glycylglycine (**6**) was dissolved in 6.0 mL of anhydrous DMF. To this solution under magnetic stirring was added 0.56 g (3.76 mmol) of 2,2,2-trichloroethanol, followed by 0.91 mL (11.26 mmol) of gold label grade pyridine. To the stirred reaction mixture was added 0.77 g (3.76 mmol) of DCC, followed by 4.0 mL of anhydrous DMF to rinse the DCC vial. The reaction mixture was stirred at room temperature for two days. Precipitated dicyclohexylurea was removed by filtration. Solvent from the filtrate was removed under vacuum leaving a crude brown oil after five days. The crude product was purified by flash chromatography on a silica gel column using 1:1 ethyl acetate:hexane as an elution solvent. Fractions containing the product were combined. Solvent was removed under vacuum to yield 0.86 g (58%) of pure white compound **7**. ^1H NMR ($\text{DMSO}-d_6$): δ 3.71 (d, 2H), 4.05 (d, 2H), 4.95 (s, 2H), 5.07 (s, 2H), 7.39 (s, 5H, C_6H_5), 7.56 (t, 1H, NH), 8.47 (t, 1H, NH).

2,2,2-Trichloroethyl Glycylglycinate (8). 0.2 g (0.503 mmol) of 2,2,2-trichloroethyl N-CBZ-glycylglycinate (**7**) was dissolved in 1.5 mL of glacial acetic acid. This reaction solution was added to a flask containing 2.0 mL of HBr in acetic acid (9.35 mmol, ~30–32 wt % HBr). The reaction mixture was allowed to stand at room temperature for 30 min. The reaction solution darkened without any evidence of carbon dioxide evolution. Following addition of 40 mL of diethyl ether, the reaction mixture was cooled in the freezer for 1 h. The white precipitate that formed was collected by filtration and dried to yield 0.148 g (86%) of pure compound **8**, mp 208 °C dec. ^1H NMR ($\text{DMSO}-d_6$): δ 3.67 (s, 2H), 4.17 (d, 2H), 4.97 (s, 2H), 8.06 (bs, 3H, $^+\text{NH}_3$), 8.93 (t, 1H, NH). IR (KBr, cm^{-1}): 3280, 3200, 3000, 1740, 1660, 1530, 1410, 1370, 1250, 1160, 1100, 1070, 1040, 980, 900, 860, 800, 720, 680, 620. HRMS: mass calcd for $\text{C}_6\text{H}_9\text{N}_2\text{O}_3\text{Cl}_3$ ($\text{M} + \text{H}$) $^+$, 262.97568; found, 262.9757005.

2,2,2-Trichloroethyl δ -Benzyl L- α -N-(carbobenzyloxy)-aminoadipoylglycylglycinate (9). 0.59 g (1.18 mmol) of compound **5** was dissolved in 4.0 mL of dry DMF. To the solution was added 0.406 g (1.18 mmol) of compound **8** followed by 2.0 mL of dry DMF. The reaction solution turned slightly yellow and became homogeneous. With stirring 164 μL of neat triethylamine was added dropwise over a 5 min time period. The reaction mixture was stirred at room temperature for two days. The DMF from the reaction mixture was removed under vacuum. The crude product was purified by flash chromatography on a silica gel column using 75% ethyl acetate–hexane as an eluting solvent. The fractions containing the desired product were pooled, and the solvent was removed under reduced pressure and dried to yield 0.55 g (75%) of product. ^1H NMR ($\text{DMSO}-d_6$): δ 1.45–1.80 (m, 4H), 2.37 (t, 2H), 3.78 (d, 2H), 4.04 (d, 3H), 4.93 (s, 2H), 5.04 (s, 2H), 5.09 (s, 2H), 7.37 (s, 5H, C_6H_5), 7.52 (d, 1H, NH), 8.27 (t, 1H, NH), 8.38 (t, 1H, NH).

2,2,2-Trichloroethyl L- α -Amino- α -adipoylglycylglycinate (10). 0.515 g (0.816 mmol) of compound **9** was dissolved in 2.0 mL of glacial acetic acid. The solution was then transferred to a Parr hydrogenation bottle containing ~100 mg of 5% palladium on activated carbon. The reaction mixture was shaken with hydrogen at 60 psi for 24 h and then filtered through a Celite pad using additional acetic acid to rinse the flask and the Celite pad. Solvent from the filtrate was removed under

reduced pressure yielding a thick residue. The residue was rotary evaporated from heptane three times and then triturated with ether and dried in vacuo to yield 0.39 g (100%) of hygroscopic white solid. ^1H NMR ($\text{DMSO}-d_6$): δ 1.45–1.65 (m, 2H), 1.65–1.80 (m, 2H), 2.25 (t, 2H), 3.70–3.90 (m, 4H), 4.0–4.10 (m, 2H), 4.93 (s, 2H), 8.64 (t, 1H, NH), 8.80–8.95 (m, 2H, NH).

2,2,2-Trichloroethyl L- α -S-(1-Ethoxyethyl)mercaptoacetamido- α -adipoylglycylglycinate (13). 0.34 g (0.729 mmol) of compound **10** was dissolved in 4.0 mL of anhydrous DMF. To the solution was added 0.2 g (0.766 mmol) of compound **12** and the mixture stirred at room temperature until dissolved. Additionally 2.0 mL of anhydrous DMF was added to rinse down the sides of the flask. To the reaction mixture was added 305 μL (2.19 mmol) of triethylamine dropwise over a period of 2 min. The reaction mixture was then stirred at room temperature overnight. The DMF was removed in vacuo. Ethyl acetate was added to the residue and the precipitated solid filtered off. The filtrate was repeatedly washed with 0.1 N HCl until no NHS remained in the organic phase as indicated by TLC in a solvent mixture of $\text{Et}_2\text{O}:\text{CH}_3\text{OH}:\text{HOAc}$ (92:4:4). The organic layer was then washed with brine and dried over anhydrous sodium sulfate. The solution was filtered and concentrated under vacuum to a solid. The solid was triturated with ether, collected by filtration, and dried under vacuum to yield 0.182 g (45%) of product. ^1H NMR ($\text{DMSO}-d_6$): δ 1.097 (t, 3H), 1.414 (2d, 3H), 2.200 (t, 2H), 3.225 (d, 2H), 3.25–3.50 (m, 4H), 3.55–3.70 (d, 1H), 3.70–3.80 (d, 2H), 4.0–4.05 (d, 2H), 4.20–4.35 (m, 1H), 4.70–4.85 (p, 1H), 4.924 (s, 2H), 8.10–8.225 (d, 1H), 8.25–8.45 (m, 2H). IR (KBr, cm^{-1}): 3300, 3280, 2980, 2920, 1760, 1720, 1640, 1540, 1520, 1450, 1400, 1380, 1220, 1180, 1110, 1060, 1030, 940, 900, 850, 820, 790, 730, 660, 630. HRMS: mass calcd for $\text{C}_{18}\text{H}_{28}\text{Cl}_3\text{N}_3\text{O}_8\text{S}$ ($\text{M} + \text{H}$) $^+$, 552.07409; found, 552.0724.

2,2,2-Trichloroethyl α -S-(1-Ethoxyethyl)mercaptoacetamido(L- δ -2,3,5,6-tetrafluorophenyl)adipoylglycylglycinate (14). 0.145 g (0.262 mmol) of compound **13** was dissolved in 4.0 mL methylene chloride. To the solution was added 65 mg (0.393 mmol) of tetrafluorophenol with stirring. 65 mg (0.315 mmol) of dicyclohexylcarbodiimide was added followed by 1.0 mL of methylene chloride to rinse DCC from the vial. The reaction mixture was stirred at room temperature overnight. The dicyclohexyl urea precipitate was removed by filtration, and the solvent from the filtrate was removed under vacuum to concentrate the residue. The crude product was purified by flash chromatography on a silica gel column using $\text{EtOAc}:\text{hexane}:\text{acetic acid}$ at a ratio of 71:25:4 as an eluting solvent. Fractions containing the product were pooled. The solvent from the combined fractions was removed under reduced pressure to yield 114 mg (62%) of desired compound **14**. ^1H NMR ($\text{DMSO}-d_6$): δ 1.111 (t, 3H), 1.447 (t, 3H), 1.50–1.90 (m, 3H), 2.822 (t, 2H), 3.255 (d, 2H), 3.342 (s, 2H), 3.55–3.75 (m, 1H), 3.80–3.90 (d, 2H), 4.0–4.10 (d, 2H), 4.30–4.45 (m, 1H), 4.75–4.90 (p, 1H), 4.934 (s, 2H), 7.85–8.1 (m, 1H), 8.20–8.30 (d, 1H, NH), 8.30–8.50 (m, 2H, NH).

L- δ -2,3,5,6-Tetrafluorophenyl α -S-(1-Ethoxyethyl)mercaptoacetamidoadipoylglycylglycinate (15). 0.107 g (0.15 mmol) of compound **14** was dissolved in 1.0 mL of THF. To the solution was added 203 μL of 1.0 M potassium dihydrogen phosphate, pH 4.26. Then 100 mg of zinc dust was added with stirring at room temperature. The progress of the reaction was monitored by TLC in a solvent mixture of ethyl acetate:acetic acid (96:4). After 30 min, an additional 203 μL of buffer and 100 mg of

zinc dust were added. Stirring was continued for 90 min. The reaction mixture was then filtered through a Celite plug using THF to rinse material from the Celite. Solvent from the filtrate was then removed under reduced pressure and the crude product purified by flash chromatography on a silica gel column using the mixture CH_2Cl_2 :IPA:HOAc at a ratio of 75:20:5 as eluting solvent. Fractions containing the product were pooled, and the solvent was removed. The resulting purified residue was rotatory evaporated three times from heptane to remove the residual acetic acid and dried in vacuo overnight. The dried residue was then triturated with diethyl ether and allowed to stand for a few minutes, yielding a fine white precipitate. The supernatant ether layer was carefully decanted and residual solvent was removed under reduced pressure. This dried residue was then triturated with fresh ether. The resulting combined white solid was dried in vacuo to yield 63 mg (72%) of pure product. ^1H NMR ($\text{DMSO}-d_6$): δ 1.090 (2t, 3H), 1.43 (2d, 3H), 1.50–1.90 (m, 4H), 2.81 (t, 2H), 3.24–3.26 (d, 2H), 3.50–3.70 (2Q, 2H), 3.69 (s, 2H), 3.76 (s, 2H), 4.25–4.45 (m, 1H), 4.70–4.85 (Q, 1H), 7.85–8.05 (m, 1H, aromatic), 8.09 (t, 1H, NH), 8.23 (d, 1H, NH), 8.34 (t, 1H, NH). MS (CI): 570 ($M + 1$). Anal. Calcd for $\text{C}_{22}\text{H}_{27}\text{N}_3\text{O}_8\text{SF}_4$: C, 46.40, H, 4.78, N, 7.38, S, 5.63. Found: C, 46.29, H, 4.73, N, 7.15, S, 5.38.

2,2,2-Trichloroethyl *L*- δ -2,3,5,6-Tetrafluorothiophenyl α -S-(1-Ethoxyethyl)mercaptoacetamidopoylglycylglycinate (**16**). ^1H NMR ($\text{DMSO}-d_6$): δ 1.118 (t, 3H), 1.426 (t, 3H), 1.45–1.80 (m, 3H), 2.895 (t, 2H), 3.224–3.238 (d, 2H), 3.324 (d, 2H), 3.55–3.75 (m, 1H), 3.70–3.80 (d, 2H), 4.0–4.10 (d, 2H), 4.25–4.40 (m, 1H), 4.70–4.85 (p, 1H), 4.914 (s, 2H), 8.05–8.25 (m, 2H, NH), 8.30–8.50 (m, 2H, NH).

L- δ -2,3,5,6-Tetrafluorothiophenyl α -S-(1-Ethoxyethyl)mercaptoacetamidopoylglycylglycine (**17**). The synthesis of compound **17** is similar to compounds **14** and **15** except for using tetrafluorothiophenol in place of tetrafluorophenol to activate the carboxylic acid as described in **14**. ^1H NMR ($\text{DMSO}-d_6$): δ 1.10 (2t, 3H), 1.45 (2d, 3H), 1.50–1.85 (m, 4H), 2.85–3.0 (t, 2H), 3.20–3.25 (d, 2H), 3.25–3.50 (m, 2H), 3.50–3.80 (2Q, 4H), 4.20–4.45 (m, 1H), 4.70–4.90 (P, 1H), 8.05–8.25 (m, 1H, aromatic), 8.15–8.25 (2d, 2H, NH), 8.25–8.40 (t, 1H, NH). Anal. Calcd for $\text{C}_{22}\text{H}_{27}\text{N}_3\text{O}_7\text{S}_2\text{F}_4$: C, 45.12, H, 4.65, N, 7.18, S, 10.95. Found: C, 45.16, H, 4.54, N, 6.97, S, 11.0.

Formulation of N_3S -Adipate-STFP Reagent. The N_3S -adipate-STFP prelyophilized reagent was formulated as a 1 mL aqueous solution containing 100 μg of N_3S -adipate-STFP, 2.5 mg of sodium gluconate, 0.1 mg of stannous chloride, 0.1 mg of gentisic acid, 40 mg of lactose filler, and 26% v/v *tert*-butyl alcohol. During preparation, N_3S -adipate-STFP was dissolved at 350 $\mu\text{g}/\text{mL}$ in an $\text{H}_2\text{O}/\text{tert}$ -butyl alcohol solution (1:9) and added to an aqueous concentrate (1.4 $\times$) of the remaining ingredients adjusted to pH 1.8 with sulfuric acid. The solution was vialled in grade A borosilicate 10 mL vials fitted with 25 mm diameter gray butyl rubber lyophilization stoppers and frozen at -40°C within 30 min of preparation. Lyophilization proceeded at 50–100 m torr vacuum with maintenance of product below a collapse temperature of -25°C for 23 h for primary drying. Secondary drying proceeded upon $0.3^\circ\text{C}/\text{min}$ ramping to -12°C , holding for 16 h, followed by ramping in like manner to 25°C and holding for 20 h. Afterwards, the vials were back-filled with nitrogen to 6 in. of Hg and stoppered. Final moisture content was $<0.4\%$ as assessed by Karl Fischer assay.

Formulation of NR-LU-10 Fab Lyophilizate. NR-LU-10 Fab fragment was prepared at 20 mg/mL in 20 mM sodium citrate buffer, pH 6.2, containing 1% sorbitol as cryogenic stabilizer. Following aseptic filtration through a 0.2 μm Sartorius filter, the solution was vialled in grade A borosilicate 2 mL vials. The vials were then fitted with 13 mm diameter gray butyl rubber lyophilization stoppers and quick frozen in liquid nitrogen prior to placement on lyophilization chamber shelves cooled to -40°C . A vacuum of 50–100 m torr was applied to achieve primary sublimation over the next 17 h. Secondary drying proceeded upon ramping the temperature at $0.5^\circ\text{C}/\text{min}$ to 25°C and holding at this temperature for the next 16 h. Afterward, the vials were back-filled with nitrogen to 6 in. of Hg and stoppered. Moisture content as assessed by Karl Fischer assay was $<1\%$.

$^{99\text{m}}\text{Tc}-\text{N}_3\text{S}$ NR-LU-10 Fab Purification Column Design. Purification of the final radiolabeled protein reaction mixture utilized a custom designed plastic cartridge packed with 5 mL of QAE Sephadex anion exchange resin conditioned in 37 mM sodium phosphate, pH 6.8, at time of packing. The cartridge included a top leir lock to accommodate leir syringe loading of the radioactive reaction mixture and a bottom leir locked 25 mm diameter Sartorius filter of 0.2 μm pore size. The cartridge contained an internal channeling spoke for uniform distribution of applied reaction mixture to the resin bed. The entire assembly was sterilized by autoclaving in a Chex-all sterilizing pouch.

Preparation of $^{99\text{m}}\text{Tc}-\text{N}_3\text{S}$ -NR-LU-10 Fab Conjugate. Radiolabeling of $^{99\text{m}}\text{Tc}$ antibody Fab conjugate by the kit procedure (Scheme 2) involved the preparation of $^{99\text{m}}\text{Tc}-\text{N}_3\text{S}$ -adipate-STFP and then conjugation to the protein followed by purification using a small QAE-Sephadex column.

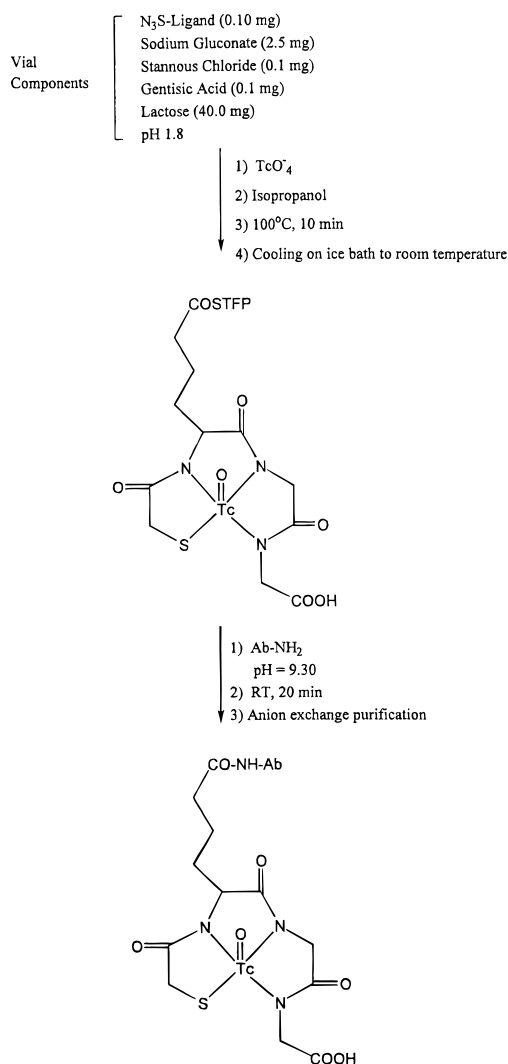
To prepare $^{99\text{m}}\text{Tc}$ N_3S -adipate-STFP, 1.0 mL of [$^{99\text{m}}\text{Tc}$] pertechnetate in saline (100 mCi) was added to the lyophilized N_3S -adipate-STFP reagent. The vial was shaken to dissolve contents and then vented to the air. Following addition of 0.4 mL of 2-propanol, the reaction vial was mixed, heated to 100°C for 10 min, and then cooled on ice.

While the radiolabeled active ester cooled, 10 mg of lyophilized protein was reconstituted in 2.0 mL of 0.25 M carbonate buffer, pH 9.3. The protein solution was then added to the $^{99\text{m}}\text{Tc}-\text{N}_3\text{S}$ -adipate-STFP reaction vial and incubated for 20 min at room temperature. The $^{99\text{m}}\text{Tc}-\text{N}_3\text{S}$ -NR-LU-10 Fab conjugate was purified by loading onto the QAE-Sephadex cartridge and subsequently eluting with 5 mL of normal saline (150 mM NaCl) solution.

Challenge Studies of $^{99\text{m}}\text{Tc}-\text{N}_3\text{S}$ -NR-LU-10 Fab Conjugate. The stability of $^{99\text{m}}\text{Tc}-\text{N}_3\text{S}$ -NR-LU-10 Fab was tested by incubation at 37°C for 2 h in the presence of various challenging agents. They included human serum (diluted to 50%), 100 mM DTPA, 250 mM carbonate, 10 mM cysteine, BSA, human transferrin, and 8 M urea. Samples were removed after 2 h and analyzed for radiochemical purity by either ITLC or by size exclusion chromatography.

Radiolabeled Cell Binding Assay. The immunoreactivity of $^{99\text{m}}\text{Tc}-\text{N}_3\text{S}$ -NR-LU-10 Fab was assessed in an antigen-excess cell binding assay and compared to NR-LU-10 Fab radiolabeled with ^{125}I -PIP reagent (**14**) as previously described (**15**).

Biodistribution Studies of $^{99\text{m}}\text{Tc}$ -labeled NR-LU-10 Fab. Time course biodistribution studies at 4 and 20 h postinjection were conducted separately with ^{125}I and $^{99\text{m}}\text{Tc}$ labeled NR-LU-10 Fab in nude mice bearing

Scheme 2. ^{99m}Tc Labeling and Ab Conjugation with N_3S -adipate-STFP

LS-180 colon carcinoma xenografts (16). Three to four mice per timepoint were injected intravenously via lateral tail vein with $10\ \mu\text{g}$ of protein in a $100\ \mu\text{L}$ volume. The volume of injectate was determined by weighing the syringe before and after injection. At the designated times post-injection, mice were weighed, bled via the retro-orbital plexus, and sacrificed by cervical dislocation. Thirteen organs and tissues were collected: blood (BL), tail (TA), tumor (TU), skin (SK), muscle (MU), bone (BO), lung (LU), liver (LI), spleen (SP), stomach (ST), neck (NE), kidneys (KI), and intestine (IN). Tissues were blotted when appropriate, weighed, and counted in a γ scintillation counter with the counting window set from 80 to 200 keV.

RESULTS

As seen in the synthesis of N_3S -adipate-TFP active ester ligands shown in Scheme 1, several steps involved novel or nonroutine peptide synthesis precursor derivatives to incorporate an adipate active ester moiety in the N_3S amide thiolate ligand. Selective protection of the δ -carboxylate group of adipic acid was achieved via differential hydrolysis of the dibenzyl ester. This allowed subsequent preparation of an NHS active ester at the α -carboxylate group. Trichloroethyl ester protected diglycine was then coupled to the NHS adipate derivative. Afterwards, N-CBZ and benzyl ester groups were re-

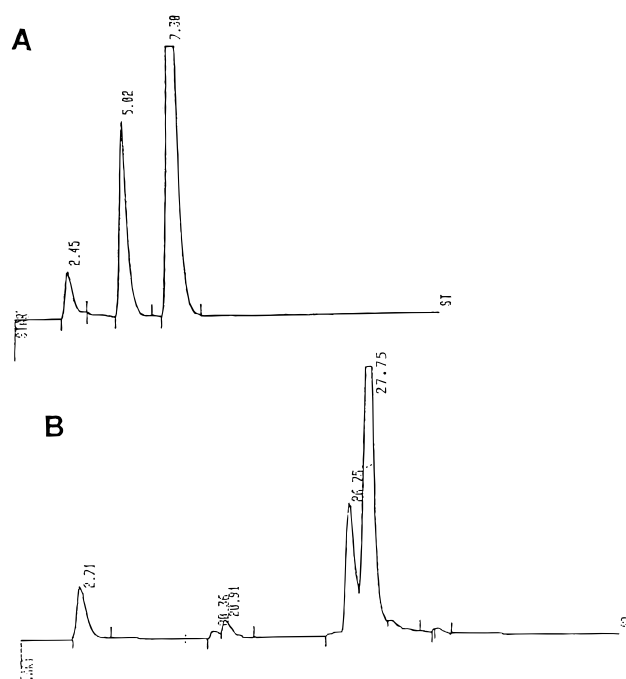


Figure 1. HPLC reverse phase isocratic (A) and gradient (B) chromatograms of ^{99m}Tc - N_3S -adipate-STFP reaction mixture. A: Retention peak at 2.45 min is a mixture of ^{99m}Tc -gluconate and ^{99m}Tc - N_3S -adipate acids while retention peaks of 5.02 and 7.30 min are ^{99m}Tc - N_3S -adipate active esters in syn/anti-epimers resulting from adipate with reference to the ^{99m}Tc oxo group (19). B: Retention peak at 2.71 min is ^{99m}Tc -gluconate; retention peaks at 20.36 and 20.91 min are syn/anti-epimers of the free acids, and retention peaks at 26.75 and 27.75 min are the syn/anti-epimers of the active esters.

moved in one step by catalytic hydrogenation. The resulting primary amine was then coupled in good yield to the S-ethoxyethyl-protected mercaptoacetate via its NHS-active ester. Final modifications to the ligand were formation of the desired TFP active ester followed by selective removal of the trichloroethyl ester protecting group using zinc dust in 1.0 M potassium hydrogen phosphate buffered solution at pH 4.26.

The ^{99m}Tc radiolabeling of the N_3S -adipate-TFP active ester requires reduction of the starting ^{99m}Tc pertechnetate, formation of an exchange ^{99m}Tc gluconate complex, transchelation of ^{99m}Tc to the N_3S -adipate-TFP active ester, conjugation of the active ester metal complex to the protein, and finally purification of the resultant product (Scheme 2). Various parameters were studied in order to optimize yields as well as minimize steps and time required for the radiolabeling process.

Stannous gluconate was used for the initial steps of pertechnetate reduction and formation of exchange gluconate chelate. Upon addition of the N_3S -adipate-TFP ligand and heating, the S-ethoxyethyl group was cleaved concomitant with ^{99m}Tc transfer into the N_3S -adipate active ester. Yields of the ^{99m}Tc chelate active ester were monitored by HPLC using isocratic and gradient systems. Elution profiles are shown in Figure 1. As the ligand contains a carboxylic acid group, it was expected that its polarity would increase water solubility, negating the need for an organic solvent such as isopropyl alcohol (IPA) (4). However, it was found that yields increased from 58% at no added IPA to 90% at 28% IPA. Low pH facilitated the loss of the ethoxyethyl hemithioacetal sulfur protecting group during chelation of the amide thiolate donor atom set. Little change in yield was noted over the pH range of 1.70–2.30. Sulfuric acid was chosen

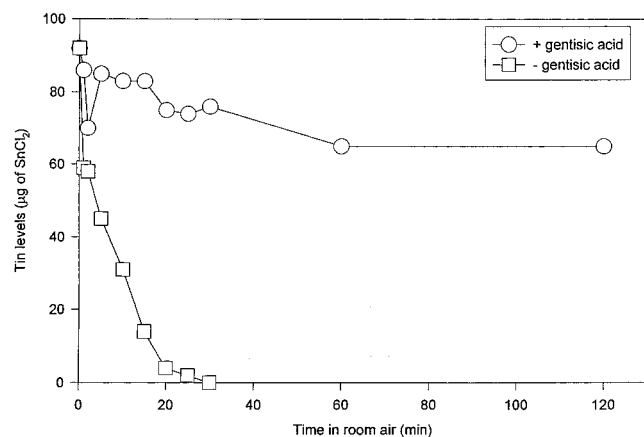


Figure 2. Stability of stannous ion in N_3S -adipate-STFP transfer agent solutions (prelyophilized).

Table 1. ^{99m}Tc Complexation as a Function of Mass of N_3S -Adipate STFP and Carrier-Added ^{99}Tc

ligand level (μg)	% ^{99m}Tc - N_3S -Adipate STFP		
	100 mCi ^a	10^{-5} M ($^{99}TcO_4^-$) ^b	10^{-6} M ($^{99}TcO_4^-$) ^c
25	76.7	26.8	85.3
50	75.4	37.1	84.9
75	75.0	38.3	84.5
100	84.1	56.4	83.2
220	87.1	63.3	83.4

^a "No-carrier-added" first elution of pertechnetate from the generator. ^b Carrier-added pertechnetate in the preparations at 10^{-5} M. ^c Carrier-added pertechnetate in the preparations at 10^{-6} M.

to adjust the pH of the prelyophilized solution of stannous gluconate and N_3S -adipate-TFP ligand since strong acid maintained pH control during the subsequent lyophilization process. Stannous ion in the prelyophilized solution was quite unstable to air (Figure 2). To stabilize this reagent during the manufacturing process, gentisic acid was incorporated at 100 μg per mL of prelyophilized solution. The time required for chelation at 100 °C was also studied. Yields increased over the first 5 min, plateaued from 6 to 10 min, and then decreased upon further heating due to ester hydrolysis. Finally, the amount of ligand needed for good ester yields was determined using 100 mCi of pertechnetate from normal $^{99}Mo/^{99m}Tc$ generator elution as well as activity spiked with $^{99}TcO_4^-$ at 10^{-5} and 10^{-6} M (Table 1). At the no carrier added and 10^{-6} M levels, little dependency was seen down to a level of 25 μg N_3S -adipate-STFP ligand. As would be expected with the 10^{-5} M level of added carrier ^{99}Tc , yields increased in a stoichiometric fashion between 25 μg to 220 μg of N_3S -adipate-STFP ligand. A level of 100 μg ligand was sufficient to chelate 5.6 nmol of $^{99}TcO_4^-$, well above that found in normal generator use. For the above parameters, no significant differences in yields of purity were seen for the OTFP and STFP active esters.

Protein Labeling with ^{99m}Tc - N_3S -Adipate-TFP Active Esters. The parameters of pH, amount of protein added, and time were evaluated. In the earlier work on the pentanoate N_2S_2 kit (4), optimal yields of protein conjugate with the oxo active ester (OTFP) were obtained at pH 10. While this high pH appears to be tolerated by antibodies and their fragments, for other applications it is desirable to carry out this step at a lower pH. Consequently the thio ester of N_3S -adipate (N_3S -adipate-STFP) was synthesized and evaluated. The dependency

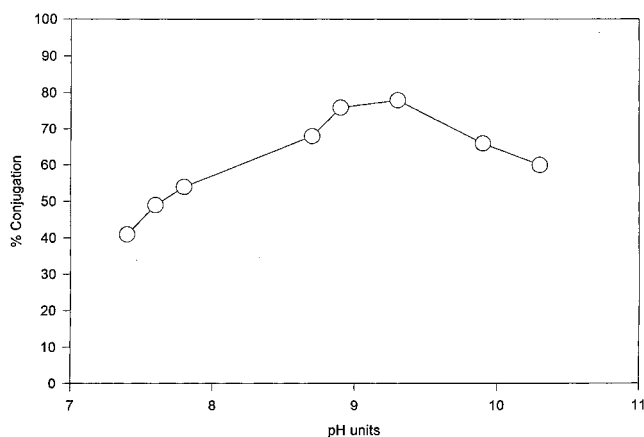


Figure 3. Conjugation of ^{99m}Tc - N_3S -adipate-STFP with NR-LU-10 Fab fragment as a function of pH.

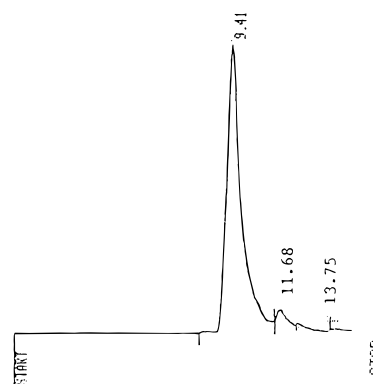


Figure 4. Radiochromatogram of ^{99m}Tc - N_3S -adipate-NR-LU-10 Fab on Zorbax HPLC following anion exchange purification. Elution conditions: 0.2 M sodium phosphate (pH 7.0) at 1.0 mL/min.

of antibody conjugation yields on pH show a rise from 40% at pH 7.5 to greater than 70% at pH 9.2. Beyond this pH, yields fell off due to competing hydrolysis (Figure 3). In comparison, the optimal yield for the N_3S oxo ester (OTFP) was at pH 10. Conjugation yields increased with increasing Fab amount, rising from 40% at 2 mg to above 70% at 10 mg. Using 10 mg of Fab fragment, conjugation required 20 min incubation at room temperature for optimal yields.

Purification of the ^{99m}Tc conjugate was carried out using a closed system anion exchange QAE Sephadex cartridge column as described (4). The ^{99m}Tc forms including gluconate, pertechnetate, and N_3S carboxylates were retained on the column while the protein conjugate was eluted at neutral pH. The radiochemical purity by ITLC was 90–96% and protein recovery was $\geq 85\%$.

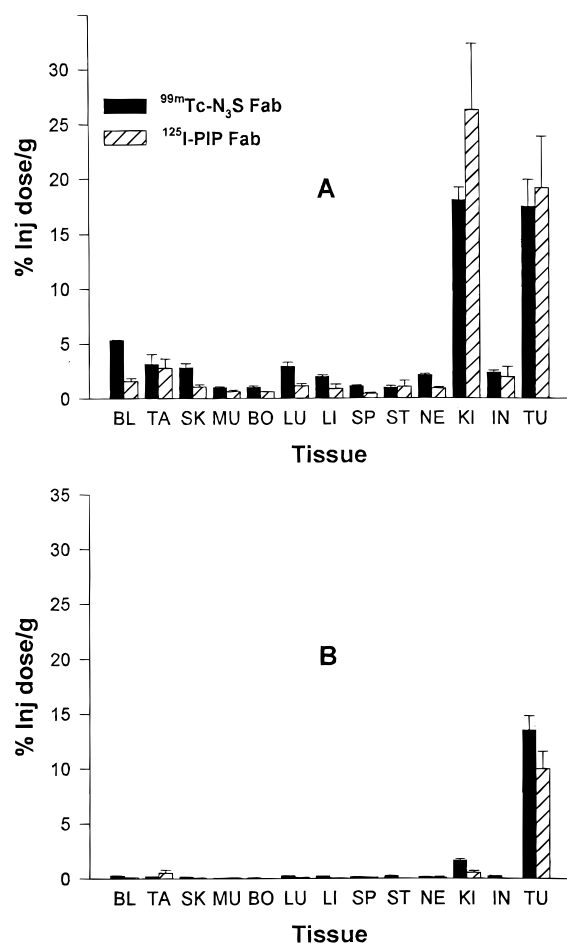
In Vitro Analysis. The ^{99m}Tc - N_3S -NR-LU-10 Fab conjugates were analyzed by various techniques. A size exclusion HPLC radiochromatogram (Figure 4) shows that greater than 90% of the ^{99m}Tc is attached to protein, eluting at a 45 kD molecular size. Of the later eluting non-protein bound radioactive species, 90% was ^{99m}Tc - N_3S -carboxylate and 10% was ^{99m}Tc -gluconate. Immunoreactivity assessment of the conjugate, using the Lindmo cell binding assay at antigen excess (15) showed 70% compared to 75% for the ^{125}I -PIP labeled conjugate standard.

The *in vitro* stability was evaluated by incubation of radiolabeled protein with various challenge conditions (Table 2). Insignificant loss of radioactivity after 2 h at 37 °C was seen with buffer (pH 9), DTPA, serum, and 8

Table 2. Stability of ^{99m}Tc -N₃S-Adipate Binding to NR-LU-10 Fab

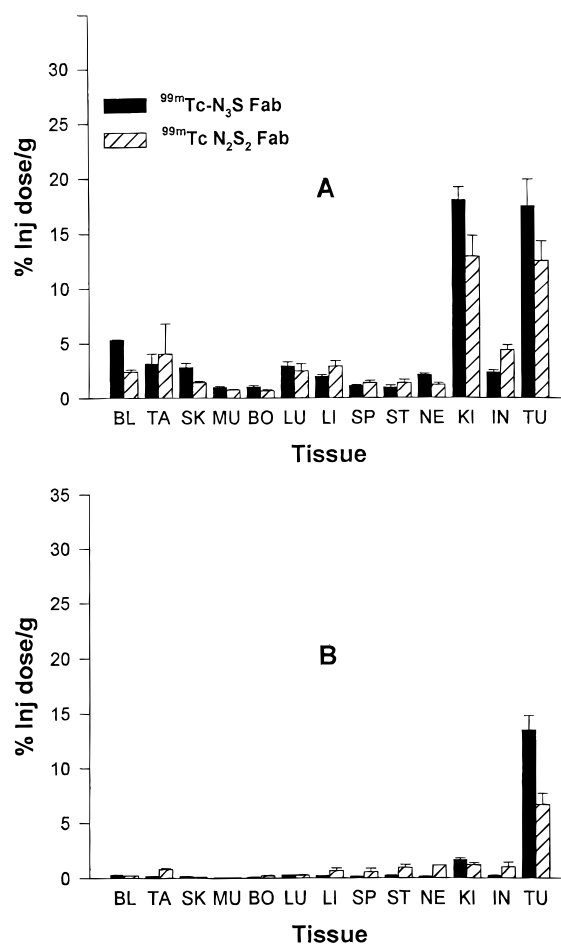
challenge agent and conditions ^a	% ^{99m}Tc -N ₃ S-adipate bound to NR-LU-10 Fab ^b
none (i.e. ^{99m}Tc -labeled NR-LU-10 Fab of 1.2 mg/mL of PBS)	90.8
PBS (10 mM sodium phosphate, 150 mM NaCl, pH 7.2)	91.5
0.25 M carbonate, pH 8.0	89.5
0.25 M carbonate, pH 9.0	87.6
10 mM cysteine, pH 8.0	77.0
100 mM DTPA, pH 8.0	92.2
8.0 M urea in PBS	91.0
1% BSA in PBS	91.0
50% human serum	90.6
human transferrin (0.5 mg/mL PBS)	91.0

^a Two hours at 37 °C; unless otherwise noted the concentration of ^{99m}Tc N₃S-adipate NR-LU-10 Fab conjugate in the challenge solutions is 0.3 mg/mL. ^b ITLC values in 12% trichloroacetic acid.

**Figure 5.** Biodistribution of ^{99m}Tc -N₃S NR-LU-10 Fab and ^{125}I -PIP NR-LU-10 Fab in tumored nude mice. Panels A and B depict 4 h and 20 h timepoints, respectively.

M urea. A loss of 14% versus control was seen from challenge at a relatively high concentration of 10 mM cysteine.

In Vivo Evaluation. The tissue biodistribution and tumor targeting ability of the ^{99m}Tc -N₃S-adipate NR-LU-10 Fab was compared with ^{125}I -PIP labeled NR-LU-10 Fab in mice bearing LS-180 xenograft colon carcinoma tumors at 4 (panel A) and 20 (panel B) h postinjection (Figure 5). In normal tissues, 20 h levels are much lower, reflecting blood disappearance of radioactivity. As the kidney is the major organ processing the Fab fragment,

**Figure 6.** Biodistribution of ^{99m}Tc -N₃S NR-LU-10 Fab and ^{99m}Tc -N₂S₂ NR-LU-10 Fab in tumored nude mice. Panels A and B depict 4 h and 20 h timepoints, respectively.

the levels are relatively high in this organ for both preparations at the 4 h timepoint, but are reduced by the 20 h timepoint. In contrast, tumor uptake is about 18% ID/g at 4 h and still nearly 14% at 20 h resulting in ~40:1 tumor to blood and other organ ratios. Although, the radioiodinated Fab control was somewhat lower in tumor at 20 h post-injection, the difference is not meaningful with the number of animals at that time point.

A comparison was also made between ^{99m}Tc N₃S Fab and N₂S₂ Fab (Figure 6) at 4 (panel A) and 20 (panel B) h post-injection. Activity levels are similar in normal tissues at all timepoints. The tumor radioactivity levels are higher for the ^{99m}Tc -N₃S-adipate labeled Fab at 20 h but due to interstudy tumor uptake differences, the observed difference is not considered meaningful.

DISCUSSION

The N₃S-adipate ligand described in this study for the attachment of ^{99m}Tc to proteins represents changes in both donor atoms and an added carboxylate group over the first generation adaptation of N₂S₂ amide thiolate ligands as bifunctional chelating agents (3, 4). These changes are in the direction of added hydrophilic character of the chelating agent as well as the ^{99m}Tc chelate and ^{99m}Tc protein catabolites. It was expected that such changes would result in an all aqueous system for preformed chelate ^{99m}Tc labeling resulting in deletion of the IPA organic solvent component of the earlier N₂S₂ kit (4). Further, it was expected that reduced intestinal radioactivity would result following catabolism of the antibody Fab fragment.

Kit simplification was achieved by combining stannous reducing agent, exchange chelate gluconate, and N_3S -adipate TFP active ester chelating agent in a single vial at acid pH. The "cosolvent" isopropyl alcohol, however, was found to be essential in maintaining high yields of the intermediate chelate active ester. Thus, the process for production of the $^{99\text{m}}\text{Tc}$ chelate active ester required only the addition of pertechnetate, IPA, and heating. This contrasts with the earlier N_2S_2 kit that involved six separate components and five transfers (4).

The conjugation of the N_3S -adipate-TFP active ester chelate was improved by using a more reactive thio TFP ester rather than the oxo analog. This change allowed the conjugation to be done at a lower pH range of 8.5 to 9.5. This pH is a milder condition for base labile proteins. It also reduced both the amount of buffer needed to raise the pH for conjugation and final pH adjustment needed for radiolabeled protein purification via the anion exchange column cartridge.

The *in vitro* evaluation indicated that a $^{99\text{m}}\text{Tc}$ protein conjugate of high purity can be obtained. The resulting $^{99\text{m}}\text{Tc}$ radiolabeled protein was stable to a variety of challenge conditions except for 10 mM cysteine which caused a partial loss of radioactivity from the protein after 2 h at 37 °C. Under these conditions, no loss was seen with N_2S_2 bifunctional chelate. It should be noted that normal cysteine concentrations in serum are 0.1–0.2 mM, 50 to 100 times lower than the 10 mM concentration used here. Thus, the cysteine challenge may be seen as an indicator of relative stability.

Although the N_3S ligand appears to result in somewhat less stable attachment of $^{99\text{m}}\text{Tc}$ to protein, the *in vivo* evaluation studies in a mouse colon carcinoma xenograft model showed the $^{99\text{m}}\text{Tc}$ – N_3S antibody conjugates to have excellent tumor uptake and retention without significant accumulation in normal organs, being slightly lower than the N_2S_2 conjugate in liver, spleen, and intestine. In rat studies in which blood time course levels were compared to radioiodinated standards, the $^{99\text{m}}\text{Tc}$ – N_3S conjugates showed similar pharmacokinetics, indicating that the N_3S amide thiolate chelating group has sufficient stability for *in vivo* conditions (11).

More detailed studies of a series of related N_3S ligands (11) indicated that in the rat model, hepatobiliary excretion was decreased by about 30% due to the additional carboxylate group and thus afforded improved imaging capability in the lower abdomen. However, the added charge resulted in increased levels of radioactivity in the kidneys which would be a concern for application of these charged ligands for ^{186}Re or ^{188}Re radioimmunotherapy (17).

Limited unpublished clinical studies with the N_3S Fab conjugate show decreased lower abdominal radioactivity compared to the N_2S_2 Fab conjugate (7, 8).

CONCLUSION

The $^{99\text{m}}\text{Tc}$ labeling of proteins such as antibody Fab fragments can be done efficiently with carboxylate-substituted N_3S ligands. A formulation was developed which combines the stannous reductant, gluconate exchange chelating agent, and ligand into one vial for simpler preparation of the $^{99\text{m}}\text{Tc}$ chelate active ester. The performance of this ligand shows promise in animal and human tumor imaging studies as well as when used for the $^{99\text{m}}\text{Tc}$ radiolabeling of IL2 for autoimmune disease evaluation (18).

ACKNOWLEDGMENT

The authors acknowledge the assistance of other NeoRx personnel for immunoassays, antibody fragmentations, purification, lyophilizations, and animal biodistribution studies. In addition, they thank Andrea Lynn Fahlenkamp for the preparation of this manuscript.

LITERATURE CITED

- (1) Davison, A., Jones, A. G., Orvig, C., and Sohn, M. (1981) A new class of oxotechnetium (5+) chelate complexes containing a TcON_2S_2 core. *Inorg. Chem.* 20, 1629–1632.
- (2) Kasina, S., Fritzberg, A. R., Johnson, D. L., and Eshima, D. (1986) Tissue distribution properties of technetium-99m-diamide-dimercaptide complexes and potential use as renal radiopharmaceuticals. *J. Med. Chem.* 29, 1933–1940.
- (3) Fritzberg, A. R., Abrams, P. G., Beaumier, P. L., Kasina, S., Morgan, A. C., Rao, T. N., Reno, J. M., Sanderson, J. A., Srinivasan, A., Wilbur, D. S., and Vanderheyden, J.-L. (1988) Specific and stable labeling of antibodies with technetium-99m with a diamide dithiolate chelating agent. *Proc. Natl. Acad. Sci. U.S.A.* 85, 4025–4029.
- (4) Kasina, S., Rao, T. N., Srinivasan, A., Sanderson, J. A., Fitzner, J. N., Reno, J. M., Beaumier, P. L., and Fritzberg, A. R. (1991) Development and biologic evaluation of a kit for preformed chelate technetium-99m radiolabeling of an antibody Fab fragment using a diamide dimercaptide chelating agent. *J. Nucl. Med.* 32, 1445–1451.
- (5) Blend, M. J., Hyun, H., Patel, B., Sullivan, K., and Salk, D. (1996) Radioimmunoscinigraphy in patients with early stage cutaneous malignant melanoma. *J. Nucl. Med.* 37, 252–257.
- (6) Balaban, E. P., Walker, B. S., Cox, J. V., Bordlee, R. P., Salk, D., Abrams, P. G., Sheehan, R. G., and Frenkel, E. P. (1992) Detection and staging of small cell lung carcinoma with a technetium-labeled monoclonal antibody. A comparison with standard staging methods. *Clin. Nucl. Med.* 17, 439–445.
- (7) Vansant, J. P., Johnson, D. H., O'Donnell, D. M., Stewart, J. R., Sonin, A. H., McCook, B. M., Powers, T. A., Salk, D. J., Frist, W. H., and Sandler, M. P. (1992) Staging lung carcinoma with a Tc-99m labeled monoclonal antibody. *Clin. Nucl. Med.* 17, 431–438.
- (8) Friedman, S., Sullivan, K., Salk, D., Nelp, W. P., Griep, R. J., Johnson, D. H., Blend, M. J., Aye, R., Suppers, V., and Abrams, P. G. (1990) Staging non-small cell carcinoma of the lung using technetium-99m-labeled monoclonal antibodies. *Hematol. Oncol. Clin. North Am.* 4, 1069–1078.
- (9) Stratton, J. R., Dewhurst, T. A., Kasina, S., Reno, J. M., Cerqueira, M. D., Baskin, D. G., and Tait, J. F. (1995) Selective uptake of radiolabeled annexin V on acute porcine left atrial thrombi. *Circulation* 92, 3113–3121.
- (10) Axworthy, D. B., Kasina, S., Rao, T. N., Srinivasan, A., and Fritzberg, A. R. (1991) Metabolite analysis, biliary and urinary excretion of Tc-99m- N_2S_2 preformed chelate labeled anti-melanoma monoclonal antibody Fab fragment in mice and rats. *J. Nucl. Med.* 32, (abstr) 915.
- (11) Srinivasan, A., Kasina, S., Fitzner, J. N., Gustavson, L. G., Reno, J. M., Rao, T. N., Sanderson, J. A., Gray, M. A., Axworthy, D. B., and Fritzberg, A. R. (1990) Modified bifunctional amide thiolate ligands; enhanced utility of Tc-99m Fab for radioimmunodetection. *J. Nucl. Med.* 31, (abstr) 747.
- (12) Bjorn, M. J., Manger, R., Sivan, G., Morgan, A. C., and Torok-Storb, B. (1990) Selective elimination of breast cancer cells from human bone marrow using an Antibody-Pseudomonas exotoxin A conjugate. *Cancer Res.* 50, 5992.
- (13) Okabe, T., Kaizu, T., Fujisawa, M., Watanabe, J., Kojima, K., Yamashita, T., and Takaku, F. (1984) Monoclonal antibodies to surface antigens of small cell carcinoma of the lung. *Cancer Res.* 44, 5273–5278.
- (14) Wilbur, D. S., Hadley, S. W., Hylarides, M. D., Abrams, P. G., Beaumier, P. L., Morgan, A. C., Reno, J. M., and Fritzberg, A. R. (1989) Development of a stable radioiodinating reagent to label monoclonal antibodies for radiotherapy of cancer. *J. Nucl. Med.* 30, 216–226.

- (15) Lindmo, T., Boven, E., Cuttitta, F., Fedorko, J., and Bunn, P. A., Jr. (1984) Determination of the immunoreactive fraction of radiolabeled monoclonal antibodies by linear extrapolation to binding at infinite antigen excess. *J. Immunol. Meth.* 72, 77–89.
- (16) Tom, B. H., Rutzky, L. P., Oyasu, R., Tomita, J. T., Goldenberg, D. M., and Kahan, B. D. (1977) Human colon adenocarcinoma cells. II. Tumorigenic and organoid expression in vivo and in vitro. *J. Natl. Cancer Inst.* 58(5), 1507–1512.
- (17) Su, F. M., Axworthy, D. B., Vanderheyden, J. L., Srinivasan, A., Fitzner, J. N., Galster, J., Kasina, S., Reno, J. M., Beaumier, P. L., and Fritzberg, A. R. (1991) Evaluation of a cleavable N₃S-hydroxyl ester ligand for improved targeting of Rhenium-186 labeled antibodies. *J. Nucl. Med.* 32, (abstr) 1020.
- (18) Chinanelli, M., Signore, A., Sobnack, R., Fritzberg, A. R., Britton, K. E., and Mather, S. J. (1994) Tc-99m-Interleukin-2: A new radiopharmaceutical for the *in vivo* detection of lymphocytic infiltration. *Technetium Rhenium Chem. Nucl. Med.* 4, 551–555.
- (19) Costello, C. E., Brodack, J. W., Jones, A. G., Davison, A., Johnson, D. L., Kasina, S., and Fritzberg, A. R. (1983) The investigation of radiopharmaceutical components by fast atom bombardment mass spectrometry: The identification of Tc-HIDA and the epimers of Tc-CO₂DADS. *J. Nucl. Med.* 24, 353–355.

BC970047I

Effect of Biodegradable Polyrotaxanes on Platelet Activation

Nobuhiko Yui,* Tooru Ooya, and Takashi Kumeno

School of Materials Science, Japan Advanced Institute of Science and Technology, 1-1 Asahidai, Tatsunokuchi, Ishikawa 923-12, Japan. Received March 10, 1997; Revised Manuscript Received October 27, 1997[®]

Cellular response to our designed biodegradable polyrotaxanes was evaluated in terms of physicochemical interaction with plasma membrane and intracellular metabolism of platelets. The polyrotaxanes, in which many hydroxypropylated (HP-) α -cyclodextrins are threaded onto a poly(ethylene glycol) chain capped with a L-phenylalanine moiety via a peptide linkage, were synthesized and characterized. The polyrotaxanes inhibited cytoplasmic calcium increase in platelets, increased plasma membrane fluidity of red blood cell ghosts, and elevated cytoplasmic cyclic-3',5'-AMP levels in platelets. Such cellular response to the polyrotaxanes was observed, which was more than that to constituent molecules. These results suggest that the supramolecular structure of the polyrotaxanes contributes to acceleration of the physicochemical interaction with plasma membrane and intracellular metabolism of platelets. Thus, biodegradable polyrotaxanes can be useful as new biomaterials for fabricating blood-contacting devices.

INTRODUCTION

Current studies in supramolecular sciences have explored fascinating fields by constructing nanoscale devices as biomimetic systems (1, 2). A polyrotaxane has been recently demonstrated to be a molecular necklace in which many α -cyclodextrins (α -CDs) are threaded onto a poly(ethylene glycol) (PEG) chain capped with bulky end groups (3, 4). From the viewpoint of a polymeric drug carrier aiming for therapeutic application, we previously synthesized a series of biodegradable polyrotaxanes in which hydroxypropylated (HP) α -CDs are threaded onto a PEG chain capped with L-phenylalanine (L-Phe) moieties via peptide linkages (5–7). It was found that the release of HP- α -CDs was achieved only when the terminal peptide moiety was degraded by a hydrolytic enzyme (7). The cellular response to synthetic polymers is of fundamental importance to their possible therapeutic application as pharmacologically active polymers and polymeric carriers for biologically active agents (8). In our previous study, we examined interaction of the polyrotaxanes with hairless rat stratum corneum to demonstrate the feasibility of them being a transdermal penetration enhancer (9, 10). The polyrotaxanes were found to interact with lipid components in the stratum corneum (9) and to enhance the permeation of indomethacin through a full-thickness rat skin (10). Considering these results, such polymers may interact with cellular systems in a certain period until the polymers disappear due to hydrolysis.

Thrombogenesis has been shown to be initiated via both activation pathways of coagulation factors and platelets. Platelet response to synthetic polymers forms an important investigative basis for the design of blood compatible polymers (11, 12). It is well-known that platelet activation by extracellular stimuli such as thrombin is mediated by an elevation of cytoplasmic free

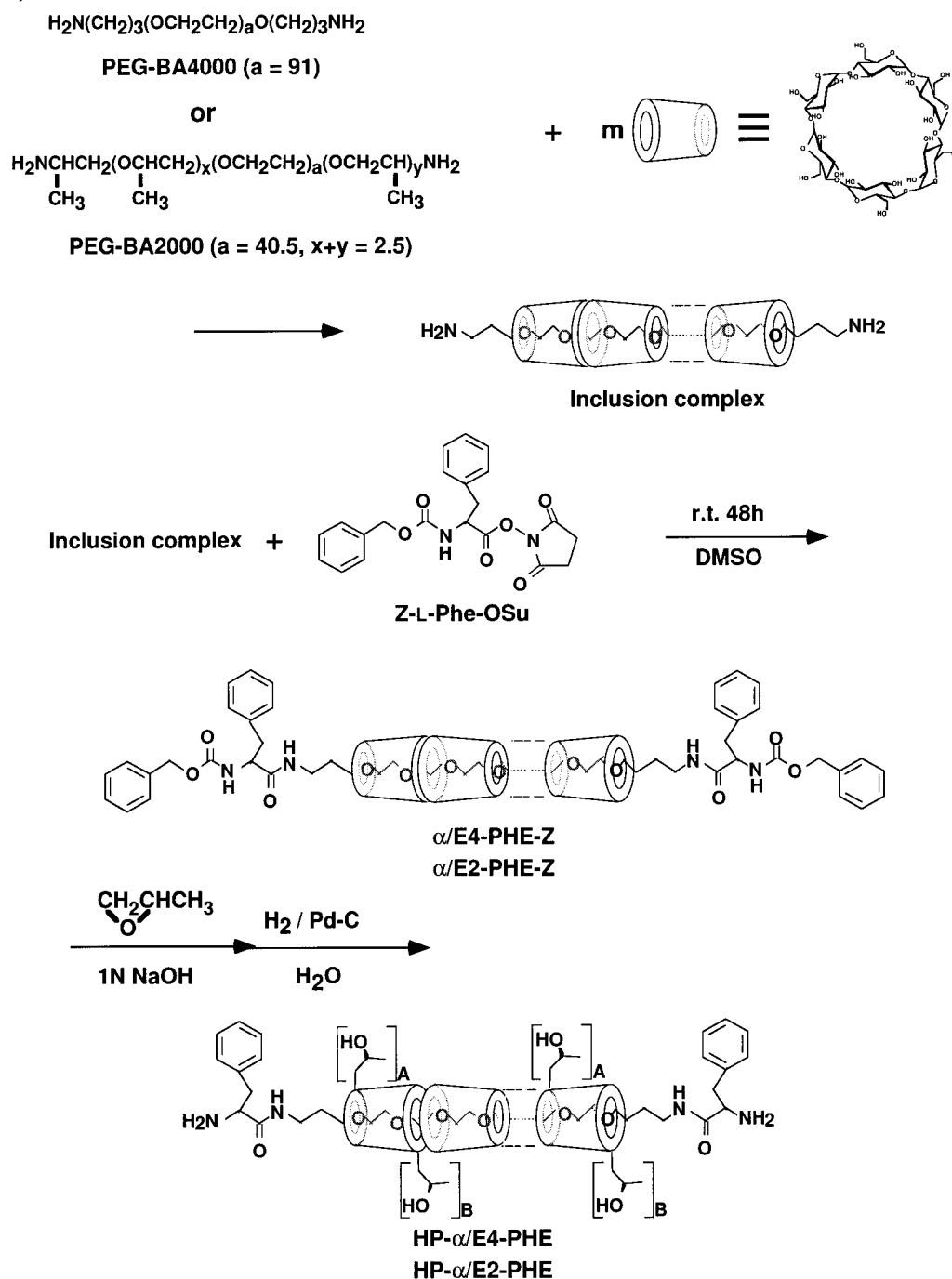
calcium concentration ($[Ca^{2+}]_i$) in relation to decreased membrane fluidity (13). Our systemic studies concerning blood-contacting properties of synthetic polymer surfaces have demonstrated that the prevention of contact-induced activation of platelets is a dominant factor for designing nonthrombogenic surfaces (14–16). Through these studies, it has been clarified that nonthrombogenic polymer surfaces showed no increase in $[Ca^{2+}]_i$ but enhanced membrane fluidity of platelets (11, 12). Thus, it is suggested that the regulation of intracellular calcium levels in platelets is enhanced by contacting the nonthrombogenic surfaces. In this paper, we report on cellular response to our designed polyrotaxanes by inhibiting thrombin-induced platelet activation. It was found that the polyrotaxanes inhibit thrombin-induced cytoplasmic calcium increase in platelets, increase plasma membrane fluidity of red blood cell ghosts, and, to a certain extent, elevate cytoplasmic cyclic-3',5'-AMP levels in platelets. This finding has many applications in polymeric supramolecular assemblies for constructing blood-contacting devices.

EXPERIMENTAL PROCEDURES

Chemicals. α -Cyclodextrin (α -CD), (benzyloxy-carbonyl)(Z)-L-phenylalanine (L-Phe), and propylene oxide were purchased from Wako Pure Chemical Co. Ltd. (Osaka, Japan) and used as received. *N*-Hydroxysuccinimide (HOSu) was purchased from Peptide Institute, Inc. (Osaka, Japan) and used as received. α -[(2-Amino-2-methylethyl)-x-oxypropyl]- ω -(amino-y-oxypropyl)polyoxyethylene ($x + y = 2.5$) with a number-average molecular weight (M_n) of 2000 (PEG-BA2000) was kindly supplied from Suntechno Chemical Co. (Tokyo, Japan) as JEF-FAMINE ED-2001. α -(3-Aminopropyl)- ω -(3-aminopropyl)polyoxyethylene with an M_n of 4000 (PEG-BA4000) was kindly supplied by Sanyo Chemical Co. Ltd. (Kyoto, Japan) as IONET YB-400. Fura 2-AM [1-[2-(5'-carboxyoxazol-2'-yl)-6-aminobenzofuran-5-oxy]-2-(2'-amino-5'-methylphenoxy)ethane-*N,N,N',N'*-tetraacetic acid pentaacetoxymethyl ester] was purchased from Dojin Chemical Co. (Kumamoto, Japan). Thrombin from bovine plasma (5000 units/bottle) was purchased from

* Author to whom correspondence should be addressed. Phone: +81-761-51-1621. Fax: +81-761-51-1625. E-mail: yui@jaist.ac.jp.

[®] Abstract published in *Advance ACS Abstracts*, December 15, 1997.

Scheme 1. Synthesis of Biodegradable Polyrotaxanes in Which the $\bar{M}_n$ of PEG Is 4000 (HP- α /E4-PHE) and 2000 (HP- α /E2-PHE)^a

^a A $\approx$ 6 and B $\approx$ 2 will be assumed when the degree of HP substitution is ca. 8.

Sankyo Co. Ltd. (Tokyo, Japan). 1,6-Diphenyl-1,3,5-hexatriene (DPH) was purchased from Wako Pure Chemical Co. Ltd. Bicinchoninic acid (BCA) protein assay reagents were purchased from Pierce (Rockford, IL). Unless otherwise noted, all chemicals were purchased from Wako Pure Chemical Co. Ltd.

Synthesis of the Polyrotaxanes. Two types of biodegradable polyrotaxanes, in which the $\bar{M}_n$ of PEGs was 2000 and 4000 (Scheme 1), were synthesized according to our previous method (5–7). The synthesis of the polyrotaxanes was as follows. The inclusion complex of α -CD and PEG-BA2000 or PEG-BA4000 was prepared as reported previously (3, 4). Z-L-Phe succinimide (Z-L-Phe-OSu), which was prepared as reported previously (17), was dissolved in dry DMSO, followed by the addition

of the inclusion complex. Then, DMSO was added to the suspension until homogeneous conditions were reached and the mixture stirred at room temperature for 48 h. After washing with acetone and water to remove unreacted Z-L-Phe-OSu and the inclusion complex, the obtained polyrotaxanes (α /E2-PHE-Z or α /E4-PHE-Z) were allowed to react with propylene oxide in a 1 N NaOH solution at room temperature for 24 h. After neutralizing with HCl solution, the solution was dialyzed against water and lyophilized. Finally, the Z group in α /E2-PHE-Zs was deprotected under a H_2 atmosphere with palladium-carbon to obtain hydroxypropylated polyrotaxanes (HP- α /E2-PHE and HP- α /E4-PHE) (yield, 40%).

As reference samples, HP- α -CD and L-Phe-terminated PEGs (E2-PHE and E4-PHE) were prepared as follows.

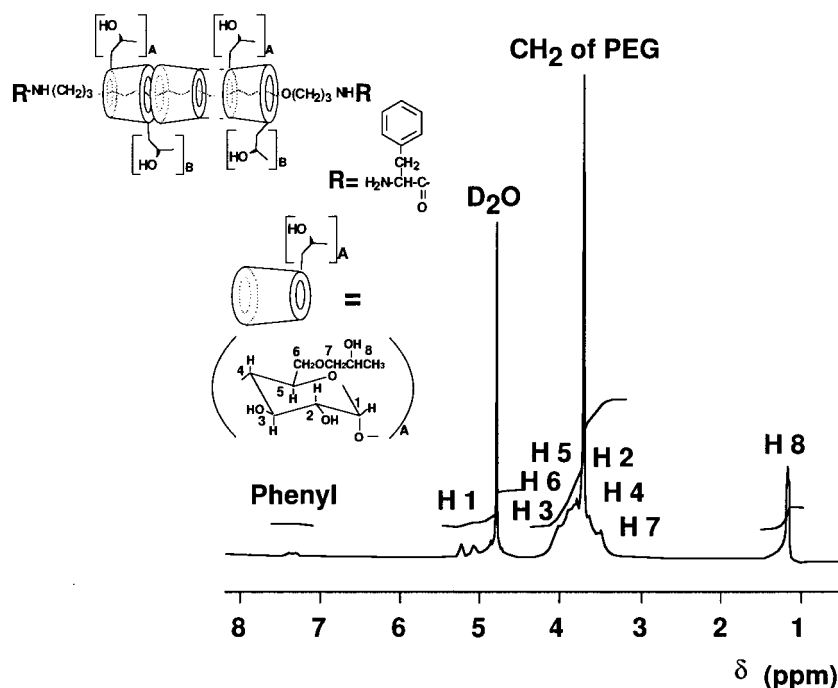


Figure 1. ^1H -NMR spectrum of HP- α /E4-PHE (in D_2O).

α -CD was hydroxypropylated by the same manner used in the case of the polyrotaxanes. E2-PHE and E4-PHE were synthesized using Z-L-Phe-OSu and PEG-BA2000 or PEG-BA4000 in DMF. The reaction mixture was poured into excess dry ether and then washed with ether to obtain pure E2-PHE or E4-PHE. The purity was checked by ^1H -NMR and GPC (yield of HP- α -CD, 64%; yield of E2-PHE, 82%; and yield of E4-PHE, 88%).

Characterization of the Polyrotaxanes. The polyrotaxanes were characterized by ^1H -NMR and high-performance size exclusion chromatography (SEC) (5–7). From ^1H -NMR spectra of both HP- α /E2-PHE and HP- α /E4-PHE, the peaks of α -CDs, PEG, L-Phe, and hydroxypropyl groups were observed. A representative ^1H -NMR spectrum of HP- α /E4-PHE is shown in Figure 1. ^1H -NMR (D_2O): δ 7.50–7.20 (m, 5H, phenyl of L-Phe), 5.22, 5.07 (s, 6H $\times$ 23, C-1 H of α -CD), 4.20–3.35 (m, 18H $\times$ 23, C-3 H, C-6 H, C-5 H of α -CD, 3H $\times$ 184, CH₂ of hydroxypropyl groups), 3.72 (s, 4H $\times$ 90, CH₂ of PEG), 1.18 (d, 3H $\times$ 184, CH₃ of hydroxypropyl groups). The average number of α -CDs threaded onto a PEG chain was determined to be ca. 11 for HP- α /E2-PHE and ca. 23 for HP- α /E4-PHE from the ^1H -NMR, comparing the integration of the signals at δ 5.22 and 5.07 (C-1 H of α -CD) with those at δ 3.72 (CH₂ of PEG). With comparison of the integration of the signals at δ 5.22 and 5.07 (C-1 H of α -CD) with those at δ 1.18 (CH₃ of hydroxypropyl groups), the average ratio of hydroxypropylation per α -CD molecule was determined to be ca. 8 in HP- α /E2-PHE, HP- α /E4-PHE, and HP- α -CD.

SEC was performed on a column of G3000SW (30 $\times$ 2.5 cm, Tosoh, Tokyo, Japan). A HPLC system (two-line DG-980–51 degasser, Intelligent HPLC PU-980 pump, Column CO-965 oven, and Chiral OR-990 detector, Jasco, Tokyo, Japan) was used at a flow rate of 1.0 mL/min at 40 $^\circ\text{C}$. The eluent was 0.05 mol/L phosphate buffer (PBS, pH 7.0) containing 0.3 mol/L NaCl. The sample solution was prepared by dissolving HP- α /E2-PHE or HP- α /E4-PHE (4 mg) in the eluent (1 mL) and passing the solution through a filter (0.45 μm). $\bar{M}_n$ and the weight-averaged molecular weight ($\bar{M}_w$) of HP- α /E2-PHE and HP- α /E4-

PHE were determined from the SEC spectra using the PEG standard.

In order to confirm the supramolecular structure (the threading of HP- α -CDs onto a PEG chain) of the polyrotaxanes, the *in vitro* degradation experiment of the polyrotaxanes by papain was carried out as follows. HP- α /E2-PHE or HP- α /E4-PHE (3.3×10^{-3} mmol) was dissolved in 10 mL of a citrate buffer (5 mmol/L citric acid, 58 mmol/L Na_2HPO_4 , 2 mmol/L ethylenediamine-tetraacetic acid (EDTA), and 10 mmol/L mercaptoethanol at pH 7.1), followed by the addition of papain (9.5 mg, 200 units per 10 mL), and stirred at 37 $^\circ\text{C}$. The reaction mixture (0.1 mL) was sampled at an appropriate time and diluted with water (0.1 mL), followed by heating to precipitate papain. The hydrolysis of the terminal L-Phe moiety and the release of HP- α -CDs were calculated by the result of HPLC analysis [SEC columns, G2500PW_{XL} (Tosoh) for monitoring the hydrolysis of the terminal L-Phe moiety and G3000SW (Tosoh) for monitoring the release of HP- α -CD measurements] (7). As to the reference experiment, the similar *in vitro* degradation of the polyrotaxanes without papain was carried out.

Measurement of Cytoplasmic Calcium Change in Platelets. A change in the cytoplasmic free calcium concentration ($[\text{Ca}^{2+}]_i$) in platelets in contact with the polyrotaxanes was examined using a Fura 2-AM-loaded platelet suspension (11, 12). A platelet suspension in Ca^{2+} - and Mg^{2+} -free Hanks' balanced salt solution (HBSS) (platelet concentration, $3 \times 10^8/\text{mL}$) was prepared from citrated blood of male Japanese white rabbits, weighing 2.5–3.0 kg. Fura 2-AM was loaded into platelets by incubating the platelet suspension with a Fura 2-AM solution at 37 $^\circ\text{C}$ for 60 min at a Fura 2-AM concentration of 5 mM. The platelets were washed with HBSS and were finally resuspended in HBSS so that the final platelet concentration was $3 \times 10^8/\text{mL}$. The platelet suspension was recalcified with CaCl_2 so that the external calcium concentration was 1 mM just prior to use in the $[\text{Ca}^{2+}]_i$ measurements.

A $[\text{Ca}^{2+}]_i$ in platelets was measured according to the method of Tsien et al. (18). HP- α /E2-PHE or HP- α /E4-

PHE in HBSS (10 wt %, 100 μ L) was mixed with a Fura 2-AM-loaded platelet suspension (400 μ L) in a fluorescence cuvette in a fluorimeter (Japan Spectroscopic Co., Tokyo, Japan, CAF-110) at 37 °C with magnetic stirring. The mixture was excited at both 340 and 380 nm, and emission was measured at 500 nm. Fluorescence intensities at each of the two wavelengths were used to determine the fluorescence dichroic ratio 340/380 (R). $[Ca^{2+}]_i$ was calculated on the basis of the R value using the following equation (18):

$$[Ca^{2+}]_i = K_d[(R - R_{min})/(R_{max} - R)]S_{f2}/S_{b2}$$

where K_d is the dissociation constant for Ca^{2+} binding to the fluorescent dye, R_{min} and R_{max} are ratios of fluorescence emission intensities at the selected excitation wavelengths (340 and 380 nm, respectively, with very low and very high calcium concentrations, and S_{f2} and S_{b2} are proportionality coefficients for free dye and Ca^{2+} -bound dye measured at the longer excitation wavelength (380 nm), respectively. The K_d for the Ca^{2+} -Fura 2 interaction is assumed to be 224 nM in the cytoplasmic environment. R_{min} , R_{max} , and S_{f2}/S_{b2} were measured under the same conditions by adding 10 μ L of a 1% Triton-X aqueous solution and then 10 μ L of a 3 M ethylene glycol bis(β -aminoethyl ether)- N,N,N',N' -tetraacetic acid (EGTA) aqueous solution. As a control, 100–200 nM $[Ca^{2+}]_i$ in unused platelets is checked prior to the mixing with the solution.

Thrombin was used as an agonist of platelets in order to evaluate the effect of the polyrotaxanes on the platelet activation. Thrombin in HBSS (4 units/mL, 10 μ L) was added to the platelet suspension 1 min after mixing with the polyrotaxane solution (final thrombin concentration, 0.1 unit/mL). The magnitude of the increase in $[Ca^{2+}]_i$ in platelets, $\Delta[Ca^{2+}]_i$, was determined by the following equation:

$$\Delta[Ca^{2+}]_i = ([Ca^{2+}]_i)_{thr} - ([Ca^{2+}]_i)_0$$

where $([Ca^{2+}]_i)_{thr}$ is the $[Ca^{2+}]_i$ in platelets after adding thrombin in HBSS and $([Ca^{2+}]_i)_0$ is the $[Ca^{2+}]_i$ in platelets before adding thrombin in HBSS. In order to minimize time-dependent effects on platelet functions or leakage of Fura 2-AM, these experiments were completed within 1 h of Fura 2-AM loading. In the same manner, $[Ca^{2+}]_i$ was calculated for constituent molecules of the polyrotaxanes and their mixture.

Fluorescence Polarization Measurement of DPH-Loaded Red Blood Cell (RBC) Ghosts. Hemoglobin-free RBC ghosts were prepared according to Dodge et al. (19). Citrated blood of male Japanese white rabbits weighing 2.5–2.8 kg was collected and centrifuged at 1000 rpm for 15 min to obtain RBCs. RBCs were washed with a 0.155 M NaCl solution at pH 7.4 and centrifuged at 1000 rpm for 10 min two times. The suspension was washed with 15–20 volumes of 0.01 M PBS at pH 7.4 several times. After centrifugation (18 000 rpm for 30 min at 4 °C), the RBC ghost suspension was adjusted at 0.1 mg of protein/mL with a 0.155 M NaCl solution at pH 7.4 by a micro BCA method (20). DPH solution in tetrahydrofuran (2 mM) was diluted into the RBC ghost suspension to 2 μ M and incubated with gentle agitation at 37 °C for 60 min in the dark (21).

Fluorescence anisotropy of DPH in RBC ghosts was measured in order to assess the membrane fluidity (22). Fluorescence emission spectra (360–500 nm) of DPH-loaded RBC ghosts were checked at an excitation wavelength of 360 nm. The polyrotaxane solution in 0.155 M

NaCl at pH 7.4 (10 wt %, 100 μ L) was mixed with 400 μ L of the DPH-loaded RBC ghost suspension in a fluorescence cuvette in a spectrofluorimeter (Japan Spectroscopic Co., FP-777) equipped with a fluorescence polarization accessory (Japan Spectroscopic Co., ADP-300) at 37 °C with magnetic stirring. DPH was excited at 360 nm, and the fluorescence was detected at 430 nm. The slit widths for both excitation and emission were 10 nm. Fluorescence intensities were measured with polarizers inserted into the excitation and emission light paths. Fluorescence anisotropy, $\langle r \rangle$, was calculated with the following equation (22):

$$\langle r \rangle = (I_H - I_{Hb}) - G(I_V - I_{Vb}) / (I_H - I_{Hb}) + 2G(I_V - I_{Vb}), \quad G = I_V'/I_H'$$

where I_H and I_V are emission intensities observed with the analyzing polarizer horizontal and vertical with respect to the polarized excitation beam. I_{Hb} and I_{Vb} are fluorescence intensities for a blank solution (0.155 M NaCl) at the same position of the polarizer as I_H and I_V . G is a correction factor, equal to I_V'/I_H' , the primes indicating excitation polarized in a horizontal direction.

In the same manner, $\langle r \rangle$ was measured for constituent molecules of the polyrotaxanes and their mixture.

Platelet Cyclic-3',5'-AMP Assay. Cytoplasmic cyclic-3',5'-AMP (cyclic AMP) levels ($[cyclic AMP]_i$) in platelets were measured by means of a ^3H -labeled cyclic AMP assay according to the method reported by Beretz et al. (23). HP- α /E2-PHE or HP- α /E4-PHE solution (0.1 g/mL, 200 μ L) in HBSS was mixed with the platelet suspension (3×10^8 /mL, 800 μ L) in HBSS at 37 °C for 1 or 2 min. Cold trichloroacetic acid (50%, 100 μ L) was added to the suspension 1 or 2 min after the polyrotaxane addition to stop the cellular metabolism. The suspension was then centrifuged at 12 000 rpm for 1 min. The supernatant was decanted and trichloroacetic acid extracted by five washings with 3 volumes of water-saturated ether. Cyclic AMP was then measured in the supernatant by the protein binding method of Gilman (24) using a commercial kit containing [^3H]cyclic AMP (Yamasa Co., Chiba, Japan).

RESULTS AND DISCUSSION

Synthesis and Characterization of the Polyrotaxanes. The hydroxypropylation of α -CDs improved the solubility of the polyrotaxanes in PBS at pH 7.4. For example, the solubility of HP- α /E4-PHE was 85 wt %, whereas that of HP- α /E2-PHE was 28 wt % in spite of similar degree of HP substitution (eight per α -CD) (7). On SEC curves of HP- α /E2-PHE and HP- α /E4-PHE, a single peak which was detected by both optical rotation (detection of α -CD) and absorbance at 280 nm (detection of the terminal L-Phe moiety) was obtained (Figure 2). Taking the obtained ^1H -NMR spectra of the polyrotaxanes into account, the observed single peak may indicate that α -CDs are threaded onto a PEG chain with the L-Phe moiety at both terminals. $\bar{M}_n$, $\bar{M}_w$, and $\bar{M}_w/\bar{M}_n$ of the polyrotaxanes were summarized in Table 1. $\bar{M}_n$ values of HP- α /E2-PHE and HP- α /E4-PHE were determined to be 1.29×10^4 and 1.06×10^4 , respectively. These values were smaller than the calculated $\bar{M}_n$ values (1.81×10^4 for HP- α /E2-PHE and 3.75×10^4 for HP- α /E4-PHE). Since these $\bar{M}_n$ and $\bar{M}_w$ values were calculated by both $\bar{M}_n$ and $\bar{M}_w$ values of PEG maintaining the random coil structure, the $\bar{M}_n$ value of the polyrotaxanes may not be accurately estimated by SEC. Furthermore, the $\bar{M}_w$ value of HP- α /E2-PHE was larger than that of HP- α /E4-

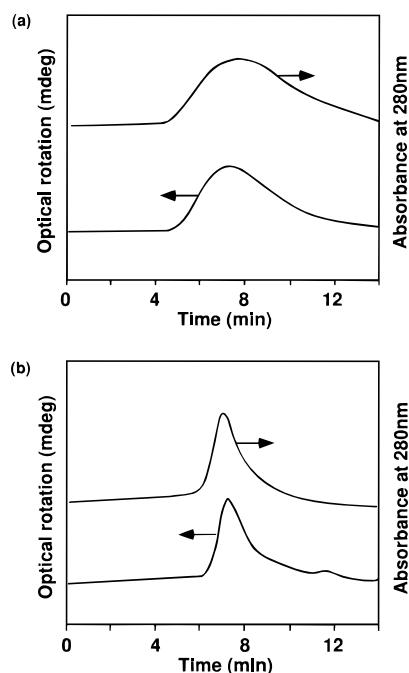


Figure 2. SEC curves of polyrotaxanes (a) HP- α /E2-PHE and (b) HP- α /E4-PHE. Column, Tosoh G3000SW 4.4 $\times$ 30 cm; eluent, 0.05 M PBS (pH 7.0) containing 0.3 M NaCl; flow rate, 1 mL/min.

Table 1. Molecular Weight of the Polyrotaxanes^a

polyrotaxane	$\bar{M}_n (\times 10^4)$	$\bar{M}_w (\times 10^4)$	$\bar{M}_w/\bar{M}_n$
HP- α /E2-PHE ^b	1.29	3.42	2.65
HP- α /E4-PHE ^b	1.06	1.23	1.16

^a Molecular weights of the polyrotaxanes were determined by SEC as described in Experimental Procedures. A calibration curve was prepared with the PEG standard. ^b The number of α -CD in HP- α /E2-PHE and HP- α /E4-PHE was determined by ¹H-NMR to be ca. 11 and 23, respectively.

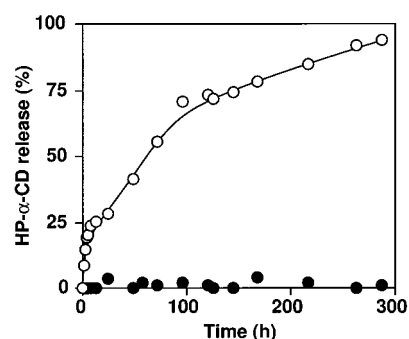


Figure 3. HP- α -CD release from HP- α /E4-PHE with (○) and without (●) 20 units/mL papain. HP- α -CD was determined by HPLC analysis (flow rate, 1 mL/min; detect, optical rotation).

PHE (Table 1). Recently, the $\bar{M}_w$ value of the polyrotaxanes was determined by static light scattering (SLS) measurements, and the $\bar{M}_w$ value of HP- α /E2-PHE was found to be larger than that of HP- α /E4-PHE (25). From these results, it is suggested that the larger $\bar{M}_w$ value of HP- α /E2-PHE was caused by the association of HP- α /E2-PHE molecules under a physiological condition (25).

Figure 3 shows HP- α -CD release from HP- α /E4-PHE by the hydrolysis of the terminal L-Phe moiety with or without papain *in vitro*. It was found that HP- α -CD was released only in the presence of papain. A similar phenomenon was observed in the case of HP- α /E2-PHE. In our previous study, it has been clarified that the HP- α -CD release was dominated by the hydrolysis of the

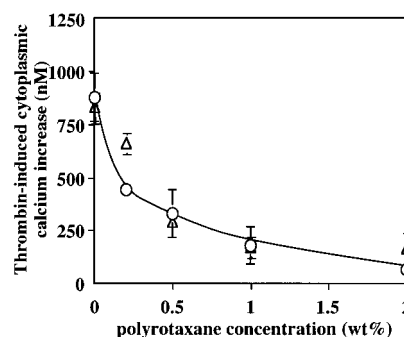


Figure 4. Relationship between the polyrotaxane concentration and thrombin-induced cytoplasmic calcium increase in platelets ($n = 4$, $\pm$ SEM): HP- α /E2-PHE (○) and HP- α /E4-PHE (Δ).

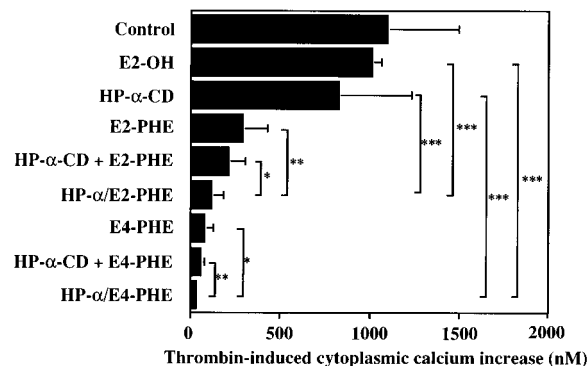


Figure 5. Inhibition of thrombin-induced cytoplasmic calcium increase in platelets by the polyrotaxanes ($n = 5$, $\pm$ SEM). The concentration of constituent molecules (HP- α -CD + E-PHEs, E-PHEs, HP- α -CD, and E2-OH) is equal to the mole percentage in the polyrotaxanes. Entries marked with *, **, and *** were significantly different at $P < 0.1$, $P < 0.05$, and $P < 0.001$, respectively, calculated using the *t*-test.

terminal L-Phe moiety (7). Therefore, the HP- α -CD release by the enzymatic hydrolysis indicates the threading of HP- α -CDs onto the PEG chain; i.e. the polyrotaxanes form a supramolecular structure.

Effect of the Polyrotaxanes on Cytoplasmic Calcium Change in Platelets. With the viewpoint of fabricating blood-contacting devices with these supramolecular-structured surfaces, platelet response to the polyrotaxanes will be the most important basis for preventing thrombus formation. Figure 4 shows a dose-response relationship between polyrotaxane concentration and thrombin-induced increase in $[Ca^{2+}]_i$. From this result, the most effective concentration of the polyrotaxanes (2 wt %) was selected to compare with the constituent molecules of the polyrotaxanes. With this concentration, an increase in $[Ca^{2+}]_i$ was not observed without thrombin, indicating no cytotoxicity and cellular activation (25). The thrombin-induced increase in $[Ca^{2+}]_i$ was significantly decreased by the addition of HP- α /E2-PHE or HP- α /E4-PHE solution, as shown in Figure 5. As to reference samples, a mixture of HP- α -CD and L-Phe-terminated PEG (HP- α -CD + E2-PHE or HP- α -CD + E4-PHE), E2-PHE, and E4-PHE showed a lower inhibitory effect on $[Ca^{2+}]_i$ increase, although HP- α -CD and PEG (E2-OH) showed no inhibitory effect (Figure 5). These results indicate that the polyrotaxanes significantly inhibited thrombin-induced platelet activation. Further, in order to confirm the inhibitory effect of the polyrotaxanes on thrombin-induced activation process, a similar experiment was carried out using sodium azide (NaN_3)-treated platelets. Here, NaN_3 was added to the recalcified platelet suspension to 40 mM, followed by incubation at 37 $^{\circ}C$ for 5 min prior to the addition of the polyrotax-

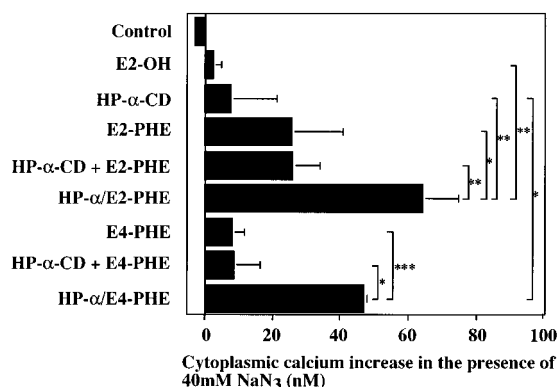


Figure 6. Effect of sodium azide on thrombin-induced cytoplasmic calcium increase in platelets by the polyrotaxanes ($n = 3$, $\pm$ SEM). Entries marked with *, **, and *** were significantly different at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively, calculated using the t -test.

ane solution or the constituent molecules. The polyrotaxanes showed a significant effect on $[Ca^{2+}]_i$ increase in NaN_3 -treated platelets compared with the constituents (Figure 6). It is recognized that NaN_3 inhibits the platelet metabolism via regulation of ATP production in cytoplasmic mitochondria (26). As far as platelet metabolism is inhibited, the interaction of the polyrotaxanes with plasma membranes may induce the increase in $[Ca^{2+}]_i$. Thus, it is suggested that regulation of $[Ca^{2+}]_i$ in platelets is enhanced by physicochemical interaction of the polyrotaxanes, the mechanism of which is metabolically directed.

Quite recently, static light scattering of the polyrotaxane solutions under a physiological condition was measured (25, 27). Rod-like structure and/or associated natures of these polyrotaxanes were observed, and the association was found to depend upon the hydrophilic-hydrophobic balance of threaded HP- α -CDs and terminal L-Phe moieties. It was found that HP- α /E2-PHE showed a loosely packed association nature under a physiological condition, although HP- α /E4-PHE showed a little association. E2-PHE and E4-PHE showed a much more closely packed association nature in the physiological condition, and HP- α -CD and E2-OH showed no association (25). From these characteristics, it is considered that supramolecular structure (rod-like structure) of HP- α /E4-PHE contributes to regulating platelet functions. In addition, supramolecular association of HP- α /E2-PHE may also contribute to physicochemical interaction with cell membrane. Our previous studies have demonstrated that poly(acrylamide-co-methacrylic acid) (PAAMMAC) microparticles exhibit a similar effect on platelet activation (11, 12). Molecular assemblies seen in the polyrotaxanes as well as PAAMMAC particles may induce local interaction with plasma membrane and/or membrane proteins.

Changes in Membrane Fluidity of RBC Ghosts Caused by the Polyrotaxanes. It has been well reported that any physicochemical changes in plasma membrane such as membrane fluidity change are related to platelet activation (13). The effect of the polyrotaxanes on the plasma membrane fluidity was examined using a DPH-loaded rabbit RBC ghost suspension. A significant decrease in $\langle r \rangle$ was observed when the HP- α /E2-PHE solution was mixed with the RBC ghost suspension (Figure 7). Here, the $\langle r \rangle$ decrease indicates an increase in membrane fluidity. It was found that the addition of HP- α -CD + E2-PHE, E2-PHE, HP- α -CD, and E2-OH showed a much smaller decrease in $\langle r \rangle$ than HP- α /E2-

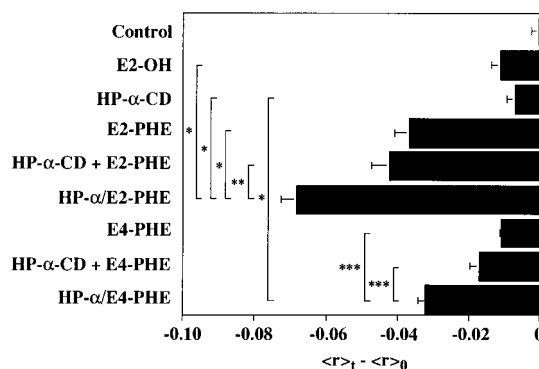


Figure 7. Changes in anisotropy of DPH fluorescent polarization in red blood cell (RBC) ghosts by the polyrotaxanes ($n = 3$, $\pm$ SEM). The concentration of constituent molecules (HP- α -CD + E-PHEs, E-PHEs, HP- α -CD, and E2-OH) is equal to the mole percentage in HP- α /E-PHEs. Entries marked with *, **, and *** were significantly different at $P < 0.05$, $P < 0.01$, and $P < 0.005$, respectively, calculated using the t -test.

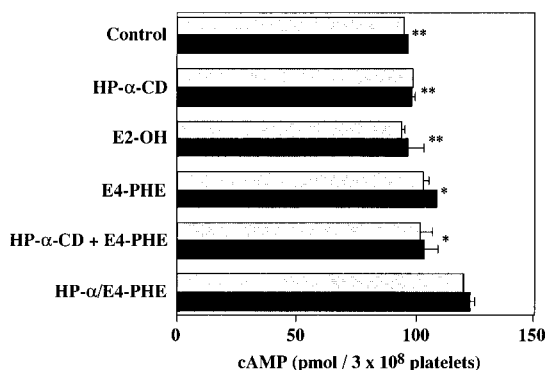


Figure 8. Increase in cytoplasmic cyclic AMP levels in platelets by the polyrotaxanes for 1 min (shaded) and 2 min (solid) ($n = 4$, $\pm$ SEM). Entries marked with * and ** were significantly different from HP- α /E4-PHE at $P < 0.002$ and $P < 0.001$, respectively, calculated using the t -test.

PHE, as shown in Figure 7. Similar phenomena were observed in the case of samples using PEG with an $\bar{M}_n$ of 4000. Since the increase in membrane fluidity was enhanced more by HP- α /E2-PHE than by HP- α /E4-PHE, the supramolecular association of HP- α /E2-PHE may lead to a much more enhanced membrane fluidity increase than that of HP- α /E4-PHE (25). This membrane fluidity change was observed within a few seconds after the polyrotaxane addition (data not shown). Taking the rapid increase in membrane fluidity and the results of the $[Ca^{2+}]_i$ change in platelets into account, the polyrotaxanes are considered to perturb membrane structure triggering membrane-dependent signaling pathways to regulate intracellular metabolism.

Effect of the Polyrotaxanes on Cyclic AMP Levels in Platelets. As mentioned above, it is suggested that the $[Ca^{2+}]_i$ increase in platelets by the polyrotaxanes is regulated by the metabolically directed mechanism in relation to the increase in membrane fluidity. One possible way will be used to explain the inhibition as follows. The polyrotaxane can accelerate the biochemical mechanism regulating $[Ca^{2+}]_i$ in platelets. A well-known mechanism for regulating $[Ca^{2+}]_i$ in platelets is calcium inhibition by cyclic-3',5'-AMP (cyclic AMP)-dependent protein kinase via the activation of adenylate cyclase or the inhibition of cyclic AMP phosphodiesterase (28). The polyrotaxane induced an increase in $[cyclic\ AMP]_i$ in platelets, although the other references showed almost the same $[cyclic\ AMP]_i$ as the control, as shown in Figure 8. This finding was statistically significant; however, the

extent of $[\text{cyclic AMP}]_i$ increase was ca. 20%. This result suggests that the polyrotaxanes inhibit the $[\text{Ca}^{2+}]_i$ increase in platelets, the mechanism of which may involve the contribution of the $[\text{cyclic AMP}]_i$ increase. Previously, it was reported that an increase in membrane fluidity of rat reticulocytes allows adenylate cyclase to be activated by enhancing the coupling with β -adrenergic receptor (29). Taking the above report into account, it is likely that the polyrotaxanes could enhance the membrane fluidity of platelets to elevate $[\text{cyclic AMP}]_i$.

CONCLUSION

Cellular response to our designed biodegradable polyrotaxanes was examined in terms of the intracellular metabolism of platelets and physicochemical interaction with plasma membrane. The polyrotaxanes were characterized by $^1\text{H-NMR}$, SEC, and an *in vitro* degradation experiment. The inhibition of thrombin-induced cytoplasmic calcium levels in platelets was observed with the addition of our designed polyrotaxanes. Further, the polyrotaxanes were found to enhance membrane fluidity in RBC ghosts. These effects of the polyrotaxanes were statistically more significant than those of their constituent molecules such as a mixture of HP- α -CDs and L-Phe-terminated PEGs. The 20% increase in cyclic AMP levels in platelets by the polyrotaxanes suggests that the polyrotaxanes inhibit the $[\text{Ca}^{2+}]_i$ increase in platelets, the mechanism of which may involve the contribution of the $[\text{cyclic AMP}]_i$ increase. Although the relation with the supramolecular structure and association is currently under investigation, these findings could contribute to new fields of polyrotaxanes in medical and therapeutic application. These biodegradable polyrotaxanes will degrade into noninteractive components (HP- α -CD, PEG, and L-Phe) within a period of time, and will be naturally removed from the body. It is believed that biodegradable polyrotaxanes can be used to fabricate blood-contacting devices which ultimately can be absorbed in the body.

ACKNOWLEDGMENT

We thank Yamasa Co. for the cyclic AMP assay. This study was partially supported by a Proposal-Based Advanced Industrial Technology R&D Program, New Energy and Industrial Technology Development Organization (NEDO), Japan.

LITERATURE CITED

- Behr, J.-P. (1994) *The Lock-and-Key Principle*, John Wiley & Sons, Chichester, U.K.
- Lehn, J.-M. (1995) *Supramolecular Chemistry*, VCH, Weinheim, Germany.
- Harada, A., and Kamachi, M. (1990) Complex formation between poly(ethylene glycol) and α -cyclodextrin. *Macromolecules* 23, 2821–2823.
- Harada, A., Li, J., and Kamachi, M. (1992) The molecular necklace: a rotaxane containing many threaded α -cyclodextrins. *Nature* 356, 325–327.
- Ooya, T., Mori, H., Terano, M., and Yui, N. (1995) Synthesis of a biodegradable polymeric supramolecular assembly for drug delivery. *Macromol. Rapid Commun.* 16, 259–263.
- Yui, N., and Ooya, T. (1996) Novel design of supramolecular-structured biodegradable polymer for drug delivery. In *Advanced Biomaterials in Biomedical Engineering and Drug Delivery Systems* (N. Ogata, S. W. Kim, J. Feijen, and T. Okano, Eds.) pp 333–334, Springer, Tokyo.
- Ooya, T., and Yui, N. (1997) Synthesis and characterization of biodegradable polyrotaxanes as a novel supramolecular-structured drug carrier. *J. Biomater. Sci., Polym. Ed.* 8, 437–455.
- Ríhová, B. (1996) Biocompatibility of biomaterials: hemocompatibility, immunocompatibility and biocompatibility of solid polymeric materials and soluble targetable polymeric carriers. *Adv. Drug Deliv. Rev.* 21, 157–176.
- Kamimura, W., Ooya, T., and Yui, N. (1997) Interaction of supramolecular assembly with hairless rat stratum corneum. *J. Controlled Release* 44, 295–299.
- Ooya, T., Sugawara, H., and Yui, N. (1997) Interaction of supramolecular-structured polyrotaxanes with hairless rat stratum corneum and its effect on indomethacin permeation. *Drug Delivery Syst.* 12, 89–94.
- Yui, N., Suzuki, K., Okano, T., Sakurai, Y., Ishikawa, C., Fujimoto, K., and Kawaguchi, H. (1993) Mechanism of cytoplasmic calcium changes in platelets in contact with polystyrene and poly(acrylamide-co-methacrylic acid) surfaces. *J. Biomater. Sci., Polym. Ed.* 4, 199–215.
- Yui, N., Suzuki, K., Okano, T., Sakurai, Y., Nakano, M., Ishikawa, C., Fujimoto, K., and Kawaguchi, H. (1995) Cytoplasmic calcium level and membrane fluidity of platelets contacting poly(acrylamide-co-methacrylic acid) particles with different surface properties. *J. Biomater. Sci., Polym. Ed.* 7, 253–264.
- Feinstein, M. B., Egan, J. J., Sha'afi, R. I., and White, J. (1983) The cytoplasmic concentration of free calcium in platelets is controlled by stimulators of cyclic AMP production (PGD₂, PGE₁, Forskolin). *Biochem. Biophys. Res. Commun.* 113, 598–604.
- Yui, N., Kataoka, K., Sakurai, Y., Aoki, T., Sanui, K., and Ogata, N. (1988) *In vitro* and *in vivo* studies on antithrombogenicity of poly(propylene oxide) segmented nylon 610 in relation to its crystalline-amorphous microstructure. *Biomaterials* 9, 225–229.
- Takei, Y. G., Yui, N., Okano, T., Haruyama, A., Sanui, K., Sakurai, Y., and Ogata, N. (1994) Postadsorptive behavior of plasma proteins on poly(propylene oxide)-segmented nylon-610 surfaces and its implication in preventing contact-induced activation of platelets on these surfaces. *J. Biomater. Sci., Polym. Ed.* 6, 149–167.
- Yui, N. (1996) Nonthrombogenic polymers (Multiblock copolymers of polyether and polyamide). In *Polymeric Materials Encyclopedia* (J. C. Salamone, Ed.) Vol. 6, pp 4618–4624, CRC Press, New York.
- Anderson, D. W., Zimmerman, J. E., and Callahan, F. M. (1964) The use of esters of *N*-hydroxysuccinimide in peptide synthesis. *J. Am. Chem. Soc.* 86, 1839–1842.
- Grynkiewicz, G., Poenie, M., and Tsien, Y. (1985) A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.* 260, 3440–3450.
- Dodge, J. T., Mitchell, C., and Hanahan, D. J. (1963) The preparation and characteristics of hemoglobin-free ghosts of human erythrocytes. *Arch. Biochem. Biophys.* 100, 119–130.
- Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., and Klenk, D. C. (1985) Measurement of protein using bicinchoninic acid. *Anal. Biochem.* 150, 76–85.
- Stoltz, J. F., and Donner, M. (1985) Fluorescence polarization applied to cellular microrheology. *Biorheology* 22, 227–247.
- Sumbilla, C., and Lakowicz, J. R. (1982) Fluorescence studies of red blood cell membranes from individuals with Huntington's disease. *J. Neurochem.* 38, 1699–1707.
- Beretz, A., Stirle, A., Anton, R., and Cazenave, J.-P. (1981) Role of cyclic AMP in the inhibition of human platelet aggregation by quercetin, a flavonoid that potentiates the effect of prostacyclin. *Biochem. Pharmacol.* 31, 3597–3600.
- Gilman, A. G. (1970) A protein binding assay for adenosine 3',5'-cyclic monophosphate. *Proc. Natl. Acad. Sci. U.S.A.* 67, 305–312.
- Ooya, T., and Yui, N. (1997) Regulation of intracellular metabolism by biodegradable polyrotaxanes. *J. Biomater. Sci., Polym. Ed.* (in press).
- Wilson, D. F., and Chance, B. (1966) Reversal of azide inhibition by uncouplers. *Biochem. Biophys. Res. Commun.* 23, 751–756.

- (27) Ooya, T., and Yui, N. (1997) Degradation kinetics and supramolecular association of biodegradable polyrotaxanes. *Proc. Int. Symp. Controlled Release Bioact. Mater.*, 24th, 461–462.
- (28) Feinstein, M. B., Zavoico, G. B., and Halenda, S. P. (1985) Calcium and cyclic AMP: Antagonistic modulators of platelet function. In *The platelets: Physiology and Pharmacology* (G. L. Longenecker, Ed.) pp 237–269, Academic, London.
- (29) Hirata, F., Strittmatter, W. J., and Axelrod, J. (1979) β -Adrenergic receptor agonists increase phospholipid methylation, membrane fluidity, and β -Adrenergic receptor-adenylate cyclase coupling. *Proc. Natl. Acad. Sci. U.S.A.* 76, 368–372.

BC970189C

TECHNICAL NOTES

A New Method for the Synthesis of Neoglycopeptides[†]

Paul R. Hansen,* Carl E. Olsen, and Arne Holm

Research Center for Medical Biotechnology, Chemistry Department, Royal Veterinary and Agricultural University, Thorvaldsensvej 40, 1871 Frederiksberg C, Denmark. Received May 21, 1997;

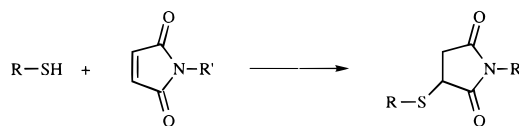
Revised Manuscript Received September 30, 1997[®]

A new method for conjugation of small carbohydrates to peptides based on maleimido–thiol chemistry is described. The reducing carbohydrate is reacted with *S*-trityl-2-mercaptoethylamine in methanol. The resulting Schiff base is then stabilized by acetylation with acetic anhydride to give an *S*-trityl-protected glycosylamide. Activation of the carbohydrate is effected by brief treatment with trifluoroacetic acid/triisopropylsilane, followed by precipitation in ether. The thiol-functionalized carbohydrate is then reacted with a maleimido-modified peptide to give a neoglycopeptide. The potential of this method was investigated using maltose as the model compound. Furthermore, we prepared five maleimido-modified model peptides. In contrast to some literature reports, we found that the maleimido group is stable to treatment trifluoroacetic acid/triisopropylsilane which was used to cleave the modified peptides from the resin.

INTRODUCTION

Glycopeptides containing an unnatural type of linkage between the saccharide moiety and peptide (e.g. a spacer) are termed neoglycopeptides. For a review of the area, see Magnusson (2) and references cited therein. Neoglycopeptides have been used for various purposes, including the determination of the influence of carbohydrate molecules on biological activity, conformation, and stability of the parent peptides (3). Neoglycopeptides have been prepared by reacting an amino group in the carbohydrate with a carboxyl group in the peptide (C-terminal, Asp,¹ Glu) (3, 4) or by reacting 3-mercaptopropionic acid spacer glycosides with the N terminus of a peptide (5). Furthermore, reductive amination (6), use of a protected valine Amadori compound in peptide synthesis (7), and

Scheme 1



periodate oxidation followed by reductive amination (8) have been described.

Boas et al. (9) reported the coupling of an unprotected sugar to the N terminus of a protected resin-bound peptide, following the method described by Blomberg et al. (10) for immobilization of reducing sugars to matrices. The formed glycosylamine was stabilized by N-acetylation and the product cleaved from the resin. However, the yield was low, and many byproducts were observed.

Schneller and Geiger (11) described a method in which a sulfhydryl group is introduced into the carbohydrate by reductive amination with cysteamine. The resulting product is then coupled to a SPDP-modified protein to form a disulfide bridge.

In this paper, we describe a new strategy for conjugation of small carbohydrates to peptides based on maleimido–thiol chemistry. Maleimido moieties react fast, selectively, and irreversibly with sulfhydryl groups to form a thioether as shown in Scheme 1.

This approach has been used for a variety of applications, including conjugation of enzymes to insulin (12), peptides to proteins (13), phosphorothioate nucleotides to proteins (14), and lipids to peptides (15), radioiodolabeling of hormones (16), and immunoassays (17). However, to the best of our knowledge, this chemistry has not been utilized for synthesis of neoglycopeptides.

RESULTS AND DISCUSSION

The principle of our approach is shown in Scheme 2 using maltose and a 6-maleimidocaproic acid-modified peptide as an example.

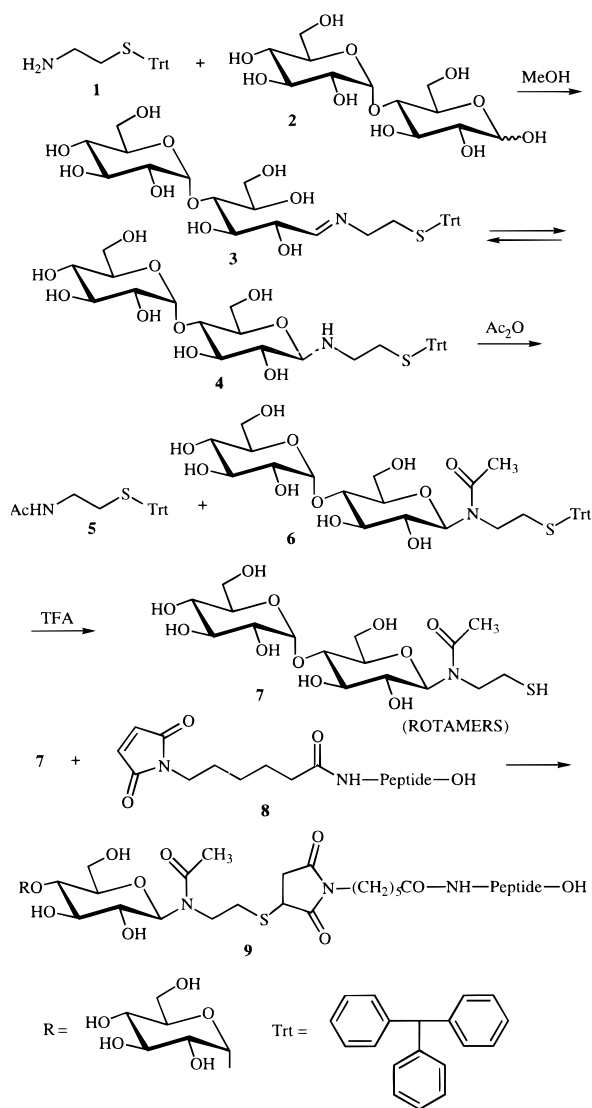
[†] This work was presented in part at the 24th European Peptide Symposium, Edinburgh, Scotland, September 8–13, 1996, and will be published in the proceedings (1).

* Author to whom correspondence should be addressed. Telephone: +45-3528-2573. Fax: +45-3528-2398. E-mail: prh@pepsyn.chem.kvl.dk.

[®] Abstract published in *Advance ACS Abstracts*, December 15, 1997.

¹ Abbreviations used for amino acids are according to the IUPAC-IUB Commission on Biochemical Nomenclature [(1985) *J. Biol. Chem.* **260**, 14–42]. Additional abbreviations: BME, β -mercaptoethanol; CCK, cholecystokinin; DCM, dichloromethane; DIC, *N,N'*-diisopropylcarbodiimide; DIEA, *N,N*-diethylisopropylamine; DMF, dimethylformamide; FAB-MS, fast atom bombardment mass spectrometry; Fmoc, 9-fluorenylmethyloxycarbonyl; HOBt, 1-hydroxybenzotriazole; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; Mca, 6-maleimidocaproyl; RP-HPLC, reversed-phase high-performance liquid chromatography; SPDP, *N*-succinimidyl-3-(2-pyridyldithio)propionate; SPPS, solid-phase peptide synthesis; TFA, trifluoroacetic acid; TIS, triisopropylsilane; TLC, thin-layer chromatography; Trt, triphenylmethyl.

Scheme 2



First, the protected thiol spacer *S*-trityl-2-mercaptoethylamine **1** is reacted with the reducing carbohydrate **2** in methanol. The resulting imine **3**, which is in equilibrium with the glycosylamine **4**, is then stabilized by *N*-acetylation with acetic anhydride. This principle was first described by Blomberg et al. (10) for immobilization of sugars to matrices. *N*-Acetyl-*S*-trityl-2-mercaptoethylamine **5** is generated as a coproduct in the reaction. When using a small carbohydrate such as **2**, the resulting spacer saccharide **6** may be purified by preparative RP-HPLC. Alternatively, the byproduct **5** may be removed from the crude reaction mixture by extraction. The *S*-trityl group, which is a commonly used protecting group for cysteine in Fmoc solid-phase peptide synthesis (18), is cleaved by brief treatment with trifluoroacetic acid/triisopropylsilane. Most glycosidic linkages and carbohydrate moieties are stable to treatment with TFA for 2 h, even with unprotected hydroxyl groups (19). Triisopropylsilane (20) was chosen as the scavenger for the trityl group, since water or thiol scavengers cannot be present during deprotection. Following precipitation in ether, the thiol-functionalized carbohydrate is then reacted with a maleimido-modified peptide² or protein to

²For obvious reasons, a cysteine residue with an unprotected thiol group cannot be present in the maleimido-modified peptide.

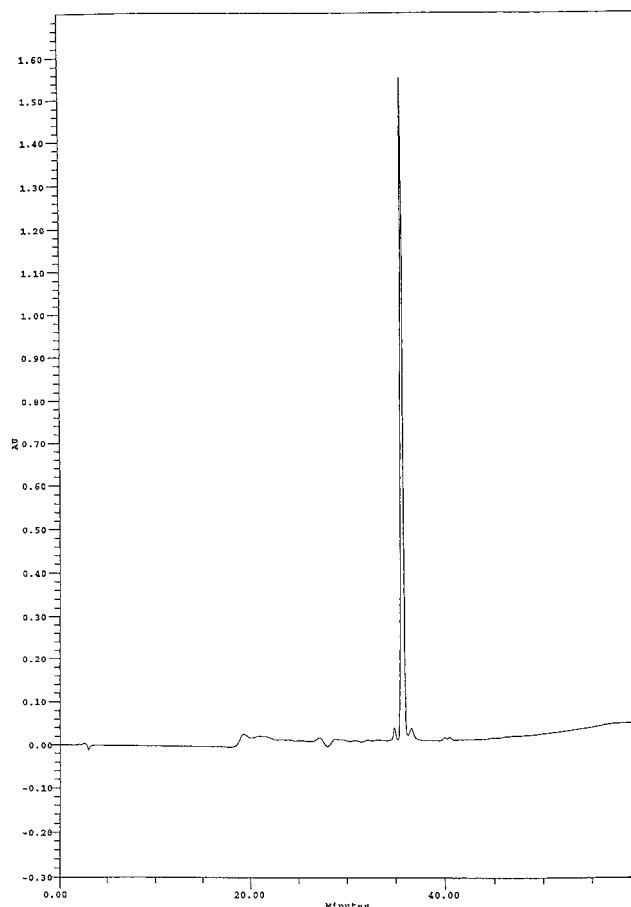


Figure 1. Analytical HPLC of **6** following purification by preparative HPLC.

give a neoglycopeptide or neoglycoprotein. When small carbohydrates are conjugated to peptides, the neoglycopeptide may be purified by preparative RP-HPLC. When small carbohydrates are conjugated to proteins, workup may be done by gel filtration.

Synthesis of a Spacer-Modified Carbohydrate Model Compound. Using the above-described procedure with maltose, we prepared the model spacer saccharide *N*-acetyl-[2-[*S*-(triphenylmethyl)mercapto]ethyl]-[α -D-glucopyranosyl(1 $\rightarrow$ 4)]- β -D-glucopyranosylamine (**6**). The course of the reaction was easily monitored by RP-HPLC. After overnight reaction, two peaks were observed. The imine **3** and starting material **1** eluted approximately after 30 and 40 min, respectively. After acetylation with a large excess of acetic anhydride and overnight reaction, the aforementioned peaks had disappeared; two new peaks appeared at approximately 37 min (**6**) and 47 min (**5**). HPLC analysis of the crude reaction mixture before workup always revealed some accompanying *O*-acetylated carbohydrate, with a t_R of $\sim$ 39 min, usually less than 5%. Attempts to recover unreacted maltose by collecting the elute from the first 10 min only resulted in minute amounts of sugar. Compound **6** was obtained in 62% yield following preparative RP-HPLC (Figure 1).

An alternative purification procedure for **6** involved washing the crude reaction mixture extensively with DCM. Using this approach, **6** could be obtained in 43.5% yield. The crude product may be used without further purification since unreacted maltose will not interfere with the subsequent conjugation reaction. When using 5 equiv of *S*-trityl-2-mercaptoethylamine, the reaction

product was completely soluble in DCM. In this case, crude **6** was obtained by washing the lyophilized mixture extensively with water.

As described by Blomberg et al. (10), we observed two methyl signals in the ^1H NMR spectrum of **6** (at 1.82 and 2.10 ppm), indicating the presence of two rotamers in the ratio 1:3.4. The $J_{1,2}$ values for the two rotamers (9.4 Hz, 5.24 ppm, H-1a; and 8.9 Hz, 4.65 ppm, H-1b) show that the glycosylamide **6** has the β -configuration. For α -linked *N*-glycopeptides, the $J_{1,2}$ value is typically 5–6 Hz (21).

The ^{13}C NMR spectrum of **6** revealed 22 signals stemming from the major rotamer. All the expected peaks were identified. As with the ^1H NMR spectrum, minor peaks indicated the presence of the other rotamer.

Synthesis of a Maleimido-Modified Model Peptide. Keller and Rudinger (22), Marburg et al. (23), and Wünsch et al. (24) have reported that the maleimido group is sufficiently stable toward TFA to allow its incorporation into a peptide chain. However, Papini et al. (25) reacted several histidine-containing porcine CCK-related peptides with *N*-hydroxy succinimido maleoyl- β -alaninate in DMF. They found that during workup under neutral conditions the *N*^{im}-imino function of the side chain of histidine reacts nearly quantitatively with the maleimido function. Furthermore, Papini and co-workers found this intramolecular cyclization reaction to proceed at remarkably higher rates than the bimolecular alkylation of histidine derivatives with *N*-ethylmaleimide.

Jensen and Jakobsen (26) reported the direct solid-phase peptide synthesis of *N*-maleoyl- β -alanyl-Leu-Ala-Gly-Val-OH using Fmoc chemistry. The product was cleaved from the resin with neat TFA, TFA/H₂O, or TFA/triethylsilane and isolated in good purity after gel filtration. Contrary to the above, Rusiecki et al. (27) claim that the maleimido group is degraded in TFA/CH₂Cl₂.

In order to investigate the stability of the maleimido group to cleavage conditions of Fmoc SPPS, the model peptide Mca-WHRQDGKL-OH was synthesized. The peptide was designed so that it would contain the most commonly used side chain protecting groups (Boc, *t*Bu, trityl, and Pmc) in Fmoc SPPS. Furthermore, we chose 6-maleimidocaproic acid (Mca) as the spacer, since this compound would be less prone to intramolecular cyclization compared to *N*-hydroxy succinimido maleoyl- β -alaninate.

After each coupling step, starting with Asp⁵, 50 mg of resin was removed and derivatized with 6-maleimidocaproic acid. The product was then cleaved from the resin with TFA/TIS (95:5) and subjected to analytical HPLC. In each case, the desired maleimido-modified peptide was obtained in >80% purity and showed the correct mass by FAB-MS. Analytical HPLC of crude Mca-WHRQDGKL-OH is shown in Figure 2. In order to establish that the maleimido function was available for conjugation, we reacted both Mca-HRQDGKL-OH and Mca-WHRQDGKL-OH with β -mercaptoethanol (BME) in 0.1 M NaOAc buffer at pH 4. After 18 h, the mixture was analyzed by HPLC and FAB-MS. In both cases, FAB-MS showed that BME had reacted with the maleimido group. Furthermore, the analytical HPLC showed no sign of starting material, but one new peak eluting 0.5 min earlier than the maleimido-modified peptide.

Conjugation of a Spacer-Modified Carbohydrate to Maleimido-Modified Peptides. Encouraged by these results, we then synthesized two maltose-containing model neoglycopeptides, α -D-Glcp(1 $\rightarrow$ 4)- β -D-Glcp-1-*N*-(Ac-(CH₂)₂-S-Mca-DRVYIHPF (**9a**) and α -D-Glcp(1 $\rightarrow$ 4)- β -D-Glcp-1-*N*-(Ac-(CH₂)₂-S-Mca-YETPTRMLVAGIAA (**9b**)

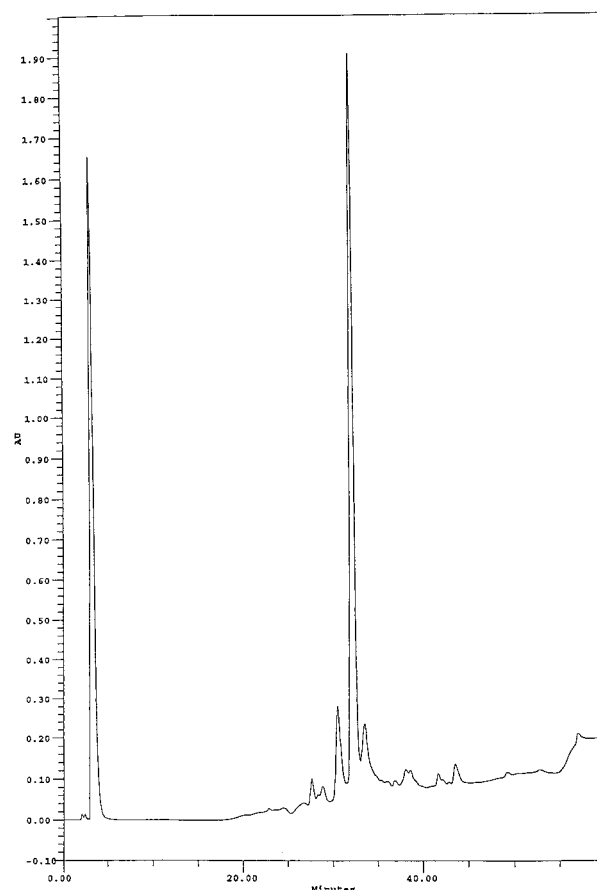


Figure 2. Analytical HPLC of crude WHRQDGKL-OH modified with 6-maleimidocaproic acid.

in a small scale using the approach outlined in Scheme 2. The crude maleimido-modified peptides Mca-DRVYIHPF and Mca-YETPTRMLVAGIAA were obtained in greater than 80% purity and used without further purification. The conjugation reaction was carried out in 0.1 M NaOAc at pH 4. The progress of the reaction was followed by analytical RP-HPLC and found to be complete after 18 h. Following purification by RP-HPLC, the neoglycopeptides were isolated in 71.2 and 55.4% yield, based on **6**. Both **9a** (Figure 3) and **9b** (Figure 4) were isolated in high purity. The products were analyzed by MALDI-TOF-MS, and in both cases, the expected mass was obtained.

In conclusion, we have investigated a new strategy for conjugation of reducing carbohydrates to peptides based on maleimido–thiol chemistry. We were able to show that this method works on a small scale for short carbohydrates by conjugating the maltose spacer saccharide *N*-acetyl-*N*-[2-[*S*-(triphenylmethyl)mercapto]ethyl]-[α -D-glucopyranosyl(1 $\rightarrow$ 4)]- β -D-glucopyranosylamine (**6**) to some maleimido-modified peptides. In both cases, very pure products were obtained, upon purification by RP-HPLC.

EXPERIMENTAL PROCEDURES

General. Analytical HPLC was performed using a Waters C₁₈ reversed-phase column (Delta-Pak, 100 Å, 15 μm , Millipore) on a Waters 600E system equipped with Millennium software. Samples were chromatographed at a flow rate of 1.5 mL/min starting with 0.1% aqueous TFA (buffer A) for 10 min and increasing over 45 min to 0.1% TFA in CH₃CN/H₂O (9:1) (buffer B), with detection at 220 nm. For preparative HPLC, a Waters C₁₈ reversed-phase column (RCM-module, Millipore) was used.

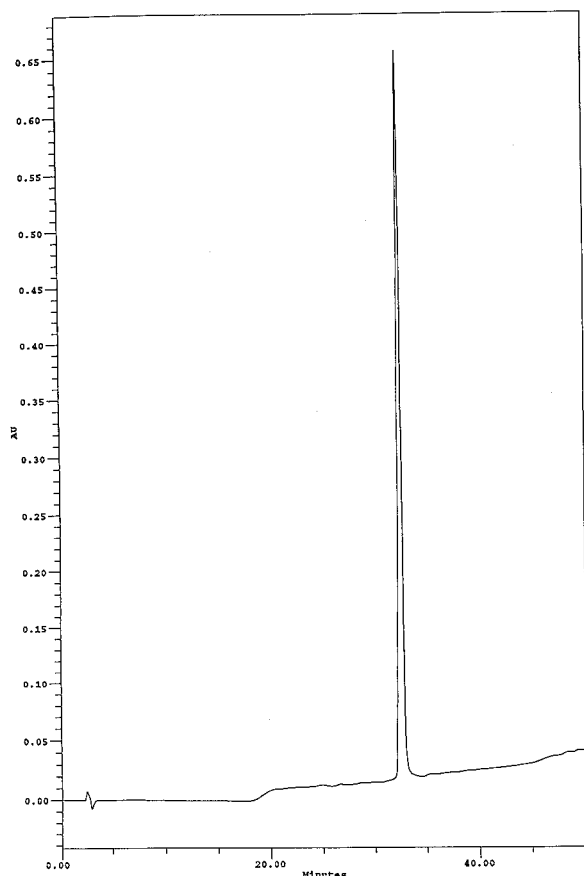


Figure 3. Analytical HPLC of **9a** following purification by preparative HPLC.

NMR spectra were recorded on a Bruker AC 250P instrument. Amino acid analyses were performed on a Waters Pico-Tag analyzer after samples were hydrolyzed with 6 N aqueous HCl/0.1% phenol at 110 °C for 18 h. MALDI-TOF-MS was carried out on a Fisons ToFSpec E instrument in the linear mode. FAB-MS was carried out on a JEOL JMS AX505W instrument. TLC was carried out on Kiesel Gel 60 F₂₅₄ Plates (Merck, Germany). Spots were visualized by UV or spraying with a dilute solution of sulfuric acid followed by heating. Melting points were determined on a Gallenkamp melting point apparatus.

Chemicals. Protected amino acids and Pepsyn KA resin were purchased from Milligen (Bedford, MA). Triisopropylsilane, DIC, MBS, and HOBt were obtained from Aldrich. 6-Maleimidocaproic acid was obtained from Fluka. DMF and DCM were from Riedel de Haën (Seelze, Germany).

Peptide Synthesis. Peptide synthesis was accomplished manually in a syringe equipped with a filter by a stepwise solid-phase peptide synthesis procedure starting with a Pepsyn K resin (0.200 g, 0.1 mequiv/g) using the Fmoc/tBu protection scheme (18). The following side chain-protected amino acid derivatives were used: Arg-(Pmc), Asp(tBu), His(Boc), Tyr(tBu), Lys(Boc), Thr(tBu), and Trp(Boc).

The amino acids were coupled in 3-fold excess using DIC (3 equiv) and HOBt (1.5 equiv) in DMF for 2 h. Fmoc removal was accomplished by treatment for 2 × 10 min with 20% piperidine in DMF.

Following peptide synthesis, 6-maleimidocaproic acid (10 equiv) was coupled overnight to the peptidyl resin using DIC (10 equiv) and HOBt (1.5 equiv) in DMF. The product was cleaved from the support with TFA/TIS (90: 5, v:v) (2 mL) for 2 h and the resin washed with TFA.

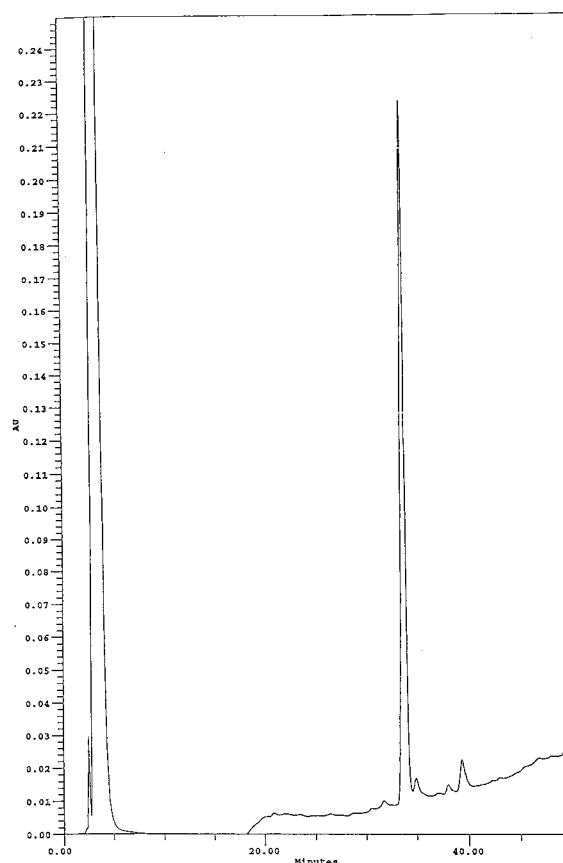


Figure 4. Analytical HPLC of **9b** following purification by preparative HPLC.

The combined TFA washes (3 × 5 mL) were evaporated *in vacuo*, and the product was precipitated in dry ether (5 mL). After centrifugation, the product was washed three times with ether (3 × 5 mL) and dried. In order to investigate the stability of the 6-maleimidocaproic acid (Mca) moiety to cleavage conditions of Fmoc SPPS, a 6-maleimidocaproic acid-modified model peptide, Mca-WHRQDGKL-OH, was synthesized. After each coupling step, starting with Asp⁵, 50 mg of resin was removed and derivatized with 6-maleimidocaproic acid as described above and subjected to analytical HPLC.

Mca-DGKL-OH HPLC purity: >95%. FAB-MS: calcd for [M + H⁺] (C₂₈H₄₄N₆O₁₀) 624, found 625. Mca-QDGKL-OH HPLC purity: >95%. Mca-RQDGKL-OH HPLC purity: >95%. Mca-HRQDGKL-OH HPLC purity: >80%. FAB-MS: calcd for [M + H⁺] (C₄₅H₇₁N₁₅O₁₄) 1046, found 1068 [M + Na]⁺. Amino acid analysis (found/theory): Asp (1.0/1), Glu (1.0/1), Gly (1.1/1), His (0.9/1), Arg (1.0/1), Leu (1.1/1), Lys (1.1/1). Mca-WHRQDGKL-OH HPLC purity: >80%. MALDI-TOF-MS: calcd for [M + H⁺] (C₅₆H₈₂N₁₇O₁₅) 1233, found 1233.

In order to establish that the maleimido function was available for conjugation, Mca-HRQDGKL-OH (1 mg, 1.0 × 10⁻³ mmol) was dissolved in 0.1 M NaOAc buffer at pH 4 (1 mL), to which was added BME (10 μL, 1.2 × 10⁻¹ mmol). After 18 h, the reaction mixture was analyzed by HPLC which showed no sign of starting material, but one new peak eluting 0.5 min earlier as compared to the maleimido-modified peptide. FAB-MS: calcd for [M + H⁺] (C₄₇H₇₆N₁₅O₁₅S) 1123, found 1124.

A similar experiment was carried out with Mca-WHRQDGKL-OH. MALDI-TOF-MS: calcd for [M + H⁺] (C₅₈H₈₇N₁₇O₁₆S) 1310, found 1308. Mca-DRVYIHPF amino acid analysis (found/theory): Asp (0.9/1), His (1.0/

1), Arg (0.8/1), Pro (0.8/1), Tyr (0.9/1), Val (1.0/1), Ile (0.9/1), Phe (1.0/1). MALDI-TOF-MS: calcd for $[M + H^+]$ ($C_{60}H_{83}N_{14}O_{15}$) 1240, found 1241. HPLC showed one major peak with a t_R of 34 min ($>85\%$ purity). Mca-YEVPTRMLVAGIAA amino acid analysis (found/theory): Glu (0.8/1), Gly (1.0/1), Arg (0.8/1), Ala (3.0/3), Thr (1.5/2), Pro (0.9/1), Tyr (0.8/1), Val (1.7/2), Met (1.0/1), Ile (0.9/1), Leu (1.0/1). MALDI-TOF-MS: calcd for $[M + H^+]$ ($C_{81}H_{130}N_{19}O_{24}S$) 1786, found 1786. HPLC showed one major peak with a t_R of min 37 ($>80\%$ purity).

Synthesis of S-Trityl-2-mercaptoethylamine (1). The title compound was prepared as described by Herman et al. (28).

Synthesis of N-Acetyl-N-[2-[S-(triphenylmethyl)mercapto]ethyl][α -D-glucopyranose(1 $\rightarrow$ 4)]- β -D-glucopyranosylamine (6). Method A. Maltose monohydrate (18 mg, 0.05 mmol) and S-trityl-2-mercaptoethylamine (32 mg, 0.1 mmol) in MeOH were refluxed for 18 h. Next, Ac_2O (500 μ L, 5.0 mmol) was added at 0 $^{\circ}C$, and the mixture was left stirring for 18 h. The solvent was then removed by evaporation and the residue dissolved in H_2O/CH_3CN (4:1) and lyophilized. The resulting white powder was dissolved in methanol (2 mL) and purified by preparative HPLC to give 21.2 mg (61.8%) of the title compound. FAB-MS: calcd for $[M + H^+]$ ($C_{35}H_{44}O_{11}NS$) 686, found 686.

Selected ^1H NMR data (a = minor rotamer, b = major rotamer; 250 MHz, CH_3OD): δ 7.3 (m, 15H, aromatic H), 5.24 ($J_{1,2} = 9.39$ Hz, β -D-Glcp, H-1a), 5.18 ($J_{1,2} = 3.9$ Hz, α -D-Glcp, H-1), 4.65 ($J_{1,2} = 8.9$ Hz, β -D-Glcp, H-1b), 3.10 (t, 2.2H, CH_2N), 2.5 (m, 2H, CH_2S), 2.10, (s, 2.3H, methyl), 1.82 (s, 0.7H, methyl). ^{13}C NMR (62 MHz, CH_3OD): δ 174.7 (CON), 146.5 (C-1 aromatic ring), 130.9, 129.1, 129.0, 128.1, 128.0 (aromatic ring C), 103.0 (C-1, α -D-Glcp), 88.6 (C-1, β -D-Glcp), 81.1 (C-4, β -D-Glcp), 79.2, 78.8 (C-5, α -D-Glcp and β -D-Glcp), 75.1, 74.9 (C-3, α -D-Glcp and β -D-Glcp), 74.2 (C-4, α -D-Glcp), 71.5, 71.3 (C-2, α -D-Glcp and β -D-Glcp), 68.0 [$SC(Ph)_3$], 62.7 (C-6, α -D-Glcp and β -D-Glcp), 45.3 (CH_2N , a), 42.1 (CH_2N , b), 32.7 (CH_2S , a), 31.5 (CH_2S , b), 22.0 (CH_3). The carbohydrate carbon atoms were assigned on the basis of literature values (29).

Anal. Calcd for $C_{35}H_{43}O_{11}NS$: C, 61.3; H, 6.32; N, 2.04; S, 4.67. Found C, 59.9; H, 5.8; N, 1.9; S, 4.2.

Method B. Maltose monohydrate (36 mg, 0.1 mmol) and S-trityl-2-mercaptoethylamine (64 mg, 0.2 mmol) were reacted in MeOH as described above. Following lyophilization, the reaction mixture was washed with DCM (10 $\times$ 2 mL) to remove excess **1** and byproduct **5**. The product was then lyophilized to give 29.8 mg of crude **6** (43.5%).

Method C. Maltose monohydrate (36 mg, 0.1 mmol) and S-trityl-2-mercaptoethylamine (160 mg, 0.5 mmol) were reacted in MeOH as described above, except that 2 mL of acetic anhydride was used. Following lyophilization, the product was extracted by washing with H_2O (250 mL) to give 32.1 mg of product (46.9% crude yield).

Synthesis of α -D-Glcp-(1 $\rightarrow$ 4)- β -D-Glcp-1-N-Ac-(CH_2)₂-S-Mca-DRVYIHPF (9a). **6** (1 mg, 1.5×10^{-3} mmol) was deprotected by treatment with TFA/TIS (95:5, 1 mL) for 5 min. The solution turned yellow upon adding the deprotection mixture. However, the color disappeared after a few seconds, indicating a very fast reaction. Following evaporation, the product was precipitated in ether and dried. In a separate experiment, HPLC analysis of the product showed no trace of the starting material **6**. The thiol-functionalized carbohydrate was then dissolved in 500 μ L of 0.1 M NaOAc buffer at pH 4, transferred to a test tube containing conjugate Mca-

DRVYIHPF (3.5 mg, 2.8×10^{-3} mmol), and diluted with buffer to a final volume of 1 mL. The reaction mixture was left on a rocking table overnight, lyophilized, and purified by preparative HPLC. The yield was 1.8 mg (71.2% based on **6**). Analytical HPLC is shown in Figure 3. MALDI-TOF-MS: calcd for $[M + H^+]$ ($C_{76}H_{113}N_{15}O_{25}S$) 1684.9, found 1685.0.

Synthesis of α -D-Glcp-(1 $\rightarrow$ 4)- β -D-Glcp-1-N-Ac-(CH_2)₂-S-Mca-YETPTRMLVAGIAA (9b). **6** (1.5 mg, 2.2×10^{-3} mmol) was conjugated to Mca-YETPTRMLVAGIAA (5 mg, 2.8×10^{-3} mmol) as described above. The product was purified using preparative HPLC (Figure 4). The yield was 2.7 mg. (55.4% based on **6**). MALDI-TOF-MS: calcd for $[M + H^+]$ ($C_{97}H_{160}N_{20}O_{35}S_2$) 2230.6, found 2229.3.

LITERATURE CITED

- (1) Hansen, P. R., Heron, I., Olsen, C. E., Jakobsen, M. H., and Holm, A. (1997) A new strategy for the synthesis of neoglycopeptides based on maleimido-thiol chemistry. In *Peptides 1996: Proceedings of the 24th European Peptide Symposium* (R. Epton, Ed.) ESCOM, Leiden, The Netherlands (in press).
- (2) Magnusson, G., Chernak, A. Y., Kihlberg, J., and Kononov, L. O. (1994) Synthesis of neoglycoconjugates. In *Neoglycoconjugates: Preparation and Applications* (Y. C. Lee and R. T. Lee, Eds.) pp 53–143, Academic Press, San Diego.
- (3) Varga-Defterdarovic, L., Horvat, S., Chung, N. N., and Shiller, P. W. (1992) Glycoconjugates of opioid peptides. *Int. J. Pept. Protein Res.* 39, 12–17.
- (4) Christiansen-Brams, I., Meldal, M., and Bock, K. (1993) Solid-phase synthesis of a di-N-glycosylated RGD analog. In *Peptides 1992. Proceedings of the Twenty-Second European Peptide Symposium* (C. H. Schneider and A. N. Eberle, Eds.) pp 365–366, ESCOM, Leiden, The Netherlands.
- (5) Eloffsson, M., Roy, S., Walse, B., and Kihlberg, J. (1993) Solid-phase synthesis and conformational studies of glycosylated derivatives of helper-T-cell immunogenic peptides from hen-egg lysozyme. *Carbohydr. Chem.* 240, 89–103.
- (6) Morehead, H., McKay, P., and Wetzel, R. (1982) High-performance liquid chromatography analysis in the synthesis, characterization, and reactions of neoglycopeptides. *Anal. Chem.* 126, 29–36.
- (7) Forrow, N. J., and Batchelor, M. J. (1990) Synthesis of the N-glycopeptide partial sequence A1-A12 of the b-Chain of glycosylated haemoglobin HbA1c. A new approach to Amadori N-glycopeptides. *Tetrahedron Lett.* 31, 3493–3495.
- (8) Sasaki-Yagi, Y., Kimura, S., Ueda, H., and Imanishi, Y. (1994) Binding of enkephalin/dextran conjugates to opioid receptors. *Int. J. Pept. Protein Res.* 43, 219–224.
- (9) Boas, U., Heegaard, P., and Jakobsen, M. H. (1996) Solid phase coupling of unprotected reducing carbohydrates to the N-terminus of peptides. In *Innovations and Perspectives in Solid Phase Synthesis* (R. Epton, Ed.) Mayflower, Andover, England (in press).
- (10) Blomberg, L., Wieslander, J., and Norberg, T. (1993) Immobilization of reducing oligosaccharides to matrices by a glycosylamide linkage. *J. Carbohydr. Chem.* 12, 265–276.
- (11) Schneller, M., and Geiger, R. E. (1992) An effective method for the synthesis of neoglycoproteins and neogangliosides by use of reductively aminated sulfhydryl-containing carbohydrate conjugates. *Biol. Chem. Hoppe-Seyler* 373, 1095–1104.
- (12) Kitagawa, T., and Aikawa, T. (1976) Enzyme coupled immunoassay of insulin using a novel coupling reagent. *J. Biochem.* 76, 233–236.
- (13) Plaué, S., Muller, S., Briand, J. P., and van Regenmortel, M. V. H. (1990) Recent advances in solid-phase peptide synthesis and preparation of antibodies to synthetic peptides. *Biologicals* 18, 147–157.
- (14) Karim, A. S., Johansson, C. S., and Weltman, J. K. (1995) Maleimide-mediated proteinconjugates of a nucleoside triphosphate gamma-S and an internucleotide phosphorothioate. *Nucleic Acids Res.* 23, 2037–2040.
- (15) Moroder, L., Romano, R., Guba, W., Mierke, D. F., Kessler, H., Delporte, C., Winand, J., and Christophe, J. (1993) New

- evidence for a membrane-bound pathway in hormone receptor binding. *Biochemistry* 32, 13551–13559.
- (16) Chorev, M., Caulfield, M. P., Roubini, E., McKee, R. L., Gibbons, S. W., Leu, C.T., Levy, J. J., and Rosenblatt, M. (1992) A novel mild, specific and indirect maleimido-based radiolabeling method. Radiolabeling of analogs derived from parathyroid hormone (PTH) and PTH-related protein (PTHrP). *Int. J. Pept. Protein Res.* 40, 445–455.
- (17) Moroder, L., Tzougraki, C., Göhring, W., Mourier, G., Musiol, H.-J., and Wünsch, E. (1987) Studies on Immunoassays of Peptide Factors V. Synthesis of cholecystokinin-58-[1-11]/iso-1-cytochrome c conjugate. *Biol. Chem. Hoppe-Seyler* 368, 855–861.
- (18) Fields, G. B., Tian, Z., and Barany, G. (1992) Principle and practice of solid-phase peptide synthesis. In *Synthetic Peptides: A User's Guide* (G. A. Grant, Ed.) pp 77–183, W. H. Freeman and Co., New York.
- (19) Urge, L., Otvos, L., Jr., Lang, E., Wroblewski, K., Laczko, I., and Hollosi, M. (1992) Fmoc-protected, glycosylated asparagines potentially useful as reagents in the solid-phase synthesis of N-glycopeptides. *Carbohydr. Res.* 235, 83–93.
- (20) Pearson, D. A., Blanchette, M., Baker, M. L., and Guidon, C. A. (1989) Trialkylsilanes as scavengers for the trifluoroacetic acid deblocking of protecting groups in peptide synthesis. *Tetrahedron Lett.* 30, 2739–2742.
- (21) Ratcliffe, A. J., Konradson, P., and Fraser-Reid, B. (1991) Application of n-pentenyl glycosides in the regio- and stereo-controlled synthesis of α -linked N-glycopeptides. *Carbohydr. Res.* 216, 323–335.
- (22) Keller, O., and Rudinger, J. (1975) Preparation of some properties of maleimido acids and maleoyl derivatives of peptides. *Helv. Chim. Acta* 58, 531–541.
- (23) Marburg, S., Neckers, A. C., and Griffin, P. G. (1996) Introduction of the maleimide function onto resin-bound peptides: A simple high-yield process useful for discriminating among several lysines. *Bioconjugate Chem.* 7, 612–616.
- (24) Wünsch, E., Moroder, L., Nyfelder, R., Kalbacher, H., and Gemeiner, M. (1985) Zur Immunbestimmung von peptidfaktoren. I. Mitteilung: Synthese von N $^{\alpha}$ -maleoyl-peptidwirkstoffen (Immunoassays of peptide factors. I. Synthesis of N $^{\alpha}$ -maleoyl-peptide derivatives). *Biol. Chem. Hoppe-Seyler* 366, 53–61.
- (25) Papini, A., Rudolph, S., Siglmüller, R. G., Musiol, H.-J., Göhring, W., and Moroder, L. (1992) Alkylation of histidine with maleimido-compounds. *Int. J. Pept. Protein Res.* 39, 348–355.
- (26) Jensen, S. P., and Jakobsen, M. H. (1994). Direct solid phase synthesis of N-maleoyl peptides. In *Innovations and Perspectives in Solid Phase Synthesis* (R. Epton, Ed.) pp 563–566, Mayflower, Andover, England.
- (27) Rusiecki, V. K., and Warne, S. A. (1993) Synthesis of N $^{\alpha}$ -Fmoc-N $^{\epsilon}$ -Nvoc-lysine and use in the preparation of selectively functionalized peptides. *Bioorg. Med. Chem. Lett.* 3, 707–710.
- (28) Herman, S., Persijn, G., Vandekerckhove, J., and Schacht, E. (1993) Synthesis of dextrane derivatives with thiol-specific reactive groups for the preparation of dextran-protein conjugates. *Bioconjugate Chem.* 4, 402–405.
- (29) Bock, K., Pedersen, C., and Pedersen, H. (1984) Carbon-13 nuclear magnetic data for oligosaccharides. *Adv. Carbohydr. Chem. Biochem.* 42, 193–225.

BC9700998

Synthesis of Carbon-3-Substituted 1,5,9-Triazacyclododecanes, RNA Cleavage Agents Suitable for Oligonucleotide Tethering

Jari Hovinen*,†

Department of Chemistry, University of Turku, FIN-20014 Turku, Finland. Received June 2, 1997;
Revised Manuscript Received September 30, 1997*

Two derivatives of 1,5,9-triazacyclododecane bearing an alkylamino tether group at carbon-3 (**7** and **13**) were synthesized. The first of them, **7**, was prepared starting from diethyl 2-(2-cyanoethyl)malonate and the second one, **13**, from glycerol. In both cases, the cyclization reaction was performed by allowing the corresponding ditosylates (**5** and **12**) to react with TBD. The guanidinium salts formed were reduced *in situ* to the orthoamides (**6** and **12**), acid-catalyzed hydrolysis of which yielded the title compounds. The suitability of the azacrowns prepared for oligonucleotide tethering is also demonstrated.

INTRODUCTION

The use of oligonucleotides in the treatment of certain viral diseases and types of cancer is based on inhibition of the unwanted protein translation by hybridization of the target mRNA molecule with a complementary oligonucleotide (*1*). When unmodified oligodeoxyribonucleotides or oligonucleotide monothioates are employed, the RNA/DNA duplex formed serves as a substrate for cellular RNase H that cleaves the mRNA strand. Direct degradation of the target RNA fragment can be achieved by oligonucleotides tethered to catalytically active groups. The oligonucleotide moiety recognizes a complementary base sequence in the target nucleic acid, and the catalytic group cleaves the chain over a narrow range of nucleotide units. Up to now, several such catalytically active oligonucleotide conjugates that hydrolyze the target RNA chain have been synthesized (2–9). However, many of them have some drawbacks; they either exhibit only moderate RNA cleaving activity or use “unnatural” metals, such as europium.

It has been shown that certain azacrowns, such as 1,5,9-triazacyclododecane, promote hydrolysis of RNA fragments in the presence of Zn²⁺ ions (10–12). The greatest rate enhancement observed was a reaction 10⁷–10⁸-fold faster compared to the uncatalyzed reaction. Thus, by linking these metal ion complexes covalently to oligodeoxyribonucleotides, we could obtain an efficient site specific hydrolysis of the complementary RNA sequence, upon hybridization.

We have recently developed a versatile strategy for oligonucleotide tethering. A thioester function is attached to the oligonucleotide during chain assembly, and upon completion of the synthesis, the desired functional group is introduced by treating the oligomer, when still anchored to the solid support, with an appropriate primary amine (9, 13). To exploit our tethering strategy

in the preparation of oligonucleotide conjugates, synthesis of azacrowns bearing an alkylamino spacer in their structure is needed. The only method reported in the literature that can be applied to our tethering strategy involves a multistep synthesis of 2-(4-aminobutyl)-1,4,7-triazacyclononane starting from L-alanine methyl ester (14). Here is presented a simple synthesis of two carbon-3-substituted 1,5,9-triazacyclododecanes (**7** and **13**). Their suitability for oligonucleotide tethering is also demonstrated.

EXPERIMENTAL PROCEDURES

Adsorption column chromatography was performed on columns packed with Kieselgel 60 (Merck). Analytical TLC was conducted on silica gel 60 F₂₅₄ plates (Merck) using the following solvent systems: (A) 9:1 CH₂Cl₂/MeOH (v:v); and (B) 99:1 CH₂Cl₂/MeOH (v:v). Compounds containing Boc-protected primary amino functions were detected using the following procedure. After normal elution with the appropriate solvent system, the TLC plate was kept on a hot plate for a few seconds. This removes the Boc group, and the spots containing primary amino groups were visualized on spraying with ninhydrin.

NMR spectra were recorded on a JEOL LA-400 spectrometer operating at 399.8 and 100.5 MHz for ¹H and ¹³C, respectively. If not otherwise stated, TMS was used as an internal reference (0.00 ppm). Coupling constants are given in hertz. Identification of NMR signals is based on ¹H, ¹H and ¹H, ¹³C COSY experiments. Mass spectra were recorded either on a VG Analytical MM 7070E (electron impact) or on a VG ZabSpec-ao TOF (fast-atom bombardment) spectrometer. IR spectra were recorded on a Mattson Instruments 6030 Galaxy Series spectrophotometer, and elemental analyses were performed on a Perkin-Elmer 2400 Series II instrument. Melting points were measured on a Büchi 510 apparatus and are uncorrected.

Oligonucleotides were assembled on an Applied Biosystems 392 DNA synthesizer using phosphoramidite chemistry and recommended protocols (DMTr-Off synthesis). Oligonucleotides were purified by HPLC techniques as described previously in detail (15). MALDI-TOF spectrometric measurements of oligonucleotides were performed on a Finnigan LaserMat mass spectrometer using protocols described by Piele et al. (16).

* Address correspondence to Dr. Jari Hovinen, Wallac Oy, P.O. Box 10, FIN-20101 Turku, Finland. Telephone: + 358 2 267 8111. Fax: + 358 2 267 8380. E-mail: jari.hovinen@wallac.fi.

† Present address: Wallac Oy, P.O. Box 10, FIN-20101 Turku, Finland.

© Abstract published in *Advance ACS Abstracts*, November 15, 1997.

tert-Butyl-N-[4-(hydroxymethyl)-5-hydroxypentyl]-carbamate (3) and tert-Butyl-N-[4-(ethoxymethyl)-5-hydroxypentyl]carbamate (4). Diethyl 2-(2-cyanoethyl)malonate (23.4 mmol, 5.0 g) was dissolved in dry THF (20 mL). The borane–methyl sulfide complex (15 mL, 10 M) was added dropwise under a flow of nitrogen. The mixture was heated at reflux with distilling off the methyl sulfide liberated. When the distillation had ceased, the reaction mixture was cooled on an ice bath, and aqueous HCl (6 M, 30 mL) was added dropwise (Caution! Vigorous liberation of H₂). The mixture was refluxed for 30 min, and the clear solution obtained was concentrated *in vacuo*. The residue was suspended in water and filtered. The filtrate was basified with solid NaOH; di-*tert*-butyl dicarbonate (5.46 g, 25 mmol) in THF (100 mL) was added, and the mixture was stirred overnight at ambient temperature. The mixture was concentrated *in vacuo*. The residue was suspended in dichloromethane and washed with water. The organic layer was separated, dried (Na₂SO₄), and concentrated. The oil obtained was dissolved in the mixture of dichloromethane and methanol (9:1, v:v) and purified on a silica gel column using the same solvent system as the eluent. Two products were separated.

Compound 4: 3.60 g (59%); *R_f* (A) 0.65; ¹H NMR (DMSO-*d*₆) δ 6.72 (1H, br t, NH), 4.32 (1H, t, *J* = 5.1, OH), 3.39–3.20 (6H, q, *J* = 7.1, OCH₂CH₃ and m, 2 × CH₂OH), 2.86 (2H, m, CH₂NH), 1.59 (1H, m, CH), 1.19 (12H, s and m, *t*-Bu and CH₂CH), 1.20 (2H, m, CH₂CH₂-CH₂), 1.08 (3H, t, *J* = 7.1, CH₂CH₃); ¹³C NMR (DMSO-*d*₆) δ 155.6, 77.2, 70.7, 65.5, 61.3, 40.8, 40.3, 28.3, 27.1, 25.3, 15.2.

Compound 3: 1.9 g (34%); *R_f* (A) 0.45; ¹H NMR (DMSO-*d*₆) δ 6.76 (1H, br t, *J* = 5.4, NH), 4.30 (2H, t, *J* = 5.1, 2 × OH), 3.40 (4H, m, overlapping with the signal of H₂O, 2 × CH₂OH), 2.87 (2H, m, CH₂NH), 1.43–1.38 (3H, m, CH and CH₂CH), 1.37 (9H, s, *t*-Bu), 1.19 (2H, p, CH₂CH₂-CH₂); ¹³C NMR (DMSO-*d*₆) δ 155.6, 77.3, 61.5, 42.9, 40.4, 28.3, 27.2, 24.9. Anal. (C₁₁H₂₃NO₄): C, H, N.

cis-2-(Cyanoethyl)-1,3-benzylidene Glycerol (9). *cis*-1,3-Benzylidene glycerol **8** (17) (20 g, 0.11 mol) and acrylonitrile (15 mL, 0.2 mol) were dissolved in dry THF (200 mL), and the mixture was cooled on an ice bath. NaH (0.1 g) was added, and the mixture was allowed to stir for 30 min, after which water (50 mL) was added. THF was evaporated *in vacuo*. Water (100 mL) was again added, and the resulting suspension was extracted with diethyl ether (2 × 100 mL). The etheral layer was dried (Na₂SO₄) and concentrated to an oil, which was crystallized from ether to give **9** (15 g, 58%); mp 54 °C; IR (KBr, cm⁻¹) 2251 (CN); ¹H NMR (CDCl₃) δ 7.45 (2H, m), 7.36 (3H, m), 5.56 (1H, s), 4.37 (2H, dd, *J* = 1.5 and 13.0), 4.10 (2H, dd, *J* = 1.7 and 13.0), 3.85 (2H, t, *J* = 6.3), 3.42 (1H, m), 2.69 (2H, t, *J* = 6.3); ¹³C NMR (CDCl₃) δ 137.8, 129.0, 128.3, 126.0, 117.8, 101.3, 71.5, 68.8, 63.6, 19.2. Anal. (C₁₃H₁₅NO₃): C, H, N.

2-[3-[N-(tert-Butoxycarbamoyl)aminolpropyl]glycerol (10). Compound **9** (14.68 g, 63.1 mmol) was dissolved in methanol (300 mL). Cobalt(II) chloride hexahydrate (30 g) was added, and the mixture was cooled on an ice bath. Sodium borohydride (24 g) was added portionwise (Caution! Exothermic reaction and vigorous foaming), and the mixture was allowed to stir for 2 h, after which concentrated aqueous ammonia (150 mL) was added. The black precipitate was filtered off cautiously (paper filter and no vacuum). Methanol was removed *in vacuo*, and the residue was extracted with dichloromethane. The organic phase was concentrated and dissolved in the mixture of 1 M HCl and THF (200

mL, 1:1, v:v) and kept overnight at ambient temperature. All volatile material was removed *in vacuo*. The residue was dissolved in water and basified with solid NaOH. Di-*tert*-butyl dicarbonate (13 g, 65 mmol) in THF (20 mL) was added, and the mixture was allowed to stir overnight at ambient temperature. The mixture was concentrated to ca. 20 mL and extracted with dichloromethane until TLC analysis showed that all the product was transferred into dichloromethane. The combined organic phases were dried (Na₂SO₄), concentrated, and purified on silica gel (eluent, 9:1 CH₂Cl₂/MeOH, v:v). Pure fractions were pooled and concentrated to give **10** as an oil (5.9 g, 38%); ¹H NMR (CDCl₃) δ 4.96 (1H, br), 3.76 (2H, dd, *J* = 4.1 and 12.0), 3.69 (2H, dd, *J* = 5.1 and 12.0), 3.62 (2H, t, *J* = 5.6), 3.44 (1H, m), 3.30 (2H, br t, *J* = 6.1), 2.60 (2H, br), 1.74 (2H, p), 1.44 (9H, s); ¹³C NMR (CDCl₃) δ 156.7, 80.3, 79.6, 66.7, 62.1, 30.6, 28.4. Anal. (C₁₁H₂₃NO₅): C, H, N.

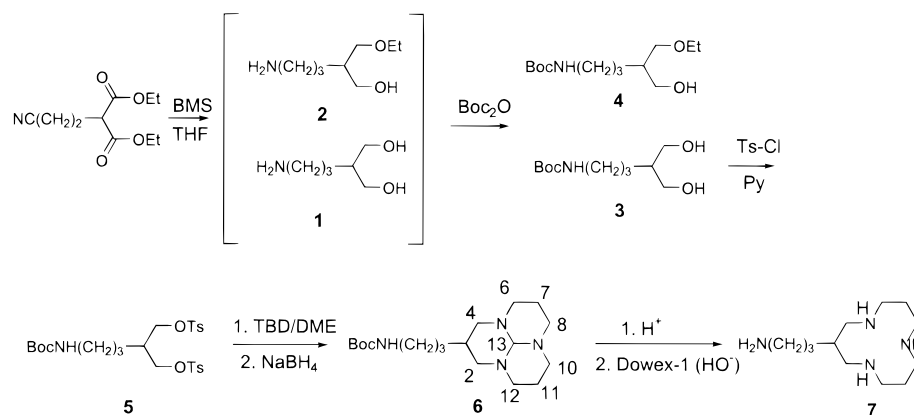
(2-Methyl-*p*-tolylsulfonyl)-5-(N-tert-butoxycarbamoyl)pentane Tosylate (5). Compound **3** (1.0 g, 4.3 mmol) was dried by coevaporations with dry pyridine and dissolved into the same solvent (15 mL). Tosyl chloride (2.4 g, 12.9 mmol) and a catalytic amount of DMAP were added, and the mixture was stirred overnight at ambient temperature. All volatile materials were evaporated off *in vacuo*, and the residue was dissolved in dichloromethane, washed with aqueous NaHCO₃, and dried (Na₂SO₄). After concentration, the residue was redissolved in CH₂Cl₂ and applied onto a silica gel column. The column was eluted initially with CH₂Cl₂ to remove the excess tosyl chloride and then with the mixture of CH₂Cl₂ and methanol (99:1, v:v) to elute the product. The yield was 1.86 g (80%); *R_f* (B) 0.38; ¹H NMR (CDCl₃) δ 7.74 (4H, d, *J* = 8.3), 7.36 (4H, d, *J* = 8.3), 4.57 (1H, br t), 3.95 (4H, m), 3.00 (2H, m), 2.50 (6H, s), 2.00 (1H, m), 1.43 (9H, s), 1.40 (2H, m), 1.32 (2H, m); ¹³C NMR (CDCl₃) δ 155.9, 145.3, 132.5, 130.0, 127.9, 79.2, 68.6, 40.1, 37.7, 28.4, 26.9, 24.2, 21.7. Anal. (C₂₅H₃₅NO₈S₂): C, H, N, S.

2-[3-(N-tert-Butoxycarbamoyl)propoxy]propane 1,3-Ditosylate (11). Compound **10** (5.0 g, 20 mmol) was tosylated and purified as described above for **5**. The yield was 9.4 g (84%); *R_f* (B) 0.39; ¹H NMR (CDCl₃) δ 7.76 (4H, d, *J* = 8.1), 7.35 (4H, d, *J* = 8.1), 4.06 (4H, m), 3.68 (1H, p, *J* = 5.1), 3.35 (2H, t, *J* = 5.9), 3.07 (2H, br q), 2.43 (6H, s), 1.60 (2H, p, *J* = 6.1), 1.43 (9H, s); ¹³C NMR (CDCl₃) δ 155.7, 144.9, 132.0, 129.7, 127.5, 78.5, 74.4, 68.0, 67.3, 37.1, 29.5, 28.0, 21.2. Anal. (C₂₅H₃₅NO₈S₂): C, H, N, S.

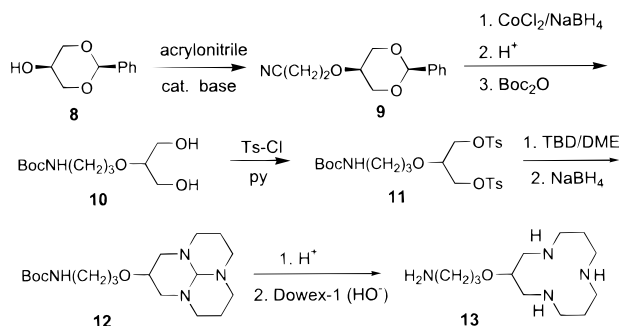
Synthesis of the Orthoamides (6 and 12). A mixture of compound **5** or **11** (3.9 mmol) and TBD (0.82 g, 7.8 mmol) in DME (20 mL) was stirred for 2 days at ambient temperature. NaBH₄ (0.74 g, 20 mmol) was added, and the mixture was allowed to stir for an additional day. Methanol was then added, and the mixture was allowed to stir for 30 min, after which volatile materials were removed *in vacuo*. The residue was suspended in aqueous NaHCO₃ with stirring. When the liberation of gas had ceased, the mixture was extracted twice with dichloromethane. The organic phase was dried (Na₂SO₄) and concentrated. The residue was dissolved in a mixture of CH₂Cl₂ and MeOH (9:1, v:v) and applied on a silica gel column. The column was eluted with the same solvent system. Fractions containing pure products were pooled and concentrated to give **16** and **17** as oils that solidified on standing.

Compound **6** (0.41 g, 31%); *R_f* (A) 0.29; ¹H NMR (CDCl₃) δ 4.71 (1H, br), 3.08 (2H, br q), 2.83 (3H, m), 2.29 (1H, s), 2.23 (4H, m), 2.00 (2H, m), 1.78 (2H, t, *J* = 11.8), 1.45 (13H, s and m), 1.12 (4H, m); ¹³C NMR (CDCl₃)

Scheme 1



Scheme 2



δ 156.0 (C=O), 99.8 (C-13), 79.0 (CMe₃), 60.0 (C-2 and C-4), 53.7 (C-6 and C-12), 53.6 (C-8 and C-10), 40.8 (NHCH₂), 33.0 (C-3), 28.9 (CH₂CH), 28.4 (CH₂CH₂CH₂), 28.0 (*t*-Bu), 24.0 (C-7 and C-10); EI (relative intensity) *m/z* 338 (20%), 337 (100%), 281 (46%); HRMS (EI) 337.2603 (*M*⁺ - H), calcd for C₁₈H₃₂N₄O₂⁺ 337.2603.

Compound **12** (0.72 g, 52%): ¹H NMR (CDCl₃) δ 4.87 (1H, br), 3.68 (1H, m), 3.55 (2H, t, *J* = 5.9), 3.20 (2H, br q), 3.07 (2H, dd, *J* = 4.4 and 10.5), 2.84 (4H, m), 2.37 (1H, s), 2.23–1.99 (6H, m), 1.75 (2H, p), 1.47 (11H, s and m); ¹³C NMR (CDCl₃) δ 156.0 (C=O), 98.9 (C-13), 79.0 (CMe₃), 70.5 (C-3), 67.1 (CH₂O), 58.1 (C-2 and C-4), 53.8–53.7 (C-6, C-8, C-10, and C-12), 38.4 (NHCH₂), 30.0 (CH₂CH₂CH₂), 28.4 (*t*-Bu), 24.0 (C-7 and C-11); EI (relative intensity) *m/z* 354 (20%), 353 (100%), 297 (46%); HRMS (EI) 353.2554 (*M*⁺ - H), calcd for C₁₈H₃₂N₄O₃⁺ 353.2552.

3-(3-Aminopropyl)-1,5,9-triazacyclododecane (7) and 3-(3-Aminopropoxy)-1,5,9-triazacyclododecane (13). Compound **6** or **12** (1.0 mmol) was dissolved in

hydrochloric acid (6 M, 50 mL), and the mixture was heated for 48 h at reflux. All volatile materials were removed *in vacuo*, and the residue was coevaporated twice with water. Precipitation from ethanol yielded the title compounds as tetrahydrochlorides.

Compound **9**: ¹H NMR (D₂O, external reference of DSS at 0.00 ppm) δ 3.45 (12H, m, H-2a/b, H-4a/b, H-6a/b, H-8a/b, H-10a/b, and H-12a/b), 3.24 (2H, t, *J* = 5.8, NH₂CH₂), 2.34 (1H, m, H-3), 2.27 (4H, H-7a/b and H-11a/b), 1.84 (2H, m, NH₂CH₂CH₂), 1.65 (2H, m, NH₂CH₂CH₂); ¹³C NMR (D₂O, external reference of DSS at 0.00 ppm) δ 46.3, 43.6, 41.2, 40.3, 32.7, 27.9, 24.9, 19.6; HRMS (EI) 228.2313 (*M*⁺), calcd for C₁₂H₂₈N₄⁺ 228.2325.

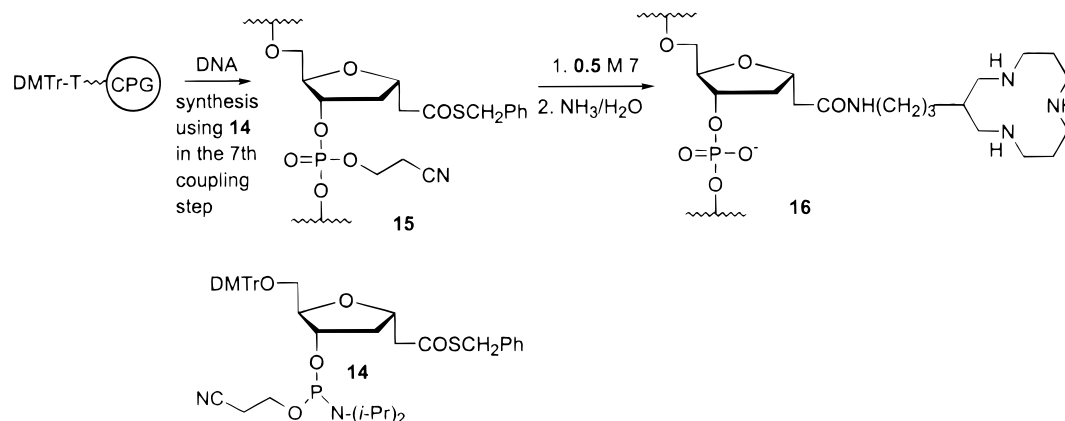
Compound **15**: ¹H NMR (D₂O, external reference of DSS at 0.00 ppm) δ 4.03 (1H, m, H-3), 3.59 (2H, t, *J* = 5.8, CH₂O), 3.34 (2H, dd, *J* = 6.6 and 14.0, H-2a/H-4a), 3.20 (10H, H-2b, H-4b, H-6a/b, H-8a/b, H-10a/b, and H-12a/b), 2.91 (2H, t, *J* = 7.3, NH₂CH₂), 2.01 (4H, p, H-7a/b and H-11a/b), 1.78 (2H, p, *J* = 6.8, NH₂CH₂CH₂-CH₂O); ¹³C NMR (D₂O, external reference of DSS at 0.00 ppm) δ 70.9 (C-3), 68.6 (CH₂O), 45.2 (C-2/C-4), 44.0 (C-6/C-12), 40.6 (C-8/C-10), 38.3 (NH₂CH₂), 27.9 (C-7/C-11), 19.0 (NHCH₂CH₂CH₂O); HRMS (FAB) 245.2341 (*M*⁺ + H), calcd for C₁₂H₂₉N₄O⁺ 245.2344.

The azacrowns **7** and **13** were converted to the free bases by passing them through a Dowex-1 resin (200 mesh, hydroxide ion form) using water as the eluent. The basic fractions were collected and concentrated to give the title compounds as oils.

Synthesis of the Oligonucleotide Conjugate (16).

The oligonucleotide d(T₆XT₇) was assembled on an ABI DNA synthesizer using standard protocols in a 0.2 mmol scale. The phosphoramidite **14** was used in the seventh coupling step (13). Upon completion of the oligonucle-

Scheme 3



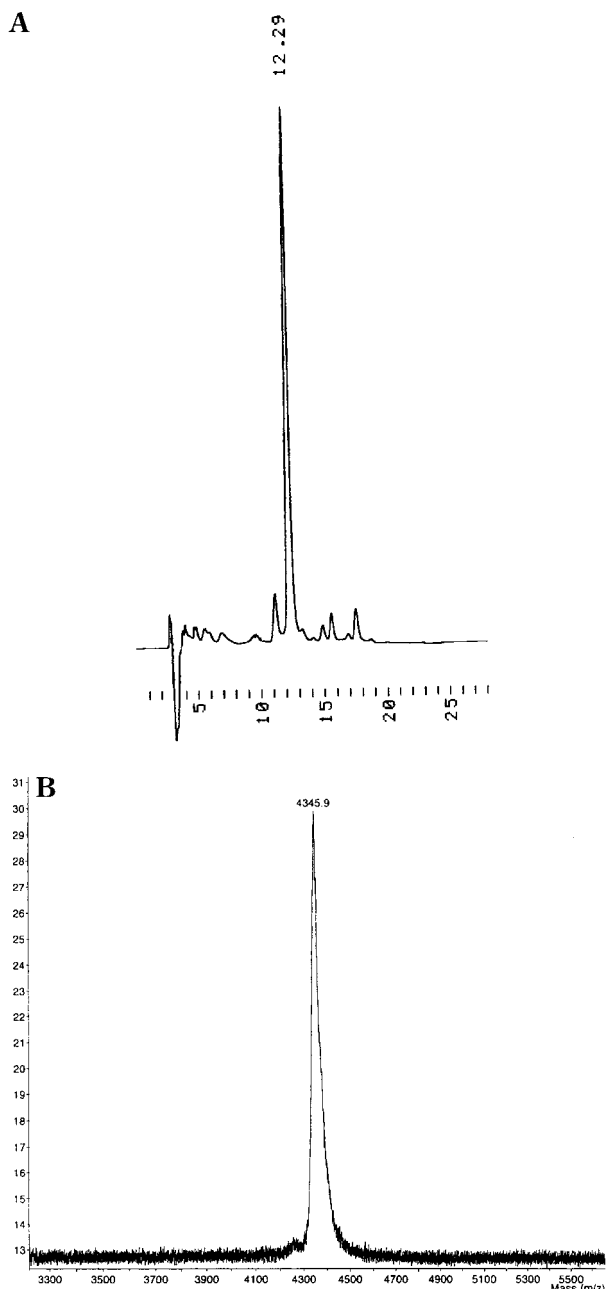


Figure 1. (A) Ion exchange HPLC profile of crude **16**: Syn-Chropak AX-300, 4.6 × 250 mm, 6 mm column, flow rate of 1 mL min⁻¹, λ = 260 nm, buffer A = 0.05 M KH₂PO₄ in 50% (v:v) aqueous formamide at pH 5.6; buffer B = buffer A + 0.6 mol dm⁻³ (NH₄)₂SO₄, from 10 to 40% B in 20 min. (B) MALDI-TOF mass spectrum of **16** after HPLC purification (negative detection mode). The spectrum was obtained by overlaying 16 laser pulses. M (calcd) 4342.9.

otide chain assembly, the protected oligonucleotide, **15**, was treated with **7** (free base; 0.5 M solution in water) for 6 h followed by ammoniolysis (13). The oligonucleotide, **16**, was purified on HPLC as described previously (15) and characterized with MALDI-TOF mass spectrometry.

RESULTS AND DISCUSSION

Synthesis of the Azacrowns. Synthesis of 3-(3-aminopropyl)-1,5,9-triazacyclododecane (**7**) is outlined in Scheme 1. Diethyl 2-(2-cyanoethyl)malonate was initially reduced into 2-(hydroxymethyl)-5-aminopentan-1-ol, **1**, by using the method of Brown (18). To simplify

the purification procedure, the primary amino group of **1** was protected as a carbamate without isolation. Surprisingly, TLC analysis of the reaction mixture showed the presence of two major products. They were separated and characterized as the desired diol, **3**, and the ether **4**, produced from **2**. The formation of **2** is quite surprising since conversion of esters into ethers has been reported only with trichlorosilane and UV light or by catalytic reduction (19). Furthermore, BMS reduction of diethyl succinate is known to produce butane-1,4-diol in good yield (18). Thus, the formation of ether seems to be a unique characteristic of malonates. Fortunately, **4** was easily separated on silica gel column chromatography, and the desired product, **3**, could be isolated in 34% overall yield. Tosylation of **3** with tosyl chloride in dry pyridine gave the key intermediate, **5**, in good yield.

From the several methods described for the preparation of azacrowns (20), the method of Alder *et al.* (21), later modified by Haseltine *et al.* (22), was chosen. In this approach, an alkane ditosylate is initially allowed to react with 1,5,7-triazabicyclo[4.4.0]dec-5-ene (TBD) to form a guanidinium salt which is reduced *in situ* to an orthoamide. Acid-catalyzed hydrolysis of the orthoamide function gives the azacrown. Accordingly, **5** was treated with a 2-fold excess of TBD in DME for 2 days at ambient temperature followed by borohydride reduction. Although the yield of **6** was considerably lower than that reported (22) for the corresponding 3-unsubstituted analogue (32% vs quantitative), the product was easily separated from the reaction mixture on silica gel. Acid-catalyzed hydrolysis of the orthoamide function and concomitant removal of the amino protecting group yielded **7** as the final product. It was converted, prior to oligonucleotide tethering, to the corresponding free base by passing it through a strong anion exchange resin.

The second azacrown, **13**, was synthesized from *cis*-1,3-benzylidene glycerol, **8**, according to Scheme 2. Michael addition of acrylonitrile to **8** in the presence of a catalytic amount of sodium hydride gave **9** in moderate yield. It is worth noting that alkylations which require stoichiometric amounts of base were not successful, in all likelihood due to epoxidation of **8**. The cyano group of **9** was reduced to the amino function with cobalt(II) chloride in the presence of sodium borohydride (23, 24). To simplify the purification procedure, the product was converted without isolation into **10** by removal of the diol protection followed by protection of the amino group. Tosylation as described above gave rise to **11**. It was then converted to the orthoamide, **12**, as above in 52% isolated yield. Acid-catalyzed hydrolysis gave rise to **13** as the final product.

Preparation of the Oligonucleotide Conjugate.

To demonstrate the suitability of the azacrowns prepared for oligonucleotide tethering, a model sequence dT₆XT₇ (X = abasic site) was synthesized (Scheme 3). The phosphoramidite **14** was used in the seventh coupling step in the conventional manner (13). When the oligonucleotide chain assembly was completed, the solid support-bound material, **15**, was treated with 0.5 M aqueous **7** for 6 h at room temperature. This introduces the tether group and removes the phosphate protections. The deprotection procedure was completed by conventional ammonolysis to release all of the oligonucleotide from the solid support. The oligonucleotide, **16**, was isolated on ion exchange HPLC, further purified on an RP column, and desalted by gel filtration (15). Its ion exchange HPLC profile (unpurified reaction mixture) is shown in Figure 1A. The identity of the oligonucleotide conjugate was confirmed on MALDI-TOF mass spectrom-

etry (Figure 1B). The observed molecular weight was in agreement with the calculated value. Studies on the ability of oligodeoxyribonucleotides tethered to azacrowns to promote sequence specific RNA hydrolysis are in progress in our laboratory.

ACKNOWLEDGMENT

Financial support from the Academy of Finland is gratefully acknowledged.

LITERATURE CITED

- (1) For reviews, see: (a) Green, P. J., Pines, O., and Inouye, M. (1986) The Role of Antisense RNA and Gene Regulation. *Annu. Rev. Biochem.* 55, 569–97; (b) Uhlman, E., and Peyman, A. (1990) Antisense Oligonucleotides: a New Therapeutic Principle. *Chem. Rev.* 90, 543–84; (c) James, W. (1991) Towards Gene-inhibition Therapy: a Review of Progress and Prospects in the Field of Antiviral Antisense Nucleic Acids and Ribozymes. *Antiviral Chem.* 2, 191–214; (d) Thuong, N. T., and Helene, C. (1993) Sequence-specific Recognition and Modification of Double-helical DNA by Oligonucleotides. *Angew. Chem., Int. Ed. Engl.* 32, 666–90; (e) Goodchild, J. (1990) Conjugates of Oligonucleotides: a Review of Their Synthesis and Properties. *Bioconjugate Chem.* 1, 165–87; (f) English, U., and Gauss, D. (1991) Chemically Modified Oligonucleotides as Probes and Inhibitors. *Angew. Chem., Int. Ed. Engl.* 30, 613–29; (g) De Mesmaeker, A., Häner, R., Martin, P., and Moser, H. E. (1995) Antisense Oligonucleotides. *Acc. Chem. Res.* 28, 366–74; (h) Eckstein, F. (1985) Nucleoside Phosphorothioates. *Annu. Rev. Biochem.* 54, 367–402; (i) Lönnberg, H., and Vuorio, E. (1996) Towards Genomic Drug Therapy with Antisense Oligonucleotides. *Ann. Med.* 28, 511–22.
- (2) Magda, D., Miller, R. A., Sessler, J. L., and Iverson, B. L. (1994) *J. Am. Chem. Soc.* 116, 5891.
- (3) Bashkin, J. K., Frolova, E. I., and Sampath, U. S. (1994) Sequence-specific Cleavage of HIV mRNA by a Ribozyme Mimic. *J. Am. Chem. Soc.* 116, 5981–2.
- (4) Komiyama, M., and Inokawa, T. (1994) Selective Hydrolysis of tRNA by ethylenediamine Bound to a DNA Oligomer. *J. Biochem.* 116, 719–20.
- (5) Kazunari, M., Endo, M., and Komiyama, M. (1994) Lanthanide Complex-Oligo-DNA Hybrid for Sequence-selective Hydrolysis of RNA. *J. Chem. Soc., Chem. Commun.*, 2019–20.
- (6) Hall, J., Husken, D., Piele, U., Moser, H. E., and Häner, R. (1994) Efficient Sequence-specific Cleavage of RNA Using Novel Europium Complexes Conjugated to Oligonucleotides. *Chem. Biol.* 1, 185–90.
- (7) Uchiyama, Y., Inoue, H., and Ohtsuka, E. (1994) DNA-Linked RNase H for Site-Selective Cleavage of RNA. *Bioconjugate Chem.* 5, 327–32.
- (8) Komiyama, M., Inokawa, T., and Yoshinari, K. (1995) Ethylenediamine-oligo DNA Hybrid as Sequence-selective Artificial Ribonuclease. *J. Chem. Soc., Chem. Commun.*, 77–8.
- (9) Hovinen, J., Guzaev, A., Azhayev, A., and Lönnberg, H. (1995) Imidazole Tethered Oligonucleotides: Synthesis and RNA Cleaving Activity. *J. Org. Chem.* 60, 2205–9.
- (10) Kuusela, S., and Lönnberg, H. (1992) Metal Ion-Promoted Hydrolysis of Uridine 2',3'-Cyclic Monophosphate: Effect of Metal Chelates and Uncomplexed Aquo ions. *J. Phys. Org. Chem.* 50, 803–11.
- (11) Kuusela, S., and Lönnberg, H. (1994) Metal-ion-promoted Hydrolysis of Polyuridylic Acid. *J. Chem. Soc., Perkin Trans.* 2, 2301–6.
- (12) Kuusela, S., Rantanen, M., and Lönnberg, H. (1995) Metal-ion-promoted Hydrolysis of Uridyl(3',5')uridine: Internal vs. External General Base Catalysis. *J. Chem. Soc., Perkin Trans.* 2, 2269–73.
- (13) Hovinen, J., and Salo, H. (1997) C-Glycoside Phosphoramidite Building Block for Versatile Functionalization of Oligonucleotides. *J. Chem. Soc., Perkin Trans.* 1, 3017–20.
- (14) Cox, J. P. L., Craig, A. S., Helps, I. M., Jankowski, K. J., Parker, D., Eaton, M. A. W., Millican, A. T., Millar, K., Beeley, N. R. A., and Boyce, B. A. (1990) Synthesis of C- and N-Functionalised Derivatives of 1,4,7-Triazacyclononane-1,4,7-triyltriacetic Acid (NOTA), 1,4,7,10-Tetra-azacyclododecane-1,4,7,10-tetrayltetraacetic Acid (DOTA), and Diethylenetriaminepenta-acetic Acid (DTPA): Bifunctional Complexing Agents for the Derivatization of Antibodies. *J. Chem. Soc., Perkin Trans.* 1, 2567–76.
- (15) Hovinen, J., Guzaev, A., Azhayev, A., and Lönnberg, H. (1994) Novel Solid Supports for the Preparation of 3'-Derivatized Oligonucleotides: Introduction of 3'-Alkylphosphate Tether Groups Bearing Amino, Carboxy, Carboxamido, and Mercapto Functionalities. *Tetrahedron* 50, 7203–18.
- (16) Piele, U., Zürcher, W., Schär, M., and Moser, H. E. (1993) Matrix-assisted Laser Desorption Ionization Time-of-flight Mass Spectrometry: a Powerful Tool for the Mass and Sequence Analysis of natural and Modified Oligonucleotides. *Nucleic Acids Res.* 21, 3191–6.
- (17) Carlsen, P. H., Sorbye, K., Ulven, T., and Aasbo, K. (1996) Synthesis of Benzylidene-Protected Dihydroxyacetone. *Acta Chem. Scand.* 50, 185–7.
- (18) Brown, H. C., Choi, T. M., and Narasimhan, S. (1982) Selective Reductions. 29. A Simple Technique to Achieve an Enhanced Rate of Reduction of Representative Organic Compounds by Borane-Dimethyl Sulfide. *J. Org. Chem.* 47, 3153–63.
- (19) March, J. (1992) *Advanced Organic Chemistry*, 4th ed., p 1213, Wiley, New York.
- (20) For reviews, see: (a) Kaden, T. A. (1984) Synthesis and Metal Complexes of Aza-Macrocycles with Pendant Arms having Additional Ligating Groups. *Top. Curr. Chem.* 121, 157–79; (b) Kimura, E. (1992) Macrocyclic Polyamines with Intelligent Functions. *Tetrahedron* 48, 6175–217.
- (21) Alder, R. W., Mowlam, R. W., Vachon, D. J., and Weisman, G. R. (1992) New Synthetic Routes to Macrocyclic Triamines. *J. Chem. Soc., Chem. Commun.*, 507–8.
- (22) Kim, R. D. H., Wilson, M., and Haseltine, J. (1994) Simple Preparation of Tricyclic Orthoamides and Macrocyclic Triamines. *Synth. Commun.* 24, 3109–14.
- (23) Satoh, T., Suzuki, S., Suzuki, Y., Miyaji, Y., and Imai, Z. (1969) Reduction of Organic Compounds with Sodium Borohydride Transition Metal Salt Systems. Reduction of Organic Nitrile, Nitro and Amide Compounds to Primary Amines. *Tetrahedron Lett.*, 4555–8.
- (24) Misiura, K., Durrant, I. m., Evans, M. R., and Gait, M. J. (1990) Biotinyl and Phosphotyrosinyl Phosphoramidite Derivatives Useful in the Incorporation of Multiple Reporter Groups on Synthetic Oligonucleotides. *Nucleic Acids Res.* 18, 4345–54.

BC970108C

Methods for the Characterization of Phosphatase-Stabilized 2–5A-Antisense Chimeras

Mark R. Player, Wei Xiao, Hagen Cramer, and Paul F. Torrence*

Section on Biomedical Chemistry, Laboratory of Medicinal Chemistry, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892-0805.

Received August 21, 1997; Revised Manuscript Received November 3, 1997[®]

We have developed chromatographic and spectrophotometric assays for determining the degree of thiolation in phosphatase-resistant 5'-monothiophosphate-capped 2–5A-antisense chimeras. Concomitantly, we have explored the reactivity of this 5'-monophosphorothioate moiety with reporter reagents such as 5-iodoacetamidofluorescein and 5,5'-dithiobis(2-nitrobenzoic acid). On the basis of these reactions, analyses for 5'-monothiophosphate-functionalized 2–5A-antisense chimeras were made possible. Kinetic experiments demonstrated that oligonucleotide backbone negative charge could retard mixed disulfide formation in the reaction of 5,5'-dithiobis(2-nitrobenzoic acid) with 5'-monothiophosphorylated 2–5A-antisense chimeras.

INTRODUCTION

Selective inhibition of gene expression with antisense oligodeoxynucleotides is possible through several pathways. Hybridization arrest by the antisense oligonucleotide and RNase H-mediated cleavage of the duplex formed between that oligonucleotide and its mRNA target have been presented as two predominant mechanisms (1). Recently, a third mechanism has been realized through a novel structural class of oligonucleotides. Conjugation of a 5'-phosphorylated 2',5'-oligoadenylate tetramer (2–5A)¹ to a 3',5'-deoxyribonucleotide complementary to a targeted sequence in an RNA provides a novel reagent which effects the selective and specific cleavage of that RNA through intervention of RNase L (EC 3.1.26.) (2–6).

The general structure of these 2–5A-antisense chimeras is pA2'p(5'A2'p)₂5'ApBupBup5'dN3'[p5'dN3']_np5'dN, where Bu is 1,4-butanediol, *n* is any integer, and dN is any common deoxyribonucleoside. The presence of a 5'-monophosphate group is necessary for this principle, as the chimera with no 5'-monophosphate (or core chimera) has been demonstrated to bind only poorly and not to activate RNase L.

As phosphatases are ubiquitous in the biological systems in which 2–5A-antisense chimeras are used, stabilization against this activity became necessary.

Although 3'-exonuclease stabilization of antisense oligonucleotides has been vigorously pursued, phosphatase stabilization is pertinent only to the 2–5A mechanism. Nucleoside 5'-monothiophosphates show dramatically increased resistance toward phosphatase compared to 5'-nucleoside monophosphates (7). We have demonstrated that an oxygen to sulfur substitution in the 5'-monophosphate to afford a 5'-monothiophosphate 2–5A trimer (ps5'A2'p5'A2'p5'A) yields several distinct benefits, including (1) dramatically increased resistance of ps5'A2'p5'A2'p5'A toward degradation by bacterial alkaline phosphatase and calf intestinal alkaline phosphatase compared to p5'A2'p5'A2'p5'A and (2) the fact that ps5'A2'p5'A2'p5'A is as efficient in binding to and activating RNase L as p5'A2'p5'A2'p5'A (8). A 2–5A-antisense chimera targeting the protein kinase PKR, ps5'A2'p(5'A2'p)₃BupBupd(GTACTACTCCCTGCTTCTG), was equipotent with its 5'-monophosphate-containing analogue (4). Finally, a 2–5A-antisense chimera, stabilized by such a 5'-thiophosphate substitution, has been found to be a potent inhibitor of respiratory syncytial virus replication (6). The vital role played by the 5'-monothiophosphate in the cellular biological activity of 2–5A-antisense chimeras, coupled with the relative paucity of data on functionalization of this moiety, led us to develop facile approaches for quantitative analysis of the 5'-monothiophosphate group in these oligonucleotides.

EXPERIMENTAL PROCEDURES

Instrumental Techniques. For HPLC analysis, a HP1050 instrument with Chemstation software and data analysis was used throughout. Four different columns and methods were used for analytical and preparative evaluations. The ion-pair method (method A) utilized a Hamilton polystyrene reverse-phase (PRP-1) 300 × 7 mm column with a flow rate of 1.5 mL/min running a convex gradient of 5 to 90% mobile phase B in A over 60 min, where A was 10 mM tetrabutylammonium phosphate (TBAP) solution (pH 7.5) and B was 10 mM TBAP (pH 7.5) in an acetonitrile/water (8:2, v:v) solution. The enzyme method (method B) used a Beckman Ultrasphere ODS (4.6 × 250 mm) column with a flow rate of 0.5 mL/min running 2% B isocratically for 20 min and then a

* To whom correspondence should be addressed. Phone: (301) 496-2653. Fax: (301) 402-0589. E-mail: torrence@helix.nih.gov.

[®] Abstract published in *Advance ACS Abstracts*, December 15, 1997.

¹Abbreviations: 2–5A, (pp)p[5'A2']_nA (*n* = 1); HRFABMS, high-resolution fast atom bombardment mass spectroscopy; MALDIMS, matrix-assisted laser-desorption ionization mass spectroscopy; 5-IAF, 5-iodoacetamidofluorescein; PRP, polystyrene reverse-phase; SVPDE, snake venom phosphodiesterase; psA, adenosine 5'-monophosphorothioate; pA, adenosine 5'-monophosphate; core chimera, A2'p(5'A2'p)₂5'ApBupBup5'dN3'-[p5'dN3']_np5'dN; 5'-ps chimera, psA2'p(5'A2'p)₂5'ApBupBup5'dN3'[p5'dN3']_np5'dN; 5'-po chimera, pA2'p(5'A2'p)₂5'ApBupBup5'dN3'[p5'dN3']_np5'dN; AUC, integrated area under the curve; MWCO, molecular weight cutoff; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); TETD, tetraethylthiuram disulfide; ddH₂O, distilled deionized water; CGE, capillary gel electrophoresis.

Table 1. High-Resolution Mass Spectral Data of Conjugates

compound	type of MS	empirical formula	calcd exact weight	obs exact weight	error
1	MALDI	C ₂₃₁ H ₃₀₃ N ₈₀ O ₁₅₂ P ₂₅	7403.2	7402.3	0.01%
2	MALDI	C ₂₃₁ H ₃₀₃ N ₈₀ O ₁₅₁ P ₂₅ S	7419.2	7419.3	0.001%
3	HRFAB (neg)	C ₃₂ H ₂₇ N ₆ O ₁₂ PS	749.1067	749.1042	-3.4, -2.5 ppm/mmu
4	MALDI	C ₂₅₃ H ₃₁₆ N ₈₁ O ₁₅₇ P ₂₅ S	7806.2	7810.2	0.05%

linear gradient of 2 to 45% B in A over 15 min, followed by isocratic conditions for another 10 min where A was 100 mM ammonium phosphate (pH 5.5) and B was methanol/water (1:1, v:v). The reverse-phase method (method C) employed a Beckman Ultrasphere ODS (10 × 250 mm) reverse-phase C₁₈ column with a flow rate of 1 mL/min running a linear gradient of 0 to 50% solvent B in A over 30 min, where A was 50 mM ammonium acetate and B was acetonitrile/water (1:1, v:v). The ion-exchange method (method D) made use of a Dionex PA-100 (4 × 250 mm) ion-exchange column with a flow rate of 1.0 mL/min running a linear gradient of 10 to 90% solvent B in A over 25 min, followed by isocratic conditions for another 25 min where A was 25 mM Tris-HCl (pH 7.0) in MeCN/H₂O (1:200, v:v) and B was 25 mM Tris-HCl and 1 M ammonium chloride (pH 7.0) in MeCN/H₂O (1:200, v:v).

Capillary gel electrophoretic (CGE) analyses and snake venom phosphodiesterase (SVPDE) digestion were performed as previously described (9). 5'-Monothiophosphate-modified 2-5A-antisense chimera syntheses were also performed as described (9) with modifications in the sulfurization step as described below. The following compounds were synthesized: **1**, p5'A2'p(5'A2'p)₃BupBupd(GTACTACTCCCTGCTTCTG); **2**, ps5'A2'p(5'A2'p)₃BupBupd(GTACTACTCCCTGCTTCTG); and **3**, A2'p(5'A2'p)₃BupBupd(GTACTACTCCCTGCTTCTG).

The 2-5A-antisense chimeras were synthesized automatically on an ABI 392 DNA/RNA synthesizer using multiple cycles and phosphoramidite chemistry. The four different domains present within 2-5A-antisense oligonucleotides, each of which requires different coupling wait times, were antisense DNA, a 1,4-butanediol linker, 2',5'-oligoadenylate, and 5'-monophosphate. The corresponding coupling wait times were 15, 300, 600, and 60 s, respectively.

Compound **2** was prepared using two different sulfurization reactions. The first used TETD with a 6 s delivery time and a 900 s wait time and generated preparation **I**; the second employed 3H-1,2-benzodithiol-3-one 1,1-dioxide (Beaucage reagent) (10) with a 30 s delivery time and a 10 s wait time which yielded preparation **II**. Preparations **I** and **II** were obtained after initial purification using method A.

Conjugation Reactions with pA, psA, I, and II. 5-Iodoacetamidofluorescein Conjugation. 5-Iodoacetamidofluorescein (30 μL of a 100 mM DMF solution) (Pierce, Rockford, IL) was added to 20 μL of either (i) a 100 mM solution of pA or psA, (ii) a 10 mM solution of **1**, or (iii) a 10 mM solution of preparation **II**, all in 100 mM Tris (pH 8) buffer. After they were vortexed, the reaction preparation was incubated at 55 °C for 18 h, fully immersed in a water bath. For workup of reaction preparations containing **1** or preparation **II**, the resultant solution was applied to a Chromaspin column (Clontech, Palo Alto, CA) and centrifuged at 700g for 5 min. The eluant from the spin column was purified using method D with the following modification. Buffer A contained 25 mM Tris-HCl (pH 7.0) in MeCN/H₂O (1:9, v:v), and buffer B contained 25 mM Tris-HCl and 1 M lithium chloride (pH 7.0) in MeCN/H₂O (1:9, v:v). The reaction

mixtures containing pA or psA were purified directly by HPLC using method C.

Manipulation of reaction conditions did not provide an increase in the percentage of conversion of compound **2** to **4**. Therefore, a correction factor of 1.25 was applied to the product peak on the basis of empirically derived reaction product yields. The A₂₆₀ of the fluoresceinated product peak was also corrected for the absorbance of fluorescein at 260 nm (Promega Technical Bulletin 213). This was determined experimentally by measurement of A₂₆₀/A₄₉₅ under the buffer conditions used during elution. Therefore, the formula used to estimate the percentage of **2** from the HPLC results was 100 - 100[AUC of all nonproduct peaks/AUC of all nonproduct peaks + 1.25-AUC of product peak - (AUC of product peak × 0.45)]].

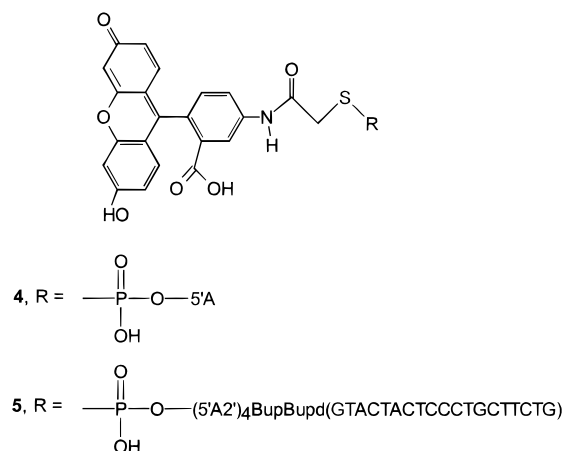
5,5'-Dithiobis(2-nitrobenzoic acid) Conjugation. A 10 mM 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) (Pierce) solution was prepared in 100 mM NaH₂PO₄ (pH 8.0) buffer. The buffer was first deaerated with Ar and extensive sonication in order to prevent reoxidation of the mixed disulfide generated during the reaction. A 45 μM psA solution was prepared in 1 mL of ddH₂O. The DTNB solution (50 μL) was added to an appropriate amount of the 45 μM stock psA solution to give five final psA concentrations ranging between 2 and 35 μM. Triplicate psA standards were prepared. The spectrophotometer was zeroed with DTNB solution (50 μL) diluted to 1 mL. Triplicate concentrations were measured at 420 nm, averaged, and plotted with the biased standard deviation serving as the error bar. With a standard curve thus constructed, the corresponding A₄₂₀ of a similarly treated 12.1 μM solution of preparation **I** was determined.

Preparation **I** was diluted to 12.1 μM, lyophilized, and rediluted in sodium phosphate buffer to 1 mL, and again with 50 μL of a DTNB solution. Measurement of the spectrum showed an A₄₂₀ of 0.0967. This corresponded to a concentration of 8.4 μM as determined by the psA concentration calibration curve.

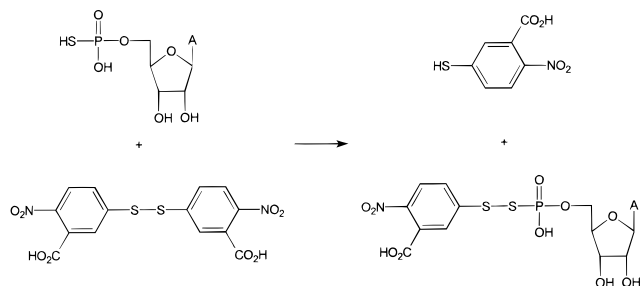
Kinetic experiments with preparation **I** and psA were performed as follows. psA or preparation **I** was prepared as a 10 μM solution and reacted with 50 μL of the DTNB solution prepared as described above, in 1 mL of phosphate buffer (pH 8). The extent of the reaction was monitored at 420 nm.

Mass Spectrometric Analysis. Advances in matrix-assisted laser desorption ionization mass spectroscopy (MALDIMS) using 6-aza-2-thiothymine (ATT) cocrystallized with ammonium citrate as a matrix (11) were used to obtain a mass spectrum of **1**, **2**, and **5** (Table 1, Scheme 1). The matrix solution was prepared with ATT (10 mg/mL) in acetonitrile/20 mM ammonium citrate (1:1, v:v). This solution (3 μL) and 10 pmol of **1**, **2**, and **5** (total volume, 4 μL) were lyophilized and then analyzed on a Kompact MALDI III instrument using a 337 nm N₂ laser. With the instrument in the linear mode, the desorption took place with a static electric potential of approximately 22 keV. High-resolution fast-atom bombardment mass spectroscopy (HRFABMS) of **4** was performed with a JEOL JMS-SX102 instrument in the negative ion mode using a glycerol matrix (Table 1).

Scheme 1



Scheme 2



Identification of Higher-Molecular Weight Contaminants in Preparations I and II. Before or after purification of preparation **I** or **II** (or any 5'-ps chimera) using method A or D, a contaminant with a higher R_t can be observed. To determine if this material was the disulfide, product with approximately 70% of higher- R_t material was applied to a prewashed Sep-Pak cartridge, and the column was washed with 20 mL of ddH₂O and eluted with methanol/ddH₂O (1:1). After evaporation of the methanol, dialysis with a 3500 MWCO membrane was performed to remove any remaining low-molecular weight impurities. After lyophilization, the oligonucleotide was dissolved in 10 mM Tris (pH 7.5) at 3.5 μM for 12 h at 4 °C with 2-mercaptoethanol concentrations of either 0, 3.5, or 35 μM .

Dionex Separation of 5'-ps and 5'-po Chimeras. Preparations **I** and **II**, which had initially been purified by method A, were analyzed for homogeneity by method D. The main product peak for preparation **I** was observed at 15.36 min (Figure 1a) and for preparation **II** at 15.37 min (Figure 1b). The primary side product for both preparations eluted with a shorter retention time and was observed at 14.86 min for preparation **I** and at 14.88 min for preparation **II**.

To demonstrate that method D was resolving **1** and **2**, a co-injection experiment was performed with **1** and preparation **II**. First, the faster-eluting peak ($R_t = 14.88$ min) and the main product peak ($R_t = 15.37$ min) of preparation **II** were purified using method D, and then collected, desalted, and quantified. The homogeneity of the method D-purified main product peak of preparation **II** and **1** was demonstrated by injection with method D. Compound **1** had a retention time of 14.35 min, and the isolated main product peak of preparation **II** had a retention time of 14.99 min. This difference of 0.64 min was approximately the same as that seen between the main product peak and the faster-eluting peak ($R_t = 14.88$ min), when preparations **I** and **II** were analyzed

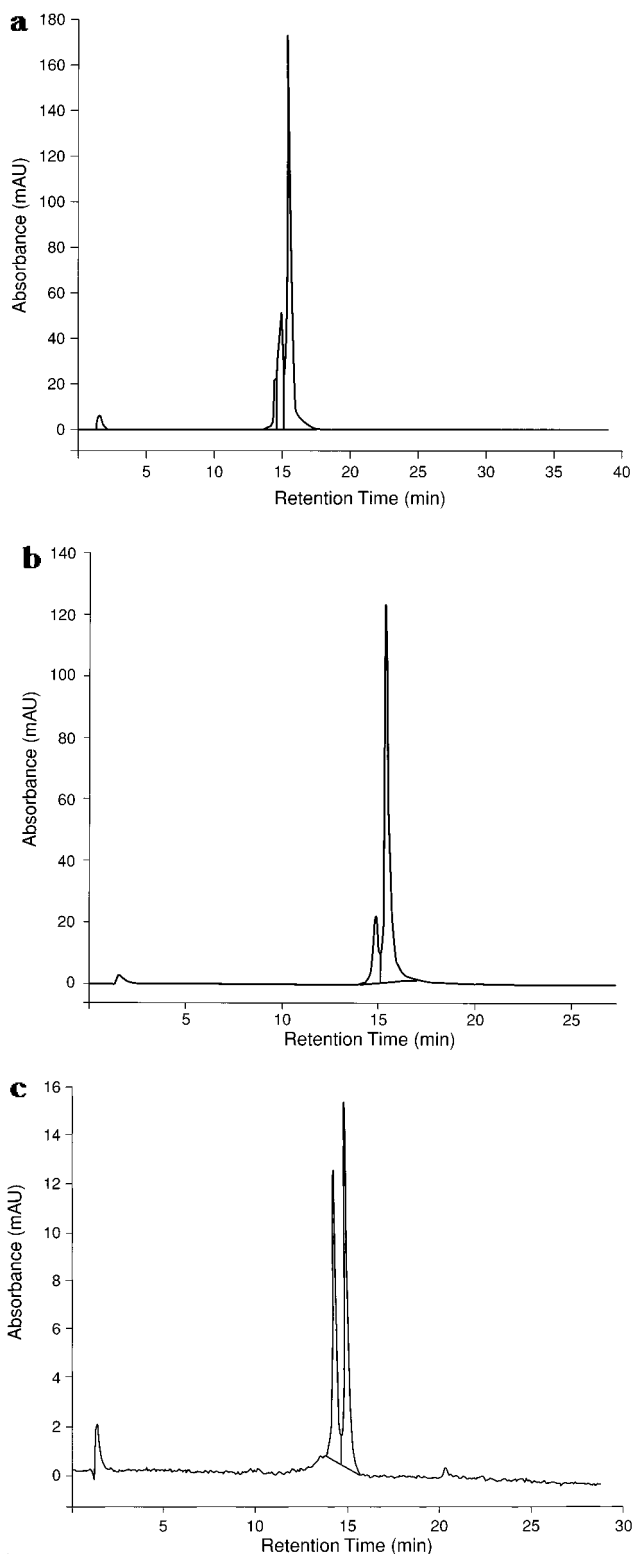


Figure 1. (a) Ion-exchange HPLC chromatogram of preparation **I** (method D), (b) ion-exchange HPLC chromatogram of preparation **II** (method D), and (c) ion-exchange HPLC chromatogram of a co-injection of **1** and preparation **I** (method D).

by method D. Next, **1** and the main product peak of method D-purified preparation **II** were mixed in an equimolar ratio and co-injected. A doublet with individual peaks at 14.38 and 14.99 min was observed (Figure 1c), again in good agreement with the retention times obtained with each compound separately.

The major and minor product peaks observed during method D purification of preparation **I** were collected as

they separated. The peak that eluted first at 14.86 min (**1**) and the main product peak which eluted later at 15.36 min (**2**) were each desalted with a Sep-Pak cartridge (Waters), lyophilized, and subjected to MALDIMS (11) (Table 1) which confirmed the identity of the peaks as monophosphate (**1**) and thiophosphate (**2**). This permitted a precise determination, in this instance, of the percentage of **2** in the compounds prepared by alternate sulfurization protocols. The percentage of **2** in preparation **I** was 67% and in preparation **II** 83%.

RESULTS AND DISCUSSION

2–5A-Antisense chimeric nucleic acids are relatively complex structures when compared with antisense nucleic acids in general since they contain several structural domains that are vital for the biological activity of these reagents. Proceeding from the 5'-terminus to the 3'-terminus, these domains are: (1) a 5'-monophosphate or 5'-monothiophosphate moiety that is required for the activation of RNase L, (2) a 2',5'-phosphodiester-linked oligoadenylate tetranucleotide (2–5A) which also is necessary for activation of RNase L, (3) a linker domain made up of two 1,4-butanediol monophosphates in tandem, and (4) an antisense domain consisting of, for instance, a 3',5'-deoxyribonucleotide that may or may not be stabilized to degradation. Thus, four distinct chemical entities must be structurally intact in order to act in harmony as biologically active 2–5A-antisense agents. The ability to adequately characterize synthetic 2–5A-antisense products is absolutely a prerequisite for their effective use in biological systems and as potential therapeutic agents.

As described previously, a combination of chromatographic analysis, MALDIMS, Maxim–Gilbert sequencing, and SVPDE digestion can be employed to characterize completely such 2–5A-antisense composite nucleic acids. Of these analytical approaches, the most informative is the latter SVPDE digestion since it dissects the purified synthetic product into nucleotidic products that reflect the structural integrity of the 2–5A-antisense molecule as described above. Specifically, the following are produced: 3 mol of 5'-AMP from the intact 5'-phosphorylated 2',5'-oligoadenylate tetramer, 1 mol of 5'-AMP still linked to the two butanediol phosphate linkers, and the various dNMPs from the antisense component. In the event that a key determinant of biological activity, the 5'-phosphate, is missing, or that 5'-phosphorylation is incomplete, then A itself is produced in the SVPDE digestion.

A second generation of 2–5A-antisense chimeras possess a 5'-monothiophosphate (e.g. **2**) that is resistant to the action of phosphatases and thereby extends the biological half-life of 2–5A-antisense chimeras. Analysis of such 5'-monothiophosphorylated 2–5A-antisense chimeras by ion-exchange HPLC using Dionex PA-100 columns frequently revealed what appears at first to be the presence of an unusually large percentage of the $n - 1$ failure sequence.² For instance, when preparation **I**, from sulfurization using TETD, was analyzed using HPLC method D, a major product peak was observed at 15.36 min (Figure 1a) with a minor product at 14.86 min. A similar chromatographic picture emerged when preparation **II** was analyzed (Figure 1b).

The major and minor peaks observed during the above HPLC method D analysis of preparation **I** were collected separately. After the homogeneity of each collected peak was checked by HPLC method D, fractions containing each peak (R_t s of 14.86 and 15.36 min, respectively) were

desalted with a Sep-Pak cartridge, lyophilized to dryness, and subjected to MALDIMS. The peak with the faster retention time possessed an observed weight consistent with structure **1**, whereas the peak with the slower R_t (15.36 min) gave an observed weight consistent with oligonucleotide **2** (Table 1). Co-injection experiments showed that the two observed peaks of both preparation **I** and **II** were identical, corresponding to oligonucleotide **1** with the faster R_t and **2** with the slower R_t .

The above Dionex ion-exchange HPLC proved to be neither a definitive nor practical methodology for routine analysis since the core chimera (**3**) also elutes earlier than the desired product peak **2**. Discrimination among free 5'-OH (**3**), 5'-monophosphate (**1**), and 5'-monothiophosphate (**2**) derivatives of the chimeric oligonucleotide would thus require independent syntheses of reference 5'-monophosphate and 5'-OH core derivatives for each 2–5A-antisense sequence prepared, an impractical requirement.

The direct application of the powerful SVPDE digestion analysis to 5'-thiophosphorylated chimeric oligonucleotide **2** was problematic. Digestion with SVPDE itself, as for the analysis of chimeric oligonucleotides such as **1**, was not successful because reliable separation of psA and pA could not be achieved using the routine HPLC method B or any variation thereof that would still allow appropriate separation of the other digestion products. In one variation of the analysis, the percentage of **2**, as compared to the percentages of **3** and **1**, might be addressed by digestion of the oligonucleotide sample with a combination of SVPDE and calf intestinal phosphatase. Since the thiophosphate, psA, was resistant to phosphatase, it would remain intact and could be directly determined. However, this approach would not permit direct estimation of the percentages of **1** and **3** in a sample since all pA, produced by SVPDE cleavage, would be converted to A itself.

Another possible strategy for analysis of the psA content of a sample of oligonucleotide **2** would be selective covalent modification of psA in the oligonucleotide so that, after SVPDE digestion, the modified psA could be readily detected and analyzed. Relevant requirements for such an acceptable reporter group would include the absence of reactivity toward pA and the other components of a 2–5A-antisense chimera and fast and complete reaction with psA.

When 5-iodoacetamidofluorescein (5-IAF), which has been shown to react with sulfhydryls (17) as well as with phosphorothioate diesters (18), was reacted with pA or oligonucleotide **1**, no detectable reaction occurred. In contrast, when 5-IAF was reacted with psA, a product was obtained which was easily distinguished by its high

²Occasionally, an additional product has been observed in some preparations of 5'-monothiophosphorylated 2–5A-antisense chimeras. It migrates in Dionex HPLC or CGE with a markedly increased retention time, indicating that it possesses a much higher molecular weight. HPLC analysis (method D) revealed that the addition of 2-mercaptoethanol effected a quantitative conversion of this high- R_t component to oligonucleotide **2**, in support of the hypothesis that this additional product corresponded to a P1,P2-bis(adenosine 5'-monophosphoryl)oligonucleotide disulfide. In further support of this, the 5'-thiophosphates of adenosine, thymidine, uridine, and inosine have all been reported to dimerize after oxidation with I_2 or $K_3Fe(CN)_6$ or when passed through a short column of activated charcoal (12–14). 5'-Thiophosphoryl and 5'-dithiophosphoryl oligodeoxyribonucleotides also have been reported to undergo dimerization in the presence of dilute $K_3Fe(CN)_6$ solution (15, 16).

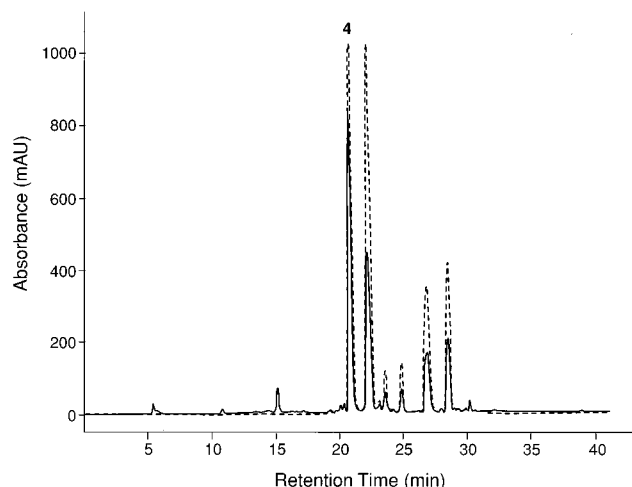


Figure 2. Reverse-phase HPLC chromatogram of the reaction mixture of psA and 5-IAF demonstrating the formation of **4**. Solid lines represent A_{260} . Dashed lines represent A_{495} .

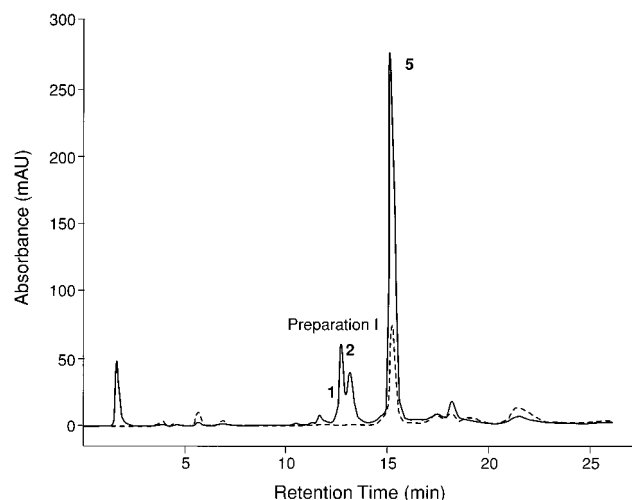


Figure 3. Reverse-phase HPLC chromatogram of the reaction mixture of preparation **I** and 5-IAF demonstrating the formation of **5**. Solid lines represent A_{260} . Dashed lines represent A_{495} .

A_{260}/A_{495} ratio (0.91) (Figure 2). HRFABMS showed that the structure of the product was consistent with **4**.

Reaction of 5-IAF with preparation **II** under the identical conditions gave a new product, consistent with structure **5**, according to MALDIMS (Table 1). SVPDE digestion of oligonucleotide adduct **5** gave, in addition to the usual nucleotidic products of digestion, a new product peak (**4**) which, however, eluted very late under method C conditions due to its hydrophobic nature.

A variation of this approach for analysis of oligonucleotide **2**-containing samples involved determination of the extent of reaction of oligonucleotide with 5-IAF. Since oligonucleotide **1** did not react with 5-IAF, the extent of reaction would provide a direct measure of the percentage of **2** in the mixture. In trial reactions of **2** with 5-IAF, the maximum conversion to **5** that could be obtained ranged from 77 to 83% as determined by HPLC method D so that a correction factor of 1.25 was applied for determination of the percentage of **2** in a product mixture. Preparation **II** analyzed for 80 and 82% **2** in two separate determinations, in good agreement with the 83% value determined by Dionex HPLC method D (Figure 3).

5, 5'-Dithiobis(2-nitrobenzoic acid) (DTNB) reacts with compounds containing a sulfhydryl moiety to generate a

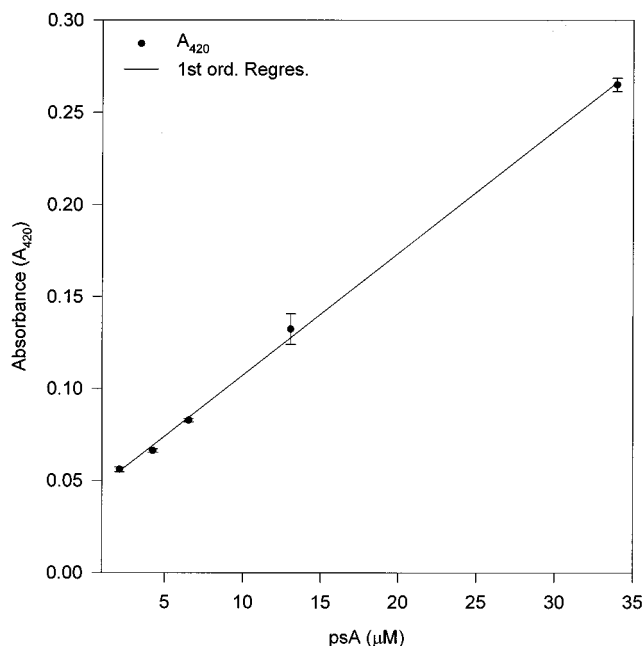


Figure 4. Standard curve of psA concentration versus A_{420} resulting from thiolate anion formation.

mixed disulfide and a thiolate dianion, the latter of which has a $\lambda_{\max}$ of 420 nm and an ϵ of about 14 000 (19). Although there are several reagents which can label sulfhydryls to produce a visible product, DTNB is the most likely to be useful for 5'-monothiophosphates due to its high reactivity at neutral pH, high ϵ , and high water solubility, the latter allowing the reaction to be performed with no cosolvent (20). The reaction with peptide and cysteine standards has been reported to follow Beer's law down to $0.41 \mu\text{M}$ (21).

When DTNB was reacted with psA, there resulted a linear ($r^2 = 0.9988$) dependence of A_{420} on the concentration of psA in the range of $2\text{--}34 \mu\text{M}$ (Figure 4). Nonlinearity at concentrations of less than $2 \mu\text{M}$ was due to an unfavorable signal:noise ratio, whereas nonlinearity above $34 \mu\text{M}$ was possibly due to an ionic strength dependence of the reaction as has been demonstrated in the case of cysteine (22). When preparation **I** was analyzed using this approach, a percentage of **2** value of 69% was obtained, in good agreement with the 67% value determined with HPLC method D.

During the above experiments, we observed that oligonucleotide **2** in preparation **I** took much longer to react with DTNB than did the psA mononucleotide standard. Specifically, the reaction with psA was 50% complete in 10 s and 100% complete in 50 s, whereas the reaction with oligonucleotide **2** was 50% complete at 200 s and 100% complete at 850 s. These results may be related to the increased negative charge on the oligonucleotide **2** substrate. Glutathione, which carries a negative charge under DTNB reaction conditions, has been observed to react more slowly with DTNB than with cysteine (21). In contrast, neighboring group positive charge greatly accelerates the rate of reaction of DTNB with protein sulfhydryls through Coulombic and inductive effects (22).

CONCLUSIONS

We have explored the ability of several methodologies to provide a convenient, facile, and reliable determination of the extent of 5'-thiolation in 2-5A-antisense chimeras. Of the various chromatographic, enzymatic, and chemical approaches examined, we found the most suitable to be

the reaction of the 2–5A-antisense chimera with 5-IAF, resulting in the conjugation of the 5'-terminal adenosine 5'-monothiophosphate residue with fluorescein. The conjugated product could be quantitated after HPLC separation. This method was superior to an alternate chemical approach involving DTNB as time was required to carry out the requisite steps of standard steps of standard curve generation and the reaction itself.

During the course of these experiments, we identified the disulfide as an occasional significant contaminant on preparation of **2**. We further provided conditions under which formation of the disulfide could be eliminated.

LITERATURE CITED

- (1) De Mesmaeker, A., Haner, R., Martin, P., and Moser, H. E. (1995) Antisense oligonucleotides. *Acc. Chem. Res.* 28, 366–374.
- (2) Lesiak, K., Khamnei, S., and Torrence, P. F. (1993) 2',5'-Oligoadenylate: Antisense Chimeras-Synthesis and Properties. *Bioconjugate Chem.* 4, 467–472.
- (3) Torrence, P. F., Maitra, R. K., Lesiak, K., Khamnei, S., Shou, A., and Silverman, R. S. (1993) Targeting RNA for Degradation with a (2'-5')Oligoadenylate-antisense Chimera. *Proc. Natl. Acad. Sci. U.S.A.* 90, 1300–1304.
- (4) Maran, A., Maitra, R. K., Kumar, A., Dong, B., Xiao, W., Li, G., Williams, B. R. G., Torrence, P. F., and Silverman, R. H. (1994) Blockage of NF- κ B Signaling by Selective Ablation of an mRNA Target by 2–5A-antisense Chimeras. *Science* 265, 789–792.
- (5) Maitra, R. K., Li, G., Xiao, W., Dong, B., Torrence, P. F., and Silverman, R. H. (1995) Catalytic Cleavage of an RNA Target by 2–5A-antisense Chimeras. *J. Biol. Chem.* 270, 15071–15075.
- (6) Cirino, N. M., Li, G., Xiao, W., Torrence, P. F., and Silverman, R. H. (1997) Targeting RNA decay in respiratory syncytial virus infected cells with 2'→5' oligoadenylate-antisense. *Proc. Natl. Acad. Sci. U.S.A.* 94, 1937–1942.
- (7) Eckstein, F. (1966) Nucleoside phosphorothioates. *J. Am. Chem. Soc.* 88, 4292–4294.
- (8) Xiao, W., Li, G., Lesiak, K., Dong, B., Silverman, R. H., and Torrence, P. F. (1994) Synthesis of a 5'-thiophosphate analogue of 2–5A, a phosphatase resistant activator of the 2–5A-dependent ribonuclease. *Bioorg. Med. Chem. Lett.* 4, 2609–2614.
- (9) Xiao, W., Player, M. R., Li, G., Zhang, W., Lesiak, K., and Torrence, P. F. (1996) Synthesis and characterization of composite nucleic acids containing 2',5'-oligoriboadenylate linked to antisense DNA. *Antisense Nucleic Acid Drug Dev.* 6, 247–258.
- (10) Iyer, R. P., Egan, W., Regan, J. B., and Beaucage, S. L. (1990) 3H-1,2-Benzodithiol-3-one 1,1-dioxide as an improved sulfurizing reagent in the solid-phase synthesis of oligodeoxyribonucleoside phosphorothioates. *J. Am. Chem. Soc.* 112, 1253–1254.
- (11) Lecchi, P., Le, H. M. T., and Pannell, L. K. (1995) 6-Aza-2-thiothymine: A matrix for MALDI spectra of oligonucleotides. *Nucleic Acids Res.* 23, 1276–1277.
- (12) Wu, T., and Orgel, L. E. (1991) Disulfide-linked oligonucleotide phosphorothioates: novel analogues of nucleic acids. *J. Mol. Evol.* 32, 274–277.
- (13) Eckstein, R. (1979) Phosphorothioate analogues of nucleotides. *Acc. Chem. Res.* 12, 204–210.
- (14) Haga, K., Kainosho, M., and Yoshikawa, M. (1971) Studies of Phosphorylation. V. The Synthesis of Inosine-5'-thiophosphate. *Bull. Chem. Soc. Jpn.* 44, 460–463.
- (15) Gryaznov, S. M., and Letsinger, R. L. (1993) Template controlled coupling and recombination of oligonucleotide blocks containing thiophosphoryl groups. *Nucleic Acids Res.* 21, 1403–1408.
- (16) Seeberger, P. H., Jorgensen, P. N., Bankaitis-Davis, D. M., Beaton, G., and Caruthers, M. H. (1996) 5'-Dithiophosphoryl deoxynucleotides: synthesis and biological studies. *J. Am. Chem. Soc.* 118, 9562–9566.
- (17) Gorman, J. J., Corino, G. L., and Mitchell, S. J. (1987) Fluorescent labeling of cysteinyl residues. Application to extensive primary structure analysis of protein on a microscale. *Eur. J. Biochem.* 168, 169–179.
- (18) Conway, N. E., Fidanza, J. A., O'Donnell, M. J., Narekian, N. D., Ozaki, H., and McLaughlin, L. W. (1991) Site specific attachment of labels to the DNA backbone. In *Oligonucleotides and Analogues: A Practical Approach* (F. Eckstein, Ed.) pp 211–239, IRL Press, New York.
- (19) Riddles, P. W., Blakely, R. L., and Zerner, B. (1983) Reassessment of Ellman's Reagent. *Methods Enzymol.* 91, 49–60.
- (20) Ellman, G. L. (1959) Tissue Sulphydryl Groups. *Arch. Biochem. Biophys.* 82, 70–77.
- (21) Shah, A., De Biasi, V., and Camilleri, P. (1995) Development of a Spectrophotometric Method for the Measurement of Thiols at Trace Levels. *Anal. Proc.* 32, 149–153.
- (22) Snyder, G. H., Cennerazzo, M. J., Karalis, A. J., and Field, D. (1981) Electrostatic Influence of Local Cysteine Environments on Disulfide Exchange Kinetics. *Biochemistry* 20, 6509–6519.

BC970157P

ARTICLES

Preparation and Photoactivation of Caged Fluorophores and Caged Proteins Using a New Class of Heterobifunctional, Photocleavable Cross-Linking Reagents

Johannes Ottl, Daniela Gabriel, and Gerard Marriott*

Biomolecular and Cellular Dynamics Research Group, Max Planck Institute for Biochemistry, 82152 Martinsried, Germany. Received August 13, 1997; Revised Manuscript Received October 13, 1997*

The design, synthesis, and spectroscopic and chemical properties of four members of a new class of heterobifunctional photocleavable (caged) cross-linking reagents were described. One of the two reactive groups of the cross-linker reacted with amino groups to form the corresponding photolabile carbamates. Amino group containing compounds or proteins caged with these reagents can be coupled through the thiol reactive oxirane group of the cross-linker to a different biomolecule or to a thiol-derivatized surface. The 3,4-dimethoxy-6-nitrophenyl photoisomerization group of the reagent was physically and chemically isolated from the cross-linking functionality, and the high extinction coefficient and red-shifted action spectrum of this chromophore make it suitable for photoactivation applications of caged compounds on surfaces or in living cells. The bifunctional, photocleavable cross-linking reagents were used to prepare a thiol reactive caged rhodamine 110. The new reagents and conjugation procedures described may be used as part of a general procedure to cage the activity of proteins by physically masking binding sites.

INTRODUCTION

Light-directed activation of caged compounds has emerged as a powerful technique for generating concentration jumps of biomolecules in complex molecular environments (1) and has provided important information on the reaction mechanism of protein activity in muscle and in living cells (1, 2). Functional groups of molecules that have been protected with caged compounds include the primary amine (3), primary and secondary alcohol (4), phosphate (5), ketone (6), thiol (7, 13), and carboxylic

acid (8). Irradiation of functional groups protected with the 2-nitrophenyl-based caged reagents with near-ultraviolet light leads to a photoisomerization reaction that cleaves the bond linking the protected functionality of interest to the caged group. Caged reagents have been used to prepare inactive yet photoactivatable enzyme substrates (5), receptor ligands (9, 10), and fluorescent probes (11, 12). In addition, caged reagents capable of labeling amino and thiol groups have been used to inhibit the activity of proteins (caged proteins) by modifying one or more essential amino acid residues (7, 13–15, 26). These caged groups can be efficiently removed from the caged protein with concomitant recovery of its activity following irradiation of the conjugate with near-ultraviolet light.

* Address correspondence to this author. Fax: +49 89 85 78 2895. E-mail: marriott@biochem.mpg.de.

® Abstract published in *Advance ACS Abstracts*, December 15, 1997.

In addition to these monofunctional caged reagents, heterobifunctional photocleavable reagents have been described to protect amino or thiol groups of small molecules (25) and proteins (7, 15) as their corresponding photolabile carbamates or thioethers, respectively (7, 15, 25). The second reactive group of the cross-linking reagent may be used to attach the caged compound to a different biomolecule; for example, an antibody molecule for targeting the caged compound to a specific site (15), or a derivatized surface, or a fluorescent dye for imaging or for probing protein–protein interactions (7, 25).

The few photocleavable cross-linking reagents described in the literature all harbor this second reactive group at the 4-position of the 2-nitrophenyl ring (7, 15, 25). In this study, we designed a new class of reagents in which the second reactive group was introduced off the benzylic carbon atom. The photocleavable cross-linking reagents were designed with the following operational considerations in mind. (1) Caged reagents should react with the functional group of interest in aqueous solution and at a slightly alkaline pH. The second reactive group, an epoxide, is stable in the absence of free thiols and can then be reacted in a second step with thiol functions. (2) The photoactivation reaction should exhibit a reasonable quantum yield and an action spectrum in the near-ultraviolet wavelength region (340–400 nm) to avoid interference with other biomolecules. (3) The caged group should exhibit a high molar absorptivity for photoactivation reactions performed on optically thin samples. (4) The photoproducts of the photoactivation reaction should not be toxic or reactive with other functional groups. The new reagents described in this report fulfill most of the requirements listed above. Suitable examples are provided to demonstrate the functional group selectivity of the two reactive groups. Members of this new class of cross-linking reagents were used for the preparation and photoactivation of caged fluorophores and caged proteins.

MATERIALS AND METHODS

3,4-Dimethoxy-6-nitrobenzaldehyde, di-*N*-succinimidylcarbonate (DSC), vinyl magnesium bromide, *m*-chloroperoxybenzoic acid (MCPBA), 4-(dimethylamino)pyridine (DMAP), and 4,6-diphenylthieno[3,4-*d*][1,3]dioxol-2-on-5,5-dioxide (TDO) were purchased from Aldrich. Bovine serum albumin (BSA), polylysine (average molecular weight of 40 000 kDa), and 2-iminothiolane were purchased from Sigma. Tetramethylrhodamine iodoacetamide (IATMR) was a gift from J. Corrie (NIMR, Mill Hill, London). Acrylodan and aminodextran were purchased from Molecular Probes (Eugene, OR). Rhodamine 110 was purchased from Lambda Physik (Göttingen, Germany). All other reagents were of the highest quality available and obtained from Sigma or Aldrich unless stated otherwise. G-Actin was prepared according to Marriott (14).

The NMR spectra were measured on a 500 MHz Bruker instrument; mass spectrophotometry was carried out on a Finnigan MAT 900 or Finnigan HSQ 30 instrument. Infrared spectra were recorded on a Perkin-Elmer 1760 X FT-IR spectrophotometer. Absorption spectra were recorded on a Hewlett-Packard 82152 diode array spectrophotometer. Fluorescence spectroscopy was performed on an SLM-AB2 fluorometer (Sopra, Buttelborn, Germany). Light-directed photoactivation of caged compounds was performed essentially as described by Marriott (14).

Syntheses. All syntheses were conducted in a darkened room or protected from room light with aluminum foil.

Preparation of 1-Hydroxy-1-(3,4-dimethoxy-6-nitrophenyl)-2-propene (I). 3,4-Dimethoxy-6-nitrobenzaldehyde (1.88 g, 8.95 mmol) freshly recrystallized out of toluene, was dissolved in 50 mL of dry THF under an argon atmosphere. The yellow solution was cooled to -70°C and 10.7 mL of a 1 M solution of vinylmagnesium bromide in THF added dropwise. The deep red solution was stirred for 3 h and slowly warmed to 20°C and then 40 mL of a saturated NH_4Cl solution added dropwise. The red solution was extracted three times with ethyl acetate and the organic phase washed five times with saturated NaCl and dried over MgSO_4 and the solvent removed. The red oily residue (2.44 g) was taken up in a little ethyl acetate and eluted through a silica gel column developed in 3:1 hexane/ethyl acetate. The solvent was removed from the product fractions producing an orange-red powder. Yield: 1.53 g (6.33 mmol, 72%). MW: 239.23 ($\text{C}_{11}\text{H}_{13}\text{NO}_5$). Mp: $101\text{--}102^{\circ}\text{C}$. $^1\text{H-NMR}$ (CDCl_3 , in ppm): 2.63 (s, broad, 1H), 3.94 (s, 3H), 3.96 (s, 3H), 5.24 (dd, $J = 11$ Hz, 2 Hz, 1H), 5.42 (dd, $J = 18$ Hz, 2 Hz, 1H), 5.92 (d, $J = 6$ Hz, 1H), 6.07 (ddd, $J = 18$ Hz, 11 Hz, 6 Hz, 1H), 7.20 (s, 1H), 7.57 (s, 1H). MS (70 eV) m/z (%): 239 (42) M^+ , 162 (100) $\text{C}_9\text{H}_8\text{NO}_2^+$.

Preparation of 1-(3,4-Dimethoxy-6-nitrophenyl)-2,3-epoxypropyl Hydroxide (II). *m*-Chloroperoxybenzoic acid (MCPBA, technical grade, about 80% pure) was washed three times with phosphate buffer at pH 7.5 and dried. Purified MCPBA (1.311 g, 7.60 mmol) was dissolved in 20 mL of CH_2Cl_2 and slowly dropped into a precooled solution of 1.80 g (7.52 mmol) of I in 15 mL of CH_2Cl_2 . The bright yellow solution was stirred for 48 h at room temperature. The white precipitate was filtered and washed with CH_2Cl_2 and then 70 mL of a saturated solution of NaHCO_3 added to the solution which was stirred for 30 min. The organic phase was washed seven times with a saturated solution of NaHCO_3 , then three times with a saturated solution of NaCl. The organic phase was dried over MgSO_4 and the solvent removed. The yellow, oily residue (1.90 g) was recrystallized out of ethyl acetate/hexane. Yield: 1.55 g (6.10 mmol, 81%, orange needles). MW: 255.23 ($\text{C}_{11}\text{H}_{13}\text{NO}_6$). Mp: $118\text{--}121^{\circ}\text{C}$. $^1\text{H-NMR}$ (CDCl_3 , in ppm): isomer a, 2.62 (s, 1H), 2.71 (dd, $J = 5$ Hz, 2 Hz, 1H), 2.90 (dd, $J = 5$ Hz, 2 Hz, 1H), 3.32 (ddd, $J = 5$ Hz, 5 Hz, 2 Hz, 1H), 3.96 (s, 3H), 3.98 (s, 3H), 5.57 (dd, $J = 5$ Hz, 2 Hz, 1H), 7.23 (s, 1H), 7.66 (s, 1H); isomer b, 2.63 (s, 1H), 2.82 (dd, $J = 5$ Hz, 2 Hz, 1H), 3.02 (dd, $J = 5$ Hz, 2 Hz, 1H), 3.62 (ddd, $J = 5$ Hz, 5 Hz, 2 Hz, 1H), 3.96 (s, 3H), 4.02 (s, 3H), 5.76 (dd, $J = 5$ Hz, 2 Hz, 1H), 7.28 (s, 1H), 7.67 (s, 1H). MS (70 eV) m/z (%): 255 (35) M^+ , 164 (100) $\text{C}_9\text{H}_{10}\text{NO}_2^+$. FAB-MS m/z : 256.1 MH^+MS .

Photolytic Isomerization of II. A 100 μM solution of II dissolved in methanol was irradiated in a quartz cuvette with ultraviolet light (350 and 420 nm) according to Marriott (14). The absorption spectrum of the sample was recorded at different irradiation times.

Preparation of 1-(3,4-dimethoxy-6-nitrophenyl)-2,3-epoxypropyl chloroformate (III). In a well-ventilated hood, 566 mg of II (2.22 mmol) dissolved in 10 mL of water free dioxane in a dried 50 mL flask was treated with 0.18 mL of water free pyridine (2.25 mmol) together with 0.26 mL of diphosgene (2.20 mmol) which was slowly dropped into the yellow solution. The reaction mixture was stirred for 2 h at room temperature, the precipitate filtered off, the solvent removed, and the flask left for 1 h under a high vacuum to remove excess diphosgene. The

oily orange-brown residue was taken up in a little ethyl acetate and eluted through a silica gel column developed in 3:1 hexane/ethyl acetate. The solvent was removed from the product fractions yielding a yellow powder. Yield: 620 mg (1.95 mmol, 88%). MW: 317.68 ($C_{12}H_{12}NO_7Cl$). Mp: 178–181 °C. ^1H -NMR ($CDCl_3$, in ppm): isomer a, 3.57 (dd, $J = 12$ Hz, 4 Hz, 1H), 3.76 (dd, $J = 12$ Hz, 4 Hz, 1H), 3.94 (s, 3H), 3.97 (s, 3H), 5.52–5.56 (m, 1H), 6.32 (d, $J = 8$ Hz, 1H), 7.20 (s, 1H), 7.71 (s, 1H); isomer b, 3.92 (s, 3H), 3.96 (s, 3H), 4.00 (dd, $J = 12$ Hz, 2 Hz, 1H), 4.19 (dd, $J = 12$ Hz, 3 Hz, 1H), 4.66–4.68 (m, 1H), 6.13 (d, 3 Hz, 1H), 7.00 (s, 1H), 7.73 (s, 1H). MS (70 eV) m/z (%): 317 (82) M^+ , 136 (100) $C_8H_8O_2^+$.

Preparation of 4-[1-(3,4-Dimethoxy-6-nitrophenyl)-2,3-epoxypropyl-1-oxycarbonyloxy]-3-oxo-2,5-diphenyl-2,3-dihydrothiophen-1,1-dioxide (IV). **II** (500 mg, 1.959 mmol) dissolved in 4 mL of water free THF was treated with 672 mg of freshly recrystallized, 4,6-diphenylthienof[3,4-*d*][1,3]dioxol-2-on-5,5-dioxide (2.062 mmol). The orange solution was refluxed for 4 h in the absence of base and the solvent evaporated to give an orange residue that was recrystallized out of dry toluene. Yield: 186.7 mg (0.321 mmol, 82%). MW: 581.56 ($C_{28}H_{23}NO_{11}$). Mp: 176–180 °C. ^1H -NMR (in $CDCl_3$, in ppm): isomer a, 2.35 (dd, $J = 4$ Hz, 3 Hz, 1H), 3.06 (dd, $J = 4$ Hz, 3 Hz, 1H), 3.53 (ddd, $J = 4$ Hz, 4 Hz, 2 Hz, 1H), 3.90 (s, 3H), 3.95 (s, 3H), 5.14 (s, 1H), 6.62 (d, $J = 4$ Hz, 1H), 7.01 (s, 1H), 7.27–7.32 (m, 4H), 7.43–7.48 (m, 4H), 7.60 (s, 1H), 7.92–7.97 (m, 2H); isomer b, 2.39 (dd, $J = 4$ Hz, 3 Hz, 1H), 3.08 (dd, $J = 4$ Hz, 3 Hz, 1H), 3.64 (ddd, $J = 4$ Hz, 4 Hz, 2 Hz, 3H), 3.92 (s, 3H), 3.97 (s, 3H), 5.15 (s, 1H), 6.67 (d, $J = 4$ Hz, 1H), 7.07 (s, 1H), 7.27–7.32 (m, 4H), 7.43–7.48 (m, 4H), 7.63 (s, 1H), 7.92–7.97 (m, 2H); isomer c, 2.83 (ddd, $J = 4$ Hz, 4 Hz, 2 Hz, 1H), 3.48 (ddd, $J = 4$ Hz, 4 Hz, 2 Hz, 1H), 3.64 (ddd, $J = 4$ Hz, 4 Hz, 2 Hz, 3H), 3.96 (s, 3H), 3.98 (s, 3H), 5.16 (s, 1H), 6.78 (d, $J = 3$ Hz, 1H), 7.08 (s, 1H), 7.43–7.48 (m, 4H), 7.52–7.57 (m, 8H), 7.65 (s, 1H), 7.92–7.97 (m, 2H); isomer d, 2.92 (ddd, $J = 4$ Hz, 4 Hz, 2 Hz, 1H), 3.48 (ddd, $J = 4$ Hz, 4 Hz, 2 Hz, 1H), 3.64 (ddd, $J = 4$ Hz, 4 Hz, 2 Hz, 3H), 3.96 (s, 3H), 3.99 (s, 3H), 5.18 (s, 1H), 6.81 (d, $J = 3$ Hz, 1H), 7.14 (s, 1H), 7.43–7.48 (m, 4H), 7.52–7.57 (m, 8H), 7.67 (s, 1H), 7.92–7.97 (m, 2H). FAB-MS m/z : 582.4 MH^+ .

Preparation of 1-(3,4-Dimethoxy-6-nitrophenyl)-2,3-epoxypropyl Carbonate (V). **IV** (150 mg, 0.258 mmol) dissolved in 0.5 mL of water free THF was treated with 65.8 mg of **II** (0.258 mmol) and 30 μ L of *N*-ethyl-diisopropylamine (0.258 mmol). The flask was subjected to bath sonification for 2 min, and the orange solid, which precipitated out of solution, was left for 1 h at room temperature. This precipitate was filtered off and the solvent evaporated to give an orange residue that was crystallized out of toluene/hexane as an orange powder. Yield: 106 mg (0.198 mmol, 77%). MW: 536.2 ($C_{23}H_{24}N_2O_7$). Mp: 115–120 °C. ^1H -NMR ($CDCl_3$, in ppm): isomer a, 2.38 (dd, $J = 6$ Hz, 3 Hz, 1H), 2.77 (ddd, $J = 6$ Hz, 6 Hz, 3 Hz, 1H), 2.98 (dd, $J = 6$ Hz, 3 Hz, 1H), 3.92 (s, 3H), 6.61 (d, $J = 3$ Hz, 1H), 7.00 (s, 1H), 7.58 (s, 1H); isomer b, 2.38 (dd, $J = 6$ Hz, 3 Hz, 1H), 2.80 (ddd, $J = 6$ Hz, 6 Hz, 3 Hz, 1H), 2.98 (dd, $J = 6$ Hz, 3 Hz, 1H), 3.93 (s, 3H), 6.64 (d, $J = 3$ Hz, 1H), 7.02 (s, 1H), 7.62 (s, 1H); isomer c, 2.41 (dd, $J = 6$ Hz, 3 Hz, 1H), 2.87 (ddd, $J = 6$ Hz, 6 Hz, 3 Hz, 1H), 3.55 (dd, $J = 6$ Hz, 3 Hz, 1H), 3.95 (s, 3H), 6.67 (d, $J = 3$ Hz, 1H), 7.05 (s, 1H), 7.63 (s, 1H); isomer d, 2.41 (dd, $J = 6$ Hz, 3 Hz, 1H), 3.48 (ddd, $J = 6$ Hz, 6 Hz, 3 Hz, 1H), 3.55 (dd, $J = 6$ Hz, 3 Hz, 1H), 3.95 (s, 3H), 6.72 (d, $J = 3$ Hz, 1H), 7.11 (s, 1H), 7.72 (s, 1H). MS (70 eV) m/z (%): 536 (20) M^+ , 238 (100) $C_{11}H_{12}NO_5^+$.

Preparation of *N*-1-[(3,4-Dimethoxy-6-nitrophenyl)-2,3-

epoxypropyloxycarbonyl]-4-(*N,N*-dimethylamino)pyridinium 3-Oxo-2,5-diphenyl-2,3-dihydrothiophen-1,1-dioxide-4-hydroxide (VIa). **IV** (150 mg, 0.258 mmol) dissolved in 0.4 mL of dry THF was treated with a solution of 35 mg of DMAP (0.285 mmol) in 0.1 mL of dry THF. After a 2 min sonification treatment, an orange precipitate formed that was left to stand for 1 h at room temperature and centrifuged, and the orange residue was washed with THF. Yield: 166 mg (0.237 mmol, 92%). MW: 704.7 (cation, 404.4; cation, $C_{19}H_{22}N_3O_7$). Mp: 85 °C (dec). ^1H -NMR ($CDCl_3$, in ppm): isomer a, 2.84 (dd, $J = 5$ Hz, 2 Hz, 1H), 3.02 (dd, $J = 5$ Hz, 2 Hz, 1H), 3.25 (s, 6H), 3.58 (ddd, $J = 5$ Hz, 5 Hz, 2 Hz, 1H), 3.93 (s, 3H), 4.04 (s, 3H), 6.63 (d, $J = 4$ Hz, 1H), 6.70 (d, $J = 7$ Hz, 2H), 7.05 (s, 1H), 7.64 (s, 1H), 8.12 (d, $J = 7$ Hz, 2H); isomer b, 2.89 (dd, $J = 5$ Hz, 2 Hz, 1H), 3.11 (dd, $J = 5$ Hz, 2 Hz, 1H), 3.25 (s, 6H), 3.63 (ddd, $J = 5$ Hz, 5 Hz, 2 Hz, 1H), 3.96 (s, 3H), 4.06 (s, 3H), 6.72 (d, $J = 7$ Hz, 2H), 6.75 (d, $J = 3$ Hz, 1H), 7.09 (s, 1H), 7.66 (s, 1H), 8.14 (d, $J = 7$ Hz, 2H); counterion, 4.91 (s, 1H), 7.10–7.40 (m, 8H), 7.95–8.30 (m, 2H). FAB-MS m/z : 404.5 M^+ (cation).

Preparation of *N*-1-[(3,4-Dimethoxy-6-nitrophenyl)-2,3-epoxypropyloxycarbonyl]-4-(*N,N*-dimethylamino)pyridinium Succinimide-*N*-hydroxide (VIb). **VII** (150 mg, 0.378 mmol) dissolved in 0.4 mL of dry THF was treated with a solution of 51 mg of DMAP (0.42 mmol) in 0.1 mL of dry THF. After a 2 min sonification treatment, an orange precipitate formed that was left to stand for 1 h at room temperature and centrifuged, and the orange residue was washed with THF. Yield: 180 mg (0.34 mmol, 90%). MW: 704.7 (cation, 404.4; cation, $C_{19}H_{22}N_3O_7$). Mp: 88 °C (dec). ^1H -NMR ($CDCl_3$, in ppm): isomer a, 2.84 (dd, $J = 5$ Hz, 2 Hz, 1H), 3.02 (dd, $J = 5$ Hz, 2 Hz, 1H), 3.25 (s, 6H), 3.58 (ddd, $J = 5$ Hz, 5 Hz, 2 Hz, 1H), 3.93 (s, 3H), 4.04 (s, 3H), 6.63 (d, $J = 4$ Hz, 1H), 6.70 (d, $J = 7$ Hz, 2H), 7.05 (s, 1H), 7.64 (s, 1H), 8.12 (d, $J = 7$ Hz, 2H); isomer b, 2.89 (dd, $J = 5$ Hz, 2 Hz, 1H), 3.11 (dd, $J = 5$ Hz, 2 Hz, 1H), 3.25 (s, 6H), 3.63 (ddd, $J = 5$ Hz, 5 Hz, 2 Hz, 1H), 3.96 (s, 3H), 4.06 (s, 3H), 6.72 (d, $J = 7$ Hz, 2H), 6.75 (d, $J = 3$ Hz, 1H), 7.09 (s, 1H), 7.66 (s, 1H), 8.14 (d, $J = 7$ Hz, 2H); counterion, 2.79 (s, 8H). FAB-MS m/z : 404.5 M^+ (cation).

Preparation of 1-(3,4-Dimethoxy-6-nitrophenyl)-2,3-epoxypropylsuccinimidyl Carbonate (VII). **II** (100 mg, 0.392 mmol) dissolved in 2 mL of acetonitrile was treated with 110 mg (0.431 mmol) of di-*N*-succinimidyl carbonate. *N*-Ethyl-diisopropylamine (70 μ L, 0.431 mmol) was added and the reaction mixture stirred for 5 h at room temperature. After evaporation of the solvent, the residue was dissolved in ethyl acetate and washed three times with 20% citric acid, sodium bicarbonate solution, and saturated sodium chloride. The organic phase was dried over magnesium sulfate, the solvent evaporated, and the yellow residue recrystallized out of chloroform/hexane producing yellow-orange crystals. Yield: 138 mg (0.348 mmol, 89%). MW: 396.32 ($C_{16}H_{16}N_2O_{10}$). ^1H -NMR ($CDCl_3$, in ppm): isomer a, 2.79 (s, 4H), 2.82 (dd, $J = 5$ Hz, 2 Hz, 1H), 3.02 (dd, $J = 5$ Hz, 2 Hz, 1H), 3.56 (ddd, $J = 5$ Hz, 5 Hz, 2 Hz, 1H), 3.95 (s, 3H), 4.04 (s, 3H), 6.66 (d, $J = 4$ Hz, 1H), 7.05 (s, 1H), 7.64 (s, 1H); isomer b, 2.79 (s, 4H), 2.90 (dd, $J = 5$ Hz, 2 Hz, 1H), 3.10 (dd, $J = 5$ Hz, 2 Hz, 1H), 3.63 (ddd, $J = 5$ Hz, 5 Hz, 2 Hz, 1H), 3.96 (s, 3H), 4.06 (s, 3H), 6.77 (d, $J = 3$ Hz, 1H), 7.09 (s, 1H), 7.67 (s, 1H). MS (70 eV) m/z (%): 396 (51) M^+ , 238 (100) $C_{11}H_{12}NO_5^+$. FAB-MS m/z : 397.4 MH^+ .

Preparation of Bis[1-(3,4-dimethoxy-6-nitrophenyl)-2,3-epoxypropyl]-*N,N'*-rhodamine Carbonate (VIII). **IV** (100 mg, 0.180 mmol) dissolved in 0.5 mL of water free THF was treated with 40 μ L of *N*-ethyl-diisopropylamine (0.350

mmol) and 32 mg of rhodamine 110 hydrochloride (0.086 mmol). An orange precipitate developed over a 2 min sonification treatment. The reaction mixture was left to stand for 4 h at room temperature and centrifuged, and the solvent was evaporated and the orange residue taken up in 5 mL of ethyl acetate. Successive washing of this organic solution with saturated NaHCO_3 , 20% citric acid, and saturated NaCl was used to remove the sulfonate. The organic phase was then dried over MgSO_4 , the solvent removed, and the product recrystallized from the residue out of toluene. Yield: 58 mg (0.065 mmol, 75%). MW: 892.5 ($\text{C}_{44}\text{H}_{36}\text{N}_4\text{O}_{17}$). Mp: 188–193 °C. $^1\text{H-NMR}$ (CDCl_3 , in ppm): isomer a, 2.35 (dd, $J = 4$ Hz, 3 Hz, 1H), 3.06 (dd, $J = 4$ Hz, 3 Hz, 1H), 3.53 (ddd, $J = 4$ Hz, 4 Hz, 2 Hz, 1H), 3.90 (s, 3H), 3.95 (s, 3H), 6.62 (d, $J = 4$ Hz, 1H), 7.01 (s, 1H), 6.68 (d, 8 Hz, 1H), 7.10 (s, 1H), 7.15 (d, 8 Hz, 1H), 7.17 (d, 7 Hz, 1H), 7.19 (dd, $J = 7$ Hz, 7 Hz, 1H), 7.30 (dd, $J = 7$ Hz, 7 Hz, 1H), 7.60 (s, 1H), 8.10 (d, $J = 7$ Hz, 1H); isomer b, 2.39 (dd, $J = 4$ Hz, 3 Hz, 1H), 3.08 (dd, $J = 4$ Hz, 3 Hz, 1H), 3.64 (ddd, $J = 4$ Hz, 4 Hz, 2 Hz, 3H), 3.92 (s, 3H), 3.97 (s, 3H), 6.65 (d, $J = 4$ Hz, 1H), 6.68 (d, $J = 8$ Hz, 1H), 7.07 (s, 1H), 7.10 (s, 1H), 7.15 (d, $J = 8$ Hz, 1H), 7.17 (d, $J = 7$ Hz, 1H), 7.19 (dd, $J = 7$ Hz, 7 Hz, 1H), 7.30 (dd, $J = 7$ Hz, 7 Hz, 1H), 7.63 (s, 1H), 8.10 (d, $J = 7$ Hz, 1H); isomer c, 2.83 (ddd, $J = 4$ Hz, 4 Hz, 2 Hz, 1H), 3.48 (ddd, $J = 4$ Hz, 4 Hz, 2 Hz, 3H), 3.64 (ddd, $J = 4$ Hz, 4 Hz, 2 Hz, 3H), 3.96 (s, 3H), 3.98 (s, 3H), 6.68 (d, $J = 8$ Hz, 1H), 6.78 (d, $J = 3$ Hz, 1H), 7.08 (s, 1H), 7.10 (s, 1H), 7.15 (d, $J = 8$ Hz, 1H), 7.17 (d, $J = 7$ Hz, 1H), 7.19 (dd, $J = 7$ Hz, 7 Hz, 1H), 7.30 (dd, $J = 7$ Hz, 7 Hz, 1H), 7.65 (s, 1H), 8.10 (d, $J = 7$ Hz, 1H); isomer d, 2.92 (ddd, $J = 4$ Hz, 4 Hz, 2 Hz, 1H), 3.48 (ddd, $J = 4$ Hz, 4 Hz, 2 Hz, 1H), 3.64 (ddd, $J = 4$ Hz, 4 Hz, 2 Hz, 3H), 3.96 (s, 3H), 3.99 (s, 3H), 6.81 (d, $J = 3$ Hz, 1H), 6.68 (d, $J = 8$ Hz, 1H), 7.10 (s, 1H), 7.14 (s, 1H), 7.15 (d, $J = 8$ Hz, 1H), 7.17 (d, $J = 7$ Hz, 1H), 7.19 (dd, $J = 7$ Hz, 7 Hz, 1H), 7.30 (dd, $J = 7$ Hz, 7 Hz, 1H), 7.67 (s, 1H), 8.10 (d, $J = 7$ Hz, 1H). FAB-MS m/z : 893.3 MH^+ .

Reaction of 2-Iminothiolane with BSA and Aminodextran. BSA (4 mg) dissolved in 1 mL of 50 mM borate buffer at pH 8.5 was treated with 40 μL of a freshly prepared solution of 3.5 mg of 2-iminothiolane in 250 μL of distilled water. The solution was left at room temperature for 1 h and then centrifuged for 10 min at 10 000 rpm. The protein conjugate was dialyzed overnight against two changes of phosphate buffer at pH 7.5, previously degassed and saturated with nitrogen gas to prevent oxidation of the thiol groups. After dialysis, the thiolated BSA conjugate was centrifuged at 14 000 rpm at 4 °C. A similar labeling procedure was used to prepare the thiolated dextran from aminodextran.

Determination of the Free Thiol Content of BSA. A 10 mM stock solution of Acrylodan (15 μL) (the thiol reactive form of the fluorescent dye Prodan) dissolved in DMF was added to 300 μL of a 60 μM solution of iminothiolane-BSA at pH 8.0. The reaction mixture was left for 2 h at room temperature, and the solution was centrifuged for 10 min at 10 000 rpm and dialyzed for 2 days against phosphate buffer at pH 7.5 and 4 °C with three changes of buffer. The protein conjugate was centrifuged for 20 min at 14 000 rpm at 4 °C before recording its absorption spectrum. The concentration of Prodan in the conjugate was calculated from the absorption value at 385 nm using an extinction coefficient of 18 500 $\text{M}^{-1} \text{cm}^{-1}$ (24).

Reaction of II with Thiolated BSA. A 60 μM solution of thiolated BSA (500 μL) in borate buffer at pH 9.0 was treated with 20 μL of a 20 mM stock solution of compound II dissolved in acetonitrile. The solution

turned yellow and was left to stand for 2 h. The BSA conjugate was centrifuged for 10 min at 10 000 rpm at 4 °C and the solution dialyzed for 2 days against phosphate buffer at pH 7.5 and 4 °C with three changes of buffer. The conjugate was then centrifuged for 20 min at 14 000 rpm at 4 °C and the absorption spectrum recorded. The labeling ratio of II:BSA was determined using an extinction coefficient for II of 5000 $\text{M}^{-1} \text{cm}^{-1}$ at 350 nm, no losses in BSA were assumed to occur during the workup of the conjugate.

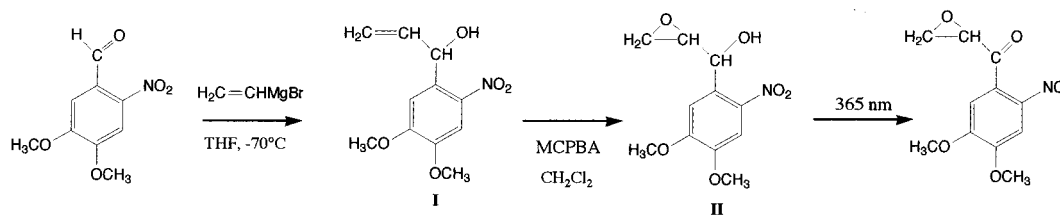
Coupling of G-Actin with Tetramethylrhodamine-Iodoacetamide. Rabbit muscle G-actin, purified according to Marriott (14), in G-buffer (2 mM Tris, 0.2 mM CaCl_2 , and 0.2 mM ATP at pH 8.0) containing 0.5 mM DTT was treated with 13 μL of a 10 mM stock solution of IATMR dissolved in DMF and the reaction mixture left for 2 h at room temperature. The protein was centrifuged for 30 min at 100 000g at 4 °C and dialyzed overnight against two changes of G-buffer without DTT at 4 °C. After a clear spin at 100 000g for 1 h, the polymerization was initiated by addition of 2 mM MgCl_2 and 0.1 M KCl to the supernatant and the mixture left for 2 h at room temperature. Ultracentrifugation at 100 000g for 1 h and resuspension of the F-actin pellet in G-buffer was followed by overnight dialysis against G-buffer without DTT. The labeled G-actin solution was clarified by centrifugation (100 000g for 1 h) and absorption spectrometry used to calculate a labeling ratio of 0.4 fluorophore per actin monomer using an extinction coefficient for TMR of 96 500 $\text{M}^{-1} \text{cm}^{-1}$ at 550 nm (26).

Labeling of TMR-G-Actin with VI. A 30 μM solution of TMR-labeled G-actin (800 μL) in G-buffer without DTT was mixed with 20 μL of a freshly prepared 20 mM stock solution of VI in acetonitrile. After a 45 min reaction at room temperature in the dark, the protein was centrifuged for 20 min at 14 000 rpm at 4 °C and dialyzed for 16 h at 4 °C against nitrogen-purged G-buffer without DTT with two buffer changes. The protein was clarified by ultracentrifugation at 100 000g for 1 h and the absorption spectrum recorded. The protein labeling ratio was calculated using an extinction coefficient for VI of 5000 $\text{M}^{-1} \text{cm}^{-1}$ at 350 nm and 3400 $\text{M}^{-1} \text{cm}^{-1}$ at 290 nm (14).

Reaction of the TMR-G-Actin Conjugate of VI with Thiolated Dextran. Aminodextran labeled with 2-iminothiolane as described earlier was added to a 20 μM solution of TMR-labeled G-actin in a borate-based G-buffer at pH 9.5. After a 1 h reaction, the complex was centrifuged for 20 min at 14 000 rpm at 4 °C and dialyzed overnight against two changes of Tris-based G-buffer with 1 mM DTT. The fluorescent actin-dextran complex was clarified by centrifugation for 20 min at 14 000 rpm at 4 °C.

Reaction of VIII with G-Actin. Two milliliters of 25 μM F-actin was depolymerized during dialysis over 2 days at 4 °C against nitrogen-purged G-buffer without DTT. The G-actin solution was clarified by centrifugation for 1 h at 100 000g at 4 °C. A 20 mM stock solution of VIII (20 μL) was added to 500 μL of the G-actin solution after adjusting its pH to 9.0. After a 1 h reaction at room temperature, the actin conjugate was clarified by centrifugation for 30 min at 10 000 rpm and dialyzed for 2 days against G-buffer (pH 8.0) containing 1 mM DTT. After a clear spin for 1 h at 100 000g at 4 °C, the labeling ratio of VIII:G-actin was determined by absorption spectroscopy using an extinction coefficient for VIII of 5000 $\text{M}^{-1} \text{cm}^{-1}$ at 350 nm and 3400 $\text{M}^{-1} \text{cm}^{-1}$ at 290 nm (14).

Scheme 1



RESULTS AND DISCUSSION

The Grignard reaction of vinylmagnesium bromide on 3,4-dimethoxy-6-nitrobenzaldehyde created a hydroxyl and vinyl group off the benzylic carbon which provided the starting point for the synthesis of the two different reactive groups of the cross-linking reagent (Scheme 1). The first group, an activated carbamate or carbonate, was used to react with and cage an amino group of the compound of interest to its corresponding photolabile carbamate, while the second group, an oxirane, was used to alkylate thiol groups and served to cross-link the caged amino compound to a different thiol-containing bio-molecule. This synthetic approach also allowed the physical and chemical separation of the two nucleophilic reactive groups from the photoisomerization functionality. This design feature permitted the use of the 3,4-dimethoxy-6-nitrophenyl group, which has a high molar absorptivity and a red-shifted action spectrum in the 340–420 nm wavelength region. These spectroscopic properties are important for applications of caged compounds in living cells or on surface monolayer (27). Since the photocleavable cross-linking reagents introduced by Senter et al. (15), Marriott et al. (7), and Olejnik et al. (25) incorporate the second reactive group at the 4-position of the nitrophenyl group, no other substitutions of the nitrophenyl ring are possible, and correspondingly, these reagents exhibit a low molar absorptivity and blue-shifted action spectra.

Reactivity of the Oxirane Group with Thiols.

Treatment of compound **I** with MCPBA generated the epoxy alcohol, compound **II**, in excellent yield (Scheme 1). Although the benzylic proton was resolved for each isomer of compound **II** using NMR spectroscopy, it was not possible to physically isolate the diastereomers. To show that the oxirane group of compound **II** reacted specifically with thiol groups, a qualitative analysis of the reactions of compound **II** with 2-mercaptoethanol was performed under various conditions of pH and solvent composition. In summary, these results showed alkylation of mercaptoethanol with compound **II** occurred in aqueous solutions at a pH value of 8.0–9.5 or in organic solvents under slightly acidic or neutral conditions. Compound **II** did not react with primary amines under these conditions (data not shown). Investigations were also made on the reactions of the oxirane group of compound **II** with reduced thiol groups of native BSA, containing a single reduced thiol group, and a 2-iminothiolane conjugate of BSA containing several reactive thiol groups. Dialysis of these reaction mixtures removed excess compound **II**, and allowed an absorption spectro-photometric analysis-based calculation of the labeling ratio of **II**:BSA for each conjugate. Native BSA was found to harbor a single molecule of **II** per BSA molecule, while the iminothiolane–BSA was labeled with six molecules of **II** per BSA (Figure 1). An independent determination of the number of reactive thiol groups in the iminothiolane–BSA conjugate using the thiol labeling reagent Acrylodan (24) also showed there were six free thiol groups (data not shown). Evidently at this slightly basic

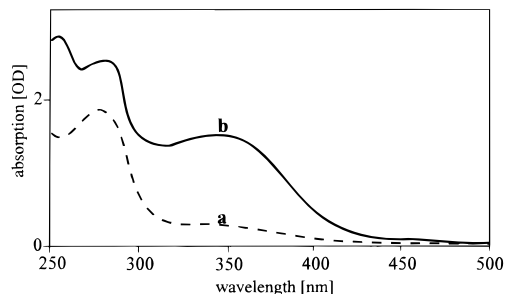


Figure 1. Absorption spectra of (a) native BSA labeled (59 μ M) with compound **II** and (b) iminothiolane-labeled BSA (59 μ M) labeled with compound **II**.

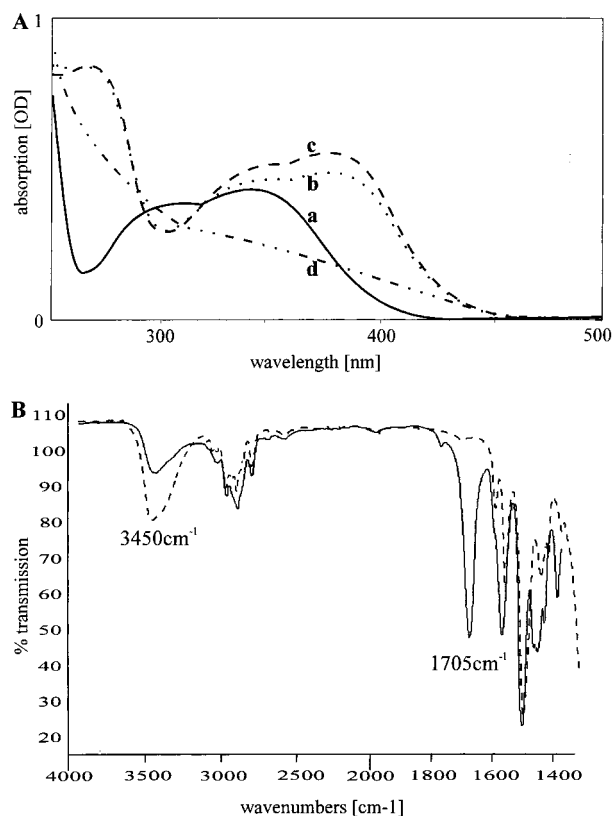
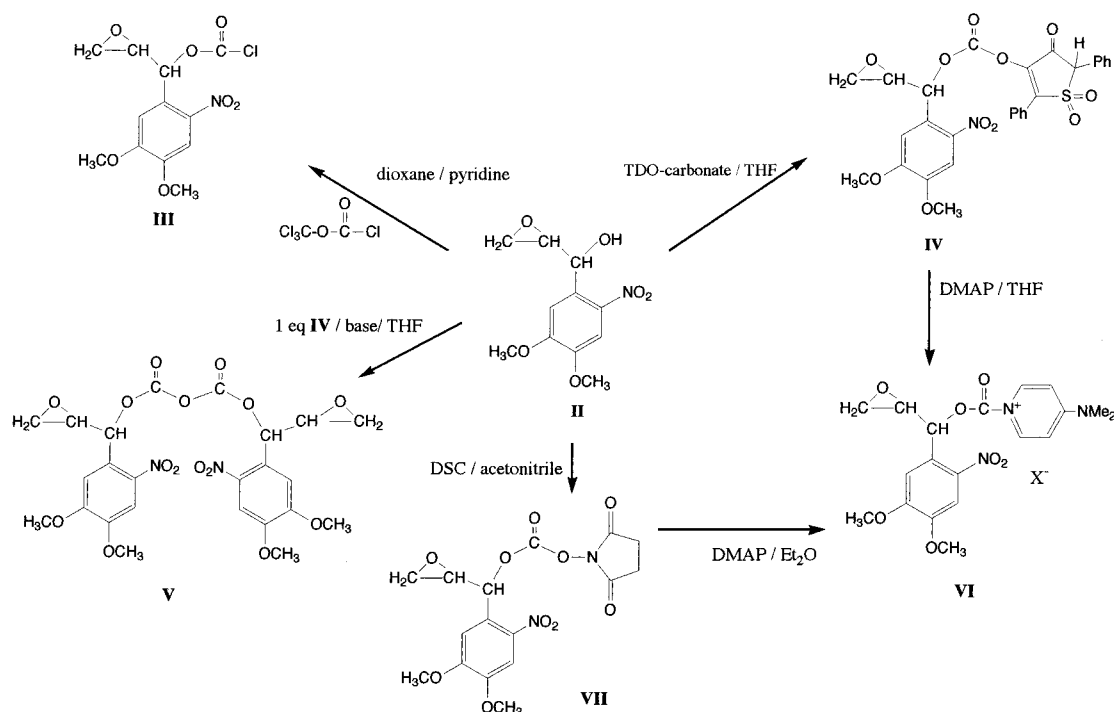


Figure 2. (A) Absorption spectra of **II** in ethanol (a) before irradiation with 365 nm light and (b) after irradiation for 30 s, (c) 90 s, and (d) 900 s. (B) Infrared spectra of **II** before (dashed line) and after a 90 s irradiation with near-ultraviolet light (solid line).

pH, the oxirane group was able to react selectively with reduced thiol groups of proteins.

Spectral Properties of II. The absorption spectrum of a defined concentration of an ethanolic solution of compound **II** (Figure 2A) showed that the chromophore had a broad $n-\pi^*$ transition centered at 350 nm and an extinction coefficient at this wavelength of $5000 \text{ M}^{-1} \text{ cm}^{-1}$. Excitation of this ethanolic solution with near-ultraviolet light (340–420 nm) led to a dose-dependent loss of the 350 nm band, while two new absorption

Scheme 2



transitions evolved with maxima at 268 and 378 nm (Figure 2A). These absorption bands most probably belong to the 3,4-dimethoxy-6-nitrosoacetophenone photoproduct. Evidence that this reaction occurred via a photoisomerization reaction of the nitrophenyl group was provided by an analysis of the infrared spectra of a film of **II** recorded before and after near-ultraviolet irradiation (Figure 2B) which showed a loss of the hydroxyl absorption band at 3450 cm^{-1} of compound **II** and a gain of a ketone absorption band at 1705 cm^{-1} . These results are consistent with the reaction pathway presented in Scheme 1.

Preparation of Photocleavable, Cross-Linking Reagents Based on Compound II. Compound **II** proved to be a key intermediate in the synthesis of the four cross-linking reagents described in this report (Scheme 2). On the basis of earlier, successful studies in which NVOC-Cl was used to cage amino groups (14, 15, 20), we decided to prepare compound **III**, the corresponding chloroformate of **II**, by activation of its benzylic hydroxyl group using diphosgene. Analysis of the reaction showed the presence of two major products which, after purification over silica gel and NMR analysis, proved to be two isomers at the benzylic carbon. Isomer **IIIa** was crystallized out of 2:1 hexane/ethyl acetate. The isomers exhibited identical absorption and infrared spectra, and they could not be distinguished by mass spectrophotometry. The photoisomerization rates of **IIIa** and **IIIb** were found to be similar as judged by a qualitative TLC analysis of the amount of product that formed upon irradiation of each isomer with 365 nm light (data not shown). Compound **III** reacted poorly with the amino groups of polylysine or G-actin in bicarbonate buffer at pH 8.5. Furthermore, although the unsubstituted chloroformate, NVOC-Cl, has been shown to react with amino groups in both organic and aqueous solvents (3, 14), this was not the case with compound **III**, and presumably, the substituent on the benzylic carbon atom deactivated the chloroformate group. These findings prompted the syntheses of more activated carbonates and

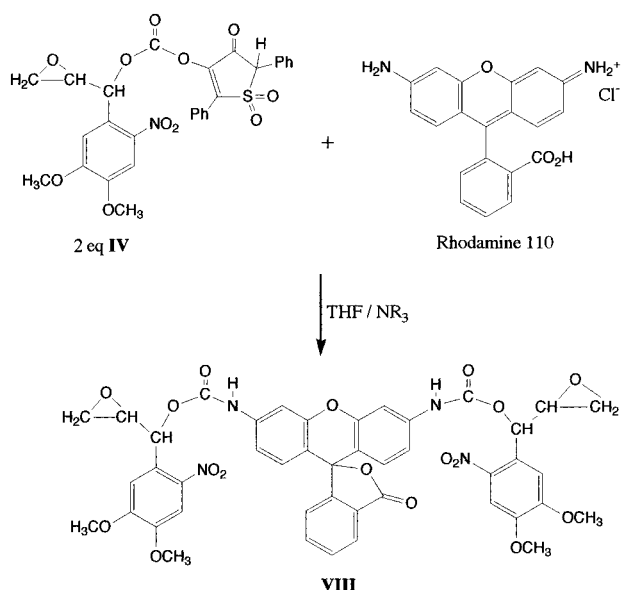
carbamates of compound **II** that could be used to protect amino groups in aqueous or organic solvents.

Steglich's reagent (TDO) (17) was chosen to make a more activated carbonate of compound **II**. This reaction proceeded smoothly in THF under reflux to produce compound **IV** in high yield. The two isomers of compound **IV** were not resolved using the purification scheme described in Materials and Methods. However, the photoisomerization rates of the isomers of compound **IV** should not differ to any significant extent given the similarity of the photoisomerization rates found for the isomers of compound **III**.

An interesting, competing side reaction was discovered in the synthesis of compound **IV**. This involved the reaction of compound **IV** with compound **II**, accelerated by the addition of catalytic amounts of base, which yielded the symmetrical carbonate **V**, a further, thiol reactive, homobifunctional, photocleavable cross-linking reagent (Scheme 2).

Photocleavable, Cross-Linking Reagents and the Preparation of a Thiol Reactive Caged Rhodamine 110. Compound **IV** was shown to react efficiently with amino groups (e.g. rhodamine 110, glycine and polylysine, data not shown) in organic solvent and in aqueous solution at pH 8.5 to produce the corresponding carbamate. Amidation of the two aromatic amino groups of the highly fluorescent rhodamine 110 fluorophore has been shown to form the nonfluorescent, lactone form of the dye which lacks a visible absorption band and fluorescence emission (18). Similarly, compound **IV** was shown to react with rhodamine 110 to produce a nonfluorescent, bis-substituted carbamate (**VIII**, Scheme 3). Irradiation of the nonfluorescent (caged) rhodamine 110 with near-ultraviolet light cleaved the two carbamate bonds and liberated free rhodamine 110 with concomitant recovery of its visible absorption band and intense fluorescence emission centered at 523 nm (Figure 3A,B). This reaction was quite rapid since the photoactivation of a $500\text{ }\mu\text{L}$

Scheme 3



sample of 50 μ M solution of caged rhodamine 110 in the presence of 5 mM DTT was complete within 240 s (Figure 3A,B).

One of the oxirane groups of compound **VII** was used to label a thiol group on a cysteine residue of G-actin in a reaction conducted in aqueous buffer at pH 8.5. A labeling ratio of 1:1 for compound **VIII**:G-actin was calculated using absorption spectroscopy, suggesting the caged fluorophore was covalently attached to cysteine-374 (24). This caged fluorescent G-actin conjugate was able to polymerize under physiological conditions as seen by a comparison of the absorption spectra of the supernatant and pellet fractions of a high-speed centrifugation run, which showed the pellet fraction contained the majority of the photoactivated actin (Figure 4). Irradiation of the nonfluorescent G-actin conjugate with near-ultraviolet light cleaved the carbamate bond that linked the caged groups to rhodamine 110 and liberated the highly fluorescent rhodamine 110, and a polymerization competent G-actin that harbored the 3,4-dimethoxy-6-nitrosophenylketone-based photoproduct (Figure 4).

Photocleavable, Cross-Linking Reagents with Improved Water Solubility and Amino Group Reactivity. To improve upon the water solubility of heterobifunctional photocleavable cross-linking reagents, and to increase their reactivity toward the ϵ -amino group of lysine residues of proteins, compound **VI**, the DMAP salt of compound **IV** was prepared via two synthetic routes outlined in Scheme 2. In the first approach, the succinimide carbonate of **II** was prepared in a reaction of **II** with di-*N*-succinimidyl carbonate in acetonitrile which produced the crystalline product **VII** in high yield. Treatment of compound **VII** in THF with DMAP led to the precipitation of the DMAP salt of compound **VI**. In the second synthetic approach, compound **VI** was prepared in a single-step reaction of compound **IV** with DMAP as described in Materials and Methods. Compound **VI**, a highly activated, water soluble carbamate reacted rapidly with amino groups of proteins at a pH between 8 and 9 (Scheme 4). Compound **VI** was found to hydrolyze in water, and consequently, its reactions with proteins should be performed using the reactants at a relatively high concentration. Compound **VI** can be added to protein labeling reactions as a solid to reduce any risk of organic solvent-based protein denaturation.

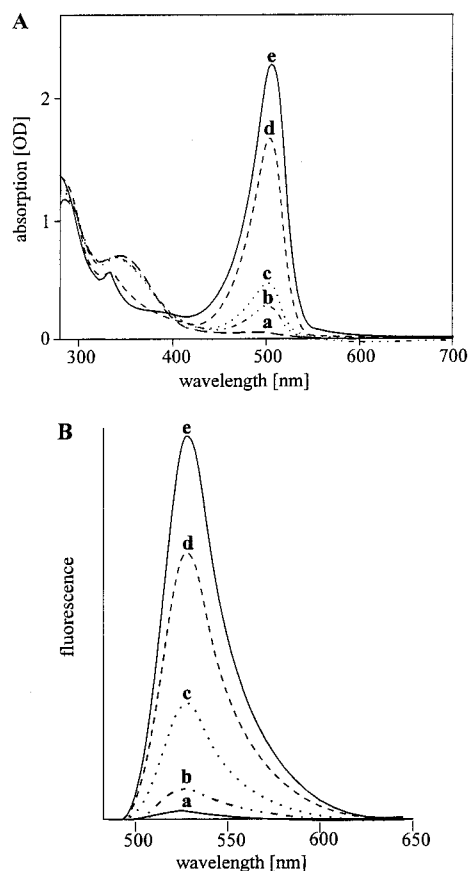


Figure 3. (A) Absorption spectra of a 50 μ M solution of caged rhodamine 110 in EtOH/H₂O (10:1) containing 2 mM DTT before irradiation (a) and after near-ultraviolet irradiation (340–400 nm) for the following times: (b) 5 s, (c) 10 s, (d) 180 s, and (e) 240 s. (B) Fluorescence emission spectra of caged rhodamine 110 with excitation at 488 nm of a 15 μ M solution of caged rhodamine 110 in EtOH/H₂O (10:1) before irradiation (a) and after near-ultraviolet irradiation (340–400 nm) for the following times: (b) 5 s, (c) 20 s, (d) 60 s, and (e) 240 s.

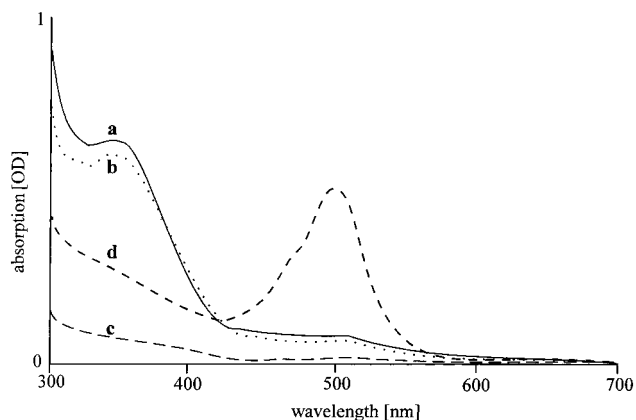


Figure 4. (a) Absorption spectrum of G-actin-labeled caged rhodamine 110 before irradiation with near-ultraviolet light in G-buffer containing 2 mM DTT. The G-actin was treated with MgCl₂ and KCl to 2 and 100 mM, respectively, and after 2 h, the protein was centrifuged at 100000*g* for 60 min. (b) Absorption spectrum of the pellet fraction of this centrifugation run after resuspension in an equal volume of F-buffer. (c) Absorption spectrum of the supernatant fraction of this centrifugation run. (d) Absorption spectrum of the resuspended pellet fraction (from part b) after irradiation with near-ultraviolet light for 10 min in the presence of 5 mM DTT.

It should be noted however that in organic solvents and in the presence of nucleophiles DMAP-activated salts such as **VI** undergo esterification or even reaction to

Scheme 4

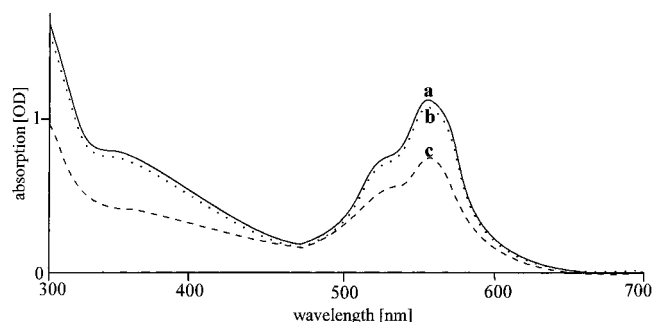
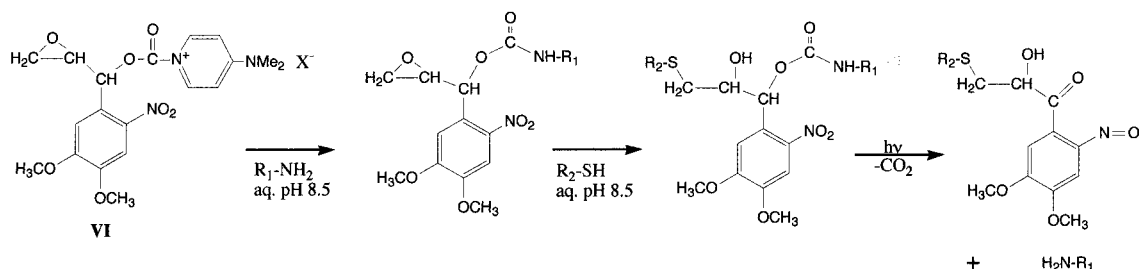


Figure 5. (a) Absorption spectra of the IATMR-labeled G-actin-dextran complex in G-buffer containing 2 mM DTT. After addition of MgCl_2 and KCl to 2 and 100 mM, respectively, and 2 h at room temperature, the sample was centrifuged at $100000g$ for 60 min. (b) Absorption spectrum of the supernatant fraction. No pellet fraction was found after the centrifugation; i.e. the actin was caged. The supernatant fraction was then irradiated with near-ultraviolet light for 10 minutes, and after 2 h at room temperature, the sample was centrifuged at $100000g$ for 60 min. (c) Absorption spectrum of the pellet fraction resuspended in an equal volume of F-buffer.

isocyanates (21), and therefore, stock solutions of **VI** made in acetonitrile should be prepared just before the protein labeling reaction.

Application of Photocleavable, Cross-Linking Reagents for the Preparation of Caged G-Actin.

Compound **VI** was used as part of a new labeling procedure to prepare caged G-actin, i.e. a G-actin conjugate that does not polymerize under physiological conditions (14). An average of 3.5 lysine residues of TMR-labeled G-actin were protected as their corresponding photolabile carbamates with compound **VI**. One or more of the oxirane groups of this fluorescent G-actin conjugate of compound **IV** were then cross-linked to a thiolated dextran. Sixty minutes after addition of physiological salt to this caged G-actin cross-linked complex, the sample was subjected to high-speed centrifugation and the absorption spectra of the supernatant and resuspended pellet fractions were recorded (Figure 5). These data showed the majority of the fluorescently labeled G-actin conjugate was contained in the supernatant fraction and was, consequently, polymerization incompetent (Figure 5). A $500\ \mu\text{L}$ sample of this caged G-actin complex in F-buffer was exposed to near-ultraviolet light for 10 min in the presence of 5 mM DTT to photocleave the cross-link between G-actin and the dextran, and then left for 60 min at room temperature to allow for polymerization. After high-speed centrifugation of the sample, absorption spectroscopic analysis now showed the majority of the actin was in the resuspended pellet fraction; i.e. the photoactivated actin had polymerized into F-actin filaments (Figure 5). The inability of the G-actin cross-linked dextran complex to polymerize in physiological salt probably arose from a physical masking of the actin binding site on the caged G-actin by the flexible, cross-linked dextran molecule. This mode of inhibition was

evidently relieved after the light-directed dissociation of the carbohydrate polymer. Importantly, the photocleavage reaction released native, fully functional G-actin, while the photoproduct remained covalently attached to the thiolated dextran. This new approach to caging the activity of G-actin required the modification of fewer lysine residues compared to the NVOC-Cl method (14).

An interesting parallel to the caged G-actin complex described above has been reported by Mann and Vannan (26). These authors demonstrated that, although a succinimidyl D-biotin conjugate of calmodulin could still activate the enzyme phosphodiesterase, this activity was completely blocked when the calmodulin conjugate was pretreated with avidin. Presumably, avidin, like the dextran in G-actin, physically masked the PDE binding site of calmodulin. Given the importance of specific interactions in protein function, targeted disruption of protein binding sites using the photocleavable reagents and the dextran conjugation methods described herein could be used as part of a general approach to caging the activity of a wide range of proteins. Native, active proteins would then be liberated from these caged dextran cross-linked complexes upon illumination with near-ultraviolet light.

SUMMARY

In this study, we designed, synthesized, and characterized four members of a new class of heterobifunctional photocleavable cross-linking reagents. Biomolecules or fluorophores caged with these reagents can be covalently cross-linked to a second thiol-containing protein, ligand, or derivatized surface through the oxirane moiety. The cross-linking reaction may be used for the following applications: (1) to prepare a caged protein by masking an active site with a thiolated dextran, (2) to target the caged compound to a specific site using a thiolated antibody (15), (3) to attach the caged compound to a thiol-derivatized surface, and (4) to render the caged compound fluorescent in order to image or to quantify the yield of the photoactivation reaction.

The activated carbonate and carbamate groups of two of the reagents described in this report can be used to protect almost any amino group as a photolabile carbamate in either water or organic solvent. The order of reactivity toward amino groups of the different activated groups in water was found to be $\text{R-DMAP}^+ > \text{R-TDO} > \text{R-OSu} > \text{R-Cl}$. Compound **IV**, the TDO carbonate of compound **II**, was used to protect amino groups in organic solvents, while compound **VI**, the DMAP salt of compound **IV**, was the most water soluble and reactive toward amino groups. The physical and chemical separation of the cross-linking functionality from the photoisomerization functionality in these reagents allowed the introduction of one or more methoxy groups into the nitrobenzyl ring which increased its absorption cross section and shifted its action spectrum to a lower energy.

One of the new bifunctional, photocleavable cross-linking reagents, compound **IV**, was used to prepare a thiol reactive, nonfluorescent (caged) rhodamine 110. Irradiation of this caged fluorescent actin conjugate with near-ultraviolet light generated free, highly fluorescent rhodamine 110 and a polymerization competent G-actin conjugate. Compound **V**, a water soluble photocleavable cross-linking reagent, was used as part of a new approach to caging the activity of G-actin by exploiting the ability of a dextran molecule cross-linked to G-actin to physically mask its actin binding site. Photocleavage of the G-actin-dextran cross-linked complex in F-buffer with near-ultraviolet light liberated native G-actin which polymerized into filaments and released the dextran conjugate of the 3,4-dimethoxy-6-nitrosoketone-based photoproduct. The new photocleavable, cross-linking reagents and conjugation methods described in this article can form the basis of a new, general approach to caging the activity of proteins.

LITERATURE CITED

- (1) Adams, S. R., and Tsien, R. Y. (1993) *Annu. Rev. Physiol.* 55, 755–784.
- (2) McCray, J. A., and Trentham, D. R. (1989) *Annu. Rev. Biophys. Chem.* 18, 239.
- (3) Cameron, J. F., and Frechet, J. (1991) *J. Am. Chem. Soc.* 113, 4303.
- (4) Amit, B., Zehavi, U., and Patchornik, A. (1974) *J. Org. Chem.* 39, 192.
- (5) Kaplan, J. H., Forbush, B., III, and Hoffman, J. F. (1978) *Biochemistry* 17, 1929.
- (6) Gravel, D., Murray, S., and Ladouceur, G. (1985) *J. Chem. Soc. Chem. Commun.*, 1828.
- (7) Marriott, G., Miyata, H., and Kinoshita, K. (1992) *Biochem. Int.* 26, 9943.
- (8) Wilcox, M., Viola, R. W., Johnson, K. W., Billington, A. P., Carpenter, B. K., McCray, J. A., Cuzikowski, A. P., and Hess, G. P. (1990) *J. Org. Chem.* 55, 1585.
- (9) Gee, K. R., Wieboldt, R., and Hess, P. (1994) *J. Am. Chem. Soc.* 116, 8366.
- (10) Corrie, J. T., and Trentham, D. R. (1993) *Bioorg. Photochem.* 2, 243.
- (11) Mitchison, T. J. (1989) *J. Cell Biol.* 109, 637.
- (12) Theriot, J. A., and Mitchison, T. J. (1991) *Nature* 352, 126.
- (13) Chang, C.-Y., Niblack, B., Walker, B., and Bayley, H. (1994) *Chem. Biol.* 2, 391.
- (14) Marriott, G. (1994) *Biochemistry* 33, 9092.
- (15) Senter, P. D., Tansey, M., Lambert, J., and Blättler, W. (1985) *Photochem. Photobiol.* 42, 231.
- (16) Gordon, E. M., Gallop, M. A., and Patel, D. V. (1996) *Acc. Chem. Res.* 29, 144.
- (17) Kirstgen, R., Olbrich, A., Rehwinkel, H., and Steglich, W. (1988) *Liebigs Ann. Chem.*, 437.
- (18) Leytus, S. P., Melhado, L. L., Mangel, W. F. (1983) *Biochem. J.* 209, 299.
- (19) Cummings, R. T., and Krafft, G. A. (1988) *Tetrahedron Lett.* 29, 65.
- (20) Fodor, S. P. A., Read, J. L. M., Pirrung, C., Stryer, L., Lu, A. T., and Solas, D. (1991) *Science* 251, 767.
- (21) Knölker, H. J., Braxmeir, T., and Schlechtingen, G. (1995) *Angew. Chem.* 107, 2746.
- (22) March, G. (1985) in *Advanced Organic Chemistry*, 3rd ed., J. Wiley & Sons, New York.
- (23) Rich, D., and Gurwara, S. K. (1975) *J. Am. Chem. Soc.* 97, 157.
- (24) Marriott, G., Zechel, K., Jovin, T. M. (1988) *Biochemistry* 27, 6214.
- (25) Olejnik, J., Sonor, S., Kryzysmanska-Olejnik, E., and Rothschild, K. (1995) *Proc. Natl. Acad. Sci. U.S.A.* 92, 7590.
- (26) Mann, D., and Vanamann, T. C. (1992) *Meth. Enzymol.* 139, 433.
- (27) Marriott, G., and Heidecker, M. (1996) *Biochemistry* 35, 3170.

BC970147O

Cell Targeting by Glycosidic Telomers. Specific Recognition of the Kb CWL1 Lectin by Galactosylated Telomers

Joël Coulon,[†] Roger Bonaly,^{*,†} Bernard Pucci,^{*,‡} Ange Polidori,[‡] Philippe Barthelemy,[‡] and Christiane Contino[‡]

Nancy 1, Faculté de Pharmacie, Laboratoire de Biochimie Microbienne, Université Henri Poincaré, 5 rue Albert Lebrun B.P. 403, 54001 Nancy Cedex, France, and Faculté des Sciences d'Avignon, Laboratoire de Chimie Bioorganique et des Systèmes Moléculaires Vectoriels, Université d'Avignon et des Pays de Vaucluse, 33, rue Louis Pasteur, 84000 Avignon, France. Received April 23, 1997; Revised Manuscript Received November 3, 1997

This work deals with the synthesis and lectinic recognition ability of galactosylated telomers. To investigate if telomeric carriers could exhibit cellular recognition properties, we have synthesized mono- and polygalactosylated tris(hydroxymethyl)acrylamidomethane (THAM) telomers. The affinity of such macromolecular drug carriers toward a receptor, the yeast Kb CWL1 lectin, was defined, and the influence of mono- or polygalactosylation of THAM units on the recognition phenomenon was assessed. The lectinic affinity of the compounds was estimated by measuring the inhibition of yeast aggregation. The average degree of polymerization as well as the hydrophilic–lipophilic balance of such galactosylated telomers affects their recognition ability for the lectin.

INTRODUCTION

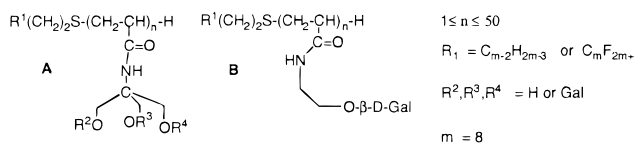
The cell–cell recognition mechanisms as well as the potentialities offered by natural or synthetic macromolecular delivery systems have been widely investigated in recent years (Duncan and Kopecek, 1984; Ouchi and Ohya, 1995).

Binding bioactive molecules to polymers is expected to modulate several disposition parameters, including solubility, absorption and desorption rates, ability to cross membranes, and also cell targeting.

In the course of our project dealing with the study of new drug carriers, we have developed the synthesis and assessments of a new class of low macromolecular carriers or telomers. These compounds were obtained by radical polymerization of an acryloyl or vinylic monomer in the presence of an alkane/perfluoroalkane thiol as the transfer reagent. The physicochemical parameters of prepared multifunctional molecules can be adjusted through both the starting material and the experimental conditions (Pucci et al., 1988). In previous reports (Pucci et al., 1990), we described the synthesis of new amphiphilic telomers derived from tris(hydroxymethyl)acrylamidomethane (THAM)¹ and showed that such compounds were able to cross physiological membranes to diffuse in all biological tissues and could be used for an in vivo drug-delayed delivery (Maurizis et al., 1994). Indeed, no toxicity was observed and, after iv (intravenous) or po (oral) administration to rat, these compounds were slowly released, i.e., within 100 h. Moreover, glycosylation of THAM hydroxyl functions affects the hydrophilic–lipophilic balance (HLB) of these macromolecules and could ensure selective cell targeting via

a specific recognition of pendent carbohydrates by membrane lectins.

The goal of this work is to demonstrate and to specify the recognition ability toward specific lectins of such glycosylated telomers.



It is known that glycosidic compounds are recognized by yeast lectins. These carbohydrate binding proteins are specific and can selectively bind galactose (Al-Mahmood et al., 1991), mannose (Straver et al., 1994), or other glucidic structures (Johnson et al., 1988). In several yeasts, lectins are anchored at the periphery of the cell membrane. The yeast *Kluyveromyces fragilis* bears on its surface a galactose specific Kb CWL1 lectin which has been isolated by Al-Mahmood et al. (1991). This lectin is involved in flocculation or aggregation of this yeast which is a specific cell–cell recognition phenomenon. We observed (Attoui et al., 1994) that the galactose-substituted cyclodextrins were able to inhibit the flocculation of these yeast cells and to change their growth properties. In these phenomena, the linkage of the galactose-substituted cyclodextrins to the cells via a lectinic mechanism seemed to be the initial but important event.

Similar results were obtained with other lectins by Lee (1978) using bis- and mono- β -D-galactopyranoside analogues of the Tris derivative suitable for attachment to proteins on solid matrixes. Kitano et al. (1995) prepared and used amphiphilic compounds bearing several galactose residues in their hydrophilic head groups. These compounds were obtained by telomerization of a galactose containing vinyl monomers using a lipophilic radical as the initiator. Plank et al. (1992) showed that in artificial ligands galactose residues were very important for their

* To whom correspondence should be addressed.

[†] Université Henri Poincaré.

[‡] Université d'Avignon et des Pays de Vaucluse.

¹ Abbreviations: THAM, tris(hydroxymethyl)acrylamidomethane; Tris, tris(hydroxymethyl)aminomethane; DP_n, average degree of polymerization; CMC, critical micellar concentration; HLB, hydrophilic–lipophilic balance.

recognition by lectin-like proteins located on the surface of parenchymal hepatocytes.

To investigate if the telomeric carriers could ensure cellular recognition properties, first, we report herein the telomer synthesis from mono- or polygalactosylated THAM and from galactosylated *N*-(acryloyl)ethanolamine. Second, we determine their cellular recognition ability of ligand using as the receptor the galactose specific Kb CWL1 lectin and as target cells the yeast *K. bulgaricus*. Like mammalian cells, yeasts are eukaryotic cells bearing at their surface lectins and ligands. As they are surrounded by a cell wall that protects them against osmotic shocks, they can easily be used in various assays without alterations of their cellular morphology to evaluate recognition properties of galactose derivatives.

EXPERIMENTAL PROCEDURES

Chemistry and Synthesis. The progress of the reaction and the homogeneity of the compounds were monitored by thin-layer chromatography (TLC, Merck F₂₅₄). Detection was achieved by either iodine absorption, exposure of plates to UV light (254 nm), or charring with a methanol/sulfuric acid (1:1) solution. Chromatographic separations were carried out on silica gel 60 (70–230 mesh, Merck), and gel permeation was carried out on Sephadex LH 60 (Pharmacia LKB). Melting points were measured on a electrothermal 9100 type apparatus and are reported uncorrected. The ¹H, ¹³C, and ¹⁹F nuclear magnetic resonance (NMR) spectra were recorded at 250 MHz (or 67.3 MHz for ¹³C) on a Bruker AC 250 apparatus in CDCl₃ or DMSO-*d*₆, and the chemical shifts are expressed in parts per million relative to tetramethylsilane as an internal reference for the ¹H and ¹³C spectra. Elemental analyses were performed by the "Service Central de Microanalyses" of the CNRS of Montpellier (France).

Reactions were performed under anhydrous conditions under dry nitrogen. All the solvents were distilled and dried according to standard procedures. For the telomerization, the solutions were previously carefully deoxygenated by nitrogen bubbling. AIBN (2,2'-azobisisobutyronitril) was purified by recrystallization from absolute ethanol (twice).

Synthesis of mono-, di-, and trigalactosylated monomers **3**, **5**, and **8** and fluorocarbonated monoadducts **15**–**17** have been previously reported (Polidori et al., 1994).

Telomerization of *N*-Tris[[[(2,3,4,6-tetra-*O*-acetyl-β-*D*-galactopyranosyl)oxy]methyl]methyl]acrylamide (3**) in the Presence of 1*H*,1*H*,2*H*,2*H*-Perfluorodecanethiol (**20**).** To a solution of trigalactosylated monomer **3** (3 g, 2.57 mmol) in anhydrous methanol (100 mL) warmed to 40–50 °C under a nitrogen atmosphere were added 1*H*,1*H*,2*H*,2*H*-perfluorodecanethiol (0.246 g, 0.514 mmol, *R*_o = [thiol]/[monomer] = 0.2) in methanol (10 mL) and freshly recrystallized AIBN (0.010 g, 6.43 × 10^{−2} mmol). The mixture was refluxed under stirring; the progress of the reaction was monitored by thin-layer chromatography (7:3 ethyl acetate/hexane). After refluxing for 12 h, the monomer **3** was entirely consumed. Solvent was evaporated, and the resulting oil was subjected to column chromatography on Sephadex LH 60 (1:1 dichloromethane/methanol) to afford the peracetylated telomer **20'** as a white powder (2.1 g, 65%). The average degree of polymerization (DP_n) (4.8) was estimated by ¹H NMR by comparing the area of the signal attributed to methylene α to the sulfur in the fluoroalkyl chain (*d* = 2.75 ppm) with the area of those ascribed to H₄ protons (*d* = 5.4 ppm) of the galactopyranosyl residues. It was con-

firmed by elemental analysis using the nitrogen-to-fluorine ratios %N/%F (=14 DP_n/(17 × 19)). Anal. Found: C, 48.02; H, 5.63; N, 1.20; F, 5.1. Anal. For a DP_n of 5.4, calcd: C, 48.60; H, 5.40; O, 39.45; N, 1.12; S, 0.49; F, 4.94 and an average degree of polymerization of approximately 5 for peracetylated telomer **20**.

Deacetylation was performed at room temperature in methanol containing a catalytic amount of sodium methoxide. After treatment with a H⁺ type resin (Amberlite IRC 50), filtration, and evaporation of the solvent, deacetylated derivative **20** was isolated in quantitative yield.

The same procedure used for the preparation of telomer **20** was applied to the synthesis of telomers **18** and **19**.

Synthesis of telomers bearing a hydrocarbon tail such as **21** was previously described (Polidori et al., 1994). The DP_n values were determined by ¹H NMR spectroscopy (CDCl₃) on telomers before deacetylation by comparing the area of the signal ascribed to the terminal methyl group (*d* = 0.85 ppm) with that of H₄ proton(s) of the galactopyranose moiety (*d* = 5.4 ppm).

Telomerization of *N*-(Acryloyl)ethanolamine **9.** Sixteen milliliters of acryloyl chloride (197 mmol) was added at 0 °C dropwise to 7.9 mL of ethanolamine (130 mmol) solubilized in 150 mL of methanol. A solution of KOH (1 N) was simultaneously added in order to maintain the pH of the methanolic solution at 8–9. The mixture was stirred for 4 h at room temperature. After filtration of the KCl salt, the solvent was evaporated under reduced pressure and the crude oil was purified by column chromatography on silica gel (eluent, 10:90 ethanol/AcOEt, v/v). Pure *N*-(acryloyl)ethanolamine **9** was isolated as an oil (10 g, 67% yield, *R*_f 0.4 with 10:90 EtOH/AcOEt). ¹H NMR (DMSO-*d*₆): δ 3.2 (2H, q, CH₂OH), 3.5 (2H, t, CH₂NH), 3.55 (1H, m, OH), 5.6 (1H, dd, CH₂=C), 6.05 (1H, dd, CH₂=C), 6.25 (1H, dd, C=CH), 8.15 (1H, m, NH). ¹³C NMR (DMSO-*d*₆): δ 164.9 (CONH), 131.81 (CH=), 125.04 (CH₂=), 59.7 (CH₂OH), 41.57 (CH₂NH).

Telomerization of *N*-[[[(2,3,4,6-Tetra-*O*-acetyl-β-*D*-galactopyranosyl)oxy]ethyl]acrylamide **10.** *N*-(Acryloyl)ethanolamine **9** (5.4 g, 43.5 mmol) and mercuric cyanide (15.4 g, 56.5 mmol) were solubilized in freshly distilled acetonitrile. After the mixture was stirred for 15 min in the presence of Drierite, acetobromogalactose (25.05 g, 56.5 mmol) was added and the mixture was sonicated under a nitrogen atmosphere for 25 min at room temperature. Then, the precipitate was filtered off and the solvent evaporated. The crude oil was solubilized in ethyl acetate and successively washed with sodium hydrogen carbonate (200 mL), 10% sodium iodide (200 mL), saturated sodium thiosulfate (200 mL), and water. The organic layer was dried over sodium sulfate and then concentrated. Monomer **10** was isolated as a white powder after chromatography on the silica gel column (13.1 g, 67.6% yield, *R*_f = 0.3 with 80:20 AcOEt/hexane, this compound is very hygroscopic). ¹H NMR (CDCl₃): δ 2.1 (12H, 3s, CH₃CO), 3.5 (2H, m, CH₂NH), 3.7 (2H, m, CH₂O), 3.9 (1H, m, H₅), 4.15 (2H, d, H₆), 4.5 (1H, d, H₁), 5.0 (1H, dd, H₃), 5.2 (1H, dd, H₂), 5.4 (1H, d, H₄), 5.65 (1H, dd, CH₂=), 6.1 (1H, dd, CH₂=), 6.25 (2H, m, CH₂=, NH). ¹³C NMR (CDCl₃): δ 170.4, 170.2, 170.1, 169.8 (CH₃CO), 165.5 (CONH), 130.7 (CH₂=), 126.7 (CH=), 101.5 (C₁, β-anomer), 70.9 (C₅ or C₃), 70.7 (C₅ or C₃), 69.1 (CH₂O or C₂), 68.9 (CH₂O or C₂), 67 (C₄), 61.4 (C₆), 39.2 (CNH), 20.5 (CH₃CO), 20.6, 20.55, 20.3.

Synthesis of *N*-[[[(2,3,4,6-Tetra-*O*-acetyl-β-*D*-galactopyranosyl)oxy]ethyl]-4-thiadodecanamide **22.** To a solution of compound **10** (0.9 g, 2.62 mmol) in anhydrous methanol (40 mL) warmed to 40–50 °C under a nitrogen

atmosphere were added 1.05 mL of octanethiol (6.066 mmol) in methanol (10 mL) and freshly recrystallized AIBN (0.010 g, 6.43×10^{-2} mmol). The mixture was refluxed under stirring and the progress of the reaction was monitored by thin-layer chromatography (7:3 ethyl acetate/hexane). After refluxing for 12 h, the monomer **10** was entirely consumed. Solvent was evaporated, and the resulting oil was subjected to column chromatography on silica gel to afford the pure peracetylated monoadduct **22'** as a white powder (0.595 g, 46.6%, hygroscopic). ^1H NMR (CDCl_3): δ 1.0 (3H, t, CH_3), 1.4 (10H, m, $(\text{CH}_2)_5$), 1.7 (H, m, $\text{CH}_2\text{CH}_2\text{S}$), 2.2 (12H, 4s, CH_3CO), 2.6 (4H, m, CH_2SCH_2), 2.85 (2H, t, CH_2CO), 3.6 (2H, m, CH_2NH), 3.9 (H, m, CH_2O), 4 (1H, m, H_5), 4.2 (2H, d, H_6), 4.6 (1H, d, H_1), 5.2 (1H, dd, H_2), 5.4 (1H, d, H_4), 6.2 (1H, t, NH). ^{13}C NMR (CDCl_3): δ 171.3, 170.4, 170.2, 170, 169.8 (CO), 101.4 ($\text{C}_1 \beta$), 70.9 (C_5 or C_3), 70.7 (C_3 or C_5), 69 (C_2 or CH_2O), 68.8 (CH_2O or C_2), 67.0 (C_4), 61.3 (C_6), 39.3 (CNH), 36.7 (CH_2CO), 22.6 to 32.4 ($(\text{CH}_2)_n$), 20.8, 20.7, 20.6, 20.5 (CH_3CO), 14.1 (CH_3).

Deacetylation was realized as above (compound **20**).

Telomerization of Monomer 10 in the Presence of Octanethiol. The procedure was similar to that described above for telomer **20**. For example, starting from monomer **10** (0.92 g, 2.68 mmol), octanethiol (0.06 g, 0.41 mmol, $\text{Ro} = [\text{thiol}]/[\text{monomer}] = 0.153$), and AIBN (0.02 g, 0.12 mmol), acetylated telomer **22'** (0.81 g, 82% yield) was isolated after purification by column chromatography on Sephadex LH₆₀. The D_pn (6.2) was obtained by comparing the area of the terminal methyl signal ($d = 0.85$ ppm) with the signal integration of the H_4 of the galactopyranosyl residues ($d = 5.4$ ppm). For acetyl hydrolysis, the same procedure as for compound **20** was applied to telomer **22'**.

Microorganisms and Culture Conditions. The flocculent yeast *K. bulgaricus* (ATCC 96631) was grown aerobically (air flow of 40 L/h) at 25 °C over 24 h in 1.5 L of a liquid medium [4% glucose, 0.4% bactopeptone (Difco), 0.1% KH_2PO_4 , 0.02% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 0.02% CaCl_2 (Prolabo, Paris, France)] contained in a 2 L capacity fermentor (LSL Biolafitte S.A., Saint Germain en Laye, France). The flocculent yeast *Saccharomyces cerevisiae* obtained from "Laboratoire de Microbiologie et Génétique ENSAM" at Montpellier (France) was grown under the same aerobic conditions but in a Sabouraud liquid medium [2% glucose and 1% bactopeptone (Difco)].

Growth Assays. The monogalactosylated and digalactosylated monomers or telomers were tested on the growth of *K. bulgaricus* and *S. cerevisiae*. The inoculum (10^7 cells/mL) was obtained from an overnight culture of the yeast in liquid medium. The cultures were grown aerobically at 25 °C in 100 mL Erlenmeyer flasks containing 20 mL of their respective liquid culture medium supplemented with compounds to test. The culture flasks were stirred on a rotary shaker for 48 h. The growth of the yeasts was followed by measuring the absorption at 620 nm with a Perkin-Elmer spectrophotometer. The growth rate calculated according the method of Libudzisz (1986) corresponds to the number of cell divisions per unit of time. All the compounds were solubilized in sterile distilled water before addition in the culture medium at appropriate concentrations.

Extraction of a Galactose Specific Lectin from the Yeast Cells. The Kb CWL1 lectin, involved in the flocculation phenomenon of *K. bulgaricus* (Al-Mahmood et al., 1991), was isolated from whole yeast cells. After growth in a 2 L fermentor under aerobic conditions *K. bulgaricus* cells were harvested by centrifugation at

3000g for 10 min at 4 °C, washed with Helm's buffer (150 mM CH_3COONa , 3.75 mM CaCl_2 , and 3 mM NaN_3 at pH 4.5), treated with a 0.2 M D-galactose solution, and extensively washed with a 0.01 M phosphate buffer (pH 7). Then, cells were suspended at a concentration of 4% (w/v) in PBS supplemented with 5 mM EDTA and incubated at 37 °C for 90 min with weak stirring. After centrifugation at 3000g for 10 min, the supernatant was collected, dialyzed at 4 °C for at least 48 h against distilled water, and then freeze-dried.

The residual flocculating activity of the yeast cells was assessed in Helm's acetate buffer.

Flocculation and Flocculation Inhibition Tests. Nonflocculating yeast cells obtained after lectin extraction were washed first with distilled water, then with a 1% EDTA aqueous solution, and then twice with distilled water. Washed cells (about 2.5×10^6 cells/mL) were suspended in 10 mL of Helm's acetate buffer.

The lectin titer determination was assayed in glass tubes by mixing together 50 μL of 2-fold serial dilutions of lectin solution in the Helm's buffer at pH 4.5 and 50 μL of yeast suspension (2.5×10^6 cells/mL) in the same buffer.

A positive reaction resulted in formation of aggregate (flocs), and the lectin titer corresponded to the reciprocal of the highest dilution of the lectin solution giving detectable aggregation of deflocculated yeasts.

Flocculation (or reflocculation) of the yeast cells was achieved in glass tubes by mixing 50 μL of lectin solution titer 4 with 50 μL of deflocculated yeast cells.

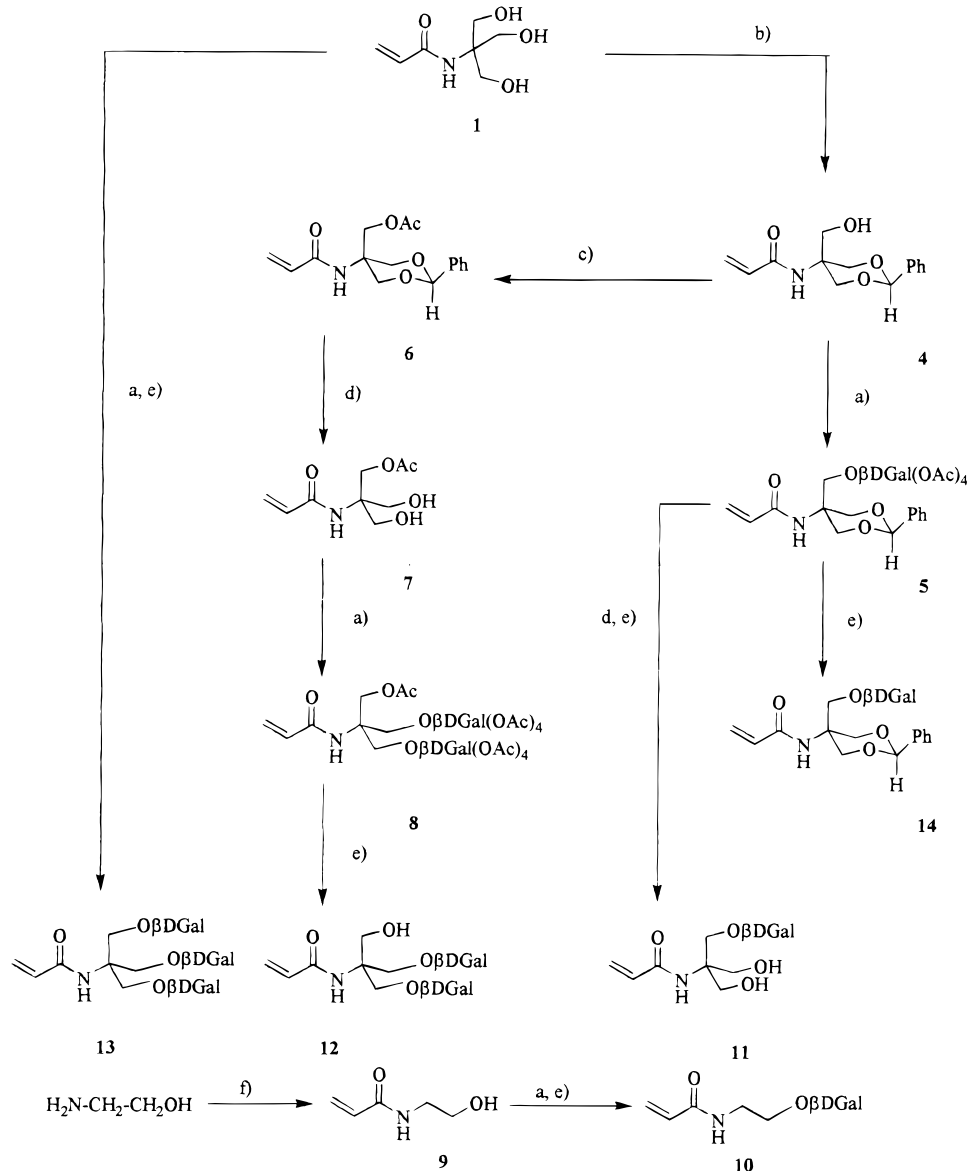
Inhibition of flocculation was assayed in glass tubes by mixing 50 μL of a 2-fold serial dilution of galactosylated substrate solutions in Helm's acetate buffer with 50 μL of lectin solution titer 4. Flocculation inhibition was controlled with galactose, methylgalactose, and *p*-nitrophenylgalactose derivatives. After incubation for 60 min at room temperature, 50 μL of the deflocculated yeast suspension was added. Yeast flocculation was estimated by visual reading or optical microscope observation after 1 h of incubation at room temperature. The inhibitory concentration of the flocculation (ICF) corresponded to the tube which shows a visible aggregation phenomenon. These assays allow us to specify the affinity of the compounds for the lectin. When the amount of compound inhibiting the reflocculation increases, its affinity for the lectin decreases.

RESULTS AND DISCUSSION

Chemistry. The synthesis of A type surfactants requires the selective glycosylation of hydroxyl groups of THAM. For the sake of convenience, we decided to achieve glycosylation on the THAM molecule and to telomerize them subsequently.

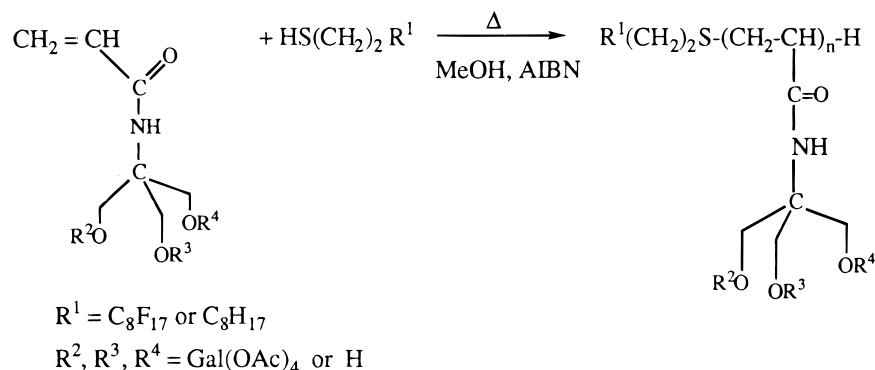
Syntheses of mono-, di-, and trigalactosylated monomers derived from THAM have been already reported (Polidori et al., 1994) and are summarized in Scheme 1.

Surfactants were prepared as previously reported (Pucci et al., 1988, 1990; Polidori et al., 1994) by radical telomerization (Scheme 2). Basically, this reaction follows the same rule as a classic radical polymerization. Both methods involve self-condensation of an unsaturated polymerizable monomer in the presence of a radical initiator. Both exhibit the conventional initiation, propagation, and termination steps. The major difference between both processes arises from the fact that telomerization involves the participation of a further chemical species, a chain transfer reagent (or telogen), in this case an alkanethiol.

Scheme 1. Synthesis of Galactosylated THAM Monomers^a

^a (a) Acetobromogalactose, $\text{Hg}(\text{CN})_2$, CH_3CN , ultrasounds, 67–90%; (b) benzaldehyde, *p*-toluenesulfonic acid, CH_3CN , 70%; (c) acetic anhydride, pyridin, 90%; (d) 7/3 $\text{CH}_3\text{COOH}/\text{H}_2\text{O}$, 50 °C, 50–90%; (e) MeONa , MeOH , 95%; (f) acryloyl chloride, MeOH , KOH , 90%.

Scheme 2. Radical Telomerization of Surfactants



The reaction was carried out in boiling methanol under a nitrogen atmosphere in the presence of 2,2'-azobisisobutyronitrile (AIBN) as the radical initiator. Telomerizations were monitored by thin-layer chromatography, and the reaction was pursued until the complete disappearance of monomer **3**, **5**, or **8** (12–24 h).

Two thiols were used as the telogen: octanethiol and the 1*H*,1*H*,2*H*,2*H*-perfluorodecanethiol leading to various monoadducts and/or telomeric surfactants of the **A** or **B** type.

Monoadducts were isolated in pure form after column chromatography on silica gel ($\text{AcOEt}/\text{hexane}$). Telomeric

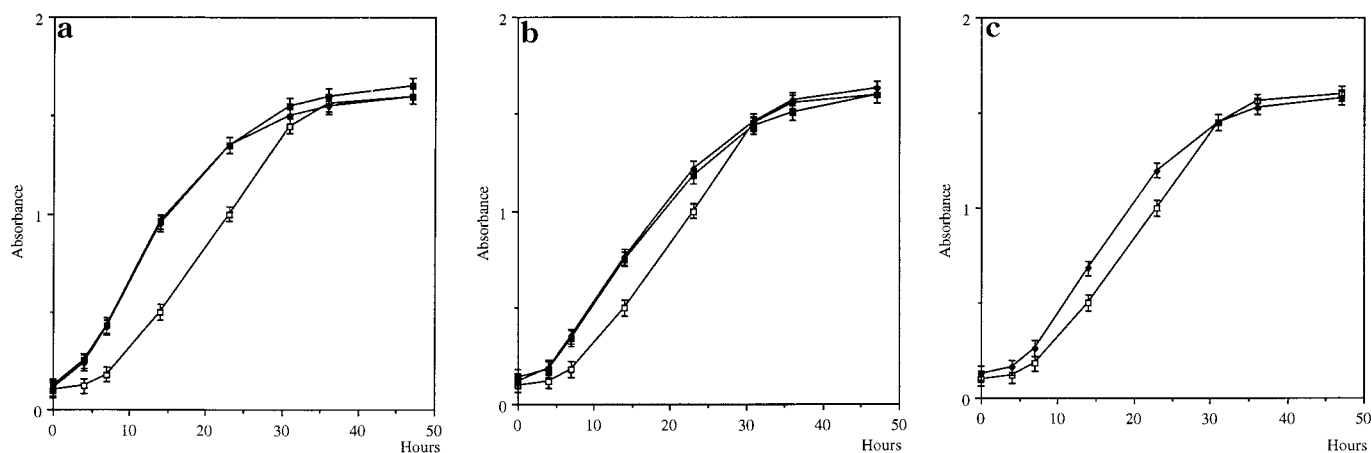


Figure 1. Growth of *K. bulgaricus* in the presence of mono- and digalactosylated monomers and telomers and galactose: (a) (□) control, (◆) monogalactosylated monomer (**11**), and (■) monogalactosylated telomer (**18**); (b) (□) control, (◆) digalactosylated monomer (**12**), and (■) digalactosylated telomer (**19**); and (c) (□) control and (◆) galactose. The monomers and telomers were tested at a concentration of 1.25 mM, and galactose was tested at a concentration of 2 mM. Each curve corresponds to the average curve of three assays, and the bar corresponds to the standard deviation.

surfactants were purified by gel filtration on Sephadex LH 60.

When the telomer bore a hydrocarbon tail, the $\overline{\text{DPn}}$ was determined by ^1H NMR spectroscopy (CDCl_3) by comparing the area of the signal ascribed to the terminal methyl group ($\delta = 0.85$ ppm) with the H_4 proton(s) signal area of the galactopyranose moiety ($\delta = 5.4$ ppm). In the case of fluorocarbon compounds, the elemental analysis allowed us to calculate %N/%F. The $\overline{\text{DPn}}$ was determined with the equation

$$\%N/\%F = 14n/(17 \times 19) \text{ where } \overline{\text{DPn}} = n$$

All compounds were isolated as white amorphous solids which were very difficult to crystallize. Acetyl deprotection with the monogalactosylated compounds (monomers or telomers), cleavage of the dioxane ring, used as hydroxyl protecting groups, was achieved in an 8:2 acetic acid/water mixture at 60°C for 1 h. Acetyl groups were entirely removed with a catalytic amount of sodium methoxide in anhydrous methanol.

Effects of Mono- and Digalactosylated Monomers and Telomers on *K. bulgaricus* Growth and Flocculation. When *K. bulgaricus* was cultivated in control medium, the exponential growth phase started after 5 h of incubation with a growth rate of 0.15 h^{-1} . Flocculation was observed 10 h after the inoculation.

The presence in the medium of monogalactosylated monomer **11** and telomer **18** (Figure 1a) or digalactosylated monomer **12** and telomer **19** (Figure 1b) at a concentration of 1.25 mM showed no toxic effect on the yeast cells. However, a slight stimulating effect on growth occurred at the beginning of the culture with growth rates of 0.11 and 0.10 h^{-1} for monogalactosylated and digalactosylated derivatives, respectively. No flocculation was observed. When the stationary phase is reached, the biomasses were identical for control and cultures realized in the presence of galactosylated monomers or telomers. This stimulation effect could be due to the inhibition of flocculation at the beginning of growth, or the lack of aggregation increasing the cell degree of freedom and thus facilitating their multiplication. A similar effect was observed when the medium was supplemented with 2 mM galactose (Figure 1c) instead of the galactosylated monomers or telomers. The growth rate in culture in the presence of galactose was 0.13 h^{-1} . Indeed, *K. bulgaricus* cells bear at their

Table 1. Inhibitory Concentration of the Flocculation (ICF) of Monosaccharides and Derivatives^a

oses and derivatives	ICF (mM)
D-galactose	3.12
methyl β -D-galactoside	1.75
PNP α -galactoside	0.40
PNP β -galactoside	0.83
lactose (Gal- β -1,4-Glc)	1.25
melibiose (Gal- α -1,6-Gal)	0.60
D-fucose (6-deoxy-Gal)	3.50
raffinose (Gal- β -1,4-Glc-1,1-Fruc)	1.20
mannose	NI ^b
D-glucose	NI

^a ICF, concentration of sugar derivatives necessary to inhibit the yeast flocculation. ^b NI, no inhibition.

periphery galactose specific Kb CWL1 lectin (Al-Mahmood et al., 1991) which is involved in yeast flocculation. Galactose molecules or galactosylated monomers and telomers inhibit this cell-cell aggregation phenomenon.

The same galactosylated monomers or telomers were tested on the yeast *S. cerevisiae*. In this case, we observed a flocculation phenomenon; furthermore, these compounds did not modify the cell growth. Indeed, this yeast species bears mannose specific lectins on its outer surface (Stratford et al., 1992).

Inhibition of Yeast Reflocculation by Monosaccharides and Derivatives. Several studies have already been carried out to determine the main structural parameters involved in the recognition phenomenon (Kornfeld and Kornfeld, 1976). In lectinic recognition mechanisms, monosaccharides characterize the specificity of the different lectins, but it is well-known that oligosaccharides could exhibit a higher lectin affinity than the free specific monosaccharides (Kawaguchi et al., 1980). In this report, the reflocculation of *K. bulgaricus* cells induced by the galactose specific Kb CWL1 lectin was used to determine the recognition affinity of different galactosylated derivatives. Their ability to inhibit the yeast cell reflocculation process is directly correlated to their lectin affinity. Results reported in Table 1 show that the functionalization of the glycoside anomeric carbon induces an increase of the affinity. Indeed, methyl- β -D-galactose, lactose, and *p*-nitrophenyl- β -D-galactose have a higher inhibitory activity of the lectin than free galactose. These results underline the fact that a hydrophobic structure grafted to the galactose anomeric

Table 2. Inhibition of *K. bulgaricus* Reflocculation by Mono- and Polygalactosylated Tris Monomers^a

N°	Product	ICF (mM)
11	$\text{CH}_2=\text{CH}-\text{CO}-\text{NH}-\text{C} \begin{array}{l} \diagup \text{O}-\beta\text{-D-Gal} \\ \diagdown \text{OH} \\ \diagdown \text{OH} \end{array}$	0.5
12	$\text{CH}_2=\text{CH}-\text{CO}-\text{NH}-\text{C} \begin{array}{l} \diagup \text{O}-\beta\text{-D-Gal} \\ \diagdown \text{O}-\beta\text{-D-Gal} \\ \diagdown \text{OH} \end{array}$	0.125
13	$\text{CH}_2=\text{CH}-\text{CO}-\text{NH}-\text{C} \begin{array}{l} \diagup \text{O}-\beta\text{-D-Gal} \\ \diagdown \text{O}-\beta\text{-D-Gal} \\ \diagdown \text{O}-\beta\text{-D-Gal} \end{array}$	0.125
14	$\text{CH}_2=\text{CH}-\text{CO}-\text{NH}-\text{C} \begin{array}{l} \diagup \text{O}-\beta\text{-D-Gal} \\ \diagdown \text{O} \diagup \text{Ph} \\ \diagdown \text{O} \diagdown \text{H} \end{array}$	0.1

^a ICF, concentration of galactosylated THAM monomers necessary to inhibit the yeast flocculation. Results are the average of three independent experiments.

position enhances the inhibitory activity, i.e., increases the affinity of these derivatives for the Kb CWL1 lectin.

Inhibitory Activity on the Reflocculation of Mono- and Polygalactosylated Tris Monomers. As reported in Table 2, all the galactosylated monomers derived from THAM showed a higher affinity for the Kb CWL1 lectin than galactose (3.12 mM) used as a control. This affinity improvement can be related to the results obtained with the α and β *p*-nitrophenyl-D-galactose and the melibiose.

The di- or trigalactosylated THAM monomers (compounds **12** and **13**) exhibited an affinity for the lectin 4 times higher than that observed with the monogalactosylated monomer (compound **11**). However, this affinity enhancement is not proportional to the number of galactose moieties. Thus, if the activity of monomer **12** bearing two galactose units is slightly higher than that observed for the monogalactosylated monomer **11**, the presence of three galactose residues did not enhance further the affinity of monomer **13**. The grafting of several sugars on the Tris central carbon involves a steric hindrance which appears to be detrimental to the recognition. Each sugar must be accessible and recognized as a guest. The presence of a hydrophobic structure near the sugar improves the affinity for the lectin; this assumption is checked with the benzylidene derivative (compound **14**). The presence of the hydrophobic character close to the sugar increases 5-fold the affinity of the ligand for the lectin.

Inhibitory Activity on the Reflocculation of Perfluoroalkylated and/or Polygalactosylated Monoadducts and Telomers. Results listed in Table 3 demonstrate that when a hydrophobic perfluoroalkylated chain is linked to the mono- or polygalactosylated monoadducts (compounds **15–17**) the affinity of such ligands for the lectin increased from 2.5- to 10-fold compared to the values for monomer derivatives (compounds **11–13**, Table 2). Taking into account the effect of this perfluoroalkyl chain on the compound's affinity, it seemed interesting to specify how the number of galactosylated structures grafted on the telomer affected this affinity. As reported in Table 4, we can observe that the inhibitory

Table 3. Inhibition of *K. bulgaricus* Reflocculation by Perfluoroalkylated Mono- and Polygalactosylated Monoadducts^a

N°	Product	ICF (mM)
15	$\text{C}_8\text{F}_{17}-\text{CH}_2-\text{CH}_2-\text{S}-\text{CH}_2-\text{CH}_2-\text{CO}-\text{NH}-\text{C} \begin{array}{l} \diagup \text{O}-\beta\text{-D-Gal} \\ \diagdown \text{OH} \\ \diagdown \text{OH} \end{array}$	0.05
16	$\text{C}_8\text{F}_{17}-\text{CH}_2-\text{CH}_2-\text{S}-\text{CH}_2-\text{CH}_2-\text{CO}-\text{NH}-\text{C} \begin{array}{l} \diagup \text{O}-\beta\text{-D-Gal} \\ \diagdown \text{O}-\beta\text{-D-Gal} \\ \diagdown \text{OH} \end{array}$	0.05
17	$\text{C}_8\text{F}_{17}-\text{CH}_2-\text{CH}_2-\text{S}-\text{CH}_2-\text{CH}_2-\text{CO}-\text{NH}-\text{C} \begin{array}{l} \diagup \text{O}-\beta\text{-D-Gal} \\ \diagdown \text{O}-\beta\text{-D-Gal} \\ \diagdown \text{O}-\beta\text{-D-Gal} \end{array}$	0.01

^a ICF, concentration of galactosylated THAM monomers necessary to inhibit the yeast flocculation. Results are the average of three independent experiments.

Table 4. Inhibition of *K. bulgaricus* Reflocculation by Perfluoroalkylated Telomers with Low DPⁿ^a

N°	Product	ICF (mM)
18	$\text{C}_8\text{F}_{17}-\text{CH}_2-\text{CH}_2-\text{S}-\left[\begin{array}{c} \text{CH}_2-\text{CH} \\ \\ \text{CO} \\ \\ \text{NH}-\text{C} \begin{array}{l} \diagup \text{O}-\beta\text{-D-Gal} \\ \diagdown \text{OH} \\ \diagdown \text{OH} \end{array} \end{array} \right]_5 \text{H}$	0.25
19	$\text{C}_8\text{F}_{17}-\text{CH}_2-\text{CH}_2-\text{S}-\left[\begin{array}{c} \text{CH}_2-\text{CH} \\ \\ \text{CO} \\ \\ \text{NH}-\text{C} \begin{array}{l} \diagup \text{O}-\beta\text{-D-Gal} \\ \diagdown \text{O}-\beta\text{-D-Gal} \\ \diagdown \text{OH} \end{array} \end{array} \right]_4 \text{H}$	0.125
20	$\text{C}_8\text{F}_{17}-\text{CH}_2-\text{CH}_2-\text{S}-\left[\begin{array}{c} \text{CH}_2-\text{CH} \\ \\ \text{CO} \\ \\ \text{NH}-\text{C} \begin{array}{l} \diagup \text{O}-\beta\text{-D-Gal} \\ \diagdown \text{O}-\beta\text{-D-Gal} \\ \diagdown \text{O}-\beta\text{-D-Gal} \end{array} \end{array} \right]_5 \text{H}$	0.06

^a ICF, concentration of galactosylated THAM monomers necessary to inhibit the yeast flocculation. Results are the average of three independent experiments.

activity of the telomers increases from the mono- to the trigalactosylated derivatives. However, it is noteworthy that, compared to that of the corresponding monoadducts (compounds **15–17**), telomerization decreases the affinity for the lectin. These results could be attributed to an alteration of the molecule hydrophilic–lipophilic balance (HLB) due to telomerization. Enhancement of THAM units or polygalactosylated moieties gives to the macromolecule a higher hydrophilic character which induces a loss of affinity. The clustering effect brought on by increasing galactose moieties does not compensate for the HLB alteration.

Effect of the Polymerization Degree on the Monogalactosylated Telomer Recognition Ability. Previous assays have shown that polygalactosylation of

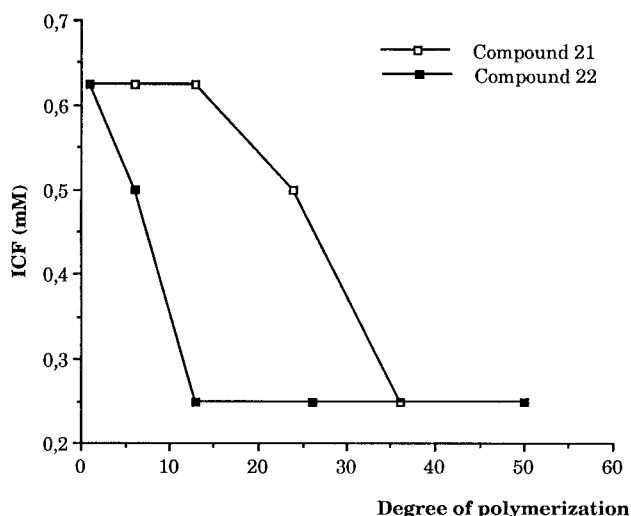


Figure 2. Relation between the degree of polymerization of the monogalactosylated telomers and their recognition ability and comparison of Tris and ethanolamine derivatives.

THAM enhanced the affinity of the derivatives for the Kb CWL1 lectin. The affinity of the molecule for this lectin is also increased in the presence of a hydrophobic moiety.

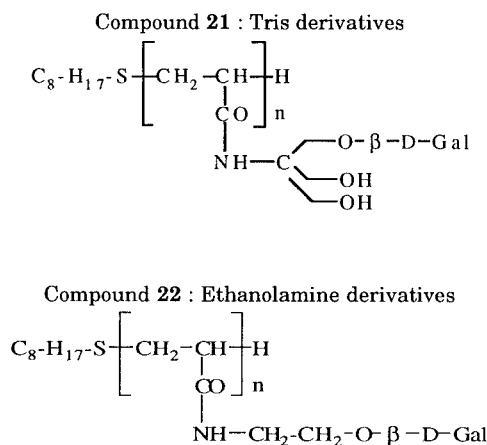
In this assay, we tried to obtain a clustering effect by polymerizing the monogalactosylated adduct. In this case, the sugar is linked to the backbone chain by either a hydrophilic Tris structure (compound **21**) or an ethanolamine moiety (compound **22**). The latter acts as a spacer and a hydrophobic structure.

Results in Figure 2 show that for both polymers, the affinity of the telomers increases with the degree of polymerization to reach an optimum value. It can be supposed that telomerization induces a clustering effect for the lectin recognition as already reported (Lee, 1978; Kornfeld and Kornfeld, 1976). However, if an equal optimum affinity was obtained with both telomers, the way to reach this optimum depends on the structure bearing the galactose unit. The ethanolamine derivatives (compound **22**) reach this optimum with a $\overline{DPn}$ of 12, while Tris derivatives reach this value when $\overline{DPn}$ is 35. These results demonstrate that clustering and hydrophobicity may have synergetic effects on affinity. Several parameters must be considered to give to a macromolecular carrier an optimum recognition efficiency for a given lectin and thus a good cell targeting. Among them, the molecular mass value, the number and kind of carbohydrate moieties, the accessibility to the sugars, and the HLB of the whole macromolecule must be carefully adjusted.

CONCLUSION

Mammalian, vegetal, and microbial lectins are able to recognize as ligands not only free mono- and disaccharides or isolated oligosaccharides but also various natural or synthetic carbohydrate-containing complex compounds. In each case, the lectins can recognize structural differences in the carbohydrate-containing target site. Moreover, the hydrophobic or hydrophilic character of the recognized epitope of the ligands seems to affect their affinity for the lectins.

Such a heterophilic recognition mechanism has been involved in specific drug targeting on cells bearing lectins on their outer surface. Glycoconjugate derivatives containing galactose residues at their nonreducing end are recognized by the lectin of hepatic parenchymatic cells.



Thus, such compounds are considered efficient antimalarial agents and antiviral nucleotide carriers. In a same way, mannose-conjugated derivatives are recognized by a macrophage peripheral lectin. They are efficient carriers of nucleotides against viruses (*Herpes*) or parasites (*Leishmania*).

Taking into account the in vivo biodistribution of THAM telomers (Zarif et al., 1993) and the present results, especially the efficient recognition of glycosylated telomers by lectin, one can consider that these compounds are valuable tools for ensuring in vivo cell targeting and drug delivery. Current studies involving antimetabolic agents such as arabinofuranosylcytosine or 5-fluorouracil substantiate the validity of this approach (Contino et al., 1996).

However, the carrier must take an appropriate structure to produce the best affinity. First, the specific sugars recognized by the cell lectins have to be accessible, and second, a balance between the clustering effect and the whole carrier HLB has to be maintained.

LITERATURE CITED

- Al-Mahmood, S., Colin, S., and Bonaly, R. (1991) *Kluyveromyces bulgaricus* yeast lectins. Isolation of two galactose-specific lectin forms from the yeast cell wall. *J. Biol. Chem.* 266, 20882–20887.
- Attoui, F., Al-Omar, A., Leray, E., Parrot-Lopez, H., Finance, C., and Bonaly, R. (1994) Recognition ability and cytotoxicity of some oligosaccharidyl substituted β -cyclodextrin. *Biol. Cell.* 82, 161–167.
- Contino, C., Ollier, M., Maurizis, J. C., Lacombe, J. M., and Pucci, B. (1996) Synthesis of cotelomers derived from Tris-(hydroxymethyl)acrylamidomethane (THAM) bearing cytosine arabinoside moieties. Preliminary investigation of their biological activity. *Tetrahedron Lett.* 37 (50), 9049–9052.
- Duncan, R., and Kopecek, J. (1984) Synthetic polymers as potential drug carriers. *Adv. Polym. Sci.* 57, 52–101.
- Johnson, B. F., Walker, T., Calleja, G. B., and Seligy, V. L. (1988) Sexual and co-flocculation and asexual self-flocculation in budding and fission yeast: experimental establishment of a fundamental difference. *Can. J. Microbiol.* 34, 1105–1107.
- Kawaguchi, K., Kuhlenschmidt, M., Roseman, S., and Lee, Y. C. (1980) Synthesis of some cluster galactosides and their effect on the hepatic galactose-binding system. *Arch. Biochem. Biophys.* 205, 388–395.
- Kitano, H., Sohda, K., and Kosaka, A. (1995) Galactose-containing amphiphiles prepared with a lipophilic radical initiator. *Bioconjugate Chem.* 6, 131–134.

- Kornfeld, R., and Kornfeld, S. (1976) Comparative aspects of glycoprotein structure. *Annu. Rev. Biochem.* 45, 217–238.
- Lee, Y. C. (1978) Synthesis of some cluster glycosides suitable for attachment to proteins or solid matrixes. *Carbohydr. Res.* 67, 509–514.
- Libudzisz, Z., Mansfeld, B., Kacki, I., and Oberman, H. (1986) Optimisation of the cultivation medium composition for lactic acid bacteria. *Milchwissenschaft* 41, 625–628.
- Maurizis, J. C., Azim, M., Rapp, M., Pucci, B., Pavia, A. A., Madelmont, J. C., and Veyre, A. (1994) Disposition in rat of a new fluorinated, biocompatible, non-ionic telomeric carrier. *Xenobiotica* 6, 535–541.
- Ouchi, T., and Ohya, Y. (1995) Macromolecule prodrugs. *Prog. Polym. Sci.* 20, 211–257.
- Plank, C., Zatloukal, K., Cotten, M., Mechtler, K., and Wagner, E. (1992) Gene transfer into hepatocytes using asialoglycoprotein receptor mediated endocytosis of DNA complexed with an artificial tetra-antennary galactose ligand. *Bioconjugate Chem.* 3, 533–539.
- Polidori, A., Pucci, B., Maurizis, J. C., and Pavia, A. A. (1994) Synthesis of nonionic glycosidic surfactants derived from tri-(hydroxymethyl) aminomethane preliminary assessment. *New J. Chem.* 18, 839–848.
- Pucci, B., Guy, E., Vial-Reveillon, F., and Pavia, A. A. (1988) Télomères et cotélomères d'intérêt biologique et biomédical I. Etude de la télomérisation radicalaire de monomères de type acrylopeptides (Telomers and cotelomers of biological and biomedical interest. I. Study of radical telomerization of acryloyl peptide monomers). *Eur. Polym. J.* 24, 1077–1086.
- Pucci, B., Maurizis, J. C., and Pavia, A. A. (1990) Télomères et cotélomères d'intérêt biologique et biomédical IV. Les télomères du tris(hydroxyméthyl) acrylamidométhane, nouveaux agents amphiphiles non ioniques (Telomers and cotelomers of biological and biomedical interest. IV. Telomers from tris-(hydroxyméthyl)acrylamidométhane. New nonionic amphiphilic agents). *Eur. Polym. J.* 27, 1101–1106.
- Stratford, M. (1992) Yeast flocculation: a new perspective. *Adv. Microb. Physiol.* 33, 1–71.
- Straver, M. H., Traas, V. M., Smit, G., and Kijne, J. W. (1994) Isolation and partial purification of mannose-specific agglutinin from brewer's yeast involved in flocculation. *Yeast* 10, 1183–1193.
- Zarif, L., Riess, J. G., Pucci, B., and Pavia, A. A. (1993) Biocompatibility of alkyl and perfluoroalkyl telomeric surfactants derived from THAM. *Biomater., Artif. Cells, Immobilization Biotechnol.* 21, 597–608.

BC970056R

A Homobifunctional Rhodamine for Labeling Proteins with Defined Orientations of a Fluorophore

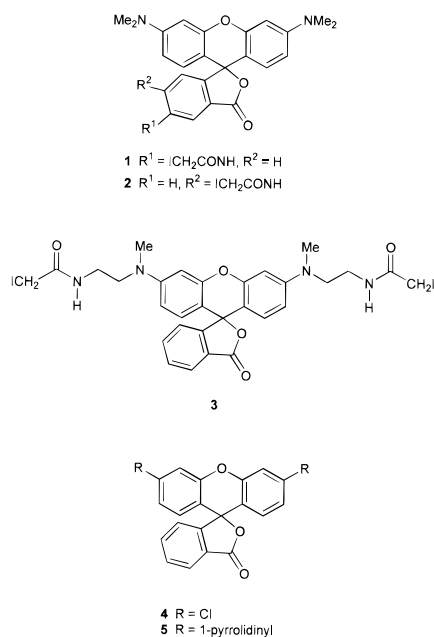
John E. T. Corrie,* James S. Craik, and V. Ranjit N. Munasinghe

National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, U.K. Received September 22, 1997; Revised Manuscript Received December 15, 1997

The synthesis and characterization of a bifunctional rhodamine dye bearing 2-(iodoacetamido)ethyl substituents on the 3'- and 6'-nitrogen atoms is described. Aspects of the conversion of chloroacetamides to iodoacetamides are discussed, including a remarkably mild dehalogenation of an aromatic haloacetamide in the presence of NaI and camphorsulfonic acid. The bifunctional rhodamine has been designed for two-site, 1:1 labeling of proteins that contain two suitably disposed cysteine residues and is intended to constrain the orientation of the rhodamine absorption and emission dipoles in a predictable relationship to the protein structure.

INTRODUCTION

We have previously described (1) the synthesis and characterization of the 5- and 6-isomers of iodoacetamidotetramethylrhodamine **1** and **2**, and these compounds



have been used to label Cys 707 (SH-1) on the myosin heavy chain of rabbit skeletal muscle and Cys 108 on the regulatory light chain of chicken gizzard myosin. Fluorescence polarization measurements with sub-millisecond time resolution on these labeled proteins in skeletal muscle fibers have provided strong evidence that the regulatory domain of myosin changes orientation during the power stroke of muscle contraction (2a) while the catalytic domain does not (2b). The probe on Cys 707 of the catalytic domain gave evidence for coupling between

the actin–myosin and the myosin–nucleotide interaction sites (2b). Despite the success of these biophysical studies, there have been residual uncertainties of interpretation because the orientation of the reporter fluorophore relative to the protein might change during the contractile cycle (3). More importantly, we envisaged that new information would become available if the rhodamine fluorophore could be linked to muscle proteins, particularly to the 20 kDa myosin regulatory light chain (RLC), by two bonds which would constrain the dye to particular orientations fixed by the termini of the two links to the protein. With the probe dipole fixed in the coordinate frame of the RLC, which is itself contained in the ordered filament lattice of muscle cells, it should be possible to measure molecular coordinates in real time in a working muscle cell and thus to bridge the gap between static crystal structures and cellular function.

Other workers have used homo- and heterobifunctional reagents as immobilized or oriented probes, with particular activity in the synthesis and use of EPR probes. Gaffney et al. (4) described a series of spin-labeled α,ω -alkanedioic acids and studied their interaction with lipid bilayers. A membrane-spanning orientation was found only when the total chain length matched the bilayer thickness. A similar strategy has been used to locate a photolabile diazirene moiety deep in a lipid bilayer, thereby providing a reagent with which to identify transmembrane domains of membrane-spanning proteins (5). In a series of papers, Beth and co-workers have used bis(*N*-sulfosuccinimidyl) esters of spin-labeled pimelic and azelaic acids to link near-neighbor lysine groups on the extracellular domains of membrane proteins (6). Two-site linkage was demonstrated, and tight immobilization of the probe relative to the protein was found for both reagents. Heterobifunctional spin-labeled reagents have been described (7) which react first with a thiol group on the protein to provide a single attachment site and are then linked nonspecifically to a second site by photolysis of an aryl azide. Rigidification of the label following formation of the second link was explicitly demonstrated.

Examples of bifunctional fluorescent probes include crabescien, a fluorescein derivative which has been used specifically to label a reduced disulfide bond in immu-

* Address correspondence to Dr. J. E. T. Corrie, National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, U.K. Phone: 011 44 181 959 3666 (extn 2276). Fax: 011 44 181 906 4419. E-mail: j-corrie@nimr.mrc.ac.uk.

noglobulin G (8), and bifunctional cyanine dyes (9) which label ϵ -amino groups of lysine residues. The attachment site of crabscein can be predicted with some accuracy when, as in immunoglobulin G, the site of the most readily reduced disulfide is known, but for the lysine-directed cyanines, there is little opportunity to control the site of labeling. Nevertheless, results with each of these probes confirmed a reduction in independent probe mobility resulting from the two-site attachment (8, 10).

The studies reviewed above indicate that two-site attachment of spectroscopic probes can provide significant information gain. It is particularly important that 1:1 probe-protein stoichiometry can be demonstrated and achieved with reasonable yields. These factors should not be taken for granted, since formation of a very large macrocycle is an implicit feature of the concept. The protein scaffold, which holds the two attachment sites on the protein in a restricted spatial distribution, evidently makes a major contribution to the experiments described above (8, 10).

A key part of our strategy was the possibility that the dye should react with its target protein at chosen sites. We anticipated this would most readily be achieved with a reagent which would alkylate two cysteine residues on the protein, and for our particular application we planned to use the known three-dimensional structures of myosin proteins (11) to select suitable sites at which to introduce these cysteine residues by site-directed mutagenesis. Thus, we hoped to create a set of labeled regulatory light chains which could carry the fluorophore with differing three-dimensional orientations into the ordered actomyosin filament lattice of muscle fibers. The emission dipole of xanthene dyes is coincident with the long axis of the xanthene (12), and we anticipated that fluorescence polarization measurements on these labeled fibers would be able to map the orientations of the dye and hence of their carrier protein in both static and dynamic experimental protocols.

This paper deals with the synthesis of the bis-iodoacetamide **3**, which was chosen with the expectation that the linker arms would substantially constrain the movement of the fluorophore. These linker arms are as short as is practical, consistent with chemical stability and maintaining the rhodamine chromophore. In accordance with our previous convention (1), all rhodamine compounds are drawn and named as their nonfluorescent tautomeric lactone form.

MATERIALS AND METHODS

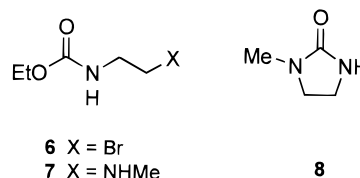
Microanalyses were carried out by the Chemical Analysis Centre (University of Kent, Canterbury, U.K.) or MEDAC Ltd. (Brunel University, Uxbridge, U.K.). Low-resolution electrospray mass spectra were determined on a VG Platform instrument, and high-resolution FAB spectra were determined on a VG ZAB-SE instrument. ^1H NMR spectra were determined in deuteriochloroform on JEOL FX90Q, Bruker WM400, and Varian Unity 500Plus spectrometers with tetramethylsilane as the internal standard unless otherwise stated. J values are given in hertz. The concentration-dependent UV-vis spectra of rhodamine derivatives were recorded on a Cary 3E spectrophotometer.

Buffer solutions were prepared from boric acid or potassium dihydrogen phosphate at the molarities specified and adjusted to the required pH value by addition of solid KOH, followed by 4 M aqueous KOH for final adjustment. Merck 9385 silica gel was used for flash chromatography. Trimethylsilyl polyphosphate was from

Fluka (Gillingham, Dorset, U.K.). Other reagents were from Aldrich (Gillingham, Dorset, U.K.). Petroleum ether was the fraction boiling at 40–60 °C. Procedures involving rhodamines were performed under subdued light. Organic extracts were dried over anhydrous sodium sulfate and evaporated under reduced pressure.

3',6'-Bis(1-pyrrolidiny)spiro[isobenzofuran-1(3H),9'-[9H]xanthen]-3-one (5). A suspension of 3',6'-dichlorospiro[isobenzofuran-1(3H),9'-[9H]xanthen]-3-one **4** (13) (0.074 g, 0.2 mmol), anhydrous ZnCl_2 (0.136 g, 1 mmol), and pyrrolidine (0.142 g, 2 mmol) in dry chlorobenzene (1 mL) was heated at reflux under nitrogen for 4 h and then cooled to room temperature. The reaction mixture was diluted with chloroform (50 mL), and the solution was washed with 2 M aqueous KOH (50 mL) and dried. Silica gel (0.5 g) was added, and the solvent was removed under reduced pressure. The silica gel containing the adsorbed reaction products was added to the top of a flash chromatography column containing silica gel (40 mL), and the column was successively eluted with chloroform (50 mL), methanol-chloroform (1:9 v/v, 100 mL), methanol-chloroform (1:4, 100 mL), and methanol-chloroform (1:3, 200 mL) to afford the rhodamine **5** as a purple glass (0.067 g, 76%): low-resolution electrospray mass spectroscopy (ES-MS) m/e ($M + H$) calcd 439, found 439; ^1H NMR (90 MHz) δ 1.86–2.06 (m, 8H, NCH_2CH_2), 3.17–3.34 (m, 8H, NCH_2CH_2), 6.23 (dd, 2H, $J = 9$ and 2.5, 2',7'-H), 6.34 (d, 2H, $J = 2.5$, 4',5'-H), 6.60 (d, 2H, $J = 9$, 1',8'-H), 7.07–7.20 (m, 1H, 7-H), 7.40–7.63 (m, 2H, 5,6-H), 7.86–8.03 (m, 1H, 4-H). This compound has previously been reported as its hydrochloride salt (**14a**) and characterized by its visible absorption spectrum and elemental analysis.

Ethyl *N*-[(2-Methylamino)ethyl]carbamate (7). Aqueous methylamine (40%, 100 mL) was added to a solution of ethyl *N*-(2-bromoethyl)carbamate **6** (25) (3.9



g, 20 mmol) in methanol (20 mL), and the mixture was stirred for 2 h at room temperature. The solvents were removed under reduced pressure, and the residue was partitioned between 5% aqueous NaHCO_3 (50 mL) and dichloromethane (50 mL). The organic extract was dried and concentrated under reduced pressure to afford the carbamate **7** as a clear liquid (1.9 g, 65%) which was used without further purification as it decomposed on distillation: ^1H NMR (90 MHz) δ 1.23 (t, 3H, $J = 7$, CH_3), 2.40 (s, 3H, NCH_3), 2.69 (t, 2H, $J = 6$, MeNHCH_2), 3.23 (q, 2H, $J = 6$, CONHCH_2), 4.08 (q, 2H, $J = 7$, OCH_2), 5.31 (br s, 2H, $2 \times \text{NH}$). The derived ethyl *N*-[(2-[*N'*-(3,5-dinitrobenzoyl)-*N'*-methylamino]ethyl)carbamate had a melting point of 125–126 °C (EtOAc–petroleum ether). Anal. ($\text{C}_{13}\text{H}_{16}\text{N}_4\text{O}_7$) C, H, N.

3-Methoxy-*N*-methylaniline (10). The preparation of the starting amine was modified from the procedure of Amery and Corbett (16) to give a convenient method with minimal manipulation. Benzenesulfonyl chloride (86 g, 487 mmol) was added over the course of 0.5 h to a mechanically stirred mixture of freshly distilled *m*-anisidine (50 g, 406 mmol) and 2 M aqueous KOH (1100 mL). The mixture was stirred for 1.25 h at room temperature and then heated on a steam bath for 1 h.

The hot solution was filtered, and the filtrate was cooled in an ice bath. Dimethyl sulfate (42.5 mL, 447 mmol; **Caution: potent alkylator**) was added, and the mixture was allowed to warm to room temperature and stirred for 1.5 h, and then kept overnight. Concentrated aqueous ammonia (50 mL) was added, and the mixture was stirred for 0.5 h and then extracted with ether (4 × 150 mL). The ether solution was washed with 2 M aqueous HCl (2 × 100 mL), dried, and evaporated under reduced pressure to give the crude *N*-methylsulfonamide (54 g). This material was refluxed with 6 M aqueous HCl (350 mL) for 7 h, then cooled to room temperature, neutralized by addition of solid KOH, and extracted with ether. The ether extract was dried and evaporated, and the residue was distilled to give the title compound as a pale yellow oil (20.55 g, 37% overall): bp 98 °C at 1.6 mmHg [lit. (17) 131 °C at 17 mmHg]; ¹H NMR (400 MHz) δ 2.76 (s, 3H, NCH₃), 3.74 (s, 3H, OCH₃), 6.13 (t, 1H, *J* = 2.2, 2-H), 6.18–6.27 (m, 2H, 4,6-H), 7.06 (t, 1H, *J* = 8, 5-H). The *N*-acetyl derivative was crystallized from petroleum ether, with a melting point of 64–65 °C [lit. (17) 64 °C].

Ethyl *N*-(3-Methoxyphenyl)-*N*-methylglycinate (11). A solution of the amine **10** (17.0 g, 124 mmol) and ethyl bromoacetate (16.5 mL, 149 mmol) in acetonitrile (175 mL) together with NaHCO₃ (10.9 g, 130 mmol) was stirred and refluxed under nitrogen for 4 h and allowed to cool. Triethylamine (9.8 mL) was added, and the mixture was stirred for 16 h at room temperature and then filtered and evaporated. The residue was dissolved in ether (250 mL), washed with water (3 × 150 mL), dried, evaporated, and distilled to give the ester **11** as a clear liquid (24.0 g, 86%): bp 130–132 °C at 0.6 mmHg; low-resolution ES-MS *m/e* (*M* + *H*) calcd 224, found 224; IR (film) 1745 cm⁻¹; ¹H NMR (400 MHz) δ 1.23 (t, 3H, *J* = 7.15, CH₂CH₃), 3.04 (s, 3H, NCH₃), 3.76 (s, 3H, OCH₃), 4.02 (s, 2H, NCH₂), 4.16 (q, 2H, *J* = 7.15, CH₂CH₃), 6.23 (br s, 1H, 2-H), 6.28–6.32 (m, 2H, 4,6-H), 7.12 (t, 1H, *J* = 8.2, 5-H).

2-[*N*-(3-Methoxyphenyl)-*N*-methylamino]ethanol (12). A suspension of sodium borohydride (7.1 g, 187 mmol) and lithium bromide (16.3 g, 187 mmol) in dry THF (188 mL) was refluxed under nitrogen for 1 h. A solution of the ester **11** (22.0 g, 98.7 mmol) in dry THF was added over the course of 10 min, and the mixture was refluxed for a further 5 h. The solvent was evaporated, and the residue was treated slowly with 1 M aqueous KH₂PO₄ (500 mL) and stirred until hydrogen evolution ceased (ca. 1 h), and then extracted with dichloromethane (5 × 100 mL). The organic extracts were washed with saturated aqueous NaHCO₃ (150 mL), dried, and evaporated. The residue was distilled to give the alcohol **12** as a colorless viscous oil (15.1 g, 84%): bp 127–128 °C at 0.7 mmHg; low-resolution ES-MS *m/e* (*M* + *H*) calcd 182, found 182; IR (film) 3350 cm⁻¹; ¹H NMR (90 MHz) δ 2.40 (br s, 1H, OH), 2.86 (s, 3H, NCH₃), 3.37 (t, 2H, *J* = 5, OCH₂CH₂N), 3.71 (s, 3H, OCH₃) superimposed on 3.70 (t, 2H, *J* = 5, OCH₂CH₂N), 6.17–6.45 (m, 3H, Ar-H), 7.06 (t, 1H, *J* = 8, 5-H). The derived 2-[*N*-(3-methoxyphenyl)-*N*-methylamino]ethyl 3,5-dinitrobenzoate was crystallized from EtOAc–petroleum ether as red needles, with a melting point of 152–153 °C. Anal. (C₁₇H₁₇N₃O₇) C, H, N.

***N*-(2-[*N'*-(3-Methoxyphenyl)-*N'*-methylamino]ethyl)phthalimide (13).** A mixture of the alcohol **12** (7.88 g, 43.5 mmol), triphenylphosphine (12.3 g, 47 mmol), 4 Å molecular sieves (4.0 g), and phthalimide (6.8 g, 46.3 mmol) in dry THF (230 mL) and dry acetonitrile (38 mL) was stirred at –78 °C under nitrogen. Diethyl

azodicarboxylate (8.1 g, 46.6 mmol) was added dropwise over a period of 5 min, and the solution was stirred for 3 h at –78 °C and then allowed to warm to room temperature and stirred overnight. The solvent was evaporated under reduced pressure, and the residue was dissolved in 1 M aqueous sulfuric acid (100 mL) and washed with ether (200 mL). The organic layer was back-extracted with 1 M aqueous sulfuric acid (5 × 50 mL), and the combined aqueous solution was stirred vigorously and basified to pH 8.5 by slow addition of 2 M aqueous KOH and solid NaHCO₃. The resulting suspension was extracted with ether (4 × 100 mL), dried, and evaporated. The residue was filtered through a short column of silica gel (flash chromatography grade) in EtOAc–petroleum ether (1:4), and the eluate was evaporated and triturated with diisopropyl ether to give the phthalimide **13** (8.37 g, 62%) as yellow needles: mp 81 °C from diisopropyl ether; IR (Nujol) 1770, 1705 cm⁻¹; ¹H NMR (400 MHz) δ 2.98 (s, 3H, NCH₃), 3.60 (t, 2H, *J* = 7, CH₂N), 3.76 (s, 3H, OCH₃), 3.88 (t, 2H, *J* = 7, CH₂N), 6.15 [dd, 1H, *J* = 8 and 1.9, 4(6)-H], 6.33 (t, 1H, *J* = 1.9, 2-H), 6.37 [dd, 1H, *J* = 8 and 1.9, 6(4)-H], 7.03 (t, 1H, *J* = 8, 5-H), 7.64–7.69 (m, 2H, phthaloyl 4- and 5-H), 7.76–7.80 (m, 2H, phthaloyl 3- and 6-H). Anal. (C₁₈H₁₈N₂O₃) C, H, N.

***N*-(2-[*N'*-(3-Acetoxyphenyl)-*N'*-methylamino]ethyl)-acetamide (14).** A solution of the phthalimide **13** (11.2 g, 36.1 mmol) in concentrated aqueous hydrobromic acid (325 mL) was refluxed under nitrogen for 2 h and allowed to cool. The solution was evaporated to dryness in vacuo (dry ice condenser), and the crude amine hydrobromide was dissolved in 2 M aqueous potassium borate (pH 9.3, 360 mL) under nitrogen and cooled in an ice bath. A solution of acetic anhydride (27 mL, 286 mmol) in dichloromethane (180 mL) was added over the course of 20 min with vigorous stirring. The aqueous phase was maintained at pH 9.0–9.5 by addition of KOH pellets as required, and after 15 min, a further portion of acetic anhydride (9 mL) in dichloromethane (18 mL) was added. The solution was stirred for 2 h with ice cooling, while the pH was maintained as described above, and then allowed to warm to room temperature and treated with a final portion of acetic anhydride (4 mL) in dichloromethane (10 mL). After a further period of 0.5 h at pH 9–9.5, the organic phase was separated and the aqueous phase was extracted with dichloromethane (3 × 100 mL). The combined organic extracts were washed with saturated aqueous sodium hydrogen carbonate (2 × 100 mL), dried, and concentrated to give a pale gum which solidified upon trituration with ether and was recrystallized from EtOAc–petroleum ether to afford the acetamide **14** (6.65 g, 74%) as clear slabs: mp 87 °C; UV (EtOH) nm (ε in M⁻¹cm⁻¹) 256 (17 700), 310 (3100); IR (Nujol) 3270, 1740, 1630 cm⁻¹; ¹H NMR (400 MHz) δ 1.91 (s, 3H, NCOCH₃), 2.29 (s, 3H, OCOCH₃), 2.93 (s, 3H, NCH₃), 3.42–3.47 (m, 4H, CH₂CH₂), 5.74 (br s, 1H, NH), 6.41–6.43 (m, 2H, Ar-H), 6.60 (1H, dd, *J* = 8.5 and 2, Ar-H), 7.23 (t, 1H, *J* = 8.5, 5-H). Anal. (C₁₃H₁₈N₂O₃) C, H, N.

2-[4'-[*N*-(2-Acetamidoethyl)-*N*-methylamino]-2'-hydroxybenzoyl]benzoic Acid (15). The acetamide **14** (1.00 g, 4 mmol) was dissolved in methanol (10 mL) containing KOH (0.5 g) and stirred at room temperature under nitrogen for 25 min. The solvent was removed under reduced pressure, and the residue was mixed with 2 M aqueous potassium phosphate (pH 8.5, 50 mL) and extracted with dichloromethane (4 × 35 mL). The combined organic extracts were dried and evaporated, and the residual gum was azeotroped with dry toluene (3 × 5 mL) to remove traces of water to leave the crude

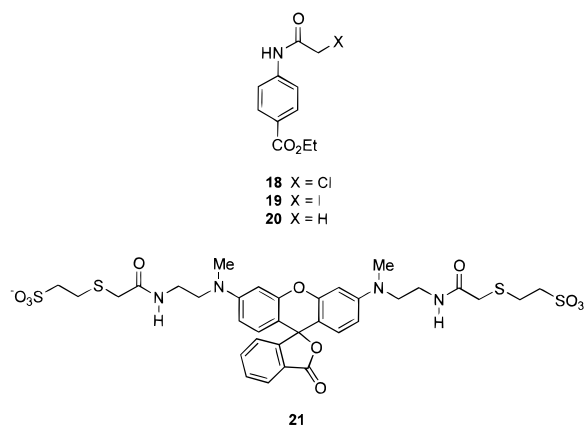
phenol **9** (0.83 g), which was dissolved in dry dichloromethane (5 mL) and transferred to a glass tube (150 × 16 mm). The solvent was removed under a current of nitrogen and finally by evacuation (~1 mmHg). Freshly sublimed phthalic anhydride (0.65 g, 4.4 mmol) was added, and the tube was flushed with nitrogen, sealed, and then heated at 135 °C for 4 h. During the first 1 h of heating, the tube was inverted each 15 min to ensure mixing of its contents. After cooling to room temperature, the contents of the tube were dissolved in a mixture of hot ethanol (200 mL) and ethylenediamine (2 mL) to quench excess phthalic anhydride. The solution was concentrated to ~5 mL, diluted with EtOAc (200 mL), and washed with 2 M hydrochloric acid (2 × 70 mL). The aqueous washings were back-extracted with EtOAc (2 × 50 mL), and the combined organic layers were extracted with 10% aqueous NaHCO₃ (5 × 50 mL). The combined aqueous extracts were acidified (pH < 2) with 6 M hydrochloric acid, saturated with NaCl, and extracted with EtOAc (4 × 100 mL). The organic extract was washed with saturated brine (30 mL), dried, and evaporated to leave the benzoylbenzoic acid **15** as a gum which solidified on trituration with ether. The pale orange solid (0.74 g) resisted crystallization and was used without further purification: high-resolution FAB mass spectroscopy (FAB-MS) *m/e* (M + H) calcd 357.1440, found 357.1450; IR (CH₃CN) 1725, 1670, 1625 cm⁻¹; ¹H NMR (400 MHz, [²H₆]DMSO, TMS as reference) δ 1.76 (s, 3H, COCH₃), 2.98 (s, 3H, NCH₃), 3.19 (q, 2H, *J* = 6, CH₂-NH), 3.39 [t, 2H, *J* = 6, ArN(Me)CH₂], 6.16 (d, 1H, *J* = 2.2, 3'-H), 6.25 (dd, 1H, *J* = 8.8 and 2.2, 5'-H), 6.80 (d, 1H, *J* = 8.8, 6'-H), 7.36 (d, 1H, *J* = 7.4, 3-H), 7.60 [t, 1H, *J* = 7.4, 4(5)-H], 7.67 [t, 1H, *J* = 7.4, 5(4)-H], 7.94–7.97 (m, 2H, 6-H and NH), 12.55 (br s, 1H, ArOH).

3',6'-Bis[*N*-(2-acetamidoethyl)-*N*-methylamino]spiro[isobenzofuran-1(3*H*),9'-[9*H*]xanthen]-3-one (16**).** The acetamide **14** (1.25 g, 5.1 mmol) was hydrolyzed as described above (compound **15**), and the crude phenol **9** was mixed with the acid **15** (0.63 g) and a solution of trimethylsilyl polyphosphate (1.54 g) in dry dimethylformamide (31 mL). The mixture was heated at 130 °C under nitrogen for 4 h, cooled, and evaporated (~1 mmHg) to ~1 mL; 1 M aqueous NaOH (50 mL) was added, and the mixture was stirred at room temperature under nitrogen for 16 h. The aqueous solution was saturated with NaCl and extracted with chloroform (5 × 70 mL). The organic extract was dried and evaporated, and the residue dissolved in CHCl₃-MeOH (9:1, 2 mL) was applied to a column of silica gel (flash chromatography grade, 50 mL). The column was successively eluted with CHCl₃-MeOH (9:1, 250 mL), CHCl₃-MeOH (3:1, 300 mL), and CHCl₃-MeOH (1:1, 600 mL) to give the bis-acetamide **16** as a dark purple glass (0.56 g, 60%): low-resolution ES-MS *m/e* (M + H) calcd 529, found 529; ¹H NMR (500 MHz) δ 1.92 (s, 6H, COCH₃), 2.95 (s, 6H, NCH₃), 3.37–3.47 (m, 8H, CH₂CH₂), 6.28 (t, 2H, *J* = 7, NH), 6.40 (dd, 2H, *J* = 9.0 and 2.6, 2',7'-H), 6.52 (d, 2H, *J* = 2.6, 4',5'-H), 6.59 (d, 2H, *J* = 9.0, 1',8'-H), 7.15 (d, 1H, *J* = 7.5, 7-H), 7.58 (t, 1H, *J* = 7.5, 5-H), 7.63 (t, 1H, *J* = 7.5, 6-H), 8.00 (d, 1H, *J* = 7.5, 4-H).

3',6'-Bis[*N*-(2-chloroacetamido)ethyl]-*N*-methylamino]spiro[isobenzofuran-1(3*H*),9'-[9*H*]xanthen]-3-one (17**).** The bis-acetamide **16** (0.264 g, 0.5 mmol) was refluxed for 8 h with 1.5 M hydrochloric acid (18 mL) and then evaporated to dryness (~1 mmHg) and azeotroped with toluene (3 × 5 mL). The residual solid was cooled in an ice bath and mixed under nitrogen with chloroacetyl chloride (0.16 mL, 2 mmol) and diisopropylethylamine (0.7 mL, 4 mmol) in dry dichloromethane (17

mL), and the solution was stirred in the ice bath for 2 h and then at room temperature for 1 h. A mixture of chloroform and methanol (9:1, 400 mL) was added, and the solution was washed with 0.5 M hydrochloric acid (15 mL). The aqueous layer was back-extracted with CHCl₃-MeOH (9:1, 2 × 10 mL), and the combined organic layers were washed with 10% aqueous NaHCO₃ and brine, dried, and evaporated. The residue was dissolved in CHCl₃-MeOH (9:1, 2 mL) and applied to a flash chromatography column (50 mL silica gel), which was successively eluted with CHCl₃-MeOH (9:1, 200 mL), CHCl₃-MeOH (4:1, 200 mL), and CHCl₃-MeOH (65:35, 600 mL) to afford the bis-chloroacetamide **17** as a purple glass (0.149 g, 50%): low-resolution ES-MS *m/e* (M + H) calcd 597, found 597; ¹H NMR (400 MHz, 9:1 CDCl₃-CD₃OD) δ 3.06 (s, 6H, NCH₃), 3.49 (t, 4H, *J* = 6, CH₂), 3.58 (t, 4H, *J* = 6, CH₂), 3.96 (s, 4H, CH₂Cl), 6.56 (dd, 2H, *J* = 9 and 2.5, 2',7'-H), 6.62 (d, 2H, *J* = 2.5, 4',5'-H), 6.77 (d, 2H, *J* = 9, 1',8'-H), 7.15–7.17 (m, 1H, 7-H), 7.60–7.66 (m, 2H, 5,6-H), 8.03–8.05 (m, 1H, 4-H).

Reaction of Ethyl 4-[*N*-(Chloroacetyl)amino]benzoate (18**) with NaI-Acetone.** (a) The chloroacetamide **18** (**18**) (0.12 g, 0.5 mmol) was dissolved in a solution of



NaI (4.1 g) in acetone (27.5 mL) under nitrogen, and the solution was kept overnight in the dark at room temperature and then diluted with EtOAc (50 mL) and washed with 5% aqueous sodium ascorbate (20 mL) and water. The organic phase was dried and evaporated to give the iodoacetamide **19** (0.22 g, 65%): mp 158 °C; δ_H 1.36 (t, 3H, *J* = 7, CH₂CH₃), 3.84 (s, 2H, CH₂I), 4.35 (q, 2H, *J* = 7, CH₂CH₃), 7.48–7.74 (m, 2H, ArH), 7.90–8.12 (m, 2H, ArH), 8.22 (br s, 1H, NH). Anal. (C₁₁H₁₂INO₃) C, H, N.

(b) The reaction was performed as in part (a) above, but with camphorsulfonic acid (0.125 g, 0.5 mmol) added to the solution at the start of the reaction. Workup as described above gave the acetamide **20** (0.058 g, 57%): mp 103 °C (benzene-cyclohexane) [lit. (19) 104 °C]. No other product was detected in the crude reaction product by ¹H NMR or TLC analysis.

3',6'-Bis[*N*-(2-(iodoacetamido)ethyl)-*N*-methylamino]spiro[isobenzofuran-1(3*H*),9'-[9*H*]xanthen]-3-one (3**).** Sodium iodide (6.6 g, 44 mmol) was dissolved in a mixture of dry acetone, methanol, and chloroform (65:25:10 v/v, 44 mL), and a stream of nitrogen was passed through the solution for 10 min. This solution was added to the bis-chloroacetamide (0.12 g, 0.20 mmol), and the flask was flushed with nitrogen and kept in the dark at room temperature for 3 days. The reaction mixture was diluted with chloroform (400 mL), washed with 5% aqueous sodium ascorbate (150 mL), and water (3 × 30 mL), and dried. The yield of recovered rhodamine

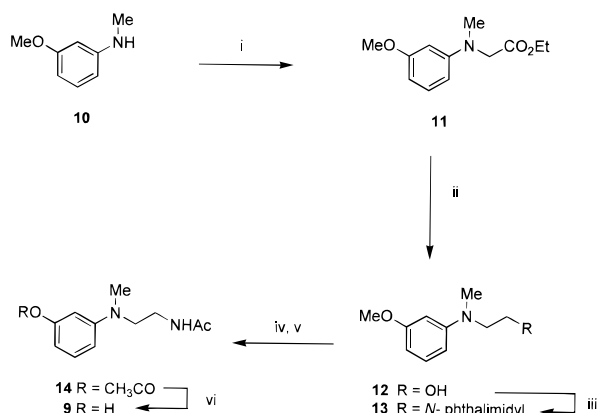
chromophore was 57%, determined by quantitative visible spectroscopy (10% aqueous EtOH) based on an ϵ_{547} of $89\,250\text{ M}^{-1}\text{ cm}^{-1}$ (see Results and Discussion). The chloroform solution was evaporated to give the bis-iodoacetamide **3** as a purple glass: high-resolution FAB-MS m/e ($M + H$) calcd 781.0384, found 781.0360; $^1\text{H NMR}$ (400 MHz, 9:1 $\text{CDCl}_3\text{--CD}_3\text{OD}$) δ 3.10 (s, 6H, NCH_3), 3.39 (t, 4H, $J = 6.5$, CH_2), 3.54 (t, 4H, $J = 6.5$, CH_2), 3.59 (s, 4H, ICH_2), 6.68–6.71 (m, 4H, 2',4',5',7'-H), 6.98 (d, 2H, $J = 9.7$, 1',8'-H), 7.12 (dd, 1H, $J = 7.2$ and 1.4, 7-H), 7.56–7.64 (m, 2H, 5,6-H) and 8.12 (dd, 1H, $J = 7.1$ and 1.4, 4-H). The product was dissolved in dry DMF and stored in aliquots (10–20 mM) at -70°C .

Concentration Dependence of Rhodamine Spectra. An aliquot (0.02 mL) of a solution of the bis-iodoacetamidorhodamine **3** (ca. 20 mM in DMF) was mixed with 200 mM aqueous sodium 2-mercaptoethanesulfonate (0.08 mL) and 200 mM potassium phosphate (pH 7.2, 0.35 mL) and allowed to stand at room temperature for 24 h. Aliquots of this solution were diluted with 25 mM potassium phosphate (pH 7.2) which contained 150 mM NaCl to give solutions of differing concentrations over the range of 5–500 μM . The UV–vis spectra were recorded using quartz cells with path lengths of 10, 1, or 0.105 mm as appropriate to the concentrations. Spectra were corrected for path length and dilution factors and plotted on the basis of an assumed extinction coefficient at the isosbestic point as outlined in Results and Discussion.

RESULTS AND DISCUSSION

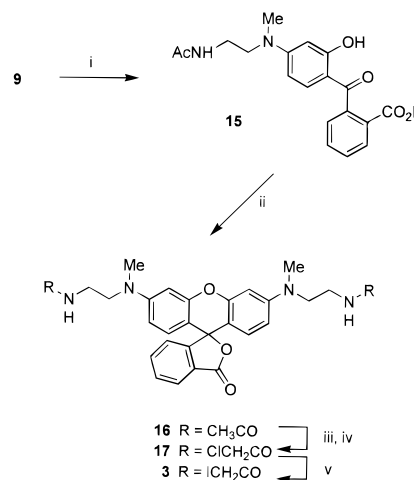
Our initial plan for synthesis of the target compound was to perform a 2-fold displacement on the dichlorofluoran **4** with an unsymmetrical derivative of ethylenediamine. Many such displacements on dichlorofluoran or the related sulfofluoran have been described for simple amines (14). In a brief study with pyrrolidine as a model amine, chlorobenzene was found to be a particularly effective solvent, and the rhodamine **5** was readily obtained. To obtain an amine reagent appropriate to our purpose, we examined displacements on a range of *N*-acyl derivatives of 2-bromoethylamine, which were often complicated by cyclization to oxazolines. We eventually found that the *N*-ethoxycarbonyl compound **6** could be efficiently converted to the secondary amine **7** by treatment with methylamine in aqueous methanol. However, treatment of dichlorofluoran **4** with amine **7** under a variety of conditions gave a poor yield of rhodamine, probably because the amine cyclized rapidly to the imidazolidinone **8**. Rather than pursue this approach, we turned instead to a conventional rhodamine synthesis, i.e., condensation of a suitable *N*-substituted *m*-aminophenol **9** with phthalic anhydride. The synthesis of compound **10** (Scheme 1) began from *N*-methyl-*m*-anisidine **10**, preparation of which was initially approached from *m*-anisidine by treatment with triethyl orthoformate under reflux to give the formimidate, followed by reduction with $\text{NaBH}_4\text{--THF}$ (20). However, the product was consistently contaminated by ~15% of the starting *m*-anisidine, and we fell back upon the less convenient methylation of *N*-(3-methoxyphenyl)benzenesulfonamide (16), which gave the required product **10** without contamination. This material was cleanly alkylated by ethyl bromoacetate to give the ester **11**, which was smoothly reduced to the alcohol **12** with $\text{NaBH}_4\text{--LiBr}$ in tetrahydrofuran (21). Treatment of the alcohol **12** with phthalimide under Mitsunobu conditions (22) gave a satisfactory yield of the substituted phthalimide **13** as bright yellow crystals. Stacking between a phthalimide and an

Scheme 1^a



^a (i) $\text{BrCH}_2\text{CO}_2\text{Et--NaHCO}_3$, (ii) $\text{NaBH}_4\text{--LiBr--THF}$, (iii) $\text{EtO}_2\text{CN=NCO}_2\text{Et--Ph}_3\text{P--phthalimide}$, (iv) concentrated aqueous HBr , (v) Ac_2O , and (vi) KOH--MeOH .

Scheme 2^a



^a (i) phthalic anhydride, (ii) **9** and TMS polyphosphate–DMF, (iii) aqueous HCl , (iv) $\text{ClCH}_2\text{COCl--DIPEA}$, and (v) $\text{NaI--acetone--CHCl}_3\text{--MeOH}$.

adjacent electron-rich aromatic system leading to a presumed charge-transfer complex has been reported previously (23). The methoxy and phthaloyl protecting groups were simultaneously removed from **13** with concentrated hydrobromic acid under reflux, and acetylation of the crude product gave the *O,N*-diacetyl derivative **14**. Although acetylation of the phenolic hydroxyl added an additional step to the synthesis, it was beneficial since it allowed isolation and storage of a stable, crystalline precursor from which the air-sensitive aminophenol **9** could be prepared as required by brief alkaline hydrolysis. An alternative sequence for synthesis of **14** in which a methoxymethyl group was used to protect the phenolic oxygen was also explored. However, the sensitivity of this group to cleavage under acidic conditions meant that the route was less satisfactory than the one described above, and it is not discussed further.

With the phenol **9** available, we turned to assembly of the rhodamine system via the two-stage process shown (Scheme 2) and were surprised to find that reaction of **9** with phthalic anhydride to give the benzoylbenzoic acid **15** was capricious. Although simple *m*-dialkylaminophenols react readily with phthalic anhydrides in toluene under reflux (1, 24), phenol **9** was poorly soluble under these conditions and failed to react. In more polar

solvents (e.g., chlorobenzene or anisole) in which simple *m*-dialkylaminophenols still reacted readily with phthalic anhydride and in which phenol **9** was soluble, the latter compound was nevertheless still unreactive. Dimethylformamide as solvent, with or without added trimethylsilyl polyphosphate (**1**), completely prevented the reaction of phthalic anhydride with all *m*-dialkylaminophenols tested. However, when **9** was heated with phthalic anhydride in the absence of solvent, the required acid **15** was obtained in reasonable yield. The origin of this poor reactivity remains obscure.

Despite the difficulties encountered above, condensation of the benzoylbenzoic acid **15** with a second molecule of aminophenol **9** proceeded smoothly in a DMF solution with trimethylsilyl polyphosphate catalysis (**1**) to give the bis-acetamidorhodamine **16**. In our previous work on monofunctional rhodamines (**1**), the rhodamine was isolated in rather poor yield after brief treatment of the reaction mixture with aqueous sodium hydroxide, which broke down a complex of the dye with the polyphosphate. We have now found that much longer exposure to alkali (see Materials and Methods) is necessary to maximize the product recovery. Acidic hydrolysis of the acetamido groups of **16**, followed by treatment of the crude product with chloroacetyl chloride–diisopropylethylamine, gave the bis-chloroacetamide **17**. Completion of the synthesis of compound **3** required only exchange of the chloro groups on **17** for iodides. The fact that the exchange was complete was thought to be important since the half-exchanged (i.e., mono-chloro and mono-iodo) intermediate would be expected to cause difficulties during the eventual use of the reagent to effect 1:1 labeling of a bis-thiol protein. This simple transformation had been easily achieved in synthesis of the isomeric iodoacetamides **1** and **2**, and we were therefore surprised to encounter considerable difficulties. Treatment of **17** with NaI–acetone gave the expected Cl → I exchange (assessed by an upfield shift of the halomethyl proton signal in the ¹H NMR spectrum), but the product was contaminated with material which appeared to be oligomeric from a number of broadened peaks in the ¹H NMR spectrum. In addition, intense singlet resonances at δ 1.18 (6H), 2.17 (3H), and 2.64 (2H) were consistent with the presence of diacetone alcohol. The latter product was not formed when a solution of NaI alone in acetone was kept for a comparable period of time, and we assume that the displaced chloride ion, acting as a base, is responsible for formation of the aldol product. This reasoning led us to believe that the chloride ion might also be responsible for formation of the apparent rhodamine oligomer(s), and we performed a series of experiments which aimed to combat the basicity of chloride ion under the aprotic conditions of the reaction. We initially used the chloroacetamide **18** as a model compound and showed that treatment with NaI–acetone at room temperature cleanly gave the expected iodoacetamide **19**, although again with contaminating diacetone alcohol. When the reaction was repeated with 1:1 chloroacetamide–camphorsulfonic acid (CSA) to neutralize the released chloride, the dehalogenated acetamide **20** was the sole product, uncontaminated by diacetone alcohol. We are not aware of previous reports of similar dehalogenation of haloacetamides, but α-haloketones are known to undergo clean reductive dehalogenation with NaI in the presence of dilute sulfuric acid (25). When the amount of CSA was progressively reduced, the proportion of the reduction product **20** decreased but remained detectable at 0.005 equiv of CSA, which was still sufficient to suppress formation of diacetone alcohol. When acetic

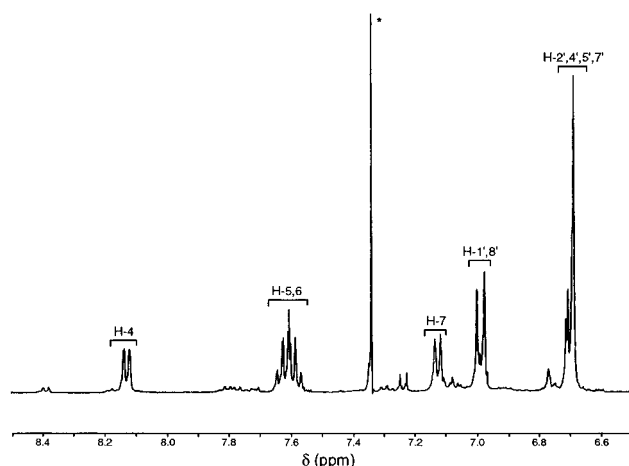


Figure 1. Partial ¹H NMR spectrum (400 MHz) of the bis-iodoacetamidorhodamine **3**. The peak marked * is from residual CHCl₃.

acid was used in place of CSA, dehalogenation was much diminished but still detectable and no diacetone alcohol was formed. These conditions were also investigated using *N*-benzylchloroacetamide, where the aliphatic amino group is more similar to those in the rhodamine **17**. With either CSA (0.1 equiv) or acetic acid, only a very low degree of dehalogenation was detectable. Nevertheless, it was undesirable to risk any dehalogenation in the rhodamine product **3**, and we abandoned these experiments. A protocol for the Cl → I exchange which minimized the formation of the apparent oligomeric material was established empirically and used a mixture of chloroform–acetone–methanol that ensured complete solubility of the rhodamine together with full exchange. The aromatic region of the ¹H NMR spectrum of typical material so obtained is shown in Figure 1 to illustrate the extent to which impurities contaminate the final product **3**. The reasons underlying the difficulties encountered in this reaction remain unclear.

Use of the bifunctional rhodamine **3** as a labeling reagent requires some information about its visible absorption spectrum for aiding quantification of labeled proteins. Rhodamine dyes are known to undergo a noncovalent concentration-dependent dimerization (26), and this property, together with the noncrystalline nature of **3**, meant that determination of an extinction coefficient was not straightforward. In our previous work (**1**) on monofunctional rhodamines, we used a radiolabeled derivative with a known specific activity to determine the extinction coefficient and, in conjunction with spectra of the products formed from each dye by displacement of the iodo group with 2-mercaptoethanesulfonate, showed that the spectra had an isosbestic point at 528 nm, where the extinction coefficient was 52 000 M⁻¹ cm⁻¹. Corresponding spectra from the aqueous solution of the species **21** formed by reaction of **3** with excess 2-mercaptoethanesulfonate were markedly less concentration-dependent than those for the derivatives of the monofunctional tetramethylrhodamines, with the extinction coefficient (547 nm) measured at 500 μM being only 6% lower than the value measured at 5 μM. As for the monofunctional rhodamines, the spectra show an isosbestic point at 528 nm, and we made the assumption that the extinction coefficient at this point was the same as that for the tetramethylrhodamines. On the basis of this assumption, the monomer spectrum of **21** has an extinction coefficient of 89 250 M⁻¹ cm⁻¹ at its maximum of 547 nm. This maximum is blue-shifted by only 2 nm from

that of the tetramethylrhodamines, while the extinction coefficient at the maximum is 92% of the value found for the tetramethylrhodamine monomers. In view of these rather small perturbations and the very similar overall appearance of the monomer spectrum for the different rhodamines, the assumptions made seem reasonable, and hence, the extinction coefficient data appear to be reliable estimates. The diminished tendency of the derivative **21** to dimerize probably reflects the presence of the extended, flexible side chains, which could be expected to interfere with face-to-face stacking of the xanthene rings.

In conclusion, we have synthesized a new rhodamine derivative suitable for two-site linkage to proteins. Data now at hand indicate that the desired 1:1 labeling of mutant myosin regulatory light chain proteins containing two cysteine residues at various positions can be achieved with acceptable yields. These results and the consequent fluorescence polarization studies have been presented as conference proceedings (27) and will be described in detail elsewhere.

ACKNOWLEDGMENT

We are grateful to the MRC Biomedical NMR Centre for access to facilities and to Dr. M. J. Gradwell for assistance with NMR spectroscopy. We thank Drs. S. Howell and K. J. Welham for mass spectroscopic determinations at low and high resolution, respectively, and Drs. Y. E. Goldman, M. Irving, and D. R. Trentham for helpful discussions.

LITERATURE CITED

- (1) Corrie, J. E. T., and Craik, J. S. (1994) Synthesis and characterisation of pure isomers of iodoacetamidotetramethylrhodamine. *J. Chem. Soc., Perkin Trans. 1*, 2967–2973.
- (2) (a) Irving, M., Allen, T. St. C., Sabido-David, C., Craik, J. S., Brandmeier, B., Kendrick-Jones, J., Corrie, J. E. T., Trentham, D. R., and Goldman, Y. E. (1995) Tilting of the light-chain region of myosin during step length changes and active force generation in skeletal muscle. *Nature* 375, 688–691. (b) Berger, C. L., Craik, J. S., Trentham, D. R., Corrie, J. E. T., and Goldman, Y. E. (1996) Fluorescence polarization of skeletal muscle fibers labeled with rhodamine isomers on the myosin heavy chain. *Biophys. J.* 71, 3330–3343.
- (3) Huxley, A. F. (1995) Crossbridge tilting confirmed. *Nature* 375, 631–632.
- (4) Gaffney, B. J., Willingham, G. L., and Schepp, R. S. (1983) Synthesis and membrane interactions of spin-label bifunctional reagents. *Biochemistry* 22, 881–892.
- (5) Delfino, J. M., Schreiber, S. L., and Richards, F. M. (1993) Design, synthesis and properties of a photoactivatable membrane-spanning phospholipidic probe. *J. Am. Chem. Soc.* 115, 3458–3474.
- (6) (a) Beth, A. H., Conturo, T. E., Venkataramu, S. D., and Staros, J. V. (1986) Dynamics and interactions of the anion channel in intact human erythrocytes: an electron paramagnetic resonance spectroscopic study employing a new membrane-impermeant bifunctional spin-label. *Biochemistry* 25, 3824–3832. (b) Anjaneyulu, P. S. R., Beth, A. H., Sweetman, B. J., Faulkner, L. A., and Staros, J. V. (1988) Bis(sulfo-*N*-succinimidyl) [¹⁵N,²H₁₆]doxyl-2-spiro-4'-pimelate, a stable isotope-substituted, membrane-impermeant bifunctional spin-label for studies of the dynamics of membrane proteins: application to the anion-exchange channel in intact human erythrocytes. *Biochemistry* 27, 6844–6851. (c) Anjaneyulu, P. S. R., Beth, A. H., Cobb, C. E., Juliao, S. F., Sweetman, B. J., and Staros, J. V. (1989) Bis(sulfo-*N*-succinimidyl) doxyl-2-spiro-5'-azelaate: synthesis, characterization and reaction with the anion-exchange channel in intact human erythrocytes. *Biochemistry* 28, 6583–6590.
- (7) (a) Keana, J. F. W., Hideg, K., Birrell, G. R., Hankovsky, O. H., Ferguson, G., and Parvez, M. (1982) New mono- and difunctionalized 2,2,5,5-tetramethylpyrrolidine- and Δ^3 -pyrroline-1-oxyl nitroxide spin labels. *Can. J. Chem.* 60, 1439–1447. (b) Wilcox, M. D., Parce, J. W., Thomas, M. J., and Lyles, D. S. (1990) A new bifunctional spin-label suitable for saturation-transfer EPR studies of protein rotational motion. *Biochemistry* 29, 5734–5743.
- (8) Packard, B., Edeidin, M., and Komoriya, A. (1986) Site-directed labeling of a monoclonal antibody: targeting to a disulfide bond. *Biochemistry* 25, 3548–3552.
- (9) Mujumdar, R. B., Ernst, L. A., Mujumdar, S. R., Lewis, C. J., and Waggoner, A. S. (1993) Cyanine dye labeling reagents: sulfoindocyanine succinimidyl esters. *Bioconjugate Chem.* 4, 105–111.
- (10) Timbs, M. M., and Thompson, N. L. (1990) Slow rotational mobilities of antibodies and lipids associated with substrate-supported phospholipid monolayers as measured by polarized fluorescence photobleaching recovery. *Biophys. J.* 58, 413–428.
- (11) (a) Xie, X., Harrison, D. H., Schlichting, I., Sweet, R. M., Kalabokis, V. N., Szent-Györgyi, A. G., and Cohen, C. (1994) Structure of the regulatory domain of scallop myosin at 2.8 Å resolution. *Nature* 368, 306–312. (b) Rayment, I., Rypniewski, W. R., Schmidt-Base, K., Smith, R., Tomchick, D. R., Benning, M. M., Winkelman, D. A., Wesenberg, G., and Holden, H. M. (1993) *Science* 261, 50–58.
- (12) van der Heide, U. A., Orbons, B., Gerdtisen, H. C., and Levine, Y. K. (1992) The orientation of transition moments of dye molecules used in fluorescence studies of muscle systems. *Eur. Biophys. J.* 21, 263–272.
- (13) Hurd, C. D., and Schmerling, L. (1937) Alkenyl derivatives of fluorescein. *J. Am. Chem. Soc.* 59, 112–117.
- (14) (a) Ioffe, I. S., Gofman, I. A., and Zalmanovich, M. Z. (1965) Rhodamine dyes and related compounds. XV. Rhodamine dyes with hydroaromatic and polymethylene radicals. *Zh. Org. Khim.* 1, 584–586. (b) Locatelli, L., Cieciuch, R. F., and Zepp, C. M. (1981) Novel xanthene compounds. British Patent GB 2 067 215; (1982) *Chem. Abstr.* 96, 70436. (c) Raue, R., and Wunderlich, K. (1983) Reactive diarylaminoxanthene dyes. German Patent DE 3 347 246; (1983) *Chem. Abstr.* 103, 197374. (d) Werner, T., He, H., Kessler, M. A., and Wolfbeis, O. S. (1992) New lipophilic rhodamines and their application to optical potassium sensing. *J. Fluoresc.* 2, 93–98.
- (15) Katchalski, E., and Ishai, D. B. (1950) 2-Oxazolidinones: synthesis from *N*-carbalkoxy- β -haloalkylamines. *J. Org. Chem.* 15, 1067–1073.
- (16) Amery, G. F., and Corbett, J. F. (1967) The synthesis and identification of some *N*-methylated aminonitrophenols. *J. Chem. Soc. C*, 1053–1057.
- (17) Millson, M. F., and Robinson, R. (1955) Methylated hexahydro-9:11-dimethylcarbazoles. *J. Chem. Soc.*, 3362–3371.
- (18) Epstein, E., and Kaminsky, D. (1957) Alkyl and diethylaminoethyl esters of *N*-substituted aminoacylamino benzoic acids. *J. Am. Chem. Soc.* 79, 5814–5817.
- (19) Foster, H. E., and Hurst, J. (1973) Pyrazolopyridines. Part II. Preparation of some 3-substituted 2-aryl-2H-pyrazolo[4,3-*b*]pyridines. Acid catalysed cyclisation of 2-arylaminoethyl-3-nitropyridines. *J. Chem. Soc., Perkin Trans. 1*, 319–324.
- (20) Crochet, R. A., and Blanton, C. D. (1974) *N*-Monoalkylation of primary aromatic and heteroaromatic amines with trialkyl orthoformates and sodium borohydride. *Synthesis*, 55–56.
- (21) (a) Brown, H. C., Narasimhan, S., and Choi, Y. M. (1982) Selective reductions. 30. Effect of cation and solvent on the reactivity of saline borohydrides for reduction of carboxylic esters—improved procedures for the conversion of esters to alcohols by metal borohydrides. *J. Org. Chem.* 47, 4702–4708. (b) Brown, H. C., Choi, Y. M., and Narasimhan, S. (1981) Convenient procedure for the conversion of sodium borohydride into lithium borohydride in simple ether solvents. *Inorg. Chem.* 20, 4454–4456.
- (22) Mitsunobu, O., Wada, M., and Sano, T. (1972) Stereospecific and stereoselective reactions. Preparation of amines from alcohols. *J. Am. Chem. Soc.* 94, 679–680.
- (23) Williams, D. M., Yakovlev, D. Y., and Brown, D. M. (1997) The synthesis of a tricyclic pyrrolopyrimidine related to *N*6-hydroxyadenine. *J. Chem. Soc., Perkin Trans. 1*, 1171–1178.

- (24) Ritchie, C. D., Wenninger, J. A., and Jones, J. H. (1959) Identification and detection of triethylrhodamine and *o*-(2-hydroxy-4-diethylamino)benzoylbenzoic acid in commercial samples of D & C Red No. 19. *J. Assoc. Off. Agric. Chem.* 42, 720–724.
- (25) Gemal, A. L., and Luche, J. L. (1980) Haloketone dehalogenation by iodide ion. *Tetrahedron Lett.* 21, 3195–3198.
- (26) Selwyn, J. E., and Steinfeld, J. I. (1972) Aggregation equilibria of xanthene dyes. *J. Phys. Chem.* 76, 762–774.
- (27) (a) Corrie, J. E. T., Brandmeier, B. D., Craik, J. S., Kendrick-Jones, J., Munasinghe, V. R. N., Howell, S., and Trentham, D. R. (1997) A new bifunctional rhodamine to label regulatory light chains of gizzard myosin. *Biophys. J.* 72, A52. (b) Ferguson, R. E., Martin, S. R., Brandmeier, B. D., Kendrick-Jones, J., Hodges, R. S., Sykes, B. D., Corrie, J. E. T., Irving, M., and Trentham, D. R. (1997) Bifunctional rhodamine-labeled regulatory light chains bind to myosin heavy chains and provide a framework of dipole orientations in fibers. *Biophys. J.* 72, A52. (c) Sabido-David, C., Ferguson, R. E., Brandmeier, B. D., Hopkins, S. C., Goldman, Y. E., Kendrick-Jones, J., Dale, R. E., Corrie, J. E. T., Trentham, D. R., and Irving, M. (1997) Orientation of bifunctional rhodamine probes on myosin regulatory light chain (RLC) in relaxed, contracting and rigor muscle. *Biophys. J.* 72, A52. (d) Hopkins, S. C., Sabido-David, C., Brandmeier, B. D., Kendrick-Jones, J., Dale, R. E., Corrie, J. E. T., Trentham, D. R., Irving, M., and Goldman, Y. E. (1997) Motions of bifunctional rhodamine probes with defined orientations on the regulatory light chain (RLC) in skeletal muscle fibers. *Biophys. J.* 72, A1.

BC970174E

Hybridization Characteristics of Biomolecular Adaptors, Covalent DNA–Streptavidin Conjugates

Christof M. Niemeyer,^{*,†} Wolfgang Bürger,[†] and Rein M. J. Hoedemakers[‡]

Department of Biotechnology and Molecular Genetics, University of Bremen, FB 2-UFT, Leobener Strasse, D-28359 Bremen, Germany, and Centre for Biomedical Technology, University of Groningen, Hanzeplein 1, 9713 HZ Groningen, The Netherlands. Received September 19, 1997;
Revised Manuscript Received December 12, 1997

Semisynthetic, covalent streptavidin–DNA adducts are versatile molecular connectors for the fabrication of both nano- and microstructured protein arrays by use of DNA hybridization. In this study, the hybridization characteristics of six adduct species, each containing a different DNA sequence of 21 or 24 bases, have been compared. First, the adducts were conjugated to biotinylated alkaline phosphatase, and their binding to immobilized oligomer complements of similar lengths was quantified in a microplate assay. The binding efficiency observed varied to a great extent with the specific sequence of the oligonucleotide attached, and could not be predicted from affiliated thermodynamic data of duplex stability. To further elucidate the hybridization properties, the hybridization rate constants of association and dissociation (k_{assn} and k_{dissn}) have been determined for both unconjugated oligonucleotides and protein adducts, using a surface plasmon resonance biosensor. The k_{assn} values observed for the oligonucleotides are in the range of 9×10^3 to $2 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ and correlate with structural properties of the probe strands. Up to 3-fold decreased k_{assn} values were obtained for the corresponding protein adducts. Likewise, values were observed for k_{dissn} ranging from 1.4×10^{-4} to $1.9 \times 10^{-5} \text{ s}^{-1}$ for the oligonucleotides. The dissociation of the analogous protein conjugates was reduced by up to 5-fold. The extent of this decrease correlates with the formation of homodimeric or intramolecular aggregation of probe strands. A mechanistic model for explaining these data is based on attractive intramolecular interaction between the nucleic acid and protein moiety.

INTRODUCTION

The generation of new biomaterials for analytical, industrial, or pharmaceutical applications is a central goal of biotechnology. Owing to its unique recognition capabilities, physicochemical stability, and mechanical rigidity, DNA is a promising candidate for the construction of nanometer-sized frameworks (1, 2) which are potentially useful for the selective positioning of molecular devices such as conducting polymers (3), proteins (4), or nanocrystal metal clusters (5, 6). Recently, hybrid molecules of short single-stranded DNA and streptavidin (STV) have been applied in the supramolecular assembly of novel bioconjugates (4). The covalent attachment of an oligonucleotide moiety to STV provides a specific recognition domain for a complementary nucleic acid sequence in addition to the four native biotin-binding sites. These bispecific binding capabilities allow the adducts to serve as versatile connectors in a variety of applications, such as the fabrication of nano- and microstructured protein arrays. While the nanostructures can be accessed by positioning several DNA–protein adducts along a single-stranded RNA or DNA carrier molecule containing a set of complementary sequences, the microstructures can be obtained from given surface-immobilized DNA arrays, such as microchip-based oligonucleotide arrangements currently under active investigation for integrated nucleic acid analysis (7–9). For example,

the “self-sorting translation” of a model DNA array into polyfunctional immunoglobulin G (IgG) arrays was attained by hybridization of a mixture of specific sequence-containing DNA–STV adducts, each previously conjugated to a particular biotinylated IgG (4).

Due to the sensitivity of most biomolecular devices, such as antibodies and enzymes, the effective employment of nucleic acid hybridization-based assembly strategies requires mild reaction conditions such as moderate temperatures and salt concentrations. Thus, binding efficiency of oligonucleotide-tagged compounds is controlled by hybridization rate constants for association, k_{assn} , and dissociation, k_{diss} , rather than thermodynamic duplex stability. Kinetic data of DNA hybridization are scarce (10–17), and it is difficult a priori to design sequences of single-stranded oligonucleotides that are optimized for fast and efficient binding to their complementary target strands. As an experimental approach for selecting appropriate sequences, the hybridization characteristics of six covalent DNA–protein adducts have been analyzed in this study. Each of these hybrids (HA through HF; for sequences of A24 through F24, see Table 1) contains a different oligonucleotide sequence of 21 or 24 bases, chosen from the antisense strand of the multiple cloning site of the bacteriophage M13mp18. As a relevant test of near-surface hybridization properties, a binding assay of the DNA–STV adducts was carried out in microplates (Figure 1). The adducts were pre-conjugated to biotinylated alkaline phosphatase (AP), and their binding to immobilized single-stranded oligonucleotide targets was quantified by colorimetric detection of enzymatic activity. The hybridization efficiency observed varied to a great extent with the specific sequence of the

* To whom correspondence should be addressed. Phone: (49) 421 218-4911. Fax: (49) 421 218-7578. E-mail: CMN@biotec.uni-bremen.de.

[†] University of Bremen.

[‡] University of Groningen.

Table 1. Oligonucleotides Used in This Study

probe	description ^a	sequence
TA16	5'-thiolated 16-mer; $T_m = 44.8$ °C; $\Delta G = -29.3$ kcal/mol	5'-thiol-G AAA TTG TTA TCC GCT-3'
TA21	5'-thiolated 21-mer; $T_m = 52.3$ °C; contains mismatch (underlined)	5'-thiol-TCC <u>TAT</u> GTG AAA TTG TTA TCC-3'
TA24	5'-thiolated 24-mer; $T_m = 65.1$ °C; $\Delta G = -43.9$ kcal/mol	5'-thiol-TCC TGT GTG AAA TTG TTA TCC GCT-3'
bcA24	5'-biotinylated 24-mer; complementary to TA24, TA21, and TA16	5'-biotin-AGC GGA TAA CAA TTT CAC ACA GGA-3'
TB21	5'-thiolated 21-mer; $T_m = 52.1$ °C; $\Delta G = -34.2$ kcal/mol	5'-thiol-GTA ATC ATG GTC ATA GCT GTT-3'
bcB21	5'-biotinylated 21-mer; complementary to B21	5'-biotin-AAC AGC TAT GAC CAT GAT TAC-3'
TC21	5'-thiolated 21-mer; $T_m = 68.0$ °C; $\Delta G = -44.7$ kcal/mol	5'-thiol-CCG GGT ACC GAG CTC GAA TTC-3'
bcC21	5'-biotinylated 21-mer; complementary to TC21	5'-biotin-GAA TTC GAG CTC GGT ACC CGG-3'
TD21	5'-thiolated 21-mer; $T_m = 58.1$ °C; $\Delta G = -37.4$ kcal/mol	5'-thiol- CAG GTC GAC TCT AGA GGA TCC-3'
bcD21	5'-biotinylated 21-mer; complementary to TD21	5'-biotin- GGA TCC TCT AGA GTC GAC CTG-3'
TE21	5'-thiolated 21-mer; $T_m = 68.5$ °C; $\Delta G = -43.2$ kcal/mol	5'-thiol-AGT GCC AAG CTT GCA TGC CTG-3'
bcE21	5'-biotinylated 21-mer; complementary to TE21	5'-biotin- CAG GCA TGC AAG CTT GGC ACT-3'
TF24	5'-thiolated 21-mer; $T_m = 65.5$ °C; $\Delta G = -43.7$ kcal/mol	5'-thiol- GTT TTC CCA GTC ACG ACG TTG TAA-3'
bcF24	5'-biotinylated 24-mer; complementary to TF24	5'-biotin- TTA CAA CGT CGT GAC TGG GAA AAC-3'

^a Melting temperatures (T_m) and free energies of duplex stability (ΔG) have been calculated for underivatized oligonucleotides at 25 °C and 150 mM NaCl using Primer Select version 3.01a (DNA-Star Inc.), following a model based on adjacent dinucleotides (19, 20).

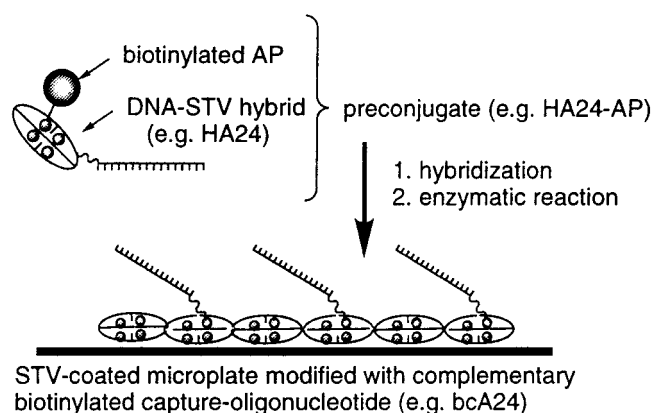


Figure 1. Schematic drawing of the enzyme-linked oligonucleotide sorbent assay (ELOSA) for the determination of hybridization efficiency of covalent DNA-STV adducts. The latter were coupled with a signal-generating enzyme by mixing with biotinylated alkaline phosphatase (AP) to form preconjugates (e.g., HA24-AP). Biotinylated capture oligonucleotides were immobilized on STV-coated microplates, and remaining biotin-binding sites of the surface-bound STV were blocked with free D-biotin, represented by shaded spheres. The preconjugates were then allowed to bind to their complement by formation of specific Watson-Crick base pairs. Subsequently, the amount of hybridized material was determined by AP-dependent color reaction using pNPP as a substrate.

oligonucleotide domain, thus, enabling a classification of strong or weak binders. To further elucidate the results observed for the enzyme conjugates, real-time biospecific interaction analysis (BIA) technology was used to quantify the hybridization kinetics of both the STV-DNA hybrid and their analogous, unconjugated oligonucleotides, TA through TF. BIA, a method based on surface plasmon resonance (SPR), has previously been used to study DNA hybridization (10, 13, 14, 18). The kinetic rate constants for association (k_{assn}) and dissociation (k_{diss}), measured here for the six sequences under investigation, confirm the hybridization characteristics previously observed in the microplate assay, and correlate with structural features of the probe strands rather than thermodynamic duplex stability (19, 20). Owing to their elevated information contents, the BIA data also reveal influences of the protein moiety on the process of nucleic acid hybridization. Both annealing and dissociation rate constants are reduced relative to those of the analogous oligonucleotides. This effect may be due to intramolecular surface interaction between the nucleic acid and, for

example, positively charged side chain groups of the protein moiety.

MATERIALS AND METHODS

Oligonucleotides. The 5'-thiol-modified oligonucleotides (TA24 through TF24; for sequences, see Table 1) were purchased from MWG-Biotech (Germany) or NAPS (Göttingen, Germany). The oligonucleotides were synthesized by standard methods using C6 Thiolmodifier reagent (Glen Research) and were purified by HPLC. The purity of the samples was determined from anion-exchange chromatography analysis on a Mono Q column (Pharmacia). The concentrations were determined by UV absorbance measurement. Biotinylated oligomer complements were also purchased from MWG-Biotech or NAPS (for sequences, see Table 1).

Synthesis of DNA-Streptavidin Adducts and ELOSA. Synthesis and purification of covalent DNA-STV adducts were carried out from thiolated oligonucleotides, TA24 through TF24, and recombinant streptavidin (Boehringer Mannheim), as previously described (4). In brief, STV (10 nmol) was derivatized with maleimido groups using a heterobispecific cross-linker (sulfo-SMPB, Pierce), reacted with a 5'-thiolated oligonucleotide (10 nmol) and subsequently purified by anion-exchange chromatography. A 1:1 molar ratio of nucleic acid and protein moiety of the hybrids was verified by gel electrophoretic and photometric analysis, and concentrations of the preparations were determined by absorbance measurements (4). Conjugates of the DNA-STV hybrids and AP were prepared by mixing 0.1 μM stock solutions of the hybrids (HA24 through HF24; for sequences, see Table 1) and a 4-fold molar excess (3.8 μM stock solution) of biotinylated AP (Sigma) in TETBS buffer [20 mM Tris-HCl buffer (pH 7.3), 150 mM NaCl, 5 mM EDTA, and 0.01% (w/v) Tween-20] containing 0.1 mg/mL bovine serum albumin. After incubation for 60 min at room temperature, the mixtures were diluted to a final concentration of 10–1 nM for the conjugates with TETBS containing 0.1 mg/mL bovine serum albumin and 10 mM D-biotin (Fluka), and the dilutions were incubated for an additional 30 min. STV-coated microplates were prepared as previously described (21), and 50 μL of a 600 nM solution of the biotinylated oligomer complements (capture oligomers) in TETBS was allowed to bind for 60 min. After the plate was washed with TETBS containing 10 mM D-biotin, 50 μL of the AP conjugate solution described above was allowed to hybridize for 60 min at room temperature. Negative controls were run

in parallel by applying preconjugates to wells, coated with noncomplementary oligonucleotides. After incubation, the plate was washed four times with TETBS and twice with TBS [Tris-buffered saline, 20 mM Tris-HCl buffer (pH 7.3) and 150 mM NaCl]. AP-dependent color reactions were carried out with 200 μ L of 1 M diethanolamine (pH 9.8) and 0.5 mM MgCl_2 containing 10 mM *p*-nitrophenyl phosphate (pNPP). The absorbance at 405 nm was determined with a Victor Multilabel-Counter (Wallac) after incubation for 10 min at 37 $^\circ\text{C}$. Relative intensities (signal to noise, *S/N* values) were calculated from the observed absolute signal intensities divided by the corresponding background signal obtained for binding to noncomplementary capture oligomers, and typically fell in the range of 2–25, depending upon the preconjugate concentration. Standard deviations were calculated from the signal intensities of interest. Typically, duplicate determination of a particular sample revealed a percent error of 15%.

BIA Measurements. Biospecific interaction analysis was performed using a BIAcore-1000 instrument from Pharmacia Biosensor AB (Uppsala, Sweden).

Immobilization of Capture Oligomers. A continuous flow of hybridization buffer [150 mM NaCl, 30 mM sodium phosphate, 3 mM EDTA, and 0.25% Triton X-100 (pH 7.4)] at 10 $\mu\text{L}/\text{min}$ and 25 $^\circ\text{C}$ was maintained throughout the entire analysis. Biotinylated capture oligomer (10 μM in hybridization buffer) was injected for 4 min over STV-coated sensorchips (SA-Chips, Pharmacia Biosensor AB). Loosely attached material was removed by three cycles of 10 μL of 50 mM NaOH. An amount of 500–2000 RU of the biotinylated oligomer complements was immobilized for kinetic analysis.

Kinetic Analysis. Oligonucleotides or DNA–STV adducts at various concentrations in hybridization buffer, typically in the range of 400–25 nM, were injected over the DNA-specific surface at 10 $\mu\text{L}/\text{min}$ for 4 min. The interaction was monitored continuously as the change in SPR signal. Dissociation was effected by injecting hybridization buffer only and was followed for 15 min. The surface was regenerated by injection of 10 μL of 50 mM NaOH. The entire process was automated. Binding and dissociation curves were recorded automatically, and analytical cycles were preprogrammed. The data analysis was carried out using BIAcore Evaluation 2.1 software (Pharmacia). The pseudo-first-order kinetic model was used, and parts of the binding curves where mass transport was evident were excluded from the calculations. A series of controls was carried out (data not shown). Injection of probes across surfaces, modified with noncomplementary oligonucleotides, revealed no significant increase in RU response, suggesting that the binding observed in the kinetic experiments is due exclusively to specific single-stranded DNA hybridization. Lowering the amount of coupling density of the biotinylated oligomer did not result in differences in kinetic constants, suggesting that any influence of rebinding (22) is negligible in these experiments.

RESULTS AND DISCUSSION

Semisynthetic hybrids of DNA and STV were synthesized from various 5'-thiolated oligonucleotides (Table 1) by means of chemical cross-linking as previously described (4). In brief, STV was derivatized with maleimido groups using a heterobispecific cross-linker, and subsequently reacted with a thiolated oligonucleotide. The chief products, hybrids containing a single oligonucleotide moiety per STV, were purified by anion-exchange chromatography.

ELOSA Analysis of Conjugate Hybridization. As a relevant test for quantifying the hybridization characteristics of the DNA–streptavidin biomolecular adaptors, a microplate assay in the form of an enzyme-linked oligonucleotide sorbent assay (ELOSA) (23) was used (Figure 1). For this purpose, DNA–STV covalent adducts were conjugated to biotinylated AP. Initially, to determine the optimal conjugation stoichiometry, a fixed amount of the hybrid HA24 was mixed with varying quantities of the biotinylated enzyme. After incubation, the reaction was quenched by addition of free D-biotin. The resulting preconjugates (HA24–AP) were then allowed to bind to their corresponding oligomer complement (bcA24), previously immobilized on a STV-coated microplate (21), by using the STV–biotin interaction (see Materials and Methods for details). After hybridization, the amount of material immobilized was estimated from the enzymatic activity of AP, using colorimetric pNPP as a substrate. Negative controls were carried out by incubation of a similar preconjugate containing a noncomplementary oligonucleotide sequence, HC21-AP (prepared from 2.5 equiv of AP), as well as by incubation of biotinylated AP or buffer only. As shown in Figure 2, the low background signal obtained for the controls confirmed that binding occurred exclusively via specific single-stranded DNA hybridization. Relative intensities (signal-to-noise, *S/N*) of about 20 were obtained for a 1:0.25 molar ratio of the biotin binding sites of STV and the biotinylated enzyme. An increase of the amount of biotinylated AP did not lead to significantly stronger signals (Figure 2). These findings indicate that an approximately equimolar conjugation stoichiometry of the two protein components occurred despite the tetravalent binding capabilities of STV, and is likely due to steric hindrance of the voluminous AP. Furthermore, variations of the concentration of the biotinylated capture oligomer during immobilization on the microplate showed that saturation of the binding capacity of single microplate wells occurred at oligonucleotide concentrations of about 60 nM (Figure 2), which corresponds to an estimated biotin-binding capacity of about 1–2 pmol/well (24).

Further experiments were carried out using DNA–STV adducts containing the various oligonucleotide sequences of A24, B21, C21, D21, E21, and F24 as indicated in Table 1. The adducts were conjugated with biotinylated AP and allowed to bind to their immobilized complement at analyte concentrations ranging from 10 to 1 nM. Negative controls were carried out for all experiments by incubation of the 10 nM preconjugate solutions in microplate wells containing noncomplementary capture oligonucleotides. The *S/N* values observed were concentration-dependent and typically in the range of 25–2. Investigation of a large number of independently synthesized preconjugates gave percent errors of about 25%, thus satisfying the requirements for the reproducibility of chemical hybrid synthesis and AP conjugation as well as the microplate binding assay protocol. Time course experiments, carried out over varying periods of hybridization, revealed that maximum signal intensities are reached within 30 min (not shown). Therefore, the signal intensities obtained after 1 h of binding reflect equilibrium binding and, thus, the stability of a DNA duplex under the given reaction conditions. As indicated in Figure 3, the amount of hybridization varied to a great extent with the oligonucleotide sequence of the conjugates. The relative fraction of binding observed for particular sequences was linearly dependent on reagent concentration in the range of 10–1 nM. A

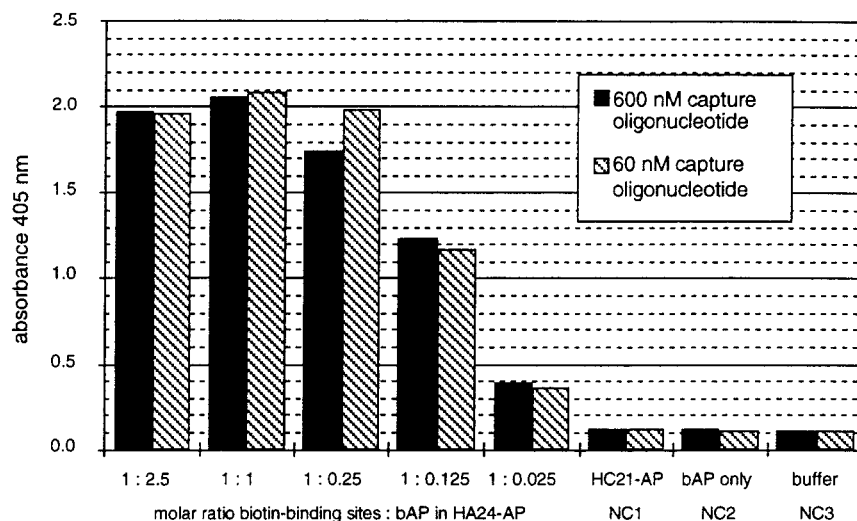


Figure 2. Optimization of the preconjugation stoichiometry for ELOSA. Fixed amounts of DNA-STV adduct HA24 were mixed with varying amounts of biotinylated AP. The molar ratio of biotin-binding sites of the adduct to biotinylated AP was in the range of 1:2.5 to 1:0.025. Negative controls (NCs) were carried out with a comparable HC21-AP preconjugate, containing a noncomplementary oligonucleotide sequence (NC1), bAP only (NC2), and buffer only (NC3). The low signal intensities observed for controls indicate that binding is exclusively due to specific DNA base pairing.

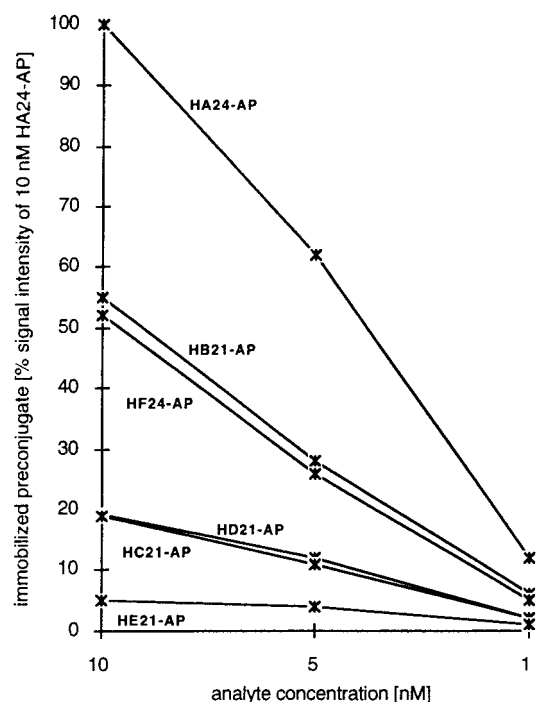


Figure 3. Quantification of the sequence-dependent hybridization efficiency, as determined from ELOSA microplate analysis. The binding of several DNA-STV AP conjugates, containing oligonucleotide sequences A through F (see Table 1), was quantified in an ELOSA assay at analyte concentrations of 10, 5, and 1 nM, respectively. The relative amounts of preconjugate immobilized were calculated from enzyme-generated signals observed and normalized as a percentage fraction of the signal intensity obtained for 10 nM HA24-AP.

comparison of the hybridization efficiency, calculated from the average slope of concentration-dependent signal increase, allows a classification of the sequences studied. The 24-mer species HA24-AP exhibited the strongest binding (relative slope of 1.00). The preconjugates HF24-AP and HB21-AP bind less efficiently (0.53 and 0.56, respectively). Species HC21-AP and HD21-AP exhibited low hybridization efficiencies (0.19 and 0.20, respectively), and the binding of HE21-AP (0.04) was barely detectable. These results could not be predicted

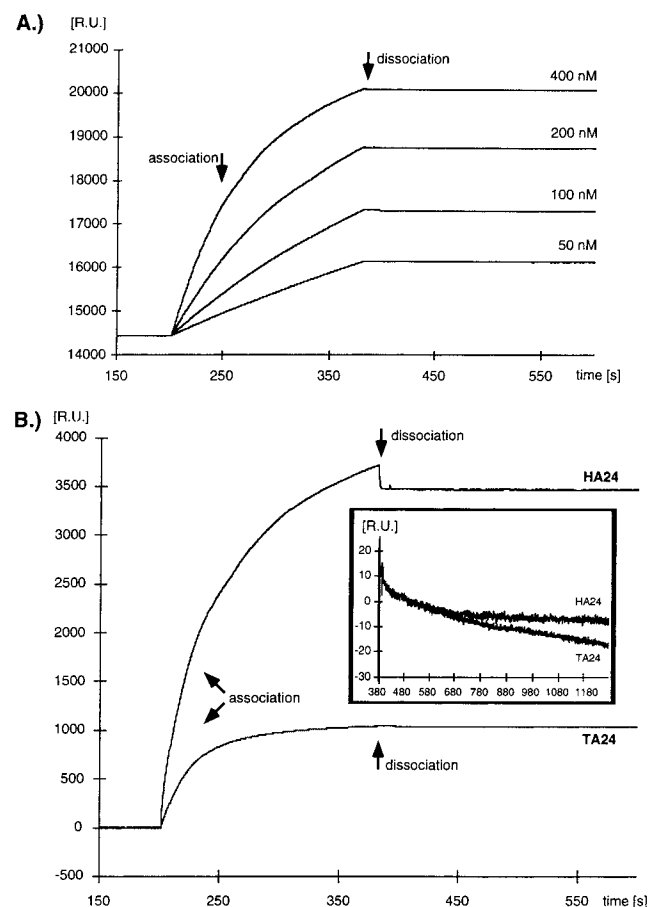
from thermodynamic data of the particular oligomer sequences (19, 20), such as the calculated melting temperatures and free energies of duplex formation (T_M and ΔG , see Table 1).

Analysis of DNA Hybridization by SPR Measurements. To elucidate further the binding characteristics, the rate constants of association (k_{assn}) and dissociation (k_{diss}) of single-stranded DNA interactions have been determined by biomolecular interaction analysis (BIA) using a biosensor based on surface plasmon resonance (SPR). With this technique, the refractive index within a gold sensor chip-immobilized dextran matrix is continuously monitored, plotted against time, and presented in a sensorgram. The change of SPR response, measured in resonance units (RU), allows a quantification of interaction between chip-immobilized and liquid phase-diluted binding partners. Here, the biotinylated oligomer complements, bcA through bcF (see Table 1), were immobilized on streptavidin-coated sensor chips, and real-time kinetic measurements at several analyte concentrations were carried out with the various oligonucleotides and the analogous DNA-STV hybrids. A series of controls was carried out by injecting oligonucleotides or DNA-STV adducts over sensor chips modified with noncomplementary capture oligomers (not shown). No significant change in SPR response indicated that the binding observed was due exclusively to specific single-stranded DNA hybridization, a result similar to that obtained from ELOSA. Although the data obtained from BIA for triplicate determinations of a particular sample were generally very reproducible (errors of 10%), deviations increased upon measuring identical samples after a certain period of storage and/or on a different sensor chip (percent error of up to 50%). As no degradation products of the probes could be detected by gel electrophoretic analysis, these alterations may reflect quality differences of the sensor surfaces or changes in, i.e., the salt concentration of the buffers that were used (10, 25). Implementation of a large number of measurements, including various ($n \geq 5$) independently synthesized analyte samples and statistical analysis of the data obtained, allowed elucidation of the trends discussed below.

To confirm the applicability of BIA, a kinetic study of the hybridization of three oligonucleotides was carried

Table 2. Kinetic Constants of Single-Stranded DNA Hybridization Obtained from BIA Measurements of the Binding of Thiolated Oligonucleotides TA16, TA21, and TA24 to the Immobilized Biotinylated Oligomer Complement bcA24^a

probe	$10^3 k_{\text{assn}} (\text{M}^{-1} \text{s}^{-1})$	$10^{-7} k_{\text{dissn}} (\text{s}^{-1})$	$10^7 K_A (\text{M}^{-1})$
TA16	148 ± 29	527 ± 41	2.8
TA21	211 ± 13	446 ± 47	4.7
TA24	216 ± 35	253 ± 63	8.6

^a For sequences, see Table 1.**Figure 4.** Representative sensorgrams obtained from BIA. (A) Binding of covalent the STV-DNA adduct HA24 to sensor-immobilized oligomer complement, bcA24, carried out at several analyte concentrations. Computational analysis of binding and dissociation phases yields their reaction rate constants, k_{assn} and k_{dissn} , respectively. (B) Overlay of sensorgrams obtained for the binding of covalent STV adduct HA24 and oligonucleotide TA24 at a concentration of 0.4 μM to sensor-immobilized bcA24 (for sequences, see Table 1). The decrease in hybridization rate constants is detectable from eye inspection of the sensorgrams. The binding of HA24 proceeds over a period of more than 200 s, while the TA24 association curve has reached saturation after this period of time. The decreased k_{dissn} of HA24 is clearly visible in a normalized overlay of sensorgrams, obtained for the dissociation phase (boxed inset).

out. The affinity constants ($K_A = k_{\text{assn}}/k_{\text{dissn}}$) of the probes investigated were expected to increase in the order TA16 < TA21 < TA24, as they contain identical sequence stretches of increasing lengths and TA21 contains a single G $\rightarrow$ A mismatch (Table 1). The kinetic rate constants observed (Table 2) were 1.5×10^5 , 2.1×10^5 , and $2.2 \times 10^5 \text{ M}^{-1} \text{s}^{-1}$ for association (k_{assn}) and 5.3×10^{-5} , 4.5×10^{-5} , and $2.5 \times 10^{-5} \text{ s}^{-1}$ for dissociation (k_{dissn}) of TA16, TA21, and TA24, respectively. Although derived from near-surface hybridization, these values are in a range comparable to the range of those obtained in

Table 3. Kinetic Constants of Single-Stranded DNA Hybridization Obtained from BIA Measurements of the Binding of Thiolated Oligonucleotides (Tx) and the Corresponding DNA-STV Hybrid Molecules (Hx), Respectively, to Their Immobilized Biotinylated Oligomer Complements^a

probe	immobilized material RU _{rel} ^b	$10^3 k_{\text{assn}} (\text{M}^{-1} \text{s}^{-1})$	$10^{-7} k_{\text{dissn}} (\text{s}^{-1})$
TA24	0.90 ± 0.06	216 ± 35	253 ± 63
HA24	3.03 ± 0.44	144 ± 44	50 ± 17
TB21	0.60 ± 0.02	69 ± 7	307 ± 77
HB21	1.72 ± 0.27	53 ± 16	90 ± 69
TC21	0.14 ± 0.02	13 ± 9	456 ± 162
HC21	0.26 ± 0.05	18 ± 9	257 ± 62
TD21	0.13 ± 0.01	14 ± 5	386 ± 26
HD21	0.19 ± 0.03	9 ± 2	330 ± 63
TE21	0.03 ± 0.01	9 ± 4	1415 ± 105
HE21	0.07 ± 0.03	12 ± 1	966 ± 54
TF24	0.65 ± 0.11	149 ± 17	193 ± 83
HF24	2.12 ± 0.71	38 ± 13	98 ± 27

^a For sequences of A24 through F24, see Table 1. ^b Relative response units (RU_{rel}) were calculated from the RU value observed for immobilization of capture oligonucleotide and the RU value observed for analyte binding at a concentration of 200 nM.

homogeneous reactions. For example, in a recent study, diffusional fluorescence correlation analysis was used to measure the hybridization of a set of six oligodeoxyribonucleotides to a complementary 101-mer target RNA (17). Different probes, designed for equal thermodynamic stability, revealed association constants between 3×10^4 and $1.5 \times 10^6 \text{ M}^{-1} \text{s}^{-1}$. The similar association rates of TA24, TA21, and TA16 presented in Table 2 are consistent with the general principle of nucleic acid hybridization. The low activation barrier of duplex formation is associated with rate-limiting nucleation of a small number of base pairs, which is then followed by rapid hybridization of the remaining base pairs (26–28). As a consequence, oligonucleotides containing identical sequence stretches may reveal similar association rates. The decrease in stability, observed in the order TA24 > TA21 > TA16, is also affected by variations in dissociation, particularly in the case of TA24 versus TA21. This result is in agreement with a recent study of homogeneous nucleic acid hybridization carried out by Kool and co-workers (15).

Sequence-Specific Hybridization Characteristics. Binding efficiency and kinetic rate constants have been determined for the hybridization of oligonucleotides and the analogous covalent STV adducts, respectively (Figure 4). The results obtained are summarized in Table 3. The amount of analyte bound to the sensor chip increased linearly with the amount of immobilized capture probe. Therefore, relative RU values (RU_{rel}; see column 2 of Table 3) can be calculated for each experiment by dividing the RU obtained from analyte by the RU obtained from capture probe binding. Owing to the higher molecular mass, the RU_{rel} values observed for the hybrids are generally greater than those of the corresponding oligonucleotide (Figure 4B). Normalization of the RU_{rel} of the particular sequences as a percentage of the binding efficiency of sequence A24 allowed a comparison of the amount of hybridization measured by ELOSA or BIA. As shown in Figure 5, the similarity of the resulting patterns of sequence-dependent binding indicates good agreement between both methods. Furthermore, attachment of a protein moiety evidently results in only minor changes of sequence-specific hybridization efficiencies.

Upon comparison of the kinetic data determined for the oligonucleotides of sequences A through F, differences

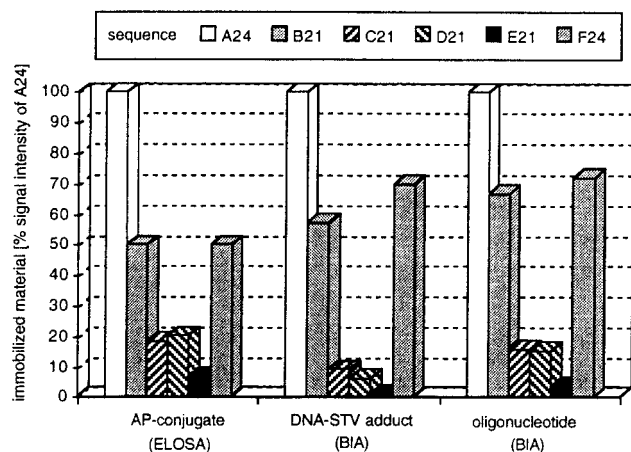


Figure 5. Comparison of the sequence-dependent hybridization efficiency obtained from ELOSA (left group of bars) and bio-specific interaction analysis (BIA) of oligonucleotides (right group) or covalent DNA-STV adducts (middle group). The signal intensities observed were normalized as a percentage fraction of the signal intensities obtained from species containing the A24 sequence. The amount of material immobilized in BIA was calculated from relative RU values (see Table 3 and the text for details).

of up to 2 orders of magnitude could be observed for both k_{assn} and k_{dissn} with no obvious correlation with thermodynamic values T_m and ΔG (see Table 1). A distinct dependency of k_{assn} and the formation of secondary structure via intramolecular hydrogen bonds competing with intermolecular hybridization has previously been reported (17, 29). The k_{assn} values of the binding of oligonucleotides to target RNA correlated with secondary structures of probes and target sites. The lowest rate constants were observed for probes exhibiting tight stem-loop structures. Within this context, the structural characteristics of the DNA sequences studied here have

been examined by software analysis, and the free energies (ΔG) for intramolecular loop formation and intermolecular self-association were calculated, following a model based on adjacent dinucleotides (19, 20). As indicated in Figure 6, sequences A24, B21, and F24 form less stable intermolecular aggregates, while the G/C-rich sequences of C21, D21, and E21 produce more stable hairpin loops and dimeric duplexes. Although no aggregates could be detected by photometric, gel electrophoretic, or chromatographic analysis (not shown), dimerization or intramolecular folding of the probes will be present in equilibrium and bias the kinetics of DNA hybridization. However, a simplistic correlation with energies of homodimeric duplex or loop formation is evidently not sufficient to explain subtle differences, such as those observed in the hybridization characteristics of, e.g., B21 and F24 or C21 and E21. More complex models, combined with computational methods (8), may assist in predicting hybridization characteristics from mere sequence information.

Influences of the STV Moiety on Hybridization Kinetics. A comparison of oligonucleotides and the analogous STV adducts (Table 3) exhibits some clearly perceptible differences. The latter exhibited reduced rate constants of hybridization which could already be detected from eye inspection of the sensorgrams (Figure 4B). The amount of decrease in the dissociation rate constant correlated with structural features of the oligonucleotide. The k_{dissn} of linear sequences, as in HA24, HB21, and HF24, decreased approximately 2–5-fold, while those of dimer-forming sequences, as in HC21, HD21, and HE21, were only affected 1–2-fold. A similar trend was evident from the comparison of k_{assn} values. The decrease was 2–3-fold for linear sequences, as in HA24, HB21, and HF24, while the annealing of HD21 was almost unaffected. The rates of structurally rich sequences, HC21 and HE21, were even slightly acceler-



Figure 6. Secondary structures of the oligonucleotides used in this study. Oligonucleotide duplexes and hairpin loops of the sequences have been calculated with Primer Select version 3.01a (DNA-Star Inc.). The numbers indicate the free energy of structure formation at 25 °C.

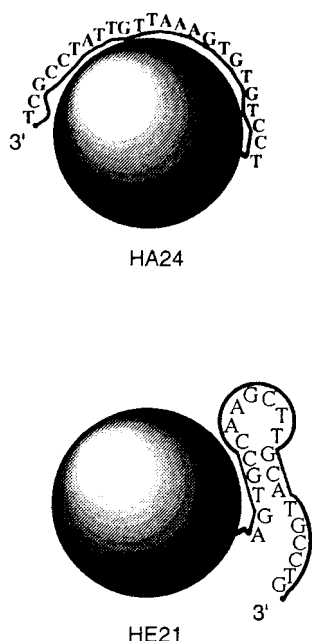


Figure 7. Schematic model of covalent DNA-STV adducts. The putative attractive interaction between surfaces of the protein and nucleic acid moiety is decreased by secondary structures of the DNA strand, e.g., in HE21. For linear sequences (e.g., in HA24), a closer contact of the surfaces influences the process of intermolecular hybridization to a greater extent.

ated by the protein attached. These phenomena were further investigated by melting temperature analysis of the duplex structures formed by DNA-STV conjugates or the analogous oligonucleotides with their oligomer complements (data not shown). T_m values in agreement with the predicted thermodynamic parameters were obtained for sequences A through F. However, a slight decrease in T_m of approximately 1–2 °C was observed for the protein conjugates, indicating a weak, duplex-destabilizing effect of the STV moiety under conditions in which the influences of DNA native structure as well as intramolecular DNA-protein interactions are reduced.

A possible model mechanism for explaining these data is based on intramolecular attractive interactions between the protein and nucleic acid moiety of the hybrid (Figure 7). An attraction of the surfaces may be mediated by positively charged lysine or arginine residues of STV and the negatively charged phosphate backbone of the DNA. This would restrict the degrees of freedom of the nucleic acid, and thus reduce the number of potential nucleation sites along the DNA strand. As a consequence, both association and dissociation velocities would be reduced. Additional steric hindrance of the protein may also affect the hybridization process. The fact that the influence of the STV molecule is lower on dimer- or loop-forming sequences is in agreement with this model; linear strands of nucleic acids might be affected to a greater extent, due to a more intimate interaction of their surface with the STV moiety (see HA24 in Figure 7). The slight increase observed for association of HC21 and HE21 suggests influences of an additional mechanism, recently suggested by Corey and co-workers, for explaining an accelerated hybridization of oligonucleotides that are conjugated with positively charged peptides or a basic protein, staphylococcal nuclease, to double-stranded DNA targets (30–32). As a result of Coulomb attraction of the protein moiety, the DNA domain of the probe is held in close proximity to the DNA template, thereby increas-

ing its effective concentration near its target site. This mechanism, however, appears to be of minor importance in the case of rapid single-stranded DNA hybridization but may be significant for slow, single-stranded DNA hybridization reactions.

We have investigated the sequence-specific near-surface hybridization of both covalent DNA-protein conjugates and their analogous oligonucleotides at nondenaturing temperatures. The hybridization efficiencies, determined for binding to surface-immobilized complements, varied to a great extent with the base composition of the nucleic acid and correlated with DNA native structure rather than standard thermodynamic parameters. As demonstrated in a microplate assay, covalent DNA-STV adducts are convenient reagents for the quantification of sequence-dependent binding efficiency. The kinetic rate constants of the covalent adducts and the analogous unmodified oligonucleotides have been determined by SPR measurements. A comparison of the data suggests intramolecular interactions between the protein and nucleic acid moiety within the adducts. This study emphasizes the importance of structural influences on hybridization reactions proceeding with kinetic control, such as the supramolecular assembly of delicate DNA-tagged molecular devices. It is difficult to draw systematic conclusions at this stage since much remains to be learned about hybridization of nucleic acids near surfaces or tagged with macromolecules. Nevertheless, binding will profit from sequences specially optimized for fast and efficient hybridization.

ACKNOWLEDGMENT

We thank D. Blohm for generous support and valuable discussion, S. Rohlfing, S. Behrens, and M. Adler for experimental help with the microplate assay, and T. Ferdelman for critical reading of the manuscript. Financial support for this work was obtained from Deutsche Forschungsgemeinschaft, Fonds der Chemischen Industrie, and Tönjes-Vagt Stiftung Bremen.

LITERATURE CITED

- (1) Seeman, N. C. (1982) Nucleic acid junctions and lattices. *J. Theor. Biol.* 99, 237–247.
- (2) Seeman, N. C. (1997) DNA components for molecular architecture. *Acc. Chem. Res.* 30, 357–363.
- (3) Robinson, B. H., and Seeman, N. C. (1987) The design of a biochip: a self-assembling molecular-scale memory device. *Protein Eng.* 1, 295–300.
- (4) Niemeyer, C. M., Sano, T., Smith, C. L., and Cantor, C. R. (1994) Oligonucleotide-directed self-assembly of proteins: Semisynthetic DNA-streptavidin hybrid molecules as connectors for the generation of macroscopic arrays and the construction of supramolecular bioconjugates. *Nucleic Acids Res.* 22, 5530–5539.
- (5) Mirkin, C. A., Letsinger, R. L., Mucic, R. C., and Storhoff, J. J. (1996) A DNA-based method for rationally assembling nanoparticles into macroscopic materials. *Nature* 382, 607–609.
- (6) Alivisatos, A. P., Johnsson, K. P., Peng, X., Wilson, T. E., Loweth, C. J., Bruchez, M. P., Jr., and Schultz, P. G. (1996) Organization of 'nanocrystal molecules' using DNA. *Nature* 382, 609–611.
- (7) Lipshutz, R. J., Morris, D., Chee, M., Hubbell, E., Kozal, M. J., Shah, N., Shen, N., Yang, R., and Fodor, S. P. A. (1995) Using oligonucleotide probe arrays to access genetic diversity. *BioTechniques* 19, 442–447.
- (8) Lockhart, D. J., Dong, H., Byrne, M. C., Follettie, M. T., Gallo, M. V., Chee, M. S., Mittmann, M., Wang, C., Kobayashi, M., Horton, H., and Brown, E. L. (1996) Expression monitoring by hybridization to high-density oligonucleotide arrays. *Nat. Biotechnol.* 14, 1675–1680.

- (9) Burke, D. T., Burns, M. A., and Mastrangelo, C. (1997) Microfabrication technologies for integrated nucleic acid analysis. *Genome Res.*, 189–197.
- (10) Wood, S. J. (1992) DNA-DNA hybridization in real time using BIAcore. *Microchem. J.* 47, 330–337.
- (11) Parkhurst, K. M., and Parkhurst, L. J. (1993) Kinetic Studies of Oligonucleotide-DNA Hybridization in Solution by Fluorescence Resonance Energy-Transfer. *Biophys. J.* 64, A266–A266.
- (12) Morrison, L. E., and Stols, L. M. (1993) Sensitive Fluorescence-Based Thermodynamic and Kinetic Measurements of DNA Hybridization in Solution. *Biochemistry* 32, 3095–3104.
- (13) Application Note 306 (1994) Kinetic characterization of DNA hybridization using real time BIA. Pharmacia Biosensor AB, Uppsala, Sweden.
- (14) Gotoh, M., Saito, K., Shimizu, M., and Tosu, M. (1994) A novel approach for determination of mismatch formation effects on DNA hybridization kinetics using a biosensor. *Nucleic Acids Symp. Ser.* 31, 121–122.
- (15) Wang, S. H., Friedman, A. E., and Kool, E. T. (1995) Origins of High Sequence Selectivity: A Stopped-Flow Kinetics Study of DNA/RNA Hybridization by Duplex-Forming and Triplex-Forming Oligonucleotides. *Biochemistry* 34, 9774–9784.
- (16) Parkhurst, K. M., and Parkhurst, L. J. (1995) Kinetic Studies by Fluorescence Resonance Energy-Transfer Employing a Double-Labeled Oligonucleotide: Hybridization to the Oligonucleotide Complement and to Single-Stranded DNA. *Biochemistry* 34, 285–292.
- (17) Schwille, P., Oehlenschläger, F., and Walter, N. G. (1996) Quantitative Hybridization Kinetics of DNA Probes to RNA in Solution Followed by Diffusional Fluorescence Correlation Analysis. *Biochemistry* 35, 10182–10193.
- (18) Nilsson, P., Persson, B., Uhlen, M., and Nygren, P. A. (1995) Real-Time Monitoring of DNA Manipulations Using Biosensor Technology. *Anal. Biochem.* 224, 400–408.
- (19) Breslauer, K. J., Frank, R., Blöcker, H., and Marky, L. A. (1986) Predicting DNA duplex stability from the base sequence. *Proc. Natl. Acad. Sci. U.S.A.* 83, 3746.
- (20) Freier, S. M., Kierzek, R., Jaeger, J. A., Sugimoto, N., Caruthers, M. H., Neilson, T., and Turner, D. H. (1986) Improved free-energy parameters for predictions of RNA duplex stability. *Proc. Natl. Acad. Sci. U.S.A.* 83, 9373–9377.
- (21) Niemeyer, C. M., Adler, M., and Blohm, D. (1997) Fluorometric Polymerase Chain Reaction (PCR) Enzyme-Linked Immunosorbent Assay for Quantification of Immuno-PCR Products in Microplates. *Anal. Biochem.* 246, 140–145.
- (22) Nieba, L., Krebber, A., and Plückthun, A. (1996) Competition BIAcore for measuring true affinities: Large differences from values determined from binding kinetics. *Anal. Biochem.* 234, 155–165.
- (23) Whetsell, A. J., Drew, J. B., Milman, G., Hoff, R., Dragon, E. A., Adler, K., Hui, J., Otto, P., Gupta, P., Farzadegan, H., and Wolinsky, S. M. (1992) Comparison of three nonradioisotopic polymerase chain reaction-based methods for detection of human immunodeficiency virus type 1. *J. Clin. Microbiol.* 30, 845–853.
- (24) Adler, M. (1996) Auswahl und Etablierung alternativer Methoden zur Detektion von Immuno-PCR Produkten. Diploma Thesis, Universität Bremen, Bremen, Germany.
- (25) Keller, G. H., and Manak, M. M. (1989) *DNA Probes*, Stockton, New York.
- (26) Wetmur, J., and Davidson, N. (1968) Kinetics of renaturation of DNA. *J. Mol. Biol.* 31, 349–370.
- (27) Pörschke, D., and Eigen, M. (1971) Co-operative non-enzymic base recognition. 3. Kinetics of the helix-coil transition of the oligoribouridylic–oligoriboadenylic acid system and of oligoriboadenylic acid alone at acidic pH. *J. Mol. Biol.* 62, 361–381.
- (28) Kool, E. T. (1997) Preorganization of DNA: Design principles for improving nucleic acid recognition by synthetic oligonucleotides. *Chem. Rev.* 97, 1473–1487.
- (29) Lima, W. F., Monia, B. P., Ecker, D. J., and Freier, S. M. (1992) Implication of RNA Structure on Antisense Oligonucleotide Hybridization Kinetics. *Biochemistry* 31, 2055–2061.
- (30) Corey, D. R. (1995) 48000-fold Acceleration of Hybridization by chemically modified Oligonucleotides. *J. Chem. Am. Soc.* 117, 9373–9374.
- (31) Iyer, M., Norton, J. C., and Corey, D. R. (1995) Accelerated Hybridization of Oligonucleotides to Duplex DNA. *J. Biol. Chem.* 270, 14712–14717.
- (32) Smulevitch, S. V., Simmons, C. G., Norton, J. C., Wise, T. W., and Corey, D. R. (1996) Enhancement of strand invasion by oligonucleotides through manipulation of backbone charge. *Nat. Biotechnol.* 14, 1700–1704.

BC970170+

Extending Insulin Action in Vivo by Conjugation to Carboxymethyl Dextran

Miroslav Baudyš,[†] Didier Letourneur,[‡] Feng Liu,[†] Don Mix,[†] Jacqueline Jozefonvicz,[‡] and Sung Wan Kim^{*†}

Department of Pharmaceutics and Pharmaceutical Chemistry/Center for Controlled Chemical Delivery, Biomedical Polymers Research Building, Room 205, University of Utah, Salt Lake City, Utah 84112, and Laboratoire de Recherches sur les Macromolécules, CNRS URA 502, Université Paris Nord, Avenue J. B. Clement, 93430 Villetaneuse, France. Received October 15, 1997; Revised Manuscript Received December 18, 1997

The biochemical and pharmacological properties of bioactive peptides and proteins can be altered by conjugation with polymers. This report describes site-specific attachment of insulin to activated carboxyl groups of carboxymethyl dextran (CMD, MW = 51 000) through the GlyA1 insulin amino group. On average, three or four insulin molecules were grafted to a CMD linear chain. Coupled insulin molecules were properly folded, and the bioactivity of conjugated insulin in the blood glucose depression assay was 9.6 IU/mg, which was only 2.6 times less than that for native insulin. The cell growth study indicated that the CMD–insulin conjugate was as mitogenic as insulin on vascular smooth muscle cells, whereas the starting CMD polymer was not. The insulin receptor binding constant of the conjugate ($3.6 \times 10^9 \text{ M}^{-1}$) compared well with that of native insulin ($7.6 \times 10^9 \text{ M}^{-1}$), indicating that the CMD chain does not present any major constraints to binding. Plasma clearance of CMD–insulin obeyed a two-compartment pharmacokinetic (PK) model with a CMD–insulin conjugate plasma elimination half-life of 114.1 min, which was significantly longer than that of soluble Zn–insulin (12.4 min). In contrast, pharmacodynamic (PD) profiles (blood glucose lowering effects) after intravenous (iv) administration of the conjugate or insulin in rats were not different. Subcutaneous (sc) administration of the conjugate resulted in a significantly prolonged plasma profile with a noncompartmental PK parameter mean residence time (MRT) of 103.5 min which was significantly longer than that of soluble Zn–insulin (40.5 min). This was reflected in the protracted PD effect of sc administered conjugate with time needed to reach minimum glucose concentration T_{nadir} of 95.7 min, which was significantly longer than that of insulin (62 min). We conclude that the conjugation of insulin to CMD leads to a bioactive conjugate with a delayed sc PD profile showing prolonged response, resembling intermediate acting insulin preparations.

INTRODUCTION

Since the 1980s, many proteins with great therapeutic potential have become available due to enormous progress in recombinant DNA techniques. Their clinical efficiency, however, is often limited (compromised) due to a short half-life in vivo, instability, or immunogenicity. One general approach for overcoming these shortcomings is conjugation of a protein drug to soluble polymeric carriers such as poly(vinyl alcohol) (1), poly(ethylene glycol) (2), or dextran (3), which usually results in a significantly longer circulatory half-life, increased stability, and attenuated immunogenicity.

Originally, unmodified dextran activated with cyanogen bromide or periodate was used for protein coupling (4). Functionalized dextran derivatives as protein carriers, such as carboxymethyl dextran (CMD)¹ or diethyl-aminoethyl dextran, were introduced and studied later (5). For instance, the superoxide dismutase–carboxymethyl dextran conjugate has been shown to have a long plasma half-life compared to that of the free enzyme because of impaired glomerular filtration and tissue

interaction (6). In this case, the enzyme was coupled by periodate oxidation. Others took advantage of functional carboxyl groups on CMD to prepare stable enzyme conjugates by using water-soluble carbodiimide as the activating agent (7–9). The use of carbodiimide activation is, however, problematic because of the well-recognized tendency of the activated intermediate to undergo an O- to N-acyl shift to form more stable nonreactive N-acyl urea derivatives (10).

Insulin, a polypeptide hormone discovered more than 70 years ago (11), is the only drug used to control type I diabetes. As for any polypeptide hormone in general, its half-life in circulation is short, ranging from about 10 min for rats to about 40 min for humans (12, 13). As a consequence, soluble insulin action after intravenous (iv) or subcutaneous (sc) administration is relatively short-

* To whom correspondence should be addressed. Telephone: (801) 581-6654. Fax: (801) 581-7848. E-mail: rburns@pharm.utah.edu.

[†] University of Utah.

[‡] Université Paris Nord.

¹ Abbreviations: CMD, carboxymethyl dextran; IBCF, isobutylchloroformate; TBA, tributylamine; MWCO, molecular weight cutoff; DMSO, dimethyl sulfoxide; FPLC, fast protein liquid chromatography; RI, refractivity index; SEC, size exclusion chromatography; PBS, phosphate-buffered saline; IR, insulin receptor; MEM, minimum essential medium; FCS, fetal calf serum; SMC, smooth muscle cell; PK, pharmacokinetic(s); PD, pharmacodynamic(s); EDTA, ethylenediaminetetraacetic acid; BGL, blood glucose level; AUC, area under the curve; AUMC, area under the first moment curve; MRT, mean residence time; MAT, mean absorption time.

lived. This makes it necessary to prolong action of exogenously given insulin in diabetic patients. The basic principle of prolonging insulin action used in current pharmaceutical formulations is to delay the absorption after sc injection by altering the solubility of insulin at physiological pH (14). In practical terms, insulin is administered as a suspension which dissolves slowly.

We decided to investigate whether the coupling of short-lived insulin to carboxymethyl dextran used as a polymeric carrier would substantially increase the insulin half-life. Early experiments with insulin conjugated to unmodified dextran activated with cyanogen bromide or periodate yielded conflicting bioactivity data (15, 16). The effect of the size of the dextran carrier was also ambiguous and contradictory (17–19). These incompatibilities, from today's point of view, can likely be explained by a rather broad molecular weight distribution of commercial dextran fractions used, chain fragmentation by aggressive activation methods, and inadequate purification and physicochemical characterization of conjugates.

By using well-defined CMD for insulin conjugation, we hoped to substantially increase insulin half-life since it has been shown that polyanionic CMD is retained in circulation for a long period of time (20). We employed a mixed anhydride method to selectively activate CMD carboxyl groups under defined conditions so that we could control the site of attachment of CMD to the insulin molecule (21). We characterized the conjugate physicochemically and studied its insulin receptor binding features as well as its effect on *in vitro* smooth muscle cell proliferation and the hypoglycemic effect *in vivo*. Finally, we evaluated the pharmacokinetic properties of the CMD–insulin conjugate in rats and found that its elimination rate is significantly smaller than that of insulin and, consequently, the sc pharmacodynamic profile shows a delayed, prolonged response, resembling the intermediate acting insulin preparations (14).

EXPERIMENTAL PROCEDURES

Materials. All chemicals were commercially available and used without further purification. Bovine Zn–insulin was obtained from Sigma Chemical Co. (St. Louis, MO) and GlyA1–glucosyl insulin (bovine) was synthesized according to ref 21. Ethylenediaminetetraacetic acid, dansyl chloride, ammonium bicarbonate, and Trizma base were also purchased from Sigma. Acetic and iodoacetic acids, isobutylchloroformate (IBCF), tributylamine (TBA), urea, and all other reagents as well as all organic solvents used were at least ACS grade and were purchased from Aldrich Chemical Co. (Milwaukee, WI). Carboxymethyl dextran was prepared from dextran T-40 (Pharmacia AB, Uppsala, Sweden) using monochloroacetic acid under alkaline treatment as described previously (22). Spectra/Por dialysis tubing (MW cutoff of 3500) was purchased from Spectrum Medical Industries (Houston, TX). Insulin radioimmunoassay kits from ICN Pharmaceuticals (Costa Mesa, CA) were used. Trypsin–ethylenediaminetetraacetic acid (EDTA), fetal calf serum (FCS), and Eagle's minimal essential medium (MEM) were from Gibco BRL (Cergy-Pontoise, France). Twenty-four-well tissue cultures dishes from Costar (Cambridge, MA) were used. 6-[³H]Thymidine (20 Ci/mmol) was from Amersham (Les Ulis, France).

Synthesis and Purification of the CMD–Insulin Conjugate. One hundred milligrams of freeze-dried CMD [degree of substitution, 0.61 mol of carboxymethyl group/mol of glucose (dextran unit), COOH form], subsequently dried under high vacuum over P₂O₅, was

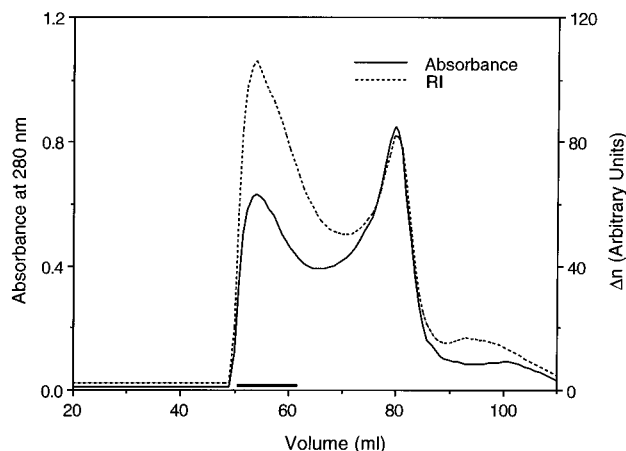


Figure 1. Purification of the CMD–insulin conjugate on a preparative 16/60 Superdex 75 column (Pharmacia AB). The pooled fraction containing the conjugate is marked by a horizontal bar. See Experimental Procedures for details.

dissolved in 2 mL of dry DMSO (three parallel experiments); 25, 50, or 75 μ mol of TBA and 25, 50, or 75 μ mol of IBCF were added at room temperature, and the mixture was stirred for 15 min under N₂. Simultaneously, three fixed amounts of insulin (145 mg, 25 μ mol) were dissolved in 3 mL of dry DMSO and mixed with the activated CMD solutions, irrespective of the IBCF amount used for activation. Additional TBA (25, 50, or 75 μ mol) was added, and the resulting solutions were stirred overnight at room temperature under N₂. The mixtures were then diluted with 3 volumes of ice cold water, dialyzed exhaustively against 0.02% NH₄HCO₃, and then freeze-dried. Separation of the conjugate and uncoupled insulin was achieved using FPLC on a HiLoad 16/60 Superdex 75 column (Pharmacia AB) in 0.05 M Tris-HCl buffer (pH 8.0) containing 0.5 M NaCl at a flow rate 1 mL/min. Fifty milligrams of the mixture in 2 mL of the buffer was injected, and the refractive index (RI) and A₂₈₀ of the eluent were continually monitored. The first major peak eluted giving both A₂₈₀ and RI response (Figure 1) was pooled, dialyzed against 0.02% NH₄HCO₃, and lyophilized. To remove traces of physically bound insulin, rechromatography under identical conditions was necessary.

CMD–Insulin Conjugate Chemical Characterization. The relative distribution of insulin covalently attached to polydisperse CMD in the purified conjugate fraction was examined by size exclusion chromatography on an analytical Superose 12 column (10 mm $\times$ 30 cm) (Pharmacia AB) using FPLC. The column was eluted with 0.05 M Tris-HCl buffer (pH 8.0) at a flow rate 0.4 mL/min at 22 $^{\circ}$ C. A 0.2 mL sample ($\sim$ 1 mg of CMD–insulin/mL) was injected, and the RI and A₂₈₀ of the eluent were continuously monitored.

UV absorption spectra were obtained on a Perkin-Elmer UV/Vis Lambda 19 spectrophotometer. CD absorption spectra for CMD–insulin conjugates were measured on a JASCO J-720 spectropolarimeter. In this case, samples (1 mg/mL or lower) were dissolved in phosphate-buffered saline (PBS) (0.01 M phosphate and 0.15 M NaCl at pH 7.4) containing 0.01% azide, and the ellipticity of the solution was measured in a cylindrical quartz cell (0.1 cm optical path length).

Free N-terminal amino acids in insulin conjugated to CMD were determined by the N-terminal dansylation technique. Released dansyl amino acids were identified after acid hydrolysis (6 N HCl, 105 $^{\circ}$ C, 8 h) by two-dimensional thin layer chromatography (23).

The insulin content in the conjugates was determined by two independent methods. First, A_{276} of the CMD–insulin conjugate of known concentration was measured and the insulin content calculated assuming that A_{276} of the 0.1% insulin solution is 1.00 (24). Alternatively, the insulin amount attached to CMD was determined by amino acid analysis.

In Vitro Cell Growth Studies. Thoracic aorta smooth muscle cells (SMCs) from Sprague-Dawley rats were isolated as previously described (25, 26). SMCs grown from aorta explants were cultured in MEM supplemented with 10% FCS and 2 mM L-glutamine at 37 °C in a humidified atmosphere containing 5% CO₂. Cultures of third and fourth passage were used throughout the study. The nature of SMCs was confirmed by the aspect of confluent cultures (hills and valleys pattern) and by positive immunofluorescence for α -smooth muscle actin (27).

SMCs (10^4) were plated into 16 mm multiwell plates in MEM containing 10% FCS. After 48 h, cell growth was arrested (G_0) by placing them in medium with 0.4% FCS for 72 h. Cell growth was initiated by changing the medium to MEM with 2% FCS and adding insulin, CMD, or the CMD–insulin conjugate (26). The control cells were exposed to medium with 2% FCS and positive control cells to 10% FCS. After 3 days, cells in quadruplicate wells were harvested after dislodging them with 0.05% trypsin/0.5 mM EDTA treatment and then counted using a Coulter Counter ZM (Coultronics, Beds, U.K.). The net growth of SMC in cultures was obtained by subtracting the cell number at the end of the experiment from the starting cell number (at the time the cells were released from G_0), and results were expressed as the net growth above the control.

The effect on cell proliferation was also assessed with the tritiated thymidine uptake method as previously described (26). Confluent SMCs were placed in MEM and 0.4% FCS for 48 h. Then, the cells were incubated with or without (control) the tested compounds for 24 or 48 h. Cells were pulsed with 1 μ Ci per well of [³H]-thymidine for the last 3 h. At the end of the assay, the plates were washed, DNA was precipitated with 10% trichloroacetic acid and solubilized, and radioactivity was determined by β -liquid scintillation counting (LKB-Pharmacia, Uppsala, Sweden).

Insulin Receptor Binding Studies. A rat insulinoma cell line (RINm5F) was used for CMD–insulin conjugate insulin receptor (IR) binding studies. The cells were grown in 75 mL flasks in MEM supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 50 units/mL penicillin, and 50 μ g/mL streptomycin. The cells were harvested at the exponential growth phase, 4 days after seeding, using a calcium- and magnesium-free medium at 4 °C. The harvested cells were washed and resuspended in the binding buffer (15 mM Tris, 120 mM NaCl, 1.2 mM MgSO₄, 5 mM KCl, 10 mM glucose, 15 mM sodium acetate, 1% bovine serum albumin, 20 μ g/mL bacitracin, and 50 μ g/mL merthiolate) at a final concentration of 1.5×10^7 cells/mL. The cell suspension (200 μ L) was mixed with 200 μ L of insulin solution, insulin concentration varying from 10^{-11} to 4×10^{-10} M. The insulin solution consisted either of pure [¹²⁵I]insulin having an activity of ~ 300 μ Ci/ μ g (CIS Biointernational, Saclay, France) or a mixture of [¹²⁵I]insulin and a particular insulin derivative, specifically unlabeled insulin, GlyA1-glucosyl insulin (28), or the CMD–insulin conjugate. The molar ratios of 2:1, 1:1, and 1:2 between unlabeled insulin or the insulin conjugate and [¹²⁵I]-insulin were usually used. The incubation was per-

formed at 4 °C for 1 h. The fractions of bound and free insulin were separated by centrifugation. The cell pellet was resuspended in 400 μ L of binding buffer and the whole procedure repeated five times to remove insulin nonspecifically adsorbed. The bound radioactivity was measured in a γ -counter (LKB-Pharmacia). First, the adsorption isotherm for pure [¹²⁵I]insulin was measured, and the results were replotted according to Scatchard (29) to determine its affinity constant for IR. In the next step, the binding isotherm for a mixture of [¹²⁵I]insulin and the insulin derivative, with their molar ratio fixed at 2:1, 1:1, or 1:2, was acquired, and the affinity constant for a particular insulin derivative was then calculated.

Blood Glucose Depression Assay. The biological activity of CMD–insulin conjugates was tested in a rat model (male Sprague-Dawley rats, 300 ± 25 g) by measuring blood glucose depression levels. All procedures in handling animals adhered to the *Principles of Laboratory Animal Care* (NIH Publication 85-23, revised in 1985). The animals fasted overnight (16 h) prior to the experiment. Water was allowed *ad libitum*. In the morning, the rats ($n = 6-9$) were anesthetized with sodium pentobarbital intraperitoneally (21). Each animal received an intravenous (iv) injection (tail vein) of insulin (3.4 nmol/mL NS/kg) or the CMD–insulin conjugate (equimolar amount attached insulin dose/mL NS/kg). Blood samples were taken through a catheter introduced into the jugular vein 15 and 5 min before injection and 15, 30, 60, 90, 120, 180, and 240 min after injection. Blood glucose levels (BGLs) were measured with an Accucheck III blood glucose monitor (Boehringer Mannheim, Indianapolis, IN). Bioactivity of CMD–insulin conjugates was calculated from AUC (area under the curve) values and the AUC value for the same dose of unmodified insulin.

Pharmacokinetic and Pharmacodynamic Studies. Male Sprague-Dawley rats weighting 250–350 g were used throughout the studies. At least six animals were used in individual experiments. The basic animal protocol was the same as that described above (see the bioactivity determination).

For the iv route of administration and pharmacokinetic (PK) and pharmacodynamic (PD) analysis of the CMD–insulin conjugate, blood samples were taken at 0, 3, 7, 15, 30, 60, 90, 120, 180, 240, and 300 min. EDTA/plasma samples were prepared and stored at -20 °C until analysis. Blood sample volumes were replaced with the same volume of saline (37 °C). Three different CMD–insulin doses of 0.125, 0.25, and 0.5 mg/mL/kg dissolved in 0.01 M PBS (pH 7.4) were administered through a tail vein of rats that fasted overnight. Insulin reference for PK analysis (0.5 IU/mL) was injected at a dose 0.5 IU/kg, and blood samples were collected at 0, 1, 3, 5, 7, 10, 15, 20, 30, and 45 min; for PD analysis, blood samples were taken at 0, 15, 30, 60, 90, 120, 180, 240, and 300 min.

For the subcutaneous (sc) route of administration and PK analysis, blood samples were taken at 0, 10, 20, 30, 45, 60, 90, 120, 180, and 240 min, while for PD analysis, blood samples were obtained at 0, 30, 60, 90, 120, 180, 240, 300, and 360 min. Three different CMD–insulin doses of 0.125, 0.25, and 0.5 mg/mL/kg in PBS were administered sc into the nape of the neck. Insulin reference was injected at a dose 0.5 and 1.0 IU/mL/kg.

Usually, both BGLs and plasma insulin concentrations (EDTA/plasma) were determined. BGLs were immediately measured using Chemstrip bG reagent strips with an Accucheck III blood glucose monitor. Plasma insulin and CMD–insulin concentrations were determined with

a commercial insulin radioimmunoassay kit (ICN Pharmaceuticals). The CMD–insulin standard curve was used for radioimmunoassay of plasma CMD–insulin.

Statistical Analysis. Both iv administered insulin and CMD–insulin appear to follow a two-compartment PK model. Thus, the data were fitted by a nonlinear least-squares fit into biexponential eq 1, where preexponential and exponential terms have the units $\mu\text{units/mL}$ and min^{-1} , respectively.

$$C = Ae^{-\alpha t} + Be^{-\beta t} \quad (1)$$

Data were analyzed using the PKAnalyst software package (MicroMath, Salt Lake City, UT) that provided basic PK parameters, including biological plasma half-life and area under the curve (AUC). Basal insulin levels were considered to be $20 \mu\text{units/mL}$, and the area below this threshold was subtracted from AUC.

To process PK data of the sc as well as iv administered CMD–insulin conjugate and reference insulin, statistical moment-derived noncompartmental analysis was applied (30). The AUCs for insulin and CMD–insulin plasma concentration–time curves above controls were calculated by the linear trapezoidal method. If the insulin levels were still higher than those of control experiments ($20 \mu\text{units/mL}$), the last two points were extrapolated to a time point where the plasma insulin concentration reached $20 \mu\text{units/mL}$ and this region was also included. The areas under the first momentum curve (AUMC) were also calculated and used to calculate the mean residence time (MRT) of CMD–insulin and insulin after iv as well as sc administration. Finally, mean absorption times (MAT) for sc administered CMD–insulin and insulin were calculated, using eq 2.

$$\text{MAT} = \text{MRT}_{\text{sc}} - \text{MRT}_{\text{iv}} \quad (2)$$

The time needed to reach the maximum plasma insulin and CMD–insulin concentration (T_{max}) after sc administration was also used as an independent PK parameter.

For PD analysis, two parameters reflecting the time dependency of BGL after CMD–insulin or insulin administration and prolongation of the blood glucose lowering effect were selected. The most important PD parameter measured was the time to reach minimum BGL (T_{nadir}). The duration over which BGL remained below 70% of the initial value ($T_{70\% \text{ BGL}}$) was taken as the second PD parameter.

For the assessment of the statistical significance of the difference, the Student's *t* test, with one- and two-way analysis of variance, was used. Results were termed significant when $P < 0.02$. To pool data for different insulin or CMD–insulin conjugate doses (see Results and Discussion), *P* had to be > 0.3 .

RESULTS AND DISCUSSION

CMD–Insulin Conjugate Synthesis, Purification, and Characterization. Previously, we have shown that the GlyA1 insulin amino group is a preferential site of reaction and attachment of any structural moiety containing a carboxylic group activated by a mixed anhydride method (21). We decided to use this activation method after having established that the free acid (COOH) form of CMD is easily soluble in dry DMSO. Exclusion of water enabled us to activate only a small molar fraction of CMD carboxylic groups. In the first experiment, only 8 mol % of available carboxyl groups in the polymer, which corresponded to about 12 groups per CMD molecule, was activated with IBCF and reacted

with an equimolar insulin amount to minimize cross-linking. No insoluble material was formed, indicating no cross-linking occurred. Separation of the conjugate from uncoupled insulin was accomplished by size exclusion chromatography on a Superdex 75 column (Figure 1). The refractive index RI (CMD) and A_{280} (insulin) of the eluent were monitored. The first peak eluted, containing the conjugate, was collected, dialyzed, freeze-dried, and rechromatographed under identical conditions to remove residual, physically bound insulin since insulin is well-known for its tendency to form physical dimers and hexamers (24). The conjugate emerged from the Superdex 75 column, however, in the void volume peak, so there was a possibility that a soluble, high-molecular-weight, insulin-cross-linked material was present in the same fraction (see below). Using analytical Superose 6 and 12 columns that have high-molecular-weight exclusion limits, for both proteins and dextrans, we established that purified conjugate is chromatographically homogeneous (single peak) and that the RI to A_{280} signal ratio was practically constant, which indicates that the insulin moiety was evenly distributed (attached) with regard to the polydispersity of CMD. This means that a constant insulin amount was attached per arbitrary unit length of linear CMD chain. We excluded the possibility that the insulin molecule behaved like a cross-linking agent bridging two or more CMD molecules, because no significant difference between the retention volume of free CMD and the CMD–insulin conjugate was found. To have definite proof that no physically bound insulin was present in the conjugate fraction, the conjugate was again examined on Superose 6 and 12 columns with the elution buffer containing 8 M urea. Under such conditions, the insulin molecule unfolds and can no longer form dimers and/or hexamers. No peak corresponding to the unfolded insulin monomer was detected. Thus, the structure of the conjugate corresponds to the linear CMD chain with grafted insulin molecules. For future preparative production, repetitive size exclusion chromatography is not an ideal method for the conjugate purification because of its limited capacity. An obvious simple and efficient alternative method is pH-dependent isoelectric precipitation of free insulin (pH range 5.0–6.0), because the solubility of the CMD–insulin conjugate is not significantly affected (as compared to the solubility at pH 8.0). By using this procedure, at least one inconvenient chromatographic step can be avoided.

The UV spectrum of the CMD–insulin conjugate was identical to the UV spectrum of insulin, so no chemical modification of aromatic amino acids occurred. The far-UV CD spectrum was indistinguishable from that of Zn-free insulin or GlyA1-glucosyl insulin (21), indicating that the attached insulin molecule is properly folded (data not shown). We have also studied the site of the insulin molecule attachment to CMD carboxyl groups with the N-terminal dansylation technique. Insulin is a two-chain molecule with two N-terminal amino acids, GlyA1 and PheB1. The third insulin amino group comes from LysB29 (24). Since only DNS-Phe and DNS- ϵ -Lys were detected by two-dimensional TLC (23), we concluded that insulin molecules were attached to the CMD chain by an amide bond through the GlyA1 amino group. Insulin molar content in the CMD–insulin conjugate was determined by UV absorption spectrometry and amino acid analysis. As an average, 3.26 mol of insulin was found per mole of CMD. Thus, only 25% of activated COOH groups reacted with insulin, even though equimolar amounts of insulin and activated COOH groups were used. To shed more light on the limiting step, we

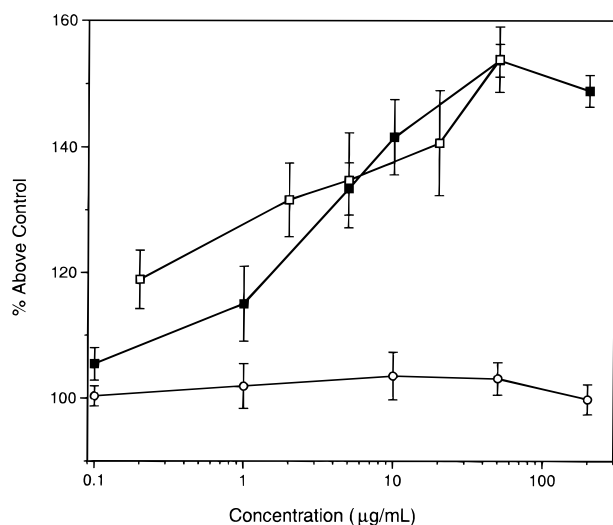


Figure 2. SMC growth stimulation by insulin and the CMD-insulin conjugate. Rat aorta SMCs were plated in 2% serum containing insulin (□), the CMD-insulin conjugate (■), or the starting CMD polymer (○). After 3 days, the cell numbers were counted. The net growth was then determined and normalized by the control values in the presence of 2% serum. Results are mean $\pm$ SD of two experiments performed in quadruplicate.

increased the amount of activated COOH groups to 16 mol % (equivalent to 24 mol % of available carboxyl groups in the polymer), keeping the insulin amount constant (equivalent to 8 mol % of available COOH groups). Surprisingly, the insulin content in the conjugates increased only marginally to 3.5–3.9 mol of insulin/mol of CMD in two separate experiments. In both cases, no cross-linking was detected. Apparently, a maximum of three or four insulin molecules can be conjugated per CMD chain, and then the remaining activated carboxylic groups become unavailable for coupling.

CMD-Insulin Conjugate Studies in Vitro. The in vitro effects of the CMD-insulin conjugate containing 3.5 mol of insulin/mol were studied with the mitogenic activity on vascular cells and the binding to the insulin receptor. The in vitro cell growth effects of the CMD-insulin conjugate were investigated by the assessment of cell counting and [^{3} H]thymidine uptake using rat aorta smooth muscle cells. The results shown in Figure 2 indicated that the CMD-insulin conjugate as well as native insulin, but not the starting CMD polymer, increased SMC growth. [^{3} H]Thymidine incorporation was used as an alternative method to measure the mitogenic effect, and also indicated after 24 and 48 h (data not shown) that the CMD-insulin conjugate enhanced cell proliferation in a concentration-dependent manner (from 0.1 to 50 μ g/mL, i.e., about 20 nM to 10 μ M). These results indicated that insulin grafted to CMD kept its mitogenic activity on cells in culture.

The characteristics of CMD-insulin binding to insulin receptor (IR) were then measured in vitro in a competitive binding assay with [^{125} I]insulin. First, the binding isotherm for [^{125} I]insulin was obtained, and the binding constant was determined from a Scatchard plot (Table 1). Its magnitude is well within the range of those published by others (32, 33). As a next step, the binding constant for native insulin was determined to show that radiolabeling of insulin did not have a substantial negative effect on IR binding. Since the effect was negligible, we proceeded with GlyA1-glucosyl insulin and, finally, with CMD-insulin binding studies (Table 1). The modification of the GlyA1 insulin amino group with succinamidophenyl glucopyranoside (GlyA1-glucosyl insulin)

Table 1. Binding Constants (Mean $\pm$ SD) for the CMD-Insulin Conjugate and GlyA1-Glucosyl Insulin and the Insulin Receptor Using the RINm5F Cell Line

insulin derivative	binding constant (M^{-1})
native insulin	$(7.64 \pm 0.17) \times 10^9$
[^{125} I]insulin	$(6.37 \pm 0.14) \times 10^9$
GlyA1-Glc-insulin	$(2.46 \pm 0.06) \times 10^9$
CMD-insulin conjugate ^a	$(1.19 \pm 0.08) \times 10^{10}$
CMD-insulin conjugate ^b	$(3.61 \pm 0.11) \times 10^9$

^a Expressed in terms of conjugate concentration. ^b Expressed in terms of insulin concentration.

decreased the binding constant 3-fold (25). This is in agreement with previous studies aimed at modification of this particular amino group with low-molecular-weight compounds (31), which established that GlyA1 is not directly involved in, but is very close to, the IR binding region of the insulin molecule. The binding constant for the CMD-insulin conjugate can be expressed in terms of either the conjugate concentration or the insulin concentration (Table 1). The two apparent affinity constants can be viewed as a reflection of two different CMD-insulin conjugate binding modes and consequently two different models of binding. The first binding constant, expressed in terms of conjugate concentration, shows that the affinity of the conjugate for IR is 4.8 times higher than that of GlyA1-glucosyl insulin and even 1.5 times higher than that of native insulin. This can be physically interpreted in such a way that only one insulin molecule in the conjugate binds IR and that a positive cooperative binding of the CMD chain increased the affinity for IR. In the second alternative, the CMD-insulin binding constant, expressed in terms of conjugated insulin concentration, is much closer to the GlyA1-glucosyl insulin binding constant (only 1.5 times higher) and likely better reflects the physical reality. Here, one can view individual conjugated insulin molecules as being statistically distributed along CMD chain and, thus, more or less independent. The insulin modification on GlyA1 in the CMD-insulin conjugate, as in the case of GlyA1-glucosyl insulin, leads to decreased affinity. However, this decrease is smaller than that for GlyA1-glucosyl insulin (Table 1), because after the binding of the first insulin molecule the remaining insulin molecules attached to the CMD chain have a higher probability of interaction with cell surface IR (entropic factor). In other words, the binding of one insulin molecule in the conjugate facilitates the binding of other insulin molecules in the conjugate to proximal IRs on the cell surface. The bioactivity data (see below), on the other hand, suggest that the first model better describes the interaction with IR on the cell surface.

Blood Glucose Depression Assay. The in vivo activity of CMD-insulin conjugates was first tested by blood glucose depression test in rats after an iv injection of 20 μ g of conjugated insulin/kg/mL. Its bioactivity was found to be 9.6 ± 2.1 IU/mg of conjugated insulin or 2.5 ± 0.5 IU if expressed per milligram of the conjugate. Consequently, conjugated insulin is 2.6 times less active than native insulin (~ 25 IU/mg) in this bioassay. The earlier studies with dextran-insulin conjugates found either a very low insulin bioactivity of about 1.0 IU/mg of conjugated insulin (15, 18) or a high insulin bioactivity (16, 17, 19) which was comparable to that of native insulin. Thus, the bioactivity value of insulin coupled to CMD is between these two extremes. For comparison, the bioactivity of structurally related GlyA1-glucosyl insulin is 22.2 ± 4.2 IU/mg (21), which is very close to that of native insulin. Apparently, the presence of the

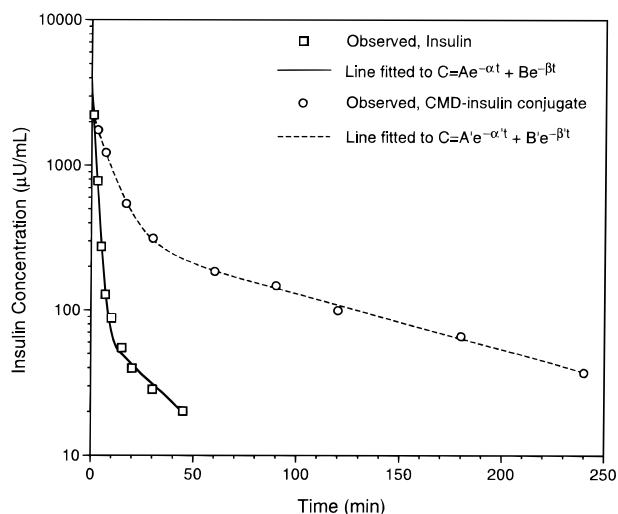


Figure 3. Fit of the observed plasma insulin concentrations and plasma CMD–insulin conjugate concentrations following intravenous administration of insulin (0.5 IU/kg) or the CMD–insulin conjugate (0.125 mg/kg) in rats ($n = 8$) to the equation for a two-compartment pharmacokinetic model: $A = 3743 \mu\text{units/mL}$, $B = 60 \mu\text{units/mL}$, $\alpha = 0.702 \text{ min}^{-1}$, and $\beta = 0.056 \text{ min}^{-1}$; and $A' = 2056 \mu\text{units/mL}$, $B' = 316 \mu\text{units/mL}$, $\alpha' = 0.124 \text{ min}^{-1}$, and $\beta' = 0.0089 \text{ min}^{-1}$.

CMD polymeric chain carrier interferes with insulin activity in vivo or a fraction of attached insulin molecules cannot exert its action. Thus, we investigated in detail the pharmacokinetic (PK) and pharmacodynamic (PD) properties of one CMD–insulin conjugate in rats using iv and sc administration routes.

Intravenous Administration of the CMD–Insulin Conjugate. Intravenous administration of soluble Zn–insulin (0.5 IU/kg) resulted in plasma concentration–time profiles characteristic of an apparent two-compartment PK model, best described by biexponential eq 1 (Figure 3). The distribution half-life was only $1.03 \pm 0.22 \text{ min}$, while the overall elimination half-life was found to be $12.4 \pm 3.4 \text{ min}$, which are consistent with results reported by others (12, 34). In contrast, iv administration of 0.125 mg/kg of the CMD–insulin conjugate (3.47 mol of insulin/mol) resulted in a significantly prolonged plasma profile (Figure 3). Another CMD–insulin dose, 0.25 mg/kg, was also tested. Since both CMD–insulin doses provided very similar constants (dose-independent) with statistically insignificant differences, the data were pooled giving a distribution half-life of $4.94 \pm 1.06 \text{ min}$ and an elimination half-life of $114.1 \pm 28.6 \text{ min}$. Thus, the main goal of the study, i.e., the substantial increase of insulin elimination half-life, was achieved. To our knowledge, this study is the first to report this effect, since all previous studies with insulin–dextran conjugates failed to investigate this important parameter (15–19). Interestingly, iv PD profiles of the CMD–insulin conjugate and insulin were not different (Figure 4), even though the CMD–insulin mean residence time (MRT) for iv administration was 57.7 min (average for 0.125 and 0.25 mg/kg doses), which was very significantly increased as compared to the native insulin MRT of 3.16 min (Table 2). Our findings differ from those of Torchilin et al. (35), who reported that the hypoglycemic effect of periodate activated dextran-bound insulin was much longer than that of native insulin after iv administration in dogs. In addition to the differences in the conjugation chemistry (chloroformate activation of carboxyl groups versus periodate activation of hydroxyl groups), another plausible explanation may be the difference in the basal metabolic

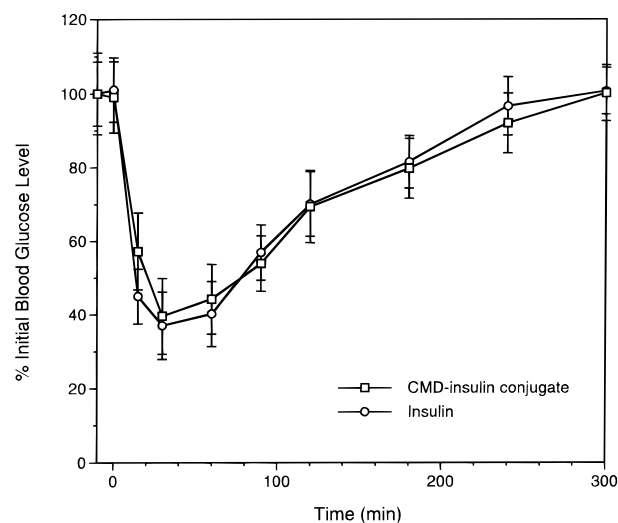


Figure 4. Blood glucose levels (mean $\pm$ SD) after intravenous administration of insulin (0.5 IU/kg, $n = 7$) and the CMD–insulin conjugate (0.25 mg/kg, $n = 8$) into the tail vein of rats that had fasted.

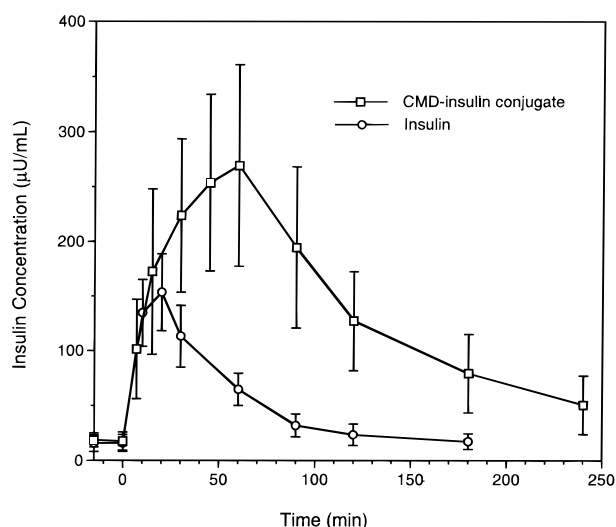
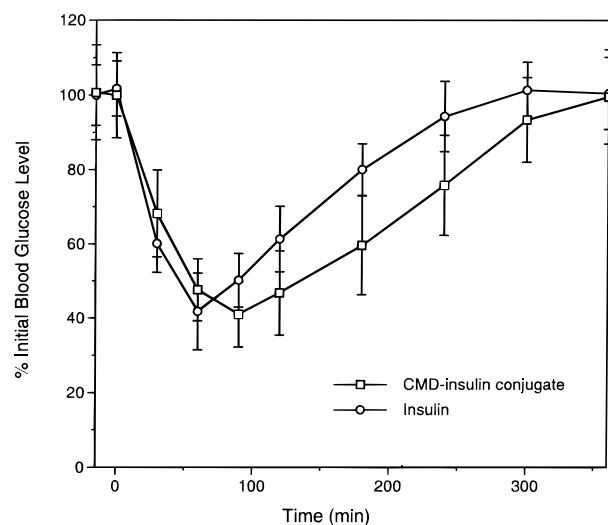
rate between the two animals (36). Rats, with a faster metabolism, also react faster to a blood glucose level drop with feedback mechanisms to bring the blood glucose concentration back to physiological levels. Consequently, blood glucose lowering effects of the conjugate in the later phase of PD response are suppressed, even though high plasma levels of the conjugate prevail.

Subcutaneous Administration of the CMD–Insulin Conjugate. Insulin administration into the back of the neck of rats (0.5 and 1.0 IU/kg) resulted in plasma insulin concentration–time profiles characteristic of a PK curve controlled by the absorption phase being a rate-limiting step (Figure 5). The insulin MRT for sc administration was 40.8 min (Table 2, pooled data for 0.5 and 1.0 IU/kg doses) and was not significantly different from that of the insulin mean absorption time (MAT), equal to 37.7 min (Table 2, average pooled data). Administration of the CMD–insulin conjugate by the sc route (0.125 mg/kg) resulted in a significantly prolonged plasma profile (Figure 5) in accord with iv data. Another CMD–conjugate dose, 0.25 mg/kg, was tested. As for the iv route, both doses provided parameters with statistically insignificant differences. All data were pooled, regardless of dose, for final PK evaluation using the statistical moment-derived noncompartmental approach. The MRT of insulin conjugate for sc administration (103.5 min) is significantly longer than that of insulin. Since MATs of insulin and CMD–insulin are not significantly different (Table 2), both free insulin and conjugated insulin are absorbed from sc depot at comparable rates, but are eliminated at different rates, similar to those found for the iv route of administration. In agreement with these results, the times T_{max} (Table 2) to reach the maximum plasma level for insulin and the conjugate differ substantially. The average T_{max} was more than 3 times longer for CMD–insulin than for free insulin. PD profiles of sc administered intravenously equipotent doses of insulin (0.5 IU/kg) and CMD–insulin (0.25 mg/kg) reflect the differences in sc PK profiles (Figure 6). The CMD–insulin conjugate showed a protracted PD profile. This differs from the iv administrative route, where no significant differences in PD profiles were observed. This finding can be explained by much lower initial phase exogenous insulin plasma concentrations, as compared with those of iv administered samples, controlled by the

Table 2. Pharmacokinetic and Pharmacodynamic Parameters (Mean $\pm$ SD) for the CMD–Insulin Conjugate and Insulin Administered by either the Intravenous (iv) or Subcutaneous (sc) Route

insulin derivative	administration route	dose	MRT ^a (min)	MAT ^b (min)	T _{max} ^c (min)	T _{nadir} ^d (min)	T _{70% BGL} ^e (min)
insulin	iv	0.5 IU/kg ⁱ	3.16 $\pm$ 1.03 ^l	NA ^f	NA ^f	30.0 $\pm$ 8.9	ND ^g
	sc	0.25 IU/kg ^h	ND ^g	ND ^g	ND ^g	59.7 $\pm$ 11.0 ^m	65.3 $\pm$ 15.9 ^m
		0.5 IU/kg ⁱ	40.5 $\pm$ 11.5 ^m	37.4 $\pm$ 10.1	18.8 $\pm$ 5.6 ^m	62.0 $\pm$ 12.2 ^m	119.3 $\pm$ 19.9 ^m
		1.0 IU/kg ⁱ	41.2 $\pm$ 10.4 ^m	38.1 $\pm$ 11.0	20.5 $\pm$ 6.7 ^m	63.8 $\pm$ 10.9 ^m	131.4 $\pm$ 22.7 ^m
CMD–insulin	iv	0.125 mg/kg ^{h,k}	53.7 $\pm$ 12.3 ^l	NA ^f	NA ^f	33.1 $\pm$ 8.7	ND ^g
		0.25 mg/kg ⁱ	59.9 $\pm$ 10.1 ^l	NA ^f	NA ^f	31.5 $\pm$ 11.1	ND ^g
	sc	0.125 mg/kg ^{h,k}	98.6 $\pm$ 24.7 ^m	44.9 $\pm$ 10.6	62.8 $\pm$ 18.3 ^m	90.5 $\pm$ 17.3 ^m	103.6 $\pm$ 26.2 ^m
		0.25 mg/kg ⁱ	106.8 $\pm$ 28.6 ^m	46.9 $\pm$ 12.1	69.0 $\pm$ 13.7 ^m	94.3 $\pm$ 20.7 ^m	209.0 $\pm$ 51.1 ^m
		0.5 mg/kg ^j	ND ^g	ND ^g	ND ^g	102.1 $\pm$ 23.6 ^m	274.4 $\pm$ 46.9 ^m

^a Mean residence time. ^b Mean absorption time. ^c Time to reach maximum plasma insulin concentration. ^d Time to reach minimum blood glucose concentration. ^e Duration over which BGL remained below 70% of the initial value. ^f Not applicable. ^g Not determined. ^h Equipotent insulin and CMD–insulin doses. ⁱ Equipotent insulin and CMD–insulin doses. ^j Equipotent insulin and CMD–insulin doses. ^k A 0.125 mg sample of the CMD–insulin conjugate contains an insulin amount equivalent to 0.72 IU. ^l Statistically different corresponding iv pairs regardless of the dose ($P < 0.02$). ^m Statistically different corresponding sc pairs regardless of the dose ($P < 0.02$).

**Figure 5.** Plasma levels (mean $\pm$ SD) of insulin (0.5 IU/kg, $n = 7$) and the CMD–insulin conjugate (0.125 mg/kg, $n = 8$) after subcutaneous administration of the derivatives into the back of the neck of rats that had fasted.**Figure 6.** Blood glucose response (mean $\pm$ SD) following subcutaneous administration of insulin (0.5 IU/kg, $n = 8$) and the CMD–insulin conjugate (0.25 mg/kg, $n = 9$) into the back of the neck of rats that had fasted.

insulin or CMD–insulin absorption rate. The first quantitative PD parameter, T_{nadir} , the time needed to reach minimum BGL, was again dose-independent, for both insulin and the insulin conjugate (Table 2). How-

ever, T_{nadir} for the CMD–insulin conjugate was significantly longer than that for Zn–insulin, resembling intermediate-acting insulin preparations (14). The second quantitative PD parameter, $T_{70\% BGL}$, the time period over which BGL remained below 70% of the initial value, was, on the other hand, dose-dependent for both insulin preparations studied. This is quite obvious since, as the dose is decreased, the blood glucose lowering effect diminishes. Thus, Table 2 shows $T_{70\% BGL}$ for three equipotent doses of insulin (0.25, 0.5, or 1.0 IU/kg) and the CMD–insulin conjugate (0.125, 0.25, or 0.5 mg/kg). In all cases, $T_{70\% BGL}$ for the CMD–insulin conjugate was about twice as long as $T_{70\% BGL}$ for the corresponding insulin dose, again demonstrating the protracted blood glucose lowering effect of the CMD–insulin conjugate. The achieved protraction of insulin action still seems to be rather short for practical application. However, as discussed previously, the rat animal model is far from ideal, and a large animal model, such as dogs or pigs, should be used. Currently, we are planning PK and PD experiments in dogs. If the protraction of the insulin effect in dogs is still insufficient, obviously, the next step is to increase the length of the CMD chain. This should result in increased MRTs and possibly also MATs, both of which should lead to a further extended and prolonged action profile similar to that of insulin basal formulations. Similarly, the extension of the time action of insulin by chemical modification has been recently achieved through acylation of human insulin with fatty acids (37). In this case, however, the binding of acylated insulin to serum albumin in the interstitial liquid at the injection side was suggested (increase in MATs) to be mainly responsible for protracted action (38).

CONCLUSION

A bioactive CMD–insulin conjugate containing three or four insulin molecules was synthesized, purified, and characterized. By coupling the insulin molecule to the CMD chain with an amide bond, we achieved a significant increase in insulin half-life and protraction of the PD effect for the sc route, while the mean absorption time of the CMD–insulin conjugate was not significantly different from that of soluble insulin. Thus, prolongation of insulin action was realized with chemical modification, not by classical physical means through the manipulation of insulin solubility and consequently absorption at the sc injection site. Further studies will focus on optimization of the synthetic procedure to prepare a 1:1 conjugate on the molar basis. The effect of CMD molecular weight (chain length) will also be studied. Finally, PK and PD

properties of the optimized conjugate will be evaluated using a large animal model.

ACKNOWLEDGMENT

This work was supported by Centre National de Recherche Scientifique (CNRS), France, and by Macro-Med, Inc. The authors acknowledge E. F. Zhu and T. Avramoglou (LRM, Villeteuse, France) for the binding experiments.

LITERATURE CITED

- Hixson, H. F. (1973) Water-soluble enzyme-polymer grafts: Thermal stabilization of glucose oxidase. *Biotechnol. Bioeng.* 15, 1011–1016.
- Abuchowski, A., McCoy, J., Palczuk, N. C., van Es, T., and Davis, F. F. (1977) Effect of covalent attachment of polyethylene glycol on immunogenicity and circulating life of bovine liver catalase. *J. Biol. Chem.* 252, 3582–3586.
- Marshall, J., Humphreys, J. D., and Abramson, L. (1977) Attachment of carbohydrate to enzymes increases their circulatory lifetimes. *FEBS Lett.* 83, 249–251.
- Mikolajczyk, S. D., Meyer, D. L., Fagnani, R., Hagan, M. S., Law, K. L., and Starling, J. J. (1996) Dextran modification of a Fab'- β -lactamase conjugate modulated by variable pretreatment of Fab' with amine-blocking reagents. *Bioconjugate Chem.* 7, 150–158.
- Takakura, Y., Mahato, R. I., Nishikawa, M., and Hashida, M. (1996) Control of pharmacokinetic profiles of drug-macromolecule conjugates. *Adv. Drug Delivery Rev.* 19, 377–399.
- Fujita, T., Nishikawa, M., Tamaki, C., Takakura, Y., Hashida, M., and Sezaki, H. (1992) Targeted delivery of human recombinant superoxide dismutase by chemical modification with mono and polysaccharide derivatives. *J. Pharmacol. Exp. Ther.* 263, 971–978.
- Kobayashi, M., Yanagihara, S., and Ichishima, E. (1989) Preparation and characterization of Taka-amylase A attached to carboxymethyl dextran by water-soluble carbodiimide. *Agric. Biol. Chem.* 53, 3133–3138.
- Hattori, M., Nagasawa, K., Ametani, A., Kaminogawa, S., and Takahashi, K. (1994) Functional changes in β -lactoglobulin by conjugation with carboxymethyl dextran. *J. Agric. Food Chem.* 42, 2120–2125.
- Hattori, M., Imamura, S., Nagasawa, K., and Takahashi, K. (1994) Functional changes of lysozyme by conjugating with carboxymethyl dextran. *Biosci., Biotechnol., Biochem.* 58, 174–177.
- Heindel, N. D., Kauffman, M. A., Akyea, E. K., Engel, S. A., Frey, M. F., Lacey, C. J., and Egolf, R. A. (1994) Carboxymethyl dextran lactone: A preactivated polymer for amine conjugations. *Bioconjugate Chem.* 5, 98–100.
- Banting, F. G., and Best, C. H. (1922) Pancreatic extracts. *J. Lab. Clin. Med.* 7, 464–472.
- Schilling, R. J., and Mitra, A. K. (1992) Pharmacodynamics of insulin following intravenous and enteral administrations of porcine zinc insulin in rats. *Pharm. Res.* 9, 1003–1009.
- Howey, D. C., Bowsher, R. R., Brunelle, R. L., and Woodworth, J. R. (1994) [Lys(B28), Pro(B29)]-human insulin; A rapidly absorbed analogue of human insulin. *Diabetes* 43, 396–402.
- Brange, J. (1987) *Galenics of Insulin*, Springer-Verlag, Berlin and Heidelberg.
- Kagedal, L., and Akerstrom, S. (1971) Binding of covalent proteins to polysaccharides by cyanogen bromide and organic cyanates. I. Preparation of soluble glycine-, insulin-, and ampicillin-dextran. *Acta Chem. Scand.* 25, 1855–1859.
- Suzuki, F., Daikuhara, Y., Ono, M., and Takeda, Y. (1972) Studies on the mode of action of insulin: Properties and biological activity of an insulin-dextran complex. *Endocrinology* 90, 1220–1230.
- Molteni, L., and Scrollini, F. (1974) Methode pour prolonger l'action des medicaments par la formation de composés macromoléculaires. *Eur. J. Med. Chem.* 9, 618–620.
- Sakamoto, Y., Akanuma, Y., Kosaha, K., and Jeanrenaud, B. (1977) Comparative effects of native insulin and insulin-dextran complexes on the metabolism of adipose tissue. *Biochim. Biophys. Acta* 498, 102–113.
- Armstrong, K. J., Noall, N. W., and Stouffer, J. E. (1972) Dextran-linked insulin: A soluble high molecular weight derivative with biological activity *in vivo* and *in vitro*. *Biochem. Biophys. Res. Commun.* 47, 354–360.
- Takakura, Y., Fujita, T., Hashida, M., and Sezaki, H. (1990) Disposition characteristics of macromolecules in tumor-bearing mice. *Pharm. Res.* 7, 339–346.
- Baudys, M., Uchio, T., Mix, D., Wilson, D., and Kim, S. W. (1995) Physical stabilization of insulin by glycosylation. *J. Pharm. Sci.* 84, 28–33.
- Mauzac, M., and Jozefonvicz, J. (1984) Anticoagulant activity of dextran derivatives. Part I: Synthesis and characterization. *Biomaterials* 5, 301–304.
- Baudys, M., and Kostka, V. (1983) Covalent structure of chicken pepsinogen. *Eur. J. Biochem.* 136, 89–99.
- Blundell, T. L., Dodson, G. G., Hodgkin, D. C., and Mercola, D. (1972) Insulin: The structure in the crystal and its reflection in chemistry and biology. *Adv. Protein Chem.* 26, 279–402.
- Letourneur, D., Logeart, D., Avramoglou, T., and Jozefonvicz, J. (1993) Antiproliferative capacity of synthetic dextrans on smooth muscle cell growth. The model of derivatized dextrans as heparin-like polymers. *J. Biomater. Sci., Polym. Ed.* 4, 431–444.
- Chytry, V., Letourneur, D., Baudys, M., and Jozefonvicz, J. (1996) Conjugates of insulin with copolymers of N-(2-hydroxypropyl) methacrylamide: Effects on smooth muscle cell proliferation. *J. Biomed. Mater. Res.* 31, 265–272.
- Skalli, O., Rapraz, P., Trzeciak, A., Benzonana, G., Gillissen, D., and Gabbiani, G. (1986) A monoclonal antibody against α smooth muscle actin. A new probe for smooth muscle cell differentiation. *J. Cell. Biol.* 103, 2783–2796.
- Baudys, M., Uchio, T., Hovgaard, L., Zhu, E. F., Avramoglou, T., Jozefowicz, M., Rihová, B., Park, J. Y., Lee, H. K., and Kim, S. W. (1995) Glycosylated insulins. *J. Controlled Release* 36, 151–156.
- Dahlquist, F. W. (1978) The meaning of Scatchard and Hill plots. *Methods Enzymol.* 48, 270–299.
- Gibaldi, M., and Perrier, D. (1982) *Pharmacokinetics*, pp 409–417, Marcel Dekker, New York.
- Gammeltoft, S. (1984) Insulin receptors: Binding kinetics and structure–function relationship of insulin. *Physiol. Rev.* 64, 1321–1378.
- Schäffer, L. (1994) A model for insulin binding to the insulin receptor. *Eur. J. Biochem.* 227, 1127–1132.
- Murray-Rust, J., McLeod, A. N., Blundell, T. L., and Wood, S. P. (1992) Structure and evolution of insulins: Implications for receptor binding. *BioEssays* 14, 325–330.
- Sato, H., Terasaki, T., Okumura, K., and Tsuji, A. (1991) Effect of receptor up-regulation on insulin pharmacokinetics in streptozotocin-treated diabetic rats. *Pharm. Res.* 8, 563–569.
- Torchilin, V. P., Il'ina, E. V., Mazaev, A. V., Lebedev, B. S., Smirnov, V. N., and Chazov, E. I. (1977) Study of modified Sephadex-bound insulin in animal experiments. *J. Solid-Phase Biochem.* 2, 187–193.
- Spector, W. S. (1956) *Handbook of Biological Data*, p 258, W. B. Saunders, Philadelphia and London.
- Kurtzhals, P., Havelund, S., Jonassen, I., Kiehr, B., Larsen, U. D., Ribøl, U., and Markussen, J. (1995) Albumin binding of insulins acylated with fatty acids: Characterization of the ligand-protein interaction and correlation between binding affinity and timing of the insulin effect *in vivo*. *Biochem. J.* 312, 725–731.
- Markussen, J., Havelund, S., Kurtzhals, P., Andersen, A. S., Halstrøm, J., Hasselager, E., Larsen, U. D., Ribøl, U., Schäffer, L., Vad, K., and Jonassen, I. (1996) Soluble fatty acid acylated insulins bind to albumin and show protracted action in pigs. *Diabetologia* 39, 281–288.

Antibody-Mediated versus Nontargeted Delivery in a Human Small Cell Lung Carcinoma Model

Edgardo Marecos, Ralph Weissleder, and Alexei Bogdanov, Jr.*

Center for Molecular Imaging Research, Department of Radiology, Massachusetts General Hospital, Building 149, 13 Street, Boston, Massachusetts 02129. Received August 1, 1997;
Revised Manuscript Received December 5, 1997

The uptake of macromolecular agents in tumor cells (LX-1, human small cell lung carcinoma) and in corresponding tumor xenografts was compared in a parallel study utilizing a long-circulating biocompatible graft copolymer, MPEGs-PL-DTPA [Bogdanov, A., Jr., et al. (1995) *Adv. Drug Delivery Rev.* 16, 335–348; Bogdanov, A., Jr., et al. (1996) *Bioconjugate Chem.* 7, 144–149] and a tumor-specific chimeric monoclonal antibody, BR96 [Hellstrom, I., et al. (1990) *Cancer Res.* 50, 2183–2190; Garrigues, J., et al. (1993) *Am. J. Pathol.* 142, 607–622]. Covalent grafted conjugates of methoxy-(polyethylene glycol)succinate and polylysine and BR96 were modified with DTPA, biotinyl, or rhodamine-X-residues. Using radionuclide and fluorescent labeled derivatives of the copolymer and the antibody, we established that (1) the copolymer does not associate with the plasma membrane in *N*-ethylmaleimide-treated cells and is slowly internalized by live cells at 37 °C; (2) the antibody binds rapidly to the surface of LX-1 cells and shows active internalization in vesicles with a subsequent slow decrease in the cell-associated antibody concentration; (3) LX-1 cells bear more than 1 million BR96 binding sites/cell (with an apparent K_d of 4.5×10^{-7} M); and (4) intravesicular fluorescence intensity in LX-1 cells was linearly dependent on copolymer concentration, suggesting fluid phase endocytosis. Tumor localization by nuclear imaging, biodistribution, microdistribution by histology, and determination of tumor cell fraction uptake was performed in LX-1 tumor xenografts. In vivo study showed that MPEGs-PL-DTPA progressively accumulates in the tumor, yielding from $2.8 \pm 1.5\%$ injected dose per gram of tissue (ID/g) at 24 h to $5.2 \pm 1.7\%$ ID/g of tissue at 48 h. The antibody accumulation peaked at 24 h ($6.0 \pm 3.2\%$ ID/g) and decreased thereafter. We determined that at 24 h $43.9 \pm 11.29\%$ of the polymer accumulated in the tumor was associated with tumor cell fraction with the remainder of the accumulated dose localized in the interstitium. Accumulation of the biotinylated graft copolymer and the antibody in LX-1 xenografts and their uptake in cells were confirmed by histology using avidin–peroxidase staining. Our study demonstrates that, although BR96 is highly specific in vitro, tumoral drug delivery in vivo can be equally high with long-circulating graft copolymers because of slow extravasation at the tumor site.

INTRODUCTION

A variety of tumor targets have been tested experimentally to deliver therapeutic or diagnostic payloads to solid tumors (5, 6). The most widely investigated approach of tumor targeting includes the conjugation of a tumor-specific antibody or antibody fragments to a given drug carrier so that the resultant conjugate specifically associates with tumor cells in vivo. Successful tumor antibody-mediated delivery to several classes of tumor-associated antigens has been achieved: (1) oncogene-encoded proteins [e.g., c-erb proto-oncogene product, an EGF-receptor like protein kinase (7–9)], (2) tissue-associated differentiation antigens, onco-fetoproteins [e.g., carcinoembryonic antigen (10–13)], (3) tumor-associated mucins (14) or glycoproteins [TAG-72 (11, 15)], (4) receptor subtypes [e.g., the folate receptor in adenocarcinomas (16–18)], or (5) antibodies against histones (TNT-1) to detect nuclei in the regions of massive cell necrosis in tumors (19, 20). Several difficulties are

associated with this targeting strategy because of three major obstacles: (1) rare occurrence of tumor-specific antigens (expression of antigen in normal tissues), (2) antigen expression heterogeneity of tumors, and (3) generation of anti-ideotype antibodies to the targeting ligand requiring the use of chimeric antibodies or small antibody fragments.

Most of the alternative approaches utilize the intravascular delivery to shuttle a given drug to the tumor and exploit elevated permeability of tumor vessels to achieve drug accumulation within the tumor. It has been demonstrated that macromolecular agents, such as linear polymer–drug conjugates (21, 22), accumulate in experimental tumors, presumably at sites of increased capillary permeability (23, 24). The permeability itself can be pharmacologically modulated, thus leading to more efficient intratumoral delivery (reviewed in ref 25). We have previously presented the evidence that MPEG-grafted long-circulating polymers and their conjugates with cisplatin efficiently accumulate in experimental mammary adenocarcinomas (2, 26). The property of tumor accumulation is shared also by other long-circulating drug carriers, such as liposomes bearing MPEG-conjugated lipids (27–32). Furthermore, it has been suggested that long-circulating polymeric agents are

* Author to whom correspondence should be addressed at Center for Molecular Imaging Research, Department of Radiology, Massachusetts General Hospital, Bldg 149, Rm 5420, 13th St., Boston, MA 02129. Telephone: (617) 726-5788. Fax: (617) 726-5708. E-mail: abogdanov@helix.mgh.harvard.edu.

taken up by tumor cells by fluid phase endocytosis (pinocytosis) (26, 33, 34), a process which is typically upregulated in rapidly proliferating cells and is cell cycle-dependent (35, 36).

In this study, we intended to compare directly the "specific" targeting of tumor-specific antibody and the "non-specific" accumulation of a long-circulating polymer in a model of human cancer *in vitro* and *in vivo*, i.e., to address the issue of "active" versus "passive" delivery of macromolecules into tumors.

MATERIALS AND METHODS

Graft Copolymer. Synthesis of a protected graft copolymer [*O*-methyl-poly(ethylene glycol)-*O'*-succinyl-*N*-*O'*-poly(L-lysine) (MPEGs-PL)¹] and its analogue modified with DTPA has been accomplished as described in refs 2 and 37. The product was purified by passing the copolymer through Sephadex A-25 and subsequent ultrafiltration (YM 50 membrane, Amicon, Beverly, MA). The duration of ultrafiltration was determined by subjecting an aliquot of purified copolymer to size-exclusion HPLC analysis (Hydropore-5-SEC column, 4.5 mm inside diameter $\times$ 25 cm long, Rainin Instrument Co., Woburn, MA) eluted with 50 mM sodium phosphate (pH 6.8) at 0.5 mL/min. The composition of the copolymer was confirmed by elemental analysis (Galbraith Labs, Knoxville, TN). The molecular mass of MPEG-PL was calculated as 410 kDa.

To label MPEGs-PL with rhodamineX-isothiocyanate (X-RITC, Molecular Probes, Eugene, OR), a solution of 20 μ M MPEGs-PL in 10 mM sodium bicarbonate and 0.15 M NaCl (pH 8.7) was treated with a 5-fold molar excess of X-RITC over MPEGs-PL. The rest of the free amino groups in MPEGs-PL were blocked with DTPA cyclic anhydride. Three or four X-RITC groups/mol of MPEGs-PL were incorporated, assuming $\epsilon = 92\,000$ at 572 nm for X-RITC.

Biotinylation of MPEGs-PL was performed by reacting 100 mg of *O*-biotinyl-poly(ethylene glycol) 3500 succinyl *N*-hydroxysuccinimide (Shearwater Polymers Inc., Birmingham, AL) with 200 mg of poly(L-lysine) [MW of 35 500, at 25 mM lysine monomer in 0.05 M sodium bicarbonate (pH 8.7)] for 3 h with subsequent modification of 30% of *N*- ϵ -amino groups of poly(L-lysine) with MPEG 5000 succinate as described previously (2, 37). The biotinylated polymer was purified using ultrafiltration as described before.

Indium (¹¹¹In) labeling of MPEGs-PL-DTPA was performed by incubating MPEGs-PL-DTPA in the presence of ¹¹¹InCl₃ (specific activity of 1.7×10^7 Ci/mol) at a ratio of 50 mCi/mg of copolymer (1 mol of In/600 mol of MPEGs-PL-DTPA) dissolved in 10 mM sodium citrate and 0.15 M NaCl at pH 6.0 (CBS), for 30 min. The yield of isotope chelation to DTPA was 85% from added ¹¹¹In. The labeled product was purified by gel filtration on Sephadex G-25m spin columns equilibrated with CBS. The properties of the labeled agent have been described in more detail previously (26).

Antibody Conjugates. Chimeric monoclonal antibody [Chi BR96 (38)] was kindly provided by P. Senter (Bristol-Myers Squibb Pharmaceutical Research Institute, Seattle, WA). Antibody was dialyzed overnight against 10 mM borate and 0.15 M NaCl (pH 8.7) and

centrifuged at 15000g for 15 min. BR96 modified with DTPA was obtained by reacting a 6-fold molar excess of DTPA cyclic anhydride per mole of antibody (9.4 mg of BR96/mL, final concentration) for 4 h with subsequent overnight dialysis against 10 mM sodium citrate and 0.15 M NaCl (pH 6) and Sephadex G-25m chromatography. Labeling of the BR96-DTPA with ¹¹¹In has been performed as described above. According to TNBS titration, three DTPA residues were conjugated with mAb on the average. Labeling with X-RITC has been performed using a ratio of 6 mol of X-RITC/mol of mAb at pH 8.7. Fluorescent antibody was purified from aggregates by centrifugation at 15000g for 30 min and passed through a column of Sephadex G-25m. The resultant preparation contained 1.2 mol of X-RITC/mol of antibody. The biotinylation of BR96 was performed with a 3-fold molar excess of biotinylation reagent per mole of antibody by reacting 65 μ M *N*-hydroxysulfosuccinimidyl[6-(biotinyl-amido)]hexanoate (Pierce Chemical Co.) with BR96 in 50 mM sodium bicarbonate and 0.1 M NaCl (pH 8). The biotinylated antibody was then purified by Sephadex G-25m column chromatography.

Nonspecific human IgG (Sigma Chemical Co. catalog no. I-4506) was purified from the aggregates and modified under conditions identical to those described above. The purity of modified antibodies was verified by SDS-PAGE using 7.5 and 10% gels and autoradiography.

Cell Culture Experiments. Human small cell lung carcinoma LX-1 (kindly provided by E. Neuwelt, University of Oregon, Portland, OR) was propagated in RPMI and 10% FBS. Cells were detached from the plastic by aspiration, sedimented, and resuspended in fresh medium at 2×10^5 cells/mL. Indium-labeled MPEGs-PL-DTPA and BR96-DTPA were added to 1 mL of cell suspension at the concentration of 0.1–70 μ g/mL and incubated with agitation at 37 °C. Cells were washed three times by centrifugation through a cushion of 30% Histopaque-1077 in HBSS (Sigma Chemical Co.) at 800g, for 10 min, and counted. The associated radioactivity was determined by γ -counting. Fluorescent microscopy was performed at 37 °C using live cells attached to the glass coverslips (39). Images were collected using a SenSys (Photometrics) CCD camera attached to a Zeiss Axiovert 100TV fluorescent microscope equipped with an XF22 filter set (Omega Optical Inc.). For intravesicular fluorescence measurements, images of 50–100 cells were digitized and segmented using a histogram method provided by IP LabSpectrum software (Signal Analytics Co., Vienna, VA). Fluorescence intensity of segments (intracellular vesicles, $n = 100$ –200 per data point) was measured. Segment areas were limited to a median of 10–20 arbitrarily chosen vesicles to exclude nonvesicular structures and cell fragments from consideration. The linearity of the CCD response was checked using standard X-RITC solutions. The response of the device was linear in the range of 50–3000 nM X-RITC.

Animal Model of Human Lung Carcinoma. Female nude mice ($n = 36$; 25–30 g; Edwin L. Steele Laboratory, MGH, Boston, MA) bearing 0.5–0.9 cm LX-1 human small cell lung carcinomas were used as a tumor model for all comparative experiments. In each animal, 1×10^6 LX-1 human lung small cell carcinoma cells were implanted subcutaneously in the left abdominal flank. Mice were used for biodistribution studies 15–17 days after implantation. Twelve were used for imaging studies. Six were used to process tumors for avidin-peroxidase histology. Six were used for the cell uptake experiment.

¹ Abbreviations: MPEGs-PL-DTPA, *O*-methyl poly(ethylene glycol)-*O'*-(succinyl)-*N*- ϵ -poly(L-lysyl)diethylenetriaminepentaacetamide; NEM, *N*-ethylmaleimide; ID, injected dose; hIgG, human immunoglobulin; PBS, 10 mM sodium phosphate and 0.15 M NaCl (pH 7.4).

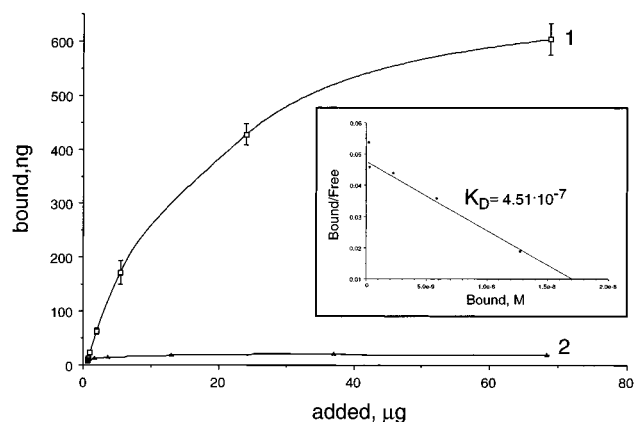


Figure 1. Binding of BR96-[^{111}In]DTPA and MPEGs-PL-[^{111}In]DTPA to LX-1 cells (1 h, 37 °C) treated in the presence of 100 μM *N*-ethylmaleimide: (1) BR96-DTPA and (2) MPEGs-PL-DTPA. (Inset) Scatchard analysis of curve 1. Results are presented as mean $\pm$ SD ($n = 3$).

Animal Experiments. Biodistribution studies in 36 mice were performed after intravenous (iv) injection of the agents via the tail vein. Animals were divided into two experimental groups of 18. Animals were injected in the tail vein with 200 μg of BR96-[^{111}In]DTPA (60 nmol of mAb/kg, 30 μCi /animal) dissolved in 100 μL of sterile HBSS or with an equimolar amount of MPEGs-PL-[^{111}In]DTPA (ca. 60 μg of polymer/animal, 25 μCi /animal). In each group, sets of three animals were sacrificed at 6, 12, 48, and 72 h time points, and a set of six was sacrificed at the 24 h time point. Samples of blood, major normal tissues, and tumors were collected, and the tissues were blotted dry and weighed. Radioactivity of the samples was measured using a well-type γ -counter (LKB model 1282, Wallac, Turku, Finland). To correct for radioactive decay and to permit the calculation of activity in each organ as a fraction of the administered dose, aliquots of standard stock solutions were counted simultaneously. Biodistribution results were expressed as a percentage of the injected dose per gram of tissue (% ID/g).

Tumor Fractionation Experiment. To determine the intracellular and extracellular fractions of the graft copolymer and monoclonal antibody within tumors, the tumor tissue obtained from six animals was processed (three for each group, 24 h postadministration). The tumor (200–300 mg of tissue) was excised, minced to small fragments on ice, suspended in 20 mL of PBS supplemented with 1 mM PMSF, 5 mM NaN_3 , and 1 mM *N*-ethylmaleimide (pH 7), and passed three times through #4 wire mesh (Sigma Chemical Co.) at 4 °C. Samples were centrifuged at 18000g for 15 min. The integrity of cells released from the tissue was verified with the trypan blue exclusion method. The radioactivity of the pellet was determined in a γ -counter and was assumed to be associated with intracellular compartment, and radioactivity of the supernatant was assumed to be contained within the extracellular fluid (40).

Scintigraphy. Mice ($n = 12$, 6 in each group) were used for imaging 6, 12, and 24 h postinjection (2 of the animals in each group were imaged at all time points). Mice were anesthetized subcutaneously (sc) using a mixture of 90 mg/mL ketamine (Ketalar, Parke Davis, Morris Plains, NJ) and 9 mg/mL xylazine (Rompun, Miles, Shawnee Mission, KA). Images were acquired by using 20% windows over a 247 keV photopeak of ^{111}In (10 min at 6 h and 20 min at 12 and 24 h). All scintigrams were obtained by using a small-field of view

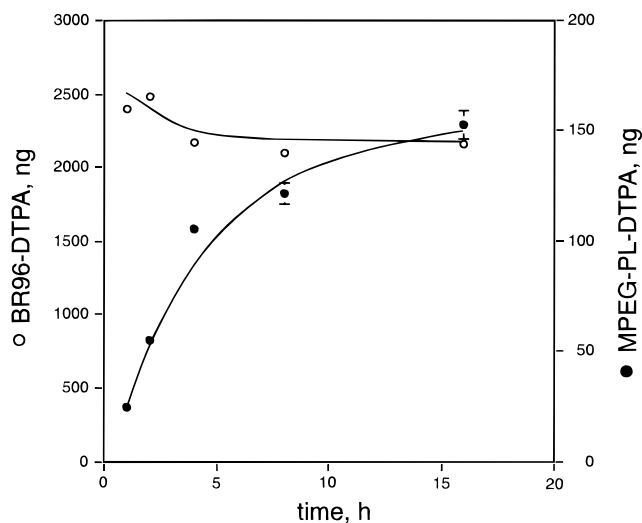


Figure 2. Time course of the uptake of BR96-[^{111}In]DTPA (open symbols) and MPEGs-PL-[^{111}In]DTPA (closed symbols) in LX-1 cells. Cells were incubated for the time indicated in the presence of equimolar concentrations (0.4 μM) of BR96 (60 μg /mL) and the copolymer (165 μg /mL) at 37 °C. Results are presented as mean $\pm$ SD ($n = 3$).

γ -camera equipped with a pinhole collimator (Sigma 410, Ohio Nuclear, Solon, OH) and analyzed using NucLear Mac 2.9 software (Scientific Imaging Inc.).

Histology. Mice (nu/nu, $n = 6$) were divided into two groups. The first group of animals received iv ca. 100 μg of BR96-biotin/animal. The animals of the second group were injected with 100–200 μg of MPEGs-PL-biotin. Animals were sacrificed at 24 h postinjection. Tumors were excised, and tumor tissue was quickly frozen and cryosectioned into 10–20 μm sections. Sections were fixed for 1 h in a mixture of ethyl acetate/acetone/acetic acid (6/3/1), washed with PBS, blocked in solution of 10% goat serum in PBS, and incubated with the avidin–peroxidase complex (ABC kit, Vector Laboratories, Burlingame, CA) as described by the manufacturer. Sections were stained using diaminobenzidine/hydrogen peroxide and counterstained with hematoxylin/eosin.

RESULTS

Interaction of the Copolymer and BR96 with LX-1 Cells. These experiments were conducted to determine whether the binding to plasma membrane may mediate the uptake of both MPEGs-PL-DTPA and BR96 in LX-1 cells. To abolish internalization, cells were preincubated in the presence of 100 mM NEM, an inhibitor of endocytosis (41). Indium-labeled BR96-DTPA showed a typical binding to *N*-ethylmaleimide-treated cells (Figure 1). Using Scatchard analysis, we established that LX-1 cells bear ca. 1 million BR96-DTPA binding sites/cell, with an apparent K_d of 4.51×10^{-7} M. The copolymer, MPEGs-PL-[^{111}In]DTPA, incubated with cells under identical conditions showed no detectable binding to the cell surface (Figure 1).

Kinetics of the labeled compound uptake by LX-1 cells was studied in live cells at equimolar concentrations (Figure 2). Labeled antibody was rapidly associated with LX-1 cells (16 pmol of antibody was taken up by 1 million cells during the first hour of incubation). The radioactivity associated with mAb-labeled cells slowly diminished with time. Graft copolymer was gradually taken up by cells, with the relatively fast initial phase (6 ng/h per 1 million cells) and slower later accumulation (Figure 2).

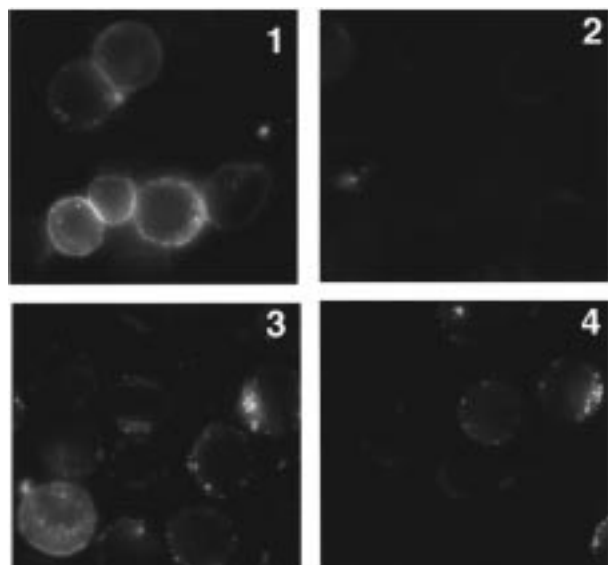


Figure 3. Fluorescent microscopy of live LX-1 cells incubated in the presence of BR96-X-RITC (1 and 3) or MPEGs-PL-X-RITC (2 and 4) for 15 min (1 and 2) or 3 h (3 and 4) at 37 °C. Equal concentrations of fluorophore were used (500 nM).

Approximately 150 ng (ca. 0.1% of added copolymer) was associated with 2×10^5 cells after incubation for 15 h at 37 °C, whereas a total of 2500 ng of BR96 (16.7 pmol, 4.1% of the added antibody) was associated with the same number of cells.

Using fluorescence microscopy and rhodamine-labeled BR96 antibody, we observed a characteristic fluorescent "halo" indicating association of antibodies with the cell surface at 15 min (Figure 3, panel 1). The majority of cells exhibited typical extracellular fluorescence with concomitant uptake in vesicles. At 3 h, most of the cell-associated fluorescence appeared to be distributed inside the vesicles (Figure 3, panel 3). For MPEGs-PL-DTPA, virtually no association of the polymer with plasma membrane was seen at the initial stage of incubation (Figure 3, panel 2). At 3 h, however, the morphology of intracellular distribution of fluorescence was almost identical to that of BR96 cells (Figure 3, panel 4). The main difference detected was in the number of fluorescent vesicles per cell which was lower in the case of the copolymer. To obtain a semiquantitative measure of the uptake of both fluorescently labeled BR96 and MPEG-PL, we digitized fluorescent images of intracellular vesicles. This made it feasible to study the concentration dependence of the uptake only and allowed the exclusion of the fraction of plasma membrane-bound antibody or copolymer that was not internalized. Normalized vesicular fluorescence intensity of the cells incubated in the presence of MPEGs-PL-X-RITC showed a nearly linear increase with the concentration of fluorophore (Figure 4, closed symbols). Vesicular fluorescence intensity of BR96-treated LX-1 cells was very similar to that of cells treated with the copolymer in the broad range of rhodamine concentrations (Figure 4). In the higher concentration range of BR96 ($>20 \mu\text{g/mL}$, $>1500 \text{ nM}$ rhodamine-X), LX-1 cells showed signs of cytotoxicity which were manifested by the presence of fragmented cells. The cytotoxicity resulted in a dramatic increase of fluorescence (Figure 4, open symbols).

Biodistribution. Biodistribution experiments were performed to compare kinetics of ^{111}In -labeled BR96-DTPA and MPEGs-PL-DTPA accumulation in LX-1 tumor xenografts (Table 1). At the dose used for intra-

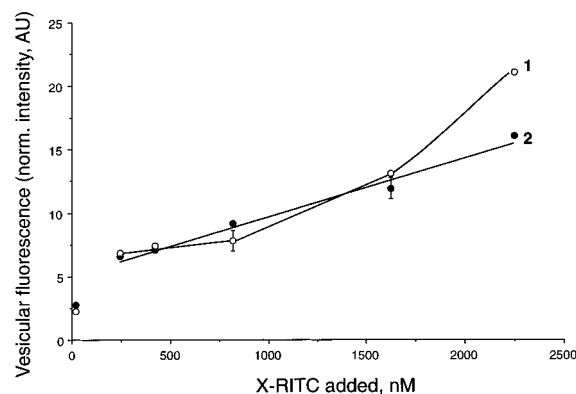


Figure 4. Concentration dependence of vesicular fluorescence intensity measured by a CCD device in LX-1 cells: BR96-X-RITC (1, open symbols) and MPEGs-PL-X-RITC (2, closed symbols). Cells were incubated with labeled compounds for 3 h at 37 °C and subjected to microfluorimetry as described in Materials and Methods.

Table 1. Biodistribution of ^{111}In -Labeled MPEGs-PL-DTPA and ^{111}In -Labeled BR96-DTPA in Mice Bearing Human Small Cell Lung Carcinoma Xenografts (in % ID/g)

tissue	24 h		48 h		
	mAb	MPEGs-PL-DTPA	mAb	MPEGs-PL-DTPA	hIgG
blood	7.7 ± 2.2	10.3 ± 1.8	4.0 ± 0.5	8.8 ± 0.3	9.3 ± 0.5
tumor	6.0 ± 3.2	2.8 ± 1.5	5.8 ± 0.4	5.2 ± 1.7	4.1 ± 1.4
liver	5.8 ± 3.7	2.7 ± 1.5	4.7 ± 0.8	3.0 ± 0.3	5.5 ± 2.9
lung	2.1 ± 1.0	3.4 ± 1.5	2.0 ± 0.2	3.7 ± 0.4	4.9 ± 3.0
heart	2.3 ± 0.3	4.6 ± 1.0	1.5 ± 0.3	3.9 ± 0.8	2.4 ± 0.4
spleen	4.2 ± 3.0	3.5 ± 2.1	3.0 ± 0.5	3.2 ± 0.5	3.9 ± 1.9
kidney	6.3 ± 2.1	4.2 ± 1.0	5.0 ± 0.8	4.5 ± 0.5	4.7 ± 1.8
muscle	0.8 ± 0.4	1.6 ± 1.6	0.8 ± 0.0	1.1 ± 0.2	2.0 ± 0.1
pancreas	1.0 ± 0.3	0.6 ± 0.4	1.1 ± 0.1	1.0 ± 0.1	1.5 ± 0.3
intestine	0.9 ± 0.7	1.6 ± 1.4	1.3 ± 0.4	1.9 ± 0.7	1.2 ± 1.0
fat	1.2 ± 1.7	0.5 ± 0.3	0.6 ± 0.5	0.8 ± 0.3	1.1 ± 0.8
bone marrow	0.3 ± 0.1	0.7 ± 0.4	0.9 ± 0.4	1.3 ± 0.3	1.7 ± 1.1

venous injections (60 nmol/kg), both ^{111}In -labeled copolymer and antibody showed very long blood residence times in nude mice, with a $T_{1/2}$ of 26.6 h for mAb (monoexponential fitting, $r^2 = 0.925$). The blood half-life of the copolymer could be approximated as 60 h. Indium-labeled BR96-DTPA accumulated in the tumor rapidly and exhibited the highest accumulation 6–12 h after the injection at 7.1–7.9% of the injected dose per gram of tissue. Graft copolymer showed a slower uptake in the tumor with the maximum accumulation reached at 48 h (Table 1). Corresponding γ -camera images reveal a clear tumor mass which could be detected by the antibody at 6 h postadministration (Figure 5), whereas the copolymer accumulation was evident at 12 h postinjection. Over time, BR96-DTPA showed good retention within the tumor with high tumor/muscle localization ratios at 48–72 h post-IV administration. The copolymer was taken up by the tumor ca. 5-fold more efficiently than in the muscle at the peak of tumor accumulation (at 48 h). At this time point, we did not observe any statistically significant differences between tumor/muscle ratios calculated for BR96 (range of 6.75–7.25) and MPEG-PL-DTPA (range of 2.7–7.7) (Table 1). Both ratios were higher than the tumor/muscle ratio of nonspecific hIgG (1.3–2.6).

Compartmental Distribution of Labeled Agents in the Tumor. Tumor compartmental distribution of labeled BR96-DTPA and MPEGs-PL-DTPA was determined by separating the cellular fraction from the extracellular fluid. Twenty-four hours after injection,

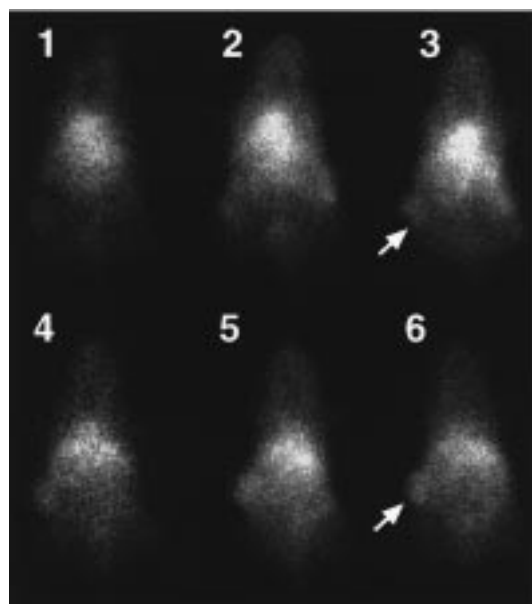


Figure 5. Nuclear imaging of mice bearing LX-1 xenografts. Animals were injected iv with MPEGs-PL-[^{111}In]DTPA (images 1–3) or BR96-[^{111}In]DTPA (images 4–6). Images collected at 6 (images 1 and 4), 12 (images 2 and 5), and 24 (images 3 and 6) h postinjection as described in Materials and Methods. Arrows indicate localization of the tumor.

$43.9 \pm 11.3\%$ of the total amount of ^{111}In -labeled copolymer located in tumor was associated with the cellular fraction. The remainder of the dose localized in the tumor was found in the extracellular compartment. The cell-associated fraction of the injected dose was $60.4 \pm 3.9\%$ of mAb, and $39.6 \pm 3.6\%$ of the antibody was located in the extracellular compartment. Histological examination of the tumor obtained from the animal injected with MPEGs-PL-biotin (Figure 6A) and biotinylated antibody (Figure 6B) showed the presence of extravasation of both antibody and the polymer in perivascular stroma and certain accumulation in cells located in the perivascular area.

DISCUSSION

This study was undertaken with the goal of comparing, in a set of parallel experiments, the ability of a cancer cell-specific monoclonal antibody (BR96) and a nonspecific long-circulating polymer to localize within cancer cells (LX-1) *in vitro* and *in vivo*. The tumor-specific monoclonal antibody used in this study binds to a variant of a Le^y antigen abundantly expressed (>200000 molecules per cell) at the plasma membrane of human colon, lung, breast, and ovary carcinomas (3, 42). This antibody rapidly internalizes in human carcinoma cells and is cytotoxic *in vitro* (3, 4). Intravenous administration of BR96 results in antitumor activity against tumor xenografts in mice (38).

The copolymer, MPEGs-PL-DTPA, was used because of its controllable size, relatively narrow molecular mass distribution, and long circulation time (1, 43). Furthermore, the copolymer is relatively inert in nature and is not immunogenic (37). Additionally, the copolymer does not induce local changes in capillary permeability and accumulates within tumors in experimental cancer (2, 26). The latter property was explained by the extravasation from vessels with abnormally elevated permeability (44, 45).

It has been shown before that intravenous administration of tumor-specific antibodies may result in a very high

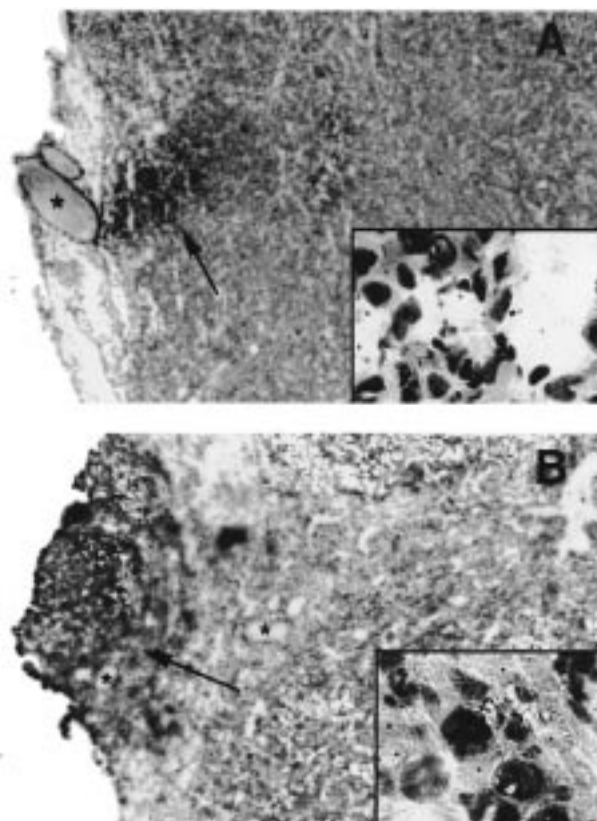


Figure 6. Extravasation and uptake of biotinylated BR96 (A) and biotin-MPEGs-PL-DTPA (B) in LX-1 xenografts by histology. Staining of tumor slices was performed with the avidin–peroxidase complex with hematoxylin/eosin counterstaining. Arrows indicate extravasation sites. Asterisks indicate vascular lumens. (Insets) Magnified image of cells positively stained by avidin–peroxidase.

percentage of dose accumulated in tumor tissue, and that this effect is not accompanied by efficient binding to tumor cells. Tumor-specific antibodies accumulate *in vivo* in tumoral perivascular stroma (40, 46), and the majority of the dose (76–80%) is not bound to cells, but rather is associated with tumor-shed antigens (40). The discovery of interstitial fluid pressure gradients in tumors has also been used to explain the low efficiency of cellular uptake of tumor-specific antibodies (47–49). These findings prompted us to compare delivery to tumors and cellular uptake of a tumor-specific antibody and a copolymer which can associate with tumor cells only as a result of endocytosis. For “inert” particulates and macromolecules which do not bind to cell surfaces, constitutive fluid phase endocytosis is the predominant pathway of uptake (33, 34, 50–52). Therefore, we hypothesized that MPEG-PL-DTPA may accumulate in tumor cells as a result of fluid phase uptake.

The results of *in vitro* experiments clearly indicate that MPEGs-PL-DTPA does not bind to the LX-1 cell plasma membrane, whereas BR96, labeled with either DTPA or rhodamine-X (Figures 1 and 3), binds to the plasma membrane at a very high density (1 million sites/cell were detected) and rapidly internalizes in live cells. After NEM treatment which blocks fluid phase uptake (41), the cells avidly bound the antibody while no binding of polymer was observed (Figure 1). Fluorescence microscopy suggested that nearly all association of MPEGs-PL with LX-1 cells is a result of fluid phase uptake; the copolymer appears within the cells at a much later stage than surface-bound antibody and associates almost ex-

clusively with intracellular vesicles (Figure 3). In addition, semiquantitative intravesicular fluorescence measurements revealed a linear dependence of fluorescence intensity on fluorophore concentration. This observation is consistent with the fluid phase uptake (Figure 4). Vesicular fluorescence of BR96-treated cells showed certain level of saturation within the range of 250–750 nM rhodamine-X, presumably because of the saturation of antibody binding sites and the predominance of membrane-bound antibodies in the vesicles. With the further increase in antibody concentration, fluorescence intensity of vesicles increased as a result of cell permeabilization by the cytotoxic antibody. It should be noted that an internalized molecule encounters a vesicular environment different from that of an extracellular medium in pH, microviscosity, and high protein concentrations. These factors can affect the quantum yield of the fluorescence of the given probe. It is also possible that the antibody and the polymer can be taken up and sorted into different vesicular compartments with different degrees of "maturation" and compaction (53). This may lead to different levels of fluorescence quenching. However, the results of measurements performed using radioactive BR96 and copolymer (Figure 2) were consistent with fluorescent microscopy data, revealing rapid association of the antibody with the cells and slow uptake of MPEGs-PL-DTPA.

The results of the *in vivo* biodistribution study performed using an LX-1 xenograft model and the antibody–copolymer pair were remarkably similar to *in vitro* data. For example, tumor accumulation kinetics and corresponding tumor/muscle ratios were consistent with rapid accumulation of BR96 in cells (Figure 5). MPEGs-PL-DTPA, lacking the ability to bind to tumor cells, showed a delayed accumulation in the tumor. At the 48 h time point, which corresponds to the peak of accumulation of MPEGs-PL-DTPA (Table 1), we observed only an insignificant difference between the antibody and copolymer accumulation (5.8 ± 0.4 vs $5.2 \pm 1.7\%$ ID/g, $P > 0.05$). These values were only marginally higher than that of hIgG (Table 1) but resulted in substantially higher localization (tumor/muscle ratios). Both BR96 and the copolymer were present in tumor interstitium and in tumor cells in animals injected with biotinylated mAb and MPEGs-PL-DTPA (Figure 6). Strongly positive cells (Figure 6, insets) were detected in both cases, suggesting the internalization of biotinylated copolymer and BR96 *in vivo*. As expected, higher amounts of the antibody were found to be associated with LX-1 cells in xenografts (more than 60% of tumor-associated antibody) than the copolymer (44% of the tumor-accumulated dose) at 24 h. This may be explained by rapid initial uptake of mAb in the tumor. We believe that the high tumor cell-associated activity cannot be explained by the uptake of plasma components labeled as a result of transchelation of ^{111}In from the copolymer to plasma proteins since ^{111}In -labeled MPEGs-PL-DTPA is stable in plasma as well as in the presence of apotransferrin for at least 48 h (26).

Our data indicate that nonspecific fluid phase uptake in tumor cells is the major pathway of tumor-directed delivery of nontargeted macromolecular agents exemplified in our study by the MPEGs-PL graft copolymer. There are obvious differences in the kinetics of tumor accumulation of BR96 and MPEGs-PL graft copolymer resulting in rapid tumor-specific accumulation of the tumor-specific antibody. However, we showed that one can achieve a progressive increase of tumor delivery with increasing blood half-lives of the drug carriers with no

specificity against tumor cells. Approximately 100 molecules of diagnostic label (e.g., DTPA) or an antitumor drug can be conjugated to a nonimmunogenic MPEGs-PL copolymer. Thus, the nontargeted approach can potentially yield a high accumulation of the drug in the tumor and concomitant uptake by proliferating cancer cells, irrespective of the tumor antigenic status.

ACKNOWLEDGMENT

This work was supported in part by the Center for Innovative Minimally Invasive Therapy, Massachusetts General Hospital (Fellowship Award to E.M. and Research Award to A.B.) and NIH Grant RO1 CA59649-01 (to R.W.).

LITERATURE CITED

- (1) Bogdanov, A., Jr., Weissleder, R., and Brady, T. (1995) Long-circulating blood pool imaging agents. *Adv. Drug Delivery Rev.* 16, 335–348.
- (2) Bogdanov, A., Jr., Martin, C., Bogdanova, A. V., Brady, T. J., and Weissleder, R. (1996) An adduct of cis-diamminedichloroplatinum(II) and poly(ethylene glycol)poly(L-lysine)-succinate: synthesis and cytotoxic properties. *Bioconjugate Chem.* 7, 144–149.
- (3) Hellstrom, I., Garrigues, H. J., Garrigues, U., and Hellstrom, K. E. (1990) Highly tumor-reactive, internalizing, mouse monoclonal antibodies to Le(y)-related cell surface antigens. *Cancer Res.* 50, 2183–2190.
- (4) Garrigues, J., Garrigues, U., Hellstrom, I., and Hellstrom, K. E. (1993) Ley specific antibody with potent anti-tumor activity is internalized and degraded in lysosomes. *Am. J. Pathol.* 142, 607–622.
- (5) Buchsbaum, D. J. (1995) Experimental approaches to increase radiolabeled antibody localization in tumors. *Cancer Res.* 55, 5729s–5732s.
- (6) von Mehren, M., and Weiner, L. M. (1996) Monoclonal antibody-based therapy. *Curr. Opin. Oncol.* 8, 493–498.
- (7) Adams, G. P., McCartney, J. E., Tai, M. S., Oppermann, H., Huston, J. S., Stafford, W. F. d., Bookman, M. A., Fand, I., Houston, L. L., and Weiner, L. M. (1993) Highly specific *in vivo* tumor targeting by monovalent and divalent forms of 741F8 anti-c-erbB-2 single-chain Fv. *Cancer Res.* 53, 4026–4034.
- (8) George, A. J., Jamar, F., Tai, M. S., Heelan, B. T., Adams, G. P., McCartney, J. E., Houston, L. L., Weiner, L. M., Oppermann, H., Peters, A. M., et al. (1995) Radiometal labeling of recombinant proteins by a genetically engineered minimal chelation site: technetium-99m coordination by single-chain Fv antibody fusion proteins through a C-terminal cysteinyl peptide. *Proc. Natl. Acad. Sci. U.S.A.* 92, 8358–8362.
- (9) Dean, C. J., Eccles, S. A., Valeri, M., Box, G., Allan, S., McFarlane, C., Sandle, J., and Styles, J. (1993) Rat MABs to the product of the c-erbB-2 proto-oncogene for diagnosis and therapy in breast cancer. *Cell Biophys.* 22, 111–127.
- (10) Biersack, H. J., Briele, B., Hotze, A. L., Oehr, P., Qian, L., Mekawy, M. A., and Shih, W. J. (1992) The role of nuclear medicine in oncology. *Ann. Nucl. Med.* 6, 131–136.
- (11) Abdel-Nabi, H. H., and Doerr, R. J. (1992) Multicenter clinical trials of monoclonal antibody B72.3-GYK-DTPA ^{111}In (111In-CYT-103; OncoScint CR103) in patients with colorectal carcinoma. *Targeted Diagn. Ther.* 6, 73–88.
- (12) Leitha, T., Walter, R., Schlick, W., and Dudczak, R. (1991) $^{99\text{m}}\text{Tc}$ -anti-CEA radioimmunoscintigraphy of lung adenocarcinoma. *Chest* 99, 14–19.
- (13) Saleh, M. N., Wheeler, R. H., Lee, J. Y., Khazaeli, M. B., Unger, M. W., Russell, C. H., and LoBuglio, A. F. (1991) In- ^{111}In labeled monoclonal anti-carcinoembryonic antigen antibody (ZCE025) in the immunoscintigraphic imaging of metastatic antigen-producing adenocarcinomas. *Clin. Nucl. Med.* 16, 110–116.
- (14) Britton, K. E., Granowska, M., and Shepherd, J. (1985) Localization of cancer of the ovary and metastases using ^{125}I -labeled monoclonal antibody HMFG-2 compared to surgical

- findings. In *Monoclonal Antibodies for Cancer Detection and Therapy*, p 10, Academic Press, New York.
- (15) Surwit, E. A., Childers, J. M., Krag, D. N., Katterhagen, J. G., Gallion, H. H., Waggoner, S., and Mann, W. J., Jr. (1993) Clinical assessment of ^{111}In -CYT-103 immunoscintigraphy in ovarian cancer. *Gynecol. Oncol.* 48, 285–292.
 - (16) Holm, J., Hansen, S. I., Hoier-Madsen, M., Sondergaard, K., and Bzorek, M. (1994) Folate receptor of human mammary adenocarcinoma. *APMIS* 102, 413–419.
 - (17) Franklin, W. A., Waintrub, M., Edwards, D., Christensen, K., Prendergrast, P., Woods, J., Bunn, P. A., and Kolhouse, J. F. (1994) New anti-lung-cancer antibody cluster 12 reacts with human folate receptors present on adenocarcinoma. *Int. J. Cancer Suppl.* 8, 89–95.
 - (18) Coney, L. R., Mezzanzanica, D., Sanborn, D., Casalini, P., Colnaghi, M. I., and Zurawski, V. R., Jr. (1994) Chimeric murine-human antibodies directed against folate binding receptor are efficient mediators of ovarian carcinoma cell killing. *Cancer Res.* 54, 2448–2455.
 - (19) Epstein, A. L., Khawli, L. A., Chen, F.-M., Hu, P., Glasky, M. S., and Taylor, C. R. (1995) Tumor necrosis imaging and treatment of solid tumors. In *Targeted Delivery of Imaging Agents*, pp 259–288, CRC Press, Boca Raton, FL.
 - (20) Epstein, A. L., Chen, F.-M., and Taylor, C. R. (1988) A novel method for the detection of necrotic lesions in human cancers. *Cancer Res.* 48, 5842.
 - (21) Pimm, M. V., Perkins, A. C., Strohm, J., Ulbrich, K., and Duncan, R. (1996) Gamma scintigraphy of a ^{123}I -labelled N-(2-hydroxypropyl)methacrylamide copolymer-doxorubicin conjugate containing galactosamine following intravenous administration to nude mice bearing hepatic human colon carcinoma. *J. Drug Targeting* 3, 385–390.
 - (22) Pimm, M. V., Perkins, A. C., Strohm, J., Ulbrich, K., and Duncan, R. (1996) Gamma scintigraphy of the biodistribution of ^{123}I -labelled N-(2-hydroxypropyl)methacrylamide copolymer-doxorubicin conjugates in mice with transplanted melanoma and mammary carcinoma. *J. Drug Targeting* 3, 375–383.
 - (23) Jain, R. (1988) Determinants of tumor blood flow: A review. *Cancer Res.* 48, 2641–2658.
 - (24) Trotter, M., Chaplin, D., Durand, R., and Olive, P. (1989) The use of fluorescent probes to identify regions of transient perfusion in murine tumors. *Int. J. Radiat. Oncol., Biol., Phys.* 16, 931–934.
 - (25) Maeda, H., and Matsumura, Y. (1989) Tumorotropic and lymphotropic principles of macromolecular drugs. *Crit. Rev. Ther. Drug Carrier Syst.* 6, 193–210.
 - (26) Bogdanov, A., Jr., Wright, S. C., Marecos, E. M., Bogdanova, A. V., Martin, C., Petherick, P., and Weissleder, R. (1997) A long-circulating co-polymer in “passive targeting” to solid tumors. *J. Drug Targeting* 4, 321–330.
 - (27) Yuan, F., Leunig, M., Huang, S. K., Berk, D. A., Papahadjopoulos, D., and Jain, R. K. (1994) Microvascular permeability and interstitial penetration of sterically stabilized (stealth) liposomes in a human tumor xenograft. *Cancer Res.* 54, 3352–3356.
 - (28) Yuan, F., Dellian, M., Fukumura, D., Leunig, M., Berk, D. A., Torchilin, V. P., and Jain, R. K. (1995) Vascular permeability in a human tumor xenograft: molecular size dependence and cutoff size. *Cancer Res.* 55, 3752–3756.
 - (29) Oku, N., Namba, Y., and Okada, S. (1992) Tumor accumulation of novel RES-avoiding liposomes. *Biochim. Biophys. Acta* 1126, 255–260.
 - (30) Litzinger, D. C., Buiting, A. M., van Rooijen, N., and Huang, L. (1994) Effect of liposome size on the circulation time and intraorgan distribution of amphipathic poly(ethylene glycol)-containing liposomes. *Biochim. Biophys. Acta* 1190, 99–107.
 - (31) Zou, Y., Ling, Y. H., Reddy, S., Priebe, W., and Perez-Soler, R. (1995) Effect of vesicle size and lipid composition on the in vivo tumor selectivity and toxicity of the non-cross-resistant anthracycline annamycin incorporated in liposomes. *Int. J. Cancer* 61, 666–671.
 - (32) Francis, G. E., Delgado, C., Fisher, D., Malik, F., and Agrawal, A. K. (1996) Polyethylene glycol modification: relevance of improved methodology to tumour targeting. *J. Drug Targeting* 3, 321–340.
 - (33) Pratten, M. K., Duncan, R., Cable, H. C., Schnee, R., Ringsdorf, H., and Lloyd, J. B. (1981) Pinocytic uptake of divinyl ether-maleic anhydride (pyran copolymer) and its failure to stimulate pinocytosis. *Chem. Biol. Interact.* 35, 319–330.
 - (34) Duncan, R., Pratten, M. K., Cable, H. C., Ringsdorf, H., and Lloyd, J. B. (1981) Effect of molecular size of ^{125}I -labelled poly(vinylpyrrolidone) on its pinocytosis by rat visceral yolk sacs and rat peritoneal macrophages. *Biochem. J.* 196, 49–55.
 - (35) Quintart, J., Leroy-Houyet, M. A., Trouet, A., and Baudhuin, P. (1979) Endocytosis and chloroquine accumulation during the cell cycle of hepatoma cells in culture. *J. Cell Biol.* 82, 644–653.
 - (36) Illinger, D., Italiano, L., Beck, J., Waltzinger, C., and Kuhry, J. (1993) Comparative evolution of endocytosis levels and of the cell surface area during the L929 cell cycle: a fluorescence study with TMA-DPH. *Biol. Cell.* 79, 265–268.
 - (37) Bogdanov, A. A., Jr., Weissleder, R., Frank, H. W., Bogdanova, A. V., Nossif, N., Schaffer, B. K., Tsai, E., Papisov, M. I., and Brady, T. J. (1993) A new macromolecule as a contrast agent for MR angiography: preparation, properties, and animal studies. *Radiology* 187, 701–706.
 - (38) Schreiber, G. J., Hellstrom, K. E., and Hellstrom, I. (1992) An unmodified anticarcinoma antibody, BR96, localizes to and inhibits the outgrowth of human tumors in nude mice. *Cancer Res.* 52, 3262–3266.
 - (39) Schulze, E., Ferrucci, J., Poss, K., Lapointe, L., Bogdanova, A., and Weissleder, R. (1995) Cellular uptake and trafficking of a prototypical magnetic iron oxide label *in vitro*. *Invest. Radiol.* 30, 604–610.
 - (40) Lin, K., Nagy, J. A., Xu, H., Shockley, T. R., Yarmush, M. L., and Dvorak, H. F. (1994) Compartmental distribution of tumor-specific monoclonal antibodies in human melanoma xenografts. *Cancer Res.* 54, 2269–2277.
 - (41) Schnitzer, J. E., Allard, J., and Oh, P. (1995) NEM inhibits transcytosis, endocytosis, and capillary permeability: implication of caveolae fusion in endothelia. *Am. J. Physiol.* 268, H48–H55.
 - (42) Trail, P., Willner, D., Lasch, S., Henderson, A., Hofstead, S., Casazza, A., Firestone, R., Hellström, I., and Hellström, K. (1993) Cure of xenografted human carcinomas by BR96-doxorubicin immunoconjugates. *Science* 261, 212–215.
 - (43) Bogdanov, A., Jr., Callahan, R., Wilkinson, R., Martin, C., Cameron, J., Fishman, A., Brady, T., and Weissleder, R. (1994) A synthetic copolymer kit for radionuclide blood pool imaging. *J. Nucl. Med.* 35, 1880–1886.
 - (44) Gupta, H., Weissleder, R., Bogdanov, A. A., Jr., and Brady, T. J. (1995) Experimental gastrointestinal hemorrhage: detection with contrast-enhanced MR imaging and scintigraphy. *Radiology* 196, 239–244.
 - (45) Gupta, H., Wilkinson, R. A., Bogdanov, A. A., Jr., Callahan, R. J., and Weissleder, R. (1995) Inflammation: imaging with methoxy poly(ethylene glycol)-poly-L-lysine-DTPA, a long-circulating graft copolymer. *Radiology* 197, 665–669.
 - (46) Shockley, T. R., Lin, K., Nagy, J. A., Tompkins, R. G., Yarmush, M. L., and Dvorak, H. F. (1992) Spatial distribution of tumor-specific monoclonal antibodies in human melanoma xenografts. *Cancer Res.* 52, 367–376.
 - (47) Baxter, L. T., and Jain, R. K. (1991) Transport of fluid and macromolecules in tumors. IV. A microscopic model of the perivascular distribution. *Microvasc. Res.* 41, 252–272.
 - (48) Baxter, L. T., and Jain, R. K. (1991) Transport of fluid and macromolecules in tumors. III. Role of binding and metabolism. *Microvasc. Res.* 41, 5–23.
 - (49) Jain, R. K. (1990) Physiological barriers to delivery of monoclonal antibodies and other macromolecules in tumors. *Cancer Res.* 50, 814–819.
 - (50) Duncan, R., Starling, D., Rypacek, F., Drobnik, J., and Lloyd, J. B. (1982) Pinocytosis of poly(α,β -(N-2-hydroxyethyl)DL-aspartamide and a tyramine derivative by rat visceral yolk sacs cultured in vitro. Ability of phenolic residues to enhance the rate of pinocytic capture of a macromolecule. *Biochim. Biophys. Acta* 717, 248–254.

- (51) Duncan, R., Kopeckova-Rejmanova, P., Strohalm, J., Hume, I., Cable, H. C., Pohl, J., Lloyd, J. B., and Kopecek, J. (1987) Anticancer agents coupled to N-(2-hydroxypropyl)methacrylamide copolymers. I. Evaluation of daunomycin and puromycin conjugates in vitro. *Br. J. Cancer* 55, 165–174.
- (52) Pratten, M., and Lloyd, J. (1986) Pinocytosis and phagocytosis: the effect of size of a particulate substrate on its mode of capture by rat peritoneal macrophages cultured in vitro. *Biochim. Biophys. Acta* 881, 307–313.
- (53) Racoosin, E. L., and Swanson, J. A. (1993) Macropinosome maturation and fusion with tubular lysosomes in macrophages. *J. Cell Biol.* 121, 1011–1020.

BC970146W

Identification of the Soluble *in Vivo* Metabolites of Indium-111-Diethylenetriaminepentaacetic Acid-D-Phe¹-Octreotide

Laura A. Bass,[†] Margaret V. Lanahan,[†] James R. Duncan,[†] Jack L. Erion,[‡] Ananth Srinivasan,[‡] Michelle A. Schmidt,[‡] and Carolyn J. Anderson^{*,†}

Division of Radiological Sciences, Washington University School of Medicine, St. Louis, Missouri 63110, and Mallinckrodt Inc., St. Louis, Missouri 63134. Received August 13, 1997; Revised Manuscript Received December 23, 1997

Indium-111-diethylenetriaminepentaacetic Acid-D-phenylalanine¹-octreotide (¹¹¹In-DTPA-octreotide) is a cyclic eight amino acid somatostatin analogue which is approved for gamma scintigraphy of neuroendocrine tumors. To address the factors that contribute to liver and kidney retention of this radiopharmaceutical, its metabolism was evaluated in normal and tumor-bearing rats. The soluble fractions from nontarget (liver and kidney) and target (tumor, pancreas, adrenals) organ homogenates were analyzed out to 21 h postinjection, and urine was analyzed out to 12 h postinjection. The blood was analyzed at shorter time intervals due to the rapid clearance of ¹¹¹In-DTPA-octreotide. Radio-TLC and HPLC were used to analyze organ homogenates, blood, and urine. By TLC, intact ¹¹¹In-DTPA-octreotide was resolved from the soluble metabolites, and a similar apparent rate of metabolism was observed in the liver, kidney, tumor, and pancreas with ~30% intact ¹¹¹In-DTPA-octreotide at 4 h postinjection. In the adrenals, metabolism occurred more slowly with ~60% intact ¹¹¹In-DTPA-octreotide at 4 h postinjection. At 4 h postinjection, the activity excreted in the urine consisted of 85% intact ¹¹¹In-DTPA-octreotide. HPLC provided resolution of the individual extractable metabolites. In an attempt to identify these metabolites, two DTPA-amino acid sequences were synthesized: DTPA-D-Phe-Cys and DTPA-D-Phe. Under the conditions used for metabolite analysis, ¹¹¹In-DTPA-D-Phe-Cys-OH eluted at 14.6 min and ¹¹¹In-DTPA-D-Phe-OH eluted at 7.0 min. Each of these standard sequences was combined with the soluble portion of the organ homogenate and was shown by HPLC to coelute with the metabolites. These data suggest that ¹¹¹In-DTPA-octreotide was initially degraded to ¹¹¹In-DTPA-D-Phe-Cys-OH and ¹¹¹In-DTPA-D-Phe-OH. The ¹¹¹In-DTPA-D-Phe-Cys-OH was further degraded to ¹¹¹In-DTPA-D-Phe-OH, which appeared to be the final metabolite that was extracted from the organs. From these results, it can be concluded that at longer time points (>2 h postinjection) a significant amount of ¹¹¹In was retained in nontarget organs as ¹¹¹In-DTPA-D-Phe-OH and ¹¹¹In-DTPA-D-Phe-Cys-OH and not as intact ¹¹¹In-DTPA-octreotide.

INTRODUCTION

Somatostatin is a naturally occurring peptide hormone consisting of 14 or 28 amino acids. It is present in the hypothalamus, the cerebral cortex, the brain stem, the gastrointestinal tract, and the pancreas and exerts an inhibitory effect on several cell functions such as secretion of peptide hormones and growth factors (1–4). Neuroendocrine tumors have been found to have an increased number of somatostatin receptors. Other common types of tumors also contain somatostatin receptors, such as those of the CNS, breast, and lung (5–10). The clinical value of somatostatin is limited due to its very short half-life *in vivo* ($t_{1/2} < 3$ min) (11). Octreotide, an eight amino acid peptide, is an analogue of somatostatin that is highly resistant to degradation by enzyme attack and more effective than the native hormone in the suppression of growth hormone secretion (12).

Somatostatin analogues, including octreotide have been labeled with a variety of radiometals such as ¹¹¹In (13, 14), ⁶⁴Cu (15), ^{67/68}Ga (16, 17), ⁹⁰Y (18), and ¹⁸⁸Re (19) for targeted radiotherapy or for imaging of somatostatin receptor positive tumors by SPECT or PET. Radiometals are linked to octreotide via a bifunctional chelate. The bifunctional chelate contains a functional group through which it is conjugated to octreotide. The chelate is chosen such that it binds the metal strongly to prevent release of the metal *in vivo*. The biodistribution of a variety of octreotide conjugates has been investigated in tumor-bearing rodents, and although receptor-mediated tumor uptake was observed, many of these agents exhibited high nontarget uptake in the kidney and liver. To alleviate nontarget accumulation, research efforts have focused on altering the stability of the metal–chelate complex or the chemistry of the chelate–octreotide bond.

Understanding the mechanism of uptake, degradation and retention of octreotide conjugates is key to designing optimal somatostatin analogues. Since ¹¹¹In-DTPA-octreotide¹ (Figure 1) is clinically used for the scintigraphic imaging of neuroendocrine tumors (20–23) and, more recently, has been studied for its therapeutic efficacy (11,

* Corresponding author. Division of Radiological Sciences, 510 S. Kingshighway, Box 8225, St. Louis, MO 63110. Phone: 314-362-8427. Fax: 314-362-9940. E-mail: andersoncj@mirlink.wustl.edu.

[†] Washington University School of Medicine.

[‡] Mallinckrodt, Inc.

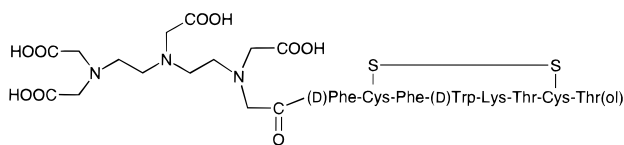


Figure 1. Structure of ^{111}In -DTPA-octreotide.

24), we studied its metabolism *in vivo* in normal and tumor-bearing rats.

There have been few studies on the metabolism of radiolabeled octreotide analogues, although many reports have been published on the *in vivo* fate of various ^{111}In -labeled polypeptides and glycoproteins (25–30). It has been demonstrated that radiolabeled antibodies bound to cell surface antigens are internalized and delivered to the lysosome for degradation (31–34). Recent studies with liver-targeted ^{111}In -DTPA-glycoproteins have shown delivery of these molecules to lysosomes with subsequent degradation of the polypeptide backbone to a low molecular weight species. Chromatographic methods were used to identify ^{111}In -DTPA-lysine as this low molecular weight metabolite (25, 26, 35). ^{111}In -DTPA-lysine was also identified as the metabolic product of ^{111}In -DTPA-MAb conjugates in the liver and kidney (30). These results indicate that the DTPA–amino acid linkage (amide or thiourea bond) is stable *in vivo* and ^{111}In is not significantly dissociated from DTPA. It was therefore proposed that retention of radioactivity in the nontarget organs was due to the inability of In-DTPA-lysine to be released from the cell using the carrier-mediated transport systems designed for the intact polypeptide/antibody.

The internalization of ^{111}In -DTPA-octreotide has been evaluated by several research groups (11, 36, 37). Bree-man et al. injected ^{111}In -DTPA-octreotide into rats and at various times postinjection challenged receptor uptake with cold octreotide (36). He concluded that ^{111}In -DTPA-octreotide was rapidly internalized (<20 min) in somatostatin receptor positive tissues and normal organs. Recently, Duncan et al. confirmed that ^{111}In -DTPA-octreotide was rapidly internalized and, using subcellular fractionation techniques, identified lysosomes as the site of delivery in pancreatic, tumor, kidney, and liver cells (37). Andersson et al. studied the internalization and release of ^{111}In -DTPA-octreotide in human glucagonoma and carcinoid cells (11). Electron microscope autoradiography was used to determine the subcellular distribution of ^{111}In in the midgut carcinoid and activity was reported to be primarily in the cytoplasm, with about 30% found in the nucleus.

In the present study, the metabolism of ^{111}In -DTPA-octreotide was evaluated *in vivo* in target (somatostatin receptor containing) and nontarget organs of normal and tumor-bearing rats. The aims of this investigation were (1) to analyze and compare metabolism patterns in target and nontarget tissues; (2) to identify the retained radiolabeled species; and (3) to gain an understanding of the factors that contribute to nontarget retention of octreotide radiopharmaceuticals.

MATERIALS AND METHODS

Materials. DTPA-octreotide and $^{111}\text{InCl}_3$ (4.2×10^5 Ci/g) were provided by Mallinckrodt Inc. (St. Louis, MO). DTPA was purchased from Sigma Chemical Co. (St. Louis, MO). Trifluoroacetic acid (TFA) was purchased

from J. T. Baker (Chicago, IL). C-18 Sep-Pak cartridges were from Waters/Millipore (Burlington, MA). Biospin-6 chromatography columns were from Bio-Rad Laboratories (Hercules, CA). Organs were prepared using a Tekmar tissue homogenizer (Cincinnati, OH), a Branson Sonifier 185 cell disrupter, and a Sorvall RC2-B centrifuge. Ultrafree-MC 0.22 μm filter units were from Millipore (Bedford, MA). Centricon-10 and -30 ultrafiltration units were from Amicon (Beverly, MA). Reversed-phase HPLC was performed on a Waters 600 system with a Waters 991 photodiode array detector (200–300 nm) and an Ortec (EG&G Instruments) radioactive detector. Reversed-phase columns used were either Vydac 218TP54 (C18, 4.6×250 mm, analytical) or Vydac 218TP1010 (C18, 10×250 mm, semi-prep). Unless otherwise indicated, a gradient elution was employed with 5% A to 70% A in 30 min (solvent A = 95% ACN:5% H_2O and solvent B = 95% H_2O :5% ACN, both solvents contained 0.1% TFA). Millenium software (Waters Corporation) was used to quantify chromatograms by integration. Reversed-phase silica gel (KC18F, 60 Å, 200 μm) TLC plates were from Whatman and the radioactive compounds were analyzed on a Bioscan System 200 imaging scanner after development in 70% MeOH:30% (5% $\text{NH}_4\text{-OAc}$). Radioactivity was quantified using a Beckman Gamma 8000 automated well-type gamma counter. Adult, female, Sprague–Dawley rats were purchased from Sasco (Omaha, NE), and female Lewis rats were purchased from Charles River Laboratories (Boston, MA). The rat pancreatic tumor CA20948 was obtained from the Tumor Bank at Biomeasure, Inc. (Hopkinton, MA). All animal experiments were performed in compliance with guidelines specified by the Washington University Animal Studies Committee.

Synthesis of DTPA-D-Phe-OH and DTPA-D-Phe-Cys-OH. Preparation of tri-*tert*-butyl-DTPA and a general method for the incorporation of DTPA by solid phase were previously described (38).

DTPA-D-Phe-OH (Figure 2A). To a solution of tri-*tert*-butyl-DTPA (42 mg, 75 μ mol) in 1 mL of anhydrous dimethylformamide 15.5 mg of dicyclohexylcarbodiimide in 500 mL was added and shaken for 30 min. A solution of 8 mg (50 μ mol) of D-phenylalanine in 1 mL of dimethylformamide containing 10 mg (12 mL, 100 μ mol) of triethylamine was added and the solution was shaken for 3–4 h at room temperature. Dimethylformamide was removed *in vacuo*, and the residue was treated with 95% trifluoroacetic acid:5% water to remove the *tert*-butyl groups. The solvents were removed *in vacuo* and DTPA-D-Phe-OH was isolated by preparative reversed-phase liquid chromatography [mobile phase, solvent A (water: 0.1% TFA) and solvent B (90% H₂O:10% acetonitrile:0.1% TFA); gradient, 95% A:5% B to 30% A:70% B in 40 min, linear gradient, detection mode UV at wavelength 214 nm] followed by lyophilization. DTPA-D-Phe-OH eluted at 6.1 min. MS, *m/z* 541.2 (M + 1).

DTPA-D-Phe-Cys-OH (Figure 2B). Solid-phase peptide synthesis (SPPS) was carried out using an Applied Biosystems Model 432 A "Synergy" Peptide synthesizer using Fmoc (9-fluorenylmethoxycarbonyl) strategy (39). The instrument protocol required 25 μmol of preloaded Fmoc amino acid to the resin and 75 μmol of subsequent Fmoc-protected amino acids. Activation was accomplished by a combination of hydroxylbenzotriazole (HOBt) and 2-(1-H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU). Tri-*tert*-butyl DTPA (75 μmol) was placed at the appropriate location in the synthesizer and activated similar to the other amino acids for coupling. Fmoc-Cys(S-Trt)-Wang resin (50 mg,

¹ Abbreviations: DTPA, diethylenetriaminepentaacetic acid; SPECT, single-photon emission computerized tomography; PET, positron emission tomography; CNS, central nervous system.

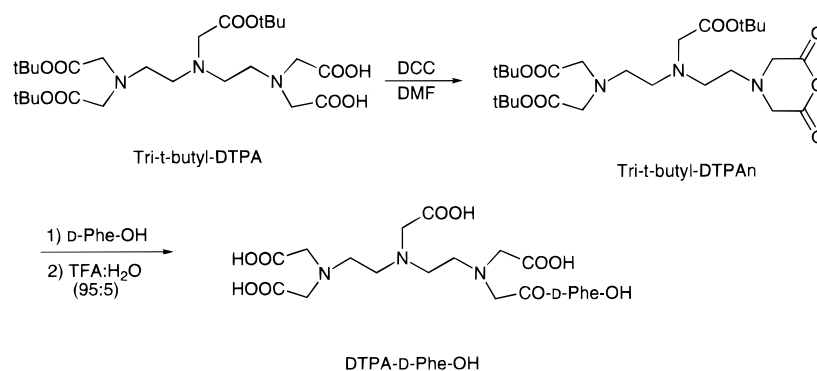
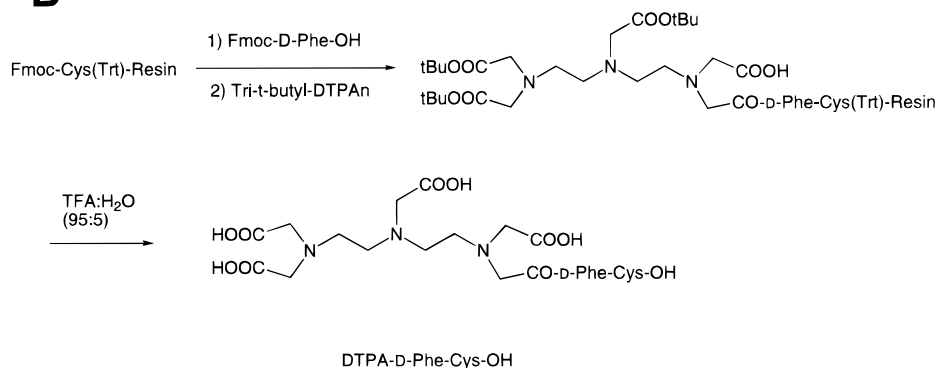
A**B**

Figure 2. Synthesis of metabolite standards. (A) Synthesis of Tri-*tert*-butyl-DTPAn and DTPA-D-Phe-OH; (B) synthesis of DTPA-D-Phe-Cys-OH.

0.5 mmol/g) was used. After successive coupling of Fmoc-D-Phe and tri-*tert*-butyl-DTPA, the peptide was cleaved and deprotected from the resin with trifluoroacetic acid: thianisole:phenol:water (85:5:5:5) for 8–10 h. The peptide was precipitated by the addition of 10 mL of *tert*-butylmethyl ether. Peptide-resin mixture was washed with 4×10 mL of *tert*-butylmethyl ether. Acetonitrile:water (2:3) was added to dissolve the peptide and the solution was filtered to remove the resin. The solution containing the crude peptide was lyophilized prior to purification by liquid chromatography. Final purification was accomplished by C-18 reversed phase chromatography [mobile phase, solvent A (water:0.1% TFA) and solvent B (90% H₂O:10% acetonitrile:0.1% TFA), gradient, 90% A:10%B to 30% A:70% B in 40 min, linear gradient, detection mode UV at wavelength 214 nm] followed by lyophilization. DTPA-D-Phe-Cys-OH eluted at 9.3 min. MS, m/z 644.2 ($M + 1$).

Radiolabeling. ¹¹¹In-DTPA-octreotide was prepared as previously described by Anderson et al. and was analyzed by radio-TLC and HPLC (15). DTPA, DTPA-D-Phe, and DTPA-D-Phe-Cys (1–3 μ g) were dissolved in NH₄OAc, pH = 5.5, 0.1 M, and incubated with ¹¹¹Indium-acetate (1–3 mCi) at room temperature for 1 h. The compounds were analyzed by radio-TLC and HPLC without purification. Transferrin was also labeled by its incubation with ¹¹¹Indium-acetate. It was purified from uncomplexed ¹¹¹In by passage through a Biospin-6 column and was analyzed by radio-TLC.

In Vivo Metabolism of ¹¹¹In-DTPA-Octreotide. Mature, female, Sprague–Dawley rats and female Lewis tumor-bearing rats ($n = 2$ –4) were injected with 200–2000 μ Ci of ¹¹¹In-DTPA-octreotide (specific activity, 1000–4000 μ Ci/ μ g) via the tail vein. Prior to injection, the dose

was diluted with 0.1% BSA in buffer or saline. At 1, 2, 4, 6, 8, 14, and 21 h postinjection the urine was collected and the liver, kidney, adrenals, pancreas, tumor, and blood removed. In the experiments, only a section of the liver was used (~1 g). All organs were rinsed with water and blotted dry. Samples were kept in an ice bath at all times.

To the organs and blood, 2.0 mL of 20% HEPES (1.0 M, pH = 7) in EtOH was added and the samples were homogenized (1–3 min), sonicated (30 s), and centrifuged (23000g for 10 min, 10 °C) to precipitate protein. This extraction was repeated with 1 mL of 20% HEPES in EtOH and the supernatants combined. Both supernatant and pellet were counted to determine extraction efficiency. Supernatants were passed through a 0.22 μ m Ultra-MC spin filter and analyzed by HPLC and/or TLC. Organ blanks, where the ¹¹¹In-DTPA-octreotide injectate was added directly to tissues prior to workup, were performed as controls.

In separate experiments, four female Sprague–Dawley rats were injected with 2 mCi ¹¹¹In-DTPA-octreotide. At 4 h postinjection, the rats were sacrificed and their livers and kidneys excised. A portion of the livers and one kidney were homogenized and extracted twice with 20% HEPES in EtOH as described above. The pellets then underwent digestion with 1.0 mg/mL collagenase at room temperature for 6 h, followed by digestion with pronase-E (0.5 mg/mL) for 6 h and trypsin (0.5 mg/mL) for 6 h as described by Paik et al. (28). Another portion of the livers and the second kidney underwent a similar procedure, except they were digested with each enzyme for 24 h at room temperature (40).

Metabolite Analysis by Radio-TLC. All samples were immediately analyzed by radio-TLC. The distribution of

Table 1. TLC and HPLC Retentions of Standard Compounds^a

standard compound	R _f	R _t (min)	radiochemical purity
¹¹¹ In-DTPA-octreotide	0.50	20.60	100%
¹¹¹ In-DTPA-D-Phe-OH	0.85	7.05	>98%
¹¹¹ In-DTPA-D-Phe-Cys-OH	0.85	14.70	>95%
¹¹¹ In-acetate	0.00	4.00 (void) ^b	ND ^c
¹¹¹ In-DTPA	1.00	4.00 (void)	ND
¹¹¹ In-transferrin	0.00	ND	ND

^a Radiochemical purity was determined by HPLC integration.

^b Void indicates that the activity was eluted in the void volume.

^c ND signifies that no data was collected.

activity was determined by integration of the chromatograms. The kidney supernatants were cospotted with the standards ¹¹¹In-DTPA-D-Phe-Cys-OH and ¹¹¹In-DTPA-D-Phe-OH and also with ¹¹¹In-DTPA and ¹¹¹Indium-transferrin for comparison. To further evaluate the metabolites by TLC, centricon filters were used to determine if organ extracts contained protein bound activity. Organ supernatants were passed through a centricon filter with a molecular mass cutoff of 30 000 Da, and the top and bottom fractions were analyzed.

Metabolite Identification by HPLC. In separate experiments, liver, kidney, tumor, and adrenal supernatants were analyzed by HPLC at 2, 6, or 8 and 24 h postinjection, and the pancreas supernatant was analyzed at 24 h. Supernatants with low amounts of radioactivity per milliliter were concentrated using a stream of nitrogen. To ensure reproducibility of retention, samples were diluted to less than 5% EtOH and the maximum volume injected was 300 μL. To verify the integrity of the standards under our extraction conditions, ¹¹¹In-DTPA-D-Phe-Cys-OH and ¹¹¹In-DTPA-D-Phe-OH were each added to a kidney, one 2 mL extraction was performed and the supernatant was analyzed by HPLC. To ensure that all activity was accounted for, fractions were collected from the 2 and 6 h analysis of the kidney, counted, and the activity compared to the amount injected. Greater than 90% of the activity was recovered from the column. To demonstrate comigration of the metabolites with each standard, the 6 h kidney supernatant was combined with ¹¹¹In-DTPA-D-Phe-Cys-OH and ¹¹¹In-DTPA-D-Phe-OH separately and then analyzed by HPLC.

RESULTS

Analysis of Standards. The standards DTPA-D-Phe and DTPA-D-Phe-Cys were analyzed by electron spray mass spectrometry and the expected mass peaks were observed at 541 and 644, respectively. The radiochemical purities of ¹¹¹In-DTPA-octreotide, ¹¹¹In-DTPA-D-Phe-Cys-OH, and ¹¹¹In-DTPA-D-Phe-OH were assessed using reversed-phase HPLC and radio-TLC. Radiochemical purities and chromatographic retentions of the ¹¹¹In labeled agents used are listed in Table 1.

Extraction of ¹¹¹In-DTPA-octreotide and Metabolites from Tissues. Organ blanks were done for every experiment in order to establish the inherent extraction efficiency of ¹¹¹In-DTPA-octreotide from the organ matter. This extraction efficiency was then used as a correction factor in determining the percent authentic intact (see Table 3).

Extraction efficiencies for each organ over the time course of an experiment are listed in Table 2. We found that, for the liver and kidney, the extraction efficiencies decreased over time, whereas for the tumor and pancreas, extraction efficiencies were initially lower than the other organs and remained fairly constant. Adrenals exhibited

Table 2. Extraction Efficiencies Are Listed for Each Organ at the Time Points Studied Postinjection^a

time (h)	liver	kidney	tumor	adrenal	pancreas
0	89.3 ± 9.7	91.6 ± 3.8	89.0 ^b	89.3 ± 12.9	99.9 ^b
1	82.5 ± 7.8	88.0 ± 1.4	62.0 ± 5.7	87.1 ± 13.6	55.8 ± 0.5
2	68.0 ± 0.0	84.5 ± 2.1	65.5 ± 13.4	86.7 ± 13.5	31.5 ± 1.9
4	64.5 ± 0.7	77.5 ± 2.1	77.5 ± 12.0	87.0 ± 9.4	20.3 ± 2.6
6	57.5 ± 7.8	66.5 ± 5.0	ND	85.4 ± 7.7	14.4 ± 6.8
8	ND ^c	ND	51.0 ± 0.0	ND	ND
21	21.0 ^b	45.0 ^b	53.0 ^b	77.0 ^b	0.0 ^b

^a Normal organs are from Sprague–Dawley rats, whereas the tumor was excised from lewis rats (*n* = 3 or 4 unless noted). The data is expressed as: % extraction = cpm in supernatant/(cpm in pellet + cpm in supernatant). ^b *n* = 2 for these data. ^c ND, no data was collected at this time postinjection.

high extraction efficiencies throughout the experiment, probably due to the small size of the organ and the larger ratio of extraction solvent to tissue used in the extraction procedure.

A digestion procedure was evaluated to maximize the amount of ¹¹¹In-labeled intact DTPA-octreotide and metabolites from liver homogenates. Paik et al. determined that this digestion procedure increased their extraction of ¹¹¹In-labeled mAb metabolites from 75 to ~95% (28). With ¹¹¹In-DTPA-octreotide, it was determined that this procedure did not appreciably increase extraction efficiency, and in all samples, the additional ¹¹¹In-labeled agents extracted increased by less than 5%. No further samples were evaluated using this procedure.

Metabolite Analysis by Radio-TLC. Following extraction, the organ supernatants were immediately analyzed by radio-TLC. The formation of metabolites with time in the kidney is shown in Figure 3. In all organs, a broad metabolite peak at an *R_f* of 0.86 was observed. By TLC, these metabolites were separated from intact ¹¹¹In-DTPA-octreotide, which eluted at an *R_f* of 0.50. A species was intermittently observed at the origin in small amounts relative to the intact compound. This species did not pass through a centricon filter membrane with a 30 000 MW cutoff, suggesting that ¹¹¹In was protein bound.

Table 3 shows the percent authentic intact compound present in the organ extracts. These data were calculated from the integration of the radio-thin-layer chromatograms and took into account the unidentified activity left in the pellet, the extraction efficiency of the ¹¹¹In-DTPA-octreotide injectate determined from the organ blank experiments and the purity of the injectate. The tumor and pancreas exhibited a similar rate of metabolism with approximately 60% of the extracted activity in the form of intact ¹¹¹In-DTPA-octreotide at 1 h postinjection and only 22% at 4 h. In the adrenals, ¹¹¹In-DTPA-octreotide appeared to metabolize much more slowly with 99% intact ¹¹¹In-DTPA-octreotide at 1 h and 60% intact at 4 h. The liver and kidney exhibited an almost identical rate of metabolism with approximately 80–90% of the activity as intact ¹¹¹In-DTPA-octreotide at 1 h and 20% at 4 h. As indicated in Table 3, radioactivity in the blood could only be detected out to 1 h and was present as mostly intact ¹¹¹In-DTPA-octreotide. ¹¹¹In-DTPA-octreotide was initially excreted mostly intact with 85% of the excreted activity in the form of intact ¹¹¹In-DTPA-octreotide at 4 h postinjection. At 12 h postinjection only 23% of the excreted activity was intact compound.

Metabolite Identification by HPLC. In separate experiments, HPLC was used to resolve the individual

Table 3. The Percent of Authentic Intact ^{111}In -DTPA-Octreotide Determined in Normal Tissues and Tumors Extracted from Sprague–Dawley (Normal Tissues) or Lewis (Tumor) Rats ($n = 3$ or 4 Unless Noted)^a

time (h)	liver	kidney	tumor	adrenals	pancreas	blood	urine
0.0	100.0 ^c	99.9 ^c	100.0 ^c	100.0 ^c	99.9 ^c	88.4 ± 4.6	ND
0.5	ND ^b	ND	ND	ND	ND	87.3 ± 4.6	ND
1.0	78.7 ± 7.3	88.5 ± 1.9	62.4 ± 2.7	99.2 ± 13.3	55.8 ± 0.5	39.9 ± 0.1	100
2.0	46.0 ± 5.3	48.1 ± 4.9	49.0 ± 4.6	75.6 ± 7.8	31.5 ± 1.9	ND	100 ^c
4.0	23.0 ± 1.9	20.4 ± 2.2	23.8 ± 1.0	60.3 ± 12.3	20.3 ± 2.6	ND	85.0 ^c
6.0	17.2 ± 2.1	15.4 ± 6.5	ND	49.3 ± 9.4	14.4 ± 6.8	ND	73.5 ^c
8.0	ND	ND	4.3 ± 0.7	ND	ND	ND	ND
12.0	ND	ND	ND	ND	ND	ND	22.9 ^c
21.0	0.0 ^c	3.3 ^c	0.0 ^c	15.2 ^c	0.0 ^c	ND	ND

^a The % authentic intact (%AI) ^{111}In -DTPA-octreotide determined from the integration of the radio-TLCs and calculated as follows: %AI = %P × %I/[1 + (%Pellet/%Super)]%E. %P = purity of injectate determined by TLC; %I = intact ^{111}In -DTPA-octreotide determined by integration of the TLC; %Pellet/%Super = the ratio of the radioactivity in the pellet and supernatant of the organ blank; %E = the extraction efficiency of the harvested organ after injection of ^{111}In -DTPA-octreotide. ^b ND signifies that no data was collected at this time postinjection. ^c $n = 2$.

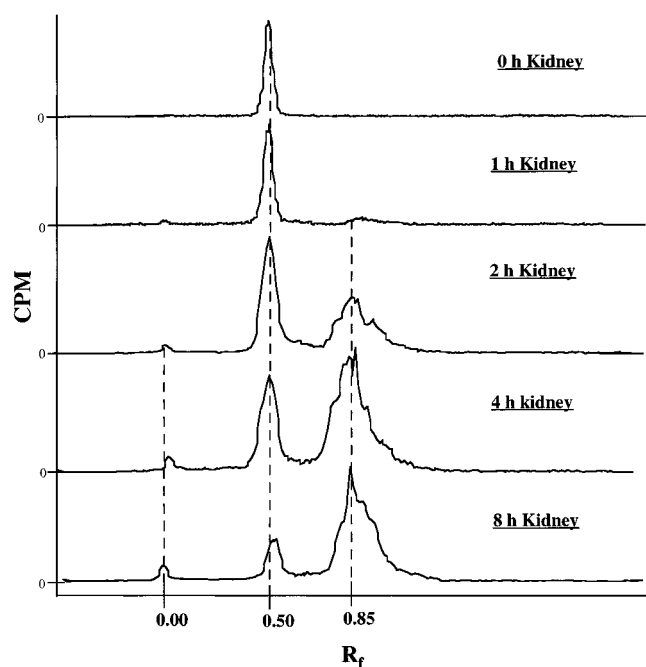


Figure 3. Radio-TLC time course of metabolite formation in the kidney shown out to 6 h postinjection. ^{111}In -DTPA-octreotide migrated with an R_f of 0.50 whereas the metabolites comigrated with an R_f of 0.85.

metabolites which, when analyzed by radio-TLC, coeluted at $R_f = 0.86$. In all organs studied two major metabolites were observed eluting at 7.0 and 14.6 min. To elucidate the identity of these metabolites the 6 h kidney supernatant was co-injected with ^{111}In -DTPA-D-Phe-Cys-OH and ^{111}In -DTPA-D-Phe-OH separately as shown in Figure 4. The species eluting at 7.0 min increased in integration area upon co-injection with ^{111}In -DTPA-D-Phe-OH and the species eluting at 14.6 min increased in area upon co-injection with ^{111}In -DTPA-D-Phe-Cys-OH. These data strongly suggest that the metabolites of ^{111}In -DTPA-octreotide were ^{111}In -DTPA-D-Phe-Cys-OH and ^{111}In -DTPA-D-Phe-OH.

The HPLC time course for formation of metabolites in the kidney is shown in Figure 5. At 24 h postinjection, no intact ^{111}In -DTPA-octreotide was observed, 85% of the activity coeluted with ^{111}In -DTPA-D-Phe-OH, and 7% coeluted with ^{111}In -DTPA-D-Phe-Cys-OH. In the adrenals at 24 h, 12% of the activity eluted as ^{111}In -DTPA-octreotide, 28% as ^{111}In -DTPA-D-Phe-Cys-OH and 60% as ^{111}In -DTPA-D-Phe-OH. These kidney and adrenal samples were analyzed immediately after organ preparation; however, other samples were stored up to 24 h at

−20 °C prior to analysis. After storage of the supernatants we observed further conversion of ^{111}In -DTPA-D-Phe-Cys-OH to ^{111}In -DTPA-D-Phe-OH (as indicated by the differing 6 h kidney chromatograms in Figure 4 versus Figure 5).

DISCUSSION

To design octreotide radiopharmaceuticals with optimal targeting (high target-to-nontarget ratios of radio-label), the mechanisms by which these agents are internalized, degraded, and released must be understood. In this study, we set out to determine if ^{111}In -DTPA-octreotide was metabolized in normal and target tissues, to compare the rates of degradation in these organs and to elucidate the identity of the metabolites.

The models used in these studies were normal and tumor-bearing rats. To obtain reproducible results, the methods for performing these *in vivo* studies had to be refined. This first involved maximizing the extraction efficiencies of ^{111}In -DTPA-octreotide and its metabolites from the selected tissues. In Table 2, the extraction efficiencies are listed for each organ over the time course studied. The same extraction procedure was used each time yet a decrease in extraction efficiencies was observed in the liver and kidney over time postinjection. The extraction efficiencies of the pancreas and tumor were initially lower than the other organs and did not vary much with time. At all times, greater than 75% of the activity was extracted from the adrenals.

The decrease in extraction efficiency with respect to time postinjection could represent the inability to extract an unidentified metabolite from the pellet, the binding of ^{111}In to intracellular proteins or the inefficient release of internalized metabolites during the extraction procedure. Paik et al. exhaustively extracted ^{111}In -DTPA-containing metabolites from the liver after injection of a ^{111}In -DTPA-labeled antibody and obtained a maximum of 75% extraction efficiency (28), whereupon with enzyme digestion of the liver homogenate they obtained greater than 90% extraction efficiency. In our experiments, exhaustive extractions did somewhat increase the activity extracted, but after the first two extractions, we did not observe appreciable increases in amounts of activity. Interestingly, radio-TLC and HPLC showed the same metabolite profile for each individual extraction, indicating that there were no new metabolites extracted in the later extractions. We observed an increase in extraction efficiency upon sonication of the organ samples, indicating the importance of bursting the intracellular vesicles; however, the digestion procedure similar to that described by Paik et al. (28) did not significantly increase the extraction efficiency in our hands.

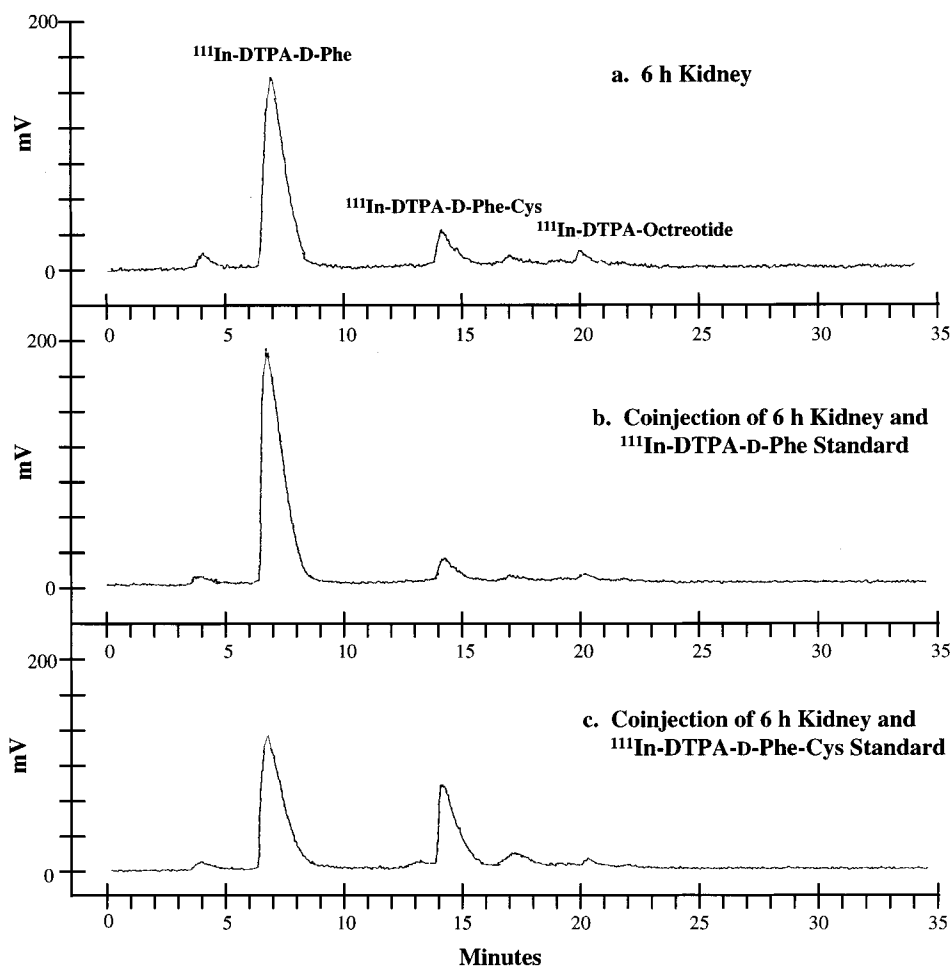


Figure 4. HPLC co-injection of the kidney supernatant with the standards ^{111}In -DTPA-D-Phe-Cys-OH and ^{111}In -DTPA-D-Phe-OH. (a) In an aliquot of the 6 h kidney supernatant the integrated area of the peak eluting at 7.0 min was 7×10^6 mV·s and that of peak eluting at 14.6 min was 9×10^5 mV·s. (b) To the same volume aliquot of the 6 h kidney supernatant ^{111}In -DTPA-D-Phe-OH was added. The integrated area of the peak eluting at 7.0 min increased to 11×10^6 mV·s whereas the area of the peak eluting at 14.6 min remained constant at 9×10^5 mV·s. (c) ^{111}In -DTPA-D-Phe-Cys-OH was added to the same volume aliquot of the 6 h kidney. The integrated area of the peak eluting at 7.0 min was equal to that in panel a (7×10^6 mV·s) whereas the area of the peak eluting at 14.6 min increased to 36×10^6 mV·s.

A low extraction efficiency may indicate that it is more difficult to extract the metabolites than to extract the intact compound, or it may reflect the possibility that ^{111}In was bound to insoluble tissue proteins and was therefore not extracted. It is unlikely that the activity in the pellet is intact ^{111}In -DTPA-octreotide; however, because the identity of this activity is unknown, the percent authentic intact data presented in Table 3 has a degree of error associated with it. It is unlikely that the pellet contains polar or nonpolar smaller molecular weight metabolites, since several different combinations of polar and nonpolar solvents were used in order to increase extraction efficiency. Different combinations of isopropyl alcohol, deionized water, ethanol, acetonitrile, and a variety of buffers (including TEA, HEPES) and detergents (Icon-x, Triton-x) were attempted, and it was determined from these studies that no additional metabolites were extracted. Maximum extraction efficiency was obtained in 20% HEPES in ethanol.

Organ supernatants were analyzed by radio-TLC, and integration of these chromatograms allowed us to compare the rates of metabolism of ^{111}In -DTPA-octreotide in the various organs studied. As Table 3 indicates, similar rates of metabolism were observed for the tumor, pancreas, liver, and kidney. HPLC analysis provided resolution of the individual metabolites and revealed that in all organs studied the chromatographic retentions of the

two major soluble metabolites were the same. To identify these metabolites two DTPA-amino acid standards were synthesized. ^{111}In -DTPA-D-Phe-OH was chosen as a standard based on the lysosomal metabolism patterns of other ^{111}In -DTPA-polypeptides (25, 26, 30, 35). We also analyzed ^{111}In -DTPA-D-Phe-Cys-OH as a metabolite standard because of the potentially higher stability of the D-Phe-Cys bond (since Phe is a D-amino acid as opposed to the naturally occurring L-amino acids). The chromatographic elutions of the observed metabolites corresponded to those for ^{111}In -DTPA-D-Phe-Cys-OH and ^{111}In -DTPA-D-Phe-OH. As shown in Figure 4, co-injection of these standards with the 6 h kidney supernatant confirmed coelution. Identity of the metabolites is not, however, proven by coelution. Future characterization studies involving LC-MS (liquid chromatography-mass spectrometry) of the organ homogenates are planned to confirm the identity of the metabolites of ^{111}In -DTPA-octreotide.

Although HPLC provided better resolution, one caveat to its use for the study of metabolism is the length of time it takes for each analysis. Due to the time required for sample preparation and analysis, certain samples had to be stored at -20°C for up to 24 h before analysis. We observed further conversion of ^{111}In -DTPA-D-Phe-Cys-OH to ^{111}In -DTPA-D-Phe-OH after 24 h of storage at -20°C with subsequent thawing at room temperature. This was

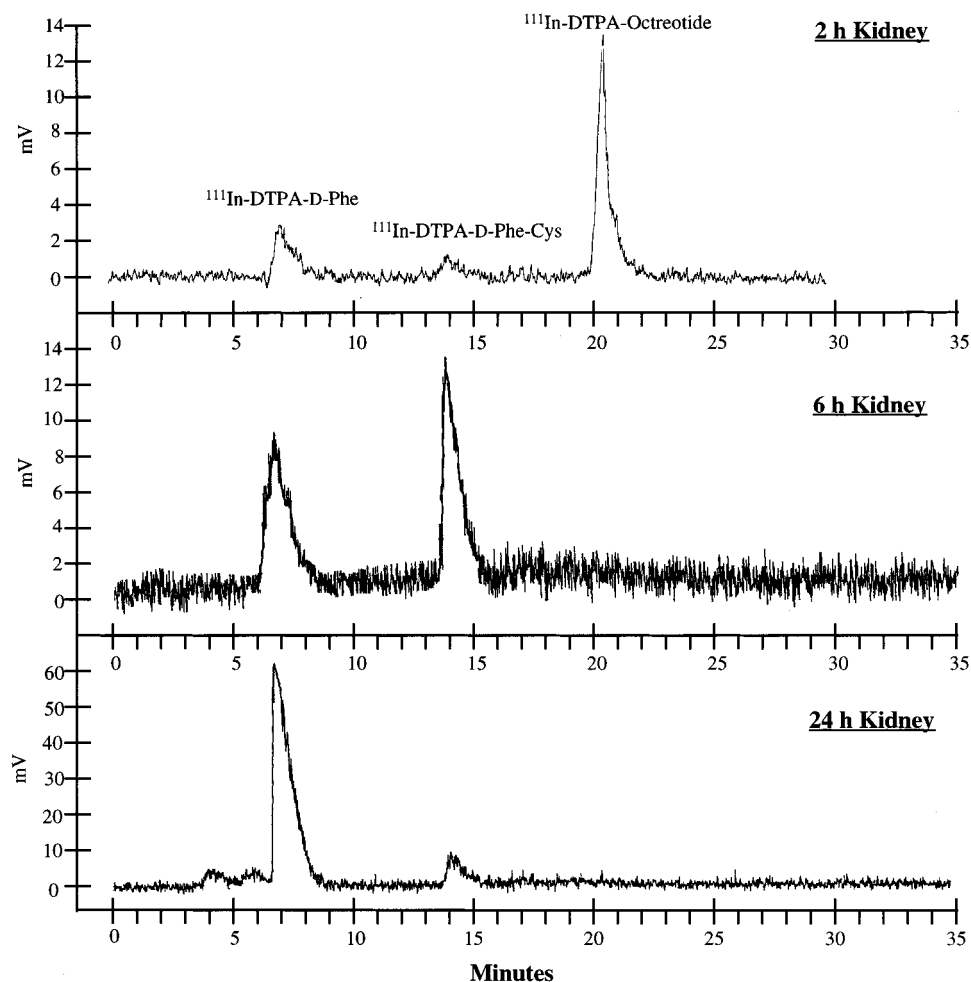


Figure 5. HPLC time course of metabolite formation in the kidney shown out to 24 h postinjection. ^{111}In -DTPA-octreotide eluted at 20.5 min, ^{111}In -DTPA-D-Phe-Cys-OH eluted at 14.6 min and ^{111}In -DTPA-D-Phe-OH eluted at 7.0 min.

apparent in the 6 h kidney supernatant which initially had 54% of the activity eluting as ^{111}In -DTPA-D-Phe-Cys-OH and 46% as ^{111}In -DTPA-D-Phe-OH (Figure 5), yet after 24 h of storage it had only 11% of the activity eluting as ^{111}In -DTPA-Phe-Cys and 84% of the activity as ^{111}In -DTPA-D-Phe-OH (Figure 4). We were therefore unable to use HPLC as a means for accurately quantifying the distribution of metabolites in the samples.

The results obtained in this study will enable a better understanding of the factors that govern uptake and retention of peptides. We observed less than 25% of the activity in the form of ^{111}In -DTPA-octreotide in the tumor, pancreas, liver, and kidney at 4 h postinjection. We showed that, out to 21 h postinjection, approximately 50% of the activity in the organs was extractable and contained the metabolites ^{111}In -DTPA-D-Phe-Cys-OH and ^{111}In -DTPA-D-Phe-OH. Therefore, it is interesting to note that, in scintigraphic-imaging experiments, which are often carried out at 24 h postinjection, the images most likely represent metabolites remaining in the tissues and not intact ^{111}In -DTPA-octreotide.

To design octreotide radiopharmaceuticals that will result in enhanced target to nontarget ratios, it is important that the identity of the retained radiolabel is known and the transport mechanisms available to the various organs are taken into account. Breeman et al. demonstrated that ^{111}In -DTPA-octreotide was taken up in the target organs in less than 20 min postinjection (36). Duncan et al. showed that ^{111}In -DTPA-octreotide was internalized into the cell and delivered to the lysosomes

within 1 h postinjection (37). A preliminary experiment was carried out to analyze by TLC the kidney lysosomal fraction at 1 h postinjection of ^{111}In -DTPA-octreotide (Duncan et al., unpublished results). The majority of the activity was in the form of intact ^{111}In -DTPA-octreotide. These results were in agreement with our studies presented here and indicate that ^{111}In -DTPA-octreotide was delivered intact to the lysosomes and then degraded.

In biodistribution studies previously carried out by our group, retention of radioactivity was observed in the kidney, liver, and target organs (Figure 6) (15). Duncan et al. showed that at 20 h ^{111}In was retained in the lysosomes in the kidney, liver, tumor, and pancreas (37). Our data presented here show that, in these organs at 20 h postinjection, ^{111}In -DTPA-octreotide was almost completely metabolized to ^{111}In -DTPA-D-Phe-OH and ^{111}In -DTPA-D-Phe-Cys-OH and other insoluble metabolites. This retention must therefore be dependent on the cellular mechanisms available for transport of these metabolites out of the lysosomes. Although the clearance of activity from the various organs differed, as verified by the biodistribution results, we found the rates of metabolism to be very similar. This implies that the mechanisms by which the degradation products of radiometal-peptide complexes are transported from the cells varies with organ type. Recently, it has been shown that these degradation products are poor substrates for the carrier-mediated transport systems found in lysosomes (26, 30, 37). Although other routes are available, they are slow, and thus, radiometal metabolites ac-

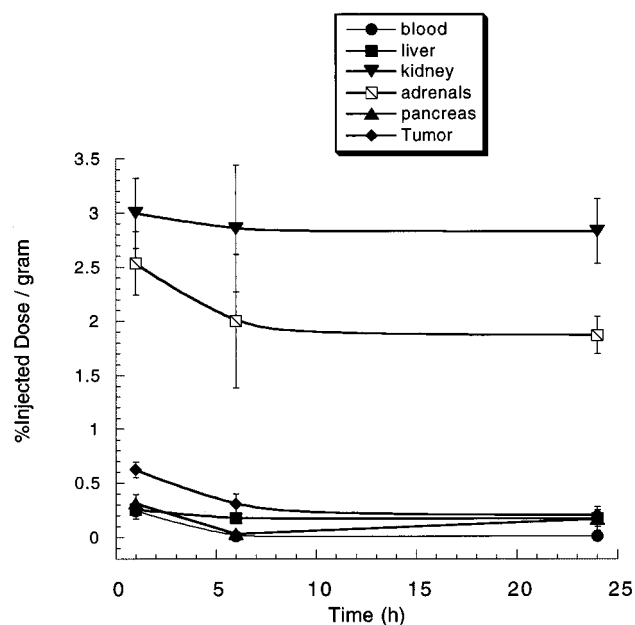


Figure 6. Biodistribution of ¹¹¹In-DTPA-octreotide in CA20948 tumor-bearing Lewis rats. The data are presented as % injected dose/gram of tissue $\pm$ standard deviation; $n = 4$.

cumulate in the lysosomes of certain organs. Understanding the chemistry of the metabolites may allow us to take advantage of the specialized transmembrane transporters found in the hepatocyte and renal tubular cells that are not present in the target organs, therefore enhancing target to nontarget ratios of radiolabel.

This study along with studies done on the metabolism of other ¹¹¹In-DTPA-polypeptides stress the importance of the terminal amino acid (or amino acid to which conjugation occurs) on the fate of the radiolabel. Therefore, one approach to exploiting the different transport mechanisms may involve altering the terminal amino acid (as long as receptor binding is not compromised). Wu et al. observed the formation of two metabolites from an antibody fragment (41). These metabolites were identified as ⁶⁷Ga-2-(p-SCN-Bz)-NOTA-Met and ⁶⁷Ga-2-(p-SCN-Bz)NOTA-Lys and it was found that the Met containing metabolite had a significantly shorter residence time in the kidney than did the Lys containing metabolite. We are currently exploring the export mechanisms available to the liver and kidney in hopes of understanding the substrates they transport.

In conclusion, we have shown that ¹¹¹In-DTPA-octreotide is metabolized in the blood, liver, kidney, adrenals, pancreas, and tumor and have compared the rate at which metabolism occurs in these tissues. There are no significant differences in the rate of metabolism between target and nontarget tissues, with the exception of slower metabolism in the adrenal glands. The HPLC data indicate that ¹¹¹In-DTPA-D-Phe-Cys-OH and ¹¹¹In-DTPA-D-Phe-OH are the major extractable metabolites in both the target and nontarget organs; however, there are unknown insoluble metabolites that were not extracted from the tissues. Upon correlation of biodistribution results with the metabolism studies, we conclude that retention of ¹¹¹In in the organs studied is likely due to these metabolites. Evaluating other bifunctional chelates or peptides with different N-terminal amino acids may aid in increasing the clearance of these metabolites from tissues. In the design of radiometal-chelate-peptide conjugates that clear normal tissues but are retained in target tissues, we are currently investigating bifunctional

chelates that have specificity for transport systems found in nontarget organs such as liver and kidney, but are absent from target tissues such as tumors.

ACKNOWLEDGMENT

The authors thank Michael E. Cristel, Lynne A. Jones, and Elizabeth L.C. Sherman for their excellent technical assistance. Funding for this project was provided by NIH CA64475 and CA68699 (C.J.A. and L.A.B.) and Mallinckrodt Medical, Inc. (C.J.A.).

LITERATURE CITED

- (1) Guillemin, R. (1978) Peptides in the brain: the new endocrinology of the neuron. *Science* 202, 390–402.
- (2) Reichlin, S. (1983) Somatostatin (part 1). *N. Engl. J. Med.* 309, 1495–1501.
- (3) Reichlin, S. (1983) Somatostatin (part 2). *N. Engl. J. Med.* 309, 1556–1563.
- (4) Reubi, J. C., Kvols, L. K., Krenning, E. P., and Lamberts, S. W. J. (1990) Distribution of somatostatin receptors in normal and tumor tissue. *Metabolism (suppl. 2)* 39, 78–81.
- (5) Reubi, J. C., Heitz, P. U., and Landolt, A. M. (1987) Visualization of somatostatin receptors and correlation with immunoreactive GH and PRL in human pituitary adenomas; evidence for different tumor subclasses. *J. Clin. Endocrinol.* 65, 65–73.
- (6) Reubi, J. C., Kvols, L. K., Waser, B., D. M., N., Heitz, P. U., Charboneau, J. W., Reading, C. C., and Moertel, C. (1990) Detection of somatostatin receptors in surgical and percutaneous needle biopsy samples of carcinoids and islet cell carcinomas. *Cancer Res.* 50, 5969–5977.
- (7) Reubi, J. C., Maurer, R., Klijn, J. G. M., Stefanko, S. Z., Foekens, J. A., Blaauw, G., Blankenstein, M. A., and Lamberts, S. W. J. (1986) High incidence of somatostatin receptors in human meningiomas: biochemical characterization. *J. Clin. Endocrinol.* 63, 433–438.
- (8) Reubi, J. C., Waser, B., Foekens, J. A., Klijn, J. G. M., S. W. J., L., and Laissue, J. (1990) Somatostatin receptor incidence and distribution in breast cancer using receptor autoradiography: relationship to EGF-receptors. *Int. J. Cancer* 46, 416–420.
- (9) Reubi, J. C., Waser, B., Sheppard, M., and Macaulay, V. (1990) Somatostatin receptors are present in small-cell but not in nonsmall-cell primary lung carcinomas: relationship to EGF receptors. *Int. J. Cancer* 45, 269–274.
- (10) Fekete, M., Wittliff, J. L., and Schally, A. V. (1989) Characteristics and distribution of receptors for [D-Trp6]-luteinizing-hormone-releasing hormone, somatostatin, epidermal growth factor and sex steroids in 500 biopsy samples of human breast cancer. *J. Clin. Lab. Anal.* 3, 137–141.
- (11) Andersson, P., Forsell-Aronsson, E., Johanson, V., Wangberg, B., Nilsson, O., Fjalling, M., and Ahlman, H. (1996) Internalization of Indium-111 into human neuroendocrine tumor cells after incubation with Indium-111-DTPA-D-Phe-Octreotide. *J. Nucl. Med.* 37, 2002–2006.
- (12) Bauer, W., Briner, U., Doepfner, W., Haller, R., Huguenin, R., Marbach, P., Petcher, T. J., and Pless, J. (1982) SMS 201–995: a very potent and selective octapeptide analogue of somatostatin with prolonged action. *Life Sci.* 31, 1133–1140.
- (13) Bakker, W. H., Albert, R., Bruns, C., Breeman, W. A. P., Hofland, L. J., Marbach, P., Pless, J., Pralet, D., Stolz, B., Koper, J. W., Lamberts, S. W. J., Visser, T. J., and Krenning, E. P. (1991) [¹¹¹In-DTPA-D-Phe-1]-Octreotide, a Potential Radiopharmaceutical for Imaging of Somatostatin Receptor-Positive Tumors: Synthesis, Radiolabeling and In Vitro Validation. *Life Sci.* 49, 1583–1591.
- (14) Krenning, E. P., Bakker, W. H., Kooij, P. P. M., Breeman, W. A. P., Oei, H. Y., de Jong, M., Reubi, J. C., Visser, T. J., Bruns, C., Kwekkeboom, D. J., Reijts, A. E. M., van Hagen, P. M., Koper, J. W., and Lamberts, S. W. J. (1992) Somatostatin Receptor Scintigraphy with Indium-111-DTPA-D-Phe-1-Octreotide in Man: Metabolism, Dosimetry and Comparison with Iodine-123-Tyr-3-Octreotide. *J. Nucl. Med.* 33, 652–658.

- (15) Anderson, C. J., Pajean, T. S., Edwards, W. B., Sherman, E. L. C., Rogers, B. E., and Welch, M. J. (1995) In Vitro and In Vivo Evaluation of copper-64-Octreotide Conjugates. *J. Nucl. Med.* 36, 2315–2325.
- (16) Smith-Jones, P. B., S., R., A., Bruns, C., and Macke, H. (1993) A gallium labeled desferrioxamine/somatostatin analogue (SDZ 216–927). *J. Lab. Comput. Radiopharm.* 32, 422.
- (17) Smith-Jones, P. M., Stolz, B., Bruns, C., Albert, R., Reist, H. W., Fridrich, R., and Macke, H. R. (1994) Gallium-67/Gallium-68-[DFO]-octreotide: a potential radiopharmaceutical for PET imaging of somatostatin receptor-positive tumors: synthesis and radiolabeling in vitro and preliminary in vivo studies. *J. Nucl. Med.* 35, 317–325.
- (18) Smith-Jones, P. M., Stolz, B., Albert, R., Ruser, G., Mäcke, H., Briner, U., Tolsvai, L., Weckbecker, G., and Bruns, C. (1995) Synthesis, Radiolabeling, and Evaluation of DTPA/octreotide Conjugates for Radiotherapy. *J. Lab. Comput. Radiopharm.* 37, 499–501.
- (19) Zamora, P. O., Guhlke, S., Bender, H., Diekmann, D., Rhodes, B. A., Biersack, H.-J., Knapp Jr., F. F. (1996) Experimental Radiotherapy of Receptor-Positive Human Prostate Adenocarcinoma with 188Re–RC-160, a Directly-Radiolabeled Somatostatin Analogue. *Int. J. Cancer* 65, 214–220.
- (20) Krenning, E. P., Kwekkeboom, D. J., Bakker, W. H., Breeman, W. A. P., Kooij, P. P. M., Oei, H. Y., van Hagen, M., Postema, P. T. E., de Jong, M., Reubi, J. C., Visser, T. J., Reijts, A. E. M., Hofland, L. J., Koper, J. W., and Lamberts, S. W. J. (1993) Somatostatin receptor scintigraphy with [111In-DTPA-d-Phe1]- and [123I-Tyr3]-octreotide: the Rotterdam experience with more than 1000 patients. *Eur. J. Nucl. Med.* 20, 716–731.
- (21) Kwekkeboom, D. J., van Urk, H., Pauw, B. K. H., Lamberts, S. W., Kooij, P. P., Hoogma, R. P., and Krenning, E. P. (1993) Octreotide scintigraphy for the detection of paragangliomas. *J. Nucl. Med.* 34, 873–878.
- (22) Maini, C. L., Togani, A., Sciuto, R., Carapella, C., Cioffi, R., and Crecco, M. (1993) Somatostatin receptors in meningiomas: a scintigraphic study using 111In-DTPA-D-Phe-octreotide. *Nucl. Med. Commun.* 14, 550–558.
- (23) Ahlman, H., Wangberg, B., Tisell, L. E., Nilsson, O., Fjalling, M., and Forsell-Aronsson, E. (1994) Clinical efficacy of 111In-DTPA-Phe-octreotide scintigraphy in patients with midgut carcinoid tumors and evaluation of intraoperative scintillation detection. *Br. J. Surg.* 81, 1144–1149.
- (24) Krenning, E. P., Kooij, P. P. M., Bakker, W. H., Breeman, W. A. P., Postema, P. T. E., Kwekkeboom, D. J., Oei, H. Y., de Jong, M., Visser, T. J., Reijts, A. E. M., and Lamberts, S. W. J. (1994) Radiotherapy with a Radiolabeled Somatostatin Analogue, 111In-DTPA-Phe1-Octreotide: A Case History. *Ann. N. Y. Acad. Sci.* 733, 496–506.
- (25) Duncan, J. R., and Welch, M. J. (1993) Intracellular Metabolism of Indium-111-DTPA-labeled Receptor Targeted Proteins. *J. Nucl. Med.* 34, 1728–1738.
- (26) Franano, F. N., Edwards, W. B., Welch, M. J., and Duncan, J. R. (1994) Metabolism of Receptor Targeted 111In-DTPA-Glycoproteins: Identification of 111In-DTPA-epsilon-lysine as the Primary Metabolic and Excretory Product. *Nucl. Med. Biol.* 21, 1023–1034.
- (27) Jones, P. L., Brown, B. A., and Sands, H. (1990) Uptake and Metabolism of 111In-labeled Monoclonal Antibody B6.2 by the Rat Liver. *Cancer Res. (suppl.)* 50, 852s–856s.
- (28) Paik, C. H., Sood, V. K., Le, N., Cioloca, L., Carrasquillo, J. A., Reynolds, J. C., Neumann, R. D., and Reba, R. C. (1992) Radiolabeled Products in at Liver and Serum after Administration of Antibody-Amide-DTPA-Indium-111. *Nucl. Med. Biol.* 19, 517–522.
- (29) Motta-Hennessey, C., Sharkey, R. M., and Goldenberg, D. M. (1990) Metabolism of Indium-111-Labeled Murine Monoclonal Antibody in Tumor and Normal Tissue of Athymic Mouse. *J. Nucl. Med.* 31, 1510–1519.
- (30) Rogers, B. E., Franano, F. N., Duncan, J. R., Edwards, W. B., Anderson, C. J., Connett, J. M., and Welch, M. J. (1995) Identification of metabolites of 111In-Diethylenetriaminepentaacetic Acid-Monoclonal Antibodies and Antibody Fragments in Vivo. *Cancer Res. (suppl.)* 55, 5714s–5720s.
- (31) Geissler, F., Anderson, S. K., and Press, O. (1991) Intracellular Catabolism of Radiolabeled Anti-CD3 Antibodies by Leukemic T Cells. *Cell. Immunol.* 137, 96–110.
- (32) Geissler, F., Anderson, S., Venkatesan, P., and Press, O. (1992) Intracellular Catabolism of radiolabeled Anti-u Antibodies by Malignant B-Cells. *Cancer Res.* 52, 2907–2915.
- (33) Hopkins, C. R., and Trowbridge, I. S. (1983) Internalization and processing of transferrin and the transferrin receptor in human carcinoma A431 cells. *J. Cell Biol.* 97, 508–521.
- (34) Press, O. W., DeSantes, K., Anderson, S. K., and Geissler, F. (1990) Inhibition of catabolism of radiolabeled antibodies by tumor cells using lysosomotropic amines and carboxylic ionophores. *Cancer Res.* 50, 1243–1250.
- (35) Arano, Y., Mukai, T., Uezono, T., Wakisaka, K., Motonari, H., Akizawa, H., Taoka, Y., and Yokoyama, A. (1994) A Biological Method to Evaluate Bifunctional Chelating Agents to Label Antibodies with Metallic Radionuclides. *J. Nucl. Med.* 35, 890–898.
- (36) Breeman, W. A. P., Kwekkeboom, D. J., Kooij, P. P. M., Bakker, W. H., Hofland, L. J., Visser, T. J., Ensing, G. J., Lamberts, S. W. J., and Krenning, E. P. (1995) Effect of Dose and specific Activity on Tissue Distribution of Indium-111-Pentetreotide in Rats. *J. Nucl. Med.* 36, 623–627.
- (37) Duncan, J. R., Stephenson, M. T., Wu, H. P., and Anderson, C. J. (1997) Indium-111-Diethylenetriaminepentaacetic acid-octreotide is delivered in vivo to pancreatic, tumor cell, renal and hepatocyte lysosomes. *Cancer Res.* 57, 659–671.
- (38) Srinivasan, A., and Schmidt, M. A. (1997) American Peptide Symposium, Nashville, TN Poster No. 110.
- (39) Atherton, E., and Sheppard, R. C. (1989) Fluorenylmethoxycarbonyl-polyamide Solid-Phase Synthesis—A Practical Approach. Oxford Information Press Ltd.
- (40) Deshpande, S. V., Subramanian, R., McCall, M. J., DeNardo, S. J., DeNardo, G. L., and Meares, C. F. (1990) Metabolism of Indium Chelates Attached to Monoclonal Antibody: minimal Transchelation of Indium from Benzyl-EDTA Chelate In Vivo. *J. Nucl. Med.* 31, 218–224.
- (41) Wu, C. C., Jagoda, E., Brechbiel, M., Webber, K. O., Pastan, I., Gansow, O., and Eckelman, W. C. (1997) Biodistribution and Catabolism of Ga-67 Labeled anti-tac ddsFv Fragment. *Bioconjugate Chem.* 8, 365–369.

BC970158H

Interleukin 7 (IL-7) Receptor-Specific Cell Killing by DAB₃₈₉ IL-7: A Novel Agent for the Elimination of IL-7 Receptor Positive Cells

E. B. Sweeney,[†] F. M. Foss,[‡] J. R. Murphy,^{*,‡} and J. C. vanderSpek[§]

Evans Department of Clinical Research and Department of Medicine, Boston Medical Center, Boston, Massachusetts 02118. Received September 26, 1997; Revised Manuscript Received December 1, 1997

Interleukin 7 (IL-7) induces the proliferation of B cell progenitors in long-term bone marrow cultures, promotes the growth of resting fetal and adult thymocytes, and costimulates mature human T cell proliferation. IL-7 also induces cell growth in hematologic malignancies such as acute lymphoblastic leukemia, chronic lymphocytic leukemia, acute myelogenous leukemia, and Sezary syndrome. We have constructed a recombinant fusion protein, DAB₃₈₉ IL-7, composed of the catalytic and transmembrane domains of diphtheria toxin (DT), fused to IL-7. We demonstrate that DAB₃₈₉ IL-7 is selectively cytotoxic for only those cells bearing the IL-7 receptor and that entry into target cells is mediated through the receptor. The nontoxic mutant, DA(E149S)B₃₈₉ IL-7, was constructed and used to demonstrate that the catalytic domain of DT is responsible for the ADP ribosylation of elongation factor 2 that results in cytotoxicity. Finally, we demonstrate that DA(E149S)B₃₈₉ IL-7 induces the growth of IL-7-dependent cells, verifying the bioactivity of the IL-7 binding domain of DAB₃₈₉ IL-7. We propose that DAB₃₈₉ IL-7 may be an important reagent in studying the IL-7–IL-7 receptor complex and may possess potential as a therapeutic agent against IL-7 receptor-bearing hematologic malignancies.

INTRODUCTION

The techniques of protein engineering have allowed the generation of cell receptor-specific, diphtheria toxin (DT)-based, fusion protein toxins (Murphy and vanderSpek, 1995). DT-based fusion toxins are composed of the catalytic and transmembrane domains of DT, genetically fused to different targeting ligands. Some of the cell surface receptor targeting ligands used to replace the native receptor binding domain of DT include α -melanocyte stimulating hormone, interleukins 2, 4, and 6, epidermal growth factor, and CD4 (Murphy et al., 1986; Williams et al., 1987; Lakkis et al., 1991; Jean et al., 1992; Shaw et al., 1991; Aullo et al., 1992). Since the targeting ligand and toxophore are joined by a peptide bond, the site of linkage between the two is precisely defined. The cytotoxic action of these fusion protein toxins has been shown to be directed to only those cells that express the cognate receptor for a given targeting ligand. The sequence of events that led to cell death are as follows: (i) binding of the targeting ligand to its specific receptor, (ii) internalization by receptor-mediated endocytosis of the ligand–receptor complex, (iii) acidification of the endocytic vesicle and facilitated delivery of the catalytic domain to the cell cytosol, and (iv) ADP ribosylation of elongation factor 2 by the catalytic domain

which results in the inhibition of protein synthesis, and cell death. One molecule of catalytic domain delivered to the cytosol will result in cell death (Pappenheimer, 1977; Yamaizumi et al., 1978; Bacha et al., 1988; Waters, et al., 1990).

Phase I clinical studies on the first-generation form of the interleukin 2 (IL-2) receptor-targeted fusion toxin, DAB₄₈₆ IL-2, were conducted on patients with refractory, hematologic malignancies. Adverse effects were shown to be mild and transient and included nausea–vomiting, fever–malaise, hypersensitivity, chills, and elevations in serum hepatic transaminases. There were no changes in lymphocyte functions or subsets, and no increased rate of opportunistic infection occurred. Pharmacokinetics did not change following multiple courses of administration. Serum levels of soluble IL-2 receptors increased in many of the patients but did not effect serum clearance of DAB₄₈₆ IL-2. The presence of anti-DT or anti-IL-2 antibodies did not interfere with the *in vivo* effects of DAB₄₈₆ IL-2 (LeMaistre et al., 1991, 1992, 1993; Schwartz et al., 1992; Hesketh et al., 1993). The second-generation form of the fusion toxin, DAB₃₈₉ IL-2, has been evaluated in a phase II study on patients with advanced refractory cutaneous T-cell lymphoma (CTCL). As a result, phase III trials for DAB₃₈₉ IL-2 have been initiated for CTCL. Preliminary studies using DAB₃₈₉ IL-2 for treatment of severe rheumatoid arthritis, recent onset insulin-dependent diabetes mellitus, and severe methotrexate resistant psoriasis have also been very promising (Woodworth, 1993; Gottlieb et al., 1995).

Interleukin 7 (IL-7) is a 25 kDa glycoprotein originally identified for its ability to sustain the growth of pre-B cells in long-term bone marrow cultures (Namen et al., 1988). In studies on human hematopoietic malignancies, IL-7 has been shown to stimulate the growth of both B and T acute lymphoblastic leukemia (ALL) cells *in vitro* (Tuow et al., 1990). Other studies have demonstrated

* To whom correspondence should be addressed: Department of Medicine, Boston Medical Center, 88 E. Newton St., Boston, MA 02118. Telephone: 617-638-6010. Fax: 617-638-6009. E-mail: jmurphy@med-med1.bu.edu.

[†] Present address: Oncogene Research Products/Calbiochem, Cambridge, MA 02142.

[‡] Supported in part by Public Health Service Grant CA-48626 from the NCI, National Institutes of Health. Present address: Section of Hematology/Oncology and Section of Biomolecular Medicine, Department of Medicine, Boston Medical Center, Boston, MA 02118.

[§] Supported by a special fellowship from the Leukemia Society of America.

Table 1. Plasmids and the Proteins They Encode

plasmid	protein product	source or reference
pJV127	DAB ₃₈₉ IL-2	vanderSpek et al., 1993
pET11d	—	Novagen
pES5	human IL-7	Cosenza et al., 1997
pESDT-IL-7	DAB ₃₈₉ IL-7	this study
pETDT-IL-7	DAB ₃₈₉ IL-7	this study
pDA(E149S)B ₃₈₉ IL-2	DA(E149S)B ₃₈₉ IL-2	this lab, unpublished
pDA(E149S)B ₃₈₉ IL-7	DA(E149S)B ₃₈₉ IL-7	this study
pETDT-IL-7 E149S	DA(E149S)B ₃₈₉ IL-7	this study

that IL-7 is also able to stimulate the proliferation of malignant cells derived from patients with chronic lymphocytic leukemia (CLL) and the cutaneous T cell lymphoma, Sezary syndrome (Digel et al., 1991; Merle-Beral et al., 1993). As anticipated from studies with normal myeloid cells in vitro, IL-7 has been shown to induce an increase in DNA synthesis in acute myelogenous leukemia (AML) cells (Digel et al., 1991).

The widespread incidence of IL-7-responsive malignant cells led us to construct an IL-7 receptor targeted fusion protein toxin, DAB₃₈₉ IL-7. We describe here the genetic construction and properties of DAB₃₈₉ IL-7 and demonstrate that this fusion protein toxin selectively inhibits protein synthesis in IL-7 receptor-positive cells. We also show that the cytotoxic activity of DAB₃₈₉ IL-7 is mediated through the IL-7 receptor binding domain portion of the fusion protein. Using an ADP ribosyltransferase defective mutant, DA(E149S)B₃₈₉ IL-7, we demonstrate that the inhibition of protein synthesis in intoxicated cells is due to the ADP ribosylation of elongation factor 2 in target cells. Finally, since DA(E149S)B₃₈₉ IL-7 stimulates the proliferation of 2E8 cells, an IL-7-dependent cell line, we conclude that the initial events following binding of these fusion proteins to the IL-7 receptor result in signal transduction and stimulation of protein synthesis.

EXPERIMENTAL PROCEDURES

Cell Lines and Culture Conditions. Since human IL-7 interacts with the murine IL-7 receptor the murine, IL-7-dependent, immature B lymphocyte cell line 2E8 was used to test the cytotoxicity of the IL-7 receptor-directed fusion toxin, DAB₃₈₉ IL-7. The cells were seeded in 75 cm² flasks at a density of 5×10^5 cells/mL. The cells were maintained in Isocove's modified Dulbecco's medium, supplemented with 0.05 mM 2-mercaptoethanol, 2 mM glutamine, 50 IU/mL penicillin, 50 µg/mL streptomycin, 5 ng/mL IL-7, and 5% fetal bovine serum (Pietrangeli et al., 1988). The 2E8 cells grew in grape-like clusters and were harvested and plated at 5×10^5 cells/mL every 2–3 days. The human T cell leukemia HUT 102/6TG cells, which do not express IL-7 receptors, were tested as a control cell line and were maintained in RPMI 1640 medium supplemented with 2 mM glutamine, 50 IU/mL penicillin, 50 µg/mL streptomycin, and 10% fetal bovine serum.

Bacterial Strains. The JM101 strain (BRL) of *Escherichia coli* was used for plasmid propagation. The HMS174(DE3)pLysS strain of *E. coli* was the host used for expression of chimeric *tox* gene products. The structural genes encoding the fusion protein toxins were cloned downstream of the T7 promoter in derivatives of the expression plasmid pET11d.

Plasmids and Fusion Toxin Products. The plasmids and the fusion protein toxins they encode are listed in Table 1. The parental plasmid encoding DAB₃₈₉ IL-2 was pJV127 (vanderSpek et al., 1993). The gene encod-

ing human IL-7 was excised from pES5 (Cosenza et al., 1997) with the restriction endonucleases *Sph*I and *Hin*dIII and vectorially cloned into the *Sph*I and *Hin*dIII sites of pJV127 to construct plasmid pESDT-IL-7(E149S). The gene encoding DAB₃₈₉ IL-7 was then recloned into *Nco*I and *Hin*dIII sites of pET11d vector construct pETDT-IL-7.

The plasmid encoding DA(E149S)B₃₈₉ IL-7 was created by an *Nco*I to *Nsi*I exchange of the gene encoding the defective C domain from pDA(E149S)B₃₈₉ IL-2 into pESDT-IL-7 to construct pDA(E149S)B₃₈₉ IL-7. The gene encoding DA(E149S)B₃₈₉ IL-7 was then recloned into the pET11d vector to form pETDT-IL-7 E149S for high-level expression.

Expression and Purification of DAB₃₈₉ IL-7 and DA(E149S)B₃₈₉ IL-7. To express the fusion toxin DAB₃₈₉ IL-7, plasmid pETDT-IL-7 was transformed into the *E. coli* host HMS174(DE3)pLysS. This bacterial host carries the pLysS plasmid which encodes the T7 polymerase gene which is under the control of LacI, and therefore can be induced with isopropyl-thiogalactoside (IPTG). A 6 mL volume of LB/Amp/Cm [LB medium containing 100 µg/mL ampicillin (Amp) and 25 µg/mL chloramphenicol (Cm)] was inoculated with a single colony of HMS174-(DE3)pLysS carrying the desired plasmid and incubated overnight with shaking at 37 °C. The following day, 5 mL of the overnight culture was used to inoculate 1 L of LB/Amp/Cm. The culture was incubated at 37 °C, with shaking, until the A₆₀₀ was 0.7, and fusion protein toxin expression was then induced by the addition of IPTG to a final concentration of 0.4 mM. Two hours after induction, another 1.5 mL aliquot was taken and the bacteria were harvested by centrifugation. The bacterial pellet was frozen at –20 °C overnight. Fusion proteins expressed in this system accumulated as inclusion bodies in recombinant *E. coli*. The pellet from the 1 L expression was resuspended in 20 mL of STET buffer [50 mM Tris-HCl (pH 8.0), 8% glucose, 10 mM EDTA, and 5% Triton X-100]. The bacteria were lysed by pulsed sonication for 4 min on ice (Branson Cell Disruptor, dial 6, 40% duty cycle). The lysate was pelleted by centrifugation at 10500g for 30 min at 4° C. The inclusion body pellet was resuspended in 10 mL of STET buffer, sonicated, and washed twice in STET buffer as described. The final inclusion body pellet was resuspended in 5 mL of denaturation buffer [50 mM Tris-HCl (pH 8.0), 5 M guanidine hydrochloride, and 5 mM EDTA], and dithiothreitol (DTT) was added to a final concentration of 6 mM. The suspension was then sonicated and placed on ice for 1 h. The denatured protein was dialyzed against 2 L of refolding buffer [0.05 M glycine, 0.03 M NaOH (pH 10), and 1 mM DTT] and the mixture stirred gently at room temperature overnight to renature the proteins. The fusion protein preparation was then exhaustively dialyzed against 2 L of PBS [10 mM phosphate buffer, 2.7 mM KCl, and 137 mM NaCl (pH 7.4)] and centrifuged at 2000g for 10 min to remove any precipitate. The protein concentration in the supernatant fluid was determined by a Bradford assay, and the fusion protein toxins were stored in aliquots at –20 °C.

SDS-PAGE and Immunoblot Analyses. SDS-PAGE was performed according to the method of Laemmli (1970) using 12% polyacrylamide and 0.1% SDS. Following electrophoresis, gels were fixed in 12.5% trichloroacetic acid (TCA) for 5 min and stained with Coomassie brilliant blue.

Immunoblot analysis was carried out essentially as described by Towbin et al. (1979). Following electrophoresis, proteins were transferred to poly(vinylidene

difluoride) (PVDF) membrane and incubated in blocking buffer B [10 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.1% Brij 58] for 1 h at room temperature. The membrane was then incubated with primary antibody: either polyclonal anti-DT antibody (1:500 dilution) or monoclonal anti-IL-7 antibody (1:1,000 dilution) for 30 min at room temperature in TNB buffer [10 mM Tris, 500 mM NaCl, and 0.1% Brij 58 (pH 7.4)]. Following incubation with the primary antibody, the appropriate secondary horseradish peroxidase-labeled (hrp) antibody was applied for another 30 min at room temperature in TNB buffer. The immunoblots were then rinsed with TNB buffer and developed by the addition of 0.5 mg/mL 3',3'-diaminobenzidine tetrahydrochloride in PBS and 5 mL of 33% H₂O₂.

Cytotoxicity Assays and Neutralization Experiments. Since the DT-based fusion proteins inhibit protein synthesis by the ADP ribosylation of elongation factor 2 (EF2), inhibition of [¹⁴C]leucine uptake was used to measure cytotoxicity. For cytotoxicity assays, 1×10^5 2E8 cells in 100 μ L of complete medium were seeded in 96-well plates. The fusion protein toxins were diluted so that the addition of 100 μ L volumes resulted in final concentrations of fusion toxin ranging from 10^{-8} to 10^{-12} M. For the neutralization experiments, 2E8 cells were also incubated with concentrations of fusion toxins ranging from 10^{-8} to 10^{-12} M and 1000 ng/mL monoclonal anti-IL-7 antibody was also added to each well. After an 18 h incubation at 37 °C in a 5% CO₂ atmosphere, the plates were centrifuged at 170g for 5 min. The medium was carefully aspirated and replaced with 200 μ L of leucine-free minimal essential medium containing 1 μ Ci/mL [¹⁴C]leucine (specific activity of >280 mCi/mmol), 2 mM L-glutamine, 50 μ g/mL streptomycin, and 50 IU/mL penicillin. The cell cultures were incubated at 37 °C for 2 h and then centrifuged at 170g for 5 min. The medium was carefully removed, and the cells were lysed by the addition of 0.4 M KOH (60 μ L/well) and incubated at room temperature for 10 min. Total protein was then precipitated by the addition of 160 μ L/well TCA. The insoluble material was collected on glass fiber filters using a cell harvester, and radioactivity was determined according to standard methods. Medium alone served as the control, and all assays were performed in quadruplicate.

Elongation Factor 2 (EF-2) ADP Ribosylation Assay. To demonstrate that the inhibition of protein synthesis, due to ADP ribosylation of EF-2, resulted from C domain activity, the levels of NAD incorporation were determined on cell-free EF-2 extracts. An EF-2-containing extract was prepared from wheat germ and stored in aliquots at -70 °C (Seal et al., 1972; Marcus et al., 1974). An assay cocktail was freshly prepared containing 5 μ Ci/mL [³²P]NAD (specific activity of 800 mCi/mmol), 2 μ M cold NAD, 40 mM DTT, and a 1:5 dilution of the EF-2 extract in TE buffer. Dilutions of the samples to be assayed were made in TE and added in 10 mL aliquots to a 96-well plate. Fifty microliters of the assay cocktail was added to each well. After gentle agitation, the plate was incubated at room temperature for 15 min, after which time the reaction was stopped by the addition of 200 μ L of 10% TCA. The precipitates were transferred to glass fiber disks. The disks were washed three times in 10% TCA and counted by liquid scintillation counting. Results were reported as the -fold increase in [³²P]NAD incorporation over the control. Assays were performed in triplicate.

To study the effects of DA(E149S)B₃₈₉ IL-7 stimulation on the level of EF-2 available for ADP ribosylation

following toxin exposure, the level of EF-2 was determined essentially as described by Moynihan and Pappenheimer (1981). The 2E8 cell line was seeded in 24-well plates at a concentration of 5×10^5 cells/well in 1 mL of medium. Wild-type DAB₃₈₉ IL-7 or the mutant DA(E149S)B₃₈₉ IL-7 toxin was added to the wells in 100 μ L of medium to yield final concentrations ranging from 10^{-8} to 10^{-13} M. After a 24 h incubation period, the cell suspension was transferred to microcentrifuge tubes and centrifuged for 4 min. The cell pellets were lysed in 20 μ L of assay buffer [10 mM Tris-HCl, 10 mM NaCl, 3 mM MgCl₂, 10 mM thymidine, 1 mM EGTA, and 1% Triton X-100 (pH 8.0)] and assayed for the level of EF-2 available for ADP ribosylation by the addition of purified CRM45 to 2 μ g/mL and [³²P]NAD (800 mCi/mmol) to 15 μ Ci/mL. After 10 min at 40 °C, the ADP-ribosyltransferase reaction was stopped by the addition of 5% TCA. The precipitated protein was then collected on glass fiber disks and treated as outlined above. The levels of EF-2 available for ADP ribosylation in toxin-treated cells are presented as the percentage of radiolabel incorporated when compared to that incorporated by untreated controls.

RESULTS

The cloning strategy used in the construction of DAB₃₈₉ IL-7 is shown in Figure 1A. A scheme representing the gene encoding DAB₃₈₉ IL-7 is shown in Figure 1B. The structural gene encoding the ADP-ribosyltransferase defective fusion protein DA(E149S)B₃₈₉ IL-7 was constructed by an *Nco*I-*Sph*I DNA fragment exchange from pDA(E149S)B₃₈₉ IL-2 into pESDT-IL-7 to form pDA(E149S)B₃₈₉ IL-7. The allele encoding the C domain in this mutant contains a Glu to Ser substitution at position 149 of the fusion toxin that inactivates the ADP-ribosyltransferase activity of the C domain (Killeen et al., 1992).

DAB₃₈₉ IL-7 was expressed in *E. coli*, HMS174(DE3)-plysS(pETDT-IL-7) as outlined in Experimental Procedures. Crude cell extracts containing DAB₃₈₉ IL-7 were analyzed on Coomassie blue-stained 12% SDS-polyacrylamide gels prior to and 2 h after IPTG induction (Figure 2A, lanes 2 and 3). It is evident that 2 h after induction there was expression of a recombinant protein with an *M_r* of approximately 60 kDa. This value is in excellent agreement with the molecular weight of 58 428 deduced from the nucleotide base sequence of the structural gene encoding DAB₃₈₉ IL-7. The cells were then lysed, and the inclusion bodies were pelleted by centrifugation, washed with STET buffer, and then solubilized and denatured in 5 M guanidine buffer (Figure 2A, lanes 4-6). Following refolding, the recombinant protein-purified inclusion body preparation is the major protein species (Figure 2, lane 7). To demonstrate that the *M_r* 60 kDa protein was the recombinant DAB₃₈₉ IL-7, proteins were analyzed by immunoblot using anti-DT and anti-IL-7 antibodies. As shown in Figure 2B, the *M_r* 60 kDa fusion protein was found to retain immunological determinants intrinsic to the DT-related component. The protein was also immunoreactive with anti-IL-7 antibodies (data not shown). Following the expression and purification of DA(E149S)B₃₈₉ IL-7, immunoblot analysis demonstrated that this recombinant protein was also immunoreactive with both anti-DT and anti-IL-7 antibodies (data not shown). In both instances, immunoblot analyses also revealed the presence of a 120 kDa dimeric species of the fusion protein as well as proteolytic degradation. The Coomassie-stained gel is more indica-

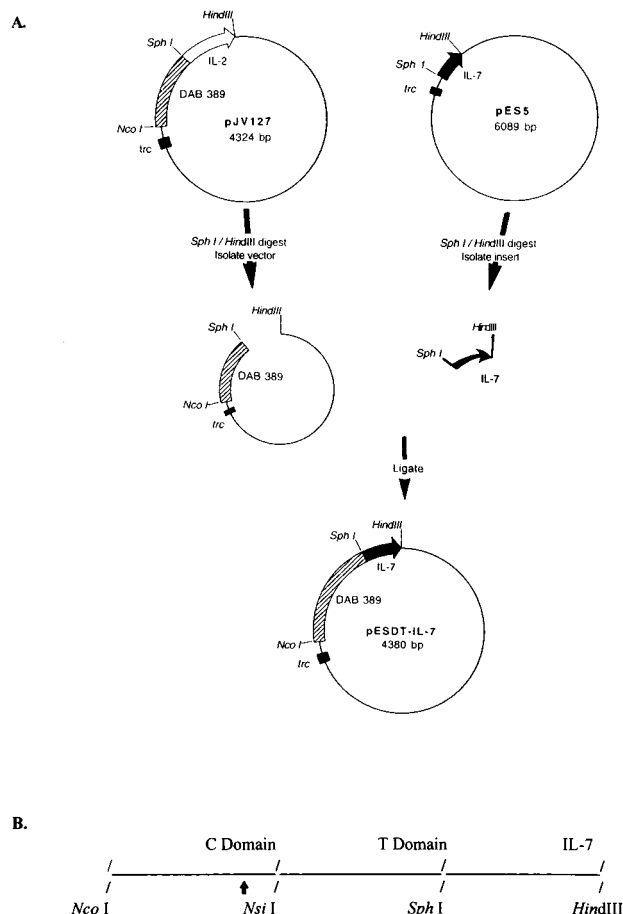


Figure 1. Cloning strategy employed to generate a DAB₃₈₉ IL-7 expression plasmid. (A) The structural gene encoding IL-7 was excised from pES5 by *Sph*I and *Hind*III digestion and cloned into pJV127, replacing the IL-2 structural gene. (B) Scheme representing the gene encoding DAB₃₈₉ IL-7. The restriction sites used for cloning are shown, and the arrow indicates the position of the Glu to Ser mutation in the C domain that is present in the nontoxic form of the fusion toxin, DA(E149S)-B₃₈₉ IL-7.

tive of the amounts of each species present however and indicates that the 60 kDa, full-length protein is the major product.

Figure 3 shows the dose-response analysis of DAB₃₈₉ IL-7 on the IL-7 receptor positive 2E8 cell line. DAB₃₈₉ IL-7 was found to have an IC₅₀ of 1×10^{-10} M on these cells. In marked contrast, DAB₃₈₉ IL-7 was not cytotoxic to the IL-7 receptor negative cell line, HUT 102/6TG. To demonstrate that the cytotoxic action of DAB₃₈₉ IL-7 on 2E8 cells was not due to receptor blockade, we constructed, expressed, purified, and tested the ADP-ribosyltransferase defective mutant DA(E149S)B₃₈₉ IL-7 on these cells. As shown, DA(E149S)B₃₈₉ IL-7 was not cytotoxic to 2E8 cells at concentrations of up to 1×10^{-9} M. Since at this concentration DAB(E149S)B₃₈₉ IL-7 stimulated protein synthesis in 2E8 cells by 2-fold compared to the control, we conclude that these fusion proteins bind to the IL-7 receptor and effect signal transduction (Figure 3).

To show that the cytotoxic effect of DAB₃₈₉ IL-7 was mediated through its IL-7 receptor binding domain, 2E8 cells were incubated with 10^{-8} – 10^{-12} M DAB₃₈₉ IL-7, in the presence and absence of 1000 ng/mL polyclonal anti-IL-7 antibody. Approximately 90% of the cytotoxic activity, at the highest concentration of DAB₃₈₉ IL-7 tested (10^{-8} M), was neutralized upon the addition of the anti-

IL-7 antibody. The presence of excess IL-7 was also found to block the cytotoxic effect of DAB₃₈₉ IL-7 in vitro (Figure 3).

We next demonstrated that the inhibition of protein synthesis in IL-7 receptor-bearing 2E8 cells treated with DAB₃₈₉ IL-7 was the result of the delivery of the C domain to the target cell cytosol. The levels of NAD incorporation on cell-free EF-2 extracts by DAB₃₈₉ IL-7 and DA(E149S)-B₃₈₉ IL-7 were determined as described in Experimental Procedures. DAB₃₈₉ IL-7 catalyzed [³²P]NAD incorporation 14-fold greater than did the control. In marked contrast, DA(E149S)B₃₈₉ IL-7 only catalyzed incorporation by 2-fold (Table 2).

To demonstrate the stimulatory effect of DA(E149S)-B₃₈₉ IL-2, 2E8 cells were incubated without fusion toxin, in the presence of 10^{-8} M DAB₃₈₉ IL-7 or in the presence of 10^{-8} M DA(E149S)B₃₈₉ IL-7 for 24 h. Cells were then lysed, and their respective levels of EF-2 available for ADP ribosylation were determined. Following incubation with DAB₃₈₉ IL-7, the level of EF-2 available for ADP ribosylation was 16% of the control, whereas following a 24 h incubation in the presence of DA(E149S)B₃₈₉ IL-7, the level of EF-2 was 135% of the control (Table 3). This observation further demonstrates that binding of the fusion protein toxins to the IL-7 receptor on 2E8 cells results in signal transduction and the stimulation of cell growth.

DISCUSSION

This work describes the genetic construction, expression, purification, and biological activities of two IL-7 diphtheria toxin-related fusion proteins: DAB₃₈₉ IL-7, a chimeric protein composed of the catalytic and transmembrane domains of DT fused to interleukin 7, and DA(E149S)B₃₈₉ IL-7, a nontoxic ADP-ribosylase-defective mutant of DAB₃₈₉ IL-7.

Following IPTG-induced expression and inclusion body purification of DAB₃₈₉ IL-7, SDS-PAGE analysis identified a major protein with an approximate molecular mass of 60 kDa which is in excellent agreement with the calculated molecular weight of 58 428 of the fusion protein toxin. Both DAB₃₈₉ IL-7 and DA(E149S)B₃₈₉ IL-7 were immunoreactive with anti-DT and anti-IL-7 antibodies.

The cytotoxic potency of DAB₃₈₉ IL-7 was determined using the IL-7-dependent murine pre-B cell line, 2E8, and the IL-7 receptor negative, human T-leukemic cell line, HUT102/6TG. As anticipated, this fusion protein toxin was selectively active against the 2E8 cells, which express the cell surface target receptor, but not the HUT102/6TG cells. Moreover, since the addition of either recombinant IL-7 or polyclonal anti-IL-7 antibody to the 2E8 cell assay mixture blocked the action of DAB₃₈₉ IL-7, we conclude that cytotoxicity is mediated through the IL-7 receptor.

The ADP-ribosyltransferase defective mutant DA(E149S)B₃₈₉ IL-2 was constructed to investigate the role of the C domain of the fusion toxin in the intoxication process. While this mutant fusion protein toxin did not inhibit protein synthesis in 2E8 cells, we found that, at a concentration of 10^{-9} M, the fusion protein induced a 2-fold increase in protein synthesis. These results indicate that the IL-7 receptor binding domain of the fusion protein is folded in a biologically active conformation and, upon binding to the IL-7 receptor, effects signal transduction and stimulates cell proliferation. This finding is analogous to that observed by vanderSpek et al. (1995), who reported that exposure to DA(E149S)B₃₈₉ IL-15

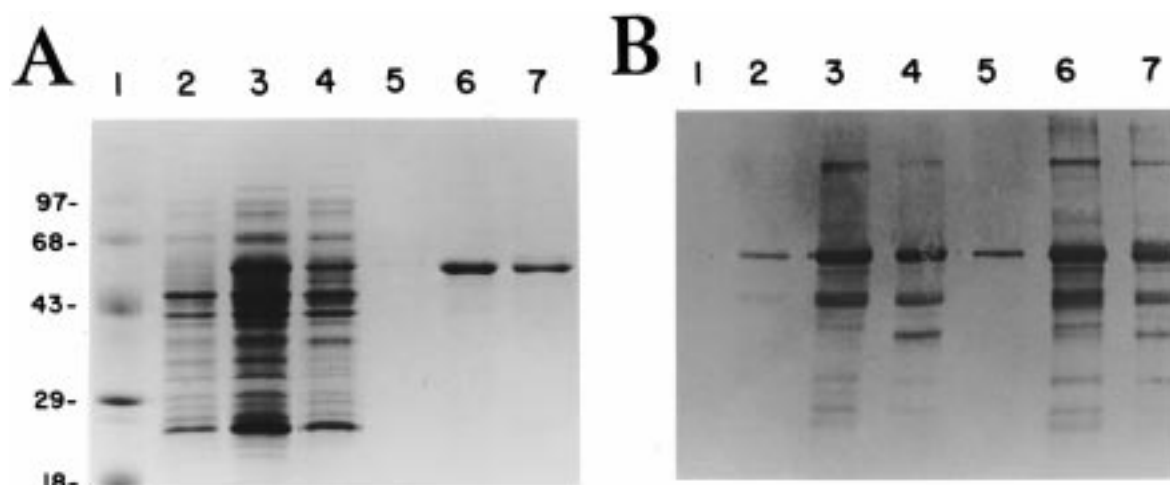


Figure 2. SDS-PAGE and Western blot analyses of DAB₃₈₉ IL-7 expression and inclusion body purification. Protein samples were separated by electrophoresis on 12% SDS-PAGE gels under denaturing conditions and analyzed by Coomassie staining (A) and DT Western blot analyses (B): lane 1, molecular weight standards; lane 2, *E. coli* cell extract before induction; lane 3, *E. coli* cell extract 2 h after induction; lane 4, cell lysate suspension; lane 5, inclusion body preparation wash; lane 6, inclusion body preparation; and lane 7, inclusion body preparation after dialysis and refolding. Apparent molecular weights are given as $10^{-3}M_r$.

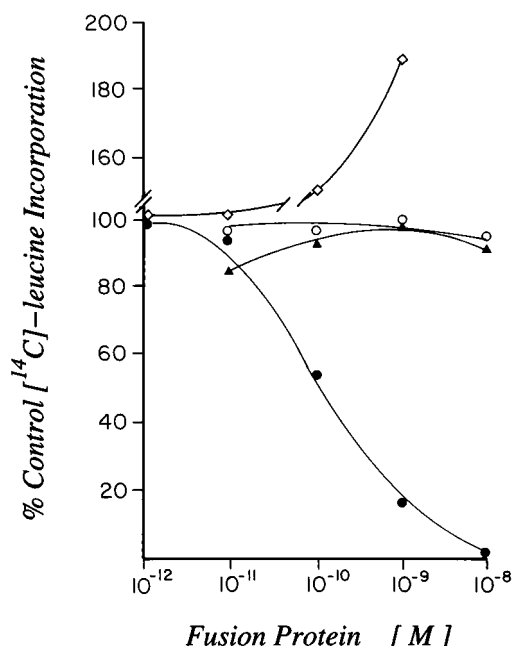


Figure 3. Dose-response analysis of IL-7 receptor positive and negative cell lines following exposure to DAB₃₈₉ IL-7. The inhibition of protein synthesis by DAB₃₈₉ IL-7 was measured by the incorporation of [¹⁴C]leucine. Data are plotted as the percentage of [¹⁴C]leucine incorporation compared to control cells treated with medium alone. The effect of DAB₃₈₉ IL-7 on IL-7 receptor positive 2E8 cells (●) and on IL-7 receptor negative HUT 102/6TG cells (○). The effect of DA(E149S)B₃₈₉ IL-7 on 2E8 cells (please notice the change in scale, ◇) and the effect of DAB₃₈₉ IL-7 on 2E8 cells in the presence of 1000 ng/mL polyclonal anti-IL-7 antibody (▲).

Table 2. ADP Ribosylation of EF-2 Extract by DAB₃₈₉ IL-7 and DA(E149S)B₃₈₉ IL-7

fusion protein	[³² P]NAD incorporation (cpm)
none	2242
DAB ₃₈₉ IL-7 (10^{-8} M)	31044
DA(E149S)B ₃₈₉ IL-7 (10^{-8} M)	4200

resulted in the induction of proliferation and activation of IL-15 receptor positive CTLL-2 cells in vitro. These results demonstrate that DAB₃₈₉ IL-7 exerts its lethal effect by virtue of the delivery of its C domain to the cytosol of target cells and, second, that the IL-7 receptor

Table 3. Murine 2E8 EF-2 Available for ADP Ribosylation Following a 24 h Exposure to DAB₃₈₉ IL-7 or DA(E149S)B₃₈₉ IL-7

fusion protein	EF-2 available for ADP ribosylation (percentage of control)
none	100
DAB ₃₈₉ IL-7 (10^{-8} M)	16
DA(E149S)B ₃₈₉ IL-7	135

binding domain of the fusion toxin is capable of binding and inducing the proliferation in the 2E8 cells.

The C domain of DAB₃₈₉ IL-7 was further proven to be catalytically active by the ADP ribosylation of EF-2. The level of [³²P]NAD incorporation in the ribosylation assay was 14-fold greater than that of the control. The DA(E149S)B₃₈₉ IL-7 C domain mutant was only 2-fold above that of the control. On the basis of the fact that DA(E149S)B₃₈₉ IL-7 did not inhibit protein synthesis, was not catalytically active in the ADP ribosylation assay, but caused the IL-7-dependent 2E8 cell line to proliferate, the amount of EF-2 available for ADP ribosylation after pretreatment with DAB₃₈₉ IL-7 and DA(E149S)B₃₈₉ IL-7 was evaluated. In DAB₃₈₉ IL-7-treated cells, only 16% of control levels of EF-2 was available for ADP ribosylation following a 24 h exposure. As anticipated, due to cell proliferation and stimulation of protein synthesis induced by exposure of 2E8 cells to DA(E149S)₃₈₉ IL-7, increased levels of EF-2 were available for ADP ribosylation in 2E8 cells treated with the mutant fusion protein.

While there has been considerable investigation of the biologic effects of IL-7, the mechanisms by which the IL-7 receptor transduces growth signals remain poorly understood. Goodwin et al. (1990) cloned cDNA encoding both a membrane-bound and a soluble form of the IL-7 receptor. Noguchi et al. (1993) later demonstrated that association of the interleukin 2 γ -chain with the IL-7 receptor resulted in better binding affinity and increased efficiency of IL-7 internalization. Binding of IL-7 to its receptor induces tyrosine kinase activity, activates phosphatidylinositol 3-kinase, and induces production of inositol 1,4,5-triphosphate (Roifman et al., 1992; Dibirdik et al., 1991; Uckun et al., 1991a,b; Dadi and Roifman, 1993; Dadi et al., 1993). IL-7 also induces activation of some Janus nonreceptor tyrosine kinases and *src* family kinases (Seckinger and Fougereau, 1994; Foxwell et al., 1995; Musso et al., 1995).

Due to the frequent expression of the IL-7 receptor on a variety of human leukemias and lymphomas (e.g., ALL, CLL, Sezary syndrome), DAB₃₈₉ IL-7 may serve as an experimental therapeutic agent for patients with IL-7 receptor-bearing hematopoietic neoplasms. Further studies on the cytotoxic activity of this fusion toxin against tumor cell lines and freshly isolated leukemic cells from such patients are needed to further define its role as a cancer therapeutic.

ACKNOWLEDGMENT

We thank Sabina Sweeney for help with preparation of the manuscript. This study was supported in part by Public Health Service Grant CA-48626 from the NCI, National Institutes of Health (to J.R.M.).

LITERATURE CITED

- Aullo, P., Alcani, J., Popoff, M. R., Klatzman, D. R., Murphy, J. R., and Boquet, P. (1992) *In Vitro* effects of a recombinant diphtheria-human CD4 fusion toxin on acute and chronically HIV-1 infected cells. *EMBO J.* 12, 921–931.
- Bacha, P., Waters, C., Williams, J., Murphy, J. R., and Strom, T. B. (1988) Interleukin-2 targeted cytotoxicity: selective action of a diphtheria toxin-related interleukin-2 fusion protein. *J. Exp. Med.* 167, 612–622.
- Cosenza, L., Sweeney, E., and Murphy, J. R. (1997) Disulfide bond assignment in human interleukin-7 by MALDI mass spectroscopy and site directed cysteine to serine mutational analysis. *J. Biol. Chem.* 272, 32995–33000.
- Dadi, H. K., and Roifman, C. M. (1993) Activation of phosphatidylinositol-3 kinase by ligation of the interleukin-7 receptor on human thymocytes. *J. Clin. Invest.* 92, 1559–1563.
- Dadi, H. K., Ke, S., and Roifman, C. M. (1993) Interleukin 7 receptor mediates the activation of a phosphatidylinositol-3 kinase in human B-cell precursors. *Biochem. Biophys. Res. Commun.* 192, 459–464.
- Dibirdik, I., Langlie, M. C., Ledbetter, J. A., Tuel-Ahlgren, L., Obuz, V., Waddick, K. G., Gajl-Peczalska, K., Schieven, G. L., and Ukun, F. M. (1991) Engagement of interleukin-7 receptor stimulates tyrosine phosphorylation, phosphoinositide turnover and clonal proliferation of human T-lineage acute lymphoblastic leukemia cells. *Blood* 78, 564–570.
- Digel, W., Schmid, M., Heil, G., Conrad, P., Gillis, S., and Porzolt, F. (1991) Human Interleukin-7 induces proliferation of neoplastic cells from chronic lymphocytic leukemia and acute leukemias. *Blood* 78, 753–759.
- Foxwell, B. M. J., Beadling, C., Guschin, D., Kerr, I., and Cantrell, D. (1995) Interleukin-7 can induce the activation of Jak 1, Jak 3 and STAT 5 proteins in murine T cells. *Eur. J. Immunol.* 25, 3041–3046.
- Goodwin, R. G., Friend, D., Ziegler, S. F., Jerzy, R., Falk, B. A., Gimpel, S., Cosman, D., Dower, S. K., March, C. J., Namen, A. E., and Park, L. S. (1990) Cloning of the human and murine Interleukin-7 receptors: Demonstration of a soluble form and homology to a new receptor superfamily. *Cell* 60, 941–951.
- Gottlieb, S. L., Gilleaudeau, P., Johnson, R., Estes, L., Woodworth, T. G., Gottlieb, A. B., and Krueger, J. G. (1995) Response of psoriasis to a lymphocyte-selective toxin (DAB₃₈₉ IL-2) suggests a primary immune, but not keratinocyte, pathogenic basis. *Nat. Med.* 1, 442–447.
- Hesketh, P., Caguioa, P., Koh, H., Dewey, H., Facada, A., McCaffrey, R., Parker, K., Nylen, P., and Woodworth, T. (1993) Clinical activity of a cytotoxic fusion protein in the treatment of cutaneous T cell lymphoma. *J. Clin. Oncol.* 11, 1628–1690.
- Jean, L.-F., and Murphy, J. R. (1992) Diphtheria toxin receptor binding domain substitution with interleukin-6: genetic construction and interleukin-6 receptor specific action of a diphtheria toxin-related interleukin-6 fusion protein. *Protein Eng.* 4, 989–994.
- Killeen, K. P., Escuyer, V., Mekalanos, J. J., and Collier, R. J. (1992) Reversion of recombinant toxoids: mutations in diphtheria toxin that partially compensate for active-site deletions. *Proc. Natl. Acad. Sci. U.S.A.* 89, 6207–6209.
- Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680–685.
- Lakkis, F., Steele, A., Pacheco-Silva, A., Rubin-Kelley, V., Strom, T. B., and Murphy, J. R. (1991) Interleukin 4 receptor targeted cytotoxicity: genetic construction and *in vivo* immunosuppressive activity of a diphtheria toxin-related interleukin 4 fusion protein. *Eur. J. Immunol.* 21, 2253–2258.
- LeMaistre, C. F., Rosenblum, M. G., Reuben, J. M., Parkinson, D. R., Meneghetti, C. M., Parker, K., Shaw, J. P., Deisseroth, A. B., and Woodworth T. (1991) Therapeutic effects of genetically engineered toxin (DAB₄₈₆ IL-2) in patients with chronic lymphocytic leukemia. *Lancet* 337, 1124–1125.
- LeMaistre, C. F., Meneghetti, C., Rosenblum, M., Reuben, J., Parker, K., Shaw, J., Woodworth, T., and Parkinson, D. (1992) Phase I trial of an interleukin-2 receptor (IL-2R) fusion toxin (DAB₄₈₆IL-2) in hematologic malignancies expressing the IL-2 receptor. *Blood* 79, 2547–2554.
- LeMaistre, C. F., Craig, F. E., Meneghetti, C., McMullin, B., Parker, K., Reuben, J., Boldt, D. H., Rosenblum, M., and Woodworth, T. (1993) Phase I trial of a 90-minute infusion of the fusion toxin DAB₄₈₆IL-2 in hematologic cancers. *Cancer Res.* 53, 3930–3934.
- Marcus, A., Seal, S., and Weeks, D. (1974) Protein chain initiation in wheat embryo. In *Methods in Enzymology* (K. Moldave and L. Grossman, Eds.) Vol. 30, Academic Press, San Diego.
- Merle-Beral, H., Schmitt, C., Mossalayi, D., Bismuth, G., Dalloul, A., and Mentz, F. (1993) Interleukin-7 and malignant T cells. *Nouv. Rev. Fr. Hematol.* 35, 231–232.
- Moynihan, M., and Pappenheimer, A. M., Jr. (1981) Kinetics of adenosinediphospho-ribosylation of elongation factor 2 in cells exposed to diphtheria toxin. *Infect. Immun.* 32, 575–582.
- Murphy, J. R., and vanderSpek, J. C. (1995) Targeting diphtheria toxin to growth factor receptors. *Cancer Biol.* 6, 259–267.
- Murphy, J. R., Bishai, W., Bowoski, M., Miyanochara, A., Boyd, J., and Nagle, S. (1986) Genetic construction, expression and melanoma-selective cytotoxicity of a diphtheria toxin α -melanocyte stimulating hormone fusion protein. *Proc. Natl. Acad. Sci. U.S.A.* 83, 8258–8262.
- Musso, T., Johnston, J. A., Linnekin, D., Varesio, L., Rowe, T. K., O'Shea, J. J., and McVicar, D. W. (1995) Regulation of JAK3 expression in human monocytes: phosphorylation in response to interleukins 2, 4, and 7. *J. Exp. Med.* 181, 1425–1431.
- Namen, A. E., Lupton, S., Hjerrild, K., Wignall, J., Mochizuki, D., Schmierer, A., Mosley, B., March, C. J., Urdal, D., Gillis, S., Cosman, D., and Goodwin, R. G. (1988) Stimulation of B cell progenitors by cloned murine interleukin-7. *Nature* 333, 571–573.
- Noguchi, M., Nakamura, Y., Russell, S. M., Ziegler, S. F., Tsang, M., Cao, X., and Leonard, W. J. (1993) Interleukin-2 receptor γ chain: a functional component of the interleukin-7 receptor. *Science* 262, 1877–1880.
- Pappenheimer, A. M. (1977) Diphtheria Toxin. *Annu. Rev. Biochem.* 46, 69–94.
- Pietrangeli, C., Hayashi, S., and Kincade, P. (1988) Stromal cell lines which support lymphocyte growth: Characterisation, sensitivity to radiation and responsiveness to growth factors. *Eur. J. Immunol.* 18, 863.
- Roifman, C. M., Wang, G., Freedman, M., and Pan, Z. (1992) IL-7 receptor mediates tyrosine phosphorylation but does not activate the phosphatidylinositol-phospholipase C- γ 1 pathway. *J. Immunol.* 148, 1136–1142.
- Schwartz, G., Tepler, I., Charette, J., Kadin, L., Parker, K., Woodworth, T., and Schnipper, L. (1992) Complete response of a Hodgkin's Lymphoma in a phase I Trial of DAB₄₈₆ IL-2. *Blood* 79, 175a.
- Seal, S., Bewley, J., and Marcus, A. (1972) Protein chain initiation in wheat embryo. Resolution and function of the soluble factors. *J. Biol. Chem.* 247, 2592–2597.

- Seckinger, P., and Fougereau, M. (1994) Activation of *src* family kinases in human pre-B cells by IL-7. *J. Immunol.* 153, 97–109.
- Shaw, J. P., Akiyoshi, D. E., Arrigo, D. A., Rhoad, A. E., Sullivan, B., Thomas, J., Genbauffe, F. S., Bacha, P., and Nichols, J. C. (1991) Cytotoxic properties of DAB₄₈₆ EGF and DAB₃₈₉ EGF, epidermal growth factor (EGF) receptor-targeted fusion toxins. *J. Biol. Chem.* 266, 12449–12455.
- Towbin, H., Staehelin, T., and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc. Natl. Acad. Sci. U.S.A.* 76, 4350–4354.
- Tuow, I., Pouwels, K., van Agthoven, T., van Gur, R., Budel, L., Hoogerbrugge, H., Delwel, R., Goodwin, R., Namen, A., and Löwenberg, B. (1990) Interleukin-7 is a growth factor of precursor B and T acute lymphoblastic leukemia. *Blood* 75, 2097–2101.
- Uckun, F. M., Dibirdik, I., Smith, R., Tuel-Ahlgren, L., Chandan-Langlie, M., Schieven, G. L., Waddick, K. G., Hanson, M., and Ledbetter, J. A. (1991a) Interleukin 7 receptor ligation stimulates tyrosine phosphorylation, inositol phospholipid turnover, and clonal proliferation of human B-cell precursors. *Proc. Natl. Acad. Sci. U.S.A.* 88, 3589–3593.
- Uckun, F. M., Tuel-Alghren, L., Obuz, V., Smith, R., Dibirdik, I., Hanson, M., Chandan-Langli, M., and Ledbetter, J. A. (1991b) Interleukin 7 receptor engagement stimulates tyrosine phosphorylation, inositol phospholipid turnover, proliferation, and selective differentiation to the CD4 lineage by human fetal thymocytes. *Proc. Natl. Acad. Sci. U.S.A.* 88, 6323–6327.
- vanderSpek, J. C., Mindell, J. A., Finkelstein, A., and Murphy, J. R. (1993) Structure/function analysis of the transmembrane domain of DAB₃₈₉–Interleukin-2, an interleukin-2 receptor-targeted fusion toxin. *J. Biol. Chem.* 268, 12077–12082.
- vanderSpek, J. C., Sutherland, J., Sampson, E., and Murphy J. R. (1995) Genetic construction and characterization of the diphtheria toxin-related interleukin 15 fusion protein DAB₃₈₉ sIL-15. *Protein Eng.* 8, 1317–1321.
- Waters, C. A., Schimke, P., Snider, C. E., Itoh, K., Smith, K. A., Nichols, J. C., Strom, T. B., and Murphy, J. R. (1990) Interleukin-2 receptor targeted cytotoxicity: receptor binding requirements for entry of IL-2 toxin into cells. *Eur. J. Immunol.* 20, 785–791.
- Williams, D. P., Parker, K., Bacha, P., Bishai, W., Borowski, M., Genbauffe, F., Strom, T. B., and Murphy, J. R. (1987) Diphtheria toxin receptor binding domain substitution with interleukin-2: Genetic construction and properties of a diphtheria toxin-related interleukin 2 fusion protein. *Protein Eng.* 1, 493–498.
- Woodworth, T. G. (1993) Early clinical studies of IL-2 fusion toxin in patients with severe rheumatoid arthritis and recent onset insulin-dependent Diabetes Mellitus. *Clin. Exp. Rheumatol.* 11 (Suppl. 8), S177–S180.
- Yamaizumi, M., Mekada, E., Uchida, T., and Okada, Y. (1978) One molecule of diphtheria toxin fragment A introduced into a cell can kill the cell. *Cell* 15, 245–250.

BC9701757

High-Affinity No-Carrier-Added ^{99m}Tc -Labeled Chemotactic Peptides for Studies of Inflammation in Vivo

Kwamena E. Baidoo,* Ursula Scheffel, Marigo Stathis, Paige Finley, Susan Z. Lever, Yougen Zhan, and Henry N. Wagner, Jr.

Division of Radiation Health Sciences, Department of Environmental Health Sciences, The Johns Hopkins University, 615 North Wolfe Street, Baltimore, Maryland 21205. Received November 13, 1997; Revised Manuscript Received January 15, 1998

N^{α} -for-Nle-Leu-Phe-Nle-Tyr-Lys, a chemotactic peptide that binds with high affinity to the chemoattractant receptor on granulocytes and monocytes, was labeled with ^{99m}Tc using the diaminedithiol (DADT) chelating system to coordinate the Tc. ^{99m}Tc labeling of the DADT-coupled peptide was accomplished in 84% overall yield (room temperature for 10 min) using [^{99m}Tc]glucoheptonate as the donor of prereduced Tc. HPLC analysis showed two major ^{99m}Tc -labeled peptide peaks, ^{99m}Tc -DADT-Pep-I and ^{99m}Tc -DADT-Pep-II, were obtained in a ratio of 1:0.85. Using an iodoacetamide-derivatized gel to remove unlabeled peptide from the ^{99m}Tc labeling mixtures, essentially no-carrier-added (nca) high-specific activity ^{99m}Tc -labeled chemotactic peptides were obtained. The ^{99m}Tc analogues of the peptides were synthesized (72% yield) in a similar fashion and correlated with ^{99m}Tc complexes I and II by HPLC. In vitro competitive receptor binding assays of the isolated ^{99m}Tc analogues were performed against the tritiated chemotactic peptide [^3H]N-for-Met-Leu-Phe ([^3H]fMLF) using isolated granulocytes. The ^{99m}Tc -derivatized peptides showed similar binding affinities to the chemoattractant receptor as the unlabeled N^{α} -for-Nle-Leu-Phe-Nle-Tyr-Lys. The nca ^{99m}Tc -labeled peptides gave high contrast images of experimental inflammation in rabbits without causing neutropenia. Thus, it is feasible to attach the Tc-DADT chelate to low-molecular weight receptor binding chemotactic peptides and retain substantial binding to the receptor. Chemotactic peptides labeled with ^{99m}Tc via the DADT ligand system have the potential for imaging focal sites of inflammation without toxic effects, an important consideration in the successful utilization of chemotactic peptide agonists.

INTRODUCTION

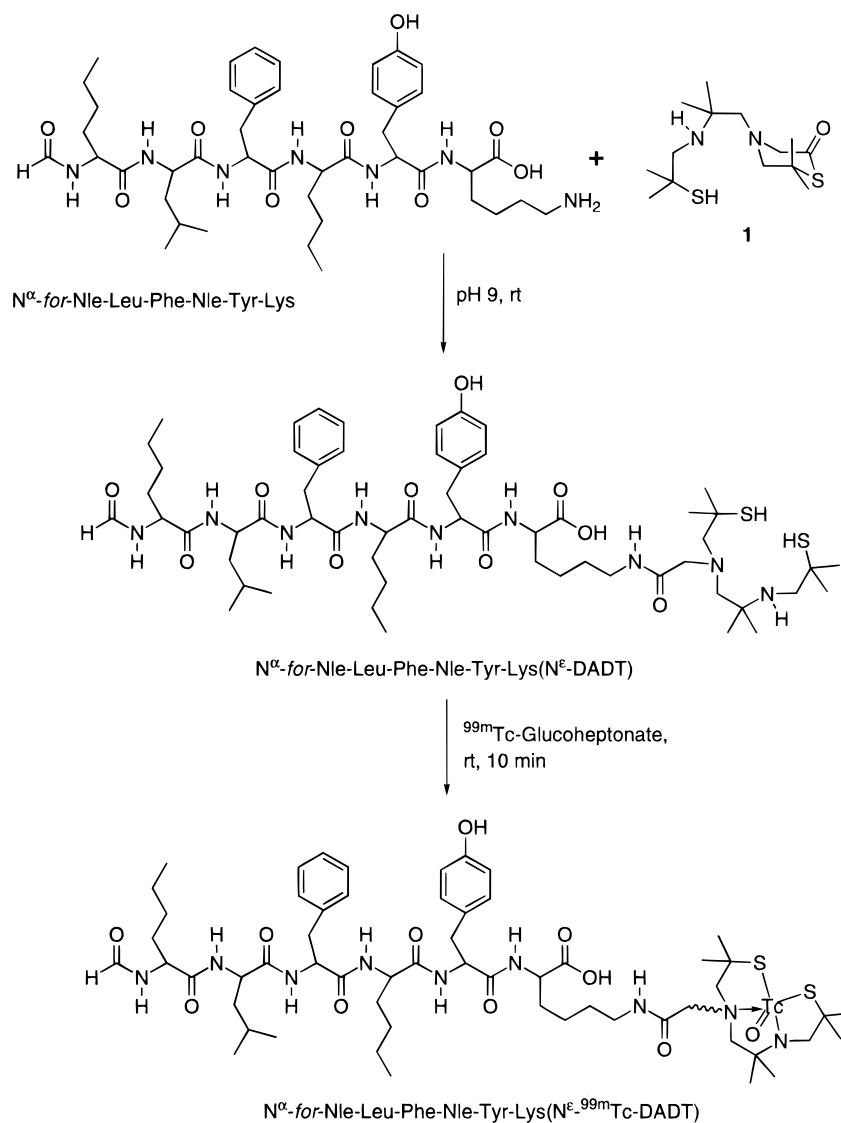
Over the past two decades, a number of radiotracers have been proposed or used for the detection of focal sites of inflammation or infection in vivo by scintigraphy. These include ^{67}Ga citrate (1), leukocytes labeled with ^{111}In (^{111}In -WBC) (2, 3) or ^{99m}Tc (^{99m}Tc -WBC) (4), ^{99m}Tc - or ^{123}I -labeled antibodies against granulocytes (5, 6), nonspecific IgG and its Fc fragment labeled with ^{99m}Tc or ^{111}In (7, 8), and ^{99m}Tc -nanocolloid (9). Among these agents, the gold standard for imaging inflammation or infection in vivo has been autologous leukocytes labeled in vitro with ^{111}In (^{111}In -WBC). Inadequacies of the various techniques especially with respect to applicability in emergency situations and ease of preparation have spurred a search for the ideal radiopharmaceutical for imaging inflammation.

The labeling of leukocytes in vitro is cumbersome and requires very mild conditions and experienced personnel to avoid inactivating the cells. Radiopharmaceuticals that are systemically injected directly into patients for imaging focal sites of infection without the need for in vitro labeling of autologous leukocytes are more advantageous as has been demonstrated in humans using the labeled antibodies against granulocytes (5, 6). Chemotactic peptides (10) are another class of compounds that

have the potential to generate radiopharmaceuticals that could be used in a similar fashion by systemic injection. These are small oligopeptides that bind with high affinity to high-density chemoattractant receptor sites on granulocytes and monocytes (11, 12). The pharmacokinetics of small peptides would allow labeled chemotactic peptides to be used even in acute cases where the answer to the clinical question is needed under 1 h, a time frame in which current clinically available scintigraphic agents are not very effective. For these reasons, a great deal of attention has recently been focused on labeled chemotactic peptides (13–17).

The use of these important peptides for imaging applications has been tempered by their highly potent neutropenic nature (18). Successful utilization of labeled chemotactic peptides, therefore, necessitates the availability of very high-specific activity formulations. To achieve high specific activity, the radionuclide used must have a relatively short half-life and must be available with little or no carrier. Of desirable radionuclides utilized for scintigraphy in vivo, ^{99m}Tc , with its 6.02 h half-life and availability in no-carrier-added (nca) form from the $^{99}\text{Mo}/^{99m}\text{Tc}$ generator, offers the potential to generate ^{99m}Tc -labeled chemotactic peptides having the required specific activity for use in imaging without accompanying neutropenia. Previous efforts to generate high-specific activity ^{99m}Tc -labeled chemotactic peptides have involved a combination of utilizing low concentrations of peptide for synthesis, elution of the generator at the time of highest specific activity, and/or the use of HPLC to separate labeled from unlabeled ligand (14).

* To whom correspondence should be addressed at Division of Radiation Health Sciences, Department of Environmental Health Sciences, The Johns Hopkins University, 615 N. Wolfe St., Room 2001, Baltimore, MD 21205. Telephone: (410) 955-7706. Fax: (410) 955-6222. E-mail: kbaidoo@jhsph.edu.

Scheme 1. Synthesis of the ^{99m}Tc-Labeled Chemotactic Peptides

These methods suffer from low labeling yields and cumbersome methodology that limits the availability of the radiopharmaceuticals for general use.

Previously, we reported the synthesis of a bifunctional chelate **1** (Scheme 1), derived from the diaminedithiol ligand system, capable of forming highly stable complexes with Tc (19). In this paper, we have utilized this bifunctional chelate in the preparation of ^{99m}Tc-labeled chemotactic peptides derived from *N*^α-for-Nle-Leu-Phe-Nle-Tyr-Lys, a potent chemotactic peptide that binds with high affinity to granulocytes (20). We have explored the chemical differences between the diaminedithiol ligand and its ^{99m}Tc complex to develop a highly efficient solid-phase separation procedure for the preparation of high-affinity and high-specific activity technetium derivatives of this chemotactic peptide devoid of contaminating unlabeled peptide, the usual culprit of low-specific activity ^{99m}Tc preparations. The results of our initial evaluation of this solid-phase technology were recently reported (21).

MATERIALS AND METHODS

Solvents and chemicals were reagent grade and used as received from the Aldrich Chemical Co. (Milwaukee, WI) unless specified. [^{99m}Tc]NH₄TcO₄ and [³H]-for-Met-Leu-Phe were purchased from NEN Life Science Products

(Boston, MA). [^{99m}Tc]Pertechnetate was purchased as a physiologic saline solution from Syncor (Timonium, MD). The Glucoscan kit was purchased from the Du Pont Radiopharmaceutical Division (Billerica, MA). Ultralink iodoacetamide gel was purchased from Pierce (Rockford, IL). Histopaque 1077, Histopaque 1119, *N*^α-for-Nle-Leu-Phe-Nle-Tyr-Lys, and *N*^α-for-Met-Leu-Phe were purchased from the Sigma Chemical Co. (St. Louis, MO). Male CD-1 mice and female New Zealand white rabbits were purchased from the Charles River Laboratories (Charles River, MA). Animal studies were performed according to a protocol approved by our institutional Animal Care and Use Committee.

HPLC was performed on either a Perkin-Elmer Series 2 instrument equipped with an LC-75 UV/visible detector or a Waters Chromatography Division system with two model 510EF pumps and an automated gradient controller model 680 equipped with a model 490 UV absorbance detector, an EG&G NaI scintillation detector, and a Hewlett-Packard HP 3392A or Shimadzu CR3A integrating recorder. [^{99m}Tc]Glucoheptonate and the diaminedithiol bifunctional chelating agent (BCA), **1**, were synthesized according to published procedures (19, 22). Infrared spectroscopic determinations of neat liquids or KBr pellets were performed on a Perkin-Elmer 399B or Nicolet 205 FTIR system. ¹H NMR spectra were recorded

in deuteriochloroform solution using a Bruker WM 300 MHz spectrometer. Elemental analyses were performed by Atlantic Microlabs (Norcross, GA). High-resolution MALDI mass spectral analyses were performed at the University of Minnesota Mass Spectrometry Laboratory (Minneapolis, MN) or Quality Controlled Biochemicals (Hopkington, MA). Amino acid analyses were performed at Quality Controlled Biochemicals or the Johns Hopkins School of Medicine Peptide Synthesis Laboratory (Baltimore, MD). Filtrations of in vitro binding assay mixtures were performed on a BRANDEL, 48M Harvester. Liquid scintillation counting was performed on an LKB Wallac instrument model 1219 Rackbeta using Ecolume (ICN Pharmaceuticals, Inc., Costa Mesa, CA) as a cocktail. The ^3H channel was corrected for $^{99\text{m}}\text{Tc}$ spillover. Leukocytes were counted with a hemacytometer from the American Optical Co. (Buffalo, NY). $^{99\text{m}}\text{Tc}$ activity was measured in a Capintec CRC-7 Dose Calibrator or an automated γ -counter (Pharmacia-Wallac model 1282). Planar scintigraphic imaging was performed with a Technicare Omega 500 single-headed SPECT system interfaced with a GE Starcam II computer.

EXPERIMENTAL PROCEDURES

Synthesis of N^α -for-Nle-Leu-Phe-Nle-Tyr-Lys(N^ϵ -DADT) (DADT-Pep). Coupling of the diaminedithiol ligand to N^α -for-Nle-Leu-Phe-Nle-Tyr-Lys was performed according to Scheme 1. A solution of N^α -for-Nle-Leu-Phe-Nle-Tyr-Lys (24 mg, 29 μmol) in THF/ H_2O (2:1 v/v, 3 mL) was adjusted to pH 9 with NaOH(aq) (70 μL , 1 M) followed by the addition of a solution of the thiolactone bifunctional chelate **1** (19) (44 mg, 145 μmol) in THF (0.5 mL). The solution was stirred at room temperature for 3 h. Solvents were removed by distillation under reduced pressure, and the solid residue was triturated with ether (4×20 mL). The crude product was purified by reversed-phase HPLC [C-18 Novapak column, 25×100 mm, 6 μm , Waters Chromatography Division; with a linear gradient from 10% solvent A (0.1% TFA in acetonitrile)/90% solvent B (0.1% TFA in water) to 90% solvent A/10% solvent B over the course of 40 min; flow rate of 6 mL/min, monitored on-line by UV at 220 nm]. The product eluting with a 31 min retention time was collected and lyophilized. High-resolution MALDI MS: calcd MW of 1127.65, found $[\text{M} + \text{H}]^+$ 1128.63. Amino acid analysis: found (expected) Nle, 2.02 (2); Leu, 0.96 (1); Phe, 1.04 (1); Tyr, 0.95 (1); Lys, 0.96 (1).

$^{99\text{m}}\text{Tc}$ Labeling of N^α -for-Nle-Leu-Phe-Nle-Tyr-Lys(N^ϵ -DADT). N^α -for-Nle-Leu-Phe-Nle-Tyr-Lys(N^ϵ -DADT) was labeled with $^{99\text{m}}\text{Tc}$ using [$^{99\text{m}}\text{Tc}$]glucoheptonate as the donor of prereduced $^{99\text{m}}\text{Tc}$ according to Scheme 1. Water (1.0 mL) was used to dissolve the contents of a glucoheptonate kit (Glucoscan). An aliquot of this solution (200 μL) was added to a [$^{99\text{m}}\text{Tc}$]pertechnetate solution (300 μL , 20 mCi) and vortexed for 1 min. The resulting [$^{99\text{m}}\text{Tc}$]glucoheptonate solution was added to a solution of N^α -for-Nle-Leu-Phe-Nle-Tyr-Lys(N^ϵ -DADT) (1.2 mg, 1.1 μmol) in THF/ H_2O (5:1 v/v, 0.6 mL), vortexed for 1 min, and then incubated at room temperature for 10 min. The reactions were followed by reversed-phase HPLC [C-18 Novapak column (8×100 mm, 4 μm , Waters Chromatography Division); 48% solvent A/52% solvent B for 20 min followed by a linear gradient to 70% solvent A/30% solvent B over the course of 15 min; flow rate of 4 mL/min, monitored on-line by UV at 220 nm and scintillation for radioactivity]. As shown in Figure 1A, two major $^{99\text{m}}\text{Tc}$ -labeled peptide peaks ($^{99\text{m}}\text{Tc}$ -DADT-Pep-I and $^{99\text{m}}\text{Tc}$ -DADT-Pep-II) were obtained in a ratio (I:II)

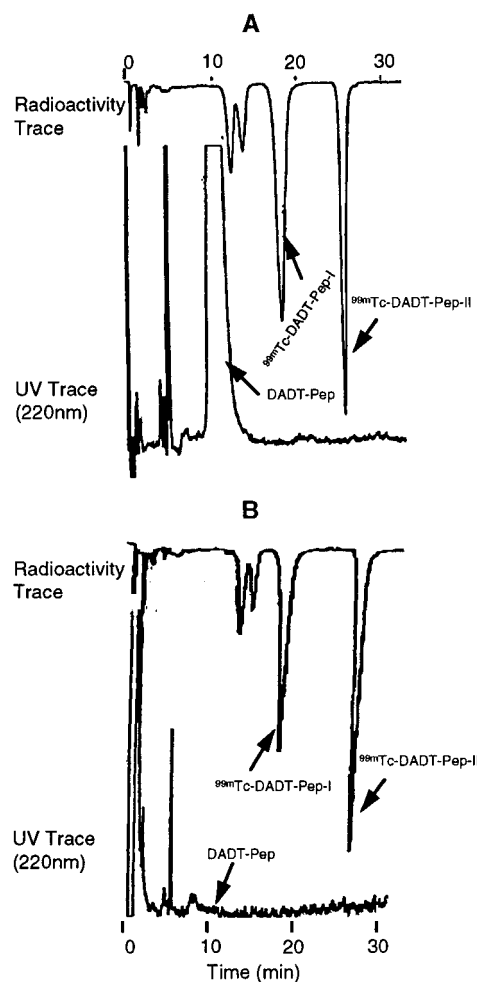
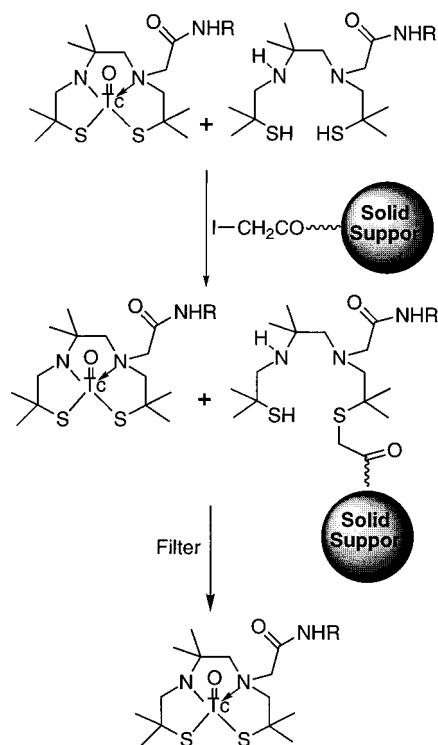


Figure 1. HPLC traces of the crude $^{99\text{m}}\text{Tc}$ labeling reaction mixture of BCA **1** coupled chemotactic peptide after incubation for 10 min (A) and after iodoacetamide gel treatment (B).

of 1:0.85. The total radiochemical yield of $^{99\text{m}}\text{Tc}$ -labeled peptide peaks I and II was 84%.

Unlabeled adduct was removed from the $^{99\text{m}}\text{Tc}$ labeling mixture according to Scheme 2. Two milliliters of iodoacetamide-derivatized gel (Ultralink iodoacetamide) was equilibrated in Tris buffer (50 mM, pH 8.5) containing 5 mM EDTA. Acetonitrile (1 mL) was added to the equilibrated gel followed by the crude labeling mixture (1 mL) containing N^α -for-Nle-Leu-Phe-Nle-Tyr-Lys(N^ϵ -DADT) (1 μmol) and the $^{99\text{m}}\text{Tc}$ -labeled peptides and the mixture incubated at room temperature for 30 min. The mixture was filtered. The gel was washed with an acetonitrile/saline mixture (3 $\times$ 2 mL, 20:80). The filtrate was concentrated into 0.4 mL of ethanol by solid-phase extraction using a Waters C-18 Sep-Pak cartridge (Waters Chromatography Division). The presence of thiol-containing compounds in the filtrate, crude reaction mixture before gel treatment, and HPLC-purified products were assayed by Ellman's method (23) using 2,2,4,9,9-pentamethyl-4,7-diaza-1,10-decanedithiol (**24**) as a standard to quantitate the thiols.

Synthesis of N^α -for-Nle-Leu-Phe-Nle-Tyr-Lys(N^ϵ - $^{99\text{m}}\text{Tc}$ -DADT). A solution of [$^{99\text{m}}\text{Tc}$]glucoheptonate (**22**) in water (1 mL, 10 μmol) was added to the purified N^α -for-Nle-Leu-Phe-Nle-Tyr-Lys(N^ϵ -DADT) (11.3 mg, 10 μmol) dissolved in THF/ H_2O (2:1 v/v, 1 mL). The mixture was stirred at room temperature for 30 min. The two major components formed, $^{99\text{m}}\text{Tc}$ -DADT-Pep-I and $^{99\text{m}}\text{Tc}$ -DADT-Pep-II, were purified by HPLC [C-18 Novapak column

Scheme 2. Solid-Phase Purification of ^{99m}Tc -Diaminedithiolate Complexes

(8 × 100 mm, 4 μm); 48% solvent A/52% solvent B for 20 min followed by a linear gradient to 70% solvent A/30% solvent B over the course of 15 min; flow rate of 4 mL/min, monitored on-line by UV at 220 nm].

High-resolution MALDI MS: calcd MW for ^{99m}Tc -DADT-Pep-I 1239.62, found $[\text{M} + \text{Na}]^+$ 1262.52. Amino acid analysis: found (expected) Nle, 1.96 (2); Leu, 0.98 (1); Phe, 1.05 (1); Tyr, 0.97 (1); Lys, 0.92 (1).

High-resolution MALDI MS: calcd MW for ^{99m}Tc -DADT-Pep-II 1239.62, found $[\text{M} + \text{Na}]^+$ 1262.51. Amino acid analysis: found (expected) Nle, 1.89 (2); Leu, 0.97 (1); Phe, 0.98 (1); Tyr, 0.94 (1); Lys, 0.97 (1).

^{99m}Tc Labeling of the Hydrolyzed Bifunctional Chelate (^{99m}Tc -DADT). NaOH (0.5 M, 250 μL) was added to a solution of **1** (5 mg, 16 μmol) in acetonitrile (250 μL) and the mixture incubated for 5 min at room temperature. The pH of the mixture was adjusted to 6 with HCl(aq) (100 μL , 1.0 M) followed by a solution of [^{99m}Tc]glucoheptonate (7 mCi, 1.5 mL). The resulting mixture was incubated at room temperature for 10 min. The reaction was monitored by reversed-phase HPLC [C-18 Novapak column (8 × 100 mm, 4 μm); linear gradient from 0% solvent A/100% solvent B to 60% solvent A/40% solvent B over the course of 30 min; flow rate of 2 mL/min, monitored on-line by UV at 254 nm and scintillation for radioactivity]. As shown in Figure 3, two main products with retention times of 22.1 and 26.2 min were obtained in a ratio of 1:3.1 and a total yield of 91%. For the *in vivo* studies, the two ^{99m}Tc -labeled peaks (^{99m}Tc -DADT) collected from HPLC purification were combined. The products were extracted from the HPLC eluate by solid-phase extraction procedures into ethanol, and the ethanol solution was diluted to 2% ethanol with saline for injection.

In Vitro Binding Assay. The binding assay was conducted according to an adaptation of the methods of Williams et al. (11) and Nidel et al. (20). Aliquots of human blood (15 mL) were gently layered over Histopaque 1077 (15 mL) underlayered with Histopaque 1119

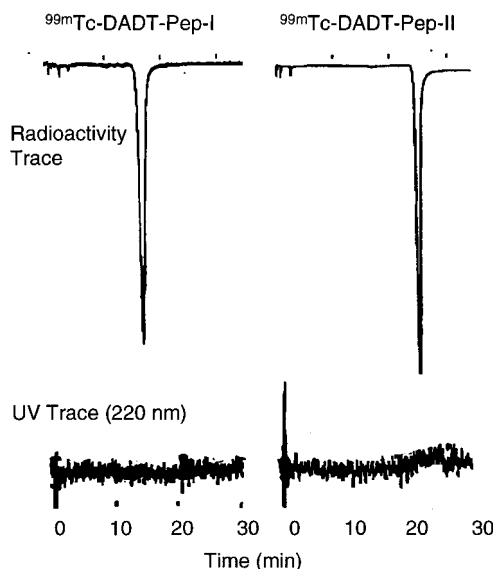


Figure 2. Analytical chromatograph of the HPLC-purified ^{99m}Tc -labeled peptides.

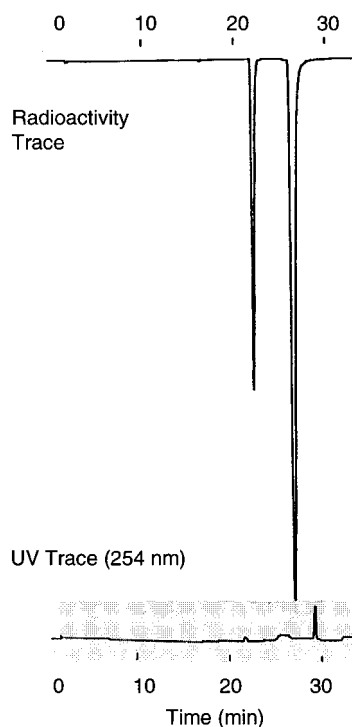


Figure 3. HPLC chromatograph of the crude ^{99m}Tc labeling reaction mixture of the hydrolyzed BCA **1** (^{99m}Tc -DADT) after incubation for 10 min.

(15 mL). The mixture was then centrifuged at 700g for 30 min. Granulocyte cells at the Histopaque 1077–1119 interface were collected and washed twice in the incubation buffer [10 mM phosphate (pH 7.2) containing 117 mM NaCl, 0.15 mM CaCl₂, and 0.5 mM MgCl₂] by centrifugation at 200g for 10 min. The final pellet was suspended in incubation buffer at a concentration of 2×10^7 cells/mL. The incubation mixture consisted of 6 nM [^3H]N $^{\alpha}$ -for-Met-Leu-Phe, 1.5×10^5 cells, in the presence or absence of 0.01–600 nM N $^{\alpha}$ -for-Nle-Leu-Phe-Nle-Tyr-Lys, ^{99m}Tc -DADT-Pep-I, or ^{99m}Tc -DADT-Pep-II in a total volume of 150 μL . The mixture was incubated at 37 $^{\circ}\text{C}$ for 15 min. Nonspecific binding was ascertained using 10 μM unlabeled N $^{\alpha}$ -for-Met-Leu-Phe. After incubation, the mixture was filtered through GF/B filters presoaked with assay buffer using a BRANDEL manifold. The cells

were washed with ice cold buffer (3×5 mL) and the filters counted with a liquid scintillation counter. The results of the assay were analyzed with the EBDA/LIGAND computer programs (25).

Biodistribution of the ^{99m}Tc -Labeled Peptides in Normal Mice. For in vivo studies, the two major products from ^{99m}Tc labeling were separated by HPLC after gel treatment. Fractions containing ^{99m}Tc -DADT-Pep-I and ^{99m}Tc -DADT-Pep-II were collected. The labeled peptides were isolated into ethanol by solid-phase extraction procedures using a C-18 Sep-Pak cartridge. The ethanol solutions were reconstituted with saline. The final injectate contained 2% ethanol.

Male CD-1 mice (average weight, 22 g) were each injected via the tail vein with ^{99m}Tc -DADT-Pep-I or ^{99m}Tc -DADT-Pep-II ($2 \mu\text{Ci}$, 0.2 mL). At 5, 30, 60, and 120 min postinjection, five mice at each time point per tracer were euthanized by cervical dislocation and 0.1 mL of blood was collected. Major organs were dissected, weighed, and counted in an automated scintillation counter along with the blood collected, and aliquots of the injectate as standards.

Imaging of Experimental Inflammation in Rabbits. Twelve female New Zealand white rabbits (2.7–3.1 kg) were each injected in the right hind leg with 0.2 mL of turpentine. Forty-eight hours after the injection of the turpentine, the rabbits were sedated with a mixture of Ketaset (100 mg, 1.0 mL) and AceProm (5 mg, 0.5 mL). The rabbits were divided into four groups of three each. Each group was injected via the left ear vein with 1 mCi (1.5 mL) no-carrier-added ^{99m}Tc -DADT-Pep-I, ^{99m}Tc -DADT-Pep-II, $^{99m}\text{TcO}_4^-$, or ^{99m}Tc -DADT. At various time intervals up to 2 h postinjection, 0.5 mL of blood was drawn from the right ear vein. Aliquots (0.1 mL) of the blood were counted in the automated γ -scintillation counter and compared with aliquots of the injectate counted at the same time.

We obtained images of the rabbits carrying the experimental inflammation on a γ -scintillation camera. Planar lateral images of 500K counts were acquired 5, 30, 60, 180, and 360 min postinjection. The images were converted into the Image Program in Macintosh format for processing and analysis. Region of interest analyses were performed over the inflamed area compared to the contralateral thigh. The average counts per pixel per millicurie injected activity were calculated and corrected for decay with respect to the time of imaging after injection of tracer. The presence of inflammation was confirmed by euthanizing the rabbits and visually confirming the presence of pus in the inflamed area at the end of the studies.

Effect of the No-Carrier-Added ^{99m}Tc -DADT-Pep-I, ^{99m}Tc -DADT-Pep-II, and fMLF on the Leukocyte Count in Rabbits. Five minutes before the injection of ^{99m}Tc -DADT-Pep-I and ^{99m}Tc -DADT-Pep-II in the imaging studies above, 0.5 mL of blood was withdrawn via the right ear vein of the rabbits. Then at 5, 10, 15, 30, and 60 min postinjection, 0.5 mL of blood was withdrawn via the right ear vein. Another group of three rabbits were treated in a similar fashion except that they were injected via the left ear vein with 2 nmol of fMLF in 1.5 mL of saline containing 2% ethanol instead of the ^{99m}Tc -labeled tracers. Aliquots (0.1 mL) of the blood samples were diluted 5-fold with saline. A 0.1 mL aliquot of the saline-diluted blood sample was mixed with an equal volume of a 2% acetic acid solution, and the number of leukocytes per milliliter was determined by counting with a hemacytometer.

RESULTS AND DISCUSSION

Peptides are capable of coordinating technetium directly through side chain functional groups and the amide nitrogen atom. For small peptides, coordination of Tc this way especially at the binding site would inactivate the peptide. To prepare the Tc-derivatized peptides, the bifunctional chelate approach in which a strong chelating agent is used to carry the metal was used (26). Of chelating agents used in the bifunctional chelate approach for coordinating Tc, the diaminedithiol chelate (DADT) system offers an excellent combination of characteristics for application to peptides. The ligand forms one of the most stable complexes of the metal (27–30). The Tc complex cores are small, compact, well-defined, and characterized. The ligand system has been used for the stable and specific incorporation of ^{99m}Tc into proteins without utilizing the peptide bonds or other side chain coordinating groups of the protein (31).

Structure–activity studies of chemotactic peptides indicate that the active site is represented by the N^α -for-tripeptide sequence at the N terminus (11, 20, 32). We chose the chemotactic hexapeptide N^α -for-Nle-Leu-Phe-Nle-Tyr-Lys which binds with high affinity to granulocytes (20) for evaluation because this hexapeptide contains a terminal lysine residue at the carboxyl-terminal portion of the molecule not required for binding to the chemoattractant receptor that can be utilized for BCA coupling. During the course of this work, this peptide was also successfully labeled with ^{18}F (17), ^{111}In , and ^{99m}Tc using a diethylenetriaminepentaacetic acid dianhydride (13) and a hydrazinonicotinamide (14) as BCAs.

BCA **1** was synthesized as previously reported (19). Coupling of BCA **1** to the hexapeptide was accomplished at room temperature and pH 9 using a BCA:peptide mole ratio of 5 (Scheme 1). The reaction was followed by reversed-phase HPLC monitored by UV at 220 nm and was practically complete after incubation for 1 h. The crude product was purified by semipreparative HPLC to >95% purity in 53% isolated yield. The product was characterized by mass spectroscopy and amino acid analysis.

Figure 1A shows the chromatographic profile after incubation for 10 min from the labeling of the purified BCA **1** coupled peptide, N^α -for-Nle-Leu-Phe-Nle-Tyr-Lys(N^ϵ -DADT), with ^{99m}Tc using [^{99m}Tc]glucoheptonate as the donor of prereduced ^{99m}Tc . The HPLC chromatogram showed two major ^{99m}Tc -labeled peptide peaks (^{99m}Tc -DADT-Pep-I and ^{99m}Tc -DADT-Pep-II) in a ratio (I:II) of 1:0.85. The total yield of the ^{99m}Tc -labeled peptide peaks I and II was 84%. The formation of the two products is in agreement with the geometric isomerism (syn and anti forms) that can result from the complexation of technetium to nitrogen-substituted as well as carbon-substituted diaminedithiol ligands (33–35). It is also possible that the products are the result of differences in the way the peptide wraps around the chelate core.

The cause of the apparent low specific activity in labeled chemotactic peptides is the presence of unlabeled material in the labeling mixture which can also bind to the receptor. Current methods of achieving high specific activity for ^{99m}Tc -labeled chemotactic peptides include the use of low concentrations of unlabeled ligand coupled with the use of $^{99m}\text{TcO}_4^-$ eluted from the generator at the time of maximum specific activity and/or the application of HPLC to separate labeled from unlabeled conjugate (14, 36). The use of low concentrations of unlabeled ligand leads to low labeling yields as well as long reaction

Table 1. Results of the Ellman's Assay for the Determination of Thiol Groups

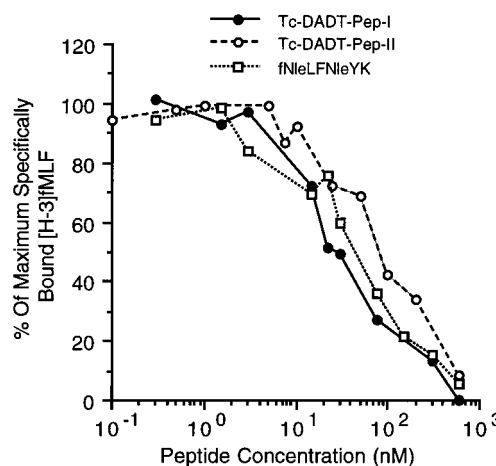
sample	peptide concentration (mM)
reaction mixture (before gel)	1.0
after gel treatment	not detectable ^a
HPLC purification	not detectable ^a

^a Minimum detection limit = 0.25 nmol.

times (the result of bimolecular reaction kinetics) and still does not produce formulations of high enough specific activity. While HPLC can potentially produce no-carrier-added formulations, the methodology is not available at the community hospital radiopharmacy level and therefore limits widespread utility. Furthermore, when high concentrations of ligand are used in order to boost labeling yield and shorten reaction time, unlabeled ligand can sometimes contaminate the labeled product peak due to insufficient separation, overloading, or tailing of the ligand peak.

Use of the DADT ligand for coordinating Tc presents a unique opportunity for chemical removal of the unlabeled conjugate. When DADT ligands form complexes with Tc, the two thiol groups participate in bond formation with the metal (Scheme 2). This allows the chemical removal of the unlabeled ligand from solution by use of an appropriately functionalized solid support (such as an iodoacetamide-derivatized gel) that reacts specifically and efficiently with free thiol groups, followed by filtration (21, 37). This simple but highly efficient procedure permits the preparation of the high-specific activity ^{99m}Tc formulations required for successful utilization of labeled chemotactic peptide agonists for imaging. Figure 1B shows the HPLC chromatogram of the gel-treated filtrate using the same HPLC conditions as depicted in Figure 1A. Compared to that in Figure 1A, the UV peak with a 10 min retention time associated with unlabeled DADT-peptide is not detectable, indicating complete removal of the unlabeled material from the gel. Table 1 shows the results of the Ellman's assay of thiol-containing compounds. There was no detectable thiol-containing peptide after gel treatment or HPLC purification, whereas the crude product showed a peptide concentration of 1 mM. These results show the powerful potential of this method to develop ^{99m}Tc -labeled chemotactic peptides in extremely high specific activity. Indeed, our initial evaluation of this procedure using model DADT compounds showed that >99.9% of the unlabeled ligand is removed after gel treatment (21). This takes into consideration a minimum detectable level of the DADT ligand by Ellman's assay of 0.25 nmol. The procedure is amenable to kit formulation since it requires no complex chromatographic procedure such as HPLC to obtain formulations of the requisite specific activity. Thus, the tracers can actually be prepared on demand from kits at community hospital radiopharmacies for use even in emergencies. We are currently developing a Sep-Pak procedure for removing the hydrophilic nonpeptide-associated UV active peaks that appear at the void volume in the chromatograph (Figure 1B) after gel treatment. The Sep-Pak procedure is fast and can be completed within 10 min. We are also investigating shorter incubation times for the iodoacetamide gel with the aim of completing the synthesis and separation procedures in <1 h.

In a recent report, Wu and co-workers (38) also reported the use of a *p*-chloromercuribenzoate gel to remove $\geq 98.3\%$ of the excess *N*¹-(2-mercapto-2-methylpropyl)-*N*²-(2-propargylthio-2-methylpropyl)-1,2-benzene-diamine (T691) after ^{99m}Tc labeling. These results sup-

**Figure 4.** Competitive binding of the ^{99m}Tc -labeled peptides versus [^3H]N-for-Met-Leu-Phe ([^3H]fMLF).

port the powerful potential of these solid-phase technologies for generating high-specific activity ^{99m}Tc radiopharmaceuticals.

To assess the receptor binding properties of the technetium-labeled peptides and to characterize the complexes conventionally, we synthesized the ^{99m}Tc analogues of the peptides (72% yield) by exchange from [^{99m}Tc]glucoheptonate. The products were characterized by amino acid analysis and high-resolution mass spectroscopy. To our knowledge, these are the first Tc derivatives of chemotactic peptides to be conventionally characterized. The ability to isolate and characterize these complexes is an advantage in toxicological studies in the FDA approval process. Previously reported Tc-derivatized chemotactic peptides did not report characterization of the Tc species (13–16). We performed in vitro competitive receptor binding assays using the isolated ^{99m}Tc analogues against the tritiated authentic chemotactic peptide [^3H]N-for-Met-Leu-Phe (fMLF) using human granulocytes according to published procedures (11, 20). Figure 4 shows that increasing concentrations of the technetium-labeled analogues efficiently inhibit the binding of the potent, authentic chemotactic peptide [^3H]fMLF, indicating competition for the same receptor. The results of the binding assays were analyzed by the EBDA/LIGAND computer program (25). As shown in Table 1, the inhibition constants, K_i , of the analogues were 13.7 ± 1.1 and 35.7 ± 9.3 nM for ^{99m}Tc -DADT-Pep-I and ^{99m}Tc -DADT-Pep-II, respectively. The unmodified peptide N^α-for-Nle-Leu-Phe-Nle-Tyr-Lys has a K_i of 21.3 ± 2.5 nM in the assay system. The ^{99m}Tc -labeled peptides therefore show comparable binding affinity to the potent chemotactic peptide N^α-for-Nle-Leu-Phe-Nle-Tyr-Lys from which they were derived. These binding experiments show that it is feasible to attach the Tc-DADT chelate to the low-molecular weight receptor binding chemotactic peptide and retain substantial binding to the receptor. The Tc chelate in these labeled peptides is sufficiently distant from the binding site so that it does not interfere with the interaction of the peptides with the receptor.

For the in vivo biological evaluation that follows, we separated the ^{99m}Tc -DADT-Pep-I and ^{99m}Tc -DADT-Pep-II by HPLC after gel treatment in order to study each product individually. ^{99m}Tc -DADT-Pep-I and ^{99m}Tc -DADT-Pep-II were each isolated to >95% radiochemical purity by HPLC. Figure 2 shows the analytical chromatograms of the HPLC-separated ^{99m}Tc -labeled peptides. There was no UV peak associated with the purified

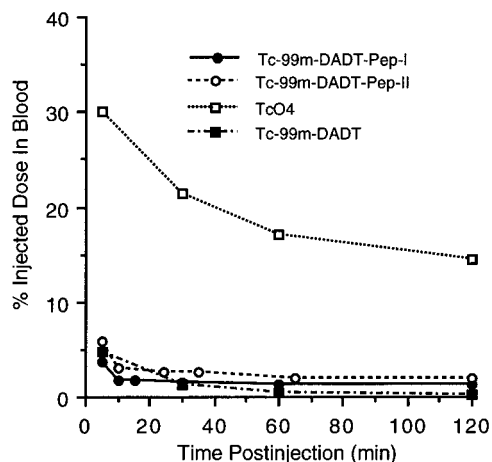
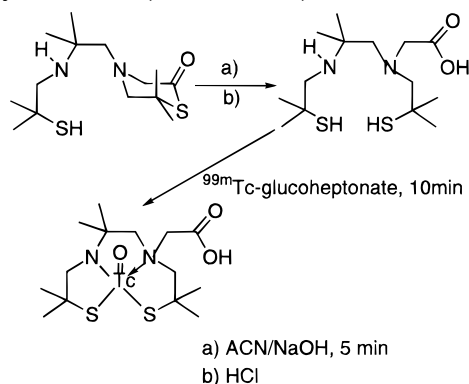


Figure 5. Blood clearance of ^{99m}Tc -DADT-Pep-I and ^{99m}Tc -DADT-Pep-II, compared to those of ^{99m}Tc -DADT and [^{99m}Tc]-pertechnetate ($^{99m}\text{TcO}_4$) in rabbits.

Scheme 3. Synthesis of the ^{99m}Tc Complex of Hydrolyzed BCA 1 (^{99m}Tc -DADT)



radioactivity peaks even at the highest sensitivity of the UV detector (0.2 nmol detection limit), indicating extremely high specific activity of the labeled peptides. As is the case for nca ^{99m}Tc preparations, it is not possible to measure the mass amount of labeled material by conventional means due to the small amount of material involved. We estimated, on the basis of the history of the ^{99m}Tc generator used, that the specific activity of the labeled peptide was at least 100 000 mCi/ μmol .

When ^{99m}Tc -DADT-Pep-I and ^{99m}Tc -DADT-Pep-II are used in vivo, one point of possible metabolic cleavage is the amide bond between the Lys residue and the DADT ligand. If this occurred, the resultant ^{99m}Tc complex would be the same as the complex derived from hydrolyzed BCA 1 (^{99m}Tc -DADT, Scheme 3). Therefore, we prepared ^{99m}Tc -DADT as depicted in Scheme 3 for comparison to the labeled peptides during in vivo imaging. Figure 3 shows the HPLC chromatogram of the labeling mixture after incubation for 10 min. Two main products were obtained in a major:minor ratio of 3.1:1. Both products were combined for in vivo imaging studies.

Figure 5 shows the blood clearance of the saline-reconstituted, no-carrier-added ^{99m}Tc -labeled peptides compared with the clearance of $^{99m}\text{TcO}_4^-$ and the ^{99m}Tc complex of the hydrolyzed BCA 1 (^{99m}Tc -DADT) injected separately in different rabbits carrying turpentine-induced sterile inflammation. $^{99m}\text{TcO}_4^-$ and ^{99m}Tc -DADT served as controls. The ^{99m}Tc -labeled peptides as well as the ^{99m}Tc -DADT complex without peptide clear rapidly from the blood with less than 3% of the injected dose remaining in blood 20 min postinjection. The fast

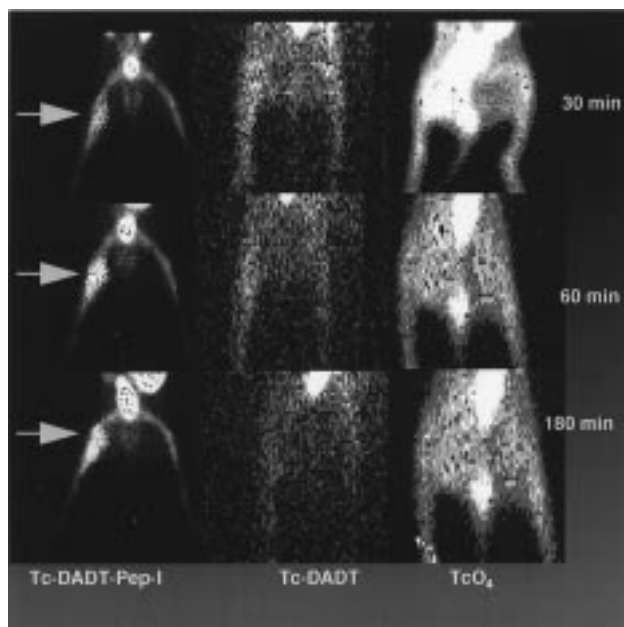


Figure 6. Images of experimental inflammation in rabbits using ^{99m}Tc -DADT-Pep-I, the ^{99m}Tc -DADT chelate, and [^{99m}Tc]pertechnetate ($^{99m}\text{TcO}_4$) at 30 min, 1 h, and 3 h postinjection.

clearance of the activity from blood is a desired property of the labeled peptides that would permit imaging at shorter times postinjection. As is usual for $^{99m}\text{TcO}_4^-$, the activity of this tracer in the blood is much higher in these studies. Of the blood activity for the labeled peptides, $26.2 \pm 6.2\%$ was cell associated 60 min postinjection.

We obtained images of the rabbits carrying the experimental inflammation on a γ -scintillation camera. We compared images from the ^{99m}Tc -DADT-Pep-I and -II with those obtained from $^{99m}\text{TcO}_4^-$ and ^{99m}Tc -DADT. The $^{99m}\text{TcO}_4^-$ and ^{99m}Tc -DADT give an indication of the leakiness of blood vessels and blood flow to the inflammation site. Figure 6 shows representative results obtained using ^{99m}Tc -DADT-Pep-I, $^{99m}\text{TcO}_4^-$, and ^{99m}Tc -DADT. The results for ^{99m}Tc -DADT-Pep-I are similar to those obtained for ^{99m}Tc -DADT-Pep-II. It is clear that as early as 30 min postinjection the site of inflammation (arrow) could be seen and persists throughout the 3 h study period shown for the ^{99m}Tc -labeled peptides. It is more difficult to identify the inflammation site on the $^{99m}\text{TcO}_4^-$ and ^{99m}Tc -DADT images 30 min postinjection. This is an indication that accumulation of the ^{99m}Tc -labeled peptides at the inflammation site is not due to leakiness of blood vessels or blood flow alone.

Analyses of the images are shown in Figures 7 and 8. Figure 7 shows that the target (inflammation site): nontarget (contralateral region) ratio for the ^{99m}Tc -labeled peptides continues to increase from 2 to 9 over the 6 h study period. This is further confirmation that the accumulation of the peptide tracers at the inflammation site is not dependent on blood flow or leakiness of blood vessels alone. For $^{99m}\text{TcO}_4^-$ and the ^{99m}Tc -DADT chelate whose accumulation at the site of inflammation is dependent on blood flow or leakiness of blood vessels, the ratio remains practically constant at about 2 over the entire study period. The behavior of ^{99m}Tc -DADT suggests that the results obtained with the ^{99m}Tc -labeled peptides are not an artifact of the Tc chelate by itself.

Panels A and B of Figure 8 show the average counts per pixel (corrected for decay) at the inflamed site (target) and the contralateral region (nontarget) as a function of

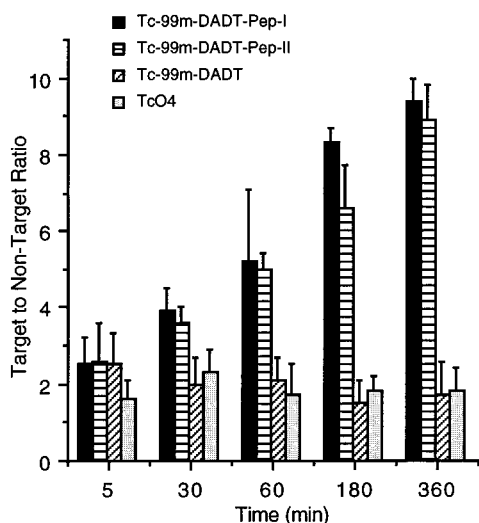


Figure 7. Target:nontarget ratios as a function of time for ^{99m}Tc -DADT-Pep-I and ^{99m}Tc -DADT-Pep-II, compared to those of ^{99m}Tc -DADT and [^{99m}Tc]pertechnetate ($^{99m}\text{TcO}_4$) in rabbits carrying experimental inflammation.

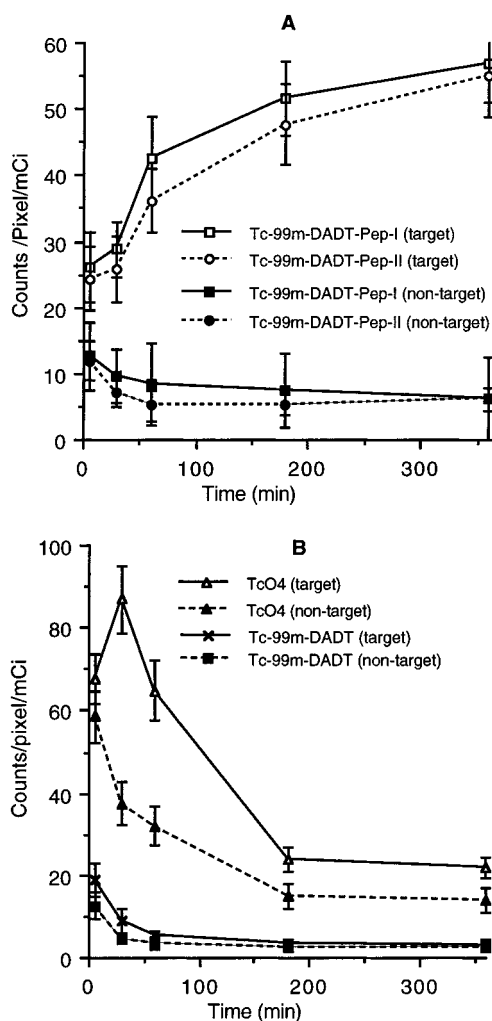


Figure 8. Activity of ^{99m}Tc -DADT-Pep-I and ^{99m}Tc -DADT-Pep-II (A) and the ^{99m}Tc -DADT chelate alone and [^{99m}Tc]pertechnetate ($^{99m}\text{TcO}_4$) (B) in target and nontarget areas as a function of time postinjection.

time postinjection for ^{99m}Tc -DADT-Pep-I and -II (Figure 8A) as well as $^{99m}\text{TcO}_4^-$ and ^{99m}Tc -DADT (Figure 8B). Figure 8A shows that for the labeled peptides, activity continues to increase with time at the inflammation site,

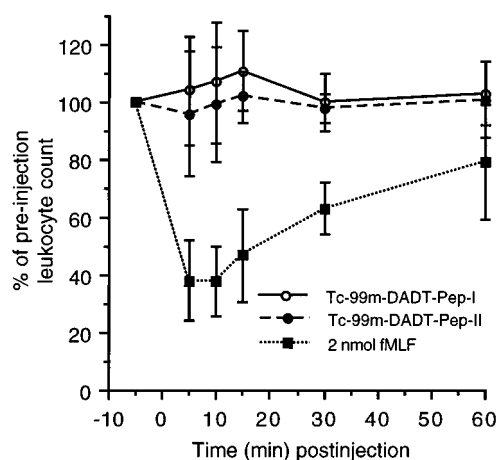


Figure 9. Effect of no-carrier-added ^{99m}Tc -labeled peptides on leukocytes in vivo compared to the effect of 2 nmol of N^α -for-Met-Leu-Phe (fMLF).

whereas the activity in nontarget areas decreases with time. This explains why the target:nontarget ratios for the ^{99m}Tc -labeled peptides increase as a function of time postinjection. On the other hand, as shown in Figure 8B, the activity in both the target and nontarget areas decreases in a parallel fashion for $^{99m}\text{TcO}_4^-$ and ^{99m}Tc -DADT as a function of time. Therefore, the target:nontarget ratios for $^{99m}\text{TcO}_4^-$ and ^{99m}Tc -DADT do not increase with time but remain relatively constant. Thus, the pharmacokinetics of the labeled peptides differ from those of either $^{99m}\text{TcO}_4^-$ or ^{99m}Tc -DADT.

Agonists of the chemoattractant receptor cause acute neutropenia (18). This could be a very unwelcome side effect in patients undergoing studies with labeled chemotactic peptide agonists. That is why the requirement of high-specific activity radiopharmaceuticals is extremely important in this system. A major hypothesis of this work is that the amount of ^{99m}Tc (picomoles) at nca levels will be too small to cause a depression of the white blood cell count. Therefore, we investigated the effect of the nca ^{99m}Tc -labeled peptides on the level of white blood cells in the rabbits carrying the experimental inflammation. We compared the total white blood cell count of blood drawn 5 min before the injection of the tracers to the cell count in blood drawn 5, 10, 15, 30, and 60 min after injection of the tracers. The effects of the tracers were compared with the injection of 2 nmol of N^α -for-Met-Leu-Phe (fMLF). Figure 9 shows that, up to the 1 h study period, there was no significant difference in the white blood cell count before and after the injection of the ^{99m}Tc -labeled peptides, indicating that the tracers are devoid of neutropenic effects. On the other hand, 2 nmol of fMLF drastically reduced the leukocyte count immediately after injection.

These results demonstrate the feasibility of utilizing the diaminedithiol ligand system for the development of chemotactic peptide analogues for use in inflammation imaging without accompanying neutropenia. The advantage of using the DADT ligand in this system is that it is feasible to prepare the nonpharmacologically active formulations needed in a simple and highly efficient manner. Second, the structure of Tc-diaminedithiolate complexes are very well-defined, making it possible to isolate the Tc species formed for conventional characterization as done in these studies.

The general biodistribution studies in mice, however, show high liver and intestinal activity of the labeled peptides, indicating that the major route of excretion for

Table 2. Results of in Vitro Competitive Binding of the Isolated ^{99}Tc Peptide Analogues to Granulocytes

compound	K_i^a (nM)
N $^{\alpha}$ -for-Nle-Leu-Phe-Nle-Tyr-Lys	21.3 $\pm$ 2.5
^{99}Tc -DADT-Pep-I	13.7 $\pm$ 1.1
^{99}Tc -DADT-Pep-II	35.7 $\pm$ 9.3

^a Average $K_i \pm$ SEM ($n = 3$).**Table 3. Biodistribution of ^{99m}Tc -DADT-Pep-I and ^{99m}Tc -DADT-Pep-II in Mice**

organ	time (min)	% injected dose per gram ^a	
		Tc-DADT-Pep-I	Tc-DADT-Pep-II
blood	5	1.44 $\pm$ 0.53	2.14 $\pm$ 0.41
	30	1.43 $\pm$ 0.25	0.30 $\pm$ 0.07
	60	0.28 $\pm$ 0.04	0.20 $\pm$ 0.09
	120	0.16 $\pm$ 0.03	0.30 $\pm$ 0.16
brain	5	0.09 $\pm$ 0.04	0.04 $\pm$ 0.01
	30	0.09 $\pm$ 0.04	0.01 $\pm$ 0.01
	60	0.02 $\pm$ 0.01	0.01 $\pm$ 0.00
	120	0.03 $\pm$ 0.01	0.01 $\pm$ 0.00
lungs	5	10.41 $\pm$ 1.32	1.22 $\pm$ 0.74
	30	9.97 $\pm$ 1.00	1.33 $\pm$ 1.03
	60	3.01 $\pm$ 2.26	0.66 $\pm$ 0.28
	120	1.25 $\pm$ 0.42	0.37 $\pm$ 0.07
liver	5	38.82 $\pm$ 11.19	43.57 $\pm$ 9.04
	30	38.06 $\pm$ 12.77	29.78 $\pm$ 5.89
	60	15.03 $\pm$ 16.31	7.38 $\pm$ 2.33
	120	5.41 $\pm$ 4.46	5.18 $\pm$ 1.50
spleen	5	5.02 $\pm$ 2.32	1.88 $\pm$ 0.72
	30	5.56 $\pm$ 2.27	1.42 $\pm$ 0.73
	60	2.85 $\pm$ 1.51	0.93 $\pm$ 0.23
	120	2.13 $\pm$ 0.29	0.61 $\pm$ 0.19
kidneys	5	6.39 $\pm$ 2.67	7.68 $\pm$ 1.98
	30	6.81 $\pm$ 2.89	3.42 $\pm$ 1.80
	60	5.96 $\pm$ 1.52	4.47 $\pm$ 1.47
	120	3.34 $\pm$ 1.18	2.28 $\pm$ 0.16
stomach	5	7.15 $\pm$ 9.40	4.19 $\pm$ 4.78
	30	8.41 $\pm$ 10.35	10.53 $\pm$ 2.83
	60	4.30 $\pm$ 3.22	3.16 $\pm$ 3.35
	120	1.33 $\pm$ 0.23	2.22 $\pm$ 0.59
skeletal muscle	5	0.45 $\pm$ 0.11	0.46 $\pm$ 0.06
	30	0.45 $\pm$ 0.13	0.16 $\pm$ 0.12
	60	0.36 $\pm$ 0.12	0.05 $\pm$ 0.04
	120	0.07 $\pm$ 0.02	0.08 $\pm$ 0.05
femur	5	0.85 $\pm$ 0.29	0.62 $\pm$ 0.06
	30	0.80 $\pm$ 0.30	0.26 $\pm$ 0.07
	60	0.37 $\pm$ 0.11	0.23 $\pm$ 0.19
	120	0.21 $\pm$ 0.03	0.13 $\pm$ 0.07
intestines	5	15.91 $\pm$ 4.97	14.39 $\pm$ 4.48
	30	15.35 $\pm$ 5.55	30.34 $\pm$ 4.25
	60	30.83 $\pm$ 3.95	30.15 $\pm$ 6.13
	120	31.34 $\pm$ 3.01	30.87 $\pm$ 3.01

^a Mean $\pm$ SEM ($n = 5$).

the labeled peptides presented in this study was the hepatobiliary system (Table 3). The DADT ligand and chemotactic peptide pair used in this study, therefore, may not be clinically useful for imaging applications in the abdominal area because of the high nonspecific abdominal activity. The hepatobiliary clearance is the result of the high lipophilicity of the neutral Tc-DADT chelate used. We are currently investigating more hydrophilic Tc-DADT chelates and chemotactic peptide combinations to obtain an optimum radiopharmaceutical that would be clinically useful for all areas of the body.

ACKNOWLEDGMENT

This work was supported by U.S. Public Health Service Grants CA-32845 and AI-38276 and U.S. Public Health Service Training Grant CA-019199.

LITERATURE CITED

- (1) Deysine, M., Robinson, R., Rafkin, H., Teicher, I., Silver, L., and Aufses, A. H. (1974) Clinical infections detected by ^{67}Ga scanning. *Ann. Surg.* 180, 897–901.
- (2) Thakur, M. L., Lavender, J. P., Arnot, R. N., Silvester, D. J., and Segal, A. W. (1977) Indium-111-labeled autologous leukocytes in man. *J. Nucl. Med.* 18, 1014–1021.
- (3) Peters, A. M., Savarymattu, S. H., Reavy, H. J., Osman, S., and Lavender, J. P. (1983) Imaging of inflammation with indium-111 tropolonate labeled leukocytes. *J. Nucl. Med.* 24, 39–44.
- (4) Peters, A. M., Osman, S., Henderson, B. L., Kelly, J. D., Danpure, H. J., Hawker, R. J., Hodgson, H. J., Neirincx, R. D., and Lavender, J. P. (1986) Clinical experience with Tc-99m hexamethyl-propyleneamineoxime for labeling leukocytes and imaging inflammation. *Lancet* 2, 946–949.
- (5) Seybold, K., Locher, J. T., Coosemans, C., Andres, R. Y., Schubiger, P. A., and Blauenstein, P. (1986) Immunoscintigraphic localization of inflammatory lesions: clinical experience. *Eur. J. Nucl. Med.* 13, 587–593.
- (6) Lind, P., Langsteger, W., Koltringer, P., Dimai, H. P., Passl, R., and Eber, O. (1990) Immunoscintigraphy of inflammatory processes with a technetium-99m-labeled monoclonal anti-granulocyte antibody (MAb BW 250/183). *J. Nucl. Med.* 31, 417–423.
- (7) Rubin, R. H., Fischman, A. J., Callahan, R. J., Khaw, B. A., Keech, F., Ahmad, M., Wilkinson, R., and Strauss, H. W. (1989) ^{111}In -labeled nonspecific immunoglobulin scanning in the detection of focal infection. *N. Engl. J. Med.* 321, 935–940.
- (8) Abrams, M. J., Juweid, M., tenKate, C. I., Schwartz, D. A., Hauser, M. M., Gaul, F. E., Fucello, A. J., Rubin, R. H., Strauss, H. W., and Fischman, A. J. (1990) Technetium-99m-human polyclonal IgG radiolabeled via the hydrazinonicotinamide derivative for imaging focal sites of infection in rats. *J. Nucl. Med.* 31, 2022–2028.
- (9) Streule, K., de Schrijver, M., and Fridrich, R. (1988) Tc-99m labeled HSA nanocolloid vs In-111 oxine labeled granulocytes in the detecting skeletal septic processes. *Nucl. Med. Commun.* 9, 59–67.
- (10) Schiffman, E., Corcoran, B. A., and Wahl, S. M. (1975) N-Formylmethionyl peptides as chemotactants for leukocytes. *Proc. Natl. Acad. Sci. U.S.A.* 72, 1059–1062.
- (11) Williams, L. T., Snyderman, R., Pike, M. C., and Lefkowitz, R. J. (1977) Specific receptor sites for chemotactic peptides on human polymorphonuclear leukocytes. *Proc. Natl. Acad. Sci. U.S.A.* 74, 1204–1208.
- (12) Aswanikumar, S., Corcoran, B. A., Schiffman, E., Day, A. R., Freer, R. J., Showell, H. J., Becker, E. L., and Pert, C. B. (1977) Demonstration of a receptor on rabbit neutrophils for chemotactic peptides. *Biochem. Biophys. Res. Commun.* 74, 810–817.
- (13) Fischman, A. J., Pike, M. C., Kroon, D., Fucello, A. J., Rexinger, D., tenKate, C., Wilkinson, R., Rubin, R. H., and Strauss, H. W. (1991) Imaging focal sites of bacterial infection in rats with indium-111-labeled chemotactic peptide analogues. *J. Nucl. Med.* 32, 483–491.
- (14) Babich, J. W., Solomon, H., Pike, M., Kroon, D., Graham, W., Abrams, M. J., Tomkins, R. G., Rubin, R. H., and Fischman, A. J. (1993) Tc-99m-labeled hydrazino nicotinamide derivatized chemotactic peptide analogs for imaging focal sites of bacterial infection in rabbits. *J. Nucl. Med.* 34, 1964–1974.
- (15) Babich, J. W., and Fischman, A. J. (1995) Effect of co-ligand on the biodistribution of Tc-99m labeled hydrazino nicotinic acid derivatized chemotactic peptides. *Nucl. Med. Biol.* 33, 25–30.
- (16) Pollak, A., Goodbody, A. E., Ballinger, J. R., Kronauge, J. F., Duncan, G. S., Tran, L. L., Dunn-Dufault, R., Meghji, K., Lau, F., Andrey, T. W., Boxen, I., and Sumner-Smith, M. (1996) Imaging inflammation with ^{99m}Tc -labeled chemotactic peptides: Analogues with reduced neutropenia. *Nucl. Med. Commun.* 17, 132–139.

- (17) Vaidyanathan, G., and Zalutsky, M. R. (1995) Fluorine-18 labeled chemotactic peptides: A potential approach for PET imaging of bacterial infection. *Nucl. Med. Biol.* 22, 759–764.
- (18) O'Flaherty, J. T., Showell, H. J., and Ward, P. A. (1977) Neutropenia induced by systemic infusion of chemotactic factors. *J. Immunol.* 118, 1586–1589.
- (19) Baidoo, K. E., Lever, S. Z., and Scheffel, U. (1994) A bifunctional chelator for facile preparation of neutral technetium complexes. *Bioconjugate Chem.* 5, 114–118.
- (20) Niedel, J., Wilkinson, S., and Cuatrecasas, P. (1979) Receptor mediated uptake and degradation of ¹²⁵I-chemotactic peptide by human neutrophils. *J. Biol. Chem.* 254, 10700–10706.
- (21) Kao, K., Baidoo, K. E., and Lever, S. Z. (1997) Validation and application of a solid-phase chemistry method for the preparation of high effective specific activity technetium-99m radiopharmaceuticals. *Nucl. Med. Biol.* 24, 499–505.
- (22) de Kieviet, W. (1981) Technetium Radiopharmaceuticals: Chemical characterization and tissue distribution of Tc-glucoseheptonate using Tc-99m and Tc-99. *J. Nucl. Med.* 22, 703–709.
- (23) Ellman, G. L. (1959) Tissue sulfhydryl groups. *Arch. Biochem. Biophys.* 82, 70–77.
- (24) Baidoo, K. E., and Lever, S. Z. (1990) Synthesis of a diaminedithiol bifunctional chelating agent for the incorporation of technetium-99m into biomolecules. *Bioconjugate Chem.* 1, 132–137.
- (25) McPherson, G. A. (1985) Analysis of radioligand binding experiments. A collection of computer programs for the IBM PC. *J. Pharmacol. Methods* 14, 213–228.
- (26) Sundberg, M. W., Meares, C. F., Goodwin, D. A., and Diamanti, C. I. (1974) Chelating agents for the binding of metal ions to macromolecules. *Nature* 250, 587–588.
- (27) Epps, L. A. (1984) The chemistry of neutral lipid soluble technetium (V) complexes of aminoalcohols and aminothiols. Ph.D. Thesis, The Johns Hopkins University, Baltimore, MD.
- (28) Lever, S. Z., Burns, H. D., Kervitsky, T. M., Goldfarb, H. W., Woo, D. V., Wong, D. F., Epps, L. A., Kramer, A. V., and Wagner, H. N., Jr. (1985) Design, preparation, and biodistribution of a technetium-99m triaminedithiol complex to assess regional cerebral blood flow. *J. Nucl. Med.* 26, 1287–1294.
- (29) Kung, H. F., Molnar, M., Billings, J., Wicks, R., and Blau, M. (1984) Synthesis and biodistribution of neutral, lipid soluble Tc-99m complexes that cross the blood-brain barrier. *J. Nucl. Med.* 25, 326–332.
- (30) Kung, H. F., Liu, B.-L., Wei, Y., and Pan, S. (1990) A study of the structure–stability relationship of technetium compounds. *Appl. Radiat. Isot.* 41, 773–781.
- (31) Baidoo, K. E., Scheffel, U., and Lever, S. Z. (1990) Technetium-99m labeling of proteins: Initial evaluation of a novel diaminedithiol bifunctional chelating agent. *Cancer Res.* 50 (Suppl.), 799s–803s.
- (32) Showell, H. J., Freer, R. J., Zigmond, S. H., Schiffmann, S., Aswanikumar, S., Corcoran, B., and Becker, E. L. (1976) Structure activity relations of synthetic peptides as chemotactic factors and inducers of lysosomal enzyme secretion for neutrophils. *J. Exp. Med.* 143, 1154–1169.
- (33) Epps, L. A., Burns, H. D., Lever, S. Z., Goldfarb, H., and Wagner, H. N., Jr. (1987) Brain imaging agents: Synthesis and characterization of (N-piperidinyloxy)-hexamethyl diamino-dithiolate oxo technetium(V) complexes. *Appl. Radiat. Isot.* 38, 361–364.
- (34) Kung, H. F., Guo, Y.-Z., Yu, C.-C., Billings, J., Subramanyam, V., and Calabrese, J. (1989) New brain perfusion agents based on the Tc-99m bis-aminoethanethiol (BAT) complexes: stereoisomers and biodistribution. *J. Med. Chem.* 32, 433–437.
- (35) Lever, S. Z., Baidoo, K. E., and Mahmood, A. (1990) Structure Proof of Syn/Anti Isomerism in N-Alkylated Diaminedithiol (DADT) Complexes of Technetium. *Inorg. Chem. Acta* 176, 183–184.
- (36) Baidoo, K. E., Scheffel, U., Lever, S. Z., and Wagner, H. N. (1993) High affinity Tc-labeled chemotactic peptides. *J. Nucl. Med.* 34, 18P.
- (37) Baidoo, K. E., Scheffel, U., Finley, P., Lever, S. Z., and Wagner, H. N. (1994) No carrier added (NCA) Tc-99m diaminedithiolate derivatives of chemotactic peptides for imaging inflammation. *J. Nucl. Med.* 35, 19P.
- (38) Wu, J.-L., White, D. S., Ma, J. M., Fourby, D. R., and Baldwin, R. M. (1995) A simple method for the preparation of ligand-free ^{99m}Tc complexes. In *Technetium and Rhenium in Chemistry and Nuclear Medicine* (M. Nicolini, G. Bandoli, and U. Mazzi, Eds.) Vol. 4, pp 441–443, SGE Ditoriali, Padova, Italy.

BC970197T

Design, Synthesis, and Initial Evaluation of High-Affinity Technetium Bombesin Analogues

Kwamena E. Baidoo,* Kuo-Shyan Lin, Yougen Zhan, Paige Finley, Ursula Scheffel, and Henry N. Wagner, Jr.

Division of Radiation Health Sciences, Department of Environmental Health Sciences, The Johns Hopkins University, 615 North Wolfe Street, Baltimore, Maryland 21205. Received November 13, 1997; Revised Manuscript Received January 15, 1998

Potent antagonists of bombesin-like peptides have shown great potential for applications in cancer therapy. A ^{99m}Tc -labeled agent capable of identifying patients who could benefit from these emerging therapies would have a great impact on patient management. This study involves the synthesis and initial evaluation of technetium diaminedithiolate analogues derived from the potent bombesin analogue Pyr-Gln-Lys-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH₂ (Lys³-bombesin). We coupled two diaminedithiol (DADT) bifunctional chelating agents (BCAs **1** and **2**) to the Lys³ residue at the N-terminal region that is not required for binding to the receptor. ^{99m}Tc labeling was performed by ligand exchange on addition of [^{99m}Tc]glucoheptonate to a solution of the adduct at room temperature. Two products were obtained from each adduct on analysis by HPLC. The major to minor product ratios of the ^{99m}Tc -labeled analogues were 3:1 for products from BCA **1** and 9:1 for the products from BCA **2**. Macroscopic amounts of the ^{99m}Tc analogues were similarly prepared using [^{99m}Tc]glucoheptonate. In this case, the major to minor ratios were 2:1 for the products from both BCAs. For initial evaluation of the binding of the Tc-labeled peptides to bombesin receptors, the ^{99m}Tc analogues were used in vitro in competitive binding assays in rat brain cortex membranes against [^{125}I -Tyr⁴]bombesin. Results of the in vitro assays showed that the inhibition constants (K_i) of the major and minor products were 3.5 ± 0.7 and 3.9 ± 1.5 nM, respectively, for the products from BCA **1**; and 7.4 ± 2.0 and 5.2 ± 1.5 nM for the products derived from BCA **2**, respectively. The high affinity exhibited by these technetium analogues is an indication of their potential for use in non-invasive in vivo biochemical characterization of cancers that possess receptors for bombesin.

INTRODUCTION

Bombesin is a 14-amino acid peptide initially isolated from frog skin (1). The main mammalian members of the bombesin family of peptides are gastrin-releasing peptide (GRP), a 27-amino acid peptide isolated from porcine stomach (2), and neuromedin B, a 10-amino acid peptide isolated from porcine spinal cord (3). Receptors for bombesin/GRP are expressed in the central nervous system as well as in peripheral tissues such as the intestines and pancreas. Many tumors are also known to express bombesin/GRP receptors. These include glioblastoma, small cell lung cancer, prostate, breast, gastric, colon, and pancreatic cancer (4–17). In many of these cancers, bombesin/GRP analogues function as autocrine growth factors. Activation of bombesin/GRP receptors on cancer cells causes a number of biochemical events such as phosphatidylinositol turnover, activation of protein kinase C, and elevation of cytosolic calcium leading to the expression of early oncogenes such as c-fos and c-jun and ultimately results in stimulated growth and proliferation of the cells (5, 18, 19). It is conceivable that disruption of the biochemical pathways of bombesin action on cancer cells would result in the inhibition of growth. This has been the basis of recent efforts to

develop therapeutic regimens in several cancers. For example, an antibody to the receptor binding C terminus of bombesin and GRP was reported to effect clinical remission of small cell lung cancer in a patient and partial remission in others (20). In another approach, several antagonists to the receptor have also been developed. The early antagonists were substance P analogues and were generally of low affinity (21, 22). The second-generation analogues, such as isobutyryl-His-Trp-Ala-Val-D-Ala-His-Leu-NH-CH₃ (23), D-Tpi⁶,Leu¹³Ψ(CH₂-NH)-Leu¹⁴-bombesin^{6–14} (RC-3095) (24), and 3-phenylpropanoyl-His²⁰,D-Ala²⁴,Leu²⁶Ψ(CH₂NH)-Phe²⁷-GRP^{20–27} (BW2258U89) (25), are highly potent. Among these, D-Tpi⁶,Leu¹³Ψ(CH₂NH)-Leu¹⁴-bombesin^{6–14} (RC-3095) has been shown to inhibit the growth of many cancers (9–14, 16, 17, 26, 27), and 3-phenylpropanoyl-His²⁰,D-Ala²⁴,Leu²⁶Ψ(CH₂NH)-Phe²⁷-GRP^{20–27} (BW2258U89) has been shown to inhibit the growth of small cell lung cancer cells (28). Furthermore, labeling bombesin analogues with therapeutic radionuclides would result in derivatives that could be used directly in cancer therapy as has been demonstrated using a ^{105}Rh -labeled bombesin analogue (29). A radiolabeled agent capable of identifying the patients that could benefit from these emerging therapies would have a great impact on patient management.

This study involves the synthesis and initial evaluation of receptor binding of technetium analogues derived from the bombesin analogue Pyr-Gln-Lys-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH₂ (Lys³-bombesin) and

* Author to whom correspondence should be addressed at Division of Radiation Health Sciences, Department of Environmental Health Sciences, The Johns Hopkins University, 615 N. Wolfe St., Room 2001, Baltimore, MD 21205. Telephone: (410) 955-7706. Fax: (410) 955-6222. E-mail: kbaidoo@jhsp.hu.edu.

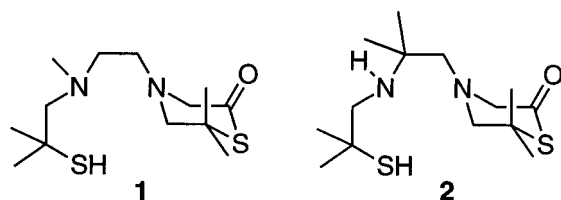


Figure 1. Diaminedithiol bifunctional chelating agents.

the diaminedithiol (DADT) bifunctional chelating agents (BCA) **1** and **2** (Figure 1) (30, 31), which are capable of forming highly stable complexes with technetium.

MATERIALS AND METHODS

Solvents and chemicals were reagent grade and used as received unless specified. [^{99}Tc] NH_4TcO_4 and [^{125}I -Tyr ^{4}]bombesin were purchased from NEN Life Science Products (Boston, MA). [$^{99\text{m}}\text{Tc}$] NaTcO_4 was purchased as a solution in physiologic saline from Syncor (Timonium, MD). The Glucoscan kit was purchased from the Du Pont Radiopharmaceutical Division (Billerica, MA). Bombesin, Lys ^{3} -bombesin, and somatostatin were purchased from Advanced ChemTech (Louisville, KY). Male CD-1 mice and Sprague-Dawley rats were purchased from the Charles River Laboratories (Charles River, MA).

The DADT bifunctional chelating agents **1** and **2** were synthesized according to our previously published procedures (30, 31). Infrared spectroscopic determinations of neat liquids or KBr pellets were performed on a Nicolet 205 FTIR system. ^1H NMR spectra were recorded in a deuteriochloroform or deuteriomethylene chloride solution using a Bruker WM 300 MHz spectrometer. Elemental analyses were performed by Atlantic Microlabs (Norcross, GA). MALDI mass spectral analyses were performed by the University of Minnesota Mass Spectrometry Service Laboratory (Minneapolis, MN).

HPLC was performed with a Waters Chromatography Division HPLC System equipped with two model 510EF pumps, a model 680 automated gradient controller, a model 490 UV absorbance detector, an EG&G NaI scintillation detector, and a Hewlett-Packard HP 3392A or Shimadzu CR3A integrating recorder. HPLC solvents consisted of acetonitrile containing 0.1% trifluoroacetic acid (solvent A) and water containing 0.1% trifluoroacetic acid (solvent B). Liquid scintillation counting for quantification of ^{99}Tc activity was performed on an LKB Wallac instrument model 1219 Rackbeta using Ecolume (ICN Pharmaceuticals, Inc., Costa Mesa, CA) as a cocktail. Filtration of in vitro binding assay mixtures was performed on a BRANDEL, 48M Harvester. ^{125}I activity on filters from in vitro assays and $^{99\text{m}}\text{Tc}$ activity of tissues from biodistribution studies were counted in an automated γ -counter (Pharmacia-Wallac model 1282). Analyses of in vitro binding assay results were performed using the EBDA/LIGAND programs from Biosoft (Milltown, NJ). $^{99\text{m}}\text{Tc}$ activity for synthetic reactions was measured in a Capintec CRC-7 Dose Calibrator.

Coupling of the Diaminedithiol Bifunctional Chelating Agents 1 and 2 to Pyr-Gln-Lys-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH $_2$ (Lys ^{3} -Bombesin). BCAs **1** and **2** were coupled to Lys ^{3} -bombesin according to Scheme 1. For coupling reactions using BCA **1** (30), **1**·2HCl (19.4 mg, 53.4 μmol) and Pyr-Gln-Lys-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH $_2$ (9.0 mg, 5.3 μmol) were dissolved in borate buffer (0.8 mL, 0.1 M, pH 9) and acetonitrile (0.9 mL) and the pH was readjusted to 9 with triethylamine. The mixture

was incubated at room temperature for 4.5 h and then extracted with ether (3×2 mL). The aqueous fraction was chromatographed by semipreparative reversed phase HPLC [linear gradient from solvent B (100%) to solvent A (80%)/solvent B (20%) over the course of 40 min at a flow rate of 6 mL/min using a C-18 Novapak cartridge (25×100 mm, 6 μm , Waters Chromatography Division, Milford, MA) monitored on-line for UV absorption at 220 nm]. The product with a retention time of 25.6 min was isolated from the HPLC eluate by solid phase extraction using a C-18 Sep-Pak cartridge (Waters Chromatography Division), followed by lyophilization. The isolated yield of the adduct named Pyr-Gln-Lys(Pm-DADT)-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH $_2$ [Lys ^{3} (Pm-DADT)-bombesin] was 5.2 mg (57%). High-resolution MALDI MS: calcd MW for Lys ^{3} (Pm-DADT)-bombesin 1880.96, found $[\text{M} + \text{Na}]^+$ 1904.05.

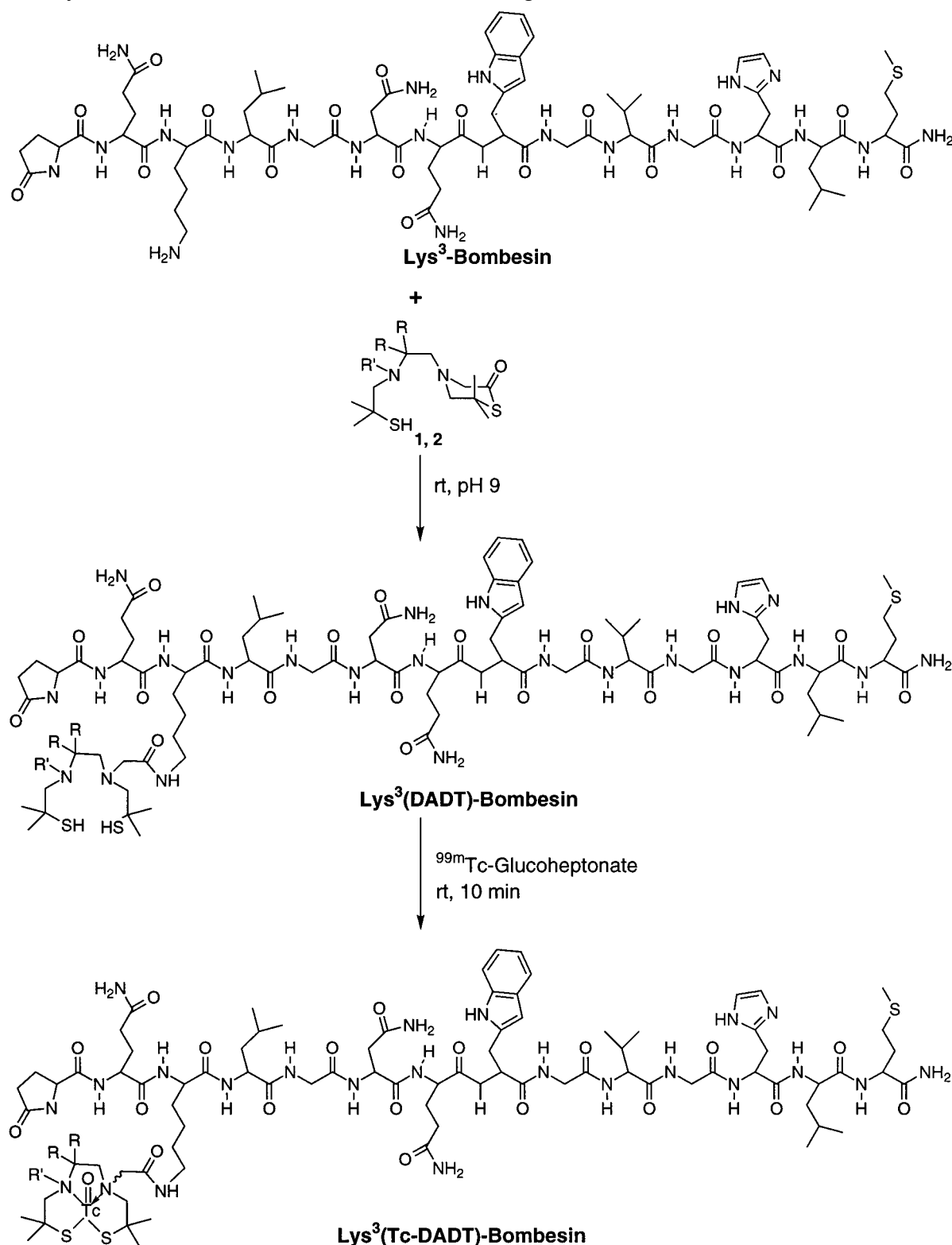
Preparation of the BCA **2** (31) adduct was similarly performed by incubating a mixture of **2** (20.0 mg, 66 μmol) and Pyr-Gln-Lys-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH $_2$ (10.0 mg, 6.3 μmol) in a borate buffer (0.7 mL, 0.1 M, pH 9) containing acetonitrile (0.9 mL) for 2.5 h at room temperature. Using the same isolation procedure as above gave the product Pyr-Gln-Lys(Hx-DADT)-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH $_2$ [Lys ^{3} (Hx-DADT)-bombesin] with a 26.7 min retention time and a 52% (6.2 mg) isolated yield. High-resolution MALDI MS: calcd MW for Lys ^{3} (Hx-DADT)-bombesin 1894.97, found $[\text{M} + \text{Na}]^+$ 1917.95.

$^{99\text{m}}\text{Tc}$ Labeling of the Lys ^{3} (DADT)-Bombesin Adducts. $^{99\text{m}}\text{Tc}$ labeling of Lys ^{3} (Pm-DADT)-bombesin and Lys ^{3} (Hx-DADT)-bombesin was performed as depicted in Scheme 1. A glucoheptonate kit (Glucoscan) was reconstituted with water (1.0 mL). From this solution, [$^{99\text{m}}\text{Tc}$]glucoheptonate was prepared by addition of an aliquot (200 μL) to a [$^{99\text{m}}\text{Tc}$]pertechnetate solution (400 μL , 8 mCi) from the generator and vortexed for 1 min. The [$^{99\text{m}}\text{Tc}$]glucoheptonate solution (300 μL) was then added to a solution of the Lys ^{3} (DADT)-bombesin (0.5 μmol) in water (500 μL) and the mixture vortexed for 1 min and then incubated at room temperature for 10 min. The reactions were followed by reversed phase HPLC. Two products were obtained from either adduct. The total yield of products in either case was >90%.

The HPLC conditions for the isolation of the products from Lys ^{3} (Pm-DADT)-bombesin were a linear gradient from solvent A (15%)/solvent B (85%) to solvent A (50%)/solvent B (50%) over the course of 70 min at a flow rate of 4 mL/min using a C-18 Novapak cartridge (8×100 mm, 4 μm , Waters Chromatography Division) monitored on-line for UV absorption at 220 nm and scintillation for radioactivity. These products were designated Lys ^{3} ($^{99\text{m}}\text{Tc}$ -Pm-DADT)-bombesin-1 and Lys ^{3} ($^{99\text{m}}\text{Tc}$ -Pm-DADT)-bombesin-2 with retention times of 41.3 and 44.5 min, respectively, and a ratio of Lys ^{3} ($^{99\text{m}}\text{Tc}$ -Pm-DADT)-bombesin-1:Lys ^{3} ($^{99\text{m}}\text{Tc}$ -Pm-DADT)-bombesin-2 of 3:1.

To isolate the products obtained from Lys ^{3} (Hx-DADT)-bombesin, the HPLC conditions were solvent A (34%)/solvent B (66%) using the same column as above at a flow rate of 4 mL/min. The products were designated Lys ^{3} ($^{99\text{m}}\text{Tc}$ -Hx-DADT)-bombesin-1 and Lys ^{3} ($^{99\text{m}}\text{Tc}$ -Hx-DADT)-bombesin-2 with retention times of 11.3 and 13.5 min, respectively, and a ratio of Lys ^{3} ($^{99\text{m}}\text{Tc}$ -Hx-DADT)-bombesin-1:Lys ^{3} ($^{99\text{m}}\text{Tc}$ -Hx-DADT)-bombesin-2 of 1:9.

Preparation of the ^{99}Tc Analogues of the Lys ^{3} (DADT)-Bombesin Adducts. The general method for the preparation of the ^{99}Tc analogues was as follows. To a solution of Lys ^{3} (Pm-DADT)-bombesin or Lys ^{3} (Hx-

Scheme 1. Synthesis of Technetium Diaminedithiolate Analogues of Bombesin

1, R = H, R' = CH₃; **2**, R = CH₃, R' = H

DADT)-bombesin (0.9 μ mol) in methanol (850 μ L) and water (200 μ L) was added a solution of [⁹⁹Tc]glucoheptonate (32) in water (45 μ L, 20 mM), and the mixture was stirred at room temperature for 1.0 h. Similar products were obtained as described above for the ^{99m}Tc analogues. The overall yield of products was 48–69%. The products were isolated by reversed phase HPLC

using the same conditions above for the separation of the ^{99m}Tc analogues. The product ratios were as follows: 2.2:1 Lys³(^{99m}Tc-Pm-DADT)-bombesin-1:Lys³(⁹⁹Tc-Pm-DADT)-bombesin-2 and 1:2.3 Lys³(⁹⁹Tc-Hx-DADT)-bombesin-1:Lys³(⁹⁹Tc-Hx-DADT)-bombesin-2. High-resolution MALDI MS: calcd MW for Lys³(⁹⁹Tc-Pm-DADT)-bombesin-1 1993.94, found [M]⁺ 1993.86; calcd

MW for Lys³(⁹⁹Tc-Pm-DADT)-bombesin-2 1993.94, found [M]⁺ 1993.82; calcd MW for Lys³(⁹⁹Tc-Hx-DADT)-bombesin-1 2006.94, found [M + Na]⁺ 2029.85; calcd MW for Lys³(⁹⁹Tc-Hx-DADT)-bombesin-2 2006.94, found [M + Na]⁺ 2029.85.

In Vitro Binding Assay of the Bombesin Analogues. In vitro binding assays were performed using an adaptation of the method of Moody et al. (33). Briefly, adult male Sprague-Dawley rats were decapitated, the brains dissected on ice, and the medulla/pons removed. The rest of the brain was weighed and homogenized in 50 volumes of Tris-HCl buffer (50 mM, pH 7.4) at 4 °C with a Brinkmann Polytron instrument (setting 5, 15 s). The homogenate was centrifuged at 30000g for 15 min and the resulting pellet resuspended in 10 volumes of Tris-HCl buffer (50 mM, pH 7.4, 4 °C) containing NaCl (100 mM) and incubated at 4 °C for 60 min. The homogenate was centrifuged again at 30000g for 15 min. The resulting pellet was resuspended in 10 volumes of the Tris-HCl buffer (50 mM, pH 7.4). The protein concentration of the membrane suspension was determined using the Pierce (Rockford, IL) BCA Protein Reagent Kit and aliquoted for storage at -70 °C at a concentration of 5 mg protein/mL.

For the receptor binding assay, 400 µg of the membrane protein was incubated for 25 min with 0.25 nM [¹²⁵I-Tyr⁴]bombesin at 4 °C in a total volume of 125 µL of assay buffer in the presence or absence of 0.01–1000 nM bombesin, Lys³-bombesin, Lys³(Pm-DADT)-bombesin, Lys³(Hx-DADT)-bombesin, or the ⁹⁹Tc-labeled bombesin analogues or 1–10⁶ nM somatostatin. The assay buffer (50 mM Tris-HCl at pH 7.4) contained 1 mg/mL BSA and 2 µg/mL bacitracin. The incubation was terminated by addition of 5 mL of ice cold Tris-HCl (50 mM, pH 7.4) containing 1 mg/mL BSA followed by rapid filtration through Whatman GF/B glass fiber filters presoaked for 20 min in Tris-HCl buffer (50 mM, pH 7.4) at 4 °C. The filters were washed twice more with 5 mL aliquots of Tris-HCl buffer (50 mM, pH 7.4) at 4 °C and then counted in an automated scintillation γ-counter. Nonspecific binding was determined in the presence of 1 µM bombesin. The inhibition constants (K_i) were calculated using the EBDA/LIGAND computer programs (35).

Biodistribution of the Lys³(⁹⁹mTc-DADT)-Bombesin Analogues in Normal Mice. The HPLC-purified Lys³(⁹⁹mTc-DADT)-bombesin analogues were isolated from the respective HPLC eluates by solid phase extraction using a C-18 Sep-Pak cartridge. The Sep-Pak cartridge was first washed with ethanol (5 mL) followed by a solution of ammonium acetate (0.05 M, 5 mL). The HPLC eluate containing the Lys³(⁹⁹mTc-DADT)-bombesin analogue was diluted five times with ammonium acetate (0.05 M) and then passed through the Sep-Pak cartridge. The Sep-Pak cartridge was then washed with an ammonium acetate solution (0.05 M, 5 mL). The Lys³(⁹⁹mTc-DADT)-bombesin analogues were then eluted with ethanol. The ethanol solution was diluted with physiologic saline. The final injectate contained 2.5% ethanol. CD-1 mice were injected via the tail vein with the HPLC-purified Lys³(⁹⁹mTc-DADT)-bombesin analogues (2 µCi, 0.2 mL per mouse). At 15 min and 3 h postinjection, four mice for each time point per tracer were euthanized by cervical dislocation. Major organs were dissected, weighed, and counted in an automated scintillation γ-counter with aliquots of the injectate (as standards) counted at the same time.

RESULTS AND DISCUSSION

The development of biomarkers for detection of cancer is important in patient management. Because they are

rapidly metabolized in blood, autocrine growth peptides such as bombesin are not suitable as biomarkers for detection. The receptors, on the other hand, could be used as biomarkers for detection if suitable tracers could be developed for identifying them in vivo. Toward this end, bombesin/GRP receptors on cancer cells may be important for early diagnosis of certain cancers. For example, in a study by Preston et al. (36), only 1 of 23 stomach mucosa samples obtained from areas uninvolved with cancer in gastric cancer patients showed high-affinity binding sites for bombesin/GRP. This patient was the only one who had Menetrier's disease, a mucosa disorder known to be premalignant. In gastric (36) as well as other cancers such as prostate (34) and small cell lung cancers (37, 38), moderate to very high receptor densities are present on the cells. This would make the system amenable to good target to nontarget differentiation that can be taken advantage of in both scintigraphic imaging and therapy.

For in vivo scintigraphic studies by planar imaging or single photon emission computed tomography (SPECT), ^{99m}Tc is the best radionuclide by virtue of its short 6.02 h half-life, 140 keV photons, a high photon yield per disintegration unaccompanied by significant particulate radiation, a relatively low dose to patients, ready availability in generator form, and low cost. In order to derivatize small biologically active peptides with the metal, a chelating ligand (39) or a specific sequence of amino acids usually including one or more cysteine residues (40) has to be introduced into the peptide to carry the metal. Care has to be taken so that the Tc chelate does not interfere with the pharmacophore of the substrate so as to preserve its biological activity. Of chelating systems available for Tc, the diaminedithiol ligand forms one of the most stable complexes (41–44). To facilitate the incorporation of Tc into biologically active compounds using the DADT ligand system, we developed the two thiolactone derivatives **1** and **2** (Figure 1) (30, 31).

We have evaluated the feasibility of applying the DADT ligand in the form of the two bifunctional chelates as the carrier of the Tc in the derivatization of Lys³-bombesin (Figure 2), a potent bombesin agonist. This analogue of bombesin contained a Lys residue at amino acid position 3 in place of Arg in natural bombesin (Figure 2). In bombesin analogues, the binding site resides in the C-terminal octapeptide sequence (23, 45). Because the Lys residue is further from the binding site, we hypothesized that a Tc chelate attached to the ε-NH₂ group of Lys³ would lead to an analogue that still maintained high-affinity binding to the receptor. Coupling of the BCAs to Lys³-bombesin was performed at pH 9 using a BCA:Lys³-bombesin ratio of 10:1 (Scheme 1). After incubation at room temperature, the excess BCA was removed by extraction with ether and the Lys³-(DADT)-bombesin adduct purified by semipreparative reversed phase HPLC. No side chain protection was necessary since the indole and imidazole groups of Trp and His, respectively, are weak nucleophiles under the coupling conditions. The purified adducts were characterized by mass spectroscopy.

We have prepared both no-carrier-added (^{99m}Tc) and milligram quantities (⁹⁹Tc) of the Tc-derivatized bombesin analogues [Lys³(Tc-DADT)-bombesin]. The compounds were prepared in ligand exchange reactions by incubation of a mixture of the Lys³(DADT)-bombesin adducts with [^{99m}Tc]- or [⁹⁹Tc]glucoheptonate (prepared from glucoheptonate, [^{99m}Tc]- or [⁹⁹Tc]pertechnetate, and stannous ion), respectively, at room temperature (Scheme 1).

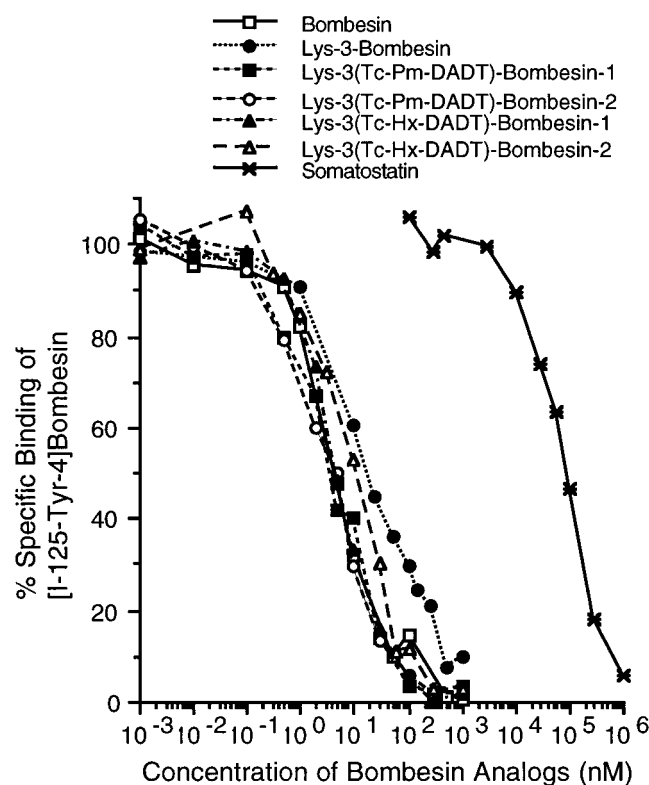


Figure 3. Inhibition of [^{125}I -Tyr ^{4}]bombesin binding to the bombesin receptor in rat brain membranes by bombesin analogues.

Table 1. Results of in Vitro Binding Studies of the ^{99}Tc Bombesin Analogues to Rat Brain Membranes

compound	K_i^a (nM)
bombesin	4.3 ± 1.0
Lys ^{3} -bombesin	21.4 ± 5.9
Lys ^{3} (Pm-DADT)-bombesin	24.2 ± 12.3
Lys ^{3} (Hx-DADT)-bombesin	17.9 ± 8.1
Lys $^3(^{99}\text{Tc-Pm-DADT})$ -bombesin-1	3.5 ± 0.7
Lys $^3(^{99}\text{Tc-Pm-DADT})$ -bombesin-2	3.9 ± 1.5
Lys $^3(^{99}\text{Tc-Hx-DADT})$ -bombesin-1	5.2 ± 1.5
Lys $^3(^{99}\text{Tc-Hx-DADT})$ -bombesin-2	7.4 ± 2.0
somatostatin	69500 ± 11200

^a Average $K_i \pm \text{SEM}$ ($n = 3$).

Lys ^{3} -bombesin or the adducts Lys ^{3} (Pm-DADT)-bombesin and Lys ^{3} (Hx-DADT)-bombesin. The affinities of the Lys $^3(^{99}\text{Tc-DADT})$ -bombesin analogues were comparable to that of bombesin ($K_i = 4.3 \pm 1.0$ nM) itself.

BCAs 1 and 2 were designed to affect the biodistribution of the Tc complexes of the resulting adducts as a result of the charge carried by the Tc complex. Tc-DADT complexes contain a $[\text{Tc}(\text{V})\text{-oxo}]^{3+}$ core. Complexes in which at least one of the coordinating nitrogens of the DADT ligand is a secondary amine such as adducts from BCA 2 have neutral complex cores because complexation of Tc results in deprotonation from the two thiols as well as a secondary amine nitrogen (30, 41–44, 46–48). Therefore, Lys ^{3} (Tc-Hx-DADT)-bombesin-1 and Lys ^{3} (Tc-Hx-DADT)-bombesin-2 which are derived from BCA 2 both have neutral Tc complex cores. When both coordinating nitrogens of the ligand are tertiary amines, as is the case for adducts from BCA 1, deprotonation from nitrogen is not possible. Therefore, complexes derived from BCA 1 adducts have a positively charged chelate core (31, 49). These differences in the charge of the Tc complex core are reflected in the biodistribution of the ^{99}Tc analogues (Figure 4A,B). Thus, Lys $^3(^{99}\text{Tc-Hx-}$

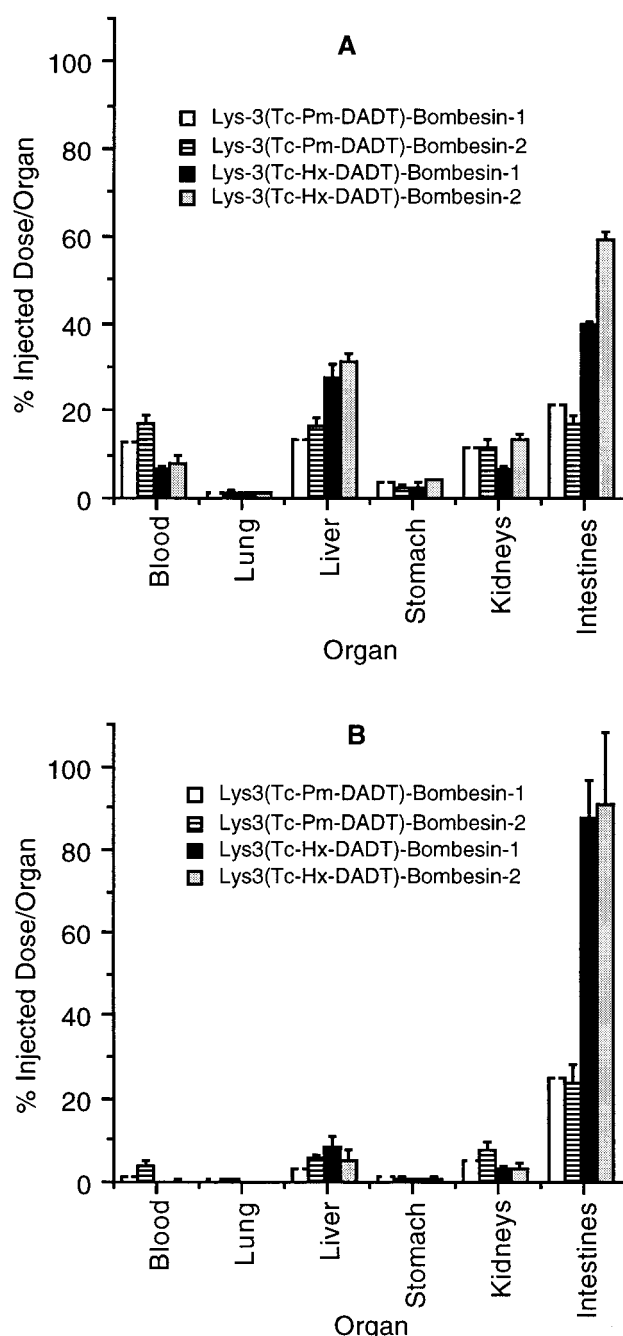


Figure 4. Biodistribution of the $^{99\text{m}}\text{Tc}$ -labeled bombesin analogues in normal mice 15 min postinjection (A) and 3 h postinjection (B).

DADT)-bombesin-1 and Lys $^3(^{99}\text{Tc-Hx-DADT})$ -bombesin-2 which are more lipophilic in character showed about 4-fold greater accumulation in the intestines than the Lys $^3(^{99}\text{Tc-Pm-DADT})$ -bombesin-1 and Lys $^3(^{99}\text{Tc-Pm-DADT})$ -bombesin-2 analogues which are derived from BCA 1 and therefore contain positively charged chelate cores. The ^{99}Tc -labeled analogues derived from BCA 1 would therefore be more suitable for imaging in the abdominal area.

The ^{99}Tc -labeled bombesin analogues described above have a potential for use in the in vivo biochemical characterization of bombesin/GRP receptors by scintigraphy. Their application in cancer may allow non-invasive identification of patients that would benefit from therapies based on the disruption of the bombesin/GRP growth promotion pathway as well as early cancer

detection. Use of the agents in this manner would greatly impact patient management. DADT ligands are also capable of forming highly stable complexes with rhenium, the congener of Tc. Therefore, the ^{186}Re and ^{188}Re analogues of the $^{99\text{m}}\text{Tc}$ -labeled bombesin compounds above would be directly applicable for the therapy of many cancers.

ACKNOWLEDGMENT

This work was supported by U.S. Public Health Service Grants CA-32845 and CA-019199.

LITERATURE CITED

- Anastasi, A., Ersparmer, V., and Bucci, M. (1973) Isolation and amino acid sequences of slytesin and bombesin: Two analogous active tetradecapeptides from skin of European discoglossid frogs. *Arch. Biochem. Biophys.* 148, 443–446.
- McDonald, T. J., Jornvall, J., Nilsson, G., Vagne, M., Ghatei, M., Bloom, S. R., and Mutt, V. (1979) Characterization of a gastrin releasing peptide from porcine non-antral gastric tissue. *Biochem. Biophys. Res. Commun.* 90, 227–233.
- Minamino, N., Kangawa, K., and Matsuo, H. (1983) Neuropeptide, B: A novel bombesin-like peptide identified in porcine spinal cord. *Biochem. Biophys. Res. Commun.* 114, 541–548.
- Alexander, R. W., Upp, J. R., Jr., Poston, G. J., Gupta, V., Townsend, C. M., Jr., and Thompson, J. C. (1988) Effects of bombesin on growth of human small cell lung carcinoma in vivo. *Cancer Res.* 48, 1439–1441.
- Moody, T. W., Carney, D. N., Cuttita, F., Quattrocchi, K., and Minna, J. D. (1985) High affinity receptors for bombesin/GRP-like peptides on human small cell lung cancer. *Life Sci.* 37, 105–113.
- Moody, T. W., Mahmoud, S., Staley, J. S., Naldini, L., Cirillo, D., South, V., Felder, S., and Kris, R. (1989) Human glioblastoma cell lines have neuropeptide receptors for bombesin/GRP. *J. Mol. Neurosci.* 1, 235–242.
- Thomas, F., Arvelo, F., Antoine, E., Jacrot, M., and Poupon, M. F. (1992) Antitumoral activity of bombesin analogues on small cell lung cancer xenografts: Relationship with bombesin receptor expression. *Cancer Res.* 52, 4872–4877.
- Farre, A., Ishizuka, J., Gomez, G., Parekh, D., Koo, J. Y., Townsend, C. M., and Thompson, J. C. (1993) Bombesin stimulates growth of colon cancer in mice and decreases their survival. *Surg. Oncol.* 2, 169–173.
- Qin, Y., Ertl, T., Cai, R. Z., Halmos, G., and Schally, A. V. (1994) Inhibitory effect of bombesin receptor antagonist RC-3095 on the growth of human pancreatic cancer cells in vivo and in vitro. *Cancer Res.* 54, 1035–1041.
- Qin, Y., Halmos, G., Cai, R. Z., Szoke, B., Ertl, T., and Schally, A. V. (1994) Bombesin antagonists inhibit in vitro and in vivo growth of human gastric cancer and binding of bombesin to its receptors. *J. Cancer Res. Clin. Oncol.* 120, 519–528.
- Pinski, J., Halmos, G., Yano, T., Szepeshazi, K., Qin, Y., Ertl, J., and Schally, A. V. (1994) Inhibition of growth of MKN human gastric adenocarcinoma xenografts in nude mice by treatment with gastrin releasing peptide antagonist (RC-3095) and somatostatin analogue RC-160. *Int. J. Cancer* 57, 54–58.
- Pinski, J., Schally, A. V., Halmos, G., Yano, T., Szepeshazi, K., and Groot, K. (1994) Somatostatin analogues and bombesin/gastrin releasing peptide antagonist RC-3095 inhibit the growth of glioblastomas in vitro and in vivo. *Cancer Res.* 54, 5895–5901.
- Yano, T., Pinski, J., Szepeshazi, K., Groot, K., and Schally, A. V. (1992) Stimulation by bombesin and inhibition by bombesin/gastrin releasing peptide antagonist RC-3095 of growth of human breast cancer cell lines. *Cancer Res.* 52, 4545–4547.
- Yano, T., Pinski, J., Szepeshazi, K., Halmos, G., Radulovic, S., Groot, K., and Schally, A. V. (1994) Inhibitory effect of bombesin/gastrin releasing peptide antagonist RC-3095 and lutenizing hormone-releasing hormone antagonist SB-75 on the growth of MCF-7 MIII human breast xenografts in athymic nude mice. *Cancer* 73, 1229–1238.
- Bold, R. J., Lowry, P. S., Ishizuka, J., Battey, J. F., Townsend, C. M., Jr., and Thompson, J. C. (1994) Bombesin stimulates the in vitro growth of a human gastric cancer cell line. *J. Cell. Physiol.* 161, 519–525.
- Radulovic, S., Miller, G., and Schally, A. V. (1991) Inhibition of growth of HT-29 human colon cancer xenografts in nude mice by treatment with bombesin/gastrin releasing peptide antagonist (RC-3095). *Cancer Res.* 51, 6006–6009.
- Radulovic, S., Schally, A. V., Reile, H., Halmos, G., Szepeshazi, K., Groot, K., Milovanovic, S., Miller, G., and Yano, T. (1994) Inhibitory effects of antagonists of bombesin/gastrin releasing peptide (GRP) and somatostatin analogue (RC-160) on growth of HT-29 human colon cancers in nude mice. *Acta Oncol.* 33, 693–701.
- Moody, T. W., Murphy, S., Mahmoud, S., and Fiskum, G. (1987) Bombesin like peptides elevate cytosolic calcium levels in small cell lung cancer cells. *Biochem. Biophys. Res. Commun.* 147, 189–195.
- Draoui, M., Chung, P., Park, M., Birrer, M., Jakowlew, S., and Moody, T. W. (1995) Bombesin stimulates c-fos and c-jun mRNA in small cell lung cancer cells. *Peptides* 16, 289–292.
- Kelley, M. J., Linnoila, R. I., Avis, I. L., Georgiadis, M. S., Cuttitta, F., Mulshine, J. L., and Johnson, B. E. (1997) Antitumor activity of a monoclonal antibody directed against gastrin-releasing peptide in patients with small cell lung cancer. *Chest* 112, 256–261.
- Jensen, R. T., Jones, S. W., Folkers, K., and Gardner, J. D. (1984) A synthetic peptide that is a bombesin receptor antagonist. *Nature* 309, 61–63.
- Woll, P. J., and Rozengurt, E. (1988) [D-Arg¹,D-Phe⁵,D-Trp^{7,9},Leu¹¹]Substance P, a potent bombesin antagonist in murine Swiss 3T3 cells, inhibits the growth of human small cell lung cancer in vitro. *Proc. Natl. Acad. Sci. U.S.A.* 85, 1859–1863.
- Camble, R., Cotton, R., Dutta, A. S., Garner, A., Hayward, C. F., Moore, V. E., and Scholes, P. B. (1989) *iso*-Butyryl-His-Trp-Ala-Val-D-Ala-His-Leu-NH-CH₃ (ICI-216140). A potent in vivo antagonist of bombesin/gastrin releasing peptide (BN/GRP) derived from the C-terminal sequence lacking the final methionine residue. *Life Sci.* 45, 1521–1527.
- Radulovic, S., Cai, R. Z., Serfozo, P., Groot, K., Redding, T. W., Pinski, J., and Schally, A. V. (1991) Biological effects and receptor binding affinities of new pseudonapeptide bombesin/GRP receptor antagonists with N-terminal D-Trp or Tpi. *Int. J. Pept. Res.* 38, 593–600.
- Singh, P., Gao, Y. S., Kull, F. C., Jr., and Leban, J. J. (1992) A novel bombesin receptor antagonist, potentially inhibits bombesin evoked release of gastrointestinal hormones from rats and dogs in vitro and in vivo. *Regul. Pept.* 40, 75–86.
- Szepeshazi, K., Schally, A. V., Cai, R. Z., Radulovic, S., Milovanovic, S., and Szoke, B. (1991) Inhibitory effect of bombesin/gastrin releasing peptide antagonist RC-3095 and high dose somatostatin analogue RC-160 on nitrosamine-induced pancreatic cancers in hamsters. *Cancer Res.* 51, 5980–5986.
- Szepeshazi, K., Schally, A. V., Halmos, G., Groot, K., and Radulovic, S. (1992) Growth inhibition of estrogen dependent and estrogen-independent MTX mammary cancers in mice by the bombesin and gastrin releasing peptide antagonist RC-3095. *J. Natl. Cancer Inst.* 84, 1915–1921.
- Moody, T. W., Venugopal, R., Zia, R., Patierno, S., Leban, J. J., and McDermid, J. (1995) BW2258U89: A GRP antagonist which inhibits small cell lung cancer. *Life Sci.* 56, 521–529.
- Li, N., Hoffman, T. J., Sieckman, G. L., Ochrymowycz, L. A., Higginbotham, C., Strutman, M., Volkert, W. A., and Ketring, A. R. (1996) In vitro and in vivo characterization of a Rh-105-Tetrathiamacrocycle conjugate of a bombesin analogue. *J. Nucl. Med.* 37 (Suppl.), 61P.
- Baidoo, K. E., and Lever, S. Z. (1990) Synthesis of a diaminedithiol bifunctional chelating agent for the incorporation of technetium-99m into biomolecules. *Bioconjugate Chem.* 1, 132–137.

- (31) Baidoo, K. E., Lever, S. Z., and Scheffel, U. (1994) A bifunctional chelator for facile preparation of neutral technetium complexes. *Bioconjugate Chem.* 5, 114–118.
- (32) de Kieviet, W. (1981) Technetium Radiopharmaceuticals: Chemical characterization and tissue distribution of Tc-glucosheptonate using Tc-99m and Tc-99. *J. Nucl. Med.* 22, 703–709.
- (33) Moody, T. W., Pert, C. B., Rivier, J., and Brown, M. R. (1978) Bombesin: Specific binding to rat brain membranes. *Proc. Natl. Acad. Sci. U.S.A.* 75, 5372–5376.
- (34) Reile, H., Armatis, P. E., and Schally, A. V. (1994) Characterization of high-affinity receptors for bombesin/gastrin releasing peptide on the human prostate cancer cell lines PC-3 and DU-145: Internalization of receptor bound ¹²⁵I-(Tyr⁴)-Bombesin by tumor cells. *Prostate* 25, 29–38.
- (35) McPherson, G. A. (1985) Analysis of radioligand binding experiments. A collection of computer programs for the IBM PC. *J. Pharmacol. Methods* 14, 213–228.
- (36) Preston, S. R., Woodhouse, L. F., Jones-Blackett, S., Wyatt, J. I., and Primose, J. N. (1993) High affinity binding sites for gastrin releasing peptides on human gastric cancer and Menetrier's disease. *Cancer Res.* 53, 5090–5092.
- (37) Sunday, M. E., Kaplan, L. M., Motoyama, E., Chin, W. W., and Spindel, E. R. (1988) Biology of disease: Gastrin-releasing peptide (mammalian bombesin) gene expression in health and disease. *Lab. Invest.* 59, 5–24.
- (38) Moody, T. W., and Cuttitta, F. (1993) Growth factor and peptide receptors in small cell lung cancer. *Life Sci.* 52, 1161–1173.
- (39) Sundberg, M. W., Meares, C. F., Goodwin, D. A., and Diamanti, C. I. (1974) Chelating agents for the binding of metal ions to macromolecules. *Nature* 250, 587–588.
- (40) Pearson, D. A., Lister-Jones, J., McBride, W. J., Wilson, D. M., Martel, L. J., Civitello, E. R., Taylor, J. E., Moyer, B. R., and Dean, R. T. (1996) Somatostatin receptor-binding peptides labeled with technetium-99m: Chemistry and initial biological studies. *J. Med. Chem.* 39, 1361–1371.
- (41) Epps, L. A. (1984) The chemistry of neutral lipid soluble technetium (V) complexes of aminoalcohols and aminothiols. Ph.D. Thesis, The Johns Hopkins University, Baltimore, MD.
- (42) Lever, S. Z., Burns, H. D., Kervitsky, T. M., Goldfarb, H. W., Woo, D. V., Wong, D. F., Epps, L. A., Kramer, A. V., and Wagner, H. N., Jr. (1985) Design, preparation, and biodistribution of a technetium-99m triaminedithiol complex to assess regional cerebral blood flow. *J. Nucl. Med.* 26, 1287–1294.
- (43) Kung, H. F., Molnar, M., Billings, J., Wicks, R., and Blau, M. (1984) Synthesis and biodistribution of neutral, lipid soluble Tc-99m complexes that cross the blood-brain barrier. *J. Nucl. Med.* 25, 326–332.
- (44) Kung, H. F., Liu, B.-L., Wei, Y., and Pan, S. (1990) A study of the structure-stability relationship of technetium compounds. *Appl. Radiat. Isot.* 41, 773–781.
- (45) Knight, M., Pineda, J. D., Burke, T. R., Jr., Cohen, S. N., Mahmoud, S., and Moody, T. W. (1990) Design of cyclic bombesin analogue. In *Peptides: Chemistry, Structure and Biology* (J. Rivier and G. Marshal, Eds.) pp 185–187, ESCOM, Leiden, The Netherlands.
- (46) Epps, L. A., Burns, H. D., Lever, S. Z., Goldfarb, H., and Wagner, H. N., Jr. (1987) Brain imaging agents: Synthesis and characterization of (N-piperidinyethyl)-hexamethyl diamino-dithiolate oxo technetium(V) Complexes. *Appl. Radiat. Isot.* 38, 361–364.
- (47) Kung, H. F., Guo, Y.-Z., Yu, C.-C., Billings, J., Subramanyam, V., and Calabresse, J. (1989) New brain perfusion agents based on the Tc-99m bis-aminoethanethiol (BAT) complexes: stereoisomers and biodistribution. *J. Med. Chem.* 32, 433–437.
- (48) Lever, S. Z., Baidoo, K. E., and Mahmood, A. (1990) Structure Proof of Syn/Anti Isomerism in N-Alkylated Diaminedithiol (DADT) Complexes of Technetium. *Inorg. Chem. Acta* 176, 183–184.
- (49) Faggiani, R., Lock, C. J. L., Epps, L. A., Kramer, A. V., Brune, D., and Burns, H. D. (1988) (3,6-Dimethyl-3,6-diaza-octan-1,8-dithiolato-S¹, N³, N⁶, S⁸) oxotechnetium(V) pertechnetate. *Acta Crystallogr. C* 44, 777–779.

BC9701959

Structure Characterization of Functional Histidine Residues and Carbethoxylated Derivatives in Peptides and Proteins by Mass Spectrometry

Markus Kalkum,[†] Michael Przybylski, and Michael O. Glocker*

Faculty of Chemistry, University of Konstanz, P.O. Box M 732, D-78457 Konstanz, Germany.
Received September 11, 1997; Revised Manuscript Received December 6, 1997

We developed a mass spectrometric method to precisely characterize the structures of the diethyl pyrocarbonate (DEP)-modified amino acid derivatives in intact peptides and proteins. Using acetate-buffered solutions for modification reactions improved the yields of DEP modification. UV quantification of carbethoxylation of angiotensin II was consistent with the degree of mass spectrometrically determined modification. Unequivocal identification of the modification sites in carbethoxylated angiotensin II derivatives was achieved by HPLC separation and mass spectrometric sequencing. With increasing concentrations of DEP, a gradual increase of carbethoxy groups, comprising biscarbethoxylation products, was detected in angiotensin II and in insulin. When using a high molar excess of DEP, histidine carbethoxylation was found together with modifications at α -amino groups and tyrosine residues. The sites of carbethoxylation in insulin were identified by MALDI-MS—peptide mapping analyses of the tryptic digestion mixtures from the nonreduced insulin derivatives and after reduction of disulfide bonds, demonstrating that histidine carbethoxylation was sufficiently stable during disulfide bond reduction and tryptic digestion at pH 7.5. The mass spectrometric identification of mono- and biscarbethoxylated histidine residues in insulin is in agreement with surface accessibilities of imidazolyl nitrogen atoms and seems to reflect the microenvironment of the protein tertiary structure. Thus, mass spectrometric peptide mapping analyses of carbethoxylated protein derivatives allowed both the simultaneous identification of histidine carbethoxylation in the presence of other modified groups and the detection of different chemical behavior of histidine residues by the unambiguous identification of mono- and bismodifications.

INTRODUCTION

Histidine residues are known to be involved in a variety of functions in different proteins such as proton donors and/or acceptors in the catalytic triade of serine proteases, or as metal chelating agents for the central ions of the heme groups, e.g. in myoglobin or in hemoglobin (Creighton, 1993). Functional protein domains and amino acid residues can be detected either by a variety of DNA cloning approaches, including deletion studies, gene fusion, and site-specific mutagenesis (Kornberg and Baker, 1992), or, alternatively, by selective chemical modification of particular amino acid residues in the “wild-type” proteins (Glazer, 1976).

The combination of limited, protein tertiary structure-selective chemical modification and mass spectrometric analyses has been developed as a new approach for both the molecular characterization of the selectivity and the determination of relative reactivities at specific modification sites (Glocker et al., 1994; Suckau et al., 1992). In the general analytical scheme, the precise number of modifications introduced and their distribution in partially modified proteins are first determined by direct mass spectrometric molecular weight analyses. Assignments of the reactivities of modification sites are then

derived from peptide mapping data using mass spectrometry (Przybylski et al., 1993). This method has been successfully employed in proteins, as well as in small peptides, with several specific modification reactions such as amino acylation, tyrosine iodination and nitration, carboxylate amidation, and the bifunctional cysteine modification by arsonous acid derivatives (Glocker et al., 1994, 1996; Przybylski, 1995; Kussmann and Przybylski, 1995; Happersberger et al., 1996; Akashi et al., 1997; Przybylski et al., 1996). Most recently, direct evidence for the conservation of tertiary structures upon selective chemical modification has been obtained for partially acylated derivatives of the ion channel protein porin from *Rhodobacter capsulatus* by mass spectrometric and X-ray crystallographic characterization (Przybylski et al., 1996).

Diethyl pyrocarbonate (DEP)¹ is a reagent commonly used for the specific modification of histidine residues in proteins (Miles, 1977). DEP has been used to demonstrate the importance of histidine residues in enzymes

* To whom correspondence should be addressed: Faculty of Chemistry, University of Konstanz, P.O. Box M 732, D-78457 Konstanz, Germany. Phone: +49-7531-882690. Fax: +49-7531-883097. E-mail: Michael.Glocker@Uni-Konstanz.de.

[†] Present address: Max-Planck-Institut für molekulare Genetik, Ihnestr. 73, D-14195 Berlin, Germany.

¹ Abbreviations: CEt-His, monocarbethoxyhistidine derivative; FCEt-His, biscarbethoxylated, ring-opened (formyl-CEt) derivative (*N,N'*-dicarbethoxy-*N*-formyl-1,2-diaminoethene derivative); UCET-His, biscarbethoxylated, deformylated (urethane-CEt) derivative (*N,N'*-dicarbethoxy-1,2-diaminoethene derivative); HPLC, high-performance liquid chromatography; ESI-MS, electrospray ionization mass spectrometry; MALDI-MS, matrix-assisted laser desorption mass spectrometry; FAB-MS, fast atom bombardment mass spectrometry; PSD, post source decay; LC-ESI-MS/MS, liquid chromatography-ESI tandem mass spectrometry; DEP, diethyl pyrocarbonate; HCCA, α -cyano-4-hydroxycinnamic acid; E:S, enzyme:substrate ratio.

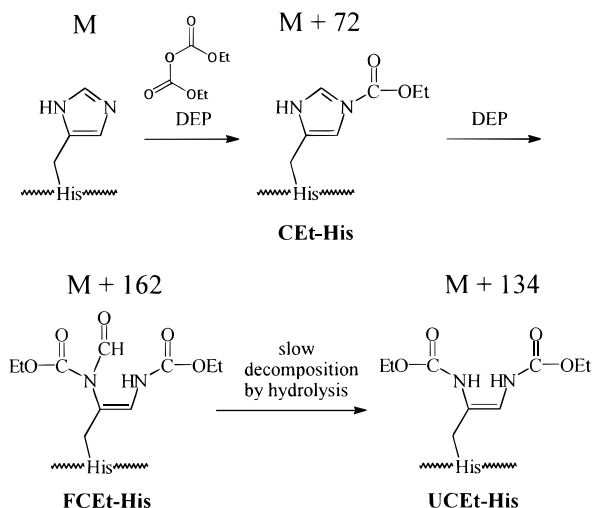


Figure 1. Pathway of the reaction of histidine imidazolyl groups with an excess of DEP. The molecular mass of the unmodified protein (M) and the additional mass increment (in daltons) caused by the carbethoxylation are indicated for the monomodification (CEt-His) and the bismodification products (FCeT-His or UCeT-His). Constitutional isomers of either CEt-His or FCeT-His carrying the carbethoxy group or the formyl on the opposite ring nitrogen atom are not distinguished by mass spectrometric molecular mass determinations and were not added to this scheme: CEt-His, monocarbethoxyhistidine derivative; FCeT-His, biscarbethoxylated, ring-opened (formyl-CEt) derivative; and UCeT-His, biscarbethoxylated, deformylated (urethane-CEt) derivative.

(Gomi and Fujioka, 1983; Vangrýsperre et al., 1988, 1989; Li et al., 1993), transport systems (Garcia et al., 1982), muscle proteins (Hegyi et al., 1974), and ligand-receptor interactions (Nishino et al., 1980; Glocker et al., 1996). However, in most of these studies, structures of modified histidine residues were not determined but were based on model studies with amino acid derivatives (Mühlrad et al., 1967), and reaction pathways from imidazole derivatives were adopted (Miles, 1977; Avaeva and Krasnova, 1975; Grace et al., 1980). Further, analyses of concurrent side reactions of DEP with other nucleophilic amino acid residues and of histidine bismodification (FCeT-His or UCeT-His) rather than selective monomodification (CEt-His) were frequently lacking in the protein work but were described to occur quite readily with histidine residues and in tri- to pentapeptides (Foti et al., 1991). Recent FAB-MS investigations with deuterium exchange reactions of carbethoxylated imidazole (Kalkum et al., 1995) support the reaction pathway (Figure 1) by which mono- and bis-modified histidine residues are also formed in proteins. The mono- and biscarbethoxylated products lead to different mass adducts so that double mono-modifications ($2 \times$ CEt) can easily be distinguished from bismodification (FCeT or UCeT) by mass spectrometry.

In this report, we describe improved reaction conditions resulting in high yields of carbethoxylated polypeptides and compare UV quantification of the carbethoxylation reaction with the degree of mass spectrometrically determined modification in peptides and proteins. We describe the mass spectrometric characterization and identification of (i) concurrent histidine mono- and bis-carbethoxylated products and (ii) the simultaneous identification of histidine carbethoxylation in the presence of other carbethoxylated amino acid residues in angiotensin II and in insulin. Angiotensin II and insulin, two well-characterized polypeptides, were chosen as models, as in both polypeptides histidine residues are crucial for

bioactivity (Spear et al., 1990; Bovy et al., 1990; Casaretto et al., 1987; Fischer et al., 1985; Nakagawa and Tager, 1986).

MATERIALS AND METHODS

All substances were of the highest grade available. Water from a Millipore water purification system (MilliQ UV Plus) was used for the preparation of aqueous solutions described below.

UV Monitoring of the Carbethoxylation Reaction with Imidazole and Histidine Residues in Angiotensin II Using Diethyl Pyrocarbonate. DEP (2.5 μ mol, Aldrich; from a 1 M stock solution in dried acetonitrile) was added to imidazole (1.24 μ mol, Aldrich), dissolved in either 5 mL of 50 mM potassium phosphate or 50 mM ammonium acetate buffer (pH 6.8). UV spectra of carbethoxylated reaction products were recorded against imidazole solutions as a reference in the range of 200–260 nm with a UV/vis spectrophotometer (UV1202, Shimadzu) at several time points until a maximum absorbance at 230 nm was reached (after 40 min). The pH in the solutions remained constant during the reactions. A molar extinction coefficient for $\lambda = 230$ nm of $2940 \text{ cm}^{-1} \text{ M}^{-1}$ was determined at pH 6.8, which is in good agreement with the molar extinction coefficient of $3000 \text{ cm}^{-1} \text{ M}^{-1}$ at pH 6 (Melchior and Fahrney, 1970). DEP modification of angiotensin II was performed in ammonium acetate buffer. DEP (1 M solution in dried acetonitrile) was added to angiotensin II (0.24 mM solution in 50 mM ammonium acetate at pH 6.8) to yield a molar ratio of 4:1. The time course of the reaction was followed by recording UV spectra in the range of 230–300 nm. The maximum absorbance was observed at 240 nm which is consistent with previous observations (Ovadi and Keleti, 1969), and a molar extinction coefficient for histidine residues from proteins of $3200 \text{ cm}^{-1} \text{ M}^{-1}$ ($\lambda = 240$ nm, pH 6) was used for calculation of the degree of modification.

DEP Modification of Angiotensin II and Insulin for MS Investigations. DEP solutions in dried acetonitrile (0.01 or 1.0 M) were added to aliquots of angiotensin II and insulin solutions in 50 mM ammonium acetate (pH 6.8) to obtain different molar ratios of DEP to polypeptide (0.5:1 to 1000:1) in the reaction mixtures. The protein concentration was 1 $\mu\text{g}/\mu\text{L}$ for angiotensin II and 2.4 $\mu\text{g}/\mu\text{L}$ for insulin. The final concentration of DEP in the reaction mixtures varied from 2 to 10 mM. After the modification reaction (1 h), the angiotensin II samples were used directly for MS analyses and insulin samples were purified from unreacted DEP by ultrafiltration through a microcon 3 microconcentrator (Amicon, MW exclusion of 3000 Da). Retentates were washed three times with 100 μL aliquots of 50 mM ammonium acetate (pH 6.8). All retentates were collected and diluted with 100 μL of 25 mM ammonium acetate.

Tryptic Digestion of Carbethoxylated Insulin Derivatives and Reduction of Disulfide Bonds. Aliquots of 60 μL of purified DEP-modified insulin derivatives and of unmodified insulin (2.2 $\mu\text{g}/\mu\text{L}$) in 50 mM ammonium acetate (pH 6.8) were mixed with 1.4 μL of a trypsin solution in 0.1 N HCl (1 $\mu\text{g}/\mu\text{L}$, trypsin from bovine pancreas, TPCK-treated; Sigma), resulting in an E:S of 1:94. The pH of each digestion mixture was adjusted to 7.5 with 0.1 M ammonium hydrogen carbonate. After 6 h of incubation at 37 $^{\circ}\text{C}$ on a thermomixer (Eppendorf), aliquots were taken for immediate MALDI-MS analyses (1.5 μL). Reduction of disulfide bonds was performed by addition of 10 μL of a 0.1 M β -mercapto-

ethanol (β -ME) solution dissolved in 25 mM ammonium hydrogen carbonate to aliquots of the tryptic peptide mixtures (10 μ L) at 37 °C for 20 min. The molar excess of the reducing agent was approximately 7-fold over thiol groups from insulin.

FAB-MS of Carbethoxylated Angiotensin II. FAB-MS spectra were obtained with a Finnigan MAT 312/AMD 5000 magnetic sector field mass spectrometer (Niehr-Johnson geometry) operated at an acceleration voltage of 3 kV for an extended mass range up to m/z 3500. Spectra were recorded with a scan rate of 14 s/decade, and resolution was set to 2500. Primary ions were produced from a 20 kV Cs⁺ thermoion source. The detector voltage was 2.4 kV. Glycerol was used as a matrix. CsI/glycerol cluster ions were used for calibration in the mass range from m/z 100 to 2500. Solutions containing 20 μ g of carbethoxylated angiotensin II were mixed with 1 μ L of the matrix and concentrated to the matrix volume by evaporation of the solvent.

ESI-MS Analyses of Carbethoxylated Angiotensin II and Insulin. ESI-MS was performed with a Vestec-201A quadrupole mass spectrometer (Vestec, Houston, TX) equipped with a "thermally-assisted" electrospray interface. The spray interface temperature was approximately 40 °C for all measurements. The mass analyzer with a nominal m/z range of 2000 was operated at unit resolution. An electrospray voltage at the tip of the stainless steel capillary needle of 2–2.2 kV and repeller voltages of typically 10–60 V were employed. Spectra were recorded with a scan rate of 7 s/scan with a mass window of m/z 200–2000. Mass calibration was performed with the 8+, 9+, and 10+ charged ions of hen egg white lysozyme, and raw data were analyzed using a Teknivent Vector2 data system (Teknivent, Houston, TX). Protein and peptide solutions were pumped with a Harvard microinfusion pump (Harvard) through a fused silica capillary (inside diameter, 75 μ m) with a flow rate of 2 μ L/min into the ion source. Samples were diluted to a final concentration of 0.1 mg/mL with 2% acetic acid/methanol (9:1, v/v) at pH 3.

On-Line LC-ESI-MS/MS of Carbethoxylated Angiotensin II. The microbore HPLC system consisted of an Applied Biosystems (Foster City, CA) 140B solvent delivery system equipped with an Applied Biosystems 759A absorbance detector which was set at 215 nm. The DEP-modified angiotensin II mixtures (5 μ L aliquots) were separated using a microbore C18 column (Spherisorb, 100 $\times$ 1 mm, 3 μ m). Samples were injected using a Rheodyne (Cotati, CA) injection port (model 8125) equipped with a 5 μ L sample loop. Solvent A was 0.1% TFA in H₂O, and solvent B was 0.07% TFA in acetonitrile. The flow rate was adjusted to 40 μ L/min. After sample injection, the solvent mixture was kept constant at 5% B for 5 min and then was raised to 70% B over a time period of 60 min. On-line ESI-MS was performed on a Perkin-Elmer Sciex API-III triple quadrupole mass spectrometer (Thornhill, ON) in the single quadrupole scanning mode, and ESI-MS/MS was carried out using argon as the collision gas.

MALDI-MS Analyses of Carbethoxylated Angiotensin II and Insulin. MALDI-MS analyses were carried out with a Bruker Biflex linear time-of-flight spectrometer (Bruker-Franzen Analytik GmbH, Bremen, Germany), equipped with a UV nitrogen laser (337 nm) and a dual microchannel plate detector. For the molecular mass determinations, the acceleration voltage was set to 25 kV and spectra were calibrated with insulin, cytochrome c, or myoglobin as internal and external standards. For peptide mapping experiments, the ac-

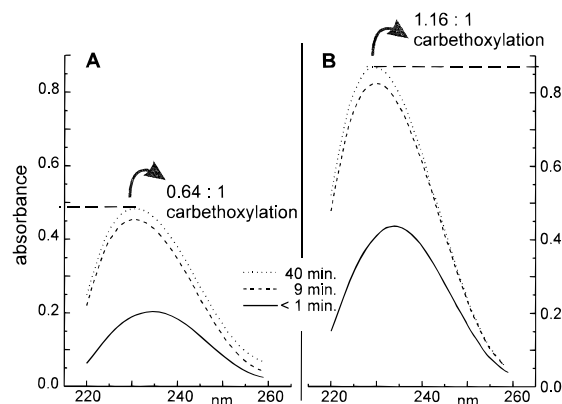


Figure 2. UV spectra of imidazole carbethoxylation. (A) Reaction in 50 mM phosphate buffer at pH 6.8. (B) Reaction in acetate buffer at pH 6.8. Spectra were recorded after reaction time intervals as indicated. A molar extinction coefficient of 2940 cm⁻¹ M⁻¹ (λ = 230 nm) was used for calculating the amount of carbethoxylated imidazole. Ratios of carbethoxylated products: educts are given.

celeration voltage was set to 10 kV and insulin and neurotensin were used for internal and external mass calibration. Samples (1.5 μ L) were diluted 10-fold with CH₃CH/0.1% TFA (2:1, v/v), and 1 μ L of this solution was mixed with 1 μ L of a saturated HCCA solution [dissolved in CH₃CH/0.1% TFA (2:1, v/v)]. Spectra were recorded after evaporation of the solvent and processed using the X-MASS data system.

PSD-MALDI-MS of Carbethoxylated Angiotensin II. All spectra were obtained using the α -cyano-4-hydroxycinnamic acid matrix and a MALDI-TOF instrument (REFLEX II, Bruker-Franzen Analytik GmbH) equipped with a gridless delayed extraction (GDE) ion source with visualization optics, ion selector, and reflector. Laser powers clearly beyond threshold were used to obtain PSD spectra. The acceleration voltage was 28.5 kV, and the reflector potential was reduced by ca. 25% in steps from 30 to 1 kV. Calibration was carried out using the ACTH clip 18–39. Spectra were recorded after evaporation of the solvent and processed using the X-MASS data system.

Determination of Partial Accessible Surfaces of Insulin from the X-ray Structure. The structure of the Zn-insulin dimer was examined using the corresponding X-ray coordinates (Protein Data Bank entry 5INS) that were obtained from the Brookhaven National Laboratory on a Silicon Graphics Indy workstation with the Molecular Surface Package (MSP) software (Connolly, 1983).

RESULTS

Stoichiometry and Quantification of Monocarbethoxylation at Histidine Residues in Angiotensin II. In initial experiments, we studied the reaction of imidazole with DEP in a commonly used potassium phosphate buffer system and, additionally, in ammonium acetate buffer, as the latter is readily compatible with mass spectrometric analyses. Both buffers were applied at pH 6.8 at a concentration of 50 mM. An imidazole: DEP molar ratio of 1:2 was adjusted, and UV spectra were recorded at several time points (Figure 2) until a maximum absorbance at 230 nm was reached. From the absorbance values of 0.483 in phosphate buffer and of 0.867 in acetate buffer, and the determined molar extinction coefficient of 2940 cm⁻¹ M⁻¹, stoichiometric ratios of carbethoxylated product to imidazole of 0.64:1 and 1.16:1 were calculated, respectively. Thus, by using

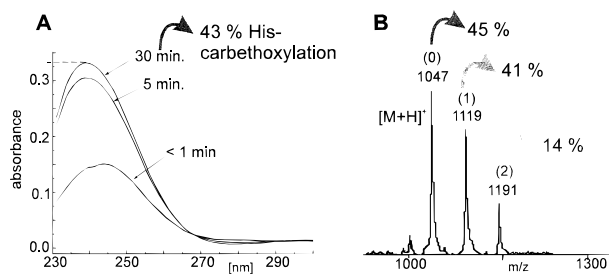


Figure 3. UV and MALDI-MS characterization of angiotensin II carboxylation. (A) UV spectra were recorded after reaction time intervals as indicated. A molar extinction coefficient of $3200 \text{ cm}^{-1} \text{ M}^{-1}$ ($\lambda = 240 \text{ nm}$) was used (Ovadi and Keleti, 1969) for calculating the amount of carboxylated angiotensin II. (B) MALDI-MS of the product mixture after 30 min of reaction time. $[M + H]^+$ ions are present for the unmodified angiotensin II, for the monocarboxylated angiotensin II derivative(s), and for the doubly monocarboxylated angiotensin II derivative. Numbers of introduced carboxy groups are given in parentheses. Spectra were recorded using HCCA as a matrix.

acetate buffer, a nearly equimolar consumption of DEP per imidazole molecules was achieved. Predominant monomodification under these conditions was ascertained by NMR analyses of reaction mixtures with equivalent stoichiometries but higher concentrations (data not shown). As quantitative reaction between imidazole and DEP was observed in acetate-buffered solutions, all carboxylation reactions with angiotensin II and insulin were carried out using this buffer system at pH 6.8.

In a first experiment, angiotensin II was carboxylated with DEP using a molar ratio of 1:4 in diluted solutions (the angiotensin II concentration was 0.24 mM), and reaction was followed spectrophotometrically. Reaction was complete after 30 min; no further increase in absorption was observed (Figure 3A). Using the molar extinction coefficient for histidine residues in proteins (Ovadi and Keleti, 1969), a conversion of 43% of histidines to carboxylated histidines was calculated. As an absorption increase at 240 nm gives information only about carboxylation of histidine imidazolyl groups, the reaction mixture was also analyzed mass spectrometrically (Figure 3B). The MALDI mass spectrum shows three signals for molecular ions at m/z 1047, 1119, and 1191. The mass differences of 72 Da are indicative of monocarboxylations (CEt); thus, the observed $[M + H]^+$ ions resemble unmodified, monocarboxylated, and doubly monocarboxylated angiotensin II, respectively. From the relative peak intensities, the respective ion abundances of 45, 41, and 14% were determined. The amount of histidine modification ($41-X\%$) cannot be determined directly by molecular weight determination of the monocarboxylated product. However, α -amino carboxylation ($X\%$) is thought to add only a minor portion ($X \leq 14\%$), since the doubly monocarboxylated derivative was found in only little amounts (14%). Thus, the determined degree of histidine carboxylation by UV (43%) and the mass spectrometrically determined degree of monocarboxylation (41%) are in good agreement.

To distinguish α -amino carboxylation from monocarboxylation at histidine, the products from the carboxylation reaction of angiotensin II were separated by HPLC (Figure 4A). For these studies, the reaction was carried out at a 4-fold higher angiotensin II concentration (0.96 mM) and a molar ratio of DEP: angiotensin II of 1:1 was chosen. Four fractions were obtained by HPLC, and the peptide derivatives were identified by on-line ESI-MS/MS analyses (Table 1).

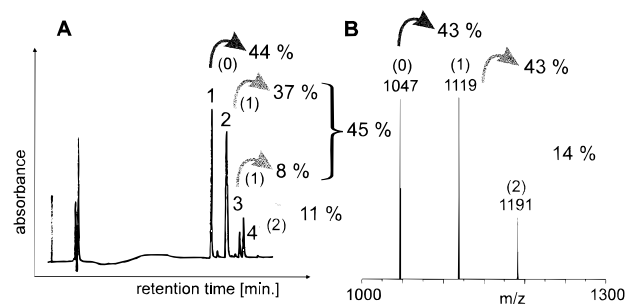


Figure 4. HPLC and ESI-MS characterization of angiotensin II carboxylation. (A) UV trace of HPLC-separated fractions from angiotensin II carboxylation monitored at 215 nm . Angiotensin II and modified derivatives were identified by on-line ESI-MS/MS analyses (Table 1). Fraction 1 contains unmodified angiotensin II, and fraction 2 contains the His monocarboxylated angiotensin II derivative; fraction 3 corresponds to the α -amino monocarboxylated angiotensin II derivative, and fraction 4 corresponds to the doubly monocarboxylated angiotensin II derivative. (B) ESI-MS of the product mixture. $[M + H]^+$ ions are present for the unmodified angiotensin II, for the monocarboxylated angiotensin II derivative(s), and for the doubly monocarboxylated angiotensin II derivative. Spectra were recorded using 2% acetic acid/methanol (9:1, v/v) at pH 3. Relative intensities of peaks are shown in percent. Numbers of introduced carboxy groups are given in parentheses.

Fraction 1 corresponds to unmodified angiotensin II and represents 44% of the products. Fraction 2 contained the monocarboxylated His derivative, accounting for 37% of the products. Fraction 3 was shown to contain the α -amino carboxylated product and represents 8% of the derivatives. This result verified the assumption that α -amino carboxylation plays a minor role and adds only little to the monocarboxylated angiotensin II derivatives. Finally, fraction 4 resembled the doubly carboxylated angiotensin II derivative (α -amino and His) and represents 11% of the products. The ESI-MS spectrum of the product mixture (Figure 4B) showed three molecular ions resembling unmodified angiotensin II (m/z 1047, 43%) and monocarboxylated (m/z 1119, 43%) and doubly monocarboxylated angiotensin II (m/z 1191, 14%). Peak heights of molecular ion signals were determined for quantification and revealed good correlation of determined degrees of modification between the two methods (Figure 4).

In our hands, DEP-modified angiotensin II derivatives, which were incubated for 48 h at 20°C in aqueous acetate buffers at pH 6–7, showed unchanged intensities of ion signals for the monocarboxylated, doubly monocarboxylated, and unmodified peptides, thus indicating that the carboxylation products were stable under these conditions, consistent with previous studies (Melchior and Fahrney, 1970; Mühlrad et al., 1967). However, at pH 8.5, DEP-modified peptides hydrolyzed to some extent, exhibiting more abundant ion signals for the unmodified rather than the DEP-modified peptides, which is also in agreement with previous results (Miles, 1977; Vangrysterpe et al., 1989).

Mass Spectrometric Identification of Histidine Biscarboxylation and Further Modification Sites in Angiotensin II. Unequivocal identification of the sites of modification in angiotensin II was obtained from the HPLC-separated carboxylated derivatives (Figure 4) by mass spectrometric sequencing using on-line LC-ESI-MS/MS. Angiotensin II and carboxylated derivatives yielded abundant b-type fragment ions (Roepstorff and Fohlmann, 1984; Biemann, 1988) covering the entire sequence (Table 1). For the α -amino carboxylated

Table 1. Identification of Modification Sites by Mass Spectrometric Sequencing of Angiotensin II and Carbethoxylated Derivatives

b-type fragment ^a	residue and/or derivative	m/z (obsd)				
		unmodified ^b	CEt-His ^b	CEt- α -amino ^b	2 $\times$ CEt ^{b,c}	3 $\times$ CEt ^c
1	CEt-D	— ^h	— ^h	— ^h	— ^h	188.2 ^e
2	D-R	272.4 ^{d-f}	272.4 ^{d-f}	— ^h	— ^h	— ^h
2	CEt-D-R	— ^h	— ^h	344.4 ^d	343.8 ^{d-f}	344.4 ^e
3	V	371.6 ^{d-f}	371.6 ^d	443.4 ^d	443.4 ^{d-f}	443.4 ^e
4	Y	534.4 ^{d-f}	534.4 ^d	606.4 ^d	606.4 ^{d-f}	— ^h
4	CEt-Y	— ^h	— ^h	— ^h	— ^h	678.3 ^e
5	I	647.6 ^{d,e,g}	647.6 ^{d,g}	719.6 ^{d,g}	719.6 ^{d,e,g}	791.2 ^e
6	H	784.8 ^{d,e,g}	— ^h	856.4 ^{d,g}	— ^h	— ^h
6	CEt-H	— ^h	856.8 ^{d,g}	— ^h	928.8 ^{d,e,g}	1000.6 ^e
molecular ion	P-F	1046.8 ^{d-f}	1118.8 ^{d,f,g}	1118.8 ^{d,f,g}	1190.8 ^{d,f,g}	1262.7 ^e

^a Nomenclature according to Roepstorff and Fohlmann (1984) and Biemann (1988). ^b A 1:1 angiotensin II:DEP molar ratio was applied. ^c A 1:10 angiotensin II:DEP molar ratio was applied. ^d Observed by LC-ESI-MS/MS; HPLC-separated fractions were analyzed (Figure 4). ^e Observed by PSD-MALDI-MS; parent ions were selected from mixtures by ion extraction (Figure 6). ^f Observed by FAB-MS of the product mixture. ^g Observed by ESI-MS skimmer fragmentation with an elevated repeller voltage (30 V) of the product mixture. ^h Not observed.

derivative (fraction 3 in Figure 4A), a characteristic b2-fragment was observed at m/z 344.4 which is 72 Da higher than that of the corresponding fragment of the unmodified angiotensin II (fraction 1 in Figure 4A, m/z 272.4) and, thus, is indicative of the CEt group attached at the N-terminal Asp residue. Similarly, His modification in the monocarbethoxylated product (fraction 2 in Figure 4A) was ascertained by the b6-fragment which was found at m/z 856.8, whereas the corresponding fragment of the unmodified angiotensin II was found at m/z 784.8. Additionally, between the His and the α -amino carbethoxylated derivative, the b5-, b4-, and b3-fragment ions displayed mass differences of 72 Da which identified the CEt groups attached to His and the α -amino group, respectively. Finally, the doubly monocarbethoxylated angiotensin II derivative (fraction 4 in Figure 4A) showed sequence fragments that were indicative of modification of both the His and the α -amino group (Table 1).

In addition to the above-described carbethoxylations of the histidine residue and the N-terminal α -amino group, a further side reaction with the tyrosine residue in position 4 was observed, when modification was carried out using a 1:10 molar ratio of angiotensin II:DEP (Figure 5A). An ion signal at m/z 1263 which is indicative of a triply monocarbethoxylated (3CEt) derivative was found under these conditions next to the monocarbethoxylated (CEt, m/z 1119) and the doubly monocarbethoxylated (2CEt, m/z 1191) derivative.

The 2CEt and the 3CEt derivatives were sequenced using PSD-MALDI-MS by extracting the precursor ions from the product mixtures prior to fragmentation (Figure 6). Specific a-type and b-type fragment ions were detected in high abundances. The mass differences of 28 Da between a-type and b-type fragment ions, leading to characteristic ion doublets that were also observed in PSD-MALDI spectra of unmodified angiotensin II, are marked by Δ . The spectrum of the doubly CEt-modified angiotensin II derivative allowed determination of the entire sequence, and carbethoxylation at His-6 was clearly identified by the mass difference of 209.2 Da between the b6- and b5-fragment ions (Figure 6A and Table 1). Carbethoxylation at Tyr-4 can be excluded in this derivative, as the mass difference between the b4- and b5-fragment ions of 163.0 Da is indicative of an unmodified Tyr residue. Further abundant ions were found at m/z 589.3, 426.5, and 326.9 which correspond to the b4-, b3-, and b2-fragment ions with an additional loss of 17 amu. The presence of y-type ions in the spectrum confirmed the carbethoxylation at His-6 (y2-

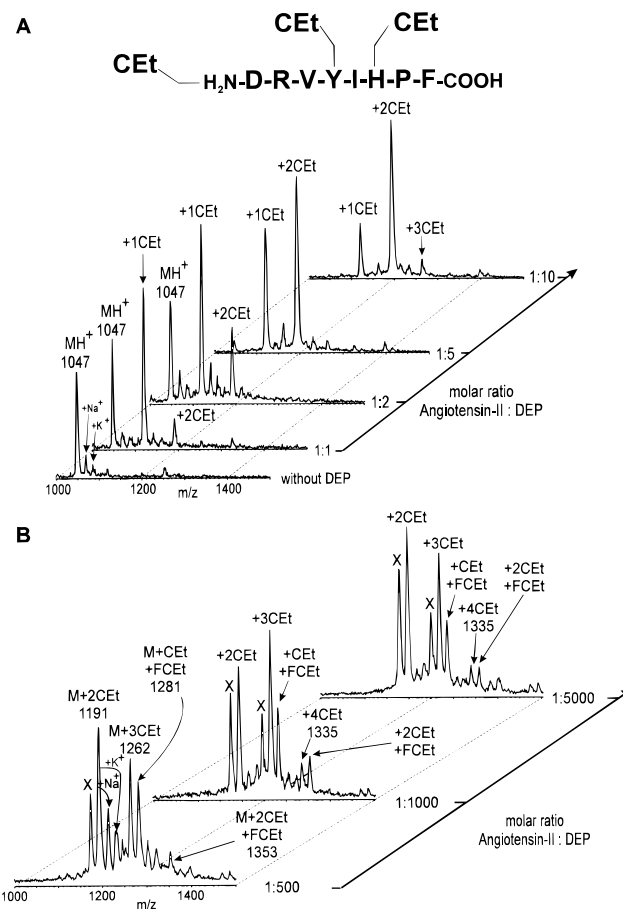


Figure 5. MALDI-MS of carbethoxylated angiotensin II using increasing molar ratios of DEP. (A) Molar ratios of angiotensin II to DEP are increasing from 1:0 up to 1:10. Monocarbethoxylation is indicated by CEt. Carbethoxylation sites are shown for the triply monocarbethoxylated angiotensin II in the sequence. (B) Molar ratios of angiotensin II:DEP are increasing from 1:500 up to 1:5000. Molecular ions due to formation of His bismodification (FCEt) are observed in addition to the monocarbethoxylation products. Signals marked with $\times$ are 18 Da lower than the corresponding $[M + H]^+$ ions. Additional minor peaks were found for sodium adducts, potassium adducts, and matrix adducts. Spectra were recorded using HCCA as a matrix.

and y3-fragment ions, Figure 6A) and also identified the second carbethoxylation site at the α -amino group of Asp-1 by the y7-fragment at m/z 1003.5. In addition to the sequence-specific ions, direct evidence for the modification sites was obtained by PSD-MALDI detection of i-type ions. The triply monocarbethoxylated angiotensin

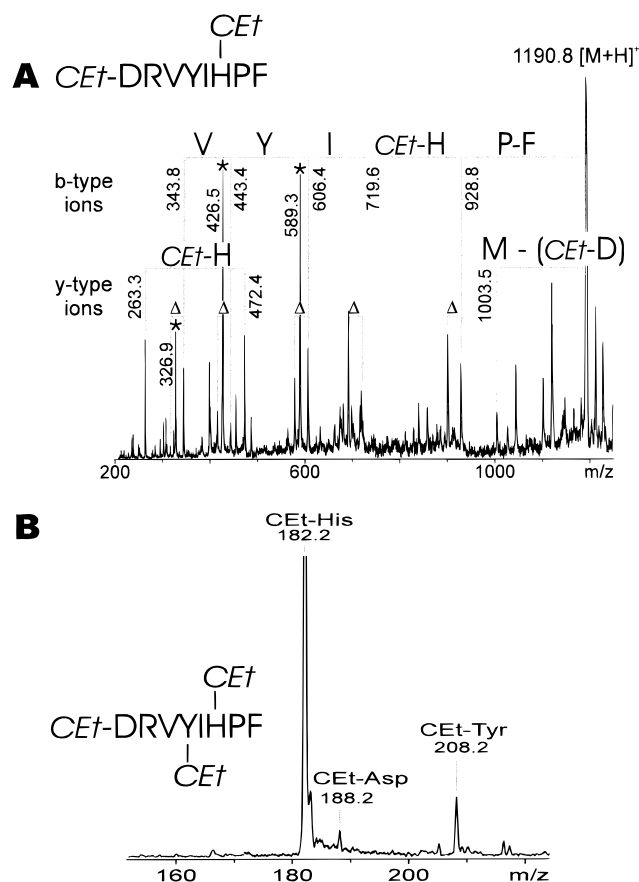


Figure 6. PSD-MALDI-MS of carbethoxylated angiotensin II. (A) The PSD-MALDI-MS spectrum of doubly monocarbethoxylated angiotensin II shows abundant a-type, b-type (top horizontal dashed line), and y-type fragment ions (bottom horizontal dashed lines) from which the sites of modification can be identified by the characteristic mass differences. The entire sequence of angiotensin II is covered, and m/z values for the most abundant fragment ions are given. The mass difference between a- and b-fragments (28 Da) is marked by Δ . Fragment ions due to additional loss of 17 amu are indicated by $*$. (B) PSD-MALDI-MS spectrum of triply monocarbethoxylated angiotensin II (i-type ion region). The signal for CEt-His is off-scale. The identified sites of modification are indicated in the sequence. Spectra were recorded using HCCA as a matrix.

II yielded i-type ions of CEt-His (m/z 182.2) and CEt-Tyr (m/z 208.2), while CEt- α -amino-Asp (m/z 188.2) was identified as a b1-fragment ion (Figure 6B). The presence of carbethoxylated i-type ions demonstrates that the modification remained stable under the MALDI-MS sequencing conditions, whereas carbethoxy groups are easily lost by using conventional peptide purification procedures for Edman sequencing (Deka et al., 1992). Characteristic fragmentation at particular amide bonds (b6- and b5-fragments, Table 1) of angiotensin II and carbethoxylated products was also observed in ESI spectra with an elevated repeller voltage (front end fragmentation) and produced additional evidence of simultaneous His monocarbethoxylation and α -amino carbethoxylation in the doubly monocarbethoxylated angiotensin II derivative.

For modification of proteins, high DEP excesses (molar ratios of approximately 1:1000 to 1:10000) usually have been chosen. MALDI mass spectra of DEP-modified angiotensin II samples revealed generally higher degrees of modification products with an increasing excess of DEP (Figure 5). When a higher excess of DEP was used for modification of angiotensin II (molar ratios of 1:500 to 1:5000), additional biscarbethoxylation of the histidine

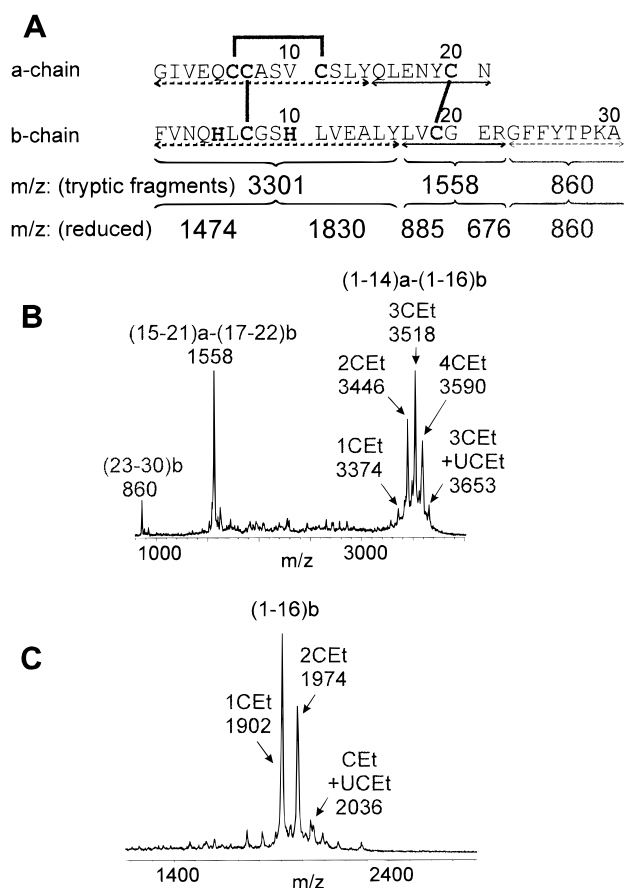
residue, leading to the ring-opened formyl-CEt derivative (FCEt), was observed (Figure 5B; cf. Figure 1). FCEt-His-modified angiotensin II derivatives were detected, together with other carbethoxylation products, at m/z 1281 and 1353, respectively. The FCEt product leads to an additional mass increment of 162 Da and is, thus, easily distinguished from double monocarbethoxylation ($\Delta m = 144$ Da) by molecular weight determination. At these high molar ratios of DEP:angiotensin II, additional, yet unidentified signals with a mass shift Δm of -18 Da were observed besides the signals of the molecular ions of highly modified angiotensin II derivatives.

Identification of Carbethoxylation Sites in Insulin, after Proteolytic Degradation, and after Disulfide Bond Reduction. DEP modification reactions of insulin were carried out using molar ratios of DEP:protein of 50:1 to 1000:1. MALDI-MS molecular weight analyses of unmodified and modified proteins gave characteristic singly and doubly charged ions, which allowed precise determinations of modification degrees. With increasing concentrations of DEP, a gradual increase of up to approximately six carbethoxy groups/insulin was detected comprising mixtures of protein derivatives that carried CEt, FCEt, and UCEt groups (data not shown). Biscarbethoxylation (FCEt and UCEt formation) is characteristic of histidine modification and is clearly identified by mass spectrometric molecular weight analyses of insulin. For the insulin derivatives obtained by carbethoxylation with a 50-fold molar excess DEP, the most abundant molecular ion was observed at m/z 6023, which corresponds to the 4CEt derivative (Table 2). In addition, a molecular ion signal at m/z 6112 was observed for the insulin derivative carrying three CEt modifications and one FCEt modification. After reduction of the disulfide bonds, three major molecular ions were detected. One signal (m/z 2414) corresponded to the a-chain, carrying one CEt group. Two signals were indicative of the b-chain derivatives at m/z 3619 (carrying three CEt groups) and at m/z 3680 (containing two CEt and one UCEt group). These results showed that the modified sites remained carbethoxylated after reduction and during mass spectrometric analyses. However, FCEt was transformed to UCEt under the slightly basic conditions (pH 7.5–8.0) necessary for reduction of disulfide bonds.

The sites of carbethoxylation were identified by MALDI-MS-peptide mapping analyses of the digestion mixtures from the nonreduced insulin derivatives after tryptic cleavage in solution (see Materials and Methods). Insulin has two cleavage sites for trypsin (Arg-22 and Lys-29 in the b-chain, Figure 7A), but additional cleavage due to intrinsic chymotryptic activity of trypsin was observed at Tyr-14 from the a-chain and at Tyr-16 from the b-chain (Figure 7B), showing that these residues were not modified. The corresponding ions of the disulfide-linked N-terminal peptide (1–14)a-S-S-(1–16)b were found modified with up to four CEt groups (m/z 3590) and with three CEt groups and one UCEt group (m/z 3653). The most abundant ion observed (m/z 3518) corresponds to this disulfide-linked peptide carrying three CEt groups. The C-terminal parts from both the a-chain and the b-chain resulted in unmodified peptide ions at m/z 1558 and 860, respectively. Hence, the mass spectrometric peptide mapping data indicated carbethoxylation at His-5, His-10, and both N termini. Under the tryptic (chymotryptic) digestion conditions applied (pH 7.5), FCEt was again transformed to UCEt and partial hydrolysis of carbethoxy groups was detected. This is consistent with previous reports (Deka et al., 1992; Hegyi et al.,

Table 2. MALDI-MS Molecular Weight Determination of Carbethoxylated Insulin^a before and after Reduction of Disulfide Bonds

protein or peptide (modified groups)	[M + H] ⁺ (calcd)	<i>m/z</i> (obsd)	highest degree of modification	most abundant modification species
insulin (2 × α-NH ₂ , 2 × His)	6023	6023	4CEt	4CEt
a-chain (1 × α-NH ₂)	6113 2413	6112 2414	3CEt + 1FCEt 1CEt	1CEt
b-chain (1 × α-NH ₂ , 2 × His)	3617 3679	3619 3680	3CEt 2CEt + UCEt	3CEt

^a Molar ratio DEP: insulin = 50:1.**Figure 7.** Structure representation and MALDI-MS of carbethoxylated insulin. (A) Schematic representation of the insulin monomer. Disulfide bonds are shown as gray bars. Tryptic fragments are indicated by underlining arrows, and calculated molecular masses of tryptic fragments are given. (B) MALDI-MS after tryptic digestion of carbethoxylated insulin at pH 7.5. Molecular ions for the tryptic peptides show multiple monocarbethoxylations (CEt) and formation of UCEt-His in the N-terminal peptide (1-14)a-(1-16)b. (C) MALDI-MS after reduction of disulfide bonds at pH 7.5. The molecular ions for the reduced tryptic peptide (1-16)b show multiple monocarbethoxylations (CEt) and formation of UCEt-His. Spectra were recorded using HCCA as a matrix.

1974; Ovadi and Keletti, 1969) in which proteolytic digestion of carbethoxylated proteins was carried out partly in the presence of a reducing agent (β -mercaptoethanol) without substantial removal of the carbethoxy groups. On the other hand, monocarbethoxylated histidines can be reversed into unmodified histidine residues by addition of excess nucleophiles, such as hydroxylamine (Deka et al., 1992; Miles, 1977). As the observed ions were not baseline-resolved, PSD-MALDI-MS sequencing of the peptide mixture was not carried out and, thus, the sites where partial losses of modifications may have

occurred were not determined. Reduction of the tryptic peptide mixture yielded mixtures of molecular ions for the modified and disulfide bond-cleaved peptides (Figure 7C). Ions for peptide (1-16)b were found at *m/z* 1902 (one CEt), at *m/z* 1974 (two CEt), and at *m/z* 2036 (one CEt and one UCEt), substantiating the fact that His residues and the N terminus were carbethoxylated.

Correlation of Chemical Reactivity with Partial Accessible Surfaces of Histidine Nitrogen Atoms in Insulin. Only one biscarbethoxylation at histidine residues was observed in insulin under the conditions employed. Hence, we assumed that the chemical reactivities of the two His residues in insulin may be different due to different surface structure features. Possible structural parameters for correlation with the relative reactivities (Glocker et al., 1994) of imidazolyl groups are relative (static) surface accessibilities (SA values) defined at a 1.4 Å van der Waals sphere of solvent and were obtained from X-ray crystallography data (Badger et al., 1991) using the insulin dimer structure (Figure 8). For monoderivatization (CEt formation) of histidine residues, only one nitrogen atom of the imidazolyl group has to be exposed on the surface, as is the case for His-5 in both b-chains of the dimer (referred to as the b- and d-chains in Table 3). While in His-5b the ND1 atom shows high solvent accessibility (total area of 11.8 Å²) and NE2 shows little total area (4.1 Å²), the situation is just the opposite for His-5d. Here the total accessible area for NE2 (10.2 Å²) is larger than that of ND1 (1.7 Å²). This suggests that the surface accessibilities of histidine nitrogen atoms in the His-5 residues resemble a "frozen" state in the insulin core structure of an otherwise flexible (but not freely rotating) histidine residue, which can be attacked by chemicals only on one side of the imidazolyl ring. Rotation may be hindered even more after carbethoxylation, and therefore, the His-5 residues may be found mostly CEt-modified. By contrast, ND1 and NE2 atoms of His-10 residues in both b-chains are equally accessible (total areas are 11.5 and 13.4 Å², respectively; Table 3). Thus, it appears likely that the observed FCEt and UCEt derivatives observed in our studies were formed by biscarbethoxylation at His-10, reflecting the chemical behavior of freely rotating histidine residues in nonstructured peptides. As His-5 and His-10 residues are close together in the sequence (Figure 8), they were not separated by proteolytic cleavage; thus, the site of the biscarbethoxylated His residue in insulin was not determined from the above-described results. However, the mass spectrometric peptide mapping analyses of carbethoxylated protein derivatives allowed the simultaneous identification of histidine carbethoxylation in the presence of other modified groups and the detection of the different chemical behavior of histidine residues, providing insight into protein surface structure features.

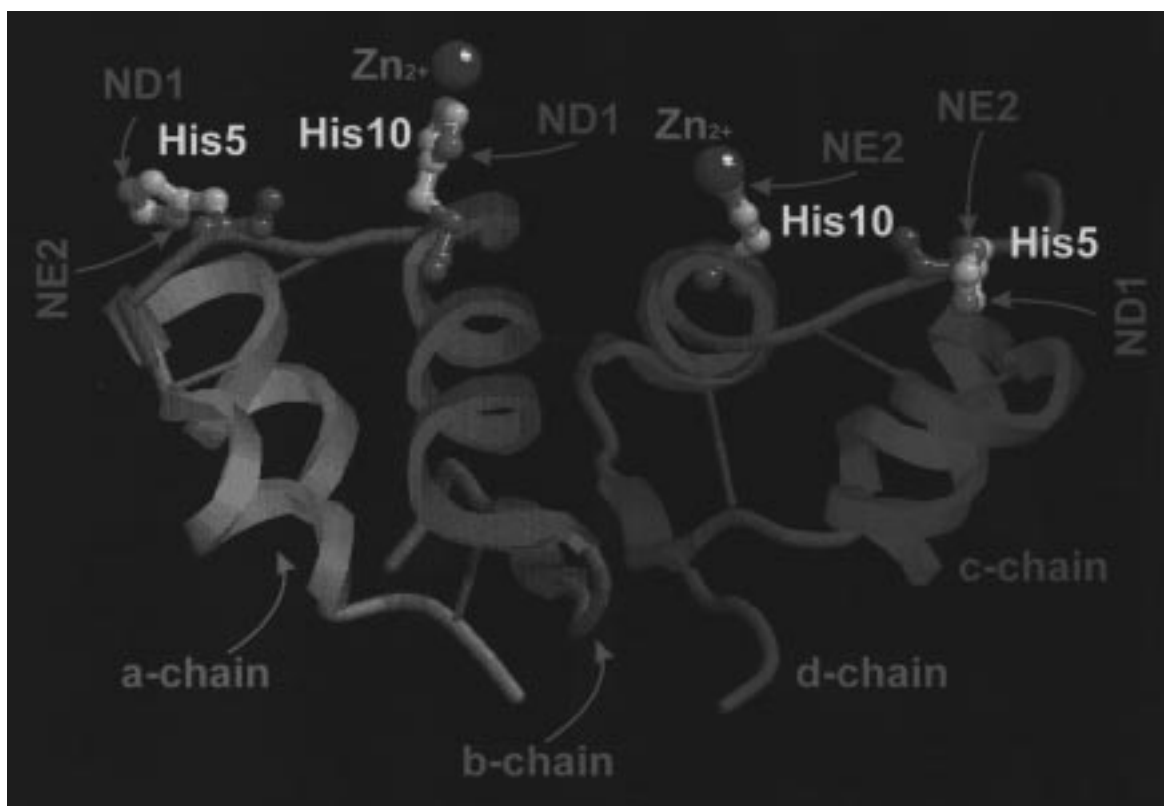


Figure 8. Schematic representation of the insulin dimer. The ribbon diagram shows the a- and b-chains and His residues are depicted as ball and stick diagrams. The NE2 atoms of His-10 are shown complexed with Zn^{2+} ions of the else freely rotatable residue. His-5 participates in the insulin core structure and shows accessible surface for either ND1 or NE2, depending on the monomer.

Table 3. Static Accessible Surface Areas of Nitrogen Atoms in Insulin His Residues^a

His residue ^b	atom ^c	contact area (Å ²)	reentrant area (Å ²)	total area (Å ²)
5b	ND1	6.4	5.4	11.8
	NE2	0.2	3.9	4.1
10b	ND1	5.2	7.8	13.0
	NE2 ^d	7.2 (0.3)	4.3 (2.3)	11.5 (2.6)
5d	ND1	0.3	1.4	1.7
	NE2	6.1	4.1	10.2
10d	ND1	5.2	8.2	13.4
	NE2 ^d	7.3 (0.5)	4.4 (1.4)	11.7 (1.9)

^a Areas are calculated from histidine residues of both monomers of the homodimeric structure. ^b Sequence positions; d represents the b-chain of the second monomer. ^c Nomenclature according to X-ray data set (Protein Data Bank entry 5INS). ^d Values in parentheses show the accessible areas in the presence of Zn^{2+} .

DISCUSSION

Angiotensin II is a linear octapeptide with a single histidine residue in position 6 that plays an important role in the regulatory angiotensin–renin system. Vasoconstrictive and blood pressure-elevating processes result upon binding of angiotensin II to its receptor. The importance of His-6 for receptor binding has been shown by using cyclized synthetic (Spear et al., 1990) and C-terminally truncated synthetic analogues (Bovy et al., 1990). Our studies show that degrees of His modification in angiotensin II, determined by UV absorption measurements, were in good agreement with modification degrees determined by MALDI-MS. Mass spectrometric determination of modification degrees with peptide mixtures of large proteins seems to have advantages over quantitation by HPLC analysis and/or amino acid analysis of separated fractions (Deka et al., 1992; Vangrýsperre et al., 1989), as complex peptide mixtures may not be

separated completely (Glocker et al., 1996) and coelution of modified and unmodified peptides may obscure quantitative results. However, MALDI-MS seems not to be generally applicable for absolute quantitation, as ion yields may be varying for different analytes. This can be overcome by using internal calibrants with similar chemical properties, providing that signal levels become independent of experimental variables such as inhomogeneous sample distribution and the dependence of laser power for analyte desorption (Tang et al., 1993). Relative quantitations show linear relationships over at least 1 order of magnitude (Nelson et al., 1994). Desorption properties of peptides may be altered upon DEP modification. Thus, to semiquantitatively analyze peptides of interest, especially for proteolytic peptide mixtures from large proteins, we suggest a comparison of the decrease of signal intensity of His-containing unmodified peptides with the ion abundance of peptides that are found unmodified throughout the course of modification experiments (Glocker et al., 1996), thereby not changing the chemical relationships, i.e. ionization efficiencies.

For protein derivatization, large excesses of DEP were usually applied but mostly reversible monomodification of histidines has been reported (Miles, 1977; Gomi and Fujioka, 1983; Garcia et al., 1982; Hegyi et al., 1974; Nishino et al., 1980). This seems in contrast to results from model peptides for which irreversible formation of UCEt and FCEt products was found (Foti et al., 1991). Since most protein modification reactions were carried out in phosphate buffers in which hydrolysis is fast, the actual excess of DEP may have been overestimated. The final degree of histidine modification depends on the total amount of reagent added and on the ratio of the rate constants between carboxylation of amino acid residues and of hydrolysis (Melchior and Fahrney, 1970). As

the rate constant for histidine modification is second-order, the initial protein concentration (i.e. the concentration of modifiable groups) plays a role in the extent of modification observed. Therefore, comparable modification degrees were obtained with angiotensin II when modification was carried out in a 1:4 molar ratio with a low angiotensin II concentration ($0.25 \mu\text{g}/\mu\text{L}$) and in a 1:1 molar ratio with a 4-fold higher angiotensin II concentration ($1 \mu\text{g}/\mu\text{L}$).

Biscarbethoxylation is indicative of histidine modification in proteins and was detected in insulin and in angiotensin II by mass spectrometry together with modification at other nucleophilic groups. This is consistent with very recent studies in which biscarbethoxylation in proteins together with monocarbethoxylation has been reported (Li et al., 1993; Glocker et al., 1996). Insulin is a very well-studied polypeptide hormone for the regulation of glucose homeostasis and is produced by the β -cells of the pancreas in response to nutritional stimuli (Lee and Pilch, 1994). Human insulin encompasses two histidine residues (His-5 and His-10) in the N-terminal part of the b-chain, and this region is necessary for receptor binding (Casaretto et al., 1987; Fischer et al., 1985; Nakagawa and Tager, 1986). In addition, His-10 was found to bind to Zn^{2+} in the crystal structure of porcine insulin (Badger et al., 1991), resulting in noncovalent dimers and hexamers which may structurally resemble the hexamer arrays in which insulin is stored in the pancreatic β -cells. For monoderivatization of histidine residues, only one nitrogen atom of the imidazolyl group needs to be exposed on the surface, as is the case for His-5 in both b-chains of the insulin dimer, as deduced from the X-ray data (Figure 8 and Table 3). By contrast, the His-10 residues of insulin appear to be more similar to histidine residues of nonstructured peptides in solution; i.e. the formation of FCET and/or UCET derivatives should occur more readily. As in our experiments, modification reactions were carried out without addition of cations, both nitrogen atoms of the imidazolyl groups of the His-10 residues should be equally accessible. Thus, it appears likely that the observed FCET and UCET insulin derivatives in our studies may be formed by biscarbethoxylation at His-10. In a recent mass spectrometric study, carbethoxylation of synthetic peptides containing His residues was carried out in the presence of metal ions (Zn^{2+} and Cu^{2+}) and revealed that His residues were partially shielded from carbethoxylation by metal complex formation (Hornshaw and Sutton, 1997). The ability of mass spectrometry to distinguish histidine residues with different chemical behaviors as shown here was also recently demonstrated in studies with a carbethoxylated cytokine and allowed us to distinguish His residues participating in the globular core structure from other His residues located in peptide-like unstructured parts of the protein (Glocker et al., 1996).

In general, chemical reactivity reflects both the protein's surface topology and the amino acid microenvironment. Hence, structural requirements by which histidine imidazolyl groups participate in intact protein core structures may lead to distinct chemical behavior. The ability of mass spectrometry to distinguish histidine residues with different chemical properties, thus, provides insight into protein structural details and structure-function relations.

ACKNOWLEDGMENT

We express our thanks to Prof. Dr. Max L. Deinzer for making his LC-ESI-MS/MS equipment available and to

Dr. Detlev Suckau for assistance with the PSD-MALDI-MS experiments. We thank Prof. Dr. Salvatore Foti for many fruitful discussions on mass spectrometry of carbethoxylated peptides. This work was supported by grants from the Deutsche Forschungsgemeinschaft and by the EU-network "Peptide and Protein Structure Elucidation by Mass Spectrometry".

LITERATURE CITED

- Akashi, S., Shirouzu, M., Terada, T., Ito, Y., Yokoyama, S., and Takio, K. (1997) Characterization of the structural difference between active and inactive forms of the Ras protein by chemical modification followed by mass spectrometric peptide mapping. *Anal. Biochem.* 248, 15–25.
- Avaeva, S. M., and Krasnova, V. J. (1975) Reaction of Diethyl Pyrocarbonate with Imidazole and with Histidine Derivatives. *Bioorg. Chem.* 1, 1151–1155.
- Badger, J., Harris, M. R., Reynolds, C. D., Evans, A. C., Dodson, E. J., Dodson, G. G., and North, A. C. T. (1991) Structure of the Pig Insulin Dimer in the Cubic Crystal. *Acta Crystallogr. B* 47, 127–136.
- Biemann, K. (1988) Contributions of Mass Spectrometry to Peptide and Protein Structure. *Biomed. Environ. Mass Spectrom.* 16, 99–111.
- Bovy, P. R., O'Neil, J. M., Olins, G. M., Patton, D. R., McMahon, E. G., Palomo, M., Koepke, J. P., Salles, K. S., Trapani, A. J., Smits, G. J., McGraw, D. E., and Hutton, W. C. (1990) Structure-Activity Relationships for the Carboxy-Terminus Truncated Analogues of Angiotensin II, a New Class of Angiotensin II Antagonists. *J. Med. Chem.* 33, 1477–1482.
- Casaretto, M., Spoden, M., Diaconescu, C., Gattner, H.-G., Zahn, H., Brandenburg, D., and Wollmer, A. (1987) Shortened Insulin with Enhanced in vitro Potency. *Biol. Chem. Hoppe-Seyler* 368, 709–716.
- Connolly, M. L. (1983) Solvent-Accessible Surfaces of Proteins and Nucleic Acids. *Science* 221, 709–713.
- Creighton, T. E. (1993) in *Proteins, Structures and molecular properties* (T. E. Creighton, Ed.) 2nd ed., Freeman and Company, New York.
- Deka, R. K., Kleanthous, C., and Coggins, J. R. (1992) Identification of the Essential Histidine Residue at the Active Site of *Escherichia coli* Dehydrogenase. *J. Biol. Chem.* 267, 22237–22242.
- Fischer, W. H., Saunders, D., Brandenburg, D., Wollmer, A., and Zahn, H. (1985) A Shortened Insulin with Full in vitro Potency. *Biol. Chem. Hoppe-Seyler* 366, 521–525.
- Foti, S., Saletti, D. M., and Petrone, G. (1991) Fast-atom Bombardment Mass Spectrometry of Peptide Derivatives with Diethylpyrocarbonate. *Rapid Commun. Mass Spectrom.* 5, 336–339.
- Garcia, M. L., Patel, L., Padan, E., and Kaback, H. R. (1982) Mechanism of Lactose Transport in *Escherichia coli* Membrane Vesicles: Evidence for the Involvement of Histidine Residue(s) in the Response of the *lac* Carrier to the Protein Electrochemical Gradient. *Biochemistry* 21, 5800–5805.
- Glazer, A. N. (1976) The Chemical Modification of Proteins by Group-Specific and Site-Specific Reagents. In *The Proteins* (H. Neurath, R. L. Hill, and C.-L. Boeder, Eds.) 3rd ed., Vol. II, pp 1–101, Academic Press, New York.
- Glocker, M. O., Borchers, C., Fiedler, W., Suckau, D., and Przybylski, M. (1994) Molecular Characterization of Surface Topology in Protein Tertiary Structures by Amino-Acylation and Mass Spectrometric Peptide Mapping. *Bioconjugate Chem.* 5, 583–590.
- Glocker, M. O., Kalkum, M., Yamamoto, R., and Schreurs, J. (1996) Selective Biochemical Modification of Functional Residues in Recombinant Human Macrophage Colony-Stimulating Factor β (rhM-CSF β); Identification by Mass Spectrometry. *Biochemistry* 35, 14625–14633.

- Gomi, T., and Fujioka, M. (1983) Evidence for an Essential Histidine Residue in S-Adenosylhomocysteinase from Rat Liver. *Biochemistry* 22, 137–143.
- Grace, M. E., Loosemore, M. J., Semmel, M. L., and Pratt, R. F. (1980) Kinetics and Mechanism of the Bamberger Cleavage of Imidazole and of Histidine Derivatives by Diethyl Pyrocarbonate. *J. Am. Chem. Soc.* 102, 6784–6789.
- Happersberger, P., Cowgill, C., Deinzer, M. L., and Glocker, M. O. (1996) Characterization of Protein-Folding Intermediates by Chemical Trapping and Mass-Spectrometry. In *Proceedings of the 44th Conference of the American Society for Mass Spectrometry*, p 1050.
- Hegyi, G., Premecz, H., Sain, B., and Mührlad, A. (1974) Selective Carbethoxylation of the Histidine Residues of Actin by Diethylpyrocarbonate. *Eur. J. Biochem.* 44, 7–12.
- Hornshaw, M. P., and Sutton, C. W. (1997) Chemical Modification of Histidine Residues and the Effect of Cu(II) Coordination Determined by MALDI-MS. In *Proceedings of the 45th Conference of the American Society for Mass Spectrometry*, p 592.
- Kalkum, M., Glocker, M. O., Jetschke, M. R., Borchers, C., Przybylski, M., Saletti, R., and Foti, S. (1995) Modification of Histidine and Arginine Residues in Peptides and Proteins with Amino acid Selective Reagents and Mass Spectrometric Characterization of the Reaction Products. *Eur. J. Clin. Chem. Clin. Biochem.* 33, A23.
- Kornberg, A., and Baker, T. A. (1992) *DNA Replication* (A. Kornberg and T. A. Baker, Eds.) Freeman and Company, New York.
- Kussmann, M., and Przybylski, M. (1995) Tertiary Structure-Selective Characterization of Protein Dithiol Groups by Phenylarsine Oxide Modification and Mass Spectrometric Peptide Mapping. *Methods Enzymol.* 251, 430–435.
- Lee, J., and Pilch, P. F. (1994) The insulin receptor: Structure, Function, and Signaling. *Am. J. Physiol.* 266, C319–C334.
- Li, C., Moore, D. S., and Rosenberg, R. C. (1993) Circular Dichroism Studies of Diethyl Pyrocarbonate-modified Histidine in Egg White Lysozyme. *J. Biol. Chem.* 268, 11090–11096.
- Melchior, W. B., Jr., and Fahrney, D. (1970) Ethoxyformylation of Proteins. Reaction of Ethoxyformyl Anhydride with α -Chymotrypsin, Pepsin, and Pancreatic Ribonuclease at pH 4. *Biochemistry* 9, 251–258.
- Miles, E. W. (1977) Modification of Histidyl Residues in Proteins by Diethylpyrocarbonate. *Methods Enzymol.* 48, 431–443.
- Mührlad, A., Hegyi, G., and Toth, G. (1967) Effect of Diethylpyrocarbonate on Proteins. *Acta Biochim. Biophys. Acad. Sci. Hung.* 2, 19–29.
- Nakagawa, S. H., and Tager, H. S. (1986) Role of the Phenylalanine B25 Side Chain in Directing Insulin Interaction with its Receptor. *J. Biol. Chem.* 261, 7332–7341.
- Nelson, R. W., McLean, M. A., and Hutchens, T. W. (1994) Quantitative Determination of Proteins by Matrix-Assisted Laser Desorption/Ionization Time-of Flight Mass Spectrometry. *Anal. Chem.* 66, 1408–1415.
- Nishino, T., Massey, V., and Williams, C. H., Jr. (1980) Chemical Modifications of D-Amino Acid Oxidase. *J. Biol. Chem.* 255, 3610–3616.
- Ovadi, J., and Keletti, T. (1969) Effect of Diethylpyrocarbonate on the Conformation and Enzymic Activity of D-Glyceraldehyde-3-phosphate Dehydrogenase. *Acta Biochim. Biophys. Acad. Sci. Hung.* 4, 365–378.
- Przybylski, M. (1995) Mass Spectrometric Approaches to the Characterization of Tertiary and Supramolecular Structures of Biomacromolecules. *Adv. Mass Spectrom.* 13, 257–283.
- Przybylski, M., Borchers, C., Suckau, D., Mák, M., and Jetschke, M. (1993) Selective Chemical Modification and Mass Spectrometric Peptide Mapping: A New Approach for the Molecular Characterization of Surface Topology and Micro-Environment in Protein Tertiary Structures. In *Peptides 1992 (Proceedings of the 22nd European Peptide Symposium)* (C. H. Schneider and A. N. Eberle, Eds.) pp 83–84, Escom Science Publishers, Amsterdam.
- Przybylski, M., Glocker, M. O., Nestel, U., Schnaible, V., Blüggel, M., Diederichs, K., Weckesser, J., Schad, M., Schmid, A., Welte, W., and Benz, R. (1996) X-Ray Crystallographic and Mass Spectrometric Structure Determination and Functional Characterization of Succinylated Porin from *Rhodobacter capsulatus*: Implications for Ion Selectivity and Single-Channel Conductance. *Protein Sci.* 5, 1477–1489.
- Roepstorff, P., and Fohlmann, J. (1984) Proposal for a Common Nomenclature for Sequence Ions in Mass Spectra of Peptides. *Biomed. Mass Spectrom.* 11, 601.
- Spear, K. L., Brown, M. S., Reinhard, E. J., McMahon, G., Olins, G. M., Palomo, M. A., and Patton, D. R. (1990) Conformational Restriction of Angiotensin II: Cyclic Analogues Having High Potency. *J. Med. Chem.* 33, 1935–1940.
- Suckau, D., Mák, M., and Przybylski, M. (1992) Protein Surface Topology-Probing by Selective Chemical Modification and Mass Spectrometric Peptide Mapping. *Proc. Natl. Acad. Sci. U.S.A.* 89, 5630–5634.
- Tang, K., Allman, S. L., Jones, R. B., and Chen, C. H. (1993) Quantitative Analysis of Biopolymers by Matrix-Assisted Laser Desorption. *Anal. Chem.* 65, 2164–2166.
- Vangrýsperre, W., Callens, M., Kersters-Hilderson, H., and DeBruyne, C. K. (1988) Evidence for an essential histidine residue in D-xylose isomerases. *Biochem. J.* 250, 153–160.
- Vangrýsperre, W., Ampe, C., Kersters-Hilderson, H., and Tempst, P. (1989) Single active-site histidine in D-xylose isomerase from *Streptomyces violaceoruber*. *Biochem. J.* 163, 195–199.

BC970162T

Molecular Characterization of a Conformational Epitope of Hen Egg White Lysozyme by Differential Chemical Modification of Immune Complexes and Mass Spectrometric Peptide Mapping

Winfried Fiedler, Christoph Borchers, Marcus Macht, Sören-Oliver Deininger, and Michael Przybylski*

Faculty of Chemistry, University of Konstanz, 78457 Konstanz, Germany. Received August 11, 1997; Revised Manuscript Received November 28, 1997

A new approach for the characterization of conformationally dependent epitope structures in protein antigens is described using differential chemical modification of immune complexes in combination with mass spectrometric peptide mapping analysis. Well-established methods for epitope characterization are frequently not applicable to conformationally dependent epitopes, and direct methods of structure analysis such as X-ray crystallography of immune complexes have been successful only in a few cases. Our approach combines tertiary structure-selective chemical modification of immune complexes with the molecular characterization of reaction products by mass spectrometric peptide mapping. The comparison of the modification pattern of free and antibody-bound antigen provides the identification of residues protected from modification by the antibody. These residues hence are characterized as part of the epitope structure. The well-characterized hen egg white lysozyme and a corresponding monoclonal IgM-type antibody were investigated as a model system. Specific modification reactions for arginine, lysine, and tyrosine residues were performed, and the modification sites in free and antibody-bound antigen were determined by mass spectrometric peptide mapping. The R14 residue and residues K13 and K96 in the antibody-bound lysozyme were found to be protected from modification, comprising a surface of spatially adjacent residues by folding of the native protein. In contrast, other K and R residues as well as Y20 and Y23 showed no significant shielding from modification in the immune complex. These results provided an estimation of the molecular epitope surface area of native lysozyme.

INTRODUCTION

Monoclonal antibodies are important tools in biochemistry, molecular biology, medical diagnostics, and immunology (1–4). Molecular structures in protein antigens recognized by antibodies (epitopes) can be classified as linear epitopes (continuous partial sequences of typically 5–10 amino acids) and epitopes dependent on the three-dimensional structure of the antigen (conformational epitopes) (5–9). Conformational epitopes occurring on the native structure of the antigen comprise a multiplicity of amino acid residues spatially assembled by the protein folding. Knowledge of epitope structures is important for predicting and identifying cross-reactivities, for the study of structure–function relationships, and for understanding molecular processes in immunology (1–3).

Several methods for the analysis of linear epitopes have been recently established such as binding analysis of synthetic partial peptides (10–12) and mass spectrometric analysis by epitope extraction or epitope excision (13–18). The characterization of conformational epitopes is considerably more difficult than the characterization of linear epitopes, and as yet, only a few methods are available. Site-specific mutagenesis approaches using binding analyses of recombinant antigen variants have been successfully employed (19, 20), and several recent

X-ray crystallographic determinations of immune complexes have provided structural details of molecular interactions (21–24). Both methods, however, comprise demanding tasks, and site-directed mutagenesis may not provide complete epitope structure data. A further approach employed previously is NMR analysis upon differential deuterium exchange of labile hydrogens in immune complexes (25); the use of synthetic peptides has been successful in a few cases. In previous publications, differential chemical modification was used as an alternative approach for the characterization of conformational epitopes (26–28). This approach applies selective chemical modification by specific reagents to intact immune complexes. As a result of the binding with the antibody, certain residues show significantly diminished reactivity in the immune complex compared to that of the free antigen as a consequence of the shielding by the antibody. However, conventional analysis of the modified antigen by proteolytic digestion and chromatographic separation has limited resolution and provides no direct molecular characterization of the epitope. In addition, this approach requires the use of radioactive reagents.

We have significantly extended this approach by using reactions specific for one type of amino acid, and the precise molecular identification of the sites of modification by mass spectrometry (29, 30). The general analytical scheme employed in this procedure is shown in Figure 1. Reactions used in this study were (i) modification of arginine residues by 1,2-cyclohexanedione (29–32), (ii) acetylation of lysine residues (30, 33, 34), and (iii) tyrosine iodination (34–36). The monoclonal antibody

* To whom correspondence should be addressed: Faculty of Chemistry, University of Konstanz, 78457 Konstanz, Germany. Fax: ++49-7531-883097. Phone: ++49-7531-882249. E-mail: Michael.Przybylski@uni-konstanz.de.

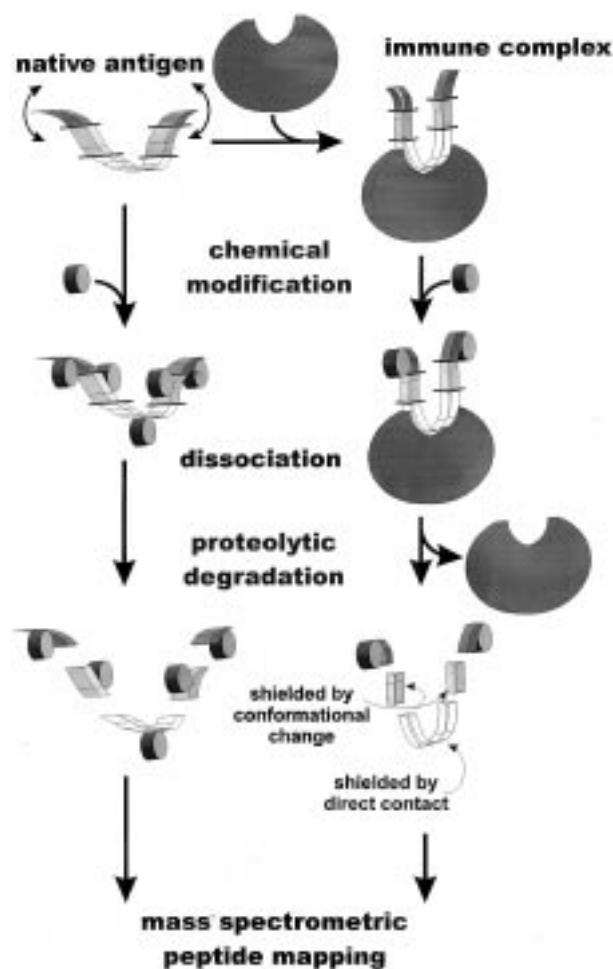


Figure 1. Analytical scheme for the characterization of conformational epitopes by differential chemical modification of antigen-antibody complexes and mass spectrometric peptide mapping. Modification groups and proteolytic cleavage sites are represented by filled circles and lines, respectively. The white areas represent shielded sequences due to direct interaction with the antibody; light gray areas represent sequences shielded by conformational effects.

employed here was produced in large amounts by cell culture techniques. Binding analysis showed its specific affinity to native HEL,¹ but no significant binding to reduced and denatured HEL which indicated a conformational epitope and native protein structure was required for recognition. This feature was confirmed in this study by the results of the differential chemical modification and mass spectrometric data.

MATERIALS AND METHODS

Materials. All chemicals were of analytical grade or the highest available purity. HEL, TPCK-treated trypsin, and α -cyano-4-hydroxycinnamic acid (HCCA) were purchased from Sigma (St. Louis, MO); cellulose nitrate was from Schleicher & Schuell (Dassel, Germany). 1,2-

Cyclohexanedione (CHD) was from Merck (Darmstadt, Germany); acetic anhydride was from Riedel-de Haën (Hannover, Germany), and iodine was from Aldrich (Milwaukee, WI). Materials and media for antibody production were purchased from Gibco (Eggenstein, Germany). NHS-activated Sepharose columns were obtained from Pharmacia (Freiburg i. Br., Germany).

Antibody Production. A monoclonal antibody (mAb-WF1; IgM) was produced by repeated immunization of Balb/c-mice with native HEL without additives. The antibody concentration in plasma was determined by ELISA. Fusion of isolated spleen cells with a myeloma cell line (X63Ag8.653) was carried out by addition of poly(ethylene glycol), and fusion cells were selected by HAT selection. Single cells of antibody-producing clones were cultivated to ensure a monoclonal cell line. Large amounts of the monoclonal antibody-producing cell line ($>2 \times 10^8$ cells) were grown by the rotating bottle technique (37).

Purification and Characterization of the Antibody. Antibody purification was carried out by affinity chromatography using a column with HEL immobilized on NHS-activated Sepharose according to the manufacturer's instructions (38). Purification was carried out by using cell culture supernatant at pH 7.5, washing with phosphate buffer at pH 7.5, and subsequent elution at pH 3–4. The purified antibody was concentrated and buffered using ultrafiltration units. The purity was assessed by SDS gel electrophoresis, and the activity was determined by ELISA. A sodium ion concentration of 100 mM was required for stable antigen-antibody binding with maximal stability of the complex at pH 7.5. Further characterization of the purified mAb-WF1 showed that it was surprisingly unstable with a complete loss of activity within 10 days. A possible explanation for the lower stability of the IgM antibody compared to IgG stabilities is its high molecular weight and supramolecular structure.

Chemical Modification of Free and Antibody-Bound Antigen. The HEL-mAb immune complex was formed by incubating 1 mg of antibody with 75 μ g of HEL in 1 mL of buffer for 1 h. A 2-fold excess of antibody binding sites was employed to prevent the presence of free antigen. Modification of R residues with 1,2-cyclohexanedione was generally carried out in 200 mM sodium borate buffer and 100 mM NaCl (pH 8). Free HEL was modified according to procedures described previously (29). The reaction was started by addition of 1.56 mmol of CHD and performed for 6 h. The reaction was quenched; the immune complex was dissociated by addition of 30 μ L of acetic acid, and the solution was stored for 12 h at 4 °C. The antibody precipitated under these conditions and was removed by centrifugation. The CHD-modified antigen was purified and isolated by ultrafiltration.

Acetylation of lysine residues was carried out in 500 mM NH_4HCO_3 and 100 mM NaCl (pH 6). The reaction was started by slow addition of acetic anhydride to the solution, and the pH due to hydrolysis of the anhydride was controlled with 1 M NaOH in an automated pH-stat (Titration Controller, Schott, Mainz, Germany). The immune complex was dissociated; the antibody was precipitated by addition of acetic acid until pH 3 was reached, and the solution was stored for 12 h at 4 °C. The precipitated antibody was centrifuged, and the modified HEL was purified by HPLC.

Modification of tyrosine residues was carried out in 100 mM NH_4OAc and 100 mM NaCl (pH 7) by addition of an iodine solution (25 mM KI and 5 mM I_2 in acetate buffer).

¹ Abbreviations: HEL, hen egg white lysozyme; MALDI-MS, matrix-assisted laser desorption/ionization mass spectrometry; ESI-MS, electrospray ionization mass spectrometry; PD-MS, plasma desorption mass spectrometry; HCCA, α -cyano-4-hydroxycinnamic acid; TFA, trifluoroacetic acid; HPLC, high-performance liquid chromatography; IgM, immunoglobulin M; mAb, monoclonal antibody; ELISA, enzyme-linked immunosorbent assay; NHS, *N*-hydroxysuccinimide; CHD, 1,2-cyclohexanedione; DHCH-R, (*N*⁷,*N*⁸-dihydroxy-1,2-cyclohexylidene)-arginine.

The mixture was stirred for 15 min. The reaction was quenched by addition of 30 μ L of acetic acid, and the solution was stored for 12 h at 4 $^{\circ}$ C. The precipitated antibody was centrifuged, and the iodinated HEL was purified by ultrafiltration.

HPLC Purification of Acetylated HEL Derivatives. HPLC purification was carried out on a Waters M510/M45 gradient instrument equipped with a 490E multiwavelength UV detector and a MAXIMA 820 data system, using a 250 $\times$ 4 mm, 6 mm C₄-RP column (Vydac, Hesperia, CA). Gradient elution was carried out with 0.04% aqueous TFA (solvent A) and 0.03% TFA in acetonitrile (solvent B) at 1.5%/min from 10% B and a flow rate of 1 mL/min. Fractions were detected at 220 nm and collected manually.

Proteolytic Digestion. Acetylated and iodinated HEL derivatives were reduced with DTT and alkylated with 4-vinylpyridine as described previously (30, 39). The reaction products were purified by HPLC (acylated derivatives) or by dialysis (iodinated derivatives). Proteolytic digestion with trypsin was carried out at pH 8 and 37 $^{\circ}$ C in the presence of 6 M urea to prevent precipitation of reduced HEL. The enzyme:substrate ratio varied from 1:10 to 1:50. Proteolytic mixtures were directly analyzed by MALDI-MS. Tryptic digestion was also carried out directly on the nitrocellulose target and analyzed by PD-MS as described previously (29, 40, 41).

Mass Spectrometry. PD-MS analysis was carried out with a BIO-ION BIN 20 TOF mass spectrometer (BIO-ION, Uppsala, Sweden) equipped with a 10 μ Ci ²⁵²Cf primary ion source. Spectra were recorded with an acceleration voltage of 17 kV and accumulation of 10⁷ single spectra. Sample preparation was carried out as previously described (16, 30, 40). MALDI-MS was performed with a Bruker Biflex linear TOF mass spectrometer equipped with a SCOUT source and video system (Bruker-Franzen, Bremen, Germany), a nitrogen UV laser (λ_{max} = 337 nm), and a dual-channel plate detector. Sample preparation was performed with 1 μ L of a freshly prepared saturated solution of HCCA in acetonitrile/H₂O (60:40) with 0.1% TFA which was mixed with 1 μ L of the peptide or protein solution. The drop was placed on the target and allowed to dry at 25 $^{\circ}$ C, and the crystals were washed several times with 2 μ L of TFA. Spectra were recorded at an acceleration voltage of 25 kV using an initial deflection pulse of 2 kV perpendicular to the ion flight path and a 1 μ s duration for deflecting matrix ions. Each spectrum was acquired by accumulating 20–100 single laser shots. Calibration was carried out using the singly and doubly protonated ions of bovine insulin as an internal standard. Electrospray ionization mass spectrometry was performed on a Vestec 201 A single quadrupole mass spectrometer (Vestec Corp., Houston, TX) with a Teknivent Vector 2 data system. A desolvation potential of 20 V at a needle voltage of 1.9–2.1 kV and a temperature of 40 $^{\circ}$ C in the spray chamber was used.

RESULTS

Molecular Weight Determination of Lysozyme Derivatives upon Differential Chemical Modification of Immune Complexes. HEL was subjected to selective chemical modification of R, K, and Y residues (i) as the free protein and (ii) as the intact immune complex bound to the antibody. After modification of the immune complexes, the antibody was generally precipitated by acidification and the purified HEL derivatives were subjected to molecular weight determination by

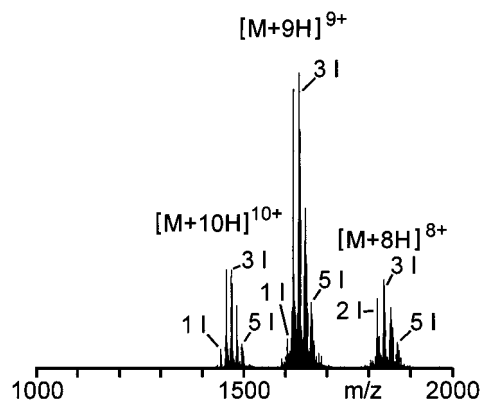


Figure 2. ESI mass spectrometric molecular weight determination of iodinated HEL upon modification of the HEL–antibody complex by I₂ (see Materials and Methods). The spectrum was obtained with a sample concentration of 0.1 μ g/ μ L (7 μ M) in 2% acetic acid containing 10% methanol.

mass spectrometry. The molecular weight determination of the iodinated HEL derivative upon modification of the antibody-bound protein with a 150-fold molar excess of iodine by ESI-MS is illustrated in Figure 2. The spectrum shows the 8–10-fold protonated molecular ions from which an extent of modification by up to five iodines was identified. The relative intensities of the molecular ions provide an estimation of an average modification extent of 2.8. Molecular weights of HEL derivatives partially modified by acetic anhydride and CHD were determined in the same manner by PD-MS and MALDI-MS, respectively.

The molecular weight increase for free and antibody-bound HEL upon iodination, acetylation, and CHD modification as a function of reaction time or molar excess of reagent is shown in Figure 3. For each reaction, the average extent of modification was directly determined from the molecular weight difference between the unmodified and modified protein. In all cases, the mass spectrometric data showed significantly lower modification of antibody-bound HEL compared to that of the free antigen (Figure 3). This result is explained well by the shielding effect of the antibody and by the lower concentration of antigen in the modification reactions with the intact immune complex.

Identification of Modification Sites in Free and Antibody-Bound HEL Derivatives. Modified and purified HEL derivatives were subjected to reductive cleavage of disulfide bonds with DTT and were then digested with trypsin under identical conditions for each protein derivative as described in Materials and Methods. The proteolytic peptide mixtures were directly analyzed by MALDI-MS and PD-MS peptide mapping. From the molecular weight determinations of peptide fragments, the modification sites were determined as described in previous studies of lysozyme derivatives (29, 36). Hence, the comparison of peptides from the free and antibody-bound proteins provided the identification of residues protected from modification by the antibody.

Lysine residues were acetylated by acetic anhydride as described previously (29, 30), with the control of the extent of modification governed by the amount of acidic anhydride present in excess. Since the reactivities of K residues have been previously shown to be primarily dependent on the relative surface accessibilities (36, 42), HEL derivatives with an average extent of modification of four acetyl groups were used to compare the modification pattern of free and antibody-bound protein. Corresponding MALDI-MS peptide mapping analyses of tryptic

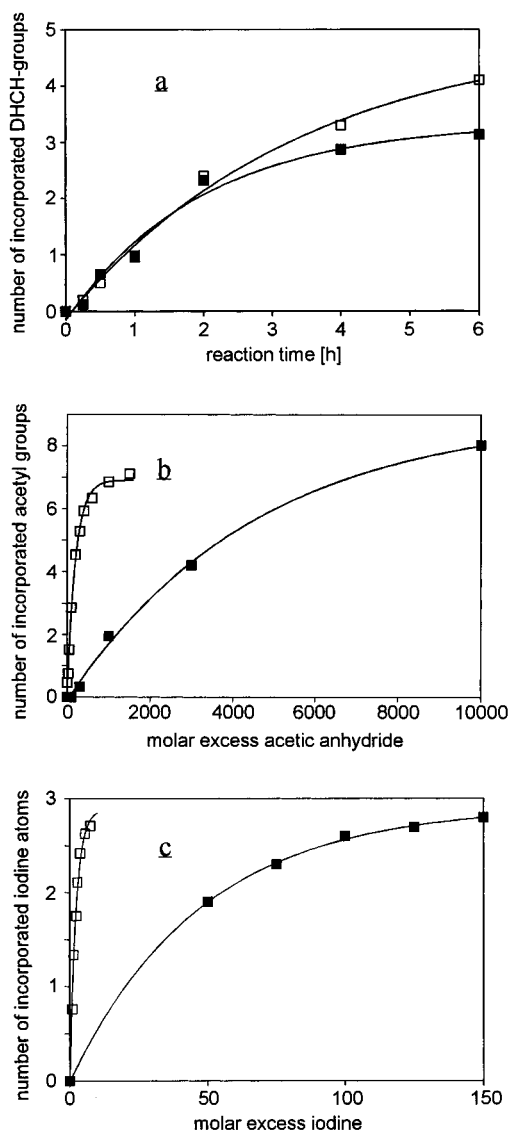


Figure 3. Molecular weight increase of HEL derivatives upon chemical modification of R, K, and Y residues as a function of reaction time or molar reagent excess. The open squares denote modification of the free protein and the filled squares the modification of the antibody-bound protein. Molecular weight determinations were performed by ESI-MS and MALDI-MS as described in Materials and Methods: (a) R modification by 1,2-cyclohexanedione, (b) K acetylation with acetic anhydride, and (c) iodination.

fragments are shown in Figure 4. In the case of the acetylated, uncomplexed HEL (Figure 4a), a partial modification of all seven amino groups (N terminus, K1, K97, K13, K33, K96, and K116) was detected by the molecular ions due to the modified peptides. In contrast, no ion signals due to acetylation at K13 and K96 were observed upon acetylation of antibody-bound HEL (indicated by arrows in Figure 4b), whereas modification of all other amino groups was found to be identical in the free protein. Furthermore, modification of the immune complex with an increasing molar excess of acetic anhydride did not yield any acylation at these lysine residues but resulted eventually in precipitation of the antibody and dissociation of the immune complex. The complete protection of these residues from acetylation of the antibody indicates their participation in the epitope structure.

Iodination of tyrosine residues was performed as described previously (35, 36), and the extent of modifica-

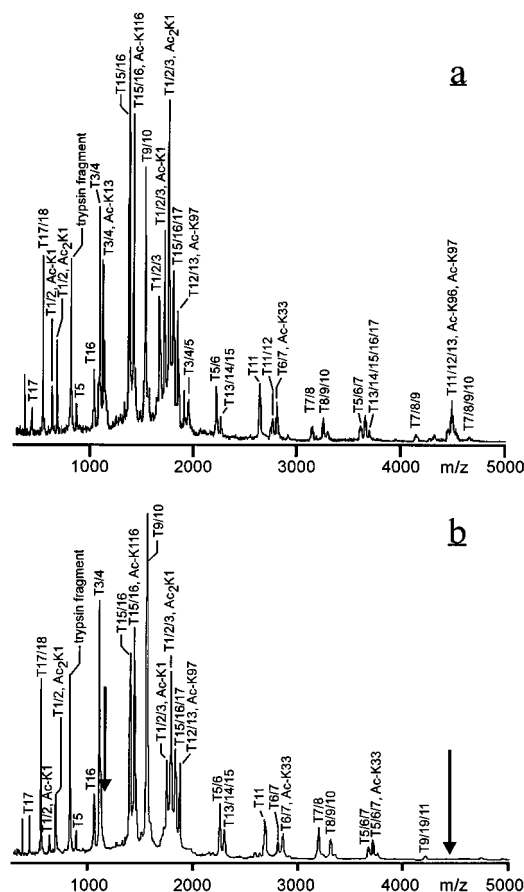


Figure 4. MALDI-MS peptide mapping analysis of tryptic peptides (T) of acetylated HEL. The numbers of fragments denote the cleavage positions as shown in Figure 6. Acetylated K residues are indicated: spectrum of (a) peptide fragments from free HEL and (b) peptide fragments from antibody-bound proteins.

tion was controlled again by the molar excess of reagent as described above. Tryptic digestion of iodinated HEL derivatives was performed with a relatively high substrate:enzyme ratio to obtain cleavage at R21 necessary to differentiate between the Y20 and Y23 residues. For comparison of the free and antibody-bound HEL, protein derivatives with an average extent of 2.8 modified residues were selected and peptide mapping analyses were performed by MALDI-MS. Both protein derivatives yielded similar spectra, and no significant differences in the modification pattern were detected (data not shown). The Y20 and Y23 residues were singly and doubly iodinated, whereas only a weak signal was identified for the iodination of Y53. These results suggested that no significant shielding of tyrosine residues was provided by the antibody and hence no participation of these residues in the epitope structure. In the case of Y53, no conclusion was possible because of its low reactivity.

The modification of arginine residues by CHD to (N^7, N^8 -dihydroxy-1,2-cyclohexylidene)arginine (DHCH-R) adducts and the selectivity and reaction mechanism have been described in detail previously (29). Under the conditions described in Materials and Methods, 4 of the 11 arginine residues were found to be modified in the uncomplexed protein. The modification of antibody-bound HEL under identical conditions provided an average extent of modification of three arginine residues, indicating selective shielding of one arginine residue by the antibody. The identification of the modified residues was performed by mass spectrometric peptide mapping

Table 1. Identification of Reactive and Shielded Residues upon Modification at Lys, Arg, and Tyr in Uncomplexed HEL and Antibody-Bound HEL

amino acid	modified residues		shielded residues
	HEL	HEL-mAb complex	
Lys ^a	1, ^d 97, 13, 33, 96, 116 ^e	1, ^d 97, 33, 116 ^e	13, 96
Arg ^b	5, 14, 73, 125 ^f	5, 125, 14	73
Tyr ^c	20, 23, 53	20, 23, (53) ^g	(53) ^g

^a Lysine acetylation with acetic anhydride. ^b Arg modification by 1,2-cyclohexanedione. ^c Tyr iodination. ^d Acetylation at the N terminus was found in uncomplexed HEL and the immune complex. ^e Relative reactivities according to surface accessibilities (28). ^f Unmodified Arg residues in HEL are Arg-21, -45, -61, -68, -112, -114, and -128. ^g Assignment not certain due to low reactivity.

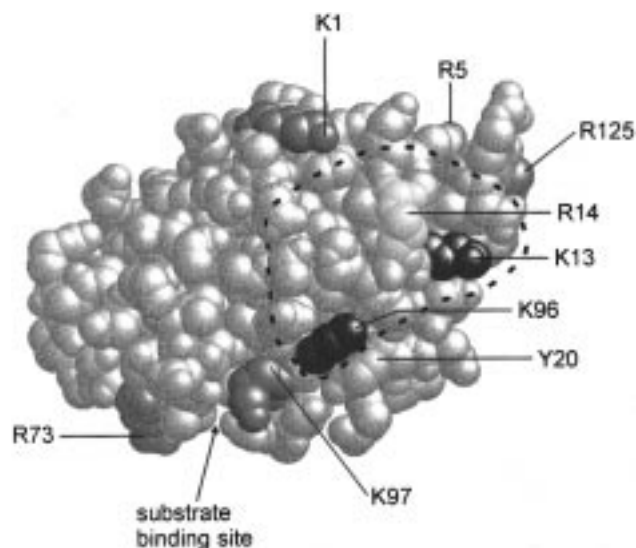


Figure 5. Structure model of HEL indicating the epitope surface estimated from the solvent accessible surface area according to ref 40. The epitope comprising about 400 Å² as a minimum area is marked by the dotted line. R and K residues as part of the epitope are shown in blue; nonepitope residues are marked in red. The Y20 residue adjacent to the epitope surface is shown in orange.

of tryptic fragments for the free and antibody-bound protein. Arginine residues 5, 14, 73, and 125 (Table 1) were selectively modified in the uncomplexed protein which was established in previous studies due to proton acceptor groups in the microenvironment of these residues (16, 29). In contrast, the peptide mapping analysis after CHD modification of antibody-bound HEL showed reactivity only for arginine residues 5, 125, and 73, whereas the ion due to the DHCH-modified peptide containing R14 had disappeared. The selective protection of R14 by the antibody again suggests a protected location of this residue in the epitope structure. However, in the case of the arginine modification, no conclusion could be made about those residues which are unreactive in the uncomplexed protein (29).

DISCUSSION

The epitope surface area defined by the binding site of the monoclonal antibody used in this model study with HEL and determined using mass spectrometric epitope mapping is shown schematically in Figure 5. A minimum surface of approximately 400 Å² was estimated from the K and R residues comprising the epitope (K13, K96, and R14), and from corresponding adjacent residues that were found unshielded and hence were modified on the surface of the immune complex (K1, K97, R5, R73, and R125).

Taken together, the results from these modifications and the additional tyrosine iodination define an epitope surface that can be described by conformational arrangement of the intact protein. Near K96, the surface epitope can be located more accurately, because nonepitope residues Y20 and K97 are in close spatial proximity. In contrast, only a rough epitope surface estimation is possible between K1 and R125 because of the lack of suitable reactive residues in the environment of these residues. The distance between the ϵ -amino and guanidino groups of K13 and K96 is 12.5 Å, from K13 to R14 (C atom in the guanidino group) is 9.1 Å, and from K96 to R14 is 13.1 Å. This yields an estimated epitope surface of about 400 Å² which is consistent with the order of epitope surfaces previously determined by X-ray crystallography (ca. 700 Å²) (22, 23, 43). Residues K13, K96, and R14 were frequently found to be involved in epitope structures of HEL with different monoclonal antibodies, and hence can be considered strongly antigenic parts in HEL (45). However, a detailed comparison of the epitope with previously published epitope surfaces is not possible due to the different (IgM) antibody employed in this study.

In conclusion, this study shows that the combination of selective protein chemical modification and mass spectrometric peptide mapping is well suited for the characterization of molecular contact areas in protein-protein interactions. Likewise, this approach can be used to study not only antigen-antibody complexes but also receptor-ligand interactions or complexes of regulatory proteins and hence should constitute a valuable approach which is complementary to X-ray crystallography (36).

ACKNOWLEDGMENT

We thank Professor Volker Ullrich, Dr. Ingrid Kuhlmann, and Renate Sprenger (Faculty of Biology, University of Konstanz) for their kind support and assistance in the preparation of monoclonal antibodies and Klaus Hägele for expert assistance in mass spectrometric procedures. We also gratefully acknowledge the advice of and discussions with Dr. Konrad Kürzinger (Boehringer Mannheim, Penzberg, Germany). This work has been supported by the Deutsche Forschungsgemeinschaft (Bonn, Germany; Forschergruppe "Struktur und Funktionssteuerung an zellulären Oberflächen") and the European Union through the EU network "Peptide and Protein Structure Elucidation by Mass Spectrometry".

LITERATURE CITED

- Owens, J., Lewis, R. M., Cantor, A., Furie, B. C., and Furie, B. (1984) Monoclonal antibodies against human abnormal (des- γ -carboxy)prothrombin specific for the calcium-free conformer of prothrombin. *J. Biol. Chem.* 259, 13800–13805.
- Maelicke, A., Plümer-Wilk, R., Fels, G., Spencer, S. R., Engelhard, M., Veltel, D., and Conti-Tronconi, B. M. (1989) Epitope mapping employing antibodies raised against short synthetic peptides: A study of the nicotinic acetylcholine receptor. *Biochemistry* 28, 1396–1405.
- Brakenhoff, J. P. J., Hart, M., de Groot, E. R., di Padua, F., and Aarden, L. A. (1990) Structure-function analysis of human IL-6. Epitope mapping of neutralizing monoclonal antibodies with amino- and carboxyl-terminal deletion mutants. *J. Immunol.* 145, 561–568.
- Burnette, W. N. (1990) The advent of recombinant pertussis vaccines. *Bio/Technology* 8, 1003–1005.
- Sela, M. (1969) Antigenicity: some molecular aspects. *Science* 166, 1365–1375.
- Atassi, M. Z., and Smith, J. A. (1978) A proposal for the nomenclature of antigenic sites in peptides and proteins. *Immunochemistry* 15, 609–610.

- (7) Getzoff, D. E., Tainer, J. A., Lerner, R. A., and Geysen, M. H. (1988) The chemistry and mechanism of antibody binding to protein antigens. *Adv. Immunol.* 43, 1–98.
- (8) Jin, L., Fendly, B. M., and Wells, J. A. (1992) High resolution functional analysis of antibody-antigen interactions. *J. Mol. Biol.* 226, 851–865.
- (9) Laver, W. G., Air, G. M., Webster, R. G., and Smith-Gill, S. J. (1990) Epitopes on protein antigens: misconceptions and realities. *Cell* 61, 553–556.
- (10) Geysen, H. M., Meloen, R. H., and Barteling, S. J. (1984) Use of peptide synthesis to probe viral antigens for epitopes to a resolution of a single amino acid. *Proc. Natl. Acad. Sci. U.S.A.* 81, 3998–4002.
- (11) Chen, J., Marechal, V., and Levine, A. J. (1993) Mapping of the p53 and mdm-2 interaction domains. *Mol. Cell. Biol.* 13, 4107–4114.
- (12) Devlin, J. J., Panganiban, L. C., and Devlin, P. E. (1990) Random peptide libraries: a source of specific protein binding molecules. *Science* 249, 404–406.
- (13) Suckau, D., Köhl, J., Karwath, G., Schneider, K., Bitter-Suermann, D., and Przybylski, M. (1990) Molecular epitope identification by limited proteolysis of an immobilized antigen-antibody complex and mass spectrometric peptide mapping. *Proc. Natl. Acad. Sci. U.S.A.* 87, 9848–9852.
- (14) Fiedler, W., Etspüler, H., Suckau, D., and Przybylski, M. (1992) A new approach for the molecular epitope identification in protein antigens by combination of partial proteolytic digestion of an immobilized immune complex with mass spectrometric peptide mapping. *Fresenius' Z. Anal. Chem.* 343, 58–59.
- (15) Macht, M., Fiedler, W., Kürzinger, K., and Przybylski, M. (1996) Mass spectrometric mapping of protein epitope structures of myocardial infarct markers myoglobin and Troponin T. *Biochemistry* 35, 15633–15639.
- (16) Przybylski, M. (1995) Mass spectrometric approaches to the characterization of tertiary and supramolecular structures of biomacromolecules. *Adv. Mass Spectrom.* 13, 257–283.
- (17) Papac, D. I., Hoyes, J., and Tomer, K. B. (1994) Epitope mapping of the gastrin-releasing peptide/anti-bombesin monoclonal antibody complex by proteolysis followed by matrix-assisted laser desorption ionisation mass spectrometry. *Protein Sci.* 3, 1485–1492.
- (18) Parker, C. E., Papac, D. I., Trojak, S. K., and Tomer, K. B. (1996) Epitope mapping by mass spectrometry: determination of an epitope on HIV-1 IIIB p26 recognized by a monoclonal antibody. *J. Immunol.* 157, 198–206.
- (19) Mizukami, T., Fürst, T. R., Berger, E. A., and Moss, B. (1988) Binding region for human immunodeficiency virus (HIV) and epitopes for HIV-blocking monoclonal antibodies of the CD4 molecule defined by site-directed mutagenesis. *Proc. Natl. Acad. Sci. U.S.A.* 85, 9273–9277.
- (20) Schejter, A., Luntz, T. L., Koshy, T. I., and Margoliash, E. (1992) Relationship between local and global stabilities of proteins: site-directed mutants and chemically-modified derivatives of cytochrome C. *Biochemistry* 31, 8336–8343.
- (21) Amit, A. G., Mariuzza, R. A., Phillips, S. E. V., and Poljak, R. J. (1986) Three-dimensional structure of an antigen-antibody complex at 2.8 Å resolution. *Science* 233, 747–753.
- (22) Wilson, I. A., and Stanfield, R. L. (1994) Antibody-antigen interactions: new structures and conformational changes. *Curr. Opin. Struct. Biol.* 4, 857–867.
- (23) Sheriff, S., Silverton, E. W., Padlan, E. A., Cohen, G. H., Smith-Gill, S. J., Finzel, B. C., and Davies, D. R. (1987) Three-dimensional structure of an antibody-antigen complex. *Proc. Natl. Acad. Sci. U.S.A.* 84, 8075–8079.
- (24) Davies, D. R., and Cohen, G. H. (1996) Interactions of protein antigens with antibodies. *Proc. Natl. Acad. Sci. U.S.A.* 93, 7–12.
- (25) Paterson, Y., Englander, S. W., and Roder, H. (1990) An antibody binding site on cytochrome C defined by hydrogen exchange and two-dimensional NMR. *Science* 249, 755–759.
- (26) Burnens, A., Demotz, S., Corradin, G., Binz, H., and Bosshard, H. R. (1987) Epitope mapping by chemical modification of free and antibody-bound protein antigen. *Science* 235, 780–783.
- (27) Oertle, M., Paterson, Y., Bosshard, H. R., and Immerglück, K. (1989) Mapping of four discontinuous antigenic determinants on horse cytochrome C. *Eur. J. Biochem.* 182, 699–704.
- (28) Maly, P., and Luthi, C. (1988) The binding sites of insulin-like growth factor I (IGF I) to type I IGF receptor and to a monoclonal antibody. Mapping by chemical modification of tyrosine residues. *J. Biol. Chem.* 263, 7068–7072.
- (29) Suckau, D., Mák, M., and Przybylski, M. (1992) Protein surface topology probing by selective chemical modification and mass spectrometric peptide mapping. *Proc. Natl. Acad. Sci. U.S.A.* 89, 5630–5634.
- (30) Glocker, M. O., Borchers, C., Fiedler, W., Suckau, D., and Przybylski, M. (1994) Molecular characterization of surface topology in protein tertiary structures by amino-acylation and mass spectrometric peptide mapping. *Bioconjugate Chem.* 5, 583–590.
- (31) Patthy, L., and Smith, E. L. (1975) Reversible modification of arginine residues. Application to sequence studies by restriction of tryptic hydrolysis to lysine residues. *J. Biol. Chem.* 250, 557–564.
- (32) Smith, E. L. (1977) Reversible blocking at arginine by cyclohexanedione. *Methods Enzymol.* 47, 156–161.
- (33) Imoto, T., and Tamada, H. (1989) Chemical Modification. In *Protein Function, a Practical Approach* (T. E. Creighton, Ed.) pp 247–277, Oxford University Press, Oxford.
- (34) Glazer, A. N. (1976) The chemical modification of proteins by group specific and site-specific reagents. In *The Proteins* (H. Neurath, and R. L. Hill, Eds.) Vol. 2, pp 1–103, Academic Press, New York.
- (35) Amico, V., Foti, S., and Saletti, R. (1988) Identification of iodination sites in cytochrome c by high-performance liquid chromatography and fast atom bombardment mass spectrometry. *Biol. Environ. Mass Spectrom.* 16, 431–437.
- (36) Przybylski, M., Schnaible, V., Kast, J., Blüggel, M., Michels, J., Wattenberg, A., Fligge, T., Forst, D., Diederichs, K., Zeth, C., Glocker, M. O., and Welte, W. (1996) Approaches to the Characterization of Tertiary and Supramolecular Protein Structures by Combination of Protein Chemistry and Mass Spectrometry. *NATO Adv. Res. Ser.* (in press).
- (37) Cleveland, W. L., Wood, I., and Erlanger, B. F. (1983) Routine large-scale production of monoclonal antibodies in a protein-free culture medium. *J. Immunol. Methods* 56, 221–234.
- (38) Product information from Pharmacia, Freiburg i. Br., Germany.
- (39) Kussmann, M., and Przybylski, M. (1995) Tertiary structure-selective characterization of protein dithiol groups by phenylarsinoxide modification and mass spectrometric peptide mapping. in *Methods in Enzymology* (L. Packer, Ed.) pp 430–435, Academic Press, San Diego.
- (40) Roepstorff, P. (1990) Sample preparation for plasma desorption mass spectrometry. *Methods Enzymol.* 193, 432–441.
- (41) Jespersen, S., Talbo, G., and Roepstorff, P. (1993) Optimization of sample recovery from the nitrocellulose support used in plasma desorption mass spectrometry and its use for multiple analyses of insulin. *Biol. Mass Spectrom.* 22, 77–83.
- (42) Lee, B., and Richards, F. M. (1971) The interpretation of protein structures: estimation of static accessibilities. *J. Mol. Biol.* 55, 379–400.
- (43) Conolly, M. L. (1983) *J. Appl. Crystallogr.* 16, 548–558.
- (44) Hopp, T. P. (1994) Different views of protein antigenicity. *Pept. Res.* 7, 229–231.
- (45) Westhof, E., Altschuh, D., Moras, D., Bloomer, A. C., Mondragon, A., Klug, A., and van Regenmortel, M. H. V. (1984) Correlation between segmental mobility and the location of antigenic determinants in proteins. *Nature* 311, 123–126.

Fluorescently Detectable Magnetic Resonance Imaging Agents

Martina M. Hüber, Andrea B. Staubli, Karen Kustedjo, Mike H. B. Gray, John Shih, Scott E. Fraser, Russell E. Jacobs,* and Thomas J. Meade*

Division of Biology and Beckman Institute, California Institute of Technology, Pasadena, California 91125. Received August 13, 1997; Revised Manuscript Received December 12, 1997

This report describes the synthesis, characterization, and in vivo testing of several bifunctional contrast-enhancing agents for optical and magnetic resonance imaging (MRI) of experimental animals. These new agents integrate the advantages of both techniques since they can be visualized simultaneously by light and MRI microscopy. Employing this strategy allows the same biological structures of a specimen to be studied at dramatically different resolutions and depths. The complexes possess a metal chelator for binding a paramagnetic ion, gadolinium (Gd^{3+}), and a covalently attached fluorescent dye. The first class of complexes are low-molecular weight species that are composed of the macrocyclic tetraamine 1,4,7,10-tetraazacyclododecane-*N,N',N'',N'''*-tetraacetic acid (DOTA) as the metal-chelating ligand coupled to tetramethylrhodamine. The second class of MRI-enhancing agents are composed of high-molecular weight polymers that are membrane impermeable and once injected into a cell or cells are trapped inside. These complexes possess multiple copies of both the metal-chelator–diethylenetriaminepentaacetic acid (DTPA) and the tetramethylrhodamine attached to a macromolecular framework of either poly(D-lysine) (pdl) or dextran. Images acquired of single cells after injection with these bifunctional agents enabled us to follow the relative motions and reorganizations of different cell layers during amphibian gastrulation and neurulation in *Xenopus laevis* embryos.

INTRODUCTION

In recent years, two imaging techniques have emerged that have had a major impact upon basic and clinical science: computer-enhanced light microscopy imaging and nuclear magnetic resonance imaging (1–3). Unlike previous approaches that required the processing of fixed tissue samples, both these techniques permit aspects of a living, intact organism to be imaged. With imaging of the organism in defined physiological states, a more realistic picture of developmental processes has begun to emerge.

Computer-enhanced light microscopy permits high-resolution imaging to be accomplished in imperfect preparations, such as whole tissues and organisms. The use of fluorescent indicator dyes visualized with a video or confocal microscope has resulted in several classes of novel experiments. For example, individual cells have been injected with membrane impermeable dyes (e.g., fluorescent dextrans) so that the only cells that are labeled are the original cell and its progeny (1).

MRI offers the potential of true three-dimensional imaging of biological structures and processes at cellular resolution ($\approx 10 \mu m$) (4–7). Typically, the image is based upon the NMR signal from the protons of water where the signal intensity in a given volume element is a function of the water concentration and relaxation times (T_1 and T_2). The paramagnetic ion gadolinium (Gd^{3+}) is used in MRI agents to decrease the local T_1 of water protons and therefore provide increased contrast. The long electron spin relaxation time and high magnetic moment of Gd^{3+} make it an efficient perturbant of T_1 . Through chelation of the Gd^{3+} aqua ion using various ligands, its toxic effects can be minimized (8–10).

While significant results have been obtained employing these techniques, each suffers from inherent constraints.

Light microscopy is limited by optical aberrations and light scattering to the outer 100–300 μm of a specimen. MRI microscopy provides a solution to this limitation by rendering three-dimensional images of opaque specimens, but sacrifices resolution by at least 10–20-fold compared to that of light microscopy. There are a significant number of MRI contrast agents that have been developed for clinical use because they enhance the local image intensity of organs and tissues. However, there are very few examples of agents that have been specifically designed for imaging experimental animals.

Therefore, we have prepared a series of bifunctional contrast-enhancing agents that are designed to integrate the advantages of both techniques. We report the synthesis and in vivo testing of a class of agents that are simultaneously detectable by fluorescence and MRI microscopy, thereby permitting multimodal imaging of experimental animals. The design of these bifunctional contrast agents included the lanthanide chelators DTPA and DOTA, which are known to form lanthanide complexes with high stability and selectivity (9, 11, 12). Conjugation of the fluorescent functionality, tetramethylrhodamine, was achieved either through coupling to the carboxylic acid group of DTPA or by functionalizing the DOTA macrocycle (Figures 1–3) (13–17).

The first class of complexes includes $Gd[p\text{-(aminobenzyl)DOTA}]\text{--tetramethylrhodamine}[Gd(\text{rhoda-DOTA})]$. This agent carries the macrocyclic tetraamine DOTA linked to the fluorochrome in a 1:1 complex and may be used to investigate membrane permeability, fusion, gap-junctional communication, and lesions. The second class includes membrane impermeable agents, such as $Gd\text{-(DTPA)--tetramethylrhodamine--hydroxypropylpoly(D-lysine)}$ (GRIP) and $Gd\text{-(DTPA)--tetramethylrhodamine--aminodextran}$ (GRID). These agents are based on a polymeric scaffold (i.e., pdl and dextran, respectively), carrying several tetramethylrhodamine molecules and $Gd\text{-(DTPA)}$ complexes.

* To whom correspondence should be addressed.

EXPERIMENTAL PROCEDURES

Materials. Unless otherwise noted, all solvents and reagents for organic synthesis were obtained from Aldrich or Fluka and were used without further purification. Dextran and pdl were purchased from Sigma; tetramethylrhodamine-5-isothiocyanate (TRITC) was obtained from Molecular Probes. 2-(*p*-Aminobenzyl)-1,4,7,10-tetraazacyclododecane-*N,N',N'',N'''*-tetraacetic acid (PABD) was synthesized as reported in the literature (13, 14). Doubly distilled deionized water was obtained from a NANO pure II filtration unit to 18 M Ω -cm resistivity and was used throughout to minimize trace-metal contamination of the ligands and chelates. Reactions were performed in oven-dried flasks under positive argon pressure and monitored by analytical thin-layer chromatography (TLC) using E. Merck silica gel 60F plates (0.25 mm). E. Merck silica gel (230–400 mesh) was used for flash chromatography. Samples were purified on PD 10 and NAP 25 prepacked, disposable columns (Pharmacia Biotech). ^1H spectra were recorded at 300 MHz on a GE300 NMR spectrometer. Analytical HPLC was performed with a Waters 600E liquid chromatograph equipped with a 994 diode array detector using a reverse phase VYDAC 201HS54 4.6 mm $\times$ 25 cm, 5 μm C18 column. Samples were eluted with 100 mM triethylammonium acetate with a gradient of 1 to 20% MeCN at a flow rate of 1 mL/min, and products were detected by UV absorption at 260 and 330 nm. FPLC was performed using a Pharmacia Liquid Chromatography Controller LCC 550 unit applying a HR 10/10 Fast Desalting Column and a Superdex 75 column (Pharmacia). Aqueous solutions were injected onto the column and eluted with a 0.15 M sodium phosphate buffer (pH 7) at a flow rate of 1 mL/min. The fluorescence of Gd^{3+} - and tetramethylrhodamine-containing derivatives was analyzed on a Hitachi F-4500 instrument (Gd^{3+} , λ_{ex} = 275 or 280 nm, λ_{em} = 313–335 nm; tetramethylrhodamine, λ_{ex} = 547 nm, λ_{em} = 572 nm). Concentrations of tetramethylrhodamine-containing compounds were determined by UV measurements at 548 nm on a Hewlett-Packard 8452A diode array spectrophotometer using an ϵ of $\approx 100 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ as the molar extinction coefficient.

Fluorescence Microscopy. Survival of the labeled cells was monitored by embryos that were placed in 30% Ringer's solution and transferred to a Sylgard-based plate (Dow Corning Sylgard 182 silicone elastomer, cured and then pretreated with 2% BSA solution) with precut triangular wells. The positions, distributions, and fates of labeled graft progeny were monitored and documented in the living whole mount using a SIT camera (Hamamatsu C2400) mounted on a Zeiss AxioPhot microscope. Bright-field transmitted images were taken on a Zeiss AxioPhot microscope using a Hamamatsu Neuvicon camera. In each case, the images were captured on a 151 image processor (Imaging Technologies, Inc.) and averaged ($n = 8$ frames) before storage on an optical memory disk (Panasonic OMDR 3038) using the VIDIM image-processing software (authored by Belford, Fraser, and Stollberg).

MRI. MR images were acquired using a Bruker AMX500 spectrometer (Bruker Instruments Inc., Billerica, MA) with a microimaging accessory, a homebuilt RF probe with 2.5 and 5 mm ID RF solenoids, a homebuilt low-noise preamplifier, and an Acustar gradient set (Bruker Instruments, Inc., Fremont, CA) capable of producing $\approx 290 \text{ G/cm}$ gradients. For MR imaging, the samples were transferred to 2.5 or 5 mm outside diameter thin-walled glass tubes and maintained at 18 $^\circ\text{C}$ in the

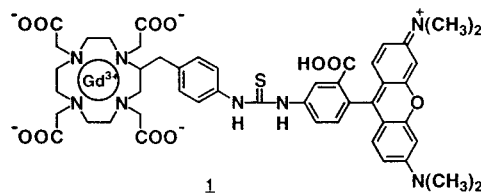


Figure 1. Gd(rhoda-DOTA) (1).

same solution used for growing the embryos. A three-dimensional steady state free precession (SSFP) spin echo imaging sequence with TR/TE = 200 ms/4.4 ms was used to obtain T_1 weighted MR images. The typical isotropic voxel size was 30 μm , although in some cases, data sets with 15 μm voxels were collected. Image visualization was performed on a SGI Indigo*2 workstation (Silicon Graphics, Inc., Mountain View, CA) using VoxelView software (Vital Images, Inc., Fairview, IA).

In Vivo MRI. Imaging studies were performed on *Xenopus laevis* (African frog) embryos. Approximately 10 nL of a buffered aqueous solution containing each compound at a known concentration was injected into the animal pole of one cell in a two-cell embryo. Alternately, ≈ 2 nL of the same solution was injected into a marginal blastomere at the 32–256-cell stage. The distribution and survival of the labeled cells were followed for up to 4 days using MRI and fluorescence microscopy.

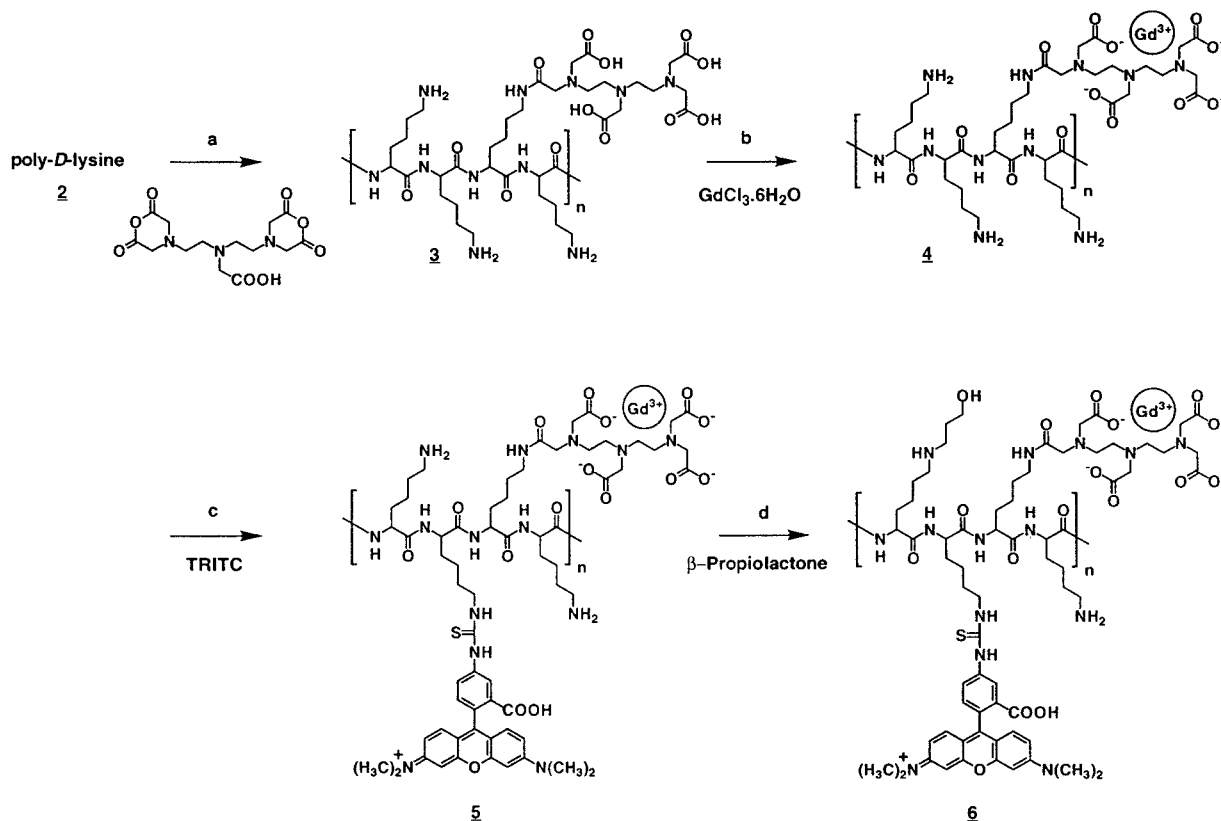
Gd(PABD). *Method A.* To a stirred solution of PABD in either water or a 100 mM Na_2CO_3 solution (pH 8) was added $\text{GdCl}_3 \cdot 6\text{H}_2\text{O}$ (0.99 equiv). The reaction mixture was stirred at 80 $^\circ\text{C}$ for 12 h. The solution was concentrated to dryness, redissolved in 100 mM triethylammonium acetate/MeCN, and purified by HPLC.

Method B. To a stirred solution of PABD in water or a 100 mM Na_2CO_3 solution (pH 8) was added Gd_2O_3 (1.3 equiv). The reaction mixture was stirred at 80 $^\circ\text{C}$ for 18 h. The solution was filtered through an Acrodisc (LC 13 PVDF, 0.2 μm) to remove unreacted Gd_2O_3 . The solution was then concentrated to dryness, redissolved in 100 mM triethylammonium acetate/MeCN, and purified by HPLC.

Gd(rhoda-DOTA) (1) (Figure 1). To a stirred solution of Gd(PABD) (1 mg, 1.46 μmol) in 250 μL of DMF was added TRITC (0.72 mg, 1.61 μmol , 1.1 equiv). The solution was covered with aluminum foil and stirred at room temperature for 10 h. The solution was concentrated to dryness, taken up in a 100 mM Na_2CO_3 solution/MeCN, and purified by HPLC. ^1H NMR (D_2O): δ 2.5–3.6 (m, 25 H), 3.25 (s, 12 H), 6.9–7.15 (m, 4 H), 7.27 (d, 2 H), 7.35–7.45 (m, 3 H), 7.5 (d, 2 H), 7.75 (d, 1 H), 8.1 (s, 1 H).

Synthesis of GRIP_c (3–6) (Scheme 1). *DTPA–pdl* (3). To a stirred solution of pdl (2) (50 mg, MW = 3500, 25 000, and 53 000 g/mol) in 12.5 mL of a NaHCO_3 solution (0.5 M, pH 9.5) was slowly added DTPA dianhydride in various amounts (100, 400, or 800 equiv). The pH was maintained at pH 9.5 by addition of a 1 M NaOH solution. After 1 h, the reaction mixture was filtered, concentrated to dryness, redissolved in 1.5 mL of water, and filtered. The solution was applied to a PD10 column and eluted with water, and the collected fractions were concentrated to dryness. The isolated products are referred to as DTPA–pdl_{100ex}, DTPA–pdl_{400ex}, or DTPA–pdl_{800ex} corresponding to the amount of DTPA dianhydride added to the reaction.

Gd–DTPA–pdl (4). To a stirred solution of DTPA–pdl (3) in 1 mL of water at 80 $^\circ\text{C}$ was added $\text{GdCl}_3 \cdot 6\text{H}_2\text{O}$ (10 equiv) dissolved in 0.5 mL of water. After 12 h, the solution was applied to a PD10 column and eluted with

Scheme 1. Synthesis of GRIP_c (6)^a

^a (a) DTPA dianhydride, pH 9.5; (b) GdCl₃·6H₂O or Gd₂O₃, 80 °C, 12 h; (d) β-propiolactone.

water, and the collected fractions were concentrated to dryness.

GRIP (5). Method A. To a stirred solution of Gd-DTPA-pdl_{100ex} (MW = 3500 g/mol) (4) (11 mg, 2.41 μmol) or Gd-DTPA-pdl_{400ex} (MW = 3500 g/mol) (4) (11 mg, 1.95 μmol) in 400 μL of a NaHCO₃ solution (0.5 M, pH 9.5) at 85 °C was added a solution of TRITC (10 equiv) in 200 μL of DMSO/pyridine (50:1). After 24 h, the solution was concentrated to dryness and redissolved in water. The solution was applied to a Sephadex G-25 column and eluted with water, and the collected fractions were concentrated to dryness.

Method B. To a stirred solution of DTPA-pdl (MW = 25 000 g/mol) (3) dissolved in 1 mL of a NaHCO₃ solution (0.5 M, pH 9.5) was added TRITC (30 equiv) dissolved in 50 μL of DMF. After 2 h, the solution was concentrated to dryness and redissolved in water. The solution was applied to a Sephadex G-25 column and eluted with water, and the collected fractions were concentrated to dryness. The intermediate was subsequently redissolved in 1.5 mL of water, and GdCl₃·6H₂O (10 equiv) dissolved in 0.5 mL of water was added. After 12 h, the solution was applied to a Sephadex G-25 column and eluted with water, and the collected fractions were concentrated to dryness.

GRIP_c (6). To a stirred solution of 5 (2.5 mg) in 2 mL of water at 0 °C was first added a 1 N NaOH solution to adjust the pH to 7 and then β-propiolactone (10 equiv) (18). After 10 min, the bath was removed and the reaction solution stirred at ambient temperature for 90 h. The solution was then applied to a PD10 column and eluted with water, and the collected fractions were concentrated to dryness. The crude reaction product was then redissolved in water and purified by FPLC.

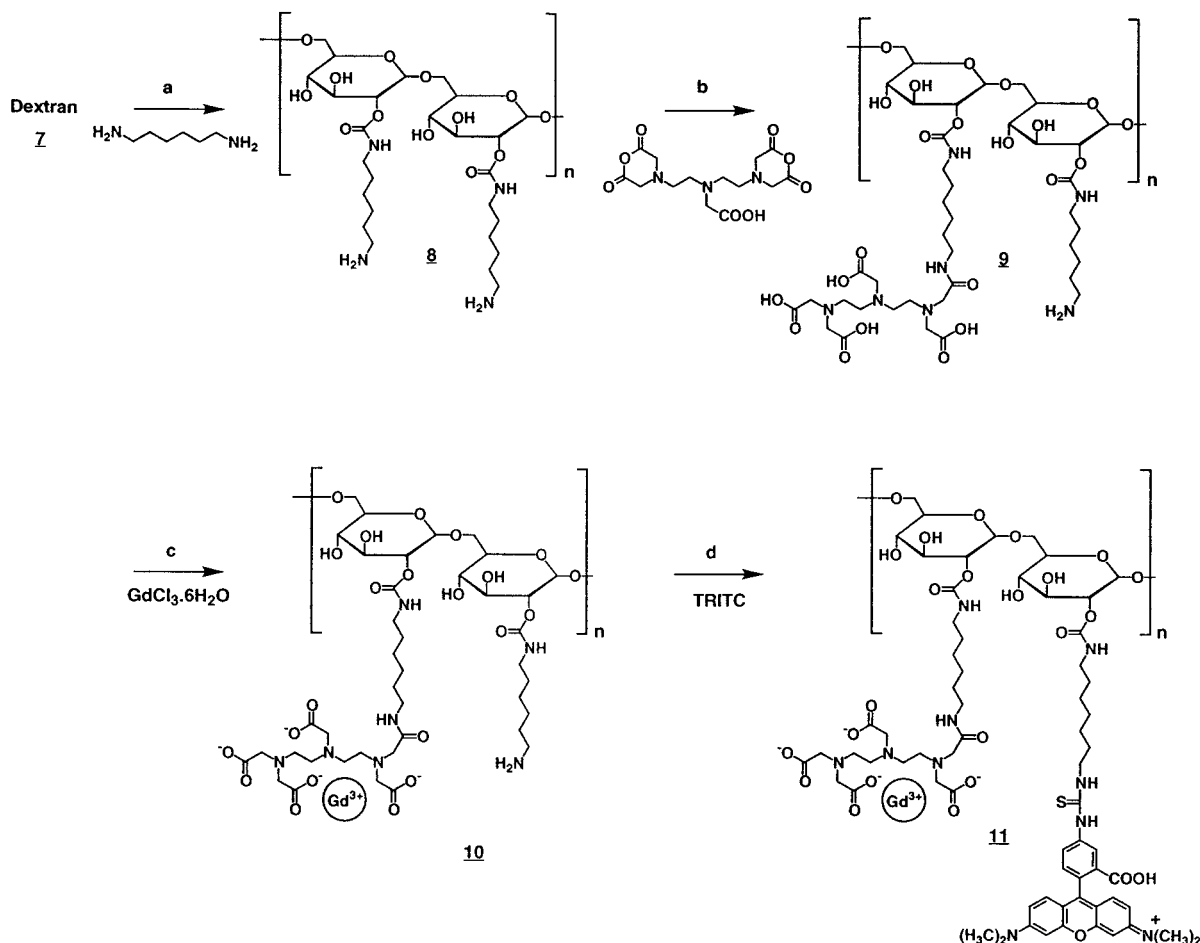
Synthesis of GRID (8–11) (Scheme 2). Dex-Am (8). The six-carbon linker 1,6-diaminohexane was at-

tached to dextran via BrCN activation following a literature procedure (19). To a stirred solution of dextran 7 (212 mg, MW = 12 000 g/mol) in 40 mL of water at pH 10.7 (0.1 N NaOH) was slowly added BrCN (50 mg, 0.24 mmol). The reaction mixture was maintained at pH 10.7 by addition of a 0.1 N NaOH solution. After 4 h, the pH of the solution was adjusted to 7.8 by the addition of a 0.1 M HCl solution. To this solution was added 1,6-diaminohexane (500 mg, 4.3 mmol) and the reaction mixture was stirred for 2 h at room temperature. The solution was concentrated to dryness, taken up in water, and purified by FPLC.

Dex-Am-DTPA (9). To a solution of DTPA (360 mg, 90 mmol) in DMSO (40 mL) was added Dex-Am (8) (114 mg, 9 μmol) dissolved in DMSO (5 mL), and the reaction mixture was stirred for 12 h. The solution was concentrated to dryness and purified by FPLC. Dextran: C, 41.3%; H, 6.2%; N, 0%. Dex-Am-DTPA: C, 43.2%; H, 5.75%; N, 6.4%.

Gd-Dex-Am-DTPA (10). To a solution of Dex-Am-DTPA (9) (86 mg, 6.36 μmol) in water (4 mL) was slowly added a solution of GdCl₃·6H₂O (47 mg, 1.27 μmol) dissolved in water (0.4 mL). The pH was adjusted to 5 by the addition of a 0.1 M NaOH solution, and the reaction mixture was stirred for 24 h. The solution was concentrated to dryness and the crude product redissolved in water and purified by FPLC.

GRID (11). TRITC was covalently attached to Gd-Dex-Am-DTPA (10). To a solution of Gd-Dex-Am-DTPA (10) (30 mg, 1.62 μmol) in DMSO (1 mL) was added a solution of TRITC (10 mg, 1.86 μmol) dissolved in DMSO (1 mL) followed by pyridine (5 μL, 0.06 mmol) and dibutyltin dilaureate (3 μL, 5 × 10⁻³ mmol). The reaction mixture was stirred at 80 °C for 24 h. The solution was concentrated to dryness, redissolved in water (2 mL), and

Scheme 2. Synthesis of GRID (11)^a

^a (a) cyanogen bromide; (b) DTPA dianhydride, 12 h; (c) $\text{GdCl}_3 \cdot 6\text{H}_2\text{O}$, 80 °C, pH 5; (d) TRITC, DMSO, dibutyltin dilaureate, 24 h.

applied to a NAP 25 column. The product was eluted with water, and the collected fractions were concentrated to dryness.

RESULTS AND DISCUSSION

PABD was synthesized as described in the literature and purified by HPLC (13–17). The macrocycle was subsequently suspended in either water or a weakly buffered medium (100 mM sodium carbonate) and treated with either Gd_2O_3 or $\text{GdCl}_3 \cdot 6\text{H}_2\text{O}$ over the course of 12–24 h at 80 °C which afforded $\text{Gd}(\text{PABD})$. This complex was then coupled to commercially available TRITC and purified by HPLC to give $\text{Gd}(\text{rhoda-DOTA})$ (**1**) (Figure 1). The highest yields were obtained if the coupling reaction was performed in organic solvents such as *N,N*-dimethylformamide. It is of interest to note that the final product could not be obtained unless the Gd^{3+} was added to PABD prior to the addition of TRITC. This result suggests that the tetramethylrhodamine ligand is sterically shielding the binding cavity of the macrocycle, preventing Gd^{3+} coordination.

GRIP (**5**) and GRIP_c (**6**) were synthesized as illustrated in Scheme 1. The progress of the reaction was monitored by FPLC. The Gd^{3+} concentration of GRIP was determined by fluorescence spectroscopy ($\lambda_{\text{ex}} = 275 \text{ nm}$, $\lambda_{\text{em}} = 313 \text{ nm}$), leading to a ratio of $2 \pm 1 \text{ Gd}^{3+}$ per 3500 MW pdl in the case of GRIP_{100ex} and to a ratio of $4 \pm 1 \text{ Gd}^{3+}$ per 3500 MW pdl in the case of GRIP_{400ex}. An average of two tetramethylrhodamine molecules per pdl were found as estimated by fluorescence and UV–vis spectroscopy.

The synthesis of GRID (**11**) is illustrated in Scheme 2. The number of DTPA chelates (9 ± 2) covalently attached to the dextran backbone was determined by elemental analysis. The number of tetramethylrhodamine molecules (9 ± 4) coupled to the dextran backbone was determined by fluorescence spectroscopy ($\lambda_{\text{ex}} = 547 \text{ nm}$, $\lambda_{\text{em}} = 572 \text{ nm}$) and UV–vis spectroscopy ($\lambda_{\text{max}} = 548 \text{ nm}$, molar extinction coefficient $\epsilon \approx 65\,000 \text{ M}^{-1} \text{ cm}^{-1}$).

In Vivo Imaging Studies with Gd(rhoda-DOTA) (1). $\text{Gd}(\text{rhoda-DOTA})$ (**1**) contains the modified macrocycle DOTA with a pendent *p*-aminobenzyl group linked to one of the methylene carbons. This functional group allows covalent attachment of fluorescent markers such as the commercially available TRITC to the amine. A series of in vitro experiments using $\text{Gd}(\text{rhoda-DOTA})$ (**1**) revealed strong fluorescent and MR signals, and the relaxivity of the complex is comparable to that of $\text{Gd}(\text{DOTA})$. Surprisingly, when similar concentrations of **1** were injected into living embryos, no MR signal was observed while the fluorescence signal from the complex was observable. This may be due to absorption of the relatively hydrophobic molecule into fatty tissues where little or no water exchange with the gadolinium can take place.

In Vivo Imaging with Gd–DTPA–pdl (4) (pdl MW = 3500 g/mol), GRIP (5), and GRIP_c (6). GRIP (**5**) and GRIP_c (**6**) are high-molecular weight, membrane impermeable agents based on pdl as a macromolecular framework. These molecules were designed to be used for embryonic cell lineage analyses. The experiments are performed by single-cell injection of the complex and

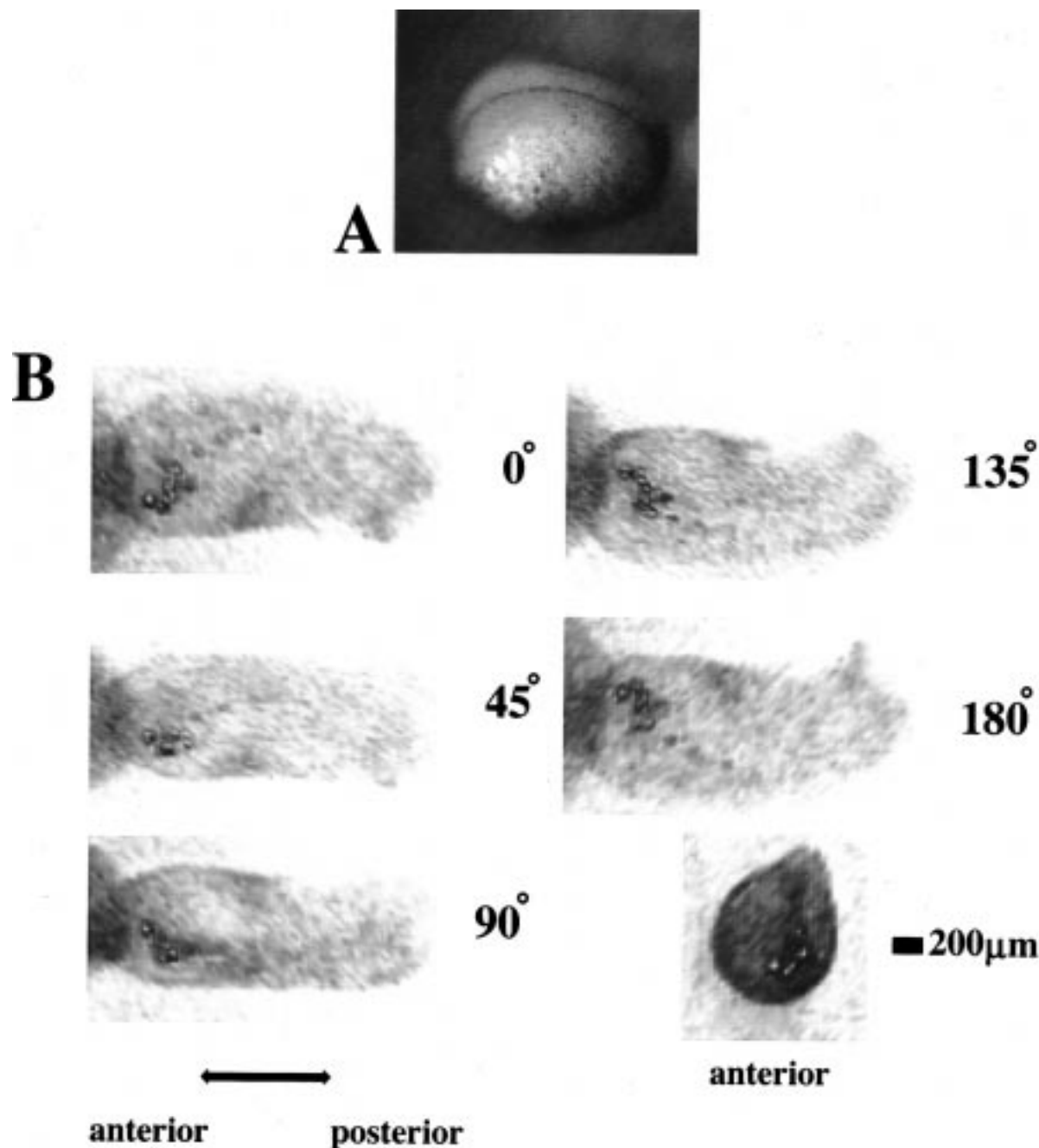


Figure 2. In vivo injections of GRIP (5) (pdl MW = 3500 g/mol). (A) *X. laevis* embryo at approximately stage 17 as seen under a fluorescence microscope at the 256-cell stage. GRIP (5) was injected into a single cell. At stage 17, six cells exhibit strong fluorescence, seen as white regions. (B) MRI of the same embryo shown in panel A; semitransparent volume renderings of the embryo, rotated along its long axis (0–180°). Twelve cells produce a strong MRI contrast revealing the presence of the agent.

subsequent monitoring of the descendants of that labeled cell. The polymer contains several DTPA and tetramethylrhodamine molecules linked to the side chain amino groups of lysine. The biodistribution of Gd-DTPA-pdl (4) was studied using *X. laevis* embryos. This system was chosen to test the in vivo behavior of the agents because *X. laevis* cells are extremely sensitive to perturbations during development. If the contrast agents exhibit even minor toxic side effects, the injected cells develop at dramatically different rates compared to neighboring cells.

At the two-cell stage, 10 nL of aqueous solutions (10 mg/mL) of Gd-DTPA-pdl_{100ex} or Gd-DTPA-pdl_{400ex} were injected into the animal pole of the embryos to determine their biodistribution. Gd-DTPA-pdl_{100ex} diffused rapidly and uniformly as revealed by epifluores-

cence microscopy during the first 30 min after injection. Gd-DTPA-pdl_{400ex} did not disperse and remained localized at the site of injection. These observations suggested that the biodistribution correlates with the observed increasing viscosity of the injected solutions (as determined by the increased pressure needed for injections), which is lowest for Gd-DTPA-pdl_{100ex} and highest for Gd-DTPA-pdl_{800ex}. No attempt was made to inject Gd-DTPA-pdl_{800ex}.

In order to evaluate these derivatives as in vivo contrast agents, fluorescence microscopy and MR imaging studies were performed using GRIP (5) and GRIP_c (6) that was microinjected into *X. laevis* embryos. Cell division was monitored, and the embryos developed normally for 24 h after injection of GRIP (5). It was observed that embryo survival could be enhanced by

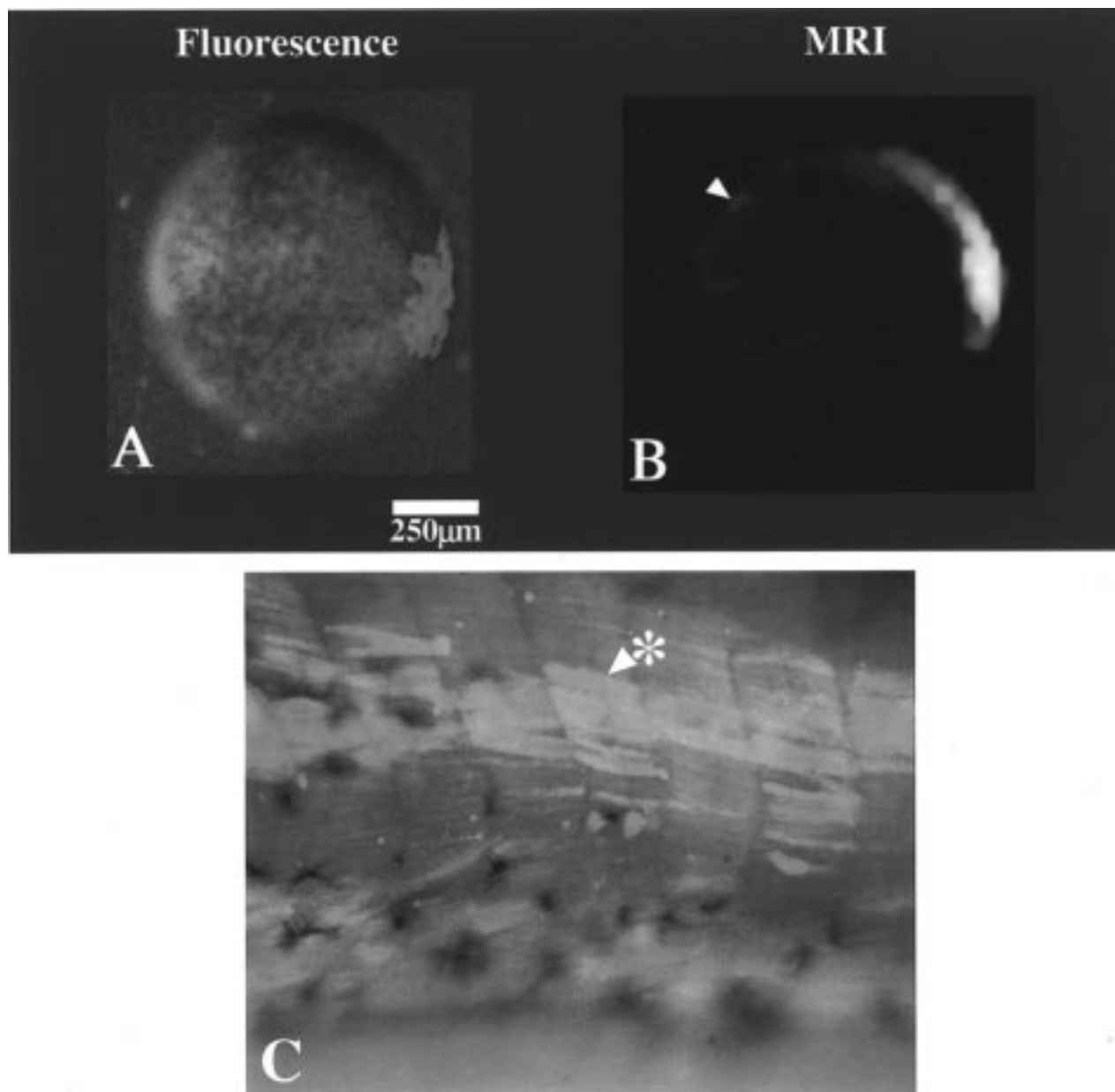


Figure 3. *X. laevis* embryo (stage 8) with GRIP_c (**6**) injected into the marginal blastomere at the 16-cell stage. (A) The labeled cells populate the prospective mesodermal regions as seen in this confocal fluorescence image. (B) MRI image showing a single slice through the middle of the same embryo shown in panel A. The distribution of labeled cells reveals features and delineates a number of cells that are unobservable by fluorescence techniques because they are too far below the surface. (C) A similarly injected embryo seen at stage 38–39 reveals labeled cells in the somatic mesoderm. The labeled cells, shown in green, are seen to span the length of the somite blocks.

protecting the free amino groups of the high-molecular weight derivatives of pdl by converting the unmodified amino side chains to terminal alcohols with β -propiolactone [GRIP_c (**6**)]. Unmodified pdl is toxic when injected into living cells, and it appears that charge distribution may effect the in vivo toxicity of the pdl derivatives. Similarly, GRIP_{100ex} (**5**) (pdl MW = 3500 g/mol) was injected into a single marginal cell of a *X. laevis* embryo at the 256-cell stage. Later, at stage 17, six fluorescent cells could be detected using fluorescence microscopy (Figure 2A). Figure 2B shows semitransparent volume renderings of MRI data rotated along the long axis of the same embryo at stage 18–19, revealing many more labeled cells at greater depth in the embryo.

Figure 3 depicts a *X. laevis* embryo that was injected with GRIP_c (**6**) (pdl MW = 25 000 g/mol) into a marginal blastomere at the 16-cell stage. Using confocal microscopy (Figure 3A), the labeled cells are seen to populate

the prospective mesodermal regions. The distribution of labeled cells was confirmed using MRI (Figure 3B). In addition, a number of cells that are not visible in the confocal are seen using MRI, demonstrating the unique ability of these agents in cell lineage analysis. The progeny of the labeled blastomere 5 days later at stage ≈ 38 –39 is shown in Figure 3C. Labeled cells in somatic mesoderm are clearly visible and span the length of each somitic block as expected. Cells in the endoderm are labeled in agreement with published fate maps of the 16-cell stage *X. laevis* embryos (20, 21).

In Vivo Imaging Studies with Gd–DTPA–Dextran [GRID (11**)].** GRID (**11**) is a high-molecular weight, membrane impermeable contrast agent based on dextran as a macromolecular framework. The backbone is multiply modified with several DTPA and tetramethylrhodamine molecules. As described above, *X. laevis* embryos were injected with GRID (**11**) and analyzed over

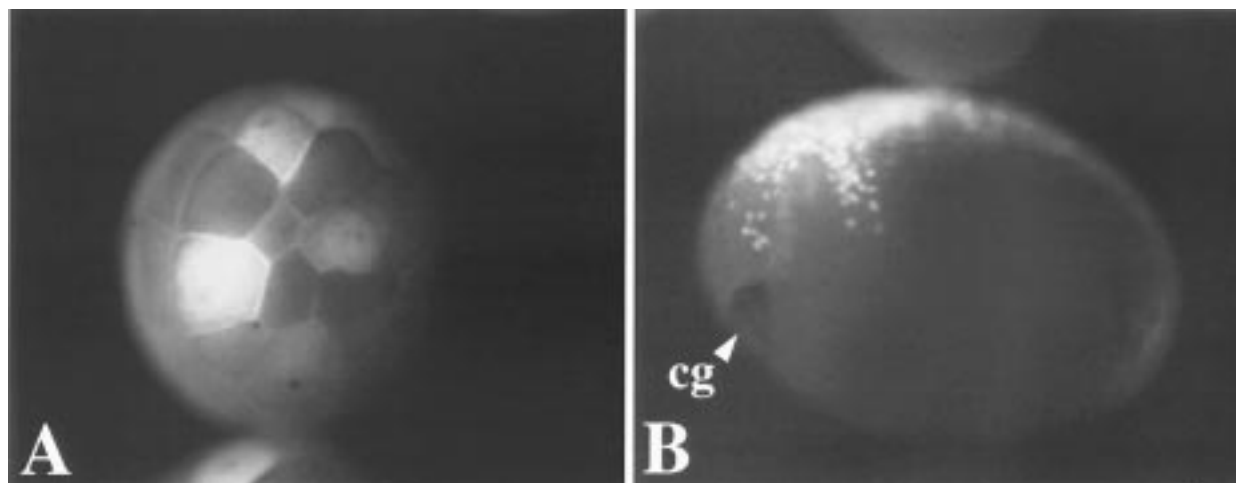


Figure 4. In vivo injections of GRID (11) in *X. laevis* as seen under the fluorescence microscope. (A) Embryo at the 32-cell stage just after injection of GRID (11) into only two cells. (B) The same embryo shown in panel A, however at a much later stage of development (stage 23–24); numerous labeled cells that are descendants of the two injected cells (panel A) can be seen in the embryo's neural axis and in the overlying head ectoderm.

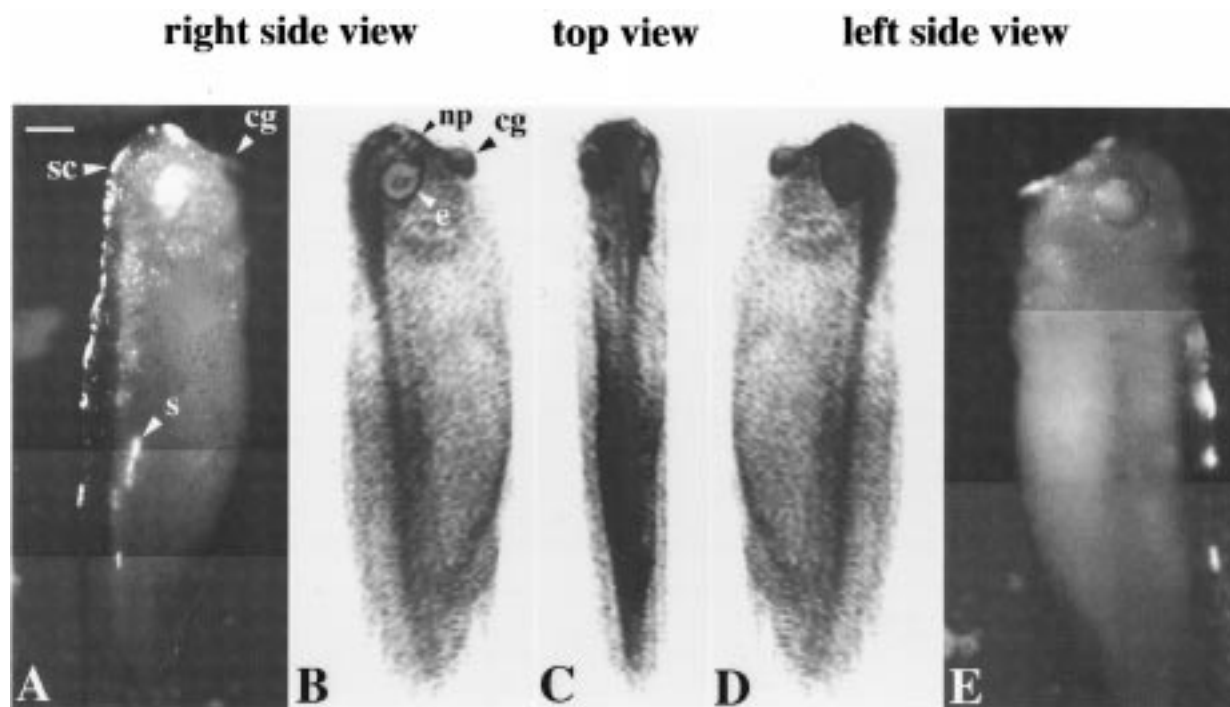


Figure 5. Late stage (26–27) *X. laevis* embryo injected with GRID (11). Panels A and E show fluorescent images of cell progeny labeled with the contrast agent during the 128-cell stage. Panels B–D show the same embryo at the same stage using MRI. Panels A and B reveal the right side of the embryo in fluorescent (A) and MRI (B) views. The eye is brightly fluorescent in panel A and displays a correspondingly strong signal in panel B (e, eye; np, nasal pit). In the top view (C), the difference in signal intensity within the neuraxis is striking. Cells on the left side of the embryo (D and E) are not labeled, as determined using MRI (D) or fluorescence microscopy (E); scale bar = 200 μm ; cg, cement gland which displays intense inherent signal unrelated to the injected contrast agent. The color scale shown in panels B–D is as follows: Yellow represents the most intense signal, red is less intense, and gray is for background. Abbreviations for anatomical structures are as follows: cg, cement gland; e, eye; np, nasal pit; s, somite cell; and sc, skin cell.

time using both fluorescence microscopy and MRI. Cell division could be successfully followed, and the embryos developed normally for several days. Figure 4A shows the *X. laevis* embryo just after injection of GRID (11) into two cells at the 32-cell stage as observed under the fluorescence microscope. The same embryo depicted in Figure 4A is shown in the tail-bud stage (stage 23–24) (Figure 4B). Labeled cells are clearly visible in the embryo's neural axis and in the overlying head ectoderm.

In Figure 5B,C,D, the distribution of the marked cells in the interior of the embryo is visualized by MRI. Figure 5A–D shows a stage 26–27 the *X. laevis* embryo lineage

labeled using GRID (11). Less than 1 nL of the bifunctional agent has been pressure injected into a single dorsal tier-2 blastomere at the 128-cell stage. On the basis of earlier cell lineage studies, the normal fate of this blastomere is expected to be largely anterior neural derivatives.

Panels A and E of Figure 5 reveal low-light-level epifluorescence microscope images that document the distribution of fluorescently labeled cells which are shown in white in this black and white image. On the right-hand side of the animal (Figure 5A), the eye (e) is brightly labeled as is the nasal pit (np) and numerous skin cells

of the head (sc). Cells of the dorsal fin-fold and a few in the somites (s) are also labeled. Because the progeny of the injected blastomere is normally restricted to the right-hand side of the animal, most structures on the left-hand side (Figure 5E), with the exception of the fin-fold, appear unlabeled.

Finally, the distribution of labeled progeny as revealed by fluorescent microscopy is consistent with MR images of the same embryo. The three-dimensional MR data set is shown in the middle three panels (Figure 5B–D) (the labeled cells that were white in the fluorescent image appear in the MR images with a reddish color; as the MR images represent three-dimensional data sets, many more cells deep within the embryo labeled with the bifunctional contrast agent are seen using MR than can be seen with fluorescence microscopy) as right, top, and left views, respectively. Cells that are labeled with the bifunctional agent appear as yellowish regions against a reddish background signal. The right eye and right nasal pit are clearly labeled. The same structures on the left side are not labeled. In the top view, the difference in contrast between the left and right sides is striking. Just below the eye, in the approximate position of the developing mouth, is a structure that displays a high signal in the MR image but appears not to be labeled in the fluorescent image. This structure is the cement gland which typically possesses high contrast and does not represent signal from the injected agent.

In summary, this report describes synthesis and in vivo testing of two new classes of bifunctional contrast-enhancing agents. The agents were designed to integrate the advantages of optical and MR microscopy for studies in experimental animals. As a result, features that are invisible to light microscopes are clearly rendered by MRI microscopy. The complexes contain either one of two Gd^{3+} chelates, DTPA or DOTA as the MRI component, and a fluorescent dye, tetramethylrhodamine. The fluorescent functionality of these agents provides the advantage of allowing us to score the accuracy of labeled specimens immediately after injection, and to follow the initial stages of development using epifluorescence microscopy. Further, after careful analysis of cell divisions and cell lineage distributions of developing embryos, the complexes were shown not to interfere with normal development. Employing this class of agents in the study of embryonic cell lineage permits the acquisition of three-dimensional data sets that are not limited by the nature of the specimen.

ACKNOWLEDGMENT

Support provided by the Human Brain Project with contributions from the National Institute on Drug Abuse, the National Institute of Mental Health, and the National Science Foundation. M.M.H. thanks the Deutsche Forschungsgemeinschaft for support.

LITERATURE CITED

- (1) Keller, H. E. (1995) in *Handbook of Biological Confocal Microscopy* (J. B. Pawley, Ed.), 2nd ed., p 111, Plenum Publishing, New York.
- (2) Jacobs, R. E., and Fraser, S. E. (1994) Magnetic Resonance Microscopy of Embryonic Cell Lineages and Movements. *Science* 263, 681–684.
- (3) Muller, R. N. (1996) in *Molecular Magnetism: From Molecular Assemblies to the Device* (E. Coronado, Ed.) pp 553–560, Kluwer Academic Publishers, Dordrecht, The Netherlands.
- (4) Moats, R. A., Fraser, S. E., and Meade, T. J. (1997) A "Smart" Magnetic Resonance Imaging Agent that Reports on Specific Enzymatic Activity. *Angew. Chem., Int. Ed. Engl.* 36, 726–728.
- (5) Liang, Z. P., and Lauterbur, P. C. (1994) An Efficient Method for Dynamic Magnetic Resonance Imaging. *IEEE Trans. Med. Imaging* 13, 677–686.
- (6) Bowtell, R. W., Sharp, J. C., Mansfield, P., Hsu, E. W., Aiken, N., and Horsman, A. (1995) NMR Microscopy of Single Neurons Using Spin-Echo and Line Narrowed 2DFT Imaging. *Magn. Reson. Med.* 33, 790–794.
- (7) Kayyem, J. F., Kumar, R. M., Fraser, S. E., and Meade, T. J. (1995) Receptor-Targeted Co-Transport of DNA and Magnetic Resonance Contrast Agents. *Chem. Biol.* 2, 615–620.
- (8) Kumar, K., and Tweedle, M. F. (1993) Macrocyclic Polyaminocarboxylate Complexes of Lanthanides as Magnetic-Resonance-Imaging Contrast Agents. *Pure Appl. Chem.* 65, 515–520.
- (9) Alexander, V. (1995) Design and Synthesis of Macrocyclic Ligands and their Complexes of Lanthanides and Actinides. *Chem. Rev.* 95, 273–342.
- (10) Jacques, V., Gilsoul, D., Comblin, V., and Desreux, J. F. (1997) Rigidified Macrocyclic Lanthanide Chelates for Magnetic-Resonance-Imaging. *J. Alloys Compd.* 249, 173–177.
- (11) Aime, S., Botta, M., Fasano, M., Marques, M. P., Gerlades, C. F. G. C., Pubatz, D., and Merbach, A. E. (1997) Conformational and Coordination Equilibria on DOTA Complexes of Lanthanide Metal Ions in Aqueous Solution Studied by ^1H -NMR Spectroscopy. *Inorg. Chem.* 36, 2059–2068.
- (12) McMurphy, T. J., Brechbiel, M., Kumar, K., and Gansow, O. A. (1992) Convenient Synthesis of Bifunctional Tetraaza Macrocycles. *Bioconjugate Chem.* 3, 108–117.
- (13) Garrity, M. L.; Brown, G. M., Ebert, J. E., and Sachleben, R. A. (1993) A New Synthetic Route to 2-(p-Nitrobenzyl)-1,4,7,10-Tetraazacyclododecane. *Tetrahedron Lett.* 34, 5531–5534.
- (14) Takenouchi, K., Tabe, M., Watanabe, K., Hatzato, A., Kato, Y., Shionoya, M., Koike, T., and Kimura, E. (1993) Novel Pendant-Type Macrocyclic Bifunctional Chelating-Agents—(Carboxymethyl)amino-1,4,7,10-tetraazacyclododecane- N,N',N'',N''' -tetraacetic acid and their Complex Formation with Yttrium(III). *J. Org. Chem.* 58, 6895–6899.
- (15) Ansari, M. H., Ahmad, M., and Dicke, K. A. (1993) Synthesis of N -[3-(p-Aminophenyl)-1-carboxypropyl] Derivatives of 1,4,7-Triazacyclononane- N,N' -Diacetic Acid and 1,4,7,10-Tetraazacyclododecane- N,N',N'',N''' -Triacetic Acid—Macrocyclic Bifunctional Chelating-Agents Useful for Antibody Labeling. *Bioorg. Med. Chem.* 3, 1067–1070.
- (16) Renn, O., and Meares, C. F. (1992) Large Scale Synthesis of the Bifunctional Chelating Agent 2-(p-Nitrobenzyl)-1,4,7,10-Tetraazacyclododecane- N,N',N'',N''' -Tetraacetic Acid, and the Determination of its Enantiomeric Purity by Chiral Chromatography. *Bioconjugate Chem.* 3, 563–569.
- (17) Kang, S. I., Ranganathan, R. S., Emswiler, J. E., Kumar, K., Gougoutas, J. Z., Malley, M. F., and Tweedle, M. F. (1993) Synthesis, Characterization, and Crystal-Structure of the Gadolinium(III) Chelate of (1*R*,4*R*,7*R*)- α,α',α'' -Trimethyl-1,4,7,10-Tetraazacyclododecane-1,4,7-Triacetic Acid (DO3MA). *Inorg. Chem.* 32, 2912–2918.
- (18) Bourat, G., and Margraff, R. (1973) Sulfonated Polymers Derived from L-Lysine. German Patent 2,322,310; U.S. Patent 3,907,755.
- (19) Bligh, S. W. A., Harding, C. T., Sadler, P. J., Bulman, R. A., Bydder, G. M., Pennock, J. M., Kelly, J. D., Latham, I. A., and Marriott, J. A. (1991) Use of Paramagnetic Chelated Metal Derivatives of Polysaccharides and Spin-Labeled Polysaccharides as Contrast Agents in Magnetic-Resonance-Imaging. *Magn. Reson. Med.* 17, 516–532.
- (20) Hirose, G., and Jacobson, M. (1979) Clonal Organization of the Central Nervous System of the Frog. I. Clonal Stemming from Individual Blastomers of the 16-Cell and Earlier Stages. *Dev. Biol.* 71, 191–202.
- (21) Moody, S. A. (1987) Fates of the Blastomers of the 16-Cell Stage *Xenopus* Embryo. *Dev. Biol.* 119, 560–578.

Synthesis of Disulfide-Containing Phospholipid Analogs for the Preparation of Head Group-Specific Lipid Antigens: Generation of Phosphatidylserine Antibodies

Cecilia Diaz, Krishnakumar Balasubramanian, and Alan J. Schroit*

Department of Cell Biology, The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030. Received August 20, 1997; Revised Manuscript Received November 14, 1997[®]

In this report, we describe a new approach for the production of lipid antigens that elicit specific immune responses against phosphatidylserine (PS). Because phospholipids are small nonimmunogenic haptens, PS analogs containing activated coupling groups were synthesized and covalently attached to carrier proteins. Sulfhydryl-reactive PS was generated by acylation of 1-oleoyl-2-(aminocaproyl)-phosphatidylcholine with *N*-succinimidyl-3-(2-pyridyldithio) propionate, converted to PS by phospholipase D-catalyzed base exchange with L-serine, and conjugated to carrier proteins by thiol–disulfide exchange. Antisera to these lipid hapten–protein carrier conjugates were developed in rabbits. Antibodies bound PS but not phosphatidylcholine (PC), phosphatidylglycerol, phosphatidic acid, or phosphatidylethanolamine (PE) when presented together with PC. Inhibition studies using water-soluble lipid analogs and sonicated vesicles indicated that antibody specificity was directed toward the lipid's polar head group. These antibodies also inhibited the PS-dependent prothrombinase activity assay by ~60%. These data show that the covalent coupling of phospholipid haptens to protein carriers via the lipid's fatty acyl side chains preserves its primary head group moiety for the production of specific lipid antibodies.

Antibodies against different membrane components have become an indispensable aid in the study of membrane structure and function. Unlike protein antigens, little attention has been given to the application of lipid-specific antibodies for studying lipid-dependent processes. Because of the inherent difficulty of producing antibodies against small highly conserved lipids, the development of lipid antibodies has progressed slowly. Nonetheless, several laboratories have produced antibodies against certain phospholipid species by immunization with liposomes (Maneta-Peyret et al., 1988, 1989; Benerji and Alving, 1990) or by adsorption of monomeric phospholipids to proteins (Tamamura et al., 1971; Maneta-Peyret et al., 1989), bacteria (Umeda et al., 1989), and acrylamide (Maneta-Peyret et al., 1988, 1989). Due to the apparent lack of specific lipid adsorption, antibodies produced by these methods may cross-react with different lipids (Umeda et al., 1989; Benerji and Alving, 1990) and other phosphate-containing moieties (Alving, 1986).

Because PS¹ can be considered to be a nonimmunogenic hapten, we reasoned that an appropriate lipid–protein

conjugate might elicit a potent and specific immune response. To address this issue and overcome the inherent problems of lipid immunogenicity and cross-reactivity, we designed a lipid–protein conjugate in which the lipid hapten reacts exclusively through a noncritical reactive site at the acyl side chain. In this paper, we report on the synthesis of a phosphatidylcholine analog that contains a “sulfhydryl-activated” coupling group at the end of the 2-position side chain by reacting SPDP with 1-oleoyl-2-(aminocaproyl)phosphatidylcholine (NH₂-PC). The thiolated lipid was then converted into an activated phosphatidylserine derivative by phospholipase D-catalyzed base exchange and then coupled to a sulfhydryl-containing protein carrier and presented to the immune system. This antigen induced the production of PS-specific antibodies which could become potentially useful for the study of lipid-dependent processes and the distribution of specific lipid species in cell membranes.

EXPERIMENTAL PROCEDURES

Materials. PS, PA, PC, PG and PE were purchased from Avanti Biochemicals (Alabaster, AL). *N*-succinimidyl-3-(2-pyridyldithio) propionate (SPDP) and 2-IT were purchased from Pierce (Rockford, IL). BSA, prothrombin, factor X, and analytical reagents were from Sigma (St. Louis, MO). Factor V was purified as previously described (Connor et al., 1989). Phospholipase D (*Streptomyces chromofuscus*) was from Calbiochem (LaJolla, CA), and S2238 was purchased from Kabi Laboratories (Franklin, OH). Human red blood cells (RBCs) were obtained from healthy volunteers by venipuncture into heparinized syringes.

General Procedures. UV adsorption was recorded on a Gilford response spectrophotometer. ELISAs were recorded on a Samtron CERES UV900C instrument at 450 nm. The molecular mass of BSA was assumed to be

* To whom correspondence should be addressed: Department of Cell Biology, Box 173, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030. Telephone: (713) 792-8586. Fax (713) 792-8394. E-mail: sa10015@odin.mdacc.tmc.edu.

[®] Abstract published in *Advance ACS Abstracts*, December 15, 1997.

¹ Abbreviations: 2-IT, 2-iminothiolane; BSA, bovine serum albumin; DTNB, dithiobis(nitrobenzoic acid); DTT, dithiothreitol; NH₂-PC, 1-oleoyl-2-(aminocaproyl)phosphatidylcholine; PA, dioleoylphosphatidic acid; PC, dioleoylphosphatidylcholine; PE, dioleoylphosphatidylethanolamine; PG, dioleoylphosphatidylglycerol; PS, phosphatidylserine; SPDP, *N*-succinimidyl-3-(2-pyridyldithio) propionate; SUV, small unilamellar vesicles; TLC, thin-layer chromatography.

65 kDa. The extinction coefficients used for DTNB and thiopyridine were 14 150 (412 nm) and 8 000 (312 nm), respectively.

The purities of the lipid compounds were assessed after TLC on precoated silica gel 60 plates (Merck). Lipids were purified by silica gel column chromatography. API electrospray mass spectra were recorded by Avanti Polar Lipids (Alabaster, AL) on a HP 5989A mass spectrometer.

Syntheses. *1-Oleoyle-2-(aminocaproyl)phosphatidylcholine (NH₂-PC).* NH₂-PC was synthesized as previously described (Schroit and Madsen, 1983). Briefly, aminocaproic acid was protected by *tert*-butoxycarbonylation, reacted with dicyclohexylcarbodiimide to yield the [(*tert*-Boc)amino]caproic anhydride, condensed with lysophosphatidylcholine to yield [[(*tert*-Boc)amino]caproyl]phosphatidylcholine, and finally deblocked with anhydrous HCl.

1-Oleoyle-2-N-succinimidyl-3-(2-pyridyldithio) Propionyl(aminocaproyl)-PC (SPDP-PC). NH₂-PC (20 μ mol) was dried under N₂ and resuspended in CHCl₃/MEOH/triethylamine (1/2/0.015) (3.0 mL) containing SPDP (40 μ mol) for 16 h at 4 °C. The lipid was extracted by adding CHCl₃ (1 mL) and water (1.8 mL). The organic extract containing the product was dried under reduced pressure. The residue was analyzed by TLC (65/25/4 CHCl₃/MEOH/H₂O) and revealed a single phosphate-positive, ninhydrin-negative spot ($R_f = \sim 0.4$). Mass analysis m/z calcd for C₄₀H₆₉N₃O₉PS₂ (M + Na⁺) 854.08, found 854.7.

1-Oleoyle-2-N-succinimidyl-3-(2-pyridyldithio) Propionyl(aminocaproyl)-PS (SPDP-PS). SPDP-PC (20 μ mol) was dried under N₂ and resuspended in 50% L-serine (1 mL) in acetate buffer (0.1 M, pH 5.6) containing CaCl₂ (0.1 M). Ether (1 mL) and phospholipase D (25 units, 70 μ L) were added, and the suspension was mixed at 45 °C for 3 h. The reaction was stopped by adding EDTA (to 0.2 M). The ether was then evaporated, and the product was resuspended in CHCl₃/MEOH/H₂O (1/2/0.8). The suspension was cooled to 4 °C, and the resulting L-serine precipitate was removed by centrifugation. The product was recovered from the organic phase after the addition of CHCl₃ and water (1 volume each). The organic phase was taken to dryness, dissolved in CHCl₃, and applied to a 2 $\times$ 30 cm column of activated pre-washed silica gel. The column was washed with CHCl₃ (100 mL), followed by 100 mL aliquots of CHCl₃ containing increasing MEOH. Analysis of the product which eluted with CHCl₃/MEOH (6/4) by TLC revealed a single phosphate- and ninhydrin-positive spot ($R_f = \sim 0.2$). The purified product was stored at -70 °C in CHCl₃. Mass analysis m/z calcd for C₃₈H₆₃N₃O₁₁PS₂ (M) 833.02, found 832.7.

Reaction of 2-IT with Carrier Proteins. BSA (10 mg) was solubilized in Tris-EDTA buffer [1 mL, 10 mM Tris (pH 8.0) and 0.1 mM EDTA]. 2-IT was added at a 100-fold molar excess, and the reaction was allowed to proceed for 1 h at 20 °C (Jue et al., 1978). The solution was then dialyzed overnight against deaerated, N₂-flushed Tris-EDTA. The proteins (1 mL) were then mixed with DTT (0.05 mL 0.1 M) for 30 min at 20 °C. The reduced proteins were then purified by exclusion chromatography on a Biogel P6 column (15 $\times$ 500 mm). Peak fractions were collected, and available sulfhydryls and protein concentrations were estimated spectrophotometrically by thiol-disulfide exchange with DTNB as described by Riddles et al. (1983) and by adsorption at 280 nm, respectively.

Coupling of SPDP-PS to Protein Carriers. Reduced, purified, 2-IT-treated BSA (63 nmol in 2 mL, 1300 nmol of SH) was immediately mixed with 1 SH equiv

molar of SPDP-PS in 1/10 volume of ethanol. The efficiency of derivatization was estimated after 10 min by assessing the stoichiometric release of 2-thiopyridine at 343 nm (Grassetti and Murray, 1967). Excess reagents were removed by dialysis against NaCl (0.15 M). The products were stored at -70 °C.

Immunization Protocol. Rabbits were injected in multiple intradermal sites with ~ 1 mg of the lipid-protein conjugates (~ 0.25 mg of lipid hapten) in complete Freund's adjuvant followed by a boost 1 month later in incomplete Freund's adjuvant. The rabbits were bled 2 weeks after the last injection.

Enzyme-Linked Immunoabsorbent Assays (ELISAs). Polystyrene microtiter plates were coated overnight with 30 μ L/well of the indicated phospholipids (200 μ g of lipid/mL) in CHCl₃/MEOH (1/50) at room temperature. The plates containing the evaporated lipids were blocked with goat serum (10% in 0.8% NaCl/20 mM Tris at pH 8.0) for 1 h at room temperature. Antiserum samples prepared in blocking solution were applied to the wells at different dilutions (reported in the figure captions) for 2 h, and binding was assessed by adding anti-rabbit (whole molecule) peroxidase conjugate (1/10 000 dilution in 0.8% NaCl/20 mM Tris at pH 8.0) for 2 h. TMB-ELISA (3,3',5,5'-tetramethylbenzidine base) was used as the substrate, and the ELISA was stopped with H₂SO₄ (1 N). Inhibition of immune serum binding to PS was determined with the head group analogs glycerophosphorylserine (GPS), phosphorylserine (PhoS), serine, glycerophosphorylethanolamine (GPE), phosphorylethanolamine (PhoE), and ethanolamine. Inhibition with SUV (0.5 mg/mL) composed of PS (40 mol % in PC), PE (40 mol % in PC), and PC was achieved by the addition of equal volumes of liposomes to antiserum samples diluted in 10% goat serum. After 1 h of incubation at 37 °C, the ELISA was carried out as described above.

Prothrombinase Activity Assay. The PS-dependent prothrombinase assay was carried out as described previously (Diaz et al., 1996) except that PS SUV were used as the procoagulant surface. Briefly, PS SUV (0.05 mL at 1 mg/mL) were incubated with antiserum or control preimmune serum (0.05 mL) for 1 h at 37 °C. The suspension was then added to prothrombinase assay buffer (0.2 mL) containing Ca²⁺ and the necessary coagulation factors for the period of time indicated. Aliquots of the suspension were then transferred to a cuvette containing EDTA buffer (1 mL) to stop the production of thrombin. The thrombin-dependent chromogen, S2238, was added to the cuvettes (to 0.2 mM), and the rate of chromophore formation was monitored at 405 nm with a Gilford response spectrophotometer employing appropriate kinetic software. The initial rate of thrombin-dependent chromophore production was determined from the slope of the absorbance curve. These rates were plotted on the ordinate against the incubation time.

RESULTS

Preparation of PS-Carrier Conjugates. Because the specificity of lipid antibodies must be directed to the lipid's polar head group, its modification by the attachment of covalent spacers and/or coupling groups will likely generate immune responses that fail to recognize the native lipid. Reactive thiols placed in the fatty acyl chain, however, would not be expected to change the structure of the polar head group and, when incorporated as a heterobifunctional intermediate, could serve as a

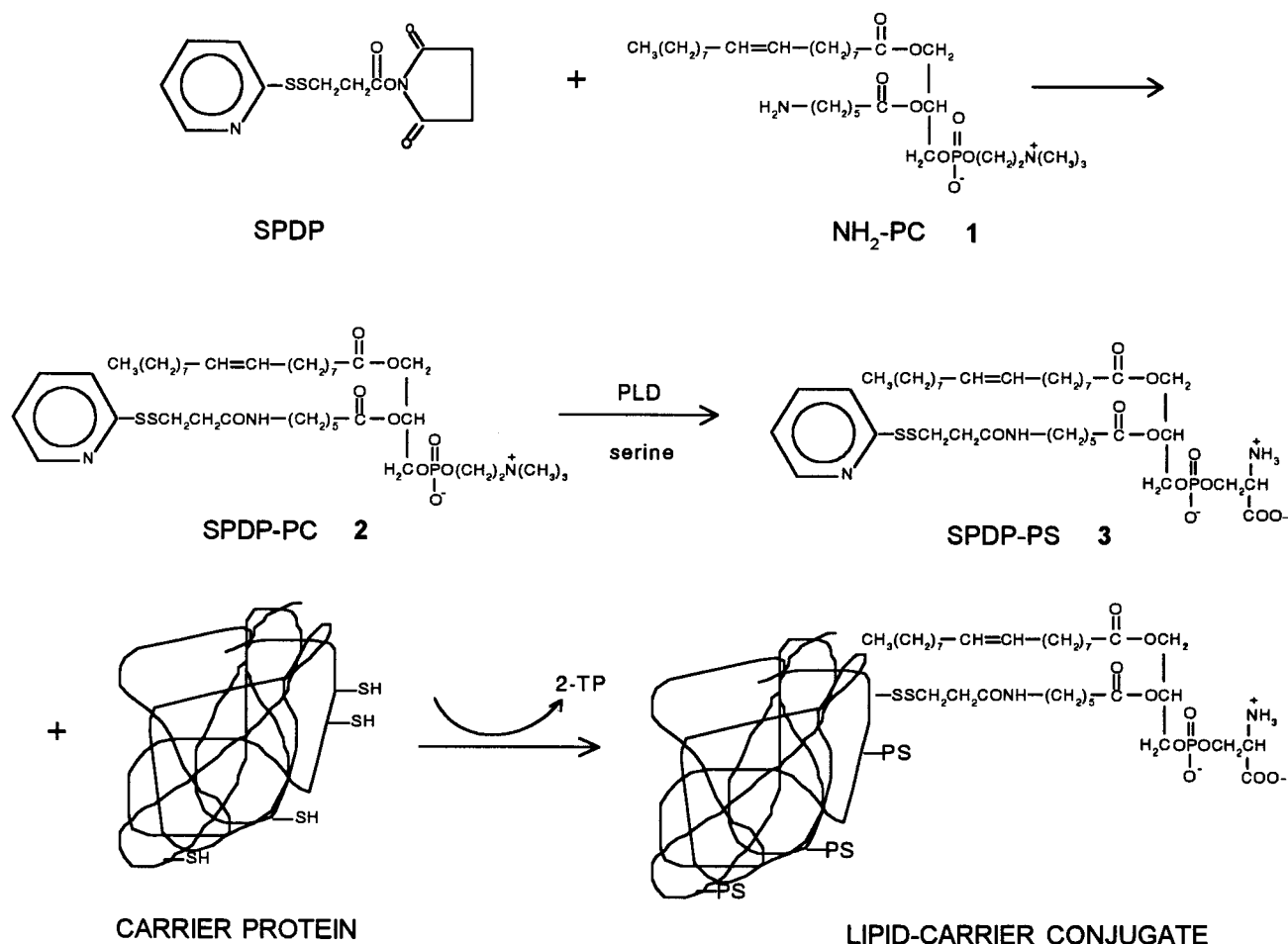


Figure 1. Synthesis of PS-carrier conjugates. $\text{NH}_2\text{-PC}$ was reacted with SPDP, converted to the PS derivative with phospholipase D, and coupled by thiol–disulfide exchange to carrier protein: PLD, phospholipase D; 2-TP, 2-thiopyridine.

convenient attachment point for various proteins or peptides via thiol–disulfide exchange reactions. On this basis, we reasoned that phosphatidylcholine could be modified to contain a single reactive amine in an acyl side chain, and that it could be derivatized with an amine- and sulfhydryl-reactive heterobifunctional reagent. Once coupled, the lipid could be readily converted to other lipid species by phospholipase D-catalyzed base exchange without activating the protected thiol-dependent exchange moiety.

To demonstrate the feasibility of this approach, SPDP was first conjugated to $\text{NH}_2\text{-PC}$ **1** as shown in Figure 1. The product, SPDP–PC **2**, containing a protected disulfide β chain, was obtained in >90% yield. This compound was then converted to the PS derivative **3** with phospholipase D in the presence of L-serine. Lipid–protein coupling was accomplished by thiol–disulfide exchange of the activated lipid haptens to proteins treated with 2-IT (1 mol of SPDP–PS per mole of SH). Coupling was stoichiometric as estimated by monitoring the release of 2-thiopyridine. Coupling ratios were typically 20/1.

Reaction of PS Antibodies with Different Lipids.

Antisera obtained from rabbits immunized against PS–BSA were tested by ELISA for their ability to bind unmodified native phospholipids. The data shown in Figure 2A indicate that the antiserum, but not the preimmune serum, reacted with PS. To determine specificity, the antibody was tested against different phospholipid species (Figure 2B). It can be seen that the antibody did not bind PC or the negatively charged phospholipids, PA and PG. The reactivity against PE

Table 1. Phosphate/Head Group Specificity

$\begin{array}{c} \text{HOCH}_2 \\ \\ \text{HOCH} \text{---} \text{O} \\ \quad \parallel \\ \text{H}_2\text{C} \text{---} \text{OPOR} \\ \\ \text{O}^- \end{array}$	glycerophospho- serine ethanolamine
$\begin{array}{c} \text{O} \\ \parallel \\ \text{HOPOR} \\ \\ \text{O}^- \end{array}$	phospho- serine ethanolamine
$\text{R} = \begin{array}{c} \text{NH}_3^+ \\ \\ \text{---CH}_2\text{CH} \\ \\ \text{COO}^- \\ \text{serine} \end{array} \quad \text{or} \quad \begin{array}{c} \text{---CH}_2\text{CH}_2\text{NH}_3^+ \\ \text{ethanolamine} \end{array}$	

was likely an artifact of lipid deposition on the ELISA plates because reactivity was abolished when the lipid was coated as a 50 mol % mixture with PC. The Activity to PS was retained under the same conditions (Figure 2c).

To determine the epitope responsible for PS binding, the reactivity of the antibodies to the lipid's polar head group was assessed by competitive inhibition with water-

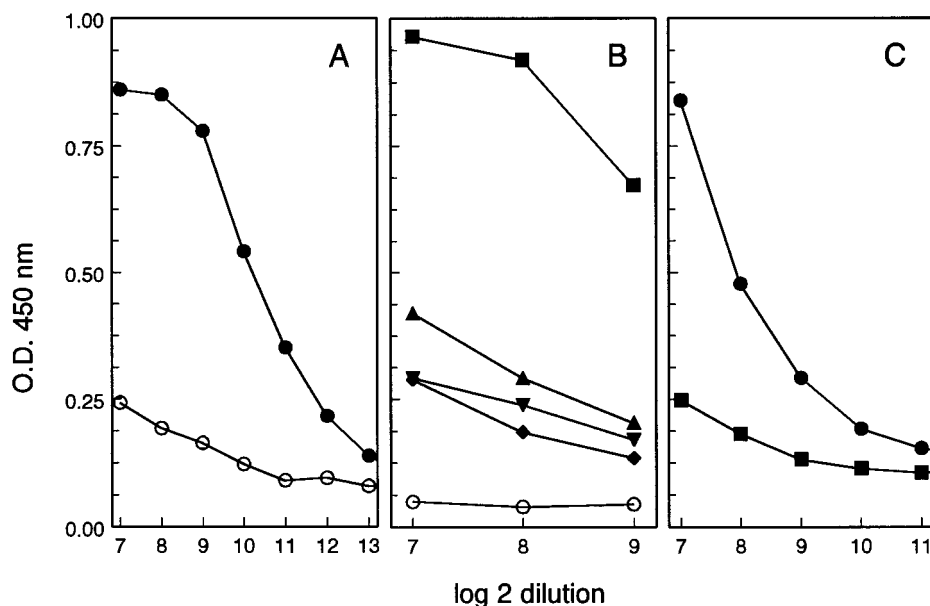


Figure 2. Reactivity of PS antiserum with phospholipids. Microtiter plates were coated with 6 μ g of lipid. Bound antibody was quantified by ELISA with peroxidase-conjugated goat anti-rabbit Ig. (A) Binding of control preimmune serum (○) and antiserum (●) to PS-coated plates. (B) Binding of PS antiserum to polystyrene plates coated with 6 μ g of PE (■), PA (▲), PG (▼), and PC (◆) and preimmune serum to PA-, PG-, PC-, and PE (○)-coated plates. (C) Antiserum binding to PS/PC (●) and PE/PC-coated (■) plates.

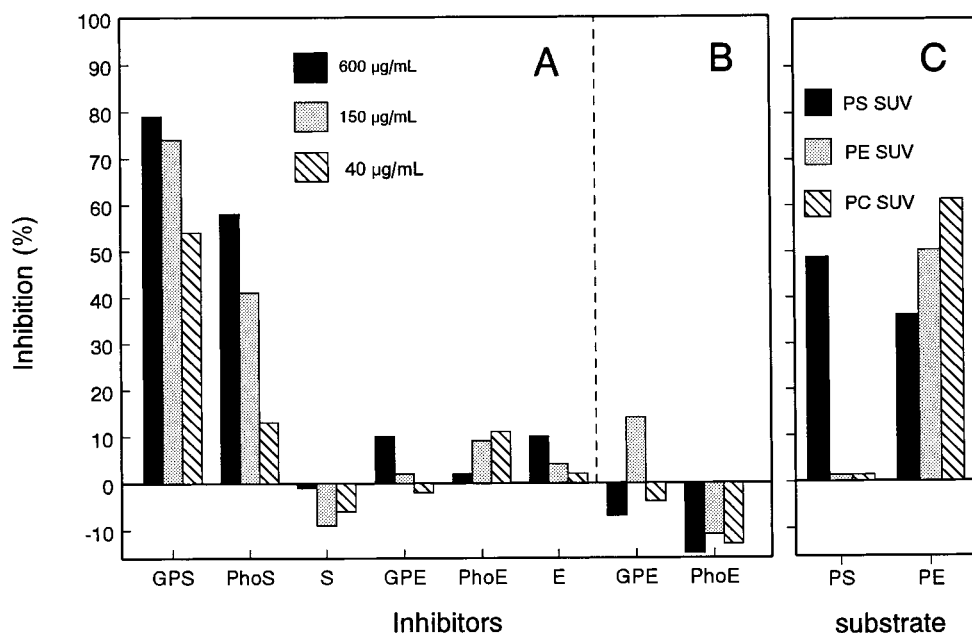


Figure 3. Inhibition of immune serum binding with soluble head group analogs and vesicles. The binding assays were carried out as described in Figure 2 at a serum dilution of 1/388 ($\log 2^{8.6}$). (A) PS- and (B) PE-coated plates in the presence of GPS, PhoS, serine (S), GPE, PhoE, and ethanolamine (E) at the indicated concentrations. (C) Plates coated with PS and PE were assessed for antibody binding in the presence of SUV (0.5 mg/mL) containing PS, PE, and PC.

soluble lipid analogs (Table 1) and liposomes of different lipid compositions. At 600 μ g/mL, GPS and PhoS inhibited binding to the PS-coated plates by ~80 and ~60%, respectively. Serine, GPE, PhoE, and ethanolamine had no effect (Figure 3A). Similar to the lipid analogs, SUV composed of PS/PC (1/1), but not PE/PC (1/1) or PC inhibited antibody binding by ~50% to PS-coated ELISA plates (Figure 3C). Consistent with the notion that antibody binding to pure PE is an artifact, GPE and PhoE did not inhibit binding to PE-coated plates (Figure 3B). Moreover, unlike the specific inhibition obtained with PS-coated plates, antibody binding to PE was inhibited with vesicles irrespective of their lipid composition.

Because these antibodies bind PS, they should also be able to interfere with PS-dependent processes such as

coagulation. To test this, PS vesicles were preincubated with the antiserum for 1 h at 37 °C and the ability of the vesicles to promote the PS-dependent prothrombinase reaction was assessed. The results presented in Figure 4 show that the initial rates of thrombin-dependent S2238 cleavage after the indicated reaction times were inhibited by ~60% in the antiserum-treated samples (calculated from the slopes of the fitted curve).

DISCUSSION

To preserve the structural integrity of phospholipids, it is important to construct conjugates that do not modify the lipid's polar head group. While the amino lipids seem to be amenable to derivatization with peptide or carrier proteins through their head group (Martin and Papa-

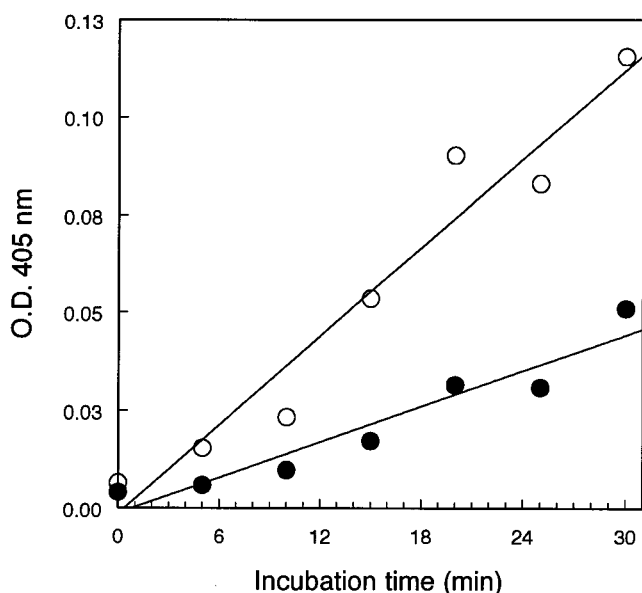


Figure 4. Inhibition of prothrombinase activity. Prothrombinase activity was assessed in the presence of PS-containing vesicles preincubated with the indicated sera. The reaction was stopped at various time points with EDTA, and thrombin production was assessed by determining the initial rates of thrombin-dependent cleavage of the chromogenic substrate. These rates were plotted on the ordinate against the incubation time: preimmune serum (○), antiserum (●).

hajopoulos, 1982; Bate et al., 1993), it is important to realize that such conjugation destroys the lipid's structure. Antibodies obtained with such conjugates cannot be expected to specifically react with natural unmodified phospholipids. Although several laboratories have tried to overcome this problem by using lipids absorbed non-covalently to carriers (Maneta-Peyret et al., 1993; Rote et al., 1993, 1995; Katsuragawa et al., 1995), many of these preparations are not specific and cross-reactivity is common. This may be due to the weak antigenic presentation of the phosphorylated head groups or to the generation of antibodies to diacylglycerol, phosphodiester, and/or fatty acid moieties that are common to all phospholipids.

In an attempt to produce specific PS antibodies, we immunized rabbits with PS covalently coupled to protein carriers via its fatty acid side chain without modifying the crucial phosphoserine moiety. Our data show that the rabbit antibodies specifically recognize PS but not other major phospholipids. The inhibition experiments (Figure 3) show that the presentation of both the serine head group and the phosphate moiety (see Table 1) are required for the specificity of binding to PS. Indeed, the recognition of the serine-phosphate is critical because ethanolamine-phosphate, which is ineffective, differs only by a single carboxyl. These antibodies therefore differ from other lipid antibodies which are partially inhibited by phosphate buffers and phosphorylated nucleotides (Alving, 1986; Banerji and Alving, 1990).

In summary, we have described the synthesis of a novel intermediate sulfhydryl-activated lipid where the coupling group is located on the fatty acyl chain distal from the head group. Thus, the native head group structure is retained even after coupling to carrier proteins. Because the protected intermediate can be used to prepare any glycerophospholipid hapten by phospholipase D-catalyzed base exchange, it should be possible to use this approach for the preparation of other lipid-specific antibodies.

ACKNOWLEDGMENT

The authors thank Dr. Jerald Killion for his kind help and critical comments and Polina Khaskina for technical assistance. This work was supported in part by National Institutes of Health Grant DK 41714.

LITERATURE CITED

- Alving, C. R. (1986) Antibodies to liposomes, phospholipids and phosphate esters. *Chem. Phys. Lipids* 40, 303–314.
- Banerji, B., and Alving, C. R. (1990) Antibodies to liposomal phosphatidylserine and phosphatidic acid. *Biochem. Cell Biol.* 68, 96–101.
- Bate, C. A. W., Taverne, J., Kwiatkowski, D., and Playfair, J. H. L. (1993) Phospholipid coupled to carrier induce IgG antibody that blocks tumor necrosis factor induction by toxic malaria antigens. *Immunology* 79, 138–145.
- Connor, J., Bucana, C., Fidler, I. J., and Schroit, A. J. (1989) Differentiation-dependent expression of phosphatidylserine in mammalian plasma membranes Quantitative assessment of outer leaflet lipid by prothrombinase complex formation. *Proc. Natl. Acad. Sci. U.S.A.* 86, 3184–3188.
- Diaz, C., Morkowski, J., and Schroit, A. J. (1996) Generation of phenotypically aged phosphatidylserine-expressing erythrocytes by dilauroylphosphatidylcholine-induced vesiculation. *Blood* 87, 2956–2961.
- Grassetti, D. R., and Murray, J. F. Jr., (1967) Determination of sulfhydryl groups with 2,2'- or 4,4'-dithiodipyridine. *Arch. Biochem. Biophys.* 119, 41–49.
- Jue, R., Lambert, J. M., Pierce, L. R., and Traut, R. R. (1978) Addition of sulfhydryl groups to *Escherichia coli* ribosomes by protein modification with 2-iminothiolane (methyl 4-mercaptopbutyrimidate). *Biochemistry* 17, 5399–5405.
- Katsuragawa, H., Rote, N. S., Inoue, T., Narukawa, S., Kanzaki, H., and Mori, T. (1995) Monoclonal anti-PS antibody reactivity against human first-trimester placental trophoblasts. *Am. J. Obstet. Gynecol.* 172, 1592–1597.
- Maneta-Peyret, L., Bessoule, J. J., Geffard, M., and Cassagne, C. (1988) Demonstration of high specificity antibodies against phosphatidylserine. *J. Immunol. Methods* 108, 123–127.
- Maneta-Peyret, L., Freyburger, G., Bessoule, J. J., and Cassagne, C. (1989) Specific immunocytochemical visualization of phosphatidylserine. *J. Immunol. Methods* 122, 155–159.
- Maneta-Peyret, L., Biron, C., Previsani, C., Moreau, P., Bezian, J. H., and Cassagne, C. (1993) Demonstration that anti-phospholipid auto-antibodies react with both anionic and zwitterionic phospholipids. *Immunol. Lett.* 35, 141–146.
- Martin, F. J., and Papahajopoulos, D. (1982) Irreversible coupling of immunoglobulin fragments to preformed vesicles. *J. Biol. Chem.* 257, 286–288.
- Riddles, P. W., Blakely, R. L. and Zerner, B. (1983) Reassessment of Ellman's reagent. *Methods Enzymol.* 91, 49–60.
- Rote, N. S., Ng, A. K., Dostal-Johnson, D. A., Nicholson, S. L., and Siekman, R. (1993) Immunologic detection of phosphatidylserine externalization during thrombin-induced platelet activation. *Clin. Immunol. Immunopathol.* 66, 193–200.
- Rote, N. S., Chang, J., Katsuragawa, H., Ng, A. K., Lynden, T. W., and Mori, T. (1995) Expression of phosphatidylserine-dependent antigens on the surface of differentiation BeWo human choriocarcinoma cells. *Am. J. Rep. Immunol.* 33, 114–121.
- Schroit, A. J., and Madsen, J. (1983) Synthesis and properties of radioiodinated phospholipid analogs that spontaneously undergo vesicle-vesicle and vesicle-cell transfer. *Biochemistry* 22, 3617–3623.
- Tamamura, S., Hashimoto, T., and Hara, I. (1971) The immunological reactions between acidic phospholipids and their antibodies. *Jpn. J. Exp. Med.* 41, 31–38.
- Umeda, M., Igarashi, K., Nam, K. S., and Inoue, K. (1989) Effective production of monoclonal antibodies against phosphatidylserine: Stereo-specific recognition of phosphatidylserine by monoclonal antibody. *J. Immunol.* 143, 2273–2279.

BC970156X

Development and Activities of a New Melphalan Prodrug Designed for Tumor-Selective Activation

David E. Kerr, Zhengong Li, Nathan O. Siemers, Peter D. Senter, and Vivekananda M. Vrudhula*

Bristol-Myers Squibb Pharmaceutical Research Institute, 3005 First Avenue, Seattle, Washington 98121.
Received September 12, 1997; Revised Manuscript Received January 5, 1998

The synthesis of C-Mel, a cephalosporin carbamate derivative of the clinically used alkylating agent melphalan, is described. C-Mel was designed as an anticancer nitrogen mustard prodrug that releases melphalan upon tumor-specific activation by targeted β -lactamase (bL). The K_m and k_{cat} values for bL hydrolysis of C-Mel were 218 μ M and 980 s^{-1} , respectively. In vitro cytotoxicity assays with 3677 human melanoma cells demonstrated that C-Mel was 40-fold less toxic than melphalan and was activated in an immunologically specific manner by L49-sFv-bL, a recombinant fusion protein that binds to the melanotransferrin antigen on melanomas and on some carcinomas. L49-sFv-bL in combination with C-Mel led to regressions and cures of established subcutaneous 3677 tumors in nude mice. The effects were significantly greater than those of melphalan, which did not result in any long-term regressions in this tumor model. The therapeutic effects were comparable to those obtained in mice treated with the previously described L49-sFv-bL/7-(4-carboxybutanamido)-cephalosporin mustard (CCM) combination. However, C-Mel may be more attractive than CCM for clinical development since the released drug is clinically approved.

INTRODUCTION

Targeting cytotoxic agents to sites of malignancy using monoclonal antibodies (mAb)¹ that bind to tumor-associated antigens has been the subject of a great deal of research. Among the cytotoxic moieties delivered using this strategy are radionuclides (1, 2), protein toxins (3), and chemotherapeutic drugs (4), all of which involve covalent attachment of the targeted molecule to the mAb. An alternative strategy is to use mAb-enzyme conjugates for the activation of anticancer prodrugs. In this two-step process, a mAb-enzyme conjugate is used to convert a prodrug substrate for the targeted enzyme to an active drug in a site-selective manner. Several reviews discussing this approach are currently available (5–7). Earlier work has demonstrated that this approach can generate higher intratumoral levels of drug (8–10) and better therapeutic effects than systemic administration of drug alone (11–15).

β -Lactamases (bL) are enzymes that have been shown to be very effective and versatile for anticancer prodrug activation (8, 11, 13, 15–21). These monomeric enzymes are abundant, available in multiple forms, inexpensive, and retain their activity in vivo. Furthermore, a variety of anticancer drugs can be appended to cephalosporin so that, upon β -lactam hydrolysis, a fragmentation reaction leads to the release of active drug (Scheme 1). Several studies have demonstrated that bL can release phenylenediamine mustard (13, 17), doxorubicin (18), mitomycin C (16), platinum compounds (19), and paclitaxel (20) from

cephalosporin prodrugs. We have previously shown that L6-bL in combination with a cephalosporin doxorubicin prodrug led to antitumor activities in two models of human lung carcinoma (8). Significantly better activities were obtained in melanoma models using either chemically conjugated L49-bL (13, 21) or the single-chain Fv-containing fusion protein L49-sFv-bL (15) in combination with CCM, a prodrug of phenylenediamine mustard. Because of the pronounced activities obtained using alkylating agents in our studies (13, 15, 21) and in others (12, 14), we were prompted to develop cephalosporin prodrugs of a clinically approved nitrogen mustard. One agent that came to our attention was melphalan, an anticancer drug that alkylates DNA, mainly at the N-7 position of guanine residues (22). Since it is known that amide derivatives of melphalan are generally less cytotoxic than the parent drug (23), we reasoned that attachment of melphalan through the primary amine to a cephalosporin via a carbamate linkage would greatly reduce its cytotoxic activity. In this article, we report the synthesis and in vitro and in vivo properties of C-Mel, a cephalosporin prodrug of melphalan.

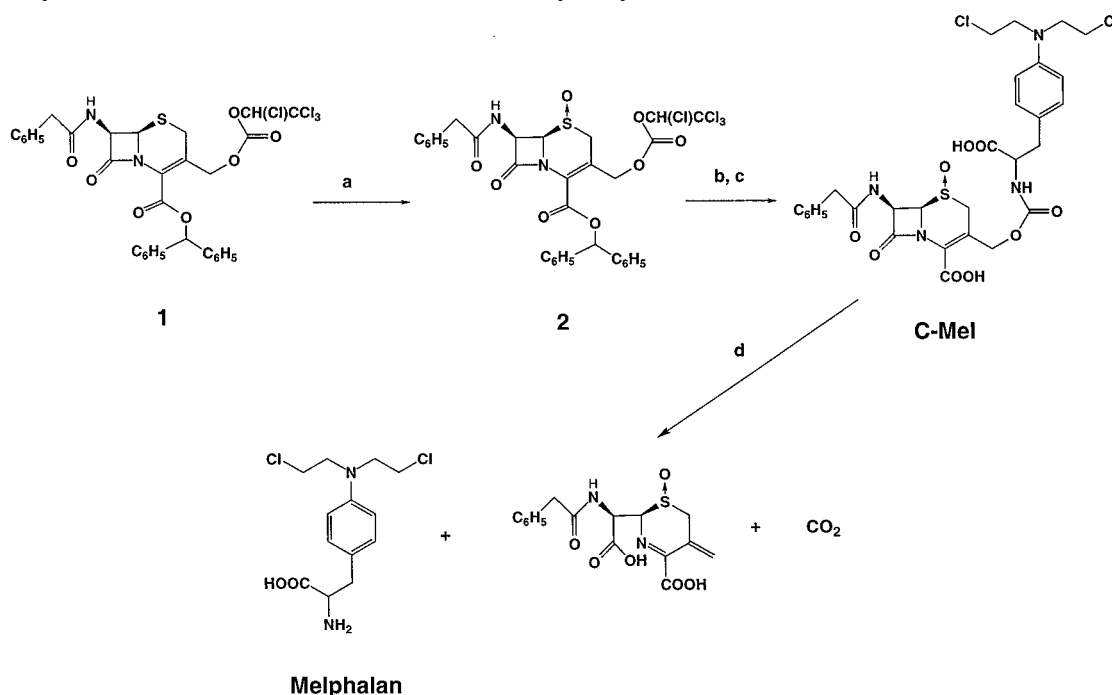
MATERIALS AND METHODS

Materials. L49 is a mAb that binds to the melanotransferrin antigen expressed on most human melanomas (15). L49-sFv-bL was expressed in *Escherichia coli* and purified as described earlier (15). The bL used was a mutated form of the enzyme from *Enterobacter cloacae* (15, 24). CCM was synthesized as described earlier (17). HPLC analyses were performed on a Perkin-Elmer LC Bio 410 instrument equipped with a 5 μ m C-18 column and a UV detector set at 264 nm. Acetonitrile/0.1 M sodium phosphate (1:1, pH 1.8) was used as the eluting solvent to monitor the enzymatic hydrolysis of the prodrug.

Preparation of 7-(Phenylacetamido)-3-[[[(1',2',2',2'-tetrachloroethoxycarbonyl)oxy]methyl]ceph-3-em-

* To whom correspondence should be addressed at Bristol Myers Squibb Pharmaceutical Research Institute, 5 Research Parkway, Wallingford, CT 06492.

¹ Abbreviations: bL, β -lactamase; CCM, 7-(4-carboxybutanamido)cephalosporin mustard; mAb, monoclonal antibody; C-Mel, cephalosporin melphalan; IC₅₀, concentration causing 50% inhibition; L49-sFv-bL, fusion protein of the L49 sFv fragment attached to *Enterobacter cloacae* bL; MTD, maximum tolerated dose; PDM, phenylenediamine mustard.

Scheme 1. Synthesis of C-Mel and bL-Mediated C-Mel Hydrolysis^a

^a (a) 3-Chloroperoxybenzoic acid/CH₂Cl₂; (b) melphalan/THF, aqueous NaHCO₃; (c) TFA–anisole/CH₂Cl₂; (d) β-lactamase.

4-carboxylic Acid 1-Oxide Diphenyl Methyl Ester (2). An ice cold solution of the corresponding thioether (**1**) (25) (7.10 g, 9.80 mmol) in methylene chloride (300 mL) was treated with 3-chloroperoxybenzoic acid (3.05 g, 80% purity). After 15 min, a cold solution containing a 10% sodium bisulfite solution (100 mL) and a 0.1 N sodium bicarbonate solution (100 mL) was added. The biphasic mixture was stirred at 0 °C for 10 min, and the layers were separated. The organic layer was washed with ice cold 0.1 N sodium bicarbonate solution (3 × 100 mL) and saturated NaCl (100 mL), dried over MgSO₄, and concentrated to approximately 80 mL, at which point crystallization occurred. After cooling in ice, the crystalline material was filtered to give 5.20 g (7.01 mmol, 72%) of sulfoxide (**2**) as a white solid. MS (*M* + NH₄⁺): 756 (found), 756 (calcd). ¹H NMR (CDCl₃): δ 7.60–7.24 (m, 15H, ArH), 6.92 (s, 1H, CHCl), 6.69 (d, 1H, *J*_{NH,7} = 9.9 Hz, 7-NH), 6.62 (s, 1H, CHPh₂), 6.11 (dd, 1H, *J*_{6,7} = 4.8 Hz, H-7), 5.61 (d, 1H, *J*_{AB} = 14.1 Hz, CH_{2A}OCO), 4.85 (d, 1H, H-6), 4.54 (d, 1H, H-6), 3.80 (d, 1H, *J*_{AB} = 18.8 Hz, H-2A), 3.67 (d, 1H, CH_{2A}Ph, *J* = 6.3 Hz), 3.60 (d, 1H, CH_{2B}Ph), 3.20 (d, 1H, H-2B). Anal. Calcd for C₃₂H₂₆N₂Cl₄O₈S: C, H, N, S, Cl, 19.15%. Found: C, H, N, S, Cl, 19.87%.

Preparation of C-Mel. To a solution of melphalan (412 mg, 1.35 mmol) in a mixture of 0.25 N sodium bicarbonate (14 mL) and THF (28 mL) was added the activated sulfoxide (**2**) (1.00 g, 1.35 mmol). The resulting suspension turned clear after sonicating at room temperature for 15 min. The reaction mixture was stirred for an additional 10 min at room temperature and cooled in an ice bath; 1 N HCl (5 mL) was added, and the reaction mixture was extracted with ethyl acetate (3 × 40 mL). The combined organic layer was washed with water (80 mL) and brine (80 mL), dried over MgSO₄, and evaporated in vacuo. The resulting residue was suspended in methylene chloride (50 mL) and anisole (5 mL) at 0 °C, and trifluoroacetic acid (5 mL) was added over a period of 5 min. The resulting reaction mixture was stirred at 0 °C for 45 min. Volatile materials were then removed under reduced pressure. The residue was

dissolved in a minimum amount of dichloromethane, and the solution was poured into ice cold hexane (100 mL). The white precipitate formed was filtered and recrystallized from absolute ethanol/methylene chloride (1:1) to give 445 mg (0.60 mmol, 44%) of the title compound as an off-white solid. MS for C₃₀H₃₃Cl₂N₄O₉S (MH⁺): 695 (calcd), 695 (found). ¹H NMR (DMSO-*d*₆): δ 13.80 (br s, 1H, COOH), 12.71 (br s, 1H, COOH), 8.44 (d, 1H, *J* = 8.3 Hz, OCONH), 7.67 (d, 1H, *J*_{NH,7} = 8.3 Hz, 7-NH), 7.32–7.20 (m, 5H, 7-ArH), 7.08 (d, 2H, *J* = 8.6 Hz, 2Ar'H), 6.64 (d, 2H, 2Ar'H), 5.79 (dd, 1H, *J*_{6,7} = 4.8 Hz, H-7), 5.06 (d, 1H, *J*_{AB} = 13.3 Hz, CH_{2A}OCONH), 4.81 (d, 1H, H-6), 4.54 (d, 1H, CH_{2B}OCONH), 4.05–4.01 (m, 1H, NHCHCOOH), 3.78–3.21 (m, 12H, H-2A, H-2B, PhCH₂-CO, 2 NCH₂CH₂Cl), 2.91 (dd, 1H, *J*_{AB} = 13.8 Hz, *J*_{A,X} = 4.4 Hz, CHCH_{2A}Ph-N), 2.70 (dd, 1H, *J*_{B,X} = 10.1 Hz, CHCH_{2B}Ph-N). Anal. Calcd for C₃₀H₃₂Cl₂N₄O₉S·1C₂H₅OH·1H₂O: C, H, N, Cl, S.

Enzyme Kinetics. The hydrolysis of C-Mel by bL was measured as described previously (17). The cleavage of the β-lactam ring results in a loss of absorbance at 260 nm. Initial velocities of β-lactam hydrolysis were determined at room temperature, and a Lineweaver–Burk plot of the data was used to estimate the *K*_m and *k*_{cat} values. The bL concentration was 25 ng/mL, and the C-Mel concentration was 20–100 μM.

In Vitro Cytotoxicity. Type 3677 cells in Iscove's modified Dulbecco's medium containing 10% fetal bovine serum, 60 μg/mL penicillin, and 100 μg/mL streptomycin were plated into 96-well microtiter plates at 10⁴ cells/mL and allowed to adhere overnight at 37 °C. For blocking experiments, the cells were incubated at 4 °C with unconjugated L49 at 2 μM (300 μg/mL) for 30 min prior to treatment with L49–sFv–bL. The cells were then treated with 2 nM L49–sFv–bL (134 ng/mL). After 30 min at 4 °C, the plates were washed four times with antibiotic-free Roswell Park Memorial Institute 1640 media with 10% fetal bovine serum, and then varying concentrations of C-Mel were added. C-Mel and melphalan were also added to cells treated with media alone. After 4 h at 37 °C, cells were washed three times with

Iscove's modified Dulbecco's medium and incubated for approximately 12 h at 37 °C. The cells were then pulsed for 18 h with [^3H]thymidine (1 $\mu\text{Ci}/\text{well}$) at 37 °C, detached by freezing at -20 °C and thawing, and harvested onto glass fiber filter mats using a 96-well harvester. Radioactivity was counted using a LKB Wallac β -plate counter.

Therapy Experiments. Type 3677 tumor-bearing mice (subcutaneous implants, five animals/group, average tumor volume of 110 mm ^{3}) were injected with 1 mg/kg L49-sFv-bL (intravenous, 8 days after tumor implant), followed 18 h later by CCM or C-Mel. Treatment with L49-sFv-bL and CCM or C-Mel was repeated 7 and 14 days later. Control mice were treated with PDM or melphalan. Animals were monitored daily for general health and one or two times per week for body weight and tumor growth. Tumor volume was estimated using the formula (longest length)[(perpendicular dimension) ^{2} /2]. Cures were defined as an established tumor that, after treatment, was not visually detectable for 10 tumor doubling periods (40 days in the 3677 tumor model). MTDs led to less than 10% weight loss and no treatment-related deaths and were within 50% of the dose where such events took place. If an animal was removed from the experiment because of tumor growth, the data set was no longer plotted, but the remaining animals were monitored for tumor size and general health.

RESULTS

Synthesis of C-Mel and Enzymatic Conversion to Melphalan. C-Mel was synthesized by condensing commercially available melphalan with the activated sulfoxide (2) as shown in Scheme 1. Cephalosporin sulfoxide (2) was prepared from 1 (25) by oxidation with 3-chloroperoxybenzoic acid. HPLC analysis revealed that C-Mel was a substrate for bL and released melphalan (data not shown). Enzymatic hydrolysis of C-Mel to melphalan by bL was investigated spectrophotometrically. The K_m was 218 μM , and the k_{cat} was 980 s $^{-1}$. The fast rate of bL hydrolysis of the sulfoxide prodrug C-Mel is comparable to those of other similar cephalosporin sulfoxides (26) and is substantially faster than those of related thioether prodrugs (16).

In Vitro Cytotoxicity. L49-sFv-bL is a recombinant fusion protein containing the sFv fragment of the anti-melanoma mAb L49 and a mutated form of the enzyme bL (24). The protein was expressed in *E. coli* as described earlier and was purified by affinity chromatography (15). The cytotoxic effects of L49-sFv-bL in combination with C-Mel were determined on 3677 human melanoma cells, which express the melanotransferrin (p97) antigen. The experiments were performed by treating the cells with the fusion protein, washing off unbound material, adding various concentrations of C-Mel for 4 h at 37 °C, and using [^3H]thymidine incorporation as a measure of cytotoxic activity. The prodrug C-Mel (IC_{50} = 53 μM) was approximately 40-fold less toxic to 3677 cells than the drug melphalan (IC_{50} = 1.3 μM). As expected, L49-sFv-bL activated C-Mel, and the combination was equivalent in activity to melphalan (Figure 1). This indicates that prodrug conversion was efficient under the conditions tested. In addition, it was found that activation was immunologically specific, since a much lower level of C-Mel activation took place on 3677 cells that were saturated with unconjugated L49 prior to addition of the fusion protein.

Therapeutic Activity. In vivo therapy experiments were performed in which the activities of L49-sFv-bL/

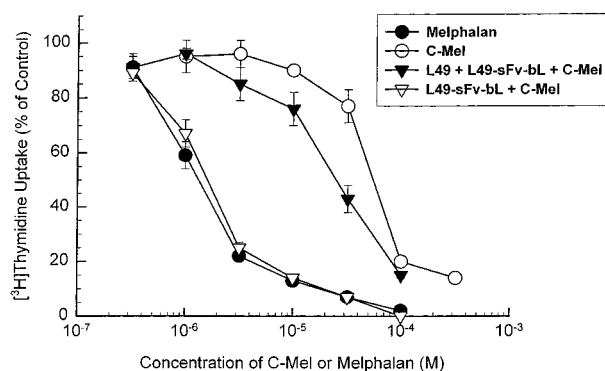


Figure 1. Cytotoxic effects of L49-sFv-bL and C-Mel on 3677 melanoma cells as measured by [^3H]thymidine incorporation. Cells were incubated with L49-sFv-bL, washed, and treated with C-Mel for 4 h. Control cells were treated with melphalan or C-Mel in the absence of L49-sFv-bL or with L49 at saturating amounts prior to L49-sFv-bL addition.

CCM and L49-sFv-bL/C-Mel were compared in nude mice with established subcutaneous 3677 tumors. This particular tumor model has previously been shown to be resistant to treatment with PDM and CCM but was responsive to treatment with the L49-sFv-bL/CCM combination (15). In the experiments reported here, conjugate treatment was initiated 8 days after tumor implant, at which time the tumors were approximately 110 mm ^{3} in volume. CCM or C-Mel was then administered 18 h later, and the treatment protocol was repeated twice more on a weekly basis. This particular time interval was chosen on the basis of the high tumor:nontumor ratios 18 h after L49-sFv-bL administration (15).

The MTD of melphalan (10 mg/kg/injection) led to a delay in tumor outgrowth of 39 days (Figure 2A). More pronounced antitumor activity was seen in mice that received C-Mel (100 mg/kg/injection) after L49-sFv-bL. Therapeutic activity was dose-dependent, as cures (three out of five mice) and complete regressions (two out of five mice) were obtained at a higher C-Mel dose (150 mg/kg/injection). Both of these doses were well tolerated. In the same experiment, it was found that PDM at 3 mg/kg (MTD) had no effect on the growth of the 3677 tumors (Figure 2B). In contrast, animals treated with L49-sFv-bL prior to CCM (100 mg/kg/injection) experienced a tumor growth delay of 63 days, with one out of five cures. L49-sFv-bL followed by CCM (150 mg/kg/injection) resulted in cures (three out of five mice) and partial regressions (two out of five mice). These doses were nontoxic. L49-sFv-bL followed by either C-Mel or CCM at 200 mg/kg was above the MTD. Thus, CCM and C-Mel are equally effective in 3677 tumor-bearing mice that were pretreated with L49-sFv-bL.

DISCUSSION

Among the many prodrugs that have been reported for tumor-selective activation by mAb-enzyme conjugates (5-7), the most pronounced activities have generally been obtained from those containing nitrogen mustards (12-15). Possible reasons for this may be due to the steep dose-response curves displayed by such agents, along with the fact that nitrogen mustards tend not to be cell cycle-specific (22). The alkylating agents employed thus far have been PDM (13, 15, 21) and several benzoic acid mustard derivatives (12, 14), none of which are clinically approved. There may be some advantages in releasing clinically approved agents since their pharmacology and toxicities are well-understood. For this reason, we

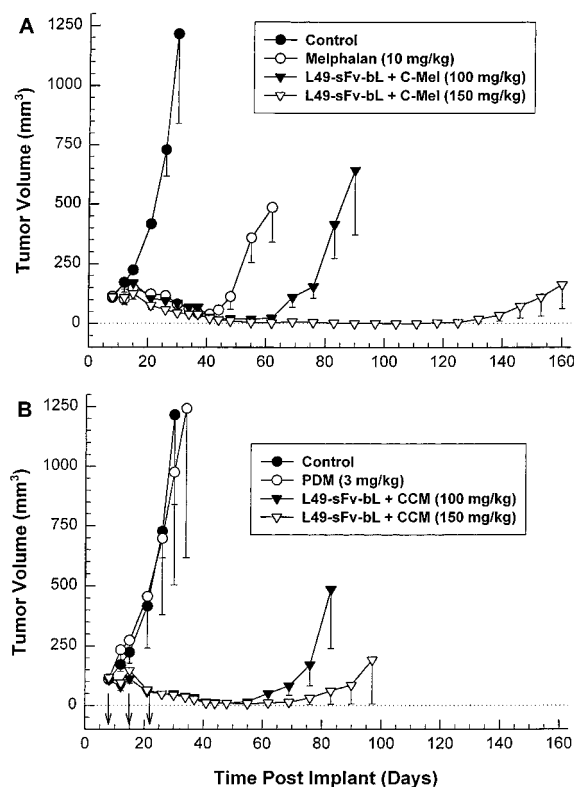


Figure 2. Therapeutic effects of L49-sFv-bL in combination with (A) C-Mel or (B) CCM. Nude mice (five mice per group) were treated with L49-sFv-bL at 1 mg/kg followed 15–18 h later by prodrugs. The average tumor volumes were plotted until one or more mice were removed from the experiment due to tumor outgrowth. Melphalan and PDM at the MTDs were also injected without prior L49-sFv-bL treatment.

selected melphalan, a drug that has proven to be useful in the clinical treatment of malignant melanoma and other neoplasias (22, 27).

Melphalan is well-suited for the targeting strategy described here, since acylation of its primary amino group has previously been shown to lead to a reduction in cytotoxic activity (23). Consequently, a prodrug of melphalan was prepared by attaching the drug via its side chain amine to a cephalosporin so that upon bL-catalyzed hydrolysis the parent drug was released. In the prodrug CCM, the primary amine of the phenylenediamine mustard was attached to cephalosporin through a stable carbamate linkage. We therefore decided to append melphalan to cephalosporin using a carbamate linkage. Our starting material for the synthesis of C-Mel was the tetrachloroethyl carbonate **1** (25) in which the cephalosporin carboxylic acid was protected as a diphenyl methyl ester to prevent the otherwise facile lactonization under basic conditions. In designing C-Mel, we decided to oxidize the cephalosporin thioether to a sulfoxide, since this reduces the isomerization of the Δ^2 double bond under the basic conditions used in the coupling reaction (28). Cephalosporin sulfoxides have been shown to be excellent substrates for bL (26), so it was not surprising to find that the k_{cat} value for bL-catalyzed hydrolysis of C-Mel (980 s^{-1}) was quite high. This is substantially higher than the previously reported turnover number of 337 s^{-1} with CCM as the substrate (17).

One of the noteworthy findings in these studies was that significant antitumor activity was obtained in animals treated with a MTD of melphalan (39 day growth delay), while the MTD of PDM was ineffective. The lack of antitumor activity of PDM has been previously noted

(13, 17, 21), and may be due to its high degree of instability under physiological conditions (29). This might lead to the conclusion that the portion of C-Mel that was systemically activated could contribute to therapeutic efficacy, while that of CCM would lead to greater toxicity.

In conclusion, we have demonstrated that C-Mel is a prodrug of melphalan that can be activated in an immunologically specific manner by L49-sFv-bL. All of the tumors in animals treated with L49-sFv-bL and C-Mel at 150 mg/kg/injection underwent complete regressions, and three out of five were eventually cured. Although the therapeutic effects of C-Mel and CCM were equivalent in the tumor model used in these studies, we feel that C-Mel is advantageous since it releases a clinically approved anticancer drug.

ACKNOWLEDGMENT

We acknowledge the contributions of Pam Gronbeck, Håkan Svensson, Steve Kloor, and David Lowe.

LITERATURE CITED

- (1) Schubiger, P. A., Alberto, R., and Smith, A. (1996) Vehicles, chelators, and radionuclides: choosing the "building blocks" of an effective therapeutic radioimmunoconjugate. *Bioconjugate Chem.* 7, 165–179.
- (2) Knox, S. J. (1995) Overview of studies on experimental radioimmunotherapy. *Cancer Res.* 55, 5832s–5836s.
- (3) Frankel, A. E., FitzGerald, D., Siegall, C., and Press, O. W. (1996) Advances in immunotoxin biology and therapy: A summary of the fourth international symposium on immunotoxins. *Cancer Res.* 56, 926–932.
- (4) Pietersz, G. A., Rowland, A., Smyth, M. J., and McKenzie, I. F. (1994) Chemoimmunoconjugates for the treatment of cancer. *Adv. Immunol.* 56, 301–387.
- (5) Melton, R. G., and Sherwood, R. F. (1996) Antibody-enzyme conjugates for cancer therapy. *J. Natl. Cancer Inst.* 88, 153–165.
- (6) Niculescu-Duvaz, I., and Springer, C. J. (1995) Antibody-directed enzyme prodrug therapy (ADEPT): A targeting strategy in cancer chemotherapy. *Curr. Med. Chem.* 2, 687–706.
- (7) Senter, P. D., and Svensson, H. P. (1997) A summary of monoclonal antibody-enzyme/prodrug combinations. *Adv. Drug Delivery Rev.* 22, 341–349.
- (8) Svensson, H. P., Vruthula, V. M., Emswiler, J. E., MacMaster, J. F., Cosand, W. L., Senter, P. D., and Wallace, P. M. (1995) In vitro and in vivo activities of a doxorubicin prodrug in combination with monoclonal antibody β -lactamase conjugates. *Cancer Res.* 55, 2357–2365.
- (9) Wallace, P. M., MacMaster, J. F., Smith, V. F., Kerr, D. E., Senter, P. D., and Cosand, W. L. (1994) Intratumoral generation of 5-fluorouracil mediated by an antibody-cytosine deaminase conjugate in combination with 5-fluorocytosine. *Cancer Res.* 54, 2719–2723.
- (10) Rogers, G. T., Burke, P. J., Sharma, S. K., Koodie, R., and Boden, J. A. (1995) Plasma clearance of an antibody-enzyme conjugate in ADEPT by monoclonal anti-enzyme: its effect on prodrug activation in vivo. *Br. J. Cancer* 72, 1357–1363.
- (11) Meyer, D. L., Jungheim, L. N., Law, K. L., Mikolajczyk, S. D., Shepherd, T. A., Mackensen, D. G., Briggs, S. L., and Starling, J. J. (1993) Site-specific prodrug activation by antibody- β -lactamase conjugates—regression and long-term growth inhibition of human colon carcinoma xenograft models. *Cancer Res.* 53, 3956–3963.
- (12) Eccles, S. A., Court, W. J., Box, G. A., Dean, C. J., Melton, R. G., and Springer, C. J. (1994) Regression of established breast carcinoma xenografts with antibody-directed enzyme prodrug therapy against c-erbB2 p185. *Cancer Res.* 54, 5171–5177.
- (13) Kerr, D. E., Schreiber, G. J., Vruthula, V. M., Svensson, H. P., Hellstrom, I., Hellstrom, K. E., and Senter, P. D. (1995) Regressions and cures of melanoma xenografts following

- treatment with monoclonal antibody β -lactamase conjugates in combination with anticancer prodrugs. *Cancer Res.* 55, 3558–3563.
- (14) Blakey, D. C., Burke, P. J., Davies, D. H., Dowell, R. I., East, S. J., Eckersley, K. P., Fitton, J. E., McDaid, J., Melton, R. G., Niculescu-Duvaz, I. A., Pinder, P. E., Sharma, S. K., Wright, A. F., and Springer, C. J. (1996) ZD2767, an improved system for antibody-directed enzyme prodrug therapy that results in tumor regressions in colorectal tumor xenografts. *Cancer Res.* 56, 3287–3292.
- (15) Siemers, N. O., Kerr, D. E., Yarnold, S., Stebbins, M., Vrudhula, V. M., Hellström, I., Hellström, K. E., and Senter, P. D. (1997) Construction, expression, and activities of L49-sFv- β -lactamase, a single chain antibody fusion protein for anticancer prodrug activation. *Bioconjugate Chem.* 8, 510–519.
- (16) Vrudhula, V. M., Svensson, H. P., and Senter, P. D. (1997) Immunologically specific activation of a cephalosporin derivative of mitomycin C by monoclonal antibody β -lactamase conjugates. *J. Med. Chem.* 40, 2788–2792.
- (17) Vrudhula, V. M., Svensson, H. P., Kennedy, K. A., Senter, P. D., and Wallace, P. M. (1993) Antitumor activities of a cephalosporin prodrug in combination with monoclonal antibody- β -lactamase conjugates. *Bioconjugate Chem.* 4, 334–340.
- (18) Vrudhula, V. M., Svensson, H. P., and Senter, P. D. (1995) Cephalosporin derivatives of doxorubicin as prodrugs for activation by monoclonal antibody- β -lactamase conjugates. *J. Med. Chem.* 38, 1380–1385.
- (19) Hanessian, S., and Wang, J. (1993) Design and synthesis of a cephalosporin-carboplatinum prodrug activatable by a β -lactamase. *Can. J. Chem.* 71, 896–906.
- (20) Rodrigues, M. L., Carter, P., Wirth, C., Mullins, S., Lee, A., and Blackburn, B. K. (1995) Synthesis and β -lactamase-mediated activation of a cephalosporin-taxol prodrug. *Chem. Biol.* 2, 223–227.
- (21) Svensson, H. P., Frank, I. S., Berry, K. K., Vrudhula, V. M., and Senter, P. D. (1998) Therapeutic effects of monoclonal antibody- β -lactamase conjugates in combination with a nitrogen mustard anticancer prodrug in models of human renal cell carcinoma. *J. Med. Chem.* (in press).
- (22) Berger, N. A. (1993) Anticancer Drugs. In *Cancer: Principles and Practice of Oncology* (V. T. J. DeVita, S. Hellman, and S. A. Rosenberg, Eds.) pp 400–407, J. B. Lippincott Co., Philadelphia.
- (23) Vrudhula, V. M., Senter, P. D., Fischer, K. J., and Wallace, P. M. (1993) Prodrugs of doxorubicin and melphalan and their activation by a monoclonal antibody-penicillin-G amidase conjugate. *J. Med. Chem.* 36, 919–923.
- (24) Siemers, N. O., Yelton, D. E., Bajorath, J., and Senter, P. D. (1996) Modifying the specificity and activity of the Enterobacter cloacae P99 β -lactamase by mutagenesis within an M13 phage vector. *Biochemistry* 35, 2104–2111.
- (25) Hudyma, T. W., Bush, K., Colson, K. L., Firestone, R. A., and King, H. D. (1993) Synthesis and release of doxorubicin from a cephalosporin based prodrug by a β -lactamase immunoconjugate. *Bioorg. Med. Chem. Lett.* 3, 323–328.
- (26) Jungheim, L. N., Shepherd, T. A., and Meyer, D. L. (1992) Synthesis of acylhydrazido-substituted cepheams. Design of cephalosporin-vinca alkaloid prodrugs: substrates for an antibody-targeted enzyme. *J. Org. Chem.* 57, 2334–2340.
- (27) Alexander, H. R., Jr., Fraker, D. L., and Bartlett, D. L. (1996) Isolated limb perfusion for malignant melanoma. *Semin. Surg. Oncol.* 12, 416–428.
- (28) Murphy, C. F., and Webber, J. L. (1972) Alteration of the dihydrothiazine ring moiety. In *Cephalosporin and Penicillins: Chemistry and Biology* (E. H. Flynn, Ed.) pp 134–182, Academic Press, New York.
- (29) Chakravarty, P. K., Carl, P. L., Weber, M. J., and Katzenellenbogen, J. A. (1983) Plasmin-activated prodrugs for cancer chemotherapy. 1. Synthesis and biological activity of peptidylacivicin and peptidylphenylenediamine mustard. *J. Med. Chem.* 26, 633–638.

BC970163L

Membrane Permeabilization and Efficient Gene Transfer by a Peptide Containing Several Histidines

Patrick Midoux,* Antoine Kichler, Valérie Boutin, Jean-Claude Maurizot, and Michel Monsigny

Centre de Biophysique Moléculaire, CNRS et Université d'Orléans, rue Charles-Sadron,

F-45071 Orléans Cedex 02, France. Received September 4, 1997; Revised Manuscript Received December 10, 1997

We designed a peptide, H5WYG (GLFHAIHFIHGGWHGLIHGWYG), that permeabilizes cell membrane at a slightly acidic pH but not at neutral pH. Absorbance, fluorescence, and circular dichroism spectra showed that H5WYG undergoes a dramatic conformational change between pH 7.0 and 6.0 that correlates with the protonation of the histidyl residues. Cell permeabilization studies monitored by flow cytometry on living cells showed that H5WYG permeabilizes the cell membrane with a great efficiency at pH 6.4 but was not active at neutral pH; at pH 6.8, the peptide permeabilized 50% of the cells at 20 °C within 10 min. H5WYG increased the expression of genes transferred to cells as glycosylated polylysine–DNA complexes, and the transfection efficiency was not impaired in the presence of serum. Therefore, this peptide containing several histidines that become positively charged when the pH decreased to less than 7.0 is a suitable helper for delivering molecules into the cytosol upon either permeabilization of the plasma membrane induced by lowering the extracellular medium to pH 6.4 or permeabilization of the endosomal membrane induced by acidification of endosomes.

INTRODUCTION

The introduction of nucleic acids into cells is of great interest for the development of gene therapy. Various nonviral vectors are available. Cationic lipids such as *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium chloride (DOTMA) (1) or dioctadecylamidoglycylspermine (DOGS) (2) and cationic polymers such as polylysine conjugates (3–6) or polyethylenimine (7) are synthetic vectors that form lipoplexes and polyplexes with DNA (8), suitable for gene delivery into eukaryotic cells. Recognition signals, including asialoglycoprotein, transferrin, insulin, immunoglobulins, growth factors, carbohydrates, and metabolites, linked to polylysine (for reviews, see refs 3–6) and to polyethylenimine (9) increased the cell specificity of polyplexes. Cationic lipids and cationic polymers are also suitable for oligonucleotide delivery; DOTMA (10, 11) and polyethylenimine (7) enhanced oligonucleotide cellular uptake. An antisense oligonucleotide complexed with polylysine substituted with either asialoglycoprotein (12), transferrin (13), folate (14), or fucose (15) inhibited hepatitis B viral, *myc*, *myb*, and ICAM-1 gene expression, respectively.

Nucleic acids must be delivered into the cytosol and/or the nucleus. To increase the transmembrane passage of the DNA into the cytosol, several helper molecules, including lipids, chloroquine, glycerol, or fusogenic peptides, were used. Dioleoylphosphatidylethanolamine (DOPE), a neutral fusogenic lipid, was mixed with DOTMA to give lipofectin, which is commonly used for gene and oligonucleotide delivery (1). Chloroquine, a weak base which prevents the delivery of the endosomal content to lysosomes, induces the formation of large endosome-derived vacuoles, and destabilizes their membrane, enhancing the polyfection with transferrin–poly-

lysine (16) and glycosylated polylysine (17, 18); in addition, chloroquine allows the dissociation of glycosylated polylysine–plasmid complexes (19). The inhibition of ICAM-1 gene expression by antisense oligonucleotide–fucosylated polylysine complexes increased drastically in the presence of chloroquine (15). Enhancement of the polyfection with transferrin–polylysine was also reported in the presence of glycerol (20). Amphiphilic peptides exhibiting fusogenic properties in acidic media were used in the development of synthetic virus-like gene-transfer vehicles. Amphiphilic anionic peptides such as GLFEA-IAGFIENGWEGMIDGGGC (21, 22), GLFEAIEGFIENGWEGMIDGWYG (23) derived from the sequence of the N-terminal segment of the HA-2 subunit of the influenza virus hemagglutinin involved in the fusion of the viral envelope with the endosomal membrane, increased the polyfection with transferrin–polylysine complexes. The amphiphilic anionic peptide GLFEAIAEFIEGGWEG-LIEG (E5) corresponding to the N-terminal segment of the HA-2 subunit of the A/PR/8/34 influenza virus hemagglutinin in which G-4, G-8, T-15, and D-19 were replaced by glutamyl residues and M-17 was replaced by a leucyl residue, which induces membrane fusion and permeabilization at acidic pH (24–26), increased the polyfection with lactosylated polylysine (17). Other amphiphilic anionic peptides such as GLFEALLELLESLWELLLEA (27), NPVENYIDEVLNEVLVVPNININSSNC [the sequence of the N-terminal segment of VP1 of HRV2 rhinovirus (28)], or WEAALAEALAEALAEHLAEALAEALAEALAA (GALA) (23, 29) were also found to enhance the gene-transfer efficiency of various polylysine conjugate–DNA complexes. These amphiphilic anionic peptides which contain acid residues (mainly glutamate) are supposed to increase the transmembrane passage of the plasmid into the cytosol upon the membrane disruption of the acidic endocytotic vesicles; this permeabilization is attributed to the protonation of the acid residues of the peptides, and the protonation allows the peptide to

* To whom correspondence should be addressed. Fax: 33 2 38 69 00 94. Phone: 33 2 38 25 55 95. E-mail: midoux@cns-orleans.fr.

change its conformation from random to α -helix as shown by circular dichroism. Similarly, amphiphilic basic peptides such as melittin (GIGAVLKVLTTGLPALISWIKRKRQQ-NH₂), a peptide isolated from the venom of the european honey bee *Apis mellifera* (30), and GLFKAIAKFIKGGWKGLIKG (K5), an amphiphilic cationic peptide in which the glutamyl residues of the anionic peptide E5 in positions 4, 8, 11, 15, and 19 were replaced by lysyl residues (25), and WKAALAKALAKALAKHLAKALAKALKALAA (KALA) (31) exhibit membrane fusion and permeabilization activities at neutral pH. Melittin coupled to dioleoylphosphatidylethanolamine and complexed with DNA was reported to be efficient in transfecting cells (32). Nevertheless, it is noteworthy to emphasize that those cationic peptides as well as the above-mentioned anionic peptides are inefficient when the transfection step is conducted in the presence of serum.

Knowing that the imidazole group of histidine has a pK of around pH 6.0 and thus becomes cationic in a slightly acidic medium, we hypothesized that changing the lysyl residues of the peptide K5 by histidine residues uncharged at neutral pH but cationic in slightly acidic medium (at a pH close to that found in the lumen of the endocytotic vesicles) will not permeabilize the plasma membrane of the cells under classical conditions, medium at pH 7.4, but will selectively disrupt the membrane of acid vesicles and will lead to the transmembrane passage of plasmids into the cytosol.

In this work, we describe the synthesis and characterization of the peptide GLFHAIHFIHGGWHGLIHGWYG (H5WYG¹), which is analogous to the N-terminal segment (GLFGAIAFGIEGGWTGMIDGWYG) of the HA-2 subunit of the influenza virus hemagglutinin in which G-4, G-8, E-11, T-15, and D-19 were replaced by histidyl residues and M-17 was replaced by a leucyl residue. We show that this peptide exhibits a very efficient membrane permeabilization activity at pH 6.8; at such a very slightly acidic pH, the amphiphilic anionic E5WYG peptide was completely ineffective. We also demonstrate that this peptide increases the gene-transfer efficiency of cells by using glycosylated polylysine-DNA complexes in a pH-dependent manner, and that the efficient gene transfer is not impaired by the presence of serum.

MATERIALS AND METHODS

Chemicals. Luciferin, chloroquine, bafilomycin A₁, and Triton X-100 were from Sigma (St. Louis, MO). L-Glutamine, dimethyl sulfoxide, ATP, glycerol, and MgCl₂ were from Merck (Darmstadt, Germany). Dithiothreitol was from Serva (Heidelberg, Germany). Poly(L-lysine) hydrobromide 30000–50000 (average degree of polymerization of 190) was from Bachem Feinchemikalien (Bubendorf, Switzerland). Poly(L-lysine) hydrobromide (1 g in 200 mL of H₂O) was transformed to its *p*-toluenesulfonic acid salt as described previously (33). Polylysine was partially substituted with an average number of 60 lactosyl residues as previously described (17).

Peptide H5WYG. H5WYG (GLFHAIHFIHGGWHGLIHGWYG) and E5WYG (GLFEAIAEFIEGGWGLIEGWYG) were purchased from Severn Biotech Ltd. (Kidderminster, Worcester, U.K.); alternatively, peptides were synthesized with the Fmoc strategy. E5WYG was

purified as previously described (17). H5WYG was purified by HPLC on a C18 Spherisorb ODS, 5 mm column by using a linear gradient in H₂O containing 0.5% trifluoroacetic acid over the course of 40 min from 0 to 80% acetonitrile. The peptide was eluted with a retention time of 23.4 min. The peptide was characterized by mass spectrometry using a matrix-assisted laser desorption/ionization (MALDI) time-of-flight mass spectrometer (calcd 2584.6 Da, found 2584.7 Da). The peptide concentration was determined by using an $\epsilon_{280\text{ nm}}$ of 12 200 M L⁻¹ cm⁻¹.

Spectrofluorimetry. Fluorescence measurements were performed at 25 °C with a Shimadzu RF-5000 spectrofluorimeter (Roucaire, Courtaboeuf, France). Solutions were contained in a 5 × 5 mm quartz cuvette; their absorbance ($A_{1\text{ cm}}$) was lower than 0.1 at the excitation wavelength.

Circular Dichroism. Circular dichroism measurements were carried out on a Jobin-Yvon Mark IV dichrograph. Data acquisition and analysis were performed on a microcomputer interfaced to the spectrometer. The instrument had been calibrated with camphor-sulfonic acid-*d*₁₀. Optical quartz cells with a path length of 0.1 cm were used under nitrogen. The temperature of the cell was maintained at 20 ± 0.5 °C with a circulating water bath. Curves were signal-averaged with a minimum of four repeats and presented with a similarly signal-averaged buffer baseline subtracted.

Cells and Cell Culture. U937 promonocytic cells (a human histiocytic lymphoma cell line, ATCC CRL 1593, ATCC, Rockville, MD) were cultured in RPMI 1640 medium (Gibco, Renfrewshire, U.K.) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco); HepG2 cells (a human hepatoma cell line, ATCC 8065 HB) in MEM (Gibco) supplemented with 10% FBS and 1% non-essential amino acids; and Rb-1 cells [rabbit smooth muscle cells, kindly provided by M. Nachtigal (University of South Carolina, Columbia, SC)] (34) and B16 cells (a murine melanoma cell line, ATCC CRL 6322) in DMEM medium (Gibco) supplemented with 10% FBS and new-born calf serum (Gibco), respectively. All the culture media were supplemented with 2 mM L-glutamine (Merck), 100 units/mL penicillin, and 0.1 mg/mL streptomycin (Eurobio, Paris, France). Cells were cultured at 37 °C in a humidified atmosphere (95% air and 5% CO₂). Cells were mycoplasma-free as shown by using the bisbenzimidazole Hoechst 33258 (35) and the BVC-kanamycin A staining methods (36).

Membrane Permeabilization Studies. The membrane permeabilization activity of H5WYG was assessed by using flow cytometry as described previously (26). U937 cells growing in RPMI complete medium were collected in the exponential-growth phase and washed in sheath fluid [134 mM NaCl, 3.7 mM KCl, 15.2 mM NaF, 1.9 mM KH₂PO₄, 16.5 mM Na₂HPO₄, and 0.2% 2-phenoxyethanol (pH 7.4)]. The sheath fluid (1 mL) containing 5 × 10⁵ U937 cells was made 12.5 μ M in ethidium bromide (BET, Molecular Probes, La Jolla, CA) (5 μ L of a 1 mg/mL stock solution in H₂O) at 20 °C and then 36 μ M in H5WYG (50 μ L of a 0.84 mM stock solution in H₂O). The pH of the cell suspension was then lowered by adding aliquots (10–70 μ L) of a 0.3 N HCl solution. The pH values were controlled by using a pH meter. After a 5–15 min incubation, the cell fluorescence intensity was measured by using a FACSort cytometer (Becton Dickinson, Sunnyvale, CA). A 488 nm excitation wavelength was produced by a 15 mW cold air argon laser. A 585 ± 44 nm long-pass filter was used to collect the emitted red fluorescence. The side scatter, the forward

¹ Abbreviations: BET, ethidium bromide; BVC, *p*-[β -[7-(dimethylamino)-1,4-benzoxazin-2-on-3-yl]vinyl]phenylpropenoic acid; E5WYG, GLFEAIAEFIEGGWGLIEGWYG; FBS, fetal bovine serum; H5WYG, GLFHAIHFIHGGWHGLIHGWYG; Lact, lactosyl residue; pLK, poly(L-lysine); RLU, relative light unit.

scatter, and the red fluorescence were simultaneously recorded in the list mode on a minimum of 10 000 cells at 300 events per second. The data were analyzed by using the Cell Quest software (Becton Dickinson).

Plasmid. pSV2LUC plasmid (5 kb), kindly provided by A. B. Brasier (Massachusetts General Hospital, Boston, MA) (37), is an expression vector encoding the firefly luciferase gene under the control of the SV40 T large antigen promoter. pUT650 plasmid (5149 bp) from CAYLA (Toulouse, France) is an expression vector encoding the firefly luciferase gene under the control of the human cytomegalovirus promoter. Supercoiled DNA plasmids were isolated by a standard alkaline lysis method followed by CsCl gradient centrifugation in the presence of BET, extensive extraction with *n*-butanol, and precipitation with ethanol.

Gene Transfer. HepG2 cells (3×10^5 cells per 4 cm² well) and Rb-1 cells (1×10^5 cells per 4 cm² well) were plated (day 0) into 12-well tissue culture plates; B16 cells (8×10^4 cells per 2 cm² well) were plated (day 0) into 24-well tissue culture plates. Polyplexes between a plasmid and lactosylated polylysine (LactpLK) were made with the lowest polymer to DNA molar ratio giving a complete retardation of all the DNA in electrophoresis and used to transfect cells as previously described (18). pSV2LUC or pUT650 (10 μ g, 3 pmol) in 0.7 mL of serum-free DMEM was mixed with LactpLK (30 μ g, 340 pmol) in 0.3 mL of serum-free DMEM, and the solution was kept for 30 min at 20 °C. Then the solution was made either 100 μ M in chloroquine, 10 μ M in E5WYG, or 10 μ M in H5WYG and then supplemented with 1, 20, or 50% FBS, unless otherwise stated. On day 1, the medium was removed and the polyfection solution was added into each well: 1 mL in the case of 12-well tissue culture plates or 0.5 mL in that of 24-well tissue culture plates. After incubation for 4 h at 37 °C in a humidified atmosphere (95% air and 5% CO₂), the medium was removed and cells were further incubated for 48 h at 37 °C in 2 mL of fresh complete culture medium containing 10% serum, in a humidified atmosphere (95% air and 5% CO₂). Experiments were carried out in triplicate.

Luciferase Assay. Luciferase gene expression was measured by monitoring its luminescence activity according to De Wet et al. (38) as previously described (18). The luminescence was recorded for 4 s in a Lumat LB 9501 luminometer (Berthold, Wildbach, Germany). Measurements were carried out in duplicate. The data shown correspond to the number of relative light units (RLU) from 10^6 cells. The number of RLU from a 1 pg/mL luciferase solution was 2000.

RESULTS

Peptide Characterization. The peptide used, GLF-HAIAHFIHGGWHGLIHGWYG (H5WYG), is an analogue of the N-terminal segment (GLFGAIAAGFIEGG-WTGMIDGWYG) of the HA-2 subunit of the influenza virus hemagglutinin in which G-4, G-8, E-11, T-15, and D-19 were replaced by histidyl residues and M-17 was replaced by a leucyl residue. The absorbance and fluorescence spectra of a 16 μ M peptide solution in various buffers in the 9.0–4.5 pH range were studied. The absorbance spectrum of H5WYG depended on the pH of the medium (Figure 1). The absorbance at the maximal wavelength (279 nm) decreased when the pH decreased with a midpoint at pH 5.7, and the ratio of the maximal absorbance at 279 nm to the minimal absorbance between 252 and 260 nm ($A_{\max}/A_{\min}$) increased when the pH decreased with a midpoint at pH 5.7. These data indicate that (i) H5WYG was slightly associated or

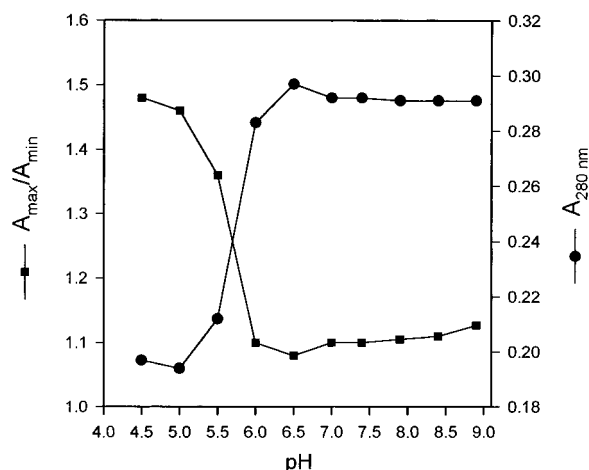


Figure 1. pH dependence of the absorbance of H5WYG. A stock solution (1 mg/mL in H₂O) was diluted to 16 μ M with the following buffers: from pH 4.5 to 7.0, 0.15 M NaCl, 50 mM citrate phosphate buffers and 0.15 M NaCl, 50 mM sodium phosphate buffer (pH 7.4); and from pH 8.0 to 9.0, 0.15 M NaCl, 50 mM Tris-HCl buffers. (●) Absorbance at 280 nm and (■) the 280 nm absorbance/255 nm absorbance ratio.

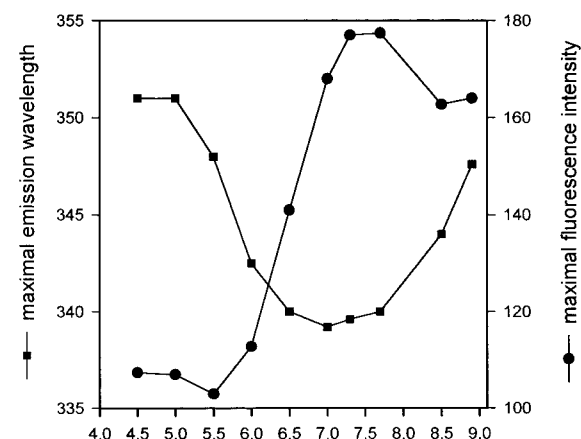


Figure 2. pH dependence of the fluorescence of H5WYG. A stock solution (1 mg/mL in H₂O) was diluted to 16 μ M with the following buffers: from pH 4.5 to 7.0, 0.15 M NaCl, 50 mM citrate phosphate buffers and 0.15 M NaCl, 50 mM sodium phosphate buffer (pH 7.4); and from pH 8.0 to 9.0, 0.15 M NaCl, 50 mM Tris-HCl buffers. The fluorescence spectra were recorded under a 290 nm excitation wavelength. (●) Maximal fluorescence intensity and (■) the wavelength at the maximal emission intensity.

aggregated at neutral pH, (ii) peptide aggregates dissociated between pH 6.5 and 5.0, and (iii) H5WYG was not associated or aggregated at pH <5.0. These statements were confirmed by fluorescence studies (Figure 2). The maximum of the fluorescence spectrum of H5WYG at pH 4.5 was centered at 350 nm, corresponding to solvated tryptophan residues. The wavelength maximum of the spectrum of the peptide shifted from 350 to 338 nm when the pH increased from 5.0 to 7.0 with a midpoint at pH 5.75, indicating the tryptophan residues were in a more hydrophobic environment at neutral pH. In agreement with the absorbance data, the fluorescence data indicated that the peptide was slightly associated or aggregated at neutral pH and not aggregated at pH <5.5. The fluorescence intensity of the peptide decreased when the pH was lowered from pH 7.0 to 5.0 with a midpoint at pH 6.5 (Figure 2). This value, different from that found for the shift of the wavelength maximum, i.e., midpoint at pH 5.75, indicated that the fluorescence quenching of the tryptophan residues (W-14 and W-21) was probably

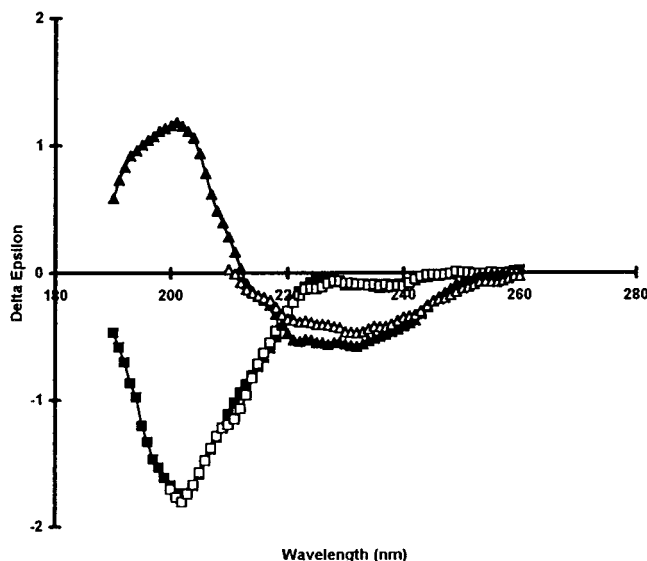


Figure 3. pH dependence of the circular dichroism of H5WYG. The spectra of 34 μ M H5WYG were measured at either (□) pH 5.0 in 0.15 M NaCl and 10 mM citrate phosphate, (■) pH 6.0 in 0.15 M NaCl and 10 mM citrate phosphate, (▲) pH 7.0 in 0.15 M NaCl and 10 mM sodium phosphate, or (△) pH 9.0 in 0.15 M NaCl and 10 mM Tris-HCl.

related to the ionization state of the surrounding amino acids and especially some of the histidyl residues in relation with the conformation of the peptide at a given pH. Circular dichroism spectra of H5WYG performed at pH 6.0 and 5.0 were similar, while those recorded at pH 7.0 or 9.0 were drastically different (Figure 3). The peptide exhibited a conformational change between pH 7.0 and 6.0, a pH range where the protonation of the histidyl residues does occur. The CD spectra showed that at acidic pH the spectra were surely of unordered structure, but those at neutral and basic pH suggested the presence of some ordered structures, most probably of β -structure.

H5WYG-Induced Membrane Permeabilization.

The membrane permeabilization activity of H5WYG was assessed by a fluorescent method (26). The permeabilization of the plasma membrane of U937 cells was measured by flow cytometry using ethidium bromide, a nonfluorescent and poorly membrane-permeant molecule, which enters the cells upon membrane permeabilization and becomes strongly fluorescent upon binding to DNA. U937 cells incubated in the presence of both BET (12.5 μ M) and H5WYG (36 μ M) became strongly labeled at slightly acidic pH. The membrane permeabilization activity of H5WYG was pH-, time-, and concentration-dependent. In the presence of 36 μ M H5WYG, within 10 min, 97% of the cells were labeled at pH 6.1, 50% at pH 6.8, and <5% at pH 7.1 (Figure 4); the ED_{50} at pH 6.45 was 2.3 μ M (Figure 4 inset), and 89 and 80% of the cells were labeled after 5 min at pH 6.45 in the presence of 36 and 10 μ M H5WYG, respectively (Figure 4 inset).

Effect of H5WYG on the Polyfection with Lactosylated Polylysine. The human hepatoma (HepG2) cells, the murine melanoma (B16) cells, and the rabbit vascular smooth muscle (Rb-1) cells were transfected by using lactosylated polylysine–DNA complexes. Depending on the cell type, complexes were formed between an expression vector encoding the firefly luciferase gene under the control of either the human cytomegalovirus (pUT650) or the large SV40 T antigen (pSV2LUC) promoters and lactosylated polylysine (LactpLK). HepG2, B16, and Rb-1 cells were then incubated for 4 h at 37 °C

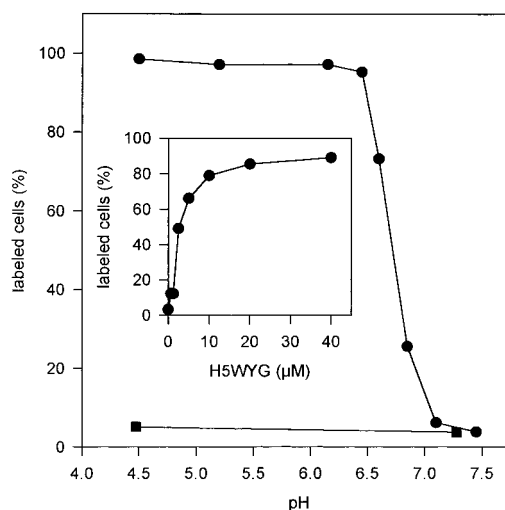


Figure 4. pH dependence of H5WYG-induced membrane permeabilization. U937 cells [5×10^5 cells in 1 mL of sheath fluid [134 mM NaCl, 3.7 mM KCl, 15.2 mM NaF, 1.9 mM KH_2PO_4 , 16.5 mM Na_2HPO_4 , and 0.2% 2-phenoxyethanol (pH 7.4)]] were incubated at 20 °C with 12.5 μ M BET in the absence (■) or in the presence (●) of 36 μ M H5WYG. The pH of the cell suspension was lowered by addition of aliquots of a 0.3 N HCl solution. After 10 min, the red cell fluorescence intensity was measured by flow cytometry ($\lambda_{exc} = 488$ nm; $\lambda_{em} = 585 \pm 44$ nm) and the percentage of red labeled cells was determined. (Inset) Concentration dependence of H5WYG-induced membrane permeabilization. U937 cells (5×10^5 cells) in 1 mL of sheath fluid were incubated at 20 °C with 12.5 μ M H5WYG. The pH of the cell suspension was lowered to 6.45 by addition of 40 μ L of a 0.3 N HCl solution. After 10 min, the red cell fluorescence intensity was measured by flow cytometry and the percentage of red labeled cells was determined.

in the presence of either 10 μ M H5WYG, 10 μ M E5WYG, or 100 μ M chloroquine. The efficiency of the gene transfer was evaluated 48 h after the transfection step by measuring the luciferase activity (RLU per 10^6 cells) in the cell lysates (Figure 5). The emitted light was low with any cell line when the transfection was performed in the absence of either H5WYG, E5WYG, or chloroquine. In contrast, when the transfection was performed in the presence of H5WYG, the luciferase activity increased 2150-, 93-, and 630-fold in B16, HepG2, and Rb-1 cells, respectively. In contrast, with E5WYG which was only efficient in HepG2 cells, H5WYG was efficient on all three cell lines; the luciferase activity in Rb-1 and B16 cells transfected in the presence of H5WYG was 63- and 29-fold greater than that in the presence of E5WYG, whereas in HepG2 cells, it was 5.8-fold lower than that in the presence of E5WYG. Depending on the cell type, H5WYG had a better effect than chloroquine; the luciferase activity in Rb-1 cells transfected in the presence of H5WYG was 5-fold greater than that in the presence of chloroquine, whereas in B16 and HepG2 cells, it was 9- and 7-fold lower than that in the presence of chloroquine, respectively. The effect of H5WYG on the transfection efficiency was concentration-dependent; the transfection efficiency of Rb-1 cells was maximal in the presence of 8 μ M H5WYG (Figure 6).

Influence of the Serum on the Polyfection Efficiency in the Presence of H5WYG. The luciferase activity in cells transfected with LactpLK–pUT650 complexes in the presence of H5WYG was not significantly affected when the transfection was performed in the presence of serum from 1 up to 50%. The luciferase activity in B16 cells transfected with H5WYG in the presence of 20 and 50% FBS was 88 and 68%, respectively, of that obtained in the presence of 1% FBS. In

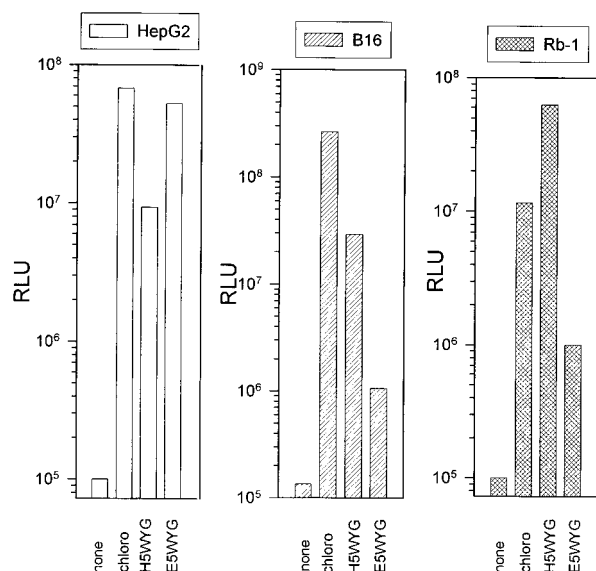


Figure 5. Effect of H5WYG on the polyfection with lactosylated polylysine. HepG2 cells (3×10^5), B16 cells (8×10^4), and Rb-1 cells (1×10^5) were incubated for 4 h at 37 °C in the presence of 1% FBS and of LactpLK–DNA complexes (5 μ g of pUT650 for HepG2 cells, 5 μ g of pSV2LUC for Rb-1 cells, and 2 μ g of pUT650 for B16 cells) in the absence or presence of either 10 μ M H5WYG, 10 μ M E5WYG, or 100 μ M chloroquine in a humidified atmosphere (95% air and 5% CO₂). The cells were washed and incubated in complete culture medium containing 10% FBS in a humidified atmosphere (95% air and 5% CO₂). The gene expression was determined 48 h later by assaying the luciferase activity in cell lysates. RLU, the number of relative light units, represents the luciferase activity in 10^6 cells.

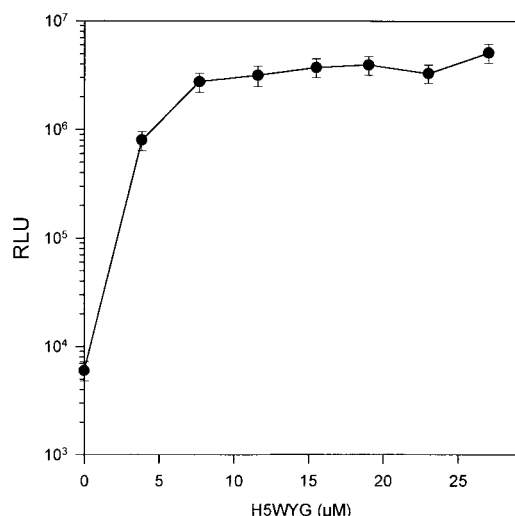


Figure 6. Influence of H5WYG concentration on the polyfection efficiency. Rb-1 cells (1×10^5) were incubated for 4 h at 37 °C in the presence of 1% FBS and LactpLK–pSV2LUC complexes (5 μ g of DNA) in the presence of H5WYG in a humidified atmosphere (95% air and 5% CO₂). The cells were washed and incubated in 2 mL of complete culture medium containing 10% FBS in a humidified atmosphere (95% air and 5% CO₂). The gene expression was determined 48 h later by assaying the luciferase activity in cell lysates. RLU, the number of relative light units, represents the luciferase activity in 10^6 cells.

contrast, the luciferase activity in B16 cells transfected in the presence of serum and of the anionic peptide E5WYG was dramatically reduced. In the presence of E5WYG and 20% FBS, it was only 7% of that obtained in the presence of 1% FBS. In the case of 100 μ M chloroquine and of 20% serum, the luciferase activity in B16 cells was slightly enhanced (120%), while in the presence of 50% serum, the activity dropped to 16%.

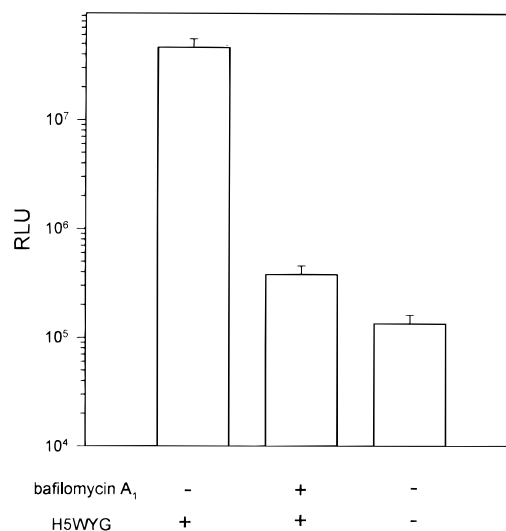


Figure 7. Influence of bafilomycin A₁ on the polyfection efficiency in the presence of H5WYG. B16 cells (8×10^4) were incubated at 37 °C with 750 μ L of DMEM containing LactpLK–pUT650 complexes (2 μ g of DNA) and 1% FBS and in the absence or presence of 19 μ M H5WYG and of 200 nM bafilomycin A₁. After 4 h, the medium was removed and cells were incubated in 1 mL of culture medium containing 10% FBS in a humidified atmosphere (95% air and 5% CO₂). The gene expression was determined 48 h later by assaying the luciferase activity in cell lysates. RLU, the number of relative light units, represents the luciferase activity in 10^6 cells.

Influence of the Endosomal pH on the Polyfection Efficiency in the Presence of H5WYG. The capacity of H5WYG, supposed to disrupt endocytotic vesicles containing LactpLK–DNA complexes, was assessed by transfecting B16 cells in the absence or in the presence of bafilomycin A₁, an inhibitor of vacuolar ATPases known to block the endosomal proton pump (39). When the transfection by LactpLK–pUT650 complexes in the presence of H5WYG was performed in the presence of 200 nM bafilomycin A₁, the luciferase activity into B16 cells was 120-fold lower than that obtained in the absence of bafilomycin A₁ and close to that obtained in the absence of H5WYG (Figure 7). Therefore, the inhibition of the endosomal proton pump activity, by impairing the acidification of the endosome lumen, decreased the transfection efficiency in the presence of H5WYG. This indicated that the endosomal membrane disrupting activity of H5WYG occurred in a pH-dependent manner.

DISCUSSION

In this report, we show that the peptide H5WYG has a powerful membrane permeabilization activity at pH 6.4 but not at neutral pH and that it strongly increases the efficiency of cell transfection by glycosylated polylysine–DNA complexes even in the presence of high concentrations of serum.

We chose to synthesize the peptide H5WYG on the following bases. (i) The peptide E5WYG was more active than the peptide E5; this enhanced activity was related to the presence of an additional tripeptide sequence WYG in a C-terminal position as in the native fusogenic peptide (GLFGAIAGFIENGWEGMIDGWYG---) of the influenza hemagglutinin subunit HA-2 (23). (ii) Histidyl residues are expected to be protonated at a pH higher than that for glutamyl residues. Therefore, H5WYG rather than H5 was expected to very efficiently destabilize the endosomal compartments very early upon uptake.

The pH dependence of the absorbance (Figure 1), of the fluorescence (Figure 2), and of the circular dichroism

(Figure 3) spectra of H5WYG indicated that (i) the peptide underwent a drastic conformational change at $\text{pH} < 7.0$, mainly between $\text{pH} 7.0$ and 6.0 , the range corresponding to the protonation of histidyl residues; (ii) at neutral pH , when the histidyl residues are not charged, the peptide was weakly water soluble and was mainly in an aggregated state; (iii) at pH close to 5.0 where all histidyl residues are expected to be protonated, the peptide had a higher solubility and was not aggregated; and (iv) the peptide had unordered structures at acidic pH .

The membrane permeabilization activity of H5WYG was studied on living cells by flow cytometry by using ethidium bromide (BET), a poorly membrane-permeant molecule which is not fluorescent as long as it is free but becomes fluorescent upon binding to nucleic acids. H5WYG permeabilized U937 cells in a pH -, concentration-, and time-dependent manner (Figure 4). It was efficient as soon as the pH was lower than 7.0 . At $\text{pH} 6.85$, the concentration required to obtain 50% permeabilized cells within 10 min, was $2.3 \mu\text{M}$ peptide. H5WYG had a better permeabilizing capacity in slightly acidic pH than the anionic E5CA peptide. By using the same method, the pH had to be lowered to $\text{pH} 6.1$ to reach 50% of permeabilized HL60 cells within 10 min in the presence of $2.5 \mu\text{M}$ E5CA (26). Comparatively, 50% of HL60 cells were permeabilized within 10 min in the presence of $0.5 \mu\text{M}$ melittin (26) as well as in the presence of $5 \mu\text{M}$ K5 (P. Midoux, unpublished results), even when the pH was kept at 7.4 .

We recently showed that the polyfection with glycosylated polylysines was efficient on various cell types. For instance, lactosylated polylysine was highly efficient in transfecting human hepatoma (HepG2) (17, 18) and human normal and cystic fibrosis airway epithelial cells (40); polyfection with mannosylated polylysine was efficient in transfecting human blood monocyte-derived macrophages (41). The capacity of H5WYG to permeabilize membrane in a slightly acid pH but not at neutral pH prompted us to investigate its ability to increase the polyfection with DNA–glycosylated polylysine complexes which are internalized by the cells via receptor (membrane lectin)-mediated endocytosis. H5WYG which permeabilized cells at a pH close to the neutrality ($\text{pH}_{50} = 6.85$) was expected to be effective on endosomal membrane and its membrane disrupting activity might occur in very early endosomes at pH s just below 7.0 , i.e., long before polyplexes could be delivered to lysosomes. The gene-transfer efficiency into a human hepatocarcinoma cell line (HepG2 cells), a murine melanoma cell line (B16 cells), and a rabbit vascular smooth muscle cell line (Rb-1 cells) by using lactosylated polylysine–DNA complexes was clearly enhanced in the presence of H5WYG (Figure 5). In contrast with the effect of the anionic E5WYG peptide which was only efficient into HepG2 cells, H5WYG was efficient on all the three lines used. Anionic peptides which are negatively charged at neutral pH can bind to polylysine-rich DNA complexes (23). DNA–lactosylated polylysine complexes, used in our study, were weakly cationic; the calculated $\pm$ charge ratio was 1.1 – 1.5 (they were made with the lowest polymer to DNA molar ratio inducing a complete retardation of all the DNA in electrophoresis) (18), and the E5WYG peptide did not ionically bind to DNA–lactosylated polylysine complexes (A. Kichler et al., manuscript in preparation). H5WYG which is not negatively charged at neutral pH cannot bind to polyplexes. Therefore, lactosylated polyplexes, E5WYG and H5WYG, were internalized by the cells without direct binding of the peptides to polyplexes.

The extent of the internalization of E5WYG and polyplexes, the luminal pH of endocytotic vesicles containing polyplexes and E5WYG, and their intracellular traffic which depends on the cell type could account for the variability of the effects of E5WYG on the transfection efficiency. HepG2 cells express the hepatocyte membrane lectin specific for β -galactose (asialoglycoprotein receptor) (42); B16 cells and Rb-1 cells do not express a galactose-specific membrane lectin but possess an endogenous lectin (called galectin 3) binding β -galactose also present at the cell surface which is different from the galactose-specific membrane lectin of hepatocytes (ref 43 and V. Boutin et al., unpublished data, respectively). The endocytosis of lactosylated polyplexes is mediated by the galactose-specific membrane lectin of HepG2 cells; the polyplexes are delivered in endosomes with an average luminal pH of 6.0 (19). The peptides H5WYG and E5WYG also reach the endosomes and, because of the low pH of endosomes, induce an efficient permeabilization. In contrast, the lactosylated polyplexes internalized into Rb-1 cells were delivered to endosomes with an average luminal pH of 6.3 (indicating that the majority of the endosomes were weakly acid) (V. Boutin et al., unpublished data), allowing their efficient permeabilization by H5WYG but not by E5WYG. B16 cells which are known to internalize molecules bearing galactose residues (43) took up lactosylated polyplexes probably by a pathway different from that induced by the galactose-specific membrane lectin of HepG2 cells; as in the case of Rb-1 cells, lactosylated polyplexes were delivered in weakly acidic endosomes, allowing their efficient permeabilization by H5WYG but not by E5WYG. Different intracellular traffic of the lactosylated polyplexes and of the peptides in these three cell lines could also account for the differences in efficiency of these two peptides. In the case of Rb-1 and B16 cells, a low amount of E5WYG could reach endosomes (late endosomes) containing polyplexes with a luminal pH , allowing their efficient permeabilization by E5WYG, while H5WYG can already permeabilize endosomal membranes at a less acidic pH (early endosomes). H5WYG elicited its helper effect in gene transfection even in the presence of a high quantity of bovine fetal serum (even at concentration as high as 50%), while E5WYG was almost inactive at a concentration of above a few percent. E5WYG negatively charged at neutral pH could ionically bind to serum proteins and as a consequence had a decreased transfection efficiency, while H5WYG not charged at neutral pH , was not trapped by serum proteins, and kept its activity in the presence of serum.

H5WYG is supposed to disrupt the endosomal membrane containing lactosylated polylysine–DNA complexes. This is supported by the fact that the enhancement of the gene-transfer efficiency was totally abolished in the presence of bafilomycin A_1 , an inhibitor of vacuolar ATPases known to block the acidification of the lumen of endosomes by the endosomal proton pump (Figure 7). The lack of acidification of endosomes prevents the protonation of the imidazole groups of H5WYG and, as a consequence, its membrane permeabilization activity. The membrane permeabilization induced by H5WYG exhibiting an unordered structure in a slightly acidic medium is probably related to its polycationic nature as in the case of the peptide K5 (GLFKAIKFIKGGWKG-LIKG) at neutral pH , an amphiphilic cationic peptide (24, 44, 45), or as in the case of polyhistidine (46, 47), a polypeptide which becomes polycationic at slightly acidic pH . The generation of a polycationic form inside

the early endosomes is supposed to induce the disruption of their membrane and to favor the delivery of large molecules into the cytosol.

Recently, dioleoylphosphatidylethanolamine substituted with an imidazole group was used to make pH sensitive cationic liposomes and to increase the gene-transfer efficiency (48). Similarly, the DNA transfer was helped by using liposomes containing imidazole-substituted lipids (49).

In conclusion, the replacement of glutamyl residues in the GLFEAIAEFIEGGWEGLIEGWYG peptide by histidyl residues leads to a peptide that becomes positively charged when the pH decreases below pH 7.0, and appears to efficiently permeabilize cell membranes at a pH higher than that of the related anionic peptide. In addition, this histidyl-based permeabilization peptide H5WYG is efficient even in cells for which its acidic counterpart E5WYG is almost inefficient. Furthermore, H5WYG keeps its efficiency in the presence of serum, making it a potential tool for transferring genes *in vivo*.

ACKNOWLEDGMENT

We are grateful to Dr. Annie Claude Roche for helpful discussions and critical reading of the manuscript. We thank Sophie Fauvin, who received a fellowship from ANRS (Agence Nationale de Recherche sur le Sida), Philippe Marceau, and Françoise Fargette for their skillful technical help in cell culture and transfection experiments, the synthesis of peptides, and the preparation of plasmids, respectively. This work was partly supported by grants from ANRS (ANRS Grant 97003), "Association pour la Recherche sur le Cancer" (ARC), "Association Française de Lutte contre la Mucoviscidose" (AFLM), "Ministère de la Recherche et de la Technologie" (ACCSV14 Grant 9514104), MERCK/INSERM, and "Ligue Nationale Contre le Cancer". P. Midoux is Research Director at INSERM. A. Kichler received a fellowship from ARC. V. Boutin received a fellowship from MENESRI. J.-C. Maurizot is Research Director at CNRS. M. Monsigny is Professor at the University of Orléans.

LITERATURE CITED

- (1) Felgner, P. L., Gadek, T. R., Holm, M., Roman, R., Chan, H. W., Wenz, M., Northrop, J. P., Ringold, G. M., and Danielsen, M. (1987) Lipofection, a highly efficient, lipid-mediated DNA transfection procedure. *Proc. Natl. Acad. Sci. U.S.A.* 84, 7413–7417.
- (2) Behr, J. P., Demeinex, B., Loeffler, J. P., and Perez-Mutul, J. (1989) Efficient gene transfer into mammalian primary endocrine cells with lipopolyamine-coated DNA. *Proc. Natl. Acad. Sci. U.S.A.* 86, 6982–6986.
- (3) Monsigny, M., Roche, A. C., Midoux, P., and Mayer, R. (1994) Glycoconjugates as carriers for specific delivery of therapeutic drugs and genes. *Adv. Drug Delivery Rev.* 14, 1–24.
- (4) Wagner, E., Curiel, D. T., and Cotten, M. (1994) Delivery of drugs, proteins and genes into cells using transferrin as a ligand for receptor-mediated endocytosis. *Adv. Drug Delivery Rev.* 14, 113–136.
- (5) Frese, J., Jr., Wu, C. H., and Wu, G. Y. (1994) Targeting of genes to the liver with glycoprotein carriers. *Adv. Drug Delivery Rev.* 14, 137–153.
- (6) Cristiano, R. J., and Curiel, D. T. (1996) Strategies to accomplish gene delivery via the receptor-mediated endocytosis pathway. *Cancer Gene Ther.* 3, 49–57.
- (7) Boussif, O., Lezoualc'h, F., Zanta, M. A., Mergny, M. D., Scherman, D., Demeinex, B., and Behr, J. P. (1995) A versatile vector for gene and oligonucleotide transfer into cells in culture and *in vivo*: polyethylenimine. *Proc. Natl. Acad. Sci. U.S.A.* 92, 7297–7301.
- (8) Felgner, P. L., Barenholz, Y., Behr, J. P., Cheng, S. H., Cullis, P., Huang, L., Jessee, J. A., Seymour, L., Szoka, F., Thierry, A. R., Wagner, E., and Wu, G. (1997) Nomenclature for synthetic gene delivery systems. *Hum. Gene Ther.* 8, 511–512.
- (9) Kircheis, R., Kichler, A., Wallner, G., Kurs, M., Ogis, M., Felzmann, T., Buchberger, M., and Wagner, E. (1997) Coupling of cell-binding ligands to polyethylenimine for targeted gene delivery. *Gene Ther.* 4, 409–418.
- (10) Capaccioli, S., Di Pasquale, G., Mini, E., Mazzei, T., and Quattrone, A. (1993) Cationic lipids improve antisense oligonucleotide uptake and prevent degradation in cultured cells and in human serum. *Biochem. Biophys. Res. Commun.* 197, 818–825.
- (11) Bennet, C. F., Chiang, M.-Y., Chan, H., Shoelaker, J. E., and Mirabelli, C. K. (1992) Cationic lipids enhance cellular uptake and activity of phosphorothioate antisense oligonucleotides. *Mol. Pharmacol.* 41, 1023–1033.
- (12) Wu, G. H., and Wu, C. H. (1992) Specific inhibition of hepatitis B viral gene expression *in vitro* by targeted antisense oligonucleotides. *J. Biol. Chem.* 267, 12436–12439.
- (13) Citro, G., Perrotti, D., Cucco, C., D'Agnano, I., Sacchi, A., Zupi, G., and Calabretta, B. (1992) Inhibition of leukemia cell proliferation by receptor-mediated uptake of c-myc antisense oligodeoxynucleotides. *Proc. Natl. Acad. Sci. U.S.A.* 89, 7031–7035.
- (14) Citro, G., Szczylik, C., Ginobbi, P., Zupi, G., and Calabretta, B. (1994) Inhibition of leukemia cell proliferation by folic acid–polylysine-mediated introduction of c-myc antisense oligodeoxynucleotides into HL60 cells. *Br. J. Cancer* 69, 463–467.
- (15) Stewart, A. J., Pichon, C., Meunier, L., Midoux, P., Monsigny, M., and Roche, A. C. (1996) Enhanced biological activity of antisense oligonucleotides complexed with glycosylated poly-L-lysine. *Mol. Pharmacol.* 50, 1487–1494.
- (16) Zenke, M., Steinlein, P., Wagner, E., Cotten, M., Beug, H., and Birnstiel, M. L. (1990) Receptor-mediated endocytosis of transferrin–polycation conjugates: an efficient way to introduce DNA into hematopoietic cells. *Proc. Natl. Acad. Sci. U.S.A.* 87, 3655–3659.
- (17) Midoux, P., Mendes, C., Legrand, A., Raimond, J., Mayer, R., Monsigny, M., and Roche, A. C. (1993) Specific gene transfer mediated by lactosylated poly-L-lysine into hepatoma cells. *Nucleic Acids Res.* 21, 871–878.
- (18) Erbacher, P., Roche, A. C., Monsigny, M., and Midoux, P. (1995) Glycosylated polylysine/DNA complexes: gene transfer efficiency in relation with the size and the sugar substitution level of glycosylated polylysines and with the plasmid size. *Bioconjugate Chem.* 6, 401–410.
- (19) Erbacher, P., Roche, A. C., Monsigny, M., and Midoux, P. (1996) Putative role of chloroquine in gene transfer into a human hepatoma cell line by DNA/lactosylated polylysine complexes. *Exp. Cell Res.* 225, 186–194.
- (20) Zauner, W., Kichler, A., Schmidt, W., Sinski, A., and Wagner, E. (1996) Glycerol enhancement of ligand–polylysine/DNA transfection. *BioTechniques* 20, 905–913.
- (21) Wagner, E., Plank, C., Zatloukal, K., Cotten, M., and Birnstiel, M. L. (1992) Influenza virus hemagglutinin HA-2 N-terminal fusogenic peptides augment gene transfer by transferrin–polylysine–DNA complexes: toward a synthetic virus-like gene-transfer vehicle. *Proc. Natl. Acad. Sci. U.S.A.* 89, 7934–7938.
- (22) Plank, C., Zatloukal, K., Cotten, M., Mechtler, K., and Wagner, E. (1992) Gene transfer into hepatocytes using asialoglycoprotein receptor mediated endocytosis of DNA complexed with an artificial tetra-antennary galactose ligand. *Bioconjugate Chem.* 3, 533–539.
- (23) Plank, C., Oberhauser, B., Mechtler, K., Koch, C., and Wagner, E. (1994) The influence of endosome-disruptive peptides on gene transfer using synthetic virus-like gene transfer systems. *J. Biol. Chem.* 269, 12918–12924.
- (24) Murata, M., Kagiwada, S., Takahashi, S., and Ohnishi, S. (1991) Membrane fusion induced by mutual interaction of the two charge-reversed amphiphilic peptides at neutral pH. *J. Biol. Chem.* 266, 14353–14358.

- (25) Murata, M., Takahashi, S., Kagiwada, S., Suzuki, A., and Ohnishi, S. (1992) pH-dependent membrane fusion and vesiculation of phospholipid large unilamellar vesicles induced by amphiphilic anionic and cationic peptides. *Biochemistry* 31, 1986–1992.
- (26) Midoux, P., Mayer, R., and Monsigny, M. (1995) Membrane permeabilization by alpha-helical peptides: a flow cytometry study. *Biochim. Biophys. Acta* 1239, 249–256.
- (27) Gottschalk, S., Sparrow, J. T., Hauer, J., Mims, M. P., Leland, F. E., Woo, S. L. C., and Smith, L. C. (1996) A novel DNA-peptide complex for efficient gene transfer and expression in mammalian cells. *Gene Ther.* 3, 448–457.
- (28) Zauner, W., Blaas, D., Kuechler, E., and Wagner, E. (1995) Rhinovirus-mediated endosomal release of transfection complexes. *J. Virol.* 69, 1085–1092.
- (29) Subbarao, N. K., Parente, R. A., Szoka, F. C., Nadasli, L., and Pongracz, K. (1987) pH-dependent bilayer destabilization by amphipathic peptide. *Biochemistry* 26, 2964–2974.
- (30) Dempsey, C. E. (1990) The actions of melittin on membranes. *Biochim. Biophys. Acta* 1031, 143–161.
- (31) Wymn, T. B., Nicol, F., Zelphati, O., Scaria, P. V., Plank, C., and Szoka, F. C. (1997) Design, synthesis, and characterization of a cationic peptide that binds to nucleic acids and permeabilizes bilayers. *Biochemistry* 36, 3008–3017.
- (32) Legendre, J. Y., Trzeciak, A., Bohrmann, B., Deuschle, U., Kitas, E., and Supersaxo, A. (1997) Dioleoylmelittin as a novel serum-insensitive reagent for efficient transfection of mammalian cells. *Bioconjugate Chem.* 8, 57–63.
- (33) Derrien, D., Midoux, P., Petit, C., Negre, E., Mayer, R., Monsigny, M., and Roche, A. C. (1989) Muramyl dipeptide bound to poly-L-lysine substituted with mannose and glucanoyl residues as macrophage activators. *Glycoconjugate J.* 6, 241–255.
- (34) Nachtigal, M., Nagpal, M. L., Greenspan, P., Nachtigal, S. A., and Legrand, A. (1989) Characterization of a continuous smooth muscle cell line derived from rabbit aorta. *In Vitro Cell. Dev. Biol.* 25, 892–898.
- (35) Chen, T. R. (1977) In situ detection of mycoplasma contamination in cell cultures by fluorescent Hoeschst 33258 stain. *Exp. Cell Res.* 104, 255–262.
- (36) Monsigny, M., Midoux, P., Depierreux, C., Bebear, C., Le Bris, M. T., and Valeur, B. (1990) Benzoxazinone kanamycin A conjugate. A new fluorescent probe suitable to detect mycoplasmas in culture. *Biol. Cell* 70, 101–105.
- (37) Brasier, A. R., Tate, J. E., and Habener, J. F. (1989) Optimized use of the firefly luciferase assay as a reporter gene in mammalian cell lines. *BioTechniques* 7, 1116–1123.
- (38) De Wet, J. R., Wood, K. V., De Luca, M., Helinsky, D. R., and Subramani, S. (1987) Firefly luciferase gene: structure and expression in mammalian cells. *Mol. Cell. Biol.* 7, 725–737.
- (39) Bowman, E. J., Siebers, A., and Altendorf, K. (1988) Bafilomycins: a class of inhibitors of membrane ATPases from microorganisms, animal cells, and plant cells. *Proc. Natl. Acad. Sci. U.S.A.* 85, 7972–7976.
- (40) Kollen, W. J. W., Midoux, P., Erbacher, P., Yip, A., Roche, A. C., Monsigny, M., Glick, M. C., and Scanlin, T. F. (1996) Glucanoylated and glycosylated polylysines as vectors for gene transfer into cystic fibrosis airway epithelial cells. *Hum. Gene Ther.* 7, 1577–1586.
- (41) Erbacher, P., Bousser, M.-T., Raimond, J., Monsigny, M., Midoux, P., and Roche, A. C. (1996) Gene transfer by DNA/glycosylated polylysine complexes into human blood monocyte-derived macrophages. *Hum. Gene Ther.* 7, 721–729.
- (42) Schwartz, A. L., Fridovich, S. E., Knowles, B. B., and Lodish, H. F. (1981) Characterization of the asialoglycoprotein receptor in a continuous hepatoma line. *J. Biol. Chem.* 256, 8878–8881.
- (43) Ras, A., and Lotan, R. (1987) Endogenous galactoside-binding lectins: a new class of functional tumor cell surface molecules related to metastasis. *Cancer Metastasis Rev.* 6, 433–452.
- (44) Takahashi, S. (1990) Conformation of membrane fusion-active 20-residue peptides with or without lipid bilayers. Implication of α -Helix formation for membrane fusion. *Biochemistry* 29, 6257–6264.
- (45) Murata, M., Shirai, Y., Ishiguro, R., Kagiwada, S., Tahara, Y., Ohnishi, S., and Takahashi, S. (1993) Fusion of dioleoylphosphatidylcholine vesicles induced by an amphiphilic cationic peptide and oligophosphates at neutral pH. *Biochim. Biophys. Acta* 1152, 99–108.
- (46) Wang, C.-Y., and Huang, L. (1984) Polyhistidine mediates an acid-dependent fusion of negatively charged liposomes. *Biochemistry* 23, 4409–4416.
- (47) Uster, P. S., and Deamer, D. W. (1985) pH-dependent fusion of liposomes using titratable polycations. *Biochemistry* 24, 1–8.
- (48) Budker, V., Gurevich, V., Hagstrom, J. E., Bortzov, F., and Wolff, J. A. (1996) pH-sensitive, cationic liposomes: a new synthetic virus-like vector. *Nat. Biotechnol.* 14, 760–764.
- (49) Hughes, J. A., Aronsohn, A. I., Avrutskaya, A. V., and Juliano, R. L. (1996) Evaluation of adjuvants that enhance the effectiveness of antisense oligodeoxynucleotides. *Pharm. Res.* 13, 404–410.

Novel Glycosynthons for Glycoconjugate Preparation: Oligosaccharylpyroglutamylanilide Derivatives

Christophe Quétard,[†] Sylvain Bourgerie,[†] Nadia Normand-Sdiqui,^{†,‡} Roger Mayer,[†] Gérard Strecker,[§] Patrick Midoux,[†] Annie-Claude Roche,[†] and Michel Monsigny^{*,†}

Glycobiologie, Centre de Biophysique Moléculaire, CNRS and Université d'Orléans, F-45071 Orléans Cedex 2, France, and Laboratoire de Chimie Biologique, CNRS, Université des Sciences et Technologies de Lille, F-59655 Villeneuve d'Ascq Cedex, France. Received July 2, 1997; Revised Manuscript Received October 15, 1997

The reducing sugar of an oligosaccharide reacting with the α -amino group of an amino acid is converted to an *N*-oligosaccharyl amino acid which can then be stabilized by *N*-acylation. Oligosaccharides in solution in *N,N*-dimethylformamide reacted with α -glutamyl-*p*-nitroanilide at 50 °C for a few hours, leading to an *N*-oligosaccharylglutamyl-*p*-nitroanilide. Then, the γ -carboxylic group of the glutamyl moiety, activated by adding (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate (BOP), reacted with the substituted α -amino group of the glutamyl residue, leading to an *N*-oligosaccharylpyroglutamyl-*p*-nitroanilide within 0.5 h. Such a one-pot two-step reaction was shown to be very efficient in the case of a disaccharide such as lactose, or pentasaccharides such as lacto-*N*-fucopentaoses, Lewis^a or Lewis^x. The glycosynthons were characterized by chromatography (HPAEC and HPLC); their molecular mass was determined by electrospray ionization mass spectrometry, and the glycosylamides were shown to have a β -anomeric configuration on the basis of their proton NMR. The *N*-oligosaccharylpyroglutamyl-*p*-nitroanilides are quite stable at room temperature over a large pH range. They are easily converted to *N*-oligosaccharylpyroglutamyl-*p*-isothiocyanatoanilides which can be used to prepare glycoconjugates such as cationic glycosylated polylysines suitable for specifically delivering genes or oligonucleotides in a sugar-dependent manner.

INTRODUCTION

The sugar moieties of glycoconjugates are recognition signals which are involved in several important physiological phenomena [for reviews, see Monsigny et al. (1988b,c, 1994b), Lis and Sharon (1991), Drickamer and Taylor (1993), Varki (1993), Karlsson (1995), and Crocker and Feizi (1996)]. To make easier the investigation of the role of those recognition signals, synthetic glycoconjugates have been prepared, including neoglycoproteins, serum albumin substituted with about 25 simple sugar residues as well as glycosylated neutral polymers [for reviews, see Aplin and Wriston (1981), Stowell and Lee (1980), Duncan and Kopecek (1984), Monsigny et al. (1983, 1989, 1994a,b), Lee and Lee (1994a–c), Wong (1995), Roy (1996), and Gabius and Gabius (1997)]. While free mono- or disaccharides usually have a low affinity for lectins, those synthetic glycoconjugates have a high apparent affinity for lectins [Privat et al., 1974; Krantz et al., 1976; Townsend and Stahl, 1981; for reviews, see Stowell and Lee (1980), Monsigny et al. (1994a), Lee and Lee (1994), and Kiessling and Pohl (1996)] because of the presence of a large number of sugar units inducing a multiplicity effect related to avidity. Similarly, complex oligosaccharides have a much higher affinity than their various components. For instance, the galactose-specific hepatocyte lectin binds triantennary oligosaccharides with three Gal β -4GlcNAc β moieties in

a terminal nonreducing position, with an affinity 1000 times higher than that of monoantennary oligosaccharides with a single Gal β -4GlcNAc β moiety in a terminal nonreducing position (Lee et al., 1983). On these bases, neoglycoproteins and glycopolymers bearing a small number of complex oligosaccharides should be more efficient and more specific than those bearing simple sugars. To prepare such glycoconjugates, several methods leading to activated oligosaccharide derivatives suitable to be covalently linked to either a protein, a polymer, or a matrix have been described. These methods belong to three main categories. The first one is a total *de novo* synthesis [for reviews, see Garg et al. (1994), Sinay (1991), Lee and Lee (1992, 1994a–c), Meldal (1994a,b), Roy (1996), Whitfield and Douglas (1996), and Von Itzstein and Colman (1996)] which requires many protection, coupling, and deprotection steps; this approach is time-consuming, and the overall yield remains relatively low. In the second approach, the oligosaccharide can be reversibly converted into a glycosylamine by incubation in the presence of a high concentration of ammonia, an ammonium salt, an aliphatic amine, or an aromatic amine [for reviews, see Ellis and Honeyman (1955), Isbell and Frush (1980), Aplin and Wriston (1981), Kallin (1994), Rice and Corradi Da Silva (1996), and Roy (1996)]. The glycosylamines must be further stabilized by acylation [see, for instance, Isbell and Frush (1958), Thomas (1970), and Likhoshershtov et al. (1986)], the acylating agent being usually an organic compound containing an activated carboxylic group. However, this method suffers from two major disadvantages. One is that it requires several intermediary reactions and purification steps. The second is that there is at least one step in alkaline medium which may release any acyl substituent borne

* To whom correspondence should be addressed. Telephone: 33 2 38 25 55 57. Fax: 33 2 38 69 00 94. E-mail: monsigny@cns-orleans.fr.

[†] CNRS and Université d'Orléans.

[‡] Current address: Innovir Ltd., Cambridge CB4 4FJ, United Kingdom.

[§] Université des Sciences et Technologies de Lille.

on the oligosaccharide. The third approach proposed by Gray (1974) is a reductive amination, which allows a one-step glycoconjugate synthesis. This method leads to an altered oligosaccharide, the reducing sugar being transformed into a linear polyol amine derivative.

In a previous paper (Sdiqui et al., 1995), we reported a one-pot two-step synthesis of *N*-glycoamino acids: the first step was the coupling of an oligosaccharide with α -glycyl-*p*-nitroanilide (Gly-*p*NA¹), and the second step was an acylation of the glycosylamine formed. Here, we describe the synthesis of novel glycosylamine derivatives by coupling α -glutamyl-*p*-nitroanilide (Glu-*p*NA) to reducing oligosaccharides. With regard to the previous method described by Sdiqui et al. (1995), this new method has two main advantages. First, the formation of the glycosylamine is faster. Second, the acylation step is an intramolecular reaction involving the γ -carboxylic group of glutamic acid, and as a consequence, it is rapid, efficient, and highly specific. The glycoamino acids obtained can be further coupled to any molecule bearing an amino group.

EXPERIMENTAL PROCEDURES

Materials. α -Glutamyl-*p*-nitroanilide (Glu-*p*NA) was purchased from Bachem (Bubendorf, Switzerland). Poly-(L-lysine) (pLK) HBr salt ($M_r = 95\,100$) with a mean degree of polymerization of 455 (pLK₄₅₅) and ϵ -aminocaproic acid (Aca) were purchased from Sigma (St. Quentin Fallavier, France); pLK, HBr was transformed into pLK, *p*-toluenesulfonate (Derrien et al., 1989) to increase the solubility of the polymer in organic solvents and to avoid the cytotoxic effect of the bromide anion. Lactose and *N,N*-diisopropylethylamine (DIEA) were from Janssen Chimica (Beerse, Belgium); imidazole and dimethyl sulfoxide (DMSO) were from Merck (Darmstadt, Germany). (Benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate (BOP) was from Richelieu Biotechnologies (Saint Hyacinthe, Canada). *N,N*-Dimethylformamide (DMF) was freshly distilled in the presence of 0.2 mg/mL (benzyloxycarbonyl)glycyl-*p*-nitrophenyl ester. *N,N'*-Thiocarbamylbisimidazole (TCBI), *p*-toluenesulfonic acid, and δ -gluconolactone were purchased from Aldrich (St. Quentin Fallavier, France); lacto-*N*-fucopentaose II [Lewis^a, Gal β -(Fuc α 4)3GlcNAc β -3Gal β -4Glc] and III [Lewis^x, Gal β -(Fuc α 3)4GlcNAc β -3Gal β -4Glc] (as a mixture) were isolated from human milk (G. Strecker, unpublished data).

Chromatography. Conjugates were purified by gel filtration on a column (2.3 $\times$ 90 cm) of Trisacryl GF 05 (Biosepra, Villeneuve-la-Garenne, France) stabilized in and eluted with 0.1 M acetic acid containing 3% *n*-butanol (flow rate, 8 mL/h).

High-performance anion-exchange chromatography (HPAEC) was performed on a Dionex DX-300 chromatography system (Sunnyvale, CA) which includes a quaternary gradient pump, an eluent degas (He) module, a (4 $\times$ 250 mm) CarboPac PA1 column with a matching guard column, a pulsed amperometric detector (PAD), and a PD40 diode array detector. The flow rate was 1

mL/min. The gradient was obtained by using two solutions (solvent A, 0.1 N sodium hydroxide; and solvent B, 1 M sodium acetate in 0.1 N sodium hydroxide). The column was equilibrated with solution A and eluted with solution A for 5 min and linear gradients of solution B up to 20% over the course of 10 min and then up to 100% over the course of 15 min.

High-performance liquid chromatography (HPLC) analysis was performed on a reverse phase (4 $\times$ 250 mm) column (LiChrospher 100 RP-18, 5 μ m, Merck) to monitor the reduction of *N*-(β -lactosyl)-pGlu-*p*NA into *N*-(β -lactosyl)-pGlu-*p*-aminoanilide and its conversion into *N*-(β -lactosyl)-pGlu-*p*-amidophenyl isothiocyanate [*N*-(β -lactosyl)-pGlu-*p*-amidoPITC]; a reverse phase (3.9 $\times$ 150 mm) cartridge column (Nova-Pak C18, 4 μ m, Waters) was used to monitor the coupling of Aca with *N*-(β -lactosyl)-pGlu-*p*-amidoPITC. The HPLC system included a dual pump system monitored by a Waters 600 Controller linked to a Waters 996 photodiode array detector. The mobile phase was solvent C [0.1 M triethylammonium acetate (TEAA, pH 7.0) and 5% acetonitrile] and solvent D [acetonitrile and 0.1 M TEAA (5%)]. The flow rate was 1 mL/min. The column was equilibrated with a mixture of solvents C and D (95:5, v/v). After the sample (20 μ L) was injected, the column was eluted for 5 min with a 95% C/5% D mixture; solvent D then increased linearly from 5 up to 30% over the course of 30 min.

Electrospray Ionization Mass Spectrometry (ESI-MS). The positive ion electrospray mass spectra were obtained using a Platform quadrupole mass spectrometer (VG Biotech, Fisons Instruments, Altrincham, U.K.) equipped with an electrospray atmospheric pressure ionization source. The capillary voltage was set at 3 kV, and the cone voltage was adjusted at 25 V. The analyzer was calibrated for *m/z* from 400 to 1200 in the positive ion mode using a cesium iodide solution. The sample was dissolved in an acetonitrile/1% formic acid mixture (1:1, v/v) at 20–50 pmol/mL.

¹H NMR Spectroscopy. For ¹H NMR analysis, compounds were dissolved at room temperature in deuterated water at 5 mg/mL, once with D₂O containing 99.9% D and, after freeze-drying, a second time with D₂O containing 99.96% D (Sigma). ¹H NMR spectroscopy was performed at 300 MHz on a Bruker AM-300 spectrometer (Wissembourg, France). Chemical shifts were measured with acetone ($\delta = 2.225$ ppm in D₂O) used as an internal reference and were expressed in parts per million downfield from internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS).

Synthesis, Purification, and Characterization of *N*-(β -Oligosaccharyl)-pGlu-*p*NA. (1) *N*-(β -Lactosyl)-pGlu-*p*NA (4). In a typical experiment, 5 mg (15 μ mol) of lactose (2, peak 2 in Figure 2A), 7.8 mg (30 μ mol) of Glu-*p*NA, and 4 mg (60 μ mol) of imidazole (1, peak 1 in Figure 2A) were dissolved in 250 μ L of DMF. The solution was kept at 50 $^{\circ}$ C for 8 h.

Without any purification, the *N*-(β -lactosyl)-Glu-*p*NA (3, peak 3 in Figure 2) was further intramolecularly *N*-acylated at room temperature by adding 14.6 mg (33 μ mol) of BOP and 4 mg (60 μ mol) of imidazole. The formation of lactosylpyroglutamyl-*p*-nitroanilide, *N*-(β -lactosyl)-pGlu-*p*NA (4, peak 4 in Figure 2), monitored by HPAEC, was found to be complete within 30 min (Figure 2).

N-(β -Lactosyl)-pGlu-*p*NA (4) was purified by gel filtration. The reaction mixture diluted up to 10 mL with solvent E (0.1 M acetic acid containing 3% *n*-butanol) was introduced onto a Trisacryl GF 05 column (2.3 $\times$ 90 cm) using solvent E as an eluent at a flow rate of 8 mL/h.

¹ Abbreviations: Aca, ϵ -aminocaproic acid; BOP, (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate; DIEA, *N,N*-diisopropylethylamine; DMF, *N,N*-dimethylformamide; DMSO, dimethyl sulfoxide; Glu-*p*NA, α -glutamyl-*p*-nitroanilide; Gly-*p*NA, α -glycyl-*p*-nitroanilide; HOBt, 1-hydroxybenzotriazole; HPAEC, high-performance anion-exchange chromatography; pA, *p*-aminoanilide; PITC, phenyl isothiocyanate; pLK, poly(L-lysine); pNA, *p*-nitroanilide; TCBI, *N,N'*-thiocarbamylbisimidazole; TEAA, triethylammonium acetate.

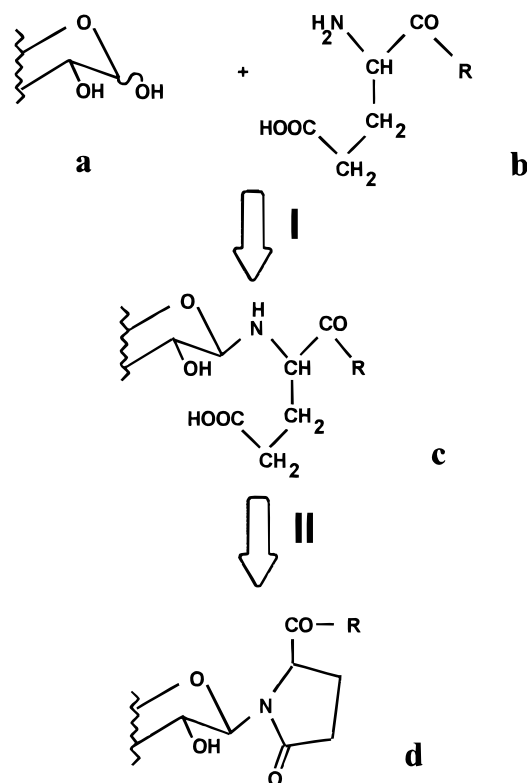


Figure 1. Synthesis of glycoamino acid species: I, coupling; and II, cyclization. (a) Reducing oligosaccharide. (b) α -Glu derivative, Glu-pNA when R is *p*-nitroanilide ($\text{NHC}_6\text{H}_4\text{NO}_2$). (c) *N*-Oligosaccharyl-Glu-pNA. (d) *N*-Oligosaccharyl-pGlu-pNA.

The glycoamino acid came out first; the other reagents and the coupling solvent came out later. As shown by HPAEC, the purified product still contained some imidazole. The glycoconjugate was made free of imidazole by precipitation from its water solution upon adding 9 volumes of ethanol. Using electrospray ionization mass spectrometry, the mass of the molecular ion was 574.2 (Figure 3A), equal to that calculated for $\text{C}_{23}\text{H}_{31}\text{N}_3\text{O}_{14}$ [($\text{M} + \text{H}$) $^+$, 574.1]: ^1H NMR (300 MHz, D_2O) δ 8.29 and 7.75 (4H, 2d, *H* aromatic), 5.23 (1H, d, $J_{1,2} = 8.25$ Hz, *H*-1, β -D-Glc), 4.65 (1H, d, $J_{1,2} = 7.83$ Hz, α CH pGlu), 4.37 (1H, d, $J_{1,2} = 7.51$ Hz, *H*-1 β -D-Gal), 2.83 (1H, m, γ CH_2 pGlu), 2.56 (2H, m, γ' CH_2 and β CH_2 pGlu), 2.28 (1H, m, β' CH_2 pGlu).

(2) *Lewis*^a/*Lewis*^x-pGlu-pNA. *Lewis*^a/*Lewis*^x oligosaccharides were treated under the conditions described for the synthesis of lactosyl-pGlu-pNA.

The mass of the molecular ion, using an electrospray ionization mass spectrometer [calculated for $\text{C}_{43}\text{H}_{64}\text{N}_4\text{O}_{28}$ ($\text{M} + \text{H}$) $^+$, 1085.4], was found to be 1085.6 (Figure 3B). The assignment of the *Lewis*^a/*Lewis*^x-pGlu-pNA ^1H NMR spectrum in D_2O was as follows: resonances common to the two glycopeptides δ 8.33 and 7.79 (4H, 2d, *H* aromatic), 4.90 (2H, m, *H*-5 α -L-Fuc), 4.67 (1H, d, $J_{1,2} = 7.32$ Hz, *H*-1 β -D-GlcNAc), 4.63 (1H, d, α CH pGlu), 4.37 (1H, d, $J_{1,2} = 7.42$ Hz, *H*-1 β -D-Gal^{im}), 4.16 (1H, s, *H*-4 β -D-Gal^{im}), 2.83 (H, m, γ CH_2 pGlu), 2.58 (2H, m, γ' and β CH_2 pGlu), 2.33 (H, m, β' CH_2 pGlu), 2.05 and 2.04 (6H, 2s, CH_3 Ac β -D-GlcNAc), 1.22 and 1.21 (6H, 2s, CH_3 α -L-Fuc); *Lewis*^a-specific δ 5.05 (1H, d, *H*-1 α -L-Fuc); 4.53 (1H, d, *H*-1 β -D-Gal); *Lewis*^x-specific δ 5.24 (1H, d, $J_{1,2} = 7.2$ Hz, *H*-1 α -L-Fuc); 4.50 (1H, d, *H*-1 β -D-Gal).

Stability of Glycosyl-pGlu-pNA. To assess the stability of the glycosyl-pGlu-pNA derivatives, we exposed the lactosyl-pGlu-pNA (4) at various pHs. The

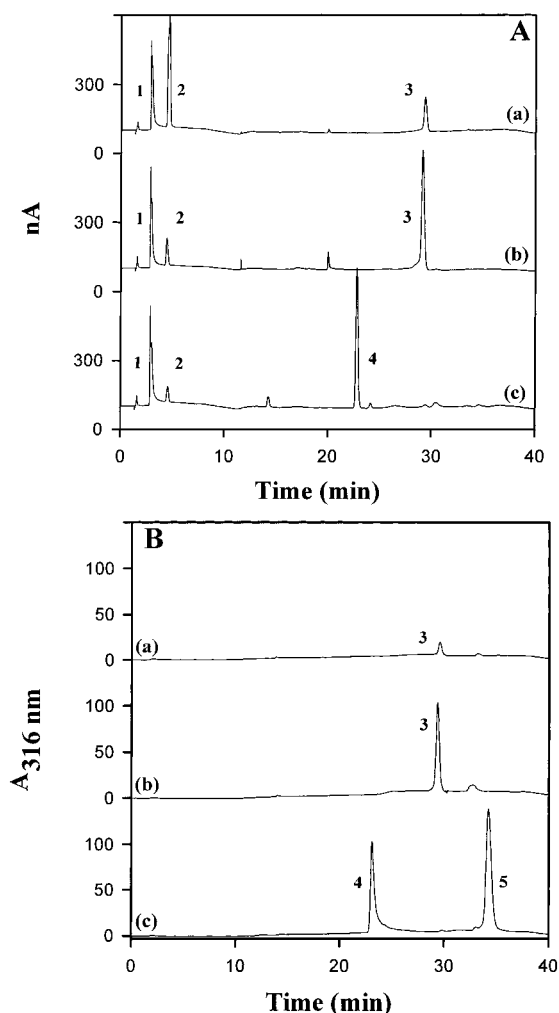


Figure 2. Conversion of lactose to *N*-(β -lactosyl)-pGlu-pNA. HPAE chromatography analysis monitored using (A) amperometry and (B) absorbance at 316 nm: (a) after 10 min, (b) after incubation for 6 h, and (c) after the cyclization step. Peak 1, imidazole; peak 2, lactose; peak 3, *N*-(β -lactosyl)-Glu-pNA; peak 4, *N*-(β -lactosyl)-pGlu-pNA; and peak 5, HOBT.

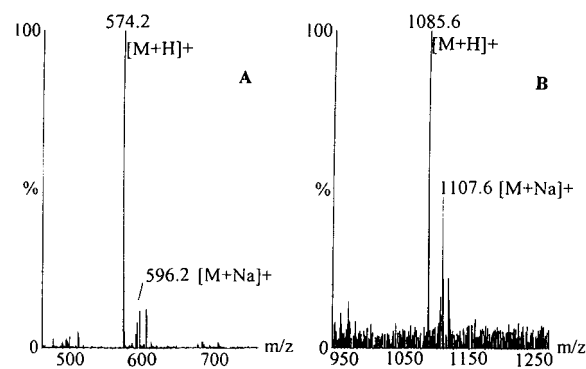


Figure 3. Electrospray ionization mass spectra of (A) *N*-(β -lactosyl)-pGlu-pNA (4) and (B) *Lewis*^a/*Lewis*^x-pGlu-pNA.

formation of putative degradation products was monitored by HPAEC, using sorbitol as an internal standard. The conjugate (1 mg/mL) and sorbitol (500 $\mu\text{g/mL}$) were maintained up to 48 h at either 25 or 90 $^{\circ}\text{C}$ in an acidic environment (50 mM sodium acetate buffer at pH 3.6), in a neutral medium (50 mM sodium phosphate buffer at pH 7.0), or in a basic environment (50 mM sodium carbonate buffer at pH 9.0).

Reduction of *N*-(β -Lactosyl)-pGlu-pNA (4) and Conversion into *N*-(β -Lactosyl)-pGlu-p-amidophen-

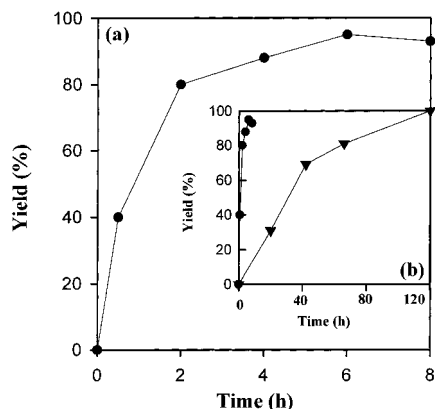


Figure 4. Time course of the formation of (●) *N*-(β-lactosyl)-Glu-pNA and comparison with the formation of (▼) *N*-(β-lactosyl)-Gly-pNA (inset). Each reaction was performed in DMF at 50 °C in the presence of imidazole. The conjugate formation was monitored by HPAE chromatography.

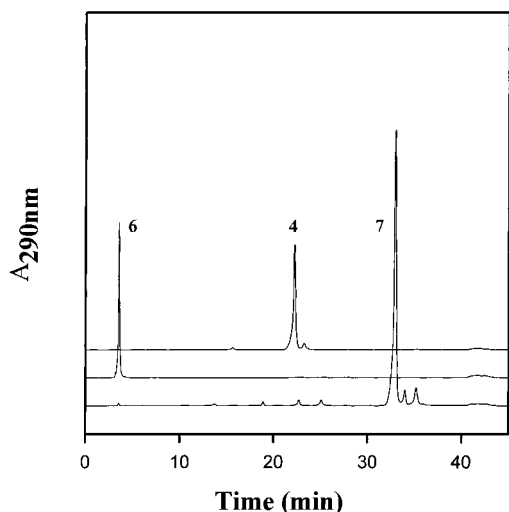


Figure 5. Preparation of *N*-(β-lactosyl)-pGlu-p-amidoPITC, monitored by HPLC analysis at 290 nm. Reduction of *N*-(β-lactosyl)-pGlu-pNA (4) into *N*-(β-lactosyl)-pGlu-paA (6) and conversion to *N*-(β-lactosyl)-pGlu-p-amidoPITC (7).

yl Isothiocyanate [7, *N*-(β-Lactosyl)-pGlu-p-amido-PITC]. *N*-(β-Lactosyl)-pGlu-pNA (4) (5 mg, 8.7 μmol) was dissolved in 1 mL of a water/methanol mixture (1:1, v/v). To this solution was added 2 mg of 10% palladium on charcoal (Merck). The suspension was stirred for 3 h under hydrogen (1 atm) at room temperature. Both *N*-(β-lactosyl)-pGlu-pNA (4, peak 4 in Figure 5) and its reduced derivative *N*-(β-lactosyl)-pGlu-p-aminoanilide [*N*-(β-lactosyl)-pGlu-paA] (6, peak 6 in Figure 5) were monitored by HPLC at 290 nm.

The palladium on charcoal was removed by centrifugation. The methanolic solution was treated at room temperature with 23 μL of a TCBI (13 μmol) solution at 100 mg/mL in chloroform. After the mixture was stirred for 30 min, the *N*-(β-lactosyl)-pGlu-p-amidoPITC (7, peak 7 in Figure 5) was obtained in a good yield, as shown by HPLC at 290 nm (Figure 5). One volume of chloroform was added to extract the excess of TCBI. The organic phase was removed upon centrifugation (3000g for 5 min). Then methanol was removed under reduced pressure.

Lewis^a/Lewis^x-pGlu-pNA was reduced according to the same procedure.

Coupling of ε-Aminocaproic Acid (Aca) with *N*-(β-Lactosyl)-pGlu-p-amidoPITC (7). Three milligrams

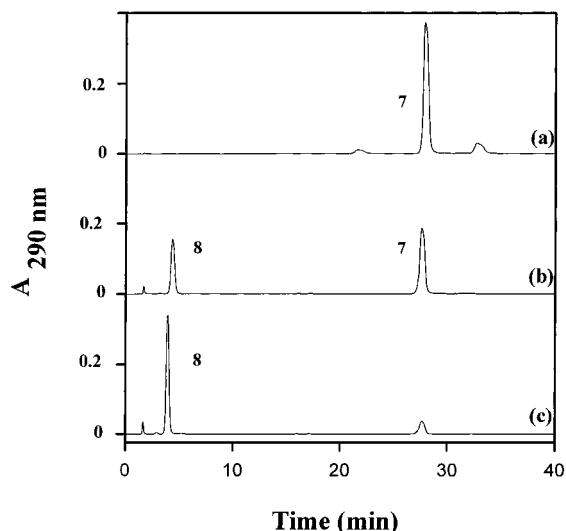


Figure 6. Time course of the coupling of ε-aminocaproic acid (5 equiv) with *N*-(β-lactosyl)-pGlu-p-amidoPITC (1 equiv) monitored by HPLC at 290 nm: after (a) 1 min, (b) 45 min, and (c) 100 min. Peak 7 is *N*-(β-lactosyl)-pGlu-p-amidoPITC, and peak 8 is ε-[*N*-(β-lactosyl)-pGlu-p-amidophenylthioureido]caproic acid.

(5.2 μmol) of *N*-(β-lactosyl)-pGlu-p-amidoPITC (7, peak 7 in Figure 6) was added to Aca (3.4 mg, 25 μmol) in 800 μL of 0.3 M NaCl, 0.1 M sodium bicarbonate buffer at pH 9.3. The mixture was stirred at room temperature and monitored by HPLC at 290 nm. The glycosylated Aca, ε-[*N*-(β-lactosyl)-pGlu-p-amidophenylthioureido]caproic acid (8, peak 8 in Figure 6) was obtained after 100 min in 90% yield as calculated from HPLC. The pure compound was isolated by collecting the corresponding HPLC peak and evaporation of the solvent. The mass of the molecular ion using an electrospray ionization mass spectrometer [calculated for C₃₀H₄₄N₄O₁₄S₁ (M + H)⁺, 717.3] was 717.2: ¹H NMR (300 MHz, D₂O) δ 7.56 and 7.34 (4H, 2d, *H* aromatic), 5.22 (1H, d, *H*-1 β-D-Glc), 4.63 (1H, d, α *CH* pGlu), 4.40 (1H, d, *H*-1 β-D-Gal), 3.57 (2H, m, CH₂-6 Aca), 2.83 (1H, m, γ CH₂ pGlu), 2.58 (2H, m, γ' CH₂ and β CH₂ pGlu), 2.28 (1H, m, β' CH₂ pGlu), 2.22 (2H, t, CH₂-2 Aca), 1.61 (4H, m, CH₂-3 and 5 Aca), 1.38 (2H, m, CH₂-4 Aca).

Preparation of the (Lewis^a/Lewis^x)₁₀₀-pLK⁴⁵⁵ Conjugate. Lewis^a/Lewis^x-pGlu-p-amidoPITC (8 mg, 7.4 μmol) was added to *p*-toluenesulfonate poly(L-lysine) (9.0 mg, 0.0655 μmol) dissolved in 1 mL of DMSO in the presence of DIEA (8.2 μL, 58 μmol). The mixture was stirred overnight at room temperature. The glycosylated pLK was precipitated by adding 10 volumes of 2-propanol and centrifuged (1800g for 15 min). The pellet was washed with 2-propanol, collected upon centrifugation (1800g for 15 min), solubilized in distilled water, and freeze-dried. The average number of Lewis^a/Lewis^x residues bound per pLK molecule was determined using the colorimetric resorcinol sulfuric acid method (Monsigny et al., 1988a).

RESULTS

Synthesis of *N*-(β-Lactosyl)-pGlu-pNA. The general scheme for the synthesis is shown in Figure 1. The first step (I) was the coupling between a reducing oligosaccharide (a) and α-glutamyl-*p*-nitroanilide (b) in the presence of imidazole. Efficient and rapid coupling between the hemiacetal hydroxyl group of the reducing saccharide and the α-amino group of glutamic acid occurred by dehydration. The second step (II) was an intramolecular *N*-acylation (d) of the glycoamino acid

derivative. The reaction was monitored by HPAEC by using two detectors (amperometric and UV). Chromatograms in panels A and B of Figure 2 showed that the amount of *N*-(β -lactosyl)-Glu-pNA (**3**, peak 3 in Figure 2A,B), eluted at 29.2 min, increased as the reaction proceeded; concomitantly, the amount of lactose (**2**, peak 2 in Figure 2A), which came out at 4.9 min, decreased. The time course of the coupling between Glu-pNA and lactose, as monitored by HPAEC, showed that the formation of *N*-lactosyl-Glu-pNA was fast; after 2 h, 80% of the lactose was already converted into lactosyl-Glu-pNA, and after 6 h, 95% was converted (Figure 4). Comparatively, the time course of the coupling between lactose and Gly-pNA was much slower (Figure 4, inset); 120 h was required to reach the 95% level of reaction (Sdiqui et al., 1995). Stabilization of the glycopeptide with 1.1 equiv of BOP and an additional 2 equiv of imidazole led to *N*-(β -lactosyl)-pGlu-pNA (**4**, peak 4 in Figure 2A,B) which came out at 23 min (Figure 2A,B, chromatograms c).

Stability of *N*-(β -Lactosyl)-pGlu-pNA (4**).** The lactosyl-pyroglutamate conjugate maintained at 25 °C for 48 h at pH 7.0, 3.6, or 9.0 was quite stable; neither a decrease in the *N*-(β -lactosyl)-pGlu-pNA (**4**) nor the appearance of a new compound could be detected. At 90 °C, the *N*-(β -lactosyl)-pGlu-pNA (**4**) was stable as long as the pH was either slightly acidic (pH 3.6) or neutral (pH 7.0), and no degradation product could be detected even after 5 h. In contrast, in alkaline medium (pH 9.0), the concentration of *N*-(β -lactosyl)-pGlu-pNA (**4**) decreased according to a first-order kinetics with a constant of $1.25 \times 10^{-4} \text{ s}^{-1}$; a second, still unidentified compound (retention time, 11.5 min) appeared, and its amount increased correlatively.

Reduction of *N*-(β -Lactosyl)-pGlu-pNA (4**), Conversion into *N*-(β -Lactosyl)-pGlu-p-amidoPITC (**7**), and Its Reaction with ϵ -Aminocaproic Acid.** The pure product **4** [*N*-(β -lactosyl)-pGlu-pNA, peak 4 in Figure 5] was quantitatively reduced into **6** [*N*-(β -lactosyl)-pyroglutamyl-*p*-aminoanilide [*N*-(β -lactosyl)-pGlu-paA, peak 6], in the presence of palladium (10% Pd) on charcoal under a hydrogen pressure of 1 atm. The reduced derivative **6** then reacted with TCBI and was converted into *N*-(β -lactosyl)-pyroglutamyl-*p*-isothiocyanatoanilide [*N*-(β -lactosyl)-pGlu-p-amidoPITC], compound **7** (peak 7 in Figure 5), within 30 min. The arylisothiocyanate group was further corroborated by infrared spectroscopy (ν , 2120 cm^{-1}) and by UV spectroscopy on the basis of its maximal absorbance at 290 nm. To test the reactivity of this compound with an ω -alkylamine, the coupling reaction between **7** [*N*-(β -lactosyl)-pGlu-p-amidoPITC] and ϵ -aminocaproic acid (Aca) was monitored by HPLC at 290 nm (Figure 6). A complete disappearance of **7**, *N*-(β -lactosyl)-pGlu-p-amidoPITC, occurred within 2 h, leading to the formation of **8** [ϵ -[*N*-(β -lactosyl)-pGlu-p-amidophenylthioureido]caproic acid]. Compound **8** was collected and characterized by electrospray ionization mass spectrometry and proton magnetic resonance (^1H NMR). Both spectra were consistent with the expected product (see Experimental Procedures).

Synthesis of Lewis^a/Lewis^x-pGlu-pNA and Preparation of pLK-Lewis^a/Lewis^x. Lewis^a/Lewis^x oligosaccharides were reacted with α -glutamyl-*p*-nitroanilide using the conditions described for the *N*-(β -lactosyl)-pGlu-pNA synthesis. This one-pot two-step procedure was also found to be very successful with such relatively complex oligosaccharides. The purified products were obtained, as described in detail in Experimental Procedures, upon gel filtration on a Trisacryl GF05 column and ethanolic

precipitation. The purified compound, Lewis^a/Lewis^x-pGlu-pNA, was shown to be the expected glycoamino acid derivative by ^1H NMR spectroscopy (see Experimental Procedures) and ESI-MS analysis, with a molecular ion peak at m/z 1085.6 (Figure 3B) corresponding to the calculated value.

Under the experimental conditions used for *N*-(β -lactosyl)-pGlu-pNA, Lewis^a/Lewis^x-pGlu-pNA was quantitatively reduced to the Lewis^a/Lewis^x-pGlu-p-aminoanilide and then, upon reaction with TCBI, was converted to the Lewis^a/Lewis^x-pGlu-p-amidoPITC.

The coupling of Lewis^a/Lewis^x-pGlu-p-amidoPITC with pLK₄₅₅ under the conditions described above was achieved with a yield leading to a poly(L-lysine) substituted with 100 Lewis^a/Lewis^x per pLK molecule.

DISCUSSION

Natural oligosaccharides isolated from biological fluids or released by enzymatic or chemical means from natural glycoconjugates can be used for many purposes, especially to synthesize conjugates such as glycopolymers, neoglycoproteins, neoglycolipids, or even glycosylated prodrugs. Among the various described methods, the transformation of reducing oligosaccharides to glycosylamines or glycosylamine derivatives is quite attractive.

Glycosylamines have been obtained as crystalline substances arising from sugars dissolved in methanol containing ammonia (Lobry de Bruyn, 1895; Lobry de Bruyn and Franchimont, 1893; Lobry de Bruyn and Van Leent, 1895, 1896). Glycosylamines were also prepared upon incubation of sugars in the presence of a saturated ammonium bicarbonate solution for up to 7 days (Likhoshervostov et al., 1986; Kallin et al., 1989; Otvos et al., 1989; Urge et al., 1991, 1992; Manger et al., 1992a,b; Vetter and Gallop, 1995) or in the presence of 0.2 M ammonium bicarbonate in 16 M aqueous ammonia for 36 h (Lubineau et al., 1995). Alternatively, β -glycosylamines may be obtained from N-type glycoproteins upon hydrolysis of the linkage between the oligosaccharylamine and the β -carboxylic group of the aspartyl residue by N-glycanase under alkaline conditions (Rasmussen et al., 1992; Tarentino et al., 1993). Unfortunately, glycosylamines are readily hydrolyzed in neutral or slightly acidic aqueous solution (Lobry de Bruyn and Van Leent, 1895; Frush and Isbell, 1951; Isbell and Frush, 1958). Glycosylamines were stabilized by acylation using various simple organic acids activated as acid chloride, anhydride, mixed anhydride, *N*-succinimide ester, etc. [for reviews, see Ellis and Honeyman (1955), Thomas (1977), and Garg et al. (1994)]. Alternatively, glycosylamines were stabilized as a glycoamino acid or a glycopeptide [for reviews, see Ellis and Honeyman (1955) and Garg et al. (1994)] by acylation with an activated amino acid (Otvos et al., 1989; Urge et al., 1991, 1992; Gupta and Suroliia, 1994; Tamura et al., 1994; Arsequel et al., 1994; Wadhwa et al., 1995) and/or a peptide bearing a single activated carboxylic group (Anisfeld and Lansbury, 1990; Cohen-Anisfeld and Lansbury, 1993; Wong et al., 1993). Glycosylamines were also stabilized by acylation with a bifunctional organic compound such as acryloyl chloride (Kallin et al., 1989) or disuccinimidyl suberate (Vetter et al., 1995) or with an activated halogeno (bromoacetic, chloroacetic, or iodoacetic) acid (Thomas, 1970; Manger et al., 1992a,b; Wong et al., 1994) or chloroacetic anhydride (Manger et al., 1992a,b), the second function being used later to achieve a further substitution. Glycosylamines may also be acylated by enzymatic β -aspartylation (Mononen et al., 1996).

Glycopeptides may also be obtained by direct coupling of a reducing sugar and a peptide. Dixon (1972) found that N_α -glucosylpeptides were formed upon incubation of glucose and short peptides such as Val-His or Ile-Tyr in the presence of pyridine and acetic acid for 3 days. The conjugate was stable enough to be separated by paper electrophoresis. The equilibrium constant for the dissociation of Glc-Val-His to Val-His in aqueous solution at pH 6.2 was found to be 0.3 M. The relatively high stability of such glycopeptides may be related to the steric hindrance of the side chain of the N-terminal amino acid (Val or Ile) substituted by the glucosyl residue or to the Amadori rearrangement [for reviews, see Hodge (1955), Hodge and Fisher (1963), and Lee and Cerami (1992)]. In contrast, glycosylamino acid such as an oligosaccharidyl-Gly-*p*NA containing a glycyl residue was found to be readily hydrolyzed in neutral aqueous medium (Normand-Sdiqui, 1995; Sdiqui et al., 1995), and an N-acylation was required to stabilize it. To simplify the stabilization step, we developed another class of glycoamino acid derivatives in which a glutamic acid derivative was used. The γ -COOH function of the glutamic acid side chain, upon activation, readily reacts with the α -amino group, leading to a pyroglutamate derivative; this intramolecular cyclization occurred with an excellent yield.

The *N*-glycopyroglutamyl conjugate is perfectly stable at room temperature in aqueous medium tested at any pH in the 3.6–9.0 range. It is also quite stable at high temperatures in a slightly acidic or a neutral medium; no degradation was observed after exposure at 90 °C for 5 h. In contrast, the compound was degraded in alkaline medium (pH 9.0) at 90 °C with a half-life of 85 min.

The main advantage of using a glutamic acid derivative is that the intramolecular cyclization step does not require any additional acylating reagent; the addition of a carboxylic group activator is sufficient. The formation of the pyroglutamyl derivative is very fast and actually stoichiometric, and no other acylation reaction could be detected. This contrasts with the N-acylation of the glyco-Gly-*p*NA which requires the addition of an acetylating agent, leading in some cases to a partial O-acetylation in addition to the expected N-acylation.

The reaction conditions used here to couple lactose with Glu-*p*NA are similar to those used previously for the coupling of lactose with Gly-*p*NA; i.e., the reaction occurs in DMF at 50 °C with 2 equiv of Glu-*p*NA and 4 equiv of imidazole. However, the coupling reaction was faster in the case of Glu-*p*NA than in that of Gly-*p*NA. Indeed, the times required to reach 50% of the reaction leading to *N*-(β -lactosyl)-Gly-*p*NA and to *N*-(β -lactosyl)-Glu-*p*NA were 30 and <2 h, respectively; those required to reach 95% were 120 and 8 h, respectively (Figure 4). Thus, unexpectedly, the use of Glu-*p*NA instead of Gly-*p*NA induced a second advantage in that both steps of the one-pot reaction appeared to be faster.

Under the conditions used, the reducing sugar reacts with the α -amino group of the α -glutamyl-*p*-nitroanilide and leads to a glycosylamine derivative in a β -configuration. Indeed, upon cyclization of the glutamic residue, the NMR spectrum shows that the *H*-1 proton resonance of the glucosyl residue is a doublet with a coupling constant $J_{1,2}$ of 8.25 Hz, in agreement with a β -configuration, and is downfield shifted to δ 5.23 ppm as expected for an *N*-acyl glucosylamide (Vliegthart et al., 1983). Furthermore, the *H*-1 proton intensity of the glucosyl residue and that of the galactosyl residue are identical, showing that the glucosyl residue is quantitatively present as an *N*-acyl- β -glucosylamide. This conclusion

is also supported by the lack of a low $J_{1,2}$ coupling constant doublet resonance between 4 and 6 ppm; such a doublet could correspond to the anomeric resonance of an *N*-acyl- α -glucosylamide or the anomeric resonance of an *N*-acyl- α -mannosylamide which is expected to be further downfield and could be derived from an isomerization process, as it occurs as a minor side product in the formation of glycosylamine in the presence of ammonium bicarbonate (Tamura et al., 1994). Another possible side reaction is the formation of diglycosylamines upon a transglycosylation process [for a review, see Ellis and Honeyman (1955), and for instance a recent paper on the structure of some *N,N*-bisglycosylamines from Linek et al. (1993) and references therein]. Such *N,N*-bis-glycosyl-glutamates, if they were formed, could not be N-acylated because the α -amino group should become a tertiary amine. Another side reaction which could occur is an Amadori rearrangement leading in the present case to the galactopyranosyl- β -4-(1-deoxy-1-pyroglutamyl-*p*-nitroanilide)fructose, in which there is only one anomeric resonance, that of the β -galactosyl residue, the fructosyl residue containing no anomeric proton. The Amadori rearrangement is known to occur when reducing sugars are incubated in the presence of ammonia, amines, amino acids, or protein [for reviews, see Hodge (1955), Hodge and Fisher (1963), and Lee and Cerami (1992)]. Under the conditions used in this paper, that type of isomerization does not occur, and this may be related to the presence of imidazole; imidazole is required to increase the rate of conjugate formation (Sdiqui et al., 1995) and also to give a clean reaction leading to a single conjugate. The quantitative analysis of the NMR spectra shows also that the sugar moiety and the pyroglutamyl anilide moiety are in equal amounts; for instance, the area of the *H*-1 proton resonance of each sugar residue at δ 5.23 or 4.37 ppm is identical to that of the α -CH proton resonance of the pyroglutamyl residue at δ 4.65 ppm.

The *N*-glycopyroglutamyl-*p*-nitroanilide derivatives were rapidly and quantitatively reduced in the presence of hydrogen and Pd on charcoal, leading to a single *p*-aminoanilide derivative, indicating that the pyroglutamyl ring was not altered during this step. The *N*-glycopyroglutamyl-*p*-aminoanilide also reacted rapidly and quantitatively with TCBI, leading to the phenyl isothiocyanate derivative without side reactions. Such a procedure described by Staab and Walther (1962) appeared to be quite efficient also in the case of these new glycoamino acid derivatives. TCBI is more convenient than the classical thiophosgene used previously by us (Roche et al., 1983; Monsigny et al., 1984) and many others because TCBI is not volatile and is less toxic.

The coupling of the glycopyroglutamyl-*p*-amidophenyl isothiocyanate with ϵ -aminocaproic acid as a model was fast and nearly quantitative with regard to the glycoside derivative. In addition, the coupling of an *N*-glycopyroglutamyl-*p*-isothiocynatoanilide to poly(L-lysine) was roughly quantitative.

The synthesis of such glycosynthons appeared to be quite efficient with either simple oligosaccharides such as lactose or more complex oligosaccharides such as fucosylpentaoses. In addition, this procedure is also suitable for the preparation of glycosynthons from oligosaccharides ending with other reducing sugars such as galactose, *N*-acetylglucosamine, and *N*-acetylgalactosamine (N. Normand-Sdiqui, unpublished results). Alternatively, other glutamyl derivatives may also be used instead of the α -glutamyl-*p*-nitroanilide; for instance, other glycosynthons were recently obtained by coupling, under the conditions described above, oligosac-

charides with glutamyl derivatives containing a protected thiol (Quétard et al., 1997).

Such *p*-nitrophenyl sugar derivatives have a large field of applications. They may be used to characterize oligosaccharides by HPLC with a sensitive detection at 375 nm, as in the case of *N*-*p*-nitrophenylglycosylamines (Kurth and Lehmann, 1986), or at 280 nm with *N*-tyrosyloligosaccharylamide [Tamura et al., 1994; see also the review of Rice et al. (1995) and references therein]. The aminophenyl sugar derivatives are suitable for being selectively acylated and converted into diazophenyl or into phenyl isothiocyanate [Buss and Goldstein, 1968; see Reichert et al. (1994) for a review]. The acylation with succinic anhydride leads to a derivative containing a carboxylic group ready for reaction with amino groups of a drug, a protein, a polypeptide, or a matrix bearing amino groups. Alternatively, the aminophenyl group may be acylated by an activated organic acid as in the case of the substitution with an aminobenzylglycoside derivative (Rafestin et al., 1974). The acylation with *N*-succinimidyl 3-(2-pyridylthio)propionate (SPDP) (Carlsson et al., 1978), or with iminothiolane (Tarentino et al., 1993) leads to a glycoconjugate bearing a thiol group suitable for a further substitution.

The diazophenyl derivatives may be used to prepare antigens (Goebel and Avery, 1929), neoglycoproteins, and glycosylated cytochemical markers suitable for visualizing lectins bound to cell surface glycoconjugates [for reviews, see Monsigny et al. (1983, 1989), Lee and Lee (1994a–c), and Gabius and Gabius (1997)]. The phenyl isothiocyanate derivatives may be used to prepare neoglycoproteins (Roche et al., 1983; Monsigny et al., 1984), neutral glycosylated polymers (Derrien et al., 1989), or cationic glycosylated polylysine [Midoux et al., 1993; for reviews, see Monsigny et al. (1984, 1988a,b, 1994a,b), Magnusson et al. (1994), Molema and Meijer (1994), and Reichert et al. (1994)]. Such glycoconjugates have been shown to be quite useful in studying the properties of endogenous lectins and in carrying drugs, oligonucleotides, and genes [for reviews, see Monsigny et al. (1994a,b) and Wadhwa and Rice (1995)]. Cationic glycosylated polylysines are efficient in increasing the biological effect of antisense oligonucleotides (Hangeland et al., 1995, 1997; Stewart et al., 1996) and in allowing gene targeting (Plank et al., 1992; Midoux et al., 1993; Wadhwa et al., 1995; Perales et al., 1994).

The one-pot two-step synthesis preparation of *N*-glycoamino acid derivatives from unprotected complex oligosaccharides bearing a reducing sugar in a terminal position described in this paper is very simple and very efficient and opens another way of preparing conjugates highly specific for endogenous lectins.

ACKNOWLEDGMENT

We thank Yves Plancke (Laboratoire de Chimie Biologique, CNRS, Université des Sciences et Technologies de Lille, Villeneuve d'Ascq, France) and Anita Caille and Henri Labbé (Centre de Biophysique Moléculaire, CNRS, Orléans, France) for recording the NMR spectra and Alain Deroussent (Institut Gustave Roussy, CNRS URA 147, Villejuif, France) for running the electrospray mass spectrometer. This work was supported by grants from the Agence Nationale de Recherches sur le SIDA (ANRS) and from the Association de Recherche sur le Cancer (Grant ARC 6132). C.Q. received a fellowship from the Région Centre Council. S.B. is the recipient of a postdoctoral fellowship from the ANRS. R.M. and G.S. are Research Directors at CNRS. A.C.R. and P.M. are

Research Directors at INSERM. M.M. is a Professor at the Université d'Orléans.

LITERATURE CITED

- Anisfeld, S. T., and Lansbury, P. T. (1990) A convergent approach to the chemical synthesis of asparagine-linked glycopeptides. *J. Org. Chem.* 55, 5560–5562.
- Aplin, J. D., and Wriston, J. C. (1981) Preparation, properties and application of carbohydrate conjugates of proteins and lipids. *Crit. Rev. Biochem.* 10, 259–306.
- Arsequell, G., Dwek, R. A., and Wong, S. Y. (1994) 9-Fluorenylmethoxycarbonyl (Fmoc)-glycine coupling of saccharide β -glycosylamines for the fractionation of oligosaccharides and the formation of neoglycoconjugates. *Anal. Biochem.* 216, 165–170.
- Buss, D. H., and Goldstein, I. J. (1968) Protein carbohydrate interaction. Part XIV. Carbohydrate containing groups for the alkylation of proteins. *J. Chem. Soc. C*, 1457–1461.
- Carlsson, J., Drevin, D., and Axen, R. (1978) Protein thiolation and reversible protein–protein conjugation. *N*-succinimidyl 3-(2-pyridylthio)propionate: a new heterobifunctional reagent. *Biochem. J.* 173, 727–737.
- Cohen-Anisfeld, S. T., and Lansbury, P. T. (1993) A practical, convergent method for glycopeptide synthesis. *J. Am. Chem. Soc.* 115, 10531–10537.
- Crocker, P. R., and Feizi, T. (1996) Carbohydrate recognition systems: functional triads in cell–cell interactions. *Curr. Opin. Struct. Biol.* 6, 679–691.
- Derrien, D., Midoux, P., Petit, C., Nègre, E., Mayer, R., Monsigny, M., and Roche, A. C. (1989) Muramyl dipeptide bound to poly-L-lysine substituted with mannose and gluconoyl residues as macrophage activators. *Glycoconjugate J.* 6, 241–255.
- Dixon, H. B. F. (1972) A reaction of glucose with peptides. *Biochem. J.* 129, 203–208.
- Drickamer, K., and Taylor, M. E. (1993) Biology of animal lectins. *Annu. Rev. Cell. Biol.* 9, 237–264.
- Duncan, R., and Kopecek, J. (1984) Soluble synthetic polymers as potential drug carriers. *Adv. Polymer Sci.* 57, 51–101.
- Ellis, G. P., and Honeyman, J. (1955) Glycosylamines. *Adv. Carbohydr. Chem.* 10, 95–168.
- Frush, H. L., and Isbell, H. S. (1951) Mutarotation, hydrolysis and structure of D-galactosylamines. *J. Res. Natl. Bur. Stand.* 47, 239–247.
- Gabius, H. J., and Gabius, S., Eds. (1997) *Glycosciences. Status and perspectives*, p 631, Chapman and Hall, Weinheim, Germany.
- Garg, H. G., von-dem Bruch, K., and Kunz, H. (1994) Developments in the synthesis of glycopeptides containing glycosyl L-asparagine, L-serine, and L-threonine. *Adv. Carbohydr. Chem. Biochem.* 50, 277–310.
- Goebel, W. F., and Avery, O. T. (1929) Chemo-immunological studies on conjugated carbohydrate proteins. IV The synthesis of *p*-aminophenol β -glucoside, *p*-aminophenol β -galactoside and their coupling with serum albumin. *J. Exp. Med.* 50, 521–531.
- Gray, G. (1974) The direct coupling of oligosaccharides to proteins and derivatized gels. *Arch. Biochem. Biophys.* 163, 426–428.
- Gupta, D., and Surolia, A. (1994) Synthesis of neoglycopeptides and analyses of their biodistribution *in vivo* to identify tissue specific uptake and novel putative membrane lectins. *Glycoconjugate J.* 11, 558–571.
- Hangeland, J. J., Levis, J. T., Lee, Y. C., and Ts'o, O. P. (1995) Cell-type specific and ligand specific enhancement of cellular uptake of oligodeoxynucleoside methylphosphonates covalently linked with a neoglycopeptide, YEE(ah-GalNAc)3. *Bioconjugate Chem.* 6, 695–701.
- Hangeland, J. J., Flesher, J. E., Deamond, S. F., Lee, Y. C., Ts'O, P. O., and Frost, J. J. (1997) Tissue distribution and metabolism of the [^{32} P]-labeled oligodeoxynucleoside methylphosphonate-neoglycopeptide conjugate, [YEE(ah-GalNAc)3]-SMCC-AET-pUmpT7, in the mouse. *Antisense Nucleic Acid Drug Dev.* 7, 141–149.
- Hodge, J. E. (1955) The Amadori rearrangement. *Adv. Carbohydr. Chem.* 10, 169–205.

- Hodge, J. E., and Fisher, B. E. (1963) Amadori rearrangement products. *Methods Carbohydr. Chem.* 2, 99–107.
- Isbell, H. S., and Frush, H. L. (1958) Mutarotation, hydrolysis and rearrangement reactions of glycosylamines. *J. Org. Chem.* 23, 1309–1319.
- Isbell, H. S., and Frush, H. L. (1980) Primary glycopyranosylamines. *Methods Carbohydr. Chem.* 8, 255–259.
- Kallin, E. (1994) Use of aminoalditols and glycosylamines in neoglycoconjugate synthesis. In *Neoglycoconjugates: preparation and applications* (Y. C. Lee and R. T. Lee, Eds.) pp 199–223, Academic Press, San Diego.
- Kallin, E., Lönn, H., Norberg, J., and Elofsson, M. (1989) Derivatization procedures for reducing oligosaccharides. Part 3: Preparation of oligosaccharide glycosylamines, and their conversion into oligosaccharide-acrylamide polymers. *J. Carbohydr. Chem.* 8, 597–611.
- Karlsson, K. A. (1995) Microbial recognition of target-cell glycoconjugates. *Curr. Opin. Struct. Biol.* 5, 622–635.
- Kiessling, L. L., and Pohl, N. L. (1996) Strength in numbers: non-natural polyvalent carbohydrate derivatives. *Chem. Biol.* 3, 71–77.
- Krantz, M. J., Holtzman, N. A., Stowell, C. P., and Lee, Y. C. (1976) Attachment of thio-glycosides to proteins. Enhancement of liver membrane binding. *Biochemistry* 15, 3963–3970.
- Kurth, H., and Lehmann, J. (1986) Efficient HPLC separation of *N*-*p*-nitrophenylglycosylamines derived from complex oligosaccharide mixtures. Human orosomucoid as a model. *Biomed. Chromatogr.* 1, 58–63.
- Lee, A. T., and Cerami, A. (1992) Role of glycation in aging. *Ann. N.Y. Acad. Sci.* 663, 63–70.
- Lee, R. T., and Lee, Y. C. (1994) Enhanced biochemical affinity of multivalent neoglycoconjugates. In *Neoglycoconjugates: preparation and applications* (Y. C. Lee and R. T. Lee, Eds.) pp 23–50, Academic Press, San Diego.
- Lee, Y. C., and Lee, R. T. (1992) Synthetic glycoconjugates. In *Glycoconjugates: composition, structure and function* (H. J. Allen and E. C. Kisailus, Eds.) pp 121–165, Marcel Dekker, New York.
- Lee, Y. C., and Lee, R. T., Eds. (1994a) Neoglycoconjugates. Part A. Synthesis. In *Methods in Enzymology*, Vol. 242, p 238, Academic Press, San Diego.
- Lee, Y. C., and Lee, R. T., Eds. (1994b) Neoglycoconjugates. Part B. Biochemical application. In *Methods in Enzymology*, Vol. 247, p 450, Academic Press, San Diego.
- Lee, Y. C., and Lee, R. T., Eds. (1994c) *Neoglycoconjugates: preparation and applications*, p 549, Academic Press, San Diego.
- Lee, Y. C., Townsend, R. R., Hardy, M. R., Lonngren, J., Arnap, J., Haraldsson, M., and Lonn, H. (1983) Binding of synthetic oligosaccharides to the hepatic Gal/GalNAc lectin. Dependence on fine structural features. *J. Biol. Chem.* 258, 199–202.
- Likhoshervostov, L. M., Novikova, O. S., Derevitskaja, V. A., and Kochetkov, N. K. (1986) A new simple synthesis of amino sugar β -D-glycosylamines. *Carbohydr. Res.* 146, c1–c5.
- Linek, K., Alfoldi, J., and Defaye, J. (1993) Structure of glycosylamines and diglycosylamines in the arabinose, mannose, and rhamnose series. *Carbohydr. Res.* 247, 329–335.
- Lis, H., and Sharon, N. (1991) Lectin-carbohydrate interactions. *Curr. Opin. Struct. Biol.* 1, 741–749.
- Lobry de Bruyn, C. A. (1895) Dérivé ammoniacal de la δ -glucose. *Rec. Trav. Chim.* 14, 98–105.
- Lobry de Bruyn, C. A., and Franchimont, A. P. N. (1893) Dérivés ammoniacaux cristallisés d'hydrate de carbone. *Rec. Trav. Chim. Pays-Bas* 12, 286–289.
- Lobry de Bruyn, C. A., and Van Leent, F. H. (1895) Dérivés ammoniacaux de quelques sucres (lactose, maltose, galactose, xylose, arabinose, rhamnose). *Rec. Trav. Chim. Pays-Bas* 14, 134–149.
- Lobry de Bruyn, C. A., and Van Leent, F. H. (1896) Dérivés ammoniacaux de la mannose, de la sorbose et de la galactose. *Rec. Trav. Chim.* 15, 81–85.
- Lubineau, A., Augé, J., and Drouillat, B. (1995) Improved synthesis of glycosylamines and a straightforward preparation of *N*-acylglycosylamines as carbohydrate-based detergents. *Carbohydr. Res.* 266, 211–219.
- Magnusson, G., Chernyak, A. Y., Kihlberg, J., and Kononov, O. (1994) Synthesis of neoglycoconjugates. In *Neoglycoconjugates* (Y. C. Lee and R. T. Lee, Eds.) pp 53–143, Academic Press, San Diego.
- Manger, I. D., Rademacher, T. W., and Dwek, R. A. (1992a) 1-*N*-glycyl β -oligosaccharide derivatives as stable intermediates for the formation of glycoconjugate probes. *Biochemistry* 31, 10724–10732.
- Manger, I. D., Wong, S. Y. C., Rademacher, T. W., and Dwek, R. A. (1992b) Synthesis of 1-*N*-Glycyl β -oligosaccharide derivatives. Reactivity of *Lens culinaris* lectin with a fluorescent labeled streptavidin pseudoglycoprotein and immobilized neoglycolipid. *Biochemistry* 31, 10733–10740.
- Meldal, M. (1994a) Glycopeptide synthesis. In *Neoglycoconjugates: preparation and applications* (Y. C. Lee and R. T. Lee, Eds.) pp 145–198, Academic Press, San Diego.
- Meldal, M. (1994b) Recent developments in glycopeptides and oligosaccharide synthesis. *Curr. Opin. Struct. Biol.* 4, 710–718.
- Midoux, P., Mendes, C., Legrand, A., Raimond, J., Mayer, R., Monsigny, M., and Roche, A. C. (1993) Specific gene transfer mediated by lactosylated poly-L-lysine into hepatoma cells. *Nucleic Acids Res.* 21, 871–878.
- Molema, G., and Meijer, D. K. (1994) Targeting of drugs to various blood cell types using (neo-)glycoproteins, antibodies and other protein carriers. *Adv. Drug Delivery Rev.* 14, 25–50.
- Mononen, I., Ivanov, G. I., Stoineva, I. B., Noronkoski, T., and Petkov, D. D. (1996) Enzymatic synthesis of the *N*-glycosidic bond by β -aspartylation of glycosylamines. *Biochem. Biophys. Res. Commun.* 218, 510–513.
- Monsigny, M., Kieda, C., and Roche, A. C. (1983) Membrane glycoproteins, glycolipids and membrane lectins as recognition signals in normal and malignant cells. *Biol. Cell* 47, 95–110.
- Monsigny, M., Roche, A. C., and Midoux, P. (1984) Uptake of neoglycoproteins via membrane lectin(s) of L 1210 cells evidenced by quantitative flow cytofluorometry and drug targeting. *Biol. Cell* 51, 187–196.
- Monsigny, M., Petit, C., and Roche, A. C. (1988a) Colorimetric determination of neutral sugars by a resorcinol sulfuric acid micromethod. *Anal. Biochem.* 175, 525–530.
- Monsigny, M., Roche, A. C., Kieda, C., Midoux, P., and Obrénovitch, A. (1988b) Characterization and biological implications of membrane lectins in tumor, lymphoid and myeloid cells. *Biochimie* 70, 1633–1649.
- Monsigny, M., Roche, A. C., and Midoux, P. (1988c) Endogenous lectins and drug targeting. *Ann. N.Y. Acad. Sci.* 551, 399–414.
- Monsigny, M., Hubert, J., Obrénovitch, A., and Schrével, J. (1989) Neoglycoproteins as tools to analyze endogenous lectins and glycoconjugates. In *Electron microscopy of subcellular dynamics* (H. Plattner, Ed.) pp 239–263, CRC Press, Boca Raton, FL.
- Monsigny, M., Roche, A. C., Midoux, P., and Mayer, R. (1994a) Glycoconjugates as carriers for specific delivery of therapeutic drugs and genes. *Adv. Drug Delivery Rev.* 14, 1–24.
- Monsigny, M., Roche, A. C., Midoux, P., and Mayer, R. (1994b) Sugar specific delivery of drugs, oligonucleotides and genes. In *Targeting of drugs, 4: Advances in system constructs* (G. Gregoriadis, B. McCormack, and G. Poste, Eds.) NATO ASI Series A: Life Sciences, Vol. 273, pp 31–50, Plenum Press, New York.
- Normand-Sdiqui, N. (1995) Synthèse de glycoconjugués spécifiques de lectines membranaires et leur utilisation pour cibler oligonucléotides et gènes. Ph.D. Thesis, University of Orléans, Orléans, France.
- Otvos, L., Wroblewski, K., Kollat, E., Perczel, A., Hollosi, M., Fasman, G. D., Ertl, H. C. J., and Thurin, J. (1989) Coupling strategies in solid-phase synthesis of glycopeptides. *Pept. Res.* 2, 362–366.
- Perales, J. C., Ferkol, T., Beegen, H., Ratnoff, O. D., and Hanson, R. W. (1994) Gene transfer *in vivo*: sustained

- expression and regulation of genes introduced into liver by receptor-targeted uptake. *Proc. Natl. Acad. Sci. U.S.A.* 91, 4086–4090.
- Plank, C., Zatloukal, K., Cotten, M., Mechtler, K., and Wagner, E. (1992) Gene transfer into hepatocytes using asialoglycoprotein receptor mediated endocytosis of DNA complexed with an artificial tetra-antennary galactose ligand. *Bioconjugate Chem.* 3, 533–539.
- Privat, J. P., Delmotte, F., and Monsigny, M. (1974) Protein-sugar interaction. Association of (β -4) linked *N*-acetyl-D-glucosamine oligomer derivatives with wheat germ agglutinin (lectin). *FEBS Lett.* 46, 224–228.
- Quétard, C., Normand-Sdiqui, N., Mayer, R., Strecker, G., Roche, A. C., and Monsigny, M. (1997) Simple synthesis of novel glycosynthons: oligosylpyroglutamyl derivatives. *Carbohydr. Lett.* 2, 415–422.
- Rafestin, M. A., Obrénovitch, A., Oblin, A., and Monsigny, M. (1974) Purification of *N*-acetyl-D-glucosamine binding proteins by affinity chromatography. *FEBS Lett.* 40, 62–66.
- Rasmussen, J. R., Davis, J., Risley, J. M., and Van Etten, R. L. (1992) Identification and derivatization of (oligosaccharyl)-amines obtained by treatment of asparagine-linked glycopeptides with *N*-glycanase enzyme. *J. Am. Chem. Soc.* 114, 1124–1126.
- Reichert, C. M., Hayes, C. E., and Goldstein, I. J. (1994) Coupling of carbohydrates to proteins by diazonium and phenylisothiocyanate reactions. In *Methods in Enzymology*, Vol. 242, pp 108–116, Academic Press, San Diego.
- Rice, K. G., and Corradi Da Silva, M. L. (1996) Preparative purification of tyrosinamide *N*-linked oligosaccharides. *J. Chromatogr. A* 720, 235–249.
- Rice, K. G., Chiu, M. H., Wadhwa, M. S., Thomas, V. H., and Stubbs, H. J. (1995) *In vivo* targeting function of *N*-linked oligosaccharides. Pharmacokinetic and biodistribution of *N*-linked oligosaccharides. *Adv. Exp. Med. Biol.* 376, 271–282.
- Roche, A. C., Barzilay, M., Midoux, P., Junqua, S., Sharon, N., and Monsigny, M. (1983) Endocytosis of glycoconjugates by Lewis lung carcinoma cells. *J. Cell Biochem.* 22, 131–140.
- Roy, C. (1996) Syntheses and some applications of chemically defined multivalent glycoconjugates. *Curr. Opin. Struct. Biol.* 6, 692–702.
- Sdiqui, N., Roche, A. C., Mayer, R., and Monsigny, M. (1995) New synthesis of Glycopeptides. *Carbohydr. Lett.* 1, 269–275.
- Sinaï, P. (1991) Recent advances in glycosylation reactions. *Pure Appl. Chem.* 63, 519–528.
- Staab, H. A., and Walther, G. (1962) Reaktionen von *N,N'*-thiocarbonyl-di-imidazol. *Ann. Chem.* 657, 98–103.
- Stewart, A. J., Pichon, C., Meunier, L., Midoux, P., Monsigny, M., and Roche, A. C. (1996) Enhanced biological activity of antisense oligonucleotides complexed with glycosylated poly-L-lysine. *Mol. Pharmacol.* 50, 1487–1494.
- Stowell, C. P., and Lee, Y. C. (1980) Neoglycoproteins. The preparation and application of synthetic glycoproteins. *Adv. Carbohydr. Chem. Biochem.* 37, 225–281.
- Tamura, T., Wadhwa, M. S., and Rice, K. G. (1994) Reducing and modification of *N*-linked oligosaccharide with tyrosine. *Anal. Biochem.* 216, 335–344.
- Tarentino, A. L., Phelan, A. W., and Plummer, T. H. (1993) 2-Iminothiolane: a reagent for the introduction of sulphydryl groups into oligosaccharides derived from asparagine-linked glycans. *Glycobiology* 3, 279–285.
- Thomas, E. W. (1970) Potential specific inhibitors of the lactose transport system of *Escherichia coli*. *J. Med. Chem.* 13, 755–756.
- Thomas, E. W. (1977) Carbohydrate binding sites. In *Methods in Enzymology*, Vol. 46, pp 362–368, Academic Press, San Diego.
- Townsend, R., and Stahl, P. (1981) Isolation and characterization of a mannose/*N*-acetylglucosamine/fucose binding protein from rat liver. *Biochem. J.* 194, 209–214.
- Urge, L., Kollat, E., Hollosi, M., Laczko, L., Wroblewski, K., Thurin, J., and Otvos, L. (1991) Solid-phase synthesis of glycopeptides: synthesis of *N*- α -fluorenylmethoxycarbonyl-L-asparagine *N*- β -glycosides. *Tetrahedron Lett.* 32, 3445–3448.
- Urge, L., Otvos, L. J. R., Lang, E., Wroblewski, K., Larzko, M., and Hollosi, M. (1992) F-moc protected, glycosylated asparagines potentially useful as reagents in the solid-phase synthesis of *N*-glycopeptides. *Carbohydr. Res.* 235, 83–93.
- Varki, A. (1993) Biological roles of oligosaccharides: all of theories are correct. *Glycobiology* 3, 97–130.
- Vetter, D., and Gallop, M. A. (1995) Strategies for the synthesis and screening of glycoconjugates. 1. A library of glycosylamines. *Bioconjugate Chem.* 6, 316–318.
- Vetter, D., Tate, E. M., and Gallop, M. A. (1995) Strategies for the synthesis and screening of glycoconjugates. 2. Covalent mobilization for flow cytometry. *Bioconjugate Chem.* 6, 319–332.
- Vliegthart, J. F. G., Dorland, L., and van Halbeck, H. (1983) High resolution, H nuclear magnetic resonance spectroscopy as a tool in the structural analysis of carbohydrates related to glycoproteins. *Adv. Carbohydr. Chem. Biochem.* 41, 209–374.
- Von Itzstein, M., and Colman, P. (1996) Design and synthesis of carbohydrate based inhibitors of protein carbohydrate interactions. *Curr. Opin. Struct. Biol.* 6, 703–709.
- Wadhwa, M. S., and Rice, K. G. (1995) Receptor mediated glycotargeting. *J. Drug Targeting* 3, 111–127.
- Wadhwa, M. S., Knoell, D. L., Young, A. P., and Rice, K. G. (1995) Targeted gene delivery with a low molecular weight glycopeptide carrier. *Bioconjugate Chem.* 6, 283–291.
- Whitfield, D. M., and Douglas, S. P. (1996) Glycosylation reaction, present status, future directions. *Glycoconjugate J.* 13, 5–17.
- Wong, S. Y. C. (1995) Neoglycoconjugates and their applications in glycobiology. *Curr. Opin. Struct. Biol.* 5, 599–604.
- Wong, S. Y. C., Guile, G. R., Rademacher, T. W., and Dwek, R. A. (1993) Synthetic glycosylation of peptides using unprotected saccharide β -glycosylamines. *Glycoconjugate J.* 10, 227–234.
- Wong, S. Y. C., Guile, G. R., Dwek, R. A., and Arsequell, G. (1994) Synthetic glycosylation of proteins using *N*-(β -saccharide) iodoacetamides: application in site-specific glycosylation and solid-phase enzymic oligosaccharide synthesis. *Biochem. J.* 300, 843–850.

BC970122P

Micropatterned Immobilization of Epidermal Growth Factor To Regulate Cell Function

Yoshihiro Ito,^{*,†,‡} Guoping Chen,[†] and Yukio Imanishi[†]

Department of Material Chemistry, Faculty of Engineering, Kyoto University, Kyoto 606-01, Japan, and PRESTO, JST, Keihanna Plaza, Hikaridai 1-7, Seika-cho, Kyoto 619-02, Japan. Received September 30, 1997; Revised Manuscript Received December 16, 1997

Photoreactive epidermal growth factor (EGF) was synthesized by conjugating mouse EGF with photoreactive polyallylamine, which was synthesized by the coupling reaction of polyallylamine with *N*-[4-(azidobenzoyl)oxy]succinimide. The EGF derivative was pattern-immobilized onto a polystyrene plate by UV irradiation in the presence of a photomask in a prescribed micropattern. The patterned immobilization of EGF on the polystyrene plate was confirmed by immunostaining with anti-EGF antibody. Chinese hamster ovary (CHO) cells overexpressing EGF receptors were cultured on the micropatterned plate. The phosphorylated tyrosine residues of signal proteins, including EGF receptors, were detected only in the cells adhered in the EGF-immobilized area, and cell growth was observed only in the EGF-immobilized area. The cells growing in the EGF-immobilized area were partially stained by anti-phosphotyrosine antibody, when the area of EGF immobilization was smaller than the cell. The partial staining of activated proteins indicates that immobilization of EGF inhibited the free lateral diffusion and internalization of the activated EGF–EGF receptor complex. The enhanced cell growth is due to juxtacrine stimulation realized by immobilized EGF.

INTRODUCTION

Natural and artificial substrata are important in basic bioscience and biotechnology, including cell culture and tissue engineering (1–4). Cellular interactions with the extracellular matrix play critical roles in various biological processes, including migration, morphogenesis, growth, differentiation, and apoptosis (5). On the other hand, selective cell attachment (6–9) and cell shape regulation (10, 11) by micropatterned surface substrata constructed by nanotechnology have been reported. However, those artificial substrata could not transduce biological signals such as for growth and differentiation. Recently, several growth factors, including the epidermal growth factor (EGF), have been reported to regulate cell functions in the transmembrane form by “juxtacrine stimulation” (12, 13). They stimulate cells without internalization. The juxtacrine mechanism was deduced from the studies of intercellular regulation by paraformaldehyde-fixed cells that express the growth factors (13).

In addition to the juxtacrine stimulation, recently some researchers, including our group, demonstrated artificial juxtacrine stimulation by interleukin 2 or insulin immobilized on artificial substrata such as polystyrene and poly(methyl methacrylate) films (14–16). In this investigation, photoreactive EGF conjugate was synthesized and immobilized on a prescribed micropattern of different size to visualize signal transduction without internalization. EGF transduced the signal through the cognate receptor to stimulate the growth of Chinese hamster ovary cells overexpressing EGF receptors (CHO-ER cells).

MATERIALS AND METHODS

Materials. *N,N*-Dimethylformamide (DMF), paraformaldehyde, Triton X-100, and sodium orthovanadate were purchased from Wako Pure Chemicals Ltd. (Osaka, Japan). Dicyclohexylcarbodiimide (DCC) and 4-azidobenzoic acid were purchased from Tokyo Kasei Co. (Tokyo, Japan). *N*-Hydroxysuccinimide was purchased from Protein Institute Inc. (Minoh, Japan). Polyallylamine hydrochloride was purchased from Nittobo (Tokyo, Japan). Bovine serum albumin (BSA) was purchased from Interger Co. (Purchase, NY). Mouse EGF was purchased from Toyobo (Osaka, Japan). Anti-EGF IgG was purchased from Becton Dickinson Labware (Bedford, MA). Anti-phosphotyrosine antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rhodamine-conjugated antibody was purchased from Protos Immunoresearch (San Francisco, CA). Vectashield mounting medium for fluorescence was purchased from Vector Laboratories, Inc. (Burlingame, CA). Tissue culture polystyrene plates with six wells (Sumilon) were purchased from Akita Sumitomo Bake Co. (Akita, Japan).

Preparation of Photoreactive Polyallylamine and Photoreactive EGF. The preparative scheme of photoreactive polyallylamine and photoreactive EGF is shown in Figure 1a. Photoreactive EGF was synthesized by conjugating mouse EGF with the photoreactive polyallylamine, which was synthesized by the coupling reaction of polyallylamine with *N*-[4-(azidobenzoyl)oxy]succinimide.

N-[4-(Azidobenzoyl)oxy]succinimide was prepared as described by Matsuda and Sugawara (17). A solution of DCC (13.3 g, 64.6 mmol) in tetrahydrofuran (THF, 50 mL) was added dropwise to a solution of *N*-hydroxysuccinimide (7.43 g, 64.6 mmol) and 4-azidobenzoic acid (9.57 g, 58.7 mmol) in THF (150 mL) and then cooled in an ice bath under stirring. After 3 h, the reaction mixture was

* Corresponding author: Department of Material Chemistry, Graduate School of Engineering, Kyoto University, Kyoto 606-01, Japan. Telephone: 81-75-753-5638. Fax: 81-75-753-4911. E-mail: H51535@sakura.kudpc.kyoto-u.ac.jp.

[†] Kyoto University.

[‡] PRESTO, JST.

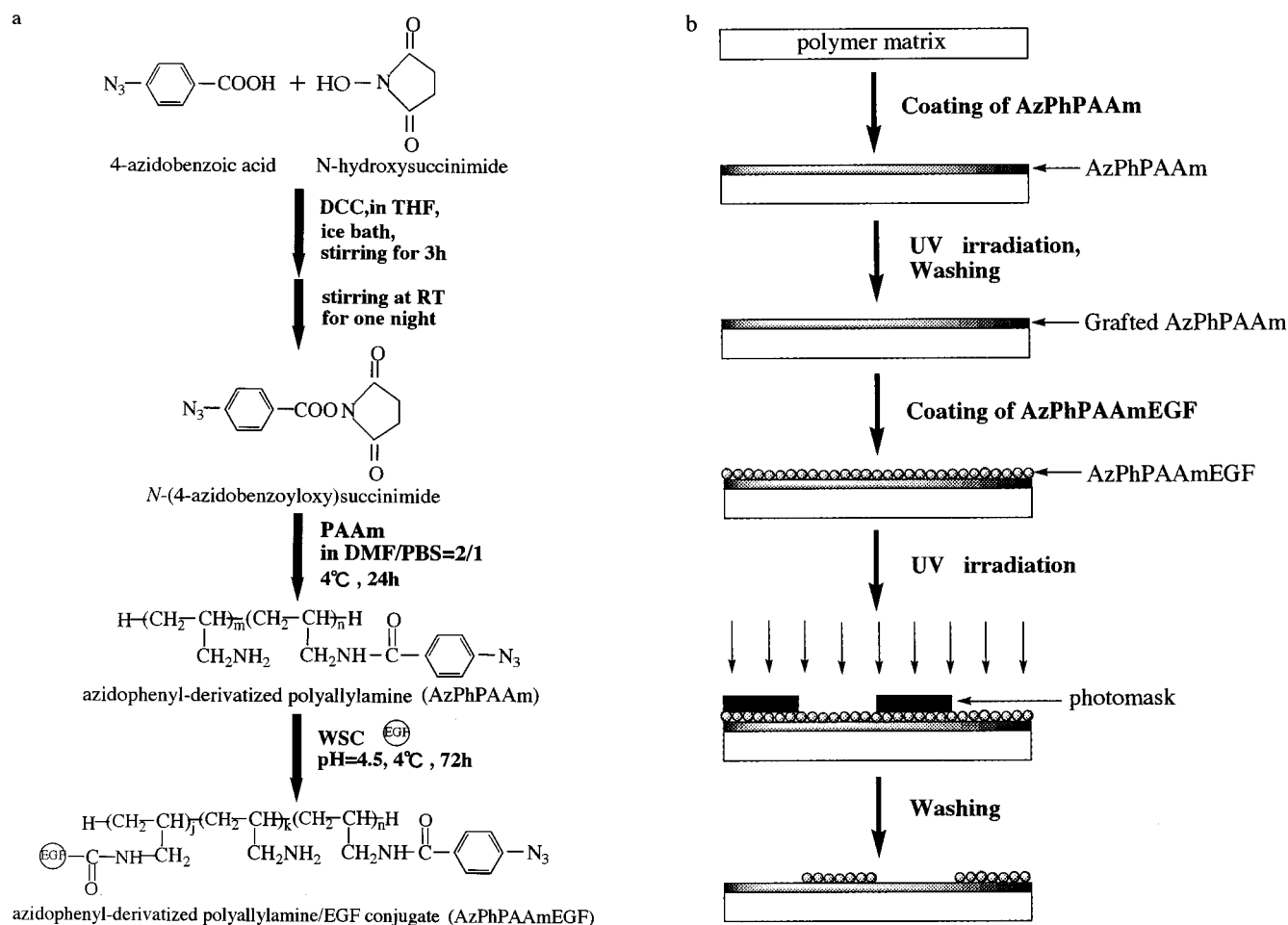


Figure 1. Synthetic scheme of photoreactive polyallylamine and photoreactive EGF (a) and microprocessing of patterned immobilization of EGF on a polystyrene plate (b). DMF and PBS represent *N,N*-dimethylformamide and phosphate-buffered saline, respectively.

warmed slowly to room temperature and then stirred overnight. The white solid that formed was filtered off, and the solvent was removed under reduced pressure. The yellow residue obtained was crystallized from isopropyl alcohol/diisopropyl ether.

Polyallylamine (MW = 60 000, 30 mg) dissolved in 10 mL of phosphate-buffered solution (pH 7.0) was added to a DMF solution (20 mL) of *N*-[4-(azidobenzoyl)oxy]succinimide (25.8 mg) being stirred on ice. After incubation at 4 °C for 24 h with stirring, the solution was ultrafiltrated (Millipore MoleCut II, cutoff below 10 kDa) and washed three times with 10 mL of distilled water. The azidophenyl-derivatized polyallylamine was referred to as AzPhPAAm. The amount of azidophenyl groups in the conjugate calculated from the absorbance at 280 nm was 65 mol/mol. The molar extinction coefficient was 1141. The azidophenyl-derivatized polyallylamine was further conjugated with EGF as follows. To a 0.1 M 2-(*N*-morpholino)ethanesulfate-buffered solution (MES, pH 4.5, 10 mL) were added 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (water-soluble carbodiimide, WSC, 10 mg), EGF (300 µg), and AzPhPAAm (600 µg), and they were allowed to react at 4 °C for 24 h while being stirred. Then the solution was ultrafiltrated (Millipore MoleCut II, cutoff below 10 kDa) and washed twice with 2 mL of distilled water. The photoreactive EGF conjugate was referred to as AzPhPAAmEGF. The amount of EGF in the AzPhPAAmEGF conjugate was determined by measuring the fluorescence intensity at 345 nm by excitation at 280 nm and by an elemental

analysis. The calibration curve of fluorescence of EGF was measured in the presence of AzPhPAAm before photoirradiation. Both measurements demonstrated that 1.4 molecules of EGF was contained in 1 molecule of AzPhPAAmEGF.

Pattern Immobilization of EGF. The procedure of pattern immobilization of EGF is shown in Figure 1b. An aqueous solution of AzPhPAAm (200 µg/mL, 200 µL) was eluted on a polystyrene plate in the shape of a circle (diameter of 10 mm) and air-dried at room temperature. Then the plate was UV-irradiated using a UV lamp (Koala, 100 W) from a distance of 5 cm for 10 s. The plate was thoroughly washed with diluted hydrochloric acid (pH 3.0) until the absence of released AzPhPAAm was confirmed by ultraviolet absorbance at 280 nm (7 days). Subsequently, the EGF conjugate (200 µg/mL, 50 µL) was cast in a circular shape (diameter of 5 mm) and air-dried at room temperature. The plate was covered with a photomask of a specific pattern and irradiated with an ultraviolet lamp from a distance of 5 cm for 10 s. Finally, the plate was washed with PBS at 4 °C until the absence of released EGF was confirmed by ultraviolet absorbance at 280 nm (7 days).

Immunostaining of Immobilized EGF. The plates immobilized with EGF were immersed in PBS containing 0.02% NaN₃ and 3% bovine serum albumin (BSA) at 4 °C for 24 h and subsequently incubated with anti-EGF IgG antibody diluted in PBS containing 0.02% NaN₃ and 3% bovine serum albumin (2 µg/mL) at 4 °C for 12 h. After being washed with PBS containing 0.02% NaN₃,

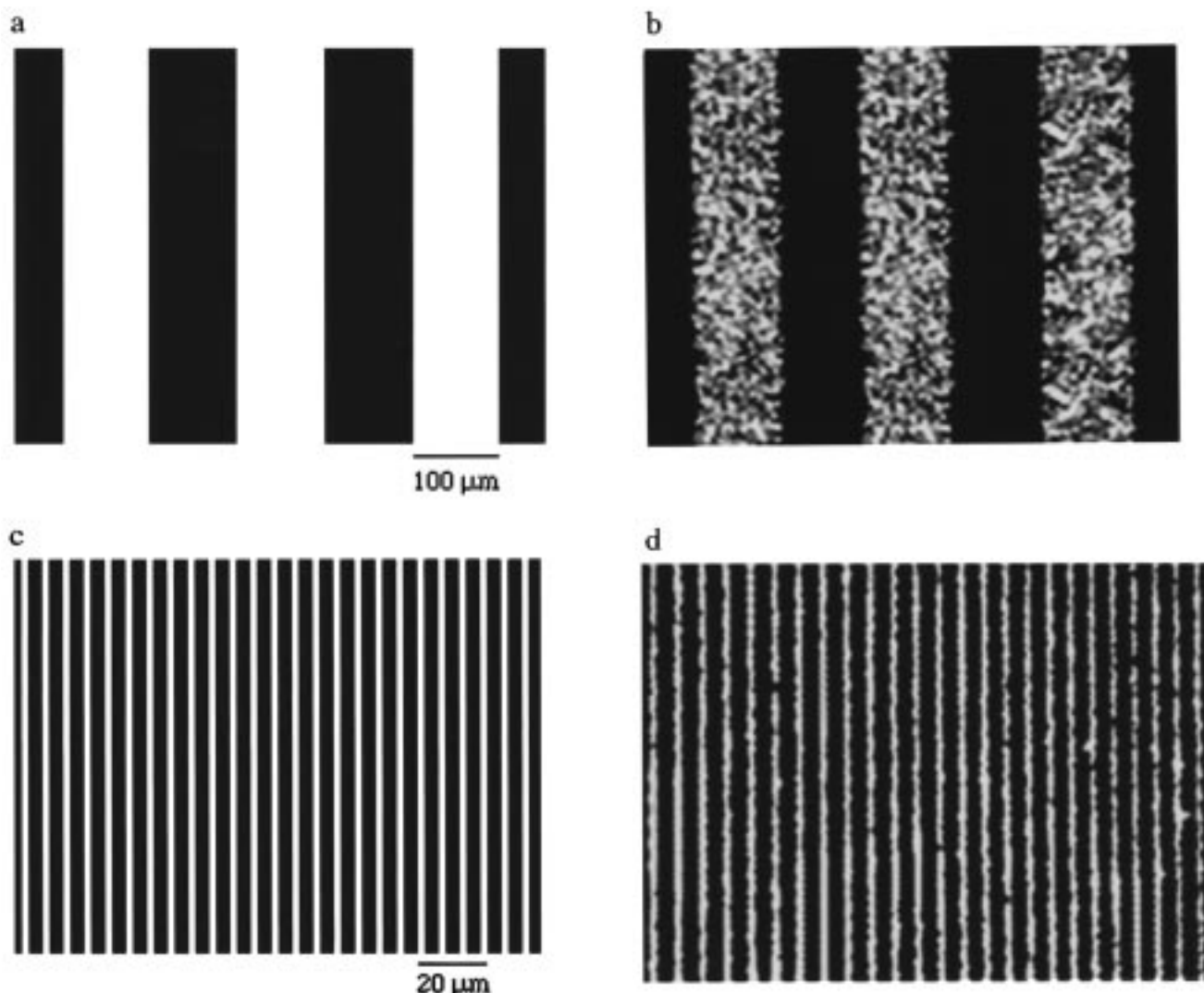


Figure 2. Micrographs of photomask (a and c) and the corresponding fluorescence micrographs of EGF immobilized in a stripe pattern (b and d).

the plate was incubated in PBS containing rhodamine-conjugated anti-mouse IgG antibody ($2 \mu\text{g/mL}$) and 0.02% NaN_3 at 4°C for 12 h. The stained plate was washed with PBS, briefly rinsed with distilled water, mounted in Vectashield mounting medium, and observed under a laser fluorescence microscope (Olympus Co., Tokyo, Japan).

Cell Culture and Treatments for Microscopic Observation. CHO-ER cells (2.5×10^5 receptor molecules per cell) were subcultured in Ham's F-12 medium containing 10% (v/v) fetal bovine serum under an atmosphere containing 5% CO_2 at 37°C . After culturing in the absence of serum for 2 days, cells were harvested by incubation at 37°C for 10 min with PBS containing 0.02% (w/v) ethylenediaminetetraacetic acid and pipetting. After being washed twice with Ham's F-12 medium, the cells were suspended in Ham's F-12 medium (1×10^6 cells/mL). The cell suspension was added to six-well tissue culture plates (0.2 mL/well) containing the EGF-immobilized polystyrene plate that had been incubated in the well in the presence of Ham's F-12 medium (5 mL) at 37°C for 2 h. The cells were cultured under a 5% CO_2 atmosphere at 37°C for 48 h and observed with a phase-contrast microscope.

To investigate the tyrosine phosphorylation of signal proteins, the cells were cultured under a 5% CO_2 atmosphere at 37°C for 30 min. Then, the cells were fixed

for 30 min at 4°C with 3% paraformaldehyde in PBS. The fixed cells were washed three times with PBS containing 1 mM Na_3VO_4 . Subsequently, the cells were permeabilized with PBS containing 0.25% Triton X-100 and 1 mM Na_3VO_4 and washed three times with 50 mM Tris-HCl-buffered solution containing 150 mM NaCl and 0.1% Triton X-100 (TBST, pH 7.4) and 1 mM Na_3VO_4 . After an overnight incubation at 4°C in TBST containing 3% BSA and 1 mM Na_3VO_4 , the treated cells were incubated for 2 h at 25°C with a solution of anti-phosphotyrosine mouse IgG diluted to 1/100 with 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.01% Tween 20, 0.02% NaN_3 , and 1 mM Na_3VO_4 (TBS) containing 3% BSA. The cells were washed once with TBS, once with TBST, and once with TBST containing 0.1% BSA. A solution of rhodamine-conjugated anti-mouse IgG antibody was diluted to 1/200 with TBS containing 3% BSA and incubated with the cells for 2 h at 25°C . The cells were washed three times for 5 min each with TBST and three times with PBS, briefly rinsed with distilled water, and then mounted in Vectashield mounting medium. The cells were observed by a laser fluorescence microscope.

RESULTS

Preparation of the Photoreactive EGF Conjugate and Pattern Immobilization of EGF. Photoreactive polyallylamine was synthesized by coupling polyallyl-

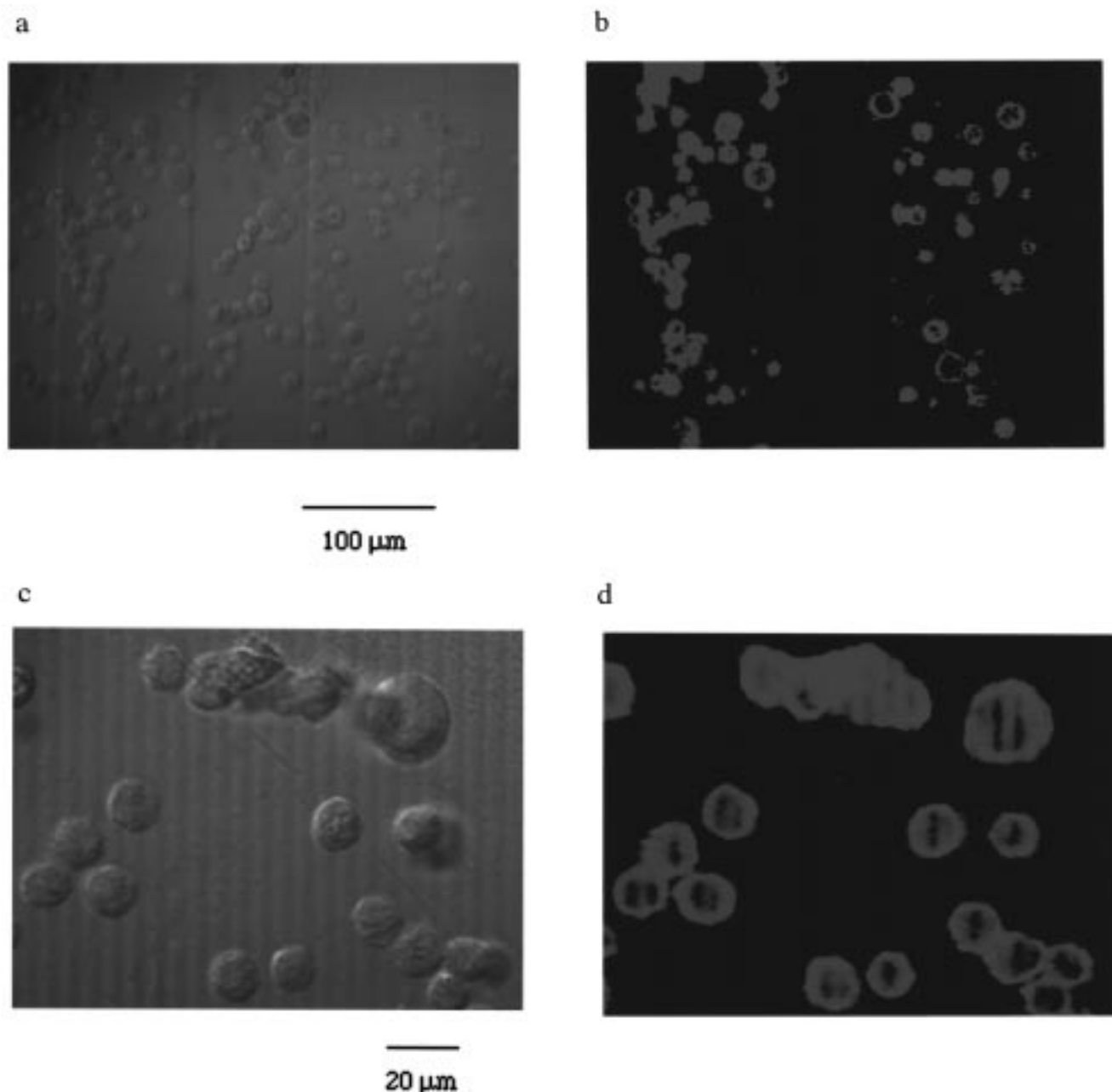


Figure 3. Optical (a and c) and the corresponding fluorescence (b and d) micrographs of CHO-ER cells cultured for 30 min on a polystyrene plate with immobilized EGF. For fluorescence microscopy, the cells were stained with anti-phosphotyrosine antibody.

amine with *N*-[4-(azidobenzoyl)oxy]succinimide. The amount of incorporated azidophenyl groups in the conjugate calculated from the absorbance at 280 nm was 65 mol/mol. The azidophenyl-derivatized polyallylamine was further conjugated with EGF to synthesize the photoreactive EGF conjugate. The content of EGF in the AzPhPAAmEGF conjugate determined by measuring the fluorescence intensity at 345 nm by excitation at 280 nm was 1.4 mol/mol. A polystyrene plate was in advance grafted with the photoreactive polyallylamine by UV irradiation. Then, an aqueous solution of the photoreactive EGF was coated on the polyallylamine-grafted polystyrene plate and air-dried, and the plate was UV-irradiated in the presence of a photomask in a prescribed micropattern. Upon UV irradiation, the azidophenyl groups were easily photolyzed to generate highly reactive nitrenes, which spontaneously formed covalent bonds with neighboring hydrocarbons on the polystyrene plate surface. The irradiated areas should be covalently cross-

linked with EGF. The photoreactive EGF in other areas should not be cross-linked and could be removed by washing. A pattern of covalently cross-linked EGF would appear on the polystyrene plate surface.

After the plate was completely washed with PBS, the patterned immobilization of EGF on the polystyrene plate was confirmed by staining with anti-EGF antibody. As shown in Figure 2, the pattern of immobilized EGF was the same as that of the photomask used.

Tyrosine Phosphorylation of Signal Proteins. CHO-ER cells were cultured on the plate pattern-immobilized with EGF for 30 min. The CHO-ER cells were fixed by paraformaldehyde and stained with anti-phosphotyrosine mouse IgG and rhodamine-conjugated anti-mouse IgG antibodies. Figure 3a shows the uniform adherence of cells on the surface of the plate pattern-immobilized with EGF, indicating that cell adhesion was not enhanced by immobilized EGF. However, Figure 3b shows that the phosphorylated tyrosine residue was

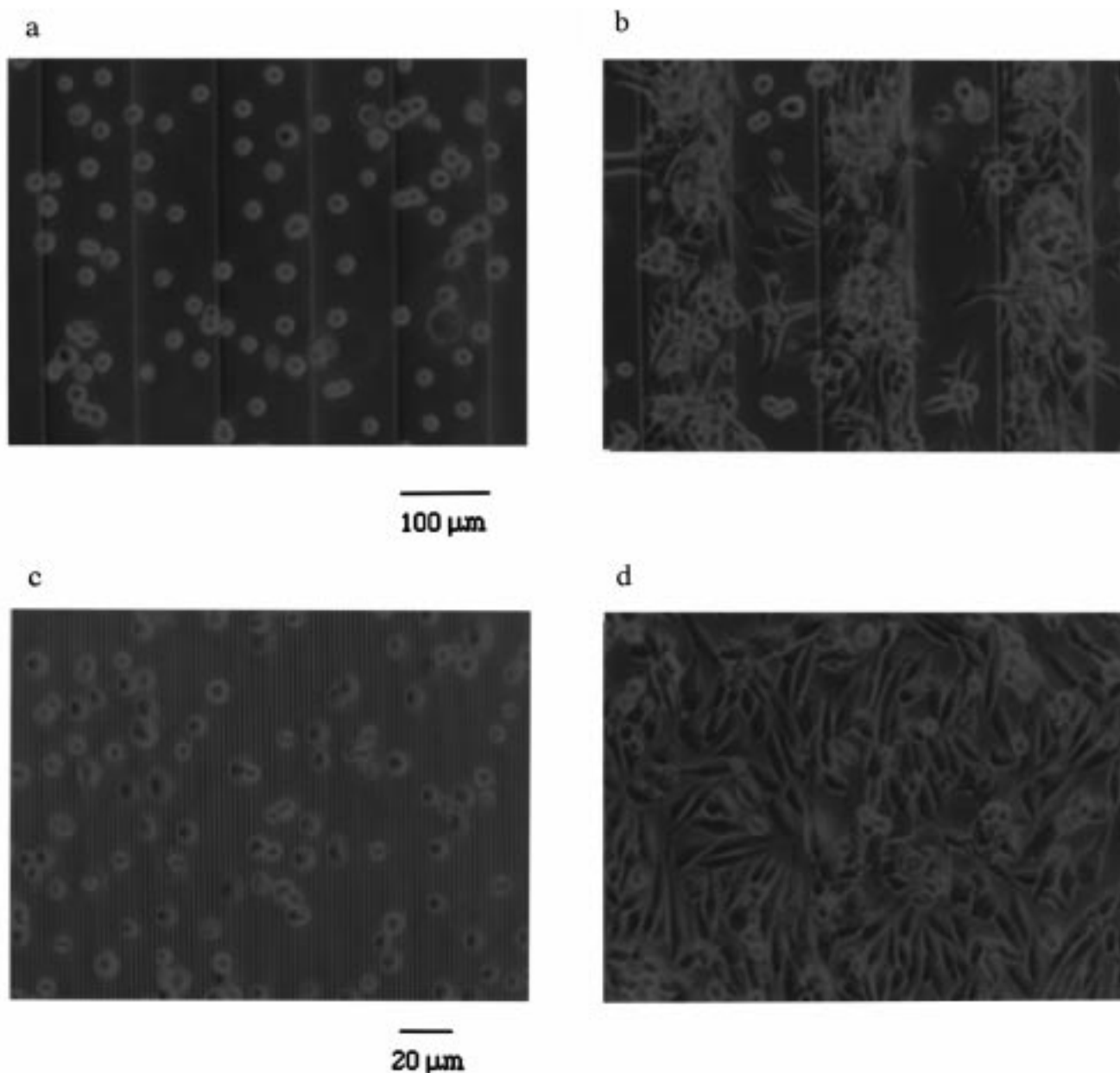


Figure 4. Phase-contrast micrograph of CHO-ER cells on EGF immobilized in a stripe pattern with a $100\ \mu\text{m}$ width before culture (a) and after a 48 h culture (b) and on a plate having EGF immobilized in a stripe pattern with a $2\ \mu\text{m}$ width before culture (c) and after a 48 h culture (d).

detected only in the cells adhered on the EGF-immobilized area, indicating a signal transduction occurring only through immobilized EGF.

Panels c and d of Figure 3 show the cells adhering on the plate pattern-immobilized with EGF in a narrow stripe pattern. The contact area (stripes $2\ \mu\text{m}$ in width) between the cells and the immobilized EGF was stained by anti-phosphotyrosine antibody. Since free lateral diffusion and internalization of the bound EGF–EGF receptor complex are prohibited by immobilization of EGF, signal proteins were partially activated. These findings indicate that the biological signal is transduced only to the cells that interact with immobilized EGF.

Cell Growth. CHO-ER cells before and after a 48 h culture on the culture plate with immobilized EGF in a micropattern (100 or $2\ \mu\text{m}$ in width) are shown in Figure 4. When the width of the stripe is larger ($100\ \mu\text{m}$) than the cell, the patterned growth of cells was observed as shown in Figure 4b. The patterned growth may have been caused by the enhancement of cell growth due to

signal transduction from the immobilized EGF. When the pattern width is smaller than the cell ($2\ \mu\text{m}$), all the cells proliferated and patterned growth did not occur (Figure 4d). Considering the absence of cell growth in the EGF-nonimmobilized area (Figure 4b), the partial and lateral diffusion-limited stimulation was sufficient for cell growth.

DISCUSSION

Although the conjugation of a biosignal with an insoluble substratum is an important technique for elucidation of the biosignaling mechanism and molecular design of drugs or medical materials, it has not sufficiently been examined after the pioneering investigation using sepharose gel with immobilized insulin (18, 19). The stimulation of the growth of only the cells on the immobilized EGF region indicated that diffusible EGF was absent in the culture system. The local concentration of EGF on the surface is sufficient to promote effective interaction with the EGF receptors of adsorbed

cells. Recent studies on overlapping of adhesion molecules and growth factors (20), the growth stimulation by noninternalizing EGF receptors (21), and various juxtacrine stimulators (12, 13) suggest that the signal of immobilized EGF was transduced and cell growth stimulated without internalization. With similar methods, it will be possible to manipulate cells and tissues using artificial substrata with covalently conjugated growth factors and cytokines.

ACKNOWLEDGMENT

We thank Dr. T. Matsuda of the National Cardiovascular Center, the Research Institute for their valuable suggestions on the microprocessing technique, Prof. M. Kasuga of Kobe University for providing the authors with CHO-ER cells, and Prof. S. Nakanishi of Kyoto University for critical suggestions on the manuscript.

LITERATURE CITED

- (1) Mrksich, M., Chen, C. S., Xia, Y., Dike, L. E., Ingber, D. E., and Whitesides, G. M. (1996) Controlling cell attachment on contoured surfaces with self-assembled monolayers of alkanethiolates on gold. *Proc. Natl. Acad. Sci. U.S.A.* 93, 10775–10778.
- (2) Peppas, N. A., and Langer, R. (1994) New challenges in biomaterials. *Science* 263, 1715–1720.
- (3) Hubbell, J. A. (1995) Biomaterials in tissue engineering. *Bio/Technology* 13, 565–576.
- (4) Gumbiner, B. M., and Yamada, K. M. (1995) Cell-to-cell contact and extracellular matrix. *Curr. Opin. Cell Biol.* 7, 615–618.
- (5) Roskelley, C. D., Srebrow, A., and Bissell, M. (1995) A hierarchy of ECM-mediated signalling regulates tissue-specific gene expression. *Curr. Opin. Cell Biol.* 7, 736–747.
- (6) Lee, J.-S., Kaibara, M., Iwaki, M., Sasabe, H., Suzuki, Y., and Kusakabe, M. (1993) Selective adhesion and proliferation of cells on ion-implanted polymer domains. *Biomaterials* 14, 958–960.
- (7) Spargo, B. J., Testoff, M. A., Nielsen, T. B., Stenger, D. A., Hickman, J. J., and Rudolph, A. S. (1994) Spatially controlled adhesion, spreading, and differentiation of endothelial cells on self-assembled molecular monolayers. *Proc. Natl. Acad. Sci. U.S.A.* 91, 11070–11074.
- (8) Connolly, P. (1994) Bioelectronic interfacing: micro- and nanofabrication techniques for generating predetermined molecular arrays. *Trends Biotechnol.* 12, 123–127.
- (9) Clemence, J.-F., Ranieri, J. P., Aebischer, P., and Sigrist, H. (1995) Photoimmobilization of a bioactive laminin fragment and pattern-guided selective neuronal cell attachment. *Bioconjugate Chem.* 6, 411–417.
- (10) Singhvi, R., Kumar, A., Lopez, G. P., Stephanopoulos, G. N., Wang, D. I. C., Whitesides, G. M., and Ingber, D. E. (1994) Engineering cell shape and function. *Science* 264, 696–698.
- (11) Chen, C. S., Mrksich, M., Huang, S., Whitesides, G. M., and Ingber, D. E. (1997) Geometric control of cell life and death. *Science* 276, 1425–1428.
- (12) Massague, J., and Pandiella, A. (1993) Membrane-anchored growth factors. *Annu. Rev. Biochem.* 62, 515–541.
- (13) Higashiyama, S., Iwamoto, R., Goishi, K., Raab, G., Taniguchi, N., Klagsbrun, M., and Mekada, E. (1995) The membrane protein CD9/DRAP27 potentiates the juxtacrine growth factor activity of the membrane-anchored heparin-binding EGF-like growth factor. *J. Cell Biol.* 128, 929–938.
- (14) Horwitz, J. I., Toner, M., Tompkins, R. G., and Yarmush, M. L. (1993) Immobilized IL-2 preserves the viability of an IL-2 dependent cell line. *Mol. Immunol.* 30, 1041–1048.
- (15) Ito, Y., Zheng, J., Imanishi, Y., Yonezawa, K., and Kasuga, M. (1996) A protein-free cell culture on an artificial substrata covalently immobilized with insulin. *Proc. Natl. Acad. Sci. U.S.A.* 93, 3598–3601.
- (16) Chen, G., Ito, Y., and Imanishi, Y. (1997) Mitogenic activities of water-soluble and -insoluble insulin conjugates. *Bioconjugate Chem.* 8, 106–110.
- (17) Matsuda, T., and Sugawara, T. (1995). Photochemical protein fixation on polymer surfaces via derivatized phenyl azido group. *Langmuir* 11, 2272–2276.
- (18) Cuatrecasas, P. (1969) Interaction of insulin with the cell membrane: the primary action of insulin. *Proc. Natl. Acad. Sci. U.S.A.* 63, 450–457.
- (19) Venter, J. C. (1982) Immobilized and insolubilized drugs, hormones, and neurotransmitters: properties, mechanisms of action and applications. *Pharmacol. Rev.* 34, 153–187.
- (20) Clark, E. A., and Brugge, J. S. (1995) Integrins and signal transduction pathways: the road taken. *Science* 268, 233–239.
- (21) Wells, A., Welsch, J. B., Lazar, C. S., Wiley, H. S., Gill, G. N., and Rosenfeld, M. G. (1990) Ligand-induced transformation by a non-internalizing epidermal growth factor receptor. *Science* 247, 962–964.

BC970190B

A Mild and Efficient Solid-Support Synthesis of Novel Oligonucleotide Conjugates

Ivan Habus, Jin Xie, Radhakrishnan P. Iyer, Wen-Qiang Zhou, Ling X. Shen, and Sudhir Agrawal*

Hybridon, Inc., 620 Memorial Drive, Cambridge, Massachusetts 02139. Received July 18, 1997;

Revised Manuscript Received December 16, 1997

Conjugates of oligodeoxyribonucleotide phosphorothioate (ODN-PS) with folic acid, retinoic acid, arachidonic acid, and methoxypoly(ethylene glycol)propionic acid have been synthesized. The procedure involved the initial solid-phase preparation of 5'-amino-functionalized ODN-PS using *N*-pent-4-enoyl-derived (PNT) nucleoside phosphoramidites followed by conjugation of the oligonucleotide either to the ligand acids, using 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide as a coupling reagent, or to their corresponding succinimidyl derivatives. Subsequent exposure of the support to aqueous ammonium hydroxide (28%, 2 h, 55 °C) resulted in the release of the fully deprotected ODN conjugates, which were purified by reversed-phase HPLC or by preparative polyacrylamide gel electrophoresis. The identity of the oligonucleotide conjugates was confirmed by MALDI-TOF mass spectral analysis.

INTRODUCTION

Oligodeoxynucleotides (ODNs) are widely used as research tools for inhibiting gene expression and as diagnostic agents and are being developed as potential therapeutic agents (1, 2). In recent years, considerable efforts have been devoted to the synthesis of chemically modified ODNs to improve their stability against nucleases, to enhance their binding affinity with the complementary target, and to facilitate their uptake by cells (3–7). In this context, phosphorothioate (PS) ODNs in which one of the nonbridging oxygens in an internucleotidic phosphodiester linkage is replaced by sulfur have emerged as a promising first-generation antisense therapeutic. Studies on the *in vivo* pharmacokinetics in rodents reveal that PS-ODNs are widely distributed in tissues and are slowly cleared primarily by urinary excretion.

In the development of antisense ODNs as potential anticancer therapeutics, it would be desirable to achieve high intracellular concentration of the ODN at the tumor site. One approach to achieve this objective is to conjugate oligonucleotides to tissue-specific carriers, which would facilitate "carrier-mediated uptake" of the oligonucleotides. In our earlier studies, we had conjugated ODNs to β -cyclodextrin, and its analogues (8, 9), as well as to adamantane (10) to facilitate cellular uptake of ODNs. In the present study, it was envisioned that the dual objectives of tissue-specific targeting and facilitated intracellular delivery could potentially be achieved by conjugating ODNs to ligands such as folic acid (FA) (1), retinoic acid (RA) (2), arachidonic acid (AA) (3), and methoxypoly(ethylene glycol)propionic acid (PEG-A) (4) (Chart 1). Indeed, several papers have described the facilitated intracellular delivery of proteins (11) and other macromolecules (12) as well as liposomes containing entrapped drugs (13) by folate complexation. Presumably, folic acid-receptor-mediated endocytosis is operative in these cases. Recently, a conceptually similar idea was explored in which antisense ODNs were complexed with FA–polylysine conjugate (14, 15). The regulation of RA-

induced proliferation and differentiation of various cancerous cells *in vitro* has been attempted by the use of antisense ODNs (16, 17), peptide nucleic acids (PNA) (18), stable transformant antisense RNA (19), and antisense-transfected clones (20). Similarly, numerous roles have been suggested for AA and its metabolites in cellular function, and antisense ODNs have been employed in studies aimed at delineating these roles (21, 22).

In analogy with these studies, we hoped to achieve tissue-specific targeting and facilitated intracellular delivery of ODN conjugates by receptor-mediated endocytosis mechanism, since the receptors for certain growth factors, vitamins, and hormones are overexpressed in rapidly dividing tumor cells (23–26). Conjugation of ODNs with the lipophilic PEG-A was expected to facilitate their cellular uptake, perhaps by passive diffusion, and reduce their protein binding. PEG is also nontoxic and is a pharmacopoeia-approved excipient for topical and oral dosage forms (27).

For our studies, we employed an 18-mer ODN sequence targeted against the RI α subunit of protein kinase A (PKA). The down-regulation of the RI α PKA isoform was reported to cause inhibition of tumor growth in a number of experimental *in vivo* tumor models (28).

RESULTS AND DISCUSSION

Synthesis of the Conjugates. A number of methods have been reported for conjugating ODNs to various ligands (29). Two approaches that are commonly employed are as follows: (a) the attachment of the ligand at the 5'-end of the ODN by incorporating the corresponding phosphoramidite during solid-phase ODN synthesis and (b) postsynthetic conjugation of an appropriately functionalized ODN with a ligand. Initially, we attempted to attach FA to the amino-functionalized ODN 5 (Chart 2) by following a procedure for preparation of liposome–FA conjugates (18). The requisite *N*-hydroxysuccinimide (NHS) ester 6 of FA (1) was prepared and isolated as reported (18). However, in our hands, attempts to synthesize the FA–ODN conjugate 7, by the reaction between 5 and 6, failed to reveal significant product formation when analyzed by reversed-phase

* Author to whom correspondence should be addressed [telephone (617) 528-7000; fax (617) 528-7692].

Chart 1

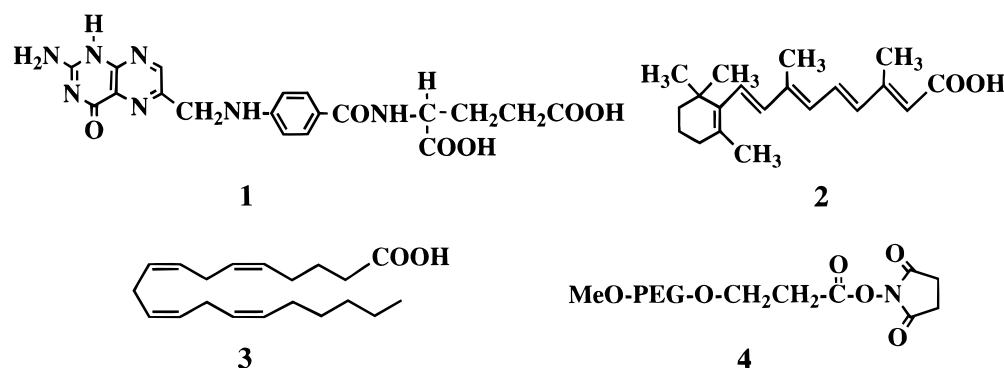
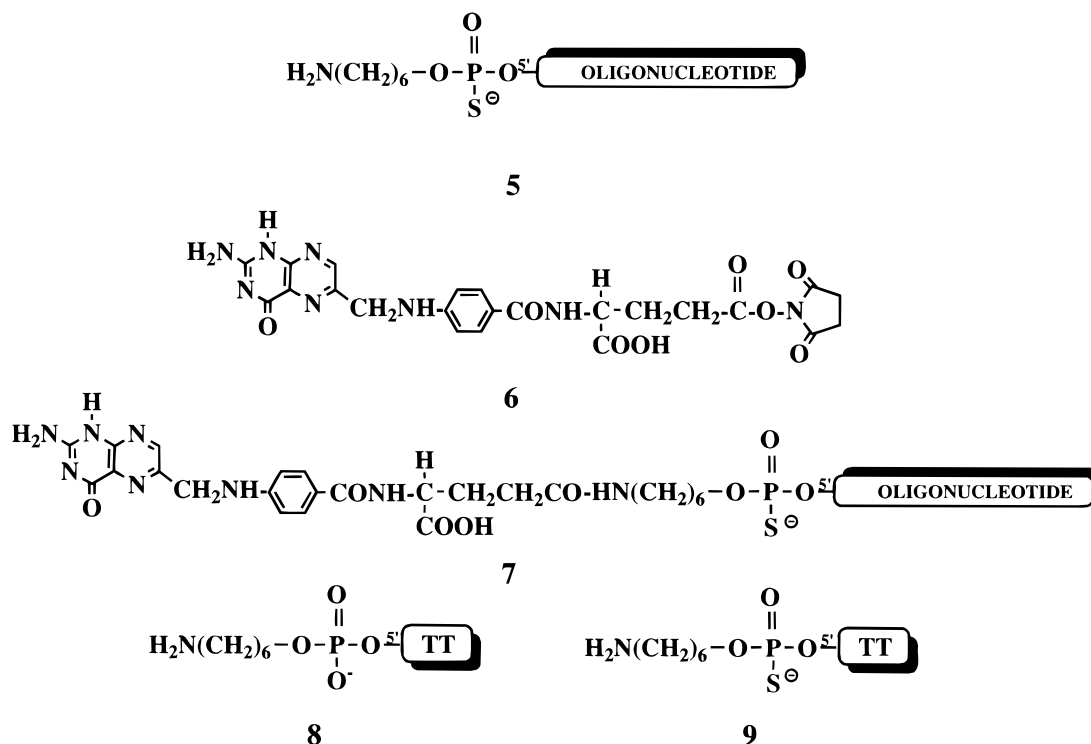


Chart 2



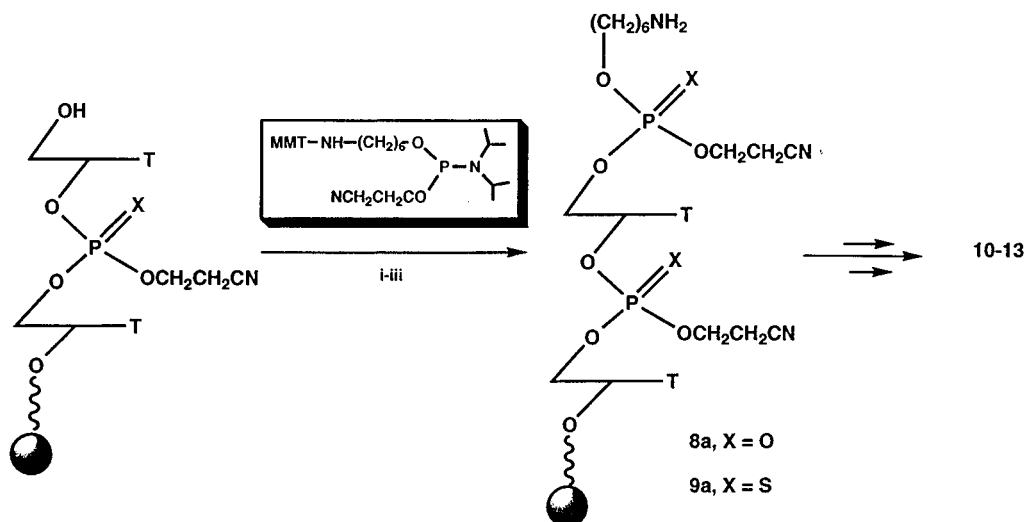
OLIGONUCLEOTIDE = ODN-PS, 5'-GCGTGCCTCCTCACTGGC-3'

HPLC and PAGE (data not shown). In a separate model study, we synthesized the amino-functionalized TT phosphodiester (TT PO) **8** and phosphorothioate (TT PS) **9** dinucleotides. Again, the reaction of **8** and **9** with the NHS ester **6** and analysis of the products by reversed-phase HPLC did not show the formation of the expected conjugate in isolable yields, but instead revealed a complex mixture of products (data not shown).

We then examined an alternate solid-phase approach in which a support-linked functionalized ODN was coupled to the ligands using 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide (DEC) as a coupling reagent. Since folic acid, arachidonic acid, and retinoic acid are known to be heat- and light-sensitive molecules, mild deprotection conditions had to be employed during the preparation of the requisite ODN conjugates. This precluded the use of nucleosides that carry conventional protecting groups for the synthesis of the ODN conjugates as they require harsh deprotection conditions for their removal. In the present study, we employed *N*-pent-4-enoyl-

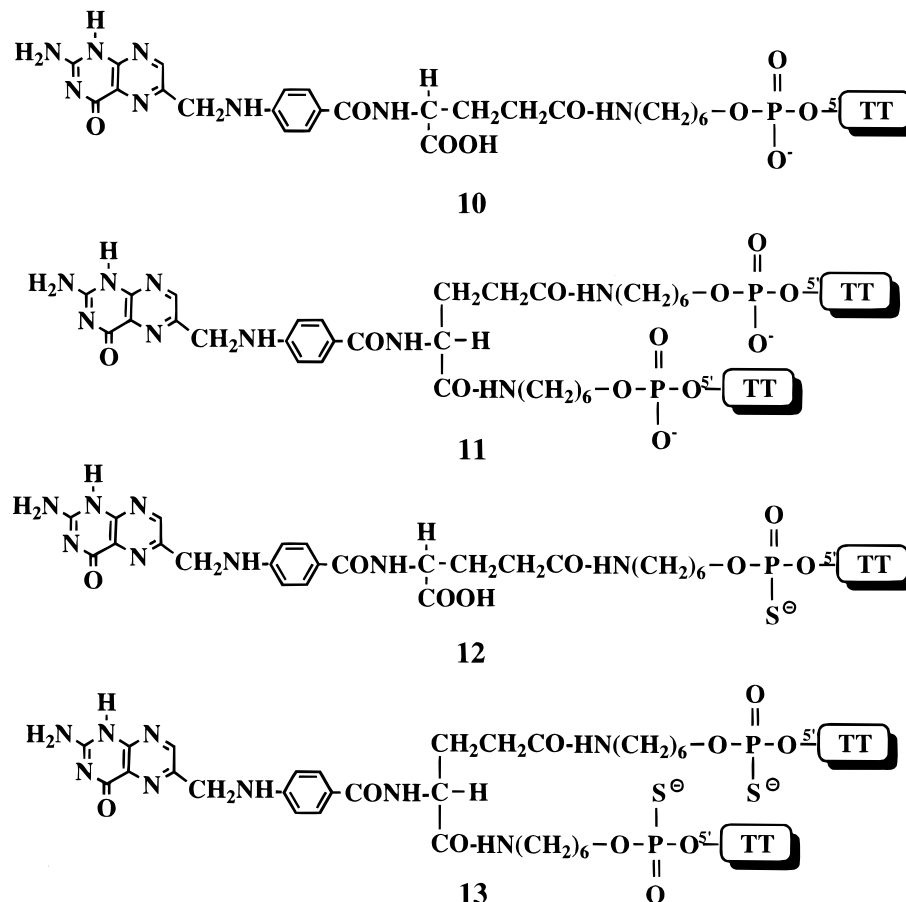
derived (PNT)-protected nucleoside monomers (31). However, nucleosides that carry other base-labile protecting groups, e.g., *N*-tert-butylphenoxyacetyl (*t*-Pac) (32), can also be employed. As a model study, we prepared the support-linked 5'-amino-terminated dinucleotides TT PO **8a** and TT PS **9a** (Scheme 1). The synthesis of **8a** and **9a** was carried out on a 10 μ mol scale in an automated DNA synthesizer. During their synthesis, the oxidation of the internucleotidic phosphite linkage was done using a solution of iodine, and the oxidative sulfurization was achieved using a 2% solution of 3*H*-1,2-benzodithiole-3-one 1,1-dioxide (30) in acetonitrile. For the attachment of the 5'-amino linker, the column was removed from the synthesizer and exposed to an acetonitrile solution of the 5'-amino-modifier phosphoramidite C6. Oxidation and capping, followed by the removal of the monomethoxytrityl (MMT) group, gave the support-linked dinucleotides **8a** and **9a**. The dimers **8a** and **9a** were then reacted with a solution of FA in anhydrous DMF in the presence of DEC as the coupling agent for 24 h.

Scheme 1



(i) Tetrazole (ii) I₂ or 3*H*-benzodithiole-3-one-1,1-dioxide (30) (iii) 2% DCA in methylene chloride

Chart 3



After successive washing with DMF and acetonitrile, the support was exposed to aqueous ammonium hydroxide (28%, 2 h, 55 °C) to release the fully deprotected dinucleotide conjugates **10–13** (Chart 3). The crude reaction mixtures were analyzed by HPLC (Figure 1) and purified by preparative HPLC. Although the analytical HPLC chromatograms of the purified conjugates revealed the apparent presence of only one product peak, derived from **8** and **9**, the MALDI-TOF mass spectra showed two sets of molecular ions presumably corresponding to mono-

and bisconjugates **10** and **11** (calcd for **10**, *m/z* 1149.0, found, *m/z* 1147.0; calcd for **11**, *m/z* 1856.6, found, *m/z* 1854.0), as well as **12** and **13** (calcd for **12**, *m/z* 1181.13, found, *m/z* 1181.8; calcd for **13**, *m/z* 1920.84, found, *m/z* 1923.5) derived from **8a** and **9a**, respectively. Also, the presence of the FA moiety in each of the conjugates **10–13** was ascertained by UV–VIS spectra, which revealed the presence of characteristic bands for folic acid chromophore at λ_{290} to λ_{390} nm (Figure 2). The formation of the monoconjugates **10** and **12** as well as the bisconju-

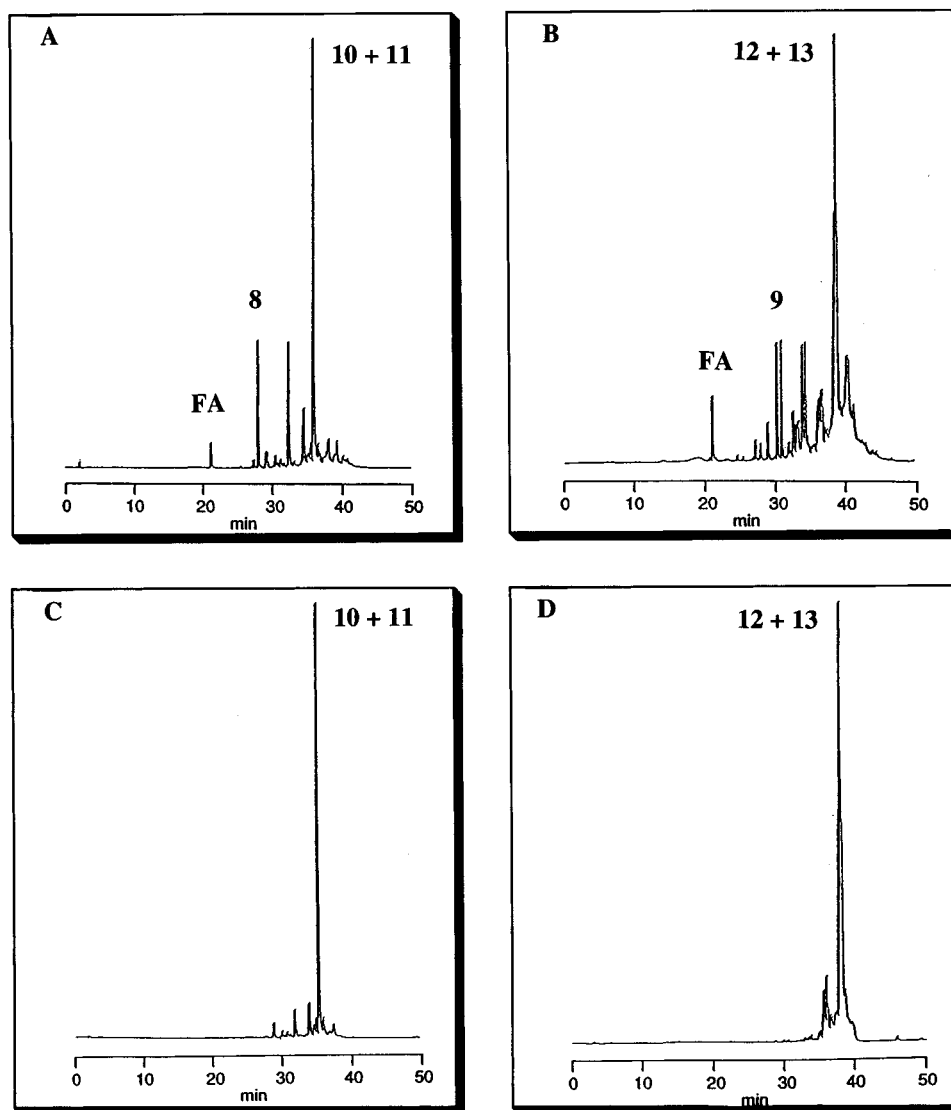


Figure 1. Reversed-phase HPLC profile of the reaction mixture between support-bound **8a** (A), **9a** (B), and between FA and isolated product mixture of **10** and **11** (C) and **12** and **13** (D). The solid-phase reaction was carried out as follows: A mixture of **8a** or **9a** on solid support (250 mg of CPG), **1** (0.5 mmol), DEC (2.5 mmol), and anhydrous DMF (10 mL) was shaken at room temperature for 24 h under nitrogen atmosphere. The CPG was filtered off and washed successively with DMF and acetonitrile. The products were released from the solid support, deprotected in ammonium hydroxide for 2 h at 55 °C, and after evaporation to dryness purified by reversed-phase HPLC. HPLC was carried out using a Waters 600E system controller, a 996 photodiode array detector, an NEC PowerMate 486/33i, and a Millennium 2010 chromatography manager. For further details see Experimental Procedures.

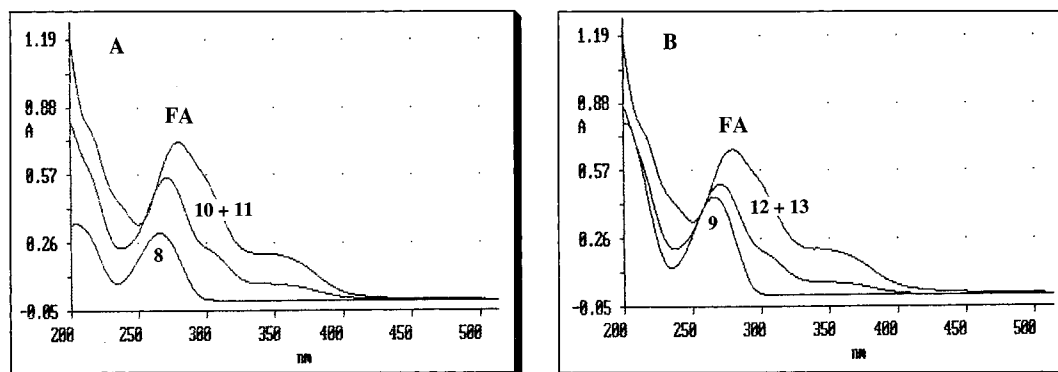
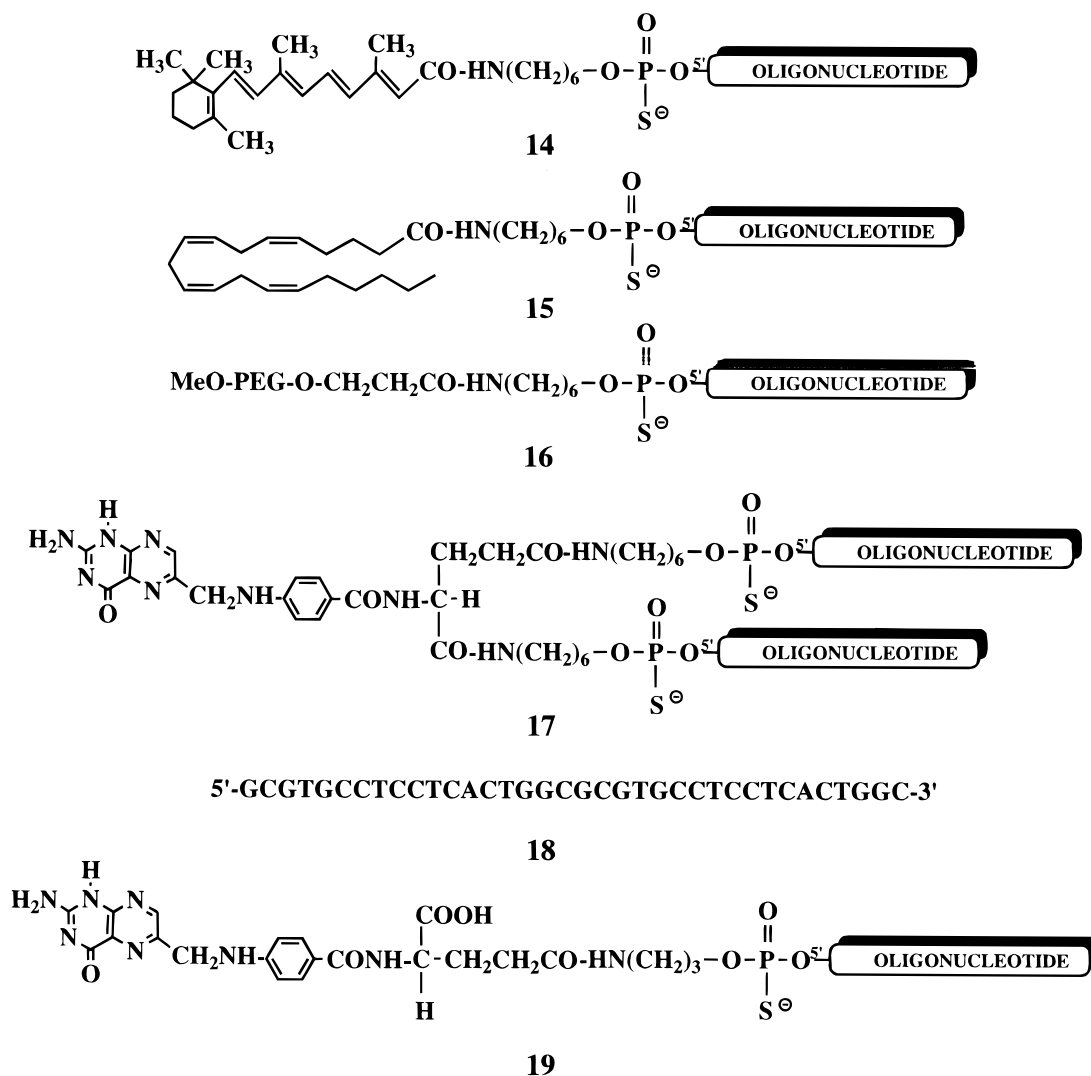


Figure 2. UV-VIS spectra of the compounds FA, **8**, and a mixture of **10** and **11** (A) and **1**, **9**, and a mixture of **12** and **13** (B). UV-VIS spectra were recorded on a Perkin-Elmer Lambda 2 spectrometer.

gates **11** and **13** is consistent with the expected reaction of **8** and **9** with one and two carboxylic functions of FA, respectively. Furthermore, according to the HPLC analysis (Figure 1A,B), the integral ratio of the area under the

respective peaks corresponding to the dinucleotides and the derived conjugates were 1:5 for **8**:(**10** + **11**) and 1:7 for **9**:(**12** + **13**), reflecting an overall conversion of 80–85% for the conjugation step.

Chart 4



At this juncture, we did not develop procedures for the separation of the mono- and bisconjugates, but applied the methodology for ODN conjugate synthesis. The principal advantage of this solid-phase approach was that all nucleophilic sites remained fully protected while the nucleophilic amino terminus reacted with the ligands in a chemoselective fashion. Consequently, a relatively high yield of the desired conjugates was obtained by this approach compared to the alternate solution-phase approach. Thus, the corresponding fully protected support-bound ODN PS **5** was synthesized on a 10 μ mol scale using PNT-protected nucleoside phosphoramidites, and dC^{PNT} nucleoside was anchored to succinylated controlled pore glass (CPG) on an automated DNA synthesizer. The attachment of the 5'-amino-modifier C6 was performed manually on a solid support. After oxidative sulfurization and capping, the MMT group was removed, and the CPG-bound ODN **5** was reacted with FA, RA, and AA in anhydrous DMF and PEG-A in anhydrous pyridine for 24–72 h. After successive washing with DMF or pyridine and acetonitrile, the support-bound conjugates were treated with aqueous ammonium hydroxide (28%, 2 h, 55 °C) and purified by reversed-phase HPLC or, alternatively, by preparative PAGE, to give the corresponding ODN conjugates **7** and **14–17** (Chart 4).

Upon analysis by PAGE, it was found that the conjugation reaction of ODN **5** with FA on solid support gave two products, **7** and **17**. The products **7** and **17** were

isolated and examined by MALDI-TOF mass spectrometry. The major product **7** (faster moving band on the PAGE, Figure 3) had one molecule of FA attached to the ODN (found, m/z 6319.1, calcd, m/z 6319.50) (Figure 4A). The minor product **17** (slower moving band on PAGE, Figure 3) had two ODNs bridged with one molecule of FA (MALDI-TOF spectrum showed a broad molecular ion between m/z 11900 and 13500 and centered approximately at m/z 12351.1; calcd, m/z 12197.54). To further ascertain their structural identity, we synthesized the ODN PS **18** (36-mer, twice as long as the sequence **5**) as a standard. Upon analysis by PAGE the electrophoretic mobilities of both **17** and the 36-mer **18** were found to be similar (Figure 3). Interestingly, the formation of the bisconjugate **17** could be minimized by reducing the reaction time from 72 to 24 h and/or by replacing the C6 amino linker with the C3 amino linker. This way, we could isolate a single product with C6 amino linker or **19** with C3 amino linker (found, m/z 6277.31, calcd, m/z 6277.40).

The crude ODN conjugates **14–16** (derived from RA, AA, and PEG-A, respectively) were also purified by preparative HPLC. The conjugates **14** and **15** showed a single sharp band by PAGE analysis (Figure 3), while the PEG oligoconjugate **16** showed a somewhat dispersed band presumably because the PEG used in the present study was a mixture of polymers with an average molecular weight of 5245. MALDI-TOF mass spectrom-

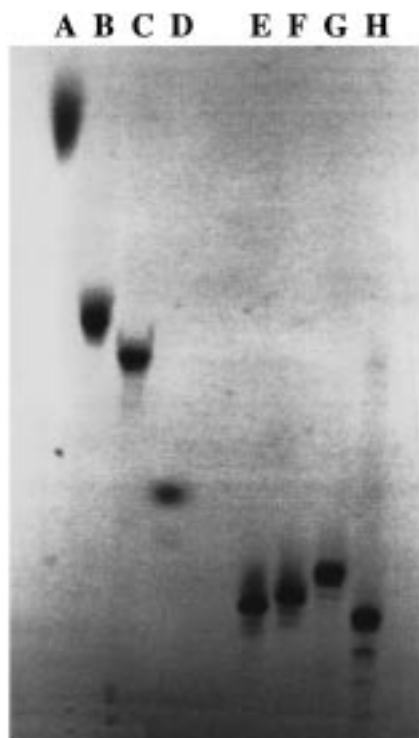


Figure 3. PAGE of the oligonucleotide conjugates (lanes): **16** (A), **17** (B), **18** (C), xylene cyanol (D) (marker dye), **15** (E), **14** (F), **7** (G), and **5** (H). The oligonucleotide conjugates (0.2 A_{260} unit) were analyzed by electrophoresis on a 20% polyacrylamide gel containing 8 M urea and visualized by UV shadowing.

etry analysis revealed the expected mass ions for **14** (found, m/z 6177.4, calcd, 6178.52) (Figure 4B) and **15** (found, m/z 6180.6, calcd, 6182.55) (Figure 4C). A sample of **16** gave a positive ion MALDI-TOF spectrum showing a broad peak unresolved pseudomolecular ion signal between approximately m/z 10000 and 13000 and centered at 11413 (calcd, 11179.07). Figure 5 shows the CGE profiles of the oligonucleotide conjugates **7**, **14**, **15**, **16**, **17**, and **19**.

The ODN conjugates **7** and **14–16** were studied for hybridization properties with the complementary RNA (30-mer) (Table 1). Hybridization studies indicated that incorporation of FA slightly increased the stability of the duplex formed ($\Delta T_m = 1.8$ °C) relative to the unconjugated control ODN, while incorporation of RA and AA had a slight destabilizing effect on duplex formation, $\Delta T_m = -3.3$ and -2.5 °C, respectively. The incorporation of PEG-A caused more significant reduction in binding ($\Delta T_m = -4.9$ °C; Table 1), presumably due to destabilization of the duplex by steric interference by the large polymer such as PEG (MW $\sim$ 5245).

While our work was in progress, a synthetic route for conjugation of ODNs, with cholic acid, FA, lipoic acid, and pantothenic acid, through thioether and disulfide bonds was reported (34), involving the initial multistep synthetic preparation of solid-support derivatized with cysteine. Also, recently, Kranz and co-workers reported successful conjugation of FA to N-terminal amino groups of protein using DEC (35).

In conclusion, we have successfully demonstrated a simple and efficient method for ODN conjugation on solid support. The combination of solid-support ODN synthesis by employing PNT-protected (or other suitable base-labile-protected) nucleoside phosphoramidites and conjugation of the ODN on the solid support has the following advantages: (a) The conjugation could be

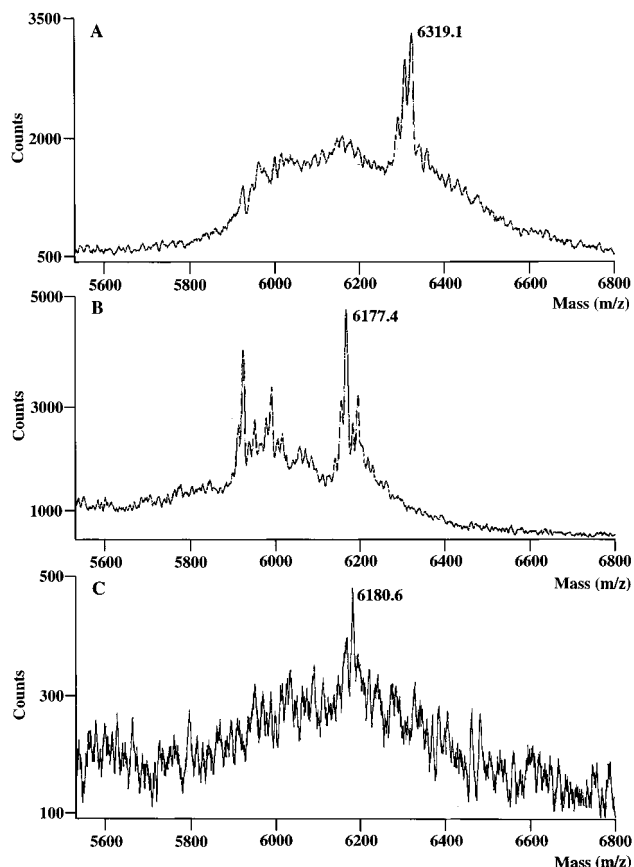


Figure 4. MALDI-TOF mass spectra of folic acid-ODN conjugate **7** (A), retinoic acid-ODN conjugate **14** (B), and arachidonic acid-ODN conjugate **15** (C). The molecular ions were at 6319.1, 6177.4, and 6180.6, respectively.

performed at essentially neutral conditions. (b) Both the 5'- and 3'-ends of an amino-linked ODN could be conjugated, which is dependent on the use of the 3'- or 5'-phosphoramidites. (c) Because the PNT group can be removed using multiple deprotection strategies ranging from neutral to mildly basic conditions (32), the use of this base-protecting group allows flexibility in the synthesis of ODN analogues, as well as in the structurally diverse molecules that could be conjugated to ODN. (d) Conjugation on solid support facilitated the removal of excess reactants and made it easier to isolate the products. The ODN conjugates, thus synthesized, formed stable duplexes with complementary RNA target.

EXPERIMENTAL PROCEDURES

Materials and Methods. MALDI-TOF mass spectrometry was performed using a PerSeptive Biosystems Voyager Elite Biospectrometry research station coupled with a delayed extraction laser desorption mass spectrometer, using hydroxypicolinic acid as a matrix.

UV-VIS measurements were recorded on a Perkin-Elmer Lambda 2 spectrometer. T_m measurements were recorded on a GBC 920 UV-VIS spectrometer, supplied with GBC Thermocell.

Oligonucleotides were assembled on CPG, carrying the leader PNT nucleoside (31), using an Expedite Nucleic Acid Synthesis System equipped with an NEC Image 466es and CTX monitor.

A Waters 650E Advanced Protein Purification System equipped with a Waters 600E system controller, a Dyanmax model UV-C absorbance detector, and 746 data module was used for purification of the oligonucleotides.

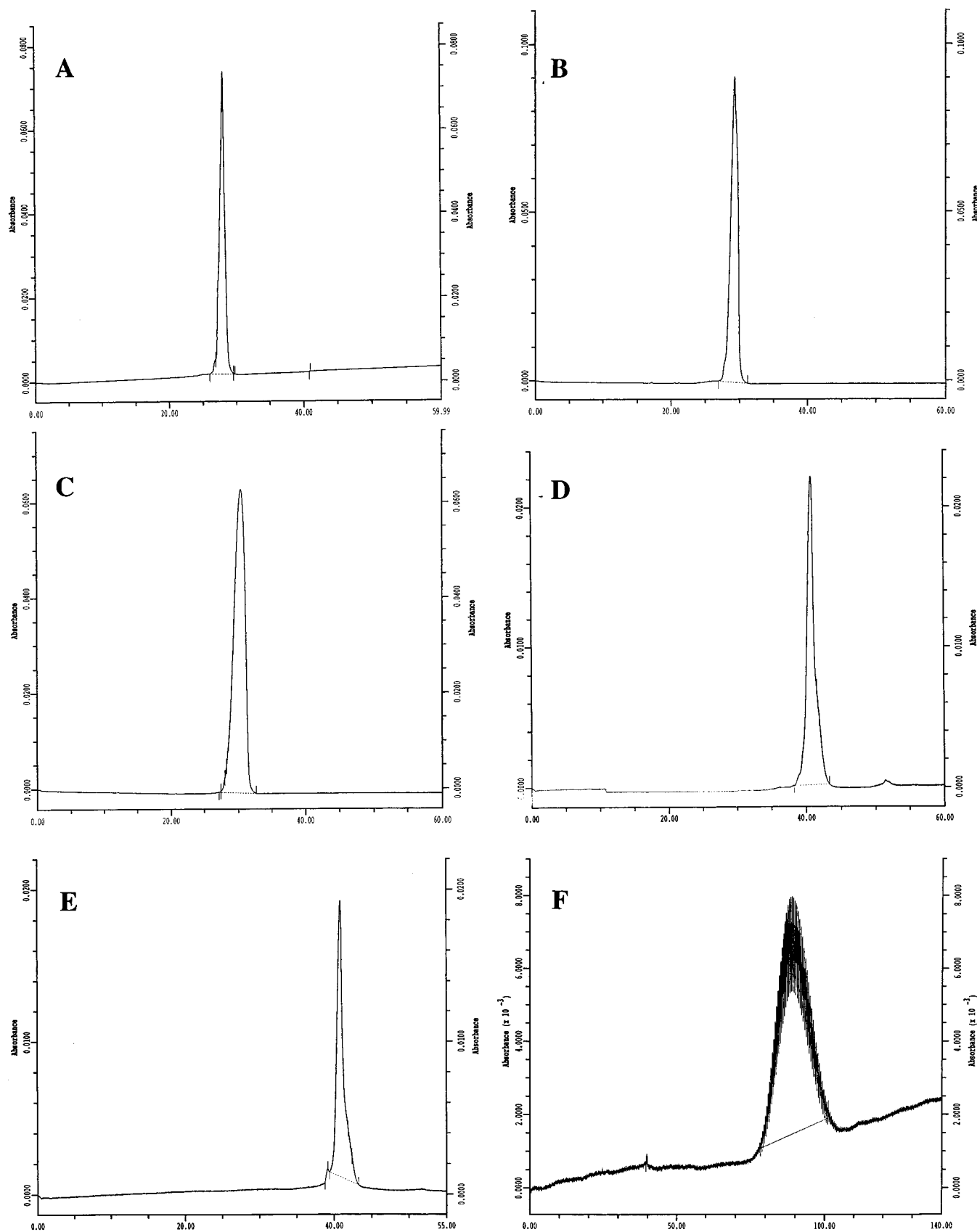
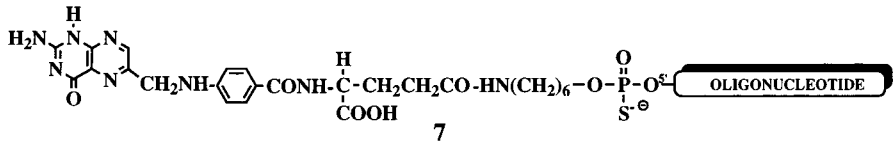
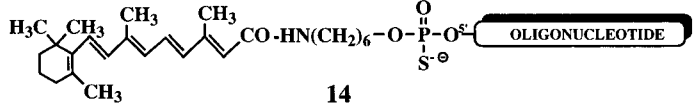
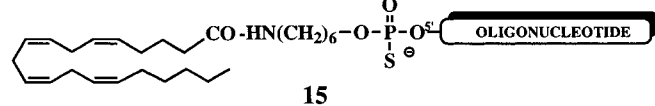
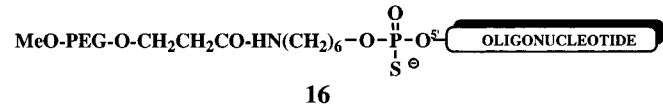


Figure 5. CGE profiles of oligonucleotide conjugates **7** (A), **17** (B), **19** (C), **14** (D), **15** (E), and **16** (F).

For the analysis of the oligonucleotides, a Waters chromatographic system equipped with a 600E system controller, a 996 photodiode array detector, an NEC Power Mate 486/33i, and a Millennium 2010 chromatography manager was used. Preparative reversed-phase HPLC

was done using a column (300 mm length, 10 mm diameter) packed with bulk preparative C_{18} matrix (125 Å, 55–105 μ m). For analytical HPLC, a Waters Radial-Pak cartridge was used. Buffers used were (A) 0.1 M ammonium acetate and (B) acetonitrile/A, 20:80. For

Table 1. T_m Data of Oligonucleotides^a

Entry	Oligonucleotide	T_m^b , (°C)	ΔT_m , (°C)
1	5'-GCGTGCCTCCTCACTGGC-3' ODN PS	62.8	-
2	 7	64.6	+1.8
3	 14	59.5	-3.3
4	 15	60.3	-2.5
5	 16	57.9	-4.9

^a T_m determinations were done using complementary RNA (5'-ACGCGCCGAGGAGGACGACGCCUU-3'). ^b Absorbance vs temperature profiles were obtained using a 1 μ M concentration for each strand, in 1 mL of buffer (100 mM Na⁺, 10 mM phosphate, 0.1 mM EDTA, pH 7.4).

preparative HPLC, a linear gradient was used: 100% A, 0% B for 5 min, 0–20% B for 15 min, 20–35% B for 30 min, 35–100% B for 15 min; flow rate of 10 mL/min. For analytical HPLC, a linear gradient was used: 100% A, 0% buffer B for 2 min, 0–20% buffer B for 30 min, 20–60% buffer B for 30 min; flow rate of 1.5 mL/min; detection at λ_{254} nm.

CGE analysis was carried out using the Beckman System Gold Personal chromatograph P/ACE system 2200, equipped with P/ACE UV absorbance detector and eCAP ssDNA 100 gel column, 47 cm in length. The oligonucleotides were electrokinetically loaded onto the column and analyzed by applying a voltage of 14.1 kV for 60–100 min and using tris–borate 7 M urea buffer for elution with the detector set at 254 nm.

DNA and RNA reagents, RNA phosphoramidite monomers (32), and the LCAA–CPG for the synthesis of oligodeoxy and oligoribonucleotides were obtained from PerSeptive Biosystems GmbH and CPG Inc., respectively. The 5'-amino modifier—C6 and C3 phosphoramidites—and 3H-1,2-benzodithiole-3-one 1,1-dioxide were purchased from Glen Research and R. I. Chemicals Inc., respectively. PNT phosphoramidites were synthesized and analyzed as described before (31). Folic acid, retinoic acid, and arachidonic acid were obtained from Aldrich Chemical Co. The HPLC grade solvents were obtained from Fisher Scientific Co. PEG-A (MW 5000) was purchased from Shearwater Polymers, Inc., and used as received.

Protocol for Oligonucleotide Conjugate Synthesis. The synthesis of the oligonucleotide phosphorothioate (ODN PS, 18-mer) sequence targeted against the RI α subunit of protein kinase A (PKA) (28) was carried out on a 10 μ mol scale on a CPG support (40 μ mol/g) in conjunction with PNT β -cyanoethyl 3'-phosphoramidites on an automated DNA synthesizer. A 2% solution of 3H-1,2-benzodithiole-3-one 1,1-dioxide in acetonitrile was used for PS oxidative sulfurization. The last DMT was manually removed on the synthesizer, and the deblocking solution was thoroughly rinsed out of the column with

acetonitrile. The CPG column was removed from the synthesizer and purged with argon. 5'-Amino modifier C6 or C3 phosphoramidite (250 mg) was dissolved in anhydrous acetonitrile (1 mL) and transferred into a luer tip syringe. The syringe was then attached onto the CPG column with another syringe containing 4 mL of standard activator solution. Both solutions of phosphoramidite and activator were swished back and forth through the column for 5 min. The CPG column was then installed back into the DNA synthesizer and washed with acetonitrile, followed by oxidation and capping. The MMT group was then removed using deblocking solution. After thorough washing with acetonitrile, followed by washing with anhydrous pyridine (20 mL) and acetonitrile, the CPG oligonucleotide was dried overnight in vacuo over P₂O₅.

(a) *Oligonucleotide Conjugates of Folic Acid, Retinoic Acid, and Arachidonic Acid.* A mixture of CPG oligonucleotide (250 mg), folic acid (1), retinoic acid (2), or arachidonic acid (3) (0.5 mmol), 4-(dimethylamino)pyridine (DMAP) (0.05 mmol), triethylamine (40 μ L), DEC (2.5 mmol), and anhydrous DMF (10 mL) was shaken in a septum-fitted round-bottom flask (25 mL) at room temperature for 24–72 h under nitrogen atmosphere. The CPG–oligonucleotide conjugate was filtered off and washed successively with DMF and acetonitrile.

(b) *PEG-A Oligonucleotide Conjugate.* A mixture of CPG-bound oligonucleotide (250 mg), methoxy-PEG succinimidyl propionate (4, 0.1 mmol), and anhydrous pyridine (5 mL) was shaken in a septum-fitted round-bottom flask (25 mL) at room temperature for 24 h under nitrogen atmosphere. The CPG–oligonucleotide conjugate was filtered off and washed successively with pyridine and acetonitrile.

The CPG-bound oligonucleotide conjugates were treated with aqueous ammonium hydroxide (28%, 2 h, 55 °C) and evaporated to dryness, and the crude conjugates **7**, **17**, **19**, and **14–16** were purified by reversed-phase HPLC and/or PAGE. Oligonucleotide conjugates **7** (257 A₂₆₀ units), **17** (55 A₂₆₀ units), and **19** (157 A₂₆₀ units) were

purified by PAGE, while **16** (172 A_{260} units, 30% overall yield) was purified by reversed-phase HPLC. Oligonucleotide-conjugates **14** (195 A_{260} units) and **15** (90 A_{260} units) were purified by both techniques to obtain pure samples.

LITERATURE CITED

- (1) Akhtar, S., and Agrawal, S. (1997) *In Vivo* Studies with Antisense Oligonucleotides. *Trends Pharmacol. Sci.* 18, 12–18.
- (2) Agrawal, S., and Iyer, R. P. (1995) Modified Oligonucleotides as Therapeutic and Diagnostic Agents. *Curr. Opin. Biotechnol.* 6, 12–19.
- (3) Agrawal, S., Ed. (1996) *Antisense Therapeutics*, Humana Press, Totowa, NJ, and references cited therein.
- (4) Akhtar, S., Ed. (1995) *Delivery Strategies for Antisense Oligonucleotide Therapeutics*, CRC Press, Boca Raton, FL, and references cited therein.
- (5) Sanghvi, Y. S., and Cook, P. D., Eds. (1994) *Carbohydrate Modifications in Antisense Research*, ACS Symposium Series 580, American Chemical Society, Washington, DC, and references cited therein.
- (6) Crooke, S. T., and Lebleu, B., Eds. (1993) *Antisense Research and Applications*, CRC Press, Boca Raton, FL, and references cited therein.
- (7) Uhlman, E., and Peyman, A. (1990) Antisense Oligonucleotides: A New Therapeutic Principle. *Chem. Rev.* 90, 543–584.
- (8) Zhao, Q., Tamsamani, J., and Agrawal, S. (1995) Use of Cyclodextrin and its Derivatives as Carriers for Oligonucleotide Delivery. *Antisense Res. Dev.* 5, 185–192.
- (9) Agrawal, S., Zhao, Q., and Habus, I. (1997) Cyclodextrin Cellular Delivery System for Oligonucleotides. U.S. Pat. 5,605,890 and 5,615,565.
- (10) Habus, I., Zhao, Q., and Agrawal, S. (1995) Synthesis, Hybridization Properties, Nuclease Stability, and Cellular Uptake of the Oligonucleotide–Amino- β -cyclodextrins and Adamantane Conjugates. *Bioconjugate Chem.* 6, 327–331.
- (11) Leamon, C. P., and Low, P. S. (1993) Membrane Folate-binding Proteins are Responsible for Folate-protein Conjugate Endocytosis into Cultured Cells. *Biochem. J.* 291, 855–860.
- (12) Leamon, C. P., and Low, P. S. (1991) Delivery of Macromolecules into Living Cells: A Method that Exploits Folate Receptor Endocytosis. *Proc. Natl. Acad. Sci. U.S.A.* 88, 5572–5576.
- (13) Lee, R. J., and Low, P. S. (1994) Delivery of Liposomes into Cultured KB Cells via Folate Receptor-mediated Endocytosis. *J. Biol. Chem.* 269, 3198–3204.
- (14) Citro, G., Szczylik, C., Ginobbi, P., Zupi, G., and Calabretta, B. (1994) Inhibition of Leukemia Cell Proliferation by Folic Acid–Polylysine-Mediated Introduction of *c-myc* Antisense Oligodeoxynucleotides into HL-60 Cells. *Br. J. Cancer* 69, 463–467.
- (15) Citro, G., Perrotti, D., Cucco, C., D'Agnano, I., Sacchi, A., Zupi, G., and Calabretta, B. (1992) Inhibition of Leukemia Cell Proliferation by Receptor-mediated Uptake of *c-myc* Antisense Oligodeoxynucleotides. *Proc. Natl. Acad. Sci. U.S.A.* 89, 7031–7035.
- (16) Baldassarre, G., Bianco, C., Tortora, G., Ruggiero, A., Moasser, M., Dmitrovsky, E., Bianco, A. R., and Ciardiello, F. (1996) Transfection with a Cripto Antisense Plasmid Suppresses Endogenous Cripto Expression and Inhibits Transformation in a Human Embryonal Carcinoma Cell Line. *Int. J. Cancer* 66, 538–543.
- (17) Manna, B., Ashbaugh, P., and Bhattacharyya, S. N. (1995) Retinoic Acid-Regulated Cellular Differentiation and Mucin Gene Expression in Isolated Rabbit Tracheal Epithelial Cells in Culture. *Inflammation* 19, 489–502.
- (18) Gambacorti-Passerini, C., Mologni, L., Bertazzoli, C., le Coutre, P., Marchesi, E., Grignani, F., and Nielsen, P. E. (1996) *In Vitro* Transcription and Translation Inhibition by Anti-Promyelocytic Leukemia (PML)/Retinoic Acid Receptor α and Anti-PML Peptide Nucleic Acid. *Blood* 88, 1411–1417.
- (19) Suzuki, T., Kim, H.-S., Kurabayashi, M., Hamada, H., Fujii, H., Aikawa, M., Watanabe, M., Watanabe, N., Sakomura, Y., Yazaki, Y., and Nagai, R. (1996) Preferential Differentiation of P19 Mouse Embryonal Carcinoma Cells Into Smooth Muscle Cells. Use of Retinoic Acid and Antisense Against the Central Nervous System-Specific POU Transcription Factor Brn-2. *Circ. Res.* 78, 395–404.
- (20) Oridate, N., Esumi, N., Lotan, D., Hong, W. K., Rochette-Egly, C., Chambon, P., and Lotan, R. (1996) Implication of Retinoic Acid Receptor γ in Squamous Differentiation and Response to Retinoic Acid in Head and Neck SqCC/Y1 Squamous Carcinoma Cells. *Oncogene* 12, 2019–2028.
- (21) Locati, M., Lamorte, G., Luini, W., Introna, M., Bernasconi, S., Mantovani, A., and Sozzani, S. (1996) Inhibition of Monocyte Chemotaxis to C-C Chemokines by Antisense Oligonucleotide for Cytosolic Phospholipase A_2 . *J. Biol. Chem.* 271, 6010–6016.
- (22) Balsinde, J., Barbour, S. E., Bianco, I. D., and Dennis, E. A. (1994) Arachidonic Acid Mobilization in P388D₁ Macrophages is Controlled by two Distinct Ca^{2+} -Dependent Phospholipase A_2 Enzymes. *Proc. Natl. Acad. Sci. U.S.A.* 91, 11060–11064.
- (23) Lacey, S. W., Sanders, J. M., Rothberg, K. G., Anderson, R. G. W., and Kamen, B. A. (1989) Complementary DNA for the Folate Binding Protein Correctly Predicts Anchoring to the Membrane by Glycosyl-phosphatidylinositol. *J. Clin. Invest.* 84, 715–720.
- (24) Selhub, J., and Franklin, W. A. (1984) The Folate-binding Protein of Rat Kidney. Purification Properties and Cellular Distribution. *J. Biol. Chem.* 259, 6601–6606.
- (25) Asok, A. C., Utley, C., Van Horne, K. C., and Kolhouse, J. F. (1981) Isolation and Characterization of a Folate Receptor from Human Placenta. *J. Biol. Chem.* 256, 9684–9692.
- (26) Rothenberg, S. P., and Da Costa, M. (1971) Further Observations on the Folate-binding Factor in Some Leukemic Cells. *J. Clin. Invest.* 50, 719–726.
- (27) Shearwater Polymers, Inc., Catalog (1996) Polyethylene Glycol Derivatives, pp 18 and 42–49, 2307 Spring Branch Rd., Huntsville, AL 35801, and references cited therein.
- (28) Nesterova, M., and Cho-Chung, Y. S. (1995) A Single-injection Protein-kinase A-directed Treatment to Inhibit Tumor Growth. *Nat. Med.* 1, 528–533.
- (29) Agrawal, S. (1994) Functionalization of Oligonucleotides with Amino Group and Attachment of Amino-specific Reporter Groups. In *Methods in Molecular Biology: Protocols for Oligonucleotide Conjugates* (Agrawal, S., Ed.) pp 93–120, Humana Press, Totowa, NJ.
- (30) Iyer, R. P., Regan, J. B., Egan, W., and Beaucage, S. L. (1990) 3H-1,2-Benzodithiole-3-one-1,1-Dioxide as an Improved Sulfurizing Reagent in the Solid-Phase Synthesis of Oligodeoxyribonucleoside Phosphorothioates. *J. Am. Chem. Soc.* 112, 1253.
- (31) Iyer, R. P., Yu, D., Habus, I., Ho, N.-H., Johnson, S., Devlin, T., Jiang, Z., Zhou, W., Xie, J., and Agrawal, S. (1997) N-Pent-4-enoyl (PNT) Group as a Universal Nucleobase Protector: Applications in the Rapid and Facile Synthesis of Oligonucleotides, Analogs, and Conjugates. *Tetrahedron* 53, 2731–2750.
- (32) Sinha, N. D., Davis, P., Usman, N., Perez, J., Hodge, R., Kremsky, J., and Casale, R. (1993) Labile exocyclic amine protection of nucleosides in DNA, RNA and oligonucleotide analog synthesis facilitating N-deacylation, minimizing depurination and chain degradation. *Biochimie* 75, 13.
- (33) Tarasow, T. M., Tinnermeier, D., and Zyzanski, C. (1997) Characterization of Oligodeoxyribonucleotide-Polyethylene Glycol Conjugates by Electrospray Mass Spectrometry. *Bioconjugate Chem.* 8, 89–93.
- (34) Harrison, J. G., and Balasubramanian S. (1997) A Convenient Synthetic Route to Oligonucleotide Conjugates. *Bioorg. Med. Chem. Lett.* 7, 1041–1046.
- (35) Cho, B. K., Roy, E. J., Patrick, T. A., and Kranz, D. M. (1997) Single-Chain Fv/Folate Conjugates Mediate Efficient Lysis of Folate-Receptor-Positive Tumor Cells. *Bioconjugate Chem.* 8, 338–346.

Characterization of Interpolyelectrolyte Complexes between Double-Stranded DNA and Polylysine Comb-Type Copolymers Having Hydrophilic Side Chains

Atsushi Maruyama,* Hiromitsu Watanabe, Anwarul Ferdous, Maiko Katoh, Tsutomu Ishihara, and Toshihiro Akaike

Department of Biomolecular Engineering, Faculty of Bioscience and Biotechnology, Tokyo Institute of Technology, 4259 Nagatsuta, Midori, Yokohama 226-8501, Japan. Received August 11, 1997; Revised Manuscript Received January 29, 1998

The polyionic interaction between DNA and polycations grafted with hydrophilic dextran side chains was evaluated. The comb-type copolymers, poly(L-lysine)-*graft*-dextran, were successfully prepared by employing a reductive amination reaction between ϵ -amino groups of poly(L-lysine) (PLL) and the reductive ends of dextran (Dex). A coupling efficacy on the order of 70% was obtained regardless of intrinsic philicities of the solvents used, either aqueous buffer or DMSO. The resulting graft copolymers, which varied in the degree of grafting and the length of hydrophilic side chains, formed a soluble complex with DNA. They also affected the melting behavior of double-stranded DNA (dsDNA) in different ways. Copolymers having a high degree of grafting thermally stabilized dsDNA without affecting its reversible transition between single-stranded and double-stranded forms. However, copolymers with a low degree of grafting or with a high degree of grafting of short dextran chains impeded the reversibility of this transition. Furthermore, highly grafted copolymers also accelerated the hybridization of DNA strands in a low-ionic strength medium. It is of particular note that these copolymers scarcely altered circular dichroic signals of dsDNA even when the copolymers were added in excess. This suggested that the copolymer interacted with dsDNA without affecting its native structure or physicochemical properties. Finally, the copolymer even formed a stable complex with a short oligonucleotide (20 bases). We, therefore, concluded that, by regulating the degree of grafting and the molecular weight of grafted side chains, it would be possible to design novel different graft copolymers capable of acting as carriers of functional genes to target cells or tissue.

INTRODUCTION

Polycations interact with polyanions strongly to form polyanion complexes (PICs) or interpolyelectrolyte complexes (IPECs). Although both polyanions and polycations are readily soluble in water, when combined in solution they form complexes, such as coacervates or precipitates, which separate out of aqueous media (1–4). IPECs have been investigated as biocompatible (5–8), membrane- (5) and enzyme-immobilization (9), and microencapsulation (10–12) materials. More recently, complexes between DNA and polycation derivatives have been used as artificial carriers of poly(oligo)nucleotides, including genes and antisense oligonucleotides (13). However, applications of IPECs are limited due to their irreversible and insoluble behavior. As a result, regulation of both properties and structures of IPECs is needed to expand the functional applications of these polymer complexes.

While complex formation in mixtures of linear polyanions and polycations has frequently been observed, little is known about IPEC formation between polyion segments in mixed block (14–16) or graft copolymers. In specific, complex formation is poorly understood between graft copolymers of one polyion and polymers of the opposite charge (17, 18). We previously reported that the graft copolymer consisting of a poly(L-lysine) (PLL)

backbone and dextran (Dex) graft chains forms a soluble complex with DNA and stabilizes DNA triple helices of poly(dA)·2poly(dT) (17). Without the copolymer, these triple helices were unstable under physiological conditions (19). Despite the graft copolymers' considerable elevation of the triple helix melting temperature (T_m), they did not disturb reversible transition between the triplex and its constituent single-stranded DNAs (ssDNAs).

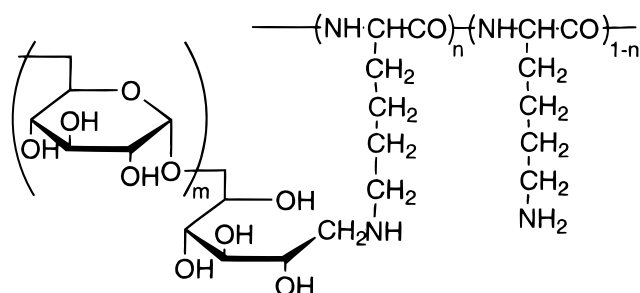
The main aim of this study was to understand and to control the interaction between polyanionic DNA and various graft copolymers consisting of a PLL backbone and hydrophilic graft side chains of Dex. PLL-*graft*-Dex (PLL-*g*-Dex) copolymers with various degrees of grafting and lengths of Dex chains were synthesized, and their interaction with DNA was evaluated by turbidity assays, UV-melting temperature assays (UV- T_m), circular dichroism (CD) measurements, and gel electrophoresis assays. We found that the graft copolymers formed basically two categories of soluble complexes. The first category of graft copolymer–DNA complexes allowed the reversible transition between duplex and its ssDNAs. Such copolymers were also capable of thermal stabilization of dsDNA. The second category included mostly irreversible complexes. Our results suggest that, by changing the molecular weight, degree of grafting, and the properties of the graft chains, we were able to control the associating properties of these polyions.

* Corresponding author. Phone: +81-45-924-5809. Fax: +81-45-924-5815. E-mail: amaruyam@bio.titech.ac.jp.

Table 1. Preparation of PLL-graft-Dex Copolymers^a

run	in feed						copolymer					
	PLL		dextran			NaBH ₃ CN (mg)	yield (%)	molecular weight		dextran content ^d		coupling efficacy ^e (%)
	$M_n^{b/10^4}$	mg	M_n^b	mg	wt %			$M_n^{b/10^4}$	M_w/M_n^b	wt %	mol % ^d	
1	3.6	92	2600	94	50.6	22.7	26	8.2	1.5	43.7	3.8	76
2	3.6	92	2600	187	67.1	45.0	78	9.5	1.4	60.3	7.5	75
3	3.6	92	2600	375	80.3	91.0	66	11.2	1.7	74.3	14.2	71
4	3.6	92	2600	937	91.1	227	71	22.7	1.4	87.3	34.0	68
5	3.6	92	5900	212	69.8	22.7	62	8.3	1.6	66.0	3.6	72
6	3.6	92	5900	425	82.2	45.0	nd	13.5	1.4	79.3	7.1	71
7	3.6	92	5900	850	90.3	91.0	71	30.5	1.2	88.3	14.0	70
8	3.6	62	5900	150	70.8	7.5	60	nd	nd	70.9	4.7	88
9	3.6	62	5900	300	82.8	15.0	77	nd	nd	83.0	9.6	90
10	3.6	62	5900	600	90.6	30.0	79	nd	nd	90.7	19.7	93
11	1.3	31	5900	300	90.9	15.0	69	15.7	1.2	90.4	17.0	82
12	1.3	31	5900	450	93.6	22.5	74	19.6	1.2	92.6	21.4	68

^a The reaction was carried out in the borate buffer (runs 1–7) or in DMSO (runs 8–12). ^b M_n = the number-average molecular weight; M_w = the weight-average molecular weight. M_n and M_w were determined as described in Materials and Methods. ^c Determined by ¹H NMR. ^d Mol % = 100(fraction of lysine units coupled with Dex). ^e Coupling efficacy = [Dex]_{copolymer}/[Dex]_{in feed}.

**Figure 1.** Structural formula of the polylysine-graft-dextran copolymer.

MATERIALS AND METHODS

Materials. Poly(L-lysine) (PLL) was purchased from Sigma-Aldrich Japan (Tokyo, Japan) or Peptide Institute, Inc. (Tokyo, Japan). Dextran T-10 (M_n = 5900), poly(dA), and poly(dT) were obtained from Pharmacia Biotech (Uppsala, Sweden). Oligonucleotides were a HPLC-purified grade obtained from Grainer Japan Co. (Tokyo, Japan). Dextran 4 (fraction with a MW of 4000–6000) was purchased from Funakoshi Chemical Co. (Tokyo, Japan). Calf thymus DNA (t-DNA) was obtained from Sigma-Aldrich Japan. Other solvents and chemicals were reagent grade, were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), and were used without further purification.

Synthesis of the Poly(L-lysine)-graft-Dextran Copolymer. Comb-type copolymers, poly[(L-lysine)-graft-(α -1,6-D-glucopyranoside)] (PLL-g-Dex), were synthesized by a reductive amination reaction of PLL·HBr with dextran (Dex) (17, 20). Either borate buffer or DMSO was used as a solvent for the reaction. The conditions for the synthesis are described in Table 1. The resulting copolymers were isolated by ultrafiltration to remove unreacted Dex. The ultrafiltration was carried out using a Millipore UFV2BGC10 centrifugal filter device (MW cutoff of 10 000, Millipore Corp., Bedford, MA) or a Q0100 ultrafilter (MW cutoff of 10 000, Advantec Toyo Filter Ltd., Tokyo, Japan). Finally, the copolymers were lyophilized.

¹H NMR of the copolymers in D₂O was recorded on a Varian VNMR 4.3 spectrometer and used to determine the composition of the resulting copolymers. The structural formulas of the comb-type copolymer are shown in Figure 1. Hereafter, we designate the copolymers as PLL-Dex(X-Y), where X and Y represent the molecular weight (number average) and the content (mole percentage) of Dex chains in the copolymers, respectively.

Gel Permeation Chromatography (GPC) and Molecular Weight Determination of Polymers.

The resulting copolymers were analyzed using a gel permeation chromatography system (GPC, Jasco model 800, Tokyo, Japan) on Waters Ultrahydrogel 250 and 500 columns connected with a multiangle light scattering detector (Dawn-DSP, Wyatt Technology, Santa Barbara, CA) and a differential refractive index (RI) detector (Jasco, model 830-RI). An aqueous solution of 0.5 M acetic acid and 0.2 M Na₂SO₄ was used as a mobile phase at 1.0 mL/min. An aliquot (200 μ L) of graft copolymer solution (1 mg/mL) was injected into the column. The molecular weight and its distribution were determined according to the instruction manual of Dawn-DSP on the basis of Zimm's equation (eq 1).

$$\frac{R_\theta}{K^*c} = MP(\theta) - 2A_2cM^2P^2(\theta) \quad (1)$$

where c is the concentration of solute molecules in the solvent r , M is the weight-average molecular weight, A_2 is the second virial coefficient, $P(\theta)$ is a theoretically derived factor and is a function of the molecule's z -average size, shape, and structure, $R(\theta)$ is the Rayleigh ratio at the individual scattering angle, and K^* is an optical constant [$=4\pi^2n_0^2(dn/dc)^2\lambda_0^{-4}N_A^{-1}$, where n_0 is the refractive index of the solvent at the incident radiation wavelength (nanometers), N_A is Avogadro's number, and dn/dc is the differential refractive index increment of the solvent/solute solution with respect to a change in solute concentration].

By extrapolating the value of θ to 0, we derived eq 2 from eq 1.

$$\frac{R_{\theta=0}}{K^*c} = M - 2A_2cM^2 \quad (2)$$

By neglecting the second term, we determined M with a R_θ/K^*c versus $\sin^2(\theta/2)$ plot (Debye plot) for θ approaching 0. M was calculated at each second of the elution period using c monitored by the RI detector. The estimated values of M at each 1 s slice in the GPC were then used to determine weight- and number-average molecular weights of polymers.

Turbidity Measurements of the DNA/Copolymer Mixture. Calf thymus DNA was dissolved in Milli Q water at 1.4 mg/mL and sonicated twice for 8 min on ice with a 80 W output at a 20% interval using a probe-type generator (Tomy UD-201, Tokyo, Japan). The approxi-

mate molecular weight of the sonicated DNA fragments was 1 kbp as determined by agarose gel electrophoresis (data not shown). The DNA solution was diluted to 30 $\mu\text{g/mL}$ with 10 mM sodium phosphate buffer solution (PBS, pH 7.2) containing 150 mM NaCl (buffer I). To the DNA solution was added a stock solution (1 mg/mL) of the graft copolymer in buffer I at various polymer/DNA charge ratios ($P/D = [\text{amino group}]_{\text{polymer}}/[\text{phosphate group}]_{\text{DNA}}$). The final DNA concentration was adjusted to 27.4 $\mu\text{g/mL}$. After the solution was vigorously mixed with a vortex mixer, it was allowed to stand at 4 °C. Turbidity measurements were carried out after 1, 4, and 14 days at 340 nm with Beckman DU 640 spectrophotometer with a 1 cm path length.

CD and UV- T_m Measurements. Stock solutions were prepared by dissolving nucleotides in 10 mM PBS (pH 7.2) containing 150 mM NaCl and 0.1 mM EDTA (buffer II). Concentrations of the stock solutions were calculated using molar extinction coefficients of 8900 at 257 nm for poly(dA) and 9000 at 265 nm for poly(dT) (21). Solutions for CD and UV- T_m measurements were prepared by diluting the stock nucleotide solutions (final concentration of 36 $\mu\text{mol/L}$) and the comb-type copolymer solution at various P/D ratios into buffer II. The mixtures were heated at 90 °C for 30 min, gradually cooled, and allowed to stand for 16 h at room temperature.

UV spectra and UV melting curves were recorded with a Beckman DU-640 spectrometer equipped with a micro-melting temperature apparatus (Beckman). The UV melting curves were recorded at 0.2 K/min as heating and cooling rates. The differential absorbance ($\Delta A = A_{260} - A_{340}$) was calculated to correct for baseline shift. The first derivative $[d(\Delta A)/dT]$ was calculated from the melting curve data. Peak temperatures in the derivative curves were designated T_m .

CD spectra were measured with a Jasco model J-600 spectropolarimeter. Each spectrum shown was the average of more than three scans that have been smoothed.

Analysis of DNA-Polymer Complex Formation by Gel Electrophoresis. An increasing amount of each of the copolymers was added to 3 μg of pSV plasmid DNA (6.8 kbp) in buffer I from a stock solution. The mixture was diluted with buffer I to a final plasmid concentration of 43.4 $\mu\text{g/mL}$, followed by incubation for 24 h at room temperature. The mixture was then analyzed by running it on a 1% agarose gel in Tris-acetate buffer (40 mM, pH 8.0) containing 1 mM EDTA. The gel was then stained with ethidium bromide to visualize the DNA bands.

The complex formation between a 20-mer oligonucleotide (ODN) and the graft copolymer was analyzed as follows. The ODN (500 ng) in 50 mM Tris-acetate (pH 7.0), 100 mM NaCl, and 10 mM MgCl_2 was heated at 65 °C for 10 min to disrupt self-associated chains and then quickly cooled on ice. The copolymer was then added at the indicated P/D ratio and the mixture incubated for 12 h at room temperature. The mixture was then electrophoresed on a 15% polyacrylamide gel for 16 h at constant voltage (8 V/cm). The resulting bands were visualized by staining with Stain-all (Nacalai Tesque, Inc., Kyoto, Japan). A competition assay was also performed as described above, except that the ODN was incubated with or without copolymer at a P/D ratio of 1. Samples were electrophoresed in the presence or absence of an excess (ca. 10 times) amount of sonicated calf thymus DNA.

RESULTS AND DISCUSSION

Successful Preparation of PLL-g-Dex with High Coupling Efficacy. Semitelechelic prepolymers which

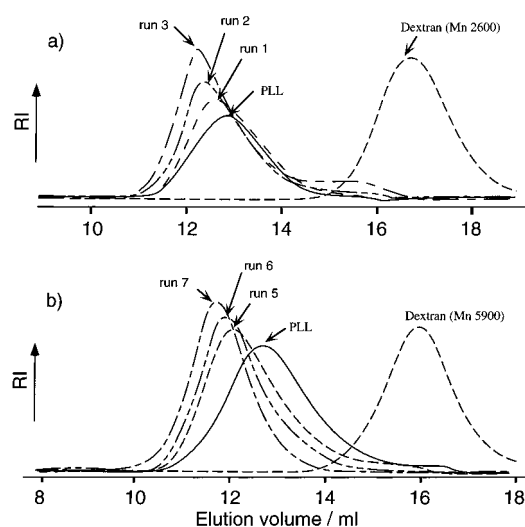


Figure 2. GPC profiles of PLL, Dex, and the resulting copolymers. Copolymers prepared from Dex having a M_n of 2600 (a) and a M_n of 5900 (b) were analyzed on Waters Ultrahydrogel 250 and 500 columns connected with a multiangle light scattering detector and a differential refractive index (RI) detector (see the text for details). An aqueous solution containing 0.5 M acetic acid and 0.2 M Na_2SO_4 was used as a mobile phase at a rate of 1.0 mL/min.

have a reactive group at their one chain end are required in the preparation of graft copolymers by the polymer coupling method. Polysaccharides are suitable prepolymers for graft copolymer preparation, since most polysaccharides possess one reductive end. Their semitelechelic character is preserved even after chemical or enzymic hydrolysis of the glycosyl bonds of the polysaccharide chains. Thus, by combining the hydrolysis and coupling reactions, we can obtain graft copolymers having polysaccharide chains with various molecular weights. In this study, the reductive end of Dex was directly coupled to the ϵ -amino groups of PLL by a reductive amination reaction using NaBH_3CN as a reductant. Figure 2 shows GPC profiles of PLL, Dex, and the copolymers. These reactions show an apparent increase in molecular weight. Furthermore, since there is no corresponding Dex peak in the copolymer formulations, they can be assumed to be free of unreacted Dex. The results of the coupling reaction are summarized in Table 1 with the number-average molecular weight of the polymers estimated by static light scattering. At a lower feed ratio (<20 mol %) of Dex/PLL, the efficacy of coupling of Dex to PLL was $>70\%$ regardless of the molecular weight of Dex and solvents used in the coupling process. The polymer composition calculated from the molecular weight was in good agreement with that determined from ^1H NMR measurements (Figure 3). This result indicated that no detectable side reactions that may cause cross-linking or degradation of the resulting copolymers occurred. As reported elsewhere, the coupling reaction between PLL and other polysaccharides, including amylose (20), hyaluronic acid (22), and arabinogalactane (manuscript in preparation), were found to be successful, indicating the validity of the coupling polysaccharide conjugates with PLL.

Turbidity of the DNA/Copolymer Mixture and the Melting Behavior of DNA in the Mixture. Polycationic homopolymers such as polylysine and polyarginine form precipitates or coacervates when they are mixed with polyanions such as DNA in aqueous media. The strong electrostatic attraction between oppositely charged macromolecules causes them to be drawn together and

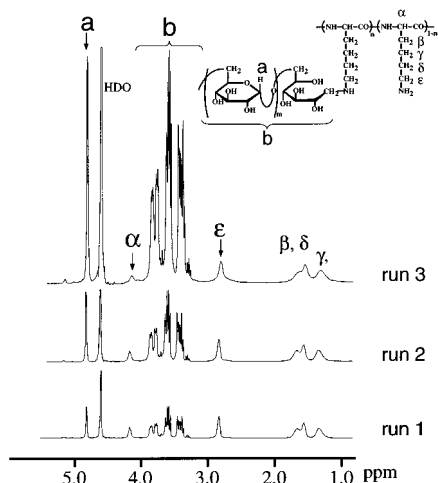


Figure 3. ^1H NMR spectra of PLL-g-Dex copolymers in D_2O . The indicated run numbers were the same as those indicated in Table 1.

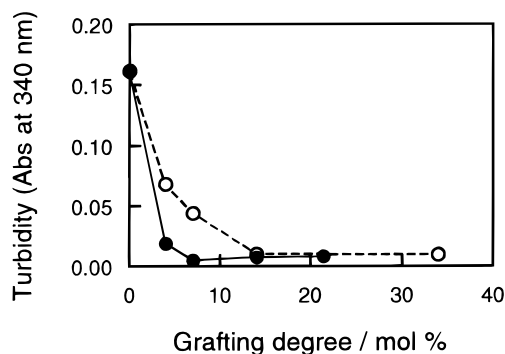


Figure 4. Turbidity of DNA/polymer mixtures (P/D ratio = 1) in phosphate-buffered saline (PBS). To a $30\text{ }\mu\text{g/mL}$ sonicated calf thymus DNA solution in PBS was added a 1 mg/mL PBS solution of graft copolymers prepared with Dextran 4 ($M_n = 2600$, $\circ$) or Dextran T-10 ($M_n = 5900$, $\bullet$) at a P/D ratio of 1. The mixture was diluted with buffer I to a final DNA concentration of $27.4\text{ }\mu\text{g/mL}$. After the mixture was vigorously mixed with a vortex mixer, the turbidity of the solution was measured as the optical density (OD) at 340 nm .

react ionically, releasing their associated counterions as free salt (1, 23). The counterion release caused a significant loss in solubility due to a loss in osmotic pressure of the complex. The important characteristic of the comb-type graft copolymer is the fact that they form soluble complexes with DNAs as estimated by turbidity measurements of DNA/graft copolymer mixtures (Figure 4). The mixture of linear polylysine and DNA shows significant turbidity in 10 mM PBS containing 150 mM NaCl; however, the turbidity decreases with an increasing degree of grafting of Dex side chains. No turbidity is observed at higher degrees of grafting even after storage for 4 weeks at $4\text{ }^\circ\text{C}$. The grafted dextran chains seem to be responsible for protecting the DNA-polycation complex from self-aggregation and precipitation. In support of our argument, we have seen, using fluorescence microscopy, that this protective effect also occurs in large (166 kbp) DNA sequences of T4 phage (data not shown).

The solubility of such complexes minimized the effect of turbidity and/or precipitation and enabled us to evaluate their interaction with DNA by UV- T_m measurements. Figure 5 shows the UV- T_m curves of double-helical poly(dA)·poly(dT) in the absence or presence of the comb-type copolymers with various Dex contents. Poly(dA)·poly(dT) alone undergoes double helix to single

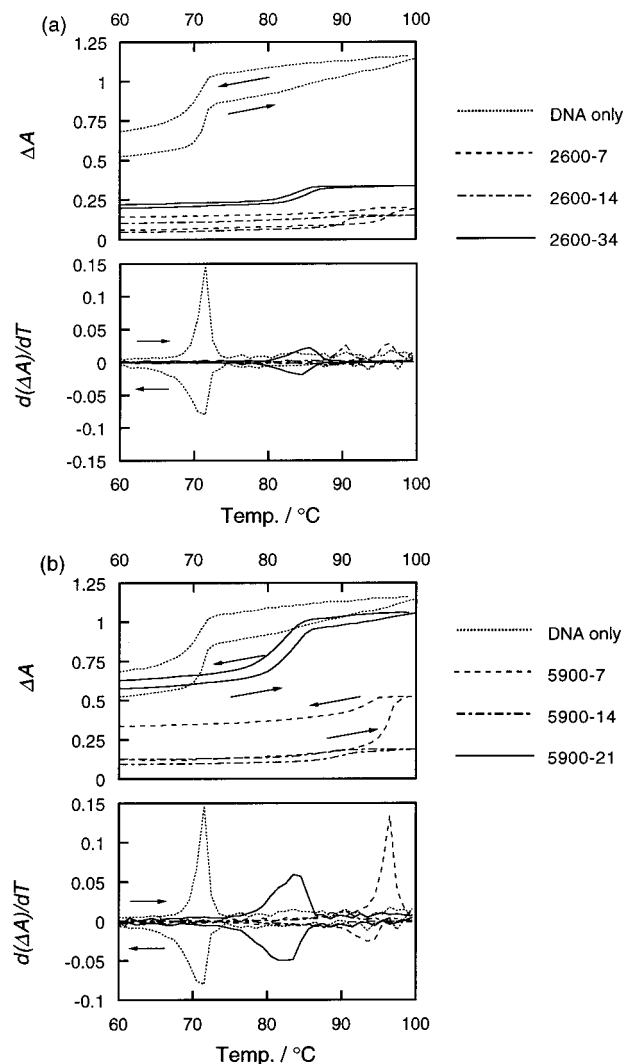


Figure 5. UV- T_m profiles (upper panel) and its derivative curves (lower panel) of the poly(dA)·poly(dT) duplex in the presence ($P/D = 1$) of PLL-g-Dex copolymers having various degrees of grafting and lengths of Dex in buffer II. The differential absorbance ($\Delta A = A_{260} - A_{340}$) was plotted. Poly(dA) and poly(dT) were dissolved in buffer II. To a 1/1 mixture of poly(dA) and poly(dT) was added the copolymer at a P/D ratio of 1. After dilution to a final DNA concentration of $36\text{ (bp) }\mu\text{mol/L}$ with buffer II, the mixtures were heated at $90\text{ }^\circ\text{C}$ for 30 min , cooled to room temperature, and allowed to stand for 16 h . The UV- T_m curves were recorded at 0.2 K/min as heating and cooling rates with a DU-640 spectrometer (Beckman) equipped with a micro-melting temperature apparatus. The differential absorbance ($\Delta A = A_{260} - A_{340}$) was used to correct for background variation. First-derivative curves were obtained from absorbance data.

strand transition at $72\text{ }^\circ\text{C}$ in PBS. In the presence of the comb-type copolymers either with lower-molecular weight Dex, PLL-Dex(2600-7, -14, and -34) (Figure 5a), or with lower Dex content, PLL-Dex(5900-7) (Figure 5b), the complete melting of the double helix was not observed. In the presence of PLL-Dex(5900-7), although partial melting of the double-helical DNA to single strands was observed, reassociation of the ssDNA to form the double helix was incomplete in the cooling process. These results suggest that copolymers having lower degrees of grafting or shorter Dex chains interact firmly with the DNAs and prevent them from reversible duplex-single strand transition. This is in agreement with the previously reported finding that polycations such as polylysine and polyarginine irreversibly interacted with DNA (3, 4). Like PLL homopolymers, the PLL backbone

in the copolymers with a lower degree of grafting is assumed to interact irreversibly with DNA, leading to considerable stabilization of DNA double helices or inhibition of ssDNAs to form double helices. In other words, the Dex chains of these copolymers do not considerably hinder the interaction between DNA and the PLL backbone; however, they effectively increase the solubility of the complex.

As seen in Figure 5, UV absorbance at 260 nm that is attributed to DNA in the mixture is decreased compared with that of free DNA. Similar behavior was previously observed in DNA/PLL mixtures (24), although significant turbidity was detected in this case. It seems that the polycationic backbone triggers a conformational change of the DNA to a dense globular structure (25–27), leading to a loss in UV absorption. However, we believe that further study on the assembly process between DNA and these copolymers is needed to understand the exact nature and behavior of the resulting complex.

In contrast to the results obtained with the graft copolymers with lower degrees of grafting, the double-helical DNA undergoes complete melting and reassociation when associated with the graft copolymer [PLL–Dex(5900–21)] with a higher degree of grafting (Figure 5b). The T_m of the dsDNA increased by more than 15 °C in the presence of an electrostatically equivalent amount ([amino group]_{polymer}/[phosphate group]_{DNA} = 1) of PLL–Dex(5900–21). It can be concluded that PLL–Dex(5900–21) is capable of thermally stabilizing the poly(dA)·poly(dT) double helices without interfering with their interpolynucleotide recognition and assembly. The abundant Dex graft chains (90 wt %) in PLL–Dex(5900–21) may prevent close association of the DNA to the PLL backbone, leading to weak PLL–DNA interactions. This weak interaction still allows the copolymer to thermally stabilize the DNA double helices possibly by reducing the electrostatic repulsion between negatively charged DNA strands. PLL–Dex(2600–34), for which the degree of grafting is higher than that of PLL–Dex(5900–21), does not allow the DNA to melt. Thus, both the degree of grafting and the length of graft chains are parameters by which we could regulate reversible melting of dsDNA.

Stabilizing Effect of PLL–Dex(5900–21) on the Poly(dA)·Poly(dT) Double Helix. The stabilizing effect of the graft copolymer, PLL–Dex(5900–21), having a higher Dex content was further evaluated by changing the polymer/DNA ratio. Figure 6 shows the copolymer/DNA ratio ([amino group]_{polymer}/[phosphate group]_{DNA} = P/D) effects on the melting of the poly(dA)·poly(dT) double helix in the presence of PLL–Dex(5900–21). The melting temperature of the dsDNA gradually increases with an increasing P/D ratio. It is notable that an excess amount of the copolymer did not affect the reversibility of the DNA melting and reassociation. Since the ionic strength of the media is a factor that affects interpolyion interaction, we assessed the melting behavior of the double helix in a low-salt medium. Figure 7 shows the melting profiles of poly(dA)·poly(dT) duplex in 1/100 diluted buffer I (0.1 mM PBS with 1.5 mM NaCl). Under these conditions, poly(dA)·poly(dT) alone melted at 48 °C and did not exhibit obvious reassociation in the cooling process, indicating a considerably slower rate in double-helix formation. Upon addition of the copolymer, the melting temperatures significantly increased and double-helix reassociation also became more prominent. We concluded that the copolymer permits reassociation of DNA even under low-ionic strength conditions and promotes annealing of dsDNA. In the presence of a small amount of the copolymer, the DNA T_m surpassed 45 °C,

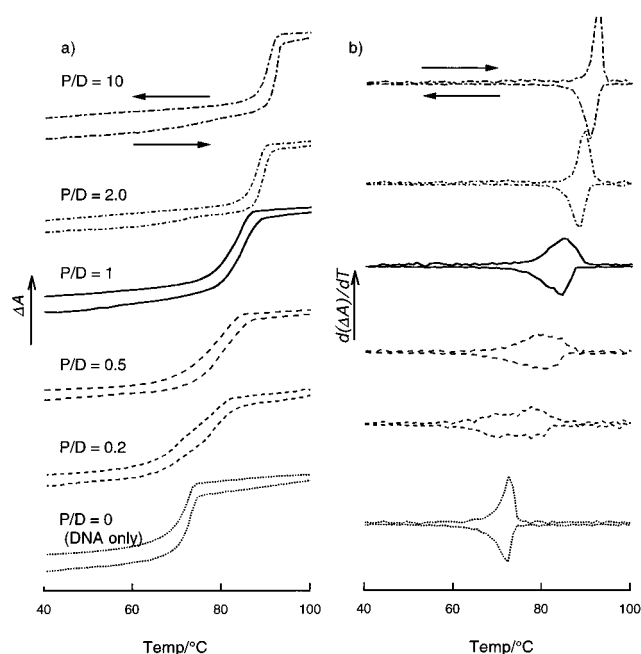


Figure 6. P/D ratio dependency of thermal dissociation and reassociation of poly(dA)·poly(dT) in the presence of PLL–Dex(5900–21) in buffer II: (a) change in absorbance at 260 nm and (b) first-derivative curves. The measurements were performed according to the same procedure described in Figure 5, except using different P/D ratios.

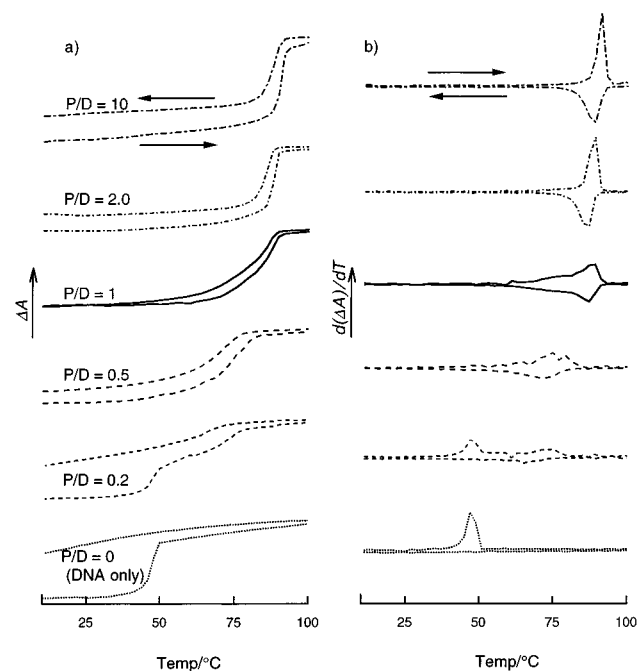


Figure 7. P/D ratio dependency of dissociation and reassociation of poly(dA)·poly(dT) in the presence of PLL–Dex(5900–21) in 1/100 diluted PBS: (a) change in absorbance at 260 nm and (b) first-derivative curves. The measurements were performed according to the same procedure as that described in Figure 6, except that the 1/100 diluted buffer II was used in these experiments.

leading to the same T_m observed in undiluted buffer I. The T_m value reached a plateau at a P/D ratio of 1, supporting the assumption that the graft copolymer and DNA form a stable association in buffer I. Further evidence supporting stable graft copolymer association is shown in Figures 6 and 7, where poly(dA)·poly(dT) melted in a bimodal manner at a P/D ratio of <1 , indicating a dissociation rate of the copolymer from DNA

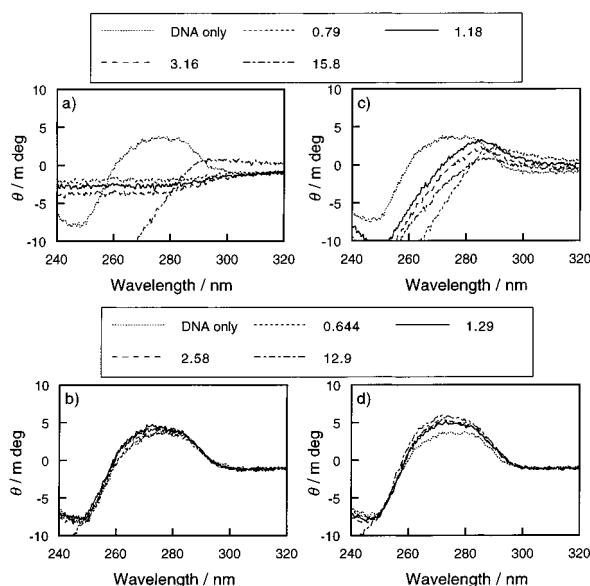


Figure 8. Circular dichroism spectra of calf thymus DNA in the presence of varying amounts of PLL (a and c) or PLL-Dex(5900-21) (b and d). To 1 mL of 100 $\mu\text{g/mL}$ calf thymus DNA in Dulbecco's PBS (a and b) or 1/100 diluted PBS (c and d) was added the appropriate amount of polymers from a 10 mg/mL stock solution at room temperature. The mixtures were diluted to 2.5 mL, followed by CD measurement at room temperature (ca. 20 $^{\circ}\text{C}$). The P/D ratios are indicated above the graphs.

considerably slower than that of the dsDNA to ssDNAs. Oligomeric cations, such as spermine, are reported to elevate the T_m of poly(dA)·poly(dT) in low-ionic strength media (21). Spermine, however, failed to stabilize the duplex in physiological saline (17), indicating a weak association to the duplex. In contrast to spermine, the graft copolymer stably associates with the duplex, leading to T_m elevation in high- and low-salt media.

The graft copolymer, PLL-Dex(5900-21), was previously reported to be an effective stabilizer of triple-helical DNA of poly(dA)·2poly(dT) (17), which is unstable under physiological conditions (19).

The Grafting Copolymer PLL-Dex(5900-21) Does Not Change the CD Signal of Duplex DNA. The unique effect of PLL-Dex(5900-21) in the DNA melting process prompted us to further analyze the polymer's effects on the secondary structure of DNA. Figure 8a shows CD spectra of calf thymus DNA mixed with various amounts of PLL homopolymer or PLL-Dex(5900-21) in buffer I. CD signals do not change for the DNA/PLL homopolymer mixture at a P/D ratio from 1 to 10 due to precipitation of the complex. Although a transparent solution of the PLL/DNA mixture was obtained at P/D ratios of >15 , the CD signal was considerably different when compared to that of free DNA. The change in CD signals in the PLL/DNA mixtures has been explained by distortion and base tilting of the B conformation (3). X-ray diffraction results, however, indicated that DNA in these complexes remains essentially in the untilted B conformation, characteristic of free DNA in solution (3). The abnormal CD pattern was attributed to micellar or liquid crystal structure in the complex. CD signals observed at a wavelength of >300 nm indicated the formation of chiral aggregates (28–31). On the other hand, the graft copolymer does not induce a significant change in CD signals as shown in Figure 8b, suggesting only a slight effect on the DNA secondary structure. Similar results were also obtained even in the presence of large excesses of the copolymers. The CD measurements were also carried out in 1/100 diluted buffer I.

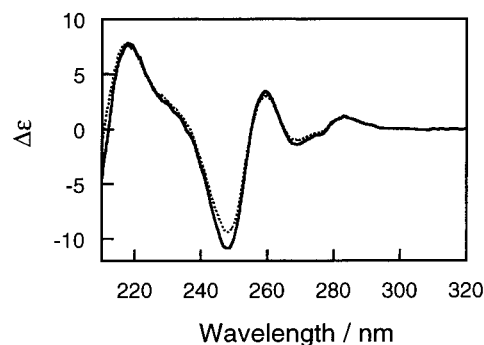


Figure 9. Circular dichroism spectra of poly(dA)·poly(dT) in the absence (dotted line) or presence (solid line) of PLL-Dex(5900-21) ($P/D = 2$). The samples were prepared according to the same procedure as that described in Figure 5. The spectra were recorded on a Jasco J-600 apparatus using a 1 cm long quartz cell with 20 nm/min as a scanning speed, 0.1 nm/point as a wavelength step, and 1 s/point as a time step. Each spectrum shown was the average of three scans and has been smoothed. The CD signal attributed to PLL-g-Dex was negligible under these conditions.

Although the PLL/DNA mixture was a transparent solution, probably due to a lower salting out effect, modified CD signals were recorded at all P/D ratios as shown in Figure 8c. The copolymer did not induce a significant change in CD signals even in low-ionic strength media (Figure 8d), in which ionic interactions should be significantly enhanced.

The CD study was further carried out with the poly(dA)·poly(dT) duplex as shown in Figure 9. Results similar to those obtained with calf thymus DNA were observed; namely, PLL-Dex(5900-21) addition did not significantly change the CD signals of poly(dA)·poly(dT). It was confirmed that the graft copolymers with higher Dex content did not change the secondary structure of DNA double helices, while it elevated the T_m .

Gel Electrophoresis of the DNA/Copolymer Mixture. The stability of the complex between DNA and the graft copolymer, PLL-Dex(5900-21), was assessed by gel electrophoresis of plasmid DNA (6.8 kbp) or a 20-mer oligodeoxynucleotide (ODN). As shown in Figure 10, migration of the plasmid DNA slowed as the P/D ratio increased. The graft copolymer completely inhibited migration of the plasmid DNA into the agarose gel at a nearly electrostatically equivalent point. Since the molecular weight of polyelectrolytes should be a factor which determines the stability of its IPECs, we then evaluated the complex of PLL-Dex(5900-21) with oligonucleotides. The migration of a 20-mer ODN (Figure 11A), which bears 19 phosphate anions in a molecule, into a 15% polyacrylamide gel was also impeded by the copolymer at a P/D ratio of 1 as shown in Figure 11B. PLL-Dex(5900-21) forms a stoichiometric complex even with a 20-mer ODN which bears considerably fewer negative charges than the plasmid DNA. Although the interaction between PLL-Dex(5900-21) and DNA seemed to be weaker than the interaction between the PLL homopolymer and the copolymers with a lower degree of grafting or length, it retained an ability to form a relatively stable complex with DNA.

The property of the complex was further estimated by a gel electrophoresis assay where an excess of thymus DNA (t-DNA) was added to the preformed complex. The ODN was completely and rapidly released from the complex by the addition of excess t-DNA (Figure 11C), while slight dissociation was observed with the PLL/ODN mixture (data not shown). Thus, PLL-Dex(5900-21)

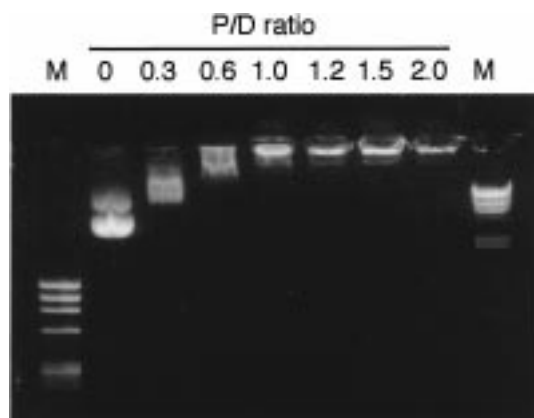


Figure 10. Analysis of complex formation between plasmid DNA (pSV) and PLL-Dex(5900-21) by agarose gel electrophoresis. The increasing amount of the copolymer was added to 3 μ g of pSV plasmid (6.8 kbp) in PBS from a stock solution. The mixture was diluted with PBS to a final plasmid concentration of 43.4 μ g/mL, followed by incubation for 24 h at room temperature. The mixture was then analyzed on a 0.8% agarose gel in Tris-acetate buffer (40 mM, pH 8.0) containing 1 mM EDTA. The plasmid was finally stained with ethidium bromide to visualize it. M marks the columns with low- and high-molecular weight markers.

stably retains the ODN but allows rapid exchange between DNA molecules. This unique behavior of the copolymer seems to be related to the reversible dissociation and association of the DNA duplex and triplex. The copolymer thus allows DNA molecules in the complex to recognize and hybridize to each other.

CONCLUSION

Although the graft copolymer forms a soluble complex with DNA, physicochemical properties of DNA in the complex were significantly changed with the degree of grafting and length of graft chains. Graft copolymers with lower degrees of grafting firmly interacted with DNA, and probably condensed DNA into a globular conformation. This phenomenon was supported by a fluorescent dye exclusion assay using ethidium bromide and fluorescence microscopic observation of giant T4 DNA (data not shown). However, graft copolymers with higher degrees of grafting increased the T_m of the poly-(dA)·poly(dT) duplex by nearly 20 °C without affecting the reversible dissociation and reassociation of dsDNA. DNA associated with graft copolymers considerably accelerated hybridization of DNA strands in low-ionic strength media. The copolymers with higher degrees of grafting did not induce severe conformational changes of DNAs; furthermore, they still maintained the ability to form stoichiometric complexes with ODN. Thus, it can be concluded that grafting hydrophilic chains to PLL allows the regulation of the physicochemical properties of DNA-PLL copolymer complexes.

Several conjugates between polycations and biological ligands have been examined as carriers of DNA, including genes and ODNs. DNA delivery by these carriers, however, lacks high efficiency. To improve DNA delivery using such conjugates, it is crucial to understand the structure-function relationships of DNA carriers and their complexes with DNA. Regulation of physicochemical properties and assembling structures of DNA-carrier complexes with graft copolymers may provide new knowledge for effective DNA delivery and may lead to remarkable progress in this field. We have recently found that the PLL graft copolymers with cell-specific polysaccharide chains permits cell-specific delivery and expression of a

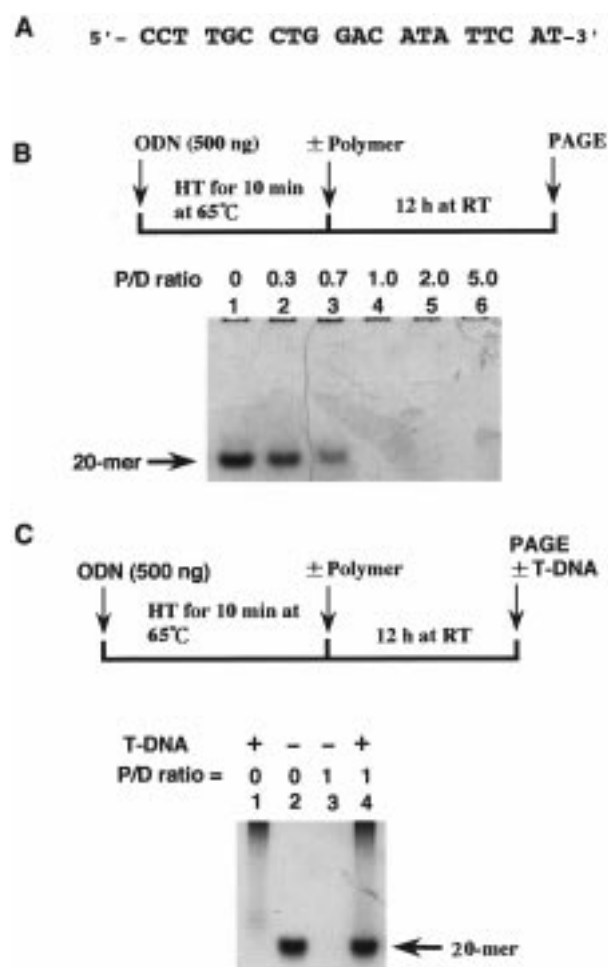


Figure 11. Analysis of complex formation between oligodeoxynucleotide and PLL-Dex(5900-21) by polyacrylamide gel electrophoresis. (A) Nucleotide sequence of the 20-mer oligonucleotide (ODN). (B) Analysis of polymer-ODN complex formation. Polymer-DNA complex formation was analyzed according to the protocol shown above the gel and described in details in Materials and Methods. The ODN was heat-treated (HT) and incubated with (lanes 2–5) or without (lane 1) PLL-Dex(5900-21) at the indicated *P/D* ratio for 12 h at room temperature (RT). The mixtures were then separated by electrophoresis on a 15% polyacrylamide gel, and the gel was stained with Stain-all. An arrow indicates the position of the 20-mer ODN. (C) Competition assay. The ODN was incubated with (+) or without (–) PLL-Dex(5900-21) at a *P/D* ratio of 1. Just before the gel electrophoretic analysis, thymus DNA (T-DNA) was added (lane 4). T-DNA alone was applied in lane 1. The position of the ODN is indicated by an arrow.

foreign gene in vivo (22). Furthermore, these copolymers may have several applications in which internucleotide recognition and/or supramolecular formation are involved.

ACKNOWLEDGMENT

We are grateful to Dr. Horst von Recum for his valuable discussion. This work was supported in part by a grant-in-aid (09750967) for scientific research from the Ministry of Education, Science, Culture, and Sports of Japan.

LITERATURE CITED

- (1) Michaels, A. S., and Miekka, R. G. (1961) Polycation-polyanion complexes: Preparation and properties of poly-(vinylbenzyltrimethylammonium) poly-(styrenesulfonate). *J. Phys. Chem.* 65, 1765–1773.

- (2) Michaels, A. S. (1965) Polyelectrolyte complexes. *Ind. Eng. Chem.* 57, 32–40.
- (3) von Hippel, P. H., and McGhee, J. D. (1972) DNA-protein interactions. *Annu. Rev. Biochem.* 41, 230–300.
- (4) Tsuboi, M. (1967) Helical complexes of poly-L-lysine and nucleic acids. In *Conformation of Biopolymers* (G. N. Ramachandran, Ed.) Vol. II, pp 689–702, Academic Press, New York.
- (5) Markley, L. L., Bixler, H. J., and Cross, R. A. (1968) Utilization of polyelectrolyte complexes in biology and medicine. *J. Biomed. Mater. Res.* 2, 145–155.
- (6) Kataoka, K., Akaike, T., Sakurai, Y., and Tsuruta, T. (1978) Effect of charge and molecular structure of polyion complexes on the morphology of adherent blood platelets. *Makromol. Chem.* 179, 1121–1124.
- (7) Kataoka, K., Maeda, M., Nishimura, T., Nitadori, Y., and Tsuruta, T. (1980) Estimation of cell adhesion on polymer surfaces with the use of "column-method". *J. Biomed. Mater. Res.* 14, 817–823.
- (8) Kataoka, K., Tsuruta, T., Akaike, T., and Sakurai, Y. (1980) Biomedical behavior of synthetic polyion complexes toward blood platelets. *Makromol. Chem.* 181, 1363–1373.
- (9) Kohjiya, S., Maeda, K., and Yamashita, S. (1991) Immobilization of catalase by polyion complex. *J. Appl. Polym. Sci.* 43, 2219–2222.
- (10) Lim, F., and Sun, A. M. (1980) Microencapsulated islets as bioartificial endocrine pancreas. *Science* 210, 908–910.
- (11) O'Shea, G. M., Goosen, M. F. A., and Sun, A. M. (1984) Prolonged survival of transplanted islets of Langerhans encapsulated in a biocompatible membrane. *Biochim. Biophys. Acta* 804, 133–136.
- (12) O'Shea, G. M., and Sun, A. M. (1986) Encapsulation of rat islets of Langerhans prolongs xenograft survival in diabetic mice. *Diabetes* 35, 943–946.
- (13) Kabanov, A. V., and Kabanov, V. A. (1995) DNA complexes with polycations for the delivery of genetic material into cells. *Bioconjugate Chem.* 6, 7–20.
- (14) Wolfert, M. A., Schacht, E. H., Toncheva, V., Ulbrich, K., Nazarova, O., and Seymour, L. W. (1996) Characterization of vectors for gene therapy formed by self-assembly of DNA with synthetic block copolymers. *Hum. Gen. Ther.* 7, 2123–2133.
- (15) Harada, A., and Kataoka, K. (1995) Formation of polyion complex micelles in an aqueous medium from a pair of oppositely-charged block copolymers with poly(ethylene glycol) segments. *Macromolecules* 28, 5294–5299.
- (16) Kataoka, K., Togawa, H., and Katayose, S. (1996) Spontaneous formation of polyion complex micelles with narrow distribution from antisense oligonucleotide and cationic block copolymer in physiological saline. *Macromolecules* 29, 8556–8557.
- (17) Maruyama, A., Katoh, M., Ishihara, T., and Akaike, T. (1997) Comb-type polycations effectively stabilize DNA triplex. *Bioconjugate Chem.* 8, 3–6.
- (18) Sawhney, A. A., and Hubbell, J. A. (1992) Poly(ethylene oxide)-graft-poly(L-lysine) copolymers to enhance the biocompatibility of poly(L-lysine)-alginate microcapsule membranes. *Biomaterials* 13, 863–879.
- (19) Frank-Kamenetskii, M. D., and Mirkin, S. M. (1995) Triplex DNA structures. *Annu. Rev. Biochem.* 64, 64–95.
- (20) Maruyama, A., Ishihara, T., Kim, J., Kim, S. W., and Akaike, T. (1997) A nanoparticle DNA carrier with poly(L-lysine) grafted polysaccharide copolymer and poly(D,L-lactic acid). *Bioconjugate Chem.* 8, 735–742.
- (21) Thomas, T., and Thomas, T. (1993) Selectivity of polyamines in triplex DNA stabilization. *J. Biochem.* 32, 14068–14074.
- (22) (a) Takei, Y., Maruyama, A., Kawano, S., Okumura, S., Asayama, S., Nogawa, M., Oshita, M., Hashimoto, M., Makino, Y., Nagai, H., Tsujii, M., Tsuji, S., Nagano, K., Kinoshita, M., Akaike, T., and Hori, M. (1998) Targeted gene delivery to liver sinusoidal endothelial cells by hyaluronan receptor-mediated endocytosis. *J. Clin. Invest.* (submitted for publication). (b) Asayama, S., Nogawa, M., Takei, Y., Akaike, T., and Maruyama, A. (1998) Synthesis of apmopholyte polylysine-graft-hyaluronic acid copolymer as a gene carrier. *Bioconjugate Chem.* (submitted for publication).
- (23) Mascotti, D. P., and Lohman, T. M. (1990) Thermodynamic extent of counterion release upon binding oligolysines to single stranded nucleic acids. *Proc. Natl. Acad. Sci. U.S.A.* 87, 3142–3146.
- (24) Olins, D. E., Olins, A. L., and von Hippel, P. H. (1967) Model nucleoprotein complexes: Studies on the interaction of cationic homopolypeptides with DNA. *J. Mol. Biol.* 24, 157–176.
- (25) Shapiro, J. T., Leng, M., and Felsenfeld, G. (1969) Deoxyribonucleic acid–polylysine-complexes. Structure and nucleotide specificity. *Biochemistry* 8, 3219–3232.
- (26) Minagawa, K., Matsuzawa, Y., Yoshikawa, K., Matsumoto, M., and Doi, M. (1991) Direct observation of the biphasic conformational change of DNA induced by cationic polymers. *FEBS Lett.* 295, 67–69.
- (27) Inoue, S., and Fuke, M. (1970) An electron microscope study of deoxyribonucleoprotamines. *Biochim. Biophys. Acta* 204, 296–303.
- (28) Haynes, M., Garrett, R. A., and Gratzer, W. B. (1970) Structure of nucleic acid-poly base complexes. *Biochemistry* 9, 4410–4416.
- (29) Maestre, M. F., and Reich, C. (1980) Contribution of light scattering to the circular dichroism of deoxyribonucleic acid films, deoxyribonucleic acid–polylysine complexes, and deoxyribonucleic acid particles in ethanolic buffers. *Biochemistry* 19, 5214–5223.
- (30) Keller, D., and Bustamante, C. (1986) Theory of the interaction of light with large inhomogeneous molecular aggregate. II. Psi-type circular dichroism. *J. Chem. Phys.* 84, 2972–2980.
- (31) Kim, M., Ulibarri, L., Keller, D., Maestre, M. F., and Bustamante, C. (1986) The psi-type circular dichroism of large molecular aggregate. III. Calculations. *J. Chem. Phys.* 84, 2981–2989.

BC9701510

TECHNICAL NOTES

Synthesis of Heterobifunctional Poly(ethylene glycol) with a Reducing Monosaccharide Residue at One End

Teruo Nakamura, Yukio Nagasaki, and Kazunori Kataoka*

Department of Materials Science and Technology, Science University of Tokyo, Noda 278, Japan.
Received September 15, 1997; Revised Manuscript Received December 15, 1997

A new synthetic method for a heterobifunctional poly(ethylene glycol) (PEG) having a monosaccharide moiety at one end was created. PEG with a reducing monosaccharide residue at the α -end, which is linked to a defined position of the sugar molecule, could be prepared via the anionic polymerization of ethylene oxide (EO) initiated with a potassium alkolate of a protected monosaccharide such as 1,2;5,6-di-*O*-isopropylidene- β -D-glucopyranose (DIGL), 1,2;3,4-di-*O*-isopropylidene- β -D-galactopyranose (DIGA), and 1,2-*O*-isopropylidene-3,5-*O*-benzylidene- β -D-glucopyranose (IBGL). The resulting PEGs possess the corresponding sugar molecule at the α -chain end and a hydroxyl group at the ω -chain end. The ω -chain end could be converted to several functional groups such as allyl, amino, and hydroxycarbonyl groups in high yield. Such heterobifunctional PEGs possessing a reducing monosaccharide residue at the α -end are one of the promising tools for bioconjugate chemistries.

INTRODUCTION

Poly(ethylene glycol) (PEG) is a polymer possessing peculiar physicochemical properties such as high water solubility and flexibility. In addition, its bioinert characteristic increases an opportunity for biomedical applications. Chemical modification of biologically active compounds with PEG, designated PEGylation, led to alteration of their pharmacokinetics (1). As we reported previously, polymeric micelles, composed of PEG–poly(peptide) block copolymers, behave as long-circulating vehicles of antitumor agents in vivo (2). The hydrophilic character of the PEG outer shell along with the small size of the micelle (approximately tens of nanometers) confers a reduced nonspecific uptake to reticuloendothelial systems. By installing homing devices onto the surface of the micelles, we enhanced cellular uptake at a target tissue. Carbohydrates are known as one of the promising candidates for a homing moiety. Recent progress in glycobiology revealed that carbohydrate–protein interaction requires a ligand with well-defined structural compatibility (3). For instance, the permeability of intestinal epithelium to macromolecular substances was improved by installing a reducing glucose residue rather than a nonreducing moiety on them (4). This enhanced permeability may be attributed to receptor-mediated endocytosis via glucose transporters, mediating the facilitative diffusion of glucose, through specific binding with the reducing glucose residue. This result suggests that the designed sugar moieties can be utilized as a targeting device. However, to design a sugar derivative as a homing unit, regiospecific conjugation as mentioned above and spatial arrangement of plural sugar residues are key factors in demonstrating the target function. If an accommodating spacer, such as PEG, can

be introduced at a sugar residue site-specifically, it will provide a superior device for incorporating a potent targeting moiety into the compounds desired to be delivered.

Thus, we report herein a new synthetic route for a heterobifunctional PEG with a reducing monosaccharide residue at one end with a defined position linkage of PEG and the sugar moiety. A functional group was introduced at the other end for further derivatization. Our strategy is to initiate anionic polymerization of ethylene oxide (EO) from the hydroxyl of site-specifically protected sugar molecules.

EXPERIMENTAL PROCEDURES

Polymerization of EO from Protected Sugars. To a solution of 1,2;5,6-di-*O*-isopropylidene- β -D-glucopyranose (DIGL) (5) (260 mg, 1 mmol) in tetrahydrofuran (THF) was added potassium naphthalene (1 mmol) as a THF solution to convert a hydroxyl group at the C-3 position to a potassium salt. EO (115 mmol) was added to the solution via a cooled syringe, and the mixture was stirred in a water bath for 2 days. The reaction mixture was then poured into ice-cold ether (approximately 1 L) to obtain a white precipitate. Polymerizations of EO with the potassium alkolate form of 1,2;3,4-di-*O*-isopropylidene- β -D-galactopyranose (DIGA) (5) and 1,2-*O*-isopropylidene-3,5-*O*-benzylidene- β -D-glucopyranose (IBGL) (6) were carried out in a procedure similar to the DIGL-initiated EO polymerization.

Removal of Protective Groups from the Sugar End Group. PEG with a sugar derivative end group was dissolved in 8:2 (v/v) trifluoroacetic acid–water and allowed to stand for 1 h at room temperature. The reaction solution was diluted with water, and the solvent was removed under reduced pressure followed by lyophilization from water.

* To whom all correspondence should be addressed.

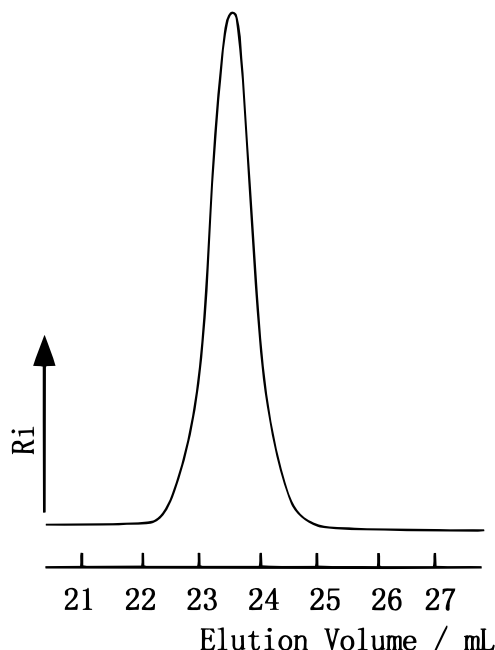


Figure 1. GPC chromatogram of PEG with a DIGL end group (the same sample as run 2 in Table 1).

ω -End Functionalization of Sugar-PEG. After the polymerization of EO (115 mmol) initiated with DIGL (130 mg, 0.5 mmol) in THF, a THF solution of potassium naphthalene (1 mmol) was added to the polymerization mixture and the mixture stirred at room temperature for 30 min. To the solution was added allyl bromide (0.43 mL, 5 mmol) via a syringe, and the mixture was stirred at room temperature for 4 h. Then, the reaction solution was mixed with chloroform and washed with a saturated NaCl aqueous solution several times to eliminate impurities from the PEG derivative. The organic phase was dried with sodium sulfate followed by concentration and then poured into ice-cold ether. The resultant white precipitate was dried in vacuo. Under these reaction conditions, the conversion of the ω -end from potassium alkoxide to the allyl group was almost complete, forming DIGL-PEG-allyl heteroPEG quantitatively.

For the introduction of an amino group at the ω -end, 100 mg of DIGL-PEG-allyl and a 100-fold molar excess of 2-aminoethanethiol hydrochloride were dissolved in degassed water containing 10 wt % sodium chloride and the mixture was stirred in a water bath at 40 °C for 3 h. Purification was carried out in the same way as the sugar-PEG-allyl preparation.

RESULTS AND DISCUSSION

As we reported previously, potassium alkoxide possessing an acetal moiety initiates the polymerization of EO to yield PEG with an acetal group at the α -end without any side reaction (7). If the potassium salt of DIGL acts as an initiator for EO polymerization, then a heterobifunctional PEG with sugar derivative at the α -end should be obtained. To confirm the hypothesis, synthesis of α -DIGL- ω -hydroxyl-PEG was attempted. The precipitate obtained after polymerization was dried in vacuo and was analyzed by gel permeation chromatography (GPC) as shown in Figure 1. The resultant material had a number average molecular weight (M_n) of 4300 with a unimodal and narrow molecular weight distribution (MWD) of 1.09 (M_w/M_n). Results of polymerization using DIGL as an initiator under several conditions are summarized in Table 1. The value of the

Table 1. Results of Polymerization of Ethylene Oxide (EO) Initiated with Potassium Alkoxide of DIGL^a

run	feed [EO] ₀ /[DIGL] ₀	time (d)	M_n		M_w/M_n	yield (%)
			calcd	obsd		
1	60	2	2600	2400	1.05	88
2	115	2	5000	4300	1.09	83
3	230	4	10000	7600	1.17	80

^a M_n and M_w/M_n were determined by GPC.

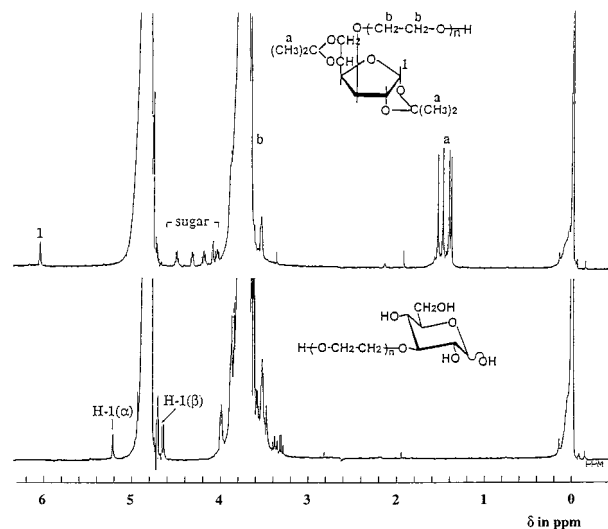


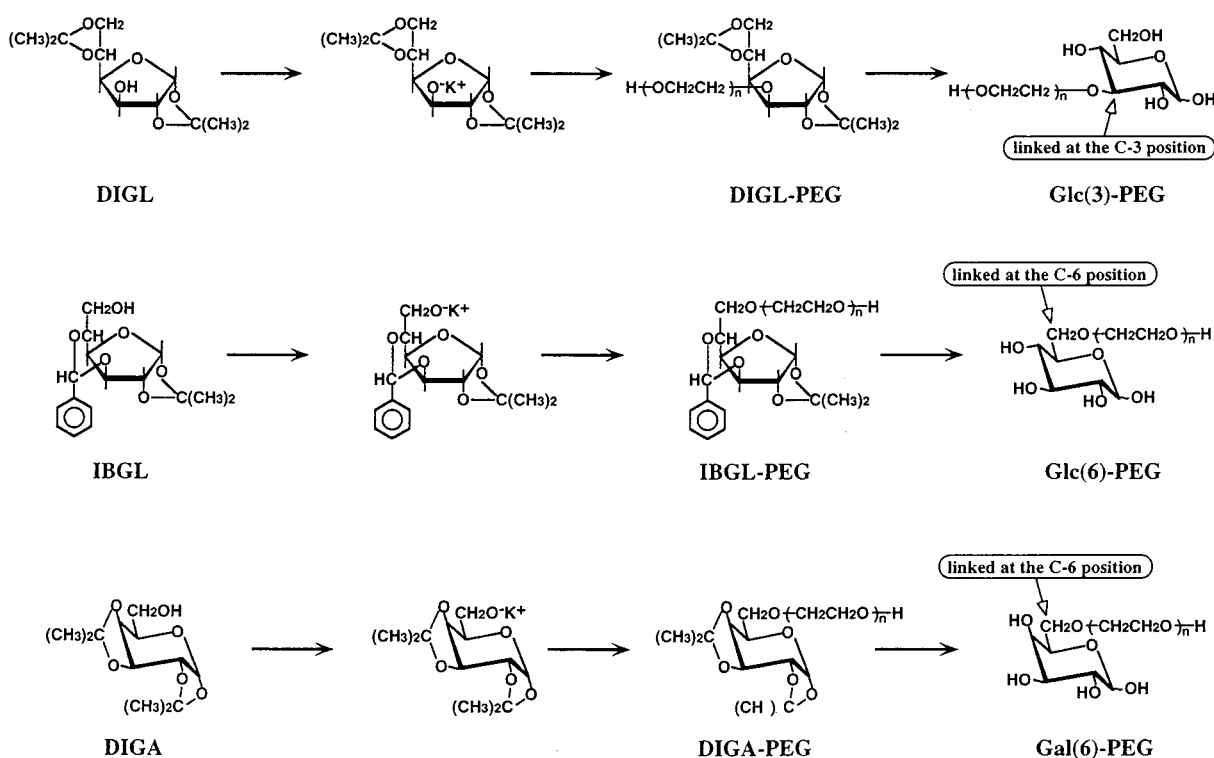
Figure 2. 400 MHz ^1H NMR spectra of PEGs with a sugar residue at the α -end in D_2O . DIGL-PEG (upper) and deprotected product Glc(3)-PEG (lower).

M_n could be controlled by the initial monomer/initiator ratio while retaining a narrow MWD.

Removal of protective groups from the sugar derivative at the PEG chain end was achieved under acidic condition (8). Figure 2 shows the ^1H NMR spectrum of the PEG before and after the TFA treatment. In the lower spectrum, anomeric protons of the reducing glucose residue at 4.66 ppm [H-1(β)] and 5.23 ppm [H-1(α)] were clearly observed at the same time as the disappearance of signals at 1.24, 1.40, 1.45, and 1.52 ppm originating from isopropylidene groups, observed in the upper spectrum, indicating the complete deprotection of the sugar derivative at the end of the polymer. Typical protons of the free sugar residue in Figure 2 (lower) further supported the achievement of cleavage. Worth noticing, revealed by the ^1H NMR spectrum of the deprotected polymer, was an increased ratio of α -anomer to β -anomer of the glucose residue on coupling with PEG. The α/β ratio from the ^1H NMR spectrum of the polymer is estimated to be 45/55; on the other hand, the ratio is 37/63 for intact glucose. The reason for this increased stability of the α -anomer may be the intramolecular interaction of the PEG chain with the sugar moiety.

EO polymerizations with IBGL and DIGA as the initiators were also carried out to obtain sugar-ended PEGs with a different linkage position and a different sugar residue, respectively (see Scheme 1). In the case of IBGL, PEG with a hydroxyl group at both chain ends was produced in approximately 35% as a byproduct due to a trace amount of water in IBGL. The larger M_n of the polymer with hydroxyl ends permitted the isolation of PEG with a sugar end group by gel filtration. In the DIGA case, the polymerization succeeded in producing the targeted compound adequately. Removal of protective groups on the sugar derivative on these polymers was also achieved by TFA treatment to convert them to

Scheme 1



reducing types. On the whole, the polymerization of EO proceeded smoothly with the sugar derivative initiators.

The chances of exploiting the sugar-PEG for drug targeting or surface modification can be improved by ω -end derivatization to an appropriate function. The living nature of the propagating end of the PEG chain allows the introduction of an additional functionality to the ω -end via a successive chemical reaction in the same reaction vessel.

The product obtained after ω -end allylation was analyzed by GPC to reveal the formation of the polymer having an M_n of 6000 with a unimodal MWD ($M_w/M_n = 1.06$) (Figure 3). In the ^1H NMR spectrum of the polymer (Figure 4), signals that can be attributed to DIGL and the allyl group were observed. The signal intensities of methyl protons in the isopropylidene unit, oxymethylene protons in PEG, and methine protons in the allyl end group along with C-1 protons in the sugar moiety were 12, 590, and 2.2, respectively. The M_n of the polymer was then calculated to be 6500, assuming one sugar residue per each polymer chain, which is in good agreement with that from the GPC measurements. This result is consistent with the formation of heterobifunctional PEG possessing sugar and allyl groups at the α - and ω -termini, respectively.

The allyl end can be converted to an amino group or a carboxyl group by radical addition of thiol compounds possessing the corresponding functional group. Although the procedure using a radical initiator at 70 °C in anhydrous DMF was previously reported for preparing amino-ended PEG (9), addition of thiols to the allyl group under mild conditions without any catalyst (10) should be attractive from the standpoint of polymer end-group derivatization. With the procedure described herein, the conversion to the amine end was accomplished in 91% yield, estimated by the intensity ratio of isopropylidene to the allyl unit from the ^1H NMR spectrum (Figure 4), which was proved by the appearance of two methylene protons of the 2-aminoethanethiol unit at 2.85 and 3.20

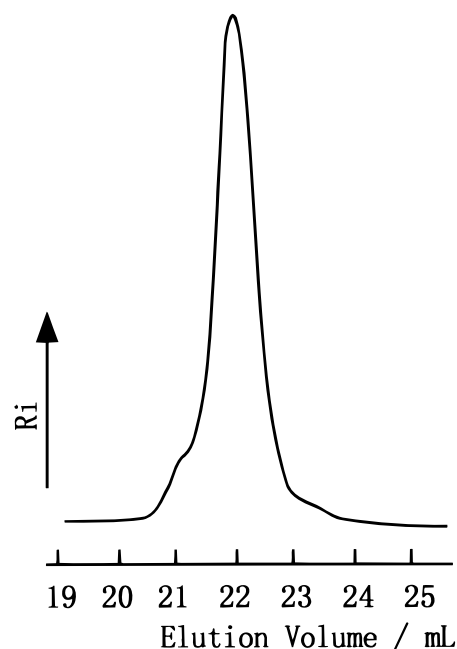


Figure 3. GPC chromatogram of PEG with α -DIGL and α -allyl end groups.

ppm with suitable intensities. On the contrary, amination in pure water was achieved in 26% conversion. Formation of a PEG-solute complex may change the chain conformation in the vicinity of the ω -end, enhancing the possibility of thiol access to the allyl group. Derivatization using mercaptoacetic acid proceeded under the same conditions. From the ^1H NMR spectrum of the resultant polymer, there was no signal to assign to the allyl group, while the methylene proton of the mercaptoacetic acid unit was clearly observed at 3.25 ppm as a singlet. A great excess of mercaptoacetic acid in the reaction solution, however, partially cleaved isopropylidene on the sugar derivative. The ω -end amina-

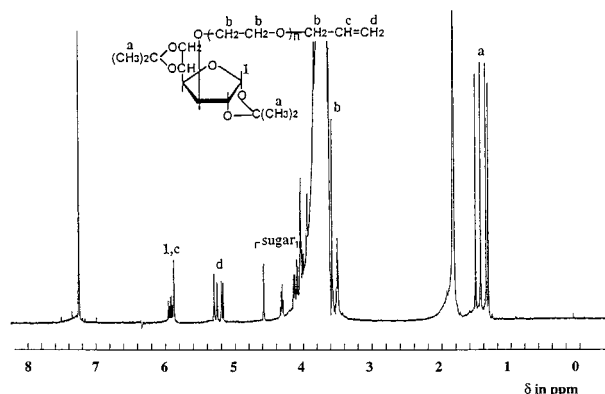


Figure 4. 400 MHz ^1H NMR spectrum of PEG with α -DIGL and ω -allyl end groups in CDCl_3 .

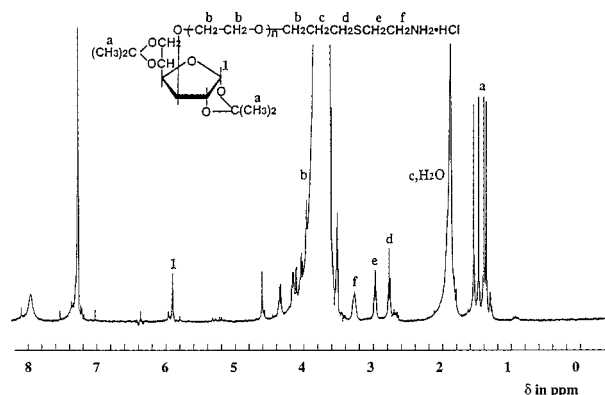


Figure 5. 400 MHz ^1H NMR spectrum of ω -aminated PEG with an α -DIGL end group in CDCl_3 .

tion or hydroxycarbonylation of sugar-PEGs increases their usefulness in the preparation of a variety of bioconjugates.

In conclusion, the successful synthesis of a heterobifunctional PEG with a sugar end via EO polymerization using a sugar derivative as an initiator was demonstrated. The sugar residue was introduced onto PEG without any linkage reagent, and the PEG linking

position on the sugar was defined by protective groups introduced to the hydroxyl on the sugar molecule. The procedure reported here can be applied to an array of carbohydrates to provide a series of sugar-PEGs. Furthermore, ω -end functionalization of the sugar-PEG was attained. These sugar-PEGs may be applicable to preparation of a wide variety of bioconjugates as well as polymeric micelles with many sugar residues on their surface, which should be quite useful in active targeting of drugs and biologically active compounds.

A part of this study was supported by a Grant-in-Aid for Scientific Research on Priority Areas of "Super-Biosystem Constructed by Cognitive Multidimensional Glyco-Molecules" (09240233), The Ministry of Education, Science, Sports and Culture, Japan.

LITERATURE CITED

- (1) Harris, J. M., Ed. (1992) *Poly(ethylene glycol) Chemistry: Biotechnical and Biomedical Applications*, Plenum Press, New York.
- (2) Kataoka, K., Kwon, G. S., Yokoyama, M., Okano, T., and Sakurai, Y. (1993) *J. Controlled Release* 24, 119–132.
- (3) Lee, Y. C., and Lee, R. T. (1995) *Acc. Chem. Res.* 28, 321–327.
- (4) Koyama, Y., Ishikawa, M., Iwamoto, M., and Kojima, S. (1992) *J. Controlled Release* 22, 253–262.
- (5) Whister, R. L., and Wolfrom, M. L., Eds. (1963) *Methods in carbohydrate chemistry*, Vol. 2, pp 318–325, Academic Press, New York.
- (6) Whister, R. L., and Wolfrom, M. L., Eds. (1963) *Methods in carbohydrate chemistry*, Vol. 1, pp 198–201, Academic Press, New York.
- (7) Nagasaki, Y., Kutsuna, T., Iijima, M., Kato, M., Kataoka, K., Kitano, S., and Kadoma, Y. (1995) *Bioconjugate Chem.* 6, 231–233.
- (8) Christensen, J. E., and Goodman, L. (1968) *Carbohydr. Res.* 7, 510–512.
- (9) Cammas, S., Nagasaki, Y., and Kataoka, K. (1995) *Bioconjugate Chem.* 6, 226–230.
- (10) (a) Lee, R. T., and Lee, Y. C. (1974) *Carbohydr. Res.* 37, 193–201. (b) Koyama, Y., Umehara, M., Mizuno, A., Itaba, M., Yasukouchi, T., Natsume, K., Suganaka, A., and Watanabe, K. (1996) *Bioconjugate Chem.* 7, 298–301.

BC970179B

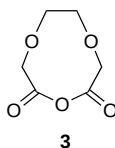
CORRECTIONS

Volume 8, Number 3, May/June 1997.

Gary M. Johnson,* James P. Albarella, and
Christoph Petry

HETEROBIFUNCTIONAL CROSS-LINKERS
CONTAINING 4,9-DIOXA-1,12-DODECANE-
DIAMINE SPACERS

Page 448. Structure **3** in Table 1 was incorrectly
depicted and should be shown as the structure below:



Page 450. The description of the preparation of **1a** should read as follows. *tert*-Butyl-*S*-(4,6-dimethylpyrimidin-2-yl)thiocarbonate (35.3 g, 0.147 mmol, 0.6 equiv) in 1,4-dioxane (100.0 mL) under argon was added dropwise via cannula to a solution of H₂N(CH₂)₃O(CH₂)₄O(CH₂)₃NH₂ (50.0 g, 0.245 mmol) in 1,4-dioxane (200.0 mL) over 60 min.

BC9800062

Published on Web 02/12/1998

COMMUNICATIONS

Cascade Liposomal Triggering: Light-Induced Ca^{2+} Release from Diplasmerylcholine Liposomes Triggers PLA_2 -Catalyzed Hydrolysis and Contents Leakage from DPPC Liposomes

Nathan J. Wymer, Oleg V. Gerasimov, and David H. Thompson*

Department of Chemistry, Purdue University, West Lafayette, Indiana 47907-1393. Received January 7, 1998;
Revised Manuscript Received February 19, 1998

We have previously reported a direct triggering approach [Thompson, D. H., et al. (1996) *Biochim. Biophys. Acta* 1279, 25–34; Gerasimov, O. V., et al. (1997) *Biochim. Biophys. Acta* 1324, 200–214] based on the facile degradation of plasmerylcholine and diplasmerylcholine vinyl ether linkages by either photooxidation or low-pH environments. This report describes a novel, cascade-type triggering technique that utilizes liposome photooxidation and contents release to activate an enzyme capable of destabilizing conventional phosphatidylcholine liposomes. Our application of this concept employs a mixture of two different liposome populations, one composed of synthetic diplasmerylcholine (1,2-dihexadec-1'-enyl-*sn*-glycero-3-phosphocholine, DPPIsCho) containing Ca^{2+} as a signaling agent for phospholipase A_2 (PLA_2) and the second composed of 1,2-dihexadecanoyl-*sn*-glycero-3-phosphocholine (DPPC) with encapsulated calcein as the reporter molecule. Bacteriochlorophyll (BChl)-sensitized photorelease of Ca^{2+} from PLA_2 -resistant DPPIsCho liposomes activates extravesicular PLA_2 , thereby promoting catalyzed DPPC hydrolysis in a secondary triggering reaction, leading to calcein release. BChl/DPPIsCho/DHC/DPPE-PEG5000/ $\text{Ca}^{2+}_{\text{IN}}$ (0.5:85:10:5) liposomes can be phototriggered using 800 nm excitation, resulting in Ca^{2+} release ($t_{50\% \text{ release}} = 15 \text{ min}$) that cocatalyzes the release of calcein ($t_{50\% \text{ release}} = 40 \text{ min}$) from DPPC liposomes (1.5 mM total lipid in DPPIsCho liposomes, 0.18 mM DPPC, 210 μM final Ca^{2+} concentration, 90 units of PLA_2/mL , 50 mM calcein, and 36 μM EDTA). No appreciable calcein release occurs in the absence of either PLA_2 or BChl/DPPIsCho/DHC/DPPE-PEG5000/ Ca_{IN} liposomes. The implications of this cascade triggering technique on drug delivery approaches are briefly discussed.

Triggered release from liposomal carriers has been the subject of numerous studies (1, 2). We have previously reported a triggering approach (3), based on the facile degradation of plasmerylcholine vinyl ether linkages by either photooxidation (3a–d) or low environmental pH (3a,d,e), that utilizes plasma-stable liposomes having long blood circulation times in a murine model (1b) and

efficiently delivers water-soluble drugs to the cytoplasm of target cells (3e). Plasmerylcholines and diplasmerylcholines, however, suffer from higher costs compared with egg or soy lecithins and from liposome processing restrictions due to the involvement of vinyl ether degradation reactions. In an attempt to mitigate these limitations, we investigated a novel, cascade-type triggering technique that utilizes liposome photorelease to activate an enzyme capable of destabilizing conventional phosphatidylcholine liposomes. Inexpensive phosphatidylcholine

* Corresponding author. Fax: (765) 496-2592. E-mail: davethom@chem.purdue.edu.

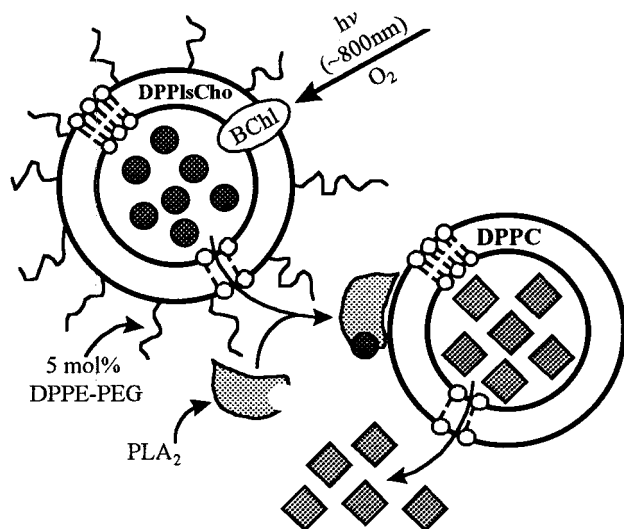


Figure 1. Schematic view of cascade triggering in a binary liposomal system. Calcium ions are shown as shaded circles, and calcein is shown as diamonds. Hydrolyzed/photolyzed lipid molecules are shown as one-tail species, as opposed to intact two-tail molecules.

(lecithin) liposomes are intended to serve as a main payload carrier, while phototriggerable liposomes containing a signaling substance will be ideally present in a small proportion. To demonstrate the feasibility of this approach, we present a system (Figure 1) which employs a mixture of two different populations of liposomes, one composed of synthetic diplasmenylcholine (4) (1,2-hexadec-1'-enyl-*sn*-glycero-3-phosphocholine, DPPIsCho¹) containing calcium ions as a signaling agent and the second composed of 1,2-dihexadecanoyl-*sn*-glycero-3-phosphocholine (DPPC) with encapsulated calcein as the reporter molecule. Photorelease of Ca²⁺ from diplasmenylcholine liposomes, therefore, should activate extravesicular calcium-dependent phospholipase A₂ (PLA₂) and promote catalyzed DPPC hydrolysis in a secondary triggering reaction, leading to calcein release (5).

Bacteriochlorophyll (BChl)-sensitized photooxidation of semisynthetic plasmenylcholine (1-alk-1'-enyl-2-hexadecanoyl-*sn*-glycero-3-phosphocholine) liposomes has previously been used as a phototriggering mechanism (3c,d); however, due to the presence of an *sn*-2 acyl substituent in this phospholipid, liposomes formed from it can also serve as a PLA₂ substrate. DPPIsCho, containing 1'-hexadecenyl ether linkages in both the *sn*-1 and *sn*-2 positions, was chosen to form the photosensitive liposome fraction since its resistance to PLA₂-catalyzed hydrolysis would help us avoid complications that could arise from PLA₂-induced background calcium leakage before illumination. In addition, a protective, steric-stabilization layer of poly(ethylene oxide) was added to the DPPIsCho liposomes, in the form of 5 mol % DPPE-PEG5000, to

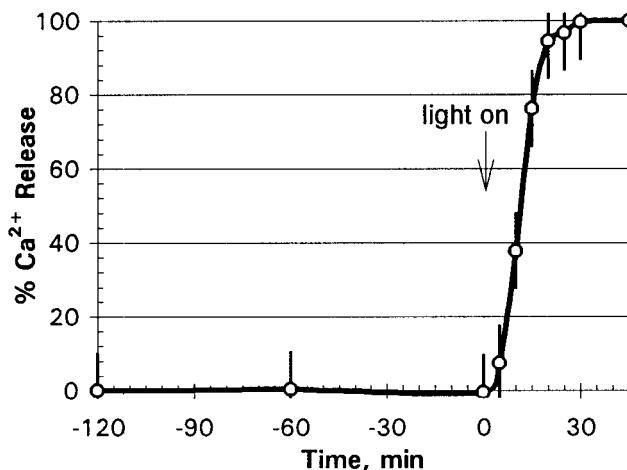


Figure 2. Photorelease of Ca²⁺ from DPPIsCho liposomes. BChl/DPPIsCho/DHC/DPPE-PEG5000 liposomes (0.5:85:10:5 mole ratio, 10 μ mol of total lipid) were prepared by extrusion (9). Briefly, lipids and BChl were dissolved in CHCl₃ in a cryovial and after chloroform removal (N₂ flow followed by ≤ 100 μ m vacuum for ≥ 6 h) were hydrated in 0.1 M CaCl₂ by multiple freeze-thaw cycles and extruded through two stacked 0.1 μ m Poretics polycarbonate filters. The extraliposomal Ca²⁺ was then replaced by 150 mM NaCl on a 1 $\times$ 20 cm Sephadex G50M column. Illumination was carried out with a Spectra Diode Labs SDL820 laser emitting at 800 nm (incident light power of 1 W/cm², 1 cm path length) in an unstoppered 3 mL (1 $\times$ 1 cm) glass cuvette thermostated at 37 $^{\circ}$ C, the contents of which were continuously stirred to aerate the liposome solution. Illumination was ceased after 15 min due to complete photobleaching of BChl. The release of Ca²⁺ was monitored by taking a 100 μ L aliquot and diluting it in 2 mL of 0.2 mM Arsenazo III solution in HBS. The absorbance of the solution at 656 nm was then measured before and after the addition of 1 drop of a 10% Triton X-100 solution. Concentrations used in the experiments presented here were as follows: lipid (total) = 1.2 mM and Ca²⁺ = 120 μ M (bulk concentration when completely released) in HBS.

prevent PLA₂ contact with them (6), since PLA₂ adsorption might, in principle, destabilize the DPPIsCho bilayer and induce contents release even in the absence of lipid hydrolysis (7). Dihydrocholesterol (DHC, 10 mol %) was also included in the DPPIsCho liposome formulation (3e) to further suppress the dark leakage rate of calcium ions (8).

The results shown in Figure 2 indicate that 0.5:85:10:5 (mole ratio) BChl/DPPIsCho/DHC/DPPE-5000/Ca²⁺_{IN} liposomes can be phototriggered using 800 nm excitation, yielding Ca²⁺ release rates that were comparable to those reported for calcein release from semisynthetic plasmalogen liposomes under similar conditions (3c). Subsequent control experiments demonstrated that pure DPPC liposomes loaded with 50 mM calcein are stable at 37 $^{\circ}$ C in the presence of PLA₂ for at least 20 min, yet they rapidly release calcein in the presence of 50 μ M Ca²⁺ (Figure 3). These results suggested that the individual liposomes in mixtures of BChl/DPPIsCho/DHC/DPPE-5000/Ca²⁺_{IN}, DPPC/calcein, and PLA₂ would retain their contents until the solution was illuminated. Liposome-compartmentalized Ca²⁺ and calcein are, in fact, retained until 800 nm irradiation induces the onset of calcein leakage (Figure 4), presumably via a cascade mechanism involving calcium ion photorelease and PLA₂-catalyzed hydrolysis. Control experiments showed that appreciable calcein release from DPPC liposomes was not observed in the absence of either PLA₂ or BChl/DPPIsCho/DHC/DPPE-5000/Ca²⁺_{IN} liposomes.

The two coupled chemical reactions of the cascade triggering mechanism (Figure 1) are DPPIsCho photooxidation and DPPC hydrolysis catalyzed by Ca²⁺-activated

¹ Abbreviations: BChl, bacteriochlorophyll (Porphyrin Products); DHC, (+)-dihydrocholesterol (Aldrich); DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (Avanti Polar Lipids); DPPIsCho, 1,2-dihexadec-1'-enyl-*sn*-glycero-3-phosphocholine; DPPE-PEG5000, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[poly(ethylene glycol) 5000] (Avanti Polar Lipids); EDTA, ethylenediaminetetraacetic acid, disodium salt (Sigma); HBS, HEPES-buffered saline, where [HEPES] = 20 mM and [NaCl] = 150 mM at pH 7.4; PLA₂, calcium-dependent bee venom phospholipase A₂ (Fluka); BChl/DPPIsCho/DHC/DPPE-PEG5000/Ca²⁺_{IN}, BChl/DPPIsCho/DHC/DPPE-PEG5000 liposomes (0.5:85:10:5 mole ratio) with encapsulated CaCl₂.

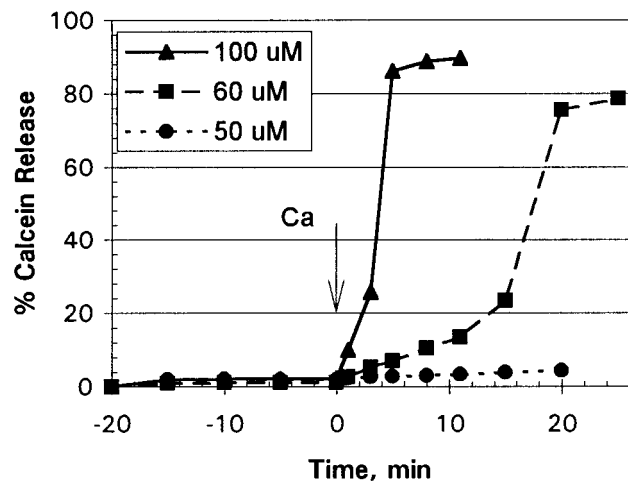
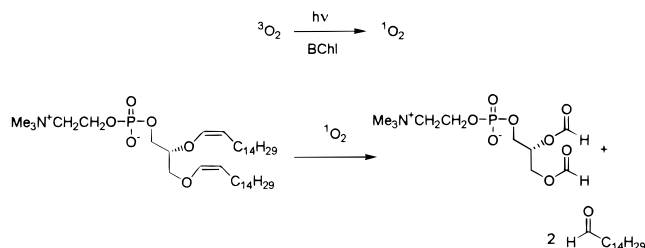


Figure 3. PLA_2 -induced calcein release from DPPC liposomes in the presence of 50 μM EDTA and varying concentrations of Ca^{2+} at 37 $^\circ\text{C}$. The DPPC liposomes were prepared by extrusion (9) (after hydrating dry DPPC in a 50 mM calcein solution), followed by gel filtration on a 1×40 cm Sephadex G50M column, equilibrated with 150 mM NaCl. Calcein release was monitored by measuring the solution luminescence intensity at 520 nm ($\lambda_{\text{ex}} = 475$ nm) (10). Concentrations (in HBS) were as follows: [DPPC] = 120 μM , [PLA_2] = 15 units/mL, [EDTA] = 50 μM , and [Ca^{2+}]_{added} = (●) 50 μM , (■) 60 μM , and (▲) 100 μM , resulting in 0, 10, and 50 μM free Ca^{2+} , respectively.

PLA_2 . BChl-sensitized photooxidation of DPPCsCho, which initiates the entire triggering sequence, is a well-documented reaction for the case of semisynthetic plasmenylcholine (3c,d). By analogy, singlet oxygen-mediated cleavage of the DPPCsCho vinyl ether bonds would produce a water-soluble bisformyl-*sn*-glycerophosphocholine fragment and 2 equiv of the single-chain surfactant, pentadecanal:



Accumulation of these surfactant photolysis products in the liposomal membrane leads to increased membrane permeability and Ca^{2+} release (11). Once the photoreleased Ca^{2+} concentration exceeds the EDTA concentration, PLA_2 activation occurs, leading to the catalytic degradation of DPPC and the production of the single-chain surfactants, lysophosphatidylcholine and hexadecanoic acid, in a subsequent consecutive reaction. DPPC hydrolysis, therefore, increases the liposome bilayer permeability (12) and initiates calcein leakage in a manner analogous to that of photooxidized DPPCsCho liposomes.

It should be noted that bee venom PLA_2 activation requires the presence of bulk calcium ion concentrations in the micromolar range (13). We achieved these relatively high concentrations of photoreleased Ca^{2+} by using concentrated solutions of DPPCsCho liposomes. Therefore, in its present form, the DPPCsCho content required to elicit a detectable cascade triggering response from DPPC liposomes is approximately 8:1; presumably, this may be improved by increasing the Ca^{2+} concentration within the DPPCsCho liposome population.

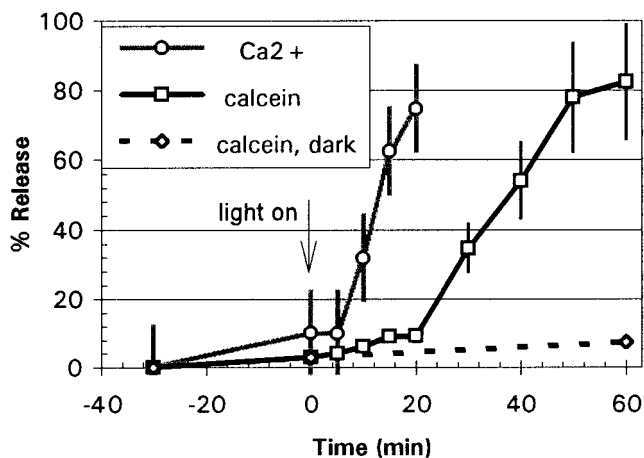


Figure 4. Kinetics of cascade-triggered calcein photorelease in the system comprised of two different liposomal populations described in Figures 2 and 3. Concentrations used were as follows: [BChl/DPPCsCho/DHC/DPE-PEG5000 (0.5:85:10:5 mole ratio)] = 1.5 mM (total lipid in the diplasmenylcholine liposome population), [Ca^{2+}] = 210 μM (total Ca^{2+} concentration), [PLA_2] = 90 units, [EDTA] = 36 μM , and [DPPC] = 0.18 mM.

While we have not yet reached our goal of reducing the amount of DPPCsCho required for photorelease of water-soluble materials from liposomal carriers (i.e. cascade amplification), these results clearly indicate new possibilities for cascade liposomal triggering. Incorporation of novel photochemical and cascade triggering properties into standard liposomal formulations that previously released their contents via passive diffusion is now feasible (14). Although this work focused on PLA_2 -mediated release from DPPC liposomes, in principle, calcium ion photorelease from DPPCsCho liposomes may be used to activate any Ca^{2+} -dependent enzyme or other calcium-requiring agent (15). It is also conceivable that this system could be used with minor modifications (i.e. encapsulation of small phototriggerable, Ca^{2+} -containing DPPCsCho liposomes within larger PLA_2 - and drug-containing phosphatidylcholine liposomes) to assemble a complete autonomous vesicular drug delivery vehicle that is photoresponsive. Structures such as these (vesicles inside vesicles with external diameters of $\sim 1 \mu\text{m}$) have been described recently by Zasadzinski and co-workers (16). Another system, based on lecithin liposomes microencapsulated in much larger particles ($> 100 \mu\text{m}$) of an alginate poly(L-lysine)-containing gel in which contents release rates on a week time scale was modulated by the presence of co-encapsulated PLA_2 , has been reported by Langer et al. (17). These and related enzyme-activated systems (18), in principle, can now be actively triggered using this cascade triggering approach.

ACKNOWLEDGMENT

We thank Dr. Y. Rui for the synthesis of diplasmenylcholine and Mr. J. Boomer for assistance with manuscript preparation. Financial support from the Purdue Research Foundation and INEX Pharmaceuticals, Inc., is gratefully acknowledged.

LITERATURE CITED

- (1) (a) O'Brien, D. F., and Tirrell, D. A. (1993) Photoinduced reorganization of bilayer membranes. In *Bioorganic Photochemistry* (H. Morrison, Ed.) pp 111–167, Wiley, New York. (b) Gerasimov, O. V., Rui, Y., and Thompson, D. H. (1996) Triggered release from liposomes mediated by physically and chemically induced phase transitions. In *Vesicles* (M. Rosoff,

- Ed.) pp 679–746, Marcel Dekker, New York, and references therein. (c) Gerasimov, O. V., Boomer, J. A., Qualls, M., and Thompson, D. H. (1998) Cytosolic drug delivery using pH- and light-sensitive liposomes. *Adv. Drug Delivery Rev.* (in press). (d) A review of other triggerable materials has been reported: Hoffman, A. S. (1995) Intelligent polymers in medicine and biotechnology. *Macromol. Symp.* 98, 645–664.
- (2) (a) Vogel, K., Wang, S., Lee, R. J., Chmielewski, J., and Low, P. S. (1996) Peptide-mediated release of folate-targeted liposome contents from endosomal compartments. *J. Am. Chem. Soc.* 118, 1581–1586. (b) Miller, C. R., Bennett, D. E., Chang, D. Y., and O'Brien, D. F. (1996) Effect of liposomal composition on photoactivated liposome fusion. *Biochemistry* 35, 11782–11790. (c) Armitage, B. A., Bennett, D. E., Lamparski, H. G., and O'Brien, D. F. (1996) Polymerization and domain formation in lipid assemblies. *Adv. Polym. Sci.* 126, 53–84. (d) Song, X., Perlstein, J., and Whitten, D. G. (1995) Photoreactive supramolecular assemblies: aggregation and photoisomerization of azobenzene phospholipids in aqueous bilayers. *J. Am. Chem. Soc.* 117, 7816–7817. (e) Chang, C., Niblack, B., Walker, B., and Bayley, H. (1995) A photogenerated pore-forming protein. *Chem. Biol.* 2, 391–400. (f) Lee, R. J., and Huang, L. (1996) Lipidic vector systems for gene transfer. *J. Biol. Chem.* 271, 8481–8487. (g) Nicol, F., Nir, S., and Szoka, F. C. (1996) Effect of cholesterol and charge on pore formation in bilayer vesicles by a pH-sensitive peptide. *Biophys. J.* 71, 3288–3301. (h) Bailey, A. L., and Cullis, P. R. (1997) Membrane fusion with cationic liposomes: Effects of target membrane lipid composition. *Biochemistry* 36, 1628–1634. (i) Bailey, A. L., Monck, M. A., and Cullis, P. R. (1997) pH-induced destabilization of lipid bilayers by a lipopeptide derived from influenza hemagglutinin. *Biochim. Biophys. Acta* 1342, 232–244. (j) Kono, K., Igawa, T., and Takagishi, T. (1997) Cytoplasmic delivery of calcein mediated by liposomes modified with a pH-sensitive poly(ethylene glycol) derivative. *Biochim. Biophys. Acta* 1325, 143–154.
 - (3) (a) Anderson, V. C., and Thompson, D. H. (1992) Triggered release of hydrophilic agents from plasmalogen liposomes using visible-light or acid. *Biochim. Biophys. Acta* 1109, 33–42. (b) Anderson, V. C., and Thompson, D. H. (1992) Photo-induced morphological changes in plasmalogen liposomes using visible-light. In *Macromolecular Assemblies* (P. Stroeve and A. Balazs, Eds.) ACS Symposium Series 493 pp 154–170, American Chemical Society, Washington, DC. (c) Thompson, D. H., Gerasimov, O. V., Wheeler, J. J., Rui, Y., and Anderson, V. C. (1996) Triggerable plasmalogen liposomes: Improvement of system efficiency. *Biochim. Biophys. Acta* 1279, 25–34. (d) Gerasimov, O. V., Schwan, A., and Thompson, D. H. (1997) Acid-catalyzed plasmenylcholine hydrolysis and its effect on bilayer permeability: a quantitative study. *Biochim. Biophys. Acta* 1324, 200–214. (e) Rui, Y., Wang, S., Low, P. S., and Thompson, D. H., (1998) *J. Am. Chem. Soc.* (submitted for publication).
 - (4) Rui, Y., and Thompson, D. H. (1994) Stereocontrolled synthesis of plasmalogen-type lipids from glyceryl ester precursors. *J. Org. Chem.* 59, 5758–5762.
 - (5) This reaction sequence is analogous to the cascade amplification pathways involving intracellular Ca^{2+} release and calcium-dependent PLA_2 activation in mammalian cell signal transduction cascades [Dennis, E. A. (1994) *J. Biol. Chem.* 269, 13057–13060; Clark, J. D., Schievella, A. R., Nalefski, E. A., and Lin, L. L. (1995) *J. Lipid Mediators Cell Signalling* 12, 83–117].
 - (6) Addition of PEG-conjugated lipids is a common strategy for reducing protein adsorption onto the liposome surface (see ref 14a).
 - (7) It has been reported that PLA_2 isozymes, while lacking PLA_2 activity, can cause leakage of carboxyfluorescein from phosphatidylcholine liposomes [Shimohigashi, Y., et al. (1995) *J. Biochem.* 118, 1037–1044]. This means that PLA_2 can, in some cases, cause leakage simply by disturbing bilayer structure upon contact. We found that the presence of PEG reduced dark leakage of Ca^{2+} from DPPHsCho liposomes but did not affect their photosensitivity.
 - (8) EDTA was present in the solution (~0.25 equiv of EDTA/equiv of Ca^{2+}) to chelate any Ca^{2+} that may have leaked passively before the illumination started. EDTA was included as a precautionary measure even though appreciable dark calcium leakage was not observed from any batches of DPPHsCho/DHC/DPPE-PEG5000/ Ca^{2+} liposomes (cf. Figure 2). Its presence did not affect the performance of either the phototriggering or PLA_2 hydrolysis reactions once the free (i.e. photoreleased) calcium ion concentration exceeded the EDTA concentration.
 - (9) Mayer, L. D., Hope, M. J., and Cullis, P. R. (1986) Vesicles of variable sizes produced by a rapid extrusion procedure. *Biochim. Biophys. Acta* 858, 161–168.
 - (10) Allen, T. M., and Cleland, L. G. (1980) Serum-induced leakage of liposome contents. *Biochim. Biophys. Acta* 597, 418–426.
 - (11) The details of this release mechanism and its quantitative correlation to the accumulation of single chain hydrolysis fragments within the liposome bilayer are described in ref 3d. In particular, it was shown that 3–5 mol % plasmenylcholine cleavage was sufficient to promote liposomal contents release within minutes in the absence of cholesterol.
 - (12) (a) Fugman, D. A., Shirai, K., Jackson, R. L., and Johnson, J. D. (1984) Lipoprotein lipase- and phospholipase A_2 -catalyzed hydrolysis of phospholipid vesicles with an encapsulated fluorescent dye. Effects of apolipoproteins. *Biochim. Biophys. Acta* 795, 191–195. (b) Eriksson, O., and Saris, N. E. (1989) The phospholipase A_2 -induced increase in the permeability of phospholipid membranes to Ca^{2+} and H^+ ions. *Biol. Chem. Hoppe Seyler* 370, 1315–1320. (c) Kaszuba, M., and Hunt, G. R. A. (1990) ^{31}P - and ^1H -NMR investigations of the effect of n-alcohols on the hydrolysis by phospholipase A_2 of phospholipid vesicular membranes. *Biochim. Biophys. Acta* 1030, 88–93.
 - (13) When completely released, Ca^{2+} concentrations were typically 150–200 μM .
 - (14) Practical methods for active liposome triggering are still sought for site-specific drug and gene delivery applications since the passive drug release-exchange mechanisms involved in standard liposome formulations (14a–e) deliver water-soluble agents inefficiently. (a) (1995) Lasic, D., and Martin, F., Eds. *Stealth Liposomes*, CRC Press Inc., Boca Raton, FL. (b) Gregoriadis, G. (1995) Engineering liposomes for drug delivery: progress and problems. *Trends Biotechnol.* 13, 527–537. (c) Chonn, A., and Cullis, P. (1995) Recent advances in liposomal drug-delivery systems. *Curr. Opin. Biotechnol.* 6, 698–708. (d) Allen, T. M. (1996) Liposomal drug delivery. *Curr. Opin. Colloid Interface Sci.* 1, 645–651. (e) Lasic, D. D., and Papahadjopoulos, D. (1996) Liposomes and biopolymers in drug and gene delivery. *Curr. Opin. Solid State Mater. Sci.* 1, 392–400.
 - (15) Calcium-containing photosensitive plasmenylcholine liposomes are a higher payload analogue of stoichiometric caged calcium reagents.
 - (16) Walker, S. A., Kennedy, M. T., and Zasadzinski, J. A. (1997) Encapsulation of bilayer vesicles by self-assembly. *Nature* 387, 61–64.
 - (17) Kibat, P. G., Igari, Y., Wheatley, M. A., Eisen, H. N., and Langer, R. (1990) Enzymatically activated microencapsulated liposomes can provide pulsatile drug release. *FASEB J.* 4, 2533–2539.
 - (18) (a) PLA_2 has also been used to activate the degradation of phospholipid tubules: Carlson, P. A., Gelb, M. H., and Yager, P. (1997) Zero-order interfacial enzymatic degradation of phospholipid tubules. *Biophys. J.* 73, 230–238. (b) Menger, F. M., and Johnston, D. E. (1991) Specific enzyme-induced decapsulation. *J. Am. Chem. Soc.* 113, 5467–5468. (c) Russo, M. J., Bayley, H., and Toner, M. (1997) Reversible permeabilization of plasma membranes with an engineered switchable pore. *Nat. Biotechnol.* 15, 278–282. (d) Pinnaduwage, P., and Huang, L. (1988) A homogeneous, liposome-based signal amplification for assays involving enzymes. *Clin. Chem.* 34, 268–272. (e) Liu, D. (1994) Enzyme-controlled release with liposomes. *J. Liposome Res.* 4, 413–425.

ARTICLES

Application of Cystamine and *N,N'*-Bis(glycyl)cystamine as Linkers in Polysaccharide–Protein Conjugation

Odo de Weers,[†] Michel Beurret, Leo van Buren, Lukas A. Oomen, Jan T. Poolman,[‡] and Peter Hoogerhout*

National Institute of Public Health and the Environment (RIVM), Laboratory of Vaccine Development and Immune Mechanisms, P.O. Box 1, 3720 BA Bilthoven, The Netherlands. Received October 31, 1997; Revised Manuscript Received February 10, 1998

Pneumococcal polysaccharide type 6B, 14, or 23F (35–70 kDa) was activated with cyanogen bromide and modified with cystamine. After reduction of the spacer, the thiol-containing (i.e. cysteamine-modified) polysaccharide obtained was added in a 5–10-fold molar excess to bromoacetylated tetanus toxoid to give thioether-linked polysaccharide–protein conjugates in a yield of 10–20%. This approach failed for preparing a type 19F polysaccharide–protein conjugate, possibly due to intramolecular elimination of cysteamine from the reduced 19F polysaccharide. When *N,N'*-bis(glycyl)cystamine was introduced as a spacer molecule, the elimination of the reduced spacer was suppressed, thus allowing preparation of a 19F polysaccharide–tetanus toxoid conjugate (15%).

INTRODUCTION

Many bacterial capsular polysaccharides (PS¹), for example, from *Haemophilus influenzae* type b, *Neisseria meningitidis*, and *Streptococcus pneumoniae*, can be used as vaccines. However, PS vaccines are poorly immunogenic in infants due to their T cell-independent nature as antigens. Conjugation of the PS to proteins (1–5) gives T cell-dependent antigens which on immunization induce anti-polysaccharide immunoglobulin G responses and immunological memory. This approach has been applied successfully for the preparation of protective vaccines, in particular against *H. influenzae* type b (6–9) and, more recently, against *S. pneumoniae* (7, 10–13).

Conjugation of PS and a protein can be performed directly, for example, by reductive amination of a terminal reducing sugar or of periodate-activated PS (14–16),

or indirectly, by first modifying activated PS with spacers such as cystamine, diaminobutane (5, 17), or 6-amino-hexanoic acid (18). Cystamine has been used for introduction of a masked thiol group into carboxylic acid-containing carbohydrates by carbodiimide-mediated coupling reactions (19–21). The compound has also been coupled to proteins (22) and synthetic oligonucleotides (23).

We have incorporated cystamine into (carboxylic acid-devoid) polysaccharides after classical activation with cyanogen bromide (24). In this paper, we illustrate this approach by preparation of cystamine-modified PS of pneumococcal types 6B,² 14, and 23F (cf. Scheme 1 for the PS structures). After reduction of the spacer, the thiol-PS obtained was coupled to bromoacetylated tetanus toxoid (BrAc-TTd) to give thioether conjugates. Furthermore, it is reported that this approach failed in preparing a type 19F PS–TTd conjugate but that this problem can be solved by using *N,N'*-bis(glycyl)cystamine as a spacer molecule.

EXPERIMENTAL PROCEDURES

Tetanus toxoid was obtained from the Laboratory of Vaccine Production of RIVM. The material met the requirements of the World Health Organization for human administration. Purified pneumococcal polysaccharides were purchased from the American Type Culture Collection (Rockville, MD) or obtained from the Laboratory of Product and Process Development of RIVM. The polysaccharides met the requirements of the European Pharmacopoeia. Solvents, reagents, and standards were purchased and were of analytical or higher grade, and used without purification.

² The Danish nomenclature for pneumococcal serotype classification is being used.

* To whom correspondence should be addressed. Telephone: +31 30 274 3327. Fax: +31 30 274 4429. E-mail: Peter.Hoogerhout@rivm.nl.

[†] Present address: Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Plesmanlaan 125, 1066 CX Amsterdam, The Netherlands.

[‡] Present address: SmithKline-Beecham, 89 rue de l'Institut, Rixensart, B 1330 Belgium.

¹ Abbreviations: ATCC, American Type Culture Collection (Rockville, MD); Boc, *tert*-butoxycarbonyl; BrAc, bromoacetyl; DMA, *N,N*-dimethylacetamide; DTE, 1,4-dithioerythritol; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid) (Ellman's reagent); EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; Gly, glycyl; GP(C), gel permeation (chromatography); HPLC, high-performance liquid chromatography; *M*_w, molecular mass; NSu, *N*-succinimidyl; ONSu, *N*-oxysuccinimidyl; PBS, phosphate-buffered saline (10 mM sodium phosphate in physiological salt at pH 7.2); Pn, pneumococcus and/or pneumococcal; PS, polysaccharide(s); RU, repeating unit(s) of the polysaccharide; TNBS, 2,4,6-trinitrobenzenesulfonic acid; TTd, tetanus toxoid.

Table 1. Overview of the Preparation of Pneumococcal Polysaccharide–Tetanus Toxoid Conjugates

pneumococcal type	PS M_w		starting amount of PS (mg)	RU/NH ₂ ^a	RU/SH ^b	amount of PS ^c (mg)	amount of protein ^c (mg)	PS:TTd ratio (mol:mol)	yield ^d (%)
	kDa	RU							
6B ^e	46	67	12.0	3.0	7.7	1.95	2.00	3.2	16.3
14 ^e	37	54	11.2	1.2	7.0	1.16	2.16	2.2	10.4
19F ^f	49	83	11.5	4.2	8.5	1.68	2.36	2.2	14.6
23F ^e	67	87	12.0	2.0	33.0	2.44 ^g	1.48	3.7 ^g	20.4

^a After incorporation of cystamine or *N,N'*-bis(glycyl)cystamine. ^b After DTE reduction of the linker. ^c In the pooled fractions (12–16) containing the conjugate, after purification over Sephacryl S-400 HR. ^d Based on the starting amount of PS. ^e Using cystamine as the linker. ^f Using *N,N'*-bis(glycyl)cystamine as the linker. ^g During purification over Sephacryl S-400 HR, about 10% of the nonconjugated PS 23F (67 kDa) coeluted with the selected conjugate-containing fractions nos. 12–16. For the three other conjugates, this was less than 2%.

Bromoacetylated Tetanus Toxoid. The cysteamine-modified Pn PS 6B [12 mg in 1.4 mL of 0.1 M sodium phosphate buffer, containing 5 mM EDTA (pH 7)] was mixed with BrAc-TTd [5 mg in 1.8 mL of 0.1 M sodium phosphate buffer, containing 5 mM EDTA (pH 7)], at a PS:TTd molar ratio of 7.5:1, at room temperature under a helium atmosphere. Conjugation was monitored by GP–HPLC analysis. After 91 h, essentially no uncoupled BrAc-TTd was left, and the main GP–HPLC peak had moved to a higher M_w . The remaining thiol groups on the PS were capped with bromoacetamide (8.1 mg, i.e. a 10-fold molar excess based on the initial amino group content, as measured on the PS before reduction) for 6 h at room temperature. The remaining bromoacetyl groups on BrAc-TTd were capped with 2-aminoethanethiol hydrochloride (17.0 mg, i.e. a 2.5-fold molar excess based on the amount of bromoacetamide added previously) overnight at room temperature. The conjugate was purified at room temperature by low-pressure GPC on a Sephacryl S-400 HR (Pharmacia) column (100 × 1.6 cm) using PBS as the eluent at a flow rate of 1 mL/min. The appropriate fractions (8 mL) were analyzed for carbohydrate (phenol/sulfuric acid assay) and protein content, sterile-filtered over a 0.2 μ m membrane, and stored at ~4 °C.

Preparation of Pn PS 14– and Pn PS 23F–TTd Conjugate Vaccines Using Cystamine. Starting from Pn PS 14 (ATCC lot 2016107) and Pn PS 23F (ATCC lot 2009269), these conjugates were prepared as described above for the Pn PS 6B–TTd conjugate. Table 1 shows the results.

***N,N'*-Bis[(*tert*-butyloxycarbonyl)glycyl]cystamine [N,N'-Bis(Boc-Gly)cystamine] (36).** *N,N*-Dimethylacetamide (42 mL) and *N,N*-diisopropylethylamine (14.6 mL, 83.8 mmol) were added to a mixture of *N'*-succinimidyl *N*-(*tert*-butyloxycarbonyl)glycinate, Boc-Gly-ONSu (11.4 g, 41.9 mmol), and cystamine dihydrochloride (3.77 g, 16.7 mmol). The suspension was stirred overnight at room temperature (cystamine dihydrochloride dissolved gradually in a few hours). The clear solution was diluted with water (16.7 mL), which caused phase separation. After the mixture was stirred for 1 h, diethyl ether (170 mL) was added and the mixture obtained was washed with water (170 mL). After phase separation, the water layer was extracted with diethyl ether (85 mL). The organic phases were combined, washed with 1 M NaHCO₃ (3 × 85 mL), water (3 × 85 mL), and 1 M NaH₂PO₄ (3 × 85 mL), successively, and dried with MgSO₄. The MgSO₄ was removed by filtration, and the filtrate was concentrated in vacuo to give *N,N'*-bis(Boc-Gly)cystamine (4.81 g, 62%) as a “glassy” solid.

***N,N'*-Bis(glycyl)cystamine (Dihydrotrifluoro Acetate) (36).** *N,N'*-Bis(Boc-Gly)cystamine (4.81 g, 10.3 mmol), as obtained in the previous step, was dissolved in dichloromethane (16.7 mL). The solution was stirred,

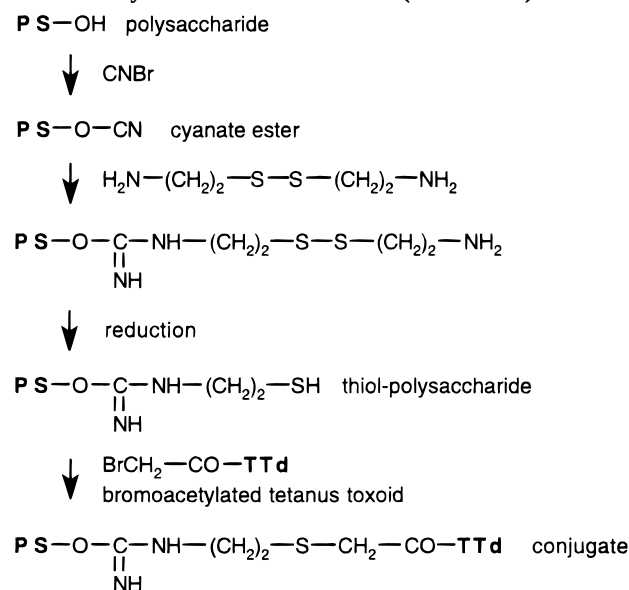
and trifluoroacetic acid (16.7 mL) was added. Formation of isobutene and carbon dioxide was visible for about 5 min. After a total period of 30 min, the reaction mixture was added dropwise to a powerfully stirred mixture of diethyl ether (170 mL) and pentane (170 mL). Stirring was ceased, and the precipitate was allowed to settle for 5 min. Diethyl ether/pentane was removed by decantation, and the solid was washed with a mixture of diethyl ether (50 mL) and pentane (50 mL). Again, diethyl ether/pentane was removed by decantation. The hygroscopic solid was dissolved in water (40 mL). Traces of diethyl ether/pentane were removed in vacuo at room temperature. Thereafter, the solution was lyophilized to give *N,N'*-bis(glycyl)cystamine dihydrotrifluoro acetate (4.93 g, 97%). Finally, the compound was dissolved in water at a concentration of 1.0 M and stored frozen at –20 °C.

Preparation of the Pn PS 19F–TTd Conjugate Vaccine Using *N,N'*-Bis(glycyl)cystamine. *Partial Depolymerization of Pn PS 19F.* Pneumococcal polysaccharide serotype 19F (RIVM, lot 19FEXP2A/S13; 275 mg, ~90% polysaccharide by colorimetric assay) was dissolved in 20 mL of water and sonicated for a total of 125 min, as described for Pn PS 6B. The M_w of the PS was reduced from ~980 to 49 kDa (from ~1700 to ~85 RU). The solution was filtered over a 0.45 μ m membrane and stored frozen at –20 °C.

*Modification of Pn PS 19F (49 kDa) with *N,N'*-Bis(glycyl)cystamine.* For the modification, 3.25 mL of a Pn PS 19F solution in water (6.15 mg/mL by colorimetric assay) was mixed with an equal volume of a 1 M sodium carbonate solution (pH 12). The mixture was cooled to ~2 °C. While the mixture was stirred, 250 μ L of the CNBr reagent (100 mg/mL in acetonitrile) was added. After 10 min, the solution was buffer-exchanged over Sephadex G-25 M with 0.2 M sodium carbonate/bicarbonate buffer at pH 9.25 and ~4 °C. The PS-containing fraction was mixed with 4.73 mL of a precooled solution of 0.2 M sodium carbonate/bicarbonate buffer (pH 9.25)/1.0 M *N,N'*-bis(glycyl)cystamine (dihydrotrifluoro acetate) (1/1, v/v). The pH dropped to 6.5 and was readjusted to 9.3 with 6 M sodium hydroxide. Over the course of 1.5 h, the pH was checked occasionally and, if necessary, readjusted. Thereafter, the mixture was kept at ~4 °C overnight. Finally, the sample was buffer-exchanged against 0.1 M sodium phosphate buffer, containing 5 mM EDTA (pH 8), and concentrated to a volume of 2 mL on a Centriprep-10 concentrator. Analysis of primary amino groups gave 1 NH₂/4.2 RU (TNBS assay).

*Reduction of *N,N'*-Bis(glycyl)cystamine-Modified Pn PS 19F.* *N,N'*-Bis(glycyl)cystamine-modified Pn PS 19F [13 mg in 1.75 mL of 0.1 M sodium phosphate buffer, containing 5 mM EDTA (pH 8)] was reduced by adding an 11-fold molar excess (based on NH₂ groups introduced into the PS) of DTE (8.9 mg). After incubation overnight at room temperature, the sample was buffer-exchanged over Sephadex G-25 M with 0.1 M sodium phosphate

Scheme 2. Activation of a Polysaccharide (PS) with Cyanogen Bromide, Followed by Modification with Cystamine, Reduction, and Conjugation to Bromoacetylated Tetanus Toxoid (BrAc-TTd)



buffer, containing 5 mM EDTA (pH 7, deaerated with helium), and concentrated to a volume of 3.5 mL on a Centriprep-10 concentrator. Analysis for sulfhydryl groups gave 1 SH/8.5 RU (Ellman assay).

Conjugation of *N*-(Glycyl)cysteamine-Modified Pn PS 19F and Bromoacetylated Tetanus Toxoid. The *N*-(glycyl)cysteamine-modified Pn PS 19F (11.5 mg) and BrAc-TTd (5 mg) were conjugated in a similar manner as described for cysteamine-modified Pn PS 6B and BrAc-TTd. The appropriate fractions (8 mL) from the Sephacryl S-400 HR purification were analyzed for carbohydrate (anthrone assay) and protein content, sterile-filtered over a 0.2 μm membrane, and stored at $\sim 4^\circ\text{C}$.

RESULTS AND DISCUSSION

A cystamine spacer was easily incorporated into the pneumococcal 6B, 14, and 23F PS. To this end, the PS was randomly activated with cyanogen bromide in sodium carbonate at pH 12 (35). Although the reaction pathway is more complicated (24) than depicted in Scheme 2, the major product (apart from immediate hydrolysis) is a highly reactive cyanate ester which, after lowering the pH to 9.25, was coupled with cystamine to give an isourea derivative. The reaction conditions applied allowed the incorporation of one cystamine per 1–3 repeating units (RU) as determined by measurement of the amino group content of the PS (Table 1).

After reduction of the disulfide bond of cystamine with an excess of dithioerythritol (DTE) and purification by ultrafiltration, thiol-PS (i.e. cysteamine-modified PS) was obtained. The thiol content of the PS varied from 1 SH per 7–33 RU (Table 1), which is lower compared to the amino content found in the previous step. A 5–10-fold molar excess of thiolated type 6B, 14, or 23F PS was used for conjugation to bromoacetylated tetanus toxoid (BrAc-TTd) at pH 7. Figure 1 shows a representative GP-HPLC analysis of the conjugation of the Pn PS 6B. The peak representing BrAc-TTd disappeared from the chromatogram in the course of about 4 days, whereas a protein-containing product (the carbohydrate is not detected by UV absorption at 280 nm) with a higher molecular mass was formed. This peak shift indicated

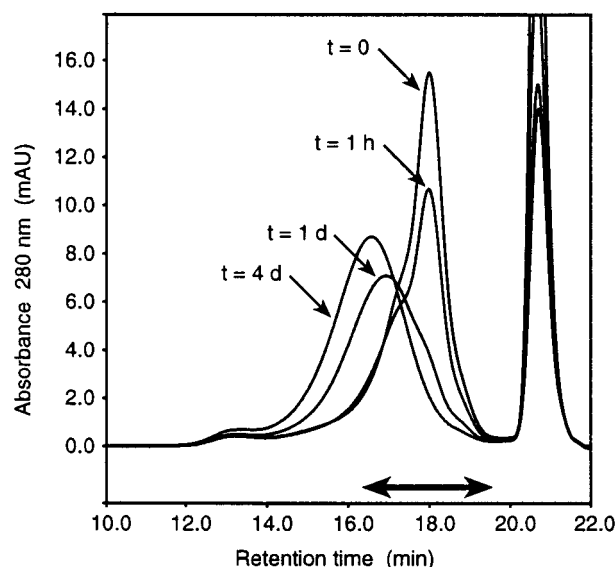


Figure 1. Gel permeation-HPLC of the conjugation of thiolated pneumococcal polysaccharide 6B (Pn PS 6B) to bromoacetylated tetanus toxoid (BrAc-TTd) at $t = 0$, 1 h, 1 day, and 4 days, monitored by UV detection at 280 nm. The bold arrow near the horizontal axis indicates where nonconjugated thiolated Pn PS 6B (as detected by refractive index in a separate run) as well as nonconjugated BrAc-TTd eluted. It should be noted that polysaccharide is not detected at 280 nm and that BrAc-TTd alone is stable for 4 days under the conditions used for the conjugation (i.e. overlapping with the conjugate mixture profile at $t = 0$).

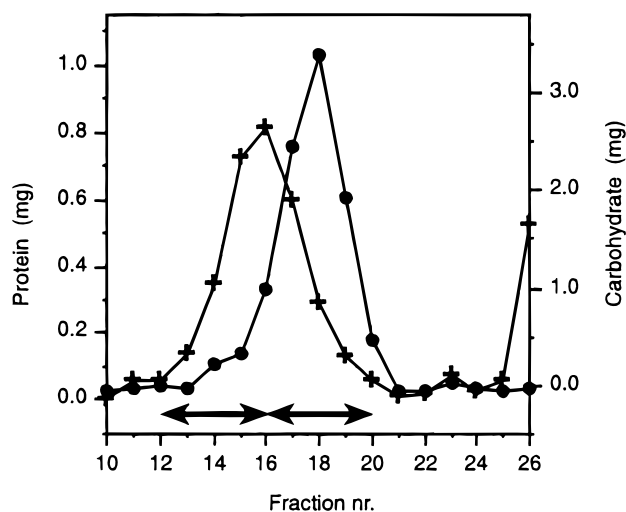
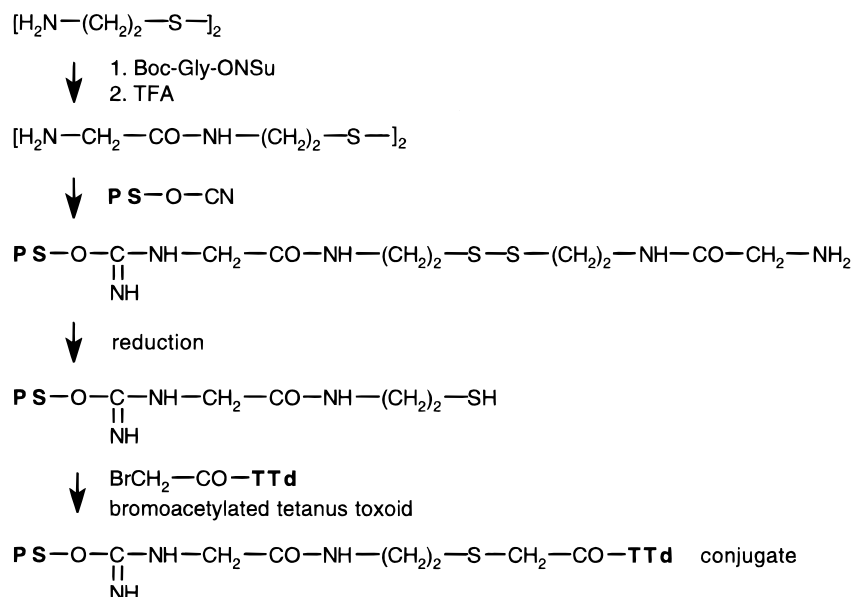


Figure 2. Purification of the pneumococcal type 6B polysaccharide-tetanus toxoid conjugate over a Sephacryl S-400 HR column (100×1.6 cm) using phosphate-buffered saline as the eluent: (+) protein content as determined by colorimetric assay and (●) carbohydrate content as determined by phenol/sulfuric colorimetric assay. The left arrow indicates which fractions (12–16) were pooled for immunological experiments. The right arrow indicates where nonconjugated Pn PS 6B eluted from this column (i.e. fractions 16–20). The conjugate content (not shown), as determined by a specific immunochemical assay, correlated with the protein content.

complete conjugation of the protein, since BrAc-TTd, in the absence of thiol-PS, does not shift to a higher molecular mass under the reaction conditions within 4 days. After the remaining thiol and bromoacetyl groups were capped, the conjugates were purified by low-pressure gel filtration over Sephacryl S-400 HR. As shown for the Pn PS 6B-TTd conjugate (Figure 2), the elution profiles of the conjugate (as determined by the protein assay) and carbohydrate (phenol/sulfuric acid

Scheme 3. Synthesis of *N,N'*-Bis(glycyl)cystamine and Its Use for Preparing Thiol-Modified Polysaccharide, Which Is Conjugated to BrAc-TTd

assay) did not coincide, indicating the presence of uncoupled PS. Samples from the fractions were also analyzed by conjugate-specific enzyme-linked immunosorbent assays (ELISA).³ An elution profile derived from the intensity of the ELISA readings coincided with the profile based on the protein content in the fractions (data not shown). Only the conjugate-containing fractions which were (almost) devoid of free PS were pooled (in all cases, fractions 12–16, Figure 2) and used for immunization experiments.⁴ Yields and PS:TTd ratios are summarized in Table 1.

Cystamine-modified Pn PS 19F containing one cystamine per 2–5 RU was easily obtained as described above for modification of type 6B, 14, and 23F PS. However, immediately after reduction with DTE and purification, a dramatically low thiol content was measured (less than 1 SH/100 RU). On incubation of the DTE-reduced Pn PS 19F with BrAc-TTd for several days, as described above, a peak shift correlated to conjugation of the protein was not observed by GP–HPLC analysis. Possible explanations for the low thiol content of the 19F PS are (a) incomplete reduction, (b) reoxidation during purification, (c) elimination of cysteamine by intermolecular attack on the isourea bond, for instance, by the excess of DTE present during reduction, and (d) intramo-

lecular elimination of cysteamine. Repeated reduction of the modified Pn PS 19F and thorough precautions to avoid reoxidation did not improve the thiol content of the PS. To rule out the third possibility, Pn PS 19F was activated with cyanogen bromide and then incubated with diaminobutane instead of cystamine. The diaminobutane-modified Pn PS 19F proved to be stable to DTE treatment; its amino content kept almost constant (loss of <5%, data not shown). Therefore, it was concluded that the loss of cysteamine might be caused by intramolecular attack of the thiol on the isourea bond. Although this hypothesis was not investigated mechanistically, intramolecular elimination of cysteamine should proceed via a favorable five-membered ring transition state with a low activation energy. This would suggest that elimination of elongated linkers should be more difficult, for instance, if a less favorable eight-membered ring transition state would be required for elimination.

This suggestion was tested by using *N,N'*-bis(glycyl)-cystamine for modification of cyanogen bromide-activated Pn PS 19F (Scheme 3). The modified PS contained an average of 1 linker per 4.2 RU. After DTE reduction, *N*-(glycyl)cysteamine-modified Pn PS 19F containing 1 thiol per 8.5 RU was obtained. This degree of functionalization proved to be satisfactory, since conjugation to BrAc-TTd was readily achieved. Elution profiles of the conjugate on GP–HPLC and Sephacryl S-400 HR were comparable to profiles for the Pn PS 6B–TTd conjugate as shown in Figures 1 and 2, respectively.

CONCLUSIONS

Type 6B, 14, and 23F pneumococcal polysaccharides were conveniently activated with cyanogen bromide and subsequently modified by coupling of cystamine. After reduction, thiolated polysaccharides were obtained and conjugated to bromoacetylated tetanus toxoid. This procedure failed for the 19F polysaccharide, possibly due to elimination of cysteamine by an intramolecular reaction. Although it remains unclear why elimination of the linker from the 19F polysaccharide is apparently much faster than that from the 6B, 14, and 23F polysaccharides, the problem could be solved by incorporating *N,N'*-bis(glycyl)cystamine into the 19F polysaccharide. The

³ Briefly, in case of types 6B, 14, and 19F, microtiter plates were coated with polyclonal sheep anti-tetanus toxoid antibodies. Thereafter, the plates were successively incubated with the sample (containing the PS–TTd conjugate), poly (type 14) or monoclonal (types 6B and 19F) mouse anti-pneumococcal PS antibodies, peroxidase-conjugated polyclonal goat anti-mouse antibodies, a substrate (3,3',5,5'-tetramethylbenzidine), and sulfuric acid. The conversion of the substrate, as measured by the optical density at 450 nm, is related to the amount of conjugate present in the sample. For 23F, a reversed assay proved to be more convenient and sensitive. In this case, polyclonal rabbit anti-PS 23F antibodies were coated onto a microtiter plate. Thereafter, the plates were successively incubated with the sample, peroxidase-conjugated polyclonal horse anti-tetanus toxoid antibodies, the substrate, and sulfuric acid.

⁴ The results of the immunological experiments will be described elsewhere. The conjugates induce anti-PS IgG antibodies and immunological memory in mice and/or rats. The first results will appear in ref 39.

use of cystamine and *N,N'*-bis(glycyl)cystamine as linkers allows preparation of a tetravalent pneumococcal polysaccharide–tetanus toxoid conjugate vaccine.

ACKNOWLEDGMENT

We thank Mrs. Danny Schijf and Dr. Wim Jiskoot from the Laboratory of Product and Process Development of RIVM for providing purified pneumococcal polysaccharide type 19F.

LITERATURE CITED

- Jennings, H. J. (1983) Capsular polysaccharides as human vaccines. *Adv. Carbohydr. Chem. Biochem.* 41, 155–208.
- Cruse, J. M., and Lewis, R. E., Jr., Eds. (1989) Conjugate Vaccines. In *Contributions to Microbiology and Immunology*, Vol. 10, Karger, Basel, Switzerland.
- Robbins, J. B., and Schneerson, R. (1990) Polysaccharide-protein conjugates: a new generation of vaccines. *J. Infect. Dis.* 161, 821–832.
- Jennings, H. J. (1990) Capsular polysaccharides as vaccine candidates. In *Current Topics in Microbiology and Immunology*, Vol. 150: *Bacterial Capsules* (K. Jann and B. Jann, Eds.) pp 97–127, Springer-Verlag, Berlin.
- Peeters, C. C. A. M., Lagerman, P. R., de Weers, O., Oomen, L. A., Hoogerhout, P., Beurret, M., and Poolman, J. T. (1996) Polysaccharide-conjugate vaccines. In *Methods in Molecular Medicine: Vaccine Protocols* (A. Robinson, G. H. Farrar, and C. N. Wibl, Eds.) pp 111–133, Humana Press, Totowa, NJ.
- Eskola, J., Käyhty, H., Peltola, H., Karanko, V., Mäkelä, P. H., Samuelson, J., and Gordon, L. K. (1985) Antibody levels achieved in infants by course of *Haemophilus influenzae* type b polysaccharide/diphtheria toxoid conjugate vaccine. *Lancet* i, 1184–1186.
- Schneerson, R., Robbins, J. B., Parke, J. C., Jr., Bell, C., Schlesselman, J. J., Sutton, A., Wang, Z., Schiffman, G., Karpas, A., and Shiloach, J. (1986) Quantitative and qualitative analyses of serum antibodies elicited in adults by *Haemophilus influenzae* type b and *Pneumococcus* type 6A capsular polysaccharide-tetanus toxoid conjugates. *Infect. Immun.* 52, 519–528.
- Madore, D. V., Johnson, C. L., Phipps, D. C., Popejoy, L. A., Eby, R., and Smith, D. H. (1990) Safety and immunologic response to *Haemophilus influenzae* type b oligosaccharide-CRM₁₉₇ conjugate vaccine in 1- to 6-month-old infants. *Pediatrics* 85, 331–337.
- Ahonkhai, V. I., Lucakcs, L. J., Jonas, L. C., and Calandra, G. B. (1991) Clinical experience with PedvaxHIB, a conjugate vaccine of *Haemophilus influenzae* type b polysaccharide-*Neisseria meningitidis* outer membrane protein. *Vaccine* 9, S38–S41.
- Siber, G. R. (1994) Pneumococcal disease: prospects for a new generation of vaccines. *Science* 265, 1385–1387.
- Vella, P. P., Marburg, S., Staub, J. M., Kniskern, P. J., Miller, W., Hagopian, A., Ip, C., Tolman, R. L., Rusk, C. M., Chupak, L. S., and Ellis, R. W. (1992) Immunogenicity of conjugate vaccines consisting of pneumococcal capsular polysaccharide types 6B, 14, 19F, and 23F and a meningococcal outer membrane protein complex. *Infect. Immun.* 60, 4977–4983.
- Åhman, H., Käyhty, H., Tamminen, P., Vuorela, A., Malinoski, F., and Eskola, J. (1996) Pentavalent pneumococcal oligosaccharide conjugate vaccine PncCRM is well-tolerated and able to induce an antibody response in infants. *Pediatr. Infect. Dis. J.* 15, 134–139.
- Anderson, E. L., Kennedy, D. J., Geldmacher, K. M., Donnelly, J., and Mendelman, P. M. (1996) Immunogenicity of heptavalent pneumococcal conjugate vaccine in infants. *J. Pediatr.* 128, 649–653.
- Anderson, P. W., Pichichero, M. E., Insel, R. A., Betts, R., Eby, R., and Smith, D. H. (1986) Vaccines consisting of periodate-cleaved oligosaccharides from the capsule of *Haemophilus influenzae* type b coupled to a protein carrier: structural and temporal requirements for priming in the human infant. *J. Immunol.* 137, 1181–1186.
- Beuvery, E. C., Roy, R., Kanhai, V., and Jennings, H. J. (1986) Characteristics of two types of meningococcal group C polysaccharide conjugates using tetanus toxoid as carrier protein. *Dev. Biol. Stand.* 65, 197–204.
- Jennings, H. J., and Lugowski, C. (1981) Immunochemistry of groups A, B, and C meningococcal polysaccharide-tetanus toxoid conjugates. *J. Immunol.* 127, 1011–1018.
- Marburg, S., Jorn, D., Tolman, R. L., Arison, B., McCauley, J., Kniskern, P. J., Hagopian, A., and Vella, P. P. (1986) Bimolecular chemistry of macromolecules: synthesis of bacterial polysaccharide conjugates with *Neisseria meningitidis* membrane protein. *J. Am. Chem. Soc.* 108, 5282–5287.
- Peeters, C. C. A. M., Tenbergen-Meekes, A.-M., Evenberg, D. E., Poolman, J. T., Zegers, B. J. M., and Rijkers, G. T. (1991) A comparative study of the immunogenicity of pneumococcal type 4 polysaccharide and oligosaccharide tetanus toxoid conjugates in adult mice. *J. Immunol.* 146, 4308–4314.
- Szu, S. C., Stone, A. L., Robbins, J. D., Schneerson, R., and Robbins, J. B. (1987) Vi capsular polysaccharide-protein conjugates for prevention of typhoid fever. Preparation, characterization, and immunogenicity in laboratory animals. *J. Exp. Med.* 166, 1510–1524.
- Verheul, A. F. M., Braat, A. K., Leenhouts, J. M., Hoogerhout, P., Poolman, J. T., Snippe, H., and Verhoef, J. (1991) Preparation, characterization, and immunogenicity of meningococcal immunotype L2 and L3,7,9 phosphoethanolamine group-containing oligosaccharide-protein conjugates. *Infect. Immun.* 59, 843–851.
- Verheul, A. F. M., Boons, G. J. P. H., van der Marel, G. A., van Boom, J. H., Jennings, H. J., Snippe, H., Verhoef, J., Hoogerhout, P., and Poolman, J. T. (1991) Minimal oligosaccharide structures required for induction of immune responses against meningococcal immunotype L1, L2, and L3,7,9 lipopolysaccharides determined by using synthetic oligosaccharide-protein conjugates. *Infect. Immun.* 59, 3566–3573.
- Lin, C., Mihal, K. A., and Krueger, R. J. (1990) Introduction of sulfhydryl groups into proteins at carboxyl sites. *Biochim. Biophys. Acta* 1038, 382–385.
- Fidanza, J. A., and McLaughlin, L. W. (1992) Use of a thiol tether for the site-specific attachment of reporter groups to DNA. *J. Org. Chem.* 57, 2340–2346.
- Wilchek, M., Miron, T., and Kohn, J. (1984) Affinity chromatography. *Methods Enzymol.* 104, 3–55.
- Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., and Smith, F. (1956) Colorimetric method for determination of sugars and related substances. *Anal. Chem.* 28, 350–356.
- Saha, S. K., and Brewer, C. F. (1994) Determination of the concentrations of oligosaccharides, complex type carbohydrates, and glycoproteins using the phenol sulfuric acid method. *Carbohydr. Res.* 254, 157–167.
- Scott, T. A., Jr., and Melvin, E. H. (1953) Determination of dextran with anthrone. *Anal. Chem.* 25, 1656–1661.
- Shields, R., and Burnett, W. (1960) Determination of protein-bound carbohydrate in serum by a modified anthrone method. *Anal. Chem.* 32, 885–886.
- Jermyn, M. A. (1975) Increasing the sensitivity of the anthrone method for carbohydrate. *Anal. Biochem.* 68, 332–335.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193, 265–275.
- Peterson, G. L. (1979) Review of the Folin phenol protein quantitation method of Lowry, Rosebrough, Farr and Randall. *Anal. Biochem.* 100, 201–220.
- Habeeb, A. F. S. A. (1966) Determination of free amino groups in proteins by trinitrobenzenesulfonic acid. *Anal. Biochem.* 14, 328–336.
- Ellman, G. L. (1959) Tissue sulfhydryl groups. *Arch. Biochem. Biophys.* 82, 70–77.
- Riddles, P. W., Blakeley, R. L., and Zerner, B. (1979) Ellman's reagent: 5,5'-dithiobis(2-nitrobenzoic acid)—a reexamination. *Anal. Biochem.* 94, 75–81.
- March, S. C., Parikh, I., and Cuatrecasas, P. (1974) A simplified method for cyanogen bromide activation of agarose for affinity chromatography. *Anal. Biochem.* 60, 149–152.

- (36) Oiry, J., Pue, J. Y., Imbach, J. L., Fatome, M., Sentenac-Roumanou, H., and Lion, C. (1986) Synthesis and radioprotective activity of new cysteamine and cystamine derivatives. *J. Med. Chem.* 29, 2217–2225.
- (37) Kenne, L., and Lindberg, B. (1983) Bacterial polysaccharides. In *The Polysaccharides* (G. O. Aspinall, Ed.) Vol. 2, pp 287–363, Academic Press, Orlando, FL.
- (38) Richards, J. C., and Perry, M. B. (1988) Structure of the specific capsular polysaccharide of *Streptococcus pneumoniae* type 23F (American type 23). *Biochem. Cell Biol.* 66, 758–771.
- (39) Rodriguez, M. E., van den Dobbelsteen, G. P. J. M., Oomen, L. A., de Weers, O., van Buren, L., Beurret, M., Poolman, J. T., and Hoogerhout, P. (1998) Immunogenicity of *Streptococcus pneumoniae* type 6B and 14 polysaccharide-tetanus toxoid conjugates and the effect of uncoupled polysaccharide on the antigen-specific immune response. *Vaccine* (in press).

BC9702011

Detection of Oligonucleotide Hybridization on a Single Microparticle by Time-Resolved Fluorometry: Quantitation and Optimization of a Sandwich Type Assay

Harri Hakala,* Esa Mäki, and Harri Lönnberg

Department of Chemistry, University of Turku, FIN-20014 Turku, Finland. Received October 24, 1997; Revised Manuscript Received January 22, 1998

Uniformly sized (50 μm) porous glycidyl methacrylate/ethylene dimethacrylate particles (SINTEF) were used as the solid phase in a sandwich type mixed-phase hybridization assay based on time-resolved fluorescence detection on a single particle. These particles were coated with oligodeoxyribonucleotide probes by conventional phosphoramidite chain assembly. An oligodeoxyribonucleotide bearing a photoluminescent europium(III) chelate, $\{2,2',2'',2'''-\{4'-\{4'''-[(4,6\text{-dichloro-}1,3,5\text{-triazin-}2\text{-yl)amino]phenyl}\}-2,2':6',2''\text{-terpyridine-}6,6''\text{-diyl}\}\text{bis(methylenenitrilo)}\}\text{tetrakis(acetato)}\}\text{europium(III)}$, was hybridized to a complementary sequence of the target oligonucleotide, and the resulting duplex was further hybridized to the particle-bound probes. The latter binding was quantified by time-resolved measurement of the emission signal of a single particle. Kinetics of hybridization and the effect of the concentration of the target oligomer and the fluorescently tagged probe on the efficiency of hybridization were studied. The intensity of the emission signal was linearly related to the concentration of the target oligomer over a range of 5 orders of magnitude. The length of the complementary region between the target oligomer and the particle-bound probe was varied, and the effect of point mutations and deletions on the hybridization efficiency was determined in each case. The maximal selectivity was observed with 10–16-base pair complementary sequences, the optimal length depending on the oligonucleotide loading on the particle. Discrimination between the complete matches and point mismatches was unequivocal, a single point mutation and/or deletion decreasing the efficiency of hybridization by more than 2 orders of magnitude.

INTRODUCTION

Solid supports bearing immobilized oligodeoxyribonucleotide probes are a useful tool in analyzing the base sequence of nucleic acids (1–10). This kind of mixed-phase assay combined with efficient amplification techniques, such as polymerase chain reaction (PCR; 11), has gained increasing popularity in clinical diagnostics. These assays are usually based on arrays of allele-specific oligonucleotides attached to a solid support, hybridization of fluorescently or radioactively labeled PCR-amplified sequences to the support-bound probes, and detection of the hybridized sequences by location of the label spots on the support (12–14). We have recently tried to evaluate the feasibility of an alternative approach based on the following principle (15). A mixture of microscopic particles, each of which bears a given allele-specific oligonucleotide and a reporter group defining the particle category, is used as the solid phase. After hybridization, individual particles are separately subjected to two parallel measurements: one identifies the particle category (reporter groups on the particle), and the other quantifies the fluorescently tagged oligonucleotide hybridized to the particle-bound probes. The fluorescent markers employed for labeling of oligonucleotides are photoluminescent lanthanide chelates. Such markers have advantages over organic fluorophores, since their long-lived fluorescence allows the usage of the time-

resolved mode in the measurement, and this, in turn, allows efficient elimination of the prompt background fluorescence. Furthermore, the difference between the wavelength of excitation and emission is large, and the emission bands are narrow (16, 17). Lanthanide chelates have earlier been utilized in highly sensitive immunoassays (18–20) and also in studying nucleic acid hybridization (19–24). However, none of the previous hybridization assays is based on a single-particle approach similar to that used in this work. In other words, they do not form a basis for development of a multiparametric assay.

We have previously described several methods for covalent immobilization of oligonucleotide probes to uniformly sized polymer particles (25) and shown that the same particles may also, after appropriate derivatization, be used as a solid support in automated oligonucleotide synthesis (26). In fact, the postsynthetic immobilization of purified oligonucleotide probes appears to offer no advantage over the more convenient direct chain assembly on the particles. With both types of particles, the intensity of the emission signal measured directly from the particle surface is proportional to the concentration of the fluorescently tagged oligomer in solution over a concentration range of 5 orders of magnitude. About 10^4 oligonucleotides hybridized to a single particle is sufficient for reliable quantitation. To show that the particles obtained by direct chain assembly also exhibit excellent selectivity of hybridization, although they bear a considerable amount of truncated sequences, we now report on quantitation of a sandwich type hybridization assay on these particles. The principle of the assay is described in Figure 1. The results show that

* Send correspondence to Harri Hakala, Department of Chemistry, University of Turku, FIN-20014 Turku, Finland. Telephone: +358-2-333 8091. Fax: +358-2-333 6700. E-mail: harri.hakala@utu.fi.

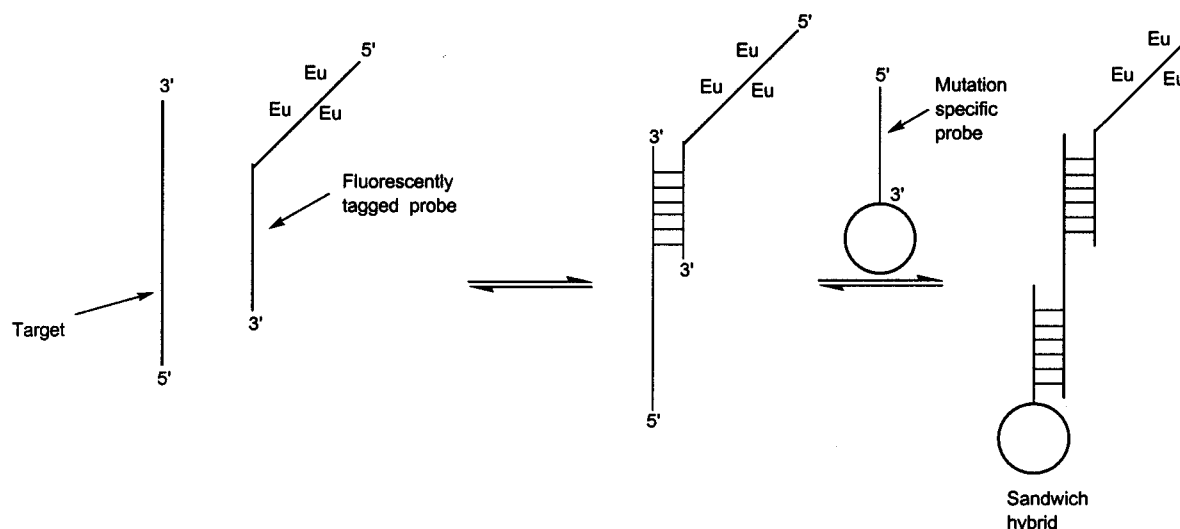


Figure 1. Principle of the sandwich hybridization assay studied.

Table 1. Target Oligodeoxyribonucleotides^a

compd	sequence
2	5'-d(CTATATTCATCATAGG AAACACCAAAGATGATATTC)-3'
3	5'-d(CTATATTAATCATAGG AAACACCAAAGATGATATTC)-3'
4	5'-d(CTATATTCCTCATAGG AAACACCAAAGATGATATTC)-3'
5	5'-d(CTATATTATCATAGG AAACACCAAAGATGATATTC)-3'
6	5'-d(ATATTCATCATAGG AAACACCAAAGATGATATTC)-3'
7	5'-d(ATATTCCTCATAGG AAACACCAAAGATGATATTC)-3'
8	5'-d(ATATTCATCATAGG AAACACCAAAGATGATATTC)-3'
9	5'-d(TATTCATCATAGG AAACACCAAAGATGATATTC)-3'
10	5'-d(TATTCAGCATAGG AAACACCAAAGATGATATTC)-3'
11	5'-d(TATTCATCATAGG AAACACCAAAGATGATATTC)-3'
12	5'-d(ATTCATCATAGG AAACACCAAAGATGATATTC)-3'
13	5'-d(ATTCATAATAGG AAACACCAAAGATGATATTC)-3'
14	5'-d(ATTCATCATAGG AAACACCAAAGATGATATTC)-3'
15	5'-d(TCATCATAGG AAACACCAAAGATGATATTC)-3'
16	5'-d(TCATCCTAGG AAACACCAAAGATGATATTC)-3'
17	5'-d(TCATCTAGG AAACACCAAAGATGATATTC)-3'
18	5'-d(CATCATAGG AAACACCAAAGATGATATTC)-3'
19	5'-d(ATCATAGG AAACACCAAAGATGATATTC)-3'

^a The sequence complementary to the particle-bound probes is indicated by bold letters and that complementary to the fluorescently tagged probe in italics. The site of possible point mutation or deletion is underlined or shown by an arrow, respectively.

proper adjustment of the length of the complementary region between the target oligonucleotide and the particle-bound probes allows an unequivocal discrimination between a complete match and a point mismatch (mutation and/or deletion) over a wide concentration range. Under optimal conditions, a single mismatch reduces the efficiency of hybridization by more than 2 orders of magnitude.

EXPERIMENTAL PROCEDURES

Oligonucleotide-Coated Particles (1). The particles were porous uniformly sized beads ($\varnothing = 50 \mu\text{m}$) made of a copolymer of glycidyl methacrylate (40%) and ethylene dimethacrylate (60%) (37% matrix) and derivatized with bis(3-aminopropyl)amine, yielding a 1 mmol g^{-1} density of primary amino functions (SINTEF). They were derivatized for oligonucleotide synthesis as described previously (26), and the sequence 5'-d(CCTATGATGAATATAGTTT)-3' was assembled with the normal phosphoramidite strategy. According to the DMTr cation response (27), the loadings of the full-length oligonucleotide sequence on three separate batches prepared (1) were 2, 5, and $10 \mu\text{mol g}^{-1}$. After conventional ammonolysis and washings, the particles were dried under reduced pressure, removed from the column, and washed with the buffer used in the hybridization assays.

Oligonucleotides and Their Fluorescent Conjugates. The unlabeled oligodeoxyribonucleotides used as targets in the sandwich assays were synthesized with the conventional phosphoramidite strategy and purified by ion exchange and RP-HPLC and desalted. Table 1 lists the oligomers studied.

Two fluorescently tagged oligonucleotides were used in the hybridization assays: 5'-d(X₅ATATCATCTTTGGTGT)-3' (**20**) and 5'-(X₂₀ATCATCTTTGGT)-3' (**21**). Here X stands for a N⁴-(6-aminoethyl)-2'-deoxycytidine unit tethered to a photoluminescent europium(III) chelate, {2,2',2'',2'''-[[{4'-{4'''-[(4,6-dichloro-1,3,5-triazin-2-yl)-amino]phenyl}-2,2':6',2''-terpyridine-6,6''-diyl}bis(methylenenitrilo)}tetrakis(acetato)]europium(III) (**28**). These oligonucleotide conjugates were prepared as described previously (29). Probes **20** and **21** were observed to bear 5 and 17 labels per oligomer, respectively, when assayed according to the DELFIA protocol (18). In other words, the extent of labeling of the aliphatic amino functions was 1.0 with **20** and 0.85 with **21**.

Hybridization Assays. The hybridization assays were carried out in a Tris buffer (50 mmol L^{-1} at pH 7.5, 0.01% Tween 20 and 0.5 mol L^{-1} NaCl). Usually, 50 particles were incubated in $10 \mu\text{L}$ of the buffer containing a known amount of the target oligonucleotide (**2–19**) and the fluorescently labeled probe (**20** or **21**). The reaction

time was typically 24 h. However, on examination of the kinetics of hybridization, 500 particles in a total volume of 100 μL was used, from which aliquots of 10 μL were withdrawn. In these experiments, the concentration of the target oligonucleotide (**2**) was 5 nmol L^{-1} and that of the labeled oligonucleotide (**20**) 17 nmol L^{-1} . The particles were washed twice with 200 μL of glycine buffer (50 mM at pH 10), containing 20% propanol, and then transferred into a quartz capillary tube for measurement. However, in the experiments of kinetics of hybridization, particles were washed once with 300 μL . The fluorescence emission of each particle was determined separately on a time-resolved microfluorometer as described previously (30). A delay time of 296 μs was used, and the signal was collected for 300 μs , which allowed complete elimination of the prompt background fluorescence. Typically, 5–10 individual particles were measured, the standard deviation ranging from 5 to 10%.

RESULTS

Quantitation of the Sandwich Type Hybridization. Particles **1** bearing the sequence 5'-d(CCTATGATGAATATAGTTT)-3' (three batches with loadings of 2, 5, and 10 $\mu\text{mol g}^{-1}$) were used as the solid support in all hybridization studies. The target oligonucleotides (**2**–**19**) were 28–36 nucleotides long. They contained at their 5'-terminus an 8–16-nucleotide sequence complementary to the particle-bound probe (indicated by bold letters in Table 1) and at their 3'-terminus a 16- or 12-nucleotide sequence (indicated in italics in Table 1) complementary to the fluorescently tagged oligomers **20** [5'-d(X₂₀ATATCATCTTTGGTGT)-3'] or **21** [5'-d(X₂₀ATCATCTTTGGT)-3'], respectively. The labeled oligomer (**20** or **21**) was used in excess compared to the target (**2**–**19**) to ensure quantitative hybridization of the target to the labeled probe. With **20**, which has a 16-nucleotide sequence complementary to the target, no excess is really needed. The emission signal measured was independent of the concentration of **20**, as long as this concentration was at least equal to that of the target. With **21**, which has only a 12-nucleotide sequence complementary to the target, a 15-fold excess was needed to obtain a maximal emission signal. For this reason, **20** was used as the fluorescent probe in most of the measurements. Unspecific adsorption of **20** or **21** to the particles was estimated by treating the particles with the labeled probe in the absence of the target oligomer. The unspecific binding represented in all the cases less than 1% of the total amount of the labeled probe. The resulting background signal was determined at each concentration of **20** (or **21**) employed and subtracted from the signal obtained in the presence of the target. Throughout this paper, only these corrected signals referring to sequence-selective hybridization are given.

To convert the microfluorometry signals to the number of fluorescently labeled oligonucleotides hybridized to the particle, a calibration line was determined. Two sets of hybridization reactions were carried out, in which the concentrations of the target (**2**) and fluorescent probe (**20**) were varied from 0.17 to 50 nM. After incubation for 20 h, one set of particles was subjected to measurement by microfluorometry, while from the other set, europium(III) ions were released in solution and determined according to the DELFIA protocol (18). The calibration line obtained (data not shown) was essentially similar to that described previously (25, 26) for the direct hybridization of a fluorescently tagged oligonucleotide to the same particles, and it was used throughout this work

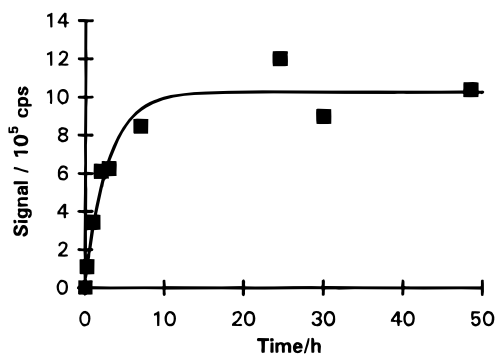


Figure 2. Kinetics of formation of a sandwich hybrid on particles **1**, having an oligonucleotide loading of 2 $\mu\text{mol g}^{-1}$. The concentration of the target oligonucleotide (**2**) was 5 nmol L^{-1} and that of the fluorescently tagged probe (**20**) 17 nmol L^{-1} . The particle density was 5 particles/ μL and the temperature 25 $^{\circ}\text{C}$.

to convert the microfluorometry signals to the number of fluorescently labeled oligonucleotide molecules.

Figure 2 shows the hybridization kinetics observed with particles **1**, having an oligonucleotide loading of 2 $\mu\text{mol g}^{-1}$. The concentration of target (**2**) was 5 nmol L^{-1} (3×10^{11} molecules in 100 μL) and that of the fluorescently tagged oligonucleotide (**20**) 17 nmol L^{-1} . The kinetics of this sandwich type hybridization are very similar to those observed previously (26) for direct hybridization of fluorescently labeled oligonucleotides to the same particles. Evidently, hybridization of **2** to **20** is, in the excess of the latter, quantitative and fast compared to the hybridization of the resulting duplex to the solid phase. The latter sandwich hybridization obeys first-order kinetics. The curve indicated in Figure 2 represents the best least-squares fit obtained on the basis of this model. The kinetics were similar also when **21** was used as the labeled probe (data not shown).

The dependence of the intensity of the emission signal on the concentration of the target oligomer was studied by keeping the concentration of the fluorescent probe (**20**) constant and varying that of the target (**2**): [**20**] = 166 pmol L^{-1} when [**2**] = 0.5–100 pmol L^{-1} , and [**20**] = 50 nmol L^{-1} when [**2**] = 0.1–166 nmol L^{-1} . The oligonucleotide loading of the particles was 10 $\mu\text{mol g}^{-1}$. This experiment mimics the real assay of amplified oligomers, because then the amount of target is unknown and a constant higher concentration of the labeled probe must be used. The results obtained are shown in Figure 3. As seen, the linear range is wide, 5 orders of magnitude. However, the entire range cannot be utilized by using a single constant probe concentration, because at the lowest target concentrations the unspecific binding of the probe becomes too high compared to the specific hybridization signal. The excess of the fluorescent probe cannot be larger than 1000-fold.

Although the intensity of the emission signal is linearly related to the concentration of the target oligomer in solution over a range of 5 orders of magnitude, the slope of this correlation line is not exactly unity but 1.05. At least apparently the efficiency of hybridization increases with the increasing concentration of the target. At the lowest concentration of **2**, the efficiency [(amount of **2** hybridized)/(total amount of **2**)] is only 16% but at the highest concentration 85%. In this respect, the sandwich hybridization seems to differ from the direct hybridization of the labeled probe to the particles. As shown previously (26), in the latter case, the hybridization efficiency remains constant over the entire concentration range of 5 orders of magnitude.

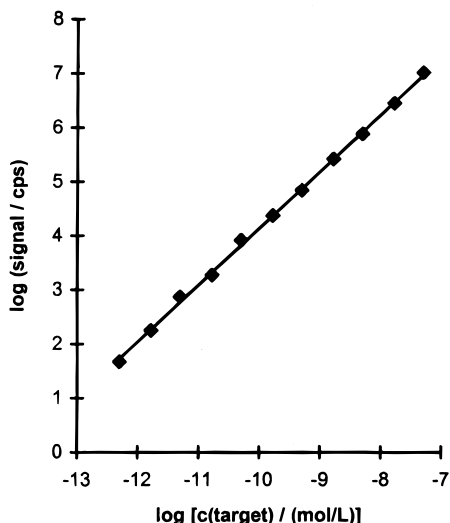


Figure 3. Intensity of the emission signal measured from a single microparticle plotted against the concentration of the target oligonucleotide (**2**). The concentration of the fluorescently tagged probe was $0.166 \text{ nmol L}^{-1}$ when $[\mathbf{2}] < 0.1 \text{ nmol L}^{-1}$ and 50 nmol L^{-1} at higher concentrations of **2**.

Effect of the Length of the Complementary Region on the Hybridization Efficiency. To determine the minimal length of the complementary sequence between the target oligonucleotide and the particle-bound probe that allows hybridization with maximal efficiency, a set of target oligomers (**2**, **6**, **9**, **12**, **15**, **18**, and **19**) having sequences of 16, 14, 13, 12, 10, 9, and 8 bases complementary to the particle-bound oligonucleotide were hybridized to **1**. Panels A and B of Figure 4 refer to oligonucleotide loadings of 10 and $2 \mu\text{mol g}^{-1}$ on the particles, respectively. With both types of particles, the intensity of the emission signal is increased linearly with the increasing concentration of target, as long as the concentration of the target oligomer remains lower than that of the labeled probe. As seen, the concentration-dependent signal levels are identical as long as the complementary region is 12 nucleotides or longer. With shorter sequences, the signal level gradually decreases. Similar results were obtained when target **2** was allowed to hybridize with particles bearing probes shorter than **1** (data not shown). Comparison of the curves in panels A and B of Figure 4 reveals that decreasing the length of the complementary sequence has a more dramatic effect on the efficiency of hybridization when the oligonucleotide loading on the particles is low. Table 2 contains the hybridization efficiencies observed at high target concentrations, i.e. at concentrations exceeding the one required to make the signal independent of the target concentration ($17\text{--}170 \text{ nmol L}^{-1}$). The data obtained with an oligonucleotide loading of $5 \mu\text{mol g}^{-1}$ are included in the same table. These results indicate that the minimum length of complementarity needed for maximal efficiency of hybridization is 10 nucleotides when the oligonucleotide loading on the particle is $5 \mu\text{mol g}^{-1}$ or higher and 12 nucleotides for particles where the oligonucleotide loading is $2 \mu\text{mol g}^{-1}$.

Selectivity of Hybridization. To determine how sensitive the efficiency of hybridization is to the presence of a single mismatch within a complementary sequence of variable length, a set of target oligonucleotides containing a single point mutation or deletion was prepared and hybridized to particles **1**. The length of the complementary region was varied from 10 to 16 nucleotides, and the mismatch was placed approximately in the center of this region to obtain a maximal discrimination between

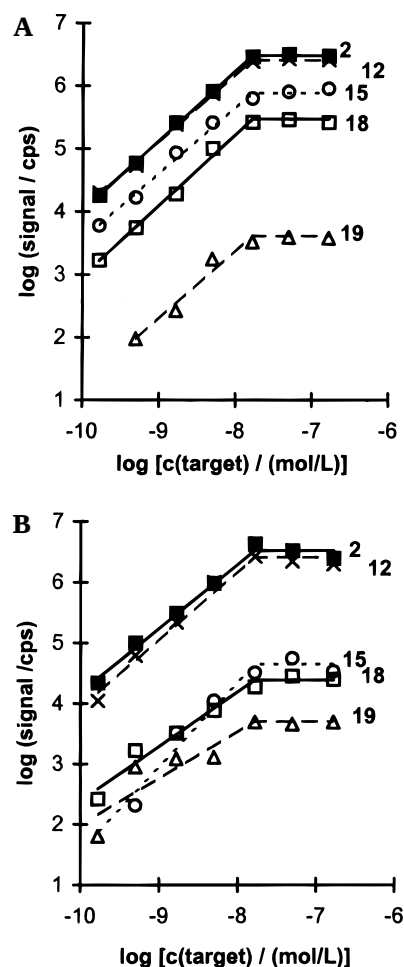


Figure 4. Effect of the length of the complementary sequence between the target and the particle-bound probes (**1**) on the hybridization efficiency. The intensity of the emission signal measured from a single microparticle plotted against the concentration of the target (**2**, **12**, **15**, **18**, and **19**). The concentration of the fluorescently tagged probe was 17 nmol L^{-1} . Panels A and B correspond to oligonucleotide loadings of 10 and $2 \mu\text{mol g}^{-1}$, respectively.

Table 2. Hybridization Efficiencies of Particles **1 Having Oligonucleotide Loadings of 2, 5, or $10 \mu\text{mol g}^{-1}$ ^a**

compd	length	efficiency (%)		
		$2 \mu\text{mol g}^{-1}$	$5 \mu\text{mol g}^{-1}$	$10 \mu\text{mol g}^{-1}$
2	16	76	71	77
6	14	78	87	76
9	13	78	75	60
12	12	50	72	61
15	10	0.7	17	18
18	9	0.4	1.6	3
19	8	0.07	0.07	0.06

^a The effect of the length of the complementary sequence between the target oligonucleotide and the particle-bound probe.

matches and mismatches (12, 31). The concentration of these targets (**3**–**5**, **7**, **8**, **10**, **11**, **13**, **14**, **16**, and **17**) was varied from 0.17 to 170 nmol L^{-1} in the hybridization assays, and that of the fluorescent probe (**20**) was 17 nmol L^{-1} . Particles **1** with all three different oligonucleotide loadings (2 , 5 , and $10 \mu\text{mol g}^{-1}$) were used as a solid phase. Figure 5 shows an example of the results: the effect of a single point mutation (**13**) and deletion (**14**) on the concentration-dependent hybridization efficiency of a target having 12 bases complementary to the particle-bound oligomers (loading of $10 \mu\text{mol g}^{-1}$). As seen, both mismatches reduce the hybridization efficiency

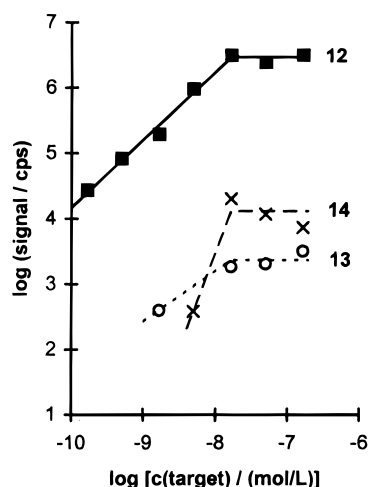


Figure 5. Effect of a single point mutation or deletion on hybridization of a target oligonucleotide (**12**) having a 12-nucleotide sequence complementary to the particle-bound probes of particles **1** (loading of $10 \mu\text{mol g}^{-1}$). The concentration of the fluorescently tagged probe was 17 nmol L^{-1} .

Table 3. Effect of a Single Point Mutation on the Hybridization Efficiency of Oligonucleotides Having a 10–16-Nucleotide Sequence Complementary to the Particle-Bound Probes on Particles **1**^a

compd	length	efficiency (%)		
		$2 \mu\text{mol g}^{-1}$	$5 \mu\text{mol g}^{-1}$	$10 \mu\text{mol g}^{-1}$
3	16	4.6	47	73
4	16	5.6	28	63
7	14	0.9	1.4	4.2
10	13	0.5	1.3	3.3
13	12	0.3	0.08	0.1
16	10	0.9	2	1

^a The results obtained with particles having oligonucleotide loadings of 2, 5, or $10 \mu\text{mol g}^{-1}$ are indicated. The hybridization efficiencies are expressed as a percentage of the value of the unmutated sequence.

Table 4. Effect of a Single Point Deletion on the Hybridization Efficiency of Oligonucleotides Having a 10–16-Nucleotide Sequence Complementary to the Particle-Bound Probes on Particles **1**^a

compd	length	efficiency (%)		
		$2 \mu\text{mol g}^{-1}$	$5 \mu\text{mol g}^{-1}$	$10 \mu\text{mol g}^{-1}$
5	16	1.4	15	50
8	14	1.4	5.2	9.6
11	13	0.3	0.9	3.8
14	12	0.3	0.05	0.2
17	10	1.1	3	1.3

^a The results obtained with particles having oligonucleotide loadings of 2, 5, or $10 \mu\text{mol g}^{-1}$ are indicated. The hybridization efficiencies are expressed as a percentage of the value of the parent sequence.

by more than 2 orders of magnitude. Table 3 summarizes the entire data for point mutations and Table 4 for point deletions. As seen, the oligonucleotide loading on particles slightly affects the discrimination of matches and mismatches. If the loading on particles is low ($2 \mu\text{mol g}^{-1}$), a point mutation may unequivocally be distinguished from the unmutated sequence when the length of the complementary sequence falls in the range of 10–14 bases, and rather clearly even when this sequence is 16 nucleotides long. With higher oligonucleotide loadings, the complementary sequence should not be longer than 13 bases, preferably 12 bases. There are no significant differences between the effects of mutations in C•G or A•T base pairs. The effects of point deletions

on the hybridization efficiency are rather similar to those of point mutations.

DISCUSSION

A sandwich type hybridization assay may be carried out on microparticles in two different manners. Either the mutation-specific probe is immobilized to the particles, as described in Figure 1, or the fluorescently tagged oligomer is mutation-specific. Of these two approaches, the former was applied in this study, since it is more convenient to coat the particles with various mutation-specific probes than to prepare a large selection of fluorescently tagged oligonucleotides, each having a different sequence.

The results presented above clearly show that porous polymer particles bearing 3'-attached oligonucleotide probes (**1**) constitute a good solid support for sandwich hybridization assays. The dynamic range achieved with the single-particle technique described is exceptionally large; the intensity of the emission signal measured from a single particle is linearly related to the concentration of the target oligonucleotide over a concentration range of 5 orders of magnitude (Figure 3). The main reasons for the wide linear range include (i) the high capacity of porous particles, (ii) the almost negligible concentration quenching of the photoluminescent europium(III) chelate used as a fluorescent marker, and (iii) the efficient elimination of the prompt background fluorescence by the use of the time-resolved mode in the measurement.

The sensitivity of the sandwich assay described is high. On using 5 particles/ μL , the detection limit for the target oligonucleotide is less than 1.7 pmol L^{-1} . In other words, about 3×10^4 probes (0.05 amol) hybridized to a single particle becomes detected (signal $> 2 \times$ standard deviation). In terms of concentration, the detection limit may still be decreased; decreasing the number of particles per volume increases the signal measured from a single particle, but the time required to reach the equilibrium is simultaneously increased. To some extent, the sensitivity may also be increased by increasing the number of lanthanide chelates per probe (21). Even as it is, the sensitivity is excellent compared to that of other reported assay systems, including those based on radioactive detection (32).

The major shortcoming of the methodology introduced is relatively slow hybridization. Up to 20 h was needed to reach the equilibrium on using 5 particles/ μL . Our previous results (26) on direct hybridization of a fluorescently tagged oligomer on the same particles, however, suggest that the reaction time may be reduced to less than $1/10$ of the value by increasing the number of particles in a given volume of the sample. Unfortunately, this simultaneously decreases the sensitivity of detection, since the same amount of target oligonucleotides is then distributed among a larger number of particles.

The selectivity of hybridization is good. However, a prerequisite for this is a proper adjustment of the length of the complementary region between the target oligonucleotide and the particle-bound probe. The minimum length required for maximal hybridization efficiency somewhat depends on the loading of the probes on particles, ranging from 10 (loading of $10 \mu\text{mol g}^{-1}$) to 12 (loading of $2 \mu\text{mol g}^{-1}$) nucleotides. When this minimal length of complementarity is used, a single point mutation or deletion near the center of the hybridizing sequence decreases the efficiency of hybridization to less than 1% of the original value. However, even complementarity 3 to 4 nucleotides longer gives selectivity that

is almost as good (Tables 3 and 4). Accordingly, the method appears not to be too sensitive to the length of the mutation-specific probes.

ACKNOWLEDGMENT

The particles employed were a generous gift of Dr. Ruth Schmid (SINTEF). The photoluminescent marker was prepared in the Laboratory of Wallac Oy (Turku, Finland) by Dr. Veli-Matti Mikkala and Dr. Harri Takalo. We also thank Mr. Harri Salo, M.Sc., for his help in oligonucleotide synthesis. Financial support from the Academy of Finland, the Research Council for Natural Sciences and Technology, is gratefully acknowledged.

LITERATURE CITED

- (1) Southern, E. M., Case-Green, S. C., Elder, J. K., Johnson, M., Mir, K. U., Wang, L., and Williams, J. C. (1994) Arrays of complementary oligonucleotides for analysing the hybridisation behaviour of nucleic acids. *Nucleic Acids Res.* 22, 1368–1373.
- (2) Maskos, U., and Southern, E. M. (1992) Oligonucleotide hybridisations on glass supports: a novel linker for oligonucleotide synthesis and hybridisation properties of oligonucleotides synthesised *in situ*. *Nucleic Acids Res.* 20, 1679–1684.
- (3) Southern, E. M., Maskos, U., and Elder, J. K. (1992) Analyzing and comparing nucleic acid sequences by hybridization to arrays of oligonucleotides: evaluation using experimental models. *Genomics* 13, 1008–1017.
- (4) Peterlinz, K. A., and Georgiadis, R. M. (1997) Observation of hybridization and dehybridization of thiol-tethered DNA using two-color surface plasmon resonance spectroscopy. *J. Am. Chem. Soc.* 119, 3401–3402.
- (5) Gingeras, T. R., Kwok, D. Y., and Davis, G. R. (1987) Hybridization properties of immobilized nucleic acids. *Nucleic Acids Res.* 15, 5373–5390.
- (6) Albrechtsen, C., Kalland, K.-H., Haukanes, B.-I., Håvarstein, L.-S., and Kleppe, K. (1990) Applications of magnetic beads with covalently attached oligonucleotides in hybridization: isolation and detection of specific measles virus mRNA from a crude cell lysate. *Anal. Biochem.* 189, 40–50.
- (7) Lund, V., Schmid, R., Rickwood, D., and Hornes, E. (1988) Assessment of methods for covalent binding of nucleic acids to magnetic beads, Dynabeads, and the characteristics of the bound nucleic acids in hybridization reactions. *Nucleic Acids Res.* 16, 10861–10880.
- (8) Stimpson, D. I., Hoijer, J. V., Hsieh, W. T., Jou, C., Gordon, J., Theriault, T., Gamble, R., and Baldeschwieler, J. D. (1995) Real-time detection of DNA hybridization and melting on oligonucleotide arrays by using optical wave guides. *Proc. Natl. Acad. Sci. U.S.A.* 92, 6379–6383.
- (9) Erout, M.-N., Troesch, A., Pichot, C., and Cros, P. (1996) Preparation of conjugates between oligonucleotides and *N*-vinylpyrrolidone/*N*-acryloxysuccinimide copolymers and applications in nucleic acid assays to improve sensitivity. *Bioconjugate Chem.* 7, 568–575.
- (10) O'Donnell-Maloney, M. J., Smith, C. L., and Cantor, C. R. (1996) The development of microfabricated arrays for DNA sequencing and analysis. *TIBTECH* 14, 401–407.
- (11) Saiki, R., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B., and Ehrlich, H. A. (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* 239, 487–491.
- (12) Yershov, G., Barsky, V., Belgovskiy, A., Kirillov, E., Kreindlin, E., Ivanov, I., Parinov, S., Guschin, D., Drobishev, A., Dubiley, S., and Mirzabekov, A. (1996) DNA analysis and diagnostics on oligonucleotide microchips. *Proc. Natl. Acad. Sci. U.S.A.* 93, 4913–4918.
- (13) Guo, Z., Guilfoyle, R. A., Thiel, A. J., Wang, R., and Smith, L. M. (1994) Direct fluorescence analysis of genetic polymorphisms by hybridization with oligonucleotide arrays on glass supports. *Nucleic Acids Res.* 22, 5456–5465.
- (14) Lamture, J. B., Beattie, K. L., Burke, B. E., Eggers, M. D., Ehrlich, D. J., Fowler, R., Hollis, M. A., Kosicki, B. B., Reich, R. K., Smith, S. R., Varma, R. S., and Hogan, M. E. (1994) Direct detection of nucleic acid hybridization on the surface of a charge coupled device. *Nucleic Acids Res.* 22, 2121–2125.
- (15) Soini, E. (1991) Biospecific multianalyte assay method. U.S. Patent 5,028,545.
- (16) Georges, J. (1993) Lanthanide-sensitized luminescence and applications to the determination of organic analytes. *Analyst* 118, 1481–1486.
- (17) Soini, E., and Lövgren, T. (1987) Time-resolved fluorescence of lanthanide probes and applications in biotechnology. *CRC Crit. Rev. Anal. Chem.* 18, 105–154.
- (18) Hemmilä, I., Dakubu, S., Mikkala, V.-M., Siitari, H., and Lövgren, T. (1984) Europium as a label in time-resolved immunofluorometric assays. *Anal. Biochem.* 137, 335–343.
- (19) Diamandis, E. P., and Christopoulos, T. K. (1990) Europium chelate labels in time-resolved fluorescence immunoassays and DNA hybridization assays. *Anal. Chem.* 62, 1149A–1157A.
- (20) Elbanowski, M., and Makowska, B. (1996) The lanthanides as luminescent probes in investigations of biochemical systems. *J. Photochem. Photobiol., A* 99, 85–92.
- (21) Kwiatkowski, M., Samiotaki, M., Lamminmäki, U., Mikkala, V.-M., and Landegren, U. (1994) Solid-phase synthesis of chelate-labelled oligonucleotides: application in triple-color ligase-mediated gene analysis. *Nucleic Acids Res.* 22, 2604–2611.
- (22) Iitiä, A., Hogdall, E., Dahlen, P., Hurskainen, P., Vuust, J., and Siitari, H. (1992) Detection of mutation $\Delta F508$ in the cystic fibrosis gene allele-specific PCR primers and time-resolved fluorometry. *PCR Methods Appl.* 2, 157–162.
- (23) Dahlen, P., Iitiä, A., Mikkala, V.-M., Hurskainen, P., and Kwiatkowski, M. (1991) The use of europium (Eu^{3+}) labelled primers in PCR amplification of specific target DNA. *Mol. Cell. Probes* 5, 143–149.
- (24) Bush, C. E., Di Michele, L. J., Peterson, W. R., Sherman, D. G., and Godsey, J. H. (1992) Solid-phase time-resolved fluorescence detection of human immunodeficiency virus polymerase chain reaction amplification products. *Anal. Biochem.* 202, 146–151.
- (25) Hakala, H., and Lönnberg, H. (1997) Time-resolved fluorescence detection of oligonucleotide hybridization on a single microparticle: covalent immobilization of oligonucleotides and quantitation of a model system. *Bioconjugate Chem.* 8, 232–237.
- (26) Hakala, H., Heinonen, P., Iitiä, A., and Lönnberg, H. (1997) Detection of oligonucleotide hybridization on a single microparticle by time-resolved fluorometry: hybridization assays on polymer particles obtained by direct solid phase assembly of the oligonucleotide probes. *Bioconjugate Chem.* 8, 378–384.
- (27) Pon, R. T. (1993) in *Methods in Molecular Biology* (S. Agrawal, Ed.) Vol. 20, p 467, Humana Press, Totowa, NJ.
- (28) Mikkala, V.-M., Helenius, M., Hemmilä, I., Kankare, J., and Takalo, H. (1993) Development of luminescent europium(III) and terbium(III) chelates of 2,2':6',2''-terpyridine derivatives for protein labelling. *Helv. Chim. Acta* 76, 1361–1378.
- (29) Lövgren, T., Iitiä, A., Hurskainen, P., and Dahlen, P. (1995) Detection of lanthanide chelates by time-resolved fluorescence. In *Blotting and Sequencing* (L. Kricka, Ed.) pp 350–358, Academic Press, San Diego, CA.
- (30) Lövgren, T., Heinonen, P., Lehtinen, P., Hakala, H., Heinola, J., Harju, R., Takalo, H., Mikkala, V.-M., Schmid, R., Lönnberg, H., Pettersson, K., and Iitiä, A. (1997) Sensitive bioaffinity assays on individual microparticles using time-resolved fluorometry. *Clin. Chem.* 43, 1937–1943.
- (31) Pease, A. C., Solas, D., Sullivan, E. J., Cronin, M. T., Holmes, C. P., and Fodor, S. P. A. (1994) Light-generated oligonucleotide arrays for rapid DNA sequence analysis. *Proc. Natl. Acad. Sci. U.S.A.* 91, 5022–5026.
- (32) Urdea, M. S., Warner, B. D., Running, J. A., Stempien, M., Clyne, J., and Horn, T. (1988) A comparison of non-radioisotopic hybridization assay methods using fluorescent, chemiluminescent and enzyme labeled synthetic oligodeoxyribonucleotide probes. *Nucleic Acids Res.* 16, 4937–4956.

Streptavidin in Antibody Pretargeting. 2. Evaluation of Methods for Decreasing Localization of Streptavidin to Kidney while Retaining Its Tumor Binding Capacity

D. Scott Wilbur,^{*,†} Donald K. Hamlin,[†] Kent R. Buhler,[‡] Pradip M. Pathare,[†] Robert L. Vessella,[‡] Patrick S. Stayton,[§] and Richard To[§]

Departments of Radiation Oncology, Urology, and Bioengineering, University of Washington, Seattle, Washington 98195. Received October 10, 1997; Revised Manuscript Received January 28, 1998

An investigation has been conducted to determine if the kidney localization of recombinant streptavidin can be decreased to improve its characteristics in pretargeting protocols. Three different methods of accomplishing this were evaluated. The first method, blocking kidney uptake with a preadministration of recombinant streptavidin in which biotin occupied all of the binding sites, was unsuccessful. In a second method, L-lysine administration was used to block kidney localization. This method worked well, decreasing the concentration to 29% of the unmodified amount at 8 h postinjection. However, this method suffered from a requirement for constant infusion of lysine during the period of observation. A third method, use of succinylated recombinant streptavidin, was found to be the best approach. Succinylation of streptavidin was readily accomplished with very good protein recovery. With the succinylated streptavidin, the kidney concentration was only 14% of that of nonmodified streptavidin at 4 h postinjection. While these results demonstrated that the concentration of streptavidin could be decreased in the kidney, it was important to assess whether the tumor colocalization of streptavidin with biotinylated antibody was affected under those conditions. As part of our continuing investigation of pretargeting, a new water-solubilized biotinidase-stabilized biotinylation reagent was prepared. Using that reagent in a pretargeting experiment, an equivalent quantity of succinylated recombinant streptavidin as biotinylated antibody Fab' was localized in a tumor xenograft model. In that experiment, the kidney concentration was decreased to less than 10% of that obtained with unmodified recombinant streptavidin at 24 h postinjection. The results of our investigation have demonstrated that succinylation of streptavidin improves its distribution characteristics for pretargeting applications. The fact that succinylated streptavidin has no specific tissue localization should allow its use as a carrier of radioactivity in "two-step" pretargeting protocols.

INTRODUCTION

A number of research groups are investigating a monoclonal antibody (mAb)-based approach for systemic delivery of therapeutic radionuclides to cancer cells in patients termed "tumor pretargeting" (1–15). The pretargeting approach uses mAb conjugates to target cancer cells *in vivo* prior to administration of the radionuclide in a "two-step" or "three-step" protocol (16). The key to the pretargeting approach is that the molecule carrying the radionuclide bind tightly to the preadministered antibody conjugate. Our pretargeting studies have fo-

cused on a three-step approach. In that approach, three reagents are administered as sequential steps in a protocol designed to maximize the tumor radiation dose while minimizing radiation doses to bone marrow and other nontarget tissues. The reagents used in our three-step pretargeting approach include a biotinylated mAb Fab' fragment, recombinant streptavidin (r-SAv), and a radiolabeled biotin derivative. Although only one of the reagents carries the radionuclide, the distributions of all three reagents are important as each reagent binds *in vivo* with the next reagent administered. Thus, the tissue distribution observed for the administered radionuclide is a composite of the combination of distributions of the three components, and the adducts they form *in vivo*.

As part of our pretargeting studies, we have investigated the tissue localization of each of the three components used. Our reasons for conducting the studies are to gain a better understanding of the distribution of radioactivity and to determine if the reagents or conditions used might be optimized to provide more favorable radiation dosimetry for patient therapy. The results obtained from the tissue distribution of one of the components used, r-SAv, indicated that localization to kidney might be a problem in the pretargeting approach (11, 17). Biodistribution studies conducted with radioiodinated r-SAv have shown that a high concentration (25–35% ID/g) is localized in kidney within 4 h of

* Address correspondence to D. Scott Wilbur, Ph.D., Department of Radiation Oncology, University of Washington, 2121 N. 35th St., Seattle, WA 98103-9103. Phone: 206-685-3085. Fax: 206-685-9630. E-mail: dswilbur@u.washington.edu.

[†] Department of Radiation Oncology.

[‡] Department of Urology.

[§] Department of Bioengineering.

¹ Abbreviations: ChT, chloramine-T; cpm, counts per minute; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; HABA, 2-(4'-hydroxyazobenzene)benzoic acid; IEF, isoelectric focusing; *ip*, intraperitoneal; mAb, monoclonal antibody; % ID/g, percent injected dose per gram; PBS, phosphate-buffered saline; *pi*, postinjection; RCC, renal cell carcinoma; r-SAv, recombinant streptavidin; *rt*, room temperature; SAv, streptavidin; *sc*, subcutaneously; *tBoc*, *tert*-butoxycarbonyl; TFA, trifluoroacetic acid; TFP, tetrafluorophenyl; TFP-OTFA, tetrafluorophenyl trifluoroacetate.

injection, and the concentration of radioactivity does not decrease appreciably over a 72 h period. Importantly, no other specific tissue localization appears to occur with r-SAv. Similar kidney localization has been observed for two r-SAv mutants and two commercially available SAv proteins (17). High kidney localization is a general characteristic of SAv proteins, although reported concentrations of SAv in kidney have varied greatly (7, 8, 18–21).

The importance of the concentration of SAv in pretargeting may be questioned since it is not radiolabeled in most protocols. Indeed, if SAv localized in kidney does not result in a specific binding of the radiolabeled biotin molecule in that tissue, then there is no problem. Unfortunately, our (unreported) studies of the localization of radiolabeled biotin molecules have not provided a clear answer to the question of kidney binding of radiolabeled biotin. The results obtained have been complicated by the issue of biotinidase cleavage (22–25) of the biotin derivatives used, by issues relating to endogenous biotin (26), and by the fact that renal clearance is the normal route of excretion of the radiolabeled biotin derivatives. This issue cannot be readily resolved from literature reports either, as many other investigators have not addressed it directly or have not provided data that could be used to assess the localization quantitatively. Recently, Sharkey et al. (15) reported that specific kidney localization occurred when large quantities (i.e. 100–500 μ g) of SAv were administered. Additional studies in our laboratory will be directed at kidney localization of radioiodinated biotin derivatives.

Aside from the issue of localization of radiolabeled biotin in the three-step pretargeting approach, the high kidney concentrations of SAv have kept it from being used as a carrier of radioactivity in a two-step pretargeting approach. Although there are distinct advantages for using radiolabeled biotin derivatives for many radionuclides, it may be advantageous to use a two-step approach with certain radionuclides if kidney localization can be circumvented. For example, the very fast blood clearance of small biotin derivatives results in a low percentage of the injected dose being delivered to the tumor. The longer serum half-life of a radiolabeled SAv molecule would likely result in more of the injected dose being bound at the tumor. This might be a favorable situation for very expensive radionuclides such as the α -emitting therapeutic radionuclide At-211 (27). Decay of the short path length α -emitter in blood might prove to be less toxic than if it is excreted in a high concentration through a critical organ such as liver or kidney.

Our concern regarding kidney localization of radiolabeled biotin, and the desire to use radiolabeled r-SAv as a carrier of radionuclides in pretargeting protocols, led to an investigation of methods of blocking or decreasing the kidney localization of SAv. In the investigation, three different methods were evaluated: (1) blocking with r-SAv that was saturated with biotin, (2) blocking by administering L-lysine, and (3) decreasing the pI of r-SAv through chemical modification. The latter two methods were found to be effective in decreasing kidney localization of radioiodinated r-SAv. On the basis of those results, additional biodistribution studies were conducted to evaluate the tissue distribution and localization of radiolabeled r-SAv to a human tumor xenograft model in athymic mice. The biodistribution results indicated that equivalent tumor concentrations were obtained with L-lysine treatment and through the use of succinylated r-SAv as obtained with the standard protocol. However, chemical modification (succinylation) provided the best

combination of retaining tumor localization while decreasing kidney localization. The results obtained from our investigation of methods for decreasing kidney localization of r-SAv are reported herein.

EXPERIMENTAL PROCEDURES

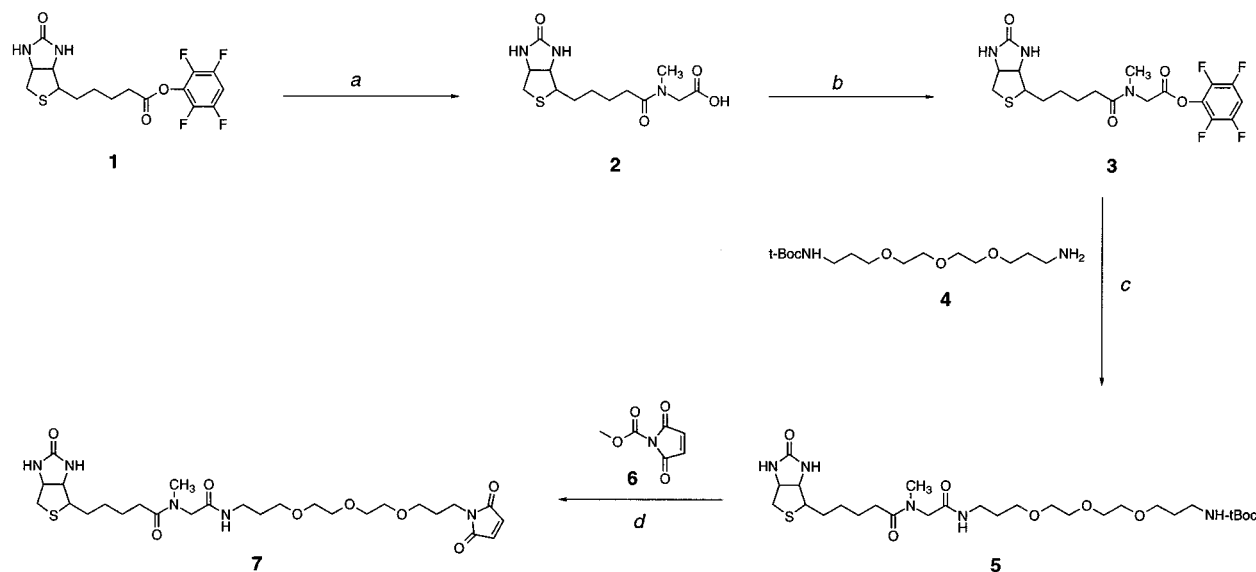
General. All chemicals purchased from commercial sources were analytical grade or better and were used without further purification unless noted. Chloramine-T (ChT), *d*-biotin, and phosphate-buffered saline (PBS) were obtained from Sigma (St. Louis, MO). HABA [2-(4'-hydroxyazobenzene)benzoic acid] and avidin were obtained from Pierce (Rockford, IL). (Methoxycarbonyl)-maleimide, **6**, was obtained from Fluka Chemical Corp. (Ronkonkoma, NY). Succinic anhydride, di-*tert*-butyl dicarbonate, and most other chemicals were obtained from Aldrich Chemical Co. (Milwaukee, WI). Solvents for HPLC analysis were obtained as HPLC grade and were filtered (0.2 μ m) prior to use. Biotin derivatives **1** and **2** (Scheme 1) were prepared as previously described (25). Tetrafluorophenyl trifluoroacetate (TFP-OTFA) was prepared as previously described (28). A6H Fab' was obtained as previously described (11). r-Streptavidin was obtained as previously described (29). Biotin deficient, egg white-enriched (avidin rich) mouse chow (catalog no. 5836 test diet from basal diet catalog no. 5755) was obtained from Purina Test Diets (Richmond, IN). Centricon-10 and Centricon-30 centrifugation concentrators were obtained from Amicon (Beverly, MA). NAP-10 (Sephadex G-25) size exclusion columns were obtained from Pharmacia Biotech AB (Uppsala, Sweden).

Na¹²⁵I and Na¹³¹I were purchased from NEN/Dupont (Billerica, MA) as high-concentration, high-specific activity radioiodide in 0.1 N NaOH. Measurement of ¹²⁵I and ¹³¹I was accomplished on the Capintec CRC-15R or a Capintec CRC-6A radioisotope calibrator. Tissue samples were counted in a LKB 1282 gamma counter with the following window settings: channels 35–102 (20–90 keV) and channels 165–185 (300–450 keV) when ¹²⁵I and ¹³¹I were counted together.

Spectroscopic Data. ¹H NMR spectra were obtained on Bruker AF-200 (200 MHz) instrument. The chemical shifts are expressed as parts per million using tetramethylsilane as an internal standard (δ = 0.0 ppm). IR data were obtained on a Perkin-Elmer 1420 infrared spectrophotometer. Mass spectral data were obtained on a VG 70SEQ mass spectrometer with a 11250J data system. Fast atom bombardment (FAB⁺) mass spectral data were obtained at 8 kV using a matrix of 90% thioglycerol, 9% DMSO, and 1% TFA (DMIX) or propylene glycol 600 containing thioglycolate.

Analytical Chromatography. Equipment. HPLC separations of compounds were obtained on a Hewlett-Packard quaternary 1050 gradient pumping system with a variable wavelength UV detector (254 nm) and a Vares ELSD MKIII evaporative light-scattering detector. Analyses of the HPLC data were conducted on Hewlett-Packard HPLC ChemStation software.

Biotin Compounds. All reactions for synthesizing the biotinylation reagent, **7**, were monitored by HPLC. Reversed-phase HPLC was carried out on an Alltech Altima C-18 column (5 μ m, 250 $\times$ 4.5 mm) using a gradient solvent system at a flow rate of 1 mL/min. Solvent A in the gradient was methanol. Solvent B was aqueous 1% HOAc. Starting from 40% solvent A, the initial solvent mixture was maintained for 2 min, then the gradient was increased linearly to 100% A over the next 10 min, and 100% A was maintained for 5 min.

Scheme 1. Synthesis of the Biotinidase-Stabilized, Water-Solubilized Maleimidobiotin Derivative Used for Biotinylation of A6H Fab' ^a

^a (a) Sarcosine methyl ester, DMF, Et₃N, rt, 2–3 h, 1 N NaOH/MeOH, rt, 1 h; (b) TFP-OAc, Et₃N, 20 min, not isolated; (c) 4, DMF, rt, 1.5 h; (d) TFA, rt, 30 min, saturated NaHCO₃, 0 °C, **6**, 10 min, 57%.

Retention times (*t_R*) for biotin derivatives using these conditions were as follows: **5**, *t_R* = 10.9 min; and **7**, *t_R* = 8.6 min (see Supporting Information).

Proteins. Size exclusion chromatography was performed on all A6H Fab' and r-SAv modifications. Proteins were evaluated on a TosoHaas G3000SW column (10 μm, 7.5 mm × 30 cm) run isocratically at 0.75 mL/min eluting with 50 mM sodium phosphate buffer (pH 6.8) which contained 300 mM NaCl, 1 mM EDTA, and 1 mM sodium azide. Retention times (*t_R*) for the various proteins using this system were as follows: biotinylated A6H Fab', *t_R* = 29.7 min; r-SAv, *t_R* = 28.7 min; perbiotinylated r-SAv, *t_R* = 28.7 min; and succinylated r-SAv, *t_R* = 27.8 min.

Radiolabeled Proteins. Size exclusion chromatography was also used to evaluate the radioiodinated proteins. The equipment and conditions for elution were as previously reported (11).

N-(tert-Butoxycarbonyl)-4,7,10-trioxa-1,13-tridecanediamine (4). To a solution of 4,7,10-trioxa-1,13-tridecanediamine (50.45 g, 229 mmol) in 200 mL of CH₂Cl₂ was added *tert*-butyl dicarbonate (2 g, 9.2 mmol). The reaction mixture was stirred at room temperature for 12 h, and the solvent was removed under vacuum. The residue was extracted into CHCl₃ (300 mL) and was washed with H₂O (2 × 25 mL). The CHCl₃ solution was dried over anhydrous Na₂SO₄, and the CHCl₃ was removed under vacuum to yield 2.74 g (92%) of **4** as an oil. ¹H NMR (MeOH-*d*₄): δ 4.9 (s, 1H), 3.1–3.3 (m, 13H), 2.7 (m, 3H), 2.5 (m, 2H), 1.3–1.5 (m, 4H), 1.1 (s, 9H). IR (Nujol): 3280, 2920, 2860, 1760, 1650, 1450, 1380, 940 cm⁻¹. HRMS: Calcd for C₁₅H₃₂N₂O₅ (M + H) 321.2389, found 321.2395.

13-N-(tert-Butoxycarbonyl)-4,7,10-trioxatridecanyl-1-N-methylglycine Biotinamide (5). To a solution of *N*-methylglycine biotinamide, **2** (0.5 g, 1.59 mmol), in 12 mL of DMF was added TFP-OTFA (0.5 g, 1.92 mmol) followed by Et₃N (200 μL). The reaction mixture was stirred at room temperature for 20 min (monitored by HPLC) to obtain **3**, and then **4** (0.509 g, 1.59 mmol) was added in 5 mL of DMF. The reaction mixture was stirred at room temperature for 0.5 h (monitored by HPLC), and the DMF was removed under vacuum. The residue was

extracted with CHCl₃ (300 mL) and washed with H₂O (2 × 25 mL). The CHCl₃ solution was dried over anhydrous Na₂SO₄ and then evaporated under vacuum. The isolated **5** was dried under vacuum to yield 0.8 g (82%) of an oil. ¹H NMR (MeOH-*d*₄): δ 4.5 (m, 1H), 4.3 (m, 1H), 4.0 (d, *J* = 9.4 Hz, 2H), 3.4–3.7 (m, 14H), 3.1–3.3 (m, 8H), 2.8–3.0 (m, 3H), 2.7 (d, *J* = 13 Hz, 1H), 2.2–2.4 (m, 2H), 1.4–1.8 (m, 19H). IR (Nujol): 3280, 2920, 2860, 1760, 1650, 1450, 1380, 940 cm⁻¹. HRMS: Calcd for C₂₈H₅₁N₅O₈S (M + H) 618.3537, found 618.3530.

13-N-Maleimido-4,7,10-trioxatridecanyl-1-N-methylglycine Biotinamide (7). A 400 mg (0.774 mmol) quantity of biotinamide **5** was dissolved in 2 mL of TFA and the mixture stirred at room temperature for 0.5 h. The TFA was removed under vacuum. The residue was dissolved in 5 mL of saturated aqueous NaHCO₃ and cooled with ice–H₂O. *N*-(Methoxycarbonyl)maleimide, **6** (0.239 g, 1.55 mmol), was added, and the reaction mixture was stirred at 0 °C for 10 min. A 25 mL aliquot of H₂O was added to the reaction mixture, and stirring was continued at room temperature for an additional 15 min. The mixture was extracted with CHCl₃ (4 × 100 mL). The combined CHCl₃ extracts were washed with H₂O (2 × 50 mL) and dried over anhydrous Na₂SO₄, and the CHCl₃ was removed under vacuum. The isolated product was dried under vacuum to yield 220 mg (57%) of **7** as an oil. ¹H NMR (MeOH-*d*₄): δ 6.7 (s, 2H), 4.4 (m, 1H), 4.2 (m, 1H), 3.9 (d, *J* = 10.6 Hz, 2H), 2.9–3.5 (m, 23H), 2.6 (d, *J* = 12.8 Hz, 1H), 2.2–2.4 (m, 2H), 1.3–1.7 (m, 11H). IR (KBr): 3280, 2920, 2860, 1760, 1650, 1450, 1380, 940 cm⁻¹. HRMS: Calcd for C₂₇H₄₄N₅O₈S (M + H) 598.2899, found 598.2910.

Biotinylation of A6H Fab'. To 120 μL of a 17 mg/mL solution of A6H F(ab')₂ (2 mg, 4 × 10⁻⁵ mmol) was added 5 μL of 100 mM DTT in H₂O. After 1 h at room temperature, the entire solution was added to a NAP-10 column that had been equilibrated in 20 mM sodium phosphate buffer (pH 6.5) containing 1 mM EDTA and was eluted with the same buffer. The protein-containing fractions were pooled (monitored by UV). The combined fractions sat at room temperature for 1 h prior to adding 0.24 mg (4 × 10⁻⁴ mmol) of **7** in 100 μL of H₂O. After 30 min at room temperature, the entire mixture was placed

in a Centricon-30 apparatus and concentrated to 200 μ L. That solution was then eluted on a NAP-10 column that had been equilibrated in 100 mM PBS. The protein-containing fractions were pooled and concentrated with a Centricon-30 concentrator. The final concentration was determined by UV (A_{280} ; 1 mg/mL = 1.1). Protein recovery yields for the A6H Fab' biotinylations with **7** have ranged from 71 to 89%. Immunoreactivities of the biotinylated Fab' were assessed as described previously (11) and were found to be equivalent to the those of nonbiotinylated A6H Fab'.

Quantitation of Biotins on A6H Fab'. The number of biotin molecules per protein was determined by measuring the decrease in absorbance at 500 nm for a solution containing avidin and HABA after the biotinylated protein was added. The HABA method used was adapted from a Pierce biotinylation kit (catalog no. 21430). Briefly, to a 1 mL aliquot of 1 mg/mL avidin in PBS was added 0.94 mL of PBS, followed by 60 μ L of 10 mM HABA in 10 mM NaOH for preparing the avidin/HABA solution. The UV absorbance at 500 nm was measured for 900 μ L of the avidin/HABA solution. Then 100 μ L of a diluted biotinylated protein was added. After 1 min, the absorbance was again measured. The number of biotin molecules was determined from the difference in absorbance measured by the following equation (simplified from the Pierce equation). The number of biotins per antibody is $(A/3400)/B$, where $A = [0.9(A_{500} \text{ of the HABA/avidin solution}) - (A_{500} \text{ of the HABA/avidin solution plus the sample})]$ and $B = \text{the sample molar concentration}$. Using this method of assay, the number of biotins per Fab' obtained with **7** ranged from 1.2 to 3.8.

Perbiotinylation of Streptavidin. To 67 μ L of a 15 mg/mL solution of r-SAv (1 mg, 1.9×10^{-5} mmol) was added 200 μ L of a 2.5 mg/mL biotin solution (0.5 mg, 2×10^{-3} mmol) in 20 mM sodium phosphate (pH 6.8). The biotin binding was allowed to proceed at room temperature for 30 min, and then the reaction mixture was added to a Centricon-10 filtration unit and concentrated to 50 μ L. PBS (500 μ L) was added, and the solution was again concentrated to 50 μ L. This wash step was repeated five times to remove excess biotin. The final concentration of perbiotinylated r-SAv was 1.3 mg/mL (A_{280} ; 1 mg/mL = 2.56) with a protein recovery of 59%.

Succinylation of Streptavidin. A 185 μ L aliquot of a 5.4 mg/mL solution of r-SAv (1 mg, 1.9×10^{-5} mmol) was added to 370 μ L of 50 mM NaHCO₃ buffer (pH 8.5). To this solution was added 95 μ g (1×10^{-3} mmol) of succinic anhydride in 20 μ L of DMSO. After 30 min at room temperature, the contents were transferred to a Centricon-10 apparatus and concentrated to 50 μ L. PBS (500 μ L) was added, and the solution was again concentrated to 50 μ L. This wash step was repeated five times. The final concentration was determined to be 3.5 mg/mL with a protein recovery of 88%.

Radioiodination of Proteins. In a typical radioiodination, 2 μ L of Na¹²⁵I or Na¹³¹I in 0.1 N NaOH was added to 25 μ L of sodium phosphate (0.5 M, pH 7.4). To this solution was added 1.0 mg of r-SAv in 185 μ L of PBS, followed by the addition of 20 μ L of a 1 mg/mL ChT solution in H₂O. After 1 min at room temperature, the reaction was quenched by the addition of 2 μ L of a 10 mg/mL aqueous sodium metabisulfite solution. The entire reaction solution was then placed on a NAP-10 column that was equilibrated in 0.9% saline and eluted with 0.9% saline. The protein fractions were combined and the concentrations and specific activities determined using UV₂₈₀ and a dose calibrator.

Labeling Yields. The radiochemical yields for five r-SAv radioiodinations ranged from 73 to 95%, and the specific activity ranged from 0.82 to 1.76 mCi/mg. Succinylated r-SAv was radioiodinated in the same manner with yields of 76–78% (specific activity of 0.47–1.1 mCi/mg). Perbiotinylated r-SAv was radioiodinated with a yield of 80% (specific activity of 1.6 mCi/mg). Radioiodinations of the biotinylated A6H Fab' were also conducted in the same manner, with radiochemical yields ranging from 82 to 87% (specific activities of 0.84–1.3 mCi/mg).

Biodistribution Studies. General. Animal use and procedures were approved by the University of Washington's Animal Care Committee. Animal care and use was conducted in accordance with the NIH guidelines.² Male athymic mice (BALB/c nu/nu), 4–5 weeks of age, were obtained from Simonsen Laboratories (Gilroy, CA). Mice were housed for 1 week in the animal facility prior to beginning the study. All injections of reagents were administered to mice in a total volume of approximately 100 μ L (injectate weighed) via the lateral tail vein. Injections and sacrifice of the animals were done at predetermined times. The tissues excised are shown in the tables, and the average animal weight in a biodistribution and average tumor weights for groups involving mice bearing tumor xenografts are provided in the table footnotes. Blood samples were obtained by cardiac puncture immediately before sacrifice. Urine samples were obtained with a syringe bladder tap at the time the tissues were excised. Excised tissues were blotted free of blood, weighed, and counted. In those studies employing human tumor xenografts, small tumor pieces (5–10 mg) of TK-82 renal cell carcinoma (RCC) (30) were implanted sc over the right shoulder of the mice. The implants were allowed to grow for 3–4 weeks or until the average size was approximately 100 mg. In the studies involving localization of r-SAv to tumors, the mice were placed on a biotin deficient diet 5 days prior to initiation. Calculation of the percent injected dose (%ID) and percent injected dose per gram (%ID/g) in the tissues was based on internal standards containing 1 μ L of the injected dose. The counts obtained were compensated for decay, and where dual radionuclide counting was required, the ¹²⁵I counts were compensated (13.5%) for spillover counts from the ¹³¹I. The total blood volume was estimated to be 8% of body weight for the calculations (31). Statistical analysis of the data was conducted using the paired Student's *t* test. Differences were considered statistically significant when the *p* value was less than 0.04.

r-SAv Blocking Study. In this study, variations of blocking were evaluated in three groups of athymic mice. In group 1, five mice were injected with 15 μ g of r-[¹²⁵I]-SAv, and then at 4 h *pi*, 15 μ g of r-[¹³¹I]-SAv was injected. In group 2, five mice were injected with 15 μ g of perbiotinylated r-[¹²⁵I]-SAv, and then at 4 h *pi*, 15 μ g of r-[¹³¹I]-SAv was injected. In group 3, five mice were co-injected with 15 μ g of perbiotinylated r-[¹²⁵I]-SAv and 15 μ g of r-[¹³¹I]-SAv was injected. Mice were sacrificed at 28 h *pi* for groups 1 and 2 and 24 h *pi* for group 3. Biodistribution results from this study are provided in Table 1.

L-Lysine Blocking Studies. In the initial study, L-lysine blocking was evaluated in two groups of five athymic mice. All mice were injected with 30 μ g of r-[¹³¹I]-SAv and sacrificed 5 h *pi*. The blocking group was treated with

² NIH guidelines are described in NIH Publication 86-23 (Guide for the Care and Use of Laboratory Animals).

Table 1. Distribution of Radioactivity in Athymic Mice after Administration of r-[¹²⁵I]SAv or Perbiotinylated r-[¹²⁵I]SAv Followed by Administration of r-[¹³¹I]SAv^a

tissue	group 1 ^b		group 2 ^c		group 3 ^d	
	r-[¹²⁵ I]SAv	r-[¹³¹ I]SAv	r-[¹²⁵ I]SAv and biotin	r-[¹³¹ I]SAv	r-[¹²⁵ I]SAv and biotin	r-[¹³¹ I]SAv
blood	1.48 ± 0.17	1.99 ± 0.35	1.56 ± 0.14	2.13 ± 0.28	2.51 ± 0.27	2.49 ± 0.26
muscle	0.57 ± 0.08	0.67 ± 0.07	0.63 ± 0.09	0.69 ± 0.07	0.85 ± 0.09	0.81 ± 0.09
lung	1.89 ± 0.26	2.19 ± 0.27	2.22 ± 0.24	2.46 ± 0.30	2.96 ± 0.51	2.86 ± 0.50
kidney	13.32 ± 2.29	18.71 ± 4.45	12.46 ± 1.91	17.63 ± 2.14	17.93 ± 2.98	21.10 ± 3.47
spleen	2.21 ± 0.27	2.56 ± 0.32	2.45 ± 0.14	2.71 ± 0.22	2.96 ± 0.47	2.86 ± 0.42
liver	1.80 ± 0.21	2.20 ± 0.29	1.92 ± 0.17	2.24 ± 0.26	2.65 ± 0.32	2.56 ± 0.31
intestine	0.66 ± 0.08	0.79 ± 0.08	0.80 ± 0.12	0.92 ± 0.10	1.35 ± 0.08	1.30 ± 0.07
urine ^e	9.78 ± 0.93	11.18 ± 1.34	6.96 ± 3.97	10.22 ± 5.7	10.41 ± 2.65	11.48 ± 2.53
neck	2.78 ± 0.54	2.95 ± 0.41	2.83 ± 0.17	3.07 ± 0.30	3.54 ± 0.44	3.93 ± 0.95
stomach	1.39 ± 0.13	1.69 ± 0.22	1.40 ± 0.09	1.72 ± 0.17	1.79 ± 0.25	2.25 ± 0.36

^a Values shown are the percentage of the injected dose per gram ± standard deviation. Mice were on a biotin deficient diet for 4 days prior to the study. Data were obtained for five mice per group; the average animal weight was 27.69 ± 1.65 g. Injections were made in the lateral tail vein; all animals received a total of 30 μg of r-SAv in approximately 100 μL of 0.9% sterile saline. ^b Each animal received 15 μg of [¹²⁵I]SAv with a specific activity of 1.76 μCi/μg and after 4 h 15 μg of [¹³¹I]SAv with a specific activity of 1.15 μCi/μg. Animals were sacrificed 24 h after the second injection. ^c Each animal received 15 μg of perbiotinylated [¹²⁵I]SAv with a specific activity of 1.6 μCi/μg and after 4 h 15 μg of [¹³¹I]SAv with a specific activity of 1.15 μCi/μg. Animals were sacrificed 24 h after the second injection. ^d Each animal received a co-injection of 15 μg of perbiotinylated [¹²⁵I]SAv and 15 μg of [¹³¹I]SAv. Animals were sacrificed at 24 h postinjection. ^e Urine was collected with a syringe bladder tap after sacrifice. Statistical analysis of the controls r-[¹³¹I]SAv (groups 1 and 2), the pretreatment reagents, r-[¹²⁵I]SAv (group 1) and r-[¹²⁵I]SAv and biotin (group 2), and the co-injected reagents, r-[¹²⁵I]SAv and biotin and r-[¹³¹I]SAv (group 3), produced no significant differences in the data when $p < 0.04$.

Table 2. Comparison of the Biodistribution of r-[¹³¹I]SAv (Study 1) with r-[¹³¹I]SAv after L-Lysine Treatment and (Study 2) with Succinylated r-[¹²⁵I]SAv in Athymic Mice^a

tissue	study 1 (lysine treatment) ^b		study 2 (succinylation) ^c	
	r-[¹³¹ I]SAv	r-[¹³¹ I]SAv (with lysine)	r-[¹³¹ I]SAv	r-[¹²⁵ I]SAv-succ
blood	11.38 ± 1.96	10.29 ± 0.48	3.86 ± 1.23	6.17 ± 0.92
muscle	1.52 ± 0.44	1.17 ± 0.26	0.88 ± 0.27	0.99 ± 0.34
lung	7.83 ± 3.39	6.15 ± 0.53	3.76 ± 0.83	4.44 ± 1.08
kidney	16.01 ± 4.70	4.67 ± 0.38	16.10 ± 2.88	2.24 ± 0.03
spleen	2.99 ± 0.61	2.79 ± 0.24	2.38 ± 0.46	2.97 ± 0.70
liver	2.67 ± 0.44	2.65 ± 0.23	1.18 ± 0.03	1.77 ± 0.20
intestine	0.79 ± 0.45	0.65 ± 0.25	1.65 ± 0.12	1.89 ± 0.06
urine ^d	6.80 ± 4.92	33.89 ± 21.46	30.48 ± 4.36	9.60 ± 2.02
neck	4.64 ± 1.60	3.57 ± 0.36	3.07 ± 0.40	3.58 ± 0.75
stomach	2.79 ± 0.83	2.11 ± 0.27	1.95 ± 0.32	2.26 ± 0.61

^a Values shown are the percentage of the injected dose per gram ± standard deviation; Injections were made in the lateral tail vein. ^b Two groups of animals (five in each group) were used; the average animal weight was 29.99 ± 2.38 g. A 30 μg quantity of r-SAv was injected into each mouse. One group (control) had no L-lysine administered, and the second group was injected intravenously with 30 mg of L-lysine at $t = 0, 1, 2$, and 3 h. All animals were sacrificed 5 h after the initial injection. ^c Data were obtained for five mice 4 h after co-injection of 15 μg of r-[¹³¹I]SAv and 15 μg of succinylated r-[¹²⁵I]SAv in a 100 μL volume; the average animal weight was 23.34 ± 1.59 g. ^d Urine was collected with a syringe bladder tap after sacrifice. Statistical analysis was not conducted on these data due to the differences in the study parameters.

30 mg of L-lysine by *ip* injection³ at 10 min prior to administration of r-[¹³¹I]SAv and at 1, 2, and 3 h *pi*. The results from this study are provided in Table 2 (study 1). A second study involved athymic mice bearing the TK-82 human RCC xenografts. Two groups of five mice were used (with and without the L-lysine treatment). All mice were administered 30 μg of biotinylated A6H [¹²⁵I]-Fab', and after 24 h, 30 μg of r-[¹³¹I]SAv was administered. Half of the mice (group 2) were treated with four injections of 30 mg of L-lysine each (in 100 μL of saline) at 10 min prior to and 1, 2, and 3 h after injection of r-[¹³¹I]SAv. All animals were sacrificed at 48 h *pi* of the

Table 3. Distribution of Radioactivity for A6H [¹²⁵I]Fab' and r-[¹³¹I]SAv with and without L-Lysine Treatment in Athymic Mice Bearing Human Tumor Xenografts^a

tissue	group 1 (no lysine) ^d		group 2 (with lysine) ^d	
	[¹²⁵ I]Fab'	r-[¹³¹ I]SAv	[¹²⁵ I]Fab'	r-[¹³¹ I]SAv
blood	2.03 ± 0.53	2.98 ± 0.70	2.03 ± 0.36	2.60 ± 0.37
tumor ^b	12.30 ± 1.41	12.11 ± 1.19	11.25 ± 1.41	10.57 ± 1.53
muscle	0.16 ± 0.03	0.62 ± 0.09	0.11 ± 0.10	0.49 ± 0.09
lung	1.02 ± 0.25	2.29 ± 0.43	0.98 ± 0.13	1.79 ± 0.19
kidney	0.40 ± 0.09	28.30 ± 6.05*	0.50 ± 0.05	11.50 ± 2.22*
spleen	0.45 ± 0.12	2.63 ± 0.49	0.46 ± 0.09	2.51 ± 0.26
liver	0.38 ± 0.09	2.37 ± 0.42	0.36 ± 0.08	2.16 ± 0.29
intestine	0.25 ± 0.05	0.88 ± 0.14 ⁺	0.21 ± 0.02	0.64 ± 0.07 ⁺
urine ^c	4.75 ± 1.45	8.72 ± 2.05 [#]	6.42 ± 2.00	6.07 ± 0.59 [#]
neck	4.67 ± 8.20	2.75 ± 0.42 [*]	3.73 ± 6.24	1.93 ± 0.42 [*]
stomach	0.92 ± 0.20	1.48 ± 0.25 [@]	1.16 ± 0.47	0.83 ± 0.10 [@]

^a Values shown are the percentage of the injected dose per gram ± standard deviation. Injections were made in the lateral tail vein. Mice were on a biotin deficient diet for 5 days prior to the study. Data were obtained for five mice per group. The average animal weight was 28.72 ± 2.04 g; the average tumor weight was 0.210 ± 0.033 g. Animals received 30 μg of biotinylated [¹²⁵I]Fab' in approximately 100 μL of 0.9% sterile saline and 24 h later 30 μg of r-[¹³¹I]SAv in 100 μL of 0.9% sterile saline. Animals in group 2 received intravenous injections of 30 mg of L-lysine in 200 μL of saline at -0.1, 1, 2, and 3 h after the injection of r-[¹³¹I]SAv. All animals were sacrificed 48 h after the Fab' injection. ^b Tumor xenografts were TK-82 renal cell carcinoma. ^c Urine was collected with a syringe bladder tap after sacrifice. ^d Statistical analysis comparing the two [¹²⁵I]Fab' data sets and comparing the r-[¹³¹I]SAv with and without L-lysine data sets indicated that there were no significant differences (where $p < 0.04$) except for the following: * $p = 0.0004$, ⁺ $p = 0.01$, [#] $p = 0.02$, ^{*} $p = 0.01$, and [@] $p = 0.0007$.

Fab' (24 h *pi* of r-SAv). The results from this study are provided in Table 3.

Succinylated r-SAv Studies. In the initial study, one group of five mice were co-injected with succinylated r-[¹²⁵I]SAv and r-[¹³¹I]SAv and then sacrificed 4 h *pi*. The biodistribution data are shown in Table 2 (study 2). A second study involved athymic mice bearing TK-82 human RCC xenografts. Two groups of five mice were used (comparing succinylated r-SAv with r-SAv). All mice were administered 30 μg of biotinylated A6H [¹²⁵I]-Fab', and after 24 h, 30 μg of r-[¹³¹I]SAv or succinylated r-[¹³¹I]SAv was administered. All animals were sacrificed at 48 h *pi* of the Fab'.

³ No difference was noted in kidney localization of r-SAv whether the L-lysine was administered by intraperitoneal or by intravenous injection (unreported results).

RESULTS

Preparation of Reagents. *Biotinylation of r-SAv.* Saturation of the biotin binding sites of r-SAv was accomplished by addition of 100 molar equiv of biotin and incubating at room temperature for 30 min. The excess biotin was removed by exchanging the solvent (PBS) five times in size exclusion ultrafiltration concentrators.

Synthesis of Biotinylation Reagent (7). We have previously described the synthesis and application of a water-solubilized, sulfhydryl-reactive biotinylation reagent (11). However, that reagent is not stabilized toward cleavage by the enzyme biotinidase (25). Due to the concern for biotinidase cleavage of our biotinylation reagent, and other commercially available reagents, we designed and synthesized another water-solubilized, sulfhydryl-reactive biotinylation reagent that also contains a *N*-methyl moiety for blocking biotinidase. The synthesis of the new biotinylation reagent is shown in Scheme 1. Synthesis of biotin derivatives 1–3 has been previously reported (25). Reaction of the tetrafluorophenyl (TFP) ester of *N*-methylglycine adduct 3 (formed in situ) with mono-*t*Boc protected 4,7,10-trioxa-1,13-tridecanediamine, 4, yielded biotin derivative 5 in high yield. Reaction of 5 with trifluoroacetic acid provided the deprotected biotin derivative with a free terminal amine, and reaction of that compound with (methoxycarbonyl)maleimide, 6, gave the desired maleimidobiotin derivative, 7, in good yield.

Biotinylation of A6H Fab'. This procedure was conducted as previously described for the non-biotinidase-stabilized biotinylation reagent (11). A6H Fab' was prepared by reductive cleavage of A6H F(ab')₂ with DTT over a 1 h period. After the excess DTT was removed and a period of 1 h passed, 10 molar equiv of the biotinylation reagent, 7, was added. Recovery of biotinylated Fab' was high. All characterizations (HPLC, SDS–PAGE, IEF, and immunoreactivity assay) of the biotinylated Fab' indicated that biotinylation had a minimal effect on it.

Succinylation of r-SAv. Succinylation of r-SAv was accomplished by reacting 50 molar equiv of succinic anhydride in aqueous solution at a pH of 8.5 or 9.5 for 30 min at room temperature. The succinylation decreased the *pI* of r-SAv from 7–7.3 (17) to 4.0–4.3 as shown in the scanned IEF gel provided as Figure 1. As no difference in *pI* or protein heterogeneity was noted between the succinylations at the two pH values, the pH of choice for running the reaction is 8.5. Good recovery of succinylated r-SAv was obtained after removing the excess succinic anhydride/succinic acid. Assessment of the biotin binding capacity of the succinylated r-SAv indicated that it had not changed from the nonmodified r-SAv (data not shown).

Kidney Blocking with r-SAv and Perbiotinylated r-SAv. Blocking of kidney localization of r-SAv was evaluated in athymic mice. Three different combinations of reagents and conditions were evaluated for decreasing kidney concentrations of r-SAv by preinjected r-SAv. These combinations included (1) pre-injection of an equal amount (i.e. 15 μ g) of r-SAv 4 h prior to a second injection, (2) pre-injection of an equal amount of r-SAv that had been saturated with biotin (perbiotinylated) 4 h prior to the second injection, and (3) co-injection of equal quantities of r-SAv and perbiotinylated r-SAv. Dual isotope labeling allowed us to distinguish the two r-SAv proteins in vivo. The distribution of radioactivity was evaluated 24 h after the first injection. The results are shown in Table 1 as group 1–3, respectively. The results in group

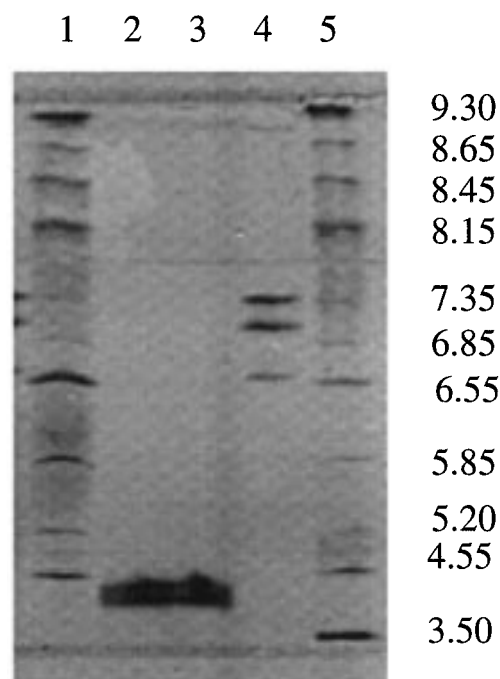


Figure 1. Digitized image of an IEF gel showing succinylated and nonsuccinylated r-SAv. The gel (PhastGel IEF 3–9) was run on a PhastSystem electrophoresis apparatus (Pharmacia LKB, Uppsala, Sweden) using the optimized method for IEF described in Separation Technique File #100. Protein staining was accomplished by the silver stain method described in Development Technique File #210: lane 1, IEF standards (Isoelectric Calibration Kit, broad *pI* of 3–10) run in the opposite direction of application of the sample (e.g. top to bottom); lane 2, r-SAv succinylated at pH 8.5; lane 3, r-SAv succinylated at pH 9.5; lane 4, r-SAv that has not been modified; and lane 5, IEF standards run in the direction of application (bottom to top).

1 show that pre-injection of r-SAv does not affect the distribution of a second injection of r-SAv 4 h later. Similarly, results from group 2 indicate that pre-injection of r-SAv which has been saturated with biotin does not affect the distribution of a second injection of r-SAv 4 h later. Co-injection of perbiotinylated r-SAv with r-SAv demonstrated that they have essentially identical distributions.

L-Lysine Blocking of Kidney Localization. Two experiments were conducted to assess blocking of kidney localization of r-SAv with the use of L-lysine administration. The first experiment was conducted to determine if four hourly injections (at *t* = –10 min and 1, 2, and 3 h) of 30 mg of L-lysine hydrochloride would decrease the kidney concentration of r-[¹³¹I]SAv in athymic mice at 5 h *pi*. A dramatic drop in kidney concentrations was obtained (16–4.7% ID/g) without affecting the distribution in other tissues (Table 2, study 1). Because of the encouraging results obtained, a second study was conducted to determine if the lysine treatment affected the tumor localization properties of r-SAv in a pretargeting protocol. In the study, two groups of athymic mice bearing RCC tumor xenografts were pretargeted with biotinylated A6H [¹²⁵I]Fab' over a 24 h period, and then both groups were administered r-[¹³¹I]SAv. In one group, L-lysine was administered just prior to the administration of r-[¹³¹I]SAv and administered again 1, 2, and 3 h later. The results of that study (Table 3) clearly demonstrated that tumor localization of r-[¹³¹I]SAv was equivalent to the tumor localization of biotinylated [¹²⁵I]Fab' with or without lysine treatment. The localization of r-[¹³¹I]SAv was also equivalent between the groups of mice in most

Table 4. Distribution of Radioactivity for A6H [¹²⁵I]Fab' and r-[¹³¹I]SAv or Succinylated r-[¹³¹I]SAv in Athymic Mice Bearing Human Tumor Xenografts^a

tissue	group 1 (no succinylation) ^d		group 2 (with succinylation) ^d	
	[¹²⁵ I]Fab'	r-[¹³¹ I]SAv	[¹²⁵ I]Fab'	r-[¹³¹ I]SAv-succ
blood	1.89 ± 0.30	2.99 ± 0.63*	1.64 ± 0.28	4.27 ± 0.84*
tumor ^b	16.48 ± 3.41	14.76 ± 3.56	17.96 ± 2.86	16.16 ± 1.94
muscle	0.15 ± 0.02	0.68 ± 0.09 ⁺	0.14 ± 0.02	1.03 ± 0.18 ⁺
lung	1.08 ± 0.26	2.53 ± 0.47	0.96 ± 0.25	3.26 ± 0.79
kidney	0.55 ± 0.09	28.69 ± 8.21 [#]	0.70 ± 0.13	2.44 ± 0.45 [#]
spleen	0.47 ± 0.07	2.88 ± 0.65	0.42 ± 0.10	3.36 ± 0.71
liver	0.39 ± 0.05 ^{&}	2.72 ± 0.22	0.29 ± 0.05 ^{&}	3.28 ± 0.56
intestine	0.30 ± 0.04	0.99 ± 0.15	0.27 ± 0.07	1.30 ± 0.34
urine ^c	4.17 ± 1.71	10.78 ± 3.42	15.64 ± 27.14	10.34 ± 6.36
neck	6.02 ± 6.09	3.38 ± 0.65	5.91 ± 6.34	4.16 ± 1.12
stomach	1.61 ± 0.21	2.25 ± 0.42	1.53 ± 0.32	2.68 ± 0.60

^a Values shown are the percentage of the injected dose per gram ± standard deviation. Injections were made in the lateral tail vein. Mice were on a biotin deficient diet for 5 days prior to the study. Data were obtained for five mice in each group. The average animal weight was 26.34 ± 2.12 g; the average tumor weight for group 1 was 0.210 ± 0.167 g, and the average tumor weight for group 2 was 0.110 ± 0.033 g. Both groups of animals received 30 μg of biotinylated [¹²⁵I]Fab' in approximately 100 μL of 0.9% sterile saline and 24 h later 30 μg of r-[¹³¹I]SAv or succinylated r-[¹³¹I]SAv in 100 μL of 0.9% sterile saline. All animals were sacrificed 48 h after the Fab' injection. ^b Tumor xenografts were TK-82 renal cell carcinoma. ^c Urine was collected with a syringe bladder tap after sacrifice. ^d Statistical analysis comparing the two [¹²⁵I]Fab' data sets and comparing the r-[¹³¹I]SAv/r-[¹³¹I]SAv-succ data sets indicated that there were no significant differences (where $p < 0.04$) except for the following: * $p = 0.03$, ⁺ $p = 0.005$, [#] $p = 0.0001$, and [&] $p = 0.02$.

other tissues. The relevant exception was the kidney, which was decreased from 28 to 11% ID/g.

Decreasing Kidney Concentration with Succinylated r-SA_v. Succinylated r-SA_v was evaluated as a reagent for decreasing kidney localization in two animal distribution studies. The initial study compared the tissue distribution of dual isotope-labeled, co-injected succinylated r-[¹²⁵I]SAv with that of unmodified r-[¹³¹I]SAv. The results of that study are shown in Table 2 (study 2). A dramatic decrease in the kidney concentration of the succinylated r-SA_v was noted in comparison with that of the unmodified r-SA_v (i.e. 16% ID/g decreased to 2% ID/g). The decreased kidney localization was accompanied by an increase in the blood concentration of the succinylated r-SA_v. In a second study, the application of succinylated r-SA_v in tumor pretargeting was compared with application of unmodified r-SA_v. The results of that study are shown in Table 4. In both study groups, the tumor localization of r-SA_v was equivalent to that of the A6H Fab'. Most importantly, the succinylated r-SA_v retained equivalent tumor localization while decreasing the kidney localization from 29% ID/g to 2% ID/g.

DISCUSSION

Streptavidin is an important component in many of the "tumor pretargeting" studies being conducted throughout the world (3, 5–13, 15). Although we use recombinant SA_v in our studies, all of the truncated or "core" SA_v proteins evaluated in our laboratory appear to have similar distributions (17). The prominent feature in the SA_v distribution is its propensity to localize in kidney. This kidney localization of SA_v is of concern when it is used in pretargeting, as it may cause an increased localization of radiolabeled biotin in that tissue. Further, the high kidney localization of SA_v has precluded its use as the carrier of radionuclides. This is unfortunate as

there may be advantages to using radiolabeled SA_v rather than a radiolabeled biotin derivative. These factors led to our investigating methods of reducing the propensity for SA_v to localize in kidney.

Renal accumulation of SA_v most likely proceeds in the same manner as that of other small proteins, i.e. glomerular filtration followed by uptake in proximal tubular cells (32). Although the tubular uptake of small proteins is considered a high-capacity process, some proteins may interact with receptors on the peritubular side. Depending on the rate of endocytosis of a receptor-bound SA_v molecule, it could be available for binding with the administered radiolabeled biotin molecule. We were particularly concerned about a slow endocytosis process as we had noted in previous biodistributions of SA_v proteins that the concentration of r-[¹³¹I]SA_v did not change appreciably from the 4 h to the 24 or 48 h time points (11, 17). While this could be due to a slow catabolism in the lysosomes of tubular cells, there could also be a receptor-mediated process for internalizing SA_v. Further, if SA_v proved to be saturable, administration of SA_v that had all the biotin binding sites occupied (perbiotinylated) would preclude binding of radiolabeled biotin molecules. Wilchek et al. (33, 34) have postulated that renal accumulation of SA_v is related to the "universal recognition sequence" (RGD) which is present in fibronectin and other adhesion proteins that bind with cell surface integrins. Their hypothesis is that the RYD sequence found in SA_v mimics the RGD receptor domain of fibronectin and could cause receptor binding with tubular cells (33). However, they also recognized that the kidney accumulation could simply be due to a slow degradation of the proteolytically stable SA_v in lysosomes (34). The results of the studies reported herein appear to indicate that the long retention time of radioiodinated SA_v in kidney is due to its resistance to degradation. The kidney blocking studies using preadministered r-SA_v clearly show that, if it is a receptor-mediated process, it is not saturable at the quantities investigated. The total amount accumulated in the kidney appears to be dependent on the initial blood concentration.

Another hypothesis for renal tubular reabsorption of proteins was presented by Mogensen and Solling (35). They believed that the "initial event in the normal tubular protein reabsorption is binding between a free positive amino- or guanidino-group in the protein molecule and a negative site on the tubular cell surface". This hypothesis led to the discovery that lysine and other positively charged substances could block tubular absorption of proteins. The fact that this methodology for blocking tubular absorption could be applied to humans without undue toxicity (36–40) provided an impetus for evaluating its use in decreasing kidney accumulation of antibody Fab (41–44) and Fv (45) fragments. Our studies demonstrated that L-lysine also blocks the absorption of SA_v. Although administration of L-lysine did not affect the tumor localization of r-SA_v, it has a major shortcoming in that a constant infusion of a high concentration is necessary to keep the kidney localization low.

Constant infusion of L-lysine was not desired, so we continued to investigate alternative methods for decreasing kidney accumulation of SA_v. We had noted in an earlier investigation of r-SA_v distribution in mice that radioiodination with *N*-hydroxysuccinimidyl *p*-[¹²⁵I]iodobenzoate ([¹²⁵I]PIB) that there was a significant decrease (i.e. by 25%) in the kidney accumulation compared to that of the same protein labeled by the chloramine-T method (17). It appeared that the only difference in these two

proteins was that one was modified by adding an iodine atom to tyrosine (ChT-labeled) and the other was modified by conjugation on a lysine amine (PIB-labeled). This led us to hypothesize that chemical modification of the surface lysine amines had resulted in decreased kidney localization. It was known that glomerular permeability of macromolecules is a function of their overall charge. Positively charged macromolecules cross the glomerular wall more readily than neutral macromolecules, and negatively charged macromolecules are restricted from crossing (46, 47). On the basis of this, we thought that chemical modification of r-SAv that makes it more anionic (i.e. lower *pI*) might block its accumulation in kidney.

Rosebrough and Hartley previously described (48) chemical modification of SAv and avidin to evaluate the effect on their *in vivo* properties. Galactosylation of amino groups on SAv was shown to decrease its *pI* to less than 5.1. They noted that galactosylated SAv had increased hepatic uptake (as expected) and retained its biotin binding capacity when less than 50 equiv of the galactosylation reagent was used, but they did not indicate how this affected kidney localization. Because of the highly positive nature of avidin [e.g. *pI* = 10 (49)], they modified the amino groups with the *N*-hydroxysuccinimide ester of acetic acid (NHS-Ac). Modification with 50–75 equiv of NHS-Ac resulted in decreasing the *pI* to below 4.7. They also evaluated the distribution of avidin that had been deglycosylated. The combination of deglycosylation and treatment of NHS-Ac resulted in low levels of avidin accumulating in liver and kidney. Further evidence that modification of the lysine amines might result in decreased kidney accumulation of SAv was obtained from studies of avidin that was modified by succinic anhydride reported by Jeong et al. (50). They demonstrated that conjugation of succinic acid moieties with lysine amines decreased the *pI* of avidin to 4.0–4.8 and decreased the kidney concentrations from 19 to 1.7% ID/g. Prior to those studies, succinylation of avidin had been shown to reduce its binding with membranes without significantly altering its biotin binding properties (51). Our studies with succinylated r-SAv demonstrated a dramatic decrease in kidney localization. Most importantly, no difference was noted in binding with biotinylated Fab' localized in a tumor xenograft model between succinylated and unmodified r-SAv.

Summary. Three methods of decreasing kidney localization of r-SAv were examined in this investigation. The first method investigated, pre-injection of a dose of r-SAv that was saturated with biotin, did not alter the kidney uptake. The second method investigated, use of four hourly injections of L-lysine, was successful in reducing kidney localization. However, when the lysine dosing was discontinued, the kidney concentrations of r-SAv rose. The third method investigated, chemical modification of r-SAv through succinylation of lysine amines, demonstrated a dramatic and prolonged decrease in kidney concentrations of r-SAv. Both lysine treatment and succinylation of r-SAv had no effect on its binding with biotinylated Fab' in the tumor. On the basis of the results of this study, succinylated r-SAv will be used in our pretargeting protocols. The decrease in kidney accumulation will alleviate the concerns about binding of radiolabeled biotin derivatives and will permit its use as a carrier of radioactivity when that is desired.

ACKNOWLEDGMENT

We are grateful for the generous financial support provided by the Department of Energy, Medical Applica-

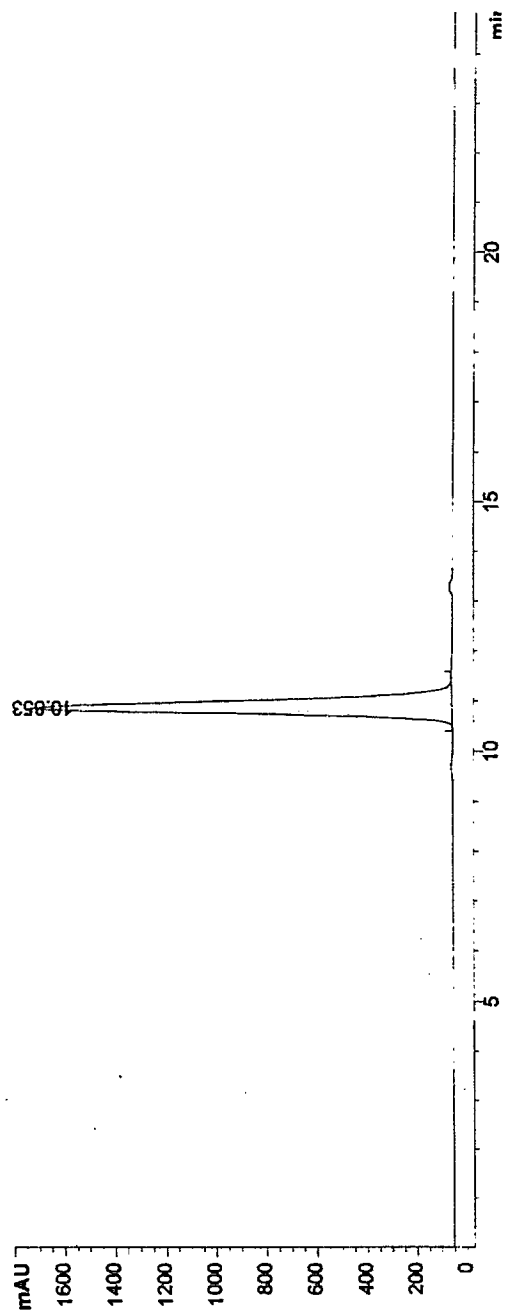
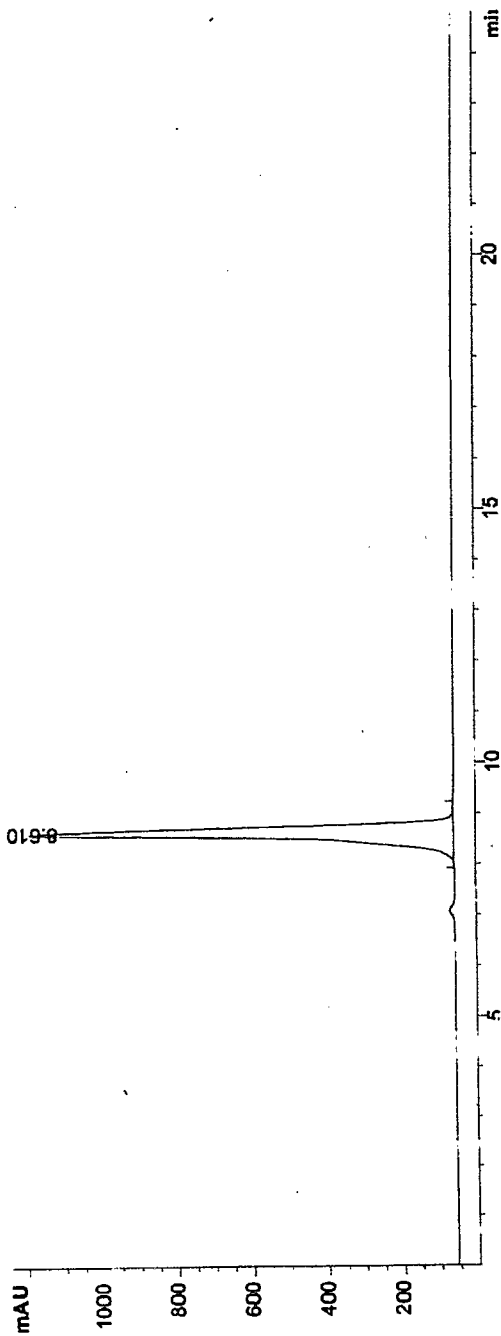
tions and Biophysical Research Division, Office of Health and Environmental Research, under Grant DE-FG06-95ER62029.

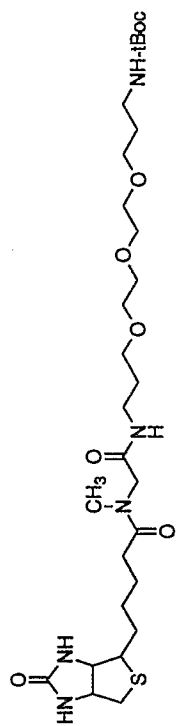
Supporting Information Available: HPLC chromatograms and ¹H NMR spectra of the new compounds (5 and 7) prepared in this paper (3 pages). Ordering information is given on any current masthead page.

LITERATURE CITED

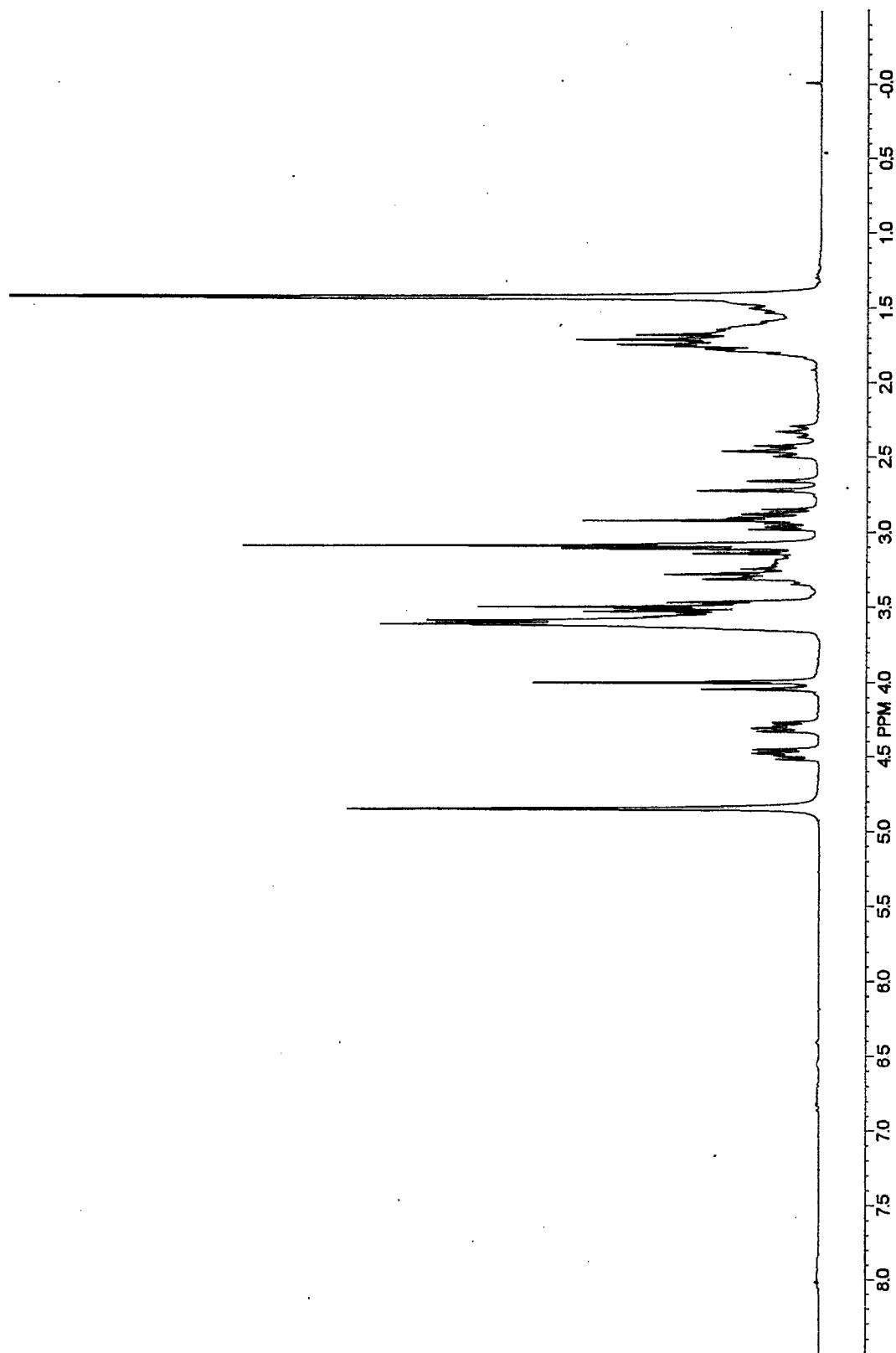
- (1) Hnatowich, D. J., Virzi, F., and Rusckowski, M. (1987) Investigations of Avidin and Biotin for Imaging Applications. *J. Nucl. Med.* 28, 1294–1302.
- (2) Goodwin, D. A., Meares, C. F., McCall, M. J., McTigue, M., and Chaovapong, W. (1988) Pre-Targeted Immunoscintigraphy of Murine Tumors with Indium-111-Labeled Bifunctional Haptens. *J. Nucl. Med.* 29, 226–234.
- (3) Axworthy, D. B., Fritzberg, A. R., Hylarides, M. D., Mallett, R. W., Theodore, L. J., Gustavson, L. M., Su, F.-M., Beaumier, P. L., and Reno, J. M. (1994) Preclinical Evaluation of an Anti-tumor Monoclonal Antibody/Streptavidin Conjugate For Pretargeted ⁹⁰Y Radioimmunotherapy in a Mouse Xenograft Model. *J. Immunother.* 16, 158.
- (4) del Rosario, R. B., and Wahl, R. L. (1993) Biotinylated Iodopolylysine for Pretargeted Radiation Delivery. *J. Nucl. Med.* 34, 1147–1151.
- (5) Oehr, P., Westermann, J., and Biersack, H. J. (1988) Streptavidin and Biotin as Potential Tumor Imaging Agents. *J. Nucl. Med.* 29, 728–729.
- (6) Hashmi, M., and Rosebrough, S. F. (1995) Synthesis, Pharmacokinetics, and Biodistribution of ⁶⁷Ga Deferoxamineacetyl-Cysteinylobiotin. *Drug Metab. Dispos.* 23, 1362–1367.
- (7) Pimm, M. V., Fells, H. F., Perkins, A. C., and Baldwin, R. W. (1988) Iodine-131 and indium-111 labelled avidin and streptavidin for pre-targeted immunoscintigraphy with biotinylated anti-tumour monoclonal antibody. *Nucl. Med. Commun.* 9, 931–941.
- (8) Khawli, L. A., Alauddin, M. M., Miller, G. K., and Epstein, A. L. (1993) Improved Immunotargeting of Tumors with Biotinylated Monoclonal Antibodies and Radiolabeled Streptavidin. *Antibody, Immunoconjugates, Radiopharm.* 6, 13–27.
- (9) Kassis, A. I., Jones, P. L., Matalka, K. Z., and Adelstein, S. J. (1996) Antibody-Dependent Signal Amplification in Tumor Xenografts after Pretreatment with Biotinylated Monoclonal Antibody and Avidin or Streptavidin. *J. Nucl. Med.* 37, 343–352.
- (10) Yao, Z., Zhang, M., Kobayashi, H., Sakahara, H., Nakada, H., Yamashina, I., and Konishi, J. (1995) Improved Targeting of Radiolabeled Streptavidin in Tumors Pretargeted with Biotinylated Monoclonal Antibodies through an Avidin Chase. *J. Nucl. Med.* 36, 837–841.
- (11) Wilbur, D. S., Hamlin, D. K., Vessella, R. L., Stray, J., Buhler, K. R., Stayton, P., Klumb, L., Pathare, P. M., and Weerawarna, S. A. (1996) Antibody Fragments in Tumor Pretargeting. Evaluation of Biotinylated Fab' Co-localization with Recombinant Streptavidin and Avidin. *Bioconjugate Chem.* 7, 689–702.
- (12) Sung, C., van Osdol, W. W., Saga, T., Neumann, R. D., Dedrick, R. L., and Weinstein, J. N. (1994) Streptavidin Distribution in Metastatic Tumors Pretargeted with a Biotinylated Monoclonal Antibody: Theoretical and Experimental Pharmacokinetics. *Cancer Res.* 54, 2166–2175.
- (13) Alvarez-Diez, T. M., Polihronis, J., and Reilly, R. M. (1996) Pretargeted Tumour Imaging with Streptavidin Immunoconjugates of Monoclonal Antibody CC49 and ¹¹¹In-DTPA-Biocytin. *Nucl. Med. Biol.* 23, 459–466.
- (14) Paganelli, G., Riva, P., Deleide, G., Clivio, A., Chiolerio, F., Scasellati, G. A., Malcovati, M., and Siccardi, A. G. (1988) In Vivo Labelling of Biotinylated Monoclonal Antibodies by Radioactive Avidin: A Strategy to Increase Tumor Radiolocalization. *Int. J. Cancer* 2, 121–125.
- (15) Sharkey, R. M., Karacay, H., Griffiths, G. L., Behr, T. M., Blumenthal, R. D., Mattes, M. J., Hansen, H. J., and Goldenberg, D. M. (1997) Development of a Streptavidin-Anti-Carcinomaembryonic Antigen Antibody, Radiolabeled Biotin

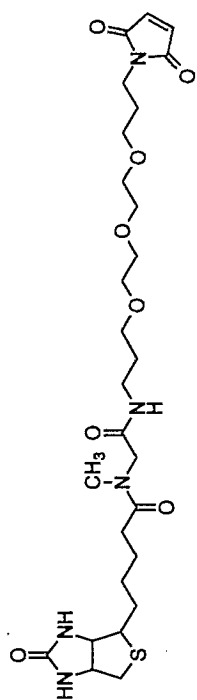
- Pretargeting Method for Radioimmunotherapy of Colon Cancer. Studies in a Human Colon Cancer Xenograft Model. *Bioconjugate Chem.* 8, 595–604.
- (16) Paganelli, G., Malcovati, M., and Fazio, F. (1991) Monoclonal Antibody pretargeting techniques for tumour localization: the avidin–biotin system. *Nucl. Med. Commun.* 12, 211–234.
- (17) Wilbur, D. S., Stayton, P. S., To, R., Buhler, K. R., Klumb, L. A., Hamlin, D. K., Stray, J. E., and Vessella, R. L. (1998) Streptavidin in Antibody Pretargeting. Comparison of a Recombinant Streptavidin with Two Streptavidin Mutant Proteins and Two Commercially Available Streptavidin Proteins. *Bioconjugate Chem.* 9, 100–107.
- (18) Rosebrough, S. F. (1993) Pharmacokinetics and Biodistribution of Radiolabeled Avidin, Streptavidin and Biotin. *Nucl. Med. Biol.* 20, 663–668.
- (19) Ngai, W. M., Reilly, R. M., Polihronis, J., and Shptiz, B. (1995) In Vitro and In Vivo Evaluation of Streptavidin Immunoconjugates of the Second Generation TAG-72 Monoclonal Antibody CC49. *Nucl. Med. Biol.* 22, 77–86.
- (20) Schechter, B., Silberman, R., Arnon, R., and Wilchek, M. (1990) Tissue distribution of avidin and streptavidin injected to mice. Effect of avidin, carbohydrate, streptavidin truncation and exogenous biotin. *Eur. J. Biochem.* 189, 327–331.
- (21) Zhang, M., Sakahara, H., Yao, Z., Saga, T., Nakamoto, Y., Sato, N., Nakada, H., Yamashina, I., and Konishi, J. (1997) Intravenous Avidin Chase Improved Localization of Radiolabeled Streptavidin in Intraperitoneal Xenograft Pretargeted with Biotinylated Antibody. *Nucl. Med. Biol.* 24, 61–64.
- (22) Pispas, J. (1965) Animal Biotinidase. *Ann. Med. Exp. Biol. Fenn.* 43, 3–40.
- (23) Chauhan, J., Ebrahim, H., Bhullar, R. P., and Dakshinamurti, K. (1985) Human Serum Biotinidase. *Ann. N.Y. Acad. Sci.* 447, 386–388.
- (24) Wolf, B., Hymes, J., and Heard, G. S. (1990) Biotinidase. *Methods Enzymol.* 184, 103–111.
- (25) Wilbur, D. S., Hamlin, D. K., Pathare, P. M., and Weerawarna, S. A. (1997) Biotin Reagents for Antibody Pretargeting. Synthesis, Radioiodination and In Vitro Evaluation of Water Soluble, Biotinidase Resistant Biotin Derivatives. *Bioconjugate Chem.* 8, 572–584.
- (26) Ruszkowski, M., Fogarasi, M., Fritz, B., and Hnatowich, D. J. (1997) Effect of Endogenous Biotin on the Applications of Streptavidin and Biotin in Mice. *Nucl. Med. Biol.* 24, 263–268.
- (27) Berei, K., Eberle, S. H., Kirby, H. W., Munzel, H., Rossler, K., Seidel, A., and Vasáros, L. (1985) Astatine. In *Gmelin Handbook of Chemistry* (H. K. Kugler and C. Keller, Eds.) 8th ed., Springer-Verlag, Berlin.
- (28) Gamper, H. B., Reed, M. W., Cox, T., Viroso, J. S., Adams, A. D., Gall, A. A., Scholler, J. K., and Meyer, R. B. (1993) Facile Preparation of Nuclease Resistant 3' Modified Oligodeoxynucleotides. *Nucleic Acids Res.* 21, 145–150.
- (29) Chilkoti, A., Tan, P. H., and Stayton, P. S. (1995) Site-directed mutagenesis studies of the high-affinity streptavidin–biotin complex: contributions of tyrosine residues 79, 108, and 120. *Proc. Natl. Acad. Sci. U.S.A.* 92, 1754–1758.
- (30) Vessella, R. L., Alvarez, V., Chious, R.-K., Rodwell, J., Elson, M., Palme, D., Shafer, R., and Lange, P. (1987) Radioimmunoscintigraphy and Radioimmunotherapy of Renal Cell Carcinoma Xenografts. *Ned. Chem. Ind. Monogr.* 3, 159–167.
- (31) Fritzberg, A. R., Beaumier, P. L., Bottino, B. J., and Reno, J. M. (1994) Approaches to improved antibody- and peptide-mediated targeting for imaging and therapy of cancer. *J. Controlled Release* 28, 167–173.
- (32) Maack, T., Johnson, V., Kau, S. T., Figueiredo, J., and Sigulem, D. (1979) Renal Filtration, Transport, and Metabolism of Low-Molecular-Weight Proteins: A Review. *Kidney Int.* 16, 251–270.
- (33) Alon, R., Bayer, E. A., and Wilchek, M. (1990) Streptavidin Contains an RYD Sequence Which Mimics the RGD Receptor Domain of Fibronectin. *Biochem. Biophys. Res. Commun.* 170, 1236–1241.
- (34) Schechter, B., Arnon, R., Colas, C., Burakova, T., and Wilchek, M. (1995) Renal accumulation of streptavidin: Potential use for targeted therapy of the kidney. *Kidney Int.* 47, 1327–1335.
- (35) Mogensen, C. E., and Sølling, K. (1977) Studies on Renal Tubular Protein Reabsorption: Partial and Near Complete Inhibition by Certain Amino Acids. *Scand. J. Clin. Lab. Invest.* 37, 477–486.
- (36) Mogensen, C. E., Vittinghus, E., and Sølling, K. (1979) Abnormal Albumin Excretion After Two Provocative Renal Tests in Diabetes: Physical Exercise and Lysine Injection. *Kidney Int.* 16, 385–393.
- (37) Sumpio, B. E., and Maack, T. (1982) Kinetics, Competition, and Selectivity of Tubular Absorption of Proteins. *Am. J. Physiol.* 243, F379–F392.
- (38) Claris-Appiani, A., Assael, B. M., Tirelli, A. S., Cavanna, G., and Corbetta, C. (1988) Proximal Tubular Function and Hyperfiltration During Amino Acid Infusion in Man. *Am. J. Nephrol.* 8, 96–101.
- (39) Olsen, N. V., Hansen, J. M., Ladefoged, S. D., Fogh-Andersen, N., Nielsen, S. L., and Leyssac, P. P. (1990) Overall Renal and Tubular Function During Infusion of Amino Acids in Normal Man. *Clin. Sci.* 78, 497–501.
- (40) Rustom, R., Maltby, P., Grim, J. S., Stockdale, H. R., Critchley, M., and Bone, J. M. (1992) Effects of Lysine Infusion on the Renal Metabolism of Aprotinin (Trasylol) in Man. *Clin. Sci.* 83, 295–299.
- (41) Behr, T. M., Sharkey, R. M., Juweid, M. E., Blumenthal, R. D., Dunn, R. M., Griffiths, G. L., Bair, H.-J., Wolf, F. G., Becker, W. S., and Goldenberg, D. M. (1995) Reduction of the Renal Uptake of Radiolabeled Monoclonal Antibody Fragments by Cationic Amino Acids and Their Derivatives. *Cancer Res.* 55, 3825–3834.
- (42) Behr, T. M., Becker, W. S., Sharkey, R. M., Juweid, M. E., Dunn, R. M., Bair, H.-J., Wolf, F. G., and Goldenberg, D. M. (1996) Reduction of Renal Uptake of Monoclonal Antibody Fragments by Amino Acid Infusion. *J. Nucl. Med.* 37, 829–833.
- (43) DeNardo, S. J., O'Donnell, R. T., and DeNardo, G. L. (1996) Potential Use of Amino Acid Cocktails to Reduce Renal Tubular Reabsorption of Radioconjugates. *J. Nucl. Med.* 37, 837–838.
- (44) Behr, T. M., and Goldenberg, D. M. (1996) Improved Prospects for Cancer Therapy with Radiolabeled Antibody Fragments and Peptides. *J. Nucl. Med.* 37, 834–836.
- (45) Kobayashi, H., Yoo, T. M., Kim, I. S., Kim, M.-K., Le, N., Webber, K. O., Pastan, I., Paik, C. H., Eckelman, W. C., and Carrasquillo, J. A. (1996) L-Lysine Effectively Blocks Renal Uptake of ¹²⁵I- or ^{99m}Tc-labeled Anti-Tac Disulfide-stabilized Fv Fragment. *Cancer Res.* 56, 3788–3795.
- (46) Rennke, H. G., Patel, Y., and Venkatachalam, M. A. (1978) Glomerular filtration of proteins: Clearance of anionic, neutral, and cationic horseradish peroxidase in the rat. *Kidney Int.* 13, 324–328.
- (47) Brenner, B. M., Hostetter, T. H., and Humes, H. D. (1978) Glomerular permselectivity: barrier function based on discrimination of molecular size and charge. *Am. J. Physiol.* 234, F455–F460.
- (48) Rosebrough, S. F., and Hartley, D. F. (1996) Biochemical Modification of Streptavidin and Avidin: In Vitro and In Vivo Analysis. *J. Nucl. Med.* 37, 1380–1384.
- (49) Green, N. M. (1975) Avidin. *Adv. Protein Chem.* 29, 85–133.
- (50) Jeong, J. M., Kinuya, S., Paik, C. H., Saga, T., Sood, V. K., Carrasquillo, J. A., Neumann, R. D., and Reynolds, J. C. (1994) Application of High Affinity Binding Concept to Radiolabel Avidin with Tc-99m Labeled Biotin and the Effect of pI on Biodistribution. *Nucl. Med. Biol.* 21, 935–940.
- (51) Hofmann, K., Titus, G., Montibeller, J. A., and Finn, F. M. (1982) Avidin Binding of Carboxyl-Substituted Biotin and Analogues. *Biochemistry* 21, 978–984.

Size exclusion HPLC chromatogram of 13-N-(*tert*-butoxycarbonyl)-4,7,10-trioxatridecanyl-1-N-methylglycine biotinamide; **5**.Size exclusion HPLC chromatogram of 13-N-maleimido-4,7,10-trioxatridecanyl-1-N-methylglycine biotinamide; **7**.

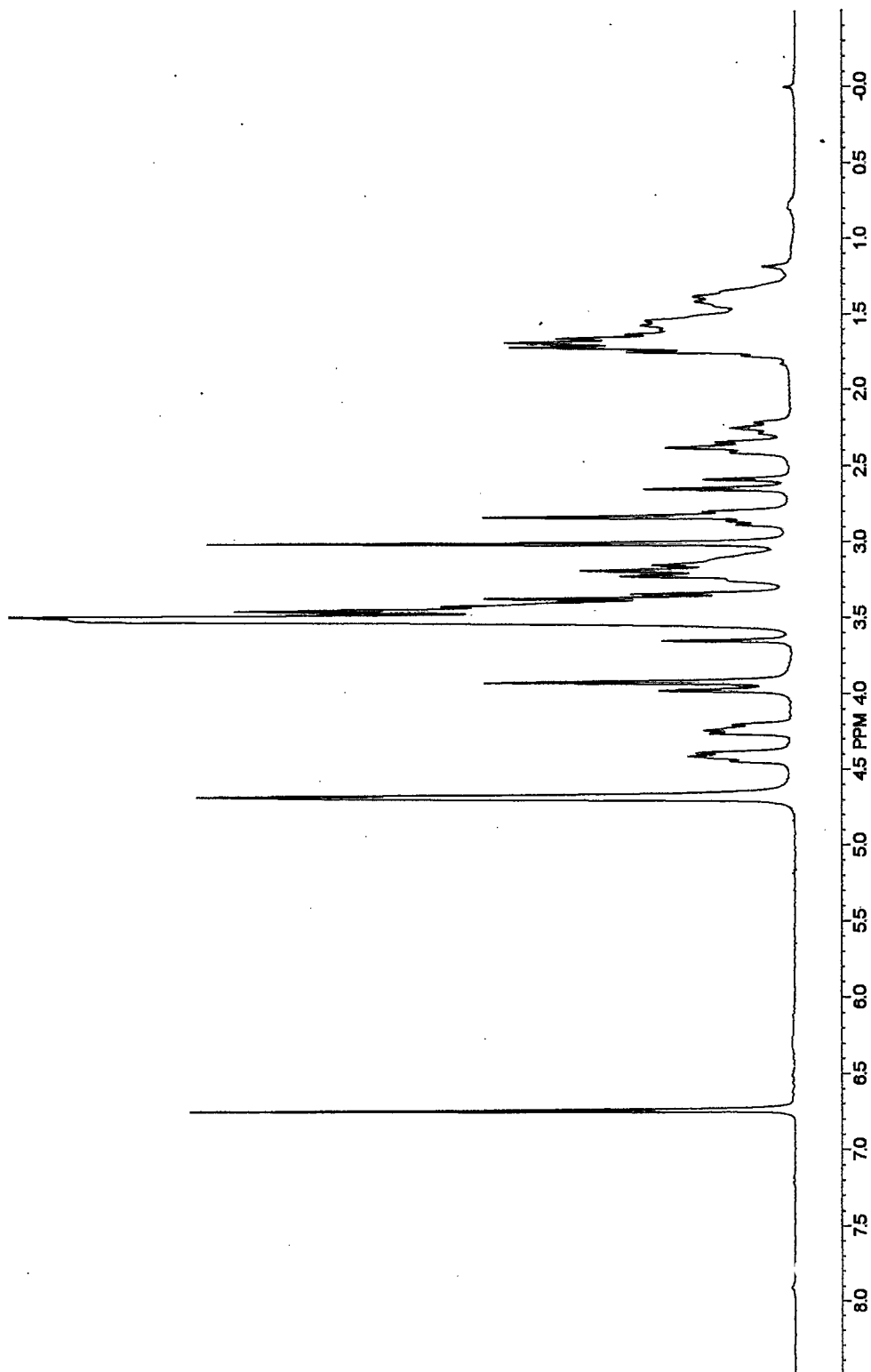


^1H NMR of **5** in MeOH-d_4 :





^1H NMR of 7 in MeOH-d_4 :



Solution Structure of a DNA Complex with the Fluorescent Bis-Intercalator TOTO Modified on the Benzothiazole Ring

Michael Petersen and Jens Peter Jacobsen*

Department of Chemistry, Odense University, Odense M, DK-5230 Denmark. Received July 21, 1997;
Revised Manuscript Received December 3, 1997

We have used two-dimensional ^1H NMR spectroscopy to determine the solution structure of the DNA oligonucleotide $d(5'\text{-CGCTAGCG-3}')_2$ complexed with the bis-intercalating dye 1,1'-(4,4,8,8-tetramethyl-4,8-diazaundecamethylene)bis-4-[3-ethyl-2,3-dihydro(benzo-1,3-thiazolyl)-2-methylidene]quinolinium tetraiodide (TOTOEt). The determination of the structure was based on a total relaxation matrix analysis of the NOESY cross-peak intensities. DQF-COSY spectra were used to obtain coupling constants for the deoxyribose ring protons. The coupling constants were transformed into angle estimates. The NOE-derived distance and dihedral restraints were applied in restrained molecular dynamics calculations. Twenty final structures each were generated for the TOTOEt complex from both A-form and B-form double-stranded (ds) DNA starting structures, giving a total of 40 final structures. Since many NOE contacts were observed between TOTOEt and dsDNA, the resulting structure has a fairly high resolution and allows determination of local features in the dsDNA structure after TOTOEt binding. The root-mean-square (rms) deviation of the coordinates for the 40 structures of the complex was 0.52 Å. The local DNA structure is distorted in the complex. The helix is unwound by 80° and has an overall helical repeat of 12 base pairs, caused by bis-intercalation of TOTOEt. The benzothiazole ring system is twisted relative to the quinoline in the uncomplexed TOTOEt molecule. The site selectivity of TOTOEt for the CTAG-CTAG site is explained by its ability to adapt to the base pair propeller twist of dsDNA to optimize stacking and the hydrophobic interaction between the thymidine methyl group and the benzothiazole ring. The polypropylene amine linker chain is located in the minor groove of dsDNA. The *N*-ethyl group on the benzothiazole of TOTOEt is placed in the major groove pointing toward the center of the oligonucleotide and the dyad symmetry axis of the complex. This orientation seems to make it feasible to create a TOTO analogue with a linker connecting the two chromophores in the major groove. The design of such an analogue and a macrocyclic analogue with a linker in both the major groove and the minor groove seems to be straightforward.

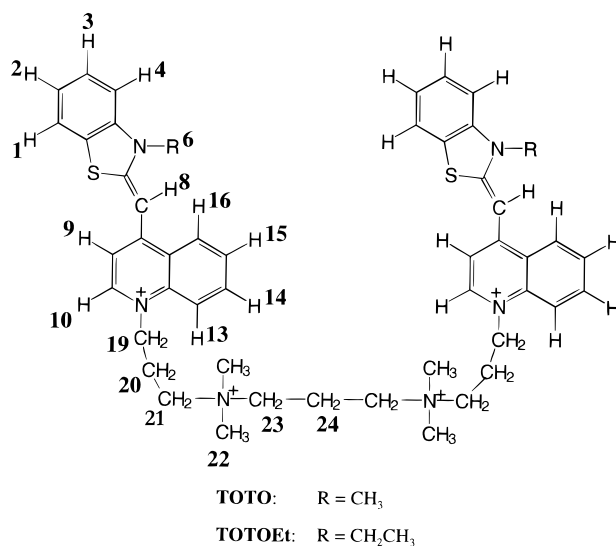
INTRODUCTION

Progress in cancer research indicates that neoplasia is a malady of genes (Bishop, 1987). Although several potent antitumor drugs do not interact with nucleic acids, there is accumulating evidence that suggests that the malignant transformation of cells is due to alterations of particular genes. Thus, the development and investigation of DNA-interacting substances is called for. A prominent class of DNA-targeting drugs are intercalators and bis-intercalators (e.g. amsacrine, daunomycin, and

* To whom correspondence should be addressed. E-mail: jppj@chem.ou.dk.

¹ Abbreviations: AMBER, assisted model building with energy refinement; DNA, deoxyribose nucleic acid; DMSO, dimethyl sulfoxide; DQF-COSY, double-quantum-filtered correlation spectroscopy; dsDNA, double-stranded DNA; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate; EDTA, ethylenediaminetetraacetic acid; HSQC, heteronuclear single-quantum coherence; MARDIGRAS, matrix analysis of relaxation for discerning the geometry of an aqueous structure; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser effect spectroscopy; RANDMARDI, randomized MARDIGRAS; RMD, restrained molecular dynamics; TOCSY, total correlation spectroscopy; TOTO, 1,1'-(4,4,8,8-tetramethyl-4,8-diazaundecamethylene)bis-4-[3-methyl-2,3-dihydro(benzo-1,3-thiazolyl)-2-methylidene]quinolinium tetraiodide; TOTOEt, 1,1'-(4,4,8,8-tetramethyl-4,8-diazaundecamethylene)bis-4-[3-ethyl-2,3-dihydro(benzo-1,3-thiazolyl)-2-methylidene]quinolinium tetraiodide; TPPI, time-proportional phase incrementation.

Scheme 1. TOTO and TOTOEt Numbering Scheme



adriamycin). To gain knowledge of the often subtle features that govern drug–DNA recognition, the need for high-resolution structures of drug–DNA complexes is imperative.

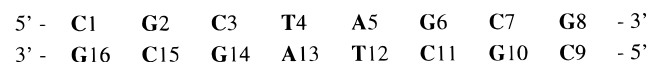
Recent research in our laboratory has focused on the bis-intercalating drug TOTO¹ (Scheme 1). We have examined both the sequence specificity and the kinetics

of association and dissociation for dsDNA complexes with both TOTO and a number of TOTO analogues (Faridi et al., 1997; Hansen et al., 1996; Jacobsen et al., 1995; Spielmann et al., 1995b; Stærk et al., 1997). TOTO bis-intercalates into dsDNA by nearest neighbor intercalation and shows sequence selectivity toward the (5'-CTAG-3')₂ site (Hansen et al., 1996; Jacobsen et al., 1995). The structure of the complex implies that modifications on the benzothiazole nitrogen would be expected to be positioned in the major groove on complex formation with dsDNA (Spielmann et al., 1995b). However, the binding of TOTO has been shown to be very sensitive to even small modifications on the oligonucleotide (Hansen et al., 1996). Consequently, there is a risk that slight changes of the chromophore of TOTO would destroy the binding affinity and selectivity of the molecule.

Our long-term objective is to design a TOTO analogue with a linker connecting the two chromophores in the major groove, subsequently with a linker in both the major groove and the minor groove. As a primary modification, the *N*-methyl group of TOTO was substituted with a *N*-ethyl group to give the compound 1,1'-(4,4,8,8-tetramethyl-4,8-diazaundecamethylene)bis-4-[3-ethyl-2,3-dihydro(benzo-1,3-thiazolyl)-2-methylidene]-quinolinium tetraiodide (TOTOEt, Scheme 1). We have used ¹H NMR spectroscopy to determine the solution structure of the DNA oligonucleotide d(5'-CGCTAGCG-3')₂ complexed with the bis-intercalating TOTOEt. The determination of the structure was based on the protocol described by James and co-workers (Schmitz and James, 1995) combined with inclusion of dihedral angle estimates obtained from coupling constants in the deoxyribose ring protons. This study was carried out to investigate whether this modification implied a dramatic change of the binding and selectivity of the molecule. Furthermore, this structural study was aimed at determining the position of the *N*-ethyl group in a dsDNA complex and the feasibility of substituting larger groups than ethyl, eventually a linker, at the benzothiazole nitrogen while still maintaining proper affinity and selectivity with respect to dsDNA.

EXPERIMENTAL PROCEDURES

Sample Preparation. TOTOEt was a gift from E. B. Pedersen (Department of Chemistry, Odense University, Denmark). The purified oligonucleotide d(CGC-TAGCG)₂ was purchased from DNA technology (Århus, Denmark) and used without further purification. The numbering scheme for the double-stranded oligonucleotide is



TOTOEt is almost insoluble in water, and the complexes with dsDNA were therefore made with the procedure described earlier (Jacobsen et al., 1995). The NMR samples were prepared by dissolving the complexes in 0.5 mL of 10 mM sodium phosphate buffer (pH 7.0), 0.05 mM Na EDTA, 0.01 mM NaN₃, and 0.1 mM DSS. For experiments carried out in D₂O, the solid complex, lyophilized three times from D₂O, was redissolved in 99.96% D₂O (Cambridge Isotope Laboratories). A mixture of 90% H₂O and 10% D₂O (0.5 mL) was used for experiments examining exchangeable protons. The sample was kept in a NMR tube under nitrogen. The final concentration of the complex was 2 mM.

NMR Experiments. NMR experiments were performed on a Varian Unity 500 spectrometer at 27 °C

unless otherwise stated. NOESY spectra of the complex were acquired in D₂O using 1024 complex points in *t*₂ and a spectral width of 5000 Hz. A total of 512 *t*₁ experiments were recorded using the States phase cycling scheme. The residual signal from HOD was removed by presaturation. NOE buildups were obtained by recording NOESY spectra with mixing times of 50, 100, 150, and 200 ms with 64 scans acquired for each *t*₁ value and a repetition delay of 2.8 s between each scan. The NOESY spectra were obtained sequentially without removing the sample from the magnet. TOCSY spectra of the complex with mixing times of 30 and 90 ms were obtained in the TPPI mode using 1024 complex points in *t*₂ and 512 *t*₁ experiments with 64 scans each. DQF-COSY spectra were recorded using the States phase cycling scheme with 1024 complex points in *t*₂ and 1024 *t*₁ experiments with 64 scans each. Removal of the residual HOD signal was in either case carried out with presaturation. A NOESY spectrum of the complex in H₂O with a mixing time of 160 ms was obtained by using a NOESY pulse sequence where the last 90° pulse is replaced by a 1-1 jump-return pulse to suppress the solvent signal (Guéron and Plateau, 1982). A Watergate NOESY spectrum in H₂O with a mixing time of 50 ms was recorded on a Varian Unity+ 500 spectrometer. Both NOESY spectra in H₂O were acquired with a spectral width of 10 000 Hz in 2048 complex points using 512 *t*₁ experiments with 64 scans acquired in each *t*₁ experiment. A ¹H, ¹³C HSQC spectrum in D₂O was recorded on a Varian Unity+ 500 spectrometer. A NOESY spectrum with mixing time of 500 ms was recorded of the free TOTOEt in DMSO-*d*₆.

The acquired data were processed using FELIX (version 2.35, Biosym/MSI, San Diego, CA). All spectra were apodized by a skewed sine bell squared function in both dimensions. The four spectra used in the buildup study were zero-filled to 2K points to enhance digital resolution and baseline corrected by first-order standard polynomial fits in both dimensions. The NOESY spectrum recorded in H₂O was baseline corrected by a third-order standard polynomial in *F*₂ and seventh-order one in *F*₁. The DQF-COSY spectrum was zero-filled to 4K points. The spectra were assigned by conventional methods (Wüthrich, 1986; Feigon et al., 1983; Hare et al., 1983; Scheek et al., 1983, 1984; Boelens et al., 1985).

Distance and Angle Restraints. The structure determination was performed using the protocol described by James and co-workers (Schmitz and James, 1995) combined with inclusion of dihedral angle estimates obtained from coupling constants in the deoxyribose ring protons in the following way. The upper and lower diagonal parts of each of the four NOESY buildup spectra were integrated separately with FELIX, yielding a total of eight peak intensity sets. The RANDMARDI procedure (Liu et al., 1995) of the complete relaxation matrix analysis method MARDIGRAS (Borgias et al., 1990; Borgias and James, 1990) was used to calculate interproton distance RMD bounds from the resulting integrated peak intensities. In the calculations, a noise level of 5 times the integrated intensity of the smallest cross-peak was used as the "noise" range. The dynamic range of observed cross-peak intensities was 2000. In the RANDMARDI procedure, 30 different intensity sets from each experimental data set were generated, and MARDIGRAS calculations were performed on all of them. Resulting distances were then averaged together to form one bounds file for each of the eight data sets. The bounds files from all of the mixing times were then combined into a single bounds file from which the RMD restraint file was generated. Upper and lower bounds

were average interproton distances $\pm$ one standard deviation calculated from all of the MARDIGRAS runs.

An additional 22 distance restraints were included to mimic Watson–Crick hydrogen bonding throughout the calculations. Three hydrogen bonds were included for each of the six G•C base pairs and two hydrogen bonds for each of the A•T base pairs with lower and upper bounds of 1.7 and 2.1 Å, respectively.

The H1' to H2' and H2'' region of the DQF-COSY spectrum was used as input to CHEOPS to obtain *J* coupling information for the H1', H2', H2'', and H3' deoxyribose ring protons (Gochin et al., 1990; Macaya et al., 1992). The ring conformations were analyzed using the PSEUROT procedure (de Leeuw and Altona, 1983).

Restrained Molecular Dynamics. TOTOEt was manually docked into the model of either A-form or B-form DNA in INSIGHTII (version 95.0, Biosym/MSI) to obtain the starting models for the structure refinements. The TOTOEt molecule was built using INSIGHTII. The potentials for both DNA and TOTOEt were assigned by the AMBER force field, modified to accommodate the benzothiazole sulfur atom of TOTOEt. Partial charges for TOTOEt were calculated using ab initio GAUSSIAN94 calculations.

The distance restraints obtained from RANDMARDI and dihedral angle restraints from PSEUROT were incorporated into energy minimization and an RMD procedure using DISCOVER (version 95.0, Biosym/MSI). An initial energy minimization was followed by 28 ps of restrained molecular dynamics, at 600 K for 4 ps, followed by cooling to 200 K in 50 K steps of 3 ps each. The final structure was then energy minimized to a maximum derivative of 0.01 Å.

The pseudo-energy terms used to enforce the restraints were

$$E_{\text{NOE}} = \begin{cases} k_1(r - r_1)^2 & \text{when } r_1 > r \\ 0 & \text{when } r_2 \geq r \geq r_1 \\ k_1(r - r_2)^2 & \text{when } r > r_2 \end{cases}$$

$$E_{\text{dihedral}} = \begin{cases} k_2(\theta - \theta_1)^2 & \text{when } \theta_1 > \theta \\ 0 & \text{when } \theta_2 \geq \theta \geq \theta_1 \\ k_2(\theta - \theta_2)^2 & \text{when } \theta > \theta_2 \end{cases}$$

where r_1 and r_2 are the lower and upper distance bounds determined from the cross-peak volumes, respectively, k_1 and k_2 are the force constants, and θ_1 and θ_2 are the lower and upper dihedral angle bounds determined from the DQF-COSY spectral simulations, respectively. For NOE-derived distance restraints a force constant k_1 of 50 kcal/(mol Å²) was employed and for dihedral angle restraints a force constant k_2 of 25 kcal/(mol Å²). The width of the flat region of the potential well reflects the accuracy of a given constraint. A distance-dependent dielectric ($\epsilon = r$) was employed to account for solvent effects. Helix parameters were calculated with the program CURVES 5.1 (Lavery and Sklenar, 1988, 1989).

RESULTS

Spectral Analysis. The 1D spectrum of the dsDNA–TOTOEt complex exhibits sharp lines, with line widths as small as those observed for the dsDNA–TOTO complex. There is no sign whatsoever of any minor forms. We therefore conclude that TOTOEt has a strong preference for the (5'-CTAG-3')₂ site. Observation of lines for just one dsDNA strand and one thiazole orange chromophore shows that the dyad symmetry of the d(CGC-

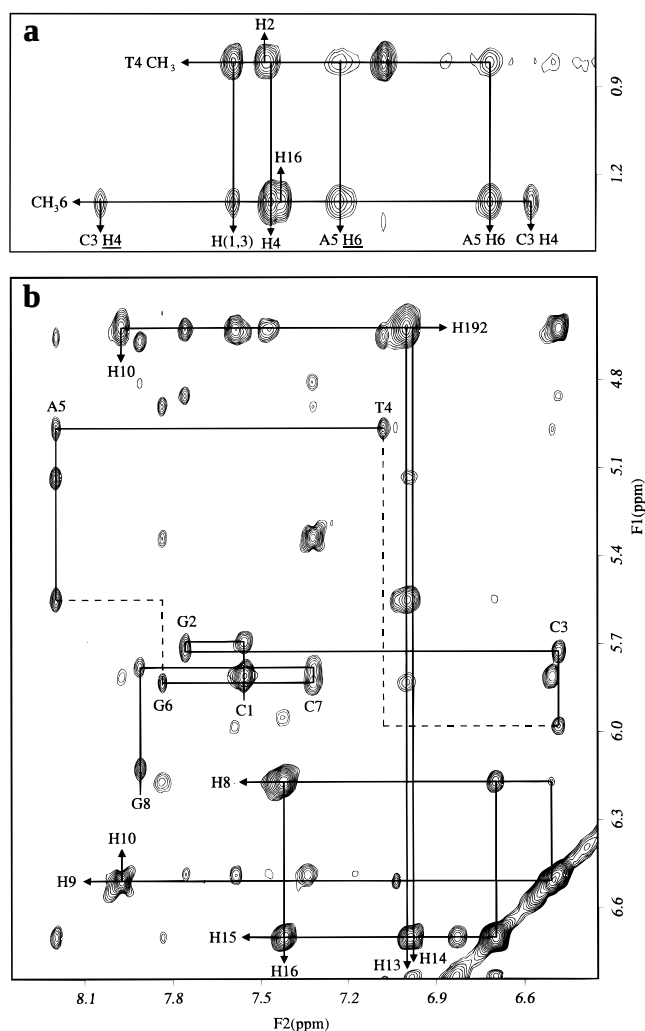


Figure 1. (a) H1'–aromatic part of the 150 ms NOESY spectrum of the TOTOEt complex. The sequential H1'–H6/H8–H1' connectivity pathway is indicated by a solid line. The interrupted connectivities at the 5'-C3pT4-3' and 5'-A5pG6-3' base pair steps due to intercalation sites are indicated by dotted lines. It is also possible to follow the H3'–H6/H8–H3' connectivity pathway with similar interrupted connectivities. A few of the observed cross-peaks between TOTOEt and the oligonucleotide are also marked. It is noteworthy that the cross-peak between TOTOEt H15 and A5 H1' has a measured volume which is approximately 100 times smaller than the observed cross-peak between TOTOEt H8 and H16. This demonstrates the large dynamic range and precision of the measured volumes that can be obtained from the spectra. (b) Methyl–aromatic part of the 160 ms NOESY spectrum recorded in H₂O. The assignments of the C3 and A5 amino protons, the thymidine methyl protons, and some of the TOTOEt protons are indicated. Cross-peaks between TOTOEt CH₃(6) and the amino protons of C3 and A5 unambiguously place the benzothiazole *N*-ethyl group in the major groove of the dsDNA.

TAGCG)₂ oligonucleotide is retained on complex formation with TOTOEt.

The NOESY spectrum of this complex exhibits the characteristic features of dsDNA sequential connectivities. However, the sequential pathways are interrupted at the 5'-C3pT4-3' and 5'-A5pG6-3' base pair steps. This is clear evidence of bis-intercalation (Hansen et al., 1996; Jacobsen et al., 1995; Spielmann et al., 1995b). Parts of the NOESY spectra are shown in Figure 1. The NOESY spectra of the complex also contain a multitude of intermolecular cross-peaks between dsDNA and TOTOEt as well as intramolecular TOTOEt cross-peaks. These were assigned in a similar way as previously

Table 1. Chemical Shift Values of the TOTOEt Complex with d(CGCTAGCG)₂ at 27 °C^a

	C1	G2	C3	T4	A5	G6	C7	G8
H8–H6	7.55 (7.65)	7.76 (7.99)	6.48 (7.44)	7.08 (7.41)	8.20 (8.23)	7.83 (7.70)	7.32 (7.30)	7.91 (7.93)
H5–CH ₃ –H2	5.80 (5.92)		4.62 (5.39)	0.82 (1.70)	7.03 (7.37)		5.33 (5.34)	
H1'	5.68 (5.81)	5.72 (5.97)	5.97 (5.98)	4.96 (5.60)	5.54 (6.02)	5.82 (5.71)	5.77 (5.76)	6.12 (6.16)
H2'	1.91 (2.00)	2.40 (2.71)	1.82 (2.07)	1.92 (2.14)	2.87 (2.74)	2.68 (2.50)	1.88 (1.87)	2.60 (2.60)
H2''	2.31 (2.45)	2.40 (2.77)	2.45 (2.51)	1.99 (2.44)	2.75 (2.88)	2.68 (2.60)	2.32 (2.33)	2.35 (2.37)
H3'	4.63 (4.76)	4.84 (5.00)	4.62 (4.78)	4.65 (4.88)	5.12 (5.05)	4.88 (4.95)	4.80 (4.79)	4.66 (4.66)
H4'	4.01 (4.09)	4.25 (4.38)	4.05 (4.24)	4.15 (4.14)	4.32 (4.40)	4.57 (4.37)	4.20 (4.14)	4.07 (4.16)
H5'	3.67 (3.74)	3.90 (4.11)	4.05 (4.18)	4.15 (4.09)	3.95 (4.15)	4.02 (4.19)	4.20 (4.10)	4.07 (4.06)
H5''	3.67 (3.74)	4.02 (4.01)	4.16 (4.26)	4.10 (4.09)	4.12 (4.06)	4.39 (4.19)	4.14 (4.19)	4.17 (4.06)
H1–H3		12.78 (13.02)		13.57 (13.76)		12.14 (12.76)		–
H4–H6	–		8.03 (8.27)		7.22 (7.81)		8.19 (8.34)	
H4–H6	–		6.54 (6.58)		6.71 (6.54)		6.43 (6.47)	

TOTOEt	shift	TOTOEt	shift	TOTOEt	shift	TOTOEt	shift
H1	7.59	H8	6.16	H16	7.42	H21''	3.73
H2	7.48	H9	6.50	H19'	4.13	CH ₃ (22)	3.31
H3	7.58	H10	7.97	H19''	4.62	H23'	3.48
H4	7.46	H13	6.99	H20'	2.49	H23''	3.48
CH ₃ (6)	1.30	H14	6.97	H20''	2.57	H24	2.49
CH ₂ (6)	4.18	H15	6.69	H21'	3.65		

^a Compared to free dsDNA chemical shifts given in parentheses. The values are given relative to DSS.

published (Jacobsen et al., 1995; Spielmann et al., 1995b). The DQF-COSY spectrum was used for the assignment of proton resonances in the linker of TOTOEt. The combined use of DQF-COSY spectra and NOESY spectra with short mixing times allows unambiguous assignments of the H2' and H2'' resonances. The HSQC spectrum was used to completely establish the assignments of the H3' and H4' protons. The chemical shift values of the protons in the complex are given in Table 1.

The NOESY spectra of the complex recorded in H₂O contain the normal Watson–Crick NOE connectivity pattern justifying the inclusion of 22 hydrogen bond distance restraints. Chemical shift values of labile protons are included in Table 1. Furthermore, the spectra in H₂O contain distinct cross-peaks between the thymidine imino proton (T4–H3) and the amino protons of the base-paired adenosine (A13–H6). These peaks are absent in normal dsDNA at room temperature due to exchange. The lowering of the exchange rates of the adenosine amino protons on complex formation is a consequence of the bis-intercalation. Similarly, exchange cross-peaks between water and the thymidine imino protons are absent at room temperature, and only very small ones are observed for the G6 and G14 imino protons. The G2 and G10 imino protons yield an exchange cross-peak to water of normal intensity.

Studies of the conformation of the free TOTOEt in DMSO-*d*₆ using NOESY and DQF-COSY spectra reveal that the relative conformation of the two ring systems in each chromophore is close to that indicated in Scheme 1. This is a consequence of a prominent NOESY cross-peak between H8 and H16. We have found an identical conformation of the aromatic ring systems in the dsDNA–TOTOEt complex. The relative conformation of the aromatic ring systems about the cyanine methine bonds in the chromophores is therefore not affected by intercalation into dsDNA.

Cross-peaks in the NOESY spectra unambiguously show that the poly(propylene amine) linker is positioned in the minor groove. This is demonstrated by a large number of cross-peaks. The H2 of the adenines gives cross-peaks to the linker protons H21, H22, and H23 and the H1' proton of G6 to the linker H19, H21, and H22 protons, and the H4' of the adenines and the guanines

Table 2. Coupling Constants for the Sugar Protons in the dsDNA–TOTOEt Complex^a

	<i>J</i> _{1'2'}	<i>J</i> _{1'2''}	<i>J</i> _{2'2''} ^b	corr ^c	%S ^d	<i>P</i> _S ^e
C1	8.0	6.2	–15.0	0.96	88	164.9
G2 ^f	–	–	–	–	–	–
C3	6.0	7.0	–15.0	0.96	<i>g</i>	–
T4	9.6	4.5	–15.0	0.95	84	134.0
A5	10.9	4.4	–15.0	0.93	87	154.2
G6 ^f	–	–	–	–	–	–
C7	7.8	6.4	–15.0	0.95	92	119.4
G8	7.8	5.7	–15.0	0.96	78	173.7

^a Derived from the H1'–H2' and H1'–H2'' cross-peaks in the DQF-COSY spectrum using CHEOPS. ^b *J*_{2'2''} coupling constants were kept fixed at –15.0 Hz. ^c Correlation coefficient (Macaya et al., 1992). ^d Percent S-type sugar conformation calculated using PSEUROT. ^e Pseudorotation angle calculated for the S-type sugar using PSEUROT. The puckering amplitude was kept constant at 36°. ^f G2 and G6's sugar pucker could not be analyzed due to spectral overlap in the DQF-COSY spectrum. ^g No minimum could be found for the sugar pucker parameters for this nucleotide; see the following discussion.

in the binding site also shows cross-peaks to linker protons.

Cross-peaks between TOTOEt CH₃(6) and CH₂(6) and the amino protons of C3 and A5 unambiguously place the benzothiazole *N*-ethyl group in the major groove of the dsDNA. The relative intensities of these cross-peaks in the NOESY spectrum recorded in H₂O with a short mixing time (50 ms) established that the *N*-ethyl group is pointing toward the center of the oligonucleotide.

Deoxyribose Ring Conformations. Due to the dyad symmetry of the TOTOEt–d(CGCTAGCG)₂ complex, only eight spectroscopically distinct deoxyribose residues are observed in the DQF-COSY spectrum. Two of these, G2 and G6, are hampered by severe overlap of the H2' and H2'' resonances, making use of cross-peaks from these residues impossible. For the remaining six residues, CHEOPS simulations of the DQF-COSY spectrum returned values that correlated well (>0.93) with the experimental spectrum (Macaya et al., 1992). The *J* coupling constants returned by CHEOPS were used as input for PSEUROT. The PSEUROT minimization converged for five of the six residues analyzed by CHEOPS, producing the values given in Table 2.

Structure Calculations. More than 450 NOE cross-peaks were observed in the NOESY spectrum with a

Table 3. Covalently Determined Interproton Distances (Å) in TOTOEt and a Comparison between Actual Values and Values Obtained from MARDIGRAS Calculations

atom	pair	actual value ^a	lower bound ^b	upper bound ^b	width ^b	violation
CH ₃ (6)	CH ₂ (6)	2.36	2.00	2.12	0.12	0.24
H9	H10	2.36	2.12	2.29	0.17	0.07
H10	H13	4.66	3.55	5.25	1.70	
H14	H16	4.22	3.57	5.69	2.12	
H15	H16	2.41	2.23	2.38	0.15	0.03

^a Obtained from GAUSSIAN94 calculations. ^b Obtained from MARDIGRAS calculations and derived from the average distance $\pm$ one standard deviation.

Table 4. Number of Distance Restraints Used in the RMD Calculations of the TOTOEt Complex^a

intraresidue						
	intra-sugar	base-sugar	total	inter-residue	dye-DNA	dye-dye
C1	3	5	8	C1-G2 6 ^b	C3-TOTOEt 5 (7)	19
G2	8	5	13	G2-C3 3	T4-TOTOEt 7 (2 ^b)	
C3	3	2	5	C3-T4 2	A5-TOTOEt 18 (3)	
T4	2	6	8	T4-A5 12 (2)	G6-TOTOEt 3 (0)	
A5	16	7	23	A5-G6 2		
G6	11	6	17	G6-C7 6		
C7	2	7	9	C7-G8 5 ^b		
G8	4	4	8			
total	49	42	91	total 38	total 45	total 19

^a The restraints were derived from MARDIGRAS calculations unless otherwise stated. Restraints derived from the NOESY spectrum recorded in H₂O are tabulated in parentheses. The 193 NOE connectivities gave a total of 386 symmetry-related distance restraints; 22 distance restraints ensuring Watson-Crick base pairing were also included in the RMD calculations. ^b One of the restraints was derived directly from inspection of the spectra and used with loose bounds.

mixing time of 200 ms. Some of these resulted predominantly from spin diffusion and were consequently not observed in NOESY spectra with shorter mixing times. The accuracy of the integration of some cross-peaks was hampered by spectral overlap. These cross-peaks were therefore not included in the MARDIGRAS calculations. The integrated intensities from a total number of 3056 in eight sets of 382 NOESY cross-peak intensities were used in the total relaxation matrix analysis. Of these, 2880 cross-peak intensities (360 per set) corresponded to interproton distances that are not covalently fixed in either the dye or the dsDNA in the complex. Cross-peak integrals that corresponded to fixed distances in the dsDNA were not converted into distance restraints for use in the RMD simulations. The RANDMARDI calculations returned 362 interproton distances based on the eight sets of 382 NOE cross-peak intensities. These were distributed as given in Table 4.

MARDIGRAS uses internal calibration when it iteratively fits the data to a model of the molecule. In particular, fixed distances were used to normalize the other distances. These fixed distances include the H5-H6 distance in cytosine, the 5-methyl-H6 distance in thymidine, and the H2'-H2'' distance in deoxyribose. NOE intensities for these known distances are used to normalize the NOE intensities in the model. The program does not reset covalently determined proton-proton distances within TOTOEt because these distances intentionally were left out of the reference file. The magnetization transfer for these protons is calculated and

provides a check of how well the RANDMARDI procedure is working. Good agreement between the actual distances of the fixed bonds and the experimentally determined distances indicates that the RANDMARDI procedure yields accurate interproton distances (Table 3).

The MARDIGRAS calculations were performed with various values of the correlation time for the complex in the range of 2–5 ns. The results were fairly insensitive to the actual value, and a correlation time of 3.75 ns was chosen for the final calculation. No symmetry was enforced during the RANDMARDI procedure. The bounds for symmetry-related restraints were calculated by averaging the symmetry-related upper and lower bounds. An additional 0.2 Å was added to all upper bounds of the distance restraints returned by RANDMARDI for use in the RMD calculations to circumvent any problem that may occur due to an incomplete description of the relaxation matrix (cf. Table 3). No additional symmetry was enforced during the RMD calculations.

The range of differences between the upper and lower bounds for the 362 NOE-derived distance restraints used was between 0.27 and 3.13 Å with an average flat well potential width of 1.19 Å. This range between the upper and lower bounds represents the accuracy of the restraints calculated from the NOESY data. Weak NOESY cross-peaks that arose primarily from spin diffusion had the greatest difference between their upper and lower bounds.

Interproton distances involving exchangeable DNA amino and imino protons were derived from the NOESY spectrum of the complex recorded in H₂O with a mixing time of 160 ms. The "jump-and-return" delay was chosen to give optimal excitation of the amino protons. The volume integral for each cross-peak was integrated and normalized for the sinusoidal excitation profile of the jump-and-return pulse sequence. The normalized volume for the cross-peak between C7H5 and C7H6 was used as a reference (2.45 Å) for calculating the interproton distances involving exchangeable protons using the isolated spin pair approach. Due to the inherent error in this approach, upper and lower bounds of ± 1 Å were assigned to all distance restraints. An additional 0.8 Å was added to the upper bounds for restraints involving methylene protons, and 1.0 Å was added to upper bounds for methyl protons. A total of 28 distance restraints derived from the NOESY spectrum recorded in H₂O were included in the RMD simulation. In addition, a few restraints with loose bounds were derived by inspection of the spectra in D₂O to give a grand total of 408 distance restraints incorporated into the RMD simulations (Table 4).

Dihedral angle restraints for the H1'-C1'-C2'-H2', H1'-C1'-C2'-H2'', H2'-C2'-C3'-H3', and H2''-C2'-C3'-H3' deoxyribose angles were obtained by the angles returned for the most populated conformation by PSEUROT. Upper and lower bounds of $\pm 3^\circ$ were assigned giving a flat well potential width of 6° . A total of 20 dihedral angle restraints were included in the RMD simulations.

Twenty final structures each were generated for the TOTOEt complex starting from either an A-form or B-form DNA. All the structures converged to one family of conformations. The root-mean-square (rms) deviation of the coordinates of the 40 structures was 0.52 Å. The sum of violations was 11.4 Å for the A-form starting structure and 10.9 Å for the B-form starting structure. We observed three violations of >0.3 Å with none being >0.6 Å. These violations involved backbone H5' and H5'' protons of A5, perhaps an indication of unresolved

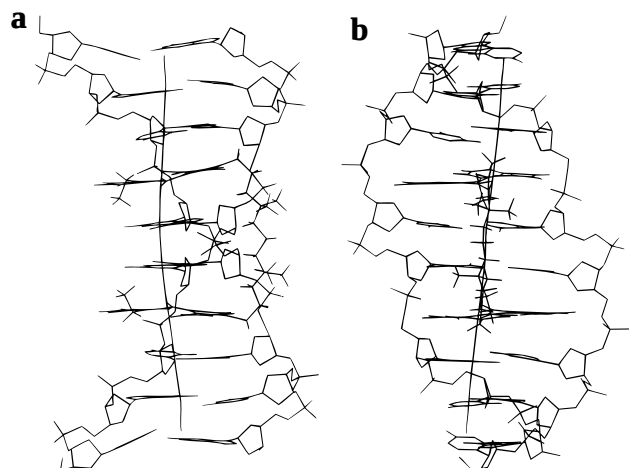


Figure 2. Stick plot of one of the calculated TOTOEt-DNA structures showing the helix axis. (a) View from the side of the helix. (b) View perpendicular to the view in panel a. Deoxyribose protons have been omitted for clarity.

dynamics of the phosphate backbone of the central part of the oligonucleotide. Different views of the structure are presented in Figures 2–4.

The reliability of the structure calculations was carefully evaluated. RMD calculations in which the force constants of the distance restraints, k_1 , were lowered to 25 kcal/Å² generated structures that converged to exactly the same family of structure as with the higher force constants, although the rmsd was slightly higher. The structure obtained is not strongly dependent on the exclusion of any of the restraints. In particular, calculations performed without including the Watson-Crick restraints yielded exactly the same structure, although the rmsd value was slightly higher.

Helix Parameters. Helical parameters for the 40 final structures were thoroughly analyzed with the program CURVES 5.1 (Lavery and Sklenar, 1988, 1989). The CURVES algorithm allows the determination of local structural parameters and the overall helical axis for irregular nucleic acid structures. Plots of some of the global helical parameters for the TOTOEt complex are shown in Figure 5. The structural parameters for canonical A-DNA and B-DNA are included in Figure 5 for comparison.

DISCUSSION

Binding Mode and Selectivity of TOTOEt. The 1D ¹H NMR spectrum of TOTOEt bound to d(CGCTAGCG)₂ exhibits sharp lines (line widths ≈ 7 Hz) indicating that there is no chemical exchange between different binding sites. TOTOEt binds to the (5'-CTAG-3')₂ site by bis-intercalating in the CT and AG base steps with an affinity more than 100 times higher than that to any other possible site in the oligomer. There seems to be some evidence that the binding of TOTOEt to dsDNA is even more selective and tighter than that to TOTO.

The chemical shift values of the dsDNA protons at the binding site in the TOTOEt complex reflect intercalation of the aromatic chromophores. Compared to the free dsDNA, the T4 CH₃(6) protons are shifted 0.8 ppm upfield. This is in agreement with the structure of the complex that shows that this methyl group is positioned exactly above the benzothiazole ring and thus exposed to a large ring current effect. Similar strong shifts are observed for A5 H1', A5 H2'', C3 H6, and T4 H6, consistent with the positions of these protons either above

or below a ring system of the intercalating chromophore or near the intercalation site.

Description of the Structure. The structure of the TOTOEt-dsDNA shows that TOTOEt bis-intercalates in the (5'-CTAG-3')₂ site with the benzothiazole ring system sandwiched between the pyrimidines and the quolinium ring system between the purines. The *N*-ethyl group on the benzothiazole is placed in the major groove pointing toward the center of the oligonucleotide and the dyad symmetry axis of the complex to avoid a steric clash with the thymidine methyl group. The linker between the two chromophores is positioned in the minor groove, crossing from one side of the groove to the other. This is similar to what has been observed in other TOTO analogues (Spielmann et al., 1995b; Stærk et al., 1997).

In the complex, the TOTOEt chromophores extend across the dsDNA helix. T4 and T12 unstack from A5 and A13 and move into the major groove to maximize van der Waals contacts with the benzothiazole ring of TOTOEt. The thymidine methyl groups are positioned right on top of the benzothiazole ring of TOTOEt. The observed imino proton cross-peaks, including the cross-peaks to the adenine amino protons and interstrand cross-peaks between adenine H2 and thymidine H1', prove the existence of normal Watson-Crick base pairing in the intercalation site. So even though the thymidines are unstacked from the neighboring adenines, the base pairings of the thymidines with the complementary adenines do not appear to be appreciably disturbed. This is similar to what was observed in the TOTO complex (Spielmann et al., 1995b).

Sugar Conformations. In B-form DNA, the deoxyribose ring adopts an *S*-type conformation described by a pseudorotation angle P of $180 \pm 90^\circ$, while the deoxyribose residues of A-form DNA adopt an *N*-type conformation described by a pseudorotation angle P of $0 \pm 90^\circ$. The results from the PSEUROT calculations resulted in *S*-type sugar conformations for all residues analyzed except for C3 in a purity above ~80% with the pseudorotation angle P_s between 120 and 175° corresponding to C1'-exo and C2'-endo conformations, respectively. For the C3 sugar part, no minimum could be found by the PSEUROT procedure, indicating that the dynamical processes governing the C3 sugar conformation are complex and cannot be described by the two-state model applied by PSEUROT. The C3 nucleotide is flanking one of the intercalated TOTOEt chromophores on the 5' side. Similar complex dynamics were found for the nucleotides flanking the intercalation side of psoralen monoadducted and cross-linked to a DNA oligomer (Emsley et al., 1993). It should be noted, though, that the sugars of the two nucleotides trapped between the TOTOEt chromophores are found to be in relatively pure *S*-type conformations (Table 2).

Helix Parameters. There are three major categories of helical parameters: axis-base pair, intra-base pair, and inter-base pair parameters (Dickerson et al., 1989). These parameters are discussed for the TOTOEt complex with respect to the calculated global helix axis below. However, the parameters reported for the terminal base pairs of the complex are not reliable because of dynamic processes and are included only for completeness. Spectroscopically, the complex was found to possess *C2* symmetry. The small deviation of the structure and the calculated helical parameters from this symmetry reflects the level of uncertainty in the data.

Global Helix Axis. The global helix axis in the dsDNA-TOTOEt complex is not straight but exhibits a pronounced kink at the intercalation site (Figure 2). The

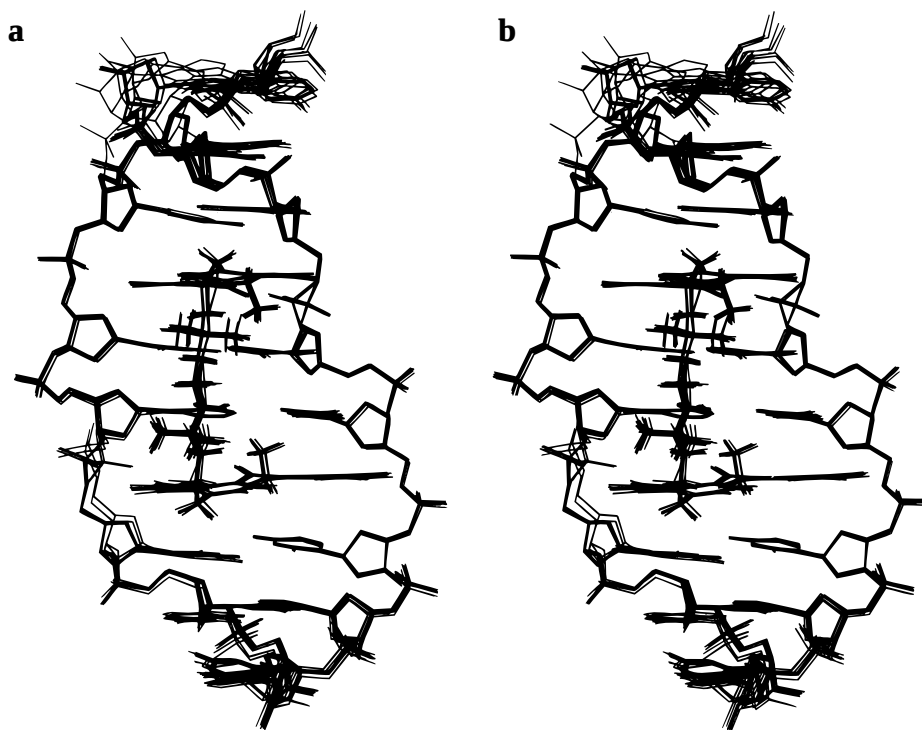


Figure 3. Stereoview of a superposition of 10 randomly chosen structures of the 40 structures obtained by RMD calculations for the TOTOEt-DNA complex. Deoxyribose protons have been omitted for clarity.

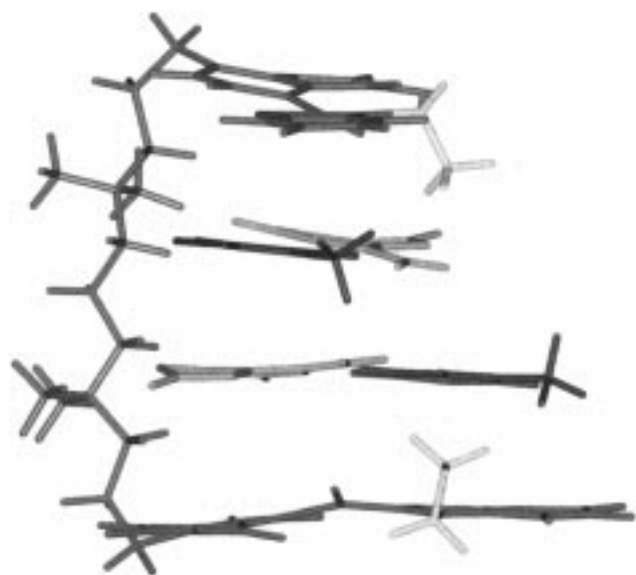


Figure 4. Stacking of the TOTOEt chromophore in the CpT-GpA binding site viewed from the side. Only nucleobases and the TOTOEt chromophores are shown. It is evident that the benzothiazole *N*-ethyl group protrudes into the major groove. The following coloring scheme is used: TOTOEt chromophore, red; benzothiazole *N*-ethyl, yellow; adenine base, green; and thymine base, blue.

helix axis can be divided into different parts. The T4-A5 segment is almost parallel to the terminal parts at both the 5' and 3' ends of the helix, while the intermediate parts, G2-T4 and A5-C7, describe a slip dislocation of these segments caused by the bis-intercalated TOTOEt. The effects of the slip dislocations of the helix axis are demonstrated by the helical parameters. Besides the pronounced kink, the helix axis of the TOTOEt complex shows a small bend ($\sim 5^\circ$) into the minor groove.

We believe that the kink of the global helix axis in the dsDNA-TOTOEt complex is not a mere artifact of the

structure determination. We support this conclusion with the fact that the artifact of distance restraints that are too short applied in the RMD protocol normally produces bends into the major groove due to the larger density of restraints in the major groove of dsDNA (Spielmann et al., 1995a). Furthermore, the distribution of restraints applied in the structure determination of the TOTOEt complex is fairly isotropic around the intercalation site, diminishing the effects of foul distance restraints.

The kink of the helix axis occurs around the TOTOEt intercalation site, and we believe the benzothiazole *N*-ethyl group is responsible for this as the dsDNA-TOTO complex shows no kinks or bends of the helix axis. It has recently been demonstrated that the placement of bulky groups in the minor groove of dsDNA can induce pronounced bending into the major groove (Chen et al., 1996). The benzothiazole ring system does not stack exactly coplanar with T4 but is twisted slightly relative to the nucleobase and thus inducing the kink in the helix axis.

Axis-Base Pair Parameters. *X*-axis displacement, *Y*-axis displacement, inclination, and tip are the parameters minimized to obtain the global helix axis (Lavery and Sklenar, 1988). These parameters thus describe how the base pairs are positioned in the helix relative to the global helix axis.

The *X*-axis displacement and *Y*-axis displacement are both close to the values for B-DNA, while inclination shows significant deviations with an evident C2 symmetry. The value for tip shows large and correlated changes from the B-DNA values, changing from negative to positive going from C3 to G6; this is a direct consequence of the geometry of the kinking helix axis.

Intra-Base Pair Parameters. The values obtained for shear, stretch, stagger, and opening are partly determined by the constraints introduced to mimic Watson-Crick base pairing. However, in the central part of the oligonucleotide, the numerous dsDNA-TOTOEt NOEs

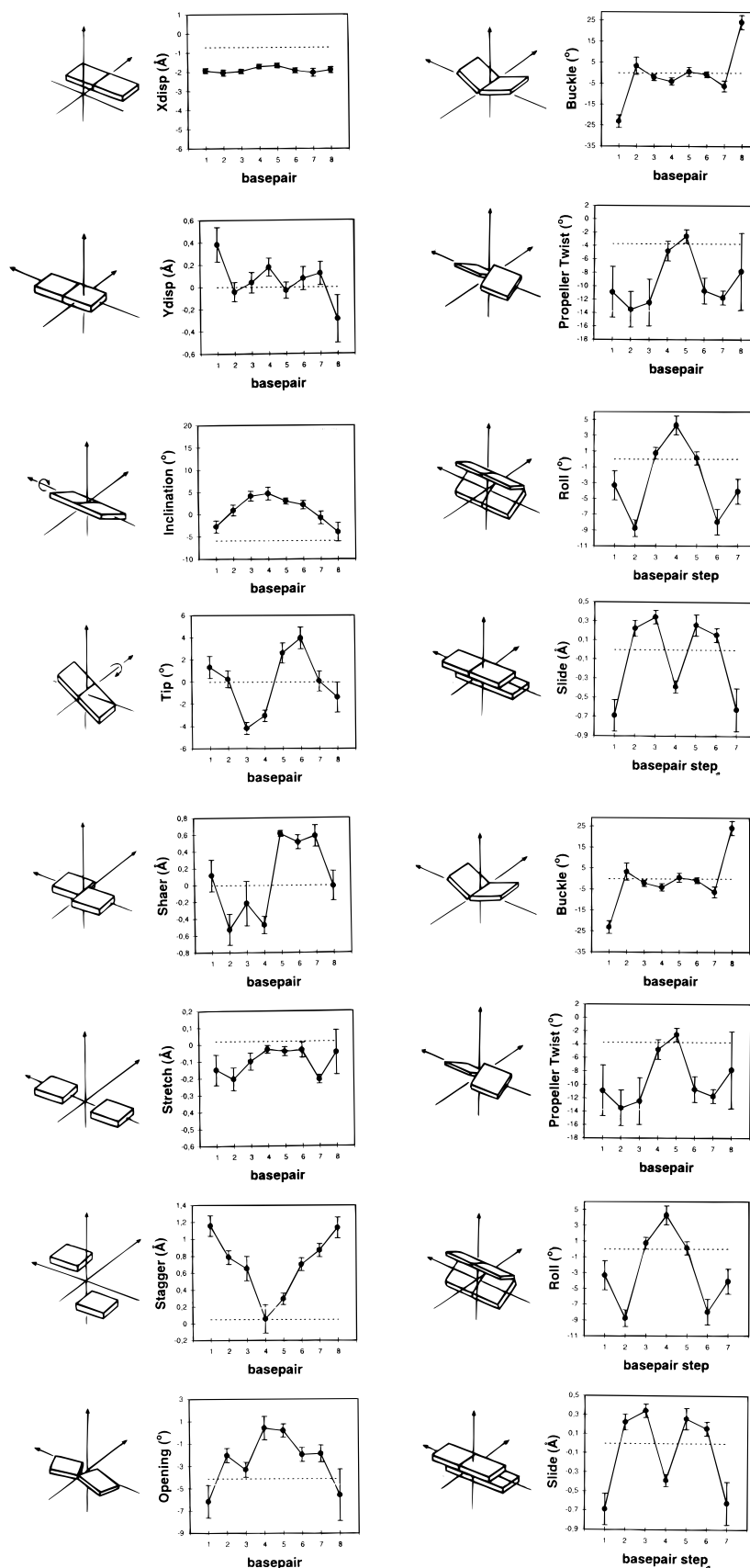


Figure 5. Helical parameters for the TOTOEt complex calculated using CURVES and compared with those of canonical A-DNA (•••) and B-DNA (---).

lock the conformation of the structure. The TOTOEt ligand extends across the duplex and hence makes NOE contacts to both strands. This contributes significantly to the determination of the above parameters.

The shear changes from negative to positive across the intercalation site, compensating for the intercalation of the TOTOEt chromophores. The shear introduced possibly stems from the A5 maximizing the overlap with one

ring of the quinolinium ring system and the T4 moving into the major groove to maximize van der Waals forces with the benzothiazole ring system. There is only a slight net shear introduced in the helix. This was also observed for the dsDNA-TOTO structure (Spielmann et al., 1995b). The stretch is essentially that of B-DNA.

Base opening is almost symmetrically disposed around the T4-A5 base step. An opening angle larger than that of standard DNA narrows the minor groove. It is seen that the T4 moves into the major groove and the minor groove subsequently narrows, probably also due to van der Waals interactions between the linker *N*-methyl groups and electrostatic attractions between the anionic DNA phosphates and the linker nitrogens. The opening observed for the central base pairs is not as large as that found in the dsDNA-TOTO complex. This is a consequence of the near coplanarity of the two ring systems of the TOTOEt chromophores. For the TOTO complex, it was found that the chromophores were twisted about the quinoline-C8-thiazole bond by an angle of 22°. In the dsDNA-TOTOEt complex, this angle is 6°. A GAUSSIAN94 calculation performed on the free TOTOEt shows an angle of 22° between the two planes of the chromophores. The decrease of this angle from the free TOTOEt to the bound is due to clamping of TOTOEt by the nucleobases in the complex.

The slight twisting of the TOTOEt chromophores is mirrored in the parameters for buckle and propeller twist. It is reported that intercalation can induce buckling in dsDNA as the base pairs adjacent to the intercalation site buckle to maximize van der Waals contacts with the chromophore(s) of the intercalating agent (Williams and Gao, 1992). TOTOEt introduces almost no buckle in the dsDNA, indicating that the nucleobases of the complex can maximize van der Waals contacts and stacking with the TOTOEt chromophores without the propensity to introduce buckling.

The propeller twists of the two central base pairs are essentially that of B-DNA. A large propeller twist is introduced into the C3-G14 and G6-C11 base pairs. This is because the G6-G14 remains coplanar with the quinolinium ring system, while C3-C11 stacks with G2-G10.

Inter-Base Pair Parameters. The roll, slide, and shift in regular A-DNA and B-DNA conformations are essentially zero. These parameters show large and correlated changes from the equilibrium values in the TOTOEt complex. There are shifts for the two intercalation sites with opposite signs that cancel each other in terms of the overall helix. The slide exhibits correlated and compensatory changes for the C3-T4 and T4-A5 base pair steps to accommodate the dye intercalation. The shift and slide values are exact mirrors of the lateral displacements of the global helix axis.

The central base pair step T4-A5 has a positive roll. Positive roll opens the angle between base pairs toward the minor groove, resulting in a bend into the major groove. A negative roll results in a bend into the minor groove. The central base pair T4-A5 is clamped between the two thiazole orange chromophores to maximize van der Waals contacts. The bases roll to open the minor groove. The base pair steps flanking the intercalation sites show almost zero rolls, while the G2-C3 and G6-C7 base pair steps one step away from the intercalation site exhibit significant negative roll. This is to compensate for the kinking of the helix axis at the C3-T4 and A5-G6 base pair steps.

The helix is unwound by 80° and has an overall helical repeat of 12 base pairs in the complex, consistent with the bis-intercalation of TOTOEt. It is noteworthy that

the unwinding is largely confined to the two base pair steps defining the intercalation site.

CONCLUSION

The results obtained in this work show that TOTOEt binds selectively to dsDNA with (5'-CTAG-3')₂ as the preferred binding site. Compared to TOTO, this implies that modification of this drug on the benzothiazole N atom system is possible while still maintaining or even enhancing the selectivity and binding affinity. Our study showed that an extremely well defined complex between TOTOEt and (5'-CGCTAGCG-3')₂ was obtained. The complex seems to be even more well defined than that in the case of TOTO, indicating that TOTOEt has a higher affinity and selectivity toward dsDNA. This is possibly caused by the benzothiazole *N*-ethyl group clamping the drug to the dsDNA.

The *N*-ethyl group on the benzothiazole of TOTOEt is placed in the major groove pointing toward the center of the oligonucleotide and the dyad symmetry axis of the complex. This is exactly the orientation that is required for a TOTO analogue with a linker connecting the two chromophores in the major groove. Consequently, it seems rather straightforward to design such an analogue, the only remaining problem being the length of the linker. Direct measurement of the structure indicates the linker in the major groove should be slightly shorter than the linker in the minor groove. After the proper linker in the major groove has been found, it seems obvious from the structure that the subsequent design of a TOTO analogue with a linker in both the major groove and the minor groove is straightforward. This would probably yield a selective dsDNA binding drug with an extremely high binding affinity and vanishing kinetic abilities.

ACKNOWLEDGMENT

We are grateful to Dr. E. B. Pedersen (Department of Chemistry, Odense University, Denmark) for providing TOTOEt. Professor H. Peter Spielmann is acknowledged for supporting this work with spectrometer time on a Varian Unity+ 500 NMR spectrometer and many helpful discussions.

LITERATURE CITED

- Bishop, J. M. (1987) The Molecular Genetics of Cancer. *Nature* 235, 305-311.
- Boelens, R., Scheek, R. M., Dijkstra, K., and Kaptein, R. (1985) Sequential Assignment of Imino- and Amino-Proton Resonances in ¹H NMR Spectra of Oligonucleotides by Two-Dimensional NMR Spectroscopy. Application to lac Operator Fragment. *J. Magn. Reson.* 62, 378-386.
- Borgias, B. A., and James, T. L. (1990) MARDIGRAS-A procedure for matrix analysis of relaxation for discerning geometry of an aqueous structure. *J. Magn. Reson.* 87, 475-487.
- Borgias, B. A., Gochin, M., Kerwood, D., and James, T. L. (1990) Relaxation Matrix analysis of 2D NMR data. *Prog. NMR Spectrosc.* 22, 83-100.
- Chen, H., Liu, X., and Patel, D. J. (1996) DNA Bending and Unwinding Associated with Actinomycin D Antibiotics Bound to Partially Overlapping Sites on DNA. *J. Mol. Biol.* 258, 457-479.
- de Leeuw, F. A. A. M., and Altona, C. (1983) Computer-Assisted Pseudorotation Analysis of Five-Membered Rings by Means of Proton Spin-Spin Coupling Constants: Program PSEUROT. *J. Comput. Chem.* 4, 428-437.
- Dickerson, R. E., Bansal, M., Calladine, C. R., Diekmann, S., Hunter, W. N., Kennard, O., von Kitzing, E., Nelson, H. C. M., Lavery, R., Olson, W. K., Saenger, W., Shakked, Z., Soumpasis, D. M., Tung, C.-S., Sklenar, H., Wang, A. H. J.,

- and Zhurkin, V. B. (1989) Definitions and nomenclature of nucleic acid structure parameters. *EMBO J.* 8, 1–4.
- Emsley, L., Dwyer, T. J., Spielmann, H. P., and Wemmer, D. E. (1993) Determination of DNA Conformational Features from Selective Two-Dimensional NMR Experiments. *J. Am. Chem. Soc.* 115, 7765–7771.
- Faridi, J., Nielsen, K. E., Stein, P. C., and Jacobsen, J. P. (1997) Bis-Intercalation of a Homodimeric Thiazole Orange Dye in DNA: Dynamic Exchange between Two Sites. *J. Biomol. Struct. Dyn.* 15, 321–331.
- Feigon, J., Leupin, W., Denny, W. A., and Kearns, D. R. (1983) Two-dimensional proton nuclear magnetic resonance investigation of the synthetic deoxyribonucleic acid decamer d(ATATCGATAT)₂. *Biochemistry* 22, 5943–5951.
- Gochin, M., Zon, G., and James, T. L. (1990) Two-Dimensional COSY and Two-Dimensional NOE Spectroscopy of d(AC)₄·d(AC)₄: Extraction of Structural Constraints. *Biochemistry* 29, 11161–11171.
- Guéron, M., and Plateau, P. (1982) Exchangeable Proton NMR without Base-Line Distortion, Using New Strong-Pulse Sequences. *J. Am. Chem. Soc.* 104, 7310–7311.
- Hansen, L. F., Jensen, L. K., and Jacobsen, J. P. (1996) Bis-intercalation of a homodimeric thiazole orange dye in DNA in symmetrical pyrimidine-pyrimidine-purine-purine oligonucleotides. *Nucleic Acids Res.* 24, 859–867.
- Hare, D. R., Wemmer, D. E., Chou, S.-H., Drobný, G., and Reid, B. R. (1983) Assignment of the nonexchangeable proton resonances of d(C-G-C-G-A-A-T-T-C-G-C-G) using two-dimensional nuclear magnetic resonance methods. *J. Mol. Biol.* 171, 319–336.
- Jacobsen, J. P., Pedersen, J. B., Hansen, L. F., and Wemmer, D. E. (1995) Site selective bis-intercalation of a homodimeric thiazole orange dye in DNA oligonucleotides. *Nucleic Acids Res.* 23, 753–760.
- Lavery, R., and Sklenar, H. (1988) The definition of generalized helicoidal parameters and for axis curvature of irregular nucleic acids. *J. Biomol. Struct. Dyn.* 6, 63–91.
- Lavery, R., and Sklenar, H. (1989) Defining the structure of irregular nucleic acids: Conventions and principles. *J. Biomol. Struct. Dyn.* 7, 655–667.
- Liu, H., Spielmann, H. P., Ulyanov, N. A., Wemmer, D. E., and James, T. L. (1995) Interproton distances bounds from 2D-NOE intensities: Effect of experimental noise and peak integrations errors. *J. Biol. NMR* 6, 390–402.
- Macaya, R. F., Schultze, P., and Feigon, J. (1992) Sugar Conformation in Intramolecular DNA Triplexes Determined by Coupling Constants Obtained by Automated Simulation of P.COSY Cross Peaks. *J. Am. Chem. Soc.* 114, 781–783.
- Scheek, R. M., Russo, N., Boelens, R., and Kaptein, R. (1983) Sequential Resonance Assignments in DNA ¹H NMR Spectra by Two-Dimensional NOE Spectroscopy. *J. Am. Chem. Soc.* 105, 2914–2916.
- Scheek, R. M., Boelens, R., Russo, N., Van Boom, J. H., and Kaptein, R. (1984) Sequential Resonance Assignments in ¹H NMR Spectra of Oligonucleotides by Two-Dimensional NMR Spectroscopy. *Biochemistry* 23, 1371–1376.
- Schmitz, U., and James, T. L. (1995) How to Generate Accurate Solution Structures of Double-Helical Nucleic Acid Fragments Using Nuclear Magnetic Resonance and Restrained Molecular Dynamics. *Methods Enzymol.* 261, 3–44.
- Spielmann, H. P., Dwyer, T. J., Hearst, J. S., and Wemmer, D. E. (1995a) Solution Structure of Psoralen Monoadducted and Cross Linked DNA Oligomers by NMR Spectroscopy and Restrained Molecular Dynamics. *Biochemistry* 34, 12937–12953.
- Spielmann, H. P., Wemmer, D. E., and Jacobsen, J. P. (1995b) Solution Structure of a DNA Complex with the Fluorescent Bis-Intercalator TOTO Determined by NMR Spectroscopy. *Biochemistry* 34, 8542–8553.
- Stærk, D., Hamed, A. A., Pedersen, E. B., and Jacobsen, J. P. (1997) Bis-Intercalation of Homodimeric Thiazole Orange Dyes in DNA: The Effect of Modifying the Linker. *Bioconjugate Chem.* 8, 869–877.
- Williams, L. D., and Gao, Q. (1992) DNA-Ditercalinium Interactions: Implications for Recognition of Damaged DNA. *Biochemistry* 31, 4315–4324.
- Wüthrich, K. (1986) in *NMR of Proteins and Nucleic Acids*, John Wiley & Sons, New York.

BC970133I

Preparation of Conjugates of Oligodeoxynucleotides and Lipid Structures and Their Interaction with Low-Density Lipoprotein

Erik T. Rump, Remco L. A. de Vruh, Leo A. J. M. Sliedregt, Erik A. L. Biessen, Theo J. C. van Berkel, and Martin K. Bijsterbosch*

Division of Biopharmaceutics, Leiden/Amsterdam Center for Drug Research, P.O. Box 9503, 2300 RA Leiden, The Netherlands. Received October 3, 1997; Revised Manuscript Received January 16, 1998

The high expression level of receptors for low-density lipoprotein (LDL) on tumor cells makes LDL an attractive carrier for selective delivery of drugs to these cells. The aim of this study is to allow incorporation of oncogene-directed antisense oligodeoxynucleotides (ODNs) into the lipid moiety of LDL. Therefore, ODNs were conjugated with oleic acid, cholesterol, and several other steroid lipids. These latter steroid lipids were synthesized starting from bile acids and were varied in lipophilicity by attaching oleic acid ester structures. The lipid structures, activated as pentafluorophenyl esters, were conjugated in solution phase to 3'-amino-tailed ODNs. The ODNs conjugated with lithocholic acid, oleic acid, and cholesterol could easily be purified by reversed phase (RP)-HPLC. However, the ODNs conjugated with the oleoyl steroid ester structures irreversibly bound to the column material. These highly lipidic ODNs were separated from the nonconjugated ODN by electrophoresis in a 1% low-melting agarose gel containing 0.1% Tween 20. This method was found to be very effective in isolating the ODNs conjugated to the oleoyl steroid ester structures. The ODNs conjugated with cholesterol and the oleoyl esters of lithocholic and cholenic acid associated readily and nearly completely with LDL. However, the less lipidic ODNs and the ODN conjugated with the dioleoyl ester of chenodeoxycholic acid did not and did incompletely associate, respectively. Lithocholic acid and oleic acid are probably not sufficiently lipophilic to induce association with LDL, whereas the dioleoyl ester structure is probably too bulky and extended to allow partitioning into the lipid moiety of LDL. We conclude that several of the lipid-ODNs can associate readily with LDL, enabling delivery of oncogene-directed antisense ODNs via the LDL receptor pathway.

INTRODUCTION

The so-called first-generation antisense ODNs,¹ mostly phosphorothiate ODNs, were primarily designed to stabilize the nuclease-sensitive phosphodiester backbone of unmodified ODNs. Numerous reports have shown their effectiveness both in vitro and in vivo (1, 2). The success in preclinical research has led to approval of several antisense ODNs for clinical trials (2). Although the phosphorothioate ODNs and other backbone-modified ODNs are very promising in antisense research, cellular uptake of these modified ODNs still remains an inefficient process.

Lipophilic moieties, conjugated to ODNs at either the 3'- or the 5'-end, stabilize the ODN and significantly enhance their cellular uptake (3–6). This can subsequently lead to improved antisense inhibition, as has been shown for cholesteryl-derivatized ODNs (7–11). The increased antisense efficacy of these ODNs may be a direct result of the affinity of the lipophilic moiety for membrane structures leading to a more efficient uptake, probably via a process of adsorptive endocytosis (4, 8, 10).

Alternatively, binding to the low-density lipoprotein (LDL) may also play a role, as it has been demonstrated that upregulation of the LDL receptor (LDLr) on cells increases the cellular uptake and effectiveness of cholesteryl-derivatized antisense ODNs (10).

LDL is the main cholesterol-transporting vehicle in the human circulatory system. It is a spherical particle with a mean diameter of 23 nm, and it consists of an apolar core of cholesteryl esters and triglycerides, which is surrounded by a shell of cholesterol and phospholipids (12). A large part of the surface is covered by apolipoprotein B100, which is responsible for recognition by the LDLr. Binding of LDL to its receptor is followed by internalization of the particle. There is evidence that many malignant cells, like leukemia cells, take up large amounts of LDL via the LDLr. The LDL particle may therefore constitute an attractive carrier for selective and highly efficient intracellular delivery of antineoplastic drugs to tumor cells. The LDLr-mediated therapeutic approach has already been explored for conventional lipophilic antitumor drugs (13–16) but may also be applied for lipophilic oncogene-directed antisense ODNs. Earlier studies established the potential of receptor-mediated endocytosis to mediate effective internalization of ODNs, thereby increasing the antisense efficacy (17, 18).

We and others have shown before that attachment of a cholesteryl moiety to the 5'-end allows association of ODNs with LDL in vitro. Association presumably occurs by partitioning of the relatively rigid cholesteryl moiety into the lipoprotein's lipid shell (10, 19). In analogy,

* Author to whom correspondence should be addressed. E-mail: bijsterb@LACDR.Leidenuniv.nl. Telephone: 31-71-5276038. Fax: 31-71-5276032.

¹ Abbreviations: BAP, 4-bromoacetophenyl; CH₃CN, acetonitrile; DCC, dicyclohexylcarbodiimide; DCE, 1,2-dichloroethane; DCM, dichloromethane; DiPEA, *N,N*-diisopropylethylamine; DMAP, (dimethylamino)pyridine; DMF, *N,N*-dimethylformamide; ODN, oligodeoxynucleotide; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; *T*_m, melting temperature.

conjugation of an alkyl chain was also shown to induce association of ODNs to LDL (20).

In this study, we examine the ability of several lipophilic moieties to induce spontaneous association of ODNs to LDL. *c-myb* antisense ODNs were conjugated in solution phase at the 3'-end with lipidic structures which are composed of oleic acid and steroids. The antisense ODNs used in this study are directed to the *c-myb* proto-oncogene which is abundantly expressed in leukemic cells (21, 22). Numerous reports already showed the therapeutic potential of these ODNs in leukemic malignancies (23–25). Association of the lipid-ODNs to LDL is anticipated to enhance the cellular uptake, leading to an improved efficacy of the antisense ODN.

EXPERIMENTAL SECTION

Reagents. An 18-mer antisense ODN complementary to the *c-myb* proto-oncogene (5'-G*T*G* CCG GGG TCT TCG GGC-3'), provided with a seven-carbon 3'-amine linker and three phosphorothioate linkages (*) at the 5'-end, and a corresponding 28-mer sense ODN (5'-CCA TGG CCC GAA GAC CCC GGC ACA GCA T-3') were from Eurogentec (Seraing, Belgium). Low-melting multipurpose (LM-MP) agarose and Agarase from *Pseudomonas atlantica* were obtained from Boehringer (Mannheim, Germany). Tween 20 was from Merck (Darmstadt, Germany). Hionic Fluor and Emulsifier Safe scintillation cocktails and Soluene-350 were from Packard (Downers Grove, IL). Pyridine and DCE were dried by refluxing with CaH₂ and were distilled. DMF was stirred with CaH₂ prior to distillation under reduced pressure. These and other solvents (analytical grade) were stored over molecular sieves (4 Å). Oleoyl chloride was distilled under reduced pressure before use. Na¹²⁵I (carrier-free) and ³H₂O were purchased from Amersham (Buckinghamshire, U.K.). DiPEA (99.5%, redistilled) was purchased from Aldrich (Milwaukee, WI). All other chemicals were of analytical grade.

General Procedures. ODN concentrations were determined by UV spectroscopy at 260 nm. All reactions were run at ambient temperature, unless otherwise stated. Reactions were monitored by TLC on silica gel 60 F₂₅₄ aluminum sheets (Merck). Compounds were visualized under UV (254 nm) or by spraying with MnCl₂ for detection of steroids (26) or cresol green for carboxylic acids. Short column chromatography was performed on silica gel 60 (Merck, 230–400 mesh). ¹H NMR spectra were measured at 200 MHz using a JEOL JNM-FX 200 spectrometer with tetramethylsilane as an internal standard. ¹³C NMR spectra were measured at 50.3 MHz on the same apparatus, with CDCl₃ as an internal standard. ¹³C NMR spectra were monitored using the attached proton test technique. ¹⁹F NMR spectra were measured at 282 MHz with CFCl₃ as an internal standard on a Bruker WM-300 spectrometer. FAB mass spectrometry was carried out using a JEOL JMS SX/SX-102A four-sector mass spectrometer.

5-Cholenic Acid 3 β -Ol (4-Bromobenzoyl)methyl Ester 1b. To a solution of 250 mg (0.67 mmol) of cholenic acid **1a** in 10 mL of DMF were added 205 mg (0.74 mmol) of dibromoacetophenone and 240 mg (0.74 mmol) of Cs₂CO₃. After completion of the reaction, monitored by TLC (DCM), the mixture was filtered, and DMF was evaporated under reduced pressure. The product was purified by column chromatography using *n*-hexane/DCM (3:7, v/v) as the eluent, yielding 364 mg (0.64 mmol, 95%) of **1b**. ¹H NMR [parts per million (ppm)]: δ 7.78, 7.63 (4H,

dd, J = 8.9 Hz, ArH), 5.30 (3H, m, COCH₂O and vinyl H), 3.51 (1H, m, H-3). ¹³C {¹H} NMR (ppm): δ 71.7 (C-3), 65.7 (COCH₂O), 121.6 (C-6), 132.2, 129.3 (CH Ar), 133.0, 129.1 (qC Ar), 173.6 (C-24), 191.5 (C=O ketone).

5 β -Cholanic Acid 3 α -Ol (4-Bromobenzoyl)methyl Ester 2b. Compound **2b** was prepared with the procedure described for **1b**, starting from 865 mg (2.30 mmol) of lithocholic acid **2a**. The product was purified by column chromatography with MeOH/DCM (1:99, v/v) as the eluent, yielding 1.31 g (2.28 mmol, 99%) of **2b**. ¹H NMR (ppm): δ 7.74 and 7.59 (4H, dd, J = 8.6 Hz, ArH), 5.26 (2H, m, COCH₂O), 3.57 (1H, m, H-3). ¹³C {¹H} NMR (ppm): δ 65.3 (COCH₂O), 70.7 (C-3), 128.5, 132.3 (qC Ar), 128.8, 131.6 (CH Ar), 173.3 (C-24), 191.4 (C=O ketone).

5 β -Cholanic Acid 3 α ,7 α -Diol (4-Bromobenzoyl)methyl Ester 3b. Compound **3b** was prepared with the procedure described for **1b**, starting from 500 mg (1.27 mmol) of chenodeoxycholic acid. The product was purified by column chromatography with DCM $\rightarrow$ MeOH/DCM (1:25, v/v) as the eluent, yielding 650 mg (1.10 mmol, 87%) of **3b**. ¹H NMR (ppm): δ 7.79, 7.67 (4H, dd, J = 8.6 Hz, ArH), 5.28 (2H, m, COCH₂O), 3.45 (1H, m, H-3). ¹³C {¹H} NMR (ppm): δ 65.4 (COCH₂O), 67.8 (C-7), 71.4 (C-3), 128.6, 132.6 (qC Ar), 129.0 and 131.8 (CH Ar), 173.2 (C-24), 191.2 (C=O ketone).

3 β -(Oleoyloxy)-5-cholenic Acid (4-Bromobenzoyl)methyl Ester 1c. To a solution of 200 mg (0.35 mmol) of **1b** and 47 mg (0.39 mmol) of DMAP in 10 mL of pyridine, kept under a N₂ atmosphere, was added dropwise 116.5 mg (0.40 mmol) of oleoyl chloride. After being stirred for 6 h, the mixture was concentrated in vacuo. The residue was dissolved in DCM and the mixture washed with 1 N HCl and brine. The organic layer was dried over MgSO₄ and evaporated to dryness. The product was purified by column chromatography using *n*-pentane/DCM (2:5, v/v) as the eluent, yielding 170 mg (0.20 mmol, 58%) of **1c**. ¹H NMR (ppm): δ 7.68, 7.52 (4H, dd, J = 8.6 Hz, ArH), 5.26 (5H, m, COCH₂O and vinyl H), 4.50 (1H, m, H-3). ¹³C {¹H} NMR (ppm): δ 14.1 (CH₃-oleoyl), 65.6 (COCH₂O), 73.6 (C-3), 122.4 (C-6), 129.2, 132.1 (CH Ar), 129.0, 132.9 (qC Ar), 129.7, 129.9 (CH=oleoyl), 139.6 (C-5), 173.2 (C=O oleoyl ester), 173.5 (C-24), 191.4 (C=O ketone).

3 α -(Oleoyloxy)-5 β -cholanic Acid (4-Bromobenzoyl)methyl Ester 2c. Compound **2c** was prepared with the procedure described for **1c**, starting from 470 mg (0.82 mmol) of **2b**. The product was purified by column chromatography using *n*-pentane/DCM (2:3, v/v) as the eluent, yielding 650 mg (0.78 mmol, 95%) of **2c**. ¹H NMR (ppm): δ 7.78 and 7.63 (4H, dd, J = 8.2 Hz, ArH), 5.34 (4H, m, COCH₂O and vinyl H), 4.75 (1H, m, H-3). ¹³C {¹H} NMR (ppm): δ 14.0 (CH₃-oleoyl), 65.6 (COCH₂O), 73.9 (C-3), 129.2, 132.1 (CH Ar), 129.0, 132.9 (qC Ar), 129.7, 129.9 (CH=oleoyl), 173.2 (C=O oleoyl ester), 173.5 (C-24), 191.3 (C=O ketone).

3 α ,7 α -Bis(oleoyloxy)-5 β -cholanic Acid (4-Bromobenzoyl)methyl Ester 3c. To a solution of 340 mg (0.58 mmol) of **3b** in 10 mL of pyridine were added 155 mg (1.3 mmol) of DMAP and 451 mg (1.5 mmol) of oleoyl chloride. After the mixture was stirred for 2 h, another portion of 100 mg of DMAP and 226 mg of oleoyl chloride were added. After being stirred overnight, the mixture was concentrated in vacuo. The residue was dissolved in DCM, and the organic layer was washed with 1 N HCl and brine. The organic layer was dried over MgSO₄. The product was purified by column chromatography using DCM as the eluent, yielding 150 mg (0.13 mmol, 23%) of the dioleate **3c** and 210 mg (0.27 mmol, 46%) of the monooleate (C-3-esterified). ¹H NMR (ppm): δ 7.78 and

7.62 (4H, dd, $J = 8.9$ Hz, ArH), 5.34 (6H, m, COCH₂O and vinyl H), 4.89 (1H, m, H-7), 4.60 (1H, m, H-3). ¹³C {¹H} NMR (ppm): δ 14.0 (CH₃-oleoyl), 65.6 (COCH₂O), 70.8 (C-7), 73.7 (C-3), 129.1, 132.1 (CH Ar), 129.0, 132.9 (qC Ar), 129.1, 129.9 (CH=oleoyl), 172.9, 173.2, 173.4 (2 $\times$ C=O oleoyl ester, C24).

3 β -(Oleoyloxy)-5-cholenic Acid 1d. To a solution of 170 mg (0.20 mmol) of **1c** in 20 mL of DCM/HAc (1:1, v/v) was added 100 mg of Zn (powder). After being stirred overnight, the mixture was filtered over Hyflo and concentrated in vacuo. The product was purified by column chromatography using MeOH/DCM (1:99, v/v) as the eluent, yielding 124 mg (0.19 mmol, 97%) of **1d**. ¹H NMR (ppm): δ 5.32 (3H, m, vinyl H), 4.60 (1H, m, H-3). ¹³C {¹H} NMR (ppm): δ 14.1 (CH₃-oleoyl), 73.6 (C-3), 122.4 (C-6), 129.7, 129.9 (CH=oleoyl), 139.6 (C-5), 173.3 (C=O oleoyl ester), 180.3 (C-24). MS(FAB): 637.5 (M - H). HRMS: Calcd for C₄₂H₆₉O₄ 637.5196, found 637.5189.

3 α -(Oleoyloxy)-5 β -cholanolic Acid 2d. Compound **2d** was prepared with the procedure described for **1d**, starting from 650 mg (0.78 mmol) of **2c**. The product was purified by column chromatography using MeOH/DCM (from 1:99 to 1:66, v/v) as the eluent, yielding 460 mg (0.72 mmol, 92%) of **2d**. ¹H NMR (ppm): δ 11.06 (1H, m, COOH), 5.33 (2H, m, vinyl H), 4.72 (1H, m, H-3). ¹³C {¹H} NMR (ppm): δ 14.0 (CH₃-oleoyl), 74.0 (C-3), 129.7, 129.9 (CH=oleoyl), 173.4 (C=O oleoyl ester), 180.5 (C-24). MS(FAB): 639 (M - H). HRMS: Calcd for C₄₂H₇₁O₄ 639.5352, found 639.5399.

3 α ,7 α -Bis(oleoyloxy)-5 β -cholanolic Acid 3d. Compound **3d** was prepared with the procedure described for **1d**, starting from 150 mg (0.13 mmol) of **3c**. The product was purified by column chromatography using MeOH/DCM (from 1:99 to 2:99, v/v) as the eluent, yielding 96 mg (0.10 mmol, 80%) of **3d**. ¹H NMR (ppm): δ 5.34 (4H, m, vinyl H), 4.89 (1H, m, H-7), 4.59 (1H, m, H-3). ¹³C {¹H} NMR (ppm): δ 13.9 (CH₃-oleoyl), 70.8 (C-7), 73.7 (C-3), 129.5, 129.8 (CH=oleoyl), 172.8, 173.1 (2 $\times$ C=O oleoyl ester), 179.5 (C-24). MS(FAB): 920 (M - H). HRMS: Calcd for C₆₀H₁₀₃O₆ 919.7755, found 919.7819.

3 β -(Oleoyloxy)-5-cholenic Acid Pentafluorophenyl Ester 1e. To a solution of 90 mg (0.14 mmol) of **1d** in 10 mL of DCE was added 30 mg (0.16 mmol) of pentafluorophenol, and the mixture was cooled to 0 °C. Subsequently, 35 mg (0.17 mmol) of DCC was added. After 0.5 h, the mixture was allowed to warm to room temperature. After precipitation of dicyclohexylurea, the mixture was filtered and concentrated in vacuo. The product was purified by column chromatography using hexane/DCM (11:9, v/v) as the eluent, yielding 110 mg (0.14 mmol, 98%) of **1e** as a colorless oil. ¹H NMR (ppm): δ 5.34 (3H, m, vinyl H), 4.59 (1H, m, H-3), 0.88 (3H, t, CH₃-oleoyl). ¹³C {¹H} NMR (ppm): δ 73.6 (C-3), 122.4 (C-6), 129.7, 129.9 (CH=oleoyl), 139.7 (C-5), 169.9 (C-24), 173.2 (C=O oleoyl ester). ¹⁹F NMR (ppm): δ -151.1 (m), -156.5 (t), -160.7 (m).

3 α -(Oleoyloxy)-5 β -cholanolic Acid Pentafluorophenyl Ester 2e. Compound **2e** was prepared with the procedure described for **1e**, starting from 260 mg (0.41 mmol) of **2d**. The product was purified by column chromatography with hexane/DCM (2:3, v/v) as the eluent, yielding 325 mg (0.40 mmol, 98%) of **2e** as a colorless oil. ¹H NMR (ppm): δ 5.34 (2H, m, vinyl H), 4.73 (1H, m), 0.88 (3H, t, CH₃-oleoyl). ¹³C {¹H} NMR (ppm): δ 14.1 (CH₃-oleoyl), 74.0 (C-3), 129.7, 130.0 (CH=oleoyl), 169.9 (C-24), 173.3 (C=O oleoyl ester). ¹⁹F NMR (ppm): δ -151.1 (m), -156.5 (t), -160.7 (m).

3 α ,7 α -Bis(oleoyloxy)-5 β -cholanolic Acid Pentafluorophenyl Ester 3e. Compound **3e** was prepared with

the procedure described for **1e**, starting from 96 mg (0.10 mmol) of **3d**. The product was purified by column chromatography with hexane/DCM (1:3, v/v) as the eluent, yielding 77 mg (0.07 mmol, 68%) of **3e** as a colorless oil. ¹H NMR (ppm): δ 5.33 (4H, m, vinyl H), 4.90 (1H, m, H-7), 4.60 (1H, m, H-3), 0.88 (3H, t, CH₃-oleoyl). ¹³C {¹H} NMR (ppm): δ 14.0 (CH₃-oleoyl), 70.8 (C-7), 73.7 (C-3), 129.6, 129.9 (CH=oleoyl), 169.8 (C-24), 172.9, 173.1 (2 $\times$ C=O oleoyl ester). ¹⁹F NMR (ppm): δ -151.1 (m), -156.5 (t), -160.7 (m).

5 β -Cholanolic Acid 3 α -Ol Pentafluorophenyl Ester 2f. To a solution of 150 mg (0.40 mmol) of lithocholic acid **2a** in 5 mL of DCE/DMF (4:1, v/v) was added 88 mg (0.44 mmol) of pentafluorophenol, and the mixture was cooled to 0 °C. Subsequently, 99 mg (0.44 mmol) of DCC was added. After 0.5 h, the mixture was allowed to warm to room temperature and stirring was continued until dicyclohexylurea precipitated. The mixture was filtered and concentrated in vacuo. The product was purified by column chromatography using DCM/MeOH (98:2, v/v) as the eluent, yielding 194 mg (0.36 mmol, 89%) of **2f** as a white solid. ¹H NMR (ppm): δ 3.73 (1H, m, H-3). ¹³C {¹H} NMR (ppm): δ 73.6 (C-3), 122.4 (C-6), 129.7, 129.9 (CH=oleoyl), 139.7 (C-5), 169.9 (C-24), 173.2 (C=O oleoyl ester). ¹⁹F NMR (ppm): δ -151.1 (m), -156.6 (t), -160.7 (m).

Oleic Acid Pentafluorophenyl Ester 4b. Compound **4b** was prepared with the procedure described for **2f**, starting from 200 mg (0.71 mmol) of oleic acid **4a**. The product was purified by column chromatography with *n*-hexane/DCM (9:1, v/v) as the eluent, yielding 230 mg (0.51 mmol, 72%) of **4b** as a colorless oil. ¹H NMR (ppm): δ 5.35 (2H, m, vinyl H), 0.88 (3H, t, CH₃-oleoyl). ¹³C {¹H} NMR (ppm): δ 14.04 (CH₃-oleyl), 129.63 and 130.06 (CH=oleyl), 169.52 (C=O). ¹⁹F NMR (ppm): δ -151.1 (m), -156.6 (t), -160.7 (m).

Radiolabeling of ODN by Hydrogen-Tritium Exchange. The 3'-amine ODN was tritiated by the procedure of Graham et al. (27), with slight modifications. After 0.50 mg of the 3'-amine ODN was dissolved in 200 μ L of 50 mM sodium phosphate buffer (pH 7.8), containing 0.1 mM EDTA, the solution was freeze-dried. The lyophilized material was dissolved in 200 μ L of ³H₂O (0.6 Ci), and 4.2 μ L of β -mercaptoethanol was added. The solution was covered with 400 μ L of mineral oil and was incubated in a gasketed vial in an oil bath kept at 90 °C. After 6 h, the radiolabeled ODN was desalted and depleted of ³H₂O by repetitive centrifugation and resuspension in ¹H₂O using a Microcon-3 concentrator (Amicon, Witten, Germany). To remove final traces of ³H₂O, the concentrated [³H]ODN was taken up in 50 μ L of H₂O and precipitated with 500 μ L of 3% LiClO₄ in acetone. Finally, the precipitate was taken up in H₂O, and after the A₂₆₀ and radioactivity were measured, the ODN was stored at -20 °C. We did not detect any significant exchange of radioactivity upon 6 months of storage at -20 °C. The total yield was 0.28 mg (56%). The specific activity of the [³H]ODN was 80 $\times$ 10⁶ dpm mg⁻¹ (0.5 $\times$ 10⁹ dpm μ mol⁻¹).

Synthesis and Purification of 3'-Lipid-ODN. The 3'-amine ODN was precipitated as a Li salt with 10 volume equiv of 3% LiClO₄ in acetone. Next, the ODN was dissolved in H₂O and precipitated again with 10 volume equiv of acetone to remove final traces of LiClO₄. In a typical derivatization experiment, 15 nmol of ODN was dissolved in 350 μ L of H₂O/DMF/dioxane (1:4:2, v/v/v). Subsequently, 1 μ mol of the activated lipid dissolved in 100 μ L of dioxane and 35 μ mol of DiPEA were added. The mixture was incubated for 48 h at 56 °C for lipids

1e, **2e**, and **3e** or at 37 °C for **2f**, **4b**, and cholesterol chloroformate. Solvents were removed in a speed-vac concentrator, and the residue was taken up in 200 μ L of DCM and 200 μ L of H₂O. The layers were separated by centrifugation, and the organic layer was washed twice with 200 μ L of H₂O. The H₂O fractions were combined and freeze-dried. Derivatization of [³H]ODN was performed as described above with 2 nmol of [³H]ODN and 4 nmol of unlabeled ODN.

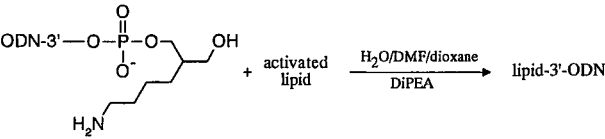
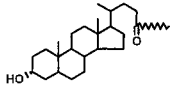
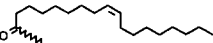
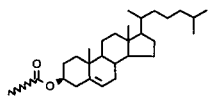
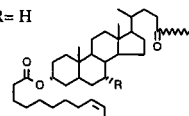
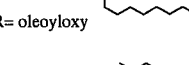
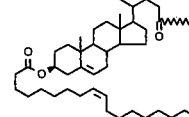
ODNs conjugated with lithocholic acid, oleic acid, or cholesterol were purified by reversed phase (RP)-HPLC on a Waters C4 column (5 μ m, 300 Å, 300 $\times$ 3.9 mm), applying a gradient of 1% CH₃CN/min in 50 mM triethylammonium acetate (pH 7.0) at a flow rate of 1 mL/min. The gradient (5 to 50%) was started after elution for 5 min at 5% CH₃CN. ODNs were detected at 260 nm. All other lipid-conjugated ODNs were purified by gel electrophoresis in a 1% LM-MP agarose gel containing 0.1% Tween 20, in 0.5 $\times$ TBE [45 mM Tris-borate and 0.1 mM EDTA (pH 8.4)]. After separation of the lipid-ODN from the nonconjugated ODN, the gel slice containing the lipid-ODN was melted for 5 min at 65 °C. The agarose was digested with Agarase (40 units per milliliter of gel) at 45 °C in 30 mM Bis-Tris and 10 mM EDTA for 2 h. The lipid-ODNs were precipitated with 10 volume equiv of acetone. To remove traces of undigested agarose, the precipitate was taken up in 200 μ L of H₂O and passed over a filter paper (no. 589, Schleicher and Schüll). Lipid-conjugated ODNs were isolated in a yield of 35–75%.

Determination of Melting Temperatures. Melting temperatures of hybrids of the antisense 3'-lipid-ODNs with an 28-mer sense ODN were determined using a Perkin-Elmer spectrophotometer equipped with a PTP-6 thermal programmer. Equimolar amounts of both ODNs were dissolved in PBS (pH 7.4) to a concentration of 3.6 μ M, denatured at 96 °C for 2 min, and subsequently cooled to room temperature to anneal slowly. Then, the temperature was adjusted to 35 °C, and hybrids were melted by increasing the temperature to 95 °C at a rate of 0.5 °C min⁻¹. Duplicate runs produced *T*_ms within 1 °C.

Isolation and Radiolabeling of LDL. LDL was isolated from the serum of fasted volunteers by density gradient ultracentrifugation (28) and was dialyzed against PBS containing 1 mM EDTA (pH 7.4). Radioiodination was performed at pH 10.0 with carrier-free ¹²⁵I as described by McFarlane (29). Protein concentrations of LDL were determined by the method of Lowry (30) with bovine serum albumin as a standard.

Determination of the Association of Lipid-ODNs with LDL. Equimolar amounts of [¹²⁵I]LDL and lipid-[³H]ODNs (1.7 μ M), dissolved in PBS and 1 mM EDTA (pH 7.4), were incubated for 2 h at 37 °C. For these experiments, the molecular weight of an LDL particle was related to the molecular weight of the apoprotein B-100 (31), as each LDL particle contains only one copy of the apoprotein. The molecular weights of the lipid-ODNs were taken from Table 1. Aliquots of the incubation mixtures were subjected to gel electrophoresis in a 0.75% (w/v) agarose gel in 75 mM Tris-hippuric acid buffer (pH 8.8). After electrophoresis, the gel was cut into slices, and after addition of 0.5 mL of Soluene-350, the ¹²⁵I radioactivity was counted. After 24 h, 3 mL of Hionic Fluor was added and samples were evaluated for ³H radioactivity. The measured values of ³H radioactivity were corrected for the contribution of ¹²⁵I radioactivity.

Table 1. Synthesis and Analysis of Lipid-ODNs

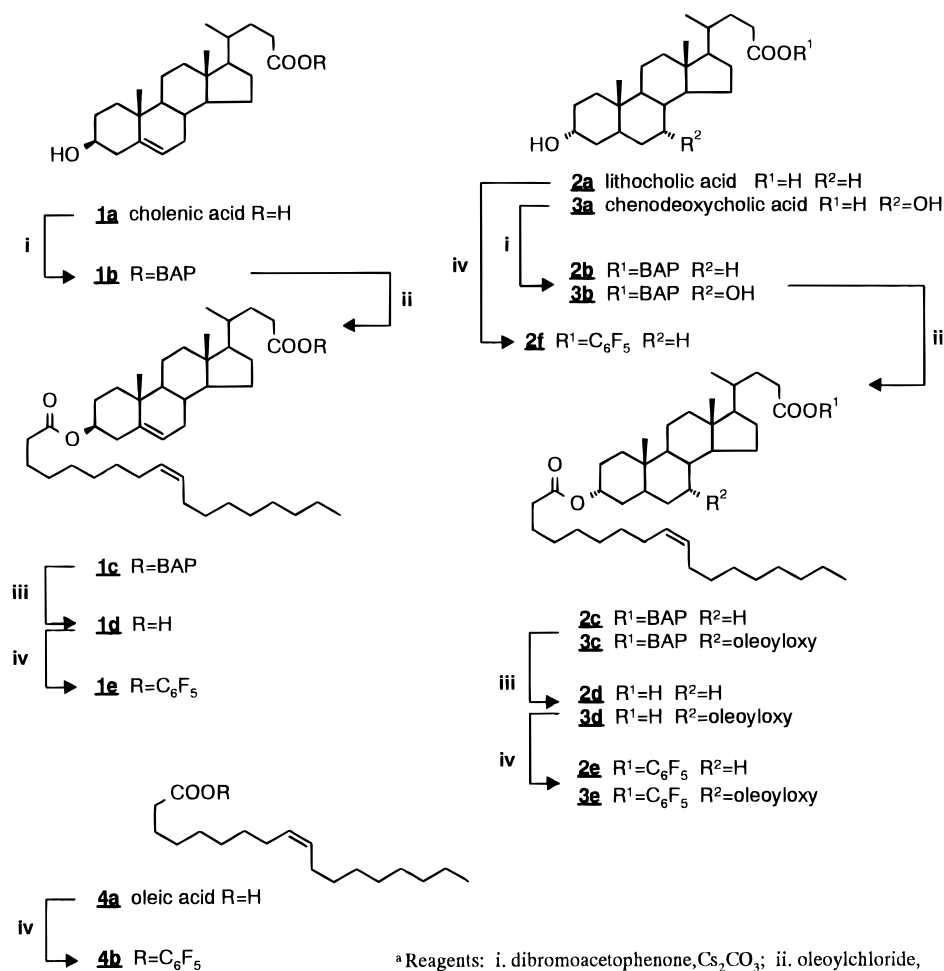
					
	3'-lipid	activated lipid	<i>T</i> _m ^a	mass calculated	mass ^b found
ODN-1	-	-	71	5821.07	5821.10
ODN-2		2f	72	6179.60	6180.83
ODN-3		4b	72	6085.48	6086.08
ODN-4		CCF ^c	73	6233.69	6235.09
ODN-5	R= H 	2e	70	6444.05	6444.34
ODN-6	R= oleoyloxy 	3e	69	6724.50	6724.41
ODN-7		1e	70	6442.03	6442.77

^a Melting temperatures (degrees Celsius) were calculated from duplicate runs with a 28-mer unmodified DNA strand as the target sequence in PBS. ^b Lipid-ODNs were analyzed by ES mass spectrometry. The exact mass data were calculated from at least four multiply charged ions. ^c Cholesteryl chloroformate.

RESULTS AND DISCUSSION

Synthesis of Activated Lipid Structures. Starting points for the design of the lipids to be conjugated to the *c-myc* specific ODNs were the studies of Firestone et al. (13, 14). These studies show that aliphatic lipids with at least one cis double bond and oleoyl steroid esters facilitate reconstitution of LDL with lysosomotropic agents and nitrogen mustards. The lipid structures in this study were synthesized, as shown in Scheme 1, starting from oleic acid **4a** and the bile acids cholenic acid **1a**, lithocholic acid **2a**, and chenodeoxycholic acid **3a**. Bile acids were selected because they contain both a carboxylic acid functionality which can be used for conjugation to ODNs bearing nucleophilic moieties and hydroxyl groups that can be esterified with fatty acids. The lipophilicity of the bile acids was enhanced by introducing one oleic acid chain (**1d** and **2d**) or two oleic acid chains (**3d**). The bile acids lithocholic acid **2a** and cholenic acid **1a** differ in the configuration of the hydroxyl at the 3 position and the rigidity of the steroid rings. First, the carboxylic acids of the bile acids were protected with 4-bromophenacyl bromide. Then, the hydroxyls were esterified with oleoyl chloride in the presence of DMAP, resulting in the oleoyl steroid esters **1c** and **2c** in a yield of 58–95%. Synthesis of **3c** afforded the dioleoyl ester in a low yield (23%), compared to those of the monooleoyl esters **1c** and **2c**.

Characterization of the reaction products formed during synthesis of **3c** revealed two main products: the dioleoyl ester **3c** and the monooleoyl steroid ester esteri-

Scheme 1. Synthesis of Activated Lipid Structures^a

^a Reagents: i. dibromoacetophenone, CS_2CO_3 ; ii. oleoylchloride, DMAP, pyridine; iii. DCM/HAc, Zn; iv. pentafluorophenol, DCC.

fied at the 3-OH. The 7-OH monooleoyl ester could not be detected as a main product. The 3-OH of chenodeoxycholic acid thus appears to react more easily with oleoyl chloride than the 7-OH, presumably because this position is more readily accessible. Upon formation of the oleic acid ester at the 3-OH, the 7-OH may become even less accessible for esterification, due to steric hindrance. We also attempted to synthesize the dioleoyl steroid ester of deoxycholic acid (3-OH and 12-OH). However, compared to the 7-OH of chenodeoxycholic acid, the 12-OH of deoxycholic acid was found to be even more difficult to esterify.

After esterification of the hydroxyls of the steroids, the bromophenacyl group could easily be removed by overnight stirring in HAc/Zn. All synthesized lipids were activated with pentafluorophenol to allow coupling to 3'-amino-tailed ODNs.

Conjugation of ODNs with Lipid Structures. We performed synthesis of the lipid-ODNs in solution phase, since in the alternative approach, solid phase synthesis, the steroid ester structures would be disrupted by the standard ammonia cleavage of the ODN from the solid support (32, 33). However, earlier studies have proven that the covalent attachment of large lipids to ODNs in solution phase is a difficult task. The lipids and the ODNs have very different solubility properties, and the amphiphilic properties of the resulting lipid-ODNs often lead to isolation problems and consequently low yields (34-36). To achieve a high conjugation efficiency for our

lipids, we have tested several protocols described in the literature. Derivatization of ODNs with lipids in aqueous buffer solutions (pH 8-10) has been reported to be the most successful derivatization method (32, 33). However, the low solubility of most of our lipids in these mixtures resulted in very low yields of the conjugated ODN. Lipophilic cations such as CTAB or PMDBD have been used to dissolve and subsequently derivatize ODNs with lipids in organic solvents such as DMSO and $CHCl_3$ (34, 35, 37, 38). Unfortunately, this approach failed for derivatization of our lipids. We found that derivatization in a mixture of H_2O and the aprotic solvents DMF and 1,4-dioxane (1:4:4, v/v/v), in which DiPEA was present to create mildly basic conditions, is far superior to the other two methods (39). The presence of dioxane in this mixture improved the solubility of the steroid lipids, and DMF appeared to be a crucial component. When DMF was replaced by dioxane, resulting in a mixture of H_2O and dioxane only (1:8, v/v), the conjugations were unsuccessful, although both the ODN and the activated lipid could be dissolved. Derivatization of the 3'-amino ODN could efficiently be accomplished in the H_2O /DMF/dioxane mixture for 48 h at 37 °C for the lipids **2f**, **4b**, and cholesteryl chloroformate. The activated lipids **1e**, **2e**, and **3e** were conjugated in the same mixture, but the temperature was raised to 56 °C to ensure complete solubility of these activated steroid lipids.

After conjugation of the ODN and removal of the reaction mixture solvents, excess reactive lipids were

extracted with DCM. Without this extraction step, recoveries of the conjugated ODNs were very low. Separation of the ODNs conjugated with lithocholic acid, oleic acid, and cholesterol (ODN-2, ODN-3, and ODN-4, respectively) from the nonconjugated ODNs was accomplished by RP-HPLC (Waters C4 column, 5 μ m, 300 Å) using an CH₃CN gradient from 5 to 50% in 50 mM triethylammonium acetate (pH 7.0). The retention time of ODNs increased significantly from 17 min for the nonconjugated ODN to 23.7 min for ODN-2 and 34.5 min for ODN-3 and ODN-4. When a C8 HPLC column was utilized, the cholesteryl-derivatized ODN had a much higher retention time than the oleic acid-derivatized ODN. The C4 column was preferred since it led to higher recoveries of the ODNs (overall recoveries of up to 80%).

Purification of the ODNs conjugated with the oleoyl steroid ester structures (ODN-5, ODN-6, and ODN-7) was not possible by RP-HPLC; no conjugated products could be recovered. Several RP-HPLC columns (C4, C8, and C18) were tested, and very high concentrations of the organic modifier (CH₃CN and tetrahydrofuran up to 95%) were used. Only small amounts (<30%) of the non-derivatized ODNs could be recovered. Attempts to purify the lipid-ODNs by PAGE gave comparable results. The lipid-ODNs are readily soluble in water after the DCM/H₂O extraction, but since no lipid-ODN could be detected by RP-HPLC, we assume that the highly lipidic ODN-5, ODN-6, and ODN-7 bind irreversibly to the column material. The limitations of RP-HPLC for purification of lipid-ODNs have already been shown by Manoharan et al. (40). These authors observed a linear correlation between the percentage of acetonitrile needed to elute lipid-ODNs from a HPLC C18 column and the number of carbon atoms in the lipid conjugated to the ODN. This indicates that RP-HPLC analysis is restricted to lipidic ODNs of limited hydrophobicity. PAGE of ODN-5, ODN-6, and ODN-7 suggests formation of large structures, since none of the conjugated ODNs migrated into the gel. Only a small amount of the underivatized ODN could be detected. This was also observed by Farooqui et al. (11) for ODNs conjugated with two cholesteryl groups. Bourtoune et al. (36) reported similar isolation problems with fullerene (C60)-conjugated ODNs by both RP-HPLC and PAGE. They solved the problem by separating the fullerene-ODN from the nonconjugated ODN by electrophoresis in a 1% agarose gel containing 0.1% Triton X-100. Separation is based on the formation of micelles between lipid-ODNs and a nonionic detergent such as Tween 20 or Triton X-100. We applied this method to separate the nonconjugated ODN from the ODNs conjugated to the oleoyl steroid esters (ODN-5, ODN-6, and ODN-7). As can be seen in Figure 1, these lipid-ODNs migrate more slowly than the nonconjugated ODNs in a gel matrix of LM-MP agarose and Tween 20. The lipid-ODNs could be isolated by treatment of the ODN-containing gel slices with Agarase and subsequent precipitation of the ODNs by acetone. The yields of the derivatized ODNs purified by this method varied from 35 to 65% (based upon starting material, as judged from both the radioactivity and the A₂₆₀ of the isolated ODNs). Surprisingly, oleic acid- and lithocholic acid-conjugated ODNs (ODN-2 and ODN-3), which were readily isolated by RP-HPLC, could not be separated from the nonconjugated ODN by this gel electrophoresis method. An interesting feature of the cholesteryl-conjugated ODNs is that they could be purified by RP-HPLC as well as by agarose/detergent gel electrophoresis. When agarose gel-purified cholesteryl-conjugated ODNs were analyzed by

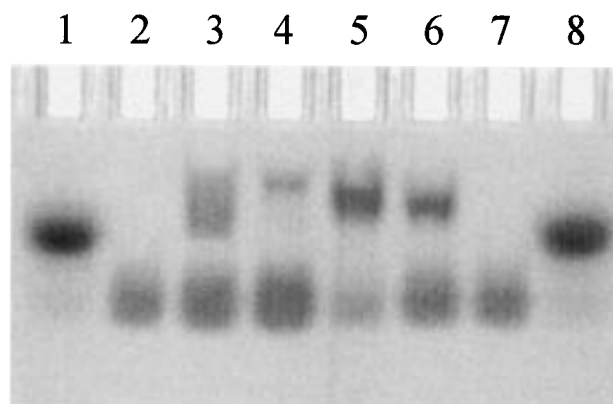


Figure 1. Analysis of reaction mixtures of ODNs conjugated with steroid lipids. Reaction mixtures were subjected to electrophoresis in a 1% agarose/0.1% Tween 20 gel in 0.5× TBE [45 mM Tris-borate and 0.1 mM EDTA (pH 8.4)]. ODNs were visualized by UV shadowing: lanes 1 and 8, xylene cyanol (marker); lanes 2 and 7, ODN-1; lane 3, reaction mixture ODN-6; lane 4, reaction mixture ODN-7; lane 5, reaction mixture ODN-5; and lane 6, reaction mixture ODN-4.

RP-HPLC, the retention time was exactly the same as that for the HPLC-purified cholesteryl-conjugated ODNs.

All purified lipid-ODNs were analyzed by electrospray mass spectrometry. The exact molecular weights were calculated from at least four multiply charged ions in the mass spectrum and are listed in Table 1. The calculated molecular weights agree very well with the measured values which confirms the chemical structures of the isolated products.

Interaction of Lipid-ODNs with Their Target Sequences. To ascertain that the attached lipid structures do not negatively interfere with the interaction of the antisense ODN with the target sequence, we determined the melting temperatures of antisense and sense hybrids. The 28-mer sense ODN possessed five overhang nucleotides at both the 3'- and 5'-end for assessing a possible interaction of the lipid with the single-stranded part of the hybrid. Although the steroid lipids, and in particular the oleoyl steroid esters, are relatively bulky, our data indicate that interference of the lipids with the overhang nucleotides of the target sequence is minimal, since only a slight difference of maximally 2 °C in T_m was observed (Table 1). These findings are consistent with other reports on lipid-ODNs (41, 42).

Interaction of Lipid-ODNs with LDL. After conjugation of the lipids to the ODN, we determined the association of these conjugates with native LDL. The ODN was specific to the *c-myc* proto-oncogene. The association of these oncogene-directed ODNs with LDL is highly relevant, as it has been shown that various tumor cells express elevated levels of the LDLr (15). The use of LDL as a carrier particle may therefore facilitate cellular uptake of oncogene-directed ODNs, and thus enhance antisense activity.

To examine which of the synthesized lipids were able to associate the ODN with native LDL, radioiodinated LDL was incubated for 2 h at 37 °C with equimolar amounts of lipid-[³H]ODN. Incubation mixtures were analyzed by agarose gel electrophoresis in 75 mM Tris-hippuric acid buffer (pH 8.8) (Figure 2). The electrophoretic mobility (R_f) of native LDL (relative to bromophenol blue) in this gel matrix is 0.2, whereas the nonassociated ODN and lipid-ODNs have R_f values close to 1.0. These complexation experiments reveal that ODNs conjugated with cholesterol and the oleoyl esters

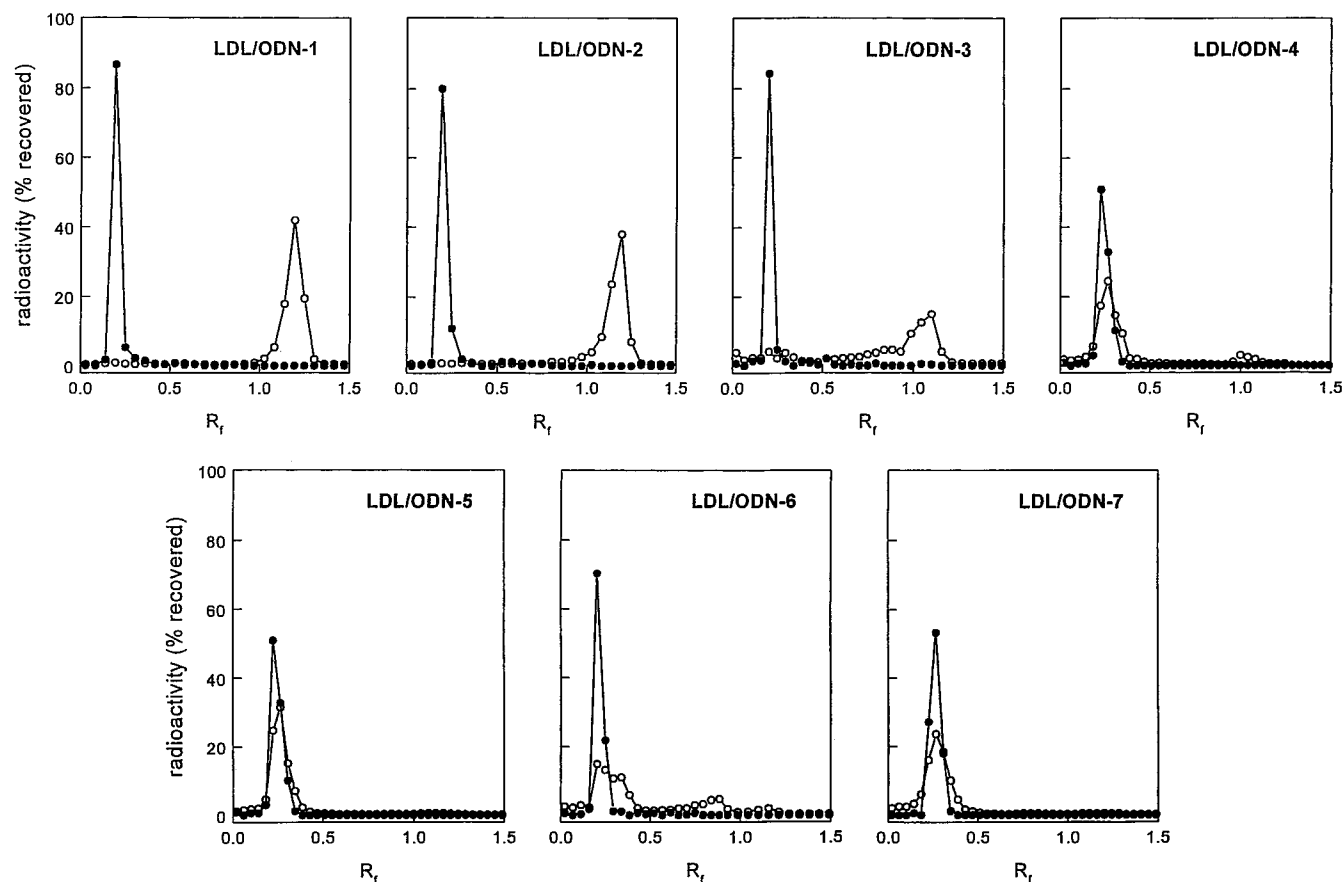


Figure 2. Association of lipid-ODNs with LDL; analysis by agarose gel electrophoresis. Equimolar amounts of [^{125}I]LDL and lipid-[^3H]ODNs (1.7 μM), dissolved in PBS and 1 mM EDTA (pH 7.4), were incubated for 2 h at 37 $^{\circ}\text{C}$. Aliquots of the incubation mixtures were subjected to gel electrophoresis in a 0.75% (w/v) agarose gel in 75 mM Tris-hippuric acid buffer (pH 8.8). After electrophoresis, the gel was cut into slices that were assayed for ^3H radioactivity ($\circ$) and ^{125}I radioactivity ($\bullet$). The results are expressed as a percentage of the recovered radioactivities. Recoveries were $>95\%$.

of lithocholic acid and cholenic acid associate spontaneously and almost completely with LDL. More than 95% of the [^3H]ODNs conjugated with these steroids comigrated in the gel with radioiodinated LDL at an R_f of 0.2. These steroids meet the structural requirements for LDL anchors as defined by Firestone (13). Association of the lipid-ODNs with LDL also slightly broadened the band of LDL in the gel. This is probably due to an increase of the overall negative charge of the complex. When ODNs were associated at higher molar ratios, the increase in the electrophoretic mobility was more clear (data not shown). The association of cholesteryl-derivatized ODNs with native LDL has been shown before (10, 19), but the use of oleoyl steroid esters to induce association of ODNs with LDL is novel.

In Figure 2, the fact that not all lipids can induce spontaneous association of the ODN with LDL is also illustrated. Lithocholic acid and oleic acid proved not to be effective as a LDL anchors in our experiments. This also applies for the bis-oleoyl steroid ester. Only a small proportion of the ODN conjugated to this steroid ester (ODN-6) comigrated with LDL, and more than 50% of the ^3H radioactivity was found at an R_f of >0.2 . The aberrant migration pattern for ODN-6 may indicate that this lipid-ODN has formed an aggregate during incubation with LDL or that it slowly dissociates from the LDL during electrophoresis. The lipid moiety of ODN-6 probably does not partition with its complete steroid structure into the lipids of LDL, but only with the two oleoyl chains. The complete bis-oleoyl steroid ester structure may be too bulky to associate spontaneously with LDL. How-

ever, this ODN might be an interesting candidate to use in the recently developed artificial LDL-like carrier systems (43, 44). In this system, lipid-ODNs are incorporated during vesicle formation, in contrast to the spontaneous association with LDL in this study. The lack of association of ODN-3 with LDL was not expected, since complexation of a 5'-palmitoylated 16-mer phosphorothioate ODN with LDL was already reported by Mishra et al. (20). The structural difference between oleic acid and palmitic acid may explain the difference in LDL association. In fact, Svinarchuk et al. (8) reported that small structural differences of fatty acids can change their binding characteristics for membrane structures significantly. Alternatively, the inability of the oleoyl chain to associate ODNs with LDL may be caused by different methods of analysis of complexation.

Concluding Remarks. We have efficiently conjugated ODNs to several lipid structures in solution phase. The conjugates could easily be purified in high yields, either by RP-HPLC or by electrophoresis in a 1% agarose gel in the presence of 0.1% detergent. Several of the lipid-ODN conjugates associate readily with LDL, which opens the possibility of utilizing the lipid structures for specific delivery of oncogene-directed ODNs to tumor cells via the LDLr pathway. To be effective, however, the ODNs need to escape from the endosomal/lysosomal compartment. We speculate that lipid-ODNs can exit these compartments, since it has been shown by several authors that lipid-ODNs can bind to membrane structures which may facilitate transport. Indeed, enhanced activity of lipid-ODNs, which may be the result of

increased transport across the cellular membrane, has been described (7, 9).

ACKNOWLEDGMENT

Muthiah Manoharan and Kathleen Tivel at ISIS Pharmaceuticals (Carlsbad, CA) and Sven Wagner at the DKFZ (Heidelberg, Germany) are acknowledged for mass analyses of the ODNs. Dr. S. van Zwanenbergstichting is acknowledged for financial support for presenting part of this work at the 12th IRT (1996, La Jolla, CA).

LITERATURE CITED

- (1) Wagner, R. W., and Flanagan, W. M. (1997) Antisense technology and prospects for therapy of viral infections and cancer. *Mol. Med. Today* 3 (1), 31–38.
- (2) Szymkowski, D. E. (1996) Developing antisense oligonucleotides from the laboratory to clinical trials. *Drug Discovery Today* 1 (10), 415–428.
- (3) Boutorine, A. S., and Kostina, E. V. (1993) Reversible Covalent Attachment of Cholesterol to Oligodeoxyribonucleotides for Studies of the Mechanisms of Their Penetration into Eucaryotic Cells. *Biochimie* 75 (1–2), 35–41.
- (4) Boutorine, A. S., Gus'kova, L. V., Ivanova, E. M., Kobetz, N. D., Zarytova, V. F., Rytte, A. S., Yurchenko, L. V., and Vlassov, V. V. (1989) Synthesis of alkylating oligonucleotide derivatives containing cholesterol or phenazinium residues at their 3'-terminus and their interaction with DNA within mammalian cells. *FEBS Lett.* 254, 129–132.
- (5) Vu, H., Singh, P., Lewis, L., Zendegeui, J. G., and Jayaraman, K. (1993) Synthesis of cholesteryl supports and phosphoramidate for automated DNA synthesis of triple-helix forming oligonucleotides (TFOs). *Nucleosides Nucleotides* 12, 853–864.
- (6) Shea, R. G., Marsters, J. C., and Bischoffberger, N. (1990) Synthesis, hybridization properties and antiviral activity of lipid-oligonucleotide conjugates. *Nucleic Acids Res.* 18, 3777–3783.
- (7) Alahari, S. K., Dean, N. M., Fisher, M. H., Delong, R., Manoharan, M., Tivel, K. L., and Juliano, R. L. (1996) Inhibition of expression of the multidrug resistance-associated P-glycoprotein by phosphorothioate and 5' cholesterol-conjugated phosphorothioate antisense oligonucleotides. *Mol. Pharmacol.* 50 (4), 808–819.
- (8) Svinarchuk, F. P., Konevets, D. A., Pliasunova, O. A., Pokrovsky, A. G., and Vlassov, V. V. (1993) Inhibition of HIV Proliferation in MT-4 Cells by Antisense Oligonucleotide Conjugated to Lipophilic Groups. *Biochimie* 75 (1–2), 49–54.
- (9) Desjardins, J., Mata, J., Brown, T., Graham, D., Zon, G., and Iversen, P. (1995) Cholesteryl-conjugated phosphorothioate oligodeoxynucleotides modulate CYP2B1 expression in vivo. *J. Drug Targeting* 2 (6), 477–485.
- (10) Krieg, A. M., Tonkinson, J., Matson, S., Zhao, Q., Saxon, M., Zhang, L.-M., Bhanja, U., Yakubov, L., and Stein, C. A. (1993) Modification of antisense phosphodiester oligodeoxynucleotides by a 5' cholesteryl moiety increases cellular association and improves efficacy. *Proc. Natl. Acad. Sci. U.S.A.* 90, 1048–1052.
- (11) Farooqui, F., Sarin, P. S., Sun, D., and Letsinger, R. L. (1991) Effect of structural variations in cholesteryl-conjugated oligonucleotides in inhibitory activity toward HIV-1. *Bioconjugate Chem.* 2, 422–426.
- (12) Brown, M. S., and Goldstein, J. L. (1986) A receptor-mediated pathway for cholesterol homeostasis. *Science* 232, 34–47.
- (13) Firestone, R. A., Pisano, J. M., Falck, J. R., McPaul, M. M., and Krieger, M. (1984) Selective delivery of cytotoxic compounds to cells by the LDL pathway. *J. Med. Chem.* 27, 1037–1043.
- (14) Dubowchik, G. M., and Firestone, R. A. (1995) Improved cytotoxicity of antitumor compounds deliverable by the LDL pathway. *Bioconjugate Chem.* 6, 427–439.
- (15) Firestone, R. A. (1994) Low-density lipoprotein as a vehicle for targeting antitumor compounds to cancer cells. *Bioconjugate Chem.* 5 (2), 105–113.
- (16) Shaw, J. M., Shaw, K. V., Yanovich, S., Jwanik, M., Futch, W. S., Rosowsky, A., and Schook, L. B. (1987) Delivery of lipophilic drugs using lipoproteins. *Ann. N. Y. Acad. Sci.* 507, 252–271.
- (17) Rojanasakul, Y., Weissman, D. N., Shi, X. L., Castranova, V., Ma, J. K. H., and Liang, W. W. (1997) Antisense inhibition of silica-induced tumor necrosis factor in alveolar macrophages. *J. Biol. Chem.* 272 (7), 3910–3914.
- (18) Rojanasakul, Y. Y. (1996) Antisense oligonucleotide therapeutics: Drug delivery and targeting. *Adv. Drug Delivery Rev.* 18 (2), 115–131.
- (19) De Smidt, P. C., Le Doan, T., De Falco, S., and van Berkel, Th. J. C. (1991) Association of antisense oligonucleotides with lipoproteins prolongs the plasma half-life and modifies the tissue distribution. *Nucleic Acids Res.* 19, 4695–4700.
- (20) Mishra, R. K., Moreau, C., Ramazeilles, C., Moreau, S., Bonnet, J., and Toulmé, J.-J. (1995) Improved leishmanicidal effect of phosphorothioate antisense oligonucleotides by LDL-mediated delivery. *Biochim. Biophys. Acta* 1264, 229–237.
- (21) Ferrari, S., Torelli, U., Selleri, L., Donelli, A., Venturelli, D., Narni, F., Moretti, L., and Torelli, G. (1985) Study of the levels of expression of two oncogenes, c-myc and c-myb, in acute and chronic leukemias of both lymphoid and myeloid lineage. *Leuk. Res.* 9, 833–842.
- (22) Gopal, V., Hulette, B., Li, Y. Q., Kuvelkar, R., Raza, A., Larson, R., Goldberg, J., Tricot, G., Bennett, J., and Preisler, H. (1992) C-myc and c-myb expression in acute myelogenous leukemia. *Leuk. Res.* 16, 1003–1011.
- (23) Ratajczak, M. Z., and Kant, J. A. (1992) In vivo treatment of human leukemia in a acid mouse model with c-myb antisense oligodeoxynucleotides. *Proc. Natl. Acad. Sci. U.S.A.* 89, 11823–11827.
- (24) Hijiya, N., Zhang, J., Ratajczak, M. Z., Kant, J. A., Deriel, K., Herlyn, M., Zon, G., and Gewirtz, A. M. (1994) Biologic and therapeutic significance of MYB expression in human melanoma. *Proc. Natl. Acad. Sci. U.S.A.* 91 (10), 4499–4503.
- (25) Gewirtz, A. M. (1994) Treatment of chronic myelogenous leukemia (CML) with c-myb antisense oligodeoxynucleotides. *Bone Marrow Transplant.* 14 (Suppl. 3), S57–S61.
- (26) Goswami, S. K., and Frey, C. F. (1970) Manganous chloride spray reagent for cholesterol and bile acids on thin-layer chromatograms. *J. Chromatogr.* 53, 389–390.
- (27) Graham, M. J., Freier, S. M., Crooke, R. M., Ecker, D. J., Maslova, R. N., and Lesnik, E. A. (1993) Tritium Labeling of Antisense Oligonucleotides by Exchange with Tritiated Water. *Nucleic Acids Res.* 21 (16), 3737–3743.
- (28) Redgrave, T. G., Roberts, D. L. K., and West, C. E. (1975) Separation of plasma lipoproteins by density gradient ultracentrifugation. *Anal. Biochem.* 65, 42–49.
- (29) McFarlane, A. S. (1958) Efficient trace-labelling of proteins with iodine. *Nature* 182, 53–54.
- (30) Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193, 265–275.
- (31) Chen, S. H., Yang, C. Y., Chen, P. F., Setzer, D., Tanimura, M., Li, W. H., Gotto, A. M., Jr., and Chan, L. (1986) The complete cDNA and amino acid sequence of human apolipoprotein B-100. *J. Biol. Chem.* 261 (28), 12918–12921.
- (32) Manoharan, M. (1993) Designer antisense oligonucleotides: conjugation chemistry and functionality placement. In *Antisense Research and Applications* (S. T. Crooke, Ed.) pp 303–349, CRC Press, Boca Raton, FL.
- (33) Goodchild, J. (1990) Conjugates of oligonucleotides and modified oligonucleotides: a review of their synthesis and properties. *Bioconjugate Chem.* 1, 165–187.
- (34) Boutorine, A. S., Le Doan, T., Battioni, J. P., Mansuy, D., Dupré, D., and Hélène, C. (1990) Rapid route of synthesis of chemically reactive and highly radioactive labeled α - and β -oligonucleotide derivatives for in vivo studies. *Bioconjugate Chem.* 1, 350–356.
- (35) Lukhtanov, E. A., Kutyavin, I. V., Gamper, H. B., and Meyer, R. B. (1995) Oligodeoxyribonucleotides with conjugated dihydropyrroloindole oligopeptides: Preparation and hybridization properties. *Bioconjugate Chem.* 6 (4), 418–426.
- (36) Boutorine, A. S., Tokuyama, H., Takasugi, M., Isobe, H., Nakamura, E., and Hélène, C. (1994) Fullerene-oligonucle-

- otide conjugates: photoinduced sequence-specific DNA cleavage. *Angew. Chem., Int. Ed. Engl.* 33, 2462–2465.
- (37) Oberhauser, B., and Wagner, E. (1992) Effective incorporation of 2'-O-methyl-oligoribonucleotides into liposomes and enhanced cell association through modification with thiocholesterol. *Nucleic Acids Res.* 20, 533–538.
- (38) Boutorine, A. S., Brault, D., Takasugi, M., Delgado, O., and Hélène, C. (1996) Chlorin-oligonucleotide conjugates: Synthesis, properties, and red light-induced photochemical sequence-specific DNA cleavage in duplexes and triplexes. *J. Am. Chem. Soc.* 118 (40), 9469–9476.
- (39) Bannwarth, W., Schmidt, D., Stallard, R. L., Hornung, C., Knorr, R., and Müller, R. (1988) Bathophenanthroline-ruthenium(II) complexes as non-radioactive labels for oligonucleotides which can be measured by time-resolved fluorescence techniques. *Helv. Chim. Acta* 71, 2085–2099.
- (40) Manoharan, M., Tivel, K. L., and Cook, P. D. (1995) Lipidic nucleic acids. *Tetrahedron Lett.* 36 (21), 3651–3654.
- (41) Reed, M. W., Adams, A. D., Nelson, J. S., and Meyer, R. B. (1991) Acridine- and cholesterol-derivatized solid supports for improved synthesis of 3'-modified oligonucleotides. *Bioconjugate Chem.* 2, 217–225.
- (42) MacKellar, C., Graham, D., Will, W. W., Burgess, S., and Brown, T. (1992) Synthesis and physical properties of anti-HIV antisense oligonucleotides bearing terminal lipophilic groups. *Nucleic Acids Res.* 20, 3411–3417.
- (43) Rensen, P. C. N., Herijgers, N., Netscher, M. H., Meskers, S. C. J., van Eck, M., and van Berkel, Th. J. C. (1997) Particle size determines the specificity of apolipoprotein E-containing triglyceride-rich emulsions for the LDL receptor versus hepatic remnant receptor in vivo. *J. Lipid Res.* 38, 1070–1084.
- (44) Rensen, P. C. N., Schiffelers, R. M., Versluis, A. J., Bijsterbosch, M. K., van Kuijk-Meuwissen, M. E. M. J., and van Berkel, Th. J. C. (1997) Human recombinant apolipoprotein E-enriched liposomes can mimic low-density lipoproteins as carriers for the site-specific delivery of anti-tumour agents. *Mol. Pharmacol.* 52, 445–454.

BC970176Z

Terbium Chelate Membrane Label for Time-Resolved, Total Internal Reflection Fluorescence Microscopy of Substrate-Adherent Cells

Sam Phimphivong and S. Scott Saavedra*

Department of Chemistry, University of Arizona, Tucson, Arizona 85721. Received September 11, 1997;
Revised Manuscript Received March 6, 1998

A chelating agent conjugated to a lipid was synthesized by reacting the dianhydride form of diethylenetriaminepentaacetic acid sequentially with 4-aminosalicylate and dioleoylphosphatidylethanolamine. The product, DOPE-YAS-Tb, exhibits photophysical properties characteristic of a chelated Tb³⁺ ion bound to an organic triplet donor: an excitation maximum at 310 nm, narrow emission bands at 490, 545, 590, and 625 nm, and a lifetime of 1.57 ms. The suitability of DOPE-YAS-Tb as a membrane-staining agent for morphological studies of cultured cells using total internal reflection fluorescence microscopy (TIRFM) was investigated. Swiss albino mouse 3T3 cells were cultured on silica and polystyrene substrates. Time-resolved detection was employed to reject short-lived background emission (autoemission from the cells and/or the polymer substrate), which allowed the long-lived Tb³⁺ emission to be selectively imaged. The results show that time-resolved TIRFM of cells stained with DOPE-YAS-Tb is an effective method of quantitatively examining the cell morphology in situations where background due to autoemission from cells and/or the substrate material is problematic.

INTRODUCTION

Total internal reflection fluorescence microscopy (TIRFM), in which an evanescent wave is used to selectively excite fluorophores near a solid-liquid interface, was first used by Axelrod to visualize cell-substrate contact regions by labeling the plasma membrane with an amphiphilic fluor (1). TIRFM has since been used widely to study cell adhesion phenomena. Examples include morphological studies of chick heart cells (2), *Dicytostelium* amoebae (3), bovine aortic endothelial cells (4, 5), and rat myotubes (6), Fc receptors in rat basophilic leukemia cells (7), the angiotensin-converting enzyme in endothelium (8), and the structural organization of interphase fibroblasts (9). A thorough review is provided in a recent paper by Burmeister et al. (10).

Several groups have shown that digital TIRFM can be used to determine separation distances between the plasma membrane and the substrate in cell-substrate contact regions (5, 9–11), which allows the technique to be used for quantitative studies of cell adhesive interactions. For example, Burmeister et al. (10) examined relationships between contact area and adhesion strength in endothelial cells cultured on biomaterial surfaces and performed real-time measurements of the cell-substrate separation distance as a function of applied shear stress.

However, quantitative conversion of a digital TIRFM image to a map of contact region morphology can be compromised by extraneous emission from the cell and/or the substrate (5). The emission spectra of conventional membrane labels such as DiIC₁₈ and lipid-conjugated fluorescein and rhodamine overlap with cellular autofluorescence at wavelengths of less than 600 nm (12, 13), which can be problematic if the label density is relatively low and/or the separation distance is large. Furthermore,

the autofluorescence and Raman scattering background from many polymers, including those of biomedical interest, is usually more intense than that of a silica substrate.

Methods for correcting steady-state fluorescence measurements on cells for autofluorescence have been described (13–15). Typically, correction factors are determined on a cell-by-cell basis by making a second measurement of emission intensity at a wavelength that is both spectrally resolved from the label emission and shown to be correlated with autoemission intensity within the label emission band.

Time-resolved detection using an extrinsic label having decay kinetics much slower than those of the sources of background emission is an alternative approach. Since the radiative lifetime of cellular autofluorescence is less than 100 ns, it can be temporally discriminated from the emission of lanthanide metals such as Eu³⁺ and Tb³⁺, which have lifetimes in the millisecond range. Principles and applications of time-resolved fluorometry using lanthanide probes have been reviewed elsewhere (16, 17). Briefly, following excitation of a sample with a light pulse, photon detection is delayed to allow the short-lived background emission to decay to a negligible level. The detection system is activated thereafter to record only the signal from the long-lived label.

We previously examined the use of two Tb chelates as staining agents for time-resolved TIRFM of substrate-adherent cells (18). The results showed that elimination of short-lived cellular autofluorescence permitted selective detection of the long-lived label fluorescence. However, the chelates were found to stain not only the plasma membrane but also cytoplasmic regions, including the nucleus. Thus, interpretation of the TIRF image was somewhat ambiguous due to the nonselective localization of the label. Here we describe the synthesis of another Tb chelate, DOPE-YAS (dioleoylphosphatidylethanolamine conjugated to diethylenetriaminepentaacetyl-4-

* Corresponding author. Phone: (520) 621-9761. Fax: (520) 621-8407. E-mail: saavedra@u.arizona.edu.

aminosalicylic acid), and its use as a long-lived probe for time-resolved TIRFM of cells. The utility of this label in cell adhesion studies where background emission is problematic is demonstrated by performing TIRFM on stained cells cultured on polystyrene. Although this material is a ubiquitous cell culture substrate, in comparison to silica, it is a relatively poor material for TIRF studies due to inherently high levels of Raman scattering and autoluminescence (19).

EXPERIMENTAL SECTION

Chemicals and Cell Culture Supplies. 1,2-Di-*cis*-9-octadecenoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), Dulbecco's modified Eagle's medium (DMEM) without phenol red, trypsin, Hank's balanced salt solution (HBSS) without phenol red, fetal bovine serum, penicillin (100 units/mL), streptomycin sulfate, and Tris hydrochloride were obtained from Sigma. Diethylenetriaminepentaacetic acid dianhydride (YdA), 4-aminosalicylate, sodium salt (pAS), pyridine, anhydrous dimethyl sulfoxide (DMSO), terbium(III) chloride hexahydrate, polystyrene (secondary standard, $M_w = 240\,000$), potassium bromide, and spectral-grade chlorobenzene were obtained from Aldrich. All chemicals were used as received. Deionized water was obtained from a Barnstead Nanopure system.

Preparation and Characterization of DOPE-YAS. YdA was reacted with pAS to produce the YAS monoanhydride, as previously described (20, 21). DOPE-YAS was synthesized by reacting YAS monoanhydride with DOPE, following the general procedure of Nayar et al. (22). DOPE (53.7 μmol) was placed in a 100 mL round-bottom flask and dried under high vacuum for 2 h to remove residual chloroform. The dried lipid was redissolved in 10 mL of freshly distilled pyridine containing 537 μmol of YAS anhydride. The reaction mixture was stirred under a N_2 atmosphere at 60 $^\circ\text{C}$ for 24 h. Excess YAS anhydride was hydrolyzed by adding 2 mL of 1:2:0.8 $\text{CHCl}_3/\text{CH}_3\text{OH}/0.58\% \text{ NaCl}$ (v/v). Thin-layer chromatography of the unpurified product (using a mobile phase of 65:25:4 $\text{CH}_3\text{Cl}/\text{MeOH}/\text{H}_2\text{O}$ and ninhydrin staining) showed a nearly complete conversion of DOPE to DOPE-YAS.

Unreacted DOPE and DOPE-YAS were recovered by repeated extraction with a 1:1 mixture of $\text{CH}_3\text{Cl}/0.58\% \text{ NaCl}$. The saline solution was buffered at pH 2 with 100 mM phosphate (note that DOPE-YAS is insoluble in water at pH 2). The CH_3Cl fractions, which contained DOPE, DOPE-YAS, methanol, and pyridine, were pooled, dried on a rotary evaporator, and redissolved in 50 mL of 1:9 $\text{CH}_3\text{Cl}/\text{CH}_3\text{OH}$. DOPE-YAS was removed from this solution by repeated extraction with a 1:1 mixture of $\text{CH}_3\text{Cl}/0.58\% \text{ NaCl}$. In this step, the saline solution was buffered at pH 8 with 100 mM phosphate (note that DOPE-YAS is slightly soluble in water at pH 8). The progress of the extraction was monitored by adding a few drops of the organic phase to a solution of TbCl_3 dissolved in Tris buffer at pH 7.4. When DOPE-YAS was present in the organic phase (i.e., had not been quantitatively extracted), the interface between the droplets and the TbCl_3 solution fluoresced green under illumination from a mercury lamp. The extraction was repeated until no fluorescence could be visually detected. The aqueous fractions were then pooled, washed three times with CH_3Cl , and lyophilized to yield the DOPE-YAS product.

The product was characterized by UV-visible and infrared absorbance spectrometry, using a Hewlett-Packard 8452A spectrophotometer and a Nicolet 510P

FT-IR spectrometer, respectively. Steady-state fluorescence spectroscopy of dissolved DOPE-YAS-Tb was performed using a Spex Fluorolog fluorometer (Spex Industries, Inc., Edison, NJ). Fluorescent lifetime measurements on samples of dissolved DOPE-YAS-Tb were performed using the time-resolved fluorescence spectroscopic system that was described previously (18).

Substrates. Fused silica slides (Dynasil, Berlin, NJ) were cleaned by light mechanical scrubbing with cotton in 2% PCC-54 detergent (Pierce) and sonicated first in hot (ca. 60 $^\circ\text{C}$) 2% PCC-54 for 15 min, then in methanol for 10 min, and finally in deionized water for 10 min. Slides were rinsed with deionized water between sonications and then dried overnight at 120 $^\circ\text{C}$. Fused silica slides coated with polystyrene (PS) were prepared by spin coating at 1500 rpm, using an 8% (w/w) solution of PS dissolved in chlorobenzene, as described previously (23). After the slides were coated, films were dried at 70 $^\circ\text{C}$ for 24 h. The thickness and refractive index were determined by measuring prism coupling angles that produced waveguided modes in the film at 514.5 nm, as described previously (23). The typical thickness and refractive index of a PS film prepared in this manner were 0.84 μm and 1.59, respectively. These values differed by no more than 5% when measured at several different spots on a single slide. Among a batch of PS-coated slides prepared on a given day, the variation in film thickness and refractive index was also no more than 5%.

Cell Culture and Labeling. Cells were cultured as described previously (18). Briefly, Swiss albino mouse 3T3 cells (American Type Culture Collection, Rockville, MD) were cultured in 90% DMEM, supplemented with 10% fetal bovine serum, 100 $\mu\text{g}/\text{mL}$ streptomycin, and 100 units/mL penicillin. Cells were grown, usually to confluence, in a 75 cm^2 culture flask (Costar, Cambridge, MA) at 37 $^\circ\text{C}$ in a 5% $\text{CO}_2/95\%$ air atmosphere. Cells were passaged by washing with HBSS, detached by incubation with 2 mL of a trypsin solution (diluted to 1:20 from the stock solution) for 5 min, and resuspended in supplemented medium at a density of ca. 10^5 cells/mL. Passage numbers 10–30 were used in experiments described herein.

For fluorescence spectroscopy and microscopy, cells were plated at a density of approximately 10^3 cells/mL on either fused silica slides or PS-coated fused silica slides, with the planar substrate mounted in a Lucite chamber which also functioned as a TIRF cell. The plating time was 24 h on fused silica slides and 48–72 h for PS-coated slides. Longer times were required on PS because the cells adhered poorly to it and growth was relatively slow, which is well-known for PS that has not been oxidized (24).

After plating, the adherent cells were washed with HBSS, fixed in 3.7% formaldehyde at room temperature for 7 min, and rinsed with deionized water. Labeling with Tb was performed using a two-step procedure. Cells were incubated with DOPE-YAS (16 μM in Tris buffer at pH 7.4) for 60 min, rinsed with deionized water, incubated with TbCl_3 (16 μM in Tris buffer at pH 7.4) for 10 min, and then rinsed with deionized water.

Fluorescence Microscopy of Cultured Cells. Fluorescence microspectroscopy and microphotography were performed using the instrumentation described previously (18). Briefly, the sample is excited in a TIR geometry at a 70 $^\circ$ incidence angle with the 325 nm beam (about 1 mW) of a helium-cadmium laser (Liconix model 3207N). The beam is mechanically chopped at 400 Hz and adjusted to transverse electric (s) polarization with

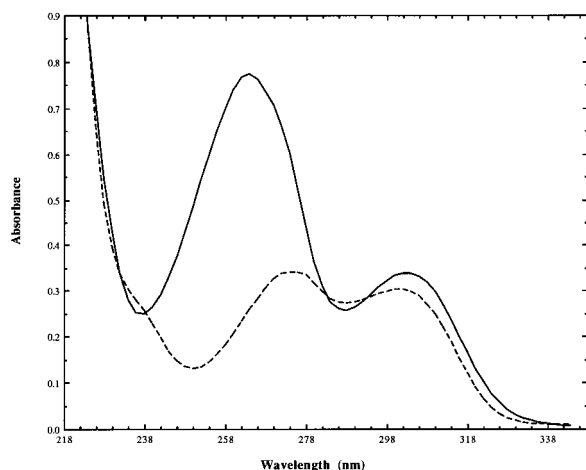


Figure 1. Absorbance spectra of pAS (8 μ M, solid line) and DOPE-YAS (84 μ M, dashed line) dissolved in 0.1 M Tris buffer at pH 7.

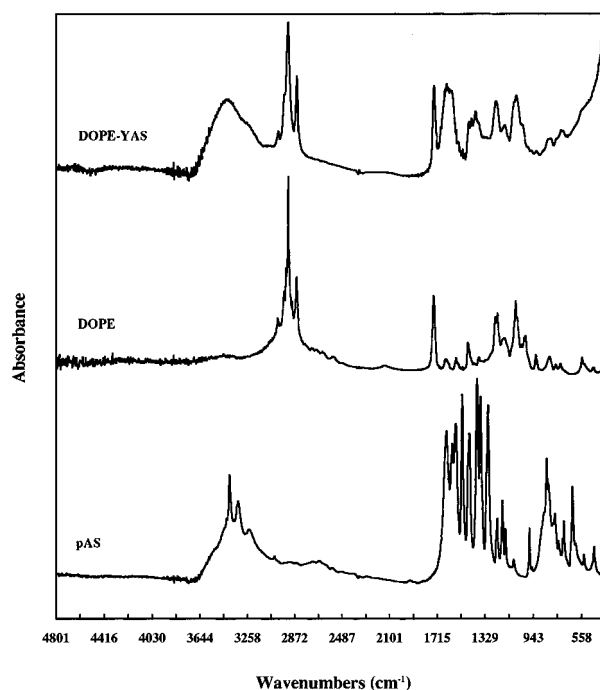


Figure 2. Infrared spectra of pAS, DOPE, and DOPE-YAS (KBr pellet). Spectra were scaled for presentation.

reaction product. Figure 2 shows IR spectra of pAS, DOPE, and DOPE-YAS. In the pAS spectrum, the phenolic O-H stretch appears as a broad band at 3650–3200 cm^{-1} and overlaps the asymmetric (3372 cm^{-1}) and symmetric (3290 cm^{-1}) N-H stretching modes of the amine. This phenolic O-H stretch is also observed in the spectrum of the product, consistent with incorporation of the pAS moiety.

In Tris buffer at pH 7.4 containing an equimolar amount of Tb^{3+} , DOPE-YAS exhibits a fluorescent excitation maximum at 308 nm, similar to its absorption spectrum, and sharp emission maxima at 490, 545, 590, and 625 nm (uncorrected) which are characteristic of the Tb^{3+} ion (data not shown). The fluorescence emission decay is first-order with a lifetime of 1.57 ms (data not shown). These properties are very similar to those of YAS-Tb (18, 20, 21), which shows that the electronic properties of YAS-Tb are not significantly altered by conjugation to DOPE. The quantum yield of DOPE-YAS-Tb was not measured. However, since the struc-

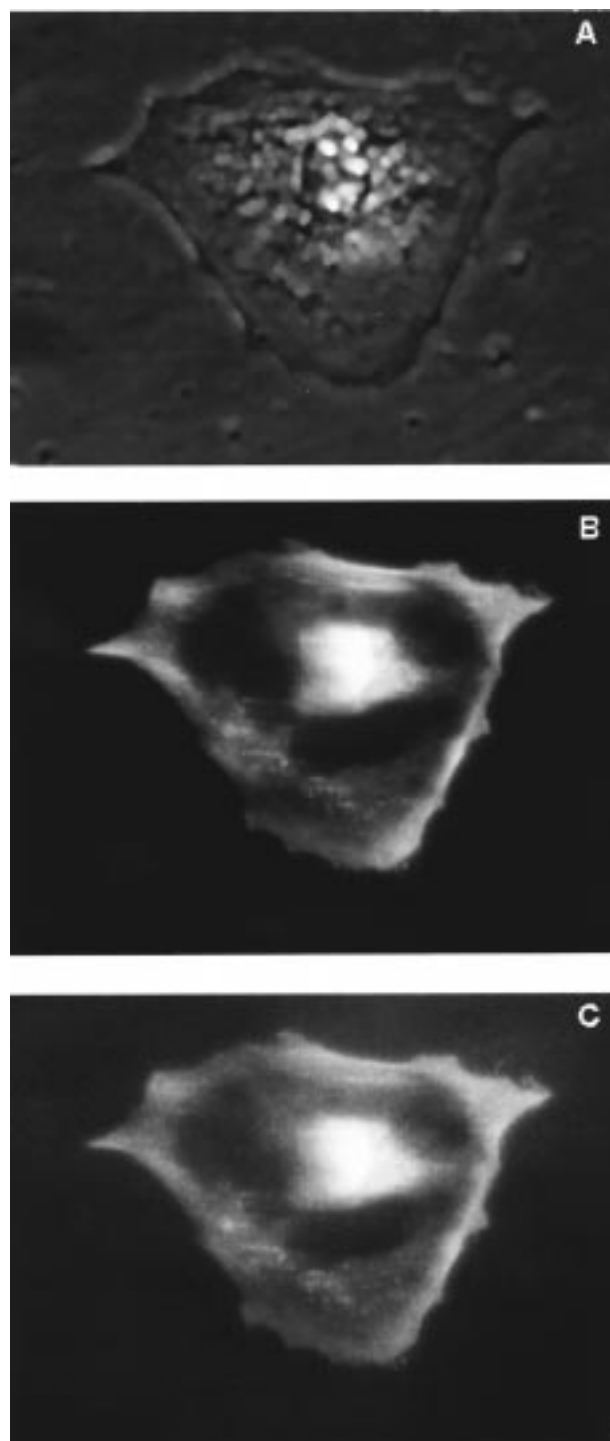


Figure 3. Images of a 3T3 cell cultured on a fused silica substrate for 24 h, fixed, and labeled with DOPE-YAS-Tb: (A) phase-contrast image, (B) steady-state TIRF image, and (C) time-resolved TIRF image. The image size is 160 $\mu\text{m} \times 118 \mu\text{m}$.

ture of the chelating group is very similar to YAS-Tb, the quantum yield of DOPE-YAS-Tb is not expected to be significantly different from 0.1, the value reported previously for YAS-Tb (21).

Fluorescence Microscopy. Cell Adhesion on Fused Silica. Shown in Figure 3 are phase-contrast and TIRF images of a 3T3 cell plated on a fused silica substrate, fixed 24 h later, and stained with DOPE-YAS-Tb. Under steady-state excitation (Figure 3B), the cell exhibits spatially distributed patches of green fluorescence. The brightest patches are in regions adjacent to the nucleus and at the cell periphery (identified from the

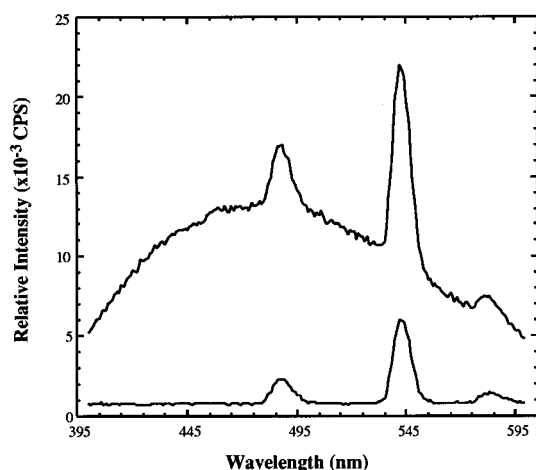


Figure 4. Steady-state (upper curve) and time-resolved (lower curve) TIRF spectra of a 3T3 cell cultured on a fused silica substrate for 24 h, fixed, and stained with DOPE-YAS-Tb.

phase-contrast image). A steady-state emission spectrum of this cell is shown in Figure 4. The emission bands at 490, 545, and 590 nm which can be attributed to the Tb label are superimposed on a broad background emission, which is due to cellular autofluorescence (18) that occurs over the entire 400–600 nm region.

The corresponding time-resolved TIRF image of the same cell is shown in Figure 3C. Although the pattern is similar, the emission intensity is reduced relative to that of the steady-state image (Figure 3B). The corresponding time-resolved TIRF spectrum is displayed in Figure 4. It is apparent that gating the detection system essentially eliminates the broad-band autofluorescence present in the steady-state spectrum (Figure 4, upper curve), leaving the characteristic Tb^{3+} emission bands superimposed on a relatively flat background. Thus, the emission pattern in the time-resolved image (Figure 3C) arises almost entirely from the DOPE-YAS-Tb label. Elimination of the autofluorescence occurs because its lifetime is considerably shorter [in the nanosecond range (18)] than the delay period before the liquid crystal shutter was opened to expose the CCD.

The percentage of DOPE-YAS incorporated into the membranes of fixed cells via the labeling procedure described above was not determined. The percentage of incorporated DOPE-YAS molecules that were complexed with Tb^{3+} was also not determined. However, on the basis of a formation constant of 10^{17} reported previously for YAS-Tb (21), the affinity of binding of Tb^{3+} to DOPE-YAS is likely to be very strong, and thus, the percentage of incorporated DOPE-YAS molecules that are complexed with Tb^{3+} is probably near unity. Furthermore, the fluorescence emission intensity from fixed cells incubated with $16 \mu\text{M}$ TbCl_3 for 10 min followed by rinsing with deionized water (i.e., the cells were not stained with DOPE-YAS) was indistinguishable from the background intensity due to cellular autofluorescence. This result shows that, in the absence of DOPE-YAS labeling, fixed 3T3 cells have relatively little binding affinity for Tb^{3+} .

Cell Adhesion on a Polystyrene-Coated Fused Silica Substrate. The steady-state TIRF spectrum of an unstained 3T3 cell that was fixed 72 h after being plated on a fused silica substrate coated with polystyrene (PS) is shown in Figure 5A. The broad emission band with a maximum near 450 nm in the steady-state spectrum is due to autoemission from both the cell and the polymer film [the emission maximum of the PS itself was near

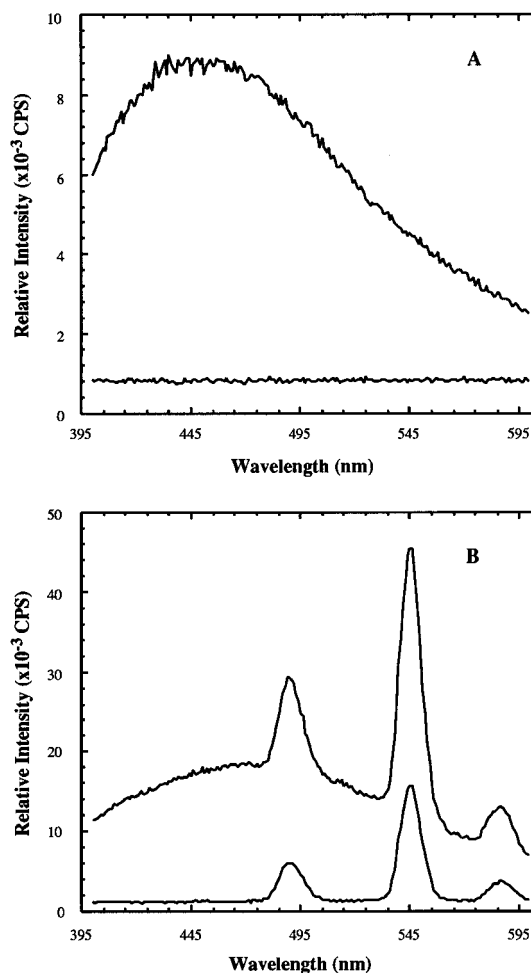


Figure 5. (A) Steady-state (upper curve) and time-resolved (lower curve) TIRF spectra of a fixed, unstained 3T3 cell cultured on a PS-coated silica substrate for 72 h. (B) Steady-state (upper curve) and time-resolved (lower curve) TIRF spectra of a 3T3 cell cultured on a PS-coated silica substrate for 72 h, fixed, and stained with DOPE-YAS-Tb.

520 nm (spectrum not shown)]. The time-resolved TIRF spectrum, also plotted in Figure 5A, shows that both sources of autoemission are essentially eliminated when time-resolved detection is implemented.

Phase-contrast and TIRF images of a 3T3 cell plated on a PS-coated fused silica substrate, fixed 72 h later, and stained with DOPE-YAS-Tb are shown in Figure 6. The steady-state TIRF image shows spatially distributed patches of green fluorescence similar to those present in the images of the stained cell that adheres to fused silica (Figure 3). The steady-state emission spectrum of the cell shown in Figure 6 is plotted in Figure 5B and shows the Tb emission bands superimposed on the autoemission background which arises from the cell and the polymer film. The corresponding time-resolved TIRF image and spectrum of the same cell are shown in Figures 6C and 5B, respectively. A comparison of the two spectra shows that time-resolved detection essentially eliminates the background. Thus, with the background removed, the time-resolved image represents the actual spatial distribution of the Tb label in the cell.

Steady-state and time-resolved TIRF spectra were also acquired from the three subcellular regions outlined by the boxes shown in Figure 6A. The spectra are plotted in Figure 7. In regions 1 and 2, the steady-state emission intensities at 545 nm are approximately 1.9×10^4 and 1.6×10^4 counts per second, respectively. However, the

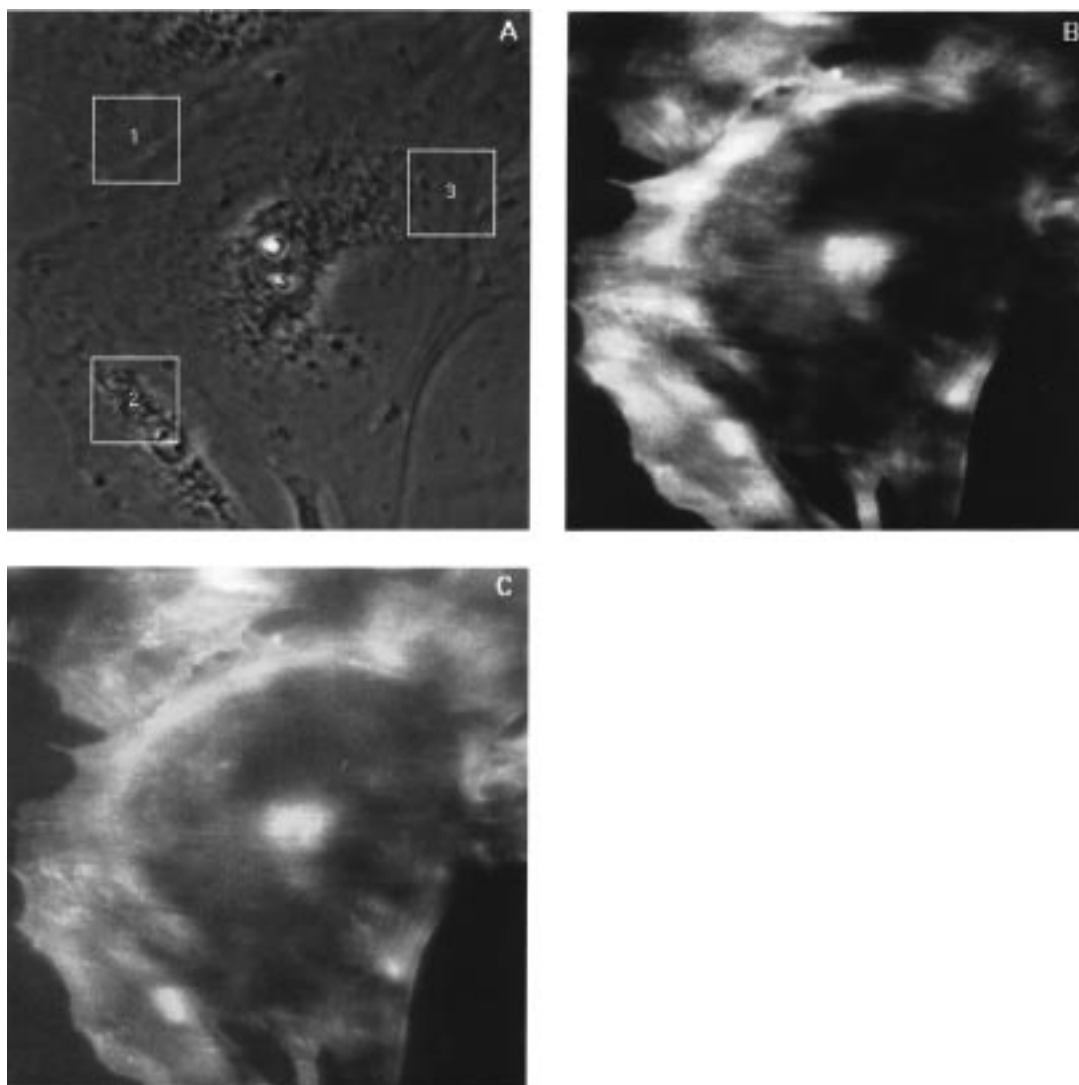


Figure 6. Images of a 3T3 cell cultured on a PS-coated silica substrate for 72 h, fixed, and labeled with DOPE-YAS-Tb: (A) phase-contrast image, (B) steady-state TIRF image, and (C) time-resolved TIRF image. The image size is $176\ \mu\text{m} \times 176\ \mu\text{m}$. The regions outlined by the boxes in panel A represent an area of $865\ \mu\text{m}^2$ each.

time-resolved emission intensities, which more accurately reflect the relative number densities of DOPE-YAS-Tb in the two regions, are nearly equal at 545 nm. Thus, the difference observed in the steady-state mode is due primarily to differences in background intensity between the two regions. This comparison illustrates the fact that quantitative conversion of a digital TIRFM image to a map of contact region morphology can be compromised by extraneous emission from the cell and/or the substrate. The severity of the problem may be greater when the label emission intensity is much lower, such as in region 3, in which the mean cell-substrate gap is presumably larger than that in regions 1 and 2.

Assessment. In a previous paper (18), we showed that background emission in TIRFM of substrate-adherent cells due to cellular autofluorescence could be rejected using a Tb chelate as a staining agent coupled with time-resolved detection. However, the Tb chelates examined in that study stained not only the plasma membrane but also cytoplasmic regions, including the nucleus, which made interpretation of the TIRF images somewhat ambiguous. Since the Tb chelate described in this study is a modified lipid, it was expected to exhibit greater selectivity for the membranes of substrate-adherent cells. The TIRF images shown in Figures 3 and 6 are consistent with this expectation. The spatial distribution of fluo-

rescence emission is similar to that observed for cells labeled with DiI (data not shown) and with TIRF images of substrate-adherent cells that have appeared in the literature (3–5, 9). Since the penetration depth of the evanescent wave used to generate the TIRF images shown in Figures 3 and 6 is less than 100 nm, the images presumably represent the ventral membrane topography of the cells. However, to identify particular regions as (for example) focal contacts, a complementary technique should be employed, such as immunofluorescence staining for a protein known to be present in these regions [e.g., vinculin (4, 26)].

Cell adhesion, growth, and motility on polymeric materials is a very active area of research because these materials are widely used in implantable devices (24, 27–30). However, the use of TIRFM in studying cell adhesion behavior on biomedical polymers has been very limited to date, which is due in part to the high background emission characteristic of these materials. As noted above, converting a digital TIRFM image to a quantitative map of cell-substrate contact region morphology requires that the background emission be minimal or be removed (5). The results described herein show that the use of DOPE-YAS-Tb as a membrane-staining agent eliminates the background problem for polystyrene, which is a relatively poor substrate material for TIRFM

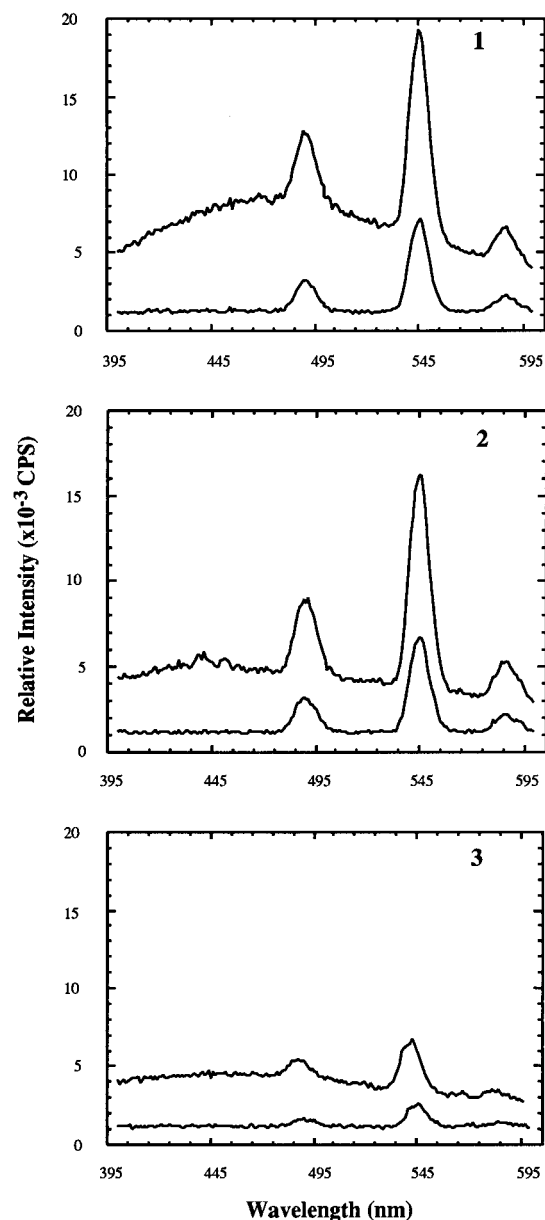


Figure 7. Steady-state and time-resolved TIRF spectra (upper and lower curves, respectively, in each pair of spectra) acquired from three subregions of the cell shown in Figure 6. The numbers on each pair of spectra correspond to the labeled subregions shown in Figure 6A.

studies due to its intrinsic autoluminescence and Raman scattering (19). This technique may therefore be useful in TIRFM studies of cell adhesion on other types of polymers, although we have not yet tested materials other than PS.

Finally, we note that the TIRFM experiments described above were performed on fixed cells. The degree to which live cells internalize DOPE-YAS was not investigated. Internalization is undesirable since it produces fluorescence emission from regions of the cell other than the plasma membrane. In the presence of significant emission originating from the cell interior, the use of digital TIRFM to quantitatively assess cell-substrate separation distances would be problematic (10).

ACKNOWLEDGMENT

This work was supported initially by a Biomedical Engineering Grant from the Whitaker Foundation and

more recently by the National Science Foundation (Grants CHE-9403896 and CHE-9726132).

LITERATURE CITED

- (1) Axelrod, D. (1981) Cell-substrate contacts illuminated by total internal reflection fluorescence. *J. Cell Biol.* 89, 141–145.
- (2) Gingell, D., Todd, I., and Bailey, J. (1985) Topography of cell-glass apposition revealed by total internal reflection fluorescence of volume markers. *J. Cell Biol.* 100, 1334–1338.
- (3) Todd, I., Smellor, J. S., and Gingell, D. (1988) Mapping cell-glass contacts of *Dicytostelium* amoebae by total internal reflection aqueous fluorescence overcomes a basic ambiguity of interference reflection microscopy. *J. Cell Sci.* 89, 107–114.
- (4) Truskey, G. A., Burmeister, J. S., Grapa, E., and Reichert, W. M. (1992) Total internal reflection fluorescence microscopy (TIRFM) II. Topographical mapping of relative cell/substratum separation distances. *J. Cell Sci.* 103, 491–499.
- (5) Burmeister, J. S., Truskey, G. A., and Reichert, W. M. (1994) Quantitative analysis of variable-angle total internal reflection fluorescence microscopy (VA-TIRFM) of cell/substrate contacts. *J. Microsc.* 173, 39–51.
- (6) Bloch, R. J., Velez, M., Krikorian, J. G., and Axelrod, D. (1989) Microfilaments and actin-associated proteins at sites of membrane-substrate attachment within acetylcholine receptor clusters. *Exp. Cell Res.* 182, 583–596.
- (7) Weis, R., Balakrishnan, K., Smith, B., and McConnell, H. M. (1982) Stimulation of fluorescence in a small contact region between rat basophil leukemia cells and planar lipid membrane targets by coherent evanescent radiation. *J. Biol. Chem.* 257, 6440–6445.
- (8) Nakache, M., Gaub, H. E., Shreiber, A. B., and McConnell, H. M. (1986) Topological and modulated distribution of surface markers on endothelial cells. *Proc. Natl. Acad. Sci. U.S.A.* 83, 2874–2878.
- (9) Lanni, F., Waggoner, A. S., and Taylor, D. L. (1985) Structural organization of interphase 3T3 fibroblasts studied by total internal reflection fluorescence microscopy. *J. Cell Biol.* 100, 1091–1102.
- (10) Burmeister, J. S., Olivier, L. A., Reichert, W. M., and Truskey, G. A. (1998) Application of total internal reflection fluorescence microscopy to study cell adhesion to biomaterials. *Biomaterials* (in press).
- (11) Gingell, D., Heavens, O., and Mellor, J. S. (1987) General electromagnetic theory of total internal reflection fluorescence: The quantitative basis for mapping cell-substratum topography. *J. Cell Sci.* 87, 677–693.
- (12) Aubin, J. E. (1979) Autofluorescence of viable cultured mammalian cells. *J. Histochem. Cytochem.* 27, 36–43.
- (13) Roederer, M., and Murphy, R. F. (1986) Cell-by-cell autofluorescence correction for low signal-to-noise systems: Application to epidermal growth factor endocytosis by 3T3 fibroblasts. *Cytometry* 7, 558–565.
- (14) Corsetti, J. P., Sotirchos, S. V., Cox, C., Cowles, J. W., Leary, J. F., and Blumberg, N. (1987) Correction of cellular autofluorescence in flow cytometry by mathematical modeling of cellular fluorescence. *Cytometry* 9, 539–547.
- (15) Steinkamp, J. A., and Stewart, C. C. (1986) Dual laser, differential fluorescence correction method for reducing cellular background autofluorescence. *Cytometry* 9, 566–574.
- (16) Soini, E., and Lovgren, T. (1987) Time-resolved fluorescence of lanthanide probes and applications in biotechnology. *CRC Crit. Rev. Anal. Chem.* 18, 105–155.
- (17) Holzwarth, A. F. (1995) Time-resolved fluorescence spectroscopy. *Methods Enzymol.* 246, 335–362.
- (18) Phimphivong, S., Kolchens, S., Edmiston, P. L., and Saavedra, S. S. (1995) Time resolved, total internal reflection fluorescence microscopy of cultured cells using a Tb chelate label. *Anal. Chim. Acta* 307, 403–417.
- (19) (a) Rabolt, J. F., Santo, R., and Swalen, J. D. (1979) Raman spectroscopy of thin polymer films using integrated optical techniques. *Appl. Spectrosc.* 33, 549–551. (b) Ives, J. T., and Reichert, W. M. (1987) Protein adsorption on the surface of a

- thin-film polymer integrated optical waveguide. *Appl. Spectrosc.* 42, 68–72.
- (20) Bailey, M. P., Rocks, B. F., and Riley, C. (1984) Terbium chelate for use as a label in fluorescent immunoassays. *Analyst* 109, 1449–1450.
- (21) Saavedra, S. S., and Picozza, E. G. (1989) Time-resolved fluorometric detection of terbium-labeled deoxyribonucleic acid separated by gel electrophoresis. *Analyst* 114, 835–838.
- (22) Najar, R., and Schroit, L. (1985) Generation of pH-sensitive liposomes: Use of large unilamellar vesicles containing *N*-succinyldioleoylphosphatidylethanolamine. *Biochemistry* 24, 5967–5971.
- (23) Saavedra, S. S., and Reichert, W. M. (1990) Prism coupling into polymer integrated optical waveguides with liquid substrates. *Appl. Spectrosc.* 44, 1210–1217.
- (24) Steel, J. G., Dalton, A. B., Johnson, G., and Underwood, P. A. (1993) Polystyrene chemistry affects vitronectin activity: An explanation for cell attachment to tissue culture polystyrene but not unmodified polystyrene. *J. Biomed. Mater. Res.* 27, 927–940.
- (25) Edmiston, P. L., Kolchens, S., and Saavedra, S. S. (1992) Temporally gating a slow-scan CCD with a liquid crystal shutter. *Appl. Spectrosc.* 47, 250–253.
- (26) Burridge, K., Faith, K., Kelly, T., Nuckolls, G., and Turner, C. (1988) Focal adhesions: Transmembrane junctions between the extracellular matrix and the cytoskeleton. *Annu. Rev. Cell Biol.* 4, 487–525.
- (27) Burmeister, J. S., Vraný, J., Reichert, W. M., and Truskey, G. A. (1996) Effect of vitronectin amount and conformation on the strength of endothelial cell adhesion to HEMA/EMA copolymers. *J. Biomed. Mater. Res.* 30, 12–22.
- (28) Horbett, T. A., and Schway, M. B. (1988) Correlations between mouse 3T3 cell spreading and serum fibronectin adsorption on glass and hydroxyethylmethacrylate-ethylmethacrylate copolymers. *J. Biomed. Mater. Res.* 22, 763–793.
- (29) Williams, S. K., Rose, D. G., and Jarrell, B. E. (1994) Microvascular endothelial cell seeding of ePTFE vascular grafts: Improved patency and stability of the cellular lining. *J. Biomed. Mater. Res.* 28, 203–212.
- (30) Schakenraad, J. M., Busscher, H. J., Wildevuur, C. R. H., and Arends, J. (1986) The influence of substratum surface free energy on the growth and spreading of human fibroblasts in the presence and absence of serum. *J. Biomed. Mater. Res.* 20, 773–784.

BC9701609

A Series of 6-(ω -Methanesulfonylthioalkoxy)-2-*N*-methyl-1,2,3,4-tetrahydroisoquinolines: Cysteine-Reactive Molecular Yardsticks for Probing α_2 -Adrenergic Receptors

Petri Heinonen,^{*,†} Katja Koskua,[†] Marjo Pihlavisto,[‡] Anne Marjamäki,[‡] Victor Cockcroft,^{*,§} Juha-Matti Savola,[§] Mika Scheinin,[‡] and Harri Lönnberg[†]

Department of Chemistry, University of Turku, FIN-20014 Turku, Finland, Department of Pharmacology and Clinical Pharmacology, University of Turku, FIN-20520 Turku, Finland, and Juvantia Pharma, FIN-20520 Turku, Finland. Received October 28, 1997; Revised Manuscript Received February 18, 1998

A series of 6-(ω -methanesulfonylthioalkoxy)-2-*N*-methyl-1,2,3,4-tetrahydroisoquinolines (**7a–d**) was prepared and characterized as SH-reactive molecular yardsticks useful in probing α_2 -adrenergic receptors. Rapid displacement of the methanesulfonyl group by a cysteine residue in dilute aqueous solution with concomitant formation of a disulfide conjugate was verified by MALDI-TOF mass spectrometric analysis of the reaction of **7a** with a cysteine-containing decapeptide. **7a–d** all showed a marked affinity for the three different variants of human α_2 -adrenergic receptors: H α_{2A} wt, H α_{2B} -wt, and mutant H α_{2A} Ser201Cys197. However, only the mutated receptor (H α_{2A} Ser201Cys197) was irreversibly inactivated, and the extent of inactivation in this case was linearly dependent on the length of the side chain of **7a–d**. These results show that the molecular yardstick approach tested here can provide useful information for modeling receptor proteins.

INTRODUCTION

The α_2 -adrenergic receptors, for which there are three distinct subtypes in humans (α_{2A} , α_{2B} , and α_{2C}) (1–3), mediate many of the physiological and pharmacological effects of norepinephrine and epinephrine. As typical G-protein-coupled receptors, each consists of a single polypeptide chain spanning the cell membrane seven times with three intra- and three extracellular loops (4). The seven transmembrane segments are believed to be conserved α -helical elements forming the binding site for not only the endogenous transmitters but also small molecule analogues, such as oxymetazoline, chlorpromazine, UK 14,304, dexmedetomidene, and alkylated tetrahydroisoquinolines. All these compounds show a different pattern of subtype selectivity, and hence different pharmacological actions (5, 6).

Elucidation of the structural details of the binding domain of receptor proteins can facilitate the development of new therapeutic agents, exhibiting improved selectivity. However, primarily because of their membrane protein nature, direct structural studies (e.g., X-ray crystallography or NMR) on these receptors have so far not yielded high-resolution data despite considerable efforts. In attempts to obtain experimental information on receptor structure, mutagenesis studies in combination with pharmacological characterization have been popular, with over 200 literature references in the area

of G-protein-coupled receptors. However, these studies suffer from the limitation that noncovalent interactions are involved which introduces uncertainty in the interpretation stage. So far and because of their importance to the pharmaceutical industry, molecular modeling by a variety of means has been used to produce crude models, but there is still no consensus as to which residues line the binding cavity.

In a previous study, a new biochemical approach for mapping the binding site crevice of the human α_2 -adrenergic receptors (α_2 -ARs) was introduced by us (7). Based on a three-dimensional (3D) receptor model, each of the residues (197–201 and 204) in the fifth transmembrane domain (TM5) of the human α_{2A} -AR was changed to cysteine (Cys). Subsequently, the irreversible binding of chloroethylclonidine (CEC), an alkylating derivative of the α_2 -adrenergic agonist clonidine, was used as a criterion for identifying the introduced Cys residues as being exposed in the binding cavity. Thus, the results supported a model in which Val197, Ser200, Cys201, and Ser204 are exposed to the binding cavity and residues Ile198, Ser199, Ile202, and Gly203 face the lipid bilayer of the plasma membrane. To extend the utility of this approach, a molecular yardstick approach series of new synthetic receptor ligands was designed and synthesized. The new compounds (**7a–d**) have a *N*-methyl-1,2,3,4-tetrahydroisoquinoline core conferring α_2 -AR affinity, a methanesulfonylthio group making them Cys-reactive, and an alkyl linker of variable length connecting the two functional moieties. The basic assumption is that varying the length of the linker affects the alkylating properties of **7a–d** by placing distance constraints on the receptor–ligand interaction. This paper describes the synthesis, molecular modeling, and initial characterization of the set of 6-(ω -methanesulfonylthioalkoxy)-2-*N*-methyl-1,2,3,4-tetrahydroisoquinolines (**7a–d**) as site-specific labeling agents providing information that is useful for further improving the reliability of 3D models

* Address correspondence to either author. Petri Heinonen, Department of Chemistry, University of Turku, FIN-20014 Turku, Finland. Telephone: +358-2-333 8092. Fax: +358-2-333 6700. E-mail: petri.heinonen@utu.fi. Victor Cockcroft, Drug Design & Bioinformatics, Juvantia Pharma, Tykistökatu 6 A, FIN-20520 Turku, Finland. Telephone or fax: +358-2-2327763.

[†] Department of Chemistry, University of Turku.

[‡] Department of Pharmacology and Clinical Pharmacology, University of Turku.

[§] Juvantia Pharma.

of α_2 -ARs, as well as other closely related receptors. A similar chemistry has very recently been applied by Berglund et al. (8) to obtain a series of chemically modified cysteine mutants of subtilisin.

EXPERIMENTAL PROCEDURES

General Methods. Chromatographic purifications were carried out on Kieselgel 60 (Merck) silica gel and TLC analyses on Alufolien Kieselgel 60 F₂₅₄ (Merck) TLC plates, using the following eluent systems: system A, 8:92 methanol/dichloromethane (v/v); system B, 1.5:98.5 methanol/dichloromethane (v/v); and system C, 7:93 triethylamine/dichloromethane (v/v). The NMR spectra were recorded on JEOL JNM-GX 400, JEOL JNM-A 500, or Bruker AW 80 NMR spectrometers. The chemical shifts are given in parts per million from internal TMS. The mass spectra of small molecular compounds were recorded on a 7070E VG mass spectrometer, and that of the peptide conjugate was recorded on a Finnigan MAT Lasermat mass spectrometer. Pyridine was dried by refluxing with CaH₂ and then stored over 4 Å molecular sieves. [³H]RX821002 [2-(2-methoxy-1,4-benzodioxan-2-yl)-2-imidazoline] was from Amersham (Buckinghamshire, U.K.; specific activity of 52 Ci/mmol). Phentolamine and CEC were from Research Biochemicals Inc. (Natick, MA). Cell culture reagents were supplied by Gibco (Gaithersburg, MD). The construction of expression vectors and receptor production have been described elsewhere (7). Saturation isotherms of [³H]RX821002 binding when analyzed by LIGAND (9) gave the following receptor affinities (K_d) and receptor densities (B_{max}): 0.60 ± 0.02 nM (B_{max} = 595 ± 2 fmol mg⁻¹) for H α_{2A} wt, 6.12 ± 0.46 nM (B_{max} = 1870 ± 111 fmol mg⁻¹) for H α_{2B} wt, and 0.53 ± 0.05 nM (B_{max} = 295 ± 22 fmol mg⁻¹) for H α_{2A} -Ser201Cys197 (7).

The radioligand binding assays were performed with cell homogenates in potassium phosphate buffer as previously described (10). Competition and inactivation studies were carried out using [³H]RX821002 concentrations that were close to its affinity constant (K_d) at each receptor and 10–13 concentrations of the competitors.

The receptor inactivation was investigated in parallel assays with CEC and **7a–d**. The concentrations used for inactivation were 2–3 K_i (inhibition constant) for each compound. The cell homogenates were first incubated with CEC (1 μ M) or **7a–d** (5 μ M) in 2.5 mL of potassium phosphate buffer for 45 min at 37 °C. The protein content was 0.3–0.5 mg/mL. The cell membranes were then pelleted at 40000g for 15 min at 4 °C, washed twice with 2.5 mL of ice-cold potassium phosphate buffer, and rehomogenized with the Ultra-Turrax homogenizer. Residual α_2 -AR binding was assessed by incubating the membranes (0.1–0.2 mg of protein/assay tube) with 2.5 nM [³H]RX821002. Nonspecific binding was determined by including 10 μ M phentolamine in parallel assays.

Molecular Modeling. The Tripos program Sybyl was used to perform molecular modeling on a Silicon Graphics Indigo 2 machine fitted with 128 megabytes of memory. The starting structure of each of the compounds in the series had the reactive side chain in a fully extended conformation and the amine nitrogen atom assigned an N4 atom type. Energy minimization was performed using the Tripos force field without electrostatics. Conformational dynamics were calculated using the Tripos force field without electrostatics, at 310 K, for a 1 ns time period, with a step size of 1 fs, saving coordinates every 50 fs. The dynamic trajectory frames were read into standard Sybyl tables, where atom–atom distances were calculated and statistical analysis was performed.

6-Methoxy-2-N-methyl-1,2,3,4-tetrahydroisoquinoline 2. 6-Methoxy-1,2,3,4-tetrahydroisoquinoline (**1**) (1.28 g, 7.8 mmol) was dissolved into a mixture of formic acid (1.5 mL) and formaldehyde (1.4 mL of a 40% aqueous solution). The reaction mixture was first warmed to 30 °C and, when the formation of CO₂ was ceased, to 80 °C. After 4 h, the reaction mixture was made acidic (pH ~ 3) with dilute aqueous hydrogen chloride and then washed with diethyl ether. The aqueous solution was made alkaline with aqueous sodium hydroxide and extracted with diethyl ether. The organic layer was separated, dried on Na₂SO₄, and evaporated to dryness. Yield: 1.1 g (80%). MS(EI) (relative intensity): 176 (100, M⁺ – 1), 161 (20), 134 (93). ¹H NMR (400 MHz, CDCl₃): 6.93 (d, 1H, *J* = 8.4 Hz), 6.69 (dd, 1H, *J* = 2.6 Hz, *J* = 8.4 Hz), 6.64 (d, 1H, *J* = 2.6 Hz), 3.77 (s, 3H), 3.52 (s, 2H), 2.90 (t, 2H, *J* = 6.0 Hz), 2.66 (t, 2H, *J* = 6.0 Hz), 2.45 (s, 3H). ¹³C NMR (100 MHz, CDCl₃): 29.5, 46.1, 52.8, 55.2, 57.5, 112.0, 113.2, 127.0, 127.3, 134.9, 157.9.

6-Hydroxy-2-N-methyl-1,2,3,4-tetrahydroisoquinoline 3. **2** (1.07 g, 6.0 mmol) was dissolved in 25 mL of 47% aqueous HBr and refluxed for 4 h under nitrogen. The solution was evaporated to dryness, and **3** was recrystallized from ethanol as hydrobromide. A total of 1.2 g (81%) of white crystals was obtained. MS(EI) (relative intensity): 162 (100, M⁺ – 1), 120 (60). ¹H NMR (400 MHz, DMSO-*d*₆): 9.88 (s, 1H), 9.53 (s, 1H), 6.99 (d, 1H, *J* = 8.4 Hz), 6.68 (dd, 1H, *J* = 2.6 Hz, *J* = 8.3 Hz), 6.62 (d, 1H, *J* = 2.5 Hz), 4.37 (d, 1H, *J* = 14.4 Hz), 4.16 (dd, 1H, *J* = 8.2 Hz, *J* = 14.4 Hz), 3.62 (m, 1H), 3.29 (m, 1H), 3.07 (m, 1H), 2.97 (m, 1H), 2.91 (d, 3H, *J* = 4.3 Hz). ¹³C NMR (100 MHz, DMSO-*d*₆): 25.1, 41.9, 50.3, 53.5, 114.3, 114.5, 118.4, 127.6, 131.9, 156.8.

The hydrobromide of **3** (1.0 g, 4.1 mmol) was dissolved in water, and 2 mol L⁻¹ aqueous sodium hydroxide (2.0 mL, 4.1 mmol) was added. The precipitated product was filtered, washed with a small amount of water, and dried under reduced pressure. Yield: 0.52 g (77%). MS(EI) (relative intensity): 162 (100, M⁺ – 1), 120 (66). ¹H NMR (400 MHz, DMSO-*d*₆): 9.07 (s, 1H), 6.80 (d, 1H, *J* = 8.3 Hz), 6.49 (dd, 1H, *J* = 2.5 Hz, *J* = 8.3 Hz), 6.46 (d, 1H, *J* = 2.2 Hz), 3.33 (s, 2H), 2.69 (t, 2H, *J* = 5.7 Hz), 2.50 (t, 2H, *J* = 5.7 Hz), 2.28 (s, 3H). ¹³C NMR (100 MHz, DMSO-*d*₆): 29.3, 46.2, 52.8, 57.5, 113.4, 114.9, 125.5, 127.4, 135.0, 155.7.

6-(Triphenylmethoxy)-1-hexanol 8d. Triphenylmethyl chloride (10.0 g, 36 mmol) was dissolved in dry pyridine, and 10.6 g (90 mmol) of 1,6-hexanediol was added. After 16 h, the reaction mixture was diluted with dichloromethane, washed with aqueous sodium bicarbonate and brine, dried on Na₂SO₄, and evaporated to dryness. The product was purified by silica gel chromatography (system B). Yield: 11.9 g (92%). ¹H NMR (80 MHz, CDCl₃): 7.10–7.50 (m, 15H), 3.60 (t, 2H, *J* = 5 Hz), 3.05 (t, 2H, *J* = 5 Hz), 1.20–1.70 (m, 8H).

5-(Triphenylmethoxy)-1-pentanol 8c was prepared from 1,5-pentanediol as previously described for **8d**. Yield: 84%. ¹H NMR (80 MHz, CDCl₃): 7.10–7.50 (m, 15H), 3.60 (t, 2H, *J* = 5 Hz), 3.05 (t, 2H, *J* = 5 Hz), 1.25–1.70 (m, 6H).

4-(Triphenylmethoxy)-1-butanol 8b was prepared from 1,4-butanediol as previously described for **8d**. Yield: 90%. ¹H NMR (80 MHz, CDCl₃): 7.10–7.50 (m, 15H), 3.60 (t, 2H, *J* = 5 Hz), 3.10 (t, 2H, *J* = 5 Hz), 1.55–1.75 (m, 4H).

3-(Triphenylmethoxy)-1-propanol 8a was prepared from 1,3-propanediol as previously described for **8d**. Yield: 65%. ¹H NMR (400 MHz, CDCl₃): 7.2–7.5 (m,

15H), 3.77 (t, 2H, $J = 5.8$ Hz), 3.28 (t, 2H, $J = 5.8$ Hz), 1.86 (m, 2H).

6-[(6-Hydroxyhexyl)oxy]-2-*N*-methyl-1,2,3,4-tetrahydroisoquinoline 5d. **3** (0.50 g, 3.1 mmol) was dissolved in THF. **8d** (1.5 g, 4.3 mmol) and triphenylphosphine (0.96 g, 3.7 mmol) were added. To this solution was added dropwise 0.57 mL (3.7 mmol) of diethyl azodicarboxylate (DEAD), and the mixture was left to stand for 24 h. After completion of the reaction (TLC, system A), dichloromethane was added and the solution was washed with aqueous sodium bicarbonate, dried with Na_2SO_4 , and evaporated. **4d** was dissolved in 25 mL of 1 mol L^{-1} aqueous hydrogen chloride, and the mixture was refluxed for 1 h, allowed to cool to room temperature, and washed with ethyl acetate. Aqueous sodium hydroxide solution was added, and the product was extracted to ethyl acetate, dried on Na_2SO_4 , and evaporated. The product was purified by silica gel chromatography (system C). Yield: 0.53 g (66%). MS(EI) (relative intensity): 262 (100, $\text{M}^+ - 1$), 220 (12), 162 (36), 120 (45). ^1H NMR (400 MHz, CDCl_3): 6.92 (d, 1H, $J = 8.5$ Hz), 6.68 (dd, 1H, $J = 2.4$ Hz, $J = 8.5$ Hz), 6.63 (d, 1H, $J = 2.2$ Hz), 3.92 (t, 2H, $J = 6.6$ Hz), 3.65 (t, 2H, $J = 6.6$ Hz), 3.51 (s, 2H), 2.89 (t, 2H, $J = 5.8$ Hz), 2.65 (t, 2H, $J = 5.8$ Hz), 2.44 (s, 3H), 1.74–1.81 (m, 2H), 1.56–1.63 (m, 2H), 1.38–1.52 (m, 4H). ^{13}C NMR (100 MHz, CDCl_3): 25.6, 25.9, 29.3, 29.5, 32.7, 46.1, 52.9, 57.5, 62.8, 67.8, 112.5, 113.9, 126.8, 127.3, 134.9, 157.5.

6-[(5-Hydroxypentyl)oxy]-2-*N*-methyl-1,2,3,4-tetrahydroisoquinoline 5c was prepared from **3c** as previously described for **5d**. Yield: 62%. MS(EI) (relative intensity): 248 (100, $\text{M}^+ - 1$), 206 (11), 162 (49), 120 (57). ^1H NMR (400 MHz, CDCl_3): 6.92 (d, 1H, $J = 8.3$ Hz), 6.68 (dd, 1H, $J = 2.4$ Hz, $J = 8.6$ Hz), 6.63 (d, 1H, $J = 2.2$ Hz), 3.92 (t, 2H, $J = 6.4$ Hz), 3.65 (t, 2H, $J = 6.4$ Hz), 3.51 (s, 2H), 2.89 (t, 2H, $J = 5.9$ Hz), 2.66 (t, 2H, $J = 5.9$ Hz), 2.44 (s, 3H), 1.75–1.82 (m, 2H), 1.59–1.66 (m, 2H), 1.45–1.55 (m, 2H). ^{13}C NMR (100 MHz, CDCl_3): 22.4, 29.1, 29.5, 32.5, 46.1, 52.8, 57.5, 62.7, 67.8, 112.5, 113.9, 126.8, 127.3, 134.9, 157.4.

6-[(4-Hydroxybutyl)oxy]-2-*N*-methyl-1,2,3,4-tetrahydroisoquinoline 5b was prepared from **3b** as previously described for **5d**. Yield: 67%. MS(EI) (relative intensity): 234 (93, $\text{M}^+ - 1$), 192 (7), 162 (63), 120 (100). ^1H NMR (500 MHz, CDCl_3): 6.92 (d, 1H, $J = 8.4$ Hz), 6.69 (dd, 1H, $J = 2.6$ Hz, $J = 8.4$ Hz), 6.64 (d, 1H, $J = 2.5$ Hz), 3.97 (t, 2H, $J = 6.3$ Hz), 3.71 (t, 2H, $J = 6.3$ Hz), 3.52 (s, 2H), 2.89 (t, 2H, $J = 6.0$ Hz), 2.67 (t, 2H, $J = 6.0$ Hz), 2.44 (s, 3H), 1.84–1.89 (m, 2H), 1.72–1.77 (m, 2H). ^{13}C NMR (100 MHz, CDCl_3): 25.9, 29.4, 29.6, 46.0, 52.7, 57.4, 62.6, 67.9, 112.5, 114.0, 126.8, 127.3, 134.9, 157.4.

6-[(3-Hydroxypropyl)oxy]-2-*N*-methyl-1,2,3,4-tetrahydroisoquinoline 5a was prepared from **3a** as previously described for **5d**. Yield: 73%. MS(EI) (relative intensity): 220 (100, $\text{M}^+ - 1$), 178 (29), 162 (46), 120 (50). ^1H NMR (400 MHz, $\text{DMSO}-d_6$): 6.87 (d, 1H, $J = 8.3$ Hz), 6.6 (m, 2H), 3.92 (t, 2H, $J = 6.3$ Hz), 3.49 (t, 2H, $J = 6.1$ Hz), 3.33 (s, 2H), 2.72 (t, 2H, $J = 5.6$ Hz), 2.49 (t, 2H, $J = 5.9$ Hz), 2.26 (s, 3H), 1.73–1.83 (m, 2H). ^{13}C NMR (100 MHz, $\text{DMSO}-d_6$): 29.0, 32.2, 45.8, 52.3, 56.9, 57.3, 64.4, 112.3, 113.5, 126.8, 127.0, 134.8, 156.8.

6-(6-Methanesulfonyloxyhexoxy)-2-*N*-methyl-1,2,3,4-tetrahydroisoquinoline 6d. **5d** (0.26 g, 1.0 mmol) was dissolved in a small amount of dry pyridine, and 10 mg of (dimethylamino)pyridine and 0.21 g (1.2 mmol) of methanesulfonyl anhydride were added. After completion of reaction (TLC, system A), dichloromethane was added and the solution was washed with aqueous sodium bicarbonate. The organic layer was dried on Na_2SO_4 and evaporated.

The product was purified by silica gel chromatography (system A). Yield: 0.20 g (59%). MS(EI) (relative intensity): 340 (100, $\text{M}^+ - 1$), 298 (19), 244 (14), 162 (35), 120 (48).

6-(5-Methanesulfonyloxypropoxy)-2-*N*-methyl-1,2,3,4-tetrahydroisoquinoline 6c was prepared from **5c** as previously described for **6d**. Yield: 61%. MS(EI) (relative intensity): 326 (100, $\text{M}^+ - 1$), 284 (21), 230 (15), 162 (47), 120 (64).

6-(4-Methanesulfonyloxybutoxy)-2-*N*-methyl-1,2,3,4-tetrahydroisoquinoline 6b was prepared from **5b** as previously described for **6d**. Yield: 52%. MS(EI) (relative intensity): 312 (100, $\text{M}^+ - 1$), 270 (23), 216 (14), 162 (38), 120 (33).

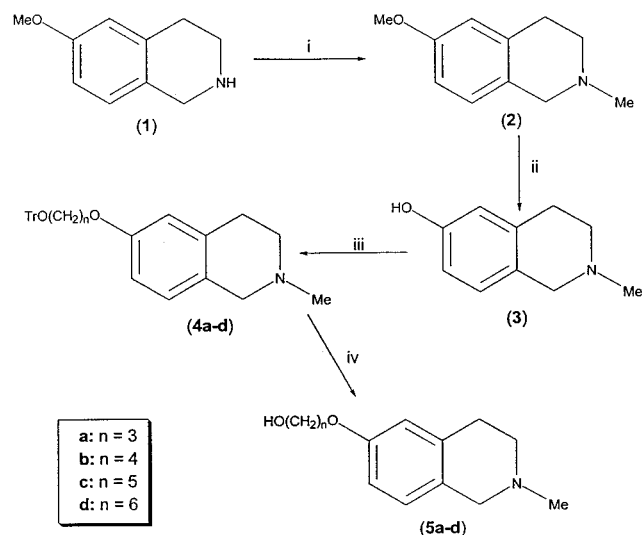
6-(3-Methanesulfonyloxypropoxy)-2-*N*-methyl-1,2,3,4-tetrahydroisoquinoline 6a was prepared from **5a** as previously described for **6d**. Yield: 62%. MS(EI) (relative intensity): 298 (100, $\text{M}^+ - 1$), 256 (15), 202 (10), 162 (40), 120 (50).

6-(6-Methanesulfonylthiohexoxy)-2-*N*-methyl-1,2,3,4-tetrahydroisoquinoline 7d. **6d** (0.18 g, 0.5 mmol) was dissolved in ethanol, and 0.15 g (1.1 mmol) of sodium methanethiosulfonate was added. The reaction mixture was incubated for 65 h at 50 °C and then diluted with dichloromethane. After being washed with aqueous sodium bicarbonate, being dried on Na_2SO_4 , and evaporation, the product was purified by silica gel chromatography (system A). Yield: 82 mg (42%). MS(EI) (relative intensity): 356 (100, $\text{M}^+ - 1$), 278 (24), 162 (33), 146 (11), 120 (39). ^1H NMR (500 MHz, CDCl_3): 6.93 (d, 1H, $J = 8.4$ Hz), 6.69 (dd, 1H, $J = 2.6$ Hz, $J = 8.4$ Hz), 6.63 (d, 1H, $J = 2.5$ Hz), 3.92 (t, 2H, $J = 6.3$ Hz), 3.59 (s, 2H), 3.32 (s, 3H), 3.18 (t, 2H, $J = 7.4$ Hz), 2.92 (t, 2H, $J = 6.1$ Hz), 2.74 (t, 2H, $J = 6.1$ Hz), 2.50 (s, 3H), 1.76–1.82 (m, 4H), 1.49–1.51 (m, 4H). ^{13}C NMR (120 MHz, CDCl_3): 25.5, 28.3, 29.0, 29.1, 29.5, 36.4, 45.7, 50.6, 52.6, 57.1, 67.6, 112.7, 113.9, 126.2, 127.4, 134.6, 157.5.

6-(5-Methanesulfonylthiopentoxo)-2-*N*-methyl-1,2,3,4-tetrahydroisoquinoline 7c was prepared from **6c** as previously described for **7d**. Yield: 39%. MS(EI) (relative intensity): 342 (100, $\text{M}^+ - 1$), 264 (36), 162 (68), 146 (18), 120 (68). ^1H NMR (400 MHz, CDCl_3): 6.93 (d, 1H, $J = 8.6$ Hz), 6.68 (dd, 1H, $J = 2.7$ Hz, $J = 8.6$ Hz), 6.63 (d, 1H, $J = 2.2$ Hz), 3.94 (t, 2H, $J = 6.3$ Hz), 3.60 (s, 2H), 3.32 (s, 3H), 3.20 (t, 2H, $J = 7.3$ Hz), 2.93 (t, 2H, $J = 6.1$ Hz), 2.75 (t, 2H, $J = 6.1$ Hz), 2.51 (s, 3H), 1.77–1.87 (m, 4H), 1.59–1.64 (m, 2H). ^{13}C NMR (120 MHz, CDCl_3): 25.2, 28.6, 29.1, 29.3, 36.3, 45.7, 50.7, 52.7, 57.2, 67.4, 112.7, 113.9, 126.2, 127.4, 134.6, 157.4.

6-(4-Methanesulfonylthiobutoxy)-2-*N*-methyl-1,2,3,4-tetrahydroisoquinoline 7b was prepared from **6b** as previously described for **7d**. Yield: 36%. MS(EI) (relative intensity): 328 (100, $\text{M}^+ - 1$), 250 (21), 162 (62), 146 (12), 120 (38). ^1H NMR (400 MHz, CDCl_3): 6.93 (d, 1H, $J = 8.3$ Hz), 6.68 (dd, 1H, $J = 2.4$ Hz, $J = 8.3$ Hz), 6.63 (d, 1H, $J = 2.4$ Hz), 3.97 (t, 2H, $J = 5.9$ Hz), 3.59 (s, 2H), 3.33 (s, 3H), 3.26 (t, 2H, $J = 7.3$ Hz), 2.93 (t, 2H, $J = 5.9$ Hz), 2.74 (t, 2H, $J = 5.9$ Hz), 2.51 (s, 3H), 1.89–2.00 (m, 4H). ^{13}C NMR (100 MHz, CDCl_3): 26.5, 28.0, 29.0, 36.2, 45.7, 50.8, 52.6, 57.1, 66.9, 112.7, 113.9, 126.4, 127.4, 134.3, 157.1.

6-(3-Methanesulfonylthiopropoxy)-2-*N*-methyl-1,2,3,4-tetrahydroisoquinoline 7a was prepared from **6a** as previously described for **7d**. Yield: 14%. MS(EI) (relative intensity): 314 (100, $\text{M}^+ - 1$), 162 (39), 146 (19), 120 (24). ^1H NMR (500 MHz, CDCl_3): 6.93 (d, 1H, $J = 8.4$ Hz), 6.68 (dd, 1H, $J = 2.6$ Hz, $J = 8.3$ Hz), 6.64 (d, 1H, $J = 2.6$ Hz), 4.05 (t, 2H, $J = 7.4$ Hz), 3.57 (s, 2H), 3.37 (t, 2H, $J = 7.1$ Hz), 3.32 (s, 3H), 2.93 (t, 2H, $J = 6.0$

Scheme 1^a

^a (i) Aqueous formaldehyde (37%)/formic acid, 80 °C, 4 h; (ii) 48% aqueous HBr, reflux, 4 h; (iii) **8a-d**, Pph₃, DEAD, THF, 24 h; (iv) 1 mol L⁻¹ aqueous HCl, reflux, 1 h.

Hz), 2.72 (t, 2H, *J* = 6.0 Hz), 2.48 (s, 3H), 2.46 (m, 2H). ¹³C NMR (125 MHz, CDCl₃): 29.2, 29.4, 33.2, 45.8, 50.4, 52.6, 57.2, 65.3, 112.6, 113.9, 126.9, 127.5, 134.9, 156.9.

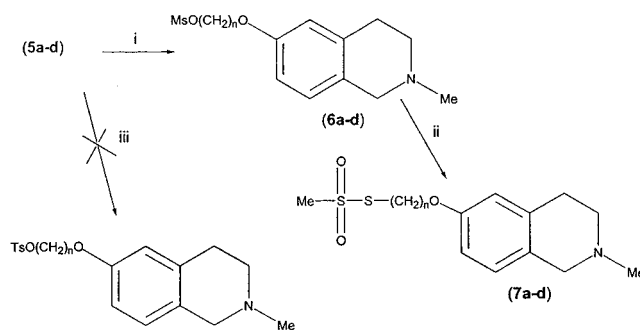
Hydrolysis of 7a-d. The rates of hydrolyses of **7a-d** were measured at 37 °C in potassium phosphate buffer (50 mM, pH 7.4). The aliquots of 40 μ L were withdrawn at suitable intervals and analyzed immediately by HPLC [Purospher RP-18e column, 0.050 mol L⁻¹ ammonium acetate buffer at pH 4.7, flow rate of 0.75 mL min⁻¹; elution, 85:15 buffer/MeCN for **7a-b**, 80:20 buffer/MeCN for **7c**, and 75:25 buffer/MeCN for **7d**; detection, 276 nm; *t_R*(**7a**) = 6.8 min, *t_R*(**7b**) = 12.6 min, *t_R*(**7c**) = 9.9 min, and *t_R*(**7d**) = 8.6 min]. The first-order rate constants were 4.3×10^{-4} , 3.7×10^{-4} , 3.2×10^{-4} , and 2.8×10^{-4} s⁻¹ for **7a-d**, respectively.

Reaction of 7a with Peptide YVISSCIGSF. One milligram (0.93 μ mol) of the 10-mer oligopeptide Tyr-Val-Ile-Ser-Ser-Cys-Ile-Gly-Ser-Phe was dissolved in 10 mL of 50 mmol L⁻¹ potassium phosphate buffer (pH 7.4 at 21 °C), and 2 equiv of **7a** (0.58 mg, 1.86 μ mol) was added. The reaction mixture was incubated for 60 min at 37 °C. Analysis by MALDI-TOF mass spectroscopy verified the conjugation. MS(MALDI): 1313.7. The reaction of **7a** with YVISSCIGSF was further studied by HPLC. The peptide (0.3 μ mol mL⁻¹) was treated with 1 equiv of **7a**, and the aliquots withdrawn were immediately chromatographed on a Purospher RP-18e column (0.050 mol L⁻¹ ammonium acetate buffer at pH 4.7, flow rate of 0.75 mL min⁻¹; elution, 85:15 buffer/MeCN; detection, 276 nm).

RESULTS

Synthesis of 6-(ω -Methanesulfonylthioalkoxy)-2-N-methyl-1,2,3,4-tetrahydroisoquinolines. 6-Methoxy-1,2,3,4-tetrahydroisoquinoline (**1**) was prepared as described earlier (11); the nitrogen atom was methylated by an Eschweiler-Clarke reaction (**2**), and the 6-methoxy group was converted to a 6-hydroxy group with boiling of aqueous 48% hydrogen bromide under nitrogen (**3**). The phenolic hydroxy function of **3** was then displaced with an appropriate monotritylated α,ω -diol by Mitsunobu reaction, and the product (**4a-d**) was detritylated to give **5a-d** (Scheme 1).

Several alkyl methanethiosulfonates have previously been prepared from alkyl halides by S_N2 substitution

Scheme 2^a

^a (i) Methanesulfonic anhydride/Py, 20 h; (ii) sodium methanethiosulfonate, EtOH, 50 °C, 65 h; (iii) *p*-toluenesulfonyl chloride/Py.

with sodium methanethiosulfonate. Accordingly, conversion of **5a-d** to the corresponding tosylates, followed by displacement with sodium methanethiosulfonate, was considered to be a viable route to **7a-d**. However, all attempts to convert **5a-d** to tosylates were unsuccessful. By contrast, less reactive mesylates **6a-d** could be easily obtained. Use of mesyl anhydride, instead of the more commonly used mesyl chloride, proved to be essential, since **6a-d** reacted surprisingly easily with the chloride ion released to the reaction mixture upon the S_N2 displacement. Compounds **6a-d** turned out to be relatively unstable. To obtain reasonable yields of **7a-d**, the reaction with sodium methanethiosulfonate had to be started immediately after silica gel purification of **6a-d** (Scheme 2).

Covalent Coupling of 7a to Model Peptide YVISSCIGSF. The applicability of **7a-d** in protein labeling, and hence as useful agents in α_2 -adrenergic receptor mapping, was demonstrated by reacting one of them with a cysteine residue of model peptide YVISSCIGSF. This peptide is part of the TM5 region of the human α_{2A} -AR. Two equivalents of **7a** was added to the solution of the model peptide in 0.050 mol L⁻¹ potassium phosphate buffer (pH 7.4 at 21 °C) at 37 °C, and after 1 h, a matrix-assisted laser desorption mass spectrum was recorded from the reaction mixture. The disappearance of the signal referring to peptide YVISSCIGSF and the appearance of a new signal at higher field by 235 mass units indicated that **7a** had formed a covalent conjugate with the peptide (Figure 1). The half-lives for the hydrolysis of **7a-d** were observed to be 27 (**7a**), 31 (**7b**), 36 (**7c**), and 41 min (**7d**) under the conditions employed in the conjugation with the peptide. When the model peptide was treated at a 0.3 mmol L⁻¹ concentration with 1 equiv of the most labile labeling agent (**7a**), the HPLC signal of **7a** qualitatively disappeared in 2 min at 37 °C. Accordingly, the hydrolysis of **7a-d** is slow enough to ensure efficient conjugation with the peptide.

Molecular Modeling. Table 1 presents a summary of intramolecular atom-atom distances between the ω -sulfur and both the phenoxyl oxygen and the quaternary ammonium nitrogen occurring in the molecular dynamics trajectory of **7a-d**. The maximal distance from the ether oxygen to the ω -sulfur in the side chain (*n* = 3–6) spans from 5.3 to 8.7 Å and that from the amine nitrogen to sulfur from 11.6 to 14.5 Å. The successive increment per methylene group is about 1 Å (the theoretical upper limit of the distance resolution by a molecular yardstick approach). Whereas the average distance for the O–S atom pairs is incremental, the average distance for the N–S atom pairs is not. This is a result of folding back of the linker in the longer (*n* = 6) side

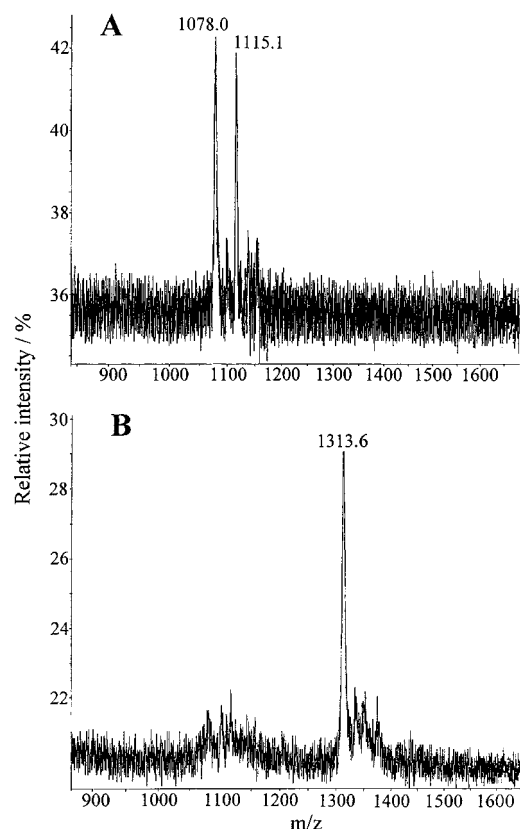


Figure 1. MALDI-TOF spectra of peptide YVSSCIGSF (A) and its conjugate formed upon reaction with **7a** (B). The peaks at m/z 1078.0 and 1115.1 represent the peptide and its potassium adduct, and that at 1313.6 represents the peptide conjugate formed by the displacement of the methanesulfonyl group of **7a** by the peptide.

Table 1. Intramolecular Atom–Atom Distances from Conformational Dynamics of 6-(ω -Methanesulfonylthioalkoxy)-2-*N*-1,2,3,4-tetrahydroisoquinolines (**7a–d**)

	O–S distance (Å)				N–S distance (Å)			
	max	min	avg	SD	max	min	avg	SD
7a	5.3	2.5	3.7	0.7	11.6	4.8	8.9	1.1
7b	6.7	3.0	4.4	0.8	12.9	4.1	9.3	2.1
7c	7.6	2.9	5.6	0.9	13.8	3.9	10.0	2.2
7d	8.7	4.1	7.0	0.7	14.5	3.4	8.6	2.3

Table 2. Binding Affinities of CEC and **7a–d** for α_2 -AR Variants, Determined by Competition Assays with [^{3} H]RX821002^a

test compound	K_i (nM)		
	H α_{2A} wt	H α_{2A} Ser201Cys197	H α_{2B} wt
CEC	578	60	1500
7a	2388	1402	2789
7b	1560	34	1530
7c	1690	34	1600
7d	1780	34	1100

^a Mean values of two or three independent experiments are shown.

chain such that the shortest N–S distance of 3.4 Å is shorter than that of the other compound ($n = 3$), 4.8 Å.

Binding Affinity of CEC and **7a–d.** The CEC-resistant wild-type receptor H α_{2B} , the mutant H α_{2A} -Ser201Cys197, and H α_{2A} wt were all capable of binding CEC and **7a–d** (approximate K_i values of 34–2789 nM). Table 2 shows the results of competition binding assays, where CEC and **7a–d** were allowed to compete with the radioligand [^{3} H]RX821002 for binding to H α_{2A} wt, H α_{2B} -wt, and H α_{2A} Ser201Cys197 receptors. The K_i values

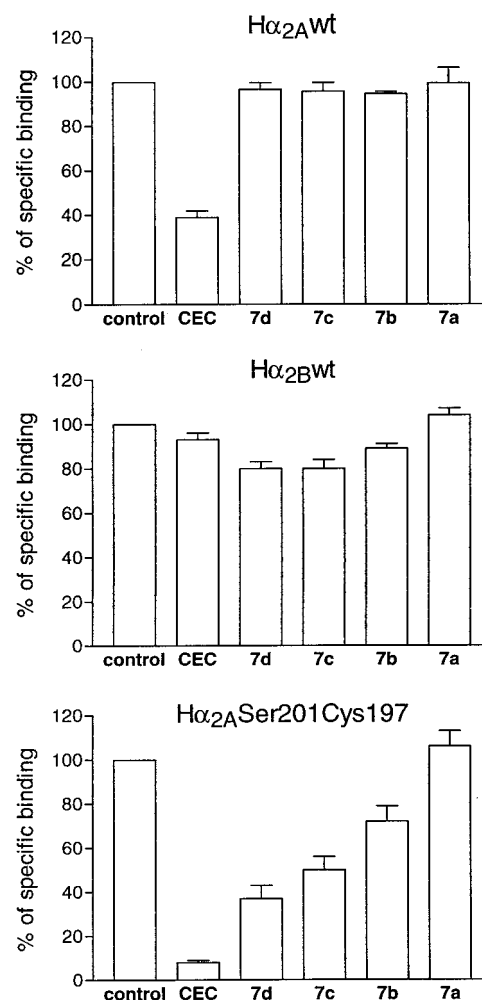


Figure 2. Effect of treatment with CEC and **7a–d** on binding activity of human α_2 -AR subtypes. CHO cells expressing human α_2 -AR subtypes H α_{2A} wt, H α_{2B} wt, and H α_{2A} Ser201Cys197 were incubated in the absence (control) and presence of CEC (1 μ M) or **7a–d** (5 μ M) for 45 min at 37 °C, followed by two washes. The residual α_2 -AR binding capacity was determined by incubation with 2.5 nM [^{3} H]RX821001. The nonspecific binding was determined with 10 μ M phentolamine. The results are expressed as the percentage of specific [^{3} H]RX821002 binding left after treatment with CEC or **7a–d**. The results are means $\pm$ SE of three or four experiments, each performed in duplicate.

obtained do not accurately represent competitive affinities of binding of the test compounds to the receptors; in the case of H α_{2A} wt and H α_{2A} Ser201Cys197 receptors, some irreversible binding is also taking place at this reaction temperature (25 °C). In any case, the results show that compounds **7a–d** have quite high affinities for the receptors tested, comparable to that of CEC.

Receptor Inactivation Studies. The alkylating effect of **7a–d** was tested on three H α_2 -AR variants (H α_{2A} wt, H α_{2B} wt, and the mutant H α_{2A} Ser201Cys197). Incubation of cell homogenates in the absence (control) and presence of CEC was performed for control purposes and as a reference for **7a–d**. CEC treatment reduced the binding capacity of H α_{2A} wt and H α_{2A} Ser201Cys197 by 61 and 91%, respectively, while H α_{2B} wt (lacking an exposed Cys residue) was not alkylated by CEC (Figure 2). In experiments with **7a–d**, the mutant H α_{2A} -Ser201Cys197 receptor was inactivated to a significant extent. Moreover, the degree of inactivation was linearly related to the length of the linker.

DISCUSSION

This study is part of a series of studies aimed at providing geometric constraints from biochemical studies for improving the structural modeling of α_2 -adrenergic receptor subtypes, which are important drug target sites. Previously, we have shown that amino acid residues Val197, Ser200, Cys201, and Ser204 of the human α_{2A} -AR are accessible inside the binding cavity using cysteine substitution and covalent labeling with chloroethylclonidine (CEC) (7). Thus, a hypothetical three-dimensional model of the receptor, albeit admittedly rather crude, was supported. As an alkylating agent, CEC reacts via a reactive aziridinium ion species and is expected to be of use in the identification of amino acid residues located inside the binding cavity within the fourth, fifth, and sixth transmembrane helical elements. The development of the 6-(ω -methanesulfonylthioalkoxy)-2-*N*-methyl-1,2,3,4-tetrahydroisoquinoline series is expected to aid in exploring regions of the receptor toward and outside the entrance of the binding cavity.

Previous chemical studies with simple alkyl methanesulfonothiolates, RSSO_2Me , show that the mercapto-methyl side chain of proteins is able to displace rapidly and quantitatively the methanesulfonyl group in dilute aqueous solution with concomitant formation of a disulfide linkage (12, 13). The specificity of this reaction for cysteine residues over other amino acid residues has also been demonstrated. Here, a pilot study was carried out on a 10-mer peptide with the sequence YVISCIGSF, part of the sequence of the fifth transmembrane-spanning segment of the human α_{2A} -AR, to determine the reaction conditions needed for biochemical inactivation studies of the receptor. The methanesulfonylthio group was the preferred labeling agent compared to more commonly used SH-reactive functions (including pyridyl disulfide, maleimido, and bromoacetyl groups) because it does not carry a formal charge, is relatively hydrophilic, and appears to be sterically less demanding. These features were attractive as they were considered less likely to affect pharmacophore interactions, as was borne out by similar binding affinities of the series.

Three receptors were chosen for these studies; $\text{H}\alpha_{2A}$ -wt (Cys in position 201), $\text{H}\alpha_{2B}$ -wt (Ser in position 177 corresponding to 201), and the mutant $\text{H}\alpha_{2A}\text{Ser201Cys197}$ (Cys in position 197 close to the extracellular surface of the plasma membrane and Ser in position 201 in place of the wild-type Cys 201) (7). Inactivation of the $\text{H}\alpha_{2A}\text{Ser201Cys197}$ indicates that the series of compounds **7a–d** bind to α_2 -ARs in a manner similar to that of CEC, with the quaternary amine group of the tetrahydroisoquinoline core forming an ion pair with Asp113 of the third transmembrane helical element (TM3), and the benzene ring occupying the cavity region formed by TM4–TM5–TM6. Assuming that the methanesulfonylthioalkyl groups of **7a–d** ($n = 3–6$) react with a cysteine thiol group at similar rates and with a similar mode of binding, then the linear relationship from $n = 3$ to $n = 6$ probably reflects a progressive distance approach of the reactive methanesulfonylthioalkyl headgroup toward Cys197. This is interpreted here as locating this residue position about 8.6–14.5 Å from the position of Asp113, with the lower and upper limit corresponding to the average and maximum distances, respectively, indicated by molecular dynamics for compound **7d** (Table 1).

We did not yet explore compounds with a chain length of greater than 6 as already at this length the molecular dynamics showed that for this chain length there is a

significant proportion of folded conformation accessed. Although a rigid side chain would have helped in the interpretation of the results, it is less likely to be accommodated by the receptor through unfavorable steric interactions.

Unlike CEC, none of the **7a–d** series inactivated the human $\text{H}\alpha_{2A}$ -AR, containing Cys201. In our previous study, we found that the relative rate of receptor inactivation by CEC was dependent on the location of the exposed Cys residue in the binding crevice. The Cys residues located close to the extracellular surface of the plasma membrane were alkylated significantly more avidly than those located deeper in the binding site pocket formed between the seven α -helical TM domains of the receptor protein. The $\text{H}\alpha_{2B}$ -wt receptor, which is resistant to the alkylating effect of CEC, was used as a negative control. However, as can be seen in Figure 2, a moderate level of inactivation was observed with the compounds where $n = 5$ and 6. This was possibly a result of covalent coupling to reactive residues in the loop region of the extracellular surface, which includes a cysteine residue in the loop between TM6 and TM7. That binding occurs before inactivation is indicated by comparison with a study on the D2 dopamine receptor by Javitch et al. (14). This study involved introduction of unique cysteine residues into the receptor followed by covalent labeling with a nonspecific cysteine methanesulfonylthio alkylating reagent. Concentrations in the millimolar range were required to cause receptor inactivation as opposed to the micromolar concentrations used in this study.

In summary, we show that the 6-(ω -methanesulfonylthioalkoxy)-2-*N*-methyl-1,2,3,4-tetrahydroisoquinolines have a binding mode similar to that of the human α_{2A} -AR as CEC, the binding mode in each case being supported by covalent coupling. In terms of a molecular yardstick approach, this preliminary study shows that compounds **7a–d** retain affinity at α_{2A} -ARs and that a linear relationship exists between linker length and target site inactivation.

LITERATURE CITED

- (1) Kobilka, B. K., Matsui, H., Kobilka, T. S., Yang-Feng, T. L., Francke, U., Caron, M. G., Lefkowitz, R. J., and Regan, J. W. (1987) Cloning, sequencing, and expression of the gene coding for the human platelet α_2 -adrenergic receptor. *Science* 238, 650–656.
- (2) Lomasney, J. W., Lorenz, L. F., Allen, K., King, K., Regan, J. W., Yang-Feng, T. L., Caron, M. G., and Lefkowitz, R. J. (1990) Expansion of the α_2 -adrenergic receptor family: cloning and characterization of a human α_2 -adrenergic receptor subtype, the gene for which is located on chromosome 2. *Proc. Natl. Acad. Sci. U.S.A.* 87, 5094–5098.
- (3) Regan, J. W., Kobilka, T. S., Yang-Feng, T. L., Caron, M. G., Lefkowitz, R. J., and Kobilka, B. K. (1988) Cloning and expression of a human kidney cDNA for an α_2 -adrenergic receptor subtype. *Proc. Natl. Acad. Sci. U.S.A.* 85, 6301–6305.
- (4) Ruffolo, R. R., Nichols, A. J., Stadel, J. M., and Hieble, J. P. (1993) Pharmacologic and therapeutic applications of α_2 -adrenoceptor subtypes. *Annu. Rev. Pharmacol. Toxicol.* 32, 243–279.
- (5) Marjamäki, A., Luomala, K., Ala-Uotila, S., and Scheinin, M. (1993) Use of recombinant human α_2 -adrenoceptors to characterize subtype selectivity of antagonist binding. *Eur. J. Pharmacol., Mol. Pharmacol. Sect.* 246, 219–226.
- (6) Jansson, C. C., Marjamäki, A., Luomala, K., Savola, J.-M., Scheinin, M., and Åkerman, K. E. O. (1994) Coupling of human α_2 -adrenoceptor subtypes to regulation of cAMP-production in transfected S115 cells. *Eur. J. Pharmacol., Mol. Pharmacol. Sect.* 266, 165–174.

- (7) Marjamäki, A., Pihlavisto, M., Cockcroft, V., Heinonen, P., Savola, J.-M., and Scheinin, M. (1998) Chloroethylclonidine binds irreversibly to exposed cysteines in the fifth membrane spanning domain of the human α_{2A} -adrenergic receptor. *Mol. Pharmacol.* (in press).
- (8) Berglund, P., DeSantis, G., Stabile, M. R., Shang, X., Gold, M., Bott, R. R., Graycar, T. P., Lay, T. H., Mitchinson, C., and Jones, J. B. (1997) *J. Am. Chem. Soc.* 119, 5265–5266.
- (9) McPherson, G. A. (1985) Analysis of radioligand binding experiments. A collection of computer programs for the IBM PC. *J. Pharmacol. Methods* 14, 213–229.
- (10) Halme, M., Sjöholm, B., Savola, J.-M., and Scheinin, M. (1995) Recombinant human α_2 -adrenoceptor subtypes: comparison of [^{3} H]rauwolscine, [^{3} H]atipamezole and [^{3} H]RX821002 as radioligands. *Biochim. Biophys. Acta* 1266, 207–214.
- (11) Helfer, L. (1924) Sur la 6-méthoxy-1,2,3,4-tétrahydro-isoquinoléine. *Helv. Chim. Acta* 7, 945–950.
- (12) Kenyon, G. L., and Bruice, T. W. (1977) Novel sulfhydryl reagents. *Methods Enzymol.* 47, 407–430.
- (13) Stauffer, D. A., and Karlin, A. (1994) Electrostatic potential of the acetylcholine binding sites in the nicotinic receptor probed by reactions of binding-site cysteines with charged methanethiosulfonates. *Biochemistry* 33, 6840–6849.
- (14) Javitch, J., Fu, A., and Chen, J. (1995) Residues in the fifth membrane-spanning segment of the dopamine D2 receptor exposed in the binding-site crevice. *Biochemistry* 34, 16433–16439.

BC970193O

Disulfide-Tethered Solid Supports for Synthesis of Photoluminescent Oligonucleotide Conjugates: Hydrolytic Stability and Labeling on the Support[†]

Harri Salo,* Andrei Guzaev,[‡] and Harri Lönnberg

Department of Chemistry, University of Turku, FIN-20014 Turku, Finland. Received November 10, 1997; Revised Manuscript Received March 11, 1998

Several new disulfide-tethered solid supports (**S1–S5**) were synthesized, and their resistance against ammonolysis was tested. Among these supports, only the one bearing an *N*-[15-[(4,4'-dimethoxytrityl)-oxy]-12,13-dithiapentadecanoyl] linker (**S4b**) tolerated ammonolysis and exhibited properties compatible with the oligonucleotide synthesis by phosphoramidite strategy. The applicability of this disulfide linker structure in postsynthetic oligonucleotide labeling on the support was demonstrated by introduction of two photoluminescent lanthanide chelates or two dansyl groups to the *N*⁴-(6-aminoethyl) amino-modified cytosine residues at the 5' end of the oligonucleotide sequence. Subsequent release of the resulting conjugates as their 3'-phosphates was achieved by reductive cleavage of the disulfide bond and precipitation of the conjugate from the solution with ethanol. The fluorescently tagged oligomer obtained showed hybridization properties similar to those of oligonucleotides labeled in solution.

INTRODUCTION

The introduction of polymerase chain reaction (PCR) (1) has been a launching pad for the development of an increasing number of hybridization assays for the detection of gene mutations. Methods utilizing fluorescent oligonucleotide conjugates have become the subject of particular interest (2–7). Besides organic fluorophores, photoluminescent lanthanide chelates have recently been exploited as markers in hybridization assays (8–13). These chelates offer some obvious advantages over conventional fluorescent dyes: (i) the luminescence lifetime is long (≈ 1 ms), allowing utilization of time-resolved measurement, (ii) the difference between the wavelength of excitation and main emission is large, (iii) the emission bands are narrow, and (iv) the concentration quenching is almost negligible (14). The most serious drawback is the rather laborious preparation of the oligonucleotide conjugates. Usually an oligonucleotide bearing a nucleophilic functionality, such as an aliphatic amino group, is reacted in solution with an isothiocyanatophenyl or 4,6-dichloro-1,3,5-triazin-2-ylaminophenyl group of the chelate (15). This necessarily leads to somewhat complicated chromatographic purifications. To simplify the purifications and to increase the efficiency of labeling, Kwiatkowski et al. (16) have prepared phosphoramidite building blocks that are compatible with the automated solid-phase synthesis of oligonucleotides and bear the desired ligand of the photoluminescent chelate in a fully protected form. After chain assembly and deprotection of the ligand and base moieties, the oligomer is released in solution, and the lanthanide ion is introduced by treat-

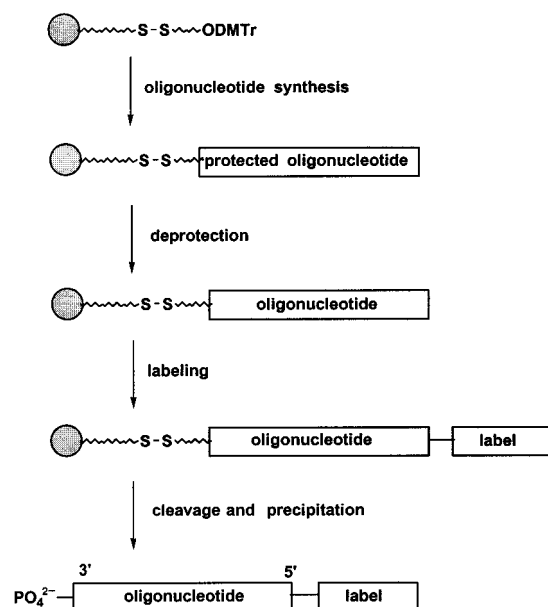
ment with lanthanide citrate. The final purification then includes desalting on NAP-10 Sephadex and RP FPLC.

While the labeling method described above undoubtedly is highly useful, it still depends on synthesis of a separate nucleosidic phosphoramidite building block for each luminescent chelate used as a marker. For some purposes, coupling of an activated chelate to a functional group of the oligomer that is still bound to the support would constitute a more versatile strategy. Obviously, the latter approach put high demands for the linker tethering the 3'-terminal nucleoside to the solid support. It should withstand, in addition to the conditions of the chain assembly, deprotection of the completed oligomer and reaction of the activated chelate with the appropriate conjugate group on the oligomer. Both of these steps are usually carried out under rather basic conditions. Furthermore, the linker should undergo cleavage under such mild conditions that the labeled oligomer is not modified upon the release in solution, and the final purification remains as simple as possible. Several linkers that allow synthesis of fully deprotected oligonucleotide on solid support and postsynthetic derivatization have been introduced, but in most cases their cleavage requires rather drastic conditions (17, 18). Disulfide-based tethers, however, may be expected to meet the requirements of solid-phase labeling with lanthanide chelates. They have been successfully used in automated oligonucleotide synthesis (19–24); they withstand basic conditions rather well, and they readily undergo reductive cleavage. Unfortunately, no data on postsynthetic transformation reactions of disulfide-immobilized oligonucleotides appear to exist. We now report on derivatization of polymeric solid supports (**S1–S5**) with a number of disulfide linkers. The stability of these linkers under basic conditions has been compared, and the best candidates (**S4b**) have been used in labeling of the *N*⁴-(6-aminoethyl)cytosine residues of the support-bound oligonucleotide with a 4,6-dichloro-1,3,5-triazin-2-ylaminophenyl-acti-

[†] Preliminary results published in a congress abstract: Salo, H., Guzaev, A., Azhaye, A., and Lönnberg, H. (1996) *Collect. Czech. Chem. Commun.* 61 (special issue), S110–S111.

* Author to whom correspondence should be addressed. Telephone: +358-2-333 8092. Fax: +358-2-333 6770. E-mail: harri.salo@utu.fi.

[‡] Current address: Isis Pharmaceuticals, Carlsbad, CA.

Scheme 1. General Scheme for Labeling of Oligonucleotides on the Support

vated europium(III) chelate. The general protocol of this idea is presented in Scheme 1.

EXPERIMENTAL PROCEDURES

General. The reagents of oligonucleotide synthesis were products of Cruachem and Glen Research. The amino-modified polystyrene and Tentagel supports were from Applied Biosystems Inc. *N*⁴-(*N*-Trifluoroacetyl-6-aminoethyl)-2'-deoxycytidine 3'-(*N,N*-diisopropyl-*O*-cyanoethyl)phosphoramidite (**13**) and the photoluminescent europium chelate {2,2',2'',2'''-[4'-{4'''-[(4,6-dichloro-1,3,5-triazin-2-yl)amino]phenyl}-2,2':6',2''-terpyridine-6,6''-diyl]-bis(methylenenitrilo)}tetrakis(acetato)}europium(III) were received from the laboratory of Wallac Oy. Adsorption column chromatography was performed on Silica Gel 60 (Merck). All the other reagents were purchased from Aldrich. NMR spectra were recorded on a JEOL GX-400 spectrometer operating at 399.8 and 100.5 MHz for ¹H and ¹³C, respectively. The oligonucleotides were analyzed by ion-exchange and RP HPLC (columns, Synchropak AX-300, 4.0 mm × 250 mm, Synchrom Inc., for the ion exchange, and Hypersil ODS 5 μm, 4.0 mm × 250 mm and Nucleosil 300-5C18, 4.0 mm × 250 mm, Macherey-Nagel, for the RP HPLC). Elemental analyses were performed on a Perkin-Elmer 2400 Series II instrument.

2-[(4,4'-Dimethoxytrityl)oxy]ethyl 2-Pyridyl Disulfide (5). Bis(2-pyridyl) disulfide (1.1 mmol, 250 mg) was dissolved in a mixture of pyridine and MeOH (10 mL, 1:99, v/v). 2-Mercaptoethanol (1.0 mmol, 72 μL) was added dropwise to the stirred solution, and the mixture was left to stand at ambient temperature until no 2-mercaptoethanol could be detected by TLC. The reaction mixture was evaporated to dryness and coevaporated three times with dry pyridine. The residue (**4**) was dissolved in dry pyridine (10 mL); 4,4'-dimethoxytrityl chloride (1.1 mmol, 375 mg) was added, and the reaction mixture was left to stand overnight at ambient temperature. The reaction was stopped by adding MeOH (0.5 mL) and aqueous NaHCO₃ (5 mL of a 5% solution). The mixture was evaporated to an oil in vacuo, dissolved in CH₂Cl₂ (40 mL), washed with water (2 × 20 mL), and dried over Na₂SO₄. The solvent was removed in vacuo, and **5** was separated on a silica gel column (30 mm ×

100 mm), eluting with a stepwise gradient system of CH₂-Cl₂ in hexane containing 0.1% pyridine (from 75 to 100% CH₂Cl₂). The overall yield was 81%. ¹H NMR (CDCl₃; δ, ppm): 8.41 (1H, m, Pyr H6), 7.68 (1H, m, Pyr H4), 7.54 (1H, m, Pyr H5), 7.43 (2H, m, DMTr), 7.32–7.15 (6H, m, DMTr), 7.04 (1H, m, Pyr H3), 6.83–6.78 (5H, m, DMTr), 3.77 (6H, s, OMe), 3.38 (2H, t, *J*³ = 6.4 Hz, DMTrOCH₂), 2.94 (2H, t, *J*³ = 6.4 Hz, PyrSSCH₂). TLC: *R*_f = 0.88 (19:1 CH₂Cl₂/MeOH), 0.62 (49:1 CH₂Cl₂/MeOH). MS: *m/z* at 489 (M⁺).

3-[(4,4'-Dimethoxytrityl)oxy]propyl 2-Pyridyl Disulfide (8). Bis(3-hydroxypropyl) disulfide (**25**) (5.76 mmol, 1.05 g) was coevaporated with pyridine (3 × 20 mL) and dissolved in dry pyridine (50 mL). 4,4'-Dimethoxytrityl chloride (10.2 mmol, 3.45 g) in pyridine (50 mL) was added to the stirred solution and the mixture left to stand overnight at ambient temperature. The resulting **6** was worked up as described for **5** and used without further purification. **6** (0.6 g, 0.8 mmol) was dissolved in CH₂Cl₂ (10 mL), and methanolic DTT (0.8 mmol in 3 mL) was added. The reductive cleavage of **6** to **7** was followed by TLC. The reaction mixture was evaporated, dissolved in CH₂Cl₂, washed with water, dried, and concentrated to an oil. The oil was immediately added to a solution of bis(2-pyridyl) disulfide (1.6 mmol, 0.35 g) in MeOH (10 mL). After being stirred for 4 days at ambient temperature, the reaction mixture was evaporated to an oil and dissolved in a mixture of hexane and CH₂Cl₂ (25:75) containing 0.1% pyridine. **8** was isolated on a silica gel column (30 mm × 100 mm), eluting with a gradient of CH₂Cl₂ (75 to 100%) in hexane. The overall yield was 40%. ¹H NMR (CDCl₃; δ, ppm): 8.44 (1H, m, Pyr H6), 7.68 (1H, m, Pyr H4), 7.59 (1H, m, Pyr H5), 7.41 (2H, m, DMTr), 7.30–7.16 (6H, m, DMTr), 7.06 (1H, m, Pyr H3), 6.84–6.80 (5H, m, DMTr), 3.78 (6H, s, MeO), 3.14 (2H, t, *J*³ = 7.3 Hz, DMTrOCH₂), 2.93 (2H, t, *J*³ = 6.0 Hz, PyrSSCH₂), 1.98 (2H, q, *J*³ = 7.3 Hz CH₂CH₂CH₂). TLC: *R*_f = 0.88 (19:1 CH₂Cl₂/MeOH), 0.60 (49:1 CH₂Cl₂/MeOH). MS: *m/z* at 503 (M⁺).

15-[(4,4'-Dimethoxytrityl)oxy]-12,13-dithiapentadecanoic Acid (11). To a solution of thiolacetic acid (13 mmol, 1.0 g) in dioxane (1 mL) was added a few crystals (1–2 mg) of dibenzoylperoxide. The solution was mixed with a solution of 10-undecenoic acid (6.6 mmol, 1.21 g) in dioxane (20 mL), and the mixture was stirred overnight at ambient temperature. The completeness of the reaction was checked by TLC; water (20 mL) was added, and the mixture was acidified with aqueous hydrogen chloride. Extraction with ethyl acetate (2 × 30 mL) gave 11-acetylthioundecanoic acid (**9**) as a white solid (1.63 g, 95%). Anal. (C₁₃H₂₄O₃S): C, H, S. ¹H NMR (CDCl₃; δ, ppm): 2.86 (2H, t, *J*³ = 7.4 Hz, SCH₂CH₂), 2.35 (2H, t, *J*³ = 7.4 Hz, CH₂COOH), 2.32 (3H, s, SC(=O)CH₃), 1.63 (2H, q, *J*³ = 7.4 Hz, CH₂CH₂COOH), 1.56 (2H, q, *J*³ = 7.0 Hz, SCH₂CH₂), 1.31 (12H, br m, (CH₂)₆CH₂COOH). TLC: *R*_f = 0.60 (1:2 EtOAc/hexane).

9 (0.50 g, 1.9 mmol) was dissolved in 20 mL of MeOH and deacetylated with an excess of 40% aqueous methanamine (5 mL), evaporated to dryness, and dissolved in water (30 mL). The pH was adjusted to 2 with aqueous hydrogen chloride, and the deprotected 11-mercaptopentadecanoic acid (**10**) was extracted in diethyl ether (3 × 30 mL) and dried with Na₂SO₄. From the crude **10**, a sample (0.070 g, 0.32 mmol) was then dissolved in a mixture of pyridine and MeOH (1:9, 0.9 mL) and added to a solution of **5** (0.2 g, 0.4 mmol) dissolved in a mixture of MeOH and MeCN (1:1, 5 mL), and the solution was stirred at room temperature overnight. The evaporation residue was dissolved in CH₂Cl₂ (5 mL) containing 5%

Et₃N and fractionated on a silica gel column, using a stepwise gradient of MeOH (0 to 5%) in Et₃N/CH₂Cl₂ (5:95) as an eluent. The yield of **11** (starting from **9**) as a triethylammonium salt was 0.15 g (72%). ¹H NMR-(CDCl₃; δ , ppm): 7.46–6.78 (13H, m, DMTr), 3.79 (6H, s, OCH₃), 3.35 (2H, t, J = 7.5 Hz, OCH₂CH₂), 2.85 (2H, t, J = 6.4 Hz, CH₂CH₂S), 2.56 (2H, t, J^3 = 7.7 Hz, CH₂-COO), 2.29 (2H, t, J^3 = 7.3 Hz, SSCH₂), 1.60 (2H, br m, SSCH₂CH₂), 1.25–1.40 (14 H, br m, 7 $\times$ CH₂). The spectrum also exhibited signals of the triethylammonium group. TLC: R_f = 0.74 (5% Et₃N in 9:1 CH₂Cl₂/MeOH), R_f = 0.33 (5% Et₃N in 19:1 CH₂Cl₂/MeOH).

N-[7-[(4,4'-Dimethoxytrityl)oxy]-4,5-dithiaheptanoyl] Supports (S1a–c). Long-chain aminoalkyl controlled pore glass (LCAA-CPG) was treated overnight with 3% dichloroacetic acid in CH₂Cl₂, subjected to successive washings with CH₂Cl₂, 20% Et₃N in MeOH, MeOH, and Et₂O, and finally dried in vacuo. Polystyrene and Tentagel supports were used as received. The supports were suspended in dry pyridine and degassed under reduced pressure (water pump). *N*-Hydroxysuccinimide (2–3 mmol) and 3,3'-dithiodipropionic acid (1–2 mmol, 20–100-fold excess with respect to the support-bound amino groups) were dissolved in pyridine, dried by coevaporation with pyridine, and dissolved again in a minimum amount of pyridine. The resulting solution was added to the suspension of the support in pyridine, and *N,N'*-diisopropylcarbodiimide (2–3 mmol) and a catalytic amount of 4-(dimethylamino)pyridine were added. The suspension was shaken overnight, and the support was washed on a filter with pyridine, CH₂Cl₂, MeOH, and Et₂O. The support dried in the air was suspended in methanol and degassed. DTT (0.30 g, 1.9 mmol) and 30 μ L of a 0.3 mol L⁻¹ solution of Et₃N in methanol were added, and the solution was shaken for several hours. After the mixture was washed with methanol, **5** (0.45 g) was immediately added to the suspension of the support in a mixture of MeOH and MeCN (1:1, 5 mL), and the suspension was shaken overnight. The support was washed on the filter with MeOH. The unreacted amino groups of the support (**S1a–c**) were finally capped by treating the support for 3 $\times$ 10 min with a 1:1 mixture of solutions A and B (A is 1:1:8 acetic anhydride/2,6-lutidine/THF and B is 16:84 *N*-methylimidazole/THF). The loading of the washed and dried support was determined by the dimethoxytrityl cation assay (26). The loadings ranged from 8 to 33 μ mol g⁻¹.

N-[8-[(4,4'-Dimethoxytrityl)oxy]-4,5-dithiaoctanoyl] Supports (S2a–c). These supports were prepared as described for **S1a–c** by using **8** instead of **5**.

N-[16-[(4,4'-Dimethoxytrityl)oxy]-12,13-dithiahexadecanoyl] Supports (S5a,b). 10-Undecenoic acid was attached to amino-modified polystyrene or Tentagel support by the *N*-hydroxysuccinimide and *N,N'*-diisopropylcarbodiimide assisted acylation as described above for supports **S1a–c**. The *N*-acylated support was suspended in dioxane, and thiolacetic acid diluted with dioxane (1:1, 800 μ L) and a few crystals (1–2 mg) of dibenzoylperoxide were added. The suspension was shaken overnight, washed with dioxane, and dried on the filter. The *S*-acetyl group was removed with 1 mol L⁻¹ butylamine in MeOH, and the released mercapto function was coupled with **8** and capped to give supports **S5a,b**, as described for supports **S1a–c**. The loading determined by the dimethoxytrityl cation assay ranged from 7 to 16 μ mol g⁻¹.

N-[15-[(4,4'-Dimethoxytrityl)oxy]-12,13-dithiapentadecanoyl] Supports (S4a,b). These supports were

prepared as described for supports **S5a,b** by using **5** instead of **8**. The support **S4b** was also obtained in two alternative manners. First, the Tentagel support was acylated with the triethylammonium salt of **11**, according to the procedure described for supports **S1a–c** and capped. Second, the Tentagel support was acylated with 11-acetylthioundecanoic acid (**9**), deacetylated with 1 mol L⁻¹ aqueous butylamine, reacted with **5**, and capped.

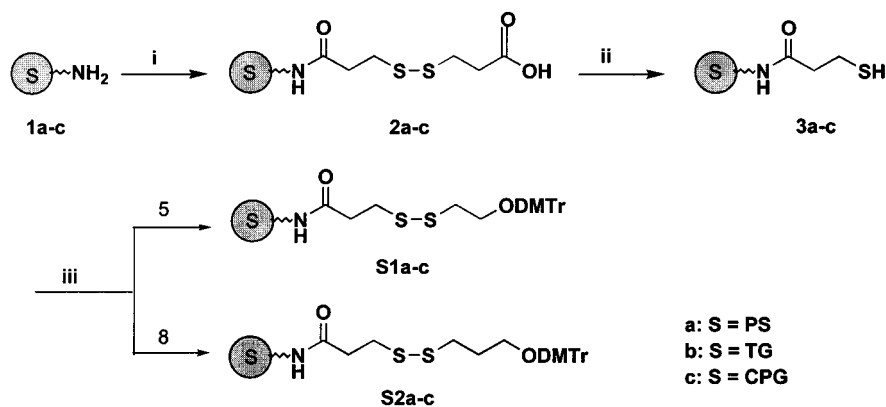
N-[10-[(4,4'-Dimethoxytrityl)oxy]-7,8-dithiadecanoyl] Support (S3). The support was prepared as described for supports **S5a,b** by using 5-hexenoic acid instead of 10-undecenoic acid and **3** instead of **7**.

Determination of the Hydrolytic Stability of S1–S5. The loading of each batch of **S1–S5** was determined by the dimethoxytrityl cation assay. The supports were then weighed into a synthesizer column and subjected to ammonolysis according to the protocol of manual deprotection and cleavage by a double-syringe method (27). The columns were kept at room temperature for 24 or 48 h. At the end of this period, the ammonia was withdrawn and the support was washed with water, aqueous MeCN, and MeCN, and dried. The dimethoxytrityl cation assay was repeated.

Oligodeoxynucleotide Synthesis. Supports **S4b** were used as supports in the automated oligonucleotide synthesis. The protected oligonucleotides were assembled on an Applied Biosystems 392 DNA synthesizer in 0.2 and 1.0 μ mol scales, using the standard phosphoramidite protocol and unaltered coupling times even when the modified building block (**13**) was introduced (Scheme 6). The support-bound oligonucleotides were deprotected under standard deprotection conditions: treatment with concentrated ammonia (33%) at 55 °C for 8 h or at room temperature for 48 h. The support, still bearing the deprotected oligonucleotide, was washed with water and acetonitrile and dried.

Labeling of the Oligonucleotides on the Solid Support. An appropriate amount of the dried support, containing ~2 OD of oligomer on 0.5 mg of the support, was weighed in a microcentrifuge tube, and the labeling reagent, either 5-(dimethylamino)-1-naphthalenesulfonyl chloride (DNSCl) (7.7 mg, 0.28 μ mol) or **14** (3.0 mg, 3.2 μ mol), from Scheme 6, in 0.05:1:2.95 DBU/MeCN/H₂O (50 μ L, v/v/v) was added (28). The tubes were shaken from 16 to 48 h in the dark at ambient temperature. The solid phase was filtered and washed with water and acetonitrile. The labeled oligonucleotides were detached from the supports with slightly basic 0.5 mol L⁻¹ aqueous DTT (100 μ L, a 3 μ mol L⁻¹ Et₃N/methanol solution in 1 mL of aqueous DTT or DTT in 0.1 mol L⁻¹ Tris buffer at pH 8.4). The reaction was performed either in a synthesizer column by a double-syringe method or in a 1.5 mL microcentrifuge tube. The support was removed by filtration and rinsed with water, aqueous MeCN, and MeCN. The filtrate and washings were combined and evaporated to dryness on a SpeedVac or rotary evaporator. The labeled oligonucleotide conjugate was precipitated with EtOH, and the quality and identity of the product were verified by HPLC. In the case of labeling with **14**, the extent of labeling was determined by releasing Eu(III) ion in solution with a fluorescence enhancement solution and measuring the fluorescence intensity on a time-resolved fluorometer against a europium(III) chloride standard solution (Delfia protocol) (29).

Hybridization Experiments. The hybridization experiments were carried out as described previously (30).

Scheme 2^a

^a (i) 3,3'-Dithiodipropionic acid/*N*-hydroxysuccinimide/*N,N'*-diisopropylcarbodiimide/Pyr/DMAP; (ii) DTT/MeOH/Et₃N; (iii) for **S1a–c**, **5**/MeOH/MeCN; for **S2a–c**, **8**/MeOH/MeCN. DTT, 1,4-dithio-D,L-threitol; DMAP, *N,N*-(dimethylamino)pyridine.

Table 1. Results of the DMTr Cation Assay of the Supports before and after Hydrolysis

Symbol	Support material	Linker structure	loading before NH ₃ -treatment μmol/g	loading after NH ₃ -treatment μmol/g (% of original)	treatment time/h
S1a	Polystyrene		7.9	5.4 (68)	24
S1b	Tentagel	"	33	15 (45)	24
S1c	CPG	"	13	3 (23)	24
S2a	Polystyrene		3.2	2.4 (75)	24
S2b	Tentagel	"	50	23 (46)	24
S2c	CPG	"	32	12 (38)	24
S3	Tentagel		43	33 (77)	48
S4a	Polystyrene		3.3	3.8 (100)	48
S4b ^a	Tentagel	"	14	13 (93)	48
S4b ^b	Tentagel	"	19	16 (84)	48
S4b ^c	Tentagel	"	41	41 (100)	48
S5a	Polystyrene		6.8	6.3 (93)	48
S5b	Tentagel	"	16	15 (94)	48

^a From Scheme 4. ^b From Scheme 5, route A. ^c Scheme 5, route B.

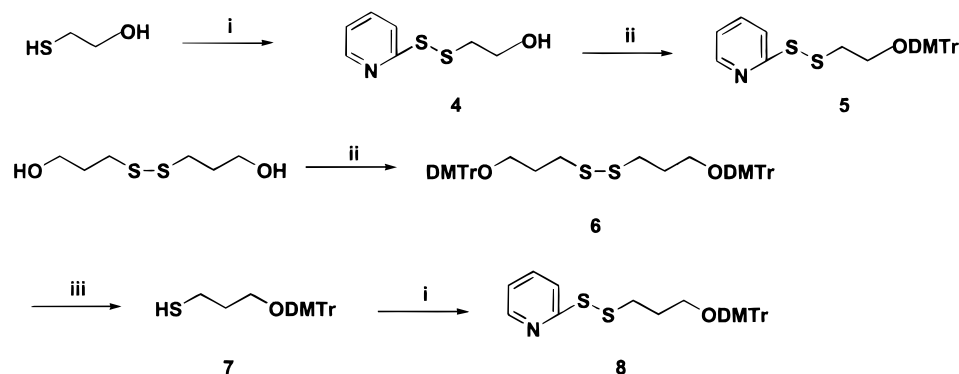
RESULTS AND DISCUSSION

Synthesis of the Supports. Solid supports **S1** and **S2** were prepared as depicted in Scheme 2. Accordingly, the amino functions of polystyrene, Tentagel, and LCAA CPG supports were first acylated with 3,3'-dithiodipropionic acid; the disulfide bond was reductively cleaved with DTT, and the released mercapto function was reacted with either 2-[(4,4'-dimethoxytrityl)oxy]ethyl 2-pyridyl disulfide (**5**; supports **S1a–c**) or 3-[(4,4'-dimethoxytrityl)oxy]propyl 2-pyridyl disulfide (**8**; supports **S2a–c**). The loadings determined by the dimethoxytrityl cation method are listed in Table 1. The 2-pyridyl disulfide-activated ω-[(4,4'-dimethoxytrityl)oxy]thiols, **5** and **8**, were prepared from 2-mercaptoethanol and bis-(3-hydroxypropyl) disulfide, as described in Scheme 3.

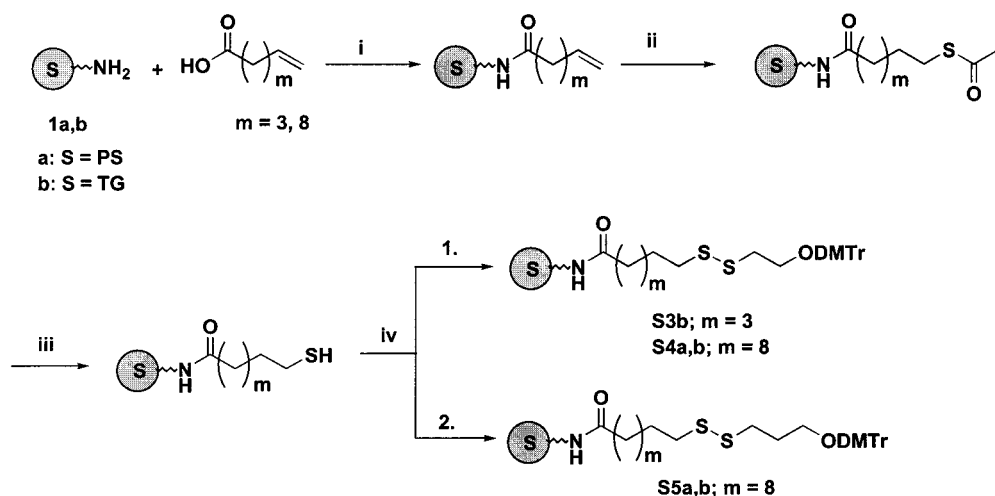
The rest of the supports employed (**S3–S5**) were obtained via initial acylation with ω-alkenoic acid, followed by radical addition of thiolacetic acid to the terminal double bond (**31**) (Scheme 4). The *S*-acetyl

group was then removed by aminolysis with butylamine, and the released mercapto group was reacted with **5**. The Tentagel-based support **S4b** was also prepared in two alternative manners shown in Scheme 5. First, the disulfide linker (**11**) was synthesized in solution and attached to the amino-modified Tentagel by an *N*-acylation assisted by *N,N*-diisopropylcarbodiimide, *N*-hydroxysuccinimide, and 4-(dimethylamino)pyridine. Second, 11-(acetylthio)undecanoic acid (**9**) was prepared by radical addition in solution, and only the aminolysis and coupling with **5** were performed on the support (for the loadings, see Table 1).

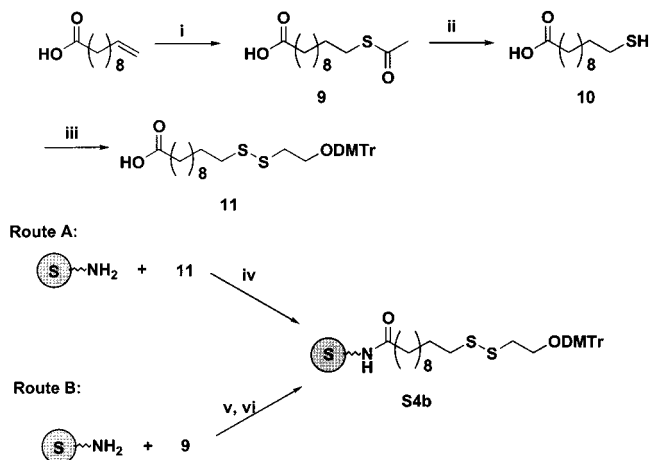
Hydrolytic Stability of the Disulfide-Tethered Supports (S1–S5). To evaluate the applicability of the supports in machine-assisted oligonucleotide synthesis, they were subjected to ammonolysis. According to the standard protocol, the base moiety protections are removed by treating the support-bound oligomer with concentrated aqueous ammonia for 48 h at room tem-

Scheme 3^a

^a (i) Bis(2-pyridyl) disulfide/MeOH/Pyr; (ii) DMTTr-Cl/Pyr; (iii) DTT/MeOH/Et₃N.

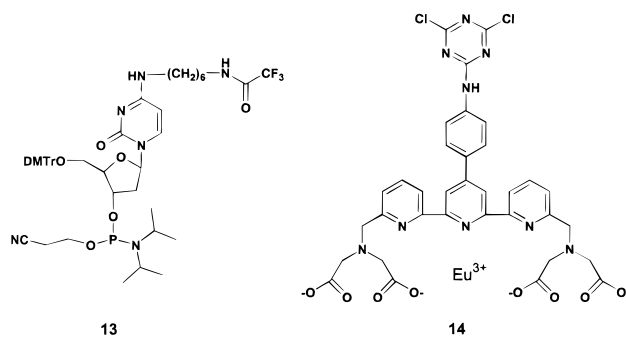
Scheme 4^a

^a (i) *N*-Hydroxysuccinimide/*N,N'*-diisopropylcarbodiimide/Pyr/DMAP; (ii) CH₃COSH/dioxane/dibenzoylperoxide; (iii) 1 M *n*-BuNH₂/MeOH; (iv) for **S3b** and **S4a,b**, **5**/MeOH/MeCN; for **S5a,b**, **8**/MeOH/MeCN.

Scheme 5^a

^a (i) CH₃COSH/dioxane/dibenzoylperoxide; (ii) aqueous MeNH₂/MeOH; (iii) **5**/MeOH/Pyr; (iv) *N*-hydroxysuccinimide/*N,N'*-diisopropylcarbodiimide/Pyr/DMAP; (v) 1 M *n*-BuNH₂/MeOH; (vi) **5**/MeOH/Pyr.

perature or for 8 h at 55 °C. We applied the double-syringe method for 24–48 h at room temperature. Table 1 records the dimethoxytrityl loadings before and after this ammonolysis. These results indicate that the material of the support is of considerable importance. The loading of the CPG supports, **S1c** and **S2c**, decreased dramatically during the ammonolysis, which in all likelihood may be attributed to partial dissolution of glass.

Scheme 6. Structures of the Modified Phosphoramidite **13** and the Photoluminescent Lanthanide Chelate **14**

Another important factor appears to be the length of the linker molecule; the resistance to ammonolysis increases with the length of the hydrocarbon chain connecting the disulfide group to the carbonyl carbon of the linker. Supports containing a dimethylene bridge are considerably more labile than those containing a nonamethylene bridge. This difference in stability is most clearly seen with the Tentagel-based supports **S2b** and **S5b**. Evidently, the carbonyl function and the disulfide group must be separated by at least three carbon atoms to prevent β -elimination. The distance between the disulfide linkage and the dimethoxytrityloxy group seems to be of lesser importance.

Synthesis of Oligonucleotides on Supports. A model sequence of T₆ was synthesized on those supports

that showed considerable resistance toward ammonolysis. The support-bound oligos were then subjected to ammonia treatment, and after 48 h the ammonia was washed from the supports and collected. These washings were then investigated by ion-exchange HPLC. The oligonucleotide that remained bound to the support was finally released with DTT under mildly basic conditions. The oligomer content of the washings was again determined by HPLC. The general conclusion of these studies was that assembly of an oligonucleotide on the deprotected hydroxy function of the supports still decreased the stability of the disulfide bond toward ammonolysis. Support **S4b**, in particular the batches obtained by route B in Scheme 5, exhibited the desired properties and was selected for further studies. More than 85% of the assembled oligomer remained bonded to the support after ammonolysis and could be released in 84% yield by subsequent reductive cleavage. Furthermore, the released oligomer was more homogeneous than with the other supports tried.

Support **S4b** prepared by route B in Scheme 5 was used to assemble a 15-mer oligonucleotide, 5'-d(GAA-CATCATGGTTCGT)-3', and ammonolyze it in the dimethoxytrityl-on mode. According to RP HPLC, 10% of the full-length tritylated sequences were released during ammonolysis and 90% by the DTT treatment. The latter treatment also resulted in cleavage of the *O*-(2-mercaptoethyl) from the 3'-terminal phosphate group of the released oligomer. This reaction has been suggested to proceed by an intramolecular nucleophilic displacement of the phosphate group by the mercapto function (32). That this really happened under the conditions employed was verified by comparing a dT₆ sequence prepared on **S4b** to an authentic sample of 3'-phosphorylated dT₆ synthesized on a commercially available universal solid support producing oligonucleotide 3'-phosphates. These two materials exhibited identical retention times on ion-exchange HPLC, and when co-injected, gave only a single peak.

Labeling of Oligodeoxyribonucleotides on the Support. The labeling experiments were carried out with an 18-mer sequence, 5'-d(X₂ATATCATCTTTGGTGT)-3', assembled on **S4b** [X = N⁴-(6-aminohexyl)-2'-deoxycytidine] (33). The synthesis gave an average stepwise yield of 99%, calculated by the dimethoxytrityl cation release after the ammonia treatment. The free aliphatic amino groups of the 5'-terminal N⁴-aminoalkylated cytosine bases were labeled either with a prompt fluorophor, 5-(dimethylamino)-1-naphthalenesulfonyl chloride, or a dichlorotriazine derivative of a photoluminescent europium(III) chelate. The appropriate labeling reagent was dissolved in a minimum amount (<50 μ L) of a mixture of DBU [1,8-diazabicyclo(5,4,0)undec-7-ene], water, and MeCN (0.05:1:2.95 v/v/v) and added to 0.5 mg of support-bound oligo (~2 OD) in a microcentrifuge tube. After incubation for 16 h in a shaker, the excess of the reagents was washed, and the support-bound, labeled oligonucleotides were released into solution with dithiothreitol. An ethanol precipitation of the oligonucleotides gave a DTT-free fraction of the labeled oligonucleotide. The extent of labeling of this oligonucleotide conjugate was determined according to the Delfia protocol (29). Accordingly, the europium(III) ion was released in solution and quantified against europium chloride standards. The amount of oligonucleotide was determined spectrophotometrically at 260 nm. The extent of labeling was 1.6, i.e., about 80% of the theoretical value, 2. The presence of the prompt fluorophor (dansyl) was, in turn, verified by measuring the UV spectrum of the ethanol-

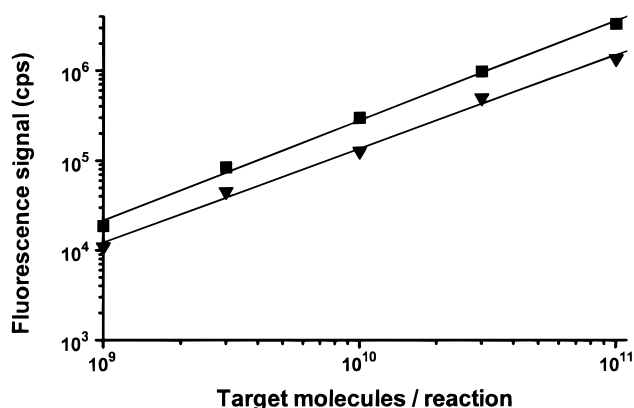


Figure 1. Sandwich hybridization on microparticles bearing a sequence complementary to labeled oligonucleotide. The fluorescence signal is measured from one particle as a function of target molecules added to the hybridization reaction mixture. A comparison of in-solution labeled oligo containing five chelates per molecule (line with squares) and solid-phase labeled oligo (maximum of two chelates per oligo) (line with triangles).

precipitated labeled oligo against water. The observed ratio of the absorbance at 360 nm (DNS moiety) and at 260 nm (base moieties) agreed with the almost theoretical extent of labeling. Additionally, the presence of a DNS oligoconjugate was verified with RP HPLC.

Hybridization Properties of the Multilabeled Oligonucleotides. The identity of the oligomer conjugate derived from the photoluminescent europium chelate was further verified by performing two parallel hybridization assays by the previously reported technique based on time-resolved fluorescence detection on microparticles (30). In one of the experiments, an oligonucleotide conjugate prepared in the present work was used. The signal intensity was then compared to that observed when an oligomer labeled previously in solution was used in the same assay (30). As seen from Figure 1, the ratio of the two sets of signals is consistent with the determined extent of labeling.

In summary, the methodology described in the present paper allows preparation of satisfactorily pure oligonucleotide 3'-monophosphates labeled with a photoluminescent europium chelate to the amino groups of N⁴-(aminoalkyl)cytosine residues. The chain assembly and labeling take place on a single solid support, and the resulting conjugate may be purified simply by washing, release in solution, and precipitation with ethanol.

ACKNOWLEDGMENT

The polystyrene and Tentagel particles were a generous gift of Dr. Alex Andrus (Applied Biosystems Inc.). The photoluminescent europium chelate was prepared in the Laboratory of Wallac Oy (Turku, Finland) by Dr. Veli-Matti Mikkala. The authors also thank Mr. Harri Hakala, M.Sc., for the hybridization measurements. Financial support from the Research Council for Natural Sciences, the Academy of Finland, is gratefully acknowledged.

LITERATURE CITED

- (1) Saiki, R., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B., and Erlich, H. A. (1988) Primer directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* 239, 487–491.
- (2) Pochet, S., Huynh-Dinh, T., and Igolen, J. (1987) Synthesis of DNA fragments linked to a solid support. *Tetrahedron* 43, 3481–3490.

- (3) Urdea, M., Warner, B., Running, J., Stempien, M., Clyne, J., and Horn, T. (1988) A comparison of nonradioisotopic hybridization assay methods using fluorescent, chemiluminescent and enzyme labeled oligonucleotide probes. *Nucleic Acids Res.* 16, 4937–4956.
- (4) Chehab, F., and Wall, J. (1992) Detection of multiple cystic fibrosis mutations by reverse dot blot hybridization: a technology for carrier screening. *Hum. Genet.* 89, 163–168.
- (5) Abel, A. P., Weller, M. G., Duveneck, G. L., Ehrat, M., and Widmer, H. M. (1996) Fiber-optic evanescent wave biosensor for the detection of oligonucleotides. *Anal. Chem.* 68, 2905–2912.
- (6) Cronin, M. T., Fucini, R. V., Kim, S. M., Masino, R. S., Wespi, R. M., and Miyada, C. G. (1996) Cystic fibrosis mutation detection by hybridization to light-generated DNA probe arrays. *Hum. Mutat.* 7, 244–255.
- (7) Mansfield, E. S., Worley, J. M., McKenzie, S. E., Surrey, S., Rappaport, E., and Fortina, P. (1995) Nucleic acid detection using nonradioactive labeling methods. *Mol. Cell. Probes* 9, 145–156.
- (8) Hakala, H., Heinonen, P., Iitiä, A., and Lönnberg, H. (1997) Detection of oligonucleotide hybridization on a single microparticle by time-resolved fluorometry: Hybridization assays on polymer particles obtained by direct solid phase assembly of the oligonucleotide probes. *Bioconjugate Chem.* 8, 378–384.
- (9) Dickson, E. F., Pollack, A., and Diamandis, E. P. (1995) Time-resolved detection of lanthanide luminescence for ultrasensitive bioanalytical assays. *J. Photochem. Photobiol. B* 27, 3–19.
- (10) Prat, O., Lopez, E., and Mathis, G. (1991) Europium(III) cryptate: a fluorescent label for the detection of DNA hybrids on solid support. *Anal. Biochem.* 195, 283–289.
- (11) Hurskainen, P., Dahlén, P., Ylikoski, J., Siitari, H., and Lövgren, T. (1991) Preparation of europium-labeled DNA probes and their properties. *Nucleic Acids Res.* 19, 1057–1061.
- (12) Lopez, E., Chypre, C., Alpha, B., and Mathis, G. (1993) Europium(III) trisbipyridine cryptate label for time-resolved fluorescence detection of polymerase chain reaction products fixed on a solid support. *Clin. Chem.* 39, 196–201.
- (13) Bush, C., Di Michele, L., Peterson, R., Sherman, D., and Godsey, J. (1992) Solid-phase time-resolved fluorescence detection of human immunodeficiency virus polymerase chain reaction amplification products. *Anal. Biochem.* 202, 146–151.
- (14) Soini, E., and Hemmilä, I. (1979) Fluoroimmunoassay: present status and key problems. *Clin. Chem.* 25, 353–361.
- (15) Lövgren, T., Iitiä, A., Hurskainen, P., and Dahlen, P. (1995) Detection of lanthanide chelates by time-resolved fluorescence. In *Blotting and Sequencing* (L. Kricka, Ed.) pp 350–358, Academic Press, San Diego, CA.
- (16) Kwiatkowski, M., Samiotaki, M., Lamminmäki, U., Mukkala, V.-M., and Landegren, U. (1994) Solid-phase synthesis of chelate-labeled oligonucleotides: application in triple-color ligase-mediated gene analysis. *Nucleic Acids Res.* 22, 2604–2611.
- (17) Sproat, B. S., and Brown, D. M. (1985) A new linkage for solid-phase synthesis of oligodeoxyribonucleotides. *Nucleic Acids Res.* 13, 2979–2987.
- (18) Stengele, K. P., and Pfeleiderer, W. (1990) Improved synthesis of oligodeoxyribonucleotides. *Tetrahedron Lett.* 31, 2549–2552.
- (19) Kumar, P., Bose, N. K., and Gupta, K. C. (1991) A versatile solid-phase method for the synthesis of oligonucleotide-3'-phosphates. *Tetrahedron Lett.* 32, 967–970.
- (20) Zuckerman, R., Corey, D., and Schultz, P. (1987) Efficient methods for attachment of thiol specific probes to the 3'-ends of synthetic oligodeoxyribonucleotides. *Nucleic Acids Res.* 15, 5305–5321.
- (21) Gupta, K. C., Sharma, P., Sathyanarayana, S., and Kumar, P. (1990) A universal solid support for the synthesis of 3'-thiol group containing oligonucleotides. *Tetrahedron Lett.* 31, 2471–2474.
- (22) Bonfils, B., and Thuong, N. T. (1991) Solid-phase synthesis of 5',3'-bifunctional oligodeoxyribonucleotides bearing a masked thiol group at the 3'-end. *Tetrahedron Lett.* 32, 3053–3056.
- (23) Asseline, U., and Thuong, N. T. (1990) New solid-phase for automated synthesis of oligonucleotides containing an amino-alkyl linker at their 3'-end. *Tetrahedron Lett.* 31, 81–84.
- (24) Kumar, A. (1994) Development of suitable linkage for oligonucleotide synthesis and preliminary hybridization studies on oligonucleotides synthesized in situ. *Nucleosides Nucleotides* 13, 2125–2134.
- (25) Guzaev, A. (1991) An efficient synthesis of 3'-mercaptoalkylated oligodeoxyribonucleotides on the universal solid support. *Nucleic Acids Res. Symp. Ser.* 24, 236.
- (26) Pon, R. T. (1993) Solid-phase supports for oligonucleotide synthesis. In *Methods in Molecular Biology Volume 20. Protocols for Oligonucleotides and Analogues* (S. Agrawal, Ed.) pp 465–496, Humana Press, Totowa, NJ.
- (27) Applied Biosystems Inc. (1992) Manual Cleavage and Deprotection of Oligonucleotides. In *Evaluating and Isolating Synthetic Oligonucleotides*, pp A-13–A-14, Applied Biosystems, Foster City, CA.
- (28) Mukkala, V.-M., Helenius, M., Hemmilä, I., Kankare, J., and Takalo, H. (1993) Development of luminescent europium(III) and terbium(III) chelates of 2,2':6',2''-terpyridine derivatives for protein labeling. *Helv. Chim. Acta* 76, 1361–1378.
- (29) Hemmilä, I., Dakubu, S., Mukkala, V.-M., Siitari, H., and Lövgren, T. (1984) Europium as a label in time-resolved immunofluorometric assays. *Anal. Biochem.* 137, 335–343.
- (30) Hakala, M., Mäki, E., and Lönnberg, H. (1998) Detection of oligonucleotide hybridization on a single microparticle by time-resolved fluorometry: quantitation and optimization of a sandwich type assay. *Bioconjugate Chem.* (in press).
- (31) Brown, R., Jones, W. G., and Pinder, A. R. (1951) The addition of thiolacetic acid to unsaturated compounds. *J. Chem. Soc.*, 2123–2125.
- (32) Connolly, B. A. (1987) Solid phase 5'-phosphorylation of oligonucleotides. *Tetrahedron Lett.* 28, 463–466.
- (33) Sund, C., Ylikoski, J., Hurskainen, P., and Kwiatkowski, M. (1988) Construction of europium (Eu³⁺)-labeled oligo DNA hybridization probes. *Nucleosides Nucleotides* 7, 655–659.

BC970194G

Role of Cross-Linking Agents in Determining the Biochemical and Pharmacokinetic Properties of Mgr6–Clavin Immunotoxins

Franco Dosio,[†] Silvia Arpicco,[†] Elena Adobati,[‡] Silvana Canevari,[‡] Paola Brusa,[†] Rita De Santis,[§] Dino Parente,[§] Paola Pignanelli,[§] Donatella R. M. Negri,[‡] Maria I. Colnaghi,[‡] and Luigi Cattel^{*,†}

Dipartimento di Scienza e Tecnologia del Farmaco, University of Torino, Torino, Italy, Divisione di Oncologia Sperimentale E, Istituto Nazionale per lo Studio e la Cura dei Tumori, Milano, Italy, and Dipartimento di Biotecnologie, Menarini Ricerche Sud, Pomezia, Italy. Received October 31, 1997; Revised Manuscript Received January 27, 1998

Several immunotoxins (ITs) were synthesized by the attachment of clavin, a recombinant toxic protein derived from *Aspergillus clavatus*, to the monoclonal antibody Mgr6 that recognizes an epitope of the gp185^{HER-2} extracellular domain expressed on breast and ovarian carcinoma cells. Conjugation and purification parameters were analyzed in an effort to optimize the antitumor activity and stability of the ITs in vivo. To modulate the in vitro and in vivo properties of the immunotoxins, different coupling procedures were used and both disulfide and thioether linkages were obtained. Unhindered and hindered disulfide with a methyl group linkage ethyl S-acetyl 3-mercaptopropionthioimide ester hydrochloride (AMPT) or ethyl S-acetyl 3-mercaptoputylthioimide ester hydrochloride (M-AMPT) were obtained by reaction with recombinant clavin, while the monoclonal antibody Mgr6 was derivatized with ethyl 3-[(4-carboxamidophenyl)dithio]propionthioimide ester hydrochloride (CDPT). To achieve higher hindrance (a disulfide bond with a geminal dimethyl group), Mgr6 was derivatized with the *N*-hydroxysuccinimidyl 3-methyl-3-(acetylthio)butanoate (SAMBA) and clavin with CDPT. To evaluate the relevance of the disulfide bond in the potency and pharmacokinetic behavior of the ITs, a conjugate consisting of a stable thioether bond was also prepared by derivatizing Mgr6 with the *N*-hydroxysuccinimidyl ester of iodoacetic acid (SIA) and clavin with AMPT. The immunotoxins were purified and characterized using a single-step chromatographic procedure. Specificity and cytotoxicity were assayed on target and unrelated cell lines. The data indicate that the introduction of a hindered disulfide linkage into ITs has little or no effect on antitumor activity and suggest that disulfide cleavage is essential for activity; indeed, the intracellularly unbreakable thioether linkage produced an inactive IT. Analysis of IT stability in vitro showed that the release of mAb by incubation with glutathione is proportional to the presence of methyl groups and increases exponentially with the increase in steric hindrance. Analysis of the pharmacokinetic behavior of ITs in Balb/c mice given intravenous bolus injections indicated that ITs with higher in vitro stability were eliminated more slowly; i.e., the disulfide bearing a methyl group doubled the β -phase half-life (from 3.5 to 7.1 h) compared with that of the unhindered, while a geminal dimethyl protection increased the elimination phase to 24 h. The thioether linkage showed its intrinsic stability with a β -phase half-life of 46 h. The thioether linkage also increased the distribution phase from 17 to 32 min. The in vitro characteristics and in vivo stability of Mgr6–clavin conjugates composed of a methyl and dimethyl steric hindered disulfide suggest clinical usefulness.

INTRODUCTION

The potent and highly specific cytotoxic activity of conjugates between monoclonal antibodies and toxins has raised the promise of effective immunotherapy for tumors and graft-versus-host disease. ITs¹ have particular potential in the treatment of leukemias and lymphomas (1, 2), although some evidence suggests they are effective against solid tumors as well (3, 4).

Extremely active ITs have been constructed by covalently linking mAb to bacterial toxins such as *Pseudomonas* exotoxin or to plant toxins such as the ricin A chain, saporin, and gelonin (5–8).

Other interesting ribosome-inactivating proteins (RIPs) with potent antitumor activity such as α -sarcin, restriction, and mitogillin have been isolated from fungi and used to prepare ITs (9–11). RIPs of fungi are extensively homologous in their primary structure (>80%) and have the same catalytic activity, i.e., hydrolysis of the phosphodiester linkage between the G residue at position 4325 and the A residue at position 4326 in 28S rRNA (12).

* Correspondence should be addressed to Luigi Cattel, Dipartimento di Scienza e Tecnologia del Farmaco, v. P. Giuria 9, 10125 Torino, Italy. Phone: ++39-11-6707697. Fax: ++39-11-6707695. E-mail: dosio@pharm.unito.it.

[†] University of Torino.

[‡] Istituto Nazionale per lo Studio e la Cura dei Tumori.

[§] Menarini Ricerche Sud.

¹ Abbreviations: ITs, immunotoxins; AMPT, ethyl S-acetyl-propionthioimide; M-AMPT, ethyl S-acetyl-3-mercaptoputylthioimide; CDPT, 3-[(4-carboxamidophenyl)dithio]propionthioimide; SAMBA, *N*-hydroxysuccinimidyl 3-methyl-3-(acetylthio)butanoate; SIA, *N*-hydroxysuccinimidyl ester of iodoacetic acid; RIP, ribosome-inactivating protein; DTNB, Ellman's reagent; DTT, dithiothreitol; TNB, 5-mercaptop-2-nitrobenzoic acid; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; GSH, reduced glutathione; SMPT, 4-[(succinimidyl-oxycarbonyl)- α -methyl- α -(2-pyridyldithio)toluene.

Recently, Parente et al. (13) described the cloning and expression of a new RIP, clavin, from *Aspergillus clavatus*. Clavin was highly active on free ribosomes, inducing low and transient systemic toxicity and a late, low-level antibody response in mice. These characteristics and its availability in large amounts identify clavin as a good candidate for IT preparation.

To selectively target clavin to breast and ovarian carcinoma cells, we used a mAb (Mgr6) that recognizes an epitope of the gp185^{HER-2} extracellular domain (14). The tissue distribution of gp185^{HER-2} in normal human tissues is restricted, and overexpression of the HER-2 oncogene is associated with poor prognosis in breast and ovarian carcinoma patients (15, 16). Existing ITs directed to gp185^{HER-2} have already shown immunotherapeutic promise (17–19).

In this study, we analyzed the conjugation and purification parameters that might yield efficient and stable ITs suitable for preclinical studies.

We prepared various ITs and characterized them with respect to coupling systems (disulfide or thioether linkage). In fact, most of the ITs prepared to date have a reducible disulfide bond which is required for the intracellular release of the toxic moiety (20). The major in vivo limitations of such ITs rest in the instability of the chemical linkage, which restricts the amount of conjugate that is able to bind to target cells (21, 22), and in the potential competition of the released antibody with the intact conjugate since the mAb persists longer in circulation. Previously released antibody may mask tumor antigens, thereby compromising IT potency during multiple-dose treatment regimens.

In an effort to prolong the in vivo half-life of ITs and enhance their antitumor potency, we prepared Mgr6–clavin conjugates using the thioimide cross-linking system which preserves the positive charge on the derivatized proteins and allows modulation of the hindrance on the chemical linkage between clavin and the mAb.

EXPERIMENTAL PROCEDURES

Materials. Anhydrous dichloromethane, Ellman's reagent (DTNB), and all other reagents were from Aldrich (Milwaukee, WI).

Diethyl ether was distilled from lithium aluminum hydride, and dry tetrahydrofuran was obtained by distillation from sodium.

General Procedures. Melting points were determined with a Reichert Kofler apparatus and are uncorrected. ¹H NMR spectra were recorded on a JEOL PMX-60 spectrometer, operating at 60 MHz, with tetramethylsilane as the internal standard. IR spectra were obtained as KBr disks on a Shimadzu FT-IR 8101 M spectrophotometer; wavelengths are given in inverse centimeters. Mass spectra were obtained with a FINNI-GAN-MAT TSQ-700 or with a VG Analytical 70-70 EQ-HF spectrometer. Ultraviolet spectra were recorded on a Beckman DU-70 spectrophotometer.

Reactions were checked on F₂₅₄ silica gel precoated sheets (Merck, Milan, Italy). Purification was carried out by column flash chromatography on silica gel 60 (Merck, 230–400 mesh).

Synthesis of Cross-Linkers. Preparation of Thioimide Ester Hydrochlorides (CDPT, AMPT, and M-AMPT). Hydrogen chloride gas was bubbled through ice-cold ethanethiol (3.25 mL, 0.0435 mol) for 1 h. The nitriles 4-[(2-cyanoethyl)dithio]benzamide, 3-(acetylthio)propionitrile, and 3-(acetylthio)butyronitrile, synthesized

as described by Arpicco (23), were diluted in anhydrous diethyl ether or dry tetrahydrofuran and quickly added to the cold solution with stirring, and the reaction mixture was left overnight at 0 °C. Anhydrous cold diethyl ether was then added, and the reaction mixture was left at –20 °C until a crystalline solid formed. The supernatant was decanted, and the precipitate was washed with anhydrous diethyl ether under argon and dried under reduced pressure at room temperature.

Ethyl 3-[(4-carboxamidophenyl)dithio]propionthioimide ester hydrochloride (CDPT): yield 0.64 g (65%); mp 110–115 °C; ¹H NMR (CD₃OD) δ 7.85 (q, 4H, Ar-H), 3.4–3.15 (m, 6H, SCH₂CH₃ and CH₂CH₂), 1.47 (t, 3H, SCH₂CH₃); IR (KBr) 3500–2200 (NH₂ and NH₂⁺), 1650 (C=O), 1620 (C=N); MS (FAB⁺) 301 (M⁺ + 1).

Ethyl S-acetyl 3-mercaptopropionthioimide ester hydrochloride (AMPT): yield 0.87 g (88%); mp 64–66 °C; ¹H NMR (CDCl₃) δ 3.48 (q, 2H, SCH₂CH₃), 3.26 (m, 4H, CH₂CH₂), 2.38 (s, 3H, SAC), 1.45 (t, 3H, SCH₂CH₃); IR (KBr) 3300–2400 (NH), 1690 (C=O), 1620 (C=N), 1360 (SAC).

Ethyl S-acetyl 3-mercaptoputyrothioimide ester hydrochloride (M-AMPT): yield 0.79 g (75%); mp 112 °C; ¹H NMR (CDCl₃) δ 3.4–3.2 (m, 5H, SCH₂CH₃, CH, and CH₂), 2.43 (s, 3H, SAC), 1.6 (d, 3H, CH₃), 1.43 (t, 3H, SCH₂CH₃); IR (KBr) 3300–2400 (NH), 1690 (C=O), 1620 (C=N), 1360 (SAC); MS-EI *m/z* (relative intensity) 206 (M⁺, 7), 162 (100), 145 (53), 130 (20), 102 (65), 89 (20), 75 (33), 61 (52), 43 (100).

Preparation of N-Hydroxysuccinimidyl 3-Methyl-3-(acetylthio)butanoate (SAMBA). The method of Carroll (24) was used with minor modifications. Briefly, 3-methyl-3-(acetylthio)butanoic acid (1.5 g, 0.0085 mol) dissolved in 12 mL of dry dichloromethane was mixed with 1.3 g of NHS (0.011 mol) in 5 mL of dichloromethane, and dicyclohexylcarbodiimide (2.3 g, 0.011 mol) dissolved in 1 mL of dry dichloromethane was added dropwise. The reaction mixture was stirred at room temperature for 22 h. After filtration and evaporation, the mixture was purified by flash chromatography with elution in hexane/EtOAc (80/20). The ester was obtained as a pale yellow oil that crystallized rapidly at room temperature: yield 1.6 g (70%); mp 63 °C; TLC (80/20 hexane/EtOAc) *R_f* = 0.15; ¹H NMR (CDCl₃) δ 3.23 (s, 2H, CH₂), 2.80 (s, 4H, NHS ester), 2.25 (s, 3H, SAC), 1.6 (s, 6H, CH₃); MS-EI *m/z* (relative intensity) 273 (10), 159 (90), 117 (30), 75 (15), 43 (55).

The N-hydroxysuccinimidyl ester of iodoacetic acid (SIA) was prepared as described (25).

Clavin and Mgr6 Production. For clavin production, a 100 mL culture of *Escherichia coli* HB101 cells carrying the pMRS38 plasmid was used to start the final cultivation step in a Chemap bioreactor with a working volume of 1.5 L. The cultivation was performed in complex medium [20 g/L glucose, 25 g/L yeast extract, 40 g/L casamino acid (Difco), 0.5 g/L NaCl, 5 g/L KCl, 2.6 g/L K₂SO₄, 0.86 g/L MgCl₂·6H₂O, 6.6 mg/L CaCl₂·6H₂O, and trace amounts of oligo elements], supplemented with 100 mg/mL ampicillin. Cultivation parameters such as temperature, pH, and oxygen dissolved in tension (DOT) were computer-controlled. The culture supernatant was recovered by centrifugation and filtered on a Millipore 0.22 μm filter. Clavin was purified as described (13).

Mgr6 hybridoma, directed against the extracellular domain of gp185^{HER-2}, was obtained by immunization of Balb/c mice with the adenocarcinoma cell line CaLu3, and its characterization is described elsewhere (14). mAb Mgr6 was obtained by cultivating hybridoma cells in a

hollow fiber bioreactor (Acusyst R, Endotronics) using a serum-free medium. Medium, prepared in our facilities, consisted of a basal mixture (1/1) of RPMI 1640 and DMEM supplemented with transferrin, insulin, albumin, and Ex-Cite (Miles Inc.) as a lipid source. Additional ingredients were 2-mercaptoethanol, ethanolamine, and sodium selenite.

Cell cultivation was performed as continuous perfusion for approximately 1 month, during which parameters such as glucose and lactic acid concentrations, pH, oxygen and carbon dioxide levels, and antibody production were monitored along with operator responses.

Conditioned media harvested during cultivation were pooled after thawing and adjusted to pH 5.5. Material was diluted and filtered on 4.5 μm filters (Millipore Co., Bedford, MA) before being loaded on a Bakerbond Abx column (Mallinckrodt Baker B. V., Deventer, The Netherlands). The eluted protein peak was desalted on a Sephadex G-25 (Pharmacia-Biotech, Uppsala, Sweden) column and loaded onto a Q-Sepharose FF column (Pharmacia-Biotech). The eluted peak was desalted as indicated above and subjected to the final purification step on a S-Sepharose FF column (Pharmacia-Biotech). Elution was obtained by applying a linear gradient from 20 to 200 mM NaCl in 20 mM phosphate buffer.

Preparation of Immunotoxins. Disulfide Bridge. In a standard preparation, a recombinant clavin solution (357 μM , 1 mL) was stirred with AMPT (122 mM, 30 μL dissolved in ethanol), M-AMPT (98 mM, 30 μL dissolved in ethanol), or CDPT (139 mM, 40 μL in dry dimethylformamide) for 30 min at 25 $^{\circ}\text{C}$ to incorporate an average of 1–1.3 linkers per mole of clavin. The mixture was purified by gel filtration on a 1×20 cm Bio-Gel P6-DG column (Bio-Rad, Hercules, CA) eluted with HBS (0.1 M, 0.2 M NaCl and 1 mM EDTA disodium salt at pH 7.4) at 20 $^{\circ}\text{C}$. The protein solution was concentrated to 1 mL with an Amicon concentrator (Amicon, Beverly, MA) equipped with a Y10 membrane.

mAb Mgr6, dissolved in HBS, was separately reacted with two different cross-linkers, CDPT and SAMBA. The reaction of Mgr6 with CDPT proceeded as follows: CDPT in dry dimethylformamide (13 mM, 40 μL) was added to the mAb in solution (33 μM , 1 mL), and the mixture was stirred for 30 min at 25 $^{\circ}\text{C}$. The Mgr6/CDPT molar ratio was 1/1.1. Derivatization of Mgr6 with SAMBA (11 mM, 30 μL) dissolved in ethanol was carried out for 30 min at 25 $^{\circ}\text{C}$ to introduce two acetylthio groups. In both cases, the mixture was purified by gel centrifugation on a 15×55 mm Bio-Gel P6-DG column preequilibrated in HBS at 20 $^{\circ}\text{C}$.

The number of thioacetylated groups linked to the protein was calculated spectrophotometrically by reaction of the sample with the deacetylating reagent hydroxylamine hydrochloride (0.5 M, 12.5 mM EDTA, pH 7.4) followed by thiol disulfide exchange with DTNB as described (26).

The number of arylthio groups linked to Mgr6 or clavin was determined following the release of the thiolated anion at 313 nm, after incubation of the protein sample (1 mL) with 2-mercaptoethanol in PBS/EDTA (11 mM sodium phosphate and 3 mM EDTA, 50 μL) and NaOH (1 M, 40 μL), to a final pH of 8.8–9.4. The molar absorptivity value of the 4-carboxamidophenylthiolate anion under these conditions at 313 nm was $15\,200 \pm 300 \text{ M}^{-1} \text{ cm}^{-1}$.

All conjugations were performed by mixing the derivatized mAb and RIP in the presence of a solution of hydroxylamine (1/10, v/v). Reactions were allowed to proceed for 5 h at 25 $^{\circ}\text{C}$ followed by 18–35 h at 4 $^{\circ}\text{C}$; at

the end of the reaction, a solution of *N*-ethylmaleimide (20 mM, 20 μL) was added to block free thiol groups.

Thioether Bridge. Clavin (357 μM , 1 mL) was derivatized with AMPT as described above; Mgr6 (33 μM , 1 mL) was reacted with SIA (5.7 mM, 30 μL) dissolved in ethanol, for 30 min at 25 $^{\circ}\text{C}$. The iodoacetyl groups inserted on Mgr6 were identified by reaction with the TNB (5-mercapto-2-nitrobenzoic acid) reagent previously prepared; briefly, 25 mg of dithiothreitol (DTT) was added to 50 mg of DTNB dissolved in 300 μL of 1 M aqueous NaOH under an inert atmosphere. After 30 min, the reaction mixture was acidified with 1 M HCl and the mixture was extracted with diethyl ether under an inert atmosphere. The organic layers were then anhydri-ficated and evaporated under reduced pressure. The residue was washed several times with ethanol to obtain TNB as a dark yellow product.

The Mgr6 derivatization degree was determined by reaction of mAb (3.3 μM , 500 μL) with TNB dissolved in ethanol (3.4 mM, 10 μL) for 90 min at 25 $^{\circ}\text{C}$. After purification by gel centrifugation, the number of iodoacetyl groups was determined spectrophotometrically (TNB molar absorption coefficient was $8800 \text{ M}^{-1} \text{ cm}^{-1}$ at 339 nm). The mean derivatization degree was 1.7.

The derivatized mAb and RIP were mixed in the presence of a solution of hydroxylamine (1/10, v/v). The conjugation reactions proceeded for 18 h at 4 $^{\circ}\text{C}$; at the end of the reaction, a solution of *N*-ethylmaleimide (20 mM, 20 μL) was added to block free thiol groups.

Immunotoxin Purification. Conjugates were purified from unconjugated clavin and Mgr6 in a one-step procedure using CM MemSep cartridges (1000 or 1010) (Millipore) for analytical or preparative application and a Merck-Hitachi 655A-12 HPLC gradient system equipped with an L-5000 LC controller. The eluting fractions were monitored at 280 nm using a L4000 UV detector. Peak heights were recorded and processed on a CBM-10A Shimadzu interface. The mobile phase was sodium acetate buffer (20 mM at pH 5.5) flushed at 1 mL/min. Fractions containing immunoconjugates were dialyzed and concentrated. The purity of the immunotoxins was monitored by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) using 4–15% precast gels (Bio-Rad) under nonreducing conditions and Coomassie blue staining.

In Vitro Evaluation of Disulfide Bond Stability. The in vitro stability of the bond in the various conjugates was evaluated as follows. Samples (1 mg/mL, 3 μL) were incubated for 1 h at 37 $^{\circ}\text{C}$ with solutions of reduced glutathione (GSH, 3 μL) in increasing excesses (from 3 to 10000-fold), and the reaction was stopped by addition of excess iodoacetamide. Following SDS–PAGE under nonreducing conditions, the 7.5% precast gels (Bio-Rad) were stained with Coomassie blue, dried, and scanned on a Compact 4800 flatbed scanner using Twain compatible software. Band densities were analyzed using ImagePC (Scion Co., Frederick, MD) to calculate the amount of Mgr6 released.

Cell Lines. Human breast carcinoma cell line SKBr3 (ErbB2+) was purchased from ATCC (Rockville, MD), and human melanoma cell line MeWo (ErbB2–) was kindly provided by the late J. Fogh (Memorial Sloan-Kettering Cancer Center, New York). Both cell lines were grown in RPMI 1640 containing 10% fetal calf serum and gentamicin (100 $\mu\text{g/mL}$).

Binding Inhibition Assay. mAb activity after conjugation was assayed as the ability to inhibit binding of [^{125}I]Mgr6 to adherent glutaraldehyde-fixed SKBr3 cells. A fixed amount of [^{125}I]Mgr6 (1 nM) was mixed with serial

dilutions of cold Mgr6 or IT, starting from a 100-fold molar excess. The mixture was added to SKBr3 fixed cells (adherent in 96-well plates) and incubated for 3 h at 37 °C. Cells were washed 10 times with PBS and incubated with 2 N NaOH (100 μ L/well) for 20 min at room temperature. The supernatant was collected and radioactivity determined in a γ -counter (Beckman Instruments, Fullerton, CA).

Percent inhibition was calculated as follows:

$$\% \text{ inhibition} = (1 - C_i/C_{ni}) \times 100$$

where C_i is the average counts per minute in the presence of cold inhibitor and C_{ni} is the average counts per minute without inhibitor.

Inhibition of Protein Synthesis. The assay was carried out essentially as described (13). Briefly, SKBr3 and MeWo cells were suspended in culture medium containing the appropriate concentration of IT, toxin, or mAb alone and incubated for 3 h at 4 °C. Control cells were incubated with medium alone. Cells were centrifuged, resuspended in fresh culture medium, and seeded in 96-well plates (3×10^5 cells/well). After incubation at 37 °C for 48 h, [^{3} H]proline (1 μ Ci/well) was added. After 48 h, cells were washed and [^{3} H]proline incorporation was determined by liquid scintillation in a β -counter. Results are expressed as a percentage of [^{3} H]proline incorporation in control cells.

Radioiodination Procedure. ITs, clavin, and Mgr6 were ^{125} I-labeled using the Iodogen method according to the manufacturer's instructions, to a mean specific activity of 1.5 mCi/mg for ITs, 8.5 mCi/mg for Mgr6, and 4.5 mCi/mg for clavin. The integrity of the radiolabeled protein was tested by ascending paper chromatography in 10% trichloroacetic acid and by SDS–PAGE analysis.

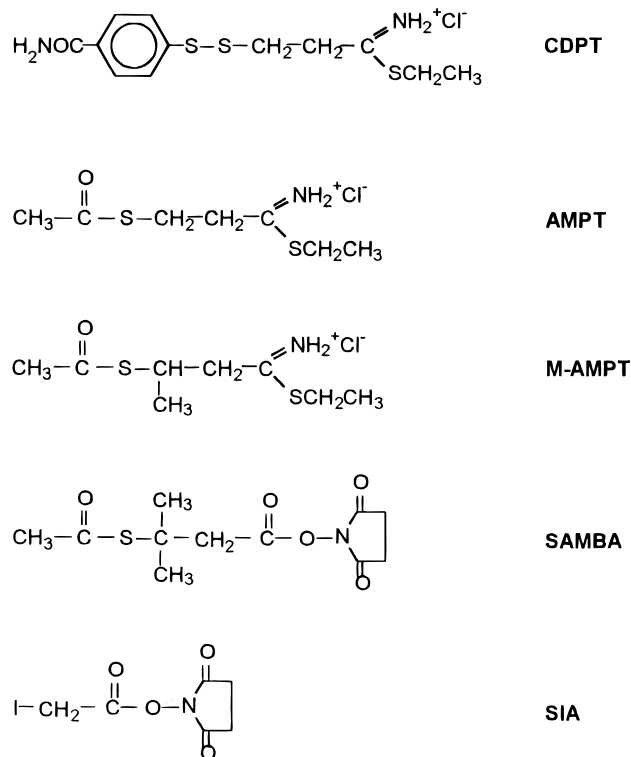
Pharmacokinetic Evaluation. Experiments were performed on 6-week-old female Balb/c mice (Charles River, Como, Italy) maintained according to the provisions of the European Economic Community Council directive 86/209 recognized and adopted by the Italian Government.

Mice received a Lugol solution (0.02% I_2) and 0.6 mg/mL $KClO_4$ in their drinking water 3 days before administration of radioiodinated ITs and throughout the experiments to block free iodine uptake by the thyroid gland and the stomach mucosa. Animals were injected intravenously with a single dose of ^{125} I-labeled IT, ranging from 1.7 to 2.5 μ g/mouse. Blood samples were drawn from the retro-orbital sinus at fixed times after injection. Pharmacokinetic analysis was carried out on serum samples. Each serum sample was diluted 1/20 and run in SDS–PAGE under nonreducing conditions. Gels were fixed, dried, and exposed in a PhosphorImager screen (Molecular Dynamics, Sunnyvale, CA). Densitometric analysis, performed using Image Quant software (Molecular Dynamics), was used to determine the percentage of intact immunoconjugates in the serum at each time point. Pharmacokinetic parameters were calculated using PCNONLIN software (SCI Software, Lexington, KY).

RESULTS

Chemistry. The cross-linkers, prepared as described (23–25), were characterized by NMR, IR, and MS. Before conjugation, the reactivity of the linkers toward the lysyl ϵ -amino groups of IgG as a model protein was evaluated to insert 1–2 groups. Scheme 1 shows the structures of the cross-linkers.

Scheme 1. Structures of Cross-Linkers Used to Produce Mgr6–Clavin ITs



Preparation of Immunotoxins. Four different coupling procedures (Schemes 2 and 3) were followed to compare the relative activity, stability, and in vivo fate of the immunotoxins.

Disulfide Bridge Immunotoxins. Linkers AMPT and M-AMPT were reacted with recombinant clavin to introduce 1–1.3 S-acetylthio groups per protein molecule, whereas a mean value of 1.1 arylthio residues was introduced into Mgr6 with CDPT; the derivatized proteins were coupled in the presence of hydroxylamine to deprotect the acetylthio groups (IT-1 and IT-2 in Scheme 2). Clavin modified with CDPT was reacted with the Mgr6 previously reacted with the hindered *N*-hydroxy-succinimidyl linker SAMBA (IT-4) (Scheme 3).

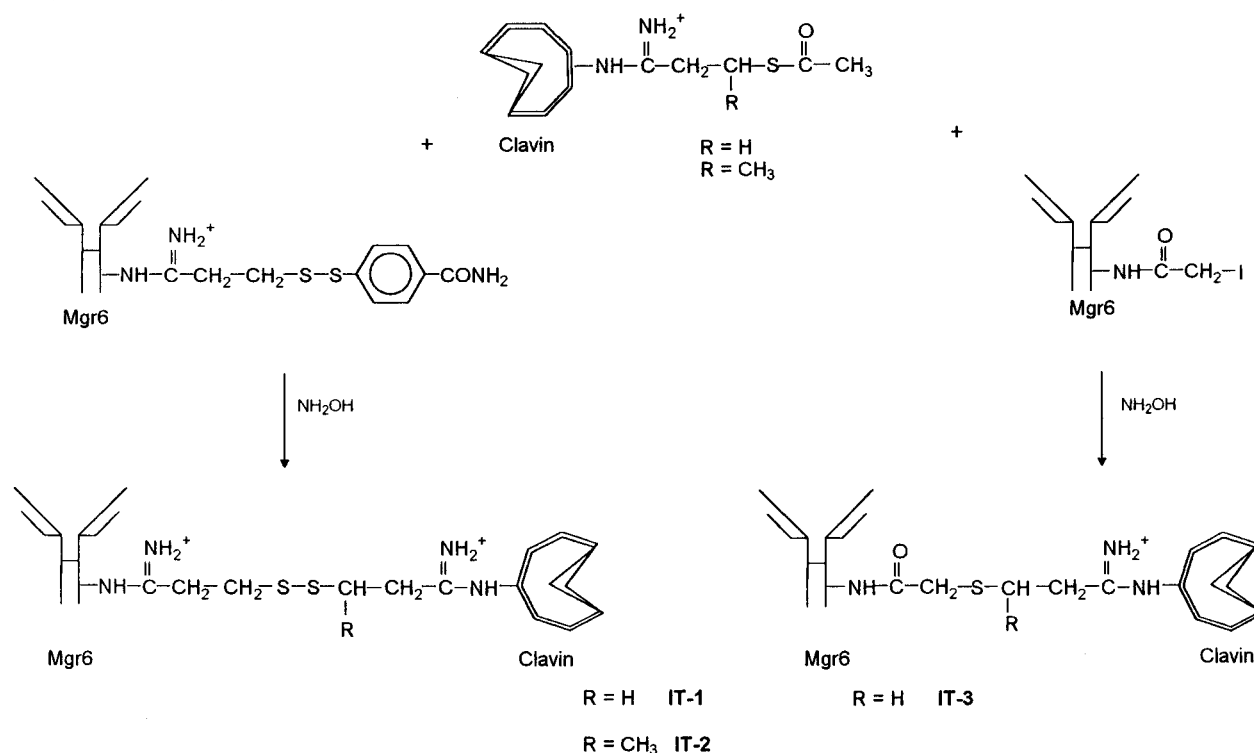
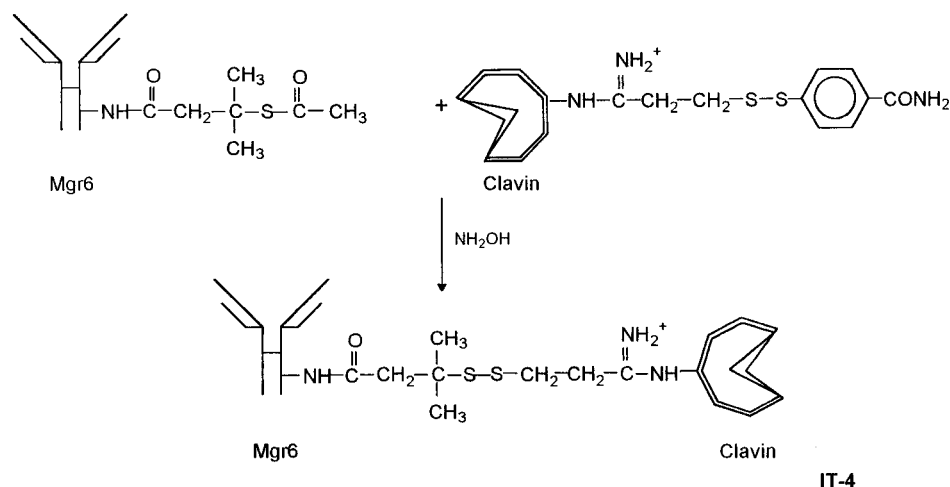
All coupling reactions were allowed to proceed for 5 h at 25 °C and for 18–35 h at 4 °C; aliquots of the reaction mixtures were analyzed by SDS–PAGE.

Thioether Bridge Immunotoxin. To obtain a thioether linkage, Mgr6 was derivatized with SIA (1.7 iodoacetyl groups) and clavin with AMPT as described above. After addition of hydroxylamine, the reaction proceeded for 18 h at 4 °C (IT-3 in Scheme 2).

The yield of conjugation for the thioether-linked IT was 12%, while for the disulfide-ITs, the yield was approximately 10% for IT-1 and IT-2 and 5% for IT-4. This suggested an inverse correlation between disulfide bond stability and conjugation efficiency. The low yield can be attributed in part to the low (range of 1–1.7) number of reactive groups per protein.

Purification of Immunotoxins. All ITs were purified from the reaction mixture in a single step by HPLC–ion exchange chromatography using a 1010 CM MemSep cartridge.

Figure 1A shows an example of the elution profile of the crude mixture eluted using a discontinuous gradient of sodium chloride ranging from 0 to 250 mM. Fractions from each of the four regions of the elution peaks were separately pooled and subjected to nonreducing SDS–

Scheme 2. Preparation of Mgr6-(CDPT)SS(AMPT)-Clavin (IT-1), Mgr6-(CDPT)SS(M-AMPT)-Clavin (IT-2), and Mgr6-(SIA)CS(AMPT)-Clavin (IT-3)**Scheme 3. Preparation of Mgr6-(SAMBA)SS(CDPT)-Clavin (IT-4)**

PAGE analysis (Figure 1B). By comparison with purified Mgr6 and clavin electrophoresed in parallel, peak 1 was composed only of unreacted Mgr6, peak 2 was 80% IT with a molecular mass of 167 kDa, peak 3 was >99% IT (167 kDa), and peak 4 contained only unreacted clavin (17 kDa).

Disulfide Bond Stability of Conjugates in Vitro.

To test the in vitro stability of the immunotoxins, samples were incubated with reduced GSH in different excesses, subjected to SDS-PAGE, and assessed densitometrically (Figure 2). The profile of the disulfide rupture as a function of GSH excess used is shown in Figure 3. An increase in disulfide stability in relation to the steric hindrance around the disulfide of the conjugates was observed. In comparison, the presence of a thioether bond precluded the breakage by GSH. The increase in stability was exponential, i.e., 10-fold for one methyl group and 100-fold for a geminal dimethyl group. When GSH was used in an excess higher than 7000-fold,

cleavage of the mAb disulfide bond was measurable (data not shown).

In Vitro Activity of ITs. To determine whether Mgr6 retained its binding capacity after derivatization and conjugation, competitive inhibition experiments were conducted with the four ITs. A small, reproducible drop in binding capacity was observed after derivatization that was independent of the cross-linker used (see Figure 4, Mgr6-CDPT curve). All ITs competed with the native [^{125}I]mAb, with a titration similar to that of cold derivatized Mgr6, and no significant differences due to the linkage method adopted were evident (Figure 4).

Each IT was tested for its ability to inhibit protein synthesis in ErbB2-positive SKBr3 cells compared with the inhibition by unconjugated clavin. All ITs specifically inhibited protein synthesis in the SKBr3 cells, whereas none did so in ErbB2-negative MeWo cell. As shown in Figure 5, the IC_{50} s of the conjugates containing a disulfide bridge were similar and ranged between 1 and 4 nM; the

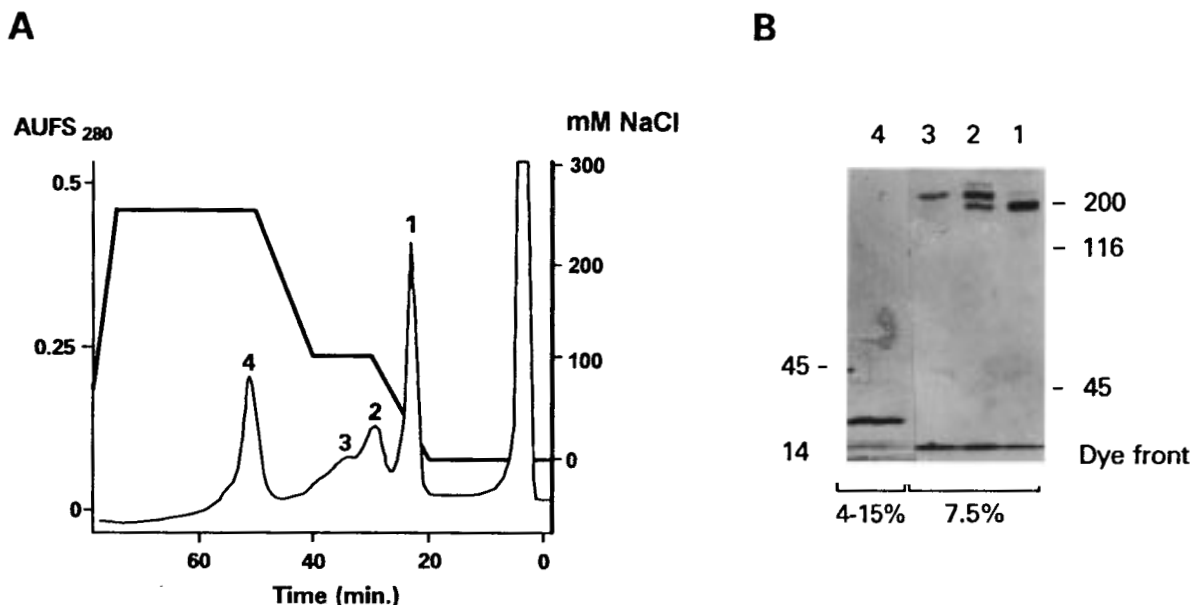


Figure 1. Elution profile of the IT reaction mixture from the CM-MemSep column (A) and SDS-PAGE analysis of eluted fractions (B). For preparative purposes, a MemSep 1010 cartridge eluted in sodium acetate buffer (20 mM at pH 5.5) was loaded with 400 μ L of conjugation mixture, and a step gradient of the same buffer and 1 M NaCl was applied, at a 0.5 mL/min flow rate for the first 12 min and at 2 mL/min thereafter. After 20 min, the gradient was raised over the course of 10 min to 100 mM NaCl and kept constant for another 10 min. To remove unconjugated clavin, the gradient was increased to 250 mM over the course of 10 min and held there for 20 min. The collected fractions (about 3.5 mL) were immediately brought to pH 7.4 by addition of a solution of 1 M PBS (pH 8).

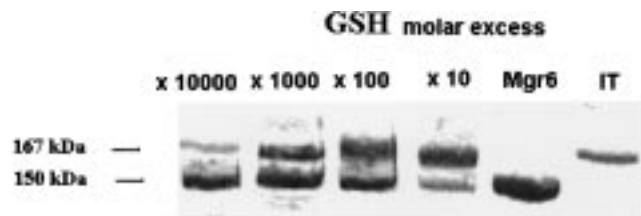


Figure 2. SDS-PAGE analysis of IT-2 incubated with GSH at different concentrations for 1 h at 37 °C.

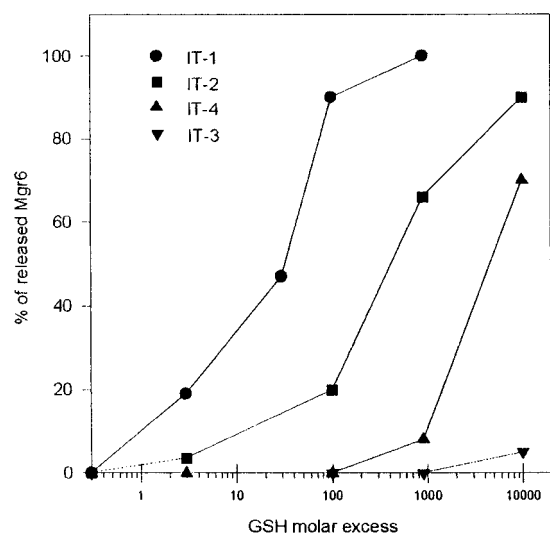


Figure 3. Release of mAb from the immunoconjugates. The conjugates were incubated with GSH at different concentrations for 1 h at 37 °C. The amounts released were measured by densitometry after SDS-PAGE. Results are the average of three independent experiments (SDs < 10% of the mean).

conjugation to the mAb resulted in a 150–650-fold increase in cytotoxic activity compared to that of clavin alone. No toxicity was observed after treatment with the conjugate containing the thioether bond (IT-3), which cannot be cleaved inside the cell. The specificity of the

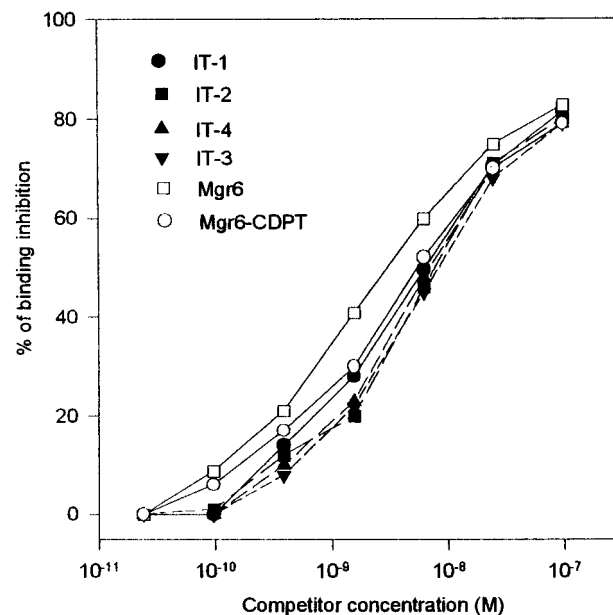


Figure 4. Competition of [¹²⁵I]Mgr6 binding to SKBr3 (HER2+) glutaraldehyde-fixed cells with increasing molar concentrations of unlabeled mAb, derivatized mAb, and ITs (SD < 5% of the means).

cytotoxicity was confirmed by the absence of an effect on MeWo cells (IC₅₀s for all ITs are > 1 μ M vs an IC₅₀ of 0.8 μ M for clavin).

Pharmacokinetics. To evaluate the in vivo stability of ITs with a hindered (IT-2 and IT-4) or an unhindered (IT-1) disulfide bond or a thioether bond (IT-3), the pharmacokinetic behavior of each species was evaluated after intravenous bolus administration of ¹²⁵I-labeled IT in groups of three Balb/c mice. Blood samples were collected at various times, and radioactivity was determined. Plasma samples were analyzed by SDS-PAGE, autoradiography, and densitometry. The unhindered disulfide conjugate (IT-1) broke down to release the free

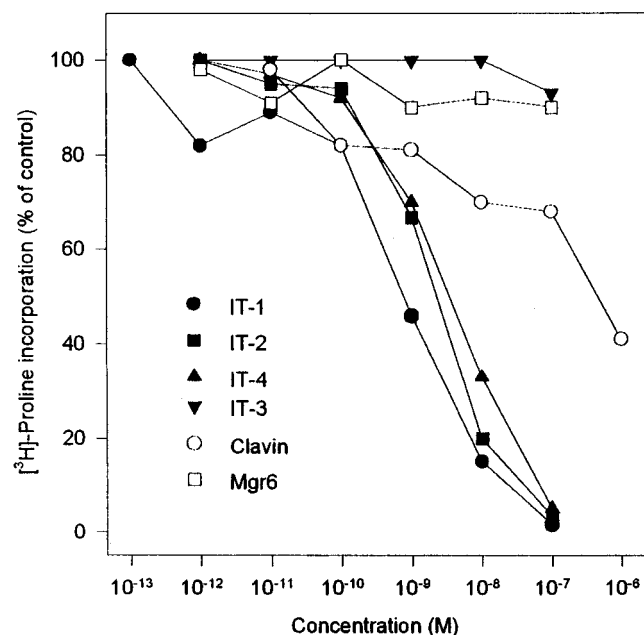


Figure 5. Inhibition of [³H]proline incorporation in SKBr3 target cells. Values represent the arithmetic means of three determinations (SDs < 10% of the means).

antibody in vivo at the fastest rate, and after 6 h, only 12% of the intact IT was present as compared with 35% of the IT-2 at the same time point. Plasma samples collected from IT-4 injected mice contained approximately equal amounts of intact immunotoxin and released mAb 47 h after injection. IT-3 appeared to be stable, and by densitometric analysis, the conjugate represented 86–90% of the total radioactivity 72 h after injection.

Plasma concentrations of IT at each time point were used to construct pharmacokinetic curves for each conjugate (Figure 6). Table 1 lists the parameters obtained from computerized analysis of the clearance data using an open two-compartment pharmacokinetic model. Data for the single components of the ITs are included for comparison. Mgr6 showed a long serum elimination phase, whereas clavin, due to its low molecular mass and relevant charge, was cleared rapidly with only 3% of the initial radioactivity present after 1 h. The clearance values for clavin are shorter than those we reported previously (13) due possibly to the different method used for evaluation.

The elimination curves of the ITs were biphasic, with a similar initial rapid α phase (about 17 min) followed by a slower β phase. The immunoconjugates with higher in vitro stability were eliminated more slowly. Indeed, IT-1 was cleared rapidly; IT-2 doubled the β -phase half-life (from 3.5 to 7.1 h), and IT-4 increased the elimination phase to 24 h. The thioether linkage showed its intrinsic stability, with a β -phase half-life of 46 h. The difference in the elimination rate (144 h for the mAb and 46 h for IT-3) is due only to the presence of the clavin molecule. The thioether linkage stability also increased the distribution phase (from 17 to 32 min).

DISCUSSION

Immunotoxins have shown promise in therapeutic applications for hematological malignancies (1, 2, 27, 28); however, the therapy of disseminated solid tumors has proven to be a greater challenge, and only few trials have provided encouraging results (3, 29). Several investigators used a reduced size IT Fab linked to the toxic domain

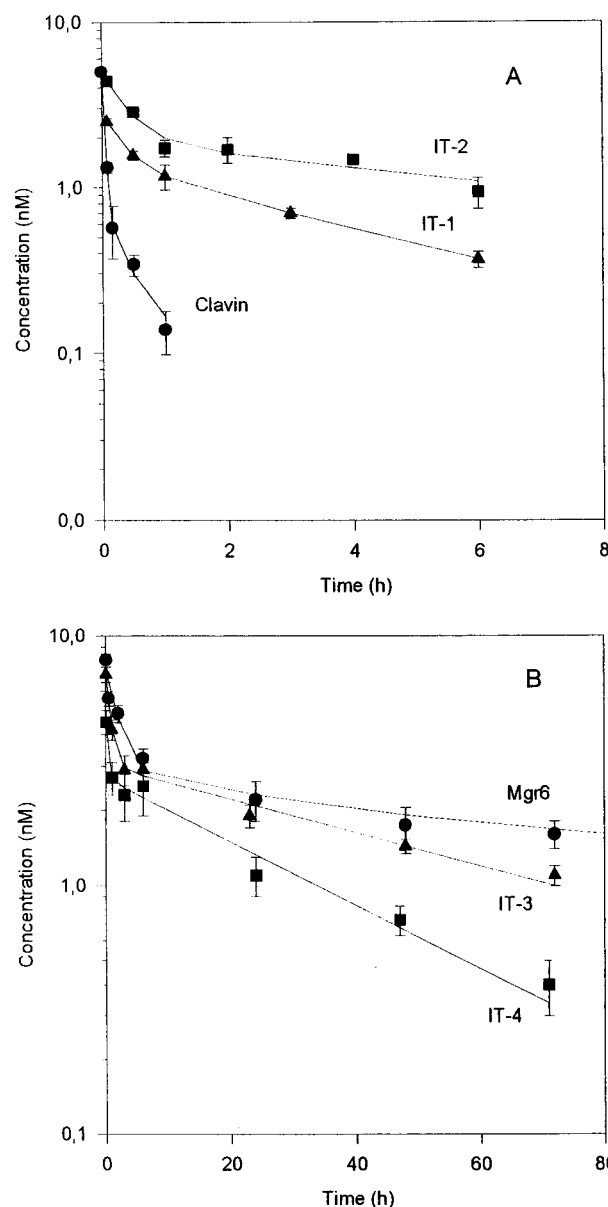


Figure 6. Pharmacokinetic serum curves for ITs, clavin, and Mgr6. Mice were injected intravenously with the proteins, and blood samples were obtained at various time intervals. Concentrations were determined by assay of ¹²⁵I radioactivity and by densitometric analysis of SDS–polyacrylamide gels. Note the different clearance times in panels A and B.

of diphtheria toxin or *Pseudomonas* exotoxin to allow sufficient tissue permeation; however, this approach was not efficient in the treatment of solid tumors due to faster increased in vivo clearance (30). We and others have used whole ITs (mAb–RIP) with high potency and good pharmacokinetic properties (6, 10, 31, 32).

In this study, we chose an anti-erbB2 mAb as the RIP targeting component. A large array of murine mAbs directed to HER2 have been generated (33), and several, including Mgr6, induce down-modulation of the oncoprotein and inhibition of tumor growth. The antitumor effect of these and related mAbs is significantly improved by chemical or molecular coupling with toxins (34, 35). A phase I study in breast cancer patients indicated the clinical feasibility of intravenous administration of up to 80 mg of Mgr6 (our unpublished results). Preliminary data in nine breast cancer patients indicate that Mgr6 has a mean elimination half-life of 37 h and does not

Table 1. Pharmacokinetic Parameters^a

	clavin	Mgr6	IT-1	IT-2	IT-4	IT-3
A ($\mu\text{g/mL}$)	0.17 $\pm$ 0.1	0.61 $\pm$ 0.06	0.26 $\pm$ 0.04	0.51 $\pm$ 0.09	0.39 $\pm$ 0.07	0.748 $\pm$ 0.03
B ($\mu\text{g/mL}$)	0.015 $\pm$ 0.007	0.34 $\pm$ 0.05	0.21 $\pm$ 0.05	0.31 $\pm$ 0.09	0.44 $\pm$ 0.03	0.495 $\pm$ 0.02
$t_{1/2}(\alpha)$ (h)	0.05 $\pm$ 0.02	3.76 $\pm$ 0.9	0.26 $\pm$ 0.06	0.28 $\pm$ 0.13	0.24 $\pm$ 0.18	0.54 $\pm$ 0.07
$t_{1/2}(\beta)$ (h)	0.7 $\pm$ 0.1	144.17 $\pm$ 12	3.58 $\pm$ 0.21	7.17 $\pm$ 1.6	24.1 $\pm$ 1.1	46.1 $\pm$ 3.1
AUC ($\mu\text{g h mL}^{-1}$) ^b	0.03 $\pm$ 0.01	73.7 $\pm$ 8.2	1.197 $\pm$ 0.22	3.49 $\pm$ 0.9	15.41 $\pm$ 1.7	33.53 $\pm$ 3.2
Cl (mL/h)	27.2 $\pm$ 4.3	0.033 $\pm$ 0.007	1.0 $\pm$ 0.18	0.46 $\pm$ 0.1	0.11 $\pm$ 0.016	0.071 $\pm$ 0.007
Vss (mL)	8.05 $\pm$ 2.1	6.74 $\pm$ 1	4.78 $\pm$ 0.87	4.46 $\pm$ 1.2	3.81 $\pm$ 0.26	4.67 $\pm$ 0.2
MRT (h)	0.28 $\pm$ 0.1	198.9 $\pm$ 20	4.77 $\pm$ 1.2	9.75 $\pm$ 2.1	34.4 $\pm$ 3.1	65.3 $\pm$ 6.2

^a Values were obtained by adopting a two-compartment open pharmacokinetic model. ^b The trapezoidal rule has been used to calculate the area under the curve values.

induce a human anti-mouse antibody response (manuscript in preparation).

As the toxic agent, we used a novel recombinant RIP, clavin, characterized by a low molecular mass (17 kDa), high in vitro activity, and reduced immunogenicity. The latter is especially relevant because almost all of the RIPs used for IT preparation such as PAP, saporin, momordin, and bryodin are highly immunogenic, and the antibody moiety of most IT conjugates elicits an even stronger immune reaction (36). Those findings are consistent with results obtained in patients receiving IT (37).

Clavin also shows a very low-level, transient, and reversible systemic toxicity on kidney and liver. This aspect is important because one of the major problems in using RIPs, such as PAP-1, PAP-S, RTA, and saporin, is related to their irreversible liver and kidney damage (38, 39).

To be therapeutically effective, ITs composed by type I RIPs must be linked in such a way that they are easily cleaved intracellularly but sufficiently stable in the blood circulation. A stable disulfide bond is essential in maintaining the half-life and potency of ITs, especially for solid tumors. The use of sterically hindered linkers such as SMTP to construct ITs with deglycosylated RTA is widely described, and these ITs are being used in phase I and II clinical studies (1, 2).

We therefore chose a series of thioimide linkers which preserve the positive charge on derivatized proteins and generate both a labile disulfide bond and different steric hindrances. The maintenance of a positive charge is especially important when using toxins such as clavin which is a relatively small molecule with a high positive charge.

Using the thioimide reagents, we prepared three ITs with a disulfide bond and one with a thioether linkage; all were expected to be stable in vivo. A geminal dimethyl hindrance was inserted using the SAMBA reagent because the analogous thioimide linker (in particular its nitrile precursor) tended to polymerize and appeared to be unsuitable for protein derivatization. Since neither SAMBA nor SIA preserved the surface charge, the protein derivatized with these linkers was the mAb which appeared to be less sensitive to surface charge modification (data not shown).

Using a derivatization degree of 1–1.7, we obtained only a 1/1 Mgr6–clavin species of conjugate, reflecting the reactivity of the carboxamidophenylthiolate leaving group presented in the linker CDPT. These conjugation procedures led to a highly homogeneous IT population, which is an advantage in accurately addressing the potency of an IT, but the presence of contaminating unconjugated mAb and RIP remains. The toxin can be removed easily by gel permeation, but this procedure fails to separate IT from free mAb. The amount of free mAb is generally too low to interfere with cytotoxicity assays; however, ITs are cleared more rapidly than unconjugated

mAb from the blood circulation. Moreover, the excess of mAb can compete with the IT for binding to the target antigen and can compromise the effectiveness of the IT in vivo. Several methods for the purification of IT from unconjugated antibody have been reported: ion exchange chromatography (40), high-performance liquid immunoaffinity chromatography (41), chromatography on immobilized Cibacron Blue F3GA (42), and, more recently, a nickel-based resin (Ni-NTA) technique (43) for purifying immunoconjugates containing 10 consecutive histidine residues at the amino terminus of the recombinant toxin.

To increase the yield of conjugate and to minimize the number of purification steps, we focused on an HPLC ion exchange procedure exploiting the high ionic charge ($pI > 10$) on the small surface of clavin, which allows interaction with weak ionic exchanger groups. In fact, isoelectrofocusing of the ITs showed an increase in the pI from 6.2 for the free Mgr6 to 8.2 pH units (data not shown). Further, an HPLC procedure with a membrane cartridge system allows the use of an increased elution flow rate, maintaining high peak resolution. The semistepwise linear salt gradient removes the low-molecular mass reactives (*N*-ethylmaleimide and leaving groups), and Mgr6 eluted in a sharp peak as well as unconjugated clavin. The IT eluted progressively in different peaks, with the first containing small amounts of Mgr6 and the final peaks containing only one pure species, 1/1 mAb–clavin IT.

As confirmed by competitive binding assay, the conjugation procedure and the nature of the linkage did not significantly modify the mAb binding capacity. Conjugates with disulfide bonds showed almost the same cytotoxic activity toward the target SKBr3 cell line, whereas they were ineffective on the nontarget MeWo cell line. The intracellular release of free clavin is necessary for its activity, and the IT linked by a thioether bond was not cytotoxic at all. These data are in agreement with previous findings that the introduction of a hindered disulfide linkage into ITs has little or no effect on pharmacological potency (23), suggesting that disulfide cleavage is not the rate-limiting step in the toxic activity of clavin conjugates on the cell. Moreover, for RIPs with a ribonuclease action, the maintenance of an intracellular easily breakable linkage between mAb and RIP is essential. This behavior is consistent with that described for gelonin and RTA (44) but in contrast with other findings (45) which suggested that gelonin can be delivered efficiently by a thioether linkage with the appropriate anti- α -fetoprotein antibody.

Comparison of the stability among unhindered and hindered disulfide- and thioether-linked ITs showed that the release of mAb by incubation with GSH is proportional to the presence of methyl groups and increased exponentially with the increase in steric hindrance. The in vivo stability of the ITs was verified by studying the

pharmacokinetic behavior after intravenous bolus administration in Balb/c mice.

An exponential correlation was also observed between the clearance rate and steric hindrance for the ITs and Mgr6. These findings underline the importance of inserting a highly stable dimethyl instead of a monomethyl or, obviously, an unhindered disulfide into the clavin IT. This was not so important in the case of RTA ITs (24) where a dimethyl group represented no advantage over a monomethyl, with respect to stability. Moreover, we previously reported (23) a linear correlation in the stability of methyl-substituted disulfide-linked gelonin ITs.

We also prepared a nonreducible thioether linkage to demonstrate the theoretical limit of in vivo linkage stability that can be obtained using Mgr6-clavin ITs. Even the presence of 1 mol of clavin per mole of mAb determined a decrease in elimination time (from 144 to 46 h) due to modification of the surface charge in mAb linked to a highly basic toxin.

The presence of a thioether linkage also affected the distribution phase (α phase) which was doubled compared to that of the three disulfide-linked ITs. In the latter, all three ITs had a similar α phase, suggesting that the reducibility of the linkage is not a limiting factor during the distribution.

We have used the most hindered cross-linking system (SAMBA and CDPT) to obtain an IT which maintains its high potency and specificity and has a slow blood clearance similar to that of RTA-containing ITs used in clinical trials (1, 2, 24). Since even a short half-life IT has been reported to be clinically effective in inducing tumor reduction (29, 30), we speculate that an increase in the half-life to 24 h renders the Mgr6-clavin IT more efficient in inducing in vivo tumor damage.

ACKNOWLEDGMENT

We thank Elena Luison for technical support and Mario Azzini for figure preparation. This work was supported by grants from the Italian Ministry of University and Scientific and Technological Research and the Italian Association for Cancer Research (AIRC), Funds for Italian Cancer Research, and CNR. E.A. is supported by a fellowship from AIRC.

LITERATURE CITED

- Engert, A., Diehl, V., Schnell, R., Radszuhn, A., Hatwig, M. T., Drillich, S., Schon, G., Bohlen, H., Tesch, H., Hansmann, M. L., Schindler, J., Ghetie, V., Uhr, J., and Vitetta, E. (1997) A phase-I study of an anti-CD5 ricin A-chain immunotoxin (RFT5-SMPT-dgA) in patients with refractory Hodgkin's lymphoma. *Blood* 89, 403–410.
- Winkler, U., Barth, S., Schnell, R., Diehl, V., and Engert, A. (1997) The emerging role of immunotoxins in leukemia and lymphoma. *Ann. Oncol.* 8 (Suppl. 1), 139–146.
- Lynch, T. J., Lambert, J. M., Coral, F., Shefner, J., Wen, P., Blattler, W. A., Collison, A. R., Ariniello, P. D., Braman, G., Esselene, D., Elias, A., Skarin, A., and Ritz, J. (1997) Immunotoxin therapy of small-cell lung cancer: a phase I study of N901-blocked ricin. *J. Clin. Oncol.* 15, 723–734.
- Frankel, A. E., Fitzgerald, D., Siegall, C., and Press, O. W. (1996) Advances in immunotoxin biology and therapy: a summary of the Fourth International Symposium on Immunotoxins. *Cancer Res.* 56, 926–932.
- Pastan, I., Willingham, M. C., and Fitzgerald, D. J. F. (1986) Immunotoxins. *Cell* 47, 641–644.
- Falini, B., Bolognesi, A., Flenghi, L., Tazzari, P. L., Broe, M. K., Stein, H., Durkop, H., Aversa, F., Cornell, P., Pizzolo, G., Barbabietola, G., Sabattini, E., Pileri, S., Matelli, M. F., and Stirpe, F. (1992) Response of refractory Hodgkin's disease to monoclonal anti-CD30 immunotoxin. *Lancet* 339, 1195–1196.
- Blakey, D. C., and Thorpe, P. E. (1988) An overview of therapy with immunotoxins containing ricin or its A chain. *Antibody, Immunoconjugates, Radiopharm.* 1, 1–15.
- Dosio, F., Brusa, P., Delprino, L., Grosa, G., Ceruti, M., and Cattell, L. (1994) A new approach in the synthesis of immunotoxins: ribosome inactivating protein noncovalently bound to monoclonal antibody. *J. Pharm. Sci.* 83, 206–211.
- Conde, F. P., Orlandi, R., Canevari, S., Mezzanzanica, D., Ripamonti, M., Munoz, S. M., Jorge, P., and Colnaghi, M. I. (1989) The Aspergillus toxin restriction is a suitable cytotoxic agent for generation of immunoconjugates with monoclonal antibodies directed against human carcinoma cells. *Eur. J. Biochem.* 178, 795–802.
- Orlandi, R., Canevari, S., Conde, F. P., Leoni, F., Mezzanzanica, D., Ripamonti, M., and Colnaghi, M. I. (1988) Immunoconjugate generation between the ribosome inactivating protein restrictocin and an anti-human breast carcinoma mAb. *Cancer Immunol. Immunother.* 26, 114–120.
- Rathore, D., and Batra, J. K. (1997) Cytotoxic activity of ribonucleolytic toxin restrictocin-based chimeric toxins targeted to epidermal growth factor receptor. *FEBS Lett.* 407, 275–279.
- Endo, Y., and Wool, I. (1982) The site of action of α -sarcin on eukaryotic ribosomes: the sequence at the α -sarcin cleavage site in 28S ribosomal ribonucleic acid. *J. Biol. Chem.* 257, 9054–9060.
- Parente, D., Raucci, P., Celano, B., Pacilli, A., Zanoni, L., Canevari, S., Adobati, E., Colnaghi, M. I., Dosio, F., Arpicco, S., Cattell, L., Mele, A., and De Santis, R. (1996) Clavin, a type I ribosome-inactivating protein from Aspergillus Clavatus IFO 8605. cDNA isolation, heterologous expression and recombinant protein characterization. *Eur. J. Biochem.* 239, 272–280.
- Centis, F., Tagliabue, E., Uppungumduri, S., Pellegrini, R., Martignone, S., Mastroianni, A., Menard, S., and Colnaghi, M. I. (1992) p185 Her2/neu epitope mapping with murine monoclonal antibodies. *Hybridoma* 11, 267–276.
- Di Fiore, P. P., Segatto, O., Taylor, W. G., Aaronson, S. A., and Pierce, J. H. (1990) EGF receptor and erbB-2 tyrosine kinase domains confer cell specificity for mitogenic signaling. *Science* 248, 79–83.
- Felip, E., Del Campo, J. M., Rubio, D., Vidal, M. T., Colomer, R., and Bermejo, B. (1995) Overexpression of c-erbB-2 in epithelial ovarian cancer. Prognostic value and relationship with response to chemotherapy. *Cancer* 75, 2147–2152.
- Wels, W., Harwerth, I. M., Mueller, M., Groner, B., and Hynes, N. E. (1992) Selective inhibition of tumor cell growth by a recombinant single-chain antibody-toxin specific for the erbB-2 receptor. *Cancer Res.* 52, 6310–6317.
- Batra, J. K., Kasprzyk, P. G., Bird, R. E., Pastan, I., and King, C. R. (1992) Recombinant anti-erbB2 immunotoxins containing Pseudomonas exotoxin. *Proc. Natl. Acad. Sci. U.S.A.* 89, 5867–5871.
- Di Lazzaro, C., Digiesi, G., Tecce, R., Lotti, L. V., Torrisi, M. R., and Natali, P. G. (1994) Immunotoxins to the HER-2 oncogene product: functional and ultrastructural analysis of their cytotoxic activity. *Cancer Immunol. Immunother.* 39, 318–324.
- Masuh, Y., Kishida, K., Saito, M., Umemoto, N., and Hara, T. (1982) Importance of the antigen-binding valency and the nature of the cross-linking bond in ricin A-chain conjugates with antibody. *J. Biochem.* 91, 1583–1591.
- Worrel, N. R., Cumber, A. J., Parnell, G. D., Ross, W. C., and Forrester, J. A. (1986) Fate of an antibody-ricin A chain conjugate administered to normal rats. *Biochem. Pharmacol.* 35, 417–423.
- Thorpe, P. E., Blakey, D. C., Brown, A. N. F., Knowles, P. P., Knyba, R. E., Wallace, P. M., Watson, G. J., and Wawrzyniak, E. J. (1987) Comparison of two anti-Thy 1.1-abrin A-chain immunotoxins prepared with different cross-linking agents: antitumor effects, in vivo fate, and tumor cell mutants. *J. Natl. Cancer Inst.* 79, 1101–1111.

- (23) Arpicco, S., Dosio, F., Brusa, P., Crosasso, P., and Cattel, L. (1997) New coupling reagents for the preparation of disulfide cross-linked conjugates with increased stability. *Bioconjugate Chem.* 8, 327-337.
- (24) Carroll, S. F., Bernhard, S. L., Goff, D. A., Bauer, R. J., Leach, W., and Kung, A. H. C. (1994) Enhanced stability in vitro and in vivo of immunoconjugate prepared with 5-methyl-2-iminothiolane. *Bioconjugate Chem.* 5, 248-256.
- (25) Higgins, W., and Miles, E. W. (1978) Affinity labeling of the pyridoxal phosphate binding site of the beta-2-subunit of *Escherichia coli* tryptophan synthase. *J. Biol. Chem.* 253, 4648-4652.
- (26) Duncan, R. J. S., Weston, P. D., and Wrigglesworth, R. (1983) A new reagent which may be used to introduce sulfhydryl groups into proteins, and its use in the preparation of conjugates for immunoassay. *Anal. Biochem.* 132, 68-73.
- (27) Grossbard, M. L., Gribben, J. G., Freedman, A. S., Lambert, J. M., Kinsella, J., Rabinowe, S. N., Eliseo, L., Taylor, J. A., Blattler, W. A., Epstein, C. L., and Nadler, L. M. (1993) Adjuvant immunotoxin therapy with anti-B4-blocked ricin after autologous bone marrow transplantation for patients with B-cell non-Hodgkin's lymphoma. *Blood* 81, 2263-2271.
- (28) Grossbard, M. L., Lambert, J. M., Goldmacher, V. S., Spector, N. L., Kinsella, J., Eliseo, L., Coral, F., Taylor, J. A., Blattler, W. A., Epstein, C. L., and Nadler, L. M. (1993) Anti-B4-blocked ricin: a phase II trial of 7-day continuous infusion in patients with B-cell neoplasms. *J. Clin. Oncol.* 11, 726-737.
- (29) Pai, H. L., Wittes, R., Setser, A., Willingham, M. C., and Pastan, I. (1996) Treatment of advanced solid tumors with immunotoxin LMB-1: an antibody linked to *Pseudomonas* exotoxin. *Nat. Med.* 2, 350-354.
- (30) Francisco, J. A., Schreiber, G. J., Comerreski, C. R., Mezza, L. E., Warner, G. L., Davidson, T. J., Ledbetter, J. A., and Siegall, C. B. (1997) In vivo efficacy and toxicity of a single-chain immunotoxin targeted to CD40. *Blood* 89, 4493-4500.
- (31) Brusa, P., Dosio, F., Pietribiasi, P., Delprino, L., Feraioni, M., Mariani, M., Bussolati, G., and Cattel, L. (1992) Antitumor activity of a sterically blocked ricin immunotoxin on a human colorectal adenocarcinoma grafted subcutaneously in nude mice. *Cancer Immunol. Immunother.* 35, 373-380.
- (32) Lambert, J. M., Goldmacher, V. S., Collinson, A. R., Nadler, L. M., and Blattler, W. A. (1991) An immunotoxin prepared with blocked ricin: a natural plant toxin adapted for therapeutic use. *Cancer Res.* 51, 6236-6242.
- (33) Dougall, W. C., and Greene, M. I. (1994) Biological studies and potential therapeutic applications of monoclonal antibodies and small molecules reactive with the neu/c-erbB-2 protein. *Cell Biophys.* 24-25, 209-218.
- (34) Tecce, R., Digiesi, G., Savarese, A., Trizio, D., and Natali, P. G. (1993) Characterization of cytotoxic activity of saporin anti-gp185/HER-2 immunotoxins. *Int. J. Cancer* 55, 122-127.
- (35) Wels, W., Beerli, R., Hellmann, P., Schmidt, M., Marte, B. M., Kornilova, E. S., Hekele, A., Mendelsohn, J., Groner, B., and Hynes, N. E. (1995) EGF receptor and p185erbB-2-specific single-chain antibody toxins differ in their cell-killing activity on tumor cells expressing both receptor proteins. *Int. J. Cancer* 60, 137-144.
- (36) Strocchi, P., Barbieri, L., and Stirpe, F. (1992) Immunological properties of ribosome-inactivating proteins and a saporin immunotoxin. *J. Immunol. Methods* 155, 57-63.
- (37) Rybak, S. M., and Youle, R. J. (1991) Clinical use of immunotoxins. Monoclonal antibodies conjugated to protein toxins. *Immunol. Allergy Clin. North Am.* 11, 359-365.
- (38) Benigni, F., Canevari, S., Gadina, M., Adobati, E., Ferrerei, A. J. M., Ferraris di Celle, E., Comolli, R., and Colnaghi, M. I. (1995) Preclinical evaluation of the ribosome inactivating proteins PAP-1, PAP-S and RTA in mice. *Int. J. Immunopharmacol.* 17, 829-839.
- (39) Stirpe, F., Derenzini, M., Barbieri, L., Fararbegoli, F., Brown, A. N. F., Knowles, P. P., and Thorpe, P. E. (1987) Hepatotoxicity of immunotoxins made by saporin, a ribosome inactivating protein from *Saponaria officinalis*. *Virchows Arch.* 53, 259-271.
- (40) Lambert, J. M., Senter, P. D., Yau-Young, A., Blattler, W. A., and Goldmacher, V. S. (1985) Purified immunotoxins that are reactive with human lymphoid cells. *J. Biol. Chem.* 260, 12035-12043.
- (41) Cumber, A. J., Henry, V. H., Parnell, G. D., and Wawrzynczak, E. J. (1990) Purification of immunotoxins containing the ribosome-inactivating protein gelonin and momordin using high performance liquid immunoaffinity chromatography compared with Blue Sepharose CL-6B affinity chromatography. *J. Immunol. Methods* 135, 15-24.
- (42) Knowles, P. P., and Thorpe, P. E. (1987) Purification of immunotoxins containing ricin A-chain and abrin A-chain using blue sepharose CL-6B. *Anal. Biochem.* 160, 440-446.
- (43) Dhanabal, M., Fryxell, D. K., and Ramakrishnan, S. (1995) A novel method to purify immunotoxins from free antibodies using modified recombinant toxins. *J. Immunol. Methods* 182, 165-175.
- (44) Wawrzynczak, E. J., and Thorpe, P. E. (1988) Effect of chemical linkage upon the stability and cytotoxic activity of A chain immunotoxins. In *Immunotoxins* (A. E. Frankel, Ed.) pp 239-251, Kluwer, Boston.
- (45) Masuda, K., Takahashi, K., Hirano, K., and Takagishi, Y. (1994) Selective antitumor effect of thioether-linked immunotoxins composed of gelonin and monoclonal antibody to alpha-fetoprotein or its F(ab')₂ fragment. *Tumor Biol.* 15, 175-183.

BC970192W

Temperature-Dependent Associating Property of Liposomes Modified with a Thermosensitive Polymer

Hiroshi Hayashi,[†] Kenji Kono,^{*,‡} and Toru Takagishi[‡]

Shiga Central Research Laboratories, Noevir, Company, Ltd., Yokaichi, Shiga 527, Japan, and Department of Applied Materials Science, College of Engineering, and Department of Applied Bioscience, Research Institute for Advanced Science and Technology, Osaka Prefecture University, Sakai, Osaka 593, Japan. Received August 6, 1997; Revised Manuscript Received December 1, 1997

Novel temperature-sensitive liposomes having surface properties that can be controlled by temperature were designed as liposomes coated with poly(*N*-isopropylacrylamide), which exhibits a hydrated coil to dehydrated globule transition at ca. 32 °C. To obtain the polymer with anchoring groups to the liposome, a copolymer of *N*-isopropylacrylamide and octadecyl acrylate (99:1, mol/mol) was synthesized by radical copolymerization. The copolymer revealed the transition near 30 °C. Liposomes made from various phospholipids were prepared by sonication and coated with the copolymer. When dipalmitoylphosphatidylcholine and distearoylphosphatidylcholine were used as liposome lipids, remarkable aggregation and fusion of the copolymer-modified liposomes took place between the transition temperature of the copolymer and the gel–liquid-crystalline transition temperature (T_c) of the lipid membranes. However, above the T_c , association between the liposomes was much less significant, although the copolymer is still hydrophobic. In the case of the copolymer-modified dilauroylphosphatidylcholine liposome, the membrane of which takes on a liquid-crystalline state under the experimental conditions, association between the liposomes also hardly occurred even when the copolymer became hydrophobic. On the other hand, below the transition temperature of the copolymer, the copolymer-modified liposomes were very stable and aggregation of the liposomes was not observed, irrespective of membrane lipid. Results obtained in this study demonstrate that the copolymer chains fixed on the surface of the liposome with a solid membrane promote aggregation and fusion of the liposomes by hydrophobic interactions between the copolymer chains and/or between the copolymer chains and the liposome membranes above the transition temperature of the copolymer.

INTRODUCTION

Since liposomes made from naturally occurring lipids are biocompatible, their application to drug delivery systems has been extensively attempted (1). To elevate their usefulness, a number of liposomes with various functionalities have been designed. For example, with respect to site specific delivery of drugs, liposomes that release their contents in response to temperature (2, 3), pH (4–6), and light (7–9) have been reported. Also, for cytoplasmic delivery of membrane-impermeable molecules, fusogenic liposomes have been developed (10–14). These liposomes are considered to fuse with the plasma membrane or the endosomal membrane and release their contents into the cytoplasm (14–16). Therefore, fusogenic liposomes are of importance as a carrier of oligonucleotides, nucleic acids, and proteins.

While several approaches to the production of liposomes with functionalities have been attempted, one of the most effective methods is their modification with polymers (17, 18). It was shown that coating of liposomes with polysaccharides improves the stability of the liposomes (19). Also, conjugation of synthetic polymers with

pH sensitivity onto liposomes was shown to provide them with pH-sensitive release and fusion properties (5, 13).

Recently, to obtain liposomes with temperature sensitivity, we (3, 20) and other groups (21–23) designed liposomes modified with poly(*N*-isopropylacrylamide) [poly(NIPAM)].¹ It is well-known that poly(NIPAM) is soluble in water and takes on a hydrated coil state below ca. 32 °C, whereas the polymer is insoluble in water and exhibits a dehydrated globule state above this temperature (24). Already, we have demonstrated that release of contents from the poly(NIPAM)-coated liposomes is enhanced above the coil–globule transition temperature of the polymer (3, 20).

So far, we have focused on the temperature-sensitive release property of the poly(NIPAM)-coated liposomes. However, fixation of the temperature-sensitive polymer onto the liposome surface is also considered to render the nature of the liposome surface temperature-sensitive. The highly hydrated polymer chains will cover the liposome surface below the transition temperature of the polymer. However, above the polymer transition temperature, the polymer chains become hydrophobic and will adsorb onto

* Address correspondence to this author at the Department of Applied Materials Science, College of Engineering, Osaka Prefecture University, 1-1 Gakuen-cho, Sakai, Osaka 593, Japan (telephone +81-722-52-1161; fax +81-722-59-3340; e-mail kono@chem.osakafu-u.ac.jp).

[†] Noevir Co. Ltd.

[‡] Osaka Prefecture University.

¹ Abbreviations: poly(NIPAM), poly(*N*-isopropylacrylamide); copoly(NIPAM–ODS), copolymer of *N*-isopropylacrylamide and octadecyl acrylate; DPPC, 1,3-bis(sn-3'-phosphatidyl)-sn-glycero-3-phosphocholine; DLPC, 1,3-bis(sn-3'-phosphatidyl)-sn-glycero-3-phosphocholine; DSPC, 1,3-bis(sn-3'-phosphatidyl)-sn-glycero-3-phosphocholine; NBD-PE, *N*-(7-nitro-2-benzoxa-1,3-diazol-4-yl)phosphatidylethanolamine; Rh-PE, *N*-(1-issamine rhodamine B sulfonyl)phosphatidylethanolamine; AIBN, azobisisobutyronitrile.

the liposome surface, making the surface hydrophobic. Thus, it is expected that hydrophilicity or hydrophobicity of the liposome surface can be controlled by the ambient temperature. Also, interaction of dehydrated polymer chains with the liposome membrane might reduce the integrity of the membrane structure above the transition temperature of the polymer, which may increase the fusion ability of the liposome.

To examine the influence of surface modification of liposomes with the temperature-sensitive polymer on the nature of the liposomes, we have synthesized a copolymer of NIPAM and octadecylacrylate [copoly(NIPAM-ODA)] as poly(NIPAM) having anchors to liposomal membranes and prepared liposomes coated with the copolymer. Temperature-dependent aggregation and fusion of the copolymer-attached liposomes have been reported.

EXPERIMENTAL PROCEDURES

Materials. L- α -Dipalmitoylphosphatidylcholine (DPPC), L- α -dilauroylphosphatidylcholine (DLPC), and L- α -distearoylphosphatidylcholine (DSPC) were purchased from Sigma. *N*-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)phosphatidylethanolamine (NBD-PE) and *N*-(lissamine rhodamine B sulfonyl)phosphatidylethanolamine (Rh-PE) were obtained from Avanti Polar Lipids. *N*-Isopropylacrylamide (NIPAM), octadecylacrylate (ODA), and azobis(isobutyronitrile) (AIBN) were from Tokyo Kasei. NIPAM was purified by recrystallization from cyclohexane/*n*-hexane (1:1) before use.

Synthesis of Copoly(NIPAM-ODA). Copoly(NIPAM-ODA) was synthesized as reported previously (3). In brief, NIPAM (9.9 mmol), ODA (0.1 mmol), and AIBN (2.4×10^{-2} mmol) were dissolved in tetrahydrofuran. The solution was degassed with N_2 and kept at 60 °C for 4 h. The copolymer was recovered by precipitation with diethyl ether. The copolymer was dissolved in tetrahydrofuran and reprecipitated with diethyl ether. The copolymer was dissolved in water and lyophilized.

Preparation of Liposomes. Liposomes were prepared as follows: a dry thin membrane of each of the phosphatidylcholines (10 mg) was dispersed in 1.0 mL of 10 mM Tris-HCl-buffered solution of pH 7.4 containing 140 mM NaCl. The lipid dispersion was sonicated for 10 min using a probe-type ultrasonic disruptor (Tomy Seiko, UR-20P). To the liposome suspension (1 mL) was added 0.5 mL of an aqueous solution in which 1 or 5 mg of the copolymer was dissolved, and the mixture was then incubated for 4 h at 5 °C with stirring. Free copolymer was removed by gel permeation chromatography on a Sephacryl S-400 column at 10 °C.

Estimation of the Amount of Copolymer Bound onto Liposome. The amount of the copolymer bound onto the liposome was estimated by high-performance liquid chromatography (HPLC) analysis on an Asahipak GF-310HQ column using methanol as an effluent, as reported previously (20). The copolymer-coated liposome was dried under reduced pressure and then dissolved in methanol. The solution (20 μ L) was injected into the column, and the effluent was monitored by absorbance at 220 nm. For calibration, the copolymer solutions of given concentrations were also injected. From the absorbance of the copolymer separated, the amount of copolymer adsorbed onto the liposome was determined using the calibration curve.

Size Change of Liposomes. Liposomes were prepared as mentioned above except that contamination of large multilamellar liposomes was eliminated by cen-

trifugation (18 000 rpm, 40 min) before incubation with the copolymer. The liposomes were suspended in 10 mM Tris-HCl and 140 mM NaCl solution (0.1 mg of lipid/mL) at pH 7.4 at desired temperatures for 3 min. Mean diameter of the liposome was measured immediately after the incubation or after an additional 10 min of incubation at 20 °C using a laser particle analyzing system (Otsuka Electronics, LPA 3100S).

Fusion of Liposomes. Fusion of liposomes was detected as reported previously (13, 25) by measuring resonance energy transfer between NBD-PE and Rh-PE as reported by Struck et al. (26). Liposomes containing 0.3 mol % of NBD-PE and Rh-PE were prepared according to the above method. The liposomes containing the fluorescent lipids were mixed in a 1:1 mole ratio with probe-free liposomes. The suspension (0.1 mg of lipid/mL) was excited at 475 nm, and fluorescence emission spectra were measured at various time intervals. The *R* value, the ratio of fluorescence intensity at 532 nm to that at 591 nm, was calculated. The ratio was converted into apparent concentration of the fluorophores in the membrane using a standard curve of the reciprocal of the concentration of the fluorophores vs *R* value. The percent fusion was defined as

$$\% \text{ fusion} = (C^0 - C^i)/(C^0 - C^f)$$

where C^0 and C^i are the initial and intermediate concentrations of the fluorophores, respectively. C^f , which equals 0.15 mol % of each fluorophore, represents the concentration at which complete fusion of the liposomes occurs.

Electron Microscopy. The copolymer-modified and the bare DPPC liposomes were suspended in 10 mM Tris-HCl and 140 mM NaCl solution (pH 7.4). The liposome samples were heated at 39 °C for 3 min and returned to room temperature (ca. 20 °C). A small drop of the liposome sample was placed on collodion-coated grids and drawn off with filter paper. A drop of 2% (w/v) phosphotungstic acid was applied to the grid, drawn off with filter paper, and then allowed to dry. The grids were viewed under an electron microscope (JEOL, JEM-2000FEX II).

Other Methods. Nuclear magnetic resonance (NMR) spectra were taken with a JEOL JNM-GX 270 MHz instrument. Differential scanning calorimetry (DSC) was performed with a Seiko Electronics DSC 120 microcalorimeter. The samples were analyzed at a heating rate of 0.5 °C/min. Phospholipid concentration was measured by an assay using Phospholipids B-Test Wako purchased from Wako Pure Chemical Industries. The molecular weight of the copolymer was estimated by HPLC analysis on a Shodex KD-803 column (Showa Denko) using *N,N*-dimethylformamide.

RESULTS AND DISCUSSION

Characterization of Copoly(NIPAM-ODA). To obtain poly(NIPAM) with anchors to liposomal membranes, we synthesized the copolymer of NIPAM and ODA, because a number of studies have shown that hydrophilic polymers with a few hydrophobic groups are fixed onto the liposome membranes through hydrophobic interactions (13, 17, 27). We have previously demonstrated that the NIPAM-ODA copolymer binds onto DPPC and egg yolk phosphatidylcholine liposomes (3).

The copolymer used here was evaluated to contain NIPAM and ODA units in the ratio of 99:1 (mol/mol) by ^1H NMR from the ratio of isopropyl groups in the NIPAM unit (δ 1.2 ppm) to *n*-alkyl groups in the ODA unit (δ 1.3

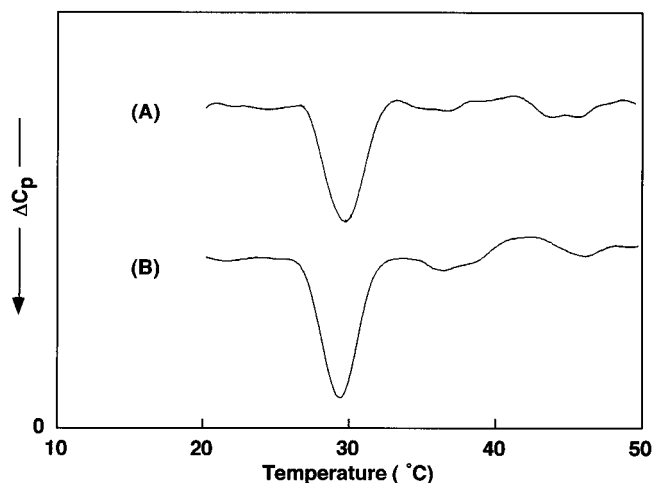


Figure 1. Microcalorimetric endotherms for aqueous copolymer samples in the absence (A) and in the presence (B) of egg yolk phosphatidylcholine liposome: (A) copolymer solution (0.4 wt %); (B) copolymer solution containing egg yolk phosphatidylcholine liposome (0.4 wt %). The samples (70 μ L) were heated at 0.5 $^{\circ}$ C/min. ΔH for curves (A) and (B) are 4.2 and 4.1 cal/g of the copolymer, respectively.

ppm). The weight-average molecular weight, the number-average molecular weight, and the heterogeneity of the copolymer were determined by gel permeation chromatography to be 12200, 5600, and 2.2, respectively.

It was shown by Schild and Tirrell (28) that the coil-globule transition of poly(NIPAM) can be detected by DSC. Therefore, a DSC measurement was performed to estimate the coil-globule transition temperature of the copolymer (Figure 1). DSC curves of the copolymer in water and in the presence of egg yolk phosphatidylcholine liposomes displayed endotherms with a peak centered at 29.4 $^{\circ}$ C and with a peak centered at 29.7 $^{\circ}$ C, respectively. These values are somewhat lower than the transition temperature of homopolymer of NIPAM, possibly due to the existence of ODA units in the copolymer. It has been previously reported that incorporation of hydrophobic groups into poly(NIPAM) lowers the transition temperature of poly(NIPAM) in water (29).

Fixation of Copoly(NIPAM-ODA) onto Liposomes. In this study, DPPC, DSPC, and DLPC were used as liposomal lipids because membranes of these lipids exhibit the gel-liquid-crystalline phase transition at different temperatures. Liposomes were prepared by sonication and coated with copoly(NIPAM-ODA) by incubation of the liposomes with the copolymer solutions and subsequent gel filtration on a Sephacryl S-400 column. Typical examples of the elution profiles for a DPPC liposome dispersion (A), for the copolymer solution (B), and for DPPC liposomes suspended in the copolymer solution (C) are shown in Figure 2. As is apparent in Figure 2A,B, the liposomes passed through the column more rapidly than the copolymer. Therefore, free copolymer can be removed from the copolymer-attached liposomes by using this column. We collected the effluent of the elution band shown in Figure 2C and used it for experiments.

The amount of the copolymer attached onto the liposome after the gel filtration was quantified by HPLC analysis on an Asahipak column for gel permeation chromatography using methanol as the eluent. Figure 3 shows typical examples of the chromatograms for the copolymer (A), DPPC (B), and copolymer-fixed DPPC liposomes (C). When an aliquot of the copolymer solution was injected into the column, two peaks (7.8 and 13.7

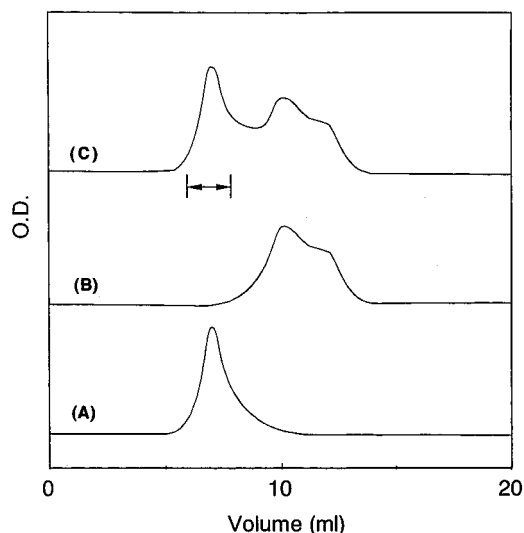


Figure 2. Typical elution profiles through a Sephacryl S-400 column for DPPC liposomes (6.7 mg/mL) (A), copolymer (3.3 mg/mL) (B), and DPPC liposomes incubated in copolymer solution (lipid = 6.7 mg/mL; copolymer = 3.3 mg/mL). Optical density at 220 nm was monitored. The samples (0.5 mL) were loaded onto the column. Flow rate of the effluent was 1 mL/min.

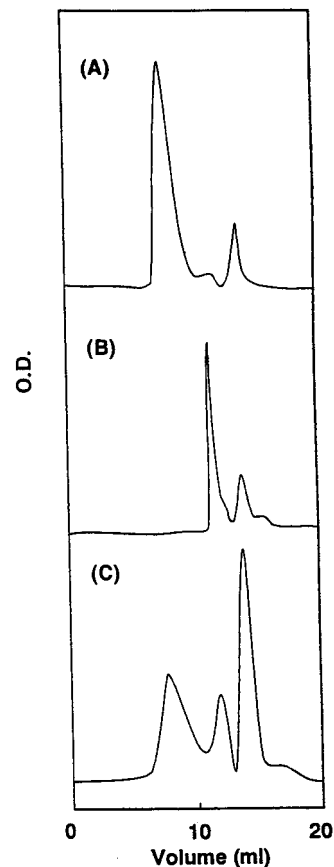


Figure 3. Typical elution profiles through an Asahipak column for copolymer (0.1 mg/mL) (A), DPPC (0.7 mg/mL) (B), and copolymer-fixed DPPC liposome (0.3 mg DPPC/mL) (C). Sample solutions (20 μ L) were injected into the column and eluted with methanol at 0.8 mL/min.

mL) were observed in the chromatogram as shown in Figure 3A. The first and second peaks are attributable to the copolymer and solvent, respectively. It was confirmed that area of the first peak is proportional to the concentration of the copolymer solution under the experimental conditions. When an aliquot of the lipid

Table 1. Preparation of Copoly(NIPAM–ODA)-Modified Liposomes

liposome	copoly(NIPAM–ODA) added (mg/mg of lipid)	copoly(NIPAM–ODA) bound (mg/mg of lipid)	NIPAM unit lipid (mol/mol)
DPPC 1	0.1	0.06	0.38
DPPC 2	0.5	0.16	1.01
DLPC	0.5	0.20	1.07
DSPC	0.5	0.15	1.02

solution was applied into the column, a new peak at 11.4 mL was seen in addition to the solvent peak (Figure 3B). As shown in Figure 3C, the chromatogram for the copolymer-fixed liposomes exhibited three peaks near 7.8, 11.4, and 13.7 mL, corresponding to the copolymer, lipids, and solvent, respectively. The amount of copolymer fixed on the liposomes was evaluated from the chromatograms using a calibration curve that shows the relationship between the area of the copolymer peak and the amount of copolymer.

When DPPC liposomes were incubated with the copolymer in the ratio 0.1:1 (copolymer/lipid, w/w), 0.06 mg of the copolymer was adsorbed onto 1 mg of the liposomal lipid. By increasing the copolymer/lipid ratio to 0.5:1 (w/w) during the incubation, the amount of copolymer adsorbed onto the DPPC liposomes increased to 0.16 mg/mg of lipid. The amounts of copolymer adsorbed on various liposomes are listed in Table 1. When these liposomes are incubated with the copolymer in the ratio 0.5:1 (copolymer/lipid, w/w), the numbers of NIPAM units adsorbed per lipid molecule for DLPC, DPPC, and DSPC liposomes are 1.07, 1.01, and 1.02, respectively, indicating that density of the copolymer on the liposome surface is nearly identical among these liposomes, despite the difference in their membrane fluidities.

Temperature-Dependent Association of Copoly(NIPAM–ODA)-Fixed Liposomes. To investigate the effect of temperature on association of the copolymer-modified liposomes, we first examined size change of liposomes depending on temperature. Liposomes were kept at various temperatures for 3 min, and then the temperature was changed to 20 °C. After 10 min of standing at 20 °C, the diameter of the liposomes was measured. The result is shown in Figure 4. The sizes of the bare DPPC and the bare DSPC liposomes hardly change throughout the range of temperatures we investigated. Also, the copolymer-modified DPPC and the copolymer-modified DSPC liposomes do not change their mean diameters below 30 °C, at which the copolymer bound on the liposomes is hydrophilic.

However, above this temperature when the copolymer is dehydrated and becomes hydrophobic, the size of these liposomes increases greatly. For the copolymer-modified DPPC liposome, a substantial increase in diameter was observed around 39 °C. This temperature appears to be slightly lower than the gel–liquid-crystalline phase transition temperature of DPPC membranes (ca. 41–42 °C) (30). However, it is known that DPPC liposomes made by sonication exhibit the phase transition in a temperature region lower than 41 °C because the membranes of the sonicated liposomes contain packing inhomogeneities caused by their high curvature (31). Thus, it is considered that aggregation of the copolymer-fixed DPPC liposomes is induced most intensively at their phase transition temperature. At the phase transition temperature, the gel and liquid-crystalline phases coexist in the membrane, and hence the membrane contains structural defects. Interaction of the copolymer with the liposome membrane should promote further generation

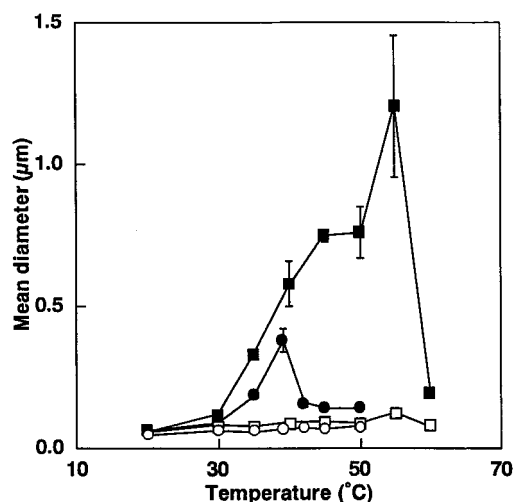


Figure 4. Mean diameter of various liposomes: (●) copolymer-modified DPPC liposome; (○) bare DPPC liposome; (■) copolymer-modified DSPC liposome; (□) bare DSPC liposome. The liposomes were kept at various temperatures for 3 min and followed by incubation at 20 °C for 10 min, and then diameters of the liposomes were measured at 20 °C. The copolymer-modified DPPC liposome 2 was used (see Table 1).

of defects in the membrane and destabilize the liposome. In addition to the hydrophobic interactions between the copolymers fixed on the liposomes, instability of the defective liposomes might enhance their aggregation, resulting in a significant increase in vesicle size.

In contrast, the increase of the vesicle size was much less significant above 42 °C. It is well-known that liposomes are more stable above the phase transition temperature than below it, where fusion of small vesicles takes place more readily (32).

On the other hand, since the copolymers fixed on the liposome are laterally mobile on the membrane surface above the gel–liquid-crystalline phase transition temperature, they will form aggregates through hydrophobic interactions within the membrane. In addition to the stability of liposomes with a fluid membrane, the difference in mobility of the copolymer on the membrane may also affect the potential of the liposome for association, as mentioned below.

A similar trend can be seen in the case of the copolymer-modified DSPC liposome, except that the most intensive increase in vesicle size occurred near 55 °C, which corresponds to the phase transition temperature of DSPC membrane. These results clearly demonstrate that the size increases of copolymer-modified liposomes are influenced by the fluidity of the liposome membrane as well as the hydrophobicity of the copolymer.

Because the copolymer alters its nature between hydrophobic and hydrophilic states reversibly, responding to the ambient temperature, it is expected that association and dissociation of the copolymer-modified liposomes occur reversibly. Therefore, the reversibility of this process for the copolymer-modified DPPC liposomes was examined. We measured the size of the liposomes immediately after a 3 min incubation at various temperatures, as well as after an additional incubation for 10 min at 20 °C. The results are shown in Figure 5. While in both cases the diameter of the liposome increases significantly near 40 °C, a much greater diameter was observed for the liposomes without additional incubation at 20 °C. This result indicates that liposomes which are associated through hydrophobic

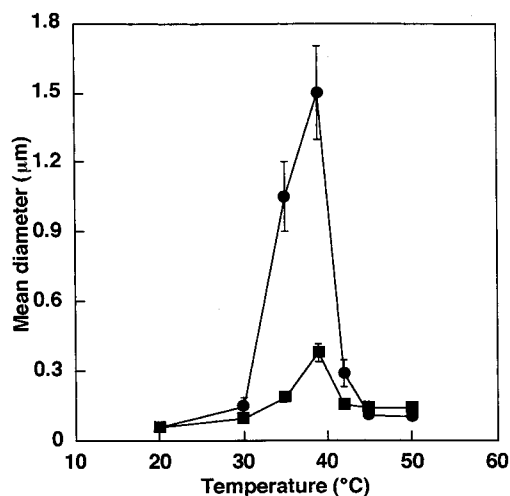


Figure 5. Mean diameter of the copolymer-modified DPPC liposomes. Diameters of the liposomes were measured immediately after 3 min of incubation at various temperatures (●) or after an additional 10 min of incubation at 20 °C (■). The copolymer-modified DPPC liposome 1 was used (see Table 1).

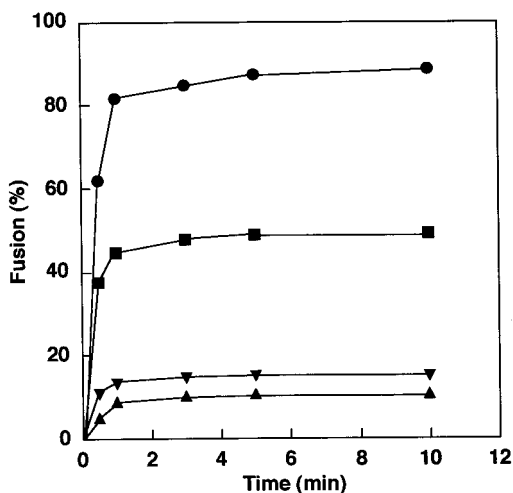


Figure 6. Time courses of fusion of the copolymer-modified DPPC liposomes in 10 mM Tris-HCl-buffered solution containing 140 mM NaCl at various temperatures: (▲) 20 °C; (▼) 30 °C; (●) 39 °C; (■) 45 °C. The copolymer-modified DPPC liposome 1 was used.

interactions between the copolymers can be dissociated when the ambient temperature is lowered below the transition temperature of the copolymer. However, the liposome does not return to its original size, ca. 60 nm, during the 10 min cooling at 20 °C, suggesting that irreversible association of the liposomes also occurs at the same time.

Temperature-Dependent Fusion of Copoly-(NIPAM-ODA)-Fixed Liposomes. As mentioned above, irreversible association between the copolymer-modified liposomes was observed. As such association, fusion of the liposomes is most likely. Thus, we examined the occurrence of fusion by the resonance energy transfer method (26).

Figure 6 shows time courses of fusion of the copolymer-modified DPPC liposomes at various temperatures. Apparently, fusion of the liposomes depends on temperature. The liposomes hardly fuse at 20 °C, whereas intensive fusion takes place at 39 °C, at which a significant increase in vesicle size occurs (Figure 4). Fusion of the liposomes is quite fast and is complete within 1 min.

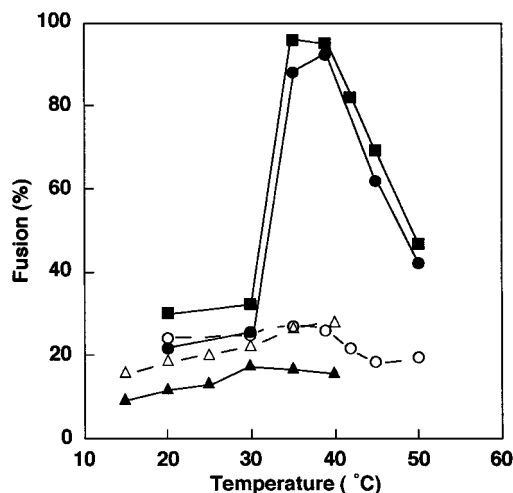


Figure 7. Percent fusion of various liposomes after 5 min of incubation as a function of temperature: (●) copolymer-modified DPPC liposome 1; (■) copolymer-modified DPPC liposome 2; (○) bare DPPC liposome; (▲) copolymer-modified DLPC liposome; (△) bare DLPC liposome.

The temperature dependence of fusion of various liposomes is shown in Figure 7. For the copolymer-modified DPPC liposomes, the liposomes hardly fuse below 30 °C, whereas above this temperature fusion occurs remarkably. Maximum fusion is seen near 39 °C, followed by a gradual decrease at temperature above 41 °C. This temperature dependence of fusion is similar to that of vesicle size shown in Figure 4. Because the bare DPPC liposomes exhibit a low percent of fusion throughout the experimental temperatures, this fusion property of the copolymer-modified liposomes is apparently controlled by the copolymer. Since the liposome membrane contains structural defects near the gel-liquid-crystalline phase transition temperature, the liposomes will be strongly destabilized by interactions with the hydrophobic copolymer. Moreover, the copolymers fixed on the liposomes facilitate aggregation of the liposomes by their hydrophobic interactions. Thus, fusion of the liposomes is promoted strongly near the transition temperature of the membrane.

When DLPC was used as the liposome lipid, enhancement of fusion hardly occurs above 30 °C. Because a DLPC membrane takes on a liquid-crystalline phase under the experimental conditions, it is suggested that the copolymer does not promote fusion of liposome with a fluid membrane.

To confirm the occurrence of fusion of the copolymer-fixed liposomes, the liposomes were observed by TEM. The copolymer-fixed and the bare DPPC liposomes were incubated at room temperature (ca. 20 °C) or at 39 °C for 3 min and then returned to room temperature and kept for 30 min. Typical TEM images of these liposomes are shown in Figure 8. For the bare DPPC liposomes, diameters of 20–70 nm were observed (Figure 8A). No apparent difference in vesicle size was seen between the liposomes incubated at room temperature and those incubated at 39 °C (result not shown). The size distribution of the liposomes is roughly consistent with the mean diameter (60–70 nm) estimated using dynamic light scattering (Figure 4).

In contrast, a significant difference in diameter was seen between the copolymer-fixed liposomes kept at room temperature and those incubated at 39 °C. When the copolymer-fixed liposomes were incubated at room temperature, liposomes with diameter of ~60 nm were

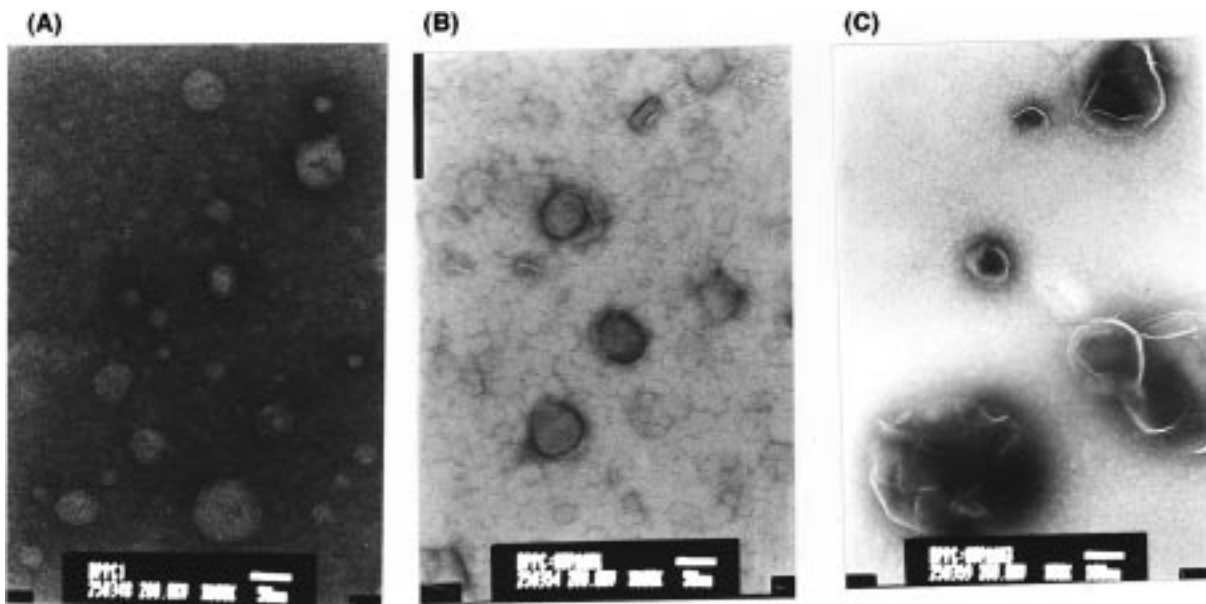


Figure 8. Negative stain electron micrographs of liposome preparations: bare DPPC liposomes incubated at room temperature (A) and copolymer-fixed DPPC liposomes incubated at room temperature (B) and at 39 °C (C). The bars shown in the (A), (B), and (C) represent 50, 50, and 100 nm, respectively.

observed as shown in Figure 8B. This diameter is similar to that of the bare DPPC liposomes. However, the size of the liposomes treated at 39 °C was quite different. The diameter of a large fraction of the liposomes was much >100 nm (Figure 8C). These results clearly indicate that fusion of the copolymer-fixed liposomes occurred during the incubation at 39 °C.

Temperature-Dependent Associating Property of the Copolymer-Modified Liposomes. As mentioned above, association of the copolymer-modified liposomes was induced above the copolymer transition temperature where the copolymer is hydrophobic. Therefore, it is considered that hydrophobic interactions play an important role in this association. However, association of the copolymer-modified liposomes is affected by the membrane fluidity of the liposomes. This association occurred only at and below the gel–liquid-crystalline transition temperature of the liposome membrane. When the copolymer is bound on the solid membrane, it may exist on the surface of the liposomes even above the transition temperature of the copolymer. However, when the copolymer is bound on the fluid membranes, it should penetrate more easily into the membrane. Therefore, the dehydrated copolymer is considered to be buried partly in the lipid membrane and may not interact with other liposomes effectively.

Ringsdorf et al. showed that hydrophobically modified poly(NIPAM) fixed on a liposome shrinks more efficiently on the surface of the liposome with a liquid-crystalline membrane than on the surface of the liposomes with a gel membrane, because the lateral diffusion of the hydrophobic groups inserted into the membrane is precluded in the gel membrane (33). Similarly, in this study, when the copolymer is fixed on the liposome with a fluid membrane, the copolymer will take a compact conformation above its transition temperature and, as a result, the bare lipid surface will be exposed. However, when the copolymer is fixed on the liposome with a solid membrane, the liposome surface will be covered more effectively by the hydrophobic copolymer chains. The position of the copolymer might also affect the association of the copolymer-modified liposomes, as indicated schematically in Figure 9.

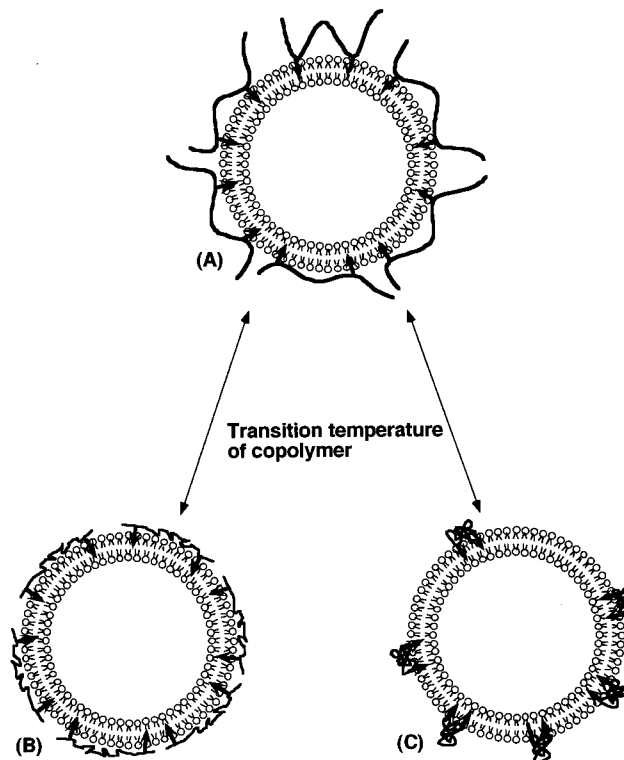


Figure 9. Schematic illustration of temperature-dependent associating property of the copolymer-modified liposomes. Below the copolymer transition temperature, the highly hydrated copolymers cover the liposome surface and stabilize liposome (A). Above this temperature, the liposome surface is covered with the dehydrated copolymers, which enhance association of the liposomes when the liposome has a solid membrane (B). When the copolymer is fixed on a liposome with a fluid membrane, the copolymers shrink effectively and the bare surface is exposed (C).

In addition, it is well-known that the gel phase and liquid-crystalline phase coexist in lipid membranes at the phase transition temperature and that, as a consequence, these membranes contain structural defects. Therefore, it is likely that the dehydrated copolymer destabilized

the liposomes at this temperature and promotes association of liposomes strongly.

CONCLUDING REMARKS

It was found that surface modification of liposomes with copoly(NIPAM-ODA) provided liposomes with a temperature-sensitive associating property: both the aggregation and the fusion of the copolymer-modified DPPC and DSPC liposomes were remarkably enhanced above the transition temperature of the copolymer when these liposomes had a solid membrane.

The copolymer-modified liposomes prepared in this study exhibited temperature response above 30 °C, corresponding to the transition temperature of the copolymer. Since the transition temperature of this type of polymer is controllable by copolymerization with monomers with varying hydrophilicity (34), it is possible to obtain temperature-sensitive polymers with the transition temperature at a desired temperature. Kim et al. recently showed that the transition temperature of copolymers of NIPAM and acrylic acid was elevated as the content of acrylic acid in the copolymers increased. They prepared the copolymer-coated liposomes and showed that release of calcein loaded within the liposomes is enhanced above the transition temperature of the copolymers to some extent (35). In addition, several polymers are known to exhibit transition temperatures slightly higher than physiological temperature (28, 36). If such polymers are fixed on the liposomes, the liposomes are expected to reveal a temperature response near the physiological temperature.

When the liposomes with the temperature-sensitive surface property are injected into the blood stream, their interaction with the blood components, such as plasma proteins and various kinds of cells, should be affected by temperature. Therefore, the distribution of the liposomes in the body may be controlled by temperature. Moreover, the liposomes may deliver their contents into the cytoplasm by fusion with the cellular membranes. Interactions of the liposomes with proteins and cells are currently under investigation.

LITERATURE CITED

- (1) Lasic, D. D. (1993) *Liposomes: From Physics to Applications*, Elsevier, Amsterdam.
- (2) Yatvin, M. B., Weinstein, J. N., Dennis, W. H., and Blumenthal, R. (1978) Design of liposomes for enhanced local release of drugs by hyperthermia. *Science* 202, 1290–1293.
- (3) Kono, K., Hayashi, H., and Takagishi, T. (1994) Temperature-sensitive liposomes: liposomes bearing poly(N-isopropylacrylamide). *J. Controlled Release* 30, 69–75.
- (4) Yatvin, M. B., Kretz, W., Horwitz, B. A., and Shinitzky, M. (1980) pH-Sensitive liposomes: possible clinical implications. *Science* 210, 1253–1255.
- (5) Maeda, M., Kumano, A., and Tirrell, D. A. (1988) H⁺-induced release of contents of phosphatidylcholine vesicles bearing surface-bound polyelectrolyte chains. *J. Am. Chem. Soc.* 110, 7455–7459.
- (6) Nayar, R., and Schriot, A. J. (1985) Generation of pH-sensitive liposomes: use of large unilamellar vesicles containing N-succinyldioleoylphosphatidylethanolamine. *Biochemistry* 24, 5967–5971.
- (7) Pidgeon, C., and Hunt, C. A. (1983) Light sensitive liposomes. *Photochem. Photobiol.* 37, 491–494.
- (8) Frankel, D. A., Lamparski, H., Liman, U., and O'Brien, D. F. (1989) Photoinduced destabilization of bilayer vesicles. *J. Am. Chem. Soc.* 111, 9262–9263.
- (9) Anderson, V. C., and Thompson, D. H. (1992) Triggered release of hydrophilic agents from plasmalogen liposomes using visible light or acid. *Biochim. Biophys. Acta* 1109, 33–42.
- (10) Straubinger, R. M., Düzgünes, N., and Papahadjopoulos, D. (1985) pH-Sensitive liposomes mediate cytoplasmic delivery of encapsulated macromolecules. *FEBS Lett.* 179, 148–154.
- (11) Connor, L., and Huang, L. (1985) Efficient cytoplasmic delivery of a fluorescent dye by pH-sensitive immunoliposomes. *J. Cell Biol.* 101, 582–589.
- (12) Düzgünes, N., Goldstein, J. A., Friend, D. S., and Felgner, L. (1989) Fusion of liposomes containing a novel cationic lipid, N-[2,3-(dioleoyloxy)propyl]-N,N,N-trimethylammonium: induction by multivalent anions and asymmetric fusion with acidic phospholipid vesicles. *Biochemistry* 28, 9179–9184.
- (13) Kono, K., Zenitani, K., and Takagishi, T. (1994) Novel pH-sensitive liposomes: liposomes bearing a poly(ethylene glycol) derivative having carboxyl groups. *Biochim. Biophys. Acta* 1193, 1–9.
- (14) Kono, K., Igawa, T., and Takagishi, T. (1997) Cytoplasmic delivery of calcein mediated by liposomes modified with a pH-sensitive poly(ethylene glycol) derivative. *Biochim. Biophys. Acta* 1325, 143–154.
- (15) Litzinger, D. C., and Huang, L. (1992) Phosphatidylethanolamine liposomes: drug delivery, gene transfer and immunodiagnostic applications. *Biochim. Biophys. Acta* 1113, 201–227.
- (16) Felgner, P. L., and Ringold, G. M. (1989) Cationic liposome-mediated transfection. *Nature* 337, 387–388.
- (17) Ringsdorf, H., Schlarb, B., and Venzmer, J. (1988) Molecular architecture and function of polymeric oriented systems: models for the study of organization, surface recognition, and dynamics of biomembranes. *Angew. Chem., Int. Ed. Engl.* 27, 113–158.
- (18) Thomas, J. L., and Tirrell, D. A. (1992) Polyelectrolyte-sensitized phospholipid vesicles. *Acc. Chem. Res.* 25, 336–342.
- (19) Sunamoto, J., Iwamoto, K., Takada, M., Yuzuriha, T., and Katayama, K. (1983) Improved drug delivery to target specific organs using liposomes as coated with polysaccharides. *Polym. Sci. Technol.* 23, 157–168.
- (20) Hayashi, H., Kono, K., and Takagishi, T. (1996) Temperature-controlled release property of phospholipid vesicles bearing a thermo-sensitive polymer. *Biochim. Biophys. Acta* 1280, 127–134.
- (21) Wu, X. S., Hoffman, A. S., and Yager, P. (1992) Conjugation of phosphatidylethanolamine to poly(N-isopropylacrylamide) for potential use in liposomal drug delivery systems. *Polymer* 33, 4659–4662.
- (22) Kitano, H., Maeda, Y., Takeuchi, S., Ieda, K., and Aizu, Y. (1994) Liposomes containing amphiphiles prepared by using a lipophilic chain transfer reagent: responsiveness to external stimuli. *Langmuir* 10, 403–406.
- (23) Winnik, F. M., Adronov, A., and Kitano, H. (1995) Pyrene-labeled amphiphilic poly(N-isopropylacrylamides) prepared by using a lipophilic radical initiator: synthesis, solution properties in water, and interactions with liposomes. *Can. J. Chem.* 73, 2030–2040.
- (24) Schild, H. G. (1992) Poly(N-isopropylacrylamide): experiment, theory and application. *Prog. Polym. Sci.* 17, 163–249.
- (25) Kono, K., Nishii, H., and Takagishi, T. (1993) Fusion activity of an amphiphilic polypeptide having acidic amino acid residues: generation of fusion activity by α -helix formation and charge neutralization. *Biochim. Biophys. Acta* 1164, 81–90.
- (26) Struck, D. K., Hoekstra, D., and Pagano, R. E. (1981) Use of resonance energy transfer to monitor membrane fusion. *Biochemistry* 20, 4093–4099.
- (27) Sunamoto, J., Sato, T., Taguchi, T., and Hamazaki, H. (1992) Naturally occurring polysaccharide derivatives which behave as an artificial cell wall on an artificial cell liposome. *Macromolecules* 25, 5665–5670.
- (28) Schild, H. G., and Tirrell, D. A. (1990) Microcalorimetric detection of lower critical solution temperatures in aqueous polymer solutions. *J. Phys. Chem.* 94, 4352–4356.
- (29) Schild, H. G., and Tirrell, D. A. (1991) Microheterogeneous solutions of amphiphilic copolymers of N-isopropylacrylamide. An investigation via fluorescence methods. *Langmuir* 7, 1319–1324.

- (30) Hinz, H.-J., and Sturtevant, J. M. (1972) Calorimetric studies of dilute aqueous suspensions of bilayers formed from synthetic L- α -lecithins. *J. Biol. Chem.* 247, 6071–6075.
- (31) Sheetz, M. P., and Chan, S. I. (1972) Effect of sonication on the structure of lecithin bilayers. *Biochemistry* 11, 4573–4581.
- (32) Larrabee, A. L. (1979) Time-dependent changes in the size distribution of distearoylphosphatidylcholine vesicles. *Biochemistry* 18, 3321–3326.
- (33) Ringsdorf, H., Venzmer, J., and Winnik, F. M. (1991) Interaction of hydrophobically modified poly-N-isopropylacrylamides with model membranes—or playing a molecular accordion. *Angew. Chem., Int. Ed. Engl.* 30, 315–318.
- (34) Taylor, L. D., and Cerankowski, L. D. (1975) Preparation of films exhibiting a balanced temperature dependence to permeation by aqueous solution: a study of lower consolute behavior. *J. Polym. Sci., Polym. Chem. Ed.* 13, 2551–2570.
- (35) Kim, J.-C., Bae, S. K., and Kim, J.-D. (1997) Temperature-sensitivity of liposomal lipid bilayers mixed with poly(N-isopropylacrylamide-co-acrylic acid). *J. Biochem.* 121, 15–19.
- (36) Kim, Y. J., Yamada, K., Ito, H., Kono, K., and Takagishi, T. (1990) Interaction of copolymers of N-acryloylpyrrolidine and N-acryloylpiperidine with small organic molecules. *Chem. Express* 5, 969–972.

BC9701454

Alkaline Phosphatase Activatable Polymeric Cross-Linkers and Their Use in the Stabilization of Proteins

Christopher Bieniarz,^{*,†} Michael J. Cornwell,[‡] and Douglas F. Young[‡]

Advanced Drug Delivery, Hospital Products Division, and Diagnostics Division, Abbott Laboratories, Abbott Park, Illinois 60064-3500. Received February 9, 1998

We report the synthesis of polymeric cross-linking agents, poly(glutamic acid) poly(phosphorothioates), and their use in the cross-linking and stabilization of proteins upon treatment with alkaline phosphatase. We have shown that poly(phosphorothioates) are excellent substrates of alkaline phosphatase, yielding thiolated polymers which react covalently with electrophilic groups introduced into the proteins. Three proteins of different structure and function were cross-linked using this method: calf intestinal alkaline phosphatase, glucose oxidase (*Aspergillus niger*), and (R)-phycoerythrin. The cross-linking of alkaline phosphatase is self-catalyzed since this enzyme catalyzes the hydrolysis of phosphates, unmasking thiols which react with the maleimide prederivatized alkaline phosphatase. Incubation of buffered solutions of native alkaline phosphatase at 45 °C for 7–14 days resulted in a 35% higher loss of enzymatic activity compared to that of cross-linked enzyme. The effect of cross-linking glucose oxidase is even more notable, ranging from 800% stabilization at 37 °C and pH 9.0 to 3000% at 37 °C and pH 7.4. (R)-Phycoerythrin cross-linked with 1–3 equiv of poly(phosphorothioates) and incubated at 45 °C for 45 days was 20% more fluorescent than the native (R)-phycoerythrin subjected to the same conditions. The stabilizing effect of cross-linking was confirmed by comparing the rate of loss of quaternary structure of the cross-linked (R)-phycoerythrin with that of the native protein.

INTRODUCTION

The stabilization of proteins is an important field of practical and fundamental research. Enhancement of protein stability against denaturing effects of temperature, pH, and chemical agents has great practical applications in the biosensors, biopharmaceuticals, food, and cosmetic industries. Some of the methods used for stabilizing proteins against unfolding consist of molecular modifications, cross-linking, physical entrapment, surface immobilization, and the use of stabilizing carbohydrates and polymer additives. There is a plethora of reports, patents, and symposia dealing with many aspects of protein stabilization consisting of chemical derivatization and inter- or intramolecular cross-linking of the reactive functionalities on the surface of the proteins, presumably leading to increased conformational stability and enhanced resistance to denaturation (1–9). Unfolding of globular proteins generally involves exposure of buried hydrophobic side chains. Chemical cross-linking of proteins appears to be a particularly efficient stabilization method for minimizing unfolding pathways and preventing changes in the quaternary structure of multimeric proteins. Such unfolding often leads to dissociation into inactive protein subunits. Cross-linking approaches were exploited successfully in the stabilization of proteases, endonucleases, and antibodies (6, 7). Similarly, multimeric enzymes have been stabilized by cross-linking with glutaraldehyde (10). Deliberate polymerization of enzymes with the aim of improving their thermal stability

has also been described (11, 12). Several bifunctional cross-linking agents used in chemical cross-linking and stabilization of proteins were reviewed (13).

The reactive polymeric cross-linkers described in the above references lead to mostly intermolecular cross-linking of the proteins. The resulting polymeric protein often has a molecular weight well in excess of that of its native state. Moreover, the large adducts often have biological activities quite different from those of the native proteins. This is particularly troublesome when the cross-linked proteins are destined for use in immunoconjugates, biosensors, or other biometric applications because of the problems with nonspecific binding and altered solubilities of the cross-linked proteins.

In this paper, we report a novel method of protein cross-linking and stabilization. The cross-linking is based on an alkaline phosphatase activatable linear poly(phosphorothioate). The method is depicted in Scheme 1.

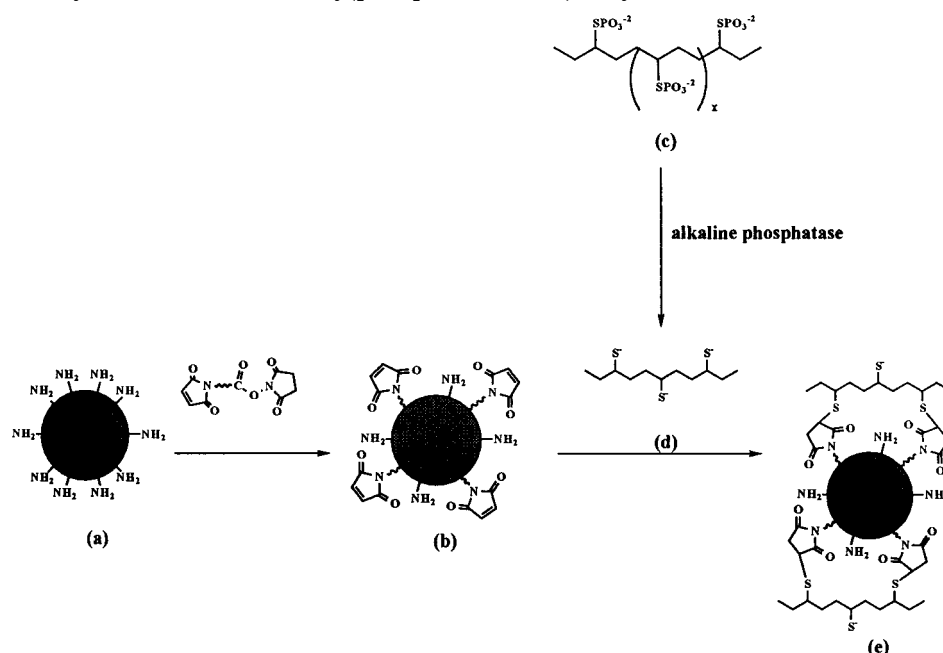
Protein (a) is first derivatized with maleimides. To the derivatized protein (b) is added a poly(phosphorothioate) (c), which is then exposed to the action of alkaline phosphatase (ALP).¹ ALP very efficiently catalyzes the

* To whom the correspondence should be addressed: Abbott Laboratories, Hospital Products Division D-97D, Abbott Park, North Chicago, IL 60064-3500. Phone: (847) 937-2239. Fax: (847) 938-3645. E-mail: bienica@hpd.abbott.com.

[†] Hospital Products Division.

[‡] Diagnostics Division.

¹ Abbreviations: ALP, alkaline phosphatase; R-PE, (R)-phycoerythrin; GOX, glucose oxidase; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); DMF, dimethylformamide; PGA, poly(L-glutamic acid); EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride; EDTA, ethylenediaminetetraacetic acid; NEM, N-ethylmaleimide; PNPP, p-nitrophenyl phosphate; BSA, bovine serum albumin; SMCC, succinimidyl 4-(N-maleimido-methyl)cyclohexane-1-carboxylate; HRPO, horseradish peroxidase; 4-AAP, 4-aminoantipyrine; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SATA, N-succinimidyl S-acetylthioacetate; SPDP, N-succinimidyl-3-(2-pyridyldithio)propionate; DTT, dithiothreitol.

Scheme 1. ALP-Catalyzed Activation of Poly(phosphorothioate) Polymers and Their Cross-Linking of Proteins

hydrolysis of phosphates, unmasking thiolates on the backbone of the polymer (d). The polymer then reacts with maleimide-derivatized enzyme, resulting in a predominantly intramolecular cross-linking and concomitant stabilization of the protein (e). When the protein (a) is alkaline phosphatase, the process is self-catalyzed in that the very enzyme to be cross-linked catalyzes the hydrolysis of the phosphorothioate bonds and cross-linking of the protein surface.

To show the generality of our cross-linking stabilization method, we present results of ALP-induced cross-linking of three proteins of very different structure and function: calf intestinal ALP, glucose oxidase, and (R)-phycoerythrin. The proteins also vary in the number of subunits, as R-PE is multimeric, ALP dimeric, and GOX monomeric.

EXPERIMENTAL SECTION

Materials. Except as noted, reagents were obtained commercially and used without further purification. All solvents were HPLC grade. Hydrazine monohydrate, ethylenediamine, DTNB, and anhydrous DMF were from Aldrich Chemical Co. PGA, cysteamine S-phosphate, EDC, EDTA, Sephadex G-25, NEM, sodium periodate, sodium cyanoborohydride, glucose, PNPP, BSA, and all buffer components were from Sigma Chemical Co. (St. Louis, MO). SMCC and Traut's reagent (2-iminothiolane hydrochloride) were from Pierce Chemical Co. (Rockford, IL). Concentration membranes, the Centriprep-30 concentrator, and the Centricon-30 concentrator were from Amicon Co. (Beverly, MD). All dialysis tubing was from Spectrum (Houston, TX). Chromatography Econo and Bio-Sil SEC-400 columns and BIO-REX MSZ(D) resin were from Bio-Rad (Hercules, CA). Bovine intestinal ALP and GOX were from Boehringer Mannheim (Indianapolis, IN). R-PE was from Molecular Probes (Eugene, OR). HRPO, 3,5-dichloro-2-hydroxybenzenesulfonic acid sodium salt, and 4-AAP were from Abbott Laboratories (Abbott Park, IL).

General Procedures. Electronic spectra were recorded on a Hewlett-Packard 8452A diode array spectrophotometer. Fluorescence spectra were recorded on a Hitachi F-4010 spectrophotometer. HPLC analyses

were performed on a Spectra-Physics instrument equipped with an SP8490 dual-wavelength detector. All enzyme activity measurements were determined with the initial rate method (14).

Synthesis of Poly(glutamic acid) Poly(phosphorothioate) PGA(SPO₃)₂₄. PGA (MW = 50000–100000, 1.00 g, 14 mmol) and cysteamine S-phosphate (0.26 g, 1.4 mmol) were dissolved in 40 mL of deionized water. EDC (1.00 g, 5.2 mmol) was added in 100 mg aliquots every 30 min for 5 h. The product was purified with a Centriprep-30 concentrator against deionized water and then lyophilized.

Phosphorothioate Analysis. Quantitation of thiolates on poly(phosphorothioates) was achieved with adaptation of Ellman's method as described previously (15, 16). To 1.00 mL of a solution of 5.0 mM PGA(SPO₃)₂₄ in 0.1 M Tris buffer, 1.0 mM MgCl₂, and 0.1 mM ZnCl₂ at pH 7.5 (buffer A) was added 30 mL of 10 mM DTNB in buffer A. The solution was incubated for 5 min, and the absorbance at 412 nm was recorded. No free thiol was detected. ALP (10 mL of a 74.1 mM solution) was added and the mixture incubated until no further increase in 412 nm absorbance was detected (30 min). The concentration of thiol (0.12 mM) was calculated from the final 412 nm absorbance (1.54 AU) and the extinction coefficient of 13 000 M⁻¹ cm⁻¹ of DTNB at pH 7.5. We found there to be 24 equiv of phosphorothioate per mole of polymer.

PGA(SPO₃)₂₄ Cross-Linking of Bovine ALP. To 0.75 mL of an aqueous solution of 74.1 mM ALP was added 1.25 mL of 0.1 M sodium phosphate, 0.1 M NaCl, 1.0 mM MgCl₂, and 0.1 mM ZnCl₂ at pH 7.0 (buffer B). The enzyme was concentrated to 0.2 mL using a Centricon-30 concentrator. The concentrate was rediluted to 2.0 mL with buffer B and then reconcentrated to 0.2 mL, and the procedure was repeated three times. The volume of the enzyme solution was increased to 1.5 mL with buffer B and the mixture placed in a vial. To 75 mL of DMF was added 0.62 mg (1.87 mmol) of SMCC. This solution was added to 1.46 mL of a 35.5 mM solution of ALP (46.7 nmol) and the mixture allowed to react for 1 h at room temperature while being rotated at 100 rpm on a rotary agitator. Coarse Sephadex G-25 that had

been previously rehydrated with 0.1 M sodium phosphate, 0.1 M NaCl, and 0.05% NaN₃ at pH 7.0 (buffer C) was poured to a bed height of 45 cm in a 1 cm × 50 cm Econo column. The column was equilibrated with buffer B. Following the incubation, SMCC-derivatized ALP was applied to the column and the column eluted with buffer B. Fractions with an A₂₈₀ of >0.5 AU were pooled, and the A₂₈₀ of the pool was used to calculate the SMCC-derivatized enzyme concentration. To 300 mL of buffer B was added 2.43 mg (34.7 nmol) of PGA(SPO₃)₂₄, and this solution was added to 1.86 mL of a 1.40 mg/mL solution of SMCC-derivatized ALP (17.4 nmol) and the mixture allowed to react overnight at 5 °C while being rotated on a rotary agitator.

Characterization of PGA-Cross-Linked ALP. PGA-cross-linked ALP was evaluated by size exclusion chromatography using a Bio-Sil SEC-400 column and SDS-PAGE, as described in the Results.

Thermal Stability Evaluation of Cross-Linked ALP. Both the native and the cross-linked enzymes were diluted to 74.1 nM (10 mg/mL) with buffer A. These dilutions were stored at 45 °C in an incubator for the duration of the study. At day 0 and various time points along the course of the study, the activity of the enzymes was evaluated. To separate 1.00 mL volumes of 7 mM PNPP in 0.5 M diethanolamine, 1.0 mM MgCl₂, and 0.1 mM ZnCl₂ at pH 10.2 (buffer D) was added 20 mL of the 74.1 nM ALP dilutions. The rate change in the 412 nm absorbance was calculated over 14 s intervals. The rate generated by the enzyme preparations at the various points was divided by the rate generated by the same enzyme preparation at day 0 to calculate the percentage of residual enzyme activity for the stressed dilution.

PGA(SPO₃)₁₉ Cross-Linking, Characterization, and Thermal Stability Evaluation of GOX from *Aspergillus niger*. GOX experiments were performed in a manner analogous to the ALP procedures except the polymer had an average of 19 phosphorothioate groups. GOX was derivatized with 25, 50, 75, 100, and 150 molar equiv of SMCC as described above for ALP. The stoichiometry of GOX/PGA(SPO₃)₁₉ was 1:1. Hydrolysis of phosphate groups on PGA(SPO₃)₁₉ was the result of externally added micromolar native ALP. Cross-linked GOX was incubated at 37 °C at pH 7.4 and 9.0 and compared to native GOX under the same conditions. Both the native enzyme and the cross-linked enzyme were diluted to 500 mg/mL with 1.0 mM EDTA and 0.1 M NaCl at pH 7.4 (buffer E). These dilutions were stored in a 37 °C incubator for the duration of the study. At day 0 and various time points along the course of the study, the activity of native and cross-linked enzymes was evaluated. Prior to evaluation, the stressed enzyme preparations were diluted to 2 mg/mL using 0.1 M NaCl and 0.1 M sodium phosphate at pH 7.0 (buffer F). Enzyme activity was monitored in a solution of 2 mM 4-AAP and 8 mM 3,5-dichloro-2-hydroxybenzenesulfonic acid in the presence of 1 unit of HRPO and 100 mM glucose in buffer F. The rate changes were monitored at A₅₅₀ over 2 min intervals. Thermal stability evaluations of cross-linked GOX at pH 9.0 were carried out like they were for the sample at pH 7.4 except both the native and the cross-linked enzymes were diluted to 500 mg/mL with 0.1 M sodium phosphate, 1.0 mM EDTA, and 0.1 M NaCl at pH 9.0 (buffer G). Of course, in this application, ALP was added externally to catalyze the hydrolysis of phosphates on PGA(SPO₃)₁₉.

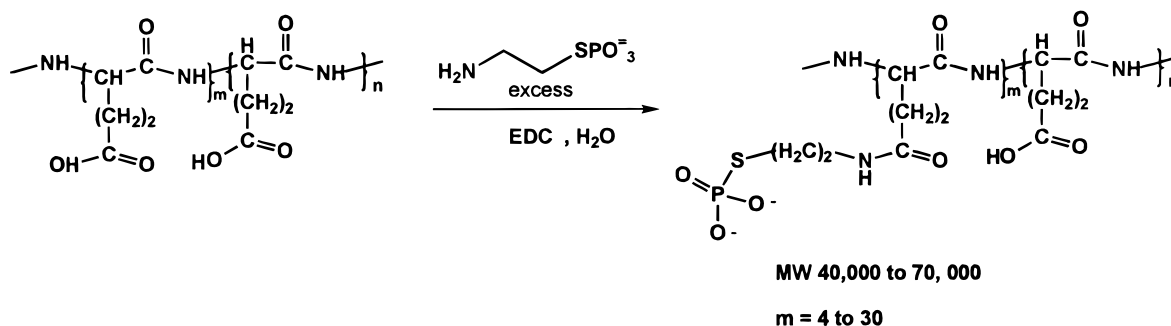
PGA(SPO₃)₁₈ and PGA(SPO₃)₂₈ Cross-Linking of R-PE from *Porphyra tenera*. To 2.5 mL of an aqueous solution of 41.7 mM R-PE was added 2 mL of buffer E.

This solution was transferred to dialysis tubing with a MW cutoff of 12000–14000 and dialyzed for 24 h each against three 4 L changes of buffer E. To a 1.21 mL aliquot of a 24.1 mM solution of dialyzed R-PE (0.292 nmol) was added a solution of 0.49 mg (1.47 nmol) of SMCC in 200 mL of DMF, and the mixture was allowed to react for 1 h at room temperature while being rotated at 100 rpm on a rotary agitator. A Sephadex G-25 column was prepared as above with buffer E, and following the incubation, the SMCC-derivatized R-PE was applied to a Sephadex G-25 column to remove unreacted SMCC. The column was eluted with buffer E, and 0.75 mL fractions were collected. Fractions with an A₅₆₆ of >1.0 AU were pooled, and the A₅₆₆ of the pool was used to calculate the protein concentration of the SMCC-derivatized R-PE. To 1 mL of buffer B was added 10 mg (167 nmol) of PGA(SPO₃)₁₈ (MW = 60 000). To this solution was added 25 mL of 10 mg/mL (1.67 nmol) ALP to deprotect the phosphorothioates. The deprotection reaction was allowed to proceed for 3 h at room temperature while the mixture was being rotated at 100 rpm on a rotary agitator. Following the incubation, a 71 mL (11.5 nmol) aliquot of this solution was added to 1.91 mL of 1.44 mg/mL (11.5 nmol) SMCC-derivatized R-PE and the mixture allowed to react overnight at 5 °C while being rotated at 100 rpm on a rotary agitator. Cross-linking at higher PGA(SPO₃)₁₈:R-PE ratios (2:1 and 3:1 molar ratios) was done analogously.

Characterization of Cross-Linked R-PE. PGA-(SPO₃)₁₈ and PGA(SPO₃)₂₈ cross-linked R-PE were evaluated by size exclusion chromatography using a Bio-Sil SEC-400 column. Detection was at 280 nm. The mobil phase was buffer E running at a flow rate of 1.0 mL/min. The primary population generated had a retention time corresponding to a singlet and doublet cross-linked protein with very little polymerization occurring. The residual fluorescence intensity of the cross-linked R-PE was measured and compared to the fluorescence of the native R-PE under the same conditions. The fluorescence generated by the cross-linked R-PE preparations was divided by the value of fluorescence of the native R-PE to calculate the percentage of residual fluorescence for the cross-linked preparations. The result of this evaluation showed that the cross-linked R-PE had retained 92% of the native protein fluorescence intensity.

Thermal Stability Evaluation of Cross-Linked R-PE by Fluorescence Measurement and by Size. The thermal stability of PGA(SPO₃)₁₈ or PGA(SPO₃)₂₈ cross-linked R-PE was evaluated by measuring fluorescence decay of samples thermally stressed at 45 °C and compared to that of native R-PE under the same conditions, using a λ_{exc} of 488 nm and a λ_{em} of 576 nm. Both the native and the cross-linked proteins were diluted to 100 mg/mL (0.42 mM) with buffer E. These dilutions were stored in a 45 °C incubator for the duration of the study. At day 0 and various time points along the course of the study, the fluorescence intensities of both preparations were evaluated. Prior to evaluation, the stressed protein preparations were diluted to 1 mg/mL (4.2 nM) using buffer E. The fluorescence of the R-PE preparations at various time points was divided by the fluorescence of the same preparation at day 0 to calculate the percentage of residual fluorescence intensity of the stressed protein.

The thermal stability of PGA(SPO₃)₁₈ and PGA(SPO₃)₂₈ cross-linked R-PE was evaluated at 45 °C and compared to that of native R-PE under the same conditions. A Bio-Sil SEC-400 column was used to follow the decomposition of the stressed R-PE into smaller subunits. Detection

Scheme 2. Synthesis of Poly(glutamic acid) Poly(phosphorothioate)

was at 280 nm. The mobile phase was buffer E running at a flow rate of 1.0 mL/min. Both the native protein and the cross-linked protein were diluted to 100 mg/mL with buffer E. These dilutions were stored in a 45 °C incubator for the duration of the study. At day 0 and various time points along the course of the study, the percentage of the total 280 nm absorbance which was due to the small components was evaluated.

RESULTS

Phosphorothioates are excellent substrates for alkaline phosphatase (17, 18). We have exploited this feature for an alkaline phosphatase-catalyzed hydrolysis of phosphorothioate-derivatized polymers. The resulting polymeric cross-linkers display many thiols which in a subsequent step may be reacted with the thiol-reactive electrophilic groups on the protein. Thus, the phosphate may be thought of as a protective group for thiols and alkaline phosphatase as the deprotection reagent. In view of the high reactivity of thiols toward oxidative coupling, the protective phosphate groups prevent disulfide bond formation. Thus, polymers derivatized with phosphorothioates allow access to a new family of cross-linking and conjugating polymers. Moreover, the high polarity of the phosphates ensures the solubility of even very water insoluble, nonpolar polymer backbones.

Although we have prepared several poly(phosphorothioate) polymers, in this work, we focus on cross-linking of three proteins of different structure and function by poly(glutamic acid) poly(phosphorothioate) shown in Scheme 2.

Cross-Linking of Bovine ALP. PGA was activated with EDC, and addition of cysteamine S-phosphate at various stoichiometries afforded polymers with various numbers of phosphorothioate groups per polymer. We found that the optimal level of phosphorothioate groups per polymer was between 18 and 24. The synthesis of $\text{PGA}(\text{SPO}_3)_x$ polymers is shown in Scheme 2.

In preliminary experiments, we ascertained that $\text{PGA}(\text{SPO}_3)_{24}$ is a good substrate for ALP. Virtually all thiols on the polymer were deprotected after 2 h at 5 °C and pH 7.0 using nanomolar quantities of ALP. This was demonstrated by quantitating the number of thiols as a function of time during the deprotection reaction, using Ellmann's reagent (DTNB). In previous work, we demonstrated that the derivatization of ALP with the maleimide heterobifunctional agents did not significantly affect the activity of the enzyme; 90–95% of the native enzyme activity remained after functionalization of the enzyme with various extended linkers (16). We confirmed this result in the current work and found that derivatization of the enzyme with 40 equiv of SMCC per enzyme resulted in a loss of only 5% of its activity. $\text{PGA}(\text{SPO}_3)_{24}$ and ALP were reacted in molar ratios of 1:1, 2:1, 3:1 or 4:1, and 6:1. The ratio of cross-linked multimeric

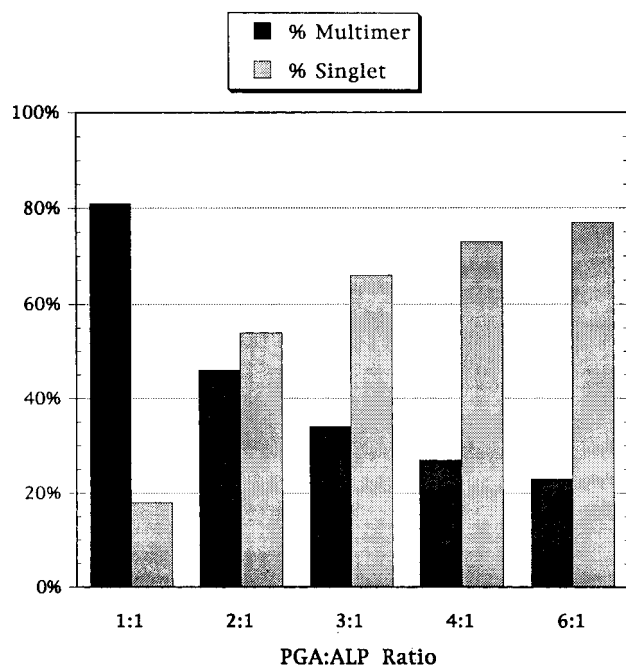


Figure 1. Dependence of the size of the cross-linked ALP on the stoichiometry of $\text{PGA}(\text{SPO}_3)_{24}$. Cross-linking ALP with 1 equiv of PGA cross-linker yields 82% of the multimeric aggregate of the enzyme (intermolecular cross-linking), while higher ratios of PGA per enzyme yield more singlet enzyme (intramolecular cross-linking).

structures to cross-linked singlets depends on the $\text{PGA}(\text{SPO}_3)_{24}$:ALP stoichiometry. Figure 1 shows that at higher $\text{PGA}(\text{SPO}_3)_{24}$:ALP ratios the cross-linking is prevalently intramolecular, resulting in higher ratios of the surface-cross-linked single enzyme molecule. At low $\text{PGA}(\text{SPO}_3)_{24}$:ALP ratios, multimeric intermolecular cross-linking predominates.

The maleimide-derivatized ALP catalyzes the cross-linking of its own surface in an autocatalytic process which, to the best of our knowledge, is unprecedented.

The cross-linked ALP was also examined by SDS-PAGE using a Phastgel system. Gradient gels of 7 to 10% polyacrylamide were run under nonreducing and reducing conditions. Results from nonreducing conditions showed that the primary population generated was singlet cross-linked enzyme with very little intermolecular cross-linking occurring. Results from the reducing conditions showed that the cross-linked ALP was not monomerized under conditions that were sufficient to monomerize the native ALP. Gel filtration HPLC analysis of the ALP cross-linked with 3 molar equiv of $\text{PGA}(\text{SPO}_3)_{24}$ per ALP using the Bio-Sil SEC-400 column in buffer B showed essentially complete replacement of the peak of the SMCC-derivatized ALP at 11.31 min with a

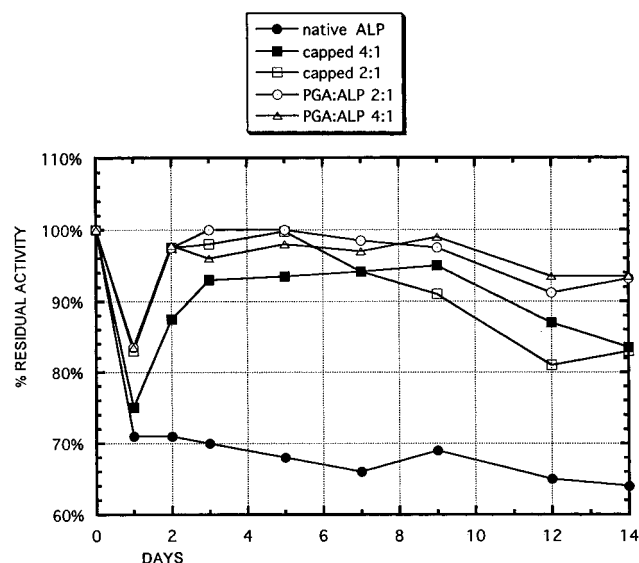


Figure 2. Thermal stability of ALP cross-linked at 2:1 and 4:1 PGA(SPO₃)₂₄:ALP molar ratios, with NEM capped and uncapped, at 45 °C in 0.1 M Tris buffer (pH 7.5), 0.1 mM ZnCl₂, and 1 mM MgCl₂. The data are normalized to 100% activity at day 0 to better visualize the changes.

new peak at 10.96 min and a small shoulder at a higher molecular weight corresponding to polymerized ALP. The UV-vis detector was set at A_{280} , and the flow rate was 1.0 mL/min. Similar results were observed at 2:1 and 4:1 polymer:enzyme stoichiometries with higher stoichiometries resulting in higher amounts of intramolecular cross-linking and lower multimeric content, as seen in Figure 1.

The cross-linked ALP is significantly stabilized against thermal denaturation. Figure 2 shows the extent of stabilization of cross-linked ALP compared to that of the native enzyme.

A conspicuous feature of the plot is a steep loss of the enzymatic activity during the first 24 h with the cross-linked and native ALP enzymes. Unlike the native enzyme whose activity decreases throughout the incubation, the PGA-cross-linked ALP recovers activity quite rapidly. This striking behavior is discussed later in the text.

The enzyme retains 75–90% of its native activity at day 0. The data in the figure are normalized to better visualize the loss of activity as the result of incubations at 45 °C. We found that *N*-ethylmaleimide capping of the unreacted thiolates left after the cross-linking was not necessary, and in fact led to 10% lower resistance to thermal denaturation. Figure 3 shows that at 5 °C the number of titratable thiols is essentially constant over time for 2:1 and 4:1 PGA(SPO₃)₂₄:ALP ratios. At 45 °C, there is a steady loss of thiolates, probably due to an oxidative dimerization process. Thus, after 5 days at 45 °C, there were only two or three titratable thiols. The PGA(SPO₃)₂₄:ALP ratio used was 2:1, 3:1, and 4:1, yielding 32, 55, or 68 free thiolates, respectively, on the cross-linked ALP at the start of the incubations (only 2:1 and 4:1 stoichiometries are shown in Figure 3). Interestingly, size exclusion HPLC analysis of the cross-linked ALP incubated at 45 °C showed no increase in the higher-molecular weight material despite the observed decrease of the titratable thiols at this higher temperature.

The nature of the maleimide heterobifunctional agent used to derivatize the enzyme is important. Shorter maleimide linkers, i.e. SMCC, resulted in better resis-

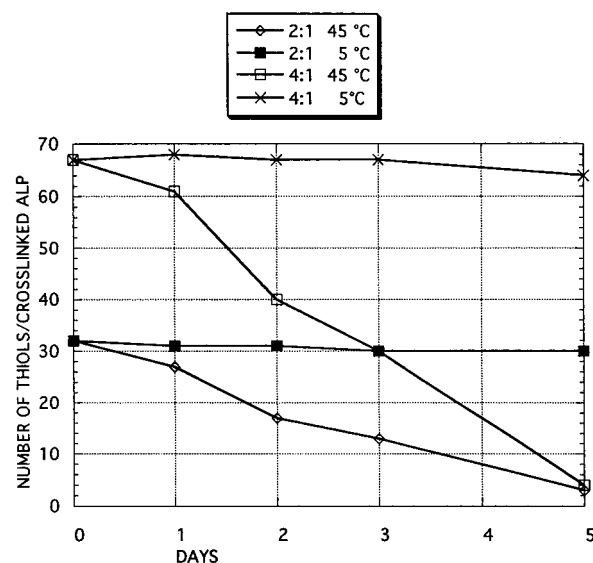


Figure 3. Number of thiols of ALP cross-linked at 2:1 and 4:1 ratios with PGA(SPO₃)₂₄ at 5 and 45 °C as a function of time.

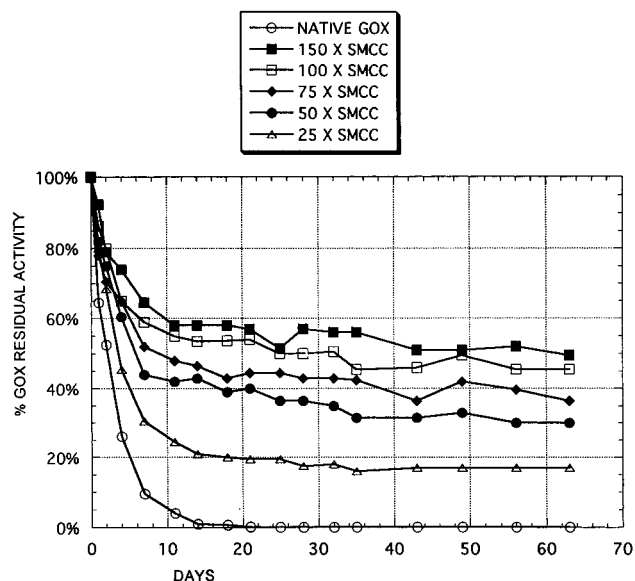


Figure 4. Thermal stability of GOX functionalized with 25, 50, 75, 100, and 150 equiv of SMCC, cross-linked at a 1:1 PGA-(SPO₃)₁₉:GOX molar ratio at 37 °C in phosphate buffer (pH 7.4), compared to that of native GOX. The data are normalized to 100% at day 0.

tance of the cross-linked protein to thermal denaturation than the longer 30-atom maleimide conjugating agents (15).

Cross-Linking of GOX. We also applied this cross-linking methodology to GOX (*A. niger*). Figures 4 and 5 show comparisons of the stabilities at 37 °C of the GOX cross-linked to the native enzyme at two different pHs.

The PGA(SPO₃)₁₉:GOX ratio was 1:1. At day 0 after cross-linking, GOX derivatized with 50 equiv of maleimide/enzyme had 85% of the native enzyme activity. The figures above show the very high degree of stabilization for GOX. As in previous figures, the data in Figure 4 are normalized to 100% at day 0 to visualize the effect of thermal stress on the various preparations. Figure 4 shows that, at pH 7.4 and at a fixed 1:1 cross-linking polymer:enzyme ratio, stabilization is dependent on the degree of derivatization of GOX by SMCC. Enzyme derivatized with the lowest stoichiometries of SMCC (25 equiv of SMCC per GOX) showed the lowest degree of

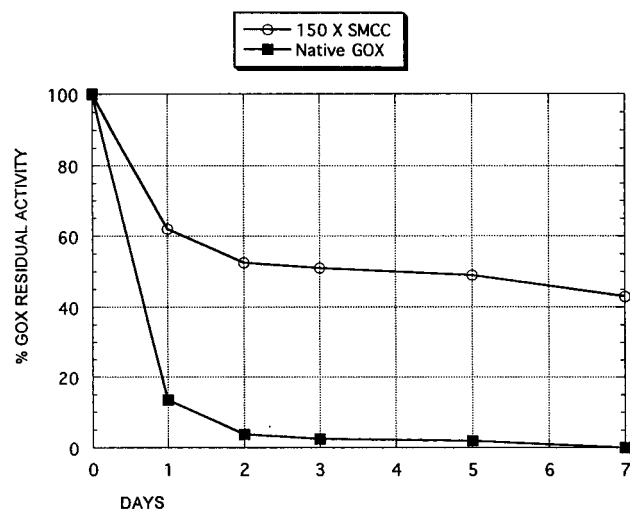


Figure 5. Thermal stability of GOX functionalized with 150 equiv of SMCC, cross-linked at a 1:1 PGA(SPO₃)₁₉:GOX molar ratio at 37 °C in phosphate buffer (pH 9.0), compared to that of native GOX. The data are normalized to 100% at day 0.

stabilization. Derivatization of GOX with a progressively higher number of equivalents of SMCC resulted in correspondingly higher degrees of stabilization.

GOX that is prefunctionalized with 150 molar equiv of SMCC and then cross-linked with PGA(SPO₃)₁₉ in a 1:1 ratio is approximately 8 times more stable than the native enzyme (*t*_{1/2} of 4 vs 0.5 day), as shown in Figure 5. The stabilizing effect of cross-linking is even more pronounced at pH 7.4, which is more compatible with GOX. The progress of the cross-linking reaction was followed by HPLC using a Bio-Rad SEC-400 column and 280 nm detection. Thus, before incubation, GOX derivatized with a 50-fold molar excess of SMCC, in the presence of 1 equiv of PGA(SPO₃)₁₉ and a micromolar level of alkaline phosphatase, had a GPC signal at 12.10 min, but that signal was completely replaced after 16 h by one at 11.37 min (70%) and at 10.25 min (30%) corresponding to a singlet cross-linked GOX and dimeric and trimeric adducts. When higher PGA(SPO₃)₁₉:GOX ratios (3:1 and 5:1) were used, the original signal of GOX was replaced with essentially a single peak at 11.41 min corresponding to a singlet intramolecular cross-linked GOX. Thus, higher ratios of cross-linker per enzyme led to increased content of intramolecular as opposed to intermolecular cross-linking. This trend is consistent with the one observed for ALP shown in Figure 1.

Cross-Linking of R-PE. R-PE, a member of the phycobiliprotein system, is a light-harvesting photosynthetic algal pigment, bilin-protein conjugate with a MW of 240 000 (19, 20). It possesses a complex quaternary structure consisting of 13 subunits organized in subunit architecture (αβ)₆γ. It has 34 bilin chromophores covalently conjugated to cystein residues in the subunits. Due to the multiple bilins, the extremely high absorbance coefficient ($\epsilon = 2.4 \times 10^6 \text{ cm}^{-1} \text{ M}^{-1}$), and the correct mutual orientation of the bilins within the protein domains, R-PE has an exceedingly high fluorescence quantum yield and very large Stokes shift ($\lambda_{\text{ex}} = 495 \text{ nm}$, $\lambda_{\text{em}} = 576 \text{ nm}$). Light absorbed by biliproteins is transferred through a network of pairwise Förster resonance processes (21, 22). The fluorescence characteristics of the phycobiliproteins depend on the mutual orientation of the chromophores within the protein domains.

The top of Figure 6 shows a GPC profile of the chromatographic changes of R-PE stressed at 45 °C with

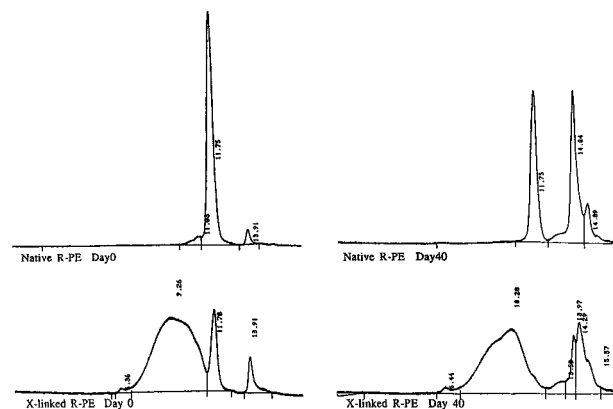


Figure 6. Chromatographic changes of R-PE stressed at 45 °C in 0.1 M sodium phosphate and 0.1 M NaCl (pH 7.0): (top) native R-PE and (bottom) R-PE cross-linked at a 1:1 PGA-(SPO₃)₁₈:R-PE molar ratio.

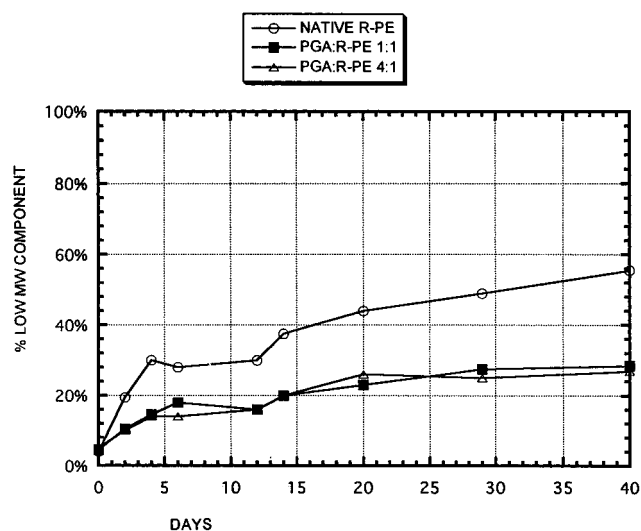


Figure 7. Thermal stability of R-PE at 45 °C and pH 7.4. Rate of formation of low-molecular weight subunits of R-PE cross-linked at 1:1 and 4:1 PGA(SPO₃)₁₈:R-PE molar ratios.

0.1 M sodium phosphate and 0.1 M NaCl at pH 7.0. A Bio-Sil SEC-400 column was used to follow the decomposition. Upon incubation, approximately 60% of the protein is fragmented into smaller subunits (α, β, and γ with MWs of 17 000, 18 000, and 30 000, respectively) (20) corresponding to peaks at 14.04 min and an earlier shoulder peak. The bottom of Figure 6 shows a GPC profile of R-PE cross-linked with PGA(SPO₃)₁₈ in 1:1 polymer:R-PE ratio and exposed to the same thermal stress as the native protein. The smaller subunit peaks correspond to approximately 40% of the material, with 60% corresponding to the higher-molecular weight cross-linked material. Thus, cross-linked material displayed a lower tendency to decompose into subunits than the native R-PE.

The changes in the quaternary structure of R-PE are accompanied by a gradual loss of its fluorescence in solution upon exposure to heat.

Figure 7 shows the dependence of the formation of the low-molecular weight fraction in the R-PE solution on the time of incubation at 45 °C and pH 7.4. Clearly, the content of the low-molecular weight component is 30% higher for native R-PE than for R-PE cross-linked with 1 or 4 equiv of PGA(SPO₃)₁₈.

Concomitant with the loss of the quaternary structure of R-PE was a loss of its fluorescence, as shown in Figure 8.

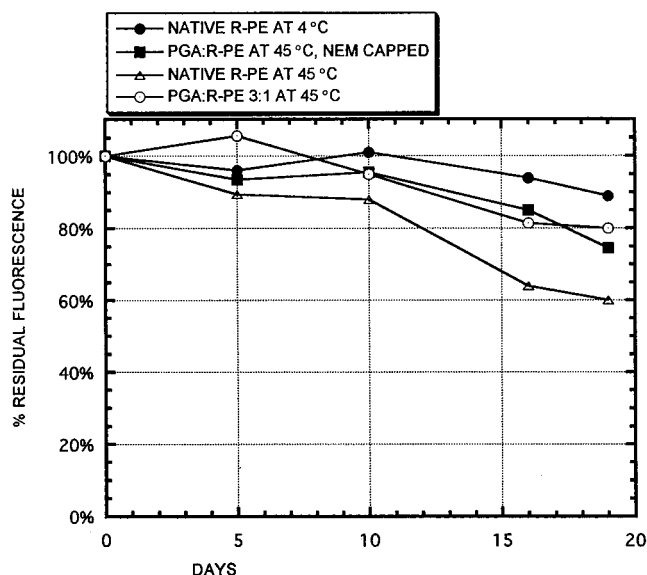


Figure 8. Thermal stability of R-PE at 45 °C and pH 7.4. Rate of fluorescence loss of R-PE cross-linked at a 3:1 PGA(SPO₃)₁₈:R-PE molar ratio. For comparison, native R-PE at 4 and 45 °C is shown. NEM-capped cross-linked R-PE is also shown.

As seen in Figure 8, the fluorescence loss is significantly lower for PGA-cross-linked R-PE than for the native R-PE at 45 °C and pH 7.4. After 18 days at 45 °C, PGA-cross-linked R-PE (open circles) retained 20% more fluorescence than the un-cross-linked, native R-PE (triangles). Capping of the sulfhydryl groups by NEM (solid squares) does not appear to have an effect on the fluorescence.

DISCUSSION

Alkaline phosphatase is frequently employed as a label in enzyme immunoassays. Its widespread use may be attributed to the very high second-order rate constant ($k_{cat}/K_m = 5 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$), the existence of excellent fluorogenic and chromogenic substrates, a pH optimum 9 to 10.5, which is compatible with an optimal signal from fluorogenic and chromogenic substrates, and reasonably good thermal stability (23–25). In this work, we describe a series of new polymeric substrates of alkaline phosphatase. The poly(phosphorothioates) reported here are excellent enzyme substrates and generate thiols upon catalysis. The byproduct of the reaction is phosphate ion, an innocuous material which may be left in the reaction medium and does not interfere with the subsequent use of the polymers as cross-linkers and stabilizers of proteins. Thus, the phosphorothioate functionality may be thought of as a protected thiolate nucleophile. While other protected thiols are widely used in bioconjugations and protein modifications, i.e. SATA and SPDP, these reagents mask thiols as thioacetyl and 2-pyridyl disulfide. The generation of thiols using these reagents is achieved by reductive cleavage of 2-pyridyl disulfide using DTT as a reducing agent which needs to be removed from the reaction medium or by using harsh reaction conditions of 0.2 N NaOH, aqueous NH₃ (26), or hydroxylamine (27) which may be incompatible with base-sensitive haptens or proteins.

The reaction of the polythiolated polymers with the electrophile-derivatized proteins cross-links the surface of the protein and results in protein stabilization. Figure 2 shows that at suitable stoichiometries of PGA(SPO₃)₂₄ the enzyme may be considerably stabilized against thermal denaturation. A striking aspect of this graph is

the precipitous loss of enzymatic activity of this homodimeric protein during the first day of incubation at 45 °C. While the native protein does not recover, the cross-linked ALP recovers its activity. After 72 h, the enzyme activity is similar to the one at the beginning of the incubation. This behavior may be explained in terms of a reversible change in the quaternary structure of the cross-linked dimer versus an irreversible loss of the activity of the native enzyme due to monomerization. Alkaline phosphatases are homodimers in their active forms, and monomers have no detectable enzymatic activity (23, 28). The rate of loss of activity after the third day appears to be similar for the cross-linked and native enzymes. This suggests that the initial loss of activity of the native enzyme is due to a monomerization of the enzyme, a process prevented in the cross-linked protein.

The quantitation of the number of thiols at 5 and 45 °C incubations of ALP cross-linked at 2:1 and 4:1 ratios with PGA is shown in Figure 3. At 5 °C, the number of thiolates measured by spectrophotometric titration with DTNB is reasonably constant for both 2:1 and 4:1 cross-linked enzymes. However, at 45 °C, the number of thiolates decreases rather rapidly, reaching essentially zero after 5 days. The rate of loss of titratable thiols is much faster than the rate of loss of the enzymatic activity shown in Figure 2. HPLC analysis showed that the process is not accompanied by the formation of larger aggregates. HPLC analysis showed that the ratios of multimers to monomeric cross-linked proteins remain constant over the course of 5 days at 45 °C. This suggests intramolecular oxidative disulfide bond formation between the contiguous thiols on the cross-links.

This method of stabilization was also applied to GOX, an oxidoreductase of great importance in biosensor technology and the diagnosis of diabetes (29). As shown in Figure 4, the degree of stabilization of this enzyme is dependent on the level of derivatization of the enzyme with maleimide. The lowest stoichiometries of electrophile resulted in relatively low levels of thermal stabilization, while the highest levels of maleimide resulted in excellent stabilization through reaction with 1:1 PGA-(SPO₃)₁₉. GOX prederivatized with 150 equiv of maleimide and reacted with 1 equiv of PGA retained 58% of the enzymatic activity after 12 days at pH 7.4 and 37 °C, while 50% of the activity remained after 60 days. Under identical conditions, the native enzyme lost 50% of its activity after 2.5 days. Even at an unfavorable pH of 9.0, cross-linking provided a very significant level of stabilization, as shown in Figure 5.

In the past decade, phycobiliproteins such as R-PE have played an increasingly important role as fluorescent labels in single-cell analysis and flow cytometry. Consequently, there has been interest in conjugates of phycobiliproteins and immunoglobulins directed against specific cellular epitopes (30). The high fluorescence of R-PE depends on the preservation of the native quaternary structure of the protein. R-PE is composed of 13 subunits called phycobilisomes organized in an ($\alpha\beta$)₆ γ complex. Each subunit carries one or two bilins for a total of 32 fluorogenic bilins in R-PE. Dissociation of the phycobilisomes occurs as the result of factors which denature the proteins, i.e. low salt, urea, or heat (31–33). The hexamers are known to break down to smaller subunits, including $\alpha\beta$ and $\alpha\beta\gamma$ (34). The effect of cross-linking R-PE is seen in Figures 7 and 8. Clearly, the rate of appearance of the low-molecular weight component is lower for the cross-linked protein than for the unmodified protein, as shown in Figure 7. Similarly, cross-linking has an effect in preserving the intrinsic

fluorescence of R-PE as shown in Figure 8. The congruence of the results in Figures 7 and 8 may be explained by the known dependence of the fluorescence energy transfer and pathways on the three-dimensional structure of phycobiliproteins (20, 35). Denaturing conditions lead to disassembly of the subunits and loss of quaternary structure of the proteins. We believe that stabilizing effects of cross-linking may be attributed to the fact that the intramolecular cross-linking of the protein allows less conformational mobility of the subunits and lessens the degree to which the protein disassembles under the denaturing conditions.

We found that in all cases the use of higher PGA-(SPO₃)_x:protein molar ratios led to increased content of intramolecular cross-linking. This is demonstrated in Figure 1 for the case of alkaline phosphatase-catalyzed auto-cross-linking but was also observed in the case of R-PE and GOX. This may be explained by entropic considerations. The ability to control the degree of cross-linking and the molecular weight of the cross-linked stabilized proteins is a distinct advantage of this method.

The cross-linking process introduces several thiolates into the stabilized protein. These thiolates may serve as functionalities, through which conjugation to other biological molecules or surfaces may be accomplished. We are describing such a bioconjugation in the following paper (36).

SUMMARY

We have presented a new, enzyme-catalyzed cross-linking method based on alkaline phosphatase-catalyzed activation of poly(phosphorothioate) polymers. The cross-linked proteins are considerably stabilized against thermal denaturation in solutions. The method is rapid and in most cases leads to quantitative cross-linking of the protein. The byproducts of the polymer activation and cross-linking are phosphate ions which do not interfere in the biological activity of most proteins. Consequently, it may be left in the reaction medium. Poly(phosphorothioate) polymers are very soluble in water which facilitates the cross-linking process and increases the solubility of the stabilized protein. The method allows easy conjugation of the stabilized cross-linked proteins through reactions of thiolates with electrophilic functionalities on other biomolecules. The chemical and biological properties of the cross-linked proteins are retained. The cross-linking process may be adjusted to individual proteins. Excellent control of the molecular weight of the cross-linked species may be exercised by adjusting the stoichiometries of the cross-linked polymer and proteins.

The method could be adapted for biomolecules other than proteins. Thus, conjugation of oligonucleotides, carbohydrates, and lipids to proteins may be implemented using alkaline phosphatase activation of phosphorothioate-derivatized species. The conjugation to surfaces, i.e. colloidal gold and microchips, in a spatially controlled manner is an intriguing possibility.

ACKNOWLEDGMENT

We thank Dr. Eric Ginsburg of the Abbott Laboratories Advanced Drug Delivery Department for his critical reading of the manuscript.

LITERATURE CITED

- (1) Mer, G., Hietter, H., and Lefevre, J.-F. (1996) Stabilization of proteins by glycosylation examined by NMR analysis of a fucosylated proteinase inhibitor. *Nat. Struct. Biol.* 3, 45–53.
- (2) Sobolov, S. B., Bartoszko-Malik, A., Oeschger, T. R., and Montalbano, M. M. (1994) Cross-linked enzyme crystals of fructose diphosphate aldolase: development as a biocatalyst for synthesis. *Tetrahedron Lett.* 35, 7751–7754.
- (3) St. Clair, N., and Navia, M. A. (1992) Cross-linked enzyme crystals as robust biocatalysts. *J. Am. Chem. Soc.* 114, 7314–7316.
- (4) Suh, J., and Hwang, B. K. (1992) Enhancement of durability of chymotrypsin through cross-linking with poly(allylamine). *Bioorg. Chem.* 20, 223–235.
- (5) Torchilin, V. P., and Martinek, K. (1979) Enzyme stabilization without carriers. *Enzyme Microb. Technol.* 1, 74–82.
- (6) Wang, P., Hill, T. G., Bednarski, M. D., and Callstrom, M. R. (1991) The catalytic formation of peptide bonds with carbohydrate protein conjugates of proteases [CPC(Proteases)]. *Tetrahedron Lett.* 32, 6827–6830.
- (7) Wang, P., Hill, T. G., Wartchow, C. A., Huston, M. E., Oehler, L. M., Bradley Smith, M., Bednarski, M. D., and Callstrom, M. R. (1992) New carbohydrate-based materials for the stabilization of proteins. *J. Am. Chem. Soc.* 114, 378–380.
- (8) Schellman, J. A. (1987) The thermodynamic stability of proteins. *Annu. Rev. Biophys. Chem.* 16, 115–137.
- (9) Pollak, A., Blumenfeld, H., Wax, M., Baughn, R. L., and Whitesides, G. M. (1980) Enzyme immobilization by condensation copolymerization into cross-linked polyacrylamide gels. *J. Am. Chem. Soc.* 102, 6324–6336.
- (10) Gottschalk, N., and Jaenicke, R. (1987) Chemically crosslinked lactate dehydrogenase: stability and reconstruction after glutaraldehyde fixation. *Biotechnol. Appl. Biochem.* 9, 389–400.
- (11) Freytag, J. W., and Ishikawa, E. (1987) Immunoassays utilizing covalent conjugates of polymerized enzyme and antibody. U.S. Patent 4,657,853.
- (12) Pedersen, S., and Jørgensen, O. B. (1991) Immobilization of enzymes by cross-linking with a cross-linking agent and a polymer containing 1-aminoethylene moiety. PCT WO 91/08287.
- (13) Wong, S. S., and Wong, L.-J. C. (1992) Chemical crosslinking and the stabilization of proteins and enzymes. *Enzyme Microb. Technol.* 14, 866–874.
- (14) Segel, I. H. (1976) *Biochemical Calculations*, 2nd ed., p 216, John Wiley & Sons, New York.
- (15) Bieniarz, C., Husain, M., Barnes, G., King, C. A., and Welch, C. J. (1996) Extended length heterobifunctional coupling agents for protein conjugation. *Bioconjugate Chem.* 7, 88–95.
- (16) Husain, M., and Bieniarz, C. (1994) Fc site-specific labeling of immunoglobulins with calf intestinal alkaline phosphatase. *Bioconjugate Chem.* 5, 482–490.
- (17) Neumann, H., Boross, L., and Katchalski, E. (1967) Hydrolysis of S-substituted monoesters of phosphorothioic acid by alkaline phosphatase from *Escherichia coli*. *J. Biol. Chem.* 242, 3142–3147.
- (18) Neumann, H. (1968) Substrate selectivity in the action of alkaline and acid phosphatases. *J. Biol. Chem.* 243, 4671–4676.
- (19) Glazer, A. N., and Stryer, L. (1984) Phycofluor probes. *Trends Biochem. Sci.*, 423–427.
- (20) Glazer, A. N. (1989) Light guides. Directional energy transfer in a photosynthetic antenna. *J. Biol. Chem.* 264, 1–4.
- (21) Lakowicz, J. R. (1983) *Principles of Fluorescence Spectroscopy*, pp 303–339, Plenum Press, New York.
- (22) MacColl, R., and Guard-Riar, D. (1987) *Phycobiliproteins*, CRC Press, Boca Raton, FL.
- (23) Butterworth, P. J. (1983) Alkaline phosphatase. *Cell Biochem. Funct.* 1, 66–70.
- (24) Maggio, E. T. (1980) *Enzyme-Immunoassay*, pp 64–65, CRC Press, Boca Raton, FL.
- (25) McComb, R. B., Bowers, G. N., and Posen, S. (1979) *Alkaline Phosphatase*, pp 289–372, Plenum Press, New York.
- (26) Greene, T. W., and Wuts, P. G. M. (1991) *Protective Groups in Organic Synthesis*, p 298, John Wiley & Sons, New York.
- (27) Duncan, R. J. S., Weston, P. D., and Wigglesworth, R. (1983) A new reagent which may be used to introduce

- sulfhydryl groups into proteins, and its use in preparation of conjugates for immunoassay. *Anal. Biochem.* 132, 68–73.
- (28) Walsh, C. (1979) *Enzymatic Reaction Mechanisms*, p 192, W. H. Freeman and Co., San Francisco.
- (29) Turner, A. P. F., Karube, I., and Wilson, G. S. (1987) *Biosensors. Fundamentals and Applications*, Oxford Science Applications, Oxford.
- (30) Oi, V. T., Glazer, A. N., and Stryer, L. (1982) Fluorescent phycobiliprotein conjugates for analyses of cells and molecules. *J. Cell Biol.* 93, 981–986.
- (31) MacColl, R., Malak, H., Cipollo, J., Label, B., Ricci, G., MacColl, D., and Eisele, L. E. (1995) Studies on the dissociation of cryptomonad biliproteins. *J. Biol. Chem.* 270, 27555–27561.
- (32) MacColl, R., and Eisele, L. E. (1996) R-Phycocerythrins having two conformations for the same aggregate. *Biophys. Chem.* 61, 161–167.
- (33) Wilbanks, S. M., and Glazer, A. N. (1993) Rod structure of a Phycocerythrin II-containing phycobilisome. *J. Biol. Chem.* 268, 1236–1241.
- (34) Gingrich, J. C., Williams, R. C., and Glazer, A. N. (1982) Rod substructure in cyanobacterial phycobilisomes: phycocerythrin assembly in *Synechocystis* 6701 phycobilisomes. *J. Cell Biol.* 95, 170–178.
- (35) Glazer, A. N., Yeh, S. W., Webb, S. P., and Clark, J. H. (1985) Disk-to-disk transfer as the rate-limiting step for energy flow in phycobilisomes. *Science* 227, 419–423.
- (36) Bieniarz, C., Young, D. F., and Cornwell, M. J. (1997) Thermally stabilized immunoconjugates: conjugation of antibodies to alkaline phosphatase stabilized with polymeric cross-linkers. *Bioconjugate Chem.* 9, 399–402.

BC9800264

TECHNICAL NOTES

Thermally Stabilized Immunoconjugates: Conjugation of Antibodies to Alkaline Phosphatase Stabilized with Polymeric Cross-Linkers

Christopher Bieniarz,^{*,†} Douglas F. Young,[‡] and Michael J. Cornwell[‡]

Advanced Drug Delivery, Hospital Products Division, and Diagnostics Division, Abbott Laboratories, Abbott Park, Illinois 60064-3500. Received February 9, 1998

A method of conjugating poly(glutamic acid) poly(phosphorothioate)-cross-linked alkaline phosphatase to maleimide-derivatized immunoglobulin is described. Intramolecular autocatalyzed cross-linking of alkaline phosphatase at 2:1 to 4:1 polymer:enzyme ratios introduced 32–68 thiols on the surface of the enzyme. Depending on the stoichiometry of polymer to enzyme, the cross-linked alkaline phosphatase retained 75–90% of its native catalytic activity. The cross-linked thiolate-functionalized alkaline phosphatase was conjugated to maleimide-derivatized immunoglobulin. Compared to a control prepared using non-cross-linked alkaline phosphatase, these conjugates were smaller in size and more stable to heat. The enzymatic activity of the cross-linked conjugates after incubation at 45 °C and pH 7.5 for 25 days was 35% higher than those of the highest-activity control conjugates. The conjugation process could be controlled by varying the stoichiometries of poly(glutamic acid) poly(phosphorothioate), alkaline phosphatase, and immunoglobulin.

INTRODUCTION

Alkaline phosphatase is one of the enzymes most frequently employed in ELISA¹ (1, 2). ALP popularity can be attributed to its relatively high stability, its high turnover number, the sensitivity of its enzyme assays (3), and the existence of excellent fluorogenic and chromogenic substrates (4, 5). Although many bioconjugation methods have been developed for efficient conjugation of ALP and other enzymes to antibodies (6–9), there is a continuing need for development of conjugation methods which preserve the chemical and biological properties of those biomolecules in the conjugate. Although ALP is considered to have good thermal stability, in our experience, prolonged storage of ALP–IgG conjugates leads to gradual loss of the enzymatic signal.

In the preceding paper (10), we describe stabilization of proteins via novel polymeric cross-linkers, poly(phosphorothioate) polymers, activated by alkaline phosphatase. The protein is first derivatized with thiol-reactive electrophiles e.g., maleimides. The poly(phosphorothioate) polymer is then exposed to the action of ALP which very efficiently catalyzes the hydrolysis of the phosphates to unmask thiols on the polymeric back-

bone. The thiols on the polymer then react with the electrophiles on the surface of the protein, resulting in predominantly intramolecular cross-linking and concomitant stabilization of the protein. When the protein is ALP, the process is self-catalyzed in that the very enzyme to be cross-linked catalyzes the hydrolysis of the phosphorothioate bonds and the cross-linking of the protein surface.

Here we describe the application of the ALP-catalyzed auto-cross-linking to the construction of stabilized alkaline phosphatase–anti-TSH conjugates. These conjugates are crucially important in enzyme immunoassays for the immunodiagnostic assessment of the function of the thyroid gland. Stabilization of these immunoconjugates against thermal denaturation is of great practical interest because of the prolonged storage requirements for such immunoreagents.

EXPERIMENTAL SECTION

Materials and General Procedures. The synthesis and analysis of poly(glutamic acid) poly(phosphorothioate) PGA(SPO₃)₂₄, materials, and other reagents were as described in the preceding paper (10). Monoclonal anti-TSH was from Abbott Laboratories (Abbott Park, IL). STMCC 30-atom heterobifunctional maleimide-active ester linker was synthesized as described previously (11).

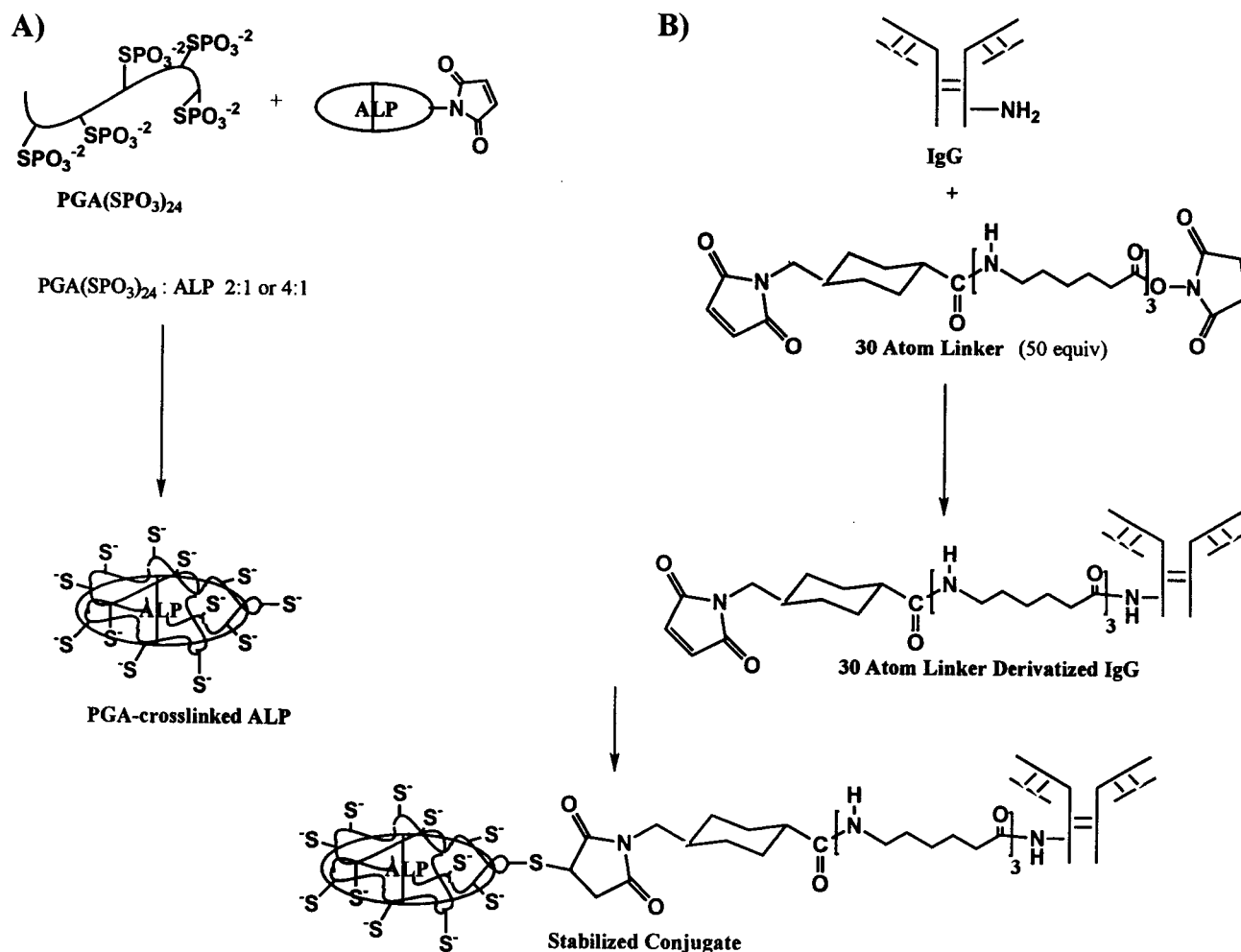
Conjugation of Anti-TSH IgG to PGA(SPO₃)₂₄ Cross-Linked Bovine Alkaline Phosphatase. (a) *Derivatization of Anti-TSH IgG.* To 1 mL of 6.6 mg/mL anti-TSH IgG (44.0 nmol) was added 1 mL of buffer composed of 0.1 M sodium phosphate, 0.1 M NaCl, 1.0 mM MgCl₂, and 0.1 mM ZnCl₂ at pH 7.0 (buffer B). The antibody was concentrated to approximately 0.2 mL using a Centricon-30 concentrator with a MW cutoff of 30 000. The concentrate was rediluted to 2 mL using

* To whom correspondence should be addressed: Abbott Laboratories, Hospital Products Division D-97D, Abbott Park, North Chicago, IL 60064-3500. Phone: (847) 937-2239. Fax: (847) 938-3645. E-mail: bienica@hpd.abbott.com.

[†] Hospital Products Division.

[‡] Diagnostics Division.

¹ Abbreviations: ELISA, enzyme-linked immunosorbent assay; ALP, alkaline phosphatase; TSH, thyroid-stimulating hormone; DMF, dimethylformamide; PGA, poly(L-glutamic acid); STMCC, succinimidyl tris(caproamido) 4-(N-maleimidomethyl)cyclohexane-1-carboxylate; SMCC, succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate.

Scheme 1. Conjugation of Poly(phosphorothioate)-Cross-Linked ALP to IgG

buffer B and then reconcentrated to 0.2 mL. This concentration–dilution cycle was repeated three times. The volume of the antibody solution was increased to 1 mL with buffer B and the mixture placed in a vial. The concentration of the protein was 5.3 mg/mL (35 mM by A_{280}). To 50 mL of DMF was added 0.56 mg (831 nmol) of succinimidyl tris(caproamido) 4-(*N*-maleimidoethyl)-cyclohexane-1-carboxylate (STMCC), a 30-atom hetero-bifunctional maleimide active ester cross-linking agent (11). This solution was added to 0.47 mL of the 35 mM solution of IgG (16.6 nmol) with a 50-fold molar excess of STMCC linker to protein. The solution was reacted for 1 h at room temperature while being rotated at 100 rpm on a rotary agitator. The reaction solution was passed through a Sephadex G-25 column which was eluted with buffer B. Fractions with an A_{280} of >0.5 were pooled. The concentration of IgG in the pooled fractions was 0.83 mg/mL (5.5 nM). The antibody pool was stored on ice until it was conjugated.

(b) *Conjugation of STMCC-Derivatized Anti-TSH IgG to PGA(SPO₃)₂₄ Cross-Linked Bovine ALP.* To a 0.72 mL aliquot of 0.83 mg/mL (4 nmol) STMCC linker-derivatized anti-TSH IgG was added 0.69 mL of 1.30 mg/mL (3.3 nmol) PGA(SPO₃)₂₄ cross-linked ALP prepared as described in the preceding paper (10). The resulting solution was allowed to react overnight at 5 °C while being rotated at 100 rpm on a rotary agitator. The progress of the conjugation was followed by gel permeation HPLC using a Bio-Rad Bio-Sil SEC-400 column and pH 7.0 buffer containing 0.1 M sodium phosphate and

0.1 M NaCl (buffer C) at a flow rate of 1.0 mL/min (detector at A_{280}). After 12 h, the reaction was essentially complete with $>80\%$ of the protein conjugated. Conjugates were concentrated to a volume of 200 mL with 30 000 MW cutoff Amicon membranes. The process was repeated three times. The concentration of the protein was 3.2 mg/mL as determined by A_{280} .

As a control experiment, the thermal stability and GPC profile of the PGA(SPO₃)₂₄-cross-linked ALP–anti-TSH conjugate was compared to that of the highest-activity and stability un-cross-linked ALP–anti-TSH control conjugate. Derivatization of ALP with iminothiolane hydrochloride and its conjugation to STMCC-derivatized anti-TSH IgG were carried out as described previously (11).

Thermal Stability Evaluation of the Conjugates.

Stock solutions of PGA(SPO₃)₂₄-cross-linked ALP–anti-TSH IgG conjugates from above were diluted with pH 7.5 buffer containing 0.1 M Tris, 1.0 mM MgCl₂, and 0.1 mM ZnCl₂ to a concentration of 10 mg/mL. These dilutions were stored at 45 °C in an incubator for the duration of the study. The values of the enzyme activity of all the conjugates were normalized to 100% at the start of the incubation. Progressive changes in the enzyme activity of the conjugates were monitored as described in the preceding paper (10).

RESULTS AND DISCUSSION

Scheme 1 shows the conjugation of PGA(SPO₃)₂₄-cross-linked, stabilized ALP to IgG derivatized with the 30-

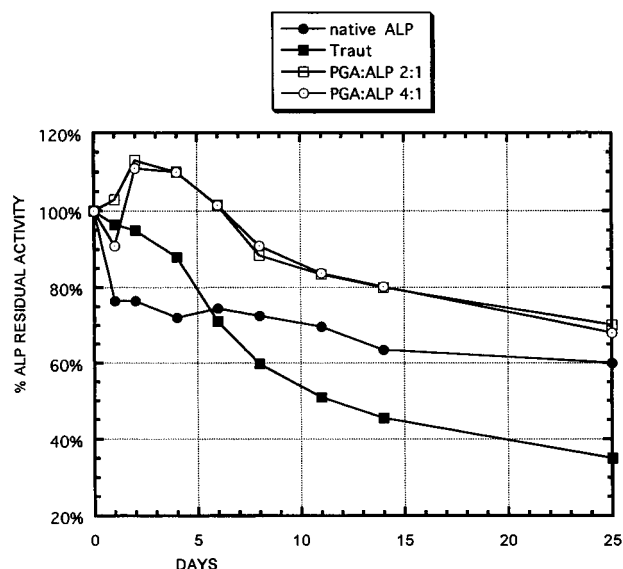


Figure 1. Thermal stability of PGA(SPO₃)₂₄-cross-linked ALP-anti-TSH IgG conjugates at 45 °C in pH 7.5 buffer containing 0.1 M Tris, 1.0 mM MgCl₂, and 0.1 mM ZnCl₂. Decay of enzyme activity in the best conjugate based on Traut reagent-derivatized ALP and conjugated to STMCC-derivatized IgG, compared with conjugates of ALP cross-linked at 2:1 and 4:1 stoichiometries with PGA(SPO₃)₂₄.

atom heterobifunctional coupling agent STMCC. We have shown before that the performance of this longer maleimide active ester is significantly better than the performance of the shorter analogues (11).

Self-catalyzed cross-linking of ALP by PGA(SPO₃)₂₄ is shown in Scheme 1A. Poly(glutamic acid) poly(phosphorothioate) is incubated in the presence of SMCC-derivatized ALP. This enzyme catalyzes the hydrolysis of phosphate and unmask thiol in the polymer, which react with the maleimides on the surface of ALP. Between 32 and 68 thiols are thus introduced into the ALP, lowering the activity of the enzyme by 10–25%. In the preceding paper, we have presented data which show that PGA-cross-linked ALP is considerably stabilized against thermal denaturation (10). The stoichiometries of PGA(SPO₃)₂₄:ALP may be varied from 1:1 to 6:1. As shown in the preceding paper (10), higher PGA(SPO₃)₂₄:ALP ratios result in predominantly intramolecular cross-linking of ALP. The cross-linking process generates a multiplicity of thiols in the cross-linked protein. These unreacted thiols are used to further conjugate the thermally stabilized enzyme to anti-TSH IgG. Scheme 1B shows derivatization of the antibody with the 30-atom heterobifunctional coupling agent SMCC. The maleimides introduced into the antibody molecule react in the final step with the thiols on the PGA-cross-linked ALP.

The thermal stability of the conjugates is shown in Figure 1. After 8 days at 45 °C in a pH 7.5 buffer containing 0.1 M Tris, 1.0 mM MgCl₂, and 0.1 mM ZnCl₂, the best cross-linked ALP-anti-TSH conjugates (2:1 and 4:1 PGA(SPO₃)₂₄:ALP) had a normalized enzyme activity 15% higher than that of the unmodified native ALP and 30% higher than that of the most active control conjugates based on un-cross-linked ALP, made using the Traut method (12). Notably, the conjugates prepared with the cross-linked ALP also had better thermal stability than the native unconjugated ALP. In our experience, the enzyme thermal stability of ALP conjugates is generally lower than that of the unconjugated ALP. Unlike PGA-cross-linked ALP, in which the cross-

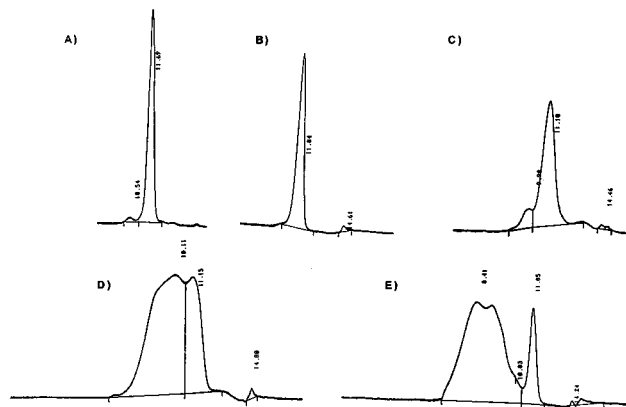


Figure 2. GPC chromatograms of the conjugation: (A) native IgG, (B) native ALP, (C) unfractionated cross-linked ALP, (D) the unfractionated cross-linked ALP-IgG conjugate, with 1:1 enzyme:IgG stoichiometry, and (E) unfractionated Traut reagent-derivatized ALP conjugated to STMCC-derivatized IgG.

linked enzyme experiences a dramatic loss of activity followed by a quick recovery within the first 72 h of incubation at 45 °C (10), the cross-linked ALP conjugates do not show similar behavior. If the loss of activity followed by the recovery, observed in the first 72 h in the cross-linked ALP, is due to the reversibility of the changes in the quaternary structure, the conjugated IgG molecule may have a stabilizing effect which prevents such transient changes in the quaternary structure possibly because of the presence of covalent cross-links to IgG and the contiguity of the IgG and ALP molecules.

All conjugates were prepared from antibodies derivatized with a 50-fold molar excess of STMCC linkers and ALP cross-linked with 2 or 4 equiv of PGA(SPO₃)₂₄. As described in the Experimental Section, the ratio of IgG to cross-linked enzyme was 1:1. The data in Figure 1 are normalized to 100% at day 0 to visualize the effect of thermal stress on various preparations. *N*-Ethylmaleimide-capped conjugates underwent thermal denaturation at a faster rate than the uncapped conjugates (data not shown).

The progress of the conjugations was followed by HPLC using a Bio-Sil SEC-400 column. The ALP enzyme activity of 1:1 ALP-IgG conjugates at day 0 was between 65 and 80% of that of the native enzyme. Panels A–E of Figure 2 show GPC profiles of IgG, native ALP, unfractionated 4:1 PGA-cross-linked ALP, the crude unfractionated IgG-cross-linked ALP conjugate, and the best crude unfractionated conjugate based on the Traut method. Chromatogram D appears to consist predominantly of the conjugated IgG. The crude conjugates at 10.11 min and higher molecular weights all show enzymatic activity, as demonstrated by careful fractionation of this material. The shoulder peak at 11.15 min shows ALP activity and has retention times virtually identical to that of PGA-cross-linked ALP.

As seen in Figures 2E and 1, the most active unfractionated Traut ALP-IgG conjugate yields material with a much higher molecular weight whose thermal stability is considerably lower than that of the cross-linked material.

Free thiols on the enzyme do not lead to aggregation of the conjugates as demonstrated by GPC analysis of the incubated materials. In view of the decrease in the number of titratable thiols in the cross-linked ALP (10), the constant size of the conjugates can possibly be attributed to the exhaustion of the thiols through some oxidative process, i.e. intramolecular disulfide bond formation.

CONCLUSIONS

Auto-catalyzed cross-linking of maleimide-derivatized alkaline phosphatase with poly(glutamic acid) poly-(phosphorothioate) stabilizes the protein against the thermal denaturation and introduces multiple thiolates on the surface of the enzyme. The stabilized enzyme is then conjugated to an the immunoglobulin molecule through the reaction of thiolates with maleimides in the IgG. The conjugation process is highly controllable; conjugate size may be adjusted by selection of appropriate stoichiometries of the cross-linking polymer, enzyme, and immunoglobulin. The method leads to thermally stabilized conjugates.

ACKNOWLEDGMENT

We thank Dr. Eric Ginsburg of the Abbott Laboratories Advanced Drug Delivery Department for his critical reading of the manuscript.

LITERATURE CITED

- (1) Butt, W. R. (1984) *Practical Immunoassay*, pp 47–50, Marcel Dekker, New York.
- (2) Maggio, E. T. (1980) *Enzyme-Immunoassay*, pp 64–65, CRC Press, Boca Raton, FL.
- (3) Ishikawa, E., Imagawa, M., Hashida, S., Yoshitake, S., Hamaguchi, Y., and Ueno, T. (1983) Enzyme-labeling of antibodies and their fragments for enzyme immunoassay and immunohistochemical staining. *J. Immunoassay* 4 (3), 209–327.
- (4) Maggio, E. T. (1980) *Enzyme-Immunoassay*, p 174, CRC Press, Boca Raton, FL.
- (5) McComb, R. B., Bowers, G. N., and Posen, S. (1979) *Alkaline Phosphatase*, pp 304–318, Plenum Press, New York.
- (6) Means, G. E., and Feeney, R. E. (1990) Chemical modification of proteins: history and applications. *Bioconjugate Chem.* 1, 2.
- (7) Wong, S. H. (1991) *Chemistry of Protein Conjugation and Cross-linking*, CRC Press, Boca Raton, FL.
- (8) Brinkley, M. (1992) A brief survey of methods for preparing protein conjugates with dyes, haptens, and cross-linking reagents. *Bioconjugate Chem.* 3, 2.
- (9) Feeney, R. E. (1987) Chemical modification of proteins: comments and perspectives. *Int. J. Pept. Protein Res.* 29, 145.
- (10) Bieniarz, C., Cornwell, M. J., and Young, D. F. (1997) Alkaline phosphatase activatable polymeric cross-linkers and their use in the stabilization of proteins. *Bioconjugate Chem.* 9, 390–398.
- (11) Bieniarz, C., Husain, M., Barnes, G., King, C. A., and Welch, C. J. (1996) Extended length heterobifunctional coupling agents for protein conjugation. *Bioconjugate Chem.* 7, 88–95.
- (12) Jue, R., Lambert, J. M., Pierce, L. R., and Traut, R. R. (1978) Addition of sulfhydryl groups to *Escherichia coli* ribosomes by protein modification with 2-iminothiolane (methyl 4-mercaptobutyrimidate). *Biochemistry* 17, 5399–5406.

BC980025B

Synthesis of 6 β -[(2'-Aminoethyl)carboxamidomethyl]estradiol and Preparation of Estradiol Probes

Maciej Adamczyk,* Donald D. Johnson, and Rajarathnam E. Reddy

Division Organic Chemistry Research (9NM, AP20), Diagnostic Division, Abbott Laboratories, 100 Abbott Park Road, Abbott Park, Illinois 60064-3500. Received November 28, 1997; Revised Manuscript Received February 13, 1998

Reformatsky reaction of 3,17 β -bis[(2-trimethylsilyl)ethoxymethyl]-1,3,5(10)-estratrien-6-one (**2**) with bromoethyl acetate and zinc gave the ester (**3**) in 60% yield which upon treatment with methanesulfonyl chloride in pyridine afforded the olefinic esters (**4** and **5**) as an endo and exo mixture (67:33 ratio) in 81% yield. Hydrolysis of the SEM protective groups in compounds **4** and **5** followed by hydrogenation of the resulting hydroxy compounds **6** and **7** using 10% Pd/C afforded an epimeric mixture (β : α = 79:21) of 6-[(ethoxycarbonyl)methyl]estradiol (**8a** and **8b**) in 95% yield. Hydrolysis of the ethyl esters (**8a** and **8b**) using sodium hydroxide gave the acid (**9a** and **9b**) in 81% yield. The epimeric mixture of acids (**9a** and **9b**) was activated, treated with *tert*-butyl-*N*-(2-aminoethyl)carbamate (**10**), and purified by HPLC to afford 6 β -[[[(2-*tert*-butoxycarbonyl)amino]ethyl]carboxamidomethyl]estradiol (**11**) in 39% yield as the major isomer. Hydrolysis of the BOC group in compound **11** using TFA afforded the desired 6 β -[(2-aminoethyl)carboxamidomethyl]estradiol **12** in 50% yield. The biotinylated estradiol probe **14**, fluorescent probe **16**, and chemiluminescent probe **18** were prepared from 6 β -[(2'-aminoethyl)-carboxamidomethyl]estradiol (**12**) and the corresponding biotin, 5-carboxyfluorescein, and 10-(3-sulfopropyl)-*N*-tosyl-*N*-(3-carboxypropyl)acridinium-9-carboxamide *N*-succinimidyl esters (**13**, **15**, and **17**) in 65–74% yield and 99% purity.

INTRODUCTION

Estradiol (E₂, **1**) is a hormone which controls the differentiation, growth, and function of the reproductive system in females. It also plays an important role in the growth of estrogen-dependent tumors and other endocrinological disorders (1–3). A variety of E₂ (**1**) derivatives have been used to study estrogen binding proteins (i.e., estrogen receptors or antibodies) and for assaying E₂ (**1**) in serum or other human body fluids (4–7). Typically, the existing hydroxyl groups at the C3 (8–10) or C17 (9) positions of the estradiol nucleus were modified. This, of course, had the disadvantage of blocking those critical binding determinants. Introduction of an auxiliary functionality at the C6 or C7 of the estradiol molecule can address this drawback. Among the many available labels, fluorescein and biotin have often been chosen for the construction of probes at the C6 (11–15), C7 (16–18), and C17 positions (19–21) of estradiol (**1**). Increasingly, chemiluminescent isoluminol (9) conjugates at the C3, C6, and C17 positions and acridinium phenyl esters at the C3 position (8–10) have been employed.

During the past few years in clinical diagnostics, nonradioactive immunoassays have gained tremendous popularity in contrast to the radioimmunoassay (RIA) (22) method, because of their simplicity and ease for automation. In nonradioactive immunoassays, a variety of probes are used for measurement of an analyte depending upon the assay format. The desired probes for the development of competitive nonradioactive immunoassays are those bearing biotin-, fluorescein-, and acridinium-labeled conjugates. A chief reason for using biotin in different assay formats is its specific and high-

affinity interaction with avidin/streptavidin (23). One of the most widely used homogeneous immunoassay formats for low-molecular weight compounds (<2000 Da) is the fluorescence polarization immunoassay (FPIA) which requires a fluorescein conjugate for signal generation (7, 24). Recently, in addition to these methods, the need for higher sensitivity and throughput has spurred the development of chemiluminescent immunoassays (CLIAs) (8–10, 25). The CLIA method is based on the measurement of light which is emitted as result of a chemical reaction, and using this technology, one can measure the analyte concentration down to high femto-gram (10⁻¹⁵ g) per milliliter levels (26). The chemiluminescent probes containing 9-acridinium derivatives have numerous advantages (26). First, the chemical reaction is simplified without the need for a catalyst. The light reaction is rapid, thereby providing an increased counting efficiency, and low background. Also, a major advantage lies in the fact that a portion of the 9-acridinium conjugate bound to the protein (antibody) is cleaved and separated from the light-emitting moiety during the oxidation process which eliminates the inhibition of the light yield and thereby maximizes the sensitivity (26). Thus, in the development of immunoassays, the choice of the label which is conjugated to the ligand will vary depending on the format and requirements of the assay.

Recently, we described the synthesis of fluorescent probes from 6 α -aminoestradiol for studies on the estrogen binding proteins (27). However, in these E₂ probes (27), the native electronic environment on the phenyl ring might be altered due to the electron-withdrawing nature of the amide functionality at the C6 position. We envisioned that, after a label is attached at the C6 position of E₂ (**1**) through an alkyl functionality, the electronic effect on the phenyl ring will be reduced and

* To whom correspondence should be addressed. Telephone: (847) 937-0225. Fax: (847) 938-8927.

the resulting probes would more closely mimic the unmodified E₂. In this paper, we describe a stereoselective synthesis of 6 β -[(2-aminoethyl)carboxamidomethyl]-estradiol (**12**) and the preparation of its biotinylated (**14**), fluorescent (**16**), and chemiluminescent (**18**) conjugates for use as estradiol probes.

EXPERIMENTAL PROCEDURES

General Methods. ¹H NMR spectra were recorded on a varian Gemini spectrometer (300 MHz). Mass spectra were obtained on a Nermang 3010 MS-50 or JEOL SX102-A mass spectrometer or Perkin-Elmer Sciex API 100 electrospray mass spectrometer. Thin-layer chromatography was performed on precoated Watman MK6F silica gel 60 Å plates (layer thickness of 250 μ m) and were visualized with UV light and/or using a KMnO₄ solution [KMnO₄ (1.0 g) and NaOH (8.0 g) in water (200 mL)] or the phosphomolybdic acid reagent (20 wt % solution in ethanol), unless otherwise noted. Column chromatography was performed on silica gel, Merck grade 60 (230–400 mesh). THF was freshly distilled from a purple solution of sodium and benzophenone, and CH₂-Cl₂ was freshly distilled from CaH₂ under nitrogen. All reagents were purchased from Aldrich Chemical Co. (Milwaukee, WI) or Sigma Chemical Co. (St. Louis, MO) and used without further purification, except where noted. All the solvents employed were of HPLC grade and purchased from EM Science (Gibbstown, NJ) and used as received. After workup, the solvent was dried using anhydrous Na₂SO₄ and concentrated on a rotary evaporator at 40 °C (bath temperature). Reverse phase (RP) analytical HPLC was performed using a Waters μ Bondapak C18 10 μ m (8 mm $\times$ 100 mm) column eluting at 2 mL/min with MeCN/0.1% aqueous trifluoroacetic acid (v/v ratio reported) with UV light at 225 nm unless otherwise stated. Preparative RP HPLC was performed using a Waters μ Bondapak C18 10 μ m (40 mm $\times$ 100 mm) column eluting at 45 mL/min with MeCN/0.1% aqueous trifluoroacetic acid (v/v ratio reported) with UV light at 225 nm unless otherwise stated.

3,17 β -Bis[(2-trimethylsilyl)ethoxymethyl]-6-hydroxy-6-[(ethoxycarbonyl)methyl]estra-1,3,5(10)-triene (3**).** In a dry single-necked round-bottom flask, 3,17 β -bis[(2-trimethylsilyl)ethoxymethyl]-1,3,5(10)-estratrien-6-one (**2**) (2.31 g, 4.22 mmol) was dissolved in a mixture of dry ether and benzene (1:1 ratio, 120 mL). Ethyl bromoacetate (1.41 mL, 12.7 mmol, 3.0 equiv) and zinc powder (1.93 g, 29.5 mmol, 7.0 equiv) were added, and the mixture was gently refluxed for 21 h under a nitrogen atmosphere. The reaction mixture was cooled to room temperature, the mixture filtered, and the filtrate concentrated. The residue was diluted with ethyl acetate (25 mL) and hexane (105 mL), and the mixture was stirred at room temperature for 15 min and filtered. The filtrate was concentrated, and the crude product was purified by silica gel column chromatography (20% ethyl acetate in hexane) to afford 1.85 g of **3** (69%) as a colorless thick oil (73:27 mixture of epimers). ¹H NMR (CDCl₃): δ 7.26–7.16 (m, 2 H), 6.97–6.90 (m, 1 H), 5.27–5.15 (m, 2 H), 4.69 (s, 2 H), 4.26–4.15 (m, 2 H), 3.74–3.58 (m, 5 H), 3.04–2.80 (m, 2 H), 2.30–1.20 (m, 16 H), 0.98–0.92 (m, 4 H), 0.82 (s, 73/100, 3 H), 0.78 (s, 27/100, 3 H), 0.22 (s, 9 H), 0.00 (s, 9 H). ESMS: 652 (M + NH₄)⁺, 1286 (2 $\times$ M + NH₄)⁺.

3,17 β -Bis[(2-trimethylsilyl)ethoxymethyl]-6-[(ethoxycarbonyl)methyl]estra-1,3,5(10),6-tetraene (4**, and Also Compound **5**).** The epimeric mixture of the hydroxy compound (**3**, 1.74 g, 2.74 mmol)

was dissolved in pyridine (35 mL), and methanesulfonyl chloride (2.12 mL, 27.4 mmol, 10.0 equiv) was added at room temperature under a nitrogen atmosphere. The mixture was heated at 60 °C for 2.5 h, and the pyridine was removed on a rotary evaporator. The residue was diluted with ethyl acetate (100 mL) and water (100 mL), and the aqueous layer was separated and extracted with ethyl acetate (2 $\times$ 100 mL). The combined organic layers were washed with brine (100 mL), dried, and concentrated. The crude compound was purified by silica gel column chromatography (10% ethyl acetate in hexane) to afford 1.34 g (81%) of compounds **4** and **5** as a thick oil (63:37 mixture of endo and exo isomers). Analytical RP HPLC (90:10 MeCN/0.1% aqueous acetic acid, 2.0 mL/min, 225 nm, relative intensity): t_R 17.63 (76%), 29.2 min (20%). ¹H NMR (CDCl₃): δ 7.32–6.90 (m, 3 H), 6.26 (s, 0.37/1 exocyclic olefinic H), 5.92 (s, 0.63/1 endocyclic olefinic H), 5.22 and 5.19 (s, s, 2 H), 4.7 (s, 2 H), 4.21–4.15 (m, 2 H), 3.80–3.25 (m, 6 H), 2.34–1.20 (m, 15 H), 0.99–0.92 (m, 4 H), 0.79 (s, 63/100, 3 H), 0.77 (s, 37/100, 3 H), 0.02 (s, 9 H), 0.00 (s, 9 H). ESMS: 634 (M + NH₄)⁺.

6-[(Ethoxycarbonyl)methyl]-3,17 β -dihydroxyestra-1,3,5(10),6-tetraene (6**, and Also Compound **7**).** The mixture of olefinic compounds (**4** and **5**, 1.24 g, 2.01 mmol) was dissolved in acetonitrile (75 mL), and 48% HF (4.2 mL) was added dropwise at room temperature. After the mixture was stirred for 2 h, it was then concentrated on a rotary evaporator to an about 20 mL volume and diluted with water (100 mL). The mixture was extracted with ethyl acetate (3 $\times$ 100 mL), and the combined organic layers were dried and concentrated. The crude compound was purified by silica gel column chromatography (5% methanol in CH₂Cl₂) to afford 0.447 g (62%) of compounds **6** and **7** present as a 2:1 mixture of endo and exo isomers. Analytical RP HPLC (60:40 MeCN/0.1% aqueous formic acid, 1.0 mL/min, 225 nm, relative intensity): t_R 6.55 (67.8%), 7.91 min (31.2%). ¹H NMR (CDCl₃): δ 7.22–6.66 (m, 3 H), 6.24 (s, 33/100, H), 5.92 (s, 67/100 H), 4.24–4.10 (m, 2 H), 3.80–3.30 (m, 2 H), 2.40–1.20 (m, 15 H), 0.76 and 0.75 (2 s, 3 H). ESMS: 357 (M + H)⁺, 374 (M + NH₄)⁺.

6 β / α -[(Ethoxycarbonyl)methyl]-3,17 β -dihydroxyestra-1,3,5(10)-triene (8a** and **8b**).** The mixture of olefinic compounds (**6** and **7**, 0.434 g, 1.22 mmol) was dissolved in a mixture of ethyl acetate/methanol (2:1, 24 mL), and 10% Pd/C (0.0130 g) was added. The mixture was stirred under a H₂ atmosphere using balloon at room temperature for 16 h. The mixture was then filtered through Celite power, and the catalyst was washed with methanol (10 mL) and the filtrate concentrated to afford 0.415 g (95%) of compounds **8a** and **8b** as an epimeric (β : α = 79:21) mixture. Analytical RP HPLC (60:40 MeCN/0.1% aqueous formic acid, 1.0 mL/min, 225 nm, relative intensity): t_R 7.49 min (91.02%). ¹H NMR (CDCl₃): δ 7.18–7.40 (m, 1 H), 6.70–6.35 (2, H), 4.24–4.12 (m, 2 H), 3.78–3.70 (m, 1 H), 3.40–3.62 (m, 1 H), 2.86–1.20 (m, 18 H), 0.81 (s, 79/100, 3 H), 0.75 (s, 29/100, 3 H). ESMS: 376 (M + NH₄)⁺.

6 β / α -(Carboxymethyl)-3,17 β -dihydroxyestra-1,3,5(10)-triene (9a** and **9b**).** Aqueous NaOH (10%, 3.6 mL, 8.96 mmol, 8.0 equiv) was added to a solution of the epimeric mixture of esters (**8a** and **8b**) (0.401 g, 1.12 mmol) in methanol (7 mL) at room temperature and the mixture stirred for 1.5 h. The reaction mixture was then diluted with water (50 mL), and the pH was adjusted to 3.0 using 1 M HCl. The mixture was extracted with ethyl acetate (3 $\times$ 50 mL), and the combined organic layers were washed with brine (50 mL), dried, and concentrated to afford 0.370 g (81%) of acid **9a** and **9b** as a mixture

(β : α = 79:21) mixture. Analytical RP HPLC (50:50 MeCN/0.1% aqueous formic acid, 1.0 mL/min, 225 nm, relative intensity): t_R 4.92 min (91.8%). ^1H NMR (CD_3OD): δ 7.10 (d, 1 H, J = 9.0 Hz), 6.68–6.55 (m, 2 H), 3.70–3.60 (m, 1 H), 3.20–3.25 (m, 1 H), 2.84–1.22 (m, 18 H), 0.80 (s, 79/100, 3 H), 0.75 (s, 29/100, 3 H). ESMS: 329 (M – H).

6 β -[[[(2-*tert*-Butoxycarbonyl)amino]ethyl]carboxamidomethyl]-3,17 β -dihydroxyestra-1,3,5 (10)-triene (11a). The epimeric mixture of acids (**9a** and **9b**) (0.360 g, 1.09 mmol) was dissolved in CH_2Cl_2 (20 mL), and *N*-hydroxysuccinimide (HOSu, 0.251 g, 2.18 mmol, 2.0 equiv) was added followed by ethyl-3-[(dimethylamino)propyl]carbodiimide (EDAC, 0.418 g, 2.18 mmol, 2.0 equiv) at room temperature under nitrogen. The mixture was stirred for 16 h and diluted with water (100 mL). The pH of the solution was adjusted to 4.0 using 1 M HCl, and the solution was extracted with CH_2Cl_2 (3 $\times$ 300 mL). The combined CH_2Cl_2 extracts were washed with brine (100 mL), dried, and concentrated to afford 0.509 g of the corresponding succinimidyl esters.

The above-prepared crude mixture of succinimidyl esters was dissolved in DMF (10.0 mL) and treated with *tert*-butyl *N*-(2-aminoethyl)carbamate (**10**, 0.179 g, 1.09 mmol, 1.0 equiv) followed by triethylamine (0.304 mL, 2.18 mmol, 2.0 equiv) at room temperature under a nitrogen atmosphere. After the mixture was stirred for 3 h, the solvent was removed on a rotary evaporator in vacuo. The residue was purified by preparative RP HPLC [Waters μ Bondpak C18 10 μm column (40 mm $\times$ 100 mm) with 60:40 MeOH/0.1% aqueous acetic acid, 25.0 mL/min, 225 nm]. The solvent was removed on a rotary evaporator and dried azeotropically using a mixture of toluene and methanol (1:1, 3 $\times$ 10 mL) to afford 0.201 g of 6 β -[[[(2-*tert*-butoxycarbonyl)amino]ethyl]carboxamidomethyl]estradiol (**11a**) in 39% yield as a colorless powder. Analytical RP HPLC (70:30 MeOH/0.1% aqueous acetic acid, 1.0 mL/min, 225 nm, relative intensity): t_R 10.02 min (93%). ^1H NMR (CD_3OD): δ 8.10 (dist t, 1 H), 7.32–7.06 (m, 1 H), 6.60–6.50 (m, 2 H), 3.65 (t, 1 H, J = 8.4 Hz), 3.22–3.36 (m, 2 H merged with CD_3OD peak), 3.20–3.10 (m, 2 H), 2.80–1.05 (m, 16 H), 1.43 (s, 9 H), 0.81 (s, 3 H). ESMS: 473 (M + H) $^+$.

Also isolated was 0.055 g (10%) of the minor isomer, 6 α -[[[(2-*tert*-butoxycarbonyl)amino]ethyl]carboxamidomethyl]estradiol (**11b**). Analytical RP HPLC (70:30 MeOH/0.1% aqueous acetic acid, 1.0 mL/min, 225 nm, relative intensity): t_R 7.68 min (95.3%). ^1H NMR (CD_3OD): δ 7.10 (d, 1 H, J = 8.4 Hz), 6.64 (d, 1 H, J = 2.5 Hz), 6.57 (dd, 1 H, J = 8.7 and 2.5 Hz), 3.63 (t, 1 H, J = 8.7 Hz), 3.22–3.36 (m, 2 H merged with CD_3OD peak), 3.14 (t, 2 H, J = 6.6 Hz), 2.77–2.72 (m, 1 H), 2.32–1.05 (m, 15 H), 1.43 (s, 9 H), 0.74 (s, 3 H). ESMS: 473 (M + H) $^+$.

6 β -[(2-Aminoethyl)carboxamidomethyl]-3,17 β -dihydroxyestra-1,3,5(10)-triene (12). Trifluoroacetic acid (4.0 mL) was added at room temperature to a solution of 6 β -[[[(2-*tert*-butoxycarbonyl)amino]ethyl]carboxamidomethyl]estradiol (**11a**, 0.180 g, 0.381 mmol) dissolved in anhydrous CH_2Cl_2 (4.0 mL). After the mixture was stirred for 30 min, the solvent was removed on a rotary evaporator to afford 0.217 g of crude amine (**12**). The crude compound (**12**) was purified by preparative RP HPLC [Waters μ Bondpak C18 10 μm column (25 mm $\times$ 100 mm) with 25:75 MeCN/0.1% aqueous trifluoroacetic acid, 9.0 mL/min, 225 nm]. The product was collected, the solvent removed on a rotary evaporator in vacuo, and the residue dried azeotropically using a mixture of toluene and methanol (1:1, 3 $\times$ 10 mL) to afford 0.092 g

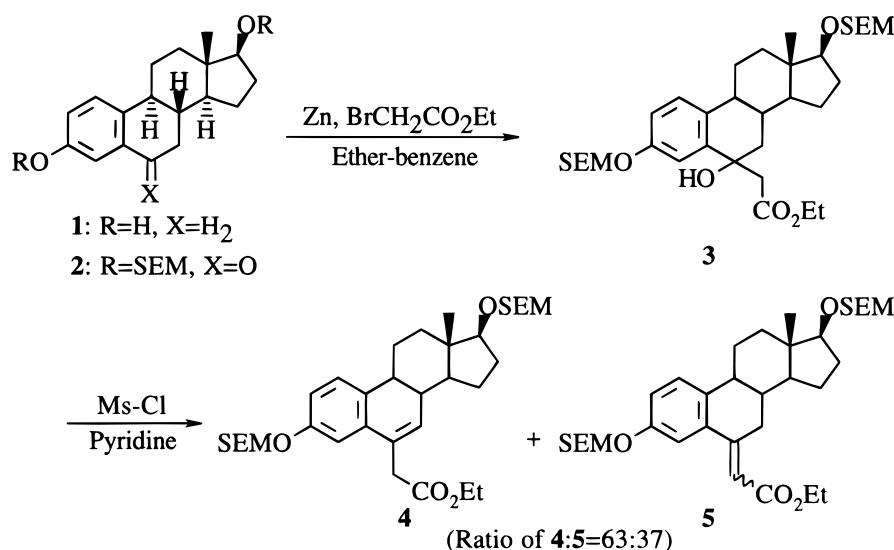
of 6 β -[(2-aminoethyl)carboxamidomethyl]-3,17 β -dihydroxyestra-1,3,5(10)-triene [**12**, 6 β -[(2'-aminoethyl)carboxamidomethyl]estradiol] in 50% yield. Analytical RP HPLC (75:25 MeCN/0.1% aqueous trifluoroacetic acid, 1.0 mL/min, 225 nm, relative intensity): t_R 8.65 min (98.6%). ^1H NMR (CD_3OD): δ 7.10 (d, 1 H, J = 8.1 Hz), 6.57–6.61 (m, 2 H), 3.68 (t, 1 H, J = 8.4 Hz), 3.58–3.38 (m, 2 H), 3.05 (t, 2 H, J = 6.3 Hz), 2.60–1.12 (m, 16 H), 0.81 (s, 3 H). ESMS: 373 (M + H) $^+$.

3,17 β -Dihydroxyestra-1,3,5(10)-triene-6 β -(Methylcarboxamidoethyl)biotinamide (14). Biotin succinimidyl ester (**23**) (**13**, 0.0102 g, 0.03 mmol, 1.2 equiv) and diisopropylethylamine (0.043 mL, 0.25 mmol, 10.0 equiv) were added sequentially to the solution of amine **12** (0.0094 g, 0.025 mmol) in anhydrous DMF (0.6 mL) at room temperature under nitrogen. After the mixture was stirred for 16.5 h, the solvent was removed on a rotary evaporator and dried on a vacuum pump. Purification of the crude product by preparative RP HPLC [Waters μ Bondpak C18 10 μm column (40 mm $\times$ 10 mm) with 25:75 MeCN/0.1% aqueous trifluoroacetic acid, 45 mL/min, 225 nm] and lyophilization afforded 0.011 g of the biotinylated probe (**14**) in 74% yield as a colorless powder. Analytical RP HPLC (30:70 MeCN/0.1% aqueous trifluoroacetic acid, 2.0 mL/min, 225 nm, relative intensity): t_R 4.56 min (99%). ^1H NMR (CD_3OD): δ 7.10 (d, 1 H, J = 9.0 Hz), 6.59–6.55 (m, 2 H), 4.44–4.41 (m, 1 H), 4.25–4.22 (m, 1 H), 3.65 (t, 1 H, J = 8.7 Hz), 3.35–3.15 (m, 4 H merged with CD_3OD), 2.90–2.65 (m, 2 H), 2.55–1.14 (m, 25 H), 0.81 (s, 3 H). ESMS: 599 (M + H) $^+$.

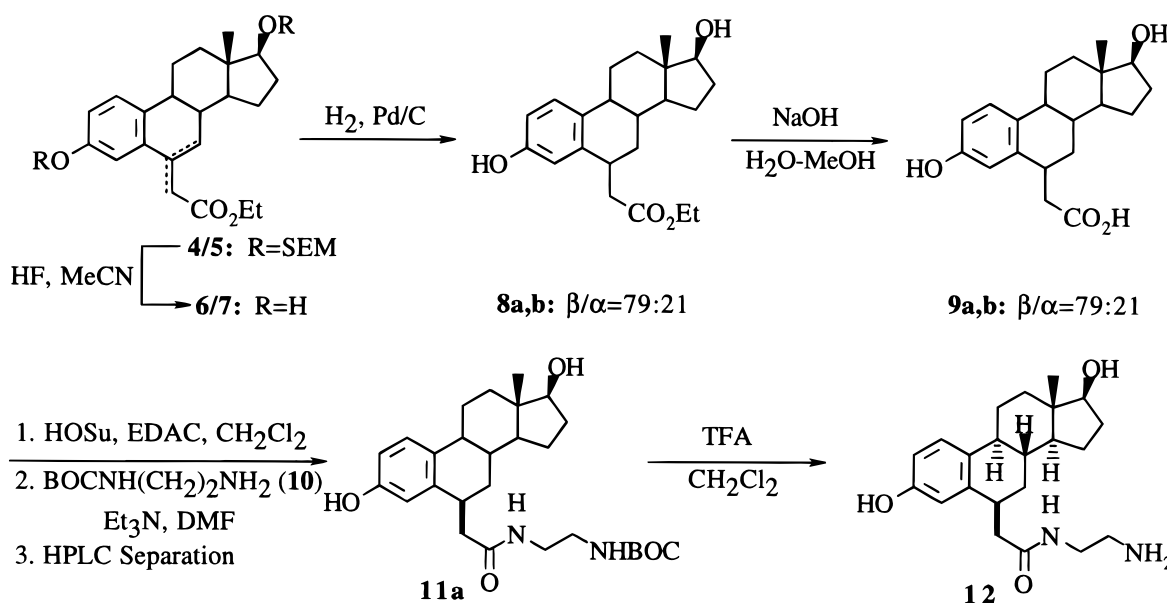
3,17 β -Dihydroxyestra-1,3,5(10)-triene-6 β -(Methylcarboxamidoethyl)-5-carboxyfluoresceinamide (16). The amine **12** (0.010 g, 0.021 mmol) was dissolved in anhydrous DMF (0.6 mL), and 5-carboxyfluorescein succinimidyl ester (**29**) (**15**, 0.010 g, 0.021 mmol, 1.0 equiv) was added followed by triethylamine (0.072 mL, 0.071 mmol, 3.4 equiv) at room temperature under a nitrogen atmosphere. The flask was covered with aluminum foil, and the mixture was stirred for 18 h. The solvent was removed on a rotary evaporator and dried on a vacuum pump. Purification of the crude product by preparative RP HPLC [Waters μ Bondpak C18 10 μm column (25 mm $\times$ 10 mm) with 40:60 MeCN/0.1% aqueous acetic acid, 9.0 mL/min, 225 nm]. The acetonitrile was removed on a rotary evaporator and the remaining material was lyophilized to afford 0.0106 g of the fluorescent probe (**16**) in 69% yield as an orange powder. Analytical RP HPLC (40:60 MeCN/0.1% aqueous acetic acid, 2.0 mL/min, 225 nm, relative intensity): t_R 11.96 min (99.9%). ^1H NMR (CD_3OD): δ 8.45 (s, 1 H), 8.24–8.18 (m, 1 H), 7.31 (d, 1 H, J = 8.1 Hz), 7.09 (d, 1 H, J = 8.4 Hz), 6.73–6.54 (m, 8 H), 3.65–3.45 (m, 5 H), 2.58–2.24 (m, 3 H), 2.10–1.90 (m, 3 H), 1.70–1.06 (m, 10 H), 0.78 (s, 3 H). ESMS: 731 (M + H) $^+$.

Acridinium-9-carboxamide Probe (18). The acridinium succinimidyl ester (**30**, **31**) (**17**, 0.014 g, 0.021 mmol, 1.0 equiv) and triethylamine (0.010 mL, 0.072 mmol, 3.4 equiv) were added to a solution of amine **12** (0.010 g, 0.021 mmol) in anhydrous DMF (0.6 mL) at room temperature under nitrogen. The flask was covered with aluminum foil, and the mixture was stirred for 15 h. The solvent was removed on a rotary evaporator and dried on a vacuum pump, and the crude product was purified by preparative RP HPLC [Waters μ Bondpak C18 10 μm column (25 mm $\times$ 10 mm) with 35:65 MeCN/0.1% aqueous formic acid, 9 mL/min, 225 nm]. The acetonitrile was removed on a rotary evaporator, and the remaining material was lyophilized to afford 0.013 g of the acridinium-9-carboxamide probe (**18**).

Scheme 1



Scheme 2



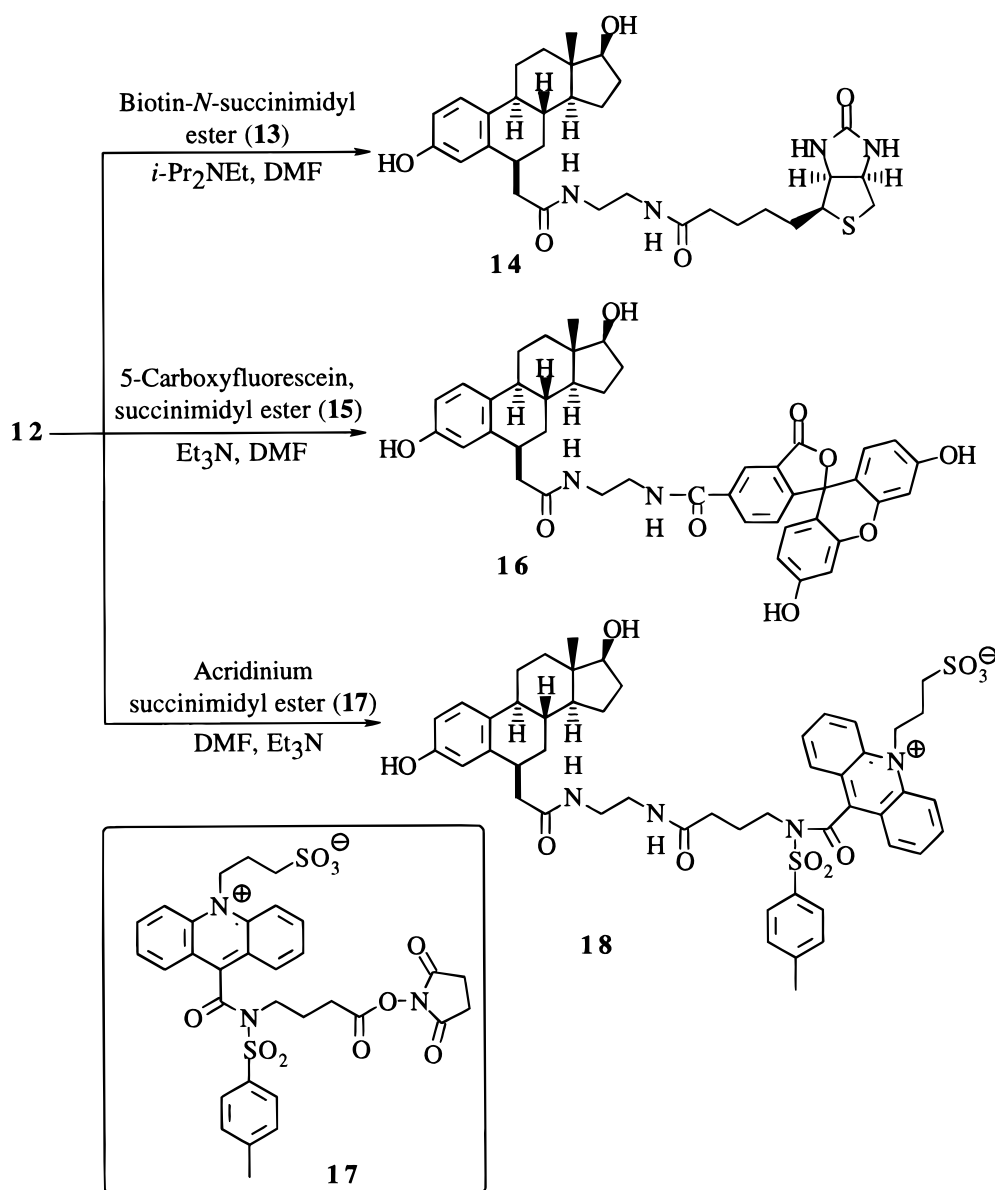
dinium probe (**18**) in 65% yield. Analytical RP HPLC (25: 75 MeCN/0.1% aqueous formic acid, 1.0 mL/min, 225 nm, relative intensity): t_R 8.02 min (>99%). ¹H NMR (CD₃-OD): δ 8.98–8.86 (m, 2 H), 8.46–8.38 (m, 2 H), 8.20–7.60 (m, 6 H), 7.18–6.98 (m, 3 H), 6.60–6.40 (m, 2 H), 5.76–5.66 (m, 2 H), 4.32–4.23 (m, 1 H), 3.68–3.12 (m, 8 H, merged with CD₃OD), 3.00–1.05 (m, 22 H), 2.35 (s, 3 H), 0.80 and 0.69 (2 s, 3 H). ESMS: 939 (M)⁺.

RESULTS AND DISCUSSION

The strategy for the synthesis of 6 β -[(2'-aminoethyl)-carboxamidomethyl]estradiol (**12**) involves (Schemes 1–3) a stereoselective introduction of carboxymethyl group at the C6 position of estradiol (**1**) and further elaboration of the spacer arm with an ethylenediamine linker. Thus, Reformatsky reaction of 3,17 β -bis[(2-trimethylsilyl)-ethoxymethyl]-1,3,5(10)-estratrien-6-one (**2**) with bromoethyl acetate and zinc and dehydration of the resulting hydroxy compound (**3**) gave the mixture of endo and exo esters (**4** and **5**) in a 67:33 ratio. Previously, Ghosh et al. (28), in their studies on pentacyclic steroids, obtained a mixture of endo and exo olefinic esters in an 85:15 ratio from 17 β -acetoxy-3-methoxyestra-1,3,5(10)-trien-6-one, a

differentially protected estradiol derivative. In the ¹H NMR spectra of **4**, the endo olefinic proton appeared at 5.92 ppm and the exo olefinic proton in **5** at 6.26 ppm which are consistent with the structures. The exo compound (**5**) appeared to be present as a single isomer by ¹H NMR (olefinic proton at 6.26 ppm), and since the double bond in ester (**5**) be reduced in the subsequent steps, no further effort was made to determine its geometry. It is noteworthy that, unlike the acetate group (28), both the C17 and C3 SEM protective groups in compound **3** were stable under the dehydration conditions; however, they were easily hydrolyzed in esters **4** and **5** using HF in MeCN (27). The resulting endo/exo mixture (**6** and **7**) was hydrogenated using 10% Pd/C to afford 6 β/α -[(ethoxycarbonyl)methyl]estradiol (**8a** and **8b**) in a 79:21 epimeric ratio as determined by ¹H NMR. The stereochemistry of the major product (**8a**) was determined to be β -isomer on the basis of the mode of hydrogenation and comparison to the previous observations by Ghosh et al. (28). In their studies (28), hydrogenation of the mixture of 17 β -hydroxy-3-methoxy-1,3,5-(10),6-estratetraene-6-acetic acid and its exo isomer gave a major β -isomer, 17 β -hydroxy-3-methoxy-1,3,5(10)-es-

Scheme 3



tratriene-6 β -acetic acid. Thus, the hydrogenation of esters **6** and **7** took place from the sterically less hindered α -face of the steroidal molecule, giving the major β -isomer. In the ¹H NMR spectra, the C17 methyl group of the β -isomer (**8a**) appeared at 0.81 ppm, whereas in the α -isomer (**9b**), the C17 methyl group appeared at 0.75 ppm.

The ethyl esters in **8a** and **8b** were hydrolyzed to the corresponding acids (**9a** and **9b**) using sodium hydroxide in 81% yield and a 79:21 ratio (β : α). Activation of the acids (**9a** and **9b**) followed by the treatment with *tert*-butyl *N*-(2-aminoethyl)carbamate (**10**) and HPLC separation afforded the pure β -isomer (**11a**) and the minor α -isomer (**11b**) in 39 and 10% yields, respectively. Finally, hydrolysis of the BOC group in **11a** using TFA in methylene chloride afforded the desired hapten, 6 β -[(2'-aminoethyl)carboxamidomethyl]estradiol (**12**), in 50% yield after purification by preparative HPLC. The hapten (**12**) was then treated with the *N*-succinimidyl active esters of biotin (**13**) (23), 5-carboxyfluorescein (**15**) (29), and 10-(sulfopropyl)-*N*-tosyl-*N*-(3-carboxypropyl)acridinium-9-carboxamide (**17**) (30, 31) in DMF to produce the corresponding biotinylated (**14**), fluorescent (**16**), and

chemiluminescent (**18**) E₂ probes, respectively, in 65–74% yield and >99% purity.

In preparation of a suitable ligand for label conjugation, the selection of the appropriate functionality on the steroidal backbone, site of substitution, and control of stereocenters all play an important role. A C6-functionalized E₂ in which the sp³ nature of C6 is retained would more closely mimic the unmodified E₂ and therefore be a more suitable hapten for preparing immunoreagents for the detection of E₂. In addition, the substitution of a label at the C6 position of E₂ (**1**) prevents the metabolic hydroxylation at this position. When a chiral center is introduced at C6, the stereochemistry and the electronic nature of the substituent at that position become critical because of its spacial disposition to the C18 methyl and C17 hydroxyl groups and the electronic effect on the phenyl ring.

Thus, in this paper, we have described the synthesis of 6 β -[(2'-aminoethyl)carboxamidomethyl]estradiol (**12**), a C6-alkyl-substituted estradiol hapten from 6-oxoestradiol (**2**). The three critically needed estradiol probes (**14**, **16**, and **18**) containing biotin, fluorescein, and acridinium-9-carboxamide, respectively, were prepared from

the amine **12** for studies on estradiol binding proteins and for development of assays for E₂ (**1**).

LITERATURE CITED

- (1) Lippman, M. E., and Allegra, J. C. (1978) Estrogen receptors and endocrine therapy of breast cancer. *N. Engl. J. Med.* 299, 930–933.
- (2) Dence, J. B. (1980) *Steroids and Peptides*, John Wiley & Sons, New York.
- (3) Top, S., El Hafa, H., Vessieres, A., Quivy, J., Vassermann, J., Hughes, D. W., McGlinchey, M. J., Mornon, J.-P., Thoreau, E., and Jaouen, G. (1995) Rhenium carbonyl complexes of β -estradiol derivatives with high affinity for the estradiol receptor: An approach to selective organometallic radiopharmaceuticals. *J. Am. Chem. Soc.* 117, 8373–8380.
- (4) Chan, D. W., and Perlstein, M. T. (1987) *Immunoassay. A Practical Guide*, Academic Press, New York.
- (5) Alberton, B. D., and Haseltine, F. P. (1988) *Non-radiometric Assays. Technology and Application in Polypeptide and Steroid Hormone Detection*, Alan R. Liss, New York.
- (6) Gosling, J. P. (1990) A decade of development in immunoassay methodology. *Clin. Chem.* 36, 1408–1427.
- (7) Wheeler, M. J. (1994) Infertility. In *The Immunoassay Handbook* (D. Wild, Ed.) pp 366–378, Stockton Press, New York.
- (8) Sato, H., Mochizuki, H., Tomita, Y., Izako, T., Sato, N., and Kanamori, T. (1996) Competitive chemiluminescent immunoassay for estradiol using an N-functionalized acridinium ester. *J. Biolumin. Chemilumin.* 11, 23–29.
- (9) De Boever, J. G., Kohen, F., and Bosmans, E. (1993) Antibody binding efficiency of differently labeled steroid hormones. *Anal. Chim. Acta* 275, 81–87.
- (10) Patel, A., Woodhead, J. S., Campbell, A. K., Hart, R. C., and McCapra, F. (1982) The preparation and properties of a chemiluminescent derivative of 17 β -estradiol. *Serono Symp. Publ. Raven Press* 1, 181–189.
- (11) Dandliker, W. B., Hicks, A. N., Levison, S. A., and Brawn, R. J. (1977) A fluorescein-labeled derivative of estradiol with binding affinity towards cellular receptors. *Biochem. Biophys. Res. Commun.* 74, 538–544.
- (12) Bodmer, D. M., Tiefenauer, L. X., and Andres, R. Y. (1989) Antigen-versus antibody-immobilized ELISA procedures based on a biotinyl-estradiol conjugate. *J. Steroid Biochem.* 33, 1161–1166.
- (13) Tiefenauer, L. X., and Andres, R. Y. (1990) Biotinyl-estradiol derivatives in enzyme immunoassays: Structural requirements for optimal antibody binding. *J. Steroid Biochem.* 35, 633–639.
- (14) Lippa, P., Birkmayer, C., and Hauptmann, H. (1994) Synthesis of 3-hydroxyestra-1,3,5(10)-trien-17-one and 3,17 β -dihydroxyestra-1,3,5(10)-triene 6 α -N-(ϵ -biotinyl)caproamide, tracer substances for developing immunoassays for estrone and estradiol. *Bioconjugate Chem.* 5, 167–171.
- (15) Mares, A., DeBoever, J., Stans, G., Bosmans, E., and Kohen, F. (1995) Synthesis of novel biotin-estradiol conjugate and its use for the development of a direct, broad range enzyme immunoassay for plasma estradiol. *J. Immunol. Methods* 183, 211–219.
- (16) Muhlenbruch, B., Kirmeier, F., and Roth, H. J. (1986) Syntheses und eigenschaften fluoreszierender estradiol-derivative (Syntheses and properties of fluorescent estradiol derivatives). *Arch. Pharm. (Weinheim, Ger.)* 319, 196–203.
- (17) French, A. N., Wilson, S. R., Welch, M. J., and Katzenellenbogen, J. A. (1993) A synthesis of 7 α -substituted estradiols: Synthesis and biological evaluation of a 7 α -pentyl-substituted BODIPY fluorescent conjugate and a fluorine-18-labeled 7 α -pentylestradiol analog. *Steroids* 58, 157–169.
- (18) Redeuilh, G., Secco, C., and Baulieu, E. E. (1990) Biotin-estradiol for purification of estrogen receptor. *Methods Enzymol.* 184, 292–300.
- (19) Barrows, G. H., Stropupe, S. B., and Riehm, J. D. (1980) Nuclear uptake of a 17 β -estradiol-fluorescein derivatives as a marker of estrogen dependence. *Am. J. Clin. Pathol.* 73, 330–339.
- (20) Daxenbichler, G., Grill, H. J., Domanid, R., Moser, E., and Dapunt, O. (1980) Receptor binding of fluorescein labeled steroids. *J. Steroid Biochem.* 13, 489–493.
- (21) Joyce, B. G., Nicholson, R. J., Morton, M. S., and Griffiths, K. (1982) Studies with steroid-fluorescein conjugates on estrogen target tissues. *Eur. J. Cancer Clin. Oncol.* 18, 1147–1155.
- (22) Alberton, B. D., and Haseltine, F. P. (1988) *Non-radiometric assays: Technology and application in polypeptide and steroid hormone detection*, Alan R. Liss, New York.
- (23) Wilchek, M., and Bayer, E. A. (1990) Biotin-containing reagents. In *Avidin-Biotin Technology* (M. Wilchek, Ed.) Vol. 184, Academic Press, New York.
- (24) Dandliker, W. B., Hsu, M.-L., and Vanderlaan, W. P. (1980) in *Immunoassays: Clinical Laboratory Techniques for the 1980's* (R. M. Nakamura, W. R. Dito, and E. S. Tucker, Eds.) pp 89–126, Alan R. Liss, New York.
- (25) Bouma, S., Worobec, S., Baker, A., Dubler, R., Frias, E., Ginsburg, S., Hehmann, M., Ishman, S., Gardiner, M., Hsu, S., Pacenti, D., Pascucci, T., Patel, C., Piechura, J., Schueller, M., Sheu, M., Wendland, D., and Wang, P. (1997) Performance of automated chemiluminescent paramagnetic micro-particle immunoassays for estradiol, progesterone, and testosterone on the Abbott i2000 system. *Clin. Chem.* 43, 295.
- (26) Dudley, R. F. (1990) Chemiluminescence immunoassay: An alternative to RIA *Lab. Med.* 21, 216–222.
- (27) Adamczyk, M., Mattingly, P. G., and Reddy, R. E. (1997) An efficient stereoselective synthesis of 6 α -aminoestradiol: preparation estradiol probes. *Steroids* 62, 462–467.
- (28) Ghosh, A. C., and Hazra, B. G. (1977) Pentacyclic steroids. Synthesis of 4,6 β -ethanoestradiol, 4,6 β -ethanoestrone, and 17 α -ethynyl-4,6 β -ethanoestradiol. *J. Org. Chem.* 42, 3091–3094.
- (29) Adamczyk, M., Fishpugh, J. R., and Heuser, K. J. (1997) Preparation of succinimidyl and pentafluorophenyl active esters of 5- and 6-carboxyfluorescein. *Bioconjugate Chem.* 8, 253–255.
- (30) Mattingly, P. G., and Barnett, L. (1995) Chemiluminescent acridinium salts. U.S. Patent 5,468,646.
- (31) Mattingly, P. G. (1991) Chemiluminescent 10-methylacridinium-9-(N-sulfonyl carboxamide)salts. Synthesis and kinetics of light emission. *J. Biolumin. Chemilumin.* 6, 107–114.

BC970203L

Photostable Chlorophyll *a* Conjugated with Poly(vinylpyrrolidone)–Smectite Catalyzes Photoreduction and Hydrogen Gas Evolution by Visible Light

Tetsuji Itoh,[†] Asako Ishii,[†] Yoh Kodera,[†] Ayako Matsushima,[†] Misao Hiroto,[†] Hiroyuki Nishimura,[†] Toshi Tsuzuki,[†] Toshiaki Kamachi,[‡] Ichiro Okura,[‡] and Yuji Inada*,[†]

Toin Human Science and Technology Center, Department of Materials Science and Technology, Toin University of Yokohama, 1614 Kurogane-cho, Aoba-ku, Yokohama 225-8502, Japan, and Department of Bioengineering, Tokyo Institute of Technology, Nagatsuta, Midori-ku, Yokohama 226-8501, Japan. Received September 12, 1997; Revised Manuscript Received January 14, 1998

Chlorophyll *a* was adsorbed to a synthetic smectite intercalated by poly(vinylpyrrolidone) (PVP) to form the chlorophyll–PVP–smectite conjugate (Chl–PVP–SME) having an absorption maximum at 677 nm. The conjugate was found to be stable toward light illumination in comparison with chlorophyll–smectite, chlorophyll–PVP, and free chlorophyll *a*. Chl–PVP–SME had a photoinduced activity for catalyzing the reduction of methyl viologen. Furthermore, the evolution of hydrogen gas was observed when an aqueous suspension containing Chl–PVP–SME, methyl viologen (an electron carrier), 2-mercaptoethanol (an electron donor), and hydrogenase was illuminated by visible light.

INTRODUCTION

Chlorophyll molecules bind to proteins to form chlorophyll–protein conjugates in the layered thylakoid membrane of chloroplasts and exhibit physiological functions: the photolysis of water and the reduction of NADP (1) under visible light illumination.

A large number of investigations on photosensitization have been published using photocatalytic inorganic compounds such as titanium oxide in the presence of ultraviolet ray (2). To enhance the efficacy of the photocatalytic reactions, Domen et al. (3, 4) demonstrated that a series of layered metal oxide, perovskite, catalyzes overall water splitting. In addition, Miyata et al. (5) prepared viologens intercalated into the layered space of bentonites in relation to their photochromic behavior.

From the viewpoint of solar energy conversion, however, photocatalysts such as chlorophyll and porphyrin derivatives which work under visible light illumination are indispensable. Okura et al. (6, 7) observed photoinduced hydrogen evolution using zinc *meso*-tris(sulfonatophenyl)porphyrin as a photosensitizer in the presence of an electron donor and an electron carrier under visible light illumination. Chlorophyll, however, has been seldom used as a photocatalyst because the natural pigment loses its photostability by extraction with an organic solvent.

We prepared chlorophyll *a* adsorbed onto bentonite (8) and onto smectite (9). Each conjugate was found to be photostable, and its adsorption maximum was shifted to a longer wavelength by increasing the amount of chlorophyll *a* adsorbed onto clay minerals. Bentonite and smectite minerals, which consist of two-dimensional silicate layers separated by hydrated exchangeable cations, swell with a variety of molecules and form inter-

calated complexes (10). Recently, we reported that the chlorophyll–bentonite conjugate exhibited a high photosensitizing activity for reducing nitro blue tetrazolium, through the formation of superoxide anion (11).

This paper deals with preparation of a chlorophyll–PVP–smectite conjugate having high photostability against light. Furthermore, the conjugate evolves hydrogen gas effectively in the presence of an electron donor, an electron carrier, and a catalyst under visible light illumination.

EXPERIMENTAL PROCEDURES

General. Chlorophyll *a* purified from *Spirulina*, poly(vinylpyrrolidone) (PVP)¹ with an average molecular weight of 10 000, and methyl viologen (1,1-dimethyl-4,4-bipyridinium chloride) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Smectite powder (hectorite), which was hydrothermally synthesized, was obtained from Co-op Chemical Co., Ltd. (Tokyo, Japan). Its properties are as follows: elemental composition Si 8.00, Mg 5.65, Li 0.70, Na 1.05; transmittance 95% in 1% aqueous solution at 500 nm; and methylene blue adsorption 101 mequiv per 100 g. Hydrogenase was isolated from *Desulfovibrio vulgaris* according to Yagi's method (12). Other reagents were of analytical grade.

Chlorophyll–PVP–Smectite. The chlorophyll–PVP–smectite conjugate (Chl–PVP–SME) was prepared to test the photostability of chlorophyll *a* as follows. At first, smectite was suspended in a 3% NaCl aqueous solution for 24 h three times to make Na-smectite. Na-smectite was then stirred in a PVP aqueous solution (5%) with a 3:2 weight ratio of PVP and Na-smectite, and the mixture was freeze-dried to form a PVP–smectite con-

* Author to whom correspondence should be addressed. Phone: +81-45-974-5060. Fax: +81-45-972-5972.

[†] Toin University of Yokohama.

[‡] Tokyo Institute of Technology.

¹ Abbreviations: PVP, poly(vinylpyrrolidone); Chl–PVP–SME, chlorophyll–poly(vinylpyrrolidone)–smectite conjugate; PVP–SME, poly(vinylpyrrolidone)–smectite conjugate; Chl–PVP, chlorophyll–poly(vinylpyrrolidone) conjugate; Chl–SME, chlorophyll–smectite conjugate.

jugate (PVP-SME) according to the method of Miyata et al. (5). Two milliliters of chlorophyll *a* dissolved in benzene (9.8 mg/mL) was added to PVP-SME (600 mg). The suspension was then shaken for 1 h at 25 °C to establish the adsorption equilibrium between chlorophyll *a* and PVP-SME. The Chl-PVP-SME thus prepared was collected by centrifugation and was dried under reduced pressure. The amount of chlorophyll *a* adsorbed to PVP-SME was spectrophotometrically determined by using the molar extinction coefficient of chlorophyll *a* ($9.25 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ at 661.6 nm in acetone) (13). One gram of smectite powder in the conjugate adsorbed 12.5 mg of chlorophyll *a* and 1.5 g of PVP. The absorption spectrum of Chl-PVP-SME in 50 mM Tris-HCl buffer (pH 7.8) was measured with a Shimadzu MPS-2000 multipurpose spectrophotometer (Kyoto, Japan) (14). The photostability of chlorophyll *a* was tested as follows. Chl-PVP-SME together with Chl-SME, Chl-PVP in water, and free chlorophyll *a* in benzene was illuminated with a 60 W incandescent lamp at a distance of 9.5 cm, with a light intensity of $200 \text{ J m}^{-2} \text{ s}^{-1}$, at 30 °C.

Chlorophyll-PVP-Smectite Intercalated by Methyl Viologen. Chl-PVP-SME intercalated by methyl viologen was prepared to test whether the conjugate causes photoreduction of methyl viologen by visible light. First, an aqueous solution (17 mL) of methyl viologen (101 mg) was added to powdered PVP-SME (580 mg), and the mixture was stirred for 48 h. After centrifugation, the precipitate was washed with methanol and dried (the conjugate contained 21 mg of methyl viologen). PVP-SME intercalated by methyl viologen was then added to chlorophyll *a* (19.6 mg) dissolved in benzene (2 mL), and the suspension was shaken for 1 h at 25 °C to establish the adsorption equilibrium between chlorophyll *a* and PVP-SME intercalated by methyl viologen. The Chl-PVP-SME intercalated by methyl viologen was collected by centrifugation and was dried under reduced pressure. The conjugate was composed of Chl, PVP, SME, and methyl viologen at a weight ratio of 1:178:130:16.

Photoreduction of Methyl Viologen. Photoreduction of methyl viologen intercalated into Chl-PVP-SME was tested in the presence of 2-mercaptoethanol. The conjugate (17.3 mg; 0.06 μmol as chlorophyll *a*, 4.6 μmol as methyl viologen) was suspended in 3 mL of 25 mM Tris-HCl buffer (pH 7.4) containing 1 mmol of 2-mercaptoethanol. The sample mixture was deaerated by repeated freeze-pump-thaw cycles and was illuminated with a 60 W incandescent lamp at a distance of 3.5 cm, with a light intensity of $1500 \text{ J m}^{-2} \text{ s}^{-1}$, at 30 °C. The photochemical reduction of methyl viologen was observed by measuring the absorbance increase at 605 nm, using the molar extinction coefficient of $1.65 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$.

Evolution of Hydrogen Gas. Hydrogen evolution by Chl-PVP-SME intercalated by methyl viologen in the presence of 2-mercaptoethanol and hydrogenase was tested according to the method of Okura et al. (6, 7). The reaction system (3 mL) consisted of 47.5 mg of Chl-PVP-SME intercalated by 2.5 mg of methyl viologen (50 mg; 0.17 μmol as chlorophyll *a*, 3 μmol as methyl viologen) suspended in 25 mM Tris-HCl buffer (pH 7.4) in the presence of 2-mercaptoethanol (1 mmol) and a hydrogenase solution (40 μL). The sample mixture in a Pyrex cell (17.5 cm^3) was deaerated by repeated freeze-pump-thaw cycles, and the upper space of the cell (14.5 cm^3) was filled with argon gas. The sample in the cell equipped with a magnetic stirrer was illuminated with a 200 W tungsten lamp (light intensity of $200 \text{ J m}^{-2} \text{ s}^{-1}$). Light at wavelengths of $<390 \text{ nm}$ was eliminated by a

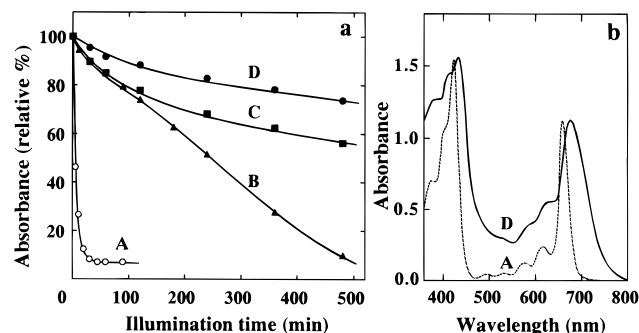


Figure 1. Photostability (a) and absorption spectra (b) of chlorophyll *a* (Chl) and chlorophyll *a* conjugated with poly(vinylpyrrolidone) (PVP) and/or smectite (SME). (a) Curve A, chlorophyll *a* in benzene; and curves B–D, Chl-PVP, Chl-SME, and Chl-PVP-SME, respectively. Each sample was illuminated at a light intensity of $200 \text{ J m}^{-2} \text{ s}^{-1}$, at 30 °C. (b) Curve A, spectrum of chlorophyll *a* in benzene (absorption maximum at 665 nm); and curve D, spectrum of Chl-PVP-SME in aqueous solution (absorption maximum at 677 nm).

Table 1. Absorption Maxima and Photostability of Chl-PVP-SME and Its Related Conjugates

	chlorophyll conjugate	absorption maximum (nm)	stabilization of chlorophyll ^a (%)
A	chlorophyll in benzene	665	<8
B	chlorophyll-PVP	672	23
C	chlorophyll-smectite	673	62
D	chlorophyll-PVP-smectite	677	80
E	chlorophylls in intact leaves	678	100

^a Relative value of absorbance at absorption maximum in the red region after illumination for 360 min. The absorbance value of each sample obtained before the illumination was regarded as 100%.

Toshiba L-39 filter (Tokyo, Japan). A portion of the evolved hydrogen gas was collected via a sampling valve of the cell and was analyzed by gas chromatography.

RESULTS AND DISCUSSION

Photostability of Chl-PVP-SME. In this study, we prepared three kinds of chlorophyll conjugates, chlorophyll-poly(vinylpyrrolidone) (Chl-PVP), chlorophyll-smectite (Chl-SME), and chlorophyll-poly(vinylpyrrolidone)-smectite (Chl-PVP-SME). These chlorophyll conjugates became colloidal solutions with green color by the addition of water. Using these chlorophyll *a* conjugates in water together with chlorophyll *a* in benzene, the photostability was tested by illumination with visible light, which is shown in Figure 1a. Chlorophyll *a* dissolved in benzene is rapidly bleached by light (curve A). On the other hand, the absorbance decreases of the conjugates are much slower as shown by curves B (Chl-PVP), C (Chl-SME), and D (Chl-PVP-SME). Among them, Chl-PVP-SME (curve D) is the most stable. The absorption spectra of chlorophyll *a* in benzene and Chl-PVP-SME in aqueous solution are shown in Figure 1b (curves A and D, respectively). The wavelength of the absorption maximum is located at 665 nm for chlorophyll *a* in benzene and at 677 nm for Chl-PVP-SME in aqueous solution. Table 1 represents the relation between the absorption maximum and the stabilization of these chlorophyll conjugates with chlorophyll in intact leaves. As is clear from the table, chlorophylls in intact leaves have the absorption maximum at 678 nm (14) and are quite stable against light illumination. On the contrary, chlorophyll *a* in benzene has the absorption maximum at 665 nm. It is noteworthy that the shift in

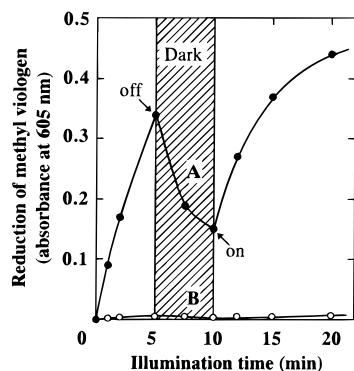


Figure 2. Photoreduction of methyl viologen by Chl-PVP-SME (light intensity of $1500 \text{ J m}^{-2} \text{ s}^{-1}$) at 30°C . (Curve A) The reaction system (3 mL) consists of methyl viologen (0.85 mg) intercalated into Chl-PVP-SME (16.45 mg) and 2-mercaptoethanol (1 mmol). (Curve B) PVP-SME (16.45 mg) was used instead of Chl-PVP-SME in the above reaction system. The hatched pattern in the figure indicates a period in the dark.

absorption maximum of each conjugate to a longer wavelength from 665 nm of chlorophyll *a* is accompanied by an increase in the stability of chlorophyll *a*.

Inamura et al. prepared water-soluble chlorophyll by the addition of macromolecules, such as poly(vinyl alcohol), poly(vinylpyrrolidone), poly(ethylene glycol), and bovine serum albumin (15–17). These chlorophyll preparations were reported to be photostable, and their absorption maxima shifted to longer wavelengths. Kathleen et al. (18) and Sakoda et al. (19) prepared smectite-porphyllin conjugates with similar shifts of the absorption maxima.

Photoreduction of Methyl Viologen and Evolution of Hydrogen Gas. A next series of experiments was conducted to test whether Chl-PVP-SME induces the reduction of methyl viologen by light illumination and also the evolution of hydrogen gas. Figure 2 represents the photoreduction of methyl viologen by Chl-PVP-SME. When a suspension containing Chl-PVP-SME intercalated by methyl viologen is illuminated in the presence of 2-mercaptoethanol, the absorbance at 605 nm increases with illumination time, indicating the reduction of methyl viologen (Figure 2, curve A). When the light is turned off, the absorbance at 605 nm decreases gradually, probably due to the oxidation of reduced methyl viologen by a trace amount of oxygen existing in the reaction system. The decreased absorbance in dark increases again by the second illumination. On the other hand, methyl viologen is hardly reduced with PVP-SME in place of Chl-PVP-SME (curve B). In the absence of 2-mercaptoethanol in the reaction system, no photoreduction of methyl viologen occurs, even by light illumination (data not shown).

As the standard oxidation–reduction potential of methyl viologen is -0.44 V , the photoreduction system containing Chl-PVP-SME intercalated by methyl viologen is expected to reduce two hydrogen ions into a hydrogen

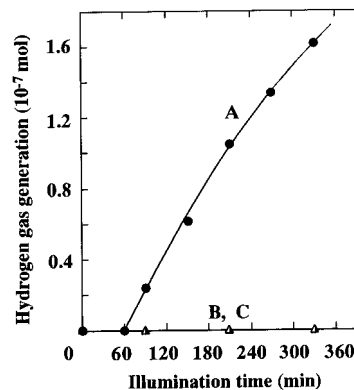
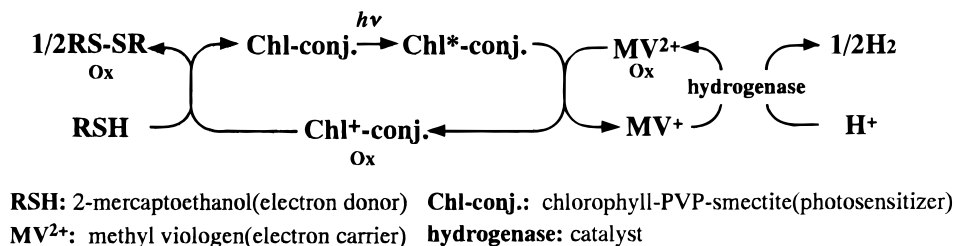


Figure 3. Hydrogen gas generation by Chl-PVP-SME intercalated by methyl viologen in the presence of 2-mercaptoethanol and hydrogenase under illumination with visible light (light intensity of $200 \text{ J m}^{-2} \text{ s}^{-1}$) at 30°C . (Curve A) The reaction system (3 mL of 25 mM Tris-HCl buffer at pH 7.4) consists of Chl-PVP-SME (47.5 mg) intercalated by methyl viologen (2.5 mg), 2-mercaptoethanol (1 mmol), and hydrogenase (40 μL). The chlorophyll content in this reaction system was $0.17 \mu\text{mol}$. (Curve B) In the absence of 2-mercaptoethanol. (Curve C) In the dark.

molecule according to Scheme 1 (6, 7). Figure 3 shows the hydrogen gas evolution by Chl-PVP-SME intercalated by methyl viologen in the presence of 2-mercaptoethanol (electron donor) and hydrogenase under illumination with visible light. The amount of hydrogen gas evolved from the reaction system increases with the illumination time, with a delay of about 60 min which is shown by curve A in Figure 3. After illumination of the mixture for 6 h, approximately $0.16 \mu\text{mol}$ of hydrogen gas is evolved from the reaction system, and the reaction is still in progress. It indicates that $0.32 \mu\text{mol}$ of hydrogen ion, which is twice the amount of chlorophyll *a* ($0.17 \mu\text{mol}$) on the smectite, is reduced to hydrogen molecules. Therefore, the chlorophyll conjugate appears to serve as a photoactivated system for transferring electrons from 2-mercaptoethanol to a more reductive hydrogen molecule (6, 7). A similar experiment is conducted in the absence of 2-mercaptoethanol or in the dark, but hydrogen gas is not evolved in each case shown by curves B and C, respectively. The reason the hydrogen evolution could not be observed during the initial 60 min is not clarified yet. It would probably take time to reduce the metal ions (Fe^{3+} , Ni^{3+}) in the active center of hydrogenase to activate the enzyme (20). From the results obtained above, the chlorophyll conjugate excited by illumination ($\text{Chl}^*\text{-conj.}$) releases an electron and changes to a cationic chlorophyll conjugate ($\text{Chl}^+\text{-conj.}$). The electron reduces methyl viologen dication (MV^{2+}) to methyl viologen cation radical (MV^+), which reduces hydrogen ion to hydrogen gas by the catalytic action of hydrogenase. On the other hand, the $\text{Chl}^+\text{-conj.}$ is reduced by 2-mercaptoethanol to reproduce chlorophyll conjugate (Scheme 1).

Scheme 1



In conclusion, chlorophyll molecules gained high stability against light illumination by conjugating with poly-(vinylpyrrolidone) and/or smectite, and the chlorophyll conjugates exhibited high photosensitizing activity during the long-period illumination. Further experiments are now in progress to stabilize the chlorophyll conjugate against light and to enhance the efficacy of photoenergy conversion. We believe that, soon, the chlorophyll molecules conjugated with multilayered compounds could be designed to allow the artificial photosynthetic reactions such as water splitting and carbon dioxide fixation.

LITERATURE CITED

- (1) Scheer, H. (1991) in *Chlorophylls* (H. Scheer, Ed.) CRC Press, London.
- (2) Yoneyama, H., Koizumi, M., and Tamura, H. (1979) Photolysis of water on illuminated strontium titanium trioxide. *Bull. Chem. Soc. Jpn.* 52, 3449–3450.
- (3) Takata, T., Shinohara, K., Tanaka, A., Hara, M., Kondo, J. N., and Domen, K. (1997) A highly active photocatalyst for overall water splitting with a hydrated layered perovskite structure. *J. Photochem. Photobiol., A* 106, 45–49.
- (4) Takata, T., Furumi, Y., Shinohara, K., Tanaka, A., Hara, M., Kondo, J. N., and Domen, K. (1997) Photocatalytic decomposition of water on spontaneously hydrated layered perovskite. *Chem. Mater.* 9, 1063–1064.
- (5) Miyata, H., Sugahara, Y., Kuroda, K., and Kato, C. (1987) Synthesis of montmorillonite-viologen intercalation compounds and their photochromic behavior. *J. Chem. Soc., Faraday Trans.* 83, 1851–1858.
- (6) Okura, I., Kaji, N., Aono, S., Kita, T., and Yamada, A. (1985) Photoinduced hydrogen evolution using bipyridinium salts as electron carrier. *Inorg. Chem.* 24, 451–453.
- (7) Okura, I., Kusunoki, S., and Aono, S. (1984) The complexation of zinc tetraphenylporphyrintrisulfonate by viologen and its role in photoredox processes. *Bull. Chem. Soc. Jpn.* 57, 1184–1188.
- (8) Kodera, Y., Kageyama, H., Sekine, H., and Inada, Y. (1992) Photo-stable chlorophylls conjugated with montmorillonite. *Biotechnol. Lett.* 14, 119–122.
- (9) Ishii, A., Itoh, T., Kageyama, H., Mizoguchi, T., Kodera, Y., Matsushima, A., Torii, K., and Inada, Y. (1995) Photostabilization of chlorophyll *a* adsorbed onto smectite. *Dyes Pigm.* 28, 77–82.
- (10) Torii, K., and Iwasaki, T. (1987) Synthesis of hectorite. *Clay Sci.* 6, 1–16.
- (11) Ishii, A., Itoh, T., Kodera, Y., Matsushima, A., Hiroto, M., Nishimura, H., and Inada, Y. (1997) Photostable chlorophyll *a*-bentonite conjugate exhibits high photosensitive activity. *Res. Chem. Intermed.* 23, 683–689.
- (12) Yagi, T. (1970) Solubilization, purification and properties of particulate hydrogenase from *Desulfovibrio vulgaris*. *J. Biochem.* 68, 649–657.
- (13) Lichtenthaler, H. K. (1987) Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. *Methods Enzymol.* 148, 350–382.
- (14) Shibata, K. (1959) Spectrophotometry of translucent biological materials—opal glass transmission method. *Methods Biochem. Anal.* 7, 77–109.
- (15) Inamura, I., Ochiai, H., Toki, K., Ishine, Y., and Araki, T. (1985) New preparation method of chlorophyll/water-soluble macromolecular complexes. *Chem. Lett.*, 381–384.
- (16) Inamura, I., Araki, T., Shei, M., Nishikawa, T., Shibata, H., and Ochiai, H. (1988) Properties of the water-soluble chlorophyll-synthetic linear macromolecular complexes. *Biochim. Biophys. Acta* 932, 335–344.
- (17) Shibata, H., Ochiai, H., Kawashima, T., Okamoto, T., and Inamura, I. (1986) Preparation and properties of the water-soluble chlorophyll-bovine serum albumin complexes. *Biochim. Biophys. Acta* 852, 175–182.
- (18) Carrado, K. A., and Winans, R. E. (1990) Interaction of water-soluble porphyrins and metalloporphyrins with smectite clay surface. *Chem. Mater.* 2, 328–335.
- (19) Sacoda, K., and Kominami, K. (1993) Photochemical hole burning of cationic porphyrin/saponite intercalation compounds. *Chem. Phys. Lett.* 216, 270–274.
- (20) Fernandez, V. M., Hatchikian, E. C., and Cammack, R. (1985) Properties and reactivation of two different deactivated forms of *Desulfovibrio gigas* hydrogenase. *Biochim. Biophys. Acta* 832, 69–79.

BC9701656

CORRECTIONS

Volume 8, Number 3, May/June 1997.

Robert S. Wu,* A. J. McNally, Ian A. Pitcher, and
Salvatore J. Salamone

SYNTHESIS OF NEW *d*-PROPOXYPHENE
DERIVATIVES AND THE DEVELOPMENT OF A
MICROPARTICLE-BASED IMMUNOASSAY FOR
THE DETECTION OF PROPOXYPHENE AND
NORPROPOXYPHENE

The authors include as one of the authors on the paper
Shaker Rashid. The authors also gratefully acknowledge
Dr. Lili Arabshahi, Mr. Stephen Vitone, and Mr. Fauzi
Rashid for their technical assistance.

BC9702009

S1043-1802(97)00200-0

Published on Web 03/20/1998

COMMUNICATIONS

A Method for the ^{32}P Labeling of Peptides or Peptide Nucleic Acid Oligomers

Igor A. Kozlov,[†] Peter E. Nielsen,[‡] and Leslie E. Orgel^{*,†}

The Salk Institute for Biological Studies, P.O. Box 85800, San Diego, California 92186, and Center for Biomolecular Recognition, IMBG, The Panum Institute, Blegdamsvej 3c, DK-2100 N Copenhagen, Denmark.
Received March 24, 1998

A novel approach to the radioactive labeling of peptides and PNA oligomers is described. It is based on the conjugation of a deoxynucleoside 3'-phosphate with the terminal amine of the substrate, followed by phosphorylation of the 5'-hydroxyl group of the nucleotide using T4 polynucleotide kinase and [γ - ^{32}P]ATP.

During the course of work on chimeras of DNA and peptide nucleic acids (PNAs), we needed to synthesize conjugates of 2'-deoxycytidine 3'-phosphate (dCp) and PNAs. Reaction between 2'-deoxycytidine 3'-phosphorimidazolid (dCpIm) and the free terminal amino group of PNA was found to proceed with almost quantitative yield. We found that the conjugate could then be phosphorylated using T4 polynucleotide kinase (Maniatis et al., 1982) as efficiently as an oligonucleotide. This is not surprising since T4 polynucleotide kinase is known to phosphorylate nucleoside-containing substrates with a free 5'-OH group and an esterified phosphate group at the 3'-position (Richardson, 1981). Our experiments show that T4 polynucleotide kinase will accept a substrate in which the 3'-phosphomonoester is replaced by

an analogous 3'-phosphoamide. We have now extended our procedure to the phosphorylation of other amine-containing molecules and have used it to label the N terminus of a peptide.

2'-Deoxycytidine 3'-phosphorimidazolid (dCpIm) was synthesized from dCp by an obvious modification of a published procedure in almost quantitative yield (Joyce et al., 1984). The PNA molecule $\text{H}_2\text{N-ATGCTCTGC-CONH}_2$ (PNA-1) with a free terminal amino group and a protected carboxyl group was obtained by solid phase synthesis using a published procedure, and its identity was confirmed by mass spectroscopy (Christensen et al., 1995; Hyrup and Nielsen, 1996). The peptide molecule $\text{H}_2\text{N-WQFEQQ-CONH}_2$ (P-1) containing a free terminal amino group and a protected carboxyl group was synthesized at the Salk Institute for Biological Studies.

To synthesize the PNA-dCp conjugate, a solution of PNA-1 (1 mM) and dCpIm (0.1 M) in 0.1 M Im buffer, at pH 7.7, was kept at room temperature for 2 days. The peptide-dCp conjugate was prepared in the same way

* Author to whom correspondence should be addressed.
Telephone: (619) 453-4100, ext 1321. Fax: (619) 558-7359.
E-mail: orgel@salk.edu.

[†] The Salk Institute for Biological Studies.

[‡] Center for Biomolecular Recognition.

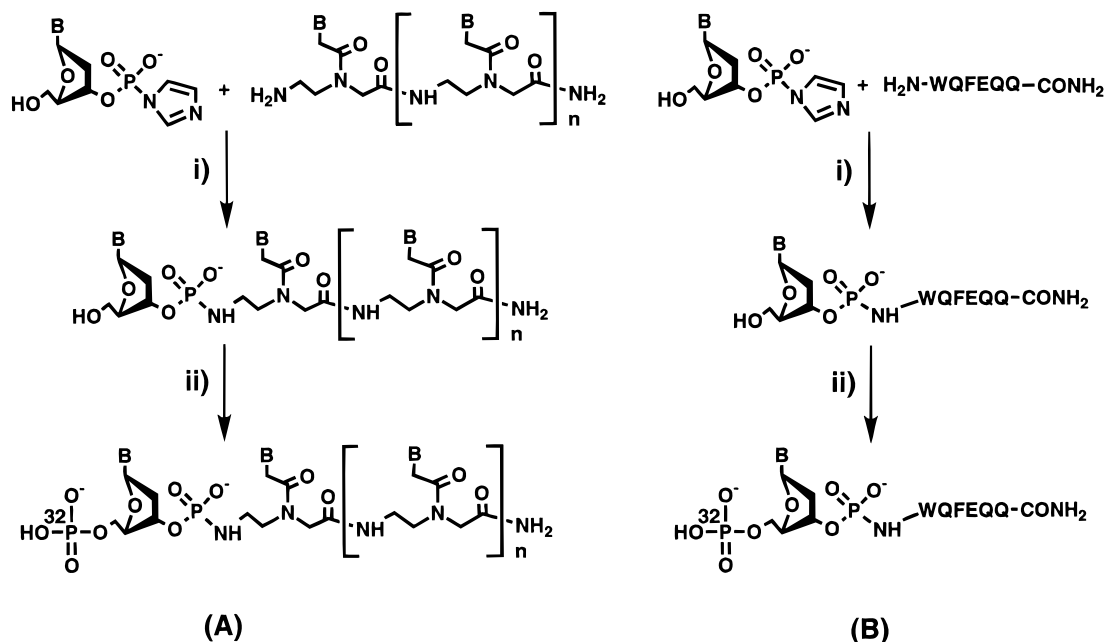


Figure 1. (A) Scheme for the synthesis of a conjugate of PNA with dCp. (B) Scheme for the synthesis of a conjugate of a peptide with dCp: (i) 0.1 M Im buffer at pH 7.7 and (ii) T4 polynucleotide kinase and ATP, under routine conditions (Maniatis et al., 1982).

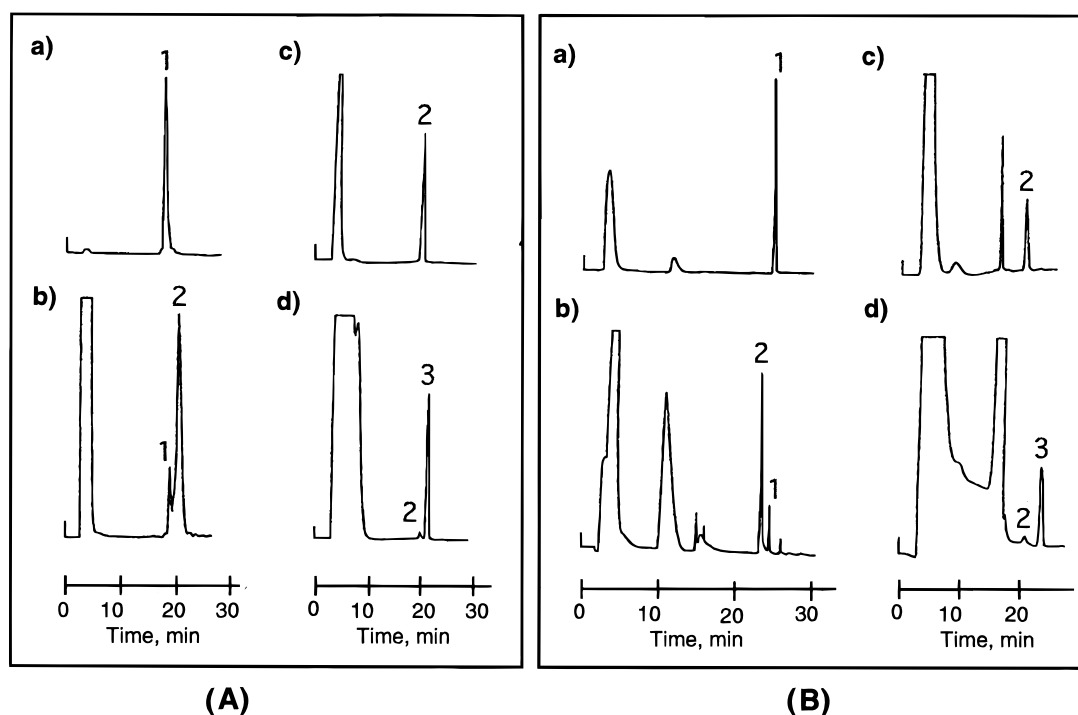


Figure 2. (A) Synthesis (a and b) and phosphorylation (c and d) of the conjugate between PNA-1 and dCp: (a) starting PNA-1, (b) reaction mixture after 2 days, (c) purified conjugate, (d) reaction mixture after phosphorylation of the conjugate, (1) starting PNA-1, (2) conjugate of PNA-1 with dCp and (3) 5'-phosphorylated conjugate. HPLC analysis was performed on an RPC5 column as previously described (Joyce et al., 1984; Schmidt et al., 1996). Reaction products were eluted with a linear gradient of NaClO_4 (pH 12, 0 to 0.06 M over the course of 60 min) and monitored by UV absorption at 254 nm. (B) Synthesis (a and b) and phosphorylation (c and d) of the conjugate of peptide P-1 with dCp: (a) starting peptide, (b) reaction mixture after 2 days, (c) purified conjugate, (d) reaction mixture after phosphorylation of the conjugate, (1) starting peptide P-1, (2) conjugate of P-1 with dCp and (3) 5'-phosphorylated conjugate. HPLC analysis (a and b) was performed on a C18 RP-HPLC column using a linear gradient of 0 to 40% acetonitrile in water (0.1% TFA) over the course of 30 min. Reaction products were monitored by UV absorption at 220 nm. HPLC analysis (c and d) was performed on an RPC5 column as previously described (Joyce et al., 1984). Reaction products were eluted with a linear gradient of NaClO_4 (pH 8, 0 to 0.06 M over the course of 60 min) and monitored by UV absorption at 220 nm. We did not study the nature of the material that eluted ahead of compound 2 in part c since the peak appears in a control experiment from which the substrate peptide was omitted. We believe that the large peak that elutes ahead of compounds 2 and 3 in part d corresponds to a component of the phosphorylation reaction mixture.

but using 3 mM peptide P-1 in place of 1 mM PNA. The reaction sequence is shown in Figure 1. The PNA-dCp conjugate was purified by HPLC on an RPC5 column at

pH 12 (Joyce et al., 1984; Schmidt et al., 1996) (Figure 2A, part b) and then dialyzed against water (Spectrum dialysis membranes). The peptide-dCp conjugate was

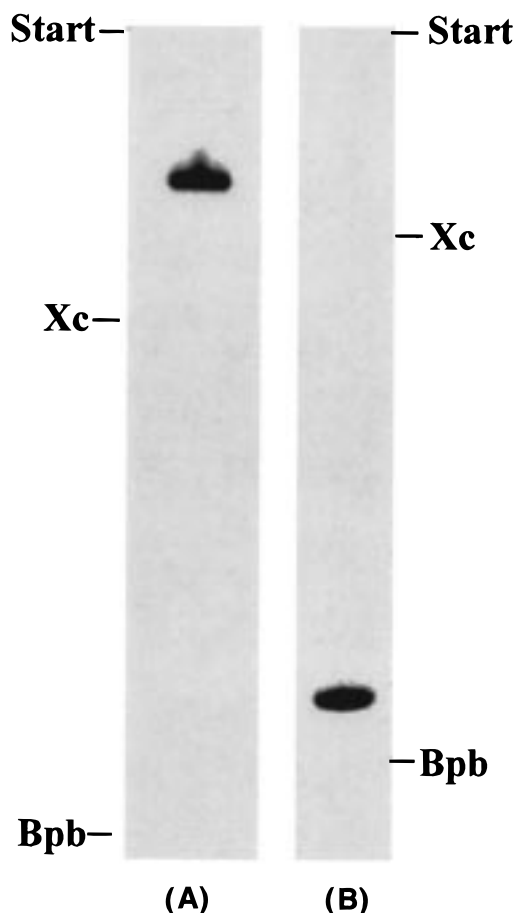


Figure 3. Analysis of ^{32}P -labeled conjugates between (A) PNA-1 and dCp and (B) peptide P-1 and dCp by electrophoresis on a 20% polyacrylamide gel. Separations were run for about 2 h at a constant current of 18 mA on a denaturing gel (8 M urea). The elution buffer was 50 mM Tris-borate (pH 8.3) containing 1 mM ethylenediaminetetraacetic acid (EDTA). Xc and Bpb are the positions of markers xylene cyanol and bromophenol blue, respectively.

purified by RP-HPLC on a C18 column (Figure 2B, part b). The fraction containing the product was neutralized with 1 N sodium hydroxide and then dialyzed against water. The molecular weights of the conjugates were determined using a Bruker Esquire-LC electrospray ionization mass spectrometer (PNA-dCp conjugate, calcd for $\text{C}_{105}\text{H}_{135}\text{N}_{52}\text{O}_{35}\text{P} - \text{H}^+$ 2714, found 2715; peptide-dCp conjugate, calcd for $\text{C}_{49}\text{H}_{65}\text{H}_{14}\text{O}_{17}\text{P} - \text{H}^+$ 1151.4, found 1150.9). The chemical structures of the conjugates were confirmed by their hydrolysis in 0.1 N HCl at 37 °C (2 h) which gave the initial PNA or peptide. These conditions are specific for the acid hydrolysis of phosphoamide linkages in nucleotide-peptide conjugates (Rjabova et al., 1965; Shabarova, 1970).

The conjugates were phosphorylated using T4 polynucleotide kinase (Maniatis et al., 1982) (Figure 1). The reaction products were analyzed by HPLC on an RPC5 column at pH 12 for PNA (Figure 2A, part d) and pH 8 for peptide (Figure 2B, part d). Virtually complete phosphorylation of the PNA-dCp conjugate was achieved in 5 min, but complete phosphorylation of the peptide-dCp conjugate required an initial 30 min of incubation with the enzyme followed by incubation for a further 30 min with a fresh portion of the enzyme. The ^{32}P -labeled PNAs and peptides could be analyzed by electrophoresis

on 20% polyacrylamide gels under standard conditions (Maniatis et al., 1982) (Figure 3). The three negative charges introduced by the terminal nucleotide are sufficient to transform a PNA or peptide molecule into a molecule suitable for electrophoretic analysis.

The procedure described above should be applicable to the ^{32}P labeling of biopolymers containing primary amino groups. dCp could be replaced by any other 2'-deoxynucleoside 3'-phosphate. Peptides that include lysyl residues are likely to undergo multiple conjugations. Some selectivity might be achieved by carrying out reactions at low pH. ^{32}P labeling of biopolymers that lack amino groups should be possible using appropriate linkers if sulfhydryl or carboxyl groups are available.

Other procedures for the ^{32}P labeling of peptides and PNA oligomers have been described. They involve the attachment of short peptides that are substrates of protein kinases to the target molecules (Kemp and Pearson, 1991; Koch et al., 1995). The nature of the potential application will determine the method of choice.

ACKNOWLEDGMENT

This work was supported by NASA Grant NAG5-4118 and NASA NSCORT/EXOBIOLGY Grant NAG5-4546. We thank Aubrey R. Hill, Jr., for technical assistance, Anthony G. Craig for mass spectrometry, Jill Meisenholder for the synthesis of the peptide, and Bernice Walker for manuscript preparation.

LITERATURE CITED

- Christensen, L., Fitzpatrick, R., Gildea, B., Petersen, K. H., Hansen, H. F., Koch, T., Egholm, M., Buchardt, O., Nielsen, P. E., Coull, J., and Berg, R. (1995) Solid-phase synthesis of peptide nucleic acids. *J. Pept. Sci.* 3, 175–183.
- Hyrup, B., and Nielsen, P. E. (1996) Peptide nucleic acids (PNA): synthesis, properties and potential applications. *Bioorg. Med. Chem.* 4, 5–23.
- Joyce, G. F., Inoue, T., and Orgel, L. E. (1984) Non-enzymatic template-directed synthesis on RNA random copolymers: poly(C,U) templates. *J. Mol. Biol.* 176, 279–306.
- Kemp, B. E., and Pearson, R. B. (1991) Design and use of peptide substrates for protein kinases. *Methods Enzymol.* 200, 121–133.
- Koch, T., Naesby, M., Wittung, P., Jorgensen, M., Larsson, C., Buchardt, O., Stanley, C. J., Norden, B., Nielsen, P. E., and Orum, H. (1995) PNA-peptide chimeras. *Tetrahedron Lett.* 36, 6933–6936.
- Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) *Molecular cloning: a laboratory manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Richardson, C. C. (1981) Bacteriophage T4 polynucleotide kinase. In *The Enzymes* (P. D. Boyer, Ed.) Academic Press, New York.
- Rjabova, T. S., Shabarova, Z. A., and Prokofiev, M. A. (1965) Selective cleavage of phosphoamide bond in adenylil-(5'-N)-peptides. *Dokl. Akad. Nauk SSSR* 162, 1068–1070.
- Schmidt, J. G., Nielsen, P. E., and Orgel, L. E. (1996) Separation of "uncharged" oligodeoxynucleotide analogs by anion-exchange chromatography at high pH. *Anal. Biochem.* 235, 239–241.
- Shabarova, Z. A. (1970) Synthetic nucleotide-peptides. In *Progress in Nucleic Acid Research and Molecular Biology*, pp 145–182, Academic Press, New York.

REVIEWS

Stabilization of Substances in Circulation

Cristina Monfardini and Francesco M. Veronese*

Department of Pharmaceutical Sciences, University of Padova, Padova, Italy. Received October 22, 1997

TABLE OF CONTENTS

1. Introduction	418
2. Polymer Conjugation	419
2.1. Introduction	419
2.2. Properties and Methods of Activation of Polymers for Drug Conjugation	419
2.2.1. Natural Polymers	419
2.2.2. Synthetic Polymers	419
2.3. Polymer–Drug Conjugates	423
2.3.1. Polymer–Protein Adducts	423
2.3.2. Polymer–Low-Molecular Mass Drug Adducts	425
2.4. General Considerations on Substance Conjugation	426
3. Liposomes	427
3.1. Introduction	427
3.2. Liposomes: Properties, Methods of Preparation, and Entrapment of Drugs	427
3.3. Liposomes as Drug Carriers and Their in Vivo Fate	427
3.4. Long-Circulating Liposomes	428
3.4.1. GM1 and Glucuronide Liposomes	429
3.4.2. PEG Liposomes	430
3.4.3. Other Polymers Used in the Preparation of Long-Circulating Liposomes	430
3.4.4. Active Targeted, Thermosensitive, pH-Sensitive Long-Circulating Liposomes	431
3.4.5. Long-Circulating Liposomes for Tumor Diagnosis and Cancer Chemotherapy	432
3.5. General Considerations on Substance Entrapment in Liposomes	432
4. Particles	433
4.1. Introduction	433
4.2. Microparticles	433
4.3. Properties and Methods of Preparation of Nanoparticles	433
4.4. Nanoparticles as Drug Carriers	434
4.5. Long-Circulating Nanoparticles	434
4.6. General Considerations on Substance Entrapment in Particles	435
5. Additional Strategies	436
6. Conclusions	437

1. INTRODUCTION

The need to maintain substances in blood is one of the main goals in therapeutic and diagnostic applications, which are often hampered by the short in vivo half-life of administered compounds. Many factors are involved in the removal of substances from the circulation: proteins and peptides are rapidly eliminated by proteolytic degradation, renal filtration, and/or immunogenic and antigenic reactions, while renal excretion and inactivation reactions by blood enzymes (especially the hydrolytic action of esterases) are the main causes for the brief plasma residence time of low-molecular mass (MW)¹ nonprotein drugs.

Many methods have been studied in the last three decades in an effort to find a means for prolonging the permanence of substances in circulation. To review this complex matter, we decided to report separately here the

* To whom correspondence should be addressed: Department of Pharmaceutical Sciences, University of Padova, Via F. Marzolo 5, 35131 Padova, Italy. Telephone: +39-49-827-5694. Fax: +39-49-827-5366. E-mail: veronese@pdfar3.dsfarm.unipd.it.

¹ Abbreviations: ADA, adenosine deaminase; ADR, adriamycin; Asp, aspartic acid; BSA, bovine serum albumin; CA, colominic acid; CAM, camptothecin; CSF, colony stimulating factor; DDP, diamminedichloroplatinum; DIVEMA, poly(divinyl ether maleic anhydride); DOX, doxorubicin; DOXeq, doxorubicin equivalent; DPP, dipalmitoylphosphatidyl; DPPG, dipalmitoylphosphatidylglycerol; DSPC, distearoylphosphatidylcholine; DSPE, distearoylphosphatidylethanolamine; EPR, enhanced permeability retention; 5FU, 5-fluorouracil; rhG-CSF, recombinant human granulocyte-colony stimulating factor; GM1, monosialoganglioside; HDL, high-density lipoproteins; HPMA, *N*-(2-hydroxypropyl)methacrylamide; HSA, human serum albumin; LM, lipid microspheres; LUV, large unilamellar vesicle; Lys, lysine; MLV, multilamellar vesicle; MW, molecular mass; 9-NC, 9-aminocamptothecin; NCS, neocarzinostatin; OXD, oxidized dextran; PACA, polyalkylcyanoacrylate; PAcM, poly(*N*-acryloyl)morpholine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PEG, poly(ethylene glycol); PEG2, branched poly(ethylene glycol); mPEG, monomethoxy-PEG; PEO, poly(ethylene oxide); PEOZ, poly(2-ethyl-2-oxazoline); PF, poly(phosphazene); PG1cUA, palmitylglucuronide; PG2, diglycerol; PG6, hexaglycerol; PHEG, PEG–poly[*N*-(2-hydroxyethyl)-L-glutamine]; PIBCA, polyisobutylcyanoacrylate; PLA, poly(lactic acid); PLGA, poly(lactic-co-glycolic acid); PLL, poly(L-lysine); PM13, 13 kDa comb-shaped PEG; PM100, 100 kDa comb-shaped PEG; PMOZ, poly(2-methyl-2-oxazoline); POP, poly(propylene oxide); PS, polystyrene; PSA, polysialic acid; PVA, poly(vinyl alcohol); PVA-R, PVA bearing a long alkyl chain; PVP, poly(*N*-vinylpyrrolidone); RES, reticuloendothelial system; SBTI, soybean trypsin inhibitor; SM, sphingomyelin; SMA, poly(styrene-co-maleic acid/anhydride); SMANCS or SMA-NCS, SMA–neocarzinostatin; SOD, superoxide dismutase; ST-PHPMA, semitelechelic poly-HPMA; SUV, small unilamellar vesicle; TMS, thermosensitive magnetoliposomes; VPA, valproic acid.

three most interesting strategies: covalent conjugation of drugs to polymers, drug encapsulation in liposomes, and drug physical entrapment in particles. As will become clearer later, polymers are currently the "last word" of all these approaches, since they are used not only for direct conjugation to drugs but also as further stabilizers of drug-loaded liposomes and particles. The additional combination of various strategies has led to the creation of drug carrier systems which are very similar to "Russian dolls", the smallest doll (represented by the innermost drug) surrounded by a larger doll (liposome or particle), in turn protected by the largest one of all (the polymer). In this review, particular emphasis is therefore given to polymers, with a brief description of their chemical structure, properties, and methods of activation.

Another approach to the stabilization of molecules in the circulation is the use of natural carriers such as cells, bacteria, or virus envelopes. These carriers will not be reviewed here, since emphasis is primarily given to strategies based on chemical approaches.

2. POLYMER CONJUGATION

2.1. Introduction. Modification of biologically active compounds with polymers is one of the methods for altering and controlling their pharmacokinetics, biodistribution, and often toxicity (1). The preparation of drug-polymer conjugates has been extensively reported in many reviews (2–14). Ringsdorf first proposed using polymers as drug carriers in the mid-1970s (15), and the possibility of creating drug delivery systems able to determine both controlled release of the drug, through optimization of its pharmacokinetics, and drug targeting, through a moiety able to drive the carried drug to specific organs and tissues, rapidly became a primary goal, especially in cancer chemotherapy. Since then, polymers with varying chemical and biological properties have been investigated in an effort to achieve long circulation times, slow release, and/or targeting of the conjugated drugs, thus improving their therapeutic use.

The following sections review (a) the properties and activation methods of polymers used for drug derivatization and (b) the characteristics of both protein and nonprotein drug conjugates. Special attention will be paid to poly(ethylene glycol) (PEG) and its adducts, since it is still the most widely used polymer for drug conjugation.

2.2. Properties and Methods of Activation of Polymers for Drug Conjugation. The general requirements for any polymer carrier (natural or synthetic) to be used for drug conjugation are water solubility, biocompatibility, lack of immunogenicity, biological inertia, and the presence of functional groups for allowing covalent conjugation to drugs and targeting moieties.

2.2.1. Natural Polymers. Natural polymers have the advantage of easy availability and biocompatibility, although their preparation may be restricted by the need for several purification steps and their use is limited by their high immunogenicity. However, polymers such as polysaccharides (dextran) and albumin are widely used.

Dextran. Dextran is an uncharged, water-soluble polymer of α -D-glucose, presenting 95% of α -1,6-linked linear units and 5% of α -1,3-linked moieties as side chains. High-molecular mass branched dextrans, first used as plasma expanders, were unfortunately associated with toxicity and allergic reactions, later avoided by using linear dextrans, obtained from *Leuconostoc mesenteroides*. However, in this case, a long purification procedure

was required, including an initial hydrolysis step for obtaining polymers with a range of different molecular masses and a size fractionation step for purifying dextrans of 40, 70, and 110 kDa. Polymers with molecular masses outside this range are useless, since the smaller ones pass through the glomerulus and are secreted and the larger ones cause erythrocyte aggregation and hypersensitivity reactions (6).

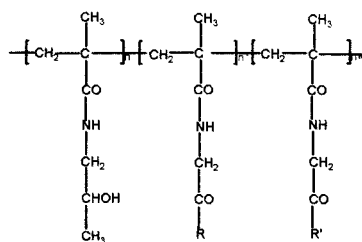
Dextrans in the 40–110 kDa molecular mass range have been studied for drug modification; for this purpose, dextran vicinal diols can be activated to electrophilic groups able to react with protein and drug amino groups. Reactive imidocarbonate groups, introduced via cyanogen bromide (16–18), or dialdehyde-dextran, obtained by oxidation with sodium periodate (19–21), are used for either protein or conventional drug modification at the amino groups. Both methods have advantages and disadvantages. Imidocarbonate-dextran reacts in a one-step reaction but is unstable in aqueous solution, and its reagents and byproducts are toxic; dialdehyde-dextran is relatively stable in aqueous solution but requires a later step for reductive amination.

Human Serum Albumin (HSA). This protein is very easily available from blood banks, and is widely used to modify proteins of therapeutic interest. Its polyfunctionality allows a variety of derivatives to be prepared, but it is also responsible for intra- and intermolecular cross-linkages, with the final formation of heterogeneous products. HSA may also sometimes confer immunogenic properties on the conjugated drug, thus limiting its use as a carrier. Drug modification by HSA is performed in aqueous buffer in the presence of cross-linking agents such as glutaraldehyde, carbodiimide, and sodium periodate.

2.2.2. Synthetic Polymers. Synthetic polymers offer the opportunity for preparing tailor-made carriers, properly designed, and presenting all the desired features and moieties, such as targeting residues, peptidyl spacers as sites for enzymatic cleavage, and pH-sensitive linkers. Although the favorable biological properties of some polymers may sometimes be combined with the main use of the carried drug to create a synergic effect, the toxicity of others often strongly limits their in vivo use. *N*-(2-Hydroxypropyl)methacrylamide copolymers (HPMA), poly(styrene-co-maleic acid/anhydride) (SMA), poly(divinyl ether maleic anhydride) (DIVEMA), poly(*N*-vinylpyrrolidone) (PVP), poly(*N*-acryloyl)morpholine (PACM), and poly(ethylene glycol) (PEG) are some of the investigated polymers, whose chemical structures are reported in Figure 1.

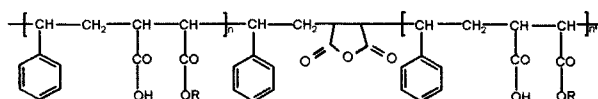
***N*-(2-Hydroxypropyl)methacrylamide Copolymers (HPMA).** HPMA is a water-soluble, biocompatible polymer, which was developed in Czechoslovakia as a plasma expander (22). It is biologically well tolerated, nonimmunogenic (23), and nontoxic at the maximum administrable dose of 30 g/kg. Conjugate synthesis (24–30) consists of reacting HPMA copolymers, containing side chains terminating in reactive ester groups, to the aliphatic amino groups on the drug. The reaction may be carried out sequentially in organic and aqueous solutions to introduce drugs, prodrugs, antibodies, and peptides, according to their solubility, along the polymer backbone (14). However, these chemical procedures do not allow one-point attachment of the polymer chain to the surface of a protein, resulting in multiple attachment reactions. To create a polymer molecule with only one point of modification, semitelechelic poly-HPMA (ST-HPMA) containing one amino endgroup is synthesized by radical polymerization in the presence of 2-amino-

HPMA



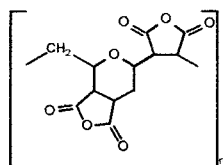
$n:n':n''=94:2:4$
R, R'=reactive groups

SMA

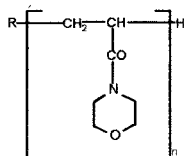


$n,n' \geq 0$
R=H, -CH₃, -C₂H₅, -C₄H₉

DIVEMA

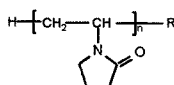


PACM



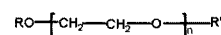
R=reactive group

PVP



R=reactive group

PEG



R=H or -CH₃
R'=reactive group

Figure 1. Chemical structures of the most used synthetic polymers.

ethanethiol as a chain transfer agent and used to modify the surface of nanospheres (31).

Poly(styrene-co-maleic acid/anhydride) (SMA). SMA is an interesting polymer introduced by Maeda and co-workers (7, 32) containing alternating linkages of styrene and maleic acid. SMA is prepared with 30–50% maleic acid in the form of reactive anhydride and half the free groups butylated to confer hydrophobicity (32, 33). The anhydride group of the polymer reacts with the amino groups of the protein, while the styrene side chain provides hydrophobicity and hindrance to the conjugate.

Poly(divinyl ether maleic anhydride) (DIVEMA). The 1:2 divinyl ether maleic anhydride cyclic alternating copolymer (DIVEMA) is well-known for the variety of its biological activities. It possesses antitumor, antiviral, antibacterial, and antifungal activity. It is an anti-coagulant, anti-inflammatory agent, and immunopoten-

tiator. It induces the formation of interferon, increases the rate of phagocytosis, activates macrophages selectively, and inhibits RNA-dependent DNA polymerase (34). Studies on the relationship between polymer MW and toxicity have been performed using DIVEMA in the 2500–44800 Da range; great differences in acute toxicity were not observed, whereas toxicity measured by other criteria (levels of serum glutamic pyruvate transaminase, inhibition of drug metabolism, and sensitization to endotoxins) increased with increasing molecular mass. All toxicity tests were positive for polymers with a molecular mass over 15000 Da (34, 35). The biocompatibility of DIVEMA in the 2500–15000 Da range, its solubility, and the numerous anhydride groups available along its backbone have allowed its use for drug modification. Because of its own pharmacological effects, it has been exploited for conjugation to biologically interesting proteins and drugs, in view of the synergic effect of the new adducts. Moreover, the high negative charge of this polymer confers on the conjugate affinity toward particular biological structures and molecules, causing a unique in vivo behavior (34–36). The synthesis of DIVEMA–protein conjugates is not simple, since the multifunctional groups of both are involved in cross-linking processes, with a possible decrease in protein activity. However, the cross-linking reaction may be minimized by a pH-reversible protective reagent for the protein amino groups, which randomly restricts the number of protein amino groups available for later conjugation to the polymer (37).

Poly(N-vinylpyrrolidone) (PVP). PVP is a synthetic polymer used in large quantities as a blood expander and is well-known for its lack of toxicity, immunogenicity, and antigenicity (38, 39). One of the methods proposed for its binding to proteins is the opening of some of the pyrrolidinone rings by alkaline treatment, followed by masking of the amino groups and activation of the carboxyl groups as succinimidyl esters for the next reaction to the protein amino groups (40). The inconvenience of this method is the formation of several potentially reactive groups along its chain, with consequent cross-linked, heterogeneous final products. The use of chain transfer techniques during the polymerization of *N*-vinylpyrrolidinones provides a method for the preparation of OH or COOH monofunctionalized PVP with better defined structure (41). The high polydispersivity of the products requires a fractionation step (performed either by gel filtration purification or by fractional precipitation) to separate the PVP oligomers of convenient molecular size (42). These end-hydroxylated oligomers are directly activated into 4-nitrophenyl carbonate derivatives or succinimidyl esters for the subsequent modification of peptide and protein amino groups. Unfortunately, attempts to convert them into carboxylated derivatives by coupling to amino acids have until now resulted in low yields (43).

Poly(N-acryloyl)morpholine (PACM). High-MW polymers of *N*-acryloylmorpholine (PACM) are amphiphilic and nontoxic polymers used in pharmaceutical applications as plasma substitutes (44) and in transdermal applications (45, 46). PACM oligomers, ending at one chain terminus with a reactive OH or COOH function and obtained by radical polymerization of the vinyl monomer *N*-acryloylmorpholine in the presence of functionalized chain transfer agents (47), are synthesized to modify proteins of therapeutic interest.

Poly(ethylene glycol) (PEG). PEG is a linear polyether diol with many useful properties such as biocompatibility (48, 49), solubility in aqueous and organic media (49, 50),

lack of toxicity, very low immunogenicity and antigenicity (51), and good excretion kinetics (52). These properties allow its use in a variety of applications (53), including the biomedical field, after FDA approval for internal administration. It is commercially available in a variety of molecular masses at low dispersivity ($M_w/M_n \leq 1.1$), and activated species are now available from specialized sources (Shearwater Polymers, Inc., Huntsville, AL; and Polymer Laboratories, Inc., Amherst, MA). The most frequently used polymer molecular masses for drug conjugation range from 1000 to 20 000 Da, although lower and higher molecular masses have also been used. The body biodistribution of PEG with different molecular masses (from 31 000 to 277 000 Da) was recently investigated after intravenous (iv) injection into mice bearing a tumor mass (54). The study indicated that PEG does not exhibit specific affinity for any organ and that its accumulation in the tumor tissue is mainly governed by the level of hyperpermeable tumor vasculature (enhanced permeability retention, EPR effect), irrespective of the molecular mass of the polymer and the tumor loading site. Conversely, the higher the molecular mass of PEG, the longer the retention time in the blood. The balance between tumor accumulation rate and blood retention results in maximal accumulation of PEG with a MW of 200 000 Da. This highlights the problem of the toxicity of high-MW PEGs (above 200 000 Da) because of their very long circulation time. However, this problem does not interfere with using PEG for drug modification, since the MWs of PEG used for this purpose are well below the critical value.

The polymer backbone is chemically inert, and the terminal primary hydroxyl groups are available for derivatization. Protein and nonprotein drug conjugates are usually prepared from monomethoxy-PEG (mPEG) which, having only one derivatizable group, minimizes the heterogeneity of conjugates which may rise from cross-linking reactions. The hydroxyl groups on the polymer are sometimes directly bound to the desired molecules in a one-step process, yielding ether (55, 56), ester (57–61), or carbonate (62) linkages. In most cases, the hydroxyl groups are first activated and then reacted with the chosen molecule. Zalipsky (63) and Herman (64) recently and exhaustively reviewed PEG activation methods.

A great deal of work has been (and still is being) carried out on PEG functionalization, for two main reasons: (1) to discover the best synthesis of activated PEGs, reactive in mild conditions and possibly also selective toward different chemical groups on the drug molecule, without affecting its activity, and (2) to investigate the various PEG–drug linkages to establish the relationship between the type of binding and the properties of the new conjugates. Briefly, the most common functionalized PEGs are obtained as follows. (a) *Chloride, bromide, and sulfonate esters* are obtained by conversion of terminal hydroxyl groups by thionyl chloride (57, 65, 66), thionyl bromide (67), or organic sulfonyl chlorides. Reactive sulfonate ester derivatives such as tosylate–PEG (68–71) and mesylate–PEG (72) are used as intermediates in the preparation of amino–PEG (68, 73), thiol–PEG (71), phenylglyoxal–PEG (74), and benzaldehyde–PEG (75). Tresylate–PEG (76) is directly used to modify the desired molecule, especially at the protein amino groups (but thiol groups can also react) (77–79), with the formation of a secondary amine between PEG and the protein (this type of bond is particularly important if the nucleophilicity of the amino group is essential for activity preservation). (b) *Amino–PEGs* can be synthesized from

one of the above intermediates with an excess of ammonia (65, 67, 80, 81) or (to avoid the secondary amine side product) by conversion of the –OH into a *p*-toluenesulfonate ester followed by nucleophilic displacement of tosyl groups by potassium phthalimide and hydrazinolysis of the phthalimide endgroups (73). For quantitative conversion, the halide group can be displaced by azide and the azide group then reduced to primary amine (82). Other methods used to introduce an amino group on PEG are ester formation between the polymer hydroxyl groups and an amino acid carboxyl group (50) or introduction of aromatic amine by reaction of potassium alkoxide–PEG with *p*-nitrobenzyl bromide (83) or chloride (84), followed by reduction of the nitro groups. Conjugates are then obtained by alkylation or acylation reactions and, in the case of aromatic amine, also by a diazo coupling reaction after conversion of the amine into diazonium salts (84). (c) *Hydrazido–PEGs* are prepared by several methods (85–87), including the reaction of alkoxide–PEG with haloacetate ester and successive hydrazinolysis of the resulting ester (88). These activated PEGs form stable hydrazones on reaction with aldehyde-containing compounds and are particularly useful for site-specific modification of glycoproteins (86). (d) *Mercapto–PEGs* are synthesized by reaction of PEG–tosylate with potassium ethyl xanthate and amination of the product by glycine (71), derivatization of mPEG–NH₂ with the heterobifunctional reagent succinimidyl 3-(2-pyridyldithio)propionate (89), or other procedures (90) and used to graft polymer chains onto maleimide-containing liposomes (89). (e) *Carboxyl–PEGs* are obtained by means of several methods: direct oxidation of the hydroxyl groups by permanganate, although unfortunately this may give rise to polymer chain cleavage as a side reaction (91), carboxymethylation with α -haloacetic acid or its esters, especially displacement of bromide in ethyl bromoacetate by PEG–alkoxide (67, 92, 93), use of succinic (or glutaric) anhydride (57, 94–96), reaction with ethyl isocyanatoacetate (71, 82, 97, 98), and hydrolysis of the resulting ethyl esters of the PEG–glycine carbamate. Biological molecules can be covalently bound to carboxyl–PEG via ester or amide bonds (57, 65), dicyclohexylcarbodiimide being used as a carboxyl activator. Succinimidyl esters of carboxymethylated and succinylated PEGs are widely used for protein, lipid, and other substrate modification (99–103), yielding an amide bond between the substrate and the succinate residue. Esters of urethane-linked amino acids are also used for protein modification, the amino acid linker being useful for conjugate characterization; a non-natural amino acid, like norleucine, allows accurate determination of the degree of protein modification by quantitative amino acid analysis of the adduct, thus avoiding the less precise colorimetric or fluorimetric evaluations based on trinitrobenzenesulfonic acid or fluorecamine, respectively (104, 105). (f) *For carbonate-, carbamate-, and chloroformate–PEGs*, PEG can be converted into (imidazolyl-carbonyl)oxy derivatives by reaction with carbonyldiimidazole and successively reacted with protein amino groups forming urethane linkages (106); mPEG carbonate derivatives of *p*-nitrophenyl, 2,4,5-trichlorophenyl (107), and succinimidyl carbonate of PEG (108–111) are also used for protein modification, the latter also having an interesting use in the synthesis of liposome-forming mPEG–stearylamine (112). (g) *Cyanuryl chloride–PEG*, obtained by reaction of mPEG with trichloro-*s*-triazine (cyanuric chloride) (113), has the disadvantage of reacting not only with the amino but also with the hydroxyl and sulfhydryl groups on proteins, thus causing loss of

selectivity and sometimes of biological activity (114). The advantage of this product is that it is obtained by replacement of two of the three reactive chlorides of trichloro-s-triazine with mPEG, leaving the third chloride available for modification of the drug amino group; this provides a branched reagent that is able to introduce double the amount of polymer per number of amino sites modified (115). This aspect is important since, as will be discussed below, some beneficial properties of PEG proteins depend directly on PEG content, but the residual activity of the conjugate may sometimes inversely depend on the number of modified amino groups. (h) *Aldehyde-PEG* is synthesized in several ways. For example, acetaldehyde residues are introduced (116, 117), and the product is used for protein conjugation via reductive amination reaction (118, 119). This procedure is an alternative to using tresylate-PEG in the formation of a secondary amine between the polymer and the protein amino group without modification of the thiol groups. Aromatic aldehydes have also been studied (120), and amination of mPEG-nitrophenyl carbonate with 3-amino-1,2-propanediol followed by periodate oxidation of the diol into aldehyde (121) is used to prepare the copolymer PEG-lysine (122). (i) *Epoxide-PEG*, obtained by reaction with epichlorohydrin (123-125), shows mild reactivity toward hydroxyl and amino groups and better reactivity with thiols, thus being a useful reagent when a low degree of conjugation is desired.

Many other interesting activation methods of PEG have been reported for special uses, such as (j) *a dimethyl anhydride analogue of PEG*, gradually cleaved from the conjugate in physiological conditions (126), (k) *a PEG-imido ester* which forms a positively charged amidine upon reaction with protein amino groups with no consequent change of the total charge balance in the conjugate (127), (l) *a maleimide-PEG* (128, 129) and (m) *a vinyl sulfone-PEG* (130) for specific reaction at the cysteine residues, (n) *a phenylglyoxal-PEG* for selective modification of arginine residues (131), and (o) *heterobifunctional-PEGs* like α -amino- ω -hydroxy-PEG (132) allowing the binding of two different molecules at the two terminal groups (e.g., a drug on one terminus and a target moiety on the other).

Fascinating new PEG derivatives have recently been developed. (p) *PEG2-Lys succinimidyl ester* (133) was synthesized to obtain a two-arm reagent or branched polymer that is able to introduce twice the amount of polymer chains without increasing the number of derivatized protein amino groups. Because of its chemical structure, the reagent avoids the problems of product heterogeneity and loss of activity encountered with the use of the PEG2-trichlorotriazine obtained by Inada and co-workers (134). Interestingly, nature provides some proteins of very complex branched carbohydrate residues which create a protective shield against degradation and inactivation processes. This branched polymer may be thought of as an artificial simulation of the glycosylated cloud naturally provided to proteins. (q) *Comb PEGs*, PEG derivatives possessing a comb-shaped conformation (PM), have been synthesized as copolymers of a PEG derivative and maleic anhydride; the reactive site of the derivative is multivalent, and the molecular mass may vary widely (PM13 and PM100 corresponding to 13 and 100 kDa, respectively, have been studied). Modification with PM introduces many more PEG chains per the number of modified amino groups than either PEG1 or PEG2 (135). To overcome the problem of the limited number of low-molecular mass drug molecules that can be linked to a single chain of PEG, new drug carriers have

been introduced, like (r) *poly(PEG-Lys)*, composed of alternating segments of PEG and lysine bound by urethane linkages (122, 136), and (s) *mPEG-poly(Asp)*, a copolymer with increased drug-loading potentiality (137).

As briefly reviewed above, most of the PEG activation methods are directed toward the amino groups of the molecule to be modified. The use of these methods is justified by the high nucleophilicity and well-defined chemistry of the amino groups and, in the case of proteins, by the high number of residues bearing amino groups present on the protein surface exposed to the solvent. Moreover, protein amino groups usually only play a structural role in conferring solubility to the macromolecule and are only seldom involved in the catalytic process. According to the procedure used for PEG activation, the final conjugate may have amino groups no longer in the nucleophilic form (as in the case of all reactions based on amino group acylation) or amino groups with lower nucleophilicity (all alkylation reactions transforming a primary amino group into a secondary one). This must be taken into consideration when amino group acylation is associated with a decrease or even loss of drug activity.

Other Polymers. Many other polymers have been studied as potential drug carriers, although most of them have not been examined in vivo studies. For some of them, acute and subacute toxicity and immunogenicity have been evaluated, but for most, only in vitro studies have been carried out. Of these, worthy of mention are natural gelatin and polyamino acids and synthetic poly(L-glutamic acid), poly(L-lysine), poly(DL-alanine), poly(hydroxyethyl-L-glutamine), poly(α -malic acid), poly(acrylic acid), poly(vinyl alcohol), polytartrate (138), and poly(1-hydroxymethylethylene hydroxymethylformal) with a hydrolyzable main chain (139). Recently, the reaction between polysuccinimide and hydrazine produced a water-soluble, nontoxic, nonimmunogenic, α,β -polyasparthydrazide polymer which was loaded with 9% of antibacterial ofloxacin via a water-soluble carbodiimide (140). α,β -Poly(*N*-hydroxyethyl)-DL-asparthamide is used to prepare micromatrices loaded with an anti-inflammatory drug, 4-biphenylacetic acid (141). New carrier molecules, $\text{NH}_2\text{OCH}_2\text{CO}-(\text{Gly})_3-[\text{Lys}(\text{H-Ser})]_5-\text{Gly-OH}$ and $\text{HCO-m-C}_6\text{H}_4\text{CH}=\text{NOCH}_2\text{CO}-(\text{Gly})_3-[\text{Lys}(\text{H-Ser})]_5-\text{Gly-OH}$, are synthesized for the preparation of protein conjugates of defined structure. These molecules are able to react either with an aldehyde group introduced on a protein, with the formation of an oxime bond, or with the carboxyl terminus of a polypeptide chain, previously modified with carbonylhydrazide, to give a hydrazone bond (142).

Copolymers. Recently, besides improvements in the activation methods for each of the above polymers, great efforts have been made to create new carriers with more versatile properties. These polymers are usually prepared by combining traditional polymers, like the *N*-(2-hydroxypropyl)methacrylate-vinylpyrrolidone copolymer, PEG-ylated polymers, and block and graft copolymers (143, 144). Among them are (a) the above-mentioned poly(Asp)-PEG copolymers and poly(PEG-Lys); (b) PEG-grafted dextran, obtained by reaction of α -methyl- ω -amino-PEG with epichlorohydrin-activated dextran (145) and similarly synthesized; (c) PEG-poly[*N*-(2-hydroxyethyl)-L-glutamine] (PHEG) (146); and (d) diblock co-poly(ethylene oxide) (PEO)-poly(propylene oxide) (POP) copolymers (proxanols), PEO and POP conferring hydrophilic and hydrophobic properties, respectively, on the copolymer and the final conjugate (144). Proteins can be linked to the copolymer either by a covalent bond (the copolymer is activated as aldehyde and reacted to protein

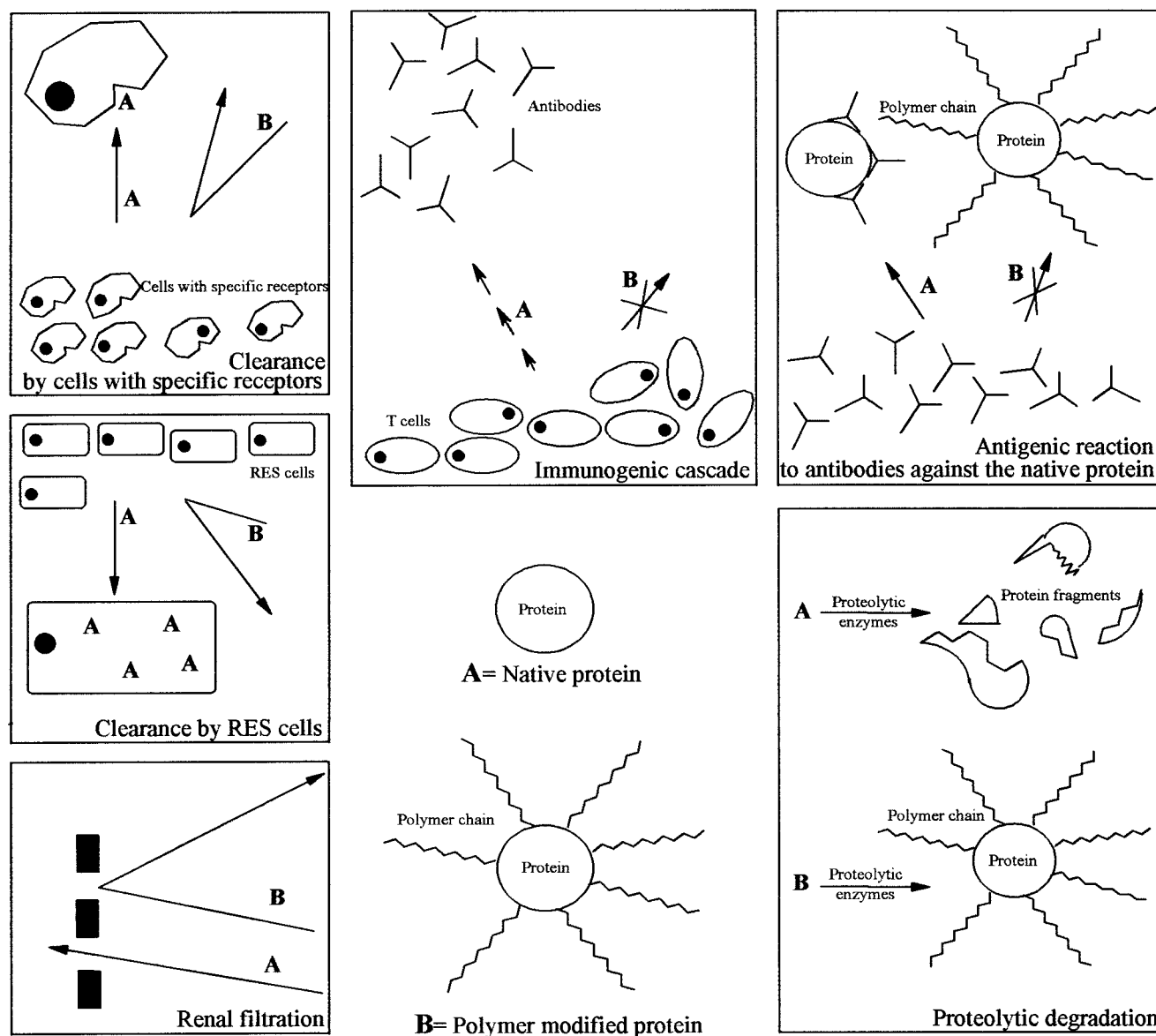


Figure 2. Potential in vivo problems connected with the therapeutic use of proteins and solutions by polymer modification.

amino groups, with later reduction of the iminium group) or by thermoinduced and high-pressure-induced complexation for a noncovalent adduct; (e) bovine serum albumin (BSA)-PEG copolymers were used to prepare controlled release systems of hydrogels with high water content (>96%) (147).

In general, the number of activatable groups along the polymer chain is of great importance when the polymers are used as drug carriers, since the presence of one or several activatable groups determines the amount of drug loaded along the polymer and therefore the characteristics of the final product. In the case of protein modification, PEG and DIVEMA are two opposite cases, PEG conjugates being easy to characterize well and DIVEMA giving rise to heterogeneous cross-linked conjugates. Although not ideal for medical use, these heterogeneous adducts are useful in biocatalysis, since a high enzyme content per molecule of polymer may be achieved. Among the many strategies studied in the biocatalysis field for increasing enzyme stability to solvents, heat, and denaturants, the multipoint attachment obtained with polyfunctional polymers is an important method.

2.3. Polymer-Drug Conjugates. The above-described polymers are used to modify proteins and non-

protein drugs to improve their therapeutic characteristics. The use of proteins in therapy is often limited by their short plasma half-life due to degradation by proteolytic enzymes, rapid clearance, and immunological reactions (when they are of heterologous origin). Instead, for low-MW nonprotein drugs, the main disadvantages are rapid metabolism by degradation reactions, toxicity, and premature excretion, which may be prevented or reduced by polymer conjugation.

2.3.1. Polymer-Protein Adducts. Peptides and proteins may acquire many advantages after polymer derivatization (Figure 2), including a plasma half-life increased by a factor of 3–500. In general, the more polymer chains attached per protein molecule, the greater the extension of the half-life (106, 148–150), although specific activity may sometimes be proportionally reduced. The increased plasma half-life is due to several mechanisms: (1) increased size of protein conjugates beyond the limit for glomerular filtration (~70 kDa), (2) reduced cell clearance through both the reticuloendothelial system (RES) and/or specific cells which interact through their specific receptors with carbohydrate chains or specific amino acid sequences on the protein (the polymer cloud around the protein masks these sites), (3)

decreased proteolysis due to polymer steric hindrance to proteolytic attack, and (4) reduced immunogenicity and antigenicity. Polymer conjugation causes reduced immunogenicity and antigenicity for polymer shielding of the epitopes and the antigenic determinants on the protein, preventing the raising of antibodies against the conjugate (8) and hindering the ability to interact with antibodies against the unmodified protein. Other new protein properties are sometimes observed upon modification, i.e., increased activity, increased solubility in aqueous and organic solvents, and increased thermal stability.

Various polymers were used to modify therapeutically useful proteins and peptides to improve their pharmacokinetic and immunogenic-antigenic properties. They are briefly reviewed here.

Dextran-Protein Conjugates. Dextran is used for derivatization of various proteins. Carboxypeptidase G and arginase (151), soybean trypsin inhibitor (SBTI) (152, 153), and superoxide dismutase (SOD) (154) conjugated to dextran all have an increased half-life in blood. The dextran-asparaginase conjugate, together with increased circulatory persistence, also shows markedly reduced antigen reactivity (6).

Gelatin-Protein Conjugates. Gelatin, used as a polymer carrier for drugs, has been proven to increase their therapeutic efficacy. Derivatization of recombinant IL-1 α to the natural polymer leads to prolonged retention in the body (155). In the same way, conjugation of succinylated gelatin to SBTI causes a 6-fold increase in circulation time compared to that of the unmodified protein (156).

Albumin-Protein Conjugates. Albumin is one of the natural polymers most frequently used for protein modification. Uricase modified by albumin has an enhanced plasma life (157), and recombinant human granulocyte-colony stimulating factor (rhG-CSF) conjugated to both rat and human serum albumin through a spacer of heterobifunctional maleimidocarboxyl PEG (1:1 albumin-rhG-CSF conjugate) increases its serum stability (158). Modification of earthworm fibrinolytic enzyme with fragmented HSA (10000–30000 Da MW) causes loss of its antigenicity, increased stability toward protease inhibitors in rat plasma, and retention of its potent proteolytic activity (159).

SMA-Protein Conjugates. SMA was developed as an ideal polymer for neocarzinostatin (NCS) modification for the properties described previously (32). NCS is a potent antitumor protein having however high toxicity (bone marrow suppression) and a very short plasma half-life. Conjugation with SMA (both 1.5 and 6 kDa) causes a longer half-life as a result of binding of SMA-NCS to albumin (33) and decreased immunogenicity and toxicity (32, 160, 161). SMA-NCS conjugates have already been evaluated in humans, and great success has been reported in many cases of liver tumors and liver tumor metastasis. Among other drugs modified by SMA, SOD has been successfully conjugated, the SMA-SOD derivative showing an increased half-life (162).

DIVEMA-Protein Conjugates. SOD-DIVEMA is prepared with minimal cross-linkage and minimal loss of activity, due to the use of 2,3-dimethylmaleic anhydride as a pH-reversible, randomly protective reagent for SOD amino groups (37). The circulation time of the conjugate has been greatly improved and immunogenicity reduced with respect to those of the native enzyme (163, 164).

PVP-Protein Conjugates. Conjugation of β -D-N-acetylhexosaminidase A to PVP extends the circulation time of the enzyme (165). Ribonuclease and uricase are

modified by a PVP-succinimidyl ester, yielding conjugates with increased thermostability, stability to proteolysis, reduced immunogenicity, and enhanced blood half-life (166). SOD-PVP has an increased body residence time, reduced antigenicity and immunogenicity, increased solubility in organic solvents, and increased stability toward thermal denaturation (167).

PACM-Protein Conjugates. Tailor-made copolymers of *N*-acryloylmorpholine and *N*-acryloxysuccinimide are prepared by radical copolymerization, the former monomer providing water solubility to the copolymer and the latter reactivity toward protein amino groups. Copolymers are successfully used to modify catalase and ribonuclease A, and the conjugates maintain full enzyme activity (168, 169).

PEG-Protein Conjugates. PEG is used to modify a variety of proteins. In particular, over the last two decades, enzymes have constituted the single largest class of proteins covalently modified by PEG (the following lists are by no means a complete bibliography). All the following proteins have an increased plasma half-life upon modification with PEG, with no or minimal loss of activity: streptokinase (170), tissue plasminogen activator (5), SOD (171, 172), arginase (173), recombinant interleukine-2 (174), tumor necrosis factor (175, 176), kallikrein and α -interferon (177), β -interferon (11), colony-stimulating factor-1 (CSF-1) (178), granulocyte-CSF (179–181), Fab-2 fragment of murine monoclonal antibody A7 (182), and serpins (a family of serine proteinase inhibitors) (183). PEG-adenosine deaminase (ADA) keeps its activity, with neither toxic effects nor hypersensitivity reactions (184, 185). PEG-uricase (a urolytic agent for treatment of hyperuricemia associated with chemotherapy) shows good activity (186). PEG-asparaginase has an increased half-life and increased antitumor activity and is nonimmunogenic, nonantigenic, and nonallergenic (187–193). Lack of immunogenicity is shown by conjugation of PEG to urokinase, cholinesterase, cellulase, and SOD (194), glutaminase-asparaginase (195), β -glucuronidase (196), urate oxidase (197), ribonuclease (107), streptokinase (198), catalase (199), arginase (200), and phenylalanine ammonia lyase (201). Asparaginase and albumin modified by PEG1-trichlorotriazine, PEG2-trichlorotriazine, PM13, and PM100 are all nonimmunogenic, although the percentage of activity depends on the type of polymer and degree of modification (202, 203).

Some important information can be drawn from the literature regarding how the number, molecular mass, and shape of the polymer chains bound to the protein influence body residence time. In SOD-PEG conjugates, there is a direct relation between residence time and number and length of PEG chains. SOD-PEG conjugates are obtained by enzyme modification with PEG of 1900 and 5000 Da. A comparison of derivatives obtained with the same molecular mass polymer but with a different degree of modification indicates that the circulation time increases with the degree of modification (204). Moreover, among the conjugates obtained with the same number of polymer chains per enzyme molecule using PEG with different molecular masses, circulation time increases with the length of the polymer chain (204). In other works, it has been shown that a single PEG chain of sufficiently high molecular mass is enough to reduce the renal excretion of the SOD-PEG conjugate to a level comparable to that obtained by several low-molecular mass polymer chains, without the disadvantage of reduced activity (205, 206). Recent investigations have shown that the molecular mass of PEG used for modification and the degree of modification have in

Table 1. Influence of the Polymer Molecular Mass, the Number of Polymer Chains Bound to the Protein, and the Polymer Shape on the Plasma Half-Life of PEG-Protein Conjugates in Rat or Mouse Circulation

conjugate	moles of PEG linked per mole of protein	half-life in rats (h)	half-life in mice (h)	reference
SOD	—	0.083	0.066	204, 206
SOD-PEG ₁₉₀₀ ^a	3	1.5	—	204
SOD-PEG ₁₉₀₀	6	3	—	204
SOD-PEG ₅₀₀₀	3	11	—	204
SOD-PEG ₅₀₀₀	7	25	24	204, 206
SOD-PEG ₅₀₀₀	18	25	—	204
SOD-PEG ₄₀₀₀₀	2	—	36	206
SOD-PEG ₇₂₀₀₀	3	—	36	206
SOD-PEG ₁₀₀₀₀₀	1	—	36	206
asparaginase	—	—	3.8	208
asparaginase— linear PEG ^b	13	—	52.7	208
asparaginase— branched PEG	13	—	65.25	208

^a The subscript number refers to the MW of PEG used in the protein conjugation. ^b Asparaginase-PEG derivatives were obtained with linear PEG with a 10 000 Da MW or branched PEG with a 2 × 5000 Da MW.

themselves a minimum effect on the half-life of SOD, demonstrating that this is mainly determined by the overall molecular size of SOD and its conjugates (207). In this respect, it is important to recall that PEG, due to its flexibility and great degree of hydration, behaves like a protein of approximately twice its molecular mass. This may be quantitatively verified by size-exclusion analysis and comparing the residence time of PEG alone or bound to a peptide or protein. When a protein-conjugate preparation is being designed, it is important to consider that a MW of over 55–60 kDa (such as the albumin MW) is enough for a protein to prolong its retention in the circulation. The shape of the polymer is also related to the final residence time of the modified protein; modification of 70% of the asparaginase amino groups with linear PEG 10000 or branched PEG 10000 (two chains of PEG 5000) indicates an increase in $t_{1/2\beta}$ (the half-time of the elimination phase) from 3.8 h for the native enzyme to 52.7 and 65.25 h for the linear and branched PEG forms, respectively (208). Table 1 shows a comparison of the factors discussed above.

The PEG length/circulation time ratio has also been demonstrated for a lower-molecular mass polypeptide, granulocyte-CSF, whose renal excretion is prevented by a single PEG chain of 30–40 kDa (209).

The great effects of PEG branching on protein residence time, resistance to proteolysis, and immunogenicity (133, 210) compared to those of the linear polymer make this PEG form of great interest for therapeutic applications.

In some cases, PEG modification causes a decrease in protein activity, especially enzymatic activity toward high- or low-MW substrates. For elastase (211), trypsin (212), and chymotrypsin (109), the hindrance created by the polymer around the active site reduces the approach of a macromolecular substrate to the active site itself. This problem may be overcome by carrying out the modification in the presence of a macromolecularized protector of the active site (such as an inhibitor); after enzyme derivatization, the protector can be removed by a change in pH, leaving the active site accessible (213, 214). Besides, some PEGylated enzymes show decreased activity toward low-MW substrates. In this case, modification of the amino groups at the active site may be responsible for the low residual activity of the conjugates. One strategy for avoiding this problem is the conjugation

of enzymes to the above-mentioned branched PEG (PEG2) which, because of its hindrance, does not have access to the amino groups of the active site cleft, leaving them unmodified. In the case of uricase, a 20% modification of its amino groups by linear PEG causes a drop in enzyme activity to 2.5%, while the conjugate with 30% of the amino groups modified by PEG2 retains 32% of the activity (208).

Other Carrier-Protein Conjugates. PEG-grafted polymers, like PEG-dextran and PEG-PHEG described above, are promising and potential carriers for protein and nonprotein molecules, since the introduction of PEG side groups results in reduced liver uptake and slow blood clearance (143). PEO-POP copolymers (2000 MW, aldehyde-activated) are used to modify soybean Bowman-Birk protease inhibitor, and the conjugate experiences a 7.3-fold decrease in total plasma clearance (215). Among the novel molecules used to modify therapeutic proteins, a succinylated keratin fragment (with affinity for cell membrane components and organelles) has been used to conjugate SOD, yielding an adduct with a longer half-life, lower immunogenicity, greater accumulation in the liver, and better therapeutic effects against paraquat toxicity compared to those of native SOD (216).

2.3.2. Polymer-Low Molecular Mass Drug Adducts. Polymer derivatization of nonprotein drugs leads to long-lasting activity due to the increased half-life in the circulation, as a result of protection toward metabolism and/or reduced kidney ultrafiltration because of the increased size of the adduct. Polymer modification also imparts decreased toxicity due to polymer shielding of "toxic" moieties, and the possibility of targeting to specific organs and tissues by introduction of a target molecule along the polymer backbone. This last issue approaches the concept of the "magic bullet" pioneered by Erlich (217) in the ideal project of delivering cancer chemotherapeutic drugs only to tumor cells. The most important examples of polymer-drug conjugates are listed here.

Dextran-Drug Conjugates. In the late 1970s, Bernstein showed that dextran oxidized to a polyaldehyde could be coupled to daunomycin, producing a conjugate with greater therapeutic activity and decreased toxicity (218). Subsequently, doxorubicin (DOX) was coupled to oxidized dextran 70 and the conjugate (OXD-DOX) had a higher plasma level, persisting for a long period, thus creating very effective passive tumor targeting (219). However, the phase I clinical trial of OXD-DOX indicated that the conjugate was significantly toxic: WHO grade IV thrombocytopenia in two of two patients treated with 40 mg/m² (milligrams per meter squared of body surface area) doxorubicin equivalent (DOXe_q), hepatotoxicity in five of five patients, and thrombocytopenia in three of five patients treated with 20 mg/m² DOX (220). Dextran of various molecular masses (10, 70, and 500 kDa) and electrical charges have been conjugated to mitomycin C and the conjugates used to investigate the charge effect on in vivo behavior and renal excretion. It was established that a negatively charged macromolecule is slowly excreted from the negatively charged basal membrane which instead enhances the passage of positively charged polymers. This holds not only for synthetic polymers but also for proteins, their pI being important in this case. Consistent with this principle, anionic conjugates of mitomycin C have a persistently high plasma level, gradually accumulating in peripheral tumors, whereas cationic ones are trapped by the liver and blood vessels and rapidly cleared from circulation, like the unmodified drug (221). Dextran (36 500 Da) is also coupled to oligopeptide derivatives of the antimicrobial

drug norfloxacin to improve their pharmacokinetics (222). The adducts, presenting tetrapeptides Gly-Phe-Ala-Leu or Gly-Phe-Leu-Gly at the piperazine amino group of the drug, show hydrolytic stability under physiological conditions but drug release in the presence of lysosomal enzymes. Dextran (500 kDa) is also used to prolong the blood half-life of the imaging agent ^{99m}Tc (223).

HPMA-Drug Conjugates. Many drugs (and targeting groups) have been linked to the HPMA copolymer backbone, including daunomycin (and galactose) (224), doxorubicin (and galactose) (225, 226), doxorubicin (and the melanocyte-stimulating hormone) (227), melphalan (and sugar residues) (228, 229), and chlorin e6 (and antibodies) (230). One of the most interesting uses of HPMA is the linkage of DOX to the polymer backbone through peptide sequences designed to be extracellularly stable but rapidly cleaved, following pinocytosis, by lysosomal enzymes (225). Targeting groups such as antibodies (231), hormones (227), and sugars (232) are also incorporated. HPMA-DOX has an extended plasma half-life responsible for passive targeting to solid tumors (233). In this case, the polymer plays an important role in inducing cell internalization by endocytosis, whereas drug release is influenced by the chemistry of the binding and the structure of the spacer, planned to be stable to blood enzymes but hydrolyzed by lysosomal cathepsins (9, 14).

DIVEMA-Drug Conjugates. DIVEMA of 7000 Da, a MW in the range of biocompatibility (34), is conjugated to DOX to produce the possible synergic effect of the antitumor properties of both polymer (234) and drug. The adduct causes prolonged retention of the drug in the body (36).

PEG-Drug Conjugates. Conjugation of drugs containing carboxylic acid groups to PEG hydroxyl groups via ester formation is used as an approach to the formulation of long-circulating, slow-released drugs, the rate of hydrolysis depending on the nature of the carboxylic acid, the MW of PEG, the pH, and the temperature. Penicillin V conjugated to mPEG (2000, 3000, and 4000 Da) (235) and poly(PEG-Lys) (50 000 Da) (122) and aspirin conjugated to PEG (400, 1000, and 3000 Da) have been studied. Ibuprofen conjugated to PEG of various MW (200, 900, and 2000 Da) yields a conjugate with long-lasting anti-inflammatory activity (58). Atropine and quinidine are linked to PEG-succinate of various molecular masses (235). In our laboratory, we synthesized a PEG-DOX conjugate with an amino acid or peptide spacer between the polymer and the drug to study tritosomic activity by lysosomal enzymes on the various derivatives. All the conjugates showed great plasma stability and a considerable decrease in toxicity, whereas activities depended on the nature of the amino acid bridge (236). PEG-5-fluorouracil (5FU) conjugates with ester, amide, or ether linkages have been studied (237), with increased stability under physiological conditions ranging from ester and amide to ether. The insoluble drug Taxol, one of the most potent chemotherapeutic agents used in the treatment of breast and ovarian cancers, has been linked to both hydroxyl ends of PEG through an ester bond, producing a highly soluble prodrug. The study indicated that the PEG molecular mass must be of such magnitude so a $t_{1/2}(\text{circulation}) > t_{1/2}(\text{hydrolysis})$ is maintained, a 40 kDa PEG being a good choice for the in vivo behavior of the prodrug (238). Similarly, the potent antitumor natural drug camptothecin has been bound to both hydroxyl groups of 40 kDa PEG, and an increased plasma half-life of the prodrug was obtained. Stability, interaction with plasma proteins, and activity of the conjugates are all strictly

influenced by the nature of the bond between the drug and the polymer (239).

One great limitation of PEG as a drug carrier resides in its mono- or bifunctionality. This characteristic, although useful in the case of PEG conjugation to polyfunctional drugs, preventing the problem of reticulation, restricts the total amount of drug molecules loaded per molecule of polymer in the case of monofunctional drugs. To circumvent this limitation and link a greater number of drug molecules per polymer chain, poly(PEG-Lys) has been synthesized and conjugated to *cis*-4-hydroxy-L-proline (240) and mPEG-poly(Asp) has been used to bind doxorubicin (241). In this latter case, the conjugate forms micelles composed of a drug-containing hydrophobic core with grafted PEG chains on their exterior, causing prolonged circulation in the bloodstream. The PEG derivative mPEG-glycol-poly(L-lysyl)diethylenetriaminepenta acetate monoamide has been used to prolong the circulation time of the imaging agent ^{99m}Tc (242).

Other Polymer-Drug Conjugates. Many other macromolecular drug conjugates have been studied. The alginate (9000 and 40000 Da MW)-*cis*-diamminedichloroplatinum (DDP) complex shows an increased plasma level, especially when high-MW alginate is used (243); the heparin-hepatocyte growth factor complex leads to improved plasma residence time for the drug (244). Other conjugates are under investigation, such as pyran copolymer-DOX (245), poly(aspartic acid)-DOX (248), poly(L-glutamic acid)-DOX, poly(hydroxyethyl-L-glutamine)-DOX (247), albumin-daunorubicin (248), *N*-(2-hydroxyethyl)methacrylate-*N*-vinylpyrrolidone copolymer-daunomycin (249), poly(L-lysine)-daunorubicin (250), polygalacturonic acid and carboxymethylated yeast β -D-glucanarabinoside C (251), poly(α -malic acid)-5-fluorouracil (252), poly(L-lysine)-methotrexate (253), block copolymers of poly(ethylenimine) and palmitic acid-cyclophosphamide derivatives (254), and DIVEMA-cyclophosphamide (255).

Special attention has been recently paid to oligonucleotide conjugates, e.g., poly(L-lysine) oligonucleotides (256), in an attempt to solve the problem of oligonucleotide delivery. The strategy combines reduced excretion of the drug, due to the increased mass of the conjugate, and mutual neutralization of polylysine and oligonucleotide, thus facilitating the crossing of cell membranes.

A potential strategy for regulating gene translation is deactivation of a target gene with oligonucleotides which form triplexes with that gene. However, triplex formation is unstable under the physiological conditions because of the repulsion among phosphate anions in DNA molecules associating in the triplex and duplex. The former use of macromolecular polycations, such as poly(L-lysine) (PLL), revealed an increase in the melting temperature (T_m) of the duplex, but it was associated with a precipitation process due to polyion formation. Comb-type copolymers of PLL with polysaccharide side chains finally proved to be a good solution to the problem (257); the hydrophilic chains of the polysaccharide grant solubility to the complex, while the polycation portion induces stability of the triplex.

2.4. General Considerations on Substance Conjugation. The conjugation of drugs to low-molecular mass molecules based on acetylation, esterification, and phosphorylation reactions is a well-established methodology in prodrug preparation. The use of polymers in drug modification is a more recent approach and, as such, is open to further developments, as described above. The two approaches increase the circulation half-life of drugs

in different ways. The old strategy causes an increase in the residence time of the drug simply by modifying its hydrophilicity and generally protecting it from metabolism. The new approach increases stability to chemicals and metabolic enzymes and prevents renal ultrafiltration because of the high molecular mass of the adduct. Moreover, the hindrance of the polymer around the drug is responsible for other specific advantages, such as the masking effect on immunogenic and antigenic sites, with consequent decreased immunogenicity and antigenicity. In addition, the increased molecular mass of the conjugate allows a targeting process to tumor tissues by means of the EPR effect.

The two main limitations of the polymer conjugation strategy are as follows: (1) the drug must possess chemical groups so that it can be derivatized by the polymer, (2) modification of those groups may lead to the decrease or loss of activity. In some lucky cases, modification does not affect drug activity, although the modified groups are the same ones involved in the explication of activity. However, more frequently, preservation of activity requires the appropriate choice of the chemical groups to be modified, since they must be different from those involved in the activity cascade (from the receptor binding step to the following phases). This choice is easier in the case of polypeptides and proteins which, due to their size, may present conjugation sites which are clearly distinguished from activity sites. Moreover, modification sites can also be introduced by substituting amino acids insignificant for the protein structure and activity with derivatizable amino acids. This explains the great efforts made in this direction by many biotechnological laboratories. The data obtained so far in polypeptide conjugation are now also paving the way for the preparation of new and original polymers that are able to modify selectively a specific amino acid. For the less versatile nonprotein drugs, the lack of anchoring groups can often make impossible the conjugation to polymers. For this case and for proteins that cannot be modified with one of the above strategies, physical entrapment in liposomes or particles is of help.

3. LIPOSOMES

3.1. Introduction. Water-insoluble polar lipids (e.g., phospholipids) in an excess of water give rise to highly ordered assemblages, which eventually arrange themselves into a system of concentric, closed, bilayered membranes called liposomes.

Since they were discovered (258), liposomes have been used as biomembrane models to provide information on the inter-relations of membrane lipid components in the basic bimolecular structure. Protein components of membranes are incorporated into liposomes to investigate lipid-protein interactions in biological membranes and the actions of membrane-active compounds such as ionophores, anesthetics, divalent cations, and antigen-antibody interactions. Liposomes have also emerged for clinical and diagnostic use as nontoxic carriers. This chapter briefly describes (a) properties and methods for entrapment of drugs, (b) liposomes as drug carriers and their *in vivo* fate, and (c) long-circulating liposomes.

3.2. Liposomes: Properties, Methods of Preparation, and Entrapment of Drugs. Various preparation procedures may be used to obtain one or more lipid lamellae liposomes, ranging from the minimum size of about 50 nm (diameter) for the smallest unilamellar vesicles (SUV) to several micrometers for the multilamellar type (MLV).

The classical procedure for preparing liposomes consists of dissolving appropriate concentrations of phos-

pholipids in an organic solvent, evaporating it, and subsequently disrupting the dry lipid layer with excess water or buffer, leading to the spontaneous formation of multilamellar liposomes of heterogeneous size (0.05–1 μm in diameter). Drugs may be entrapped either in the aqueous phase or in the lipid bilayers, depending on their hydrophilicity/hydrophobicity ratio. Substances that are soluble in organic solvents can be mixed with the lipids, ending up as constituents of the membrane structure in the final product, whereas water-soluble substances added to the solution, used in the dry lipid layer disruption step, end up in the aqueous compartment. Subsequent sonication of multilamellar liposomes yields unilamellar liposomes (50–100 nm in diameter) which are separated from the contaminating large ones by gel filtration (259) or ultracentrifugation (260). However, sonication has some disadvantages, like oxidation of unsaturated bonds in the fatty acid chains of phospholipids, with the formation of lyso compounds and free fatty acids, and denaturation and/or inactivation of entrapped molecules (proteins and nucleic acids). SUV may also be prepared by physical procedures, like the passage of large unilamellar vesicles (LUV) through acetylcellulose membranes, forced dialysis, or the French press process. An alternative method for preparing unilamellar liposomes is injection of an organic solution of the lipids into a large aqueous volume containing the substance for entrapment (261). For the preparation of SUV, repeated freezing and thawing may be employed, while LUV (diameter of $\geq 1 \mu\text{m}$) may be obtained from SUV by the addition of a divalent salt (e.g., calcium) which fuses the SUVs and subsequent removal of Ca^{2+} by EDTA.

The stability of phospholipid bilayers may be altered in terms of both rigidity and permeability by incorporating into the liposome structure other lipid soluble compounds such as sterols; alternatively, a charged amphiphile can both render the liposomal surface positively or negatively charged and increase the distance between the bilayers, giving rise to an increased aqueous volume. Size, rigidity, charge, and composition determine liposome fate and residence time in the body.

Depending on the hydrophilic/hydrophobic volume ratio, micelles may alternatively be formed and exploited for drug delivery and diagnostic purposes.

3.3. Liposomes as Drug Carriers and Their *In Vivo* Fate. Liposome carriers can alter drug biodistribution and pharmacokinetics when administered to human or animal models. Since liposomes can be modified at their surface by specific ligands, antigens, antibodies, sugars, and other targeting moieties, the entrapped drug may have quite different *in vivo* properties with regard to the site and rate of disposition and targeting. For this reason, liposomes are used to modify the properties of drugs and diagnostic agents; they can prolong their circulation time, isolate them from the systemic background, reduce their toxicity or side effects, and target them to the desired tissue with increased clinical efficacy (262–269).

The use of liposomes as drug carriers is supported by the following properties. (1) Intravenously administered liposomes are captured by monophagocytes in the reticuloendothelial system (RES) (270, 271) and can be used for efficient delivery of antiparasitic and antimicrobial drugs in treating infections localized in this system (e.g., antimonial drugs against leishmaniasis) (272) or for encapsulation of immunomodulators in activating macrophages to become tumoricidal in tumor models (273, 274). However, the efficient uptake of liposomes by the

RES, probably due to their opsonization by plasma proteins (275), results in their rapid removal from circulation and is the main disadvantage for the possible use of liposomes as delivery systems when the target site is beyond the RES. (2) Thanks to the different disposition and pharmacokinetics of the entrapped drug, liposome formulations can prevent the drug side effects such as the cardiotoxicity of doxorubicin (276, 277) and the nephrotoxicity of amphotericin B (278). (3) Both water-insoluble and amphiphilic drugs entrapped in liposome lipid bilayers have a longer half-life after intravenous administration and can be carried through the bloodstream to the target (279, 280). (4) Water-soluble macromolecules such as cytokines, plasmids, and ribozymes, which are easily inactivated and metabolized in the liver and other tissues, have an increased half-life when encapsulated in the aqueous compartment of liposomes. Liposomal monensin remains in circulation for 2 h, while the free form is cleared in only 15 min (281). Although anticancer agents such as topotecan, camptothecin (CAM), 9-aminocamptothecin (9-NC), and CTP-11 are interesting drugs capable of arresting the growth of a variety of human tumors, the rapid hydrolysis, at physiological pH, of their α -hydroxy lactone ring moiety, essential for biological activity, greatly limits their therapeutic use. Packaging of topotecan in the aqueous compartment (adjusted to pH 5) of distearoylphosphatidylcholine (DSPC) liposomes dramatically enhances its half-life in blood (282). Liposome formulations of CAM and 9-NC have a biological half-life which is 11 times longer than that of the free compounds (283). The use of other compounds such as valproic acid (VPA), an antiepileptic drug capable of crossing the placenta and producing fetal malformations, together with other toxic effects, must be avoided during pregnancy. However, encapsulation of VPA into phosphatidylcholine (PC)/cholesterol/ α -tocopherol liposomes significantly reduces the amount of exposure to the fetus (284). (5) Besides the use of liposome formulations for the reasons described above, many efforts are also directed toward modulating their permeability for sustained or local release of drugs (not discussed in this paper).

The *in vivo* properties of liposomes are determined by administration route, dose, size, formulation, surface charge, and the presence of specific ligands, sugars, antibodies, and other moieties appended along their structure.

Orally administered liposomes protect the encapsulated substances from enzymatic or pH degradation, but they may be partly lysated at pH 2.5, with 10 mM bile salts, by pancreatin and inside enterocytes (285). Local administration routes, intramuscular or subcutaneous, allow slow and sustained release of drugs at the injection site (286), especially from large multilamellar liposomes, whereas small RES-avoiding liposomes pass quickly to the bloodstream when injected subcutaneously (287).

The main liposome applications consist of drug targeting to the site of disease via circulation and controlled drug release at that site. In these applications, the characteristics of liposomes—(1) stability in plasma, (2) sufficient circulation time to interact with target tissues, (3) targeting, and (4) delivery of entrapped drugs into target cells—are important factors to be considered. In general, drugs entrapped in liposomes and retained in circulation are more stable than free drugs because of the liposome coating and stabilization by plasma proteins bound to their surface (288). However, high-density lipoproteins (HDL) are the main cause of liposome instability in plasma because of the transfer of liposomal

lipids to the HDL, with consequent release of the encapsulated material. This transfer is mediated by phospholipid transfer proteins present in plasma (289–294). Low-density lipoproteins may also increase liposomal permeability, taking lipid components from the liposomal membrane, especially when large doses of liposomes are used (295, 296). To solve this problem, several strategies, aimed at manipulating lipid composition and the fluidity of the lipid bilayer, have been used in the preparation steps of liposomes. (1) The incorporation of cholesterol, causing increased packing of phospholipids in the lipid bilayer, reduces the transfer of phospholipids to HDL (297, 298). (2) Liposomes obtained by PC with saturated fatty acyl chains (with a high liquid crystalline transition temperature) or sphingomyelin (SM) are more stable in blood than liposomes prepared by PC with unsaturated fatty acyl chains (299). (3) The use of lipid derivatives, such as PC with carbamyl analogues at the C-2 region (300, 301) or hydroxycholesterol (302), also stabilizes liposomes in the blood.

Once the problem of the release of drugs in plasma has been solved with one of the above strategies, due to their new acquired stability, clearance of the encapsulated drug depends on the clearance of the liposome itself (303). Liposomes administered systemically tend to accumulate in the RES (especially liver and spleen), with consequent rapid removal from the blood (304–307).

It is well-known that liposome composition, size, and charge can influence RES uptake; cholesterol-rich liposomes have low uptake by the RES (308), and SUV have a half-life longer than the 500–5000 nm MLV. Negatively charged liposomes have a shorter half-life in blood than neutral liposomes (289, 309) (although several studies report the opposite), due to immunological recognition (310–312) or activation of the complement system [fragments of complement component C3 may be deposited on the surface of unmodified liposomes, thus acting as opsonins (313, 314)]. Instead, positively charged liposomes are toxic and quickly removed from circulation (315).

3.4. Long-Circulating Liposomes. RES trapping of liposomes is the main problem in distributing encapsulated drugs to various organs. The first approach to the development of stabilized liposomes, later called “stealth liposomes” or “ninjasomes”, used SUV composed of saturated phospholipids such as DSPC and cholesterol (289, 309, 316, 317). Since they had the disadvantage of low aqueous entrapment volume, two more successful strategies are used to reduce internalization [due to one of the following processes: (1) immune recognition of negatively charged liposomes, (2) liposome opsonization, and (3) receptor-mediated phagocytosis] and consequent RES trapping. The first strategy is to prepare liposomes mimicking the erythrocyte membrane to be recognized by the RES as “self” instead of “foreign” structures; since the sialic acid portion of erythrocyte glycoproteins is thought to be important for immune recognition, liposomes are modified by gangliosides and sialic acid derivatives such as monosialoganglioside (GM1) (318–321). The second strategy consists of increasing the hydrophilicity of the liposomal surface, with a consequent decrease in opsonization, receptor-mediated phagocytosis, and RES entrapping (Figure 3); for this goal, poly(ethylene glycol) (PEG) was successfully introduced into liposome structures as PEG–phosphatidylethanolamine (PE) (102, 322, 323). Although PEG is still the best-known material for this aim, many other polymers have been investigated as liposome stabilizers.

Long-circulating liposomes are particularly important

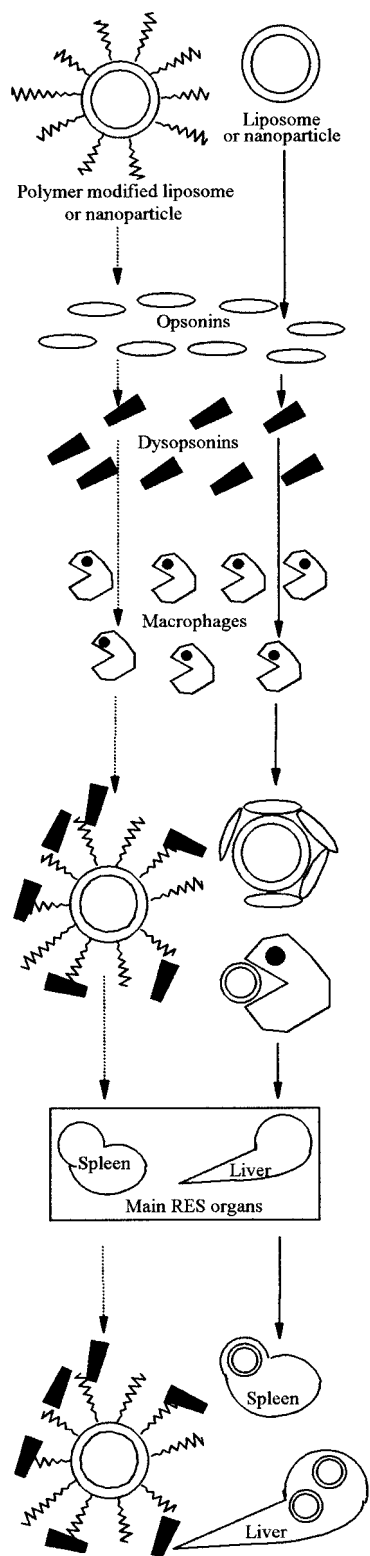


Figure 3. Different behaviors of plain and polymer-modified liposomes or nanoparticles toward proteins (opsonins and/or dysopsonins) and/or cells (macrophages) involved in the pre-RES uptake phases.

for passive targeting to tumor tissues. Because of their longer circulation time and the very permeable vasculature and low development of lymph channels (which recover extravasated molecules) in tumor tissues, long-circulating liposomes can pass through leaky endothelium tissue by passive convective transport better than conventional liposomes. This behavior is the basis for

the numerous studies now in progress in tumor targeting for both therapy and imaging.

3.4.1. GM1 and Glucuronide Liposomes. Several glycolipids are used to prepare liposomes to be studied for their RES uptake after iv injection. Only GM1 causes a decrease in RES uptake. The addition of GM1 to liposomes composed of rigid lipids increases the blood/RES ratio by 1 order of magnitude. It should be noted that rigid liposomes themselves increase this ratio by 1 order of magnitude compared to fluid lipids of all sizes (321). The effect of size in the biodistribution of GM1-containing liposomes was studied in mice bearing EMT6 solid tumors (324). Liposomes with a diameter in the 90–200 nm range have longer blood retentions, with consequent accumulation in tumor tissues, than those on either side of this range (324). Large liposomes with a diameter of >300 nm preferentially accumulate in the spleen, whereas those with a diameter of <40 nm probably deeply penetrate the interstitial spaces of liver. Unfortunately, the effect of GM1 liposomes in prolonging half-life in blood has only been observed in the mouse system, no long-circulating activity being shown in rats (325) and rabbits (326). Among other gangliosides, only GM3 prolongs liposome circulation in the rat system (327).

Studies of GM1 liposomes are an important step in the understanding and development of long-lasting liposomes. To investigate the mechanism of GM1 liposome RES avoidance, liposomes containing various gangliosides at 10 mol % (GM1, GM2, GM3, GD1a, and GD1b) and asialoganglioside GM1 liposomes, obtained by the sialidase treatment, were incubated with cultured macrophages (328). It was demonstrated that only GM1 reduced macrophage uptake, whereas the other gangliosides neither reduced nor stimulated the uptake. The degree of macrophage uptake depended on the concentration of GM1 in PC liposomes; a concentration of 10 mol % decreased RES uptake by 90%. This RES-avoiding effect was reversed by the removal of the sialic acid moiety, demonstrating the important role played by this molecule in RES-trapping avoidance. Sialic acid-modified liposomes prolong blood retention not only in mice but also in rats (325).

From all the above results, two possibilities were proposed to explain the mechanism of RES avoidance by ganglioside liposomes. (1) Specific moieties (i.e., GM1, but not other gangliosides in mice) bound to the liposome surface reduce the opsonization which occurs in plasma prior to recognition by the RES. (2) These moieties bind dysopsonins, reducing recognition by the RES. It was also suggested that the topology of carboxyl groups on the liposome surface plays an important role in liposome circulation time (329).

One problem for the extensive use of sialic acid is its cost, a fact which stimulated the investigation of other derivatives, including glucuronic acid. Palmitylglucuronide (PG1cUA) and palmitylgalacturoide were synthesized for incorporation into liposomes and to test whether RES-avoiding activity was specific to uronates in general or to glucuronate in particular (330, 331). It was demonstrated in mice that RES-avoiding activity is specific to uronates in general (331), that the liposome structure remains intact during their long half-life in blood (303), and that the mouse macrophage cell line J774 neither binds nor incorporates PG1cUA liposomes (332–334). Doubly labeled PG1cUA liposomes ($[^3\text{H}]$ -inulin in the inner aqueous space and cholesteryl $[^{14}\text{C}]$ -oleate in the bilayer) accumulate in tumor tissues (303) as a result of passive targeting, although the specific

interaction of tumor tissues and glucuronate cannot be excluded; this interaction is not due to a lower RES function in tumor-bearing mice, since liver accumulation is similar to that seen in normal mice. These long-circulating liposomes are probably not taken up by tumor cells but concentrated around them, as suggested by the comparable recovery of ^{14}C and ^3H , indicating that cholesteryl oleate is not degraded in the tumor.

3.4.2. PEG Liposomes. On the basis of the fact that increased hydrophilicity of the liposome surface prevents opsonization by serum proteins and interaction of liposomes with macrophages, liposomes have been modified with PEG in several laboratories to increase their circulation time (102, 322, 323). PEG prevention of serum protein and opsonin binding to the liposome surface and consequent RES trapping are related to its ability to mask the liposome surface under a hydrophilic, water-rich layer. X-ray diffraction analysis is used to measure structural organization and interbilayer repulsion for lipid/cholesterol (2/1) bilayers by incorporating 4 mol % of a PEG 1900 lipid. Analysis shows that the grafted polymer moiety extends ~ 50 Å from the lipid surface and gives rise to strong, slowly decaying, repulsive pressure between membranes, thus hindering their close approach (335). Recent studies indicate that the critical protective property of a PEG-coated liposome surface lies in its "self-similarity", i.e., the fractal characteristics of the spatial variations or fluctuations in the density of the polymer cloud. Self-similarity means that parts of the surface must resemble the whole surface over a prescribed range of spatial scales (336). The steric barrier created by PEG is also proven by the use of biotinyl-PE liposomes; the addition of avidin causes liposomes, but not PEG 5000-modified liposomes, to aggregate (337). However, PEG 2000 and PEG 550 provide only partial steric inhibition to the avidin-induced aggregation, while GM1-modified liposomes freely aggregate after the addition of avidin (338). The stability conferred on liposomes by PEG incorporation is due not only to the MW of the bound polymer but also to its considerable conformational flexibility (free rotation of individual units around interunit linkages) (339); a more rigid polymer like dextran, grafted to liposomes and used in similar quantities, does not cause liposome-protein interaction.

Distearoylphosphatidyl-*N*-(methoxy polyoxyethylene succinyl)ethanolamine-PEG 1900 (DSPE-PEG, 10 mol %) modification of SM/PC/cholesterol, DSPC/cholesterol, or PC/cholesterol liposomes produces increased blood residence time in all cases. It also indicates that the in vivo fate of PEG liposomes is affected little by the fluidity, the net charge on the liposome surface, the coupling method, or the hydrophobic anchoring group of PEG lipids; residence time is affected by the density of sterically active hydrophilic headgroups (340). More detailed studies testing the influence of the lipid anchor and the chemical stability of succinate, carbamate and amide linkages between mPEG derivatives and the amino headgroup of PE on circulation lifetime shows that a strong membrane anchor (DSPE) and a chemically stable conjugate bond (amide) result in retention of the polymer coating and greatly enhance circulation lifetimes (341).

The amount of PEG necessary to avoid RES activity is evaluated in the range of 5–7 mol % of PEG-DSPE in SM/PC/cholesterol liposomes, with no changes up to 20 mol %. The dependence of circulation time on PEG molecular mass indicates that a MW of 1000–2000 Da prolongs the circulation time of fluid lipid liposomes while

one of 5000–12000 Da is necessary to prolong the blood half-life of solid lipid liposomes (342). The size effect of 1/1/0.2 SM/PC/cholesterol/PEG 1900-DSPE liposomes on blood level shows that large (diameter of >300 nm) and small (diameter of <70 nm) liposomes accumulate in spleen and liver, respectively, while those of intermediate size remain in the blood (343).

The effect of PEG in liposome interactions with macrophages has been studied in vitro and indicates reduced uptake beginning at 5 mol % of PEG 1900-PE and reaching 95% reduction with 10 mol % (328). Liposomes uptake by human monocytes is also suppressed by PEG modification. These modified liposomes adhere to the cells, few being internalized by endocytosis (322).

The pharmacokinetics and biodistribution of PEG-DSPE liposomes have also been examined after subcutaneous administration. This route of administration may become very important, especially for patient self-administration and sustained release of drugs in vivo (287). Small liposomes (<120 nm diameter) appear intact in circulation after moving down lymph node chains (draining the site of injection), with up to 30% of in vivo label appearing in the blood 12–24 h postinjection. In the absence of PEG-DSPE, 4-fold lower levels of small liposomes are found in blood, probably due to macrophage uptake at the site of injection or along the lymph nodes, particularly evident when low doses are administered. These results therefore promote further investigations on the use of PEG liposomes for this new administration route of the liposome pharmaceutical form.

These novel PEG derivatives are regarded as key components of second-generation long-circulating liposomes (344). Liposomes are prepared from DSPC and cholesterol (1/1) containing 6 mol % of various PEG derivatives. The activity of the cholesterol derivative of PEG in prolonging the circulation time of liposomes is proportional to the average PEG molecular mass, i.e., 4800 Da $>$ 2600 Da $>$ 1700 Da $\gg$ 800 Da; the blood level increases from 7.8 to 32.9% as the polymer MW increases from 800 to 4800 Da. α -Methoxy- ω -(1,2-dioctadecenoyloxy glyceryl)polyoxyethylene (DO-PEG) 1000 and α -methoxy- ω -(1,2-ditetradecenoyloxy glyceryl)polyoxyethylene 1000, in which PEG is directly linked to glycerol, prolong circulation time as effectively as DSPE-PEG. Liposomes containing PEG derivatives with a functional group at the PEG terminal, such as distearoylphosphatidyl-*N*-(3-carboxypropionyl polyoxyethylene succinyl)ethanolamine (DSPE-PEG-COOH 3600 and 2400) and α -(dipalmitoylphosphatidyl)- ω -hydroxypolyoxyethylene (DPP-PEG-OH 1000), show higher blood levels than those containing DSPE-PEG 1000 (50.9, 43.3, 39.1, and 25%, respectively), suggesting that increased hydrophilicity and/or the presence of a steric barrier at the liposome surface, provided by PEG, play an important role. It should be noted that the incorporation of PEG derivatives does not change membrane fluidity and decreases the uptake by the murine macrophage-like cell line J774.

3.4.3. Other Polymers Used in the Preparation of Long-Circulating Liposomes. Other flexible, amphiphilic, functionalized oligomers are studied as PEG substitutes to obtain long-circulating liposomes. Among these, PVP and PAcM are activated as succinimidyl esters and bound to PE for liposome preparation. The plasma half-lives of unmodified liposomes, linear PEG, branched PEG (PEG2), PAcM, and PVP-modified liposomes (3% of polymer) are 10, 80, 140, 90, and 40 min, respectively (345). For all of the modified forms, a

comparable polymer molecular mass of around 5000–6000 Da (2×5000 Da for PEG2) is used. Captation by the liver follows the same pattern.

SUVs with 100 nm diameters are coated with poly(vinyl alcohol) (PVA) or a modified PVA bearing a long alkyl chain at the end of the molecule (PVA-R) to improve stability. Doxorubicin is encapsulated as a model drug to study the *in vivo* stability of polymer-coated liposomes. Both PVA- and especially PVA-R-coated liposomes are more stable in serum and have a longer survival time in systemic circulation than noncoated liposomes (346). Similar PVA and PVA-R mucoadhesive liposomes (347) are loaded with insulin (348); the faster decrease of glucose in blood and the long-lasting lowered glucose level caused by coated liposomes versus uncoated ones indicate good mucoadhesion and longer stability of modified liposomes.

Polyglycerols of various molecular masses are also investigated as potential liposome stabilizers. Polyglycerols are bound to dipalmitoylphosphatidic acid (DPP) and used for liposome preparation. Modified liposomes have high RES avoidance, which depends on the length of the polymer chains (349).

The lipid conjugates of two long-known but little exploited amphipathic polymers, poly(2-methyl-2-oxazoline) (PMOZ) and poly(2-ethyl-2-oxazoline) (PEOZ), are obtained by reaction of the glutarate esters of the polymers with DSPE or by termination of the polymerization process with DSPE. The products are used to prepare surface-modified liposomes, conferring low liver uptake and long circulation time on them to an extent similar to that for PEG, so far the best-known material for this purpose (350).

One of the newest methods proposed for protecting liposomes from external stimuli and degradation by lipases, lipooxidases, and/or serum proteins is coating their surface with hydrophobized polysaccharides, a method which mimics the role of polysaccharides in the wall of plant cells and bacterial cell membranes. Preliminary studies include the use of cholesterol-bearing pullulan with various degrees of substitution, monoalkyl (hexadecyl)-bearing pullulan, dialkyl (α,α' -dodecyl diglycerol diether)-bearing pullulan, cholesterol-bearing dextran, and cholesterol-bearing mannan. The cholesteryl moiety is the best hydrophobic anchor for such polysaccharides (351).

It has recently been found that injected polysialic acids (PSA) have long half-lives in the circulation of mice and may be used to extend the half-life of conjugated drugs (352). A low-molecular mass PSA (colominic acid, $M_n = 17000$ or 7800), already coupled to asparaginase and catalase to prolong circulation time, is coupled to the surface of SUV, increasing the liposome plasma half-life after intravenous injection (353).

A comparison of long-circulating liposomes coated with various types of polymers is reported in Table 2.

Last, a new strategy for further improving liposome pharmacokinetics is transient suppression of macrophage functions obtained by preadministration of liposome-encapsulated drugs, especially clodronate, which extends the circulation time of subsequently administered liposomes, even when they are long-circulating (354).

3.4.4. Active Targeted, Thermosensitive, pH-Sensitive Long-Circulating Liposomes. Long-circulating liposomes are ideal for active targeting. Monoclonal antibody 3A4, which binds a glycoprotein antigen, gp112, on the luminal surface of mouse lung capillary endothelium, is incorporated into PC/cholesterol liposomes containing 4 mol % of either GM1 or PEG 5000–

Table 2. Influence of the Type and Amount of Polymer Used for Liposome Coating on the Retention of Liposomes in Rat or Mouse Circulation

liposomes	retention in mouse circulation	retention in rat circulation	reference
plain	10	—	345 ^a
3 mol % PEG-PE	80	—	345
3 mol % PEG2-PE	140	—	345
3 mol % PVP-PE	40	—	345
3 mol % PACM-PE	90	—	345
7 mol % PEG-PE	230	—	345
7 mol % PEG2-PE	230	—	345
7 mol % PVP-PE	130	—	345
7 mol % PACM-PE	170	—	345
plain	18.3	—	349 ^b
6 mol % DPP-PG2	24.95	—	349
6 mol % DPP-PG6	38.11	—	349
plain	—	~1	350 ^c
5 mol % PEOZ-DSPE	—	>15	350
5 mol % PMOZ-DSPE	—	>15	350
5 mol % PEG-DSPE	—	>15	350
plain	0.5	—	353 ^d
SUV (20 mol % CA ₁₇₀₀₀ -PE)	3	—	353
SUV (20 mol % CA ₇₈₀₀ -PE)	3	—	353

^a The molecular masses of PEG, PEG2 (branched PEG), PVP, and PACM used in this preparation were 5000, 10000 (2×5000), 6000, and 6000 Da, respectively. The polymer content (3 or 7 mol %) is calculated as a fraction of PE residues. The retention in mouse circulation is expressed as half-life (minutes) (345). ^b Diglycerol (PG2) with a MW of 166 Da and hexaglycerol (PG6) with a MW of 500 were used for the preparation of dipalmitoylphosphatidylpolyglycerols (DPP-PG) of different length chains. The retention in mouse circulation is expressed as the percent injected dose in blood 6 h postinjection (349). ^c The MWs of PEOZ, PMOZ, and PEG used in this experiment were 5000, 4000, and 2000 Da, respectively. The retention in rat circulation is expressed as half-life (hours) (350). ^d The two small poly(sialic acid)s used (colominic acid, CA) had MWs of 17000 and 7800 Da, respectively. The retention in mouse circulation is expressed as half-life (hours) (353).

PE (355–357). GM1 incorporation enhances lung binding up to 52% and decreases liver uptake to 25%, indicating that GM1 is a suitable molecule for prolonging the blood life of immunoliposomes. Instead, PEG 5000 creates a general steric barrier, preventing both lung and liver uptake (decreased to 25%), PEG chain length thus turning out to be an important parameter in immunoliposome design (337). The problem may be overcome by (1) using shorter PEG chains [PEG 1900 (358) and PEG 2000 (359)], (2) introducing a long spacer arm between the liposome surface and the antibody, or (3) conjugating the antibody directly to the ends of long PEG chains (360). Many methods are available for attaching proteins to the liposome surface, a convenient one being the formation of a thioether bond (359).

Poly(ethylene glycol)-grafted liposomes with oligopeptide or oligosaccharide ligands appended to the termini of the polymer chains have recently been synthesized (361). In this case, the structure of long-circulating liposomes, obtained using DSPE as a lipid anchor and heterobifunctional PEG (2000 Da) as a linking moiety, is exploited to bind a cell-adhesive ligand, like YIGSR peptide and the Sialyl Lewis^x oligosaccharide, ligands for laminin cell surface receptors involved in metastasis and angiogenesis processes and for E-, P-, and L-selectin involved in inflammation-related tissue injury, respectively. This study highlights the fact that long-circulating liposomes may be used as a basic structure on whose external portion biologically active ligands can be attached. In general, this approach is useful both as a ligand-mediated targeting system and as a carrier for

small biologically active substances, which, in their free form, have high clearance from circulation.

Liposomes modified with a bioadhesive polymer are proposed as carriers for drugs to be driven to lymphocyte-rich sites such as inflamed sites. Cyclosporin is encapsulated in liposomes modified with various types of bioadhesive polymers (Carbopol 941, 940, 1342, and 934P). The drug has enhanced residence time in systemic circulation and delivery to spleen and liver which is twice as effective as that of the drug without liposome encapsulation (362).

Thermosensitive liposomes may be obtained by adding to the lipid mixture a high percentage of a component with a transition temperature slightly above body temperature. Delivery of encapsulated drugs to the target site may thus be enhanced, since warming above the critical transition temperature promotes drug leakage. Local hyperthermia, already used to promote tumor regression, may be combined with long-circulating thermosensitive liposomes, which are able to accumulate in tumor by penetration through the leaky vasculature of the tumor tissues. Thermosensitive GM1 liposomes (363) and PEG liposomes (364, 365), both containing doxorubicin and tested on C-26 colon carcinoma, show tumor regression and an increase in life span when the hyperthermia is applied at the tumor site to release the liposome content. Thermosensitive magnetoliposomes (TMs) are a further development of thermosensitive liposomes. In the attempt to treat diseases such as malignant tumors by warming humans and animals systemically or locally, capacitive heating is normally used. Since this method can achieve only broadly distributed and nonselective heating, hyperthermic materials such as magnetic particles, seeds, and needles have been used for more selective heating. The combination of this new technology with the use of thermosensitive liposomes has successfully led to dextran magnetite-incorporating thermosensitive liposomes, namely TMs (366).

pH-sensitive (367–369) and target-sensitive immunoliposomes (370, 371) have also been developed, the former entering cells via endocytosis and releasing the entrapped drug after fusion of liposomal and endosomal membranes at the acidic pH of the endosomes and the latter releasing their contents upon destabilization induced by liposomes binding to target cells. Sterically stabilized pH-sensitive liposomes are prepared by addition of PEG–PE to the membrane of cholesteryl hemisuccinate/dioleoylphosphatidylethanolamine liposomes, encapsulating a charged water-soluble fluorophore, calcein (372). These liposomes have better stability than those lacking PEG–PE and an unaltered ability to release calcein intracellularly. The antitumor agent methotrexate is entrapped into pH-sensitive long-circulating liposomes prepared with PEG–PE. Their plasma stability increases with the percentage of PEG–PE introduced, without losing pH sensitivity. Liposomes are destabilized in cancer tissues by the low pH in cells, with consequent release of the drug (373). Antisense oligonucleotides usually show decreased activity in biological fluids because of the strong lytic process by nucleases. To avoid this problem, they are encapsulated into pH-sensitive liposomes (374) that protect a 15-mer phosphodiester oligonucleotide from degradation in plasma and cause increased plasma concentrations. Sterically stabilized pH-sensitive liposomes may therefore be potentially useful for the *in vivo* intracellular delivery of highly negatively charged molecules such as genes, antisense oligonucleotides, and ribozymes for the treatment of various diseases (375).

3.4.5. Long-Circulating Liposomes for Tumor Diagnosis and Cancer Chemotherapy. The passive targeting of long-circulating liposomes to tumor tissues strongly supports their use in tumor imaging. [^{67}Ga]-Desferoxamine or [^{111}In]bleomycin is incorporated into hydrogenated PC/cholesterol liposomes containing a small molar fraction of GM1 or hydrogenated phosphatidylinositol. The *iv* injection of these liposomes into mice bearing B16 melanoma, J6456 lymphoma, or LS174T human colon carcinoma causes a tumor accumulation increase of up to 40-fold, 24 h after injection (376). Effective tumor imaging is successful because of the site receiving the implanted tumor cells (e.g., a hind leg), since most of the radioactivity accumulates in the bulk of the body, including the RES. [^{67}Ga]Desferoxamine is also encapsulated into PEG liposomes (377). The level of [$^{99\text{m}}\text{Tc}$]diethylenetriaminetetraacetic acid encapsulated into PG1cUA liposomes detected in blood and consequently in tumor tissue is higher than that of the marker in dipalmitoylphosphatidylglycerol (DPPG) liposomes, which preferentially accumulate in liver (378), indicating that DPPG liposomes are not suitable for tumor imaging when the tumor is close to liver.

The positive correlation between liposome circulation time and their localization in tumors makes the use of long-circulating liposomes for delivery of cancer drugs very promising. Enhanced tumor accumulation and antitumor activity, together with decreased toxicity, are reported for liposomal doxorubicin and epirubicin in mice bearing intraperitoneally injected lymphoma (379), subcutaneously (sc) injected colon carcinoma (380, 381), or sc injected Meth A sarcoma (382). Doxorubicin entrapped in PEG liposomes suppresses metastasis (383, 384) and shows a therapeutic effect in models of human ovarian carcinoma xenografts, HEY (385), and human prostate carcinoma PC-3 xenografts (386). To understand the mechanism of liposome uptake in various tissues, sterically stabilized liposome preparations with encapsulated colloidal gold particles and applied silver enhancement of the colloidal gold have been prepared to monitor the *in vivo* fate of liposomes by optical and electron microscopy (387). Results indicated that sterically stabilized small liposomes containing DSPE–PEG or GM1, with a half-life of 12–24 h, are able to traverse the endothelium of small blood vessels in tumors and extravasate into interstitial spaces; in liver and bone marrow, they are endocytosed primarily by cells of the mononuclear phagocyte system. Furthermore, the encapsulated drug, being able to diffuse much more through the tumor mass than the macromolecular carrier itself, can probably reach distant tumor cells within the same area where liposomes have extravasated and exert its cytotoxic effect there.

3.5. General Considerations on Substance Entrapment in Liposomes. Entrapment of substances in liposomes is a general procedure for obtaining long-circulating pharmaceutical or diagnostic forms. From a technological point of view, it also is an easier procedure when compared with the conjugation strategy, since chemical steps, available only to specialists, are avoided. Moreover, the drug undergoes more delicate treatment particularly important for substances which are easily susceptible to degradation.

However, when the use of liposome formulations is planned, the fact that great changes in drug pharmacokinetics and biodistribution and a limited increase in circulation half-life are often observed must be taken into consideration.

Moreover, the main limitations of this strategy are (1)

the reduced level of drug loading, especially in the case of lipophilic drugs which may disrupt the liposome double layer, and (2) the difficulty of obtaining a general viable form of liposomes for delivery and marketing. Many of these disadvantages may be overcome by the potentials of stealth liposomes and the new synthetic phospholipids which are becoming available.

In the delivery of biotechnological drugs, one problem which may remain is their antigenicity, which is not prevented but enhanced by liposome entrapment. Polymer conjugation is the only way to solve this problem, and the possibility of entrapping polymer-conjugated polypeptides in liposomes, thus exploiting the best of both techniques, must be suggested. This procedure is still to be properly investigated.

4. PARTICLES

4.1. Introduction. The use of particles as drug carriers has been developed as a method for improving drug bioavailability and for reaching two important therapeutic aims: controlled drug release and drug targeting. Both natural (e.g., red and white blood cells) and artificial particles (mostly obtained by synthetic polymers) have been used as drug carriers. Artificial particles are classified as microparticles (diameter of 1–50 μm) or nanoparticles (diameter of 10–1000 nm) according to their size and as spheres or capsules according to the presence of the polymer in the whole structure of the particle or only in its exterior portion. It is important to note that in literature the words microspheres and microcapsules are often used as synonyms.

Microparticles belong to the first generation of drug carriers. Although they deliver the active substance to the target, they cannot be considered true carriers, since they must be implanted as close as possible to their target (388). Instead, nanoparticles belong to the second generation of particulate drug carriers, since they are capable of not only releasing the active molecule at the target site but also carrying it there. Their size makes them suitable for systemic use, as they can pass capillaries. This review briefly describes the use of microparticles; a more extensive report on nanoparticles involves (a) their properties and methods of preparation, (b) nanoparticles as drug carriers, and (c) long-circulating nanoparticles.

4.2. Microparticles. Microparticles may circulate in the blood and pass through the heart, but they cannot pass through capillaries because of their size. On the basis of this property, biodegradable microspheres prepared from starch (389), albumin (390), or poly(lactic acid) (391) and nonbiodegradable microcapsules of ethylcellulose (392, 393) have been studied for chemoembolization; with this procedure, drugs localize in the nearby tissue surrounding the capillaries where microparticles accumulate by upstream intra-arterial injection. Their spherical shape, calibrated size, and capacity to carry and protect the desired drug are the major advantages of microparticles compared with natural emboli.

Microspheres are also used as depot systems, especially subcutaneous but also intravenous or intraperitoneous. In this case, the advantage of their size allows easy administration by injection, while the entrapped drug is slowly and continuously released into circulation from a system that protects it from degradation. The rate of drug release may be designed by properly choosing the polymer, i.e., pore size, degradation, and swelling properties. Polymer hydrophobicity is also an important issue since it protects the drug from hydrolytic or oxidative degradation. The best example of this application is the release from polylactide microcapsules of peptides used

to treat prostate cancer, both somatostatin analogue RC-160 (394) and LH-RH antagonist SB-75 (395). Systems releasing contraceptive drugs for more than 1 year have also been described.

Poly(lactic-co-glycolic acid) (PLGA) microspheres loaded with recombinant human growth hormone stabilize the protein and allow continuous drug release for 30 days under in vitro physiological conditions (396). PLGA is also used to prepare injectable gel implants of methotrexate. When PLGA is injected into sarcoma 180-bearing mice, drug plasma levels are sustained for more than 2 weeks (397). Somatostatin analogue RC-160 is encapsulated in PLGA microspheres for sustained drug release (398). Microspheres, recently prepared from new biodegradable polyesters with the ABA block structure, containing hydrophobic poly(lactide-co-glycolide) (A) blocks and hydrophilic poly(ethylene oxide) (B) blocks are now under therapeutic investigation (399). To stabilize microparticles for drug delivery and vaccine formulation, poly(ethylene glycol)-dextran (PEG-DEX) conjugates have been used as combined stabilizers and surface modifiers to produce reabsorbable PLGA microparticles, in which the PEG component provides an anchor to the microparticle surface while the dextran component extends from the particle surface, providing steric stabilization (400). A novel lipid microsphere (LM) emulsified by lecithin with saturated fatty acids was investigated as a drug carrier (401). Compared with yolk phospholipids LM, much slower plasma clearance was observed when dipalmitoylphosphatidylcholine LM was administered iv to rats.

4.3. Properties and Methods of Preparation of Nanoparticles. Various methods and polymers can be used for particle preparation (388). (A) The first is polymerization of a monomer. Polyalkylcyanoacrylate (PACA) nanospheres of around 150 nm can be prepared by an emulsion polymerization process, in which droplets of water-insoluble monomers are emulsified in an aqueous phase (402). Anionic polymerization is initiated by water hydroxyl ions and takes place in micelles where monomer molecules diffuse through the water phase. Drugs dissolved in the polymerization medium can be entrapped in nanospheres either before or after polymerization. PACA and polyisobutylcyanoacrylate (PIBCA) nanocapsules of 200–300 nm are formed by interfacial polymerization, in which an organic phase (including an ethanol solution of the monomer, the oily core material, the lipophilic drug to be encapsulated, and soybean lecithin as an additional surfactant) is slowly dropped into a double volume of an aqueous phase (solution of an anionic surfactant) (403, 404). (B) The second is use of macromolecules. Submicronic macromolecular particulates can be obtained from preformed (natural or synthetic) macromolecules such as polymers, proteins, or polysaccharides. Nanospheres are prepared according to one of the following procedures. (1) The solution emulsification technique produces polymer (e.g., polylactide) (405, 406) and nonpolymer (albumin) nanospheres (407). In the former case, the polymer is dissolved in an organic volatile solvent immiscible in water and the organic phase is dispersed in an aqueous phase by conventional emulsification techniques. The polymer particles are formed from oil-in-water droplets by removal of the organic solvent following distillation under reduced pressure. In the latter case, albumin is dissolved in water and the solution dispersed in an oily phase to form a W/O emulsion which, poured into oil heated to more than 100 °C, causes denaturation of the protein and evaporation of the aqueous phase. The limitations of these methods

are the difficulty of entrapping lipophilic drugs in the first case and thermolabile drugs in the second. (2) The desolvation method, consisting of the precipitation of polymers from their solution by addition of a third component (nonsolvent or salts), is optimized to yield polymer and nonpolymer nanospheres. For polymer nanospheres, the first simple method proposed consists of preparing low-dispersed 200 nm particles by adding an organic solution of the polymer to a miscible nonsolvent and evaporating the volatile solvent (408). This method is successfully applied to polymers such as polylactide, polylactide-co-glycolide, poly- ϵ -caprolactone, ethylcellulose, cellulose acetophthalate, PACA, poly(vinyl chloride)-co-acetate, and polystyrene. Nonpolymer gelatin nanospheres are prepared by controlled desolvation of an aqueous solution of gelatin by adding a desolvating agent (409). Albumin nanospheres are obtained by adapting Fessi's method (410). Both procedures are mild, and labile drugs can be entrapped in the nanoparticles. (3) The spontaneous emulsification technique (411) is adapted (412) to yield polymer nanocapsules with 200–5000 nm diameters; the polymer is dissolved in an organic solvent in association with an oily phase, which then becomes the core of the nanocapsule, and with the drug to be loaded. This organic solution is then poured into a nonsolvent of both polymer and oily core, leading to the formation of nanocapsules. (4) The recently described emulsification–diffusion technique (413) involves the formation of an O/W emulsion with a partially water-soluble solvent containing the polymer and an aqueous phase containing a stabilizer. The subsequent addition of water to the system causes the solvent to diffuse into the external phase, with consequent aggregation of the polymer held in the globules for forming nanoparticles.

4.4. Nanoparticles as Drug Carriers. The reticuloendothelial system (RES) is responsible for eliminating nanoparticle drug carriers from circulation with the following biodistribution: 80–90% in liver, 5–8% in spleen, and 1–2% in bone marrow. Many drugs have been loaded into nanoparticles to be targeted to the above organs. Examples are PACA nanospheres-DOX, used for M5076 hepatic metastases, with a higher plasma level (414, 415), lower toxicity, and stronger uptake by the liver, associated with lower clearance from liver (416), than those of the free drug; and the lipophilic immunomodulator MDP–L-alanylcholesterol (MDP–Chol), entrapped in PIBCA nanocapsules to prolong its biological half-life (417).

However, nanoparticle uptake by the phagocytic system puts limits on a more general applicability, as already described for liposomes. Formulations of long-lasting nanoparticles are therefore under investigation.

4.5. Long-Circulating Nanoparticles. Many attempts have been made to prolong the circulation time of drugs entrapped in particulate systems after iv injection. The main strategy is reduction of particle affinity to RES cells (particularly Kupffer cells), a phenomenon that greatly limits their use to tissues other than liver, spleen, and lung.

The removal of drugs entrapped in particles from circulation depends mostly on the parameters regulating the removal of the carrier system itself. Particles with polar and charged surfaces have an increased circulation time and reduced uptake by the RES organs (418). Amino-modified polystyrene nanoparticles ranging from 100 to 1000 nm are labeled with [^{14}C]formaldehyde, and their fate in blood, liver, spleen, and lung is monitored (419). Half-life in blood ranges from 1.36 to 4.92 min;

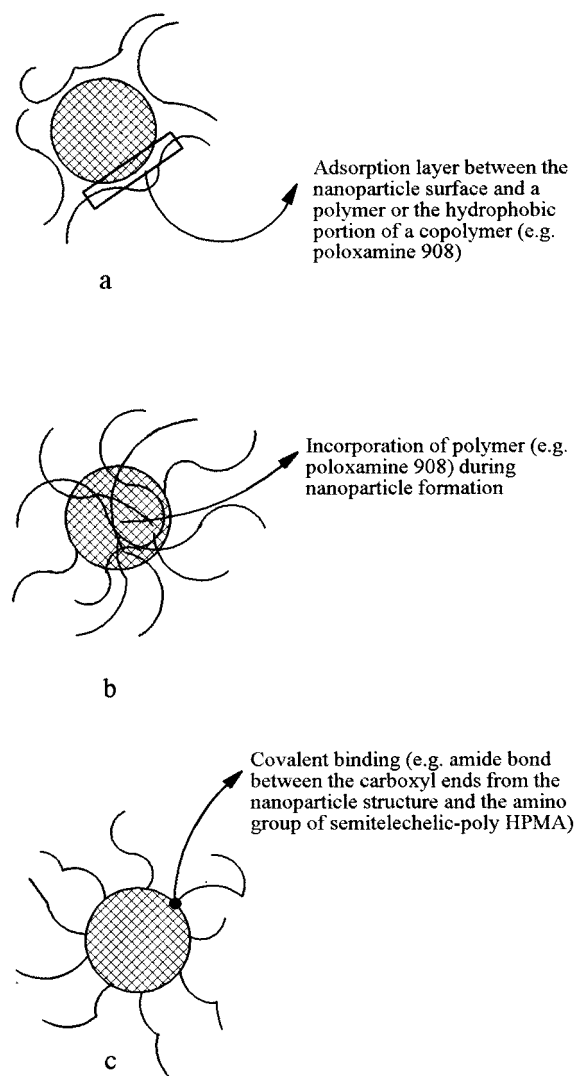


Figure 4. Methods for particle surface modification by polymers: (a) adsorption, (b) incorporation, and (c) covalent binding.

particles are mainly taken up by liver, larger particles being taken up faster than smaller ones. Amino-bearing polystyrene particles stimulate the phagocytic uptake mechanism to a lesser extent than polystyrene particles without amino groups, and consequently have a longer half-life (419).

One of the most successful strategies for obtaining long-circulating nanoparticles is polymer anchoring, especially poly(ethylene glycol) (PEG), at the particle surface, for creating a hydrophilic cloud reducing interactions with the proteins and cells responsible for their low blood residence time. The modification of nanoparticle surface by polymers can be performed by polymer adsorption, incorporation during the production of nanoparticles, or covalent attachment to the nanoparticle surface (Figure 4).

Uptake of particulate carriers by liver and/or isolated Kupffer cells may be prevented by coating particles with a hydrophilic surface based on PEG-type materials, like block copolymers of ethylene oxide/propylene oxide (POE/POP) or poloxamine-908 (420–424), which are either adsorbed or covalently bound to the particles. Adsorption of poloxamine-908 to polystyrene microspheres prolongs microsphere circulation time after iv injection into rabbits (420). Possible important factors for such increased retention in blood are (1) the thickness of the adsorbed layer (425); (2) the decrease in surface hydrophobicity,

causing limited phagocytosis (426); (3) the reduced opsonization due to the hydrophilic coating layer, with consequent decrease of particle recognition by RES phagocytes (427–429); and (4) the binding of plasma dysopsonins (suppressive substances from plasma and serum) which minimizes interaction with phagocytes (430–432) (Figure 3).

To investigate all these hypotheses, colloidal gold particles (117 nm diameter) and polystyrene microspheres (60 nm diameter) are used as models for poloxamine-908 coating (433). Since preopsonization of gold particles in plasma results in immobilization of fibrinectin on their surface (429, 434), stimulating the uptake of gold particles by isolated Kupffer cells via a peripheral membrane-bound C-reactive protein [a particle-galactose-specific receptor (434, 435)], coating them with poloxamine-908 reveals how the steric barrier of poloxamine affects the opsonization process. Moreover, since the uptake of latex microspheres by macrophages in the presence of plasma or serum is suppressed and regulated by dysopsonins (426, 430), their use in this kind of experiment highlights the role of dysopsonins in clearing poloxamine-coated particles. The study indicates that poloxamine-908 coating blocks opsonization but still allows dysopsonization (433).

Poly(lactic acid) (PLA)–monomethoxypoly(ethylene glycol) (PEG) copolymers (PLA–PEG) are used to modify the surface of biodegradable poly(lactide-co-glycolide) (PLGA) and polystyrene (PS) nanoparticles (used as controls) and to compare these particles with those obtained by modification with poloxamine-908 (436). PLA–PEG copolymer (3/4) (PEG MW of 2000 Da), 2/5 PLA–PEG (PEG MW of 5000 Da) copolymers, and poloxamine-908 produce coating layers of ~3, 7, and 9 nm, respectively. Coating particles with any of the stabilizing copolymers significantly decrease albumin adsorption, which is attributed to folding of the PEG chain into the hydrated layer. For polystyrene nanoparticles, coating with either 2/5 PLA–PEG or 3/4 PLA–PEG reduces the rapid clearance from circulation observed for uncoated nanoparticles, although the reduction is greater for poloxamine-908. PLA–PEG-coated PS nanoparticles are removed from circulation by liver to the same extent as uncoated ones, but higher clearance by spleen is observed. Coating PLGA nanoparticles with PLA–PEG copolymers results in extended circulating levels, comparable with those obtained with poloxamine-908, and decreased liver uptake. The different clearance and organ distributions of polystyrene and PLGA nanoparticles, both coated with PLA–PEG, may be attributed to the difference in stability of PLA–PEG coatings on polystyrene and PLGA nanoparticles. The PLA portion of PLA–PEG copolymers is a better anchor group when they are adsorbed on the surface of a similar structure, i.e., that of PLGA nanoparticles, providing a coating layer stable to desorption and displacement which prevents any gradual adsorption of plasma proteins (436). The lymph node localization of biodegradable PLGA nanoparticles may be dramatically enhanced by coating their surface with PLA–PEG copolymers or by producing coprecipitate nanoparticles of PLGA and PLA–PEG (437).

Other copolymers are being investigated for coating nanoparticles, like a nontoxic PEG–polycyanoacrylate copolymer for preparing PEG-coated PACA nanoparticles (438). Nanospheres are prepared by emulsion–polymerization of isobutylcyanoacrylate in water in the presence of PEG of different chemical structure (PEG, methoxy-PEG, and bimethoxy-PEG) to evaluate the role

Table 3. Influence of Different Polymer Coatings of Polystyrene (PS) and/or PLGA Nanoparticles on Their Permanence in Rat Circulation

particles	MW of the copolymer (Da)	retention in rat circulation	reference
PS	—	6	436 ^a
PS/PLA–PEG ₅₀₀₀ (2/5)	9500	36	436
PS/PLA–PEG ₂₀₀₀ (3/4)	5400	58	436
PS/poloxamine-908	25000	108	436
PLGA	—	4.8	436
PLGA/PLA–PEG ₅₀₀₀ (2/5)	9500	13.2	436
PLGA/PLA–PEG ₂₀₀₀ (3/4)	5400	60	436
PLGA/poloxamine	25000	93	436
PLGA	—	3.7	437 ^b
75% PLGA/PLA–PEG ₇₅₀ (1.5/0.75)	2219	7.0	437
65% PLGA/PLA–PEG ₇₅₀ (1.5/0.75)	2219	8.7	437
55% PLGA/PLA–PEG ₇₅₀ (1.5/0.75)	2219	12.0	437
PF	—	—	438 ^c
PF/poloxamine-908	—	0.75	438
PF/PF–PEG ₅₀₀₀	—	3	438

^a PEG 5000 and 2000 were used for the preparation of 2/5 and 3/4 PLA–PEG, respectively. The coated nanospheres were obtained by the precipitation–solvent evaporation method. The retention in rat circulation is expressed as half-life (minutes) (436).

^b The nanospheres were obtained by coprecipitation of a mixture of PLGA and PLA–PEG, in which PLGA ranged from 75 to 55%. The retention in rat circulation is expressed as the percent injected dose in blood 6 h postinjection (437). ^c The nanoparticles were coated with the poloxamine-908 (MW of 25000 Da) or PF–PEG (5% PEG chains of 5000 Da) copolymer. The retention in rat circulation is expressed as half-life (hours) (438).

of the various configurations of PEG on these potential stealth-type, rapidly biodegradable carriers (439).

The poly(phosphazene)–poly(ethyleneoxide) (PF–PEO₅₀₀₀) copolymer is used for surface modification of poly(phosphazene) nanoparticles (440). In this case, poly(phosphazene) nanoparticles are modified with both PF–PEO and poloxamine-908. Biodistribution in rat has been studied; nanoparticles with a poloxamine-908 coating are mainly captured by liver, although delayed clearance from systemic circulation was noted. In contrast, nanoparticles coated with PF–PEO have a prolonged blood circulation profile, and only small amounts of nanoparticles are sequestered by liver. This stresses the importance of the nature of both the anchoring group and the particle surface in determining the biological behavior of the system.

ST-PHPMA polymers with different molecular masses and containing an amino end group (31) are covalently bound via amide bonds to the surface of nanospheres based on copolymers of methyl methacrylate, maleic anhydride, and methacrylic acid. Such modified nanospheres show decreased *in vitro* adsorption to proteins, decreased accumulation in liver after *iv* administration in rats, and increased intravascular half-life. Changes in these properties increase with increasing polymer molecular mass (31).

Retention in blood of various long-circulating nanoparticles is reported in Table 3.

4.6. General Considerations on Substance Entrapment in Particles. Encapsulation of drugs in micro- or nanoparticle appears to be the simplest and technologically most suitable method for prolonging the body residence time of drugs, as compared with polymer conjugation and liposome entrapment. This is particularly due to the availability of many new tailor-made polymers. However, there are many problems in encaps-

sualting biotechnological products, since the organic solvents and heating procedures, often required in the entrapment step, may denature the proteins. This limitation does not apply to polymer conjugation and liposome strategies.

Once the drug has been encapsulated, two other main problems may be encountered. (1) Hydrolytically labile compounds may be damaged by water diffusing inside the matrix, unless very hydrophobic polymers are used. (2) The release of large peptides and proteins may be limited by the matrix pore size of biodegradable polymers used.

As for liposomes, the new biodistribution pattern acquired by the encapsulated drug must be taken into consideration in planning particle use, especially for microparticles which do not pass through capillaries.

The use of microparticles may become a very attractive strategy if new polymers are introduced, conferring specific characteristics on the system, e.g., adhesive polymers for direct delivery of drugs to deep aerial pathways.

5. ADDITIONAL STRATEGIES

Other strategies are applied to increase the stability of substances in circulation. For a brief review of what has been achieved in various fields and for very different purposes, a few examples are given here.

Other Long-Circulating Polymer-Coated Systems. Lipid emulsions stabilized with emulsifiers, such as phospholipids, represent an interesting system for delivering lipophilic drugs. Unlike liposomes, O/W emulsions have only one layer of amphipathic molecules (e.g., phosphatidylcholine) and a hydrophobic core which can be filled with a considerable amount of lipophilic drug. Moreover, together with the characteristics of biodegradability, biocompatibility, and physical stability, they can be prepared on a large industrial scale and are relatively stable below 25 °C for prolonged periods (441). Rhizoxin palmitoyl derivative RS-1541 in O/W emulsions has a lengthy circulation time in blood after iv injection and stability against lipoprotein lipase (442). The main problem in using O/W emulsions is rapid clearance from blood by the RES system after parental administration (443, 444), a process involving apolipoprotein E (445). PEG is again proposed as a solution to this problem. Inclusion of PEG derivatives such as Tween-80 or dioleoyl *N*-[monomethoxypoly(ethylene glycol) succinyl]phosphatidylethanolamine (mPEG-PE) as a coemulsifier in emulsions composed of Castor oil and phosphatidylcholine decreases their RES uptake and prolongs circulation in blood. PEG derivatives increase circulation time depending on the chain length of the polymer (PEG 2000 $\geq$ PEG 5000 > PEG 1000 and Tween-80) and PEG density on the emulsion surface (446). A submicrometer lipid emulsion coated with amphipathic poly(ethylene glycol) for parental administration of paclitaxel (Taxol) is an alternative formulation for the drug (447), which is usually prepared in ethanol and Cremophor EL (Diluent 12), a solvent system associated with severe side effects.

Polymer micelles are another interesting class of potentially useful drug carriers. Although their structure is quite different from that of liposomes, they are related to them by the similarity in structural elements, and their formation is based on a thermodynamic equilibrium. The small size of micelles, between that of single molecules and that of the smallest liposomes (50 nm), means that they are of great interest as carriers for drugs or imaging agents, since they can reach body sites not accessible to any other particle system. Micelles based

on the copolymers of poly(ethylene oxide) (PEO) and poly(aspartic acid) [p(Asp)] with covalently bound adriamycin (ADR) are prepared (241, 448) and have long circulation times, which increase according to the PEO MW/p(Asp) MW ratio: 1000/4800 < 5000/8700 < 5000/2100 < 12000/2100. This behavior is attributed to the low level of interaction of the PEO corona region of the micelles with various biocomponents (proteins and cells). Low uptake levels of PEO-[p(Asp)-(ADR)] in liver and spleen are reported. Biodistribution depends on micelle stability, since unstable micelles form free polymer chains which undergo renal excretion.

Micelles are also prepared from comb copolymers based on stearyl methacrylate and acrylate derivatives of PEG. Micelles may be formed by homogeneous dispersion of comb copolymers in aqueous solutions and the sizes adjusted by sonication. These micelles are now being investigated to confirm their expected high circulation half-lives due to the attachment of PEG on the copolymers (449).

Novel reactive polymer micelles for potential use as drug carriers are obtained with heterobifunctional AB block copolymers consisting of PEG and PLA containing an acetal group at the PEG chain end. This block copolymer forms polymer micelles (30 nm diameter) in aqueous media. The acetal groups are transformed into aldehyde groups, which conjugate with targeting moieties such as sugar molecules and antibodies (450).

Micelles with 10–50 nm diameters are obtained from PEO and successfully incorporated with amphiphilic ¹¹¹In and gadolinium chelates for percutaneous lymphography (451). New trends in preparing contrast-loaded micelles are also under investigation, e.g., the application of amphiphilic polychelating polymers (452).

Glycosylation. Glycosylation of SOD with monosaccharides such as galactose and mannose influences enzyme renal disposition, reducing tubular reabsorption and enhancing exposure to the luminal surface (453).

Hydrolytically Stable Peptide Analogues. Many peptide analogues have been prepared to improve the pharmacokinetics of natural peptides. An example is somatostatin, a peptide which binds to specific receptors in various target tissues and inhibits the release of growth hormone from pituitary cells. Several analogues have been synthesized, including the octapeptide analogue octreotide (Sandostatin). This has a similar high affinity for specific receptors with an IC₅₀ in the subnanomolar range but is much better under in vivo conditions, having a longer duration of action, specificity, and potency, unlike its different distribution, metabolism, and excretion behavior (454).

Antibody Fragments. In the development of tumor diagnosis and drug-targeting strategies, monoclonal antibodies play an important role as agents in directing drugs to specific tumor tissues (455). Limitations connected with using intact antibodies, like long half-life (which does not allow discrimination between tumor target and background in tumor imaging) and relatively large molecular mass (which inhibits the rate of immunoconjugate extravasation and the extent of tumor penetration), are avoided by using antibody fragments such as F(ab) or F(ab')₂ (which associates better with target cells than the monovalent fragment). These antibody fragments are generated by proteolysis of intact Ig or Fv fragments, comprising the variable domains only of H and L chains, approximately half the size of a F(ab) fragment, obtained by genetic engineering (456). In the latter case, the spontaneous separation of VH and VL chains, which are in dynamic equilibrium in solution, are

deleterious to stability, probably because the isolated VH chain is highly susceptible to denaturation (457). Bis-FvCys conjugates prepared from murine anti-lysozyme Fv fragments (458) and antcarbohydrate antigen, occurring in many human tumors (459, 460), both have better stability and a significantly longer serum half-life than univalent Fv analogues.

Lipophilization. ^{99m}Tc is an important imaging agent in nuclear medicine as mentioned above. [^{99m}Tc]Exam-etazime and its stabilized form containing CoCl_2 (461) are well-established radiopharmaceutical drugs, mainly used to study regional cerebral blood flow by single-photon emission tomography and to radiolabel leukocytes for imaging focal sites of infection and/or inflammation (462–465). On its first pass through the brain, lipophilic [^{99m}Tc]exametazime is removed from the blood by cerebral extraction and rapidly converted to a more hydrophilic species which is retained in cerebral blood long enough for the acquisition of images over the following few hours (462).

Lecithinization. Lecithinization has been investigated as an alternative method for improving protein pharmacokinetics; both SOD (466) and interleukine-6 (467) derivatives have longer persistence in blood than unmodified forms.

Lipidization. A new technique recently introduced in the drug delivery field is reversible lipidization of polypeptides. Conjugation with fatty acids decreases the level of proteolytic degradation (468) and increases the plasma half-life of polypeptide drugs (469, 470). This method seems very innovative, although critical aspects such as immunogenicity and toxicity are still to be properly investigated.

Microemulsions. The formation of macroscopically homogeneous, stable, fluid, optically transparent W/O microemulsions is being evaluated for the production of new drug carriers. The influence of various counterions added to W/O microemulsions (containing lecithin as a surfactant and water/propylene glycol at various pH values as an internal phase) on the permeation of methotrexate across intact hairless mouse skin has been studied (471). Increased permeation of the drug as a consequence of methotrexate lipophilization due to ion pair formation is noticed when dodecyl sulfate and dioctyl sulfosuccinate are used.

Dendrimers. Many efforts have been made in the field of dendrimer synthesis, characterization, and modification to obtain polymer “boxes” for potential use as nanoscopic molecular containers (472). The latest dendritic boxes are formed by the reaction of a fifth-generation poly(propylene imine) dendrimer with a bulky, hydrogen-bonding group such as a protected phenylalanine, which forms a rigid shell around the dendrimer. Modification of the shell gives boxes of various porosity, and shape-selective release of “guests” is possible. The reaction of terminal amino groups with alkanolic acids yields medium-tunable boxes, which are closed in polar media (collapsed alkyl chains) and open in apolar media (swollen alkyl chains) (473). Complexes of oligonucleotides with polyamidoamine starburst dendrimers show that dendrimers cause 50-fold enhancement in the level of cell uptake of oligonucleotides, supporting further evaluation of dendrimers for oligonucleotide delivery in cell cultures and in vivo (474).

Cyclodextrins. Inclusion complexation of unstable guests with cyclodextrins has also been proposed as a new method for stabilizing and solubilizing a variety of molecules. At this stage, researchers are still studying the kinetic parameters regulating complex formation and

degradation (475), but the system potentially represents one of the future methods for carrying molecules in circulation while avoiding degradation processes.

Niosomes. To reduce renal clearance of doxorubicin modified with HPMA and thus increase tumor accumulation, a nonionic surfactant vesicle (niosome) formulation has been developed (476). These DOX–HPMA-containing niosomes are stable in the presence of plasma, releasing only 0.2% DOX over 72 h; in the presence of a lysosomal enzyme preparation (tritosomes), the polymer conjugate is cleaved, releasing 8% DOX over 72 h.

Biological Carriers. This review does not include reports on natural carriers, since attention is focused on methods that involve chemical approaches. However, one example must be briefly reported, since it is an interesting combination of biological and chemical procedures in the preparation of a carrier. Among potential natural carriers, erythrocytes are the most widely used, since they are easy to obtain and can be pretreated to inactivate their two major proteolytic systems, Ca^{2+} -dependent neutral proteinases and acid endopeptidases (477). The specific phosphatases in their structure are exploited for slow release of prodrugs entrapped in the cells. Erythrocytes loaded with azidothymidine derivatives act in vitro as bioreactors, ensuring sustained and potentially useful release of drugs (478). Chemical modification of the erythrocyte surface may also prolong and/or target entrapped drugs to different organs. These positive advantages have already allowed successful clinical testing.

6. CONCLUSIONS

Retention of drugs in circulation is a key step in drug design and delivery. Even the most active compound in vitro is useless if it does not reside in vivo in the blood long enough to reach its target, while managing to avoid to some extent premature metabolism, immunological reactions, toxicity, rapid excretion, and captation by undesired tissues. In the last few decades, the combination of a better understanding of cell and tissue properties and the development of tailor-made polymers, liposomes, and nanoparticles has led to the production of interesting systems which have increased drug residence time in circulation.

Starting from the pioneering proposal of multivalent polymers by Ringsdorf in the 1970s, many complex tools were later developed in the design of drugs covalently bound to polymers. Great importance was given to drug stability, polymer properties and targeting to specific tissues, and protease susceptibility of the peptide spacer between polymer and drug. This approach has already yielded successful clinical results, especially with the two antitumor drug carriers SMA and HPMA, and it is now therefore considered a general strategy which may be applied to new polymers and drugs.

So far, the most widely studied polymer is PEG, which is certainly still the best characterized material for this goal. PEG has a much simpler structure than SMA, HPMA, or other polymers, and it possesses incredibly favorable in vivo properties which have encouraged many studies especially with it as a protein and peptide carrier. Many tests have been carried out on the chemistry of this polymer, reported in both academic and proprietary publications, particularly with regard to activation methods which allow its binding not only to protein and nonprotein drugs but also to liposomes and particles, to convey long-lasting properties. Chemistry is still the important issue for new discoveries in this approach to

drug delivery; it is essential both for the synthesis of new polymers and for the preparation of copolymers, in which the properties of hydrophilicity and hydrophobicity, charge, and shape of different polymers can be variously combined.

In the field of drug encapsulation, besides the classical liposomes, particles, and their long-circulating versions obtained by polymer coatings, interesting new structures are being developed, such as niosomes and dendrimers. The latter structures have rapidly demonstrated their great potential in drug delivery, thanks to the complex but extremely precise architecture of their huge synthetic structure. Another area of study, which only chemical investigations can bring to mature application, is the derivatization of fullerene for yielding products of precise size but with still undiscovered although fascinating delivery potentials.

The development of biological studies is also of primary importance, not only for evaluation of the *in vivo* behavior of the carried drugs but also for further experimentation with cells and viruses as carriers.

In conclusion, although many important goals have been already reached in the stabilization of substances in circulation, many other fascinating chemical and biological combinations must be explored, with the final goal of reaching the ambitious possibility of tailoring the most suitable strategy for any drug delivery or targeting need.

ACKNOWLEDGMENT

We thank the CNR Target Project on Biotechnology for financial support.

LITERATURE CITED

- (1) Duncan, R., and Kopecek, J. (1984) Soluble synthetic polymers as potential drug carriers. *Adv. Polym. Sci.* 57, 53–101.
- (2) Robert, K. Y., Cheng, Z., and Cheng, C. C. (1989) Delivery of anticancer drugs. *Methods Find. Exp. Clin. Pharmacol.* 11, 439–529.
- (3) Goddard, P. (1991) Therapeutic proteins—A pharmaceutical perspective. *Adv. Drug Delivery Rev.* 6, 103–131.
- (4) Nucci, M. L., Shorr, R., and Abuchoski, A. (1991) The therapeutic value of poly(ethylene glycol)-modified proteins. *Adv. Drug Delivery Rev.* 6, 133–151.
- (5) Pizzo, S. V. (1991) Preparation, *in vivo* properties and proposed clinical use of polyoxyethylene-modified tissue plasminogen activator and streptokinase. *Adv. Drug Delivery Rev.* 6, 153–166.
- (6) Wileman, T. E. (1991) Properties of asparaginase-dextran conjugates. *Adv. Drug Delivery Rev.* 6, 167–180.
- (7) Maeda, H. (1991) SMANCS and polymer conjugated macromolecular drugs: advantages in cancer chemotherapy. *Adv. Drug Delivery Rev.* 6, 181–202.
- (8) Sehon, A. H. (1991) Suppression of antibody responses by conjugates of antigens and monomethoxypoly(ethylene glycol). *Adv. Drug Delivery Rev.* 6, 203–217.
- (9) Duncan, R. (1992) Drug-polymer conjugates: potential for improved chemotherapy. *Anti-Cancer Drugs* 3, 175–210.
- (10) Delgado, C., Francis, G. E., and Fisher, D. (1992) The uses and properties of PEG-linked proteins. *Crit. Rev. Ther. Drug Carrier Syst.* 9, 91–114.
- (11) Katre, N. V. (1993) The conjugation of proteins with polyethylene glycol and other polymers. Altering properties of proteins to enhance their therapeutic potential. *Adv. Drug Delivery Rev.* 10, 91–114.
- (12) Zalipsky, S. (1995) Chemistry of polyethylene glycol conjugates with biologically active molecules. *Adv. Drug Delivery Rev.* 16, 157–182.
- (13) Duncan, R., and Spreafico, F. (1994) Polymer Conjugates. Pharmacokinetic considerations for design and development. *Clin. Pharmacokinet.* 27, 290–306.
- (14) Putnam, D., and Kopecek, J. (1995) Polymer conjugates with anticancer activity. *Adv. Polym. Sci.* 122, 55–123.
- (15) Ringsdorf, H. (1975) Structure and properties of pharmacologically active polymers. *J. Polym. Sci., Polym. Symp.* 51, 135–153.
- (16) Marshall, J. J., Humpreys, J. D., and Abramson, S. L. (1977) Attachment of carbohydrate to enzymes increases their circulation lifetime. *FEBS Lett.* 83, 249–252.
- (17) Melton, R. G., Wiblin, C. N., Foster, R. L., and Sherwood, R. F. (1987) Covalent linkage of carboxypeptidase G₂ to soluble dextran. (i). Properties of conjugates and effects on plasma persistence in mice. *Biochem. Pharmacol.* 36, 105–112.
- (18) Melton, R. G., Wiblin, C. N., Baskerville, A., Foster, R. L., and Sherwood, R. F. (1987) Covalent linkage of carboxypeptidase G₂ to soluble dextran. (ii). *In vivo* distribution and fate of conjugates. *Biochem. Pharmacol.* 36, 113–121.
- (19) Sherwood, R. F., Baird, J. K., Atkinson, T., Wiblin, C. N., Rutte, D. A., and Ellwood, D. C. (1977) Enhanced plasma persistence of therapeutic enzymes by coupling to soluble dextran. *Biochem. J.* 164, 461–464.
- (20) Wileman, T. E., Foster, R. L., and Elliott, P. N. C. (1986) Soluble asparaginase-dextran conjugates show increased circulatory persistence and lowered antigen reactivity. *J. Pharm. Pharmacol.* 38, 264–271.
- (21) Wileman, T., Bennet, M., and Lilleyman, J. (1983) Potential use of an asparaginase-dextran conjugate in acute lymphoblastic leukemia. *J. Pharm. Pharmacol.* 35, 762–765.
- (22) Sprinck, L., Exner, J., Sterba, O., and Kopecek, J. (1976) New types of synthetic infusion solutions III. Elimination and retention of poly[N-(2-hydroxypropyl)methacrylamide] in a test organism. *J. Biomed. Mater. Res.* 10, 953–963.
- (23) Rihova, B., Kopecek, J., Ulbrich, K., and Chytrý, V. (1985) Immunogenicity of N-(2-hydroxypropyl)methacrylamide copolymers. *Makromol. Chem. (Suppl.)* 9, 13–24.
- (24) Kopecek, J., and Bazilova, H. (1973) Poly[N-(2-hydroxypropyl)methacrylamide] 1. Radical polymerisation and copolymerisation. *Eur. Polym. J.* 9, 7–14.
- (25) Strohalm, J., and Kopecek, J. (1978) Poly[N-(2-hydroxypropyl)methacrylamide] IV. Heterogeneous polymerisation. *Angew. Makromol. Chem.* 70, 109–118.
- (26) Rejmanova, P., Labsky, J., and Kopecek, J. (1977) Aminolysis of monomeric and polymeric *p*-nitrophenyl esters of methacryloylated amino acids. *Makromol. Chem.* 178, 2159–2168.
- (27) Ulbrich, K., Zacharieva, E. I., Obereigner, B., and Kopecek, J. (1980) Polymers containing enzymatically degradable bonds 5. Hydrophilic polymers degradable by papain. *Biomaterials* 1, 199–204.
- (28) Kopecek, J., and Duncan, R. (1987) Targetable polymeric prodrugs. *J. Controlled Release* 6, 315–327.
- (29) Rihova, B., Ulbrich, K., Strohalm, J., Vetrík, V., Bilej, M., Duncan, R., and Kopecek, J. (1989) Biocompatibility of N-(2-hydroxypropyl)methacrylamide copolymers containing adriamycin. Immunogenicity, effect on hematopoietic stem cells in bone marrow *in vivo* and effect of mouse splenocytes and human peripheral blood lymphocytes *in vitro*. *Biomaterials* 10, 335–342.
- (30) Kopecek, J. (1990) The potential of water-soluble polymeric carriers in targeted and site-specific drug delivery. *J. Controlled Release* 11, 279–290.
- (31) Kamei, S., and Kopecek, J. (1995) Prolonged blood circulation in rats of nanospheres surface-modified with semitelechelic poly[N-(2-hydroxypropyl)methacrylamide]. *Pharm. Res.* 12, 663–668.
- (32) Maeda, H., Ueda, M., Morinaga, T., and Matsumoto, T. (1985) Conjugation of poly(styrene-co-maleic acid) derivative to the antitumor protein neocarzinostatin: pronounced improvements in pharmacological properties. *J. Med. Chem.* 28, 455–461.
- (33) Kobayashi, A., Oda, T., and Maeda, M. (1988) Protein binding of macromolecular anticancer agent SMANCS: characterization of poly(styrene-co-maleic acid) derivatives as an albumin binding ligand. *J. Bioact. Compat. Polym.* 3, 319–333.

- (34) Breslow, D. S. (1976) Biologically active synthetic polymers. *Pure Appl. Chem.* 46, 103–113.
- (35) Breslow, D. S., Edwards, E. I., and Newborg, N. R. (1973) Divinyl ether maleic anhydride (pyran) copolymer used to demonstrate the effect of molecular weight on biological activity. *Nature* 246, 160–162.
- (36) Hirano, T., Ohashi, S., Morimoto, K., Tsuda, T., Kobayashi, T., and Tsukagoshi, S. (1986) Synthesis of antitumor-active conjugates of adriamycin or daunomycin with the copolymer of divinyl ether and maleic anhydride. *Makromol. Chem.* 187, 2815–2824.
- (37) Hirano, T., Todoroki, S., Kato, H., Yamamoto, H., Caliceti, P., Veronese, F. M., Maeda, H., and Ohashi, S. (1994) Synthesis of the conjugate of superoxide dismutase with the copolymer of divinyl ether and maleic anhydride retaining enzymatic activity. *J. Controlled Release* 28, 203–209.
- (38) Ravin, H. A., Seligman, A. M., and Fine, J. (1952) Polivinylpyrrolidone as plasma expander: studies on its excretion, distribution and metabolism. *N. Engl. J. Med.* 247.24, 921–929.
- (39) Haaf, A. S., and Straub, F. (1985) Polymers of N-vinylpyrrolidone: synthesis, characterization and uses. *Polym. J.* 17.1, 143–152.
- (40) Von Specht, B. W., Seinfeld, H., and Brendel, W. (1973) Polivinylpyrrolidone as a soluble carrier of proteins. *Hope-Seyler's Z. Physiol. Chem.* 354, 1659–1660.
- (41) Andreani, F., Salatelli, E., and Ferruti, P. (1986) Synthesis of end-capped functionalized PVP oligomers. *J. Bioact. Compat. Polym.* 1, 72–80.
- (42) Veronese, F. M., Sartore, L., Caliceti, P., Schiavon, O., Ranucci, E., and Ferruti, P. (1990) Hydroxy-terminated polivinylpyrrolidone for the modification of polypeptides. *J. Bioact. Compat. Polym.* 5, 167–178.
- (43) Sartore, L., Ranucci, E., Ferruti, P., Caliceti, P., Schiavon, O., and Veronese, F. M. (1994) Low molecular weight end-functionalized poly(N-vinylpyrrolidone) for the modification of polypeptide amino groups. *J. Bioact. Compat. Polym.* 9, 411–427.
- (44) Kopeček, J., Bacik, J., and Sprinck, L. (1973) Soluble hydrophilic polymers. German Patent 2,309,894.
- (45) Nitto Electric Industrial Co., Ltd. (1985) Hydrophilic materials for transdermal patches. JP 6041,968.
- (46) Takashi, K., Saburo, O., Shoichi, T., and Keisuke, S. (1985) Transdermal pharmaceutical containing dihydropyridine derivatives. JP 60,166,61.
- (47) Ranucci, E., Spagnoli, G., Sartore, L., and Ferruti, P. (1994) Synthesis and molecular weight characterization of low molecular weight end-functionalized poly(4-acryloylmorpholine). *Makromol. Chem. Phys.* 195, 3469–3479.
- (48) Bailey, J., and Koleske, J. V., Eds. (1976) *Poly(ethyleneoxide)*, Academic Press, New York.
- (49) Powell, G. M. (1980) Polyethylene glycol. In *Handbook of Water Soluble Gums and Resins* (R. L. Davidson, Ed.) pp 18–1–18–31, McGraw-Hill, New York.
- (50) Mutter, M., and Bayer, E. (1979) The liquid-phase method for peptide synthesis. In *The Peptides* (E. Gross and J. Meienhofer, Eds.) pp 285–332, Academic Press, New York.
- (51) Dreborg, S., and Akerblom, E. B. (1990) Immunotherapy with monomethoxypolyethylene glycol modified allergens. *Crit. Rev. Ther. Drug Carrier Syst.* 6, 315–365.
- (52) Yamaoka, T., Tabata, Y., and Ikada, Y. (1994) Distribution and tissue uptake of poly(ethylene glycol) with different molecular weights after intravenous administration in mice. *J. Pharm. Sci.* 83, 601–606.
- (53) Harris, J. M., Ed. (1992) *Poly(ethylene glycol) Chemistry: Biotechnical and Biomedical Applications*, Plenum Press, New York.
- (54) Murakami, Y., Tabata, Y., and Ikada, Y. (1997) Tumor accumulation of poly(ethylene glycol) with different molecular weights after intravenous injection. *Drug Delivery* 4, 23–31.
- (55) Johansson, G., and Joelsson, M. (1985) Preparation of Cibacron Blue F3G-A-polyethylene glycol in large scale for use in affinity partitioning. *Biotechnol. Bioeng.* 27, 621–625.
- (56) Khachadurian, A. K., Fung, C. H., Van Es, T., and Davis, F. F. (1981) Polyoxyethylated cholesterol derivatives: organic synthesis, cellular uptake and effect on metabolism in cultured skin fibroblasts. *Biochim. Biophys. Acta* 665, 434–441.
- (57) Zalipsky, S., Gilon, C., and Zilkha, A. (1983) Attachment of drugs to polyethylene glycols. *Eur. Polym. J.* 19, 1177–1183.
- (58) Cecchi, R., Rusconi, L., Tanzi, M. C., Danusso, F., and Ferruti, P. (1981) Synthesis and pharmacological evaluation of poly(oxyethylene) derivatives of 4-isobutylphenyl-2-propionic acid (Ibuprofen). *J. Med. Chem.* 24, 622–625.
- (59) Ghedini, N., Zecchi, V., Tartarini, A., Scapini, G., Andrisano, V., and Ferruti, P. (1986) Synthesis and partition profiles of nicotinic acid derivatives with oligomeric carriers. *J. Controlled Release* 3, 185–191.
- (60) Dal Pozzo, A., Acquasaliente, M., Donzelli, G., Delor, F., and Ferruti, P. (1986) Synthesis and antimicrobial properties of new aroyl-acrylic esters of polyethylene glycols. *Farmaco* 41, 622–629.
- (61) Zalipsky, S., Gilon, C., and Zilkha, A. (1984) Esterification of polyethylene glycols. *J. Macromol. Sci. Chem.* A21, 839–845.
- (62) Khue, N. V., and Galin, J. C. (1985) Antiinflammatory polymer-bound steroids for topical applications I. Synthesis and characterization. *J. Appl. Polym. Sci.* 30, 2761–2778.
- (63) Zalipsky, S. (1995) Functionalized poly(ethylene glycol) for preparation of biologically relevant conjugates. *Bioconjugate Chem.* 6, 150–165.
- (64) Herman, S., Hooftman, G., and Schacht, E. (1995) Poly(ethylene glycol) with reactive endgroups: I. Modification of proteins. *J. Bioact. Compat. Polym.* 10, 145–187.
- (65) Buckmann, A. F., Morr, M., and Kula, M. R. (1987) Preparation of technical grade polyethylene glycol (PEG) (Mr 20,000)-N6-(2-aminoethyl)-NADH by a procedure adaptable to large scale synthesis. *Biotechnol. Appl. Biochem.* 9, 258–268.
- (66) Bayer, E., Zheng, H., and Geckeler, K. (1982) Functionalization of soluble polymers 4. Synthesis of dichloro- and di-(4-formylphenoxyethyl) poly(oxyethylene). *Polym. Bull.* 8, 585–592.
- (67) Buckmann, A., Morr, M., and Johansson, G. (1981) Functionalization of poly(ethylene glycol) and monomethoxy-poly(ethylene glycol). *Makromol. Chem.* 182, 1379–1384.
- (68) Ziegast, G., and Pfannemüller, B. (1984) Linear and star-shaped hybrid polymers 1. A new method for the conversion of hydroxyl end groups of poly(oxyethylene) and other polyols into amino end groups. *Makromol. Chem. Rapid Commun.* 5, 363–371.
- (69) Swamikannu, A. X., and Litt, M. H. (1984) Preparation and characterization of *p*-toluene sulfonyl ester and amino derivatives of tri- and poly(ethylene glycol). *J. Polym. Sci., Polym. Chem. Ed.* 22, 1623–1632.
- (70) De Vos, R. J., and Goethals, E. J. (1985) Convenient synthesis of α -tosyl- ω -tosyloxypoly(oxyethylene). *Makromol. Chem. Rapid Commun.* 6, 53–56.
- (71) Zalipsky, S., Albericio, F., Slomczynska, U., and Barany, G. (1987) A convenient general method for synthesis of N α - or N ω -dithiasuccinoyl (Dts) amino acids and dipeptides: Application of polyethylene glycol as a carrier for functional purification. *Int. J. Pept. Protein Res.* 30, 740–783.
- (72) Harris, J. M., Struck, E. C., Case, M. G., Paley, M. S., Yalpani, M., Van Alstine, J. M., and Brooks, D. E. (1984) Synthesis and characterization of poly(ethylene glycol) derivatives. *J. Polym. Sci., Polym. Chem. Ed.* 22, 341–352.
- (73) Pillai, V. N. R., Mutter, M., Bayer, E., and Gatfield, I. (1980) New, easily removable poly(ethylene glycol) supports for the liquid-phase method of peptide synthesis. *J. Org. Chem.* 45, 5364–5370.
- (74) Sano, A., Maeda, H., Kai, Y., and Ono, K. (1989) Polyethylene glycol derivatives, modified peptides and production thereof. *Eur. Pat. Appl.* 0340741.
- (75) Harris, J. M., Dust, J. M., McGill, A., Harris, P. A., Edgel, M. J., Herrati, R. S., Karr, L. J., and Donnelly, D. L. (1991) New polyethylene glycol for biomedical applications. In *Water-Soluble Polymers* (S. W. Shalaby, C. L. McCormic, and G. B. Butler, Eds.) pp 418–429, ACS Symposium Series, American Chemical Society, Washington, DC.

- (76) Nillsson, K., and Mosbach, K. (1984) Immobilization of ligands with organic sulfonyl chlorides. *Methods Enzymol.* 104, 56–69.
- (77) Delgado, C., Patel, J. N., Francis, G. E., and Fisher, D. (1990) Coupling of poly(ethylene glycol) to albumin under very mild conditions by activation with tresyl chloride: characterization of the conjugate by partitioning in aqueous two-phase systems. *Biotechnol. Appl. Biochem.* 12, 119–128.
- (78) Knusli, C., Delgado, C., Malik, F., Domine, M., Tejedor, C., Irvine, A. E., Fisher, D., and Francis, G. E. (1992) Polyethylene glycol modification of granulocyte-macrophage colony stimulating factor (GM-CSF) enhances neutrophil priming activity but not colony stimulating activity. *Br. J. Haematol.* 82, 654–663.
- (79) Yoshinga, K., and Harris, J. M. (1989) Effects of coupling chemistry on the activity of poly(ethylene glycol)-modified alkaline phosphatase. *J. Bioact. Compat. Polym.* 4, 17–24.
- (80) Locufier, J., Crommen, J., Vandorpe, J., and Schacht, E. (1991) End-group modification of α -hydro- ω -methoxypoly(oxyethylene), 2: Facile methods for the introduction of an α -amino end-group. *Makromol. Chem. Rapid Commun.* 12, 159–165.
- (81) Leonard, M., and Dellacherie, E. (1988) Synthesis and characterization of a polyoxyethylene derivative for the affinity labeling of human hemoglobin. *Makromol. Chem.* 189, 1809–1817.
- (82) Zalipsky, S., Chang, J. L., Albericio, F., and Barany, G. (1994) Preparation and applications of polyethylene glycol-polystyrene graft resin supports for solid-phase peptide synthesis. *React. Polym.* 22, 243–258.
- (83) Pollak, A., and Witeside, G. M. (1976) Organic synthesis using enzymes in two-phase aqueous ternary systems. *J. Am. Chem. Soc.* 98, 289–291.
- (84) Rozzell, D. (1987) Immobilized aminotransferases for amino acid production. *Methods Enzymol.* 136, 479–497.
- (85) Persson, L. O., and Olde, B. (1988) Synthesis of ATP-polyethylene glycol and ATP-dextran and their use in the purification of phosphoglycerate kinase from spinach chloroplasts using affinity partitioning. *J. Chromatogr.* 457, 183–193.
- (86) Zalipsky, S., Lee, C., and Menon-Rudolph, S. (1992) Hydrazine containing conjugates of polypeptides and glycopeptides with polymers. PCT Int. Appl. WO 9216555.
- (87) Zalipsky, S., and Menon-Rudolph, S. (1997) Hydrazide derivatives of poly(ethylene glycol) and their bioconjugates. In *Poly(ethylene glycol): Chemistry and Biological Applications* (J. M. Harris and S. Zalipsky, Eds.) pp 318–341, American Chemical Society, Washington, DC.
- (88) Andresz, H., Richter, G. C., and Pfannemuller, B. (1978) Chemische Synthese verzweigter Polysaccharide, 5: Kopplung von Oligosacchariden und Amylose an verschiedene Trager durch Hydrazonbindung. (Chemical synthesis of branched polysaccharides, 5. Coupling of oligosaccharides and amylose to various carriers by hydrazone linkage). *Makromol. Chem.* 179, 301–312.
- (89) Herron, J. N., Gentry, C. A., Davies, S. S., and Lin, J. N. (1994) Antibodies as targeting moieties: affinity measurements, conjugation chemistry, and applications in immunoliposomes. *J. Controlled Release* 28, 155–166.
- (90) Harris, J. M., Herati, M. R. S., Sather, P. J., Brooks, D. E., and Fyles, T. M. (1992) Synthesis of new poly(ethylene glycol) derivatives. In *Poly(ethylene glycol) Chemistry: Biotechnical and Biomedical Applications* (J. M. Harris, Ed.) pp 371–381, Plenum Press, New York.
- (91) Johansson, G. (1986) Effects of poly(ethylene glycol)-bound alcohols and amines on the partition of albumins and thylakoid membranes in an aqueous two-phase system. *J. Chromatogr.* 368, 309–317.
- (92) Zalipsky, S., and Barany, G. (1990) Facile synthesis of α -hydroxy- ω -carboxymethylpolyethylene oxide. *J. Bioact. Compat. Polym.* 5, 227–231.
- (93) Royer, G., and Anantharmaiah, G. M. (1979) Peptide synthesis in water and the use of immobilized carboxypeptidase Y for deprotection. *J. Am. Chem. Soc.* 101, 3394–3396.
- (94) Geckeler, K., and Bayer, E. (1980) Functionalization of soluble polymers 3. Preparation of carboxy-telechelic polymers. *Polym. Bull.* 3, 347–352.
- (95) Joppich, M., and Luisi, P. L. (1979) Peptides flanked by two polymers chains, 1. Synthesis of glycyl-L-tryptophylglycine substituted by poly(ethylene oxide) at both the carboxy and amino end groups. *Makromol. Chem.* 180, 1381–1384.
- (96) Ferruti, P., Tanzi, M. C., Rusconi, L., and Cecchi, R. (1981) Succinic half-esters of poly(ethylene glycols) and their benzotriazole and imidazole derivatives as oligomeric drug-binding matrices. *Makromol. Chem.* 182, 2183–2192.
- (97) Zalipsky, S. (1993) Synthesis of an end-group functionalized polyethylene glycol-lipid conjugate for preparation of polymer-grafted liposomes. *Bioconjugate Chem.* 4, 296–299.
- (98) Zalipsky, S., and Barany, G. (1986) Preparation of polyethylene glycol derivatives with two different functional groups at the termini. *Polym. Prepr. (Am. Chem. Soc., Div. Polym. Chem.)* 27 (1), 1–2.
- (99) Ouchi, T., Banba, T., Masuda, H., Matsumoto, T., Suzuki, S., and Suzuki, M. (1991) Design of chitosan-5FU conjugate exhibiting antitumor activity. *J. Macromol. Sci., Chem.* 28, 959–975.
- (100) Zalipsky, S., and Lee, C. (1992) Use of functionalized polyethylene glycols for modification of polypeptides. In *Poly(ethylene glycol) Chemistry: Biotechnical and Biomedical Applications* (J. M. Harris, Ed.) pp 347–370, Plenum Press, New York.
- (101) Boccu', E., Largajolli, R., and Veronese, F. M. (1983) Coupling of monomethoxypolyethylene glycols to proteins via active esters. *Z. Naturforsch.* 38C, 94–99.
- (102) Klivanov, A. L., Maruyama, K., Torchilin, V. P., and Huang, L. (1990) Amphipathic polyethylene glycols effectively prolong the circulation time of liposomes. *FEBS Lett.* 268, 235–237.
- (103) Blume, G., Cevc, G., Grommelin, M. D. J. A., Bakker-Woudenberg, I. A. J. M., Kluft, C., and Storm, G. (1993) Specific targeting of poly(ethylene glycol)-modified liposomes: coupling of homing devices to the ends of the polymeric chains combines effective target binding with long circulation times. *Biochim. Biophys. Acta* 1149, 180–184.
- (104) Sartore, L., Caliceti, P., Schiavon, O., and Veronese, F. M. (1991) Enzyme modification by MPEG with an amino acid or peptide as spacer arms. *Appl. Biochem. Biotechnol.* 27, 45–54.
- (105) Sartore, L., Caliceti, P., Schiavon, O., Monfardini, C., and Veronese, F. M. (1991) Accurate evaluation method of the polymer content in monomethoxy(polyethylene glycol) modified proteins based on amino acid analysis. *Appl. Biochem. Biotechnol.* 31, 213–222.
- (106) Beauchamp, C. O., Gonias, S. L., Menapace, D. P., and Pizzo, S. V. (1983) A new procedure for the synthesis of polyethylene glycol-protein adducts. Effects on function, receptor recognition, and clearance of superoxide dismutase, lactoferrin and α 2-macroglobulin. *Anal. Biochem.* 131, 25–33.
- (107) Veronese, F. M., Largajolli, R., Boccu', E., Benassi, C. A., and Schiavon, O. (1985) Surface modification of proteins: activation of monomethoxy-polyethylene glycols by phenylchloroformates and modification of ribonuclease and superoxide dismutase. *Appl. Biochem. Biotechnol.* 11, 141–152.
- (108) Zalipsky, S., Seltzer, R., and Menon-Rudolph, S. (1992) Evaluation of a new reagent for covalent attachment of polyethylene glycol to proteins. *Biotechnol. Appl. Biochem.* 15, 100–114.
- (109) Chiu, H. C., Zalipsky, S., Kopeckova, P., and Kopecek, J. (1993) Enzymatic activity of chymotrypsin and its poly(ethylene glycol) conjugates toward low and high molecular weight substrates. *Bioconjugate Chem.* 4, 290–295.
- (110) Zalipsky, S., Seltzer, R., and Nho, K. (1991) Succinimidyl carbonates of polyethylene glycol: useful reactive polymers for preparation of protein conjugates. In *Polymeric Drugs and Drug Delivery Systems* (R. L. Dunn and R. M. Ottenbrite, Eds.) pp 91–100, American Chemical Society, Washington, DC.

- (111) Sirokman, G., and Fasman, G. D. (1993) Refolding and proton pumping activity of a polyethylene glycol-bacteriorhodopsin water-soluble conjugate. *Protein Sci.* 2, 1161–1170.
- (112) Poiani, J. G., Gean, K. F., Fox, J. D., Kohn, J., and Riley, D. J. (1993) Antifibrotic effect of a proline analogue delivered in liposomes to cells in culture. *Amino Acids* 4, 237–248.
- (113) Abuchowski, A., Van Es, T., Palczuk, N. C., and Davis, F. F. (1977) Alteration of immunological properties of bovine serum albumin by covalent attachment of polyethylene glycol. *J. Biol. Chem.* 252, 3578–3581.
- (114) Ashihara, Y., Kono, T., Yamazaki, S., and Inada, Y. (1978) Modification of *E. coli* L-asparaginase with polyethylene glycol: Disappearance of binding ability to anti-asparaginase serum. *Biochem. Biophys. Res. Commun.* 83, 385–391.
- (115) Ono, K., Kai, Y., Maeda, H., Samizo, F., Sakurai, K., Nishimura, H., and Inada, Y. (1991) Selective synthesis of 2,4-bis(O-methoxypolyethylene glycol)-6-chloro-s-triazine as a protein modifier. *J. Biomater. Sci., Polym. Ed.* 2, 61–65.
- (116) Paley, M. S., and Harris, J. M. (1987) Synthesis of the aldehyde of oligomeric polyoxyethylene. *J. Polym. Sci., Part A: Polym. Chem.* 25, 2447–2454.
- (117) Topchieva, I. N., Kuzaev, A. I., and Zubov, V. P. (1988) Modification of polyethylene glycol. *Eur. Polym. J.* 24, 899–904.
- (118) Wirth, P., Soupe, J., Tritsch, D., and Biellmann, J. F. (1991) Chemical modification of horseradish peroxidase with ethanal-methoxypolyethylene glycol: solubility in organic solvents, activity, and properties. *Bioorg. Chem.* 19, 133–142.
- (119) Chamow, S. M., Kogan, T. P., Venuti, M., Gadek, T., Harris, R. J., Peers, D. H., Mordenti, J., Shak, S., and Ashkenazi, A. (1994) Modification of CD4 immunoadhesin with monomethoxypoly(ethylene glycol)aldehyde via reductive alkylation. *Bioconjugate Chem.* 5, 133–140.
- (120) Bayer, E., Zheng, H., and Geckeler, K. (1985) Functionalization of soluble polymers. 6. Preparation and kinetic aspects of linear, hydrophilic aldehydes based on poly(oxyethylene). *Polym. Bull.* 13, 431–434.
- (121) Vandoorne, F., Loccufier, J., and Schacht, E. (1989) Functionalization of α -hydrogen- ω -methoxypoly(oxyethylene). 1: A new method for the conversion of hydroxyl end groups into aldehyde groups. *Makromol. Chem. Rapid Commun.* 10, 271–275.
- (122) Nathan, A., Zalipsky, S., Erthel, S. I., Agathos, S. N., Yarmusch, M. L., and Kohn, J. (1993) Copolymers of lysine and polyethylene glycol: A new family of functionalized drug carriers. *Bioconjugate Chem.* 4, 54–62.
- (123) Mathis, R., Hubert, P., and Dellacherie, E. (1985) Polyethylene oxide: a ligand for mild hydrophobic interaction chromatography? *J. Chromatogr.* 347, 291–296.
- (124) Bergstrom, K., Holmberg, K., Safrani, A., Hoffman, A., Edgell, M. J., Kozlowski, A., Hovanes, B. A., and Harris, J. M. (1992) Reduction of fibrinogen adsorption on PEG-coated polystyrene. *J. Biomed. Mater. Res.* 26, 779–790.
- (125) Pitha, J., Kociolek, K., and Caron, M. G. (1979) Detergents linked to polysaccharides: Preparation and effects on membranes and cells. *Eur. J. Biochem.* 94, 11–18.
- (126) Garman, A. J., and Kalindjian, S. B. (1987) The preparation and properties of novel reversible polymer-protein conjugates. 2- ω -methoxypolyethylene (5000) glycoxymethylene-3-methylmaleyl conjugates of plasminogen activator. *FEBS Lett.* 223, 361–365.
- (127) Ueno, H., and Fujino, M. (1987) Chemically modified protein and production thereof. *Eur. Pat. Appl.* 0236987.
- (128) Kuan, C. T., Wang, Q. C., and Pastan, I. (1994) Replacement of surface exposed residues in domain II with cysteine residues that can be modified with polyethylene glycol in a site-specific manner. *J. Biol. Chem.* 269, 7610–7616.
- (129) Goodson, R. J., and Katre, N. V. (1990) Site-directed PEGylation of recombinant interleukin-2 at its glycosylation site. *Bio/Technology* 8, 343–346.
- (130) Morpurgo, M., Veronese, F. M., Kachensky, D., and Harris, J. M. (1996) Preparation and characterization of poly(ethylene glycol) vinyl sulfone. *Bioconjugate Chem.* 7, 363–368.
- (131) Atassi, M. Z. (1977) Chemical modification and cleavage of proteins and chemical strategy in immunochemical studies of proteins. In *Immunochemistry of Proteins* (M. Z. Atassi, Ed.) pp 1–161, Plenum Press, New York.
- (132) Huang, Y. H., Li, Z. M., and Morawetz, H. (1985) The kinetics of the attachment of polymer chains to reactive latex particles and the resulting latex stabilization. *J. Polym. Sci., Polym. Chem. Ed.* 23, 795–799.
- (133) Monfardini, C., Schiavon, O., Caliceti, P., Morpurgo, M., Harris, J. M., and Veronese, F. M. (1995) A branched monomethoxypoly(ethylene glycol) for protein modification. *Bioconjugate Chem.* 6, 62–69.
- (134) Matsushima, A., Sasaki, H., Koder, Y., and Inada, Y. (1992) Reduction of immunoreactivity of bovine serum albumin conjugated with polyethylene glycol in relation to its esterase activity. *Biochem. Int.* 26, 485–490.
- (135) Inada, Y., Furukawa, M., Sasaki, H., Koder, Y., Hiroto, M., Nishimura, H., and Matsushima, A. (1995) Biomedical and biotechnological applications of PEG- and PM-modified proteins. *Trends Biotechnol.* 13, 86–91.
- (136) Nathan, A., Bolikal, D., Vyavahare, N., Zalipsky, S., and Kohn, J. (1992) Hydrogels based on water-soluble poly(ether urethane) derived from L-lysine and poly(ethylene glycol). *Macromolecules* 25, 4476–4484.
- (137) Yokoyama, M., Kwon, G. S., Okano, T., Sakurai, Y., Seto, T., and Kataoka, K. (1992) Preparation of micelles-forming polymer-drug conjugates. *Bioconjugate Chem.* 3, 295–301.
- (138) Bengs, H., Bayer, U., Krone, V., Lill, N., Sandow, J., and Walch, A. (1996) Polytartrate-a new biodegradable polymer. *Proc. Int. Symp. Controlled Release Bioact. Mater.*, 23rd, 23–24.
- (139) Papisov, M., Garrido, L., Poss, K., Wright, C., Weisslender, R., and Brady, T. J. (1996) A long-circulating polymer with hydrolyzable main chain. *Proc. Int. Symp. Controlled Release Bioact. Mater.*, 23rd, 107–108.
- (140) Giammona, G., Carlisi, B., Cavallaro, G., Pitarresi, G., and Spampinato, S. (1994) A new water-soluble synthetic polymer, α,β -polyasparthydrazide, as potential plasma expander and drug carrier. *J. Controlled Release* 29, 63–72.
- (141) Pitarresi, G., Tomarchio, V., Cavallaro, G., and Giammona, G. (1996) α,β -poly(N-hydroxyethyl)-DL-asparthamide hydrogels as drug delivery devices. *J. Bioact. Compat. Polym.* 11, 328–340.
- (142) Vilaseca, L. A., Rose, K., Werlen, R., Meunier, A., Offord, R. E., Nichols, C. L., and Scott, W. L. (1993) Protein conjugates of defined structure: synthesis and use of a new carrier molecule. *Bioconjugate Chem.* 4, 515–520.
- (143) Schacht, E. H., and Hoste, K. (1997) Poly(ethylene glycol)-grafted polymers as drug carriers. In *Poly(ethylene glycol): Chemistry and Biological Applications* (J. M. Harris and S. Zalipsky, Eds.) pp 297–315, American Chemical Society, Washington, DC.
- (144) Topchieva, I. N. (1997) Covalent and noncovalent adducts of proteins with water-soluble poly(alkylene oxides). In *Poly(ethylene glycol): Chemistry and Biological Applications* (J. M. Harris and S. Zalipsky, Eds.) pp 193–206, American Chemical Society, Washington, DC.
- (145) Hoste, K., Bruneel, D., De Marre, A., De Schrijver, F., and Schacht, E. (1994) Synthesis and characterization of poly(oxyethylene) modified dextrans. *Macromol. Chem. Rapid Commun.* 15, 697–704.
- (146) De Marre, A., Hoste, K., Bruneel, D., Schacht, E., and De Schrijver, F. (1996) Synthesis, characterization, and in vitro biodegradation of poly(ethylene glycol) modified poly-[^{5} N-(2-hydroxyethyl)-L-glutamine]. *J. Bioact. Compat. Polym.* 11, 85–99.
- (147) Gayet, J. C., and Fortier, G. (1996) High water content BSA-PEG hydrogels for controlled release device: evaluation of the drug release properties. *J. Controlled Release* 38, 177–184.
- (148) Ajisaka, K., and Iwashita, Y. (1980) Modification of human hemoglobin with polyethylene glycol: a new candidate for blood substitute. *Biochem. Biophys. Res. Commun.* 97, 1076–1081.
- (149) Boccu, E., Velo, G. P., and Veronese, F. M. (1982) Pharmacokinetic properties of polyethylene glycol derivatized superoxide dismutase. *Pharmacol. Res. Commun.* 14, 113–120.

- (150) Rajagopalan, S., Gonias, S. L., and Pizzo, S. V. (1985) A nonantigenic covalent streptokinase-polyethylene glycol complex with plasminogen activator function. *J. Clin. Invest.* **75**, 413–419.
- (151) Sherwood, R. F., Baird, J. K., Atteinson, T., Wiblin, C. N., Rutter, D. A., and Ellwood, D. C. (1977) Enhanced plasma persistence of therapeutic enzymes by coupling to soluble dextran. *Biochem. J.* **164**, 461–464.
- (152) Takakura, T., Kanedo, Y., Fujita, T., Hashida, M., Maeda, H., and Sezaki, H. (1989) Control of pharmaceutical properties of soybean trypsin inhibitor by conjugation with dextran. I. Synthesis and characterization. *J. Pharm. Sci.* **78**, 117–121.
- (153) Takakura, T., Fujita, T., Hashida, M., Maeda, H., and Sezaki, H. (1989) Control of pharmaceutical properties of soybean trypsin inhibitor by conjugation with dextran. II. Biopharmaceutical and pharmacological properties. *J. Pharm. Sci.* **78**, 219–222.
- (154) Fujita, T., Nishikawa, M., Tamaki, C., Takakura, Y., Hashida, M., and Sezaki, H. (1992) Targeted delivery of human recombinant superoxide dismutase by chemical modification with mono- and polysaccharides derivatives. *J. Pharmacol. Exp. Ther.* **263**, 971–978.
- (155) Tabata, Y., Uno, K., Ikada, Y., Kishida, T., and Shigeru, M. (1993) Potentiation of in vivo antitumor effects of recombinant interleukin-1 α by gelatin conjugation. *Jpn. J. Cancer Res.* **84**, 681–688.
- (156) Shin, Y. H., Kojima, Y., Maeda, H., and Otagiri, M. (1996) Conjugation of succinylated gelatin to soybean trypsin inhibitor. *J. Bioact. Compat. Polym.* **11**, 3–16.
- (157) Remy, M. H., and Poznansky, M. J. (1978) Immunogenicity and antigenicity of soluble cross-linked enzyme/albumin polymers: advantages for enzyme therapy. *Lancet* **2**, 68–70.
- (158) Paige, A. G., Whitcomb, K. L., Liu, J., and Kinstler, O. (1995) Prolonged circulation of recombinant human granulocyte-colony stimulating factor by covalent linkage to albumin through a heterobifunctional polyethylene glycol. *Pharmacol. Res.* **12**, 1883–1888.
- (159) Nakajima, N., Ishihara, K., Sugimoto, M., Sumi, H., Mikuni, K., and Hamada, H. (1996) Chemical modification of earthworm fibrinolytic enzyme with human serum albumin fragment and characterization of the protease as a therapeutic enzyme. *Biosci., Biotechnol., Biochem.* **60**, 293–300.
- (160) Maeda, H., Takeshita, J., and Kanamaru, R. (1979) A lipophilic derivative of neocarzinostatin. A polymer conjugation of antitumor protein antibiotic. *Int. J. Pept. Protein Res.* **14**, 81–87.
- (161) Maeda, H. (1997) Pharmacological uniqueness and clinical effects of neocarzinostatin. In *Neocarzinostatin: the past, present, and future of an anticancer drug* (H. Maeda, K. Edo, and N. Ishida, Eds.) pp 205–226, Springer-Verlag, Tokyo.
- (162) Ogino, T., Inoue, M., Ando, Y., Arai, M., Maeda, H., and Morino, Y. (1988) Chemical modification of superoxide dismutase. Extension of plasma half-life of the enzyme through its reversible binding to the circulating albumin. *Int. J. Pept. Protein Res.* **32**, 153–159.
- (163) Maeda, H., Oda, T., Matsumura, Y., and Kimura, M. (1988) Improvement of pharmacological properties of protein drug by tailoring with synthetic polymers. *J. Bioact. Compat. Polym.* **3**, 27–43.
- (164) Caliceti, P., Schiavon, O., Hirano, T., Ohashi, S., and Veronese, F. M. (1996) Modification of physico-chemical and biopharmaceutical properties of superoxide dismutase by conjugation to the co-polymer of divinyl ether and maleic anhydride. *J. Controlled Release* **39**, 27–34.
- (165) Geiger, B., von Specht, B. U., and Arnon, R. (1977) Stabilization of human beta-D-N-acetylhexosaminidase A towards proteolytic inactivation by coupling it to poly(N-vinylpyrrolidone). *Eur. J. Biochem.* **73**, 141–147.
- (166) Veronese, F., Schiavon, O., and Caliceti, P. (1997) Protein delivery: PVP as new polymer for protein conjugation. *Proc. Int. Symp. Controlled Release Bioact. Mater.*, **24th**, 507–508.
- (167) Caliceti, P., Schiavon, O., Morpurgo, M., and Veronese, F. M. (1995) Physico-chemical and biological properties of monofunctional hydroxy terminating poly(N-vinylpyrrolidone) conjugated superoxide dismutase. *J. Bioact. Compat. Polym.* **10**, 103–120.
- (168) Veronese, F. M., Largajolli, R., Visco, C., Ferruti, P., and Mucci, A. (1985) Surface modification of proteins by covalent binding of acrylic polymers. *Appl. Biochem. Biotechnol.* **11**, 269–277.
- (169) Veronese, F. M., Visco, C., Massarotto, S., Benassi, C. A., and Ferruti, P. (1987) New acrylic polymers for surface modification of enzymes of therapeutic interest and for enzyme immobilization. *Ann. N.Y. Acad. Sci.* **501**, 444–448.
- (170) Brucato, F. H., and Pizzo, S. V. (1990) Catabolism of streptokinase and polyethylene glycol-streptokinase: evidence for transport of intact forms through the biliary system in the mouse. *Blood* **76**, 73–79.
- (171) Pyatak, P. S., Abuchowski, A., and Davis, F. F. (1980) Preparation of polyethylene glycol: superoxide dismutase adduct, and an examination of its blood circulatory life and anti-inflammatory activity. *Res. Commun. Chem. Path. Pharmacol.* **29**, 113–127.
- (172) Maksimenko, A. V., Petrov, A. D., Caliceti, P., Konovalova, G. G., Grigoryeva, E. L., Schiavon, O., Tischenko, E. G., Lankin, V. Z., and Veronese, F. M. (1995) Biodistribution of a polyethylene glycol-modified superoxide dismutase in mice and its effect on myocardial ischemia treatment in rats. *Drug Delivery* **2**, 39–43.
- (173) Savoca, K. V., Davis, F. F., van Es, T., McCoy, J. R., and Palczuk, N. C. (1984) Cancer therapy with chemically modified enzymes. II. The therapeutic effectiveness of arginase and arginase modified by covalent attachment of polyethylene glycol, on the Taper liver tumor and the L5178Y murine leukemia. *Cancer Biochem. Biophys.* **7**, 261–268.
- (174) Knauf, M. J., Bell, D. P., Hirtzer, P., Luo, Z. P., Young, J. D., and Katre, N. V. (1988) Relationship of effective molecular size to systemic clearance in rats of recombinant interleukine-2 chemically modified with water-soluble polymers. *J. Biol. Chem.* **263**, 15064–15070.
- (175) Katre, N. V., Knauf, M., and Thomson, J. (1987) Tumor Necrosis Factor-Formulation and preparation. EP 0247860.
- (176) Tsutsumi, Y., Nakagawa, S., and Mayumi, T. (1996) Molecular design of hybrid cytokines with water soluble polymers. *Proc. Int. Symp. Controlled Release Bioact. Mater.*, **23rd**, 871–872.
- (177) Hiratani, H. (1986) Long-Acting Composition. U.S. Patent 4,609,546.
- (178) Shadle, P. J., Koths, K. E., Moreland, M., and Katre, N. (1989) Conjugation of polymer to colony stimulating factor-1. U.S. Patent 4,847,325.
- (179) Tanaka, H., Satake-Ishikawa, R., Ishikawa, M., Matsuki, S., and Asano, K. (1991) Pharmacokinetics of recombinant human granulocyte colony stimulating factor conjugated to polyethylene glycol in rats. *Cancer Res.* **51**, 3710–3714.
- (180) Jensen-Pipito, K. E., Withcomb, K. L., DePrince, R. B., Ralph, L., and Habberfield, A. D. (1996) Enteral bioavailability of human granulocyte colony stimulating factor conjugated with poly(ethylene glycol). *Pharmacol. Res.* **13**, 102–107.
- (181) Yamasaki, M., Asano, M., Okabe, M., Morimoto, M., and Yokoo, Y. (1994) Modification of recombinant human granulocyte colony-stimulating factor (rhG-CSF) and its derivative ND 28 with polyethylene glycol. *J. Biochem.* **115**, 814–819.
- (182) Kitamura, K., Takahashi, T., Takashina, K., Yamaguchi, T., Noguchi, A., Tsurumi, H., Toyokuni, T., and Hakomori, S. (1990) Polyethylene glycol modification of the monoclonal antibody A7 enhances its tumor localization. *Biochem. Biophys. Res. Commun.* **171**, 1387–1394.
- (183) Mast, A. E., Slvensen, G., Brucato, F. H., Schnebli, H. P., and Pizzo, S. V. (1990) Polyethylene glycol modification of Serpins improves therapeutic potential. *Biol. Chem. Hoppe-Seyler* **371** (Suppl.), 101–109.
- (184) Hershfield, M. S., Buckley, R. H., Greenberg, M. L., Melton, A. L., Schiff, R., Hatem, C., Kurtzberg, J., Matkert, M. L., Kobayashi, R. H., Kobayashi, A. L., and Abuchowski, A. (1987) Treatment of adenosine deaminase deficiency with polyethylene glycol-modified adenosine deaminase. *N. Engl. J. Med.* **316**, 589–596.

- (185) Hershfield, M. S. (1997) Biochemistry and immunology of poly(ethylene glycol)-modified adenosine deaminase (PEG-ADA). In *Poly(ethylene glycol): Chemistry and Biological Applications* (J. M. Harris and S. Zalipsky, Eds.) pp 145–154, American Chemical Society, Washington, DC.
- (186) Fuertges, F., and Abuchowski, A. (1990) The clinical efficacy of poly(ethylene glycol)-modified proteins. *J. Controlled Release* 11, 139–148.
- (187) Ho, D. H., Wang, C. Y., Lin, J. R., Brown, N., Newman, R. A., and Krakoff, I. H. (1988) Polyethylene glycol-L-asparaginase and L-asparaginase studies in rabbits. *Drug Metab. Dispos.* 16, 27–29.
- (188) Park, Y. K., Abuchowski, A., Davis, S., and Davis, F. F. (1981) Pharmacology of *Escherichia coli* L-asparaginase polyethylene glycol adduct. *Anticancer Res.* 1, 373–376.
- (189) Abuchowski, A., Kazo, G. M., Verhoest, C. R., Jr., van Es, T., Kafkewitz, D., Nucci, M. L., Viau, A. T., and Davis, F. F. (1984) Cancer therapy with chemically modified enzymes I. Antitumor properties of polyethylene glycol-asparaginase conjugates. *Cancer Biochem. Biophys.* 7, 175–186.
- (190) Abuchowski, A., van Es, T., Palczuk, N. C., McCoy, J. R., and Davis, F. F. (1979) Treatment of L5178Y tumor-bearing BDF1 mice with a nonimmunogenic L-glutaminase-L-asparaginase. *Cancer Treat. Rep.* 63, 1127–1132.
- (191) Kamisaki, Y., Wada, H., Yagura, T., Nishimura, H., Matsushima, A., and Inada, Y. (1982) Increased antitumor activity of *Escherichia coli* L-asparaginase by modification with monomethoxypolyethylene glycol. *Jpn. J. Cancer Res.* 73, 470–474.
- (192) Jurgens, H., Schwamborn, D., Korholz, D., Wahn, V., and Gobel, U. (1988) Clinical experiences with polyethylene glycol-bound *E. coli* L-asparaginase in patients with multiple recurrences of acute lymphoblastic leukemia. *Klin. Padiatr.* 200, 300–306.
- (193) Veronese, F. M., Monfardini, C., Caliceti, P., Schiavon, O., Scrawen, M. D., and Beer, D. (1996) Improvement of pharmacokinetic, immunological and stability properties of asparaginase by conjugation to linear and branched monomethoxy poly(ethylene glycol). *J. Controlled Release* 40, 199–209.
- (194) Cao, S. G., Zhao, Q., Ding, Z. T., Ma, L., Yu, T., Wang, J. H., Feng, Y., and Cheng, Y. H. (1990) Chemical modification of enzyme molecules to improve their characteristics. *Ann. N.Y. Acad. Sci.* 613, 460–467.
- (195) Abuchowski, A., Davis, F. F., and Davis, S. (1981) Immunosuppressive properties and circulating life of *Achromobacter* glutaminase-asparaginase covalently attached to polyethylene glycol in man. *Cancer Treat. Rep.* 65, 1077–1081.
- (196) Lisi, P. J., van Es, T., Abuchowski, A., Palczuk, N. C., and Davis, F. F. (1982) Enzyme therapy: polyethylene glycol-glucuronidase conjugates as potential therapeutic agents in acid mucopolysaccharidosis. *J. Appl. Biochem.* 4, 19–33.
- (197) Cheng, R. H. L., Abuchowski, A., van Es, T., Palczuk, N. C., and Davis, F. F. (1981) Properties of two urate oxidases modified by the covalent attachment of polyethylene glycol. *Biochim. Biophys. Acta* 660, 293–298.
- (198) Newmark, J., Abuchowski, A., and Murano, G. (1982) Preparation and properties of adducts of streptokinase and streptokinase-plasmin complex with polyethylene glycol and pluronic polyol F38. *J. Appl. Biochem.* 4, 185–189.
- (199) Abuchowski, A., McCoy, J. R., Palczuk, N. C., van Es, T., and Davis, F. F. (1977) Effect of covalent attachment of polyethylene glycol on immunogenicity and circulating life of bovine liver catalase. *J. Biol. Chem.* 252, 3582–3586.
- (200) Savoca, K. V., Abuchowski, A., van Es, T., Davis, F. F., and Palczuk, N. C. (1979) Preparation of a non-immunogenic arginase by the covalent attachment of polyethylene glycol. *Biochim. Biophys. Acta* 578, 47–53.
- (201) Wieder, K. J., Palczuk, N. C., van Es, T., and Davis, F. F. (1979) Some properties of polyethylene glycol:phenylalanine ammonia-lyase adducts. *J. Biol. Chem.* 254, 12579–12587.
- (202) Kodera, Y., Sekine, T., Yasukohchi, T., Kiriu, Y., Hiroto, M., Matsushima, A., and Inada, Y. (1994) Stabilization of L-asparaginase modified with comb-shaped poly(ethylene glycol) derivatives, in vivo and in vitro. *Bioconjugate Chem.* 5, 283–286.
- (203) Sasaki, H., Ohtake, Y., Matsushima, A., Hiroto, M., Kodera, Y., and Inada, Y. (1993) Reduction of immunoreactivity of bovine serum albumin conjugated with comb-shaped polyethylene glycol derivatives. *Biochem. Biophys. Res. Commun.* 197, 287–291.
- (204) Boccu, E., Velo, G. P., and Veronese, F. M. (1982) Pharmacokinetic properties of polyethylene glycol derivatized superoxide dismutase. *Pharmacol. Res. Commun.* 14, 113–120.
- (205) Somack, R., Saifer, M. G. P., and Williams, L. D. (1991) Preparation of long-acting superoxide dismutase using high molecular weight polyethylene glycol (41,000–72,000 Daltons). *Free Radical Res. Commun.* 12–13, 553–562.
- (206) Saifer, M. G., Somack, R., and Williams, L. D. (1994) Plasma clearance and immunologic properties of long-acting superoxide dismutase prepared using 35,000 to 120,000 dalton poly-ethylene glycol. *Adv. Exp. Med. Biol.* 366, 377–387.
- (207) Nakaoka, R., Tabata, Y., Yamaoka, T., and Ikada, Y. (1997) Prolongation of the serum half-life period of superoxide dismutase by poly(ethylene glycol) modification. *J. Controlled Release* 46, 253–261.
- (208) Veronese, F. M., Caliceti, P., and Schiavon, O. (1997) Branched and linear poly(ethylene glycol): influence of the polymer structure on enzymological, pharmacokinetic, and immunological properties of protein conjugates. *J. Bioact. Compat. Polym.* 12, 196–207.
- (209) Sherman, M. R., Williams, L. D., Saifer, M. G. P., French, J. A., Kwak, L. W., and Oppenheim, J. J. (1997) Conjugation of high-molecular weight poly(ethylene glycol) to cytokines: granulocyte-macrophage colony-stimulating factors as model substrates. In *Poly(ethylene glycol): Chemistry and Biological Applications* (J. M. Harris and S. Zalipsky, Eds.) pp 155–169, American Chemical Society, Washington, DC.
- (210) Wada, H., Himamura, I., Sako, M., Katagiri, M., Tarui, S., Nishimura, H., and Inada, Y. (1990) Antitumor enzymes: polyethylene glycol-modified asparaginase. *Ann. N.Y. Acad. Sci.* 613, 95–108.
- (211) Koide, A., and Kobayashi, S. (1983) Modification of amino groups in porcine pancreatic elastase with polyethylene glycol in relation to binding activity towards anti-serum and to enzymatic activity. *Biochem. Biophys. Res. Commun.* 111, 659–667.
- (212) Abuchowski, A., and Davis, F. F. (1979) Preparation and properties of polyethylene glycol-trypsin adducts. *Biochim. Biophys. Acta* 578, 41–46.
- (213) Caliceti, P., Morpurgo, M., Schiavon, O., Monfardini, C., and Veronese, F. M. (1994) Preservation of thrombolytic activity of urokinase modified with monomethoxypoly(ethylene glycol). *J. Bioact. Compat. Polym.* 9, 252–266.
- (214) Caliceti, P., Schiavon, O., Sartore, L., Monfardini, C., and Veronese, F. M. (1993) Active site protection of proteolytic enzymes by poly(ethylene glycol) surface modification. *J. Bioact. Compat. Polym.* 8, 41–50.
- (215) Larionova, N. I., Gladysheva, I. P., Polekhina, O. V., and Garbatova, E. N. (1997) An ethylene oxide-propylene oxide block copolymer/Bowman-Birk proteinase inhibitor conjugate. *Proc. Int. Symp. Controlled Release. Bioact. Mater.*, 24th, 491–492.
- (216) Noda, J., Otagiri, M., Akaike, T., and Maeda, H. (1996) Pharmacological advantages of conjugation of superoxide dismutase with succinylated keratin fragment. *Proc. Int. Symp. Controlled Release Bioact. Mater.*, 23rd, 867–868.
- (217) Ehrlich, P. (1956) A general review of the recent work in immunity. In *Collected papers of Paul Ehrlich, volume 2, Immunology and cancer research*, pp 442–447, Pergamon Press, London.
- (218) Bernstein, A., Huewitz, E., Maron, R., Arnon, R., Sela, M., and Wilchek, M. (1978) Higher antitumor efficacy of daunomycin when linked to dextran: In vivo and in vitro studies. *J. Natl. Cancer Inst.* 60, 379–384.
- (219) Fujita, H., Okamoto, M., and Takao, A. (1991) Pharmacokinetics of adriamycin-oxidized dextran conjugate (ADM-

- OXD) in experimental animals (in Japanese). *Drug Delivery Syst.* 6, 133–138.
- (220) Danhauser-Riedl, S., Hausmann, E., Schick, H. D., Bender, R., Dietzfelbinger, H., Rastetter, J., and Hanauske, A. R. (1993) Phase I clinical and pharmacokinetic trial of dextran conjugated doxorubicin (AD-70, DOX-OXD). *Invest. New Drugs* 11, 187–195.
- (221) Takakura, T., Kitajima, M., Matsumoto, S., Hashida, M., and Sezaki, H. (1987) Development of a novel polymeric prodrug of mitomycin C, mitomycin C-dextran conjugate with anionic charge. I. Physicochemical characteristics and in vivo and in vitro antitumor activities. *Int. J. Pharm.* 37, 135–143.
- (222) Coessens, V., Schacht, E. H., and Domurado, D. (1997) Synthesis and in vitro stability of macromolecular prodrugs of norfloxacin. *J. Controlled Release* 47, 283–291.
- (223) Kellaway, I. W., Seale, L., and Spencer, P. S. J. (1995) The in vitro characterization and biostability of ^{99m}Tc -dextran and its accumulation with the inflamed paws of adjuvant-induced arthritic rats. *Pharmacol. Res.* 12, 588–593.
- (224) Duncan, R., Kopeckova-Rejmanova, P., Strohal, J., Hume, I. C., Lloyd, J. B., and Kopecek, J. (1988) Anticancer agents coupled to N-(2-hydroxypropyl)methacrylamide copolymers. 2. Evaluation of daunomycin conjugates in vivo against L1210 leukaemia. *Br. J. Cancer* 57, 147–156.
- (225) Duncan, R., Seymour, L. W., O'Hare, K. B., Flanagan, P. A., Wedge, S., Ulbrich, K., Strohal, J., Subr, V., Spreafico, F., Grandi, M., Ripamonti, M., Farao, M., and Suarato, A. (1992) Preclinical evaluation of polymer-bound doxorubicin. *J. Controlled Release* 18, 123–132.
- (226) Duncan, R., Hume, I. C., Kopeckova, P., Ulbrich, K., Strohal, J., and Kopecek, J. (1989) Anticancer agents coupled to N-(2-hydroxypropyl)methacrylamide copolymers. 3. Evaluation of adriamycin conjugates against mouse leukaemia L1210 in vivo. *J. Controlled Release* 10, 51–63.
- (227) O'Hare, K. B., Duncan, R., Strohal, J., Ulbrich, K., and Kopeckova, P. (1993) Polymeric drug carriers containing doxorubicin and melanocyte-stimulating hormone: in vitro and in vivo evaluation against murine melanoma. *J. Drug Targeting* 1, 217–229.
- (228) Duncan, R., Hume, I. C., Yardley, H. J., Flanagan, P. A., Ulbrich, K., Subr, V., and Strohal, J. (1991) Macromolecular prodrugs for use in targeted cancer chemotherapy: melphalan covalently coupled to N-(2-hydroxypropyl)methacrylamide copolymers. *J. Controlled Release* 16, 121–136.
- (229) Ulbrich, K., Zacharieva, E., Kopecek, J., Hume, I. C., and Duncan, R. (1987) Polymer bound derivatives of sarcosyl and their antitumor activity against mouse and human leukaemia in vitro. *Makromol. Chem.* 188, 2497–2509.
- (230) Krinick, N. L., Rihova, B., Ulbrich, K., Strohal, J., and Kopecek, J. (1990) Targetable photoactive drugs 2. Synthesis of N-(2-hydroxypropyl)methacrylamide copolymer-anti-Thy-1.2 antibody-chlorin e6 conjugates and a preliminary study of their photodynamic effect on mouse splenocytes in vitro. *Makromol. Chem.* 191, 839–856.
- (231) Seymour, L. W., Flanagan, P. A., Al-Shamkhani, A., Subr, V., Ulbrich, K., Cassidy, J. A., and Duncan, R. (1991) Synthetic polymers conjugated to monoclonal antibodies: Vehicles for tumor-targeted drug delivery. *Sel. Cancer Ther.* 7, 59–73.
- (232) Duncan, R., Seymour, L. W., Scarlett, L., Lloyd, J. B., Rejmanova, P., and Kopecek, J. (1986) N-(2-Hydroxypropyl)-methacrylamide copolymers with pendant galactosamine residues. Fate after intravenous administration to rats. *Biochim. Biophys. Acta* 880, 62–71.
- (233) Seymour, L. W., Ulbrich, K., Strohal, J., Kopecek, J., and Duncan, R. (1990) Pharmacokinetics of polymer-bound adriamycin. *Biochem. Pharmacol.* 39, 1125–1131.
- (234) Merigan, T. C., and Regelson, W. (1967) Interferon induction in man by a synthetic polyanion of defined composition. *N. Engl. J. Med.* 277, 1283–1287.
- (235) Zalipski, S., Gilon, C., and Zilkha, A. (1983) Attachment of drugs to polyethylene glycols. *Eur. Polym. J.* 19, 1177–1183.
- (236) Caliceti, P., Monfardini, C., Sartore, L., Schiavon, O., Baccichetti, F., Carllassare, F., and Veronese, F. M. (1993) Preparation and properties of monomethoxypoly(ethylene glycol) doxorubicin conjugates linked by an amino acid or peptide as spacer. *Farmaco* 48, 919–932.
- (237) Ouchi, T., Yuyama, H., and Vogl, O. (1987) Synthesis of 5-fluorouracil-terminated monomethoxypoly(ethylene glycol)s, their hydrolysis behaviour, and their antitumor activities. *J. Macromol. Sci. Chem.* A24, 1011–1032.
- (238) Greenwald, R. B., Gilbert, C. W., Pendri, A., Conover, C. D., Xia, J., and Martinez, A. (1996) Drug-delivery systems: water soluble taxol 2'-poly(ethylene glycol) ester prodrugs-Design and in vivo effectiveness. *J. Med. Chem.* 39, 424–431.
- (239) Greenwald, R. B., Pendri, A., Conover, C., Gilbert, C., Yang, R., and Xia, J. (1996) Drug delivery systems. 2. Camptothecin 20-O-poly(ethylene glycol) ester transport forms. *J. Med. Chem.* 39, 1938–1940.
- (240) Poiani, G. J., Riley, D. J., Fox, J. D., Kemnitzer, J. E., Gean, K. F., and Kohn, J. (1994) Conjugates of cis-4-hydroxy-L-proline and poly(PEG-Lys), a water soluble poly(ether urethane): synthesis and evaluation of antifibrotic effects in vitro and in vivo. *Bioconjugate Chem.* 5, 621–630.
- (241) Kwon, G., Suwa, S., Yokoyama, M., Okano, T., Sakurai, Y., and Kataoka, K. (1994) Enhanced tumor accumulation and prolonged circulation times of micelle-forming poly(ethylene oxide-aspartate) block copolymer-adriamycin conjugates. *J. Controlled Release* 29, 17–23.
- (242) Bogadnov, A. A., Jr., Callahan, R. J., Qwilkinson, R. A., Martin, C., Camero, J. A., Fischman, A. J., Brady, T. J., and Weissleder, R. (1994) Synthetic copolymer kit for radionuclide blood-pool imaging. *J. Nucl. Med.* 35, 1880–1886.
- (243) Fujii, K., Imai, T., and Otagiri, M. (1996) Control of pharmacokinetics and nephrotoxicity of cis-DDP by alginate. *Proc. Int. Symp. Controlled Release Bioact. Mater.*, 23rd, 5018–5019.
- (244) Kato, Y., Liu, K., Nakamura, T., and Sugiyama, Y. (1996) Pharmacokinetic analysis of heparin-hepatocyte growth factor complex. *Proc. Int. Symp. Controlled Release Bioact. Mater.*, 23rd, 647–648.
- (245) Zunino, F., Pratesi, G., and Pezzoni, G. (1987) Increased therapeutic efficacy and reduced toxicity of doxorubicin linked to pyran copolymer via the side-chain of the drug. *Cancer Treat. Rep.* 71, 367–373.
- (246) Bogliolo, G., Muzzolini, C., Lerza, R., and Pannacchiulli, I. (1986) Activity of doxorubicin linked to poly-L-aspartic acid on normal murine hematopoietic progenitor cells. *Cancer Treat. Rep.* 70, 1275–1281.
- (247) Hrdina, R., Bogusova, T. A., Kunova, A., and Kvetina, J. (1991) Changes in the toxicity and therapeutic efficacy of daunorubicin linked with a biodegradable carrier. *Neoplasma* 38, 265–273.
- (248) Trouet, A., Masquelier, M., Baurain, R., and Campeneere, D. D. (1982) A covalent linkage between daunorubicin and proteins that is stable in serum and reversible by lysosomal hydrolases, as required for a lysosomotropic drug-carrier conjugate: in vitro and in vivo studies. *Proc. Natl. Acad. Sci. U.S.A.* 79, 626–629.
- (249) Decher, G., Emmelius, M., and Ringsdorf, H. (1984) Synthesis and antitumor activity of daunorubicin-containing polymers. In *26th Microsymposium on Polymers in Medicine and Biology*, Prague, Czechoslovakia, pp 40–41.
- (250) Zunino, F., Savi, G., Giuliani, F., Gambetta, R., Supino, R., Tinelli, S., and Pezzoni, G. (1984) Comparison of antitumor effects of daunorubicin linked covalently to poly-L-amino acid carriers. *Eur. J. Cancer Clin. Oncol.* 20, 421–425.
- (251) Kery, V., Novotny, L., Tihlarik, K., Haplova, J., Kacurakova, M., Sandula, J., and Balazova, E. (1990) Preparation properties and antileukaemic activity of arabinosylcytosine polysaccharide conjugates. *Int. J. Biochem.* 22, 1203–1207.
- (252) Ouchi, T., Fujino, A., Tanaka, K., and Banba, T. (1990) Synthesis and antitumor activity of conjugates of poly(α -malic acid) and 5-fluorouracils bound via ester, amide and carbamoyl bonds. *J. Controlled Release* 12, 143–153.
- (253) Chu, B. C. F., and Howell, S. B. (1981) Pharmacological and therapeutic properties of carrier bound methotrexate against tumor confined to a third space body compartment. *J. Pharmacol. Exp. Ther.* 219, 389–393.

- (254) Horpel, G., Klesse, W., and Ringsford, H. (1982) Micelle forming co- and block polymers for sustained drug release. *IUPAC Proceedings of the International Symposium on Macromolecules*, Amherst, MA, p 346.
- (255) Hirano, T., Ringsford, H., and Zaharko, D. (1980) Antitumor activity of monomeric and polymeric cyclophosphamide derivative compound with in vitro hydrolysis. *Cancer Res.* 40, 2263–2267.
- (256) Wu, G. Y., and Wu, C. H. (1988) Receptor-mediated gene delivery and expression *in vivo*. *J. Biol. Chem.* 263, 14621–14624.
- (257) Maruyama, A., Katoh, M., Ishihara, T., and Akaike, T. (1997) Comb-type polycations effectively stabilize DNA triplex. *Bioconjugate Chem.* 8, 3–6.
- (258) Bangham, A. D., and Horne, R. W. (1964) Negative staining of phospholipids and their structural modification by surface-active agents as observed in the electron microscope. *J. Mol. Biol.* 8, 660–668.
- (259) Huang, C. (1969) Studies of phosphatidylcholine vesicles. Formation and physical characteristics. *Biochemistry* 8, 344–352.
- (260) Barenholtz, Y., Gibbes, D., Litman, B. J., Goll, J., Thompson, T. E., and Carlson, F. D. (1977) A simple method for the preparation of homogeneous phospholipid vesicles. *Biochemistry* 16, 2806–2810.
- (261) Batzri, S., and Korn, E. D. (1975) Interaction of phospholipid vesicles with cells. Endocytosis and fusion as alternative mechanisms for the uptake of lipid-soluble and water-soluble molecules. *J. Cell. Biol.* 66, 621–634.
- (262) Rolland, A., Ed. (1993) *Pharmaceutical Particulate Carriers: Therapeutic Applications*, Marcel Dekker, New York.
- (263) Gregoriadis, G., Ed. (1992) *Liposome Technology*, CRC Press, Boca Raton, FL.
- (264) Lopez-Berestein, G., and Fidler, I. J., Eds. (1989) *Liposomes: the therapy of infectious diseases and cancer*, Alan R. Liss, New York.
- (265) Roerdink, F. H., and Kroon, A. M., Eds. (1989) *Drug Carrier Systems*, Wiley & Sons, New York.
- (266) Gregoriadis, G., Ed. (1988) *Liposomes as Drug Carriers: Recent Trends and Progress*, Wiley & Sons, New York.
- (267) Machy, P., and Leserman, L., Eds. (1987) *Liposomes in Cell Biology and Pharmacology*, Wiley & Sons, London.
- (268) Ostro, M. J., Ed. (1987) *Liposomes from Biophysics to Therapeutics*, Dekker, New York.
- (269) Yagi, K., Ed. (1986) *Medical Applications of Liposomes*, Japan Scientific Society Press, Tokyo.
- (270) Gregoriadis, G., and Neerunjun, E. D. (1974) Control of the rate of hepatic uptake and catabolism of liposome-entrapped proteins injected into rat: possible therapeutic applications. *Eur. J. Biochem.* 47, 179–185.
- (271) Scherphof, G. L., Dijkstra, J., Spanjer, H. H., Derksen, J. K., and Roerdink, T. (1985) Uptake and intracellular processing of targeted and nontargeted liposomes by rat Kupffer cells in vivo and in vitro. *Ann. N.Y. Acad. Sci.* 446, 368–384.
- (272) Alving, C. R., Steck, E. A., Chapman, W. L., Jr., Waits, V. B., Hendricks, L. D., Schwartz, G. M., Jr., and Hanson, W. L. (1978) Therapy of leishmaniasis: superior efficacies of liposome-encapsulated drugs. *Proc. Natl. Acad. Sci. U.S.A.* 75, 2959–2963.
- (273) Sone, S., Utsugi, T., Tandon, P., and Ogawara, M. A. (1986) A dried preparation of liposomes containing muramyl tripeptide phosphatidylethanolamine as a potent activator of human blood monocytes to the anticancer state. *Cancer Immunol. Immunother.* 22, 191–196.
- (274) Pak, R. R., and Fidler, I. J., (1991) Liposomal delivery of biological response modifiers to macrophages. *Biotherapy* 3, 55–64.
- (275) Hoekstra, D., and Scherphof, G. (1979) Effect of fetal calf serum protein fractions on the uptake of liposomal phosphatidylcholine by rat hepatocytes in primary monolayer. *Biochim. Biophys. Acta* 551, 109–121.
- (276) Rahman, A., More, N., and Schein, P. S. (1982) Doxorubicin-induced chronic cardiotoxicity and its protection by liposomal administration. *Cancer Res.* 42, 1817–1825.
- (277) Van Hoesel, Q. G. C. M., Steerenberg, P. A., Crommelin, D. J. A., Van Dijk, A., Van Oort, W., Klein, S., Douze, J. M. C., De Wildt, D. J., and Hillen, F. C. (1984) Reduced cardiotoxicity and nephrotoxicity with preservation of antitumor activity of doxorubicin entrapped in stable liposomes in the LOU/M Ws1 rat. *Cancer Res.* 44, 3698–3705.
- (278) Lopez-Berestein, G. (1988) Liposomal amphotericin B in antimicrobial therapy. In *Liposomes as Drug Carriers* (G. Gregoriadis, Ed.), pp 345–352, Wiley & Sons, New York.
- (279) Lopez-Berestein, G., Fainstein, V., Hopfer, R., Mehta, K., Sullivan, M. P., Keating, M., Luna, M., Hersh, E. M., Reuben, J., Juliano, R. L., and Bodey, G. P. (1985) Liposomal amphotericin for the treatment of systemic fungal infections in patients with cancer. A preliminary study. *J. Infect. Dis.* 151, 704–710.
- (280) Lopez-Berestein, G., Bodey, G. P., Frankel, L. S., and Mehta, K. (1987) Treatment of hepato-splenic fungal infections with liposomal amphotericin B. *J. Clin. Oncol.* 5, 310–317.
- (281) Vasandani, V. M., Madan, S., and Ghosh, P. C. (1992) In vivo potentiation of ricin toxicity by monensin delivered through liposomes. *Biochim. Biophys. Acta* 1116, 315–323.
- (282) Burke, T. G., and Gao, X. (1994) Stabilization of topotecan in low pH liposomes composed of distearylphosphatidylcholine. *J. Pharm. Sci.* 83, 967–968.
- (283) Chow, D. S. L., Chen, G., Gong, L., and Giovannella, B. C. (1997) Liposomal camptothecin and 9-nitro-camptothecin: formulation, pharmacokinetics and preclinical anti-tumor activity. *Proc. Int. Symp. Controlled Release Bioact. Mater.*, 24th, 919–920.
- (284) Barzago, M. M., Bortolotti, A., Stellari, F. F., Diomede, L., Algeri, M., Efrati, S., Salmona, M., and Bonati, M. (1996) Placental transfer of valproic acid after liposome encapsulation during *in vitro* human placenta perfusion. *J. Pharmacol. Exp. Ther.* 277, 79–86.
- (285) Arien, A., Goigoux, C., Baquey, C., and Dupuy, B. (1993) Study of in vivo stability of oral liposomes loaded with calcitonin or indium in the gastrointestinal tract. *Life Sci.* 53, 1279–1290.
- (286) Arrowsmith, M., Hadgraft, J., and Kellaway, I. W. (1984) The in vivo release of cortisone esters from liposomes and the intramuscular clearance of liposomes. *Int. J. Pharm.* 20, 347–362.
- (287) Allen, T. M., Hansen, C. B., and Guo, L. S. S. (1993) Subcutaneous administration of liposomes: a comparison with the intravenous and intraperitoneal routes of injection. *Biochim. Biophys. Acta* 1150, 9–16.
- (288) Lelkes, P. I., and Friedmann, P. (1984) Stabilization of large multilamellar liposomes by human serum in vitro. *Biochim. Biophys. Acta* 775, 395–401.
- (289) Senior, J. (1987) Fate and behaviour of liposomes *in vivo*: a review of controlling factors. *Crit. Rev. Ther. Drug Carrier Syst.* 3, 123–193.
- (290) Allen, T. M. (1981) A study of phospholipids interactions between high-density lipoproteins and small unilamellar vesicles. *Biochim. Biophys. Acta* 640, 385–397.
- (291) Senior, J., Gregoriadis, G., and Mitropoulos, K. (1983) Stability and clearance of small unilamellar liposomes: studies with normal and lipoprotein deficient mice. *Biochim. Biophys. Acta* 760, 111–118.
- (292) Patel, H. M., Tuzel, N. S., and Ryman, B. E. (1983) Inhibitory effect of cholesterol on the uptake of liposomes by liver and spleen. *Biochim. Biophys. Acta* 761, 142–151.
- (293) Damen, J., Regts, J., and Scherphof, G. L. (1982) Transfer of [¹⁴C]phosphatidylcholine between liposomes and human plasma high-density lipoproteins: partial purification of a transfer stimulating factor using a rapid transfer assay. *Biochim. Biophys. Acta* 712, 444–452.
- (294) Damen, J., Dijkstra, J., Regts, J., and Scherphof, G. L. (1980) Effect of lipoprotein-free plasma on the interaction of human plasma high-density lipoprotein with egg yolk phosphatidylcholine liposomes. *Biochim. Biophys. Acta* 620, 90–99.
- (295) Shahrokh, Z., and Nichols, A. V. (1982) Particle size interconversion of human low-density lipoproteins during incubation of plasma with phosphatidylcholine vesicles. *Biochem. Biophys. Res. Commun.* 108, 888–895.

- (296) Comiskey, S. J., and Heath, T. D. (1990) Serum-induced leakage of negatively charged liposomes at nanomolar lipid concentration. *Biochemistry* 29, 3626–3631.
- (297) Damen, J., Regts, J., and Scherphof, G. L. (1981) Transfer and exchange of phospholipid between small unilamellar liposomes and rat plasma high-density lipoproteins: dependence on cholesterol and phospholipid composition. *Biochim. Biophys. Acta* 665, 538–545.
- (298) Hunt, C. A. (1982) Liposomes disposition *in vivo* V. Liposomes stability in plasma and implications for drug carrier function. *Biochim. Biophys. Acta* 719, 450–463.
- (299) Senior, J., and Gregoriadis, G. (1982) Is half-life of circulating liposomes determined by changes in their permeability? *FEBS Lett.* 145, 109–114.
- (300) Bali, A., Dhawan, S., and Gupta, C. M. (1983) Stability of liposomes in circulation is markedly enhanced by structural modification of their phospholipid component. *FEBS Lett.* 154, 373–377.
- (301) Agarwal, K., Bali, A., and Gupta, C. M. (1986) Influence of the phospholipid structure in the stability of liposomes in serum. *Biochim. Biophys. Acta* 856, 36–40.
- (302) Patel, K. R., Li, M. P., Schuh, J. R., and Baldeschwielder, J. D. (1985) Modification of vesicles surface with amphiphilic sterols. Effect on permeability and *in vivo* tissue distribution. *Biochim. Biophys. Acta* 814, 256–264.
- (303) Oku, N., Namba, Y., and Okada, S. (1992) Tumor accumulation of novel RES-avoiding liposomes. *Biochim. Biophys. Acta* 1126, 255–260.
- (304) Kao, Y. J., and Juliano, R. L. (1981) Interactions of liposomes with the reticuloendothelial system: effects of reticuloendothelial blockade on the clearance of large unilamellar vesicles. *Biochim. Biophys. Acta* 677, 453–461.
- (305) Lopez-Berenstein, G., Kasi, L., Rosenblum, M. G., Haynie, T., Jahns, M., Glenn, H., Mehta, R., Mavligit, G. M., and Hersh, E. M. (1984) Clinical pharmacology of ^{99m}Tc -labeled liposomes in patients with cancer. *Cancer Res.* 44, 375–378.
- (306) Poste, G., Bucana, C., Raz, A., Bugelski, P., Kiesh, R., and Fidler, I. J. (1982) Analysis of the fate of systemically administered liposomes and implications for their use in drug delivery. *Cancer Res.* 42, 1412–1422.
- (307) Spanier, H. H., Van Galen, M., Roerdink, F. H., Regts, J., and Scherphof, G. L. (1986) Intrahepatic distribution of small unilamellar liposomes as a function of liposomal lipid composition. *Biochim. Biophys. Acta* 863, 224–230.
- (308) Patel, H. M., Tuzel, N. S., and Ryman, B. E. (1983) Inhibitory effect of cholesterol on the uptake of liposomes by liver and spleen. *Biochim. Biophys. Acta* 761, 142–151.
- (309) Senior, J., Crawley, J. C. W., and Gregoriadis, G. (1985) Tissue distribution of liposomes exhibiting long half-lives in the circulation after intravenous injection. *Biochim. Biophys. Acta* 839, 1–8.
- (310) Nishikawa, K., Arai, H., and Inoue, K. (1990) Scavenger receptor-mediated uptake and metabolism of lipid vesicles containing acidic phospholipids by mouse peritoneal macrophages. *J. Biol. Chem.* 265, 5226–5231.
- (311) Tanaka, Y., and Schroit, A. J. (1983) Insertion of fluorescent phosphatidylserine into the plasma membrane of red blood cells: recognition by autologous macrophages. *J. Biol. Chem.* 258, 11335–11343.
- (312) Abra, R. M., Hunt, C. A., and Lau, D. T. (1984) Liposome disposition *in vivo* VI: delivery to the lung. *J. Pharm. Sci.* 73, 203–206.
- (313) Funato, K., Yoda, R., and Kiwada, H. (1992) Contribution of complement system on destabilization of liposomes composed of hydrogenated egg phosphatidylcholine in rat fresh plasma. *Biochim. Biophys. Acta* 1103, 198–204.
- (314) Chonn, A., Semple, S. C., and Cullis, P. R. (1992) Association of blood proteins with large unilamellar liposomes *in vivo*. *J. Biol. Chem.* 267, 18759–18765.
- (315) Senior, J. H., Trimble, K. R., and Maskiewicz, R. (1991) Interaction of positively charged liposomes with blood: implications for their application *in vivo*. *Biochim. Biophys. Acta* 1070, 173–179.
- (316) Chow, D. D., Essien, H. E., Padki, M. M., and Hwang, K. J. (1989) Targeting small unilamellar liposomes to hepatic parenchymal cells by dose effect. *J. Pharm. Exp. Ther.* 248, 506–513.
- (317) Ogihara-Umeda, I., and Kojima, S. (1988) Increased delivery of gallium-67 to tumors using serum-stable liposomes. *J. Nucl. Med.* 29, 516–523.
- (318) Goto, M., Arakawa, M., Sato, T., Kondo, H., and Sunamoto, J. (1987) Specific rejection of glycophorin-reconstituted liposomes by human phagocytes. *Chem. Lett.* 2, 1935–1938.
- (319) Allen, T. M., and Chonn, A. (1987) Large unilamellar liposomes with low uptake into the reticuloendothelial system. *FEBS Lett.* 223, 42–46.
- (320) Gabizon, A., and Paphadjopoulos, D. (1988) Liposome formulations with prolonged circulation time in blood and enhanced uptake by tumors. *Proc. Natl. Acad. Sci. U.S.A.* 85, 6949–6953.
- (321) Allen, T. M., Hansen, C., and Rutledge, J. (1989) Liposomes with prolonged circulation times: factors affecting uptake by reticuloendothelial and other tissues. *Biochim. Biophys. Acta* 981, 27–35.
- (322) Blume, G., and Cevc, G. (1990) Liposomes for the sustained drug release *in vivo*. *Biochim. Biophys. Acta* 1029, 91–97.
- (323) Allen, T. M., Hansen, G., Martin, F., Redeman, C., and Yau-Young, A. (1991) Liposomes containing synthetic lipid derivatives of poly(ethyleneglycol) show prolonged circulation half-lives *in vivo*. *Biochim. Biophys. Acta* 1066, 29–36.
- (324) Liu, D., Mori, A., and Huang, L. (1992) Role of liposome size and RES blockade in controlling biodistribution and tumor uptake of GM1-containing liposomes. *Biochim. Biophys. Acta* 1104, 95–101.
- (325) Yamauchi, H., Yano, T., Kato, T., Tanaka, I., Nakabayashi, S., Higashi, K., Miyoshi, S., and Yamada, H. (1994) Effects of sialic acid derivative on long circulating time and tumor concentration of liposomes. *Int. J. Pharmacol.* 113, 141–148.
- (326) Tilcock, C., Ahkong, Q. F., and Fisher, D. (1993) Polymer-derivatized technetium ^{99m}Tc -labeled liposomal blood pool agents for nuclear medicine applications. *Biochim. Biophys. Acta* 1148, 77–84.
- (327) Yamauchi, H., Kikuchi, H., Yachi, K., Sawada, M., Tomikawa, M., and Hirota, S. (1993) Effects of glycophorin and ganglioside GM3 on the blood circulation and tissue distribution of liposomes in rats. *Int. J. Pharmacol.* 90, 73–79.
- (328) Allen, T. M., Austin, G. A., Chonn, A., Lin, L., and Lee, K. C. (1991) Uptake of liposomes by cultured mouse bone marrow macrophages: influence of liposome composition and size. *Biochim. Biophys. Acta* 1061, 56–64.
- (329) Park, Y. S., Maruyama, K., and Huang, L. (1992) Some negatively charged phospholipid derivatives prolong the liposome circulation *in vivo*. *Biochim. Biophys. Acta* 1108, 257–260.
- (330) Namba, Y., Sakakibara, T., Masada, M., Ito, F., and Oku, N. (1990) Glucuronate-modified liposomes with prolonged circulation time. *Chem. Pharm. Bull.* 38, 1663–1666.
- (331) Woodle, M. C., Storm, G., Newman, M. S., Jekot, J. J., Collins, L. R., Martin, F. J., and Szoka, F. C., Jr. (1992) Prolonged systemic delivery of peptide drugs by long-circulating liposomes: illustration with vasopressin in the Brattleboro rat. *Pharmacol. Res.* 9, 260–265.
- (332) Namba, Y., Oku, N., Ito, F., Sakakibara, T., and Okada, S. (1992) Liposomal modification with uronate, which endows liposomes with long circulation *in vivo*, reduces the uptake of liposomes by J774 cells *in vitro*. *Life Sci.* 50, 1773–1779.
- (333) Juliano, R. L., Hsu, M. J., Regen, S. L., and Singh, M. (1984) Photopolymerized phospholipid vesicles: stability and retention of hydrophilic and hydrophobic marker substances. *Biochim. Biophys. Acta* 770, 109–114.
- (334) Juliano, R. L., Hsu, M. J., and Regen, S. L. (1985) Interaction of polymerized phospholipid vesicles with cells: uptake, processing and toxicity in macrophages. *Biochim. Biophys. Acta* 812, 42–48.

- (335) Needham, D., McIntosh, T. J., and Lasic, D. D. (1992) Repulsive interactions and mechanical stability of polymer-grafted lipid membranes. *Biochim. Biophys. Acta* 1108, 40–48.
- (336) Hoop, B., and Torchilin, V. P. (1996) Theoretical considerations on self-similarity of the polyethylene glycol-coated liposomal surface. *Proc. Int. Symp. Controlled Release Bioact. Mater.*, 23rd, 785–786.
- (337) Klivanov, A. L., Maruyama, K., Beckerleg, A. M., Torchilin, V. P., and Huang, L. (1991) Activity of amphipathic poly(ethylene glycol) 5,000 to prolong the circulation time of liposomes depends on the liposome size and is unfavorable for immunoliposomes binding to target. *Biochim. Biophys. Acta* 1062, 142–148.
- (338) Loughrey, H. C., Ferrarretto, A., Cannon, A. M., Acerbis, G., Sudati, F., Bottiroli, G., Masserini, M., and Soria, M. R. (1993) Characterization of biotinylated liposomes for *in vivo* targeting applications. *FEBS Lett.* 332, 183–188.
- (339) Torchilin, V. P., Omelyanenko, V. G., Papisov, M. I., Bogdanov, A. A., Trubetskoy, V. S., Herron, J. N., and Gentry, C. A. (1994) Poly(ethylene glycol) on the liposome surface: on the mechanism of polymer-coated liposomes longevity. *Biochim. Biophys. Acta* 1195, 11–20.
- (340) Blume, G., and Cevc, G. (1993) Molecular mechanism of the lipid vesicle longevity *in vivo*. *Biochim. Biophys. Acta* 1146, 157–168.
- (341) Parr, M. J., Ansell, S. M., Lewis, S. C., and Cullis, P. R. (1994) Factors influencing the retention and chemical stability of poly(ethylene glycol)-lipid conjugates incorporated into large unilamellar vesicles. *Biochim. Biophys. Acta* 1195, 21–30.
- (342) Maruyama, K., Yuda, T., Okamoto, A., Kojima, S., Suginata, A., and Iwatsuru, M. (1992) Prolonged circulation time *in vivo* of large unilamellar liposomes composed of distearoylphosphatidylcholine and cholesterol containing amphipathic poly(ethylene glycol). *Biochim. Biophys. Acta* 1128, 44–49.
- (343) Litzinger, D. C., Buiting, A. M. J., van Rooijen, N., and Huang, L. (1994) Effect of liposome size on the circulation time and intraorgan distribution of amphipathic poly(ethylene glycol)-containing liposomes. *Biochim. Biophys. Acta* 1190, 99–107.
- (344) Yuda, T., Maruyama, K., and Iwatsuru, M. (1996) Prolongation of liposome circulation time by various derivatives of polyethylene glycols. *Biol. Pharm. Bull.* 19, 1347–1351.
- (345) Torchilin, V. P., Trubetskoy, V. S., Whiteman, K. R., Caliceti, P., Ferruti, P., and Veronese, F. M. (1995) New synthetic amphiphilic polymers for steric protection of liposomes *in vivo*. *J. Pharm. Sci.* 84, 1049–1053.
- (346) Takeuchi, H., Toyoda, T., Toyobuku, H., Yamamoto, H., Hino, T., and Kawashima, Y. (1996) Improved stability of doxorubicin-loaded liposomes by polymer coating. *Proc. Int. Symp. Controlled Release Bioact. Mater.*, 23rd, 409–410.
- (347) Takeuchi, H., Yamamoto, H., Niwa, T., Hino, T., and Kawashima, Y. (1994) Mucoadhesion of polymer-coated liposomes to rat intestine *in vitro*. *Chem. Pharm. Bull.* 42, 1954–1956.
- (348) Takeuchi, H., Yamamoto, H., Niwa, T., Hino, T., and Kawashima, Y. (1996) Enteral absorption of insulin in rats from mucoadhesive chitosan-coated liposomes. *Pharm. Res.* 13, 896–901.
- (349) Maruyama, K., Okuizumi, S., Ishida, O., Yamauchi, H., Kikuchi, H., and Iwatsuru, M. (1994) Phosphatidylpolyglycerols prolong liposome circulation *in vivo*. *Int. J. Pharm.* 111, 103–107.
- (350) Woodle, M. C., Engbers, C. M., and Zalipsky, S. (1994) New amphipathic polymer–lipid conjugates forming long-circulating reticuloendothelial system-evading liposomes. *Bioconjugate Chem.* 5, 493–496.
- (351) Kang, E. C., Akiyoshi, K., and Sunamoto, J. (1997) Surface coating of liposomes with hydrophobized polysaccharides. *J. Bioact. Compat. Polym.* 12, 14–26.
- (352) Gregoriadis, G., McCormack, B., Wang, Z., and Lifely, R. (1993) Polysialic acids: potential in drug delivery. *FEBS Lett.* 315, 271–276.
- (353) Zhang, X., and Gregoriadis, G. (1997) Polysialylated liposomes: preparation and fate *in vivo*. *Proc. Int. Symp. Controlled Release Bioact. Mater.*, 24th, 861–862.
- (354) van Rooijen, N., Bakekr, J., and Sander, A. (1997) Transient suppression of macrophage function by liposomes-encapsulated drugs. *Trends Biotechnol.* 15, 178–185.
- (355) Kennel, S. J., Lankford, T., Hughes, B., and Hotchkiss, J. (1988) Quantitation of a murine lung endothelial cell protein, p112, with a double monoclonal antibody assay. *Lab. Invest.* 59, 692–701.
- (356) Maruyama, K., Kennel, S., and Huang, L. (1990) Lipid composition is important for highly efficient target binding and retention of immunoliposomes. *Proc. Natl. Acad. Sci. U.S.A.* 87, 5744–5748.
- (357) Mori, A., Klivanov, A. L., Torchilin, V. P., and Huang, L. (1991) Influence of the steric barrier activity of amphipathic poly(ethylene glycol) and ganglioside GM1 on the circulation time of liposomes and the target binding of immunoliposomes *in vivo*. *FEBS Lett.* 284, 263–266.
- (358) Holmberg, E., Maruyama, K., Litzinger, D., Wright, S., Davis, M., Kabalka, G. W., Kennel, S. J., and Huang, L. (1989) Highly efficient immunoliposomes prepared with a method which is compatible with various lipid compositions. *Biochem. Biophys. Res. Commun.* 165, 1272–1278.
- (359) Emanuel, N., Kedar, E., Bolotin, E. M., Smorodinsky, N. I., and Barenholz, Y. (1996) Preparation and characterization of doxorubicin-loaded sterically stabilized immunoliposomes. *Pharm. Res.* 13, 352–359.
- (360) Blume, G., Cevc, G., Crommelin, M. D., Bakker-Woudenberg, I. A., Kluft, C., and Storm, G. (1993) Specific targeting with poly(ethylene glycol)-modified liposomes: coupling of homing devices to the ends of the polymeric chains combines effective target binding with long circulation times. *Biochim. Biophys. Acta* 1149, 180–184.
- (361) Zalipsky, S., Mullah, N., Harding, J. A., Gittelma, J., Guo, L., and DeFrees, S. A. (1997) Poly(ethylene glycol)-grafted liposomes with oligopeptide or oligosaccharide ligands appended to the termini of the polymer chains. *Bioconjugate Chem.* 8, 111–118.
- (362) Yoshikawa, Y., Miyazaki, M., Houjou, T., Komuta, Y., Yoshikawa, H., and Takada, K. (1997) Biodistribution of cyclosporin encapsulated in liposomes modified with bioadhesive polymer. *J. Pharm. Pharmacol.* 49, 661–668.
- (363) Maruyama, K., Unezaki, S., Takahashi, N., and Iwatsuru, M. (1993) Enhanced delivery of doxorubicin to tumor by long circulating thermosensitive liposomes and local hyperthermia. *Biochim. Biophys. Acta* 1149, 209–216.
- (364) Huang, S. K., Stauffer, P. R., Hong, K., Guo, J. W. H., Phillips, T. L., Huang, A., and Papahadjopoulos, D. (1994) Liposomes and hyperthermia in mice: increased tumor uptake and therapeutic efficacy of doxorubicin in sterically stabilized liposomes. *Cancer Res.* 54, 2186–2191.
- (365) Unezaki, S., Maruyama, K., Takahashi, N., Koyama, M., Yuda, T., Suginata, A., and Iwatsuru, M. (1994) Enhanced delivery and antitumor activity of doxorubicin using long-circulating thermosensitive liposomes containing amphipathic polyethylene glycol in combination with local hyperthermia. *Pharm. Res.* 11, 1180–1185.
- (366) Masuko, Y., Tazawa, K., Sato, H., Viroonchatapan, E., Takemori, S., Shimizu, T., Ohkami, H., Nagae, H., Fujimaki, M., Horikoshi, I., and Weinstein, J. N. (1997) Antitumor activity of selective hyperthermia in tumor-bearing rats using thermosensitive magnetoliposomes as a new hyperthermic material. *Drug Delivery* 4, 37–42.
- (367) Connor, J., Yatvin, M. B., and Huang, L. (1984) pH-sensitive liposomes: acid-induced liposomes fusion. *Proc. Natl. Acad. Sci. U.S.A.* 81, 1715–1718.
- (368) Connor, J., and Huang, L. (1986) pH-sensitive immunoliposomes as an efficient and target-specific carrier for antitumor drugs. *Cancer Res.* 46, 3431–3435.
- (369) Oku, N., Shibamoto, S., Ito, F., Gondo, H., and Nango, M. (1987) Low pH induced membrane fusion of lipid vesicles containing proton-sensitive polymer. *Biochemistry* 26, 8145–8150.

- (370) Ho, R. J. Y., Rouse, B. T., and Huang, L. (1986) Target-sensitive immunoliposomes: preparation and characterization. *Biochemistry* 25, 5500–5506.
- (371) Ho, R. J. Y., Rouse, B. T., and Huang, L. (1987) Target-sensitive immunoliposomes as an efficient drug carrier for antiviral activity. *J. Biol. Chem.* 262, 13973–13978.
- (372) Slepishkin, V. A., Simoes, S., Dazin, P., Newman, M. S., Guo, L. S., Pedroso de Lima, M. C., and Duzgunes, N. (1997) Sterically stabilized pH-sensitive liposomes. *J. Biol. Chem.* 272, 2382–2388.
- (373) Hong, M. S., and Kim, C. K. (1996) Preparation and evaluation of pH-sensitive long-circulating liposomes entrapped methotrexate. *Proc. Int. Symp. Controlled Release Bioact. Mater.*, 23rd, 923–924.
- (374) Deverre, J. R., Bouteet, V., de Oliveira, M. C., Fattal, E., Boquet, D., Grogne, J. M., and Couvreur, P. (1997) Plasma stability and pharmacokinetic profile of phosphodiester antisense oligonucleotide encapsulated in pH sensitive liposomes. *Proc. Int. Symp. Controlled Release Bioact. Mater.*, 24th, 663–664.
- (375) Couvreur, P., Fattal, E., Malvy, C., and Dubernet, C. (1997) pH-sensitive liposomes: an intelligent system for the delivery of antisense oligonucleotides. *J. Liposome Res.* 7, 1–18.
- (376) Gabizon, A., Prince, D. C., Huberty, J., Bresalier, R. S., and Papahadjopoulos, D. (1990) Effect of liposome composition and other factors on the targeting of liposomes to experimental tumors: biodistribution and imaging studies. *Cancer Res.* 50, 6371–6378.
- (377) Woodle, M. C. (1993) ⁶⁷Gallium-labeled liposomes with prolonged circulation: preparation and potential as nuclear imaging agents. *Nucl. Med. Biol.* 20, 149–155.
- (378) Oku, N., Namba, Y., Takeda, A., and Okada, S. (1993) Tumor imaging with technetium-99m-DTPA encapsulated in RES-avoiding liposomes. *Nucl. Med. Biol.* 20, 407–412.
- (379) Gabizon, A. (1992) Selective tumor localization and improved therapeutic index of anthracyclines encapsulated in long-circulating liposomes. *Cancer Res.* 52, 891–896.
- (380) Papahadjopoulos, D., Allen, T. M., Gabizon, A., Mayhew, E., Matthey, K., Huang, K., Lee, K. D., Woodle, M. C., Lasic, D. D., Redemann, C., and Martin, F. J. (1991) Sterically stabilized liposomes: improvements in pharmacokinetics and antitumor therapeutic efficacy. *Proc. Natl. Acad. Sci. U.S.A.* 88, 11460–11464.
- (381) Huang, S. K., Mayhew, E., Gilani, S., Lasic, D. D., Martin, F. J., and Papahadjopoulos, D. (1992) Pharmacokinetics and therapeutics of sterically stabilized liposomes in mice bearing C-26 colon carcinoma. *Cancer Res.* 52, 6774–6781.
- (382) Oku, N., Doi, K., Namba, Y., and Okada, S. (1994) Therapeutic effect of adriamycin encapsulated in long-circulating liposomes on Meth-A-sarcoma-bearing mice. *Int. J. Cancer* 58, 415–419.
- (383) Vaage, J., Mayhew, E., Lasic, D., and Martin, F. (1992) Therapy of primary and metastatic mouse mammary carcinomas with doxorubicin encapsulated in long circulating liposomes. *Int. J. Cancer* 51, 942–948.
- (384) Vaage, J., Donovan, D., Loftus, T., Abra, R., Working, P., and Huang, A. (1994) Chemoprevention and therapy of mouse mammary carcinomas with doxorubicin encapsulated in sterically stabilized liposomes. *Cancer* 73, 2366–2371.
- (385) Vaage, J., Donovan, D., Mayhew, E., Abra, R., and Huang, A. (1993) Therapy of human ovarian carcinoma xenografts using doxorubicin encapsulated in sterically stabilized liposomes. *Cancer* 72, 3671–3675.
- (386) Vaage, J., Barbera-Guillem, E., Abra, R., Huang, A., and Working, P. (1994) Tissue distribution and therapeutic effect of intravenous free or encapsulated liposomal doxorubicin on human prostate carcinoma xenografts. *Cancer* 73, 1478–1484.
- (387) Huang, S. C., Lee, K. D., Friend, D. S., and Papahadjopoulos, D. (1992) Microscopic localization of sterically stabilized liposomes in colon carcinoma-bearing mice. *Cancer Res.* 52, 5135–5143.
- (388) Puisieux, F., Barrat, G., Courraze, G., Couvreur, P., Devissaguet, J. P., Dubernet, C., Fattal, E., Fessi, H., Vauthier, C., and Benita, S. (1994) Polymeric micro- and nanoparticles as drug carriers. In *Polymeric Biomaterials* (S. Dumitriu, Ed.) pp 749–794, Marcel Dekker, New York.
- (389) Dakhil, S., Ensminger, W., Cho, K., Neiderhuber, J., Doan, K., and Wheeler, R. (1982) Improved regional selectivity of hepatic arterial BCNU with degradable microspheres. *Cancer* 50, 631–635.
- (390) Gupta, P. K. (1990) Drug targeting in cancer chemotherapy: a clinical perspective. *J. Pharm. Sci.* 79, 949–962.
- (391) Flandroy, P., Grandfils, C., Colligon, J., Thibaut, A., Nihant, N., Barbette, S., Jerome, R., and Teyssie, P. (1990) D,L-Polylactide microspheres as embolic agent. A preliminary study. *Neuroradiology* 32, 311–315.
- (392) Kato, T., Nemoto, R., Mori, H., Takahashi, M., and Tamakawa, Y. (1981) Transcatheter arterial chemoembolization of renal cell carcinoma with microencapsulated mitomycin C. *J. Urol.* 125, 19–24.
- (393) Nemoto, R., Kato, T., Iwata, K., Mori, H., and Takahashi, M. (1981) Evaluation of therapeutic arterial embolization in renal cell carcinoma using microencapsulated mitomycin C. *Urology* 17, 315–319.
- (394) Pinski, J., Reile, H., Halmos, G., Groot, K., and Schally, A. V. (1994) Inhibitory effect of somatostatin analogue RC-160 and bombesin/gastrin-releasing peptide antagonist RC-3095 on the growth of the androgen-independent Dunning R-3327-AT-1 rat prostate cancer. *Cancer Res.* 54, 169–174.
- (395) Yano, T., Pinski, J., Szepeshazi, K., Milovanovic, S. R., Groot, K., and Schally, A. V. (1992) Effect of microcapsules of luteinizing hormone-releasing hormone antagonist SB-75 and somatostatin analogue RC-160 on endocrine status and tumor growth in the Dunning R-3327H rat prostate cancer model. *Prostate* 20, 297–310.
- (396) Cleland, J. L., Mac, A., Boyd, B., Yang, J., Duenas, E. T., Yeung, D., Brooks, D., Hsu, C., Chu, H., Mukku, V., and Jones, A. J. S. (1997) The stability of recombinant human growth hormone in poly(lactic-co-glycolic acid) (PLGA) microspheres. *Pharm. Res.* 14, 420–425.
- (397) Singh, U. V., Udupa, N., Kamath, R., and Umadevi, P. (1997) Enhanced antitumor efficacy of methotrexate poly(lactic-co-glycolic) acid injectable gel implants in mice bearing sarcoma-180. *Pharm. Sci.* 3, 133–136.
- (398) Blanco-Prieto, M. J., Orsolini, P., Heimgartner, F., Besseghir, K., Merkle, H. P., and Gander, B. (1997) In vitro and in vivo release kinetics of a somatostatin analogue from PLGA microspheres. *Proc. Int. Symp. Controlled Release Bioact. Mater.*, 24th, 953–954.
- (399) Kissel, T., Li, Y. X., Volland, C., Gorich, S., and Koneberg, R. (1996) Parental protein delivery systems using biodegradable polyesters of ABA block structure, containing hydrophobic poly(lactide-co-glycolide) A blocks and hydrophilic poly(ethylene oxide) B blocks. *J. Controlled Release* 39, 315–326.
- (400) Coombes, A. G. A., Tasker, S., Lindblad, M., Holmgren, J., Hoste, K., Toncheva, V., Schacht, E., Davies, M. C., Illum, L., and Davis, S. S. (1997) Biodegradable polymeric micro-particles for drug delivery and vaccine formulation: the surface attachment of hydrophilic species using the concept of poly(ethylene glycol) anchoring segments. *Biomaterials* 18, 1153–1161.
- (401) Inoue, T., Nawa, Y., Tsutsumiuchi, R., Imaeda, N., Matsuda, H., and Ueda, Y. (1997) A novel lipid microsphere emulsified by lecithin with saturated fatty acids for prolonged parental drug delivery. *Proc. Int. Symp. Controlled Release Bioact. Mater.*, 24th, 983–984.
- (402) Couvreur, P., Roland, M., and Speiser, P. (1982) Biodegradable submicroscopic particles containing a biologically active substance and compositions containing them. U.S. Patent 4,329,332.
- (403) Alkouri-Fallouh, N., Roblot-Treupel, L., Fessi, H., Devissaguet, J. P., and Puisieux, F. (1986) Development of a new process for the manufacture of polyisobutylcyanoacrylate nanocapsules. *Int. J. Pharmacol.* 28, 125–132.
- (404) Rollot, J. M., Couvreur, P., Roblot-Treupel, L., and Puisieux, F. (1986) Physicochemical and morphological characterization of polyisobutylcyanoacrylate nanocapsules. *J. Pharm. Sci.* 75, 133–165.
- (405) Gurny, R., Peppas, N. A., Harrington, D. D., and Banker, G. S. (1981) Development of biodegradable and injectable

- latites for controlled release of potent drugs. *Drug. Dev. Ind. Pharm.* 7, 1–25.
- (406) Krause, H. J., Schwartz, A., and Rohdelwald, P. (1985) Polylactic acid nanoparticles, a colloidal drug delivery system for lipophilic drugs. *Int. J. Pharm.* 27, 145–155.
- (407) Kramer, P. A. (1974) Albumin microspheres as vehicles for achieving specificity in drug delivery. *J. Pharm. Sci.* 63, 1646–1647.
- (408) Fessi, H., Devissaguet, J. P., Puisieux, F., and Thies, C. (1986) Procédé de préparation des systèmes colloïdaux dispersibles d'une substance sous forme de nanocapsules (Procédure for the preparation of dispersable colloidal systems of a substance in the form of nanocapsules). Fr. Pat. Appl. 8,618,446.
- (409) Marty, J. J., Oppenheim, R. C., and Speiser, P. (1978) Nanoparticles: a new colloidal drug delivery system. *Pharm. Acta Helv.* 53, 17–22.
- (410) Stainmesse, S., Fessi, H., Devissaguet, J. P., and Puisieux, F. (1988) Procédé de préparation des systèmes colloïdaux dispersibles d'une protéine sous forme de nanoparticules (Procédure for the preparation of dispersable colloidal systems of a protein in the form of nanoparticles). First Addition to Fr. Pat. Appl. 8,618,446.
- (411) Sterling, C. V., and Scriven, L. E. (1959) Interfacial turbulence: hydrodynamic instability and Marangoni effect. *AIChE J.* 5, 514–523.
- (412) Fessi, H., Devissaguet, J. P., and Puisieux, F. (1986) Procédé de préparation des systèmes colloïdaux dispersibles d'une substance sous forme de nanocapsules (Procédure for the preparation of dispersable colloidal systems of a substance in the form of nanocapsules). Fr. Pat. Appl. 8,618,444.
- (413) Quintanar-Guerrero, D., Fessi, H., Allemann, E., and Doelker, E. (1996) Influence of stabilizing agents and preparative variables on the formation of poly(D,L-lactic acid) nanoparticles by an emulsification-diffusion technique. *Int. J. Pharm.* 143, 133–141.
- (414) Couvreur, P., Kante, B., Grislain, L., Roland, M., and Speiser, P. (1982) Toxicity of polyalkyl-cyanoacrylate nanoparticles. II. Doxorubicin-loaded nanoparticles. *J. Pharm. Sci.* 71, 790–792.
- (415) Verdun, C., Brasseur, F., Vranckx, H., Couvreur, P., and Roland, M. (1990) Tissue distribution of doxorubicin associated with polyisohexylcyanoacrylate nanoparticles. *Cancer Chemother. Pharmacol.* 26, 13–18.
- (416) Chiannikulchai, N., Driouich, Z., Benoit, J. P., Parodi, A. L., and Couvreur, P. (1990) Doxorubicin loaded nanoparticles: increased efficiency in murine hepatic metastases. *Sel. Cancer Ther.* 5, 1–11.
- (417) Barrat, G. M., Yu, W. P., Fessi, H., Devissaguet, J. P., Puisieux, F., Petit, J. F., Tenu, J. P., Israel, L., and Morere, J. F. (1989) Delivery of MDP-L-alanyl-cholesterol to macrophages: comparison of liposomes and nanocapsules. *Cancer J.* 2, 439–443.
- (418) Wilkins, D. J., and Myers, P. A. (1966) Studies on the relationship between the electrophoretic properties of colloids and their blood clearance and organ distribution in the rat. *Br. J. Exp. Pathol.* 47, 568–576.
- (419) Simon, B. H., Ando, H. Y., and Gupta, P. K. (1995) Circulation time and body distribution of ¹⁴C-labeled amino-modified polystyrene nanoparticles in mice. *J. Pharm. Sci.* 84, 1249–1253.
- (420) Illum, L., Davis, S. S., Muller, R. H., Mak, E., and West, P. (1987) The organ distribution and circulation time of intravenously injected colloidal carriers sterically stabilized with a block copolymer-ploxamine 908. *Life Sci.* 40, 367–374.
- (421) Moghimi, S. M., Hedeman, H., Muir, I. S., Illum, L., and Davis, S. S. (1993) An investigation of the filtration capacity and the fate of large filtered sterically-stabilized microspheres in rat spleen. *Biochim. Biophys. Acta* 1157, 233–240.
- (422) Moghimi, S. M., Porter, C. J. H., Muir, I. S., Illum, L., and Davis, S. S. (1991) Non-phagocytic uptake of intravenously injected microspheres in rat spleen: influence of particle size and hydrophilic coating. *Biochem. Biophys. Res. Commun.* 177, 861–866.
- (423) Porter, C. J. H., Moghimi, S. M., Illum, L., and Davis, S. S. (1992) The polyoxyethylene/polyoxypropylene block copolymer poloxamer-407 selectively redirects intravenously injected microspheres to sinusoidal endothelial cells of rabbit bone marrow. *FEBS Lett.* 305, 62–67.
- (424) Moghimi, S. M., Hedeman, H., Christy, N. M., Illum, L., and Davis, S. S. (1993) Enhanced hepatic clearance of intravenously administered sterically stabilized microspheres in zymosan-stimulated rats. *J. Leukocyte Biol.* 54, 513–517.
- (425) Illum, L., Jacobsen, L. O., Muller, R. H., Mak, E., and Davis, S. S. (1987) Surface characteristics and the interaction of colloidal particles with mouse peritoneal macrophages. *Biomaterials* 8, 113–117.
- (426) Van Oss, C. F., Gillman, C. F., and Neuman, A. W., Eds. (1975) *Phagocytic engulfment and cell adhesiveness*, Marcel Dekker, New York.
- (427) Moghimi, S. M., and Patel, H. M. (1989) Differential properties of organ-specific serum opsonins for liver and spleen macrophages. *Biochim. Biophys. Acta* 984, 379–383.
- (428) Patel, H. M. (1992) Serum opsonins and liposomes: their interaction and opsonophagocytosis. *Crit. Rev. Ther. Drug Carrier Syst.* 9, 39–90.
- (429) Kolb-Bachofen, V., and Abel, F. (1991) Participation of D-galactose-specific receptors of liver macrophages in recognition of fibronectin-opsonized particles. *Carbohydr. Res.* 213, 201–213.
- (430) Ulrich, F., and Meier, F. M. (1973) Inhibition of phagocytosis: effect of normal rabbit serum on *E. coli* uptake by rabbit alveolar macrophages. *J. Reticuloendothel. Soc.* 14, 8–17.
- (431) Moghimi, S. M., and Patel, H. M. (1989) Serum opsonins and phagocytosis of saturated and unsaturated phospholipid liposomes. *Biochim. Biophys. Acta* 984, 384–387.
- (432) Nicholas, A. R., and Jones, N. M. (1991) The effect of blood on the uptake of liposomal lipid by perfused rat. *Biochim. Biophys. Acta* 1074, 105–111.
- (433) Moghimi, S. M., Muir, I. S., Illum, L., Davis, S. S., and Kolb-Bachofen, V. (1993) Coating particles with a block copolymer (poloxamine-908) suppresses opsonization but permits the activity of dysopsonins in the serum. *Biochim. Biophys. Acta* 1179, 157–165.
- (434) Kolb-Bachofen, V., Schlepper-Schafer, J., Vogell, W., and Kolb, H. (1982) Electron microscopic evidence for an asialoglycoprotein receptor on Kupffer cells: localization of lectin-mediated endocytosis. *Cell* 29, 859–866.
- (435) Kolb-Bachofen, V. (1992) Uptake of toxic silica particles by isolated rat liver macrophages (Kupffer cells) is receptor mediated and can be blocked by competition. *J. Clin. Invest.* 90, 1819–1824.
- (436) Stolnik, S., Dunn, S. E., Garnett, M. C., Davies, M. C., Coombes, A. G. A., Taylor, D. C., Irving, M. P., Purkiss, S. C., Tadros, T. F., Davis, S. S., and Illum, L. (1994) Surface modification of poly(lactide-co-glycolide) nanospheres by biodegradable poly(lactide)-poly(ethylene glycol) copolymers. *Pharmacol. Res.* 11, 1800–1808.
- (437) Hawley, A. E., Illum, L., and Davis, S. S. (1997) Preparation of biodegradable, surface engineered PLGA nanospheres with enhanced lymphatic drainage and lymph node uptake. *Pharm. Res.* 14, 657–661.
- (438) Peracchia, M. T., Desmaele, D., Appel, M., d'Angelo, J., and Couvreur, P. (1997) PEG-coated nanoparticles from a novel poly(MePEG cyanoacrylate-co-alkyl cyanoacrylate) amphiphilic copolymer. *Proc. Int. Symp. Controlled Release Bioact. Mater.*, 24th, 124–125.
- (439) Peracchia, M. T., Vauthier, C., Puisieux, F., and Couvreur, P. (1996) PEG-PIBCA nanoparticles with different PEG-coating configurations formed by chemical coupling of PEG. *Proc. Int. Symp. Controlled Release Bioact. Mater.*, 23rd, 395–396.
- (440) Vondorpe, J., Schacht, E., Dunn, S., Hawley, A., Stolnik, S., Davis, S. S., Garnett, M. C., Davies, M. C., and Illum, L. (1997) Long circulating biodegradable poly(phosphazene) nanoparticles surface modified with poly(phosphazene)-poly(ethyleneoxide) copolymer. *Biomaterials* 18, 1147–1152.

- (441) Hansrani, P. K., Davis, S. S., and Groves, M. J. (1983) The preparation and properties of sterile intravenous emulsions. *J. Parenter. Sci. Technol.* 37, 145–150.
- (442) Hisaoka, M., Kurihara, A., Kasuya, A., and Ikeda, M. (1997) Pharmacokinetics of highly lipophilic antitumor agents incorporated in lipid emulsions. *Proc. Int. Symp. Controlled Release Bioact. Mater.*, 24th, 1049–1050.
- (443) Singh, M., and Ravin, L. J. (1986) Parental emulsions as drug carrier system. *J. Parenter. Sci. Technol.* 40, 34–41.
- (444) Poste, G., Bucana, C., Raz, A., Bugelski, P., Kirsh, R., and Fidler, I. J. (1982) Analysis of the fate of systemically administered liposomes and implications for their use in drug delivery. *Cancer Res.* 42, 1412–1422.
- (445) Sonoke, S., Saheki, A., Yoshikawa, H., Nagata, T., Fukui, H., and Seki, J. (1996) Apolipoprotein E plays a key role in the hepatic clearance of exogenous lipid emulsions. *Proc. Int. Symp. Controlled Release Bioact. Mater.*, 23rd, 667–668.
- (446) Liu, F., and Liu, D. (1995) Long-circulating emulsions (oil-in-water) as carriers for lipophilic drugs. *Pharmacol. Res.* 12, 1060–1064.
- (447) Lundberg, B. (1997) A submicron lipid emulsion coated with amphipathic polyethylene glycol for parental administration of Paclitaxel (Taxol). *J. Pharm. Pharmacol.* 49, 16–21.
- (448) Kwon, G. S., Yokoyama, M., Okano, T., Sakurai, Y., and Kataoka, K. (1993) Biodistribution of micelles forming polymer-drug conjugates. *Pharmacol. Res.* 10, 970–974.
- (449) Chiu, H. C., Chern, C. S., Lee, C. K., and Chang, H. F. (1996) Synthesis and characterization of poly(ethylene glycol) derived comb copolymers. *Proc. Int. Symp. Controlled Release Bioact. Mater.*, 23rd, 757–758.
- (450) Nagasaki, Y., Scholz, C., Iijima, M., Kato, M., and Kataoka, K. (1996) A novel reactive polymeric micelles for an intelligent drug carrier. *Proc. Int. Symp. Controlled Release Bioact. Mater.*, 23rd, 779–780.
- (451) Trubetskoy, V. S., Frank-Kamenetsky, M. D., Whiteman, K. R., Wolf, G. L., and Torchilin, V. P. (1996) Stable polymeric micelles: lymphangiographic contrast media for gamma scintigraphy and magnetic resonance imaging. *Acad. Radiol.* 3, 232–238.
- (452) Torchilin, V. P. (1997) Pharmacokinetic considerations in the development of labeled liposomes and micelles for diagnostic imaging. *Q. J. Nucl. Med.* 41, 141–153.
- (453) Takakura, Y., Mihara, K., and Hashida, M. (1994) Control of the disposition profiles of proteins in the kidney via chemical modification. *J. Controlled Release* 28, 111–119.
- (454) Marbach, P., Briner, U., Lemaire, M., Schweitzer, A., and Terasaki, T. (1993) From somatostatin to sandostatin: pharmacodynamics and pharmacokinetics. *Digestion* 54, 9–13.
- (455) Baldwin, R. W., Byers, V. S., and Mann, R. D., Eds. (1990) *Monoclonal antibodies and immunoconjugates*, Partheneon, Carnforth, U.K.
- (456) Winter, G., and Mielstein, C. (1991) Man-made antibodies. *Nature* 349, 293–299.
- (457) Glockshuber, R., Malia, M., Pfitzinger, I., and Pluckthum, A. (1990) A comparison of strategies to stabilize immunoglobulin Fv fragments. *Biochemistry* 29, 1362–1367.
- (458) Cumber, A. J., Ward, E. S., Winter, G., Parnell, G. D., and Wawrzynczak, J. (1992) Comparative stabilities in vitro and in vivo of a recombinant mouse antibody FvCys fragment and a bisFvCys conjugate. *J. Immunol.* 149, 120–126.
- (459) Yokota, T., Milenic, D. E., Withlow, M., and Scholm, J. (1992) Rapid tumor penetration of a single-chain Fv and comparison with other immunoglobulin forms. *Cancer Res.* 52, 3402–3408.
- (460) Reiter, Y., Pai, L. H., Brinkmann, U., Wang, Q., and Pastan, I. (1994) Antitumor activity and pharmacokinetics in mice of a recombinant immunotoxin containing a disulfide-stabilized Fv fragment. *Cancer Res.* 54, 2714–2718.
- (461) Gartshore, G., Bannan, P., Patterson, J., Highley, B., and McCulloch, J. (1994) Evaluation of technetium-99m exametazime stabilised with cobalt chloride as a blood flow tracer in focal cerebral ischemia. *Eur. J. Nucl. Med.* 21, 913–923.
- (462) Andersen, A. R. (1989) ^{99m}Tc -D,L-hexamethylene-propyleneamine oxime (^{99m}Tc -HPMAO): basic kinetic studies of a tracer of cerebral blood flow. *Cerebrovasc. Brain Metab. Rev.* 1, 288–318.
- (463) Peters, A. M., Osman, S., Henderson, B. L., Kelly, J. D., Danrupe, H. J., Hawker, R. J., Hodgson, H. J., Neirinckx, R. D., and Lavender, J. P. (1986) Clinical experience with Tc-99m-HPMAO for labeling leucocytes and imaging inflammation. *Lancet* 2, 926–949.
- (464) Feldman, M., Voth, E., Dressler, D., Henze, T., and Felgenhauer, K. (1990) ^{99m}Tc -hexamethylpropylene amine oxime SPECT and X-ray CT in acute cerebral ischemia. *J. Neurol.* 237, 475–479.
- (465) Sharp, P. F., Smith, F. W., Gemmell, H. G., Lyall, D., Evans, N. T. S., Gvozdanovic, D., Davidson, J., Tyrrell, D. A., Pickett, R. D., and Neirinckx, R. D. (1986) Technetium-99m HM-PAO stereoisomers as potential agents for imaging regional cerebral blood flow: human volunteer studies. *J. Nucl. Med.* 27, 171–177.
- (466) Igarashi, R., Hoshino, J., Ochiai, A., Morizawa, Y., and Mizushima, Y. (1994) Lecithinized superoxide dismutase enhances its pharmacologic potency by increasing its cell membrane affinity. *J. Pharmacol. Exp. Ther.* 271, 1672–1677.
- (467) Igarashi, R., Tsutsumi, Y., Fujii, H., Tsunoda, S., Ochiai, A., Takenaga, M., Morizawa, Y., Mayumi, T., and Mizushima, Y. (1997) Lecithinization of IL-6 enhances its thrombopoietic activity in mice. *J. Pharm. Pharmacol.* 49, 113–118.
- (468) Todoya, E., Uemura, K., Tenma, T., Fujita, T., Murakami, M., Yamamoto, A., and Muranishi, S. (1994) Enhanced permeability of tetragastrin across the rat intestinal membrane and its reduced degradation by acylation with various fatty acids. *J. Pharmacol. Exp. Ther.* 271, 1509–1513.
- (469) Honeycutt, L. R., Wang, J., Ekrami, H., and Shen, W. C. (1996) Comparison of pharmacokinetic parameters of a polypeptide, the Bowman-Birk protease inhibitor (BBI), and its palmitic acid conjugate. *Pharm. Res.* 13, 1373–1377.
- (470) Shen, W. C., Wang, J., and Shen, D. (1997) Reversible lipidization of polypeptides in drug delivery. *Proc. Int. Symp. Controlled Release Bioact. Mater.*, 24th, 202–203.
- (471) Trotta, M., Pattarino, F., and Gasco, M. R. (1996) Influence of counter ions on the skin permeation of methotrexate from water-oil microemulsions. *Pharm. Acta Helv.* 71, 135–140.
- (472) Jansen, J. F. G. A., de Brabander-van der Berg, E. M. M., and Meijer, E. W. (1994) Encapsulation of guest molecules into a dendritic box. *Science* 266, 1226–1229.
- (473) van Hest, J. C. M., Baars, M. W. P. L., Elissen-Roman, C., van Genderen, M. H. P., and Meijer, E. W. (1995) Acid functionalized amphiphiles derived from polystyrene-poly(propylene imine) dendrimers, with a pH dependent aggregation. *Macromolecules* 28, 6689–6691.
- (474) DeLong, R., Stephenson, K., Loftus, T., Fisher, M., Alahari, S., Nolting, A., and Juliano, R. L. (1997) Characterization of complexes of oligonucleotides with polyamidoamine starburst dendrimers and effects on intracellular delivery. *J. Pharm. Sci.* 86, 762–764.
- (475) Loukas, Y. L. (1997) Inclusion complexation of unstable guests with cyclodextrins: improved kinetic studies for calculation of the binding constants in guest-cyclodextrin systems. *Pharm. Sci.* 3, 343–346.
- (476) Uchegbu, I. F., Gianasi, E., Cociacinch, F., Florence, A. T., and Duncan, R. (1996) Niosome encapsulation of a doxorubicin polymer conjugate. *Proc. Int. Symp. Controlled Release Bioact. Mater.*, 23rd, 182–183.
- (477) Grasso, M., Morelli, A., and De Flora, A. (1986) Calcium-induced alterations in the levels and subcellular distribution of proteolytic enzymes in human red blood cells. *Biochem. Biophys. Res. Commun.* 138, 87–94.
- (478) Benatti, U., Giovine, M., Damonte, G., Gasparini, A., Scarfi, S., De Flora, A., Fraternali, A., Rossi, L., and Magnani, M. (1996) Azidothymidine homodinucleotide-loaded erythrocytes and bioreactors for slow delivery of the antiretroviral drug azidothymidine. *Biochem. Biophys. Res. Commun.* 220, 20–25.

ARTICLES

Liposomal Formulations of Synthetic MUC1 Peptides: Effects of Encapsulation versus Surface Display of Peptides on Immune Responses

Holly H. Guan,[†] Wladyslaw Budzynski,[‡] R. Rao Koganty,[‡] Mark J. Krantz,[‡] Mark A. Reddish,[‡] James A. Rogers,[†] B. Michael Longenecker,[‡] and John Samuel^{*,†}

Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta, Edmonton, Alberta, Canada T6G 2N8, and Biomira Inc., Edmonton, Alberta, Canada. Received October 20, 1997;
Revised Manuscript Received April 6, 1998

Synthetic human MUC1 peptides are important candidates for therapeutic cancer vaccines. To explore whether a human MUC1 peptide BP25 (STAPPAHGVTSPAPDTRPAPGSTAPP) can be rendered immunogenic by incorporation in liposomes, the effects of physical association of the peptide with liposomes on immune responses were investigated. Lipid conjugated and nonconjugated MUC1 peptides were incorporated in liposomes with a composition of distearoylphosphatidylcholine/cholesterol/dimyristoylphosphatidylglycerol (3:1:0.25, molar ratio) containing monophosphoryl lipid A (1% w/w of the total lipids). Liposomes were characterized for peptide retention by HPLC and for surface peptide display of MUC1 epitopes by flow cytometry. C57BL/6 mice were immunized with lipopeptide alone, peptide mixed with peptide-free liposomes, and peptide associated with liposomes in entrapped or surface-exposed forms. T cell proliferative responses, cytokine patterns, and antibody isotypes were studied. Results showed that immune responses were profoundly influenced by the liposome formulations. Physically associated, either encapsulated or surface-exposed, peptide liposomes elicited strong antigen-specific T cell responses, but not lipopeptide alone or peptide mixed with peptide-free liposomes. Analysis of the cytokines secreted by the proliferating T cells showed a high level of IFN- γ and undetectable levels of IL-4, indicating a T helper type 1 response. Thus, physical association of the peptide with liposomes was required for T cell proliferative responses, but the mode of association was not critical. On the other hand, the nature of the association significantly affected humoral immune responses. Only the surface-exposed peptide liposomes induced MUC1-specific antibodies. A domination of anti-MUC1 IgG2b over IgG1 (94 versus 6%) was observed. Our results support the hypothesis that different immune pathways are stimulated by different liposome formulations. This study demonstrated that a liposome delivery system could be tailored to induce either a preferential cellular or humoral immune response.

INTRODUCTION

MUC1 mucin is a high-molecular weight glycoprotein consisting of tandem repeats of the 20-amino acid unit PDTRPAPGSTAPPAHGVTSA (1). It is expressed by normal secretory epithelial cells and adenocarcinomas (2). MUC1 mucin produced by adenocarcinomas is underglycosylated in comparison to its normal counterpart. This results in exposure of certain core peptide epitopes of MUC1 to the immune system (3–6). Such epitopes may elicit anticancer immune responses in cancer patients, providing a rationale for the design of therapeutic MUC1 peptide cancer vaccines (7–9). Evidence for the protection against breast cancer through natural immunization by MUC1 epitopes during pregnancy suggests such vaccines may also have prophylactic applications (10–12). However, the problem of low immuno-

genicity of synthetic MUC1 peptides must be overcome for the development of effective cancer vaccines. Synthetic MUC1 peptides coupled with a carrier protein have been shown to induce anticancer immune responses in mice and in cancer patients in phase I clinical studies (13; unpublished results). We have chosen liposomal delivery of MUC1 synthetic peptides as an alternate approach for enhancing immunogenicity.

The immunoadjuvant properties of liposomes have long been recognized (14) and repeatedly demonstrated for humoral and cell-mediated immunity (15, 16). The nature of the physical association of protein antigens with liposomes can influence immune responses to a significant degree depending on the route of administration and antigen characteristics (17–20). In contrast to many studies on liposome-associated protein antigens, little work has been done with synthetic peptides, especially linear and hydrophilic peptides. Recently, a liposomal MUC1 peptide antigen delivery system containing monophosphoryl lipid A (MPLA) has been developed (21). It elicited strong antigen-specific T cell responses; however,

* Corresponding author. Telephone: (403) 492-7469. Fax: (403) 492-1217. E-mail: jsamuel@pharmacy.ualberta.ca.

[†] University of Alberta.

[‡] Biomira Inc.

no MUC1-specific antibodies were induced. Our working hypothesis is that the nature of physical association of MUC1 peptide with liposomes will influence the type of immune responses. The goal of this work was to evaluate the effects of the association of the MUC1 peptide with liposomes on antibody production and T cell responses.

EXPERIMENTAL PROCEDURES

Materials. Mouse MUC1 peptide SP1-020 (DSTSS-PVHSGTSSPATSAPEDSTS) and human MUC1 peptides BP16 (GVTSAPDTRPAPGSTA), BP24 (TAPPAHGVT-SAPDTRPAPGSTAPP), and BP25 (STAPPAHGVTSA-PDTRPAPGSTAPP) were constructed using the solid-phase approach of Merrifield (22) on an automated peptide synthesizer (MilliGen/Bioscience model 9050). LCP25 (laurylcys-STAPPAHGVTSA-PDTRPAPGSTAPP) was synthesized using laurylcysteine as the N-terminal amino acid. Laurylcysteine was presynthesized in solution phase by coupling lauric acid to the amino group of cysteine. Monoclonal antibodies (mAb) B248.7R2 specific for MUC1 peptide sequence APPAHGVTS and B80.3 (specific for PSA, prostate-specific antigen) were produced and provided by Biomira Inc. (Edmonton, AB). Distearoylphosphatidylcholine (DSPC), cholesterol (CHOL), and dimyristoylphosphatidylglycerol (DMPG) were obtained from Princeton Lipids (Princeton, NJ).

Peptide Liposome Formulations. Five formulations with equimolar peptide content (2 nmol per 50 μ L of liposomes; 5 μ g of BP25 or 5.6 μ g of LCP25) were prepared for immune response studies. Multilamellar vesicles with a final lipid concentration of 30 mM, composed of DSPC/CHOL/DMPG (3:1:0.25, molar ratio) and MPLA (1% w/w of the total lipids) (Ribi Immunochem Inc., Hamilton, MT), were prepared by a modified freeze-and-thaw (FAT) method (23, 24). Briefly, lipids were dissolved in chloroform and coated onto the walls of a 100 mL round-bottom flask by rotary evaporation, and the residual solvent was removed by overnight vacuum desiccation at 43 °C. The desiccated lipid film was dispersed in filter-sterilized PBS containing BP25 to form "liposomal BP25" at 65 °C followed by five cycles of FAT. Freezing was carried out in a dry ice/acetone bath for 5 min, and thawing occurred at room temperature for 50 min followed by incubation for 5 min at 65 °C. Free peptides in supernatants were removed after centrifugation at 150000g for 12 min (Beckman model L8-55 ultracentrifuge). Lipopeptide LCP25 was incorporated by mixing it with the lipids in chloroform prior to rotary evaporation, followed by hydration of the film with sterile PBS, forming liposomal LCP25. The entrapment of BP25 and LCP25 in liposomes was quantified by HPLC. Peptide-free liposomes ("empty") containing 1% MPLA were similarly prepared, except that only PBS was used for hydration. The "BP25 mixed" group was formulated by adding a specific amount of BP25 to empty liposomes. A control formulation of lipopeptide LCP25 ("LCP25 alone") was made by mixing it with 1% (w/v) MPLA in a PBS solution containing 0.1% (w/v) triethylamine.

Determination of Peptide Leakage from Liposomes. Aliquots of 200 μ L of liposome-encapsulated BP25 or LCP25 were incubated in a shaking water bath at 37 °C for 24 h. At various time intervals, liposome samples were centrifuged and analyzed by HPLC for MUC1 peptide in the supernatant and the pellet.

Liposome Size Determination. Liposome size was determined by dynamic light scattering (Brookhaven Instruments, Holtsville, NY). Samples were diluted 300 times with buffer and measured at 25 °C.

HPLC Analysis. Liposome pellets were dissolved in 25% absolute ethanol and then mixed with the internal standard phenol solution and PBS. A Waters 625-746 HPLC system with a C-18 reverse-phase radial-pak cartridge (8 mm $\times$ 100 mm) was used for HPLC analysis. Peptides were eluted using a gradient aqueous mobile phase consisting of eluent A [10% acetonitrile and 0.1% trifluoroacetic acid (TFA)] and eluent B (70% acetonitrile and 0.085% TFA). The flow rate was 1 mL/min. Peptides were detected by UV absorbance at 210 nm. Quantification of MUC1 peptides was done using a calibration curve based on peak areas ($R^2 > 0.9991$).

Flow Cytometric Analysis. To determine the surface exposure of MUC1 peptides, liposome samples (empty liposomes, BP25 mixed with empty liposomes, BP25 liposomes, and LCP25 liposomes) were incubated with B248.7R2 (MUC1-specific mAb) or B80.3 (negative control mAb) for 45 min at 4 °C, washed, and incubated with FITC-conjugated goat anti-mouse IgG1 (Southern Biotechnology Associates Inc., Birmingham, AL) at 4 °C for 30 min. After washing, liposomes were analyzed using a FACSsort flow cytometer (Becton Dickinson, Mountain View, CA) under standard conditions.

Immunization Procedures. Normal serum samples were collected from C57BL/6 mice (female, 7–8-week-old) 1 day before immunization by tail vein bleeding.

Single Immunization. On day 0, five groups of mice (seven in each group) were immunized subcutaneously (sc) in the left hind footpad with 50 μ L of the following five formulations: empty, BP25 mixed with empty liposomes, LCP25 alone, liposomal BP25, and liposomal LCP25. On day 10, blood was collected from each mouse by cardiac puncture and the draining popliteal lymph nodes were collected for the T cell proliferation assay.

Two Immunizations. On day 0, another five groups of mice were immunized sc in the left thigh with the same formulations as above. On day 14, an identical second immunization was given intraperitoneally (ip). On day 26, blood and the draining inguinal lymph nodes were collected.

T Cell Proliferation Assay and Cytokine Collection. Chemically inactivated syngenic spleen cells from naïve mice were used as antigen-presenting cells (APCs). Mitomycin C (Sigma Chemical Co., Mississauga, ON) was added at a dose of 60 μ g per 20 million spleen cells and the mixture incubated for 100 min at 37 °C in 5% CO₂. T cells were isolated from the lymph node cells by the nylon wool purification method (25). T cells (2.5×10^5 /well) and APCs (5×10^5 /well) were cultured in a total volume of 300 μ L in 96-well flat-bottom Costar microtiter plates (Fisher Scientific, Fair Lawn, NJ) in the presence of media alone (peptide-free background), SP1-020 (negative control), and BP24 or BP25 at 20 μ g/well for a period of 3 days at 37 °C in 5% CO₂. Each group was cultured in quadruplicate. On day 3, the cultures were pulsed with 1 μ Ci of [³H]thymidine (Amersham Canada Ltd., Oakville, ON) at 50 μ L/well for 21 h. Incorporation of [³H]-thymidine into the proliferating cells was measured after harvesting the cells on filters (Skatron model 11055 microcell harvester) and counting the filters (Beckman model LS 60001C liquid scintillation counter) in the presence of 4 mL of scintillation fluid (Cytoscint Research Production Division, Costa Mesa, CA). The stimulation index (SI) was defined as the ratio of the counts per minutes (cpm) of the peptide-stimulated cultures to the background cpm of peptide-free cultures. Separate parallel cultures under identical conditions were set up for collection of cytokines produced during T cell prolifera-

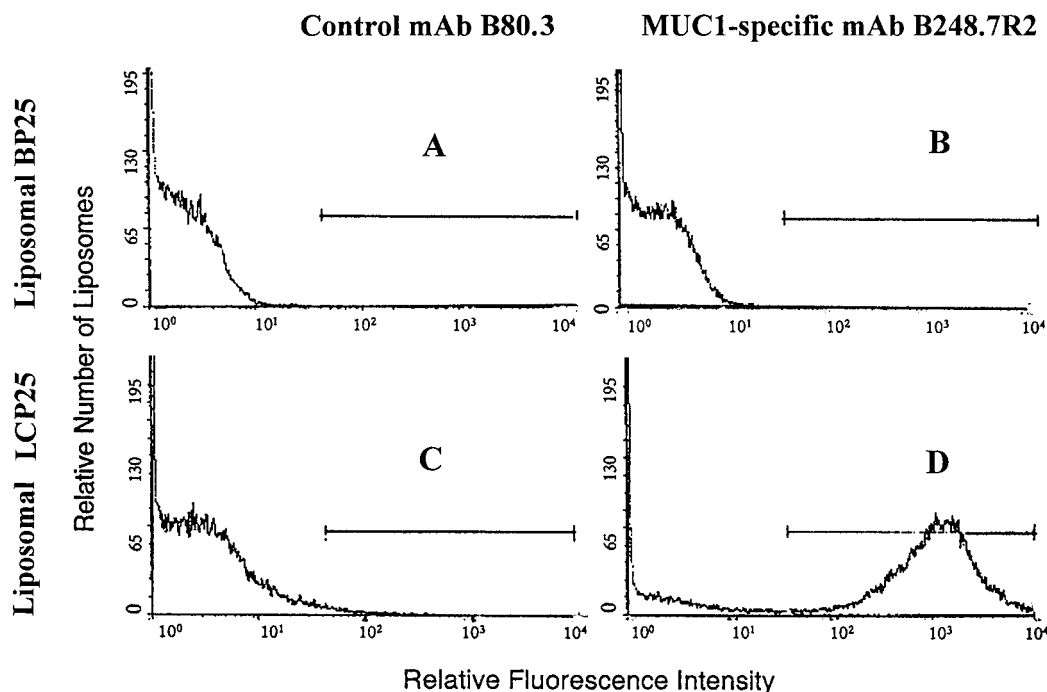


Figure 1. FACS analysis of liposomal BP25 (A and B) and LCP25 (C and D) for the presence of MUC1 epitopes on the surface. Liposomes were incubated with negative control mAb B80.3 specific for PSA (A and C) or MUC1-specific mAb B248.7R2 specific for the amino acid sequence APPAHGVSTA (B and D), subsequently incubated with an appropriate FITC-labeled secondary antibody, and analyzed by FACS. The bars represent fluorescence signal above background noise.

tion. Culture supernatant was collected from separate plates at 24, 48, and 72 h.

Cytokine Analysis. Levels of INF- γ and IL-4 in the above culture supernatants were separately estimated at 24, 48, or 72 h by a sandwich type enzyme-linked immunosorbent assay (ELISA) (26, 27). The 96-well Nunc ELISA plates were coated with antibodies R46.A2 and 11B11 (Biomira Inc.) for IFN- γ and IL-4, respectively, by incubation at 37 °C for 30 min, followed by washing with TPBS (PBS with 0.05% Tween 20 at pH 7.4). Culture supernatants or recombinant reference standards (rIFN- γ , concentration range of 5000–156 pg/mL; rIL-4, 1666–52 pg/mL; PharMingen, San Diego, CA) were added and the mixtures incubated at 37 °C for 45 min. After washing with TPBS, second antibodies (for IFN- γ , XMGI.2 biotinylated, Biomira Inc.; for IL-4, BVD6-24G2 biotinylated, PharMingen) were added and the mixtures incubated for 45 min at 37 °C. After washing, the wells were incubated with horseradish peroxidase (HRPO)-conjugated streptavidin (Jackson ImmunoResearch Lab Inc., Bar Harbor, ME) at 37 °C for 30 min and washed in TPBS. Finally, a HRPO substrate solution freshly prepared by mixing equal volumes of a TMB (3,3',5,5'-tetramethylbenzidine) solution [0.4 g/L in an organic base, Kirkegaard and Perry Laboratories Inc. (KPL), Gaithersburg, MD] and a H₂O₂ solution (0.02% in citric acid buffer, KPL) were added to the wells (100 μ L/well). The optical density (OD) at 650 nm was read immediately using a microplate reader (Molecular Devices, Menlo Park, CA) in a kinetic mode. A final OD reading was made at 450 nm after adding 100 μ L of 1 M phosphoric acid to each well. Cytokine levels in the culture supernatants were determined on the basis of the standard curves generated for the reference standards.

Antibody Analysis. Nunc maxisorb microplates were coated with 100 μ L/well BP16-HSA (human serum albumin) and HSA (negative control) at a concentration of 5 μ g/mL at 4 °C overnight. After washing with TPBS, the wells were blocked by incubation with bovine serum

albumin (1% in phosphate buffer, 200 μ L/well, KPL) at 37 °C for 30 min and washing with TPBS. Then serial dilutions of test serum samples (pooled for each immunization group) were added to the wells (100 μ L/well) and the mixtures incubated at room temperature for 1 h. A pooled normal serum sample (from unimmunized mice) was used as the negative control. Serum samples containing anti-MUC1 IgG and IgM from mice immunized with BP-16 conjugated to KLH (13) were used as positive controls (serial dilutions for IgM, 1:80 to 1:5120; for IgG, 1:2500 to 1:160000). The plates were then washed with TPBS. Goat anti-mouse IgG and IgM antibodies labeled with HRPO (KPL) at a 1:2000 dilution in PBS or subtype-specific goat anti-mouse IgG1, IgG2a, IgG2b, and IgG3 antibodies labeled with HRPO (Southern Biotechnology Associates Inc.) at a 1:4000 dilution in PBS were added to each well (100 μ L/well). After incubation at room temperature for 1 h and subsequent washing with TPBS, a HRPO substrate solution freshly prepared by mixing equal volumes of an ABTS [2,2'-azinobis(3-ethylbenzthiazoline sulfonate)] solution (0.6 g/L in glycine buffer, KPL) and a H₂O₂ solution (0.02% in citric acid buffer, KPL) were added to the wells (100 μ L/well). The wells were incubated at room temperature for 15 min, and the OD was read at 405 nm using a microplate reader (Molecular Devices).

RESULTS

Physical Characterization of Liposomes Containing Peptide versus Lipopeptide Antigen. FACS analysis of liposomal LCP25 with a mAb B248.7R2 (specific for the amino acid sequence APPAHGVSTA of human MUC1 mucin) clearly showed that the MUC1 peptide epitopes were present on the surfaces of liposomes, whereas no MUC1 epitopes were detected on liposome surfaces in the rest of the formulations (Figure 1). An irrelevant mAb B80.3 (specific for PSA) did not bind with LCP25 liposomes, establishing the antigen specificity of the FACS analysis. BP25 and LCP25

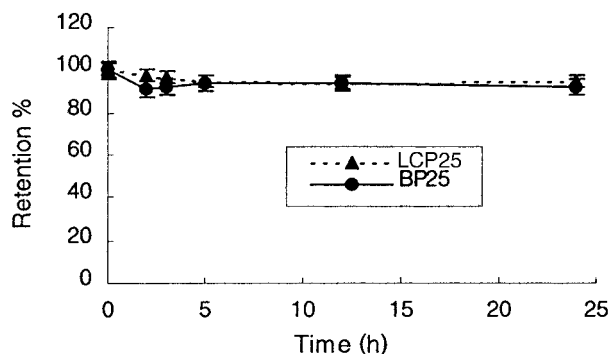


Figure 2. Retention of BP25 and LCP25 in liposomes with a lipid composition of 3:1:0.25 DSPC/CHOL/DMPG at 37 °C in PBS over the course of 24 h. Error bars indicate the standard deviation (SD) ($n = 3$).

liposomes leaked less than 10% of the entrapped peptides over the course of 24 h at 37 °C (Figure 2). The mean diameters of BP25 and LCP25 liposomes were 0.8 ± 0.1 and 0.9 ± 0.1 μm , respectively.

Physical Association of the MUC1 Peptide with Liposomes Was Required for T Cell Responses. Antigen-specific T cell priming induced by liposomal formulations was studied by T cell proliferation assays. T cell responses (SI) were significantly stronger ($P < 0.01$) in groups of mice immunized with the peptide-associated liposomes (encapsulated or surface-exposed) than in the rest of the formulations (Figure 3). There were no significant differences ($P > 0.01$) between the encapsulated and surface-exposed liposomes or between the two different immunization procedures (Figure 3). Antigen specificity was established by the observation that T cells responded to both recall human MUC1 peptides BP24 and BP25 but not to the irrelevant mouse MUC1 peptide SP1-020.

Cytokine Analysis Indicated a T Helper Type 1 (Th1) Response. T cell responses were further characterized by analyzing the cytokine secretion profiles of the proliferating T cells. No evidence of IL-4 was present in the T cell cultures following one or two immunizations, at 24, 48, or 72 h (all below the detectable level of 52 pg/mL; data not shown). However, there were significant levels of IFN- γ in the cell cultures that correlated with T cell responses (Figures 3 and 4). The amount of IFN- γ was found to be highest at 72 h. These results are indicative of Th1 responses (28).

Surface-Exposed but Not Encapsulated MUC1 Peptide Liposomes Induced Antibody Production. MUC1-specific humoral immune responses were evaluated by ELISA using BP16-HSA as the solid phase. BP16 contains the immunodominant B cell epitopes of MUC1 tandem repeat sequences in mice (13). The positive control serum for IgG and IgM was from mice immunized with BP16-KLH (keyhole limpet hemocyanin). There were no detectable levels of IgG or IgM after one immunization (Figure 5). However, high titers of IgG and IgM were detected in the sera pooled from the group of mice immunized twice with liposomal LCP25, but not with other formulations (Figure 6). This serum was further analyzed for IgG subclasses IgG1, IgG2a, IgG2b, and IgG3. Results showed a domination of IgG2b over IgG1 (94 versus 6%) (Figure 7). There were no detectable levels of IgG2a or IgG3.

DISCUSSION

Our results showed that physical association of the MUC1 peptide with liposomes containing MPLA was

necessary for induction of immune responses. Both encapsulated and surface-displayed formulations of peptides induced T cell responses, whereas an aqueous solution of lipopeptide containing MPLA or free peptide mixed with liposomes failed to induce antibodies or T cell responses. For most protein antigens, potent humoral responses may be induced by a variety of liposomal formulations, where protein antigens are entrapped, surface-linked, or even mixed with liposomes (29–31). Protein antigens containing hydrophobic domains may physically associate with lipid bilayers even by simple mixing with liposomes. Hydrophilic peptides such as MUC1 peptides used in our study are unlikely to associate with lipid bilayer. This is confirmed by the lack of MUC1-specific antibody binding with BP25 encapsulated liposomes. Since short peptides are quickly degraded by peptidases and rapidly cleared, they are unlikely to induce immune responses unless they are delivered in physical association with liposomes. The ability of the liposomes to retain more than 90% of the MUC1 peptides at 37 °C suggests that a major fraction of the peptides will be delivered to the immune system in an associated rather than released form.

MUC1 peptide delivery by both encapsulated or surface-exposed liposome formulations was equally effective in priming T cell responses *in vivo*. Induction of T cell responses requires antigen uptake by professional APCs and presentation of the antigenic peptide in association with MHC molecules: MHC class II presentation for CD4⁺ T helper (Th) cells and MHC class I presentation for CD8⁺ cytotoxic T lymphocytes (CTLs) (32). Liposomes are efficient vehicles for antigen delivery to professional APCs (33). Phospholipids have been shown to facilitate antigen presentation by enhancing peptide binding to MHC class II molecules possibly by inducing conformational changes in MHC class II molecules (34). Liposomes are also efficient vehicles for induction of CTLs (35, 36). The strong antigen-specific T cell proliferation in response to MUC1 peptides *ex vivo* is an indication of successful *in vivo* priming of Th cells by immunization with the liposomal formulations. It is possible that CD8⁺ CTLs also might have contributed to the proliferative responses *ex vivo*. However, further studies on the CD4/CD8 profile of the activated T cells and MHC-restricted lysis of MUC1⁺ target cells are required to confirm *in vivo* priming of CTLs by immunization with the liposomal MUC1 peptide formulations.

The cytokine secretion pattern (IFN- γ but not IL-4) of the MUC1-specific T cells indicates that the immune responses induced by both liposomal formulations were of the T helper 1 (Th1) type (28). It has been previously proposed that liposome surface-exposed antigen preferentially follows a Th1 and encapsulated antigen follows a Th2 activation pathway on the basis of the immunoglobulin (Ig) isotype profiles (37). Another report has suggested that both encapsulated and surface-linked formulations induce a Th1 type immunity (31). The exact mechanisms by which our formulations bias the immune responses toward a Th1 type have not been examined. We hypothesize that antigen delivery by these formulations results in induction of IL-12, a key cytokine initiator of the Th1 pathway of immune response. Phagocytosis of liposomes by professional APCs may induce secretion of IL-12. Uptake of particulate materials has been reported to induce IL-12 secretion by macrophages and dendritic cells (38). Further, MPLA adjuvant may favor Th1 responses by induction of IL-12 secretion by APCs (39).

The mode of physical association of MUC1 peptide with

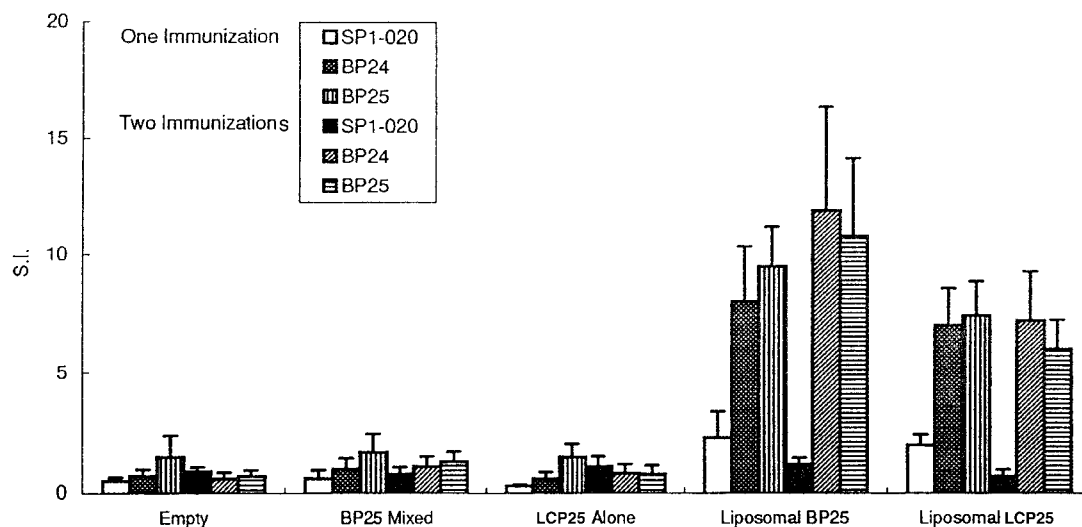


Figure 3. T cell responses induced by MUC1 peptide formulations after one or two immunizations (peptide dose, 2 nmol/mouse). T cells purified from lymph nodes were cultured along with antigen-presenting cells alone or in the presence of human MUC1 peptides (BP24 or BP25) or an irrelevant mouse MUC1 peptide (SP1-020) as shown. The stimulation index (SI) was defined as the ratio of counts per minute (cpm) of the peptide-stimulated cultures to the background cpm of peptide-free cultures. Error bars indicate the SD ($n = 4$).

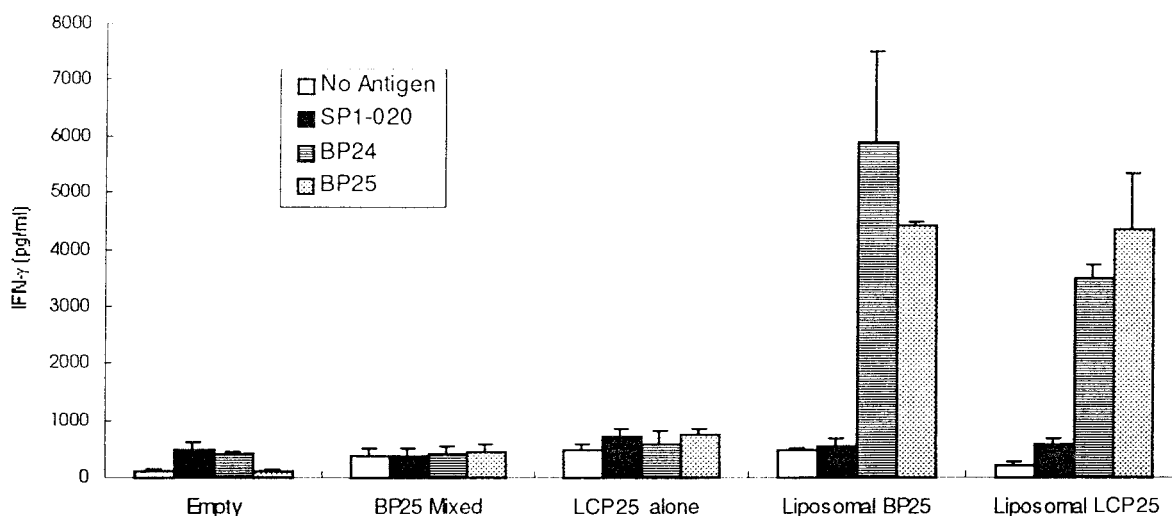


Figure 4. Levels of IFN- γ in the 72 h T cell cultures from groups of mice immunized with the MUC1 peptide formulations. T cells purified from lymph nodes were cultured along with antigen-presenting cells alone or in the presence of human MUC1 peptides (BP24 or BP25) or an irrelevant mouse MUC1 peptide (SP1-020) as shown. Error bars indicate the SD ($n = 4$).

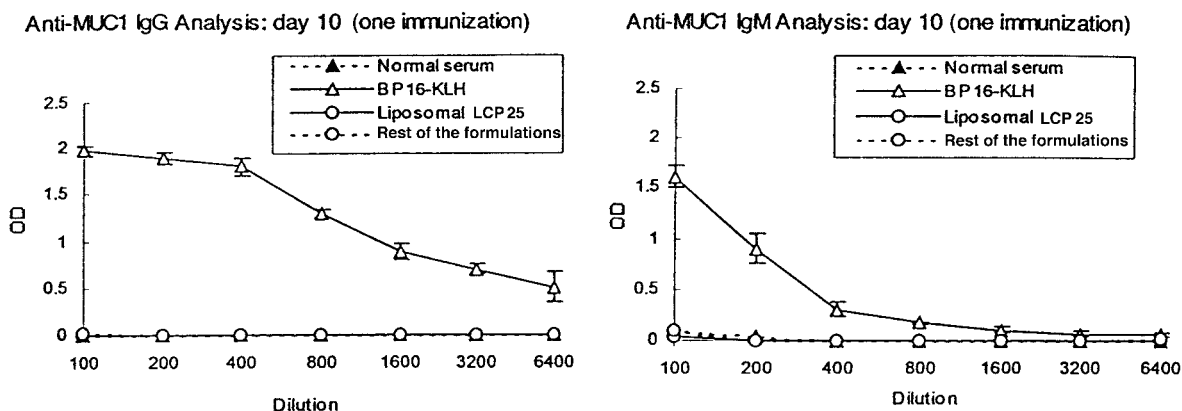


Figure 5. Anti-MUC1 IgG and IgM analysis from sera pooled from each group of mice immunized once (sc) with MUC1 peptide formulations. The polyclonal antiserum from BP16-KLH-immunized mice served as the positive control. The optical density (OD) was determined at a wavelength of 405 nm. Error bars indicate the SD ($n = 3$).

liposomes was found to be a key element for the humoral immune response induction. Only one formulation, LCP25 (N-terminally acylated BP25) liposomes with

surface-exposed MUC1 epitopes, elicited antibody production. No detectable antibody responses were induced by the BP25 encapsulated formulation, even though the

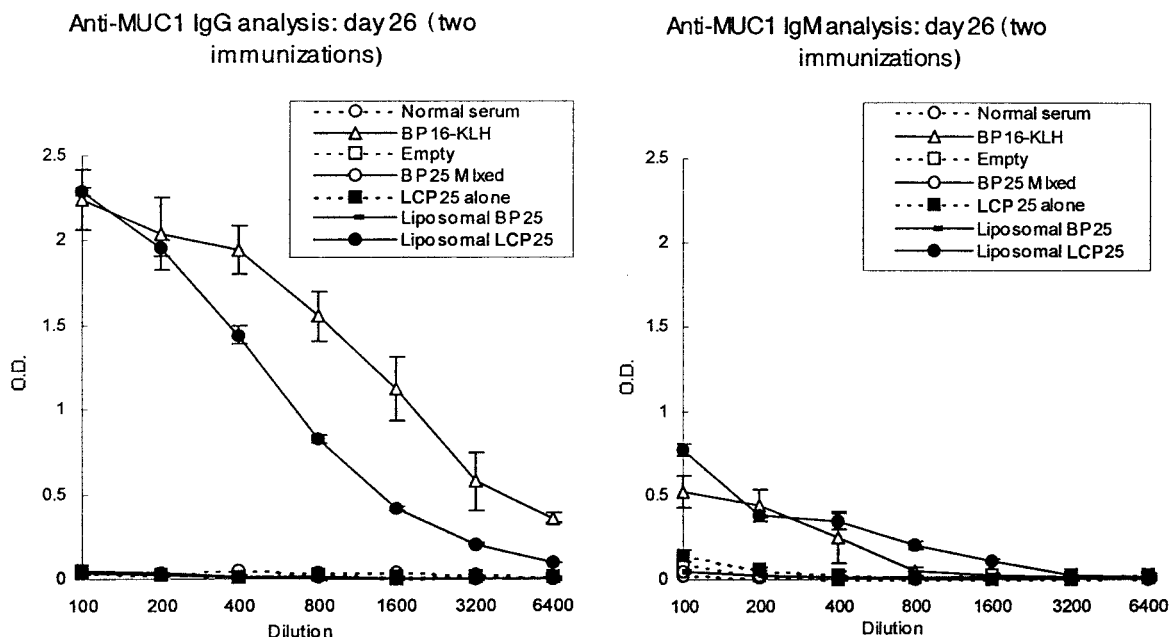


Figure 6. Anti-MUC1 IgG and IgM analyses from sera pooled from each group of mice immunized twice (sc and ip, 14 days apart) with MUC1 peptide formulations. The polyclonal antiserum from BP16-KLH-immunized mice served as the positive control. The optical density (OD) was determined at a wavelength of 405 nm. Error bars indicate the SD ($n = 3$).

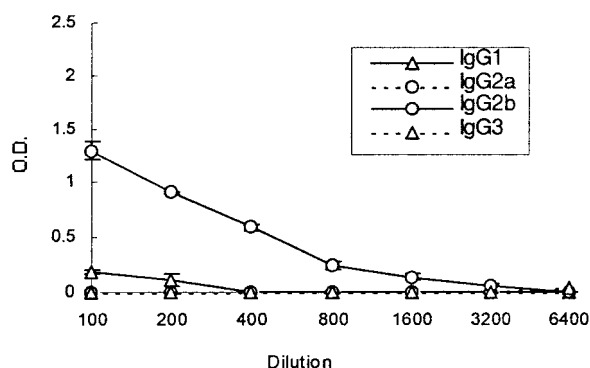


Figure 7. Anti-MUC1 IgG isotype analyses from sera of the group of mice immunized twice with liposomal LCP25. The optical density (OD) was determined at a wavelength of 405 nm. Error bars indicate the SD ($n = 3$).

immunization routes (sc followed by an ip booster) were chosen to favor antibody production. These results are consistent with our previous studies where a MUC1 24mer peptide (BP24) encapsulated within liposomes also failed to induce detectable antibodies at a comparable dose ($5 \mu\text{g}/\text{mice}$) after three sc immunizations (21). White et al. (35) reported that a gp120 V3 loop peptide (P18) encapsulated in liposomes induced CTL responses, but not detectable antibody responses (35). Induction of antibody responses against V3 loop epitopes required presentation of the epitopes on the liposome surface. Antibody responses are initiated by B cell recognition of antigens through Ig receptors on their surface (40, 41). The lack of antibody responses against liposome-encapsulated MUC1 peptide may be due to the lack of antigen accessibility to the Ig molecules on B cells. Both encapsulated and surface-exposed peptide liposomes would be expected to be phagocytosed and processed by professional APCs such as dendritic cells and macrophages; however, only the liposomes with surface-exposed peptide would interact with B cells. Dal Monte and Szoka demonstrated that soluble and liposome surface-exposed but not encapsulated pigeon cytochrome c (PCC) antigen was processed and presented by B cells *in vitro* (42), whereas the encapsulated PCC was phagocytosed and

presented by macrophages (43). Thus, the lack of antibody responses against the liposome-encapsulated MUC1 peptide may be due to the lack of antigen accessibility to the Ig molecules on B cells. Another important factor influencing antibody responses is the epitope repetitiveness. Repetitiveness of viral epitopes is a key factor responsible for efficient cross linking of surface Ig receptors leading to strong B cell responses, including isotype switching (44). The LCP25 liposomes would be expected to present multiple surface-exposed MUC1 epitopes to B cells, leading to efficient B cell activation through cross-linking of surface Igs. B cell recognition of MUC1 epitopes in BP25 liposomes would be dependent on the slow release of the encapsulated peptide, which would be detected only as monomers. Another factor influencing the differential B cell responses might be the higher *in vivo* stability of the lipopeptide in comparison to the unmodified peptide (BP25) (45, 46).

Immunoglobulins induced by liposomal LCP25 included IgM and IgG, with a domination of IgG2b over IgG1 (94 versus 6%) and no detectable levels of IgG2a or IgG3. The induction of Ig isotypes is influenced by many factors, including antigen location, adjuvants, mice strain, and route of administration (18, 19, 47). Immunomodulatory properties of adjuvants can affect Ig isotype distribution. Enhanced expression of IgG2b over IgG1 was observed by using lipid-soluble MDP (muramyl dipeptide) liposome-encapsulated protein antigens, but not with water-soluble MDP liposomes (48). Using liposomal surface-conjugated peptides, Friede et al. also observed a predominant production of IgG2b over IgG1 (49). Dominance of IgG2a and/or IgG2b over IgG1 is associated with Th1 type immune responses and is augmented by IFN- γ (50, 51). The ratio of IgG2a:IgG2b titers is influenced by the mouse strain, with a predominance of IgG2a in A/J mice and a predominance of IgG2b in C57BL/6 mice (47, 52). The gene corresponding to IgG2a in C57BL/6 mice (IgG2c) is significantly different from that in Balb/c, and the commercial reagents for murine IgG2a might not cross-react substantially with this isotype in C57BL/6 strains (53, 54). Therefore, IgG2b responses are a better indicator of Th1 responses

in C57BL/6 mice. Thus, the isotype distribution of humoral immune responses also confirmed that Th1-associated immune responses were induced by liposomal LCP25.

In summary, immune responses were profoundly influenced by characteristics of the liposome formulations. This study demonstrated that liposome delivery systems could be tailored to induce preferential cellular or humoral immune response. Our results suggest that encapsulation of peptide antigens in liposomes offers an approach to selective induction of T cell responses without activation of the humoral responses. Alternatively, surface display of peptides on liposomes would allow activation of humoral responses without compromising T cell responses. Such selective immune manipulations are useful in defining the protective immune responses against infections and cancer and are of special significance to therapeutic cancer vaccines. The results of this study are also relevant to vaccine design in general.

ACKNOWLEDGMENT

We thank Ms. Deborah Sosnowski, Ms. Ann Burrell, Ms. Brenda Christian, and Mr. Aldo Ritacco for their excellent technical assistance. Financial support for Ms. Holly Guan was provided by a Province of Alberta Postgraduate Fellowship. We thank the Medical Research Council of Canada for financial support of this work through a research grant to Dr. John Samuel (MT-13261).

LITERATURE CITED

- Gendler, S., Taylor-Papadimitriou, J., Duhig, T., Rothbard, J., and Burchell, J. (1988) A highly immunogenic region of a human polymorphic epithelial mucin expressed by carcinomas is made up of tandem repeats. *J. Biol. Chem.* 263, 12820–12823.
- Zotter, S., Hageman, P. C., Lossnitzer, A., Mooi, W. J., and Hiljers, J. (1988) Tissue and tumor distribution of human polymorphic epithelial mucin. *Cancer Rev.* 11, 55–58.
- Burchell, J., Gendler, S., Taylor-Papadimitriou, J., Girling, A., Lewis, A., Millis, R., and Lampton, D. (1987) Development and characterization of breast cancer reactive monoclonal antibodies directed to the core protein of the human milk mucin. *Cancer Res.* 47, 5476–5482.
- Hull, S. R., Bright, A., Carraway, K. L., Abe, M., Hayes, D. F., and Kufe, D. W. (1989) Oligosaccharide differences in the DF3 sialomucin antigen from normal human milk and the BT-20 human breast carcinoma cell line. *Cancer Commun.* 1, 261–267.
- Finn, O. J. (1992) Antigen-specific, MHC-unrestricted T cells. *Biotherapy* 4, 239–249.
- Jerome, K. R., Domenech, N., and Finn, O. J. (1993) Tumor-specific cytotoxic T cell clones from patients with breast and pancreatic adenocarcinoma recognize EBV-immortalized B cells transfected with polymorphic epithelial mucin complementary DNA. *J. Immunol.* 151, 1654–1662.
- Longenecker, B. M., and MacLean, G. D. (1993) Prospects for mucin epitopes in cancer vaccines. *Immunologist* 1, 89–93.
- Samuel, J., and Longenecker, B. M. (1995) Development of Active Specific Immunotherapeutic Agents Based on Cancer-associated Mucins. In *Vaccine design—the subunit and adjuvant approach* (M. F. Powell and M. J. Newman, Eds.) pp 875–890, Plenum Press, New York.
- Finn, O. J., Jerome, K. R., Henderson, R. A., Pecher, G., Domenech, N., Magarian-Blander, J., and Barratt-Boyes, S. M. (1995) MUC-1 epithelial tumor mucin-based immunity and cancer vaccines. *Immunol. Rev.* 145, 61–89.
- Agrawal, B., Reddish, M. A., Krantz, M. J., and Longenecker, B. M. (1995) Does pregnancy immunize against breast cancer? *Cancer Res.* 55, 2257–2261.
- Agrawal, B., Reddish, M. A., and Longenecker, B. M. (1996) In vitro induction of MUC-1 peptide-specific type 1 T lymphocyte and cytotoxic T lymphocyte responses from healthy multiparous donors. *J. Immunol.* 157, 2089–2095.
- Jerome, K. R., Kirk, A. D., Pecher, G., Ferguson, W. W., and Finn, O. J. (1997) A survivor of breast cancer with immunity to MUC-1 mucin, and lactational mastitis. *Cancer Immunol. Immunother.* 43, 355–360.
- Ding, L., Lalani, E. N., Reddish, M., Koganty, R., Wong, T., Samuel, J., Yacyshyn, M. B., Meikle, A., Fung, P. Y. S., Taylor-Papadimitriou, J., and Longenecker, B. M. (1993) Immunogenicity of synthetic peptides related to the core peptide sequence encoded by the human MUC1 mucin gene: effect of immunization on the growth of murine mammary adenocarcinoma cells transfected with the human MUC1 gene. *Cancer Immunol. Immunother.* 36, 9–17.
- Allison, A. G., and Gregoriadis, G. (1974) Liposomes as immunological adjuvants. *Nature* 252, 252.
- Alving, C. R. (1991) Liposomes as carriers of antigens and adjuvants. *J. Immunol. Methods* 140, 1–13.
- Gregoriadis, G. (1990) Immunological adjuvants: a role for liposomes. *Immunol. Today* 11, 89–97.
- Aramaki, Y., Fujii, Y., Yachi, K., Kikuchi, H., and Tsuchiya, S. (1994) Activation of systemic and mucosal immune response following nasal administration of liposomes. *Vaccine* 12, 1241–1245.
- Glenn, G. M., Rao, M., Richards, R. L., Matyas, G. R., and Alving, C. R. (1995) Murine IgG subclass antibodies to antigens incorporated in liposomes containing lipid A. *Immunol. Lett.* 47, 73–78.
- Naito, S., Horino, A., Nakayama, M., Nakano, Y., Nagai, T., Mizuguchi, J., Komuro, K., and Uchida, T. (1996) Ovalbumin-liposome conjugate induces IgG but not IgE antibody production. *Int. Arch. Allergy Immunol.* 109, 223–228.
- Therien, H. M., Lair, D., and Shahum, E. (1990) Liposomal vaccine: influence of antigen association on the kinetics of the humoral response. *Vaccine* 8, 558–562.
- Samuel, J., Budzynski, W. A., Reddish, M., Ding, L., Zimmermann, G., Krantz, M., Koganty, R. R., and Longenecker, B. M. (1998) Immunogenicity and antitumor activity of a liposomal MUC1 peptide based vaccine. *Int. J. Cancer* 75, 295–302.
- Merrifield, R. B. (1963) Solid-phase protein synthesis: I. The synthesis of a tetrapeptide. *J. Am. Chem. Soc.* 85, 2149–2154.
- Ohsawa, T., Miura, H., and Harada, K. (1985) Improvement of encapsulation efficiency of water-soluble drugs in liposomes formed by the freeze-thawing method. *Chem. Pharm. Bull.* 33, 3945–3952.
- Mayer, L. D., Hope, M. J., Cullis, P. R., and Janoff, A. S. (1985) Solute distributions and trapping efficiencies observed in freeze-thawed multilamellar vesicles. *Biochim. Biophys. Acta* 817, 193–196.
- Hathcock, K. S. (1991) T cell enrichment by non-adherence to nylon. In *Current Protocols in Immunology* (J. E. Coligan, A. M. Kruisbeek, D. H. Margulies, E. M. Shevach, and W. Strober, Eds.) p 3.2.1, Green Publishing and Wiley-Interscience, New York.
- Schreiber, R. D. (1991) Measurement of mouse and human interferon-gamma. In *Current Protocols in Immunology* (J. E. Coligan, A. M. Kruisbeek, D. H. Margulies, E. M. Shevach, and W. Strober, Eds.) p 6.8.1, Green Publishing and Wiley-Interscience, New York.
- Chatelain, R., Varkila, K., and Coffman, R. L. (1992) IL-4 induces a Th2 response in Leishmania major-infected mice. *J. Immunol.* 148, 1182–1187.
- Mosmann, T. R., Cherwinski, H., Bond, M. W., Giedlin, M. A., and Coffman, R. L. (1986) Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J. Immunol.* 136, 2348–2357.
- Van Rooijen, N., and Van Nieuwmegen, R. (1983) Association of an albumin antigen with phosphatidylcholine liposomes alters the nature of immunoglobulins produced during the immune response against the antigen. *Biochim. Biophys. Acta* 755, 434–438.

- (30) de Haan, A., Groen, G., Prop, J., van Rooijen, N., and Wilschut, J. (1996) Mucosal immunoadjuvant activity of liposomes: role of alveolar macrophages. *Immunology* 89, 488–493.
- (31) Shahum, E., and Therien, H. M. (1995) Liposomal adjuvanticity: effect of encapsulation and surface-linkage on antibody production and proliferative response. *Int. J. Immunopharmacol.* 17, 9–20.
- (32) Germain, R. N. (1994) MHC-dependent antigen processing and peptide presentation: providing ligands for T lymphocyte activation. *Cell* 76, 287–299.
- (33) Alving, C. R. (1995) Liposomal vaccines: clinical status and immunological presentation for humoral and cellular immunity. *Ann. N.Y. Acad. Sci.* 754, 143–152.
- (34) Roof, R. W., Luescher, I. F., and Unanue, E. R. (1990) Phospholipids enhance the binding of peptides to class II major histocompatibility molecules. *Proc. Natl. Acad. Sci. U.S.A.* 87, 1735–1739.
- (35) White, W. I., Cassatt, D. R., Madsen, J., Burke, S. J., Woods, R. M., Wassef, N. M., Alving, C. R., and Koenig, S. (1995) Antibody and cytotoxic T-lymphocyte responses to a single liposome-associated peptide antigen. *Vaccine* 13, 1111–1122.
- (36) Zhou, F., Rouse, B. T., and Huang, L. (1992) Induction of cytotoxic T lymphocytes in vivo with protein antigen entrapped in membranous vehicles. *J. Immunol.* 149, 1599–1604.
- (37) Shahum, E., and Therien, H. M. (1994) Correlation between in vitro and in vivo behaviour of liposomal antigens. *Vaccine* 12, 1125–1131.
- (38) Scheicher, C., Mehlig, M., Dienes, H. P., and Reske, K. (1995) Uptake of microparticle-adsorbed protein antigen by bone marrow-derived dendritic cells results in up-regulation of interleukin-1 alpha and interleukin-12 p40/p35 and triggers prolonged, efficient antigen presentation. *Eur. J. Immunol.* 25, 1566–1572.
- (39) Ulrich, J. T., and Myers, K. R. (1995) Monophosphoryl Lipid A as an Adjuvant: past experiences and new directions. In *Vaccine design—the subunit and adjuvant approach* (M. F. Powell, and M. J. Newman, Eds.) p 516, Plenum Press, New York and London.
- (40) Kurosaki, T. (1997) Molecular mechanisms in B cell antigen receptor signaling. *Curr. Opin. Immunol.* 9, 309–318.
- (41) Clark, E. A., and Ledbetter, J. A. (1994) How B and T cells talk to each other. *Nature* 367, 425–428.
- (42) Dal Monte, P. R., and Szoka, F. C., Jr. (1989) Antigen presentation by B cells and macrophages of cytochrome c and its antigenic fragment when conjugated to the surface of liposomes. *Vaccine* 7, 401–408.
- (43) Dal Monte, P., and Szoka, F. C., Jr. (1989) Effect of liposome encapsulation on antigen presentation in vitro. Comparison of presentation by peritoneal macrophages and B cell tumors. *J. Immunol.* 142, 1437–1443.
- (44) Bachmann, M. F., and Zinkernagel, R. M. (1997) Neutralizing antiviral B cell responses. *Annu. Rev. Immunol.* 15, 235–270.
- (45) Setoh, K., Murakami, M., Araki, N., Fujita, T., Yamamoto, A., and Muranishi, S. (1995) Improvement of transdermal delivery of tetragastrin by lipophilic modification with fatty acids. *J. Pharm. Pharmacol.* 47, 808–811.
- (46) Powell, M. F., Grey, H., Gaeta, F., Sette, A., and Colon, S. (1992) Peptide stability in drug development: a comparison of peptide reactivity in different biological media. *J. Pharm. Sci.* 81, 731–735.
- (47) Phillips, N. C., and Emili, A. (1992) Enhanced antibody response to liposome-associated protein antigens: preferential stimulation of IgG2a/b production. *Vaccine* 10, 151–158.
- (48) Phillips, N. C. (1992) Liposomes and macrophage functions. *Res. Immunol.* 143, 251–252.
- (49) Friede, M., Van Regenmortel, M. H., and Schuber, F. (1993) Lyophilized liposomes as shelf items for the preparation of immunogenic liposome-peptide conjugates. *Anal. Biochem.* 211, 117–122.
- (50) Germann, T., Bongartz, M., Dlugonska, H., Hess, H., Schmitt, E., Kolbe, L., Kolsch, E., Podlaski, F. J., Gately, M. K., and Rude, E. (1995) Interleukin-12 profoundly up-regulates the synthesis of antigen-specific complement-fixing IgG2a, IgG2b and IgG3 antibody subclasses in vivo. *Eur. J. Immunol.* 25, 823–829.
- (51) Guery, J. C., Galbiati, F., Smioldo, S., and Adorini, L. (1996) Selective development of T helper (Th)2 cells induced by continuous administration of low dose soluble proteins to normal and beta(2)-microglobulin-deficient BALB/c mice. *J. Exp. Med.* 183, 485–497.
- (52) Schmeer, N., Hoffmeister, E., Weiss, R., Hofer, R., and Baumgartner, W. (1997) Analysis of immunoglobulin classes and subclasses in response to infection of Balb/cJ and C57BL/6J mice with *Coxiella burnetii*. *J. Vet. Med., Ser. B* 44, 49–59.
- (53) Jouvin-Marche, E., Morgado, M. G., Leguern, C., Voegtli, D., Bonhomme, F., and Cazenave, P. A. (1989) The mouse Igh-1a and Igh-1b H chain constant regions are derived from two distinct isotypic genes. *Immunogenetics* 29, 92–97.
- (54) Martin, R. M., and Lew, A. M. (1998) Is IgG2a a good Th1 marker in mice? *Immunol. Today* 19, 49.

BC970183N

Kinetic Studies on the Immobilization of Antibodies to High-Performance Liquid Chromatographic Supports

Matthew R. Oates, William Clarke, Elizabeth M. Marsh, and David S. Hage*

Department of Chemistry, University of Nebraska, Lincoln, Nebraska 68588-0304. Received August 17, 1997; Revised Manuscript Received January 14, 1998

Several factors can potentially affect the rate of immobilization of proteins onto solid supports, such as those used in affinity-based high-performance liquid chromatography. This study examined several of these factors and their influence on the coupling of periodate-treated rabbit immunoglobulin G antibodies to dihydrazide-activated silica. Items considered included the number of potential coupling sites on the antibodies, the density of activated sites on the support, the relative amount of antibody combined with the support, and the density of the overall reaction slurry. In each case, the rate of change in the solution-phase antibody concentration gave biphasic behavior which could be described by two competing pseudo-first-order reactions. The overall immobilization rate was essentially independent of the density of the support's activated sites (when present at a coverage of 0.1–0.4 $\mu\text{mol}/\text{m}^2$) but was strongly influenced by the number of available coupling groups on the antibodies. Increasing the slurry density had no appreciable effect on the immobilization rate, and the reaction rate showed only a small change when using different types of reagents for support activation (e.g., adipic vs oxalic dihydrazide). These results are consistent with a mechanism in which the rate-limiting step during immobilization is the covalent attachment of antibodies to the support and not mass transfer of antibodies to the support's surface.

INTRODUCTION

Affinity chromatographic supports, such as those based on immobilized antibodies, are playing an ever-increasing role in the development of new separation and analysis methods for compounds of biological, industrial, or environmental interest (1–5). In recent years, the development of high-efficiency supports with good biocompatibility (e.g., diol-bonded silica or perfusion-type media) has promoted the use of various affinity ligands in high-performance liquid chromatography (HPLC)¹ (1–7). This, in turn, has made affinity supports even more attractive for advanced analytical methods such as immuno-analytical assays, on-line monitoring methods for biotechnology or environmental testing, and multidimensional schemes in which affinity extraction is coupled with methods like reversed-phase or size-exclusion chromatography (3–5). There are a variety of procedures available for attaching proteins and other ligands to HPLC supports for use in affinity columns (1–3, 7). However, there is little information available in the literature regarding the immobilization kinetics of these methods. Such data would be valuable in the design and optimization of new HPLC affinity media, particularly as the demand for more efficient supports and more rapid method development increases. There have been numerous studies in the past that have examined the kinetic processes behind the physical adsorption of proteins to various solid matrixes (8–21), including chromatographic materials such as silica (e.g., see refs 17–21). The general kinetic model used in these studies involves two

initial steps: (1) mass transfer of protein to the liquid–solid interface and (2) adsorption of the protein to unoccupied regions on the support's surface; a similar model would be expected to hold for covalent protein immobilization but with the adsorption step now being replaced with one involving the coupling of protein to the surface of the support. In many cases, the physical adsorption of proteins to silica and other supports has been reported to occur at a rate that is limited mainly by protein mass transfer to the surface (i.e., a diffusion-limited system) (8, 14–17), but there have also been cases in which adsorption has been noted to be the rate-limiting step (10, 12). The relative importance of these steps in determining the overall rate for the covalent immobilization of antibodies will be one item investigated in this work.

This study will examine the rate of antibody immobilization onto HPLC-grade media by using periodate-treated rabbit immunoglobulin G as the model ligand and dihydrazide-activated silica as the model support. This particular system is of practical importance since antibodies are frequently used as ligands in affinity-based HPLC (3–5), and dihydrazide-activated supports are commonly used for their immobilization (7, 22–25). Furthermore, previous work has shown that several important experimental variables, such as the number of potential coupling sites available on each antibody and the surface coverage of active sites on the support, can be controlled in this system through the proper selection of conditions for the periodate oxidation of antibodies (24, 25) or for the preparation of dihydrazide-activated silica (23). Along with these variables, other factors that will be considered in this study with regard to immobilization rates will include the effect of varying the relative amount of antibody in contact with the support and the density of the overall reaction slurry. Such work should

* To whom correspondence should be addressed.

¹ Abbreviations: BCA, bicinchoninic acid; FIA, flow injection analysis; HPLC, high-performance liquid chromatography; IgG, immunoglobulin G; LyCH, lucifer yellow CH; TNBS, 2,4,6-trinitrobenzenesulfonic acid.

provide information on the overall importance of these factors in determining the net rate of antibody immobilization. The same data should also provide clues regarding the roles played by mass transfer versus covalent attachment in the immobilization kinetics of antibodies and other proteins.

EXPERIMENTAL PROCEDURES

Reagents. Oxalic dihydrazide, adipic dihydrazide, and Lucifer yellow CH (LyCH) were from Aldrich (Milwaukee, WI). HPLC-grade Nucleosil Si-1000 silica (7 μ m particle diameter, 1000 Å pore size, 25 m²/g surface area) was obtained from Alltech (Deerfield, IL). The rabbit immunoglobulin G (IgG), *p*-periodic acid reagent (i.e., periodic acid), and 2,4,6-trinitrobenzenesulfonic acid (TNBS) were from Sigma (St. Louis, MO). Ethylene glycol and Triton X-100 were obtained from Fisher Scientific (Pittsburgh, PA). Reagents for the bicinchoninic acid (BCA) protein assay were from Pierce (Rockford, IL). All other chemicals were reagent-grade or better. All aqueous solutions were prepared using deionized water from a Nanopure water system (Barnstead, Dubuque, IA).

Apparatus. Samples for the manual BCA protein assay and TNBS assay were analyzed using disposable polystyrene cuvettes (Fisher Scientific) and a Shimadzu UV160U absorbance spectrophotometer (Kyoto, Japan). The degree of antibody oxidation was determined by labeling the oxidized antibodies with LyCH (26) and analyzing the degree of LyCH conjugation by flow injection analysis (FIA), as described previously (27). The application buffer for the FIA system was delivered by a Xper-Chrom model 400 pump from P. J. Cobert (St. Louis, MO); the BCA reagent for the same system was delivered by a model J120 cassette pump from Manostat (New York, NY). The LyCH label was detected at 428 nm on the FIA system by using a SM3100X absorbance detector from LDC/Milton Roy (Riviera Beach, FL), and the colored BCA product was detected at 562 nm using a V⁴ absorbance detector from ISCO (Lincoln, NE). The FIA samples were injected using a Rheodyne 7012 injection valve (Cotati, CA) equipped with a 20 μ L sample loop. The BCA reagent was combined with samples in the FIA system by using a standard mixing tee (Upchurch, Oak Harbor, WA), followed by passage of the mixture through a serpentine type II reaction chamber made from 1.0 m $\times$ 0.5 mm inner diameter PTFE tubing (28). This reaction chamber was immersed in an adjustable water bath set at 80 °C.

Methods. The diol-bonded silica and dihydrazide-activated supports were prepared from Nucleosil Si-1000, according to the scheme shown in Figure 1. The final diol coverage of the Nucleosil prior to activation by dihydrazide groups was 62 ± 4 μ mol of diol per gram of silica, as determined in triplicate by an iodimetric capillary electrophoresis method (29). The oxalic and adipic dihydrazide-activated supports were both prepared from the same batch of diol-bonded silica. Various degrees of dihydrazide coverage were obtained on these supports by controlling the amount of diol oxidation that was used during support preparation, as described in ref 23. The final coverage of activated sites on each support was determined by reacting the final material with an excess of TNBS, followed by washing away of the excess reagent and measurement of the resulting colored product by suspending the treated silica in a saturated sucrose solution and determining the absorbance of the suspension at 425 nm (23).

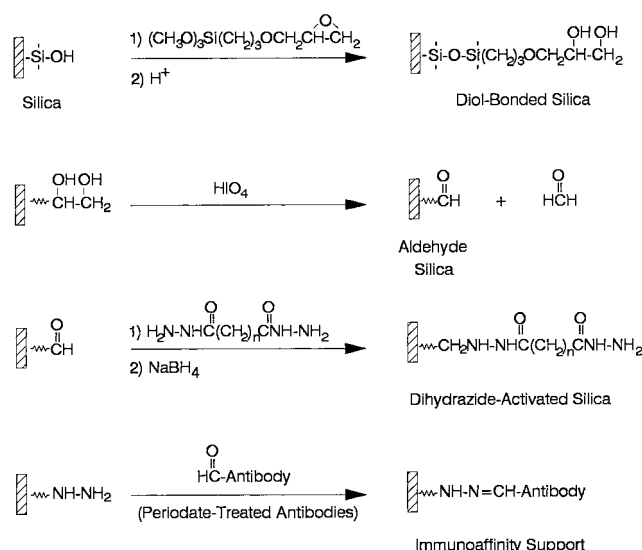


Figure 1. Reactions for the synthesis of dihydrazide-activated silica (23).

Table 1. Oxidation Conditions Used To Generate Rabbit IgG Antibodies with Various Levels of Potential Coupling Sites

pH	[periodic acid] (mM)	oxidation time (min)	degree of oxidation (mol of LyCH/mol of IgG) ^a
5.0	0.05	10	0.6 (± 0.1)
7.0	10.0	10	0.9 (± 0.1)
5.0	10.0	30	2.0 (± 0.1)
4.0	10.0	60	3.6 (± 0.1)
3.0	10.0	60	4.6 (± 0.1)

^a The numbers in parentheses represent a range of ± 1 SD. The value of moles of LyCH per mole of IgG is a measure of the average number of reactive aldehyde groups per IgG molecule that are available for coupling to a hydrazide-containing reagent (24).

The number of potential coupling sites on the rabbit IgG antibodies was varied by altering the pH and time used to treat the antibodies with periodic acid (24). All antibody oxidation was carried out at 25 °C using a rabbit IgG concentration of 1 mg/mL in 0.02 M sodium acetate buffer containing 0.15 M sodium chloride; Table 1 lists the other conditions that were used for antibody oxidation. After each oxidation mixture had been reacted for the appropriate length of time, ethylene glycol was added to quench the reaction. The ethylene glycol and remaining periodate were then removed by size-exclusion chromatography and dialysis, as described earlier (26); during this process, the antibodies were placed into a pH 6.0, 0.10 M phosphate buffer for the immobilization step. The final oxidized antibody preparations were either used immediately or stored for short periods of time at 4 °C prior to being used.

Antibody immobilization was carried out by mixing each oxidized antibody preparation with a fixed amount of dihydrazide-activated silica suspended in pH 6.0, 0.10 M phosphate buffer. This mixture was vortex-mixed, placed onto an inversion shaker, and allowed to react for various lengths of time at 4 °C. Aliquots were periodically removed from this mixture and centrifuged to remove the supernatant from the silica. The protein content in the supernatant was then determined in duplicate by a manual BCA assay (30), with rabbit IgG being used as the standard. Similar control studies were performed with nonoxidized antibodies in the presence of diol-bonded or dihydrazide-activated silica and with oxidized antibodies in the presence of diol-bonded silica.

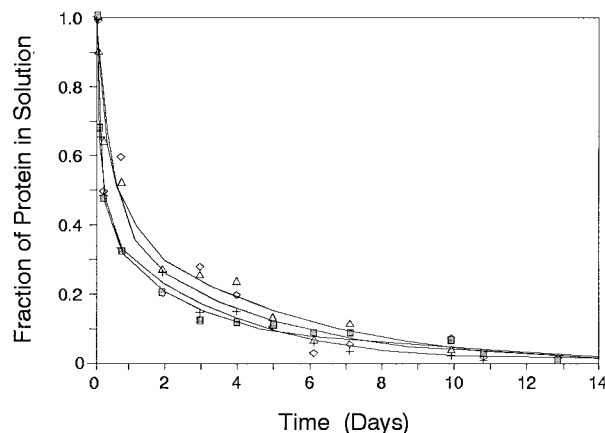


Figure 2. Immobilization rate of rabbit IgG when the coverage of activated groups on the surface of adipic dihydrazide-activated silica was (Δ) 3.0, ($\diamond$) 5.8, (+) 7.3, or ($\blacksquare$) 10.5 μmol of reactive hydrazide groups per gram of silica.

RESULTS AND DISCUSSION

Effect of Support Active Site Coverage on Antibody Immobilization Rate. The first series of studies examined the effect on the rate of antibody immobilization when the support's surface coverage of active sites to which the antibody could be attached was changed. In our model system, the coverage of active hydrazide groups was varied by adjusting the amount of periodate used in converting diol-bonded silica into an aldehyde-activated form (i.e., see the second step in Figure 1) prior to reaction of this support with a dihydrazide reagent (23). The conditions used in this work involved converting 25–100% of the available diol groups into aldehydes for reaction with adipic dihydrazide; under these conditions, the final coverages obtained for active hydrazide groups ranged from 3 to 10.5 $\mu\text{mol/g}$ of silica, or 0.12–0.42 $\mu\text{mol/m}^2$, in agreement with previously reported results (23).

Figure 2 shows the results obtained when a constant amount of oxidized rabbit IgG was reacted with fixed amounts of several silica samples that had various surface coverages of active hydrazide groups. The IgG was oxidized under conditions that produced two available aldehyde groups per antibody (see conditions in Table 1 for 2.0 mol of LyCH/mol of IgG); the total amount of added antibody was 2.2 $\mu\text{mol/L}$ or 0.33 g/L (5 mg in 15 mL), and the amount of silica in the reaction slurry was 13.3 g/L (200 mg in 15 mL). Under these conditions, the active hydrazide groups were present in a 13–67-fold molar excess versus the antibodies, and the maximum degree of antibody immobilization that could be achieved was approximately 0.3 monolayer. It can be seen from Figure 2 that essentially the same rate of antibody immobilization was observed for each surface coverage of active hydrazide groups. The consistency of these results implies that, under these particular conditions, the immobilization rate of IgG was independent of the silica's active group coverage. In other words, the immobilization rate appears to have had a zero-order dependence on the surface concentration of the support's active sites. Similar control studies performed with nonoxidized rabbit IgG in the presence of either diol-bonded silica or the dihydrazide-activated silica did not give rise to any observable immobilization; this indicated that the results in Figure 2 were due to specific covalent attachment rather than a process involving nonspecific physical adsorption of the antibodies to the silica surface (23).

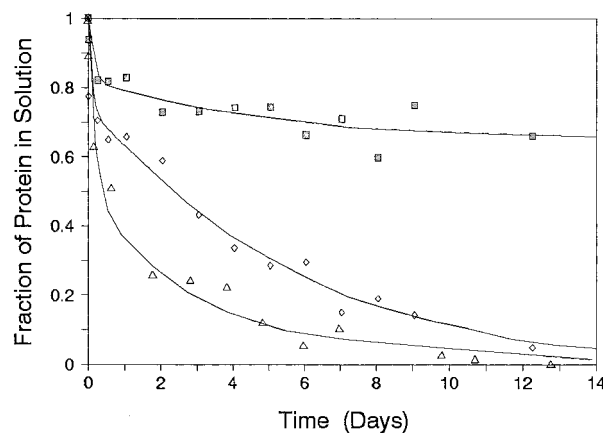


Figure 3. Immobilization rate observed for rabbit IgG for reaction mixtures containing ($\blacksquare$) 2.2, ($\diamond$) 1.1, or (Δ) 0.33 g/L IgG and 13.3 g/L adipic dihydrazide-activated silica. The total amount of IgG used in these experiments corresponded to maximum theoretical coverages on the support of approximately 2.0, 1.0, or 0.3 monolayers, respectively.

Although it may at first be surprising that the immobilization rate is essentially independent of the support's active group coverage, this can be explained by considering the relative size of an antibody versus the average area occupied by an active site on the support. For example, rabbit IgG (MW of 150 kDa) has a Stoke's diameter of approximately 100 Å (31), which gives this protein a cross-sectional area of about 7900 Å² when it is described as a spherical molecule. In contrast to this, a support with an active site coverage of 0.1–0.4 $\mu\text{mol/m}^2$, as used in this work, would have an average area per active site ranging from 1600 to 400 Å², respectively. This means that each rabbit IgG molecule will cover an area occupied by about 19–20 active sites (at 400 Å²/site) or 4–5 active sites (at 1600 Å²/site). The fact that there is a large molar excess of these active sites versus each antibody, and that these active groups are nonmobile, supports a model in which the coverage of these groups is not an important factor in limiting the number of effective collisions between the antibodies and the support during the immobilization process. At much lower surface coverages of the active sites, some dependence of the immobilization rate on active site coverage would be expected, but it is anticipated that the conditions required to achieve this are well below those typically used in affinity supports for HPLC (e.g., 0.02 μmol of active groups/ m^2 would be needed to obtain an average of only one active hydrazide site per 7900 Å², the approximate cross-sectional area of rabbit IgG).

Effect of Antibody Concentration on Immobilization Rate. A second factor considered in these immobilization studies was the role played by antibody concentration in determining the net rate of reaction. Figure 3 illustrates how antibody concentration affected the immobilization rate for rabbit IgG on adipic dihydrazide-activated silica. As in the previous section, the oxidized IgG contained about two available aldehyde groups per antibody and the amount of silica in the reaction slurry was 9–13 g/L; the degree of support activation was 3.0 μmol of hydrazide groups/g of silica for the 0.33 g/L IgG data and 10–11 $\mu\text{mol/g}$ for the 1.1–2.2 g/L IgG results. The initial amount of rabbit IgG in solution for the experiments shown in Figure 3 ranged from 0.33 to 2.2 g/L; these levels correspond to maximum theoretical coverages on the support of 0.3–2 monolayers (i.e., antibody amounts that ranged from below that

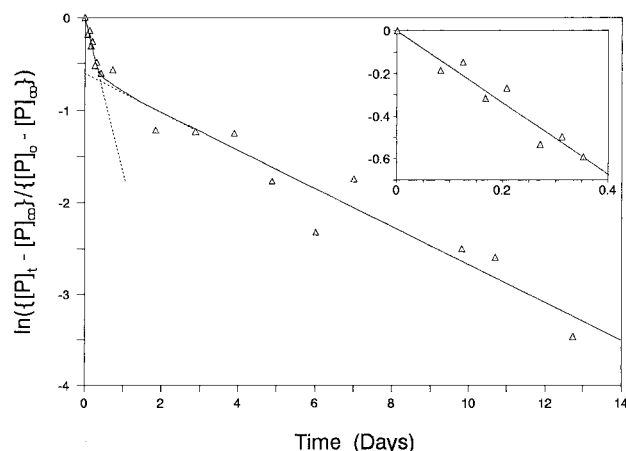


Figure 4. Relative change in solution-phase antibody concentration as a function of time for the immobilization of rabbit IgG to adipic dihydrazide-activated silica. The initial IgG concentration was 0.33 g/L; the level of silica was 13.3 g/L, and the degree of antibody oxidation was 2.0 reactive aldehydes/mol of IgG. In this graph, $[P]_t$ represents the molar concentration of protein in the supernatant at time t , $[P]_0$ is the initial protein concentration in the supernatant, and $[P]_\infty$ is the amount of protein remaining in the supernatant after the immobilization reaction has reached completion. The inset shows an enlarged view of the data points obtained over the first 10 h of the reaction.

needed to saturate the support's surface to amounts in excess of the surface area available for immobilization).

The reaction profiles in Figure 3 (and also those shown earlier in Figure 2) are typical of those seen throughout our immobilization studies with rabbit IgG. Such profiles generally included a fast rate of antibody immobilization during the first day of the reaction, followed by a slower rate that proceeded over much longer times. This type of biphasic behavior has been noted over a shorter time scale for the physical adsorption of human serum albumin (9), lysozyme, β -lactoglobulin, and hemoglobin to silica (21) and for the adsorption of bovine serum albumin, IgG, fibrinogen, and polylysine to poly(vinyl chloride) or methylacrylic-methacrylate copolymers (13).

The immobilization reaction in this study was essentially complete after 12–14 days when working with antibody levels at or below a theoretical support coverage of 1 monolayer (see Figure 2 and data in Figure 3 at 0.33 and 1.1 g/L IgG). For higher amounts of antibody (see data in Figure 3 for 2.2 g/L IgG), the reaction was also complete after 12–14 days, with the excess antibodies remaining in solution as a nonimmobilized fraction. This time frame is several orders of magnitude longer than that observed in any previous studies for the physical adsorption of proteins (including antibodies and silica supports) under mass-transfer-limited conditions (8, 14–21) and suggests a reaction mechanism in which the overall immobilization rate is controlled mainly by the rate of covalent attachment of antibodies to the support.

A more detailed analysis of the antibody immobilization data by using log concentration versus time plots consistently gave a response with two linear regions (see Figure 4). This fits a model in which the net rate of antibody immobilization to dihydrazide-activated silica can be described by two competing pseudo-first-order processes; a similar model has previously been used by others to describe the physical adsorption of various proteins to silica (21). The data obtained by these plots for studies at relatively low antibody levels (e.g., the results in Figure 4 at 0.33 g/L rabbit IgG and 13.3 g/L

silica) gave an apparent first-order rate constant for the fast immobilization reaction of approximately (1.9 ± 0.3) (one SD) $\times 10^{-5} \text{ s}^{-1}$ (half-life, 10 h); the same data gave an apparent first-order rate constant for the second, slower immobilization reaction of (4 ± 1) (one SD) $\times 10^{-6} \text{ s}^{-1}$ (half-life, 2 days). Similar rate constants were obtained at each of the other antibody levels that were tested in this study.

As mentioned earlier, control experiments with diol-bonded or dihydrazide-activated silica and nonoxidized rabbit IgG did not give rise to any observable antibody immobilization. This indicated that neither the fast nor slow immobilization reactions seen in Figure 4 were due to physical adsorption of the antibodies to the support. However, it was found that the combination of oxidized antibodies with diol-bonded silica did give rise to antibody attachment. In this case, a plot of the log of the antibody concentration versus time gave linear behavior (correlation coefficient = 0.9813 for 18 data points obtained over 0–1.2 days), as seen earlier for the dihydrazide-activated supports, indicating that the reaction of oxidized antibodies with diol-bonded silica was also a pseudo-first-order process. Furthermore, the apparent rate constant for the oxidized antibody–diol-bonded silica reaction $[(1.7 \pm 0.1) \times 10^{-5} \text{ s}^{-1}]$ was statistically identical to the value obtained in Figure 4 for the fast reaction of oxidized antibodies with dihydrazide-activated silica. This suggests that it is the remaining diol groups on the silica (and also perhaps primary alcohols that are generated in the third step of the reaction scheme in Figure 1) that are responsible for the fast immobilization process on both types of supports. Although further work is needed to conclusively identify the mechanism for this fast process, one strong possibility is that the aldehyde groups on the oxidized antibodies are forming acetal or hemiacetal linkages with secondary and primary alcohols on the surface of the silica. Regardless of whether this is the actual mechanism for the fast reaction, the results of this control study do indicate that the second, slower immobilization process seen for dihydrazide-activated silica is the one that actually represents the attachment of oxidized antibodies to active hydrazide groups on this support.

Effect of Antibody Coupling Sites on Immobilization Rate. Besides antibody concentration, this report also considered how varying the number of potential coupling sites on an antibody affected its net rate of immobilization. This factor was controlled by varying the oxidation conditions used during the periodate treatment of antibodies prior to immobilization. As discussed in detail previously (24, 25), using different times, temperatures, pHs, or periodate concentrations for antibody oxidation can lead to the production of various amounts of active aldehyde groups on an antibody's carbohydrate residues. This, in turn, leads to different numbers of sites at which hydrazide groups on the support can attach to the antibodies for immobilization.

Figure 5 shows the results obtained for adipic and oxalic dihydrazide-activated silica supports when they were reacted with rabbit IgG containing an average of 0.6–4.6 available aldehyde groups per antibody molecule, as prepared according to the oxidation conditions shown in Table 1. The total amount of antibody used in each case was 0.33 g/L, and the silica content in the reaction slurry was 13.3 g/L. The coverages of active hydrazide groups on the adipic and oxalic dihydrazide-activated supports were both in the range of 5–6 $\mu\text{mol/g}$ of silica (23). The general behavior of the plots in Figure 5 was the same as that noted in the previous studies (see

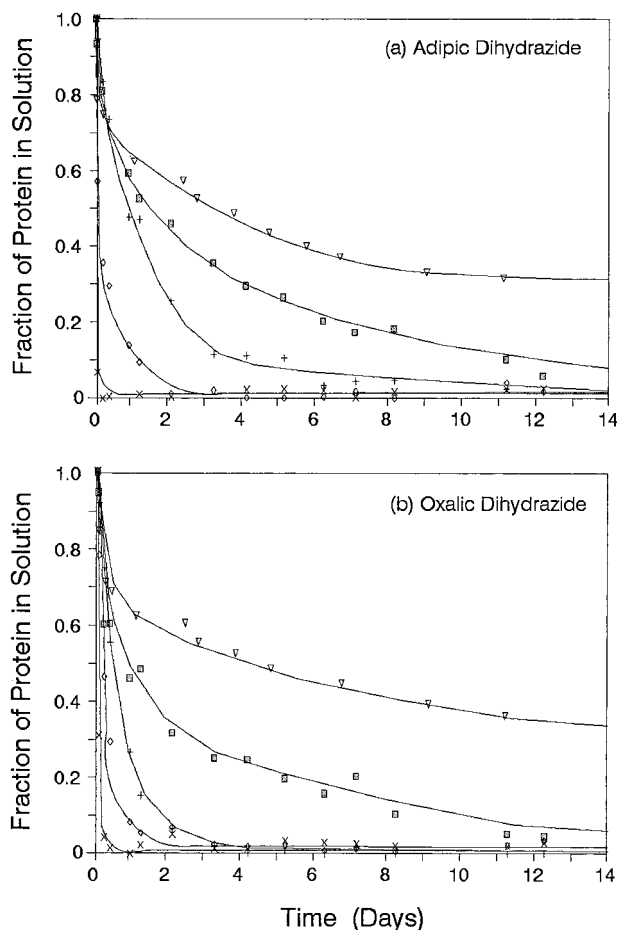


Figure 5. Immobilization rates observed for rabbit IgG containing various amounts of potential coupling sites in the presence of (a) adipic dihydrazide-activated silica or (b) oxalic dihydrazide-activated silica. In panels a and b, the number of potential coupling sites on the antibodies was (∇) 0.6, ($\blacksquare$) 0.9, (+) 2.0, ($\diamond$) 3.6, or ($\times$) 4.6 aldehydes per IgG.

Figures 2–4) in which a fast rate of antibody immobilization occurred during the first day of the reaction, followed by a slower immobilization rate over longer reaction times. Similar rates were observed for the oxalic and adipic dihydrazide supports when antibodies containing 0.6 available aldehyde per IgG were used, but a slightly faster immobilization rate was seen for the oxalic dihydrazide-activated support with antibodies that contained an average of 0.9–4.6 aldehydes per IgG. The exact reason for this apparent difference is still under investigation, but it is probably related to the different local environments that are known to be present for the immobilized oxalic and adipic dihydrazide groups. For example, it has been shown previously that these two reagents yield different fractions of active hydrazide residues as the density of aldehyde groups on silica surface is varied; this behavior is, in turn, related to the different chain lengths of these dihydrazide reagents and their different tendencies to undergo bifunctional attachment to aldehyde-activated silica (23).

It can be seen in Figure 5 that, when the average number of aldehyde groups per antibody was less than one (e.g., 0.6 or 0.9 aldehyde/IgG), a significant fraction of antibodies remained in solution after 14 days of reaction. This occurred because not all of the antibodies possessed coupling sites (i.e., aldehyde groups) under such conditions. For example, the data obtained when working with antibodies that had an average of 0.6 aldehyde per IgG might represent a situation in which

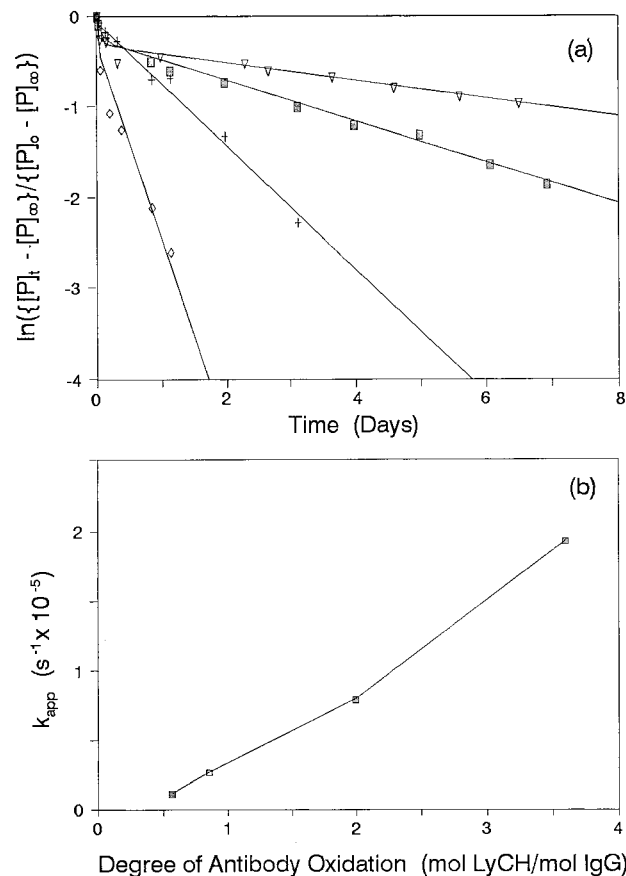


Figure 6. Plots of (a) the relative change in solution-phase antibody concentration as a function of time for the immobilization of rabbit IgG to adipic dihydrazide-activated silica for antibodies containing (∇) 0.6, ($\blacksquare$) 0.9, (+) 2.0, or ($\diamond$) 3.6 available aldehyde groups per IgG and (b) the change in the apparent pseudo-first-order rate constant (k_{app}) for the slow immobilization reaction as a function of the degree of antibody oxidation. The data used in generating these plots were obtained from Figure 5a. All abbreviations used on the y-axis in panel a are the same as those defined in Figure 4.

just under half of the antibodies had no aldehyde groups and the remainder had only one such group per IgG (e.g., if all oxidation took place at only terminal sialic acid residues) (24). Because of this, it was only at higher degrees of oxidation (2.0–4.6 aldehydes per IgG) that essentially complete immobilization of all antibodies was observed.

Examples of first-order kinetic plots that were obtained for the data in Figure 5 are shown in Figure 6. As discussed earlier, the general trend seen was one in which the reaction obeyed pseudo-first-order kinetics with respect to antibody concentration, with two competing reactions (one fast and one slow) being present. However, one interesting feature now observed was the fact that the reaction rates showed a strong dependence on the number of aldehyde groups available on the antibodies for coupling to the dihydrazide-activated support. This was most clearly seen for the slower immobilization process, in which the apparent pseudo-first-order rate constant increased with the average number of aldehyde groups per IgG (see Figure 6b). Such behavior is an indication of the importance of an antibody having the correct orientation in obtaining an effective collision with the support for immobilization. In addition, this result further supports a mechanism in which the net rate of immobilization is controlled by the rate of covalent coupling between the antibody and the support.

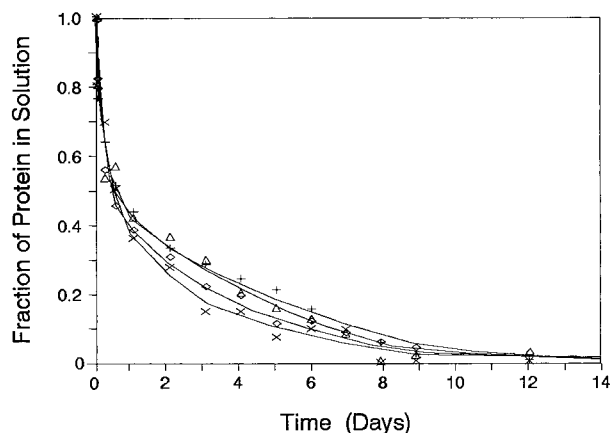


Figure 7. Change in the immobilization rate for rabbit IgG when using silica amounts in the reaction slurry of (+) 20, (◇) 13, (x) 6.7, or (Δ) 3.3 g/L.

Effect of Slurry Density on Antibody Immobilization Rate. A final study was performed to determine the effect of varying the slurry density of the reaction mixture on the observed rate of antibody immobilization. The results are shown in Figure 7. These graphs were generated using antibodies containing an average of 2 aldehyde groups per IgG. The support was adipic dihydrazide-activated silica that contained 10–11 μmol of hydrazide groups per gram of silica. The slurry density was varied by adjusting the volume of buffer present in the reaction mixture while keeping a constant mass of added antibodies and silica (i.e., a ratio of 1 mg of rabbit IgG per 40 mg of silica or a maximum theoretical coverage of 0.3 monolayer for IgG on the silica's surface).

The data in Figure 7 show that only random changes occurred in the immobilization rate when support concentrations of 3.3–20 g/L and IgG concentrations of 0.08–0.5 g/L were used. Kinetic plots made with these data gave the same type of biphasic behavior seen in Figures 4 and 6a; the pseudo-first order rate constants obtained also agreed with those given in the previous sections. The consistency of the results in Figure 7 indicates that the slurry density was not an important factor in controlling the rate of antibody immobilization. This is again consistent with a reaction mechanism in which the net rate of rate of immobilization is controlled mainly by the rate of antibody attachment rather than by antibody mass transfer to the surface of the support.

CONCLUSIONS

This work considered several factors that can potentially affect the immobilization rate of antibodies onto HPLC supports, using rabbit IgG and dihydrazide-activated silica as a model system. The overall immobilization rate was essentially independent of the density of the support's activated sites (when present at a coverage of 0.1–0.4 $\mu\text{mol}/\text{m}^2$) but was strongly influenced by the number of available coupling groups on the antibodies. Increasing the slurry density of the support and antibodies in the reaction mixture had little or no effect on the immobilization rate, and the reaction rate showed only a small change when different types of reagents were used for support activation (e.g., adipic vs oxalic dihydrazide).

In each case studied, the rate of change in the solution-phase antibody concentration gave biphasic behavior which could be described by two competing pseudo-first-order reactions. One of these reactions was relatively fast (half-life, 10 h), while the second proceeded at a much

slower rate (half-life, 2 days). Through control experiments, it was found that both processes required the presence of antibody oxidation, indicating that these reactions were due to covalent linkages rather than due to simple physical adsorption. It was further determined that the fast and slow reactions were most likely the result of the coupling of oxidized antibodies with (1) secondary or primary alcohols on the support and (2) active hydrazide residues on the support, respectively.

The results of this study provide some interesting insights into the kinetics of antibody immobilization and the factors which are most important in influencing the rates of such reactions. For example, the results of this work consistently indicated that antibody attachment (vs mass transfer) was the rate-limiting step in controlling the overall immobilization process; this behavior is much different from that generally seen for the physical adsorption of proteins to surfaces, a reaction which often tends to be mass-transfer-limited (8, 14–17). It is expected that many of the trends reported in this work apply not only to antibodies but also to other proteins during their immobilization to small diameter supports. The data provided in this study should also be useful in providing some initial guidelines regarding the selection of conditions for the preparation of affinity-based HPLC media.

ACKNOWLEDGMENT

This work was supported in part by the National Institutes of Health through Grant RO1 GM44931. M.R.O. was supported in part by a fellowship from the University of Nebraska Center for Biotechnology.

LITERATURE CITED

- (1) Larsson, P.-O. (1984) High-performance liquid affinity chromatography. *Methods Enzymol.* 104, 212–223.
- (2) Walters, R. R. (1985) Affinity chromatography. *Anal. Chem.* 57, 1099A–1114A.
- (3) Phillips, T. M. (1985) High performance immunoaffinity chromatography. *LC Mag.* 3, 962–972.
- (4) Phillips, T. M. (1989) High-performance immunoaffinity chromatography. *Adv. Chromatogr.* 29, 133–173.
- (5) De Frutos, M., and Regnier, F. E. (1993) Tandem chromatographic-immunological analyses. *Anal. Chem.* 65, 17A–25A.
- (6) Afeyan, N. B., Gordon, N. F., Mazsaroff, I., Varady, L., Fulton, S. P., Yang, Y. B., and Regnier, F. E. (1990) Flow-through particles for the high-performance liquid chromatographic separation of biomolecules: perfusion chromatography. *J. Chromatogr.* 519, 1–29.
- (7) Hermanson, G. T., Mallia, A. K., and Smith, P. K. (1992) *Immobilized affinity ligand techniques*, Academic Press, New York.
- (8) Lok, B. K., Cheng, Y. L., and Robertson, C. R. (1982) Protein adsorption on crosslinked polydimethylsiloxane using total internal reflection fluorescence. *J. Colloid Interface Sci.* 91, 104–116.
- (9) Van Dulm, P., and Norde, W. (1983) The adsorption of human plasma albumin on solid surfaces, with special attention to the kinetic aspects. *J. Colloid Interface Sci.* 91, 248–255.
- (10) Cheng, Y. L., Darst, S. A., and Robertson, C. R. (1986) Bovine serum albumin adsorption and desorption rates on solid surfaces with varying surface properties. *J. Colloid Interface Sci.* 118, 212–223.
- (11) Hlady, V., Reinecke, D. R., and Andrade, J. D. (1986) Fluorescence of adsorbed protein layers. I. Quantitation of total internal reflection fluorescence. *J. Colloid Interface Sci.* 111, 555–569.
- (12) Young, B. R., Pitt, W. G., and Cooper, S. L. (1988) Protein adsorption on polymeric biomaterials. II. Adsorption kinetics. *J. Colloid Interface Sci.* 125, 246–260.

- (13) Golander, C.-G., and Kiss, E. (1988) Protein adsorption on functionalized and ESCA-characterized polymer films studied by ellipsometry. *J. Colloid Interface Sci.* 121, 240–253.
- (14) Karamushka, V. I., Denisova, T. I., Sklyarov, A. G., Gurzina, T. G., Meleshevich, S. I., and Ul'berg, Z. R. (1989) Kinetics of sorption immobilization of serum albumin on silicopolymethylsiloxane. *Zh. Prikl. Khim. (S.-Peterburg)* 62, 561–564.
- (15) Norde, W., and Rouwendal, E. (1990) Streaming potential measurements as a tool to study protein adsorption kinetics. *J. Colloid Interface Sci.* 139, 169–176.
- (16) Ramsden, J. J., Roush, D. J., Gill, D. S., Kurrat, R., and Willson, R. C. (1995) Protein adsorption kinetics drastically altered by repositioning a single charge. *J. Am. Chem. Soc.* 117, 8511–8516.
- (17) Voegel, J. C., De Baillou, N., Sturm, J., and Schmitt, A. (1984) Adsorption kinetics of plasma proteins onto silica. *Colloid Surf.* 10, 9–19.
- (18) Hage, D. S., Walters, R. R., and Hethcote, H. W. (1986) Split-peak affinity chromatographic studies of the immobilization-dependent adsorption kinetics of protein A. *Anal. Chem.* 58, 274–279.
- (19) Hage, D. S., and Walters, R. R. (1988) Non-linear elution effects in split-peak chromatography. I. Computer simulations for the cases of irreversible diffusion- and adsorption-limited kinetics. *J. Chromatogr.* 436, 111–135.
- (20) Larew, L. A., and Walters, R. R. (1987) A kinetic, chromatographic method for studying protein hydrodynamic behavior. *Anal. Biochem.* 164, 537–546.
- (21) Sarkar, D., and Chatteraj, D. K. (1993) Activation parameters for kinetics of protein adsorption at silica-water interface. *J. Colloid Interface Sci.* 157, 219–226.
- (22) O'Shannessy, D. J. (1990) Hydrazido-derivatized supports in affinity chromatography. *J. Chromatogr.* 510, 13–21.
- (23) Ruhn, P. F., Garver, S., and Hage, D. S. (1994) Development of dihydrazide-activated silica supports for high-performance affinity chromatography. *J. Chromatogr. A* 669, 9–19.
- (24) Wolfe, C. A. C., and Hage, D. S. (1995) Studies on the rate and control of antibody oxidation by periodate. *Anal. Biochem.* 231, 123–130.
- (25) Hage, D. S., Wolfe, C. A. C., and Oates, M. R. (1997) Development of a kinetic model to describe the effective rate of antibody oxidation by periodate. *Bioconjugate Chem.* 8, 914–920.
- (26) Keener, C. R., Wolfe, C. A. C., and Hage, D. S. (1994) Optimization of oxidized antibody labeling with lucifer yellow CH. *BioTechniques* 16, 894–897.
- (27) Wolfe, C. A. C., and Hage, D. S. (1994) Automated determination of antibody oxidation using flow injection analysis. *Anal. Biochem.* 219, 26–31.
- (28) Curtis, M. A., and Shahwan, G. J. (1988) A high-efficiency open-tubular HPLC reactor using PTFE tubing in a modified serpentine geometry. *LC-GC* 6 (2), 158–164.
- (29) Chattopadhyay, A., and Hage, D. S. (1997) Determination of the diol content of chromatographic supports by capillary electrophoresis. *J. Chromatogr. A* 758, 255–261.
- (30) Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., and Klenk, D. C. (1985) Measurement of protein using bicinchoninic acid. *Anal. Biochem.* 150, 76–85.
- (31) Walters, R. R., Graham, J. F., Moore, R. M., and Anderson, D. J. (1984) Protein diffusion coefficient measurements by laminar flow analysis: method and applications. *Anal. Biochem.* 140, 190–195.

BC970177R

Use of N^{α} -Fmoc-cysteine(*S*-thiobutyl) Derivatized Oligodeoxynucleotides for the Preparation of Oligodeoxynucleotide–Peptide Hybrid Molecules

Sommay Soukchareun,* Jim Haralambidis, and Geoffrey Tregear

The Howard Florey Institute of Experimental Physiology and Medicine, University of Melbourne, Parkville, Victoria 3052, Australia. Received January 14, 1998; Revised Manuscript Received April 17, 1998

The chemical modification of antisense oligodeoxynucleotides (ODNs) by conjugating synthetic peptides of known membranotropic activities to the 3' and/or 5' terminus of ODNs may serve two functions that are important for increasing their bioavailability by protecting the ODNs from exonuclease digestion and facilitated delivery into cells. We have previously reported the preparation of ODN–peptide conjugates by the total synthesis approach. However, by such technology the preparation of ODN–peptide conjugates in amounts sufficient for in vitro functional analysis is at present limited to the syntheses of peptides containing residues without acidolytic deprotection. Requisite to the alternative method of site-specific conjugation, the segment coupling approach is the derivatization of an ODN with a nucleophilic moiety. In this paper, we describe a novel method of functionalizing synthetic ODNs by incorporating *S*-thiobutyl-protected N^{α} -Fmoc-cysteine to aminopropyl-functionalized CPG by standard N^{α} -Fmoc SPPS methodology. The derivatized solid support can be used to synthesize an ODN of any sequence by the phosphoramidite chemistry, and the removal of the *S*-thiobutyl side chain function can be conveniently affected by the standard aminolytic deprotection of ODNs containing 1 M DTT. The purified cysteine-derivatized ODN was shown to react specifically and efficiently with two types of synthetic peptides corresponding to regions within the glycoprotein (gp) of HIV that have been shown to have membranotropic activities: a 18 residue maleimide-derivatized Tat peptide of the transactivator (*tat*) of HIV and a 22 residue peptide corresponding to the carboxyl terminus of gp41(Ca-gp41).

INTRODUCTION

The potential of antisense oligodeoxynucleotides (ODNs) as chemotherapeutic agents for the treatment of AIDS as well as for the control of other viral infections lies in the predictability and specificity by which the antisense ODNs interact with the target mRNA to inhibit protein expression (1). Many instances of specific antisense inhibition have been documented (2, 3). However, the low intracellular bioavailability of ODNs, due to both their susceptibility to enzymatic degradation and their low penetration into cells, are two well-recognized factors that limit their therapeutic potential. To overcome these limitations, a class of chemically modified antisense ODNs was designed that contained peptides covalently linked at the 3'- or 5'-terminus of ODNs. The tethered peptides, usually chosen for their membranotropic activities, serve to protect ODNs from exonuclease digestion and to facilitate intracellular uptake.

There are two general methods for the preparation of structurally discrete and well-characterized ODN–peptide hybrid conjugates: the fragment coupling strategy and the total synthesis method, which requires the consecutive assembly of the peptide and then the ODN on a solid support that has been functionalized with a base-labile handle. With few exceptions (4), the assembly of the peptide is achieved by the N^{α} -fluoren-9-ylmethoxycarbonyl solid-phase peptide synthesis (Fmoc SPPS)

chemistry (5, 6). After the peptide is assembled and the N^{α} -Fmoc group of the terminal amino acid residue is deprotected, reaction with a heterobifunctional linker converts the N-terminus to a protected hydroxyl functionality suitable for solid-phase DNA synthesis. Using such method, we recently reported the synthesis of ODN–peptide conjugates containing the gp41 fusion peptide by the use of α,ω -hydroxycarboxylic acid linker (7). De la Torre et al. (4) extended this approach to the synthesis of a hybrid conjugate containing a nuclear targeting signal peptide. However, one of the major shortcomings of the total synthesis approach is that the chemistry used for the deprotection and cleavage of the peptide and ODN is often incompatible. Thus, the deprotection of peptides by acidolysis, essential to the chemical syntheses of peptides prepared by the N^{α} -Fmoc or N^{α} -Boc chemistry, may also induce depurination and degradation of the preformed ODNs (8, 9).

One way of circumventing the problems associated with the incompatible deprotection chemistry is to prepare conjugates by the classical segment coupling strategy whereby the peptides and ODNs are separately synthesized by standard solid-phase methods and derivatized with functionalities that are mutually reactive. The reaction between the functionalized ODN and peptide components is achieved in solution, after which the conjugate is purified. Methods for the derivatization of synthetic ODNs at the 5'- or 3'-terminus follow two basic routes. The first is performed largely in aqueous solutions, and it involves the conversion of the 5'-hydroxyl groups of the deprotected ODNs to an amino (10, 11), sulfhydryl (12, 13), aldehyde (14), or hydrazide group (15)

* Corresponding author and present address: Department of Immunology, IMM-3, The Scripps Research Institute, 10550 N. Torrey Pines Rd., La Jolla, CA 92037. Telephone: (619) 784-9741. Fax: (619) 784-8361. E-mail: sommay@hermes.scripps.edu.

by postsynthetic derivatization reaction. In an alternative approach, a suitably protected linker containing a masked nucleophilic function such as 5'-mercapto-5'-deoxynucleoside phosphoramidite (16), aminoalkyl alcohol derivatives (17, 18), or triphenylmethyl-*O*-methoxymorpholinophosphite derivatives of 3-mercaptopropanol and 6-mercaptohexanol (19) is incorporated on the 5'-terminus of the ODNs in the last cycle of DNA synthesis.

Although many combinations of chemical groups can be used to achieve chemoselective coupling, the nucleophilic power and specificity of reaction of the thiol functional groups have been used to afford specific and rapid reaction with a wide variety of substrates that have been derivatized with electrophilic groups such as maleimides, isothiocyanates, bromomethyl aromatics, and haloacetamides (20).

Although recent evidence indicates that endonuclease activity in cells is also a significant determinant of ODN stability, the investigations of the stability of antisense oligonucleotides in the presence of cell culture media (21, 22) and cellular lysates (22) indicated that the predominant degradative activities were mediated by enzymes directed toward the 3'-terminus of ODNs. In the development of ODN–peptide conjugates as improved antisense inhibitors, chemical modification strategies that prolong the biological half-life of ODNs may be achieved by ligating a peptide to the 3'-terminus of ODNs. However, existing methods used to introduce a nucleophilic function at the 3'-terminus of synthetic ODNs are laborious. Zuckerman et al. described the synthesis of 3'-thiolated ODNs by linking 3,3'-dithiodipropanol derivatives of thymidine to AP-CPG solid support by a disulfide exchange reaction (23). The modified thiol nucleoside used in this reaction was prepared from the 5'-dimethoxytrityl (DMT) protected thymidine. Phosphorylation of the nucleoside with 2-chlorophenyl-*O,O*-bis(1-benzotriazolyl) phosphate activated the nucleoside for subsequent reaction with 3,3'-dithiodipropanol giving rise to a thiol-containing thymidine (protected as a disulfide), which was subsequently coupled to aminopropyl CPG that was then used to synthesize DNA by the phosphoramidite or phosphotriester chemistry. After DNA synthesis, the exocyclic amino protecting groups of the bases of the 3'-disulfide-containing ODN were deprotected by standard methods. The purified 3'-thiolated ODN was further reacted with 2,2'-dithiodipyridine to form the 3'-*S*-thiopyridyl oligonucleotide derivative, which was subsequently shown to react efficiently with thiolated fluorescein or to cross-link with cysteine-derivatized *Staphylococcal* nuclease protein to generate a hybrid deoxyribonuclease. Functionalizing the 3'-terminus of synthetic ODNs by this technique is laborious since the protocol requires the preparation of four different solid supports, each individually derivatized with a disulfide-linked nucleotide. A similar and more versatile method is the coupling of an activated AP-CPG support with an *O*-DMT-6-mercaptohexanol derivative via a disulfide linkage. Activation of the CPG starts with the reaction of the thiol-derivatized CPG with 2,2'-dithiobis(5-nitropyridine) (DNTP). The DNTP-activated support is then reacted with *O*-DMT-6-mercaptohexanol, giving rise to 3'-thiolated ODNs that are subsequently used to synthesize DNA. The reactivity of the 3'-thiol-derivatized ODN was demonstrated by its efficient conjugation to pyrenyl maleimide (24, 25). The method of native chemical ligation by chemoselective coupling of two protected peptides has been described (26). In this approach, the carboxyl terminus of one peptide is functionalized with a thioester, and the other is functionalized with a cysteine

residue. Thioester exchange produces an unstable thioester-linked intermediate that subsequently rearranges to form a stable peptide bond. An extension of this technique was described that involved the template-directed fusion of 3'-amine-terminated DNA with the DNA–peptide intermediate containing a thioester linkage, resulting in an oligonucleotide–peptide hybrid containing an amide bond (27).

In this paper, we describe the development of a novel strategy for incorporating the thiol nucleophile at the 3'-terminus of an ODN. This method offers several advantages over existing approaches. The masked sulphydryl function is incorporated onto the solid support by standard *N*^α-Fmoc SPPS, using a commercially available *N*^α-Fmoc derivative of thiobutyl-protected cysteine. The cysteine-derivatized solid supports can then be used to synthesize ODNs of any sequence. After DNA synthesis, the protecting groups on the nucleotide bases of the ODNs and the pendant cysteine residue are conveniently removed by treating the support with a mixture of DTT in concentrated aqueous ammonia.

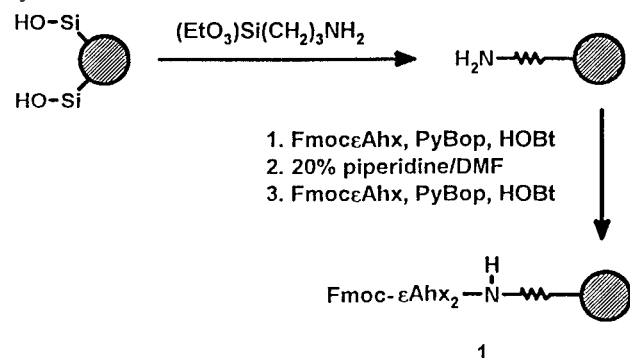
EXPERIMENTAL PROCEDURES

General. Derivatization of AP-CPG as well as the synthesis of the Ca-gp41 and the Tat peptide were performed on the Biolynx 4175 peptide synthesizer (LKB Biochrom, Cambridge, England) using continuous-flow solid-phase peptide synthesis (SPPS) protocols. Automated DNA synthesis was carried out on an Applied Biosystems 380A DNA synthesizer using standard β -cyanoethyl nucleoside phosphoramidites. Amino acid analyses were carried out on a Beckman system 6300 analyzer after hydrolysis of the samples in evacuated, sealed tubes for 24 h at 130 °C with 6 N hydrochloric acid/0.1% phenol. Preparative and analytical reversed-phase high-performance liquid chromatography (RP-HPLC) was carried out on a Waters liquid chromatography system consisting of a Waters 600 multisolvent delivery system connected to a variable wavelength detector. The ODN-(ϵ Ahx)₂-Cys were purified with either a Synchropak semipreparative RP-C18 column (10 × 250 mm, 300 Å pore diameter, 6.5 μ m particle size, Synchrom, Lafayette, IN) with flow rates of 3 mL/min at 260 nm detection. The peptides were purified using a Vydac semipreparative C18 column (10 × 250 mm, 300 Å pore diameter, 6.5 μ m particle size) with buffers composed of aqueous trifluoroacetic acid (0.1% TFA) and acetonitrile in 0.1% TFA. The gel filtration column was connected to an LKB 2138 UV-ICORD detector, and the eluate was dispensed directly into an LKB 7000 Ultrafrac fraction collector. The ODN–peptides were 5'-end labeled using polynucleotide kinase and γ -³²P-[ATP] according to established procedures (7, 28) and analyzed by polyacrylamide gel electrophoresis (PAGE).

Materials. All reagents, unless otherwise stated, were of analytical grade. Controlled pore glass resin (CPG, 200–400 mesh, pore size of 500 Å, Catalog No. 27720) was obtained from Fluka. *N*^α-Fmoc pentafluorophenyl (pfp) esters of amino acids, *N*-Fmoc-cysteine(*S*-thiobutyl)-OH, benzotriazol-1-yl-oxy-tris-pyrrolidinophosphonium hexafluorophosphate (PyBOP), DMF (peptide synthesis grade), and trifluoroacetic acid (TFA, peptide synthesis grade) were from Auspep (Melbourne, Australia).

Derivatization of Controlled Pore Glass Resin with Fmoc- ϵ Ahx. Controlled pore glass (CPG) was first aminated with 3-aminopropyltriethoxysilane (Scheme 1), then two residues of aminohexanoic acid (ϵ Ahx) were coupled to this aminopropyl-CPG (AP-CPG) using Fmoc-

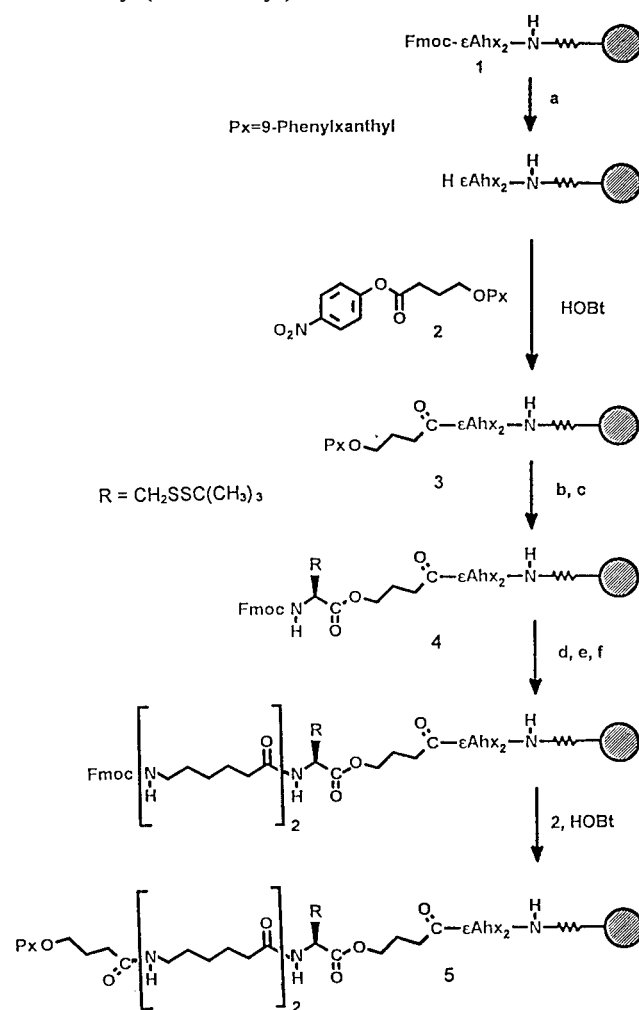
Scheme 1. Preparation of the CPG Resin for Peptide Synthesis



ϵ Ahx-OH by *in situ* activation of the amino acid using PyBOP (benzotriazol-1-yl-oxy-tris-pyrrolidinophosphonium hexafluorophosphate) by continuous-flow Fmoc SPPS as described (7). Briefly, the AP-CPG resin (**1**, Scheme 1) (6 g, 0.9 mmol of amino groups) was coupled *in situ* with a mixture of Fmoc- ϵ Ahx (2.7 mmol, 3 equiv), PyBOP (2.7 mmol, 3 equiv), 1-hydroxybenzotriazole (HOBt) (2.7 mmol, 3 equiv), and *N*-methylmorpholine (NMM) (4.5 mmol, 5 equiv) for 30 min. The resin was washed with dimethylformamide (DMF, 10 min), treated with 20% (v/v) piperidine in DMF (10 min), and washed again prior to coupling with Fmoc- ϵ Ahx. After 30 min acylation, the loading was determined to be 250 μ mol/g by Fmoc test. After the resin was washed, any free residual amino groups were acetylated with acetic anhydride (250 μ L, 1.25 mmol) and 4-*N,N*-(dimethylamino)pyridine (DMAP, 50 mg, 0.4 mmol) in pyridine (1 mL). The resin was washed, deprotected (20% piperidine, 10 min), and reacted with the *p*-nitrophenyl ester of *O*-pixyl-4-hydroxybutyric acid (**2**, Scheme 2), thereby converting the amino terminus to a protected primary aliphatic hydroxyl group. Reaction with the pixyl linker synthon **2** (3 g, 0.45 mmol) was achieved in the presence of HOBt (1.3 mmol, 3 equiv). A pixyl assay (7) performed on a small amount of resin (**3**) indicated a loading of 75 μ mol/g after coupling for 1–2 h. Any free residual amino groups were blocked by acetylation as described above and then washed. Prior to the coupling with *N* $^{\alpha}$ -Fmoc-cysteine(*S*-thiobutyl), removal of the pixyl protecting group was afforded by treatment with 3% (v/v) dichloroacetic acid in dichloromethane (DCM, 10 min). The resin was washed extensively with DCM (10 min) and then DMF.

Coupling of N^α -Fmoc-Cysteine (S-thiobutyl) and Fmoc- ϵ Ahx spacer residues. N^α -Fmoc-cysteine(S-thiobutyl)-OH (0.45 mmol, 6 equiv) was introduced to the ϵ Ahx-derivatized resin **3** (1 g) as the symmetrical anhydride, preactivated with diisopropyl carbodiimide (DIC) (0.225 mmol, 3 equiv) as described previously (7). DMAP (0.045 mmol, 0.3 equiv) dissolved in 0.5 mL of DMF was used as a catalyst. Fmoc test of aliquots of resin **4** gave a loading of $42 \pm 1 \mu\text{mol/g}$ after 1 h acylation. The resin was washed (DMF, 10 min), and then the residual resin-bound hydroxyl groups were inactivated by acylation with DMAP and acetic anhydride as described above. The resin was washed and then deprotected. Fmoc- ϵ Ahx-Pfp (0.168 mmol, 4 equiv) was then coupled in the presence of 4 equiv of HOBt (0.168 mmol, 30 min). The resin was acetylated as described above. Another Fmoc- ϵ Ahx-Pfp residue was coupled in the same way. After Fmoc deprotection, the pixyl linker synthon (**2**) (0.168 mmol, 4 equiv) was coupled in the presence of equimolar amounts of HOBt (0.168 mmol). The pixyl test of resin **5** was determined to be $26 \pm 1 \mu\text{mol/g}$ after 1 h coupling. The

Scheme 2. Functionalization of the CPG Resin with *N*-FmocCys(*S*-thiobutyl)OH^a

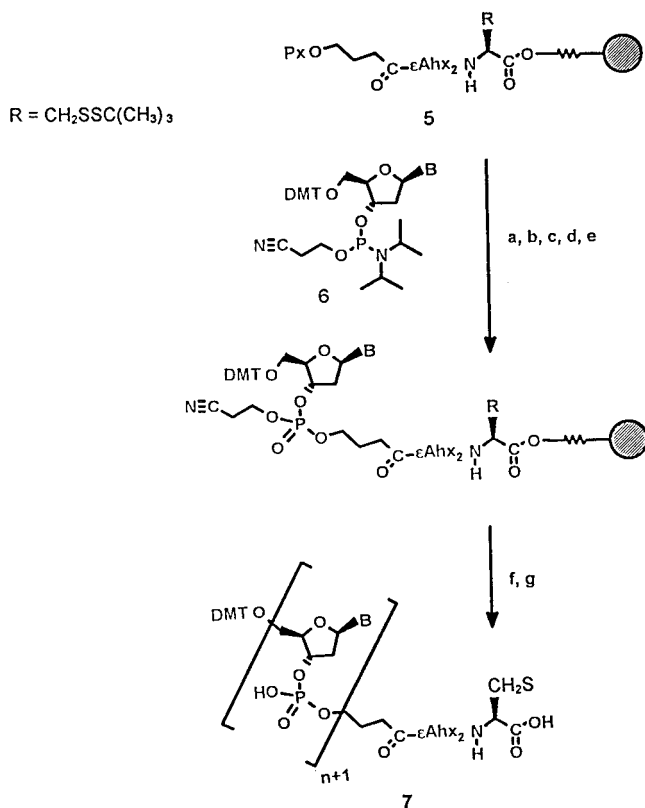


^a Key: (a) 20% piperidine/DMF, (b) 3% DCA/CH₂Cl₂, (c) (*N*-FmocCys(*S*-thiobutyl)-OH)/DIC/DMAP, (d) 20% piperidine/DMF, (e) Fmoc-εAhx₂-OPfp, HOBT, (f) repeat steps (d) and (e).

resin was acetylated as before, washed (DMF, 10 min) and then DCM, and dried under reduced pressure.

DNA Synthesis. The Cys(S-thiobutyl)-functionalized solid support **5** was used directly in solid-phase DNA synthesis using β -cyanoethyl phosphoramidite by the trityl-on method (Scheme 3). Acetylation was affected by 0.1 M acetic anhydride/tetrahydrofuran (THF) and 0.1 M imidazole/THF. After DNA synthesis, the resin was recovered by filtration and treated with concentrated ammonia containing 1 M DTT at 55 °C for 6 h to simultaneously affect deprotection of the side chain of the cysteine residue as well as cleavage of the ODN- ϵ Ahx₂Cys (**7**) from the resin. The resin was removed by filtration and rinsed with concentrated ammonia. In vacuo evaporation of the combined filtrate resulted in a clear residue containing the ODN- ϵ Ahx₂Cys and DTT, which was subsequently redissolved in water prior to purification by HPLC.

Peptide Synthesis. The Tat and Ca-gp41 peptides were synthesized by continuous-flow SPPS on ethylenediamine-treated PepsynK resin (Auspep, Melbourne, Australia) derivatized with hydroxymethylphenolacetic acid using established protocols (29, 30). All acylations were of 30 min duration, and *N*-Fmoc deprotection was affected by 20% (v/v) piperidine in DMF for 10 min. The first residue of the Tat peptide (PTSQSRGDPTGPKE) as well

Scheme 3. DNA Synthesis and Deprotection^a

^a Key: (a) 3% DCA/ CH_2Cl_2 ; (b) **6**, tetrazole; (c) Ac_2O , DMAP; (d) I_2 , H_2O ; (e) repeat steps a–d n times, (f) concentrated NH_3 , rt, 6 h. (g) 1 M DTT, concentrated NH_3 , 55 °C, 6 h.

as the Ca-gp41 peptide (DRVIEVVQGAYRAIRNIPRR-IRQG) were coupled as its symmetrical anhydride using established protocols (30). With the exception of Fmoc-Arg(Pmc)-OH, which was coupled by in situ activation using PyBop, NMM, and HOBt, the remaining amino acids were coupled as their Pfp esters in the presence of HOBt (1.18 mmol, 4 equiv of each). The side chain protecting groups were as follows: *tert*-Bu for Glu, Thr, Asp, and Ser; Boc for Lys, and Pmc for Arg. All acylation reactions were essentially complete within 30 min (as determined by TNBSA test of aliquots of the resin), with the exception of Ile and Val (residues 21 and 22) on the Ca-gp41 peptide, which required at least two consecutive couplings of 30 min duration each (7). The peptide was derivatized by reacting with succinimido-3-maleimidopropanoate (**31**) (1.18 mmol, 4 equiv) in the presence of HOBt (1.18 mmol, 4 equiv) for 1 h. The Ca-gp41 peptide was deprotected and cleaved by a mixture that consisted of ethane dithiol, H_2O , thioanisole, and TFA (5:5:5:85 v/v), while deprotection and cleavage of the Tat peptide was affected by thioanisole in TFA (5:95, v/v). Both deprotection reactions were at room temperature for 2 h.

Both the maleimide-derivatized Ca-gp41 and Tat peptides were purified by RP-HPLC using aqueous 0.1% (v/v) TFA and 0.1% (v/v) TFA in acetonitrile. RP-HPLC purification of the cysteine-derivatized ODNs used buffers consisting of triethylammonium acetate (0.1 M TEAA, pH 7.0) and acetonitrile using a modified protocol (7) that was found to be critical for the efficient removal of DTT: after the sample was injected, an isocratic mode (100% buffer A) was maintained for 15 min before initiating the programmed gradient. After the target peak was collected, two aliquots (200 μL each) of the fraction were put aside. One was diluted with buffer A to 1.5 mL, and the purified ODN was quantified by spectrophotometric

absorbance at 260 nm. The other aliquot was rechromatographed to assess homogeneity. The remainder of the purified cysteine-derivatized ODN was immediately added to the maleimide-derivatized peptide.

Conjugation of Cysteine-Derivatized ODNs with Maleimide–Peptides. Accurately weighed amounts of the maleimide-derivatized Ca-gp41 or Tat peptides were dissolved in TEAA (0.1 M, pH 7.0) at a concentration of 10 mg/mL. For the maleimide-derivatized Ca-gp41 peptide, SDS was added at the concentration of 0.25–0.5% (w/v) prior to the addition of cysteine-derivatized ODN since this was necessary to prevent the nonspecific polymerization of the peptide to the negatively charged backbone of the ODN, with the resultant precipitation of the complex (results not shown). The HPLC eluate containing the cysteine-derivatized ODN (typically 1 mg/10 mL, based on spectrophotometric reading of the purified ODN) was mixed with approximately 10–15 molar excess of maleimide-derivatized peptide. The 10–15 molar excess of maleimide–peptide was found to be optimal for rapid reaction. To monitor the reaction, at timed intervals, aliquots of the reaction mixture (typically 7 μg of the reaction mixture) were lyophilized, resuspended in 30 μL of a solution containing equal volume of H_2O and formamide, and fractionated by denaturing PAGE. When the reaction was deemed complete, the entire reaction mixture was transferred to a stoppered flask and detritylated by treating the reaction mixture with an equal volume of glacial acetic acid at room temperature for 20 min with occasional agitation. The solvents were evaporated in vacuo and dissolved in 3 mL water.

Polyacrylamide Gel Electrophoresis. Assessment of the efficiency of the conjugation reaction was carried out on denaturing 20% (w/v) acrylamide gel containing 0.089 M Tris, 0.089 M boric acid, 2 mM EDTA (TBE), and 7 M urea. The gel was stained with 0.05% (w/v) methylene blue dye (30 min) and washed by immersion in water over 8 h duration (with five changes of water) before being photographed.

Purification of Conjugates by Gel Filtration. The H1- ϵAhx_2 -Cys(Tat) conjugate was purified using a gel filtration column (2 cm width $\times$ 45 cm height) containing Sephadex G-75 gel matrix equilibrated with TEAA (0.1 M, pH 6.7). The flow rate was 250 $\mu\text{L}/\text{min}$.

RESULTS

Derivatization of ODNs with a N^α -Fmoc-Cys(S-thiobutyl) residue. The demonstration that ODN–peptide conjugates was efficiently incorporated to the 3'-terminus of ODNs by the total synthesis method (7) prompted us to investigate the feasibility of synthesizing a simple 3'-polyamide containing a cysteine residue on a CPG support as a novel approach to the derivatization of ODNs with a sulfhydryl function. The success of the strategy depends on several factors: the stability of the S-thiobutyl group to the conditions of both peptide and DNA synthesis and the ease by which it was removed under mild conditions at the end of DNA synthesis. The efficient DNA synthesis (by the phosphoramidite chemistry) onto an AP-CPG support that was functionalized with the 3,3'-dithiodipropyl derivative (**23**) or *O*-dimethoxytrityl-6-mercaptohexanol (**24**) containing a disulfide bond were previously reported, suggesting that protection of the sulfhydryl function by a disulfide bond was stable to conditions of SPSS and DNA synthesis.

In the technique described herein, the AP-CPG support was first derivatized with an acid-labile linker, such as the α,ω -hydroxybutyric acid derivative (**2**, Scheme 3).

Standard *N*^α-Fmoc SPPS was used to incorporate *N*-Fmoc-Cys(*S*-thiobutyl) onto the resin (**4**). At this point, the resin can be coupled with the pixyl linker synthon, thereby converting the amino terminus to an aliphatic hydroxyl group suitable for automated DNA synthesis. However, we discovered that the conjugation reaction between the resultant deprotected cysteine-derivatized ODN (ODN-Cys) and maleimide-derivatized peptide was sluggish (results not shown), possibly because the pendant ODN sterically hindered the accessibility of the thiol group on the side chain of cysteine residue. To investigate this possibility, two aminohexanoic spacer residues were coupled to the *N*-terminal of the Cys(*S*-thiobutyl) residue, prior to reacting with the pixyl linker synthon **2**, as described in the Experimental Procedures. The derivatized support was then used to synthesize a 20mer (GCGTACTCACCAGTCGCCGC) complementary to the primer binding site of HIV-1 mRNA (**7**) by solid-phase DNA synthesis. After DNA synthesis, the resin was treated with concentrated ammonia in the presence of DTT to affect the simultaneous deprotection of the exocyclic bases of the ODNs and the thiol side chain protection of the pendant cysteine (Scheme 3). Removal of DTT is achieved by RP-HPLC, after which the ODN-(ϵ Ahx)₂Cys can be used to ligate with a maleimide peptide in solution. DNA synthesis of resin **5** appeared to proceed well with high coupling efficiency (typically 99%). Chromatographic analysis of the crude product produced a clean profile with the predominant trityl-on peak eluting at 25 min (34% CH₃CN) (Figure 1, panel I) and the detritylated product emerging at 22 min (Figure 1, panel II). The overall yield of ODN-(ϵ Ahx)₂Cys, after RP-HPLC was typically 20 ± 4% based on the initial resin substitution. Ion-spray mass spectrometry of the purified ODN-(ϵ Ahx)₂Cys gave the correct molecular ion (results not shown), suggesting that disulfide protection of the cysteine by the *S*-thiobutyl group was stable to conditions of solid-phase peptide and DNA synthesis. The purified ODN-(ϵ Ahx)₂Cys fraction was used in the solution-phase conjugation with maleimide-derivatized peptides.

Derivatization of Peptides with Succinimido-3-maleipropanoate. Analytical RP-HPLC of the purified Ca-gp41peptide (peak at 20 min, panel I) and that of the crude Tat peptide (peak at 20 min, panel II) are represented in Figure 2. Amino acid analyses of the purified Ca-gp41 peptide and that of the crude Tat peptide showed the correct peptide composition (Tables 1 and 2). These peptides were subsequently used to conjugate with the cysteine-derivatized oligonucleotides.

Conjugation of H1-(ϵ Ahx)₂Cys with the Maleimide-Derivatized Tat Peptide. Evidence for the rapid conjugation reaction of the H1-(ϵ Ahx)₂Cys (1 mg, 10 mL) with approximately 15-fold molar excess of crude maleimide-derivatized Tat peptide is presented in Figure 3. Fractionation of the reaction mixture on denaturing PAGE showed that after 1 min (lane A) virtually all of the H1-(ϵ Ahx)₂Cys had disappeared and a slower migrating band, presumably corresponding to the H1-(ϵ Ahx)₂Cys(Tat) conjugate, had appeared (lane A, arrow 2). In addition, another weaker band (lane A, arrow 1) was obvious, migrating slightly slower than the putative conjugate band. Subsequent studies using a mock reaction with purified H1-(ϵ Ahx)₂Cys (without the peptide nor DTT) (results not shown) indicated that this band corresponds to a small proportion of H1-(ϵ Ahx)₂Cys that had dimerized. That the slower migrating band was ODN dimers was confirmed by the apparent absence of this putative dimerized ODN in lane B, which contains purified H1-(ϵ Ahx)₂Cys in the presence of DTT. Lane

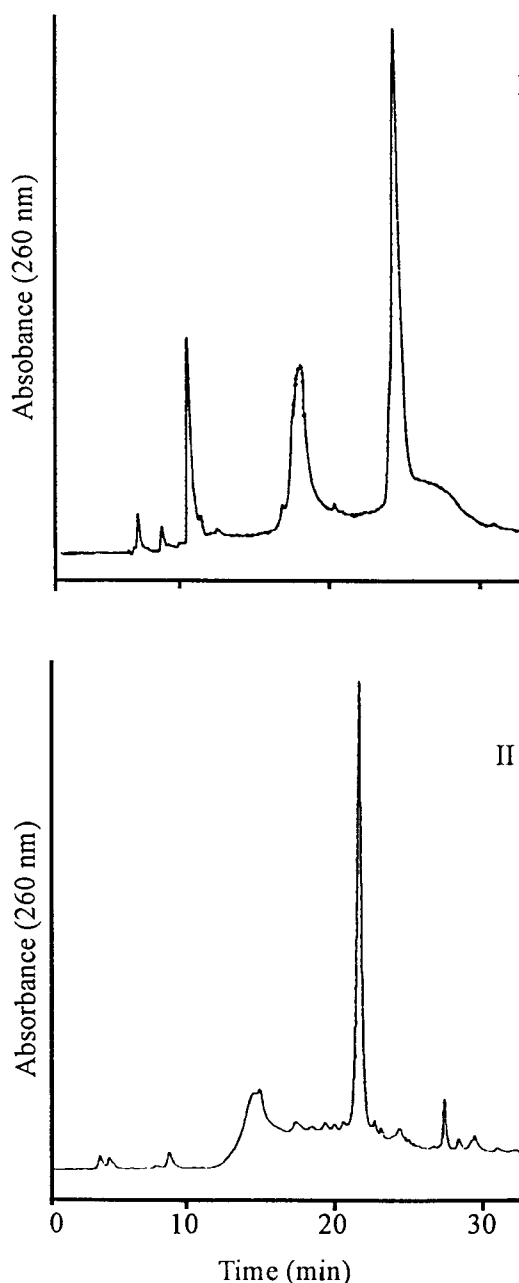


Figure 1. Reversed-phase chromatogram of the crude H1-(ϵ Ahx)₂Cys (trityl-on, panel I) and after detritylation with glacial acetic acid (panel II). Chromatographic conditions: Synchropak C18 column. Flow rate, 1.5 mL/min. Buffer A, TEAA (0.1 M, pH = 7). Buffer B, acetonitrile. Gradient, 0–40% B over 30 min.

C correspond to the normal, unmodified ODN (i.e., H1 ODN with no 3'-polyamide). These results indicate that the ODN-(ϵ Ahx)₂Cys was reactive, and emphasize the importance of immediately mixing the reactive thiol-ODN (after RP-HPLC purification) to the maleimide-peptide solution in order to minimize the loss of the H1-(ϵ Ahx)₂Cys through dimerization. Amino acid analysis of the putative H1-(ϵ Ahx)₂Cys(Tat) product (lane A, arrow 2) after being purified by denaturing PAGE indicates that the amino acid composition was consistent with that of the Tat peptide (Table 1). These results indicate that the solution-phase segment coupling of the H1-(ϵ Ahx)₂Cys with the maleimide-Tat peptide was rapid, specific, and efficient. However, an approximately 2-fold molar excess of the peptide (quantified by amino acid analysis) was observed as compared to the ODN content. The disproportionate peptide-ODN ratio de-

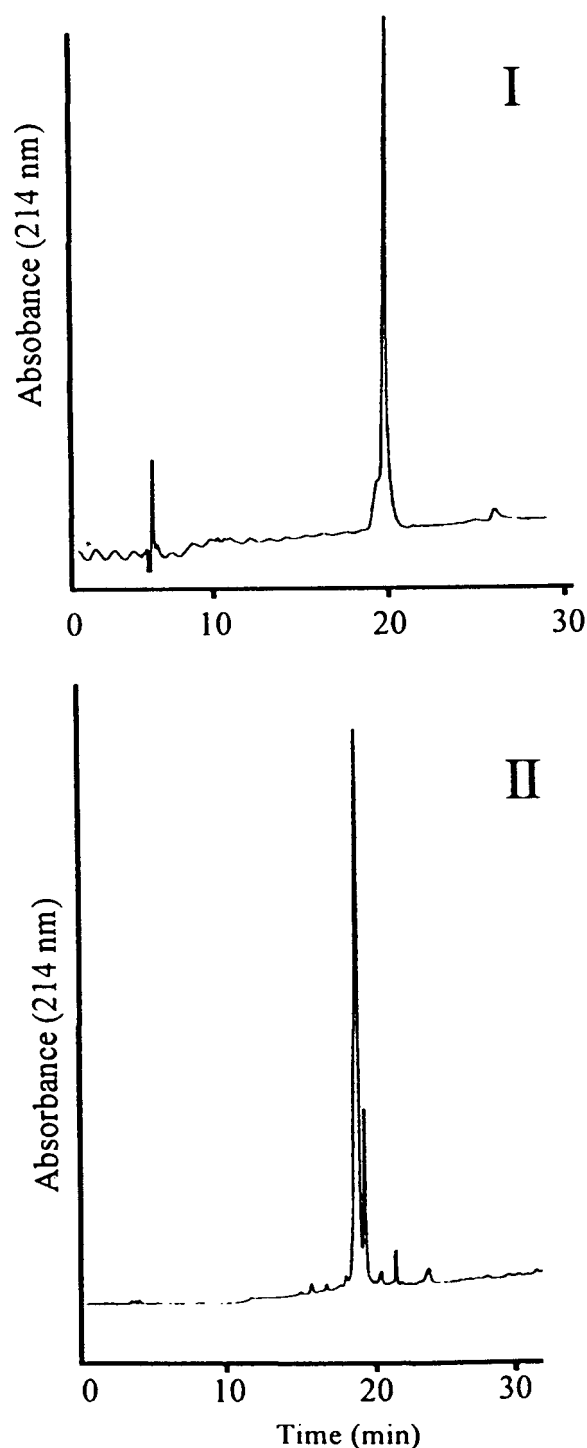


Figure 2. Reversed-phase chromatogram of purified, maleimide-derivatized Ca-gp41 peptide (panel I) and crude maleimide-derivatized Tat peptide (panel II). Chromatographic conditions: Vydak C18 column. Flow rate, 1.5 mL/min. Buffer A, 0.1% aqueous TFA. Buffer B, 0.1% aqueous TFA in acetonitrile. Gradient, 20–60% B over 30 min (top panel), 0–50% B over 30 min (bottom panel).

rived from amino acid hydrolysates of the ODN–peptide does not necessarily indicate that a 1:1 peptide–ODN ratio does not exist. In our previous paper, we also observed disproportionate amino acid to ODN ratios, yet ion-spray mass spectroscopic analyses of the conjugates unequivocally indicated the correct molecular weight (7). The disparity in the peptide and ODN ratio may be related to the different techniques used to quantify the

Table 1. Amino Acid Analyses (24 h Hydrolysis) of Crude Maleimide-Derivatized tat Peptide and Purified H1–(ϵ Ahx) $_2$ Cys (tat) Conjugates

peptide/conjugate	amino acid analyses results
maleimide–Tat peptide (crude)	Asp 1.02 (1), Thr 2.01 (2), Ser 2.01 (2), Glu and Gln 2.10 (2), Pro 2.99 (3), Gly 2.02 (2), Lys 0.83 (1), Arg 1.01 (1)
H1–(ϵ Ahx) $_2$ Cys(tat) ^a	Asp 1.20 (1), Thr 1.94 (2), Ser 2.10(2), Glu and Gln 2.26 (2), Pro 2.88 (3), Gly 2.68 (2), Lys 0.95 (1), Arg 0.96 (1)
H1–(ϵ Ahx) $_2$ Cys(tat) ^b (2:1) ^c	Asp 1.0 (1), Thr 1.78 (2), Ser 2.00(2), Glu and Gln 1.98 (2), Pro 2.42 (3), Gly 2.82 (2), Lys 0.89 (1), Arg 1.0 (1)

^a The conjugate was purified by PAGE. The expected amino acid values are in parentheses. ^b Peak D from gel filtration with Sephadex-75 (from Figure 4). ^c ODN–peptide ratios, quantified by spectrophotometric absorbance and amino acid analysis, respectively.

Table 2. Amino Acid Analyses (24 h Hydrolysis) of Maleimide-Derivatized Ca-gp41 Peptide and H1–(ϵ Ahx) $_2$ Cys (Ca-gp41) Conjugates Purified by PAGE

peptide/conjugate	amino acid analyses results
Maleimide–Ca-gp41 peptide (crude)	Asn and Asp 2.10 (2), Gln and Glu 3.25(3), Pro 0.94 (1), Gly 2.23 (2), Ala 2.25 (2), Val 2.22 (3), Ile 3.47 (4), Tyr 1.11 (1), Arg 6.34 (6)
H1–(ϵ Ahx) $_2$ Cys (Ca-gp41) (1:1) ^a	Asn and Asp 2.45 (2), Gln and Glu 3.41(3), Pro 1.54 (1), Gly 5.6 (2) ^b , Ala 2.36 (2), Val 2.23 (3) ^c , Ile 3.45 (4), Tyr 0.88 (1), Arg 5.53 (6)

^a ODN–peptide ratios, quantified by spectrophotometric absorbance and amino acid analysis, respectively. The expected amino acid values are in parentheses. ^b High glycine value due to hydrolyzed DNA, see text for explanation. ^c Incomplete hydrolysis.

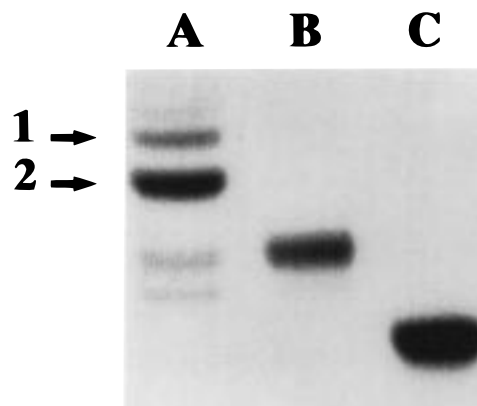


Figure 3. Denaturing PAGE analysis of the conjugation of the H1– ϵ Ahx $_2$ Cys and maleimide Tat peptide. Lane A: crude reaction mixture after 1 min. Lane B: purified H1– ϵ Ahx $_2$ Cys, in the presence of DTT. Lane C: Normal ODN. Arrow 2 corresponds to the putative H1– ϵ Ahx $_2$ Cys(Tat) conjugate, and arrow 1 corresponds to the H1– ϵ Ahx $_2$ Cys dimer. The gel was stained with methylene blue dye and photographed.

two components of the hybrid molecule, i.e., amino acid analyses for peptides and spectrophotometric absorbance of ODNs.

The RP-HPLC chromatogram of the reaction mixture taken at different time points showed a peak that eluted at the same time as the starting H1–(ϵ Ahx) $_2$ Cys (results not shown), thus making the purification of the H1–(ϵ Ahx) $_2$ Cys(Tat) conjugates by RP-HPLC problematic. The coelution of the H1–(ϵ Ahx) $_2$ Cys(Tat) conjugate and H1–(ϵ Ahx) $_2$ Cys under RP-HPLC conditions is perhaps not surprising given that the Tat peptide is hydrophilic and uncharged. Because the Tat peptide is electrically

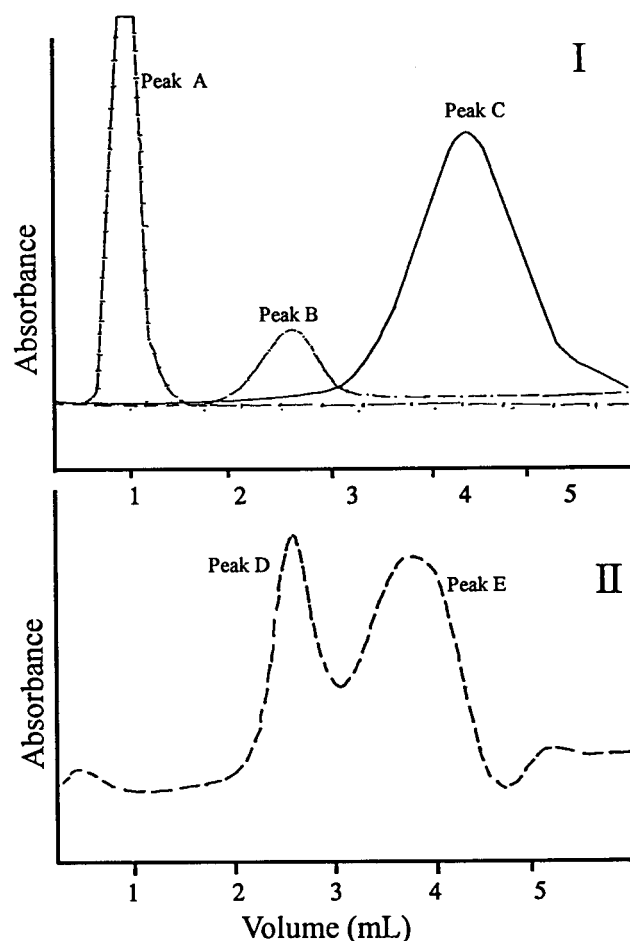


Figure 4. Size-exclusion chromatography using Sephadex G-75. Panel I: The samples (shown superimposed) were analyzed separately. Dextran sulfate (peak A, $\lambda = 220$ nm), H1-(ϵ Ahx)₂Cys (peak B, $\lambda = 254$ nm), maleimide-Tat peptide (peak C, $\lambda = 220$ nm). Panel II: The crude reaction mixture containing H1-(ϵ Ahx)₂Cys and maleimide Tat peptide ($\lambda = 220$ nm). Peak D is the H1-(ϵ Ahx)₂Cys(Tat) conjugate, and peak E corresponds to excess peptide. Chromatographic conditions: Buffer, TEAA (0.1 M, pH = 6.7). Flow rate, 250 μ L/mL.

neutral, the use of ion-exchange liquid chromatography to isolate the conjugate product from the unmodified cysteine-derivatized ODN is also precluded. Taking advantage of the large MW difference between the H1-(ϵ Ahx)₂Cys(Tat) conjugate (approximate MW of 8500 Da) and the maleimide-derivatized Tat peptide (approximate MW of 1400), the removal of excess peptide from the reaction mixture by size exclusion gel filtration was attempted. The separate analyses of the ODN and the tat peptide using identical chromatographic conditions showed that the ODN was well separated before the Tat peptide (Figure 4, panel I), suggesting that it was feasible to use size-exclusion chromatography to purify the H1-(ϵ Ahx)₂Cys(Tat). Importantly, the gel filtration profile of the reaction mixture containing the H1-(ϵ Ahx)₂Cys and the Tat peptide with detection at 220 nm clearly showed two well resolved peaks (Figure 4, panel II). One peak, D, presumably corresponded to the higher MW components of the reaction mixture [i.e., the conjugates and any remaining unreacted H1-(ϵ Ahx)₂Cys], and the other, peak E, corresponded to excess maleimide-Tat peptide. Denaturing PAGE analyses of the purified fractions showed that peak D contained ODN material, presumably the H1-(ϵ Ahx)₂Cys(Tat) conjugate (Figure 5, lane C) that migrated with lower electrophoretic mobility than the H1-(ϵ Ahx)₂Cys reactant (lane A). To

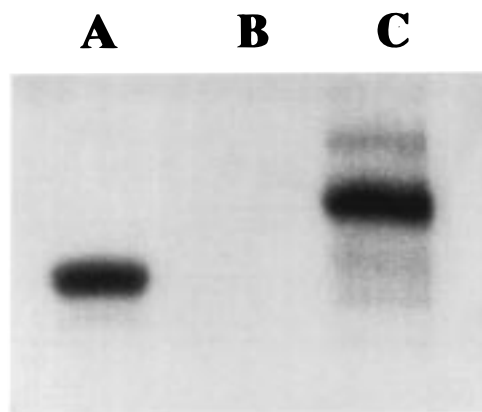


Figure 5. Denaturing PAGE analysis of fractions from Sephadex G-75 gel filtration. Lane A: H1-(ϵ Ahx)₂Cys, in the presence of DTT. Lane B: Peak E from Figure 4 (presumably containing only the Tat peptide) loaded at 5 times the concentration of the sample shown on lane C. Lane C: Peak D from Figure 4 is the putative H1-(ϵ Ahx)₂Cys(Tat) conjugate. The gel was stained with methylene blue dye and photographed.

further demonstrate that the peptides were efficiently removed from the conjugate, peak E (theoretically containing only the maleimide-Tat peptide) was worked up to the same concentration as peak A, based on absorbance reading at 260 nm but loaded on the gel at 5-fold the amount of peak D (Figure 5, lane B). The absence of a band in this lane clearly indicates that no ODN was present in this fraction. However, since the peptide is not visible under these denaturing PAGE conditions, the presence of a band with a lower electrophoretic mobility alone, although suggestive of the presence of H1-(ϵ Ahx)₂Cys(Tat) conjugate (Figure 5, lane C), does not necessarily demonstrate complete removal of the excess peptide by gel filtration. Amino acid analysis of fraction D showed that the amino acid composition (Table 1) as well as the peptide and ODN molar ratio (2:1) were within acceptable levels. However, the comparative ratio of ODN to peptide, for reasons already discussed, does not provide unambiguous determination of the actual ratio present. We attempted to analyze the purified conjugate by ion-spray mass spectrophotometry. However, due to reasons that are not entirely clear to us, we did not obtain a usable spectra. The yield of conjugates after gel filtration purification, as assessed by spectrophotometric absorbance of the products at 260 nm, was typically 34–42%. This yield is perhaps low, but since it was determined by spectrophotometric absorbance of the resulting conjugate after purification, the lower than optimal yields may also reflect inevitable losses due to sample isolation and handling.

To further demonstrate the reactivity of the cysteine-derivatized ODNs, the H1-(ϵ Ahx)₂Cys (200 μ g, 2 mL) was also reacted with 14 molar excess of the maleimide-derivatized Ca-gp41 peptide [500 μ g in 500 μ L 0.1 M TEAA containing 0.3% (w/v) SDS]. In this reaction, it was necessary to add 0.3% (w/v) SDS to the peptide mixture prior to the addition of the ODN reactant in order to abrogate the electrostatic interaction of the positively charged Ca-gp41 peptide and the negatively charged ODN, which otherwise would lead to the aggregation and insolubility of the ODNs (results not shown). Due to problems associated with aggregation and insolubility of the ODN-peptide, assessment of the reaction kinetics by RP-HPLC was problematic (results not shown). Twenty-four hours after the commencement of the reaction, an aliquot of the reaction mixture (after ethanol precipitation to remove SDS, which inhibits the

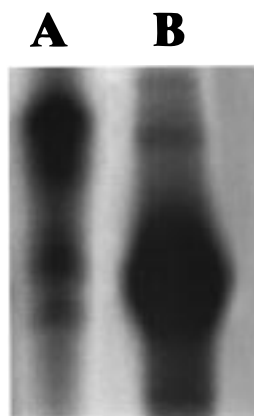


Figure 6. Denaturing PAGE analysis of 5'-end labeled H1- ϵ Ahx₂Cys (lane A) and that of the reaction mixture consisting of H1- ϵ Ahx₂Cys and maleimide Ca-gp41 peptide (containing 0.5% (w/v) SDS after ethanol precipitation, lane B).

PNK reaction) was labeled with γ -[³²P]-ATP and fractionated by denaturing PAGE. The loss of a band that comigrated with ODN-(ϵ Ahx)₂Cys (Figure 6, lane B), concomitant with the emergence of a band that migrated with retarded mobility (Figure 6, lane A) suggested complete reaction. Amino acid analysis of the putative conjugate product (i.e., the band with retarded mobility) after purification by PAGE showed an amino acid composition that was consistent with the Ca-gp41 peptide (Table 2). The low Ile and Val values are probably due to incomplete hydrolysis; the high glycine is probably related to the presence of the ODN (7). The purified sample, after extensive dialyses, was subjected to mass spectrophotometric analyses. However after several attempts, a reliable spectrum could not be attained. It should be noted that at the time that this study was undertaken, mass spectrophotometric analysis of ODNs of this length was still experimental, and the failure to obtain a reliable spectrum might be related to technical difficulties in analyzing this type of ODN–peptide conjugates.

DISCUSSION

Two different types of peptides, the 14 residue segment of the HIV transactivator (*tat*) protein (Tat), and the 24 residue segment of the carboxyl terminus of the gp41 of HIV (Ca-gp41) were used to conjugate with cysteine-functionalized ODNs. Although the precise function of the highly conserved Ca-gp41 peptide remains to be clarified, several lines of evidence suggested a role for this peptide in HIV-mediated cytopathy (32–34). Furthermore, the Ca-gp41 peptide is among the most highly amphiphilic α -helices documented to date (35), and this structural feature was shown to impart membranotropic activities by causing the fusion and lysis of artificial membranes (36–38). The 86 residue *tat* protein of HIV is produced in the cytoplasm of the HIV-infected cell and subsequently translocated into the nucleus where it binds to the long terminal repeats (LTR) of the provirus and promotes transcription of HIV genes (39). The *tat* protein consists of several structurally well-defined domains. A 14 residue segment at the carboxyl terminus of *tat* was shown to be important in promoting the virus' internalization into cells (40), and conjugation of this 14 residue synthetic peptide corresponding to immunoglobulin Fab fragments, which are normally poorly internalized, enhanced the cellular internalization of the pendant Fab fragments in carcinoma cells (41). The known membranotropic activity of the Ca-gp41 and *tat* peptide suggested

that conjugating these peptides to cysteine-derivatized ODNs might enable the passage of ODNs across cell membranes.

The preparation of ODN–peptide hybrid conjugates of a defined structure by the solution-phase chemical ligation approach necessitates the functionalization of ODNs and peptides with mutually reactive moieties. Because solid-phase DNA synthesis normally proceeds in the 3' to 5' direction, documented procedures describing the functionalization of the 3'-terminus of ODNs with a sulfhydryl group are tedious and require the derivatization of the solid support with a chemical synthon that contains a suitably protected sulfhydryl function. At present, techniques suitable for the preparation of 3'-thiol functionalized ODNs are relatively rare as compared to the plethora of techniques describing the functionalization of synthetic ODNs at the 5'-terminus. Furthermore, existing methodologies describing the introduction of a sulfhydryl function at the 3'-termini of ODNs are arduous. For example, the method described by Zuckerman et al. required multiple steps, involving the derivatization of nucleotide monomers with 3,3'-dithiodipropyl followed by its reaction with S-2-thiopyridyl-2-mercaptoethanol (23). Functionalizing the 3'-terminus of synthetic ODNs by this technique requires the preparation of four different solid supports, each individually derivatized with a disulfide-linked nucleotide (i.e., deoxyadenosine, deoxyguanosine, deoxycytidine, and thymidine). A simpler method of functionalizing synthetic ODNs involves the derivatization of CPG with mercaptohexyl derivatives reported by Kumar et al. (24, 25). Another method of synthesizing ODN–peptide hybrids whereby the peptide was ligated to 3'-end of ODNs was by the template-directed two-step reaction involving site-specific ligation of amine-terminated ODN to ODN–peptide linked by thioester linkage (27).

As a novel method for derivatizing synthetic ODNs, the thiobutyl-protected cysteine-derivatized CPG resin described in this study offers several distinct advantages. The derivatized resin can be used to efficiently synthesize ODN of any sequence by standard methods of solid-phase DNA synthesis. The ability to synthesize the ODN by the trityl-on method and the ease by which the DMT group is removed by mild acid treatment also enable ready purification without the need for additional treatment with silver nitrate to affect removal of the trityl group as described for the synthesis of C6 modifier developed by Conolly et al. (19). In addition, *N*^α-Fmoc-thiobutyl-protected Cys is commercially available, is stable to the conditions of DNA synthesis and base deprotection, and can be efficiently incorporated into the 3'-polyamide by standard *N*^α-Fmoc SPPS chemistry.

The HI ODN-(ϵ Ahx)₂Cys described in this study was shown to react specifically and rapidly with maleimide-derivatized peptides. We also attempted to conjugate the 20 residue highly hydrophobic maleimide-derivatized gp41 peptide with the H1 ODN-(ϵ Ahx)₂Cys. However, attempts at the conjugation were not successful due to the insolubility of the peptide. Results of the conjugation reactions described in this study highlight a well recognized caveat of the solution-phase segment coupling approach to the chemical synthesis of ODN–peptide conjugates, that is, the poor solubility properties of some peptides may undermine the efficiency of the conjugation reaction. The conjugation of the Ca-gp41 peptide exemplifies this. Although soluble in aqueous solvents, in the absence of SDS the Ca-gp41 peptide nevertheless interacts with and precipitates the cysteine–ODN. Furthermore, the precipitation of ODNs induced by the Ca-gp41

peptide which is not hydrophobic illustrates the possibility that precipitation of the ODN-peptide conjugate is not necessarily only confined to hydrophobic, poorly soluble peptides per se but extends also to peptides that bind avidly to ODNs.

The present study demonstrated that the success of the segment coupling approach depends on the establishment of appropriate chromatographic systems that are capable both of monitoring the progress of the conjugation reactions but can also be used as a purification tool to remove excess peptide reactants from the conjugates. Although methodologies for RP-HPLC of peptides and ODNs are well established, thus far techniques for the efficient purification of all types of ODN-peptide hybrids are not established. The major problem associated with adapting currently accepted protocols for HPLC purification of peptides and ODNs to the purification of ODN-peptide conjugates is that RP-HPLC analyses of ODNs and peptides uses incompatible buffers. Whereas isolation of peptides by RP-HPLC uses acidic TFA-based buffers with detection at 214 nm/220 nm, RP-HPLC of ODNs uses TEAA at neutral pH with detection at 260 nm. Furthermore, since RP-HPLC fractionate compounds based predominantly on hydrophobic interactions, for conjugate products in which the peptide moiety is small and/or hydrophilic, RP-HPLC analysis may be of limited value (7). Although gel filtration has certain disadvantages (slow flow rate, poor resolution), it offers the advantage of having a chromatographic support and buffer system (TEAA) that can accommodate monitoring and separation of both peptides and ODNs simultaneously. As demonstrated in this paper, for the purification of the H1 ODN-(ϵ Ahx)₂Cys(Tat) conjugate, size-exclusion chromatography may be useful in isolating the conjugate from excess peptide.

ACKNOWLEDGMENT

This project was funded by the Commonwealth AIDS Research Grants Council by a postgraduate scholarship to Sommay Soukchareun and by a project grant. The Howard Florey Institute is supported by a block grant from the National Health and Medical Research Council of Australia.

LITERATURE CITED

- (1) Uhlman, E., and Peyman, A. (1990) Antisense oligonucleotides: A new therapeutic principle. *Chem. Rev.* 90, 543-584.
- (2) Wagner, R. W., Matteucci, M. D., Lewis, J. C., Gutierrez, A. J., Moulds, C., and Froehler, B. C. (1993). Antisense gene inhibition by oligonucleotides containing C-5 propyne pyrimidines. *Science* 260, 1510-1513.
- (3) Wagner, R. W. (1994) Gene inhibition using antisense oligodeoxynucleotides. *Nature* 372, 333-335.
- (4) de la Torre, B. G., Avino, A., Tarrason, G., Piulats, J., Albericio, F., and Eritja, R. (1994) Stepwise solid-phase synthesis of oligonucleotide-peptide hybrids. *Tetrahedron Lett.* 35, 2733-2736.
- (5) Juby, C. D., Richardson, C. D., and Brousseau, R. (1991) Facile preparation of 3' oligodeoxynucleotide-peptide conjugates. *Tetrahedron Lett.* 32 (7), 879-882.
- (6) Robles, J., Pedrosa, E., and Grandas, A. (1994) Stepwise solid-phase synthesis of the nucleopeptide Phac-Phe-Val-Ser-(p3'-ACT)-Gly-OH. *J. Org. Chem.* 59, 2482-2486.
- (7) Soukchareun, S., Tregear, G. W., and Haralambidis, J. (1995) The preparation and characterization of antisense oligodeoxynucleotide-peptide hybrids containing viral fusion peptides. *Bioconjugate Chem.* 6, 43-53.
- (8) Eritja, R., Pons, A., Escarceller, M., Giral, E., and Albericio, F. (1991) Synthesis of defined peptide-oligonucleotide hybrids containing a nuclear transport signal sequence. *Tetrahedron* 47, 4113-4120.
- (9) Hakala, H., Oiranen, M., Saloniemi, E., Gouzaev, A., and Lonnberg, H. (1992) Acid-catalyzed depurination of di-, tri- and poly deoxyribonucleotides: effect of molecular environment on the cleavage of adenine residue. *J. Phys. Org. Chem.* 5, 824-828.
- (10) Chollet, A., and Kawashima, E. H. (1985) Biotin-labeled synthetic oligodeoxyribonucleotides: chemical synthesis and uses as hybridization probes. *Nucleic Acids Res.* 13, 1529-1541.
- (11) Chu, B. C. F., Wahl, G. M., and Orgel, L. E. (1983) Derivatization of unprotected polynucleotides. *Nucleic Acids Res.* 11, 6513-6529.
- (12) Bischoff, R., Coull, J. M., and Regnier, F. E. (1987) Introduction of 5'-terminal functional groups into synthetic oligonucleotides for selective immobilization. *Anal. Biochem.* 164, 336-344.
- (13) Chanh T. C., Dreesman, G. R., Kanda, P., Linette, G. P., Sparrow, J. T., Ho, D. D., and Kennedy, R. C. (1986) Induction of anti-HIV neutralizing antibodies by synthetic peptides. *EMBO J.* 5, 3065-3071.
- (14) Kremsky, J. N., Wooters, J. L., Dougherty, J. P., Meyer, R. E., Collins, M., and Brown, E. L. (1987). Rapid hybridization kinetics of DNA attached to submicron latex particles. *Nucleic Acids Res.* 15, 2891-2909.
- (15) Ghosh, S. S., Kao, P. M., McCue, A. W., and Chappelle, H. L. (1990) Use of maleimide-thiol coupling chemistry for efficient syntheses of oligonucleotide-enzyme conjugate hybridization probes. *Bioconjugate Chem.* 1, 71-76.
- (16) Tung, C. H., Rudolph, J. M., and Stein, S. (1991). Preparation of oligonucleotide-peptide conjugates. *Bioconjugate Chem.* 2, 464-465.
- (17) Agrawal, S., Christodoulou, C., and Gait, M. J. (1986) Efficient methods for attaching nonradioactive labels to the 5' ends of synthetic oligodeoxyribonucleotides. *Nucleic Acids Res.* 14, 6227-6245.
- (18) Emson, P. C., Arai, H., Agrawal, S., Christodoulou, C., and Gait, J. (1989) Nonradioactive methods of in situ hybridization: visualization of neuroendocrine mRNA. *Methods Enzymol.* 168, 753-761.
- (19) Conolly, B. A. (1985) Chemical synthesis of oligonucleotides containing a free sulphhydryl group and subsequent attachment of thiol specific probes. *Nucleic Acids Res.* 13 (12), 4486-4503.
- (20) Agrawal, S. (1994) in *Protocols for Oligonucleotide Conjugates: Synthesis and Analytical techniques* (Agrawal, S., Ed.) Vol. 26, pp 93-117, Humana Press, Totowa, NJ.
- (21) Morvan, F., Porumb, H., Degols, G., Lefebvre, I., Pompon, A., Sproat, B. S., Rayner, B., Malvy, C., Lebleu, B., and Imbach, J. L. (1993) Comparative evaluation of seven oligonucleotide analogues as potential antisense agents. *J. Med. Chem.* 36 (2), 280-287.
- (22) Wickstrom, E. (1986) Oligodeoxynucleotide stability in subcellular extracts and culture media. *J. Biochem. Biophys. Methods* 13, 97-102.
- (23) Zuckerman, R., Corey, D., and Schultz, P. (1987) Efficient methods for attachment of thiol specific probes to the 3'-ends of synthetic oligodeoxyribonucleotides. *Nucleic Acids Res.* 15 (13), 5305-5321.
- (24) Kumar, A. (1993) A rapid solid-phase method for the synthesis of 3'-thiol group containing oligonucleotides. *Nucleosides Nucleotides* 12 (7), 729-736.
- (25) Kumar, A. (1993). A versatile solid-phase method for the synthesis of masked 3'-thiol group containing oligonucleotides. *Nucleosides Nucleotides* 10 (12), 1047-1059.
- (26) Dawson, P. E., Muir, T. W., Clark-Lewis, I., and Kent, S. B. H. (1994) Synthesis of proteins by native chemical ligation. *Science* 266, 776-779.
- (27) Bruick, R. K., Dawson, P. E., Kent, S. B. H., Usman, N., and Joyce, G. F. (1996) Template-directed ligation of peptides to oligonucleotides. *Chem. Biol.* 3, 49-56.
- (28) Maniatis, T., Fritsch, E. F., and Sambrook, J. (1989) *Molecular cloning: a laboratory manual*, Cold Spring Harbor University Press, Cold Spring Harbor, NY.

- (29) Sarin, V. K., Kent, B. H., Tam, J. P., and Merrifield, R. B. (1981) Quantitative monitoring of solid-phase peptide synthesis by the ninhydrin reaction. *Anal. Biochem.* 117, 147–157.
- (30) Atherton, E., and Sheppard, R. C. (1989) *Solid-phase Peptide Synthesis: A practical approach using the Fmoc-Polyamide Technique*, IRL Press, Oxford.
- (31) Ede, N. J., Tregear, G. W., and Haralambidis, J. (1994) Routine preparation of thiol oligonucleotides: application to the synthesis of oligonucleotide–peptide hybrids. *Bioconjugate Chem.* 5, 373–378.
- (32) Fisher, A. G., Ratner, L., Mitsuya, H., Marselle, L. M., Harper, M. E., Broder, S., Gallo, R. C., and Wong-Staal, F. (1986) Infectious mutants of HTLV–III with changes in the 3' region and markedly reduced cytopathic effects. *Science* 233, 655–659.
- (33) Kowalski, M., Potz, J., Basiripour, L., Dorfmann, T., Goh, W. C., Terwilliger, E., Dayton, A., Rosen, C., Hasseltine, W., and Sodroski, J. (1987). Functional regions of the envelope glycoprotein of human immunodeficiency virus type 1. *Science* 237, 1351–1355.
- (34) Lee, S.-J., Hu, W., Fisher, A. G., Looney, D. J., Kao, V. F., Mitsuya, H., Ratner, L., and Wong-Staal, F. (1989) Role of the carboxy-terminal portion of the HIV-1 transmembrane protein in viral transmission and cytopathogenicity. *AIDS Res. Hum. Retroviruses* 5, 441–449.
- (35) Eisenberg, D., and Wesson, M. (1990) The most highly amphiphilic alpha-helices include two amino acid segments in human immunodeficiency virus glycoprotein 41. *Biopolymers* 29, 171–177.
- (36) Fujii, G., Horvath, S., Woodward, S., Eiserling, F., and Eisenberg, D. (1992) A molecular model for membrane fusion based on solution studies of an amphiphilic peptide from HIV gp41. *Protein Sci.* 1, 1454–1464.
- (37) Gawrisch, K., Han, K.-H., Yang, J.-S., Bergelson, L. D., and Ferretti, J. A. (1993) Interaction of peptide fragment 828–848 of the envelope glycoprotein of human immunodeficiency virus type I with lipid bilayers. *Biochemistry* 32, 3112–3118.
- (38) Kamata, H., Yagisawa, H., Takahashi, S., and Hirata, H. (1994) Amphiphilic peptides enhance the efficiency of liposome-mediated DNA transfection. *Nucleic Acids Res.* 22 (3), 536–537.
- (39) Vaishnav, Y. N., and Wong-Staal, F. (1991) The biochemistry of AIDS. *Annu. Rev. Biochem.* 60, 577–630.
- (40) Brake, D. A., Debouck, C., and Biesecker, G. (1990) Identification of an Arg-Gly-Asp (RGD) cell adhesion site in human immunodeficiency virus type 1 transactivation protein, tat. *J. Cell Biol.* 111, 1275–1281.
- (41) Anderson, D. C., Nichols, E., Manger, R., Woodle, D., Barry, M., and Fritzberg, A. R. (1993) Tumor cell retention of antibody Fab fragments is enhanced by an attached HIV TAT protein-derived peptide. *Biochem. Biophys. Res. Commun.* 194 (2), 876–884.

BC980004H

Synthesis of Novel Polyampholyte Comb-Type Copolymers Consisting of a Poly(L-lysine) Backbone and Hyaluronic Acid Side Chains for a DNA Carrier

Shoichiro Asayama,[†] Masayuki Nogawa,[†] Yoshiyuki Takei,^{‡,§} Toshihiro Akaike,[†] and Atsushi Maruyama^{*,†}

Department of Biomolecular Engineering, Tokyo Institute of Technology, 4259 Nagatsuta, Midori, Yokohama 226-8501, Japan, and First Department of Medicine, Osaka University School of Medicine, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan. Received December 22, 1997; Revised Manuscript Received April 3, 1998

The polyampholyte comb-type copolymers consisting of a poly(L-lysine) (PLL) main chain, a DNA binding site, and hyaluronic acid (HA) side chains, cell-specific ligands, have been prepared as the DNA carrier targeting sinusoidal endothelial cells of liver. The reducing end of HA and ϵ -amino groups of PLL were covalently coupled by reductive amination to obtain the resulting comb-type copolymers (PLL-graft-HA). The chain length of HA was controlled by the enzymatic hydrolysis of high-molecular weight HA. Since HA formed polyion complexes with PLL, the coupling reaction was carried out with high-ionic strength media to suppress polyion complex formation. The reaction proceeded in a homogeneous system, leading to a high efficiency of coupling (>70%) of HA onto the PLL backbone. By using the enzymatic hydrolysis of HA and the reductive amination reaction between HA and PLL with high-ionic strength media, it is possible to prepare the various comb-type copolymers with a defined density and a defined length of HA side chains. Furthermore, we also find that these polyampholyte comb-type copolymers vary their assembling structure in water in response to two kinds of environmental factors, i.e., ionic strength and pH. Finally, a ¹H NMR study reveals that the PLL backbone efficiently interacts with DNA molecules despite the presence of HA side chains having negative charges.

INTRODUCTION

Polycations have been used for the nonviral gene carriers because the polycations and DNAs form stable complexes in a noncovalent manner (1–3). The polycations, e.g., poly(L-lysine) (PLL¹), are reported to be conjugated with several ligands for targeted gene delivery (4–8). The physicochemical properties of the DNA complexes have been described as factors which influence transfection activity (9–11). However, the general method controlling and optimizing the complex properties has not been established yet.

We have reported on several comb-type copolymers consisting of a PLL backbone and hydrophilic dextran side chains for controlling the assembling structure of DNA–copolymer complexes (12, 13). The dextran chains grafted onto PLL are found to reduce the aggregation of the resulting complexes and increase the solubility of the complexes. Furthermore, the grafting degree of the

copolymer affects the DNA conformation in the complex, enabling us to regulate DNA compaction. The comb-type copolymers with a higher degree of grafting induce little compaction of DNA and stabilize DNA duplexes and triplexes by shielding the repulsion between phosphate anions of DNA. Moreover, the grafted chains reduce the nonspecific interaction of the PLL backbone with proteins (14). The comb-type copolymers therefore fulfill several requirements for the cell-specific carrier of DNA, if the copolymers are provided with cell-specific ligands.

Hyaluronic acid (HA) is an unbranched high-molecular weight polysaccharide consisting of alternating *N*-acetyl- β -D-glucosamine and β -D-glucuronic acid residues linked at the 1–3 and 1–4 positions, respectively (15). Liver sinusoidal endothelial cells (SECs) possess the receptors that recognize and internalize HA (16, 17). More than 90% of HAs in the blood stream are known to be taken up and metabolized by SECs. We have therefore chosen HA as the ligand for delivering the DNA to the SEC. Our recent study shows that the complexes between PLL–HA conjugates and reporter genes were distributed exclusively in SECs, leading to gene expression in vivo (18).

The binding affinity of the HA receptor is reported to increase as the chain length of HA is increased (19). In addition, the density and length of HA chains grafted onto PLL are expected to be the factors which affect the assembling structure of PLL–DNA complexes in the context of the efficiency of both targeting and transfection in vivo. The various comb-type copolymers consisting of a PLL backbone and HA side chains would be useful in

* To whom correspondence should be addressed. Telephone: +81-45-924-5122. Fax: +81-45-924-5815. E-mail: amaruyam@bio.titech.ac.jp.

[†] Tokyo Institute of Technology.

[‡] Osaka University School of Medicine.

[§] Present address: Department of Gastroenterology, Juntendo University School of Medicine, 2-1-1 Hongo, Bunkyo, Tokyo 113-0033, Japan.

¹ Abbreviations: PLL, poly(L-lysine); HA, hyaluronic acid; SEC, liver sinusoidal endothelial cell; GPC, gel permeation chromatography; RI, refractive index; LS, light scattering; M_n , number-average molecular weight; M_w , weight-average molecular weight.

investigating the structure–function relationship and optimizing the complex structure.

In this study, we have prepared the PLL-*graft*-HA comb-type copolymers with various chain lengths of both PLL and HA segments and the densities of HA *grafts*. For the synthesis of the comb-type copolymers, high-molecular weight HA was hydrolyzed, and then the HAs were covalently coupled with ϵ -amino groups of PLL at their reducing end by reductive amination. This paper describes the synthesis and characterization of the PLL-*graft*-HA comb-type copolymers in the context of their physicochemical properties and complex formation with DNA.

EXPERIMENTAL PROCEDURES

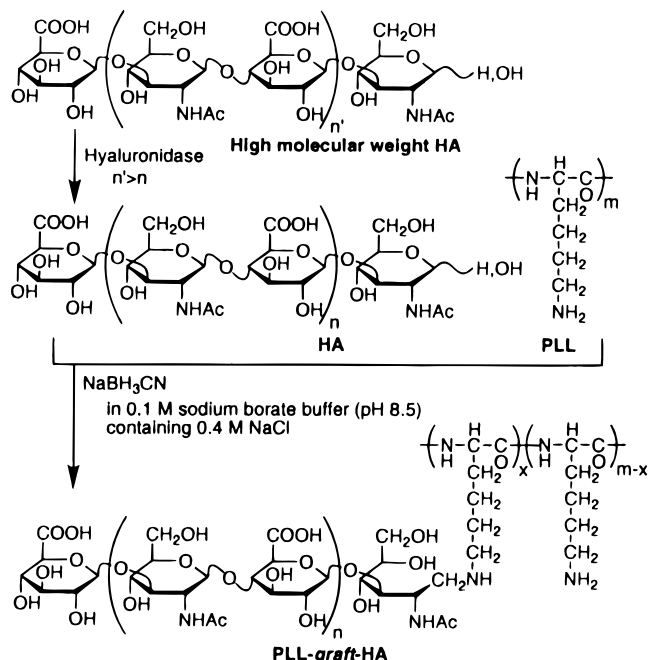
Materials. PLL, obtained as its chloride or bromide salt, was purchased from Peptide Institute, Inc. (Osaka, Japan). High-molecular weight HA (5.9×10^5), obtained as its sodium salt (sodium hyaluronate), was kindly provided by Denki Kagaku Kogyo Co., Ltd. (Tokyo, Japan). Bovine testicular hyaluronidase (EC 3.2.1.35, type I-S) was purchased from Sigma Chemical Co. (St. Louis, MO), and sodium cyanoborohydride (NaBH_3CN) was from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Salmon sperm DNA (approximately 300 base pairs) was kindly provided by Nissan Chemical Industries, Ltd. (Tokyo, Japan). All other chemicals of a special grade were used without further purification.

Enzymatic Hydrolysis of HA. High-molecular weight HA was partially degraded by hyaluronidase to low-molecular weight fragments (20). Hyaluronidase (20 mg) and HA (1 g) were added together in 120 mL of water. The solution was stirred at 50 °C for a desired time (1–20 h). Then, the solution was boiled for 5 min to terminate the reaction and allowed to cool to room temperature. After the filtration of the cooled mixture through a 0.45 μm filter (New Steradisc 25, KURABO, Osaka, Japan), the resulting HA fragments were obtained by freeze-drying.

Synthesis of PLL-*graft*-HA Comb-Type Copolymers. The obtained HA fragments were conjugated to PLL by reductive amination using NaBH_3CN as a reducing agent (Scheme 1). The NaBH_3CN was added to the mixture of HA (100–300 mg) and PLL (60–120 mg) in 15 mL of sodium borate buffer (0.1 M, pH 8.5) containing 0.4–1 M NaCl at a final concentration of 0.3 M. The reaction mixture was incubated at 40 °C for 3 days while it was stirred constantly, followed by dialysis against a 0.5 M NaCl aqueous solution using a Spectra/Por 7 membrane (molecular weight cutoff of 25 000) to remove unreacted HA. Then, the solution was desalted by dialysis against distilled water, and the resulting copolymer was obtained by freeze-drying.

Gel Permeation Chromatography (GPC). GPC was carried out using a JASCO 880-PU pumping system (Tokyo, Japan) at the flow rate of 1.0 mL/min at 25 °C with Ultrahydrogel 500 and Ultrahydrogel 250 columns (Japan Waters Ltd., Tokyo, Japan). The aqueous solution containing 0.2 M Na_2SO_4 and 5 mM sodium phosphate buffer (pH 8.0) was used as a mobile phase. Two hundred microliters of each sample was picked up from the reaction mixture and was injected into the columns. Eluate was detected by a refractive index (RI) detector (830-RI, JASCO) and a multiangle light scattering (LS) detector (Dawn-DSP, Wyatt Technology Co., Santa Barbara, CA). RI and LS signals were transferred to the computer to calculate the number-average (M_n) and

Scheme 1. Synthesis of PLL-*graft*-HA Comb-Type Copolymers



weight-average (M_w) molecular weight according to the instruction manual (Wyatt Technology Co.) for Dawn-DSP.

^1H NMR Spectroscopy. Each copolymer was dissolved in D_2O (99.95 at. % D; Merck, Darmstadt, Germany) containing 0.35 M NaCl. ^1H NMR spectra (400 MHz) were obtained with a Varian Unity 400plus spectrometer (Palo Alto, CA), at a probe temperature of 298 K. The chemical shifts are expressed as parts per million using internal HDO molecules ($\delta = 4.7$ ppm in D_2O) as a reference.

Turbidity Measurement of PLL-*graft*-HA Solutions. The copolymer was dissolved in 3 mL of distilled water at a concentration of 1.0 mg/mL. The ionic strength of the solution was increased by the stepwise addition of a 5 M NaCl solution. The pH of the solution was varied by adding a 0.1 M solution of HCl or NaOH. The turbidity change of the solution at different ionic strengths or pHs was monitored as the absorbance at 500 nm with a Beckman DU-640 spectrophotometer (Fullerton, CA).

Estimation of the DNA-PLL-*graft*-HA Interaction by ^1H NMR Spectroscopy. The stock solution of DNA (1 mg/mL) in 10 mM sodium phosphate buffer (pH 7.2) containing 0.5 mM EDTA and 1 M NaCl was mixed with the PLL-*graft*-HA solution (1 mg/mL) in 1 M NaCl at a copolymer/DNA charge ratio ($[\text{amino group}]_{\text{copolymer}}/[\text{phosphate group}]_{\text{DNA}}$) of 1.0. The mixture was then desalted by step-down dialysis using a Spectra/Por 7 membrane (molecular weight cutoff of 1000), followed by freeze-drying. The resulting sample was dissolved in D_2O . The ^1H NMR spectrum was obtained as described above.

RESULTS AND DISCUSSION

Synthesis of PLL-*graft*-HA Comb-Type Copolymers. Most polysaccharides are useful for the synthesis of comb-type copolymers using the coupling reaction because they have one reducing end per molecule; namely, they are semitelechelic polymers (21, 22). The polysaccharides whose glycosyl bonds are hydrolyzed

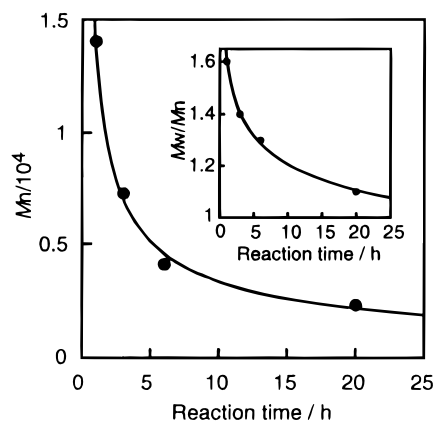


Figure 1. Time course of the hydrolysis of HA by hyaluronidase. HA (5.9×10^5 ; 1 g) was hydrolyzed by hyaluronidase (20 mg) at 50 °C. The M_n of HA was determined by GPC. The inset shows the change in the molecular weight distribution (M_w/M_n).

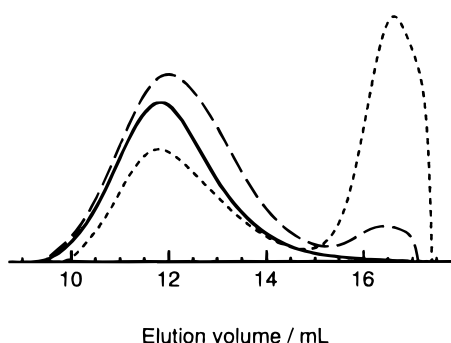


Figure 2. Gel permeation chromatograms of the isolated PLL-graft-HA copolymer (solid line) and the reaction mixture of PLL ($M_n = 9.7 \times 10^4$; 39 mg) and HA ($M_n = 2.2 \times 10^3$; 94 mg) at 0 h (dotted line) or 9 days (dashed line): column, Ultrahydrogel 500 and 250; eluent, 0.2 M Na_2SO_4 and 5 mM sodium phosphate buffer (pH 8.0); flow rate, 1.0 mL/min; temperature, 25 °C; and detection, RI.

enzymatically or chemically are also semitelechelic. Therefore, the comb-type copolymers with the different densities and lengths of polysaccharide side chains would be available by combining enzymatic hydrolysis and the conjugation of the reducing end to the main chain polymer (Scheme 1).

To regulate the molecular weight of HA, we hydrolyzed high-molecular weight HA with hyaluronidase for the indicated time period (Figure 1). The molecular weight of HA decreased and the molecular weight distribution narrowed with the increase in digestion time. When the time for enzyme reaction was controlled, the HAs with the desired molecular weight, i.e., chain length, were isolated.

The hydrolyzed HA fragment was then grafted onto PLL by reductive amination between the reducing end of HA and ϵ -amino groups of PLL using NaBH_3CN as a reducing agent. The HA polyanion can form the polyion complex, which prevents the conjugating reaction, with the PLL polycation. To suppress this polyion complex formation, the ionic strength of the medium was increased (0.4–1 M NaCl). The increase in the NaCl concentration resulted in the reaction proceeding in a homogeneous system (see Figure 4a).

Figure 2 shows the representative GPC profile of the reaction mixture and the resulting copolymer. After the reaction, the peak area of PLL increased while that of HA decreased. Although an obvious elution volume shift

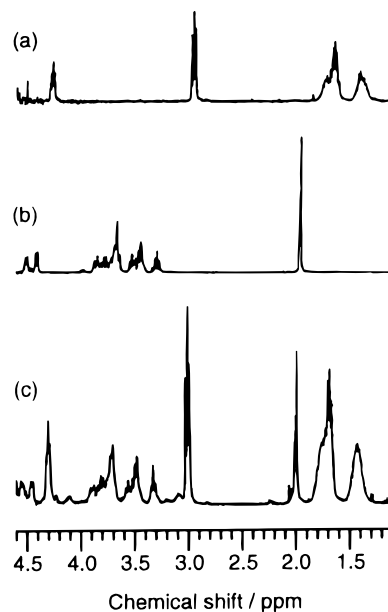


Figure 3. ^1H NMR spectra of PLL (a), HA (b), and PLL-graft-HA (c) in D_2O . For the PLL-graft-HA, D_2O containing 0.35 M NaCl was used.

was not observed in the chromatogram owing to some interaction between the polymers and column packing, the molecular weight determined by the LS detector increased drastically after the reaction, being 2.3×10^5 for the resulting copolymer in comparison with 9.7×10^4 for the original PLL. The incubation of HA or PLL alone with NaBH_3CN under the same conditions caused no change in GPC profiles, which suggested that neither degradation nor intermolecular side reaction occurred (results not shown). When HA was incubated with PLL in the presence of a 10-fold molar excess of lactose or sucrose with respect to the reducing end of HA, not sucrose, the nonreducing sugar, but lactose, the reducing one, inhibited HA consumption (results not shown). Therefore, it can be concluded that the HA was conjugated with PLL at its reducing end through the reductive amination reaction (23). Figure 2 also shows that the comb-type copolymer was isolated from unreacted HA by dialysis.

As shown in Figure 3, the ^1H NMR spectra of the comb-type copolymer showed the characteristic signals of both PLL and HA moieties: PLL, δ 1.4–1.8 (β , γ , δ - CH_2), 3.0 (ϵ - CH_2), 4.3 (α -CH); HA, δ 2.0 (NAc- CH_3), 3.3–3.9 (H-2,3,4,5,6), 4.4–4.6 (H-1). From the signal ratio of methyl protons (2.0 ppm) of the *N*-acetyl groups of the HA-grafts to ϵ -methylene protons (3.0 ppm) of the PLL backbone, the content (wt % and grafting %) of HA in the copolymer was determined. The results of the synthesis of PLL-graft-HA comb-type copolymers are summarized in Table 1. The coupling efficiency was more than about 70%. Consequently, we have easily prepared the various PLL–HA conjugates with a well-defined comb-type structure by combining enzymatic hydrolysis and reductive amination.

Polyampholyte Properties of the PLL-graft-HA Comb-Type Copolymer. The resulting PLL-graft-HA comb-type copolymer consists of a polycation backbone and polyanion side chains. The polyampholyte property of the comb-type copolymer was examined by measuring the turbidity of the copolymer solution. Figure 4a shows that the turbidity of the PLL-graft-HA solution varied with the change of NaCl concentration at neutral pH; namely, the solubility of the PLL-graft-HA markedly

Table 1. Synthesis of PLL-*graft*-HA Comb-Type Copolymers^a

sample	in feed					copolymer						
	PLL		HA			molecular weight ^b		HA content ^c			coupling efficiency ^d (%)	yield (%)
	$M_n^b/10^4$	mg	$M_n^b/10^3$	mg	wt %	$M_n/10^4$	M_w/M_n	wt %	grafting %	charge ratio		
1	4.2	61.2	2.3	94.3	61	8.5	1.5	50	5.7	0.34	66	67
2	4.2	61.2	2.3	189	76	11	1.4	63	9.4	0.56	55	57
3	7.2	122	3.8	189	61	15	1.4	53	3.8	0.40	73	55
4	7.2	61.2	3.8	189	76	23	1.6	69	7.6	0.83	73	83
5	7.2	61.2	3.8	283	82	31	1.5	77	11	1.3	71	57
6	4.2	61.2	1.6	94.3	61	9.3	1.4	55	9.8	0.49	80	38
7	7.2	122	1.6	94.3	44	12	1.5	38	4.9	0.22	80	51
8	7.2	61.2	1.6	189	76	24	1.4	71	19	1.0	77	85

^a Reducing reagent, 0.3 M NaBH₃CN; reaction temperature, 40 °C; reaction time, 56 h (samples 1 and 2), 80 h (samples 3–5), and 75 h (samples 6–8); solvent, 0.1 M sodium borate buffer (pH 8.5) containing 0.4 M NaCl (samples 1 and 2) or 1 M NaCl (samples 3–8). ^b The molecular weight and its distribution (M_w/M_n) were determined by GPC. ^c Determined by ¹H NMR; grafting % = (the mole fraction of the lysine residues grafted with HA) × 100%; charge ratio = [carboxyl group]_{HA}/[amino group]_{PLL} in the copolymer. ^d $[HA]_{\text{copolymer}}/[HA]_{\text{in feed}} \times 100\%$.

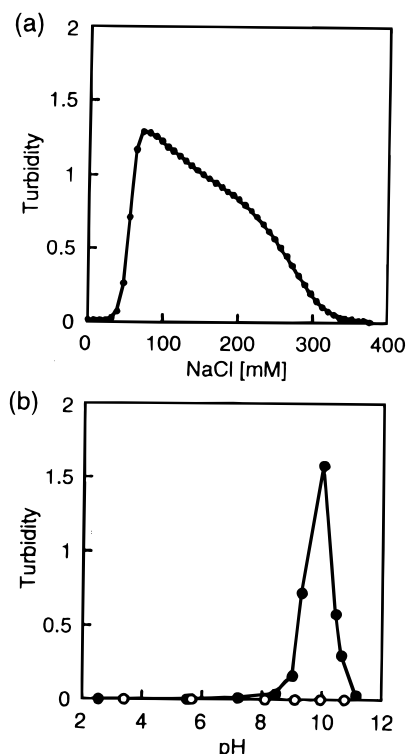


Figure 4. Polyampholyte properties of the PLL-*graft*-HA comb-type copolymer (sample 1). (a) Effect of ionic strength on the solubility of the copolymer at neutral pH. (b) Effect of pH on the solubility of the copolymer in the absence (●) or presence (○) of 400 mM NaCl. The turbidity was monitored as the absorbance at 500 nm.

varied in water in response to the ionic strength of the medium. The PLL-*graft*-HA suddenly precipitated out of the aqueous medium at a particular ionic strength but became soluble again at higher ionic strengths.

It should be noted that the PLL-*graft*-HA seems to be soluble at lower ionic strengths where the comb-type copolymers should form polyion complexes. Since the net charge of the PLL-*graft*-HA used in the experiment is positive at neutral pH, the copolymers were probably dispersed in the medium as colloidal particles without undergoing particle aggregation. The increase in the ionic strength of the medium presumably reduced the electrostatic repulsion among the colloidal particles, inducing particle aggregation (salting out) to exhibit turbidity. However, the further increase in the ionic strength of the medium over 350 mM NaCl probably

dissociated the polyion complex between PLL and HA chains, resulting in the complete disappearance of turbidity. To assess the solution behavior at the lower ionic strength, we varied the pH of the solution (24). As seen in Figure 4b, the sudden increase in turbidity was observed when the pH of the medium was increased to 9.0, where the deprotonation of the ϵ -amino groups of PLL was initiated (25). The partial deprotonation of the PLL moiety presumably decreased the repulsive force among colloidal particles and increased their hydrophobicity, triggering interparticle aggregation. Finally, the solution became transparent again above pH 11, owing to the complete dissociation of the polyion complex, due to the thorough deprotonation of PLL, between PLL and HA. These results suggest that the PLL-*graft*-HA formed its intramolecular polyion complex under low-ionic strength conditions (in 0 mM NaCl) and dissociated its polyion complex under high-ionic strength conditions (>350 mM NaCl).

DNA-PLL-*graft*-HA Polyion Complex. The DNA was mixed with the PLL-*graft*-HA ($[\text{amino group}]_{\text{copolymer}}/[\text{phosphate group}]_{\text{DNA}} = 1$), and the ionic strength of the medium was gradually decreased from 1 to 0 M NaCl by step-down dialysis (26). The transparent solution obtained after dialysis was then lyophilized, dissolved in D₂O, and analyzed by ¹H NMR. The ¹H NMR spectra of the copolymer with or without DNA are shown in Figure 5. It is worth noting that the signals of ϵ -methylene protons of the PLL moiety in the PLL-*graft*-HA broadened in the presence of DNA (Figure 5c). This is probably caused by polyion complex formation between the PLL moiety and DNA. Although the PLL signals broadened, the HA signals remained unchanged, or rather became sharper. The sharp signals of HA in the presence of DNA under low-ionic strength conditions (Figure 5c) seem to be similar to those of PLL-*graft*-HA alone at high ionic strengths (Figures 3c and 5a), where the PLL-*graft*-HA did not form polyion complex; i.e., HA chains were free. These results suggest that the PLL backbone selectively formed the polyion complex with DNA even in the presence of the HA side chains. The preferential complex formation was further demonstrated by the acrylamide gel electrophoresis analysis of a PLL-*graft*-HA-oligoDNA mixture, as will be reported elsewhere (27). In addition, the PLL-*graft*-HA-DNA complex may form the multiphase structure in which the hydrophobic PLL-DNA complex is surrounded by the hydrated shell of free HA (28, 29). Complex formation with free HA chains is considered to be essential for directing the complex to target cells.

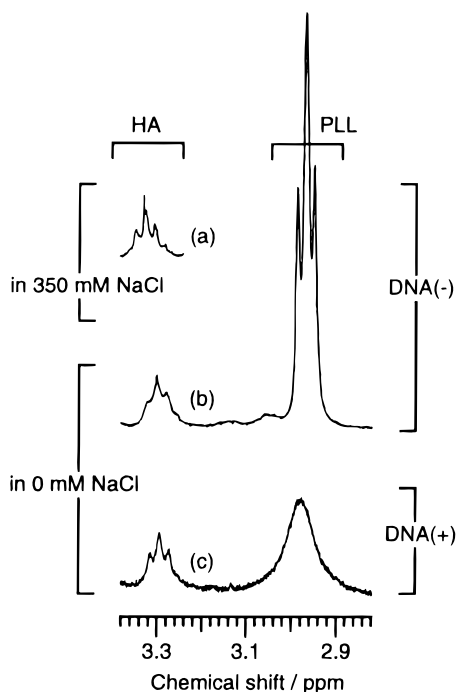


Figure 5. Effect of DNA on the ^1H NMR spectra of the PLL-graft-HA comb-type copolymer: PLL-graft-HA alone in D_2O containing 350 mM NaCl (a) and PLL-graft-HA in the absence (b) or presence (c) of DNA in D_2O without NaCl. The signal of ϵ -methylene protons of PLL (3.0 ppm) and the H-2 signal of glucuronic acid of HA (3.3 ppm) are represented.

Conclusion. In this study, we have prepared the various PLL-graft-HA comb-type copolymers with a defined density and a defined length of HA side chains. The synthesis of the comb-type copolymers has been achieved by (1) the control of the HA chain length by enzymatic hydrolysis, (2) the suppression of formation of a polyion complex between HA and PLL in high-ionic strength media during the coupling reaction, and (3) the relatively high coupling efficiency of the reductive amination between the reducing end of HA and ϵ -amino groups of PLL.

By using the various PLL-graft-HA comb-type copolymers, it would be possible to control the properties of the DNA complex such as complex size, solubility, and the degree of DNA compaction (13, 30). The resulting comb-type copolymers are, therefore, useful in investigating the relationship between DNA complex properties and the efficiency of both targeting and transfection. Furthermore, the efficient conjugation of the HA chains to other materials (31–40) can offer unique tools for studying the biological and biochemical properties of HA.

ACKNOWLEDGMENT

We are grateful to Dr. Teruzo Miyoshi and Dr. Koichi Takahashi of Denki Kagaku Kogyo Co., Ltd., for supplying hyaluronic acid and for their help in the preparation of the manuscript. We thank Dr. Anwarul Ferdous of the Tokyo Institute of Technology for his helpful discussions.

LITERATURE CITED

- (1) Kabanov, A. V., Astafyeva, I. V., Chikindas, M. L., Rosenblatt, G. F., Kiselev, V. I., Severin, E. S., and Kabanov, V. A. (1991) DNA interpolyelectrolyte complexes as a tool for efficient cell transformation. *Biopolymers* 31, 1437–1443.
- (2) Boussif, O., Lezoualc'h, F., Zanta, M. A., Mergny, M. D., Scherman, D., Demeneix, B., and Behr, J. P. (1995) A versatile vector for gene and oligonucleotide transfer into cells in culture and *in vivo*: Polyethylenimine. *Proc. Natl. Acad. Sci. U.S.A.* 92, 7297–7301.
- (3) Page, R. L., Butler, S. P., Subramanian, A., Gwazdauskas, F. C., Johnson, J. L., and Velander, W. H. (1995) Transgenesis in mice by cytoplasmic injection of polylysine/DNA mixtures. *Transgenic Res.* 4, 353–360.
- (4) Wu, G. Y., and Wu, C. H. (1987) Receptor-mediated *in vitro* gene transformation by a soluble DNA carrier system. *J. Biol. Chem.* 262, 4429–4432.
- (5) Wagner, E., Cotten, M., Foisner, R., and Birnstiel, M. L. (1991) Transferrin-polycation-DNA complexes: The effect of polycations on the structure of the complex and DNA delivery to cells. *Proc. Natl. Acad. Sci. U.S.A.* 88, 4255–4259.
- (6) Hockett, B., Ariatti, M., and Hawtrey, A. O. (1990) Evidence for targeted gene transfer by receptor-mediated endocytosis: Stable expression following insulin-directed entry of *neo* into HepG2 cells. *Biochem. Pharmacol.* 40, 253–263.
- (7) Trubetskoy, V. S., Torchilin, V. P., Kennel, S. J., and Huang, L. (1992) Use of N-terminal modified poly(L-lysine)-antibody conjugate as a carrier for targeted gene delivery in mouse lung endothelial cells. *Bioconjugate Chem.* 3, 323–327.
- (8) Martinez-Fong, D., Mullersman, J. E., Purchio, A. F., Armendariz-Borunda, J., and Martinez-Hernandez, A. (1994) Nonenzymatic glycosylation of poly-L-lysine: A new tool for targeted gene delivery. *Hepatology* 20, 1602–1608.
- (9) Perales, J. C., Grossmann, G. A., Molas, M., Liu, G., Ferkol, T., Harpst, J., Oda, H., and Hanson, R. W. (1997) Biochemical and functional characterization of DNA complexes capable of targeting genes to hepatocytes via the asialoglycoprotein receptor. *J. Biol. Chem.* 272, 7398–7407.
- (10) Wolfert, M. A., Schacht, E. H., Toncheva, V., Ulbrich, K., Nazarova, O., and Seymour, L. W. (1996) Characterization of vectors for gene therapy formed by self-assembly of DNA with synthetic block co-polymers. *Hum. Gene Ther.* 7, 2123–2133.
- (11) Kabanov, A. V., and Kabanov, V. A. (1995) DNA complexes with polycations for the delivery of genetic material into cells. *Bioconjugate Chem.* 6, 7–20.
- (12) Maruyama, A., Katoh, M., Ishihara, T., and Akaike, T. (1997) Comb-type polycations effectively stabilize DNA triplex. *Bioconjugate Chem.* 8, 3–6.
- (13) Maruyama, A., Watanabe, H., Ferdous, A., Katoh, M., Ishihara, T., and Akaike, T. (1998) Characterization of interpolyelectrolyte complexes between double-stranded DNA and polylysine comb-type copolymers having hydrophilic side chains. *Bioconjugate Chem.* 9, 292–299.
- (14) Maruyama, A., Ishihara, T., Kim, J. S., Kim, S. W., and Akaike, T. (1997) Nanoparticle DNA carrier with poly(L-lysine) grafted polysaccharide copolymer and poly(D,L-lactic acid). *Bioconjugate Chem.* 8, 735–742.
- (15) Balazs, E. A., Laurent, T. C., and Jeanloz, R. W. (1986) Nomenclature of hyaluronic acid. *Biochem. J.* 235, 903.
- (16) Forsberg, N., and Gustafson, S. (1991) Characterization and purification of the hyaluronan-receptor on liver endothelial cells. *Biochim. Biophys. Acta* 1078, 12–18.
- (17) Yannariello-Brown, J., Frost, S. J., and Weigel, P. H. (1992) Identification of the Ca^{2+} -independent endocytic hyaluronan receptor in rat liver sinusoidal endothelial cells using a photoaffinity cross-linking reagent. *J. Biol. Chem.* 267, 20451–20456.
- (18) Takei, Y., Maruyama, A., Kawano, S., Okumura, S., Asayama, S., Nogawa, M., Oshita, M., Hashimoto, M., Makino, Y., Nagai, H., Tsujii, M., Tsuji, S., Nagano, K., Kinoshita, M., Akaike, T., and Hori, M. (1998) Targeted gene delivery to liver sinusoidal endothelial cells by hyaluronan receptor-mediated endocytosis. *J. Clin. Invest.* (submitted for publication).
- (19) Laurent, T. C., Fraser, J. R. E., Pertoft, H., and Smedsrød, B. (1986) Binding of hyaluronate and chondroitin sulphate to liver endothelial cells. *Biochem. J.* 234, 653–658.
- (20) Cramer, J. A., Bailey, L. C., Bailey, C. A., and Miller, R. T. (1994) Kinetic and mechanistic studies with bovine testicular hyaluronidase. *Biochim. Biophys. Acta* 1200, 315–321.

- (21) Ulaneck, C. A., Hsieh, H. L., and Buck, O. G. (1960) Telechelic Polymers. *J. Polym. Sci.* 46, 535–539.
- (22) Swann, D. A., Silver, F. H., Sotman, S. L., and Hermann, H. (1982) Measurements of reducing end groups on bovine vitreous-humour hyaluronic acid by reaction with [^{14}C]-cyanide. *Biochem. J.* 207, 409–414.
- (23) Shen, T., Bogdanov, A., Bogdanova, A., Poss, K., Brady, T. J., and Weissleder, R. (1996) Magnetically labeled secretin retains receptor affinity to pancreas acinar cells. *Bioconjugate Chem.* 7, 311–316.
- (24) Chen, G., and Hoffman, A. S. (1995) Graft copolymers that exhibit temperature-induced phase transitions over a wide range of pH. *Nature* 373, 49–52.
- (25) Means, G. E., and Feeney, R. E. (1971) Proteins and their properties. *Chemical Modifications of Proteins*, pp 3–20, Holden-Day, San Francisco.
- (26) Perales, J. C., Ferkol, T., Molas, M., and Hanson, R. W. (1994) An evaluation of receptor-mediated gene transfer using synthetic DNA-ligand complexes. *Eur. J. Biochem.* 226, 255–266.
- (27) Maruyama, A., Nogawa, M., Asayama, S., Takei, Y., and Akaike, T. (1998) (manuscript in preparation).
- (28) Ito, K., Masuda, Y., Shintani, T., Kitano, T., and Yamashita, Y. (1983) Synthesis and interfacial characterization of graft and random copolymers of styrene and 2-hydroxyethyl methacrylate. *Polym. J.* 15, 443–448.
- (29) Hrkach, J. S., Peracchia, M. T., Domb, A., Lotan, N., and Langer, R. (1997) Nanotechnology for biomaterials engineering: structural characterization of amphiphilic polymeric nanoparticles by ^1H NMR spectroscopy. *Biomaterials* 18, 27–30.
- (30) Asayama, S., Maruyama, A., Cho, C. S., and Akaike, T. (1997) Design of comb-type polyamine copolymers for a novel pH-sensitive DNA carrier. *Bioconjugate Chem.* 8, 833–838.
- (31) West, D. C., and Kumar, S. (1989) The effect of hyaluronate and its oligosaccharides on endothelial cell proliferation and monolayer integrity. *Exp. Cell Res.* 183, 179–196.
- (32) Zhang, L., Underhill, C. B., and Chen, L. (1995) Hyaluronan on the surface of tumor cells is correlated with metastatic behavior. *Cancer Res.* 55, 428–433.
- (33) Rooney, P., Kumar, S., Ponting, J., and Wang, M. (1995) The role of hyaluronan in tumour neovascularization (review). *Int. J. Cancer* 60, 632–636.
- (34) McCourt, P., Ek, B., Forsberg, N., and Gustafson, S. (1994) Intercellular adhesion molecule-1 is a cell surface receptor for hyaluronan. *J. Biol. Chem.* 269, 30081–30084.
- (35) Thomas, L., Byers, H. R., Vink, J., and Stamenkovic, I. (1992) CD44H regulates tumor cell migration on hyaluronate-coated substrate. *J. Cell Biol.* 118, 971–977.
- (36) McGary, C. T., Yannariello-Brown, J., Kim, D. W., Stinson, T. C., and Weigel, P. H. (1993) Degradation and intracellular accumulation of a residualizing hyaluronan derivative by liver endothelial cells. *Hepatology* 18, 1465–1476.
- (37) Yannariello-Brown, J., and Weigel, P. H. (1992) Detergent solubilization of the endocytic Ca^{2+} -independent hyaluronan receptor from rat liver endothelial cells and separation from a Ca^{2+} -dependent hyaluronan-binding activity. *Biochemistry* 31, 576–584.
- (38) Yui, N., Nihira, J., Okano, T., and Sakurai, Y. (1993) Regulated release of drug microspheres from inflammation responsive degradable matrices of crosslinked hyaluronic acid. *J. Controlled Release* 25, 133–143.
- (39) Drobnik, J. (1991) Hyaluronan in drug delivery. *Adv. Drug Delivery Rev.* 7, 295–308.
- (40) Pouyani, T., and Prestwich, G. D. (1994) Functionalized derivatives of hyaluronic acid oligosaccharides: Drug carriers and novel biomaterials. *Bioconjugate Chem.* 5, 339–347.

BC970213M

Recombinant Immunocytokines Targeting the Mouse Transferrin Receptor: Construction and Biological Activities

Torsten Dreier,[†] Holger N. Lode,[†] Rong Xiang,[†] Carrie S. Dolman,[†] Ralph A. Reisfeld,^{*,†} and Angray S. Kang[‡]

Department of Immunology and Department of Molecular Biology, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, California 92037. Received February 16, 1998; Revised Manuscript Received May 7, 1998

Localized cytokine therapies with recombinant monoclonal antibody-cytokine fusion proteins, designated immunocytokines, have become of increasing interest for tumor immunotherapy, since they direct immunomodulatory cytokines into the tumor microenvironment. To investigate their mechanisms of action in a variety of syngeneic tumor models, recombinant mouse cytokines IL2 and GM-CSF were engineered as fusion proteins to the carboxyl terminus of a chimeric rat/mouse antitransferrin receptor antibody, ch17217 and expressed in stable-transfected Chinese hamster ovary cells. The recombinant immunocytokines were readily purified by affinity chromatography and their binding characteristics were identical to those shown for the ch17217 antibody. The IL2 immunocytokine had an activity similar to recombinant mouse IL2, whereas the GM-CSF immunocytokine had enhanced cytokine activity relative to recombinant mouse GM-CSF. The clearance rates of ch17217 and the GM-CSF and IL2 immunocytokines were relatively similar with elimination phases ($t_{1/2\alpha}$) of 1.8 h and distribution phases ($t_{1/2\beta}$) of 83, 88, and 91 h, respectively. Both immunocytokines demonstrated effective antitumor activity by suppressing the growth of hepatic metastases of mouse neuroblastoma and pulmonary metastases of mouse colon carcinoma in syngeneic A/J and BALB/c mice, respectively. These results indicate that biologically effective IL2 and GM-CSF immunocytokines combine the targeting ability of an antitransferrin receptor monoclonal antibody with the immunomodulatory functions of each cytokine. Because of the universal expression of the transferrin receptor on mouse tumor cell lines, these constructs should prove useful to determine their efficacy in a wide variety of syngeneic mouse tumor models and to perform detailed studies of their modes of action.

INTRODUCTION

The increased availability of recombinant cytokines in pharmacologic quantities has stimulated a number of research efforts to assess the effect of these immunomodulatory molecules on the host immune response to cancer. Among a variety of cytokines, IL2 has been most widely applied, since it is one of the most potent antitumor cytokines with biological activities that include the stimulation of proliferation of such antitumor effectors as cytotoxic T cells, natural killer (NK) cells, and macrophages (1, 2). This cytokine has been applied with some success for the treatment of patients with melanoma and renal carcinoma (3). However, the systemic administration of IL2 is limited by serious side effects, even at nanomolar concentrations (4). Therefore, investigators started to focus on local cytokine treatments, e.g., intratumoral cytokine injection or cytokine gene therapy which, in contrast to systemic application, comply with the paracrine mode of action of these immunomodulators being optimally effective within a few cell diameters from their cell of origin (5).

Gene therapy, i.e., the *ex vivo* transfection of cytokine genes into autologous tumor cells that are subsequently injected into a host, has been widely applied in clinical trials and preclinical animal models. When such cyto-

kines, including IL2 and GM-CSF, are produced by the transfected tumor cells, they induce a local inflammatory response, resulting in some cases, in a systemic immune response effective against challenge with wild-type tumor cells (6, 7). Some encouraging preclinical and early clinical data were obtained with this strategy (8); however, the feasibility of its broad clinical application as a cancer therapy is limited by its patient-specific nature.

We have developed an alternative approach for tumor immunotherapy by using immunocytokines with the unique ability to direct multifunctional cytokines to the tumor microenvironment (9). This approach conforms with the paracrine function of most cytokines and is not limited by systemic toxicities or patient specificity. We previously demonstrated in syngeneic mouse tumor models of neuroblastoma (10), colon carcinoma (11), and melanoma (12) that recombinant immunocytokines comprised of different tumor-specific antibodies and IL2 can eradicate established disseminated tumor metastases, followed in some cases by a long-lived tumor-protective immune response (11). However, the antibody specificity for one distinct target antigen limited the use of such constructs to only a small number of syngeneic mouse tumor models.

Here, we describe the construction, characterization and biological activity of two recombinant immunocytokines comprised of a monoclonal antibody against mouse transferrin receptor and mouse IL2 and GM-CSF, respectively. In both cases, the antigen-binding capacity and cytokine functions were maintained, and effective suppression of metastasis was demonstrated in two

* To whom correspondence and reprints should be addressed. Phone: 619-784-8105. Fax: 619-784-2708. E-mail: reisfeld@scripps.edu.

[†] Department of Immunology.

[‡] Department of Molecular Biology.

syngeneic models of mouse neuroblastoma and colon carcinoma. The universal expression of the transferrin receptor on mouse tumor cell lines should make these immunocytokines applicable to ascertain their antitumor activity in all syngeneic mouse tumor models and to perform in-depth studies of their underlying effector mechanisms.

EXPERIMENTAL PROCEDURES

Antibodies and Cell Lines. The rat hybridoma R17217, secreting an antibody of IgG2b isotype, directed against the mouse transferrin receptor (Tf-R), was kindly made available by Dr. Jayne F. Lesley (Salk Institute, La Jolla, CA). This antibody was extensively characterized and demonstrated not to affect functions of Tf-R involved in iron transport into cells or to inhibit the growth of tumor cells (13, 14).

The GM-CSF-dependent acute mouse hematopoietic stem cell line 32D(G) was kindly provided by Dr. D. Santoli (Wistar Institute, Philadelphia, PA). The IL-2-dependent mouse leukemia CTLL-2 and CHO cell lines were obtained from the American Type Culture Collection (Rockville, MD).

Mice. Syngeneic female A/J and BALB/c mice were obtained at 6–8 weeks of age from Jackson Laboratory (Bar Harbor, ME). The mice were maintained under specific pathogen-free conditions. All experiments were performed according to NIH Guidelines for Care and Use of Laboratory Animals.

Construction of Immunocytokines. The variable region of the rat immunoglobulin chains were amplified by RT-PCR using mRNA isolated from R17217 hybridoma as a template. To access the VL chain, a 5'-primer was designed according to sequence information available for these regions (15). We selected GAYGTNCARATGACNCARTCNCC as the 5'-primer for amplification of the variable region of the rat VL. The 3'-primer was designed to anneal to the rat κ light chain region (GGATGATGTCTTATGAACAA). In a second PCR reaction, the OKT3-leader sequence was added upstream and a *Nar*I site downstream of the primary VL PCR product. The construction of the chimeric rat-mouse light chain was completed by joining this VL via the *Nar*I site with a mouse C κ fragment (Figure 1A).

The variable heavy chain (VH) was amplified in a similar manner. Briefly, we used the 5'-primer GAGGTGCARCTNGTNGAATCNGGAGGAGG and selected the 3'-primer from the region of the rat J-minigene (GGTGACCATGGTTCCTGGGCCCCAGAAGTC). In a second PCR, the N418 hamster immunoglobulin-derived leader sequence was added upstream and a *Csp*45I site downstream of the original VH-PCR-product, respectively (Figure 1A). This VH construct was ligated to a mouse IgG2a constant heavy-chain backbone via the *Csp*45I site, which had been engineered at the 5' end of the IgG2a. The mouse IgG2a backbone was constructed with an *Sma*I site located seven amino acids upstream of the original 3' end of mouse IgG2a. In the case of the recombinant antibody, we introduced a synthetic oligonucleotide in this *Sma*I site, providing a stop codon followed by an *Eco*RI site. For the construction of the immunocytokines, the mature sequences of the mouse cytokines IL-2 and GM-CSF were amplified from the RNA of Concanavalin-A stimulated mouse splenocytes by RT-PCR with the following primers: GGACCCCGG-GAGCACCCACTTCAAGCTCC (IL-2 5'); CTGAATTCT-TATTGAGGGCTTGTTGAGATGATGC (IL-2 3'); GGAC-CCCGGGAGCACCCACCCGCTCACCC (GM-CSF 5'); and

CTGAATTCTGGGCTTCCTCATTTCCTGGCTTGG (GM-CSF 3'). The resulting PCR products were inserted as *Sma*I-*Eco*RI fragments at the 3' end of the heavy chain construct.

Construction of the Expression Vectors. For expression purposes, the light-chain construct was cloned into the multiple cloning site of the pcDNA3.1/zeo vector (Invitrogen, Carlsbad, CA) using the *Nhe*I and *Xho*I restriction sites. The heavy-chain constructs were cloned as *Nhe*I to *Eco*RI fragments into the pBK-CMV vector (Stratagene, La Jolla, CA). The entire heavy-chain expression unit was isolated after digestion with *Nsi*I followed by blunt ending with mung bean nuclease and a *Mlu*I digest. A double expression vector was constructed by cloning the heavy-chain expression unit into the pcDNA3.1/zeo vector containing the light-chain expression unit using *Sca*I and *Mlu*I restriction sites (Figure 1B), thus sacrificing the ampicillin gene used for selection in prokaryotic cells. However, the selectable zeocin marker is retained.

Cell Culture and Transfection. Chinese hamster ovary (CHO) cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and 1% nonessential amino acid solution (Biowhittaker, Walkersville, MD). Transfection with the double expression vectors was done at cell densities of 70–80% using SuperFect (Qiagen, Chatsworth, CA) according to the manufacturer's protocol. Twenty-four h after transfection, the cells were transferred to 96-well plates and drug selection was initiated by supplementing the media with 200 μ g/mL zeocin (Invitrogen, Carlsbad, CA). Zeocin-resistant colonies developed within 2 weeks after transfection. The supernatants of these colonies were assayed for mouse IgG2a production by ELISA and dot blot analyses. The highest producers (10–20 μ g/mL) were expanded to produce the desired immunocytokines.

Production and Purification of Immunocytokines. For ease of purification, the medium of 90% confluent cells cultured in roller bottles was changed to serum-free IS-CHO medium (Irvine Scientific, Santa Ana, CA) and culture continued in this medium for another 10 days. The immunocytokines produced were isolated from the spent culture media by protein A Sepharose chromatography and concentrated by ultrafiltration to a final concentration of 1–2 mg/mL. The molecular size and purity of the proteins was analyzed by SDS-PAGE.

Immunoassays. Identification of the chimeric antibodies or immunocytokines was performed by ELISA and dot blot analyses. The capture antibody used in the ELISA was an Fc-specific goat anti-mouse antibody, and the detection antibody was an alkaline phosphatase-labeled κ -chain-specific goat anti-mouse antibody (Sigma, St. Louis, MO). An alkaline phosphatase-labeled goat anti-mouse antibody was used for dot blots. The mouse IgG2a antibody UPC10 (Sigma, St. Louis, MO) served as a positive control and the supernatant of nontransfected CHO cells as a negative control.

Cytokine Activity Assays. IL-2 activity was measured by a mouse T-cell proliferation assay (16). Briefly, the IL-2 dependent murine leukemia cell line CTLL-2 (ATCC) was cultured for 48 h without IL-2. Then, 3×10^4 cells were incubated for 24 h with serial dilutions of either recombinant murine (rm) IL-2 or the ch17217-IL-2 immunocytokine, respectively, in a total volume of 200 μ L. GM-CSF activity was detected in a proliferation assay with the GM-CSF-dependent cell line, 32D(G). The cells were added to 96-well plates (5000 cells/well) together with serial dilutions of either rmGM-CSF or the

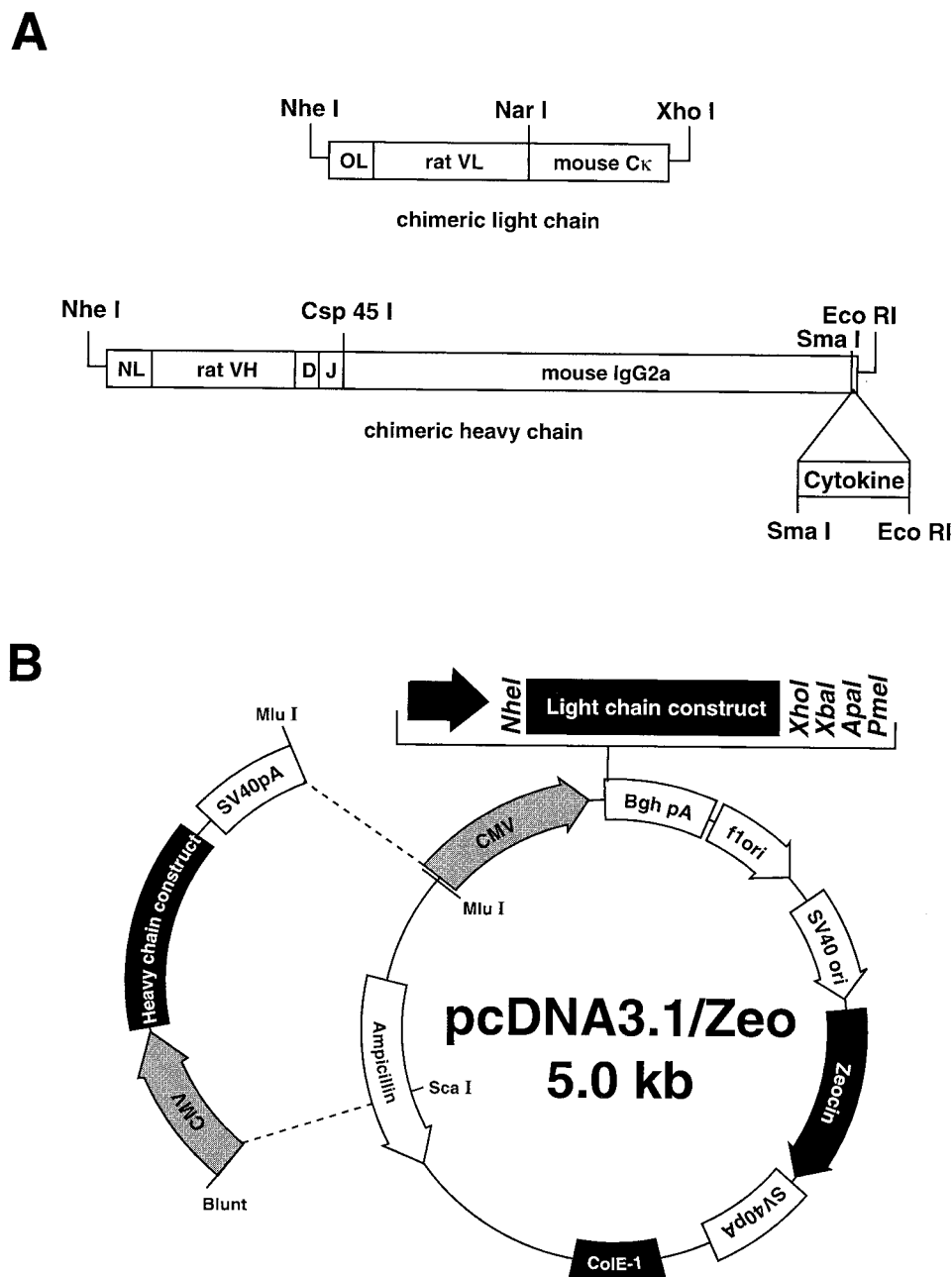


Figure 1. Schematics depicting DNA constructs encoding for the ch17217-IL2 and GM-CSF immunocytokines and their expression vector. (A) Schematic of heavy and light chains; the variable region of the rat R17217 light chain (VL) was constructed downstream of an OKT3 leader sequence (OL) and subsequently cloned upstream of the constant region of a mouse κ light chain ($C\kappa$) using the *NarI* restriction site. The variable region of the rat R17217 heavy chain (VH) was combined with a hamster-derived N418 leader sequence (NL) and introduced in the heavy-chain backbone of mouse Ig2a by using the *Csp 45I* restriction site. (B) Double expression vector with heavy and light chains. The ch17217 light-chain expression unit was cloned into the multiple cloning site of the pcDNA 3.1/zeo vector as an *NheI XhoI* fragment. Heavy-chain expression units for each fusion protein were introduced as *ScaI MluI* fragments, generated as described in the Experimental Procedures. The zeocin resistance gene remained intact as a functional selection marker in both eukaryotic and prokaryotic cells.

ch17217-GM-CSF immunocytokine and incubated for 24 h. [*methyl*- ^3H]Thymidine uptake was measured 16 h after addition of 0.5 μCi /well. All samples were tested in triplicate.

Radiolabeling. Radiolabeling with ^{125}I was performed, as previously described (17). Briefly, 1 mg of the respective protein was incubated for 30 min on ice with 1 mCi of ^{125}I (100 mCi, 3.75 Gbq/mL (Amersham, Arlington Heights, IL) in polystyrene tubes coated with 100 μg of Iodo-Gen reagent (Pierce, Rockford, IL). Non-incorporated ^{125}I was removed by gel filtration on PD10 columns (Pharmacia, Piscataway, NJ).

Saturation Binding Assays. Binding assays were performed with ch17217 and both immunocytokines on several mouse tumor cell lines (RENCA, NXS2, CT26). Although all of these cell lines expressed Tf-R, we selected RENCA mouse renal carcinoma cells for our analyses, since these were adherent and provided the most reproducible saturation binding data. For that purpose, 9×10^4 cells were added to 24-well microtiter plates and cultured for 24 h. Saturation binding was then determined by adding the respective ^{125}I -radiolabeled proteins at various concentrations to the cells followed by incubation on ice for 2 h. Cells were washed three

times with ice cold PBS and 1% BSA, and cell-bound radiolabel was determined in a γ -scintillation counter. Nonspecific binding was determined for all proteins in the presence of a 200-fold excess of unlabeled mAb R17217. The number of binding sites and the binding constants for each construct were determined by Scatchard plot analysis (18).

Pharmacokinetics. Six week old BALB/c mice were used to determine the blood clearance of mAb ch17217 and immunocytokines, ch17217-IL2 and ch17217-GM-CSF. Groups of four mice each were injected into the lateral tail vein with 25 μ g of each of these radiolabeled proteins (specific activity of 0.2 μ Ci/ μ g). Blood samples were collected from the orbital sinus at 0.5, 1, 2, 4, 8, 24, 48, and 72 h after injection of the radiolabeled proteins and assayed for ^{125} I activity in a γ -scintillation counter. Elimination ($t_{1/2\alpha}$) and distribution ($t_{1/2\beta}$) phase half-lives were calculated assuming exponential clearance according to the formula $A = A_0 e^{-kt}$ using the two earliest and latest time points, respectively (19).

Syngeneic Tumor Models. Pulmonary colon carcinoma and hepatic neuroblastoma metastases were induced in syngeneic BALB/c and A/J mice, respectively, as described previously (10, 11). Briefly, 1×10^5 or 1×10^4 NXS2 mouse neuroblastoma cells were injected into the lateral tail vein of A/J mice to induce hepatic metastases. CT26 mouse colon carcinoma cells (1×10^4) were injected intravenously (i.v.) for induction of pulmonary metastases. Mice were treated 24 h after tumor cell inoculation with daily i.v. injections ($\times 7$) of either PBS, 5 and 30 μ g ch17217-IL2, or 30 μ g of ch17217-GM-CSF. Macroscopic metastases to livers and lungs were determined 24–28 days after tumor cell inoculation. Since the metastatic foci appeared fused, metastases were scored according to the percentage of lung and liver surface involvement.

Statistics. The statistical significance of differential findings between experimental groups of mice was determined by the nonparametric Mann–Whitney rank sum test. Results were regarded significant if two-tailed P values were <0.05 .

RESULTS

Expression of Immunocytokines. The construction of immunocytokines between the ch17217 antibody and mouse IL2 or mouse GM-CSF is described in the Experimental Procedures and shown schematically in Figure 1. The mature IL2 or GM-CSF protein sequences were directly fused to the carboxyl terminus of the CH3 exon of the mouse C γ 12a gene from which the three penultimate amino acids were deleted. The proteins secreted by these transfectants assembled into complete antibody molecules with the cytokine linked to the IgH chains. In both cases, mouse IL2 and GM-CSF retained their biological activities. The expression levels of the transfectants ranged from 10 to 20 μ g/mL.

Purification and Analysis of Immunocytokines. Purification was achieved by passing spent culture medium of stable-transfected CHO cells over a protein A Sepharose column. The purity of both immunocytokines was documented by SDS–PAGE analysis for chain composition and assembly under reducing conditions. Figure 2A indicates that under reducing conditions the ch17217 antibody dissociated into heavy (H) and light (L) chains of the size corresponding to that of the R17217 antibody. The H chains of the GM-CSF and IL2 immunocytokines showed higher apparent molecular masses of ~68 and ~62 kDa, consistent with the fusion of GM-

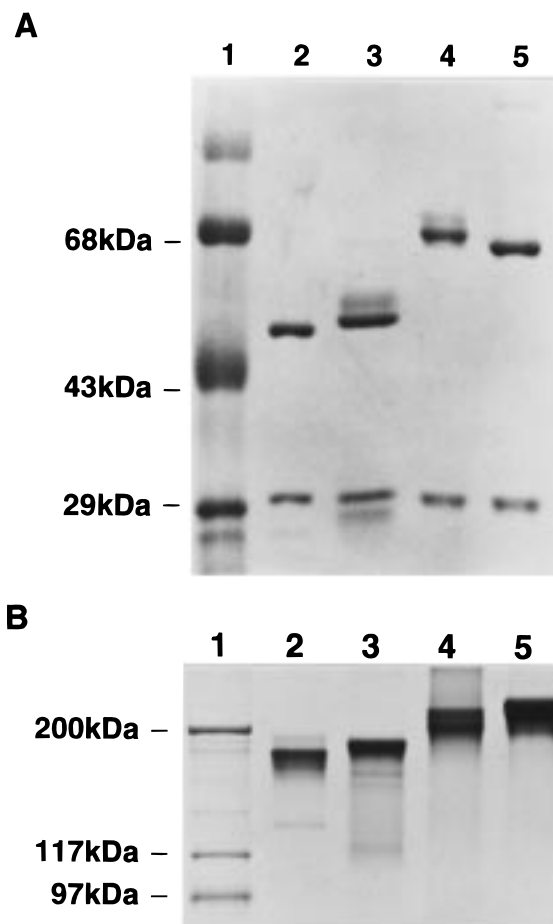


Figure 2. Analysis of ch17217 anti-mouse transferrin receptor antibody and its IL2 and GM-CSF immunocytokines. (A) Proteins were diluted in gel sample buffer, boiled under reducing conditions and analyzed on a 10% polyacrylamide gel containing SDS. Purified R17217 (lane 2) and ch17217 (lane 3) were compared to immunocytokine ch17217-GM-CSF (lane 4) and ch17217-IL2 (lane 5). Molecular weight markers are shown in lane 1. (B) Samples were also analyzed on SDS–PAGE under nonreducing conditions. Purified R17217 (lane 2) and ch17217 (lane 3) were compared to immunocytokines ch17217-IL2 (lane 4) and ch17217-GM-CSF (lane 5).

CSF and IL2, respectively. Figure 2B shows the electrophoretic profile of the same constructs on SDS–PAGE under nonreducing conditions. The molecular size of each immunocytokine and antibody was as expected, with ch17217-GM-CSF showing the highest molecular size, followed by ch17217-IL2 and the antibodies in that order.

The effect which fusion of the cytokines exerted on the antigen-binding activity of ch17217 was determined by flow cytometry and direct binding in saturation-binding assays. Flow cytometry using NXS2 neuroblastoma cells indicated equal binding of ch17217 and its IL2 and GM-CSF fusion protein to Tf-R expressed on these cells (data not shown). A representative saturation-binding assay with RENCA cells, followed by Scatchard analysis using all three constructs, is depicted in Figure 3. Essentially identical binding constants (K_D) for ch17217 and its IL2 and GM-CSF immunocytokines were demonstrated with K_D values ranging from $(2 \text{ to } 4.5) \times 10^{-9}$ M and the number of Tf-R binding sites expressed on RENCA cells ranging from $(3 \text{ to } 5) \times 10^5$ per cell in three separate experiments.

Biological Activities of Immunocytokines. The IL2 activity of ch17217-IL2 was measured in a proliferation assay with the IL2-dependent mouse CTLL-2 cell

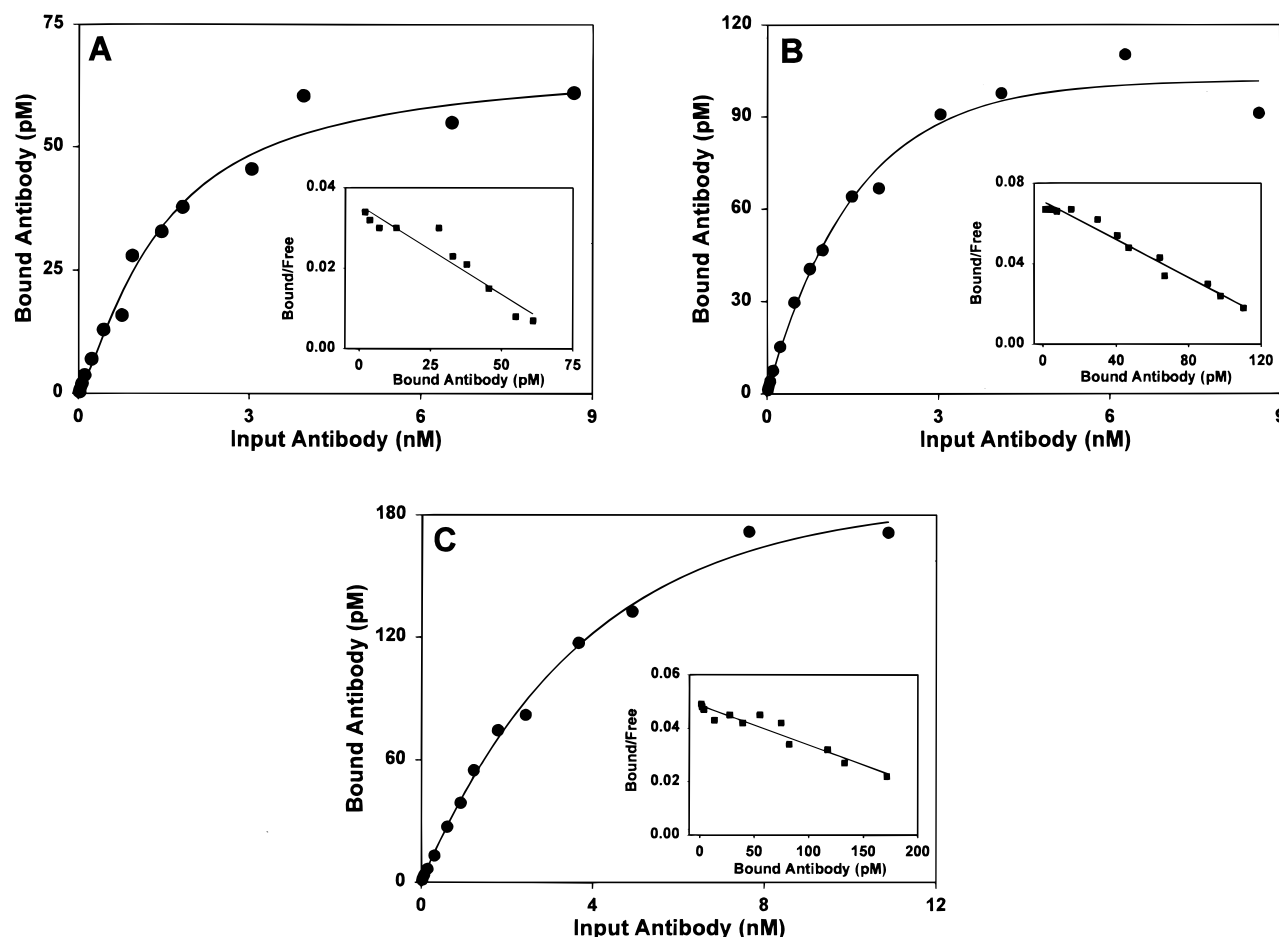


Figure 3. Scatchard plot of saturation binding analysis. Serial dilutions of ^{125}I -labeled ch17217-IL2 (A) and GM-CSF (B) immunocytokines were compared with ch17217 antibody (C) in a saturation-binding assay using mouse RENCA cells. Binding constants of each construct was calculated from corresponding Scatchard plots. Inserts depict the respective Scatchard plots.

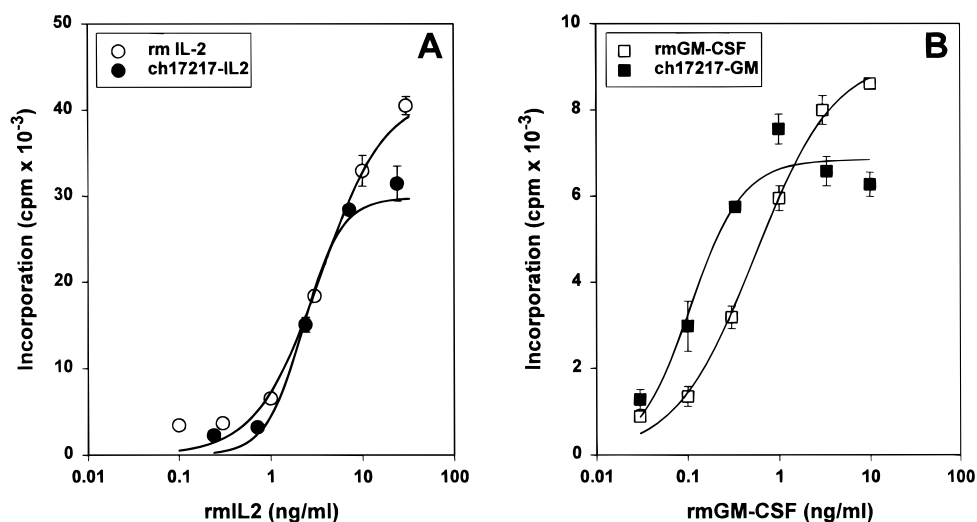


Figure 4. Biological activity of immunocytokines. (A) Proliferation activity assays of recombinant mouse (rm) IL2 standard and purified ch17217-IL2 immuno-cytokine were performed at several dilutions using the IL2-dependent mouse leukemia cell line CTLL-2. (B) Proliferation activity assay of recombinant mouse (rm) GM-CSF standard and purified ch17217-GM-CSF immunocytokine. This was performed as above with the GM-CSF-dependent mouse hematopoietic stem cell line 32D (G).

line. When compared on a protein basis to recombinant mouse IL2, produced in bacteria, the immunocytokine required essentially equal concentration to achieve the same level of proliferation when assayed in the range of 1–10 ng/mL (Figure 4A). The GM-CSF activity of the ch17217-GM-CSF immunocytokine, tested in a proliferation assay with the GM-CSF-dependent mouse hemo-

poietic stem cell line 32D(G), required less material than the rmGM-CSF standard (produced in bacteria) when assayed in the range 0.1–1 ng/mL.

Pharmacokinetics. In vivo blood clearance was measured by intravenous injection of ^{125}I -labeled, purified immunocytokines into BALB/c mice. Analyses of blood samples collected at various time intervals (see Experi-

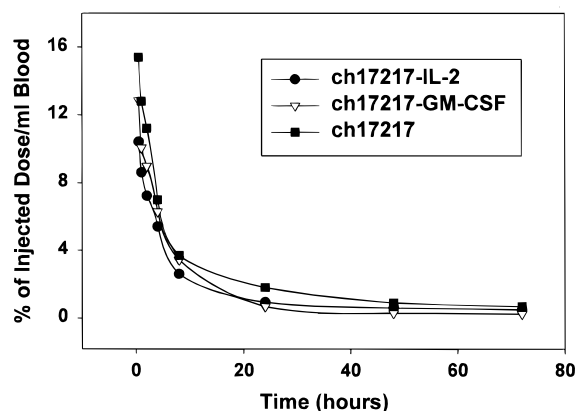


Figure 5. Blood clearance of ch17217 antibody and immunocytokines. Groups of four mice were injected with 25 μ g of ^{125} I-labeled ch17217 antibody or each immunocytokine. Blood samples were collected at various time intervals and assayed for ^{125} I activity in a γ -scintillation counter. Each data point represents the mean value of four individual mice. Standard deviations were always less than $\pm 2\%$ of injected dose/mL blood.

Table 1. Half-Lives of the α and β Phases of the Free Antibody and Immunocytokines

	$t_{1/2}$, h	
	α	β
ch17217	1.9	83
ch17217-GM-CSF	1.8	88
ch17217-IL2	1.8	91

mental Procedures) indicated very similar clearance rates for ch17217 and the GM-CSF and IL2 immunocytokines (Figure 5). Table 1 indicates that the elimination ($t_{1/2\alpha}$) and distribution ($t_{1/2\beta}$) phases for all three constructs are in a similar range.

Antitumor Activity of Immunocytokines. The antitumor activities of the IL2 and GM-CSF immunocytokines were demonstrated by their ability to effectively suppress the growth of either hepatic metastases of NXS2 mouse neuroblastoma in syngeneic A/J mice or pulmonary metastases of CT26 mouse colon carcinoma in syngeneic BALB/c mice. Treatment with daily i.v. injections ($\times 7$) was initiated 24 h after i.v. injection of the respective tumor cells, and animals were sacrificed and analyzed for macroscopic tumor growth 24–28 days after tumor cell inoculation. The data shown in Figure 6A indicate that treatment with 30 μ g ($\times 7$) of ch17217-IL2 completely suppressed the growth of hepatic neuroblastoma metastases in 6/6 animals, whereas 5 μ g ($\times 7$) was only partially effective ($p < 0.02$). This is in contrast to control animals injected with PBS that uniformly showed extensive hepatic metastases. Administration of 30 μ g ($\times 7$) of the ch17217-GM-CSF immunocytokine completely suppressed the growth of hepatic neuroblastoma metastases in 5/6 mice. By comparison, all control mice injected with PBS exhibited extensive metastases. The data shown in Figure 6B indicate that administration of 30 μ g ($\times 7$) of the ch17217-IL2 immunocytokine completely suppressed pulmonary metastases of colon carcinoma in 6/8 mice, and an equal dose of the ch17217-GM-CSF immunocytokine achieved this same effect in 5/8 animals. In both cases, two mice exhibited partial suppression of pulmonary metastases following administration of ch17217-GM-CSF immunocytokine, as compared to all control animals injected with PBS that showed extensive metastases.

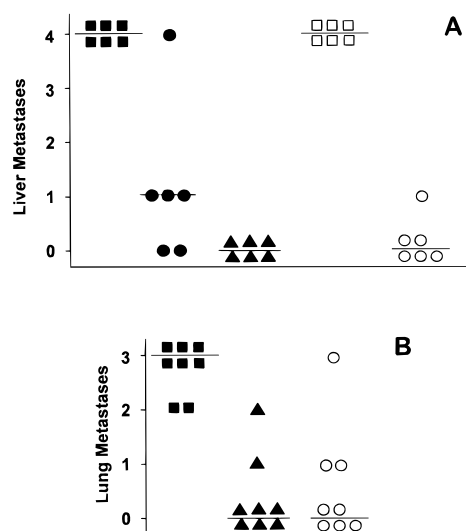


Figure 6. Antitumor activity of ch17217 immunocytokines in syngeneic tumor models of mouse neuroblastoma and colorectal carcinoma. Mice were injected intravenously with 1×10^5 (closed symbols) or 1×10^4 (open symbols) NXS2 mouse neuroblastoma cells (A) or 1×10^4 CT26 mouse colorectal carcinoma cells (B). Treatment with either PBS(n), ch17217-IL2 (l, 5 μ g; s, 30 μ g) or ch17217-GM-CSF fusion protein (m, 30 μ g) was initiated 24 h after inoculation by daily intravenous injections ($\times 7$). Animals were sacrificed 24–28 days after inoculation. Liver or lung metastases were staged according to the percentage of metastatic liver or lung surface: Liver: 0 = 0%, 1 = 0–25%, 2 = 25–50%, 3 = 50–75%, 4 = $\geq 75\%$; Lung: 0 = 0%, 1 = 0–5%, 2 = 5–50%, 3 = $\geq 50\%$. Differences in metastatic scores between mice treated with fusion protein compared to untreated controls were all statistically significant ($P < 0.02$).

DISCUSSION

The rapid development of recombinant DNA technologies during the past decade facilitated the construction of antitumor antibody-cytokine fusion proteins (20). These constructs, called immunocytokines, combine the unique targeting ability of monoclonal antibodies with the multifunctional biological activities of cytokines and effectively direct these immunomodulatory molecules to the tumor microenvironment. Since most cytokines are paracrine factors that function optimally within a few cell diameters from their cell of origin, they are most effective in activating and proliferating immune effector cells to kill tumor cells when concentrated in the tumor microenvironment.

The systemic administration of cytokines, such as IL2, has led to improvements in the treatment of some patients with melanoma and renal carcinoma (1–4); however, a number of toxic side reactions were dose limiting (4) and led investigators to focus on local cytokine treatment strategies. This included the development of cytokine gene therapy largely by ex vivo transfection of a variety of cytokine genes, including IL2 and GM-CSF, into patients' autologous tumor cells or fibroblasts (6–8). Unlike systemic administration of cytokines, gene therapy with cytokines takes advantage of their paracrine nature. In a number of early clinical trials, this type of immunotherapy resulted in improvements in the treatment of some cancer patients (8). In preclinical experiments, gene therapy with cytokines induced an inflammatory response at the site where transduced tumor cells were injected, resulting in their rejection, which, in some cases, was followed by a systemic immune response capable of rejecting a challenge of wild-type tumor cells (5–7). However, the

patient-specific nature of this type of therapy will likely limit its widespread, broad clinical application.

We (9–12) and others (20, 21) developed several recombinant immunocytokines as an alternative approach for cancer immunotherapy that conforms with the paracrine nature of cytokines and is not limited by being patient specific. In all of these experiments, an antigen expressed preferentially on human or mouse tumor cells served as a docking site for directing immunocytokines to the tumor microenvironment. In a number of experiments, the gene encoding for a human tumor-associated antigen was transduced into mouse tumor cells which then became uniquely specific targets for immunocytokines in syngeneic hosts that completely lacked this same tumor-associated antigen in their organs and tissues (11, 12).

Here, we applied a distinctly different strategy by selecting the mouse transferrin receptor (Tf-R) as a target, which is universally expressed on most mouse tumor cells, as well as on a variety of normal organs and tissues. These include, in particular, liver, kidney, and spleen. In fact, the anti-mouse Tf-R antibody R17217 used in this study was shown to follow this pattern of biodistribution and was predominantly detected in the spleen. It is presently not known whether this is entirely due to its specific binding to Tf-R or to uptake by the reticuloendothelial system (13).

We wanted to test the hypothesis that even under these somewhat unfavorable conditions, anti-TfR antibody-IL2 and GM-CSF immunocytokines could still effectively suppress the growth of pulmonary and hepatic metastases of mouse colon carcinoma and neuroblastoma in syngeneic mice. We purposely selected the rat anti-mouse Tf-R antibody R17217 of IgG2b isotype, since it was shown *not* to block Tf-R functions involved in iron transport and thus proved incapable of inhibiting the growth of mouse tumor cells (14).

We demonstrated purity of the IL2 and GM-CSF immunocytokines by SDS–PAGE analysis under reducing conditions and showed that these constructs secreted by transfected CHO cells were capable of assembling into complete antibody molecules with IL2 and GM-CSF fused to the Ig heavy chain (Figure 2). Construction of these immunocytokines did not affect their antigen-binding affinities as saturation-binding studies and Scatchard plot analyses of these data indicated that the binding constant (K_D) of the chimeric anti-Tf-R antibody ch17217 was in the same range, i.e., $(2–4.5) \times 10^{-9}$ M, as those of its GM-CSF and IL2 immunocytokines. When tested on a mouse renal carcinoma cell line (RENCA), these immunocytokines bound Tf-R sites expressed on these cells essentially in the same range of $(3–5) \times 10^5$ sites/cell as ch17217. Apparently, the deletion of the three penultimate carboxyl terminal amino acids of Ig H chains of the two immunocytokines did not measurably affect their binding affinities.

The IL2 activity of ch17217-IL2 was found to be essentially the same as that of the recombinant mouse IL2 standard produced in bacteria. This was true for a concentration range of 1–10 ng/mL required to achieve proliferation of the IL2-dependent mouse leukemia cell line CTLL-2 (Figure 4A). By contrast, the GM-CSF activity of ch17217-GM-CSF was greater in the 0.1–1 ng concentration range than that of the recombinant mouse GM-CSF standard produced in nonglycosylated form in bacteria (Figure 4B). Apparently, the GM-CSF moiety of the ch17217-GM-CSF immunocytokine has more activity than the nonglycosylated GM-CSF standard. This

could be due to its expression in CHO cells where it becomes fully glycosylated (22).

An examination of the pharmacokinetics of ch17217 and its GM-CSF and IL2 immunocytokines in BALB/c mice revealed quite similar values for $t_{1/2\alpha}$ (elimination phase) and the $t_{1/2\beta}$ (distribution phase). These data are of interest when compared with those obtained previously by us in athymic *nu/nu* mice with human/mouse chimeric anti-ganglioside GD₂ antibody (ch14.18) and its respective immunocytokines with recombinant human GM-CSF and human IL2. In this case, the $t_{1/2\alpha}$ phases were 7.5, 1.4, and 0.3 h, and the $t_{1/2\beta}$ phases were 59, 30, and 28 h, respectively (23). In the present study, $t_{1/2\beta}$ (distribution phase) values were considerably extended, ranging from 83 to 91 h. Importantly, these values far exceed those of native GM-CSF, which was reported to be 50–85 min following bolus injection into humans (24) and that of rhIL2 reported as 13–24 min under the same conditions (25). The increase in $t_{1/2\beta}$ phases of ch17217-mouse GM-CSF over that of the ch14.18-huGM-CSF immunocytokine is noteworthy, since mouse GM-CSF has considerably higher affinity for the mouse GM-CSF receptor than human GM-CSF (26). The more extended $t_{1/2\beta}$ phases of the ch17217-IL2 and GM-CSF immunocytokines may also be influenced by their accumulation in the liver, kidney and spleen of mice (13).

However, the most important question to answer was whether the two immunocytokines under study were effective in directing IL2 and GM-CSF to the tumor microenvironment to mediate cellular immune responses capable of suppressing the growth and dissemination of tumor metastases. It is quite evident from our data (Figure 6) that this is indeed the case in neuroblastoma and colon carcinoma metastasis models in syngeneic mice. It is noteworthy that growth and dissemination of hepatic neuroblastoma metastases and pulmonary colon carcinoma metastases could be effectively suppressed in contrast to all control animals that developed extensive metastases.

On the basis of the data obtained thus far, it is most likely that the two immunocytokines described here can be used in a wide variety of syngeneic mouse tumor models to critically evaluate their efficacy in suppressing growth and dissemination of tumor metastases. More importantly, it is likely that these immunocytokines will lend themselves very well for in-depth studies of immunological effector mechanisms involved in their anti-tumor activities.

ACKNOWLEDGMENT

We wish to thank Dr. Ekke Liehl (NOVARTIS, Vienna, Austria) for a generous gift of recombinant murine GM-CSF. The authors express their appreciation to Ms. Lynne Kottel for preparation of this manuscript and to Ms. Lucia Morkes for technical assistance. This work was supported by NIH Grant CA37641. T.D. and H.N.L. were supported by a training grant of the Deutsche Forschungsgemeinschaft. This is The Scripps Research Institute's manuscript 11419-IMM.

LITERATURE CITED

- (1) Rosenberg, S. A. (1988) Immunotherapy of cancer using interleukin-2: Current status and future prospects. *Immunol. Today* 9, 58–62.
- (2) Rosenberg, S. A. (1992) The immunotherapy and gene therapy of cancer. *J. Clin. Oncol.* 10, 180–199.
- (3) Rosenberg, S. A., Yanneli, J. R., Yang, J. C., Topalian, S. L., Schwartzentraber, D. S., Weber, J. S., Parkinson, D. R., Seipp, C. A., Einhorn, J. D., and White, D. E. (1994)

- Treatment of patients with metastatic melanoma with autologous tumor-infiltrating lymphocytes and interleukin-2. *J. Natl. Cancer Inst.* 86, 1159–1166.
- (4) Rosenberg, S. A., Lotze, M. T., Yang, J. C., Aebersold, P. M., Linehan, W. M., Seipp, C. A., and White, D. E. (1989) Experience with the use of high-dose interleukin-2 in the treatment of 652 cancer patients. *Ann. Surg.* 210, 474–485.
- (5) Maas, R. A., Dullens, H. F. J., and Otter, W. D. (1993) IL2 in cancer treatment. *Cancer Immunol. Immunother.* 36, 141–148.
- (6) Colombo, M. P., and Forni, G. (1994) Cytokine gene transfer in tumor inhibition and tumor therapy: Where are we now? *Immunol. Today* 15, 48–51.
- (7) Schmidt-Wolf, G., and Schmidt-Wolf, I. G. H. (1995) Cytokines and clinical gene therapy. *Eur. J. Immunol.* 25, 1137–1140.
- (8) Roth, J. A., and Cristiano, R. J. (1997) Gene therapy for cancer: What have we done and where are we going? *J. Natl. Cancer Inst.* 89, 21–39.
- (9) Gillies, S. D., Reilly, E. B., Lo, K.-M., and Reisfeld, R. A. (1992) Antibody-targeted interleukin-2 stimulates T-cell killing of autologous tumor cells. *Proc. Natl. Acad. Sci. U.S.A.* 89, 1428–1432.
- (10) Lode, H. N., Xiang, R., Dreier, T., Varki, N. M., Gillies, S. D., and Reisfeld, R. A. (1998) NK-cell-mediated eradication of neuroblastoma metastases to bone marrow by targeted interleukin-2 therapy. *Blood* (In press).
- (11) Xiang, R., Lode, H. N., Dolman, C. S., Dreier, T., Varki, N. M., Qian, X., Lo, K.-M., Lang, Y., Super, M., Gillies, S. D., and Reisfeld, R. A. (1997) Elimination of established mouse colon carcinoma metastases by antibody-interleukin 2 fusion protein therapy. *Cancer Res.* 57, 4948–4955.
- (12) Becker, J. C., Varki, N., Gillies, S. D., Furukawa, K., and Reisfeld, R. A. (1996) Long-lived and transferable tumor immunity in mice after targeted interleukin-2 therapy. *J. Clin. Invest.* 98 (12), 2801–2804.
- (13) Trowbridge, I. S., Lesley, T., and Schulte, R. (1982) Mouse cell surface transferrin receptor: Studies with an anti-receptor monoclonal antibody. *J. Cell Physiol.* 112, 403–410.
- (14) Lesley, J. F., Schulte, R. A., and Woods, J. (1989) Modulation of transferrin receptor expression and function by anti-transferrin receptor antibodies and antibody fragments. *Exptl. Cell Res.* 182, 215–233.
- (15) Kabat, E. A., Wu, T. T., Perry, H., M., Gottesman, K. S., and Foeller, C. (1991) *Sequences of proteins of immunological interest*, 5th ed., Bethesda, MD, U. S. Department of Health and Human Services, NIH, NIH Publication 91-3242.
- (16) Gillis, S., Ferm, M. M., Ou, W., and Smith, K. A. (1978) T-cell growth factor: Parameters of production and quantitative microassay for activity. *J. Immunol.* 120, 2027–2032.
- (17) Mueller, B. M., Reisfeld, R. A., and Gillies, S. D. (1990) Serum half-life and tumor localization of a chimeric antibody deleted of the CH2 domain and directed against the disialoganglioside GD2. *Proc. Natl. Acad. Sci. U.S.A.* 87, 5702–5705.
- (18) Scatchard, O. (1949) The attraction of proteins for small molecules and ions. *Ann. N.Y. Acad. Sci.* 51, 660–669.
- (19) Slater, J. B., Slater, J. M., Gridley, D. S., and Kirk, G. A. (1996) Relationship between 2CE 025 blood clearance and liver accumulation in patients with colorectal carcinoma. *Immunopharmacol.* 35, 111–118.
- (20) Reisfeld, R. A., and Gillies, S. D. (1996) Recombinant antibody fusion proteins for cancer immunotherapy. *Current Topics in Microbiology and Immunology* (U. Günthert, P. Schlag, and W. Birchmeier, Eds.) Vol. 213III, pp 27–53, Springer-Verlag, Berlin/Heidelberg.
- (21) Hornick, J. L., Khawli, L. A., Hu, P., Lynch, M., Anderson, P. M., and Epstein, A. L. (1997) Chimeric CLL-1 antibody fusion proteins containing granulocyte-macrophage colony-stimulating factor or interleukin-2 with specificity for B-cell malignancies exhibit enhanced effector functions while retaining tumor targeting properties. *Blood* 89 (12), 4437–4447.
- (22) Moonen, P., Mermod, J.-J., Ernst, J. F., Herschi, M., and DeLamar, J. F. (1987) Increased biological activity of deglycosylated recombinant human granulocyte/macrophage colony-stimulating factor produced by yeast or animal cells. *Proc. Natl. Acad. Sci. U.S.A.* 84, 4428–4432.
- (23) Gillies, S. D., Young, D., Lo, K.-M., and Robert, S. (1993) Biological activity and in vivo clearance of antitumor antibody/cytokine fusion proteins. *Bioconjugate Chem.* 4, 230–235.
- (24) Cebron, J., Dempsey, P., Fox, R. R., Kanomakis, G., Bonnem, E., Burgess, A. W., and Morstyn, G. (1988) Pharmacokinetics of human granulocyte-macrophage colony stimulating factor using a sensitive immunoassay. *Blood* 72, 1340–1345.
- (25) Konrad, M. W., Hemstreet, G., Hersh, E. M., Mansell, P. W. A., Mertelsmann, R., Kolitz, J. E., and Bradley, E. C. (1990) Pharmacokinetics of recombinant interleukin 2 in humans. *Cancer Res.* 50, 2009–2015.
- (26) Kaushansky, K., Shoemaker, S. G., Alfaro, S., and Brown, C. (1989) Hematopoietic activity of granulocyte/macrophage colony stimulating factor is dependent upon two distinct regions of the molecule: Functional analysis based upon the activities of interspecies hybrid growth factors. *Proc. Natl. Acad. Sci. U.S.A.* 86, 1213–1218.

BC980020E

Cell-Specific Modulation of Drug Resistance in Acute Myeloid Leukemic Blasts by Diphtheria Fusion Toxin, DT388-GMCSF

Arthur E. Frankel,^{*,†} Philip D. Hall,[‡] Chris McLain,[†] Ahmad R. Safa,[§] Edward P. Tagge,^{||} and Robert J. Kreitman[⊥]

Departments of Medicine, Surgery, Experimental Oncology, and Pharmaceutical Sciences, Medical University of South Carolina, Charleston, South Carolina 29425, and Laboratory of Molecular Biology, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892. Received February 4, 1998; Revised Manuscript Received April 10, 1998

Radiochemotherapy-resistant blasts commonly cause treatment failure in acute myeloid leukemia (AML), and their resistance is due, in part, to overexpression of multidrug resistance (mdr) proteins. We reasoned that targeted delivery of protein synthesis inactivating toxins to leukemic blasts would reduce the cellular concentrations of relatively short half-life resistance proteins and sensitize the cells to cytotoxic drugs. To test this hypothesis, we employed human granulocyte-macrophage colony-stimulating factor fused to truncated diphtheria toxin (DT388-GMCSF). The human AML cell line HL60 and its vincristine-resistant sublines, HL60Vinc and HL60VCR, were incubated *in vitro* for 24 h with varying concentrations of toxin. Doxorubicin was added for an additional 24 h, and cell cytotoxicity was assayed by thymidine incorporation and colony formation in semisolid medium. DT388-GMCSF sensitized HL60Vinc and HL60VCR but not HL60 to doxorubicin. Combination indices for three log cell kill varied from 0.2 to 0.3. In contrast, pretreatment with doxorubicin followed by toxins failed to show synergy. At least in the case of the vincristine-resistant cell lines, modulation of drug resistance correlated with reduction in membrane P-glycoprotein concentrations based on immunoblots with C219 antibody, flow cytometry with MRK16 antibody, and cell uptake of doxorubicin. These observations suggest clinical trials of combination therapy may be warranted in patients with refractory AML. Further, targeted toxins may represent a novel class of cell-specific modulators of drug resistance for a number of malignancies.

INTRODUCTION

There were 9200 new cases of acute myeloid leukemia (AML) in the U.S. in 1996 (1), and, even with complete remission rates of 50–70% and effective consolidation and intensification regimens including allogeneic bone marrow transplantation, over 80% of patients will die from complications of the disease and its treatment (2). Radiochemotherapy resistant blasts are a frequent cause of treatment failure in AML patients (3). In many cases, multidrug resistance (mdr) is due in part to overexpression of resistance proteins which alter drug accumulation, metabolism, and apoptotic thresholds. Modification of resistance protein function has been hampered by the finding that many resistance proteins are expressed by normal human tissues, and noncell specific modulation leads to significant toxicities. For example, the substrate analogues (quinine, cyclosporine, and PSC833) of the P-glycoprotein drug efflux transporter resistance protein have been tested in AML therapy trials and have produced toxicities to marrow and other organs, marked alterations in cytotoxic drug pharmacodynamics and minimal effects on disease-free survival or overall sur-

vival (4). Hence, there is a need for new reagents with a unique mechanism of action to modulate drug resistance specifically in AML blasts.

Three resistance proteins (P-glycoprotein, glutathione S-transferase π , and Bcl2) have been shown to have rapid turnover (24 h or less) (5–7), and the blast concentrations of these proteins correlate with clinical response rates and remission durations (8–10). These proteins could serve as targets for cell-specific inhibitors of protein synthesis such as targeted toxins. Targeted toxins are polypeptide drugs consisting of tumor cell-directed ligands covalently linked to protein synthesis inactivating peptide toxins.

A number of studies both *in vitro* and *in vivo* have shown supraadditive cell killing with combinations of immunotoxins and cytotoxic drugs (11–20). Anti-CD19-ricin A chain and anti-CD22-ricin A chain conjugates with either cyclophosphamide, doxorubicin, or camptothecin incubated with Burkitt's lymphoma cells show synergistic cell kill (11). Similarly, anti-CD19-blocked ricin conjugate combined with etoposide or doxorubicin yielded synergistic cell killing of a B-lymphoma cell line and a retrovirally transfected B-lymphoma cell line expressing mdr-1 (12). Finally, anti-p43 epithelial antigen-recombinant ricin A chain conjugate combined with thiotepa or cisplatin showed synergistic cell killing of ovarian carcinoma cell lines (13). When anti-CD19-ricin A chain and anti-CD22-ricin A chain were administered with doxorubicin, cyclophosphamide, or camptothecin to scid mice bearing Burkitt's lymphoma cells, schedule dependence for supraadditive cell killing was observed

* Address correspondence to this author at the following address: Hollings Cancer Center Room 311, 86 Jonathan Lucas St., Charleston, SC 29401. Tel: 803-792-1450. Fax 803-792-3200.

[†] Department of Medicine.

[‡] Department of Pharmaceutical Sciences.

[§] Department of Experimental Oncology.

^{||} Department of Surgery.

[⊥] Laboratory of Molecular Biology.

(14). Only when immunotoxins were given before or at the same time as cytotoxic drugs was synergy seen. With modulating doses of anti-p43-ricin A chain conjugate on ovarian carcinoma cells and anti-CD19-blocked ricin on *mdr-1* expressing B-lymphoma cells, concentrations of resistance proteins decreased (12, 13). The ovarian carcinoma cells showed decreases in glutathione S-transferase activity, and the B-lymphoma had decreases in membrane P-glycoprotein. While additional studies report coalitive interactions of immunotoxins with cytotoxic drugs in T-acute lymphoblastic leukemia (15), B-lymphoma (16), colon carcinoma (17), and B-acute lymphoblastic leukemia (18), no similar efforts to modulate drug resistance in AML with targeted toxins have been reported.

We chose to use human granulocyte-macrophage colony-stimulating factor fused to truncated diphtheria toxin (DT388-GMCSF) to inhibit protein synthesis in myeloid blasts. We and others had previously reported potent protein synthesis inhibition by this fusion toxin on leukemic blasts (19–21). We reasoned that short half-life resistance protein concentrations should be reduced, sensitizing the cells to cytotoxic drugs. Further, GMCSF receptors are expressed on the majority of AML patients' blasts (24). We chose doxorubicin as the cytotoxic drug for study since anthracyclines are a critical component of AML induction regimens. The cell lines selected were HL60 and its vincristine-resistant sublines HL60Vinc and HL60VCR (25, 26). HL60 is a well-characterized human AML cell line displaying GMCSF receptors. The latter two sublines show different degrees of multidrug resistance, and each overexpresses P-glycoprotein. The goals of the present study were to examine the ability of the fusion toxin to modulate doxorubicin sensitivity in the three cell lines, determine the schedule dependency of any observed modulation, and correlate modulation with changes in P-glycoprotein concentration. We believed such a study was a requisite step prior to examination of resistance modulation with fresh leukemic blasts.

MATERIALS AND METHODS

Materials. DT388-GMCSF was prepared as previously described (20) and stored at -20°C until used. DT390mGM-CSF was a kind gift of Dr. Daniel Vallera and was stored at -20°C until used (38).

Cell Culture. HL60 human leukemia cells were a gift from M. Center, Kansas State University. The cells were grown in RPMI1640 plus 15% fetal calf serum. All media and components were purchased from Irvine Scientific (Santa Ana, CA). HL60Vinc, a vincristine-resistant subline of HL60 (26), was a gift from M. Center and maintained in RPMI1640 plus 15% fetal calf serum. The HL60VCR vincristine-resistant subline was developed by stepwise exposure of HL60Vinc to increasing concentrations of vincristine (A. R. Safa, unpublished data) and maintained in RPMI1640 plus 15% fetal calf serum plus $1\text{ }\mu\text{g/mL}$ vincristine (Eli Lilly, Indianapolis, IN). WT19 mouse leukemia cells were a gift of Dr. Andrew Kraft, University of Colorado Medical Center (41). The cells were grown in RPMI1640 plus 15% fetal calf serum with 10% WEHI cell conditioned medium.

GMCSF Receptor Density. Ten aliquots of two million cells in RPMI1640 plus 2.5% bovine serum albumin (BSA) plus 0.2% sodium azide plus 20 mM Hepes, pH 7.2, in a total volume of $150\text{ }\mu\text{L}$ were incubated with different concentrations of [^{125}I]GMCSF (Amersham, Arlington Heights, IL) in the presence or absence of 1500

ng of unlabeled human GMCSF (Immunex, Seattle, WA) for 30 min at 37°C with shaking. The cell suspensions were then layered onto $200\text{ }\mu\text{L}$ of phthalate oil mixture (1.5 parts dibutylphthalate and 1 part dioctylphthalate) in 1.5 mL Eppendorf tubes, centrifuged at 12 000 rpm in a microfuge at room temperature for 1 min and both cell pellets and cell supernatants counted in an LKB-Wallac 1260 multi-gamma counter gated for ^{125}I with 50% counting efficiency. Scatchard plots of specific bound/free versus specific bound counts per minute were made. Receptor density/cell was calculated as the value for the x-intercept divided by (specific activity in microcuries per microgram times the cell number times 4.2×10^{-8}). K_d was calculated as the x-intercept times 2.7×10^{-13} divided by the product of the y-intercept and the specific activity.

Thymidine Incorporation Cytotoxicity Assays. A total of 5×10^4 cells (HL60, HL60Vinc, HL60VCR, or WT19) were incubated in $100\text{ }\mu\text{L}$ RPMI1640 plus 15% fetal calf serum in Costar 96 well flat-bottomed plates. Twelve different concentrations of DT388-GMCSF or DT390mGM-CSF were added to each column in $50\text{ }\mu\text{L}$ of media and the cells maintained at $37^{\circ}\text{C}/5\%\text{ CO}_2$ for 24 h. Then, eight different concentrations of doxorubicin (Pharmacia, Kalamazoo, MI) were added to each row in a total volume of $25\text{ }\mu\text{L}$, and incubation continued for another 24 h at $37^{\circ}\text{C}/5\%\text{ CO}_2$. Then $1\text{ }\mu\text{Ci}$ [^3H]thymidine (NEN DuPont, Boston, MA) in $25\text{ }\mu\text{L}$ of medium was added to each well and incubated at $37^{\circ}\text{C}/5\%\text{ CO}_2$ for an additional 18 h. Cells were then harvested by a Skatron Cell Harvester (Skatron Instruments, Lier, Norway) onto glass fiber mats and ^3H counts per minute was counted in an LKB liquid scintillation counter gated for ^3H . The calculated IC_{90} , IC_{99} , and $\text{IC}_{99.9}$ were the concentrations of toxin, doxorubicin, or combination which inhibited thymidine incorporation by 90, 99, or 99.9% compared to control wells. To determine whether the cytotoxic effect of combination treatment was additive, antagonistic, or synergistic, an analysis using the combination index (CI) for each level of cytotoxicity was calculated using the equation: $\text{CI} = A/A_e + B/B_e$, where A and B are the concentrations of toxin and drug that in combination kill 90, 99, or 99.9% of cells and A_e and B_e are the concentrations of the toxin and drug, respectively, extrapolated from their individual killing curves capable of killing 90, 99, or 99.9% of cells alone (27). For $\text{CI} > 1$, antagonism is present; for $\text{CI} = 1$, additivity is present; for $\text{CI} < 1$, synergy is present.

Clonogenic Cytotoxicity Assays. To evaluate inhibition of colony formation as another measure of cell kill, $70\text{ }\mu\text{L}$ aliquots containing 2×10^4 cells were collected from the wells with toxin and doxorubicin described above after 48 h incubation. The cells were dispersed in 3 mL of RPMI1640 plus 15% fetal calf serum with 50 ng/mL GMCSF (Immunex), GCSF (Amgen, Thousand Oaks, CA) and 0.2% agarose (SeaPlaque, FMC Bioproducts, Rockland, ME) in 35 mm gridded polystyrene Petri dishes. After 20 min of solidification at 4°C , dishes were incubated 7 days at $37^{\circ}\text{C}/5\%\text{ CO}_2$ in a humidified incubator. Colonies containing >20 cells were counted on an inverted microscope. IC_{90} , IC_{99} , and $\text{IC}_{99.9}$ were the concentrations of toxin, doxorubicin, or combination, which inhibited colony formation by 90, 99, or 99.9% compared to control wells. CIs were calculated as for thymidine incorporation inhibition.

Reversed Schedule Thymidine Assay. Aliquots of $100\text{ }\mu\text{L}$ of medium with 5×10^4 cells were incubated first with $25\text{ }\mu\text{L}$ of medium containing eight different concentrations of doxorubicin. After 24 h, $50\text{ }\mu\text{L}$ of medium

containing 12 different concentrations of DT388-GMCSF was added, and incubation continued another 24 h at 37 °C/5% CO₂. [³H]Thymidine was then added (1 μ Ci in 25 μ L/well) and incubation and harvesting done as described above. CIs were again calculated as above.

Cell Uptake of Doxorubicin. Aliquots of one million cells in 100 μ L RPMI1640 plus 15% fetal calf serum with or without 50 μ M verapamil (Sigma) were mixed with or without 2.8 μ g/mL doxorubicin for 60 min at 37 °C/5% CO₂ and then washed with PBS/BSA/azide and then resuspended in 500 μ L of PBS/BSA/azide and run on the EPICS-XL with scatter gating and rhodamine fluorescence detection.

P-Glycoprotein Content of Cells. A total of 5×10^6 HL60, HL60Vinc or HL60VCR cells in 10 mL of RPMI1640 plus 15% fetal calf serum with or without modulating concentrations of toxin (4×10^{-9} M DT388-GMCSF) were incubated 48 h at 37 °C/5% CO₂. Cells were then harvested by centrifugation at 1000 rpm for 7 min, washed twice with 10 mL of PBS, and resuspended in 10 mM NaCl, 10 mM Tris-HCl, pH 8.0, 1 mM PMSF, and 50 μ g/mL leupeptin, and left on ice for 10 min. Cells were then sonicated, and nuclei and debris removed by centrifugation at 2500 rpm for 15 min. Membranes were isolated by centrifugation at 25 000 rpm for 60 min and resuspended in 1 mM Tris, pH 8.0, 0.1 mM EDTA, 1 mM PMSF, and 50 ng/mL leupeptin. Protein concentrations were determined by Bradford assay using the Bio-Rad protein assay kit and BSA standards. Aliquots equivalent to 1.3×10^6 cells (50–100 μ g of protein) were diluted in reducing sample buffer and subjected to 8% reducing SDS-PAGE along with Bio-Rad kaleidoscope high molecular weight protein standards. Proteins were transferred to nitrocellulose sheets using a Bio-Rad Semi-dry Transblot apparatus in Towbin buffer following the recommendations of the manufacturer. The blots were blocked with 10% nonfat dry milk solution/PBS/0.1% Tween-20/0.1% BSA/0.1% sodium azide for 3 h at room temperature with gentle shaking, washed three times with PBS plus 0.05% Tween-20 and once with PBS, incubated with 2 μ g/mL C219 anti-P-gp monoclonal antibody (Signet Labs, Dedham, MA) or 1:1000 rabbit anti-GLUT1 polyclonal antibody (a kind gift of Dr. Ian Simpson, NIDDK) in PBS plus 0.05% BSA overnight, rewashed, and incubated with 1:2000 goat anti-mouse Ig conjugated to horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA) or 1:3000 donkey anti-rabbit Ig conjugated to horseradish peroxidase (Amersham). The blots were again washed, and immune complexes detected by chemiluminescence (Amersham) following the manufacturer's recommendations. Lumograms were scanned on an IBAS automatic image analysis system (Kontron, Germany) and relevant band intensities compared. KB-V1 vincristine-resistant epidermoid carcinoma cells were used as a control for P-gp and were a gift of Dr. Mark Willingham, Wake Forest University, Winston-Salem, NC.

A separate assay for the concentration of P-glycoprotein on the surface of leukemic blasts was performed by flow cytometry as previously described (39). Briefly, one million HL60 and HL60VCR cells in 5 mL of RPMI1640 plus 15% fetal calf serum with or without 4×10^{-8} M DT388-GMCSF were incubated 60 h at 37 °C/5% CO₂. Cells were harvested by centrifugation at 1000 rpm for 7 min, washed once with 0.5 mL of Hank's balanced salt solution (Gibco) with 1% fetal calf serum and 0.05% sodium azide (HFN), reacted in 100 μ L of HFN with or without 4 μ L MRK16 antibody (Kamiya, Seattle, WA) for 20 min on ice, rewashed, resuspended in 100 μ L HPN

with 2 μ L biotinylated goat anti-mouse Ig (Southern Biotech, Birmingham, AL), and incubated for 20 min on ice. The cells were washed again, resuspended in 1 mL of goat serum for 10 min on ice, rewashed in HFN, resuspended in 100 μ L of HFN plus 1.5 μ L of Texas Red-streptavidin (Molecular Probes, Eugene, OR), and incubated 20 min on ice. Finally, the cells were washed and suspended in 1 mL of PBS plus 0.2% bovine serum albumin and 0.01% sodium azide and run on an EPICS XL flow cytometer (Coulter, Hialeah, FL) with gating for live cells and detection of the FL2 fluorescence. Background fluorescence with isotype matched control antibody was subtracted.

RESULTS

To evaluate the ability of targeted toxins to modulate doxorubicin resistance in AML cell lines, we first had to define the variables that affect sensitivity of AML blasts to toxins and drug. In this study, parameters that we evaluated included the cell surface GMCSF receptor density and the amounts and function of P-glycoprotein on the three cell lines.

GMCSF Receptor Density and Ligand Affinity. Scatchard plots of [¹²⁵I]GMCSF binding to AML cell lines revealed high-affinity receptor densities of 320/cell, 123/cell, and 172/cell for HL60, HL60Vinc, and HL60VCR based on linear regression (Figure 1). The K_d s were calculated as 2×10^{-11} , 1.4×10^{-11} , and 8×10^{-12} M for HL60, HL60Vinc, and HL60VCR, respectively.

P-Glycoprotein Concentrations. Immunoblots with C219 included KB-V1 control and showed relative densities for HL60, HL60Vinc, and HL60VCR of 0.07, 0.28, and 2.75 based on equal cell loading on SDS-PAGE (Figure 2 and Table 5). Immunoblots of control protein, GLUT1, revealed bands at 45 and 55 kDa for HL60, HL60Vinc, and HL60VCR and bands at 43 and 53 kDa for KB-V1. Flow cytometry of HL60 and HL60VCR cells for surface P-gp showed minimal P-gp on HL60 cells (20% above background) and marked P-gp on HL60VCR cells (360% above background).

Doxorubicin Uptake by AML Cell Lines. The mean channel rhodamine fluorescence of AML cell lines increased from 3 without doxorubicin incubation (auto-fluorescence) to 15 for HL60 cells, 10 for HL60Vinc, and 7 for HL60VCR cells with 1 h incubation with 5×10^{-6} M doxorubicin.

Cell Cytotoxicity Measured by Thymidine Incorporation of DT388-GMCSF, DT390mGM-CSF, and Doxorubicin Alone. Table 1 shows the IC₅₀, IC₉₉, and IC_{99.9} for inhibition of thymidine incorporation after one or two day incubations with DT388-GMCSF and doxorubicin alone for each of the three AML cell lines. Doxorubicin sensitivity is most for HL60 cells, intermediate for HL60Vinc cells, and least for HL60VCR cells. Forty-eight hour incubation increased toxicity of DT388-GMCSF but not doxorubicin. DT390mGM-CSF was nontoxic to HL60 and HL60VCR cells (IC₅₀ > 4×10^{-8} M), but showed potent cytotoxicity to WT19 mouse leukemia cells (IC₅₀ = 5×10^{-12} M).

Cell Cytotoxicity Measured by Clonogenic Assay with DT388-GMCSF and Doxorubicin Alone. Table 2 displays the IC₅₀, IC₉₉, and IC_{99.9} for inhibition of colony formation in semisolid medium for DT388-GMCSF and doxorubicin on each of the AML cell lines. There was an excellent correlation with the 48 h thymidine incorporation inhibition assay results.

Modulation of Doxorubicin Resistance with DT388-GMCSF. The effects of preincubation with

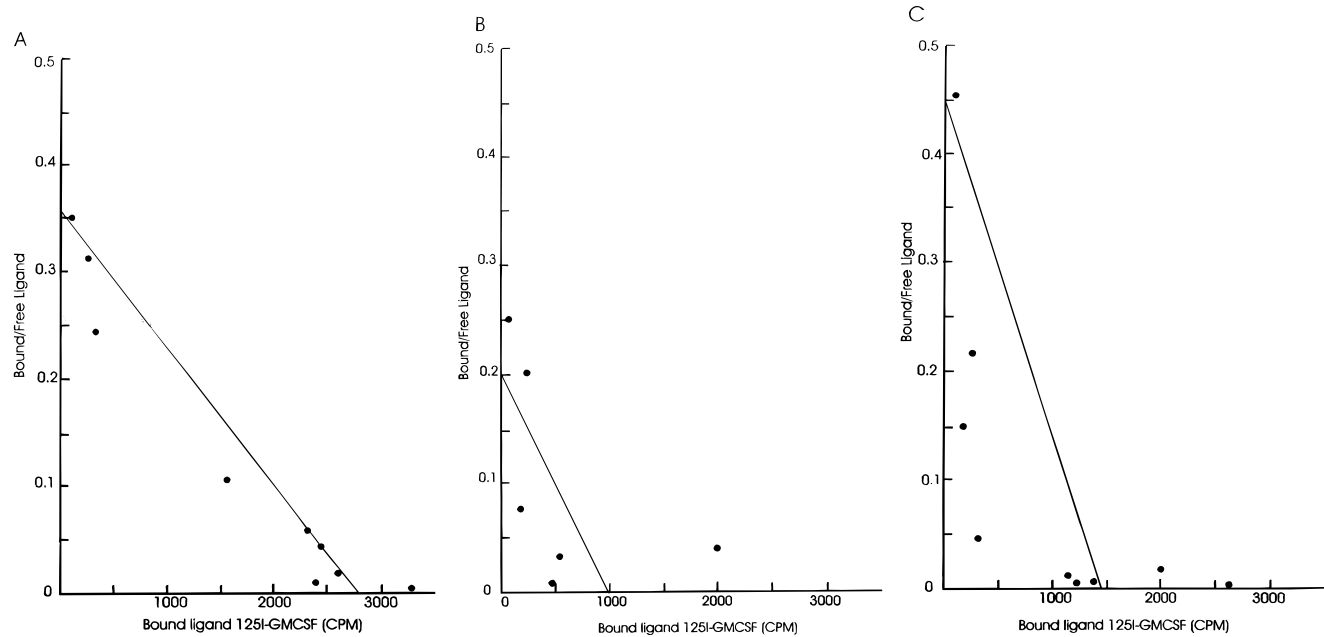


Figure 1. Scatchard plots of [¹²⁵I]GMCSF binding to AML cells. The experiment was performed as described in the text. The specific activity of the [¹²⁵I]GMCSF used was 97 μ Ci/ μ g at the test date. A, HL60; B, HL60Vinc; C, HL60VCR. K_d s were 2×10^{-11} M, 1.4×10^{-11} M, and 8×10^{-12} M for HL60, HL60Vinc, and HL60VCR, respectively.

Table 1. Inhibition of Thymidine Incorporation of AML Cell Lines by DT388-GMCSF and Doxorubicin^a

toxin/drug	24 h incubation (M)			48 h incubation (M)		
	HL60	HL60Vinc	HL60VCR	HL60	HL60Vinc	HL60VCR
DT388-GMCSF						
IC90	4×10^{-11}	1×10^{-8}	4×10^{-7}	6×10^{-11}	2×10^{-9}	1×10^{-10}
IC99	6×10^{-8}	8×10^{-8}	1×10^{-6}	6×10^{-9}	4×10^{-8}	5×10^{-9}
IC99.9	4×10^{-7}	4×10^{-7}	3×10^{-6}	4×10^{-8}	4×10^{-7}	4×10^{-8}
doxorubicin						
IC90	7×10^{-8}	1×10^{-6}	5×10^{-6}	2×10^{-8}	7×10^{-7}	5×10^{-6}
IC99	2×10^{-7}	1×10^{-5}	2×10^{-4}	2×10^{-7}	3×10^{-6}	9×10^{-5}
IC99.9	5×10^{-7}	5×10^{-5}	3×10^{-4}	7×10^{-7}	5×10^{-5}	3×10^{-4}

^a Each assay performed at least twice. IC values above 4×10^{-8} M for DT388-GMCSF and 2×10^{-4} M for doxorubicin projected from curves. Methods as in text.

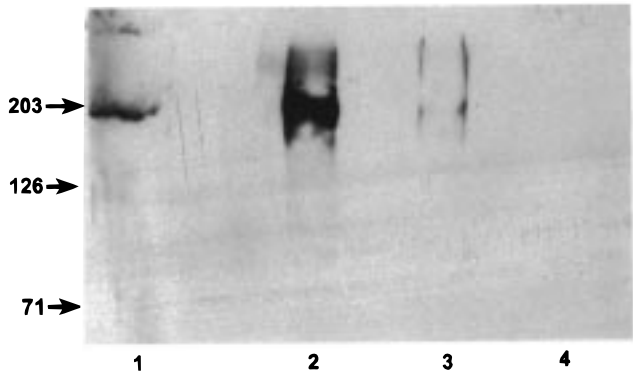


Figure 2. Immunoblot of AML cell membrane extracts reacted with C219 monoclonal anti-P-glycoprotein antibody. The experimental method is described in the text. Kladeiscope high molecular mass marker migration is depicted by arrows (203, 126, and 71 kDa). Lane 1, KB-V1 cells; lane 2, HL60VCR cells; lane 3, HL60Vinc cells; lane 4, HL60 cells.

DT388-GMCSF on doxorubicin sensitivity of thymidine incorporation and colony formation for the AML cell lines was analyzed by isobolograms (Figure 3) and calculation of the combination index (CI). CIs for colony inhibition and thymidine incorporation inhibition were similar. Modulation was greater for three log cell kill than one or two log cell kill. Concave isoboles were observed for three log cell kill of HL60Vinc and HL60VCR. CIs for

Table 2. Inhibition of Colony Formation by AML Cell Lines by DT388-GMCSF and Doxorubicin^a

toxin/drug	(M)		
	HL60	HL60Vinc	HL60VCR
DT388-GMCSF			
IC90	8×10^{-12}	2×10^{-9}	5×10^{-10}
IC99	4×10^{-10}	4×10^{-9}	6×10^{-9}
IC99.9	4×10^{-8}	2×10^{-8}	6×10^{-8}
doxorubicin			
IC90	5×10^{-8}	5×10^{-7}	9×10^{-6}
IC99	9×10^{-8}	1×10^{-6}	2×10^{-5}
IC99.9	1×10^{-7}	5×10^{-6}	4×10^{-5}

^a Assays performed as described in text. IC values above 4×10^{-8} M for DT388-GMCSF, and 2×10^{-4} M for doxorubicin extrapolated from curves.

the different cell lines are shown in Table 3. In each case, the CI was less for three log cell kill than one log cell kill.

The scheduling of toxin and drug were critical. Higher CIs at three log cell kill were observed when drug treatment preceded toxin exposure except in the case of HL60 cells (Table 4).

Effects of Modulating Concentrations of DT388-GMCSF on P-gp Concentrations. At modulating concentrations of toxins (4×10^{-9} M DT388-GMCSF), the concentrations of P-gp in HL60Vinc and HL60VCR were reduced 6–27-fold relative to untreated cells based on

Table 3. Combination Indices for AML Cell Cytotoxicity of DT388-GMCSF and Doxorubicin^a

cell line	thymidine incorporation	colony formation
HL60		
IC90	0.7	1
IC99	0.8	0.7
IC99.9	1	0.5
HL60Vinc		
IC90	0.4	1
IC99	0.2	0.2
IC99.9	0.2	0.2
HL60VCR		
IC90	0.6	0.5
IC99	0.2	0.4
IC99.9	0.2	0.3

^a Assays and calculations of CIs as described in text.**Table 4. Schedule Dependency of Modulation^a**

cell line	ratio CI drug first/ CI toxin first
HL60	
IC90	1.4
IC99	0.5
IC99.9	0.4
HL60Vinc	
IC90	1.0
IC99	0.7
IC99.9	5.0
HL60VCR	
IC90	1.3
IC99	5.0
IC99.9	5.0

^a Values for CI with toxin first or drug first obtained as described in text. IC99.9 ratios of CI for HL60Vinc and HL60VCR were reproducibly lower than IC99.9 ratios of CI for HL60.**Table 5. P-Glycoprotein Concentrations at Modulating Doses of DT388-GMCSF^a**

cell line	ratio density with and without toxin
HL60Vinc	0.17
HL60VCR	0.06

^a Immunoblots developed with C219 anti-P-gp monoclonal antibody and calibrated with 1.3×10^6 cell membrane extract of untreated cells. Modulating concentrations were 4×10^{-9} M for DT388-GMCSF for 48 h incubation. HL60 cells had no detectable P-gp under all incubation conditions.

densitometry of immunoblots (Figure 4 and Table 5). Control protein GLUT1 concentrations were altered by toxins under the conditions producing maximal modulation of drug resistance but to a much lesser degree (Figure 5).

Flow cytometry showed a 2-fold reduction in P-gp surface membrane concentration of HL60VCR cells after fusion toxin exposure.

Examination of toxin- and doxorubicin-treated apoptotic cell populations revealed that toxin pretreatment was associated with increased intracellular concentrations of doxorubicin (Table 6).

DISCUSSION

Novel treatment strategies for patients with relapsed or refractory AML have focused on circumventing multidrug resistance. Allogeneic bone marrow transplant has produced a significant fraction of durable remission in patients less than 55 years old, in part due to a graft-versus-leukemia effect (28). However, most AML patients are older than 55 years old. Chemotherapeutic agents which are not substrates for the common resis-

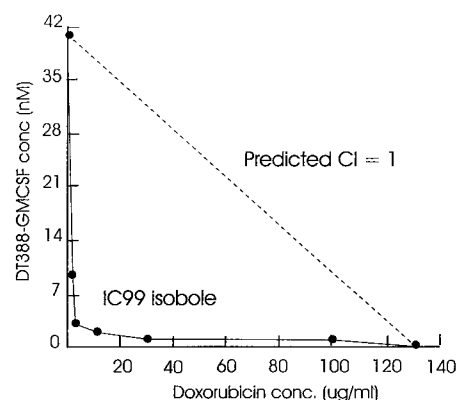


Figure 3. Isobologram for concentrations of DT388-GMCSF and doxorubicin yielding a 99% reduction in thymidine incorporation of HL60VCR cells. Cells were incubated for 24 h with DT388-GMCSF and then doxorubicin added for an additional 24 h, followed by an 18 h labeling with [³H]thymidine, harvesting and counting. Thymidine incorporation was compared to untreated cells. The concentration of DT388-GMCSF alone producing 99% reduction in thymidine incorporation, A_e , was 4×10^{-8} M. The concentration of doxorubicin yielding a 99% reduction in thymidine incorporation, B_e , was projected at 130 μ g/mL. The dotted line shows the additivity isobole. Concave lines beneath the additivity isobole show synergy, and convex lines above the additivity isobole show antagonism. The CI was 0.2.

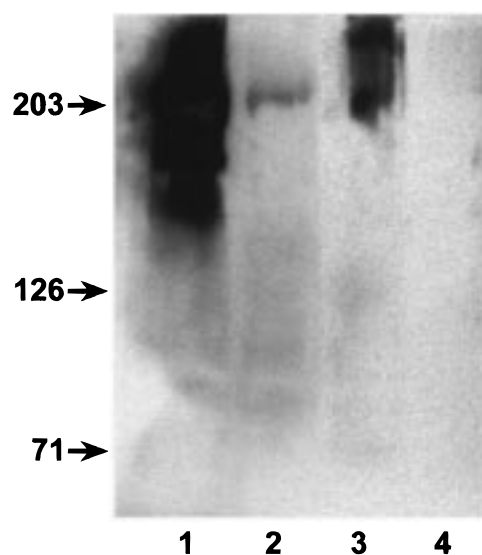


Figure 4. Immunoblot of AML cell membrane extracts reacted with C219 antibody. Standard protein markers were the same as Figure 4. Lane 1, HL60VCR control; lane 2, HL60VCR treated with 4×10^{-9} M DT388-GMCSF for 48 h; lane 3, HL60Vinc control; lane 4, same as lane 2 with HL60Vinc.

tance phenotypes have been tested. The nucleoside analogue fludarabine produced responses in relapsed patients in combination with cytosine arabinoside (29). The topoisomerase I inhibitor topotecan showed activity as a salvage agent alone and with cytarabine, but at the cost of moderate to severe oropharyngeal mucositis (30). The hypomethylating agent decitabine yielded remissions in drug-resistant AML patients, but the patients had prolonged myelosuppression (31). Finally, nonspecific modulators of P-gp such as cyclosporine have been combined with anthracyclines as salvage therapy (32). Responses were seen, but the modulation of P-gp in liver and kidney led to altered drug pharmacokinetics and enhanced toxicities to marrow, kidney, and central nervous system. The novel antibody-drug conjugate, anti-CD33-calicheamicin, showed minimal toxicities with

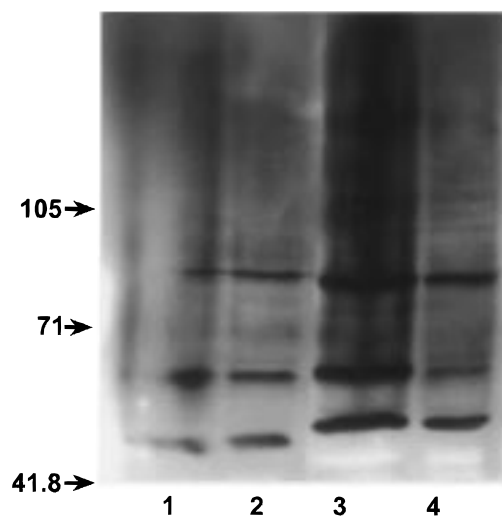


Figure 5. Immunoblot of AML membrane extracts detected with anti-GLUT antibody as described in the text. Low molecular mass prestained protein standards (105, 71, and 41.8 kDa) are shown by arrows. Membrane extracts in lanes 1–4 are the same as in Figure 4. Relative densities of the 45 kDa GLUT1 bands were 1, 1.5, 1, and 0.8, respectively, for lanes 1–4.

Table 6. Doxorubicin Intracellular Concentrations in AML Cell Lines

cell line	condition	mean rhodamine fluorescence channel ^a		
		media alone	doxorubicin	doxorubicin + verapamil
HL60	control	3	15	15
	DT388-GMCSF	4	34	32
HL60Vinc	control	4	10	13
	DT388-GMCSF	3	17	25
HL60VCR	control	3	7	19
	DT388-GMCSF	4	61	68

^a Cells were incubated for 60 min with 5 μ M doxorubicin with or without 50 μ M verapamil, washed, and run on an EPICS-XL gated for apoptotic cells by forward and 90° light scatter. Modulating concentrations of DT388-GMCSF as described in Table 5.

complete remissions in 4/35 patients, but most patients failed to respond due to active drug efflux (35). Thus, new reagents with unique mechanisms of action which can directly modify the apoptotic threshold of resistant malignant blasts are needed.

Targeted toxins are a novel class of therapeutics which may be useful for relapsed/refractory AML. Previous clinical trials of targeted toxins in T-cell and B-cell lymphomas have shown a number of partial and complete responses; however, clinical benefit has been limited by toxicity to vascular endothelium (33).

We had previously engineered a targeted toxin for AML, DT388-GMCSF (19, 20), but we anticipated vascular leak syndrome as the dose-limiting toxicity in man (34). We sought to overcome both the problem of drug-resistant leukemic blasts and endothelial injury of targeted toxins by administering combinations of targeted toxins and cytotoxic drugs. We hypothesized that targeted toxins would be synergistic with cytotoxic drugs based on previous studies in vitro with lymphoma and ovarian carcinoma cells (11–14). Thus, with combination therapy, lower doses of both targeted toxin and cytotoxic drug would be needed. Both vascular leak and some of the toxicities of cytotoxic drugs may be ameliorated. To test our hypothesis, we performed a systematic study of the optimal delivery of DT388-GMCSF with doxorubicin on three AML cell lines.

The first interesting finding was the variations in sensitivity to fusion toxin independent of receptor status. The resistance of the HL60Vinc cells to DT388-GMCSF may be due to antiapoptotic protein expression as we have previously reported (19). The results of the clonogenic assays in our study paralleled the results of the thymidine assays. Specificity was confirmed by the lack of toxicity of the diphtheria fusion toxin, DT390mGM-CSF, for HL60 and HL60VCR cells.

Synergy was observed primarily at higher log cell kill ($IC_{99.9}$) reflecting a mechanism requiring significant inhibition of protein synthesis. This observation is consistent with the requirement to reduce the intracellular concentrations of some short half-life proteins to modulate resistance.

The less pronounced synergy on HL60 cells versus HL60Vinc and HL60VCR suggests a significant impact of *mdr-1* on this process. This finding is also compatible with our model of modulation by reducing short half-life resistance proteins. Further support for the model was observed with the immunoblots, flow cytometry, and doxorubicin uptake studies showing reduced antigen and functional activity for *mdr-1* in the toxin modulated cells. The differences in the concentrations of P-gp after fusion toxin exposure assayed by immunoblots or flow cytometry may reflect different rates of metabolism of intracellular and cell surface protein.

The dependence of synergy on toxin followed by cytotoxic drug, rather than the reverse, is also evidence for the proposed molecular mechanism. Our findings confirm the schedule dependency in vivo reported by Ghetie et al. (14).

Our experiments suggest a potential for targeted toxins such as DT388-GMCSF as cell-specific modulators of drug resistance in relapsed/refractory AML patients. However, further studies will be needed to confirm these observations on fresh resistant AML blasts.

Since drug resistance contributes to treatment failure for a number of human malignancies (36) and targeted toxins have been designed for many different cell types (37), this approach, both as detailed in this study and in previous reports (11–20), may yield additional cancer therapeutic strategies.

ACKNOWLEDGMENT

We wish to thank James Nicholson for imaging analysis and Dr. Ian Simpson, NIDDK, for antibody to GLUT1.

LITERATURE CITED

- (1) Parker, S., Tong, T., Bolden, S., and Wingo, P. (1997) Cancer statistics, 1997. *Ca—Cancer J. Clin.* 47, 5–12.
- (2) Schiffer, C. (1997) Acute myeloid leukemia in adults. *Cancer Medicine* (J. Holland, E. Frei, R. Bast, D. Kufe, D. Marton, and R. Weichselbaum, Eds.) pp 2617–2633, Lea & Febiger, Philadelphia.
- (3) List, A. (1996) Role of multidrug resistance and its pharmacological modulation in acute myeloid leukemia. *Leukemia* 10, 937–944.
- (4) Solary, E., Witz, B., Caillot, D., Moreau, P., Desablens, B., Cahn, J. Y., Sadoun, A., Pignon, B., Berthou, C., Maloisel, F., Guyotat, D., Casassus, P., Ifrah, N., Lamy, Y., Audhuy, B., Colombat, P., and Harousseau, J. L. (1996) Combination of quinine as a potential reversing agent with mitoxantrone and cytarabine for the treatment of acute leukemias: a randomized multicenter study. *Blood* 88, 1198–1204.
- (5) Muller, C., Laurent, G., and Ling, V. (1995) P-glycoprotein stability is affected by serum deprivation and high cell density in multidrug-resistant cells. *J. Cell Physiol.* 163, 538–544.
- (6) Shen, H., Ranganathan, S., Kuzmich, S., and Tew, K. D. (1995) Influence of ethacrynic acid on glutathione S-transferase pi transcript and protein half-lives in human colon cancer cells. *Biochem. Pharm.* 50, 1233–1245.

- (7) Blagosklonny, M., Alvarez, M., Fojo, A., and Neckers, L. (1996) Bcl-2 protein downregulation is not required for differentiation of multidrug resistant HL60 leukemia cells. *Leuk. Res.* 20, 101–112.
- (8) Pirker, R., Wallner, J., Geissler, K., Kinkesch, W., Haas, O., Bettelheim, P., Hopfner, M., Scherrer, R., Valent, P., Havelec, L., Ludwig, H., and Lechner, K. (1991) MDR1 gene expression and treatment outcome in acute myeloid leukemia. *J. Natl. Cancer Inst.* 83, 708–712.
- (9) Russo, D., Marie, J. P., Zhou, D. C., Faussat, A. M., Melli, C., Damiani, D., Michelutti, A., Michieli, M., Fanin, R., and Baccarani, M. (1994) Evaluation of the clinical relevance of the anionic glutathione S-transferase (GST pi) and multidrug resistance (mdr-1) gene coexpression in leukemias and lymphomas. *Leuk. Lymph.* 15, 453–461.
- (10) Maung, Z., MacLean, F., Reid, M., Pearson, A., Proctor, S., Hamilton, P., and Hall, A. (1994) The relationship between bcl-2 expression and response to chemotherapy in acute leukaemia. *Br. J. Haematol.* 88, 105–115.
- (11) Ghetie, M. A., Tucker, K., Richardson, J., Uhr, J. W., and Vitetta, E. S. (1994) Eradication of minimal disease in severe combined immunodeficient mice with disseminated Daudi lymphoma using chemotherapy and an immunotoxin cocktail. *Blood* 84, 702–718.
- (12) O'Connor, R., Liu, C., Ferris, C. A., Guild, B. C., Teicher, B. A., Corvi, C., Liu, Y., Arceci, R. J., Goldmacher, V. S., Lambert, J. M., and Blattler, W. A. (1995) Anti-B4-blocked ricin synergizes with doxorubicin and etoposide on multidrug-resistant and drug-sensitive tumors. *Blood* 86, 4286–4294.
- (13) Lidor, Y. J., O'Brian, K. C., Xu, F. J., Hamilton, T. C., Ozols, R. F., and Bast, R. C. Alkylating agents and immunotoxins exert synergistic cytotoxic activity against ovarian cancer cells. (1993). *J. Clin. Invest.* 92, 2440–2453.
- (14) Ghetie, M. A., Podar, E. M., Gordon, B. E., Pantazis, P., Uhr, J. W., and Vitetta, E. (1996) Combination immunotoxin treatment and chemotherapy in scid mice with advanced, disseminated Daudi lymphoma. *Int. J. Cancer* 68, 93–105.
- (15) Uckun, F., Gajl-Peczalska, K., Myers, D., Ramsay, N., Kersey, J., Colvin, M., and Valleria, D. (1987) Marrow purging in autologous bone marrow transplantation for T-lineage acute lymphoblastic leukemia: efficacy of ex vivo treatment with immunotoxins and 4-hydroperoxy-cyclophosphamide against fresh leukemic marrow progenitor cells. *Blood* 69, 361–377.
- (16) Weil-Hillman, G., Uckun, F., Manske, J., and Valleria, D. (1987) Combined immunochemotherapy of human solid tumors in nude mice. *Cancer Res.* 47, 579–588.
- (17) Pearson, J., FitzGerald, D., Willingham, M., Wiltrout, R., Pastan, I., and Longo, D. (1989) Chemoimmunotoxin therapy against a human colon tumor (HT-29) xenograft in nude mice. *Cancer Res.* 49, 3562–3571.
- (18) Jansen, B., Kersey, J., Jaszczyk, W., Gunther, R., Nguyen, D., Chelstrom, L., Tuelahlgren, L., and Uckun, F. (1993) Effective immunochemotherapy of human t(4;11) leukemia in mice with severe combined immunodeficiency (SCID) using B43 (anti-CD19)-pokeweed antiviral protein immunotoxin plus cyclophosphamide. *Leukemia* 7, 290–301.
- (19) Frankel, A. E., Hall, P. D., Burbage, C., Vesely, J., Willingham, M., Bhalla, K., and Kreitman, R. J. (1997) Modulation of the apoptotic response of human myeloid leukemia cells to a diphtheria toxin-granulocyte-macrophage colony stimulating factor (GM-CSF) fusion protein. *Blood* 90, 3654–3661.
- (20) Kreitman, R. J., and Pastan, I. (1997) Recombinant toxins containing human GM-CSF and either *Pseudomonas* exotoxin or diphtheria toxin kill gastrointestinal cancer and leukemia cells. *Blood* 90, 242–250.
- (21) Perentesis, J., Waddick, K., Bendel, A., Shao, Y., Warman, B., Chandan-Langlie, M., and Uckun, F. M. (1997) Induction of apoptosis in multidrug-resistant and radiation-resistant acute myeloid leukemia cells by a recombinant fusion toxin directed against the human granulocyte-macrophage colony-stimulating factor receptor. *Clin. Cancer Res.* 3, 347–360.
- (22) Roy, D., Griffin, J., Belvin, M., Blattler, W., Lambert, J., and Ritz, J. Anti-MY9-blocked ricin: an immunotoxin for selective targeting of acute myeloid leukemia cells. (1991) *Blood* 77, 2404–2410.
- (23) Kabral, A., Bradstock, K. F., Grimsley, P., Favaloro, E. J. (1988) Immunophenotype of clonogenic cells in myeloid leukemia. *Leuk. Res.* 12, 51–58.
- (24) Kelleher, C., Wong, G., Clark, S., Schendel, P., Minden, M., and McCulloch, E. (1988) Binding of iodinated recombinant human GM-CSF to the blast cells of acute myeloblastic leukemia. *Leukemia* 2, 211–231.
- (25) Dalton, W. T., Ahearn, M., McCredie, K. B., Freireich, E. J., Stass, S. A., and Trujillo, J. M. (1988) HL-60 cell line was derived from a patient with FAB-M2 and not FAB-M3. *Blood* 71, 242–245.
- (26) McGrath, T., Latoud, C., Arnold, S. T., Safa, A. R., Felsted, R. L., and Center, M. S. (1989) Mechanism of multidrug resistance of HL60 cells. Analysis of resistance associated membrane proteins and levels of mdr gene expression. *Biochem. Pharm.* 38, 3611–3618.
- (27) Berenbaum, M. J. (1989) What is synergy? *Pharm. Rev.* 41, 93–123.
- (28) Forman, S. J., Schmidt, G. M., and Nademanee, A. P. (1991) Allogeneic marrow transplantation as therapy for primary induction failure of patients with acute leukemia. *J. Clin. Oncol.* 9, 1570–1585.
- (29) Gandhi, V., Estey, E., Keating, M. J., and Plunkett, W. (1993) Fludarabine potentiates metabolism of cytarabine in patients with acute myelogenous leukemia during chemotherapy. *J. Clin. Oncol.* 11, 116–129.
- (30) Seiter, K., Feldman, E. J., Halicka, H. D., Traganos, F., Darzynkiewicz, Z., Lake, D., and Ahmed, T. (1997) Phase I clinical and laboratory evaluation of topotecan and cytarabine in patients with acute leukemia. *J. Clin. Oncol.* 15, 44–59.
- (31) Kantarjian, H. M., Estey, E. H., and Keating, M. A. (1996) New chemotherapeutic agents in acute myeloid leukemia. *Leukemia* 10 (Suppl. 1), S4–S8.
- (32) List, A. F., Spier, C., Greer, J., Wolff, S., Hutter, J., Dorr, R., Salmon, S., Futscher, B., Baier, M., and Dalton, W. (1993) Phase I/II trial of cyclosporine as a chemotherapy-resistance modifier in acute leukemia. *J. Clin. Oncol.* 11, 1652–1667.
- (33) Frankel, A. E., FitzGerald, D., Siegall, C., and Press, O. W. (1996) Advances in immunotoxin biology and therapy: a summary of the fourth international symposium on immunotoxins. *Cancer Res.* 56, 926–936.
- (34) Hall, P. D., Kreitman, R. J., Willingham, M. C., and Frankel, A. E. (1998) Pharmacology and pharmacokinetics of DT388-GM-CSF, a fusion toxin consisting of a truncated diphtheria toxin (DT388) linked to human granulocyte-macrophage colony stimulating factor (GM-CSF), in C57BL/6 mice. *Toxicol. Appl. Pharm.* (In press).
- (35) Sievers, E., Appelbaum, F., Spielberger, R., Forman, S., Flowers, D., Berger, M., Shannon-Doras, K., and Bernstein, I. (1997) Dose escalation phase I study of recombinant engineered human anti-CD33 antibody-calicheamicin drug conjugate (CMA-676) in patients with relapsed or refractory acute myeloid leukemia (AML). *Blood* 90, 504a.
- (36) O'Brien, M. L., and Tew, K. D. (1996) Glutathione and related enzymes in multidrug resistance. *Eur. J. Cancer* 32A, 967–980.
- (37) Pastan, I. H. (1997) Tumor immunotoxins: technology closes in on potential. *Adv. Oncol.* 13, 3–12.
- (38) Chan, C. H., Blazar, B. R., Eider, C. R., Kreitman, R. J., Valleria, D. A. (1995) A murine cytokine fusion toxin specifically targeting the murine granulocyte-macrophage colony-stimulating factor (GM-CSF) receptor on normal committed bone marrow progenitor cells and GM-CSF-dependent tumor cells. *Blood* 86, 2732–2740.
- (39) Leith, C. P., Chen, I. M., Kopecky, K. J., Appelbaum, F. R., Head, D. R., Godwin, J. E., Weick, J. K., and Willman, C. L. (1995) Correlation of multidrug resistance (MDR1) protein expression with functional dye/drug efflux in acute myeloid leukemia by multiparameter flow cytometry: identification of discordant MDR-/Efflux+ and MDR+/Efflux- cases. *Blood* 86, 2329–2342.

BC980015A

Assessment of the Radiochemical Design of Antibodies with a Metabolizable Linkage for Target-Selective Radioactivity Delivery

Yasushi Arano,^{*,†} Kouji Wakisaka,[†] Hiromichi Akizawa,[†] Masahiro Ono,[†] Keiichi Kawai,[‡] Morio Nakayama,[§] Harumi Sakahara,^{||} Junji Konishi,^{||} and Hideo Saji[†]

Department of Patho-Functional Bioanalysis, Graduate School of Pharmaceutical Sciences, and Department of Nuclear Medicine and Diagnostic Imaging, Graduate School of Medicine, Kyoto University, Yoshida-Shimoadachi-cho, Sakyo-ku, Kyoto 606-8501, Central Research Laboratories, Miyazaki Medical College, 5200 Kihara, Kiyotake-cho, Miyazaki-gun 889-1601, and Faculty of Pharmaceutical Sciences, Kumamoto University, 5-1 Oe-Honmachi, Kumamoto 862-0973, Japan. Received December 26, 1997; Revised Manuscript Received April 15, 1998

Interposition of a metabolizable linkage has been performed to reduce the hepatic radioactivity levels of radiolabeled antibodies. To estimate the validity of this strategy, a radioiodination reagent (HML) that provides a stable attachment for *m*-iodohippuric acid with proteins in plasma while facilitating rapid and selective release of the compound after lysosomal proteolysis in the liver was conjugated with a monoclonal antibody (mAb) against osteogenic sarcoma (OST7, IgG₁). Radiolabeled OST7 conjugates with a plasma-labile ester bond for releasing *m*-iodohippuric acid (MIH), plasma-stable amide bonds for releasing radiometabolites of hepatobiliary excretion (MPH), or slow elimination rates from hepatocytes ([¹¹¹In]EMCS-Bz-EDTA) were prepared with similar conjugation chemistry. The four radiolabeled OST7 conjugates were characterized both in vitro and in vivo. All the radiolabeled OST7 conjugates had similar radiochromatograms on size-exclusion HPLC and similar antigen binding affinities. While MIH-OST7 indicated accelerated clearance of radioactivity from the blood due to the release of *m*-iodohippurate, the rest of the three radiolabeled OST7 conjugates remained stable in serum incubation studies and had similar radioactivity elimination from the blood in vivo. When injected into normal mice, HML-OST7 demonstrated tissue-to-blood ratios of radioactivity similar to those of MIH-OST7 and significantly lower than those of the other two radiolabeled OST7 conjugates. In biodistribution studies in nude mice, both HML-OST7 and MIH-OST7 exhibited tumor-to-liver or tumor-to-intestine ratios of radioactivity higher than those of [¹¹¹In]EMCS-Bz-EDTA-OST7 or MPH-OST7, respectively. HML-OST7, MPH-OST7, and [¹¹¹In]EMCS-Bz-EDTA-OST7 indicated there were no changes in the radioactivity levels in the tumor between 24 and 48 h postinjection, whereas MIH-OST7 significantly decreased the radioactivity levels in the tumor at these time points. HML reduced the radioactivity levels in nontarget tissues without impairing the tumor radioactivity levels delivered by OST7. These findings indicated that the design of a radiolabeled mAb that is stable in plasma and liberates the radiometabolite of rapid urinary excretion constitutes an effective strategy for achieving target-selective radioactivity delivery.

INTRODUCTION

The localization of radioactivity in nontarget tissues such as the liver after administration of radiolabeled monoclonal antibodies (mAbs)¹ constitutes a serious problem for clinical applications of radiopharmaceuticals. Although the precise mechanisms of mAb accumulation in the liver are still uncertain, target-selective radioactivity localization could be amplified if the radiolabeled mAbs bound to the target remain stable while the radioactivity in nontarget tissues is excreted in the urine.

This approach was first reported by Haseman et al., who introduced a diester bond between an antibody and an ¹¹¹In chelate of benzyl-EDTA as a "metabolizable linkage" to liberate the radiolabeled compound with a low molecular weight after lysosomal proteolysis in nontarget tissues (1). A variety of radiolabeled mAbs with metabolizable linkages have been developed (2–8), and some

* Address correspondence to Yasushi Arano, Department of Patho-Functional Bioanalysis, Graduate School of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto 606-8501, Japan. Telephone: 81-75-753-4608. Fax: 81-75-753-4568. E-mail: arano@pharm.kyoto-u.ac.jp.

[†] Graduate School of Pharmaceutical Sciences, Kyoto University.

[‡] Miyazaki Medical College.

[§] Kumamoto University.

^{||} Graduate School of Medicine, Kyoto University.

¹ Abbreviations: mAb, monoclonal antibody; OST7, mAb against osteogenic sarcoma; NGA, galactosylneoglycoalbumin; PB, phosphate buffer; PBS, phosphate-buffered saline; MES, 2-(*N*-morpholino)ethanesulfonic acid; 2-ME, mercaptoethanol; NCS, *N*-chlorosuccinimide; HML, 3'-iodohippuryl *N*^ε-maleoyl-L-lysine; MIH, maleimidoethyl 3'-iodohippurate; MPH, *N*-(5-maleimidopentyl) 3'-iodohippuric acid amide; SHML, 3'-(tri-*n*-butylstannyl)hippuryl-*N*^ε-maleoyl-L-lysine; SMIH, maleimidoethyl 3-(tri-*n*-butylstannyl)hippurate; SMPH, *N*-(5-maleimidopentyl) 3'-(tri-*n*-butylstannyl)hippuric acid amide; EMCS-Bz-EDTA, 1-[4-[(5-maleimidopentyl)amino]benzyl]ethylenediamine-tetraacetic acid; HPLC, high-performance liquid chromatography; RP-HPLC, reversed-phase high-performance liquid chromatography; TLC, thin-layer chromatography; CAE, cellulose acetate electrophoresis; PC, paper chromatography.

studies demonstrated enhanced target-to-nontarget ratios of the radioactivity in animal models.

In most studies, ester bonds have been used as the metabolizable linkage of choice due to rapid cleavage of the linkage in nontarget tissues such as the liver and kidney where high esterase activity levels have been reported (9). However, since ester bonds are cleaved by both chemical and enzymatic hydrolyses not only in nontarget tissues but also in plasma, use of ester bonds as the metabolizable linkage impaired the radioactivity levels in the target (1, 5, 10, 11). In addition, while the cleavage of the ester bond in plasma may cause only a slight decrease in the radioactivity levels in the target tissues in animal models, due to the presence of large tumor masses in a small body, the plasma-labile characteristics of ester bonds may significantly decrease the target radioactivity levels in humans where much smaller tumor masses are located in much higher distribution volumes.

On the other hand, recent metabolic studies of radiolabeled polypeptides demonstrated that the radiometabolites generated after lysosomal proteolysis of polypeptides play a critical role in the radioactivity levels in the liver (12–17). These findings support the rationale of the radiochemical design of antibodies using a metabolizable linkage to reduce the nontarget radioactivity levels. Furthermore, recent advances in genetic engineering have provided mAbs with less immunogenicity (e.g., humanized mAbs) and more favorable pharmacokinetic characteristics (e.g., single-chain Fv fragment) (18–21). Monoclonal antibodies directed against endothelial cells in solid tumors may also facilitate higher accumulation in target tissues within shorter postinjection intervals (22–24). These classes of mAbs would potentially solve various problems associated with the clinical applications of radiolabeled murine mAbs. These findings and developments prompted us to further assess the validity of the radiochemical design of mAbs with metabolizable linkages for reducing the nontarget radioactivity levels of radiolabeled mAbs.

To estimate the validity of the design, the metabolizable linkages should be stable enough in plasma to deliver radioactivity to both target and nontarget tissues equivalent to nonmetabolizable linkages. Furthermore, the radiometabolite generated after cleavage of the metabolizable linkage should be excreted rapidly from the nontarget tissues into urine without redistribution into other tissues. We have recently developed a radioiodination reagent for proteins with L-lysine as the metabolizable linkage, 3'-iodohippuryl *N*^ε-maleoyl-L-lysine (HML). In our previous studies using galactosyl-neoglycoalbumin (NGA) as a model polypeptide, [¹³¹I]-HML-NGA selectively liberated *m*-iodohippuric acid after lysosomal proteolysis in hepatocytes. In addition, the peptide bond of [¹³¹I]HML-NGA remained stable in human serum and murine plasma (25). Such chemical and biological characteristics would render HML useful for estimating the validity of the chemical design of mAbs for reducing the nontarget radioactivity localization.

In this study, HML was radioiodinated with Na¹²⁵I or Na¹³¹I and conjugated with a mAb against osteogenic sarcoma (OST7, IgG₁). For comparison, radioiodinated OST7 conjugates with an ester bond or an amide bond were prepared using maleimidoethyl 3'-iodohippurate (MIH) or *N*-(5-maleimidopentyl) 3'-iodohippuric acid amide (MPH), respectively. ¹¹¹In-labeled OST7 was also prepared using 1-[4-[(5-maleimidopentyl)amino]benzyl]-ethylenediaminetetraacetic acid (EMCS-Bz-EDTA) as a bifunctional chelating agent. The chemical structures of

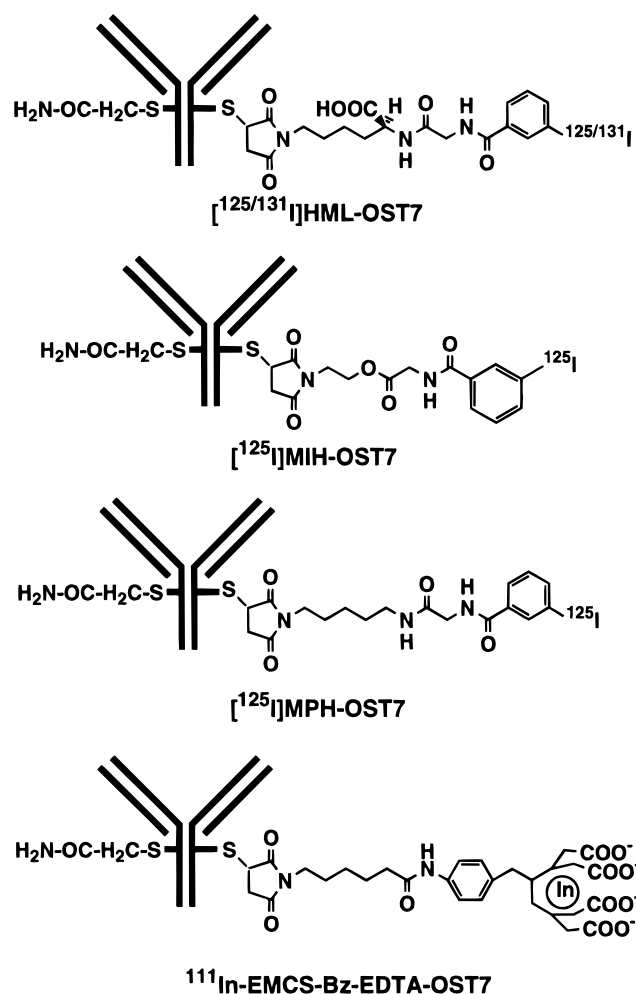


Figure 1. Chemical structures of radiolabeled OST7 conjugates. All the reagents were conjugated with OST7 molecules with maleimide–thiol chemistry after reducing the disulfide bonds in OST7.

radiolabeled OST7 conjugates used in this study are illustrated in Figure 1. All the reagents were attached to OST7 with similar conjugation chemistry using maleimide–thiol chemistry. Prior studies showed that all the reagents provided radiolabeled polypeptides stable against *in vivo* deiodination or transchelation (14, 25–27). Radiometabolites generated after lysosomal proteolysis of radiolabeled NGAs using the respective reagent were well characterized in previous studies (11, 14, 25, 26). While MIH liberates *m*-iodohippuric acid as the sole radiometabolite in hepatic parenchymal cells at a rate similar to that of HML, the ester bond in MIH is hydrolyzed with time in buffered solutions at neutral pH and in plasma. MPH generates radiometabolites with hepatobiliary excretion after lysosomal proteolysis in the murine liver. NGA-[¹¹¹In]EMCS-Bz-EDTA generates a cysteine adduct as the predominant radiometabolite in hepatocytes, and the metabolite was eliminated from the liver at a rate that was the slowest of the four reagents. All the radiolabeled OST7 conjugates were characterized *in vitro*, and the radioactivity distribution was compared in normal mice and nude mice bearing osteogenic sarcoma. The validity of the radiochemical design of mAbs using the metabolizable linkage to achieve target-selective radioactivity localization will be discussed.

MATERIALS AND METHODS

Reagents and Chemicals. Na¹³¹I and Na¹²⁵I were obtained from Daiichi Radioisotopes Laboratories (Tokyo)

and Daiichi Kagaku (Tokyo), respectively, and were diluted with 0.1 M PB (pH 7.4) to 7.4 MBq (0.2 mCi)/ μ L and 3.7 MBq (0.1 mCi)/ μ L, respectively. $^{111}\text{InCl}_3$ [74 MBq (2 mCi)/mL in 0.02 N HCl] was kindly supplied by Nihon Medi-Physics (Nishinomiya, Japan). The stannyl precursors of HML, 3'-(tri-*n*-butylstannyl)hippuryl-*N* ϵ -maleoyl-L-lysine (SHML), MPH, *N*-(5-maleimidopentyl) 3'-(tri-*n*-butylstannyl)hippuric acid amide (SMPH), and MIH, maleimidoethyl 3-(tri-*n*-butylstannyl)hippurate (SMIH), were synthesized as reported previously (25, 26). EMCS-Bz-EDTA was purchased from Dojindo Laboratories (Kumamoto, Japan). Size-exclusion HPLC and RP-HPLC were performed using Cosmosil Diol-300 (7.5 $\times$ 600 mm, Nacalai Tesque, Kyoto, Japan) and Cosmosil 5C₁₈-AR (4.6 $\times$ 250 mm, Nacalai Tesque) columns, respectively. Size-exclusion HPLC and RP-HPLC columns were equilibrated and eluted with 0.1 M PB (pH 6.8) and with a mixture of 0.1% aqueous phosphoric acid/acetonitrile (7:3), respectively, at a flow rate of 1 mL/min. Other reagents were of reagent grade and were used as received.

Tumor and mAbs. KT005-cloned human osteogenic sarcoma was maintained by serial subcutaneous transplantation in athymic mice. Pieces (1–2 mm³) of tumor tissue (ca. 0.3–0.9 g) at 2 weeks postplantation were used for the *in vivo* study. Single-cell suspensions from xenografted tumors were used for the *in vitro* study. The mAb against osteogenic sarcoma (OST7, IgG₁), generated by standard hybridoma techniques, was purified by ammonium sulfate precipitation with subsequent protein A affinity chromatography (Pharmacia Biotech Co., Ltd., Tokyo) as reported previously (2, 11, 28).

Preparation of Radioiodinated OST7 Conjugates. SHML, SMPH, and SMIH were radioiodinated in the presence of NCS as previously described (11, 25, 26). Briefly, SHML was dissolved in methanol containing 1% acetic acid (0.64 mg/mL), and 22 μ L (0.021 μ mol) of this solution was mixed with 6.04 μ L of NCS in methanol (0.5 mg/mL) in a sealed vial, followed by an addition of Na¹³¹I (3 μ L). After incubation at room temperature for 45 min, the reaction was quenched with aqueous sodium bisulfite (2.92 μ L, 0.72 mg/mL). The radiochemical yields of [¹³¹I]-HML were determined by TLC developed with a mixture of chloroform/methanol/acetic acid (40:5:2). The solvent was removed by a flow of N₂ prior to the subsequent conjugation reaction with OST7. Radioiodination of SHML with Na¹²⁵I was performed as described above except using Na¹²⁵I instead of Na¹³¹I.

Conjugation of [¹³¹I]HML with OST7 was performed by reducing the disulfide bonds of OST7, as described previously (2, 11). Briefly, OST7 (250 μ L, 5 mg/mL) in well-degassed 0.1 M PB (pH 7.0) containing 2 mM EDTA was allowed to react with a 1000-fold molar excess of 2-ME at room temperature for 30 min. Excess 2-ME was removed by a centrifuged column procedure (29) using Sephadex G-50 (Pharmacia Biotech Co., Ltd.) equilibrated and eluted with 0.1 M PB (pH 6.0) containing 2 mM EDTA. Aliquots of this mixture were sampled for estimation of the number of thiol groups with 2,2'-dithiopyridine (11, 30, 32). The filtrate (90 μ L) was then added to a reaction vial containing crude [¹³¹I]HML. After gentle agitation of the reaction mixture for 1.5 h at room temperature, 39 μ L of iodoacetamide (10 mg/mL) in 0.1 M PB (pH 6.0) was added. The reaction mixture was further incubated for 30 min to alkylate the unreacted thiol groups. [¹³¹I]HML-OST7 was subsequently purified by the centrifuged column procedure, equilibrated, and eluted with 0.1 M PBS (pH 6.0). [¹²⁵I]HML-OST7 was prepared with similar procedures.

[¹²⁵I]MIH-OST7 and [¹²⁵I]MPH-OST7 were also prepared by experimental procedures similar to those used for [^{125/131}I]HML-OST7 except using SMIH or SMPH in place of SHML. Direct radioiodination of OST7 was performed by the chloramine T method (11, 31). To 200 μ L aliquots of OST7 (0.25 mg/mL) in 0.3 M PB (pH 7.5) was added 5 μ L of Na¹²⁵I. Chloramine T (0.1 mg/mL, 25 μ L), freshly prepared in the same buffer, was then added. After incubation of the mixture for 10 min at room temperature, the reaction was terminated by addition of 6 μ L of aqueous sodium bisulfite (0.7 mg/mL). [¹²⁵I]OST7 was then purified by the centrifuged column procedure.

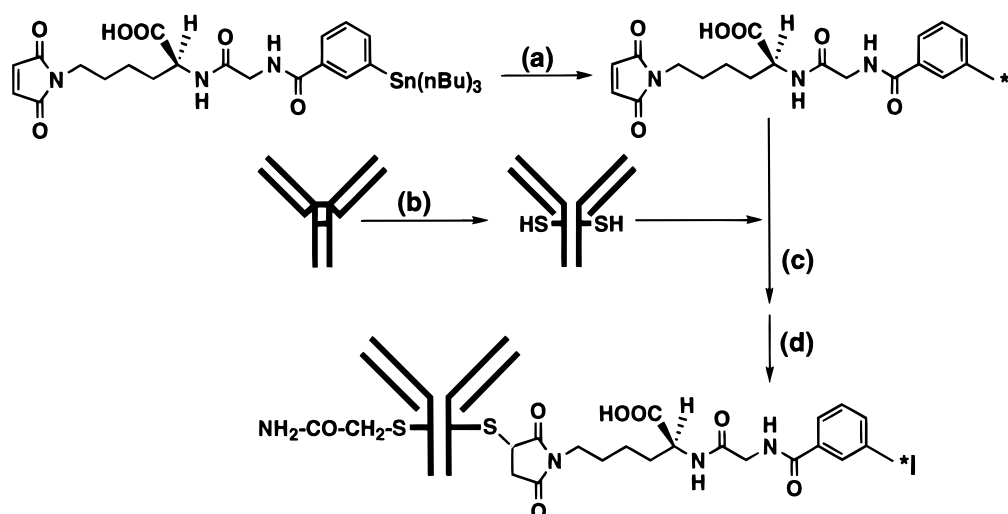
Radiochemical purities of the radioiodinated OST7 conjugates were determined by size-exclusion HPLC, TLC, and CAE. CAE was run at an electrostatic field of 0.8 mA/cm for 45 min in veronal buffer (*I* = 0.06, pH 8.6).

Preparation of [¹¹¹In]EMCS-Bz-EDTA-OST7. OST7 was also labeled with ¹¹¹In using EMCS-Bz-EDTA as the bifunctional chelating agent. EMCS-Bz-EDTA was conjugated to OST7 by reducing the disulfide bonds in OST7 with procedures similar to those used for [¹³¹I]HML conjugation, as previously reported (11). EMCS-Bz-EDTA-OST7 was purified from unreacted low-molecular weight compounds by the centrifuged column procedure using Sephadex G-50 equilibrated and eluted with 20 mM MES-buffered saline (pH 6.0). The number of EMCS-Bz-EDTA molecules introduced per molecule of OST7 was determined by measuring the thiol groups before and after conjugation reaction using 2,2'-dithiopyridine, as previously described (11, 32).

The purified EMCS-Bz-EDTA-OST7 was labeled with ¹¹¹In according to the procedure of Brechbiel et al. (33) with slight modifications as previously noted (12, 32). Briefly, 5 μ L of ¹¹¹InCl₃ in 0.02 N HCl was added to a mixed solution (5 μ L) of 1 M sodium acetate and 1.75 N HCl (4:1). After 3 min, 40 μ L of EMCS-Bz-EDTA-OST7 (1 mg/mL) was added, and the reaction solution was kept at room temperature (23 $^{\circ}$ C) for 1 h. A 13 μ L aliquot of 2 mM EDTA in 0.1 M acetate buffer (pH 5.0) was then added to the reaction solution. After incubation for an additional 30 min at room temperature, [¹¹¹In]EMCS-Bz-EDTA-OST7 was purified by the centrifuged column procedure using Sephadex G-50 equilibrated with 0.1 M PBS (pH 6.0). The radiochemical purity of [¹¹¹In]EMCS-Bz-EDTA-OST7 was determined by CAE and size-exclusion HPLC.

Immunoreactivity Measurement. The immunoreactivity of the four radiolabeled OST7 conjugates was determined as described previously (2, 11, 28). Tumor cells (2 $\times$ 10⁴ to 1 $\times$ 10⁷) suspended in 100 μ L of Dulbecco's PBS were incubated with 100 μ L of radiolabeled OST7 in microcentrifugation tubes (5.7 mm $\times$ 46 mm) for 2 h at 4 $^{\circ}$ C. After centrifugation at 10000*g* for 5 min, the supernatant was discarded, and the radioactivity was determined using a well counter (ARC 2000, Aloka, Tokyo).

Stability Estimation of Radiolabeled OST7 Conjugates in Human Serum. Each radiolabeled OST7 was diluted to 0.5 mg/mL with 0.1 M PBS (pH 6.0), and 20 μ L of each solution was added to 230 μ L of freshly prepared human serum (11). After incubation for 1, 3, 6, or 24 h at 37 $^{\circ}$ C, samples were taken from the solutions, and the percentages of radioactivity released from the three radioiodinated OST7 conjugates were determined by PC (no. 50, Advantec Toyo, Tokyo) and TLC. The percentages of the radioactivity released from [¹¹¹In]EMCS-Bz-EDTA-OST7 were determined by size-

Scheme 1^a

^a (a) *N*-Chlorosuccinimide, Na^{131/125}I, 1% AcOH/MeOH, room temperature, 45 min; (b) 2-mercaptoethanol, 0.1 M PBS/2 mM EDTA (pH 6.0), room temperature, 30 min; (c) 0.1 M PBS/2 mM EDTA (pH 6.0), 1.5 h; (d) iodoacetamide, 0.1 M PB (pH 6.0), room temperature, 30 min.

exclusion HPLC, as reported previously (32). Each value was calculated by dividing the antibody-bound radioactivity at various intervals by the radiochemical purity of the respective freshly prepared antibody. PC was developed with a mixture of methanol/water (8:2), and TLC was developed with a mixture of chloroform/methanol/water (15:8:1). Under these conditions, protein-bound radioactivity, free iodine, *m*-iodobenzoic acid, and *m*-iodohippuric acid had *R_f* values of 0, 0.6–0.65, 0.9–0.95, and 0.9–0.95 on PC and 0, 0.1, 0.4, and 0.25 on TLC, respectively.

In Vivo Studies. In vivo studies were performed after simultaneous administration of a mixed solution of [¹³¹I]-HML-OST7 and [¹²⁵I]MIH-OST7 or [¹²⁵I]MPH-OST7. A 3 mL solution of 0.1 M PB (pH 6.0) containing 1.12 mg of unmodified OST7 was mixed with 12 μ L each of [¹³¹I]HML-OST7 (14.6 μ Ci) and [¹²⁵I]MIH-OST7 or [¹²⁵I]MPH-OST7 (9.3 or 10.0 μ Ci) prior to administration. For comparison of the radioactivity distribution of [¹²⁵I]HML-OST7 and [¹¹¹In]EMCS-Bz-EDTA-OST7, 12 μ L (10.9 μ Ci, 42 μ g) of [¹²⁵I]HML-OST7 and 63 μ L (10.5 μ Ci, 42 μ g) of [¹¹¹In]EMCS-Bz-EDTA-OST7 were added to 3 mL of unmodified OST7 (1.12 mg) prior to administration. Blood clearance of radioactivity after intravenous administration of each mixture in 6-week-old male ddY mice (34) was determined. Groups of five mice, each receiving 100 μ L of the radiolabeled OST7 conjugate solution, were sacrificed 1, 3, 6, 24, 48, 72, and 96 h postinjection. Organs of interest were removed and weighed, and the radioactivity was determined with a well counter (ARC 2000). A window from 29 to 97 keV was used for counting ¹²⁵I while one from 280 to 440 keV for ¹³¹I and from 168 to 568 keV for ¹¹¹In. Correlation factors for eliminating crossover of ¹³¹I or ¹¹¹In activity into ¹²⁵I were determined by counting the ¹³¹I or ¹¹¹In standard in each window. The crossover of ¹²⁵I into the ¹³¹I or ¹¹¹In channels was negligible. Samples of urine and feces were collected for 96 h, and the radioactivity was determined. Twenty-four hours after injection of [¹²⁵I]HML-OST7, urine samples were collected and analyzed by TLC as described above. The urine samples were also analyzed by RP-HPLC after filtration through a 10 kDa cutoff ultrafiltration membrane (Microcon 10, Amicon Grace, Tokyo). In RP-HPLC analysis, free iodine, *m*-iodohippuric acid, and *m*-iodobenzoic acid had retention times of 2.9, 9.4, and 30.8 min, respectively.

Athymic mice bearing osteogenic sarcoma were also treated intravenously with 100 μ L of a mixed solution of [¹³¹I]HML-OST7 and [¹²⁵I]MIH-OST7, [¹³¹I]HML-OST7 and [¹²⁵I]MPH-OST7, and [¹²⁵I]HML-OST7 and [¹¹¹In]-EMCS-Bz-EDTA-OST7, prepared as described above. The animals were sacrificed 24 and 48 h postinjection (five mice in each group). Organs of interest were removed and weighed, and the radioactivity in each tissue was determined (ARC 2000).

Statistical Analysis. Data are expressed as means $\pm$ standard deviation where appropriate. Results were statistically analyzed using the unpaired *t* test for in vitro studies and the paired *t* test for in vivo studies. Differences were considered statistically significant when the *p* value was less than 0.05.

RESULTS

Preparation of Radiolabeled OST7 Conjugates.

Radioiodination of SHML was performed in 1% AcOH/MeOH in the presence of NCS as an oxidant (Scheme 1). After reaction for 45 min at room temperature, [¹²⁵I]HML and [¹³¹I]HML were obtained with radiochemical yields of 80.2 and 78.9%, respectively, as determined by TLC. Similar procedures were employed for the radioiodination of SMIH and SMPH, which provided [¹²⁵I]MIH and [¹²⁵I]MPH with radiochemical yields of 79.8 and 88.7%, respectively. All the radioiodinated compounds were treated with sodium bisulfite to quench any residual NCS, preventing the iodine species from reacting with tyrosine residues of the antibody molecule. Neutralization of NCS also prevented exposure of the protein to the oxidant.

Conjugation of [^{125/131}I]HML with OST7 was performed by reactions of thiolated antibodies with the maleimide group of [^{125/131}I]HML. 2-ME reduction of OST7 exposed 6.2 thiol groups per molecule of OST7, and subsequent conjugation reactions with [^{125/131}I]HML were performed at a maleimide-to-thiol molar ratio of 1:1. After the conjugation reaction, the unreacted thiol groups were capped with iodoacetamide. The reaction steps are summarized in Scheme 1. The radiochemical yield of [¹³¹I]HML-OST7 was 46.8%, as determined by CAE. After purification by the centrifuged column procedure, [¹³¹I]HML-OST7 was obtained with a radiochemical purity and specific activity of 96.0% and 348 μ Ci/mg,

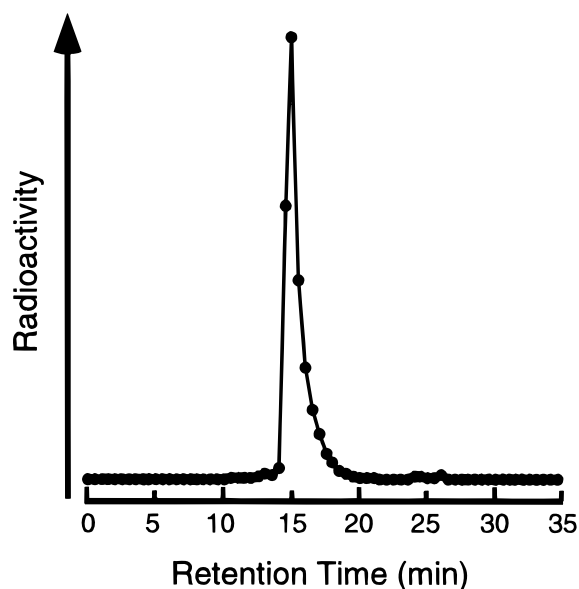


Figure 2. Size-exclusion HPLC profile of the radioactivity of [^{131}I]HML-OST7. More than 97% of the radioactivity was eluted at a retention time identical to that of unmodified OST7. Under these conditions, thyroglobulin (670 000), OST7 (IgG), bovine serum albumin (68 000), and cytochrome *c* (13 000) were eluted at retention times of 11.4, 15.4, 16.8, and 21.0 min, respectively.

respectively, for subsequent studies. With similar procedures, [^{125}I]HML-OST7, [^{125}I]MIH-OST7, and [^{125}I]MPH-OST7 were obtained with radiochemical yields of 67.1, 72.3, and 81.9%, respectively. The radiochemical purity and specific activity were 95.1% and 261 $\mu\text{Ci}/\text{mg}$, 98.7% and 223 $\mu\text{Ci}/\text{mg}$, and 99.4% and 238 $\mu\text{Ci}/\text{mg}$ for [^{125}I]HML-OST7, [^{125}I]MIH-OST7, and [^{125}I]MPH-OST7, respectively. Direct radioiodination of OST7 with Na^{125}I produced [^{125}I]OST7 with a radiochemical purity and specific activity of 97.3% and 74 $\mu\text{Ci}/\text{mg}$, respectively.

The EMCS-Bz-EDTA-OST7 conjugate was prepared with maleimide-thiol chemistry using the maleimide group of EMCS-Bz-EDTA and the thiol groups of OST7, generated by reducing the disulfide bonds. The number of EMCS-Bz-EDTA molecules attached per molecule of OST7 was 3.7, as determined by measuring the thiol groups before and after the conjugation reaction. After the centrifuged column procedure, [^{111}In]EMCS-Bz-EDTA-OST7 was obtained with a radiochemical purity and specific activity of 91.9% and 250 $\mu\text{Ci}/\text{mg}$, respectively, as determined by CAE.

Figure 2 shows the size-exclusion HPLC profile of [^{131}I]HML-OST7. More than 97% of the radioactivity was observed in fractions similar to those of unmodified OST7. Similar results were observed with all the radiolabeled OST7 conjugates used in this study (data not shown).

Serum Stability of Radiolabeled OST7 Conjugates. Table 1 shows the percentages of the protein-bound radioactivity of the respective radiolabeled OST7 conjugate after incubation in freshly prepared human serum at 37 $^{\circ}\text{C}$. [^{131}I]HML-OST7, [^{125}I]MPH-OST7, and [^{111}In]EMCS-Bz-EDTA-OST7 released only ca. 2–3% of the initial antibody-bound radioactivity after incubation for 24 h. However, [^{125}I]MIH-OST7 released a significantly higher amount of the radioactivity with incubation time, and half of the initial radioactivity was released from OST7 after incubation for 24 h. The radioactivity released from [^{125}I]MIH-OST7 was an indication of R_f values identical to those of *m*-iodohippuric acid when analyzed by TLC and PC.

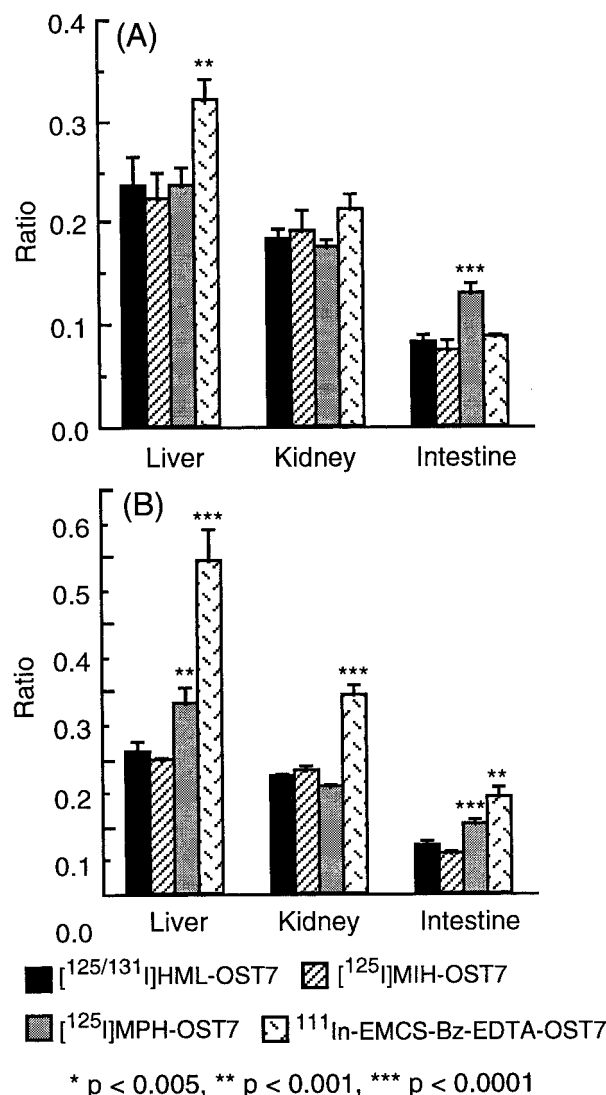


Figure 3. Organ-to-tumor ratios of the radioactivity 6 h (A) and 72 h (B) after injection of [^{125}I]HML-OST7, [^{131}I]HML-OST7, [^{125}I]MIH-OST7, [^{125}I]MPH-OST7, and [^{111}In]EMCS-Bz-EDTA-OST7 in normal mice. Since no significant differences were observed between [^{125}I]HML-OST7 and [^{131}I]HML-OST7, these results were combined and expressed as the results for [$^{125/131}\text{I}$]HML-OST7.

Immunoreactivity. The binding of [^{131}I]HML-OST7, [^{125}I]HML-OST7, [^{125}I]MIH-OST7, [^{125}I]MPH-OST7, [^{111}In]EMCS-Bz-EDTA-OST7, and [^{125}I]OST7 to KT005 cells was examined as a function of cell number. No significant differences were observed in the cell-bound radioactivity among the six radiolabeled OST7 conjugates (data not shown).

Biodistribution Studies in ddY Mice. The time courses of the radioactivity in the blood after intravenous administration of radiolabeled OST7 conjugates in ddY mice are summarized in Table 2. Since [^{131}I]HML-OST7 and [^{125}I]HML-OST7 had similar radioactivity distributions in the three experiments, these results were combined and presented as [$^{125/131}\text{I}$]HML-OST7 in Table 2. While no significant differences were observed in the radioactivity elimination from the blood up to 96 h postinjection among [$^{125/131}\text{I}$]HML-OST7, [^{125}I]MPH-OST7, and [^{111}In]EMCS-Bz-EDTA-OST7, [^{125}I]MIH-OST7 showed a significantly rapid disappearance of the radioactivity from the blood from 24 h postinjection onward. The liver-to-blood and intestine-to-blood ratios of the radioactivity 6 (A) and 72 h (B) postinjection of

Table 1. Stabilities of Radiolabeled OST7 Conjugates in Human Serum at 37 °C^a

reagent	% of protein-bound radioactivity			
	1 h ^b	3 h ^b	6 h ^b	24 h ^b
[¹³¹ I]HML-OST7 ^c	100.00 (0.42)	99.41 (0.23)	99.26 (0.23)	97.45 (0.36)
[¹²⁵ I]MPH-OST7 ^c	100.37 (0.61)	98.15 (3.83)	99.81 (0.23)	98.38 (0.35)
[¹²⁵ I]MIH-OST7 ^c	98.49 (0.18)	95.35 (0.16)	85.72 (1.21)	49.21 (4.85)
[¹¹¹ In]EMCS-Bz-EDTA-OST7 ^c	nd ^d	nd	nd	102.45 (3.49)

^a Mean (SD) of four experiments for each point. ^b Incubation time. ^c Values determined by PC and TLC for radioiodinated OST7 conjugates and by HPLC for the ¹¹¹In-labeled OST7 conjugate. ^d nd, not determined.

Table 2. Radioactivity Clearance from the Blood after Intravenous Administration of Radiolabeled OST7 Conjugates in ddY Mice^a

	1 h ^b	6 h ^b	24 h ^b	48 h ^b	72 h ^b	96 h ^b
[¹²⁵ / ¹³¹ I]HML-OST7	25.13 (1.69)	19.70 (2.34)	12.16 (0.95)	9.65 (0.78)	7.71 (0.69)	6.44 (0.53)
[¹²⁵ I]MIH-OST7	25.83 (3.04)	16.90 (0.96)	8.99 ^c (0.30)	7.24 ^c (0.68)	5.23 ^c (0.44)	4.18 ^c (0.60)
[¹²⁵ I]MPH-OST7	25.50 (2.15)	21.78 (0.83)	12.85 (1.02)	9.45 (0.74)	7.86 (0.98)	6.45 (0.85)
[¹¹¹ In]EMCS-Bz-EDTA-OST7	24.87 (1.45)	19.41 (1.75)	12.15 (1.31)	9.75 (1.33)	7.94 (1.13)	5.97 (0.22)

^a Expressed as the percentage of the injected dose per gram of blood. Mean (SD) for four to ten animals for each point. ^b Time after administration. ^c $p < 0.0005$ compared to that for [¹³¹I]HML-OST7.

Table 3. Cumulative Radioactivity Excreted in the Urine and Feces 96 h after Injection of Radiolabeled OST7 Conjugates in ddY Mice^a

	urine	feces
[¹²⁵ / ¹³¹ I]HML-OST7	45.94 (8.66)	2.17 (0.22)
[¹²⁵ I]MIH-OST7	58.92 (1.40) ^b	1.91 (0.33)
[¹²⁵ I]MPH-OST7	27.13 (3.93) ^b	23.43 (0.91) ^c
[¹¹¹ In]EMCS-Bz-EDTA-OST7	26.94 (4.88) ^b	9.83 (2.81) ^b

^a Expressed as the percentage of the injected dose. Mean (SD) for four to ten animals for each point. ^b $p < 0.01$ compared to that for [¹²⁵/¹³¹I]HML-OST7. ^c $p < 0.0001$ compared to that for [¹²⁵/¹³¹I]HML-OST7.

the four radiolabeled OST7 conjugates in ddY mice are shown in Figure 3. No significant differences in the radioactivity ratios were observed between [¹³¹I]HML-OST7 and [¹²⁵I]MIH-OST7 at either postinjection time point. However, [¹²⁵I]MPH-OST7 had significantly higher liver-to-blood ratios at 72 h and intestine-to-blood ratios at both 6 and 72 h than [¹³¹I]HML-OST7. Significantly higher radioactivity ratios were also observed with [¹¹¹In]EMCS-Bz-EDTA-OST7 at 6 h for the liver and at 6 and 72 h for the intestine when compared with those of simultaneously administered [¹²⁵I]HML-OST7.

Figure 4 shows the RP-HPLC and TLC radioactivity profiles of urine obtained 24 h after injection of [¹³¹I]HML-OST7. RP-HPLC indicated that more than 93% of the radioactivity in the urine was eluted at a retention time identical to that of *m*-iodohippuric acid. TLC analysis also indicated a single radioactivity peak (>98%) at an *R_f* value identical to that of *m*-iodohippuric acid.

Table 3 summarizes the cumulative radioactivity excreted in the urine and feces 96 h after injection of the five radiolabeled OST7 conjugates. [¹²⁵I]MIH-OST7 showed the fastest excretion rate of radioactivity from the body. While both [¹²⁵/¹³¹I]HML-OST7 and [¹²⁵I]MIH-OST7 showed more than 95% excreted radioactivity in the urine, [¹²⁵I]MPH-OST7 excreted an almost equal amount of radioactivity in urine and feces. [¹¹¹In]EMCS-Bz-EDTA-OST7 exhibited the slowest excretion rate of radioactivity from the body.

Biodistribution Studies in Nude Mice. Table 4 shows the biodistribution of radioactivity after simultaneous administration of [¹³¹I]HML-OST7 and [¹²⁵I]MIH-OST7 in athymic mice bearing osteogenic sarcoma. Although similar radioactivity levels in the tumor were observed with both radioiodinated OST7 conjugates 24 h postinjection, [¹²⁵I]MIH-OST7 indicated a significant

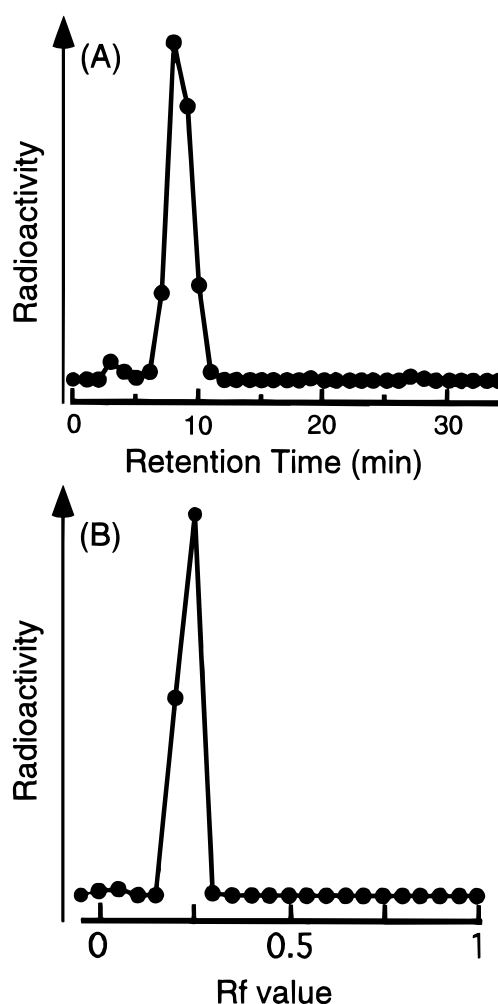


Figure 4. Radioactivity profiles of murine urine 24 h after injection of [¹³¹I]HML-OST7 on RP-HPLC (A) and TLC (B). The major radioactivity (>93% for RP-HPLC and >98% for TLC) was detected at a retention time and *R_f* value similar to those of *m*-iodohippuric acid.

decrease in the radioactivity levels in the tumor 48 h postinjection. Although [¹²⁵I]MIH-OST7 indicated significantly higher tumor-to-liver and tumor-to-intestine ratios of radioactivity at both postinjection time points, no significant differences were observed between the two

Table 4. Comparative Radioactivity Localization after Simultaneous Administration of [¹³¹I]HML-OST7 and [¹²⁵I]MIH-OST7 in Athymic Mice Bearing Osteogenic Sarcoma^a

	time (h)	% ID/gram		tumor-to-organ		organ-to-blood	
		tumor	blood	liver	intestine	liver	intestine
[¹³¹ I]HML-OST7	24	32.65 (1.54)	9.39 (1.50)	12.93 (2.38)	41.04 (8.72)	0.23 (0.04)	0.10 (0.01)
	48	30.33 (1.46)	6.20 (1.39)	28.76 (4.63)	65.40 (9.25)	0.23 (0.02)	0.09 (0.01)
[¹²⁵ I]MIH-OST7	24	32.41 (2.99)	8.36 (1.40)	15.89 ^b (3.27)	52.73 ^b (13.78)	0.21 (0.04)	0.10 (0.01)
	48	23.13 ^d (3.57)	3.46 ^c (0.98)	46.52 ^c (10.35)	132.96 ^c (31.96)	0.23 (0.04)	0.10 (0.01)

^a Values are the means (SD) of nine animals for each point. Mean weights (SD) of mice and tumors in grams: 19.62 (1.26) and 0.46 (0.11) at 24 h and 18.92 (0.61) and 0.85 (0.07) at 48 h. ^b $p < 0.05$ compared to that for [¹³¹I]HML-OST7. ^c $p < 0.01$ compared to that for [¹³¹I]HML-OST7. ^d $p < 0.005$ compared to that for [¹³¹I]HML-OST7.

Table 5. Comparative Radioactivity Localization after Simultaneous Administration of [¹³¹I]HML-OST7 and [¹²⁵I]MPH-OST7 in Athymic Mice Bearing Osteogenic Sarcoma^a

	time (h)	% ID/gram		tumor-to-organ	
		tumor	blood	liver	intestine
[¹³¹ I]HML-OST7	24	33.72 (3.21)	9.37 (0.71)	15.86 (1.24)	60.10 (4.63)
	48	31.31 (5.40)	8.09 (0.23)	24.48 (5.85)	72.42 (24.22)
[¹²⁵ I]MPH-OST7	24	32.50 (2.75)	8.52 (0.56)	15.75 (3.39)	38.10 ^b (4.63)
	48	29.75 (3.83)	7.53 (0.27)	24.52 (8.37)	32.43 ^b (9.26)

^a Values are the means (SD) of five animals for each point. Mean weights (SD) of mice and tumors in grams: 20.69 (0.77) and 0.54 (0.22) at 24 h and 20.46 (1.92) and 0.51 (0.21) at 48 h, respectively. ^b $p < 0.005$ compared to that for [¹³¹I]HML-OST7.

radioiodinated OST7 conjugates when the liver-to-blood and intestine-to-blood ratios of the radioactivity were calculated.

The biodistributions of radioactivity after simultaneous administration of [¹³¹I]HML-OST7 and [¹²⁵I]MPH-OST7 in athymic mice bearing osteogenic sarcoma are summarized in Table 5. Both radioiodinated OST7 conjugates showed no changes in radioactivity levels in the tumor between the two postinjection time points. No significant differences were observed in the radioactivity levels in the tumor and in the blood when the two radioiodinated OST7 conjugates were compared 24 and 48 h postinjection. However, while [¹³¹I]HML-OST7 and [¹²⁵I]MPH-OST7 had similar tumor-to-liver ratios of radioactivity, significantly higher tumor-to-intestine ratios were achieved by [¹³¹I]HML-OST7 at both postinjection time points.

The biodistributions of radioactivity after simultaneous administration of [¹²⁵I]HML-OST7 and [¹¹¹In]EMCS-Bz-EDTA-OST7 in athymic mice are shown in Table 6. [¹²⁵I]HML-OST7 and [¹¹¹In]EMCS-Bz-EDTA-OST7 had similar radioactivity levels in the tumor and blood both

24 and 48 h postinjection. However, [¹²⁵I]HML-OST7 had significantly higher tumor-to-liver ratios of radioactivity both 24 and 48 h postinjection and tumor-to-intestine ratios 48 h postinjection when compared with [¹¹¹In]EMCS-Bz-EDTA-OST7.

DISCUSSION

All the radiolabeled OST7 conjugates were prepared using similar conjugation chemistry. All exhibited similar radiochromatograms on size-exclusion HPLC (Figure 2) and similar affinities for KT005 cells. In addition, high stability against in vivo deiodination or transchelation of all the radiolabeled OST7 conjugates was well demonstrated in this study and in previous studies (11, 12, 14, 25–27, 32, 35). Thus, the differences in the localization of the radioactivity from the four radiolabeled OST7 conjugates in vivo could be primarily attributed to the differences in stability of the metabolizable linkage of the respective radiolabeled OST7 and the in vivo behavior of radiometabolites derived from each reagent.

The plasma-labile characteristic of the ester bond in MIH (Table 1) was reflected in the biodistribution studies in normal mice, and the fastest elimination rate of radioactivity from the blood (Table 2) and the highest excretion of the radioactivity from the body (Table 3) were observed with [¹²⁵I]MIH-OST7. Despite this, however, no significant differences were observed in the organ-to-blood ratios of the radioactivity between [¹³¹I]HML-OST7 and [¹²⁵I]MIH-OST7 (Figure 3), indicating that [¹³¹I]HML-OST7 liberated *m*-iodohippuric acid after lysosomal proteolysis in nontarget tissues at a rate similar to that of [¹²⁵I]MIH-OST7. This was supported by the results of the urine analyses where the radioactivity was excreted as *m*-iodohippuric acid after administration of [¹³¹I]HML-OST7 (Figure 4).

Since similar radioactivity levels in the blood were observed after simultaneous administration of [¹³¹I]HML-OST7 and [¹²⁵I]MPH-OST7 (Table 2), similar amounts of radiolabeled OST7 would be delivered to the liver. Thus, significantly higher radioactivity levels in

Table 6. Comparative Radioactivity Localization after Simultaneous Administration of [¹³¹I]HML-OST7 and [¹¹¹In]EMCS-Bz-EDTA-OST7 in Athymic Mice Bearing Osteogenic Sarcoma^a

	time (h)	% ID/gram		tumor-to-organ	
		tumor	blood	liver	intestine
[¹³¹ I]HML-OST7	24	37.14 (2.94)	10.07 (1.70)	12.59 (2.37)	54.64 (5.80)
	48	29.61 (9.51)	7.19 (1.43)	32.82 (14.88)	82.14 (34.38)
[¹¹¹ In]EMCS-Bz-EDTA-OST7	24	39.53 (2.93)	8.15 (0.61)	3.86 ^c (1.09)	54.21 (6.17)
	48	33.39 (7.06)	5.47 (1.02)	8.41 ^c (3.09)	75.68 ^b (10.06)

^a Values are the means (SD) of five animals for each point. Mean weights (SD) of mice and tumors in grams: 20.44 (1.41) and 0.37 (0.10) at 24 h and 20.88 (0.52) and 0.47 (0.25) at 48 h, respectively. ^b $p < 0.05$ compared to that for [¹³¹I]HML-OST7. ^c $p < 0.005$ compared to that for [¹³¹I]HML-OST7.

these tissues after administration of [^{125}I]MPH-OST7 (Figure 3) would reflect the slower hepatic elimination rate of the radiometabolites derived from MPH and subsequent hepatobiliary excretion, as also observed in a previous study using NGA (25). Similarly, the slowest hepatic elimination rate of the radiometabolites derived from [^{111}In]EMCS-Bz-EDTA-OST7 would be responsible for the highest liver-to-blood ratios of the radioactivity of this radiolabeled OST7. This was supported by the lowest radioactivity excretion levels of this radiolabeled OST7 (Table 3). These findings using OST7 were consistent with those of previous studies using NGA as a model polypeptide, reinforcing the fact that *in vivo* behavior of radiometabolites generated after lysosomal proteolysis of radiolabeled mAbs plays a critical role in the hepatic radioactivity levels. These findings also indicated that, when applied to mAbs, HML provided rapid and selective release of *m*-iodohippuric acid after accumulation in nontarget tissues such as the liver, while providing a stable attachment for *m*-iodohippuric acid with the mAb in plasma.

To further estimate the validity of the radiochemical design of mAbs with metabolizable linkages, two radiolabeled OST7 conjugates that liberate *m*-iodohippuric acid as the sole radiometabolite after lysosomal proteolysis at a similar rate, [^{131}I]HML-OST7 and [^{125}I]MIH-OST7, were simultaneously administered to nude mice bearing osteogenic sarcoma. Similar radioactivity levels in the tumor 24 h after injection of the two radiolabeled OST7 conjugates would be attributable to the similar radioactivity levels in the blood at this postinjection time and the similar affinities for the tumor cells of the two radioiodinated OST7 conjugates. The significantly lowered radioactivity levels in the blood 48 h after injection of [^{125}I]MIH-OST7 would be caused by the enzymatic and chemical hydrolysis of the ester bond in the blood, followed by the urinary excretion of the resulting *m*-iodohippuric acid. On the other hand, the formation of antigen-antibody complexes on the tumor cell surface provides steric hindrance to esterase access, which stabilized the ester bond of [^{125}I]MIH-OST7 (11). Thus, while chemical hydrolysis of the ester bond in [^{125}I]MIH-OST7 would be predominantly responsible for the elimination of radioactivity from the tumor, both chemical and enzymatic hydrolyses takes place in the blood, which enhances the target-to-nontarget ratios of the radioactivity with time after injection (Table 4). Since the peptide bond of [^{131}I]HML-OST7 was stable both in the blood and on the tumor cell surface, neither accelerated clearance of the radioactivity from the blood nor decreased radioactivity levels in the tumor were observed. Although [^{125}I]MIH-OST7 provided significantly higher tumor-to-nontarget ratios of the radioactivity, these results would be most likely caused by the presence of large tumor masses compared to their small distribution volume in the animal model. When the much lower and delayed accumulation of the radioactivity of radiolabeled mAbs in clinical studies is considered (36-39), the plasma-labile characteristics of the ester bond in MIH may cause a significant decrease in the radioactivity levels in the tumor in clinical studies. Thus, the animal model may have underestimated the plasma-labile characteristics of the ester bond in MIH in the radioactivity delivery to the target by the mAb. Similar nontarget-to-blood ratios of the radioactivity after administration of [^{131}I]HML-OST7 and [^{125}I]MIH-OST7 in normal and nude mice also suggested that the higher tumor-to-nontarget ratios of the radioactivity with [^{125}I]MIH-

OST7 are characteristic of the animal model (Figure 3 and Table 4).

The ability of HML to provide target-selective radioactivity localization using mAbs as vehicles was clearly demonstrated in the comparative biodistribution studies of [$^{125/131}\text{I}$]HML-OST7 with [^{125}I]MPH-OST7 or [^{111}In]EMCS-Bz-EDTA-OST7 (Tables 5 and 6). Similar radioactivity levels in the blood and the tumor both 24 and 48 h after injection of the three radiolabeled OST7 conjugates coincided well with the similar plasma stabilities and tumor cell affinities of the three radiolabeled OST7 conjugates. Significantly higher radioactivity levels in the intestine of [^{125}I]MPH-OST7 and in the liver and intestine of [^{111}In]EMCS-Bz-EDTA-OST7 reflected the *in vivo* behavior of radiometabolites derived from the respective reagent. These findings indicated that the radiochemical design of mAb conjugates that provides a stable attachment for the radiolabel in plasma while facilitating rapid and selective release of the radiometabolite of urinary excretion would be useful in achieving target-selective radioactivity localization using mAbs as vehicles.

Recently, efforts have been directed toward increasing the accumulation of radiolabeled mAbs by hyperthermia or biological response modifiers (40-43). Antibody fragments and mAbs that recognize the antigens present on the vascular wall in the tumoral tissues offer promise for achieving target-selective radioactivity localization within short postinjection intervals (18, 20-24). Since the radiochemical design of mAb conjugates using metabolizable linkages is complementary to these approaches, combinations of these different approaches would render the radiolabeled mAbs attractive radiopharmaceuticals for both diagnostic and therapeutic nuclear medicine.

In conclusion, the peptide bond of HML-conjugated OST7 remained stable in plasma but rapidly liberated *m*-iodohippuric acid after lysosomal proteolysis in nontarget tissues. As a result, HML-conjugated OST7 decreased the radioactivity levels in nontarget tissues without impairing those in the target delivered by the mAb. These findings strongly supported the validity of the radiochemical design of antibodies using metabolizable linkages that provide a stable attachment of radiolabels with mAbs in plasma while facilitating selective release of radiometabolites of rapid urinary excretion. The well-correlated hepatic radioactivity levels between radiolabeled mAbs and radiolabeled NGAs also indicated that the biological method using NGA as the model polypeptide would be highly useful in estimating newly designed radiolabeling reagents for reducing hepatic radioactivity levels of radiolabeled mAbs.

ACKNOWLEDGMENT

We are grateful to Dr. I. Yomoda of Daiichi Radioisotope Laboratories (Tokyo) for providing Na^{131}I . We also thank Nihon Medi-Physics for their kind gift of $^{111}\text{InCl}_3$. This study was supported in part by Grants-in-Aid for Developing Scientific Research (08557135 and 09672279) from the Ministry of Education, Science, Culture and Sports of Japan.

LITERATURE CITED

- (1) Haseman, M. K., Goodwin, D. A., Meares, C. F., Kaminski, M. S., Wensel, T. G., McCall, M. J., and Levy, R. (1986) Metabolizable ^{111}In Chelate Conjugated Anti-Idiotypic Monoclonal Antibody for Radioimmunodetection of Lymphoma in Mice. *Eur. J. Nucl. Med.* 12, 455-460.

- (2) Arano, Y., Inoue, T., Mukai, T., Wakisaka, K., Sakahara, H., Konishi, J., and Yokoyama, A. (1994) Discriminated Release of a Hippurate-Like Radiometal Chelate in Nontarget Tissues for Target-Selective Radioactivity Localization Using pH-Dependent Dissociation of Reduced Antibody. *J. Nucl. Med.* 35, 326–333.
- (3) Bridger, G. J., Abrams, M. J., Padmanabhan, S., Gaul, F., Larsen, S., Henson, G. W., Schwartz, D. A., Longley, C. B., Burton, C. A., and Ultee, M. E. (1996) A Comparison of Cleavable and Noncleavable Hydrazinopyridine Linkers for the Tc-99m Labeling of Fab' Monoclonal Antibody Fragments. *Bioconjugate Chem.* 7, 255–264.
- (4) Deshpande, S. V., DeNardo, S., Meares, C. F., McCall, M. J., Adams, G. P., and DeNardo, G. L. (1989) Effect of Different Linkages Between Chelates and Monoclonal Antibodies on Levels of Radioactivity in the Liver. *Nucl. Med. Biol.* 16, 587–597.
- (5) Faivre-Chauvet, A., Gestin, J. F., Mease, R. C., Sai-Maurel, C., Thedrez, P., Slinkin, M., Meinken, G. E., Srivastava, S. C., and Chatal, J. F. (1993) Introduction of Five Potentially Metabolizable Linking Groups Between In-111-Cyclohexyl EDTA Derivatives and F(ab')₂ Fragments of Anti-Carcinoembryonic Antigen Antibody. 2. Comparative Pharmacokinetics and Biodistribution in Human Colorectal Carcinoma-Bearing Nude Mice. *Nucl. Med. Biol.* 20, 763–771.
- (6) Paik, C. H., Yokoyama, K., Reynolds, J. C., Quadri, S. M., Min, C. Y., Shin, S. Y., Maloney, P. J., Larson, S. M., and Reba, R. C. (1989) Reduction of Background Activities by Introduction of a Diester Linkage Between Antibody and a Chelate in Radioimmunodetection of Tumor. *J. Nucl. Med.* 30, 1693–1701.
- (7) Quadri, S. M., Vriesendorp, H. M., Lechner, P. K., and Williams, J. R. (1993) Evaluation of Indium-111- and Yttrium-90-Labeled Linker-Immunoconjugates in Nude Mice and Dogs. *J. Nucl. Med.* 34, 938–945.
- (8) Weber, R. W., Boutin, R. H., Nedelman, M. A., Lister-Jones, J., and Dean, R. T. (1990) Enhanced Kidney Clearance with an Ester-Linked ^{99m}Tc-Radiolabeled Antibody Fab'-Chelator Conjugate. *Bioconjugate Chem.* 1, 431–437.
- (9) Heymann, E. (1982) Hydrolysis of Carboxylic Ester and Amides. *Metabolic Basis of Detoxification: Metabolism of Functional Group* (W. B. Jakoby, J. R. Bend, and J. Caldwell, Eds.) pp 229–241, Academic Press, New York.
- (10) Arano, Y., Matsushima, H., Tagawa, M., Inoue, T., Koizumi, M., Hosono, M., Sakahara, H., Endo, K., Konishi, J., and Yokoyama, A. (1994) A Newly Designed Radioimmunoconjugate Releasing a Hippurate-Like Radiometal Chelate for Enhanced Target/Non-Target Radioactivity. *Nucl. Med. Biol.* 21, 63–69.
- (11) Arano, Y., Wakisaka, K., Ohmono, Y., Uezono, T., Akizawa, H., Nakayama, M., Sakahara, H., Tanaka, C., Konishi, J., and Yokoyama, A. (1996) Assessment of Radiochemical Design of Antibodies Using an Ester Bond as the Metabolizable Linkage: Evaluation of Maleimidoethyl 3-(Tri-*n*-butylstannyl)hippurate as a Radioiodination Reagent of Antibodies for Diagnostic and Therapeutic Applications. *Bioconjugate Chem.* 7, 628–637.
- (12) Arano, Y., Mukai, T., Uezono, T., Wakisaka, K., Motonari, H., Akizawa, H., Taoka, Y., and Yokoyama, A. (1994) A Biological Method to Evaluate Bifunctional Chelating Agents to Label Antibodies with Metallic Radionuclides. *J. Nucl. Med.* 35, 890–898.
- (13) Arano, Y., Mukai, T., Uezono, T., Motonari, H., Wakisaka, K., and Yokoyama, A. (1994) Biological Comparison of DTPA and SCN-Bz-EDTA as a Chelating Agent for Indium Labeling of Antibodies. *J. Labelled Compd. Radiopharm.* 35, 381–383.
- (14) Arano, Y., Mukai, T., Akizawa, H., Uezono, T., Motonari, H., Wakisaka, K., Kairiyama, C., and Yokoyama, A. (1995) Radiolabeled Metabolites of Proteins Play a Critical Role in Radioactivity Elimination from the Liver. *Nucl. Med. Biol.* 22, 555–564.
- (15) Duncan, J. R., and Welch, M. J. (1993) Intracellular Metabolism of Indium-111-DTPA-Labeled Receptor Targeted Proteins. *J. Nucl. Med.* 34, 1728–1738.
- (16) Franano, F. N., Edwards, W. B., Welch, M. J., and Duncan, J. R. (1994) Metabolism of Receptor Targeted ¹¹¹In-DTPA-Glycoproteins: Identification of ¹¹¹In-DTPA- ϵ -lysine as the Primary Metabolic and Excretory Product. *Nucl. Med. Biol.* 21, 1023–1034.
- (17) Rogers, B. E., Franano, F. N., Duncan, J. R., Edwards, W. B., Anderson, C. J., Connett, J. M., and Welch, M. J. (1995) Identification of Metabolites of In-111-Diethylenetriamine-pentaacetic Acid Monoclonal Antibodies and Antibody Fragments In Vivo. *Cancer Res.* 55, S5714–S5720.
- (18) Begent, R. H. J., Verhaar, M. J., Chester, K. A., Casey, J. L., Green, A. J., Napier, M. P., Hope-Stone, L. D., Cushen, N., Keep, P. A., Johnson, C. J., Hawkins, R. E., Hilson, A. J. W., and Robson, L. (1996) Clinical Evidence of Efficient Tumor Targeting Based on Single-Chain Fv Antibody Selected From a Combinatorial Library. *Nat. Med.* 2, 979–984.
- (19) Co, M. S., Baker, J., Bednarik, K., Janzek, E., Neruda, W., Mayer, P., Plot, R., Stumper, B., Vasquez, M., Queen, C., and Loibner, H. (1996) Humanized Anti-Lewis Y Antibodies: In Vitro Properties and Pharmacokinetics in Rhesus Monkeys. *Cancer Res.* 56, 1118–1125.
- (20) Yokota, T., Milenic, D. E., Whitlow, M., and Schlom, J. (1992) Rapid Tumor Penetration of a Single-Chain Fv and Comparison with Other Immunoglobulin Forms. *Cancer Res.* 52, 3402–3408.
- (21) Zhang, H., Lake, D. F., Barbutto, J. A. M., Bernstein, R. M., Grimes, W. J., and Hersch, E. M. (1995) A Human Monoclonal Antimelanoma Single-Chain Fv Antibody Derived from Tumor-Infiltrating Lymphocytes. *Cancer Res.* 55, 3584–3591.
- (22) Fox, S. B., and Harris, A. L. (1997) Markers of Tumor Angiogenesis: Clinical Applications in Prognosis and Anti-Angiogenic Therapy. *Invest. New Drugs* 15, 15–28.
- (23) Ke Lin, Q. H., Nagy, J. A., Eckelhoefer, I. A., Mase, E. M., Dvorak, A. M., and Dvorak, H. F. (1996) Vascular Targeting of Solid and Ascites Tumours with Antibodies to Vascular Endothelial Growth Factor. *Eur. J. Cancer* 32A, 2467–2473.
- (24) Thorpe, P. E., and Burrows, F. J. (1995) Antibody-Directed Targeting of the Vasculature of Solid Tumors. *Breast Cancer Res. Treat.* 36, 237–251.
- (25) Wakisaka, K., Arano, Y., Uezono, T., Akizawa, H., Ono, M., Kawai, K., Ohmomo, Y., Nakayama, M., and Saji, H. (1997) A Novel Radioiodination Reagent for Protein Radiopharmaceuticals with L-Lysine as a Plasma-Stable Metabolizable Linkage to Liberate *m*-Iodohippuric Acid after Lysosomal Proteolysis. *J. Med. Chem.* 40, 2643–2652.
- (26) Arano, Y., Wakisaka, K., Ohmomo, Y., Uezono, T., Mukai, T., Motonari, H., Shiono, H., Sakahara, H., Konishi, J., Tanaka, C., and Yokoyama, A. (1994) Maleimidoethyl 3-(tri-*n*-butylstannyl)hippurate: A Useful Radioiodination Reagent for Protein Radiopharmaceuticals to Enhance Target Selective Radioactivity Localization. *J. Med. Chem.* 37, 2609–2618.
- (27) Deshpande, S. V., Subramanian, R., McCall, M. J., DeNardo, S. J., DeNardo, G. L., and Meares, C. F. (1990) Metabolism of Indium Chelates Attached to Monoclonal Antibody: Minimal Transchelation of Indium from Benzyl-EDTA chelate In Vivo. *J. Nucl. Med.* 31, 218–224.
- (28) Koizumi, M., Endo, K., Kunitatsu, M., Sakahara, H., Nakashima, T., Kawamura, Y., Watanabe, Y., Saga, T., Konishi, J., Yamamuro, T., Hosoi, S., Toyama, S., Arano, Y., and Yokoyama, A. (1988) 67-Ga-Labeled Antibodies for Immunosintigraphy and Evaluation of Tumor Targeting of Drug-Antibody Conjugates in Mice. *Cancer Res.* 48, 1189–1194.
- (29) Meares, C. F., McCall, M. J., Reardan, D. T., Goodwin, D. A., Diamanti, C. I., and McTigue, M. (1984) Conjugation of Antibodies with Bifunctional Chelating Agents: Isothiocyanate and Bromoacetamide Reagents, Methods of Analysis, and Subsequent Addition of Metal Ions. *Anal. Biochem.* 142, 68–78.
- (30) Grassetti, D. R., and Murray, J. F., Jr. (1967) Determination of Sulfhydryl Groups with 2,2'- or 4,4'-Dithiopyridine. *Arch. Biochem. Biophys.* 119, 41–49.
- (31) Wilbur, D. S., Hadley, S. W., Grant, L. M., and Hylarides, M. D. (1991) Radioiodinated Iodobenzoyl Conjugates of a

- Monoclonal Antibody Fab Fragment. In Vivo Comparisons with Chloramine-T-Labeled Fab. *Bioconjugate Chem.* 2, 111–116.
- (32) Arano, Y., Uezono, T., Akizawa, H., Ono, M., Wakisaka, K., Nakayama, M., Sakahara, H., Konishi, J., and Yokoyama, A. (1996) Reassessment of Diethylenetriaminepentaacetic Acid (DTPA) as a Chelating Agent for Indium-111 Labeling of Polypeptides Using a Newly Synthesized Monoreactive DTPA Derivative. *J. Med. Chem.* 39, 3451–3460.
- (33) Brechbiel, M. W., Gansow, O. A., Atcher, R. W., Schlom, J., Esteban, J., Simpson, D. E., and Colcher, D. (1986) Synthesis of 1-(*p*-Isothiocyanatobenzyl) Derivatives of DTPA and EDTA. Antibody Labeling and Tumor-Imaging Study. *Inorg. Chem.* 25, 2772–2781.
- (34) Imai, S., Morimoto, J., Tsubura, T., Esaki, K., Michalides, R., Holms, R. S., Deimling, O., and Higlgers, J. (1986) Genetic Marker Patterns and Endogenous Mammary Tumor Virus Genes in Inbred Mouse Strains in Japan. *Exp. Anim.* 35, 263–276.
- (35) Motta-Hennessy, C., Sharkey, R. M., and Goldenberg, D. M. (1990) Metabolism of Indium-111-Labeled Murine Monoclonal Antibody in Tumor and Normal Tissue of the Athymic Mouse. *J. Nucl. Med.* 31, 1510–1519.
- (36) Dykes, P. W., Bradwell, A. R., Chapman, C. E., and Vaughan, A. T. M. (1987) Radioimmunotherapy of Cancer: Clinical Studies and Limiting Factor. *Cancer Treat. Rev.* 14, 87–106.
- (37) Larson, S. M., Divgi, C. R., Scott, A., Sgouros, G., Graham, M. C., Kostakoglu, L., Scheinberg, D., Cheung, N. K. V., Schlom, J., and Finn, R. D. (1994) Current status of radioimmunotherapy. *Nucl. Med. Biol.* 21, 785–792.
- (38) Reilly, R. M., Sandhu, J., Alvarezdiez, T. M., Gallinger, S., Kirsh, J., and Stern, H. (1995) Problems of Delivery of Monoclonal Antibodies: Pharmaceutical and Pharmacokinetic Solutions. *Clin. Pharmacokinet.* 28, 126–142.
- (39) Sands, H. (1988) Radioimmunoimaging: An Overview of Problems and Promises. *Antibody, Immunoconjugates, Radiopharm.* 1, 213–226.
- (40) Greiner, J. W., Ullmann, C. D., Nieroda, C., Qi, C. F., Eggersperger, D., Shimada, S., Steinberg, S. M., and Schlom, J. (1993) Improved Radioimmunotherapeutic Efficacy of an Anticarcinoma Monoclonal Antibody (^{131}I -CC49) When Given in Combination with Gamma-Interferon. *Cancer Res.* 53, 600–608.
- (41) Hosono, M. N., Hosono, M., Endo, K., Ueda, R., and Onoyama, Y. (1994) Effect of Hyperthermia on Tumor Uptake of Radiolabeled Anti-Neural Cell Adhesion Molecule Antibody in Small-Cell Lung Cancer Xenografts. *J. Nucl. Med.* 35, 504–509.
- (42) Kinuya, S., Yokoyama, K., Konishi, S., Tonami, N., and Hisada, K. (1996) Effect of Induced Hypertension with Angiotensin II Infusion on Biodistribution of ^{111}In -Labeled Monoclonal Antibody. *Nucl. Med. Biol.* 23, 137–140.
- (43) Schuster, J. M., Zalutsky, M. R., Noska, M. A., Dodge, R., Friedman, H. S., Bigner, D. D., and Dewhirst, M. W. (1995) Hyperthermic Modulation of Radiolabelled Antibody Uptake in a Human Glioma Xenograft and Normal Tissues. *Int. J. Hyperthermia* 11, 59–72.

BC970220A

A New Photoactivatable Reagent Capable of Transferring a Radiolabel to Target Proteins. Application to the Human Growth Hormone–Rat Liver Prolactin Receptor Interaction

Néstor T. H. Masckauchán, José M. Delfino,* and Horacio N. Fernández†

Instituto de Química y Físicoquímica Biológicas, Universidad de Buenos Aires, CONICET, Buenos Aires, Argentina. Received November 14, 1997; Revised Manuscript Received March 15, 1998

A new photoactivatable cross-linking reagent, 1-(2'-dithiopyridyl)-2-(5'-azidosalicylamido)ethane (ASDPE), was synthesized. This probe can be easily labeled with ^{125}I in the azidosalicylamido ring and contains an activated disulfide bridge. After reaction of [^{125}I]ASDPE with proteins, the radiolabeled moiety of the probe becomes attached to cysteine residues. Upon partial reduction of human growth hormone (hGH) with dithiothreitol, its C-terminal disulfide bond between residues 182 and 189 was cleaved and the nascent thiol groups were modified with [^{125}I]ASDPE to yield [^{125}I]ASET–hGH [1-(thio-hGH)–2-(3'-[^{125}I]iodo-5'-azidosalicylamido)ethane]. After binding of this hormone derivative to rat liver microsomes, followed by photolysis and subsequent reduction of disulfide bridges, the specific transfer of the radiolabeled moiety to prolactin receptor (PRL-R) was achieved. Partial purification of the radiolabeled receptor by size exclusion chromatography was performed. We anticipate that [^{125}I]ASDPE will be generally useful in pursuing structural and functional studies of target proteins which interact specifically with protein ligands.

INTRODUCTION

After their introduction as photoaffinity reagents in 1969 (1), aryl azides became the most commonly used photoreactive compounds in biochemistry, mostly due to the simplicity of their synthesis (2). Cleavability of the reagent is an additional advantage, because it allows further analysis of the target of the cross-link by separating the photoreacted (and radiolabeled) head, which remains attached to the acceptor molecule, from the rest of the ligand. Certain photoactivatable probes of the azidosalicylamido kind, which may be cleaved by reduction of a disulfide bridge, have been employed to transfer a radioactive label between peptidic species (3, 4). This technique shows great promise for both identification and purification of ligand-binding proteins and further study of the binding site by fragmentation and peptide analysis. Herein, we synthesized and characterized ASDPE,¹ a new photoreagent useful for these purposes.

Rat liver PRL-R consists of a single polypeptide chain with an M_r of 42K. This species, by far the most abundant PRL-R form present in this tissue, has been the subject of previous cross-linking studies (5, 6). At present, a complete view of the transduction pathways following the initial step of binding of a lactogenic hormone to the PRL-R still has to be elucidated. In this work, we demonstrated the transfer of a radiolabel from hGH, a natural ligand of PRL-R, derivatized with [^{125}I]ASDPE to this receptor. This opens the possibility of

studying modifications of the PRL-R occurring along its intracellular transit, including degradation and/or phosphorylation. In addition, a receptor radiolabeled in this way could serve as a tracer in pursuing its purification and in studying the association with other membrane or intracellular molecules.

MATERIALS AND METHODS

General Procedures. The ^1H NMR spectra were recorded on a Bruker WM 300 NMR spectrometer, FTIR spectra on a Bruker IFS 25 infrared spectrometer, mass spectra on a Shimadzu GCMS QP-1000 mass spectrometer, and UV/visible spectra on a JASCO 7850 spectrophotometer. Thin layer chromatography (TLC) was routinely performed on 5.0 cm $\times$ 1.6 cm plates of silica gel 60 F₂₅₄ (E. Merck, Darmstadt, Germany). Different solvent systems were employed: solvent 1, benzene/acetic acid (9:1, v/v); solvent 2, tetrahydrofuran/ethanol/acetic acid (4.5:4.5:1); and solvent 3, dichloromethane/ethanol/acetic acid (6:3:1). All reagents employed were of analytical grade and were purchased from Aldrich, Sigma, or Fluka. Na ^{125}I (15–17 Ci/mg) was purchased from New England Nuclear (Boston, MA). Samples were irradiated at 254 nm in quartz cuvettes or in a Petri dish with a Desaga UVIS setup 3 cm from the lamp for the times indicated.

Synthesis and Characterization of Compounds. **5-Azidosalicylic Acid (3).** 5-Aminosalicylic acid (**1**, 200 mg, 1.3 mmol) was dissolved in 7.5 mL of 0.94 M H₂SO₄ and the mixture cooled to 4 °C, and NaNO₂ (110 mg, 1.6 mmol) dissolved in 1 mL of distilled water was then added. The reaction mixture was gently shaken at 0 °C for 20–30 min. The resulting diazonium salt (**2**) was not isolated. NaN₃ (420 mg, 6.5 mmol) dissolved in 10 mL distilled water was added next. From this step on, care was taken to avoid direct sunlight; thus, the following synthetic procedures were performed under dim yellow light. The reaction was allowed to proceed for 48 h at 4

* To whom correspondence should be addressed: IQUIFIB (UBA-CONICET), Facultad de Farmacia y Bioquímica, Junín 956, 1113 Buenos Aires, Argentina. Fax: (54-1) 962-5457. E-mail: rtdelfin@criba.edu.ar.

† Died August 2, 1995.

¹ Abbreviations: ASDPE, 1-(2'-dithiopyridyl)-2-(5'-azidosalicylamido)ethane; PRL-R, prolactin receptor; hGH, human growth hormone; [^{125}I]ASET–hGH, 1-(thio-hGH)–2-(3'-[^{125}I]iodo-5'-azidosalicylamido)ethane.

°C with gentle stirring. After the test for diazonium salt turned negative, compound **3** was filtered and purified by recrystallization from ethanol. After 24 h at 4 °C, the crystalline material was filtered and dried. The yield of product was 67%. TLC (silica, solvent 1): $R_f = 0.34$. MS (EI, 70 eV): m/e (relative intensity) 180 (4.9, $M + 1$), 179 (4.6, M), 152 (3.2, $M + 1 - N_2$), 151 (3.3, $M - N_2$). UV (1 mM HCl in methanol): λ (nm) 371 (min), 330 (max), 290 (min), 257 (max).

1-(2'-Dithiopyridyl)-2-aminoethane (6). 2,2'-Dithiodipyridine (200 mg, 0.91 mmol) was dissolved in 0.5 mL of 10% (v/v) acetic acid in ethanol, and 2-aminoethanethiol hydrochloride (**5**, 50 mg, 0.44 mmol) dissolved in 1 mL of ethanol was then added. The reaction mixture was incubated at 25 °C for 16–24 h under a nitrogen atmosphere. At the end, the mixture was centrifuged and the clear supernatant was dried under vacuum and dissolved in 0.25 mL of methanol. The product **6** was precipitated by the addition of 0.5–0.75 mL of diethyl ether and left overnight at –20 °C. After filtration and drying under vacuum, large prismatic crystals of compound **6** were obtained. Further purification was achieved by an additional recrystallization step from the same solvent mixture. The yield of pure product was 55%. TLC (silica, solvent 2): $R_f = 0.57$. MS (EI, 70 eV): m/e (relative intensity) 157 (5.5), 143 (16.4), 112 (6.3), 111 (34.2).

1-(2'-Dithiopyridyl)-2-(5'-azidosalicylamido)ethane (ASDPE, 7). A fresh batch of ASDPE can be synthesized starting from compounds **3** and **6**, which can be stored for months in the dark at 4 °C. Compound **3** (40 mg, 0.22 mmol) was dissolved in 0.35 mL of tetrahydrofuran while the mixture was gently shaken. To this solution were added *N*-hydroxysuccinimide (27 mg, 0.24 mmol) and dicyclohexylcarbodiimide (50 mg, 0.24 mmol), and the reaction mixture was stirred for 24 h at room temperature. At the end, the precipitate was separated by filtration or centrifugation, and to the clear supernatant containing the *N*-hydroxysuccinimidyl ester of 5-azidosalicylic acid (**4**) were added pyridine (20 μ L, 0.25 mmol) and a solution of compound **6** (49 mg, 0.22 mmol). The reaction was allowed to proceed under a nitrogen atmosphere while the mixture was gently shaken for 1 week at 25 °C. The progress of the reaction was followed by TLC using solvent 3. The workup procedure was as follows. The mixture was centrifuged, and the supernatant was separated. The remaining residue was washed with 0.4 mL of tetrahydrofuran, and this solution was pooled with the supernatant. Finally, the solvent was evaporated under reduced pressure. Purification was achieved by column silica gel chromatography employing straight chloroform as the solvent. Fractions (2–3 mL) were collected and analyzed by TLC; those containing compound **7** were pooled together, and the solvent was evaporated under reduced pressure. The yield of pure product was 91%. TLC (silica, solvent 3): $R_f = 0.95$. ^1H NMR (CDCl_3 , 300 MHz): δ 3.22 (m, 2H), 3.98 (m, 2H), 7.23–7.68 (complex, 5H), 7.85 (m, 1H), 8.74 (m, 1H), 9.28 (s broad, 1H), 12.53 (s broad, 1H). ^1H NMR (CDCl_3 and D_2O): δ 3.22 (t, $J = 5.4$ Hz, 2H), 3.97 (t, $J = 5.4$ Hz, 2H), 7.23–7.68 (complex, 5H), 7.85 (m, 1H), 8.73 (m, 1H). FTIR (film): wavenumber (cm^{-1}) 3337 (br, m), 2115 (s), 1643 (m), 1597 (m), 1577 (m), 1558 (m), 1492 (m), 1418 (s), 1291 (m). After photolysis in a chloroform solution, the bands at 2115 and 1291 cm^{-1} diminished significantly. MS (EI, 70 eV): m/e (relative intensity) 319 (4.1, $M - N_2$), 176 (3.2), 135 (3.8), 134 (2.9), 112 (4.8), 111 (26.5), 79 (5.3), 78 (8.2). UV (ethanol): λ (nm) 338 (max), 312 (min), 275 (sh), 233 (max). Changes that occurred

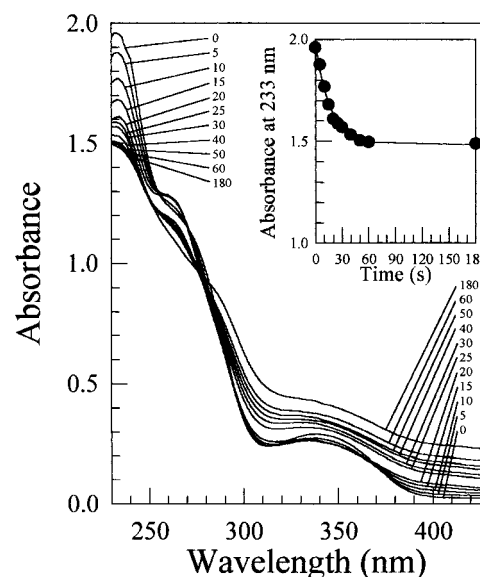


Figure 1. Time course of photolysis of ASDPE. UV spectra of compound **7** ($t = 0$ s) and the photolysis reaction mixture at the times (in seconds) indicated with numbers. The sample was irradiated at 254 nm in ethanol. The inset shows the decay of the absorbance at 233 nm as a function of the time of photolysis. The data were adjusted with the equation $A = A_1 \exp(-kt) + A_2$, where A_1 is the difference between the absorbance values at 233 nm for $t = 0$ and $t = \infty$, A_2 is the absorbance at $t = \infty$, and k is the apparent rate constant for the decay in absorbance where $A_1 = 0.496 \pm 0.015$, $A_2 = 1.483 \pm 0.010$, and $k = 0.060 \pm 0.004 \text{ s}^{-1}$.

upon photolysis of this compound (Figure 1) are discussed in the Results and Discussion.

1-(2'-Dithiopyridyl)-2-(3'-[^{125}I]iodo-5'-azidosalicylamido)ethane ([^{125}I]ASDPE, 8). ASDPE (2 μ L of a 2.5 mM solution in CHCl_3) was dried in the bottom of a 3 mm $\times$ 40 mm tube under a nitrogen stream, and 10 μ L of a 0.3 M sodium phosphate buffer (pH 7.4), 0.5 mCi of Na^{125}I (1.5 μ L), 2 μ L of distilled water, and 2 μ L of 1.2 mg/mL chloramine T were successively added. The mixture was stirred, and the reaction was allowed to proceed for 15 min. Then, 2 μ L of dimethylformamide and 30 μ L of benzene were added. After vigorous shaking and centrifugation, the organic phase containing the product (**8**) was separated carefully and dried under a nitrogen stream. The solid residue was dissolved in 100 μ L of anhydrous ethanol. There was no need for purification, since an autoradiography of a TLC analysis of the reaction mixture showed a single spot (**8**) with a mobility similar to that of unlabeled ASDPE. The radioiodinated probe (35 Ci/mmol) was used immediately for the derivatization of hGH.

Modification of hGH with [^{125}I]ASDPE. hGH (200 μ g) was dissolved in 200 μ L of 10 mM sodium phosphate buffer (pH 8.4), and 2 μ L of 25 mM dithiothreitol was added under an inert atmosphere. After 40 min at room temperature, the reaction mixture was sampled on a Sephadex G-100 column ($V_t = 0.9$ mL) using 10 mM sodium phosphate buffer (pH 7.0) and 0.01 mM EDTA as the eluent. Fractions of 200 μ L were collected under nitrogen. A sample of the fraction (50 μ L) containing reduced hGH was tested for the content of thiol groups by estimating the absorbance at 343 nm after (i) the addition of 5 μ L of 5 mM 2,2'-dithiodipyridine, (ii) incubation for 15–20 min, and (iii) dilution with water to a 600 μ L final volume. The remaining partially reduced hGH (150 μ L) was reacted with iodoacetamide (5.5 μ L of a 1 mM solution, equivalent to a 0.5-fold molar

content of thiol groups) over the course of 30 min at room temperature. The resulting partially reduced and blocked hGH was modified with [^{125} I]ASDPE as follows. The probe (100 μ L of the ethanolic solution) was dried under a nitrogen stream; the residue was dissolved in anhydrous ethanol (1 μ L) and propylene glycol (1 μ L), and the hGH solution (10 μ L) was then added. After being carefully shaken, the mixture was incubated at room temperature for 1 h under nitrogen. Next, 60 μ L of iodoacetamide-treated BSA [1 mg/mL BSA in 10 mM sodium phosphate buffer (pH 7.0) and 0.01 mM EDTA, reacted with a 0.05 mM iodoacetamide solution] was added. Finally, [^{125} I]ASET-hGH was purified by gel filtration on a Sephadex G-100 column (V_i = 100 mL) equilibrated with 10 mM sodium phosphate buffer (pH 6.0) and 0.05 mM EDTA. To minimize radiolysis and/or reduction, this photoderivative was prepared immediately before use. For the purpose of characterization, ASET-hGH was synthesized from unlabeled ASDPE and hGH in the same fashion. UV (25 mM NaHCO₃, pH 8.0): λ (nm) 351 (max), 311 (min), 270 (max), 257 (min).

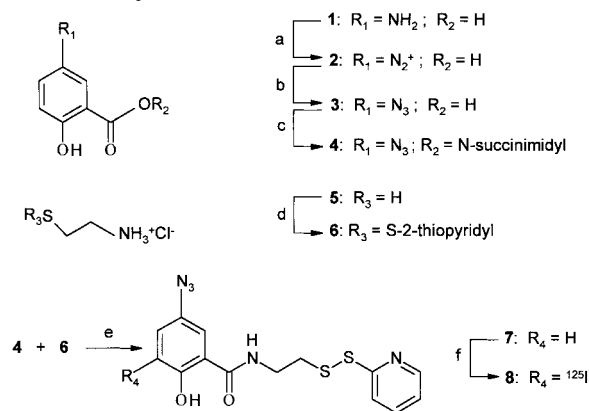
Binding of [^{125} I]ASET-hGH to Rat Liver Mitochondria. Mitochondria (1.2 mg of protein), prepared as described by Bonifacino et al. (7), were incubated in flat-bottom plastic tubes in the presence of 3.5 ng of [^{125} I]ASET-hGH (approximately 9×10^4 cpm) in a total volume of 160 μ L with or without a 1000-fold molar excess of unlabeled hGH. Samples were run in triplicate. The incubation medium was 25 mM Tris-HCl, 10 mM CaCl₂, 10 mM MgCl₂, and 0.25 mg/mL BSA (pH 7.5). Samples were incubated in the dark for 3 h at 25 $^{\circ}$ C. Binding was stopped by addition of 0.5 mL of cold buffer, and the suspension was then irradiated at 254 nm for 6 min while it was gently shaken and finally centrifuged for 5 min at 2500g. The resulting pellet was suspended in 0.5–1 mL of buffer and kept at -20° C until it was used. The protein content was evaluated (8) after boiling a sample for 20 min in 1 M NaOH.

Partial Purification of PRL-R after Radiolabel Transfer with [^{125} I]ASET-hGH. Mitochondria (0.5 mL, 0.9 mg of protein) bound to [^{125} I]ASET-hGH were centrifuged, and the pellet obtained was dissolved in 0.5 mL of 42 mM Tris-HCl buffer (pH 7.0), 1.8% (w/v) SDS, 3 mM EDTA, and 0.8% (v/v) 2-mercaptoethanol. The mixture was incubated overnight at room temperature and then applied to a Sephadex G-100 column (V_i = 14 mL), equilibrated with 3.5 mM Tris-HCl buffer (pH 7.0), 0.15% (w/v) SDS, and 0.25 mM EDTA. Fractions (220 μ L) were collected, freeze-dried, and analyzed by SDS-PAGE.

Gel Electrophoresis and Autoradiography. Samples (0.10–0.15 mg of protein) were boiled at 100 $^{\circ}$ C for 3 min in the presence of sample buffer [1.5% (w/v) SDS, 5% (v/v) glycerol, 25 mM EDTA, and 35 mM Tris-HCl buffer (pH 7.3)] and 5% (v/v) 2-mercaptoethanol. Proteins were separated by SDS-PAGE on 6.5 to 12% T [1.5% C and 0.2% (w/v) SDS] gradient gels (9). Gels were then stained with Amido Black, destained, and dried. Radioactive bands were visualized by exposure of gels on Kodak X-Omat XAR-5 films at -70° C for 5–20 days with the aid of intensifying screens.

Western Blot Analysis. After SDS-PAGE [10% T, 1.5% C, and 0.1% (w/v) SDS], rat liver mitochondria were blotted onto a nitrocellulose membrane for 2 h at 100 V in 25 mM Tris and 193 mM glycine buffer with 20% (v/v) methanol. After being blocked for 2–3 h with 4% (w/v) dry fat-free milk in buffer [PBS and 0.1% (v/v) Tween 20 (pH 7.3)], the nitrocellulose membrane was rinsed four times for 10 min with buffer and incubated overnight at

Scheme 1. Synthesis of [^{125} I]ASDPE^a



^a (a) NaNO₂, aqueous H₂SO₄, 0 $^{\circ}$ C; (b) NaN₃, aqueous H₂SO₄, 4 $^{\circ}$ C; (c) N-hydroxysuccinimide, dicyclohexylcarbodiimide, tetrahydrofuran, 25 $^{\circ}$ C; (d) 2,2'-thiopyridine, acetic acid/ethanol, 25 $^{\circ}$ C; (e) pyridine/tetrahydrofuran, 25 $^{\circ}$ C; (f) Na¹²⁵I, chloramine T, sodium phosphate buffer, pH 7.4.

room temperature with T6 mouse IgG anti-PRL-R mAb (4 g/mL, purchased from Affinity Bioreagents, Golden, CO) (10) in buffer with 1% (w/v) BSA. Next, the membrane was rinsed four times for 15 min with buffer and incubated for 1 h with a donkey anti-immunoglobulin G antibody [1/5000 dilution in buffer and 2% (w/v) BSA]. Finally, the membrane was rinsed in PBS and revealed by an enzymic chemiluminescent assay (ECL, Amersham International).

RESULTS AND DISCUSSION

Among the wide variety of photoactivatable reagents available, only a relatively limited set has the added capability of carrying a radioactive label and of transferring it between molecules. Herein, we describe the synthesis and use of the novel photoactivatable probe ASDPE (7, Scheme 1). ASDPE includes the photoreactive azidosalicylamido headgroup at one end and the thiol-reactive function dithiopyridine at the other end. The linkage between these moieties is accomplished through a short ethylene bridge. ASDPE can be radiolabeled with ¹²⁵I to yield [^{125} I]ASDPE (8, Scheme). In turn, this compound is reacted with natural cysteine residues present in proteins or those introduced by site-directed mutagenesis, to yield a stable disulfide bridge. Next, the derivatized protein interacts with its target molecule, and upon photolysis, the azido group generates a highly reactive nitrene species which inserts readily into different functional groups present in the environment around the headgroup. At variance with other reagents of this class (3, 4, 11), it is noteworthy that the phenol group para with respect to the azido group orients the substitution of the bulky ¹²⁵I in meta, thus minimizing steric hindrance in the addition reactions of the nitrene. The expectation is that this feature will result in improved yields of photoadduct.

A convergent strategy for the synthesis of ASDPE is described in Scheme 1. The starting materials were 5-aminosalicylic acid (1) and mercaptoethylamine (5). We attempted to optimize yields at each step to avoid unnecessary purifications whenever possible. For analytical purposes, a small sample of each compound was routinely purified before the final characterization was carried out. The azido group was built in place of the amino substituent in 1 through a diazonium salt intermediate 2. On the other hand, 5 was reacted with 2,2'-

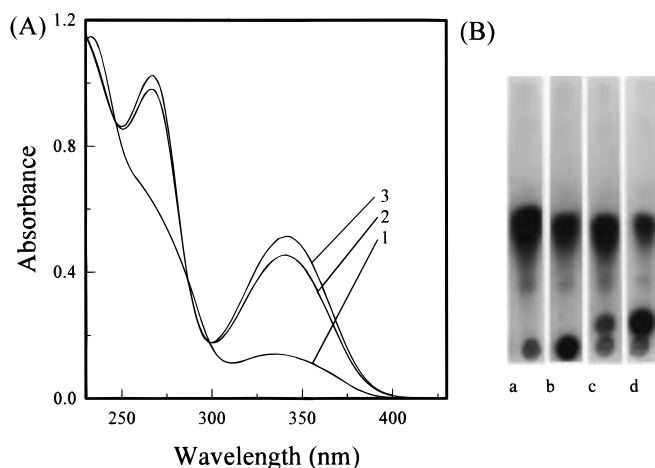


Figure 2. Reductive cleavage of ASDPE. (A) UV spectrum of a 0.05 mM solution of ASDPE in 5 mM sodium phosphate buffer (pH 7.4) (curve 1). To this solution was added 2-mercaptoethanol (1 mM final concentration), and the scanning of the spectrum was repeated after 1 min (curve 2) and 16 min (curve 3). (B) Autoradiography of a thin layer chromatogram (silica, chloroform) of a 0.2 mM solution of [^{125} I]ASDPE in ethanol ($R_f = 0.35$, lane a) and its reaction mixtures with 0.2 mM 2-mercaptoacetic acid ($R_f = 0.0$, lane b) or 0.2 or 2 mM 2-mercaptoethanol ($R_f < 0.1$, lanes c and d, respectively).

dithiodipyridine and the resulting amine **6** was coupled to 5-azidosalicylic acid (**3**), conveniently activated as the *N*-hydroxysuccinimidyl ester **4**, to yield ASDPE (**7**). The reagent, as a dry powder, is stable in the dark for months. Finally, an efficient protocol, involving a two-phase reaction, for radiolabeling ASDPE was developed to yield **8**. Thus, our reagent is able to withstand the oxidative conditions of this reaction medium with little or no appearance of byproducts (Figure 2B, lane a).

The UV spectrum of ASDPE (Figure 1) showed characteristic absorption bands with maxima at 233 and 338 nm and a shoulder at 275 nm, all these transitions related to the azido group (2). Upon irradiation, the first band decayed exponentially with a half-life of 11.6 ± 0.8 s in our photolysis setup (Figure 1, inset), while there were lesser changes in the region of 250–290 nm. Although there was a concomitant decrease of the band at 338 nm, accurate measurement in this spectral region was hampered by the appearance of UV-absorbing byproducts of the photolysis at longer irradiation times (>20 s). This is consistent with the lack of a clear isosbestic point at around 280 nm. The FTIR bands at 2115 and 1291 cm^{-1} , characteristic of the azido group (12), disappeared after photolysis (not shown).

The reactivity of ASDPE with thiol groups was tested against selected mercaptans. The reaction of ASDPE with 2-mercaptoethanol produced an almost immediate (<1 min) increase in the absorbance at 342 nm (Figure 2A), corresponding to the generation of thiopyridone. This arises as a consequence of the nucleophilic attack of the thiol group on the disulfide bridge of the probe. This reaction was routinely performed as a control assay for the integrity of the disulfide bond in the reagent, prior to the radioiodination step. The TLC analysis of the reaction mixture of [^{125} I]ASDPE and 2-mercaptoacetic acid (equimolar amount) or 2-mercaptoethanol (equimolar and 10-fold excess amounts) allowed the direct determination of the mixed disulfide products (Figure 2B). The appearance of new spots of lower mobility, consistent with the higher polarity of the parent thiol compounds, was evident, an indication of the generation of stable disulfides.

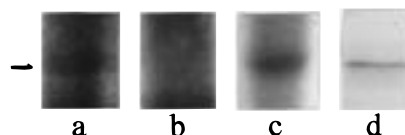


Figure 3. Radiolabel transfer from [^{125} I]ASET-hGH to rat liver PRL-R as analyzed by SDS-PAGE and autoradiography. Microsomes (1.2 mg of protein) were incubated with 1×10^{-9} M [^{125} I]ASET-hGH in the absence (lane a) or in the presence of a 600-fold molar excess of unlabeled hGH (lane b), as described in Materials and Methods. The values of radioactivity bound to microsomes were 37.4 and 12.1%, respectively. After photolysis, transfer of radioactive label occurred when, prior to electrophoresis, microsomes were incubated with sample buffer combined with 2-mercaptoethanol. Partially purified PRL-R from a sample of microsomal proteins reacted with [^{125} I]ASET-hGH and further reduced before gel filtration through Sephadex G-100 is shown (lane c). Western blot analysis of rat liver microsomal proteins incubated with an anti-PRL-R mAb (T6) is also shown (lane d). See Materials and Methods for details. The bar on the left indicates the 42 kDa band.

A suitable target protein for modification with [^{125} I]-ASDPE was partially reduced hGH, which yields [^{125} I]-ASET-hGH. Under the conditions described in Materials and Methods, reduction of the C-terminal disulfide bridge between residues Cys 182 and Cys 189 takes place. In addition, treatment of this sample with an equimolar amount of iodoacetamide with respect to hGH, prior to modification with [^{125} I]ASDPE, blocked on average one of these nascent thiol groups. The purpose of this was to minimize restoration of the disulfide link between the Cys residues, and therefore increase the stability of the resulting photoderivative. In addition to the absorption bands characteristic of the protein chromophores, the UV spectrum of purified ASET-hGH had an absorption band with a maximum at 351 nm, indicative of the presence of the azidosalicylamido group (results not shown).

The new photoderivative presented herein, i.e., [^{125} I]ASET-hGH, was successfully cross-linked, and able to transfer, after reduction, the radioactive label to PRL-R present in rat liver microsomes (42 kDa band in Figure 3, lane a). This band disappeared when the photoderivative was incubated in the presence of a 600-fold molar excess of unlabeled hGH (lane b), indicating that the interaction between these proteins is specific. After solubilization of microsomes, purification of PRL-R thus labeled was achieved by gel filtration. The analysis by SDS-PAGE of pooled fractions containing the receptor showed a single band corresponding to a labeled protein of 42 kDa (lane c). Its identity was assessed by Western blot analysis and reaction with the anti-PRL-R monoclonal antibody T6 (lane d).

Consistent with results presented herein, a noncleavable photoderivative of hGH, involving the modification of the same cysteine residues, namely, azidophenacyl-hGH ([^{125} I]AP-hGH, 21 kDa), was capable of cross-linking specifically to PRL-R in rat liver membranes to yield a 63 kDa adduct (13) as well as to a receptor-associated protein (14). To pursue future studies on the supramolecular structure of PRL-R, we are currently preparing a new photoderivative starting from a single cysteine mutant of hGH (C182S-hGH).

The novel photoactivatable reagent ASDPE can be easily synthesized in very good yield. Furthermore, it is radioiodinated at a high specific activity in a straightforward manner and can be linked to free thiol groups conveniently placed at selected locations in proteins. Cross-linking and transfer of the radioactive label to a specific target molecule make it possible to trace it along its purification. A related reagent, [^{125} I]-*N*-[4-(*p*-azido-

salicylamido)butyl]-3'-(2'-pyridyldithio)propionamide ([¹²⁵I]-APDP), was tested before as a valuable tool in the characterization of neighboring proteins in a complex such as the *Escherichia coli* ribosome (4, 15, 16). This molecule exhibits a cross-linking length of 21.1 Å in its fully extended form (4), comparable to the radius of an average protein (for hGH, this value is around 23 Å). Conceivably, the capabilities of ASDPE extend beyond its use in tagging the target protein after radiolabel transfer, including the potential for a detailed analysis of protein-protein interfaces. Given its short span (the distance between the Cβ of the modified cysteine and the proximal nitrogen of the azido group is 10.4 Å), this reagent may enhance the geometrical resolution around the site of the cross-link. Such analysis would involve radiolabel transfer, subsequent fragmentation of the target protein, and microsequencing of the resulting peptides.

In conclusion, we believe that ASDPE presents useful traits which complement the set of known photoactivatable cross-linking reagents.

ACKNOWLEDGMENT

This work was supported by grants from CONICET, Fundación Antorchas, the European Economic Community, and the University of Buenos Aires (Buenos Aires, Argentina). We are specially indebted to M. Micucci, S. Longhi, K. Gomez, and L. Retegui for their experimental contributions and to M. Kogan for interpretation of mass spectra.

LITERATURE CITED

- (1) Fleet, G., Porter, R., and Knowles, J. (1969) Affinity labelling of antibodies with aryl nitrene as reactive group. *Nature* 224, 511–512.
- (2) Bayley, H. (1983) in *Photogenerated Reagents in Biochemistry and Molecular Biology* (T. S. Work and R. H. Burdon, Eds.) pp 29 and 31, Elsevier Science Publishers, Amsterdam.
- (3) Sorensen, P., Farber, N., and Krystal, G. (1986) Identification of the Interleukin-3-receptor using an iodinated, cleavable, photoreactive cross-linking agent. *J. Biol. Chem.* 261, 9094–9097.
- (4) Zecherle, G., Oleinikov, A., and Traut, R. (1992) The C-terminal domain of *Escherichia coli* ribosomal protein L7/L12 can occupy a location near the factor-binding domain of the 50S subunit as shown by cross-linking with N-[4-(p-azidosalicylamido)butyl]-3-(2'-pyridyldithio)propionamide. *Biochemistry* 31, 9526–9532.
- (5) Caamaño, C., Fernández, H., and Paladini, A. (1983) Specificity of covalently stabilized complexes of ¹²⁵I-labeled human somatotropin and components of the lactogenic binding sites of rat liver. *Biochem. Biophys. Res. Commun.* 115, 29–37.
- (6) Borst, D., Sayare, M., and Posner, B. (1985) Characterization of the prolactin receptor in the cell fractions of rat liver. *Mol. Cell. Endocrinol.* 39, 125–130.
- (7) Bonifacino, J., Sánchez, S., and Paladini, A. (1981) Characterization of human somatotropin binding to detergent solubilized lactogenic receptors from rat liver. *Biochem. J.* 194, 385–394.
- (8) Lowry, O., Rosebrough, N., Farr, A., and Randall, R. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193, 265–275.
- (9) Laemmli, U. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680–685.
- (10) Okamura, H., Raguette, S., Bell, A., Gagnon, J., and Kelly, P. (1989) Purification and protein sequence analysis of rat liver prolactin receptor. *J. Biol. Chem.* 264, 5904–5911.
- (11) Ebright, Y., Chen, Y., Kim, Y., and Ebright, R. (1996) S-[2-(4-Azidosalicylamido)ethylthio]-2-thiopyridine: radioiodinatable, cleavable, photoactivatable cross-linking agent. *Bioconjugate Chem.* 7, 380–384.
- (12) Scriven, E. (1984) *Azides and Nitrenes, Reactivity and Utility*, p 96, Academic Press, Orlando, FL.
- (13) Robetto, E., Caamaño, C., Fernández, H., and Dellacha, J. (1989) Proteins associated with somatogenic and lactogenic receptors in microsomal membranes and intact hepatocytes. *Biochim. Biophys. Acta* 1013, 223–230.
- (14) Masckauchán, N., Delfino, J., and Fernández, H. (1998) A membrane protein associated with the prolactin receptor. Studies with a photoactivatable human growth hormone derivative. *Life Sci.* 62, 1069–1079.
- (15) Traut, R., Dey, D., Bochkariov, D., Oleinikov, A., Jokhadze, G., Hamman, B., and Jameson, D. (1995) Location and domain structure of *Escherichia coli* ribosomal protein L7/L12: site specific cysteine crosslinking and attachment of fluorescent probes. *Biochem. Cell. Biol.* 73, 949–958.
- (16) Dey, D., Bochkariov, D., Jokhadze, G., and Traut, R. (1998) Cross-linking of selected residues in the N- and C-terminal domains of *Escherichia coli* protein L7/L12 to other ribosomal proteins and the effect of elongation factor Tu. *J. Biol. Chem.* 273, 1670–1676.

BC970202T

REVIEWS

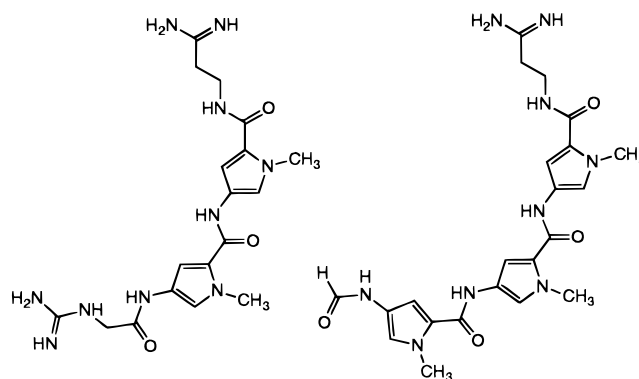
Sequence-Specific DNA Minor Groove Binders. Design and Synthesis of Netropsin and Distamycin Analogues

Christian Bailly^{*,†} and Jonathan B. Chaires[‡]

INSERM U-124 et Laboratoire de Pharmacologie Antitumorale Moléculaire du Centre Oscar Lambret, IRCL, Place de Verdun, 59045 Lille, France, and Department of Biochemistry, University of Mississippi Medical Center, 2500 North State Street, Jackson, Mississippi 39216-4505. Received January 13, 1998; Revised Manuscript Received May 5, 1998

Over the past two decades, impressive advances have occurred in the understanding of how a normal cell transforms into a tumor cell. Molecular and cellular studies have shown that point mutations, deletions, translocations, and other types of rearrangements in DNA affect either the expression or the biochemical function of specific genes, be it an oncogene or a tumor-suppressing gene. By designing a drug capable of recognizing a specific sequence in DNA, it may be possible to specifically inhibit the expression of certain oncogenes and thereby to control the development of tumor cells. The message provided by the molecular biologist is that specific DNA sequence changes can switch on the malignant transformation of a cell. The goal of the pharmacologist is to find a drug to switch it off.

The possibility that low molecular weight ligands might bind to specific sequences in DNA was raised long ago (1, 2). The antiviral antibiotics netropsin (Net) and distamycin (Dst) were arguably the first drugs discovered that bound selectively to AT-rich sequences in the minor groove of DNA. So far, >20 high-resolution structures obtained by NMR and X-ray crystallography have been reported for a variety of oligonucleotide sequences complexed with netropsin, distamycin, and related minor groove binders (Table 1) (3–19). Quantitative footprinting methods have been used to analyze the sequence



netropsin

distamycin

preference (20–25). Computational studies have contributed information that provides a rational explanation for the selective fit of these crescent-shaped ligands into the minor groove of DNA (26, 27). Thermodynamic studies of Net and Dst binding to DNA, and their effects on the formation of protein/DNA complexes, have also been thoroughly investigated (28–40). Since the pioneer discoveries of the AT-selective binding of Net and Dst to DNA (41), a considerable number of physicochemical, biochemical, and biological studies have been reported to increase our understanding of the details of the mechanisms by which these two antibiotics bind to and recognize double-stranded DNA sequence (42). This detailed information has been exploited by medicinal chem-

* Author to whom correspondence should be addressed [fax (+33) 320 16 92 29; e-mail bailly@lille.inserm.fr].

[†] INSERM U-124.

[‡] University of Mississippi Medical Center.

ists to develop molecules capable of reading specific DNA sequences and, most importantly, to establish molecular rules for the design of sequence recognition elements. Although studies are still in progress, it is already clear that the design of sequence-specific ligands promises to be one of the great success stories of pharmacology. A brief survey of the story is reviewed here. This review is mainly concerned with the different categories of netropsin and distamycin derivatives synthesized during the past 15 years.

1. GENERAL MECHANISM OF GROOVE BINDING

The general mechanism of groove binding is shown schematically in Figure 1. Conceptually, the binding process may be divided into at least two parts. First, the groove binding agent undergoes a hydrophobic transfer from solution into the DNA minor groove. If the groove binder is positively charged, this event will be accompanied by the release of condensed counterions that surround the DNA. Once in the minor groove, specific molecular interactions may then form, including van der Waals attraction and the formation of hydrogen bonds.

The detailed energetics of groove binding reactions have yet to be fully elucidated, but the following elements are minimal contributions that must be considered. The formation of any bimolecular complex contains a substantial unfavorable free energy contribution resulting from the loss of rotational and translational degrees of freedom as two reactants form a single complex. Other, energetically favorable, contributions must then balance and overwhelm this entropic cost of forming the complex. The favorable contribution from the hydrophobic transfer process to the free energy is expected to be large and may largely balance the entropic penalty. Smaller, but no less important, favorable contributions come from the polyelectrolyte contribution to the free energy, arising from counterion release and from the formation of the various noncovalent molecular interactions. Whereas such molecular interactions are usually very much the focus of high-resolution structural studies, it must always be kept firmly in mind that such interactions are the culmination of a complex, multistep reaction mechanism. The stability of the final complex arises from an often delicate balance of favorable and unfavorable free energy contributions at each step along the reaction pathway.

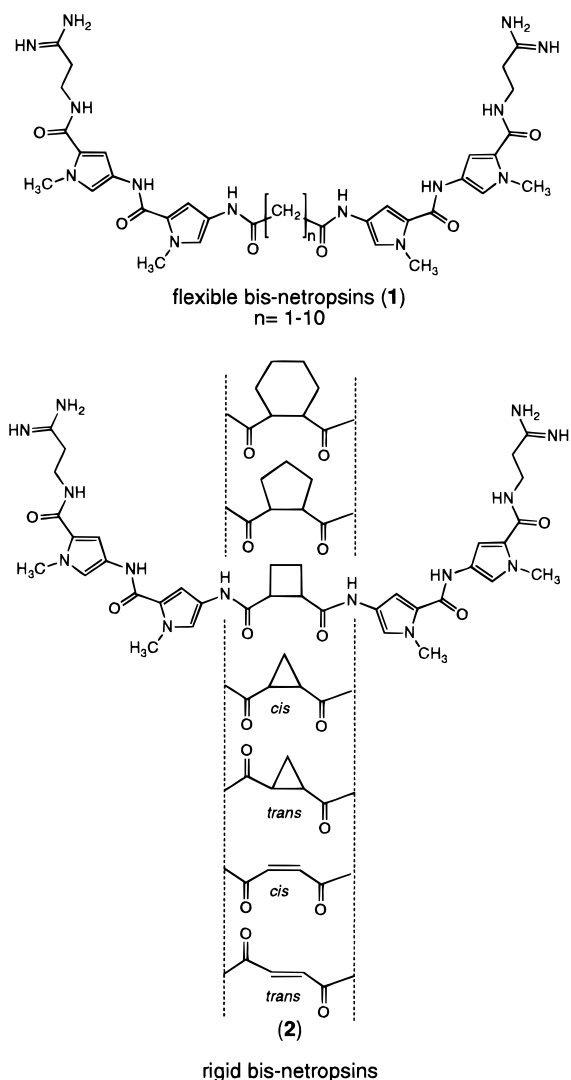
2. THE LEXITROPSINS: MONOMERS AND DIMERS

Three main factors are thought to contribute favorably to the stability between AT sequences and netropsin or distamycin: (i) hydrogen bonding between the amide NH of the drug and thymine O-2 and adenine N-3 atoms; (ii) an overall shape complementarity resulting in close ligand-DNA van der Waals contacts; and (iii) polyelectrolyte interactions between the polyanionic DNA and the cationic drugs. It is assumed that the van der Waals interactions are pivotal in the sequence recognition (43).

The first design strategy employed to generate new compounds was to extend the length of the groove binding agents in hopes of extending the length of the binding site. Tris, tetra, penta, and hexa *N*-methylpyrrolecarboxamide derivatives of Net and Dst can bind to sequences containing five, six, seven, and eight contiguous A•T base pairs, respectively. The binding site size for an analogue containing $n - 1$ pyrrole rings or n amide bonds is $n + 1$ base pairs long in terms of occluding base pairs (or n in terms of contacted base pairs) (44, 45). This rule is valid for analogues containing up to six *N*-methylpyrrolecarboxamide residues but not for longer

molecules. A hepta *N*-methylpyrrolecarboxamide derivative does not fit very well with the natural twist of the DNA, presumably because the molecule gets out of phase with the base pairs along the minor groove floor of the double helix. Indeed, the pyrrolecarboxamide unit is $\approx 20\%$ longer than required to match perfectly the base pair rise in the minor groove (46). Two alternative strategies have been envisaged to circumvent the poor phasing between the DNA and *N*-methylpyrrolecarboxamide-containing ligands.

The first consists of joining two netropsin or distamycin molecules by a linker of suitable length to permit bidentate binding to DNA. A considerable number of bis-(netropsin)s containing different types of linkers have been elaborated (47–51). Bis(netropsin)s coupled by a polymethylene tether (**1**) can engage in bidentate binding



providing that the connector contains at least three methylene groups. However, owing to the flexibility of the aliphatic connector, monodentate binding of such bis-(netropsin)s is also possible. In contrast, bis(netropsin)s possessing conformationally rigid linkers (**2**) can readily bind to sequences containing 8–10 consecutive AT base pairs without the unwanted monomer binding to shorter sequences (52, 53). For chiral bis(netropsin)s, the stereochemistry of the linker is critical and may be used for controlling the directionality in binding to DNA (54). Recently, two bis(distamycin) derivatives possessing a 3,5-*m*-pyridyl or a *trans*-vinyl linker were found to be the

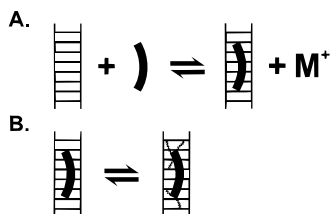
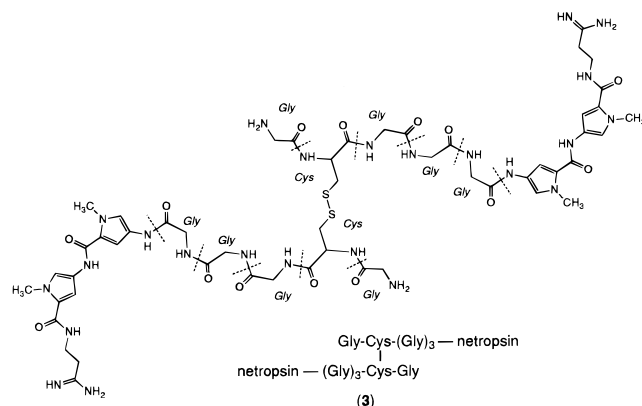


Figure 1. Schematic representation of hypothetical steps in the reaction pathway from DNA groove binding: (A) A groove binding agent is hydrophobically transferred from solution into the minor groove. If the ligand is positively charged, condensed counterions territorially bound to the DNA will be released. (B) Once in the minor groove, the ligand can form a variety of molecular interactions, including hydrogen bonds and van der Waals attractions.

Table 1. Structures of Oligonucleotide/Netropsin and/or Distamycin Complexes Determined to Atomic Resolution

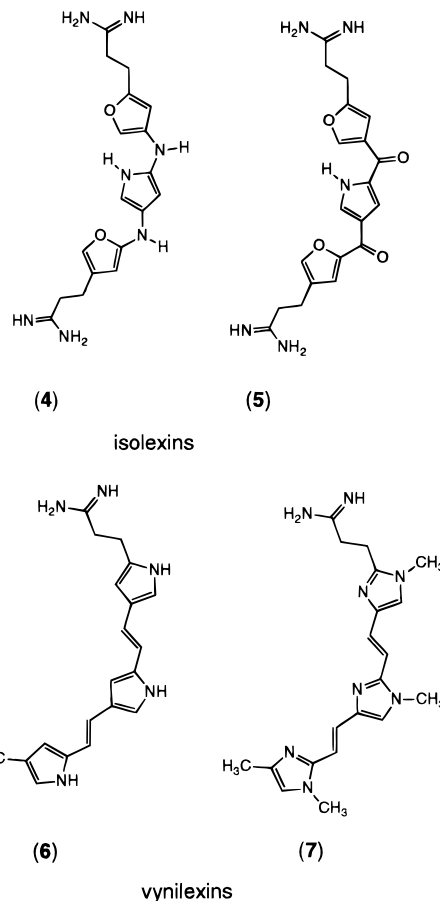
drug/DNA complex	reference
netropsin/d(CGCGAATTCGCG) ₂	3
netropsin/d(CGCGAATT ⁵ BrCGCG) ₂	4, 5
distamycin/d(CGCGAATTCGCG) ₂	6
distamycin/d(CGCAAATTTGCG) ₂	7
netropsin/d(CGCGATATCGCG) ₂	8
2 distamycin/d(CGCAAATTTGGC)	9, 10
netropsin/d(CGC[e ⁶ G]AATTCGCG)	11
netropsin/d(CGCAAATTTGCG) ₂	12
2 distamycin/d(CGCAAGTTGGC)	13
netropsin/d(CGCGAATTCGCG) ₂	14
distamycin/d(ICICICIC) ₂	15
distamycin/(ICICICIC) ₂	16
2 distamycin/d(ICITACIC) ₂	17
2 distamycin/d(ICATATIC) ₂	17

most effective bis-linked lexitropsins at inhibiting transcription by HIV-1 reverse transcriptase (55). The linker can also play an active role in the DNA recognition process. For example, the bis(netropsin) analogue **3**, in



which the two netropsin residues are connected by a disulfide bond between two Gly-Cys-Gly peptides, binds more strongly to sequences containing a central GC step such as (AT)₃(GC)₂(AT)₃ than to strictly homologous (AT)_n sequences (56, 57).

The second strategy consists of replacing the carboxamide bond in netropsin and distamycin with shorter keto or amino linkages. Molecular modeling predicts that isohelical molecules such as **4** and **5** (called isolexins) would bind more tightly in the minor groove than their corresponding carboxamide analogues (46, 58). As for the lexitropsins (see below), the use of imidazole or furan rings would favor recognition of GC sequences. In the same way, the computational studies predict that the replacement of the carboxamide bond of netropsin with an ethylene bond (compound **6**) would permit an optimum



fit to the minor groove surface (59). According to the computational measurements, molecules called vinyllexins containing both $-C=C-$ linkages and imidazole and/or furan heterocycles (e.g., compound **7**) would display a considerable preference for GC-rich sequences (60). Although these suggestions are very promising, so far neither the isolexin nor the vinyllexin strategy has been experimentally tested.

The guanine 2-amino group protrudes into the minor groove and obstructs the access of drugs to the floor of the groove. The fact that the 2-amino group constitutes a critical negative recognition element for binding of small molecules, including Net and Dst, in the minor groove of DNA has now been unambiguously demonstrated using DNA molecules in which that group has been either deleted from guanines and/or added to adenines (61–65). Given both the strategic position of the guanine 2-amino group in the minor groove and its hydrogen bonding capacity (it is the only H bond donor exposed in the minor groove), it was proposed that the introduction of an H bond acceptor heteroatom in the pyrrole rings of netropsin might permit the drug to bind to GC sequences (46). Lown and co-workers have extensively exploited this concept and synthesized an impressive number of molecules christened “lexitropsins” or information reading oligopeptides structurally related to netropsin and distamycin (66, 67). By substituting imidazole, thiazole, triazole, pyrazole, or oxazole heterocycles for the *N*-methylpyrrole rings of netropsin, one can design drugs capable of binding to sequences containing one or two G·C pairs embedded in an AT sequence (Figure 2). Among the numerous lexitropsins synthesized so far, imidazole lexitropsins such as compounds **8–10** display the most pronounced capacity for binding

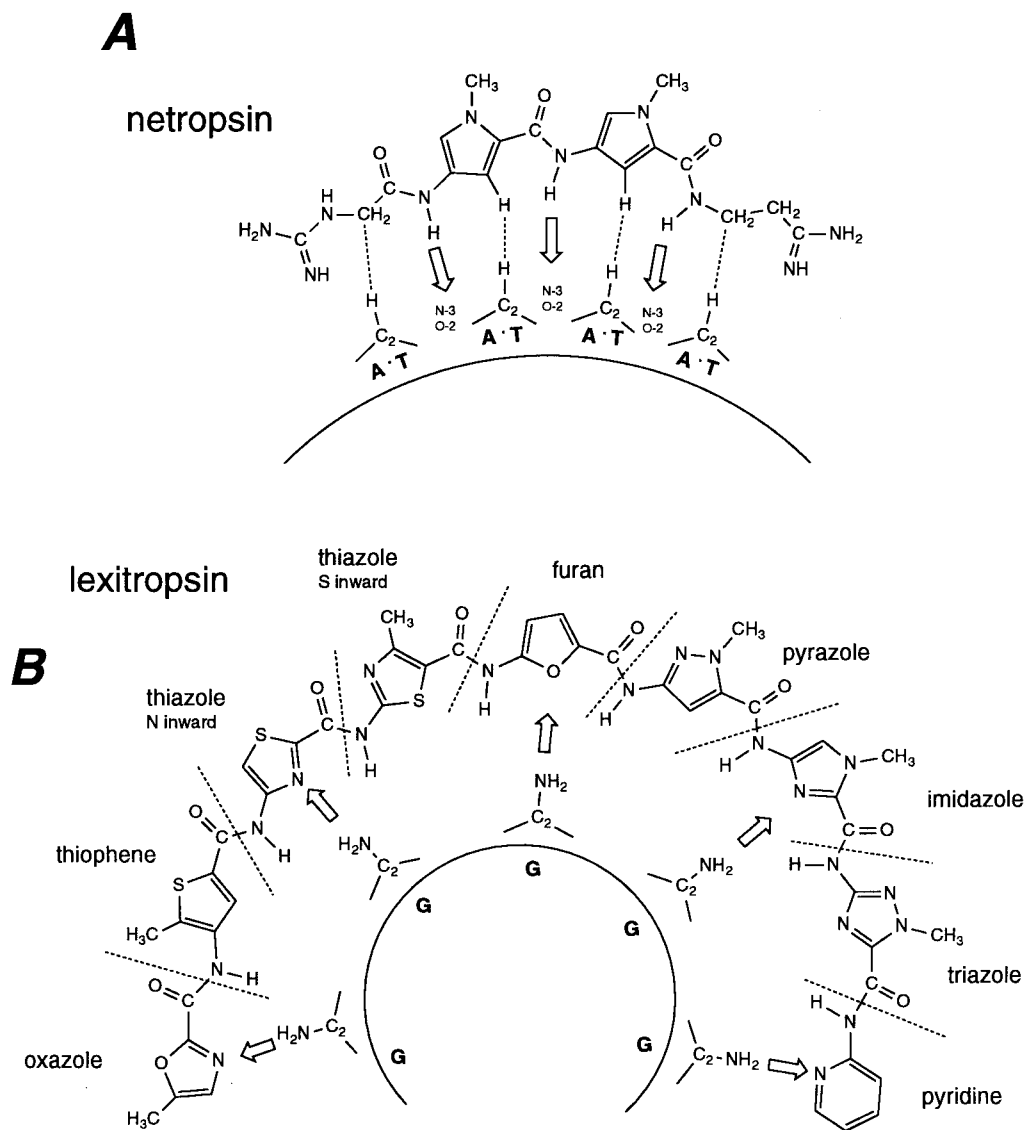
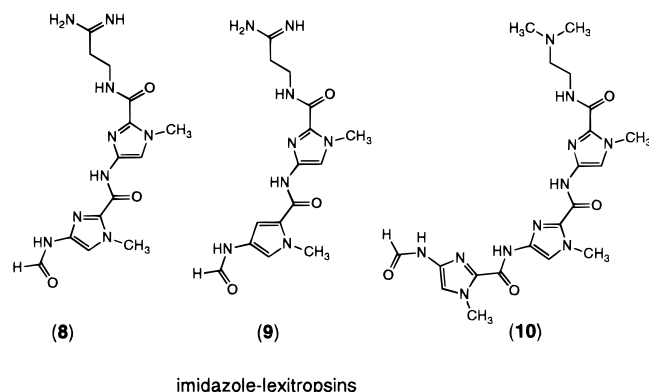
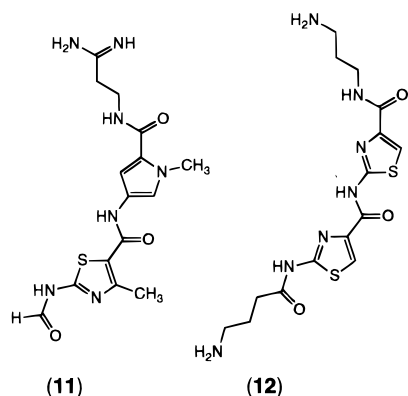


Figure 2. (A) Representation of the binding to DNA of netropsin; (B) proposed representation of a model lexitropsin molecule with guanine residues in DNA. Heavy arrows are hydrogen bonds, from donor to acceptor. Dashed lines mark close van der Waals nonbonded contacts between DNA and drug.

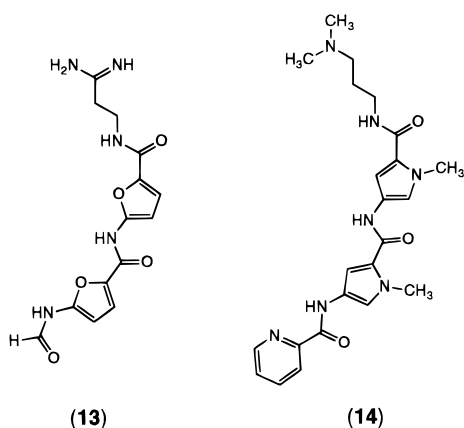


to GC-containing sequences (68–79). Thiazole lexitropsins either accept or avoid a G·C base pair in their binding sites, depending on the position of the sulfur atom (80–83). For example, the lexitropsin **11** with the sulfur atom directed into the minor groove does not bind to GC-containing sites, whereas the lexitropsin **12** containing two thiazole rings with the sulfur atoms directed outward from the minor groove binds best to alternating purine–pyrimidine sequences such as 5'-TATGAC and

5'-TGCATGC (84). The same type of result was obtained with the furan-containing lexitropsin **13** (85). To summarize, the lexitropsin approach based on a 1:1 complex has led to minor groove binders with increased tolerance for G·C base pairs in the binding site but did not lead to the design of a purely GC-specific molecule. According to computational studies, the observation that most lexitropsins can accommodate both AT- and GC-containing sequences comes in part from the fact that the electrostatic potential in the minor groove of AT-rich regions is very negative (86). The electrostatic interactions between AT sites and the positively charged end groups in the lexitropsins presumably provide the initial attraction. The binding of mono- and dicationic minor groove binders to AT-rich regions has a significant electrostatic component (87–89). However, the fact that neutral lexitropsins show the same interaction with AT sequences as the monocationic distamycin tripeptide argues against the dominant role of electrostatics in sequence selectivity (90). A recent comprehensive spectroscopic and thermodynamic study of the interaction of the minor groove binder Hoechst 33258 with the d(CG-CAAATTTGCG)₂ duplex suggested that the hydrophobic

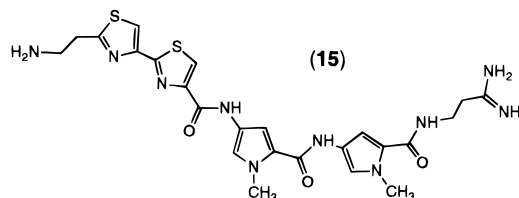


thiazole-lexitropsins



furan-lexitropsin

pyridine-lexitropsin

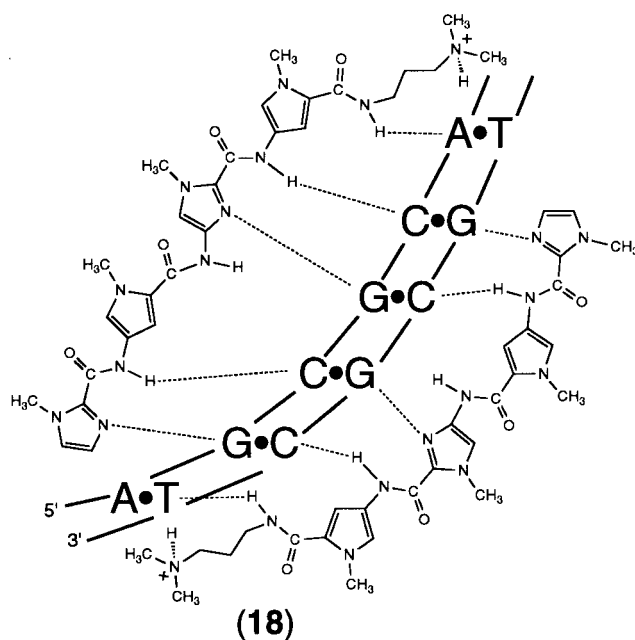
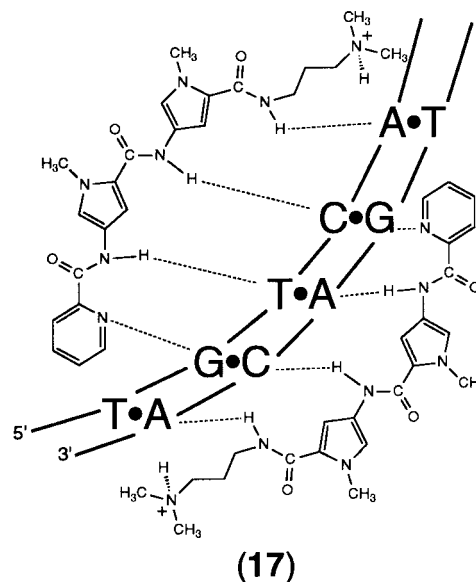
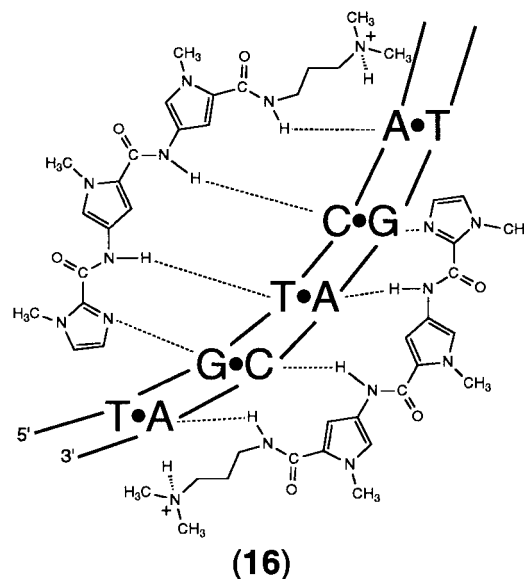


transfer of the drug from solution into the duplex binding site provides the major driving force for the binding reaction (91).

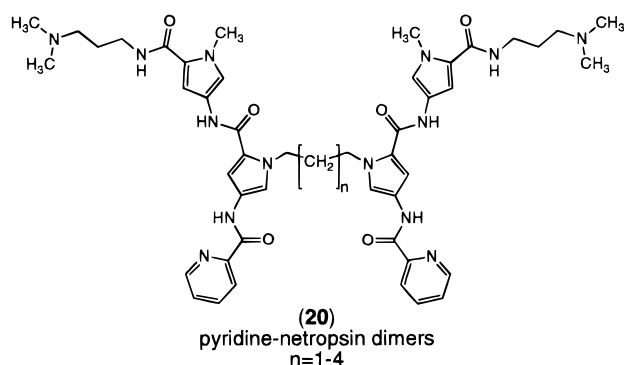
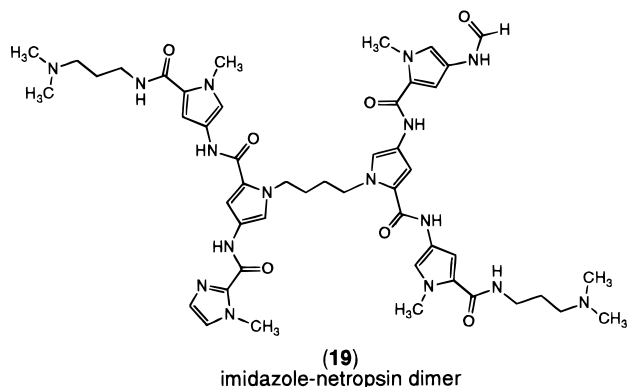
An alternative approach to design minor groove binders capable of binding GC pairs is to connect a netropsin-like molecule with GC-recognizing elements. For example, substitution of the terminal formamido-methylpyrrole group of distamycin for a pyridine group (compound **14**) permits interaction with a G•C base pair. However, as for the imidazole lexitropsin, the binding to pure AT sequences is not abolished (92, 93). The bithiazole moiety of the antitumor drug bleomycin has offered opportunities for binding to GC sites. The netropsin moiety of the conjugate **15** drives the drug to AT sites and allows the appended bithiazole unit to contact a pyrimidine-G-pyrimidine motif (94).

The discovery in 1990 that the minor groove of DNA can expand slightly to accommodate two distamycin molecules associated side-by-side in an antiparallel head-to-tail orientation has rekindled interest in the design of lexitropsin compounds (9, 10, 15–17, 95). The 2:1 drugs/DNA motif has been observed with different distamycin analogues including a carbamoyl tetrapyrrole derivative (96). This landmark discovery immediately inspired the design of homo- and heterodimeric ligands.

Imidazole- and pyridine-containing lexitropsins (compounds **16** and **17**) were combined side-by-side so as to

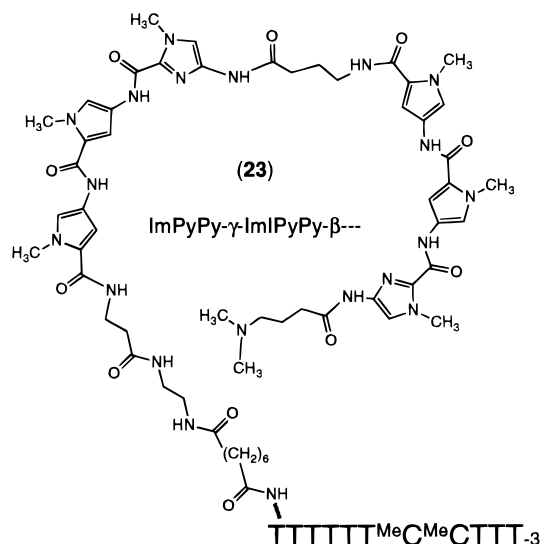
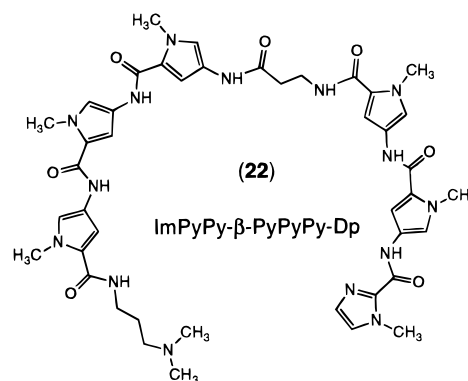
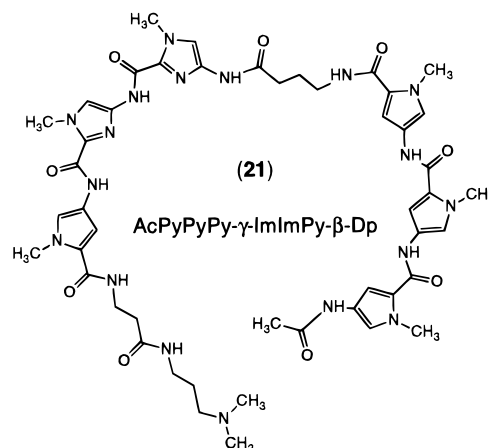


permit direct interaction with G•C pairs via hydrogen bonding with the 2-amino group of guanines (13, 97–99). The imidazole-containing homodimer (**18**) binds specifically to GCGC sequences. This was the first instance of a minor groove binding lexitropsin being directed uniquely to GC sites (100). Combination of side-by-side pyrrole and imidazole rings permits G•C base pairs to be differentiated from C•G base pairs (101). The sequence selectivity, affinity, and geometry of the drug/DNA 2:1 complex can be optimized by choosing appropriate pairs of ligand molecules with complementary recognition properties and by covalently linking the two DNA reading elements (compounds **19** and **20**) (102–111).



3. HAIRPIN POLYAMIDES: A TRIUMPH IN THE DESIGN OF SEQUENCE-SPECIFIC DNA MINOR GROOVE BINDERS

The ambitious goal of rationally designing ligands capable of binding tightly and specifically to any desired target sequence of double-stranded DNA has recently been realized. Hairpin polyamides that consist of two covalently linked lexitropsin molecules connected end-to-end via a γ -aminobutyric acid linker residue, offer promising possibilities for high-affinity specific recognition of a broad sequence repertoire in the minor groove of DNA (112–121). For examples, hairpin pyrrole–imidazole polyamides **21** and **22** bind specifically to 5'-(A,T)GG(A,T)₂ (112, 113) and 5'-AAAAAGACAAAAA (107), respectively. Hairpin polyamides can exhibit affinities and specificities for DNA comparable with transcription factors and other DNA binding regulatory proteins (122). These molecules, which can be built by solid-phase synthesis (123), can discriminate between GC-rich sequences such as GCGC and GGCC, for example (124, 125). The specificity is sufficiently stringent to target a 7 base pair sequence in the minor groove of DNA (126). Antiparallel pairing of an imidazole (Im) opposite a pyrrole (Py) residue recognizes a G•C base



pair, whereas a Py/Im combination recognizes a C•G pair (Figure 3) (127).

Discrimination of T•A versus A•T base pair was an obstacle because a pyrrole/pyrrole (Py/Py) pair is degenerate. The problem has now been resolved. A major breakthrough has been achieved with the rational design of new series of hairpin polyamides that can efficiently discriminate T•A from A•T base pairs (128). Replacement of the pyrrole unit with a bulkier 3-hydroxypyrrole enables hairpin polyamide molecules to break the degeneracy, allowing the ligand to distinguish A•T and T•A pairs (Figure 3). Apparently the increased specificity of hydroxypyrrole(Hp)-containing hairpin polyamides does not arise from enhanced interactions at specific sites but mainly comes from enhanced destabilization of interactions on unselected sequences. Placement of Hp

opposite an adenine residue provokes steric hindrance that destabilizes polyamide binding. In addition, the newly introduced OH group of Hp may engage in hydrogen-bonding interaction with O-2 of a thymine (128).

Judicious combinations of the three aromatic amino acids pyrrole (Py), imidazole (Im), and hydroxypyrrole (Hp) should permit precise recognition of A·T (Py/Hp), T·A (Hp/Py), G·C (Im/Py), and C·G (Py/im) base pairs (Figure 3) and therefore the targeting of specific sequences. Moreover, the affinity and specificity of hairpin polyamides can be further enhanced via optimization of the γ -turn linker that tethers the two minor groove binding units placed face to face. Replacement of the conventional γ -aminobutyric acid linker with analogues equipped with functional groups (e.g., amino group) can reinforce significantly the affinity as well as the specificity. Stereochemical control of the ligand–DNA interaction has recently been reported using (R)- and (S)-diaminobutyric acid linker, as depicted in Figure 4 (129). Studies of hairpin polyamides are close to success in manipulating gene expression with small molecules. A few problems remain to be solved (e.g., targeting of longer sequences, cellular uptake), but there is no doubt that polyamides are on their way to a promising future as gene-specific control agents (130–134).

By combining the end-to-end with the side-by-side dimeric motifs, one will perhaps succeed in targeting 15–17 base pairs of unique sequence in a biological system (135, 136). For the first time, it has been shown that an eight-ring hairpin polyamide [ImPyPy- γ -ImPyPyPy- β -Dp] targeted to the binding site of the transcription factor TFIIIA entered into the nucleus of cultured frog fibroblast cells and specifically inhibited transcription of the 5S RNA gene (137). Without doubt, with the demonstration of such precise engineering of the hairpin polyamides, a decisive step has been realized toward the targeting of any designated DNA sequence with small molecules.

The repertoire of sequences targeted by artificial ligands can be further extended by combining an oligonucleotide with a netropsin-like (138) or a hairpin polyamide derivative (139). For example, the affinity of the conjugate (23) for the 18 base pair target site 5'-TGACATTAAAAAGAAA-3' is 150-fold higher than that of the unlinked subunits (140). A related ligand consisting of a pyrimidine oligonucleotide 11 bases in length covalently tethered to an imidazole-containing polyamide cooperatively binds as a dimer to 27 noncontiguous base pairs of double-helical DNA via the formation of a 2:1 ligands/DNA complex (139). In both cases, the recognition process involves simultaneous recognition of the minor and major grooves of the double helix, thus mimicking the function of certain sequence-specific DNA binding proteins.

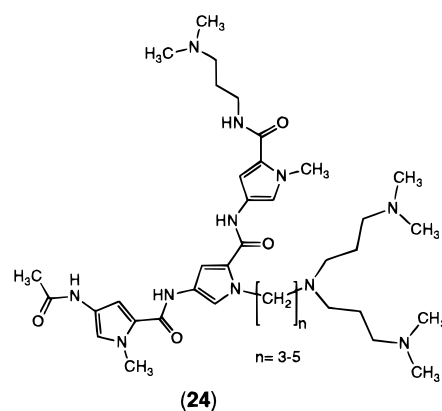
Despite the elegant design strategy for lexitropsin/DNA complexes, the biological activity of minor groove binders has not been much improved. So far, the lexitropsin approach has not led to clinically useful drugs, although certain monomeric and dimeric lexitropsins exhibit interesting antiviral or anticancer activities in vitro and sometimes in vivo (55). Some retroamide distamycin analogues exhibit promising antiviral and antiprotozoal activities (141).

A considerable number of antitumor agents, including some of clinical value, induce DNA damage either directly by alkylation or cleavage of DNA or indirectly via inhibition of topoisomerase activities. The DNA lesions induced by these different categories of drugs are presumably responsible for their cellular toxicity. Irrevers-

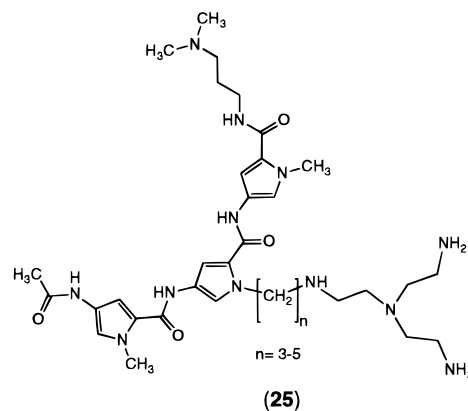
ible damage of the genetic material in cells has been considered as the basis of the antitumor effect, but it is more likely responsible, to some extent, for the toxic side effects toward normal cells (genotoxicity). Consequently, there is currently intense interest in the development of sequence-directed DNA damaging agents with a view to improving the therapeutic value of the drugs by virtue of a gene-specific recognition. The following sections describe some examples of netropsin/distamycin conjugates rationally designed to bind tightly to DNA and/or to produce irreparable damages at precisely defined genomic locations.

4. LINKAGE TO POLYAMINES

Bruce and co-workers have elaborated a series of molecules they call *microgonotropens* in which the methyl substituent on the central pyrrole ring of distamycin is replaced with a branched polyamine (142–148). The polyamine substituent on the dien- (24) and tren-microgonotropens (25) projects outward from the minor



dien-microgonotropen



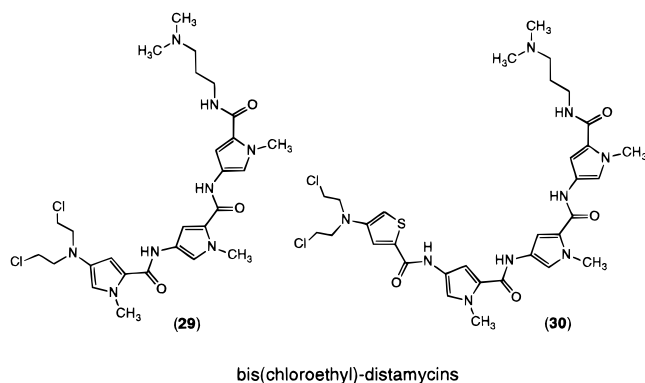
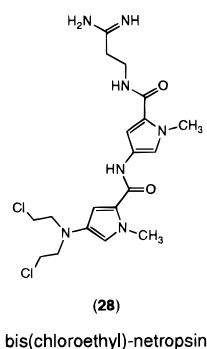
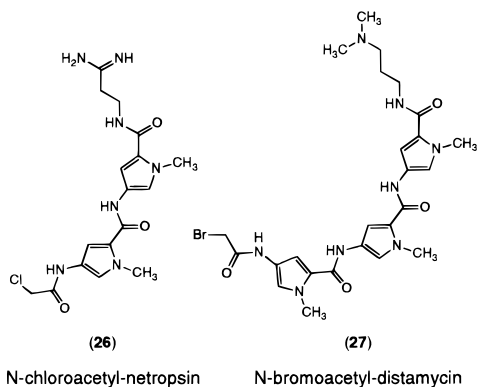
tren-microgonotropen

groove and acts as a hook to increase the affinity for DNA via interaction with the phosphodiester backbone or with the major groove of DNA. The tren [$-\text{NHCH}_2\text{CH}_2\text{N}(\text{CH}_2\text{CH}_2\text{NH}_2)_2$] microgonotropen (25, $n = 4$) is more effective than distamycin in promoting DNA bending and straightening (149). Such compounds can also efficiently compete with the sequence-specific binding of regulatory proteins to DNA (150). The microgonotropen strategy represents a new avenue in the design of minor groove reading ligands having a very high affinity for DNA. In addition, the metal-chelating properties (151) of the dien and tren substituents may be exploited for the design of catalysts for sequence-selective hydrolysis of DNA, that is, for the design of artificial nucleases (152).

5. LINKAGE TO DNA-ALKYLATING AGENTS

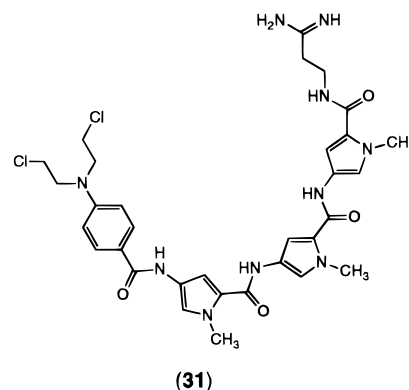
Most alkylating drugs react with DNA via an electrophilic attack on purine residues, in particular on guanines. Classical alkylating agents such as chlorambucil (153) and *cis*-platin as well as the pluramycin antibiotics (154) react within the major groove of DNA at the N-7 position of guanines. In contrast, mitomycin C (155) and anthramycin (156, 157) form adducts with the exocyclic amino group of guanine. Minor groove interactions also occur with the adenine-specific alkylators duocarmycin A and CC-1065 (158–160).

The accessibility of the major groove and the high intrinsic nucleophilicity of the N-7 heteroatom position contribute to the preferential alkylation of guanine residues by nitrogen mustards. However, the alkylation patterns of nitrogen mustards and nitrosoureas can be modified by linking the reactive group to a DNA reading element. The first generation of alkylating lexitropsins consisted of netropsin and distamycin analogues equipped with *N*-chloroacetyl (26) and *N*-bromoacetyl (27) substituents

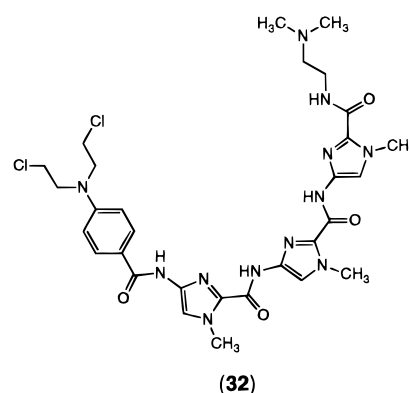


agents or with bis(chloroethyl)amino substituents (e.g., 28–30) (161, 162). *N*-Bromoacetyl distamycin (35) reacts with a single adenine in the sequence 5'-GTTTA•5'-TA•AAC within a 167 base pair restriction fragment

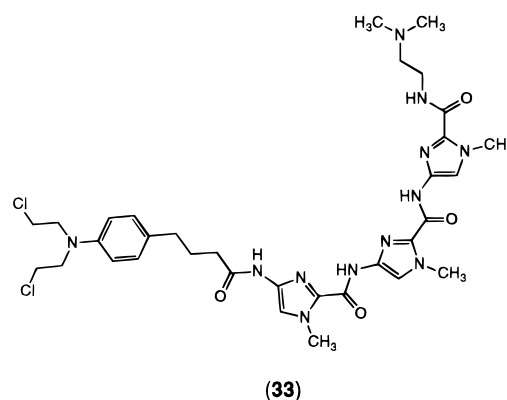
(163, 164). The second generation of alkylating lexitropsins contains benzoyl (31, 32) or benzyl mustards (33,



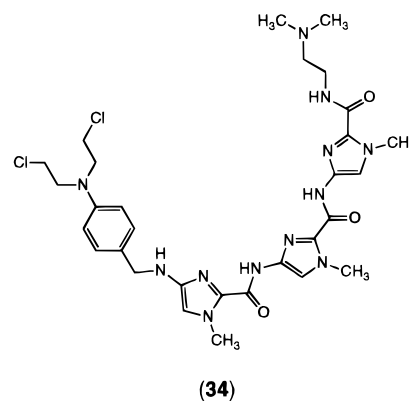
(N-benzoyl-mustard)-distamycin
FCE 24517



(N-benzoyl-mustard)-lexitropsin



chlorambucil-lexitropsin



(benzyl-mustard)-lexitropsin

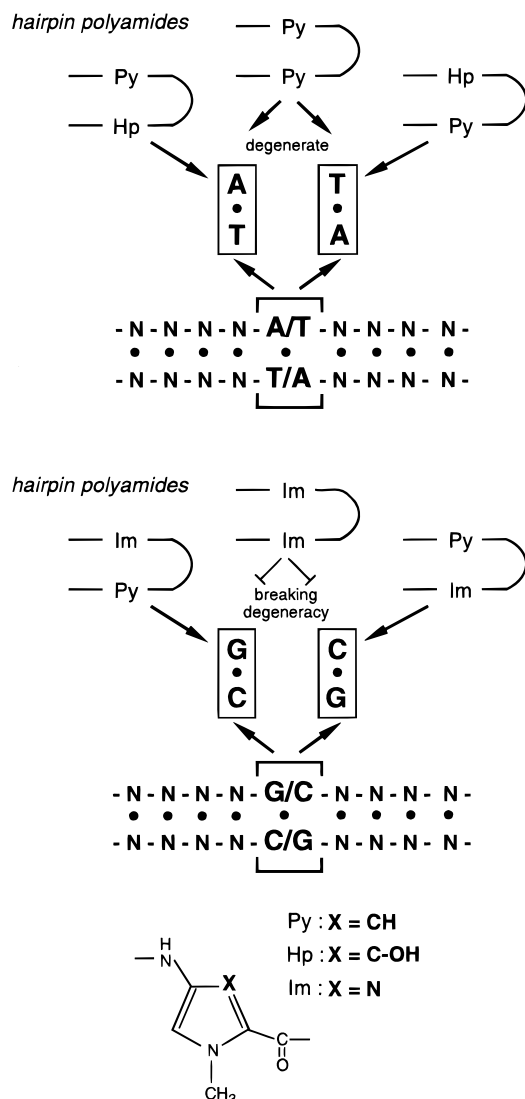
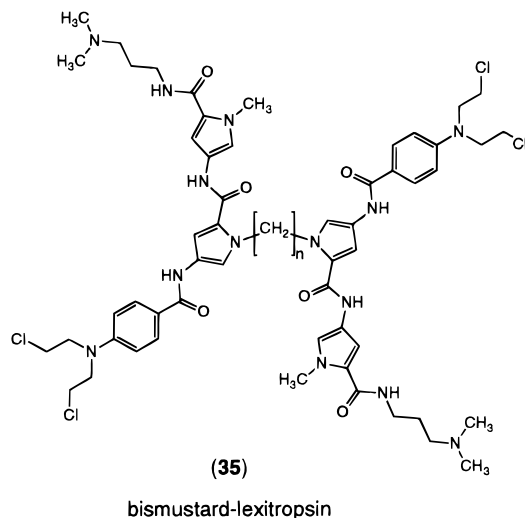


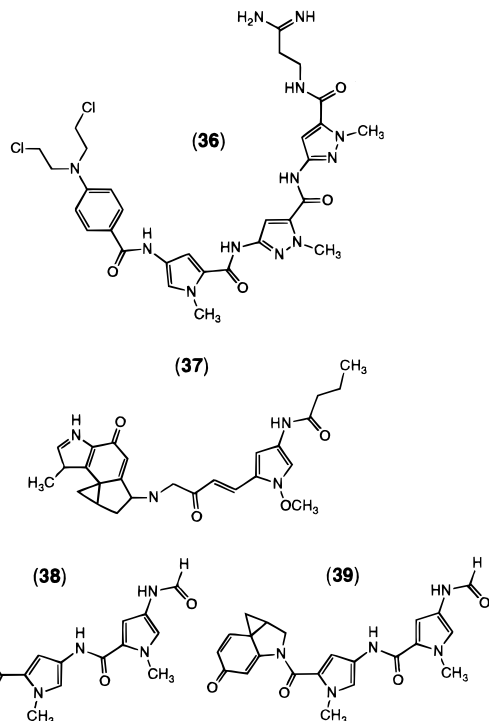
Figure 3. Schematic recognition model of the base pairs by hairpin polyamides containing imidazole (Im), pyrrole (Py), and hydroxypyrrole (Hp) motifs.

34) (165–170). Following the discovery of the aforementioned antiparallel side-by-side motif, bis(mustard) cross-linked lexitropsins such as **35** were synthesized (171).



Distamycin analogues equipped with DNA alkylating

functionalities such as compounds **31–34** show a remarkable sequence selectivity with, in some cases, an almost exclusive alkylation of adenines in the minor groove with no detectable guanine N-7 reaction. The lead compound in the series is the bis(2-chloroethyl)aminobenzoyl derivative of distamycin FCE24517 (**31**), also known as tallimustine, which demonstrates significant anticancer activity in animal models and is currently undergoing clinical trials (172–178). The antitumor activity of tallimustine is dependent on its capacity to interact with DNA. The drug alkylates the 3'-purine residue within the consensus sequence 5'-TTTTGPy-3' (167, 175). Recently, novel tallimustine derivatives have been synthesized, and some of them, such as the pyrazole derivative **36**, proved to be significantly more active than

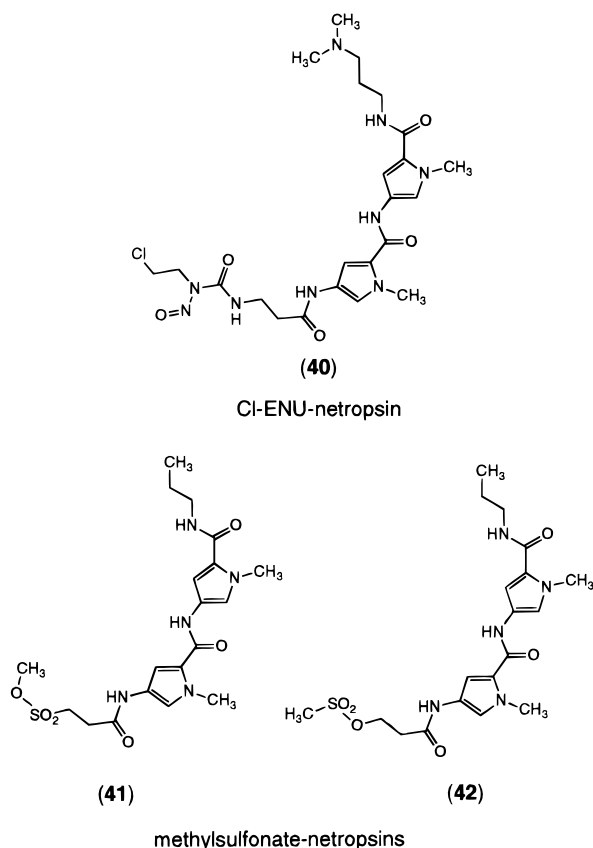


tallimustine (179, 180). Fifty years after they were introduced into medical practice in the treatment of neoplastic diseases (181), nitrogen mustards are still among the most clinically useful anticancer drugs. With the rational design of tumor active drugs such as FCE24517, there is good reason to believe that nitrogen mustards will remain of major clinical importance for a considerable time.

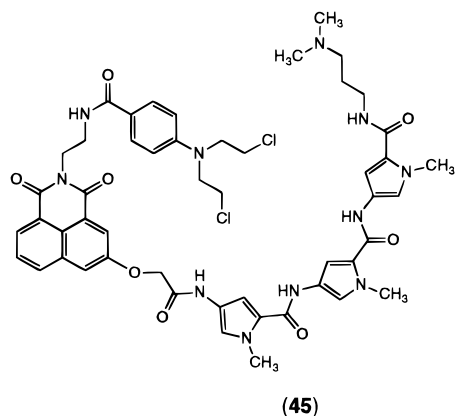
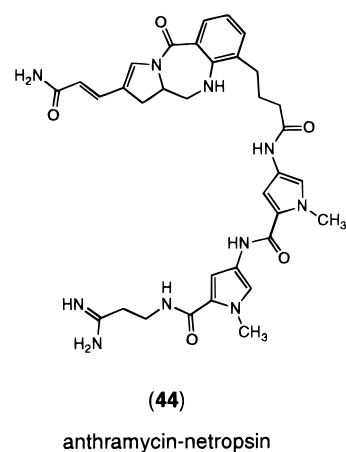
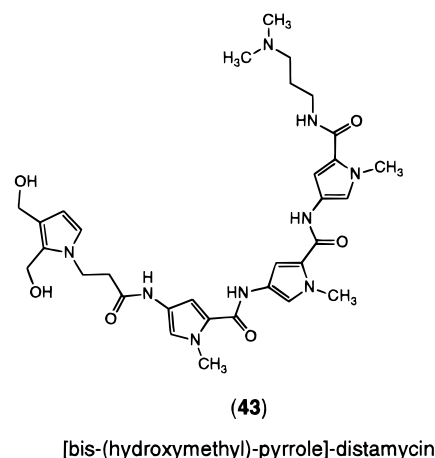
Other series of distamycin conjugates equipped with alkylating functionalities have been synthesized. Lown and collaborators have synthesized a series of lexitropsin cyclopropylpyrroloindole (CPI) hybrids (182). The CPI-*N*-methylpyrrolocarboxamide derivative (**37**) exhibits an exceptional cytotoxic potency against KB tumor cells in vitro (IC₅₀ = 0.76 fg/L) and forms stable covalent adducts in the minor groove of DNA (183). The pharmacophore found in CC-1065 and the duocarmycins has been exploited for the design of conjugates **38** and **39**. Compound **38**, containing a stable 3-(chloromethyl)-6-hydroxy-2,3-dihydroindole, binds covalently to AT-rich sequences in DNA (184). Church et al. (185) have synthesized chloroethylnitrosourea-lexitropsin conjugates such as **40**, which alkylates adenine residues in the minor groove. Zhang et al. (90) have prepared a series of noncationic *N*-methylpyrrole dipeptides incorporating sulfonate ester terminal groups (**41**, **42**). Here also, efficient alkylation

target sequence			
5'- TGTTA			
3'- ACAAT			
		<i>K_a</i> (M ⁻¹)	affinity ratio
Im – Py – Py	 <i>γ</i> -turn	2.9 x 10 ⁸	1
Dp – β – Py – Py – Py			
Im – Py – Py	 (<i>R</i>)	3.8 x 10 ⁹	13.1
Dp – β – Py – Py – Py			
Im – Py – Py	 (<i>S</i>)	2.2 x 10 ⁷	0.076
Dp – β – Py – Py – Py			
chiral <i>γ</i> -turn			

Figure 4. Influence of the linker. Replacement of the γ -aminobutyric acid linker of the hairpin polyamide ImPyPy- γ -PyPyPy- β -Dp targeting the sequence 5'-TGTTA with a chiral 2,4-diaminobutyric acid linker changes considerably the equilibrium binding constants K_a , increasing (R) or decreasing (S) binding affinity relative to the nonchiral γ -turn linker.



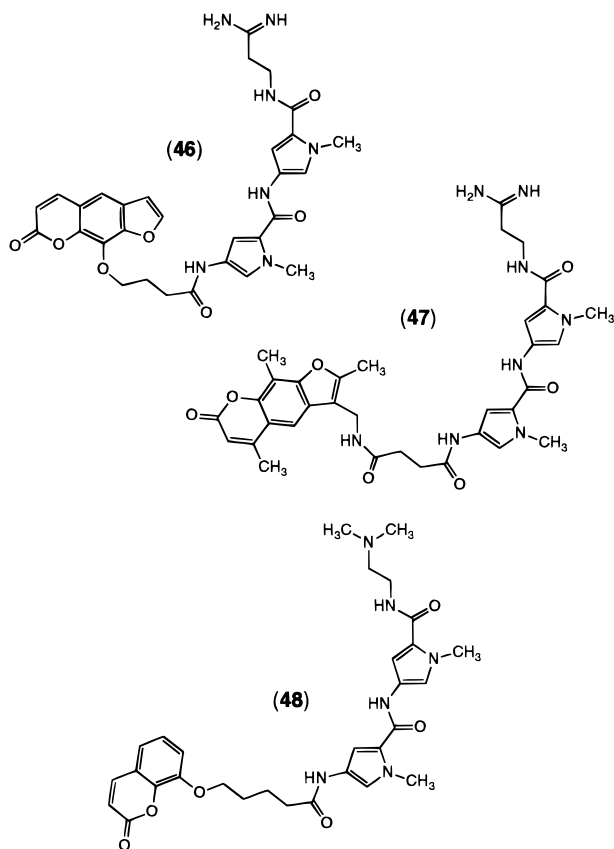
at adenine N-3 in the minor groove was observed. An elegant way to provoke DNA-DNA cross-links has been reported using a distamycin analogue coupled to a 2,3-bis(hydroxymethyl)pyrrole function, which in part mimics the functionality present in reductively activated mitomycins or oxidatively activated pyrrolizidine alkaloids. Compound **43** efficiently produces interstrand cross-links by bridging the 2-amino groups of two paired CpG steps (186, 187). More recently, Walker et al. (188) have designed netropsin-anthracycline conjugates such as the chimera **44**, which is expected to recognize the sequence RGAAAA from the HIV-1 polypurine tract. Gupta et al. recently reported the synthesis, DNA binding, sequence specificity, and biological evaluation of DNA-directed alkylating agents comprising naphthalimide,



nitrogen mustard, and lexitropsin moieties (e.g., compound **45**). However, in this case alkylation still occurred at N-7 guanine, indicating that the bulky intercalative naphthalimide mustard does not enhance sequence specificity (189).

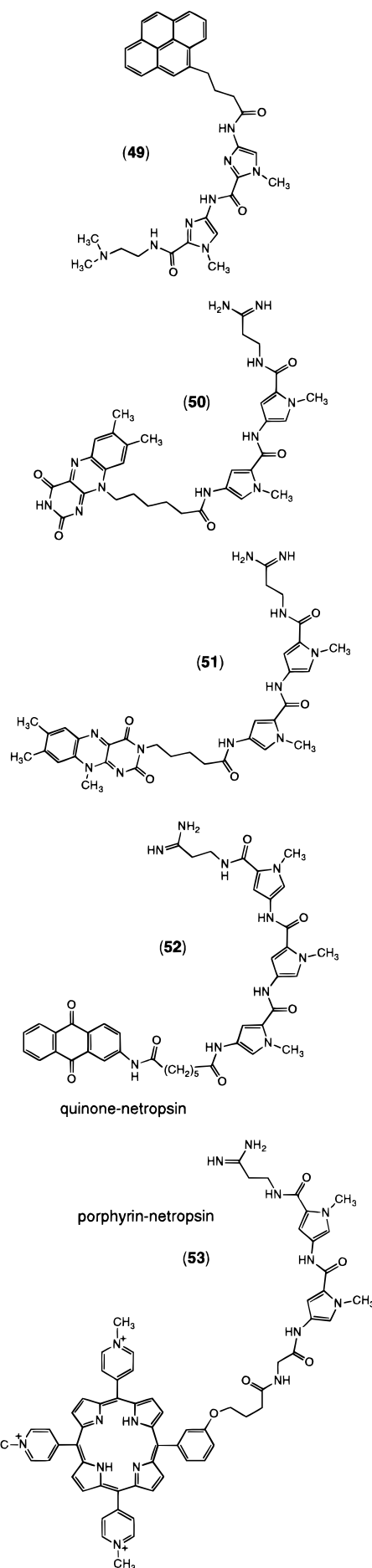
6. LINKAGE TO A PHOTSENSITIVE GROUP

A few psoralen derivatives (e.g., 8-methoxypsoralen) are used in phototherapy for the treatment of human skin diseases, chronic leukemia, and some infections connected with AIDS. The biological effect probably arises from intercalation into DNA. When exposed to UV light, intercalated molecules react covalently with DNA to form cyclobutane links to pyrimidine bases, predominantly at the 5'-TpA step (190). Psoralen (**46**, **47**) and coumarin (**48**) derivatized minor groove binding oligopeptides have been used to induce light-dependent sequence-specific reaction with DNA (191, 192). Pyrene-lexitropsin con-

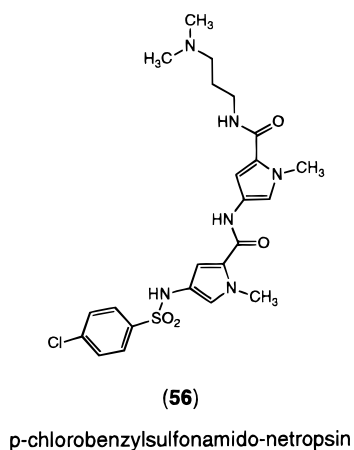
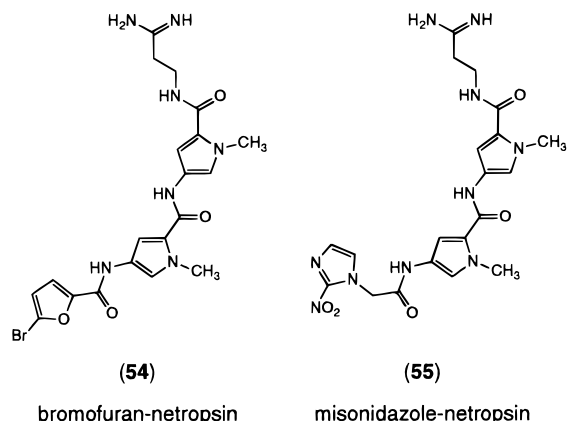


jugates such as (49) were also synthesized (193). The cytotoxicity of this sequence-selective photosensitizer is significantly enhanced upon irradiation. The photoinduced DNA lesions apparently result from the production of singlet oxygen. Using a similar approach, Herfeld et al. (194) have synthesized netropsin–isoalloxazine conjugates such as compounds 50 and 51. Upon photoactivation in the presence of molecular oxygen, the flavin chromophore oxidizes and generates oxy radicals capable of causing DNA breaks. The linkage of netropsin to a flavin chromophore leads to AT selective strand cleavage reaction (195, 196). Quinone–netropsin hybrids (e.g., 52) have been also designed. These conjugates are capable of inducing UV-mediated strand breaks (197).

Cationic porphyrins can also act as DNA photosensitizers (198). Molecular modeling predicts that the porphyrin moiety of the conjugate 53 intercalates into DNA (199, 200). UV-sensitive *p*-nitrobenzoyl groups attached to netropsin–acridine hybrids also act as photocleavers. Matsumoto et al. (201) have synthesized a series of oligo-(*N*-methylpyrrolecarboxamide) derivatives linked to halogenated heteroaromatic groups. Photoinduced DNA cleavage by the bromofuran–netropsin conjugate 54 is not mediated via active oxygen species ($\text{OH}^\bullet$) but may be due to the reaction of an aryl radical produced upon photolysis of the carbon–halogen bond (201). A similar mechanism with drug radical production has been proposed for X-ray-sensitive nitroaromatic compounds. Metronidazole and misonidazole are typical examples of radiosensitizing agents used in the treatment of anaerobic infections and are under continuing investigation regarding their use in cancer therapy, acting as markers for hypoxic regions in tumors (202). These two sensitizers only react only weakly with DNA, presumably via an electron-seeking radical (203, 204). Targeting of a nitroarene moiety to DNA via a minor groove binder is expected to increase the sensitizer concentrations at

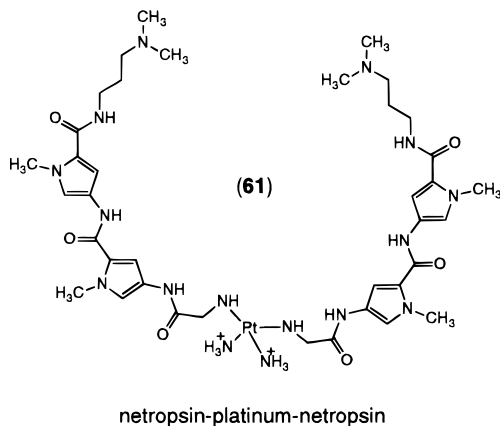
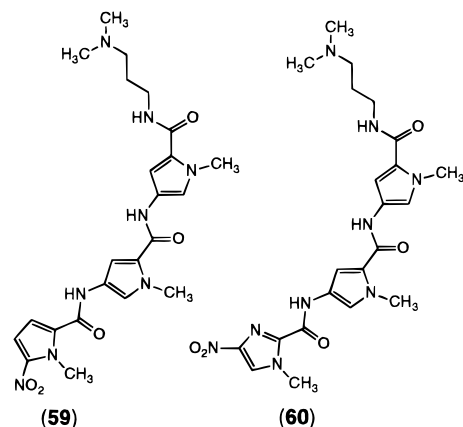
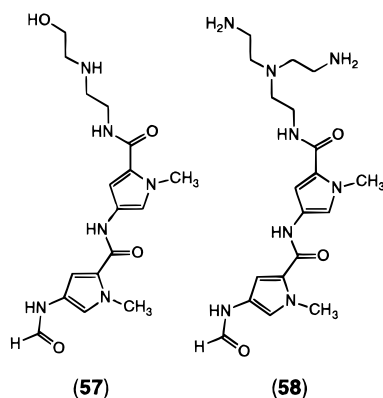


defined sites on DNA (205–207). The affinity of the 2-nitroimidazole–netropsin conjugate (**55**) for DNA is



~200-fold superior to that of misonidazole, but despite the improved interaction with DNA and improved cellular uptake capacity, the radiosensitization efficiency remains relatively poor and not better than that of misonidazole (206). Photoinduced DNA cleavage has been reported with oligo-*N*-methylpyrrolecarboxamide derivatives substituted with a benzylsulfonamido group such as the *p*-chlorobenzenesulfonamide–netropsin hybrid **56**. In this case, the efficiency of single-strand cleavage under UV-A irradiation clearly depends on the length of the pyrrolecarboxamide chain. Tetrapyrrole-sulfonamide conjugates are more efficient DNA cleavers than the corresponding analogues containing three or two pyrrole units, and conjugates with only one pyrrole are practically inactive (208). The same conclusion was drawn for simple oligopeptides such as **57** and **58**, which do not possess special side chains sensitive to UV light but which, nevertheless, can induce DNA cleavage under UV-A irradiation (209). Conversely, for nitrated oligopyrrolecarboxamide derivatives such as **59** and **60**, the DNA photocleavage efficiency is higher for the monopyrrole compounds than for the bis- and tris(peptide)s (210).

The netropsin–platinum–netropsin conjugate (**61**) represents another type of netropsin conjugate capable of inducing DNA cleavage upon X-ray ionization (211). According to footprinting experiments, the drug binds selectively to 9 base pair long AT-rich sites with the two netropsin moieties extended along the minor groove. Recently it was shown that along with the extended conformation, hairpin conformation can coexist with the pyrrole rings of one netropsin-like element stacked on the pyrrole rings of the other. This structure is remi-

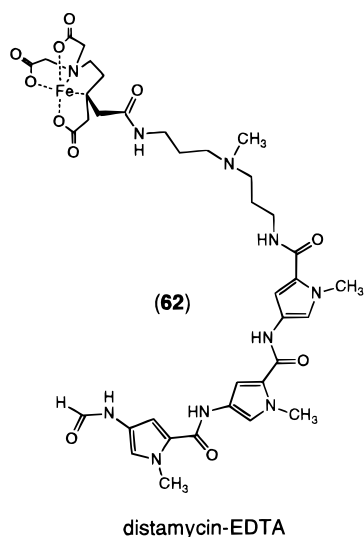


niscant of the parallel hairpin polyamide motif described above (212). X-irradiation of drug/DNA complexes yields discrete cutting sites near the center of the platinum–bis(netropsin) binding sites. The cleavage would result from the rupture of the deoxyribose residues upon attack by Auger electrons generated by the irradiated platinum atom. Such conjugate compounds capable of triggering sequence-specific DNA degradation might be of interest for X-ray therapy of tumors.

7. LINKAGE TO A METAL/COMPLEX

Endonucleases, such as DNase I, are generally employed to map ligand binding sites on DNA (213, 214), but the preequilibrated ligand/DNA complex can be subjected to the nicking activity of a chemical nuclease, such as Dervan's reagent methidiumpropyl-EDTA complexed with iron (MPE·Fe^{II}). It is also possible to equip the ligand with its own DNA-cleaving functionality, for example, to attach an Fe·EDTA complex to the test drug. This method, termed DNA-affinity cleaving, has proved

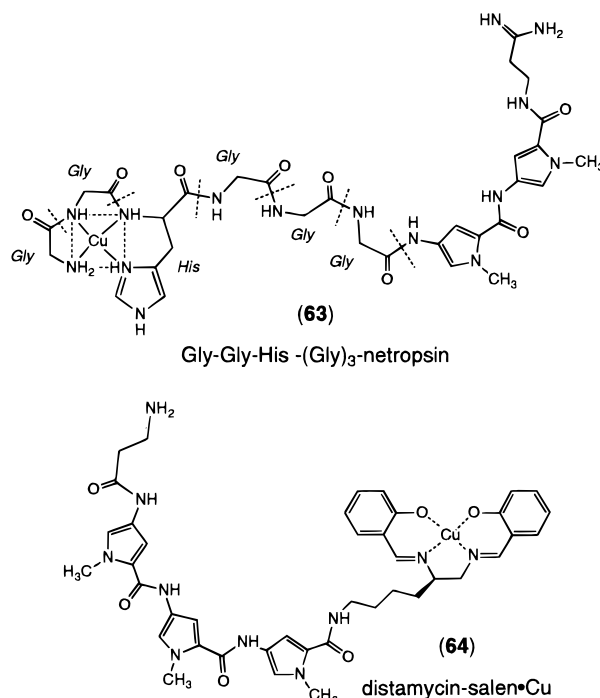
to be very successful in analyzing binding sites for distamycin via the use of EDTA–distamycin conjugates such as compound **62** (214–216). The technique has been



extensively exploited to study oligo(*N*-methylpyrrolecarboxamide) derivatives containing up to 12 pyrrole units as well as a variety of monomer and dimer lexitropsins (217–222). Upon chemical activation with a reducing agent (e.g., dithiothreitol) the iron–EDTA portion generates hydroxyl radicals, which react with the deoxyribose residues to produce DNA strand cleavage. In contrast to Fe•EDTA footprinting, the active oxygen species are produced directly in the vicinity of the ligand binding sites on DNA and so are less susceptible to diffusion.

Metal complexing peptides can serve as a source of oxygen radicals. The growth-modulating tripeptide glycyl–histidyl–lysyl (GHK) and the related tripeptide glycyl–glycyl–L-histidine (GGH) both form complexes with copper and upon activation can generate oxygen active species (223–226). Linkage of the metal-complexed GHK peptide to minor groove binding drugs has been considered as a means of inducing sequence selective cleavage. Compound **63** was synthesized. The peptide moiety not only cleaves DNA but also contributes positively to the DNA binding reaction (227, 228). Non-peptidic metal complexes have been utilized. Recently, Dst was linked to a bis(salicylidene)ethylenediamine derivative to give the distamycin–salen conjugate **64**. However, the cleavage remained largely nonspecific (229).

The best characterized natural model for sequence-specific DNA cleavage is bleomycin, which remains one of the most useful antitumor drugs. The bleomycin•Fe(II) complex combines with O₂ to produce a reactive oxygenated metalbleomycin species, which is capable of abstracting a hydrogen atom from the C4' position of deoxyriboses in DNA. Bleomycin generates mainly single-strand breaks at pyrimidine residues 3' to a guanine residue (i.e., at 5'-GpC and 5'-GpT sequences) (230). The long established clinical utility of bleomycin sparked tremendous interest in its mechanism of action and in the design of compounds based on its structure. A considerable number of bleomycin analogues and related structures have been designed, including some equipped with DNA reading elements based on netropsin and distamycin. The structure of the antibiotic has been simplified to yield analogues such as PYML (231), and AMPHIS (232–234), which mimic efficiently the metal-chelating/oxygen activation domain of bleomycin.

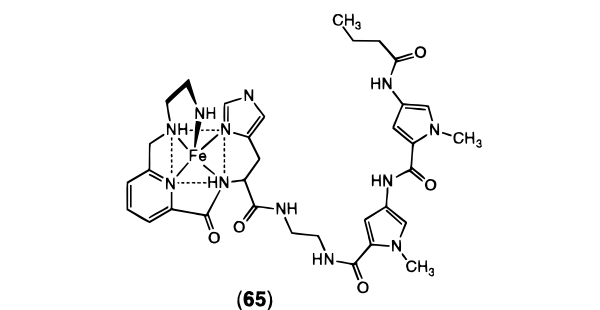


Attachment of PYML (235, 236) and AMPHIS (237–241) to lexitropsin carriers gave bleomycin-like conjugates such as compounds **65–68** endowed with interesting sequence-specific DNA recognition properties. For bleomycin, the whole molecule spanning from the pyrimidine region to the bithiazole terminus appears to be responsible for specific recognition of DNA (242–244). In contrast, the lexitropsin moiety is only responsible for specific base recognition of man-designed bleomycin conjugates. The fact that the synthetic PYML–distamycin hybrid **67** is more toxic than bleomycin itself toward L1210 leukemia cells in vitro encourages the design of other related bleomycin-like molecules tailored with DNA reading elements.

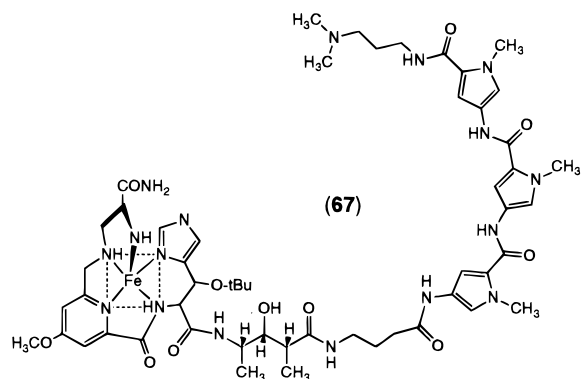
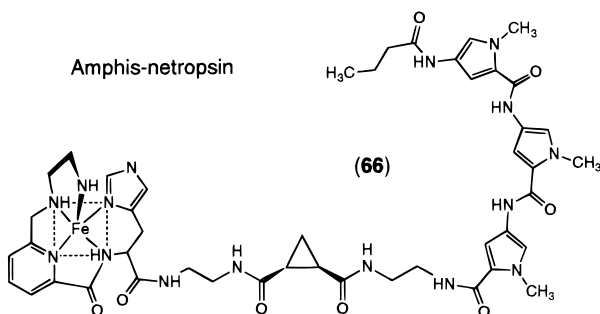
8. LINKAGE TO AN ENEDIYNE LIGAND

The discoveries in the late 1980s of the antitumor activity of enediyne antibiotics rapidly elicited extensive research activity into their chemistry. An impressive number of synthetic analogues of dynemicin, neocarzinostatin, calicheamicin, and esperamicin have been described (245–247). Upon activation, enediynes trigger a Bergman reaction, which leads to highly reactive benzenoid diradicals and causes severe DNA damage (247). Enediyne antibiotics are among the most cytotoxic compounds known so far, and their activity is likely attributable to their effect on DNA. The excessive reactivity of the enediyne moiety prompted the development of analogues containing DNA delivery systems. To this end, netropsin was coupled with the neocarzinostatin chromophore (**69**) (248) and distamycin was attached to a dynemicin model (**70**) (245). More recently, the synthesis and biological activity of new netropsin–enediyne hybrids such as **71** was reported. The addition of the binding domain improved the DNA cleavage potency considerably (up to 100-fold), but a concomitant increase in potency against tumor cells was not observed (249).

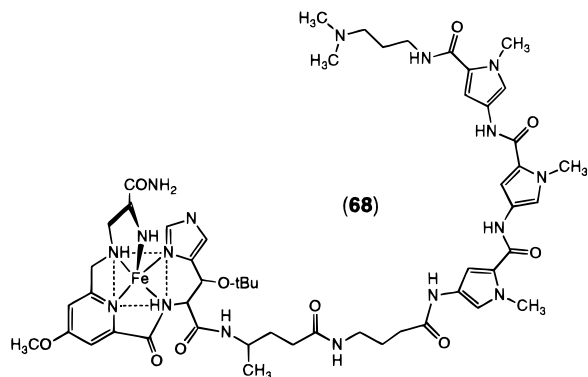
Simple cyclic enediynes attached to netropsin moieties have also been elaborated (250). The linkage of the two functionalities via acetate and crotonate (**72**) tethers results in a hybrid series in which the cleavage efficiency is strongly enhanced (up to 1000-fold compared to the



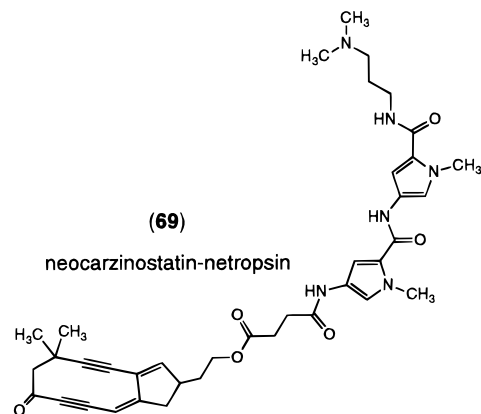
Amphis-netropsin



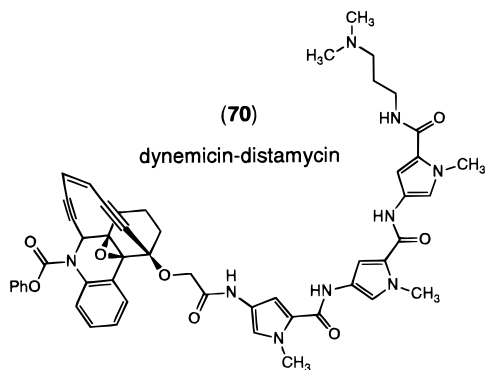
PYML(6)-(AHM)-distamycin



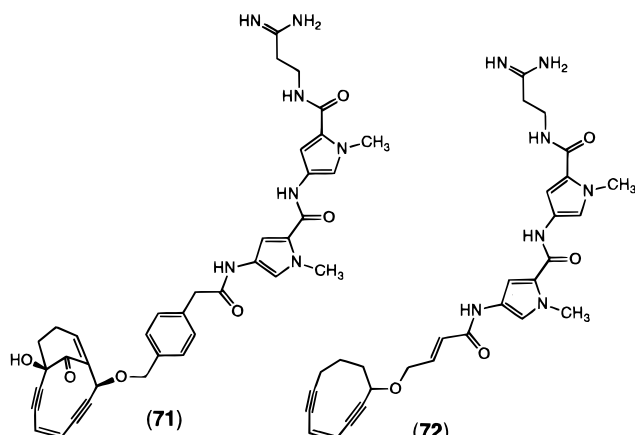
PYML(6)-(APA)-distamycin



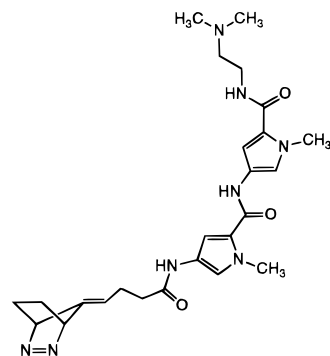
neocarzinostatin-netropsin



dymecicin-distamycin



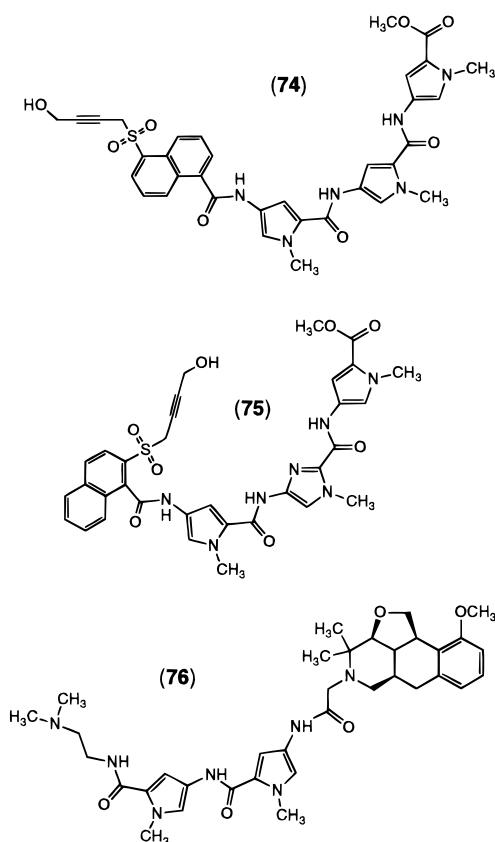
(72)



TMM-netropsin

enediyne alone) (251). These encouraging results pave the way for the design of simpler related structures capable of triggering effective DNA cleavage via a radical mechanism. In this context, Bregant et al. (252) synthesized the netropsin analogue **73** equipped with a trimethylenemethane group (TMM), which can undergo cycloaddition to electron deficient alkenes. Upon photolysis of the diazene moiety, the TMM-netropsin conjugate can transform to a diyl radical and cleaves DNA, predominantly at AT-rich regions (253). Propargylic sulfones are small synthetic molecules that mimic the

chemical action of enediynes. They can cleave DNA in a pH-dependent fashion. Here again, linkage of lexitropsin carriers to propargylic sulfones (**74**, **75**) may permit the cleavage to be directed to specific sequences in DNA (254). It is worth mentioning here the netropsin-



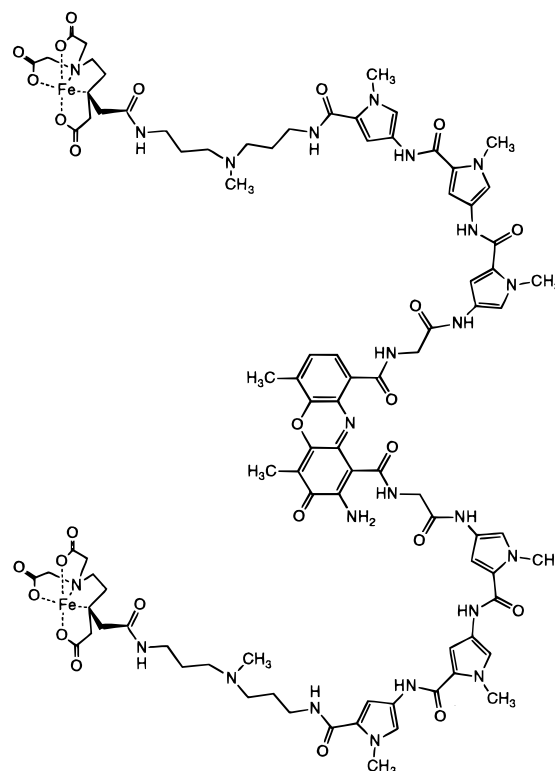
quinocarcin conjugate **76**, which can efficiently cleave DNA at AT-rich sequences via the production of a nondiffusible oxidant (255).

9. LINKAGE TO AN INTERCALATING AGENT: THE COMBILEXINS

A large number of intercalating drugs, including some of major clinical value, possess a heterocyclic chromophore substituted with peptide, alkyl, or carbohydrate side chains that participate in DNA recognition. These chains represent a kind of hook for sequence-selective interaction within the minor groove of DNA. For example, the antitumor drug actinomycin intercalates between base pairs, leaving the two pentapeptide lactones lying in the minor groove of the double helix (256–258). The symmetrically disposed cyclic peptides participate in the specific recognition of GpC sites by actinomycin. Similar observations can be made for a variety of tumor active intercalating drugs. Anthracyclines such as daunomycin and nogalamycin contain carbohydrate residues that serve as DNA recognition elements (259, 260). The peptide rings of the quinoxaline antibiotics echinomycin and triostin A are prime determinants for sequence-specific recognition by the drug (261). The enediyne antibiotics dynemicin A (262) and neocarzinostatin (263) bind to DNA by intercalation of their chromophore (naphthoate for neocarzinostatin and anthraquinone for dynemicin), placing their reactive enediyne-containing bicyclic core moiety in the minor groove in a suitable position for selective DNA cleavage. Such considerations indicate that in many cases drugs usually referred to as “intercalators” in fact exhibit mixed modes of binding to DNA and therefore should be considered as intercalator–minor groove reading hybrid molecules or naturally occurring *combilexins* (thus called by analogy with the *lexitropsins*) (264, 265).

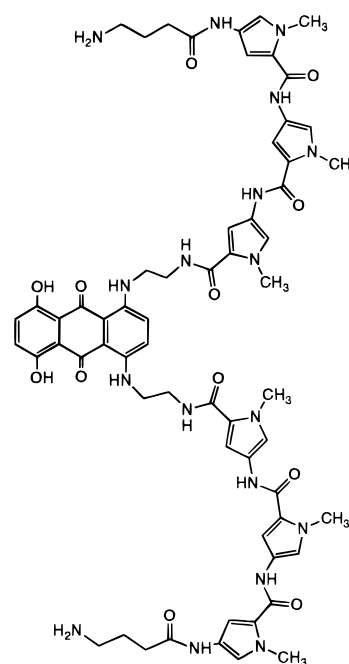
Using actinomycin D as a model compound, Krivtsora et al. (266) designed a series of hybrid molecules called

distactins in which the phenoxazine chromophore is substituted at positions 1 and 9 with one, two, or three *N*-methylpyrrolecarboxamide units. Bidentate reaction with DNA involving intercalation of the chromophore and minor groove binding of the DNA reading element was observed with distactins bearing one or two pyrrole rings but not with the analogues having three units. The model was reconsidered two years later (45). DNA affinity cleaving studies of the bis(EDTA–distamycin)–phenoxazine conjugate molecule **77** revealed a major



distactin (**77**)

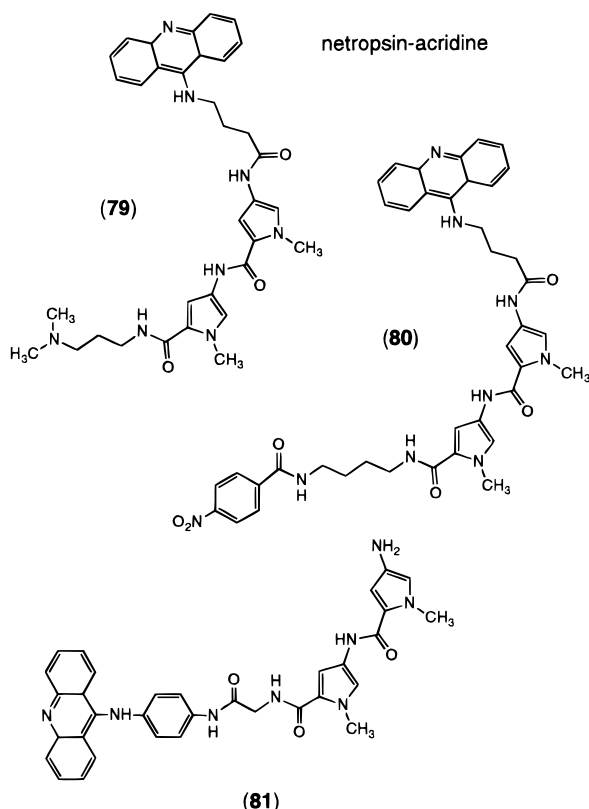
[bis-(EDTA–distamycin)]–phenoxazine



NetMitox (**78**)

cleavage site flanking the 10 base pair sequence 5'-TATAGGTAA, suggesting thus that intercalation of the tricyclic nucleus at the central GG step is accompanied with minor groove binding of the distamycin moieties at the flanking (A·T)₄ sites. Additional single cleavage loci were also observed. Depending on the recognized sequence, only one or both recognition elements engage in contact with DNA (45). Similar results were recently obtained with the bis(netropsin)-anthraquinone combilexin **78**. Intercalation of the mitoxantrone-derived chromophore is hindered by the appended minor groove elements. The anthraquinone not only provides extra strength of binding but notably influences the DNA recognition by the minor groove element (267).

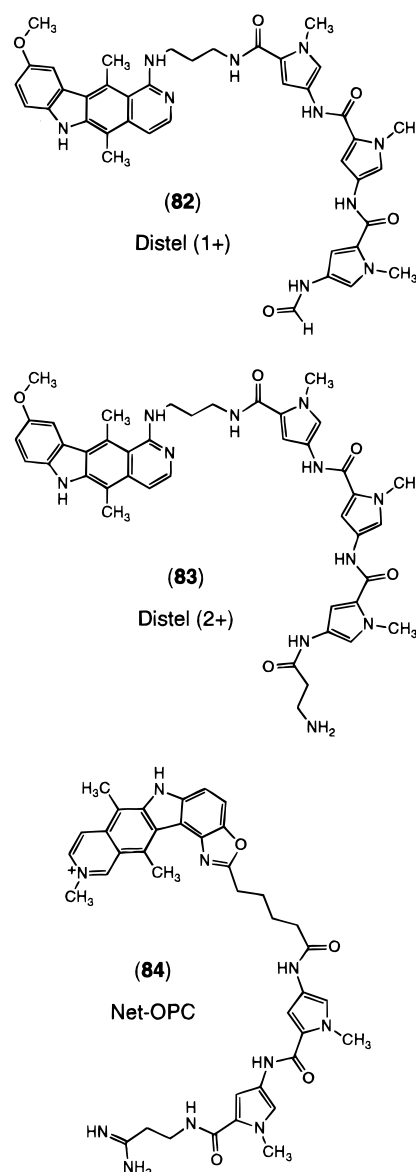
Bis(pyrrolicarboxamide) moieties were linked to a 9-aminoacridine chromophore by alkyl linkers of variable length (268). Optimum fit to DNA was obtained with the combilexin **79** with a butyryl tether. This biscationic hybrid exhibits a strict AT preference as for distamycin. A structurally related acridine-distamycin ligand equipped with a photoactivatable *p*-nitrobenzoyl (**80**)



group has been synthesized (269). This conjugate can cut DNA upon activation with UV light (310 nm) with a preference for AT sequences (270). The selectivity for AT sites was found to be significantly decreased when a truncated netropsin moiety was linked to a glycyanilinoacridine chromophore structurally related to the antileukemic drug amsacrine. In this case, the rigid connector between the bis(pyrrole) unit and the acridine nucleus of the combilexin molecule **81** does not permit optimal intercalation of the acridine and apparently slightly restricts the netropsin moiety fitting deeply into the minor groove (271, 272). However, this compound is a potent topoisomerase II inhibitor and exhibits moderate antitumor activities in vivo (273, 274). Two functional domains were identified in the combilexin **81**: the anilino group can be regarded as a skeletal core to which are

connected, on the one side, the tricyclic acridine moiety, which represents the DNA binding domain, and, on the other side, the *N*-methylpyrrolicarboxamide moiety, which constitutes the topoisomerase II-targeted domain (275).

Covalent linkage of distamycin to a GC-selective ellipticine derivative (276) afforded a monocationic hybrid molecule Distel(1+) (**82**), capable of bidentate binding to DNA. The reaction with DNA was primarily driven by the charged ellipticine moiety of the hybrid (277–279). The ellipticine chromophore has markedly reinforced the affinity of the ligand for DNA, but the effect is at the expense of DNA sequence selectivity. Computational studies suggested that the addition of a positively charged group on the distamycin terminal group would favor binding to AT sequences (277). The calculation proved to be correct. Indeed, the substitution of the terminal formamido group of Distel(1+) for an aminopropionamido group charged at neutral pH [Distel(2+) (**83**)] was the



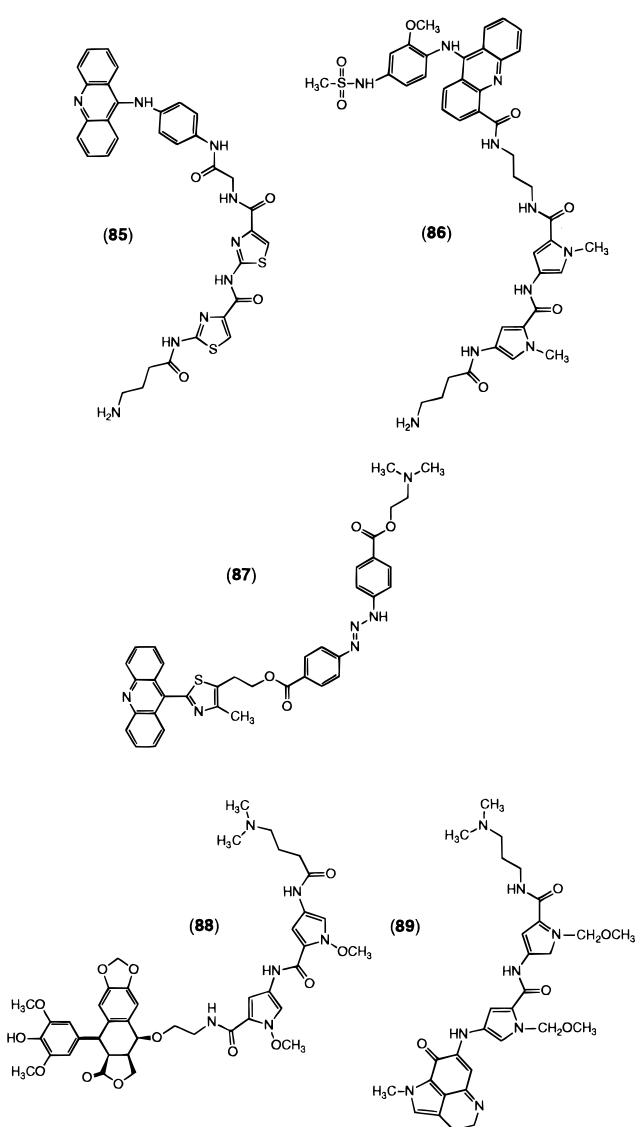
correct way to proceed to convert a nonspecific conjugate into a highly AT-specific DNA reader (280). In contrast to Distel(1+), the interaction of Distel(2+) with DNA seems to be driven as much by the distamycin moiety as by the ellipticine residue. Of the two distamycin-

ellipticine hybrids **82** and **83**, only the monocationic hybrid Distel(1+) (**82**) proved to be a topoisomerase inhibitor. Its biscationic analogue Distel(2+) (**83**) showed practically no effect on both topoisomerase I and topoisomerase II, despite its superior DNA binding properties (281). The poisoning of topoisomerase I by Distel(1+) contributes to the cytotoxic effect since P388CPT5 cells resistant to camptothecin (a powerful topoisomerase I inhibitor) display a notable cross-resistance to Distel(1+) (281).

A different situation has been reported with a biscationic netropsin conjugate containing an oxazolopyridocarbazole chromophore derived from the ellipticines. The Net-OPC (**84**) hybrid ligand adopts different configurations according to the sequence to which it binds. At GC sites, the OPC chromophore intercalates into DNA but the netropsin remains unbound. At AT sites, the most energetically favored complex has both the netropsin and the OPC moieties inserted in the minor groove of a 7 base pair long sequence. In contrast, the second favored complex at (A·T)₄ sites involved intercalation of the OPC ring and minor groove recognition by the bispyrrole moiety (282–285). A somewhat similar coexistence of an intercalative and a nonintercalative binding mode was reported for the netropsin–porphyrin conjugate **53** (199). The connecting group between the two DNA binding elements plays a critical role in the DNA recognition process (200). These typical examples illustrate the difficulties encountered in designing combilexin molecules. It is a challenging exercise that demands consideration of the notions of geometrical compatibility, hydrogen bonding capability, and the overall electronic properties of the interacting species.

A moderate antitumor activity *in vivo* was observed with the bifunctional molecule **85**, which combines features of both the lexitropsin and combilexin approaches. This hybrid ligand, in which are conjugated the thiazole lexitropsin (**12**) and the intercalating anilinoacridine chromophore, binds to DNA via a bimodal process involving minor groove binding of the lexitropsin moiety and intercalation of the acridine moiety (286).

A second generation of combilexins has now been designed. Unlike compound **81**, the netropsin–amsacrine hybrid (**86**) bears a positively charged terminal side chain, which contributes significantly to the ATT selectivity of such ligands and retains the *m*-methoxy and methanesulfonamide substituents on the anilino ring, which constitute key elements for the interference with topoisomerases, the maintenance of redox properties, and the biological properties of amsacrine. The netropsin moiety is connected to the acridine ring via a 4-carboxamide side chain, whereas it was previously attached directly to the anilino group. Linkage of a carboxamide side chain to position 4 of the acridine ring of amsacrine has earlier been shown to convert the drug from a classical intercalator to a threading intercalator (287, 288). Structural and kinetic studies have revealed that the conjugate threads through the DNA double helix so as to intercalate its acridine chromophore, leaving the netropsin moiety and the methanesulfonanilino group positioned within the minor and major grooves of the double helix, respectively (289). In addition, the hybrid retains the susceptibility to copper-dependent oxidation and to generate DNA-damaging oxygen radicals (290). It also stimulates the formation of cleavable complexes with DNA in the presence of topoisomerase II, but its netropsin-like moiety confers little or no influence on the reaction with the topoisomerase (290).



Thus far, the combilexin strategy has concerned netropsin- or lexitropsin-like minor groove binders attached to different intercalating chromophores. In 1995, the strategy was extended to another category of minor groove binders, namely, 1,3-diaryltriazenes derived from the antiviral and antiprotozoal drug berenil. The acridine–triazene combilexin (**87**) exhibits a distinct preference for AT base tracts rather than GC-rich sequences and is ~40-fold more cytotoxic than the triazene or acridine subunits toward L1210 mouse leukemia and A2780 human colon cancer cell lines (291). These results on combilexin molecules obtained so far encourage us to believe that this approach to DNA-targeted pharmacology has the potential to yield important developments in the search for new classes of topoisomerase inhibitors and perhaps for new and better anticancer drugs.

The epipodophyllotoxins etoposide (VP-16-213) and teniposide (VM-26) are very potent topoisomerase II inhibitors and are mainly used in chemotherapy for the treatment of small cell lung cancer, testicular cancer, lymphoma, and leukemia. These compounds stabilize DNA/topoisomerase II complexes but interact only weakly, if at all, with DNA in the absence of the enzyme. Lown and co-workers have attempted to direct epipodophyllotoxins to specific sequences via the conjugation with lexitropsins. A series of 4'-demethylepipodophyllotoxin–lexitropsin conjugates such as compound **88** were syn-

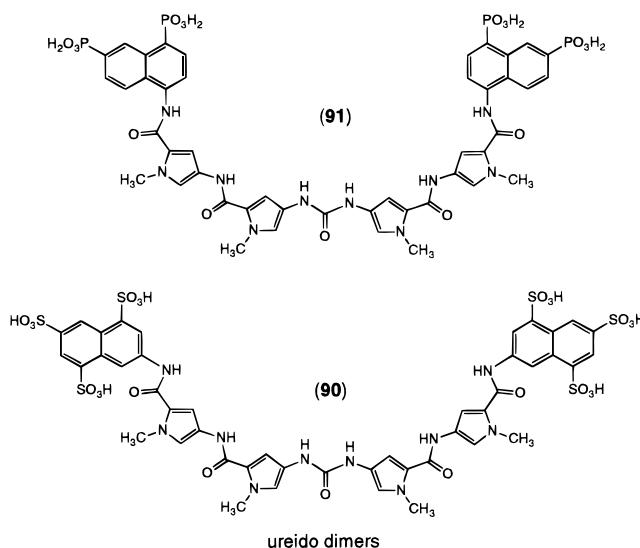
thesized, but these compounds were found to be much less cytotoxic than the parent compounds (292). In the same vein, a pyrroloquinoline nucleus of the topoisomerase II inhibitor makaluvamine A has been linked to mono-, bis-, and tris(pyrrolocarboxamide) moieties. Hybrids such as compound **89** show significant cytotoxicity against different tumor cell lines (293).

10. CONCLUSION

Netropsin and distamycin have greatly contributed to our understanding of the molecular basis of drug–DNA recognition. The understanding of how these two antibiotics recognize and bind to AT sequences in DNA provided perceptions of how to design sequence-specific DNA binding molecules and stimulated the synthesis of several classes of new compounds of potential use in cancer chemotherapy. As shown in this review, the chemical structure of netropsin-like DNA reading elements may be varied in many synthetically convenient fashions to produce different repertoires of sequence-selective DNA binding molecules with a range of functionalities. Sterically demanding groups can be attached to netropsin and distamycin without abolishing their sequence recognition properties. The fact that certain netropsin analogues can readily recognize a specific sequence in DNA (e.g., lexitropsins forming 2:1 complexes and hairpin polyamides) shows that DNA targeting with minor groove binding drugs is no longer merely a possibility but a practical reality. The fact that certain netropsin conjugates capable of inducing DNA damage at specific sequences exhibit potent antitumor activities (e.g., netropsin–nitrogen mustard conjugates) shows that this DNA-targeting strategy has the potential to yield new and efficient antitumor agents. There is good reason to believe that netropsin and distamycin will continue to inspire the development of new anticancer agents (294–297). However, it is important to bear in mind that at the present day no-one can certify that chemotherapeutic selectivity will be improved by virtue of an increase in sequence selectivity.

In the many examples presented in this review, netropsin and distamycin are almost always used to deliver cytotoxic drugs to specific sequences in DNA, but they can also be used to deliver DNA to cells. Gene transfer with netropsin–lipid conjugates has been attempted (298). Moreover, netropsin and distamycin analogues can also be used independently of their capacity to bind to AT-rich DNA sequences. For example, sulfonated and phosphonated ureido dimers of netropsin such as compound **90**, containing six sulfonic acid units, and the phosphonic acid-containing ligand **91** represent a new class of surface acting antiviral agents. Their mechanism of action apparently does not require binding to DNA but may involve the disruption of the virus attachment to CD4⁺-susceptible cells via an effect on CD4-gp120 interactions (299).

Although there are a few netropsin conjugates under clinical or preclinical evaluation, medicinal chemists still have much to do to design new clinically useful drugs endowed with the aptitude for reading any given DNA sequence and inducing the required DNA modification to obtain the desired biological response. Computer-based methods will increasingly serve to delineate more precisely the molecular rules that govern drug–DNA recognition. The increasing evidence that DNA binding proteins such as topoisomerases and transcription factors mediate the effects of drugs suggests that in addition to elucidating the structures of drug/DNA complexes it will be necessary to examine in detail the effects of drugs on



protein/DNA complexes. Close collaboration between medicinal chemists and molecular biologists will be necessary if we are to gain an improved understanding of the mechanisms whereby antitumor drugs affect the function of particular oncogenes.

Over the past 15 years, research on small molecules acting on nucleic acids has not only led to therapeutically useful drugs but also provided an invaluable source of structural and biological information on nucleic acids. The recent discovery of new tumor active compounds, such as those based on netropsin and distamycin reported here, has perhaps restored small molecules to the forefront of cancer therapy. It is still premature to state that netropsin and distamycin derivatives will provide new generations of sequence-specific antitumor drugs. However, the results obtained thus far are suggestive and promising. They augur exciting developments for years to come.

ACKNOWLEDGMENT

C.B. thanks the Association pour la Recherche contre le Cancer and the Ligue Nationale Française Contre le Cancer for financial support. J.B.C. is supported by Grant CA35635 from the National Cancer Institute.

LITERATURE CITED

- (1) Waring, M. J. (1981) DNA modification and cancer. *Annu. Rev. Biochem.* 50, 159.
- (2) Hurley, L. H. (1989) DNA and associated targets for drug design. *J. Med. Chem.* 32, 2027.
- (3) Patel, D. J. (1982) Antibiotic-DNA interactions: intermolecular nuclear overhauser effects in the netropsin-d(CGCGAATTCGCG) complex in solution. *Proc. Natl. Acad. Sci. U.S.A.* 79, 6424.
- (4) Kopka, M. L., Yoon, C., Goodsell, D., Pjura, P., and Dickerson, R. E. (1985) Binding of an antitumor drug to DNA: Netropsin and CGCGAATT^{B+}CGCG. *J. Mol. Biol.* 183, 553.
- (5) Kopka, M. L., Yoon, C., Goodsell, D., Pjura, P., and Dickerson, R. E. (1985) The molecular origin of DNA-drug specificity in netropsin and distamycin. *Proc. Natl. Acad. Sci. U.S.A.* 82, 1376.
- (6) Klevit, R. E., Wemmer, D. E., and Reid, B. R. (1986) ¹H NMR studies on the interaction between distamycin A and a symmetrical DNA dodecamer. *Biochemistry* 25, 3296.
- (7) Coll, M., Frederick, C. A., Wang, A. H. J., and Rich, A. (1987) A bifurcated hydrogen bonded conformation in the d(A.T) base pairs of the DNA dodecamer d(CGCAAATTTGCG) and its complex with distamycin. *Proc. Natl. Acad. Sci. U.S.A.* 84, 8385.

- (8) Coll, M., Aymami, J., van der Marel, G. A., van Boom, J. H., Rich, A., and Wang, A. H.-J. (1989) Molecular structure of the netropsin-d(CGCGATATCGCG) complex: DNA conformation in an alternating AT segment. *Biochemistry* 28, 310.
- (9) Pelton, J. G., and Wemmer, D. E. (1989) Structural characterization of a 2:1 distamycin A·d(CGCAAATTTGGC) complex by two-dimensional NMR. *Proc. Natl. Acad. Sci. U.S.A.* 86, 5723.
- (10) Pelton, J. G., and Wemmer, D. E. (1990) Binding modes of distamycin A with d(CGCAAATTTGGC)₂ determined by two-dimensional NMR. *J. Am. Chem. Soc.* 112, 1393.
- (11) Sriram, M., van der Marel, G. A., Roelen, H. L. P. F., van Boom, J. H., and Wang, A. H.-J. (1992) Structural consequences of a carcinogenic alkylation lesion on DNA: Effect of O⁶-ethylguanine on the molecular structure of the d(CGC-[e⁶G]AATTCGCG)-netropsin complex. *Biochemistry* 31, 11823.
- (12) Tabernero, L., Verdaguer, N., Coll, M., Fita, I., van der Marel, G. A., van Boom, J. H., Rich, A., and Aymami, J. (1993) Molecular structure of the A-tract DNA dodecamer d(CGCAAATTTGGC) complexed with the minor groove binding drug netropsin. *Biochemistry* 32, 8403.
- (13) Geierstanger, B. H., Dwyer, T. J., Bathini, Y., Lown, J. W., and Wemmer, D. E. (1993) NMR characterization of a heterocomplex formed by distamycin and its analog 2-ImD with d(CGCAAGTTGGC):d(GCCAACTTGCG): Preference for the 1:1:1 2-ImD:DNA complex over the 2:1 2-ImD:DNA and the 2:1 Dst:DNA complexes. *J. Am. Chem. Soc.* 115, 4474.
- (14) Goodsell, D. S., Kopka, M. L., and Dickerson, R. E. (1995) Refinement of netropsin bound to DNA: Bias and feedback in electron density map interpretation. *Biochemistry* 34, 4983.
- (15) Chen, X., Ramakrishnan, B., Rao, S. T., and Sundaralingam, M. (1994) Binding of two distamycin A molecules in the minor groove of an alternating B-DNA duplex. *Struct. Biol.* 1, 169.
- (16) Chen, X., Ramakrishnan, B., and Sundaralingam, M. (1995) Crystal structures of B-form DNA-RNA chimers complexed with distamycin. *Nat. Struct. Biol.* 2, 733.
- (17) Chen, X., Ramakrishnan, B., and Sundaralingam, M. (1997) Crystal structures of the side-by-side binding of distamycin to AT-containing DNA octamers d(ICITACIC) and d(ICATATIC). *J. Mol. Biol.* 267, 1157.
- (18) Wemmer, D. E., Geierstanger, B. H., Fagan, P. A., Dwyer, T. J., Jacobsen, J. P., Pelton, J. G., Ball, G. E., Leheny, A. R., Chang, W.-H., Bathini, Y., Lown, J. W., Rentzeperis, D., Marky, L. A., Singh, S., and Kollma, P. (1994) Minor groove Recognition of DNA by distamycin and its analogs. In *Structural Biology: The State of Art* (R. H. Sarma and M. H. Sarma, Eds.) pp 301–323, Adenine Press, New York.
- (19) Neidle, S. N. (1997) Crystallographic insights into DNA minor groove recognition by drugs. *Biopolymers* 44, 105.
- (20) Van Dyke, M. W., Hertzberg, R. P., and Dervan, P. B. (1983) Map of distamycin, netropsin and actinomycin binding sites on heterogeneous DNA: DNA cleavage-inhibition patterns with methidiumpropyl-EDTA·Fe(II). *Proc. Natl. Acad. Sci. U.S.A.* 79, 5470.
- (21) Portugal, J., and Waring, M. J. (1987) Comparison of binding sites in DNA for berenil, netropsin and distamycin. A footprinting study. *Eur. J. Biochem.* 167, 281.
- (22) Portugal, J., and Waring, M. J. (1987) Hydroxyl radical footprinting of the sequence-selective binding of netropsin and distamycin to DNA. *FEBS Lett.* 225, 195.
- (23) Ward, B., Rehfuess, R., and Dabrowiak, J. C. (1987) Quantitative footprinting analysis of the netropsin-DNA interaction. *J. Biomol. Struct. Dyn.* 4, 685.
- (24) Ward, B., Rehfuess, R., Goodisman, J., and Dabrowiak, J. C. (1988) Determination of netropsin-DNA binding constants from footprinting data. *Biochemistry* 27, 1198.
- (25) Abu-Daya, A., Brown, P. M., and Fox, K. R. (1995) DNA sequence preferences of several AT-selective minor groove binding ligands. *Nucleic Acids Res.* 23, 3385.
- (26) Singh, S. B., Wemmer, D. E., and Kollman, P. A. (1994) Relative binding affinities of distamycin and its analog to d(CGCAAGTTGGC)·d(GCCAACTTGCG): Comparison of simulation results with experiment. *Proc. Natl. Acad. Sci. U.S.A.* 91, 7673.
- (27) Tabernero, L., Bella, J., and Aleman, C. (1996) Hydrogen bond geometry in DNA-minor groove binding drug complexes. *Nucleic Acids Res.* 24, 3458.
- (28) Rentzeperis, D., and Marky, L. A. (1995) Interaction of minor groove ligands to an AAATT/AATTT site: Correlation of thermodynamic characterization and solution structure. *Biochemistry* 34, 2937.
- (29) a. Bruzik, J. P., Auble, D. T., and DeHaseth, P. L. (1987) Specific activation of transcription initiation by the sequence specific DNA-binding agents distamycin A and netropsin. *Biochemistry* 26, 950. b. Fesen, M., and Pommier, Y. (1989) Mammalian topoisomerase II activity is modulated by the DNA minor groove binder distamycin in simian virus 40 DNA. *J. Biol. Chem.* 264, 11354.
- (30) Woynarowski, J. M., McHugh, M., Sigmund, R. D., and Beerman, T. A. (1989) Modulation of topoisomerase II catalytic activity by DNA minor groove binding agents distamycin, Hoechst 33258, and 4',6-diamidino-2-phenylindole. *Mol. Pharmacol.* 35, 177.
- (31) Woynarowski, J. M., Sigmund, R. D., and Beerman, T. A. (1989) DNA minor groove binding agents interfere with topoisomerase II mediated lesions induced by epipodophyllotoxin derivative VM-26 and acridine derivative m-AMSA in nuclei from L1210 cells. *Biochemistry* 28, 3850.
- (32) McHugh, M. M., Woynarowski, J. M., Sigmund, R. D., and Beerman, T. A. (1989) Effect of minor groove binding drugs on mammalian topoisomerase I activity. *Biochem. Pharmacol.* 38, 2323.
- (33) Broggin, M., Ponti, M., Ottolenghi, S., D'Incalci, M., Mongelli, N., and Mantovani, R. (1989) Distamycins inhibit the binding of OTF-1 and NFE-1 transactors to their conserved DNA elements. *Nucleic Acids Res.* 17, 1051.
- (34) Levy, A., Weisman-Shomer, P., and Fry, M. (1989) Distamycin paradoxically stimulates the copying of oligo(dA)·Poly(dT) by DNA polymerases. *Biochemistry* 28, 7262.
- (35) McHugh, M., Sigmund, R. D., and Beerman, T. A. (1990) Effects of minor groove binding drugs on camptothecin-induced DNA lesions in L1210 nuclei. *Biochem. Pharmacol.* 39, 707.
- (36) Gambari, R., Barbieri, R., Nastruzzi, C., Chiorboli, V., Feriotto, G., Natali, G. P., Giacomini, P., and Arcamone, F. (1991) Distamycin inhibits the binding of a nuclear factor to the -278/-256 upstream sequence of the human HLA-DRα gene. *Biochem. Pharmacol.* 41, 497.
- (37) Dorn, A., Affolter, M., Muller, M., Gehring, W. J., and Leupin, W. (1992) Distamycin-induced inhibition of homeo-domain-DNA complexes. *EMBO J.* 11, 279.
- (38) Ueno, A., Baek, K., Jeon, C., and Agarwal, K. (1992) Netropsin specifically enhances RNA polymerase II termination at terminator sites in vitro. *Proc. Natl. Acad. Sci. U.S.A.* 89, 3676.
- (39) Oakley, M. G., Mrksich, M., and Dervan, P. B. (1992) Evidence that a minor groove-binding peptide and a major groove-binding protein can simultaneously occupy a common binding site on DNA. *Biochemistry* 31, 10969.
- (40) Chai, S., and Alonso, J. C. (1996) Distamycin-induced inhibition of formation of a nucleoprotein complex between the terminase small subunit G1P and the nonencapsidated end (pacL site) of *Bacillus subtilis* bacteriophage SPP1. *Nucleic Acids Res.* 24, 282.
- (41) Wartell, R. M., Larson, J. E., and Wells, R. D. (1974) Netropsin. A specific probe for A-T regions of duplex deoxyribonucleic acid. *J. Biol. Chem.* 249, 6719.
- (42) Zimmer, C., and Wahnert, U. (1986) Non-intercalating DNA-binding ligands: specificity of the interaction and there use as tools in biophysical, biochemical and biological investigations of the genetic material. *Prog. Biophys. Mol. Biol.* 47, 31.
- (43) Kopka, M. L., and Larsen, T. A. (1992) Netropsin and the lexitropsins. The search for sequence-specific minor-groove-binding ligands. In *Nucleic Acid Targeted Drug Design* (C. L. Propst and T. J. Perun, T. J., Eds.) pp 303–374, Dekker, New York.
- (44) Youngquist, R. S., and Dervan, P. B. (1985) Sequence specific recognition of B-DNA by oligo(N-methylpyrrole carbamides). *Proc. Natl. Acad. Sci. U.S.A.* 82, 2565.

- (45) Dervan, P. B. (1986) Design of sequence-specific DNA-binding molecules. *Science* 232, 464.
- (46) Goodsell, D., and Dickerson, R. E. (1986) Isohelical analysis of groove-binding drugs. *J. Med. Chem.* 29, 727.
- (47) Khorlin, A. A., Krylov, A. S., Grokhovsky, S. L., Zhuze, A. L., Zasedatelev, A. S., Gursky, G. V., and Gottikh, B. P. (1980) A new type of AT-specific ligand constructed of two netropsin-like molecules. *FEBS Lett.* 118, 311.
- (48) Lown, J. W., Krowicki, K., Balzarini, J., Newman, R. A., and De Clercq, E. (1989) Novel linked antiviral and antitumor agents related to netropsin and distamycin: synthesis and biological evaluation. *J. Med. Chem.* 32, 2368.
- (49) Wang, W., and Lown, J. W. (1992) Anti-HIV-I activity of linked lexitropsins. *J. Med. Chem.* 35, 2890.
- (50) Burckhardt, G., Simon, H., Störl, K., Triebel, H., Walter, A., Lown, J. W., and Zimmer, Ch. (1997) DNA binding studies and influence on the activity of DNA topoisomerases of bis-netropsin: different effects of analogues containing cis and trans ethylene linkers. *J. Biomol. Struct. Dyn.* 15, 81.
- (51) Wang, Z., Zimmer, C., Lown, J. W., and Knippers, R. (1997) Effects of bifunctional netropsin-related minor groove-binding ligands on mammalian type I topoisomerase. *Biochem. Pharmacol.* 53, 309.
- (52) Kissinger, K. L., Dabrowiak, J. C., and Lown, J. W. (1990) Molecular recognition between oligopeptides and nucleic acids: DNA binding specificity of a series of bis-netropsin analogues deduced from footprinting analysis. *Chem. Res. Toxicol.* 3, 162.
- (53) Rao, K. E., Zimmermann, J., and Lown, J. W. (1991) Sequence-selective DNA binding by linked bis-*N*-methylpyrrole dipeptides: an analysis by MPE footprinting and force field calculations. *J. Org. Chem.* 56, 786.
- (54) Singh, M. P., Plouvier, B., Hill, G. C., Gueck, J., Pon, R. T., and Lown, J. W. (1994) Isohelicity and strand selectivity in the minor groove binding of chiral (1R,2R)- and (1S,2S)-bis(netropsin)-1,2-cyclopropanedicarboxamide ligands to duplex DNA. *J. Am. Chem. Soc.* 116, 7006.
- (55) Filipowsky, M. E., Kopka, M. L., Brazil-Zison, M., Lown, J. W., and Dickerson, R. E. (1996) Linked lexitropsins and the *in vitro* inhibition of HIV-1 reverse transcriptase RNA-directed DNA polymerization: A novel induced-fit of 3,5-*m*-pyridyl bisdistamycin to enzyme-associated template-primer. *Biochemistry* 35, 15397.
- (56) Leinsoo, T. A., Nikolaev, V. A., Grokhovskii, S. L., Surovaya, A. M., Sidorova, N. Y., Streltsov, S. A., Zasedatelev, A. S., and Zhuze, A. L. (1989) Synthetic DNA-binding ligands containing reaction centers specific for AT and GC base pairs. *Mol. Biol.* 23, 1616.
- (57) Nikolaev, V. A., Grokhovsky, S. L., Surovaya, A. N., Leinsoo, T. A., Sidorova, N. Y., Zasedatelev, A. S., Zhuze, A. L., Strahan, G. A., Shafer, R. H., and Gursky, G. V. (1996) Design of sequence-specific DNA binding ligands that use a two-stranded peptide motif for DNA sequence recognition. *J. Biomol. Struct. Dyn.* 14, 31.
- (58) Zakrzewska, K., and Pullman, B. (1988) Theoretical study of the sequence selectivity of isoxans, isohelical DNA groove binding ligands. Proposal for GC minor groove specific compounds. *J. Biomol. Struct. Dyn.* 5, 1043.
- (59) Pullman, B. (1989) Molecular mechanisms of specificity in DNA-antitumor drug interactions. In *Perspectives in Quantum Chemistry* (J. Jortner and B. Pullman, Eds.) Kluwer Academic Publishers, Dordrecht.
- (60) Zakrzewska, K., Randrianarivelino, M., and Pullman, B. (1988) Drug recognition of DNA. Proposal for GC minor groove specific ligands: vinyloxins. *J. Biomol. Struct. Dyn.* 6, 331.
- (61) Marchand, C., Bailly, C., McLean, M. J., Moroney, S. E., and Waring, M. J. (1992) The 2-amino group of guanine is absolutely required for specific binding of the anti-cancer antibiotic echinomycin to DNA. *Nucleic Acids Res.* 20, 5601.
- (62) Bailly, C., Marchand, C., and Waring, M. J. (1993) New binding sites for antitumor antibiotics created by relocating the purine 2-amino group in DNA. *J. Am. Chem. Soc.* 115, 3784.
- (63) Waring, M. J., and Bailly, C. (1994) The purine 2-amino group as a critical recognition element for binding of small molecules to DNA. *Gene* 149, 69.
- (64) Bailly, C., and Waring, M. J. (1995) Transferring the purine 2-amino group from guanines to adenines in DNA changes the sequence-specific binding of antibiotic. *Nucleic Acids Res.* 23, 885.
- (65) Bailly, C., Payet, D., Travers, A. A., and Waring, M. J. (1996) PCR-based development of DNA substrates containing modified bases: an efficient system for investigating the role of the exocyclic groups in chemical and structural recognition by minor groove binding drugs and proteins. *Proc. Natl. Acad. Sci. U.S.A.* 93, 13623–13628.
- (66) Lown, J. W. (1988) Lexitropsins: rational design of DNA sequence reading agents as novel anti-cancer agents and potential cellular probes. *Anti-Cancer Drug Des.* 3, 25.
- (67) Lown, J. W. (1994) DNA recognition by lexitropsins, minor groove binding agents. *J. Mol. Recogni.* 7, 79.
- (68) Lown, J. W., Krowicki, K., Balzarini, J., and De Clercq, E. (1986) Structure–activity relationship of novel oligopeptide antiviral and antitumor agents related to netropsin and distamycin. *J. Med. Chem.* 29, 1210.
- (69) Lown, J. W., Krowicki, K., Bhat, U. G., Skorobogaty, A., Ward, B., and Dabrowiak, J. C. (1986) Molecular recognition between oligopeptides and nucleic acids: novel imidazole-containing oligopeptides related to netropsin that exhibit altered DNA sequence specificity. *Biochemistry* 25, 7408.
- (70) Krowicki, K., and Lown, J. W. (1987) Synthesis of novel imidazole-containing DNA minor groove binding oligopeptides related to the antiviral antibiotic netropsin. *J. Org. Chem.* 52, 3493.
- (71) Kissinger, K. L., Krowicki, K., Dabrowiak, J. C., and Lown, J. W. (1987) Molecular recognition between oligopeptides and nucleic acids: monocationic imidazole lexitropsins that display enhanced GC sequence dependent DNA binding. *Biochemistry* 26, 5590.
- (72) Lee, M., Pon, R. T., Krowicki, K., and Lown, J. W. (1988) Structural and dynamic aspects of the sequence specific binding of netropsin and its bis-imidazole analogue on the decaoxyribonucleotide d(CGCAATTGCG)₂. *J. Biomol. Struct. Dyn.* 5, 939.
- (73) Lee, M., Coulter, D. M., Pon, R. T., Krowicki, K., and Lown, J. W. (1988) Sequence specific molecular recognition and binding of a monocationic bis-imidazole lexitropsin to the decaoxyribonucleotide d[(GATCCGTATG):(CATACGGATC)]: structural and dynamic aspects of intermolecular exchange studied by ¹H NMR. *J. Biomol. Struct. Dyn.* 5, 1059.
- (74) Lee, M., Hartley, J. A., Pon, R. T., Krowicki, K., and Lown, J. W. (1988) Sequence specific molecular recognition by a monocationic lexitropsin of the decaoxyribonucleotide d(CATGGCCATG)₂: structural and dynamic aspects deduced from high field ¹H NMR studies. *Nucleic Acids Res.* 16, 665.
- (75) Lee, M., Chang, D. K., Hartley, J. A., Pon, R. T., Krowicki, K., and Lown, J. W. (1988) Structural and dynamic aspects of binding of a prototype lexitropsin to the decaoxyribonucleotide d(CGCAATTGCG)₂ deduced from high resolution ¹H NMR studies. *Biochemistry* 27, 445.
- (76) Lee, M., Krowicki, K., Hartley, J. A., Pon, R. T., and Lown, J. W. (1988) Molecular recognition between oligopeptides and nucleic acids: influence of Van der Waals contacts in determining the 3'-terminus of DNA sequences read by monocationic lexitropsins. *J. Am. Chem. Soc.* 110, 3641.
- (77) Burckhardt, G., Luck, G., Zimmer, C., Störl, J., Krowicki, K., and Lown, J. W. (1989) Variation of DNA sequence specificity of DNA-oligopeptide binding ligands related to netropsin: imidazole-containing lexitropsins. *Biochim. Biophys. Acta* 1009, 11.
- (78) Lee, M., Rhodes, A. L., Wyatt, M. D., Forrow, S., and Hartley, J. A. (1993) GC base sequence recognition by oligo-(imidazolecarboxamide) and C-terminus-modified analogues of distamycin deduced from circular dichroism, proton nuclear magnetic resonance, and methidiumpropylethylenediamine-tetraacetate-iron(II) footprinting studies. *Biochemistry* 32, 4237.

- (79) Wyatt, M. D., Garbiras, B. J., Lee, M., Forrow, S. M., and Hartley, J. A. (1994) Synthesis and DNA binding properties of a series of N to C linked and imidazole containing analogues of distamycin. *Bioorg. Med. Chem. Lett.* 4, 801.
- (80) Rao, K. E., Bathini, Y., and Lown, J. W. (1990) Synthesis of novel thiazole containing DNA minor groove binding oligopeptides related to the antibiotic distamycin. *J. Org. Chem.* 55, 728.
- (81) Rao, K. E., Shea, R. G., Yadagiri, B., and Lown, J. W. (1990) Molecular recognition between oligopeptides and nucleic acids: DNA sequence specificity and binding properties of thiazole-lexitropsins incorporating the concepts of base site acceptance and avoidance. *Anti-Cancer Drug Des.* 5, 3.
- (82) Kumar, S., Jaseja, M., Zimmermann, J., Yadagiri, B., Pon, R. T., Sapse, A. M., and Lown, J. W. (1990) Molecular recognition and binding of a GC site-avoiding thiazole-lexitropsin to the decaoxyribonucleotide d(CGCAATTGCG)₂: ¹H NMR evidence for thiazole intercalation. *J. Biomol. Struct. Dyn.* 8, 99.
- (83) Kumar, S., Bathini, Y., Joseph, T., Pon, R. T., and Lown, J. W. (1991) Structure and dynamics aspects of non-intercalative (1:1) binding of a thiazole-lexitropsin to the decaoxyribonucleotide d(CGCAATTGCG)₂: an ¹H NMR and molecular modeling study. *J. Biomol. Struct. Dyn.* 9 (1).
- (84) Plouvier, B., Bailly, C., Houssin, R., Rao, K. E., Lown, J. W., Hénichart, J.-P., and Waring, M. J. (1991) DNA-sequence specific recognition by a thiazole analogue of netropsin: a comparative footprinting study. *Nucleic Acids Res.* 19, 5821.
- (85) Lee, M., Krowicki, K., Shea, R. G., Lown, J. W., and Pon, R. T. (1989) Molecular recognition between oligopeptides and nucleic acids. Specificity of binding of a monocationic bis-furan lexitropsin to DNA deduced from footprinting and ¹H NMR studies. *J. Mol. Recognit.* 2, 84.
- (86) Pullman, A., and Pullman, B. (1981) Molecular electrostatic potential of the nucleic acids. *Q. Rev. Biophys.* 14, 289.
- (87) Marky, L. A., and Breslauer, K. J. (1987) Origins of netropsin binding affinity and specificity: correlation of thermodynamic and structural data. *Proc. Natl. Acad. Sci. U.S.A.* 84, 4359.
- (88) Singh, S. B., Wemmer, D. E., and Kollman, P. A. (1994) Relative binding affinities of distamycin and its analog to d(CGCAAGTTGGC)-d(GCCAACTTGCG): Comparison of simulation results with experiment. *Proc. Natl. Acad. Sci. U.S.A.* 91, 7673.
- (89) Goodsell, D. S., Leung Ng, H., Kopka, M. L., Lown, J. W., and Dickerson, R. E. (1995) Structure of a dicationic mono-imidazole lexitropsin bound to DNA. *Biochemistry* 34, 16654.
- (90) Zhang, Y., Chen, F.-X., Mehta, P., and Gold, B. (1993) Groove- and sequence-selective alkylation of DNA by sulfonate esters tethered to lexitropsins. *Biochemistry* 32, 7954.
- (91) Haq, I., Ladbury, J. E., Chowdhry, B. Z., Jenkins, T. C., and Chaires, J. B. (1997) Specific binding of Hoechst 33258 to the d(CGCAAATTTGCG)₂ duplex: calorimetric and spectroscopic studies. *J. Mol. Biol.* 271, 244.
- (92) Wade, W. S., and Dervan, P. B. (1987) Alteration of the sequence specificity of distamycin on DNA by replacement of an N-methylpyrrole carboxamide with pyridine-2-carboxamide. *J. Am. Chem. Soc.* 109, 1574.
- (93) Bielawski, K., Bartulewicz, D., Krajewska, D., and Rozanski, A. (1996) Synthetic analogues of netropsin and distamycin. III. Synthesis of a pyridine analogue of the antitumor antibiotics. *Rocz. Akad. Białymst.* 41, 293–304.
- (94) Bailly, C., Colson, P., Houssier, C., Houssin, R., Mrani, D., Gosselin, G., Imbach, J. L., Waring, M. J., Lown, J. W., and Hénichart, J. P. (1992) Binding properties and DNA sequence-specific recognition of two bithiazole-linked netropsin hybrid molecules. *Biochemistry* 31, 8349.
- (95) Fagan, P., and Wemmer, D. E. (1992) Cooperative binding of distamycin-A to DNA in the 2:1 mode. *J. Am. Chem. Soc.* 114, 1080.
- (96) Animati, F., Arcamone, F. M., Conte, M. R., Felicetti, P., Galeone, A., Lombardi, P., Mayol, L., Paloma, L. G., and Rossi, C. (1995) Synthesis of two distamycin analogues and their binding mode to d(CGCAAATTTGCG)₂ in the 2:1 solution complexes as determined by two-dimensional ¹H NMR. *J. Med. Chem.* 38, 1140.
- (97) Wade, W. S., Mrksich, M., and Dervan, P. B. (1993) Binding affinities of synthetic peptides, pyridine-2-carboxamidone-tropsin and 1-methylimidazole-2-carboxamidonetropsin, that form 2:1 complexes in the minor groove of double-helical DNA. *Biochemistry* 32, 11385.
- (98) Geierstanger, B. H., Jacobsen, J. P., Mrksich, M., Dervan, P. B., and Wemmer, D. E. (1994) Structural and dynamic characterization of the heterodimeric and homodimeric complexes between distamycin and 1-methylimidazole-2-carboxamine-netropsin bound to the minor groove of DNA. *Biochemistry* 33, 3055.
- (99) Mrksich, M., Wade, W. S., Dwyer, T. J., Geierstanger, B. H., Wemmer, D. E., and Dervan, P. B. (1992) Antiparallel side-by-side dimeric motif for sequence-specific recognition in the minor groove of DNA by the designed peptide 1-methylimidazole-2-carboxamide netropsin. *Proc. Natl. Acad. Sci. U.S.A.* 89, 7586.
- (100) Geierstanger, B. H., Mrksich, M., Dervan, P. B., and Wemmer, D. E. (1994) Design of a G·C-specific DNA minor groove-binding peptide. *Science* 266, 646.
- (101) Kopka, M. L., Goodsell, D. S., Han, G. W., Chiu, T. K., Lown, J. W., and Dickerson, R. E. (1997) Definign GC-specificity in the minor groove: side-by-side binding of di-imidazole lexitropsin to CATGGCCATG. *Structure* 5, 1033.
- (102) Mrksich, M., and Dervan, P. B. (1993) Enhanced sequence specific recognition in the minor groove of DNA by covalent peptide dimers: (bis(pyridine-2-carboxamidonetropsin))(CH₂)_{3–6}. *J. Am. Chem. Soc.* 115, 9892.
- (103) Mrksich, M., and Dervan, P. B. (1994) Design of a covalent peptide heterodimer for sequence-specific recognition in the minor groove of double-helical DNA. *J. Am. Chem. Soc.* 116, 3663.
- (104) Dwyer, T. J., Geierstanger, B. H., Mrksich, M., Dervan, P. B., and Wemmer, D. E. (1993) Structural analysis of covalent peptide dimers. Bis(pyridine-2-carboxamidonetropsin)-(CH₂)_{3–6}, in complex with 5'-TGACT-3' sites by two-dimensional NMR. *J. Am. Chem. Soc.* 115, 9900.
- (105) Mrksich, M., and Dervan, P. B. (1994) Hairpin peptide motif. A new class of oligopeptides for sequence-specific recognition in the minor groove of double-helical DNA. *J. Am. Chem. Soc.* 116, 7983.
- (106) Cho, J., Parks, M. E., and Dervan, P. B. (1995) Cyclic polyamides for recognition in the minor groove of DNA. *Proc. Natl. Acad. Sci. U.S.A.* 92, 10389.
- (107) Trauger, J. W., Baird, E. E., Mrksich, M., and Dervan, P. B. (1996) Extension of sequence-specific recognition in the minor groove of DNA by pyrrole-imidazole polyamide to 9–13 base pairs. *J. Am. Chem. Soc.* 118, 6160.
- (108) Al-Said, N. H., and Lown, J. W. (1994) Synthesis of novel cross-linked bis-lexitropsins. *Tetrahedron Lett.* 35, 7577.
- (109) Chen, Y.-H., and Lown, J. W. (1994) A new DNA minor groove binding motif: cross-linked lexitropsins. *J. Am. Chem. Soc.* 116, 6995.
- (110) Chen, Y.-H., Yang, Y., and Lown, J. W. (1996) Optimization of cross-linked lexitropsins. *J. Biomol. Struct. Dyn.* 14, 341.
- (111) Chen, Y.-H., Yang, Y., and Lown, J. W. (1996) Design of distamycin analogues to probe the physical origin of the antiparallel side by side oligopeptide binding motif in DNA minor groove recognition. *Biochem. Biophys. Res. Commun.* 220, 213.
- (112) Parks, M. E., Baird, E. E., and Dervan, P. B. (1996) Optimization of the hairpin polyamide design for recognition of the minor groove of DNA. *J. Am. Chem. Soc.* 118, 6147.
- (113) Parks, M. E., Baird, E. E., and Dervan, P. B. (1996) Recognition of 5'-(A,T)GG(A,T)₂-3' sequences in the minor groove of DNA by hairpin polyamides. *J. Am. Chem. Soc.* 118, 6153.
- (114) Kelly, J. J., Baird, E. E., and Dervan, P. B. (1996) Binding site size limit of the 2:1 pyrrole-imidazole polyamide-DNA motif. *Proc. Natl. Acad. Sci. U.S.A.* 93, 6981.
- (115) Pilch, D. S., Poklar, N., Gelfand, C. A., Law, S. M., Breslauer, K. J., Baird, E. E., and Dervan, P. B. (1996) Binding of a hairpin polyamide in the minor groove of DNA: sequence-specific enthalpic discrimination. *Proc. Natl. Acad. Sci. U.S.A.* 93, 8306.

- (116) White, S., Baird, E. E., and Dervan, P. B. (1996) Effects of the A·T/T·A degeneracy of pyrrole-imidazole polyamide recognition in the minor groove of DNA. *Biochemistry* 35, 12532.
- (117) Trauger, J. W., Baird, E. E., and Dervan, P. B. (1996) Extended hairpin polyamide motif for sequence-specific recognition in the minor groove of DNA. *Chem. Biol.* 3, 369.
- (118) Walker, W. L., Landaw, E. M., Dickerson, R. E., and Goodsell, D. S. (1997) Estimation of the DNA sequence discriminatory ability of hairpin-linked lexitropsins. *Proc. Natl. Acad. Sci. U.S.A.* 94, 5634.
- (119) Lamanie de Clairac, R. P., Geierstanger, B. H., Mrksich, M., Dervan, P. B., and Wemmer, D. E. (1997) NMR characterization of hairpin polyamide complexes with the minor groove of DNA. *J. Am. Chem. Soc.* 119, 7909.
- (120) White, S., Baird, E. E., and Dervan, P. B. (1997) Orientation preferences of pyrrole-imidazole polyamides in the minor groove of DNA. *J. Am. Chem. Soc.* 119, 6953.
- (121) White, S., Baird, E. E., and Dervan, P. B. (1997) On the pairing rules for recognition in the minor groove of DNA by pyrrole-imidazole polyamides. *Chem. Biol.* 4, 569.
- (122) Geierstanger, B. H., Mrksich, M., Dervan, P. B., and Wemmer, D. E. (1996) Extending the recognition site of designed minor groove binding molecules. *Nat. Struct. Biol.* 3, 321.
- (123) Baird, E. E., and Dervan, P. B. (1997) Solid-phase synthesis of polyamides containing imidazole and pyrrole amino acids. *J. Am. Chem. Soc.* 118, 6141.
- (124) Swalley, S. E., Baird, E. E., and Dervan, P. B. (1997) Discrimination of 5'-GGGG-3', 5'-GCGC-3', and 5'-GGCC-3' sequences in the minor groove of DNA by eight-ring hairpin polyamides. *J. Am. Chem. Soc.* 119, 8756.
- (125) Swalley, S. E., Baird, E. E., and Dervan, P. B. (1997) Recognition of a 5'(A,T)GGG(A,T)₂-3' sequence in the minor groove of DNA by a eight-ring hairpin polyamide. *J. Am. Chem. Soc.* 119, 8198-8206.
- (126) Turner, J. M., Baird, E. E., and Dervan, P. B. (1997) Recognition of seven base pair sequences in the minor groove of DNA by ten-ring pyrrole-imidazole polyamide hairpin. *J. Am. Chem. Soc.* 119, 7636.
- (127) Kielkopf, C. L., Baird, E. E., Dervan, P. B., and Rees, D. C. (1998) Structural basis for G·C recognition in the DNA minor groove. *Nat. Struct. Biol.* 5, 104.
- (128) White, S., Szewczyk, J. W., Turner, J. M., Baird, E. E., and Dervan, P. B. (1998) Recognition of the four Watson-Crick base pairs in the DNA minor groove by synthetic ligands. *Nature* 391, 468.
- (129) Herman, D. M., Baird, E. E., and Dervan, P. B. (1998) Stereochemical control of the DNA binding affinity, sequence specificity, and orientation preference of chiral hairpin polyamides in the minor groove. *J. Am. Chem. Soc.* 120, 1382.
- (130) Weisz, K. (1997) Polyamides as artificial regulators of gene expression. *Angew. Chem., Int. Ed. Engl.* 36, 2592.
- (131) Walker, W. L., Landaw, E. M., Dickerson, R. E., and Goodsell, D. S. (1997) Estimation of the DNA sequence discriminatory ability of hairpin-linked lexitropsins. *Proc. Natl. Acad. Sci. U.S.A.* 94, 5634.
- (132) Wemmer, D. (1998) Reading DNA. *Nat. Struct. Biol.* 5, 169.
- (133) Hélène, C. (1998) Reading the minor groove. *Nature* 391, 43.
- (134) Chaires, J. B. (1998) Drug-DNA interactions. *Curr. Opin. Struct. Biol.* (in press).
- (135) Trauger, J. W., Baird, E. E., and Dervan, P. B. (1996) Recognition of DNA by designed ligands at subnanomolar concentrations. *Nature* 382, 559.
- (136) Wemmer, D. E., and Dervan, P. B. (1997) Targeting the minor groove of DNA. *Curr. Opin. Struct. Biol.* 7, 355.
- (137) Gottesfeld, J. M., Neely, L., Trauger, J. W., Baird, E. E., and Dervan, P. B. (1997) Regulation of gene expression by small molecules. *Nature* 387, 202.
- (138) Sinyakov, A. N., Lokhov, S. G., Kutayvin, I. V., Gamper, H. B., and Meyer, R. B., Jr. (1995) Exceptional and selective stabilization of A-T rich DNA-DNA duplexes by N-methylpyrrole carboxamide peptides conjugated to oligodeoxynucleotides. *J. Am. Chem. Soc.* 117, 4995.
- (139) Szewczyk, J. W., Baird, E. E., and Dervan, P. B. (1996) Cooperative triple-helix formation via a minor groove dimerization domain. *J. Am. Chem. Soc.* 118, 6778.
- (140) Szewczyk, J. W., Baird, E. E., and Dervan, P. B. (1996) Sequence-specific recognition of DNA by a major and minor groove binding ligand. *Angew. Chem., Int. Ed. Engl.* 35, 1487.
- (141) Alfieri, A., Animati, F., Arcamone, F., Bailly, C., Dentini, M., Felicetti, P., Iafrate, E., Lombardi, P., Manzini, S., Rossi, C., and Waring, M. J. (1997) Biological activity and DNA sequence specificity of synthetic carbamoyl analogues of distamycin. *Antiviral Chem. Chemother.* 8, 243.
- (142) Browne, K. A., He, G.-X., and Bruice, T. C. (1993) Microgonotropens and their interactions with DNA. 2. Quantitative evaluation of equilibrium constants for 1:1 and 2:1 binding of dien-microgonotropen-a, -b, and -c as well as distamycin and Hoechst 33258. *J. Am. Chem. Soc.* 115, 7072.
- (143) Blaskó, A., and Bruice, T. C. (1993) Stoichiometry and structure of complexes of DNA oligomers with microgonotropens and distamycin by ¹H NMR spectroscopy and molecular modeling. *Proc. Natl. Acad. Sci. U.S.A.* 90, 10018.
- (144) Blaskó, A., Browne, K. A., and Bruice, T. C. (1994) Microgonotropens and their interactions with DNA. 5. Structural characterization of the 1:1 complex of d(CGCAAATTGCG)₂ and tren-microgonotropen-b by 2D NMR spectroscopy and restrained molecular modeling. *J. Am. Chem. Soc.* 116, 3726.
- (145) He, G.-X., Browne, K. A., Blaskó, A., and Bruice, T. C. (1994) Microgonotropens and their interactions with DNA. 4. Synthesis of the tripyrrole peptides tren-microgonotropen-a, -b, and -c and characterization of their interactions with dsDNA. *J. Am. Chem. Soc.* 116, 3716.
- (146) He, G.-X., Browne, K. A., Groppe, J. C., Blaskó, A., Mei, H.-Y., and Bruice, T. C. (1993) Microgonotropens and their interactions with DNA. 1. Synthesis of the tripyrrole peptides dien-microgonotropen-a, -b, and -c and characterization of their interactions with dsDNA. *J. Am. Chem. Soc.* 115, 7061.
- (147) Blaskó, A., Browne, K. A., and Bruice, T. C. (1995) NMR structure of d(CGCA₃T₃GCG)₂: Tren-microgonotropen-b:Zn(II) complex and solution studies of metal ion complexes of Tren-microgonotropen-b interacting with DNA. *BioOrg. Med. Chem.* 3, 631.
- (148) Xue, T., Browne, K. A., and Bruice, T. C. (1995) A novel minor groove binding reagent designed to serve as a "truck" to carry DNA modifying moieties into the major groove. *Bioconjugate Chem.* 6, 82.
- (149) Hansma, H. G., Browne, K. A., Groppe, J. C., Bezanilla, M., and Bruice, T. C. (1994) Bending and straightening of DNA induced by the same ligand: characterization with atomic force microscope. *Biochemistry* 33, 8436.
- (150) Bruice, T. C., Sengupta, D., Blaskó, A., Chiang, S.-Y., and Beerman, T. A. (1997) A microgonotropen branched cecaaza decabutylamine and its DNA and DNA/transcription factor interactions. *BioOrg. Med. Chem.* 5, 685.
- (151) Browne, K. A., and Bruice, T. C. (1992) Chemistry of phosphodiester, DNA and models. 2. The hydrolysis of bis-(8-hydroxyquinoline) phosphate in the absence and presence of metal ions. *J. Am. Chem. Soc.* 114, 4951.
- (152) Bruice, T. C., Mei, H.-H., He, G.-X., and Lopez, V. (1992) Rational design of substituted trispyrrole peptides that complex with DNA by both selective minor-groove binding and electrostatic interaction with the phosphate backbone. *Proc. Natl. Acad. Sci. U.S.A.* 89, 1700.
- (153) Hartley, J. A. (1993) Selectivity in alkylating agent-DNA interactions. In *Molecular Aspects of Anticancer Drug-DNA Interactions* (S. Neidle and M. J. Waring, Eds.) Vol. 1, pp 1-31, Macmillan, London.
- (154) Hansen, M., and Hurley, L. (1996) Pluramycins. Old drugs having modern friends in structural biology. *Acc Chem. Res.* 29, 249.
- (155) Tomasz, M. (1994) The mitomycins: natural cross-linkers of DNA. In *Molecular Aspects of Anticancer Drug-DNA Interactions* (S. Neidle and M. J. Waring, Eds.) Vol. 2, pp 312-349, Macmillan, London.

- (156) Boyd, F. L., Cheatham, S. F., Remers, W., Hill, G. C., and Hurley, L. H. (1990) Characterization of the structure of the anthramycin-d(ATGCAT)₂ adduct by NMR and molecular modeling studies. Determination of the stereochemistry of the covalent linkage site, orientation in the minor groove of DNA and effect on local DNA structure. *J. Am. Chem. Soc.* **112**, 3279.
- (157) Kopka, M. L., Goodsell, D., Baikalov, I., Grzeskowiak, K., Cascio, D., and Dickerson, R. E. (1994) Crystal structure of a covalent DNA-drug adduct: anthramycin bound to CCAACGT-TGG and a molecular explanation of specificity. *Biochemistry* **33**, 13593.
- (158) Hurley, L. H., and Draves, P. H. (1993) Molecular aspects of the interaction of (+)-CC-1065 with DNA. In *Molecular Aspects of Anticancer Drug-DNA Interactions* (S. Neidle and M. J. Waring, Eds.) Vol. 1, pp 89–133, Macmillan, London.
- (159) Hurley, L. H., and Sun, D. (1994) (+)-CC-1065 as a probe for intrinsic and protein-induced bending of DNA. *J. Mol. Recognit.* **7**, 123.
- (160) Boger, D. L., and Johnson, D. S. (1996) CC-1065 and the duocarmycins: Understanding their biological function through mechanistic studies. *Angew. Chem., Int. Ed. Engl.* **35**, 1438.
- (161) Krowicki, K., Balzarini, J., De Clercq, E., Newman, R. A., and Lown, J. W. (1988) Novel DNA groove binding alkylators: design, synthesis, and biological evaluation. *J. Med. Chem.* **31**, 341.
- (162) Arcamone, F. A., Animati, F., Barbieri, B., Configliacchi, E., D'Alesio, R., Geroni, C., Giuliani, F. C., Lazzari, E., Menozzi, M., Mongelli, N., Penco, S., and Verini, M. A. (1989) Synthesis, DNA-binding properties, and antitumor activity of novel distamycin derivatives. *J. Med. Chem.* **32**, 774.
- (163) Baker, B. F., and Dervan, P. B. (1985) Sequence-specific cleavage of double-helical DNA. N-bromoacetyldistamycin. *J. Am. Chem. Soc.* **107**, 8266.
- (164) Baker, B. F., and Dervan, P. B. (1989) Sequence-specific cleavage of DNA by N-bromoacetyldistamycin. Product and kinetic analyses. *J. Am. Chem. Soc.* **111**, 2700.
- (165) Lee, M., Rhodes, A. L., Wyatt, M. D., Forrow, S., and Hartley, J. A. (1993) Design, synthesis, and biological evaluation of DNA sequence and minor groove selective alkylating agents. *Anti-Cancer Drug Des.* **8**, 173.
- (166) Xie, G., Gupta, R., and Lown, J. W. (1995) Design, synthesis, DNA sequence preferential alkylation and biological evaluation of N-mustard derivatives of distamycin and netropsin analogues. *Anti-Cancer Drug Des.* **10**, 389.
- (167) Wyatt, M. D., Lee, M., Garbiras, B. J., Souhami, R. L., and Hartley, J. A. (1995) Sequence specificity of alkylation for a series of nitrogen mustard-containing analogues of distamycin of increasing binding site size: Evidence for increased cytotoxicity with enhanced sequence specificity. *Biochemistry* **34**, 13034.
- (168) Wyatt, M. D., Garbiras, B. J., Haskell, M. K., Lee, M., Souhami, R. L., and Hartley, J. A. (1994) Structure-activity relationship of a series of nitrogen-mustard- and pyrrole-containing minor groove-binding agents related to distamycin. *Anti-Cancer Drug Des.* **9**, 511.
- (169) Wyatt, M. D., Lee, M., and Hartley, J. A. (1997) Alkylation specificity for a series of distamycin analogues that tether chlorambucil. *Anti-Cancer Drug Des.* **12**, 49.
- (170) Ciucci, A., Manzini, S., Lombardi, P., and Arcamone, F. (1996) Backbone and benzoyl mustard carrying moiety modifies DNA interactions of distamycin analogues. *Nucleic Acids Res.* **24**, 311.
- (171) Chen, H. Y., Liu, J. X., and Lown, J. W. (1995) Design, synthesis and evaluation of novel bismustard cross-linked lexitropsins. *BioOrg. Med. Chem. Lett.* **5**, 2223.
- (172) Broggin, M., Erba, E., Ponti, M., Ballinari, D., Geroni, C., Spreafico, F., and D'Incalci, M. (1991) Selective DNA interaction of the novel distamycin derivative FCE 24517. *Cancer Res.* **51**, 199.
- (173) Montecucco, A., Fontana, M., Focher, F., Lestingi, M., Spadari, S., and Ciarrochi, G. (1991) Specific inhibition of human DNA ligase adenylation by a distamycin derivative possessing antitumor activity. *Nucleic Acids Res.* **19**, 1067.
- (174) Fontana, M., Lestingi, M., Mondello, C., Braghet, A., Montecucco, A., and Ciarrochi, G. (1992) DNA binding properties of FCE24517, an electrophilic distamycin analogue. *Anti-Cancer Drug Des.* **7**, 131.
- (175) Broggin, M., Coley, H. M., Mongelli, N., Pesenti, E., Wyatt, M. D., Hartley, J. A., and D'Incalci, M. (1995) DNA sequence-specific adenine alkylation by the novel drug tallimustine (FCE 24517), a benzoyl nitrogen mustard derivative of distamycin. *Nucleic Acids Res.* **23**, 81.
- (176) Broggin, M., Moncollin, V., D'Incalci, M., Mongelli, N., and Mantovani, R. (1995) Distamycin A and tallimustine inhibit TBP binding and basal in vitro transcription. *Nucleic Acids Res.* **23**, 1657.
- (177) Pezzoni, G., Grandi, M., Biasoli, G., Capolongo, L., Ballinari, D., Giuliani, F. C., Barbieri, B., Pastori, A., Pesenti, E., and Mongelli, N. (1991) Biological profile of FCE 24517, a novel benzoyl mustard analogue of distamycin A. *Br. J. Cancer* **64**, 1047.
- (178) Coley, H. M., Mongelli, N., and D'Incalci, M. (1993) The effects of a benzoic acid mustard derivative of distamycin A (FCE 24517) and related minor groove-binding distamycin analogues on the activity of major groove-binding alkylating agents. *Biochem. Pharmacol.* **45**, 619.
- (179) Baraldi, P. G., Beria, I., Cacciari, B., Capolongo, L., Cozzi, P., Mongelli, N., Romagnoli, R., and Spalluto, G. (1996) Structure-activity relationship of novel tallimustine derivatives: synthesis and antitumor activity. *BioOrg. Med. Chem. Lett.* **6**, 1247.
- (180) Brooks, N., Hartley, J. A., Simpson, J. E., Wright, S. R., Woo, S., Centioni, S., Fontaine, M. D., McIntyre, T. E., and Lee, M. (1997) Structure-activity relationship of a series of C-terminus modified aminoalkyl, diaminoalkyl- and anilino-containing analogues of the benzoic acid mustard distamycin derivative tallimustine: synthesis, DNA binding and cytotoxicity studies. *BioOrg. Med. Chem.* **5**, 1497.
- (181) Rhoads, C. J. (1946) Nitrogen mustards in treatment of neoplastic disease. *J. Am. Med. Assoc.* **131**, 656.
- (182) Wang, Y., Gupta, R., Huang, L., Luo, W., and Lown, J. W. (1996) Design, synthesis, cytotoxic properties and preliminary DNA sequencing evaluation of CPI-N-methylpyrrole hybrids. Enhancing effect of a trans double bond linker and role of the terminal amide functionality on cytotoxic potency. *Anti-Cancer Drug Des.* **11**, 15.
- (183) Fregeau, N. L., Wang, Y., Pon, R. T., Wylie, W. A., and Lown, J. W. (1995) Characterization of a CPI-lexitropsin conjugate-oligonucleotide covalent complex by ¹H NMR and restrained molecular dynamics simulation. *J. Am. Chem. Soc.* **117**, 8917.
- (184) Shishido, K., Haruna, S., Yamamura, C., Iitsuka, H., Nemoto, H., Shinohara, Y., and Shibuya, M. (1997) Synthesis and evaluation of the hybrid molecules possessing DNA-cleaving activity. *BioOrg. Med. Chem. Lett.* **7**, 2617.
- (185) Church, K. M., Wurdeman, R. L., Zhang, Y., and Gold, B. (1990) N-(2-chloroethyl)-N-nitrosoureas bound to nonionic and monocationic lexitropsin dipeptides. Synthesis, DNA affinity binding characteristics, and reactions with ³²P-end-labeled DNA. *Biochemistry* **29**, 6827.
- (186) Sigurdsson, S. T., Rink, S. M., and Hopkins, P. B. (1993) Affinity cross-linking of duplex DNA by a pyrrole-oligopeptide conjugate. *J. Am. Chem. Soc.* **115**, 12633.
- (187) Fagan, P. A., Spielmann, H. P., Sigurdsson, S. Th., Rink, S. M., Hopkins, P. B., and Wemmer, D. E. (1996) An NMR study of [d(CGCGAATTCGCG)]₂ containing an interstrand cross-link derived from a distamycin-pyrrole conjugate. *Nucleic Acids Res.* **24**, 1566.
- (188) Walker, W. L., Kopka, M. L., Filipowsky, M. E., Dickerson, R. E., and Goodsell, D. S. (1995) Design of B-DNA cross-linking and sequence-reading molecules. *Biopolymers* **35**, 543.
- (189) Gupta, R., Liu, J., Xie, G., and Lown, J. W. (1996) Novel DNA-directed alkylating agents consisting of naphthalimide, nitrogen mustard and lexitropsin moieties: synthesis, DNA sequence specificity and biological evaluation. *Anti-Cancer Drug Des.* **11**, 581.
- (190) Nielsen, P. E. (1990) Chemical and photochemical probing of DNA complexes. *J. Mol. Recognit.* **3**, 1.

- (191) Hartley, J. A., McAdam, S. R., Das, S., Roldan, M. C., Haskell, M. K., and Lee, M. (1994) Molecular and cellular pharmacology of novel photoactive psoralen and coumarin conjugates of pyrrole- and imidazole-containing analogues of netropsin. *Anti-Cancer Drug Des.* 9, 181.
- (192) Rao, K. E., Gosselin, G., Mrani, D., Périgaud, C., Imbach, J. L., Bailly, C., Hénichart, J. P., Colson, P., Houssier, C., and Lown, J. W. (1994) Psoralen-lexitropsin hybrids: DNA sequence selectivity of photoinduced cross-linking from MPE footprinting and exonuclease II stop assay, and mode of binding from electric linear dichroism. *Anti-Cancer Drug Des.* 9, 221.
- (193) Hartley, J. A., Webber, J., Wyatt, M. D., Bordenick, N., and Lee, M. (1995) Novel cytotoxic DNA sequence and minor groove targeted photosensitizers: Conjugates of pyrene and netropsin analogues. *BioOrg. Med. Chem. Lett.* 3, 623.
- (194) Herfeld, P., Helissey, P., and Giorgi-Renault, S. (1994) Poly(pyrrolicarboxamides) linked to photoactivable chromophore isoalloxazine. Synthesis, selective binding, and DNA cleaving properties. *Bioconjugate Chem.* 5, 67.
- (195) Bouziane, M., Ketterlé, C., Helissey, P., Herfeld, P., Le Bret, M., Giorgi-Renault, S., and Auclair, C. (1995) Sequence-directed single-strand cleavage of DNA by a netropsin-flavin hybrid molecule. *Biochemistry* 34, 14051.
- (196) Ketterlé, C., Gabarro-Arpa, J., Ouali, M., Bouziane, M., Auclair, C., Helissey, P., Giorgi-Renault, S., and Le Bret, M. (1996) Binding of Net-Fla, a netropsin-flavin hybrid molecule, to DNA: Molecular mechanics and dynamics studies *in vacuo* and in water solution. *J. Biomol. Struct. Dyn.* 13, 963.
- (197) Helissey, P., Bailly, C., Vishwakarma, J. N., Auclair, C., Waring, M. J., and Giorgi-Renault, S. (1996) DNA minor groove cleaving agents: synthesis, DNA binding and DNA cleaving properties of anthraquinone-oligopyrrolicarboxamide hybrids. *Anti-Cancer Drug Des.* 11, 527–551.
- (198) Meunier, B. (1992) Metalloporphyrins as versatile catalysts for oxidation reactions and oxidative DNA cleavage. *Chem. Rev.* 92, 141.
- (199) Anneheim-Herbelin, G., Perrée-Fauvet, M., Gaudemer, A., Helissey, P., and Giorgi-Renault, S. (1993) Porphyrin-netropsin: a potential ligand of DNA. *Tetrahedron Lett.* 34, 7263.
- (200) Perrée-Fauvet, M., and Gresh, N. (1994) Structure and energetics in the complexes of a double-stranded B-DNA dodecamer with netropsin derivatives of a tricationic water-soluble porphyrin: a theoretical investigation. *J. Biomol. Struct. Dyn.* 11, 1203.
- (201) Matsumoto, T., Utsumi, Y., Sakai, Y., Toyooka, K., and Shibuya, M. (1992) Synthesis of halogenated oligo-*N*-methylpyrrole-carboxamide derivatives and their photochemical DNA cleavage activities. *Heterocycles* 34, 1697.
- (202) Wilson, W. R. (1992) Tumour hypoxia: challenges for cancer chemotherapy. In *The Search for New Anticancer Drugs* (M. J. Waring and B. A. J. Ponder, Eds.) pp 87–131, Kluwer Academic Publishers, London.
- (203) Tocher, J. H., and Edwards, D. I. (1994) Evidence for the direct interaction of reduced metronidazole derivatives with DNA bases. *Biochem. Pharmacol.* 48, 1089.
- (204) Kappen, L. S., Lee, T. R., Yang, C.-C., and Goldberg, I. H. (1989) Oxygen transfer from the nitro group of a nitroaromatic radiosensitizer to a DNA sugar damage product. *Biochemistry* 28, 4540.
- (205) Nishiwaki, E., Lee, H., Matsumoto, T., Toyooka, K., Sakurai, H., and Shibuya, M. (1990) Synthesis of nitrated oligo-*N*-methylpyrrole carboxamide derivatives and their photochemical DNA cleaving properties. *Tetrahedron Lett.* 31, 1299.
- (206) Parrick, J., Porssa, M., Davies, L. K., Dennis, M. F., Patel, K. B., Stratford, M. R. L., and Wardman, P. (1993) Targeting radiosensitizers to DNA by minor groove binding: nitroarenes based on netropsin and distamycin. *Bioorg. Med. Chem. Lett.* 3, 1697.
- (207) Jenkins, T. C., Parrick, J., and Porssa, M. (1994) DNA-binding properties of nitroarene oligopeptides designed as hypoxia-selective agents. *Anti-Cancer Drug Des.* 9, 477.
- (208) Matsumoto, T., Sakai, Y., Toyooka, K., and Shibuya, M. (1992) Synthesis of sulfonamido oligo-*N*-methylpyrrole-carboxamide derivatives and their photochemical DNA cleavage activities. *Heterocycles* 33, 135.
- (209) Nishiwaki, E., Nakagawa, H., Takasaki, M., Matsumoto, T., Sakurai, H., and Shibuya, M. (1990) Synthesis of oligo-*N*-methylpyrrolicarboxamide derivatives and their photochemical DNA cleaving activities. *Heterocycles* 31, 1763.
- (210) Nishiwaki, E., Lee, H., Matsumoto, T., Toyooka, K., Sakurai, H., and Shibuya, M. (1990) Synthesis of nitrated oligo-*N*-methylpyrrole carboxamide derivatives and their photochemical DNA cleaving activities. *Tetrahedron Lett.* 31, 1299.
- (211) Grokhovsky, S. L., and Zubarev, V. E. (1991) Sequence-specific cleavage of double-stranded DNA caused by X-ray ionization of the platinum atom in the Pt-bis-netropsin-DNA complex. *Nucleic Acids Res.* 19, 257.
- (212) Surovaya, A. N., Burckardt, G., Grokhovsky, S. L., Birch-Hirschfeld, E., Gursky, G. V., and Zimmer, C. (1997) Hairpin polyamides that use parallel and antiparallel side-by-side peptide motifs in binding to DNA. *J. Biomol. Struct. Dyn.* 14, 595.
- (213) Dabrowiak, J. C., Stankus, A. A., and Goodisman, J. (1992) Sequence-specificity of drug-DNA interactions. In *Nucleic Acid Targeted Drug Design* (C. L. Propst and T. J. Perun, Eds.) pp 93–149, Dekker, New York.
- (214) Bailly, C., and Waring, M. J. (1995) Comparison of different footprinting methodologies for detecting binding sites for a small ligand on DNA. *J. Biomol. Struct. Dyn.* 12, 869.
- (215) Taylor, J. S., Schultz, P. G., and Dervan, P. B. (1984) DNA affinity cleaving. Sequence specific cleavage of DNA by distamycin-EDTA-Fe(II) and EDTA-distamycin-Fe(II). *Tetrahedron* 40, 457.
- (216) Schultz, P. G., and Dervan, P. B. (1983) Sequence-specific double-strand cleavage of DNA by penta-*N*-methylpyrrole-carboxamide-EDTA-Fe(II). *Proc. Natl. Acad. Sci. U.S.A.* 80, 6834.
- (217) Schultz, P. G., Taylor, J. S., and Dervan, P. B. (1982) Design and synthesis of a sequence-specific DNA cleaving molecule. (distamycin-EDTA)iron(II). *J. Am. Chem. Soc.* 104, 6861.
- (218) Schultz, P. G., and Dervan, P. B. (1983) Sequence-specific double-strand cleavage of DNA by bis(EDTA-distamycin-Fe^{II}) and EDTA-bis(distamycin)Fe^{II}. *J. Am. Chem. Soc.* 105, 7748.
- (219) Youngquist, R. S., and Dervan, P. B. (1985) Sequence-specific recognition of B-DNA by bis(EDTA-distamycin)-fumaramide. *J. Am. Chem. Soc.* 107, 5528.
- (220) Youngquist, R. S., and Dervan, P. B. (1987) A synthetic peptide binds 16 base pairs of A,T double helical DNA. *J. Am. Chem. Soc.* 109, 7564.
- (221) Wade, W. S., Mrksich, M., and Dervan, P. B. (1992) Design of peptides that bind in the minor groove of DNA at 5'-(A,T)G(A,T)C(A,T)-3' sequences by a dimeric side-by-side motif. *J. Am. Chem. Soc.* 114, 8783.
- (222) Mrksich, M., and Dervan, P. B. (1993) Antiparallel side-by-side heterodimer for sequence-specific recognition in the minor groove of DNA by distamycin/1-methylimidazole-2-carboxamide-netropsin pair. *J. Am. Chem. Soc.* 115, 2572.
- (223) Pickart, L., Freedman, J. H., Loker, W. J., Peisach, J., Perkins, C. M., Stenkamp, R. E., and Weinstein, B. (1980) Growth-modulating plasma tripeptide may function by facilitating copper uptake into cells. *Nature* 288, 715.
- (224) Freedman, J. H., Pickart, L., Weinstein, B., Mims, W. B., and Peisach, J. (1982) Structure of the glycyl-L-histidyl-L-lysine-copper(II) complex in solution. *Biochemistry* 21, 4540.
- (225) Chikira, M., Sato, T., Antholine, W. E., and Petering, D. H. (1991) Orientation of non-blue cupric complexes on DNA fibers. *J. Biol. Chem.* 266, 2859.
- (226) Sigman, D. S., Mazumder, A., and Perrin, D. M. (1993) Chemical nucleases. *Chem. Rev.* 93, 2295.

- (227) Bailly, C., Sun, J. S., Colson, P., Houssier, C., Hélène, C., Waring, M. J., and Hénichart, J. P. (1992) Design of a sequence-specific DNA-cleaving molecule which conjugates a copper-chelating peptide, a netropsin residue, and an acridine chromophore. *Bioconjugate Chem.* 3, 100.
- (228) Grokhovskii, S. L., Nikolaev, V. A., Zubarev, V. E., Surovaya, A. N., Zhuze, A. L., Chernov, B. K., Sidorova, N. Y., Zasedatelev, A. S., and Gurskii, G. V. (1993) Specific DNA cleavage by a netropsin analog containing a copper(II)-chelating peptide Gly-Gly-His. *Mol. Biol.* 6, 839.
- (229) Routier, S., Bernier, J. L., Catteau, J. P., and Bailly, C. (1997) Recognition and cleavage of DNA by a distamycin-salen-copper conjugate. *BioOrg. Med. Chem. Lett.* 7, 1729.
- (230) Stubbe, J., Kozarich, J. W., Wu, W., and Vanderwall, D. E. (1996) Bleomycins: a structural model for specificity, binding, and double strand cleavage. *Acc. Chem. Res.* 29, 322.
- (231) Farinas, E., Tan, J. D., Baidya, N., and Mascharak, P. K. (1993) A designed synthetic analogue of Co(III)-bleomycin with enhanced DNA-binding and photocleaving activity. *J. Am. Chem. Soc.* 115, 2996.
- (232) Hénichart, J. P., Houssin, R., Bernier, J. L., and Catteau, J. P. (1982) Synthetic model of a bleomycin metal complex. *J. Chem. Soc., Chem. Commun.* 1295.
- (233) Hénichart, J. P., Bernier, J. L., Houssin, R., Lohez, M., Kénani, A., and Catteau, J. P. (1985) Copper(II)- and iron(II)-complexes of methyl-2-(2-aminoethyl)-aminomethylpyridine-6-carboxyl-histidinate (AMPHIS), a peptide mimicking the metal-chelating moiety of bleomycin. An ESR study. *Biochem. Biophys. Res. Commun.* 126, 1036.
- (234) Kohda, J., Shinozuka, K., and Sawai, H. (1995) A novel bleomycin model compound bearing hydrophilic steric factor exhibited high oxygen activating capacity. *Tetrahedron Lett.* 36, 5575.
- (235) Otsuka, M., Masuda, T., Haupt, A., Ohno, M., Shiraki, T., Sugiura, Y., and Maeda, K. (1990) Man-designed bleomycin with altered sequence specificity in DNA cleavage. *J. Am. Chem. Soc.* 112, 838.
- (236) Owa, T., Haupt, A., Otsuka, M., Kobayashi, S., Tomioka, N., Itai, A., Ohno, M., Shiraki, T., Uesugi, M., Sugiura, Y., and Maeda, K. (1992) Man-designed bleomycins: significance of the binding sites as enzyme models and the stereochemistry of the linker chain. *Tetrahedron* 48, 1193.
- (237) Huang, L., Morgan, R., and Lown, J. W. (1993) Design of DNA-cleaving molecules which incorporate a simplified metal-complexing moiety of bleomycin and lexitropsin carriers. *Bioorg. Med. Chem. Lett.* 3, 1751.
- (238) Huang, L., Quada, J. C., and Lown, J. W. (1994) Design and synthesis of DNA cleaving bleomycin models: 1,2-trans disubstituted cyclopropane units as novel linkers. *Tetrahedron Lett.* 35, 5323.
- (239) Huang, L., Quada, J. C., Jr., and Lown, J. W. (1995) Design, synthesis, and sequence selective DNA cleavage of functional models of bleomycins. 1. Hybrids incorporating a simple metal-complexing moiety of bleomycin and lexitropsin carriers. *Bioconjugate Chem.* 6, 21.
- (240) Huang, L., Quada, J. C., Jr., and Lown, J. W. (1995) Design, synthesis, and sequence selective DNA cleavage of functional models of bleomycins. II. 1,2-trans-disubstituted cyclopropane units as novel linkers. *BioOrg. Med. Chem.* 3, 647.
- (241) Yang, Y., Huang, L., Pon, R. T., Cheng, S.-F., Chang, D.-K., and Lown, J. W. (1996) Solution structure studies of the cobalt complex of a bleomycin functional model bound to d(CGCAATTGCG)₂ by two-dimensional nuclear magnetic resonance methods and restrained molecular dynamics simulation. *Bioconjugate Chem.* 7, 670.
- (242) Kane, S. A., Natrajan, A., and Hecht, S. M. (1994) On the role of the bithiazole moiety in sequence-selective DNA cleavage by Fe-bleomycin. *J. Biol. Chem.* 269, 10899.
- (243) Manderville, R. A., Ellena, J. F., and Hecht, S. M. (1995) Interaction of Zn(II)-bleomycin with d(CGCTAGCG)₂. A binding model based on NMR experiments and restrained molecular dynamics calculations. *J. Am. Chem. Soc.* 117, 7891.
- (244) Wu, W., Vanderwall, D. E., Stubbe, J., Kozarich, J. W., and Turner, C. J. (1994) Interaction of Co-bleomycin A2 (green) with d(CCAGGCCTGG)₂: Evidence for intercalation using 2D NMR. *J. Am. Chem. Soc.* 116, 10843.
- (245) Nicolaou, K. C., Dai, W.-M., Tsay, S.-C., Estevez, V. A., and Wrasidlo, W. (1992) Designed enedynes: a new class of DNA-cleaving molecules with potent and selective anticancer activity. *Science* 256, 1172.
- (246) Nicolaou, K. C., Smith, A. L., and Yue, E. W. (1993) Chemistry and biology of natural and designed enedynes. *Proc. Natl. Acad. Sci. U.S.A.* 90, 5881.
- (247) Nicolaou, K. C., and Dai, W.-M. (1991) Chemistry and biology of the enediyne anticancer antibiotics. *Angew. Chem., Int. Ed. Engl.* 30, 1387.
- (248) Tokuda, M., Fujiwara, K., Gomibuchi, T., Hirama, M., Uesugi, M., and Sugiura, Y. (1993) Synthesis of a hybrid molecule containing neocarzinostatin chromophore analogue and minor groove binder. *Tetrahedron Lett.* 34, 669.
- (249) Wittman, M. D., Kadow, J. F., Langley, D. R., Vyas, D. M., Rose, W. C., Solomon, W., and Zein, N. (1997) The synthesis and biological activity of enediyne minor groove binding hybrids. *BioOrg. Med. Chem. Lett.* 5, 1049.
- (250) Xie, Y., Miller, G. G., Cubitt, S. A., Soderlind, K.-J., Allalunis-Turner, M. J., and Lown, J. W. (1997) Enediyne-lexitropsin DNA-targeted anticancer agents. Physicochemical and cytotoxic properties in human neoplastic cells *in vitro*, and intracellular distribution. *Anti-Cancer Drug Des.* 12, 169.
- (251) Semmelhack, M. F., and Gallagher, J. J. (1994) The effect on DNA cleavage potency of tethering a simple cyclic enediyne to a netropsin analog. *J. Org. Chem.* 59, 4357.
- (252) Bregant, T. M., Groppe, J., and Little, R. D. (1994) New class of DNA-cleaving agents based on trimethylenemethane. *J. Am. Chem. Soc.* 116, 3635.
- (253) Spielmann, H. P., Fagan, P. A., Bregant, T. M., Little, R. D., and Wemmer, D. E. (1995) The binding modes of a rationally designed photoactivated DNA nuclease determined by NMR. *Nucleic Acids Res.* 23, 1576.
- (254) Xie, G., Morgan, A. R., and Lown, J. W. (1993) Synthesis and DNA cleaving properties of hybrid molecules containing propargylic sulfones and minor groove binding lexitropsins. *Bioorg. Med. Chem. Lett.* 3, 1565.
- (255) Flanagan, M. E., Rollins, S. B., and Williams, R. M. (1995) Netropsin and spermine conjugates of a water-soluble quinocarcin analog: analysis of sequence-specific DNA interactions. *Chem. Biol.* 2, 145.
- (256) Kamitori, S., and Takusagawa, F. (1992) Crystal structure of the 2:1 complex between d(GAAGCTTC) and the anticancer drug actinomycin D. *J. Mol. Biol.* 225, 445.
- (257) Kamitori, S., and Takusagawa, F. (1994) Multiple binding modes of anticancer drug actinomycin D: X-ray, molecular modeling, and spectroscopic studies of d(GAAGCTTC)₂-actinomycin D complexes and its host DNA. *J. Am. Chem. Soc.* 116, 4154.
- (258) Chen, H., Liu, X., and Patel, D. J. (1996) DNA bending and unwinding associated with actinomycin D antibiotics bound to partially overlapping sites on DNA. *J. Mol. Biol.* 258, 457.
- (259) Wang, A. H. J. (1992) Intercalative drug binding to DNA. *Curr. Opin. Struct. Biol.* 2, 361.
- (260) Chaires, J. B. (1990) Daunomycin binding to DNA: from the macroscopic to the microscopic. In *Molecular Basis of Specificity in Nucleic Acid-Drug Interactions* (B. Pullman and J. Jortner, Eds.) pp 225–245, Kluwer Academic Publishers, Dordrecht, The Netherlands.
- (261) Waring, M. J. (1993) Echinomycin and related quinoxaline antibiotics. In *Molecular Aspects of Anticancer Drug-DNA Interactions* (S. Neidle and M. J. Waring, Eds.) Vol. 1, pp 213–242, Macmillan, London.
- (262) Sugiura, Y., Shiraki, T., Konishi, M., and Oki, T. (1990) DNA intercalation and cleavage of an antitumor antibiotic dymycin that contains anthracycline and enediyne cores. *Proc. Natl. Acad. Sci. U.S.A.* 87, 3831.
- (263) Lee, S. H., and Goldberg, I. H. (1989) Sequence-specific, strand-selective, and directional binding of neocarzinostatin chromophore to oligodeoxyribonucleotides. *Biochemistry* 28, 1019.

- (264) Bailly, C., and Hénichart, J. P. (1991) DNA recognition by intercalator-minor groove binder hybrid molecules. *Bioconjugate Chem.* 2, 379.
- (265) Bailly, C., and Hénichart, J. P. (1994) Molecular pharmacology of intercalator-minor groove binder hybrid groove binder hybrid molecules. In *Molecular Aspects of Anticancer Drug-DNA Interactions* (S. Neidle and M. J. Waring, Eds.) Vol. 2, pp 162–196, Macmillan, London.
- (266) Krivtsora, M. A., Moroshkina, E. B., and Glibin, E. (1984) DNA interaction with low molecular weight ligands with different structures. III. Complexes of DNA with distastins. *Mol. Biol.* 18, 950.
- (267) Boitte, N., Pommery, N., Colson, P., Houssier, C., Waring, M. J., Hénichart, J.-P., and Bailly, C. (1997) Synthesis, DNA-binding and cytotoxic properties of a bis(netropsin)-anthracenedione conjugate. *Anti-Cancer Drug Des.* 12, 481.
- (268) Eliadis, A., Phillips, D. R., Reiss, J. A., and Skorobogaty, A. (1988) The synthesis and DNA footprinting of acridine-linked netropsin and distamycin bifunctional mixed ligands. *J. Chem. Soc., Chem. Commun.* 1049.
- (269) Shinomiya, M., and Kuroda, R. (1992) Synthesis of novel DNA photocleaving agents with potent DNA cleaving activity. *Tetrahedron Lett.* 33, 2697.
- (270) Kuroda, R., Satoh, H., Shinomiya, M., Watanabe, T., and Otsuka, O. (1995) Novel DNA photocleaving agents with high DNA sequence specificity related to the antibiotic bleomycin A2. *Nucleic Acids Res.* 23, 1524.
- (271) Bailly, C., Pommery, N., Houssin, R., and Hénichart, J. P. (1989) Design, synthesis, DNA-binding and biological activity of a series of DNA minor groove binding intercalating drugs. *J. Pharm. Sci.* 78, 910.
- (272) Bailly, C., Helbecque, N., Hénichart, J. P., Colson, P., Houssier, C., Rao, K. E., Shea, R. G., and Lown, J. W. (1990) Molecular recognition between oligopeptides and nucleic acids. DNA sequence specificity and binding properties of an acridine-linked netropsin hybrid ligand. *J. Mol. Recognit.* 3, 26.
- (273) Fossé, P., René, B., Saucier, J. M., Hénichart, J. P., Waring, M. J., Colson, P., Houssier, C., and Bailly, C. (1994) Stimulation of site-specific topoisomerase II-mediated DNA cleavage by an *N*-methylpyrrolicarboxamide-anilinoacridine conjugate: relation to DNA binding. *Biochemistry* 33, 9865.
- (274) Bailly, C., Collyn-d'Hooghe, M., Lantoiné, D., Fournier, C., Hecquet, B., Fossé, P., Saucier, J. M., Colson, P., Houssier, C., and Hénichart, J. P. (1992) Biological activity and molecular interaction of a netropsin-acridine hybrid ligand with chromatin and topoisomerase II. *Biochem. Pharmacol.* 43, 457.
- (275) René, B., Fossé, P., Khélifa, T., Jacquemin-Sablon, A., and Bailly, C. (1996) The 1'-substituent on the anilino ring of the antitumor drug amsacrine is a critical element for topoisomerase II inhibition and cytotoxicity. *Mol. Pharmacol.* 49, 343.
- (276) Bailly, C., OhUigin, C., Rivalle, C., Bisagni, E., Hénichart, J. P., and Waring, M. J. (1990) Sequence-selective binding of an ellipticine derivative to DNA. *Nucleic Acids Res.* 18, 6283.
- (277) Bourdouxhe, C., Colson, P., Houssier, C., Sun, J.-S., Montenay-Garestier, T., Hélène, C., Rivalle, C., Bisagni, E., Waring, M. J., Hénichart, J.-P., and Bailly, C. (1992) Binding of a distamycin-ellipticine hybrid molecule to DNA and chromatin: spectroscopic, biochemical and molecular modeling investigations. *Biochemistry* 31, 12385.
- (278) Bailly, C., Leclère, V., Pommery, N., Colson, P., Houssier, C., Rivalle, C., Bisagni, E., and Hénichart, J. P. (1993) Binding to DNA, cellular uptake and biological activity of a distamycin-ellipticine hybrid molecule. *Anti-Cancer Drug Des.* 8, 145.
- (279) Bailly, C., OhUigin, C., Houssin, R., Colson, P., Houssier, C., Rivalle, C., Bisagni, E., Hénichart, J. P., and Waring, M. J. (1992) DNA-binding properties of a distamycin-ellipticine hybrid molecule. *Mol. Pharmacol.* 41, 845.
- (280) Bailly, C., Michaux, C., Colson, P., Houssier, C., Sun, J. S., Garestier, T., Hélène, C., Hénichart, J. P., Rivalle, C., Bisagni, E., and Waring, M. J. (1994) Reaction of a bis-cationic distamycin-ellipticine hybrid ligand with DNA. Mode and sequence specificity of binding. *Biochemistry* 33, 15348.
- (281) Riou, J. F., Grondard, L., Naudin, A., and Bailly, C. (1995) Effects of two distamycin-ellipticine hybrid molecules on topoisomerase I and II-mediated DNA cleavage: relation to cytotoxicity. *Biochem. Pharmacol.* 50, 424.
- (282) Mrani, D., Gosselin, G., Auclair, C., Balzarini, J., De Clercq, E., Paoletti, C., and Imbach, J. L. (1991) Synthesis, DNA binding and biological activity of oxazolopyridocarbazole-netropsin hybrid molecules. *Eur. J. Med. Chem.* 26, 481.
- (283) Subra, F., Carteau, S., Pager, J., Paoletti, J., Paoletti, C., Auclair, C., Mrani, D., Gosselin, G., and Imbach, J. L. (1991) Bis(pyrrolicarboxamide) linked to intercalating chromophore oxazolopyridocarbazole (OPC): selective binding to DNA and polynucleotides. *Biochemistry* 30, 1642.
- (284) Subra, F., Mouscadet, J. F., Lavignon, M., Roy, C., and Auclair, C. (1993) Inhibition of the moloney murine leukemia virus cycle at a post reverse transcriptional step by the netropsin-intercalating hybrid molecule netropsin-oxazolopyridocarbazole. *Biochem. Pharmacol.* 45, 93.
- (285) Goulaouic, H., Carteau, S., Subra, F., Mouscadet, J. F., Auclair, C., and Sun, J.-S. (1994) Selective binding to polynucleotides of the hybrid intercalating groove binder bis-(pyrrolicarboxamide)-oxazolopyridocarbazole: a molecular modeling study. *Biochemistry* 33, 1412.
- (286) Plouvier, B., Houssin, R., Hecquet, B., Colson, P., Houssier, C., Waring, M. J., Hénichart, J. P., and Bailly, C. (1994) Antitumor combilexin. A thiazole-containing analogue of netropsin linked to an acridine chromophore. *Bioconjugate Chem.* 5, 475.
- (287) Denny, W. A., and Wakelin, L. P. G. (1986) Kinetic and equilibrium studies of the interaction of amsacrine and anilino ring-substituted analogues with DNA. *Cancer Res.* 46, 1717.
- (288) Bailly, C., Denny, W. A., Mellor, L., Wakelin, L. P. G., and Waring, M. J. (1992) Sequence-specificity of the binding of 9-aminoacridine- and amsacrine-4-carboxamides to DNA studied by DNase I footprinting. *Biochemistry* 31, 3514.
- (289) Bourdouxhe-Housiaux, C., Colson, P., Houssier, C., Waring, M. J., and Bailly, C. (1996) Interaction of DNA-threading netropsin-amsacrine combilexin with DNA and chromatin. *Biochemistry* 35, 4251.
- (290) Hénichart, J. P., Waring, M. J., Riou, J. F., Denny, W. A., and Bailly, C. (1997) Copper-dependent oxidative and topoisomerase II-mediated DNA cleavage by a Netropsin-Amsacrine Combilexin. *Mol. Pharmacol.* 51, 448–461.
- (291) McCaunaughie, A. W., and Jenkins, T. C. (1995) Novel acridine-triazenes as prototype combilexins: synthesis, DNA binding, and biological activity. *J. Med. Chem.* 38, 3488.
- (292) Gupta, R., Al-Said, N. H., Oreski, B., and Lown, J. W. (1996) Design, synthesis and to poisomerase II inhibition activity of 4'-demethylepipodophyllotoxin-lexitropsin conjugates. *Anti-Cancer Drug Des.* 11, 325.
- (293) Zhao, R., Oreski, B., and Lown, J. W. (1996) Synthesis and biological evaluation of hybrid molecules containing the pyrroloquinoline nucleus and DNA-minor groove binders. *BioOrg. Med. Chem. Lett.* 6, 2169.
- (294) Lown, J. W. (1995) Design and development of sequence selective lexitropsin DNA minor groove binders. *Drug Dev. Res.* 34, 145.
- (295) Geierstanger, B. H., and Wemmer, D. E. (1995) Complexes of the minor groove of DNA. *Annu. Rev. Biophys. Biomol. Struct.* 24, 463.
- (296) Zunino, F., Animati, F., and Capranico, G. (1995) DNA minor groove binding drugs. *Curr. Pharm. Des.* 1, 83.
- (297) Kahne, D. (1995) Strategies for the design of minor groove binders: a re-evaluation based on the emergence of site-selective carbohydrate binders. *Chem. Biol.* 2, 7.
- (298) Remy, J.-S., Sirlin, C., Vierling, P., and Behr, J.-P. (1994) Gene transfer with a series of lipophilic DNA-binding molecules. *Bioconjugate Chem.* 5, 647.
- (299) Clanton, D. J., Buckeit, R. W., Jr, Terpening, S. J., Kiser, R., Mongelli, N., Borgia, N. L., Schultz, R., Narayanan, V., Bader, J. P., and Rice, W. G. (1995) Novel sulfonated and phosphonated analogues of distamycin which inhibit the replication of HIV. *Antiviral Res.* 27, 335.

ARTICLES

Synthesis and in Vitro T-Cell Immunogenicity of Conjugates with Dual Specificity: Attachment of Epitope Peptides of 16 and 38 kDa Proteins from *Mycobacterium tuberculosis* to Branched Polypeptide

Katalin A. Wilkinson, Hans Vordermeier,[†] R. J. Wilkinson,[†] Juraj Ivanyi,[†] and Ferenc Hudecz*

Research Group of Peptide Chemistry, Hungarian Academy of Sciences, Eötvös L. University, H-1518 Budapest, Hungary, and Tuberculosis & Related Infections Unit, MRC Clinical Sciences Center, Imperial College School of Medicine, Hammersmith Hospital, London W12 0NN, U.K. Received August 21, 1997; Revised Manuscript Received January 16, 1998

T-cell epitope containing peptides covalently attached to macromolecular carriers can be considered as synthetic immunogens for the development of skin-test diagnostics and of vaccines. As a carrier, an amphoteric branched chain polypeptide, poly[Lys-(Glu₇-DL-Ala_m)] (EAK) with poly(L-lysine) backbone has been used. This polypeptide with free α -amino and γ -carboxyl groups at the end of the side chains was conjugated with peptides representing two immunodominant regions of the 16 and 38 kDa proteins of *Mycobacterium tuberculosis*, respectively. Peptide C⁹¹SEFAYGSFVRTVSLPVGAD¹¹⁰ was elongated by Cys at the N-terminal and attached to the carrier containing protected SH groups to form disulfide bridges. Peptide ⁶⁵FNLWGPAFHERYPNVTITA⁸³ was conjugated to the 3-(2-pyridyldithio)propionic acid *N*-hydroxysuccinimide ester (SPDP) modified and acetylated EAK by introducing amide bond between the free α -amino group of peptide and the free γ -COOH group of Glu at the terminal position of the branches. This strategy lead to chemically well-defined synthetic immunogens that contain two different epitopes in multiple copies covalently linked to a synthetic branched polypeptide carrier. In vitro T-cell immunogenicity of a prototype conjugate was studied using T-cell hybridomas, lymph node cells from 38 kDa protein immunized mice, and human peripheral blood mononuclear cell (PBMC) cultures from sensitized individuals. These data document that the specific T-cell stimulatory effect of each mycobacterial epitope was maintained in this conjugate. Taken together, these findings suggest that it is feasible to use a biodegradable polymeric polypeptide for producing macromolecular bioconjugates for the stimulation of T-cell responses.

INTRODUCTION

Immunogenic peptides covalently attached to macromolecular carriers have extensively been used in experimental animals to elicit antibody responses or to induce protection against microbial or parasitic pathogens (1, 2, 3). However, this approach was applied to enhance or modulate T-cell responses sporadically (4). Recently, T-cell epitope containing peptides conjugated to various compounds have been used for the induction of antibody responses against lysozyme (5), gangliosides (6), or polysaccharides of bacterial origin (7, 8).

As a carrier, a synthetic, biodegradable branched polypeptide with poly(L-lysine) backbone has been used (9, 10). Linear and branched (multichain) polymeric peptides with controlled composition was first suggested by Sela (11), while branched polypeptides with simplified chemical structure invented by Szekerke (12, 13) provide a rational basis for its use as an immunological carrier. Recently, new bioconjugates were prepared and used successfully for constructing synthetic immunogens/antigens (14) with a phenyl-oxazolone hapten (15, 16),

B-cell epitope peptides of epithelial mucin (17), or herpes simplex virus (type I) glycoprotein D (18).

In tuberculosis, both protection and pathogenesis have generally been attributed to the actions of host T-cells (19). It is, therefore, of interest to construct synthetic immunogens with a capability to dissect *Mycobacterium tuberculosis* specific T-cell responses and to use for the development of skin-test diagnostics or vaccine. A partially purified protein mixture with lipophilic adjuvant (20) or cell wall components entrapped in liposomes (21) or the recombinant 38 kDa protein included into poly(DL-lactide-co-glycolide) microparticles (22) has been studied as subunit vaccine candidates. Bioconjugate immunogens containing T-cell epitope peptides of *M. tuberculosis* origin can be considered as specific skin-test reagents.

The 38 kDa glyco-lipoprotein of *M. tuberculosis* is a major secreted antigen with known amino acid sequence (23, 24). This protein is recognized prominently by sera from patients with multibacillary (19) or pulmonary tuberculosis (25). Several T-cell stimulatory regions were identified within the 374 amino acid residue protein using synthetic 20-mer peptides in murine (26, 27), bovine (28), and human (29) systems. Peptide 65–83 (⁶⁵FNLWGPAFHERYPNVTITA⁸³) stimulated strong proliferative responses of T-cells from mice which had been

* Author to whom correspondence should be addressed. Fax: 36-1-2090602. Tel: 36-1-2090555.

[†] Imperial College School of Medicine.

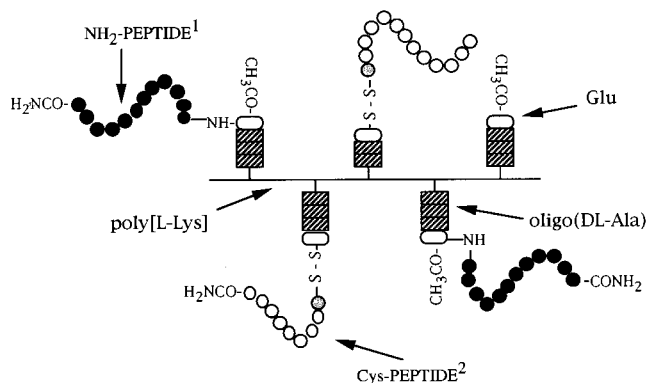


Figure 1. Schematic representation of conjugate K2 containing two different epitope peptides from *M. tuberculosis* proteins $^{65}\text{FNLWGPAFHERRYPNVTITA}^{83}$ and $^{\text{C}91}\text{SEFAYGSFVRTVSLPVGAD}^{110}$ attached to branched polypeptide EAK by amide and disulfide bond, respectively.

presensitized with either live or killed organisms or with the recombinant 38 kDa protein (26). This peptide is also recognized by T-cells from PPD¹-positive humans (29).

The 16 kDa protein of 144 amino acid residues (30) is a major membrane constituent (31). It serves as a molecular chaperone and may be involved in bacterial stasis (32). It can induce in vitro T-cell proliferative responses as well as DTH reactions in mice and guinea pigs (33). T-cell responses with murine lymph node cells suggested that peptide 91–110 ($^{\text{C}91}\text{SEFAYGSFVRTVSLPVGAD}^{110}$) contains a T-cell epitope (34), which was stimulatory also for human PBMC from PPD sensitized healthy subjects and patients with active tuberculosis (35).

For the construction of conjugates to be utilized as potentially useful *M. tuberculosis* specific skin test diagnostics, poly[Lys-(X_i-DL-Ala_m)] (XAK) type poly- α -amino acids were used as synthetic carriers (9). X is either glutamic acid (EAK) (13) or *N*- α -acetyl glutamic acid (AcEAK) (36). EAK is an amphoteric polypeptide in which two types of functional groups (α -amino and γ -carboxyl groups) are available for conjugation. Consequently, this molecule seems to be ideal for well-defined attachment of two different entities with suitable selected reactive groups (Figure 1).

In this paper we describe (i) the synthesis and characterization of the epitope-EAK conjugate with dual specificity and of several epitope-AcEAK conjugates and (ii) the epitope-related T-cell immunoreactivity of the conjugates. The results presented here demonstrate that the strategy outlined leads to chemically well-defined synthetic antigens which contain two different epitopes in multiple copies covalently linked to a synthetic branched polypeptide carrier. In vitro data from assays using a specific murine hybridoma T-cell line (38.H6), mouse

lymph node cells and human PBMC cultures from sensitized individuals indicate that T-cell stimulatory capacity of each mycobacterial epitope was maintained or even enhanced in the prototype conjugate.

MATERIAL AND METHODS

Chemicals. DL-Alanine, glutamic acid, *N* ϵ -Z-lysine, imidazole, and acetic anhydride were purchased from Reanal (Budapest, Hungary). *N,N*-Diisopropylethylamine (DIEA), 1-hydroxy benzotriazole (HOBt) and bis-(trichloromethyl) carbonate were Fluka products (Buchs, Switzerland), while benzotriazol-1-yl-oxy-tris(dimethylamino)phosphonium hexafluoro-phosphate (BOP) was from Bachem (Bubendorf, Switzerland). Dimethylformamide (DMF) was stored over molecular sieves. Protected amino acids, peptide 38.G, and resins were purchased from Bachem (Bubendorf, Switzerland) or Novabiochem (Laenfeldingen, Switzerland), unless otherwise stated.

Synthetic Peptides. Peptides $^{\text{C}91}\text{SEFAYGSFVRTVSLPVGAD}^{110}$ (16.10), $^{\text{C}91}\text{SEFAYGSFVRTVSLPVGAD}^{110}$ (C-16.10), and $^{65}\text{FNLWGPAFHERRYPNVTITA}^{83}$ (38.I) were synthesized in our laboratory by the solid-phase methodology using 4-methyl-benzhydrylamine resin. All amino acids were coupled as *N* α -tert-butyloxycarbonyl derivatives. As side-chain protecting groups cyclohexyl for Glu and Asp, benzyloxymethyl for His, benzyl for Ser, and Thr and mesitylene sulfonyl for Arg were applied. Coupling was carried out using 1-hydroxy-benzotriazole/diisopropylcarbodiimide method in DMF:DCM = 1:1 (v/v). The reactions were monitored by ninhydrin assay (37) or bromophenol-blue method (38). After the completion of the synthesis, the α -amino groups were deprotected with 33% TFA/DCM and peptides were cleaved from the resin using hydrogen fluoride containing *p*-cresol and DL-dithiothreitol (DTT) [peptide: *p*-cresol:DTT = 1:1:0.1 (mol/mol/mol)].

The crude products were purified by RP-HPLC using a DELTA-PACK column (7.8 mm $\times$ 30 cm, C18-300 Å, 15 μm , Spherical, Nixon Waters Ltd., Tokyo, Japan) and gradient elution (0.1%TFA/H₂O as eluent A and 0.1%TFA/80%MeCN/20% H₂O as eluent B). Homogeneity of the pure peptides was confirmed by analytical RP-HPLC and the primary sequences were verified by amino acid analysis and mass spectrometry measured on a VG-ZA-2SEQ tandem mass spectrometer (Fisons, Loughborough, U.K.) equipped with a Cs⁺ gun (25 keV).

Branched Polypeptides. Abbreviations for amino acids and their derivatives follow the revised recommendation of the IUPAC-IUB Committee on Biochemical Nomenclature, entitled Nomenclature and Symbolism for Amino Acids and Peptides (39). The nomenclature of branched polypeptides is used in accordance with the recommended nomenclature of graft polymers (40). For the sake of brevity, codes of branched polypeptides were constructed by us using the one-letter symbols of amino acids. All amino acids are of the L-configuration unless otherwise stated.

The average degree of polymerization ($\overline{\text{DP}}_n$) of the polylysine used for the synthesis of branched polypeptides and conjugates was estimated by sedimentation analysis (41). Sedimentation analysis was carried out in a MOM3170 ultracentrifuge. The average relative molar mass of the branched polypeptides (EAK and AcEAK) was calculated from $\overline{\text{DP}}_n$ of the poly[Lys] backbone and from the amino acid ratios Glu:Ala:Lys as described earlier (12, 41). Visking tubes with a 12 000–14 000 Da

¹ Abbreviations: AcEAK, poly[Lys-[Ac-Glu_{0.96}-DL-Ala_{3.1}]]; AK, poly[Lys-(DL-Ala_m)]; BOP, benzotriazol-1-yl-oxytris(dimethylamino)phosphonium hexafluorophosphate; DCM, dichloromethane; DIEA, *N,N*-diisopropylethylamine; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; $\overline{\text{DP}}_n$, number average degree of polymerization; DS, average degree of substitution; DTH, delayed type hypersensitivity; DTT, DL-dithiothreitol; EAK, poly[Lys-(Glu_{0.96}-DL-Ala_{3.1})] FCS, fetal calf serum; HOBt, 1-hydroxybenzotriazole; IFA, incomplete Freund's adjuvant; IL2, interleukin 2; LN, lymph node; PBMC, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; PPD, purified protein derivative; SPDP, 3-(2-pyridyldithio)propionic acid *N*-hydroxysuccinimide ester; XAK, poly[Lys-(X_i-DL-Ala_m)]; Z, benzyloxycarbonyl.

Table 1. Characteristics of Synthetic Peptides Corresponding to Regions 65–83 and 350–369 from the 38 kDa Protein and to Region 91–110 of 16 kDa Protein of *M. Tuberculosis*

code	peptide sequence	t_R^a (min)	amino acid analysis found (expected)	MH ⁺ ^b found [calculated]
65–83 (38.1)	FNLWGPAFHERYPNVTITA	24.5	F1.77(2), N1.98(2), L0.94(1), W(1), G1.04(1), P2.04(2), A2.11(2), H1.4(1), E1.17(1), R1.00(1), Y0.70(1), V1.00(1), T1.98(2), I0.85(1)	2232.7(2231.1)
350–369 (38.G)	DQVHFQPLPPAVVKLSDALI	32.0*	nt	nt
91–110 (16.10)	SEFAYGSFVRTVSLPVGAD	25.5	D1.03(1), T0.95(1), S2.81(3), E2.23(2), P1.11(1), G2.12(2), A2.04(2), V2.97(3), L1.0(1), Y0.83(1), F1.93(2), R1.03(1)	2130.5(2131.1)
C-91–110 (C-16.10)	CSEFAYGSFVRTVSLPVGAD	25.5	D1.03(1), T0.95(1), S2.81(3), E2.23(2), P1.11(1), G2.12(2), A2.04(2), C0.56(1), V2.97(3), L1.0(1), Y0.83(1), F1.93(2), R1.03(1)	2233.5(2234.1)

^a HPLC conditions using a Knauer system with Delta Pak C₁₈ column (3.9 mm × 30 cm, 300 Å, 15 μm): using 10–100% eluent B, in 35 min except (*) when 5–55% eluent B in 30 min. ^b Molecular mass determined by FAB mass spectrometry.

cutoff (Fisons, Loughborough, U.K.) were used for the dialysis of polymer products.

Poly[Lys-(Glu_{1,0}-DL-Ala_{3,1})] (EAK), was synthesized as previously described (13). Briefly, poly[L-Lys] ($\overline{DP}_n = 70$) was prepared by the polymerization of *N*^α-carboxy-*N*^ε-(benzyloxycarbonyl)-lysine anhydride. After cleavage of the protecting groups with HBr in glacial acetic acid, poly[Lys-(DL-Ala_{3,1})] (AK) was formed by grafting of short oligomeric DL-alanine side chains onto the ε-amino groups of polylysine using *N*-carboxy-DL-alanine anhydride. Attachment of Z-Glu(OBzl)-OPcp to AK was carried out in a water/DMF solvent mixture in the presence of HOBt and 4-methyl morpholine at pH 7–8. The protecting groups were cleaved with a HBr/acetic acid mixture, and their absence was verified by UV spectroscopy. EAK was purified by extensive dialysis and then freeze-dried.

Poly[Lys-(AcGlu_{1,0}-DL-Ala_{3,1})] (AcEAK) (36) was obtained by the use of acetic anhydride and imidazole in DMF in the presence of triethylamine (42). Briefly, EAK was dissolved in water/DMF (1:5, v/v) and equimolar TEA was added. Mixture of acetic anhydride and imidazole [1:1 (mol/mol)] was added to the polymer solution. After 1 h at 0 °C and overnight at room temperature, the product was purified by dialysis as described above. No ninhydrin positive reaction was observed in the ninhydrin test (37).

Synthesis of Epitope Peptide-AcEAK Conjugates.

AcEAK (10 mg, 20 μmol) was dissolved in 1 mL of deionized water and diluted with 4 mL of DMF. BOP reagent (8.9 mg, 20 μmol), 3.3 mg (25 μmol) of HOBt, and 7 μL (40 μmol) of *N,N*-diisopropylethylamine (DIEA) were dissolved in 1 mL of DMF, representing a molar ratio of BOP:HOBt:DIEA = 1:1:2 (mol/mol/mol). The activating mixture was added to the polymer solution and stirred for 20 min. Epitope peptide (0.5 mmol) (calculated for the side chains of the polymer) was dissolved in 3 mL of DMF at a concentration of 2.5 mg/mL, and 0.5 mmol of DIEA was added to this solution followed by addition to the preactivated polymer solution. The coupling was performed at room-temperature overnight under stirring. The solution was then dialyzed against deionized water for 3 days. Finally, the product was isolated by freeze-drying. The average molar substitution ratio was calculated according to the amino acid composition of the conjugate obtained from amino acid analysis (Table 1).

Synthesis of Epitope Peptides-EAK Conjugate with Amide and Disulfide Bonds. This was achieved in four steps: first, the α-amino group of the glutamic

acid side chains in the EAK polypeptide was modified by 3-(2-pyridyldithio)propionic acid *N*-hydroxy-succinimide ester (SPDP) (17,43) to obtain (SSP)EAK, and second, the remaining free α-amino groups were acetylated (42). In the third and fourth steps the conjugation of peptides was performed.

Poly[Lys-(SSP_x-Glu_i-DL-Ala_m)]_n, (SSP)EAK. EAK (100 mg, 215 μmol) was dissolved in 7.5 mL of PBS (0.1 M, pH 8, adjusted with 1 M NaOH). SPDP (20 mg, 64.0 μmol) SPDP (Sigma, Poole, U.K.) was dissolved in 2 mL of abs. methanol and added dropwise to the solution of the polypeptide. The reaction mixture was stirred for 30 min at room temperature and dialyzed for 24 h against distilled water. To determine the degree of 2-pyridyl-disulfide group incorporation, 400 μL of (SSP)EAK solution was reacted with 200 μL of DL-dithiothreitol (DTT) (2.5 mg/mL PBS), and after 5 min, the absorbance of the released pyridine-2-thione was measured at λ = 343 nm and ε = 8080 M⁻¹cm⁻¹ (44). The calculated degree of substitution was 19.3%. (SSP)EAK was isolated by freeze-drying.

Poly[Lys-(Ac_y,SSP_x-Glu_i-DL-Ala_m)]_n, (Ac,SSP)EAK. (SSP)EAK (110 mg, 235 μmol) was dissolved in 1 mL of distilled water and diluted to 5 mL with DMF at 4 °C. In the meantime, 312 μL (3.3 mmol) of acetic anhydride and 224 mg (3.3 mmol) of imidazole were mixed in 1 mL of DMF at 4 °C and stirred for 10 min. The two solutions were then mixed, and acetylation was continued for 2 h at room temperature. After dialysis against distilled water, the product was isolated by freeze-drying. The blocking of free amino groups was verified by the ninhydrin assay.

Poly[Lys-(Ac_y,SSP_x-Glu_i(Peptide₁)-DL-Ala_m)]_n, (Ac,SSP)E(Peptide₁)AK. (Ac,SSP)EAK (10 mg, 19 μmol) (calculated on the basis of 19.3% 2-pyridyl-disulfide group content, and 80.7% acetyl group content) was dissolved in 1 mL of water and diluted five times with DMF. BOP (8.9 mg, 20 μmol), 3.3 mg (25 μmol) of HOBt, and 7 μL (40 μmol) of DIEA were dissolved in 1 mL of DMF. The activating mixture was added to the polymer solution and stirred for 20 min. Peptide 38.I (10 mg, 4.5 μmol) (representing 0.25 mol of peptide/polymer side chain) was dissolved in 4 mL of DMF at a concentration of 2.5 mg/mL DMF, and mixed with 4.5 μmol of DIEA.

The peptide solution was added to the preactivated polymer solution and allowed to couple for 24 h at room temperature under stirring. The solution was then dialyzed against distilled water for 48 h as described

above, and the conjugate was isolated by freeze-drying. The average degree of substitution was calculated from amino acid analysis shown in Table 1.

The presence of the 2-pyridyl-disulfide groups was verified by reacting 400 μ L of (Ac,SSP)E(38.I)AK with 200 μ L of DTT (2.5 mg/1 mL of PBS) as described above and proved to be unmodified (19.5%). The preparation of (Ac,SSP)E(38.G)AK was performed according to a similar protocol.

Poly[Lys-(Ac₂,Peptide₂)-Glu₁(Peptide₁)-DL-Ala_m], (Ac,Peptide₂)E(Peptide₁)AK. (Ac,SSP)E(38.I)-AK conjugate (10 mg) (containing 3 μ mol protected thiol groups) was dissolved in 1 mL of distilled water. Peptide (10 mg, 4.5 μ mol) peptide C-⁹¹SEFAYGSFVRTVSLPVG-AD¹¹⁰ (C-16.10) dissolved in 1 mL of PBS (pH 8.0, adjusted with 1 M NaOH) was added to the conjugate solution. The reaction mixture was stirred for 30 min at room temperature, followed by dialysis against distilled water for 48 h and freeze-dried. The average degree of substitution was calculated from amino acid analysis and presented in Table 1.

(Ac,16.10)E(38.G)AK conjugate was prepared in a similar way. The characteristic data of these conjugates are shown in Table 1.

Amino Acid Analysis. The amino acid composition of peptides, branched polypeptides (EAK, AcEAK), and conjugates was determined from amino acid analysis using a Beckman (Fullerton, USA) model 6300 amino acid analyzer. Prior to analysis, samples were hydrolyzed in 6 M HCl in sealed and evacuated tubes at 110 °C for 24 h.

Analysis of Conjugates by RP-HPLC. The composition of the conjugates was tested by RP-HPLC, using a Delta-Pak RP C₁₈ column (7.8 mm $\times$ 30 cm) packed with spherical 15 μ m silica of 300 Å pore size (Nixon Waters Ltd., Tokyo, Japan) and the eluent system of 0.1% TFA in water (eluent A) and 0.1% TFA in acetonitrile/water = 80/20 v/v (eluent B) using high-purity (Analar) solvents and distilled, deionized water. The gradient elution was applied between 10 and 100% eluent B, for 35 min. The injection volume was 100 μ L containing 10 μ g of conjugate, polymer, or peptide. Compounds were dissolved in eluent immediately before application and filtered through 0.45 μ m Spartan 13 (Schleicher and Schuell, Dassel, Germany) filters. Free peptide or polymer polypeptide samples were run as standards, and their retention times were determined. UV absorbance was monitored at a wavelength of 220 nm. All analyses were carried out at ambient temperature with a flow rate of 1.0 mL/min. As a control, in some cases, the HPLC profile of peptide/conjugate mixture was also recorded.

The HPLC system consisted of a M600E quaternary gradient solvent delivery system with PowerLine control of programmable UV detector (model 490E), autosampler (model 717 plus), and of a Millennium 2010 chromatography manager (all from Waters, Milford, USA).

Recombinant Proteins. The recombinant 38 kDa protein produced in *Escherichia coli* and purified as described previously (45) was obtained from the WHO protein bank (Braunschweig, Germany). It is neither glycosylated nor lipoylated and lacks the first 11 amino acids of the native protein.

Cells, Mice. The HT2 cells were obtained from the European Cell Culture Collection (Porton Down, U.K.). The BW5147 cells were a gift from Dr. W. Born, Denver, CO. C57BL/10 (H-2^b) mice were obtained from Olac Harlem Ltd. (Shaws Farm, Bicester, Oxon, U.K.). Mice were age (6–10 weeks old) and sex (female) matched for each experiment.

Murine Hybridoma T-Cell Proliferation Assay. T-cell hybridomas specific for peptide 65–83 of 38 kDa protein [38.H6/1] and for peptide 91–110 of 16 kDa protein [16.10.7] were established and tested as described earlier (27, 46). Prior to use, hybridoma cells were washed with medium to remove HT and then assayed by incubating 5×10^4 cells/well of 96-well microtiter plates in the presence of 3×10^5 /well irradiated antigen presenting spleen cells and compounds at appropriate concentrations [$(5 \text{ and } 25) \times 10^{-6}$ M, calculated on the basis of epitope content]. After 24 h, culture supernatants (100 μ L/well) were removed and tested for IL-2 content using the IL-2-dependent, indicator cell line HT2 (10^4 cells/well, 100 μ L). Twenty-four hours later, 0.5 μ Ci/well [³H]thymidine (Amersham, U.K.) was added to the cells and harvested 4 h later onto glass fiber paper. The radioactivity was determined by standard liquid scintillation counting. Results are expressed as means of triplicates, in counts per minute (cpm).

Immunization and Mouse Lymph Node (LN) Proliferation Assay. Mice (5 animals/group) were immunized subcutaneously in each hind footpad with 40 μ g of recombinant 38 kDa protein emulsified in incomplete Freund's adjuvant (IFA, Difco, Detroit). Control animals were immunized with phosphate-buffered saline (PBS) emulsified in IFA. After 8 days, draining popliteal lymph nodes were removed and cell-suspensions prepared in culture medium [RPMI-1640 medium supplemented with 10% fetal calf serum (FCS, Gibco, Paisley, U.K.), 5×10^{-5} M 2-mercaptoethanol, 2 mM L-glutamine, 100 units/mL penicillin, 100 μ g/mL streptomycin sulfate, and 1% HEPES buffer 1 M (Gibco BRL, U.K.)]. Triplicate cultures of 4×10^5 cells/well were incubated in the presence of conjugate, free synthetic peptides and branched polypeptide ($c = 25 \times 10^{-6}$ M calculated on the basis of epitope content) in flat bottomed 96-well plates (Nunc, Roskilde, Denmark) at 37 °C in an atmosphere of 5% CO₂. [³H]thymidine (0.5 μ Ci/well) (Amersham International, Amersham, U.K.) were added to the cells on the third day of culture and harvested 6 h later onto glass fiber filter paper. The incorporated radioactivity was determined by standard liquid scintillation counting. The proliferation experiments were performed in triplicate, and the average value obtained from these three results was calculated. Standard deviation was <20%. The obtained data were represented as stimulation indices (SI) (cpm with peptide/cpm without peptide).

Human T-Lymphocyte Proliferation Assay. Blood was drawn from healthy PPD positive individuals. Peripheral blood mononuclear cells (PBMCs) were isolated by diluting whole citrated blood, 1:1 (v/v) in PBS and centrifuged on a Ficoll-Hypaque (Pharmacia, Sweden) density gradient. PBMCs were suspended in RPMI-1640 supplemented with 2 mM L-glutamine, 100 units/mL penicillin, 100 μ g/mL streptomycin, 5% AB positive human serum, and 0.025 M HEPES buffer (Gibco BRL, U.K.). Cells were cultured at 1.5×10^5 /well in 96-well round-bottomed microtiter plates (Nunc, Denmark) in the presence of the test peptide [$(0.2\text{--}25) \times 10^{-6}$ M]. Purified protein derivative (PPD) at 10 units/mL was used as a specific, and ConA at 5 μ g/mL was applied as a nonspecific positive control. After 6 days of incubation at 37 °C in 5% CO₂ cells were labeled with 1 μ Ci of [³H]thymidine (Amersham International, Amersham, U.K.) and harvested after 16 h onto glass fiber filters. The incorporated radioactivity was determined by standard liquid scintillation counting. The proliferation experiments were performed in triplicate and the average value obtained

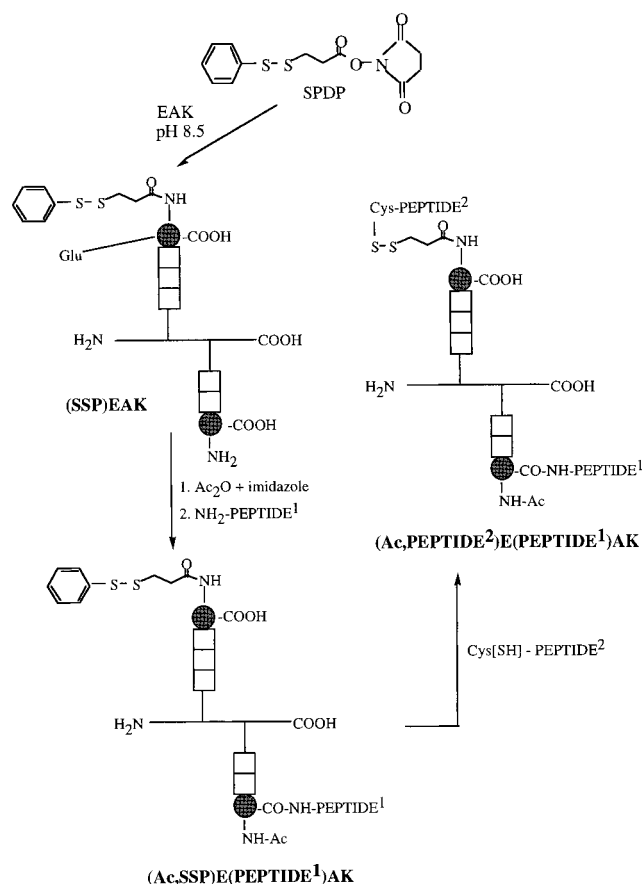


Figure 2. Outline of the synthesis of conjugates containing two different epitope peptides (peptide 1 and peptide 2) and branched polymeric polypeptide EAK.

from these three results was calculated and expressed as stimulation indices (SI) (cpm with peptide/cpm without peptide).

In Vivo Toxicity Assay. In vivo toxicity of AcEAK was evaluated by observing mice using specified pathogen free BDF1 for 20 days after single i.p. injections at doses of up to 50 mg/kg body weight. All mice were alive at the end of this period, and no body weight loss or any noticeable toxic effect was detected.

RESULTS AND DISCUSSION

Preparation of Conjugates Containing Two Different Epitope Peptides and Branched Polypeptide EAK. To prepare structurally well-defined conjugates with two different and uniformly oriented peptide epitopes, the following strategy was developed (Figure 2). As carrier, branched polypeptide EAK containing free α -amino and γ -carboxyl groups was selected and two epitope peptide amides from *M. tuberculosis* proteins were prepared by solid-phase methodology (Table 1). For conjugation, peptide ⁶⁵FNLWGPAFHERYPNVTITA⁸³ (peptide 1) from 38 kDa protein possessing free α -amino group at the N-terminal was used without modification, while the second epitope peptide originating from 16 kDa protein was elongated with an N-terminal Cys [⁹¹SEFAYGSFVRTVSLPVGAD¹¹⁰] (peptide 2).

Our aim was to form two different types of covalent linkage between the polymer and the peptides. One of these bonds was an amide between the γ -COOH of EAK and α -NH₂ of peptide 1, while the second was a disulfide bridge introduced between the α -NH₂ of EAK and SH of peptide 2 (Figure 1).

In the first step, the α -amino-group of Glu in the side chain of EAK was modified by the heterobifunctional reagent SPDP to introduce protected SH groups into the polymer structure [(SSP)EAK] (43,17). The extent of 2-pyridyldisulfide group incorporation was determined spectrophotometrically from the amount of pyridine-2-thione released by reduction with DTT (44), and the average degree of substitution was 19.3%. To produce polypeptide (Ac,SSP)EAK, the free α -amino groups of Glu in the branches were blocked by acetylation using a mixture of acetic anhydride and imidazole (42). In the next step, peptide 1 was coupled to (Ac,SSP)EAK. This was achieved by the BOP reagent-based activation method in which the γ -carboxyl group of glutamic acid of (Ac,SSP)EAK was linked to the α -amino group of phenylalanine residue in peptide 1 to provide isopeptide (γ , α -type amide) bonding. After isolation of the product, the presence of the 2-pyridyldisulfide groups was verified as described above and proved to be unchanged (19%).

In the last step, disulfide bridge was introduced by the substitution of the protecting group of SH of the polymer by peptide 2 (C⁹¹SEFAYGSFVRTVSLPVGAD¹¹⁰). The completion of the reaction between (Ac,SSP)E(peptide1)-AK and peptide 2 was checked spectrophotometrically as mentioned above. The average degree of incorporation of peptide 2 was 19.5%. Data obtained from amino acid analysis suggest that the protected thiol groups of the polymer were fully transformed into disulfide link between peptide 2 and branches of the polymer (Table 2). Similar procedure was applied for the preparation of conjugate K1, which contains peptide ³⁵⁰DQVHFQPLP-PAVVKLSDALI³⁶⁹ from 38 kDa protein. However, it should be noted that the Lys residue in position 363 of the peptide ³⁵⁰DQVHFQPLPPAVVKLSDALI³⁶⁹ was unprotected during the coupling. Therefore, this conjugate might have not only γ , α -bond between γ -COOH of the polymer and α -NH₂ of the peptide but also γ , ϵ -type linkage with ϵ -NH₂ of Lys³⁶³ in the peptide (Table 2).

Preparation of Epitope Peptide-AcEAK Conjugate. Conjugates containing multiple copies of only one kind of epitope peptide (⁶⁵FNLWGPAFHERYPNVTITA⁸³, ³⁵⁰DQVHFQPLPPAVVKLSDALI³⁶⁹, or ⁹¹SEFAYGSFVRTVSLPVGAD¹¹⁰) and branched polypeptide AcEAK derived from EAK were also prepared and used in control immunological experiments.

In AcEAK, the α -amino group of Glu in the branches are acetylated, and therefore, this polypeptide contains γ -carboxyl groups suitable for reacting with amino groups of the peptide to be attached. Coupling was performed by activation of COOH groups using BOP reagent-based method as described earlier for a GnRH antagonist peptide (42). In conjugates with peptides 16.10 or 38.I, γ , α amide bonds are expected. Due to the presence of unprotected ϵ -NH₂ of Lys³⁶³ in ³⁵⁰DQVHFQPLPPAVVKLSDALI³⁶⁹ (Table 2), an additional γ , ϵ -type peptide linkage cannot be excluded in the 38.G-AcEAK conjugate.

Characterization of the Conjugates. After extensive dialysis, products were isolated and RP-HPLC studies were carried out to access free peptides in the conjugate preparation. The retention time for peptides (Table 1) as well as for conjugates and carriers, absorbing at 220 nm, was determined. Free AcEAK was detected at 21.5 min, whereas the *t_R* values for K1 and K2 were 23.5 and 23 min, respectively. Quantitative analysis indicated less than 1% of free peptide in the conjugate samples.

The amino acid composition of conjugates K1 and K2 after the third and the last step of the synthesis and of

Table 2. Characteristics of Branched Polypeptide and Their Epitope-Peptide Conjugates

compd ($i = 1, m = 3.1$)	peptide sequence	code ^a	peptide/polymer [mol/mol] ^b (DS in %) ^c		$\overline{M}_w^b$ ($\pm 5\%$)
			j	k	
poly[Lys-(Glu _i -DL-Ala _m)]	-	EAK			32 000
poly[Lys-(Ac-Glu _i -DL-Ala _m)]	-	AcEAK			35 000
poly{Lys-[Ac-Glu _i (16.10 _j)-DL-Ala _m]}	SEFAYGSFVRTVSLPVGAD	16.10-AcEAK	23.5(33.5)		85 000
poly{Lys-[Ac-Glu _i (38.1 _j)-DL-Ala _m]}	FNLWGPAFHERYPNVTITA	38.1-AcEAK	9.8(14.0)		57 000
poly{Lys-[Ac-Glu _i (38.1 _j)-DL-Ala _m]}	DQVHFQPLPPAVVKLSDALI	38.1-AcEAK	15.4(22.0)		68 800
poly[Lys-(16.10 _k)-Glu _i (38.1 _j)-DL-Ala _m]	DQVHFQPLPPAVVKLSDALI (38.1) and CSEFAYGSFVRTVSLPVGAD (16.10)	K1	8.9(12.7)	12.7(18.1)	82 500
poly[Lys-(Ac _y , 16.10 _k)-Glu _i (38.1 _j)-DL-Ala _m]	FNLWGPAFHERYPNVTITA (38.1) and CSEFAYGSFVRTVSLPVGAD (16.10)	K2	4.9(7.0)	13.7(19.5)	76 000

^a On the basis of the single letter code of branched polypeptides (EAK, AcEAK) and on the peptide-codes (16.1, 38.1, 38.G). ^b Peptide: polymer molar ratio calculated from the amino acid composition determined by amino acid analysis. ^c Average degree of substitution expressed as percent of modified side chains of the carrier polypeptide calculated from the average degree of polymerization of the polylysine ($DP_n = 70$) and from the side chain composition of the conjugate. ^d Average molecular weight of the macromolecule, calculated from $DP_n = 70$ for polylysine and from the side-chain composition.

all purified AcEAK conjugates was determined by amino acid analysis.

On the basis of the 2-pyridyldisulfide group content of conjugates K1 and K2 after the third step of the synthesis and on the amino acid composition data obtained from conjugates, free carrier, and of the free peptides, the average degree of molar substitution (DS) was calculated.

The characteristic data are shown in Table 2. DS values for AcEAK-based conjugates show that, depending on the amino acid sequence and composition of the epitope peptide, 14–33.5% of the side chains can be modified by this method under conditions applied. These values are in good agreement with our previous experience with GnRH antagonist (42) and mucin 1 peptides (17). It is interesting to observe that the DS values combined for the two different epitope peptides in conjugates K1 and K2 fall also into this range (30.8 and 26.5%, respectively). It should be noted that the incorporation of peptide ³⁵⁰DQVHFQPLPPAVVKLSDALI³⁶⁹ in conjugate K1 was higher than that of peptide ⁶⁵FNLWGPAFHERYPNVTITA⁸³ in conjugate K2 (12.7 vs 7.0%). This variation might be attributed again to the possibility of an additional amide bond formation between the ϵ -NH₂ Lys³⁶³ of 38.G and the carboxyl group of the polymer in conjugate K1.

Immune Responses to Conjugates Containing Two Different Peptides. To investigate the effect of covalent attachment, specific T-cell responses were analyzed with one selected prototype conjugate K2. In this conjugate peptide ⁹¹SEFAYGSFVRTVSLPVGAD¹¹⁰ from the 16 kDa protein and peptide ⁶⁵FNLWGPAFHERYPNVTITA⁸³ from the 38 kDa protein were coupled with polymeric branched polypeptide, EAK. This macromolecular construct together with relevant AcEAK-based conjugates was tested by using (1) murine T-cell hybridomas (clones 16.10.7 and 38H6/1), (2) lymph node (LN) cells proliferation assays, and (3) PBMC cultures of PPD-positive individuals.

Stimulation of T-Cell Hybridomas with Conjugate K2. Conjugate K2, the free peptides (⁹¹SEFAYGSFVRTVSLPVGAD¹¹⁰ and ⁶⁵FNLWGPAFHERYPNVTITA⁸³), their AcEAK conjugates, and unsubstituted branched polypeptides (AcEAK and EAK) were tested using the murine T-cell hybridomas 38H6 and 16.10.7. The results expressed as stimulation indices at two concentrations [$c = (5 \text{ and } 25) \times 10^{-6} \text{ M}$] are summarized in Figures 3 and 4. The concentration of conjugates was calculated according to respective peptide content.

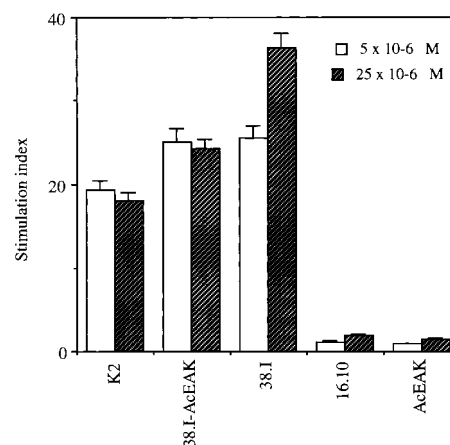


Figure 3. T-cell recognition of conjugate K2 by hybridoma [38.H6/1] specific for ⁶⁵FNLWGPAFHERYPNVTITA⁸³ (38.1). Cells were incubated with conjugate K2 and control compounds (peptides 38.1 and 16.10, conjugate 38.1-AcEAK and AcEAK) at $c = 5$ and $25 \times 10^{-6} \text{ M}$ in the presence of irradiated C57BL/10 spleen cells. Culture supernatants were tested with the cell line HT2. Results are expressed as stimulation indices (SI = cpm with peptide/cpm without peptide).

The 65–83 specific T-cell hybridoma, 38.H6/1 obtained from recombinant 38 kDa protein immunized mice was recognized by conjugates K2 and 38.1-AcEAK as well as free ⁶⁵FNLWGPAFHERYPNVTITA⁸³ peptide (Figure 3). As expected, peptide ⁹¹SEFAYGSFVRTVSLPVGAD¹¹⁰ corresponding to the immunodominant region of the 16 kDa protein did not induce response. The synthetic branched polypeptides AcEAK (Figure 3) and EAK (data not show), used for the preparation of conjugates, were not found stimulatory at the concentrations studied. These findings suggest that the 38 kDa protein-specific T-cell epitope in peptide ⁶⁵FNLWGPAFHERYPNVTITA⁸³ was efficiently recognized not only in its free but also in its polypeptide conjugated form.

The activity of peptide ⁹¹SEFAYGSFVRTVSLPVGAD¹¹⁰ from the 16 kDa protein was investigated using T-cell hybridomas obtained from peptide 91–110 immunized mice. ⁹¹SEFAYGSFVRTVSLPVGAD¹¹⁰ conjugated either to EAK through a disulfide bond (in conjugate K2) or to AcEAK with an amide bond was able to induce T-cell stimulation (Figure 4). Specificity has been proved by the lack of effect of free 38.1 or branched polypeptides (AcEAK or EAK) on these cells.

These findings suggest that the conjugate K2 containing two distinct T-cell epitopes are able to induce epitope-specific responses in hybridoma cells. Thus, the capa-

Table 3. Proliferative Response of Human Peripheral Blood Mononuclear Cells (PBMC) from PPD Positive Individuals to Peptide Conjugate K2^a

conjugates	stimulation index (SI) ^c											responder/ total
	BRCA; 6623 cpm ^d	MAGR; 3158 cpm ^d	MAMA; 664 cpm ^d	SMME; 3358 cpm ^d	RAJE; 4015 cpm ^d	MNG; 1261 cpm ^d	MRA; 1332 cpm ^d	KPI; 1503 cpm ^d	HKE; 413 cpm ^d	KASHE; 874 cpm ^d	MOKH; 1843 cpm ^d	
K2	0.8	8.6	1.8	5.7	1.2	2.6	10.0	4.0	7.4	0.7	1.1	6/11
38.I-AcEAK	0.4	2.1	1.1	1.9	1.1	2.8	5.0	4.0	6.5	0.8	0.7	4/11
16.10-AcEAK	1.0	15.1	0.8	3.7	1.6	3.1	7.1	0.9	6.1	0.6	0.8	5/11

^a Cells were incubated with conjugate K2 and control compounds (16.10-AcEAK and 38.I-AcEAK) at $c = 25 \times 10^{-6}$ M (calculated on the basis of epitope content). ^b The background cpm observed in the absence of peptide treatment. ^c Results are expressed as stimulation index (SI) = cpm with peptide/cpm without peptide and represent the average of triplicate cultures. Values > 2.5 were considered as responder. Purified protein derivative (PPD, 10U) was used as positive control, and a response of SI > 10 was obtained in each case. ^d Background.

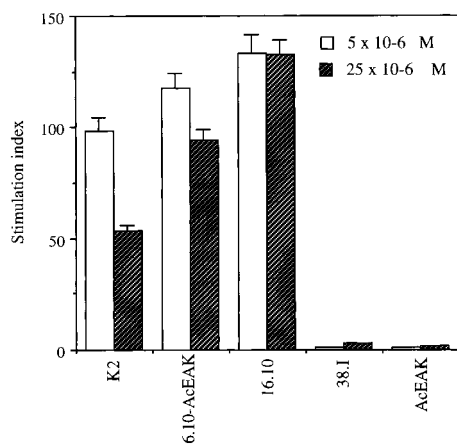


Figure 4. T-cell recognition of conjugate K2 by hybridoma [16.10.7] specific for ⁹¹SEFAYGSFVRTVSLPVGAD E¹¹⁰ (16.10). Cells were incubated with conjugate K2 and control compounds (peptides 16.10 and 38.I, conjugate 16.10-AcEAK and AcEAK) at $c = 5$ and 25×10^{-6} M in the presence of irradiated C57BL/10 spleen cells. Culture supernatants were tested with the cell line HT2. Results are expressed as stimulation indices (SI = cpm with peptide/cpm without peptide).

bility of epitope peptides to initiate T-cell stimulation was preserved after their well-defined covalent attachment to a synthetic polymeric polypeptide.

Proliferative Response of Lymph Node Cells from Mice. The prototype conjugate K2 was also tested in a proliferation experiments using LN cells from mice immunized with the recombinant 38 kDa protein. Draining popliteal LN cells were stimulated in vitro by conjugate K2, the free peptides (⁶⁵FNLWGPAFHERYP-NVTITA⁸³, ⁹¹SEFAYGSFVRTVSLPVGAD E¹¹⁰) and the unconjugated branched polypeptide, EAK at $c = 25 \times 10^{-6}$ M. As negative control, LN cells from PBS immunized mice were used and tested with the same set of compounds resulting SI values of <1.3 (data not shown). LN cells of 38 kDa immunized mice were stimulated with the conjugate K2 and the peptide from the 38 kDa protein (38.I) used as a positive controls (SI = 5.2 for K2 and SI = 5.7 for 38.I) (Figure 5). No T-cell stimulation was observed using peptide ⁹¹SEFAYGSFVRTVSLPVGAD E¹¹⁰ from the 16 kDa protein or by the free polypeptide.

Proliferative Response of Human Peripheral Blood Mononuclear Cells. We have studied conjugate K2 as well as AcEAK-based derivatives of peptides 38.I and 16.10 in proliferation assays using PBMC cultures of PPD-positive individuals (Table 3). Cells were stimulated in vitro by conjugates at $c = 25 \times 10^{-6}$ M or free carrier. Incubation with unsubstituted branched polypeptide resulted in SI values of <1.5 (data not shown). Using SI > 2.5 as cutoff, 6 out of 11 subjects responded to conjugate K2, while AcEAK conjugates of 38.I or 16.10

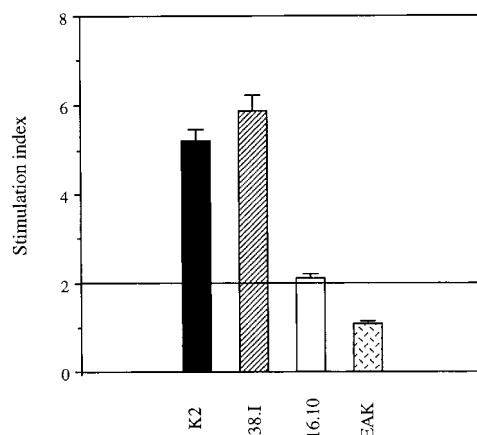


Figure 5. T-cell recognition of peptide conjugate K2 by LN cells from mice immunized by recombinant 38 kDa protein. LN cells were stimulated with conjugate K2 and control compounds (peptides 38.I and 16.10 and branched polypeptide EAK) at $c = 25 \times 10^{-6}$ M (calculated on the basis of epitope content). Results are expressed as stimulation indices (SI = cpm with peptide/cpm without peptide). PBS/IFA injected mice gave SI < 1.3. Values > 2 were considered as positive.

were recognized slightly less efficiently (4 out of 11 and 5 out of 11, respectively). The analysis of these cultures has also indicated differences among individuals in recognition of conjugates containing 16 or 38 kDa related epitopes. Interestingly, only three individuals responded to both 38.I-AcEAK and 16.10-AcEAK conjugates, but PBMC samples from each group were capable to proliferate upon incubation with conjugate K2 possessing dual specificity.

CONCLUSION

Taken together, these data suggest that the strategy outlined results in bioconjugates in which two different peptides are attached covalently and uniformly in multiple copies to a biodegradable polymeric polypeptide possessing free amino and carboxyl groups. The immunological analysis presented suggest that the prototype conjugate bearing two different T-cell epitope peptides from *M. tuberculosis* proteins were well recognized and that the epitopes sustained the specific T-cell stimulatory potential after attachment to EAK. In light of these promising findings, it is feasible to develop further macromolecular branched polypeptide bioconjugates for T-cell response based immunodiagnosis.

ACKNOWLEDGMENT

Thanks are due to Dr. D. Gaál (National Institute of Oncology, Hungary) for performing the in vivo toxicity assays. This work was supported by grants from WHO

(T9/181/133), the Hungarian Academy of Sciences (OTKA T 014964, W 015596), and from the Ministry of Education (FKFP 0101/1997).

LITERATURE CITED

- (1) Del Giudice, G. (1992) New carriers and adjuvants in the development of vaccines. *Curr. Opin. in Immunol.* 4, 454–459.
- (2) Hudecz, F., and Tóth, G. K. (1994) Synthetic peptide constructs to increase the immunogenicity of B-cell epitopes. *Synthetic peptides in the search for B- and T-cell epitopes* (É. Rajnavölgyi, Ed.) pp 97–119, R. G. Landes Company, Austin.
- (3) Delpierre, C., Martin, C., Gequiere, J. C., Tartar, A., Cachera, C., Puchois, P., Fievet, C., and Fruchart, J. C. (1987) Chemical approach to synthetic immunogens in clinical chemistry. *Int. J. Rad. Appl.* 14, 281–288.
- (4) Wan, A. M., Langton, B. C., Andria, M. L., and Benjamini, E. (1986) Antigenic requirements for T-cell activation. *Mol. Immunol.* 23, 467–474.
- (5) Scheerlinck, J. P., Michel, A., and De Baetselier, P. (1994) Grafting of a hepatitis B S-preS(2) T-cell epitope on lysozyme enhances the immunogenicity of lysozyme in responder mice primed with the T-cell epitope. *Immunol. Lett.* 41, 25–32.
- (6) Helling, F., Shang, A., Calves, M., Zhang, S., Ren, S., Yu, R. K., Oettgen, H. H., and Livingston, P. O. (1994) GD3 vaccines for melanoma: superior immunogenicity of keyhole limpet hemocyanin conjugate vaccines. *Cancer Res.* 54, 197–203.
- (7) Konen-Waisman, S., Fridkin, M., and Cohen, I. R. (1995) Self- and foreign 60-kDa heat shock protein T-cell epitope peptides serve as immunogenic carriers for a T-cell-independent sugar antigen. *J. Immunol.* 154, 5977–5985.
- (8) Lett, E., Klopstein, C., Klein, J. P., Scholler, M., and Wachsmann, D. (1995) Mucosal immunogenicity of polysaccharides conjugated to a peptide or multiple-antigen peptide containing T- and B-cell epitopes. *Infect. Immun.* 63, 2645–2651.
- (9) Hudecz, F., Votavova, H., Gaál, D., Sponar, J., Kajtár, J., Blaha, K., and Szekerke, M. (1985) Branched polypeptides with a poly(L-lysine) backbone: synthesis, conformation and immunomodulation. *Polymeric Materials in Medication* (Ch. G. Gebelein, and Ch. E. Carraher, Eds.) pp 265–289, Plenum Press, New York.
- (10) Hudecz, F. (1995) Design of synthetic branched-chain polypeptides as carriers for bioactive molecules. *Anti-Cancer Drugs* 6, 171–193.
- (11) Sela, M. (1980) Chemical synthesis for the understanding of immune response phenomena and for their medical application. *Molecules, cells, and parasites in immunology* (C. Larralde, K. Wills, L. Ortiz-Ortiz, and M. Sela, Eds.) pp 215–228, Academic Press, New York.
- (12) Hudecz, F., and Szekerke, M. (1980) Investigation of drug-protein interactions and the drug-carrier concept by the use of branched polypeptides as model systems. Synthesis and characterisation of the model peptides. *Collect. Czech. Chem. Commun.* 45, 933–940.
- (13) Hudecz, F., and Szekerke, M. (1985) Synthesis of new branched polypeptides with poly(lysine) backbone. *Collect. Czech. Chem. Commun.* 50, 103–113.
- (14) Hudecz, F. (1995) Alteration of immunogenicity and antibody recognition of B-cell epitopes by synthetic branched polypeptide carriers with poly[L-lysine] backbone. *Biomed. Pept., Proteins Nucleic Acids* 1, 213–220.
- (15) Hudecz, F., Kajtár, J., Kurucz, I., and Szekerke, M. (1988) Investigation of the carrier function of polypeptides. Synthesis, conformation and cytotoxicity of oxazolone conjugates of poly[Lys] and poly[Lys-(DL-Ala)]. *Makromol. Chem. Macromol. Symp.* 19, 107–124.
- (16) Rajnavölgyi, É., Lányi, A., Hudecz, F., Kurucz, I., Kiss, K., László, G., Szekerke, M., and Gergely, J. (1989) Structural characteristics influencing the carrier function of synthetic branched polypeptides based on poly[Lys-(DL-Ala)]_n backbone. *Mol. Immunol.* 26, 949–958.
- (17) Hudecz, F., and Price, M. R. (1992) Monoclonal antibody binding to peptide epitopes conjugated to synthetic branched polypeptide carriers. Influence of the carrier upon antibody recognition. *J. Immunol. Methods* 147, 201–210.
- (18) Hudecz, F., Hilbert, A., Mezö, G., Mucsi, I., Kajtár, J., Bösze, Sz., Kurucz, I., and Rajnavölgyi, É. (1993) Epitope mapping of the 273–284 region of HSV glycoprotein D by synthetic branched polypeptide carrier conjugates. *Peptide Res.* 6, 263–271.
- (19) Ivanyi, J., and Thole, J. (1994) Specificity and function of T- and B-cell recognition in tuberculosis. *Tuberculosis: Pathogenesis, Protection and Control* (B. R. Bloom, Ed.) pp 437–458, ASM Press, Washington, DC.
- (20) Andersen, P. (1994) Effective vaccination of mice against *M. tuberculosis* infection with a soluble mixture of secreted mycobacterial proteins. *Infect. Immun.* 62, 2526–2544.
- (21) Chugh, I. B., and Khuller, G. K. (1993) Immunoprotective behaviour of liposome entrapped cell wall subunit of *M. tuberculosis* against experimental infection in mice. *Eur. Respir. J.* 6, 811–815.
- (22) Vordermeier, H. M., Coombes, A. G., Jenkins, P., McGee, J. P., O'Hagan, D. T., Davis, S. S., and Singh, M. (1995) Synthetic delivery system for tuberculosis vaccines: immunological evaluation of the *M. tuberculosis* 38 kDa protein entrapped in biodegradable PLG microparticles. *Vaccine* 13, 1576–1582.
- (23) Harboe, M., and Wiker, H. G. (1992) The 38 kDa protein of *M. tuberculosis*: a review. *J. Infect. Dis.* 166, 874–884.
- (24) Andersen, A. B., and Hansen, E. B. (1989) Structure and mapping of antigenic domains of protein antigen b, a 38,000 molecular weight protein of *M. tuberculosis*. *Infect. Immun.* 57, 2481–2488.
- (25) Cole, R. A., Lu, H. M., Shi, Y. Z., Wang, J., De-Hua, T., and Zhou, A. T. (1996) Clinical evaluation of a rapid immunochromatographic assay based on the 38 kDa antigen of *Mycobacterium tuberculosis* on patients with pulmonary tuberculosis in China. *Tubercle Lung Dis.* 77, 363–368.
- (26) Vordermeier, H. M., Harris, D. P., Roman, E., Lathigra, R., Moreno, C., and Ivanyi, J. (1991) Identification of T-cell stimulatory peptides from the 38 kDa protein of *M. tuberculosis*. *J. Immunol.* 147, 1023–1029.
- (27) Vordermeier, H. M., Harris, D. P., Moreno, C., Singh, M., and Ivanyi, J. (1995) The nature of the immunogen determines the specificity of antibodies and T-cells to selected peptides of the 38 kDa mycobacterial antigen. *Int. Immunol.* 7, 559–566.
- (28) Pollock, J. M., Douglas, A. J., Mackie, D. P., and Neill, S. D. (1995) Peptide mapping of bovine T-cell epitopes for the 38 kDa tuberculosis antigen. *Scand. J. Immunol.* 41, 85–93.
- (29) Vordermeier, H. M., Harris, D. P., Friscia, G., Roman, E., Surcel, H. M., Moreno, C., Pasvol, G., and Ivanyi, J. (1992) T-cell repertoire in tuberculosis: selective anergy to an immunodominant epitope of the 38-kDa antigen in patients with active disease. *Eur. J. Immunol.* 22, 2631–2637.
- (30) Jackett, P. S., Bothamley, G. H., Batra, H. V., Mistry, A., Young, D. B., and Ivanyi, J. (1988) Specificity of antibodies to immunodominant mycobacterial antigens in pulmonary tuberculosis. *J. Clin. Microbiol.* 26, 2313–2318.
- (31) Lee, B. Y., Hefta, S. A., and Brennan, P. J. (1992) Characterization of the major membrane protein of virulent *Mycobacterium tuberculosis*. *Infect. Immun.* 60, 2066–2074.
- (32) Chang, Z., Primm, T. P., Jakana, J., Lee, I. H., Serysheval, I., Chiu, W., Gilbert, H. F., and Quijcho, F. A. (1996) *M. tuberculosis* 16 kDa antigen (HSP16.3) functions as an oligomeric structure in vitro to suppress thermal aggregation. *J. Biol. Chem.* 271, 7218–7223.
- (33) Kingston, A. E., Padmini, R. S., Mitchison, N. A., and Colston, M. J. (1987) Immunological activity of a 14-kilodalton recombinant protein of *Mycobacterium tuberculosis* H37Rv. *Infect. Immun.* 55, 3149–3154.
- (34) Vordermeier, H. M., Harris, D. P., Lathigra, R., Roman, E., Moreno, C., and Ivanyi, J. (1993) Recognition of peptide epitopes of the 16,000 MW antigen of *Mycobacterium tuberculosis* by murine T-cells. *Immunology* 80, 6–12.
- (35) Friscia, G., Vordermeier, H. M., Pasvol, G., Harris, D. P., Moreno, C., and Ivanyi, J. (1995) Human T-cell responses to peptide epitopes of the 16-kD antigen in tuberculosis. *Clin. Exp. Immunol.* 102, 53–57.

- (36) Hudecz, F., Kojima, Y., Miyamoto, Y., Kajtár, J., and Maeda, H. (1994) The effect of charge on the biodistribution of synthetic branched polypeptides in tumour bearing mice. *J. Controlled Release* 28, 301–302.
- (37) Kaiser, E., Colescott, R. L., Bossinger, C. D., and Cook, P. I. (1970) Color test for detection of free terminal amino groups in the solid-phase synthesis of peptides. *Anal. Biochem.* 34, 595–598.
- (38) Krchnak, V., Vagner, J., Safar, P., and Lebl, M. (1988) Noninvasive continuous monitoring of solid-phase peptide synthesis by acid–base indicator. *Collect. Czech. Chem. Commun.* 53, 2542–2548.
- (39) IUPAC–IUB Commission on Biochemical Nomenclature (1972) *Biochem. J.* 127, 753–756.
- (40) IUPAC–IUB Commission on Biochemical Nomenclature (1984) *Eur. J. Biochem.* 138, 9–37.
- (41) Hudecz, F., Kovács, P., Kutassi-Kovács, S., and Kajtár, J. (1984) GPC, CD and sedimentation analysis of poly-Lys and branched chain poly-Lys-poly-DL-Ala polypeptides. *Colloid Polymer Sci.* 262, 208–212.
- (42) Mezö, G., Mezö, I., Pimm, M. V., Kajtár, J., Seprödi, J., Teplán, I., Kovács, M., Vincze, B., Pályi, I., Idei, M., Szekerke, M., and Hudecz, F. (1996) Synthesis, conformation, biodistribution and hormone-related in vitro antitumor activity of a gonadotropin-releasing hormone antagonist–branched polypeptide conjugate. *Bioconjugate Chem.* 7, 642–650.
- (43) Carlsson, J., Drevin, H., and Axen, R. (1978) Protein thiolation and reversible protein–protein conjugation. N-succinimidyl-3-(2-pyridyl-dithio)propionate, a new heterobifunctional reagent. *Biochem. J.* 173, 723–728.
- (44) Stuchbury, T., Shipton, M., Norris, R., Malthouse, J.P.G., Brocklehurst, K., Hebert, J. A. L., and Suschitzky, H. (1975) A reporter group delivery system with both absolute and selective specificity for thiol groups and an improved fluorescent probe containing the 7-nitrobenzo-2-oxa-1,3-diazole moiety. *Biochem. J.* 151, 417–432.
- (45) Singh, M., Andersen, A. B., McCarthy, J. E. G., Rhode, M., Schütte, H., Sanders, E., and Timmis, K. N. (1992) The *Mycobacterium tuberculosis* 38 kDa antigen: overproduction in *Escherichia coli*, purification and characterisation, *Gene* 117, 53–60.
- (46) Vordermeier, H. M., Arya, A., Harris, D. P., Moreno, C., and Ivanyi, J. (1995) Abundance of H-2 promiscuous T-cells specific for mycobacterial determinants in H-2b/d F1 hybrid mice. *Eur. J. Immunol.* 25, 2770–2774.

BC970159+

Catalytic Properties of Galactose Oxidase to Liposome-Forming Amphiphiles Which Have Many Pendent Galactose Residues[†]

Kohji Ohno and Hiromi Kitano*

Department of Chemical and Biochemical Engineering, Toyama University, Toyama 930, Japan.

Received September 15, 1997; Revised Manuscript Received May 5, 1998

A galactose-carrying vinyl monomer [2-(methacryloyloxy)ethyl β -D-galactopyranoside, MEGal] was polymerized by using a lipophilic radical initiator. The amphiphiles obtained (DODA-PMEGal) formed stable liposomes by mixing with phospholipids, and the galactose residues on the liposome surface were effectively recognized and oxidized by galactose oxidase. The affinity (estimated by the $1/K_m$ value) of galactose oxidase for the galactose residues on the liposomes was higher than those for free galactose and MEGal and dependent on the length of galactose-carrying polymer chains on the liposome surface and the fluidity of the membranes, whereas not significantly influenced by the surface density of galactose residues on the liposomes. The affinity of galactose oxidase for the galactose-carrying linear polymers, which were prepared by using an ordinary azo-type radical initiator and a chain-transfer reagent, was also higher than those for free galactose and MEGal and dependent on the degree of polymerization of MEGal. The affinity was, however, relatively much smaller than those for DODA-PMEGals incorporated in liposomes.

INTRODUCTION

Recently, studies on carbohydrate chains have been making rapid progress, and the importance of carbohydrate chains in vivo (e.g., fertilization, immunological protection system, nervous system, virus infection) has become well understood (1–4). Carbohydrates conjugated to a variety of materials, including proteins, lipids, and synthetic polymers, have been also studied and applied in a wide area of disciplines (5, 6). For instance, novel techniques of medical treatments such as artificial organs or drug delivery system (DDS) have been developed by ingeniously taking advantage of the recognition processes of carbohydrates by cell in vivo, and by extensively investigating their therapeutic effects on diseases (7, 8). We have been studying the recognition between sugar-carrying liposomes and sugar receptors at liquid–lipid and solid–lipid interfaces (9, 10).

Galactose oxidase oxidizes exposed primary hydroxyl groups in nonreducing, terminal galactose and *N*-acetyl-galactosamine residues (11). The galactose oxidase/ NaB^{3}H_4 method is well-known and widely used as a technique to label cell-surface glycoconjugates (12–14). It has been found that only a portion of glycolipids is available to galactose oxidase, and different glycolipids vary in their susceptibilities to the enzyme (15–18). Lampio et al. and Masserini et al. previously reported the interaction of galactose oxidase with the major red-cell glycolipid (globoside) and GM_1 ganglioside, respectively, in liposome suspensions (17, 18).

Recently, using a lipophilic radical initiator and galactose-carrying vinyl monomer, 2-(methacryloyloxy)ethyl β -D-galactopyranoside (MEGal),¹ we prepared novel galactose-carrying amphiphiles (galactolipids) with various degrees of polymerization (10). By incorporating the galactose-carrying amphiphiles in liposome, we could

examine the recognition of amino group-carrying liposomes by galactose-carrying liposomes that had been treated with galactose oxidase beforehand, as a model system of induction of lymphocyte cytotoxicity (19). In that experiment, we found a very interesting property of galactose oxidase that its affinity for the galactose residues in the polymer chains on the liposome surface was much higher than that for free galactose.

To verify what happens in the catalytic reaction of enzyme to the carbohydrate chains on the membrane surfaces, galactose-carrying homopolymers and copolymers and galactose-carrying amphiphiles were prepared by using the galactose-carrying vinyl monomer (MEGal) in this study. We examined kinetic analyses on the catalytic behavior of galactose oxidase at various reaction fields including liposome surfaces. It will be described below why galactose oxidase exhibits enhanced affinity to the galactose residues on the membrane surfaces.

EXPERIMENTAL PROCEDURES

Materials. A lipophilic radical initiator (DODA-501, Figure 1a) was synthesized from *N,N*-dioctadecylamine (DODA, Fluka, Switzerland) and 4,4'-azobis(cyanovaleric

[†] Presented at the 45th Annual Meeting of the Society of Polymer Science, Japan, at Nagoya, in May 1996.

* To whom correspondence should be addressed.

¹ Abbreviations: AAm, acrylamide; DDPC, bis(*trans,trans*-2,4-octadecadienyl)phosphatidylcholine; DHDG-Gal, 1,3-di-*O*-hexadecyl-2-*O*-(β -D-galactopyranosyl)glycerol; DLS, dynamic light scattering; DMF, *N,N*-dimethylformamide; DODA, dioctadecylamine; DODA-PMEGal, dioctadecylamine-poly[2-(methacryloyloxy)ethyl β -D-galactopyranoside] conjugate; DODA-501, azo radical initiator with dioctadecyl groups; DP, degree of polymerization; DSC, differential scanning calorimetry; GPC, gel permeation chromatography; HEMA, 2-hydroxyethyl methacrylate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; HRP, horseradish peroxidase; MEGal, 2-(methacryloyloxy)ethyl β -D-galactopyranoside; MG, methyl β -D-galactopyranoside; PMEGal, poly[2-(methacryloyloxy)ethyl β -D-galactopyranoside]; PNPG, *p*-nitrophenyl β -D-galactopyranoside; THF, tetrahydrofuran; T_m , temperature of the midpoint of gel-liquid crystal transition; V-501, 4,4'-azobis(cyanovaleric acid).

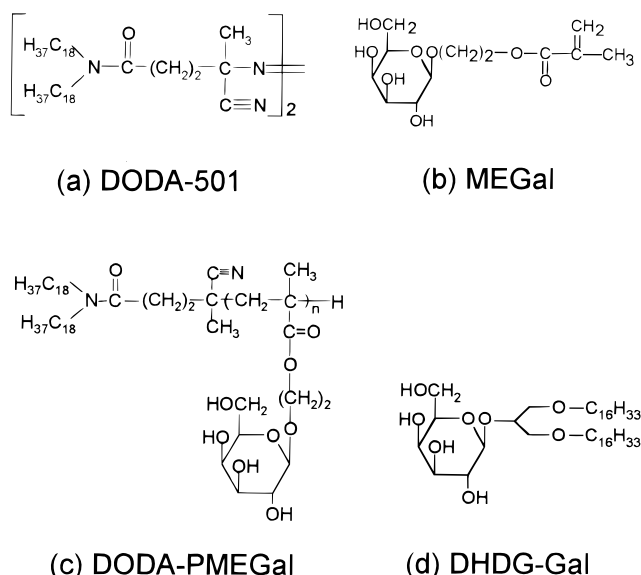


Figure 1. Chemical structures of DODA-501, MEGal, DODA-PMEGal, and DHDG-Gal.

acid) (V-501, Wako Pure Chemicals, Osaka, Japan) as described previously (20). A galactose-carrying vinyl monomer, 2-(methacryloyloxy)ethyl β -D-galactopyranoside (MEGal, Figure 1(b)), was prepared by trans-glycosylation between lactose and 2-hydroxyethyl methacrylate (HEMA) catalyzed by β -galactosidase (from *Escherichia coli*, 340 units/mg, Sigma, St. Louis, MO) in a phosphate buffer (1/15 M, pH 6.4) (21) in the presence of hydroquinone (polymerization inhibitor). 1,3-Di-O-hexadecylglycerol was synthesized by the reaction of epichlorohydrin and 1-hexadecanol in the presence of sodium hydride at 80 °C overnight (22). A polymerizable lipid, bis(*trans,trans*-2,4-octadecadienyl)-phosphatidylcholine (DDPC), was kindly donated by the Japan Fat and Oil Co., Tokyo, Japan. Galactose oxidase (from *Dactylium dendroides*, 240 units/mg) was from Sigma. Other reagents were commercially available. Deionized water was distilled before use.

Preparation of Galactose-Carrying Homopolymer. A homogeneous polymerization of MEGal was carried out using 4,4'-azobis(cyanovaleric acid) (V-501) and cysteamine hydrochloride as initiator and chain-transfer reagent, respectively, in methanol-water (1:3) at 70 °C for 24 h. After evaporation of the solvent, the oily product was washed several times with acetone and dried in vacuo. The slightly yellow powder obtained was dissolved in water again and repeatedly ultrafiltered with water (Amicon Model 8010; membrane, YM-1; exclusion limit, 1000) to remove other small impurities. The solution retained above the membrane was lyophilized. Further, the filtrate which contained the products with a lower degree of polymerization was concentrated, purified by passage through a GPC column (Sephadex G-10, 1.5 cm i.d. $\times$ 25 cm; mobile phase, water), and lyophilized. The syntheses of polymer and/or oligomer with different degrees of polymerization (DPs) were carried out under conditions with fixed concentrations of initiator and monomer and varying amount of chain-transfer agent. The DP values of MEGal homopolymers were determined by the conductometric titration of an amino group at the end of the polymers with a N/100 NaOH aqueous solution.

Preparation of Poly[acrylamide-co-2-(methacryloyloxy)ethyl β -D-galactopyranoside]. A copolymerization of MEGal (151 mg, 0.514 mmol) and acrylamide

(AAM, 37 mg, 0.514 mmol) was carried out using V-501 (2.9 mg, 0.01 mmol) and cysteamine hydrochloride (5.8 mg, 0.051 mmol) as initiator and chain-transfer reagent, respectively, in methanol-water (1:3) at 70 °C. The polymers with different DP values were synthesized as described in the preparation of homopolymer, namely, under conditions with fixed concentrations of initiator and monomer and varying amount of chain-transfer agent. The product was purified in the same way as the homopolymers described above. The molecular weight of the copolymer was determined conductometrically. The monomer ratio (MEGal:AAM) in the copolymers was evaluated by elemental analyses and ¹H NMR (in D₂O): After the molecular weight of a copolymer sample was estimated to 1200 by conductometric titration, the monomer ratio (MEGal:AAM) in the copolymer was determined to be 1:7.6 by the ratio of integrals of ¹H NMR (calculated from the signals of 1.1–1.3 and 1.4–1.9 ppm corresponding to methyl protons of MEGal unit and methylene protons of AAM, respectively). Furthermore, the validity of supposed chemical structure was clarified by elemental analysis. Anal. Calcd. for C₂H₈NCIS(C₁₂H₂₀O₈)_{1.3}(C₃H₅-ON)_{9.7}·4H₂O: C, 44.67; H, 7.29; N, 12.00. Found: C, 44.09; H, 7.27; N, 12.20.

Preparation of Galactose-Carrying Amphiphiles by Polymerization. Details of the preparation were described elsewhere (9, 10, 19). Briefly, MEGal and DODA-501 were dissolved in THF, and after N₂ gas was bubbled for several minutes, the solution mixture was incubated in a tightly sealed test tube at 70 °C for 24 h. After evaporation of the solvent in vacuo, the viscous oil was washed with *n*-hexane several times to remove unreacted initiator by centrifugation at 15 000 rpm and 20 °C for 15 min. After decantation, the precipitate was dried in vacuo and dispersed in water. The suspension was purified by passage through a GPC column (Sephacryl S-200, 2 cm i.d. $\times$ 18 cm; mobile phase, water) and lyophilized. The DP value of the amphiphile (Figure 1c) was determined by elemental analysis. DODA-PMEGal (DP = 8.5) Anal. Calcd. for C₄₂H₈₂N₂O(C₁₂H₂₀O₈)_{8.5}: C, 55.51; H, 8.15; N, 0.90. Found: C, 55.43; H, 8.42; N, 0.91.

Preparation of Galactose Lipid by Using Trichloroacetimidate of Carbohydrate. The preparation procedure followed the method of Minamikawa et al. (23): To molecular sieves 4 Å (200 mg, dried in vacuo prior to use), 1,3-di-O-hexadecylglycerol (300 mg, 0.55 mmol) and 2,3,4,6-tetra-O-D-acetylgalactopyranosyl 1- α -trichloroacetimidate (273 mg, 0.55 mmol) in dry dichloromethane (10 mL) were added. The solution was cooled at 0 °C, and trimethylsilyl trifluoromethanesulfonate (123 mg, 0.55 mmol) was added dropwise to the solution. After the solution was stirred at 0 °C for 6 h, anhydrous pyridine (40 mL) was added dropwise at 0 °C and the solution was diluted with ethyl acetate (20 mL) at room temperature. After filtration, the filtrate was washed with a saturated sodium bicarbonate solution (30 mL) and saturated aqueous sodium chloride solution (30 mL) successively. The organic layer was dried over anhydrous sodium sulfate, and the solvent was removed under reduced pressure. The residual oil was purified by silica gel chromatography (toluene:acetone = 4:1) to give 1,3-di-O-hexadecyl-2-O-(β -D-tetraacetylgalactopyranosyl)-glycerol (157 mg). A 28% methanol solution of sodium methoxide (50 mL) was added to 1,3-di-O-hexadecyl-2-O-(β -D-tetraacetylgalactopyranosyl)glycerol (150 mg) in a mixture of dry methanol (2.5 mL) and dry DMF (2 mL). The reaction was complete after 2 h at room temperature. The solution was neutralized with ion-exchange resins (Dowex 50W-X4, H⁺ form), filtered, and evaporated. The

residue was chromatographed by silica gel chromatography (chloroform:methanol = 7:1). The compound was further purified by recrystallization from methanol to give 1,3-di-*O*-hexadecyl-2-*O*-(β -D-galactopyranosyl)glycerol (DHDG-Gal, Figure 1d) as a white powder (54 mg). ^1H NMR (CDCl_3): δ 0.88 (t, 6H, CH_3), 1.1–1.4 (m, 52H, CH_2 of alkyl chains), 1.57 (br, 4H, OCH_2CH_2), 2.8–4.1 (m, 19H, protons of galactose moiety, that of glycerol moiety, and OCH_2CH_2), 4.43 (d, $J = 7.6$ Hz, 1H, anomeric proton of pyranose ring). IR (KBr, cm^{-1}): O–H stretching of galactose moiety, 3350–3500; ν_{as} C–H, 2917; ν_{s} C–H, 2850; C–O–C stretching of glycerol moiety, 1040–1130. Anal. Calcd. for $\text{C}_{41}\text{H}_{82}\text{O}_8$: C, 70.02; H, 11.78. Found: C, 70.01; H, 11.83.

Preparation of Galactose-Carrying Liposomes. DDPC (24 mg) was dissolved in chloroform (2 mL) in a small, round-bottomed flask. After evaporation of the solvent, the thin lipid membrane was dispersed in an *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer (pH 6.5, 10 mM, 3 mL) in which DODA-PMEGal (6 mg) had been dispersed beforehand. The dispersion was sonicated (Astrason W-385, Heat System-Ultrasonics, Inc., New York) for 3 min while N_2 gas was passed through the suspension. To remove large aggregates, the liposome suspension obtained was passed through a membrane filter (SLGV025LS, Gelman Sciences; pore size, $0.22\ \mu\text{m}$) and irradiated in a quartz cell by a UV lamp (HB-251A, Ushio, Tokyo, Japan) at room temperature for 4 h to polymerize the dienoyl groups of DDPC. The progress of polymerization was followed by the decrease in absorbance at 258 nm by using a UV–vis spectrophotometer (Ubest-35, Japan Spectroscopic Co., Tokyo, Japan). Unpolymerized galactose-carrying liposome was also prepared without UV irradiation.

Characterization of Liposomes. Formation of liposomal structure was confirmed by the fluorescence dye incorporation technique using Eosin Y as a probe (20, 24) (excitation at 305 nm, emission at 571 nm, FP-777, Japan Spectroscopic Co.). Hydrodynamic diameter of the liposomes was estimated as 1200 Å on average by the dynamic light scattering (DLS) technique (DLS-7000, Otsuka Electronics, Hirakata, Japan; light source He–Ne laser, 632.8 nm).

Enzyme Activity Measurements. The oxidation reaction of galactose residues catalyzed by galactose oxidase was followed using a spectrophotometric method (25, 26): Hydrogen peroxide produced by the oxidation of galactose was reduced by the reaction with 4-aminoantipyrine and phenol catalyzed by horseradish peroxidase (HRP), and the increase in optical density at 500 nm due to the production of quinoneimine dye was measured for 5 min by the spectrophotometer thermostated at 25 °C. The initial increase in optical density per minute was calculated from the initial linear portion of the curve ($\epsilon = 12\ 000$). One unit of galactose oxidase causes the formation of $1\ \mu\text{mol}$ of hydrogen peroxide/min at 25 °C. The stock solution of galactose oxidase was diluted to the appropriate concentration, and the catalytic activity of the enzyme was checked just prior to use.

Kinetic Analyses. The initial velocities of oxidation of galactose at various reaction fields were measured by using the spectrophotometric method described above, in which sufficient amount of HRP, aminoantipyrine, and phenol were added in the reaction system, that is, the reaction of galactose oxidase to galactose residues is the rate-determining step. The substrate concentrations at various reaction fields were standardized on the basis of the number of galactose residues. Previous report showed that the galactose-carrying amphiphiles were preferen-

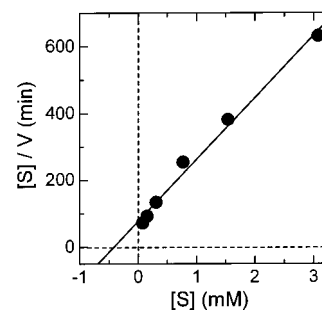


Figure 2. Hanes-Woolf plot for the catalytic reaction of galactose oxidase to a galactose-carrying homopolymer (PMEGal, DP = 16). In the HEPES buffer (10 mM, pH 6.5). [Enzyme] = 0.05 units/mL.

tially (about 80%) distributed in the outer layer of DDPC liposomes (19). Therefore, in the case using sugar-carrying liposomes as a substrate, we determined the substrate concentration based on the number of all galactose-carrying lipids incorporated inside and outside the liposome. The V_{max} and K_m values of the enzyme reaction were determined by the Hanes-Woolf plot (eq 1) (27):

$$[S]/v = (1/V_{\text{max}})[S] + K_m/V_{\text{max}} \quad (1)$$

where $[S]$ is the concentration of substrate (total concentration of galactose residues in the reacting solution), v is the initial reaction velocity, V_{max} is the limiting value of initial reaction velocity, and K_m is the Michaelis constant. Graphing $[S]/v$ vs $[S]$ yields a straight line where the slope is $1/V_{\text{max}}$, the y-intercept is K_m/V_{max} , and the x-intercept is $-K_m$. The data in the figures and table represent the mean values of two measurements. The uncertainties of the kinetic parameters were within 10%.

RESULTS AND DISCUSSION

A. Catalytic Behavior of Galactose Oxidase for Galactose-Carrying Polymers. After the incubation of galactose-carrying homopolymer with a solution of galactose oxidase, the production of H_2O_2 was confirmed by the spectrophotometric method. Figure 2 shows a typical example of Hanes-Woolf plot for the catalytic reaction of galactose oxidase to a galactose-carrying homopolymer (PMEGal, DP = 16). The affinity (estimated by $1/K_m$ values) of galactose oxidase for PMEGal was larger than those for free galactose and β -D-galactopyranosides (MEGal, lactose, PNPG and MG) (Table 1). Furthermore, by varying the degree of polymerization of PMEGal, we could examine the effect of the length of polymer chains on K_m (Figure 3a). The larger the molecular weight of PMEGal was, the larger the affinity of galactose oxidase. These results suggest that galactose oxidase can effectively recognize and oxidize galactose residues which are suspended repeatedly from the polymer chain, probably because the enzyme can easily access the galactose moiety in the vicinity of galactose which has been just oxidized. This phenomenon is consistent with the previous report that the galactose oxidase from *Polyporus circinatus* has a high affinity for galactomannan guaran (polysaccharide constituted from galactose and mannose) (26).

To verify the enhancement of the affinity of galactose oxidase for the substrates sequentially distributed, the similar experiment was carried out by using copolymers of MEGal and acrylamide (inert monomer for galactose oxidase). The affinity of galactose oxidase for the galactose residues in the copolymers with large DP values was

Table 1. Kinetic Constants of Galactose Oxidase for Various Galactose Residues at Different Reaction Fields^a

substrate	DP ^b	K_m (mM)	k_{cat} (mM mL/min units)	$10^2 k_{cat}/K_m$ (mL/min units)
galactose		58	1.0	1.7
MEGal	1	4.2	0.018	0.44
lactose		528	0.39	0.081
PNPG		22	0.40	1.8
MG		52	1.6	3.0
homopolymer	2.1 ^c	2.6	0.23	8.8
	16 ^c	0.35	0.091	26
	39 ^c	0.13	0.090	69
	53 ^c	0.13	0.14	110
copolymer	11 ^{c,d}	3.0	0.18	6.0
	13 ^{c,e}	0.76	0.24	32
	23 ^{c,f}	0.16	0.14	88
	54 ^{c,g}	0.18	0.042	23
polymerized liposome	1 ^h	ND ⁱ	ND ⁱ	ND ⁱ
unpolymerized liposome	8.5 ^j	0.0030	0.012	400
	8.5 ^j	0.0027	0.011	390

^a In the HEPES buffer (10 mM, pH 6.5) at 25 °C. The ranges of substrate concentration in the experiments using low molecular weight substrates, polymers, and liposomes were 1–100 mM, 0.01–10 mM, and 10^{-4} –0.1 mM, respectively. In the case of lactose, the range was 1 mM to 1 M. Substrate concentrations were standardized on the basis of the number of galactose residues (see Experimental Procedures). The concentrations of enzyme in the experiment using liposomes and other all substrates were 0.25 and 0.05 units/mL, respectively. ^b Degree of polymerization.

^c Determined by the conductometric method. ^d MEGal:AAM = 1:7.6. ^e MEGal:AAM = 1:1:1. ^f MEGal:AAM = 1:1:1. ^g MEGal:AAM = 1:5.7. ^h DHDG-Gal was used in place of DODA-PMEGal.

ⁱ Not detected. ^j Determined by the elemental analysis.

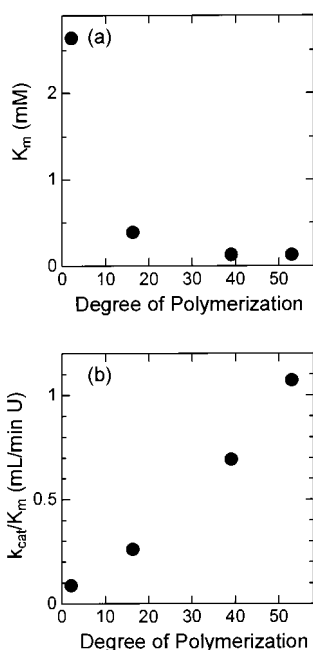


Figure 3. Effect of the degree of polymerization of homopolymer (PMEGal) on K_m and k_{cat}/K_m values at 25 °C. In the HEPES buffer (10 mM, pH 6.5). [Enzyme] = 0.05 units/mL.

comparable to those in the homopolymers with comparable DP values (Table 1), whereas the affinity for the galactose residues in the copolymer with a small DP value seemed to be smaller than that in the homopolymer with a comparable DP value. Since the spatial density of galactose residues existing in the copolymer chain is smaller than that in the homopolymer, the unexpectedly large affinity of the enzyme for the galactose residues in the copolymers with a large DP value shows that the distance between MEGal residues in the copolymers (as

for the copolymer with the monomer ratio 1:5.7, for example, 17 Å on average) is still small enough for the enzyme to access the galactose moieties one after another.

Furthermore, the k_{cat}/K_m value which represents the catalytic efficiency of galactose oxidase for PMEGal increased with the degree of polymerization of homopolymers (Figure 3b), which strongly suggests that the catalytic behavior of galactose oxidase represents an increased efficiency for the PMEGal due to the localization of substrates along the polymer chain.

B. Catalytic Behavior of Galactose Oxidase for Galactose-Carrying Liposomes. Next, to examine catalytic behavior of galactose oxidase at lipid bilayer–liquid interfaces, we prepared the galactose-carrying amphiphiles (DODA-PMEGal), which have galactose-carrying polymer chain as a hydrophilic group, from the lipophilic radical initiator and MEGal. A lipid carrying only one galactose residue (DHDG-Gal) was used as a reference.

When the galactose-carrying liposome consisted of DHDG-Gal and polymerized lipid (DDPC) was incubated with the solution of galactose oxidase, no production of H_2O_2 was confirmed by the spectrophotometric method, which is in a contrast to the results for β -D-galactopyranosides with small molecular weights (Table 1). Previously, Lampio et al. showed that galactose oxidase cannot oxidize the galactosyl residue of ceramide dihexoside in liposomes, probably because its carbohydrate moiety is too close to the membrane surface (17). Since the galactose residue of DHDG-Gal is directly attached to the glyceryl group, galactose oxidase would not easily approach the substrate, and therefore, our result is understandable.

When the similar experiment was carried out on the liposomes carrying the DODA-PMEGal, on the other hand, the production of H_2O_2 was definitely confirmed spectrophotometrically, which shows that the galactose-carrying polymer chain functions as a substrate for galactose oxidase not only in bulk solution but also at the lipid bilayer–liquid interface. The K_m value for the galactose-carrying liposome [DODA-PMEGal (DP = 8.5), 0.0030 mM] was much lower than that for the galactose-carrying homopolymer [PMEGal (DP = 16), 0.35 mM] (Table 1). Despite the steric disadvantage that the reaction field is above the lipid bilayer surface, the affinity of galactose oxidase for the galactose-carrying polymer chains on the liposome surface is much higher than that for PMEGal in bulk solution.

The galactose-carrying liposomes prepared here probably bring about the phase separation (or in other words, cluster formation) of the galactose-carrying amphiphiles in the liposomes by the two-dimensional photopolymerization of DDPC molecules (28). To verify clustering of galactose-carrying polymers on the liposome surface, we carried out a differential scanning calorimetry (DSC) with DODA-PMEGal/DDPC (unpolymerized) mixed liposomes. This experiment showed a sharp calorific change around 25 °C which is the temperature of the midpoint of gel–liquid crystal transition (T_m) of DDPC, indicating the phase separation due to the respective lipid molecules. If the different lipid molecules are compatible, the DSC curve would exhibit a broad calorific change. Therefore, the DODA-PMEGal/DDPC mixed liposome system is expected to show the phase-separated structure below 25 °C, even in the case when DDPC molecules are not polymerized. More importantly, when the DDPC molecules are polymerized, the Gal-carrying liposomes would show the phase-separated structure. This phase separation might make galactose oxidase, which has attacked

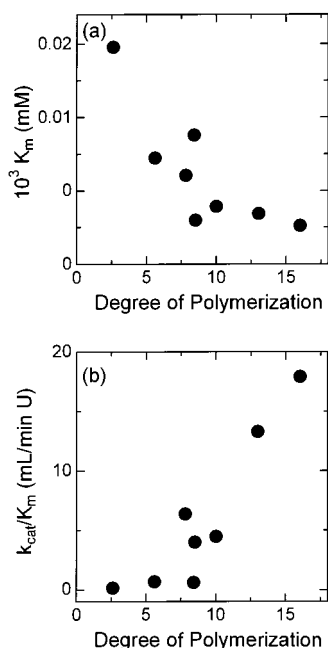


Figure 4. Effect of the degree of polymerization of DODA-PMEGal on K_m and k_{cat}/K_m values at 25 °C. [Enzyme] = 0.25 units/mL.

the amphiphile, surrounded by the galactose residues localizing on the liposome surface and, consequently, decrease the K_m value. This result is in agreement with the report by Masserini et al. (18) that the K_m value of G_{M1} ganglioside in vesicle was smaller than that of Des G_{M1} (oligosaccharide portion of G_{M1} ganglioside) in bulk solution.

The k_{cat} value, which is defined as the number of substrate molecules converted into product per enzyme molecule per unit time, of galactose oxidase for the DODA-PMEGal/polymerized DDPC liposomes was lower than those of free galactose and PMEGals, whereas the k_{cat}/K_m value, which represents the catalytic efficiency of an enzyme, of galactose oxidase for the liposomes was larger than those for free galactose and MEGal and comparable to that for PMEGals (Table 1). Since the galactose-carrying polymer chains at liquid-polymerized liposome interface are clustered, the motion of the enzyme molecule attaching the PMEGal domain above the liposome surface might be restricted, and therefore, it is understandable that the turnover number (k_{cat}) of galactose oxidase for DODA-PMEGal was smaller than those for galactose, MEGal and PMEGal. However, the clustering of the galactose-carrying polymer chains might enhance further the affinity of the enzyme for the substrate, which would consequently make the catalytic efficiency (k_{cat}/K_m) of galactose oxidase for the galactose-carrying liposomes larger than those for small molecular weight β -D-galactopyranosides.

Furthermore, the effect of the degree of polymerization of DODA-PMEGal on the catalytic behavior of galactose oxidase was examined. As shown in Figure 4a, the K_m value decreased with the degree of polymerization of DODA-PMEGal. Since the distance from the liposome surface to the end galactose residues increased with the DP value, galactose oxidase would be able to more easily approach the substrates above the liposome surface as the DP value becomes larger. It is worth noting that the increase in the degree of polymerization enhanced the affinity of galactose oxidase for galactose residue not only in the bulk solution but also at the lipid bilayer-liquid interfaces. The k_{cat}/K_m showed an increase with the DP

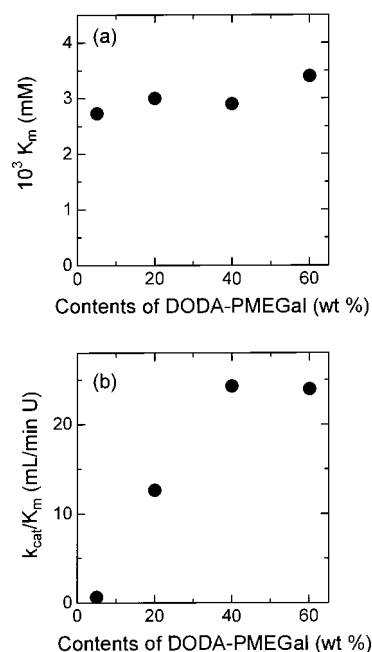


Figure 5. Effect of the content of DODA-PMEGal in the liposome on K_m and k_{cat}/K_m values at 25 °C. [Enzyme] = 0.25 units/mL.

value of DODA-PMEGal (Figure 4b), due to the decrease in the K_m value with DP.

Next, by varying the weight percents of DODA-PMEGal (DP = 8.5) included in the liposome, we could examine the effect of surface concentration of galactose-carrying amphiphiles on the affinity of galactose oxidase. Figure 5a shows that the K_m value was not significantly dependent on the surface concentration of galactose-carrying amphiphiles (DP = 8.5) above 10 wt % (3.4 mol %). Previously, Masserini et al. and Cestaro et al. investigated the catalytic properties of galactose oxidase and neuraminidase, respectively, for gangliosides on the liposome surface (18, 29). They showed that the greatest rates of enzymatic reaction were obtained at an optimal proportion of gangliosides in the liposome. These phenomena were explained by the enhanced hydrogen-bonding interaction between the carbohydrate residues of adjacent ganglioside molecules with an increase in the percentage of the ganglioside. The hydrophilic group of DODA-PMEGal used in our experiment is a polymethacrylate chain and would be more flexible than ganglioside. Therefore, the interaction between carbohydrate residues on the liposomes composed of DODA-PMEGal is probably smaller than that between gangliosides, thus, the optimum content of DODA-PMEGal in the liposome for the enzymatic reaction would not be observed in our experiment. We also think that this phenomenon is partly because the local concentration of galactose residue which the galactose oxidase can recognize is similar at any surface concentration due to the phase separation of DODA-PMEGal on the surface of polymerized DDPC liposome.

Furthermore, the k_{cat}/K_m value increased with the content of DODA-PMEGal in the galactose-carrying liposome (Figure 5b), which means that the enzyme effectively and repeatedly catalyzes oxidation of the substrates which exist on the surface of the same liposome. This is probably because it is not necessary for the enzyme molecule to migrate urgently from the liposome surface catalyzing presently to other liposome surfaces, and the enzyme only needs to oxidize the galactose residues on the same liposome.

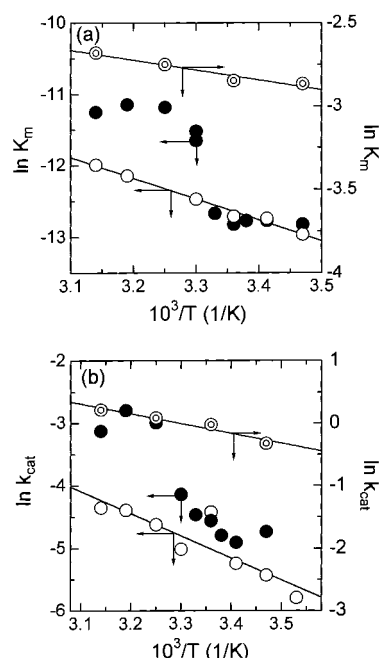


Figure 6. van't Hoff and Arrhenius plots of enzymatic reaction for various galactose residues. In the HEPES buffer (10 mM, pH 6.5). [Enzyme] = 0.25 and 0.05 units/mL for liposomes and free galactose, respectively. Contents of DODA-PMEGal (DP = 8.5) in the liposome, 20 (w/w) %. (○) polymerized liposome. (●) unpolymerized liposome; (○) free galactose. The units of K_m and k_{cat} are (molarity) and (millimolar milliliters per minute units), respectively.

Moreover, by using galactose-carrying liposome with unpolymerized DDPC, we could examine the effect of membrane fluidity on the catalytic behavior of galactose oxidase. As shown in Figure 6a, the K_m value of galactose oxidase for the unpolymerized galactose-carrying liposome was larger than that for the polymerized galactose-carrying liposome above the T_m of unpolymerized DDPC (25 °C) and comparable below 25 °C. The similar tendency was observed in the Arrhenius plots of k_{cat} (Figure 6b).

The membrane fluidity of the unpolymerized galactose-carrying liposome above 25 °C would be higher than that of polymerized one, which makes it difficult for galactose oxidase to face the domain of the galactose-carrying polymer chains on the unpolymerized fluid liposome surface, because the PMEGal chains would not be localized above 25 °C. Previously, we examined the effect of temperature on the inhibition of trypsin by soybean trypsin inhibitor at lipid–lipid interfaces (30). The recognition processes at interfaces were strongly dependent on the gel–liquid crystal-phase transition temperature of the liposomes, which supports the importance of fluidity of liposome membranes in the oxidation of MEGal residues by the enzyme at lipid bilayer–liquid interfaces.

In conclusion, the recognition of galactose residues on the liposome surface by galactose oxidase was more effective compared to those of free galactose and other small β -D-galactopyranosides. The affinity of galactose oxidase for sugar residues on the surface of liposomes was dependent on the length of galactose-carrying polymer chain and cluster formation of the amphiphiles on the liposome surface, but not significantly influenced by the surface density of galactolipids on liposomes. The present study indicates that the spatial distribution of substrates significantly affects the catalytic behavior of the enzyme. More importantly, the clustering-effect of sugar residues enhances the affinity of sugar-recognizing

proteins not only lectins but also enzymes, which would be valuable information in carbohydrate chain chemistry and polymer science.

ACKNOWLEDGMENT

We thank Misses Y. Murasaki and A. Sugayama of this department for their technical assistance. We are grateful to Wako Pure Chemicals for the kind donation of V-501. The Japan Fat and Oil Co. is greatly appreciated for the kind donation of DDPC. This work was supported by Grants-in-Aid for Scientific Research on Priority Areas (08246222, 09240213, 09232223, 10126221) from the Ministry of Education, Science, Sports and Culture.

LITERATURE CITED

- (1) Wassarman, P. M. (1987) The Biology and Chemistry of Fertilization. *Science* 235, 553–560.
- (2) Imai, Y., Lasky, L. A., and Rosen, S. D. (1993) Sulphation Requirement for GlyCAM-1, an Endothelial Ligand for L-Selectin. *Nature* 361, 555–557.
- (3) Bliss, T. V. P., and Collingridge, G. L. (1993) A Synaptic Model of Memory: Long-Term Potentiation in the Hippocampus. *Nature* 361, 31–39.
- (4) Mizuuchi, T., Matthews, T. J., Kato, M., Hamako, J., Titani, K., Solomon, J., and Feizi, T. (1990) Diversity of Oligosaccharide Structures on the Envelope Glycoprotein gp 120 of Human Immunodeficiency Virus 1 from the Lymphoblastoid Cell Line H9: Presence of Complex-Type Oligosaccharides with Bisecting *N*-Acetylglucosamine Residues. *J. Biol. Chem.* 265, 8519–8524.
- (5) Lee, Y. C., and Lee, R. T., Eds. (1994) Neoglycoconjugates: Part A Synthesis. *Methods Enzymol.* Vol. 242, Academic Press, New York.
- (6) Lee, Y. C., and Lee, R. T., Eds. (1994) Neoglycoconjugates: Part B Biomedical Applications. *Methods Enzymol.* Vol. 247, Academic Press, New York.
- (7) Kim, S. W., Pai, C. M., Makino, K., Seminoff, L. A., Holmberg, D. L., Gleeson, J. M., Wilson, D. E., and Mack, E. J. (1990) Self-Regulated Glycosylated Insulin Delivery. *J. Controlled Release* 11, 193–201.
- (8) Ouchi, T., and Ohya, Y. (1994) in *Neoglycoconjugates: Preparation and Applications* (Y. C. Lee, and R. T. Lee, Eds.) pp 465–498, Academic Press, New York.
- (9) Kitano, H., and Ohno, K. (1994) Sugar-Containing Lipids Prepared by Using a Lipophilic Radical Initiator: Interfacial Recognition by Lectin as Studied by Using the Multiple Internal Reflection Fluorescence Method. *Langmuir* 10, 4131–4135.
- (10) Kitano, H., Sohda, K., and Kosaka, A. (1995) Galactose-Containing Amphiphiles Prepared with a Lipophilic Radical Initiator. *Bioconjugate Chem.* 6, 131–134.
- (11) Suzuki, Y., and Suzuki, K. (1972) Specific Radioactive Labeling of Terminal *N*-Acetylgalactosamine of Glycosphingolipids by the Galactose Oxidase-Sodium Borohydride Method. *J. Lipid Res.* 13, 687–690.
- (12) Eccleston, E. D., White, T. W., Howard, J. B., and Hamilton, D. W. (1994) Characterization of a Cell Surface Glycoprotein Associated with Maturation of Rat Spermatozoa. *Mol. Reprod. Dev.* 37, 110–119.
- (13) Enrich, C., and Gahmberg, C. G. (1985) Characterization of Plasma-Membrane Glycoproteins from Functional Domains of the Rat Hepatocyte. *Biochem. J.* 227, 565–572.
- (14) Kumarasamy, R., and Blough, H. A. (1985) Galactose-Rich Glycoproteins Are on the Cell Surface of Herpes Virus-Infected Cells. *Arch. Biochem. Biophys.* 236, 593–602.
- (15) Stewart, R. J., and Boggs, J. M. (1993) Exposure of Galactosylceramide to Galactose Oxidase in Liposomes: Dependence of Lipid Environment and Ceramide Composition. *Biochemistry* 32, 5605–5614.
- (16) Lampio, A., Rauvala, H., and Gahmberg, C. G. (1986) Exposure of Major Neutral Glycolipids in Red Cells to Galactose Oxidase: Effect of Neuraminidase. *Eur. J. Biochem.* 157, 611–616.

- (17) Lampio, A., Siissalo, I., and Gehmberg, C. G. (1988) Oxidation of Glycolipids in Liposomes by Galactose Oxidase. *Eur. J. Biochem.* 178, 87–91.
- (18) Masserini, M., Sonnino, S., Ghidoni, R., and Chigormo, V. (1982) Galactose Oxidase Action on G_{M1} Ganglioside in Micellar and Vesicular Dispersions. *Biochim. Biophys. Acta* 688, 333–340.
- (19) Ohno, K., Sohda, K., Kosaka, A., and Kitano, H. (1995) Galactose-Containing Amphiphiles Prepared with a Lipophilic Radical Initiator: Association Processes between Liposomes Triggered by Enzymatic Reaction. *Bioconjugate Chem.* 6, 361–366.
- (20) Kitano, H., Akatsuka, Y., and Ise, N. (1991) pH-Responsive Liposomes Which Contain Amphiphiles Prepared by Using Lipophilic Radical Initiator. *Macromolecules* 24, 42–46.
- (21) Matsumura, S., Kubokawa, H., and Toshima, K. (1993) Enzymatic Synthesis of Novel Vinyl Monomers Bearing β -D-Galactopyranoside Residue. *Makromol. Chem., Rapid Commun.* 14, 55–58.
- (22) Kuwamura, T. (1961) Long Chain Aliphatic Derivatives of Glyceryl α, α' -Diethers. *Kogyo Kagaku Zasshi* 64, 1958–1964.
- (23) Minamikawa, H., Murakami, T., and Hato, M. (1994) Synthesis of 1,3-Di-O-alkyl-2-O-(β -glycosyl)glycerols Bearing Oligosaccharides as Hydrophilic Groups. *Chem. Phys. Lipids* 72, 111–118.
- (24) Kitano, H., Wolf, H., and Ise, N. (1990) pH-Responsive Release of Fluorophore from Homocysteine-Carrying Polymerized Liposomes. *Macromolecules* 23, 1958–1961.
- (25) Cooper, J. A. D., Smith, W., Bacila, M., and Medina, H. (1959) Galactose Oxidase from *Polyporus circinatus*. *Fr. J. Biol. Chem.* 234, 445–448.
- (26) Avigad, G., Amaral, D., Asensio, C., and Horecker, B. L. (1962) The D-Galactose Oxidase of *Polyporus circinatus*. *J. Biol. Chem.* 237, 2736–2743.
- (27) Gerrett, R. H., and Grisham, C. (1995) *Biochemistry*, Saunders College Publishing, Orlando.
- (28) Wagner, N., Dose, K., Koch, H., and Ringsdorf, H. (1981) Incorporation of ATP Synthetase into Long-Term Stable Liposomes of a Polymerizable Synthetic Sulfolipid. *FEBS Lett.* 132, 313–318.
- (29) Cestaro, B., Barenholz, Y., and Gatt, S. (1980) Hydrolysis of Di- and Trisialo Gangliosides in Micellar and Liposomal Dispersion by Bacterial Neuraminidases. *Biochemistry* 19, 615–619.
- (30) Kitano, H., Kato, N., Tanaka, N., and Ise, N. (1988) Mutual Recognition between Polymerized Liposomes: Enzyme and Enzyme Inhibitor System. *Biochim. Biophys. Acta* 942, 131–138.

BC970166Y

CORRECTIONS

Volume 9, Number 5, September/October 1998.

Kohji Ohno and Hiromi Kitano*

CATALYTIC PROPERTIES OF GALACTOSE OXIDASE TO LIPOSOME-FORMING AMPHIPHILES WHICH HAVE MANY PENDENT GALACTOSE RESIDUES

Page 552. The y-axis of Figure 4a should appear as follows.

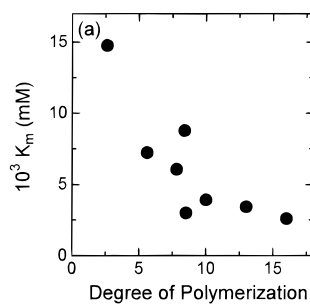


Figure 4.

BC9801981

10.1021/bc9801981

09/26/1998

Sequence-Specific Photomodification of DNA by an Oligonucleotide-Phenanthrodihydrodioxin Conjugate

Kestutis G. Bendinskas,* Andreas Harsch,^{†‡} R. Marshall Wilson,[‡] and W. Robert Midden

Center for Photochemical Sciences, Department of Chemistry, Bowling Green State University, Bowling Green, Ohio 43403 . Received December 19, 1997; Revised Manuscript Received May 22, 1998

We introduce a new member of a family of photochemically active oligonucleotide conjugates. A Phenanthrodihydrodioxin (PDHD)-based agent was synthesized and covalently linked to a 5'-end of the 9-mer oligonucleotide via a hexamethylene linker. The conjugate hybridized to a complementary 30-nucleotide-long target and efficiently cleaved it in a sequence specific manner. Up to 67% of target was specifically damaged (51% cross-links and 16% direct cleavage). While the photosensitizer alone nonspecifically damaged only Gs in a single-stranded target, its conjugate cross-linked to and damaged also A, T, and C sites in a target in agreement with duplex and triplex formation.

INTRODUCTION

Therapeutic potential of covalently modified oligonucleotides has driven the search for agents that can be coupled to DNA, are easy to control, and damage specific DNA target sites (1). One of the most frequently used classes of such agents has been photosensitizers since they offer the advantage of controlling the timing of the chemical inactivation event (2).

Several photoreactive groups have been covalently linked to oligonucleotides and their analogues (3). For example, psoralens (Pso)¹ that are able to form inter-strand cross-links or monoadducts in double-stranded DNA (4), often have been used as photosensitizers coupled to oligonucleotides. Pso-pN₁₃ was covalently linked to target DNA and irreversibly inhibited *bla* gene transcription in *Escherichia coli* in vitro (5). Psoralen-methylphosphonate conjugate cross-linked with rabbit globin mRNA 50–100% and specifically inhibited translation to the extent of 40–60% at oligomer concentrations of 5–20 μ M (6). Pso-pN₁₆ inhibition of *Dra*I digestion of HIV-I DNA was 95% at 7 μ M oligonucleotide concentration (7, 8). Pso-pN₁₀ induced 100-fold more frequent mutations in a specific region of the λ genome (9). After UV irradiation, Pso-pN₁₅ cross-linked and inhibited transcription of the α -subunit of the interleukin 2 receptor in vivo (10).

Another class of photosensitizers, porphyrins, linked to oligonucleotides, produced various types of photodamage on a complementary target DNA (11–13). This included oxidation of G and cross-linking of the porphyrin conjugate to the target sequence. Both reactions gave 57% yield of total modification (20% oxidized G, and 37%

cross-linking). Guanines located close to the porphyrin macrocycle were the most altered (11). Up to 7% of target DNA was cross-linked, and up to 23% was cleaved by the conjugate of sapphyrin, an extended porphyrin that damages DNA upon irradiation with 300 and >620 nm light (13). The conjugates of 3-azidoflavine (14) and *p*-azidophenacyl (15) were photo-cross-linked to the single- and double-stranded 27-mers and induced cleavage after piperidine treatment. A small fraction of ellipticine derivative photo-cross-linked with double-stranded DNA (16). Eosin has been linked to oligonucleotides and produced damage in double-stranded targets (17). Proflavin-linked oligonucleotide was able to induce 12% cleavage after irradiation followed by piperidine treatment (18). Uranyl UO₂²⁺ photooxidation induced cleavage at the ends of oligodeoxynucleotides. However, its conjugates failed to do so (19). Aryl azide/biotin/oligonucleotide derivative cross-linked to its target and was used to quantitate triple-helix formation (20).

Considering potential benefits of photosensitizer-DNA conjugates, it is important to search for and study new kinds of photoreactive groups that could give high yields of specific target DNA inactivation.

The purpose of this investigation was to prepare a PDHD–DNA conjugate and investigate its sequence-specific DNA damage potency. PDHDs were designed as photoactivatable DNA-damaging agents (21–26), and this study is a first attempt to couple them to a molecule that will give them sequence specificity.

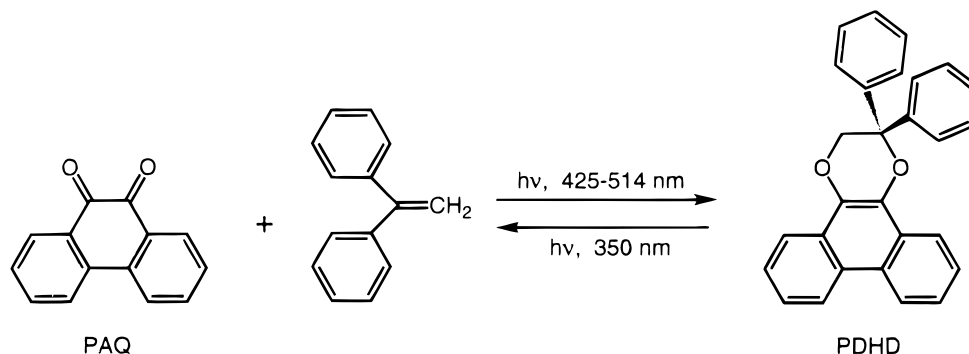
PDHDs are masked *o*-quinones: they can be made from and release phenanthrenequinone (PAQ) when irradiated with 425–514 and 350 nm light, respectively (Scheme 1) (21). This release of parent PAQ is believed to be responsible for DNA damage and cleavage. Several PDHD analogues carrying ionic groups to make them soluble in water have been prepared. PDHDs with cationic groups bind to DNA with typical binding constants $K_{\text{ass}} = 2.5 \times 10^4 \text{ M}^{-1}$. They show hyperchromism and a 3–7 nm bathochromic shift in the presence of DNA which is consistent with intercalation and/or groove binding. Cationic PDHDs efficiently relax supercoiled Φ X 174 FR I DNA upon irradiation with low doses of 365 nm light under both oxygen-rich and oxygen-free conditions. Cationic PDHDs inactivate up to 4 logs of

* Author to whom correspondence should be addressed. Present address: Department of Chemistry and Biochemistry, Notre Dame University, Notre Dame, Indiana 46556. Phone: (219)-631-8313. Fax: (219)-631-6652. E-mail: Bendinskas.1@nd.edu.

[†] Present address: Barnett Institute, Northeastern University, 360 Huntington Avenue, Boston, Massachusetts 02115.

[‡] Department of Chemistry, University of Cincinnati, Cincinnati, Ohio 45221.

¹ Abbreviations: PDHD, phenanthrodihydrodioxin; Pso, psoralen; PAQ, phenanthrenequinone; DTT, dithiothreitol; TCA, trichloroacetic acid; THF, tetrahydrofuran; σ , standard deviation; SCP, sodium chloride in sodium phosphate buffer.

Scheme 1. Schönberg Reaction: Forward Reaction with Visible Light (425–514 nm), Reverse Reaction with UV Light (350 nm)

Vaccinia virus. Results of transient spectroscopy, quenching of DNA cleavage with dithiothreitol (DTT), and irradiation of PDHDs in the presence of cytochrome *c* and superoxide dismutase support a mechanism of DNA damage which involves the release of excited PAQ that is capable of hydrogen abstraction or electron transfer and the involvement of superoxide and other radicals (21, 27). Other mechanisms of DNA damage by cationic PDHDs, including involvement of singlet oxygen, are possible. The relative efficiencies of different DNA-cleaving mechanisms for different PDHDs is being investigated and will be a subject of a separate publication.

No information was available about sequence specificity of PDHDs. Due to the small size, little if any specificity was expected. Because of the relatively low DNA binding affinity [10 times lower than ethidium bromide, 1000 times lower than tripyridyl ruthenium (27)] PDHDs are good candidates for use as DNA conjugates since they should allow sequence-specific recognition by the targeting oligonucleotide, increasing its binding slightly, but not so much as to alter the site-specificity provided by the sequence-recognizing oligonucleotide group.

EXPERIMENTAL PROCEDURES

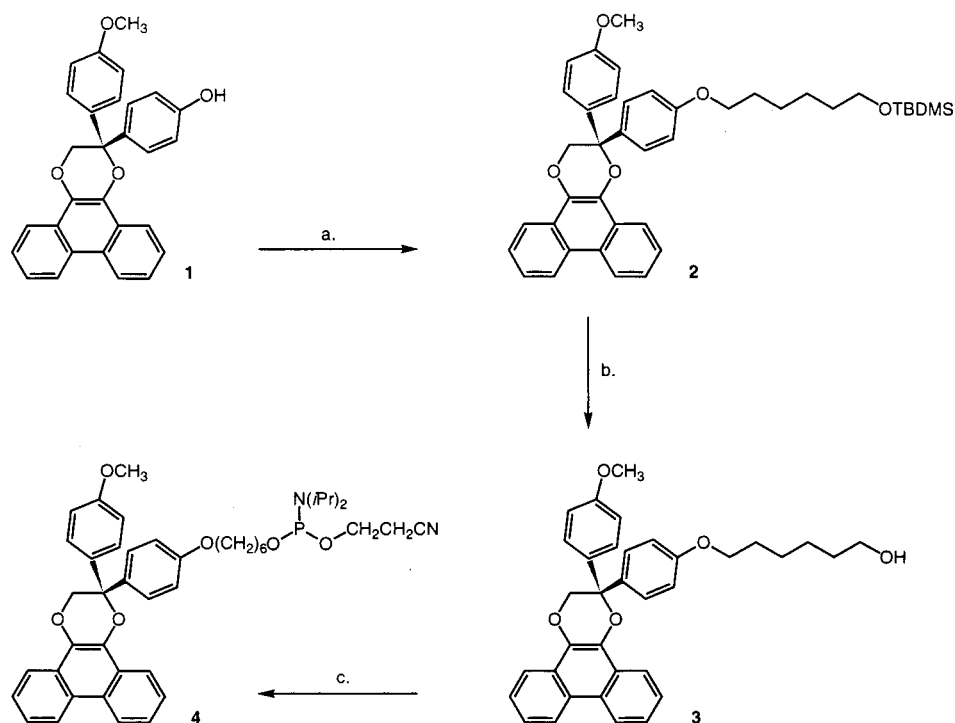
Preparation of Oligonucleotides. Regular oligonucleotides were synthesized on PerSeptive Biosystems Expedite Synthesizer using standard phosphoramidite chemistry at the DNA Core Facility at the University of Cincinnati. All oligos were purified by reverse-phase columns and PAGE and dissolved in water to make a 20 μ M solution. Target DNA was labeled at the 5'-end using [γ - ^{32} P]ATP and T4 polynucleotide kinase. Its purity and labeling efficiency were checked by 20% polyacrylamide gel electrophoresis.

Synthesis of 2-[4-(6-(*tert*-Butyldimethylsilyloxy)-hexyloxy)phenyl]-2-[4-methoxy-phenyl]-2,3-dihydrophenanthro-[9.10-b]-1,4-dioxin (2, Scheme 2). Dihydrodioxin **1** (Scheme 2) (0.0827 g, 0.19 mmol) was placed in a 5 mL one-necked flask and dissolved in 1.5 mL of HPLC-grade acetonitrile, followed by the addition of 0.06 g (0.23 mmol) 18-crown-6, 0.032 g (0.23 mmol) of potassium carbonate, and 0.078 g (0.23 mmol) of 6-(*tert*-butyldimethylsilyloxy)-1-iodohexane. The reaction was allowed to stir under nitrogen for 12 h and was then diluted with chloroform. The solids were removed by filtration and the solvent was evaporated. The crude reaction mixture was purified by rotary chromatography (7.5% ethyl acetate, 92.5% hexanes). The product **2** was isolated as a colorless oil. Product $C_{41}H_{48}O_5Si$; molar mass: 648.9 g/mol; yield 0.0993 g (0.153 mmol), 80.5%. ^1H NMR ($CDCl_3$) δ (ppm): 8.56 (m, 2H), 8.43 (d, J = 7.5

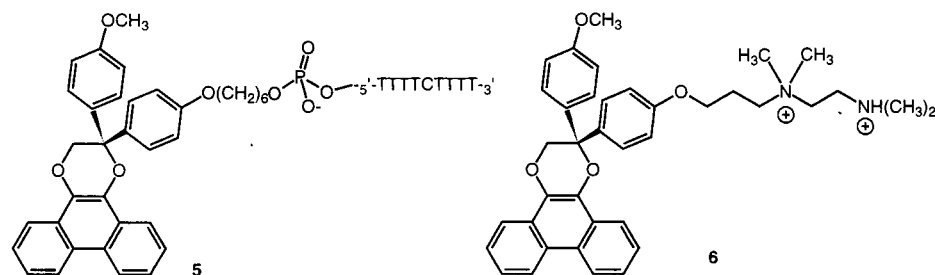
Hz, 1H), 8.12 (m, 1H), 7.67–7.42 (m, 8H), 6.83 (d, J = 8.6 Hz, 2H), 6.81 (d, J = 8.6 Hz, 2H), 4.75 (s, 2H), 3.88 (t, J = 6.5 Hz, 2H), 3.74 (s, 3H), 3.59 (t, J = 6.2 Hz, 2H), 1.72 (m, 2H), 1.54–1.35 (m, 6H), 0.88 (s, 9H), 0.03 (s, 6H). ^{13}C NMR ($CDCl_3$) δ (ppm): 158.8, 133.4, 133.1, 132.8, 132.5, 128.2, 128.1, 127.9, 126.9, 126.7, 126.6, 126.5, 126.2, 124.7, 122.4, 122.3, 120.8, 120.7, 114.3, 113.8, 79.3, 70.4, 67.8, 63.1, 55.2, 32.8, 29.2, 25.9, 25.8, 25.6, 18.4, –5.3. IR ($CHCl_3$): 2933, 2858, 1633, 1610, 1583, 1512, 1462, 1335, 1251, 1178, 1089, 1009, 978, 834 cm^{-1} . MS (m/z) 440, 208, 180, 152, 133 (base peak).

Synthesis of 2-[4-(6-Hydroxyhexyloxy)phenyl]-2-[4-methoxy-phenyl]-2,3-dihydrophenanthro-[9.10-b]-1,4-dioxin (3, Scheme 2). In a 5 mL round-bottom flask, 0.0993 g (0.153 mmol) of **2** was dissolved in distilled THF. The solution was cooled in an ice bath, then 0.184 mL of a 1 M solution of tetrabutylammonium fluoride in hexanes (0.184 mmol) was added dropwise via syringe. The reaction mixture was allowed to stir for 4 h at 0 $^{\circ}C$. Thin-layer chromatography showed complete disappearance of the protected alcohol. The solution was diluted with 10 mL of chloroform and washed twice with water. The organic layer was collected and filtered through magnesium sulfate, and the solvent was evaporated. Purification was achieved by rotary chromatography (70% ethyl acetate, 30% hexanes). The product was isolated as a highly viscous oil. Product $C_{35}H_{34}O_5$; molar mass 534.7 g/mol; yield 0.0664 g (0.124 mmol), 81.2%. ^1H NMR ($CDCl_3$) δ (ppm): 8.56 (m, 2H), 8.43 (d, J = 8.3 Hz, 1H), 8.11 (m, 1H), 7.67–7.41 (m, 8H), 6.83 (d, J = 8.3 Hz, 2H), 6.79 (d, J = 8.3 Hz, 2H), 4.74 (s, 2H), 3.86 (t, J = 6.3 Hz, 2H), 3.72 (s, 3H), 3.61 (t, J = 6.4 Hz, 2H), 1.77–1.49 (m, 8H), 1.27 (s, 1H). ^{13}C NMR ($CDCl_3$) δ (ppm): 159.2, 158.7, 133.4, 133.1, 132.8, 132.5, 128.2, 127.8, 126.9, 126.7, 126.6, 126.4, 126.2, 124.8, 122.5, 122.4, 120.8, 120.7, 114.3, 113.7, 79.3, 70.4, 67.7, 62.8, 55.2, 32.7, 29.2, 25.9, 25.5. IR ($CHCl_3$): 3622, 3080, 2938, 2881, 1633, 1610, 1512, 1454, 1350, 1335, 1250, 1178, 1118, 1089, 1040, 949, 832 cm^{-1} .

Synthesis of 2-[4-(6-(*N,N*-Diisopropyl)[cyanoethyloxy]phosphoroxy-hexyloxy)phenyl]-2-[4-methoxy-phenyl]-2,3-dihydrophenanthro-[9.10-b]-1,4-dioxin (4, Scheme 2). A solution of 0.0296 g (0.055 mmol) of **3** and 0.019 mL (0.11 mmol) *N,N*-diisopropylethylamine in 0.5 mL of distilled methylene chloride was placed in a 2 mL microscale flask. The flask was purged with argon, then 0.013 mL (0.058 mmol) of 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite was added via syringe. The reaction was stirred at room temperature for 10 min, then diluted with chloroform, and the organic layer was washed twice with small portions of water. The organic solution was filtered through magnesium sulfate,

Scheme 2. Synthesis of PDHD–Phosphoramidite Precursor **4**^a

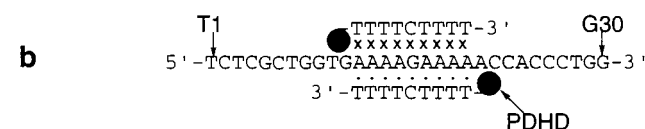
a. $[(CH_2)_6OTBDMS, K_2CO_3, 18\text{-crown-}6]$
 b. $Bu_4N^+F^-$
 c. $ClP[N(iPr)_2]OCH_2CH_2CN, EtN(iPr)_2$



^a **1**, phenanthrodihydrodioxin (PDHD) derivative; **2**, 2-[4-(6-(*tert*-butyldimethylsilyloxy)hexyloxy)phenyl]-2-[4-methoxy-phenyl]-2,3-dihydrophenanthro-[9.10-*b*]-1,4-dioxin; **3**, 2-[4-(6-hydroxyhexyloxy)phenyl]-2-[4-methoxy-phenyl]-2,3-dihydrophenanthro-[9.10-*b*]-1,4-dioxin; **4**, 2-[4-(6-([*N,N*-diisopropyl][cyanoethyloxy]phosphoroxy-hexyloxy)phenyl)-2-[4-methoxy-phenyl]-2,3-dihydrophenanthro-[9.10-*b*]-1,4-dioxin; **5**, the PDHD conjugate; and **6**, water soluble photosensitizer PDHD.

Scheme 3^a

30A 5'-TCTCGCTGGTGAAGAAAAACCACCCTGG-3'
a **30T** 3'-AGAGCGACCACTTTTCTTTTGGTGGGACC-5'
9T 5'-TTTTCTTTT-3'



^a (a) Sequences of the target DNA (**30A**) and the complementary oligonucleotides **30T** and **9T**; (b) alignment of the target and the conjugates. The bottom strand binds to the target in antiparallel orientation via Watson–Crick base-pairing. The third strand binds in parallel orientation via Hoogsteen base-pairing (**5**). The bases in the target are numbered from the 5'-end.

and the desired product **4** was isolated by rotary chromatography (50% ethyl acetate, 48.5% hexanes, 1.5%

triethylamine). Product $C_{44}H_{51}N_2O_6P$; molar mass 734.9 g/mol; yield 0.0338 g (0.046 mmol), 83.6%. ^1H NMR ($CDCl_3$) δ (ppm): 8.56 (m, 2H), 8.43 (d, $J = 8.2$ Hz, 1H), 8.11 (m, 1H), 7.67–7.41 (m, 8H), 6.83 (d, $J = 8.4$ Hz, 2H), 6.80 (d, $J = 8.7$ Hz, 2H), 4.74 (s, 2H), 3.89–3.49 (m, 11H, $-OCH_3$, 2 $-CH=$, 3 $-CH_2$), 2.57 (t, $J = 6.3$ Hz, 2H), 1.75–1.25 (m, 8H), 1.15 (dd, $J = 6.5$ Hz, $J' = 4.6$ Hz, 12H). ^{13}C NMR ($CDCl_3$) δ (ppm): 159.1, 158.7, 133.3, 133.1, 132.8, 132.5, 128.1, 126.9, 126.7, 126.6, 126.5, 126.1, 124.7, 122.4, 122.3, 120.8, 120.7, 117.6, 114.2, 113.7, 79.3, 70.4, 67.7, 63.6, 63.4, 58.4, 58.1, 55.1, 43.1, 42.8, 31.1, 31.0, 29.7, 29.1, 25.7, 25.6, 24.6, 24.5, 24.4, 20.3, 20.2. ^{31}P NMR ($CDCl_3$) δ (ppm): (external standard, H_3PO_4) 148.0. IR ($CHCl_3$): 3080, 2968, 2936, 2248, 1633, 1610, 1512, 1461, 1335, 1250, 1179, 1088, 1040, 978 cm^{-1} . MS (m/z) ($M + H^+$) observed, 735.3; calculated: 735.3. After its synthesis, the phosphoramidite **4** was tested on TLC plates for its stability in 30% ammonia and 20 mM I_2 in

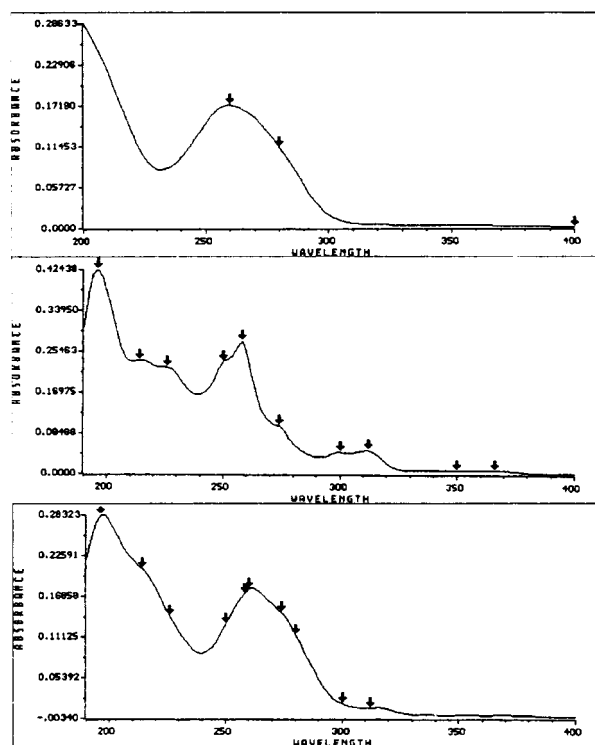


Figure 1. Absorbance spectra of 1 μ M 9T DNA (top panel), 5 μ M PDHD 6 (middle panel), and 1 μ M conjugate 5 (bottom panel).

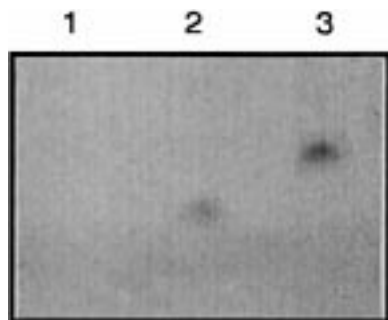


Figure 2. A photograph of the UV-shadowing experiment. Lane 1: 1 nmol of PDHD 6; lane 2: 1 nmol of PDHD 6 + 1 nmol of 9T DNA, gray spot; lane 3: 1 nmol of the conjugate 5, bright blue spot.

THF/pyridine/water, then carefully dehydrated, and dissolved in acetonitrile to make a 0.1 M solution.

Synthesis of an Oligonucleotide–PDHD Conjugate (5, Scheme 2). The 9-mer TTTTCTTTT was synthesized in the DNA Core Facility at the University of Cincinnati by standard synthesis on a 1 μ M scale using a ABI 394 synthesizer with the following changes: an easily deprotected phosphoramidite of dC was used, and, after the 5'-end deprotection step of the oligonucleotide, TCA was washed from the column with acetonitrile for 2 min (4 times longer than usual) to minimize exposure of the photosensitizer to acid. The coupling of this resin-bound oligonucleotide to PDHD–phosphoramidite was performed in 0.5 M tetrazole in acetonitrile for 3 min (6 times longer than usual) to increase coupling efficiency. Instead of standard 0.1 M I_2 , 20 mM I_2 in THF/pyridine/water was used for the oxidation step. The detritylation step, since it involves the use of TCA, was excluded at the end of the program. After cleaving from the column in aqueous ammonia, the conjugate was deprotected for 1 h at room temperature, purified using a reversed-phase

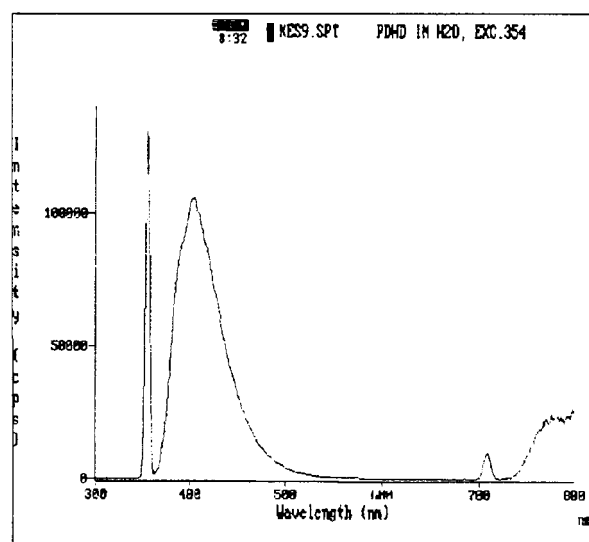


Figure 3. Fluorescence spectra of 2.5 μ M PDHD, excitation at 354 nm.

cartridge, and checked using PAGE. The conjugate's UV–vis and fluorescence spectra were recorded with a Hewlett Packard 8452A Diode Array Spectrophotometer and SPEX 1680 0.22M Double Spectrometer, respectively. Mass spectrum of the conjugate was taken on PerSeptive Biosystems Voyager-DE MALDI-TOF as the sum of 64 nitrogen laser shots at 336 nm using 3-hydroxypicolinic acid as a matrix.

Unless stated otherwise, all chemicals for syntheses were purchased from Aldrich. Solvents were purified according to standard procedures. ^1H and ^{13}C and ^{31}P -NMR spectra were recorded with a Bruker AC 250. IR spectra were recorded with a Perkin-Elmer 1600 Series FTIR in a solution cell (path length, 0.2 mm). Low resolution mass spectra were taken with a Hewlett-Packard HP 5995, electron impact 70 eV or a Finnigan Mat LCQ Ion Trap, high-resolution mass spectra were obtained on a Kratos MS 801-DS 55. Unless stated otherwise, preparative chromatography separations were performed using centrifugal chromatography with a Chromatotron and plates were coated with E. Merck silica gel 60-PF254.

UV-Melting Experiments. The change of absorbance at 260 nm was measured with a Cary 219 Spectrophotometer interfaced to an IBM computer with an Intel 80386 processor that also controlled a Haake A80 circulating water bath and collected temperature data from a thermocouple using custom software. The sample chamber was purged with N_2 . DNA was dissolved in SCP (1 M NaCl, 10 mM sodium phosphate, pH 7.4) buffer, heated to 98 $^{\circ}C$, and cooled slowly, at 0.5 $^{\circ}C/min$ rate, to 4 $^{\circ}C$.

Maxam and Gilbert Chemical Sequencing. The target DNA was sequenced using standard procedures (28) with the following changes: both, GA and TC reactions with formic acid and hydrazine, respectively, were incubated for 2 h at room temperature, and the treatment with 10% piperidine was performed at 90 $^{\circ}C$ for 60 min for GA and for 50 min for TC reaction.

Photochemical Cleavage Reaction. Radioactively labeled target DNA at 10 nM concentration was mixed with PDHD or PDHD-conjugate at 0–10 μ M in 20 μ L of SCP buffer. Carrier calf thymus DNA (2 μ g) was included where indicated. Samples were heated at 55 $^{\circ}C$ for 1 min, cooled to 4 $^{\circ}C$ at the rate of 1 $^{\circ}C/min$ in a thermal cycler, and incubated for 1 h at 4 $^{\circ}C$. All of the

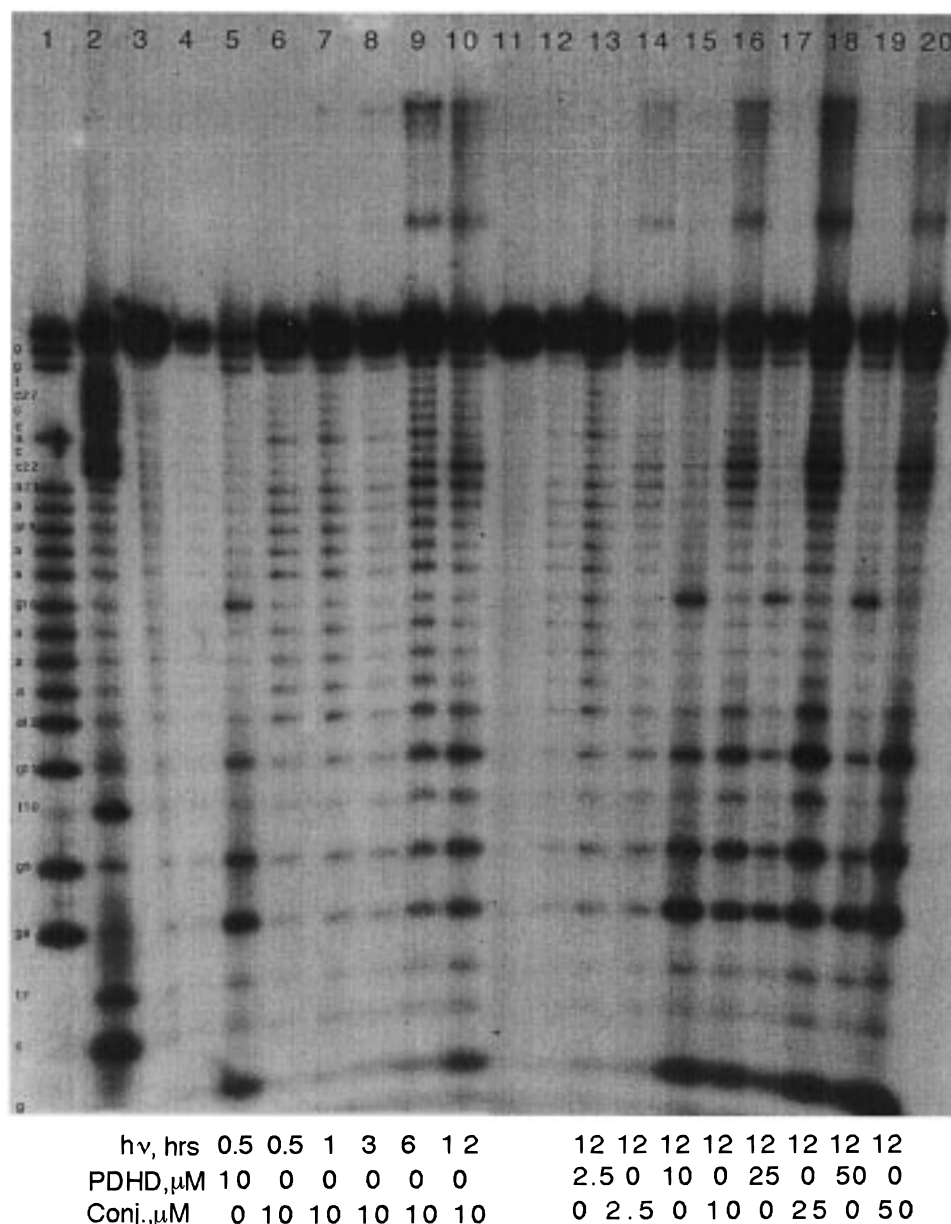


Figure 4. Irradiation of the target **30A** DNA with and without PDHD **6**/conjugate **5** for different times and concentrations. Lanes: (1) GA Maxam-Gilbert reaction of the target, (2) TC Maxam-Gilbert reaction, (3 and 12) target irradiated for 12 h and treated with piperidine (as the rest of the lanes, unless otherwise indicated), (4 and 11) target not irradiated and not treated, (5–10 and 13–20) target irradiated ($h\nu$) for indicated times with indicated concentrations of PDHD or PDHD-conjugate.

above were performed with dimmed lights. Samples were placed on Parafilm on a glass table surrounded by SCP buffer and enclosed in a Pyrex glass dish. Samples were irradiated for 1–12 h at 4 °C in a RAYONET Preperative Type RS Photochemical Reactor equipped with a cooling fan and heated-cathode 350 nm emission lamps from Southern New England Ultraviolet Co. Irradiance at the sample was 2 mW/cm². The reaction was stopped by turning off the lamps. Radicals were quenched by addition of 2 μ L of 0.1 M DTT. DNA was treated in 10% piperidine for 1 h, triple-lyophilized, redissolved in 10 μ L of 80% formamide loading buffer, and run on 20% polyacrylamide, 8 M urea gel. After electrophoresis, the gel was covered with saran wrap and exposed to Kodak X-ray film. Films were scanned using a Bio-Rad GS-670 imaging densitometer. The data were processed on an IBM computer with an Intel 80386 processor using Molecular Analyst software.

RESULTS AND DISCUSSION

The M13 genome was chosen as a target for the studies. The sequence AAAAGAAAA (between bases 5983 and 5992 of M13, traditional numbering) was selected for several reasons. It is a unique site in the genome. It is a potential site not only for double-strand but also for triple-strand formation. It is a part of the *lac i* gene which enables blue/white plaque screening for later studies. A 30-mer 5'-TCTCGCTGGTGAAAAGA-AAAACCACCCTGG-3' (**30A**, Scheme 3a) containing the target sequence AAAAGAAAA was synthesized and labeled with ³²P at the 5'-end. The conjugate that binds to such a target should have a polypyrimidine sequence 5'-TTTTCTTTT-3' to be capable of both double- and triple-strand formation (Scheme 3b). The hexamethylene linker was used to give the photosensitizer PDHD some degree of freedom to interact with the target. Two oligonucleotides complementary to the target were syn-

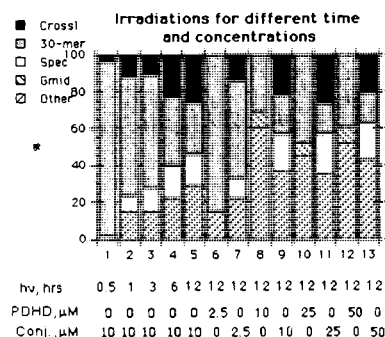


Figure 5. Quantitation of product formation for Figure 4. The amount of cross-links (Crossl), undamaged 30-mer (30-mer), specific cleavage in the region between bases A19 and C27 (Spec), damage to G16 (Gmid), and damage in the region between bases G8 and G11 (Other) was measured by scanning densitometry. Irradiation times and reagent concentrations are as indicated.

thesized, a 30-mer **30T** and a 9-mer **9T** (Scheme 3a) for the appropriate control experiments.

The PDHD derivative **1** (Scheme 2) was synthesized in a five step procedure (27). This synthesis will be reported elsewhere. Subsequent functionalization of **1** was achieved using an etherification method described by Fedorynski et al. (29). The reaction was carried out in the presence of potassium carbonate and 18-crown-6, allowing mild deprotonation of the phenolic compound **1**. The alkylating agent was 6-(*tert*-butyldimethylsiloxy)-1-iodohexane. It was prepared according to a published procedure by Heslin et al. (30). Introduction of the hexyl side chain yielded the PDHD derivative **2** (Scheme 2) in approximately 80% yield. Subsequent cleavage of the silyl ether by treatment with tetrabutylammonium fluoride afforded the alcohol **3** (Scheme 2) in good yield. This compound was then reacted with a 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite (**31**) in the presence of *N,N*-diisopropylethylamine, yielding the desired PDHD-phosphoramidite **4** (Scheme 2) in 84% yield. Its structure was verified by complete spectral characterization. Due to the lability of the dihydrodioxin ring system, the M^+ ion of **4** could not be observed using EI or FAB. However, ESI provided the molecular ion peak, $(M + H)^+$.

The PDHD oligonucleotide conjugate **5** (Scheme 2) was prepared by standard synthesis of TTTTGTTTT at 1 μ M scale and coupling this oligonucleotide to PDHD-phosphoramidite using special precautions and the manual adjustment of standard synthesis procedures to avoid any damage of the conjugate by light, acid, or harsh treatment during deprotection (see Experimental Procedures). The yield after purification through a reversed-phase column was 191 μ g.

The PDHD dication **6** (Scheme 2) was prepared for comparison of the effects of the free photosensitizer versus the effects of the conjugate. This synthesis will be reported elsewhere.

The synthesis of the conjugate **5** was confirmed using UV-vis, fluorescence, PAGE, and MALDI-TOF. The 200–400 nm spectra of **9T** DNA alone, PDHD **6**, and the conjugate **5** were compared. The spectra of the conjugate has the peaks of both DNA and PDHD (Figure 1), indicating the presence of both substances in the product. Covalent linkage of PDHD to the oligonucleotide was confirmed by electrophoresis of PDHD **6**, PDHD **6** with **9T** DNA and the conjugate **5** on 8% polyacrylamide 8M urea gel (Figure 2). DNA and PDHD were visualized by UV-shadowing. As expected, PDHD in the first lane migrated out of the gel because it has a positive charge.

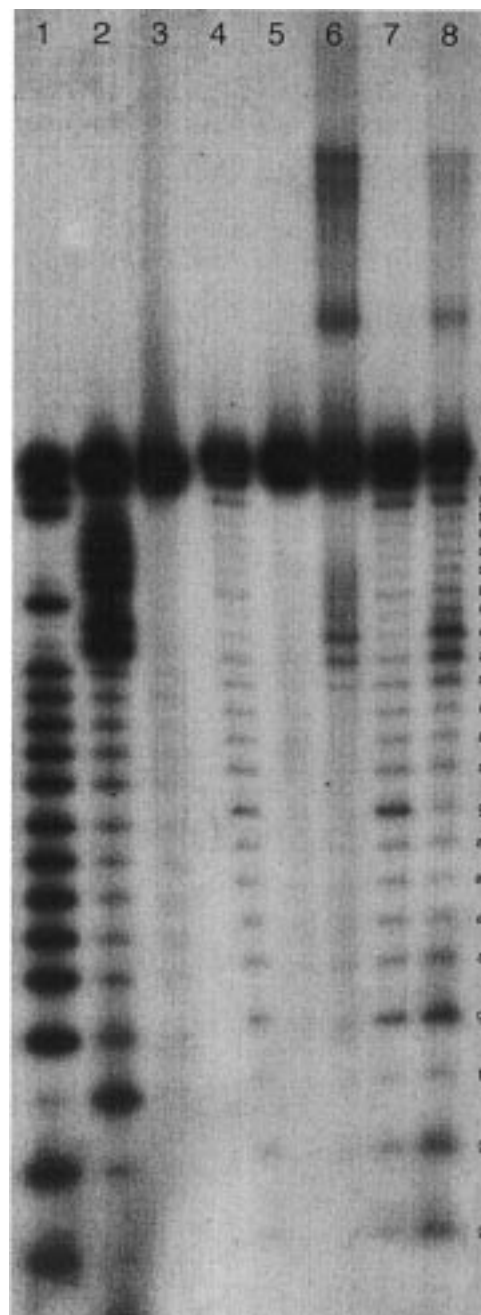


Figure 6. Irradiation of the target **30A** DNA with and without PDHD **6**/conjugate **5** with and without piperidine treatment. Lanes: (1) GA Maxam-Gilbert reaction of the target, (2) TC Maxam-Gilbert reaction, (3) target not treated, (4) target irradiated for 12 h (as the rest of the samples) and treated with piperidine, (5 and 6) without piperidine treatment, (7 and 8) with piperidine treatment, (5 and 7) with 10 μ M PDHD, and (6 and 8) with 10 μ M conjugate.

Although it is electrostatically bound to DNA, it also migrated out of the second lane that contained both PDHD and **9T** DNA. The gray band in this lane is **9T** DNA. Only the lane containing the conjugate exhibited the strong dark blue (about 400–440 nm) fluorescence that is observed at 420 nm with PDHD alone (Figure 3). This proves that PDHD is covalently linked to **9T** DNA in the conjugate. Notably, the conjugate migrates more slowly on the gel because of the higher molecular weight and because of the lower charge to molecular weight ratio (PDHD is an approximate equivalent of one and a half nucleotides with no charge) (Figure 2). The covalent linkage was clearly confirmed by the MALDI-TOF spec-

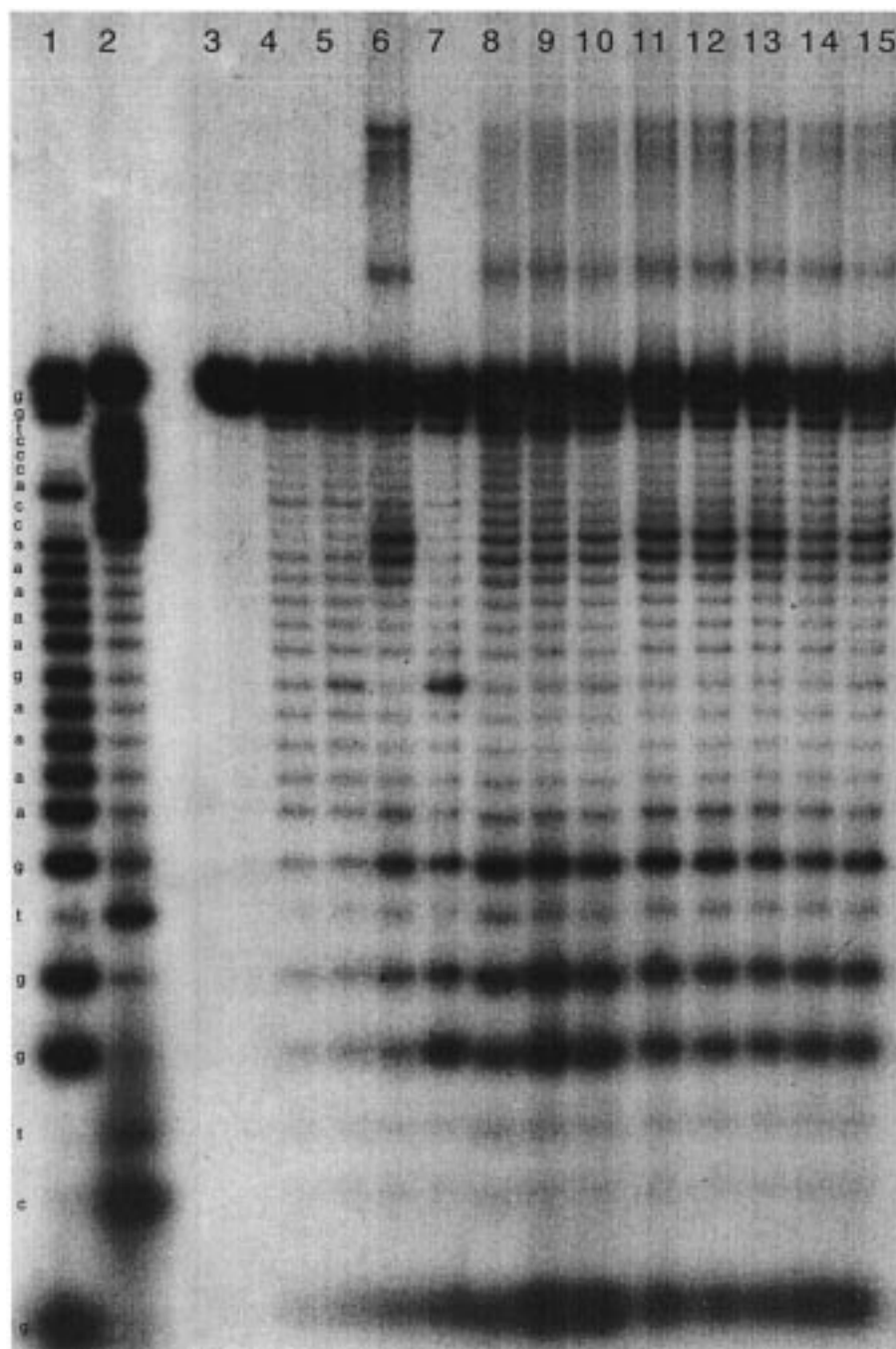


Figure 7. Irradiation of the target **30A** DNA with and without PDHD **6**/conjugate **5** in the presence of carrier DNA, at different concentrations of NaCl and different pH. Lanes: (1) GA Maxam–Gilbert reaction of the target, (2) TC Maxam–Gilbert reaction, (3) target not treated, (4–6) with 2 μ g of calf thymus carrier DNA, (4) target irradiated for 12 h and treated with piperidine (as the rest of the lanes, unless otherwise indicated), (5 and 7) with 10 μ M PDHD, (6 and 8–13) with 10 μ M conjugate, (8) 0 M NaCl, (9) 0.1 M NaCl, (10) 0.25 M NaCl, (11) 0.5 M NaCl, (12) 0.75 M NaCl, (13) 1.0 M NaCl, pH 7.4, (14) pH 6.0, (15) pH 8.8.

trum of the conjugate $[(m/z) (M - H)^-]$ observed, 3255.1; calculated, 3256.3].

To assess how PDHD in a conjugate affects DNA–DNA binding, UV-melting experiments were performed. **30A** was premixed with **30T**, **9T**, and a conjugate **5** at 1:1 molar ratio in SCP buffer, heated to 98 °C, and cooled slowly to 4 °C to anneal. Multiple UV-melting curves were collected, and melting temperatures of duplexes were determined at the maximum of the calculated dA/dT curve [data not shown, (32)]. The melting temperature for the duplex of **30A** and **30T** was 80.8 °C ($\sigma = 0.1$ °C). The duplex of **30A** and **9T** melted at 20.1 °C ($\sigma = 0.5$ °C). The duplex of **30A** and a conjugate **5** had a melting temperature of 21 °C ($\sigma = 0.5$ °C). This is the

expected result indicating very little, if any, stabilization of the duplex by the conjugated PDHD.

Irradiation of the ss 30-mer target **30A** with and without a PDHD conjugate **5** and dication PDHD **6** was performed in SCP buffer for 1–12 h at 350 nm at about 2 mW/cm² at 4 °C. The concentration of the radioactively labeled target was constant at 10 nM. The concentration of the conjugate and photosensitizer was varied from 0 to 10 μ M. The reactants were premixed, heated to 55 °C, and cooled to 4 °C at a rate of 1 °C/min and incubated at 4 °C for 1 h in the dark before irradiation. After irradiation, the radical reaction was quenched by 10 mM DTT. Then the sample was treated with 10% piperidine at 90 °C to cleave the single stranded target at damaged

bases. Results were analyzed by electrophoresis on denaturing polyacrylamide gels. To determine which bases were damaged, Maxam–Gilbert chemical sequencing was performed. Two controls were also included: the target that was not irradiated and the target that was irradiated and treated with piperidine, to determine the background cleavage that is not due to PDHD and PDHD conjugate.

The following can be concluded from Figure 4. Free PDHD damages specifically only Gs in ss DNA (lane 5 vs lane 3). In contrast, the conjugate specifically cleaves A21 and C22 (lane 10 vs lane 5). This can be explained only by ds formation (Scheme 3b). Notably, the conjugate also specifically damages T7, T10, and A12 (lane 18 vs lane 17), sites that are consistent with triplex formation (Scheme 3b). The conjugate protects from nonspecific cleavage G16, which is in the middle of the binding region (lane 16 vs lane 15). The conjugate nonspecifically damages other Gs (G8, G9, and G11 in lane 16 vs lane 15). The conjugate also forms cross-links with the target forming two new bands of higher MW (**30A** with a conjugate via ds formation and **30A** with two conjugates via triplex formation, lane 9).

Damage to the target was quantitated by scanning densitometry. The total amount of cross-links, undamaged 30-mer, specific cleavage in the region between bases A19 and C27, and damage of G16 and damage in the region between bases G8 and G11 was estimated (Figure 5). As time of irradiation was increased from $\frac{1}{2}$ to 12 h, cross-links increased from 4 to 26%, specific damage increased from 2 to 17% (Figures 4 and 5). The irradiation time for all further studies was set at 12 h. The concentration of PDHD and the conjugate were varied in the range 2.5–50 μ M. There was little specific damage and cross-links by the conjugate formed at 2.5 μ M concentrations. These products increased at 10 μ M concentrations. Further increases in concentration only increased nonspecific damage (Figures 4 and 5). Thus, the concentration of damaging agents was set at 10 μ M for all further experiments.

When ss target was irradiated with PDHD or PDHD conjugate, but not treated with piperidine, little cleavage at Gs was observed with free PDHD (lane 5 vs lane 7, Figure 6), and there were more cross-links than cleavage with the conjugate (lane 6 vs lane 8, Figure 6). This indicates that PDHD is damaging Gs, but not cleaving them, and that the conjugate primarily forms cross-links that are later partially cleaved by piperidine.

When ss target was irradiated with a large amount of carrier DNA (2000 carrier, 1 target), PDHD cleaved the target severalfold less (lane 5 vs lane 7, Figure 7), indicating complete nonspecificity, while the conjugate cleaved with almost the same efficiency as it cleaved in the absence of carrier DNA (lane 6 vs lane 13, Figure 7). Specific cleavage increased when the concentration of NaCl was increased (0, 0.1, 0.25, 0.5, 0.75, and 1.0 M in lanes 8–13, Figure 7, and lanes 10–15, Figure 8). This is consistent with increased double-strand stability at higher salt concentrations. Slight variation of pH (7.4, 6.0, and 8.8 in lanes 13–15, Figure 7) did not affect cleavage significantly. Triplex cleavage was not detected under these conditions. The maximum site-specific modification achieved was 67% (51% of cross-links and 16% of direct cleavage) in a sample that was not treated with piperidine (lane 3, Figure 8).

Four separate irradiations of the target with the conjugate at standard conditions (10 nM target, 10 μ M conjugate, 12 h of irradiation, piperidine treated) gave an average of 40% site-specific modifications: 23% ($\sigma =$

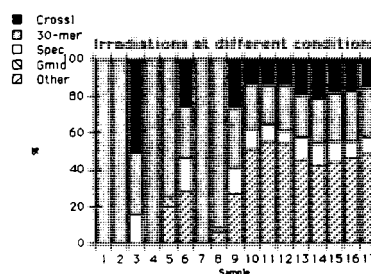


Figure 8. Quantitation of product formation for Figures 6 and 7. Samples: (1–3) DNA alone, with PDHD and with the conjugate, no piperidine treatment, (4–6) DNA alone, with PDHD and with the conjugate, piperidine treated, (7–9) DNA alone, with PDHD and with the conjugate, with 2 μ g of calf thymus carrier DNA present, 10–15 NaCl concentrations of 0, 0.1, 0.25, 0.5, and 1 M, respectively, (15) pH 7.4, (16) pH 6, (17) pH 8.8.

3%) cross-links and 17% ($\sigma = 3\%$) direct cleavage (lanes 5 and 9, Figure 5, and lanes 6 and 15, Figure 8).

While the damage at double-strand formation sites was always consistent, the damage at triple-strand sites was observed in a few cases, in conditions where it was strongly favored. Some evaporation of the samples, even though compensated by the addition of water, may have affected product formation.

As compared to other photosensitizers that were conjugated to DNA (2, 3), PDHD seems to have desirable qualities. The wavelength of excitation is 350 nm, above the absorbance of DNA and proteins although still much shorter than the 700 nm that is more useful for phototherapy. PDHD conjugate gives high yields (up to 67%) of specific damage, which places it second after psoralens (6) in efficacy of damage. PDHD conjugate is capable of photo-cross-linking to or damaging all four bases in a target DNA, while most other sensitizers damage only a limited set of bases (4, 13). Besides the site-specific damage, the current generation of PDHD conjugates induce nonspecific damage of Gs around the targeted sequence. This can hopefully be eliminated by attaching the sequence-recognizing unit to the quinone rather than the olefin-derived moiety, thus preventing damage to nearby sequences through migration of released PAQ.

In conclusion, the photochemical cleavage studies prove that the PDHD–oligonucleotide conjugate binds to the complementary target. It efficiently and site-specifically cleaves the designated target, thus, the incorporation of the oligonucleotide sequence recognizing unit onto the PDHD converts a nonspecific photochemical reagent into a sequence specific one.

In future studies, the ss PDHD conjugate will be tested as a selective photochemical M13 virus inactivating agent by measuring inactivation of the *lac i* gene. A variety of improvements of PDHDs are currently under investigation.

ACKNOWLEDGMENT

We sincerely thank the DNA Core Facility at the University of Cincinnati for providing all oligonucleotides and performing the DHD–oligonucleotide coupling reaction. R.M.W. thanks the NSF (CHE-9122141) for financial support.

LITERATURE CITED

- (1) Goodchild, J. (1990) Conjugates of oligonucleotides and modified oligonucleotides: a review of their synthesis and properties. *Bioconjugate Chem.* 1 (3), 165–186.

- (2) Knorre, D. G., and Zarytova, V. F. (1991) Novel antisense derivatives: antisense DNA intercalators, cleavers, and alkylators. in *Prospects for antisense nucleic acid therapy of cancer and AIDS* (E. Wickstrom, Ed.) pp 195–218, Wiley-Liss, New York.
- (3) Thoung, N. T., and Asseline, U. (1991) Oligonucleotides attached to intercalators, photoreactive and cleavage agents. in *Oligonucleotides and analogues* (F. Eckstein, Ed.) pp 283–306, IRL-Press, Oxford.
- (4) Cimino, G. D., Gamper, H. B., Isaacs, S. T., and Hearst, J. E. (1985) Psoralens as photoactive probes of nucleic acid structure and function: Organic chemistry, photochemistry, and biochemistry. *Annu. Rev. Biochem.* 54, 1151–1193.
- (5) Duval-Valentin, G., Thoung, N. T., and Helene, C. (1992) Specific inhibition of transcription by triple helix-forming oligonucleotides. *Proc. Natl. Acad. Sci. U.S.A.* 89, 504–508.
- (6) Kean, J. M., Murakami, A., Blake, K. B., Cushman, C. D., and Miller, P. S. (1988) Photochemical cross-linking of psoralen-derivatized oligonucleoside methylphosphonates to rabbit globin messenger RNA. *Biochemistry* 27, 9113–9121.
- (7) Giovannangeli, C., Rougee, M., Garestier, T., Thoung, N. T., and Helene, C. (1992) Triple-helix formation by oligonucleotides containing the three bases thymine, cytosine, and guanine. *Proc. Natl. Acad. Sci. U.S.A.* 89, 8631–8635.
- (8) Giovannangeli, C., Thoung, N. T., and Helene, C. (1992) Oligonucleotide-directed photoinduced cross-linking of HIV proviral DNA via triple-helix formation. *Nucleic Acids Res.* 20 (16), 4275–4281.
- (9) Havre, P. A., Gunther, E. J., Gasparro, F. P., and Glazer, P. M. (1993) Targeted mutagenesis of DNA using triple helix-forming oligonucleotides linked to psoralen. *Proc. Natl. Acad. Sci. U.S.A.* 90, 7879–7883.
- (10) Grigoriev, M., Praseuth, D., Guieysse, A. L., Robin, P., Thoung, N. T., Helene, C., and Harel-Bellan, A. (1993) Inhibition of gene expression by triple helix-directed DNA cross-linking at specific sites. *Proc. Natl. Acad. Sci. U.S.A.* 90, 3501–3505.
- (11) Doan, T. L., Praseuth, D., Perrouault, L., Chassignol, M., Thoung, N. T., and Helene, C. (1990) Sequence-targeted photochemical modifications of nucleic acids by complementary oligonucleotides covalently linked to porphyrins. *Bioconjugate Chem.* 1, 108–113.
- (12) Vlasov, V. V., Deeva, E. A., Ivanova E. M., Knorre, D. G., Maltseva, T. V., and Frolova, E. I. (1991) Photoactivatable porphyrin oligonucleotide derivatives for sequence specific chemical modifications and cleavage of DNA. *Nucleosides Nucleotides* 10 (1–3), 641–643.
- (13) Sessler, J. L., Sansom, P. I., Kral, V., O'Connor, D., and Iverson, B. L. (1996) Sapphyrin-oligonucleotide conjugates. Novel sequence-specific DNA photomodifying agents with increased binding affinity. *J. Am. Chem. Soc.* 118, 12322–12330.
- (14) Doan, T. L., Perrouault, L., Praseuth, D., Decout, J.-L., Habboub, N., Lhomme, J., Thoung, N. T., and Helene, C. (1987) Sequence-specific recognition, photo-cross-linking and cleavage of the DNA double helix by an oligo-[α]-thymidylate covalently linked to an azidoproflavine derivative. *Nucleic Acids Res.* 19, 7749–7760.
- (15) Praseuth, D., Perrouault, L., Doan, T. L., Chassignol, M., Thoung, N. T., and Helene, C. (1988) Sequence-specific binding and photo-cross-linking of α - and β -oligodeoxynucleotides to the major groove of DNA via triple-helix formation. *Proc. Natl. Acad. Sci. U.S.A.* 85, 1349–1353.
- (16) Perrouault, L., Asseline, U., Rivalle, C., Thoung, N. T., Bisagni, E., Giovannangeli, C., Doan, T. L., and Helene, C. (1990) Sequence-specific artificial photoinduced endonucleases based on triple helix-forming oligonucleotides. *Nature* 344, 358–360.
- (17) Durland, R. H., Kessler, D. J., and Hogan, M. (1991) Antiparallel triplex formation at physiological pH. in *Prospects for antisense nucleic acid therapy of cancer and AIDS* (E. Wickstrom, Ed.) pp 219–226, Wiley-Liss, New York.
- (18) Praseuth, D., Doan, T. L., Chassignol, M., Decout, J.-L., Habboub, N., Lhomme, J., Thoung, N. T., and Helene, C. (1988) Sequence-targeted photosensitized reactions in nucleic acids by oligo- α -deoxynucleotides covalently linked to proflavin. *Biochemistry* 27, 3031–3038.
- (19) Hill, A. R., Orgel, L. E. (1991) Photooxidation of oligodeoxynucleotides by UO_2^{2+} . *Bioconjugate Chem.* 2, 431–434.
- (20) Geselowitz, D. A., Neumann, R. D. (1995) Quantitation of triple-helix formation using a photocross-linkable aryl azide/biotin/oligonucleotide conjugate. *Bioconjugate Chem.* 6, 502–506.
- (21) Wilson, R. M., Schnapp, K. A., Harsch, A., Keller, S. J., and Schlemm, D. J. (1996) Preparation of 1,4-dihydrodioxins and related compounds as DNA cleaving reagents. *PCT Int. Appl. WO 96 20, 205*, (Cl C07h21/00), 4 Jul 1996, US Appl. 365,091, 28 Dec 1994; 67 pp. (Eng.), and personal communications.
- (22) Koch, T., Ropp, J. D., Sligar, S. G., and Schuster, G. B. (1993) Photocleavage of DNA: Irradiation of quinone-containing reagents converts supercoiled to linear DNA. *Photochem. Photobiol.* 58 (4), 554–558.
- (23) Armitage, B., You, C., Devadoss, C., and Schuster, G. B. (1994) Cationic anthraquinone derivatives as catalytic DNA photonucleases: mechanism for DNA damage and quinone recycling. *J. Am. Chem. Soc.* 116 (22), 9847–9859.
- (24) Breslin, D. T. and Schuster, G. B. (1996) Anthraquinone photonucleases: mechanisms for GC-selective and nonselective cleavage of double-stranded DNA. *J. Am. Chem. Soc.* 118 (10), 2311–2319.
- (25) Ly, D., Kan, Y., Armitage, B., and Schuster, G. B. (1996) Cleavage of DNA by irradiation of substituted anthraquinones: intercalation promotes electron transfer and efficient reaction at GG steps. *J. Am. Chem. Soc.* 118 (36), 8747–8748.
- (26) Breslin, D. T., Coury, J. E., Anderson, J. R., McFail-Isom, L., Kan, Y., Williams, L. D., Bottomley, L. A., and Schuster, G. B. (1997) Anthraquinone photonuclease structure determines its mode of binding to DNA and the cleavage chemistry observed. *J. Am. Chem. Soc.* 119 (21), 5043–5044.
- (27) Harsch, A. (1996) Masked *ortho*-quinones as photoactivated DNA cleaving agents. *Diss. Abstr. Int.* 57 (10B), 6263.
- (28) Maxam, A. M., and Gilbert W. (1980) Sequencing end-labeled DNA with base-specific chemical cleavages. *Methods Enzymol.* 65, 499.
- (29) Fedorynski, M., Wojciechowski, K., Matacz, Z., and Makosza, M. (1978) Sodium and Potassium Carbonates: Efficient Strong Bases in Solid-Liquid Two-Phase Systems. *J. Org. Chem.* 43, 4682–4684.
- (30) Heslin, J. C. and Moody, C. J. (1988) Rhodium Carbenoid Mediated Cyclisations. Part 2. Synthesis of Cyclic Ethers. *J. Chem. Soc., Perkin Trans. 1*, 6, 1417–1423.
- (31) Sinha, N. D., Biernat, J., and Koester, H. (1983) b-CYANOETHYL *N,N*-Dialkylamino/*N*-Morpholinomethyl Phosphoramidites, New Phosphorylating Agents Facilitating Ease of Deprotection and Workup of Synthesized Oligonucleotides. *Tetrahedron Lett.* 24, 5843–5846.
- (32) Bendinskas, K. G. (1996) Towards directing photosensitizers to key viral targets using combinatorially selected oligonucleotides. *Diss. Abstr. Int.* 58 (03B), 1262.

BC970209A

Polycaprolactone-*b*-poly(ethylene Oxide) Block Copolymer Micelles as a Novel Drug Delivery Vehicle for Neurotrophic Agents FK506 and L-685,818

Christine Allen,[†] Yisong Yu,[†] Dusica Maysinger,^{*,‡} and Adi Eisenberg^{*,†}

Department of Chemistry, McGill University, 801 Sherbrooke Street West, Montreal P.Q, Canada H3A 2K6, and Department of Pharmacology and Therapeutics, McGill University, 3655 Drummond St., Montreal, P.Q. Canada H3G 1Y6. Received December 19, 1997; Revised Manuscript Received May 7, 1998

Micelles formed from polycaprolactone-*b*-poly(ethylene oxide) (PCL-*b*-PEO) diblock copolymers were investigated as a novel drug delivery system. The affinity of the micelles for hydrophobic solubilizates was assayed by determining the partition coefficient for the lipophilic compound, pyrene, between the micelles and water; the partition coefficient was found to be on the order of 10^2 . The Trypan blue and Alamar blue survival assays were used to assess the in vitro biocompatibility of the micelles with PC 12 cells, MCF-7 breast cancer cells, and primary cultures of human microglia, astrocytes, and cortical neurons. The micelles were then studied as a delivery vehicle for the neurotrophic agents FK506 and L-685,818 in PC 12 cell cultures. In both cases, the micelle-incorporated drugs, in the presence of nerve growth factor (5 ng/mL), were able to promote the degree of differentiation of the PC 12 rat pheochromocytoma cells.

INTRODUCTION

Colloidal drug delivery vehicles such as liposomes, microspheres, nanospheres, and block copolymer micelles have recently been shown to increase the therapeutic index and improve the selectivity of various potent drugs (1–8). These vehicles have been shown to optimize the therapeutic efficacy of drugs by preventing their rapid elimination from the body (4), reducing their systemic toxicity (4), delaying their degradation, and optimizing their metabolism (3, 4). In addition, they also provide for effective delivery of drugs to specific target sites (3) and aid in overcoming both transport limitations and defense mechanisms associated with the multidrug resistance phenotype (5).

In the recent past, there has been increasing interest in the use of micellar delivery systems formed from block copolymers (4, 6, 8). The use of block copolymers in drug delivery was first proposed by Ringsdorf's group in the early 1980s (11). Recently, the work of Kabanov's group and Kataoka's group has greatly enhanced the development of block copolymer micelles as drug delivery vehicles (4–6). These micelles are formed from individual block copolymer molecules, each of which contains a hydrophobic block and a hydrophilic block. The amphiphilic nature of the block copolymers enables them to self-assemble to form nanosized aggregates of various morphologies in aqueous solution such that the hydrophobic blocks form the core of the micelle which is surrounded by the hydrophilic blocks, which form the outer shell (12). The inner core of the micelle creates a hydrophobic microenvironment for the nonpolar drug, while the hydrophilic shell provides a stabilizing interface between the micelle core and the aqueous medium. The properties of the hydrophilic shell, which in most cases

consists of poly(ethylene oxide), can be adjusted to both maximize biocompatibility and avoid reticuloendothelial system uptake. The size of the micelles is usually between 10 and 100 nm (4). This size is small enough to allow access to small capillaries while avoiding reticuloendothelial system uptake (4). Micelles in this size range are also large enough to escape renal filtration, which increases their blood circulation time. Most of the existing block copolymer micelle systems are based on poly(ethylene oxide)-*b*-polypropylene oxide-*b*-poly(ethylene oxide) triblock copolymer or from block copolymers which have a polypeptide or polylactic acid core forming block and a poly(ethylene oxide) block which forms the hydrophilic corona (4–6, 8–10).

In this paper, we introduce a novel block copolymer micelle system, formed from polycaprolactone-*b*-poly(ethylene oxide) (PCL-*b*-PEO) diblocks as a potential drug delivery vehicle. The individual block components, polycaprolactone and poly(ethylene oxide), have been explored previously for a variety of biomedical applications (13–19). Polycaprolactone, the hydrophobic core-forming block of the micelles is a synthetic semicrystalline biodegradable polymer. Due to the biodegradability of the polycaprolactone homopolymer, it has been tried both as a structural material in the production of medical devices, such as implants, sutures, stents, and prosthetics, and as a carrier for a variety of drugs (13, 14). Polycaprolactone pastes have been developed as a drug delivery system for the anti-cancer agent taxol (15) and the antineoplastic agent bis(maltolato)oxovanadium (16). Nanoparticle, nanocapsule, and microparticle drug carriers made of polycaprolactone have been assayed for the ocular delivery of indomethacin (17). Poly(ethylene oxide), the hydrophilic shell forming block of the micelles, is commonly used to impart blood compatibility to a material surface (18, 19).

Triblocks of polycaprolactone-*b*-poly(ethylene oxide)-*b*-polycaprolactone have been used to form tablets, which were studied in terms of their biocompatibility and

* To whom the correspondence should be addressed.

[†] Department of Chemistry.

[‡] Department of Pharmacology and Therapeutics.

biodegradation (20). In addition, recently matrixes to be used as implantable drug delivery systems have been formed from the triblock copolymer polycaprolactone-*b*-poly(ethylene oxide)-*b*-polycaprolactone (PCL₆-*b*-PEO₉₀-*b*-PCL₆) (21).

To our knowledge, there has only been one previous report on the use of diblock copolymer micelles of polycaprolactone-*b*-poly(ethylene oxide) as a drug delivery system (22). The novelty associated with the present application is largely due to the length of the polycaprolactone blocks. To date, diblocks with long blocks of polycaprolactone had not been synthesized; now, however, owing to a new synthetic method, longer blocks are possible (23). The longer caprolactone blocks should create a larger hydrophobic microreservoir enabling the incorporation of a greater amount of lipophilic drug. It is also anticipated that control over the degree of incorporation and the release kinetics can be gained by varying the length of the polycaprolactone block. The degree of drug incorporated and the release kinetics of that drug are largely a function of the interaction between the drug and the core forming block. Since polycaprolactone is more hydrophobic than other polymers, commonly used as core-forming blocks (e.g., polypropylene oxide), this system may be have a larger capacity for the most hydrophobic drugs.

The polycaprolactone micellar core is suitable for the incorporation of a variety of neutral, lipophilic drugs. In this paper, we focus on the drugs FK506 and its analogue, L-685,818. FK506, otherwise known as tacrolimus, has been effectively used to achieve immunosuppression in organ transplant recipients (24). FK506 binds to the immunophilin FKBP12 to form a complex which binds to and inhibits calcineurin; this, in turn, results in immunosuppression (25). Recently, FK506 has been found to promote neuronal outgrowth, which has been demonstrated in terms of the enhancement of neurite extension in PC 12 (rat pheochromocytoma) cell cultures (26) and explant cultures of rat sensory ganglia (27). The use of FK506 for the treatment of neurodegenerative diseases is limited by its immunosuppressant activity. However, L-685,818, the structural analogue to FK506, has retained the neurotrophic action of FK506 but lost its immunosuppressant action (27). The structural difference between FK506 and L-685,818 is only in the addition of a hydroxyl group on the analogue at C-18 and the substitution of an ethyl group for an allyl group at C-21 (27). Recently, L-685,818 has been of interest for its potential use in the treatment of neurodegenerative diseases, as it has been shown to "enhance functional and morphologic recovery in rats with crushed sciatic nerves" (27).

At present, FK506 is administered either orally, in a capsule, or by injection as a sterile solution. In addition to this, a microemulsion formulation of FK506 has also been developed (24). However, to our knowledge, this is the first delivery system which has been introduced for the FK 506 analogue, L-685,818.

In this communication, we report on studies which explore the potential of the PCL-*b*-PEO copolymer micelles as drug delivery vehicles. To establish this system as a suitable drug carrier, we studied a range of the micelle's characteristics including effective diameter, size population distributions, as well as their in vitro stability in various cell culture media. The micelle's affinity for lipophilic compounds was evaluated by determining the partition coefficient for the highly lipophilic compound, pyrene, between the PCL-*b*-PEO micelles and water. Though each of the components, polycaprolactone and

poly(ethylene oxide), are biocompatible, it was also necessary to assess the biocompatibility of the block copolymer as a whole. The in vitro biocompatibility of the copolymer was assayed by incubation of the micelles with PC 12 cells (26) and MCF-7 breast cancer cells as well as primary cultures of human microglia, astrocytes, and cortical neurons. Finally, the neurotrophic drugs FK506 and L-685,818 were incorporated into the PCL-*b*-PEO micelles, and the in vitro delivery was assayed by monitoring the degree of differentiation achieved in cultures of PC 12 rat pheochromocytoma cells.

2. EXPERIMENTAL SECTION

2.1. Materials. The block copolymers were synthesized by anionic polymerization, the method has been described in detail elsewhere (23). GPC results revealed that the polydispersity (PD) of the block copolymers was 1.16, with a symmetrical mono modal distribution. In all cases, following purification of the block copolymer, there was no homopolymer present. The polycaprolactone-*b*-poly(ethylene oxide) copolymers employed have identical poly(ethylene oxide) block lengths (44 ethylene oxide units) but different polycaprolactone block lengths; 14 or 20 units. These polymers are designated as PCL₁₄-*b*-PEO₄₄ and PCL₂₀-*b*-PEO₄₄, respectively. All chemicals for the synthesis were purchased from Aldrich Chemical Co.

FK 506 (Prograf) was purchased from Fujisawa Pharmaceutical, Osaka, Japan. The FK 506 analogue L-685 818 was supplied by Merck Inc. The MCF-7 cells and primary cultures of human microglia, astrocytes, and cortical neurons were supplied by Dr. Antel at the Montreal Neurological Institute. The tissue culture reagents were all purchased from Gibco BRL. The Alamar blue was purchased from Biosource Camarillo and the Trypan blue from Gibco BRL.

2.2. Methods. **2.2.1. Characterization of the PCL-*b*-PEO Micelles.** **2.2.1.1. Micelle Preparation.** The PCL-*b*-PEO copolymer was dissolved in DMF and stirred overnight at room temperature. Micellization was achieved by the addition of water in a dropwise fashion to the block copolymer solution to form a micelle solution. The micelle solution was stirred overnight and then dialyzed against milli-Q distilled water using dialysis tubing. The water was changed every hour for the first 4 h and then every 3 h for the next 12 h.

2.2.1.2. Micelle Size Distribution. Dynamic laser light scattering (DLS) measurements on the PCL-*b*-PEO block copolymer micelles were carried out using a Brookhaven laser light scattering instrument (Brookhaven Instruments Corporation, New York), with a uniphase 125 mW micro green laser at a wavelength of 532 nm at 25 °C. A scattering angle of 90° was used for all measurements. The concentration of the samples ranged from 0.01 to 0.2% w/w in filtered (0.8 μm filter) deionized distilled Milli Q water.

2.2.1.3. In Vitro Micelle Stability Studies. The 1% w/w PCL-*b*-PEO block copolymer micelle solutions prepared as described above were freeze-dried and dissolved in Dulbecco's Modified Eagle Medium (DMEM, Gibco), RPMI medium, and water to produce both 0.01 and 0.1% w/w solutions. Dynamic laser light scattering (DLS) measurements on the solutions were carried out on a weekly basis over a 3 month period.

2.2.2. Determination of the Partition Coefficient for Pyrene between the PCL-*b*-PEO Block Copolymer Micelles and a DMF/H₂O Solvent Mixture. The fluorescence method used to determine the partition

coefficient for pyrene between a mixed water/DMF solvent mixture (60–90% water content) and block copolymer micelles (concentration PCL₂₀-*b*-PEO₄₄ ranges from 1×10^{-6} to 1×10^{-2} g/g), at 25 °C, has been described in detail elsewhere (5b, 28, 29a). The incorporation of pyrene into the micelle core has been described as a partition equilibrium between a micellar phase and a solvent phase (29). Briefly, the intensity ratio of vibrational band three to vibrational band one (I_3/I_1) in the fluorescence emission spectrum of pyrene reflects the polarity of the surrounding environment of the probe molecules. The I_3/I_1 ratio can thus be used to calculate the concentration of pyrene incorporated into the micelles and from this concentration as well as the total amount of pyrene in solution we can calculate the partition coefficient (K_v) for pyrene between the PCL-*b*-PEO micelles and the H₂O/DMF solvent mixtures. The semi-logarithmic plot of the K_v versus the water content can then be extrapolated to 100% water content.

2.2.2.1. Preparation of Solutions for the Determination of the Partition Coefficient. Sample solutions were prepared by adding known amounts of pyrene in acetone to each of a series of empty vials, following which the acetone was evaporated. The amount of pyrene was chosen so as to give a pyrene concentration in the final solution of 1.5×10^{-9} mol/g. Different amounts of PCL₁₄-*b*-PEO₄₄ copolymer in DMF were then added. The initial volumes of the solutions were ca. 2 mL. Micellization of the polymer was achieved by adding water at a rate of 1 drop every 10 s. The addition of water was continued until the desired water content was reached. All the sample solutions were stirred overnight before fluorescence measurements were taken.

2.2.2.2. Fluorescence Measurements. Steady-state fluorescent spectra were measured using a SPEX Fluorolog 2 spectrometer in the right-angle geometry (90° collecting optics). For the fluorescence measurements, ca. 3 mL of solution was placed in a 1.0 cm square quartz cell. All spectra were run on air-equilibrated solutions. For fluorescence emission spectra, λ_{ex} was 339 nm. Spectra were accumulated with an integration time of 1 s/0.5 nm.

2.2.3. Studies Involving the PCL-*b*-PEO Micelles and PC 12 Cells. **2.2.3.1. Cell Cultures.** PC 12 cells were maintained in DMEM supplemented with 10% horse serum, 5% fetal calf serum, 500 µg/mL penicillin, 500 µg/mL streptomycin, and 290 µg/mL.

2.2.3.2. In vitro Biocompatibility of the PCL-*b*-PEO Micelles. PC 12 cells and the MCF-7 breast cancer cells were maintained at 37 °C in 5% CO₂. The cells were plated in 96-well plates and cultured in RPMI medium supplemented with 5% fetal bovine serum. Aliquots of the 10% and 1% PCL-*b*-PEO micelle solutions (PCL₁₄-*b*-PEO₄₄ and PCL₂₀-*b*-PEO₄₄) were added to the wells to make the final concentration of micelles in the wells to range from 0.02 to 1% (w/v). In each case, there were controls which consisted of cells without any micelles added to the wells. The cells were incubated with the micelles for both a 24 and 48 h period at which point the Trypan blue and Alamar blue survival assays were employed to assess cell viability in the presence of the PCL-*b*-PEO micelles.

2.2.3.3. Cell Survival Assays: (a) Trypan Blue Exclusion Assay. Following the 24 or 48 h incubation period, the cells were detached with 0.025% Trypsin. The cell suspension was mixed with 75 µL of PBS and 25 µL of Trypan blue. The number of viable cells was determined by using a light microscope (Nikon, objective 20×). The percentage of viable cells was expressed as the number of viable cells divided by the total number of cells.

(b) Alamar Blue Assay. A 10 µL aliquot of the Alamar blue was added to each cell well following a 24 or 48 h incubation period. Following incubation with the dye, 50 µL of the contents of each well was then transferred to a new 96-well plate and the absorbance at 570 nm was measured by a microplate reader (Bio-rad, Mode 550). Cell viability was expressed as the inverse of the absorbance. The value obtained for the controls was considered to be 100% survival.

2.2.3.4. Cell Uptake of Pyrene-Incorporated Micelles. Pyrene-incorporated micelles were prepared such that a total of 2×10^{-7} mol/L of pyrene had been added to the 1% (w/w) micelle (PCL₁₄-*b*-PEO₄₄) solution. An aliquot of the micelle solution was incubated with PC 12 cells cultured in DMEM at 37 °C, 5% CO₂. At time 0, 4, 8, and 24 h, the fluorescence intensity (Perkin-Elmer LS 50) of the supernatant was measured at 390 nm in order to determine the amount of pyrene remaining in the supernatant. Controls consisting of medium or medium and micelles alone were also measured. Following the removal of the aliquot of supernatant at each time point, the remaining supernatant was removed and the cells were washed three times with 100 µL of phosphate buffer saline. The cells were then gently scraped and fixed with aquamount on a slide and analyzed using a fluorescence microscope (Olympus BH-2).

2.2.4. Effect of the FK 506- and L-685,818-incorporated PCL-*b*-PEO Micelles.

2.2.4.1. Preparation of the FK506 and L-685,818-Incorporated PCL-*b*-PEO Micelles. Stock solutions of each drug were prepared by dissolving the drug in acetone. An aliquot of the stock solution was then added to an empty sample vial, the acetone was evaporated, and preparation proceeded as described above. The FK506 drug was received as a sterile solution (Prograf, Fujisawa Pharmaceutical, Osaka, Japan), and thus, it had to be isolated prior to use. The drug was then dissolved in acetone to produce a 5.6×10^{-4} mol/L stock solution. An aliquot of the stock solution was added to an empty sample vial, and the acetone was evaporated off. The PCL₂₀-*b*-PEO₄₄ block copolymer was added to the vial along with DMF, then water was added dropwise and the solution was dialyzed (as described above) to produce a 1.1×10^{-5} mol/L concentration of drug in a 1% (w/w) block copolymer solution. For the L-685,818 analogue, the concentration of the stock solution was 5.6×10^{-4} mol/L. The L-685,818 micelle-incorporated solution was produced in the same manner but the final concentration of the analogue was 2.3×10^{-5} mol/L in the 1% (w/w) PCL₂₀-*b*-PEO₄₄ micelle solution. It should be noted that, for both the FK506 and L-685,818 micelle solutions, the concentration mentioned is the maximum quantity of drug incorporated into the micelles; some will be lost in the dialysis process.

2.2.4.2. In Vitro Studies of Free Form FK506 and L-685,818 and Micelle-Incorporated FK506 and L-685,818. Cell Differentiation Assay. PC 12 cells were plated in 24-well plates at 37 °C in 5% CO₂ and incubated in DMEM supplemented with 5% fetal bovine serum and the treatments described below. Neurite-like outgrowth in PC 12 cells was detected after a 48 h period using a Nikon light microscope. For each data point, five fields of 250–300 cells/well were analyzed. For the quantitation of neurite-like outgrowth the PC 12 cells were considered to have outgrowth if their processes were longer than the length of the diameter of the cell.

2.2.4.3. Dose-Response Curves for NGF, FK 506 or L-685,818 Alone and the Combinations of FK 506 or L-685,818 with NGF. A range of concentrations of either NGF (0.1–50 ng/mL), FK506 (1–500 nM), or L-685–818

(1–500 nM) alone or combinations of 0.5 or 5 ng/mL of NGF with either FK506 (5–500 nM) or L-685,818 (50 nM to 10 μ M) were incubated with the PC 12 cells for a 48 h incubation period. The wells with untreated cells were used as controls.

2.2.4.4. In Vitro Effect of Micelle-Incorporated FK506 and L-685,818 along with NGF. The unfiltered solution of the micelle incorporated drug was added to the cell wells at different concentrations: a 10 μ L aliquot of a FK506-micelle solution was added to each 0.5 mL cell well. The 10 μ L aliquot was produced by diluting the 1.1×10^{-5} mol/L FK506-PCL₂₀-*b*-PEO₄₄ micelle (1% w/w) stock solution. The final concentration of FK506 in the cell well was made to range 1–1000 nM. In the same manner, a 10 μ L aliquot of the L-685,818 micelle solution was added to each 0.5 mL cell well, such that final concentration in the well was made to range from 10 nM to 1 μ M. In each case, 5 ng/mL NGF was also added to each cell well. Several controls were also used where the cells were treated with 5 ng/mL of NGF alone, 1% (w/w) PCL₂₀-*b*-PEO₄₄ micelles alone, 1% (w/w) PCL₂₀-*b*-PEO₄₄ micelles containing 1 nM FK 506 without NGF or 1% (w/w) PCL₂₀-*b*-PEO₄₄ micelles containing 10 nM L-685,818 without NGF.

3. RESULTS

3.1. Micelle Size Distributions. The Dynamic Light Scattering instrument was used to measure the effective diameter of the micelles and Contin Analysis was employed to determine the population distribution of the micelles in terms of their size. The effective diameter of 0.1% (w/w) solutions of the micelles when a 0.45 μ m filter is used is 62 nm for the PCL₂₀-*b*-PEO₄₄ and 55 nm for the PCL₁₄-*b*-PEO₄₄. Figure 1 shows the population distributions obtained for unfiltered solutions of the PCL₂₀-*b*-PEO₄₄ micelles (0.02 and 0.1% w/w, panels a and b in Figure 1, respectively). Figure 1a shows the bimodal population distribution present in the unfiltered micelle solution; the small aggregates were approximately 50 nm in diameter and the larger aggregates were $\sim$ 500 nm (Figure 1a). The effect of dilution, following the preparation, on this bimodal population distribution was examined as the concentration was varied from 0.01 to 0.2% (w/w). In all cases, the 500 nm population was most prevalent at the higher concentration range, while dilution resulted in a gradual shift in favor of the 50 nm population.

3.2. In Vitro Stability Studies. The micelles were monitored on a weekly basis by DLS in order to determine their relative stability in different sterile media. Over a 3 month period, both the effective diameter of the micelles and the population size distribution remained unchanged (as per above).

3.3. Determination of the Partition Coefficient for Pyrene. Pyrene was used as a model hydrophobic compound. Figure 2a shows the I_3/I_1 ratio of pyrene in micelle solutions of different PCL-*b*-PEO copolymer concentrations over a range of water contents in the DMF/H₂O solvent mixtures. The I_3/I_1 ratio (Figure 2a) increases with increasing water content within the solvent mixture for a constant copolymer concentration. Likewise, for a particular water content within the solvent mixture, the I_3/I_1 ratio increases as the copolymer concentration is increased. Figure 2b shows the partition coefficients calculated for pyrene between the PCL-*b*-PEO micelles and the DMF/H₂O solvent mixtures. The extrapolation of this plot to 100% water content gives a value of the order of 10^2 for the partition coefficient for pyrene between the PCL-*b*-PEO (20-*b*-44) micelles and H₂O.

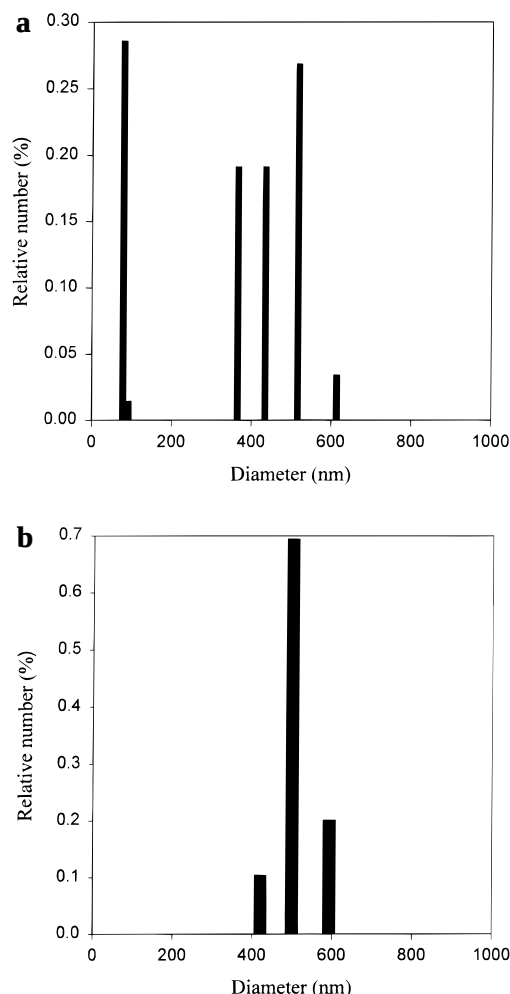


Figure 1. (a) Particle size distribution of a 0.02% (w/w) solution of the PCL₂₀-*b*-PEO₄₄ copolymer micelles. (b) Particle size distribution of a 0.1% (w/w) solution of the PCL₂₀-*b*-PEO₄₄ copolymer micelles.

3.4. Cell Survival in the Presence of the PCL-*b*-PEO Micelles. The incubation of the micelles with several different cell lines and primary cultures was employed as a means of testing the biocompatibility of the PCL-*b*-PEO micelles. Primary cultures of human microglia, astrocytes, and cortical neurons were incubated with micelles formed from PCL₁₄-*b*-PEO₄₄ and PCL₂₀-*b*-PEO₄₄. In the cell cultures tested, the PCL₁₄-*b*-PEO₄₄ induced 10–20% cell death whereas the micelles formed from the larger polycaprolactone block (PCL₂₀-*b*-PEO₄₄) did not cause any noticeable damage. For the incubation of the micelles with the PC 12 and MCF-7 cells, the results from the Trypan blue and Alamar blue survival assays found the micelles to cause little or no cell death when compared with the control wells of untreated cells. Figure 3 shows the results of the Alamar blue assay for the incubation of the PCL-*b*-PEO micelles with PC 12 cells over a 48 h period. At almost every concentration, the micelles formed from the PCL₁₄-*b*-PEO₄₄ block copolymer appeared to have a slightly lower cell survival than the 20-*b*-44 micelles.

3.5. Cell Uptake of Pyrene Incorporated Micelles. Figure 4 shows the fluorescence intensity found for both the supernatant at 390 nm and the cells at the 0, 4, 8, and 24 h time points. The fluorescence intensity of pyrene in the various aliquots of supernatant was found to decrease over the 24 h incubation period, indicating a decrease in the amount of pyrene remaining

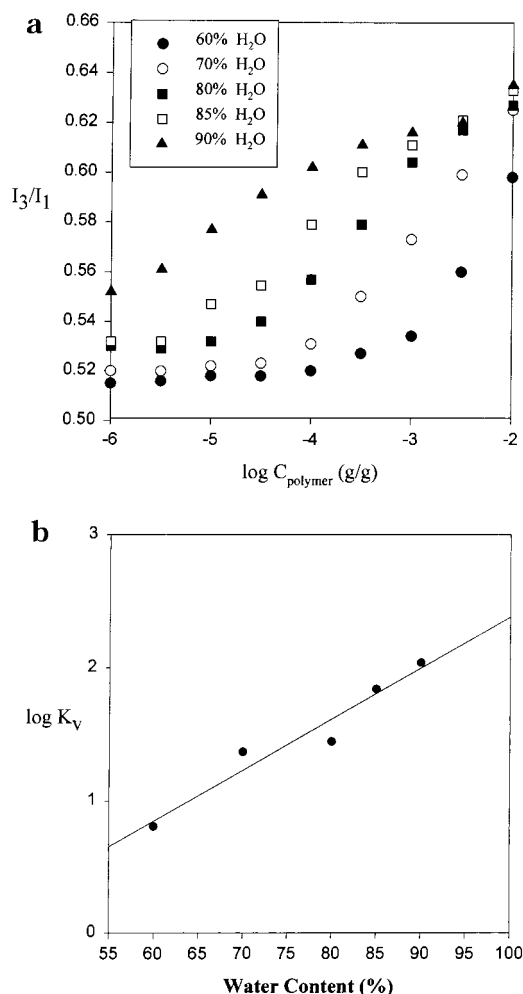


Figure 2. (a) The I_3/I_1 ratio for pyrene in a solution of PCL₁₄-*b*-PEO₄₄ copolymer micelles and a DMF/H₂O solvent mixture. (b) The semilogarithmic plot of the partition coefficient for pyrene in a solution of the PCL-*b*-PEO micelles and a range of H₂O/DMF solvent mixtures.

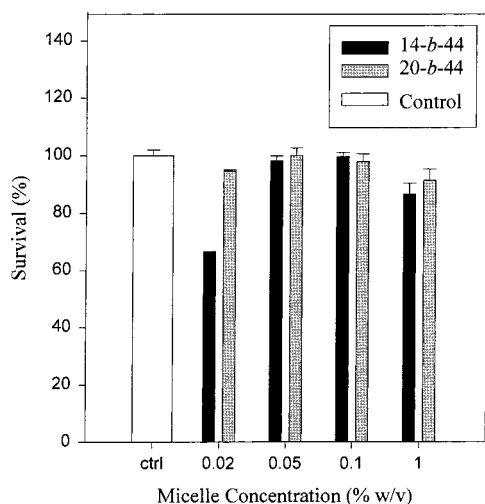


Figure 3. The in vitro biocompatibility of the PCL-*b*-PEO (14-*b*-44 and 20-*b*-44) micelles (0.02–1% w/v) as determined by the Alamar Blue survival assay. The results were confirmed by the Trypan Blue assay. Each data point represents the average of three independent experiments, from 6 to 8 wells.

in the external medium. The relative fluorescence intensity at zero time was taken as 1 while, after 4 h, it had decreased to 0.70 (± 0.05), after 8 h, 0.60 (± 0.05),

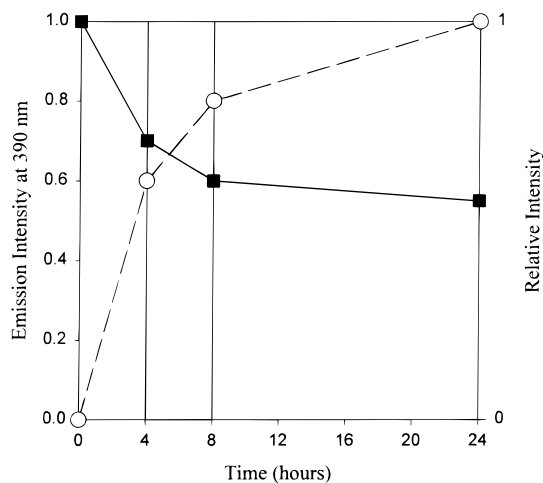


Figure 4. The change in the relative emission intensity of pyrene in the supernatant (■) and cells (○) at 390 nm, over a 24 h period. PC 12 cells were exposed to PCL₁₄-*b*-PEO₄₄ micelles containing pyrene and the change in the fluorescence intensity was measured at 4, 8, and 24 h time points.

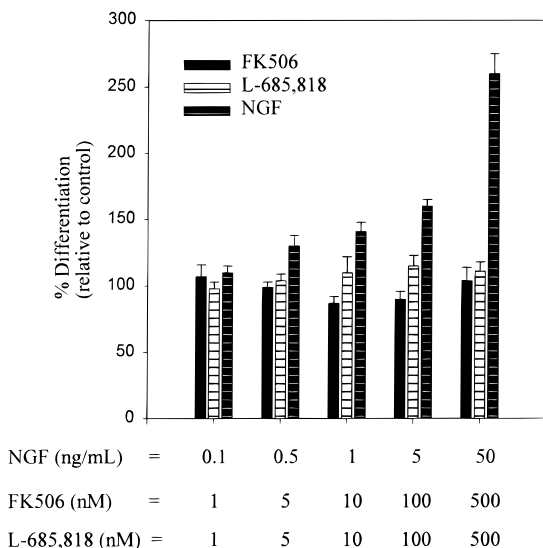


Figure 5. The degree of neurite-like outgrowth in PC 12 cells incubated with either NGF, FK506 or L-685,818, over a 48 h period ($n = 3-4$). The concentration of NGF incubated with the cells was C1 = 0.1, C2 = 0.5, C3 = 1, C4 = 5, C5 = 50 ng/mL, the concentrations of FK506 or L-685,818 were C1 = 1, C2 = 5, C3 = 10, C4 = 100, C5 = 500 nM. The degree of differentiation in the control (absence of any treatment) is taken to be 100% differentiation.

and finally, after 24 h, the intensity was 0.55 (± 0.05) (Figure 4). The cells were isolated at each time point and analyzed by fluorescence microscopy. The contrast relative to the intensity of fluorescence in the cells increased over the 24 h period, from nondetectable at time zero to the maximum (arbitrarily set at 1) after 24 h.

3.6. In Vitro Studies of Free Form FK506 and L-685,818 and Micelle-Incorporated FK506 and L-685,818. In each case, the degree of neurite-like outgrowth was taken to be 100% in the control wells of untreated PC 12 cells. The degree of differentiation in cell wells in the presence of NGF and/or drug is expressed as a percentage relative to 100% for the control.

3.7. Dose-Response Curves for NGF, FK506 and L-685,818 without Micelles. Figure 5 shows the degree of neurite-like outgrowth obtained over 48 h incubation of PC 12 cells with either NGF, FK506, or L-685,818. The

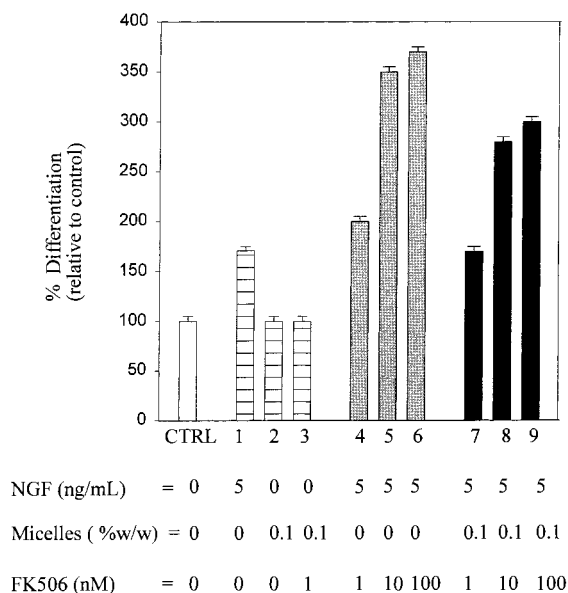


Figure 6. The effect of micelle-incorporated FK 506 and NGF (5 ng/mL) on the neurite-like outgrowth in PC 12 cells over a 48 h period ($n = 3-4$). Control = PC 12 cells in the absence of any treatment. Bars 1-9 are PC 12 cells treated with the following: bar 1 = 5 ng/mL NGF, bar 2 = PCL₂₀-*b*-PEO₄₄ micelles, bar 3 = micelle-incorporated FK506 (1 nM), bars 4-6 = FK506 (bars 4, 5, and 6 = 1, 10, 100 nM, respectively) along with 5 ng/mL NGF; bars 7-9 = micelle-incorporated FK506 (bars 7, 8, and 9 = 1, 10, 100 nM, respectively) along with 5 ng/mL NGF.

degree of outgrowth in the wells where NGF had been added was found to increase from 110 to 260% as the concentration of NGF incubated with the cells increased. In wells incubated with FK506 or L-685,818 alone, the degree of outgrowth did not vary with the concentration of drug added, rather it oscillated slightly around the 100% value.

3.8. Dose-Response Curves for the Combined Effect of NGF and FK506 or L-685,818 without Micelles. The neurite-like outgrowth for cells incubated with 5 ng/mL of NGF was found to be $180 \pm 6\%$. As shown in Figure 6, the degree of outgrowth in cells incubated with NGF (5 ng/mL) along with FK506 (1-100 nM) increased from 220 to 380% with increasing concentration of FK506. Figure 7, shows that in cells incubated with NGF (5 ng/mL) along with L-685,818 (10-1000 nM) the degree of outgrowth increased from 200 to 350% as the concentration of drug was increased.

3.9. In Vitro Effect of Micelle-Incorporated FK506 and L-685,818 along with NGF. Figure 6 shows the degree of differentiation in PC 12 cells which have been incubated with PCL-*b*-PEO-incorporated FK506 and 5 ng/mL NGF following a 48 h incubation period. The degree of neurite-like outgrowth in cells incubated with 5 ng/mL of NGF was $160 \pm 5\%$. In cell wells incubated with micelle-incorporated FK506 (1 to 100 nM) along with 5 ng/mL of NGF the neurite-like, outgrowth ranged 170-300%. In Figure 6, we see no significant difference between untreated cells and cells treated with either PCL-*b*-PEO micelles without drug or cells treated with micelle-incorporated FK506 (control vs bars 2 and 3). There was also no significant difference between cells treated with 5 ng/mL NGF and those treated with micelle incorporated FK506 (1 nM) along with 5 ng/mL NGF (bar 1 vs bar 7). However, there was a significant difference ($p < 0.01$) between cells treated with 5 ng/mL NGF and cells treated with 10 or 100 nM of micelle incorporated FK506 (bar 1 vs bars 8 and 9).

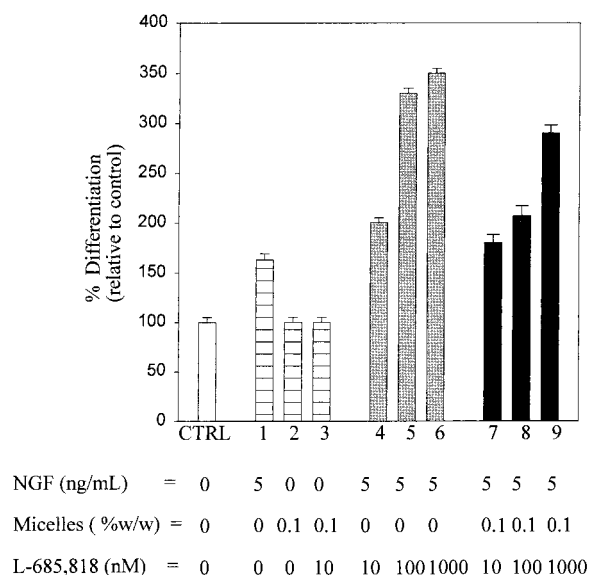


Figure 7. The effect of micelle-incorporated L-685,818 and NGF (5 ng/mL) on the neurite-like outgrowth in PC 12 cells over a 48 h period ($n = 3-4$). Control = PC 12 cells in the absence of any treatment. Bars 1-9 are PC 12 cells treated with the following: bar 1 = 5 ng/mL NGF, bar 2 = PCL₂₀-*b*-PEO₄₄ micelles, bar 3 = micelle-incorporated L-685,818 (10 nM), bars 4-6 = L-685,818 (bars 4, 5, and 6 = 10, 100, 1000 nM, respectively) along with 5 ng/mL NGF; bars 7-9 = micelle-incorporated L-685,818 (bars 7, 8, and 9 = 10, 100, 1000 nM, respectively) along with 5 ng/mL NGF.

Figure 7 shows the degree of differentiation in PC 12 cells which have been incubated with PCL-*b*-PEO incorporated L-685,818 and 5 ng/mL NGF following a 48 h incubation period. The degree of differentiation seen in cells incubated with the micelle incorporated L-685,818 along with 5 ng/mL NGF increased from 163 to 290% as the concentration of drug increased from 10 nM to 1 μ M. In Figure 7, there is no significant difference between the untreated cells and the cells treated with micelles alone or 10 nM of micelle incorporated L-685,818 (control vs bar 2 or bar 3). Also, no significant difference was found between cells treated with 5 ng/mL of NGF and 10 nM micelle incorporated L-685,818. There was a significant difference ($p < 0.01$) between cells treated with 5 ng/mL NGF and cells treated with 100 nM and 1 μ M of micelle incorporated L-685,818 (bar 1 vs bars 8 and 9).

4 DISCUSSION

4.1. Characterization of the PCL-*b*-PEO Micelles.

In this study, we explored the potential of the novel PCL-*b*-PEO micelles as a drug delivery vehicle for lipophilic drugs. The PCL-*b*-PEO micelles cannot be prepared in water alone due to the hydrophobicity of the polycaprolactone core-forming block. For this reason, the block copolymer is first dissolved in DMF, and micellization is induced by the dropwise addition of water, followed by dialysis. The micelles formed by this method of preparation were studied by dynamic light scattering in order to measure their effective diameter and their population distribution in terms of size. Figure 1 shows the population distribution of the micelles calculated by Contin analysis of the dynamic light scattering measurements. Figure 1a shows the bimodal population distribution found in a 0.02% (w/w) of PCL₂₀-*b*-PEO₄₄. The larger population, at approximately 500 nm, consists of aggregates of the small individual micelles, which we see at 50 nm. Figure 1b shows the population distribution

in terms of size of a 0.1% (w/w) of PCL₂₀-*b*-PEO₄₄. Comparing panels a and b in Figure 1, we see that as the solution is diluted, decreasing the concentration from 0.1 to 0.02%, the population of the larger aggregates is decreased in favor of the smaller (50 nm) population. This suggests that the larger aggregates are composed of aggregates of small individual micelles. Micelles with a coronal shell formed from PEO are frequently seen to form larger aggregates in solution. Winnik's group suggested the large associates of PS-*b*-PEO to be either onion-like aggregates or aggregates of small individual micelles (29). Kataoka's group agreed with the latter suggestion when explaining the large associates they observed with the PEO-*b*-PBLA micelles (6b). They suggest the aggregates of individual micelles may form due to either hydrophobic-hydrophobic interactions or van der Waals interactions between the exposed cores of the individual micelles.

In our case, we had specifically chosen the PEO block to be 44 units in length, since this has most commonly been the length of the PEO stabilizing moieties employed for the steric stabilization of liposomes. However, most likely the length of the PEO blocks is too short to outweigh the hydrophobic-hydrophobic or van der Waals interactions between the exposed polycaprolactone cores. We are now studying the relationship between the PEO chain length and the degree of aggregation achieved in the micelle solution.

Dynamic light scattering measurements were also used to monitor the in vitro stability of the micelles in water and the cell culture media DMEM and RPMI. Over a 3 month period, the micelles were still intact and the population distribution of the micelles in terms of size remained relatively constant. These studies are still ongoing since the in vitro stability of the micelles will determine the extent to which they are suitable for various applications.

4.2 Determination of the Partition Coefficient for Pyrene between the PCL-*b*-PEO Micelles and Water. The affinity of the PCL-*b*-PEO micelles for lipophilic compounds was assayed by the determination of the K_v for the hydrophobic model compound, pyrene, between the PCL-*b*-PEO micelles and water. This method was described in an earlier communication where it had been used to determine the K_v of pyrene between polystyrene-*b*-poly(acrylic acid) micelles and water. This fluorescence method is based on pyrene's sensitivity to the hydrophobicity of its microenvironment. This is reflected in changes in the ratio of the I_3/I_1 bands of its emission spectrum. The method requires the measurement of the I_3/I_1 ratio for pyrene in micelle solutions of various DMF/H₂O solvent mixtures. Figure 2a shows the increase in the I_3/I_1 ratio which accompanies an increase in the water content in the solvent mixture. The increase in the water content within the solvent mixture provides a driving force promoting the entry of pyrene into the more hydrophobic environment of the polycaprolactone micelle core. The I_3/I_1 ratio for pyrene in the many solutions was then used to calculate the partition coefficient for pyrene in each solution. Figure 2b shows the partition coefficients obtained for pyrene in the various micelle solutions, extrapolation of this plot to 100% water content provides the value for the K_v of pyrene between the PCL-*b*-PEO micelles and water. The partition coefficient of pyrene in this micelle system was found to be on the order of 10^2 ; this is low in comparison to a partition coefficient of the order of 10^5 found for pyrene between the polystyrene-*b*-poly(acrylic acid) (PS-*b*-PAA) micelles and water (28). The high value for the K_v of pyrene between the

PS-*b*-PAA micelles and water is thus due to the highly hydrophobic nature of both pyrene and the polystyrene core. The polycaprolactone core, though hydrophobic, is clearly less hydrophobic than polystyrene. However, this value of K_v for pyrene between the PCL-*b*-PEO micelles and water is on the same order of magnitude as that for pyrene in a solution of PEO_{*m*/2}-*b*-PPO_{*n*}-*b*-PEO_{*m*/2} ($m = 51$, $n = 38$) and water at the same temperature (20 °C) (4b).

It should be noted that our measurements for the determination of the partition coefficient for pyrene between the PCL-*b*-PEO micelles and water were all performed at 20 °C. Previously, it was found that the partition coefficient for pyrene between micelles formed from PEO-*b*-PPO-*b*-PEO and water increased by an order of magnitude when the temperature was increased from 20 to 37 °C. This increase is said to be due to the decrease in the degree of hydration of the core-forming block at the higher temperature. Due to the nature of the polycaprolactone core-forming block, we also anticipate an increase in the partition coefficient for pyrene at higher temperatures.

Many studies, have revealed that the greatest degree of solubilization of a compound within a micelle core is achieved when there is a close match between the degree of the polarity and hydrophobicity of the compound and the micelle core (30–32). For this reason, we are now exploring the incorporation of various hydrophobic drugs into micelles with a longer polycaprolactone core forming block. Overall, the incorporation of the highly hydrophobic compound, pyrene, reveals the suitability of this micelle system as a potential carrier for even the most lipophilic compounds.

4.3. In Vitro Biocompatibility of the PCL-*b*-PEO Micelles. The in vitro biocompatibility of the PCL-*b*-PEO micelles (14-*b*-44, 20-*b*-44) was tested by incubating the micelles with a wide range of cell lines for both 24 and 48 h periods. The Trypan blue and Alamar blue survival assays were then used to quantitate the survival rates in the presence of the PCL-*b*-PEO micelles. Figure 3 shows the percent survival obtained for the incubation of the PCL-*b*-PEO micelles [0.02–1% (w/v)] with PC 12 cells over a 24 h period. The survival in the presence of micelles was between 85 and 100%, except for the 70% survival obtained for the incubation of the PCL₁₄-*b*-PEO₄₄ micelles at the lowest concentration. In Figure 3, we see that at several concentrations the survival obtained with the PCL₁₄-*b*-PEO₄₄ is slightly lower than that obtained with the PCL₂₀-*b*-PEO₄₄. For this reason, the 20-*b*-44 PCL-*b*-PEO system, rather than the 14-*b*-44 system, is investigated as the delivery system for FK506 and L-685,818.

The in vitro biocompatibility studies have shown us that addition of a PCL₂₀-*b*-PEO₄₄ micelle solution to the cell well causes little or no cell death. However, the actual destination of the micelles following their addition to the cell well is still unknown. There are several possibilities; they all remain in the supernatant, they all enter the cell, possibly only the small micelles can enter the cell while the larger aggregates remain in the supernatant, or the micelles may aggregate at the cell surface. We are currently investigating the destination of the micelles since this will largely affect the types of drugs which will most effectively be delivered by this system.

4.4. Cell Uptake of Pyrene-Incorporated Micelles. The capability of the PCL-*b*-PEO micelle system to deliver a hydrophobic compound was first tested in vitro using pyrene as a model compound. The pyrene-incorporated PCL-*b*-PEO micelles were incubated with

PC 12 cells over a 24 h period. At 0, 4, 8, and 24 h, samples of the supernatant were studied by fluorescence in order to determine the quantity of pyrene remaining in the supernatant at each time point. Likewise, at each time point, cells were isolated and their fluorescence was examined under the fluorescence microscope, to determine if the decrease in the fluorescence within the supernatant was accompanied by an increase in fluorescence within the cells. Figure 4 shows the pronounced decrease in the fluorescence of the supernatant at the 4 h time point; this decrease was found to be more gradual over the rest of the 24 h period. A similar trend was also seen for the fluorescence of the isolated cells where the fluorescence increases gradually over the 24 h period. At the 24 h time point, the fluorescence is quite intense and appears to be localized in small spherical aggregates.

The cell uptake studies reveal that the addition of pyrene incorporated micelles to the cells does, in fact, result in the accumulation of pyrene within the cells. However, once again, it is unknown whether the micelles have simply remained in the supernatant or have somehow facilitated the entry of pyrene into the cells. Since the partition coefficient for pyrene between water and the PCL-*b*-PEO micelle system is on the order of 10^2 , it seems likely that the micelles either enter the cells themselves or possibly attach themselves to the surface of the cell, enabling the drug to enter the cell without having to enter the hydrophilic cell medium.

4.5. PCL-*b*-PEO Micelles as a Delivery System for FK506 and L-685,818. The size of the PCL-*b*-PEO micelles as well as their in vitro stability, in vitro biocompatibility, and their loading capacity for lipophilic compounds such as pyrene merited further study of this system as a delivery system for hydrophobic drugs. We have chosen FK506 and L-685,818 as the first hydrophobic candidate drugs to be delivered by the PCL-*b*-PEO micellar system.

As mentioned, FK506 and L-685,818 have both been shown to potentiate the effect of NGF on the outgrowth of PC 12 cells (24). To compare the effect of the micelle-incorporated FK506 and L-685,818 with the free drugs, it was necessary to construct dose-response curves for NGF, FK506, and L-685,818 alone as well as the combinations of NGF with FK506 or L-685,818. Figure 5 shows the dose-response curves for each of NGF, FK506, and L-685,818 alone. Clearly, the degree of differentiation of the PC 12 cells increases with increasing concentration of NGF; however, the concentration of either FK506 or L-685,818 alone was found to have no effect on the degree of differentiation (Figure 5). These findings support previous results which have been reported in the literature (27).

Figure 6 shows the degree of differentiation obtained in cells treated with micelle-incorporated FK506 along with NGF and Figure 7 shows that for cells treated with micelle-incorporated L-685,818. The PCL-*b*-PEO-incorporated FK506 and L-685,818 were both found to potentiate the effect of NGF on neurite-like outgrowth in PC 12 cell cultures. Thus, the ability of both FK506 and L-685,818 to potentiate the effect of NGF on neuronal outgrowth was retained despite their incorporation into the PCL-*b*-PEO copolymer micelles (Figures 6 and 7, bars 7–9). The degree of neurite-like outgrowth achieved in cell cultures treated with 5 ng/mL NGF and either micelle-incorporated FK506 or L-685,818 (Figure 6 and 7, bars 7–9) was in both cases less than that obtained when the cells were treated with 5 ng/mL NGF and free FK506 or L-685,818 (Figure 6 and 7, bars 4–6). This may be due to the limited 48 h incubation period over

which time only a fraction of the drug will be released from the micelles. It should be noted that, in vitro, a dose of FK506 as low as 1 nM, is adequate to potentiate neurite-like outgrowth in PC12 cell cultures (Figure 6). Also, in several in vivo studies on rats, it was found that 1 mg/kg of FK506 was an effective dose (33). This micellar delivery system is able to incorporate much higher concentrations of FK506, which will later be released slowly over time. In this way, it will prevent some of the damaging effects that high doses would induce.

In summary, the preliminary results obtained for the novel PCL-*b*-PEO micelle system reveal it to be a promising drug delivery vehicle for the delivery of various lipophilic drugs. The small size and indefinite in vitro stability of the micelles suggest that they may be useful for a wide variety of biomedical applications. Their loading capacity for the very hydrophobic compound, pyrene, encourages the incorporation of many different compounds with a high degree of hydrophobicity. Studies involving the degree of aggregation encountered in the micelle solutions are now underway with special effort placed on limiting the degree of aggregation. Also, now that the in vitro biocompatibility of the micelles has been established, it will be assayed in vivo.

ACKNOWLEDGMENT

The authors would like to thank NSERC for support of this research; in addition, C.A. is grateful to NSERC for financial assistance provided in form of a fellowship. Thanks are also due to Andrea Bardel for her help with the measurement of neurite outgrowth in PC 12 cells. Also the help of Dr. Matthew Moffitt in teaching C.A. the use of dynamic light scattering is deeply appreciated.

LITERATURE CITED

- (1) Maysinger, D. and Morinville, A. (1997) Drug Delivery to the Nervous System *Trends Biochem. Sci.* 15, 410–418.
- (2) Gregoriadis, G. (1995) Engineering Liposomes for Drug Delivery: progress and problems. *Trends Biochem. Sci.* 13, 527–537.
- (3) Muller, R. H. (1991) *Colloidal Carriers for Controlled Drug Delivery and Targeting: Modification, Characterization and In vivo Distribution*, CRC Press Inc., Florida.
- (4) Kabanov, A. V., and Alakhov, V. Y. (1997) Micelles of Amphiphilic Block Copolymers as Vehicles for Drug Delivery In *Amphiphilic Block Copolymers: Self-Assembly and Applications* (P. Alexandris, and B. Lindman, Eds.) Elsevier, Netherlands.
- (5) (a) Alakhov, V. Y., Moskaleva, E. Y., Batrakova, E. V., and Kabanov, A. V. (1996) Hypersensitization of Multidrug Resistant Human Ovarian Carcinoma Cells by Pluronic P85 Block Copolymer. *Bioconjugate Chem.* 7, 209–216. (b) Kabanov, A. V., Nazarova, I. R., Astafieva, I. R., Batrakova, E. V., Alakhov, V. Y., Yarostavov, A. A., and Kabanov, V. A. (1995) Micelle Formation and Solubilization of Fluorescent Probes in Poly(oxyethylene-*b*-oxypropylene-*b*-oxyethylene). *Macromolecules* 28, 2303–2314.
- (6) (a) Kwon, G., Naito, M., Yokoyama, M., Okano, T., Sakurai, Y., and Kataoka, K. (1997) Block Copolymer Micelles for Drug Delivery: Loading and Release of Doxorubicin. *J. Controlled Release*, 48, 195–201. (b) La, S. B., Okano, T., and Kataoka, K. (1996) Preparation and Characterization of the Micelle-Forming Polymeric Drug Indomethacin-Incorporated Poly(ethylene oxide)-Poly(β -benzyl L-aspartate) Block Copolymer Micelles. *J. Pharm. Sci.* 85, 85–90. (c) Kataoka, K., Kwon, G., Yokoyama, M., Okano, T., and Sakurai, Y. (1992) Block Copolymer Micelles as Vehicles for Drug Delivery. *J. Controlled Release* 24, 119–132.
- (7) Allen, T. M. (1994) Long-circulating (sterically stabilized) liposomes for targeted drug delivery. *Trends Pharmacol. Sci.*, 15, 215–220.

- (8) Gref, R., Minamitake, Y., and Peracchia, M. T. (1994) Biodegradable long-circulating polymeric nanospheres. *Science* 263, 1600–3.
- (9) Sahli, H., Tapon-Brethaudiere, J., Fischer, A. M., Sternberg, C., Spenlehauer, G., Verrecchia, T., and Labarre, D. (1997) Interactions of poly(lactic acid) and poly(lactic-co-ethylene oxide) nanoparticles with plasma factors of the coagulation system. *Biomaterials* 18, 281–8.
- (10) Zhang, X., Burt, H. M., Von Hoff, D., Dexter, D., Mangold, G., Degen, D., Oktaba, A. M., and Hunter, W. L. (1997) An investigation of the antitumor activity and biodistribution of polymeric micellar paclitaxel. *Cancer Chemother. Pharmacol.* 40, 81–86.
- (11) (a) Bader, H., Ringsdorf, H., and Schmidt, B. (1984) Watersoluble Polymers in Medicine *Angew. Makromol. Chem.* 123, 457–485. (b) Pratten, M. K., Lloyd, J. B., Horpel, G., and Ringsdorf, H. (1985) Micelle-Forming Block Copolymers—Pinocytosis by Macrophages and Interaction with Model Membranes. *Makromol. Chem.* 186, 725–733.
- (12) (a) Zhang, L., and Eisenberg, A. (1995) Multiple Morphologies of “Crew-Cut” Aggregates of Polystyrene-*b*-Poly(acrylic acid) Block Copolymers. *Science* 268, 1728–1731. (b) Zhang, L., Yu, K., and Eisenberg, A. (1996) Ion-Induced Morphological Changes in Crew-Cut Aggregates of Amphiphilic Block Copolymers. *Science* 272, 1777–1779.
- (13) Woodward, S. C., Brewer, P. S., Moatamed, F., Shindler, A., and Pitt, C. G. (1985) The intracellular degradation of poly(ϵ -caprolactone). *J. Biomed. Mater. Res.* 19, 437–444.
- (14) van der Giessen, W. J., Lincoff, A. M., Schwartz, R. S., van Beusekom, H. M. M., Serruys, P. W., Holmes, D. R., Ellis, S. G., and Topol, E. J. (1996) Marked Inflammatory Sequelae to Implantation of Biodegradable and Non biodegradable Polymers in Porcine Coronary Arteries. *Circulation* 94, 1690–1697.
- (15) Winternitz, C. I., Jackson, J. K., Oktaba, A. M. C., and Burt, H. M. (1996) Development of a polymeric surgical paste formulation for taxol. *Pharm. Res.* 13, 368–375.
- (16) Jackson, J. K., Min, W., Cruz, T. F., Cindric, S., Arseneault, L., Von Hoff, D. D., Hunter, W. L., and Burt, H. M. (1997) A polymer-based drug delivery system for the antineoplastic agent bis(maltolato)oxovanadium in mice *Br. J. Cancer* 75, 1014–1020.
- (17) Calvo, P., Alfonso, M. J., Vila-Jato, J. L., and Robinson, J. R. (1996) Biodegradable InjecTable Implant Systems for Long Term Drug Delivery Using Poly(Lactic-co-glycolic) Acid Copolymers. *J. Pharm. Pharmacol.* 48, 1147–1152.
- (18) Elbert, D. L., and Hubbell, J. A. (1996) Surface Treatments of Polymers for Biocompatibility. *Annu. Rev. Mater. Sci.* 26, 365–394.
- (19) Lee, J. H., Lee, H. B., and Andrade, J. D. (1995) Blood Compatibility of Poly(ethylene oxide) Surfaces. *Prog. Polym. Sci.* 20, 1043–1079.
- (20) Bei, J., Wang, Z., and Wang, S., (1995) Polycaprolactone-poly(ethylene glycol) Block Copolymer III. Drug Release Behavior. *Chin. J. Polym. Sci.* 2, 154–161.
- (21) Martini, L., Collett, J. H., and Attwood, D. (1997) The influence of Gamma Irradiation on the Physicochemical Properties of a Novel Triblock Copolymer of ϵ -caprolactone and ethylene oxide. *J. Pharm. Pharmacol.* 49, 601–605.
- (22) Kim, Y. S., Shin, I. G., Lee, Y. M., Cho, C. S., and Sung, Y. K., (1998) Methoxy poly(ethylene glycol) and ϵ -caprolactone amphiphilic block copolymeric micelle containing indomethacin. II. Micelle Formation and drug release behaviours. *J. Controlled Rel.* 51, 13–22.
- (23) Yu, Y., and Eisenberg, A. (1998) Manuscript in preparation.
- (24) Goral, S., and Helderman, J. H. (1997) The Evolution and Future of Immunosuppression in Renal Transplantation. *Sem. Nephrol.* 17 (H), 364–372.
- (25) Marks, A. R. (1996) Cellular Functions of Immunophilins. *Physiol. Rev.* 76, 631–648.
- (26) Steiner, J. P., Connolly, M. A., Valentine, H. L., Hamilton, G. S., Dawson, T. M., Hester, L., and Snyder, S. H. (1997) Neurotrophic actions of nonimmunosuppressive analogues of immunosuppressive drugs FK 506, rapamycin and cyclosporin A. *Nat. Med.* 3, 421–428.
- (27) Greene, L. A., and Tischler, A. S. (1976) Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. *Proc. Natl. Acad. Sci. U.S.A.* 73, 2424–2428.
- (28) Zhao, J., Allen, C., and Eisenberg, A. (1997) Partitioning of Pyrene between “Crew Cut” Block Copolymer Micelles and H₂O/DMF Solvent Mixtures. *Macromolecules* 30, 7143–7150.
- (29) (a) Wilhelm, M., Zhao, C-L., Wang, Y., Xu, R., Winnik, M. A., Mura, J. L., Riess, G., and Croucher, M. D. (1991) Poly(styrene-ethylene oxide) Block Copolymer Micelle Formation in Water: A Fluorescence Probe Study. *Macromolecules* 24, 1033–1040. (b) Xu, R., Winnik, M. A., Hallett, F. R., Riess, G., and Croucher, M. D. (1991) Light-Scattering Study of the Association Behavior of Styrene–Ethylene Oxide Block Copolymers in Aqueous Solution. *Macromolecules* 24, 87–93.
- (30) Nagarajan, R., and Ganesh, K. (1996) Comparison of Solubilization of Hydrocarbons in (PEO–PPO) Diblock versus (PEO–PPO–PEO) Triblock Copolymer Micelles. *J. Colloid Interface Sci.* 184, 489–499.
- (31) Tian, M., Arca, E., Tuzar, Z., Webber, S. E., and Munk, P. (1995) Light Scattering Study of Solubilization of Organic Molecules by Block Copolymer Micelles in Aqueous Media. *J. Polym. Sci., Part B: Polym. Phys.* 33, 1713.
- (32) Moroi, Y., Mitsunobu, K., Morisue, T., Kadobayashi, Y., and Sakai, M. (1995) Solubilization of Benzene, Naphthalene, Anthracene and Pyrene in 1-Dodecanesulfonic acid Micelles. *J. Phys. Chem.* 99, 2372–2376.
- (33) Butcher, S. P., Henshall, D. C., Teramura, Y., Iwasaki, K., and Sharkey, J. (1997) Neuroprotective Actions of FK506 in Experimental Stroke: In Vivo Evidence against an Antiepileptotoxic Mechanism. *J. Neurosci.* 17, 6939–6946.

BC9702157

Liposome-Anchored Vascular Endothelial Growth Factor Aptamers[§]

Michael C. Willis,*[†] Brian Collins,[‡] Tong Zhang,[‡] Louis S. Green,[†] David P. Sebesta,[†] Carol Bell,[†] Elizabeth Kellogg,[†] Stanley C. Gill,[†] Anna Magallanez,[†] Susan Knauer,[†] Ray A. Bendele,[†] Parkash S. Gill,[‡] and Nebojša Janjić*[†]

NeXstar Pharmaceuticals, 2860 Wilderness Place, Boulder, Colorado 80301, and Norris Cancer Hospital, School of Medicine, University of Southern California, Los Angeles, California 90033. Received January 7, 1998; Revised Manuscript Received April 30, 1998

Nuclease-resistant aptamers identified from randomized nucleic acid libraries represent a novel class of drug candidates. Aptamers are synthesized chemically and therefore can be readily modified with functional groups that modulate their properties. We report here on the preparation, initial characterization, and functional properties of a nuclease-resistant vascular endothelial growth factor (VEGF) aptamer anchored in liposome bilayers through a lipid group on the aptamer. While the high-affinity binding to VEGF is maintained, the plasma residence time of the liposome-anchored aptamer is considerably improved compared with that of the free aptamer. The lipid group attachment and/or liposome anchoring leads to a dramatic improvement in inhibitory activity of the aptamer toward VEGF-induced endothelial cell proliferation in vitro and vascular permeability increase and angiogenesis in vivo.

INTRODUCTION

Nucleic acids are capable of folding into stable and intricate three-dimensional structures. Randomized nucleic acid libraries therefore contain molecules with many shapes in which functional groups capable of hydrogen bonding, hydrophobic, and charge–charge interactions are arranged in a defined spatial orientation. By taking advantage of iterative cycles of affinity selection and amplification, the SELEX¹ process has emerged in recent years as a highly efficient method of identifying rare nucleic acid ligands (aptamers) from random libraries that bind to other molecules with high affinity and specificity (1–4). The advent of nuclease-stabilized libraries, such as the 2'-amino- or 2'-fluoropyrimidine RNA, has recently increased the utility of the SELEX process as a tool for identifying drug candidates (5–8).

We have recently used the SELEX process to identify 2'-aminopyrimidine RNA aptamers that bind to vascular endothelial growth factor (VEGF) (9), a potent inducer of angiogenesis. Angiogenesis, the growth of new blood vessels from existing vessels, accompanies physiological processes such as wound healing and reproductive cycles and is tightly controlled in healthy adults by opposing effects of positive and negative regulators. Under certain pathological conditions, including proliferative retinopathies, rheumatoid arthritis, psoriasis, and cancer, positive

regulators prevail and angiogenesis contributes to disease progression (reviewed in ref 10). VEGF is unique among angiogenic growth factors described to date in that it specifically stimulates endothelial cell proliferation (11–13) and induces a transient increase in blood vessel permeability with respect to macromolecules (hence, its alternative and original name, vascular permeability factor) (14–16). Increased vascular permeability and the resulting deposition of plasma proteins in the extravascular space assist the new vessel formation by providing a provisional matrix for the migration of endothelial cells (17). Hyperpermeability is indeed a characteristic feature of new vessels (17). In addition, compensatory angiogenesis induced by tissue hypoxia is now known to be mediated by VEGF (18, 19). Most tumors and tumor cell lines are capable of producing and secreting VEGF, and inhibition of VEGF activity leads to the reduced rate of tumor growth as well as the reduced incidence of metastases. Together, these observations have led to the assertion that VEGF is the crucial positive regulator of angiogenesis (20, 21). VEGF is a secreted disulfide-linked homodimer that occurs in four forms (VEGF-121, VEGF-165, VEGF-189, and VEGF-206) as a result of alternative splicing of the VEGF gene, with VEGF-165 being the most abundantly occurring form (22, 23). The biological effects of VEGF are mediated through two tyrosine kinase receptors (Flt-1 and Flk-1/KDR) whose expression is highly restricted to cells with endothelial and hematopoietic origins (24–27).

In the 2'-aminopyrimidine RNA aptamer, the subnanomolar binding affinity for VEGF-165 is encoded in a sequence of 24 nucleotides (9). We have achieved additional resistance to nuclease degradation by adding short phosphorothioate caps at the 3' and 5' ends and by substituting 2'-O-methylpurine nucleotides at all purine positions (10 out of 14) that tolerate such a substitution without loss of binding affinity. Such an aptamer (NX213 = 5'-dT^sdT^sdT^sdT^smAaCaCaCaUrGrAaUmGrGaUmAmGrAaCmGaCaCmGmG mGmGaUmGdT^sdT^sdT^sdT^sdT-3', where prefixes d, r, a, and m denote deoxy-, ribodeoxy-, 2'-amino-, and 2'-O-methylnucleotides and a superscript

[§] This paper is dedicated to Prof. Y. Pocker on the occasion of his 70th birthday.

* Address correspondence to these authors.

[†] NeXstar Pharmaceuticals.

[‡] University of Southern California.

¹ Abbreviations: AUC, area under the curve; CAM, chorioallantoic membrane; DAG–NX213-L, liposome-anchored DAG-modified VEGF aptamer; DAG–NX213, DAG-modified VEGF aptamer; DAG–scNX213-L, liposome-anchored DAG–scNX213; DAG–scNX213, DAG-modified scNX213; DAG, dialkylglycerol; DSPC, distearoylphosphatidylcholine; NX213, 2'-aminopyrimidine RNA VEGF aptamer; PBS, phosphate-buffered saline; scNX213, sequence-scrambled analogue of the VEGF aptamer; SELEX, systematic evolution of ligands by exponential enrichment; VEGF, vascular endothelial growth factor.

s denotes phosphorothioate internucleoside linkages) inhibited the binding of [^{125} I]VEGF to receptors expressed on human umbilical vein endothelial cells (HUVECs) with an EC_{50} of ≈ 1 nM, suggesting that the aptamer could be an inhibitor of VEGF-mediated effects (9). However, compared to its very good stability in biological fluids in vitro (9), the residence time of NX213 in plasma in vivo was found to be very short (see below), mostly as a consequence of rapid renal clearance (S. C. Gill and R. Fielding, unpublished results). Since aptamers in the 20–35-nucleotide size range have molecular masses of ≈ 7 –12 kDa, one obvious way of increasing their plasma residence times is by associating them with large, inert entities. Liposomes represent a class of supramolecular assemblies that could be used for this purpose. We report here on the synthesis and initial characterization of a lipid (dialkylglycerol, DAG) derivative of the VEGF aptamer (DAG–NX213) in which the lipid group is used to anchor the aptamers in liposome bilayers. The preparation of liposome-anchored aptamers is essentially as straightforward as the preparation of unloaded liposomes. Importantly, both the addition of the DAG group and liposome anchoring do not interfere with the ability of the aptamer to bind to VEGF with a high affinity. The plasma residence time of the liposome-anchored aptamer (DAG–NX213-L) is considerably longer than that of NX213. We also show that DAG–NX213 and/or DAG–NX213-L exhibit enhanced inhibitory activity compared with the free aptamer toward VEGF-induced endothelial cell proliferation in vitro and vascular permeability increase and CAM angiogenesis in vivo.

EXPERIMENTAL PROCEDURES

Preparation of the DAG Phosphoramidite Reagent. Tetraethylene Glycol Monotosylate (2). Tetraethylene glycol (200 mL, 1.15 mol) was dissolved in 500 mL of pyridine, cooled to 0 °C, and treated with 22.0 g (0.115 mol) of *p*-toluenesulfonyl chloride. Once the solution was formed, the reaction mixture was stored in the refrigerator overnight, and then concentrated in vacuo. The residue was dissolved in 800 mL of EtOAc and extracted with 3 $\times$ 600 mL of H₂O. The H₂O fractions were back-extracted with EtOAc, and the combined EtOAc fractions were extracted with saturated aqueous Na₂HPO₄. The organic phase was dried over MgSO₄ and concentrated to a colorless oil. The oil was purified by flash chromatography using 800 mL of silica gel and eluting with hexane, then 25% EtOAc/50% EtOAc in hexane, then EtOAc, and then 10% MeOH/20% MeOH in EtOAc to afford 23.7 g (60%) of pure product and 11% of product containing a minor impurity: ^1H NMR (300 MHz, CDCl₃) δ 7.77 (d, J = 8.1 Hz, 2H), 7.32 (d, J = 8.1 Hz, 2H), 4.13 (t, J = 4.8 Hz, 2H), 3.68–3.53 (m, 14H), 2.58 (t, J = 5.6 Hz, 1H), 2.42 (s, 3H); ^{13}C NMR (75 MHz, CDCl₃) δ 168.2, 158.3, 144.8, 135.9, 133.8, 132.0, 129.9, 128.0, 127.7, 126.6, 123.1, 113.0, 85.9, 73.0, 70.6, 70.4, 70.0, 69.7, 67.8, 64.4, 55.1, 37.1; low-resolution MS m/z calculated for C₁₅H₂₄O₈S (M + 1) 349.1 Da.

Tetraethylene Glycol Monophthalimide (3). To a stirred solution of 31.96 g (0.092 mol) of **2** in 400 mL of anhydrous DMF were added 14.2 g (1.05 equiv) of phthalimide and 14.4 mL (1.05 equiv) of 1,8-diazabicyclo[5.4.0]undec-7-ene. The solution was heated at 70 °C for 18 h and then concentrated in vacuo. The crude yellow oil was purified by flash chromatography using 1600 mL of silica gel and eluting with 25% EtOAc/50% EtOAc/75% EtOAc in hexane, then EtOAc, and then 10% MeOH/20% MeOH in EtOAc to afford 23.8 g (80%) of **3** as an oil. Upon standing, **3** became a waxy white solid: ^1H NMR (300

MHz, CDCl₃) δ 7.84–7.78 (m, 2H), 7.70–7.66 (m, 2H), 3.86 (t, J = 5.6 Hz, 2H), 3.70 (t, J = 5.6 Hz, 2H), 3.64–3.51 (m, 12H), 2.67 (bs, 1H); ^{13}C NMR (75 MHz, CDCl₃) δ 168.2, 133.8, 132.0, 123.1, 72.4, 70.5, 70.4, 70.2, 70.0, 67.8, 61.6, 37.2.

Synthesis of Compound 4. A solution of 15 g (0.0464 mol) of **3** in 150 mL of THF and 15 mL of DMF was cooled to 0 °C under Ar. Allyl bromide (6.0 mL, 1.5 equiv) was added to the solution, followed by addition of 1.76 g (1.5 equiv) of NaH as a solid. The opaque yellow suspension was stirred at 0 °C for 30 min and then at room temperature for 18 h. MeOH (50–100 mL) was added, and the mixture was concentrated in vacuo. The crude material was purified by flash chromatography using 1500 mL of silica gel and eluting with 25% EtOAc/50% EtOAc/75% EtOAc in hexane, then EtOAc, and then 10% MeOH in EtOAc to afford 11.05 g (65%) of **4** as a yellow oil: ^1H NMR (300 MHz, CDCl₃) δ 7.84–7.80 (m, 2H), 7.72–7.67 (m, 2H), 5.94–5.84 (m, 1H), 5.28–5.14 (m, 2H), 3.99 (d, J = 5.61 Hz, 2H), 3.88 (t, J = 5.85 Hz, 2H), 3.72 (t, J = 5.76 Hz, 2H), 3.64–3.54 (m, 13H); ^{13}C NMR (75 MHz, CDCl₃) δ 168.0, 134.6, 133.7, 131.9, 123.0, 116.9, 72.0, 70.4, 69.9, 69.2, 67.7, 37.0.

1-(Dimethoxytrityl)-3-(phthalimidotetraethylene glycolyl)-rac-glycerol (5). To a stirred solution of **4** (10.13 g, 0.0279 mol) in 100 mL of acetone and 1 mL of H₂O was added 3.98 g (1.22 equiv) of *N*-methylmorpholine *N*-oxide. To this suspension was added 1.75 mL (0.005 equiv) of OsO₄ as a 2.5% solution in i -PrOH. After addition of the OsO₄ solution, the reaction mixture became clear yellow. After TLC analysis indicated complete conversion of **4** (ca. 16 h), the reaction mixture was treated with 1.5 g of sodium hydrosulfite and 5.0 g of florisil and stirred for 30 min. The suspension was filtered through florisil, and the filtrate was concentrated to an oil. This crude product was combined with another batch prepared in the same manner from 1.0 g of **4**. Two 100 mL portions of pyridine were coevaporated from the combined lots, and the residue was dissolved in 300 mL of pyridine. The solution was cooled to 0 °C, and 10.89 g (1.05 equiv) of 4,4'-dimethoxytrityl chloride was added. A drying tube was placed into the flask, and the reaction mixture was stirred at room temperature for 16 h. The solution was treated with 20 mL of MeOH and concentrated in vacuo, keeping the temperature of the water bath below 40 °C. The crude oil was purified by flash chromatography using 1100 mL of silica gel (wet-packed onto the column using 3% triethylamine in hexane) and eluting with 10–100% EtOAc in hexane (all containing 3% triethylamine) to give 21.3 g (89% after two steps) of **5** as a yellow oil: ^1H NMR (300 MHz, CDCl₃) δ 7.80–7.77 (m, 2H), 7.66–7.64 (m, 2H), 7.39–7.22 (m, 9H), 7.20–6.76 (m, 4H), 3.97 (bs, 1H), 3.84 (t, J = 5.97 Hz, 2H), 3.74 (s, 6H), 3.68 (t, J = 5.7 Hz, 2H), 3.60–3.49 (m, 14H), 3.13–2.76 (m, 2H), 2.00 (bs, 1H); ^{13}C NMR (75 MHz, CDCl₃) δ 168.2, 158.3, 144.8, 135.9, 133.8, 132.0, 129.9, 128.0, 127.7, 126.6, 123.1, 113.0, 85.9, 73.0, 70.6, 70.4, 70.0, 69.7, 67.8, 64.4, 55.1, 37.1; low-resolution MS m/z calculated for C₄₀H₄₅O₁₀N (M + NH₄⁺) 717.5 Da.

1-(Dimethoxytrityl)-3-(aminotetraethylene glycolyl)-sn-glycerol (6). Compound **5** (5.2 g, 7.2 mmol) was taken up in 50 mL of 40% methylamine in H₂O, and 10 mL of methanol was added to solubilize the starting material. The reaction mixture was heated at 50 °C for 5 h and then concentrated in vacuo and coevaporated with toluene. The crude material was purified by flash chromatography on 200 mL of silica gel, eluting with 15% methanolic ammonia in dichloromethane. **6** (3.94 g, 96%) was collected as a pale yellow oil: ^1H NMR (300 MHz,

CDCl_3) δ 7.46–7.21 (m, 9H, DMT), 6.81 (d, 4H, DMT), 4.00 (m, 1H), 3.80 (s, 6H), 3.70–3.49 (overlapping m, 18H), 3.20 (dd, $J = 9.24, 5.49$ Hz, 1H), 3.12 (dd, $J = 9.21, 6.0$ Hz, 1H), 2.84–2.80 (m, 3H); ^{13}C NMR (75 MHz, CDCl_3) δ 158.30, 144.82, 136.01, 129.95, 128.04, 127.66, 126.61, 112.95, 85.85, 73.46, 72.85, 70.55, 70.45, 69.99, 69.51, 64.43, 55.10, 41.40; low-resolution MS m/z calculated for $\text{C}_{32}\text{H}_{44}\text{O}_8\text{N}$ ($M + 1^+$) 570.353 Da, found 570.4 Da.

Chloroformate 7. To a stirred solution of 3 g (5.03 mmol) of 1,2-di-*O*-octadecyl-*sn*-glycerol in 60 mL of toluene was added 20 mL of a 1.93 M solution of phosgene. Additional phosgene solution (2×10 mL, total of 15.4 equiv of phosgene) was added until no further alcohol starting material remained (by ^1H NMR analysis of concentrated aliquots). The excess phosgene and HCl was removed with an aspirator, and the reaction mixture was concentrated in vacuo to afford 3.3 g (98%) of the desired chloroformate **7** as a white powder: ^1H NMR (300 MHz, CDCl_3) δ 4.45 (dd, $J = 11.22, 3.69$ Hz, 1H), 4.34 (dd, $J = 11.22, 6.15$ Hz, 1H), 3.65 (m, 1H), 3.56–3.40 (m, 6H), 1.53 (m, 4H), 1.24 (m, 62H), 0.87 (t, $J = 6.36$ Hz, 6H); ^{13}C NMR (75 MHz, CDCl_3) δ 75.90, 71.91, 71.35, 70.93, 69.36, 31.99, 29.96–29.44 (overlapping signals from hydrocarbon chains), 26.13, 26.04, 22.76, 14.18.

Conjugate 8. To a stirred solution of 2.25 g (3.95 mmol) of **6** in 60 mL of pyridine was added 2.6 g of the distearyl glycerol chloroformate **7**. ^1H NMR analysis of a concentrated aliquot after 2 h revealed no remaining chloroformate, and the mixture was concentrated in vacuo. The crude residue was combined with material similarly prepared from 0.5 g (0.88 mmol) of **6** and 0.58 g of the chloroformate **7**, and the combined lots were purified by flash silica gel chromatography on a column of 100 mL of silica gel (packed in hexanes containing 2% triethylamine) eluting with 200 mL hexanes, and then 250 mL each of 10–20 and 30% EtOAc in hexanes and 500 mL of 40% EtOAc in hexanes, then 250 mL each of 50, 60, 70, and 80% EtOAc in hexanes, and finally with 250 mL of EtOAc. The product-containing fractions were concentrated to afford 3.3 g (57%) of the conjugate **8**.

Phosphoramidite 9. To a stirred solution of 3.8 g (3.26 mmol) of conjugate **8** in 25 mL of CH_2Cl_2 were added 1.14 mL (6.52 mmol) of diisopropylethylamine and then 1.09 mL (4.88 mmol) of 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite. After 2 h, the mixture was diluted with CH_2Cl_2 and washed with a saturated NaHCO_3 solution, dried over Na_2SO_4 , and concentrated. The crude residue was purified by flash silica gel chromatography on a column of 125 mL of silica gel (packed in hexanes containing 2% triethylamine) eluting with 100 mL hexanes, then 250 mL each of 10 and 20% EtOAc in hexanes and 500 mL of 30% EtOAc in hexanes, and then 250 mL of 50% EtOAc in hexanes. The product-containing fractions were concentrated to afford 4.2 g (95%) of the phosphoramidite **9**: ^{31}P NMR (CDCl_3) δ 151.52, 151.08.

Preparation of DAG–Aptamers. The VEGF aptamer (NX213) and its scrambled analogue (scNX213) were prepared by standard solid phase oligonucleotide synthesis protocols as described (ref 9 and references therein). For DAG-conjugated aptamers, phosphoramidite **9** dissolved in CH_2Cl_2 (acetonitrile was not an adequate solvent) was added as the last coupling step to the 5' end of the aptamers (two 30 min coupling cycles using a 4-fold excess of the amidite; coupling efficiency $\approx 90\%$, by dimethoxytrityl absorbance). The DAG–aptamer was purified by reverse phase HPLC (PRP-1 column, Hamilton, Reno, NV) using a 100 mM triethylammonium acetate/acetonitrile gradient followed by desalting and

salt (Na^+ for triethylammonium) exchange (PRP-1 column). Following purification, $>99\%$ of the oligonucleotide contained the DAG moiety. Electrospray mass spectroscopic analysis of DAG–NX213 indicated two major components with m/z values of 11 714.2 and 11 699.2 Da (calculated mass of DAG–NX213 = 11 715.3 Da). The m/z 11 699.2 Da component (≈ 16 Da less than calculated) is probably due to incomplete oxidation by the sulfurizing reagent (Glen Research, Sterling, VA), resulting on average in seven instead of eight phosphorothioate internucleoside linkages per molecule.

Preparation of Liposome-Associated Aptamers.

A spray-dried mixture of 1,2-distearoyl-*sn*-glycero-3-phosphatidylcholine (DSPC) and cholesterol (2 mol:1 mol) was placed in a 14 mL Pyrex glass centrifuge tube (50 mg of total lipid) and suspended in a 1 mL solution containing 1 mg/mL (0.12 mol %) DAG–NX213 or DAG–scNX213 in 9% sucrose buffered to pH 7.4 with 25 mM sodium phosphate. The lipid suspension was incubated for 5–15 min at 65 °C, gently mixed until evenly suspended, and sonicated with a 0.125 in. microtip probe at 65 °C with an approximately 215 W input power using a model 475 Virsonic power supply (Virtis, Gardiner, NY). The progress of the sonication was followed by particle sizing using dynamic light scattering (Leeds Northrop MicroTrac Model 150 Particle Analyzer, Honeywell, Ft. Washington, PA). Liposome sizing was determined by the volume of distribution method using the manufacturer's protocol (MicroTrac). Sonication was typically stopped when the liposomes reached an essentially unimodal distribution with a mean vesicle diameter of approximately 50–65 nm. The final liposome product is sterilized by filtration through a 0.2 μm syringe filter (Supor Acrodisc 25, Gelman Sciences, Ann Arbor, MI). The total concentration of aptamer in the liposomal formulation was determined from the absorbance value at 260 nm ($\epsilon_{260} = 27 \text{ au mg}^{-1} \text{ mL}^{-1}$) in a 0.1% sodium dodecyl sulfate solution. In all experiments with the liposome-anchored aptamer, the total aptamer concentration was used without a correction for the orientation of the aptamer in the liposome bilayer (see below).

Ribonuclease T₁ Digestion of the Free and Liposomal Aptamers. All digests were performed with ^{32}P 3' end-labeled DAG–NX213 at 8 pmol/ μL and 20 units/ μL RNase T₁ in 25 mM sodium phosphate buffer (pH 7.4) containing 9% sucrose. The digests were terminated by adding an equal volume of gel loading solution (80% formamide, 89 mM Tris borate, 1 mM EDTA, 2% sodium dodecyl sulfate, and 0.7 M β -mercaptoethanol), followed by heating at 95 °C for 2 min and cooling on ice before loading on a 20% polyacrylamide/7 M urea gel.

Plasma Pharmacokinetics. The pharmacokinetic properties of VEGF aptamers NX213, DAG–NX213, and DAG–NX213-L were determined in Sprague-Dawley rats. In each study, the aptamer was diluted in PBS to a concentration of 1.0 mg/mL on the basis of UV absorption at 260 nm and an extinction coefficient of 0.037 mg of oligo/mL. In all studies, six rats received 1.0 mg of oligonucleotide/kg of animal weight by bolus tail vein injection and EDTA plasma samples were taken at various times from 2 min to 4 h. The standards, plasma samples, and quality control samples were analyzed using a hybridization assay. The hybridization assay utilized a capture oligonucleotide that contains a complementary sequence at the 5' end of the VEGF aptamer conjugated to an iron oxide (FeO) bead [FeO -spacer-3'-d(GCCTTAGTCACTT)-5' where spacer = (dT)₈] and a detection oligonucleotide containing two biotin molecules at the 5' end [biotin-biotin-5'-d(spacer-CGGATGTATAAG-

CA)-3', where spacer = (dT)₈]. After incubation of the capture and detection probes with a plasma sample containing VEGF aptamers, the amount of the biotin oligonucleotide hybridized to the bead was quantitated with the streptavidin-linked alkaline phosphatase, using CSPD-Sapphire (Tropix, Bedford, MA) as the luminescent substrate. For each aptamer, the standard curve and quality control samples were matched to the aptamer being analyzed. Standard curves were fit using a four-parameter logistic equation (PRISM, version 2.00, Graph-Pad, San Diego, CA). Sample and quality control concentrations were extrapolated from the standard curve and corrected for dilution. Data for each formulation were pooled, and the pharmacokinetic parameters were analyzed using two-compartment compartmental analysis (weighting $1/y^2$, WinNonlin Pro, version 1.1.12, Scientific Consulting, Inc., Apex, NC).

Competition Electrophoretic Mobility Shift Assay. ³²P 5' end-labeled NX213 (1.5 nM) was incubated in binding buffer (phosphate-buffered saline with 0.01% human serum albumin) at 37 °C for 20 min in the presence of VEGF-165 (R&D Systems, Minneapolis, MN; 0.33 nM) and competitor oligonucleotide (5 pM to 0.33 μM). The [³²P]NX213-VEGF complex was resolved from the free [³²P]NX213 by electrophoresis on an 8% polyacrylamide gel (19:1 acrylamide/bisacrylamide, Tris-borate, 89 mM, and 1 mM EDTA as the running buffer). The intensity of the band corresponding to the [³²P]-NX213-VEGF complex at varying competitor concentrations was quantitated by phosphorimager analysis. Data normalized for the amount of complex formed in the absence of competitor were fitted by the least-squares method to a competition binding equation that describes the mutually exclusive binding of two species to the same target (28).

Receptor Binding Inhibition. Confluent cultures of HUVECs in 24-well plates were incubated with [¹²⁵I]-VEGF-165 (Amersham, Arlington Heights, IL; 20 ng/mL) and varying amounts of aptamers for 2 h at 4 °C. At the end of the 2 h incubation period, the supernatant was aspirated and the wells were washed two times with cold PBS. HUVECs were then lysed with 1% Triton X-100, and the amount of cell-associated [¹²⁵I]VEGF was determined by γ counting.

[³H]Thymidine Incorporation Assay. HUVECs (population doubling of 4–12) were plated at a density of 10⁴/well on 96-well plates in MEM supplemented with 10 mM HEPES (pH 7.4) and 1 μg/mL heparin (from porcine lungs, Sigma, St. Louis, MO). Following a 4–5 h attachment period, quiescence was induced by overnight incubation in MEM containing 0.5% fetal bovine serum and 1 μg/mL heparin. VEGF was then added to the cell cultures in the presence or absence of aptamers for a period of 48 h. [³H]Thymidine was added in the last 24 h of incubation, and the amount of DNA-associated [³H]thymidine was determined by scintillation counting.

Dermal Vascular Permeability Assay. The ability of the test aptamers to attenuate VEGF-induced changes in the permeability of the dermal vasculature (Miles assay) was performed as previously described (15, 29) with minor modifications. Briefly, adult female guinea pigs (three per study) were anesthetized with isoflurane, and the hair on the dorsal and lateral back areas was removed with clippers. Evans Blue dye (2.5 mg/guinea pig) was administered intravenously. Injection solutions (PBS, VEGF, and aptamers) were prepared ≥30 min in advance and comixed where indicated. Each solution shown was then injected intradermally (duplicate injec-

tions for each guinea pig, 40 μL/site) in a randomized manner in a grid pattern drawn on the clipped area. Guinea pigs were allowed to recover from anesthesia and were sacrificed by CO₂ exposure 30 min after completion of the intradermal injections. The skin was harvested, trimmed free of subcutis, and transilluminated. Images were then captured using a color CCD camera (Hitachi Denshi KP-50U) and Image-Pro Plus software (version 3.1, Media Cybernetics, Silver Springs, MD). Each skin sample was normalized for intensity with each injection site analyzed for optical density and the area involved.

Chicken Allantoic Membrane (CAM) Assay. The effect of various VEGF aptamers on VEGF-induced angiogenesis was tested in CAMs. Angiogenesis was induced in CAMs by placing filter disks saturated with VEGF on 10-day-old embryos. Coded test compounds (1 μM) or an equal amount of the carrier buffer (100 μL) was injected into the vessels on the following day. CAMs were resected on day 3 and analyzed using an Olympus stereomicroscope. The number of branching blood vessels infiltrating under the disk were counted and photographed. The average number of blood vessels under the disks soaked in buffer alone were subtracted from all groups.

RESULTS

Preparation of Liposome-Anchored VEGF Aptamers. To allow stable membrane anchoring, we used the solid phase phosphoramidite method to prepare a lipid derivative of the VEGF aptamer in which two 18-carbon saturated unbranched hydrocarbon chains were attached via glycerol ether linkages. The dialkylglycerol (DAG) phosphoramidite containing a tetraethylene glycol spacer was synthesized in seven steps and introduced at the 5' end of the VEGF aptamer (Figure 1A,B). DAG-NX213 was readily purified by reverse phase HPLC since the retention time of the lipid-containing aptamer is considerably greater than that of the aptamer without the lipid group. To incorporate DAG-NX213 into the bilayer of liposomes, we sonicated a solution of DAG-NX213 (1 mg/mL) in 25 mM sodium phosphate buffer (pH 7.4) containing 9% sucrose in the presence of a 50 mg/mL spray-dried hydrated mixture of distearoylphosphatidylcholine (DSPC) and cholesterol (2 mol:1 mol). The ratio of DAG-NX213 to lipids was chosen arbitrarily and is not optimized for any purpose. On the basis of size exclusion chromatography, all of the DAG-aptamer was found to be associated with the liposome fraction following sonication. Importantly, aptamer fragments were not observed following liposome preparation, either by size exclusion chromatography or by HPLC, indicating that the DAG-aptamer is stable to sonication conditions (data not shown). In a typical preparation, liposomes with a mean diameter of ≈50–65 nm were obtained (Figure 2A). Liposomes prepared in this manner were visualized by electron microscopy in freeze-fracture preparations (30) (Figure 2B). Assuming surface areas of 44.5 Å for DSPC and 39 Å for cholesterol (31), for spherical liposomes with a homogeneous lipid distribution [2 mol of DSPC (MW = 790.2) per mole of cholesterol (MW = 386.7)] and a membrane thickness of 50 Å, we calculated the number of lipids per liposome to be 3.02×10^4 and 4.88×10^4 for liposomes with 50 and 65 nm diameters, respectively. On the basis of these values, we estimate that there are on average approximately 34–60 aptamers per liposome for 50–65 nm liposomes.

In the absence of the DAG-NX213 aptamer, liposomes with a comparable size distribution were obtained using

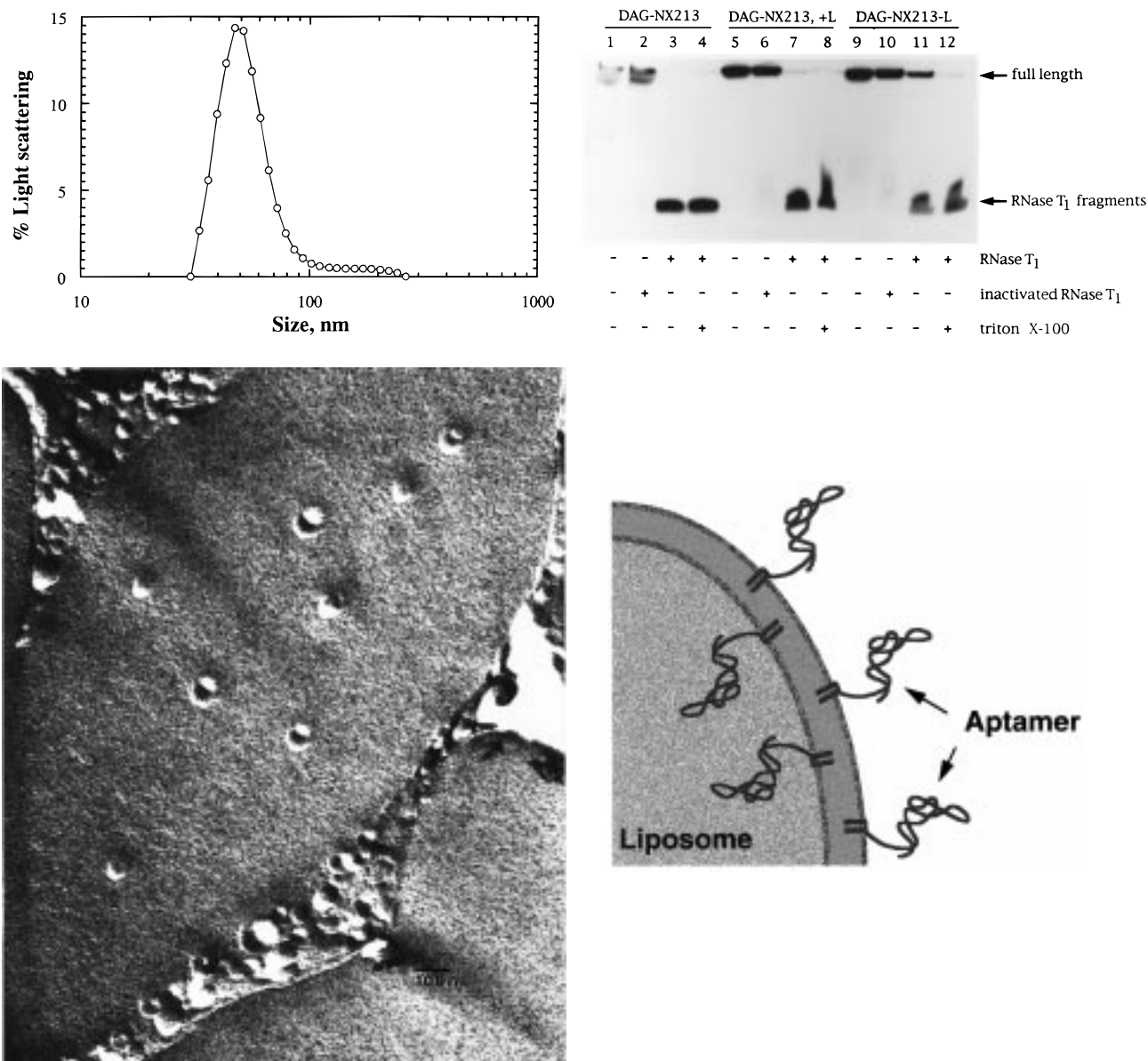


Figure 2. Characterization of physical properties of the liposomal VEGF aptamer (DAG-NX213-L). (A, top left) The size of DAG-NX213-L particles was determined by quasi-elastic light scattering using a Leeds & Northrup Model Microtrack UPA 150 particle analyzer (Horsham, PA). The distribution by liposome volume as a function of diameter is shown. (B, bottom left) Freeze-fracture electron micrograph of DAG-NX213-L. (C, top right) Accessibility of DAG-NX213 to ribonuclease T₁ as a function of formulation. RNase T₁ digests of DAG-NX213 alone (lanes 1–4), DAG-NX213 mixed with preformed liposomes (lanes 5–8), and DAG-NX213 incorporated into liposomes by sonication (DAG-NX213-L, lanes 9–12) are shown resolved on a 20% polyacrylamide/7 M urea gel. In the first lane of each set (lanes 1, 5, and 9), no RNase was added. In the second lane of each set (lanes 2, 6, and 10), the gel loading solution (which inactivates the RNase) was added to the samples prior to adding RNase. In the third lane of each set (lanes 3, 7, and 11), the samples were incubated with RNase T₁ for 30 min at 20 °C. In the fourth lane of each set (lanes 4, 8, and 12), the samples were incubated with RNase T₁ for 30 min at 20 °C in the presence of 0.1% Triton X-100. (D, bottom right) Schematic representation of liposome-anchored aptamers.

increase was studied in guinea pigs (Figure 7). Test aptamers at various concentrations were coinjected intradermally with VEGF (20 nM) in guinea pigs preinjected with Evans blue dye. Because the recovery of the dye by organic solvent extraction is generally very inefficient, we have developed a quantitation method that measures the absorption of light through the skin. NX213 at concentrations up to 10 μ M was not able to inhibit the vascular permeability activity of VEGF. DAG-NX213 and DAG-NX213-L, on the other hand, inhibited about 60 and 90% of the VEGF-induced permeability increase, respectively, at the highest aptamer concentration tested (10 μ M), with comparable IC₅₀ values of 0.3–0.5 μ M. Sequence-scrambled analogues of these compounds were not inhibitory (data not shown).

Effect of VEGF Aptamers on VEGF-Induced Angiogenesis in Chicken Chorioallantoic Membranes (CAMs). VEGF is a potent inducer of angiogenesis in a number of in vivo model systems. The effect of various VEGF aptamers on VEGF-induced angiogenesis was tested in CAMs. Capillary vessel formation within the CAMs has been used extensively as a convenient in vivo model system for studying the angiogenic and angiostatic activity of various compounds (32). Angiogenesis was induced in CAMs by placing filter disks saturated with VEGF on 10-day-old embryos. Coded test compounds (1 μ M) or an equal amount of the carrier buffer (100 μ L) was injected into the vessels on the following day. CAMs were resected on day 3 and analyzed using a stereomicroscope. The number of branching blood vessels infil-

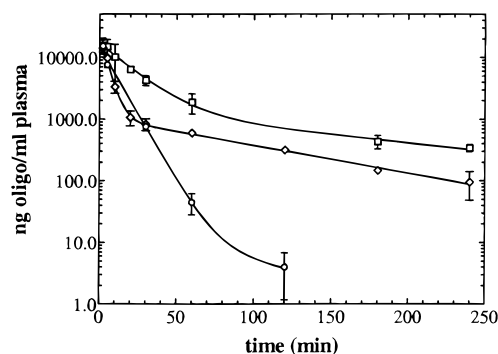


Figure 3. Plasma pharmacokinetic properties of NX213, DAG-NX213, and DAG-NX213-L in Sprague-Dawley rats. Aptamers were administered by intravenous injection via the tail vein, at a dose of 1 mg of aptamer per kilogram of animal weight. EDTA plasma samples were analyzed using a double-hybridization assay: NX213 (○), DAG-NX213 (◇), and DAG-NX213-L (□). Data from two to five animals were used per data point ($\pm$ SEM).

Table 1. Summary of Aptamer Pharmacokinetic Parameters in Plasma after Intravenous Bolus Administration in Sprague-Dawley Rats Determined from the Data Shown in Figure 3 (Compartmental Analysis)

parameter	NX213	DAG-NX213	DAG-NX213-L
total AUC ($\mu\text{g min}^{-1} \text{mL}^{-1}$) ^a	147	202	531
C, $t = 0$ min ($\mu\text{g/mL}$)	14.59	23.16	16.95
C, $t = 2$ min ($\mu\text{g/mL}$)	15.31	14.08	15.74
$\alpha(t_{1/2})$ (min)	7	3	13
$\beta(t_{1/2})$ (min)	49	67	113
clearance ($\text{mL kg}^{-1} \text{min}^{-1}$)	6.80	4.95	1.88
V_{ss} (mL/kg)	72	251	152

^a AUC = area under the curve.

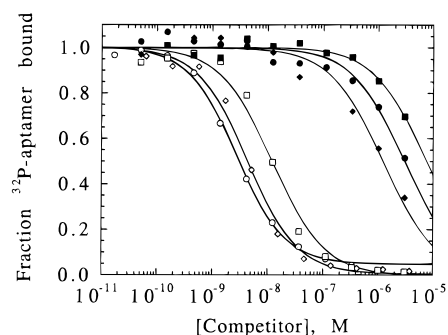


Figure 4. Binding affinities of NX213, DAG-NX213, and DAG-NX213-L and their sequence-scrambled analogues for VEGF. A competition electrophoretic mobility shift assay was used to determine the binding affinities of NX213 (○), DAG-NX213 (◇), and DAG-NX213-L (□) and their sequence-scrambled analogues scNX213 (●), DAG-scNX213 (◆), and DAG-scNX213-L (■). In all cases, competitor concentration refers to the total concentration of oligonucleotide (without a correction for aptamer accessibility).

trating under the disk were counted and photographed. The average number of blood vessels under the disks soaked in buffer alone was subtracted from all groups. The only compound that effectively blocked VEGF-induced angiogenesis was DAG-NX213-L, while NX213, DAG-NX213, and DAG-scNX213-L had a smaller effect or no effect (Figure 8).

DISCUSSION

The SELEX process has recently emerged as an efficient method of identifying aptamers with high affinities

Table 2. Relative Binding Affinities of NX213, DAG-NX213, DAG-NX213-L, and Their Sequence-Scrambled Analogues^a

aptamer	$K_d^{\text{aptamer}}/K_d^{\text{NX213}}$ ^a
NX213	(1.0)
DAG-NX213	1.6 ± 0.3
DAG-NX213-L	4.7 ± 0.7
scNX213	$(1.3 \pm 0.9) \times 10^3$
DAG-scNX213	$(5.0 \pm 0.7) \times 10^2$
DAG-scNX213-L	$(3.1 \pm 3.4) \times 10^3$

^a The value of K_d for NX213 binding to VEGF calculated from the competition binding experiment is $(3.2 \pm 0.2) \times 10^{-10}$ M.

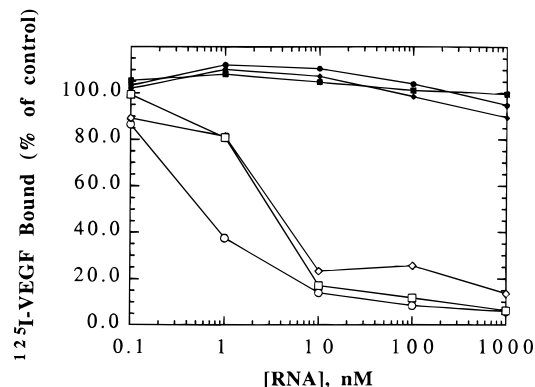


Figure 5. Effect of NX213, DAG-NX213, and DAG-NX213-L aptamers and their sequence-scrambled analogues on the binding of [¹²⁵I]VEGF to cell surface receptors expressed on human umbilical vein endothelial cells (HUVECs). Confluent cultures of HUVECs were incubated with 20 ng/mL [¹²⁵I]VEGF and varying amounts of NX213 (○), DAG-NX213 (◇), and DAG-NX213-L (□) and their sequence-scrambled analogues scNX213 (●), DAG-scNX213 (◆), and DAG-scNX213-L (■). Data points represent averages of two determinations.

for a variety of molecular targets. As chemically synthesized molecules, aptamers are uniquely amenable to a wide range of chemical modifications. In attempts to develop aptamer-based drug candidates, many such modifications have been aimed at improving the functional efficacy of the aptamers in vivo. For example, the affinity, specificity, and nuclease resistance have been substantially improved by the introduction of a series of 2'-O-methylpurine substitutions (9). Hicke et al. have recently shown that poly(ethylene glycol)-derivatized L-selectin DNA aptamers have improved pharmacokinetic properties and that this translated into more effective inhibition of lymphocyte trafficking in vivo (33).

Here we have shown that a lipid group such as DAG can be introduced into a prototypic aptamer with minimal effects on the binding affinity. Although we have employed the solid phase phosphoramidite method to conjugate the DAG group to the aptamer, solution coupling methods as well as other types of coupling chemistries are clearly feasible. Indeed, it has been demonstrated previously that hydrophobic groups can be introduced into oligonucleotides by a variety of methods and that such lipid-oligonucleotide conjugates have altered physical and pharmacokinetic properties (34–38). Lipid groups such as DAG permit stable anchoring into bilayers of liposomes. This notion has been recognized previously and exploited for improving the circulating levels in plasma of low-molecular weight molecules such as small peptides and carbohydrates (39, 40).

The plasma circulating time of the lipid-modified VEGF aptamer (DAG-NX213) is somewhat improved compared to that of the underivatized aptamer (NX213),

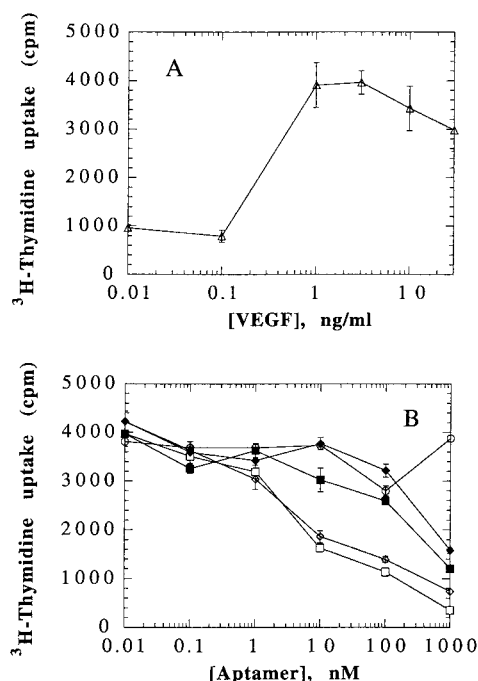


Figure 6. Effect of NX213, DAG-NX213, and DAG-NX213-L aptamers and their sequence-scrambled analogues on VEGF-induced HUVEC proliferation. (A) Dose response of VEGF added to the culture medium. (B) The effect of NX213 (○), DAG-NX213 (◇), DAG-NX213-L (□), DAG-scNX213-L (◆), and DAG-NX213-L (■) on HUVEC proliferation induced with 3 ng/mL VEGF. Values are the mean (five determinations) $\pm$ SEM.

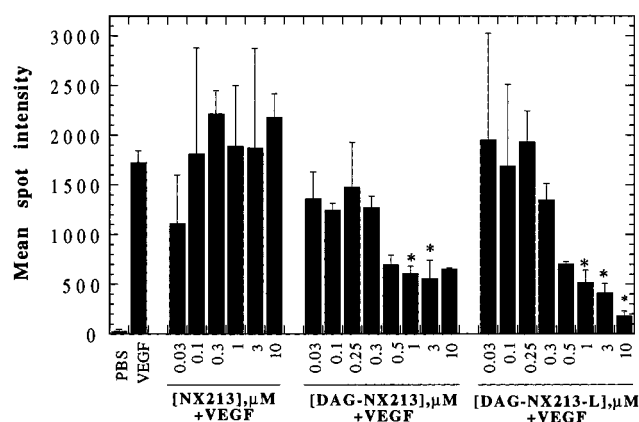
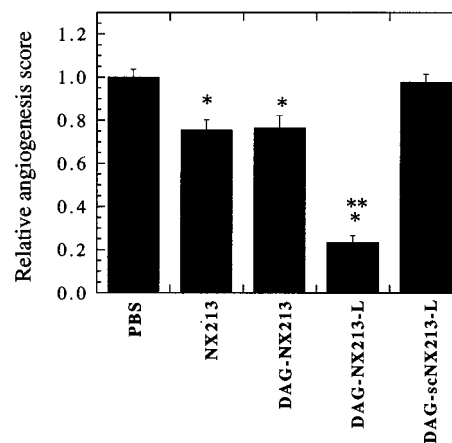
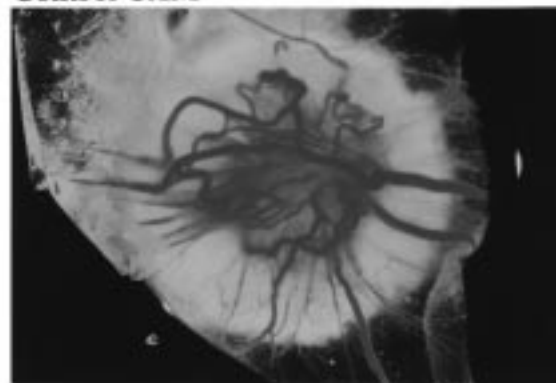


Figure 7. Effect of NX213, DAG-NX213, and DAG-NX213-L aptamers on VEGF-induced vascular permeability increase. The local extravasation of Evans blue dye was determined 30 min after injection of VEGF (0.8 pmol) in the presence or absence of aptamers by transillumination of harvested skin. Values are the mean (two to ten determinations) $\pm$ SEM. An asterisk indicates that $P < 0.05$ compared with that for VEGF alone.

as has been observed with other lipid-modified nucleic acids (38). This is probably the result of the association of DAG-NX213 with lipoproteins in plasma (38). Liposome anchoring of the DAG-NX213 aptamer, however, clearly leads to a further substantial increase in its plasma lifetime. Liposomes with the same lipid composition and size as those used in this work in which the unconjugated aptamer (NX213) was encapsulated in the interior of the liposome exhibit considerably longer plasma residence times (total AUC $\approx 4900 \mu\text{g min}^{-1} \text{mL}^{-1}$, clearance $\approx 0.2 \text{ mL kg}^{-1} \text{min}^{-1}$; see Table 1) compared with liposomes that carry DAG-NX213 in their bilayers (data not shown). Thus, like peptides and



Control CAM



DAG-NX213-L-treated CAM

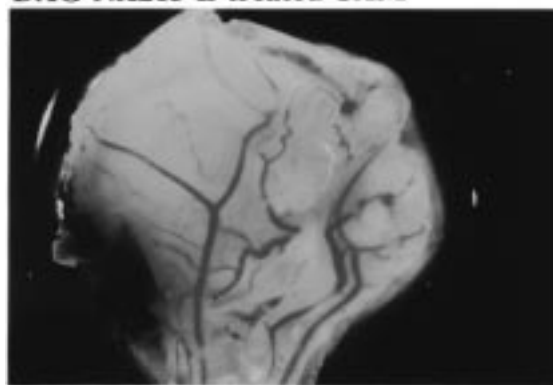


Figure 8. Effect of NX213, DAG-NX213, and DAG-NX213-L aptamers and their sequence-scrambled analogues on VEGF-induced angiogenesis in CAMs. VEGF-saturated filter disks were used on 10-day-old CAMs to induce angiogenesis. Test compounds or buffer (PBS) was injected intravascularly on the following day, and the number of branching blood vessels infiltrating the disks were counted on day 3. Five to twelve CAMs were studied for each compound, and the experiments were repeated at least two times with similar results. (A, top) Relative angiogenesis scores normalized to the number of new blood vessels observed in the group of CAMs containing the VEGF-saturated disks and treated with PBS alone ($\pm$ SEM). An asterisk indicates that $P < 0.05$ compared with that of the control (PBS) group alone, and two asterisks indicate that $P < 0.05$ compared with that of NX213 or DAG-NX213. (B, bottom) Representative CAMs containing VEGF-saturated disks (covering the entire CAM) treated with buffer alone (upper panel) or DAG-NX213-L (lower panel).

antibodies (39, 41), surface-immobilized aptamers appear to reduce the plasma residence times of liposomes.

Liposome anchoring of DAG-NX213 causes a small apparent reduction in the binding affinity for VEGF (Figure 4) which is probably due in part to the fact that

some of the aptamer is anchored in the inner leaflet of the bilayer and is therefore inaccessible to VEGF in solution. However, since liposome anchoring also creates a multivalent display of the aptamer (which is expected to increase the apparent affinity) in addition to imposing steric and orientational constraints on binding (which may reduce the apparent affinity), a straightforward interpretation of affinity is not possible from the data. Like NX213, DAG-NX213 and DAG-NX213-L aptamers inhibited the binding of VEGF to its endothelial cell receptors, albeit at ≈ 5 -fold reduced potency compared with that of NX213. In contrast to the modest reduction in the binding affinity for VEGF and in the receptor binding inhibitory activity, DAG-NX213 and DAG-NX213-L exhibited a considerably greater potency in inhibiting VEGF-induced DNA synthesis in endothelial cells compared with NX213 (NX213 showed no inhibition up to $1 \mu\text{M}$). Similar inhibitory activity enhancement was observed with DAG-NX213 and DAG-NX213-L compared to that with NX213 in the vascular permeability assay, with DAG-NX213-L showing the most complete inhibition and NX213 being ineffective up to $10 \mu\text{M}$. In the CAM angiogenesis assay, DAG-NX213-L was the only aptamer that showed effective inhibition of VEGF-induced angiogenesis.

The reason for the inhibitory activity enhancement of DAG-NX213 and DAG-NX213-L in vitro and in vivo remains to be elucidated. It will be important to establish whether DAG-NX213 and DAG-NX213-L (or similar preparations) associate with cellular plasma membranes in the mitogenicity assay and whether the larger size of these aptamers (especially of the liposomal aptamer) results in slower diffusion and therefore greater concentration at the site of injection in the Miles assay. In the mitogenicity and Miles assays, since VEGF is administered along with the aptamers in a confined space, the longer plasma circulating times of DAG-NX213 and DAG-NX213-L compared with that of NX213 are obviously not relevant. However, this may be important in the CAM assay where the aptamers were administered intravenously.

The association of aptamers with liposomes in a manner described here may have several applications of general utility. The advantages of such formulations include (i) improving the plasma pharmacokinetics of aptamers, (ii) presenting aptamers in a multivalent array with the aim of increasing the avidity of interaction with their targets, (iii) combining two or more aptamers with different specificities in the same liposome particle, (iv) enhancing the delivery of aptamers to tumors by taking advantage of the intrinsic tumor targeting properties of liposomes, and (v) using the high affinity and specificity of aptamers to guide liposomal contents to specific targets, which has to date primarily been accomplished with antibodies (41–43). Aptamers are well-suited for the kinds of preparations described here since, unlike most proteins, the denaturation of aptamers by heat, various molecular denaturants, and organic solvents is readily reversible. Given the rapidly increasing availability of aptamers with different specificities, similar liposome-anchored aptamers to the ones described here will be tested soon.

ACKNOWLEDGMENT

We thank Drs. Paul Schmidt, Eric Forssen, and Gary Fujii for many useful discussions. We also thank Jeffrey Walenta, Joelle Brown, and Sarah O'Rourke for expert technical assistance, Chandra Vargeese and Philippe

Bridonneau for assistance with large-scale oligonucleotide synthesis and purification, Julie Wolf for performing statistical analyses, and David Tinnermeier for performing electrospray mass spectroscopy analyses.

LITERATURE CITED

- (1) Tuerk, C., and Gold, L. (1990) Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase. *Science* 249, 505–510.
- (2) Ellington, A., and Szostak, J. (1990) In vitro selection of RNA molecules that bind specific ligands. *Nature* 346, 818–822.
- (3) Gold, L. (1995) Oligonucleotides as research, diagnostic, and therapeutic agents. *J. Biol. Chem.* 270, 13581–13584.
- (4) Gold, L., Polisky, B., Uhlenbeck, O. C., and Yarus, M. (1995) Diversity of oligonucleotide functions. *Annu. Rev. Biochem.* 64, 763–797.
- (5) Lin, Y., Qiu, Q., Gill, S. C., and Jayasena, S. (1994) Modified RNA sequence pools for in vitro selection. *Nucleic Acids Res.* 22, 5229–5234.
- (6) Latham, J. A., Johnson, R., and Toole, J. J. (1994) The application of a modified nucleotide in aptamer selection: novel thrombin aptamers containing 5-(1-pentynyl)-2'-deoxyuridine. *Nucleic Acids Res.* 22, 2817–2822.
- (7) Jellinek, D., Green, L. S., Bell, C., Lynott, C. K., Gill, N., Vargeese, C., Kirschenheuter, G., McGee, D. P. C., Abesinghe, P., Pieken, W. A., Shapiro, R., Rifkin, D. B., Moscatelli, D., and Janjić, N. (1995) Potent 2'-amino-2'-deoxyuridine RNA inhibitors of basic fibroblast growth factor. *Biochemistry* 34, 11363–11372.
- (8) Pagratis, N. C., Bell, C., Chang, Y.-F., Jennings, S., Fitzwater, T., Jellinek, D., and Dang, C. (1997) Potent 2'-amino- and 2'-fluoro-2'-deoxyribonucleotide RNA inhibitors of keratinocyte growth factor. *Nat. Biotechnol.* 15, 68–73.
- (9) Green, L. S., Jellinek, D., Bell, C., Beebe, L. A., Feistner, B. D., Gill, S. C., Jucker, F. M., and Janjić, N. (1995) Nuclease-resistant nucleic acid ligands to vascular permeability factor/vascular endothelial growth factor. *Chem. Biol.* 2, 683–695.
- (10) Folkman, J. (1995) Angiogenesis in cancer, vascular and other disease. *Nat. Med.* 1, 27–31.
- (11) Conn, G., Soderman, D. D., Schaeffer, M.-T., Wile, M., Hatcher, V. B., and Thomas, K. A. (1990) Purification of a glycoprotein vascular endothelial cell mitogen from a rat glioma-derived cell line (GS-9L). *Proc. Natl. Acad. Sci. U.S.A.* 87, 1323–1327.
- (12) Gospodarowicz, D., Abraham, J. A., and Schilling, J. (1989) Isolation and characterization of a vascular endothelial cell mitogen produced by pituitary-derived folliculo stellate cells. *Proc. Natl. Acad. Sci. U.S.A.* 86, 7311–7315.
- (13) Ferrara, N., and Henzel, W. J. (1989) Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells. *Biochem. Biophys. Res. Commun.* 161, 851–858.
- (14) Dvorak, H. F., Orenstein, N. S., Carvalho, A. C., Churchill, W. H., Dvorak, A. M., Galli, S. J., Feder, J., Bitzer, A. M., Rypysc, J., and Giovenco, P. (1979) Induction of fibrin-gel investment: an early event in line 10 hepatocarcinoma growth mediated by tumor-secreted products. *J. Immunol.* 122, 166–174.
- (15) Senger, D. R., Galli, S. J., Dvorak, A. M., Peruzzi, C. A., Harvey, V. S., and Dvorak, H. F. (1983) Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. *Science* 219, 983–985.
- (16) Senger, D. R., Peruzzi, C. A., Feder, J., and Dvorak, H. F. (1986) A highly conserved vascular permeability factor secreted by a variety of human and rodent tumor cell lines. *Cancer Res.* 46, 5629–5632.
- (17) Dvorak, H. F., Brown, L. F., Detmar, M., and Dvorak, A. M. (1995) Vascular permeability factor/vascular endothelial growth factor, microvascular hyperpermeability, and angiogenesis. *Am. J. Pathol.* 146, 1029–1039.
- (18) Shweiki, D., Itin, A., Soffer, D., and Keshet, E. (1992) Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. *Nature* 359, 843–845.

- (19) Levy, A. P., Levy, N. S., and Goldberg, M. A. (1996) Post-transcriptional regulation of vascular endothelial growth factor by hypoxia. *J. Biol. Chem.* 271, 2746–2753.
- (20) Thomas, K. A. (1996) Vascular endothelial growth factor, a potent and selective angiogenic agent. *J. Biol. Chem.* 271, 603–606.
- (21) Brown, L. F., Detmar, M., Claffey, K., Nagy, J. A., Feng, D., Dvorak, A. M., and Dvorak, H. F. (1997) Vascular permeability factor/vascular endothelial growth factor: a multifunctional angiogenic cytokine. In *Regulation of Angiogenesis* (I. D. Goldberg and E. M. Rosen, Eds.) pp 233–269, Birkhauser, Basel, Switzerland.
- (22) Houck, K. A., Ferrara, N., Winer, J., Cachianes, G., Li, B., and Leung, D. W. (1991) The vascular endothelial growth factor family: identification of a fourth species and characterization of alternative splicing of RNA. *Mol. Endocrinol.* 5, 1806–1814.
- (23) Tischer, E., Mitchell, R., Hartman, T., Silva, M., Gospodarowicz, D., Fiddes, J. C., and Abraham, J. A. (1991) The human gene for vascular endothelial growth factor. Multiple protein forms are encoded through alternative exon splicing. *J. Biol. Chem.* 266, 11947–11954.
- (24) Millauer, B., Witzigmann-Voos, S., Schnurch, H., Martinez, R., Moller, N. P. H., Risau, W., and Ullrich, A. (1993) High affinity vascular endothelial growth factor binding and developmental expression suggest flk-1 as a major regulator of vasculogenesis and angiogenesis. *Cell* 72, 835–846.
- (25) Terman, B. I., Carrion, M. E., Kovacs, E., Rasmussen, B. A., Eddy, R. L., and Shows, T. B. (1991) Identification of a new endothelial cell growth factor receptor tyrosine kinase. *Oncogene* 6, 519–524.
- (26) de Vries, C., Escobendo, J. A., Ueno, H., Houck, K., Ferrara, N., and Williams, L. T. (1992) The fms-like tyrosine kinase, a receptor for vascular endothelial growth factor. *Science* 255, 989–991.
- (27) Mustonen, T., and Alitalo, K. (1995) Endothelial receptor tyrosine kinases involved in angiogenesis. *J. Cell Biol.* 129, 895–898.
- (28) Gill, S. C., Weitzel, S. E., and von Hippel, P. H. (1991) E. coli Sigma70 and NusA proteins. Binding interactions with core RNA polymerase in solution and within the transcription complex. *J. Mol. Biol.* 220, 307–324.
- (29) Miles, A. A., and Miles, E. M. (1952) Vascular reactions to histamine, histamine-liberator, and leukotaxine in the skin of guinea pigs. *J. Physiol. (London)* 118, 228–257.
- (30) Hope, M. J., Wong, K. F., and Cullis, P. R. (1989) Freeze-fracture of lipids and model membrane systems. *J. Electron Microsc. Tech.* 13, 277–287.
- (31) Jain, M. K., and Wagner, R. C. (1980) *Introduction to Biological Membranes*, p 63, Wiley, New York.
- (32) Auerbach, R., Auerbach, W., and Polakowski, I. (1991) Assays for angiogenesis: a review. *Pharmacol. Ther.* 51, 1–11.
- (33) Hicke, B. J., Watson, S. R., Koenig, A., Lynott, C. K., Bargatze, R. F., Chang, Y.-F., Ringquist, S., Moon-McDermott, L., Jennings, S., Fitzwater, T., Han, H.-L., Varki, N., Albinana, I., Willis, M. C., Varki, A., and Parma, D. (1996) DNA aptamers block L-selectin function in vivo. Inhibition of human lymphocyte trafficking in SCID mice. *J. Clin. Invest.* 98, 2688–2692.
- (34) Letsinger, R. L., Zhang, G., Sun, D. K., Ikeuchi, T., and Sarin, P. S. (1989) Cholesteryl-conjugated oligonucleotides: synthesis, properties, and activity as inhibitors of replication of human immunodeficiency virus in cell culture. *Proc. Natl. Acad. Sci. U.S.A.* 86, 6553–6556.
- (35) Saison-Behmoaras, T., Tocqué, B., Rey, I., Chassignol, M., Thuong, N. T., and Hélène, C. (1991) Short modified antisense oligonucleotides directed against Ha-ras point mutations induce selective cleavage of the mRNA and inhibit T24 cells proliferation. *EMBO J.* 10, 1111–1118.
- (36) Shea, R. G., Marsters, J. C., and Bischofberger, N. (1990) Synthesis, hybridization properties and antiviral activity of lipid-oligodeoxynucleotide conjugates. *Nucleic Acids Res.* 18, 3777–3783.
- (37) MacKellar, C., Graham, D., Will, D. W., Burgess, S., and Brown, T. (1992) Synthesis and physical properties of anti-HIV antisense oligonucleotides bearing terminal lipophilic groups. *Nucleic Acids Res.* 20, 3411–3417.
- (38) de Smidt, P. C., Le Doan, T., de Falco, S., and van Berkel, T. J. C. (1991) Association of antisense oligonucleotides with lipoproteins prolongs the plasma half-life and modifies the tissue distribution. *Nucleic Acids Res.* 19, 4695–4700.
- (39) Zalipsky, S., Puntambekar, B., Boulikas, P., Engbers, C. M., and Woodle, M. C. (1995) Peptide attachment to extremities of liposomal surface grafted PEG chains: preparation of the long-circulating form of laminin pentapeptide, YIGSR. *Bioconjugate Chem.* 6, 705–708.
- (40) DeFrees, S. A., Phillips, L., Guo, L., and Zalipsky, S. (1996) Sialyl Lewis x liposomes as a multivalent ligand and inhibitor of E-selectin mediated cellular adhesion. *J. Am. Chem. Soc.* 118, 6101–6104.
- (41) Allen, T. M., Brandeis, E., Hansen, C. B., Kao, G. Y., and Zalipsky, S. (1995) A new strategy for attachment to sterically stabilized liposomes resulting in efficient targeting to cancer cells. *Biochim. Biophys. Acta* 1237, 99–108.
- (42) Kirpotin, D., Park, J. W., Hong, K., Zalipsky, S., Li, W.-L., Carter, P., Benz, C. C., and Papahadjopoulos, D. (1997) Sterically stabilized anti-HER2 immunoliposomes: design and targeting to human breast cancer cells in vitro. *Biochemistry* 36, 66–75.
- (43) Spragg, D. D., Alford, D. R., Greferath, R., Larsen, C. E., Lee, K.-D., Gurtner, G. C., Cybulsky, M. I., Tosi, P. F., Nicolau, C., and Gimbrone, M. A. (1997) Immunotargeting of liposomes to activated vascular endothelial cells: a strategy for site-selective delivery in the cardiovascular system. *Proc. Natl. Acad. Sci. U.S.A.* 94, 8795–8800.

BC980002X

CORRECTIONS

Volume 9, Number 5, September/October 1998.

Michael C. Willis,* Brian Collins, Tong Zhang,
Louis S. Green, David P. Sebesta, Carol Bell,
Elizabeth Kellogg, Stanley C. Gill, Anna
Magallanez, Susan Knauer, Ray A. Bendele,
Parkash S. Gill, and Nebojša Janjić*

LIPOSOME-ANCHORED VASCULAR ENDOTHE- LIAL GROWTH FACTOR APTAMERS

Page 573. Brian Collins is affiliated with NeXstar
Pharmaceuticals and has the middle initial D.

BC980200O

S1043-1802(98)00200-6

Published on Web 08/22/1998

A Novel Ternary Ligand System for ^{99m}Tc -Labeling of Hydrazino Nicotinamide-Modified Biologically Active Molecules Using Imine-N-Containing Heterocycles as Coligands

Shuang Liu,* D. Scott Edwards,* and Anthony R. Harris

The DuPont Merck Pharmaceutical Company, Radiopharmaceuticals Division 331 Treble Cove Road, North Billerica, Massachusetts 01862. Received January 25, 1998; Revised Manuscript Received June 8, 1998

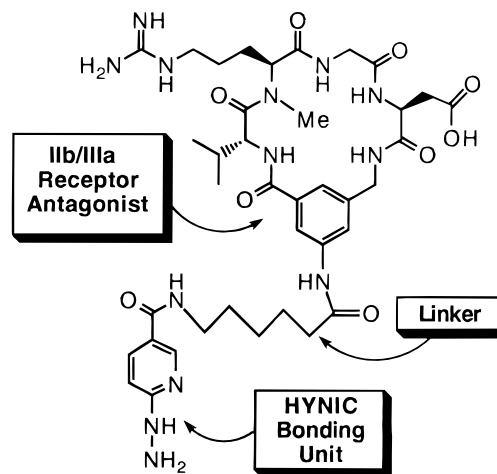
A hydrazinonicotinamide-functionalized cyclic platelet glycoprotein IIb/IIIa (GPIIb/IIIa) receptor antagonist [HYNICTide, cyclo(D-Val-NMeArg-Gly-Asp-Mamb(5-(6-(6-hydrazinonicotinamido)hexanamide)))] was labeled with ^{99m}Tc using tricine and a series of imine-N-containing heterocycles as coligands. The imine-N-containing heterocycles include *N*- ω -Acetylhistamine (HIS-AC), *N*-(2-hydroxyethyl)isonicotinamide (ISONIC-HE), isonicotinic acid (ISONIC), isonicotinoyl-L-aspartic acid dimethyl ester (ISONIC-L-Asp-OMe₂), 4-methyl-5-thiazoleethanol (MTE), nicotinic acid (NIC), 3-nitro-1,2,4-triazole (NTZ), 4-pyridylacetic acid (PA), 4-pyridineethanesulfonic acid (PES), and 3-pyridinesulfonic acid (PSA). The synthesis of these new ternary ligand [^{99m}Tc]HYNICTide complexes can be performed in one or two steps in high yield and high specific activity ($\geq 10\,000$ Ci/mmol HYNICTide). For example, the reaction of HYNICTide, [^{99m}Tc]pertechnetate, nicotinic acid, stannous chloride, and tricine at pH ~ 5 and 100 $^{\circ}\text{C}$ for 20 min results in the complex [^{99m}Tc (HYNICTide)(tricine)(NIC)] in $\geq 90\%$ yield as determined by radio-HPLC. It was found that ternary ligand technetium complexes, [^{99m}Tc (HYNICTide)(tricine)(L)] (L = ISONIC, ISONIC-L-Asp-OMe₂, ISONIC-HE, MTE, PA, PES, and PSA) are formed as equal mixtures of two isomeric forms. Complex [^{99m}Tc (HYNICTide)(tricine)(L)] (L = HIS-AC and NTZ) showed more than two well-resolved radiometric peaks at the retention times of interest, suggesting that they may have more than two forms in solution due to different bonding modalities of HIS-AC and NTZ. By a chirality experiment, it was found that the presence of two radiometric peaks is a result of the resolution of the two diastereomers which are formed by the combination of the chiral HYNICTide and the chiral technetium chelate. The formation of two diastereomers was also observed when a chiral imine-N-containing coligand was used for the radiolabeling of HYNIC-BA. The new ternary ligand [^{99m}Tc]HYNICTide complexes were found to be stable for up to 6 h in the reaction mixture. The high solution stability is attributed to their kinetic inertness. The composition of these complexes was determined to be 1:1:1:1 for Tc:HYNICTide:L:tricine (L = imine-N-containing heterocycles) through a series of mixed ligand experiments on the tracer (^{99m}Tc) level. The lipophilicity of the ternary ligand [^{99m}Tc]HYNICTide complexes can be systematically varied by the choice of polyaminocarboxylate and imine-N-containing coligands. Using the combination of tricine and an imine-N-containing coligand, HYNIC-derivatized peptides or other small molecules can be labeled with ^{99m}Tc in high specific activity and high stability for potential use as radiopharmaceuticals.

INTRODUCTION

Recently, Abrams and co-workers (1, 2) reported the use of organic hydrazines such as hydrazinonicotinamide (HYNIC) in labeling polyclonal IgG with ^{99m}Tc for imaging focal sites of infection. This technology has also been used for the ^{99m}Tc -labeling of chemotactic peptides (3–9), somatostatin analogues (10, 11), and antisense DNA oligonucleotides (12–14). Since the HYNIC group can only occupy one or two coordination sites in the technetium coordination sphere, a coligand such as glucoheptonate or tricine is required. It has been shown that the specific activity of the ^{99m}Tc -labeled polyclonal IgG using tricine as a coligand is much higher than that obtained using glucoheptonate (15, 16).

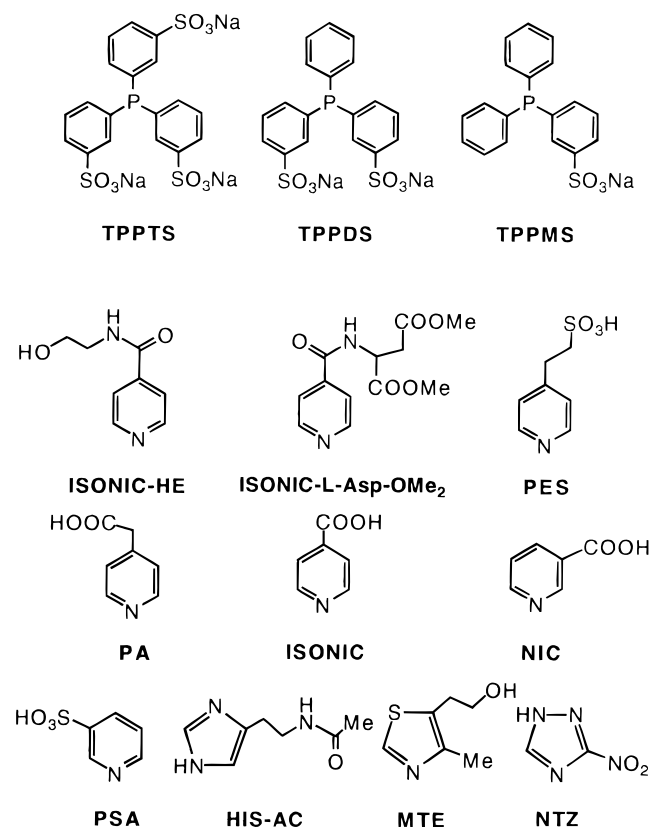
We have used tricine as the coligand in the ^{99m}Tc -labeling of a HYNIC-modified cyclic platelet glycoprotein IIb/IIIa (GPIIb/IIIa) antagonist (HYNICTide, Chart 1).

Chart 1. HYNICTide



* To whom correspondence should be addressed. Tel: 978-671-8696 (S.L.) and 978-671-8311 (D.S.E.). Fax: 978-436-7500.

Very high specific activity ($\geq 20\,000$ mCi/ μmol) can be achieved for the [^{99m}Tc]HYNICTide complex when tricine

Chart 2. Water-Soluble Phosphines and Imine-N-Containing Heterocyclic Coligands

is used as the coligand (17). However, the use of tricine as the coligand suffers two major disadvantages: the solution instability of the complex [^{99m}Tc(HYNICtide)-(tricine)₂], and the presence of many isomeric forms in solution. Similar phenomena were seen when glucoheptonate was used as a coligand in the ^{99m}Tc-labeling of a HYNIC-modified chemotactic peptide (18).

To minimize the number of isomeric forms and increase the solution stability of the [^{99m}Tc]HYNICtide complex, we have used water soluble phosphines (Chart 2: TPPTS, trisodium triphenylphosphine-3,3',3''-trisulfonate; TPPDS, disodium triphenylphosphine-3,3'-disulfonate; TPPMS, sodium triphenylphosphine-3-monosulfonate) as second coligands (19). We found that the combination of HYNICtide, tricine, and a monodentate phosphine produces a unique and versatile ternary ligand system which forms technetium complexes [^{99m}Tc(HYNICtide)-(tricine)(L)] (L = TPPTS, TPPDS, and TPPMS) with high solution stability and only two detectable isomeric forms. As a continuation of this study, we now present a new ternary ligand system, in which imine-N-containing heterocycles (Chart 2) are used as second coligands instead of water-soluble phosphines and its application in the ^{99m}Tc-labeling of a cyclic platelet GPIIb/IIIa HYNICtide. We describe the synthesis, HPLC characterization, and stability of new ternary ligand complexes, [^{99m}Tc(HYNICtide)(tricine)(L)] (L = HIS-AC, ISONIC, ISONIC-L-Asp-OMe₂, ISONIC-HE, MTE, NIC, NTZ, PA, PES, and PSA). This new ternary ligand system can also be used for the ^{99m}Tc-labeling of other HYNIC-conjugated biologically active small molecules.

EXPERIMENTAL SECTION

Materials. *N*-ω-Acetylhistamine (HIS-AC), *N*-(2-hydroxyethyl)isonicotinamide (ISONIC-HE), isonicotinic

acid (ISONIC), isonicotinoyl chloride hydrochloride, 4-methyl-5-thiazoleethanol (MTE), nicotinic acid (NIC), 3-nitro-1,2,4-triazole (NTZ), 4-pyridylacetic acid (PA), 4-pyridineethane-sulfonic acid (PES), 3-pyridinesulfonic acid (PSA), salicylaldehyde, tricine, 2-amino-2-(hydroxymethyl)-1,3-dihydroxypropane (tris), and trisodium triphenylphosphine-3,3',3''-trisulfonate (TPPTS) were purchased from Aldrich Chemical Co. TPPTS was purified according to the literature method (20) before use for radiolabeling. Succinimidyl 6-(2-sulfobenzaldehydehydrazono)nicotinate sodium salt was prepared according to the literature method (21). ¹H-NMR spectra were recorded on a 270 MHz Bruker spectrometer. The ¹H-NMR data were reported as δ (ppm) relative to TMS. FAB-MS analyses were performed using a Finnigan MAT 8230 mass spectrometer. Electrospray MS analyses were performed using a VG Quattro mass spectrometer. Na^{99m}TcO₄ was obtained from a Technelite ⁹⁹Mo/^{99m}Tc generator, DuPont Merck Pharmaceutical Co., North Billerica, MA. Deionized water was obtained from a Millipore MilliQ Water System and was of >18 MΩ quality. The synthesis and biological properties of the cyclic GPIIb/IIIa HYNICtide have been reported elsewhere (22).

Instruments and Methods. The high-performance liquid chromatography (HPLC) methods used a Hewlett-Packard Model 1050 instrument with radiometric detector using a sodium iodide probe. The instant thin-layer chromatography (ITLC) method used Gelman Sciences silica gel strips and a 1:1 mixture of acetone and saline as eluant.

HPLC method 1 used a Zorbax C₁₈ column (4.6 mm × 250 mm, 80 Å pore size) at a flow rate of 1 mL/min with a gradient mobile phase starting from 88% A (0.01 M phosphate buffer, pH 6) and 12% B (acetonitrile) to 85% A and 15% B at 30 min.

HPLC method 2 used a Zorbax C₁₈ column (4.6 mm × 250 mm, 80 Å pore size) at a flow rate of 1 mL/min with a gradient mobile phase starting from 88% A (0.01 M phosphate buffer, pH 6) and 12% B (acetonitrile) to 80% A and 20% B at 30 min.

HPLC method 3 used a Zorbax C₁₈ column (4.6 mm × 250 mm, 80 Å pore size) at a flow rate of 1 mL/min with a gradient mobile phase starting from 88% A (0.01 M phosphate buffer, pH 6) and 12% B (acetonitrile) to 86% A and 14% B at 20 min and to 80% A and 20% B at 30 min.

HPLC method 4 used a Hewlett-Packard model 1090 instrument, a Zorbax C₁₈ column (4.6 mm × 250 mm, 80 Å pore size) at a flow rate of 1 mL/min with a gradient mobile phase starting from 80% A (0.01 M phosphate buffer, pH 6) and 20% B (acetonitrile) to 60% A and 40% B at 30 min.

Synthesis of N-Benzyl-6-(2-sulfobenzaldehydehydrazono)nicotinamide Sodium Salt (HYNIC-BA).

To a solution of succinimidyl 6-(2-sulfobenzaldehydehydrazono)nicotinate sodium salt (1.0 g, 2.27 mmol) in dry DMF (20 mL) was added benzylamine (0.243 g, 2.27 mmol). The resulting mixture was stirred at room temperature for 24 h. Solvent was removed under reduced pressure, and the residue was triturated with methanol (3 mL) to give a pale-yellow solid. The solid was collected by filtration, washed with diethyl ether, and dried under vacuum. The yield was 0.9 g (92%). The title compound was recrystallized twice from methanol before use for radiolabeling. Electrospray MS (in negative mode): *m/z* 432.1 (M - 1, [C₂₀H₁₇N₄O₄NaS]⁺) and *m/z* 409.1 (M - Na, [C₂₀H₁₇N₄O₄S]⁻). ¹H NMR (DMSO-d₆): 4.48 (d, 2H, PhCH₂, *J* = 5.6 Hz); 7.20–7.41 (m, 8H,

Ph); 7.80 (dd, 1H, Py); 8.05 (d, 1H); 8.14 (dd, 1H, Py); 8.72 (d, 1H, $J = 2.0$ Hz, Py); 8.98 (t, 1H, amide NH); 9.04 (s, 1H, N=CH); and 11.40 (s, 1H, hydrazone NH).

Synthesis of *N*-((*R*)-(+)- α -Methylbenzyl)-6-(2-sulfobenzaldehyde hydrazone)nicotinamide Sodium Salt (HYNIC-MBA). To a solution of succinimidyl 6-(2-sulfobenzaldehydehydrazone)nicotinate sodium salt (1.0 g, 2.27 mmol) in dry DMF (10 mL) was added (*R*)-(+)- α -methylbenzylamine (0.35 g, 2.89 mmol). The resulting mixture was stirred at room temperature for 24 h. Removal of the solvent under reduced pressure gave an oily residue, which was redissolved in ethanol (5 mL). Trituration with diethyl ether gave a pale-yellow precipitate. The precipitate was collected by filtration, washed with diethyl ether, and dried in air. The yield was 0.95 g (94%). Recrystallization of the title compound from methanol gave an off-white powder. Electrospray MS (in negative mode): m/z 445.1 ($M - 1$, $[\text{C}_{21}\text{H}_{19}\text{N}_4\text{O}_4\text{NaS}]^-$) and m/z 423.1 ($M - \text{Na}$, $[\text{C}_{21}\text{H}_{19}\text{N}_4\text{O}_4\text{S}]^-$). ^1H NMR ($\text{DMSO}-d_6$): 1.48 (d, 3H, CH_3 , $J = 7.1$ Hz); 5.16 (m, 1H, PhCHMe); 7.20–7.41 (m, 8H, Ph); 7.76 (dd, 1H, Py); 8.05 (d, 1H, Ph); 8.10 (dd, 1H, Py); 8.69 (s, 1H, Py); 8.69 (t, 1H, amide NH); 9.01 (s, 1H, N=CH); and 11.38 (s, 1H, hydrazone NH).

Synthesis of Isonicotinoyl-L-aspartic Acid Dimethyl Ester (ISONIC-L-Asp-OMe₂). To a solution of L-aspartic acid dimethyl ester hydrochloride (2.0 g, 10 mmol) in acetonitrile (100 mL) were added isonicotinoyl chloride hydrochloride (1.8 g, 10 mmol) and triethylamine (3.0 mL, 21.5 mmol). The reaction mixture was stirred at room temperature for 24 h and was then refluxed for 2 h. The solution was filtered and the filtrate was evaporated under reduced pressure to give a whitish oil. This residue was extracted with diethyl ether (2×75 mL). The organic fractions were combined, and the solvent was removed to give the product as a whitish oil. Electrospray MS: m/z 267 ($M + 1$, $[\text{C}_{12}\text{H}_{14}\text{N}_2\text{O}_5]^+$). ^1H NMR (CDCl_3): 2.91–3.17 (m, 2H, CH_2), 3.69 (s, 3H, COOCH_3); 3.78 (s, 3H, COOCH_3); 4.99–5.05 (m, 1H, CH); 7.38–7.41 (d, 1H, NH); 7.62–7.65 (m, 2H, aromatic H) and 7.82–7.84 (m, 2H, aromatic H).

Synthesis of 2-Salicylimino-2-hydroxymethyl-1,3-propanediol (Saltris). To a solution of tris (2.4 g, 19 mmol) in hot methanol (30 mL) was added slowly salicylaldehyde (2.5 g, 20.5 mmol) in the same solvent (10 mL). The resulting mixture was refluxed for 30 min and was then cooled to room temperature. Upon addition of diethyl ether (50 mL), the reaction mixture was kept standing at room temperature for 2–3 h, during which time bright-yellow microcrystals were formed. The bright-yellow solid was collected by filtration, washed with diethyl ether, and dried in a vacuum. The yield was 4.0 g (~86%). The schiff base was used for further reaction without further purification.

Synthesis of 2-(2-Hydroxybenzyl)amino-2-hydroxymethyl-1,3-propanediol (Hbtris). Saltris (5.0 g, 21.6 mmol) was suspended in 50 mL of methanol. Potassium borohydride (KBH_4) was added in small portions until the yellow color totally disappeared. The reaction mixture was refluxed for an additional 10 min. The solvent was removed under reduced pressure. To the residue was added ammonium chloride (2 g) in 15 mL of water to give a milky solution, which became clear upon heating. The mixture was condensed on a rotary evaporator until a thick milky liquid was formed. Upon cooling in an ice-bath, the aqueous solution was discarded. To the residue was added 30 mL of absolute ethanol while a white solid formed. The solid was collected by filtration, washed with cold ethanol (5 mL) and diethyl ether

Table 1. Radio-HPLC Data for [^{99m}Tc]HYNICTide Complexes

complex	yield (%)	retention time (min)
$^{99m}\text{Tc}(\text{HYNICTide})(\text{tricine})(\text{NIC})$	90–95	13.2/14.8 ^a
$^{99m}\text{Tc}(\text{HYNICTide})(\text{tricine})(\text{ISONIC})$	85–90	11.6/12.8 ^a
$^{99m}\text{Tc}(\text{HYNICTide})(\text{tricine})(\text{ISONIC-L-Asp-OMe}_2)$	90–95	12.5/15.3 ^b
$^{99m}\text{Tc}(\text{HYNICTide})(\text{tricine})(\text{ISONIC-HE})$	90–95	26.0/27.0 ^a
$^{99m}\text{Tc}(\text{HYNICTide})(\text{tricine})(\text{PA})$	90–95	17.4/17.8 ^a
$^{99m}\text{Tc}(\text{HYNICTide})(\text{tricine})(\text{PA})$	85–90	15.6/16.5 ^a
$^{99m}\text{Tc}(\text{HYNICTide})(\text{tricine})(\text{PES})$	85–92	14.2/15.0 ^a
$^{99m}\text{Tc}(\text{HYNICTide})(\text{tricine})(\text{PSA})$	85–90	16.0/16.7 ^a
$^{99m}\text{Tc}(\text{HYNICTide})(\text{tricine})(\text{MTE})$	90–95	21.3/22.1 ^c
$^{99m}\text{Tc}(\text{HYNICTide})(\text{tricine})(\text{NTZ})$	~85	17.2/18.5 ^c
$^{99m}\text{Tc}(\text{HYNICTide})(\text{tricine})(\text{Ac-HIS})$	~90	14.2/15.5/16.0 ^c

^a HPLC method 1. ^b HPLC method 4. ^c HPLC method 2.

(10 mL), and dried in air. The product was purified by recrystallization from a mixture of water and methanol (50:50). The yield was 2.6 g (~52%). FAB-MS: m/z 228 ($M + 1$, $[\text{C}_{11}\text{H}_{18}\text{NO}_4]^+$). ^1H NMR (CD_3OD): 3.70 (s, 6H, CH_2); 3.80–4.20 (m, 2H, benzyl- CH_2); and 6.50–7.40 (m, 4H, aromatic hydrogens).

Synthesis of Ternary Ligand [^{99m}Tc]HYNICTide Complexes. Two-Step Synthesis. To a 10 mL vial was added 0.2–0.4 mL of the HYNICTide solution (50–100 $\mu\text{g/mL}$ in 25 mM succinate buffer, pH 5.0), 0.4 mL of tricine solution (100 mg/mL in 25 mM succinate buffer, pH 5.0), 0.2–0.5 mL of $^{99m}\text{TcO}_4^-$ solution (100–200 mCi/mL in saline), and 25 μL of $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$ solution (1.0 mg/mL in 0.1 N HCl). The reaction mixture was allowed to stand at room temperature for 30 min. The HPLC (method 1) showed radiochemical purity (RCP) of $\geq 95\%$ for the resulting complex [$^{99m}\text{Tc}(\text{HYNICTide})(\text{tricine})_2$]. To the solution was added 0.2 mL of the imine-N-containing coligand solution (10–50 mg/mL in 25 mM succinate buffer, pH 5.0). The reaction mixture was heated at 100 °C for 15–20 min. After cooling at room temperature for 10 min, an aliquot of the reaction mixture was analyzed by radio-HPLC and ITLC. The [^{99m}Tc]HYNICTide complex migrates while the [^{99m}Tc]colloid remains at the origin of the ITLC strip.

One-Step Synthesis. To a 10 mL vial was added 0.4 mL of tricine solution (50–100 mg/mL in 25 mM succinate buffer, pH 5.0), 0.2–0.4 mL of HYNICTide solution (50–100 $\mu\text{g/mL}$ in 25 mM succinate buffer, pH 5.0), 0.2 mL of coligand solution (10–50 mg/mL in 25 mM succinate buffer, pH 5.0), 0.2–0.5 mL of $^{99m}\text{TcO}_4^-$ solution (100–200 mCi/mL in saline), and 25 μL of $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$ solution (1.0 mg/mL in 0.1 N HCl). The reaction mixture was heated at 100 °C for 15–20 min. After cooling at room temperature for 10 min, the reaction mixture was analyzed by radio-HPLC and ITLC. The [^{99m}Tc]HYNICTide complexes prepared by this approach were shown by HPLC to be identical to those prepared by the two-step synthesis. The radiolabeling yields for [^{99m}Tc]HYNICTide complexes along with their HPLC data are summarized in Table 1.

Composition Studies of [^{99m}Tc]HYNICTide Complexes. Determination of the Number of HYNICTide Ligands. To a 10 mL vial was added 0.4 mL of tricine solution (100 mg/mL in 25 mM succinate buffer, pH 5.0), followed by 0.2 mL of PSA solution (30 mg/mL in 25 mM succinate buffer, pH 5.0), 0.4 mL of HYNICTide solution (50 $\mu\text{g/mL}$ in 25 mM succinate buffer, pH 5.0), 0.1 mL of *N*-(6-hydrazinonicotinyl)-(*R*)-(+)- α -methylbenzylamine solution (50 $\mu\text{g/mL}$ in 25 mM succinate buffer, pH 5.0), 0.2 mL of $^{99m}\text{TcO}_4^-$ solution (250 mCi/mL in saline), and 25

μL of $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$ solution (1.0 mg/mL in 0.1 N HCl). The reaction mixture was heated at 100 °C for 20 min, and was then analyzed by HPLC (method 1).

Determination of the Number of Heterocyclic Coligands. To a 10 mL vial was added 0.4 mL of HYNICTide solution (50 $\mu\text{g}/\text{mL}$ in 25 mM succinate buffer, pH 5.0), 0.4 mL of tricine solution (100 mg/mL in 25 mM succinate buffer, pH 5.0), 0.2 mL of PSA solution (25 mg/mL in 25 mM succinate buffer, pH 5.0), 0.2 mL of ISONIC-HE solution (25 mg/mL in 25 mM succinate buffer, pH 5.0), 0.2 mL of $^{99\text{m}}\text{TcO}_4^-$ solution (250 mCi/mL in saline), and 25 μL of $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$ solution (1.0 mg/mL in 0.1 N HCl). The reaction mixture was heated at 100 °C for 20 min, and was then analyzed by radio-HPLC (method 1).

Determination of the Number of Tricine Coligands. To a 10 mL vial was added 0.4 mL of HYNICTide solution (100 $\mu\text{g}/\text{mL}$ in 25 mM succinate buffer, pH 5.0), 20 μL of tricine solution (100 mg/mL in 25 mM succinate buffer, pH 5.0), 0.5 mL of Hbtris [10 mg/mL in a mixture of 50:50 (v:v) ethanol and 25 mM succinate buffer, pH 5.0], 0.2 mL of NIC solution (25 mg/mL in 25 mM succinate buffer, pH 5.0), 0.3 mL of $^{99\text{m}}\text{TcO}_4^-$ solution (~170 mCi/mL in saline), and 25 μL of $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$ solution (1.0 mg/mL in 0.1 N HCl). The reaction mixture was heated at 100 °C for 20 min. After cooling to room temperature, the reaction mixture was filtered using a Millex-GV 0.22 μm filter. The filtrate was analyzed by radio-HPLC (method 3).

Chirality Experiments. *Chiral Center on the HYNIC Ligand.* To a 10 mL vial was added 0.4 mL of tricine solution (100 mg/mL in 25 mM succinate buffer, pH 5.0), followed by 0.2 mL of PSA solution (30 mg/mL in 25 mM succinate buffer, pH 5.0), 0.2 mL of HYNIC-BA solution (100 $\mu\text{g}/\text{mL}$ in 25 mM succinate buffer, pH 5.0), 0.2 mL of HYNIC-MBA solution (100 $\mu\text{g}/\text{mL}$ in 25 mM succinate buffer, pH 5.0), 0.2 mL of $^{99\text{m}}\text{TcO}_4^-$ solution (250 mCi/mL in saline), and 25 μL of $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$ solution (1.0 mg/mL in 0.1 N HCl). The reaction mixture was heated at 100 °C for 20 min, and was then analyzed by HPLC (method 1).

Chiral Center on the Imine-N-Containing Coligand. To a 10 mL vial was added 0.4 mL of tricine solution (100 mg/mL in 25 mM succinate buffer, pH 5.0), followed by 0.2 mL of HYNIC-BA solution (100 $\mu\text{g}/\text{mL}$ in 25 mM succinate buffer, pH 5.0), 0.1 mL of ISONIC-HE solution (40 mg/mL in 25 mM succinate buffer, pH 5.0), 0.2 mL of ISONIC-L-Asp-OMe₂ solution (10 mg/mL in 50% EtOH), 0.2 mL of $^{99\text{m}}\text{TcO}_4^-$ solution (250 mCi/mL in saline), and 25 μL of $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$ solution (1.0 mg/mL in 0.1 N HCl). The reaction mixture was heated at 100 °C for 10 min, and was then analyzed by HPLC (method 4).

Solution Stability Studies. *Solution Stability of [$^{99\text{m}}\text{Tc}$]HYNICTide Complexes in Kit Matrix.* [$^{99\text{m}}\text{Tc}$]HYNICTide complexes in the kit matrix were analyzed by HPLC (method 1 or method 2) without dilution. Aliquots of the reaction mixture were placed in six autosampler vials and analyzed sequentially over 6 h.

Solution Stability of Complexes [$^{99\text{m}}\text{Tc}$ (HYNICTide)-(tricine)(L)] (L = ISONIC and ISONIC-HE) in the Presence of Cysteine in Kit Matrix. Complexes [$^{99\text{m}}\text{Tc}$ (HYNICTide)(tricine)(L)] (L = ISONIC and ISONIC-HE) were synthesized using the procedure above. To the reaction mixture was added cysteine in succinate buffer (pH 5.0) to give a concentration of 1 mg/mL. The resulting solutions were kept at room temperature and were analyzed by radio-HPLC (method 1) with four injections over 6 h.

Solution Stability of HPLC Purified [$^{99\text{m}}\text{Tc}$]HYNICTide Complexes. The [$^{99\text{m}}\text{Tc}$]HYNICTide complexes were pre-

pared first and were then purified by HPLC using either method 1 or method 2. The collected fractions were evaporated to dryness under reduced pressure, and the residues were diluted to 10 mCi/mL with deionized water. Aliquots of the diluted solution were placed in six autosampler vials and analyzed sequentially over 6 h.

Solution Stability of HPLC Purified [$^{99\text{m}}\text{Tc}$]HYNICTide Complexes in the Presence of Excess TPPTS. The complex [$^{99\text{m}}\text{Tc}$ (HYNICTide)(tricine)(PSA)] was prepared, and purified by HPLC using method 1. Volatiles from the collected fraction were removed under reduced pressure. The residue was diluted to 5 mCi/mL with a TPPTS (1.0 mg/mL) solution in water. The resulting solutions were kept at room temperature, and were analyzed at $t = 0, 1, 2,$ and 3 h by radio-HPLC.

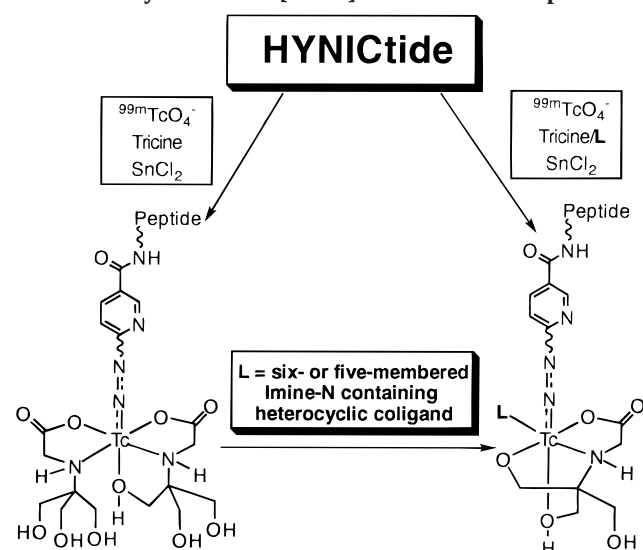
Solution Stability of the Two Isomeric Forms of the Complex [$^{99\text{m}}\text{Tc}$ (HYNICTide)(tricine)(NIC)]. The complex was prepared, and the two radiometric peaks were collected into two separate flasks. After removal of solvents, NIC solution in phosphate buffer (pH 6) was added to each flask to give a concentration of 1 mg/mL for NIC and 3–4 mCi/mL for the complex [$^{99\text{m}}\text{Tc}$ (HYNICTide)(tricine)(NIC)]. These two solutions were kept at room temperature and monitored by HPLC (method 1).

RESULTS AND DISCUSSION

There is a great current interest in the development of receptor-based target specific radiopharmaceuticals by the $^{99\text{m}}\text{Tc}$ -labeling of biologically active molecules such as antibodies (1, 2) and small peptides. Peptides used for $^{99\text{m}}\text{Tc}$ -labeling include chemotactic peptides (3–9, 18) and tuftsin receptor antagonists (22–24) for imaging focal infection or inflammation, somatostatin analogues for imaging tumors (10, 11, 26–30), and platelet GPIIb/IIIa receptor antagonists for imaging thrombosis (31–38). The $^{99\text{m}}\text{Tc}$ -labeling of highly potent small peptides (39), and the pharmacokinetics of $^{99\text{m}}\text{Tc}$ -labeled small peptides (40) have been reviewed recently.

Small peptides are of interest because of their favorable metabolic and pharmacokinetic characteristics as compared to antibodies and their fragments. Peptides are necessary elements in many fundamental biological processes, and in some cases, the affinities of small peptides for their receptors are significantly greater than that of antibodies or their fragments. Small peptides are easy to synthesize and modify, less likely to be immunogenic, and can have rapid blood clearance. The faster blood clearance results in adequate T/B ratios earlier so that it is practical to use $^{99\text{m}}\text{Tc}$, which is the preferred radionuclide for diagnostic nuclear medicine.

To label a small peptide with $^{99\text{m}}\text{Tc}$, a bifunctional coupling agent (BFCA) is often needed. The BFCA can be attached to the C-terminus or N-terminus via a linker, if needed. It can also be attached to the side-chain of the peptide or incorporated in the peptide backbone (31–34) provided that incorporation of the BFCA does not significantly affect the receptor-binding affinity of the peptide. The radiolabeling can be achieved either by direct reduction of $^{99\text{m}}\text{TcO}_4^-$ in the presence of a BFCA-peptide conjugate or by ligand exchange with an intermediate $^{99\text{m}}\text{Tc}$ complex such as [$^{99\text{m}}\text{Tc}$]glucoheptonate. For very potent small peptides with IC₅₀ values in the nanomolar range, the $^{99\text{m}}\text{Tc}$ -labeling must be accomplished with high specific activity. Therefore, the choice of a BFCA is very important for the success of the radiolabeling. As a matter of fact, one of the challenges for the development of peptide-based radiopharmaceuticals is to design new ligand systems that have very high

Chart 3. Synthesis of [^{99m}Tc]HYNICTide Complexes

labeling efficiency (rapid labeling and high yield) and form technetium complexes with high solution stability and less isomerism.

Various chelators have been used as BFCAs in labeling proteins and biologically active small molecules with ^{99m}Tc and ^{186}Re . These include DTPA (41), N_2S triamidedithiols (42–44), N_2S_2 diamidedithiols (44–47), N_2S_2 monoamidemonoaminedithiols (48), N_2S_2 diaminedithiols (49–54), BATOs (55, 56), PnAO (26), tetraamines (27), and HYNIC (1–18). Among these BFCAs, HYNIC is of particular interest due to its very high labeling efficiency. Also, the use of various coligands with HYNIC allows the hydrophilicity of the technetium complex to be modified in order to improve the pharmacokinetics of the ^{99m}Tc -labeled peptide.

Recently, we reported the use of tricine and water soluble phosphines (Chart 2: TPPTS, TPPDS, and TPPMS) as the coligands to label a cyclic GPIIb/IIIa HYNICTide with ^{99m}Tc (19). The combination of HYNICTide, tricine, and a monodentate phosphine (TPPTS, TPPDS, and TPPMS) produces a unique and versatile ternary ligand system that forms [^{99m}Tc]HYNICTide complexes with high solution stability and only two detectable isomeric forms. It is amazing that the use of a simple monodentate phosphine ligand dramatically reduces the number of isomeric forms from more than 10 for [^{99m}Tc](HYNICTide)(tricine) $_2$ to only two for [^{99m}Tc](HYNICTide)(tricine)(TPPTS)] and significantly increases the solution stability of the [^{99m}Tc]HYNICTide complex at the same time.

Phosphine-P is known as a soft donor, and binds strongly to metal ions in their lower oxidation state, such as Tc(III). When bonded to the Tc, it uses its 3p electron lone-pair to form a Tc–P σ -bond. The empty 3d orbitals can be used to accept the electrons from 4d orbitals of the Tc and form $d\pi$ – $d\pi$ back-bonding. This suggests that monodentate ligands with other soft donors such as imine-N may also be used as the second coligands to stabilize [^{99m}Tc]HYNICTide complexes. We now report the discovery of a new ternary ligand system, in which five- or six-membered imine-N-containing heterocycles, such as thiazole and pyridine derivatives (Chart 2), are used as the second coligands in the ^{99m}Tc -labeling of a GPIIb/IIIa receptor antagonist, HYNICTide (Chart 1).

Synthesis of [^{99m}Tc]HYNICTide Complexes. Synthesis (Chart 3) of ternary ligand [^{99m}Tc]HYNICTide complexes can be achieved either in two steps, in which

the complex [^{99m}Tc](HYNICTide)(tricine) $_2$ is formed first and then reacts with a imine-N-containing coligand, or in one step by direct reduction of [^{99m}Tc]pertechnetate with stannous chloride in the presence of HYNICTide, tricine, and an imine-N-containing coligand. For example, the reaction of HYNICTide, [^{99m}Tc]pertechnetate, nicotinic acid, stannous chloride, and tricine at pH $\sim$ 5 and 100 $^\circ\text{C}$ for 20 min results in the complex [^{99m}Tc](HYNICTide)(tricine)(NIC)] in $\geq$ 90% yield as determined by radio-HPLC. Successful radiolabeling at lower temperatures (50–80 $^\circ\text{C}$) requires longer heating time ($>$ 30 min). The tricine concentration can range from 20 to 60 mg/mL. Using lower tricine concentration ($<$ 20 mg/mL) may result in the formation of a significant amount of [^{99m}Tc]colloid. For pyridine analogues, the coligand concentration is 5–10 mg/mL while the concentration of imidazole, thiazole or triazole analogues is 1–5 mg/mL. Low concentration ($<$ 1.0 mg/mL) of imine-N-containing coligand may result in either incomplete conversion of [^{99m}Tc](HYNICTide)(tricine) $_2$ to the corresponding ternary ligand [^{99m}Tc]HYNICTide complexes in the two-step synthesis or the formation of [^{99m}Tc](HYNICTide)(tricine) $_2$ as a radioimpurity in the one-step synthesis. The concentration of the HYNICTide can range from 5 to 50 $\mu\text{g/mL}$. When pyridine derivatives, particularly those with strong electron-withdrawing groups, are used as coligands, the higher HYNICTide concentration may result in formation of the bis-HYNICTide complex since HYNICTide itself contains a pyridine-N donor and a hydrazine group. The labeling yields (Table 1) are usually 85–95% for complex [^{99m}Tc](HYNICTide)(tricine)(L)] (L = ISONIC, ISONIC-L-Asp-OMe $_2$, ISONIC-HE, MTE, PA, PES, and PSA) as determined by HPLC. Very high specific activity ($\geq$ 10 000 mCi/ μmol HYNICTide) can be achieved without post labeling purification. Therefore, the procedure described in this report for the preparation of ternary ligand [^{99m}Tc]HYNICTide complexes is amenable for clinical application.

HPLC Characterization. The HPLC methods for the characterization of these new ternary ligand [^{99m}Tc]HYNICTide complexes are very similar to those used for the complex [^{99m}Tc](HYNICTide)(tricine)(TPPTS)] (19). Like the phosphine coligands such as TPPTS, imine-N-containing heterocycles react with [^{99m}Tc](HYNICTide)(tricine) $_2$ and form a ternary ligand complex [^{99m}Tc](HYNICTide)(tricine)(L)] (L = ISONIC, ISONIC-L-Asp-OMe $_2$, ISONIC-HE, MTE, PA, PES, and PSA), which show only two radiometric peaks in their HPLC chromatograms. Table 1 summarizes the radio-HPLC data for these ternary ligand [^{99m}Tc]HYNICTide complexes. Under ideal conditions, most of them show two well-resolved peaks with the area ratio being approximately 1:1. Figure 1 shows two representative HPLC chromatograms for [^{99m}Tc](HYNICTide)(tricine)(ISONIC)] (bottom) and [^{99m}Tc](HYNICTide)(tricine)(PES)] (top). Chromatograms of complex [^{99m}Tc](HYNICTide)(tricine)(L)] (L = HIS-AC and NTZ) showed more than two well-resolved radiometric peaks (Figure 2) at the retention times of interest, suggesting that these complexes may have more than two forms in solution, probably due to different bonding modalities of the substituted imidazole and 1,2,4-triazole coligands. It should be noted that the retention time and separation of the two radiometric peaks for complexes [^{99m}Tc](HYNICTide)(tricine)(L)] (L = ISONIC, ISONIC-L-Asp-OMe $_2$, ISONIC-HE, MTE, PA, PES, and PSA) are very much dependent on the model of HPLC, column type, column age, and HPLC method used for the characterization of these [^{99m}Tc]HYNICTide complexes.

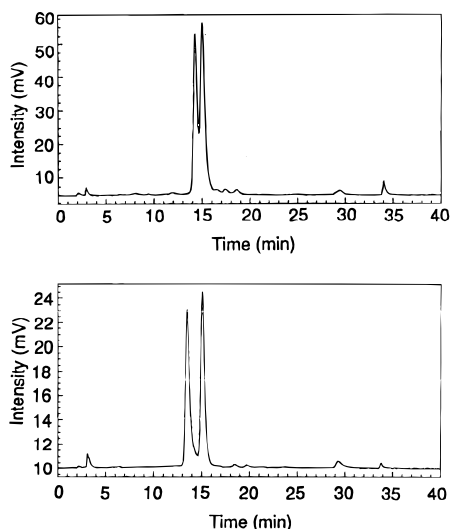


Figure 1. Radio-HPLC chromatograms (method 1) of ternary ligand technetium complexes: $[^{99m}\text{Tc}(\text{HYNICTide})(\text{tricine})(\text{ISONIC})]$ (top) and $[^{99m}\text{Tc}(\text{HYNICTide})(\text{tricine})(\text{PES})]$ (bottom).

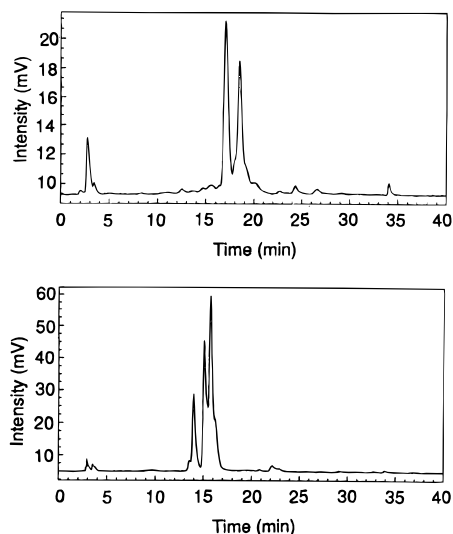


Figure 2. Radio-HPLC (method 2) chromatograms of ternary ligand technetium complexes: $[^{99m}\text{Tc}(\text{HYNICTide})(\text{tricine})(\text{NTE})]$ (top) and $[^{99m}\text{Tc}(\text{HYNICTide})(\text{tricine})(\text{HIS-AC})]$ (bottom).

Composition Studies of $[^{99m}\text{Tc}]\text{HYNICTide}$ Complexes. A mixed ligand experiment was performed to determine the number of HYNICTides bonded to the Tc. In this experiment, the HYNICTide and a model compound, N-(6-hydrazinonicotiny)-(R)-(+)- α -methylbenzylamine (HYNIC-MBA), were used in the same reaction mixture. After the radiolabeling, the reaction mixture was analyzed by radio-HPLC (method 1). If only one HYNICTide is bonded to the Tc, the chromatogram is expected to show two sets of peaks, one from $[^{99m}\text{Tc}(\text{HYNICTide})(\text{tricine})(\text{PSA})]$ and the other from $[^{99m}\text{Tc}(\text{HYNIC-MBA})(\text{tricine})(\text{PSA})]$. If there were two HYNIC-containing ligands in each $[^{99m}\text{Tc}]\text{HYNICTide}$ complex, a third set of peaks from the mixed ligand complex $[^{99m}\text{Tc}(\text{HYNICTide})(\text{HYNIC-MBA})(\text{tricine})(\text{PSA})]$ would be expected. The presence of only two pairs of peaks in the radio-HPLC chromatogram (Figure 3) demonstrates clearly that there is only one HYNICTide bonded to the Tc in $[^{99m}\text{Tc}(\text{HYNICTide})(\text{tricine})(\text{PSA})]$, and by inference in the other $[^{99m}\text{Tc}]\text{HYNICTide}$ complexes with tricine and an imine-N-containing heterocycle as coligands.

The number of PSA coligands in $[^{99m}\text{Tc}(\text{HYNICTide})(\text{tricine})(\text{PSA})]$ was determined in a fashion similar to

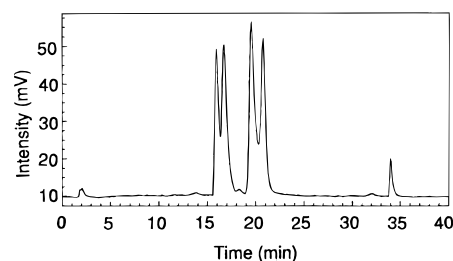


Figure 3. Radio-HPLC chromatogram (method 1) for the mixed HYNIC-ligand experiment. The peaks at 16.2 and 17.0 min are due to the complex $[^{99m}\text{Tc}(\text{HYNICTide})(\text{tricine})(\text{PSA})]$, and the peaks at 19.6 and 20.8 min are due to the complex $[^{99m}\text{Tc}(\text{HYNIC-MBA})(\text{tricine})(\text{PSA})]$.

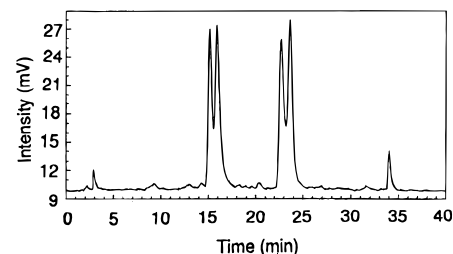


Figure 4. Radio-HPLC chromatogram (method 1) for the mixed ligand experiment. The peaks at 16.2 and 17.0 min are due to the complex $[^{99m}\text{Tc}(\text{HYNICTide})(\text{tricine})(\text{PSA})]$, and the peaks at 22.5 and 23.8 min are due to the complex $[^{99m}\text{Tc}(\text{HYNICTide})(\text{tricine})(\text{ISONIC-HE})]$.

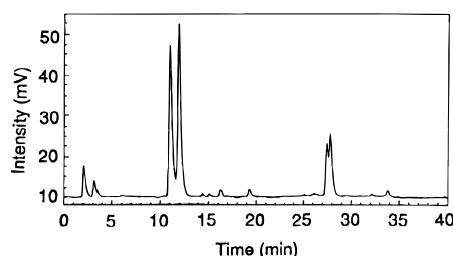


Figure 5. Radio-HPLC chromatogram (method 1) for the mixed ligand experiment. The peaks at 11.2 and 12.0 min are due to the complex $[^{99m}\text{Tc}(\text{HYNICTide})(\text{tricine})(\text{NIC})]$, and the peaks at 27.6 and 28.1 min are due to the complex $[^{99m}\text{Tc}(\text{HYNICTide})(\text{Hbtris})(\text{NIC})]$.

that for the HYNICTide using PSA and ISONIC-HE as competing coligands. Figure 4 shows the radio-HPLC chromatogram of the resulting reaction mixture. The presence of two sets of distinctive peaks at ~ 16.0 and ~ 23.5 min for $[^{99m}\text{Tc}(\text{HYNICTide})(\text{tricine})(\text{PSA})]$ and $[^{99m}\text{Tc}(\text{HYNICTide})(\text{tricine})(\text{ISONIC-HE})]$, respectively, demonstrates clearly that there is only one PSA coligand in the complex $[^{99m}\text{Tc}(\text{HYNICTide})(\text{tricine})(\text{PSA})]$, one ISONIC-HE coligand in the complex $[^{99m}\text{Tc}(\text{HYNICTide})(\text{tricine})(\text{ISONIC-HE})]$, and by inference, one imine-N-containing coligand in other $[^{99m}\text{Tc}]\text{HYNICTide}$ complexes.

The number of tricine coligands in $[^{99m}\text{Tc}(\text{HYNICTide})(\text{tricine})(\text{PSA})]$ was determined similarly using tricine and Hbtris as competing coligands. Hbtris was prepared by KBH_4 reduction of Saltris, which was synthesized from the reaction of salicylaldehyde with tris in methanol. Hbtris has been characterized by ^1H NMR and mass spectroscopy. We used a 2:1 molar ratio of Hbtris to tricine in the reaction mixture because of the low labeling efficiency of Hbtris as compared to tricine. Figure 5 shows the radio-HPLC chromatogram (method 3) of the resulting reaction mixture. The peaks at 11.2 and 12.0 min are due to $[^{99m}\text{Tc}(\text{HYNICTide})(\text{tricine})(\text{NIC})]$ and the

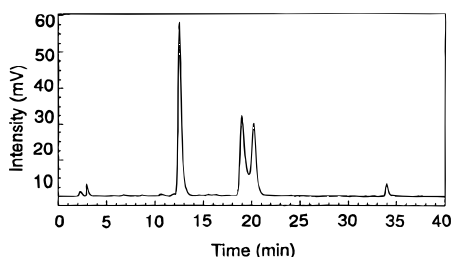


Figure 6. Radio-HPLC chromatogram (method 1) for the chirality experiment. The single peak at 12.5 min is due to the complex $^{99m}\text{Tc}(\text{HYNIC-BA})(\text{tricine})(\text{PSA})$, and the peaks at 19.8 and 20.4 min are due to the complex $^{99m}\text{Tc}(\text{HYNIC-MBA})(\text{tricine})(\text{PSA})$.

peaks at 27.6 and 28.1 min are from $^{99m}\text{Tc}(\text{HYNICTide})(\text{Hbtris})(\text{NIC})$. This clearly demonstrates that there is only one tricine coligand in the complex $^{99m}\text{Tc}(\text{HYNICTide})(\text{tricine})(\text{NIC})$, one Hbtris coligand in the complex $^{99m}\text{Tc}(\text{HYNICTide})(\text{Hbtris})(\text{NIC})$, and by inference, one tricine or Hbtris in the other ternary ligand ^{99m}Tc -HYNICTide complexes.

On the basis of these data, we conclude that these ternary ligand technetium complexes contain one HYNICTide, one tricine, and one imine-N-containing heterocyclic coligand.

Chirality Experiment. For quite a long time, we have been puzzled by the presence of two radiometric peaks in the radio-HPLC chromatograms of complex $^{99m}\text{Tc}(\text{HYNICTide})(\text{tricine})(\text{L})$ ($\text{L} = \text{ISONIC}$, $\text{ISONIC-L-Asp-OMe}_2$, ISONIC-HE , MTE , PA , PES , and PSA). The ratio of these two forms is always close to 1:1. Several possible explanations can be envisaged for the existence of two isomeric forms of these complexes, including the resolution of diastereomers resulting from the chiral centers on both the cyclic peptide backbone and the technetium chelate and different geometric orientations of the diazenido ligand due to restricted rotation around the $\text{Tc}=\text{N}$ and $\text{N}=\text{N}$ bonds.

To understand the presence of these two isomeric forms, a chirality experiment was designed. In this experiment, two closely related HYNIC derivatives, *N*-benzyl-6-(2-sulfobenzaldehydehydrazono)nicotinamide (HYNIC-BA) and *N*-((*R*)-(+)- α -methylbenzyl)-6-(2-sulfobenzaldehydehydrazono)nicotinamide (HYNIC-MBA), were used in the same reaction mixture and PSA was used as coligand. HYNIC-MBA contains a chiral center, and HYNIC-BA has no chiral center. They were prepared by reacting the corresponding free amine with succinimidyl 6-(2-sulfobenzaldehydehydrazono)nicotinate sodium salt in DMF. The ternary ligand complexes $^{99m}\text{Tc}(\text{HYNIC-BA})(\text{tricine})(\text{PSA})$ and $^{99m}\text{Tc}(\text{HYNIC-MBA})(\text{tricine})(\text{PSA})$ were synthesized by reacting ^{99m}Tc pertechnetate, tricine, PSA, HYNIC-BA and HYNIC-MBA in the presence of stannous chloride at 100 °C for 20 min. The resulting solution was analyzed by HPLC (method 1).

If the existence of two isomeric forms were due to different geometric orientations of the substituted pyridine ring in the HYNICTide because of restricted rotation around the $\text{Tc}=\text{N}$ and $\text{N}=\text{N}$ bonds, there would be two pairs of peaks in the radio-HPLC chromatogram: one from $^{99m}\text{Tc}(\text{HYNIC-BA})(\text{tricine})(\text{PSA})$ and the other from $^{99m}\text{Tc}(\text{HYNIC-MBA})(\text{tricine})(\text{PSA})$. On the contrary, the radio-HPLC chromatogram (Figure 6) of the reaction mixture shows a single peak at 12.5 min due to the complex $^{99m}\text{Tc}(\text{HYNIC-BA})(\text{tricine})(\text{PSA})$, and a pair of peaks at 19.8 and 20.4 min due to the complex $^{99m}\text{Tc}(\text{HYNIC-MBA})(\text{tricine})(\text{PSA})$. These data provide strong

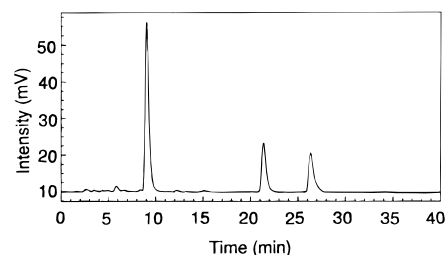


Figure 7. Radio-HPLC chromatogram (method 4) for the chirality experiment. The single peak at 11.9 min is due to $^{99m}\text{Tc}(\text{HYNIC-BA})(\text{tricine})(\text{ISONIC-HE})$, and the doublet at 21.5 and 26.5 min is from the complex $^{99m}\text{Tc}(\text{HYNIC-BA})(\text{tricine})(\text{ISONIC-Asp-OMe}_2)$.

evidence that the isomerism seen in these ternary ligand ^{99m}Tc -HYNICTide complexes is due to the formation of two diastereomers.

In another chirality experiment, HYNIC-BA was used as the ligand and the two pyridine analogues (ISONIC-HE without a chiral center and $\text{ISONIC-L-Asp-OMe}_2$ with a chiral center) were used as coligands. Synthesis of $\text{ISONIC-L-Asp-OMe}_2$ was carried out by reacting isonicotiny chloride hydrochloride with aspartic acid dimethyl ester hydrochloride in acetonitrile in the presence of excess triethylamine. $\text{ISONIC-L-Asp-OMe}_2$ was isolated as a whitish oil and has been characterized by $^1\text{H-NMR}$ and electrospray mass spectroscopy. The ternary ligand complexes $^{99m}\text{Tc}(\text{HYNIC-BA})(\text{tricine})(\text{ISONIC-HE})$ and $^{99m}\text{Tc}(\text{HYNIC-BA})(\text{tricine})(\text{ISONIC-L-Asp-OMe}_2)$ were prepared by reacting ^{99m}Tc pertechnetate, tricine, HYNIC-BA, ISONIC-HE , and $\text{ISONIC-L-Asp-OMe}_2$ in the presence of stannous chloride at 100 °C for 10 min. The resulting solution was analyzed by HPLC (method 4). The radio-HPLC chromatogram (Figure 7) shows a single peak at 9.0 min due to the complex $^{99m}\text{Tc}(\text{HYNIC-BA})(\text{tricine})(\text{ISONIC-HE})$ and a pair of peaks at 21.4 and 26.4 min due to the complex $^{99m}\text{Tc}(\text{HYNIC-MBA})(\text{tricine})(\text{ISONIC-L-Asp-OMe}_2)$. The area ratio of these two peaks is always close to 1:1, suggesting that they are indeed a result of resolution of the two diastereomers of the complex $^{99m}\text{Tc}(\text{HYNIC-MBA})(\text{tricine})(\text{ISONIC-L-Asp-OMe}_2)$.

The technetium chelate moiety is chiral, due to the tricine ligand being pro-chiral, and should be formed in an equal mixture of *D* and *L* enantiomers. These enantiomers in combination with the chiral centers on cyclic peptide backbone of HYNICTide or on the imine N-containing coligand result in the formation of diastereomers. If there is no chiral center on the imine N-containing coligand or the HYNIC-conjugated small molecules, the corresponding ternary ligand ^{99m}Tc -HYNICTide complex is expected to show only one radiometric peak unless a chiral chromatographic method is employed.

Solution Stability of ^{99m}Tc -HYNICTide Complexes. We examined the solution stability of four ^{99m}Tc -HYNICTide complexes, $^{99m}\text{Tc}(\text{HYNICTide})(\text{tricine})(\text{L})$ ($\text{L} = \text{ISONIC}$, ISONIC-HE , PES , and PSA), in the kit matrix. The stability was assessed by radio-HPLC (method 1 or 2) by performing six injections over 6 h. Figure 8 shows the plot of RCP (radiochemical purity) vs time for these four ^{99m}Tc -HYNICTide complexes. In all the cases, there is no significant RCP drop over 6 h within the experimental error. It is clear that the ternary ligand complex $^{99m}\text{Tc}(\text{HYNICTide})(\text{tricine})(\text{L})$ ($\text{L} = \text{ISONIC}$, ISONIC-HE , PES , and PSA) is stable for ≥ 6 h in solution. It is amazing that three different ligands (HYNICTide, tricine, and imine-N-containing heterocycle)

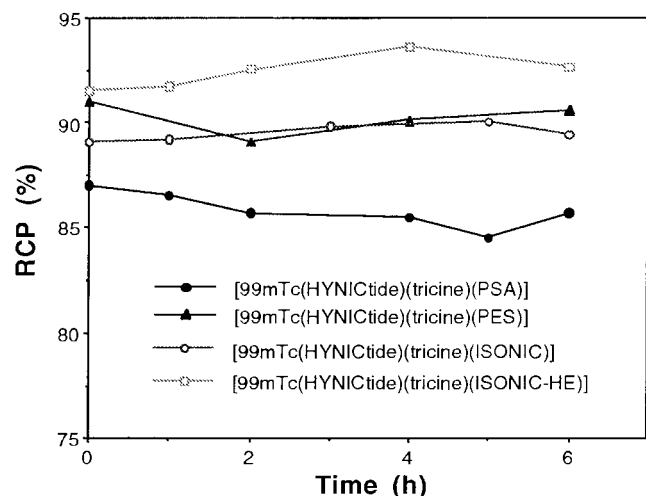


Figure 8. Plot of RCP (%) versus time (h) for $[^{99m}\text{Tc}]\text{HYNICTide}$ complexes in the kit matrix.

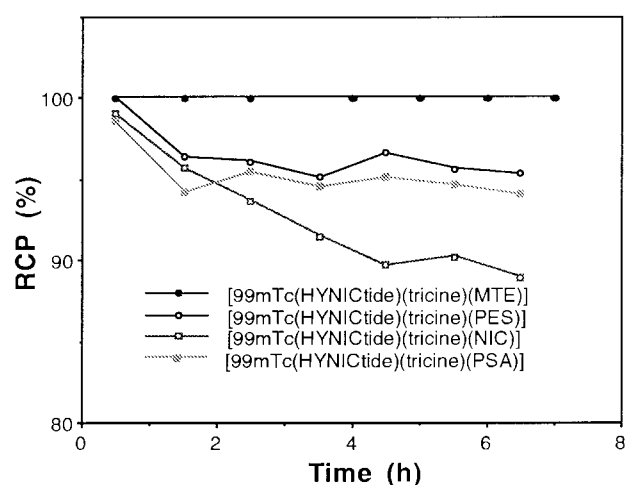


Figure 9. Plot of RCP (%) versus time (h) for $[^{99m}\text{Tc}]\text{HYNICTide}$ complexes in the presence of cysteine in the kit matrix.

combine with Tc and form a technetium complex with only two detectable isomers and with high solution stability.

Since cysteine is a native chelating ligand in biological systems, we decided to study the solution stability of two $[^{99m}\text{Tc}]\text{HYNICTide}$ complexes, $[^{99m}\text{Tc}(\text{HYNICTide})(\text{tricine})(\text{L})]$ (L = ISONIC and ISONIC-HE), in the presence of cysteine in the kit matrix. The cysteine challenging experiment is rather aggressive because of the high affinity of technetium in its intermediate or lower oxidation state for thiol ligands. In this experiment, the two $[^{99m}\text{Tc}]\text{HYNICTide}$ complexes were prepared first and then cysteine was added to the resulting reaction mixture to give a concentration of 1 mg/mL. The resulting solutions were kept at room temperature and monitored by radio-HPLC (method 1). Figure 9 shows the plot of RCP of the two $[^{99m}\text{Tc}]\text{HYNICTide}$ complexes in the presence of cysteine. It is quite clear that both complexes remain stable for ≥ 6 h in the kit matrix in the presence of cysteine.

We also examined the stability of four HPLC-purified ternary ligand complexes, $[^{99m}\text{Tc}(\text{HYNICTide})(\text{tricine})(\text{L})]$ (L = NIC, MTE, PES, and PSA). The collected fractions were evaporated under reduced pressure, and the residues were redissolved in water to a concentration of 10 mCi/mL. Since the HPLC mobile phase contains phosphate buffer (pH 6.0), no additional buffer was used for

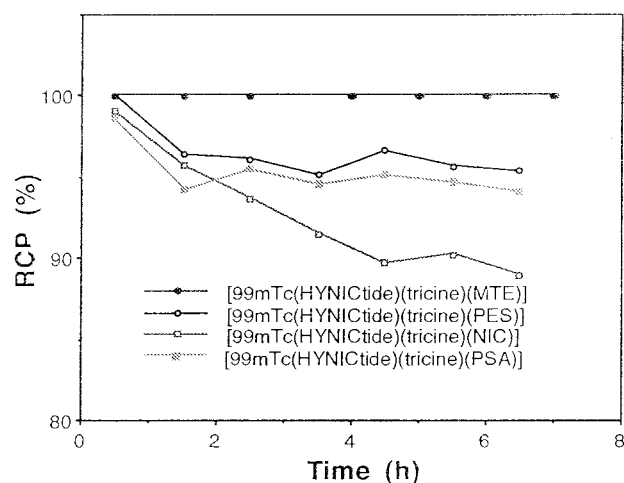


Figure 10. Plot of RCP (%) versus time (h) for the HPLC purified $[^{99m}\text{Tc}]\text{HYNICTide}$ complexes in succinate buffer (pH 5).

dilution. These solutions were monitored over 6 h by radio-HPLC (method 1 or 2). Figure 10 shows the plot of RCP vs time for the HPLC-purified complexes. It was found that the complex $[^{99m}\text{Tc}(\text{HYNICTide})(\text{tricine})(\text{MTE})]$ is stable for at least 6 h in solution (pH 6.0). For complex $[^{99m}\text{Tc}(\text{HYNICTide})(\text{tricine})(\text{L})]$ (L = PSA and PES), there was a slight drop in the RCP in the first hour, and it remained relatively unchanged over the next 5 h. For the complex $[^{99m}\text{Tc}(\text{HYNICTide})(\text{tricine})(\text{NIC})]$, the RCP drops steadily over 6 h from 100% to about 90%.

Compared to TPPTS, PSA is a much weaker coligand in binding to the Tc. For example, when PSA (15–20 mg/mL, ~ 100 mM) and TPPTS (1–2 mg/mL, 2–3 mM) were used in the same reaction mixture, the yield for $[^{99m}\text{Tc}(\text{HYNICTide})(\text{tricine})(\text{PSA})]$ is only 8–10% and the major product is $[^{99m}\text{Tc}(\text{HYNICTide})(\text{tricine})(\text{TPPTS})]$ with $\sim 80\%$ yield. Considering the electron-withdrawing sulfonate group on the pyridine ring and the overall weak donating capability of PSA, it is surprising that the HPLC purified $[^{99m}\text{Tc}(\text{HYNICTide})(\text{tricine})(\text{PSA})]$ remained stable in solution for more than 6 h. That lead us to examine the solution stability of $[^{99m}\text{Tc}(\text{HYNICTide})(\text{tricine})(\text{PSA})]$ in the presence of excess TPPTS.

The complex $[^{99m}\text{Tc}(\text{HYNICTide})(\text{tricine})(\text{PSA})]$ was first synthesized and HPLC purified. Volatiles in the collected fraction were removed under reduced pressure, and the residue was redissolved in phosphate buffer (pH 6.0) to give a concentration of about 5 mCi/mL $[^{99m/99}\text{Tc}(\text{HYNICTide})(\text{tricine})(\text{PSA})] \approx 3 \times 10^{-8}$ M. Upon addition of TPPTS (1 mg/mL $\approx 2 \times 10^{-3}$ M), the solution was kept at room temperature and monitored by radio-HPLC (method 1). To our surprise, there is no ligand exchange between TPPTS and PSA at room temperature over 3 h.

The fact that $[^{99m}\text{Tc}(\text{HYNICTide})(\text{tricine})(\text{PSA})]$ remains intact in the presence of ≥ 50000 -fold excess of TPPTS, which is a much stronger coligand than PSA, is significant. This suggests that the ligand-exchange reaction is not dependent on the concentration of the entering ligand and dissociation of the Tc–N (PSA) bond is the rate-limiting step. Therefore, the high stability of these new ternary ligand $[^{99m}\text{Tc}]\text{HYNICTide}$ complexes is probably due to their kinetic inertness. It also suggests that these complexes most likely have a common distorted octahedron coordination geometry around the Tc since 5- or 7-coordinated complexes are often kinetically labile and undergo facile associative-addition or dissociative-elimination reactions.

For the complex $^{99m}\text{Tc}(\text{HYNICTide})(\text{tricine})(\text{NIC})$, we also isolated the two peaks. Solutions of the two isolated peaks were kept at room temperature and monitored by HPLC (method 1) over 6 h. It was found that there is no interconversion between the two isomeric forms although the typical decomposition seen for the HPLC purified $^{99m}\text{Tc}(\text{HYNICTide})(\text{tricine})(\text{NIC})$ was observed. This suggests that as soon as the NIC coligand is dissociated the intermediate complex, $^{99m}\text{Tc}(\text{HYNICTide})(\text{tricine})$ decomposes. Should the coordinated tricine undergo the conformational change via partial dissociation, there would be interconversion between the two isomeric forms of the complex $^{99m}\text{Tc}(\text{HYNICTide})(\text{tricine})(\text{NIC})$.

It should be noted that the solution stability of a ternary ligand ^{99m}Tc HYNICTide complex depends on the nature of the imine-N-containing coligand. For MTE, which contains a thiazole ring, the complex $^{99m}\text{Tc}(\text{HYNICTide})(\text{tricine})(\text{MTE})$ remains stable in solution for over 6 h without any decomposition. For a pyridine analogue such as NIC, however, the complex $^{99m}\text{Tc}(\text{HYNICTide})(\text{tricine})(\text{NIC})$ decomposes slowly in solution. In general, the solution stability of ternary ligand ^{99m}Tc HYNICTide complexes follows the order of phosphines $\geq$ thiazoles/imidazoles $\gg$ pyridines. This is consistent with the σ -bonding capability of phosphine-P, thiazole-N, and pyridine-N.

Like that of phosphines, the bonding of the imine-N-containing coligand to the Tc dramatically reduces the number of isomeric forms of the ^{99m}Tc HYNICTide complexes and significantly increases the solution stability of ^{99m}Tc HYNICTide complexes. The hydrophilicity of ^{99m}Tc HYNICTide complexes with the new ternary ligand system can be tuned by changing the substituents on the heterocyclic ring. Among the coligands used in this study, ISONIC-HE and MTZ are of particular interest because of their neutrality. If the peptide is not charged, the resulting ternary ligand complex will be neutral, which may allow the complexes to cross the cell membranes and potentially bind to intracellular receptors. However, there are also limitations using this new ligand system. For example, this new ligand system may not be suitable for ^{99m}Tc -labeling of biologically active molecules containing one or more disulfide linkages because the use of SnCl_2 and high-temperature heating may destroy the S–S disulfide bonds and cause adverse effect on the biological properties of the HYNIC conjugate.

When a heterocycle such as MTE contains only one imine-N donor atom, it forms a ternary ligand complex $^{99m}\text{Tc}(\text{HYNICTide})(\text{tricine})(\text{MTE})$ with two isomeric forms. Thiazole-S is a much weaker donor than imine-N and does not impose a challenge in bonding to the Tc. However, if a heterocyclic coligand such as NTZ contains more than one imine-N donor, it may form a ternary ligand ^{99m}Tc HYNICTide complex with more than two isomeric forms because of different bonding modalities of the heterocyclic coligand. In addition, when triazole or imidazole is bonded to the Tc center, the hydrogen on the secondary amine-N may become deprotonated. An ideal imine-N-containing coligand is that which forms a ternary ligand technetium complex with only two diastereomers if the HYNIC-conjugated small molecule has a chiral center.

The HYNICTide contains a pyridine-N donor atom and a hydrazine group. Theoretically, HYNICTide itself can act as a monodentate coligand with the pyridine-N bonding to the Tc or as bidentate ligand using both pyridine-N and terminal hydrazine-N to form a five-membered chelate ring with Tc. It is not surprising that

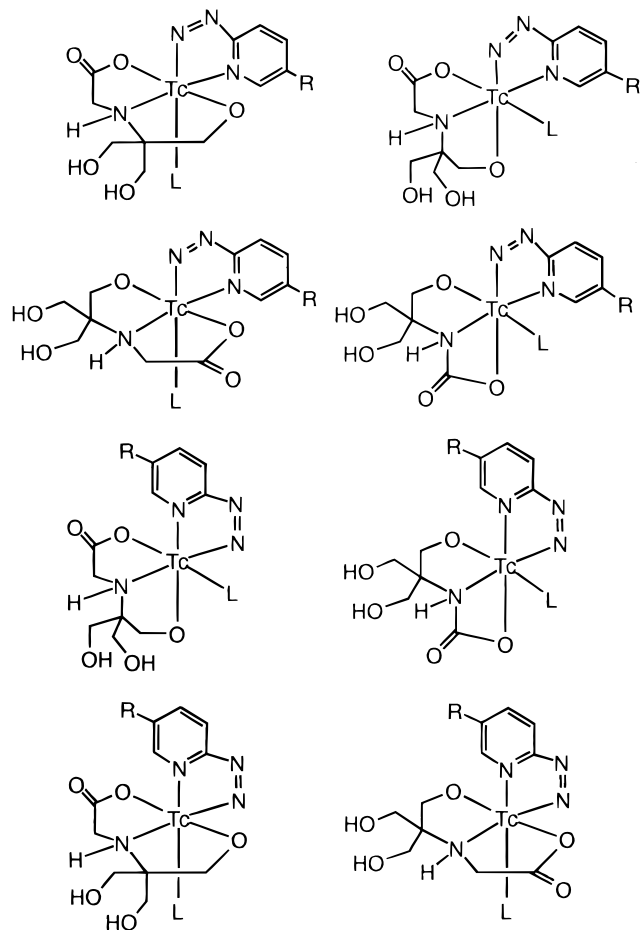
on the macroscopic (^{99}Tc) level the reaction of ^{99}Tc tricline complex with 2 equiv of HYNIC-D-Phe-OMe results in formation of two technetium complexes: $^{99}\text{Tc}(\text{HYNIC-D-Phe-OMe})(\text{tricine})^-$ and $^{99}\text{Tc}(\text{HYNIC-D-Phe-OMe})_2(\text{tricine})_2^-$ (57). It was also reported that reaction of 2-hydrazinopyridine, a model compound for HYNIC, in its dihydrochloride form with pertechnetate or perrhenate in methanol produced bis-hydrazide complexes (58). A structural study of $[\text{ReCl}_3(\text{HN}=\text{NC}_5\text{H}_4\text{N})(\text{N}=\text{NC}_5\text{H}_4\text{NH})]$ showed that the $\text{HN}=\text{NC}_5\text{H}_4\text{N}$ moiety is a neutral and bidentate pyridyldiazene while the $\text{N}=\text{NC}_5\text{H}_4\text{NH}$ moiety is a monodentate pyridiniumdiazenido.

On the tracer level, however, the complex $^{99m}\text{Tc}(\text{HYNICTide})(\text{tricine})(\text{NIC})$ is formed in $\sim 90\%$ yield from the reaction of HYNICTide, ^{99m}Tc pertechnetate, tricine, and NIC, which is a close analogue of HYNIC. In the radio-HPLC chromatogram (Figure 1), the two peaks at 13.2 and 14.8 min are due to the two isomeric forms of the complex $^{99m}\text{Tc}(\text{HYNICTide})(\text{tricine})(\text{NIC})$ while some of the later peaks at 18–30 min are probably due to the bis-HYNICTide complex. The area percentage of these peaks is usually $\leq 2\%$. This is due to the fact that the NIC concentration ($5 \text{ mg/mL} = 4 \times 10^{-2} \text{ M}$) is ~ 1000 -fold in excess over that of the HYNICTide ($40 \text{ }\mu\text{g/mL} \approx 4 \times 10^{-5} \text{ M}$). Since these later peaks are less than 2%, no further characterization was performed.

On the basis of the mixed-ligand experiments, it is quite clear that the ternary ligand ^{99m}Tc HYNICTide complexes described in this study contain one HYNICTide, one tricine, and one imine-N-containing coligand. The technetium oxidation state and the exact nature of bonding between these ligands (HYNIC, tricine, and imine-N-containing heterocycle) and the Tc metal center still remains unclear. It has been proposed that the technetium oxidation state in the ^{99m}Tc tricline complex is +5 (15, 16, 57). However, the oxidation state might change when the HYNIC group and imine-N are bonded to the Tc center since 2-hydrazinopyridine, which is a close analogue of HYNIC, has been shown to act as a reducing agent for pertechnetate or perrhenate (58, 59). Furthermore, the technetium oxidation state largely depends on how the HYNIC group binds to the Tc and how one counts the charge on the HYNIC ligand.

Complexes containing Tc-hydrazido and Tc-diazenido bonds have been previously reported and characterized by X-ray crystallography (58–66). Recently, Davison and co-workers (58, 59) reported a series of technetium(III) and rhenium(III) complexes of 2-hydrazinopyridine. It was found that, unlike phenylhydrazine, 2-hydrazinopyridine has several bonding modalities: neutral bidentate pyridyldiazene (58, 59), neutral monodentate pyridiniumdiazenido (58), and anionic monodentate pyridyldiazenido (59). Various Tc–Re complexes can be prepared depending upon the starting material and reaction conditions. For example, the reaction of $\text{NH}_4[\text{TcO}_4]$ with 2-hydrazinopyridine dihydrochloride in methanol produces a complex $[\text{TcCl}_3(\text{HN}=\text{NC}_5\text{H}_4\text{N})(\text{N}=\text{NC}_5\text{H}_4\text{NH})]$. The reaction of the complex $[\text{ReCl}_3(\text{HN}=\text{NC}_5\text{H}_4\text{N})(\text{N}=\text{NC}_5\text{H}_4\text{NH})]$ with triphenylphosphine (PPh_3) in the presence of a base such as diisopropylethylamine gives a complex $[\text{ReCl}_2(\text{PPh}_3)(\text{N}=\text{NC}_5\text{H}_4\text{N})(\text{N}=\text{NC}_5\text{H}_4\text{NH})]$, in which the $\text{N}=\text{NC}_5\text{H}_4\text{N}$ moiety was found to be an anionic monodentate pyridyldiazenido (67).

When the HYNICTide binds to the Tc, it likely occupies one site of the Tc coordination sphere by forming either a Tc–hydrazido or Tc–diazenido bond. If one assumes that the coordination geometry around the Tc is distorted octahedral, the tricline coligand is expected to be tetradentate. The technetium chelate is chiral, due to the

Chart 4. Eight Isomeric Forms from Bidentate HYNICTide and Tridentate Tricine in the Ternary Ligand [^{99m}Tc]HYNICTide Complexes

asymmetrical binding of the tricine coligand and should be formed in an equal mixture of D and L enantiomers. These enantiomers in combination with the chiral centers on the cyclic peptide backbone of HYNICTide (DLL) result in the diastereomers DDLL and LDLL. Therefore, it is not surprising that HYNIC-conjugated small molecules with one or more chiral centers form ternary ligand [^{99m}Tc]-HYNICTide complexes showing two radiometric peaks in their radio-HPLC chromatograms, as demonstrated in the chirality experiment, while HYNIC-conjugated small molecules without a chiral center form the corresponding ternary ligand [^{99m}Tc]HYNICTide complexes showing only one radiometric peak in their radio-HPLC chromatograms, as was observed for [^{99m}Tc](HYNIC-BA)(tricine)-(PSA)].

If HYNICTide bonds to the Tc center as a bidentate ligand (Chart 4), the tricine coligand has to be tridentate to complete the octahedral coordination sphere. Due to the relative geometric arrangement of these three different ligands, there are eight possible isomeric forms: four facial and four meridional (Chart 4). Each of these eight isomers has its enantiomer. This, in combination with the chiral centers of the HYNICTide, would result in total of 16 diastereomers, which should be at least partially resolvable under the chromatographic conditions used in this study. The fact that ternary ligand complexes [^{99m}Tc](HYNICTide)(tricine)(L)] (L = ISONIC, ISONIC-HE, MTE, PA, PES, and PSA) show only two radiometric peaks in their radio-HPLC chromatograms strongly suggests that these complexes have a common 6-coordinate distorted octahedron coordination geometry

with a monodentate HYNICTide, a tetradentate tricine, and a monodentate imine-N-containing coligand.

Recently, HYNIC has been used for the ^{99m}Tc -labeling of somatostatin analogues with a β -alanine or diaminobutanesuccinyl linker (10, 11). Tricine was used as the only coligand. It was found that these ^{99m}Tc -labeled somatostatin analogues are specific for the SRIF receptor, and show comparable tumor uptake to ^{111}In -DTPA-D-Phe-1-Octreotide (^{111}In -OctreoScan). One major drawback is the high level of blood activity. It was proposed that the high blood activity might be due to serum protein binding (11). The serum protein binding was also observed for the ^{99m}Tc -labeled DNA oligonucleotides when HYNIC was used as a bifunctional coupling agent (14). However, there was no evidence to prove the assumption. In this study, we clearly demonstrated that imine-N-containing heterocycles such as HIS-AC can react with the binary ligand complex [^{99m}Tc](HYNICTide)(tricine) $_2$ and form the stable ternary ligand technetium complex [^{99m}Tc](HYNICTide)(tricine)(HIS-AC)]. Since blood proteins contain many histidine amino acid residues, it is quite possible for the binary complex [^{99m}Tc](HYNIC-linker-Octreotide)-(tricine) $_2$ to react with the imidazole moiety of histidine residues from blood proteins, and form a stable bonding between the [^{99m}Tc]HYNIC-Octreotide complex and protein molecules. The results from this study provide direct evidence to support the assumption that the high level blood activity is indeed due to serum protein binding.

The coligand, ISONIC-Asp-OMe $_2$, is of particular interest because it contains two ester groups which can be enzymatically hydrolyzed in biological systems. Since ISONIC-Asp-OMe $_2$ carries no charge, it forms neutral technetium complexes, which can cross the cell membrane and potentially bind to intracellular receptors, if the biologically active molecule carries no charge. Once inside the cell, enzymatic hydrolysis of the ester groups forms negatively charged ^{99m}Tc species, which cannot be easily washed out. In this way, the target uptake may be significantly improved. If ester groups are hydrolyzed in the blood, the negatively charged ^{99m}Tc species is expected to have faster renal clearance than the neutral precursor. Therefore, the introduction of two ester groups has two potential advantages: increasing target uptake and decreasing background.

CONCLUSIONS

In this report, we described the use of a new ternary ligand system in the ^{99m}Tc labeling of a cyclic GPIIb/IIIa HYNICTide. The new ternary ligand system is composed of HYNICTide, tricine, and an imine-N-containing heterocycle. The imine-N-containing heterocyclic coligands include *N*- ω -acetylhistamine, *N*-(2-hydroxyethyl)isonicotinamide, isonicotinic acid, 4-methyl-5-thiazoleethanol, nicotinic acid, 3-nitro-1,2,4-triazole, pyridine, 4-pyridineethanesulfonic acid, 3-pyridinesulfonic acid, and 4-pyridylacetic acid. The ternary ligand complex [^{99m}Tc](HYNICTide)(tricine)(L)] (L = HIS-AC, ISONIC, ISONIC-HE, MTE, NTZ, PA, PES, PSA, and PY) was prepared in high yield and high specific activity ($\geq 10\,000$ Ci/mmol HYNICTide). We found that the ternary ligand complex [^{99m}Tc](HYNICTide)(tricine)(L)] (L = ISONIC, ISONIC-HE, MTE, PA, PES, PSA, and PY) is formed as equal mixtures of two isomeric forms. Using a chirality experiment, we demonstrated that the presence of two equal intensity radiometric peaks in their HPLC chromatograms is due to the resolution of two diastereomers which are formed as a result of the chiral centers on the cyclic peptide backbone and the formation of a mixture of

enantiomers for the technetium chelate when tricine is bonded to the Tc as a tetradentate ligand. We also found that these new ternary ligand [^{99m}Tc]HYNICTide complexes are stable for up to 6 h in the reaction mixture, and the high solution stability was due to their kinetic inertness. The composition of these complexes was determined to be 1:1:1:1 for Tc:HYNICTide:L:tricine (L = imine-N-containing heterocycle) through a series of mixed ligand experiments on the tracer (^{99m}Tc) level. The lipophilicity of the [^{99m}Tc]HYNICTide complexes can be systematically varied by the choice of polyaminocarboxylate and imine-N-containing coligands depending on the pharmacokinetic requirements. Using the combination of tricine and an imine-N-containing coligand, HYNICT-derivatized peptides or other small molecules can be labeled with ^{99m}Tc in high specific activity and high stability for potential use as radiopharmaceuticals.

ACKNOWLEDGMENT

Acknowledgment is made to Neal E. Williams for the mass spectrum of HYNIC-BA, HYNIC-MBA, ISONIC-L-Asp-OMe₂, and Hbtris, to John Pietryka for the ^1H -NMR spectra, and to Dr. T. D. Harris, Dr. M. Rajopadhye, Dr. D. Glowacka, J. P. Bourque, P. R. Damphousse, and K. Yu for the synthesis of the cyclic GPIIb/IIIa HYNICTide, cyclo(D-Val-NMeArg-Gly-Asp-Mamb(hydrazinonicotinyl-5-(6-aminocaproamide))).

LITERATURE CITED

- (1) Schwartz, D. A., Abrams, M. J., Hauser, M. M., Gaul, F. E., Larsen, S. K., Rauh, D., and Zubieta, J. A. (1991) Preparation of hydrazino-modified proteins and their use for the synthesis of ^{99m}Tc -protein conjugates. *Bioconjugate Chem.* 2, 334–336.
- (2) Abrams, M. J., Juweid, M., tenKate, C. I., Schwartz, D. A., Hauser, M. M., Gaul, F. E., Fuccello, A. J., Rubin, R. H., Strauss, H. W., and Fischman, A. J. (1990) Technetium-99m-human polyclonal IgG radiolabeled via the hydrazino nicotinamide derivative for imaging focal sites of infection in rats. *J. Nucl. Med.* 31, 2022–2028.
- (3) Babich, J. W., Solomon, H., Pike, M. C., Kroon, D., Graham, W., Abrams, M. J., Tompkins, R. G., Rubin, R. H., and Fischman, A. J. (1993) Technetium-99m-labeled hydrazino nicotinamide derivatized chemotactic peptide analogues for imaging focal sites of bacterial infection. *J. Nucl. Med.* 34, 1964–1974.
- (4) Babich, J. W., and Fischman, A. J. (1995) Effect of "coligand" on the biodistribution of ^{99m}Tc -labeled hydrazino nicotinic acid derivatized chemotactic peptides. *Nucl. Med. Biol.* 22, 25–30.
- (5) Fischman, A. J., Babich, J. W., and Rubin, R. H. (1994) Infection imaging with technetium-99m-labeled chemotactic peptide analogues. *Semin. Nucl. Med.* 24, 154–168.
- (6) Fischman, A. J., Babich, J. W., and Strauss, H. W. (1993) A ticket to ride: peptide radiopharmaceuticals. *J. Nucl. Med.* 34, 2253–2263.
- (7) Fischman, A. J., Rauh, D., Solomon, H., Babich, J. W., Tompkins, R. G., Kroon, D., Strauss, H. W., and Rubin, R. H. (1993) In vivo bioactivity and biodistribution of chemotactic peptide analogues in nonhuman primates. *J. Nucl. Med.* 34, 2130–2134.
- (8) Babich, J. W., Graham, W., Barrow, S. A., and Fischman, A. J. (1995) Comparison of the infection imaging properties of a ^{99m}Tc labeled chemotactic peptide with ^{111}In IgG. *Nucl. Med. Biol.* 22, 643–648.
- (9) Babich, J. W., Solomon, H., Pike, M. C., Kroon, D., Graham, W., Abrams, M. J., Tompkins, R. G., Rubin, R. H., Barrow, and Fischman, A. J. (1993) Technetium-99m-labeled hydrazino nicotinamide derivatized chemotactic peptide analogues for imaging focal sites of bacterial infection. *J. Nucl. Med.* 34, 1964–1974.
- (10) Mäcke, H. R., and Behe, M. (1996) New octreotide derivatives labeled with technetium-99m. *J. Nucl. Med.* 37, 29P (abstract 107).
- (11) Behe, M., and Mäcke, H. R. (1995) New somatostatin analogues labeled with technetium-99m. *Eur. J. Nucl. Med.* 22, 791 (abstract 267).
- (12) Dewanjee, M. K., Ghalfouripour, A. K., Subramanian, M., Hanna, M., Kapadvanjwala, M., Serafini, A. N., Ezuddin, S., Lopez, D., and Sfakianakis, G. N. (1994) Labeling antisense deoxyoligonucleotide with Tc-99m and hybridization with c-myc oncogene mRNA in P388 leukemic cells. *J. Labelled Compd. and Radiopharm.* 35, 40–42.
- (13) Dewanjee, M. K., Abrams, M. J., Wu, S. M., Kapadvanjwala, M., Serafini, A. N., and Sfakianakis, G. N. (1995) Technetium-99m labeled single-strand DNA probe for imaging thrombin in viscera formed during cardiopulmonary bypass. *J. Nucl. Med.* 36, 16P (abstract 54).
- (14) Hnatowich, D. J., Winnard, P., Jr., Virzi, F., Fogarsi, M., Sano, T., Smith, C. L., Cantor, C. L., and Rusckowski, M. (1995) Technetium-99m labeling of DNA oligonucleotides. *J. Nucl. Med.* 36, 2306–2314.
- (15) Larson, S. K., Abrams, M. J., Higgins, J. D., III, Solomon, H. F., Babich, J. W., and Fischman, A. J. (1994) Technetium complexes of tricine: useful precursor for the ^{99m}Tc labeling of hydrazino nicotinamide modified human polyclonal IgG. *J. Nucl. Med.* 35, 105P (abstract 418).
- (16) Larson, S. K., Solomon, H. F., Caldwell, G., and Abrams, M. J. (1995) [^{99m}Tc]Tricine: a useful precursor complex for the radiolabeling of hydrazinonicotinate protein conjugates. *Bioconjugate Chem.* 6, 635–638.
- (17) Liu, S., Edwards, D. S., Looby, R. J., Harris, A. R., Poirier, M. J., Barrett, J. A., Heminway, S. J., and Carroll, T. R. (1996) Labeling a hydrazino nicotinamide-modified cyclic IIb/IIIa receptor antagonist with ^{99m}Tc using aminocarboxylates as coligands. *Bioconjugate Chem.* 7, 63–71.
- (18) Edwards, D. S., Barrett, J. A., Liu, S., Ziegler, M. C., Mazaika, T., Vining, M., Bridger, G., Higgins, J., III, and Abrams, M. J. (1996) A stabilized Tc-99m complex of a chemotactic peptide-HYNIC conjugate for imaging infection. *Eur. J. Nucl. Med.* 23, 1142 (abstract Omo440).
- (19) Edwards, D. S., Liu, S., Harris, A. R., Looby, R. J., Ziegler, M. C., Heminway, S. J., Barrett, J. A., and Carroll, T. R. (1997) A new and versatile ternary ligand system for technetium radiopharmaceuticals: water soluble phosphines and tricine as coligands in labeling a hydrazino nicotinamide-modified cyclic glycoprotein IIb/IIIa receptor antagonist with ^{99m}Tc . *Bioconjugate Chem.* 8, 146–154.
- (20) Kuntz, E. (1981) Catalytic hydroformylation of olefins. U.S. Patent 4,248,802.
- (21) Sworin, M., Rajopadhye, M., Harris, T. D., Edwards, D. S., Cheesman, E., and Liu, S. (1996) Stable reagents for the preparation of radiopharmaceuticals. *PCT International Application*, WO 96/40637.
- (22) Rajopadhye, M., Harris, T. D., Yu, K., Glowacka, D., Damphousse, P. R., Barrett, J. A., Heminway, S. J., Edwards, D. S., and Carroll, T. R. (1997) Synthesis, evaluation and Tc-99m complexation of a hydrazinonicotinyl conjugate of a GP IIb/IIIa antagonist cyclic peptide for the detection of deep vein thrombosis. *Bioorg. Med. Chem. Lett.* 7, 955–960.
- (23) Goodbody, A. E., Ballinger, J., Tran, L. L., Sumner-Smith, M., Lau, F., Meghji, K., and Pollak, A. (1994) A new Tc-99m labeled peptide inflammation imaging agent. *Eur. J. Nucl. Med.* 21, 790 (abstract 262).
- (24) Caveller, V., Goodbody, A. E., Tran, L. L., Bossuyt, A., and Thornback, J. (1996) Human dosimetry of Tc99m-RP128, a potential inflammation imaging agent. *Eur. J. Nucl. Med.* 23, 1131 (abstract OMO398).
- (25) Peers, S. H., Tran, L. L., Eriksson, S. J., Ballinger, J., and Goodbody, A. E. (1995) Imaging a model of colitis with RP128, a Tc-99m-chelated tuftsin Antagonist. *J. Nucl. Med.* 36, 114P (abstract 465).
- (26) Maina, T., Stolz, B., Albert, R., Bruns, C., Koch, P., and Mäcke. (1994) Synthesis, radiochemistry and biological evaluation of a new somatostatin analogue (SDZ 219-387) labeled with technetium-99m. *Eur. J. Nucl. Med.* 21, 437–444.

- (27) Maina, T., Stolz, B., Albert, R., Nock, B., Bruns, C., and Mäcke, H. (1995) Synthesis, radiochemical and biological evaluation of $^{99m}\text{Tc}[\text{N4-(D)PHE}^1]\text{-octreotide}$, a new octreotide derivative with high affinity for somatostatin receptors. in *Technetium and Rhenium in Chemistry and Nuclear Medicine* (M. Nicolini, G. Banoli, and U. Mazzi, Eds.) pp 395–400, Vol. 4, SGEEditoriali, Padova.
- (28) Kolan, H., Li, J.-H., and Thakur, M. L. (1996) Sandostatin labeled with ^{99m}Tc : In vitro stability, in vivo validity and comparison with ^{111}In -DTPA-octreotide. *Pept. Res.* 9, 144–150.
- (29) Vallabhajosula, S., Moyer, B. R., Lister-James, J., McBride, W. J., Lipszyc, H., Lee, H., Bastidas, D., and Dean, R. T. (1996) Preclinical evaluation of technetium-99m-labeled somatostatin receptor binding peptides. *J. Nucl. Med.* 37, 1016–1022.
- (30) Pearson, D. A., Lister-James, J., McBride, W. J., Wilson, D. M., Martel, L. J., Civitello, E. R., Taylor, J. E., Moyer, B. R., and Dean, R. T. (1996) Somatostatin receptor-binding peptides labeled with technetium-99m: chemistry and initial biological studies. *J. Med. Chem.* 39, 1361–1371.
- (31) Liu, S., Edwards, D. S., Looby, R. J., Harris, A. R., Poirier, M. J., Rajopadhye, M., and Bourque, J. P. (1996) Labeling cyclic IIB/IIIA receptor antagonists with ^{99m}Tc by the pre-formed chelate approach: effects of chelators on properties of [^{99m}Tc]chelator-peptide conjugate. *Bioconjugate Chem.* 7, 196–202.
- (32) Barrett, J. A., Damphousse, D. J., Heminway, S. J., Liu, S., Edwards, D. S., Looby, R. J., and Carroll, T. R. (1996) Biological evaluation of ^{99m}Tc -labeled cyclic GPIIb/IIIA receptor antagonists in the canine arteriovenous shunt and deep vein thrombosis models: effects of chelators on biological properties of [^{99m}Tc]chelator-peptide conjugates. *Bioconjugate Chem.* 7, 203–208.
- (33) Barrett, J. A., Bresnick, M., Crocker, A. C., Damphousse, D. J., Heminway, S. J., Mazaika, T. J., Kagan, M., Lazewatsky, J. L., Edwards, D. S., Liu, S., Harris, T. D., Rajpadhye, M., and Carroll, T. R. (1998) RP431: a technetium-99m-labeled platelet GP IIB/IIIA receptor antagonist as a thrombus imaging agent. *J. Nucl. Med.* (In press).
- (34) Barrett, J. A., Crocker, A. C., Damphousse, D. J., Heminway, S. J.; Liu, S.; Edwards, D. S., Harris, A. R., Looby, R. J., Lazewatsky, J. L., Kagan, M., Mazaika, T. J.; Carroll, T. R. (1997) Biological evaluation of thrombus imaging agents utilizing water soluble phosphines and tricine as coligands to label a hydrazinonicotinamide-modified cyclic glycoprotein IIB/IIIA receptor antagonist with ^{99m}Tc . *Bioconjugate Chem.* 8, 155–160.
- (35) Lister-James, J., Knight, L. C., Mauer, A. H., Bush, L. R., Moyer, B. R., and Dean, R. T. (1996) Thrombus imaging with technetium-99m-labeled, activated platelet receptor binding peptide. *J. Nucl. Med.* 37, 775–781.
- (36) Muto, P., Lastoria, S., Varrella, P., Vergara, E., Salvatore, M., Morgano, G., Lister-James, J., Bernardy, J. D., Dean, R. T., Wencker, D., and Boer, J. S. (1995) Detecting deep venous thrombosis with technetium-99m-labeled synthetic peptide P280. *J. Nucl. Med.* 36, 1384–1391.
- (37) Pearson, D. A., Lister-James, J., McBride, W. J., Wilson, D. M., Martel, L. J., Civitello, E. R., and Dean, R. T. (1996) Thrombus imaging using technetium-99m labeled high potency GPIIb/IIIA receptor antagonists. Chemistry and initial biological studies. *J. Med. Chem.* 39, 1372–1382.
- (38) Lister-James, J., Vallabhajosula, S., Moyer, B. R., Pearson, D. A., McBride, B. J., De Rosch, M. A., Bush, L. R., Machac, J., and Dean, R. T. (1997) Pre-clinical evaluation of technetium-99m platelet receptor-binding peptide. *J. Nucl. Med.* 38, 105–111.
- (39) Liu, S., Edwards, D. S., and Barrett, J. A. (1997) ^{99m}Tc -labeling of highly potent small peptides. *Bioconjugate Chem.* 8, 621–636.
- (40) Lister-James, J., Moyer, B. R., and Dean, R. T. (1996) Small peptides radiolabeled with ^{99m}Tc . *Q. J. Nucl. Med.* 40, 221–233.
- (41) Lanteingne, D., and Hnatowich, D. J. (1984) The labeling of DTPA coupled proteins. *Int. J. Radiat. Isot.* 35, 617–621.
- (42) Breitz, H. B., Weiden, P. L., Vanderheyden, J.-L., Appelbaum, J. W., Bjorn, M. J., Fer, M. F., Wolf, S. B., Ratliff, B. A., Seiler, C. A., Foisie, D. C., Fisher, D. R., Schroff, R. W., Fritzberg, A. R., and Abrams, P. G. (1992) Clinical experience with rhenium-186-labeled monoclonal antibodies for radioimmunotherapy: results of phase I trials. *J. Nucl. Med.* 33, 1099–1112.
- (43) Schroff, R. W., Weiden, P. L., Appelbaum, J. W., Fer, M. F., Breitz, H. B., Vanderheyden, J.-L., Ratliff, B. A., Fisher, D. R., Foisie, D. C., Hanelin, L. G., Morgan, A. C., Jr., Fritzberg, A. R., and Abrams, P. G. (1990) Rhenium-186 labeled antibody in patients with cancer: Report of a pilot phase I study. *Antibody, Immunoconjugates, Radiopharm.* 3, 99–111.
- (44) Liu, S., and Edwards, D. S. (1995) New N_2S_2 diamidedithiol and N_3S triamidedithiols as bifunctional chelating agents for labeling small peptides with technetium-99m. in *Technetium and Rhenium in Chemistry and Nuclear Medicine* (M. Nicolini, G. Banoli, and U. Mazzi, Eds.) pp 383–393, Vol. 4, SGEEditoriali, Padova.
- (45) Fritzberg, A. R., Abrams, P. G., Beaumier, P. L., Kasina, S., Morgan, A. C., Rao, T. N., Reno, J. M., Sanderson, J. A., Srinivasan, A., and Wilbur, D. S. (1988) Specific and stable labeling of antibodies with technetium-99m with a diamide dithiolate chelating agent. *Proc. Natl. Acad. Sci. U.S.A.* 85, 4025–4029.
- (46) Eary, J. F., Schroff, R. W., Abrams, P. G., Fritzberg, A. R., Morgan, A. C., Kasina, S., Reno, J. M., Srinivasan, A., Woodhouse, C. S., Wilbur, D. S., Natale, R. B., Collins, C., Stehlin, J. S., Mitchell, M., Nelp, W. B. (1989) Successful imaging of malignant melanoma with technetium-99m-labeled monoclonal antibodies. *J. Nucl. Med.* 30, 25–32.
- (47) Kasina, S., Rao, T. N., Srinivasan, A., Sanderson, J. A., Fitzner, J. N., Reno, J. M., Beaumier, P. L., and Fritzberg, A. R. (1991) Development and biological evaluation of a kit for preformed chelate technetium-99m radiolabeling of an antibody Fab fragment using a diamide dimercaptide chelating agent. *J. Nucl. Med.* 32, 1445–1451.
- (48) Kung, H. F., Bradshaw, J. E., Chumpradit, S., Zhuang, Z. P., Kung, M. P., Mu, M., and Frederik, D. (1995) New TCO(III) and ReO(III) N_2S_2 complexes as potential CNS 5-HT_{1A} receptor imaging agents. in *Technetium and Rhenium in Chemistry and Nuclear Medicine* (M. Nicolini, G. Banoli, and U. Mazzi, Eds.) pp 383–393, Vol. 4, SGEEditoriali, Padova.
- (49) Eisenhut, M., Mißfeldt, M., Lehmann, W. D., and Karas, M. (1991) Synthesis of a bis(aminoethanethiol) ligand with an activated ester group for protein conjugation and ^{99m}Tc -labeling. *J. Labelled Compd. Radiopharm.* 29, 1283–1291.
- (50) Eisenhut, M., Lehmann, W. D., Becker, W., Behr, T., Elser, H., Strittmater, W., Steinsträsser, A., Baum, R. P., Valerius, T., Repp, R., and Deo, Y. (1996) Bifunctional NHS–BAT ester for antibody conjugation and stable technetium-99m labeling: conjugation chemistry, immunoreactivity and kit formulation. *J. Nucl. Med.* 37, 362–370.
- (51) Baidoo, K. E., and Lever, S. Z. (1990) Synthesis of a diaminedithiol bifunctional chelating agent for incorporation of technetium-99m into biomolecules. *Bioconjugate Chem.* 1, 132–137.
- (52) DiZio, J. P., Fiashi, R., Davison, A., Jones, A. G., and Katzenellenbogen, J. A. (1991) Progesterone–rhenium complexes: metal labeled steroids with high receptor binding affinity, potential receptor-directed agents for diagnostic imaging or therapy. *Bioconjugate Chem.* 2, 352–366.
- (53) DiZio, J. P., Anderson, C. J., Davison, A., Ehrhardt, G. J., Carlson, K. E., Welch, M. J., and Katzenellenbogen, J. A. (1992) Technetium- and rhenium- labeled progestins: synthesis, receptor binding and in vivo distribution of an β -substituted progesterone labeled with technetium-99 and rhenium-186. *J. Nucl. Med.* 33, 558–569.
- (54) Baidoo, K. E., Scheffel, U., Finley, P., Lever, S. Z., and Wagner, H. N., Jr. (1994) No carrier added (NCA) Tc-99m-diaminedithiolate derivatives of chemotactic peptides for imaging inflammation. *J. Nucl. Med.* 35, 19P (abstract 67).
- (55) Linder, K. E., Wen, M. D., Nowotnik, D. P., Malley, M. F., Gougoutas, J. Z., Nunn, A. D., and Eckelman, W. C. (1991)

- Technetium labeling of monoclonal antibodies with functionalized BATO's: 1. $\text{TcCl}(\text{DMG})_3$ PITC. *Bioconjugate Chem.* 2, 160–170.
- (56) Linder, K. E., Wen, M. D., Nowotnik, D. P., Ramalingam, K., Sharkey, R. M., Yost, F., Narra, R. K., Nunn, A. D., and Eckelman, W. C. (1991) Technetium labeling of monoclonal antibodies with functionalized BATO's: 2. $\text{TcCl}(\text{DMG})_3$ CPITC labeling of B72.3 and NP-4 whole antibodies and NP-4 F(ab')_2 . *Bioconjugate Chem.* 2, 407–414.
- (57) Larson, S. K., Caldwell, G., Higgins, J. D., III, Abrams, M. J., and Solomon, H. F. (1994) Technetium complex of tricine: useful precursor for the ^{99m}Tc labeling of hydrazino nicotinamide modified proteins. *J. Labelled Compd. Radiopharm.* 35, 1–2.
- (58) Nicholson, T., Cook, J., Davison, A., Rose, D. J., Maresca, K. P., Zubieta, J. A., and Jones, A. J. (1996) The synthesis and characterization of $[\text{MCl}_3(\text{N}=\text{NC}_5\text{H}_4\text{NH})(\text{HN}=\text{NC}_5\text{H}_4\text{N})]$ from $[\text{MO}_4]^-$ (where $\text{M} = \text{Re}, \text{Tc}$) organodiazenido, organodiazene-chelate complexes. The X-ray structure of $[\text{ReCl}_3(\text{N}=\text{NC}_5\text{H}_4\text{NH})(\text{HN}=\text{NC}_5\text{H}_4\text{N})]$. *Inorg. Chim. Acta* 252, 421–426.
- (59) Nicholson, T., Cook, J., Davison, A., Rose, D. J., Maresca, K. P., Zubieta, J. A., and Jones, A. J. (1996) The synthesis, characterization and X-ray crystal structure of the rhenium organodiazenido, organodiazene complex of $[\text{ReCl}_2(\text{PPh}_3)(\text{N}=\text{NC}_5\text{H}_4\text{N})(\text{HN}=\text{NC}_5\text{H}_4\text{N})]$. *Inorg. Chim. Acta* 252, 427–430.
- (60) Abrams, M. J., Larsen, S. K., Shaikh, S. N., and Zubieta, J. (1991) Investigation of technetium-organohydrazine coordination chemistry. The crystal and molecular structures of $[\text{TcCl}_2(\text{C}_6\text{H}_5\text{N}_4)(\text{PPh}_3)_2] \cdot 0.75\text{C}_7\text{H}_8$ and $[\text{TcNCl}_2(\text{PPh}_3)_2] \cdot 0.25\text{CH}_2\text{Cl}_2$. *Inorg. Chim. Acta* 185, 7–15.
- (61) Archer, C. M., Dilworth, J. R., Jobanputra, P., Thompson, R. M., McPartlin, M., Povey, D. C., Smith, G. W., and Kelly, J. D. (1990) Development of new technetium cores containing technetium–nitrogen multiple bonds. Synthesis and characterization of some diazenido-, hydrazido- and imido- complexes of technetium. *Polyhedron* 9, 1497–1502.
- (62) Dilworth, J. R., Jobanputra, P., Thompson, R. M., Archer, C. M., Povey, D. C., Kelly, J. D., and Hiller, W. (1992) Crystal structure of a diazenido- dithiocarbamate complex of technetium, $[\text{Tc}(\text{NNC}_6\text{H}_4\text{Cl})((\text{CH}_3)_2\text{NCS}_2)_2(\text{PPh}_3)]$. *Z. Naturforsch.* 46, 449–452.
- (63) Archer, C. M., Dilworth, J. R., Jobanputra, P., Thompson, R. M., McPartin, M., and Hiller, W. (1993) Technetium diazenido complexes. Part 1. Synthesis and structures of $[\text{TcCl}(\text{NNC}_6\text{H}_4\text{Cl-4})_2(\text{PPh}_3)_2]$ and $[\text{TcCl}(\text{NNPh})(\text{Ph}_2\text{PCH}_2\text{CH}_2\text{PPh}_2)_2][\text{PF}_6] \cdot \text{H}_2\text{O}$. *J. Chem. Soc., Dalton Trans.* 897–904.
- (64) Dilworth, J. R., Jobanputra, P., Thompson, R. M., Povey, D. C., Archer, C. M., and Kelly, J. D. (1994) Technetium diazenido complexes. Part 2. Substitution chemistry of structures of $[\text{TcCl}(\text{NNC}_6\text{H}_4\text{Cl-4})_2(\text{PPh}_3)_2]$ and the synthesis of technetium diazenido-complexes directly from $[\text{NH}_4][\text{TCO}_4]$. *J. Chem. Soc., Dalton Trans.* 1251–1256.
- (65) Nicholson, T., de Vries, N., Davison, A., and Jones, A. G. (1989) Synthesis and characterization of aryldiazenido technetium complexes and their protonation reactions. The X-ray structure of $[\text{TcCl}(\text{PPh}_3)_2(\text{NNC}_6\text{H}_4\text{Br})_2]$. *Technetium and Rhenium in Chemistry and Nuclear Medicine* (M. Nicolini, G. Bandoli, and U. Mazzi, Eds.) pp 95–108, Vol. 3, Cortina International, Verona.
- (66) Cook, J., Davison, A., Jones, A. J., and Davis, W. M. (1990) The reaction chemistry of $\text{HTc}(\text{CO})_3(\text{PPh}_3)_2$. *Technetium and Rhenium in Chemistry and Nuclear Medicine* (M. Nicolini, G. Bandoli, and U. Mazzi, Eds.) pp 65–68, Vol. 3, Cortina International, Verona.
- (67) Liu, S and Edwards, D. S. Unpublished results.

BC9800116

Optimization and Immunological Characterization of a Photochemically Coupled Lysergic Acid Diethylamide (LSD) Immunogen

Sarah Kerrigan[†] and Donald. E. Brooks*

Departments of Chemistry and Pathology and Laboratory Medicine, 2211 Wesbrook Mall, University of British Columbia, Vancouver, British Columbia, Canada V6T 2B5 . Received March 18, 1998; Revised Manuscript Received June 30, 1998

A photoreactive heterobifunctional linker was used to prepare an immunogen in which lysergic acid diethylamide was indirectly coupled to keyhole limpet hemocyanin at multiple sites on the drug. It was possible to attach approximately 35 drug molecules to each protein using approximately equal amounts of both species during the reaction. The presence of buffer components or water severely compromised reaction efficiency, as estimated from the molar substitution ratio. Factors such as excess linker, pH, irradiation of dry matrix in the absence of buffer, and the drug/protein ratio used during photolysis were shown to have pronounced effects on reaction efficiency. Structural insights regarding immunogen coupling were obtained by determining the specificities of antibodies which were raised against the immunogen. Cross-reactivity data indicated that haptenation of protein likely occurred at positions N1 and N6 of lysergic acid diethylamide, which is plausible given the electrophilicity of the photogenerated aryl nitrene.

INTRODUCTION

The haptenation of protein for the purpose of raising antibodies to small molecules such as drugs or toxins influences the immune response at the T-cell level (1). The production of antibody is sterically influenced by the site of attachment between drug and carrier molecule. In practice, this effect is generally observed in cross-reactivity studies as decreased antibody specificity in the vicinity of the linkage. The chemistry of the linker moiety and the hapten density affect both the intensity and the character of the immune response, as does the nature of the carrier molecule (2). In theory, a superior antiserum is obtained following immunization with a highly immunogenic conjugate in which drug is attached to the carrier protein via an extended spacer arm to improve specificity. This approach allows the hapten to be held a short distance away from the protein surface, thus increasing the possibility that lymphocytes can distinguish the haptenic determinant at the site of attachment. Immunogen design, therefore, has a profound effect on the affinity, specificity, and overall characteristics of the antiserum. The quality of the antibody reagent can have a pronounced effect on the performance of immunoassays, which are frequently used to determine the concentration of a drug in a biological specimen.

Lysergic acid diethylamide (LSD)¹ is a controlled drug which is abused for its potent psychoactive properties. A typical dose is only between 50 and 100 μ g as a result of its unusually high potency, relative to other common hallucinogens such as psilocybin or mescaline. As a result of the small dose ingested and its rapid biotransformation, LSD detection in biological matrixes can be a formidable task for drug detection agencies. Immunoassay methodologies which rely on competitive binding principles require highly sensitive antibodies for successful measurements in the sub-microgram-per-liter region of forensic interest.

There have been surprisingly few developments in LSD immunogen design spanning the history of the drug's use. By far the most successful and widely used technique has been to couple the indole nitrogen of LSD to a carrier protein using a type of Mannich condensation, first described 25 years ago (3, 4). Protein haptenation has also been accomplished via carbodiimide-mediated linkage at the carboxyl group of lysergic acid (5, 6). The incorporation of a carbon spacer between the drug and carrier has been described for chemical linkages at both these reactive sites on the drug molecule (7, 8). At the present time, there are no known reports of LSD immunogens which utilize chemical linkages at sites other than N1 or C8, due to the complex chemistry of the drug and the limited number of reactive sites.

Recently, a new LSD immunogen was prepared using a novel haptenation procedure, whereby drug was coupled to the carrier protein in a nonspecific manner at a number of possible sites (9). Polyclonal antibodies raised against this immunogen were used to detect picogram quantities of LSD in urine by enzyme-linked immunosorbent assay (ELISA) (10). It is believed that the improved detection limits toward unmetabolized LSD were the results of multisite attachment of drug to carrier protein, which produces a theoretically superior antiserum (11). When LSD is coupled to keyhole limpet hemocyanin (KLH) using a photoreactive heterobifunc-

* Author to whom correspondence should be addressed, at the Department of Pathology and Laboratory Medicine. E-mail: don@pathology.ubc.ca. Tel: 604 822 7081. Fax: 604 822 7635.

[†] Department of Chemistry.

¹ BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; ELISA, enzyme-linked immunosorbent assay; IgG, immunoglobulin G; KLH, keyhole limpet hemocyanin; LAMPA, lysergic acid methyl-*N*-propylamide; LSD, lysergic acid diethylamide; MSR, molar substitution ratio; NHS, *N*-hydroxysuccinimide; PBS, phosphate-buffered saline; SASD, sulfosuccinimidyl-2-(*p*-azidosalicylamido)ethyl-1, 3'-dithiopropionate; TMB, 3,3',5,5'-tetramethylbenzidine.

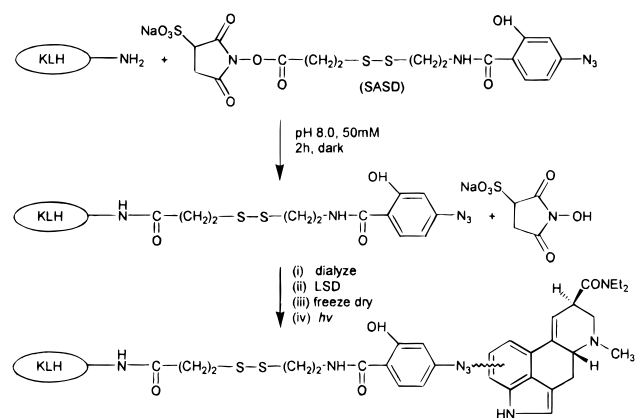


Figure 1. Schematic illustration of immunogen synthesis. The *N*-hydroxysuccinimide ester end of the photoreactive heterobifunctional linker, SASD, was coupled to amine residues on the protein, KLH, by nucleophilic substitution in the dark. After removal of excess hydrolyzed linker and buffer components by dialysis, LSD was added. Derivatized KLH and LSD were freeze-dried and the aryl azide was photoactivated under UV light. After the reaction, excess unbound drug was removed by dialysis.

tional linker, covalent attachments are the result of the relatively nonselective reactivity of the aryl azide moiety. Upon photoactivation, a highly reactive aryl nitrene is produced, which can react with neighboring drug molecules in a number of ways. This approach facilitates multisite attachments at positions on the drug molecule which would not normally be possible using group-specific coupling reagents. This technique reduces the chance that epitopes at the site of attachment are not recognized by the immune system, making more epitopes available for recognition and, therefore, increasing antiserum sensitivity.

Using the heterobifunctional linker sulfo succinimidyl-2-(*p*-azidosalicylamido)ethyl-1,3'-dithiopropionate (SASD), a two-step conjugation procedure was described for the synthesis of the LSD immunogen, KLH-SASD-LSD (9). In the first step, primary amines in KLH were coupled to the *N*-hydroxysuccinimide ester end of the linker in the absence of light. Following removal of excess linker, LSD was photochemically coupled to the aryl azide moiety of the modified protein, as outlined schematically in Figure 1.

This work describes a number of variables which were found to affect the conjugation procedure, in particular the overall efficiency of the reaction which was estimated from the molar substitution ratio (MSR). A theoretical discussion of feasible linkages is provided with respect to aryl azides. Finally, immunological characterization of the antiserum in a cross-reactivity study was used to obtain some structural insight into the possible sites of attachment during haptenation.

EXPERIMENTAL SECTION

Materials. Keyhole limpet hemocyanin, sulfo succinimidyl-2-(*p*-azidosalicylamido)ethyl-1,3'-dithiopropionate, and Slide-A-Lyzer dialysis cassettes were obtained from Pierce (Rockford, IL). Coomassie Brilliant Blue G-250 was supplied by Eastman Kodak (Rochester, NY). Fluorescamine [4-phenylspiro[furan-2(3H),1'-phthalan]-3,3'-dione], dithiothreitol (DTT), 3,3',5,5'-tetramethylbenzidine (TMB), and goat anti-rabbit IgG peroxidase were obtained from Sigma (St. Louis, MO). ACS grade inorganic salts, glycine, acids, organic solvents, and hydrogen peroxide were supplied from Fisher Scientific

(Edmonton, AB). Dimethyl sulfoxide (DMSO) was dried over molecular sieves prior to use.

Iso-LSD and lysergic acid methyl-*N*-propylamide (LAM-PA) were supplied by Dr. Kevin Gormley through the National Institute on Drug Abuse (NIDA) Drug Supply Program (Rockville, MD). Dr. Haro Avdovich of the Bureau of Drug Research (BDR), Health Canada, (Ottawa, ON) supplied *d*-lysergic acid diethylamide, dimethyltryptamine, *d*-lysergic acid, and lysergic acid amide. Ergonovine maleate, α -ergocryptine, ergotamine tartarate, lysergol, and *N*-demethyl-LSD (nor-LSD) were purchased from Sigma (St. Louis, MO). Research Biochemicals Inc. (Natick, MA) supplied ergocornine, ergocryptine, methylegonovine maleate, methysergide maleate, and *d*-lysergic acid diallylamide tartarate. Finally, 2-oxo-3-hydroxy-LSD was purchased from Radian International (Austin, TX).

Disposable flat-bottomed 96-well polystyrene microtiter plates were purchased from Corning (New York, NY). A solution of Carnation nonfat skim milk powder (5%) in 150 mM, pH 7.4, phosphate-buffered saline (PBS) was routinely used as the blocking solution in enzyme-linked immunosorbent assays. The TMB substrate solution (12), which was prepared immediately before use was made up as follows. Deionized water (16 mL) was added to pH 6.0 acetate/citrate buffer (4 mL) after which 200 μ L of 3,3',5,5'-tetramethylbenzidine in dimethyl sulfoxide (10 mg/mL) and 20 μ L hydrogen peroxide (3%) were added. The acetate/citrate buffer was made by adding 200 mM citric acid to 200 mM sodium acetate to give a final pH of 6.0. The Bradford dye binding reagent (13) was prepared by dissolving Coomassie Brilliant Blue G-250 (10 mg) in 5 mL of ethanol after which 10 mL of phosphoric acid (85%) was added and the total volume made up to 100 mL with deionized water.

Modification of Protein with SASD at Varying pH. The reaction efficiency of SASD with protein at different pH values was estimated by measuring the decrease in the number of amines before and after the reaction. Quantitative determination of primary amine residues was performed using fluorescamine as previously described (14). Briefly, a sample containing primary amines (1.5 mL) was vortex mixed with 0.5 mL fluorescamine (0.3 mg/mL) in acetone. After 10 min, the fluorescence was measured using a Turner model 430 Spectrofluorometer at excitation and emission wavelengths 395 and 475 nm, respectively. Glycine was routinely used as the standard to compensate for changes in fluorescence intensity over a range of pH.

It was necessary to remove the photoreactive aryl azide group of the derivatized protein prior to the fluorescamine assay. This was achieved by cleavage of the disulfide group using the reducing agent dithiothreitol (DTT). The percent of protein residues which were modified with SASD over a range of pH was measured using BSA in place of KLH, to minimize solubility problems. In the absence of light, 100 μ g of BSA was reacted with SASD (0.14 mg in dry DMSO) for 2 h in 50 mM phosphate or carbonate buffer between pH 7.0 and 9.5. Samples containing BSA in the absence of SASD were run in parallel for use as the blanks. DTT (0.06 mg in deionized water) was added to each sample and reacted for 1 h, after which excess linker and the photoreactive end were removed by centrifugal filtration (Centricon-10, Amicon, Oakville, ON). After isolation of the high molecular weight fraction of interest, the number of lysines which were underivatized was estimated from the ratio of fluorescence measurements before and after reaction at

different pH values. The percent of amines that were coupled to SASD was calculated by difference.

Derivatization of KLH with SASD. The *N*-hydroxysuccinimide ester of SASD was coupled to the carrier protein KLH by nucleophilic substitution at optimal pH. An improvement in yield was observed when the first conjugation step was performed directly in the dialysis cassette. This obviates the need for sample transfer after the reaction. The same cassette was rinsed and reused for dialysis in the second conjugation step to reduce additional sample loss due to adsorption on the membrane.

All steps preceding photolysis were performed in complete darkness or in the presence of a red safety light. SASD (5.0 mg) was dissolved in 50 μ L of DMSO which had been dried over molecular sieves. KLH (2.0 mg) dissolved in 1.5 mL of 100 mM, pH 8.0, phosphate buffer was injected into a 3 mL Slide-A-Lyzer dialysis cassette. SASD in DMSO was injected through a second port into the cassette with gentle agitation. A small air bubble (0.5 mL) was injected to facilitate mixing, and the cassette was taped to an oscillating table and allowed to react for 2 h in the dark. The cassette was then mounted on a floatation device and dialyzed against deionized water or PBS in the dark for 2.5 h with half-hourly water changes to remove the excess unreacted linker or its hydrolysis products. The high molecular weight solution containing the modified protein was removed from the dialysis cassette and stored in the dark until use. LSD (2.2 mg) was added and the mixture was freeze-dried in the absence of light prior to the photoactivation step.

Photoactivation with LSD. KLH which had been derivatized with SASD was photoactivated with LSD using different conditions. Due to the nonselective nature of the photocoupling reaction, the molar ratio of LSD to KLH during the reaction was investigated. The reaction was performed using modified KLH and LSD which had been freeze-dried in the absence of light. The photocoupling step was also performed in solution, in the presence of either water or buffer (PBS). Photoactivation was performed using a Chromato-Vue C3 viewing box (UltraViolet Products Inc., San Gabriel, CA). Samples were placed 10 cm from two long wave (General Electric G15T8, 15W) germicidal lamps and two short wave (Sylvania Blacklite F15T8-BL, 15W) phosphor-coated lamps, which emit light at 254 and 365 nm, respectively. A reaction time of 20 min was sufficient for photoactivation of the aryl azide of SASD, as determined by the 45% decrease in the absorption maximum (10) in accordance with the literature (15–17). Unbound LSD was removed by extensive dialysis over 24 h in a Slide-A-Lyzer cassette. TLC of the purified immunogen indicated that noncovalent incorporation of LSD into the protein was negligible.

Estimation of the Molar Substitution Ratio (MSR). Concentrations of LSD and KLH in the antigen were estimated using spectrofluorometry and colorimetric protein assays, respectively, as previously described (9). The purified immunogen was poorly soluble so antigen preparations were allowed to stand overnight at 4 °C, after which the clear supernatant layer containing the soluble fraction was removed for analysis. Quantitative estimates of LSD in the immunogen were based on the assumption that there was no change in the native fluorescence of LSD before and after reaction. The fluorescence spectrum of the soluble immunogen indicated the excitation and emission wavelengths were unchanged ($\lambda_{\text{ex}} = 322$ nm, $\lambda_{\text{em}} = 412$ nm), although it is not known how the intensity of the LSD fluorescence is

affected once the drug was bound. However, there was a good correlation between this method and the more widely used UV absorption method as demonstrated previously (3, 9). The drug content, therefore, was estimated directly from the spectrofluorometric calibration of LSD. The concentration of KLH in the soluble antigen was estimated by the Bradford method (13) in which dye-binding reagent (200 μ L) and sample (100 μ L) were mixed in a microtiter plate. The absorbance was measured using an SLT microtiter plate reader EAR 400 AT (SLT-Labinstruments, Austria) at 550 nm and the concentration of KLH in the immunogen was interpolated directly from the calibration graph.

Investigation of Immunogen Structure. Structural insights regarding the sites at which LSD was coupled to KLH in the immunogen were obtained by measuring the cross-reactivity of the polyclonal antiserum produced in rabbits which were immunized with the conjugate. A standard immunization protocol was used whereby 150 μ g of immunogen was injected subcutaneously in Freund's complete adjuvant followed by intramuscular booster injections in Freund's incomplete adjuvant at monthly intervals (18). The specificity of the antiserum toward a number of structurally related analogues to LSD was measured using a competitive binding ELISA (10). Polystyrene microtiter plates were coated overnight at 4 °C with 100 μ L BSA–LSD conjugate (10 mg/L in PBS) prepared by the Mannich reaction (3, 4). After treating uncoated sites on the well with 150 μ L of blocking solution (5% skim milk powder in PBS) for 30 min at 37 °C, wells were washed thoroughly with PBS. LSD or related compounds were diluted in blocking solution (10^{-7} – 10^{-12} M), and 50 μ L was added to the plate. An additional 50 μ L of rabbit anti-LSD in blocking solution was added, such that the overall serum dilution was 1:50. After incubation for 2.5 h at 37 °C, the plate was rinsed with PBS and 100 μ L goat anti-rabbit IgG peroxidase (1:1000) in blocking solution was added. Following a 30 min incubation at 37 °C, wells were washed with PBS and 100 μ L TMB substrate solution was added. After 5 min, the reaction was stopped using 50 μ L of 1 M sulfuric acid and the absorbance was measured at 450 nm using 620 nm as the reference using a microtiter plate reader.

RESULTS AND DISCUSSION

Derivatization of Protein with Linker. The incorporation of sufficient LSD for immune recognition is dependent on the successful modification of KLH with aryl azides. Only the unprotonated amines on the protein are reactive toward the NHS ester region of the linker; therefore, it is necessary to use a pH at which a significant number of amines are uncharged. Fluorescamine was used to determine the pH conditions at which the availability of unprotonated amines was maximized (Figure 2). A shift in pH from neutral to alkaline pH increased fluorescence, which is linearly related to the number of primary amines, more than 3-fold. This is in accordance with the deprotonation of lysine residues ($pK_a \approx 10$) which is the principal source of primary amines in the protein.

Fluorescence measurements before and after reaction were used to estimate the number of lysines which had been derivatized with SASD at different pH values. Figure 2 illustrates the delicate balance between competing aminolysis and hydrolysis reactions. Increasing stoichiometry with increasing pH is the result of deprotonation of amine groups whereas the decrease in reac-

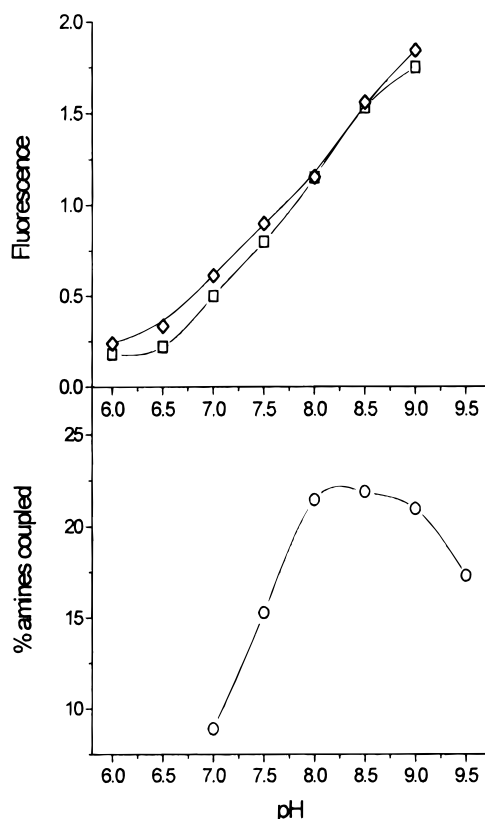


Figure 2. Effect of pH on availability of primary amines using the fluorescamine assay. BSA, 6.25 μg (diamonds), KLH, 25 μg (squares). The fluorescence signal, which is linearly related to the number of amines, was calculated relative to glycine, at the same pH. (Below) Effect of pH on reaction efficiency using BSA and SASD (circles). The percentage of amines coupled to linker was determined by difference using fluorescamine, before and after reaction.

tion efficiency at elevated pH is the result of NHS ester hydrolysis. A shift of 1 pH unit from 7.0 to 8.0 increased the derivatization of lysines by more than a factor of 2. Therefore, a pH of 8.0 was chosen as a compromise between the number of available amines and the lability of the ester. The ester moiety of SASD although stable at acid pH, hydrolyzes to *p*-azidosalicylamido-1,3'-dithiopropionate and *N*-hydroxysuccinimide (NHS) under alkaline conditions. The half-life of NHS-esters at pH 9, 7.5, and 5 is reported to be 1, 14, and >90 min, respectively (19). Optimum substitution is achieved when a large molar excess of linker is used to overcome inefficiencies due to hydrolysis (20, 21). When a 20 molar excess of linker to primary amines on KLH was used at pH 8.0, approximately 45% of the lysine residues were coupled to SASD. Assuming there to be about 689 lysine residues in the KLH molecule, this corresponds to a total of about 310 photoreactive linker moieties attached to each protein, assuming a molecular mass of 3×10^6 Da (9, 22).

Photochemical Coupling and the Molar Substitution Ratio. Estimation of the molar substitution ratio using LSD fluorescence and protein quantitation provided useful insight into the effect of photocoupling conditions on reaction efficiency. Most of the typical methods used for determining substitution ratios could not be used due to the very large size of KLH [350–13 000 kDa (23)] and the poor solubility of the immunogen ($\sim 5 \mu\text{g/mL}$). UV spectroscopic determination was not possible due to the overlapping absorption of SASD with both KLH and LSD. Indirect spectroscopic techniques,

such as the use of trinitrobenzene sulfonic acid (TNBS) allow the colorimetric determination of unreacted amino groups in the milligram per milliliter concentration range. Radiolabeled haptens can be used to measure the extent of coupling directly, by measuring the associated radioactivity of the conjugate. However, this technique is not advocated for photochemical coupling due to non specific ("dark") labeling or photodeiodination (15, 24). Mass spectrometry, particularly electrospray and matrix-assisted laser desorption ionization techniques, have become increasingly useful for the characterization of conjugates which are soluble at sufficient (approximate micromolar) concentration (25–27).

Characterization of the LSD immunogen in which relatively few drug molecules are attached to a protein with a molecular mass of several million is not an easy task. The fluorescence technique was the only method suitable for sensitive and specific estimation of LSD bound to KLH in the immunogen (9).

The combined fluorescence and Bradford dye-binding methods provides only a comparative approximation of the LSD content in the soluble immunogen fraction since it is dependent on a number of assumptions. The photolytic approach to immunogen synthesis likely results in a number of high molecular weight products with varying degrees of substitution. The first assumption is that the LSD content of the soluble fraction is representative of the entire sample, which might not be true. Solubility is likely to decrease with increasing substitution of protein which might result in the least soluble complexes (which have higher MSRs) precipitating out.

Optimization of Photocoupling Conditions. As expected, removal of excess unreacted linker prior to photoactivation was essential. Its presence in the reaction mixture severely compromised reaction efficiency by competing with drug for the limited number of aryl azide moieties. Liquid-phase photoactivation also decreased the MSR of the immunogen. When photolysis was performed in aqueous solution rather than on a freeze-dried sample, there was more than a 40% decrease in efficiency. Removal of water from the reaction mixture is preferred because the nitrene can react with water to form hydroxylamine (28), which decreases the substitution ratio. Despite reports of poor reaction efficiencies due to photolysis in aqueous solvents (16), there are no known reports in the literature of conjugation by dry photoactivation, as was routinely employed in this study.

Molar substitution ratios were measured over 2 orders of magnitude using dry photoactivation under various conditions. The covalent coupling of aryl azides on KLH with neighboring molecules is nonspecific. Therefore, to increase the chance of reaction with LSD, a large excess of drug was used. Figure 3 summarizes the results of 12 immunogen preparations performed over a number of weeks in which the molar ratio of LSD to KLH employed during photoactivation was varied. Dried down buffer components were present in the reaction matrix during irradiation. Clearly, the initial LSD/KLH ratio employed during photolysis determined the degree of substitution in the immunogen. There was a good correlation between increased concentration of LSD during photolysis and increasing molar substitution ratio ($r = 0.958$). The use of large excesses of drug during photolysis is a definite drawback, however, considering that LSD is a controlled substance which is available in limited supply. Reaction conditions which produced optimum substitution under these conditions could not

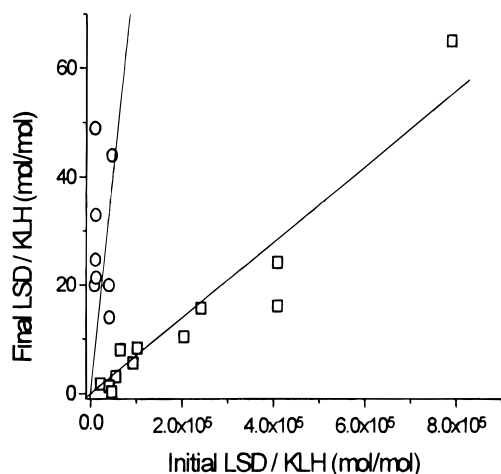


Figure 3. Effect of excess LSD on molar substitution ratio (MSR). Immunogen was prepared by irradiation of freeze-dried modified protein and LSD in the presence of dried buffer components (squares) and in the absence of buffer (circles).

be employed on a preparative scale for immunization of rabbits, because the quantity of drug required was too large.

However, dialysis of the immunogen matrix against deionized water prior to photoactivation showed improved performance (Figure 3). A dramatic increase in reaction efficiency was observed when buffer components were removed prior to irradiation. By this method, molar substitution ratios of 20–50 were attainable using only slight excesses of drug. This suggests that buffer components in the reaction matrix compete with LSD for the limited number of reactive nitrenes on the protein. In the absence of buffer, photoactivation of approximately equal amounts of KLH and LSD by weight produced conjugates with average substitution ratios of about 35.

Reaction Efficiency. The efficiency of the reaction was calculated from the percentage of lysine residues on KLH that were eventually coupled to LSD. The average two-step reaction efficiency for the injected immunogen was 5% assuming 35 LSDs attached to each KLH molecule, which contains about 689 lysine residues/ 3×10^6 Da molecule (9). It was estimated that only about 45% of the total lysines on KLH were derivatized with SASD. Therefore, the reaction efficiency of the photolysis step toward LSD using optimized conditions was approximately 11%, which is quite favorable considering the nonselective nature of the photochemical coupling. This is likely the result of solvent and buffer removal prior to photolysis and the presence of nucleophilic groups on LSD which attract the electrophilic nitrene. Typical efficiencies of photoreactive linkers which are used to couple high molecular weight species are generally low due to the nonspecific nature of the reaction. Reaction efficiencies of 15% using conventional aryl azide photoprobes such as SASD are considered good (29).

Reaction efficiencies vary greatly, depending on the type of linker used. The recent introduction of polyfluorinated phenyl azide heterobifunctional linkers, which generate strongly electrophilic nitrenes, result in greater efficiencies (30, 31) typically reported between 20 and 60% (32). The use of a fluorinated photoreactive linker and a carrier protein which contains more lysine residues would improve efficiency. The availability of primary amines on KLH and BSA at pH 8.0 was determined using the fluorescamine assay. Following linear regression analysis, a direct comparison of the slopes for BSA (0.233 ± 0.009 , $r = 0.997$) and KLH (0.057 ± 0.004 , $r = 0.987$)

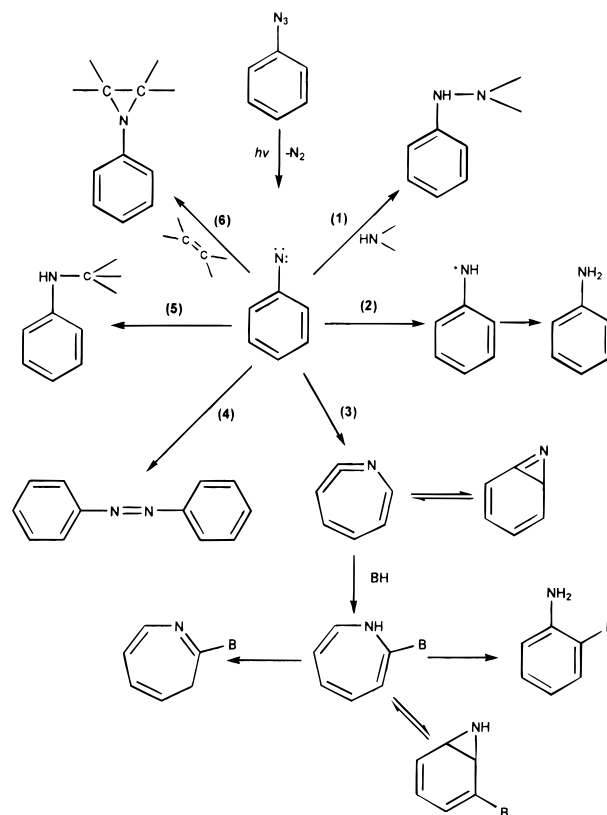


Figure 4. Important reactions of the aryl azide group.

indicated that KLH had only $25 \pm 3\%$ of the lysines available on BSA by weight. This finding suggests that overall higher reaction efficiencies might be achieved using albumin, although the immunogenicity of the derivatized BSA conjugate may be lower than that of KLH.

Theoretical Considerations. Insertion of aryl azides in model systems such as cyclohexane or diethylamine is well documented (33), although investigation of protein conjugates is rarely attempted. The relationship between insertion characteristics of the nitrene in a model system compared to a macromolecule is extraordinarily difficult due to the variety of functional groups and environments in the protein (16). The complex photochemistry of aryl azides involves three excited states and at least six short-lived ground-state intermediates. Photolysis of phenyl azides usually results in multiple reaction pathways of highly amorphous products which make interpretation and identification often difficult. To complicate things further, the products are highly variable, depending on reaction conditions such as an inert or reactive solvent systems, characteristics of the light source and substituent groups on the aryl azide ring. To gain some insight into the reactivity of the derivatized protein toward neighboring molecules, some important reactions of aryl azides are summarized in Figure 4. These include insertion into NH bonds to form hydrazines (1), reduction of the azido group (2), nucleophilic ring expansion to form azepines (3), formation of azobenzene (4), insertion into CH bonds (5), and addition across double bonds to form aziridines (6).

It should be noted, however, that the most favorable reaction pathways are primarily dependent on the electrophilic character of the nitrene and its environment. CH insertion products are produced using very strongly electrophilic nitrenes, such as polyfluorinated phenyl azides. Strong electron-withdrawing groups increase the

reactivity of the nitrene toward CH bond insertion. Aryl azides without such substituents are more likely to react with other groups, such as nucleophiles, especially if they are embedded in a nucleophilic environment, such as a protein molecule.

The lifetime of a reactive nitrene is about 10^{-4} s (34), although this depends on the substituent groups attached to the aryl ring (35). Aryl nitrenes or their intermediates may react with C—H, N—H, and aryl groups, which are present in the LSD molecule. The nitrene is one of the few reactive species that can react with C—H by abstraction and insertion, therefore allowing covalent attachment in the absence of any particular functional group. Because the species is particularly reactive toward nucleophilic groups, it is likely that a large part of the nitrene reactivity on the modified protein will be self-directed. However, the 19 Å spacer arm of SASD increases the likelihood of a bimolecular reaction (35).

An inherent disadvantage of the photocoupling technique is its low efficiency compared to most site-specific bioconjugation techniques. Yields resulting from photo-reactive linking of unsubstituted aryl azides are generally low, and efficiencies less than 10% are considered acceptable (20). Efficient coupling requires not only a reactive nitrene but also a favorable orientation toward the target molecule. To partly compensate for this, it is necessary to use a large excess of LSD to improve the probability that the photogenerated species is in proximity to a drug molecule.

Perhaps the most important difference between the literature reports of aryl nitrene chemistry and the system we describe is the use of a solid matrix for photoactivation. There have been only a few reports of the effect of temperature and phase on aromatic nitrene chemistry. Although the simple systems examined do not resemble our immunogen reaction matrix, a number of interesting observations have been made. In a restricted environment, such as solid state or ligand bound to macromolecule, radical coupling may predominate due to limited movement of the reactive species (36). It was suggested that the increase in CH insertion products via radical recombination in a "rigid" matrix was due to trapping aryl azides in the hydrophobic crevices of the protein, which resulted in higher protein labeling efficiencies (16).

Photoactivation of the aryl azides on KLH in the presence of LSD likely results in a great many products given the complexity of the reaction matrix and the diversity of functional groups which are present. A mixture of both favorable and unfavorable reaction products are contained in the high molecular weight fraction.

The most desirable reactions are those which take place between modified KLH and LSD, of which there are a number of hypothetical products (Figure 4). Azepine type conjugates (3) may result from the reaction between the electrophilic nitrene and nucleophilic substituents on LSD. Lone pairs on amino nitrogen and other nucleophiles are known to attract the nitrene. In LSD, the nitrogen in the 6 position is strongly basic due to the presence of three electron-donating alkyl groups. The indole nitrogen, which is less basic due to the aromaticity of the indole ring, could also react with a nitrene to form an azepine. Addition across double bonds (6) could also occur at a number of sites on the LSD molecule, although there are very few reports of stable aziridine products from such reactions (28). Singlet nitrenes can insert into CH bonds (5) with varying efficiencies depending on the CH bond strength. A possible site for this reaction could

be the acidic hydrogen in the 2 position, which would lead to formation of an amine. Phenyl azides are also capable of NH insertion reactions (1) which could take place at the indole nitrogen to produce a hydrazine product. In summary, there are a number of hypothetical reaction sites on LSD which would result in covalent attachment of the drug to the modified protein.

However, there are many undesirable reactions which might occur in the reaction matrix between aryl azide and neighboring amino acid residues. The reactivity of aryl nitrenes toward functional groups found in biological molecules is unquestionable. In particular, reactivity with aryl (Phe, Tyr, Trp, His), N—H (Lys, His, *N*-termini), O—H (Ser, Thr, Try), and S—H (Cys) has been suggested (37). In one report, photolysis of [^{125} I]SASD in the presence of BSA indicated incorporation of radiolabel into His, Thr, Lys, Cys, Glu, and Asp (38). It is quite likely that the bulk of the nitrene reactivity is directed toward these residues and not the target molecule, LSD. For this reason, it is necessary to use an excess of drug during photolysis, so that LSD is utilized as a "scavenger". These species are used in conventional photoaffinity labeling to preferentially react with photogenerated intermediates at places other than the ligand-binding site between protein and receptor. Nucleophilic molecules such as tris and *p*-aminobenzoic acid have been used for this purpose (35). In a sense, LSD which contains a number of groups which are reactive toward electrophilic nitrenes, is utilized as a scavenger to bind nonspecifically with photolabeled protein.

Poor efficiencies of photoreactive conjugation processes have been attributed to the unwanted reactions which occur between the aryl azide and water or buffer components in the mixture (35). Other unwanted reactions involve the formation of aniline (2) or azobenzene (4) type products. With respect to the latter case, the reaction between two aryl azide moieties in the immunogen is unlikely due to the limited number of reactive nitrenes and the restricted mobility of the derivatized protein in the solid-phase matrix. However, intramolecular cross-linking may occur if the two aryl azide groups are in close enough proximity.

Antibody Specificity and Immunogen Structure.

The cross-reactivity of anti-LSD antisera raised against the photocoupled immunogen was measured by ELISA. Regions of the drug molecule which demonstrate reduced specificity indicate that molecular transformation or conjugation may have taken place. An antibody will not recognize a particular epitope if it has not been exposed to it during immunization. This occlusion may occur as a result of the chemical linkage which exists between the hapten and carrier. Depending on the size of the protein and the distance between it and the hapten, areas surrounding the point of attachment may be obscured from the immune system. This is of particular importance for small molecules such as drugs of abuse, for which immunogen design plays a fundamental role in determining the overall antibody specificity (39–41). As a result, cross-reactivity studies can provide valuable insights into possible structural attachments which have taken place in the immunogen.

Antiserum cross-reactivity toward LSD analogues is illustrated in Figure 5. The approximate percent cross-reactivity was estimated from the concentration of analogue, which produced a signal equivalent to 1.55 nM LSD (0.5 ng/mL), which is the generally accepted cutoff concentration for LSD by immunoassay. The highest cross-reactivity was toward BSA-LSD coupled via the indole nitrogen (100%), 6-nor-LSD (52%) and LAMPA

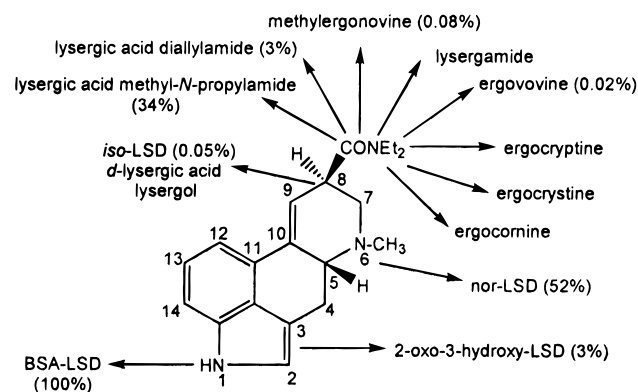


Figure 5. The specificity of the antibody raised against the immunogen was measured by ELISA. The approximate percent cross-reactivity was calculated from the concentration of analogue which produced a signal equivalent to 1.5 nM LSD. Measured cross-reactivities of 0.08% or greater are shown in parentheses.

(34%). In LAMPA, the two ethyl substituents on the amide side chain of LSD are replaced with propyl and methyl groups which do not significantly change the electronic properties of the drug. It is, therefore, no surprise that LAMPA cross-reacts with the antiserum to such an extent. A number of other compounds tested had structural differences at the amide position. When ethyl groups were replaced with hydrogen, in lysergic acid amide, no recognition occurred. In the same way, α -ergocryptine, ergocornine, ergocryptine, ergonovine, ergotamine, and methylethylgonovine, all of which are modified with large hydrocarbon groups at the amide position, do not significantly cross-react with the antiserum. When C_2H_5 is replaced with $CH_2-CH=CH_2$ in lysergic acid diallylamide, only minimal cross-reactivity was observed (<3%). The replacement of electron-donating ethyl groups with electron-withdrawing allyl groups makes the nitrogen less basic and significantly changes the properties of the molecule in such a way as to prevent effective antibody recognition.

Further insight is provided by analogues which differ from LSD at the C8 position. Replacement of the amide substituent with carboxylic acid or alcohol in *d*-lysergic acid and lysergol produced no cross-reaction and *iso*-LSD, which is a stereoisomer of LSD, cross-reacted only 0.05%. The antiserum recognizes the change in spatial arrangement of diethylamide in *iso*-LSD, thus producing a poor fit between the antibody-binding site and the drug. Overall, the antiserum exhibits good specificity in the vicinity of C8 and the amide. This suggests that, not only did conjugation not occur here but an epitope exists in this region.

Significant cross-reactivity was observed with nor-LSD which is modified in the N6 position. However, it has been shown that replacement of alkyl groups with hydrogen in the amide substituent sufficiently changed the properties of the drug, preventing effective antibody recognition. This not being the case in the N6 region might be suggestive of a covalent attachment having taken place. The reactive nitrene tends to be electrophilic in nature, thus having a preference for regions of high electron density, such as double bonds or lone pairs (28). The nitrogen at the 6 position is a tertiary amine, with three electron-donating groups. It is feasible that the electron-deficient nitrene is attracted to the lone pair on this basic nitrogen which subsequently results in covalent attachment at this point. Further evidence for the proposed reactivity between the aryl azide and nitrogen

is provided by the cross-reactivity of antibody with BSA-LSD, in which LSD is derivatized at the indole nitrogen (3). The indole nitrogen is less basic than the tertiary amine because of the electron-withdrawing double bonds of the indole ring. However, if the nitrene was attracted to the lone pair of electrons on the indole nitrogen, the subsequent chemical linkage would explain the failure of the antibody to distinguish the derivative from the parent drug. In addition to this, the hydrogen atom adjacent to the indole nitrogen is reactive. The removal of this acidic hydrogen results in a nucleophilic carbon in the 2 position which could also attract an electrophilic nitrene. A small cross-reactivity (3%) toward 2-oxo-3-hydroxy-LSD was observed. However, given the likelihood of an attachment at the indole nitrogen, it is possible that this cross-reaction is the result of the nearby linkage which depreciates immune recognition of the drug at adjacent positions.

Structural attachments within the immunogen are almost impossible to determine by conventional means due to the size and poor solubility of the immunogen. Therefore, the cross-reactivity of LSD-like substances provided valuable insight into possible sites of attachment in the immunogen. These data suggest that multisite attachment did occur between LSD and KLH, most likely at positions 1 and 6 of the drug, which is plausible given the reactivity of the photogenerated nitrene.

ACKNOWLEDGMENT

This work was supported by the Medical Research Council of Canada (Grant number MT5759 to D.E.B.).

LITERATURE CITED

- (1) Delmas, A., Brack, A., and Trudelle, Y. (1992) Studies of the Influence of Different Cross-linking Reagents on the Immune Response against a B-Epitope. *Bioconjugate Chem.* 3, 80–84.
- (2) Klaus, G. G. B., and Cross, A. M. (1974) The Influence of Epitope Density on the Immunological Properties of Hapten-Protein Conjugates. *Cell. Immunol.* 14, 226–241.
- (3) Taunton-Rigby, A., Sher, S. E., and Kelley, P. R. (1973) Lysergic Acid Diethylamide: Radioimmunoassay. *Science* 181, 165–166.
- (4) Twitchett, P. J., Fletcher, S. M., Sullivan, A. T., and Moffat, A. C. (1978) Analysis of LSD in Human Body Fluids By High-Performance Liquid Chromatography, Fluorescence Spectroscopy and Radioimmunoassay. *J. Chromatogr.* 150, 73–84.
- (5) Loeffler, L. J., and Pierce, J. V. (1973) Radioimmunoassay for Lysergide (LSD) in Illicit Drugs and Biological Fluids. *J. Pharm. Sci.* 62 (11), 1817–1820.
- (6) Van Vunakis, H., Farrow, T. T., Gjika, H. B., and Levine, L. (1971) Specificity of the Antibody Receptor Site to *d*-Lysergamide: Model of a Physiological Receptor for Lysergic Acid Diethylamide. *Proc. Natl. Acad. Sci. U.S.A.* 68 (7), 1483–1487.
- (7) Ratcliffe, W. A., Fletcher, S. M., Moffat, A. C., Ratcliffe, J. G., Harland, W. A., and Levitt, T. E. (1977) Radioimmunoassay of Lysergic Acid Diethylamide (LSD) in Serum and Urine by Using Antisera of Different Specificities. *Clin. Chem.* 23 (2), 169–174.
- (8) McNally, A. J., Goc-Szkatnicka, K., Li, Z., Pilcher, I., Polakowski, S., and Salamone, S. J. (1996) An OnLine Immunoassay for LSD: Comparison with GC-MS and the Abuscreen RIA. *J. Anal. Toxicol.* 20, 404–408.
- (9) Kerrigan, S. (1997) Immunochemical Detection of Lysergic Acid Diethylamide Using A Photochemically Linked Immunogen. Ph.D. Thesis, The University of British Columbia, Vancouver, Canada.

- (10) Kerrigan, S., and Brooks, D. E. (1998) Indirect Enzyme-Linked Immunosorbent Assay for the Quantitative Estimation of Lysergic Acid Diethylamine in Urine. *Clin. Chem.* 44, 985–990.
- (11) Strahilevitz, M. (1986) Treatment Methods, Particularly for Psychoactive Drug Dependence. United States Patent Number 4,620,977.
- (12) Bos, E. S., van der Doelen, A. A., van Rooy, N., and Schuur, A. H. W. M. (1981) 3,3',5,5'-Tetramethylbenzidine as a Ames Test Negative Chromogen for Horseradish Peroxidase in Enzyme Immunoassay. *J. Immunoassay* 2 (3/4), 187–204.
- (13) Bradford, M. M. (1976) A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein utilizing the Principle of Protein Dye Binding. *Anal. Biochem.* 72, 248–254.
- (14) Stocks, S. J., Jones, J. M., Ramey, C. W., and Brooks, D. E. (1986) A Fluorometric Assay of the Degree of Modification of Protein Primary Amines with Polyethylene Glycol. *Anal. Biochem.* 154, 232–234.
- (15) Koch, T., Suenson, E., Korsholm, B., Henriksen, U., and Buchardt, O. (1994) Pitfalls in Characterization of Protein Interactions Using Radioiodinated Cross-linking Reagents. Preparation and Testing of Novel Photochemical 125I-Label Transfer Reagent. *Bioconjugate Chem.* 5, 205–212.
- (16) Pandurang, R. S., Karra, S. R., Kuntz, R. R., and Volkert, W. A. (1995) High Efficiency Photolabeling of Human Serum Albumin and Human gamma-globulin with [¹⁴C]Methyl 4-azide-2,3,5,6-tetrafluorobenzoate. *Bioconjugate Chem.* 6, 630–634.
- (17) Wollenweber, H. W., and Morrison, D. C. (1985) Synthesis and Biochemical Characterization of a photoactivatable Iodinated, Cleavable Bacterial Lipopolysaccharide Derivative. *J. Biol. Chem.* 260 (28), 15068–15074.
- (18) Harlow, E., and Lane, D. (1988) *Antibodies: A Laboratory Manual*, pp 53–139, Cold Spring Harbour Laboratory, Plainville, NY.
- (19) Carlsson, J., Drevin, H., and Axen, R. (1978) Protein Thiolation and Reversible Protein-Protein Conjugation. N-Succinimidyl 3-(2-Pyridyldithio)propionate, A new Heterobifunctional Reagent. *Biochem. J.* 173, 723–737.
- (20) Mattson, G., Conklin, E., Desai, S., Nielander, G., Savage, M. D., and Morgensen, S. (1993) A Practical Approach to Cross-linking. *Mol. Biol. Rep.* 17, 167–183.
- (21) De-Villiers, M., Parkin, D., Van Jaarsveld, P., and Van der Walt, B. (1987) A Radioimmunoassay for Metoclopramide. *J. Immunol. Methods* 103, 33–39.
- (22) Shuler, K. R., Dunham, R. G., and Kanda, P. (1992) A Simplified Method for Determination of Peptide-Protein Molar Ratios Using Amino Acid Analysis. *J. Immunol. Methods* 156, 137–149.
- (23) van Brugen, E. F. J. (1980) Hemocyanin: the mystery of blue blood. *Trends Biochem. Sci.* 5, 185–188.
- (24) Cai, S., Glenn, D. J., and Keana, J. F. W. (1992) Toward the Development of Radiolabeled Fluorophenyl Azide-Based Photolabeling Reagents: Synthesis and Photolysis of Iodinated 4-Azidoperfluorobenzoates and 4-Azido-3,5,6-trifluorobenzoates. *J. Org. Chem.* 57, 1299–1304.
- (25) Adamczyk, M., Buko, A., Chen, Y., Fishpaugh, J. R., Gebler, J. C., and Johnson, D. D. (1994) Characterization of Protein-Hapten Conjugates. 1. Matrix Assisted Laser Desorption Ionization Mass Spectrometry of BSA-Hapten Conjugates and Comparison with Other Characterization Methods. *Bioconjugate Chem.* 5, 631–635.
- (26) Straub, K. M., and Levy, M. J. (1994) Characterization of Hapten Binding to Immunoconjugates by Electrospray Ionization Mass Spectrometry. *Bioconjugate Chem.* 5, 194–198.
- (27) Adamczyk, M., Gebler, J. C., and Mattingly, P. G. (1996) Characterization of Protein-Hapten Conjugates. Electrospray Mass Spectrometry of Bovine Serum Albumin-Hapten Conjugates. *Bioconjugate Chem.* 7, 475–481.
- (28) Patai, S. (1971) *The Chemistry of the Azido Group*, Interscience, New York.
- (29) Thevenin, B. J. M., Shahrokh, Z., Williard, R. L., Fujimoto, E. K., Kang, J. J., Ikemoto, N., and Shohet, S. B. (1992) A Novel Photoactivatable Cross-linker for the Functionally-Directed Region-Specific Fluorescent Labeling of Proteins. *Eur. J. Biochem.* 206, 471–477.
- (30) Brinkley, M. (1992) A Brief Survey of Methods for Preparing Protein Conjugates with Dyes, Haptens, and Cross-linking Reagents. *Bioconjugate Chem.* 3, 2–13.
- (31) Yan, M., Cai, S. X., Wybourne, M. N., and Keana, F. W. (1994) N-Hydroxysuccinimide Ester Functionalized Perfluorophenyl Azides as Novel Photoactive Heterobifunctional Cross-Linking Reagents. The Covalent Immobilization of Biomolecules to Polymer Surfaces. *Bioconjugate Chem.* 5, 151–157.
- (32) Kym, P. R., Carlson, K. E., and Katzenellenbogen, J. A. (1995) Evaluation of a Highly Efficient Aryl Azide Photoaffinity Labeling Reagent for the Progesterone Receptor. *Bioconjugate Chem.* 6, 115–122.
- (33) Schrock, A. K., and Schuster, G. B. (1984) Photochemistry of Phenyl Azide: Chemical Properties of the Transient Intermediates. *J. Am. Chem. Soc.* 106, 5228–5234.
- (34) Wilson, D. F., Miyata, Y., Ericiniska, M., and Vanderkooi, J. M. (1975) An Aryl Azide Suitable for Photoaffinity Labeling of Amine Groups in Proteins. *Arch. Biochem. Biophys.* 171, 104–107.
- (35) Das, M., and Fox, C. F. (1979) Chemical Cross-linking in Biology. *Annu. Rev. Biophys. Bioeng.* 8, 165–193.
- (36) Keana, J. F. W., and Cai, S. (1990) New Reagents for Photoaffinity Labeling: Synthesis and Photolysis of Functionalized Perfluorophenyl Azides. *J. Org. Chem.* 55, 3640–3647.
- (37) Bayley, H., and Staros, J. V. (1984) Photoaffinity Labeling and Related Techniques. *Azides and Nitrenes. Reactivity and Utility* (Scriven, E. F. V. Ed.) pp 439–460, Academic Press, Orlando.
- (38) Ueno, H., Masuko, T., Wang, J., and Hashimoto, Y. (1994) Epitope Mapping of Bovine Serum Albumin Using Monoclonal Antibodies with a Photoreactive Cross-linker. *J. Biochem.* 115, 1119–1127.
- (39) Adamczyk, M., Fishpaugh, J., Harrington, C., Hartter, D., Johnson, D., and Vanderbilt, A. (1993) Immunoassay Reagents for Psychoactive Drugs. I. The Method for the Development of Antibodies Specific to Amitriptyline and Nortriptyline. *J. Immunol. Methods* 162, 47–58.
- (40) Adamczyk, M., Grote, J., Douglas, J., Dubler, R., and Harrington, C. (1997) Synthesis of Conjugates for a Barbiturate Screening Assay. *Bioconjugate Chem.* 8, 281–288.
- (41) Schoneberg, T., Erhard, M. H., Kellner, J., Kuhn, S. O., and Losch, U. (1995) The Specificity and Affinity of Polyclonal Antibodies Raised in Rabbits Against a Hapten Conjugated to a Rabbit Serum Albumin. *J. Immunoassay* 16 (1), 17–35.

Preparation of a Spermine Conjugate of the Bis-phenanthroline Ligand Clip-Phen and Evaluation of the Corresponding Copper Complex

Marguerite Pitié and Bernard Meunier*

Laboratoire de Chimie de Coordination du CNRS, 205 route de Narbonne, 31077 Toulouse Cedex 4, France.
Received March 23, 1998; Revised Manuscript Received July 2, 1998

We described the synthesis of conjugates of *N,N*-diethylethylenediamine and spermine with Clip-Phen, a new ligand with two 1,10-phenanthrolines linked by serinol. The ability of the copper complexes of these Clip-Phen conjugates to cleave Φ X174 DNA when activated by a reductant in the presence of air was compared to that of 1,10-phenanthroline copper complexes. The results indicated that Clip-Phen–spermine with copper was the most active DNA cleaver. Comparison with a conjugate having only one phenanthroline unit linked to spermine showed that the high activity of Clip-Phen–spermine–Cu was not only due to the presence of spermine, which increases the affinity of the Clip-Phen entity for the DNA, but was effectively due to the presence of the two bridged phenanthroline entities. The DNA cleavage activity of (Clip-Phen–spermine) CuCl_2 was analyzed on a 167 bp restriction fragment and a 45-mer double-stranded oligonucleotide.

INTRODUCTION

Transition-metal complexes endowed with redox properties and DNA affinity have been developed as chemical cleavers of nucleic acids for the last two decades. Their ability to cleave DNA has several possible applications that include probing structural variations in nucleic acids, identification of binding sites of DNA ligands, design of artificial DNA cleavers, and potential chemotherapeutic agents. Typical examples are Fe^{III} -EDTA, manganese^{III}-porphyrin, Co, Rh or Ru complexes of phenanthroline or bipyridine, and the classical $[(\text{Phen})_2\text{Cu}^{\text{II}}]^{2+}$ complex (1–4).

1,10-Phenanthroline is a versatile ligand able to chelate copper to give mono-Phen or bis-Phen copper complexes at the oxidation state I or II (5). The $(\text{Phen})_2\text{Cu}^{\text{I}}$ complex in the presence of hydrogen peroxide efficiently cleaves DNA by oxidative attack on deoxyribose units from the DNA minor groove (6, 7). The Phen– Cu^{I} complex is less efficient (8). DNA cleavage products include 5'- and 3'-monophosphate ester termini, free bases, 5-methylenefuranone, and a small amount of 3'-phosphoglycolate (9–11). Hydrogen peroxide can be generated closely to the DNA by the $(\text{Phen})_2\text{Cu}^{\text{II}}$ itself in the presence of a reductant and molecular oxygen. $(\text{Phen})_2\text{Cu}$ cleaves double-stranded DNA at all nucleotides, but some preferences have been observed for particular conformations of B-DNA, lac-operon control region, or TAT triplets (12–15).

Different conjugates between phenanthroline and targeting DNA ligands have been prepared in order to enhance the cleavage specificity but also the reactivity

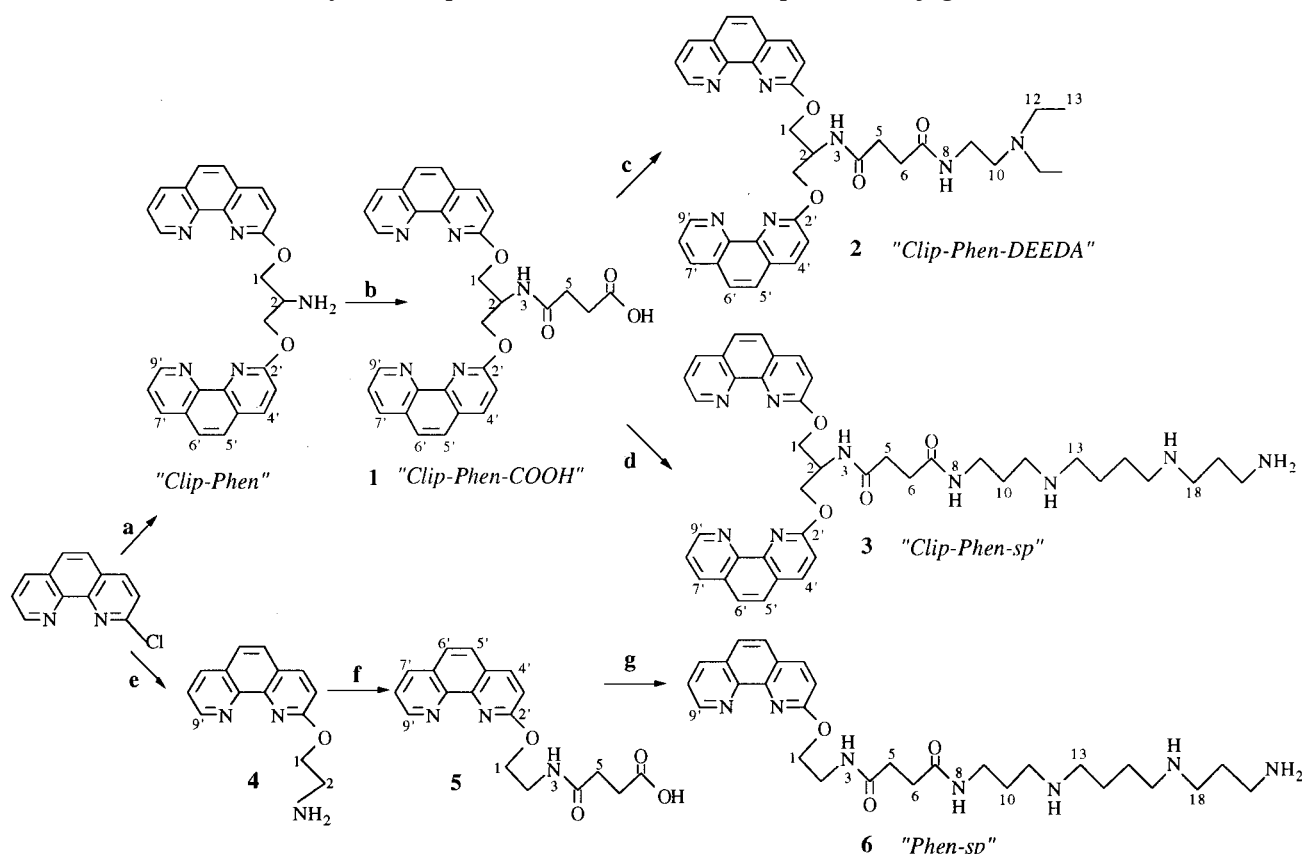
of the corresponding copper complexes by increasing their affinity for the DNA. For example, 1,10-phenanthroline has been conjugated to oligonucleotides, minor groove binders such as Hoescht-33258 or DNA binding proteins (16–22). But all these chimeric compounds contain only one phenanthroline unit, which is not the best situation for DNA cleavage, since the higher cleavage activities have been observed with a Phen/Cu ratio of 2–1.

We have recently prepared Clip-Phen (Scheme 1), a new ligand with two 1,10-phenanthroline entities linked via their C2-carbon by a short flexible arm adapted for having both phenanthrolines on the same copper ion (23). In the presence of copper(II) and a reductant and air, we observed on Φ X174 a better DNA cleavage activity for Clip-Phen than for 1,10-phenanthroline. Furthermore, this ligand has an exogenous primary amine to facilitate the attachment of different possible vectors in order to increase and to modulate the binding domain of this DNA cleaver.

We report here the synthesis of new conjugates of Clip-Phen with *N,N*-diethylethylenediamine (DEEDA) or spermine (sp) and the DNA cleavage activity of the corresponding copper complexes. We chose DEEDA in order to have a new amine-containing ligand with a terminal tertiary amine attached to Clip-Phen. Since DEEDA is easily protonated in physiological conditions, the presence of the terminal tertiary amine should be an additional favorable factor for DNA interactions of this molecule (Clip-Phen-DEEDA **2**, Scheme 1). We also used spermine to create another Clip-Phen conjugate (Clip-Phen-sp **3**, Scheme 1) which should have an enhanced DNA affinity compared to Clip-Phen itself, since this natural polyamine, protonated in physiological conditions, exhibits a strong DNA affinity (24–28). Spermine has been successfully used in different conjugates to enhance their affinity for DNA and consequently their reactivity (29–33). Since the conjugate Clip-Phen-spermine was particularly active (see below), we also decided to prepare a Phen–spermine conjugate (Phen-sp **6**, Scheme 1) with only one phenanthroline unit linked to

* Author to whom correspondence should be addressed.
E-mail: bmeunier@lcc-toulouse.fr.

¹ Abbreviations: BOP, benzotriazol-1-yloxy-tris(dimethylamino)phosphonium hexafluorophosphate; bp, base pair; DEEDA, *N,N*-diethylethylenediamine; HOBt, 1-hydroxybenzotriazole; MPA, mercaptopropionic acid; ODN, oligodeoxyribonucleotide; Phen, 1,10-phenanthroline (also abbreviated as oP); sp, spermine = *N,N'*-bis(3-aminopropyl)-1,4-butanediamine.

Scheme 1. Reaction Pathways for Preparation of the Different Clip-Phen Conjugates^a

^a Yields of each steps are given in brackets. Numbering corresponds to NMR assignments (a) Serinol, NaH in DMF (72%). (b) Succinic anhydride in chloroform (79%). (c) BOP, HOBT, *N*-methylmorpholine, DEEDA, in dichloromethane (100%). (d) BOP, HOBT, *N*-methylmorpholine in DMF, then spermine in DMF/water (86%). (e) Ethanolamine, NaH in DMF (55%). (f) Succinic anhydride in chloroform (88%). (g) BOP, HOBT, *N*-methylmorpholine in DMF, then spermine in DMF/water (63%).

spermine in order to confirm that the high DNA cleavage activity of Clip-Phen-sp was effectively due to the presence of the two phenanthroline entities. The DNA cleavage activity of (Clip-Phen-spermine) Cu^{II} was analyzed with different modes of activation (dioxigen and a reductant, hydrogen peroxide, etc.) on phage ΦX174 , a restriction fragment and an oligonucleotide and was compared to that of (Clip-Phen) Cu^{II} and (Phen) $_2\text{Cu}^{\text{II}}$ complexes.

MATERIALS AND METHODS

General Methods. Proton NMR spectra were recorded on Bruker 250 MHz. UV-vis spectra were recorded on a Hewlett-Packard 8452A diode array spectrophotometer using cuvettes of 1 cm path length. Syntheses has been monitored by thin-layer chromatography on Macherey-Nagel Alugram Sil G/UV 254, eluted by toluene/methanol (7:3, v/v) with saturation of the cuve by ammoniac (3 mL of concentrated NH_4OH in a small open flask), and spots were visualized with UV-light (violet spots at 254 nm and blue fluorescence at 365 nm for derivatives of the Clip-Phen family). DMF was dried on calcium sulfate and CH_2Cl_2 on basic aluminum oxide. Other commercial reagents were used without purification.

Synthesis. 2-Chloro-1,10-phenanthroline (34, 35) and Clip-Phen (23) have been prepared as previously described.

2-Succinoyl-amino-1,3-bis(1',10'-phenanthrolin-2'-yl-oxy)propane [Clip-Phen-COOH (1)]. To a solution of Clip-Phen (396 mg, 0.88 mmol) in chloroform (100 mL) was added succinic anhydride (254 mg, 2.4 mmol) and the

mixture was heated at reflux for 1 h. After evaporation of the solvent, the crude product was redissolved in hot methanol (250 mL), precipitated at -20°C , and centrifuged for 20 min at 3000 rpm. The product was then dissolved in chloroform and precipitated with diethyl ether to give Clip-Phen-COOH (1) as a white powder (377 mg, 79%). ^1H NMR (CDCl_3 , 250 MHz) δ 2.69 (m, 4H, H5 and H6), 5.00 (m, 5H, H1 and H2), 7.12 (d, 2H, J = 8.7 Hz, H3'), 7.59 (dd, 2H, J = 8.1, 4.5 Hz, H8'), 7.64 (d, 2H, J = 8.7 Hz, H6'), 7.72 (d, 2H, J = 8.7 Hz, H5'), 8.07 (d, 2H, J = 8.7 Hz, H4'), 8.23 (dd, 2H, J = 8.1, 1.65 Hz, H7'), 8.96 (d, 1H, J = 6.6 Hz, H3), 9.13 (dd, 2H, J = 4.5, 1.65 Hz, H9'). MS (CDI, NH_3): m/z (%) 548 ($M + 1$, 1.3), 530 (4.5), 504 (7.5), 448 (3), 352 (4), 308 (3), 252 (31.5), 197 (100). UV (CH_3OH): 226 (ϵ = 78 400 $\text{M}^{-1}\text{cm}^{-1}$), 274 nm (ϵ = 53 000).

2-Succinoyl-amino-1,3-bis(1',10'-phenanthrolin-2'-yl-oxy)propane-*N,N*-diethyl Ethylenediamine Conjugate [Clip-Phen-DEEDA (2)]. To 1 (210 mg, 0.38 mmol) in dry CH_2Cl_2 (20 mL), *N*-methylmorpholine (120 μL , 1.09 mmol), BOP (485 mg, 1.09 mmol), HOBT (200 mg, 1.48 mmol) and *N,N*-diethylethylenediamine (508 μL , 3.61 mmol) was added. After stirring 40 min at ambient temperature, triethylamine (508 μL , 3.64 mmol) was added and the mixture was stirred at room temperature for 20 h. The reaction mixture was diluted with water and the product was extracted three times with CH_2Cl_2 (3×100 mL). The organic phase was dried with sodium sulfate, and after concentration under vacuum, the product was precipitated with diethyl ether to give Clip-Phen-DEEDA 2 as a yellow powder (254 mg, 100%). ^1H NMR (CD_2Cl_2 , 250 MHz): δ 1.16 (t, 6H, J = 7.5 Hz, H13), 2.49 and 2.94

(2t, 2 × 2H, H5 and H6), 3.19 (m, 6H, H10 and H12), 3.66 (dd, 2H, H9), 4.78 (m, 2H, H1), 4.98 (m, 3H, H1 and H2), 7.25 (d, 2H, $J = 9$ Hz, H3'), 7.51 (m, 1H, H8), 7.74 (dd, 2H, $J = 8.0, 4.0$ Hz, H8'), 7.78 and 7.87 (AX, 2 × 2H, $J = 9.0$ Hz, H5' and H6'), 8.25 (d, 2H, $J = 9.0$ Hz, H4'), 8.42 (dd, 2H, $J = 8.0, 2.0$ Hz, H7'), 9.03 (dd, 2H, $J = 4.0, 2.0$ Hz, H9'), 10.00 (d, 1H, $J = 6.0$ Hz, H3). MS (ES, positive mode) m/z 646.3 ($M + 1$) (calcd for $C_{37}H_{39}N_7O_4 = 645.76$). UV (CH_3OH): 226 ($\epsilon = 58\,800$), 274 nm ($\epsilon = 38\,200$).

2-Succinoyl-amino-1,3-bis(1',10'-phenanthroline-2'-yl-oxy)propane-spermine Conjugate [Clip-Phen-sp (3)]. To **1** (200 mg, 0.36 mmol) in dry DMF (40 mL), *N*-methylmorpholine (2 mL, 18.2 mmol), BOP (485 mg, 1.09 mmol), and HOBT (200 mg, 1.48 mmol) were added, and after 40 min at 50 °C, this mixture was added during 20 min on a solution of spermine (1.5 g, 7.74 mmol) in water (40 mL) and stirred at 50 °C for 15 h. The reaction mixture was diluted with water (60 mL) and extracted with CH_2Cl_2 (3 × 100 mL). The organic phase was dried on sodium sulfate and evaporated. The product was redissolved in CH_2Cl_2 and precipitated with diethyl ether to give Clip-Phen-sp (**3**) as a white powder (230 mg, 86%). ^1H NMR (CD_2Cl_2 , 250 MHz): δ 1.59 and 1.70 (2 m, 2 × 4H, H10, H19 and H14, H15), 2.49 (t, 2H), 2.55–3.10 (m, 10H), 3.28 (m, 2H, H9), 4.83 (m, 5H, H1 and H2), 7.12 (d, 2H, $J = 6.5$ Hz, H3'), 7.47 (m, 1H, H8), 7.64 (dd, 2H, $J = 8.0, 4.5$ Hz, H8'), 7.70 and 7.77 (AX, 2 × 2H, $J = 8.5$ Hz, H5' and H6'), 8.16 (d, 2H, $J = 8.5$ Hz, H4'), 8.27 (dd, 2H, $J = 8.5, 1.5$ Hz, H7'), 9.09 (dd, 2H, $J = 4.5, 1.5$ Hz, H9'), 9.27 (d, 1H, $J = 6.5$ Hz, H3). MS (ES, positive mode): m/z 732.5 ($M + H$) (calcd for $C_{41}H_{49}N_9O_4 = 731.90$). UV (CH_3OH): 226 ($\epsilon = 70\,700$), 274 nm ($\epsilon = 46\,800$).

2-Amino-1-(1',10'-phenanthroline-2'-yloxy)ethane (4). To sodium hydride (688 mg, 17.17 mmol) in dry DMF (10 mL) on ice bath were added 2-chloro-1,10-phenanthroline (500 mg, 2.33 mmol) and ethanolamine (200 μ L, 3.26 mmol), and the mixture was stirred 24 h with return to room temperature. The crude product was then dissolved with DMF (30 mL), ethanol (10 mL), and water (100 mL), and the volume was reduced to 75 mL before to be extracted with CH_2Cl_2 (3 × 75 mL). The product was dried under vacuum, redissolved in CH_2Cl_2 and precipitated with diethyl ether to give **4** as a pale yellow powder (304 mg, 55%). ^1H NMR ($CDCl_3$, 250 MHz): δ 3.17 (t, 2H, $J = 5.3$ Hz, H2), 4.77 (t, 2H, $J = 5.3$ Hz, H1), 7.14 (d, 1H, $J = 8.5$ Hz, H3'), 7.56 (dd, 1H, $J = 8.0, 4.4$ Hz, H8'), 7.64 and 7.73 (AX, 2H, $J = 8.6$ Hz, H5' and H6'), 8.11 (d, 1H, $J = 8.5$ Hz, H4'), 8.21 (dd, 1H, $J = 8.0, 1.6$ Hz, H7'), 9.14 (dd, 1H, $J = 4.4, 1.6$ Hz, H9'). MS (CDI, NH_3): m/z (%) = 240 ($M + 1$, 100), 197 (10.8). UV (CH_3OH): 228 ($\epsilon = 40\,200$), 274 ($\epsilon = 26\,900$). Anal. Calcd for $C_{14}H_{13}N_3O \cdot 1/2H_2O$: C, 67.73; H, 5.68; N, 16.92. Found: C, 67.59; H, 5.31; N, 17.31.

2-Succinoyl-amino-1-(1',10'-phenanthroline-2'-yloxy)ethane (5). To a solution of **4** (200 mg, 0.84 mmol) in chloroform (180 mL) was added succinic anhydride (232 mg, 2.2 mmol), and the mixture was heated at reflux for 1 h. A precipitate was formed during the reaction which was filtrated after cooling, dried under vacuum, and reprecipitated in hot methanol to give **5** as a white powder (231.5 mg, 88%). ^1H NMR (DMSO- d_6 , 250 MHz): δ 2.56 (s, 4H, H5 and H6), 3.70 (dd, 2H, $J = 5.5, 5.3$ Hz, H2), 4.71 (t, 2H, $J = 5.5$ Hz, H1), 7.39 (d, 1H, $J = 8.6$ Hz, H3'), 7.86 (dd, 1H, $J = 8.3, 4.3$ Hz, H8'), 7.98 and 8.08 (AX, 2H, $J = 8.7$ Hz, H5' and H6'), 8.53 (d, 1H, $J = 8.6$ Hz, H4'), 8.59 (dd, 1H, $J = 8.3, 1.7$ Hz, H7'), 8.64 (t, 1H, $J = 5.2$ Hz, H3), 9.23 (dd, 1H, $J = 4.3, 1.7$ Hz,

H9'), 12.21 (s, 1H, COOH). MS (CDI, NH_3): m/z (%) 340 ($M + 1$, 100), 296 (3), 240 (4), 197 (10). UV (CH_3OH): 226 ($\epsilon = 47\,100$), 274 nm ($\epsilon = 32\,700$). Anal. Calcd. for $C_{18}H_{17}N_3O_3 \cdot 1/2H_2O$: C, 62.01; H, 5.16; N, 12.05. Found: C, 61.84; H, 4.50; N, 11.99.

2-Succinoyl-amino-1-(1',10'-phenanthroline-2'-yloxy)ethane-spermine Conjugate (Phen-sp (6)). To **5** (200 mg, 0.59 mmol) in dry DMF (68 mL), *N*-methylmorpholine (3.4 mL, 30.92 mmol), BOP (822 mg, 1.85 mmol), and HOBT (340 mg, 2.50 mmol) were added. After 40 min at 50 °C, the mixture was added during 20 min on a solution of spermine (2.54 g, 12.53 mmol) in water (68 mL) and the mixture was stirred at 50 °C for 15 h. The reaction mixture was diluted with water (100 mL) and alcalinized to pH 13 with NaOH before extraction with $CHCl_3$. After evaporation of the organic phase, the product was redissolved in $CHCl_3$ and precipitated with diethyl ether to give Phen-sp **6** as a white wax (195 mg, 63%). ^1H NMR (DMSO- d_6 , 250 MHz): δ 1.48 (m, 4H, H14 and H15), 1.58 (m, 2H, H8 and H19), 2.45 (m, 2H), 2.55 (m, 8H), 3.16 (m, 4H), 3.69 (m, 2H, H2), 4.70 (t, 2H, $J = 5.5$ Hz, H1), 7.37 (d, 1H, $J = 8.6$ Hz, H3'), 7.86 (dd, 1H, $J = 8.0, 4.3$ Hz, H8'), 7.98 (m, 1H, H8), 7.98 and 8.08 (AX, 2H, $J = 8.8$ Hz, H5' and H6'), 8.53 (d, 1H, $J = 8.6$ Hz, H4'), 8.59 (dd, 1H, $J = 8.0, 1.7$ Hz, H7'), 8.66 (t, 1H, $J = 5.13$ Hz, H3), 9.23 (dd, 1H, $J = 4.3, 1.7$ Hz, H9'). MS (ES, positive mode): $m/z = 524.3$ ($M + H$) (calcd for $C_{28}H_{41}N_7O_3 = 523.68$). UV (CH_3OH): 226 ($\epsilon = 36\,400$), 276 nm ($\epsilon = 24\,300$).

DNA Cleavage Experiments. Metalations by $CuCl_2$ of the different ligands used in DNA cleavage experiments were performed by using 1 or 2 equiv of the desired ligand in a DMF/water mixture (1:1, v/v; the final copper concentration being 1 mM) during 4 h at room temperature before dilution of the corresponding complex with water.

Comparison of the DNA Cleavage Activity of the Copper Complexes of 2, 3, and 6 by Cleavage of Φ X174. To 10 μ L of Φ X174 (7 nM, 40 μ M in bp) in 80 mM sodium phosphate buffer (pH 7.2), 100 mM NaCl, 20 mM $MgCl_2$ were added 5 μ L of the desired complex (4 μ M). After 30 min at room temperature, DNA cleavage was initiated by addition of an aqueous solution of MPA (5 μ L, 20 mM) and samples were incubated for 1 h at 37 °C. Of a solution of 50% (v/v) glycerol/40 mM Tris-Cl buffer (pH 8) and 0.05% bromophenol blue (w/v), 75 μ L, was then added, and samples were immediately loaded on agarose gel (0.8%) containing 1 μ g/mL of ethidium bromide. Electrophoresis was run at constant current (25 mA for 15 h) in TBE buffer. Bands were located by UV light (254 nm), photographed and quantified by microdensity. The correction coefficient 1.47 was used for the decrease of stainability of form I DNA versus forms II and III (36). A Poisson distribution was used to describe cleavage reactions. The average number of strand scissions per DNA phage, μ , is equal to $-\ln$ (fraction of form I). When only DNA forms I and II are formed, this number equals the average number of single-stranded cuts per molecule of phage DNA. When form I was not observable, μ was considered to be superior to 4.6 (value corresponding to 1% of form I).

Cleavage of a Restriction Fragment by (Clip-Phen-sp)Cu. pBR322 plasmid (12.5 μ g) was opened with *Eco*RI (100 units) and labeled on the 5'-termini with T4 polynucleotide kinase (10 units) and [γ - ^{32}P]ATP (10 pmol) in an exchange buffer. A 167 bp fragment, uniquely labeled on the template strand, was created by secondary restriction with *Rsa*I (100 units) and purified on 8% polyacrylamide gel before to be desalted on G25

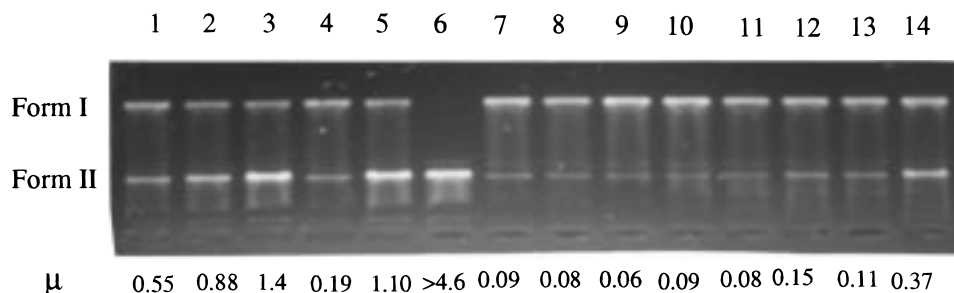
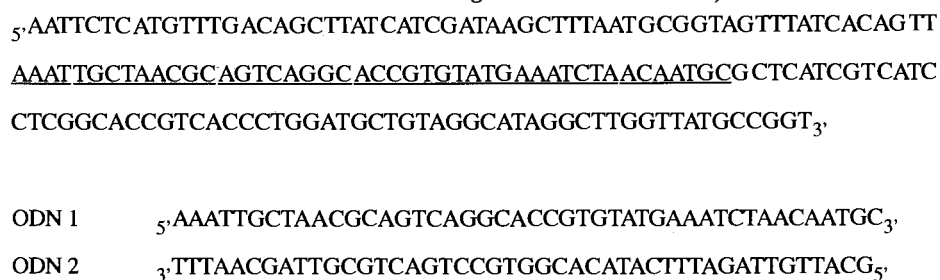


Figure 1. Comparison of Φ X174 cleavage efficiency between Phen, Clip-Phen, Clip-Phen-COOH, Clip-Phen-DEEDA, Clip-Phen-sp and Phen-sp in the presence of CuCl_2 1 μM and MPA 5 mM. Lane 1: Phen 1 μM . Lane 2: Phen 2 μM . Lane 3: Clip-Phen 1 μM . Lane 4: Clip-Phen-COOH 1 μM . Lane 5: Clip-Phen-DEEDA 1 μM . Lane 6: Clip-Phen-sp 1 μM . Lane 7: Phen-sp 1 μM . Lane 8: Phen-sp 2 μM . Lane 9: control DNA. Lane 10: control DNA plus MPA 5 mM. Lane 11: CuCl_2 1 μM , MPA 5 mM without ligand. Lanes 12, 13, and 14: Phen 2 μM , Clip-Phen 1 μM and Clip-Phen-sp 1 μM , respectively, with MPA 5 mM but without CuCl_2 . Single-strand breaks were expressed as μ values representing $-\ln(\text{fraction of form I})$.

Scheme 2. Sequence of the *Eco*RI–*Rsa*I Restriction Fragment (167 bp) and of the ODN 1-ODN 2 Duplex (45-mer) (the ODN 1 Sequence in the *Eco*RI–*Rsa*I Restriction Fragment Is Underlined)



superfine Sephadex by elution with water. The sequence of the restriction fragment is indicated in Scheme 2.

Cleavage experiments were performed as follows: to a 8 μL solution of [^{32}P]DNA fragment, 40 μM per base pair of sonicated calf thymus DNA in 80 mM sodium phosphate buffer (pH 8), 100 mM NaCl, and 20 mM MgCl_2 was added 4 μL of (Clip-Phen-sp)· CuCl_2 complex 4, 2, 0.4, or 0.2 μM . After 1 h at room-temperature cleavages were initiated by addition of 2 μL of ascorbate (0.8 mM) and 2 μL of H_2O_2 (0.8 mM) aqueous solutions and samples were incubated 1 h at 37 $^\circ\text{C}$. Sodium acetate buffer (20 μL) (3 M, pH 5.2) and 0.5 μL of yeast tRNA (10 mg/mL) were then added and samples were precipitated with 200 μL of ethanol. Pellets were rinsed with ethanol and lyophilized. Fragments of DNA were analyzed by denaturing 8% polyacrylamide gel electrophoresis.

After collecting the data on the 167 bp restriction fragment, we decided to investigate in more detail the sequence specificity of these DNA cleavers on the section of the restriction fragment corresponding to the more intense breaks. This sequence (45 bases, named ODN 1 and ODN 2 for its complementary strand) has been underlined on the sequence of the 167 bp fragment in Scheme 2.

Comparison of the DNA Cleavage Activity of Clip-Phen-sp, Clip-Phen, and 1,10-Phenanthroline Complexes of CuCl_2 . The 5'-end of ODN 1 was labeled by ^{32}P using a standard procedure with T4 polynucleotide kinase and [γ - ^{32}P]ATP. All oligonucleotides were desalted on columns of G25 Sephadex DNA grade equilibrated with water. ODN 1 (labeled on its 5'-end) and ODN 2 were annealed in 312 μL of sodium phosphate buffer (0.1 M, pH 7.2) by heating at 90 $^\circ\text{C}$ for 5 min followed by slow cooling to room temperature. 5 M NaCl (8 μL) and 80 μL of 0.1 M MgCl_2 were then added. Double-stranded oligonucleotide concentration was 0.2 μM .

For cleavage experiments, to an 8 μL solution of the ODN 1-ODN 2 duplex was added 4 μL of the desired complex (4 μM). After 30 min at room temperature, cleavages were initiated by addition of 4 μL of an aqueous solution of MPA (20 mM) or ascorbate (0.4 mM) or by addition of 2 μL of ascorbate (0.8 mM) and 2 μL of H_2O_2 (0.8 mM) aqueous solutions and samples were incubated for 1 h at 37 $^\circ\text{C}$. 20 μL of sodium acetate buffer (3 M, pH 5.2) and 0.5 μL of yeast tRNA (10 mg/mL) were then added and samples were precipitated with 200 μL of ethanol. Pellets were rinsed with ethanol and lyophilized. One part of the samples was then redissolved in 50 μL of piperidine 1 M and heated 30 min at 90 $^\circ\text{C}$ before to be precipitated with 50 μL of sodium acetate buffer (3 M, pH 5.2) and 500 μL of ethanol, rinsed with ethanol, and lyophilized. Fragments of DNA were analyzed by denaturing 15% polyacrylamide gel electrophoresis.

RESULTS AND DISCUSSION

Synthesis. To enhance the DNA cleavage activity of Clip-Phen, a new ligand with two connected 1,10-phenanthrolines (23), we prepared two different conjugates: Clip-Phen-sp (3) and Clip-Phen-DEEDA (2), having a spermine or a *N,N*-diethylethylenediamine motif, respectively. Phen-sp (6) was prepared to compare its cleavage activity with Clip-Phen-sp in order to measure the benefit of having a chelating ligand containing two Phen entities linked together. The different steps of the preparation of these conjugates are described in Scheme 1.

The introduction of a carboxylic function and additional flexibility on Clip-Phen was easily performed by using succinic anhydride to give Clip-Phen-COOH (1). This precursor was conjugated with DEEDA (which has a rather low affinity for nucleic acids) to give Clip-Phen-DEEDA (2) after activation of the carboxylic function of 1 by BOP/HOBT. The yield of the coupling reaction was quantitative (nearly 100% after an easy purification step

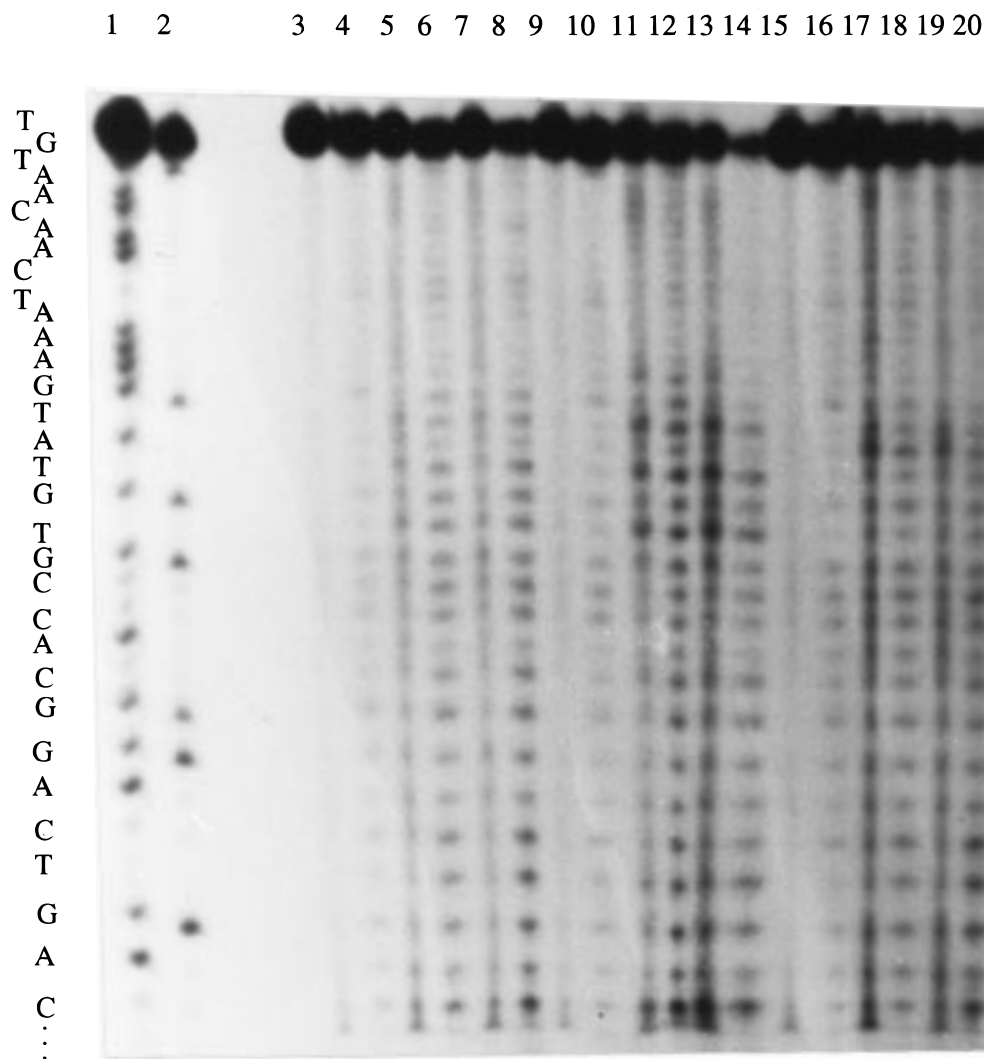


Figure 3. Cleavage of ODN 1 × ODN 2 by CuCl₂ complexes of Clip-Phen, Clip-Phen-sp and Phen. The target, labeled on the 5'-end of ODN 1, was 0.1 μM. CuCl₂ was 1 μM. Lanes 1 and 2: Maxam–Gilbert, A + G and G, respectively. Lanes 3, 5, and 7: Clip-Phen 1 μM with MPA 5 mM, ascorbate 0.1 mM, or ascorbate 0.1 mM plus H₂O₂ 0.1 mM, respectively. Lanes 9, 11, and 13: Clip-Phen-sp 1 μM with MPA 5 mM, ascorbate 0.1 mM, or ascorbate 0.1 mM plus H₂O₂ 0.1 mM, respectively. Lanes 15, 17, and 19: Phen 2 μM with MPA 5 mM, ascorbate 0.1 mM or ascorbate 0.1 mM plus H₂O₂ 0.1 mM, respectively. Lanes 4, 6, 8, 10, 12, 14, 16, 18, and 20: as lanes 3, 5, 7, 9, 11, 13, 15, 17, and 19 with a piperidine treatment, respectively.

specific site of cleavage might generate additional single-strand breaks in the vicinity of the initial DNA cleavage sites leading to form III before the total disappearance of form I). To know if the high DNA cleavage activity of Clip-Phen-sp resulted only from a better affinity for DNA due to the presence of spermine, Phen-sp (**6**), a conjugate with a spermine but only one phenanthroline entity, was prepared and its DNA cleavage activity compared to **3** (lanes 7 and 8 for a 1:1 and a 2:1 ligand/copper ratio, respectively). In the same experimental conditions used for **3**, no DNA cleavage was observed with **6**. The absence of activity of compound **6** could not be explained by the fact that the spermine was linked to phenanthroline by the C2-carbon, a position known to decrease dramatically the reactivity of substituted phenanthroline copper complexes (17), since Clip-Phen-sp with a linker between the C2-carbons had a good DNA cleavage activity. So, this result indicated that the activity of Clip-Phen-sp was not only due to spermine but was also due to the chelating properties of the two phenanthrolines of the Clip-Phen entity.

So, the Clip-Phen entity has an affinity for DNA and is able to chelate efficiently copper, and its copper complex has a redox activity leading to single-strand

breaks on DNA. Its DNA cleavage activity is increased by using ascorbate or the association of a reductant with H_2O_2 (data not shown). When MPA was replaced by the more active reductant ascorbate (0.1 mM) or by the association ascorbate/ H_2O_2 (0.1 mM for both concentrations), the obtained results indicated clearly that (Phen-sp)Cu had a detectable DNA cleavage activity at $1\ \mu\text{M}$ concentration. But in the same conditions, only 50 nM of (Clip-Phen-sp)Cu was necessary to cleave efficiently ΦX174 (data not shown). However, it must be noted that, when using ascorbate/ H_2O_2 conditions of activation, metal ion traces present in buffer solutions or in the agarose gel were able in the presence of metal-free Clip-Phen derivatives to induce a weak DNA cleavage activity that complicated the comparative studies. This last phenomenon can also explain the low DNA cleavage activity observed with Phen-sp was used in control experiments without addition of a copper salt (lane 14).

Cleavage of a Restriction Fragment by (Clip-Phen-sp)Cu. To further characterize the sequence specificity aspects of the DNA cleavage by the most active conjugate, (Clip-Phen-sp)Cu was used to cleave a double-stranded DNA fragment corresponding to a *EcoRI*-*RsaI* 167 bp restriction fragment of pBR322 plasmid labeled

by ^{32}P at the 5'-end. The target was incubated in the presence of a random double-stranded calf thymus DNA to have the same base pair concentration (20 μM) than for ΦX174 cleavage experiments. Various concentrations of (Clip-Phen-sp)Cu ranging from 1 to 0.05 μM were tested. Cleavage was initiated by addition of ascorbate (0.1 mM) and H_2O_2 (0.1 mM) because preliminary results showed that the cleavage pattern was the same with MPA (5 mM), ascorbate (0.1 mM), or ascorbate/ H_2O_2 (0.1 mM/0.1 mM), but increased with this last mode of activation (results not shown). Cleavage experiments were conducted for 1 h at 37 °C, and cleavage patterns were analyzed by high-resolution gel electrophoresis. Cleavage sites were determined with a Maxam–Gilbert sequencing reaction (results are shown in Figure 2).

Cleavage was observed at nearly all nucleotides of the sequence with a small preference for some sites. From these data, it was not easy to propose a rule for this limited sequence preference since these breaks were observed on TAT, CATGT, or TGT sequences which are known to be preferential cleavage sequence for phenanthroline copper complexes (14, 15) or near a py-pu-py triplet. (Clip-Phen-sp)Cu cleaved double-stranded DNA in a roughly nonspecific fashion as generally observed for Phen–Cu complexes. These results could be also explained by the nonspecific interaction of spermine within the minor groove of DNA (24, 26, 27, 29).

Comparison of the DNA Cleavage Specificity of Clip-Phen, Clip-Phen-sp, and Phen copPer Complexes. The DNA cleavage activity of (Clip-Phen)Cu, (Clip-Phen-sp)Cu, and (Phen) $_2$ Cu complexes were compared with the ODN 1-ODN 2 target. This double-stranded 45-mer was selected from the 167 bp restriction fragment because of some specific breaks observed with (Clip-Phen-sp)Cu $^{\text{II}}$ on this restriction fragment. The length of this oligonucleotide was small enough to allow a high-resolution analysis of the extremities of cleavage fragments by electrophoresis on denaturing polyacrylamide gel. For these experiments, the target (0.1 μM) labeled on the 5'-end of ODN 1 was incubated with 1 μM (in copper) of each different complex. The activation was performed with MPA (5 mM), ascorbate (0.1 mM), or ascorbate (0.1 mM) plus H_2O_2 (0.1 mM) for 1 h at 37 °C. Figure 3 summarizes the obtained results.

The three conjugates induced roughly the same cleavage patterns: a smear and a cleavage at all nucleotides of the target with just a little preference for some sequences. More intense breaks were observed at T_{26} , T_{28} , and T_{30} with (Clip-Phen-sp)Cu (Figure 3, lanes 11 and 13), as previously observed on the restriction fragment, and at G_{29} with (Phen) $_2$ Cu (lanes 17 and 19, for example). Since all these direct cleavage fragments migrated like the Maxam–Gilbert scale, it can be assumed that they have a 3'-phosphate extremity and resulted from deoxyribose oxidation as previously observed for 1,10-phenanthroline–copper complexes (1).

To study the smears, we decided to further analyze the cleavage patterns after piperidine treatment (30 min at 90 °C) of the nicked DNA samples. The smears of cleavage products were transformed into discrete bands with 3'-phosphate extremity and the intensity of the band corresponding to the uncleaved target was decreased. Further studies should show if this phenomenon results of oxidation of the sugars or of alkali-sensitive base damage of the DNA target.

Moreover, these experiments allowed comparison of the efficiency of the different activation modes of these copper complexes. MPA was clearly the less active activator. As previously observed for (Phen) $_2$ Cu, ascorbate was

more efficient (8). Addition of H_2O_2 increased the DNA cleavage activity of the reduced complex. This result could be explained by the fact that activation of the copper(II) complex by a reductant generates first a low-valent Cu(I) species which reacts then with dioxygen to produce superoxide and then hydrogen peroxide via a copper-catalyzed dismutation of superoxide. These catalytic cycles reduce the concentration of the copper(I) complex able to react with hydrogen peroxide to generate hydroxyl radicals via a Fenton-type reaction. So, when H_2O_2 is initially present in solution the DNA cleavage efficiency increased because of the higher concentration of H_2O_2 compared to that obtained by copper-catalyzed processes.

In conclusion, we described the preparation of phenanthroline conjugates based on the Clip-Phen entity which containing two covalently linked Phen units via a serinol bridge. An elongation of the tether with a terminal carboxylic function allowed us to prepare amine conjugates (with a spermine or a diethylethylenediamine, respectively Clip-Phen-sp and Clip-Phen-DEEDA). The copper derivative of Clip-Phen-sp is a highly efficient DNA cleaver. The nonspecific cleavage observed with these Clip-Phen derivatives will make them suitable for specific DNA cleavage after attaching the Clip-Phen motif to oligonucleotides or proteins, since the recognition of nucleic acids by these vectors will not be perturbed by the cleaver itself.

LITERATURE CITED

- (1) Sigman, D. S., Mazumder, A., and Perrin, D. M. (1993) Chemical nucleases. *Chem. Rev.* 93, 2295–2316.
- (2) Dervan, P. B. (1986) Design of sequence-specific DNA-binding molecules. *Science* 232, 464–471.
- (3) Meunier, B., Ed. (1996) *DNA and RNA Cleavers and Chemotherapy of Cancer and Viral Diseases*, Kluwer, Dordrecht.
- (4) Pratviel, G., Bernadou, B., and Meunier, B. (1995) Carbon–hydrogen bonds of DNA sugar units as targets for chemical nucleases and drugs. *Angew. Chem., Int. Ed. Engl.* 34, 746–769.
- (5) James, B. R., and Williams, R. J. P. (1961) The oxidation–reduction potentials of some copper complexes. *J. Chem. Soc.* 2007–2019.
- (6) Marshall, L. E., Graham, D. R., Reich, K. A., and Sigman, D. S. (1981) Cleavage of deoxyribonucleic acid by the 1, 10-phenanthroline-cuprous complex. Hydrogen peroxide requirement and primary and secondary structure specificity. *Biochemistry* 20, 244–250.
- (7) Veal, J. M., Merchant, K., and Rill, R. L. (1991) Noncovalent DNA binding of bis(1,10-phenanthroline)copper(I) and related compounds. *Biochemistry* 30, 1132–1140.
- (8) Veal, J. M., Merchant, K., and Rill, R. L. (1991) The influence of reducing agent and 1,10-phenanthroline concentration on DNA cleavage by phenanthroline + copper. *Nucleic Acids Res.* 19, 3383–3388.
- (9) Kuwabara, M., Yoon, C., Goyne, T. E., Thederahn, T., and Sigman, D. S. (1986) Nuclease activity of 1,10-phenanthroline-copper ion: reaction with CGCGAATTCGCG and its complexes with netropsin and *EcoRI*. *Biochemistry* 25, 7401–7408.
- (10) Goyne, T. E., and Sigman, D. S. (1987) Nuclease activity of 1,10-phenanthroline-copper ion. Chemistry of deoxyribose oxidation. *J. Am. Chem. Soc.* 109, 2846–2848.
- (11) Zelenko, O., Gallagher, J., and Sigman, D. S. (1997) Scission of DNA with bis(1,10-phenanthroline)copper without intramolecular hydrogen migration. *Angew. Chem., Int. Ed. Engl.* 36, 2776–2778.
- (12) Pope, L. E., and Sigman, D. S. (1984) Secondary structure specificity of nuclease activity of the 1, 10-phenanthroline-copper complex. *Proc. Natl. Acad. Sci. U.S.A.* 81, 3–7.

- (13) Spassky, A., and Sigman, D. S. (1985) Nuclease activity of 1,10-phenanthroline-copper ion. Conformational analysis and footprinting of the lac operon. *Biochemistry* 24, 8050–8056.
- (14) Veal, J. M., and Hill, R. L. (1988) Sequence specificity of DNA cleavage by bis(1,10-phenanthroline)copper(I). *Biochemistry* 27, 1822–1827.
- (15) Yoon, C., Kuwabara, M. D., Spassky, A., and Sigman, D. S. (1990) Sequence specificity of the deoxyribonuclease activity of 1,10-phenanthroline-copper ion. *Biochemistry* 29, 2116–2121.
- (16) Sigman, D. S., Bruice, T. W., Mazumder, A., and Sutton, C. L. (1993) Targeted chemical nucleases. *Acc. Chem. Res.* 26, 98–104.
- (17) Gallagher, J., Chen, C. B., Pan, C. Q., Perrin, M., Cho, Y. M., and Sigman, D. S. (1996) Optimizing the targeted chemical nuclease activity of 1,10-phenanthroline-copper by ligand modification. *Bioconjugate Chem.* 7, 413–420.
- (18) François, J. F., Saison-Behmoaras, T., Chassignol, M., Thuong, N. T., and Hélène, C. (1989) Sequence-targeted cleavage of single-stranded DNA by oligothymidilates covalently linked to 1,10-phenanthroline. *J. Biol. Chem.* 264, 5891–5898.
- (19) Chen, C. B., Mazumder, A., Constant, J. F., and Sigman, D. S. (1993) Nuclease activity of 1,10-phenanthroline-copper. New conjugates with low molecular weight targeting ligands. *Bioconjugate Chem.* 4, 69–77.
- (20) Sutton, C., Mazumder, A., Chen, C. B., and Sigman, D. S. (1993) Transforming the *Escherichia coli* Trp repressor into site-specific nuclease. *Biochemistry* 32, 4225–4230.
- (21) Pendergrast, P. S., Ebright, Y. W., and Ebright, R. H. (1994) High-specificity DNA cleavage agent: design and application to kilobase and megabase DNA substrates. *Science* 265, 959–962.
- (22) Pan, C. Q., Feng, J., Finkel, S. E., Landgraf, R., Johnson, R., and Sigman, D. S. (1994) Structure of the *Escherichia coli* Fis-DNA complex probed by protein conjugated with 1, 10-phenanthroline copper(I) complex. *Proc. Natl. Acad. Sci. U.S.A.* 91, 1721–1725.
- (23) Pitié, M., and Meunier, B. (1998) Preparation of a new bis-phenanthroline ligand “Clip-Phen” and evaluation of the nuclease activity of the corresponding copper complex. *Inorg. Chem.* 37, 3486–3489.
- (24) Igarashi, K., Sakamoto, I., Goto, N., Kashiwagi, K., Honma, R., and Hirose, S. (1982) Interaction between polyamines and nucleic acids or phospholipids. *Arch. of Biochem. Biophys.* 219, 438–443.
- (25) Morgan, J., Blankenship, J. W., and Matthews, H. R. (1986) Association constants for the interaction of double-stranded and single-stranded DNA with spermine, spermidine, putrescine, diaminopropane, N1- and N8-acetylspermine, and magnesium: determination from analysis of the broadening of thermal denaturation curves. *Arch. Biochem. Biophys.* 246, 225–232.
- (26) Feuerstein, B. G., Pattabiraman, N., and Marton, J. (1990) Molecular mechanisms of the interactions of spermine with DNA: DNA bending as a result of ligand binding. *Nucleic Acids Res.* 18, 1271–1282.
- (27) Edwards, M. L., Snyder, R. D., and Stermerick, D. M. (1991) Synthesis and DNA-binding properties of polyamine analogues. *J. Med. Chem.* 34, 2414–2420.
- (28) Musso, M., Thomas, T., Shirahata, A., Sigal, L. H., Van Dyke, M. W., and Thomas, T. J. (1997) Effects of chain length modification and bis(ethyl)substitution of spermine analogues on purine-purine-pyrimidine triplex DNA stabilization, aggregation, and conformational transitions. *Biochemistry* 36, 1441–1449.
- (29) Schmid, N., and Behr, J. P. (1991) Location of spermine and other polyamines on DNA as revealed by photoaffinity cleavage with polyaminobenzenediazonium salts. *Biochemistry* 30, 4357–4361.
- (30) Loeffler, J. P., and Behr, J. P. (1993) Gene transfer into primary and established mammalian cells lines with lipopolyamine-coated DNA. *Methods Enzymol.* 217, 599–618.
- (31) Tung, C. H., Breslauer, K. J., and Stein, S. (1993) Polyamine-linked oligonucleotides for DNA triple helix formation. *Nucleic Acids Res.* 21, 5489–5494.
- (32) Bigey, P., Pratviel, G., and Meunier, B. (1995) Cleavage of double-stranded DNA by “metalloporphyrin-linker-oligonucleotide” molecules: influence of the linker. *Nucleic Acids Res.* 23, 3894–3900.
- (33) Pitié, M., and Meunier, B. (1996) Cleavage of double-stranded DNA by manganese tris(methylpyridinium)porphyrin linked to 3'-spermine oligonucleotides. *J. Biol. Inorg. Chem.* 1, 239–246.
- (34) Lewis, J., and O'Donoghue, T. D. (1980) Chemistry of polydentate ligand. Part 5. Complexes of 2,9-dihydrazinoderivatives of 1,10-phenanthroline. Dependence of coordination number of a ligand on the anion present. *J. Chem. Soc., Dalton Trans.* 736–742.
- (35) Halcrow, B. E., and Kermack, W. O. (1946) Attempts to find new nantimalarials. Part XXIV. Derivatives of o-phenanthroline (7:8:3':2'-pyridoquinoline). *J. Chem. Soc.* 155–157.
- (36) Bernadou, J., Pratviel, G., Bennis, F., Girardet, M., and Meunier, B. (1989) Potassium monopersulfate and a water-soluble manganese porphyrin complex, (Mn(TMPyP))(OAc)₅, as an efficient reagent for the oxidative cleavage of DNA. *Biochemistry* 28, 7268–7275.
- (37) Holley, J. L., Mather, A., Wheelhouse, R. T., Cullis, P. M., Harley, J. A., Bingham, J. P., and Cohen, G. M. (1992) Targeting of tumor cells and DNA by a chlorambucil-spermidine conjugate. *Cancer Res.* 52, 4190–4195.
- (38) Mansouri, S., Fery-Forgues, S., Meunier, B., and Paillous, N. (1996) Organisation processes of a pyropheophorbide-spermidine conjugate in the presence or absence of DNA. *J. Chem. Soc., Perkin Trans. 2*, 1649–1654.

BC9800365

A Polyethylene Glycol Copolymer for Carrying and Releasing Multiple Copies of Cysteine-Containing Peptides

Shaei-Yun Huang,^{†,‡} Shahriar Pooyan,^{†,§} Jihong Wang,^{†,‡} Indrani Choudhury,[§] Michael J. Leibowitz,^{§,||} and Stanley Stein^{*,†,‡,§}

Center for Advanced Biotechnology and Medicine and Chemistry Department, Rutgers University, New Brunswick, New Jersey 08903-2101, Department of Molecular Genetics and Microbiology, Robert Wood Johnson Medical School-UMDNJ, Piscataway, New Jersey 08854, and Cancer Institute of New Jersey, New Brunswick, New Jersey 08903. Received April 6, 1998; Revised Manuscript Received July 6, 1998

Two different methods were developed to prepare an adduct of a poly(ethylene glycol)-lysine copolymer with either cysteamine or 1-amino-2-methyl-2-propanethiol. Cysteine-containing peptides could then be disulfide-linked to the thiol groups on the polymer in a facile manner. In the described procedures, a coupling ratio of about 8 peptides/molecule of poly(ethylene glycol)-lysine copolymer ($M_w = 27\,000$) was typically attained. The products were stable at neutral pH, but the peptides could be released from the polymer in a physiologically relevant reducing environment. The release rate was highly dependent on the linker used for forming the disulfide bond. To illustrate the potential biomedical usefulness of this polymer carrier, a Tat peptide-PEG conjugate was shown to inhibit expression of a reporter gene fused to the TAR element of human immunodeficiency virus in a model cell assay.

INTRODUCTION

Covalent attachment of water-soluble polymers can be used to manipulate the pharmacologic properties of a drug (1). Poly(ethylene glycol) (PEG)¹ can be derivatized with various functional groups for appending drug molecules (2). For example, appending PEG chains to a protein can increase its circulating half-life and minimize its immunogenic properties (3). With regard to low-molecular-weight drugs, Kohn and colleagues have designed a PEG-lysine copolymer having multiple attachment sites (4). They then prepared conjugates through either biostable or biodegradable linkages (4), but they did not investigate the biodegradable disulfide linkage. The disulfide linkage might be especially useful for drug delivery into cells, due to the stronger reducing environment within cells than in extracellular fluids. Stein and colleagues prepared a PEG derivative having a terminal thiol protected by disulfide linkage with 2-thiopyridine (5). They showed its usefulness by making a conjugate with papain, which was linked to PEG through its active-site cysteine residue, thereby keeping this enzyme in an inactive state until release from the polymer by a reducing agent. We now report the preparation and release

properties of a PEG-lysine copolymer having multiple 2-thiopyridine-protected attachment sites for cysteine-containing peptides, including those having appended biotin moieties as cellular uptake enhancers.

MATERIALS AND METHODS

Materials. Cysteamine hydrogen chloride, Ellman's reagent [5,5'-dithiobis(2-nitrobenzoic acid)], and glutathione (reduced form) were obtained from Sigma Chemicals (St. Louis, MO). Dithiothreitol (DTT) was obtained from Pierce (Rockford, IL). 2,2'-Dipyridyl disulfide, 1-amino-2-methyl-2-propanethiol, diisopropylethylamine (DIEA), and silica gel, 70–230 mesh, 60A were obtained from Aldrich Chemical (Milwaukee, WI). Hydroxybenzotriazole (HOBt), benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate (BOP), and dimethylformamide (DMF) were obtained from PerSeptive Biosystems (Boston, MA). Dichloromethane (DCM) and methanol (MeOH) were HPLC grade and obtained from Fisher Scientific (Pittsburgh, PA). Sephadex was obtained from Pharmacia LKB Biotechnology (Piscataway, NJ). Poly(ethylene glycol)-lysine copolymer ($M_w = 2.69 \times 10^4$, 2198/repeating unit), which was synthesized according to a published procedure (4), was kindly supplied by John Kemnitzer and Joachim Kohn.

Peptide Synthesis. The peptide *N*-acetyl-Phe-Arg-Arg-Arg-Cys-NH₂ (abbreviated as FR₃C) was synthesized at the Louisiana State University (LSU) core facility using Fmoc chemistry, purified by reversed-phase HPLC, and confirmed for structure by mass spectral analysis. The peptides *N*-acetyl-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Cys-NH₂ (Tat9-Cys) and *N*-acetyl-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Lys(ϵ -biotin)-Cys-NH₂ [Tat9K(bio)-Cys] were manually synthesized using Fmoc chemistry. For radiolabeling, the assembled peptide on the solid support was allowed to react with tritiated acetic anhydride in the presence of the coupling activation reagents BOP, HOBt, and DIEA. Acetylation of the peptide was com-

* Corresponding author. E-mail: stein@mbcl.rutgers.edu. Phone: 732-235-5319. Fax: 732-235-4850.

[†] Center for Advanced Biotechnology and Medicine.

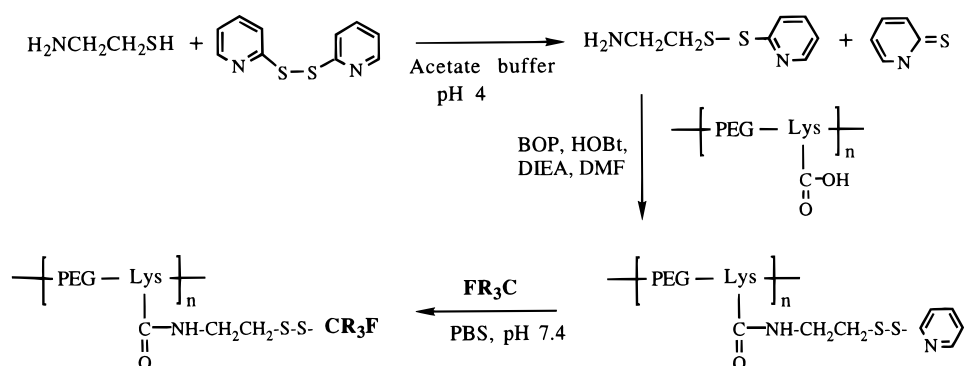
[‡] Chemistry Department.

[§] Department of Molecular Genetics and Microbiology.

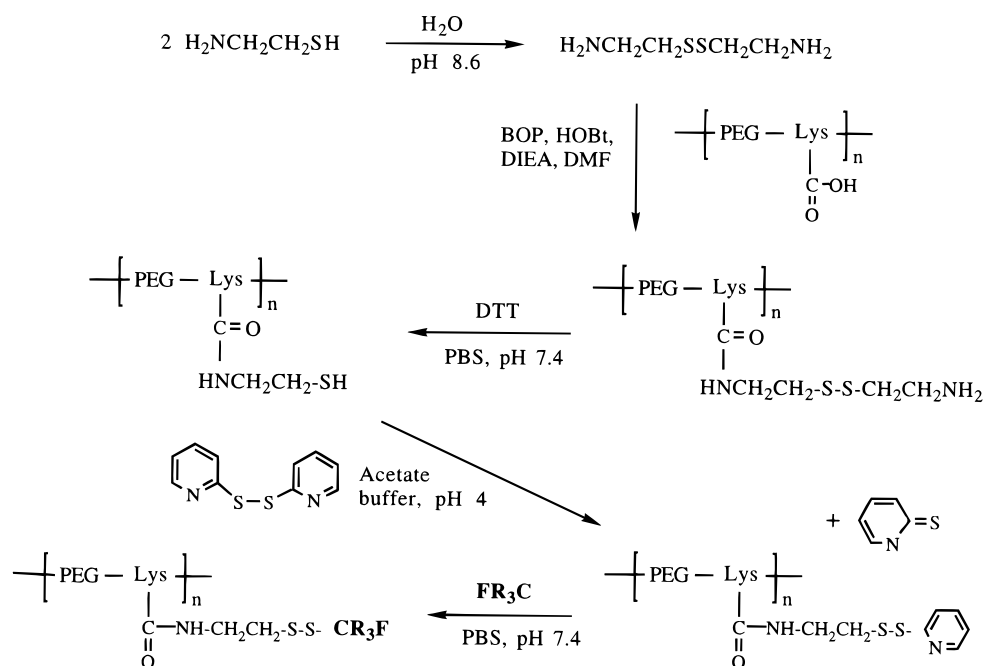
^{||} Cancer Institute of New Jersey.

¹ Abbreviations: BOP, benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate; CAT, chloramphenicol acetyltransferase; DIEA, diisopropylethylamine; DTT, dithiothreitol; FR₃C, *N*-acetyl-Phe-Arg-Arg-Arg-Cys-NH₂; HOBt, hydroxybenzotriazole; LTR, long terminal repeat; PBS, phosphate-buffered saline; PEG, poly(ethylene glycol); Tat9-Cys, *N*-acetyl-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Cys-NH₂; Tat9K(bio)-Cys, *N*-acetyl-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Lys(ϵ -biotin)-Cys-NH₂.

Scheme 1



Scheme 2



pleted by chasing with an excess of nonradioactive acetic anhydride. After cleavage from the solid support and ether precipitation, the radiolabeled peptide was purified by chromatography on Sephadex G-10 using PBS (0.15 M NaCl, 20 mM potassium phosphate buffer, pH 7.4) for elution.

Preparation of PEG-Cysteamine-TP by Scheme 1. To a solution of cysteamine hydrogen chloride (0.50 g, 4.4 mmol) dissolved in 15 mL of degassed buffer (0.1 N sodium acetate adjusted to pH 4.0 with acetic acid, 0.3 M sodium chloride, and 1 mM EDTA) was added 2,2'-dithiodipyridine (3.5 g, 15 mmol) dissolved in 18 mL of MeOH. The mixture was stirred at room temperature under N₂. The reaction, which was monitored with thin-layer chromatography (ammonium hydroxide/MeOH/DCM, 2/10/90), was complete after 4 h. The MeOH was removed in vacuo and the residue was made basic by saturating with sodium carbonate. The residue was extracted into DCM (3 × 50 mL) and the combined DCM was washed with water (2 × 40 mL) and brine (40 mL) and then dried over magnesium sulfate. The magnesium sulfate was filtered and the filtrate was concentrated in vacuo to an oily residue. The product, cysteamine-TP, was purified by flash chromatography on a silica gel column (ammonium hydroxide/MeOH/DCM, 2/10/90). Yield: 0.30 g (yellow oil) (36%). ¹H NMR (CDCl₃): δ 8.4–8.5 ppm (m, 1H), 7.7–7.6 ppm (m, 2H), 7.15–7.0 ppm (m, 1H), 3.0–2.7 ppm (m, 4H), 0.9 ppm (b, 2H).

PEG-lysine copolymer (0.20 g, 91 μmol of lysine) was dissolved in 2.5 mL of degassed DMF. Cysteamine-TP (0.5 mmol), BOP (0.5 mmol), HOBT (0.5 mmol), and DIEA (25 μL) were added to the PEG copolymer solution and stirred under N₂ overnight. The product was precipitated with cold ether, washed with cold ether, and then dried by Speedvac (Savant Instrument Co., Farmingdale, NY) to a gellike residue. The product was dissolved and purified on a Sephadex G-75 column (48 mL bed volume) using 0.1 N acetic acid. Appropriate fractions were combined and lyophilized. Yield: 0.11 g (colorless solid).

Preparation of PEG-Cysteamine-TP by Scheme 2. Cysteamine hydrogen chloride was first converted to the disulfide-linked dimer by overnight stirring of an aqueous solution (0.3 g in 15 mL) adjusted to pH 8.6 with triethylamine. Ellman's assay indicated less than 1% remaining free thiol. To the mixture was added a saturated solution of sodium carbonate to pH 10, followed by extracting into DCM (5 × 20 mL), drying over magnesium sulfate, filtering, and concentrating on a rotary evaporator. PEG-lysine copolymer (0.20 g, 91 μmol of lysine) was dissolved in 2.5 mL of degassed DMF. Cysteamine dimer (650 μmol), BOP (650 μmol), HOBT (650 μmol), and DIEA (25 μL) were added to the PEG copolymer solution and stirred under N₂ overnight. The product was precipitated with cold ether, washed with cold ether, and then dried by Speedvac to a gellike residue. The product was purified on a Sephadex G-75

gel filtration column, as above. Appropriate fractions were combined and lyophilized to yield 70 mg of colorless solid, which was dissolved in degassed PBS (pH 7.4). DTT (10 molar equivalents based on lysine) was then added and the reduction reaction proceeded under N_2 overnight. The product was purified on a G-75 column (48 mL bed volume) using 0.1 N acetic acid containing 1 mM EDTA for elution. Appropriate fractions were combined and lyophilized to yield 60 mg of colorless solid, which was dissolved in 4 mL of degassed buffer (0.1 N sodium acetate adjusted to pH 4 with acetic acid, 0.3 M sodium chloride, 1 mM EDTA). 2,2'-Dithiodipyridine (270 μ mol) dissolved in 3 mL of degassed MeOH was added, and the reaction mixture was stirred under N_2 for 2 h. The MeOH was removed on a rotary evaporator, and the product was purified on a Sephadex G-75 column (bed volume 48 mL) using 0.1 N acetic acid for elution. Appropriate fractions were combined and lyophilized to yield 30 mg of colorless solid. A similar procedure was used to prepare the PEG derivative of the sterically hindered cysteamine analogue 1-amino-2-methyl-2-propanethiol.

Determination of the 2-Thiopyridine Content in PEG-Cysteamine-TP. The concentration of a PEG-cysteamine-TP solution was determined by acid hydrolysis and measurement of the released lysine using an Applied Biosystems amino acid analyzer (LSU core facility). Varying amounts of PEG-cysteamine-TP (100, 200, and 400 μ L) of a 4 mg/mL (ca. 2 μ mol/mL of lysine) solution in water were reacted separately with an excess of DTT (200 μ L of 26 μ mol/mL in water) and diluted to a final volume of 1.0 mL with PBS. The amount of 2-thiopyridone liberated was quantitated using a molar extinction coefficient of 7.06×10^3 at 343 nm (6).

Preparation of PEG-cysteamine-Peptide. PEG-cysteamine-TP, dissolved in water (8 μ mol/mL of lysine), was mixed with an equal volume of PBS containing 1 mM EDTA. To 3 mL of this solution was added 1 equiv [as determined by Ellman's assay for thiols (7)] of the peptide FR_3C . The reaction, which was monitored by absorbance at 343 nm on aliquots of the mixture, was complete after 15 min of stirring under N_2 . The product was purified on a Sephadex G-75 column, using PBS as eluent. Appropriate fractions were pooled. The extent of peptide derivatization of the copolymer was determined by amino acid analysis (LSU core facility). Other combinations of a PEG carrier and a peptide, including radiolabeled peptides, were prepared similarly.

Release of Peptides from PEG-S-S-Peptide Conjugates. The disulfide-linked conjugate $PEG-(cysteamine-FR_3C)_n$ was added to a solution of reduced glutathione (3 mM in PBS) to a final peptide concentration of 0.3 mM and then incubated under N_2 at room temperature. A sample was taken at each time point, acidified by adding an aliquot of 5% TFA, and applied to a G-75 column in 0.1% TFA (7 mL bed volume) to separate PEG-conjugated peptide from free peptide. Appropriate fractions in the high-molecular-weight region were pooled, and the extent of peptide derivatization at each time point was determined by amino acid analysis. For measuring the release of radiolabeled peptide, each time point aliquot was acidified to pH 3.5 with sulfuric acid. The PEG-conjugated peptide and the free peptide were then separated using ultrafiltration on Centricon-10 (Amicon/Millipore, Bedford, MA). To ensure complete separation, the retentate was diluted and ultrafiltered two more times. Radioactivity in the retentate and combined filtrate was quantitated by scintillation counting.

Inhibition of Protein Expression from a TAR-CAT Plasmid. Details of this assay have been published (8). Briefly, Jurkat cells were stably transfected with a recombinant plasmid comprising the HIV-1 long terminal repeat (LTR), which contains the TAR (transactivator response element) domain, linked to the chloramphenicol acetyltransferase (CAT) gene on the pRep10 episomal vector (Invitrogen, Carlsbad, CA) (S. Stein, B. Beiss, and A. B. Rabson, unpublished results). For expression of Tat protein, which transactivates transcription after binding to the TAR RNA domain of nascent transcripts, cells were transfected with pAR(Tat), which comprises the HIV-1 LTR and viral sequences encoding the 72 amino acid form of Tat. CAT protein in harvested cells was measured using an immunoassay kit from Boehringer-Mannheim (Indianapolis, IN). In this assay, cells were transfected with pAR(Tat) at $t = 0$, and the cells were incubated in 10% fetal calf serum under 95% air/5% carbon dioxide. The Tat-peptide inhibitor, either free or appended to the PEG carrier, was added at various concentrations at $t = 18$ h, and the cells were harvested for CAT immunoassay at $t = 42$ h.

RESULTS

Preparation of PEG-S-S-Peptides. The synthetic routes are given in Schemes 1 and 2. In Scheme 1, cysteamine was first converted into the mixed disulfide with 2-thiopyridine. The cysteamine-TP reagent was purified by silica gel chromatography, and its structure was confirmed by ^1H NMR. It was necessary to use this reagent immediately for coupling to the PEG-lysine copolymer, since the 2-thiopyridine group would react back to 2,2'-dipyridyl disulfide, as evaluated by NMR, at a rate of about 15%/day. Amide bond formation between the carboxylate groups on the PEG-lysine copolymer and the amino group on cysteamine was accomplished using reagents typically used for coupling in peptide synthesis (Scheme 1). In Scheme 2, cysteamine was first converted to the symmetric disulfide, then appended to the PEG-lysine copolymer, and finally converted to the 2-thiopyridine derivative. Preparation of the PEG-1-amino-2-methyl-2-propanethiol-TP derivative by Scheme 2 was as facile as that of the cysteamine version.

The disulfide bond between 2-thiopyridine and cysteamine appended to PEG was stable to cleavage in PBS (pH 7.4), as determined by absence of absorbance at 343 nm after 24 h at room temperature. However, upon addition of a cysteinyl-peptide, FR_3C , reaction was complete within several minutes, as determined by the released 2-thiopyridone measured at 343 nm (Figure 1). This coupling reaction was about 100 times slower for the polymer in which 1-amino-2-methyl-2-propanethiol was substituted for cysteamine.

To determine the stoichiometry, the amount of 2-thiopyridone released from a given amount of PEG-cysteamine-TP solution by an excess of reducing agent (DTT) in PBS was measured. Then, 1 equiv (as defined by thiol groups measured by Ellman's assay) of the peptide, FR_3C , was added to an aliquot of PEG-cysteamine-TP solution, and essentially complete release of 2-thiopyridone was observed. Purification of the product, PEG-S-S-peptide, by gel filtration chromatography gave complete separation from released 2-thiopyridone and, presumably, from any residual unreacted peptide (Figure 2).

Acid hydrolysis, followed by amino acid analysis, was used to determine the concentration of the PEG-lysine

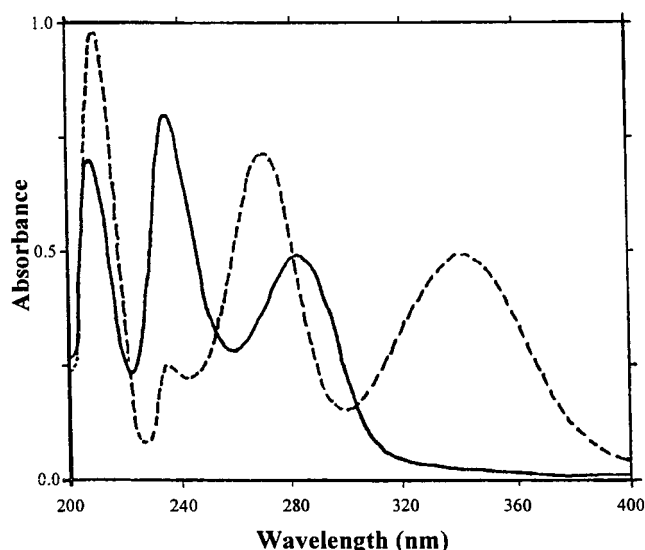


Figure 1. Spectroscopic analysis of the reaction of PEG-cysteamine-TP with the peptide FR₃C. The UV spectrum of a solution of PEG-cysteamine-TP is shown (solid line). Addition of the peptide results in release of 2-thiopyridone, which is observed as a peak with a maximum at 343 nm (dashed line).

copolymer, and treatment with an excess of DTT was used to determine the amount of coupled cysteamine-TP on that same sample. By this procedure, it was determined that $66 \pm 8\%$ of the lysine carboxylate groups had been derivatized with cysteamine-TP on a sample of PEG-cysteamine-TP prepared by Scheme 1. Acid hydrolysis, followed by amino acid analysis was also used to determine the extent of derivatization by the peptide, FR₃C. Duplicate analyses gave a ratio of Phe:Lys of 0.64 and 0.55 and a ratio of Arg:Lys of 2.0 and 1.8. Therefore, the extent of peptide derivatization, based on lysine groups in the copolymer backbone, was $62 \pm 4\%$. In another sample prepared by Scheme 1, the peptide coupling ratio was found to be $66 \pm 5\%$. In a sample prepared by Scheme 2, the peptide coupling ratio was found to be $78 \pm 9\%$.

Release Studies. Release of the peptide FR₃C by reductive cleavage of its disulfide linkage to PEG-cyste-

amine was evaluated using 3 mM glutathione in PBS (pH 7.4) at room temperature. At each time point, an aliquot was withdrawn and acidified to halt the disulfide interchange reaction. Released peptide was removed from PEG-peptide conjugate by gel filtration chromatography, and the ratio of PEG (i.e., Lys residues) to peptide (i.e., Phe or Arg residues) was determined by amino acid analysis. Release was found to be relatively rapid, with a half-time of about 3 min (data not shown). The presence of glycine and glutamic acid in the high-molecular-weight fraction from glutathione-treated samples (data not shown) indicates that glutathione replaces the released peptide on the PEG copolymer.

Release kinetics were also studied using the disulfide-linked radiolabeled peptides, Tat9-Cys and Tat9K(bio)-Cys. Released peptide was removed from PEG-peptide conjugate by ultrafiltration. The half-time of release in PBS containing 3 mM glutathione was found to also be about 3 min for either peptide (Figure 3).

To achieve a slower release rate, as previously suggested for linking antibodies to toxins (9), the PEG derivative of the sterically hindered cysteamine analogue, 1-amino-2-methyl-2-propanethiol, was synthesized. Analysis of the release rate of radiolabeled Tat9K(bio)-Cys gave a half-time of about 40 min in the presence of 30 mM glutathione (Figure 4). Since the disulfide cleavage rate is proportional to glutathione concentration (10), the sterically hindered linker has a release rate about 100-times slower than does the cysteamine linker. Thus, the release rate can be readily controlled by selection of the linker between the PEG backbone and the appended peptide.

Cell Assay. The ability to achieve a biological effect was tested using the peptide Tat9K(bio)-Cys, which represents the TAR RNA-binding domain of the Tat protein encoded in the human immunodeficiency virus (HIV-1) genome. The rationale is that Tat protein interacts with TAR, which comprises the 5'-terminus of nascent transcripts, to potentiate transcription elongation, and that a Tat-binding domain peptide might interfere with this transactivation process (8). Indeed, the Cys(S-biotin) analogue of this peptide has been shown to inhibit viral replication in infected cells, thereby being a candidate therapeutic agent for AIDS (8). The ap-

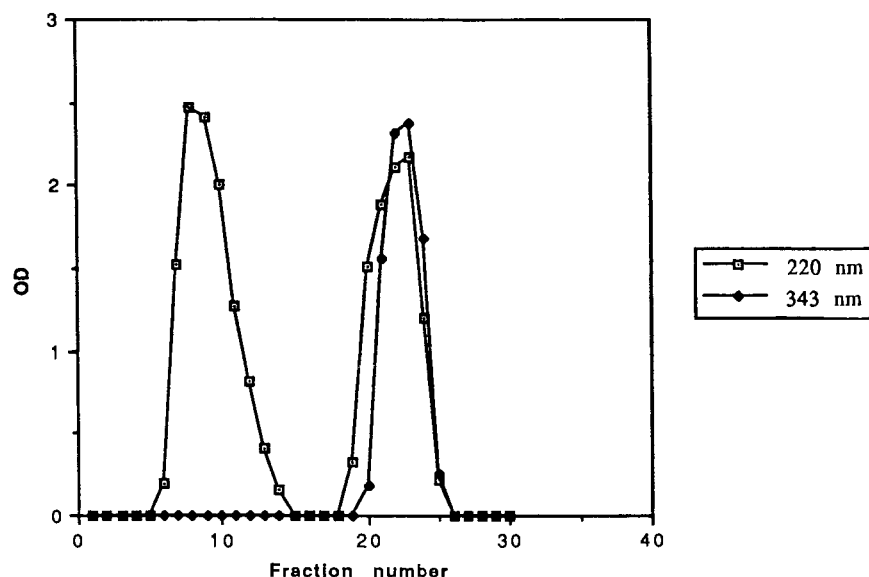


Figure 2. Purification of the product from a reaction mixture of PEG-cysteamine-TP and the peptide FR₃C by gel filtration chromatography. Note the presence of the released 2-thiopyridone in the low-molecular-weight fractions according to absorbance at 343 nm.

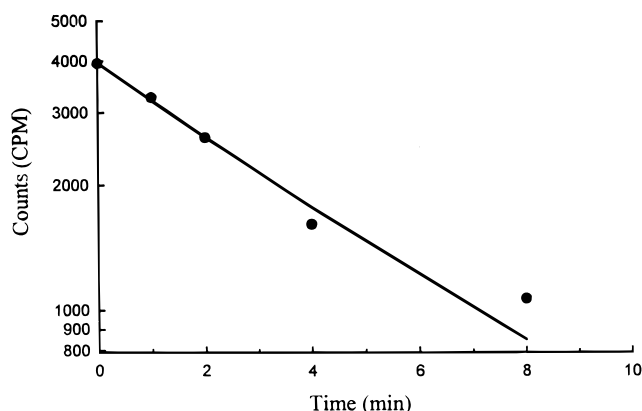


Figure 3. Time course of release of the radiolabeled peptide Tat9K(bio)-Cys from PEG-cysteamine-peptide by 3 mM glutathione in PBS. The data are presented as a semilog plot. Essentially the same release kinetics were obtained in two additional experiments.

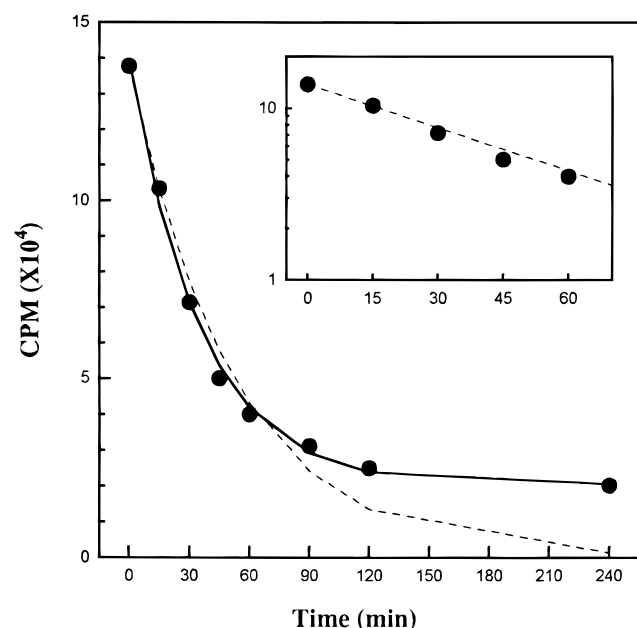


Figure 4. Time course of release of the radiolabeled peptide, Tat9K(bio)-Cys, from PEG-1-amino-2-methyl-2-propanethiol-peptide in PBS by 30 mM glutathione. The dashed line is a theoretical plot of $C = C_{\text{initial}}e^{-kt}$. A semilog plot of the early time point data is shown in the inset. Essentially the same release kinetics were obtained in two additional experiments.

pendent biotin moiety has been found to increase cell uptake of the peptide (8). The Lys(ϵ -biotin) analogue of this peptide, used in the present study, has been found to bind to TAR RNA with similar avidity and specificity as the Cys(S-biotin) analogue and to inhibit gene expression (Figure 5) in a model cell assay (8), and it would be expected to inhibit HIV-1 replication similarly. A conjugate comprising multiple copies of this peptide disulfide-linked to PEG, PEG-[cysteamine-Tat9K(bio)-Cys]_n, was not only active in the model cell assay but also resulted in an increase in potency (Figure 5).

DISCUSSION

Although peptides and peptide mimetics are potential therapeutic agents, a major limitation to their utility is the requirement of parenteral rather than oral administration, due to degradation and/or poor absorption in the gastrointestinal tract. The necessity of frequent injections of a therapeutic peptide, due to the rapid

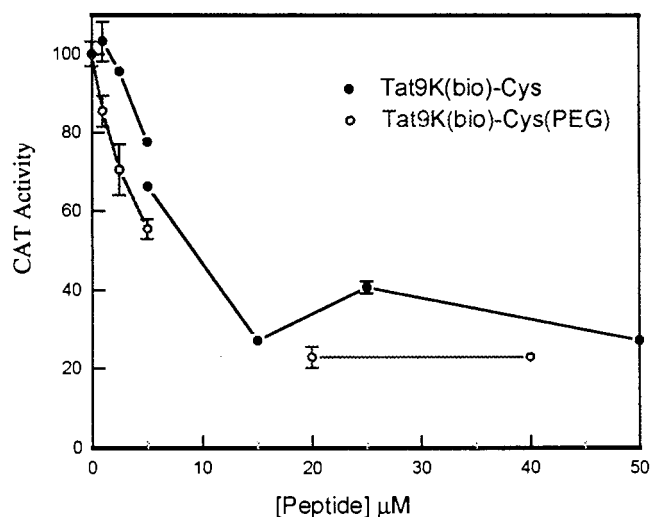


Figure 5. Inhibition of protein expression from a TAR-CAT plasmid. The biotinylated Tat-peptide inhibitor, either free or appended to the PEG carrier, was added at each indicated concentration. Each point is the average of three separate cell cultures. The data for the low concentrations of free and appended peptide were obtained in a side-by-side experiment, whereas the data for the high concentrations were obtained in separate experiments. Note the repetition of one concentration of free Tat-peptide in the high and low concentration sets of experiments; this is indicative of the assay-to-assay variability. The remaining 20–25% of CAT Activity at high inhibitor concentrations most likely represents CAT protein already synthesized prior to addition of the inhibitor to the culture medium and its uptake into the cells.

clearance of low-molecular-weight compounds, can be overcome by using PEG as a carrier (4). In this report, we describe an approach for preparing a reversible conjugate of a peptide on a PEG polymer. The reversible linkage is the disulfide bond, which can be cleaved by glutathione, a physiologically relevant reducing agent. This concept has recently been reported using hemoglobin as the carrier, with disulfide linkage to a cysteine residue in the peptide (10). In that study (10), mathematical modeling was presented to show the influence of various kinetic parameters on the blood level of released peptide.

In our studies, release of the peptide from the polymer was studied in PBS containing reduced glutathione, which is present in most tissues at a concentration of approximately 3 mM (11). Since the concentration of reduced glutathione in blood is on the order of 10 μM (12), the appended peptide molecules should remain mostly bound to the PEG carrier in the extracellular environment, but they should be released from the carrier upon entry into cells. Thus, this disulfide-based PEG delivery approach may be especially advantageous for intracellularly targeted drugs.

We now speculate that a PEG carrier loaded with multiple copies of the peptide, Tat9K(bio)-Cys (or a peptidase-resistant version), could be a particularly useful therapeutic product. Owing to its low clearance rate and resistance to disulfide exchange in extracellular fluids, this conjugate would be parenterally administered at, perhaps, daily or weekly intervals. The multiple biotin moieties appended to each PEG molecule, as well as the high density of positive charges on the side chains of the basic amino acids, might have an additive or synergistic effect on cell uptake efficiency. Indeed, preliminary cell culture experiments indicate that the peptide is more potent when added to the culture medium as a disulfide-linked PEG conjugate. Although we have not yet experimentally determined the basis for this apparent increase

in potency, we hypothesize that it is due to enhanced cellular uptake. Whatever the reason, it is clear that both an uptake enhancer (biotin) and a PEG carrier contribute markedly to enhanced potency for a peptide that has an intracellular target. In conclusion, our approach provides a method for reaching intracellular disease-related targets using peptide mimetics and other polar organic compounds that would otherwise not have the requisite pharmacologic properties.

ACKNOWLEDGMENT

We thank Dr. Joachim Kohn and Dr. John Kemnitzer for their advice and for supplying the PEG-lysine copolymer. S.P. was supported by NIH training grant GM55145.

LITERATURE CITED

- (1) Duncan, R., and Kopacek, J. (1984) Soluble synthetic polymers as potential drug carriers. *Adv. Polym. Sci.* 57, 53–101.
- (2) Zalipsky, S. (1995) Functionalized poly(ethylene glycol) for preparation of biologically relevant conjugates. *Bioconj. Chem.* 6, 150–165.
- (3) Davis, S., Abuchowski, A., Park, Y. K., and Davis, F. F. (1981) Alteration of the circulating half-life and antigenic properties of bovine adenosine deaminase in mice by attachment of poly(ethylene glycol). *Clin. Exp. Immunol.* 46, 649–652.
- (4) Nathan, A., Zalipsky, S., Ertel, S. I., Agathos, S. N., Yarmush, M. L., and Kohn, J. (1993) Copolymers of lysine and poly(ethylene glycol): A new family of functionalized drug carriers. *Bioconj. Chem.* 4, 54–62.
- (5) Woghiren, C., Sharma, B., and Stein, S. (1993) Protected thiol-poly(ethylene glycol): A new activated polymer for reversible protein modification. *Bioconj. Chem.* 4, 314–318.
- (6) Grassetti, D. R., and Murray, J. R. (1967) Determination of sulfhydryl groups with 2, 2'- or 4, 4'-dithiodipyridine. *Arch. Biochem. Biophys.* 119, 41–49.
- (7) Riddles, P. W., Blakeley, R. L., and Zerner, B. (1979) Ellman's reagent: 5,5'-Dithiobis(2-nitrobenzoic acid)-a reexamination. *Anal. Biochem.* 94, 75–81.
- (8) Choudhury, I., Wang, J., Rabson, A. B., Stein, S., Pooyan, S., Stein, S., and Leibowitz, M. J. (1998) Inhibition of HIV-1 replication by a Tat RNA-binding domain peptide analogue. *J. Acquired Immune Defic. Syndr.* 17, 104–111.
- (9) Goff, D. A., and Carroll, S. F. (1990) Substituted 2-iminothiolanes: Reagents for the preparation of disulfide cross-linked conjugates with increased stability. *Bioconjugate Chem.* 1, 381–386.
- (10) Trimble, S. P., Marquardt, D., and Anderson, D. C. (1997) Use of designed peptide linkers and recombinant hemoglobin mutants for drug delivery: In vitro release of an angiotensin II analogue and kinetic modeling of delivery. *Bioconj. Chem.* 8, 416–423.
- (11) Meister, A., Griffith, O. W., and Tate, S. S. (1979) New aspects of glutathione metabolism and translocation in mammals. *Ciba Found. Symp.* 72, 135–161.
- (12) Meister, A. (1991) Glutathione deficiency produced by inhibition of its synthesis and its reversal; applications in research and therapy. *Pharmacol. Ther.* 51, 155–194.

BC980038P

Cathepsin Substrates as Cleavable Peptide Linkers in Bioconjugates, Selected from a Fluorescence Quench Combinatorial Library

James J. Peterson and Claude F. Meares*

Department of Chemistry, University of California, One Shields Avenue, Davis, California 95616-5295.
Received June 4, 1998

Several extended peptide substrates for the human liver enzymes cathepsin B and cathepsin D have been selected as cleavable linkers for lysosomal proteolysis of bioconjugates. A one-bead-one-peptide combinatorial library of 9⁴ fluorogenic substrates was employed. We designed this library to explore a set of substrates containing nonionizable/nonoxidizable groups to meet the requirements of prelabeling [Li et al. (1994) *Bioconjugate Chem.* 5, 101–104] as well as to yield stable conjugates whose preparation is straightforward.

INTRODUCTION

Insertion of a labile lysosomal endoprotease substrate site into drug delivery agents has found several applications (1–5). For example, there is promise that insertion of a peptide substrate between a radiolabeled chelate and its targeting moiety (e.g., an antibody) may lead to expedited clearance of undesirable radioactivity from the liver during radioimmunotherapy and imaging (6, 7). Further development of the peptide linkers to include compatible amino acid residues beyond the simple Gly₃-Phe sequence introduced by Li and Meares (6) is warranted. Two likely candidates for intracellular cleavage of peptide linkers are the cysteine protease cathepsin B (8) and the aspartic acid protease cathepsin D (9, 10).

Our initial aim was to select a number of appropriate substrates as lead compounds, so that some would ultimately satisfy the additional constraints posed by radioimmunoconjugate synthesis, solubility, and metabolism. We excluded ionizable and oxidizable residues from this study in order to simplify incorporation of chelate and isothiocyanate functionalities as well as to conform to the requirements of "prelabeling" (11), which enables removal of nonradiolabeled chelate via ion exchange prior to conjugation of radiolabeled chelate to a targeting moiety. The literature is rich in ionizable cathepsin B substrates (12–16) and cathepsin D substrates (17, 18), and a few nonionizable substrates also have been identified (4, 19).

Rational, or iterative, design of protease substrates or inhibitors can yield unexpected results: the interdependency effects of perturbing existing proteolytic substrates with different amino acids have been documented (20, 21). Similarly, rational designs based on the crystal structure of the protease active site are not always successful (45).

Combinatorial strategies, wherein all positions are varied independently, offer a promising alternative. Sensitive intramolecular fluorescence quenching experiments using resonance energy-transfer donor/acceptor

pairs (22–24) are well-suited to the identification of endoprotease substrates where the features imparting lability are not fully characterized. This is particularly true with substrates for enzymes having extended binding sites. We have employed the one-bead-one-peptide strategy developed by Meldal et al. (25), which combines powerful target isolation (26) with the sensitivity of intramolecular fluorescence quenching using the anthranilamide/3-nitrotyrosine donor/acceptor pair (24). After partial digestion of the peptide bead, cleavage is signaled by visible blue fluorescence, but enough of the unmodified peptide remains on the bead so that all of the information is conserved and decodable via Edman sequencing. Substrates with donor/acceptor pairs having critical distances for energy transfer (R_0) as large as 50 Å have been studied (24). Related variations of the procedure have been used to optimize substrates for subtilisin Carlsberg (25) and Cruzipain (27).

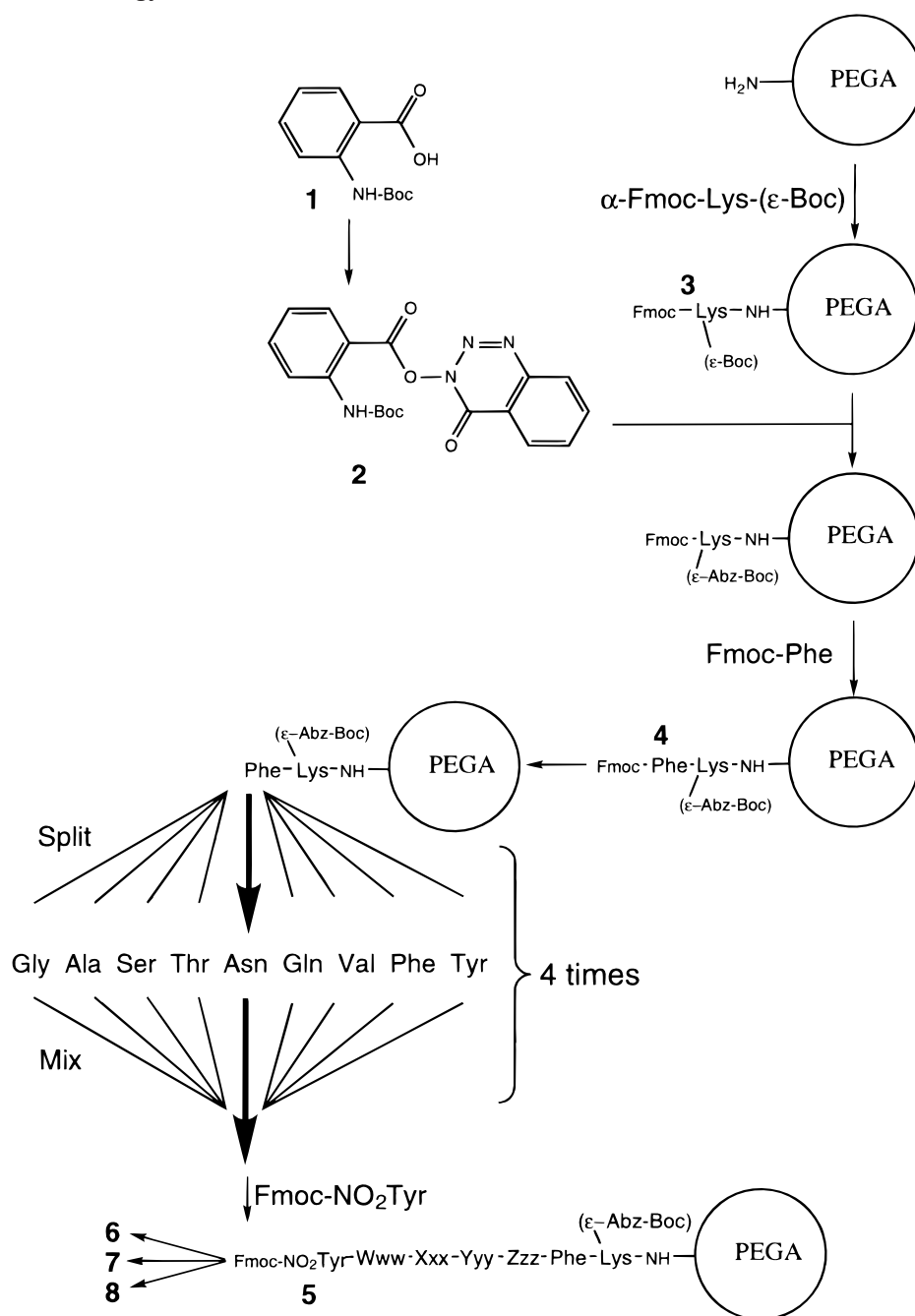
The lysosomal endoproteases cathepsin B and D are not absolutely dependent on the identity of any particular amino acid at any given subsite,¹ P_n-P_n', although some trends have been described (17, 28, 29, 45). Full characterization of lysosomal endoprotease substrates requires study of an extended substrate-binding site—particularly in the case of cathepsin D, which is known to be unresponsive to substrate motifs of <5 residues (29). Study of extended substrates is particularly appropriate in our case, since it can be inferred that increased length of the substrate is a favorable characteristic for linkers (4, 30).

The solubility properties of the PEGA² support allow enzyme permeability in an aqueous environment and are critical to the success of this combinatorial library technique (25, 27). This support consists of bis(2-acrylamidoprop-1-yl) poly(ethylene glycol) cross-linked dimethyl acrylamide and mono-2-acrylamidoprop-1-yl [2-aminoprop-1-yl] poly(ethylene glycol) (800), which we have confirmed is fully accessible to cathepsins B and D.

* Author to whom correspondence should be addressed.
Phone: 530-752-0936. Fax: 530-752-8938. E-mail: cfmeares@ucdavis.edu.

¹ For example, a peptide substrate NH₂-G-A-L-T-CO₂H, if cleaved between A and L, would be described in terms of the following subsites: G is in P₂; A is in P₁; L is in P₁'; T is in P₂'.

Scheme 1. Synthesis Strategy

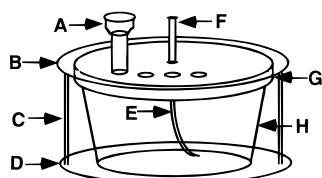


This study incorporates one of the following amino acids at each of four variable positions: Phe, Tyr, Ala, Gly, Asn, Gln, Ser, Thr, Val (Scheme 1).

² Abbreviations: Abz, o-aminobenzoyl; Abz-OH, o-aminobenzoic acid; BTEE, N-benzoyl tyrosine ethyl ester; Bop, (benzotriazolyl)tris-(dimethylamino)phosphonium hexafluorophosphate; Boc, tert-butoxycarbonyl; t-Bu, tert-butyl; DCC, N,N'-dicyclohexylcarbodiimide; dhbt, 3,4-dihydro-4-oxo-1,2,3-benzotriazo-3-yl; DIEA, diisopropylethylamine; DMF, N,N-dimethylformamide; DTT, dithiothreitol; DOTA, 1,4,7,10-tetraazacyclododecane-N,N',N'',N'''-tetraacetic acid; EtOH, ethanol; EtOAc, ethyl acetate; EDTA, ethylenediaminetetraacetic acid; Fmoc, fluorene-9-ylmethoxycarbonyl; HOBt, 1-hydroxybenzotriazole; NO₂Phe, p-nitrophenylalanine; NO₂Tyr, 3-nitrotyrosine; NMP, 1-methyl-2-pyrrolidone; PEGA, bis(2-acrylamido-1-yl)poly(ethylene glycol) (800) cross-linked dimethyl acrylamide and mono-2-acrylamidoprop-1-yl [2-aminoprop-1-yl]poly(ethylene glycol); pfp, pentafluorophenol; THF, tetrahydrofuran; trt, trityl.

MATERIALS AND METHODS

Reagents. Anhydrous DMF, anhydrous NMP, HOBt, Boc₂O, Et₃N, piperidine, redistilled DIEA, anhydrous CH₂Cl₂, DCC, dhbt-OH, anthranilic acid, CF₃COOH, fluorescamine, and bovine pancreas α -chymotrypsin type II were purchased from Aldrich Chemical Co. EtOAc, CH₃CN, CH₃OH, and THF were purchased from Fisher; THF was freshly redistilled over Na(s) before use. Bop, Fmoc-Ala-OH, Fmoc-Asn(trt)-OH, Fmoc-Gly-OH, Fmoc-Gln(trt)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Phe-OH, Fmoc-Ser(t-Bu)-OH, Fmoc-Thr(t-Bu)-OH, Fmoc-Tyr(t-Bu)-OH, Fmoc-NO₂Tyr-OH, and Fmoc-Val-OH were purchased from Bachem California. Amino-PEGA resin and human liver cathepsin D were purchased from Calbiochem-Novabiochem Corp. The solid-phase assay ninhydrin analytical reagent kit was purchased from ABI. Human liver cathepsin B was purchased from Sigma.

Scheme 2. Multiple Peptide Synthesis Apparatus^a

^a (A) For <0.25 g of resin, use Bio-Rad catalog no. 731-1550; punch a 1/4 in. hole in Chemglass catalog no. 3022-06 septum as seal/reducer. For <0.05 g of resin, use Bio-Rad catalog no. 732-6008; (B) aluminum donut (top of clamp); I.D. = 16.3 cm, O.D. = 24 cm, thickness = 7 mm (1/4 in.); (C) 8 1/4 in. lag bolts with wing nuts; (D) aluminum base (bottom of clamp); D = 24 cm, thickness = 7 mm; (E) inner flexible Teflon tubing leading to solvent recovery reservoir; (F) outer glass inlet for inert gas; (G) machined 3/4 in. Teflon plate (For column orifices, use a 5/16 in. drill followed by a 21/32 in. mill. Do not mill through. Leave a minimum of 3/16 in. step for Bio-Rad catalog no. 732-6008); (H) desiccator/primary reservoir.

Spectroscopy. NMR spectra were recorded on a General Electric QE 300 spectrometer at 300 MHz. IR spectra were recorded on a Mattson Galaxy Series FTIR 300. UV spectroscopy was performed on a Hewlett-Packard model 8450 UV-vis spectrophotometer.

Adaptable Scale Multiple Column Peptide Synthesizer. This apparatus (Scheme 2) was designed to be economical, scale-versatile, and simple. A 160 mm Pyrex glass vacuum desiccator was used as the primary solvent recovery reservoir. A 3/4 in. thick sheet of machine-grade Teflon was shaped and milled to allow interface with a variety of polypropylene-fritted "Bio-spin" and "econo-pac" chromatography vessels purchased from Bio-Rad. Aluminum plates were cut and drilled to provide the sealing clamp. Positive N₂ pressure regulated at <5 psig was used to bubble-stir reactions and washes. Soft Teflon forms an excellent seal with snugly fitting columns and glass inlets, avoiding the need for rubber seals.

Sequencing. Edman sequencing was performed on an ABI model 477A sequencer equipped with an ABI 120 HPLC column. Data were analyzed using ABI system 610 software.

Peptide Synthesis and Analysis. 2-*tert*-Butyloxy-carbonylamino benzoic acid (*Boc*-Abz-OH) (**1**) was synthesized according to literature (24). The reaction and purification were monitored by TLC. Stationary phase: Kieselgel 60 F₂₅₄, EM Science. Mobile phase EtOAc:CHCl₃:CH₃OH, 1:1:1, *R*_f = 0.8; 25.7% yield. ¹H NMR (CDCl₃): δ 1.55 (s, 9H), 7.03 (m, 1H), 7.56 (m, 1H), 8.10 (d, 1H), 8.46 (d, 1H), 10.03 (s, 1H), 10.9 (broad s, 1H). IR (KBr pellet): Broad acid-OH from 2500 to 3500 cm⁻¹. Carbamate CO stretch at 1667 and 1731 cm⁻¹. Sharp NH stretch at 3320 cm⁻¹. These properties conform to those previously reported (24).

3,4-Dihydro-4-Oxo-1,2,3-benzotriazol-3-yl 2-*tert*-butyloxycarbonylamino benzoate (*Boc*-Abz-ODhbt) (**2**) was synthesized according to literature (24), 77.5% yield. TLC (CH₂Cl₂:CHCl₃ 50:50) *R*_f = 0.5. ¹H NMR (CDCl₃): δ 1.47 (s, 9H), 7.12 (m, 1H), 7.66 (m, 1H), 7.85 (m, 1H), 8.02 (m, 1H), 8.25 (d, 1H), 8.35 (d, 1H), 8.40 (d, 1H), 9.55 (s, 1H); IR (KBr pellet): intense ester CO stretch centered at 1724 cm⁻¹. Disappearance of broad acid OH stretch from 2500 to 3500 cm⁻¹. These properties conform to those previously reported (24).

Fmoc-(*L*)-Lys-(*ε*-*Boc*)-PEGA (**3**). Amino-PEGA resin (1 g, 0.3 mmol) was delivered to a 30 mL fritted reaction vessel. (*Precoupling wash*) The slurry was washed 4 times (5 min each) with 15 mL aliquots of reagent grade

DMF. A final wash with dry DMF was carried out, and excess DMF was pushed from the swollen resin with dry N₂. (*Preactivation*) To a separate 15 mL pear-bottom flask fitted with a rubber septum, 1.2 mmol of α-Fmoc-(*L*)-Lys-(*ε*-*Boc*)-OH, 1.2 mmol of HOBt, and 1.8 mmol of DIEA were dissolved in 2.4 mL of dry DMF. Bop (0.531 g, 1.2 mmol) was dissolved separately in 0.8 mL of DMF, then added at RT. (*Coupling*) After 10 min of preactivation (31), all but ~50 μL of the HOBt ester was added via baked syringe to the swollen resin. With the remaining 50 μL, an approximate concentration of active species of 0.24–0.35 M was confirmed via amine-masking fluorescamine assay (see below). Coupling was allowed to proceed for 2 h. Complete acylation of free amines was confirmed by ninhydrin analysis. The resin was given a *postcoupling* wash of 5 vol of DMF and 5 vol of CH₂Cl₂.

Fmoc-(*L*)-Phe-(*L*)-Lys-(*ε*-Abz-*Boc*)-PEGA (**4**). The *ε*-Boc group was removed from **3** with 15 mL of 50% CF₃COOH in CH₂Cl₂ for 20 min at RT. The resin was immediately washed twice with 10 mL aliquots of CH₂Cl₂, twice with 15 mL aliquots of 2.5% DIEA in CH₂Cl₂, and once with 15 mL of CH₂Cl₂. The amine substitution was measured by quantitative ninhydrin assay (230 μmol/g). The resin was then given a *precoupling* wash.

Boc-Abz-ODhbt, 0.382 g (**2**, 1 mmol), and DIEA, 87 μL (0.5 mmol), were dissolved in 7 mL of DMF and added to α-Fmoc-Lys-PEGA just swollen in dry DMF. After 2 h, complete coupling was confirmed by ninhydrin assay. The resin was then washed 5 times with 15 mL aliquots of DMF, treated with 20 mL of 20% piperidine in DMF for 20 min, and given a *precoupling* wash.

Fmoc-Phe was coupled via the above Bop-preactivation procedure. The resin was then given a *postcoupling* wash and stored overnight in CH₂Cl₂ at 4 °C.

Fmoc-NO₂Tyr-Www-Xxx-Yyy-Zzz-Phe-Lys-(*ε*-Abz-*Boc*)-PEGA (**5**). Resin **4** was divided equally into nine separate 10 mL graduated polypropylene Bio-Rad "Bio-spin" columns, washed once with 5 mL of DMF/column, treated with 5 mL of 20% piperidine in DMF for 20 min, and given a *precoupling* wash. A common Bop/DMF solution (4.5 mL, 0.444 M) was prepared and metered from a 15 mL polypropylene conical tube fitted with a rubber septum. For each 25 μmol peptide-resin slurry, a preactivated HOBt ester (2 mL, 0.222 M) was prepared from its respective Fmoc-amino acid as before and, after 10 min, added to the appropriate column. Coupling times ranged 1.5–2 h. After checking for complete coupling, the resins were given a *postcoupling* wash, and if necessary, stored overnight at 4 °C in CH₂Cl₂. The resin then was pooled, thoroughly mixed in a 50 mL polypropylene conical tube, redistributed into the nine columns, and the deprotection/coupling cycle repeated. This was accomplished at a rate of 1–2 residues/day. Finally, *Fmoc*-NO₂Tyr was coupled to all the peptides.

Fmoc-NO₂Tyr-Www-Xxx-Yyy-Zzz-Phe-Lys-(*ε*-Abz)-PEGA (**6**). A 500 μL sample of **5** (equilibrated in CH₃OH) was treated with 1 mL of 50% CF₃COOH/45% CH₂Cl₂/5% thioanisole for 30 min and washed with 5 mL of CH₂Cl₂.

CF₃CO-NO₂Tyr-Www-Xxx-Yyy-Zzz-Phe-Lys-(*ε*-Abz)-PEGA (**7**). A 700 μL sample of **5** (equilibrated in DMF) was first treated with 1.1 mL of 20% piperidine, washed, and swollen in 1.2 mL of CH₂Cl₂. Pentafluorophenol (1 g, 5.4 mmol), DIEA (1.5 mL) and trifluoroacetic anhydride (0.7 mL, 4.2 mmol) were dissolved in 2 mL of DMF in a separate flask. After 15 min, the active solution was added to the swollen resin. After 1 h, the resin was completely negative to ninhydrin. Boc then was removed as above.

$\text{NO}_2\text{Tyr-Www-Xxx-Yyy-Zzz-Phe-Lys}(\epsilon\text{-Abz})\text{-PEGA}$ (**8**). The remaining PEGA library **5** was treated first with 20 mL of 20% piperidine for 20 min, washed 5 times with 15 mL of DMF, 5 times with 15 mL of CH_2Cl_2 , then treated with $\text{CF}_3\text{COOH}/\text{CH}_2\text{Cl}_2$ /thioanisole as above for 1 h.

Amine-Masking Active Ester Assay. Active esters were assayed for acylating activity as follows: 5 μL of a solution of 0.30 M alanine amide and 0.3 M DIEA in NMP was added to 10 conical wells of a polypropylene microtiter plate. After preactivation, 1–10 μL of active ester solution was added to each well and allowed to react for 20 min. Then, 10 μL of 350 μM fluorescamine in acetone was added and allowed to react for 10 min. Under a 365 nm UV lamp, bright green fluorescence indicates any deficiency of acylating activity and permits an estimate of active species concentration.

Enzymatic Fluorometric Analysis and Bead Capture. Throughout the experiments, the library was stored in CH_3OH at 4 $^\circ\text{C}$, and amounts were measured by spinning aqueous suspensions of beads down to a swollen volume of 25–250 μL as measured on a graduated spin filter, catalog no. 2000–600, purchased from Bio-101 Inc.³

False-positive, blue-glowing beads were carefully removed from the library of quenched, dark beads prior to each enzymatic assay. This was accomplished with a pipet while suspending a portion of the library in 60% $\text{EtOH}/5\text{mM}$ Et_3N ⁴ in a screening dish (polypropylene pipet tip box lid) under a UV lamp.

A shielded UV-254 short wave lamp was used in conjunction with a Nikon SU-B model S microscope to visualize and collect enzymatically cleaved, fluorescing beads. Positive beads were manipulated, first via polypropylene pipet, into a 5 cm Petri dish, and finally captured using microtweezers. Tweezer ends were flared slightly to grip the bead firmly without damaging it. Beads were placed onto 1.2 cm CF_3COOH -pretreated glass fiber filters, coated with 15 μL of dilute biobrene solution (20 $\mu\text{g}/\mu\text{L}$) to firmly fix the bead, and submitted for sequencing.

Library Quality Control Test. One untreated, fully quenched bead was chosen at random, removed from the library, and submitted for Edman sequencing. The bead was found to contain the sequence $\text{NO}_2\text{Tyr-Asn-Tyr-Ala-Ser-Phe-Lys}$. The quantity of peptide on this single bead was estimated as $\leq 10^{-9}$ mol.

Enzyme Preparation and Storage. Human liver cathepsin B (25 μg) in 50 mM NaOAc and 1 mM EDTA, pH 5, was diluted to 1 mL with the same buffer. The solution was divided into 100 μL portions, snap-frozen with liquid N_2 , and stored at -80°C . An aliquot was thawed and its activity determined to be 1.4 units/mL (13).

Lyophilized human liver cathepsin D (25 μg) was dissolved in 600 μL deionized water. Aliquots were snap frozen and stored at -80°C . An aliquot was thawed and its activity determined to be 0.95 units/mL (32, 33).

α -Chymotrypsin Control Digestions. Fmoc-protected resin **6** (50 μL) was divided equally into two separate spin eppendorfs and washed 5 times with 200 μL buffer A (50 mM Tris(hydroxymethyl)aminomethane-

HCl, pH 8.1 and 10 mM CaCl_2). One reaction was carried out in buffer A alone at RT. A second reaction was carried out in 200 μL of buffer A containing α -chymotrypsin [0.94 mg/mL; 52 units/mg BTEE (34)] at RT. After 3 h, the resins were washed 5 times with 500 μL of H_2O , and 5 times with 500 μL of 10% CH_3CN in 0.1% aqueous CF_3COOH , delivered to a polypropylene screening dish, dissolved in 60% $\text{EtOH}/5\text{mM}$ Et_3N , and visualized under 254 nm. None of the beads in either experiment were positive. After 15 h, two of the buffer control beads were faintly positive. Of the chymotrypsin-treated beads, only one clearly positive bead and 25–37 faintly positive blue-rimmed beads were found. The same procedure was repeated on the *N*-trifluoroacetylated resin **7**; the resulting supernatant was faintly colored and only about 10% of the beads were positive after digestion.

Fmoc was cleaved from remaining resin **6** with 20% piperidine to yield beads **8** that were bright orange at pH 8. Another 25 μL of resin was treated as above with chymotrypsin and washed. Within 1.25 h, the supernatant turned distinctly yellow, and over 85% of the beads was clearly fluorescent. This experiment was repeated using a lower enzyme concentration. Two separate 200 μL aliquots of PEGA-library **8** were used. One aliquot was treated with 32 μL of buffer A alone; the other was treated with chymotrypsin (0.48 $\mu\text{g}/\mu\text{L}$) in 32 μL of buffer A and gently stirred overnight. After 1 h, hundreds of beads had become fluorescent in the chymotrypsin treated aliquot; after 15 h, over 90% of the chymotrypsin beads were fluorescent, but none of those in the control. The final absorbance at 426 nm of the chymotrypsin treatment supernatant was 0.3, and that of the buffer-only control was <0.1 .

Cathepsin B Assays. Library resin **8** ($2 \times 250 \mu\text{L}$) was washed 5 times with 500 μL of buffer B (50 mM NaOAc and 1 mM EDTA, pH 5.4). To one of the centrifuged/swollen resin suspensions, 195 μL of buffer B and 5 μL of 1 M DTT were added and the resin incubated at 37 $^\circ\text{C}$. To the other resin bed, 100 μL of freshly thawed cathepsin B, 95 μL of buffer B, and 5 μL of 1 M DTT were added and incubated at 37 $^\circ\text{C}$. After the first 1.5 h of treatment, the resin beds were centrifuge filtered, the supernatant saved, and the resin washed 5 times with 0.5 mL of 0.1 N NaOAc, pH 6; 5 times with 0.5 mL of 20% $\text{CH}_3\text{CN}/80\%$ 0.1 N NaOAc, pH 6; and 5 times with 0.5 mL of H_2O . No positive beads were found during treatment of resin with buffer alone, while the cathepsin-treated resin bed yielded two positive beads, which were submitted for sequencing. A 100 μL aliquot of the slightly colored supernatant from this treatment was diluted to 1.1 mL with 0.1 N KHCO_3 , pH 9, and found to have an absorbance of 0.11 at 426 nm. The remaining unreacted beads from this batch of resin were recovered, and another round of proteolytic treatment was repeated for an additional 195 min, yielding an absorbance at 426 nm of <0.1 for the diluted supernatant, and two more positive beads. A final round was performed over 17 h: the diluted supernatant gave an absorbance of 0.16, and 6 positive beads were collected. Results from these and subsequent treatments are summarized in Tables 1 and 2.

Cathepsin D Assays. Treatment of 250 μL resin beads **8** with 180 μL of freshly thawed enzyme and 20 μL buffer C (1 N sodium formate, pH 3.8) for 24 h at 37 $^\circ\text{C}$ resulted in >100 positive beads for the enzymatic resin bed and three positives for the buffer control. A milder treatment on a fresh library sample was performed using 50 μL of freshly thawed enzyme, 20 μL of buffer C, and 130 μL of water at 37 $^\circ\text{C}$ for 15.5 h. This yielded eight

³ Using a small mechanic's rule, swollen beads were estimated to have a range of diameters from 75 to 200 μm in 60% EtOH . Accordingly, a 200 μL packed resin bed represents $\approx 80\,000$ beads.

⁴ The pH must be >6 to deprotonate the NO_2Tyr and completely quench the fluorophore.

Table 1. Results from Edman Degradation of Beads Collected from Library Digests (Identified Cleavage Sites Are Indicated by (-))

human cathepsin B	human cathepsin D
NO ₂ Tyr-Gln-Gly-Val-Gln-Phe-Lys(Abz) ^{a,g}	NO ₂ Tyr-Asn-Gly-Thr-Gly-Phe-Lys(Abz)
NO ₂ Tyr-Ser-Val-Val-Phe-Phe-Lys(Abz)	NO ₂ Tyr-Val-Gln-Ser-Ala-Phe
Multiple-Val-Gln-Phe-Val ^g	NO ₂ Tyr-Gly-Val-Phe-Gln-Phe
NO ₂ Tyr-Gly-Thr-Val-Ala-Phe-Lys(Abz) ^{e,f}	NO ₂ Tyr-Ala-Thr-Ala-Phe-Phe-Lys(Abz) ^d
NO ₂ Tyr-Gly-Ser-Val-Gln-Phe-Lys(Abz) ^e	NO ₂ Tyr-Gly-Gly-Gln-Phe-Phe-Lys(Abz) ^d
NO ₂ Tyr-Gln-Ser-Val-Gly-Phe-Lys(Abz) ^{e,f,g}	NO ₂ Tyr-Gly-Ser-Thr-Phe-Phe-Lys(Abz) ^{d,h}
NO ₂ Tyr-Gly-Thr-Val-Gln-Phe-Lys(Abz) ^e	NO ₂ Tyr-Gly-Ser-Thr-Phe-Phe-Lys(Abz) ^{d,h}
NO ₂ Tyr-Gly-Val-Ala-Gly-Phe-Lys(Abz) ^e	NO ₂ Tyr-Gly-Ser-Thr-Phe-Phe-Lys(Abz) ^{d,h}
NO ₂ Tyr-Ala-Ala-Gly-Thr-Phe-Lys(Abz)	NO ₂ Tyr-Val-Ala-Gln-Phe ^c
NO ₂ Tyr-Gln-Gly-Val-Gly-Phe-Lys(Abz) ^{e,g}	NO ₂ Tyr-Val-Asn-Asn-Asn-Phe-Lys(Abz)
NO ₂ Tyr-Ala-Ser-Ala-Asn-Phe-Lys(Abz)	NO ₂ Tyr-Phe-Gln-Thr-Gln-Phe-Lys(Abz)
NO ₂ Tyr-Ala-Ala-Ala-Ser-Phe-Lys(Abz)	NO ₂ Tyr-Gln-Tyr-Ser-Gly-Phe-Lys(Abz)
NO ₂ Tyr-Ala-Ala-Thr-Ala-Phe-Lys(Abz)	NO ₂ Tyr-Ala-Thr-Gln-Phe-Phe-Lys(Abz) ^d
NO ₂ Tyr-Gln-Ser-Ala-Ser-Phe-Lys(Abz)	NO ₂ Tyr-Gly-Thr-Ser-Phe-Phe-Lys(Abz) ^d
NO ₂ Tyr-Thr-Ala-Gly-Ala-Phe-Lys(Abz)	NO ₂ Tyr-Ala-Thr-Thr-Phe-Phe-Lys(Abz) ^d
NO ₂ Tyr-Ala-Ser-Gly-Ser-Phe-Lys(Abz)	NO ₂ Tyr-Gly-Thr-Thr-Phe-Phe-Lys(Abz) ^d
NO ₂ Tyr-Gly-Ala-Ala-Gly-Phe-Lys(Abz) ^f	NO ₂ Tyr-Gly-Thr-Gln-Phe-Phe-Lys(Abz) ^{d,h}
NO ₂ Tyr-Ala-Ala-Thr-Gly-Phe-Lys(Abz) ^f	NO ₂ Tyr-Gly-Thr-Gln-Phe-Phe-Lys(Abz) ^{d,h}
NO ₂ Tyr-Gln-Thr-Val-Gly-Phe-Lys(Abz) ^{e,f,g}	NO ₂ Tyr-Gly-Thr-Gln-Phe-Phe-Lys(Abz) ^{d,h}
NO ₂ Tyr-Ala-Ser-Ala-Gly-Phe-Lys(Abz)	NO ₂ Tyr-Gly-Gln-Ser-Phe-Phe-Lys(Abz) ^d
NO ₂ Tyr-Thr-Ser-Ala-Thr-Phe-Lys(Abz)	NO ₂ Tyr-Gly-Thr-Val-Ala-Phe-Lys(Abz)
NO ₂ Tyr-Thr-Ala-Gln-Ala-Phe-Lys(Abz)	NO ₂ Tyr-Gly-Val-Ala-Ala-Phe-Lys(Abz)
NO ₂ Tyr-Val-Ala-Ser-Ala-Phe-Lys(Abz)	NO ₂ Tyr-Gln-Gly-Ser-Phe-Phe-Lys(Abz) ^d
NO ₂ Tyr-Thr-Ala-Thr-Asn-Phe-Lys(Abz)	NO ₂ Tyr-Ala-Thr-Ser-Phe-Phe-Lys(Abz) ^d
NO ₂ Tyr-Thr-Gly-Val-Gly-Phe-Lys(Abz) ^e	NO ₂ Tyr-Gly-Thr-Ala-Phe-Phe-Lys(Abz) ^d
NO ₂ Tyr-Gln-Val-Ala-Gly-Phe-Lys(Abz)	
NO ₂ Tyr-Gly-Ser-Ala-Gln-Phe-Lys(Abz) ^f	
NO ₂ Tyr-Val-Ala-Ala-Gln-Phe-Lys(Abz)	
NO ₂ Tyr-Gln-Thr-Ala-Thr-Phe-Lys(Abz)	
NO ₂ Tyr-Thr-Gly-Tyr-Thr-Phe-Lys(Abz)	
NO ₂ Tyr-Ser-Ala-Gly-Thr-Phe-Lys(Abz)	
NO ₂ Tyr-Val-Tyr-Tyr-Val-Phe ^b	
NO ₂ Tyr-Ala-Ser-Tyr-Gly-Phe ^b	

^a Lysine with free ϵ -amine is detected (25). ^b Cathepsin B from human placenta. ^c Edman incomplete. Very low level of peptide detected. Doublet of Phe is inferred, since a Phe was inserted into the mother resin Phe-Lys(Abz)-PEGA. ^d Cleavage between two Phe residues. ^e Valine in position P2. ^f NO₂Tyr in P2 of second cleavage site. ^g QXV detected (19). ^h Three beads with identical sequences isolated.

clearly positive beads, and no positive beads for the buffer control. Unfortunately, only two of these contained sufficient peptide to be sequenced. The assay was repeated, using 50 μ L of cathepsin D, 20 μ L of buffer C, and 170 μ L of water for 14 h at 37 °C. At this time, six positive beads were collected, five of which could be sequenced. These procedures were repeated as necessary to accumulate a statistically relevant number of sequences.

Substrate Sequence Analysis. Pratt version 2.1 sequence pattern analysis software (52) was utilized online to search for consensus sequences for cathepsin B and cathepsin D substrates selected from the library. Amino-terminal fragments (Table 2) of substrates for both enzymes were entered as variable amino acids, except for NO₂Tyr, which was entered as an ancillary letter code to set off the relative position of the N-terminus of the aligned results without being considered as part of any pattern by the algorithm. There were too few carboxyl-terminal fragments composed of variable amino acids to be statistically significant. The "C-option" stringency was set to 25% for cathepsin B and 67% for cathepsin D. Two patterns were identified and scored for cathepsin B: G-X(0,1)-V-[AGQ]⁵ where V occupies P2 (fitness score = 10.5), and A-X(0,1)-G where A occupies P3 and/or P2 (fitness score = 7.8). Two patterns were identified and scored for cathepsin D: G-X(1,2)-F, where Gly occupies either P4 or P3, and Phe occupies P1 (fitness

score = 7.8), and T-X(0,1)-F, where Thr occupies P3 or P2, and Phe occupies P1 (fitness score = 7.8).

RESULTS AND DISCUSSION

During control experiments, we found that a free amino-terminus on the peptide bead was essential for cleavage with chymotrypsin. All peptides in the library were potential chymotrypsin substrates, yet Fmoc-protected resin-peptides were not digested by chymotrypsin. Acylation of the N-terminus with the less hydrophobic trifluoroacetyl group did not result in a significant improvement. In contrast, the library with free N-termini was efficiently hydrolyzed by chymotrypsin. These observations correlate with the findings of others using this technique. For example, Del Nery et al. (27) discovered that an unbiased library of peptide nonamers was poorly hydrolyzed by Cruzipain and ionizable substrates seemed to be preferred. In our library of heptamers, chymotrypsin was capable of cleaving nearly all the peptides, and biases in enzyme accessibility due to the presence of ionizable groups were avoided because such residues were not included in the library. Even the comparatively large cathepsin D, at 42 kDa, was capable of cleaving substrates in a way that is characteristic of this enzyme.

It should be noted that all of the peptide beads were not indefinitely stable in aqueous media. Nitrotyrosine-containing chromophores elute from the resin over time, and batches of resin incubated only with buffer produce a small but significant number of beads that become fluorescent without intentional exposure to enzymes. Therefore, any set of beads collected from proteolytic

⁵ The nomenclature for this pattern is translated as follows: Gly is followed by either 0 or 1 variable amino acids, which is followed by Val, which is followed by either Ala, Gly, or Gln.

Table 2. Cleavage Sites Identified^a

cut	P5	P4	P3	P2	P1	P1'	P2'	P3'	P4'	P5'
cathepsin B										
1	NO ₂ Tyr	Gln	Gly	Val	Gln	Phe*	Lys(Abz)			
2	NO ₂ Tyr	Gly	Thr	Val	Ala	Phe*	Lys(Abz)			
3				NO ₂ Tyr	Gly	Thr	Val	Ala	Phe*	Lys(Abz)
4	NO ₂ Tyr	Gly	Ser	Val	Gln	Phe*	Lys(Abz)			
5	NO ₂ Tyr	Gln	Ser	Val	Gly	Phe*	Lys(Abz)			
6				NO ₂ Tyr	Gln	Ser	Val	Gly	Phe*	Lys(Abz)
7	NO ₂ Tyr	Gly	Val	Ala	Gly	Phe*	Lys(Abz)			
8				NO ₂ Tyr	Gly	Val	Ala	Gly	Phe*	Lys(Abz)
9			NO ₂ Tyr	Ala	Ala	Gly	Thr	Phe*	Lys	
10	NO ₂ Tyr	Gly	Thr	Val	Gln	Phe*	Lys(Abz)			
11	NO ₂ Tyr	Gln	Gly	Val	Gly	Phe*	Lys(Abz)			
12	NO ₂ Tyr	Gly	Ala	Ala	Gly	Phe*	Lys(Abz)			
13				NO ₂ Tyr	Gly	Ala	Ala	Gly	Phe*	Lys(Abz)
14	NO ₂ Tyr	Gln	Thr	Val	Gly	Phe*	Lys(Abz)			
15				NO ₂ Tyr	Gln	Thr	Val	Gly	Phe*	Lys(Abz)
16	NO ₂ Tyr	Ala	Ala	Thr	Gly	Phe*	Lys(Abz)			
17				NO ₂ Tyr	Ala	Ala	Thr	Gly	Phe*	Lys(Abz)
18	NO ₂ Tyr	Thr	Ser	Ala	Thr	Phe*	Lys(Abz)			
19	NO ₂ Tyr	Ala	Ser	Ala	Gly	Phe*	Lys(Abz)			
20	NO ₂ Tyr	Thr	Gly	Val	Gly	Phe*	Lys(Abz)			
21	NO ₂ Tyr	Gly	Ser	Ala	Gln	Phe*	Lys(Abz)			
22				NO ₂ Tyr	Gly	Ser	Ala	Gln	Phe*	
23	NO ₂ Tyr	Val	Ala	Ala	Gln	Phe*	Lys(Abz)			
24	NO ₂ Tyr	Gln	Val	Ala	Gly	Phe*	Lys(Abz)			
25	NO ₂ Tyr	Gln	Thr	Ala	Thr	Phe*	Lys(Abz)			
26	NO ₂ Tyr	Thr	Gly	Tyr	Thr	Phe*	Lys(Abz)			
27					NO ₂ Tyr	Ser	Ala	Gly	Thr	Phe
cathepsin D										
1	NO ₂ Tyr	Ala	Thr	Ala	Phe	Phe*	Lys(Abz)			
2	NO ₂ Tyr	Gly	Gly	Gln	Phe	Phe*	Lys(Abz)			
3	NO₂Tyr	Gly	Ser	Thr	Phe	Phe*	Lys(Abz)			
4	NO ₂ Tyr	Ala	Thr	Gln	Phe	Phe*	Lys(Ant)			
5	NO ₂ Tyr	Gly	Thr	Ser	Phe	Phe*	Lys(Abz)			
6	NO ₂ Tyr	Ala	Thr	Thr	Phe	Phe*	Lys(Abz)			
7	NO ₂ Tyr	Gly	Thr	Thr	Phe	Phe*	Lys(Abz)			
8	NO₂Tyr	Gly	Thr	Gln	Phe	Phe*	Lys(Abz)			
9	NO ₂ Tyr	Gly	Gln	Ser	Phe	Phe*	Lys(Abz)			
10	NO ₂ Tyr	Gly	Thr	Val	Ala	Phe*	Lys(Abz)			
11	NO ₂ Tyr	Gly	Val	Ala	Ala	Phe*	Lys(Abz)			
12	NO ₂ Tyr	Gln	Gly	Ser	Phe	Phe*	Lys(Abz)			
13	NO ₂ Tyr	Ala	Thr	Ser	Phe	Phe*	Lys(Abz)			
14	NO ₂ Tyr	Gly	Thr	Ala	Phe	Phe*	Lys(Abz)			

^a Phe*-Lys(Abz) is present at the C-terminus of all members of the library. Substrates in **Bold** were selected independently three times.

digests may contain a small percentage of false positive sequences, underscoring the need for appropriate buffer-only controls.

About 10% of the beads submitted for Edman sequencing contained too little peptide to be sequenced. Beads collected from our first cathepsin D digestion contained particularly small amounts of peptide. A strategy where the supernatant absorbance at 426 nm was repeatedly monitored and positive beads periodically removed was found useful.

It was possible that extensive proteolysis might result in the total loss of the N-terminal portion of the peptide. On the other hand, insufficient cleavage might not produce enough product to reveal the cleavage site. Fortunately, 50–60% of beads submitted for Edman degradation contained the proper balance of cleaved and uncleaved peptide to identify both uncleaved peptide and sites of cleavage. Indeed, in some cases, two sites of cleavage were discernible from the pattern of multiple peaks resulting from Edman sequencing.

Cathepsin B Results. The results in Tables 1 and 2 correlate well with the literature on substrate specificity. For example, the presence of a hydrophobic residue in position P2 is a frequently cited characteristic of papain-like proteinases such as cathepsin B (3, 35, 46). In 27

clearly assignable cleavage sites, only one anomalous hydrophilic Thr is found here. P2 is shared about equally by Val, NO₂Tyr, and Ala. A modestly preferred N-terminal substrate consensus sequence was derived (52): G-X-V-[AGQ], where Gly occupies P4, P3 has high variability, Val occupies P2, and Ala, Gly, or Gln occupies P1. The aromatic residues Phe and Tyr were not found at P2 as might have been expected (46).

We infer from our cathepsin B results that small side chains are somewhat favored in P1, with the following ranking: Gly > Gln > Ala. Our literature search revealed that Phe and Tyr are disfavored in P1, while the equally hydrophobic O-benzyl-Thr derivative is beneficial here, for detailed steric reasons (41).⁶

Tyr, Phe, and Asn are all distinctly underrepresented for cathepsin B in *all* variable positions in our combinatorial context. Amino acid analysis of a sample of untreated library shows that Asn, Phe, and Tyr each fall within 1 standard deviation ($\pm 11\%$) of the average level of incorporation. This particular bias was not observed during cathepsin D treatment of the same library (where, for example, Phe dominates P1). These observations are

⁶ It has since been shown that the benzyl group probably occupies the adjacent S1' pocket (42).

consistent with the expectation that this procedure reveals the intrinsic preferences of the cathepsins.

Among cathepsin B substrates, the preference for the constant penultimate C-terminal Phe at P1' was notable. Menard et al. (12) note a preference for large hydrophobic residues such as Phe and Leu at P1', and this is supported by evidence from the crystal structure (42, 43). However, this alone may not explain the fact that library cleavage occurs primarily toward the C-terminus of the substrate, where only two residues occupy the S' sites. Table 2 shows that when cleavages occur elsewhere, they are almost exclusively found *in addition* to cleavages at this position for the same substrate and two residues distant.⁷ Most of the multiply cut cathepsin B substrates in Table 2 have the following characteristics: another hydrophobic residue (NO₂Tyr) occupies P2, a small residue is again found in P1, and a small residue is found in P3'. The crystal structure for cathepsin B suggests that there is narrowing of the active-site cleft at S3', which is nearly spanned by Leu181 on one side and His111 of the "occluding loop" on the other (42, 43). His110 and His111 side chains in this loop are implicated in cathepsin B's pH-controlled carboxydipeptidase activity (44). The almost exclusive preference for the C-terminal 2-amidoprop-1-yl-PEG interface, or small residues Gly, Gln, and Ala at P3' in the solution set is consistent with a model whereby the preferred site of endoprotease activity is influenced by the occlusion at S3'.

The sequence Q-X-V occurs at least 5 of 27 times in the N-terminal portions of the selected substrates for cathepsin B (Table 1). This is reminiscent of the highly conserved QXVXG motif found in the hydrophobic loops of cystatin inhibitors of papain-like proteases (36). Labile substrates based on the crystal structure of cathepsin B and certain "wedge" regions of the cystatin inhibitors have been characterized (37, 38). Substrates based on the conserved segments of the cystatins were discovered by chance and were unexpected (19) because the cystatin inhibition mechanism is reversible and does not involve peptide cleavage of the inhibitor. A literature substrate based on the central portion of this wedge, Abz-QVVAG-EDDnp, has $k_{\text{cat}}/K_m = 2.5 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ for cathepsin B (19). Gln occupies P4 in this substrate, as it does in at least six of our selected substrates for cathepsin B.

Cathepsin D Results. Cathepsin D proved to be a far more selective enzyme, as expected (46–48). Pratt sequence software was able to identify two conserved patterns in 67% of the solution set in Table 2, as compared to only 25% for cathepsin B. *Identical* substrates were selected independently on three different beads (Table 1) for each of two unique sequences, -Gly-Thr-Gln-Phe-Phe and -Gly-Ser-Thr-Phe-Phe. Cleavage of these substrates occurs between the doublet of Phe residues. It should be remembered that the library contained an invariant Phe at its C-terminus, which appears at P1' in these sequences. Both triply selected substrates are closely related to the N-terminal consensus sequences derived from Pratt analysis: G-X(1,2)-F and T-X(0,1)-F. The preferential cleavage between Phe residues in substrates of pepsin-like proteinases such as cathepsin D is well established (29, 35, 39). For example, cathepsin D substrates based upon this common (but not exclusive) phenomenon have been characterized: Lys-Pro-Ile-Leu-Phe-NO₂Phe-Arg-Leu, $k_{\text{cat}}/K_m = 1.61 \times 10^6$

$\text{M}^{-1} \text{ s}^{-1}$ (17); and Phe-Ala-Ala-NO₂Phe-Phe-Val-Leu, $k_{\text{cat}}/K_m = 4.1 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ (40). Like cathepsin B, cathepsin D cleaves primarily at the position prior to the constant Phe. This position in the library is unique in that one-ninth of all library substrates will have a doublet of Phe here. A preference for Thr in P3 is consistent with preferences as found for both human cathepsin D (49) and the homologous porcine pepsin (39), but the predicted preference for Val, Ala, and Asn at P2 was not observed. Instead hydrophilic, hydrogen-bonding residues seem to be preferred despite the large, mainly hydrophobic pocket at S2 (51). Others have found that inhibitors designed to fill this pocket with correspondingly large hydrophobic side chains failed to produce the desired result, the explanation being attributed to overall entropic penalties for the enzyme-inhibitor complex (45).

Another behavior that has eluded prediction by analogy (39), modeling assumptions (45), and historical techniques (17, 49) is the clear preference for small or hydrophilic residues Gly > Ala > Gln in P4. This again suggests a lack of complementarity to the hydrophobicity of the putative enzyme subsite.

CONCLUSIONS

The scope of this peptide library ($9^4 = 6561$) was necessarily limited due to the practical constraints imposed by constructing a peptide-linked radiolabeled chelate with protein modification functionality and by prelabeling antibodies for radiopharmaceutical use. Even so, the results were found to be not only consistent with, but supplementary to many characteristics outlined in the literature.

For cathepsin B, we found that (1) the size of the hydrophobic residue at P2 was not as important as expected, as demonstrated by the frequent acceptance of Ala here; (2) the preference for a small residue in P1 is confirmed; (3) Asn, Phe, and Tyr are statistically under-represented in positions P4–P1; (4) there may be a preference for Gln or Gly in P4; and (5) bioconjugates dependent upon the endoprotease activity of cathepsin B may benefit from a substrate design which considers the "occlusion" at the predicted P3' position.

For cathepsin D, cleavage between a doublet of Phe residues is the dominant characteristic, in accord with both statistical prediction for the homologous pepsin (39) and experiment (17, 49). The preference for Thr in P3 is similarly expected. However, our results are not in total agreement with predictions based on either the homologous porcine pepsin or structure-based modeling. We observe a preference for hydrogen-bonding residues in P2 and a distinct preference for Gly > Ala > Gln in P4. This demonstrates that even the more remote subsites may have a significant effect on substrate properties.

These results must be interpreted with deference to the unquantified role of solubility in this selection process. Study of two enzymes was found useful in this regard: substrates with clearly different sequences were selected. So while differences in solubility might not be negligible for these experiments, they do not mask the specificity of the enzymes.

The fluorescence quench one-bead-one peptide methodology is sufficiently simple and straightforward to explore new alternatives even in highly specialized drug targeting contexts such as radioimmunotherapy, and it may provide improvements on the simple Gly₃Phe linker

⁷ A similar substrate "shift" has been proposed for penicillopepsin (50). A pattern of multiple cleavage was not observed for cathepsin D.

used previously (6). To this end, we are preparing several new DOTA-peptide-antibody conjugates for biological tests.

ACKNOWLEDGMENT

This work was supported by NIH Research Grants CA16861 (C.F.M.) and CA47829 (G.L. DeNardo P.I.). We thank Dr. Douglas P. Greiner for helpful discussions.

LITERATURE CITED

- (1) Borissova, R., Stjarnkvist, P., Karlsson, M. O., and Sjöholm, I. (1995) Biodegradable Microspheres. 17. Lysosomal degradation of Primaquine-Peptide Spacer Arms. *J. Pharm. Sci.* 84, 256–262.
- (2) Hsin, C. C., Zalipsky, S., Kopeckova, P., and Kopecek, J. (1993) Enzymatic Activity of Chymotrypsin and Its Poly(ethylene glycol) Conjugates toward Low and High Molecular Weight Substrates. *Bioconjugate Chem.* 4, 290–295.
- (3) Putnam, D., and Kopecek, J. (1995) Enantioselective Release of 5-Fluoro-uracil from N-(2-Hydroxypropyl)methacrylamide-Based Copolymers via Lysosomal Enzymes. *Bioconjugate Chem.* 6, 483–492.
- (4) Putnam, D., Shiah, J.-G., and Kopecek, J. (1996) Intracellularly Biorecognizable Derivatives of 5-Fluoro-uracil. *Biochem. Pharm.* 52, 957–962.
- (5) Studer, M., Kroger, L. A., DeNardo, S. J., Kukis, D. L., and Meares, C. F. (1992) Influence of a Peptide Linker on Biodistribution and Metabolism of Antibody-Conjugated Benzyl-EDTA. Comparison of Enzymatic Digestion in Vitro and in Vivo. *Bioconjugate Chem.* 3, 424–429.
- (6) Li, M., and Meares, C. F. (1993) Synthesis, Metal Chelate Stability Studies, and Enzyme Digestion of a Peptide-Linked DOTA derivative and Its Corresponding Radiolabeled Immunoconjugates. *Bioconjugate Chem.* 4, 275–283.
- (7) DeNardo, S. J., Kukis, D. L., Kroger, L. A., O'Donnell, R. T., Lamborn, K. R., Miers, L. A., DeNardo, D. G., Meares, C. F., and DeNardo, G. L. (1997) Synergy of Taxol and radioimmunotherapy with yttrium-90-labeled chimeric L6 antibody: Efficacy and toxicity in breast cancer xenografts. *Proc. Natl. Acad. Sci. U.S.A.* 94, 4000–4004.
- (8) Huisman, W., Lanting, L., Doddema, H. J., Bouma, J. M. W., and Gruber, M. (1974) Role of Individual Cathepsins in Lysosomal Protein Digestion as Tested by Specific Inhibitors. *Biochim. Biophys. Acta* 370, 297–307.
- (9) Blum, J. S., Fiani, M. L., and Stahl, P. D. (1991) Localization of cathepsin D in endosomes: characterization and biological importance. *Adv. Exp. Med. Biol.* 306, 281–287.
- (10) Dingle, J. T., Poole, R. A., Lazarus, G. S., and Barrett, A. J. (1973) Immunoinhibition of Intracellular Protein Digestion in Macrophages. *J. Exp. Med.* 137, 1124–1141.
- (11) Li, M., Meares, C. F., Zhong, G.-R., Miers, L., Xiong, C.-Y., and DeNardo, S. J. (1994) Labeling Monoclonal Antibodies with ⁹⁰Yttrium- and ¹¹¹Indium-DOTA Chelates: A Simple and Efficient Method. *Bioconjugate Chem.* 5, 101–104.
- (12) Menard, R., Carmona, E., Plouffe, C., Bromme, D., Konoshi, Y., Lefebvre, J., and Storer, A. (1993) The Specificity of the S1' Subsite of Cysteine Proteases. *FEBS Lett.* 328 (1, 2), 107–110.
- (13) Bajkowski, A. S., and Frankfater, A. (1975) Specific Spectrophotometric Assays for Cathepsin B. *Anal. Biochem.* 68, 119–127.
- (14) Barrett, A. J. (1980) Fluorimetric Assays for Cathepsin B and Cathepsin H with Methylcoumarinylamide Substrates. *Biochem. J.* 187, 909–912.
- (15) Knight, C. G. Application of the Substrate N-Benzoyloxycarbonyl-L-arginyl-L-arginyl-Arginine 2-Naphthylamide to a Study of the Inhibition by Leupeptin. (1980) *Biochem. J.* 189, 447–453.
- (16) Tarlap, A., Kaplan, H., Sytwu, I., Vlattas, I., Bohacek, R., Knap, A., Hirama, T., Huber, C. P., and Hasnain, S. (1995) Characterization of the S3 Subsite Specificity of Cathepsin B. *J. Biol. Chem.* 270, 18036–18043.
- (17) Scarborough, P., Topham, C., Richo, G. R., Conner, G. E., Blundell, T. L., and Dunn, B. M. (1993) Exploration of Subsite Binding Specificity of Human Cathepsin D Through Kinetics and Rule-based Molecular Modeling. *Protein Sci.* 2, 264–276.
- (18) Scarborough, P., and Dunn, B. M. (1994) Redesign of Substrate Specificity of Human Cathepsin D: The Dominant Role of Position 287 in the S2 subsite. *Protein Eng.* 7 (4), 495–502.
- (19) Lalmanach, G., Serveau, C., Brillard-Bourdet, M., Chagas, J. R., Mayer, R., Juliano, L., and Gaurthier, F. (1995) Conserved Cystatin Segments as Models for Designing Specific Substrates and Inhibitors of Cysteine Proteinases. *J. Protein Chem.* 14, 645–653.
- (20) Gron, H., and Breddam, K. (1992) Interdependency of the Binding Sites in Subtilisin. *Biochemistry* 31, 8967–8971.
- (21) Schellenberger, V., Turck, C. W., and Rutter, W. J. (1994) Role of S' Subsites in Serine Protease Catalysis. Active-Site Mapping of Rat Chymotrypsin, Rat Trypsin, α -Lytic Protease, and Cercarial Protease from *Schistosoma mansoni*. *Biochemistry* 33, 4251–4257.
- (22) Chagas, J. R., Juliano, L., and Prado, E. S. (1991) Intramolecularly Quenched Fluorogenic Tetrapeptide Substrates for Tissue and Plasma Kallikreins. *Anal. Biochem.* 192, 419–425.
- (23) Maggiora, L. L., Smith, C. W., and Zhang, Z.-Y. (1992) A General Method for the Preparation of Internally Quenched Fluorogenic Protease Substrates Using Solid-Phase Peptide Synthesis. *J. Med. Chem.* 35, 3727–3730.
- (24) Meldal, M., and Breddam, K. (1991) Anthranilamide and Nitrotyrosine as a Donor–Acceptor Pair in Internally Quenched Fluorescent Substrates for Endopeptidases: Multicolumn Peptide Synthesis of Enzyme Substrates for Subtilisin Carlsberg and Pepsin. *Anal. Biochem.* 195, 141–147.
- (25) Meldal, M., Svendsen, I., Breddam, K., and Auzanneau, F.-I. (1994) Portion-mixing Peptide Libraries of Quenched Fluorogenic Substrates for Complete Subsite Mapping of Endoprotease Specificity. *Proc. Natl. Acad. Sci.* 91, 3314–3318.
- (26) Lebl, M., Krchnak, V., Sepetov, N. F., Seligmann, B., Strop, P., Felder, S., and Lam, K. S. (1995) One-Bead-One-Structure Combinatorial Libraries. *Biopolymers (Pept. Sci.)* 37, 177–198.
- (27) Del Nery, E., Juliano, M. A., Meldal, M., Svendsen, I., Scharfstein, J., Walmsley, A., and Juliano, L. (1997) Characterization of the Substrate Specificity of the Major Cysteine Protease (Cruzipain) from *Trypanosoma cruzi* Using a Portion-Mixing Combinatorial Library and Fluorogenic Peptides. *Biochem. J.* 323, 427–433.
- (28) Barrett, A. J., Ed. (1977) in *Proteinases in Mammalian Cells and Tissues*, Vol. 2, pp 181–207, Elsevier/North-Holland Biomedical Press, Amsterdam.
- (29) Barrett, A. J., Ed. (1977) in *Proteinases in Mammalian Cells and Tissues*, Vol. 2, pp 209–248, Elsevier/North-Holland Biomedical Press, Amsterdam.
- (30) Rejmanova, P., Kopecek, J., Pohl, J., Baudys, M. and Kostka, V. (1983) Polymers Containing Enzymatically Degradable Bonds. 8. Degradation of Oligopeptide Sequences in N-(2-hydroxypropyl)methacrylamide Copolymers by Bovine Spleen Cathepsin B. *Makromol. Chem.* 184, 2009–2020.
- (31) Hudson, D. (1988) Methodological Implications of Simultaneous Solid-Phase Peptide Synthesis. 1. Comparison of Different Coupling Procedures. *J. Org. Chem.* 53, 617–624.
- (32) Barrett, A. J. (1967) Lysosomal Acid Proteinase of Rabbit Liver. *Biochem. J.* 104, 601.
- (33) Barrett, A. J. (1970) Purification of Isoenzymes from Human and Chicken Liver. *Biochem. J.* 117, 601–607.
- (34) Hummel, B. (1959) A Modified Spectrophotometric Determination of Chymotrypsin, Trypsin, and Thrombin. *Can. J. Biochem. Physiol.* 37, 1393.
- (35) Keil, B. (1992) *Specificity of Proteolysis*, Springer-Verlag, Berlin.
- (36) Auerswald, E. A., Genenger, G., Assfalg-Machleidt, I., Machleidt, W., Engh, R. A., and Fritz, H. (1992) Recombinant Chicken Egg White Cystatin Variants of the QLVSG region. *Eur. J. Biochem.* 209, 837–845.

- (37) Bode, W., Engh, R., Musil, D., Thiele, U., Huber, R., Karshikov, A., Brzin, J., Kos, J., and Turk, V. (1988) The 2.0 Å X-ray Crystal Structure of chicken Egg White Cystatine and its Possible Mode of Interaction with Cysteine Proteinases. *EMBO J.* 7, 2593–2599.
- (38) Stubbs, M. T., Laber, B., Bode, W., Huber, R., Jerala, R., Lenarcic, B., and Turk, V. (1990) The Refined 2.4 Å X-ray crystal structure of Recombinant Human Stefin B in Complex with the Cysteine Proteinase Papain: A Novel Type of Proteinase Inhibitor Interaction. *EMBO J.* 9, 1939–1947.
- (39) Powers, J. C., Harley, D. A., and Myers, D. V. (1977) Subsite Specificity of Porcine Pepsin. *Adv. Exp. Med. Biol.* 95, 141–57.
- (40) Agarwal, N., and Rich, D. H. (1983) An Improved Cathepsin D Substrate and Assay Procedure. *Anal. Biochem.* 130, 158–165.
- (41) Shaw, E., Wilkstrom, P., Ruscica, J. (1983) An Exploration of the Primary Specificity Site of Cathepsin B. *Arch. Biochem. Biophys.* 222, 424–429.
- (42) Jia, Z., Hasnain, S., Hiram, T., Lee, X., Mort, J., To, R., Huber, C. P. (1995) Crystal Structures of Recombinant Rat Cathepsin B and a Cathepsin B–Inhibitor Complex. *J. Biol. Chem.* 270, 5527–5533.
- (43) Musil, D., Zucic, D., Turk, D., Engh, R. A., Mayr, I., Huber, R., Popovic, T., Turk, V., Towatari, T., Katunuma, N., Bode, W. (1991) The Refined 2.15 Å X-ray Crystal Structure of Human Liver Cathepsin B: The Structural Basis for its Specificity. *EMBO J.* 10, 2321–2330.
- (44) Polgar, L., Csoma, C. (1987) Dissociation of Ionizing Groups in the Binding Cleft Inversely Controls the Endo- and Exopeptidase Activities of Cathepsin B. *J. Biol. Chem.* 262, 14448–14453.
- (45) Majer, P., Collins, J. R., Gulnik, S. V., Erickson, J. W. (1997) Structure Based Subsite Specificity Mapping of Human Cathepsin D Using Statine-based Inhibitors. *Protein Sci.* 6, 1458–1466.
- (46) Barrett, A. J. and Kirschke, H. (1981) Cathepsin B, Cathepsin H, and Cathepsin L. *Methods Enzymol.* 80, 535–561.
- (47) Barrett, A. J. (1977) *Proteinases of Mammalian Cells and Tissues*, North-Holland Publ., Amsterdam.
- (48) Kirschke, H., Langner, J., Riemann, S., Wiederanders, B., Ansorge, S., Bohley, P. (1980) *Protein Degradation in Health and Disease*, Excerpta Medica, Amsterdam.
- (49) Dunn, B. M., Jimenez, M., Parten, B. F., Valler, M. J., Rolph, C. E., Kay, J. (1986) A Systematic Series of Synthetic Chromophoric Substrates For Aspartic Proteinases. *Biochem. J.* 237, 899–906.
- (50) Blum, M., Cunningham, A., Bendiner, M., Hofmann, T. (1985) Penicillopepsin, the Aspartic Proteinase from *Penicillium janthinellum*:: Substrate Binding Effects and Intermediates in Transpeptidation Reactions. *Biochem. Soc. Trans.* 13, 1044–1046.
- (51) Baldwin, E. T., Bhat, T. N., Gulnik, S., Hosur, M. V., Sowder, R. C., Cachau, R. E., Collins, J., Silva, A. M., Erickson, J. W. (1993) Crystal Structures of Native and Inhibited Forms of Human Cathepsin D: Implications for Lysosomal Targeting and Drug Design. *Proc. Natl. Acad. Sci.* 90, 6796–6800.
- (52) Jonassen, I., Collins, J., Higgins, D. (1995) Finding Flexible Patterns in Unaligned Protein Sequences. *Protein Sci.* 4, 1587–1595.

BC980059J

TECHNICAL NOTES

Modification of the Thiourea Linkage of a Fluorescein–Oligonucleotide Conjugate to a Guanidinium Motif during Ammonia Deprotection

Igor Dubey, Geneviève Pratviel, and Bernard Meunier*

Laboratoire de Chimie de Coordination du CNRS, 205 route de Narbonne, 31077 Toulouse Cedex 4, France.
Received December 30, 1997; Revised Manuscript Received June 8, 1998

During the preparation of a fluorescent conjugate based on a manganese cationic porphyrin carboxylate derivative linked to a 5'-amino-3'-fluorescein-labeled oligonucleotide, we observed the chemical transformation of the thiourea linkage to a guanidinium function during the deprotection in ammonia of the 3'-fluorescein–oligonucleotide from CPG support. We also noted a secondary reactivity of the activated carboxylate group of the metalloporphyrin precursor with the fluorescein entity of the conjugated oligonucleotide.

INTRODUCTION

Fluorescein is one of the most sensitive and popular fluorescent dyes and a usual nonradioactive label for nucleic acids. Modifications of oligonucleotides (ODN)¹ with this fluorescent compound are now well-established (1–8). To monitor the cell penetration of manganese-porphyrin–oligonucleotide conjugates, we decided to use 3'-fluorescein labeled oligonucleotides with the 5'-end available for the linkage of the metalloporphyrin derivative (for recent references on metalloporphyrin conjugate, see ref 9–12). In the present work, the fluorescent label was introduced at the 3'-end of an oligonucleotide by using a polymer support for solid-phase synthesis of 3'-fluorescein-labeled oligonucleotides that is commercially available. We used a polymer from Glen Research with a thiourea linker Flu-NH-CS-NH designed to provide the same fluorescein-type structure (Figure 1) as that obtained with standard fluorescein isothiocyanate reagent (FITC). However, during the synthesis of these 5'-Mn-Por-ODN-3'-Flu conjugates (and also for ODN-3'-Flu conjugates), we found that the classical thiourea linkage of the ODN-3'-Flu was modified to a guanidinium function during the deprotection step in ammonia. This not yet published modification has been evidenced by electrospray ionization mass spectrometry (ESI-MS).

EXPERIMENTAL PROCEDURE

Synthesis and Purification of 3'-Fluorescein-5'-amino-link Oligonucleotide. Solid-phase oligonucleo-

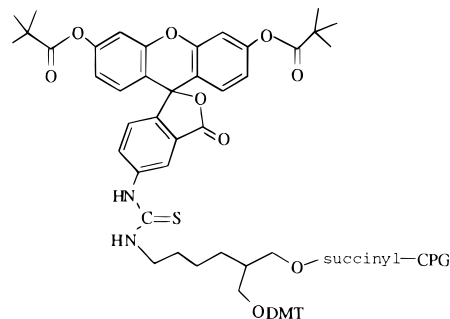


Figure 1. Structure of the commercial fluorescein-CPG support used in the present study.

tide synthesis was performed on a Cyclone Plus DNA synthesizer (Milligen/Bioscience) using a standard β -cyanoethyl phosphoramidite approach and all the necessary commercially available reagents (Perseptive). Cleavage of ODN from support required 2 h at room temperature with 32% ammonium hydroxide and base deprotection was performed by a further overnight incubation at 55 °C in the same ammonium hydroxide solution. Analyses were performed by anion-exchange HPLC using a Protein Pak DEAE 8HR column (Waters) followed by a diode-array detector (DAD 440, Kontron) with a linear gradient 0–500 mM NaCl in 25 mM Tris/HCl buffer, pH 8.5, over 45 min, at a flow rate of 0.7 mL/min. Fluorescein CPG support (0.2 μ mol cartridge from Glen Research) was used for the synthesis of 3'-fluorescein-labeled oligonucleotide. After chain elongation was over, 5'-functionalization with 1,6-diaminohexane as aminoalkyl linker was performed under usual conditions (13). The 3'-fluorescein-labeled and the 5'-amino-linked corresponding oligonucleotides were purified by anion-exchange HPLC using a Protein Pak DEAE 8HR column under the same condition as that described for analysis (R_t , 45 and 43 min for ODN–Flu and 5'-amino-linked ODN–Flu, respectively). Desalting of the product was performed

* Author to whom correspondence should be addressed.

¹ Abbreviations: HOBt, 1-hydroxy-benzotriazole; CDI, 1,1'-carbonyldiimidazole; BOP, benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate; CPG, controlled-pore glass; Flu, fluorescein; MOPS, 3-(*N*-morpholino)propanesulfonic acid; OD, optical density; TEAA, triethylammonium acetate; ODN, oligodeoxynucleotide; ESI-MS, electrospray ionization mass spectrometry; Mn-Por, manganese tris(4-*N*-methylpyridiniumyl)porphyrin motif.

Table 1. UV-Vis Data of Oligonucleotides and Porphyrin-Oligonucleotide Conjugates

	λ (nm)	ϵ ($10^3 \text{ M}^{-1} \text{ cm}^{-1}$)	vis/UV ^a
ODN	260	185	
ODN-3'-Flu	494/260	75/210	0.36 (0.35)
5'-Mn-Por-ODN-3'-Flu	468/260	130/240	0.55 (0.60)
	494/260	75/240	0.32 (0.30)

^a Calculated absorbance ratio (observed).

on Sep-Pak cartridges (Waters): elution of salts with 25 mM TEAA buffer, pH 6.5, followed by washing with a mixture of 100 mM TEAA, pH 6.5, with (70/30, v/v) acetonitrile. Spectrophotometric data of the 3'-fluorescein-modified oligonucleotide were as follows: the OD₄₉₄/OD₂₆₀ nm absorbance ratio at pH 8 for the fluorescein-oligonucleotide conjugate was 0.35, which is in full agreement with calculated values (ϵ of fluorescein at 494 and 260 nm: 75×10^3 and $25 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ respectively) (8). Concentrations of oligonucleotides were determined by UV measurements from the calculated molar extinction coefficient (14) (ϵ at 260 nm: $185 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ for ODN or $210 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ for ODN-Flu) (Table 1).

Synthesis of the Metalloporphyrin Precursor.

The manganese(III) 5-[(4-(5-carboxy-1-butoxy)phenyl)-10,15,20-tris(4-*N*-methylpyridiniumyl)-porphyrin] precursor was prepared as previously described (15). UV-vis spectral data of the manganese-porphyrin precursor in water are as follows: λ nm (ϵ , $\text{M}^{-1} \text{ cm}^{-1}$): 260 (3×10^4) and 468 (1×10^5).

Synthesis of the Mn(III) Porphyrin Conjugate of 3'-Fluorescein-Labeled Oligonucleotide. According to published procedures (16–18), activation of the manganese(III) 5-[(4-(5-carboxy-1-butoxy)phenyl)-10,15,20-tris(4-*N*-methylpyridiniumyl)porphyrin] precursor (0.038 mmol) was performed in the presence of BOP (0.27 mmol, 7 eq), HOBT (0.27 mmol, 7 eq) and *N*-methyldimorpholine (3.75 mmol, 15 eq) in 25 mL of dry DMF for 45 min at 40 °C. To hydrolyze the excess of BOP/HOBT reagent, 10 mL of 20 mM MOPS buffer (pH 7.5) was added and the mixture was warmed for 10 min at 40 °C. Then this solution was mixed with 40 μL of the purified 5'-amino-3'-fluorescein-labeled oligonucleotide (7.5 nmol, 1.4 OD₂₆₀) dissolved in 20 mM MOPS buffer (pH 7.5). Coupling reaction was allowed to proceed for 45 min at 40 °C. Addition of 150 μL of methanol and incubation at –20 °C for 20 min precipitated the conjugated product. After a centrifugation step, the pellet was washed twice with 50 μL of methanol, dried, and dissolved in 50 μL of 0.5 M sodium acetate (pH 5) at 60 °C. The product was then precipitated with 180 μL of ethanol, cooled to –20 °C and centrifuged again. The pellet was washed twice with 50 μL of ethanol, dried under vacuum, and dissolved in water. The crude product was purified by anion-exchange HPLC (R_t , 33 min for 5'-Mn-Por-ODN-3'-Flu), desalted on Sep-Pak cartridge and dissolved in water for quantification. After purification and desalting the product yield was about 40% (based on starting 5'-amino-linked oligonucleotide). The vis/UV absorbance ratios of conjugate were OD₄₆₈/OD₂₆₀ = 0.6 to account for the covalent attachment of one manganese(III) porphyrin moiety onto oligonucleotide and OD₄₉₄/OD₂₆₀ = 0.3 due to 3'-fluorescein chromophore that is in good agreement with calculated values (Table 1).

Preparation of a Fluorescein Isothiocyanate Adduct with 4-Aminobutanol and Behavior in Ammonia. Fluorescein isothiocyanate (29 mg, 0.075 mmol) was dissolved in 300 μL of freshly distilled DMF, and 4-aminobutanol (0.15 mmol, 14 μL) was added. After 4

h at ambient temperature, acetonitrile (3 mL) was added, the mixture cooled, and precipitate centrifuged. The dye material was dissolved in 5% NaHCO₃ and precipitated carefully with diluted HCl, centrifuged, washed with water, and dried. It was purified by chromatography on a silica gel column (elution with chloroform/acetone, 1:1). Yield = 19 mg (61%). TLC (Kieselgel 60F254 plates from Merck): R_f = 0.13 (CHCl₃/MeOH, 9/1, v/v), 0.80 (CH₃CN/water, 5/1), 0.82 (EtOH). ESI-MS: m/z 479.1 ($\text{M} + \text{H}^+$), 389.8 [$\text{M} - \text{NH}(\text{CH}_2)_4\text{OH}$]⁺. Fourteen milligrams of this compound (0.03 mmol) was dissolved in 1 mL of 32% ammonia and kept at 55 °C for 24 h. All starting material was transformed into a product having a value of R_f = 0.05 (CH₃CN/water, 5/1), 0.32 (EtOH). The reaction mixture was dried by Speed-Vac; the remaining material was dissolved in a small volume of diluted ammonia, precipitated with diluted HCl, and centrifuged. The pellet was washed with cold water ($3 \times 300 \mu\text{L}$) and dried. Nine milligrams of orange powder were recovered (yield = 67%). ESI-MS: m/z 462.2 ($\text{M} + \text{H}^+$), 373 [$\text{M} - \text{NH}(\text{CH}_2)_4\text{OH}$]⁺.

Electrospray MS. Electrospray ionization mass spectra were obtained using a quadrupole LC-MS spectrometer (Perkin-Elmer SCIEX API 100) equipped with an ionspray source. The ionization voltage was 5 kV in the positive mode and –4.5 kV in the negative mode. The orifice voltage was set to +50 or –50 V in the positive or negative mode, respectively. Sample solutions were introduced into the ionspray source at a rate of 5 $\mu\text{L}/\text{min}$ using a Harvard Apparatus syringe pump. The background noise recorded with the solvent used for ESI-MS analysis was subtracted when specified in the figure legends.

Electrospray MS of Oligonucleotides. Purified oligonucleotides were treated with ammonium acetate and precipitated with ethanol for displacement of sodium ions as described in ref 19. Analysis was performed in the negative mode. The injected solution consisted of 50 μL of at least 25 pmol/ μL concentration of ODN derivative dissolved in a water/acetonitrile mixture (50/50) containing 0.1% triethylamine.

Electrospray MS of the Fluorescein Isothiocyanate Adduct with 4-Aminobutanol and Its Guanidinium Analogue. Analysis was performed in the positive mode. The injected solution consisted of 50 μL of at least 25 pmol/ μL concentration of the adduct dissolved in a water/methanol mixture (50/50) containing 0.1% of trifluoroacetic acid.

RESULTS AND DISCUSSION

Preparation of the 5'-Mn-Por-ODN-3'-Flu Conjugate. In the present work, modified oligonucleotides were prepared having a fluorescent marker and/or an artificial nuclease moiety. The covalent attachment of a cationic porphyrin moiety at the 5'-end of an oligonucleotide labeled at the 3'-end by a fluorescein was performed via an amide bond resulting from the reaction in solution between an activated ester of the porphyrin carboxylate precursor and a 5'-amino-linked oligonucleotide (Figure 2). The sequence of the oligonucleotide was 5'-GTTGTAAACGACGCGCAT-3'. It is directed toward the coding region of the *Escherichia coli* β -galactosidase messenger RNA (349–411 region). The absorption maximum of fluorescein dye (492–494 nm at pH 8) is situated in a region close to that of the manganese-porphyrin core (Mn-Por) (468 nm), but the fluorescence maximum (520 nm) is far from the absorption maximum of Mn-Por.

The synthesis of the 5'-amino-linked ODN-3'-Flu was performed using the phosphoramidite method with the

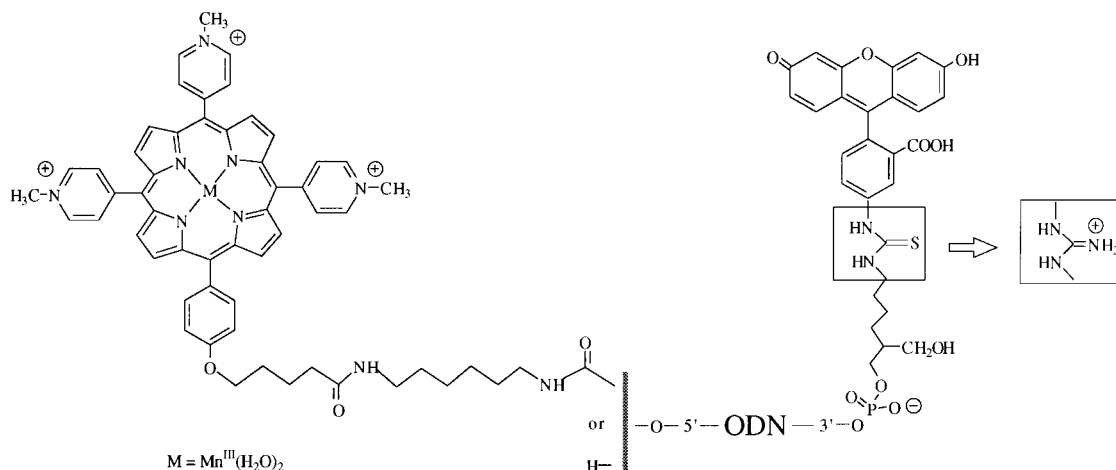


Figure 2. Structure of 3'-fluorescein oligonucleotide (ODN-3'-Flu) and 5'-manganese porphyrin-3'-fluorescein oligonucleotide (5'-Mn-Por-ODN-3'-Flu) conjugates. The oligonucleotide sequence is 5'-GTTGTAAAACGACGGCAT-3'.

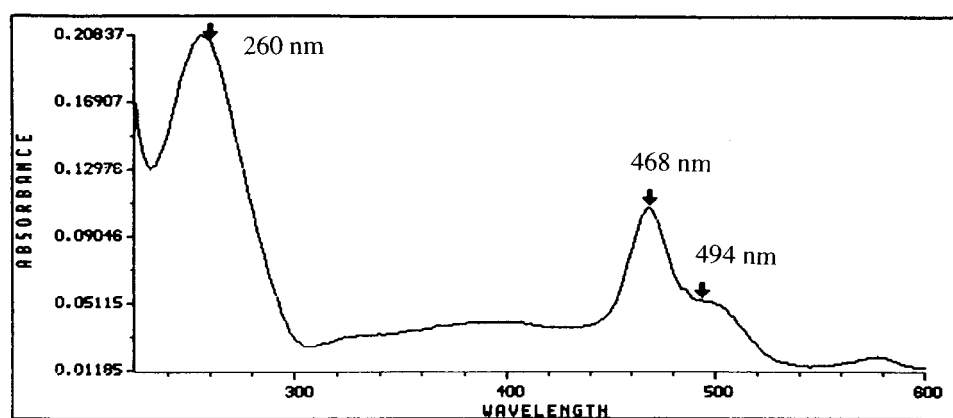


Figure 3. UV-vis spectrum of 5'-Mn-Por-ODN-3'-Flu.

fluorescein-CPG support shown in Figure 1. Derivatization on the 5'-position by an amino-linker was performed on the ODN still attached on the CPG cartridge, and the resulting 5'-amino-linked-ODN-3'-Flu product was cleaved from support, deprotected by ammonolysis, and purified before further reaction with the activated ester of Mn-Por precursor. We hydrolyzed the excess BOP/HOBT reagent after activation of the Mn-Por precursor, but before its addition to the 5'-amino-linked ODN-3'-Flu. During the coupling reaction, the starting 5'-amino-linked-ODN-3'-Flu was consumed to completion, and a side product corresponding to two Mn-Por moieties covalently bound to the ODN was observed. It could be transformed to the desired conjugate by mild acidic treatment, e.g., solubilization of the methanol precipitate of the coupling reaction in 0.5 M sodium acetate (pH 5). So, the reprecipitation of coupling reaction mixture after incubation in sodium acetate (pH 5) is essential to obtain good coupling product yield. The most probable way of formation of this side product is the covalent modification of fluorescein entity by the activated ester of Mn-Por during the coupling reaction. We ruled out some strong noncovalent interaction between porphyrin and fluorescein by control experiments where the ODN-3'-Flu and the nonactivated Mn-Por precursor were incubated and submitted to chromatography together. Fluorescein has two nucleophilic centers that are able to react with the excess of the activated ester of Mn-Por: the OH group of the carboxylic or phenolic functions. But the reaction of the fluorescein carboxylic group would lead to a mixed anhydride which should be unstable and would hydrolyze immediately during the isolation of the product. The

second possibility is the fixation of a second Mn-Por on the phenolic group of the 3'-fluorescein. In peptide synthesis, active esters of carboxylic acids are known to acylate phenolic hydroxyl of tyrosine and even aliphatic OH-group of serine, especially in the presence of HOBT as powerful catalyst of O-acylation (20–22). Furthermore, efficient acylation of alcohols by BOP/HOBT-activated carboxylic acids was recently described (23, 24). Since phenolic OH are more reactive than aliphatic alcohol functions, the reaction of the phenolic group of fluorescein with the activated ester of the Mn-Por precursor is highly probable. Its product, an aryl ester of carboxylic acid, should be rather stable under neutral conditions, but could be prone to acid or basic hydrolysis. Under acidic conditions, the protonation of the oxygen atom of the pyrone ring of fluorescein makes the fluorescein residue a good leaving group, and this may account for the transformation of the side product containing two Mn-Por entities into the desired conjugate with one Mn-Por per oligonucleotide. The UV-vis spectra of the 5'-Mn-Por-ODN-3'-Flu conjugate nicely showed the manganese-porphyrin (468 nm), oligonucleotide (260 nm), and fluorescein (492–494 nm) absorption bands (Figure 3).

Modification of the Linker between the 3'-Flu Entity and the ODN. Electrospray mass spectra were recorded in order to confirm the structure of ODN-3'-Flu and 5'-Mn-Por-ODN-3'-Flu conjugates. The mass spectra are shown in Figure 4. Figure 4B is the spectrum of 5'-Mn-Por-ODN-3'-Flu. In the mass range m/z 600–1500, four ions of the series $(M - nH)^{-n}$ appear for charged states $n = 5$ –8: 1423.2, 1186.2, 1016.5, and

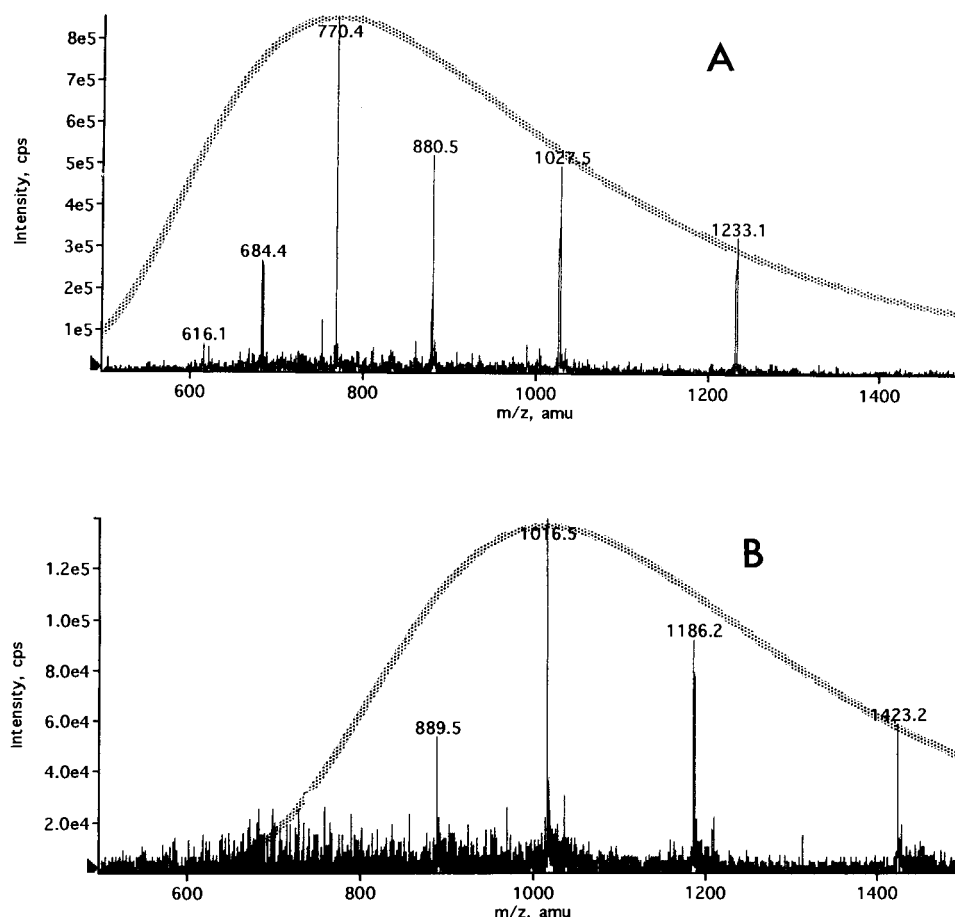


Figure 4. ESI-MS spectra of ODN-3'-Flu (A) and 5'-Mn-Por-ODN-3'-Flu (B).

Table 2. Multiply Negative Ions Observed (or Calculated), Related Calculated Mass, and Corresponding Formula of Oligonucleotide Conjugates ODN-3'-Flu (A) or 5'-Mn-Por-ODN-3'-Flu (B)

	<i>m/z</i>				mass	formula
	<i>z</i> = 8 [−]	<i>z</i> = 7 [−]	<i>z</i> = 6 [−]	<i>z</i> = 5 [−]		
A						
NH-CS-NH, calcd	772.3	882.7	1030.0	1236.2	6186.3	C ₂₀₆ H ₂₄₈ N ₇₆ O ₁₁₃ P ₁₈ S
NH-C(NH ₂) ⁺ -NH, calcd	770.2	880.3	1027.2	1232.8	6170.3	C ₂₀₆ H ₂₅₀ N ₇₇ O ₁₁₃ P ₁₈
ODN-Flu, observed	770.4	880.5	1027.5	1233.1	6170.7 ± 0.2	
B						
NH-CS-NH, calcd	891.3	1018.8	1188.7	1426.6	7142.4	C ₂₆₂ H ₃₀₂ N ₈₅ O ₁₁₆ P ₁₈ MnS
NH-C(NH ₂) ⁺ -NH, calcd	889.9	1016.9	1186.2	1423.3	7126.3	C ₂₆₂ H ₃₀₄ N ₈₆ O ₁₁₆ P ₁₈ Mn
Mn-Por-ODN-Flu, observed	889.5	1016.5	1186.2	1423.2	7126.3 ± 0.9	

889.5, respectively (see Table 2). The calculated mass of the analyzed product was 7126.3 ± 0.9 . The calculated mass of the conjugate containing a thiourea linkage corresponding to C₂₆₂H₃₀₂N₈₅O₁₁₆P₁₈MnS is 7142.4. The calculated *m/z* values of this compound for *n* = 5–8 are 1426.6, 1188.7, 1018.8, and 891.3, respectively (Table 2). These values differ from the observed ones by ~0.25%, which is above the experimental errors expected for ESI-MS (19, 25). We suspected that the difference was due to the possible transformation of the thiourea function -NH-CS-NH- in the fluorescein linker on the CPG support into a guanidinium group -NH-C(NH)-NH- during the ammonolysis of the 3'-fluorescein labeled ODNs, with a loss of molecular mass of 17 or 16 whether the guanidine function is neutral or protonated, respectively. Indeed the calculated mass corresponding to the guanidinium-linked product, C₂₆₂H₃₀₃₍₄₎N₈₆O₁₁₆P₁₈Mn, is 7125.3 (7126.3 if guanidinium is protonated) and should show *m/z* peaks at the values listed in Table 2 (1423.3, 1186.2, 1016.9, and 889.9) that differ by only 0.02% from the observed values. The same discrepancy between observed spec-

trum and theoretical values was noted for the simple ODN-3'-Flu (Figure 4A). The observed *m/z* values were 1233.1, 1027.5, 880.5, 770.4, 684.4, and 616.1 for charged states *n* = 5–10, respectively, and led to a molecular mass of 6170.7 ± 0.2 . These values could not account for a product with a thiourea linkage C₂₀₆H₂₄₈N₇₆O₁₁₃P₁₈S that has a calculated molecular mass of 6186.3 but rather to a guanidinium-linked conjugate of molecular formula of C₂₀₆H₂₄₉₍₂₅₀₎N₇₇O₁₁₃P₁₈ and of calculated molecular mass of 6169.3 (6170.3 if protonated) (Table 2). It is known that thioureas react with ammonia or amines to form guanidines (26–28). To check this possibility, we have synthesized the conjugate of fluorescein isothiocyanate (FITC) with 4-aminobutanol (mass of the thiourea fluorescein isothiocyanate adduct = 478) and applied it to the ammonolysis procedure (the molecular mass of this fluorescein-aminobutanol adduct was 462 after protonation). So, electrospray mass spectra confirmed the guanidine structure of the fluorescein linker after ammonolysis with a reduction of molecular mass of 16 (or 17 if the guanidine is not protonated) units

compared to the starting thiourea fluorescein derivative. So, the same process also takes place during the deprotection of fluorescein-oligonucleotide conjugates explaining the difference between calculated and observed molecular mass of fluorescein-ODN conjugates. However, we verified that the thiourea function was completely stable when incubated for 24 h in a 32% ammonia solution used for deprotection at room temperature.

In conclusion, this chemical transformation should appear each time NH-CS-NH linkage of modified nucleotides is subjected to a 55 °C overnight deprotection in ammonia, in particular when using 3'-fluorescein-CPG supports. It may not affect the general properties of conjugated molecules, yet introduces an extra positive charge on it and the change in its molecular mass may be misleading. This chemical transformation can be avoided for fluorescein label when the coupling of the dye can be performed after deprotection (29, 30) or when deprotection is performed under mild conditions (24 h in ammonia at room temperature). Fluorescein activated by succinimidyl esters of 5- (or 6-) carboxyfluorescein is also treated under appropriate deprotection conditions (31, 32).

ACKNOWLEDGMENT

The authors thank Catherine Claparols and Suzy Richelme for ESI-MS data and fruitful discussions. I.D. is highly indebted to a grant of the joint program between the Academy of Sciences of Ukraine (Institute for Bioorganic Chemistry and Petrochemistry at Kiev) and the University Paul Sabatier organized by Dr. Justin Tessié (CNRS, Toulouse).

LITERATURE CITED

- (1) Mundy, C. R., Cunningham, M. W., and Read, C. A. (1991) Nucleic acids labeling and detection. in *Essential molecular biology* (T. A. Brown, Ed.) Vol. 2, pp 57-109, IRL, Oxford.
- (2) Smith, L. M., Fung, S., Hunkapiller, M. W., Hunkapiller, T. J., and Hood, L. E. (1985) The synthesis of oligonucleotides containing an aliphatic amino group at the 5'-terminus: synthesis of fluorescent DNA primers for use in DNA sequence analysis. *Nucleic Acids Res.* 13, 2399-2412.
- (3) Agrawal, S., Christodoulou, C., and Gait, M. J. (1986) Efficient methods for attaching nonradioactive labels to the 5'-ends of synthetic oligodeoxyribonucleotides. *Nucleic Acids Res.* 14, 6227-6245.
- (4) Ansorge, W., Sproat, B., Stegeman, J., Schwager, C., and Zenke, M. (1987) Automated DNA sequencing: ultrasensitive detection of fluorescent bands during electrophoresis. *Nucleic Acids Res.* 15, 4593-4602.
- (5) Telser, J., Cruickshank, K. A., Morrison, L. E., and Netzel, T. L. (1989) Synthesis and characterization of DNA oligomers and duplexes containing covalently attached molecular labels: comparison of biotin, fluorescein and pyrene labels by thermodynamic and optical spectroscopic measurements. *J. Am. Chem. Soc.* 111, 6966-6976.
- (6) Schubert, F., Ahlert, K., Cech, D., and Rosenthal, A. (1990) One-step labeling of oligonucleotides with fluorescein during automated synthesis. *Nucleic Acids Res.* 18, 3427.
- (7) Sinha, N. D., and Striepeke, S. (1991) Oligonucleotides with reporter groups attached to the 5'-terminus. in *Oligonucleotides and Analogues, A Practical Approach* (F., Eckstein Ed.) pp 185-210, I. R. L. Press at Oxford University Press, Oxford, New York.
- (8) Conway, N. E., Fidanza, J. A., O'Donnel, M. J., Narekian, N. D., Ozaki, H., and Mc Laughlin L. W. (1991) Site-specific attachment of labels to the DNA backbone. in *Oligonucleotides and Analogues, A Practical Approach* (F. Eckstein, Ed.) pp 211-239, I. R. L. Press at Oxford University Press, Oxford, New York.
- (9) Mestre B., Pitié, M., Loup, C., Claparols C., Pratviel, G., and Meunier, B. (1997) Influence of the nature of the porphyrin ligand on the nuclease activity of metalloporphyrin-oligonucleotide conjugates designed with cationic, hydrophobic or anionic metalloporphyrins. *Nucleic Acids Res.* 25, 1022-1027.
- (10) Bigey, P., Sönnichsen, S. H., Meunier, B., and Nielsen, P. E. (1997) DNA binding and cleavage by a cationic manganese porphyrin-peptide nucleic acid conjugate. *Bioconjugate Chem.* 8, 267-270.
- (11) Duarte, V., Pratviel, G., Meunier, B., Berton, M., Sixou, S., and Favre, G. (1997) Inhibition of in vitro translation by antisense oligonucleotides covalently linked to a nucleic acid cleaver based on a cationic manganese porphyrin motif, Mn-trisMPyP. *New J. Chem.* 21, 55-60.
- (12) Duarte, V., Sixou, S., Favre, G., Pratviel, G., and Meunier, B. (1997) Oxidative damage on RNA mediated by cationic metalloporphyrin-antisense oligonucleotide conjugates. *J. Chem. Soc., Dalton Trans.* 4113-4118.
- (13) Wachter, L., Jablonski, J., and Ramachandran, K. L. (1986) A simple and efficient procedure for the synthesis of 5'-aminoalkyl oligodeoxynucleotides. *Nucleic Acids Res.* 14, 7985-7994.
- (14) Fasman, G., Ed. (1975) *Handbook of biochemistry and molecular biology-Nucleic Acids*, 3rd ed., p 175, CRC Press, Boca Raton, FL.
- (15) Casas, C., Saint-Jalmes, B., Loup, C., Lacey, C. J., and Meunier, B. (1993) Synthesis of cationic metalloporphyrin precursors related to the design of DNA cleavers. *J. Org. Chem.* 58, 2913-2917.
- (16) Mestre, B., Pratviel, G., and Meunier, B. (1995) Preparation and nuclease activity of hybrid metallotris(methylpyridinium)porphyrin oligonucleotide molecules having a 3'-loop for protection against 3'-exonucleases. *Bioconjugate Chem.* 6, 466-472.
- (17) Mestre, B., Jakobs, A., Pratviel, G., and Meunier, B. (1996) Structure/nuclease activity relationship of DNA cleavers based on cationic metalloporphyrin-oligonucleotides conjugates. *Biochemistry* 35, 9140-9149.
- (18) Casas, C., Lacey, C. J., and Meunier, B. (1993) Preparation of hybrid DNA cleaver-oligonucleotide molecules based on a metallotris(methylpyridinium)porphyrin motif. *Bioconjugate Chem.* 4, 366-371.
- (19) Stults, J. T. and Marsters, J. C. (1991) Improved electrospray ionization of synthetic oligodeoxynucleotides. *Rapid Commun. Mass. Spectrom.* 5, 359-363.
- (20) Bodanszky, M. (1984) *Principles of peptide synthesis*, pp 177, 224, Springer-Verlag, Berlin.
- (21) Klausner, Y. S., and Chorev, M. (1975) Synthesis of decapeptides by catalysis of active esters with 1-hydroxybenzotriazole. *J. Chem. Soc., Chem. Commun.* 24, 973-974.
- (22) Ramachandran, J., and Li, C. H. (1963) The synthesis of l-Valyl-l-Lysyl-l-Valyl-l-Tyrosyl-l-Proline. *J. Org. Chem.* 28, 173-177.
- (23) Kim, M. H., and Patel, D. V. (1994) BOP as a reagent for mild and efficient preparation of esters. *Tetrahedron Lett.* 35, 5603-5606.
- (24) Coste, J., and Campagne, J.-M. (1995) A propos de l'estérification des acides carboxyliques par le BOP ou le PyBOP. *Tetrahedron Lett.* 36, 4253-4256.
- (25) Reddy, D. M., Rieger, R. A., Torres, M. C., and Iden, C. R. (1994) Analysis of synthetic oligodeoxynucleotides containing modified components by electrospray ionization mass spectrometry. *Anal. Biochem.* 220, 200-207.
- (26) Watt G. W., and Makosky R. C. (1954) Synthesis of guanidine and nitroguanidine. Interaction of thiourea or ammonium thiocyanate and ammonia. Interaction of nitro-urea and gaseous or liquid ammonia. *Ind. Eng. Chem.* 46, 2599-2604.
- (27) Duus F. (1979) Thiocarbonyl compounds. in *Comprehensive Organic Chemistry* (D. Barton, W. D. Ollis, Vol. 3, pp 459-460 Pergamon Press, Oxford, New York.

- (28) Dempcy, R. O., Luo, J., and Bruice, T. C. (1996) Design and synthesis of ribonucleic guanidine: a polycationic analogue of RNA. *Proc. Natl. Acad. Sci. U.S.A.* 93, 4326–4330.
- (29) Dahlén, P., Liukkonen, L., Kwiatkowski, M., Hurskainen, P., Iitiä, A., Siitari, H., Ylikoski, J., Mikkala, V.-M., and Lövgren, T. (1994) Europium-labeled oligonucleotide hybridization probes: preparation and properties. *Bioconjugate Chem.* 5, 268–272.
- (30) Guzaev, A., Azhayeva, E., Hovinen, J., Azhayev, A., and Lönnberg, H. (1994) Synthesis and primer properties of oligonucleotides containing 3'-deoxypsicothymidine units, labeled with fluorescein at the 1'-position. *Bioconjugate Chem.* 5, 501–503.
- (31) Potier, N., Van Dorsselaer, A., Cordier, Y., Roch, O., and Bischoff, R. (1994) Negative electrospray ionization mass spectrometry of synthetic and chemically modified oligonucleotides. *Nucleic Acid Res.* 22, 3895–3903.
- (32) Fisher, T. L., Terhorst, T., Cao, X. and Wagner, R. W. (1993) Intracellular disposition and metabolism of fluorescently labeled unmodified and modified oligonucleotides microinjected into mammalian cells. *Nucleic Acid Res.* 21, 3857–3865.

BC9702213

CORRECTIONS

Volume 9, Number 5, September/October 1998.

Michael C. Willis,* Brian Collins, Tong Zhang,
Louis S. Green, David P. Sebesta, Carol Bell,
Elizabeth Kellogg, Stanley C. Gill, Anna
Magallanez, Susan Knauer, Ray A. Bendele,
Parkash S. Gill, and Nebojša Janjić*

LIPOSOME-ANCHORED VASCULAR ENDOTHE- LIAL GROWTH FACTOR APTAMERS

Page 573. Brian Collins is affiliated with NeXstar
Pharmaceuticals and has the middle initial D.

BC980200O

S1043-1802(98)00200-6

Published on Web 08/22/1998

Volume 8, Number 2, March/April 1997.

D. Scott Edwards,* Shuang Liu,* John A. Barrett,
Anthony R. Harris, Richard J. Looby, Marisa C.
Ziegler, Stuart J. Heminway, and Timothy R.
Carroll

NEW AND VERSATILE TERNARY LIGAND
SYSTEM FOR TECHNETIUM RADIOPHARMA-
CEUTICALS: WATER SOLUBLE PHOSPHINES
AND TRICINE AS COLIGANDS IN LABELING A
HYDRAZINONICOTINAMIDE-MODIFIED CYCLIC
GLYCOPROTEIN IIb/IIIa RECEPTOR ANTAGO-
NIST WITH ^{99m}Tc

Page 150. The caption for Figure 6 should read as follows. Radio-HPLC chromatogram (method 3) for complex **1** and [$^{99m}\text{Tc}(\text{HYNICtide})(\text{TPPTS})(\text{glycine})_2$]. The caption for Figure 7 should read as follows. Radio-HPLC chromatogram (method 4) for complexes **1** and **2**.

BC980199T

S1043-1802(98)00199-2

Published on Web 08/27/1998

REVIEWS

Bispecific Antibodies as Novel Bioconjugates

Y. Cao and M. R. Suresh*

Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta,
Edmonton, Alberta, Canada, T6G 2N8. Received April 30, 1998

Bispecific antibodies are unique macromolecular heterobifunctional cross-linkers with two different binding specificities within a single molecule. As ideal bioconjugates, they can specifically glue any two different molecules together without the need for chemical conjugation. With this unique feature, they have immense potential in biological and immunological fields. Their applications range from immunohistochemistry, immunoassays, radioimmunodiagnosis, radioimmunotherapy, and immunotherapy. Recently, a new second generation of bispecific molecules, bispecific single chain Fv and diabodies, has been produced by DNA recombinant technology. They can be considered as the ultimate magic bullets for in vivo applications. They may theoretically improve tumor or pathogen targeting and minimize side effects, eventually replacing the full-length bispecific antibodies. Emphasizing on developmental methodology and clinical applications of bispecific antibodies, this review gives a bird's-eye view of these unique bioconjugates.

INTRODUCTION

Monospecific antibodies exemplified by the naturally occurring major IgG class have two identical antigen-binding paratopes due to their makeup with two identical heavy and light chains. On the contrary, bispecific antibodies are artificially engineered immunoglobulins with two distinct binding specificities. They were first generated 37 years ago by chemical methods (Nisonoff and Rivers, 1961). In the past two decades, hybridoma technology established itself as one of the cornerstones of modern biotechnology (Kohler and Milstein, 1975), and bispecific monoclonal antibody (bsMAb) represents second generation monoclonals developed for a variety of in vivo and in vitro uses.

A number of applications using monoclonal antibody (MAb) involve chemical manipulation of the antibody to create covalent immunoconjugates with radioisotopes, enzymes, toxins, drugs, and a variety of other haptens. Early chemical conjugation methods using homobifunctional cross-linkers generated complex aggregates of the two entities. Refinement in chemical conjugation methods, employing heterobifunctional cross-linkers, has mitigated the problems but not entirely eliminated them (Hermanson, 1996). BsMAb with intrinsic binding sites to any two antigens has the capability to form uniform, homogeneous, and reproducible immunoconjugates with one or two entities in a predetermined order (Suresh et al., 1986a). Since such binding is noncovalent and dependent on the affinity of the epitope-paratope interaction, bsMAb has a singular advantage to cross-link two cells for targeting in vivo (Staerz and Bevan, 1989). The

* Author to whom correspondence should be addressed. Tel: 403-492-9233. Fax: 403-492-1217. E-mail: msuresh@pharmacy.ualberta.ca.

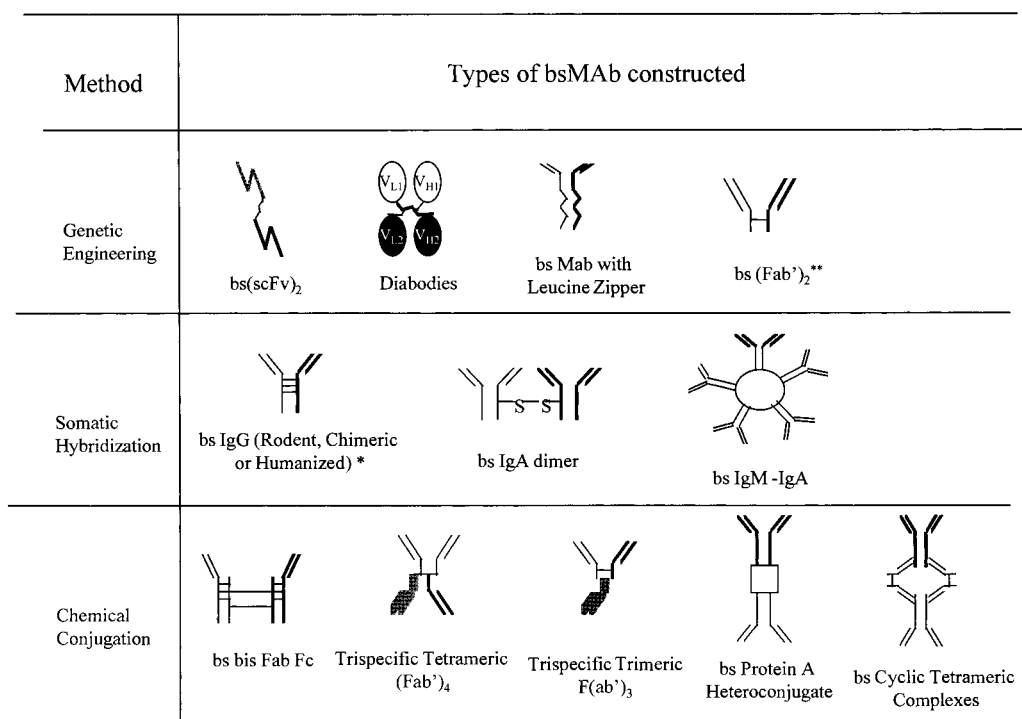


Figure 1. A schematic representation of a variety of bsMAbs. (*) This has also been constructed by chemical and genetic methods. (**) This has also been constructed by chemical methods.

latter feature has not been described to date using chemical conjugation for *in vivo* applications.

Current applications of bsMAb range from immuno-histochemistry (Milstein and Cuello, 1983), immunoassays (Kreutz and Suresh, 1995, 1997; Suresh et al., 1986a; Cao et al., 1998), complement-mediated cytotoxicity (Wong and Colvin, 1987), and *in vivo* retargeting (Fanger et al., 1995; Demanet et al., 1996; Somasundaram et al., 1996). BsMAb has great potential in a wide variety of research and eventually, clinical uses. Some of these exciting explorations include redirecting cytotoxicity to tumor cells, HIV virus, and infectious agents, targeting enzyme and site-specific activation of anticancer prodrugs and localizing fibrinogen activator to dissolve fibrin clots.

PRODUCTION OF BISPECIFIC ANTIBODIES

Many different types of bsMAb have been made (Figure 1) by one of the three general methods: (1) chemically cross-linking two antibody molecules or antibody fragments, (2) fusion of two different cell lines to form a quadroma or trioma, (3) recombinant DNA based approaches.

1. Chemically Coupling. More than 30 years ago, the first bispecific polyclonal antibodies were produced by chemically coupling two different polyclonal antibodies (Nisonoff and Rivers, 1961). This chemical manipulation involved the dissociation of the two different antibodies and reassociation of the two-half molecules. A portion of the reassembled antibodies was shown to be bispecific in nature by immunohistochemistry. With the advent of MAb and improvements in the chemistry of preferential formation of heteroconjugates, developing bispecific monoclonals became a reality eliminating the heterogeneous polyclonal reagents.

To prepare bsMAb, a large number of bifunctional reagents reactive with ϵ -amino groups or hinge region thiol groups have been used. These cross-linkers can be classified into two categories, homo- and heterobifunc-

tional reagents. Homobifunctional reagents react with the free thiols generated upon reduction of inter heavy chain disulfide bonds. 5,5'-Dithiobis(2-nitrobenzoic acid) (DTNB) or *o*-phenylenedimaleimide (O-PDM) can activate thiol groups on Fab' fragments of MAb (Brenner et al., 1985; Glennie et al., 1987; Shalaby et al., 1992). DTNB acts to regenerate disulfide bonds between the two Fabs, whereas O-PDM acts to form a thioether bond between the two Fab'. Generally, the thioether bond of O-PDM could be more stable than the disulfide bond regenerated by DTNB (Glennie et al., 1987). In addition to dimeric bsMAbs, trimeric and tetrameric antibodies with two or three specificities have been constructed using O-PDM and shown to have more potent redirecting effector cell tumor cytotoxicity than corresponding dimeric antibodies (Tutt et al., 1991a,b).

Heterobifunctional reagents can introduce a reactive group onto a protein that will enable it to react with a second protein. *N*-Succinimidyl-3-(2-pyridyldithio) propionate (SPDP) has been used to react with primary amino groups to introduce free thiol groups (Segal et al., 1988; Jung et al., 1991). SPDP can combine any two proteins that have exposed amino groups including antibodies and Fab' fragments, regardless of class or isotype (Van Dijk et al., 1989). Unfortunately, this approach causes random cross-linking of the molecules and hence exhibits batch to batch variations.

The improvements in cross-linking technology has led at least one biotechnology company (Medarex Inc., NJ) to develop bispecific (Fab')₂ antibodies and evaluate their utility in FDA-approved human clinical trials. Chemical linking that has been highly refined is more straightforward and faster, produces higher yields, and the products are comparatively easier to purify (Glennie et al., 1987). However, denaturation of either protein or loss of antibody activity are concerns during these types of chemical reactions.

2. Somatic Hybridization. Somatic hybridization for generating bispecific antibodies first appeared in 1973

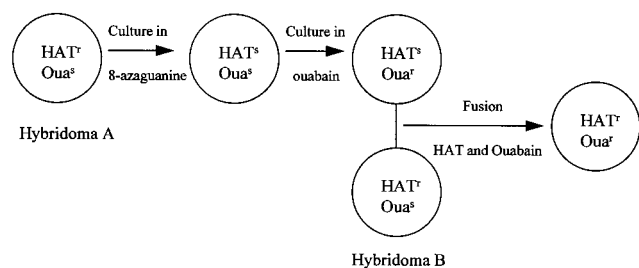


Figure 2. Production of quadromas. A HAT sensitive (HAT^s) clone is isolated from one parental hybridoma by culturing in 8-azaguanine. This clone is subsequently made resistant to ouabain (Oua^r) by mutant selection. After fusion with a wild-type hybridoma that produces a different antibody, quadromas are selected by culturing the cells in HAT medium supplemented with ouabain. The unfused parental cells will die due to sensitivity to HAT and lack of resistance to ouabain, respectively.

(Cotton and Milstein, 1973), although the term and concept of bispecific antibodies were not appreciated. Basically, somatic hybridization included fusion of two hybridomas and purification of the bsMAB secreted by the resulting quadromas. Two different methods have been described: (1) fusion of two established hybridomas generates a quadroma (Milstein and Cuellar, 1983; Suresh et al., 1986b), and (2) fusion of one established hybridoma with lymphocytes derived from a mouse immunized with a second antigen generates trioma (Nolan and Kennedy, 1990).

Somatic hybridization for development of bsMAB involves methods similar to those for preparing hybridomas (Kohler and Milstein, 1975). It has been greatly facilitated by the use of drug resistance markers introduced into cell lines by conventional and molecular genetic techniques. First, each hybridoma cell line is made resistant to a specific drug-resistant marker. Quadroma cells are then selected after fusion using a culture medium containing both drugs which can kill both parental cells allowing only hybrid-hybridoma to survive (De Lau et al., 1989; Bos and Nieuwenhuizen, 1992). Alternatively, a universal fusion partner could be developed by sequentially culturing in 8-azaguanine and ouabain to generate a HAT sensitive and ouabain resistant cell line (Figure 2). After it is fused with a second normal hybridoma, which is HAT resistant and ouabain sensitive, only the fused quadromas survive in the combined HAT and ouabain medium. The advantage of using drug resistance markers is that the selected fused cells are functionally stable synkarions (fused nuclei).

Somatic hybrids can also be selected by sorting out the heterofluorescent cells without relying on drug selection (Karawajew et al., 1987, 1988). Hybridomas were pre-labeled with either fluorescein isothiocyanate (FITC) or tetramethylrhodamine isothiocyanate (TRITC) via the cell membrane marker octadecylamine. Differentially labeled hybridomas are fused, and the cells staining for both fluorochromes are isolated using a fluorescence-activated cell sorter (FACS). Electrofusion technique was developed as an alternative to conventional PEG fusion as it is innocuous and easy to perform and produces high yield (Chang, 1989). We have developed microelectrofusion and electrofusion plus FACS selection methodology to generate the desired hybrid-hybridoma with no laborious drug selection procedures (Cao et al., 1995; Kreutz et al., 1998b). Such protocols could also be predictably adopted to generate human hybridomas with few B cells isolated from peripheral blood lymphocytes enriched by antigen specific panning or affinity microelectrofusion.

The activity of bsMAB secreted by quadroma clones should be tested as quickly as possible. A bridge ELISA is a general way to screen bsMAB (Suresh et al., 1986a; Koolwijk et al., 1988; De Lau et al., 1989). Double antigen ELISA may be employed if sufficient quantities of the respective antigens are available. When two different H chain isotypes are present in the bsMAB, isotype specific reagents could be used for detection of hybrid molecules. Last, the supernatants of clones putatively containing bsMAB to redirect T cell cytotoxicity could be tested utilizing cytotoxic T cell clones and target cell lines (Haagen et al., 1992). Biologically produced bsMAB is synthesized, assembled and secreted by the same process as native immunoglobulins, and thus they have similar characteristics in terms of stability and pharmacokinetics (Songsivilai and Lachmann, 1990). Since individual light chains and heavy chains are transcribed separately and assembled randomly, the desired bsMAB produced by the quadroma could range 10–50% (Suresh et al., 1986a). Hence, purification of this bsMAB is essential and has been achieved by hydroxylapatite chromatography (Karawajew et al., 1987; De Lau et al., 1992), ion-exchange chromatography (Suresh et al., 1986b; Auriol et al., 1994) double affinity chromatography (Sahin et al., 1990; Smith et al., 1992), mimetic affinity methods (Xu et al., 1998), and gradient thiophilic T-gel chromatography (Kreutz et al., 1998b).

3. Genetic Engineering. Chemical conjugation or cell fusion generates a heterogeneous mixture, requiring labor-intensive methods to purify the bispecific form from the other homo and hetero aggregated species. Many of the limitations of generating bsMAB through these traditional methods may be potentially overcome by genetic engineering techniques (Pluckthun and Pack, 1997). This new technique was first described by Songsivilai and colleagues to generate transfectants for secreting humanized bsMAB (Songsivilai et al., 1989). Generally, the antibody or its fragment of different specificities was expressed as distinct polypeptides from the same transcript or from separate plasmid constructs, followed by combining different antibody fragments through binding motif (Shalaby et al., 1992). Recently, bsMAB molecules have been made as a single covalent structure by combining two single chain Fv (scFv) fragments using polypeptide linker (Winter and Milstein, 1991; Holliger et al., 1993; Kranz et al., 1995; Schmidt et al., 1996; Thirion et al., 1996). Different spacers have been tried to increase the bispecific yield (Gruber et al., 1994; Mallender et al., 1994). It is essential in this strategy to ensure that the two single-chain antibodies exhibit affinities similar to the parental MABs.

Ridgway and associates reported a novel “knobs into holes” method of making bsMAB (Ridgway et al., 1996). In this method, the knobs were created by replacing small amino acid side chains at the interface between C_H3 domains with larger ones, whereas the holes were generated by replacing large side chains with smaller ones. This engineering of the C_H3 domains of heavy chains resulted in the formation of heterodimers (Ab1-knob-hole-Ab2) more favorably than homodimerization (Ab1-Ab1 and Ab2-Ab2). This elegant strategy has been extended to make full-length bispecific human IgG, which could be used in immunotherapeutic strategies (Merchant et al., 1998).

Leucine zipper-based dimerization of MAB fragments has also been introduced (Kostelny et al., 1992). The leucine zippers are sequences derived from regions of the transcription factors *fos* and *jun*. A murine anti-CD3 Fab'-*fos* and anti-Tac Fab'-*jun* were individually ex-

pressed as homodimers in SP2/0 cells. When they were reduced, mixed, and reoxidized, the formation of *fos* and *jun* leucine zipper resulted in the generation of bsMAB fragment Fab' (anti-CD3 x anti-Tac). De Kruif and Logtenberg also described the fusion of *fos* or *jun* leucine zipper and a truncated mouse IgG3 hinge region to scFv proteins (De Kruif and Logtenberg, 1996). In their method, two cysteine residues were engineered into the zipper domains to produce disulfide-stabilized homodimers. Using scFv isolated from a phage display library and these modified zipper cassettes, they have produced functional stable secreted homodimers in *Escherichia coli*. After reduction, mixing, and reoxidation of the homodimers, functional bispecific scFv molecules were developed. Rheinhecker also utilized a similar approach to create scFv dimers by fusion of an artificial helix-turn-helix dimerization domain of p53 to a long IgG3 hinge-scFv construct (Rheinhecker et al., 1996).

Holliger and colleagues developed a diabody method to generate bsMAB fragments (Holliger et al., 1993). In the diabody method, constructing scFv with short linkers can prevent intrachain pairing but allow interchain pairing to form bivalent fragments. The scFv containing V_L and V_H with two different paratopes was assembled with the reciprocal scFv pair to form bsMAB fragments. Recently, a phage display diabody approach was developed to generate and screen bispecific scFv (McGuinness et al., 1996). Although the diabody approach appears promising with previously cloned scFv molecules, construction of diabody libraries from large Ab repertoires requires several cloning steps and the stability of the library in bacteria has not yet been extensively evaluated (Hayden et al., 1997).

Among a plethora of approaches to genetically engineer bsMAB, the most promising approaches appear to be bispecific scFv and bispecific diabodies. This is due to their small size and hence is more attractive for in vivo applications exhibiting faster clearance, better tissue penetration, and decreased immunogenicities.

APPLICATIONS OF BISPECIFIC ANTIBODIES

1. Immunohistochemistry and Enzyme Immunoassays. The advent of MAb technology was instrumental in the development and refinement of immunoassays using chemically linked radioisotopes, enzymes, fluorochromes, or others detection moieties. Despite the surge in these new applications, chemical cross-linking has many disadvantages: (1) chemical cross-linking is random generally resulting in batch to batch variations in reagent quality; (2) the size of conjugates or complexes obtained are not uniform thus influencing their penetration in immunohistochemical applications; (3) active site or binding site inactivation of markers and/or immunoglobulins may be encountered; (4) mostly irreversible covalent linkages are formed which are advantageous for in vitro applications but are less suitable for in vivo therapeutic studies. These drawbacks of covalent cross-linking of antibodies to enzyme or radioactive markers often influence the linearity and reproducibility of the assay method. On the contrary, bsMAB designed to specifically cross-link the antigen with a signal generating marker such as an enzyme eliminates the need for chemical conjugation, generating a greater degree of linearity, sensitivity, and reproducibility (Suresh, 1991).

Milstein and Cuello first (Milstein and Cuello, 1983) developed a bsMAB (anti-somatostatin x anti-peroxidase) used in immunohistochemistry. Suresh and co-workers (Suresh et al., 1986a) established a hybridoma-

secreting anti-peroxidase x anti-substance P, which simplified the immunohistochemical procedures and giving a better sensitivity. A bsMAB reacting with both horseradish peroxidase (HRPO) and fluorescein isothiocyanate (FITC) was used to label different MABs in enzyme immunoassay systems (Karawajew et al., 1988). Many investigators have developed bsMABs directed against enzyme (e.g., HRPO, alkaline phosphatase, and β -galactosidase) and a second antigen (e.g., tumor specific antigen, peptide, or hormone) for use in enzyme immunoassay or immunohistochemistry (Takahashi and Fuller, 1988; Wognum et al., 1989; Tada et al., 1989; Ribeiro-da-Silva et al., 1991; Allard et al., 1992; Kontseikova et al., 1992; Jantscheff et al., 1993; Piran et al., 1993; Auriol et al., 1994; Liu et al., 1995; Kreutz and Suresh, 1995, 1997; Berkova et al., 1996; Cao et al., 1998). Rylatt developed a homogeneous immunoassay system to detect circulating antigens in whole blood. The bsMAB used here cross-linked specific antigen of interest and red blood cells, causing agglutination of a patient's own blood sample (Rylatt et al., 1990).

2. Radioimaging and Radioimmunotherapy. Conventional radioimmunoimaging and, in particular, radioimmunotherapy with MAB conjugates is severely limited by radiation toxicity to normal organs, especially blood, liver, kidney, and bone marrow. This is largely due to the high radiation dose from prolonged circulating radiolabeled antibody to these organs, from normal tissue accumulation of the antibody, or from free radionuclide deposited in the organs. Pretargeted imaging and radioimmunotherapy with bsMAB is a two or three step strategy for delivering radioisotope (^{99m}Tc, ⁹⁰Y, ⁶⁷Ga, and ¹¹¹In) to a tumor quickly and specifically with minimal radiation exposure and hence lowered dosimetry to normal organs (Chetanneau et al., 1994; Chatal et al., 1995; Dillehay et al., 1995; Kranenborg et al., 1995; Schuhmacher et al., 1995).

Pretargeting involves administration of a bsMAB with high affinity for a small rapidly excreted effector molecule, which is given as a second step after the bsMAB has concentrated in the target tumor. Removal of the excess bsMABs from the circulation with a clearing agent is an optional step before giving the effector molecule for greatly improved target-to-blood ratio. Soon after the clearing agent, the effector molecule is given, and the maximum tumor concentration and tumor-to-normal tissue ratio is achieved in 1–3 h. Unbound radiolabel is rapidly excreted via the kidney, by glomerular filtration mechanism, leading to greatly decreased radiation exposure to normal tissue. Several different targeting macromolecule and effector small molecule pairs have been proposed in the literature (Goodwin, 1987; Goodwin et al., 1994).

Pretargeting combines the pharmacokinetics of long circulating MAB with rapidly excreted small effector molecules to give both high tumor concentration and high tumor-to-normal tissue ratios. The two or three step methods eliminate radiation exposure during the MAB localizing phase, which can take up to several days. Nonspecific localization at this stage in liver, spleen, and bone marrow does not contribute to normal tissue radiation since the radioactive hapten is only injected later. The total radiation dose to normal tissue is greatly reduced in the pretargeting method by two mechanisms: (1) absence of radiation during the localization phase, since the bsMAB is not radioactive at this time; (2) rapid renal excretion of excess radiolabeled hapten that is not bound to the bsMAB in the tumor. An obvious limitation of this two or three step pretargeting strategy

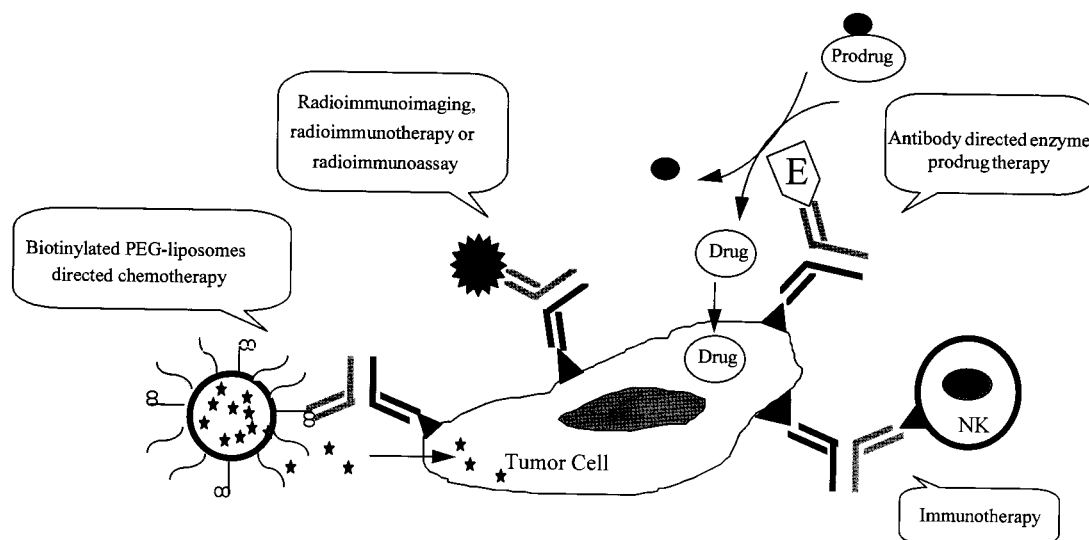


Figure 3. Schematic representation of different applications of bsMab in therapeutics.

is that bsMAbs directed to rapidly internalizing antigens would fail to capture and localize the radiolabeled hapten. Hence, poorly internalized bsMab is most useful in this strategy. An additional limitation of this strategy is that its practice requires several steps. From the perspective of drug development, this places an additional manufacturing burden and significantly complicates the design of clinical trials.

One bifunctional antibody currently under clinical evaluation was made by the chemical coupling of two Fab' fragments of anti-carcinoembryonic antigen (CEA) antibody and anti-DTPA-indium antibody. In patients with colorectal or medullary thyroid cancer, clinical studies were performed with this anti-CEA/anti-DTPA-indium bifunctional antibody and an indium-111-labeled bis-DTPA bivalent hapten. The results showed that tumor uptake was not altered compared to the results for F(ab')₂ fragments of the same anti-CEA antibody directly labeled with indium-111, whereas the radioactivity of normal tissues was significantly reduced (3–6-fold). The fast tumor uptake kinetics and high tumor-to-normal tissue ratios obtained with bifunctional antibody techniques exhibit parameters favorable for efficient radioimmunotherapy (Chatal et al., 1995).

Pretargeting methods have also incorporated the use of biotin or avidin labeled monospecific antibody followed by radiolabeled avidin or biotin respectively (Van Osdol et al., 1993). In this approach, however, the immunogenicity of the hen egg avidin or streptavidin could be a potential limitation.

3. Immunotherapy. BsMab is capable of activating and targeting the cellular immune defense system to kill tumor cells or other pathogens (Figure 3). Many groups have already demonstrated lysis of target cells by T effector cells using bsMab in a non-MHC restricted fashion. (Canevari et al., 1988; Roosnek and Lanzavecchia, 1989; Roosnek et al., 1990). The primary cytotoxic trigger complex on T cells is the TcR complex, which consists of a noncovalent association of Ti heterodimers with the CD3 molecular complex. The TcR complex is normally antigen specific, and major histocompatibility complex (MHC) restricted. However, a bsMab can react with this complex and initiate targeted cytotoxicity by activating and targeting the T-cell in a non-MHC restricted manner. Many efforts have been made to target bsMab to surface molecules such as TcR heterodimer (Staerz and Bevan, 1985), CD3 complex (Liu et al., 1985;

Rammensee et al., 1987) and CD2 molecule (Bolhuis et al., 1991; Tutt et al., 1991b). Cytotoxicity is not due to bystander lysis, since direct contact between effector and target cells is required (Barr et al., 1987; Lanzavecchia and Scheidegger, 1987). This in vivo cross-linking of specific targets with components of the cell-mediated immune response such as T-cells, natural killer cells, macrophages, and large granular lymphocytes is a unique feature outside the realms of chemical cross-linking methods.

Cancer immunotherapeutic strategies involving bsMab-mediated killing have been explored by several groups including anti-epidermal growth factor receptor for breast cancer (Knuth et al., 1994), anti-sialyl Lewis^x for colon cancer (Ohta et al., 1995), anti-ovarian cancer (Bolhuis et al., 1992; Canevari et al., 1995), anti-renal cell carcinoma (Van Dijk et al., 1991), anti small cell lung carcinomas (Azuma et al., 1992), anti-CD19 for B lymphoma (Anderson et al., 1992; De Gast et al., 1995), anti-CD13 for acute myeloid leukemia (Kaneko et al., 1993), and anti-tenascin for gliomas (Davico Bonino et al., 1995). The therapeutic success was mainly based on the retargeting of the relative small cytotoxic T cell pools which already exist and are further augmented by anti-CD28 costimulation (Demanet et al., 1996). Other types of effector molecules which have been used are FcγRIII receptor (CD16) (Hsieh-Ma et al., 1992; Weiner et al., 1995), complement receptor CR3 of macrophage (Somasundaram et al., 1996), Fc receptor (FcγRI) (Deramoudt et al., 1992), and anti-gamma/delta T cell receptor (Ferrini, 1989).

The exciting preclinical studies have prompted several clinical studies using bsMAbs. The toxicities associated with this immunotherapeutic approach appears to be minimal, and early results show promising clinical benefit as well. Guyre and Franger have developed a bsMab, MDX-210, which recognizes FcγR1 on monocytes and macrophages and the cell surface product of the HER-2/neu oncogene overexpressed on some breast and ovarian cancers. Clinical trials have demonstrated that treatment with MDX-210 is well tolerated and MDX-210 is both immunologically and clinically active. Optimization of the dose and schedule of MDX-210 and development of combination treatments with cytokines that modulate immune effector cells will greatly enhance the efficacy of this novel bsMab for treatment of tumors that overexpress HER-2/neu (Valone, 1995). Another bsMab

2B1 with specificity for the c-erbB-2 and Fc γ RIII extracellular domains has also been tested in humans. This bsMAB promotes the targeted lysis of malignant cells overexpressing the c-erbB-2 gene product of the HER2/neu proto-oncogene by human natural killer cells and mononuclear phagocytes expressing the Fc γ RIII A isoform. In a phase I clinical trial, the 2B1 treatment induced more than 100-fold increases in circulating levels of tumor necrosis factor- α , interleukin 6, and interleukin 8 and lesser rises in granulocyte-monocyte colony-stimulating factor and IFN- γ (Weiner, 1995).

BsMAB has also been tested for its immunotherapeutic applications in viral infections (Berg et al., 1991). Chamow and associates have developed a humanized bsMAB, comprised of CD4-IgG and humanized anti-CD3-IgG, to target and kill HIV-infected cells. First, the bsMAB can target to HIV-infected cells by the natural affinity of CD4 for gp120. Second, it recruits and activates, through its anti-CD3 moiety, cytotoxic T lymphocytes (CTL) to lyse target cells in a non-MHC restricted manner. They demonstrated that bsMAB could specifically lyse HIV-infected target cells using purified CTL and whole peripheral blood lymphocyte (PBL). In contrast, a human anti-gp120 monospecific antibody can lyse HIV-infected target cells only with PBL fractions and not with purified CTL. Moreover, the lytic activity of anti-gp120 antibody was completely blocked by the human serum (which competes for Fc gamma receptor binding with abundant human IgG), whereas bsMAB-mediated lysis of target cells was not affected. This has reflected a potential advantage of bsMAB over monospecific antibodies for HIV-directed therapy (Chamow et al., 1994, 1995).

4. Drug Delivery. BsMAB could be used as an effective drug delivery system for cancer treatment. BsMABs have been made with one binding site to methotrexate (Affleck and Embleton, 1992), saporin (Bonardi et al., 1992 and 1993; French et al., 1995), doxorubicin (Reddy and Ford, 1993), or vinca alkaloids (Corvalan et al., 1987). Clinical studies have been described using a combination of two bsMABs to deliver the ribosome-inactivating protein, saporin, in the treatment of low-grade, end-stage B-cell lymphoma. Both bsMABs have one arm directed at saporin and one at the CD22 on target B cells. However, the two bsMABs recognized different, nonoverlapping epitopes on each molecule, a strategy which allowed high-avidity double attachment of saporin to the target. Five patients were treated, mostly with weekly doses of between 2 and 4 mg of saporin for a period of up to 6 weeks. Toxicity was minimal and all patients showed a rapid and beneficial response to treatment (French et al., 1996).

The advantage of using bsMAB to deliver high molecular weight toxins or drugs to tumors, comparing to conjugating such moieties to MAb, is that chemical conjugations are totally avoided. This provides a more uniform cross-linking between the target and the effector molecule. However, if the toxin or drug is a low molecular weight effector molecule, the advantage is outweighed by the dose limitation of this approach. To circumvent this limitation, a strategy combining drug attached to polymeric biodegradable carriers or encapsulated in liposomes with bsMAB may be adopted.

BsMAB can also be used to activate a prodrug in vivo to cause localized targeted cytotoxicity. In this approach, a bsMAB has been made against a tumor marker human placental alkaline phosphatase and *E.coli* β -lactamase as the prodrug activating group. Activation of cephalosporin-based anti-cancer prodrugs at the tumor site can be achieved with this bsMAB (De Sutter and Fiers, 1994).

Another bsMAB against CD30 antigen and alkaline phosphatase was also used to activate the relatively nontoxic prodrug, mitomycin phosphate, to mitomycin alcohol, which is 100-fold more toxic (Sahin et al., 1990). Alternative strategies to antibody directed enzyme prodrug therapy (ADEPT) include the development of bifunctional antibody-enzyme fusion proteins. The advantage of bsMAB plus enzyme and prodrug approach over fusion protein is that the pharmacokinetics and targeting site accumulation of each component could be controlled separately. In addition, PEG-enzyme could be used for reduced immunogenicities. However, the limitation of this approach is the requirement of multistep injection.

5. Other Applications. Tissue plasminogen activator (TPA) triggers the conversion of plasminogen to the fibrinolytic enzyme plasmin, which causes dissolution of thrombi. BsMAB can be used to improve fibrinolytic efficacy of TPA by binding simultaneously to human fibrin and TPA in thrombosis therapy (Kurokawa et al., 1991). One study has shown that, in the presence of TPA, bsMAB caused a 10-fold more fibrinolysis in vitro than with TPA alone (Branscomb et al., 1990). Some researchers have also tried to use bsMAB to target urokinase-type plasminogen activator (UPA) to fibrin-containing clots (Charpie et al., 1990). It caused 13-fold more fibrinolysis in vitro than urokinase alone and 6-fold more clot lysis in vivo. In another study using baboon bearing ^{125} I-fibrin clots in femoral vein, continuous intravenous infusion of a bsMAB resulted in a 5-fold enhanced thrombolytic potency of UPA over that of unconjugated UPA (Imura et al., 1992).

BsMAB could be generated to improve the binding specificity and avidity by recognizing two different adjacent epitopes in the same antigen. This type of bsMAB has been made against two different subunits of the hormones such as human chorionic gonadotropin hormone (HCG), creatine kinase (CKMB), and adrenocorticotrophic hormone (ACTH) (Cheong et al., 1990; Cook and Wood, 1994). A bsMAB against polyhedral boron anion and a tumor associated antigen has been made to deliver a large amount of boron atoms to tumor cells for a novel radiotherapeutic application. The alpha particles can be released to destroy tumors locally when boron-10, a stable isotope, is irradiated with low-energy thermal neutrons (Pak et al., 1995; Liu et al., 1995).

The plethora of applications of bsMAB as unique macromolecular heterobifunctional cross-linkers is expanding with promising prospects for clinical uses as well. The sophistication of producing bsMAB will grow hand in hand with the increased utility of these molecules as tools for research and as therapeutic agents. Recent advances in molecular biology and cancer research have added new dimensions to our investigations and understanding of how the bsMAB works and the novel applications where it has been employed.

Addendum. Two recent reports have appeared adding to the spectrum of novel genetically engineered bispecific constructs. In the first strategy, the light chain constant domain is fused to one scFv and the first heavy chain constant domain fused to a second scFv of different specificity to form bispecific miniantibodies. This novel approach exploits the natural heterodimerization of the two constant domains. In another report, the same group link two different scFv's by a helical dimerization domain to express dimeric bispecific miniantibodies. This unique construct has not only two specificities but also incorporates bivalency to preserve avidity naturally found in immunoglobulins [Muller, K. M., Arndt, K. M., Stritt-

matter, W. and Pluckthun, A. (1998) The first constant domain(CH1 and CL) of an antibody used as heterodimerization domain for bispecific miniantibodies. *FEBS Lett.* 422, 259–264; Muller, K. M., Arndt, K. M. and Pluckthun, A. (1998) A dimeric bispecific miniantibody combines two specificities with avidity. *FEBS Lett.* 432, 45–49].

ACKNOWLEDGMENT

M.R.S. thanks MRC and Biomira for chair support and NSERC for grant funding. This review is dedicated to Dr. Cesar Milstein on the occasion of his retirement from the MRC Laboratory of Molecular Biology, Cambridge, U.K., in whose laboratory MRS was trained in the field of bispecific monoclonals. Thanks are also due to Dr. J. Kriangkum for help in preparing the cover figure.

LITERATURE CITED

- (1) Affleck, K., and Embleton, M. J. (1992) Monoclonal antibody targeting of methotrexate (MTX) against MTX-resistant tumour cell lines. *Br. J. Cancer* 65, 838–44.
- (2) Allard, W. J., Moran, C. A., Nagel, E., Collins, G., and Largen, M. T. (1992) Antigen binding properties of highly purified bispecific antibodies. *Mol. Immunol.* 29, 1219–27.
- (3) Anderson, P. M., Crist, W., Hasz, D., Carroll, A. J., Myers, D. E., and Uckun, F. M. (1992) G19.4(alpha CD3) x B43(alpha CD19) monoclonal antibody heteroconjugate triggers CD19 antigen-specific lysis of t(4;11) acute lymphoblastic leukemia cells by activated CD3 antigen-positive cytotoxic T cells. *Blood* 80, 2826–34.
- (4) Auriol, J., Guesdon, J. L., Mazie, J. C., and Nato, F. (1994) Development of a bispecific monoclonal antibody for use in molecular hybridisation. *J. Immunol. Methods* 169, 123–33.
- (5) Azuma, A., Yagita, H., Matsuda, H., Okumura, K., and Niitani, H. (1992) Induction of intercellular adhesion molecule 1 on small cell lung carcinoma cell lines by gamma-interferon enhances spontaneous and bispecific anti-CD3 x antitumor antibody-directed lymphokine activated killer cell cytotoxicity. *Cancer Res.* 52, 4890–94.
- (6) Barr, I. G., MacDonald, H. R., Buchegger, F., and von Flidner, V. (1987) Lysis of tumor cells by the retargeting of murine cytolytic T lymphocytes with bispecific antibodies. *Int. J. Cancer* 40, 423–9.
- (7) Berg, J., Lotscher, E., Steimer, K. S., Capon, D. J., Baenziger, J., Jack, H. M., and Wabl, M. (1991) Bispecific antibodies that mediate killing of cells infected with human immunodeficiency virus of any strain. *Proc. Natl. Acad. Sci. U.S.A.* 88, 4723–7.
- (8) Berkova, N., Karawajew, L., Korobko, V., Behrsing, O., Micheel, B., Shamborant, O., Stukatcheva, E., and Shingarov, L. (1996) Development of an enzyme immunoassay for the measurement of human tumour necrosis factor-alpha (hTNF-alpha) using bispecific antibodies to hTNF-alpha and horseradish peroxidase. *Biotechnol. Appl. Biochem.* 23, 163–71.
- (9) Bolhuis, R. L., Sturm, E., and Braakman, E. (1991) T cell targeting in cancer therapy. *Cancer Immunol. Immunother.* 34, 1–8.
- (10) Bolhuis, R. L., Lamers, C. H., Goey, S. H., Eggermont, A. M., Trimbois, J. B., Stoter, G., Lanzavecchia, A., di Re, E., Miotti, S., and Raspagliesi, F., et al. (1992) Adoptive immunotherapy of ovarian carcinoma with bs-MAb-targeted lymphocytes: a multicenter study. *Int. J. Cancer (Suppl.)* 7, 78–81.
- (11) Bonardi, M. A., Bell, A., French, R. R., Gromo, G., Hamblin, T., Modena, D., Tutt, A. L., and Glennie, M. J. (1992) Initial experience in treating human lymphoma with a combination of bispecific antibody and saporin. *Int. J. Cancer (Suppl.)* 7, 73–7.
- (12) Bonardi, M. A., French, R. R., Amlot, P., Gromo, G., Modena, D., and Glennie, M. J. (1993) Delivery of saporin to human B-cell lymphoma using bispecific antibody: targeting via CD22 but not CD19, CD37, or immunoglobulin results in efficient killing. *Cancer Res.* 53, 3015–21.
- (13) Bos, R., and Nieuwenhuizen, W. (1992) Enhanced transfection of a bacterial plasmid into hybridoma cells by electroporation: application for the selection of hybridoma (quadroma) cell lines. *Hybridoma* 11, 41–51.
- (14) Branscomb, E. E., Runge, M. S., Savard, C. E., Adams, K. M., Matsueda, G. R., and Haber, E. (1990) Bispecific monoclonal antibodies produced by somatic cell fusion increase the potency of tissue plasminogen activator. *Thromb. Haemost.* 64, 260–6.
- (15) Brenner, M. B., Trowbridge, I. S., and Strominger, J. L. (1985) Cross-linking of human T cell receptor proteins: association between the T cell idiotype beta subunit and the T3 glycoprotein heavy subunit. *Cell* 40, 183–90.
- (16) Canevari, S., Menard, S., Mezzanzanica, D., Miotti, S., Pupa, S. M., Lanzavecchia, A., and Colnaghi, M. I. (1988) Anti-ovarian carcinoma anti-T3 heteroconjugates or hybrid antibodies induce tumor cell lysis by cytotoxic T-cells. *Int. J. Cancer (Suppl.)* 2, 18–21.
- (17) Canevari, S., Stoter, G., Arienti, F., Bolis, G., Colnaghi, M. I., Di Re, E. M., Eggermont, A. M., Goey, S. H., Gratama, J. W., and Lamers, C. H., et al. (1995) Regression of advanced ovarian carcinoma by intraperitoneal treatment with autologous T lymphocytes retargeted by a bispecific monoclonal antibody. *J. Natl. Cancer Inst.* 87, 1463–9.
- (18) Cao, Y., Vinayagamorthy, T., Noujaim, A. A., and Suresh, M. R. (1995) A rapid nonselective method to generate quadromas by microelectrofusion. *J. Immunol. Methods* 187, 1–7.
- (19) Cao, Y., Christian, S., and Suresh, M. R. (1998) Development of bsMab anti-biotin x anti-HRPO as a universal immunoprobe for detecting biotinylated macromolecules. *J. Immunol. Methods* (in press).
- (20) Chamow, S. M., Zhang, D. Z., Tan, X. Y., Mhatre, S. M., Marsters, S. A., Peers, D. H., Byrn, R. A., Ashkenazi, A., and Junghans, R. P. (1994) A humanized, bispecific immunoadhesin-antibody that retargets CD3+ effectors to kill HIV-1-infected cells. *J. Immunol.* 153, 4268–80.
- (21) Chamow, S. M., Zhang, D., Tan, X. Y., Mhatre, S. M., Marsters, S. A., Peers, D. H., Byrn, R. A., Ashkenazi, A., and Junghans, R. P. (1995) A humanized, bispecific immunoadhesin-antibody that retargets CD3+ effectors to kill HIV-1-infected cells. *J. Hematother.* 4, 439–46.
- (22) Chang, D. C. (1989) Cell poration and cell fusion using an oscillating electric field. *Biophys. J.* 56, 641–52.
- (23) Charpie, J. R., Runge, M. S., Matsueda, G. R., and Haber, E. (1990) A bispecific antibody enhances the fibrinolytic potency of single-chain urokinase. *Biochemistry* 29, 6374–8.
- (24) Chatal, J. F., Faivre-Chauvet, A., Bardies, M., Peltier, P., Gautherot, E., and Barbet, J. (1995) Bifunctional antibodies for radioimmunotherapy. *Hybridoma* 14, 125–8.
- (25) Cheong, H. S., Chang, J. S., Park, J. M., and Byun, S. M. (1990) Affinity enhancement of bispecific antibody against two different epitopes in the same antigen. *Biochem. Biophys. Res. Commun.* 173, 795–800.
- (26) Chetanneau, A., Barbet, J., Peltier, P., Le Doussal, J. M., Gruaz-Guyon, A., Bernard, A. M., Resche, I., Rouvier, E., Bourguet, P., and Delaage, M., et al. (1994) Pretargeted imaging of colorectal cancer recurrences using an ¹¹¹In-labeled bivalent hapten and a bispecific antibody conjugate. *Nucl. Med. Commun.* 15, 972–80.
- (27) Cook, A. G., and Wood, P. J. (1994) Chemical synthesis of bispecific monoclonal antibodies: potential advantages in immunoassay systems. *J. Immunol. Methods* 171, 227–37.
- (28) Corvalan, J. R., Smith, W., Gore, V. A., and Brandon, D. R. (1987) Specific in vitro and in vivo drug localisation to tumour cells using a hybrid-hybrid monoclonal antibody recognising both carcinoembryonic antigen (CEA) and vinca alkaloids. *Cancer Immunol. Immunother.* 24, 133–7.
- (29) Cotton, R. G., and Milstein, C. (1973) Letter: Fusion of two immunoglobulin-producing myeloma cells. *Nature* 244, 42–3.
- (30) Davico Bonino, L., De Monte, L. B., Spagnoli, G. C., Vola, R., Mariani, M., Barone, D., Moro, A. M., Riva, P., Nicotra, M. R., and Natali, P. G., et al. (1995) Bispecific monoclonal antibody anti-CD3 x anti-tetanus: an immunotherapeutic

- agent for human glioma [published erratum appears in *Int. J. Cancer* (1995) 62 (3), 364]. *Int. J. Cancer* 61, 509–15.
- (31) De Gast, G. C., Van Houten, A. A., Haagen, I. A., Klein, S., De Weger, R. A., Van Dijk, A., Phillips, J., Clark, M., and Bast, B. J. (1995) Clinical experience with CD3 x CD19 bispecific antibodies in patients with B cell malignancies. *J. Hematother.* 4, 433–7.
 - (32) De Kruif, J., and Logtenberg, T. (1996) Leucine zipper dimerized bivalent and bispecific scFv antibodies from a semi-synthetic antibody phage display library. *J. Biol. Chem.* 271, 7630–4.
 - (33) De Lau, W. B., Van Loon, A. E., Heije, K., Valerio, D., and Bast, B. J. (1989) Production of hybrid hybridomas based on HAT(s)-neomycin(r) double mutants. *J. Immunol. Methods* 117, 1–8.
 - (34) De Lau, W. B., Boom, S. E., Heije, K., Griffioen, A. W., Braakman, E., Bolhuis, R. L., Tax, W. J., Clevers, H., and Bast, B. J. (1992) Heterodimeric complex formation with CD8 and TCR by bispecific antibody sustains paracrine IL-2-dependent growth of CD3+ CD8+ T cells. *J. Immunol.* 149, 1840–6.
 - (35) Demanet, C., Brissinck, J., De Jonge, J., and Thielemans, K. (1996) Bispecific antibody-mediated immunotherapy of the BCL1 lymphoma: increased efficacy with multiple injections and CD28-induced costimulation. *Blood* 87, 4390–8.
 - (36) De Sutter, K., and Fiers, W. (1994) A bifunctional murine: human chimeric antibody with one antigen-binding arm replaced by bacterial beta-lactamase. *Mol. Immunol.* 31, 261–7.
 - (37) Deramoudt, E. X., Gilard, C., Lepine, N., Alonso, J. M., and Romet-Lemonne, J. L. (1992) Bispecific anti-human red blood Rhesus-D antigen x anti Fc gamma RI targeted antibody-dependent cell-mediated cytotoxicity and phagocytosis by mononuclear leucocytes. *Clin. Exp. Immunol.* 89, 310–4.
 - (38) Dillehay, L. E., Mayer, R., Zhang, Y. G., Shao, Y., Song, S. Y., Mackensen, D. G., and Williams, J. R. (1995) Prediction of tumor response to experimental radioimmunotherapy with 90Y in nude mice. *Int. J. Radiat. Oncol. Biol. Phys.* 33, 417–27.
 - (39) Fanger, M. W., Ball, E. D., and Drakeman, D. L. (1995) Comments on the Fourth International Conference on Bispecific Antibodies and Cellular Cytotoxicity, Duck Key, Florida, March 1–5, 1995 (editorial). *J. Hematother.* 4, 345–9.
 - (40) Ferrini, S., Prigione, I., Mammoliti, S., Colnaghi, M. I., Menard, S., Moretta, A., and Moretta, L. (1989) Retargeting of T-cell receptor gamma/delta+ lymphocytes against tumor cells by bispecific monoclonal antibodies. *Int. J. Cancer* (Suppl.) 4, 53–55.
 - (41) French, R. R., Hamblin, T. J., Bell, A. J., Tutt, A. L., and Glennie, M. J. (1995) Treatment of B-cell lymphomas with combination of bispecific antibodies and saporin. *Lancet* 346, 223–4.
 - (42) French, R. R., Bell, A. J., Hamblin, T. J., Tutt, A. L., and Glennie, M. J. (1996) Response of B-cell lymphoma to a combination of bispecific antibodies and saporin. *Leuk. Res.* 20, 607–17.
 - (43) Glennie, M. J., McBride, H. M., Worth, A. T., and Stevenson, G. T. (1987) Preparation and performance of bispecific F(ab')₂ antibody containing thioether-linked Fab' gamma fragments. *J. Immunol.* 139, 2367–75.
 - (44) Goodwin, D. A. (1987) Pharmacokinetics and antibodies. *J. Nucl. Med.* 28, 1358–62.
 - (45) Goodwin, D. A., Meares, C. F., Watanabe, N., McTigue, M., Chaovapong, W., Ransone, C. M., Renn, O., Greiner, D. P., Kukis, D. L., and Kronenberger, S. I. (1994) Pharmacokinetics of pretargeted monoclonal antibody 2D12.5 and 88Y-Janus-2-(p-nitrobenzyl)-1,4,7,10-tetraazacyclododecanetetraacetic acid (DOTA) in BALB/c mice with KHJJ mouse adenocarcinoma: a model for 90Y radioimmunotherapy. *Cancer Res.* 54, 5937–46.
 - (46) Gruber, M., Schodin, B. A., Wilson, E. R., and Kranz, D. M. (1994) Efficient tumor cell lysis mediated by a bispecific single chain antibody expressed in *Escherichia coli*. *J. Immunol.* 152, 5368–74.
 - (47) Haagen, I. A., van de Griend, R., Clark, M., Geerars, A., Bast, B., and de Gast, B. (1992) Killing of human leukaemia/lymphoma B cells by activated cytotoxic T lymphocytes in the presence of a bispecific monoclonal antibody (alpha CD3/alpha CD19). *Clin. Exp. Immunol.* 90, 368–75.
 - (48) Hayden, M. S., Gilliland, L. K., and Ledbetter, J. A. (1997) Antibody engineering. *Curr. Opin. Immunol.* 9, 201–212.
 - (49) Hermanson, G. T. (1996) *Bioconjugate techniques*, Academic Press, NY.
 - (50) Holliger, P., Prospero, T., and Winter, G. (1993) "Diabodies": small bivalent and bispecific antibody fragments. *Proc. Natl. Acad. Sci. U.S.A.* 90, 6444–8.
 - (51) Hsieh-Ma, S. T., Eaton, A. M., Shi, T., and Ring, D. B. (1992) In vitro cytotoxic targeting by human mononuclear cells and bispecific antibody 2B1, recognizing c-erbB-2 protooncogene product and Fc gamma receptor III. *Cancer Res.* 52, 6832–9.
 - (52) Imura, Y., Stassen, J. M., Kurokawa, T., Iwasa, S., Lijnen, H. R., and Collen, D. (1992) Thrombolytic and pharmacokinetic properties of an immunconjugate of single-chain urokinase-type plasminogen activator (u-PA) and a bispecific monoclonal antibody against fibrin and against u-PA in baboons. *Blood* 79, 2322–9.
 - (53) Jantschkeff, P., Winkler, L., Karawajew, L., Kaiser, G., Bottger, V., and Micheel, B. (1993) Hybrid hybridomas producing bispecific antibodies to CEA and peroxidase isolated by a combination of HAT medium selection and fluorescence activated cell sorting. *J. Immunol. Methods* 163, 91–7.
 - (54) Jung, G., Freimann, U., Von Marschall, Z., Reisfeld, R. A., and Wilmanns, W. (1991) Target cell-induced T cell activation with bi- and trispecific antibody fragments. *Eur. J. Immunol.* 21, 2431–5.
 - (55) Kaneko, T., Fusauchi, Y., Kakui, Y., Masuda, M., Akahoshi, M., Teramura, M., Motoji, T., Okumura, K., Mizoguchi, H., and Oshimi, K. (1993) A bispecific antibody enhances cytokine-induced killer-mediated cytotoxicity of autologous acute myeloid leukemia cells. *Blood* 81, 1333–41.
 - (56) Karawajew, L., Micheel, B., Behrsing, O., and Gaestel, M. (1987) Bispecific antibody-producing hybrid hybridomas selected by a fluorescence activated cell sorter. *J. Immunol. Methods* 96, 265–70.
 - (57) Karawajew, L., Behrsing, O., Kaiser, G., and Micheel, B. (1988) Production and ELISA application of bispecific monoclonal antibodies against fluorescein isothiocyanate (FITC) and horseradish peroxidase (HRP). *J. Immunol. Methods* 111, 95–9.
 - (58) Knuth, A., Bernhard, H., Jager, E., Wolfel, T., Karbach, J., Jaggle, C., Strittmatter, W., and Meyer zum Buschenfelde, K. H. (1994) Induction of tumour cell lysis by a bispecific antibody recognising epidermal growth factor receptor (EGFR) and CD3. *Eur. J. Cancer* 30A, 1103–7.
 - (59) Kohler, G., and Milstein, C. (1975) Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* 256, 495–7.
 - (60) Kontsekova, E., Novak, M., and Kontsek, P. (1992) Bispecific antibodies: present status and possible applications. *Bratis. Lek. Listy* 93, 405–10.
 - (61) Koolwijk, P., Rozemuller, E., Stad, R. K., De Lau, W. B., and Bast, B. J. (1988) Enrichment and selection of hybrid hybridomas by Percoll density gradient centrifugation and fluorescent-activated cell sorting. *Hybridoma* 7, 217–25.
 - (62) Kostelny, S. A., Cole, M. S., and Tso, J. Y. (1992) Formation of a bispecific antibody by the use of leucine zippers. *J. Immunol.* 148, 1547–53.
 - (63) Kranenborg, M. H., Boerman, O. C., Oosterwijk-Wakka, J. C., de Weijert, M. C., Corstens, F. H., and Oosterwijk, E. (1995) Development and characterization of anti-renal cell carcinoma x antichelate bispecific monoclonal antibodies for two-phase targeting of renal cell carcinoma. *Cancer Res.* 55, 5864–7.
 - (64) Kranz, D. M., Gruber, M., and Wilson, E. R. (1995) Properties of bispecific single chain antibodies expressed in *Escherichia coli*. *J. Hematother.* 4, 403–8.
 - (65) Kreutz, F. T., and Suresh, M. R. (1995) Bispecific monoclonal anti-CA125 X anti-peroxidase antibodies in the me-

- surement of the ovarian carcinoma antigen. *J. Tumor Marker Oncol.* 10, 45–53.
- (66) Kreutz, F. T., and Suresh, M. R. (1997) Novel bispecific immunoprobe for rapid and sensitive detection of prostate-specific antigen. *Clin. Chem.* 43, 649–56.
- (67) Kreutz, F. T., Wishart, D. S., and Suresh, M. R. (1998a) An efficient bispecific monoclonal antibody purification using gradient thiophilic affinity chromatography. *J. Chromatogr., B* (in press).
- (68) Kreutz, F. T., Xu, D. Z., and Suresh, M. R. (1998b) A new method to generate quadromas by electrofusion and FACS sorting. *Hybridoma* 17, 267–73.
- (69) Kurokawa, T., Iwasa, S., Kakinuma, A., Stassen, J. M., Lijnen, H. R., and Collen, D. (1991) Enhancement of clot lysis in vitro and in vivo with a bispecific monoclonal antibody directed against human fibrin and against urokinase-type plasminogen activator. *Thromb. Haemost.* 66, 684–93.
- (70) Lanzavecchia, A., and Scheidegger, D. (1987) The use of hybrid hybridomas to target human cytotoxic T lymphocytes. *Eur. J. Immunol.* 17, 105–11.
- (71) Liu, L., Barth, R. F., Adams, D. M., Soloway, A. H., and Reisfeld, R. A. (1995) Bispecific antibodies as targeting agents for boron neutron capture therapy of brain tumors. *J. Hematother.* 4, 477–83.
- (72) Liu, M. A., Kranz, D. M., Kurnick, J. T., Boyle, L. A., Levy, R., and Eisen, H. N. (1985) Heteroantibody duplexes target cells for lysis by cytotoxic T lymphocytes. *Proc. Natl. Acad. Sci. U.S.A.* 82, 8648–52.
- (73) Mallender, W. D., Ferreira, S. T., Voss, E. W., Jr., and Coelho-Sampaio, T. (1994) Inter-active-site distance and solution dynamics of a bivalent-bispecific single-chain antibody molecule. *Biochemistry* 33, 10100–8.
- (74) McGuinness B. T., Walter G., Fitzgerald K., Schuler P., Mahoney, W., Duncan A. R., Hoogenboom H. R. (1996) Phage diadory repertoire for selection of large numbers of bispecific antibody fragments *Natl. Biotechnol.* 14, 1149–53.
- (75) Merchant, A. M., Zhu, Z. P., Yuan, J. Q., Goddard, A., Adams, C. W., Presta, L. G., and Carter, P. (1997) An efficient route to human bispecific IgG. *Nat. Biotechnol.* 16, 677–81.
- (76) Milstein, C., and Cuello, A. C. (1983) Hybrid hybridomas and their use in immunohistochemistry. *Nature* 305, 537–40.
- (77) Nisonoff, A., and Rivers, M. M. (1961) Recombination of a mixture of univalent antibody fragments of different specificity. *Arch. Biochem. Biophys.* 93, 460–62.
- (78) Nolan, O., and Kennedy O. R. (1990) Bifunctional antibodies: concept, production and applications. *Biochim. Biophys. Acta* 1040, 1–11.
- (79) Ohta, S., Tsukamoto, H., Watanabe, K., Makino, K., Kuge, S., Hanai, N., Habu, S., and Nishimura, T. (1995) Tumor-associated glycoantigen, sialyl Lewis(a) as a target for bispecific antibody-directed adoptive tumor immunotherapy. *Immunol. Lett.* 44, 35–40.
- (80) Pak, R. H., Primus, F. J., Rickard-Dickson, K. J., Ng, L. L., Kane, R. R., and Hawthorne, M. F. (1995) Preparation and properties of nido-carborane-specific monoclonal antibodies for potential use in boron neutron capture therapy for cancer. *Proc. Natl. Acad. Sci. U.S.A.* 92, 6986–90.
- (81) Piran, U., Silbert-Shostek, D., and Barlow, E. H. (1993) Role of antibody valency in hapten-heterologous immunoassays. *Clin. Chem.* 39, 879–83.
- (82) Pluckthun, A. and Pack, P. (1997) New protein engineering approaches to multivalent and bispecific antibody fragments. *Immunotechnology* 3, 83–105.
- (83) Rammensee, H. G., Julius, M. H., Nemazee, D., Langhorne, J., Lamers, R., and Kohler, G. (1987) Targeting cytotoxic T cells to antigen-specific B lymphocytes. *Eur. J. Immunol.* 17, 433–6.
- (84) Reddy, V. S., and Ford, C. H. (1993) Production of hybrids secreting bispecific antibodies recognising CEA and doxorubicin. *Anticancer Res.* 13, 2077–83.
- (85) Rheinacker M, Hardt C, Liag L. L., Kufer P, Gruber R., Hoess A, Lupas A., Rottenberger C, Pluckthun A. And Pack P (1996) Multivalent antibody fragments with high functional affinity for a tumor associated carbohydrate antigen. *J. Immunol.* 157, 2989–2997.
- (86) Ribeiro-da-Silva, A., Pioro, E. P., and Cuello, A. C. (1991) Substance P- and enkephalin-like immunoreactivities are colocalized in certain neurons of the substantia gelatinosa of the rat spinal cord: an ultrastructural double-labeling study. *J. Neurosci.* 11, 1068–80.
- (87) Ridgway, J. B., Presta, L. G., and Carter, P. (1996) Knobs-into-holes engineering of antibody CH3 domains for heavy chain heterodimerization. *Protein Eng.* 9, 617–21.
- (88) Roosnek, E., and Lanzavecchia, A. (1989) Triggering T cells by otherwise inert hybrid anti-CD3/antitumor antibodies requires encounter with the specific target cell. *J. Exp. Med.* 170, 297–302.
- (89) Roosnek, E., Tunnacliffe, A., and Lanzavecchia, A. (1990) T cell activation by a bispecific anti-CD3/anti-major histocompatibility complex class I antibody. *Eur. J. Immunol.* 20, 1393–6.
- (90) Rylatt, D. B., Kemp, B. E., Bundesen, P. G., John, M. A., EJ, O. R., Cottis, L. E., Miles, S. J., Khan, J. M., Dinh, D. P., and Stapleton, D., et al. (1990) A rapid whole-blood immunoassay system. *Med. J. Aust.* 152, 75–7.
- (91) Sahin, U., Hartmann, F., Senter, P., Pohl, C., Engert, A., Diehl, V., and Pfreundschuh, M. (1990) Specific activation of the prodrug mitomycin phosphate by a bispecific anti-CD30/anti-alkaline phosphatase monoclonal antibody. *Cancer Res.* 50, 6944–8.
- (92) Schmidt, M., Hynes, N. E., Groner, B., and Wels, W. (1996) A bivalent single-chain antibody-toxin specific for ErbB-2 and the EGF receptor. *Int. J. Cancer* 65, 538–46.
- (93) Schuhmacher, J., Klivenyi, G., Matys, R., Stadler, M., Regiert, T., Hauser, H., Doll, J., Maier-Borst, W., and Zoller, M. (1995) Multistep tumor targeting in nude mice using bispecific antibodies and a gallium chelate suitable for immunoscintigraphy with positron emission tomography. *Cancer Res.* 55, 115–23.
- (94) Segal, D. M., Qian, J. H., Garrido, M. A., Perez, P., Winkler, D. F., Wunderlich, J. R., Snider, D. P., Valdayo, M. J., and Titus, J. A. (1988) Targeting of cytotoxic cells against tumors with heterocross-linked, bispecific antibodies. *Princess Takamatsu Cancer Res. Fund, Proc. Int. Symp.* 19, 323–31.
- (95) Shalaby, M. R., Shepard, H. M., Presta, L., Rodrigues, M. L., Beverley, P. C., Feldmann, M., and Carter, P. (1992) Development of humanized bispecific antibodies reactive with cytotoxic lymphocytes and tumor cells overexpressing the HER2 protooncogene. *J. Exp. Med.* 175, 217–25.
- (96) Smith, W., Jarrett, A. L., Beattie, R. E., and Corvalan, J. R. (1992) Immunoglobulins secreted by a hybrid-hybridoma: analysis of chain assemblies. *Hybridoma* 11, 87–98.
- (97) Somasundaram, C., Arch, R., Matzku, S., and Zoller, M. (1996) Development of a bispecific F(ab')₂ conjugate against the complement receptor CR3 of macrophages and a variant CD44 antigen of rat pancreatic adenocarcinoma for redirecting macrophage-mediated tumor cytotoxicity. *Cancer Immunol. Immunother.* 42, 343–50.
- (98) Songsivilai, S., and Lachmann, P. J. (1990) Bispecific antibody: a tool for diagnosis and treatment of disease. *Clin. Exp. Immunol.* 79, 315–21.
- (99) Songsivilai, S., Clissold, P. M., and Lachmann, P. J. (1989) A novel strategy for producing chimeric bispecific antibodies by gene transfection. *Biochem. Biophys. Res. Commun.* 164, 271–6.
- (100) Staerz, U. D., and Bevan, M. J. (1985) Cytotoxic T lymphocyte-mediated lysis via the Fc receptor of target cells. *Eur. J. Immunol.* 15, 1172–7.
- (101) Staerz, U. D., and Bevan, M. J. (1986) Hybrid hybridoma producing a bispecific monoclonal antibody that can focus effector T-cell activity. *Proc. Natl. Acad. Sci. U.S.A.* 83, 1453–7.
- (102) Staerz, U. D., and Bevan, M. J. (1989) Redirecting the cellular immune response. *Int. Rev. Immunol.* 4, 159–73.
- (103) Suresh, M. R. (1991) Immunoassays for cancer-associated carbohydrate antigens. *Semin. Cancer Biol.* 2, 367–77.
- (104) Suresh, M. R., Cuello, A. C., and Milstein, C. (1986a) Advantages of bispecific hybridomas in one-step immunocytochemistry and immunoassays. *Proc. Natl. Acad. Sci. U.S.A.* 83, 7989–93.

- (105) Suresh, M. R., Cuello, A. C., and Milstein, C. (1986b) Bispecific monoclonal antibodies from hybrid hybridomas. *Methods Enzymol.* 121, 210–28.
- (106) Tada, H., Toyoda, Y., and Iwasa, S. (1989) Bispecific antibody-producing hybrid hybridoma and its use in one-step immunoassays for human lymphotoxin. *Hybridoma* 8, 73–83.
- (107) Takahashi, M., and Fuller, S. A. (1988) Production of murine hybrid-hybridomas secreting bispecific monoclonal antibodies for use in urease-based immunoassays. *Clin. Chem.* 34, 1693–6.
- (108) Thirion, S., Motmans, K., Heyligen, H., Janssens, J., Raus, J., and Vandevyver, C. (1996) Mono- and bispecific single-chain antibody fragments for cancer therapy. *Eur. J. Cancer Prev.* 5, 507–11.
- (109) Tutt, A., Greenman, J., Stevenson, G. T., and Glennie, M. J. (1991a) Bispecific F(ab'γ)3 antibody derivatives for redirecting unprimed cytotoxic T cells. *Eur. J. Immunol.* 21, 1351–8.
- (110) Tutt, A., Stevenson, G. T., and Glennie, M. J. (1991b) Trispecific F(ab'γ)3 derivatives that use cooperative signaling via the TCR/CD3 complex and CD2 to activate and redirect resting cytotoxic T cells. *J. Immunol.* 147, 60–9.
- (111) Valone, F. H., Kaufman, P. A., Guyre, P. M., Lewis, L. D., Memoli, V., Ernstoff, M. S., Wells, W., Barth, R., Deo, Y., and Fisher, J., et al. (1995) Clinical trials of bispecific antibody MDX-210 in women with advanced breast or ovarian cancer that overexpresses HER-2/neu. *J. Hematother.* 4, 471–5.
- (112) Van Dijk, J., Tsuruo, T., Segal, D. M., Bolhuis, R. L., Colognola, R., van de Griend, R. J., Fleuren, G. J., and Warnaar, S. O. (1989) Bispecific antibodies reactive with the multidrug-resistance-related glycoprotein and CD3 induce lysis of multidrug-resistant tumor cells. *Int. J. Cancer* 44, 738–43.
- (113) Van Dijk, J., Zegveld, S. T., Fleuren, G. J., and Warnaar, S. O. (1991) Localization of monoclonal antibody G250 and bispecific monoclonal antibody CD3/G250 in human renal-cell carcinoma xenografts: relative effects of size and affinity. *Int. J. Cancer* 48, 738–43.
- (114) Van Osdol, W. V., Sung, C., Dedrick, R. L. and Weinstein, J. N. (1993) A distributed pharmacokinetic model of two-step imaging and treatment protocols: Application to streptavidin-conjugated monoclonal antibodies and radiolabeled biotin. *J. Nucl. Med.* 34, 1552–64.
- (115) Weiner, L. M., Clark, J. I., Ring, D. B., and Alpaugh, R. K. (1995) Clinical development of 2B1, a bispecific murine monoclonal antibody targeting c-erbB-2 and Fc gamma RIII. *J. Hematother.* 4, 453–6.
- (116) Winter, G., and Milstein, C. (1991) Man-made antibodies. *Nature* 349, 293–9.
- (117) Wognum, A. W., Lansdorp, P. M., Eaves, A. C., and Krystal, G. (1989) An enzyme-linked immunosorbent assay for erythropoietin using monoclonal antibodies, tetrameric immune complexes, and substrate amplification. *Blood* 74, 622–8.
- (118) Wong, J. T., and Colvin, R. B. (1987) Bi-specific monoclonal antibodies: selective binding and complement fixation to cells that express two different surface antigens. *J. Immunol.* 139, 1369–74.
- (119) Xu, D. Z., Leveugle, B., Kreutz, F. T. and Suresh, M. R. (1997) Mimetic ligand-based affinity purification of immune complexes and immunoconjugates. *J. Chromatogr., B* 706, 217–29.

BC980044L

ARTICLES

Synthesis of Water-Soluble, Nonimmunogenic Polyamide Cross-Linking Agents[†]

Ton That Hai, David E. Pereira,* and Deanna J. Nelson

Hemoglobin Therapeutics Division, Baxter Healthcare Corp., 25212 West State Route 120, Round Lake, Illinois 60073. Received November 17, 1997; Revised Manuscript Received August 15, 1998

Novel polyamides were developed that can be used as cross-linking agents for proteins such as hemoglobin. Water-soluble, nonimmunogenic polyamides containing oxygen and sulfur atoms in the backbone were prepared by the polycondensation of the diacids bis(carboxymethoxyacetyl)-1,4-diaminobutane (**1a**) or 3,3'-thiodipropionic acid (**1b**) with diethylene glycol bis(3-aminopropyl) ether (**2**). The resulting α,ω -diacids were converted to the corresponding activated esters using any of a variety of carboxylic acid activating reagents including the novel reagent diphenyl(1-methylimidazol-2-thiyl)phosphonate (**9**). The resulting polyamides could be activated with a broad spectrum of groups that allow for the cross-linking and surface modification of proteins.

INTRODUCTION

Cross-linking reagents that react with the amino or sulfhydryl groups of proteins have a broad range of applications (1–3). While some applications, such as the identification of binding sites (receptors) for ligands, the introduction of fluorescent or spin labels, or intramolecular cross-linking of neighboring functional groups, have few associated spatial requirements, many other applications require that the cross-linking agent have substantial length to prevent steric hindrance to cross-linking.

To reduce the resulting heterogeneity of the cross-linked proteins and to maximize the cross-linking efficiency, it is important that the size of the cross-linking agent be defined and restricted to as narrow a range as can be achieved. Concomitantly, it is important the size of the cross-linker be controlled and limited, so that water solubility of the product is maintained. Similarly, the ability to activate the termini of the cross-linker using a spectrum of approaches is an important feature of useful cross-linking agents, since there may be some variability from a general pattern in the effectiveness of modification of a specific protein.

One important application of cross-linking agents is to decrease immunogenicity and to increase the lifetime of the cross-linked product in the blood stream. Therefore, it is key that the cross-linker be nontoxic and nonimmunogenic.

Finally, most protein modification reactions are completed in aqueous media. Thus, it is key that the cross-linking agent be water soluble. Likewise, to reduce the opportunistic denaturation of the protein during reaction, it is desirable that the cross-linking agent not behave like a detergent or chaotropic agent.

The purpose of our current work was to synthesize novel polyamides that could act as cross-linking agents for hemoglobin in particular and proteins in general. The resulting modified hemoglobins or proteins could be candidates as blood substitutes or therapeutic agents, respectively. In addition to meeting the requirements defined above, we anticipated that cross-linking agents having molecular masses in the range 1000–7600 Da would provide the most useful tether length while minimizing undesirable and unnecessary additions to overall molecular mass or viscosity of the resulting cross-linked products.

EXPERIMENTAL PROCEDURES

General. NMR spectra were recorded on a Bruker 270 MHz or a Bruker 300 MHz spectrometer. ¹H and ¹³C chemical shifts were measured relative to partially deuterated solvent peaks but are reported relative to tetramethylsilane. ³¹P chemical shifts are reported relative to H₃PO₄ (85%). (NMR chemical shifts for the compounds and polymers described herein are included in the Supporting Information to this paper.) Thin-layer chromatography (TLC) of the polyamides was completed using Whatman 250 μ m silica gel TLC plates and 2-propanol/NH₄OH/water, 10:1:2, by volume, as the eluent. Melting points were determined on a Fisher-Johns melting point apparatus and are uncorrected. Elemental analyses were performed by Oneida Research Services, Whitesboro, NY, or Quantitative Technologies, Inc., Whitehouse, NJ.

Nomenclature. For simplicity, the following acronyms are employed as designating nomenclature. The acronym descriptions are presented in Table 1. The term polyamide is abbreviated "PA" (or "PAT" if one component of the polyamide is 3,3'-thiodipropionate). If each end of the polyamide terminates in a succinate residue, the letter "S" is added to the acronym. Finally, if the activating group is N-oxysuccinimide, a second letter "S"

[†] This article is dedicated to Nelson J. Leonard, Faculty Associate in Chemistry, California Institute of Technology.

* Author to whom correspondence should be addressed. Phone: (847) 270-5838. Fax: (847) 270-5897. E-mail: pereird@baxter.com.

Table 1. Polyamide Acronym Definitions and Potential Reactivity With Proteins

acronym	acronym definition	reactivity with proteins
PA	polyamide	not activated
PAT	polyamide containing 3,3'-thiodipropionate residues	not activated
PAS	functional end group is a succinate residue	not activated
PATS		
PAD	functional end group is an amine residue	not activated
PATD		
PASS	polyamide activated as bis(<i>N</i> -oxysuccinimidyl ester)	cross-linking achieved by reaction with amines to form amides
PATSS		
PADSC	polyamide activated as bis(<i>N</i> -oxysuccinimidyl urethane)	cross-linking achieved by reaction with amines to form ureas
PATDSC		
PADMP	polyamide activated as bis(maleimide)	cross-linking achieved by reaction with amines and/or thiols
PATDMP		
PADA	polyamide activated as bis(aldehyde)	cross-linking achieved by reaction with amines and reduction of the imine intermediates
PATDA		

is added. The approximate molecular mass of the polyamide follows a hyphen. Thus, the acronym PASS-7600 describes the bis(*N*-oxysuccinimidyl) activated ester of a polyamide having a molecular mass of about 7600 Da.

If the polyamide end groups are amino groups, these are designated by the acronym PAD (or PATD for thioether-containing analogues). The polyamide bis-(amines) were converted to reactive urethane, maleimide, or aldehyde end groups. The acronyms for urethane, maleimide, or aldehyde end groups are SC, MP, or A, respectively. Thus, the acronym PATDSC-5700 denotes a urethane-activated, thiodipropionate-containing polyamide having a molecular mass of about 5700 Da.

Size-Exclusion Chromatography (SEC). Analytes were eluted from a Superose-12 column (Pharmacia) with 50 mM phosphate buffer, pH 6.5, delivered at a flow rate of 0.4 mL/min, with analyte detection at 220 nm.

***M_r* Determination of PAS Polymers by ¹H NMR.**

An interval of approximately 6.3 s between excitation pulses was used; this interval was sufficient to realize a complete return of the spin system to the equilibrium magnetization state. The average number of repeating units per molecule of polymer was determined by comparing the area of the resonances attributable to protons attached to the repeating unit with the area of the resonances attributable to the protons attached to the succinic acid terminal group. The areas were normalized, as appropriate, for the relative number of equivalent protons generating each resonance. A total of six resonances attributable to the repeating unit was resolved and identified. Hence, it was possible to make six estimates of the number of repeating units per polymer molecule. It should be stressed that the six estimates were not completely independent: all were derived from a single estimate of the area of the resonance of the protons attached to the terminal unit. The *n* value was obtained from the average of the six estimates of the number of repeating units per polymer molecule. This method was used to determine the average *M_r* of PAS-7600, PAS-5600, PAS-4000, and PAS-2800 (Supporting Information, Table S-2).

***M_r* Determination of PATS Polymers by ¹³C NMR.**

Composite-pulse (WALTZ-16) decoupling was employed. Artificial enhancements of resonance areas due to the nuclear Overhauser mechanism were suppressed by the use of an inverse gating technique. An interval of approximately 22 s between excitation pulses was used; this interval was sufficient to realize a complete return of the spin system to the equilibrium magnetization state. The average number of repeating units per molecule of polymer was determined by comparing the area of the resonances attributable to carbons attached to the repeating unit with the area of the resonances attributable

to the carbons attached to the terminal units. The observed resonance areas were normalized, as appropriate, for the relative number of equivalent carbons generating each resonance. A total of three resonances attributable to eight carbon atoms on the terminal units were resolved. Because only one of the three resonances could be identified unambiguously, the total area for these eight carbon atoms was obtained by summation and treated as a single area measurement. A total of seven resonances attributable to the repeating unit were resolved. Three resonances attributable to carbon atoms adjacent to oxygen in the repeating unit of **2** were resolved but not individually identified. In addition, baseline resolution of these three resonances was not achieved. Because of these two factors, the total area for the three resonances was obtained by summation and treated as a single area. Hence, it was possible to make five estimates of the number of repeating units per polymer molecule. It should be stressed that the five estimates were not completely independent: all were derived from a single estimate of the area of the resonance of the carbons attached to the terminal unit. The average of the five estimates of the number of repeating units per polymer molecule gave the degree of polymerization *n*. This method was used to determine the average *M_r* of PATSS-5700, PATSS-5200, and PATSS-2800 (Supporting Information, Table S-5).

Bis(carboxymethoxyacetyl)-1,4-diaminobutane (1a). Diglycolic anhydride (1524 g, 13 mol) was dissolved in 3 L of *N,N*-dimethylformamide (DMF). The solution was heated to 60 °C, and the heating mantle was replaced with an ice bath. A solution of 1,4-diaminobutane (529 g, 6 mol) in 1.2 L of DMF was added at such a rate that the temperature was kept between 70 and 75 °C. After all of the 1,4-diaminobutane had been added, an additional 100 g of the anhydride was added to the reaction solution. The mixture was then cooled in an ice bath for 1.5 h, and a solid was formed. The DMF solution was decanted from the solid, and the solution volume was reduced to about 500 mL. The residue was combined with the solid, and 3 L of chloroform (CHCl₃) was added to the ice-bath-cooled mixture. The mixture was stirred, and the solid was collected by filtration, washed with 1 L of CHCl₃, and dried under vacuum to give 1882 g of **1a** (98%). Mp: 168–169 °C. Anal. (C₁₂H₂₀N₂O₈) C, H, N.

***N,N'*-Bis(methoxycarbonylmethoxyacetyl)-1,4-diaminobutane (10a).** Compound **1a** (250 g, 0.8 mol) was combined with methanol (1 L) and 50 mL of 4.0 M HCl in 1,4-dioxane. The mixture was stirred overnight. The solvent was removed by rotary evaporation under high vacuum to give a solid. The solid was dried over NaOH under high vacuum. The solid was dissolved in

500 mL of warm acetone (dried over anhyd K_2CO_3) and filtered. Ethyl ether was slowly added to the filtrate, and an oil separated. Stirring with a glass rod caused the oil to solidify. The solid was collected under a flow of nitrogen. The solid was dried under a vacuum over NaOH to give 248 g (88%) of **10a**. Mp: 76–77 °C. Anal. ($C_{14}H_{24}N_2O_8 \cdot 0.5H_2O$) C, H, N.

***N,N'*-Bis(carboxymethoxyacetyl)-1,4-diaminobutane Bis(*p*-nitrophenyl ester) (10b).** *p*-Nitrophenyl trifluoroacetate (2491 g, 10.6 mol) was combined with a solution of DMF (2 L) and 2,6-lutidine (4 L). Compound **1a** (1411 g, 4.4 mol) was added as a solid in one portion to the solution of *p*-nitrophenyl trifluoroacetate. After 2 h, an additional 1 L of DMF was added to the resulting mixture. After stirring an additional hour, 2 L of ethyl ether was added. The mixture was stirred for 30 min, and the solid was collected. The solid was then combined with 4 L of ethyl ether with mixing. The solid was collected and air-dried. The ethyl ether washing was repeated to give 1997 g (80%) of **10b**. Mp: 141–142 °C. Anal. ($C_{24}H_{26}N_4O_{12}$) C, H, N.

***N,N'*-Bis[(2,3-dihydro-2-thioxo-3-benzoxazolyl)-carbonylmethoxyacetyl]-1,4-diaminobutane (10d).** Compound **8** (12.7 g, 33 mmol) was added to a stirred suspension of **1a** (4.8 g, 15 mmol) and 2,6-lutidine (4 mL, 34 mmol) in DMF (25 mL). The resulting solution was stirred for 2 h. The precipitate was collected and washed with ethyl ether to give 6.30 g of **10d**. The filtrate was evaporated to dryness, and the residue was triturated with methanol to precipitate 0.53 g of additional product for a total yield of 6.83 g (78%) of **10d**. Mp: 140–141 °C. Anal. ($C_{26}H_{26}N_4O_8S_2 \cdot 2H_2O$) C, H, N, S.

***N,N'*-(3,3'-Thiodipropionyl)bis(benzoxazoline-2-thione) (14b).** Compound **8** (843 g, 2.2 mol) was added as a solid in one portion to a stirred solution of 3,3'-thiodipropionic acid (178 g, 1.0 mol) and triethylamine (224 g, 2.2 mol) in 3.4 L of $CHCl_3$. After 1.5 h, methanol (3 L) was added, and the mixture was cooled in an ice bath. After 10 min, the solid was collected and washed with ethyl ether (1 L). The solid was dried under house vacuum to give 356 g (80% yield) of **14b**. Mp: 168–170 °C. Anal. ($C_{20}H_{16}N_2O_4S_3 \cdot 0.25H_2O$) C, H, N, S.

Diphenyl(1-methylimidazol-2-thiyl)phosphonate (9). A solution of diphenyl chlorophosphate (**11**; 76.6 g, 0.285 mol) in $CHCl_3$ (100 mL) was added in one portion to a solution of 2-mercapto-1-methylimidazole (**12**; 22.0 g, 0.19 mol) and triethylamine (29.1 g, 0.285 mol) in $CHCl_3$ (200 mL). The solution was heated at reflux overnight. The solvent was evaporated, and the residue was dissolved in toluene (250 mL). The insoluble material was removed by filtration. The filtrate was passed through a pad of silica gel. The product was eluted with a solution of chloroform:acetone (5:1, v/v) (2×250 mL). The eluent was evaporated to an oil. Hexane (150 mL) was added to the oil, and a solid was obtained. The solid was collected, washed with hexane (50 mL), and dried to give 48.2 g (68%) of **9**. Mp: 113–114 °C. Anal. ($C_{16}H_{15}N_2O_3SP$) C, H, N, S.

***S,S'*-Bis(1-methyl-2-imidazolyl)-3-thiapentane-1,5-dithiocarboxylate (14c).** Compound **9** (8.00 g, 22.0 mmol) was added to a solution of 3,3'-thiodipropionic acid (1.77 g, 10.0 mmol) and triethylamine (3.1 mL, 22.0 mmol) in $CHCl_3$ (50 mL). The solution was heated at reflux overnight. The solution was cooled to ambient temperature (RT) and passed through a pad of silica gel. The silica gel was washed with a solution of chloroform:acetone (5:1, v/v) (100 mL). The filtrate was evaporated to a yellow solid. The solid was dissolved in acetone (10 mL), and ethyl ether (15 mL) was added to precipitate

Table 2. Synthesis of Polyamide from 10b and 2^a

molar ratio of 2 to 10b	polyamide characteristics	solvent	
		DMF	chloroform
1.1:1	molecular mass ^b	7.6 kDa	7.6 kDa
	SEC ^c retention time (min)	34.7	34.1
	product distribution ^d		
	PA bis(amine) 5a	75%	>95%
	α-amino-ω-acid PA 5b	25%	<5%
1.2:1	PA bis(acid) 5c	none detected	none detected
	molecular mass ^b	5.6 kDa	5.6 kDa
	SEC ^c retention time (min)	35.5	34.7
	product distribution ^d		
	PA bis(amine) 5a	>95%	ca. 100%
1.3:1	α-amino-ω-acid PA 5b	<5%	trace
	PA bis(acid) 5c	none detected	none detected
	molecular mass ^b	5.6 kDa	4.0 kDa
	SEC ^c retention time (min)	35.5	35.8
	product distribution ^d		
1.4:1	PA bis(amine) 5a	>95%	ca. 100%
	α-amino-ω-acid PA 5b	<5%	trace
	PA bis(acid) 5c	none detected	none detected
	molecular mass ^b	5.6 kDa	2.8 kDa
	retention time (min)	35.5	37.2
	product distribution ^d		
	PA bis(amine) 5a	>95%	ca. 100%
	α-amino-ω-acid PA 5b	<5%	trace
	PA bis(acid) 5c	none detected	none detected

^a Effects of reactant molar ratio and solvent. ^b Molecular mass was determined by ^{13}C - and ^1H -NMR. ^c SEC was performed using a Superose-12 column (Pharmacia). Polyamide was eluted with 50 mM phosphate buffer, pH 6.5, delivered at 0.4 mL/min; analytes were detected at 220 nm. SEC profiles were broad for PA obtained from DMF and narrow for PA obtained from chloroform. ^d The product distribution was estimated from the thin layer chromatographic response after silica gel plates were eluted with 2-propanol/ NH_4OH/H_2O (10:1:2, volume ratio) and stained with iodine or ninhydrin.

the product. The solid was collected to give 2.3 g (68%) of **14c**. Mp: 159–161 °C. Anal. ($C_{14}H_{18}N_4O_2S_3$) C, H, N, S.

***N* Anal. ($C_{14}H_{24}N_2O_8 \cdot 0.5H_2$) C, H, N. -(2,2-Dimethoxyethyl)succinamic Acid (18).** A solution of aminoacetaldehyde dimethylacetal (5.3 g, 50.4 mmol) in 20 mL of $CHCl_3$ was added dropwise to a solution of succinic anhydride (5.1 g, 51.0 mmol) in 50 mL of $CHCl_3$. The mixture was stirred for 1 h at RT. An additional 0.2 g of succinic anhydride was added to consume all of the amine. The mixture was filtered, and the filtrate was evaporated to provide 10.1 g (98%) of **18** as an oil. The oil was used without further purification. Anal ($C_8H_{15}NO_5$) Calcd: C, 46.82; H, 7.37; N, 6.83. Found: C, 44.85; H, 6.96; N, 6.33.

***N*-(2,2-Dimethoxyethyl)succinamic Acid Succinimide Ester (17).** 1,3-Dicyclohexylcarbodiimide (12.5 g, 61.0 mmol) in 25 mL of chloroform was added to a solution of **18** (11.4 g, 56 mmol) and *N*-hydroxysuccinimide (7.0 g, 61 mmol) in 90 mL of chloroform and 10 mL of DMF. The solution was stirred for 2 h and then filtered. The filtrate was evaporated to an oil. Due to its instability, the compound was used without further purification. (Analytical data are included in the Supporting Information.)

Synthesis of Polyamides. The following procedures serve as representative preparations of the described polyamides. The molar ratios, solvents, bases (if needed), and temperatures to prepare a certain M_r range of a polyamide are listed in Tables 2–5. Tabulated ^1H -NMR and ^{13}C -NMR data can be found in the Supporting Information.

Synthesis of Polyamide Bis(amine) (PAD-5600). Compound **10b** (102.6 g, 0.18 mol) was suspended in 330 mL of chloroform. The resulting slurry was cooled in an

Table 3. Polycondensation of 2 and 14b in Chloroform in the Presence of Triethylamine at 25 °C

molar Ratio of 2 to 14b	SEC ^a retention time of product (min)	yield (%)
1:1	32.6	44
1.2:1	37.6	54
1.2:1	32.5	45
1.3:1	35.9	59
1.3:1	31.8	42

^a SEC was performed using a Superose-12 column (Pharmacia). Polyamide was eluted with 50 mM phosphate buffer, pH 6.5, delivered at 0.4 mL/min; analytes were detected at 220 nm.

Table 4. Polymerization of 2 and 14b in Chloroform

molar ratio 2:14b	base	SEC ^a retention time of product (min)	yield (%)
Reaction Temp 0 °C			
1.3:1	none	39.9	57
1.3:1	2,6-lutidine	39.9	41
1.2:1	none	38.7 ^b	50
1.2:1	2,6-lutidine	38.6	41
1.1:1	none	37.8	48
1:1	none	32.7	44
Reaction Temp 25 °C			
1.3:1	none	38.7 ^b	47
1.2:1	none	38.2	48
1.1:1	none	37.6 ^c	42
1:1	none	30.4	48

^a SEC was performed using a Superose-12 column (Pharmacia). Polyamide was eluted with 50 mM phosphate buffer, pH 6.5, delivered at 0.4 mL/min; analytes were detected at 220 nm.

^b Molecular mass of 2800 as determined by ¹³C-NMR. ^c Molecular mass of 5200 as determined by ¹³C-NMR.

Table 5. Polyamides Resulting from Polycondensation of 14c and 2 under Various Reaction Conditions

molar ratio 2:14c	reaction temp (°C)	SEC ^a retention time (min)	yield (%)
Solvent: Chloroform			
1:1	25	33.6	46
1:1	reflux	32.8	45
1.1:1	25	33.9	43
1.2:1	25	36.1	48
1.3:1	25	36.4	30
Solvent: DMF			
1.2:1	25	31.4	61
1.3:1	25	31.6	57
1.2:1	0	33.9	60
1.3:1	0	35.5	54
1.4:1	0	35.4	54
1.5:1	0	37.7	49
1.2:1	-50	37.5	76
1.3:1	-50	38.2	57

^a SEC was performed using a Superose-12 column (Pharmacia). Polyamide was eluted with 50 mM phosphate buffer, pH 6.5, delivered at 0.4 mL/min; analytes were detected at 220 nm.

ice bath to 5 °C and stirred at 131 rpm. A solution of 2,6-lutidine (42 mL, 0.36 mole) and diethylene glycol bis-(3-aminopropyl)ether (2) (47.6 g, 0.22 mol) in 85 mL of CHCl₃ was added to the stirred solution. After 15 min of stirring, the solvent was removed by rotary evaporation. The resulting oil was dissolved in 100 mL DMF, and ethyl acetate (600 mL) was added to precipitate the polymer as a yellow solid. The solid was washed with ethyl acetate (3 × 100 mL) and dried under vacuum to give 81.3 g of the polymer as the *p*-nitrophenol salt.

Synthesis of PADMP-5600. *N*-Succinimidyl 3-maleimidopropionate (16) (6.0 g, 24 mmol) was dissolved in 500 mL of CHCl₃. PAD-5600 (20.1 g, 4 mmol) and triethylamine (1.6 g) in 300 mL of CHCl₃ were slowly

added dropwise to the solution of 16. The addition was complete in 6 h. The solvent was removed to give an oil. The oil was dissolved in a solution of DMF (40 mL) and CHCl₃ (40 mL). Acetone was added to precipitate the product. The mixture was allowed to stand for 3 h, and the product was collected by filtration. The product was reprecipitated from chloroform:DMF one additional time. The precipitate was collected and dried to give 15.8 g of PADMP-5600.

Synthesis of PADSC-3000. To a stirred suspension of *N,N'*-disuccinimidyl carbonate (15) (5.86 g, 22.86 mmol) in a mixture of DMF (60 mL) and CHCl₃ (60 mL) was added dropwise a solution of PAD-2800 (8.00 g, 2.86 mmol) in CHCl₃ (65 mL) containing triethylamine (3.19 mL, 22.86 mmol) over 60 min. The mixture was stirred until all solid had dissolved (~2 h), and then stirring was continued for a further 1 h. The reaction mixture was evaporated to dryness; the residue was dissolved in DMF (50 mL), and the stirred solution was slowly diluted with acetone (400 mL) to precipitate the product, which was collected by filtration, washed with acetone and dried to provide 5.9 g of PADSC-3000 (74%).

Bis[*N*-(2,2-dimethoxyethyl)succinamyl]polyamide: PADA-2800 Bis(acetal). A solution of 10a (9.75 g, 28 mmol) and 2 (8.02 g, 36.4 mmol) in methanol (25 mL) was heated at reflux for 24 h to give crude PAD having a *M_r* of about 2800. Methanol was completely removed by evaporation, and the residue was dissolved in CHCl₃ (35 mL). To this solution was added a solution of *N*-(2-dimethoxyethyl)succinamic acid *N*-oxysuccinimide ester (17; 7.55 g, 25 mmol) and triethylamine (3.48 g, 25 mmol) in CHCl₃ (50 mL). The mixture was stirred until it became negative to ninhydrin (2 h). The solvent was removed by evaporation, and the residue was dissolved in methanol (100 mL). AG50-8X resin (Bio-Rad; 20 mL) was added until the solution pH reached 3.0. The mixture was filtered, and AG1-X8 resin (Bio-Rad; 40 mL) was gradually added to remove *N*-hydroxysuccinimide. The mixture was filtered and evaporated to dryness. The residue was triturated with acetone to precipitate bis-[*N*-(2-dimethoxyethyl)succinamyl]polyamide PADA-2800 bis(acetal) in a yield of 14.1 g after drying.

Bis[*N*-(2-formylethyl)succinamyl]polyamide: PA-DA-2800. PADA-2800 bis(acetal) (3.0 g) was dissolved in water (20 mL), and the solution was cooled in an ice-water bath. Trifluoroacetic acid (20 mL) was added gradually. After the addition was completed, the bath was removed, and the reaction mixture was stirred at room temperature for 2 h. The reaction mixture was evaporated under reduced pressure, and the residue was successively evaporated with water (50 mL) followed by acetone (3 × 50 mL). The residue was dried under high vacuum and then triturated with acetone to precipitate 2.25 g of PADA-2800 as a mixture of the aldehyde and hydrated aldehyde.

Synthesis of PAS-4200 Using 10a. Compound 10a (247 g, 0.7 mol) was dissolved in 400 mL of methanol. A solution of 2 (200 g, 0.9 mol) was added in one portion to the methanol solution. The resulting solution was heated at reflux for 24 h. The solvent was evaporated to give an oil (447 g, 0.12 mol). The oil was dissolved in CHCl₃ (1.5 L) and was added dropwise to a mixture of succinic anhydride (48.3 g, 0.48 mol) in CHCl₃ (500 mL). The mixture was stirred overnight. An additional portion of succinic anhydride (10 g) was added to the solution. The solution was stirred for 2 h, and the CHCl₃ was removed by rotary evaporation. The mixture was dissolved in water (1 L), and the mixture was placed on a rotary evaporator to remove residual chloroform. The aqueous

solution was treated with 500 g of AG50-8X resin (the resin was pretreated with 1.0 N HCl). The resin was collected and washed with 1.0 N NaOH, water, and then 1.0 N HCl. The aqueous solution was treated again with the resin. This was repeated one additional time. The filtered aqueous solution was evaporated to an oil. The oil was combined with DMF (200 mL), and the solution was evaporated to an oil. This was repeated to remove residual water. The resulting oil was dissolved in 400 mL of DMF, and acetone was slowly added to cause precipitation. A total of 4.5 L of acetone was used. By stirring the mixture with a glass rod, a solid was obtained. The solid was collected and dried under high vacuum to give 244 g of PAS-4200.

Synthesis of PAS-5600. Compound **10b** (912 g, 1.6 mol) was suspended in 3 L of CHCl_3 and cooled to 5 °C. A solution of **2** (429 g, 2.0 mol) and 2,6-lutidine (375 mL, 3.2 mol) in CHCl_3 (300 mL) was added in one portion to the suspension. The suspension was stirred at 131 rpm. After 15 min, the ice bath was removed, and succinic anhydride (26 g, 0.25 mol) was added. After 1 h, an additional 5 g of succinic anhydride was added. The solution was stirred for 45 min, and the solvents were removed by rotary evaporation. One-half of the resulting oil was dissolved in 800 mL of DMF. Acetone (5–6 L) was added to the solution to cause precipitation of the product. This was repeated with the other half of the oil. The precipitated solid was dissolved in 1.6 L of CHCl_3 , and acetone (5 L) was added to cause precipitation of the product. The solid was collected and washed with 500 mL of acetone to give 686 g of PAS-5600.

Synthesis of PAS Using 7. *N*-Methylmorpholine (726 μL , 6.6 mmol) was added to a stirred suspension of **1a** (0.961 g, 3 mmol) in CHCl_3 (15 mL). The mixture was stirred at room temperature for 10 min and then cooled to 0 °C. Compound **7** (1.155 g, 6.6 mmol) was added, and the mixture was stirred at 0 °C for 2.5 h. The cooling bath was removed, and the reaction was allowed to warm to room temperature for 1 h. A solution of **2** (0.529 g, 2.4 mmol) in CHCl_3 (2 mL) was added, and the solution was stirred overnight. The mixture was filtered, and to the filtrate was added succinic anhydride (0.140 g, 1.4 mmol) in DMF (2 mL). After 2 h, the solution was evaporated to dryness. The residue was dissolved in DMF (15 mL) and allowed to stand at room temperature for 30 min. The mixture was filtered, and the filtrate was slowly diluted with ethyl acetate (50 mL) to precipitate 1.14 g of PAS (SEC retention time, 37.5 min).

Synthesis of PAS-5600 Using 9. A solution of **2** (0.440 g, 2 mmol) in DMF (3 mL) was added to a stirred of **1a** (0.641 g, 2.4 mmol) and **9** (1.528 g, 4.4 mol) in DMF (10 mL). The solution became turbid. The solution was heated at 70 °C for 3 h and then at 50 °C overnight. The solution was evaporated to dryness. The residue was dissolved in DMF (5 mL), and the solution was slowly diluted with ethyl acetate (70 mL) to precipitate the polymer. The solid was collected, washed with acetone, and dried to give 0.680 g of PAD-5600.

Synthesis of PASS-5600. Polyamide PAS-5600 (1063 g, 0.2 mol) was dissolved in a solution of CHCl_3 (2 L) and DMF (700 mL). *N*-Hydroxysuccinimide (130 g, 1.1 mol) was added as a solid. 1,3-Dicyclohexylcarbodiimide (233 g, 1.12 mol) in DMF (500 mL) was then added. After stirring for 3 h, the mixture was filtered, and the filtrate was reduced in volume to about 2 L by rotary evaporation. The product was precipitated by the addition of acetone (8 L) while stirring. The solid was dissolved in CHCl_3 (3 L), and the product was precipitated by the

addition of acetone (6 L). The solid was collected and dried to provide 926 g of PASS-5600.

Synthesis of PATS-5200. Compound **14b** (193.0 g, 0.43 mol) was suspended in 1.6 L of CHCl_3 . A solution of **2** (99.1 g, 0.45 mol, 1.05 equiv) in 300 mL of CHCl_3 was added in one portion to the suspension. The resulting solution was stirred at room temperature overnight. Succinic anhydride (10.1 g) in 100 mL of DMF was added to the warmed reaction solution. After stirring for 2 h at room temperature, the solvent was removed. DMF (200 mL) was added, and the solvent was evaporated. DMF (350 mL) was added to the residue, followed by 2 L of ethyl acetate. The mixture was allowed to stand for 2 h, and the solid was collected by filtration. The solid was washed with acetone and dried under vacuum to give 73.2 g of PATS-5200.

Synthesis of PATSS-5200. PATS-5200 (100.0 g, 19 mmol) was dissolved in 1.8 L of CHCl_3 with warming. After cooling, triethylamine (22 mL, 155 mmol) was added followed by 300 mL of DMF. *N,N*-Disuccinimidyl carbonate (39.8 g, 155 mmol) was added as a solid. After stirring for 2 h, the solution was filtered, and the filtrate was reduced to a semisolid. The solid was dissolved in 500 mL of DMF with warming. Ethyl acetate was added in 500 mL portions with mixing. A total of 1.5 L of ethyl acetate was used. The resulting mixture was allowed to stand at room temperature for 1 h, and then the solid was collected, washed with ethyl acetate, and dried to give 79.3 g of PATS-5200.

Synthesis of PATS Using 7. A solution of 3,3'-thiodipropionic acid (0.535 g, 3 mmol) and *N*-methylmorpholine (0.726 mL, 6.6 mmol) in CHCl_3 (10 mL) was cooled to 0 °C in an ice bath. To this solution was added **7** (1.115 g, 6.6 mmol). The mixture was stirred at 0 °C. After 2 h, the temperature was allowed to warm to room temperature over 1 h. Compound **2** (0.595 g, 2.7 mmol) in CHCl_3 (2 mL) was added, and the reaction mixture was stirred at room temperature. The reaction mixture was filtered to remove the precipitate. The filtrate was treated with succinic anhydride (0.140 g) in DMF (2 mL). The solution was stirred at room temperature for 30 min and evaporated to dryness. The residue was evaporated with DMF to remove residual chloroform. The residue was dissolved in DMF (15 mL) and left at room temperature for 30 min. The mixture was filtered, and the filtrate was slowly diluted with ethyl acetate (50 mL) to precipitate PATS. The product was collected, washed with acetone, and dried to give 0.850 g of PATS having an SEC retention time of 37.9 min.

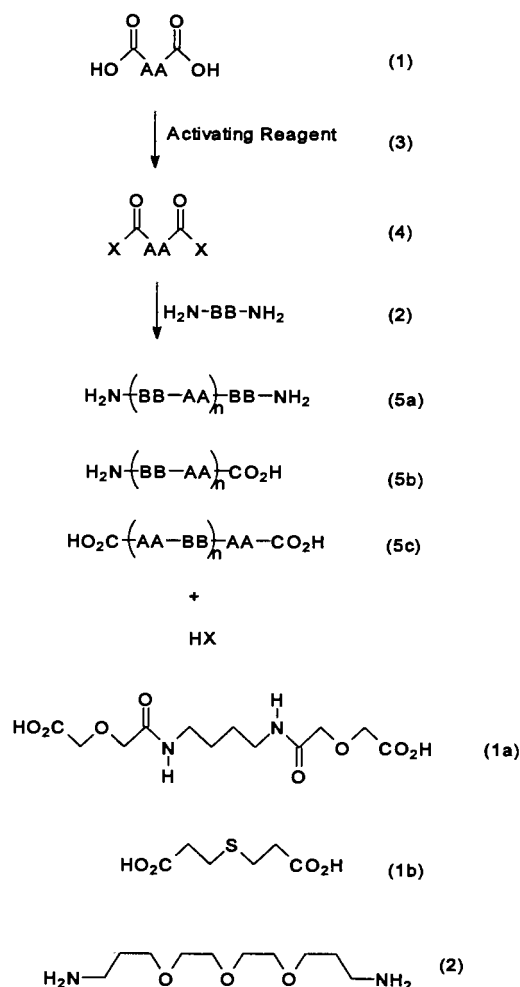
RESULTS AND DISCUSSION

General Synthetic Strategies. Incorporating oxygen atoms into the polymer backbone was deemed essential to achieve water solubility and practical non-toxicity and nonimmunogenicity in the polyamide polymers. This was accomplished by choosing ether-containing diacids and diamines as the monomers for polymerization.

Similarly, it was hypothesized that incorporation of sulfur atoms into the polymer backbone would enhance the antioxidant activity of the polyamides. Sulfur was conveniently incorporated as a thioether moiety in a diacid monomer.

It was also recognized that the number of heteroatoms in the backbone influences the water solubility and the melting point of the resulting polymers. For instance, a large number of oxygen atoms in the backbone would produce a very water-soluble but waxy polyamide that

Scheme 1



would be very difficult to purify. Conversely, the presence of few heteroatoms would give crystalline polyamides that would be much less soluble in water but easier to purify by recrystallization.

To achieve a balance between water solubility and crystallinity, both the effective length and heteroatom content of both the diacid and the diamine were critical. Altering the properties of the diacid was the strategy most frequently used in this work to achieve this balance. For example, the short diacid diglycolic acid yielded relatively low molecular mass, waxy polymers. Therefore, instead of using diglycolic acid as the diacid monomer, it was converted to the longer diamide bis(carboxymethoxyacetyl)-1,4-diaminobutane (**1a**) through the reaction of 1,4-butanediamine with diglycolic anhydride (see Scheme 1). Similarly, to obtain sulfur-containing polyamides, the sulfur was incorporated into the polymer backbone by using 3,3'-thiodipropionic acid (**1b**) as the diacid monomer. To further increase the oxygen content of the polymer backbone, the diamine diethylene glycol bis(3-aminopropyl) ether (**2**) was chosen as the diamine monomer for the polycondensation reactions.

Polycondensations of diacids and diamines can be performed at moderate temperatures if the diacids are activated (4). The general synthetic route that we chose to prepare the desired polyamides is summarized in Scheme 1. The first step in the polymerization is the conversion of a diacid **1** to an activated diacid **4** by employing an activating reagent **3**. Next, the activated diacid **4** is condensed with diamine **2** to give polyamides comprising bis(amine) end groups **5a**, monocarboxy

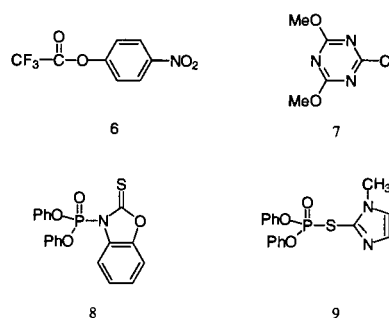


Figure 1. Carboxylic acid activating reagents.

monoamino polyamides **5b**, and polyamides with bis-(carboxy) end groups **5c**, together with the byproduct of polycondensation, HX (see Scheme 1).

The criteria that were used for selecting the diacid activating reagents were as follows: (a) the conversion of the diacid to the activated diacid **4** must be quantitative if the activated acid could not be isolated; (b) the byproduct HX must be eliminated from the product either by evaporation or through a simple recrystallization of the product mixture from an organic solvent system. The ease of removal of the byproduct HX was crucial for the purification of the polyamides.

Both traditional and novel reagents were studied for the successful activation of the diacids for polycondensation. The traditional activating agents that were studied included methanol (5), *p*-nitrophenyl trifluoroacetate (**6**), 2-chloro-4,6-dimethoxy-1,3,5-triazine (**7**), and diphenyl(2,3-dihydro-2-thioxo-3-benzoxazolyl)-phosphonate (**8**). We also studied the utility of a novel activating agent, diphenyl(1-methylimidazol-2-ylthio)-phosphonate (**9**) (see Figure 1).

In general, polycondensation conditions were varied in an attempt to optimize the yield of a single type of polyamide (**5a** or **5c**, preferentially, or **5b**) having a narrow range of molecular masses centered on a desirable value. If bis(carboxy-terminated) polyamides **5c** were obtained, these could be converted to any of a broad spectrum of activated esters. If bis(amine-terminated) polyamides **5a** were obtained, these could be activated directly to aldehyde, urethane, or maleimide derivatives or allowed to react with succinic anhydride to convert them to bis(carboxy-terminated) polyamides.

Analytical Methods. The polyamide condensation products were characterized by three methods. Size-exclusion chromatographic (SEC) analyses were performed using a Superose-12 column and 50 mM phosphate buffer, pH 6.5, as the mobile phase. SEC confirmed that polymerized products were formed and permitted the approximation of the molecular mass range of the polyamide product.

Thin-layer chromatographic (TLC) analysis was used to separate the polyamides and characterize the end-group functionality. The end-group functionality of each component was assigned on the basis of its relative migration (R_f) and reactivity toward ninhydrin spray reagent. Under the TLC conditions, polyamides with dicarboxy end groups have the largest R_f value. The polyamides with a monocarboxy and a monoamine end group have an intermediate R_f value, and the polyamides with diamine end groups have the smallest R_f value.

Finally, the molecular mass of the polyamide was determined by ^1H -NMR and ^{13}C -NMR analysis. Theoretical molecular masses were calculated by the comparison of the end group resonances to the polymer backbone resonances.

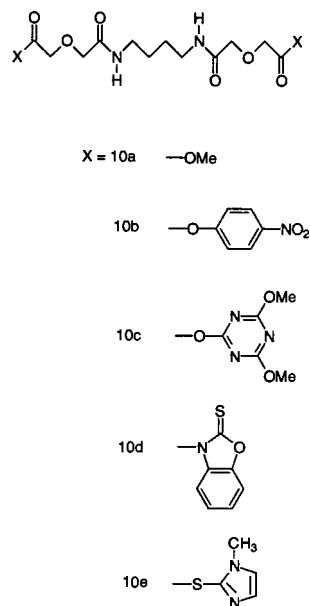


Figure 2. Activated esters of 1a.

Polyamides with Oxygen-Containing backbones.

The activated diester bis(methoxycarbonylmethoxyacetyl)-1,4-diaminobutane (**10a**) was selected initially as the diacid component for the synthesis of polyamides by a method described by Ogata et al. (5a) and Sato et al. (5b) (See Figure 2). Methanol, the byproduct of polycondensation, could easily be removed. However, we found that polycondensation with **10a** yielded a polyamide having a maximum molecular mass of about 4200 Da (by SEC retention volume). The conditions required for polycondensation were too strenuous and did not permit the production of polyamides having a range of molecular masses. Thus, methanol was not a sufficiently strong activating group to be the activating reagent of choice.

Treatment of diacid **1a** with *p*-nitrophenyl trifluoroacetate (**6**) quantitatively converted the diacid to the activated diester, **10b**. Polycondensation of PNP-activated diesters with diamines yields *p*-nitrophenol as the byproduct. This phenol is fairly soluble in organic solvents, and we anticipated that the resulting polyamides could be purified by simple recrystallization. This expectation was realized.

Compound **10b** condensed smoothly with the diamine **2** to give polyamides having molecular masses greater than 4000 Da, as indicated by SEC analysis. Under the reaction conditions employed in these studies, condensation of **2** and **10b** gave mixtures of three types of polyamide product: a polyamide bis(amine) **5a**; an α -carboxy- ω -aminopolyamide **5b**; and a polyamide bis(carboxylic acid) **5c**. The distribution and molecular masses of these polymers were found to be greatly affected by (a) the mixing rate; (b) the reaction temperature; (c) the base employed; (d) the molar ratio of **2** to **10b** employed; and (e) the solvent employed for the condensation. These parameters were studied, and the experimental results are summarized in Table 2.

The data indicate that under the same set of polycondensation conditions, chloroform was a superior solvent to *N,N*-dimethylformamide (DMF). Condensations that were performed in chloroform with various molar ratios of **10b** to **2** yielded polyamides having four different molecular masses ranging from 2.8 to 7.6 kDa and containing only the polyamide bis(amine) **5a**. In contrast,

polycondensation in DMF provided polyamides having only two different molecular masses (5.6 and 7.6 kDa). In addition, the latter polyamides were mixtures of **5a** and **5b**, and their SEC profiles were broad. With respect to the other parameters, we found the most useful polymerization conditions were a mixing rate of 130–135 rpm, 2,6-lutidine as the base, chloroform as the solvent, and -50°C as the reaction temperature.

Although *p*-nitrophenyl trifluoroacetate (**6**) was a very useful activating group, the synthesis of *p*-nitrophenyl trifluoroacetate is tedious and uses a large amount of trifluoroacetic anhydride. Furthermore, *p*-nitrophenol, the byproduct of the polycondensation, has a poor safety profile. For these reasons, we wanted to assess the utility of other activating groups.

Peptide synthesis using the coupling reagent 2-chloro-4,6-dimethoxy-1,3,5-triazine (**7**) was first proposed by Kaminski (**7**) in 1987. We reasoned that **7** might be a useful activating reagent for the polycondensation reaction, since the byproduct of the polycondensation reaction would be 2-hydroxy-4,6-dimethoxy-1,3,5-triazine, a reagent that is quite soluble in DMF and other organic solvents. This suggested that the desired polyamide could be purified easily by recrystallization of the product mixture from organic solvents.

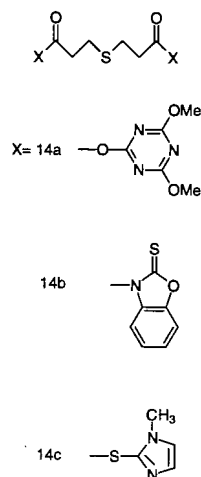
The activation of **1a** was achieved by adding **7** to a mixture of the diacid **1a** and *N*-methylmorpholine (NMM) in chloroform at 0°C . However, the activated acid **10c** could not be isolated. Alternatively, polycondensation of **1a** and **2** could be achieved by the in situ activation of the diacid **1a** with **7**. Thus, the diamine **2** was added to a solution of the diacid **1a**, **7**, and NMM at 25°C , and the reaction was allowed to proceed overnight to produce a polyamide bis(amine) (PAD derivative).

We found that the choice of solvents and bases was critical for the polycondensation of **1a** and **2** with **7** to be successful. The combination of chloroform and NMM was found to be the best solvent and base pair for the polycondensation of **1a** with **2**. Employing chloroform as the solvent and triethylamine as the base did not produce a polyamide. The substitution of DMF for chloroform as the solvent and NMM as the base gave polyamides with much lower molecular masses.

The effect of the molar ratio of **2:1a** was also studied and was found to have little effect on the molecular mass and yield of the polyamide. For example, activation of **1a** with **7** followed by polycondensation with **2** under conditions where the molar ratio of **2:1a** was in the range 0.8–1.0 gave a high yield (80%) of polyamides having a molecular mass of approximately 2800 Da (estimated by SEC retention volume).

There were three major drawbacks to the use of **7** as the activation reagent. (1) The selection of solvents and bases for the activation step apparently is limited. In our hands, only chloroform and NMM may be used for this purpose. (2) Isolation and purification of the dimethoxytriazine-activated diester (**10c**) prior to addition of the diamine was prohibitively difficult. (3) The observation that polyamides cannot be prepared in a wide range of molecular masses argued against the use of this activating agent.

Diphenyl(2,3-dihydro-2-thioxo-3-benzoxazolyl)phosphonate (**8**) (**8**) was selected as an activating agent for evaluation for the following reasons. (1) This reagent is expected to have a better safety profile than *p*-nitrophenyl trifluoroacetate or PNP-esters. (2) The reagent is easily synthesized at large scales. (3) The leaving group, 2-mercaptobenzoxazole, is very soluble in organic

**Figure 3.** Activated esters of **1b**.

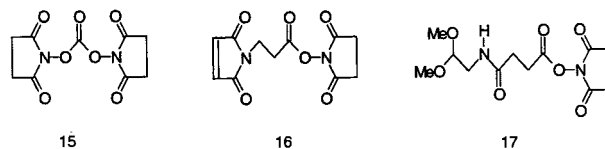
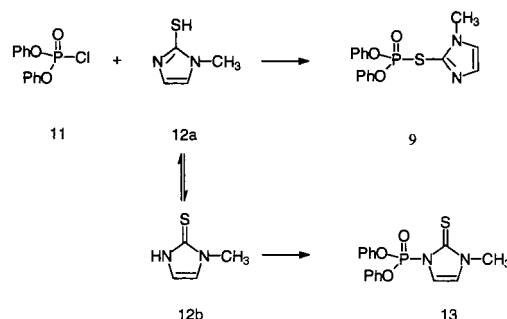
solvents; thus, polyamide products could be purified by simple recrystallization.

Treatment of **1a** with **8** provided the activated acid **10d** as an isolatable solid. However, polycondensation of **10d** with **2** met with only moderate success. The best results were obtained in DMF as the solvent and without the addition of a base. A polyamide of the PAS type with a molecular mass of about 4 kDa was obtained. Changes in the ratio of **10d**:**2** or the addition of a base did not change the outcome of the reaction. The use of chloroform as the solvent provided only very low molecular mass polymers under all reaction conditions tested.

Since the synthesis of PAS-type polyamides was not efficient with **10d**, a new activating reagent was designed. We used the strategy of substituting the benzoxazolin-2-thione group in **8** with 2-mercapto-1-methylimidazole (methimazole) (**9**). Preparation of the new activating reagent diphenyl(1-methylimidazol-2-thiyl)-phosphonate (**9**) is summarized in Scheme 2. The synthesis is based on the literature preparation of **8** (**8**). 2-Mercapto-1-methylimidazole (**12**) was treated with diphenyl chlorophosphate (**11**) in the presence of triethylamine. In theory, the product could be either the S-phosphoryl or the N-phosphoryl derivative (**9** or **13**, respectively), due to the tautomerization of 2-mercapto-1-methylimidazole. Both the ^{13}C -NMR and IR spectra of the isolated product established that it was the S-phosphoryl product **9**. The ^{13}C -NMR spectrum had a doublet, due to coupling with phosphorus, at 166.5 ppm that is representative of a carbon of the C=N bond type. Absent was a resonance in the region 180–200 ppm that would correspond to a carbon of the C=S bond type. The IR spectrum had an absorption at 1298 cm^{-1} of the P=O bond and no strong absorption in the 1340 cm^{-1} region indicative of a C=S bond (**10**).

Attempts to activate **1a** by heating at reflux with **9** in the presence of triethylamine in chloroform failed to give the desired activated diester **10e**. However, when **9** was added to a solution of **2**, **1a**, and triethylamine in DMF at 60–70 °C, a good yield (65%) of PAS that has an SEC profile similar to that of PAS-5600, was obtained. In contrast, one-step polycondensation of **2** and **1a** in the presence of **9** using triethylamine in chloroform at reflux gave only low molecular mass PAS.

Thio-Containing Polyamides. Sulfur-containing polyamides were obtained by the activation of 3,3'-thiodipropionic acid (**1b**) and subsequent treatment with **2** (see Figure 3). Compound **7** was the first activating reagent that was used in the preparation of thio-contain-

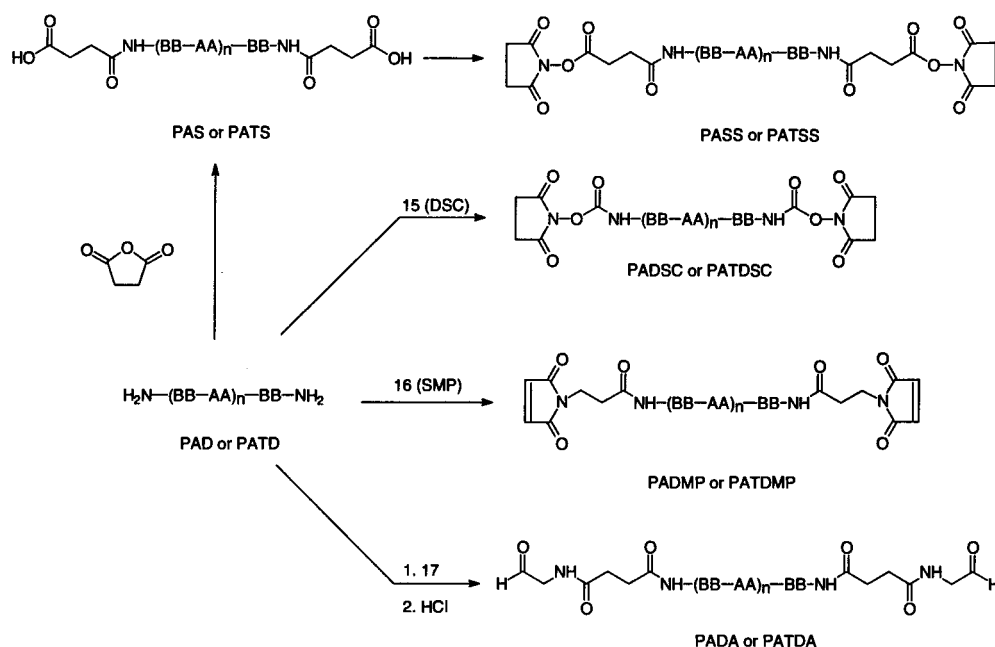
**Figure 4.** Polyamide activating reagents.**Scheme 2**

ing polyamides. As was seen with **1a**, the activated acid **14a** of **1b**, could not be isolated. Therefore, **2** was added to a solution of **7**, **1b**, and *N*-methylmorpholine at 25 °C. The reaction was allowed to proceed overnight to provide an amino-terminated, thio-containing polyamide (PATD). Variation of **2**:**1b** molar ratios from 0.8 to 1.1 gave only PATS having a molecular mass of 4000–5000 Da in good yields (73–88%). The PATS obtained using **7** has a narrow molecular mass distribution as indicated by SEC and contains only 3.5% of a lower molecular mass byproduct. The solvent of choice for the polymerization was chloroform, and the most suitable base was *N*-methylmorpholine.

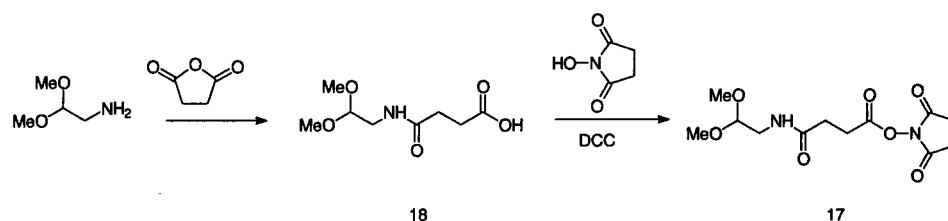
Greater success was obtained with activation of 3,3'-thiodipropionic acid with **8**. Activation of **1b** with **8** was accomplished in chloroform in the presence of triethylamine to give a 76% yield of *N,N'*-(3,3'-thiodipropionyl) bis(benzoxazolin-2-thione) (**14b**). Polycondensation of **14b** and **2** to obtain polyamides having the desired molecular masses and end groups was found to depend on five experimental parameters: (a) the molar ratio of **14b** and **2**; (b) the solvent (CHCl_3 or DMF); (c) the temperature, (d) the base (triethylamine or 2,6-lutidine); and (e) the mixing rate. For all the following experiments, the mixing rate was maintained at a moderate rate to simplify the studies.

The data indicate the following. (a) Polycondensation in chloroform in the presence of triethylamine did not give reproducible results (Table 3). Employing the same molar ratio of **2** and **14b** did not yield polyamides of similar molecular mass. Usually, high molecular mass polyamides having a retention time of 31–32 min by SEC were obtained. This suggests that triethylamine was too strong of a base. (b) Polycondensation in chloroform in the absence of base or in the presence of a weak base such as 2,6-lutidine produced polyamides in a predictable manner (Table 4). For instance, a decrease in the **2**:**14b** molar ratio gave polyamides having higher molecular masses. Thus, using molar ratio of **2**:**14b** of 1.2:1 at 0 °C or 1.3:1 at 25 °C in the absence of base during the polycondensation furnished a polyamide having a molecular mass of about 2800 Da (a retention time of 38.7 min by SEC). (c) Polycondensation in DMF in the presence or in the absence of a weak base such as 2,6-lutidine gave polyamides of similar molecular mass. Polycondensation at 0 °C gave a polyamide with a very broad molecular mass distribution, as indicated by multiple peaks in the SEC profile of the product. Em-

Scheme 3



Scheme 4



employing **2** and **14b** in a molar ratio of 1.3:1 at 25 °C in the absence of base gave two distinct polyamides: one having molecular mass of 5700 Da that is insoluble in DMF and a second having molecular mass of 2800 Da that is soluble in DMF. It was found that simple filtration could be used to separate these two polyamides. (d) Polycondensation of **2** and **14b** in chloroform using a molar ratio of 1.2:1 at 0 °C or 1.3:1 at 25 °C yielded exclusively polyamide bis(amine) having a molecular mass of 2800 Da, whereas employing a molar ratio of 1.3:1 in DMF at 25 °C gave mostly polyamide monocarboxy monoamine having a molecular mass of 5700 Da.

Synthesis of PAT polyamides was also achieved using **9**. Synthesis of PAT derivatives using **9** was carried out in two steps. In the first step, activation of **1b** was achieved by reaction of **1b** with **9** in chloroform containing triethylamine. The reaction was sluggish at 25 °C; however, heating the reaction under reflux gave the activated acid **14c** in good yield (70%). On the basis of its ¹³C-NMR and IR spectra, the activated diester **14c** was assigned the structure of an S-acyl derivative. In the second step, polycondensation was achieved by simply mixing **2** and **14c** in a suitable solvent to give a PATD.

The effects of variation in solvent, reaction temperature and **2**:**14c** molar ratio on the polycondensation are summarized in Table 5. In general, polycondensations in DMF gave a higher yield (50–76%) and cleaner PAT than those in chloroform (30–46%). In addition, increasing the **2**:**14c** molar ratio decreased the yield and the molecular mass of PAT. Finally, polymerization in DMF at 0 and 25 °C gave PAT having a broad molecular mass distribution by SEC analysis, but at –50 °C, a narrow molecular mass distribution PAT was obtained. PAT

having a molecular mass range 2800–5000 Da may be obtained in 57–76% yield by polycondensation of **2** and **14c** at –50 °C in DMF.

Activation of the Polyamide End Groups. The polyamides obtained by the synthetic methods described above contain either bis(amine) **5a**, α-carboxy-ω-amino **5b**, or bis(carboxylic acid) **5c** end groups. Each of these polyamides could be activated for reaction with proteins using various strategies (Scheme 3). For example, both a bis(amine) polyamide **5a** and a α-carboxy-ω-amino polyamide **5b** were converted to bis(carboxylic acid) end-capped polyamides (PAS or PATS derivatives) by treatment with succinic anhydride. The resulting PAS and PATS polyamide dicarboxylic acids were efficiently converted to the corresponding activated bis(N-oxy succinimide) diesters (PASS and PATSS derivatives, respectively) using *N,N*-disuccinimidyl carbonate (**15**) (11).

Similarly, the PAD or PATD bis(amines) **5a** were activated in three ways. First, the amine end groups were converted to maleimide end groups (PADMP and PATDMP derivatives, respectively) by treatment of the bis(amines) **5a** with *N*-oxy succinimidyl 2-maleimidopropionate (**16**) (12). Alternatively, the PAD or PATD bis(amines) **5a** were activated with **15** to yield the corresponding urethane-terminated polyamides PADSC and PATDSC that contain succinimidyl oxycarbonyl end groups. Finally, the bis(amines) were activated with *N*-(2,2-diethyloxyethyl)succinamic acid *N*-oxy succinimide ester (**17**) to furnish the polyamide bis(aldehydes) PADA and PADTA, respectively. The synthesis of **17** is described in Scheme 4.

Conclusions. Water-soluble polyamides having well-defined molecular masses and end groups have been

synthesized. Acute toxicity screening of these polyamides in rodents suggests that these materials are neither toxic nor immunogenic (13). Both the carboxyl and amino end groups of the various polyamides can be converted to several types of activated end groups. This provides a spectrum of activated polyamides that can be useful in the cross-linking or modification of proteins such as hemoglobin. The polyamides described herein have been used for the synthesis of polymerized DCLHb (14) (DCLHb, α,α -cross-linked hemoglobin) (15); this reactivity will be the subject of a subsequent paper.

ACKNOWLEDGMENT

We thank Dr. Walter Smith for his help with the MW determination by NMR of the polyamides and Ms. Ana Srnak for performing the SEC analyses.

Supporting Information Available: Tables S1–S7 provide the ^1H - and ^{13}C -NMR data for the polyamides (9 pages). Ordering information is given on any current masthead page.

LITERATURE CITED

- (1) Ji, T. H. (1983) Bifunctional Reagents. *Methods Enzymol.* 91, 580–609.
- (2) Means, G. E., and Feeney, R. E. (1990) Chemical modifications of proteins: history and applications. *Bioconjugate Chem.* 1, 2–12.
- (3) Wong, S. (1991) "Chemistry of Protein Conjugation and Cross-linking", CRC Press, Inc.
- (4) Ogata, N., Sanui, K., and Okouchi, K. (1973) Active polycondensation of dicarboxylic acid derivatives having β -heteroatoms. *Polym. J.* 5, 186–194.
- (5) (a) Ogata, N., and Hastate, Y. (1978) Synthesis of water-soluble polyamide by polycondensation in solution. *J. Polym. Sci., Polym. Chem. Ed.* 16, 1159–1162. (b) Sat, H., Abbacy, S., Boomer, V., and Kern, W. (1981) Preparation of regularly sequenced polyamides containing definite numbers of oxyethylene units. *Macromol. Chem.* 182, 755–762.
- (6) Sakibara, S., and N. Inukai, N. (1965) The trifluoroacetate method of peptide synthesis. I. The synthesis and use of trifluoroacetate reagents. *Bull. Chem. Soc. Jpn.* 38, 1979–1984.
- (7) Kaminsky, Z. J. (1987) 2-Chloro-4,6-dimethoxy-1,3,5-triazine. A new coupling reagent for peptide synthesis. *Synthesis* 917–920.
- (8) Ueda, M., Kameyama, A., and Hashimoto, K. (1988) Diphenyl (2,3-dihydro-2-thioxo-3-benzoxazolyl)phosphate: a new, reactive activating agent for the synthesis of amides and polyamides. *Macromolecules* 21, 19–24.
- (9) Cooper, D. S. (1984) Antithyroid Drugs. *New Engl. J. Med.* 311, 1353–1362.
- (10) Ueda, M., Seki, K., and Iami, Y. (1981) S- and N-acyl derivatives of 2-mercaptobenzoxazole: new, highly reactive acylating agents for synthesis of amides and esters. *Synthesis* 991–993.
- (11) Ogura, H., Kobayashi, T., Shimzu, K., Kawabe, K., and Takeda, K. (1979) A novel active ester synthesis reagent (*N,N'*-disuccinimidyl carbonate). *Tetrahedron Lett.* 4745–4746.
- (12) Buchardt, O. and Nielsen, O. (1991) Facile synthesis of reagents containing a terminal maleimide ligand linked to an active ester. *Synthesis* 819–821.
- (13) Unpublished results.
- (14) Hai, T. T., and Nelson, D. J. (1994) Water soluble nonimmunogenic polyamide cross-linking agents. World patent, WO 94/12220.
- (15) (a) Hai, T. T., Nelson, D. J., and Srnak, A. (1992) Process for the production of cross-linked hemoglobin in the presence of sodium tripolyphosphate. U.S. patent, 5,128,452. (b) Chatterjee, R., Welty, E. V., Walder, R. Y., Pruitt, S. L., Rogers, P. H., Arnone, A., and Walder, J. A. (1986) Isolation and characterization of a new hemoglobin derivative cross-linked between the α chains (Lysine 99 $_{\alpha 1}$ -Lysine 99 $_{\alpha 2}$). *J. Biol. Chem.* 261, 9929–9937.

BC970199D

Synthesis of Water Soluble, Non-immunogenic Polyamide Cross-linking Agents

Ton That Hai, D. Pereira, and D. Nelson

Hemoglobin Therapeutics Division, Baxter Healthcare Corp., Round Lake, IL 60073 USA

Supporting Information.

Table S-1. NMR Data for Polyamide Precursors

Polyamide	¹ H-NMR Chemical Shifts	¹³ C-NMR Chemical Shifts
1a	(In DMSO-d ₆) 1.40 (br. s, 2H), 2.61 (br. s, 2H), 3.95 (s, 2H), 4.13 (s, 2H), 7.89 (s, 1H), 12.81 (s, 1H) ppm.	(In DMSO-d ₆) 26.7, 38.1, 68.0, 70.8; 168.8, 172.1 ppm.
10a	(In CDCl ₃) 1.52 (s, 2H), 3.25 (m, 2H), 3.70 (s, 3H), 3.99 (s, 2H), 4.08 (s, 2H), 6.98 (s, 1H) ppm.	(In CDCl ₃) 26.6, 38.3, 51.9, 68.4, 71.2; 168.8, 170.2 ppm.
10b	(In DMSO-d ₆) 1.38 (br.s, 2H), 3.10 (br s, 2H), 4.18 (s, 2H), 4.52 (s, 2H), 7.48 (d, 2H, J = 9.0 Hz), 7.85 (t, 1H, J = 5.6 Hz), 8.30 (d, 2H, J = 9.0 Hz) ppm.	(In DMSO-d ₆) 26.6, 37.9, 67.8, 70.1, 123.1, 125.4, 126.2, 145.2, 152.0, 154.9, 168.4 ppm.
10d	(In DMSO-d ₆) 1.39 (br.s, 2H), 3.10 (br s, 2H), 3.94 (s, 2H), 4.09 (s, 2H), 7.21 (m, 3H), 7.48 (m, 1H), 7.85 (t, 1H, J = 5.7 Hz) ppm.	(In DMSO-d ₆): 26.6, 37.9, 67.9, 70.2, 110.1, 123.9, 125.2, 148.4, 168.7, 171.5 ppm.
14b	(In CDCl ₃): 3.07 (t, 2H, J = 6.8 Hz), 3.89 (t, 2H, J = 6.8 Hz), 7.32 (m, 3H), 8.08 (m, 1H) ppm.	(In CDCl ₃): 22.7, 39.9, 109.6, 116.4, 125.5, 126.2, 129.6, 146.4, 172.2, 178.5 ppm.
9	(In CDCl ₃) 3.55 (s, 3H), 6.61 (m, 1H), 7.08 (t, 1H, J = 2.5 Hz), 7.15-7.50 (m, 9H) ppm.	(In CDCl ₃): 34.7, 117.7 (d,), 119.8 (d,), 120.2 (d,), 120.3., 129.5, 149.2 (d,), 166.4 (d,) ppm. ³¹ P-NMR (CDCl ₃): -15.2 ppm.
14c	(In CDCl ₃): 3.00 (t, 2H, J = 6.9 Hz), 3.57 (s, 3H), 3.92 (t, 2H, J = 6.9 Hz), 6.68 (d, 1H, J = 2.8 Hz), 7.45 (d, 1H, J = 2.8) ppm.	(In CDCl ₃): 26.4, 35.3, 38.9, 113.4, 119.4, 164.7, 171.0 ppm.
18	(In CDCl ₃) 2.50 (m, 2H), 2.69 (m, 2H), 3.37 (s, 6H), 3.40 (t, 2H, J = 5.4 Hz); 4.38 (t, 1H, J = 5.4); 6.21 (s, 1H) ppm.	(In CDCl ₃) 28.3, 29.5, 41.0, 54.4, 102.4, 172.3, 176.7 ppm.
17	(In CDCl ₃) 2.52 (t, 2H, J = 7.0 Hz), 2.73 (s, 4H), 2.87 (t, 2H, J = 7.0 Hz); 3.28 (s, 6H), 3.30 (m, 2H); 4.38 (t, 1H, J = 5.0 Hz); 6.21 (s, 1H) ppm.	(In CDCl ₃) 28.3, 29.5, 41.0, 54.4, 102.4, 172.3, 176.7 ppm.

Table S-2. ¹H-NMR Assignments for PAS Polymers

δ (ppm) (CDCl ₃)				
¹ H	PAS-7600	PAS-5600	PAS-4000	PAS-2800
CONH	7.57, 7.14	7.57, 7.14 (t, J=5 Hz)	7.59, 7.14	7.61, 7.15 (t, J=5 Hz)
e, f	4.04	4.04, 4.02	4.04	4.04, 4.03
c, d	3.61- 3.54	3.62 - 3.52	3.59	3.62- 3.52
a, p	3.36- 3.29	3.38-3.28	3.35- 3.29	3.36-3.28
s/t	2.62 (poorly defined t), 2.49, poorly defined t)	2.62 (t, J=6.5 Hz), 2.49 (t, J=6.5 Hz)	2.49	2.62 (t, J=6.5 Hz), 2.49 (t, J=6.5 Hz)
b	1.81 (m)	1.78 (m)	1.78 (m)	1.78 (m)
q	1.55	1.55	1.55	1.55

Table S-3. ^{13}C -NMR Assignments for PAS Polymers

δ (ppm) (CDCl_3)				
^{13}C	PAS-7600	PAS-5600	PAS-4000	PAS-2800
-CO-	174.3, 172.0, 168.9, 168.6	174.4, 172.1, 168.9, 168.7	174.4, 172.1, 169.0, 168.7	174.3, 172.1, 169.0, 168.8
e, f	70.8	70.8	70.8	70.7
c, d	70.0, 69.8, 69.3	70.0, 69.8, 69.3	70.0, 69.8, 69.7, 69.3, 69.2	70.1, 70.0, 69.8, 69.3, 69.1
a, p	38.4, 37.8, 37.0	38.4, 37.8, 37.0	38.5, 37.8, 37.0	38.5, 37.8, 37.0, 36.9
s/t	29.5	30.7	37.0	30.7
b	26.6	26.6	26.6	26.6
s/t	28.6	28.6	28.6	29.0
q	29.0	29.0	29.0	29.0

Table S-4. ¹H -NMR Assignments for PATSS Polymers

δ (ppm) (CDCl ₃)			
¹ H	PATSS-5700	PATSS-5200	PATSS-2800
CONH	6.89	6.89	6.96, 6.85, 6.79
c, d	3.61-3.48	3.58-3.47	3.57,-3.47
a	3.27 (q, J=6.3 Hz)	3.26 (q, J=6.2 Hz)	3.28 (q, J=5.8 Hz)
s/t	2.93 (t, J=7.2 Hz)	2.91 (t, J=7.2 Hz)	2.91 (t, J=7.2 Hz)
r	2.80	2.78	2.78
g	2.75 (t, J=7.2 Hz)	2.74 (t, J=7.2 Hz)	2.74 (t, J=6.7 Hz)
s/t	2.5 4(t, J=7.2 Hz)	2.52 (t, J=7.2 Hz)	2.52 (t, J=7.2 Hz)
h	2.41 (t, J=7.2 Hz)	2.39(t, J=7.2 Hz)	2.39(t, J=7.2 Hz)
b	1.73 (q, J=6.3 Hz)	1.71 (q, J=6.2 Hz)	1.71 (q, J=5.8 Hz)

Table S-5. ^{13}C -NMR Assignments for PATSS Polymers

^{13}C	δ (ppm) (CDCl_3)		
	PATSS-5700	PATSS-5200	PATSS-2800
-CO-	172.3, 171.3, 169.8, 169.1, 168.3	171.2, 169.8, 169.0, 168.2	171.3, 171.1, 169.8, 168.2
c, d	70.4, 70.0, 69.6	70.3, 69.9, 69.8, 69.7, 69.5	70.3, 70.1, 69.9, 69.8, 69.7, 69.7, 69.6, 69.5
a	38.0, 37.6	37.9, 37.6, 37.5	37.6, 37.5
g	36.5	36.4	36.5
s/t	30.4	30.3	30.4
b	30.0, 28.8	28.9, 28.8	28.9, 28.8, 28.0
h	27.7	27.7, 27.6	27.7
s/t	26.7	26.7	26.7
r	25.5	25.5	25.5

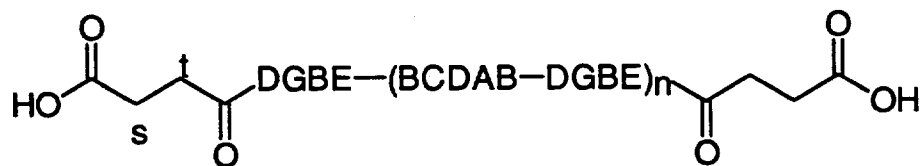
Table S-6. ^1H -NMR Assignments for PADSC and PADA Polymers

δ (ppm) (CDCl_3)			
^1H	PADCS-3000	PADA-2800 Bis(Acetal)	PADA-2800
CHO	NA	NA	9.56
CONH	6.90, 7.33, 7.50	6.70, 6.90, 7.51	7.58
$(\text{MeO})_2\text{CH}$	NA	4.30 (t, $J = 4.8$ Hz)	NA
e, f	4.03	3.96	4.02
c, d	3.52- 3.58	3.45- 3.54	3.54- 3.66 (m)
a, p, u	3.27- 3.35 (m)	3.23- 3.29 (m)	3.27-3.35 (m)
r	2.80	NA	NA
s/t	NA	2.44	2.50
b	1.74, 1.76, 1.78	1.71 (m)	1.76 (m)
q	1.53	1.48	1.53
MeO-	NA	3.29	NA

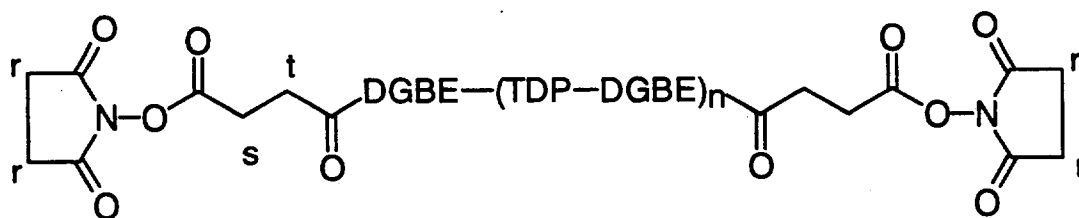
Table S-7. ^{13}C -NMR Assignments for PADSC and PADA Polymers

δ (ppm) (CDCl_3)			
^{13}C	PADSC-3000	PADAS-2800	PADA-2800
CHO	NA	NA	195.5
-CO-	172.5, 172.1, 170.2, 169.1, 168.8, 168.7	172.5, 172.1, 170.2, 169.0, 168.8, 168.7	172.2, 169.2, 168.9
CO (urethane)	151.6	NA	NA
v	NA	102.3	100.5
c, d, e, f	70.9, 70.2, 70.1, 70.0, 69.9, 69.7, 69.4, 69.3	70.9, 70.2, 70.1, 69.9, 69.7, 69.4, 69.3	70.9, 70.3, 70.2, 69.9, 69.5
u	NA	40.8	50.1
a, p	38.5, 37.3, 37.2	38.5, 37.7, 37.1, 37.0	38.6, 37.9, 37.2
q	29.1, 29.0	29.0	29.1
s/t	NA	31.4	31.5
b	26.7	26.6	26.7
s/t	NA	28.8	26.7
r	25.5	NA	NA

Figure 1-S



PAS



PATSS

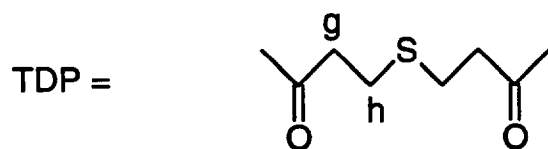
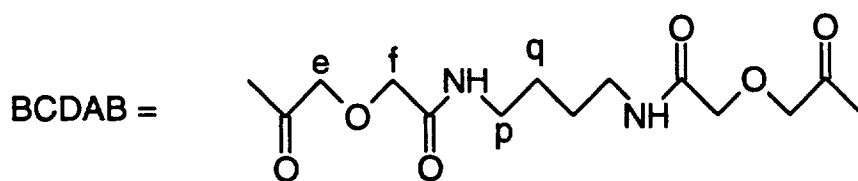
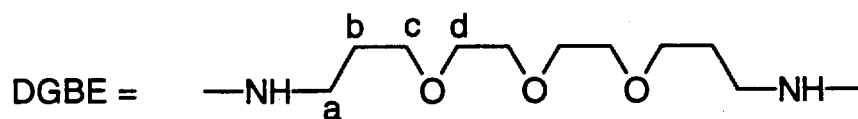
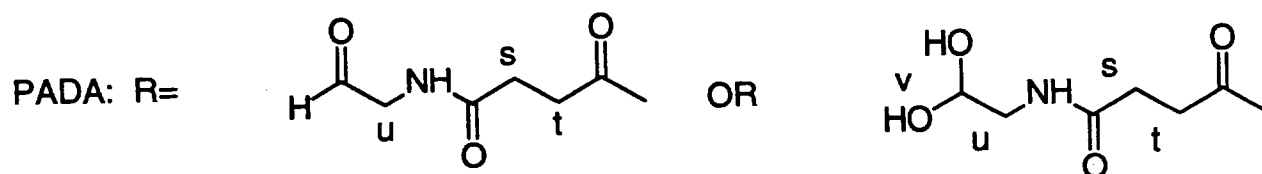
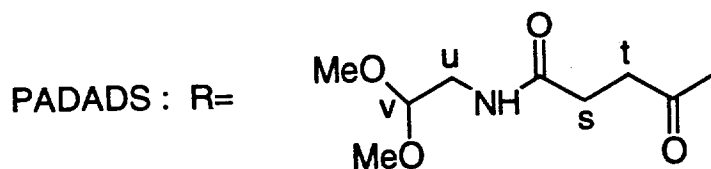
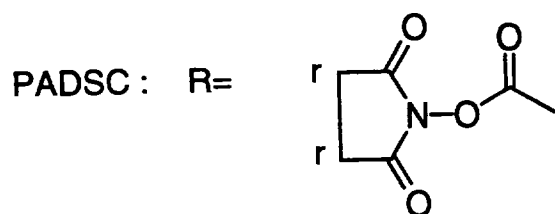
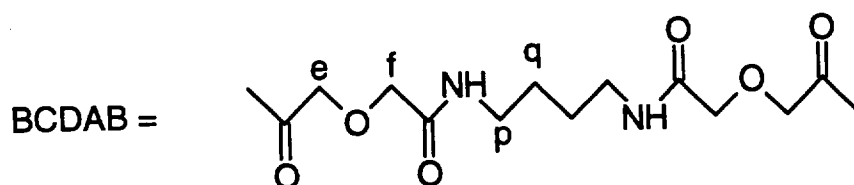
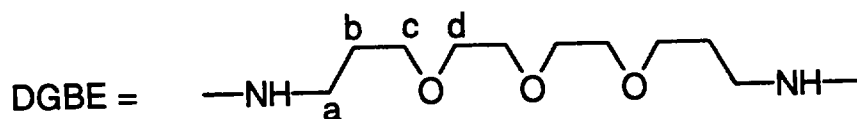


Figure 2-S



Covalent Immobilization of Proteins onto (Maleic Anhydride-*alt*-methyl Vinyl Ether) Copolymers: Enhanced Immobilization of Recombinant Proteins

Catherine Ladavière,[†] Thierry Delair,^{*,†} Alain Domard,[‡] Armelle Novelli-Rousseau,[†] Bernard Mandrand,[†] and François Mallet[†]

Unité Mixte UMR-103, CNRS-bioMérieux, ENS-Lyon, 46, allée d'Italie, 69364 Lyon, France, and Unité Mixte CNRS-Université Claude Bernard, UMR-5627, LEMPB, Université Claude Bernard, 43, Bd. du 11 Novembre 1918, 69612 Villeurbanne, France. Received November 28, 1997; Revised Manuscript Received August 1, 1998

Two genetically modified HIV-1 capsid p24 proteins, RH24 and RH24K, were covalently bound to maleic anhydride-*alt*-methyl vinyl ether (MAMVE) copolymer, under aqueous conditions. We demonstrated that the addition of a six lysine unit tag at the COOH-terminus of RH24K greatly improved the grafting reaction which could take place under many different experimental conditions. The course of the reaction was controlled by electrostatic attractive forces between the protein and the negatively charged polymer, as the chemical binding was more efficient at low ionic strength. The maximum loading capacity of the polymer depended on whether the protein bore the lysine tag (RH24K) or not (RH24). Twenty-four molecules of RH24 could be immobilized per polymer chain and 49 for RH24K. Such a difference could be explained by a difference of orientation of the protein on the polymer, side-on for RH24 and end-on for RH24K to account for the observed high packing density.

INTRODUCTION

Molecular recognition is a fundamental means of conveying information in living organisms. For applications such as in diagnostics, it may be required to immobilize proteins onto polymers, via the formation of covalent bonds, with ideally no loss of biological properties. To attain this goal, one strategy consists of directing the chemical reaction involved in the grafting, toward a specific site of the protein that is not essential for the molecular recognition process of interest. For instance, Rodwell et al. (1), used the carbohydrate moieties located at the hinge region of the immunoglobulin to target a chemical modification.

Protein modifications can be used to introduce a functional group on a specific site of the macromolecule. Enzymatic digestion of antibodies with subsequent reduction of disulfide bonds leads to the formation of thio groups exactly on the opposite end of the recognition site of the immunoglobulin (2). Genetic engineering allows the introduction or the substitution, at a well-defined position on the protein, of amino acids that bear the ad hoc functional group for further chemical modification, such as coupling. Chilkoti et al. (3), for instance, incorporated a unique cysteine residue in mutant cytochrome *b*₅ for conjugation to maleimide-terminated oligo(*N*-isopropylacrylamide). A closely related approach consists of introducing an oligo(amino acid) sequence, tags, at a defined position on the protein. For instance, oligo-(histidine) tags were introduced in recombinant HIV-1 p24 capsid protein, to allow its purification via coordination interactions between histidines and metal ion immobilized on a solid phase (4, 5). An oligo(arginine) tag

was reported (6) for solid-phase immobilization of the protein via electrostatic interactions with a negatively charged surface.

In the lab, we used a strategy related to the second approach described above. A six lysine residue tag was added at the COOH position of a recombinant p24 HIV-1 capsid protein. The rationale was that an increase of the density of amine groups at a particular position of the protein molecule, should enhance the efficiency of the immobilization reaction, as a whole, and would direct the grafting to the lysine-enriched site of the biological molecule. In the absence of the tag, the grafting onto the polymer would take place at random, on any of the 10 lysine residues available on the macromolecule. The great advantage in using amide bond formation to tether biomolecules to carriers is that the chemistry is simple to run, reproducible, and can sustain a diversity of experimental conditions which is a prerequisite for the development of a generic grafting method. Thiol chemistry, though very chemiospecific and regiospecific, suffers from the dimerization of the reactants by disulfide bond formation which easily occurs. To suppress this side reaction, strict experimental conditions have to be used (absence of traces of oxygen and divalent cations and no basic pH), which on an every day basis make this chemistry less attractive.

The p24 protein, and its recombinant equivalents, are commonly used in diagnostics tests for the detection of HIV antibodies in sera. On the basis of results obtained for the hepatitis B virus (HBV) DNA detection (7), the use of polymeric p24 in diagnostics tests should allow us to increase the sensitivity of the kits, for a still more reliable investigation of HIV infected patients.

We wish to report here on the comparative results obtained in the immobilization of two recombinant proteins: one with a six lysine residue tag and the other deprived of lysine tag.

* To whom correspondence should be addressed.

[†] CNRS-bioMérieux.

[‡] Université Claude Bernard.

EXPERIMENTAL PROCEDURES

Materials. Recombinant proteins were obtained in *Escherichia coli* from a procedure derived from ref 5. These proteins were termed RH24 and RH24K for, respectively, recombinant p24 with a six histidine residue tag (H) for metal chelate purification and recombinant p24 with a histidine (H) tag and the six lysine (K) residue tag. For both proteins, the histidine tags were at the NH₂ termini. For RH24K, the lysine tag was at the COOH-terminus. The amino acid sequence for each protein is reported as follows:

p24.HXB2	PIVQNIQQQMVHQIAISPTLNAAWVQVVEEKAFSPEVIMPFSALSE	
RH24	MRGSHHHHHHGSVDSESM.....	
RH24K	MRGSHHHHHHGSVDSESM.....	
p24.HXB2	GATPQDLNTMLNTVGHHQAAMQLKETINEEAAEWDRVHPVHAGPIAPGQMREPRGSDIA	
RH24		
RH24K		
p24.HXB2	GTSTLQEQIGWMNNPPIPVGEIYKRWIILGLNGIVRMYSPSILDIRQGPKEPFRDYY	
RH24		
RH24K		
p24.HXB2	DRFYKTLRAEQASQEVKNWMTETLLVQVANPDCKTILKALGPAATLEEMMTACQGVGGPG	
RH24	G	
RH24K	G	
p24.HXB2	HKARVL	231
RH24	DLV	243
RH24K	KKKKKKSVDSESL	252

The lysine groups are randomly dispersed along the protein macromolecules. NMR (8) and crystal structure data (9, 10) demonstrated that, in the 3D structure of the protein, the 10 lysine groups were accessible for grafting. Theoretical molecular weights and isoelectric points were determined with the MacVector software (version 6.01).

The maleic anhydride-*alt*-methyl vinyl ether copolymer [P(MAMVE): M_n = 67 000 g/mol] was from Poly-science Inc. and used as received. Other chemicals were from Aldrich and used as received. Milli-Q grade (Millipore SA, France) deionized water was used for all buffers.

Coupling of Proteins to Copolymers. The appropriate amounts of polymers were dissolved in anhydrous dimethyl sulfoxide (DMSO) at 37 °C. To a protein buffer solution at the appropriate concentrations was added the required volume of polymer solution in DMSO. The reaction mixture was stirred for 3 h at 37 °C. The pH of the buffers was measured and monitored using an Ingold HA405-DXK-58/120 electrode and a Minis 8000 pH-meter (Taccussel-Radiometer, France). As stated by the supplier, the electrode was Tris compatible.

Kinetics of the Coupling Reaction. To monitor the course of the coupling of proteins onto polymers, 20 μ L of the reaction mixture was pipetted off and the reaction was quenched by addition of 5 μ L of 15% ammonium hydroxide solution.

Protein Interaction with the Hydrolyzed Copolymer. The copolymer sample was hydrolyzed at the same concentration as for coupling (stock concentration, 1 g/L), during 2 h at 67 °C, then 48 h at 37 °C in various buffers (pH 4.0, 6.8, 7.8, 8.2, 9.2, and 10.7). Then, the coupling reaction was performed as described above.

Stability of Proteins and Conjugates. This study was carried out for the proteins RH24 and RH24K and for the conjugates in the coupling medium (DMSO/ Tris buffer, pH 7, 5/95, [protein] = 0.95 g/L, [P(MAMVE)] = 0.048 g/L) at 37 °C, for 7 days. Prior to use, the buffer was filtered on a 0.22 μ m membrane and pipet tips and Eppendorf tubes were autoclaved (3 h at 120 °C).

Analyses of the Coupling Reactions. The crude coupling mixture was analyzed by size-exclusion chro-

matography (SEC) using a Shodex Protein KW-803 (Waters) or a Protein-Pak 125 (Waters), a Kontron HPLC 422 pump, a Kontron HPLC autosampler 465, and a Kontron UV diode array detector. Purifications were run in a 0.1 M phosphate buffer, pH 6.8, containing 0.5% (w/v) of sodium dodecyl sulfate (SDS) with a flow rate of 0.5 mL/min. Detection was achieved by measuring the absorbance at 280 nm corresponding to the proteins (at the concentrations used, the polymer had no absorption). The ratio of the peak area corresponding to the polymer-bound RH24(K) versus the sum of the two peaks corresponding to the unbound and to the bound RH24(K) (i.e., the total amount of protein involved in the reaction) gave the coupling yield (Y). The extinction coefficients of the bound and unbound proteins were checked by measuring the OD at 280 nm of crude conjugates obtained in a carbonate buffer compared to the reference-free protein in the same mixture. The OD data were found identical in a 10% range. Furthermore, for each HPLC analysis, the sum of the areas of the bound and unbound proteins was routinely compared to that of a reference sample recorded in the absence of polymer. To assess the average amount of protein molecules per polymer chain, $\bar{N} = nY/n'$ where n and n' , respectively, correspond to the number of protein molecules and the number of polymer chains in the reaction mixture; as a first approximation, we assumed, that each polymer chain reacted according to the same pattern [which is the case for oligonucleotide immobilization (7)]. Trying to assess the molecular weight of the conjugates by acrylamide gel electrophoresis was a failure probably because of polymer-gel interactions. Using viscometry on-line with SEC, was not possible due to the presence of SDS in the mobile phase (unstable signals were obtained). To calculate $\bar{N}$, which gives access to the level of loading of the polymer, we assumed that no cross-linking had occurred. This assumption seems realistic given that the protein to polymer molar ratio is 50:1.

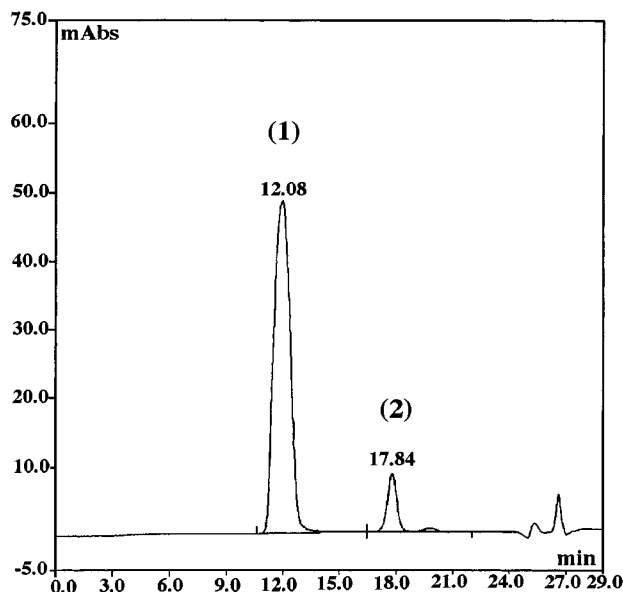
Dynamic Light-Scattering Experiments. These experiments were performed on-line using a Protein-Pak 200 SW column (Waters) and a Waters 510 high-performance liquid chromatography pump, running in a 0.1 M phosphate buffer, pH 6.8, containing 0.5 M NaCl with a flow rate of 0.5 mL/min. The refractive index increment of proteins is ca. 0.19 mL/g (11). For the detection part, a Waters 484 absorbance detector, a Waters 410 differential refractometer, and a three angles Mini Dawn F detector (WYATT Technology) at 690 nm were used on-line.

Mass Spectrometry (LC-ESI-MS). Analyses were performed with a single quadrupole API 100 mass spectrometer, 140 B pumps, and a 785 A detector (Perkin-Elmer). Reversed-phase liquid chromatography was performed on a C18 column (Vydac, 5 μ m particle size). Solvent A was 0.1% (v/v) trifluoroacetic acid in water and solvent B was 0.1% (v/v) trifluoroacetic acid in a water/acetonitrile mixture (20:80 v/v). A gradient from 45 to 70% of B was used.

Circular Dichroism (CD). Analyses were performed in a 0.05 M Tris-HCl buffer, pH 7, with a protein concentration between 30 and 50 mM. CD measurements were performed with a Jobin-Yvon model CD6 spectrophotometer at 25 °C and with a Hellma cuvette; path length-0.2 cm. To eliminate the DMSO used for the coupling (high absorption of solvent), the conjugates were analyzed after dialysis on a polycarbonate membrane with a cutoff of 3000 g/mol (AMICON). This study was carried out with a conjugate of RH24K because the

Table 1. Molecular Weight and Isoelectric Point of HIV-1 Capsid Recombinant Proteins

	theoretical MW (g/mol)	LC-ESI-MS (g/mol)	SEC-MALLS (g/mol)	theoretical IP ^a
R24	24 736		24 680	5.5
RH24	26 950	26 959	27 500	5.9
RH24K	28 022	28 026	29 100	6.9

^a IP = isoelectric point.**Figure 1.** SEC trace of the coupling reaction mixture showing two kinds of peaks corresponding to (1) conjugate and (2) unreacted RH24K molecules.

coupling yield is high (~88%) and as a consequence the unbound protein does not perturb the analysis.

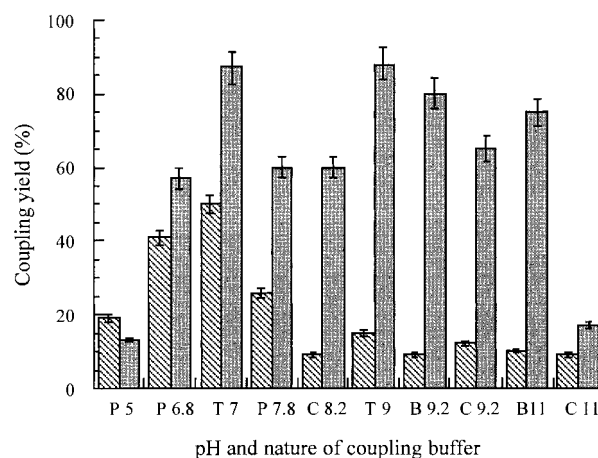
RESULTS AND DISCUSSION

Protein Characterizations. The molecular weight of each protein was determined by size-exclusion chromatography-multiangle laser light scattering (SEC-MALLS) and reversed-phase liquid chromatography coupled with electrospray ionization mass spectrometry (LC-ESI-MS).

Data depicted in Table 1 show a good correlation between the two techniques and the theoretical value deduced from the amino acid composition. In comparison to RH24 and RH24K proteins, data concerning R24 that bear no tag are shown as well.

CD spectra of RH24 and RH24K exhibited the same pattern as for the R24 protein reported in the literature (12), and the conformation corresponded to 43% α -helices and 17% β -sheets. This is an important result which demonstrated that no major conformational modification was induced by the addition of one or two tags.

Characterization of the Protein/Polymer Conjugates. Conjugates were analyzed and purified by size-exclusion HPLC. As shown in Figure 1, the polymer protein conjugates were recovered in the first elution peak and the unbound proteins in the second. We noticed a tendency of the protein to stick to many solid phases. That can probably be explained considering the role of a p24 protein, which is to protect the genetic code of the virus by aggregating to form a capsid. To overcome this autoassociation problem, whose dissociation constant of corresponding dimer, measured by Rosé et al. (13), was $(3-4) \times 10^{-5}$ M at pH 7, we added 0.5% (w/v) of sodium dodecyl sulfate in the HPLC mobile phase.

**Figure 2.** Coupling yield of the RH24 (hatched bar) and RH24K (stitched bar) to P(MAMVE) copolymer at 37 °C, 3 h [protein] = 0.95 g/L, [P(MAMVE)] = 0.048 g/L (protein to polymer molar ratio: 50:1). P, 0.1 M phosphate buffer; T, 0.05 M Tris buffer; C, 0.1 M carbonate buffer; B, 0.1 M borate buffer, pH 9, 0.01 M borate pH 11.

Determination of the Coupling Conditions. On the basis of results obtained in a preliminary study on bovine serum albumin, the grafting of recombinant proteins onto MAMVE copolymer was tested in aqueous buffers containing 5% (v/v) of DMSO (14). The results obtained, depicted in Figure 2, show that, in most cases, the grafting of the lysine-tagged protein, RH24K, was more efficient than the immobilization of the RH24 HIV-1 capsid protein. In the latter case, the coupling yields were higher at pHs close to neutrality and fell with increasing pH as the hydrolysis rate increased (15). The lysine-tagged protein reacted with the anhydride moieties of the MAMVE copolymer over a wide range of experimental conditions, demonstrating the enhancing effect of the tag on the course of the reaction. Coupling yields were over 50% except for extreme conditions, i.e., low (pH 5) or high (pH 11) pHs. The Tris buffer proved the most efficient medium for the covalent grafting of both proteins, demonstrating the poor reactivity of the amine of the Tris buffer toward acylation. It is worth noting that, in the phosphate buffer, pH 5, the conjugates precipitated but the proteins did not. The effect of the lysine tag on the course of the coupling reaction was drastically marked at pHs over 7. Above pH 7, the immobilization yields of RH24 leveled off at ca. 10%, whereas 60–88% yields were obtained for RH24K.

To explain the difference in behavior between both proteins, one can consider two factors: (i) different conformations, one of which would favor the coupling reaction via steric effects, and (ii) difference in reactivities due to the presence of the lysine tag in RH24K. CD spectra of RH24 and RH24K were identical, proving that the introduction of the lysine tag entailed no modification of the secondary structure of the protein. Therefore, conformational factors cannot be regarded responsible for the difference in behavior between the two proteins. Hence, reactivity factor must be evoked to account for such a large variation in behavior. The immobilization reaction onto the MAMVE copolymer occurs by nucleophilic attack of the reactive anhydride moieties by the primary amine groups of the proteins, as depicted in Figure 3. Since the reaction takes place in a mostly aqueous medium, the hydrolysis of the anhydride functions is very likely to occur. Therefore, the grafting pathway of the protein onto the polymer has to compete the hydrolysis reaction. The kinetics data reported in

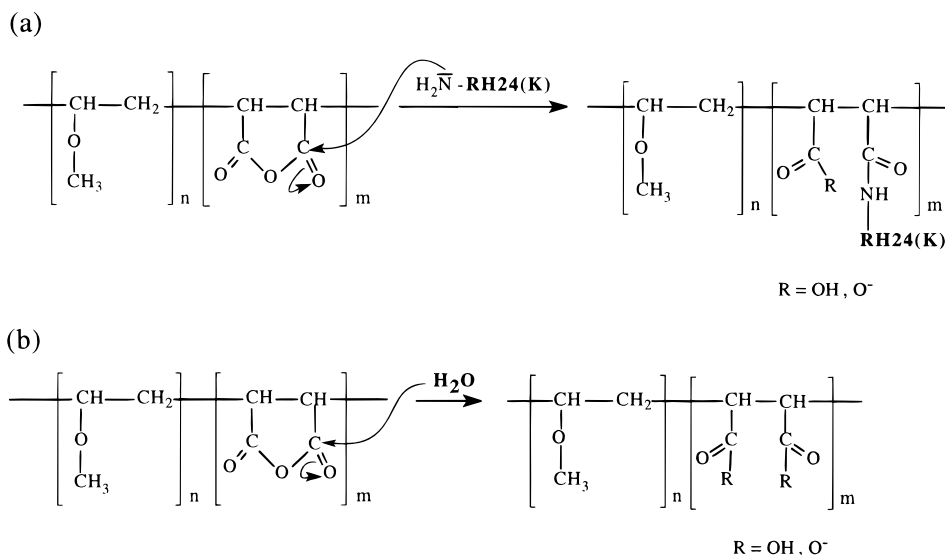


Figure 3. Coupling reaction between RH24(K) molecule and copolymer P(MAMVE) (a) and hydrolysis reaction of copolymer (b).

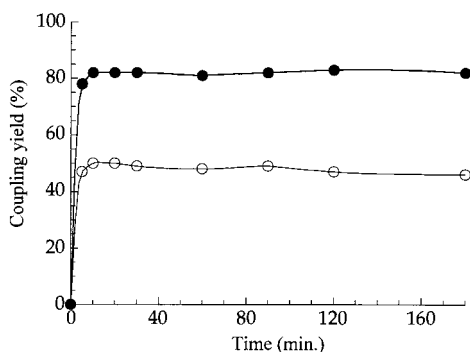


Figure 4. Kinetics of RH24 (○) and RH24K (●) coupling reaction onto P(MAMVE) sample. Buffer: 0.05 M Tris, pH 7.

Table 2. RH24K Coupling Yields vs Buffer Ionic Strength^a

[carbonate], pH 8.2 (mol/L)	coupling yield (%)	
	RH24	RH24K
0.1	18	53
0.05	21	85
0.02	26	94

^a [protein] = 0.95 g/L; [polymer] = 0.048 g/L; protein/polymer molar ratio = 50:1.

Figure 4 were obtained in a 50 mM Tris buffer, pH 7, at 37 °C, and the coupling yields were measured after quenching the reaction with excess ammonia. The presence of ammonia did not alter the stability of the amide bond formed during acylation with the polymer as the amount of bound protein did not decrease over the analysis period (illustrated by the presence of plateaus in Figure 4). Furthermore, the plateau values of the coupling yields obtained in the presence of ammonia perfectly matched the coupling yields obtained without ammonia as seen in Table 4, run 5, and Table 5, run 5. After 10 min of reaction, the coupling yields did not evolve any more, meaning that the reaction had reached a plateau and that all reactive groups on the polymer were consumed, either to form an amide bond with the protein or by hydrolysis by water molecules. For both proteins, the reaction did not proceed any further after 10 min. Nevertheless, for the same time period, more RH24K molecules were bound to the polymer than RH24.

It was checked that no coupling between the polymer and RH24 occurred when all the anhydride groups of the

Table 3. Coupling Yields of RH24 to MAMVE Copolymer as a Function of Ionic Strength (Two Different Experiments)

[NaCl] (mol/L)	coupling yield (%) 0.05 M Tris buffer pH 7	
	exp 1	exp 2
1		3
0.75		12
0.5	22	21
0.25	33	33
0.1	43	-
0	52	50

^a [protein] = 0.95 g/L; [polymer] = 0.048 g/L; protein/polymer molar ratio = 50:1.

Table 4. Effect of the Protein Concentration on the Coupling Yields in 0.05 M Tris Buffer, pH 7

run	[protein] ^a (g/L)	coupling yield (%) ^b	
		RH24	RH24K
1	0.095	100	96
2	0.19	94	95
3	0.48	75	95
4	0.76	58	92
5	0.95	47	88
6	1.90	24	50

^a Final concentration. [polymer] = 0.048 g/L; 37 °C, 3 h. ^b Mean value of two experiments.

Table 5. Polymer Concentration Effect on the Coupling Yields

run	[P(MAMVE)] ^a (g/L)	coupling yield (%) ^b	
		RH24, Tris, pH 7	RH24K, carbonate, pH 8.2
1	0.0048	11	11.5
2	0.0095	18	22
3	0.024	32	50.5
4	0.038	40	70
5	0.048	46	80

^a Final concentration, [protein] = 0.95 g/L; 37 °C, 3 h. ^b Mean value of two experiments.

polymer were hydrolyzed (at different pHs: 4.0, 6.8, 8.2, and 10.6). Actually, the mixture of hydrolyzed polymer and RH24K always led to a precipitate (used buffers pHs: 4.0, 6.8, 7.8, 8.2, 9.2, and 10.7). This precipitate could be explained by electrostatic interactions between the negative charges of polymer chains and the cationic sites

of the protein molecules, leading to highly cross-linked polyelectrolyte complexes insoluble in water. When the ratio polymer/protein was increased, no precipitation was observed as more polymer was available. Under these conditions, no conjugation occurred as observed by HPLC.

The reproducibility of the grafting experiments is quite good, based on the following results obtained on different days. The immobilization yields of RH24K were 82, 80, and 88% for experiments described respectively in Figure 4, Table 5 (run 5), and Table 4 (run 4). For the same series of experiments, coupling yields of RH24 were 48, 46, and 47%.

Role of the Ionic Strength. With the lysine-tagged protein, a series of experiments was run in a carbonate buffer in which the immobilization yield was only 53% and the coupling yield was measured as the buffer ionic strength was lowered. The results reported in Table 2 show a drastic increase in coupling yields when lowering the ionic strength and clearly emphasize the major role played by electrostatic interactions in the immobilization process of RH24K. It is worth noting that, for a 20 mM carbonate buffer, the pH of the coupling mixture only decreased 0.15 pH unit in 53 min, proving that the buffer was not exhausted by the hydrolysis of the anhydride groups of the polymer.

Though at pH 8.2 the protein net charge is negative, some protonated amine groups remain. Since negative charges are formed on the polymer, either from the hydrolysis of the anhydride moieties or from the coupling reaction, attractive electrostatic forces can arise by interaction with the positive charges borne by the protein. These forces allow the macromolecular counterparts to get in contact for the chemical reaction to take place. Finally, the results obtained in Table 2 point out a fundamental difference in behavior of the two proteins. Lowering the ionic strength had a slight effect on the coupling yield for RH24, whereas a drastic increase in coupling yields was observed with RH24K. To observe any ionic strength effect with RH24, sodium chloride was added to a Tris buffer, pH 7. No data were available for the RH24K protein because, in this experiment, precipitation was observed on adding sodium chloride (the protein alone precipitated too). From the results shown in Table 3, an increase of ionic strength by adding sodium chloride resulted in a decrease of the grafting efficiency. A slight precipitation was observed in the Tris buffer at sodium chloride concentrations higher than 0.5 M. In another set of experiments, the ionic strength of the Tris buffer, pH 7, was reduced. No improvement of the immobilization yields was obtained, neither for the lysine-tagged protein, which was not surprising since the coupling yields were already as high as 80%, nor for the RH24 sample for which the maximum coupling yield was limited to ca. 40% (data not shown). This behavior was not expected on account of results obtained with BSA, for which coupling efficiency was improved by reduction of the buffer ionic strength (14).

From these results, one may conclude that the covalent immobilization of RH24K is mostly governed by electrostatic interactions. These interactions are attractive forces between the negatively charged polymer and the positive charges on the protein. The role of these forces is to bring together the macromolecular reactants in order to allow the chemical reaction to take place. For RH24, the coupling process is controlled by a diffusional process as well as by the differences in reactivities between the amino groups of the protein and the water molecules of the reaction mixture that hydrolyze the reactive groups on the polymer.

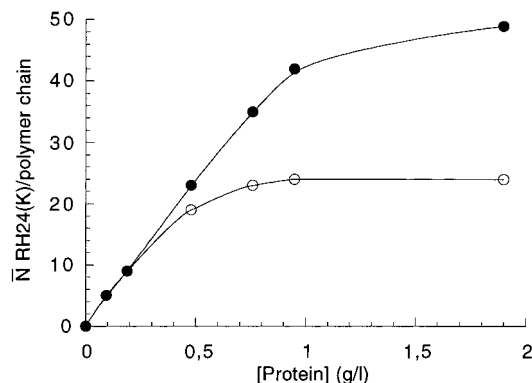


Figure 5. Number of protein molecules bound per polymer chain (N) for RH24 (○) and RH24K (●) as a function of protein concentration during coupling. Buffer: 0.05 M Tris, pH 7. Reaction time and temperature 3 h, 37 °C.

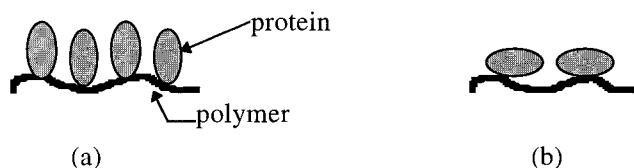


Figure 6. Representation of end-on (a) and side-on (b) orientations.

Role of the Reactant Concentration. The relative concentrations of both reactants are factors that can have an effect on the course of the binding reaction.

The increase of the protein concentration in the reaction mixture resulted in a more marked decrease of the immobilization yields of RH24 than of RH24K as seen in Table 4. More striking is the effect on the average amount of protein loaded per polymer chain, as represented by N in Figure 5. The amount of bound RH24 per polymer chain leveled off at 24 protein molecules, whereas no plateau value was reached yet for RH24K, though coupling yields started to significantly decrease at a concentration of 0.95 g/L (Table 4). The plateau value, obtained on increasing RH24 concentrations, suggests that the grafting reaction is controlled by a steric effect: the polymer chain is covered with proteins which adopted some sort of side-on orientation. For the same polymer chain, more than 49 molecules of RH24K can be tethered, which must have adopted an end-on type orientation. Due to the presence of positive charges on the six lysine residue terminus of RH24K, the tag favors the approach of a protein molecule toward a negatively charged P(MAMVE) chain, in some sort of an oriented manner. In other words, the tag acts as a leash to bind the protein to the polymer. The amino groups of the lysine tag are the first to get in contact, and react, with some anhydride moieties of the polymer which ensures an oriented immobilization of RH24K. When the concentration of RH24K is high, many proteic species can be grafted onto one polymer chain, preventing, thus, the protein to adopt a side-on orientation (Figure 6).

The next factor to be investigated was the polymer concentration in the coupling medium. This factor was investigated in a carbonate buffer for RH24K, in which RH24 very poorly binds (Table 2), which makes this buffer more selective toward the orientation of the grafting of the lysine-tagged protein. For RH24, a Tris buffer was selected which afforded a high grafting efficiency, whose variations should be quite significant. The coupling yields increased with the polymer concentrations, as reported in Table 5, and concomitant decrease

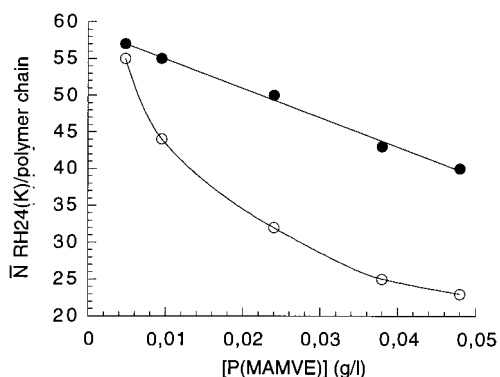


Figure 7. Amount of RH24(K) molecules bound per polymer chain ($\bar{N}$) in a 0.05 M Tris buffer, pH 7, for RH24 (○) and in a 0.02 M carbonate buffer pH 8.2 for RH24K (●) as a function of the polymer concentration. [protein] = 0.95 g/L.

of $\bar{N}$ was observed for both proteins as shown in Figure 7. It is worth noting that the maximum amount of RH24(K) bound to the polymer (i.e., at the lowest polymer concentration, run 1 in Table 5) was similar in both cases. The maximum loading capacity of ca. 58 protein molecules chain (Figure 7) could be reached when the reaction was run with a large excess of proteins (protein to polymer molar ratio of 500:1, run 1 in Table 5). At first, this result could challenge the side-on/end-on hypothesis postulated in the preceding paragraph. But one has to take into account that in run 1, Table 5, the protein to polymer molar ratio was 5-fold higher than in run 6, Table 4. Thus, under an extremely high concentration in protein (run 1, Table 5), RH24 was forced to adopt an end-on orientation during coupling. When the immobilization conditions were less drastic (run 6, Table 4, protein to polymer molar ratio of 100:1), the high level of loading of RH24K onto the polymer (49 proteins/chain) can only be explained if the protein binds in an end-on orientation. This orientation arises from attractive electrostatic forces generated by the interaction of the lysine tag and the polymer. This result clearly demonstrates that orientation of RH24K on binding to the polymer is achieved via electrostatic factors, and that the coupling reaction is indeed governed by these factors. In other words, binding of RH24 in an end-on orientation is under kinetic control, when a very large excess of proteins is used, the side-on orientation being obtained when the reaction is under thermodynamic control, i.e., at the lowest protein concentration.

When the polymer concentration in the reaction mixture was increased, more polymer chains were available for the same initial amount of protein and we observed a decrease in $\bar{N}$ as already observed with BSA (14). But the decrease in the case of RH24K is slower than for RH24. This difference in behavior could be explained by the already suspected higher reactivity of RH24K compared to the non-lysine-tagged protein. An increase in the polymer concentration of the reaction mixture results in an increase of the hydrolysis rate of the reactive groups (15) of the polymer. RH24 does not compete with hydrolysis as well as RH24K. When the polymer concentration was high, in large excess to the protein, the immobilization yields were maximum, and once again, higher for RH24K. It is worth mentioning that with RH24K three buffers were investigated in this series of experiments: 0.02 M carbonate buffer, pH 8.2, 0.05 M Tris buffer, pH 7, and 0.1 M borate buffer, pH 9.2. At low polymer concentrations in the Tris and borate buffers, an insoluble precipitate was formed. When enough polymer was present (0.024 g/L in Tris buffer, 0.038 g/L

in the borate buffer), no insoluble precipitate was formed, and the amounts of bound proteins in the three buffers were closely related.

Stability of Proteins and Conjugates. When RH24 and RH24K were incubated at 37 °C in the coupling medium (DMSO/Tris buffer, pH 7, 5/95), new peaks at higher elution times appeared, corresponding to degradation fragments of the proteins. The degradation yield increased with time at 37 °C, to reach 16% for RH24 and 20% for RH24K after 7 days.

The protein/polymer conjugates remained stable at 37 °C over the same time period. In the meantime, the unbound protein contained in the crude conjugate underwent degradation. So the conjugation of the proteins to the polymer was a means of stabilizing them and protecting them from degradation.

CONCLUSION

This work demonstrated that the introduction of a six lysine residue tag into a recombinant protein drastically modified the immobilization conditions onto a (maleic anhydride-*alt*-methyl vinyl ether) copolymer, though it only corresponded to ca. 3% of the global amino acid composition.

The nontagged protein (RH24) was immobilized at buffer pHs close to neutrality in order to disfavor the competing hydrolysis of the polymer reactive groups. For the lysine-tagged protein (RH24K), the hydrolysis reaction had less impact, and the protein could be tethered under a wide variety of experimental conditions, including high pH values. For a sufficient polymer concentration, the presence of the polylysine end allowed the immobilization of up to 50 protein molecules per polymer chain. When densely packed, the protein has probably adopted an end-on orientation. The maximal density of polymer-bound nontagged protein was shown to be lower than for its tagged counterpart. This upper limit was due to steric effects as an increase of the protein concentration in the coupling medium did not allow it to bind more RH24. Taking this last result into account, one may think that the orientation of the nontagged protein on the polymer is more of a side-on type. For a weak polymer concentration (protein-to-polymer ratio of 500:1), the maximal amount of polymer-bound protein was independent of the protein nature and attained 58 protein molecules/polymer chain. So when proteins are in large excess, they are forced to bind in an end-on orientation, affording the high loading value of 58 proteins per polymer chain. When coupling conditions were less drastic (protein to polymer molar ratio of 100:1), discrimination between the nature of the two proteins can occur. Thus, RH24K only, can bind in an end-on orientation, as shown by the high loading value of 49 proteins/chain, because of the orientation effect due to interaction of the tag with the copolymer. In the absence of these interactions, as in the case of RH24, the proteins bind according to a random orientation, which corresponds to a side-on orientation as shown by the reduced loading value of RH24 molecules.

The study on the stability of protein/polymer conjugates at 37 °C showed that the polymer-bound proteins were more stable than the unbound ones.

Finally, on a conformational standpoint, CD spectra of the conjugates with RH24K were identical to those of the unbound proteins, so the conjugation process occurred with no modification of the secondary structure of the bioactive species. Therefore, the evaluations of the

biological properties of the conjugates could take place and will be reported in due course.

ACKNOWLEDGMENT

The authors are grateful to Dr. L. Ladavière for CD measurements, Dr. G. Gervasi for analyses of proteins by mass spectrometry, and C. Hebrard for the preparation of proteins. C.L. is thankful to Fondation Mérieux for financial support.

LITERATURE CITED

- (1) Rodwell, J. D., Alvarez, V. L., Lee, C., Lopes, A. D., Goers, J. W. F., King, H. D., Powsner, H. J., and McKearn, T. J. (1986) Site-specific covalent modification of monoclonal antibodies: in vitro and in vivo evaluations. *Proc. Natl. Acad. Sci.* 83, 2632–2636.
- (2) Delair, Th., Pichot, C., and Mandrand, B., (1994) Synthesis and characterization of cationic latexes particles bearing sulfhydryl groups and their use in the immobilization of Fab antibody fragments. *Colloid Polym. Sci.* 272, 72–81.
- (3) Chilkoti, A., Chen, G., Stayton, P. S., and Hoffman, A. (1994) Site-specific conjugation of a temperature-sensitive polymer to a genetically engineered protein. *Bioconjugate Chem.* 5, 504–507.
- (4) Ng, K., Pack, D. W., Sasaki, D. Y., and Arnold, F. H. (1995) Engineering protein–lipid interactions: targeting of histidine-tagged proteins to metal-chelating lipid monolayers. *Langmuir* 11, 4048–4055.
- (5) Cheynet, V., Verrier, B., and Mallet, F. (1993) Overexpression of HIV-1 proteins in *Escherichia coli* by a modified expression vector and their one-step purification. *Protein Expression Purif.* 4, 367–372.
- (6) Stempfer, G., Holl-Neugebauer, B., Kopetzki, E., and Rudolph, R. (1996) A fusion protein designed for noncovalent immobilization: stability, enzymatic activity, and use in an enzyme reactor. *Nat. Biotechnol.* 14, 481–484.
- (7) Erout, M. N., Troesch, A., Kurfurst, R., Pichot, C., and Cros, P. (1996) Preparation of conjugates between oligonucleotides and *N*-vinylpyrrolidone/*N*-acryloxysuccinimide copolymers and applications in nucleic acid assays to improve sensitivity. *Bioconjugate Chem.* 5, 568–575.
- (8) Gitti, R. K., Lee, B. M., Walker, J., Summers, M. F., Yoo, S., and Sundquist, W. I. (1996) Structure of the amino-terminal core domain of the HIV-1 capsid protein. *Science* 273, 231–235.
- (9) Gamble, T. R., Vajdos, F. F., Yoo S., Worthylake, D. K., Houseweart, M., Sundquist, W. I., and Hill, C. P. (1996) Crystal structure of human Cyclophilin A bound to the amino-terminal domain of HIV-1 capsid. *Cell* 87, 1285–1294.
- (10) Gamble, T. R., Yoo, S., Vajdos, F. F., von Schwedker, U.K., Worthylake, D. K., Wang, H., McCutcheon, J. P., Sundquist, W. I., and Hill, C. P. (1997) Structure of the carboxyl-terminal dimerization domain of the HIV-1 capsid protein. *Science* 278, 849–853.
- (11) Brandrup, J., Immergut, E. H. (1989) *Polymer Handbook*, 3rd ed.; p 461. Wiley, New York.
- (12) Misselwitz, R., Hausdorf, G., Welfle, K., Hohne, W. E., and Welfle, H. (1995) Conformation and stability of recombinant HIV-1 capsid protein p24 (rp24). *Biochim. Biophys. Acta* 1250, 9–18.
- (13) Rosé, S., Hensley, P., O'Shannessy, D. J., Culp, J., Debouck, C., and Chaiken, I. (1992) Characterization of HIV-1 p24 self-association using analytical affinity chromatography. *Proteins: Struct., Funct., and Genet.* 13, 112–119.
- (14) Ladavière, C., Delair, Th., Domard, A., Pichot C., Mandrand B. Covalent immobilization of Bovine Serum Albumin onto (maleic anhydride-*alt*-methyl vinyl ether) copolymers. *J. Appl. Polym. Sci.* (in press).
- (15) Ladavière, C., Delair, Th., Domard, A., Pichot, C., and Mandrand, B. Covalent immobilization of biological molecules to maleic anhydride and methyl vinyl ether copolymers. A physicochemical approach. *J. Appl. Polym. Sci.* (in press).

BC970208I

Renal Metabolism of ^{111}In -DTPA-D-Phe¹-Octreotide in Vivo

Hiromichi Akizawa,[†] Yasushi Arano,^{*,†} Takashi Uezono,[†] Masahiro Ono,[†] Yasushi Fujioka,[†] Tomoya Uehara,[†] Akira Yokoyama,[†] Kenichi Akaji,[‡] Yoshiaki Kiso,[‡] Mitsuru Koizumi,[§] and Hideo Saji[†]

Department of Patho-Functional Bioanalysis, Graduate School of Pharmaceutical Sciences, Kyoto University, Yoshida-Shimoadachi-cho, Sakyo-ku, Kyoto 606-8501, Japan, Department of Medicinal Chemistry, Kyoto Pharmaceutical University, Yamashina-ku, Kyoto 607-8414, Japan, and Cancer Institute Hospital, Toshima-ku, 170-0012, Tokyo, Japan. Received December 31, 1997; Revised Manuscript Received June 5, 1998

The persistent localization of radioactivity in the kidney after administration of ^{111}In -DTPA-D-Phe¹-octreotide impairs the diagnostic accuracy of this radiopharmaceutical. To better understand the mechanisms responsible for the renal radioactivity levels of ^{111}In -DTPA-D-Phe¹-octreotide, the renal metabolism of this compound was compared with ^{111}In -DTPA-L-Phe¹-octreotide, where the N-terminal D-phenylalanine was replaced with L-phenylalanine to facilitate metabolism. DTPA-D-Phe¹-octreotide and DTPA-L-Phe¹-octreotide were synthesized by solid-phase methods. Both ^{111}In -DTPA-conjugated octreotide analogues were prepared with radiochemical yields of over 96%, and both remained stable after a 3 h incubation in murine serum at 37 °C. When injected into mice, the two ^{111}In -DTPA-conjugated octreotide analogues showed similar radioactivity elimination rates from the blood and accumulation in the kidney with about 60% injected radioactivity being excreted in the urine by 24 h postinjection. Over 85% of the radioactivity in the urine existed as intact peptides for both analogues. Despite the similar renal radioactivity levels, significant differences were observed in the radiolabeled species remaining in the kidney between the two; while ^{111}In -DTPA-L-Phe¹-octreotide was rapidly metabolized to the final radiometabolite, ^{111}In -DTPA-L-Phe, the metabolic rate of ^{111}In -DTPA-D-Phe¹-octreotide was so slow that various intermediate radiolabeled species were observed. However, both ^{111}In -DTPA-D-Phe and ^{111}In -DTPA-L-Phe remained in the lysosomal compartment of the renal cells as the final radiometabolites for long periods. These findings indicated that although the metabolic stability of ^{111}In -DTPA-D-Phe¹-octreotide in the renal cells may be partially involved, the slow elimination rate of the radiometabolite derived from ^{111}In -DTPA-D-Phe¹-octreotide from the lysosomal compartment of renal cells would be predominantly attributable to the persistent renal radioactivity levels of ^{111}In -DTPA-D-Phe¹-octreotide.

INTRODUCTION

Oncophilic low molecular weight peptides and polypeptides have attracted attention as new vehicles to deliver radioactivity to target tissues within a short postinjection period. In particular, the ^{111}In -labeled synthetic somatostatin analogue, ^{111}In -DTPA-D-Phe¹-octreotide (Figure 1), has been used clinically for imaging somatostatin receptor-positive tumors such as gastrointestinal pancreatic tumors, neuroblastoma, pheochromocytoma, breast cancer, Hodgkin's lymphoma, and small cell lung cancer (1). Besides being prepared by a simple kit formulation with specific activities and radiochemical purities sufficient for clinical applications, ^{111}In -DTPA-D-Phe¹-octreotide reduces upper abdominal radioactivity levels that are observed with a radioiodinated octreotide analogue (2). After administration, the ^{111}In -labeled peptide was cleared from the body predominantly by the kidney, 50% within the first 4.5 h; however, by 4 h postinjection, about 7% of the injected radioactivity was taken up into the renal parenchyma, and about 5% remained at 48 h, which eventually reduced the sensitivity of detection for small

^{111}In -DTPA-D-Phe-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr(ol)

^{111}In -DTPA-D-Phe¹-octreotide

^{111}In -DTPA-L-Phe-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr(ol)

^{111}In -DTPA-L-Phe¹-octreotide

Figure 1. Chemical structures of ^{111}In -DTPA-D-Phe¹-octreotide and ^{111}In -DTPA-L-Phe¹-octreotide.

tumors located in the perirenal region in the abdomen (2). Since reduction of the renal radioactivity levels of ^{111}In -DTPA-D-Phe¹-octreotide would markedly improve diagnostic accuracy, further clarification of the mechanisms responsible for the persistent renal localization of the radioactivity of this compound is warranted.

Recent studies indicated that the renal radioactivity levels of ^{111}In -DTPA-D-Phe¹-octreotide can be reduced by infusion of basic amino acids such as L-lysine (3, 4). A previous study of ^{111}In -DTPA-D-Phe¹-octreotide in rats indicated that the radioactivity is localized in the lysosomal compartments in the pancreas, tumor, and kidney

* To whom correspondence should be addressed at. Phone: 81-75-753-4608. Fax: 81-75-753-4568. E-mail: arano@pharm.kyoto-u.ac.jp.

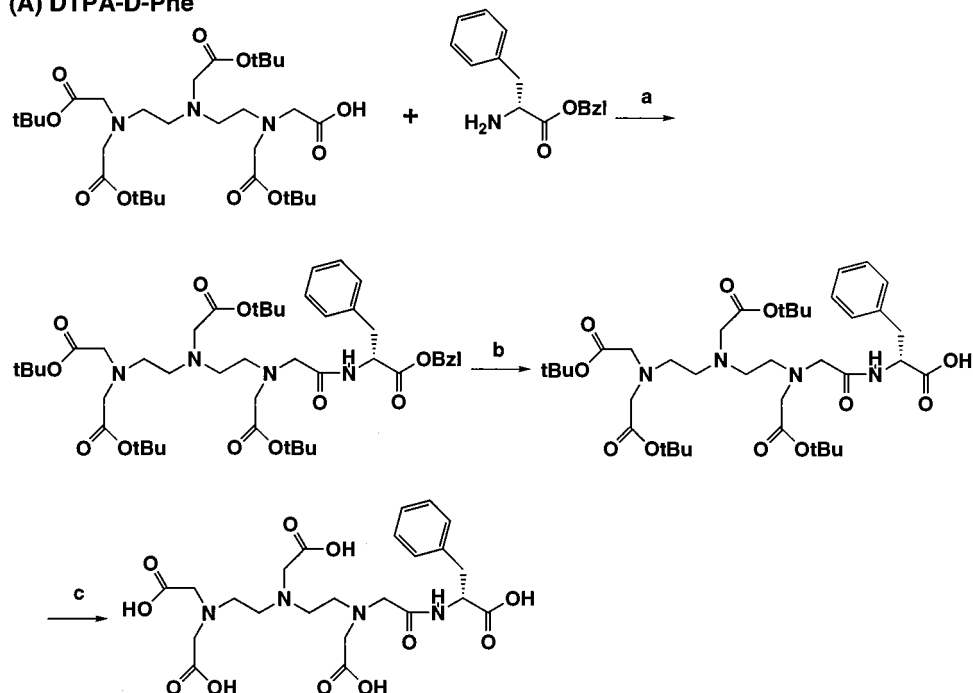
[†] Kyoto University.

[‡] Kyoto Pharmaceutical University.

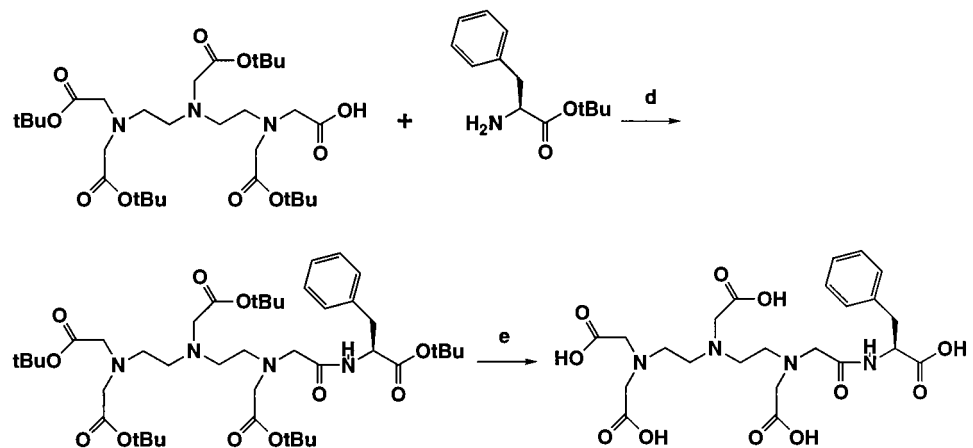
[§] Cancer Institute Hospital.

Scheme 1

(A) DTPA-D-Phe



(B) DTPA-L-Phe



Reagents: (a) dicyclohexylcarbodiimide, *N*-hydroxysuccinimide; (b) H_2 gas, 10 % palladium on carbon; (c) trifluoroacetic acid, anisole; (d) dicyclohexylcarbodiimide, *N*-hydroxysuccinimide; (e) trifluoroacetic acid, anisole

even early postinjection (5). These findings suggested that ^{111}In -DTPA-D-Phe¹-octreotide would be reabsorbed at the renal tubules after glomerular filtration, followed by transfer to the lysosomal compartment, as has been documented with many polypeptides metabolized in the kidney (6, 7).

Although these findings portray *in vivo* behaviors of ^{111}In -DTPA-D-Phe¹-octreotide, further studies are required to determine what happens to the radiolabel after incorporation of this compound in the renal cells. In addition, since the parent peptide, octreotide, is originally designed to possess high metabolic stability against *in vivo* degradation, the metabolic stability of octreotide may also be responsible for the persistent radioactivity levels in the kidney. The answers to these questions would provide an insight for better understanding of the persistent renal radioactivity levels of ^{111}In -DTPA-D-Phe¹-

octreotide and would also contribute to the design of radiolabeled somatostatin analogues more suitable for diagnosis and applicable to therapy.

In the present study, ^{111}In -DTPA-D-Phe¹-octreotide was administered to mice, and the radiolabeled species remaining in the kidney and excreted in the urine were analyzed. To investigate the role played by the metabolic stability of the parent peptide, octreotide, in renal radioactivity levels, the ^{111}In -labeled octreotide analogue, ^{111}In -DTPA-L-Phe¹-octreotide, where the N-terminal D-phenylalanine (D-Phe) of DTPA-D-Phe¹-octreotide was replaced with L-phenylalanine (L-Phe) (Figure 1), was synthesized to facilitate metabolic rate. The biodistribution of the radioactivity and the radiolabeled species remaining in the kidney and excreted in the urine after administration of ^{111}In -DTPA-L-Phe¹-octreotide were analyzed and compared with those of ^{111}In -DTPA-D-Phe¹-

octreotide. The subcellular localization of the radioactivity after administration of each of the two ^{111}In -labeled octreotide analogues was also determined.

MATERIALS AND METHODS

Reagents and Chemicals. $^{111}\text{InCl}_3$ (2.5–129.5 MBq/mL in 0.02 N HCl) was kindly supplied by Nihon-Medical Physics (Tokyo, Japan). Nonradioactive indium chloride was purchased from Nacalai Tesque as $\text{InCl}_3 \cdot 4\text{H}_2\text{O}$ (Kyoto, Japan). Reversed-phase high-performance liquid chromatography (RP-HPLC) was performed with a Cosmosil 5C₁₈-MS column (4.6 $\times$ 150 mm, Nacalai Tesque, Kyoto). ^{111}In -DTPA-D-Phe¹-octreotide was eluted with 5% methanol in 0.05 M acetate buffer (pH 5.5) for the first 15 min, followed by a linear gradient of 5 to 55% methanol in 0.05 M acetate buffer (pH 5.5) in 10 min. The final solvent composition was maintained for a further 25 min. ^{111}In -DTPA-L-Phe¹-octreotide was eluted with 5% methanol in 0.05 M acetate buffer (pH 5.5) for the first 15 min, followed by a linear gradient of 5 to 60% methanol in 0.05 M acetate buffer (pH 5.5) in 10 min. The final solvent composition was maintained for a further 25 min. Cellulose acetate electrophoresis (CAE; Joko Co. Ltd., Tokyo) was run at an electrostatic field of 0.8 mA/cm for 40 min in veronal buffer ($I = 0.06$, pH 8.6; Nacalai Tesque). 1-*tert*-Butyl hydrogen 3,6,9-tris(*tert*-butoxycarbonyl)methyl-3,6,9-triazaundecanedioic acid (mDTPA) was synthesized as reported previously (8). Proton nuclear magnetic resonance (^1H NMR) spectra were recorded on a Bruker AC-200 spectrometer, and the chemical shifts are reported in parts per million downfield from an internal tetramethylsilane standard. Fast-atom bombardment mass spectra (FABMS) were obtained with a JMS-HX/HX110 A model (JEOL Ltd., Tokyo). Other reagents were of reagent grade and used as received. To facilitate collection of urine and feces by 24 h postinjection of ^{111}In -labeled peptides, mice were housed in metabolic cages (Metabolica, MM type; Sugiyama-Gen Iriki Co. Ltd., Tokyo).

DTPA-D-Phe. To a solution of mDTPA (536 mg, 0.868 mmol) and *N*-hydroxysuccinimide (109.84 mg, 0.954 mmol) in 0.5 mL of dimethylformamide (DMF) was added a solution of dicyclohexylcarbodiimide (196.2 mg, 0.954 mmol) in 0.5 mL of DMF at 0 °C, and the reaction mixture was stirred for 4 h at room temperature. After cooling to 0 °C again, a mixed solution of *D*-phenylalanine benzyl ester *p*-toluenesulfonate (370.93 mg, 0.868 mmol) and *N,N*-diisopropylethylamine (112.25 mg, 0.868 mmol) in 0.5 mL of DMF was then added slowly to maintain the temperature. The resulting reaction mixture was warmed to room temperature, and stirring was continued for 18 h. After filtration of the precipitate, the filtrate was extracted with 200 mL of ethyl acetate, and the organic layer was washed with a saturated aqueous solution of NaHCO_3 (150 mL $\times$ 3). After drying over anhydrous calcium sulfate, the solvent was removed in vacuo. The residue was chromatographed on silica gel using a mixture of chloroform-methanol (25:1) as an eluent to yield DTPA-D-Phe with the four carboxylates of DTPA protected with *tert*-butyl groups, while the carboxylate of *D*-Phe was protected with a benzyl group as a pale yellow oil (411.4 mg; 55.5%).

A mixture of the intermediate compound and 10% palladium on carbon (0.14 g) in ethyl acetate (4 mL) was hydrogenated at room temperature for 60 h. The catalyst was removed by filtration, and the filtrate was evaporated in vacuo to provide DTPA-D-Phe with the four carboxylates of DTPA protected with *tert*-butyl groups, as a pale yellow oil (166.8 mg, 45.3%).

A mixture of anisole (51 μL , 0.471 mmol) and trifluoroacetic acid (TFA, 1.5 mL) was added to the final intermediate at 0 °C. The reaction mixture was stirred for 5 min at 0 °C and then warmed to room temperature, and stirring was continued for further 7 h. After removing TFA in vacuo, ether (5 mL) was added to the residue to precipitate DTPA-D-Phe as a colorless solid (43.0 mg, 90.4%). ^1H NMR (DMSO-*d*₆): 2.84–3.20 (10H, m, $\text{CH}_2\text{CH}_2\text{-CH}_2\text{C}_6\text{H}_5$), 3.30 (2H, s, NCH_2CO), 3.31 (2H, s, NCH_2CO), 3.49 (4H, s, NCH_2CO), 3.96 (2H, s, NCH_2CO), 4.42–4.51 (1H, m, NHCH), 7.16–7.31 (5H, m, aromatic), 8.28 (1H, d, CONH). FABMS for $\text{C}_{22}\text{H}_{31}\text{N}_4\text{O}_9$ (MH^+): m/z calcd, 541; found, 541. Anal. Calcd. for $\text{C}_{22}\text{H}_{30}\text{N}_4\text{O}_9 \cdot 3/2\text{H}_2\text{O} \cdot 2/5\text{CF}_3\text{COOH} \cdot 1/5\text{C}_2\text{H}_5\text{OC}_2\text{H}_5$: C, 47.05; H, 6.00; N, 8.92; F, 3.63. Found: C, 47.09; H, 5.85; N, 8.70; F, 3.42.

DTPA-L-Phe. *L*-Phenylalanine *tert*-butyl ester hydrochloride (199.2 mg, 0.818 mmol) and the mDTPA (252.5 mg, 0.409 mmol) were condensed according to the procedure similar to those described above using acetonitrile as a solvent. The reaction gave DTPA-L-Phe with all the carboxylates being protected with *tert*-butyl groups, as a pale yellow oil (97.7 mg, 29.1%). The oily product was treated with TFA-anisole according to the procedure described above, and DTPA-L-Phe was obtained as a colorless solid (41.8 mg, 81.3%). ^1H NMR (DMSO-*d*₆): 2.84–3.17 (10H, m, $\text{CH}_2\text{CH}_2\text{-CH}_2\text{C}_6\text{H}_5$), 3.26 (2H, s, NCH_2CO), 3.29 (2H, s, NCH_2CO), 3.47 (4H, s, NCH_2CO), 3.77 (2H, s, NCH_2CO), 4.41–4.50 (1H, m, NHCH), 7.16–7.30 (5H, m, aromatic), 8.24 (1H, d, CONH). FABMS for $\text{C}_{22}\text{H}_{31}\text{N}_4\text{O}_9$ (MH^+): m/z calcd, 541; found, 541. Anal. Calcd. for $\text{C}_{22}\text{H}_{30}\text{N}_4\text{O}_9 \cdot \text{H}_2\text{O} \cdot 3/10\text{CF}_3\text{COOH} \cdot 1/4\text{C}_2\text{H}_5\text{O-C}_2\text{H}_5$: C, 48.17; H, 6.04; N, 9.11; F, 3.09. Found: C, 48.19; H, 6.01; N, 9.20; F, 2.91.

DTPA-D-Phe¹-Octreotide and DTPA-L-Phe¹-Octreotide. DTPA-D-Phe¹-octreotide was synthesized using mDTPA, as described previously (9). DTPA-L-Phe¹-octreotide was synthesized according to the procedures similar to those used for DTPA-D-Phe¹-octreotide except for the use of *N*^α-Fmoc-L-Phe in place of *N*^α-Fmoc-D-Phe (where Fmoc is 9-fluorenylmethoxycarbonyl). The amino acid ratios after 6 N HCl hydrolysis of DTPA-L-Phe¹-octreotide were as follows: Thr $\times$ 1, 1.03; Phe $\times$ 2, 2.00; Lys $\times$ 1, 1.10. The matrix-assisted laser-desorption/ionization time-of-flight mass spectra (Voyager-DE RP; PerSeptive Biosystems, Tokyo) for $\text{C}_{63}\text{H}_{88}\text{N}_{13}\text{O}_{19}\text{S}_2$ (MH^+): m/z calcd, 1394.57; found, 1394.94.

Radiolabeling of DTPA-D-Phe¹-Octreotide and DTPA-L-Phe¹-Octreotide. Two lyophilized kits (one containing DTPA-D-Phe¹-octreotide, 10 μg , and the other containing DTPA-L-Phe¹-octreotide, 10 μg), with trisodium citrate (4.91 mg), citric acid (0.37 mg), inositol (10.0 mg), and gentisic acid (2.0 mg) were prepared using ultrapure water (Milli Q, Millipore Japan, Tokyo). The ^{111}In radiolabeling of DTPA-conjugated octreotide analogues was performed by addition of 1 mL solution of $^{111}\text{InCl}_3$ (2.5–129.5 MBq) to each kit. After standing at room temperature for 1 h, ^{111}In -DTPA-D-Phe¹-octreotide and ^{111}In -DTPA-L-Phe¹-octreotide were each subjected to RP-HPLC purification to exclude non-peptide-bound ^{111}In species and unchelated peptides. An appropriate amount of DTPA-D-Phe¹-octreotide or DTPA-L-Phe¹-octreotide was then added to fractions containing the respective ^{111}In -labeled peptide, and the solvent was removed in vacuo. A HEPES [2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid]-buffered solution containing 0.1% human serum albumin (HSA) was added to the residue to prepare ^{111}In -labeled octreotide analogues each containing 0.1 μg of the DTPA-peptide in 100 μL for biodistribution studies and 150 μL for metabolic

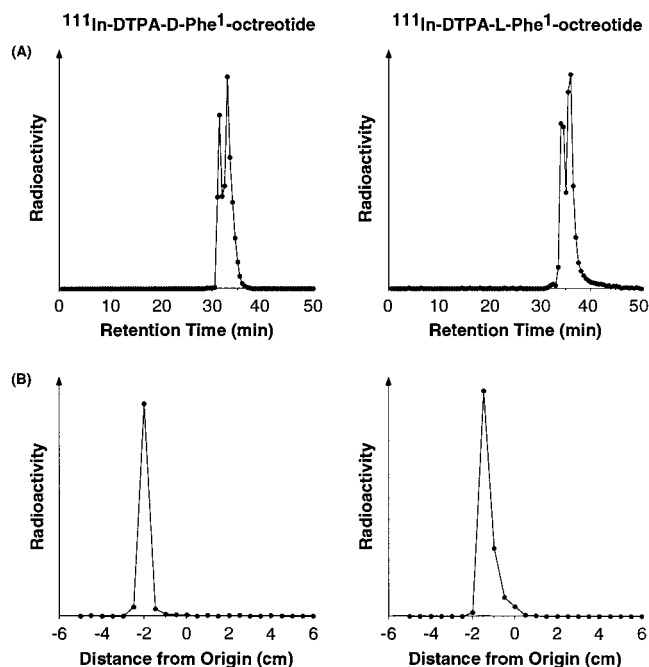


Figure 2. Radioactivity profiles of ^{111}In -DTPA-D-Phe¹-octreotide and ^{111}In -DTPA-L-Phe¹-octreotide by (A) RP-HPLC and (B) CAE.

studies. The radiochemical purities of ^{111}In -DTPA-D-Phe¹-octreotide and ^{111}In -DTPA-L-Phe¹-octreotide exceeded 96% as determined by RP-HPLC and CAE analyses. Under the analytical conditions, both ^{111}In -DTPA-D-Phe¹-octreotide and ^{111}In -DTPA-L-Phe¹-octreotide showed two radioactivity peaks on the RP-HPLC (Figure 2), as reported previously (10).

Preparation of ^{111}In -DTPA-D-Phe, ^{111}In -DTPA-L-Phe, and ^{111}In -DTPA. The ^{111}In radiolabeling of DTPA-D-Phe, DTPA-L-Phe, and DTPA was performed according to the procedure described previously (8). Briefly, a solution of sodium acetate (1 M, 10 μL) was mixed with 40 μL of a diluted solution of $^{111}\text{InCl}_3$ (0.37–0.74 MBq/mL) in 0.02 N HCl. After the mixture was allowed to stand for 5 min, a 10 μL aliquot of DTPA-D-Phe, DTPA-L-Phe, or DTPA (20 μM) in 20 mM MES [2-(*N*-morpholino)ethanesulfonic acid]-buffered saline (pH 6.0) was added, and the reaction mixture was incubated at room temperature for 1 h. ^{111}In -DTPA-D-Phe, ^{111}In -DTPA-L-Phe, and ^{111}In -DTPA were obtained with radiochemical yields of over 97% by RP-HPLC and CAE analyses and used without further purification. ^{111}In -DTPA-D-Phe gave two peaks at retention times of 5 and 10 min on RP-HPLC, whereas ^{111}In -DTPA-L-Phe gave two peaks at retention times of 6 and 11 min. On the other hand, both ^{111}In -DTPA-D-Phe and ^{111}In -DTPA-L-Phe gave a single peak at a migration distance of 2 cm to the anode by CAE analysis. ^{111}In -DTPA had a retention time of 2 min on the RP-HPLC and showed a single peak at a migration distance of 2.5 cm to the anode by CAE analysis.

To further characterize the standards, nonradioactive indium was reacted with DTPA-D-Phe or DTPA-L-Phe as follows: a solution of $\text{InCl}_3 \cdot 4\text{H}_2\text{O}$ (3.4 mg/100 μL) in 0.05 M acetic acid was added to a solution of DTPA-D-Phe or DTPA-L-Phe (6.2 mg/100 μL) in 0.05 M acetic acid. After incubation for 30 min at room temperature, the reaction mixture was analyzed by FABMAS and RP-HPLC under the conditions described above. FABMAS of both In -DTPA-D-Phe and In -DTPA-L-Phe showed a peak at m/z 653 in the positive ion mode and 651 in the negative ion mode, respectively. The RP-HPLC trace (254 nm) of In -

DTPA-L-Phe showed two peaks at retention times of 6 and 11 min, while that of In -DTPA-D-Phe showed two peaks at retention times of 5 and 10 min. RP-HPLC analysis of one component (5 min) of In -DTPA-D-Phe showed two peaks at retention times of 5 and 10 min, indicating that In -DTPA-D-Phe eluted as well-separated isomers under the analytical conditions. Similar findings were obtained with another component of In -DTPA-D-Phe and with each component of In -DTPA-L-Phe.

Serum Stability of ^{111}In -DTPA-D-Phe¹-Octreotide and ^{111}In -DTPA-L-Phe¹-Octreotide. Aliquots of 1.5 μL solutions of ^{111}In -DTPA-D-Phe¹-octreotide or ^{111}In -DTPA-L-Phe¹-octreotide (1 ng, 71.7 kBq) were added to freshly prepared murine serum (400 μL), and the mixtures were incubated at 37 $^\circ\text{C}$. After incubation for 1 and 3 h, 50 μL aliquots of the samples were drawn, and the radioactivity was analyzed by CAE. The radioactivity in the serum was also analyzed by RP-HPLC after filtering through a 10 kDa cutoff ultrafiltration membrane (Microcon-10, Amicon Inc., Beverly, MA).

In Vivo Studies. Biodistributions of radioactivity after intravenous administration of ^{111}In -DTPA-D-Phe¹-octreotide or ^{111}In -DTPA-L-Phe¹-octreotide into 6-week-old male ddY normal mice (11) weighing 28–30 g were monitored at 10 and 30 min and 1, 3, 6, and 24 h postinjection. Groups of five mice which received 0.1 μg of radiolabeled peptide were used for the experiments. The organs of interest were removed and weighed, and the radioactivity was determined with a well counter (Aloka ARC 2000, Tokyo).

To determine the amounts and routes of the radioactivity excreted from the body, mice were housed in metabolic cages for 24 h after administration of the respective preparations, and urine and feces were collected and the radioactivity was determined.

The radiolabeled species remaining in the kidney at 1, 3, and 24 h postinjection and excreted in the urine by 24 h postinjection were analyzed according to the procedure described previously (12). Briefly, each ^{111}In -labeled octreotide analogue (0.1 μg , 37–45 MBq) was administered intravenously into 6-week-old male ddY mice. At 1, 3, and 24 h postinjection, both kidneys were perfused in situ with cold 0.1 M Tris-citrate buffer (pH 6.5) containing 0.15 M NaCl, 0.02% sodium azide, 1 TIU/mL aprotinin, 2 mM benzamidine-HCl, 2 mM iodoacetamide, 1 mM phenylmethanesulfonyl fluoride, and 5 mM diisopropyl fluorophosphate before being removed. Each tissue sample was placed in a test tube and subjected to three cycles of freezing (dry ice–acetone bath) and thawing. After addition of 5 vol of the same buffer containing an additional 35 mM β -octyl-glucoside, the sample was homogenized with a Polytron homogenizer (PT10-35, Kinematica GmgH Littau, Switzerland) at full speed with three consecutive 30 s bursts on ice before centrifugation at 48000g for 20 min at 4 $^\circ\text{C}$ (Himac CS-120 Centrifuge, Hitachi Co., Tokyo). The supernatant was separated from the pellet, and the radioactivity was counted. The kidney supernatant and the urine samples were analyzed immediately by CAE after filtration through a polycarbonate membrane with a pore diameter of 0.45 μm (Myrex, Millipore). The samples were also analyzed immediately by RP-HPLC after filtering through a 10 kDa cutoff ultrafiltration membrane (Microcon-10, Amicon). Each analysis was also performed by co-chromatography with ^{111}In -DTPA-D-Phe, ^{111}In -DTPA-L-Phe, or ^{111}In -DTPA.

The subcellular distribution of radioactivity in murine kidney after administration of ^{111}In -DTPA-D-Phe¹-octreotide or ^{111}In -DTPA-L-Phe¹-octreotide was determined

by perfusing the organ in situ with cold 0.25 M sucrose buffered with 10 mM phosphate buffer (pH 7.4) at 1 and 24 h postinjection. The kidney was treated according to the procedure described previously (13). Briefly, the kidney was suspended in 4 vol of 0.25 M sucrose buffered with 10 mM phosphate buffer (pH 7.4) and homogenized with a Dounce homogenizer by hand (20 strokes), followed by two final strokes in an ice-cooled Potter-Elvehjem homogenizer at 800 rpm. The 20% homogenate was centrifuged twice for 5 min each time at 340g for 4 °C. The isolated supernatant (0.45 mL) was layered onto iso-osmotic (0.25 M sucrose) 37.5% Percoll (9 mL, Pharmacia Biotech Co., Tokyo) at a density of 1.08 g/mL. After centrifugation at 20000g (RP 30 Rotar; Hitachi Co., Tokyo) for 90 min at 4 °C, the gradient (1.02 to 1.16 g/mL) was collected in 14 fractions before analyses of β -galactosidase (14), alkaline phosphodiesterase I activities (15), density, and the radioactivity of each fraction.

Statistical Analyses. Data are expressed as means (standard deviation) where appropriate. The results were analyzed using the unpaired Mann-Whitney test. Differences between $^{111}\text{In-DTPA-L-Phe}^1\text{-octreotide}$ and $^{111}\text{In-DTPA-D-Phe}^1\text{-octreotide}$ results were considered significant when the *p* value was less than 0.05.

RESULTS

Serum Stability. When incubated in freshly prepared murine serum at 37 °C for 3 h, 95.5 and 96.5% of the initial radioactivity were detected by RP-HPLC as the intact form for $^{111}\text{In-DTPA-D-Phe}^1\text{-octreotide}$ and $^{111}\text{In-DTPA-L-Phe}^1\text{-octreotide}$, respectively. No significant differences were observed in the serum stability between the two $^{111}\text{In-DTPA}$ -conjugated octreotide analogues. Similar results were obtained with CAE analyses.

In Vivo Studies. The biodistributions of radioactivity after administrations of $^{111}\text{In-DTPA-D-Phe}^1\text{-octreotide}$ and $^{111}\text{In-DTPA-L-Phe}^1\text{-octreotide}$ are shown in Table 1. Both $^{111}\text{In-DTPA}$ -conjugated peptides displayed rapid clearance of the radioactivity from the blood with similar elimination rates. The two peptides also showed similar tissue distributions of the radioactivity from 10 min to 6 h postinjection, although slight differences were observed in some tissues. At 24 h postinjection, no significant differences were observed in the radioactivity levels remaining in the tissues and excreted from the body between the two peptides.

The supernatant of kidney homogenate was extracted with efficiencies of over 90% for both $^{111}\text{In-DTPA-D-Phe}^1\text{-octreotide}$ and $^{111}\text{In-DTPA-L-Phe}^1\text{-octreotide}$ at all postinjection times. The RP-HPLC radioactivity profiles of the kidney supernatants at 1, 3, and 24 h postinjection and those of the urine by 24 h postinjection of the two $^{111}\text{In-DTPA}$ -peptides are shown in Figure 3. In the kidney, $^{111}\text{In-DTPA-D-Phe}^1\text{-octreotide}$ was gradually metabolized to the final radiometabolite via various intermediate species. At 24 h postinjection, the major radioactivity in the kidney had retention times identical to those of $^{111}\text{In-DTPA-D-Phe}$, which was confirmed by co-chromatographic analysis using $^{111}\text{In-DTPA-D-Phe}$ as a standard. Even at this postinjection time point, the intermediate radiolabeled species were still observed in the kidney. RP-HPLC analysis of the urine by 24 h postinjection of $^{111}\text{In-DTPA-D-Phe}^1\text{-octreotide}$ showed that the major radioactivity was observed with retention times identical to those of the intact $^{111}\text{In-DTPA-D-Phe}^1\text{-octreotide}$. Similar results were obtained by CAE analyses of the kidney homogenates. The radiolabeled species in the kidney and

Table 1. Biodistribution of Radioactivity after Intravenous Administration of ^{111}In -Labeled-Octreotide Analogues in Mice^a

organs	time after administration					
	10 min	30 min	1 h	3 h	6 h	24 h
$^{111}\text{In-DTPA-D-Phe}^1\text{-Octreotide}$						
blood	4.11 (0.50)	1.71 (0.14)	0.51 (0.07)	0.16 (0.01)	0.06 (0.01)	0.03 (0.01)
liver	2.24 (0.13)	1.52 (0.32)	1.65 (0.55)	1.48 (0.32)	1.07 (0.51)	0.81 (0.04)
kidney	20.24 (5.97)	22.90 (4.72)	19.73 (4.94)	16.91 (2.45)	13.96 (3.15)	2.93 (0.72)
intestine	1.07 (0.16)	0.71 (0.07)	0.54 (0.12)	0.80 (0.10)	0.92 (0.18)	1.45 (0.62)
spleen	2.04 (0.14)	1.70 (0.75)	1.48 (0.55)	2.06 (0.69)	1.22 (0.66)	1.17 (0.31)
pancreas	2.31 (0.09)	1.16 (0.08)	0.59 (0.06)	0.35 (0.08)	0.31 (0.08)	0.15 (0.08)
lung	4.82 (0.44)	2.78 (0.72)	1.18 (0.18)	0.76 (0.23)	0.55 (0.07)	0.39 (0.11)
urine ^b						58.40 (2.66)
feces ^b						19.00 (0.53)
$^{111}\text{In-DTPA-L-Phe}^1\text{-Octreotide}$						
blood	4.49 (0.25)	2.02 (0.38)	0.53 (0.11)	0.08 ^c (0.01)	0.05 (0.01)	0.03 (0.01)
liver	2.06 (0.17)	1.85 (0.37)	1.26 (0.13)	1.48 (0.24)	1.50 (0.47)	0.81 (0.13)
kidney	17.71 (4.05)	18.28 (2.99)	15.89 (3.45)	12.82 ^c (1.80)	9.44 (1.80)	2.83 (1.00)
intestine	0.91 (0.05)	0.72 (0.11)	0.66 (0.24)	0.67 (0.10)	1.03 (0.31)	0.87 (0.54)
spleen	3.18 (0.95)	2.23 (0.57)	1.74 (0.62)	1.88 (0.58)	1.31 (0.54)	1.21 (0.82)
pancreas	1.67 ^d (0.29)	1.04 (0.17)	0.43 ^c (0.03)	0.20 ^c (0.04)	0.14 ^c (0.05)	0.16 (0.01)
lung	4.74 (0.63)	2.59 (0.27)	0.85 (0.08)	0.47 (0.16)	0.33 (0.12)	0.27 (0.05)
urine ^b						57.54 (5.64)
feces ^b						22.81 (4.08)

^a Expressed as a percentage of injected dose per gram. Mean (sd) of five animals for each point. ^b Expressed as a percentage of injected dose (sd). Differences between $^{111}\text{In-DTPA-L-Phe}^1\text{-octreotide}$ and $^{111}\text{In-DTPA-D-Phe}^1\text{-octreotide}$ were analyzed by the unpaired Mann-Whitney test. ^c *p* < 0.05. ^d *p* < 0.01.

urine determined by RP-HPLC are summarized in Table 2, where the radiolabeled species are classified into four groups; intact $^{111}\text{In-DTPA-D-Phe}^1\text{-octreotide}$, intermediate species eluted at the retention times between the intact peptide and the final radiometabolite, final radiometabolite (coeluted with $^{111}\text{In-DTPA-D-Phe}$), and others that represented radiolabeled species eluted earlier than the final radiometabolite (e.g., $^{111}\text{In-DTPA}$). At 3 h postinjection, the intermediate radiolabeled species represented about 60% of the radioactivity in the kidney. At 24 h postinjection, about 77% of the radioactivity in the kidney was present as the final radiometabolite. In the urine, over 85% and about 10% of the radioactivity was associated with the intact peptide and the intermediate species, respectively, whereas the final radiometabolite represented only about 3% of the radioactivity.

The radiolabeled species found in the kidney at 1, 3, and 24 h postinjection of $^{111}\text{In-DTPA-L-Phe}^1\text{-octreotide}$ are illustrated in Figure 3. The radioactivity profiles for the kidney supernatants showed much less radiolabeled species when compared with those of $^{111}\text{In-DTPA-D-Phe}^1\text{-octreotide}$. At 3 h postinjection, most of the radioactivity was detected at retention times identical to those of $^{111}\text{In-DTPA-L-Phe}$, which was confirmed by the co-

Table 2. Radiolabeled Species in the Kidney and Urine after Administration of ^{111}In -DTPA-D-Phe¹-Octreotide in Mice

fraction	percentage of radioactivity ^a			
	kidney (1 h)	kidney (3 h)	kidney (24 h)	urine (24 h)
intact	58.08 (0.58)	20.03 (1.96)	1.87 (0.75)	85.46 (0.31)
intermediates	36.39 (0.44)	57.69 (0.38)	17.71 (0.23)	10.13 (0.22)
final metabolite	3.86 (0.04)	18.30 (0.61)	77.47 (0.35)	3.28 (0.13)
others ^b	0.56 (0.01)	0.99 (0.55)	2.36 (0.23)	0.99 (0.17)

^a Radiolabeled species in each fraction determined by RP-HPLC analyses. Mean (sd) of three experiments. ^b Radiolabeled species eluted earlier times than ^{111}In -DTPA-D-Phe (e.g. ^{111}In -DTPA).

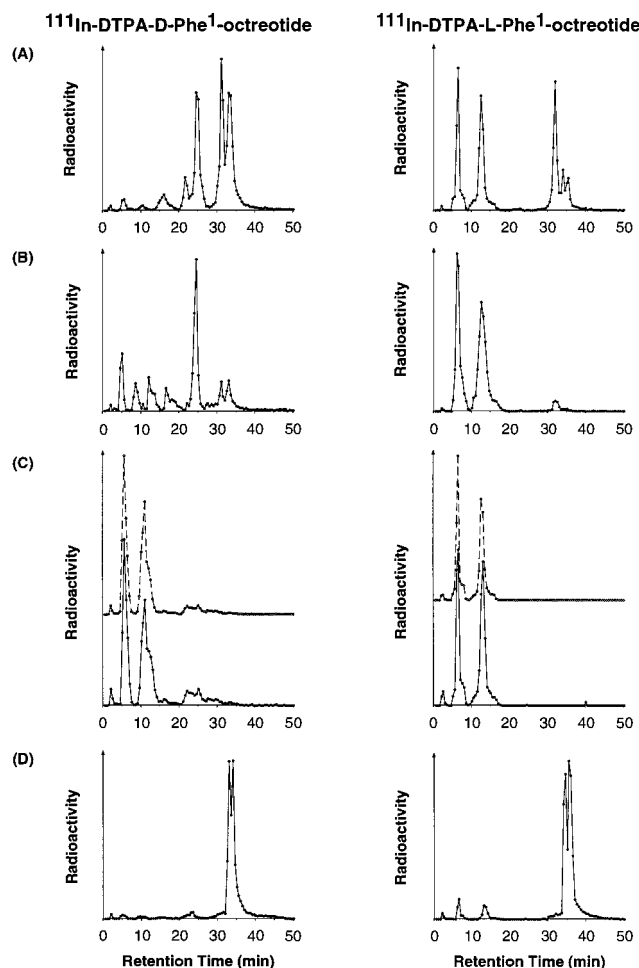


Figure 3. RP-HPLC radioactivity profiles of kidney homogenates at 1 (A), 3 (B), and 24 h (C) and urine samples by 24 h (D) postinjection of ^{111}In -DTPA-D-Phe¹-octreotide and ^{111}In -DTPA-L-Phe¹-octreotide. The kidney homogenates at 24 h (C) were eluted in the absence (●) or in the presence (○) of ^{111}In -DTPA-D-Phe or ^{111}In -DTPA-L-Phe for ^{111}In -DTPA-D-Phe¹-octreotide or ^{111}In -DTPA-L-Phe¹-octreotide, respectively.

chromatographic analysis using ^{111}In -DTPA-L-Phe as a standard. At 24 h, the radioactivity profiles of the kidney were almost identical to those at 3 h except that the radioactivity peak at around 32 min disappeared and a new small peak was observed at a retention time of 2 min. The analysis of urine by 24 h postinjection of ^{111}In -DTPA-L-Phe¹-octreotide showed that the major radioactivity was observed at retention times identical to those of the intact ^{111}In -DTPA-L-Phe¹-octreotide, and small radioactivity peaks corresponding to the final radiometabolites were also detected. Similar results were obtained by CAE analyses of the kidney homogenates. The radiolabeled species in the kidney and the urine determined by RP-HPLC are summarized in Table 3. The final radiometabolite represented over 57 and 93% of the radioactivity in the kidney at 1 and 3 h

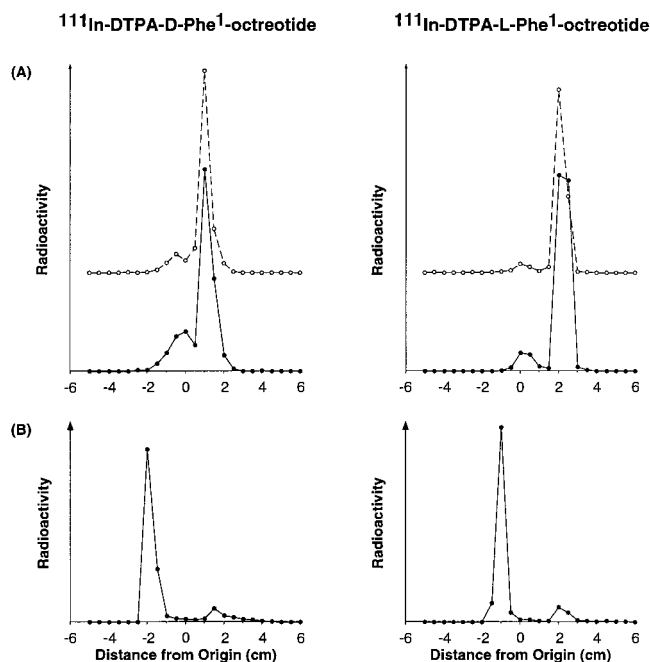


Figure 4. CAE radioactivity profiles of kidney homogenates at 24 h (A) and urine samples by 24 h (B) postinjection of ^{111}In -DTPA-D-Phe¹-octreotide and ^{111}In -DTPA-L-Phe¹-octreotide. The kidney homogenates were co-analyzed in the absence (●) or in the presence (○) of ^{111}In -DTPA-D-Phe or ^{111}In -DTPA-L-Phe for ^{111}In -DTPA-D-Phe¹-octreotide or ^{111}In -DTPA-L-Phe¹-octreotide, respectively.

postinjection, respectively. At 24 h postinjection, the final radiometabolite represented over 96% of the radioactivity in the kidney. However, the intact peptide was the predominant radiolabeled species in the urine where the final radiometabolite represented less than 10% of the radioactivity.

Figure 4 illustrates the CAE radioactivity profiles of the kidney supernatant obtained at 24 h postinjection and the urine samples collected by 24 h postinjection of ^{111}In -DTPA-D-Phe¹-octreotide and ^{111}In -DTPA-L-Phe¹-octreotide, respectively. In the kidney supernatant, two radioactivity peaks were observed and the majority of the radioactivity was detected at a position similar to that of ^{111}In -DTPA-D-Phe or ^{111}In -DTPA-L-Phe. Analyses of urine samples indicated one major and one minor radioactivity peak with the majority of the radioactivity being detected at positions similar to those of the intact ^{111}In -labeled peptides.

The Percoll density gradient centrifugation profiles of the radioactivity in the kidney at 1 and 24 h postinjection of ^{111}In -DTPA-D-Phe¹-octreotide and ^{111}In -DTPA-L-Phe¹-octreotide are illustrated in Figure 5. Each kidney homogenate indicated a similar radioactivity profile with a major radioactivity peak being observed at a density of about 1.10 g/mL, which correlated well with the β -galactosidase activity profiles.

Table 3. Radiolabeled Species in the Kidney and Urine after Administration of ^{111}In -DTPA-L-Phe¹-Octreotide in Mice

fraction	percentage of radioactivity ^a			
	kidney (1 h)	kidney (3 h)	kidney (24 h)	urine (24 h)
intact	14.36 (0.26)	0.67 (0.52)	0.15 (0.22)	85.41 (1.83)
intermediates	25.77 (0.62)	3.88 (0.21)	0.00 (0.01)	3.04 (0.26)
final metabolite	57.14 (0.69)	93.65 (0.42)	96.55 (0.03)	9.54 (0.77)
others ^b	0.65 (0.04)	0.54 (0.02)	3.17 (0.20)	1.34 (0.22)

^a Radiolabeled species in each fraction determined by RP-HPLC analyses. Mean (sd) of three experiments. ^b Radiolabeled species eluted earlier times than ^{111}In -DTPA-L-Phe (e.g., ^{111}In -DTPA).

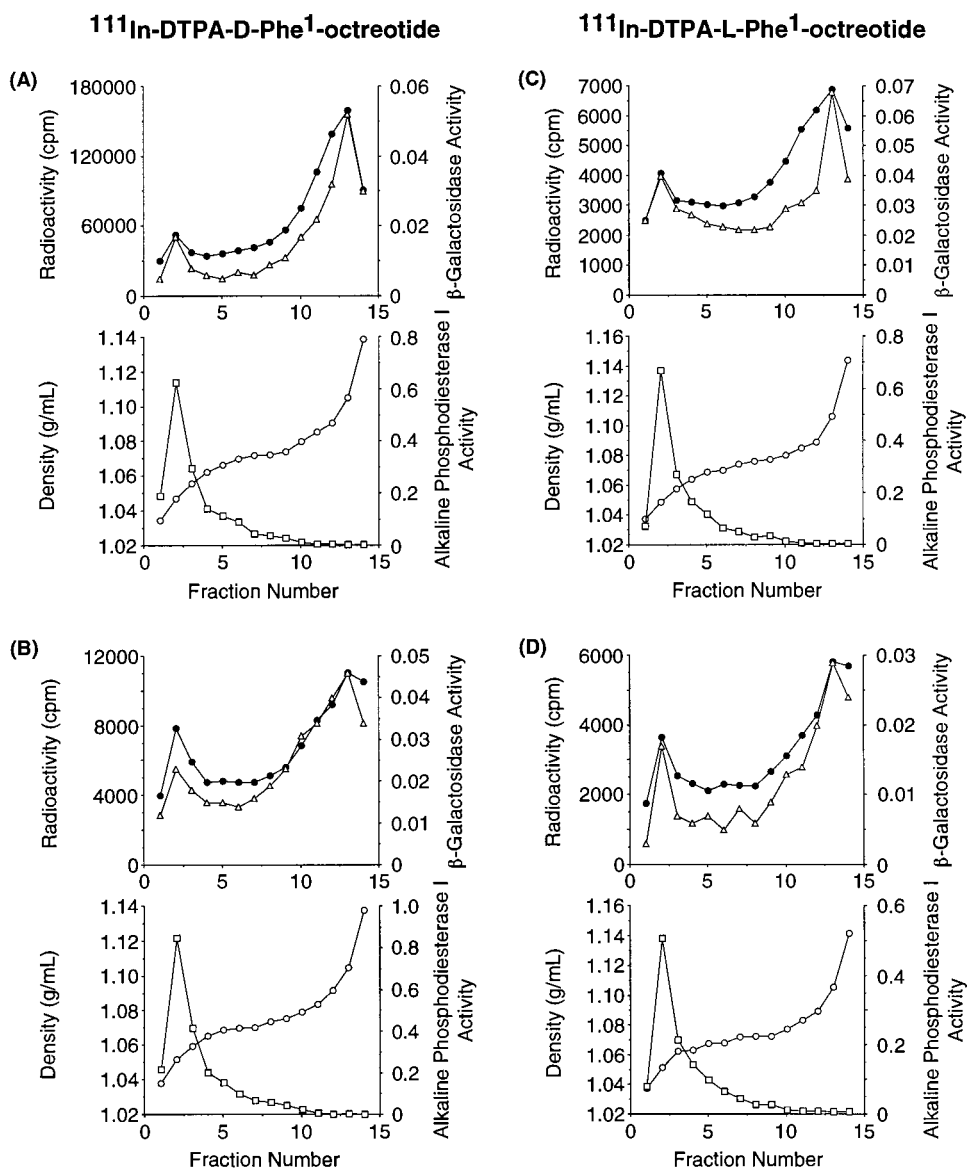


Figure 5. Subcellular fractionation of the kidney at 1 (A) and 24 h (B) postinjection of ^{111}In -DTPA-D-Phe¹-octreotide and 1 (C) and 24 h (D) postinjection of ^{111}In -DTPA-L-Phe¹-octreotide in mice. Fractions were collected from the top of the gradient and assayed for radioactivity (●); lysosomal enzyme, β -galactosidase (Δ); plasma membrane enzyme, alkaline phosphodiesterase I ($\square$); density ($\circ$).

DISCUSSION

To identify the fate of the radiolabel after incorporation of ^{111}In -DTPA-D-Phe¹-octreotide in the renal cells, radiolabeled species remaining in the kidney and excreted in the urine were investigated after administration of ^{111}In -DTPA-D-Phe¹-octreotide and ^{111}In -DTPA-L-Phe¹-octreotide in experimental animals. After radiolabeling with ^{111}In , free DTPA-peptides were separated by RP-HPLC to prepare ^{111}In -DTPA-D-Phe¹-octreotide and ^{111}In -DTPA-L-Phe¹-octreotide with similar specific activity and radiochemical purity for subsequent studies.

The unchanged radioactivity profiles of ^{111}In -DTPA-D-

Phe¹-octreotide and ^{111}In -DTPA-L-Phe¹-octreotide after incubation in freshly prepared murine serum indicated that both ^{111}In -DTPA-conjugated octreotide analogues remained stable against transchelation with serum proteins. These findings also demonstrated that the replacement of the N-terminal D-Phe of ^{111}In -DTPA-D-Phe¹-octreotide with L-Phe did not result in any proteolytic degradation by serum enzymes.

In mouse biodistribution studies, both ^{111}In -DTPA-D-Phe¹-octreotide and ^{111}In -DTPA-L-Phe¹-octreotide showed rapid clearance of the radioactivity from the blood at similar rates (Table 1). In light of the high serum

stability and rapid blood clearance of the two ^{111}In -labeled peptides (Table 1), both ^{111}In -DTPA-D-Phe¹-octreotide and ^{111}In -DTPA-L-Phe¹-octreotide appeared to accumulate in the kidney and other tissues as their intact structures. Thus, the replacement of the N-terminal D-Phe of ^{111}In -DTPA-D-Phe¹-octreotide with L-Phe induced little changes in the *in vivo* behavior. This was supported by the similar amounts and routes of the radioactivity excreted from the body by 24 h postinjection of the ^{111}In -DTPA-peptides. In addition, ^{111}In -DTPA-D-Phe¹-octreotide and ^{111}In -DTPA-L-Phe¹-octreotide demonstrated similar subcellular localization patterns of the radioactivity at 1 and 24 h postinjection (Figure 5). The major radioactivity peak was observed in the lysosomal compartment, reinforcing that ^{111}In -DTPA-D-Phe¹-octreotide is rapidly delivered to the lysosomal compartment after incorporation into renal cells (5).

However, marked differences were observed between the two ^{111}In -labeled peptides when the radiolabeled species remaining in the kidney were compared. While ^{111}In -DTPA-D-Phe¹-octreotide and ^{111}In -DTPA-L-Phe¹-octreotide generated ^{111}In -DTPA-D-Phe and ^{111}In -DTPA-L-Phe, respectively, as the final radiometabolite in the kidney (Figures 3 and 4), different metabolic rates of the two ^{111}In -labeled peptides were observed in the kidney (Tables 2 and 3). ^{111}In -DTPA-D-Phe¹-octreotide was degraded to ^{111}In -DTPA-D-Phe via various intermediate species that represented over 57% of the radioactivity in the kidney even at 3 h postinjection. In contrast, the metabolic rate of ^{111}In -DTPA-L-Phe¹-octreotide was so rapid that over 93% of the radioactivity in the kidney was in the form of the final radiometabolite at the same postinjection timepoint. In addition, ^{111}In -DTPA-L-Phe¹-octreotide generated much fewer intermediate radiometabolites in the kidney. Thus, while replacement of the N-terminal D-Phe of ^{111}In -DTPA-D-Phe¹-octreotide with L-Phe preserved the metabolic stability in serum, this modification markedly impaired the metabolic stability of the resulting peptide in the lysosomes. The presence of the D-Phe-L-Cys sequence in ^{111}In -DTPA-D-Phe¹-octreotide was probably resistant to lysosomal proteolysis, which would have produced multiple intermediate radiometabolites along with the slow generation rate of the final radiometabolite. This was supported by recent findings of Bass et al. who demonstrated that the major intermediate species of ^{111}In -DTPA-D-Phe¹-octreotide in the rat kidney was ^{111}In -DTPA-D-Phe-Cys (16). When the N-terminal D-Phe was replaced with L-Phe, lysosomal proteolysis of ^{111}In -DTPA-L-Phe¹-octreotide proceeded much more rapidly, although ^{111}In -DTPA chelate was attached to the N-terminal L-Phe. These findings suggested that replacement of the N-terminal D-amino acid with the L-form would facilitate lysosomal proteolysis without impairing serum stability when ^{111}In -DTPA chelate is attached to the α -amino group of the N-terminal amino acid of peptides.

Despite the significant differences in the metabolic rates between the two ^{111}In -labeled peptides, only a slight difference was observed in the radioactivity levels between ^{111}In -DTPA-D-Phe¹-octreotide and ^{111}In -DTPA-L-Phe¹-octreotide in the kidney (Table 1). In addition, similar amounts of radioactivity were retained in the kidney at 24 h and excreted in the urine by 24 h postinjection of the respective ^{111}In -labeled peptide. The intact ^{111}In -labeled peptide represented as much as 85% of the radiolabeled species in both urine samples (Tables 2 and 3). Thus, while replacement of the N-terminal D-Phe with L-Phe accelerated the metabolic rate of ^{111}In -labeled octreotide analogues in the lysosomes, little

improvement was achieved in the renal radioactivity levels by this modification, due to the slow elimination rate of the final radiometabolite, ^{111}In -DTPA-L-Phe, from the renal cells.

Recent metabolic studies of ^{111}In -DTPA-labeled polypeptides in the liver indicated that the final radiometabolites of the polypeptides were observed as the ^{111}In -DTPA-adduct of L-lysine, and that the radiometabolite is retained in the lysosomal compartment of the tissues for a long time postinjection (17–19). A previous study by Rogers et al. also suggested that even when ^{111}In -DTPA was attached to an α amino group of the N-terminal aspartic acid of an antibody, ^{111}In -DTPA-aspartic acid was generated as the final radiometabolite and the radiometabolite showed persistent localization within the tissues (20). In the present study, ^{111}In -DTPA-D-Phe and ^{111}In -DTPA-L-Phe were generated as the final radiometabolites after administration of ^{111}In -DTPA-D-Phe¹-octreotide and ^{111}In -DTPA-L-Phe¹-octreotide, respectively, and both radiometabolites remained in the kidney for long periods postinjection. Thus, although the slow metabolic rate of ^{111}In -DTPA-D-Phe¹-octreotide may be partially involved, the generation of the ^{111}In -DTPA-adduct of an amino acid as the final radiometabolite would be predominantly responsible for the prolonged retention of the radioactivity in the kidney. Since both ^{111}In -DTPA-L-Phe and ^{111}In -DTPA-D-Phe were retained in the kidney, the generation of D-amino acid adduct of ^{111}In -DTPA would have little effect on the radioactivity levels in the kidney. These findings suggested that the ^{111}In -DTPA-adduct of acidic, basic, and aromatic amino acid would be barely excreted extracellularly. However, a recent study by Wu et al. indicated that when a ^{67}Ga -labeled antibody fragment using 2-(*p*-isothiocyanatobenzyl)-1,4,7-triazacyclononane-1,4,7-triacetic acid (SCN-Bz-NOTA) as the bifunctional chelating agent was administered, methionine-SCN-Bz-NOTA- ^{67}Ga and lysine-SCN-Bz-NOTA- ^{67}Ga were generated as radiometabolites in the kidney and the former had significantly shorter residence time than the latter in mouse renal cells (21). Thus, further studies are warranted to elucidate whether the long residence times of ^{111}In -DTPA-adduct of amino acids are attributable to inherent characteristics of ^{111}In -DTPA chelate or to the biological characteristics of amino acids conjugated with ^{111}In -DTPA chelate. Such studies would provide an insight for design of ^{111}In -labeled peptides and polypeptides using DTPA as the chelating agent.

In conclusion, the present findings combined with those of previous studies (3–5, 16) indicated the following *in vivo* behavior of ^{111}In -DTPA-D-Phe¹-octreotide: after administration, the majority of ^{111}In -DTPA-D-Phe¹-octreotide is excreted into urine as the intact structure following glomerular filtration. However, some of the glomerularly filtered ^{111}In -DTPA-D-Phe¹-octreotide is reabsorbed into the renal parenchyma and transported to the lysosomal compartment. Although the transportation of ^{111}In -DTPA-D-Phe¹-octreotide to the lysosomal compartment proceeds rapidly, the ^{111}In -labeled peptide is degraded to the final radiometabolite, ^{111}In -DTPA-D-Phe, slowly, presumably due to the presence of the D-Phe-L-Cys sequence in the molecule. The final radiometabolite is excreted from the lysosomal compartment of the renal cells at a slow rate. The latter process would be predominantly responsible for the persistent localization of the radioactivity in the kidney, considering the rapid generation of the radiometabolite from ^{111}In -DTPA-L-Phe¹-octreotide and the slow elimination rate of ^{111}In -DTPA-L-Phe from the renal cells. The present findings

also indicated that while replacement of the N-terminal D-Phe of ^{111}In -DTPA-D-Phe¹-octreotide with L-Phe facilitated metabolic rate in the lysosomes, little improvement was achieved in the renal radioactivity levels. Since ^{111}In -DTPA-D-Phe¹-octreotide is incorporated into tumor tissue (5, 22), ^{111}In -DTPA-D-Phe may also be generated as the final radiometabolite in the tumors. This may account for the prolonged radioactivity levels observed in the tumor tissue. A recent study of radiolabeled antibody fragments suggested that the renal radioactivity levels of radiolabeled polypeptides and peptides may be reduced by releasing radiolabeled compound of urinary excretion by the action of brush border enzymes before they are incorporated into the renal cells (23). Thus, the findings of the present study would facilitate the design of radiolabeled somatostatin analogues with lower renal radioactivity levels without impairment of the tumor radioactivity level.

ACKNOWLEDGMENT

The authors thank Nihon Medi-Physics Co. Ltd. Tokyo, Japan, for their kind gift of $^{111}\text{InCl}_3$. This work was supported in part by Grants-in-Aid for Developing Scientific Research (08557135 and 09672279) from the Ministry of Education, Science, Culture and Sports of Japan.

LITERATURE CITED

- (1) Krenning, E. P., Kwekkeboom, D. J., Bakker, W. H., Breeman, W. A. P., Kooij, P. P. M., Oei, H. Y., van Hagen, M., Postema, P. T. E., de Jong, M., Reubi, J. C., Visser, T. J., Reijs, A. E. M., Hofland, L. J., Koper, J. W., and Lamberts, S. W. J. (1993) Somatostatin receptor scintigraphy with [^{111}In -DTPA-D-Phe¹]- and [^{123}I -Tyr³]-octreotide: The Rotterdam experience with more than 1000 patients. *Eur. J. Nucl. Med.* 20, 716–731.
- (2) Krenning, E. P., Bakker, W. H., Kooij, P. P. M., Breeman, W. A. P., Oei, H. Y., de Jong, M., Reubi, J. C., Visser, T. J., Bruns, C., Kwekkeboom, D. J., Reijs, A. E. M., van Hagen, P. M., Koper, J. W., and Lamberts, S. W. J. (1992) Somatostatin receptor scintigraphy with indium-111-DTPA-D-Phe¹-octreotide in man: metabolism, dosimetry and comparison with iodine-123-Tyr³-octreotide. *J. Nucl. Med.* 33, 652–658.
- (3) Hammond, P. J., Wade, A. F., Gwilliam, M. E., Peters, A. M., Myers, M. J., Gilbey, S. G., Bloom, S. R., and Calam, J. (1993) Amino acid infusion blocks renal tubular uptake of an indium-labeled somatostatin analogue. *Br. J. Cancer* 67, 1437–1439.
- (4) de Jong, M., Rolleman, E. J., Bernard, B. F., Visser, T. J., Bakker, W. H., Breeman, W. A. P., and Krenning, E. P. (1996) Inhibition of renal uptake of indium-111-DTPA-octreotide in vivo. *J. Nucl. Med.* 37, 1388–1392.
- (5) Duncan, J. R., Stephenson, M. T., Wu, H. P., and Anderson, C. J. (1997) Indium-111-diethylenetriaminepentaacetic acid-octreotide is delivered in vivo to pancreatic, tumor cell, renal, and hepatocyte lysosomes. *Cancer Res.* 57, 659–671.
- (6) Bianchi, C., Donadio, C., Tramonti, G., Auner, I., Lorusso, P., Deleide, G., Lunghi, F., and Salvadori, P. (1988) Renal handling of cationic and anionic small proteins: experiments in intact rats. *Contrib. Nephrol.* 68, 37–44.
- (7) Silbernagl, S. (1988) The renal handling of amino acids and oligopeptides. *Physiol. Rev.* 68, 911–1007.
- (8) Arano, Y., Uezono, T., Akizawa, H., Ono, M., Wakisaka, K., Nakayama, M., Sakahara, H., Konishi, J., and Yokoyama, A. (1996) Reassessment of diethylenetriaminepentaacetic acid (DTPA) as a chelating agent for indium-111 labeling of polypeptides using a newly synthesized monoreactive DTPA derivative. *J. Med. Chem.* 39, 3451–3460.
- (9) Arano, Y., Akizawa, H., Uezono, T., Akaji, K., Ono, M., Funakoshi, S., Koizumi, M., Yokoyama, A., Kiso, Y., and Saji, H. (1997) Conventional and high-yield synthesis of DTPA-conjugated peptides: Application of a monoreactive DTPA to DTPA-D-Phe¹-octreotide synthesis. *Bioconjugate Chem.* 8, 442–446.
- (10) Albert, R., Miller, K., Chong, R., Hirth, W., Vanderheyden, J.-L., Deutsch, K., and Deutsch, E. (1992) Characterization of the two diastereomers of octreoscan-111, an In-111 labeled peptide for use in cancer imaging. *J. Nucl. Med.* 33, 900 (abstract).
- (11) Imai, S., Morimoto, J., Tsubura, T., Esaki, K., Michalides, R., Holms, R. S., Deimling, O., and Higlers, J. (1986) Genetic marker patterns and endogenous mammary tumor virus genes in inbred mouse strains in Japan. *Exp. Anim.* 35, 263–276.
- (12) Arano, Y., Mukai, T., Uezono, T., Wakisaka, K., Motonari, H., Akizawa, H., Taoka, Y., and Yokoyama, A. (1994) A biological method to evaluate bifunctional chelating agents to label antibodies with metallic radionuclides. *J. Nucl. Med.* 35, 890–898.
- (13) Arano, Y., Mukai, T., Akizawa, H., Uezono, T., Motonari, H., Wakisaka, K., Kairiyama, C., and Yokoyama, A. (1995) Radiolabeled metabolites of proteins play a critical role in radioactivity elimination from the liver. *Nucl. Med. Biol.* 22, 555–564.
- (14) Wallner, S. J., and Walker, J. E. (1975) Glycosidases in cell wall-degrading extracts of ripening tomato fruits. *Plant Physiol.* 55, 93–98.
- (15) Razzell, W. E. (1963) Phosphodiesterases. *Methods Enzymol.* 6, 236–258.
- (16) Bass, L. A., Lanahan, M. V., Duncan, J. R., Erion, J. L., Srinivasan, A., Schmidt, M. A., and Anderson, C. J. (1998) Identification of the soluble in vivo metabolites of indium-111-diethylenetriaminepentaacetic acid-D-Phe¹-octreotide. *Bioconjugate Chem.* 9, 192–200.
- (17) Arano, Y., Mukai, T., Motonari, H., Akizawa, H., Uezono, T., and Yokoyama, A. (1994) Biological comparison of DTPA and SCN-benzyl-EDTA as chelating agents for indium labeling of antibodies. *J. Labeled Compd. Radiopharm.* 35, 381–383.
- (18) Duncan, J. R., and Welch, M. J. (1993) Intracellular metabolism of indium-111-DTPA-labeled receptor targeted proteins. *J. Nucl. Med.* 34, 1728–1738.
- (19) Franano, F. N., Edwards, W. B., Welch, M. J., and Duncan, J. R. (1994) Metabolism of receptor targeted In-111-DTPA-glycoproteins: identification of ^{111}In -DTPA- ϵ -lysine as the primary metabolic and excretory product. *Nucl. Med. Biol.* 21, 1023–1034.
- (20) Rogers, B. E., Franano, F. N., Duncan, J. R., Edwards, W. B., Anderson, C. J., Connett, J. M., and Welch, M. J. (1995) Identification of metabolites of ^{111}In -diethylenetriaminepentaacetic acid-monoconal antibodies and antibody fragments in vivo. *Cancer Res.* 55 (suppl.), S5714–S5720.
- (21) Wu, C., Jagoda, E., Brechbiel, M., Webber, K. O., Pastan, I., Gansow, O., and Eckelman, W. C. (1997) Biodistribution and catabolism of Ga-67-labeled anti-Tac dsFv fragment. *Bioconjugate Chem.* 8, 365–369.
- (22) Andersson, P., Forsell-Aronsson, E., Johanson, V., Wängberg, B., Nilsson, O., Fjälling, M., and Ahlman, H. (1996) Internalization of indium-111 into human neuroendocrine tumor cells after incubation with indium-111-DTPA-D-Phe¹-octreotide. *J. Nucl. Med.* 37, 2002–2006.
- (23) Arano, Y., Wakisaka, K., Akizawa, H., Ono, M., Fujioka, Y., Uehara, T., Saga, T., Sakahara, H., Konishi, J., and Saji, H. (1998) A chemical strategy to reduce renal radioactivity levels of antibody fragments. *Synthesis and Applications of Isotopes and Isotopically Labeled Compounds 1997*. (J. R. Heys, and D. G. Melillo, Ed.) pp 307–310, John Wiley & Sons, Ltd., Chichester, U.K.

BC9702258

Molecular Hybridization Probes Prepared with 4-Aminooxybutylamine

Vyacheslav A. Adarichev, Sergey M. Kalachikov, Anna V. Kiseliyova, and Grigory M. Dymshits*

Institute of Cytology and Genetics SD RAS, 10 Lavrentyeva Avenue, Novosibirsk 630090, Russia.
Received January 14, 1998; Revised Manuscript Received September 15, 1998

A versatile method is described for preparing nonradioactive DNA probes for molecular hybridization. The method is based on the transamination reaction of double-stranded DNA with 4-aminooxybutylamine (ABA). To optimize the procedure for obtaining stable and sensitive hybridization probes, time of modification, pH, and reaction temperature were varied. The optimal reaction conditions allowed the preparation of nonradioactive-labeled DNA probes that met demands for optimal length, modification degree, stability, and sensitivity. The use of 4-aminooxybutylamine as a bifunctional reagent for DNA modification allowed the possibility of choosing an appropriate reporter group: biotin or one of the fluorochromes. For probes carrying biotin, a high specificity and high sensitivity detection limit of 1 pg of target DNA were demonstrated. In addition, the applicability of probes carrying fluorochromes for multicolor direct fluorescence in situ hybridization was described.

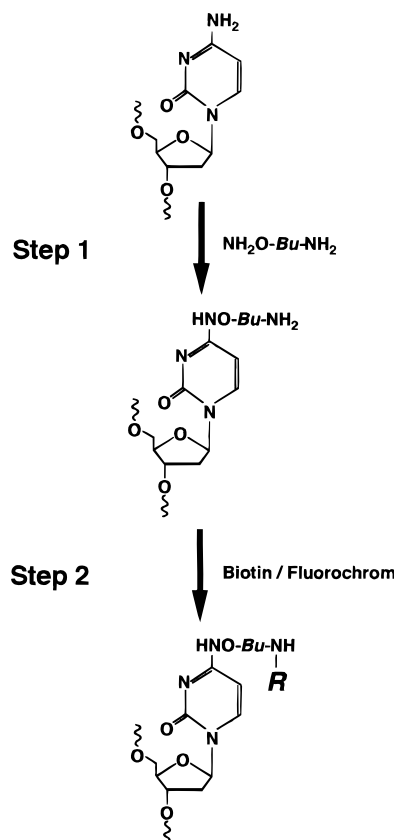
INTRODUCTION

Nucleophilic reagents are broadly used to introduce nonradioactive labels into polynucleotides: bisulfite (Lebacqz et al., 1988), aliphatic amines (Turchinskii et al., 1988), hydrazides (Reisfeld et al., 1987; Turchinskii et al., 1989; Karpyshev et al., 1990), and substituted hydroxylamines (Adarichev et al., 1987; Brosalina et al., 1987). Within this range of chemicals, hydroxylamine and its derivatives display the highest rate of nucleophilic substitution and best stability (Kochetkov et al., 1971, 1972). Therefore, we tested 4-aminooxybutylamine (ABA)¹ as a carrier of nonradioactive labels. This bifunctional agent carries an aminooxy group on one end and an amino group on the other. On the first step of DNA modification (Scheme 1), the aminooxy group reacts selectively with cytosine, and the amino group allows a variety of reporter compounds such as biotin and different fluorochromes to be attached on the second step. We reported earlier that ABA can be used for introduction of amino groups into extended double-stranded polynucleotides (Adarichev et al., 1987). The aim of the present work was to determine the optimal conditions for the reaction of DNA with ABA in order to obtain highly sensitive nonradioactive probes. The probes were characterized for their application in molecular hybridization.

EXPERIMENTAL PROCEDURES

ABA was synthesized by Vector Inc., Koltsovo, Russia. Streptavidin-alkaline phosphatase conjugate was purchased from Boehringer Mannheim, Switzerland; agarose, *n*-nitrotetrasolium blue (NBT), 5-bromo-4-chloro-3-indolyl phosphate toluidine salt (BCIP), *N*-hydroxysuccinic ether *D*-biotinylaminocaproate, DMSO, and salmon sperm DNA were from Sigma and SDS, RNase, tetramethyl-

Scheme 1. Synthesis of Biotin and/or Fluorescein-Labeled DNA Probes in Two-Step Modification Procedure^a



^a At Step 1, cytosine of DNA reacts with ABA, and the major product of ABA incorporation into DNA is shown. The structure based on temperature, concentration, and pH dependencies of cytosine modification with O-alkyl derivatives of hydroxylamine (Budowsky et al., 1971; Kochetkov et al., 1971, 1972; Petrenko and Spirin, 1982). At Step 2, ABA-DNA reacts with activated derivatives of biotin or fluorochromes (**R**).

rhodamine isothiocyanate, fluorescein isothiocyanate isomer I were from Fluka, Switzerland.

* To whom correspondence should be addressed. Tel: (3812) 35-4743. Fax: (3812) 35-6558. E-mail: dimshits@niboch.nsc.ru.

¹ Abbreviations: 1×SSC, 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0; ABA, 4-aminooxybutylamine; SDS, Na dodecyl sulfate; kb, kilobases.

Modification of Polynucleotides. Step 1. Reaction of DNA with ABA. Chemical modification of polynucleotides was performed with 20 μL of 1 $\mu\text{g}/\mu\text{L}$ plasmid DNA and 10 μL of 1.0 M ABA at pH 3.6. The final volume was adjusted with water to 40 μL ; the mixture was incubated for 1–40 min at 65, 80, or 100 $^{\circ}\text{C}$. Acidity of ABA solution was adjusted to the required pH value by titration at 65 or 80 $^{\circ}\text{C}$. Reaction pH at 100 $^{\circ}\text{C}$ was calculated based on pH at 65 $^{\circ}\text{C}$ and dependency of the ABA ionization constant with temperature. The value $\Delta K_a/\Delta\text{C}$ was -0.03 deg^{-1} for 1.0 M ABA solution and was not affected by temperature over the range 30–80 $^{\circ}\text{C}$. Removal of an uncoupled reagent was performed on Sephadex G-25 gel filtration by elution with 20 mM NaHCO_3 . DNA solution was concentrated by successive extractions with *n*-butanol and diethyl ether. Ether was removed by vacuum. Modified DNA was stored at $-20\text{ }^{\circ}\text{C}$.

Step 2. Attachment of reporter compounds to modified polynucleotides was done as described earlier (Frumgarts et al., 1986). The reaction mixture consisted of 10 mM of *N*-hydroxysuccinic ether *D*-biotinylaminocaproate, tetramethylrhodamine isothiocyanate, or fluorescein isothiocyanate and 0.1 M NaHCO_3 , 0.2 $\mu\text{g}/\mu\text{L}$ ABA-DNA, and 10% DMSO. After incubation for 2 h at 37 $^{\circ}\text{C}$, the mixture was loaded onto Sephadex G-25 gel and eluted with 20 mM NaHCO_3 to remove uncoupled reagents. DNA samples labeled with fluorescein were characterized by absorption at 260 and 490 nm to calculate the ratio of bases carrying fluorochrome. Labeled DNA was stored at $-20\text{ }^{\circ}\text{C}$.

To evaluate the percentage of nucleotide bases of DNA carrying ABA or biotin (degree of modification), an aliquot of ABA-DNA after first step of modification was labeled with fluorescein. The degree of modification for fluorescein-labeled DNA was determined as a percentage of labeled bases over all bases using the 490/260 nm absorption ratio. The extinction coefficient of fluorescein in 20 mM NaHCO_3 was $42\,000\text{ M}^{-1}\text{ cm}^{-1}$ at 490 nm and $14\,400\text{ M}^{-1}\text{ cm}^{-1}$ at 260 nm, and the extinction coefficient of DNA was $6500\text{ M}^{-1}\text{ cm}^{-1}$ at 260 nm.

The sensitivity of biotin-labeled DNA probes was estimated by homologous dot-blot hybridization. Decreasing amounts of target DNA were immobilized onto nylon membrane, and DNA probe was labeled in a two-step procedure prior to hybridization with DNA on membrane. The probe sensitivity (in picograms) was defined as a lowest detectable amount of target DNA.

Distribution of labeled DNA fragments was confirmed by agarose gel electrophoresis in 30 mM NaOH . Fluorescein-labeled DNA in agarose gel was photographed under ultraviolet illumination using "Photodyne" camera with a green filter. Biotin-labeled DNA was transferred from agarose gels under vacuum onto nylon membranes and immobilized by ultraviolet illumination (Kalachikov et al., 1992). Biotin was detected with streptavidin-alkaline phosphatase conjugate as described earlier (Langer et al., 1981).

Chromosome In Situ Hybridization Using Fluorochrome-Labeled Probes. Slides with chromosomes of mink, mouse, and vole *Microtus arvalis* were kindly provided by Dr. S. M. Zakian and Dr. O. L. Serov (Institute of Cytology and Genetics, Novosibirsk, Russia). Slides were denatured with 70% formamide in $2\times\text{SSC}$ at 70 $^{\circ}\text{C}$ for 2 min and immediately transferred into 70, 80, and 96% ethanol series for 5 min each at 2 $^{\circ}\text{C}$, and slides were then air-dried. Fluorochrome-labeled probe (1 μg) was combined with 2 μg of sheared salmon sperm DNA and 20 μg of sheared *Escherichia coli* DNA in 40

μL of hybridization buffer containing 50% formamide and 10% dextran sulfate in $2\times\text{SSC}$. The mixture was denatured at 90 $^{\circ}\text{C}$ for 3 min, immediately chilled on ice for 5 min, and then added to the slides. Hybridization was carried out overnight in a moist chamber at 37 $^{\circ}\text{C}$. Slides were washed twice in 50% formamide in $2\times\text{SSC}$, twice in $2\times\text{SSC}$ at 42 $^{\circ}\text{C}$, stained with propidium iodide, and mounted in 5 mM *p*-phenylenediamine, 70% glycerol, and $2\times\text{SSC}$. Fluorescent signal was detected with luminescent microscope "Lumam I-3" (LOMO, Russia).

RESULTS AND DISCUSSION

Specifying the Reaction Conditions. Hydroxylamine and its derivatives possess a high specificity for single-stranded nucleic acids. Phage M13 single-stranded DNA can be modified by 10% for 20 h in 1.0 M ABA, pH 4.5, at 37 $^{\circ}\text{C}$. No breaks in DNA were detected in the course of modification, and the corresponding biotin probes displayed a high sensitivity upon homologous dot-blot hybridization. Under identical conditions, double-stranded DNA can be modified by less than 1% (data not shown). To denature DNA and promote transamination, the temperature was raised. The rate of ABA incorporation was sharply increased with the reaction temperature, and DNA probes obtained at 100 $^{\circ}\text{C}$ demonstrated a higher degree of modification with ABA/biotin compared with probes obtained at 80 and 65 $^{\circ}\text{C}$ (Figure 1). Nevertheless, despite the significantly higher quantity of biotin moieties, the probes produced at 100 $^{\circ}\text{C}$ demonstrated the least sensitivity among all tested samples. Thus, 80 $^{\circ}\text{C}$ was the optimal temperature for the first step of DNA modification due to the combination of high rate of ABA incorporation and high sensitivity of probes in homologous dot-blot hybridization.

DNA modification with ABA at 80 $^{\circ}\text{C}$ (Figure 2) showed a steady increase in ABA incorporation with time. However, the results from homologous dot-blot hybridization of corresponding biotinylated DNA probes, produced under these conditions, demonstrated that sensitivity was maximal for probes incubated for 5–10 min with ABA, and longer incubation decreased the hybridization signal. In addition, the portion of the probe bound nonspecifically to the membrane increased under extended incubation. The level of DNA probe sensitivity achieved after 5–10 min of modification was greater than 1 pg. This level of sensitivity was comparable to that of DNA probes prepared by other nonradioactive-labeling methods, both chemical and enzymatic (Langer et al., 1981; Reisfeld et al., 1987; Lebacqz et al., 1988; Turchinskii et al., 1989; Karpyshev et al., 1990).

Characterization of the Probes Produced by DNA Transamination. It was shown that *O*-alkylhydroxylamines demonstrate high specificity to cytosine, does not react with guanine and thymidine, and reacts minimally with adenine nucleus (Budowsky et al., 1971a,b; Budowsky et al., 1972; Kochetkov et al., 1971, 1972). Despite ABA belonging to *O*-alkylhydroxylamines, its reactive specificity may differ under particular conditions we used for modification. To determine the reactivity of ABA to different DNA bases, we incubate the reagent with single-stranded homopolynucleotides under conditions optimal for DNA modification (0.25 M ABA pH 3.6, 80 $^{\circ}\text{C}$, 10 min). Indeed, ABA showed reactivity for each of the DNA bases. The portion of modified cytosine among all the four bases was about 70%, but ABA reacted also with guanine (16%), adenine (10%), and thymidine (4%). The resulting ratio of rates ($G > A > T$) was identical for DNA depurination upon heating in acidic

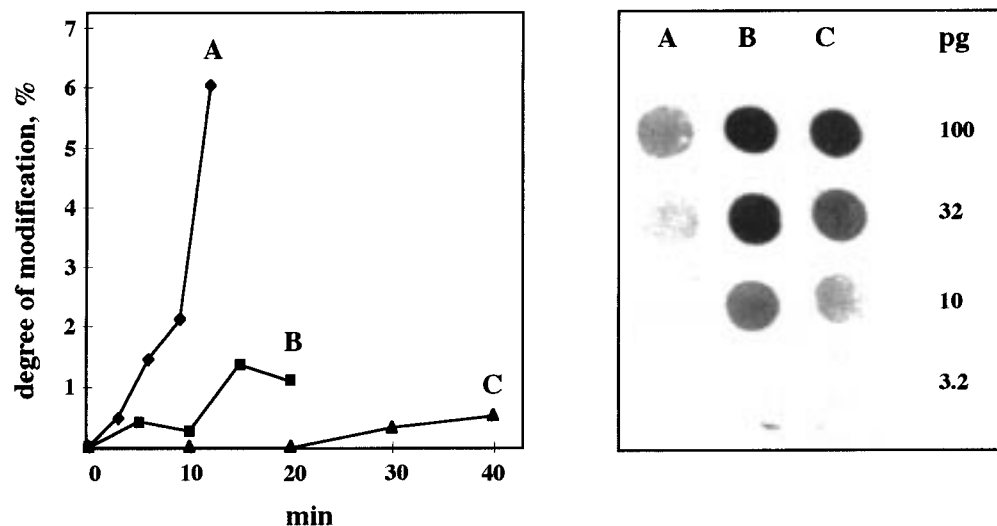


Figure 1. Time course of the ABA incorporation into double-stranded DNA at different temperatures: (A) 100 °C; (B) 80 °C; (C) 65 °C, on left. Homologous dot-blot hybridization of the probes modified for 10 min, on right.

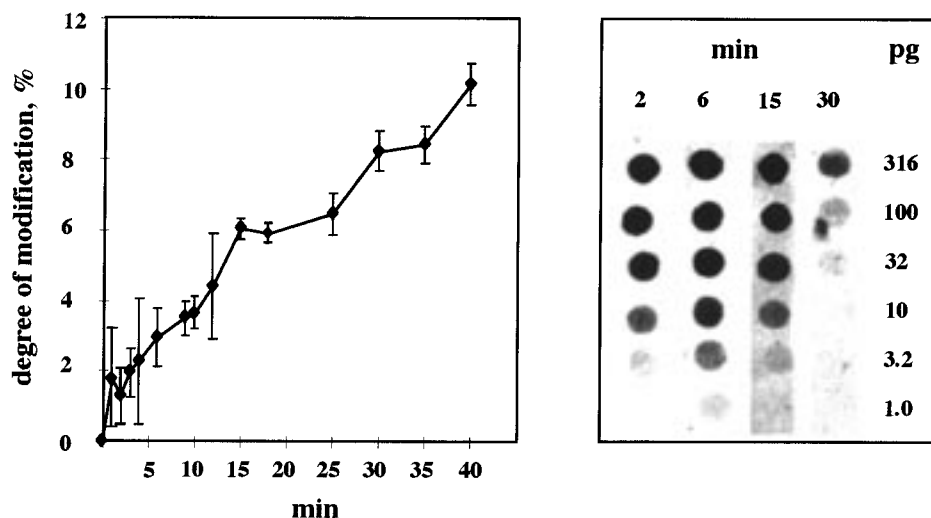


Figure 2. Time course of the ABA incorporation into plasmid DNA at 80 °C, on left. Homologous dot-blot hybridization of the produced probes, on right. Labels 2, 6, 15, 30 min, duration of the modification.

conditions (Kochetkov et al., 1971, 1972). Obviously, detachment of DNA bases followed by coupling the ABA with C1-deoxyribose aldehydes groups might explain homopolynucleotides reactivity.

Depurination at the C1 atom can lead to disintegration of polynucleotides and subsequent changes in the hybridization properties of the probe. For that reason, DNA probes obtained in the two-step labeling procedure were tested by gel electrophoresis under alkali conditions, which allowed us to detect all anucleated sites. Plasmid DNA was incubated with ABA and modified with biotin. DNA sample was fractionated on alkaline agarose gel and transferred onto nylon membrane. Following these procedures, the 6.0 kb plasmid DNA demonstrated some degree of degradation (Figure 3). Nevertheless, a final length of 1–5 kb remained optimal for hybridization. A similar size distribution pattern was also obtained for the fluorescein-labeled DNA.

The optimal degree for DNA probe labeling was studied by homologous dot-blot hybridization using plasmid DNAs carrying 0.2–8% biotin. Target DNA was spotted onto nylon membrane in decreasing amounts, and each biotinylated DNA sample was hybridized with target under standard conditions. Dependence of sensitivity at

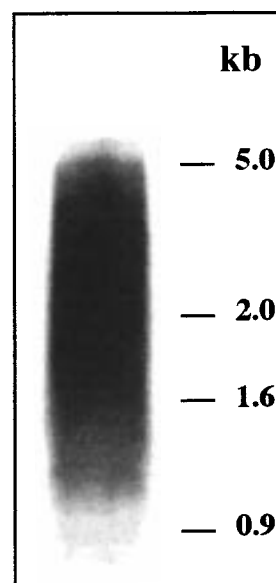


Figure 3. Size distribution for DNA probes labeled with ABA and biotin after gel electrophoresis in alkaline conditions.

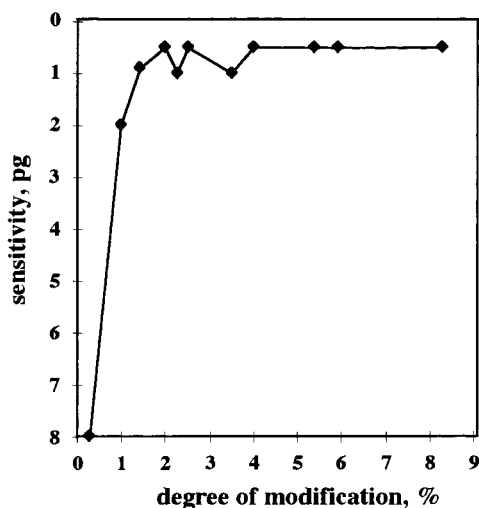


Figure 4. Sensitivity of biotinylated DNA probes with different degree of modification in homologous dot-blot hybridization.

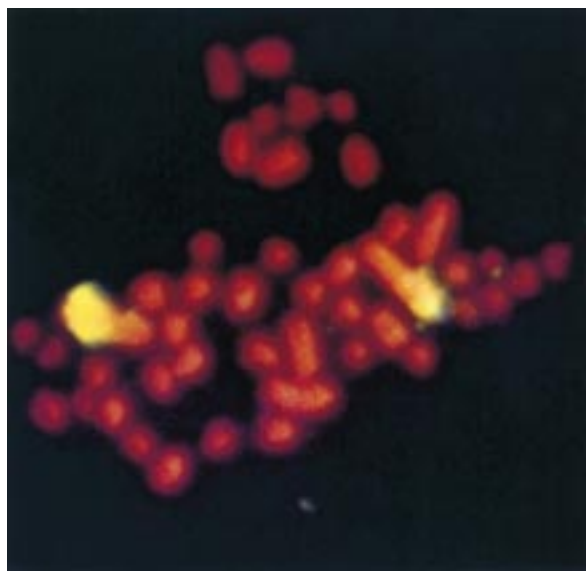


Figure 5. ABA-modified DNA probes in fluorescent in situ hybridization with chromosomes of the vole *Microtus arvalis*. Transaminated and labeled with fluorescein plasmid carrying species-specific repeated sequences was used as a probe. Hybridization signal was detected by fluorescent microscopy (green-yellow). Chromosomes were contrasted with propidium iodide staining (red).

degree of modification demonstrated plateau started from 1.5% biotinylated bases (Figure 4). Biotinylated DNA probes that carried 1.5–8% biotin demonstrated equal sensitivity, which dropped abruptly if DNA probes carried less than 1% biotin. Thus, the maximum probe sensitivity for biotin-ABA label was at 1.5% of modified DNA bases. Higher degree of modification resulted in higher density of biotin labels on the DNA strand, and obviously, steric problems arose when the sizable streptavidin-alkaline phosphatase conjugate interacted with the biotin residues hindering increased sensitivity. In the case of fluorochrome-labeled probes, the dependence was also asymptotic, but maximal sensitivity was reached at 4% modified DNA bases. In the latter case, the mechanism of quenching of fluorescent signal was responsible for sensitivity hindering (Frumgarts et al., 1986).

The application of ABA-modified DNA probes was demonstrated for in situ hybridization. Plasmid DNA carrying species-specific sequences were modified with ABA, and either biotin or fluorescein was attached. The

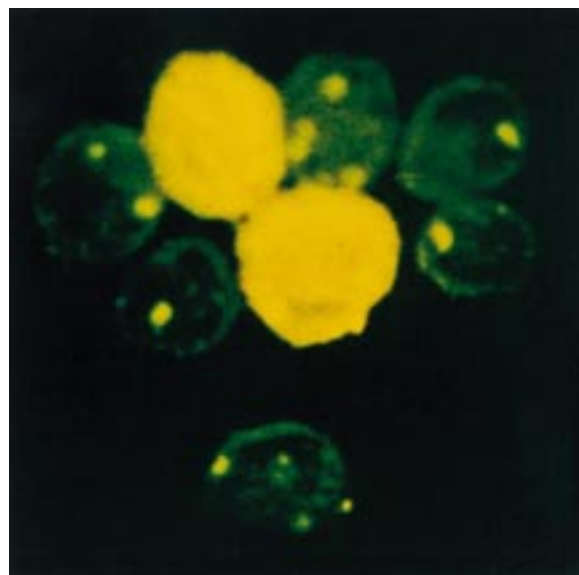


Figure 6. Direct multicolor fluorescent in situ hybridization with a mixture of mink and mouse cell nuclei. The probe for mink carried tetramethylrhodamine (orange), the probe for mice carried fluorescein (green).

species-specific repeat was localized in the heterochromatin arm of the sex chromosomes of the vole *Microtus arvalis* by direct signal detection from fluorescein-labeled DNA probes (Figure 5). Nonradioactive hybridization of the biotinylated plasmid demonstrated identical chromosomal localization (data not shown).

The described two-step labeling procedure with ABA as the first modifying agent allows the choice of the most suitable label for the task. In the frame of one method, different fluorochromes can be attached to DNA probes opening the possibility of simultaneous hybridization of several probes carrying fluorochromes with different emission spectra. Figure 6 presents the results of multicolor fluorescent hybridization with a mixture of mink and mouse cells. Total mink DNA, as a first DNA probe, was labeled with ABA and tetramethylrhodamine (orange-yellow nuclei), and total mouse DNA, as a second probe, carried ABA-fluorescein label (green nuclei).

The described procedure for the preparation of molecular hybridization probes does not require enzymes or unstable compounds. In one round of two-step modification, a large quantity up to hundred microgram of fluorochrome- or biotin-labeled probe can be produced starting from double-stranded plasmid DNA. These probes can be used in all fields of molecular hybridization.

ACKNOWLEDGMENT

The authors acknowledge the helpful comments of Drs. E. I. Budowsky and B. P. Ulanov as well as collaboration of Drs. A. S. Grafodatsky, S. M. Zakian, G. A. Zayniyev, L. P. Zakharenko, L. P. Matiakhina, O. V. Sablina, and O. L. Serov and the support from Programs "Human Genome" and "Frontiers in Genetics" of Russian Academy of Science. The editorial assistance of Vladimir Filonenko is greatly appreciated.

LITERATURE CITED

- Adarichev, V. A., Dymshits, G. M., Kalachikov, S. M., Pozdnyakov, P. I., and Salganik, R. I. (1987) DNA Carrying Aliphatic Amino Groups and Use of Its Fluorescent Derivative as a Probe in Molecular Hybridization. *Bioorg. Khim.* 13 (8), 1066–9.

- Brosalina, E. B., Vlasov, V. V., Grachev, S. A., and Demchenko, E. N. (1987) Construction of DNA-Probes and Their Immunochemical Detection Using Nonradioactive Hybridization Tests. *Bioorg. Khim.* 13 (12), 1644–54.
- Budowsky, E. I., Sverdlov, E. D., and Monastyrskaya, G. S. (1971a) Mechanism Of the Mutagenic Action Of Hydroxylamine. IV. Reaction Of Hydroxylamine And O-Methylhydroxylamine With Adenine Nucleus. *Biochim. Biophys. Acta* 246 (2), 320–8.
- Budowsky, E. I., Sverdlov, E. D., Shibaeva, R. P., Monastyrskaya, G. S., and Kochetkov N. K. (1971b) Mechanism of the Mutagenic Action of Hydroxylamine. III. Reaction of Hydroxylamine and O-methylhydroxylamine with the Cytosine Nucleus. *Biochim. Biophys. Acta* 246 (2), 300–19.
- Budowsky, E. I., Turchinsky, M. F., Domkin, V. D., Pogorelov, A. G., Pisarenko, V. N., Kusova, K. S., and Kochetkov, N. K. (1972) Mechanism of the Mutagenic Action of Hydroxylamine. VI. Reaction of Hydroxylamine with the Uracil Nucleus. *Biochim. Biophys. Acta* 277 (2), 412–27.
- Frumgarts, L. A., Kipriianov, S. M., Kalachikov, S. M., Dudareva, N. A., and Dymshits, G. M. (1986) Preparation of Fluorescent-Labeled DNA and Its Use as a Probe in Molecular Hybridization. *Bioorg. Khim.* 12 (11), 1508–13.
- Kalachikov, S. M., Adarichev, V. A., and Dymshits, G. M. (1992) Ultraviolet Cross-linking of DNA to Microporous Membranes. *Bioorg. Khim.* 18 (1), 52–62.
- Karpyshev, N. N., Bondarenko, Tlu., and Kipriianov, S. M. (1990) Evaluation of the Effectiveness of Hybridization Probes Prepared by Action of Hydrazides of Biotin Derivatives on DNA. *Bioorg. Khim.* 16 (5), 605–9.
- Kochetkov, N. K., Budowsky, E. I., Sverdlov, E. D., Simukova, N. A., Turchinskii, M. F., and Shibaev, V. N. (1971, 1972) *Organic Chemistry of Nucleic Acids* (N. K. Kochetkov, and E. I. Budowsky, Eds.) Plenum Press, New York, London.
- Langer, P. R., Waldrop, A. A., and Ward, D. C. (1981) Enzymatic Synthesis of Biotin-Labeled Polynucleotides: Novel Nucleic Acids Affinity Probes. *Proc. Natl. Acad. Sci. U.S.A.* 78 (11), 6633–7.
- Lebacqz, P., Squalli, D., Duchenne, M., Pouletty, P., and Joannes, M. (1988) A New Sensitive Non-Isotopic Method Using Sulfonated Probes to Detect Picogram Quantities of Specific DNA Sequences on Blot Hybridization. *J. Biochem. Biophys. Methods* 15, 255–66.
- Petrenko, V. A., and Spirin, G. V. (1982) Reaction of cytidylic acid with O-delta-aminooxybutylhydroxylamine. *Mol. Biol. (Moscow)* 16 (3), 644–8.
- Reisfeld, A., Rothenberg J. M., Bayer, E. A., and Wilchek, M. (1987) Nonradioactive Hybridization Probes Prepared by the Reaction of Biotin Hydrazide with DNA. *Biochem. Biophys. Res. Commun.* 142 (2), 519–26.
- Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Plainview, NY.
- Turchinskii, M. F., Aibinder, E. I., and Sverdlov, E. D. (1988) Multiple Hybridization in Genome Analysis. 1. Reaction of Diamine and Bisulfite with Cytosine for the Introduction of Nonradioactive Markers into DNA. *Mol. Biol. (Moscow)* 22 (6), 1545–52.
- Turchinskii, M. F., Aibinder, E. I., Knorre, V. D., and Scherbo, S. N. (1989) Modification of DNA with Dihydrazide of Succinic Acid for Preparation of Hybridization Probes. *Bioorg. Khim.* 15 (10), 1341–5.

BC980007U

Novel Derivatives of Cyclodextrins, Modified with Poly(ethylene Oxide) and Their Complexation Properties

Irina N. Topchieva,* Petra Mischnick,[†] Gerhard Kühn,[‡] Vladimir A. Polyakov,[§] Svetlana V. Elezkaya,^{||} Georgy I. Bystryzky,^{||} and Konstantin I. Karezin^{||}

Lomonosov Moscow State University, Department of Chemistry, 119899, Lenin Hills, Moscow, Russia, Institut für Organische Chemie der Universität Hamburg, Martin-Luther-King-Platz 6, D-20146 Hamburg, Berlin, Bundesanstalt für Materialforschung und -prüfung, Rudower Chaussee 5, Haus 10.1, D-12489 Berlin, Germany, Russian Mendeleev University of Chemical Technology, Miusskaya, 9, 125820, Moscow, Russia, and State Scientific Center "NIOPIK", B. Sadovaya, 1, bd.4, 113787 Moscow, Russia. Received January 15, 1998; Revised Manuscript Received May 7, 1998

A new family of bouquet-like molecules based on cyclodextrins is described. These compounds were obtained by polymerization of ethylene oxide. This reaction was initiated by primary and secondary hydroxyl groups of cyclodextrins, which constitute an organizing core. Analysis of structure and composition of conjugates based on α -CD and β -CD was performed using the data of MALDI-MS, GC-MS, and ¹³C-NMR spectra. Glass transition behavior of the conjugates shows that the above compounds are amorphous. Complexation properties of the conjugates are described with respect to sodium 4-nitrophenolate and calcium acetylhomotaurinate, which are used as guest molecules. Binding interaction between cyclodextrins or their conjugates and sodium 4-nitrophenolate was studied using differential absorption spectra. Association constants K_a between CD hosts and calcium acetylhomotaurinate composed of two equal anionic moieties were studied using ¹H-NMR spectroscopy. The values of binding constant for β -CD were found to increase by more than 2 orders of magnitude than that of the corresponding system based on α -CD. α -CD was shown to form the inclusion complex with one anionic moiety, whereas β -CD produces a ternary complex with two anionic moieties of CAHT. For PEO derivatives of CDs, K_a decreases as compared with that of parent CD for both guests. These conjugates may be used as potent drug-delivery systems.

INTRODUCTION

Cyclodextrins (CD)¹ have been extensively studied because of a unique ability of these cyclic starches to produce dynamic molecular inclusion complexes with various compounds including pharmaceuticals. The ability of CD for molecular recognition is controlled by hydrophobicity of a guest and fitness between the shape of guest species and a CD cavity (1). To change the molecular recognition ability of CDs, many derivatives of CDs containing various groups on both sides of CD have been investigated (1, 2). In this respect CDs, modified with water-soluble polymers, are of a great interest. At the present time, these investigations are

in their infancy. The advantages of polymer derivatives of CD were demonstrated for CDs modified with poly(*N*-isopropylacrylamide) chains (3). In these systems, polymer chains experience the coil-globule transition upon heating, and the inclusion of a guest molecule is controlled by temperature. Therefore, polymer chains are sensitive to the external action. More interesting polymer derivatives of CD are the conjugates of CD with poly(ethylene oxide), which may be presented as a microcapsule consisting of a CD core with short poly(ethylene oxide) chains attached to primary and secondary hydroxyl groups of CD (bouquet-shaped structures) (4). Even though modification of CD by PEO chains decreases the size of CD cavity and binding constant, one may anticipate that this modification would give rise to new properties such as amphiphilic character of CD derivatives (5) and the adaptation of guest accommodation due to the additional noncovalent fixation of the ligands in polymer surroundings.

In this work, the synthesis of the above compounds are described, and effect of polymer on the complexation with two guests, sodium 4-nitrophenolate (SN) and calcium acetylhomotaurinate (CAHT) was examined.

EXPERIMENTAL PROCEDURES

Chemicals. In this work, we used α - and β -cyclodextrins (Cyclolab, Hungary) and 2,5-dihydroxybenzoic acid (Aldrich); SN and CAHT² were analytically grade reagents.

* Address correspondence to this author at Moscow State University. Tel: 007-(095)-939-5583. Fax: 7-(095)-939-01-74.

[†] Institut für Organische Chemie der Universität Hamburg.

[‡] Bundesanstalt für Materialforschung und -prüfung.

[§] Russian Mendeleev University of Chemical Technology.

^{||} State Scientific Center "NIOPIK".

¹ Abbreviations: CD, cyclodextrin; CI, chemical ionization; EI, electron impact; EO, ethylene oxide; GC-MS, gas chromatography-mass spectrometry; *M*, molecular mass; *M_w*, weight average molecular mass; *M_n*, number average molecular mass; MMD, molecular mass distribution; MALDI-MS, matrix-assisted laser desorption ionization-mass spectrometry; MS, molar degree of substitution; PEO, poly(ethylene oxide); NP, natrium 4-nitrophenolate; CAHT, calcium acetylhomotaurinate; PEO- α -CD, conjugate of PEO and α -CD; PEO- β -CD, conjugate of PEO and β -CD; *T_g*, temperature of glass transition.

Methods. Gas chromatography (GC) was performed with a Carlo Erba GC 6000 Vega Series 2 equipped with CPSi18 CB (Chrompack) and retention gap, on-column injector, and a flame ionization detector. Hydrogen was used as a carrier gas.

GC-MS. Mass spectra were recorded with a Hewlett-Packard 5840 A-5985A instrument (CI, ammonia, and EI) and VG/70-250S GC-MS-system (VG analytical).

MALDI-mass spectra were recorded using the TOF laser Kratos Compact MALDI III mass spectrometer (Kratos, Shimadzu, U.K.) in a reflection mode. Samples were prepared by casting the matrix compound (2,5-dihydroxybenzoic acid, 10 mg/mL in ethanol/water, 1:1, v/v) onto the slide. After evaporation of the solvent, the solution of conjugate (in methanol or for permethylated samples methanol-dichloromethane, 2:1, v/v, 1 mg/mL) was deposited on the matrix. The solvent was evaporated. Ions were produced by laser ionization/desorption at 337 nm (3 ns pulse width). Ionization was accelerated with 20 kV in the positive ion mode.

Differential absorption spectra were measured by a Hitachi 557 model UV spectrophotometer. ^1H -NMR spectra were recorded on a CXP-200 Bruker spectrometer. ^{13}C -NMR spectra were recorded on AM-300 Bruker spectrometer. Chemical shifts are given in parts per million. Sodium (4,4'-dimethyl-4-)-silapentane sulfonate and dimethylsulphoxide are used as internal references.

Glass transition temperatures were measured with a Mettler TA 400 thermal analyzer using a DSC-30 unit. Scanning rate was 20 K/min.

Synthesis. β -CD (3.5 g, 0.003 mol) in 50 mL of 0.9% NaCl solution (pH 11.2) was placed in the reaction stainless vessel. Ethylene oxide (EO) was added at 80 °C under a pressure of 0.145–0.196 MPa. After stirring for 4–10 h, the reaction mixture was cooled down to 40 °C and unloaded. The product was dissolved in water. The content of insoluble product was estimated to be ~5%. This product was separated by filtration. The dialysis of the solution was carried out using Seamless tubing (Sigma). The completion of dialysis was controlled using thin layer chromatography on Silufol plates in $\text{CHCl}_3:\text{C}_2\text{H}_5\text{OH}:\text{H}_2\text{O}$ (36:12:1). The spots were revealed by iodine. The solution was filtered through a glass fiber filter (Sigma) and lyophilized. A repeated drying of the conjugates was carried out by heating in vacuum in the presence of phosphorous pentoxide at 95–100 °C for 1 h. Water content in the lyophilized samples of the conjugates was estimated to be 10–13%. For the PEO- β -CD conjugate, calcd: C, 45.7%; H, 8.7%. Found: C, 45.8%; H, 8.5%.

Chemical Modification of PEO-CD Conjugates. PEO-CD conjugates were permethylated using powdered sodium hydroxide and methyl iodide (6). The permethylated products were extracted with dichloromethane. Hydrolysis of the conjugates was carried out in 2 N trifluoroacetic acid in a screw cap vial at 120 °C for 2 h. The solvent was evaporated under nitrogen flow. The traces of acid were removed by codistillation with toluene. Remethylation of glucose derivatives of PEO (hydrolysates) was performed using $\text{CD}_3\text{I}/\text{NaOH}$ in DMSO according to ref 6.

Complexation Properties. Complexation between SN and CD or their conjugates was studied by the measurements of differential absorption spectra in 0.05 M phosphate buffer (pH 9.5). Equilibrium constants of complexation between CD or their conjugates with CAHT

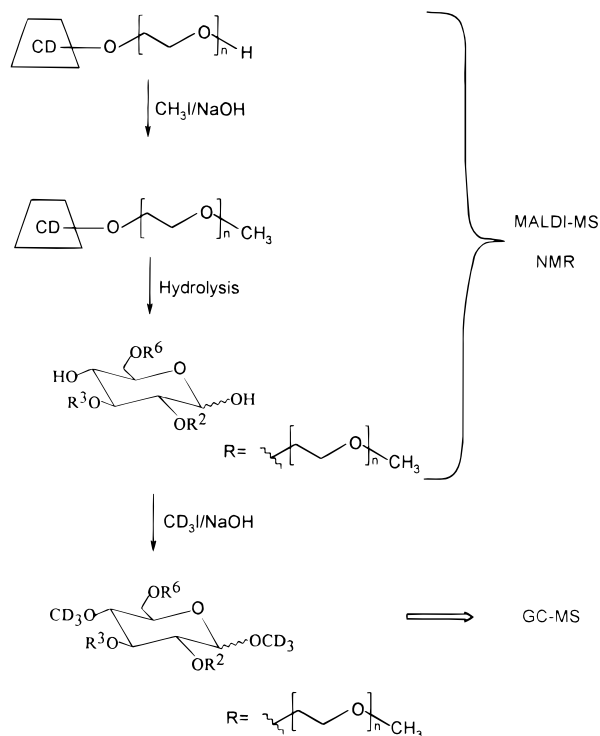


Figure 1. Analysis of the PEO-CD conjugates and their degradation products.

were estimated from ^1H -NMR spectra. The analysis of binding curves was carried out using computer program Microcal Origin 3.5. Thus, values δ_1 (the limiting value of chemical shift of methyl protons of CAHT in the presence of CD derivatives) were calculated. Correlation between chemical shifts of methyl protons of CAHT and concentration of CDs or their derivatives was described by the following equation:

$$\delta_{\text{obs}} = \delta_1 + \frac{\delta_0 - \delta_1}{1 + K_a} [\text{B}] \quad (1)$$

where δ_{obs} stands for the chemical shift of methyl protons of CAHT; δ_1 is the limiting value of chemical shift; δ_0 is the value of the chemical shift of the initial CAHT; [B] is the concentration of CDs or their derivatives; K_a is the microscopic binding constant of CDs or their derivatives with one binding center (anionic moiety of CAHT). This equation holds when both anionic moieties of CAHT (centers) are equivalent and do not interact with each other and $[\text{B}] \gg [\text{CAHT}]$.

RESULTS AND DISCUSSION

Synthesis of Conjugates. PEO-CD conjugates were synthesized in one-pot manner by polymerization of EO. The reaction was initiated by hydroxyl groups of multifunctional core of CD. This method was developed for the synthesis of graft copolymers of EO on starch (7). PEO, the main byproduct of this reaction, was produced by the polymerization of ethylene oxide initiated by hydroxide ions (8).

Characteristics of Conjugates. Characteristics of the conjugates (molecular mass, molecular mass distribution, M_w/M_n , molar degree of substitution, MS, and mode of substitution) were obtained through the procedure of successive permethylation, hydrolysis, and perdeutero-methylation (6). The consequence of the reactions and the methods of investigation are presented in Figure 1.

² CAHT was kindly presented by Dr. R. N. Alyautdin (Moscow Sechenov Medical Academy).

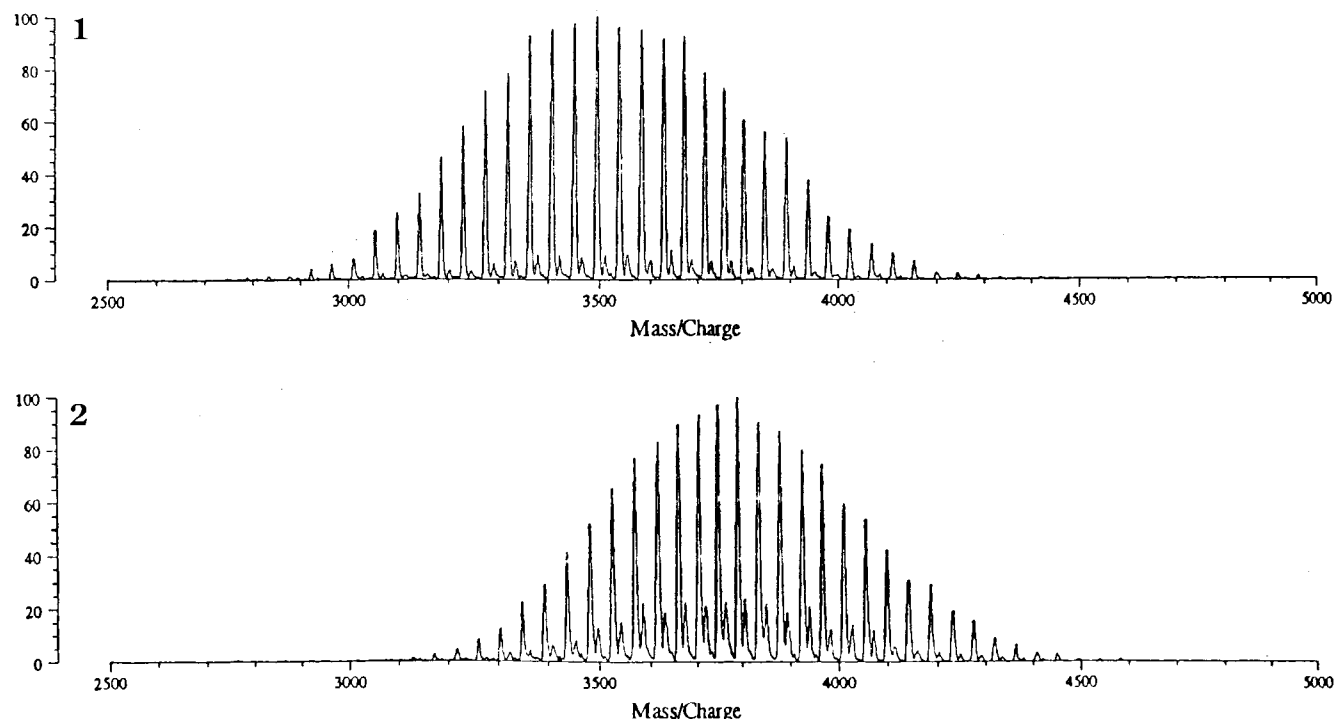


Figure 2. MALDI-mass spectra of PEO- β -CD (1) and its permethylated derivative (2). Sodium adducts are detected (Minor series of homologous present sodium adducts).

Table 1. Characteristics of PEO-CD Conjugates

CD	$\bar{M}_w$		$\bar{M}_w/\bar{M}_n$		MS-range/ CD	MS _{av}
	con- jugate	hydrol- ysate	con- jugate	hydrol- ysate		
α	3025	608	1.004	1.04	35–60	8
β	3540	778	1.005	1.09	40–70	8

Mass Spectrometry. Three parameters of the conjugates, molecular mass, $\bar{M}_w/\bar{M}_n$, and MS were determined by MALDI-MS method (Table 1). The MALDI-mass spectrum of the conjugates exhibit the distribution of weight molecular mass M_w . Using graphical method (9) values of average weight and number molecular masses, M_w and M_n , were calculated. Note that the MALDI-mass spectra are characterized by a narrow and symmetrical distribution of the components (Figure 2) with $\bar{M}_w/\bar{M}_n$ close to unity. This is characteristic of anionic polymerization. Mass distribution of the permethylated PEG-CD conjugates agrees with that of the original samples, $\Delta M = n42$ (42 is 3×14 for three methyl groups per glucose unit) where n is a number of glucose units per CD (Figure 2). To estimate molecular mass and $\bar{M}_w/\bar{M}_n$ of PEO substituents, the permethylated conjugates were hydrolyzed. The MALDI-mass spectra of the resultant PEO-containing glucose derivatives are presented in Figure 3. As it is seen, these products are characterized by a narrow interval of $\bar{M}_w/\bar{M}_n$ values (1.04–1.09). These values are slightly higher than those of the initial conjugates but MMD is still narrow. This evidence suggests that the degree of polymerization of PEO substituents attached to different positions of glucose units of CD does not differ substantially. The total number of ethylene oxide units in the conjugates was obtained from molecular mass of the conjugate minus molecular mass of the initial CD divided by 44 (molecular mass ethylene oxide unit) (Table 1). An average numbers of monomer units in each PEO substituent (MS_{av})

presented in Table 1 were calculated using the following formula:

$$\text{MS}_{\text{av}} = (M_{w,\text{hyd}} - 245)/44$$

where 245 is the total mass of all non-PEO fragments of oligomer (180 for glucose fragment, 42 for 3 methyl groups, and 23 for sodium).

Mode of Substitution. Additional information concerning the number of substituents in PEO-CD conjugates was obtained using GLC-MS method. This method allows one to estimate the number of substituents in a given compound (from the molecular mass of separate peaks). For example, using both EI and CI modes, it was shown that methyl glucosides with MS from 0 to 11 present in PEO- β -CD. This implies that, even at these high values of MS, unmodified groups in CD still exist. From the fragmentation patterns of the EI mass spectra, preferred 6-O-hydroxyethylation was demonstrated in the fraction with MS = 1 ($6 \gg 2$). For the fraction with MS = 2 the order was $66' \gg 22' > 26$. For methyl glucosides with MS = 3, the patterns $66'6''$, $266'$, $22'2''$, and $22'3$ are detected. At higher MS, the formation of oligoether at 2-OH dominates. However, it seems difficult to differentiate between longer PEO chains because the structure of isomers becomes very complex.

As known, the relative reactivities of hydroxyl groups of CD or starch during the kinetically controlled etherification are controlled by the concentration of base (10). Polymerization proceeds without any addition of base but due to nucleophilic opening of oxirane by chloride ion of NaCl pH of the reaction media achieves about 11. Under these conditions, the reaction at the most acidic 2-OH, supported by the axial glucoside oxygen, dominates. In general, a poor reactivity of secondary 3-OH in CDs prevents peretherification. Formation of oligoether in 2 and 6 positions is preferable and makes the sterically

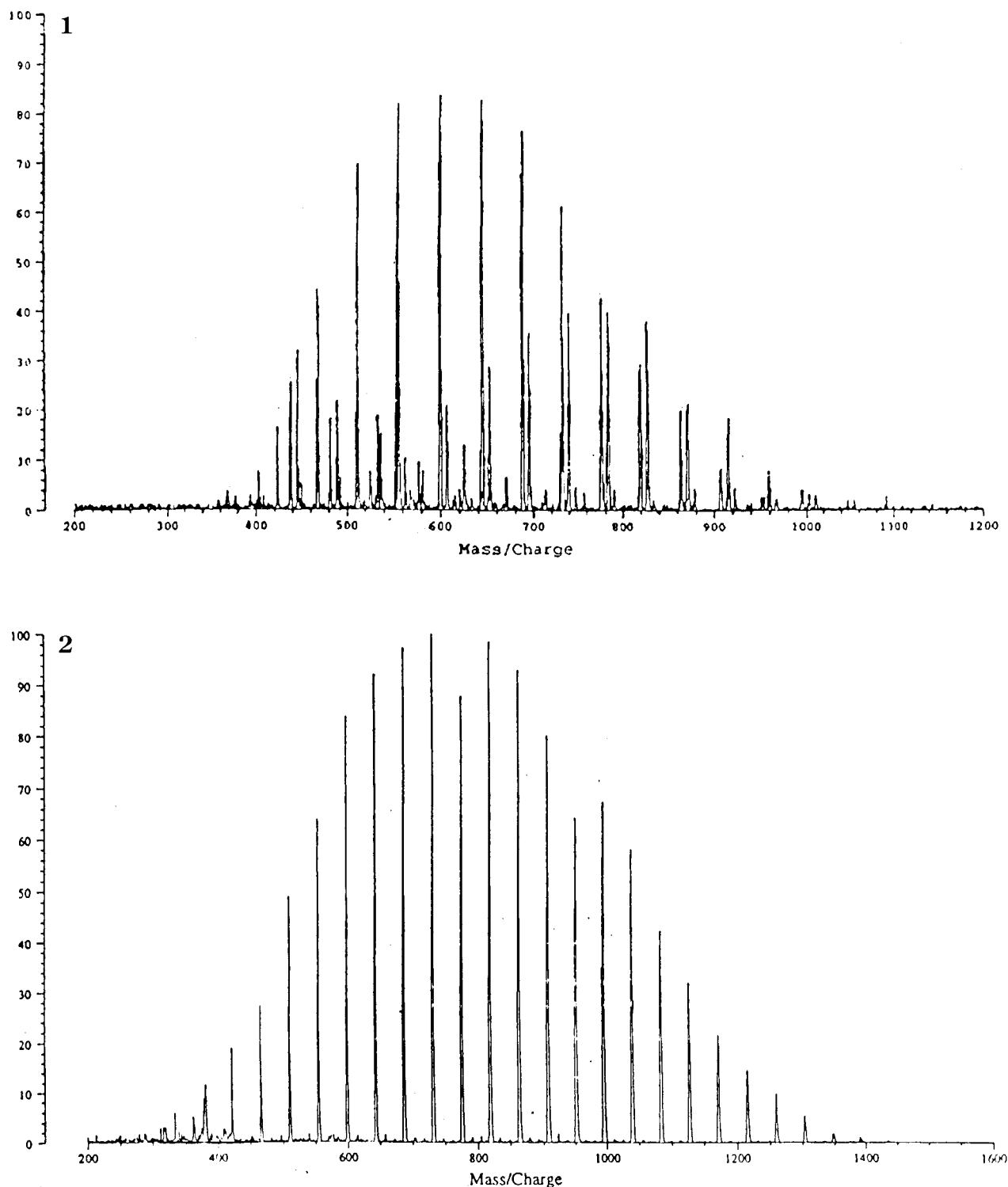


Figure 3. MALDI-mass spectra of glucose derivatives of PEO (hydrolysates): (1) From PEO- α -CD; (2) from PEO- β -CD.

hindered 3-OH even less accessible. Therefore, for the various hydroxyl groups in the glucose unit of PEG-CDs, molar degree of substitution changes in the following order: $2 > 6 \gg 3$. Polymerization of ethylene oxide in the presence of CDs yields randomly distributed PEO chains attached to CD with $MS_{av} = 8-11$ per glucose unit. In this case, MMD is symmetrical and narrow, and M_w/M_n is close to unity.

NMR Spectra. PEO-CD conjugates were studied using ^1H - and ^{13}C -NMR spectra. In the ^1H -NMR spectra, the signals of protons of glucose rings were shielded by a strong signal from PEO protons. In the ^{13}C -NMR

spectra, one may distinguish well-defined signals corresponding to carbon atoms of polymer chains which are characterized by the following chemical shifts: 62.173, 71.315, and 73.477 ppm. Furthermore, the spectrum shows broad signals of carbon atoms corresponding to CD moiety (62, 71-75, 80-84, and 100-103 ppm). Our efforts to achieve a better resolution of spectral lines by heating the sample up to 90 °C failed. This is likely to be associated with the statistical character of substitution at different positions of hydroxyl groups and a crowding effect of PEO substituents.

Thus, the formula of conjugates may be presented as

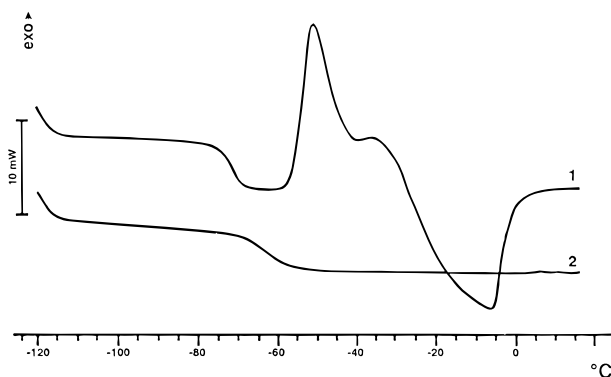


Figure 4. Curves of DSC of glass transition of PEG 300 (1) and PEO- β -CD conjugate (preparation 4). (2) The rate of heating is 10 °C/min.

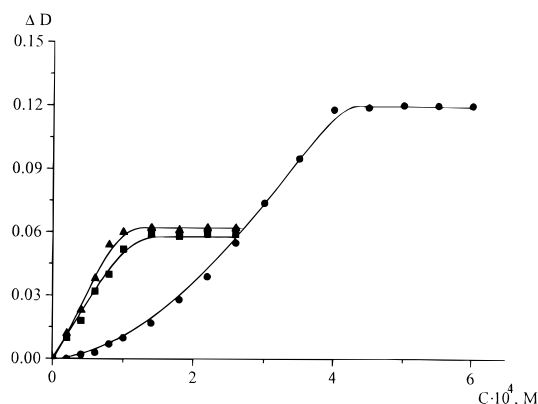
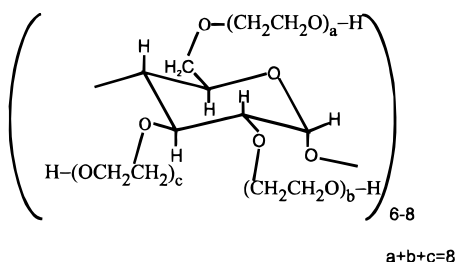


Figure 5. Binding curves for the systems based on SN and α -CD (■), PEO- β -CD (▲), PEO- α -CD (preparation 4) (●). Concentration of SN = 5×10^{-5} M; $T = 297$ K.



Additional information concerning the physical state of the conjugates was obtained studying of glass transition behavior and glass transition temperatures T_g (Figure 4). Figure 4 shows the DSC scan for PEG 300. Note that the parent β -CD is a crystalline compound with melting temperature of about -50 °C. As is seen, T_g of PEG 300 is close to T_g of PEO- β -CD conjugate and lies in the range from -75 to -70 °C. At the same time, the temperature interval of transition depending on the degree of cooperativity is much wider for bound polymer chains as compared with that of initial polymer. As follows from Figure 4, the DSC scans of PEG 300 are characterized by both picks of crystallization and melting, whereas the corresponding DSC scans of the conjugates show only the region of glass transition.

Complexation Properties of the Conjugates with Sodium 4-Nitrophenolate. The fundamental property of PEO-CD derivatives concerns their complexation ability. In this work, we used two organic compounds SN and CAHT as guest molecules. Upon the addition of α -CD, SN in aqueous solutions exhibits a spectral shift of about 15 nm (11). Figure 5 shows the binding curves for α -CD and for two conjugates with SN. As is seen, an

increase in the concentration of host molecule leads to an abrupt linear increase in the value of optical absorbance, then this value levels off when the guest:host ratio varies in the range from 1:1 to 1:2. One may conclude that (1) the affinity between the ligand and PEO-CD conjugates decreases as compared with that of the system based on parent α -CD; (2) the binding curve for PEO- α -CD conjugate shows an S-shaped profile which suggests a far more complex character of binding as compared with that of the native α -CD.

As compared to parent α -CD a decrease in the complexation ability of this conjugate is likely to be related to a decrease of the cavity size. The same effect was observed for other types of CD derivatives (12). As was shown in ref 13, the binding constant of β -CD with respect to SN is by 1 order of magnitude lower than that of the system based on α -CD. This is likely to be related to the fact that an increase in the size of β -CD cavity decreases the complex stability. The complexation between β -CD and SN was not identified by differential UV spectroscopy. At the same time, as follows from the binding curve presented in Figure 5, PEO- β -CD forms the complex with SN. For the systems based on PEO- β -CD and α -CD, the comparison between the slopes of linear portions of the corresponding binding curves shows that both hosts are characterized by similar binding constants. Therefore, flexible oligo(ethylene oxide) chains may facilitate the adaptation of guest accommodation because of the additional noncovalent fixation of guests by noncovalent forces.

As known, PEO forms inclusion compounds with α -CD (14). It seems important to understand whether self-inclusion of the grafted PEO chains in PEO- α -CD conjugates is possible. Our experiments within the framework of Stuart-Breigleb models show that oligo(oxyethylene) chains grafted to CD core are able to form complexes with one grafted oligo(oxyethylene) chain incorporated in the cavity of the same molecule (auto-complexes). Actually, these structures should show poor complexation properties in respect to the external guest. This conclusion is proved by a low value of the slope of the first portion of the binding curve (Figure 6). Probably, an increase in the affinity of components at a higher concentration of the conjugate is provided by the destruction of autocomplexes and formation of amphiphilic type associates (15).

Complexation Properties of the Conjugates with Calcium Acetylhomotaurinate. Taking into account the ability of PEO-CD conjugates for drug targeting (16), it seems interesting to study the complexation between these conjugates and biologically active ligands. As one of these substances, we have chosen CAHT [$\text{CH}_3\text{CONH}(\text{CH}_2)_3\text{SO}_3^-$] Ca^{2+} , which is used in the clinic to prevent relapses in weaned alcoholics (17). This ligand is also interesting as a guest molecule containing two anionic moieties in one molecule. Modeling of complexes based on CAHT and α -CD using Stuart-Breigleb models shows that the most stable structure is formed by the incorporation of hydrophobic fragment of CAHT to the cavity of CD, whereas the charged sulfonate groups stay outside of the cycle. For complexes based on β -CD, both of anionic moieties may be incorporated into the cavity. In both cases, the molar stoichiometry of complexes is 1:1.

The complexation of CAHT was studied by ^1H -NMR spectroscopy. When the guest molecule is accommodated in CD, hydrogen atoms of CAHT located in the channel of CD should be shifted upfield (18). Figure 6 presents ^1H -NMR spectra of free CAHT and its complex with α -CD. As follows from this figure, the character of

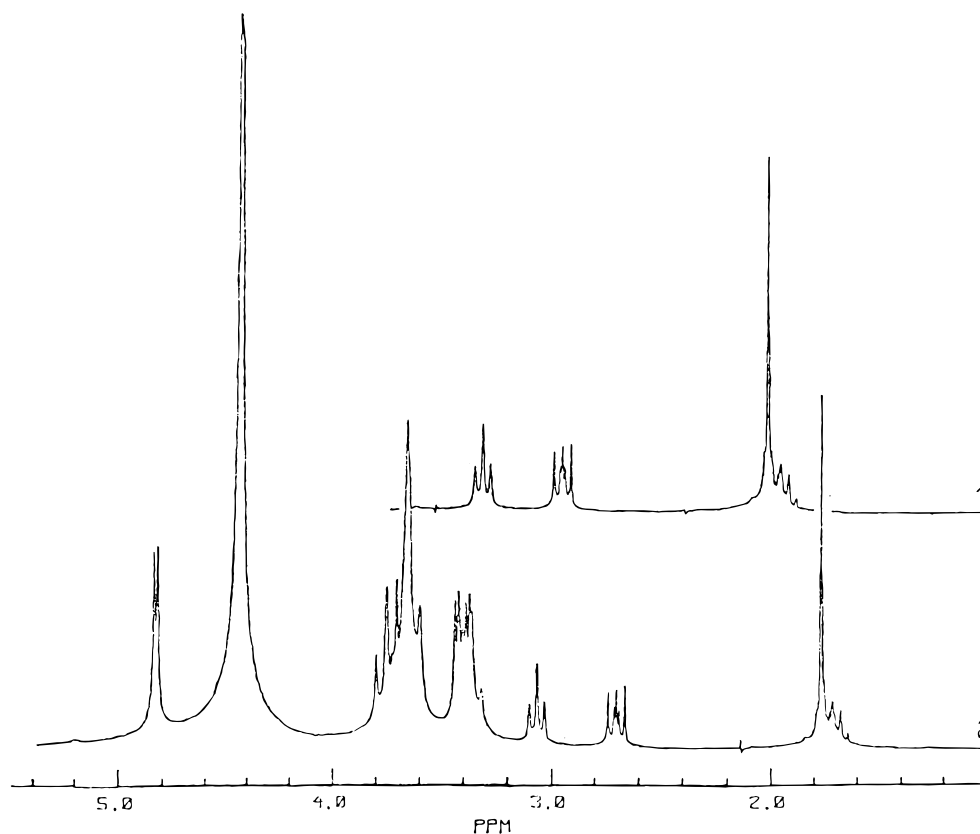


Figure 6. ^1H -NMR spectra of CAHT (1) and CAHT in the presence of α -CD. (2) Concentration of CAHT = 1.25×10^{-2} M, concentration of α -CD = 0.12 M.

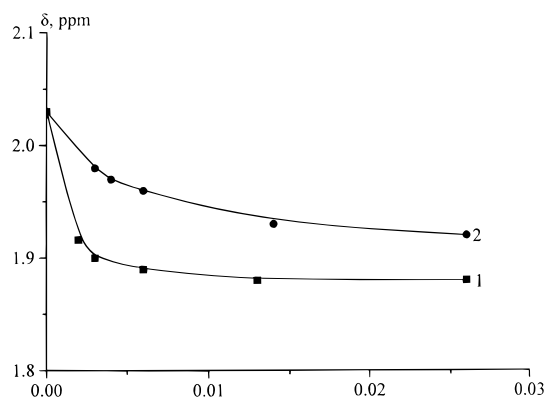


Figure 7. Binding curves for the system based on CAHT and β -CD (■), and PEO- β -CD (preparation 4) (●). Concentration of CAHT is 1.25×10^{-2} M, $T = 303$ K.

changes in ^1H -chemical shifts of methylene and methyl group of CAHT suggests the formation of inclusion complexes. Note that, in the presence of α -CD, the value of ^1H -chemical shift of CH_3 proton is higher than that of methylene groups. These values were used to estimate of K_a . At the same time, as follows from the spectra of the conjugates, C3-H and C5-H of α -CD are also shifted upfield. However, these shifts cannot be used to estimate K_a of the conjugate because of the complex character of the corresponding ^1H -NMR spectrum of CD core. Binding constants K_a were determined from the binding curves (change of ^1H -chemical shifts of methyl protons of CAHT, D , as a function of concentration of CDs) by a curve-fitting analysis using eq 1 (Figure 7 and Table 2).

For complexes based on α - and β -CD, the comparison between K_a shows that K_a for β -CD-CAHT complex increases by more than 2 orders of magnitude as com-

Table 2. Binding Properties of System Based on CDs and Their PEO Derivatives Toward Calcium Acetylhomotaurinate

	CDs or their derivatives	temp K	$D = \delta_0 - \delta_1$	K_a , 1/mol
1	α -CD	313	0.0360	4.6 ± 0.4
		323	0.0335	3.0 ± 0.2
		333	0.0320	2.1 ± 0.2
2	PEO- α -CD	313	0.0360	2.1 ± 0.1
3	β -CD	303	0.0148	1800 ± 200
		323	0.0150	1060 ± 20
		343	0.0124	860 ± 40
4	PEO- β -CD	303	0.0195	320 ± 20
		323	0.0148	168 ± 10
		343	0.0179	63 ± 2

pared this the corresponding constant of α -CD-CAHT. This situation seems to be rather unusual for the complexation of the guests with CDs, which is characterized by a decrease in the K_a with the increase of the size of the cavity (1). This behavior may be explained as follows: β -CD forms a complex with two anionic moieties of CAHT in contrast to α -CD. Both types of PEO-CD conjugates were found to be less effective hosts with respect to CAHT as compared with parent CDs.

Upon binding of CAHT with conjugates, no self-inclusion phenomenon was observed. This is likely to be related to the fact that the interval of concentrations of the conjugate was by 2-3 orders of magnitude higher than that of the corresponding systems based on SN. Taking into account the fact that the formation of autocomplexes proceeds at low concentrations, it seems evident that their effect on binding properties of the conjugates is absent at high concentrations of host molecule. As follows from the Table 2, for all systems under study, the values of K_a decrease with increasing of temperature.

Therefore, a facile procedure for synthesis of new derivatives of CD modified with PEO chains was advanced. These compounds present randomly substituted CD molecules with PEO chains predominantly linked at 2 and 6 positions of glucoside units. Note, that PEO chains are characterized by a narrow MMD. Analysis of the binding properties of PEO-modified derivatives of CD reveals some characteristic features of these hosts. In addition, trivial steric effects associated with the decrease in the dimensions of the cavity of CD-PEO chains grafted to the CD were shown to assist the adaptation due to an additional noncovalent fixation of the ligands in polymer surrounding. The application of biologically active compound CAHT composed of two anionic moieties allows one to identify new types of topologic structures of inclusion complexes of CAHT with CD and PEO-CD conjugates. These investigations provide the basis for a new strategy of targeting of this neuroleptic to brain (16). The conjugates may be used as potent drug delivery systems.

ACKNOWLEDGMENT

We would like to thank Prof. A. S. Shashkov for his valuable assistance in recording and interpretation of NMR spectra, Dr. T. E. Grokhovskaya, F. A. Kalashnikov, and E. I. Popova for their assistance in experimental work and Prof. V. I. Sokolov and Prof. B. I. Kurganov for their interest and fruitful discussion. This work was supported by the Russian Foundation of Basic Research (Project 93-03-33519a) and International Science Foundation (MPE 300).

LITERATURE CITED

- (1) Bender, M. L., and Komiyama, M. (1978) *Cyclodextrin Chemistry*, Springer-Verlag, Berlin, Heidelberg, New York.
- (2) Szejtly, J. *Cyclodextrins and their inclusion complexes*, Akademiai Kiado, Budapest.
- (3) Nozaki, T., Maeda, Y., Ito, K., and Kitano, H. (1995) Cyclodextrins modified with polymer chains which are responsive to external stimuli. *Macromolecules* 28, 522–524.
- (4) Canceill, J., Jullien, L., Lacombe, L., and Lehn, J.-M. (1992) Channel type molecular structures. Part 2. Synthesis of bouquet-shaped molecules based on β -cyclodextrin core. *Helv. Chim. Acta* 75, 791–812.
- (5) Topchieva, I. N., Elezkaya, S. V., Polyakov, V. A., and Karezin, K. I. (1996) Cyclodextrins modified with poly(ethylene oxide) as complexation and transport agents. Abstracts of the 8th International Symposium on Cyclodextrins, Budapest, Hungary, 2–20.
- (6) Mischnick, P. (1989) Determination of the patterns of substitution of hydroxyethyl and hydroxypropyl-cyclomaltoheptaoses. *Carbohydr. Res.* 192, 233–241.
- (7) Tahan, M., and Zilkha, A. (1969) Anionic graft polymerization of ethylene oxide on starch. *J. Polym. Sci.* 7, A-1, 1815–1824.
- (8) Enyeart, C. R. (1966) Polyoxyethylene alkylphenols. in *Nonionic detergents* (M. J. Schick, Ed.) p 62, Marcel Dekker, New York.
- (9) Ke, B. Ed. (1964) Newer methods of polymer characterization p 370, Interscience Publishers, New York.
- (10) Trinadha Rao, C., Lindberg, B., Lindberg, J., and Pitha, J. (1991) Substitution in β -cyclodextrin directed by basicity: preparation of 2-O- and 6-O-[(R)- and (S)-2-hydroxypropyl] derivatives. *J. Org. Chem.* 56, 1327–1329.
- (11) Cramer, F., Saenger, W., and Spatz, H.-Ch. (1967) Inclusion compounds. XIX. The formation of inclusion compounds of α -cyclodextrin in aqueous solutions. Thermodynamics and kinetics. *J. Am. Chem. Soc.* 89, 14–20.
- (12) Suzuki, J., Sakurai, Y., Ohkubo, M., Osa, T., and Ueno, A. (1994) Molecular recognition ability of cyclodextrin derivatives modified at secondary hydroxyl side. *Proc. 7th Int. Symp. Cyclodextrins* Tokyo, Japan, pp 222–225.
- (13) Tabushi, J., Shimizu, N., Sugimoto, T., Shiozuka, M., and Jamamura, K. (1977) Cyclodextrin flexible capped with metal ion. *J. Am. Chem. Soc.* 99, 7100–7102.
- (14) Harada, A., Li, J., Kamachi, M. (1993) Preparation and properties of inclusion complexes of poly(ethylene glycol) with α -cyclodextrin. *Macromolecules* 26, 5698–5703.
- (15) Topchieva, I. N., Elezkaya, S. V., Polyakov, V. A., and Say, S.B. (1993) Conjugates of cyclodextrins with poly(ethylene glycol) as complexation agents and emulgators and the method of their synthesis. Russian patent 93043686.
- (16) Alyautdin, R. N., Petrov, V. E., Elezkaya, S. V., Polyakov, V. A., and Topchieva, I. N. (1993) Transport of neuroleptics to the brain. Russian patent 93043262.
- (17) Zeise, M. L., Kasparov, S., Capogna, M., Zieglansberger, W. (1993) Acamprosate (calciumacetylhomotaurinate) decreases postsynaptic potentials in the rat neocortex: possible involvement of excitatory amino acid receptors, *Eur. J. Pharm.* 231, 47–52.
- (18) Connors, K. A. (1997) Stability of cyclodextrin complexes in solution. *Chem. Rev.* 97, 1325–1357.

BC980005+

Self-Complementary Oligodeoxyribonucleotides Incorporating L-Related Isodeoxynucleosides: Synthesis, Physical Characterization, Enzymology, and CD Studies

Thomas Wenzel and Vasu Nair*

Department of Chemistry, The University of Iowa, Iowa City, Iowa 52242. Received January 15, 1998; Revised Manuscript Received May 22, 1998

The synthesis of self-complementary oligodeoxyribonucleotides (**14–16, 18**) incorporating the L-related isodeoxynucleotides, isodA (**1**) and/or isodT (**2**), is described. For this purpose, the fully protected phosphoramidite building blocks **10** and **12** were synthesized. The conceptually new oligomers exhibit high resistance towards exonucleases. Temperature- and concentration-dependent CD spectral data and accompanying thermodynamic parameters of the oligomers show that isodA-isodT or mixed dA-isodT/isodA-dT base pairs participate in duplex stabilization.

INTRODUCTION

Naturally occurring oligonucleotides are readily degraded by extra- and intracellular nucleases that limit their usefulness in vivo applications. A number of modifications are known that increase the enzymatic stability of oligonucleotides (*1–9*), and most of them are at the phosphate backbone or at the sugar moiety. Unfortunately, in many cases, the increase in stability toward nucleases is accompanied by a decrease in the binding affinity to the target. Hitherto, unknown are oligodeoxynucleotides which incorporate nucleoside components in which the nucleobase at the natural C-1' position is transposed to the isomeric 2'-position (also equivalent to transposition of oxygen), while maintaining the cis relationship of the base with the hydroxymethyl group (Scheme 1).

Recently, isomeric deoxy- and dideoxynucleosides including (S,S)-isodideoxyadenosine [(S,S)-isodda] were synthesized in our laboratory (*10–13*). [S,S]-Isodda exhibited very high resistance toward chemical and enzymatic degradation and was also endowed with significant antiviral activity against HIV-1, HIV-2, and HIV clinical isolates (*14*). Furthermore, (S,S)-isodda triphosphate was a potent inhibitor of HIV reverse transcriptase with K_i values in the nanomolar range (*14*). In order to gain a better understanding of the special characteristics that would be acquired by oligodeoxynucleotides incorporating isomeric nucleosides, including those isonucleosides that exhibit antiviral activity through viral DNA chain termination, we have investigated the synthesis, enzymology, and properties of such conceptually new, self-complementary oligodeoxynucleotides.

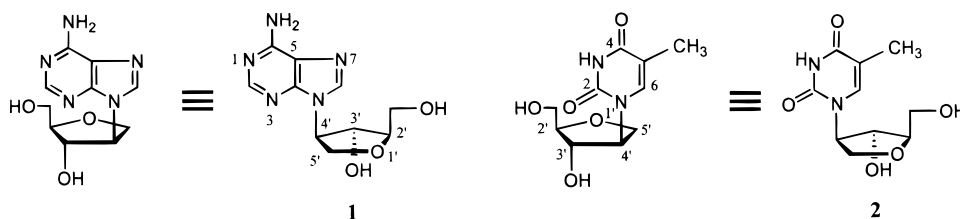
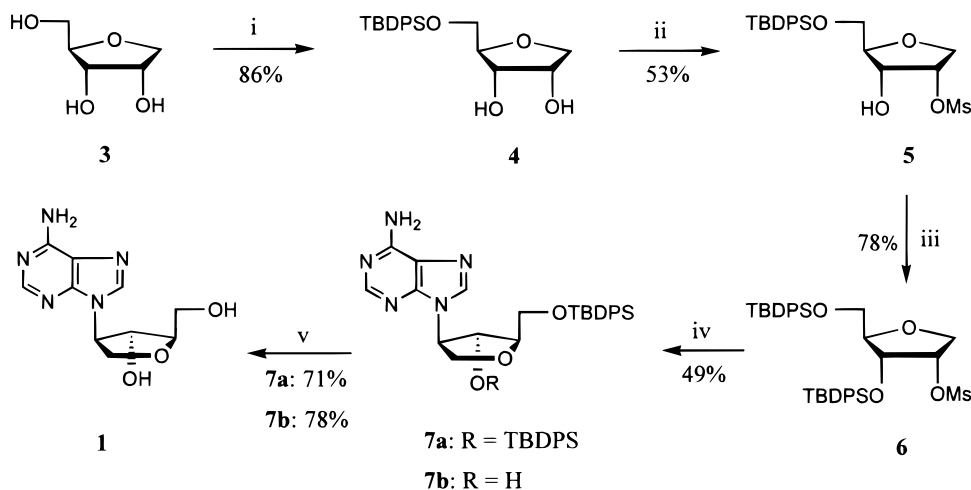
EXPERIMENTAL PROCEDURES

Materials. Melting points are uncorrected and were determined on a Thomas Hoover apparatus fitted with a microscope. ^1H - and ^{13}C -NMR spectra were recorded on a Bruker WM 360 in CDCl_3 or $\text{Me}_2\text{SO}-d_6$. Chemical shift values are reported in δ , parts per million, relative to the internal standard (TMS). UV spectra were recorded on a Varian Cary 3 spectrophotometer (Melbourne, Australia). CD spectra were measured on an AVIV Circular Dichroism Spectrometer, model 62DS (AVIV, Lakewood, NJ). Elemental analysis was per-

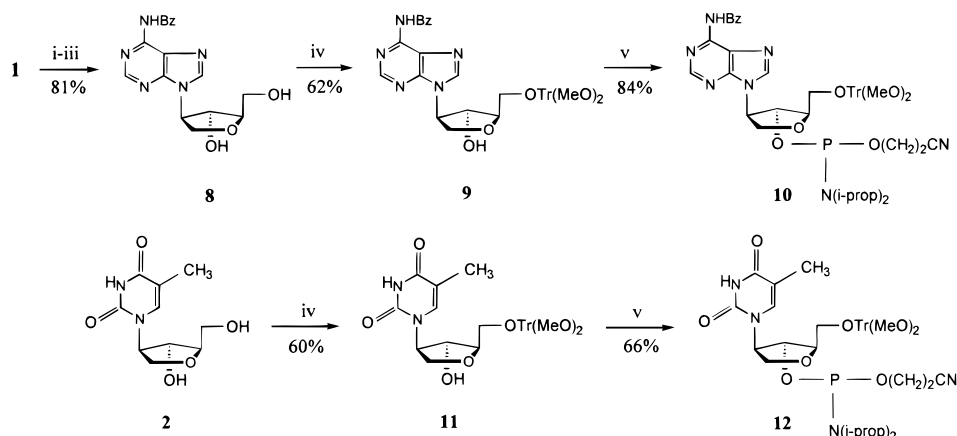
formed at NuMega Resonance Labs, Inc. (San Diego, CA). Thin-layer chromatography (TLC) was carried out on Polygram Sil G/UV₂₅₄ (0.25 mm, Machery-Nagel, Düren, Germany). TLC plates were visualized by ultraviolet absorbance or charring for several minutes after exposure to a 10% sulfuric acid/ethanol solution. Flash chromatography (FC) was carried out using glass columns packed with 230–400 mesh silica gel. Reversed-phase HPLC separations (4.6 $\times$ 250 mm, RP-18, 10 μm , Li-Chromosorb column, Merck, Darmstadt, Germany) were achieved using a Waters automated 600E system (Milford, MA) interfaced with a photodiode array detector (Waters 990) and a Satellite WISP (Waters 700) for automated sampling. The solid-phase synthesis of oligonucleotides was performed on an automated DNA-synthesizer (Applied Biosystems, Foster City, CA, model AB 391, PCR-MATE for phosphoramidite chemistry). The β -cyanoethyl phosphoramidites of regular 2'-deoxynucleosides and the Fractosil-linked 2'-deoxynucleosides were also products of Applied Biosystems. Snake-venom phosphodiesterase (EC 3.1.4.1., *Crotallus durissus*), calf-spleen phosphodiesterase (EC 3.1.16.1.), alkaline phosphatase (EC 3.1.3.1., *Escherichia coli*), and Eco R1 (*E. coli* BS 5) were purchased from Boehringer Mannheim (Mannheim, Germany).

Synthesis (Schemes 2 and 3). **4(R)-(6-Amino-9H-purin-9-yl)-2(R)-(hydroxymethyl)tetrahydrofuran-3(R)-ol (1).** To an ice-cold solution of **3** (4.00 g, 29.8 mmol) in anhydrous pyridine (80 mL) was added, under N_2 , *tert*-butyldiphenylsilyl chloride (7.8 mL, 29.8 mmol). The mixture was stirred for 3 h at 0 $^\circ\text{C}$, quenched with H_2O (10 mL), evaporated, and the oily residue applied on the top of a silica gel column (column, 25 $\times$ 5 cm; solvent, $\text{CHCl}_3/\text{MeOH}$ 95:5). Evaporation of the separated product yielded **4** (9.50 g, 86%) as a colorless solid. mp 75 $^\circ\text{C}$. TLC ($\text{CHCl}_3/\text{MeOH}$): R_f 0.3. ^1H -NMR (CDCl_3): δ 1.09 [s, 9H, $(\text{CH}_3)_3\text{C}$], 3.20 [s (br), 1H, OH-3], 3.39 [s (br), 1H, OH-4], 3.75–3.83 (m, 3H, H β -5/ CH_2), 3.86 (m, 1H, H-2), 4.15 (dd, 1H, J = 4.7, 9.1 Hz, H α -5), 4.20–4.26 (m, 2H, H-3/H-4), 7.34–7.42 (m, 6H, arom. H), 7.65–7.70 (m, 4H, arom. H). ^{13}C -NMR (CDCl_3): δ 19.4 [$(\text{CH}_3)_3\text{C}$], 27.0 [$(\text{CH}_3)_3\text{C}$], 64.5 (CH_2), 71.7 (C-3), 73.2 (C-4, C-5), 83.1 (C-2). A solution of **4** (5.00 g, 13.4 mmol) in dry CH_2Cl_2 (125 mL) was cooled down to 0 $^\circ\text{C}$. At this

Scheme 1

Scheme 2^a

^a (i) TBDPSCl, pyridine, 0 °C, 3 h. (ii) MsCl, Et₃N, CH₂Cl₂, 0 °C, 3 h. (iii) TBDPSCl, imidazole, DMF, rt, 24 h. (iv) Adenine, K₂CO₃, 18-crown-6, DMF, 120 °C, 60 h. (v) 0.5 M NH₄F/MeOH, reflux 8 h.

Scheme 3^a

^a (i) Me₃SiCl, pyridine, rt 0.5 h. (ii) BzCl, 0 °C, 2 h. (iii) NH₃/H₂O. (iv) (MeO)₂TrCl, Et(i-prop)₂N, pyridine, rt, 2 h. (v) Chloro-(2-cyanoethoxy)(diisopropylamine)phosphine, Et(i-prop)₂N, rt 1.5 h.

temperature, Et₃N (3.9 mL, 26.7 mmol) and methanesulfonyl chloride (1.0 mL, 13.4 mmol) were added and the mixture was stirred for 3 h at 0 °C under N₂. Water (10 mL) was added, the solvent was evaporated, and the oily residue was purified by FC (column, 30 × 3 cm; solvent, CHCl₃) yielding compound **5** (3.2 g, 53%) as a colorless oil. TLC (CHCl₃/MeOH 95:5): *R*_f 0.6. ¹H-NMR (CDCl₃): δ 1.09 [s, 9H, (CH₃)₃C], 2.71 (d, 1H, *J* = 3.6 Hz, OH-3), 3.13 (s, 3H, CH₃), 3.82 (dd, 1H, *J* = 3.3, 11.2 Hz, CH₂), 3.89 (m, 1H, CH₂), 3.94 (m, 1H, H-2), 4.08 (dd, 1H, *J* = 3.6, 10.5 Hz, Hβ-5), 4.24 (dd, 1H, *J* = 4.9, 10.5 Hz, Hα-5), 4.47 (m, 1H, H-3), 5.22 (m, 1H, H-4), 7.39–7.46 (m, 6H, arom. H), 7.71 (m, 4H, arom. H). ¹³C-NMR (CDCl₃): δ 19.4 [(CH₃)₃C], 27.0 [(CH₃)₃C], 38.7 (SO₂CH₃), 63.9 (CH₂), 70.5 (C-5), 72.0 (C-3), 80.4 (C-4), 82.8 (C-2). To a solution of **5** (2.1 g, 4.7 mmol) in dry DMF (35 mL) were added imidazole (930 mg, 14.0 mmol) and *tert*-butyldiphenylsilyl chloride (1.23 mL, 4.7 mmol) and the

reaction mixture was stirred for 18 h at rt under N₂. Then, *tert*-butyldiphenylsilyl chloride (1.23 mL, 4.7 mmol) was added again and the stirring maintained for another 6 h at rt. The solvent was removed under high-vacuum and the crude product was purified by FC (column, 20 × 3 cm; solvent, (CHCl₃). Collection and evaporation of the main zone afforded **6** (2.5 g, 78%) as a viscous, colorless oil. TLC (CHCl₃): *R*_f 0.3. ¹H-NMR (CDCl₃): δ 0.99 [s, 9H, (CH₃)₃C], 1.12 [s, 9H, (CH₃)₃C], 2.84 (s, 3H, CH₃), 3.33 (dd, 1H, *J* = 3.2, 11.4 Hz, CH₂), 3.64 (dd, 1H, *J* = 2.6, 11.4 Hz, CH₂), 4.02 (m, 1H, H-2), 4.15 (m, 2H, H-5), 4.55 (t, 1H, *J* = 4.6 Hz, H-3), 4.88 (dd, 1H, *J* = 4.9, 9.5 Hz, H-4), 7.31–7.46 (m, 12H, arom. H), 7.54–7.66 (m, 6H, arom. H), 7.74 (m, 4H, arom. H). ¹³C-NMR (CDCl₃): δ 19.3, 19.5 [(CH₃)₃C], 26.9, 27.0 [(CH₃)₃C], 38.3 (SO₂CH₃), 63.8 (CH₂), 69.8 (C-5), 72.9 (C-3), 78.3 (C-4), 84.6 (C-2). A mixture of **6** (2.50 g, 3.6 mmol), K₂CO₃ (990 mg, 7.2 mmol), 18-crown-6 (960 mg, 3.6 mmol), and adenine (970

mg, 7.2 mmol) in dry DMF (120 mL) was heated for 60 h at 120 °C. The solvent was evaporated under reduced pressure. The residue was dissolved in H₂O (400 mL) and extracted with CHCl₃ (3 × 200 mL). Then, the combined organic layers were extracted with brine (2 × 200 mL), dried (Na₂SO₄), evaporated and purified by FC (column, 20 × 3 cm, solvent, CHCl₃/MeOH 95:5). The faster migrating zone furnished **7a** (580 mg, 22%) as a light yellow oil. TLC (CHCl₃/MeOH 95:5): *R_f* 0.5. ¹H-NMR (CDCl₃): δ 0.98 [s, 9H, (CH₃)₃C], 1.01 [s, 9H, (CH₃)₃C], 3.63 (dd, 1H, *J* = 4.4, 11.6 Hz, CH₂), 3.75 (dd, 1H, *J* = 2.4, 11.6 Hz, CH₂), 4.08 (m, 1H, H-2'), 4.13 (dd, 1H, *J* = 4.5, 9.7 Hz, Hβ-5'), 4.29 (dd, 1H, *J* = 6.5, 9.7 Hz, Hα-5'), 4.78 (m, 1H, H-3'), 5.13 (m, 1H, H-4'), 6.13 [s (br), 2H, NH₂-6], 7.07–7.47 (m, 16H, arom. H), 7.54–7.62 (m, 4H, arom. H), 7.74 (m, 4H, arom. H), 8.01 (s, 1H, H-2), 8.22 (s, 1H, H-8). ¹³C-NMR (CDCl₃): δ 19.1, 19.3 [(CH₃)₃C], 26.9, 27.0 [(CH₃)₃C], 63.0 (C-3'), 63.2 (CH₂), 71.2 (C-5'), 78.0 (C-4'), 86.9 (C-2'), 119.4 (C-5), 138.8 (C-8), 149.8 (C-4), 152.7 (C-2), 155.6 (C-6). The slower migrating zone afforded **7b** (470 mg, 27%) as light yellow foam. TLC (CHCl₃/MeOH 95:5): *R_f* 0.4. ¹H-NMR (CDCl₃): δ 1.02 [s, 9H, (CH₃)₃C], 3.88 (dd, 1H, *J* = 4.1, 11.3 Hz, CH₂), 3.93 (dd, 1H, *J* = 3.5, 11.4 Hz, CH₂), 4.06 (m, 1H, H-2'), 4.33 (dd, 1H, *J* = 6.4, 9.4 Hz, Hβ-5'), 4.49 (dd, 1H, *J* = 6.6, 9.4 Hz, Hα-5'), 4.65 (m, 1H, H-3'), 4.88 (m, 1H, H-4'), 5.75 [s (br), 1H, OH-3'], 5.98 [s (br), 2H, NH₂-6], 7.32–7.43 (m, 6H, arom. H), 7.65 (m, 4H, arom. H), 7.75 (s, 1H, H-2), 8.28 (s, 1H, H-8). ¹³C-NMR (CDCl₃): δ 19.4 [(CH₃)₃C], 27.0 [(CH₃)₃C], 63.3 (CH₂), 64.0 (C-3'), 69.7 (C-5'), 77.0 (C-4'), 85.1 (C-2'), 119.7 (C-5), 138.6 (C-8), 150.2 (C-4), 152.8 (C-2), 155.9 (C-6).

To a solution of **7a** (500 mg, 0.68 mmol) in MeOH (10 mL) was added 0.5M NH₄F (20 mL). The mixture was refluxed for 18 h and the solvent was evaporated and the residue was purified by FC (column, 10 × 3 cm, solvent: CHCl₃/MeOH 8:2) to give **1** (120 mg, 71%). Compound **7b** (400 mg, 0.82 mmol) was similarly deprotected to give **1** (160 mg, 78%); mp 227 °C (EtOH). TLC (CHCl₃/MeOH 8:2), *R_f* 0.2. UV (MeOH): λ 260 nm (ε 14 800). ¹H-NMR (Me₂SO-*d*₆): δ 3.57–3.68 (m, 2H, CH₂), 3.70 (m, 1H, H-2'), 4.10 (dd, 1H, *J* = 4.9, 9.6 Hz, Hβ-5'), 4.49 (dd, 1H, *J* = 6.6, 9.6 Hz, Hα-5'), 4.40 (m, 1H, H-3'), 4.85–4.91 (m, 2H, H-4'/CH₂OH), 5.76 (d, 1H, *J* = 3.9 Hz, OH-3'), 7.23 [s (br) 2H, NH₂-6], 8.15 (s, 1H, H-2), 8.19 (s, 1H, H-8). ¹H-NOE-difference NMR (D₂O): Irradiation at H-4': Observed NOEs at H-1'α (2.8%), H-2' (3.2%), H-2 (0.6%), H-8 (4.3%). ¹³C-NMR (Me₂SO-*d*₆): δ 60.9 (CH₂), 61.9 (C-3'), 69.5 (C-5'), 75.7 (C-4'), 85.9 (C-2'), 118.7 (C-5), 139.2 (C-8), 149.4 (C-4), 152.4 (C-2), 156.0 (C-6). Anal. Calcd for C₁₀H₁₃N₅O₃: C, 47.81; H, 5.22; N, 27.87. Found: C, 47.46; H, 5.34; N, 27.76.

4(R)-(6-Benzoylamino-9H-purin-9-yl)-2(R)-(hydroxymethyl)tetrahydrofuran-3(R)-ol (8). To a solution of **1** (200 mg, 0.80 mmol) in dry pyridine (20 mL) was added, under N₂, Me₃SiCl (510 μL, 3.97 mmol). The mixture was stirred for 30 min at rt. After cooling down to 0 °C, benzoyl chloride (460 μL, 3.97 mmol) was added and the reaction mixture was stirred for 2 h. Excess of benzoyl chloride was destroyed by adding H₂O (5 mL). After 10 min, 25% aqueous NH₃ (10 mL) was added, and the reaction mixture was stirred for another 30 min. The solution was evaporated and the residue was purified by FC (column, 15 × 3 cm; solvent, CHCl₃/MeOH 9:1). Isolation of the main zone yielded **8** (230 mg, 81%); mp 198–199 °C (colorless plates from acetone/MeOH). TLC (CHCl₃/MeOH 9:1): *R_f* 0.4. UV (MeOH): λ 280 nm (ε 19 000). ¹H-NMR (Me₂SO-*d*₆): δ 3.57–3.66 (m, 2H, CH₂), 3.73 (m, 1H, H-2'), 4.21 (m, 2H, H-5'), 4.44 (m, 1H, H-3'),

4.92 (t, 1H, *J* = 5.5 Hz, CH₂OH), 5.03 (m, 1H, H-4'), 5.81 (d, 1H, *J* = 5.4 Hz, OH-3'), 7.52–7.65 (m, 3H, arom. H), 8.04 (d, 2H, *J* = 7.1 Hz, arom. H), 8.53 (s, 1H, H-2), 8.74 (s, 1H, H-8), 11.14 [s (br), 1H, NH-6]. ¹³C-NMR (Me₂SO-*d*₆): δ 60.7 (CH₂), 62.2 (C-3'), 69.4 (C-5'), 75.7 (C-4'), 86.0 (C-2'), 125.4 (C-5), 143.0 (C-8), 150.2 (C-4), 151.3 (C-6), 152.3 (C-2). Anal. Calcd for C₁₇H₁₇N₅O₄: C, 57.46; H, 4.82; N, 19.71. Found: C, 57.19; H, 4.97; N, 19.61.

4(R)-(6-Benzoylamino-9H-purin-9-yl)-2-[O-(4,4'-dimethoxytriphenylmethyl)-2(R)-(hydroxymethyl)-tetrahydrofuran-3(R)-ol (9). To a solution of **8** (200 mg, 0.56 mmol) in anhydrous pyridine (5 mL) were added, under N₂, ethyldiisopropylamine (370 μL, 2.28 mmol) and 4,4'-dimethoxytrityl chloride (290 mg, 0.86 mmol). The mixture was stirred for 2 h at rt. The excess of acid chloride was destroyed with 5% aqueous NaHCO₃ solution (5 mL). The aqueous layer was extracted with CH₂Cl₂ (5 × 5 mL), the solvent was evaporated, and the oily residue was applied on the top of a silica gel column (column, 20 × 3 cm; solvent, CHCl₃/MeOH 95:5). Evaporation of the main zone furnished **9**, which precipitated from light petroleum ether as a colorless solid (230 mg, 62%); mp 119–122 °C. TLC (CHCl₃/MeOH 95:5): *R_f* 0.4. UV (MeOH): λ 270 nm (ε 26 000). ¹H-NMR (Me₂SO-*d*₆): δ 3.19 (m, 2H, CH₂), 3.73 (s, 3H, OCH₃), 3.74 (s, 3H, OCH₃), 3.94 (m, 1H, H-2'), 4.30–4.37 (m, 2H, H-5'), 4.56 (m, 1H, H-3'), 5.05 (m, 1H, H-4'), 5.81 (d, 1H, *J* = 5.4 Hz, OH-3'), 6.88 (d, 4H, *J* = 8.9 Hz, arom. H), 7.20–7.40 (m, 7H, arom. H), 7.42 (m, 2H, arom. H), 7.55 (t, 2H, *J* = 7.2 Hz, arom. H), 7.63 (t, 1H, *J* = 7.3 Hz, arom. H), 8.05 (d, 2H, *J* = 7.3 Hz, arom. H), 8.50 (s, 1H, H-2), 8.74 (s, 1H, H-8), 11.16 [s (br), 1H, NH-6]. ¹³C-NMR (Me₂SO-*d*₆): δ 54.9, 55.0 (OCH₃), 62.1 (C-3'), 63.5 (CH₂), 68.5 (C-5'), 75.0 (C-4'), 83.3 (C-2'), 125.7 (C-5), 143.2 (C-8), 150.3 (C-4), 151.3 (C-6), 152.4 (C-2). Anal. Calcd for C₃₈H₃₅N₅O₆: C, 69.39; H, 5.36; N, 10.65. Found: C, 68.28; H, 5.31; N, 10.79.

4(R)-(6-Benzoylamino-9H-purin-9-yl)-2-[O-(4,4'-dimethoxytriphenylmethyl)-2(R)-(hydroxymethyl)-tetrahydrofuran-3(R)-yl-(2-cyanoethyl-N,N-diisopropylphosphoramidite (10). A solution of **9** (200 mg, 0.30 mmol) in dry CH₂Cl₂ (7 mL) was kept under N₂. Then, chloro(2-cyanoethoxy)(diisopropylamino)phosphine (200 μL, 0.91 mmol) together with ethyldiisopropylamine (160 μL, 0.93 mmol) was added under N₂. After stirring for 90 min at rt, 5% aqueous NaHCO₃ solution (7 mL) was added and the mixture was extracted with CH₂Cl₂ (5 × 5 mL). The extract was dried (Na₂SO₄), evaporated, and purified by FC (column, 10 × 3 cm; solvent, CH₂Cl₂/EtOAc/Et₃N 49:49:2). Isolation of the overlapping main zones yielded the diastereoisomeric mixture **10** (220 mg, 84%) as an oil. TLC (CH₂Cl₂/EtOAc/Et₃N 49:49:2): *R_f* 0.3, 0.4. ¹H-NMR (CDCl₃): δ 0.96–1.12 [m, 14H, (CH₃)₂CH], 2.28 (m, 2H, CH₂CH₂CN), 2.53 (m, 2H, CH₂CH₂CN), 3.44–3.57 (m, 2H, CH₂), 3.76 (s, 3H, OCH₃), 3.77 (s, 3H, OCH₃), 4.10–4.18 (m, 1H, H-2'), 4.38 (m, 2H, H-5'), 4.80 (m, 1H, H-3'), 5.24 (m, 1H, H-4'), 6.77–6.83 (m, 4H, arom. H), 7.18–7.32 (m, 7H, arom. H), 7.38–7.44 (m, 2H, arom. H), 7.53 (t, 2H, *J* = 7.7 Hz, arom. H), 7.60 (t, 1H, *J* = 7.3 Hz, arom. H), 8.03 (d, 2H, *J* = 7.1 Hz, arom. H), 8.23 (s, 1H, H-2), 8.80 (s, 1H, H-8), 9.02 [s (br), 1H, NH-6]. ³¹P-NMR (CDCl₃): δ 151.2, 151.4. Anal. Calcd for C₄₇H₅₂N₇O₇P: C, 65.80; H, 6.11; N, 11.43. Found: C, 64.74; H, 6.30; N, 11.32.

4(R)-[3,4-Dihydro-2,4-dioxo-5-methyl-1(2H)-pyrimidinyl]-2-[O-(4,4'-dimethoxytriphenylmethyl)-2(R)-(hydroxymethyl)-tetrahydrofuran-3(R)-ol (11). Compound **2** (200 mg, 0.83 mmol) was dissolved in dry pyridine (10 mL) and stirred with 4,4'-dimethoxytri-

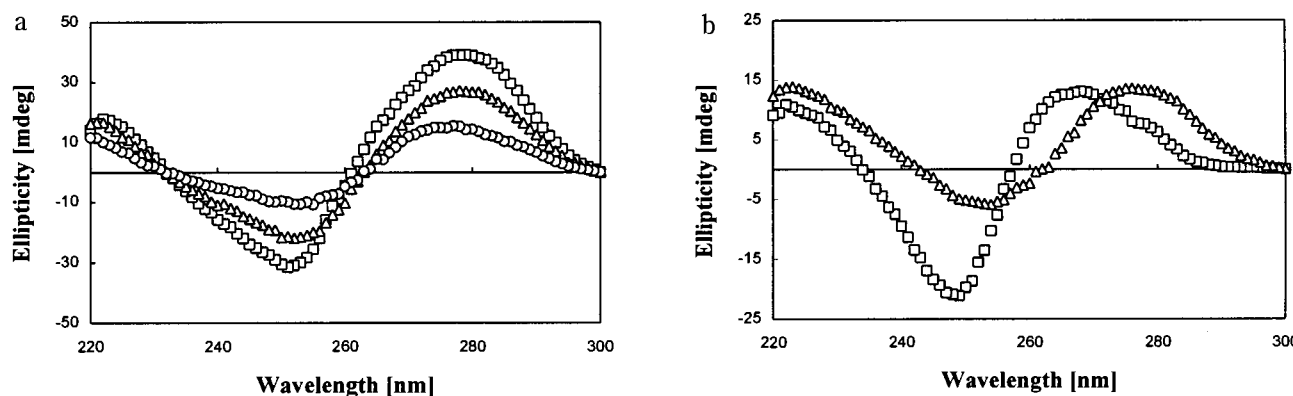


Figure 1. (a) Temperature-dependent CD-spectra of d(G-T-A-G-iA-iA-iT-iT-C-T-A-C) (**15**) in 60 mM sodium-cacodylate (pH 7.0, 100 mM MgCl₂, 1 M NaCl); oligomer concentration, 5.0 μ M; ($\square$) 10 $^{\circ}$ C; (Δ) 40 $^{\circ}$ C; ($\circ$) 70 $^{\circ}$ C. (b) CD spectra of d(A-T)₆ (**17**, $\square$) and d(A-iT)₆-T (**18**, Δ) at 10 $^{\circ}$ C; conditions, see panel a; oligomer concentration, 5.6 μ M.

phenylmethyl chloride (420 mg, 1.26 mmol) in the presence of ethyldiisopropylamine (540 μ L, 3.32 mmol) for 3 h at rt under N₂. The solution was quenched with 5% aqueous NaHCO₃ (10 mL), extracted with CH₂Cl₂ (5 $\times$ 10 mL), and the combined organic layers were dried (Na₂SO₄), and the solvent was evaporated. The residue was purified by FC (column, 15 $\times$ 3 cm; CHCl₃/MeOH 95:5). Evaporation of the main zone afforded **11** (270 mg, 60%) as a colorless foam. TLC (CHCl₃/MeOH 95:5): *R*_f 0.4. UV (MeOH): λ 232, 273 nm, (ϵ 24 400, 11 900). ¹H-NMR (Me₂SO-*d*₆): δ 1.60 (s, 3H, CH₃), 3.12 (dd, 1H, *J* = 5.8, 10.4 Hz, CH₂OH), 3.18 (dd, 1H, *J* = 2.5, 10.4 Hz, CH₂OH), 3.71 (s, 3H, OCH₃), 3.72 (s, 3H, OCH₃), 3.75 (m, 1H, H-2'), 3.94 (dd, 1H, *J* = 5.0, 10.1 Hz, H β -5'), 4.04 (dd, 1H, *J* = 7.3, 10.1 Hz, H α -5'), 4.13 (m, 1H, H-3'), 4.77 (m, 1H, H-4'), 5.61 (d, 1H, *J* = 5.8 Hz, OH-3'), 6.87 (d, 4H, *J* = 7.8 Hz, arom. H), 7.19–7.31 (m, 7H, arom. H), 7.38 (d, 2H, *J* = 7.1 Hz, arom. H), 7.44 (s, 1H, H-4), 11.29 (s, 1H, NH-1). ¹³C-NMR (Me₂SO-*d*₆): δ 55.0, 55.1 (OCH₃), 62.5 (C-3'), 63.2 (CH₂), 68.5 (C-5'), 75.3 (C-4'), 83.5 (C-2'), 109.3 (C-5), 137.6 (C-6), 151.2 (C-2), 163.7 (C-4). Anal. Calcd for C₃₁H₃₂N₂O₇: C, 68.37; H, 5.92; N, 5.14. Found: C, 67.97; H, 5.64; N, 4.99.

4(R)-[3,4-Dihydro-2,4-dioxo-5-methyl-1(2H)-pyrimidinyl]-2-[O-(4,4'-dimethoxytriphenylmethyl)]-2(R)-(hydroxymethyl)-tetrahydrofuran-3(R)-yl-(2-cyanoethyl-N,N-diisopropylphosphoramidite) (12**).** A solution of **11** (200 mg, 0.37 mmol) and ethyldiisopropylamine (190 μ L, 1.08 mmol) in dry CH₂Cl₂ (10 mL) was treated with chloro(2-cyanoethoxy) (diisopropylamino)phosphine (230 μ L, 1.02 mmol) at rt under N₂. After stirring for 1 h, the reaction was quenched by adding 5% aqueous NaHCO₃ solution (10 mL). The mixture was extracted with CH₂Cl₂ (5 $\times$ 10 mL), the organic layer was dried (Na₂SO₄) and evaporated and the residue was purified by FC (column, 10 $\times$ 3 cm; solvent, CH₂Cl₂/EtOAc/Et₃N 49:49:2). Isolation of the overlapping main zones furnished **12** (180 mg, 66%) as a diastereoisomeric mixture. TLC (CH₂Cl₂/EtOAc/Et₃N 49:49:2): *R*_f 0.6, 0.8. ¹H-NMR (CDCl₃): δ 1.04–1.25 [m, 14H (CH₃)₂CH], 1.54 (s, 3H, CH₃), 2.20 (m, 2H, CH₂CH₂CN), 2.56 (m, 2H, CH₂CH₂CN), 3.41–3.51 (m, 2H, CH₂OH), 3.71 (s, 3H, OCH₃), 3.72 (s, 3H, OCH₃), 3.82–3.88 (m, 1H, H-2'), 3.98–4.15 (m, 2H, H-5'), 4.52 (m, 1H, H-3'), 5.09 (m, 1H, H-4'), 6.75 (m, 4H, arom. H), 7.14–7.26 (m, 7H, arom. H), 7.34 (m, 3H, H-4/arom. H), 8.30 (s, 1H, NH-1). ³¹P-NMR (CDCl₃): 151.6, 151.9. Anal. Calcd for C₄₀H₄₉N₄O₈P: C, 64.50; H, 6.63; N, 7.52. Found: C, 63.63; H, 6.46; N, 7.45.

Solid-Phase Synthesis of the Oligonucleotides 13–18. Synthesis of the oligomers **13–18** was carried

out on a 1 μ mol scale using the 3'-cyanoethyl phosphoramidites of the following protected deoxynucleosides: [(MeO)₂Tr]dT, [(MeO)₂Tr]Bz⁴dC, [(MeO)₂Tr]Bz⁶dA, [(MeO)₂Tr]ib²dG, and compounds **1** and **2**. Deprotection of the oligonucleotides was performed in 25% aqueous NH₃ at 60 $^{\circ}$ C for 24 h. The 5'-[(MeO)₂Tr]-oligomers were purified, detritylated and desalted using OPC (ABI, Masterpiece). The oligonucleotides were lyophilized, dissolved in H₂O (200 μ L), and stored frozen at –20 $^{\circ}$ C.

Enzymatic Hydrolysis of the Oligomers. The self-complementary oligonucleotides **13–17** (0.2 A₂₆₀ units) were dissolved in 0.1 M Tris-HCl buffer (pH 8.3; 200 μ L) and treated with snake-venom phosphodiesterase (6 μ g) for 24 h at 37 $^{\circ}$ C followed by alkaline phosphatase (2 μ g) for 30 min at 37 $^{\circ}$ C. The mixture was analyzed using RP-18 HPLC (gradient II, followed by gradient I, see below). The quantification of the oligomers was made on the basis of the peak areas which were divided by the extinction coefficients of the nucleoside constituents.

Enzymatic Hypochromicity of the Oligonucleotides 13–17. The enzymatic hypochromicities were determined by enzymatic degradation of 0.2 A₂₆₀ units of the corresponding oligomer as described above.

Time Course of Phosphodiester Hydrolysis of the Oligomers 13–18. For these experiments, the corresponding oligonucleotide (2.25 A₂₆₀ units) was dissolved in 0.1 M Tris-HCl buffer (900 μ L, pH 8.3), and alkaline phosphatase (22.5 μ g) was added; two stock solutions were prepared. The reaction was started by addition of either snake-venom phosphodiesterase (67.5 μ g) or calf-spleen phosphodiesterase (135 μ g). From the stock solutions, samples (100 μ L) were taken after a certain interval of time. An aliquot (80 μ L) was examined by HPLC (gradient II, followed by I, see below). The UV absorbance was measured at 260 nm and quantification was made on the basis of peak areas and extinction coefficients.

HPLC Separation. The following solvent systems consisting of 0.1 M (Et₃NH)OAc (pH 7)/MeCN 95:5 (A) and MeCN (B) were used: gradient I, 20 min 0–30% B in A, 5 min 30% B in A, flow rate, 1 mL/min; gradient II, 30 min 100% A, flow rate, 0.6 mL/min.

CD Spectral Data. Temperature-dependent CD spectra were obtained under the conditions described in Figures 1 and 2.

RESULTS AND DISCUSSION

Synthesis of Monomers. While two methods are available for the synthesis of isomeric deoxyadenosine

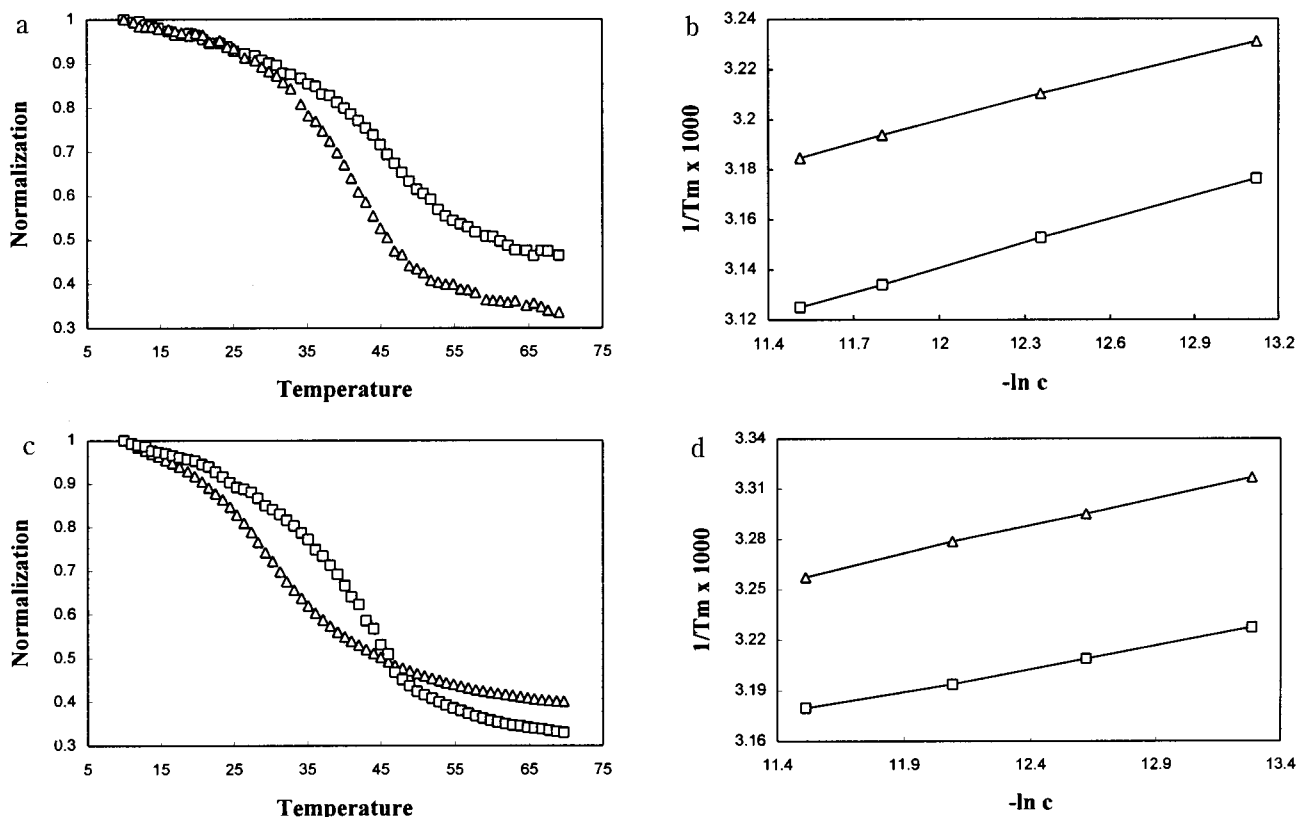


Figure 2. (a) Normalized melting profiles of d(G-T-A-G-iA-iA-T-T-C-T-A-C) (**14**, □) and d(G-T-A-G-iA-iA-iT-T-C-T-A-C) (**15**, △); conditions, see Figure 1a; oligomer concentration 5 μ M. (b) $- \ln c$ vs $1/T_m$ plot, **14** (□) and **15** (△). (c) Normalized melting profiles of d(G-iT-iA-G-A-A-T-T-C-iT-iA-C) (**16**, □) and d(A-iT)₆-T (**18**, △); conditions, see Figure 1a. (d) $- \ln c$ vs $1/T_m$ plot, **16** (□) and **18** (△).

(**15**, **16**), these either give low yields of product or have been developed for very small scale synthesis. As the synthesis of oligodeoxyribonucleotides requires the availability of isodeoxyadenosine in gram quantities, we developed a synthesis strategy for isodA (**1**) starting from 1-deoxyribose (**3**) (**17**). This approach was based on previous work from our laboratory (**18**). Selective protection of the primary hydroxyl group by treatment of **3** with *tert*-butyldiphenylsilyl chloride (TBDPS-Cl) in anhydrous pyridine at 0 °C afforded **4** in 86% yield. Methanesulfonylation of **4** at 0 °C led to a mixture of mesylates from which **5** was isolated in 53% yield. The 3-mesylate was the minor product (21%). Following protection of the 3-hydroxyl group in **5** (78%), the resulting compound **6** was coupled with adenine in the presence of K₂CO₃ and 18-crown-6 in dry DMF. However, this alkylation step required drastic conditions (120 °C, 60 h) and compound **7a** and its partially deprotected derivative **7b** were obtained in only 49% yield. Deprotection of nucleosides **7a** and **7b** was carried out using 0.5 M methanolic NH₄F solution to furnish **1** in high yields.

The isomeric thymine analog, isodT, could not be prepared in good yields using the alkylation step described above because of complications associated with O-alkylation. However, this intermediate **2** was prepared using modification of a procedure developed previously in our laboratory recently (**18**).

Nucleosides **1** and **2** were converted into the β -cyanoethyl phosphoramidite building blocks **10** and **12**. As *N*⁶-benzoyldeoxyadenosine has a half-life of 71 min under mild alkaline conditions (25% aqueous NH₃ solution (**19**), we protected the exocyclic amino group of **1** with the benzoyl group. Reaction of **1** with benzoyl chloride, after temporary protection of the sugar hydroxyls with Me₃Si

residues (transient protection) (**20**), afforded compound **8** in 81% yield. Compounds **8** and **2** were converted into the 4,4'-dimethoxytrityl [(MeO)₂Tr] derivatives **9** and **11** under standard conditions (60–62%). Subsequent phosphorylation of **9** and **11** with chloro(diisopropylamino)- β -cyanoethoxyphosphine furnished the phosphoramidites **10** and **12** as diastereoisomeric mixtures in good yields. These new compounds were characterized by ¹H-, ¹³C-, and ³¹P-NMR spectral data (see Experimental Procedures). Assignment of the protons was made on the basis of COSY spectra, whereas the unequivocal assignment of the carbons was aided by [¹H,¹³C]-2D-correlation or [¹H,¹³C]-gated decoupled spectra. The anomeric configuration and the alkylation position of compound **1** were assigned by ¹H-NOE difference spectroscopy (see Experimental Procedures) (**21**).

Synthesis of Oligonucleotides. The β -cyanoethyl phosphoramidites **10** and **12** were used in automated solid-phase synthesis of oligonucleotides (**22**). However, the incorporation of the isomeric DNA building blocks **10** and **12** required 10 times longer coupling times than the corresponding 2'-deoxyribophosphoramidites. Furthermore, the coupling yields determined on the liberation of the 4,4'-dimethoxytrityl cation at 498 nm were significantly lower than those of the regular DNA-building blocks (**10**, 70%; **12**, 80%). After treatment with concentrated aqueous NH₃ solution (24 h at 60 °C), the 5'-(MeO)₂Tr-protected oligomers were purified, detritylated, and desalted on oligonucleotide-purification cartridges (Perkin Elmer, Applied Biosystem Division) to yield the oligomers **13–18** (Table 1). The content of the modified oligonucleotides was determined by tandem hydrolysis with snake-venom phosphodiesterase followed by alkaline phosphatase (Table 2).

Table 1. Yields and Retention Times of the Oligomers 13–18

oligomer	yield A ₂₆₀	units (%)	retention time ^a (min)
d(G-T-A-G-A-A-T-T-C-T-A-C) (13)	34.4	36.5	18.6
d(G-T-A-G-iA-iA-T-T-C-T-A-C) (14)	24.0	21.9	19.5
d(G-T-A-G-iA-iA-iT-iT-C-T-A-C) (15)	14.8	12.7	20.5
d(G-iT-iA-G-A-A-T-T-C-iT-iA-C) (16)	10.8	9.5	21.4
d(A-T) ₆ (17)	36.8	33.3	21.6
d(A-iT) ₆ ^T (18)	7.7	5.0	22.2

^a Gradient I; flow rate, 1.0 mL/min, column: 4.6 × 250 mm.

d(G-T-A-G-A-A-T-T-C-T-A-C) (**13**)

d(G-T-A-G-iA-iA-T-T-C-T-A-C) (**14**)

d(G-T-A-G-iA-iA-iT-iT-C-T-A-C) (**15**)

d(G-iT-iA-G-A-A-T-T-C-iT-iA-C) (**16**)

d(A-T)₆ (**17**)

d(A-iT)₆-T (**18**)

Duplex Stability of Oligodeoxyribosides Incorporating L-Related Isodeoxynucleosides. Figure 1a displays the temperature-dependent CD spectra of d(G-T-A-G-iA-iA-iT-iT-C-T-A-C) (**15**) measured in 1 M NaCl. As can be seen, the change from the low- to the high-temperature profile led to significant decrease of the ellipticity of the CD minima and maxima, whereas increasing of the temperature did not alter the wavelength of the Cotton effects. Furthermore, the temperature-dependent CD profiles exhibit well-defined isodichroic points, an experimental finding which is in good agreement with our results on the dinucleotide, 2'-deoxyadenylyl-(3'→5')-isodideoxyadenosine (23). The CD spectra of the self-complementary alternating oligomers **17** and **18** were also measured (Figure 1b). While the unmodified oligomer **17** shows a B_{1u} transition at 246 nm with a negative sign and a B_{2u} transition at 268 nm with positive sign, the corresponding π - π^* transitions of oligonucleotide **18** exhibit similar Cotton effects but with a strong bathochromic shift of the B_{1u} and B_{2u} transitions (8 nm). Moreover, the ellipticity value of the B_{1u} transition is markedly reduced, whereas the intensity of the B_{2u} band is almost the same.

The structural stability of the modified oligonucleotides **14–16** and **18** using temperature-dependent CD spectroscopy was also investigated by measuring both π - π^* transitions (B_{1u}, B_{2u}) as a function of temperature (10 and 70 °C). In all cases, sigmoidal monophasic melting profiles were obtained (Figure 2, panels a and c). Furthermore, both π - π^* transitions exhibited almost the same *T_m* value. To discriminate between duplex and hairpin formation, the thermal denaturation curves of **14–16** and **18** as a function of oligomer concentration were determined (Figure 2, panels b and d). Reduction of the oligomer concentration from 10 to 2 μ mol led to a significant decrease of the *T_m* values by 4–6 °C. Therefore, in all cases, duplex melting was observed; the formation of hairpin structures could be excluded.

Surprisingly, the modified oligonucleotide d(A-iT)₆-T (**18**) showed exactly the same *T_m* value as determined for the regular counterpart **17**. Moreover, the oligomers **14–16** exhibited only a moderate decrease of the *T_m* values compared to the parent duplex **13**. These experimental findings encouraged us to study the thermodynamic parameters for the conceptually novel oligonucleotides. From the ln *c* vs 1/*T_m* plot (Figure 2, panels b and d), ΔH and ΔS of the melting process can be determined according to Borer et al. (Table 3) (24).

The experimentally determined ΔH values of the duplexes **15** and **16** were slightly reduced compared to the parent duplex **13**. Surprisingly, the incorporation of two isodA residues opposite of dT within the oligonucleotide **14** led to an increase of the enthalpy. The measured ΔH values of all self-complementary oligonucleotides (**13–16**) were in good agreement with calculated values ($\Delta H = -95$ kcal/mol for **13**) using published increments (25). These results indicate that incorporation of L-related isodeoxynucleosides does not disturb duplex formation to any significant extent; the isodA-dT base pair (oligomer **14**) is enthalpically favored but entropically unfavored. The incorporation of 4 isodA-isodT base pairs in the innermost part (**15**) or at the termini (**16**) of **13** led to stable duplex structures. However, the contribution of each isodA-isodT base pair to ΔH (−4.0 to −4.5 kcal/mol) is lower than that of the dA-dT base pair (−6 kcal/mol). Evaluations of the thermodynamics of duplex formation of the alternating self-complementary oligomers **17** and **18** exhibited a significant difference. While the enthalpy of duplex formation for d(A-iT)₆-T (**18**) is 16% lower than that of the regular counterpart, d(A-T)₆

Table 2. Composition of the Oligomers 14–16 and 18 Determined by Tandem-Hydrolysis with Snake-Venom Phosphodiesterase and Alkaline Phosphatase in 0.1 M Tris-HCl Buffer, pH 8.3

oligomer	dA/iA _d	dC	dG	dT/iT _d
d(GTAGiAiATTCTAC) (14)	4.3 (4.0)	1.8 (2.0)	1.9 (2.0)	4.0 (4.0)
d(GTAGiAiAiTiTCTAC) (15)	4.0 (4.0)	2.2 (2.0)	2.0 (2.0)	3.7 (4.0)
d(GiTiAGAATTCiTiAC) (16)	3.6 (4.0)	1.8 (2.0)	2.0 (2.0)	3.7 (4.0)
d(AiT) ₆ T ^a (18)				

^a No significant enzymatic hydrolysis.

Table 3. *T_m* Values and Thermodynamic Data of the Oligomers 13–18^a

oligomer	<i>h</i> _{enzyme} ^b (%)	<i>T_m</i> (°C)	ΔH (kcal/mol)	ΔS (cal/K mol)	ΔG° (kcal/mol)
d(GTAGAATTCTAC) (13)	31	48	−90.1	−280	−6.6
d(GTAGiAiATTCTAC) (14)	18	45	−97.7	−307	−6.2
d(GTAGiAiAiTiTCTAC) (15)	12	39	−86.2	−276	−3.9
d(GiTiAGAATTCiTiAC) (16)	16	40	−88.6	−283	−4.3
d(AT) ₆ (17)	28	32	−59.5	−195	−1.4
d(AiT) ₆ T ^c (18)	<i>c</i>	32	−50.0	−164	−1.1

^a Measured in 1 M NaCl containing 100 mM MgCl₂ and 60 mM sodium-cacodylate (pH 7.0); single strand concentration 5 μ M. ^b Enzymatic hypochromicity (snake-venom phosphodiesterase). ^c No significant hydrolysis of the phosphodiester backbone.

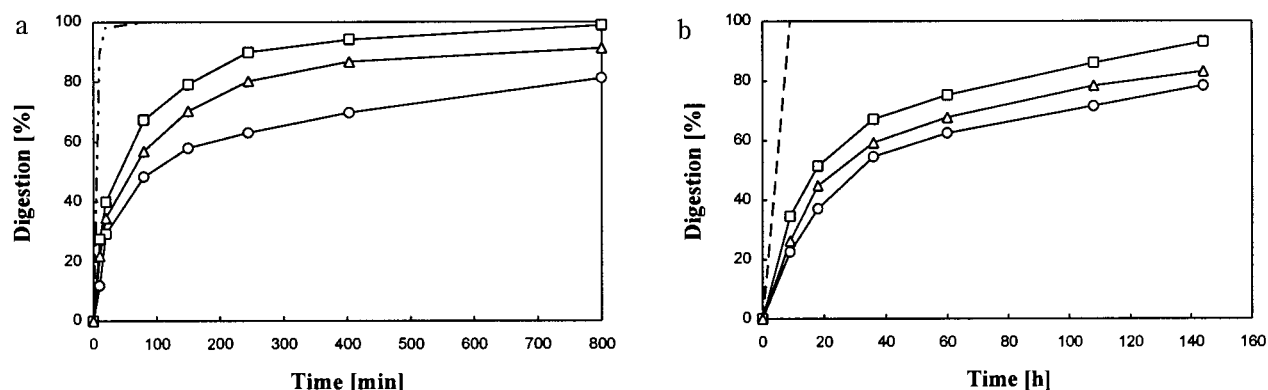


Figure 3. Time course of phosphodiester hydrolysis of the oligonucleotides **13–16** in 0.1 M Tris-HCl buffer (pH 8.3) at 37 °C; (---) d(G-T-A-G-A-A-T-T-C-T-A-C) (**13**), (□) d(G-T-A-G-iA-iA-T-T-C-T-A-C) (**14**), (○) d(G-T-A-G-iA-iA-iT-iT-C-T-A-C) (**15**), (△) d(G-iT-iA-G-A-A-T-T-C-iT-iA-C) (**16**); (a) snake-venom phosphodiesterase (3',5'-exonuclease); (b) calf-spleen phosphodiesterase (5',3'-exonuclease), both followed by alkaline phosphatase treatment.

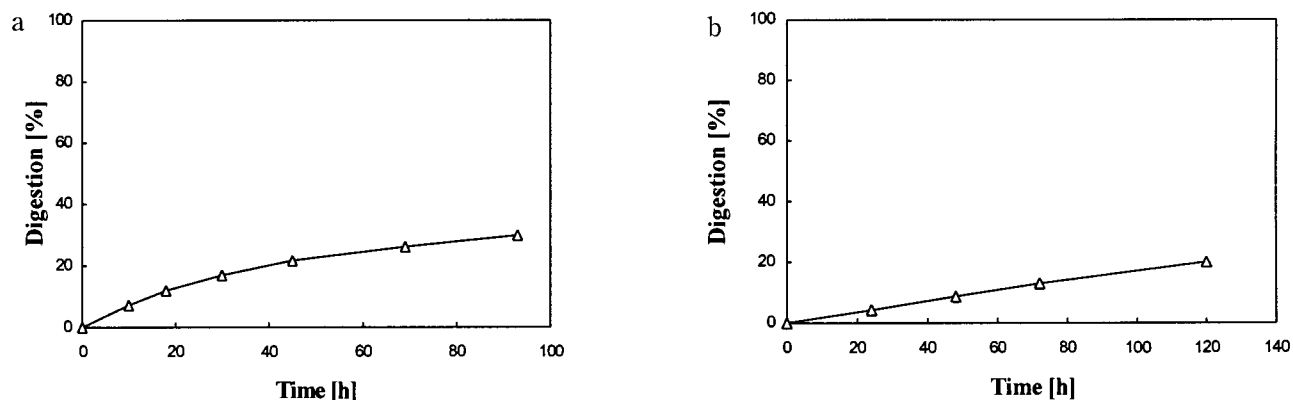


Figure 4. Time course of phosphodiester hydrolysis of d(A-iT)₆-T (**18**); conditions, see Figure 3; (a) snake-venom phosphodiesterase; (b) calf-spleen phosphodiesterase, both followed by alkaline phosphatase treatment.

(**17**), the entropical term is favored by about 18%. In that case, the contribution of each dA-isodT base pair to ΔH is still approximately -4.2 kcal/mol.

Finally, these studies show that the novel synthesized oligonucleotides **14–16** and **18** form stable Watson–Crick duplex structures in which formation of the hydrogen bonds between isodA-dT or isodA-isodT or dA-isodT is not disturbed.

Enzymology. Nuclease stability of the phosphodiester backbone is an important requirement for the development of therapeutic oligonucleotides. To test this behavior of the modified oligomers, **14–16** and **18**, we carried out enzymatic studies using snake-venom phosphodiesterase as well as calf-spleen phosphodiesterase. Figure 3 shows the rates of phosphodiester bond hydrolysis of the oligonucleotides **13–16** in the presence of the exonucleases. As can be seen, incorporation of L-related isodeoxynucleosides within the parent oligomer **13** led to significant resistance of the phosphodiester bonds towards exonucleases. Furthermore, the resistance increased with the number of isomeric nucleosides within the self-complementary oligonucleotides. Exchange of the innermost part of the oligomer by isomeric nucleosides (oligomer **15**) exhibited the highest stability toward exonucleases. The enzyme kinetics follows a two-step mechanism in which the unmodified nucleotides are hydrolyzed first with rates almost identical to the parent sequence, **13**. In the second step the modified inner part is hydrolyzed at a much lower rate.

Figure 4 shows the enzymatic degradation of the phosphodiester backbone of the alternating self-complementary d(A-iT)₆-T (**18**). The kinetic studies show that

oligomer **18** is very resistant toward enzymatic hydrolysis, toward either snake-venom or calf-spleen phosphodiesterase. Even after 100–120 h (Figure 4) only minimal amounts of oligonucleotide **18** had hydrolyzed.

Summary and Conclusion. In summary, the L-related isodeoxynucleosides **1** and **2** were successfully incorporated within self-complementary oligonucleotides, (**14–16**, **18**). These conceptually novel oligomers, and especially the alternating self-complementary oligonucleotide, d(A-iT)₆-T (**18**), exhibit high resistance toward nucleases. Comprehensive CD spectral studies and the resulting thermodynamic parameters indicate the participation of the isodA-isodT or mixed dA-isodT/isodA-dT base pairs in duplex stabilization.

ACKNOWLEDGMENT

We thank Dr. G. Pearson for technical assistance with several 2D-NMR spectra and Dr. R. Forsyth of the Protein Structure Facility for help with the operation of the CD spectrometer. This research work was supported by the National Institutes of Health (NIAID).

LITERATURE CITED

- (1) De Mesmaeker, A., Häner, R., Martin, P., and Moser, H. E. (1995) Antisense oligonucleotides. *Acc. Chem. Res.* 28, 366–374.
- (2) Van Aerschot, A., Verheggen, I., Hendrix, C., and Herdewijn, P. (1995) 1,5-Anhydrohexitol nucleic acids, a new promising antisense construct. *Angew. Chem., Int. Ed. Engl.* 34, 1338–1339.
- (3) Seela, F., Heckel, M., and Rosemeyer, H. (1996) Xylose-DNA containing the four natural bases. *Helv. Chim. Acta* 79, 1451–1461.

- (4) Jones, G. D., Lesnik, E. A., Owens, S. R., Risen, L. M., and Walker, R. T. (1996) Investigation of some properties of oligonucleotides containing 4'-thio-2'-deoxynucleotides: duplex hybridization and nuclease sensitivity. *Nucleic Acids Res.* 24, 4117-4122.
- (5) Jones, G. D., Altmann, K.-H., Hüsken, D., and Walker, R. T. (1997) Duplex- and triplex-forming properties of 4'-thio-modified oligodeoxynucleotides. *Bioorg. Med. Chem. Lett.* 7, 1275-1278.
- (6) Hess, M. T., Schwitter, U., Petretta, M., Giese, B., and Naegeli, H. (1997) DNA synthesis arrest at C-4'-modified deoxyribose residues. *Biochemistry* 36, 2332-2337.
- (7) Hamm, M. L., and Piccirilli, J. A. (1997) Incorporation of 2'-deoxy-2'-mercaptocytidine into oligonucleotides via phosphoramidite chemistry. *J. Org. Chem.* 62, 3415-3420.
- (8) Damha, M. J., Giannaris, P. A., and Marfey, P. (1994) Antisense L/D-oligonucleotide chimeras: nuclease stability, base-pairing properties, and activity at directing ribonuclease H. *Biochemistry* 33, 7877-7885.
- (9) Blommers, M. J. J., Tondelli, L., and Garbesi, A. (1994) Effects of the introduction of L-nucleosides into DNA. Solution structure of the heterochiral duplex d(G-C-G-(L) T-G-C-G). d(C-G-C-A-C-G-C) studied by NMR spectroscopy. *Biochemistry* 33, 7886-7896.
- (10) Bolon, P. J., and Nair, V. (1996) Solution conformation of the anti-HIV active compound (S,S)-isodideoxyadenosine. *Magn. Reson. Chem.* 34, 243-248.
- (11) Nair, V., and Nuesca, Z. M. (1992) Isodideoxynucleosides: A conceptually new class of nucleoside antiviral agents. *J. Am. Chem. Soc.* 114, 7951-7953.
- (12) Bolon, P. J., Sells, T. B., Nuesca, Z. M., Purdy, D. F., and Nair, V. (1994) Novel isomeric dideoxynucleosides as potential antiviral agents. *Tetrahedron* 50, 7747-7764.
- (13) Nair, V., St. Clair, M. H., Reardon, J. E., Krasny, H. C., Hazen, R. J., Paff, M. T., Boone, L. R., Tisdale, M., Najera, I., Dornsife, R. E., Averett, D. R., Borroto-Esoda, K., Yale, J. L., Zimmerman, T. P., and Rideout, J. L. (1995) Antiviral, metabolic, and pharmacokinetic properties of the isomeric dideoxynucleoside, 4(S)-(6-amino-9H-purin-9-yl)tetrahydro-2(S)-furanmethanol. *Antimicrob. Agents Chemother.* 39, 1993-1999.
- (14) Nair, V., and Jahnke, T. (1995) Antiviral activity of isomeric dideoxynucleosides of D- and L-related stereochemistry. *Antimicrob. Agents Chemother.* 39, 1017-1029.
- (15) Montgomery, J. A. and Thomas, H. J. (1978) Isonucleosides. 2. Purine and pyrimidine derivatives of 1,4-anhydro-2-deoxy-D-arabinitol. *J. Org. Chem.* 43, 541-544.
- (16) Kakefuda, A., Shuto, S., Nagahota, T., Seki, J., Sasaki, T., and Matsuda, A. (1994) Nucleosides and nucleotides. 132. Synthesis and biological evaluations of ring-expanded oxetanocin analogues: purine and pyrimidine analogues of 1,4-anhydro-2-deoxy-D-arabitol and 1,4-anhydro-2-deoxy-3-hydroxymethyl-D-arabitol. *Tetrahedron* 34, 10167-10182.
- (17) Barker, R., and Fletcher, H. G., Jr. (1961) 2,3,5-Tri-O-benzyl-D-ribosyl and -L-arabinsyl bromides. *J. Org. Chem.* 26, 4605-4609.
- (18) Purdy, D. F., Zintek, L. B., and Nair, V. (1994) Synthesis of isonucleosides related to AZT and AZU. *Nucleosides Nucleotides* 13 (1-3), 109-126.
- (19) Seela, F., and Wenzel, T. (1992) 1,7-Dideaza-2'-deoxyadenosine: building blocks for solid-phase synthesis and secondary structure of base-modified oligodeoxyribonucleotides. *Helv. Chim. Acta* 75, 1111-1122.
- (20) Ti, G. S., Gaffney, B. L., and Jones, R. A. (1982) Transient protection: efficient one-flask syntheses of protected deoxynucleosides. *J. Am. Chem. Soc.* 104, 1316-1319.
- (21) Rosemeyer, H., Toth, G., and Seela, F. (1989) Assignment of anomeric configuration of D-ribo-, arabino-, 2'-deoxyribo-, and 2',3'-dideoxyribonucleosides by NOE difference spectroscopy. *Nucleosides Nucleotides* 8 (4), 587-597.
- (22) Beaucage, S. L., and Caruthers, M. H. (1981) Deoxynucleoside phosphoramidites. A new class of key intermediates for deoxypolynucleotide synthesis. *Tetrahedron Lett.* 22, 1859-1862.
- (23) Jahnke, T. S., and Nair, V. (1995) 2'-Deoxyadenylyl-(3'→5')-isodideoxyadenosine, a unique dinucleotide: synthesis, enzymology, and conformational studies. *Bioorg. Med. Chem. Lett.* 5, 2235-2238.
- (24) Borer, B. N., Dengler, B., Uhlenbeck, O. C., and Tinoco, I., Jr. (1974) Stability of ribonucleic acid double-stranded helices. *J. Mol. Biol.* 86, 843-853.
- (25) Breslauer, K. J., Frank, R., Blöcker, H., and Marky, L. A. (1986) Predicting DNA duplex stability from the base sequence. *Proc. Natl. Acad. Sci. U.S.A.* 83, 3746-3750.

BC980009E

Derivatives of 1,3,5-Triamino-1,3,5-trideoxy-*cis*-inositol as Versatile Pentadentate Ligands for Protein Labeling with Re-186/188. Prelabeling, Biodistribution, and X-ray Structural Studies

Andreas Kramer,^{†,‡} Roger Alberto,^{†,§} André Egli,[†] Ilse Novak-Hofer,[†] Kaspar Hegetschweiler,^{*,||} Ulrich Abram,[⊥] Paul V. Bernhardt,[#] and P. August Schubiger^{*,†}

Laboratory of Inorganic Chemistry, ETH-Zentrum, Universitätstrasse 6, CH-8092 Zürich, Switzerland, Center for Radiopharmaceutical Science, Paul Scherrer Institute, CH-5232 Villigen, Switzerland, Anorganisch-Chemisches Institut, Universität Zürich, Winterthurerstrasse 190, CH-8057 Zürich, Switzerland, Forschungszentrum Rossendorf, Institute of Radiochemistry, c/o Dresden University of Technology, Institute of Analytical Chemistry, D-01062 Dresden, Germany, Anorganische Chemie, Universität des Saarlandes, D-66041 Saarbrücken, Germany, and Department of Chemistry, University of Queensland, 4072 Brisbane, Australia. Received January 23, 1998; Revised Manuscript Received July 14, 1998

The pentadentate H₃bhci [1,3,5-trideoxy-1,3-bis((2-hydroxybenzyl)amino)-*cis*-inositol] and its bifunctionalized analogue H₃bhci-glu-H [1,3,5-trideoxy-1,3-bis((2-hydroxybenzyl)amino)-5-glutamido-*cis*-inositol] were synthesized, and their coordination chemistry was investigated with inactive rhenium, with no carrier added Re-188 and with carrier added Re-186. The neutral Re(V) complexes [ReO(bhci)] and [ReO(bhci-glu-H)] are formed in good yields starting from [ReOCl₃(P(C₆H₅)₃)₂] or in quantitative yield directly from [^{186/188}ReO₄][−] in aqueous solution by reduction with Sn(II) or Sn(0). The X-ray structures of [ReO(bhci)] and [ReO(bhci-glu-H)] were elucidated revealing pentadentate “side on” coordination of the ligands to the “Re=O” core. The basic cyclohexane frame adopts a chair form in the case of [ReO(bhci)] and a twisted boat form in the case of [ReO(bhci-glu-H)]. [ReO(bhci)] crystallizes in the monoclinic space group *C2/c* with *a* = 27.425(3), *b* = 14.185(1), *c* = 19.047(2) Å, and β = 103.64(2)° and [ReO(bhci-glu-H)] in the monoclinic space group *P2₁/c* with *a* = 13.056(3), *b* = 10.180(1), *c* = 22.378(5) Å, and β = 98.205(9)°. Both ¹⁸⁸Re complexes are stable in human serum for at least 3 days without decomposition. After injection into mice, [ReO(bhci-glu)][−] is readily excreted through the intestines, while [ReO(bhci)] is excreted by intestines, liver, and the kidneys. TLC investigations of the urine showed exclusively the complexes [ReO(bhci-glu-H)] and [ReO(bhci)], respectively, and no decomposition products. For derivatization of antibodies, the carboxylic group of [ReO(bhci-glu-H)] was activated with *N*-hydroxysuccinimide, which required unusually vigorous reaction conditions (heating). The anti colon cancer antibody mAb-35 [IgG and F(ab')₂ fragment] was labeled with [^{186/188}ReO(bhci-glu)] to a specific activity of up to 1.5 mCi/mg (55 MBq/mg) with full retention of immunoreactivity. Labeling yields followed pseudo-first-order kinetics in antibody concentration with the ratio of rates between aminolysis and hydrolysis being about 2. Biodistributions of ¹⁸⁶Re-labeled intact mAb-35 as well as of its F(ab')₂ fragment in tumor-bearing nude mice revealed good uptake by the tumor with only low accumulation of radioactivity in normal tissue.

INTRODUCTION

Labeling of tumor-seeking monoclonal antibodies with radioactive nuclides is an important method in nuclear medicine for the diagnosis of small cancer metastases or for therapy (1–6). One of the most widely applied isotopes for diagnosis is ^{99m}Tc due to its favorable γ -decay characteristics, its convenient availability, and its chemical properties. The β -emitting rhenium isotopes ¹⁸⁶Re and ¹⁸⁸Re are suited for therapeutic purposes. On the basis of the chemical relationship between rhenium and technetium, it has been proposed that the same ligands

and protocols that are used with ^{99m}Tc for diagnostic purposes should be applicable to ^{186/188}Re for therapy (7, 8). There is increasing evidence, however, that this strategy suffers from the observation that the thermodynamic, kinetic, and electrochemical properties of rhenium and technetium are more different than previously expected and, as a consequence, Re(V) complexes tend to be reoxidized to [ReO₄][−] much faster than Tc(V) complexes to [TcO₄][−] (9). The facile reduction of [^{99m}TcO₄][−] to lower oxidation states at ambient conditions, for instance with S₂O₄^{2−}, allows convenient postlabeling techniques, whereas under equivalent conditions, reduction and subsequent complex formation are barely possible with [^{186/188}ReO₄][−]. Therefore, the use of ^{186/188}Re requires the application of prelabeling techniques with the disadvantage of further purification steps (10). A widely applied ligand in tumor therapy with ^{186/188}Re is MAG3, a tetradentate N₃S ligand originally developed for renal function measurement with ^{99m}Tc. The ^{186/188}Re-MAG3 system was applied in phase I radioimmuno-therapy studies and showed very promising results (11–

* To whom correspondence should be addressed. (P.A.S.) Phone: ++41/56 310 28 13. Fax: ++41/56 310 28 49. (K.H.) Phone: ++49/681 302 27 15. Fax: ++49/681 302 26 63.

[†] Paul Scherrer Institute.

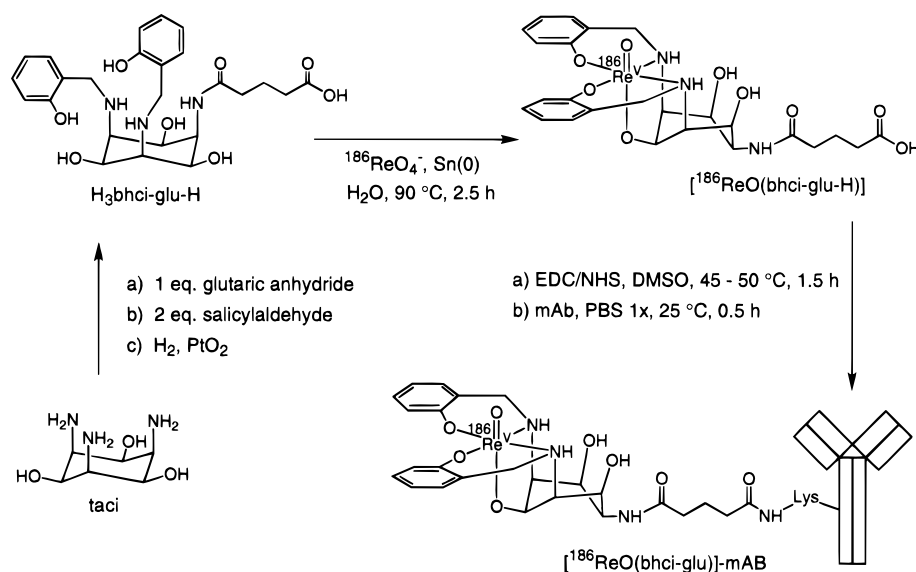
[‡] ETH-Zentrum.

[§] Universität Zürich.

^{||} Universität des Saarlandes.

[⊥] Dresden University of Technology.

[#] University of Queensland.

Scheme 2. Prelabeling Protocol with H₃bhci-glu-H

generated (8 atm) for 2 days at ambient temperature in the presence of $\text{PtO}_2 \cdot \text{H}_2\text{O}$ (0.15 g) as catalyst. Methanol was added until the solution turned clear, and the solution was separated from the catalyst. The solvent was removed in vacuo, and the residue extracted twice with hot ethanol to remove impurities and byproducts. The remaining white powder was dried in vacuo to yield 1.50 g (35%) of analytically pure H_3bhci . IR (KBr, cm^{-1}):

3404 s, 3366 s, 3242 s, 2928 w, 1654 m, 1600 m, 1572 m, 1484 s, 1452 s, 1258 s, 1234 m, 1094 m, 1066 s, 980 m, 950 s, 880 m, 860 m, 826 m, 768 s. FAB-MS (m/z , M^+): 390.1 (calcd for $\text{C}_{20}\text{H}_{27}\text{N}_3\text{O}_5$ 389.46). Anal. Calcd for $\text{C}_{20}\text{H}_{27}\text{N}_3\text{O}_5$: C, 61.68; H, 6.99; N, 10.79. Found: C, 61.86; H, 6.94; N, 10.52. ^1H NMR (d^6 -DMSO) δ (ppm): 7.10–7.03 (m, 4H), 6.75–6.67 (m, 4H), 5.5–3.5 (br m, 9H), 4.03 (t, 1H), 3.95 (s, 4H), 3.80 (t, 2H), 2.42 (t, 3H). ^{13}C NMR (d^6 -DMSO) δ (ppm): 157.59, 128.43, 127.80, 123.83, 118.40, 115.38, 72.22, 68.24, 56.99, 52.74, 46.88.

1,3,5-Trideoxy-1,3-bis[(2-hydroxybenzyl)amino]-5-glutaramido-*cis*-inositol ($\text{H}_3\text{bhci-glu-H}$). Glutaric anhydride (0.495 g, 4.33 mmol) was added to a solution of taci (0.762 g, 4.3 mmol) in 300 mL of methanol. A white precipitate readily formed and the suspension was stirred for another 30 min at ambient temperature. Salicylaldehyde (1.138 g, 9.31 mmol) was added to this suspension, which resulted in an instantaneous color change to yellow, and the solid dissolved completely after brief heating. $\text{PtO}_2 \cdot \text{H}_2\text{O}$ (0.08 g, catalyst) was added and the mixture was hydrogenated (8 atm) for 2 days at ambient temperature. The grayish suspension was taken to dryness, 200 mL of hot water was added to dissolve the solid, and the catalyst was filtered off. The slightly yellowish solution was taken to dryness again and treated twice with boiling ethanol. After filtration, the white powder was dried in vacuo to yield 1.330 g (2.64 mmol, 61%) of analytically pure $\text{H}_3\text{bhci-glu-H}$. IR (KBr, cm^{-1}): 3326 s, 2964 s, 1636 vs, 1544 s, 1460 s, 1406 s, 1252 s, 1188 w, 1158 w, 1118 m, 1068 w, 1004 w, 916 w, 874 w, 758 m, 572 w. ES-MS (m/z , M^+): 504 (calcd for $\text{C}_{25}\text{H}_{33}\text{N}_3\text{O}_8$ 503.55). Anal. Calcd for $\text{C}_{25}\text{H}_{33}\text{N}_3\text{O}_8 \cdot 2\text{H}_2\text{O}$: C, 55.65; H, 6.61; N, 8.34. Found: C, 55.51; H, 7.19; N, 8.02. ^1H NMR (d^6 -DMSO) δ (ppm): 7.23–7.16 (m, 4H), 7.85–6.80 (m, 4H), 4.19 (br s, 1H), 4.04 (s, 4H), 4.01 (br s, 2H), 3.81 (br s, 1H), 2.71 (br s, 2H), 2.34–2.30 (m, 4H), 1.88–1.79 (q, 2H). ^{13}C NMR (d^6 -DMSO) δ (ppm): 175.06,

171.46, 157.64, 128.75, 128.07, 124.14, 118.68, 115.58, 69.80, 68.72, 56.87, 51.03, 46.75, 34.62, 33.72, 20.99

[$\text{ReO}(\text{bhci})$]. H_3bhci (0.134 g, 0.35 mmol) and 1.1 mL of a 1 M NaOH solution were added to a suspension of $[\text{ReOCl}_3(\text{P}(\text{C}_6\text{H}_5)_3)_2]$ (0.281 g, 0.34 mmol) in methanol. The mixture was refluxed for 30 min, and the violet precipitate was removed by filtration. The solid was washed first with methanol, then with ether, and dried in vacuo to yield 0.143 g (72%) of analytically pure $[\text{ReO}(\text{bhci})]$. IR (KBr, cm^{-1}): 3396 br, 2894 m, 1598 m, 1570 m, 1482 s, 1446 m, 1364 w, 1258 s, 1234 s, 1098 m, 1068 m, 1048 m, 948 m, 912 m, 880 m, 862 m, 774 s, 766 s, 722 s. FAB-MS (m/z , M^+): 589.0 (calcd for $\text{C}_{20}\text{H}_{24}\text{N}_3\text{O}_6\text{-Re}$ 588.63). Anal. Calcd for $\text{C}_{20}\text{H}_{24}\text{N}_3\text{O}_6\text{Re}$: C, 40.81; H, 4.11; N, 7.14. Found: C, 40.81; H, 4.05; N, 7.01. ^1H NMR (d^7 -DMF) δ (ppm): 9.08 (m, 2H), 7.20 (dd, 2H), 7.04 (d, 2H), 6.96 (d, 2H), 6.60 (dd, 2H), 6.10 (m, 2H), 5.33, (d, 2H), 4.70 (dd, 2H), 4.52 (t, 1H), 3.99 (t, 2H), 3.86 (t, 1H), 2.89 (t, 2H), 2.72 (m, 2H). ^{13}C NMR (d^7 -DMF) δ (ppm): 172.35, 133.14, 129.91, 122.97, 120.73, 118.33, 81.81, 66.04, 64.53, 64.30, 57.37.

[$\text{ReO}(\text{bhci-glu-H})$]. NEt_3 (249 μL , 0.181 g, 1.789 mmol), concentrated NH_3 (300 μL) and $[\text{ReOCl}_3(\text{P}(\text{C}_6\text{H}_5)_3)_2]$ (0.486 g, 0.583 mmol) were added to a solution of $\text{H}_3\text{bhci-glu-H}$ (0.300 g, 0.596 mmol) in 15 mL of MeOH and 1 mL of doubly distilled water. The suspension was refluxed for 30 min, giving a deep violet solution which was evaporated to dryness. The residue was washed three times with ether and CHCl_3 . The crude product (0.363 g) was dissolved in 10 mL of MeOH/ H_2O 2/1 (v/v) and allowed to stand for 2 weeks to evaporate the MeOH. The precipitated violet solid was removed by filtration and washed twice with 2 mL of water and THF. The crystals were dried in vacuo to yield 0.110 g (26%) of analytically pure $[\text{ReO}(\text{bhci-glu-H})]$. IR (KBr, cm^{-1}): 3396 br, 2894 m, 1598 m, 1570 m, 1482 s, 1446 m, 1364 w, 1258 s, 1234 s, 1098 m, 1068 m, 1048 m, 948 m, 912 m, 880 m, 862 m, 774 s, 766 s, 722 s. ES-MS (m/z , M^+): 702.0 (calcd for $\text{C}_{25}\text{H}_{30}\text{N}_3\text{O}_9\text{Re}$ 702.73). Anal. Calcd for $\text{C}_{25}\text{H}_{30}\text{N}_3\text{O}_9\text{Re} \cdot 3.25\text{H}_2\text{O}$: C, 39.44; H, 4.83; N, 5.52. Found: C, 39.55; H, 4.88; N, 5.46. ^1H NMR (d^6 -DMSO) δ (ppm): 7.61 (d, 1H), 7.33 (t, 2H), 7.29 (br., 1H), 7.21 (d, 2H), 6.94 (d, 2H), 6.77 (t, 2H), 6.08 (br., 1H), 4.92 (t, 1H), 4.58 (dd, 4H), 4.42 (dd, 2H), 3.76 (br s, 1H), 3.33 (dd, 2H), 2.35 (t, 4H), 1.86 (q, 2H). ^{13}C NMR (d^6 -DMSO)

δ (ppm): 174.54, 171.52, 131.97, 129.55, 123.46, 118.45, 118.16, 77.97, 65.47, 62.79, 61.64, 48.49, 34.58, 33.05, 20.74.

[$^{186/188}\text{ReO}(\text{bhci-glu-H})$] (A). NaCl solution (1 mL of 0.9%) containing [$^{186/188}\text{ReO}_4$] $^-$ [up to 4.3 mCi (160 MBq)] was added to a solution of $\text{H}_3\text{bhci-glu-H}$ (2 mg) and of $\text{Na}_3\text{citrate}$ (40 mg) in 1 mL of doubly distilled water. The solution was heated to 90 °C and SnCl_2 (12 mg) was added. After 30 min, the solution was cooled to ambient temperature. Monitoring the reaction by TLC [stationary phase, silica gel; mobile phase, $\text{CH}_3\text{CN}/\text{H}_2\text{O}$ 4/1 (v/v)] revealed that complex formation was complete to >98% ($R_f = 0.45$). The mixture was purified using a preconditioned reversed-phase Sep-Pak column, eluting inorganic residues with 25 mL of water, and the complex was finally eluted with 2 mL of $\text{CH}_3\text{CN}/\text{H}_2\text{O}$ 10/1 (v/v). TLC revealed radiochemical purity to be better than 99%.

[$^{186/188}\text{ReO}(\text{bhci-glu-H})$] (B). [$^{186/188}\text{ReO}_4$] $^-$ [up to 16.2 mCi (600 MBq)] in 0.5–1 mL 0.9% NaCl solution was added to a solution of $\text{H}_3\text{bhci-glu-H}$ (1.3 mg) in 1 mL of doubly distilled water. The solution was heated to 90 °C and small pieces of metallic Sn(0) (about 250 mg) were added. After 60 min ([$^{188}\text{ReO}_4$] $^-$) or 150 min ([$^{186}\text{ReO}_4$] $^-$), the solution was allowed to cool to ambient temperature. TLC (see above) showed that complex formation was >95%. HCl (0.1 M, 1 mL) was added, and the solution was purified using a column containing 0.75 g of cation-exchange resin (Dowex 50W, X8 50–100 mesh; ionic form, H^+) and a preconditioned reversed-phase Sep-Pak column. Inorganic and organic residues were eluted with 10 mL of 0.1 M HCl. After removal of the cation-exchange column, the Sep-Pak column was rinsed with 10 mL of doubly distilled water and subsequently dried using a N_2 -stream. A second preconditioned reversed-phase Sep-Pak column was attached to the one loaded with the radioactive complex, and the complex was eluted with 2 mL of $\text{CH}_3\text{CN}/\text{H}_2\text{O}$ 10/1 (v/v). TLC revealed radiochemical purity to be better than 99%.

[$^{186/188}\text{ReO}(\text{bhci-glu-NHS})$]. *N*-(3-Dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC, 8 mg) and *N*-hydroxysuccinimide (NHS, 6 mg) were dissolved in 0.5 mL of dry DMSO. The fraction from the Sep-Pak column containing the complex was dried under a stream of N_2 , and 0.2 mL of the EDC/NHS mixture was added. This solution was heated to 45–55 °C and the activation monitored by TLC (see above; R_f [$\text{ReO}(\text{bhci-glu-H})$] = 0.45 and R_f [$^{186/188}\text{ReO}(\text{bhci-glu-NHS})$] = 0.65). After 70–80 min, more than 80% of the complex was in its activated form. The solution containing [$^{186/188}\text{ReO}(\text{bhci-glu-NHS})$] was diluted with 5 mL of doubly distilled water and purified using a preconditioned reversed-phase Sep-Pak column, eluting EDC, NHS and nonactivated complex with 10 mL of doubly distilled water, 8 mL of a 1/9 (v/v) mixture of ethanol and 0.01 M phosphate buffer pH 7.0, and 10 mL of doubly distilled water. The Sep-Pak column was dried using a N_2 -stream and the [$^{186/188}\text{ReO}(\text{bhci-glu-NHS})$] was eluted with 1.3 mL of CH_3CN . TLC showed radiochemical purity to be better than 90%, the remainder being in its nonactivated form. Aliquots of this solution were used for the protein-labeling experiment.

Labeling Experiments. The solution containing [$^{186/188}\text{ReO}(\text{bhci-glu-NHS})$] was taken to dryness under a stream of N_2 . DMSO (50 μL) was added to dissolve the activated complex, then 0.25 mg of mAb-35 antibody [IgG or $\text{F}(\text{ab}')_2$ fragment] in 0.15 mL PBS 1 $\times$ pH 7.4 and 0.3 mL 0.9% NaCl solution were added ([mAb] = 0.5 mg/mL). After thorough mixing, the solution was allowed to stand at ambient temperature for 30 min, then diluted to 1 mL

with doubly distilled water and purified by gel filtration chromatography using a PD-10 column and by FPLC (Superose 12 column, Pharmacia) with PBS 1 $\times$ as mobile phase. Labeling yield was up to 15% (see Results and Discussion).

Serum Stability Studies. An aliquot (20 μL) of the radiolabeled conjugate containing approximately 2.7 μCi (0.1 MBq) of ^{188}Re was added to 1 mL of fresh human serum. The mixture was incubated at 37 °C for 3 days and then analyzed by TLC.

Tumor Cell Binding Assays. The immunoreactivity of the labeled antibody was determined following a procedure described by T. Lindmo et al. (28), using the CEA-expressing LoVo (human colon adeno carcinoma) cell line (American Type Culture Collection). Six serial dilutions (2×10^6 to 6.25×10^4 cells) of LoVo cells were dried onto a 24-well cell-culture plate. After presaturation of the cells with 0.5% HSA in PBS 1 $\times$, 10 ng of $^{186/188}\text{Re}$ -labeled mAb-35 [IgG or $\text{F}(\text{ab}')_2$ fragment] in 0.5 mL of 0.5% HSA in PBS 1 $\times$ was added to each well. Equivalent samples containing a 100-fold excess of unlabeled mAb-35 were assayed for each cell concentration to determine nonspecific binding. The plate was incubated for 16 h at 37 °C, wells were washed twice with ice-cold PBS 1 $\times$ and the cells were digested using 0.5 mL 0.1 M NaOH. Radioactivity bound to the LoVo cells was determined in a β -counter. Data were analyzed according to the method of Lindmo.

Biodistributions of [$^{188}\text{ReO}(\text{bhci})$] and [$^{188}\text{ReO}(\text{bhci-glu})$] in White Mice and of [$^{186}\text{ReO}(\text{bhci-glu})$]-Labeled mAb-35 [IgG and $\text{F}(\text{ab}')_2$ fragment] in Tumor-Bearing Nude Mice. The complexes [$^{188}\text{ReO}(\text{bhci})$] and [$^{188}\text{ReO}(\text{bhci-glu-H})$] and the radioimmunoconjugate [$^{186}\text{ReO}(\text{bhci-glu})$]-mAb-35 were synthesized and purified as described above. ^{188}Re complex [ca. 30 μCi (1.1 MBq)] in 100 μL of PBS 1 $\times$, pH = 7.4, and ca. 10 μCi (0.4 MBq) of [$^{186}\text{ReO}(\text{bhci-glu})$]-mAb-35 [intact antibody or $\text{F}(\text{ab}')_2$ fragment] in 150 μL of PBS 1 $\times$, pH 7.4, were injected in the tail venes of groups of three white mice or three nude mice bearing LoVo tumor-xenografts (mean tumor weights, 50–150 mg). The mice were sacrificed and dissected at the indicated time points, the organs were removed, and the activity was measured in a γ -counter using an energy window between 145 and 165 keV. Results are expressed as a percentage of injected dose per gram of tissue (% ID/g).

X-ray Crystallography. Structure Analysis of [$\text{ReO}(\text{bhci})$]. A violet crystal ($0.4 \times 0.4 \times 0.3$ mm) was mounted on an Enraf Nonius CAD4 diffractometer. Reflections were collected using graphite monochromated $\text{MoK}\alpha$ radiation ($\lambda = 0.71073$ Å) and operating in the ω scan mode. The unit cell was determined by the angular setting of 25 high angle reflections ($8.59 < \theta < 14.57$). Intensity data were collected in the range $6.12 < 2\theta < 50.0$. Every 300 reflections, three reflections were measured for decay control, with an observed decrease of intensity of 5.1%. A total of 6744 reflections were measured at $T = 208$ K from which 3746 were observed [$I \geq 2.0\sigma(I)$]. A Ψ -scan absorption correction was applied ($T_{\min} = 0.742$ and $T_{\max} = 0.955$), and the structure solved by direct methods. Subsequent refinements were performed by full-matrix least-squares calculations with SHELXL 97 (29). The maximum residual electron density ($1.20 \text{ e}^-/\text{\AA}^3$) was found close to the heavy-atom site. A ZORTEP representation of the complex is given in Figure 1, crystallographic data is summarized in Table 1, and important bond lengths and angles are presented in Table 2.

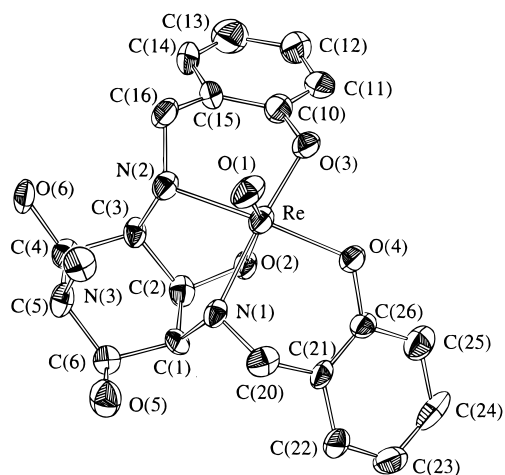


Figure 1. ORTEP presentation of [ReO(bhci)].

Table 1. Crystallographic Data for the Complexes [ReO(bhci)] and [ReO(bhci-glu-H)]

	[ReO(bhci)] ^a	[ReO(bhci-glu-H)]
formula	C ₃₀ H ₄₇ N ₃ O _{11.5} ReS	C ₂₅ H ₃₀ N ₃ O ₉ Re
fw	850.96	702.72
space group	C2/c (no. 15)	P2 ₁ /c (no. 14)
a (Å)	27.425(3)	13.056(3)
b (Å)	14.185(1)	10.180(1)
c (Å)	19.047(2)	22.378(5)
β (deg)	103.64(2)	98.205(9)
V (Å ³)	7200.8(12)	2943.9(10)
Z	8	4
T (°C)	−65	20
ρ _{calcd} (g cm ^{−3})	1.570	1.586
μ (mm ^{−1})	3.493	4.179
F(000)	3440	1392
R ₁ (I > 2σ(I)) ^b	0.0568	0.0674
wR ₂ (all data)	0.1558 ^c	0.2555 ^d
GOF	1.063	1.036

^a [ReO(bhci)]·DMSO·2dioxane·0.5H₂O. ^b $R_1 = \sum ||F_o| - |F_c|| / \sum |F_o|$, $wR_2 = (\sum w(F_o^2 - F_c^2)^2 / \sum w(F_o^4))^{1/2}$. ^c Weighting scheme: $w = 1/[\sigma^2(F_o^2) + (0.0632P)^2 + 48.7902P]$, where $P = (F_o^2 + 2F_c^2)/3$. ^d Weighting scheme: $w = 1/[\sigma^2(F_o^2) + (0.102P)^2 + 11.97P]$, where $P = (F_o^2 + 2F_c^2)/3$.

Table 2. Selected Bond Lengths (Å) and Angles (deg) for the Complex [ReO(bhci)] with esds in Parentheses

Re—O(1)	1.693(8)	N(2)—C(16)	1.493(14)
Re—O(2)	1.975(7)	N(2)—C(3)	1.487(13)
Re—O(3)	2.003(7)	N(3)—C(5)	1.445(14)
Re—O(4)	1.982(8)	O(2)—C(2)	1.402(13)
Re—N(2)	2.163(9)	O(6)—C(4)	1.413(14)
Re—N(1)	2.146(9)	O(5)—C(6)	1.437(12)
N(1)—C(20)	1.501(14)	O(3)—C(10)	1.360(12)
N(1)—C(1)	1.505(13)	O(4)—C(26)	1.375(14)
O(4)—Re—N(1)	93.5(4)	C(1)—N(1)—Re	110.3(6)
N(2)—Re—N(1)	86.8(3)	C(16)—N(2)—C(3)	114.2(8)
O(2)—Re—O(3)	88.0(3)	C(16)—N(2)—Re	112.6(7)
O(2)—Re—O(4)	90.0(3)	C(3)—N(2)—Re	111.1(7)
O(2)—Re—N(2)	76.4(3)	C(2)—O(2)—Re	108.3(7)
O(2)—Re—N(1)	77.0(3)	C(10)—O(3)—Re	122.1(7)
C(20)—N(1)—C(1)	114.6(9)	C(26)—O(4)—Re	124.0(7)
C(20)—N(1)—Re	112.2(7)		

Structure Analysis of [ReO(bhci-glu-H)]. A violet crystal (0.2 × 0.1 × 0.1 mm) of [ReO(bhci-glu-H)] was obtained by slow evaporation of MeOH from a solution of the complex in MeOH/H₂O 2:1 (v/v). X-ray data were collected on a CAD4 diffractometer (Enraf Nonius) using graphite-monochromated MoKα radiation (λ = 0.710 73 Å) and operating in the ω — θ scan mode. Intensity data were collected at 293 K with ω-scans in the range 3.16 ≤ θ ≤ 49.96°. Of the 5378 measured reflections, 5171

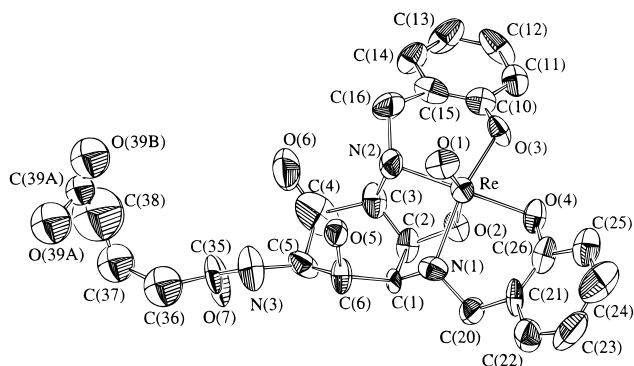


Figure 2. ORTEP presentation of [ReO(bhci-glu-H)].

Table 3. Selected Bond Lengths (Å) and Angles (deg) for the Complex [ReO(bhci-glu-H)] with esds in Parentheses

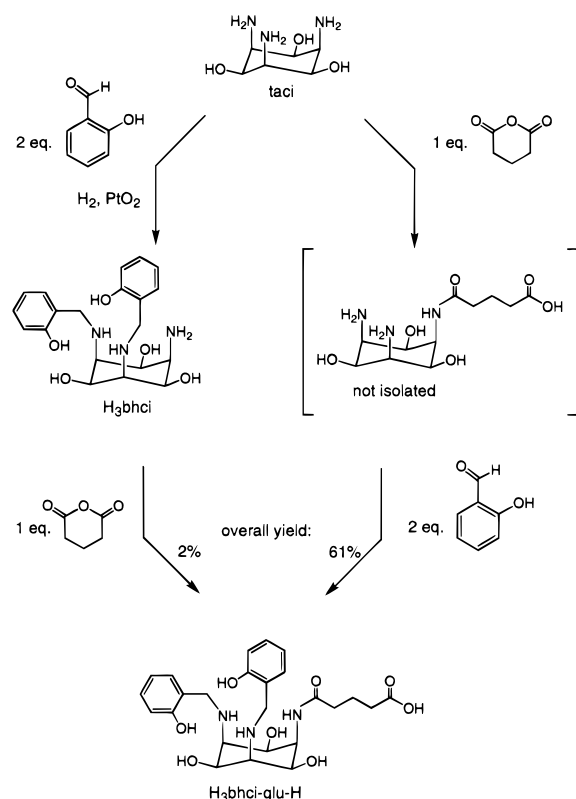
Re—O(1)	1.71(2)	N(2)—C(3)	1.53(3)
Re—O(2)	1.98(2)	N(3)—C(35)	1.26(3)
Re—O(3)	1.99(2)	N(3)—C(5)	1.46(3)
Re—O(4)	2.04(2)	O(2)—C(2)	1.36(3)
Re—N(2)	2.12(2)	O(6)—C(4)	1.42(5)
Re—N(1)	2.15(2)	O(5)—C(6)	1.43(3)
N(1)—C(20)	1.46(3)	O(3)—C(10)	1.33(3)
N(1)—C(1)	1.52(3)	O(4)—C(26)	1.36(3)
N(2)—C(16)	1.49(3)	O(7)—C(35)	1.26(4)
O(4)—Re—N(1)	90.4(8)	C(1)—N(1)—Re	107.5(2)
N(2)—Re—N(1)	92.1(8)	C(16)—N(2)—C(3)	114(3)
O(2)—Re—O(3)	89.1(7)	C(16)—N(2)—Re	115(2)
O(2)—Re—O(4)	93.8(7)	C(3)—N(2)—Re	110(2)
O(2)—Re—N(2)	76.0(9)	C(35)—N(3)—C(5)	123(2)
O(2)—Re—N(1)	73.0(7)	C(2)—O(2)—Re	107.3(2)
C(20)—N(1)—C(1)	112(2)	C(10)—O(3)—Re	125(2)
C(20)—N(1)—Re	115(2)	C(26)—O(4)—Re	121(2)

were independent and 1879 had $I \geq 2\sigma(I)$. The unit cell parameters were obtained through a least-squares analysis of 25 reflections ($10^\circ < \theta < 14^\circ$). Data reduction and empirical absorption corrections were applied with the XTAL package (30) (Ψ -scans, $T_{\min} = 0.81$ and $T_{\max} = 1.00$). No corrections for decay were employed. The structure was solved by heavy atom methods with SHELXS 86 (31) and refined by full-matrix least-squares analysis with SHELXL 93 (32, 33). All non-H atoms were refined with anisotropic thermal parameters, whereas H-atoms were included at estimated positions. A ZORTEP representation of the complex is given in Figure 2, crystallographic data is summarized in Table 1, and important bond lengths and angles are presented in Table 3. Disorder of the carboxylic acid group over three sites was modeled by restraining the occupancies of three independent C-atoms: C(39A), C(39B), and C(39C). Only carboxyl O-atoms attached to C(39A) were modeled.

RESULTS AND DISCUSSION

The stable complexation of metal centers under in vivo conditions demands ligands providing high thermodynamic stability and kinetic inertness. It is well-known that, for a particular metal center, ligands of higher denticity usually give rise to complexes of higher stability. Therefore, ligands with high denticity should be of particular value for in vivo applications. In the case of rhenium and technetium, practically all the known complexes involve tetradentate open-chained ligand systems with nitrogen and/or sulfur as donor atoms. Most of these complexes have tetragonal pyramidal geometry with four ligand atoms in the basal plane and the terminal oxo in the apical position (34–38).

We recently described the preparation and structure of the complex [ReO(thci)] (Scheme 1), which demon-

Scheme 3. Two Synthetic Ways to H₃bhci-glu-H

strates this coordination mode (39). Although H₃bhci is potentially a hexadentate ligand, the Re=O moiety was maintained during coordination, leading to pentadentate coordination with one of the pendant arms remaining uncoordinated. This uncoordinated amine functionality of the taci framework is convenient for the attachment of linking groups of choice without influencing the coordinating properties of the metal-binding site. This expectation was confirmed by H₃bhci and in particular by H₃bhci-glu-H.

Ligand Synthesis. The ligand H₃bhci was synthesized by the condensation of 2 equiv of salicylaldehyde with taci and subsequent reduction of the imines to amines, which gives rise to a statistical mixture of 1:1, 1:2, and 1:3 products from which the 1:2 product was extracted (Scheme 3). Due to the various nucleophilic functionalities of H₃bhci, derivatization of H₃bhci with glutaric anhydride and subsequent chromatographic purification resulted in a very poor yield of the desired product. Therefore, to bifunctionalize H₃bhci by the introduction of a glutaric acid group as a linker to the protein at the underivatized amine function of H₃bhci, the derivatization of the taci framework with the linker was performed prior to the attachment of the two other pendant arms. In this case, the 1:1 product precipitated from solution and the formation of a mixture of the statistically distributed 1:1, 1:2, and 1:3 products is avoided. The subsequent addition of 2 equiv of salicylaldehyde and reduction of the imines was easily performed and good yields of the bifunctional ligand H₃bhci-glu-H were achieved (Scheme 3).

This procedure allows the introduction of linkers with varying chain lengths and other biological characteristics such as lipophilicity, charge, or cleavable groups.

Structure of [ReO(bhci)] and [ReO(bhci-glu-H)]. Complex formation on the nonradioactive level was achieved by reaction of [ReOCl₃(P(C₆H₅)₃)₂] with H₃bhci

Table 4. Comparison of Structural Parameters in ReO(bhci-glu-H), ReO(bhci), ReO(thci),^a and [Co(taci)₂]³⁺^b

parameter	[ReO(bhci-glu-H)]	[ReO(bhci)]	[ReO(thci)]	[Co(taci) ₂] ³⁺
bond lengths within the coordination sphere (Å) ^c				
Re—O _{oxo}	1.71	1.69	1.70	
Re—O _{phen}	2.02	2.00	1.96	
Re—O _{alkoxo}	1.98	1.98	2.03	
Re—N	2.14	2.16	2.16	
1,2- and 1,3-nonbonding interactions (Å) ^c				
N...N ^d	3.07	2.97	2.95	2.83
N...N' ^d	4.74	2.98	2.98	
N...O _{alkoxo} ^d	2.50	2.58	2.62	
N...O _{hydroxo} ^d	2.82	2.83	2.88	2.82
N'...O _{hydroxo} ^d	2.83	2.77	2.84	
ring puckering of the cyclohexane ring ^e				
Q (Å)	0.72	0.51	0.50	0.57
θ (deg)	83.49	9.7	5.6	1.5
φ (deg)	30.1	45.2	68.1	59.7

^a H₃thci = 1,3,5-trideoxy-1,3,5-tris(2-hydroxybenzyl)amino-*cis*-inositol, data from ref 39. ^b Data from ref 27. ^c Estimated standard deviations are 0.02 Å or less. ^d N refers to the coordinating, N' to the noncoordinating amino group. ^e According to Cremer and Pople (42).

or H₃bhci-glu-H in methanol and addition of base to neutralize the released protons.

The crystal structures of both [ReO(bhci)] and [ReO(bhci-glu-H)] were determined by X-ray diffraction (Figures 1 and 2). It can be seen that the primary amino group of [ReO(bhci)] and the carboxylic group of [ReO(bhci-glu-H)] do not participate in the Re(V) coordination. This is crucial for the linkage of the complex to the protein. Some structural parameters of [ReO(bhci)], [ReO(bhci-glu-H)], and of the previously reported [ReO(thci)] (39) are presented in Table 4. They all show the same "asymmetric" Re(V)—N₂O₃ coordination (40). The distances between Re and the donor atoms of the ligands fall in the expected ranges (41), and differences between the three complexes are generally not significant. In contrast to the well-known, symmetric M—N,N,N coordination of taci, the asymmetric Re—N,N,N,O coordination causes some characteristic distortion of the ligand backbone. This is evident when comparing interatomic, nonbonding distances between the substituents in the 1,2- and the diaxial 1,3-positions. The 1,2-interactions between the coordinated donor atoms of the Re complexes all lie between 2.50 and 2.62 Å. This is remarkably short when compared to the free ligand or to the symmetric hexamine [Co(taci)₂]³⁺ (27) and indicates the considerable strain inherent in this coordination mode.

Although the three rhenium complexes have related coordination spheres, it is noteworthy that a significantly different conformation is observed between the cyclohexane rings of [ReO(thci)] and [ReO(bhci)] on one hand and for [ReO(bhci-glu-H)] on the other. Inspection of the puckering parameters presented in Table 4 shows that a slightly flattened chair is present in [ReO(thci)] and [ReO(bhci)]. However, for [ReO(bhci-glu-H)] the puckering parameters correspond to an almost ideal twisted boat (42). In the chair arrangement of [ReO(thci)] and [ReO(bhci)], the N—H bonds of the coordinated amines are oriented directly toward the lone pair of the noncoordinating amine, and the structure is stabilized by N—H...N hydrogen bonding (43). In contrast, no such stabilization would be possible in the corresponding chair conformation of [ReO(bhci-glu-H)]. However, the observed twisted boat conformation in the latter complex allows hydrogen bonding between a coordinated N—H group and a noncoordinated hydroxy group. Hence, there is evidence that hydrogen bonding between the coordi-

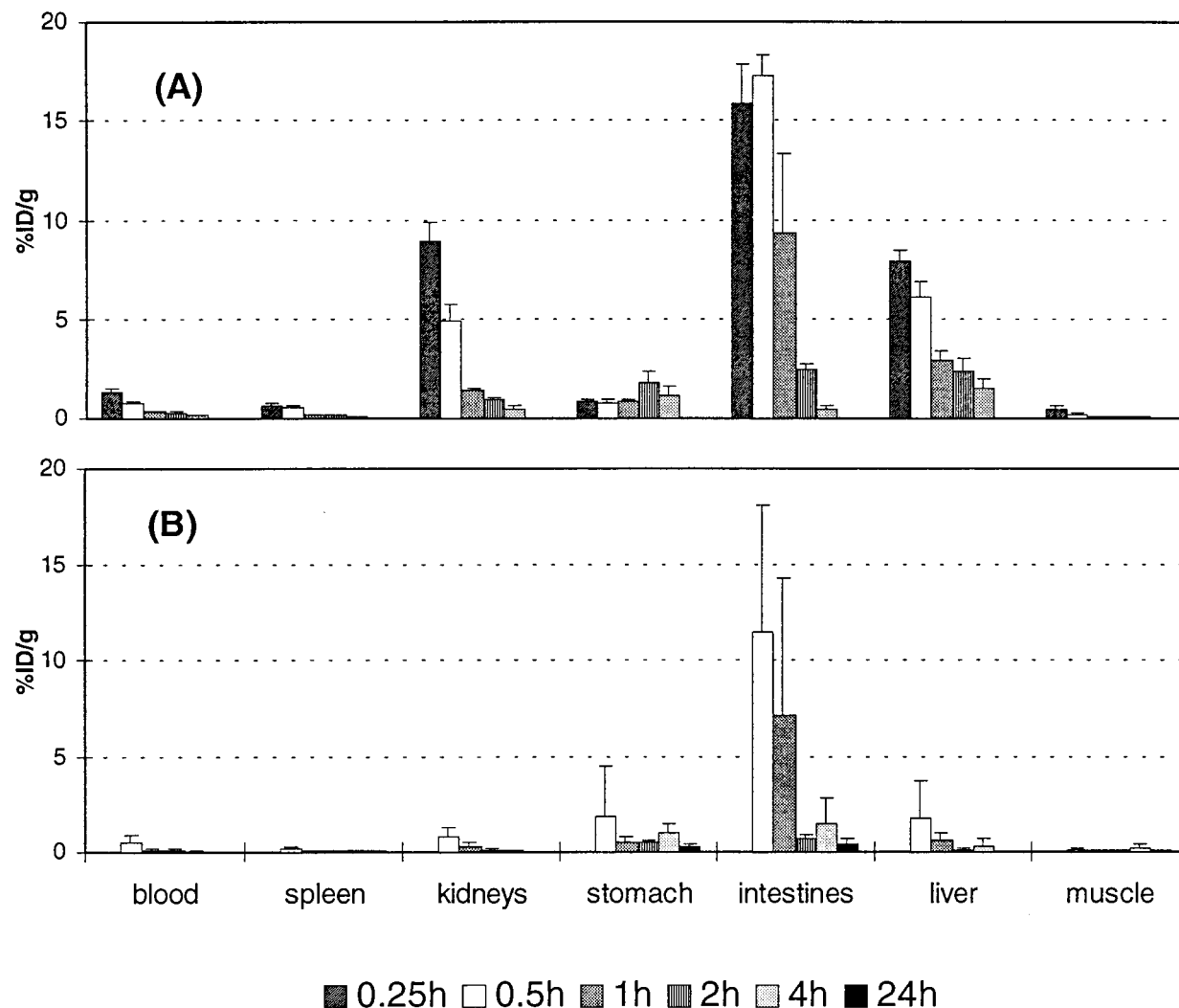


Figure 3. Biodistribution studies of $[^{188}\text{ReO}(\text{bhci})]$ and $[^{188}\text{ReO}(\text{bhci-glu})]^-$ in white mice. Organ uptake was determined at 0.25, 0.5, 1, 2, and 4 h postinjection for $[\text{ReO}(\text{bhci})]$ (A) and at 0.5, 1, 2, 4, and 8 h postinjection for $[\text{ReO}(\text{bhci-glu})]^-$ (B).

nated amines and additional noncoordinating substituents is one of the key factors responsible for the observed differences in the conformation of these complexes.

Synthesis of $[^{186/188}\text{ReO}(\text{bhci})]$ and $[^{186/188}\text{ReO}(\text{bhci-glu-H})]$. For the preparation of the radioactive complexes, $[^{186/188}\text{ReO}_4]^-$ was reduced with Sn(II) in citrate buffer or with Sn(0) in water in the presence of small amounts of ligand to yield the analogous $^{186/188}\text{Re}$ complex in quantitative yield. TLC/HPLC were used to establish that the radioactive and nonradioactive products were equivalent.

Serum and in Vivo Stability of the Complexes. The complexes $[^{188}\text{ReO}(\text{bhci})]$ and $[^{188}\text{ReO}(\text{bhci-glu-H})]$ contain ligand groups in the coordination sphere which can easily be protonated since the corresponding pK_a values are rather high. This is in particular the case for the aliphatic alkoxo-group trans to the terminal oxo function.

To investigate the stability of the two complexes, the corresponding ^{188}Re compounds were dissolved in freshly prepared human serum at 37 °C, and the solution was checked by TLC over a 3 day period. Potential decomposition products such as $^{188}\text{ReO}_2$ or $[^{188}\text{ReO}_4]^-$ can easily be detected by this method. Decomposition amounting to less than 5% was found under these conditions, clearly demonstrating the excellent stability of both of these compounds.

Furthermore, the two complexes were injected intravenously into mice in order to check their in vivo stability and behavior. The results of the biodistribution experiments are shown in Figure 3.

It was found that the uncharged complex $[^{188}\text{ReO}(\text{bhci})]$ is excreted via the liver, the kidneys, and the intestines, while $[^{188}\text{ReO}(\text{bhci-glu})]^-$, which is anionic at physiological pH values, is mainly excreted via the intestines. As $[^{188}\text{ReO}_4]^-$ is excreted via kidneys into urine, the urine was checked for the presence of this potential decomposition product using TLC/HPLC. Essentially, the total activity in the urine samples was accounted for as $[^{188}\text{ReO}(\text{bhci-glu})]^-$ with less than 5% in the form of $[^{188}\text{ReO}_4]^-$. On the other hand, samples obtained at later times revealed the presence of another $[^{188}\text{Re}]$ complex with an R_f of 0.1, which was obviously neither $[^{188}\text{ReO}_4]^-$ nor ReO_2 . It is not possible to say whether the degradation process leading to this species was already occurring in the serum or only in the urine. However, since a similar compound was not detected during in vitro incubation, it seems to be more likely that transformation occurred only in the urine. Furthermore, the R_f value indicates a degradation at the backbone rather than at the complex itself. The study shows that there is no decomposition of the complexes and that the complexes were essentially excreted in the same form as injected. The high in vivo stability and rapid renal clearance of

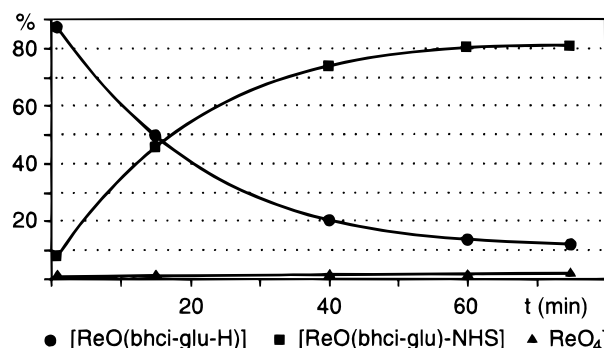


Figure 4. Formation of the [ReO(bhci-glu)-NHS] active ester at 50 °C.

[ReO(bhci-glu)]⁻ indicate that this may be a suitable complex for use in antibody labeling and RIT applications.

Studies on Active Ester Formation. Activation of the carboxylic acid function by esterification with strongly acidic alcohols is a technique that is widely applied for the covalent attachment of molecules to proteins by amide formation with the ϵ -amino group of lysine. Optimal coupling conditions are particularly important if prelabeling studies are intended. The competition between aminolysis and hydrolysis often results in poor labeling yields on the *nca* level. Accurate quantification of coupling conditions is relatively rare in the literature since they vary considerably from system to system. The EDC/NHS approach with activation after complex formation was chosen mainly due to the enhanced reactivity of the NHS esters (e.g., compared to tetrafluorophenol) toward amines, and since activation prior to coordination would probably result in cross linking to secondary amine groups in the ligand itself. Although most of the activations with NHS described in the literature were carried out under ambient conditions, no activation could be observed under these conditions with the [ReO(bhci-glu-H)] complex. The formation of the NHS active ester could only be achieved by increasing the temperature (80% yield after 60 min at 50 °C). The progress of the activation process was monitored by TLC, where [¹⁸⁸ReO(bhci-glu-H)] and [¹⁸⁸ReO(bhci-glu-NHS)] could be readily distinguished. A typical profile for the formation of [¹⁸⁸ReO(bhci-glu-NHS)] as a function of time is given in Figure 4.

The profile shows first-order kinetic behavior which is reasonable considering the large excess of NHS and EDC over the rhenium complex. Thus, the concentrations of the former compounds can be considered to remain constant over the reaction period. Quantitative activation could not be achieved under any conditions, but this did not appear to be simply an analytical problem. Deactivation on the silica gel plates would result in significant tailing of the peaks, but these were in fact fully symmetrical.

Labeling Experiments. Aliquots of [¹⁸⁸ReO(bhci-glu-NHS)], prepared as described above, were used for the labeling of the intact antibody mAb-35 and its F(ab')₂ fragment. Prelabeling often gives poor yields, so efforts were made to optimize the reaction conditions by varying the pH, mAb concentration, and reaction volume. The progress of the labeling reaction was monitored by TLC. The labeled protein remained at the origin while the complexes had defined *R_f* values (see Experimental Procedures). The final labeling yield was determined after separation of protein from small molecular weight materials and aggregates by FPLC gel chromatography.

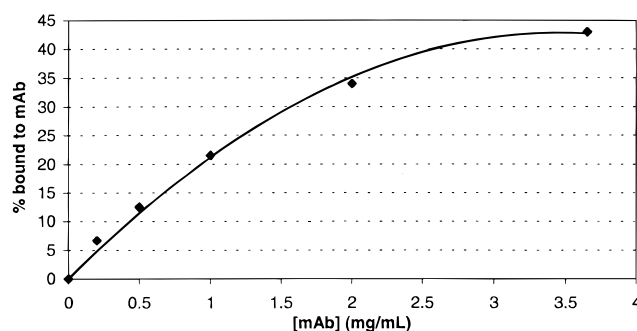


Figure 5. Labeling yield as a function of mAb concentration.

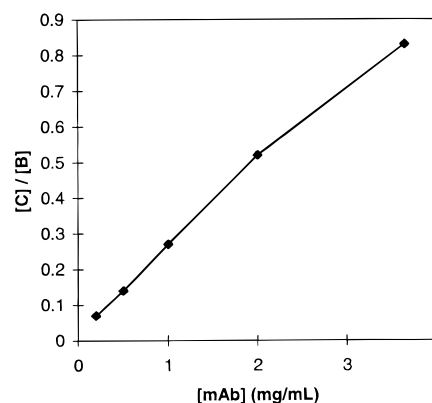


Figure 6. Protein concentration dependent ratio of hydrolysis of the active ester [ReO(bhci-glu)-NHS] (B) and binding of the complex to the antibody (C).

Labeled mAb appeared as a single peak in the same fractions as unlabeled IgG or F(ab')₂ fragment. A typical curve for the labeling yield as a function of protein concentration is depicted in Figure 5.

Yields ranged from about 7% at mAb concentrations of 0.2 mg/mL to 43% at 3.65 mg/mL. The latter value seems to represent a kinetic maximum since the curve approaches a horizontal line at this concentration. At even higher protein concentrations, there was no further increase in labeling yield, but there was a decrease in specific activity of the radioimmunoconjugate due to the larger amount of protein. Specific activities of up to 1.5 mCi/mg (55 MBq/mg) could be achieved.

The two main reactions are (a) hydrolysis of the activated metal complex [ReO(bhci-glu-NHS)] (A) with a first-order rate k_1 ($d[A]/dt = -k_1[A]$) to [ReO(bhci-glu)]⁻ (B) and (b) the effective labeling of the protein (mAb) with a rate constant k_2 . Assuming only [ReO(bhci-glu-NHS)] on the *nca* level and no free activated ligand, the rate law of the reaction b must be pseudo-first-order in protein concentration ($d[A]/dt = -k_2[A][mAb]$) since the available amine groups are in excess relative to [¹⁸⁸ReO(bhci-glu-NHS)] and can be considered as having a constant concentration. The yield of labeled protein (C) under this assumption would depend only on k_1 , k_2 , and [mAb], giving rise to the expression $[C]/[B] = k_2 [mAb]/k_1$. It is not possible to determine individual values for k_1 and k_2 , although graphical analysis of $[C]/[B]$ vs [mAb] showed that $k_2/k_1 \approx 2$ (44). The graphical representation is given in Figure 6. The linearity of the curve supports the formulated pseudo-first-order model, the invariance of protein concentration, and importantly, the absence of significant amounts of free, activated ligand. From this idealized model, it is to be expected that the labeling yield will not depend on the initial amount of metal complex, and this was indeed confirmed for the investi-

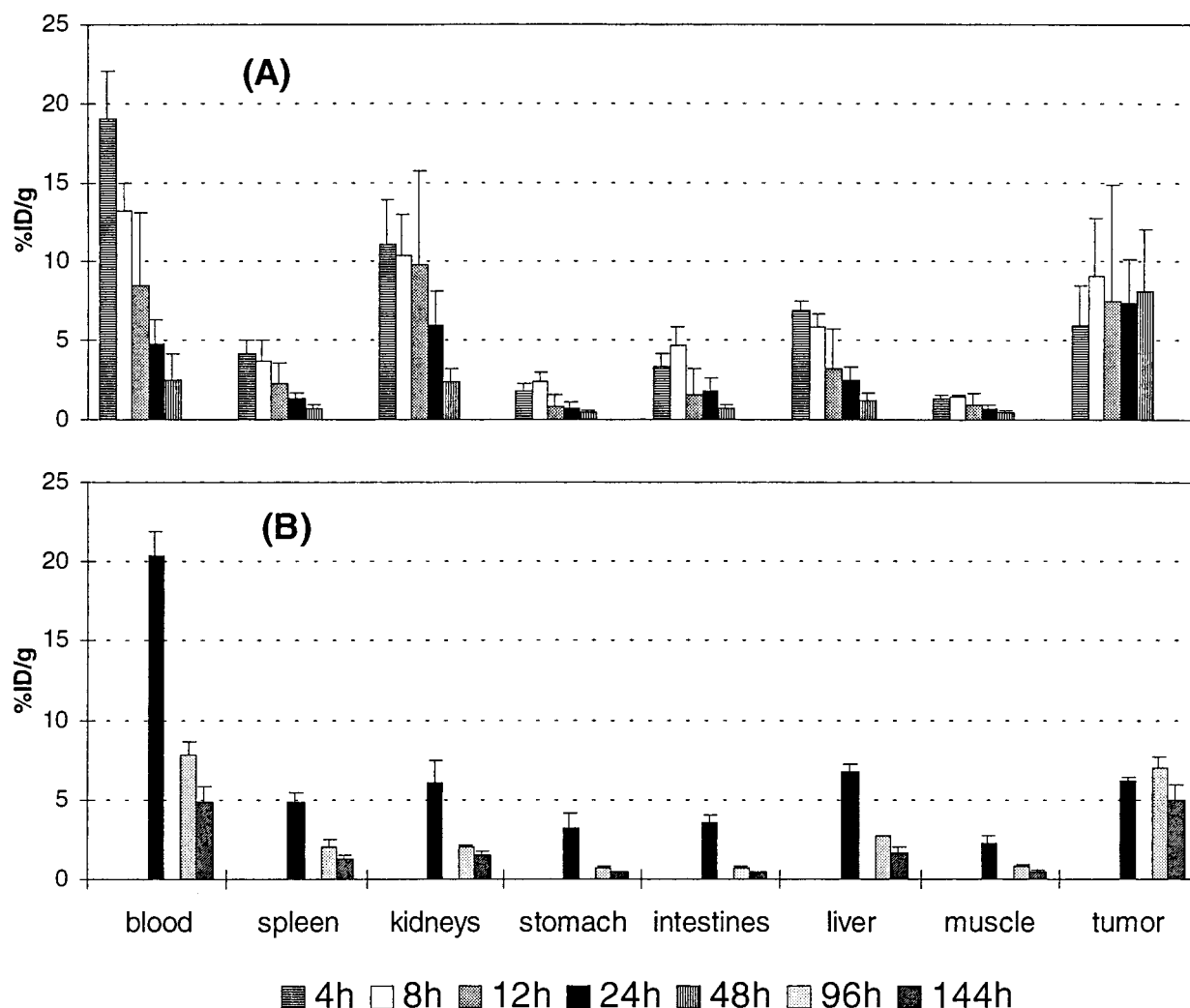


Figure 7. Biodistribution studies of [$^{186}\text{ReO}(\text{bhci-glu})$]-labeled mAb-35 in tumor-bearing nude mice. Organ and tumor uptake was determined at 4, 8, 12, 24, and 48 h postinjection for the labeled $\text{F}(\text{ab}')_2$ fragment (A) and at 24, 96, and 144 h postinjection for the labeled IgG (B).

gated range. However, when the concentration of the rhenium complex was increased by a factor of 10 or more, the yield decreased by a factor of 2 but the absolute specific activity of the radioimmunoconjugate increased by a factor of 5. It can be concluded from this observation that small amounts of free, activated ligands are nevertheless present, and these change the available amine concentration upon coupling to the protein. The pseudo-first-order kinetics are then transformed into true second-order kinetic behavior. This dependence clearly demonstrated the importance of purifying the complex prior to activation. This purification is not only essential to achieve good labeling yields and consequently high specific activities but also to maintain the immunoreactivity of the labeled mAb-35.

Samples of mAb-35 were labeled with [$^{186}\text{ReO}(\text{bhci-glu})$] as described above. The labeling yields with carrier added ^{186}Re (0.4% radioactive ^{186}Re , 99.6% "cold" Re) were ca. 30% lower than in case of *nca* ^{188}Re from the $^{188}\text{W}/^{188}\text{Re}$ generator. Calculations revealed that, at a specific activity of the radioimmunoconjugates of 1 mCi/mg (37 MBq/mg), the complex:antibody molar ratio is 1:1230 for *nca* ^{188}Re and about 1:1 for carrier added ^{186}Re .

Serum Stability and Immunoreactivity. The radioimmunoconjugate thus prepared with a specific activity of 1 mCi/mg (37 MBq/mg) was stable in serum over

at least 72 h (>95% ^{186}Re bound to the protein after 72 h in blood serum at 37 °C). Assessment by Lindmo assay (28) of the immunoreactivity of the ^{186}Re -labeled preparations of both intact and fragmented mAb-35 showed them to have an immunoreactivity of over 90%. Nonspecific binding was determined by competition with excess unlabeled mAb-35 and was less than 5% in all cases. These results are in good correlation with the results obtained in the literature using other methods for the labeling of mAb-35, such as ^{131}I -labeling (iodogen-method) or ^{67}Cu -labeling (21), where immunoreactivities of over 80% were determined.

Biodistribution of [$^{186}\text{ReO}(\text{bhci-glu})$]-Labeled mAb-35 [IgG and $\text{F}(\text{ab}')_2$] in Tumor-Bearing Nude Mice. Free [$^{186/188}\text{ReO}(\text{bhci})$] and [$^{186/188}\text{ReO}(\text{bhci-glu})$] $^-$, respectively, are rapidly cleared from the body with a maximum of 1.5% ID/g remaining in the intestines and the liver after 4 h (Figure 3). The ^{186}Re bound to intact mAb-35 is cleared much more slowly due to the pharmacokinetic behavior of the antibody. Biodistributions of ^{186}Re -mAb-35 (IgG) in tumor-bearing mice were measured over a period of 6 days. The results are shown in Figure 7B. It was found that tumor uptake reached a maximum after 4 days with 7% ID/g, and after 6 days still more than 5% ID/g was bound to the tumor. Clearance of radioactivity from all organs, with the exception of blood, was rapid.

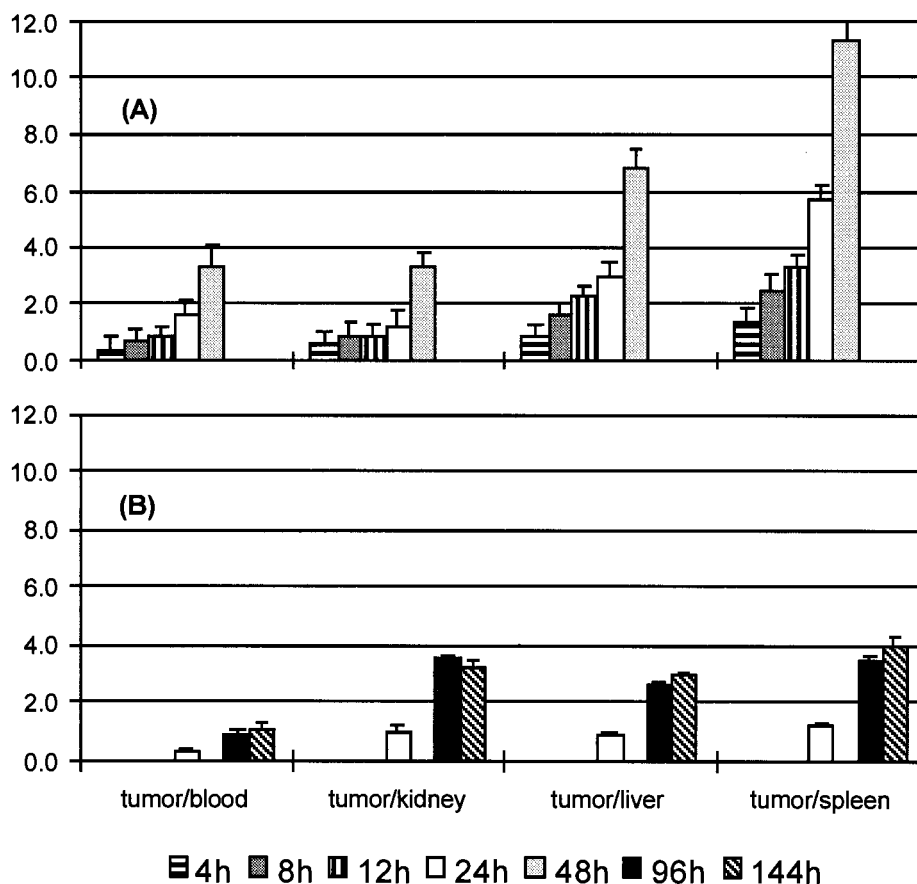


Figure 8. Tumor-to-organ ratios of $[^{186}\text{Re}(\text{bhci-glu})]$ -labeled mAb-35 in tumor-bearing nude mice. The ratios were determined at 4, 8, 12, 24, and 48 h postinjection for the labeled $\text{F}(\text{ab}')_2$ fragment (A) and at 24, 96, and 144 h postinjection for the labeled IgG (B).

Tumor-to-organ ratios, which were achieved after 24, 96, and 144 h, are shown in Figure 8B. As negative control for the specific binding of ^{186}Re -labeled mAb-35, the non-CEA-specific antibody chCE7 (an internalizing, neuroblastoma-specific mAb) was labeled and purified in the same way as mAb-35, and consecutively injected in mice bearing LoVo-cell xenografts. After 4 days, tumor-to-organ ratios of the chCE7 antibody were generally three to four times lower than with mAb-35, demonstrating the specific binding of labeled mAb-35 to CEA antigen presented on LoVo cells. The labeled $\text{F}(\text{ab}')_2$ fragment showed much faster clearance from the body than the intact mAb-35 antibody. Biodistributions of ^{186}Re - $\text{F}(\text{ab}')_2$ in tumor-bearing mice were measured over a period of 2 days. The corresponding results are shown in Figure 7A. The maximum tumor uptake was reached after 12 h (9% ID/g), and after 48 h, the tumor accumulation was still 8% ID/g.

Tumor-to-organ ratios 24 h postinjection were better for ^{186}Re - $\text{F}(\text{ab}')_2$ than for intact mAb-35 (Figure 8A). MAb-35 has been evaluated in both radioiodinated (21, 25, 45) and radiocopper labeled (21) form in nude mice bearing LoVo cell xenografts. The ^{186}Re -labeled mAb-35 [IgG and $\text{F}(\text{ab}')_2$] shows similar results as its ^{131}I -labeled analogues in terms of tumor uptake and tumor/organ ratios. Biodistributions of ^{67}Cu -labeled mAb-35 are characterized by higher levels of radioactivity in tumors but also by considerably higher uptake of the label in liver and spleen. In the case of ^{67}Cu -labeled $\text{F}(\text{ab}')_2$, fragment accumulation of radioactivity in the kidneys is very prominent and the biodistributions of ^{186}Re - $\text{F}(\text{ab}')_2$ appear to be more favorable.

Samples of blood from the dissected mice were checked by TLC, revealing that 100% of radioactivity found in the

blood was still bound to the antibody. To determine in which form the radioactivity was excreted in the urine, samples were checked by TLC. After 1 day, the radioactivity consisted of 50% $[^{186}\text{ReO}(\text{bhci-glu})]^-$, 20% $[^{186}\text{ReO}_4]^-$, and 30% $[^{186}\text{ReO}(\text{bhci-glu})]$ bound to low molecular protein, while after 4 and 6 days, most of the ^{186}Re was excreted in the form of low molecular protein bound $[^{186}\text{ReO}(\text{bhci-glu})]$ with only roughly 5% $[^{186}\text{ReO}_4]^-$. Preliminary experiments to establish the composition of this low molecular protein bound $[^{186}\text{ReO}(\text{bhci-glu})]$ showed that on TLC it has the same R_f (0.15) as $[^{186}\text{ReO}(\text{bhci-glu-Lys})]$. The results show that these metabolites are characterized by rapid renal and hepatic clearance. In summary, our data show that the labeling of monoclonal antibodies with $[^{186/188}\text{ReO}(\text{bhci-glu})]$ leads to stable, fully immunoreactive mAb conjugates having favorable biodistributions in tumor-bearing mice, and may have potential for application in RIT.

CONCLUSION

The novel pentadentate ligands H_3bhci and $\text{H}_3\text{bhci-glu-H}$ have been prepared. These ligands form the very stable Re(V) complexes $[\text{ReO}(\text{bhci})]$, and $[\text{ReO}(\text{bhci-glu-H})]$ which showed no detectable decomposition under in vivo conditions. Ligands with a denticity greater than four provide significantly better stability than the more common tetradentate systems. Furthermore, the underivatized ligand H_3bhci offers the possibility of introducing various linking systems and allows the systematic study of the influence of this part of a protein label on tumor accumulation of radioimmunoconjugates. Activation of the carboxylic acid group in $[\text{ReO}(\text{bhci-glu-H})]$ with the widely applied EDC/NHS method was found to require increased temperature and time to achieve a

reasonable yield of the activated complex form. Labeling yields depended strongly on protein concentration, the latter influencing the rate in a pseudo-first-order kinetic fashion in the absence of excess ligand, otherwise the labeling yields are dramatically reduced. It was found that it is essential to purify the activated complex before the labeling of the antibody to retain full immunoreactivity of the immunoconjugate. Animal experiments showed promising results in terms of biodistribution and clearance from the body, especially of the labeled mAb-35 F(ab')₂ fragments. The results with the novel [ReO-(bhci-glu)] label indicate its potential for the ¹⁸⁶Re labeling of antitumor antibodies for the use in RIT.

ACKNOWLEDGMENT

Thanks to C. De Pasquale and C. Ruch for their help with the biodistribution studies and to P. Osvath for the correction of this publication.

Supporting Information Available: Tables giving details of data collection, atomic coordinates, bond distances and angles, anisotropic thermal parameters, and hydrogen atom positions for the complexes [ReO(bhci)] and [ReO(bhci-glu-H)] are available (14 pages). Ordering information is given on any current masthead page.

LITERATURE CITED

- (1) Eary, J. F. (1991) Fundamentals of Radioimmunotherapy. *Nucl. Med. Biol.* 18, 105–108.
- (2) Pimm, M. V., and Baldwin, R. W. (1987) Comparative tumour localization properties of radiolabeled monoclonal antibody preparations of defined immunoreactivities. *Eur. J. Nucl. Med.* 13, 348–352.
- (3) Langmuir, V. K. (1992) Radioimmunotherapy: Clinical Results and Dosimetric Considerations. *Nucl. Med. Biol.* 19 (2), 213–225.
- (4) Hazra, D. K., Britton, K. E., Lahiri, V. L., Gupta, A. K., Khanna, P., and Saran, S. (1995) Immunotechnological Trends in Radioimmunotargeting: From Magic Bullet to Smart Bomb. *Nucl. Med. Commun.* 16, 66–75.
- (5) Srivastava, S. C., and Mease, R. (1991) Progress in Research on Ligands, Nuclides and Techniques for Labeling Monoclonal Antibodies. (1991) *Nucl. Med. Biol.* 18 (6), 589–603.
- (6) Schubiger, P. A., Alberto, R., and Smith, A. (1996) Vehicles, Chelators and Radionuclides: Choosing the Building Blocks of an Effective Radioimmunoconjugate. *Bioconjugate Chem.* 7, 165–179.
- (7) Cotton, F. A., and Wilkinson, G. (1980) Technetium and Rhenium. *Advanced Inorganic Chemistry*, 4th ed., pp 883–901, Wiley-Interscience, New York.
- (8) Deutsch, E., Libson, K., Vanderheyden, J.-L., Ketrang, A., and Maxon, H. R. (1986) The Chemistry of Rhenium and Technetium as Related to the Use of Isotopes of these Elements in Therapeutic and Diagnostic Nuclear Medicine. *Nucl. Med. Biol.* 13, 465–477.
- (9) Jurisson, S., Berning, D., Jia, W., and Ma, D. (1993) Coordination Compounds in Nuclear Medicine. *Chem. Rev.* 93, 1137–1156.
- (10) Beaumier, P. L., Venkatesan, P., Vanderheyden, J.-L., Burgua, W. D., Kunz, L. L., Fritzberg, A. R., Abrams, P. G., and Morgan, A. C., Jr. (1991) ¹⁸⁶Re Radioimmunotherapy of Small Cell Lung Carcinoma Xenografts in Nude Mice. *Cancer Res.* 51, 676–681.
- (11) Visser, G. W. M., Gerretsen, M., Herscheid, J. D. M., Snow, G. B., and van Dongen, G. (1993) Labeling of Monoclonal Antibodies with Rhenium-186 Using the MAG3 Chelate for Radioimmunotherapy of Cancer: A Technical Protocol. *J. Nucl. Med.* 34, 1953–1963.
- (12) Schroff, R. W., Weiden, P. L., Appelbaum, J., Fer, M. F., Breitz, H., Vanderheyden, J.-L., Ratliff, B., Fisher, D., Foisie, D., Hanelin, L. G., Morgan, A. C., Fritzberg, A. R., and Abrams, P. G. (1990) Rhenium-186 labeled antibody in patients with cancer: report on a pilot Phase I study. *Antibody, Immunoconjugates, Radiopharm.* 3, 99–110.
- (13) Breitz, H. B., Weiden, P. L., Vanderheyden, J.-L., Appelbaum, J. W., Bjorn, M. J., Mehmet, F. F., Wolf, S. B., Ratliff, B. A., Seiler, C. A., Foisie, D. C., Fisher, D. R., Schroff, R. W., Fritzberg, A. R., and Abrams, P. G. (1992) Clinical Experience with Rhenium-186 Labeled Monoclonal Antibodies for Radioimmuno-therapy: Results of Phase I Trials. *J. Nucl. Med.* 6, 1099–1112.
- (14) Li, M., and Meares, C. F. (1993) Synthesis, Metal Chelate Stability Studies, and Enzyme Digestion of Peptide Linked DOTA Derivative and its Corresponding Radiolabeled Immunoconjugates. *Bioconjugate Chem.* 4, 275–283.
- (15) DeNardo, S. J., Richman, C. M., Goldstein, D. S., Shen, S., Salako, Q., Kukis, D. L., Meares, C. F., Yuan, A., Welborn, J. L., and DeNardo, G. L. (1997) Yttrium-90/Indium-111 DOTA-Peptide-Chimeric L6-Pharmacokinetics, Dosimetry and Initial Results in Patients with Incurable Breast Cancer. *Anticancer Res.* 17, 1735–1744.
- (16) Haseman, M. K., Goodwin, D. A., Meares, C. F., Kaminski, M. S., Wensel, T. G., McCall, M. J., and Levy, R. (1986) Metabolizable ¹¹¹In-chelate Conjugated Anti-idiotypic Monoclonal Antibody for Radioimmunodetection of Lymphoma in Mice. *Eur. J. Nucl. Med.* 12, 455–460.
- (17) Deshpande, S. V., DeNardo, S. J., Meares, C. F., McCall, M. J., Adams, G. P., and DeNardo, G. L. (1989) Effect of Different Linkage Between Chelates and Monoclonal Antibodies on Levels of Radioactivity in Liver. *Nucl. Med. Biol.* 16, 587–597.
- (18) Marzilli, L. G., Banaszcyk, M. G., Hansen, L., Kuklenyik, Z., Cini, R., and Taylor, A., Jr. (1994) Linking Deprotonation and Denticity of Chelate Ligands. Rhenium(V) Oxo Analogues of Technetium-99m Radiopharmaceuticals Containing N₂S₂ Chelate Ligands. *Inorg. Chem.* 33, 4850–4860.
- (19) Franklin, K. J., Howard-Lock, H. E., and Lock, C. J. L. (1982) Preparation, Spectroscopic Properties and Structure of 1-Oxo-2,3,6-(D-penicillaminato-N,S,O)-4,5-(D-penicillaminato-N,S)technetium(V). *Inorg. Chem.* 21, 1941–1946.
- (20) Buchegger, F., Haskell, C. M., Schreyer, M., Scazziga, B. R., Randin, S., Carrel, S., and Mach, J.-P. (1983) Radiolabeled Fragments of Monoclonal Antibodies against Carcinoembryonic Antigen for Localization of Human Colon Carcinoma Grafted into Nude Mice. *J. Exp. Med.* 158, 413–427.
- (21) Smith, A., Alberto, R., Bläuenstein, P., Novak-Hofer, I., Mäcke, H. R., and Schubiger, P. A. (1993) Preclinical Evaluation of ⁷⁶Cu-labeled Intact and Fragmented Anti-Colon Carcinoma Monoclonal Antibody Mab35. *Cancer Res.* 53, 5727–5733.
- (22) Buchegger, F., Pèlegri, A., Delaloye, B., Bischof-Delaloye, A., and Mach, J.-P. (1990) Iodine-131-Labeled mAb F(ab')₂ Fragments are More Efficient and Less Toxic Than Intact Anti-CEA Antibodies in Radioimmunotherapy of Large Human Colon Carcinoma Grafted in Nude Mice. *J. Nucl. Med.* 31, 1035–1044.
- (23) Humm, J. L. (1986) Dosimetric Aspects of radiolabeled antibodies for tumor therapy. *J. Nucl. Med.* 27, 1490–1497.
- (24) Yorke, E. D., Beaumier, P. L., Wessels, B. W., Fritzberg, A. R., and Morgan, A. C., Jr. (1991) Optimal Antibody-Radionuclide Combinations for Clinical Radioimmunotherapy: a Predictive Model Based on Mouse Pharmacokinetics. *Nucl. Med. Biol.* 18 (8), 827–835.
- (25) Smith, A., Alberto, R., and Schubiger, P. A. (1994) Influence of Radiolabel on the In Vivo Processing of Intact and Fragmented Anti-Tumour Monoclonal Antibody. *J. Nucl. Biol. Med.* 38 (Suppl. 1 to No. 4), 54–58.
- (26) Callahan, A. P., Rice, D. E., and Knapp, F. F., Jr. (1989) Re-188 for Therapeutic Applications from an Alumina Based W-188/Re-188 Radionuclide Generator System. *NucCompact* 20, 3–7.
- (27) Ghisletta, M., Jalett, H. P., Gerfin, T., Gramlich, V., and Hegetschweiler, K. (1992) 1,3,5-Triamino-1,3,5-trideoxy-cis-inositol, a New Ligand with a Remarkable Versatility for Metal Ions. Part 2: Safe and Efficient Ligand Preparation and Structure of the Free Ligand and the Co(III) Complex. *Helv. Chim. Acta* 75, 2233–2242.

- (28) Lindmo, T., Boven, E., Cuttitta, F., Fedorko, J., and Bunn, P. A., Jr. (1984) Determination of the Immuno-reactive Fraction of Radiolabeled Monoclonal Antibodies by Linear Extrapolation to Binding at Infinite Antigen Excess. *J. Immunol. Methods* 72, 77–89.
- (29) Sheldrick, G. M. *SHELXL97—A program for the refinement of X-ray crystal data*, University of Göttingen, Germany 1997.
- (30) Hall, S. R., and Flack, H. D., Eds. *The XTAL3.2 User's Manual*, Universities of Western Australia, Geneva, and Maryland, 1992.
- (31) Sheldrick, G. M. *SHELXS86—A program for the solution of crystal structures*, University of Göttingen, Germany 1986.
- (32) Sheldrick, G. M. *SHELXL93—A program for the refinement of crystal structures*, University of Göttingen, Germany 1993.
- (33) Scattering factors, including f' and f'' , were taken from *International Tables for Crystallography* (1974) Vol. IV, Kynoch Press, Birmingham, England.
- (34) Bryson, N., Dewan, J. C., Lister-James, J., Jones, A. G., and Davison, A. (1988) Neutral Technetium(V) Complexes with Amide-Thiol-Thioether Chelating Ligands. *Inorg. Chem.* 27, 2154–2161.
- (35) Davison, A., Jones, A. G., Orvig, C., and Sohn, M. (1981) A new class of Oxotechnetium(5+) Chelate complexes Containing a TcON2S2 Core. *Inorg. Chem.* 20, 1629–1632.
- (36) Brenner, D., Davison, A., Lister-James, J., and Jones, A. G. (1984) Synthesis and Characterization of a Series of Isomeric Oxotechnetium(V) Diamido Dithiolates. *Inorg. Chem.* 23, 3793–3797.
- (37) Fritzberg, A. R., Kasina, S., Eshima, D., and Johnson, D. L. (1986) Synthesis and Biological Evaluation of Technetium-99m MAG3 as a Hippuran Replacement. *J. Nucl. Med.* 27, 111–116.
- (38) Bläuenstein, P., Pfeiffer, G., Schubiger, P. A., Anderegg, G., Zollinger, K., May, K., Proso, Z., Ianovici, E., and Lerch, P. (1985) Chemical and Biological Properties of a Cationic Tc–Tetraamine Complex. *Int. J. Appl. Radiat. Isot.* 36 (4), 315–317.
- (39) Hegetschweiler, K., Egli, A., Alberto, R., and Schmalte, H. W. (1992) 1,3,5-Trideoxy-1,3,5-tris((2-hydroxybenzyl)amino)-cis-inositol, a Novel Multidentate Ligand Providing Various N,O-Coordination Sites. Structure of the Rhenium(V) Complex. *Inorg. Chem.* 31, 4027–4028.
- (40) Hegetschweiler, K., Hancock, R. D., Ghisletta, M., Kradolfer, T., Gramlich, V., and Schmalte, H. W. (1993) 1,3,5-Triamino-1,3,5-trideoxy-cis-inositol, a Ligand with a Remarkable Versatility for Metal Ions. 5. Complex Formation with Magnesium(II), Calcium(II), Strontium(II), Barium(II), and Cadmium(II). *Inorg. Chem.* 32, 5273–5284.
- (41) Holloway, C. E., and Melnik, M. (1989) Rhenium coordination compounds: analysis and classification of crystallographic and structural data. *Rev. Inorg. Chem.* 10, 235 and references therein.
- (42) Cremer, D., and Pople, J. A. (1975) A General Definition of Ring Puckering Coordinates. *J. Am. Chem. Soc.* 97, 1354–1358.
- (43) Liang, C., Ewig, C. S., Stouch, T. R., Hagler, A. T. (1994) Ab initio studies of lipid model species. Conformational Analysis of Inositols. *J. Am. Chem. Soc.* 116, 3904–3911.
- (44) Connors, K. A. (1990) *Chemical Kinetics*, VCH Weinheim.
- (45) Smith-Jones, P. M., Fridrich, R., Kaden, A. T., Novak-Hofer, I., Siebold, K., Tschudin, D., and Mäcke, H. R. (1991) Antibody Labeling with Copper-67 Using the Bifunctional Macrocycle 4-[(1,4,8,11-Tetraazacyclotetradec-1-yl)methyl]-benzoic Acid. *Bioconjugate Chem.* 2, 415–421.

BC980014I

Table 1 : Crystal data and structure refinement for ReO(bhci).

Compound	ReO(bhci)
Empirical formula	C30 H46 N3 O11.50 Re S
Formula weight	850.96
Temperature	208(2) K
Wavelength / Radiation	0.71073 Å / MoK α
Crystal system / Spacegroup	Monoclinic/ C2/c
Unit cell dimensions	a = 27.425(3)Å alpha = 90 deg. b = 14.185(1)Å beta = 103.64(2) deg. c = 19.047(2)Å gamma = 90 deg.
Volume	7200.8(12) Å ³
Z	8
Density (calculated)	1.570 Mg/m ³
Absorption coefficient	3.493 mm ⁻¹
F(000)	3440
Crystal description	pale violet block
Crystal size	0.40 x 0.40 x 0.30 mm
No. of reflns. (lattice)	25
Theta range (lattice)	8.59 to 14.57 deg.
Theta range for data collection	3.06 to 25.00 deg.
Index ranges	-1<=h<=32, 0<=k<=16, -22<=l<=22
Reflections collected	6744
Independent reflections	6321 [R(int) = 0.0456]
Reflections observed	3746
Criterion for observation	>2sigma(I)
Absorption correction	Psi scans
Max. and min. transmission	0.9549 and 0.7422
Measurement method	omega scans
No. of standard reflections	3
Interval count/time	300/3600
Decay	5.1 %
Structure solution	SHELXS-97 (Sheldrick, 1990)
Structure refinement	
Molecular graphics	SCHAKAL (Keller,1995), ZORTEP(Zsolnai,1997)
Publication material	SHELXL-97 (Sheldrick, 1997)
Refinement method	Full-matrix least-squares on F ²
Weighting scheme	calc w=1/[\s ² (Fo ²)+(0.0632P) ² + 48.7902P] where P=(Fo ² +2Fc ²)/3
Solution primary	Patterson
Hydrogen treatment	mixed
Data / restraints / parameters	6321 / 0 / 434
Final R indices [I>2sigma(I)]	R1 = 0.0568, wR2 = 0.1284
Goodness-of-fit on F ²	1.063
Final R indices (all) R1 / wR2	0.1369 / 0.1558
Largest diff. peak and hole	1.200 and -0.673 e.Å ⁻³

Table 2: Atomic coordinates and equivalent isotropic displacement parameters ($\text{\AA}^2 \times 10^3$) for $\text{ReO}(\text{bhci})$. ($U(\text{eq})$ is defined as one third of the trace of the orthogonalized U_{ij} tensor).

	x	y	z	$U(\text{eq})$
Re	0.7303(1)	0.4133(1)	0.7779(1)	34(1)
O(1)	0.6866(4)	0.4711(6)	0.7150(4)	47(2)
O(2)	0.7613(3)	0.3142(5)	0.8469(4)	34(2)
O(3)	0.7621(3)	0.5090(5)	0.8519(4)	36(2)
O(4)	0.7941(3)	0.4282(6)	0.7473(4)	39(2)
O(5)	0.6566(3)	0.1112(5)	0.6759(4)	38(2)
O(6)	0.5906(3)	0.2674(6)	0.8552(4)	51(2)
N(1)	0.7103(4)	0.2860(6)	0.7172(5)	32(2)
N(2)	0.6721(4)	0.3784(6)	0.8330(4)	30(2)
N(3)	0.6050(4)	0.2619(7)	0.7176(5)	44(3)
C(1)	0.7144(5)	0.2028(8)	0.7672(6)	31(3)
C(2)	0.7262(4)	0.2415(8)	0.8441(6)	35(3)
C(3)	0.6799(5)	0.2825(8)	0.8653(6)	33(3)
C(4)	0.6336(5)	0.2198(9)	0.8448(6)	36(3)
C(5)	0.6221(4)	0.1849(8)	0.7669(6)	35(3)
C(6)	0.6684(5)	0.1399(8)	0.7504(6)	35(3)
C(10)	0.7565(5)	0.5063(8)	0.9210(6)	31(3)
C(11)	0.7980(6)	0.5332(9)	0.9752(6)	46(3)
C(12)	0.7949(6)	0.5313(9)	1.0473(6)	52(4)
C(13)	0.7514(6)	0.5000(9)	1.0656(7)	44(4)
C(14)	0.7114(5)	0.4750(8)	1.0131(6)	39(3)
C(15)	0.7128(5)	0.4777(7)	0.9389(6)	32(3)
C(16)	0.6646(5)	0.4536(8)	0.8843(6)	39(3)
C(20)	0.7381(5)	0.2750(9)	0.6585(6)	44(3)
C(21)	0.7940(5)	0.2796(9)	0.6837(6)	38(3)
C(22)	0.8214(6)	0.2110(9)	0.6616(7)	46(4)
C(23)	0.8731(6)	0.2154(10)	0.6791(8)	61(4)
C(24)	0.8974(6)	0.2912(10)	0.7155(7)	53(4)
C(25)	0.8700(6)	0.3608(10)	0.7383(6)	47(4)
C(26)	0.8189(5)	0.3554(9)	0.7230(6)	36(3)
S(31A)	0.1405(2)	0.7684(4)	0.0500(2)	47(2)
O(32)	0.0984(4)	0.8113(7)	-0.0066(5)	66(3)
C(33)	0.1392(6)	0.8264(10)	0.1304(6)	55(4)
C(34)	0.1227(6)	0.6554(11)	0.0691(7)	72(5)
S(31B)	0.0966(5)	0.7671(10)	0.0613(6)	55(5)
O(41)	0.5967(10)	0.4135(13)	0.4759(12)	186(8)
C(42)	0.6262(10)	0.351(2)	0.5233(17)	205(18)
C(43)	0.5990(10)	0.2537(16)	0.5102(14)	136(10)
O(44)	0.5624(8)	0.2694(11)	0.5448(9)	148(7)
C(45)	0.5307(9)	0.332(2)	0.494(2)	260(3)
C(46)	0.5583(11)	0.4291(19)	0.509(2)	208(19)
O(51)	0.4460(6)	0.9520(12)	0.0792(9)	132(6)
C(52)	0.4452(7)	1.0170(16)	0.1287(11)	104(7)
C(53)	0.4893(8)	1.0811(17)	0.1375(19)	188(16)
O(54)	0.5324(6)	1.0247(17)	0.1568(10)	183(9)
C(55)	0.5337(8)	0.9560(15)	0.1047(10)	98(7)
C(56)	0.4905(7)	0.8946(14)	0.1041(15)	124(9)
O(61)	0.4560(10)	0.5250(17)	0.253(2)	138(13)

Table 3: Full bond lengths [Å] and angles [deg] for ReO(bhci).

Re-O(1)	1.693(8)
Re-O(2)	1.975(7)
Re-O(4)	1.982(8)
Re-O(3)	2.003(7)
Re-N(1)	2.146(9)
Re-N(2)	2.163(9)
O(2)-C(2)	1.402(13)
O(3)-C(10)	1.360(12)
O(4)-C(26)	1.375(14)
O(5)-C(6)	1.437(12)
O(6)-C(4)	1.413(14)
N(1)-C(20)	1.501(14)
N(1)-C(1)	1.505(13)
N(2)-C(3)	1.487(13)
N(2)-C(16)	1.493(14)
N(3)-C(5)	1.445(14)
C(1)-C(6)	1.517(16)
C(1)-C(2)	1.526(14)
C(2)-C(3)	1.534(16)
C(3)-C(4)	1.523(16)
C(4)-C(5)	1.523(15)
C(5)-C(6)	1.519(16)
C(10)-C(15)	1.384(16)
C(10)-C(11)	1.397(17)
C(11)-C(12)	1.396(17)
C(12)-C(13)	1.392(19)
C(13)-C(14)	1.347(17)
C(14)-C(15)	1.422(15)
C(15)-C(16)	1.514(16)
C(20)-C(21)	1.496(18)
C(21)-C(22)	1.356(17)
C(21)-C(26)	1.393(16)
C(22)-C(23)	1.379(19)
C(23)-C(24)	1.365(18)
C(24)-C(25)	1.373(18)
C(25)-C(26)	1.365(18)
S(31A)-O(32)	1.508(11)
S(31A)-C(34)	1.738(16)
S(31A)-C(33)	1.748(13)
O(41)-C(46)	1.36(3)
O(41)-C(42)	1.38(3)
C(42)-C(43)	1.56(3)
C(43)-O(44)	1.34(2)
O(44)-C(45)	1.45(3)
C(45)-C(46)	1.57(4)
O(51)-C(52)	1.32(2)
O(51)-C(56)	1.45(2)
C(52)-C(53)	1.49(3)
C(53)-O(54)	1.40(3)
O(54)-C(55)	1.40(2)
C(55)-C(56)	1.47(3)
O(1)-Re-O(2)	159.7(4)
O(1)-Re-O(4)	105.5(4)
O(2)-Re-O(4)	90.0(3)
O(1)-Re-O(3)	106.8(4)
O(2)-Re-O(3)	88.0(3)
O(4)-Re-O(3)	82.3(3)
O(1)-Re-N(1)	88.7(4)
O(2)-Re-N(1)	77.0(3)
O(4)-Re-N(1)	93.5(4)
O(3)-Re-N(1)	164.5(3)
O(1)-Re-N(2)	88.6(4)
O(2)-Re-N(2)	76.4(3)
O(4)-Re-N(2)	166.0(3)
O(3)-Re-N(2)	93.6(3)
N(1)-Re-N(2)	86.8(3)
C(2)-O(2)-Re	108.3(7)
C(10)-O(3)-Re	122.1(7)
C(26)-O(4)-Re	124.0(7)
C(20)-N(1)-C(1)	114.6(9)
C(20)-N(1)-Re	112.2(7)
C(1)-N(1)-Re	110.3(6)
C(3)-N(2)-C(16)	114.2(8)

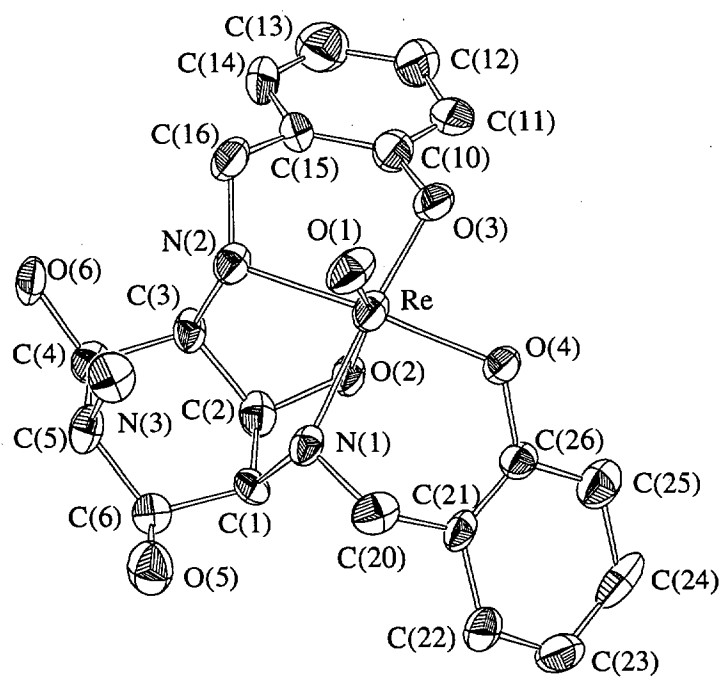
C(3)-N(2)-Re	111.1(7)
C(16)-N(2)-Re	112.6(7)
N(1)-C(1)-C(6)	113.2(9)
N(1)-C(1)-C(2)	107.0(9)
C(6)-C(1)-C(2)	113.1(10)
O(2)-C(2)-C(1)	106.9(9)
O(2)-C(2)-C(3)	108.3(9)
C(1)-C(2)-C(3)	112.8(10)
N(2)-C(3)-C(4)	113.4(10)
N(2)-C(3)-C(2)	106.5(9)
C(4)-C(3)-C(2)	113.7(9)
O(6)-C(4)-C(5)	107.6(10)
O(6)-C(4)-C(3)	110.9(10)
C(5)-C(4)-C(3)	114.2(10)
N(3)-C(5)-C(6)	110.0(10)
N(3)-C(5)-C(4)	110.4(10)
C(6)-C(5)-C(4)	110.3(9)
O(5)-C(6)-C(1)	111.0(9)
O(5)-C(6)-C(5)	108.6(9)
C(1)-C(6)-C(5)	113.8(10)
O(3)-C(10)-C(15)	123.3(10)
O(3)-C(10)-C(11)	116.7(11)
C(15)-C(10)-C(11)	120.0(10)
C(12)-C(11)-C(10)	119.5(13)
C(13)-C(12)-C(11)	120.6(13)
C(14)-C(13)-C(12)	119.5(12)
C(13)-C(14)-C(15)	121.6(13)
C(10)-C(15)-C(14)	118.7(11)
C(10)-C(15)-C(16)	124.2(10)
C(14)-C(15)-C(16)	117.0(11)
N(2)-C(16)-C(15)	112.3(10)
C(21)-C(20)-N(1)	114.7(9)
C(22)-C(21)-C(26)	119.0(12)
C(22)-C(21)-C(20)	118.0(11)
C(26)-C(21)-C(20)	122.7(12)
C(21)-C(22)-C(23)	120.4(12)
C(24)-C(23)-C(22)	120.6(14)
C(23)-C(24)-C(25)	119.2(14)
C(26)-C(25)-C(24)	120.3(13)
C(25)-C(26)-O(4)	116.8(11)
C(25)-C(26)-C(21)	120.3(12)
O(4)-C(26)-C(21)	122.8(12)
O(32)-S(31A)-C(34)	108.5(7)
O(32)-S(31A)-C(33)	105.4(7)
C(34)-S(31A)-C(33)	100.7(7)
C(46)-O(41)-C(42)	102(2)
O(41)-C(42)-C(43)	106(2)
O(44)-C(43)-C(42)	99(2)
C(43)-O(44)-C(45)	100(2)
O(44)-C(45)-C(46)	103(2)
O(41)-C(46)-C(45)	100(3)
C(52)-O(51)-C(56)	108.2(16)
O(51)-C(52)-C(53)	110.9(18)
O(54)-C(53)-C(52)	107(2)
C(55)-O(54)-C(53)	112.0(18)
O(54)-C(55)-C(56)	105.2(18)
O(51)-C(56)-C(55)	106.5(17)

Table 4. Anisotropic displacement parameters ($\text{\AA}^2 \times 10^3$) for $\text{ReO}(\text{bhci})$.
 The anisotropic displacement factor exponent takes the form:
 $-2 \pi^2 [h^2 a^{*2} U_{11} + \dots + 2 h k a^* b^* U_{12}]$

	U11	U22	U33	U23	U13	U12
Re	54(1)	22(1)	23(1)	1(1)	5(1)	-4(1)
O(1)	77(7)	33(5)	29(4)	0(4)	6(4)	8(5)
O(2)	49(6)	23(4)	26(4)	1(3)	2(4)	-8(4)
O(3)	52(6)	30(5)	25(4)	-3(4)	9(4)	-7(4)
O(4)	58(6)	33(5)	26(4)	-2(4)	12(4)	-7(4)
O(5)	34(5)	35(5)	41(4)	-13(4)	1(4)	-12(4)
O(6)	36(6)	54(6)	60(6)	-5(5)	8(5)	-4(5)
N(1)	41(7)	26(6)	27(5)	0(4)	4(5)	0(5)
N(2)	42(6)	24(5)	21(5)	1(4)	-2(4)	-2(4)
N(3)	39(7)	38(6)	57(7)	-1(5)	14(5)	8(5)
C(1)	40(9)	18(6)	31(6)	3(5)	0(6)	-4(5)
C(2)	37(8)	29(7)	34(6)	4(5)	0(6)	-3(6)
C(3)	49(9)	23(6)	28(6)	8(5)	10(6)	-11(6)
C(4)	37(8)	37(7)	36(7)	-2(6)	9(6)	-5(6)
C(5)	27(7)	33(7)	41(7)	-9(6)	-3(6)	0(6)
C(6)	42(8)	29(7)	32(6)	2(5)	5(6)	1(6)
C(10)	45(8)	25(6)	23(6)	2(5)	7(6)	-2(6)
C(11)	62(10)	35(7)	39(7)	-5(6)	7(7)	2(7)
C(12)	70(11)	45(8)	30(7)	-16(6)	-9(7)	13(8)
C(13)	63(10)	41(8)	33(7)	-3(6)	20(7)	3(8)
C(14)	58(9)	23(6)	39(7)	-10(5)	18(7)	-3(6)
C(15)	51(9)	18(6)	24(6)	-3(5)	3(6)	-4(6)
C(16)	49(9)	30(6)	38(7)	1(6)	12(6)	13(6)
C(20)	71(11)	35(7)	26(6)	-7(5)	8(7)	-12(7)
C(21)	46(9)	34(7)	35(7)	-4(6)	13(6)	-17(7)
C(22)	57(10)	33(8)	51(8)	-17(6)	19(7)	-18(7)
C(23)	79(13)	48(9)	63(10)	-5(8)	32(9)	-1(9)
C(24)	47(9)	67(10)	49(8)	-15(8)	17(7)	-15(8)
C(25)	68(11)	42(8)	34(7)	2(6)	21(7)	-14(8)
C(26)	45(9)	31(7)	32(6)	1(6)	8(6)	-1(6)
S(31A)	48(4)	55(3)	37(3)	6(2)	10(2)	-3(3)
O(32)	78(8)	80(8)	40(5)	2(5)	12(5)	8(6)
C(33)	71(11)	51(9)	40(7)	-6(7)	7(7)	0(8)
C(34)	87(13)	71(12)	48(9)	-20(8)	-3(9)	-5(10)
S(31B)	47(10)	72(9)	49(7)	-2(6)	14(6)	1(7)
O(41)	230(2)	124(15)	210(2)	93(15)	68(18)	30(16)
C(42)	130(2)	150(3)	270(4)	120(3)	-100(2)	-90(2)
C(43)	180(3)	93(17)	180(2)	38(17)	120(2)	51(18)
O(44)	230(2)	91(11)	147(14)	38(11)	92(15)	10(13)
C(45)	63(17)	230(4)	450(6)	240(4)	-20(3)	-20(2)
C(46)	180(3)	110(2)	400(5)	80(3)	210(4)	80(2)
O(51)	86(11)	151(14)	147(13)	-84(12)	5(10)	-4(10)
C(52)	51(12)	137(19)	130(18)	-51(16)	35(12)	5(13)
C(53)	50(14)	120(2)	380(5)	-120(3)	20(2)	6(16)
O(54)	91(13)	280(3)	162(16)	-136(17)	3(11)	-25(14)
C(55)	89(16)	111(16)	88(14)	-9(12)	11(12)	46(13)
C(56)	36(11)	87(16)	240(3)	-39(17)	23(14)	-11(11)
O(61)	100(2)	71(17)	290(4)	-10(2)	150(3)	16(16)

Table 5. Hydrogen coordinates ($\times 10^4$) and isotropic displacement parameters ($\text{\AA}^2 \times 10^3$) for $\text{ReO}(\text{bhci})$.

	x	y	z	U(eq)
H(5)	6314	768	6678	57
H(6)	5659	2322	8437	76
H(1)	6769	2915	6944	39
H(2)	6427	3755	7978	37
H(3A)	6210	2759	6846	53
H(3B)	5786	2942	7208	53
H(1A)	7434	1645	7618	37
H(2A)	7410	1911	8785	42
H(3)	6883	2899	9185	40
H(4)	6393	1640	8769	44
H(5)	5952	1369	7606	42
H(6)	6762	824	7804	42
H(11)	8278	5525	9632	56
H(12)	8223	5513	10837	62
H(13)	7500	4963	11144	53
H(14)	6819	4553	10257	47
H(16A)	6395	4325	9099	46
H(16B)	6517	5105	8569	46
H(20A)	7289	2143	6345	53
H(20B)	7270	3246	6225	53
H(22)	8050	1601	6341	55
H(23)	8918	1657	6658	73
H(24)	9326	2956	7248	64
H(25)	8865	4123	7646	56
H(33A)	1485	8920	1270	82
H(33B)	1627	7966	1703	82
H(33C)	1056	8230	1386	82
H(34A)	1221	6146	280	108
H(34B)	896	6575	789	108
H(34C)	1466	6310	1111	108
H(42A)	6295	3720	5733	247
H(42B)	6597	3472	5138	247
H(43A)	5856	2411	4587	163
H(43B)	6211	2019	5321	163
H(45A)	4969	3351	5022	313
H(45B)	5283	3108	4439	313
H(46A)	5369	4814	4860	249
H(46B)	5705	4412	5603	249
H(52A)	4456	9863	1750	125
H(52B)	4142	10538	1145	125
H(53A)	4886	11144	922	226
H(53B)	4890	11278	1753	226
H(55A)	5651	9201	1176	117
H(55B)	5309	9850	572	117
H(56A)	4902	8410	715	148
H(56B)	4919	8703	1527	148



ReO(bhci)

Table 1. Crystal data and structure refinement for ReO(bhci-glu).

Identification code	ReO(bhci-glu)
Empirical formula	C ₂₅ H ₃₀ N ₃ O ₉ Re
Formula weight	702.72
Temperature	293(2) K
Wavelength / Radiation	0.71073 Å / MoK α
Crystal system	P2 ₁ /c
Space group	monoclinic
Unit cell dimensions	a = 13.056(3) Å alpha = 90 deg. b = 10.180(1) Å beta = 98.205(9) deg. c = 22.378(5) Å gamma = 90 deg.
Volume	2943.8(10) Å ³
Z	4
Density (calculated)	1.586 Mg/m ³
Absorption coefficient	4.179 mm ⁻¹
F(000)	1392
Crystal size	0.2 x 0.1 x 0.1 mm
Theta range for data collection	1.58 to 24.98 deg.
Index ranges	-15 ≤ h ≤ 15, 0 ≤ k ≤ 12, 0 ≤ l ≤ 26
Reflections collected	5378
Independent reflections	5171 [R(int) = 0.0637]
Refinement method	Full-matrix least-squares on F ²
Data / restraints / parameters	5169 / 0 / 322
Goodness-of-fit on F ²	1.036
Final R indices [I > 2sigma(I)]	R ₁ = 0.0674, wR ₂ = 0.1772
R indices (all data)	R ₁ = 0.2326, wR ₂ = 0.2555
Largest diff. peak and hole	1.853 and -0.909 e.Å ⁻³

Table 2. Atomic coordinates ($\times 10^4$) and equivalent isotropic displacement parameters ($\text{\AA}^2 \times 10^3$) for $\text{ReO}(\text{bhci-glu})$. $U(\text{eq})$ is defined as one third of the trace of the orthogonalized U_{ij} tensor.

	x	y	z	U(eq)
Re(1)	514(1)	-1(2)	1929(1)	41(1)
N(1)	-144(17)	412(19)	2736(9)	50(6)
N(2)	-941(16)	194(30)	1380(9)	55(7)
N(3)	-3865(16)	1354(22)	2124(12)	70(8)
O(1)	704(15)	1616(17)	1766(8)	57(5)
O(2)	-256(12)	-1593(16)	2115(8)	44(4)
O(3)	1094(12)	-1009(18)	1295(8)	49(5)
O(4)	1863(14)	-588(17)	2436(9)	53(5)
O(5)	-1677(13)	1978(19)	2147(8)	48(5)
O(6)	-3129(18)	635(31)	1077(12)	116(11)
O(7)	-5135(13)	-93(31)	1948(11)	94(7)
C(1)	-1235(16)	-134(34)	2648(10)	40(7)
C(2)	-1235(18)	-1209(24)	2177(12)	42(6)
C(3)	-1723(19)	-742(29)	1604(14)	58(8)
C(4)	-2811(23)	-122(53)	1602(17)	110(17)
C(5)	-3046(21)	366(28)	2168(12)	59(10)
C(6)	-2010(18)	955(27)	2516(14)	52(7)
C(10)	537(22)	-1595(28)	823(12)	53(7)
C(11)	917(21)	-2764(26)	585(14)	53(7)
C(12)	377(28)	-3349(41)	73(16)	73(14)
C(13)	-582(35)	-2944(36)	-196(16)	81(13)
C(14)	-957(23)	-1774(29)	56(12)	59(8)
C(15)	-386(24)	-1176(26)	560(12)	53(7)
C(16)	-914(23)	106(45)	720(10)	73(9)
C(20)	463(15)	-46(42)	3295(10)	52(6)
C(21)	1578(19)	328(20)	3364(14)	48(8)
C(22)	2000(26)	917(32)	3905(14)	67(9)
C(23)	3025(38)	1083(43)	4047(18)	106(15)
C(24)	3673(33)	841(41)	3597(19)	101(14)
C(25)	3269(24)	278(26)	3096(16)	68(10)
C(26)	2202(17)	-7(44)	2976(11)	53(6)
C(35)	-4816(19)	1075(31)	2003(14)	58(8)
C(36)	-5551(30)	2207(42)	2075(18)	107(13)
C(37)	-6626(29)	1966(40)	1602(17)	96(12)
C(38)	-6660(50)	2696(75)	1084(30)	189(26)
C(39A)	-6527(50)	4011(68)	905(29)	61(16)
O(39A)	-6961(45)	4767(76)	1251(26)	116(21)
O(39B)	-6086(44)	4465(55)	643(25)	109(19)
C(39B)	-7097(88)	4151(122)	1175(52)	45(29)
C(39C)	-6207(79)	3166(111)	591(48)	48(26)

Table 3. Bond lengths [Å] and angles [deg] for ReO(bhci-glu).

Re(1)-O(1)	1.71(2)
Re(1)-O(2)	1.98(2)
Re(1)-O(3)	1.99(2)
Re(1)-O(4)	2.04(2)
Re(1)-N(2)	2.12(2)
Re(1)-N(1)	2.15(2)
N(1)-C(20)	1.46(3)
N(1)-C(1)	1.52(3)
N(2)-C(16)	1.49(3)
N(2)-C(3)	1.53(3)
N(3)-C(35)	1.26(3)
N(3)-C(5)	1.46(3)
O(2)-C(2)	1.36(3)
O(3)-C(10)	1.33(3)
O(4)-C(26)	1.36(3)
O(5)-C(6)	1.43(3)
O(6)-C(4)	1.42(5)
O(7)-C(35)	1.26(4)
C(1)-C(6)	1.50(3)
C(1)-C(2)	1.52(4)
C(2)-C(3)	1.43(4)
C(3)-C(4)	1.55(5)
C(4)-C(5)	1.43(4)
C(5)-C(6)	1.58(4)
C(10)-C(15)	1.33(4)
C(10)-C(11)	1.42(4)
C(11)-C(12)	1.39(4)
C(12)-C(13)	1.37(5)
C(13)-C(14)	1.43(4)
C(14)-C(15)	1.40(3)
C(15)-C(16)	1.54(4)
C(20)-C(21)	1.49(3)
C(21)-C(26)	1.32(4)
C(21)-C(22)	1.39(4)
C(22)-C(23)	1.34(5)
C(23)-C(24)	1.43(5)
C(24)-C(25)	1.30(4)
C(25)-C(26)	1.41(4)
C(35)-C(36)	1.52(4)
C(36)-C(37)	1.65(5)
C(37)-C(38)	1.37(7)
C(38)-C(39C)	1.41(11)
C(38)-C(39A)	1.42(9)
C(38)-C(39B)	1.61(13)
C(39A)-O(39B)	0.99(7)
C(39A)-O(39A)	1.28(8)
O(1)-Re(1)-O(2)	157.5(8)
O(1)-Re(1)-O(3)	105.2(8)
O(2)-Re(1)-O(3)	89.1(7)
O(1)-Re(1)-O(4)	105.1(8)
O(2)-Re(1)-O(4)	93.8(7)
O(3)-Re(1)-O(4)	81.9(7)
O(1)-Re(1)-N(2)	86.1(10)
O(2)-Re(1)-N(2)	76.0(9)
O(3)-Re(1)-N(2)	92.0(8)
O(4)-Re(1)-N(2)	168.4(10)
O(1)-Re(1)-N(1)	94.5(8)
O(2)-Re(1)-N(1)	73.0(7)
O(3)-Re(1)-N(1)	160.0(7)
O(4)-Re(1)-N(1)	90.4(8)
N(2)-Re(1)-N(1)	92.1(8)
C(20)-N(1)-C(1)	112(2)
C(20)-N(1)-Re(1)	115(2)
C(1)-N(1)-Re(1)	107.5(14)
C(16)-N(2)-C(3)	114(3)
C(16)-N(2)-Re(1)	115(2)
C(3)-N(2)-Re(1)	110(2)
C(35)-N(3)-C(5)	123(2)
C(2)-O(2)-Re(1)	107.3(14)
C(10)-O(3)-Re(1)	125(2)
C(26)-O(4)-Re(1)	121(2)
C(6)-C(1)-N(1)	110(3)
C(6)-C(1)-C(2)	118(2)
N(1)-C(1)-C(2)	105(2)

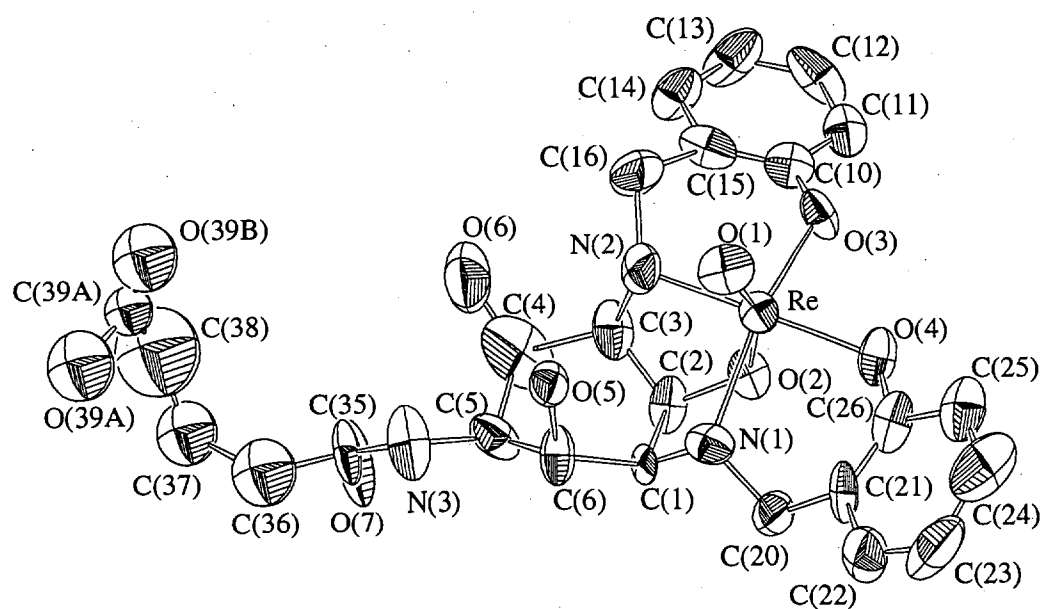
O(2)-C(2)-C(3)	108(2)
O(2)-C(2)-C(1)	112(2)
C(3)-C(2)-C(1)	110(2)
C(2)-C(3)-N(2)	106(2)
C(2)-C(3)-C(4)	115(3)
N(2)-C(3)-C(4)	114(3)
O(6)-C(4)-C(5)	118(4)
O(6)-C(4)-C(3)	113(3)
C(5)-C(4)-C(3)	117(3)
C(4)-C(5)-N(3)	115(3)
C(4)-C(5)-C(6)	107(2)
N(3)-C(5)-C(6)	110(2)
O(5)-C(6)-C(1)	113(2)
O(5)-C(6)-C(5)	108(2)
C(1)-C(6)-C(5)	109(2)
O(3)-C(10)-C(15)	124(3)
O(3)-C(10)-C(11)	119(3)
C(15)-C(10)-C(11)	116(3)
C(12)-C(11)-C(10)	120(3)
C(13)-C(12)-C(11)	124(4)
C(12)-C(13)-C(14)	115(3)
C(15)-C(14)-C(13)	120(3)
C(10)-C(15)-C(14)	124(3)
C(10)-C(15)-C(16)	125(3)
C(14)-C(15)-C(16)	111(3)
N(2)-C(16)-C(15)	111(3)
N(1)-C(20)-C(21)	114(2)
C(26)-C(21)-C(22)	119(3)
C(26)-C(21)-C(20)	123(3)
C(22)-C(21)-C(20)	117(3)
C(23)-C(22)-C(21)	121(3)
C(22)-C(23)-C(24)	119(4)
C(25)-C(24)-C(23)	119(4)
C(24)-C(25)-C(26)	121(4)
C(21)-C(26)-O(4)	123(2)
C(21)-C(26)-C(25)	121(3)
O(4)-C(26)-C(25)	117(3)
O(7)-C(35)-N(3)	122(3)
O(7)-C(35)-C(36)	121(3)
N(3)-C(35)-C(36)	115(3)
C(35)-C(36)-C(37)	108(3)
C(38)-C(37)-C(36)	113(4)
C(37)-C(38)-C(39C)	152(8)
C(37)-C(38)-C(39A)	140(6)
C(37)-C(38)-C(39B)	111(6)
O(39B)-C(39A)-O(39A)	115(8)
O(39B)-C(39A)-C(38)	135(8)
O(39A)-C(39A)-C(38)	108(7)

Table 4. Anisotropic displacement parameters ($\text{\AA}^2 \times 10^3$) for $\text{ReO}(\text{bhci-glu})$.
 The anisotropic displacement factor exponent takes the form:
 $-2 \pi^2 [h^2 a^{*2} U_{11} + \dots + 2 h k a^* b^* U_{12}]$

	U11	U22	U33	U23	U13	U12
Re(1)	42(1)	33(1)	48(1)	2(1)	10(1)	-1(1)
N(1)	76(15)	28(14)	51(13)	11(9)	28(12)	6(10)
N(2)	63(13)	46(21)	54(12)	-6(14)	4(9)	26(14)
N(3)	43(14)	29(13)	138(24)	-4(15)	12(14)	-10(11)
O(1)	85(14)	29(10)	55(12)	-9(9)	8(10)	-12(10)
O(2)	41(10)	31(9)	62(11)	-10(9)	9(8)	-8(8)
O(3)	34(9)	53(11)	60(12)	-25(10)	13(8)	2(9)
O(4)	45(10)	38(10)	72(13)	0(9)	-6(9)	3(8)
O(5)	38(10)	50(12)	58(11)	11(10)	9(8)	-2(9)
O(6)	63(15)	175(33)	105(20)	-15(20)	-2(14)	37(16)
O(7)	34(9)	68(16)	181(22)	-18(23)	18(11)	5(17)
C(1)	42(12)	31(17)	52(12)	24(17)	20(10)	29(18)
C(2)	32(14)	23(14)	68(18)	-13(13)	-12(13)	-11(11)
C(3)	29(14)	56(18)	87(22)	0(17)	4(14)	-14(13)
C(4)	58(19)	153(45)	120(29)	-69(37)	16(19)	-76(30)
C(5)	59(16)	72(30)	52(15)	-22(14)	24(13)	-2(14)
C(6)	28(13)	41(16)	82(21)	-5(15)	-5(13)	-3(12)
C(10)	61(18)	46(17)	54(17)	-2(14)	13(14)	-11(15)
C(11)	47(16)	39(16)	77(22)	13(16)	17(15)	-7(13)
C(12)	62(21)	80(29)	84(27)	-69(22)	31(21)	-24(17)
C(13)	129(37)	57(26)	47(22)	-20(20)	-26(24)	22(24)
C(14)	70(20)	58(19)	42(16)	-16(14)	-19(14)	-2(16)
C(15)	78(21)	31(15)	57(18)	-12(13)	31(16)	-15(15)
C(16)	85(19)	94(25)	35(13)	-8(28)	-4(12)	9(31)
C(20)	42(12)	63(15)	51(13)	15(29)	6(10)	0(28)
C(21)	37(13)	21(20)	83(19)	-14(12)	-3(13)	17(10)
C(22)	69(21)	68(22)	64(21)	-20(18)	4(17)	18(18)
C(23)	138(41)	98(32)	71(27)	14(24)	-28(26)	40(30)
C(24)	97(29)	102(31)	88(28)	7(25)	-39(24)	-40(26)
C(25)	65(18)	36(28)	101(23)	20(17)	2(17)	1(14)
C(26)	49(13)	26(10)	78(17)	-2(31)	-15(12)	-4(29)
C(35)	18(12)	46(17)	112(25)	10(17)	16(14)	3(13)

Table 5. Hydrogen coordinates ($\times 10^4$) and isotropic displacement parameters ($\text{\AA}^2 \times 10^3$) for $\text{ReO}(\text{bhci-glu})$.

	x	y	z	U(eq)
H(1)	-180(17)	1300(19)	2779(9)	65
H(3)	-1182(16)	999(30)	1466(9)	71
H(5)	-3704(16)	2163(22)	2077(12)	91
H(4)	-2699(18)	1224(31)	1090(12)	151
H(6)	-1108(18)	1808(31)	2063(12)	151
H(1A)	-1328(16)	-565(34)	3025(10)	52
H(2)	-1616(18)	-1972(24)	2295(12)	55
H(3A)	-1766(19)	-1510(29)	1338(14)	75
H(4A)	-3281(23)	-865(53)	1498(17)	143
H(5A)	-3262(21)	-378(28)	2397(12)	77
H(6A)	-2175(18)	1315(27)	2894(14)	67
H(11)	1521(21)	-3154(26)	776(14)	69
H(12)	666(28)	-4077(41)	-89(16)	96
H(13)	-937(35)	-3343(36)	-537(16)	106
H(14)	-1596(23)	-1416(29)	-97(12)	77
H(16A)	-545(23)	872(45)	607(10)	94
H(16B)	-1616(23)	127(45)	507(10)	94
H(20A)	417(15)	-997(42)	3276(10)	68
H(20B)	173(15)	245(42)	3645(10)	68
H(22)	1556(26)	1128(32)	4177(14)	88
H(23)	3284(38)	1577(43)	4384(18)	138
H(24)	4382(33)	883(41)	3721(19)	131
H(25)	3708(24)	206(26)	2808(16)	89
H(36A)	-5230(30)	3025(42)	1980(18)	139
H(36B)	-5704(30)	2253(42)	2486(18)	139
H(37A)	-6660(29)	1035(40)	1509(17)	125
H(37B)	-7209(29)	2167(40)	1811(17)	125


$$\text{ReO}(\text{bhci-glu})$$

Conformational Transition of Restriction Endonuclease *MvaI*–Substrate Complex under the Influence of Mg^{2+} Probed by DNA–Protein Cross-Linking Studies

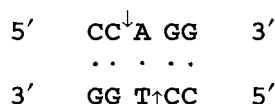
G. Y. Sheflyan,[†] E. A. Kubareva, E. S. Gromova, and Z. A. Shabarova*

A.N. Belozersky Institute of Physical and Chemical Biology, Department of Chemistry, M.V. Lomonosov Moscow State University, 119899 Moscow, Russia. Received February 10, 1998; Revised Manuscript Received May 22, 1998

The method of protein affinity modification by DNA analogues was used to study the characteristic features of restriction endonuclease *MvaI* interaction with DNA. Oligonucleotide duplexes containing a monosubstituted pyrophosphate internucleotide bond were used for cross-linking to the enzyme. The conditions of the reaction of *MvaI* endonuclease with these reagents were investigated. On the basis of data obtained, the model of successive inclusion of two Mg^{2+} ions into *MvaI* endonuclease–substrate complex was proposed and confirmed by the kinetic scheme of the process.

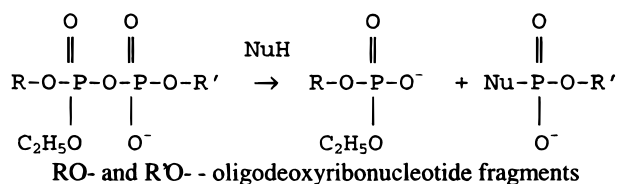
INTRODUCTION

Restriction endonuclease *MvaI* recognizes the DNA sequence



and cleaves it, as shown by the arrows, Mg^{2+} being the cofactor of this reaction. The interaction of this enzyme with DNA was probed via the use of modified DNA substrates (1). We use the method of DNA–protein cross-linking to reveal the characteristic features of substrate scission by R.*MvaI*.¹

In our laboratory, the novel method of affinity modification of proteins by modified DNA was developed (2). DNA analogues contain a monosubstituted pyrophosphate internucleotide bond in the definite position of sugar–phosphate backbone. This method was successfully applied for the cross-linking of restriction-modification enzymes *EcoRI* and *RsrI* (3) and restriction endonucleases *EcoRII* (4, 5), *MvaI* (5), and *SsoII* (6). Cross-linking follows the scheme:



* To whom correspondence should be addressed. Phone (095) 939-5411. Fax (095) 939-3181. E-mail zoeshab@nuclacid.genebee.msu.su.

[†] Present address: 1028 Pharmacy, College of Pharmacy, University of Michigan, Ann Arbor, MI 48109-1065.

¹ Abbreviations: R.*MvaI*, restriction endonuclease *MvaI*; NuH, nucleophilic agent; MeIm, *N*-methylimidazole; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

The aim of this paper is to extend the application of this cross-linking technique toward the probing of conformational changes of enzyme in the enzyme–substrate complex on the example of restriction endonuclease *MvaI*.

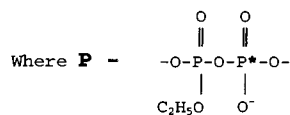
MATERIALS AND METHODS

R.*MvaI* was purchased from NPO "Fermentas" (Lithuania). Its preparation had the specific activity of 250 units/ μL and concentration of 46 $\mu\text{g}/\text{mL}$. Oligodeoxyribonucleotides were synthesized by T. S. Oretskaya, E. M. Volkov, and E. A. Romanova as described in ref 7. DNA duplexes with a monosubstituted pyrophosphate internucleotide bond for cross-linking to R.*MvaI* were synthesized as described in ref 2. DNA duplexes were ^{32}P -labeled by T4 polynucleotide kinase. ^{32}P label was at the disubstituted phosphate group of the modified internucleotide linkage within the DNA duplexes I and II modified strands (see formulas) and at the 5'-end of the nonmodified duplex IV unless otherwise stated. Cross-linking of R.*MvaI* (138 ng, concentration per monomer 2.5×10^{-7} M) to ^{32}P -labeled DNAs (100 000 cpm, concentration per duplex, C_D , 1.8×10^{-7} M) in 20 μL of buffer X (10 mM Tris-HCl, pH 8.5, 150 mM NaCl, 1 mM dithiotreitol, and 0.1 mg/mL albumin) or buffer Y (10 mM MeIm-HCl, pH 8.5, 150 mM NaCl, 1 mM dithiotreitol, and 0.1 mg/mL albumin) was performed at 37 °C as described in ref 5. Concentrations of Mg^{2+} ions have been varied. Incubation time was 18 h. Cross-linking reaction was monitored by SDS–PAGE (5, 6). The enzymatic hydrolysis was performed under the same conditions except that radioactivity of DNA per reaction was 20 000 cpm, $C_D = 3.5 \times 10^{-7}$ M, incubation time varied from 1 to 18 h, and the reaction was followed by 20% PAGE containing 7 M urea.

RESULTS AND DISCUSSION

We used the following modified DNA duplexes as R.*MvaI* substrates or their analogues:

Duplex	#	Strand
5' ACCTACC- P -TGGTGGT 3'	I	Ia
3' TGGATGG---ACCACCA 5'		Ib
5' GTCACCT- P -CCTGGATCCG 3'	II	IIa
3' CAGTGA---GGACCTAGGCA 5'		IIb
5' GCCAACCTGGCTCT 3'	III	IIIa
3' CGGTTGGACCGAGA 3'		IIIb
5' ACCTACCTGGTGGT 3'	IV	IVa
3' TGGATGGACCA 5'		IVb



* - location of ^{32}P -label

In duplex I, the modified group was located in the middle of *R.MvaI* recognition site and coincided with the scissile bond. In duplex II, we probed protein contacts with sugar-phosphate backbone on the border between the flanking sequence and the recognition site (5). Nonmodified duplexes III and IV were used in control experiments.

The monosubstituted pyrophosphate internucleotide bond in DNA duplexes does not interfere with their binding to the enzyme (5). Previously, we demonstrated (5) that the cross-linking reaction of *R.MvaI* to duplex I required Mg^{2+} ions in the concentration of $37.5 \mu\text{M}$. At higher as well as lower concentrations of Mg^{2+} , modification of *R.MvaI* proceeded with low yield. The increase of nucleophilicity of reaction buffer replacing Tris-HCl (buffer X) by MeIm (buffer Y) did not result in the increase of a cross-linking product unlike the data previously observed for *R.EcoRII* (4). The cross-linking extent of duplex II to *R.MvaI* did not exceed 2% in the absence of Mg^{2+} ions nor in their presence till the Mg^{2+} concentration $150 \mu\text{M}$ (5).

The specificity of covalent attachment of duplex I to *R.MvaI* was established through the standard method of competitive inhibition of the affinity modification reaction by the increasing amounts of duplex III (Figure 1). This reaction proved to be specific. Therefore, it can reflect the structure of DNA-protein interface and its changes during the course of enzymatic hydrolysis.

To explain the results of *R.MvaI* affinity modification by duplexes I and II we checked (i) the ability of *R.MvaI* to hydrolyze duplex I and II in comparison with duplex IV; (ii) *R.MvaI* activity dependence on the Mg^{2+} concentration on the example of duplex IV; and (iii) *R.MvaI* activity dependence on the buffer nucleophilicity.

The results of hydrolysis of duplexes I, II and IV by *R.MvaI* are adduced in Table 1. Hydrolysis of duplex I in buffer containing 10 mM MgCl_2 results in the number of products corresponding to the weak enzymatic hydrolysis of intact strand Ib as well as the monosubstituted pyrophosphate bond degradation. Since the active group coincides with the *R.MvaI* scissile bond, it is difficult to explain the degradation of the modified strand Ia by either enzymatic or chemical cleavage. However, in ref 1, it was shown that *R.MvaI* cleaves only the intact strand of the DNA duplex containing a pyrophosphate group instead of the scissile phosphodiester bond. This allows us to suggest that the enzymatic hydrolysis of a

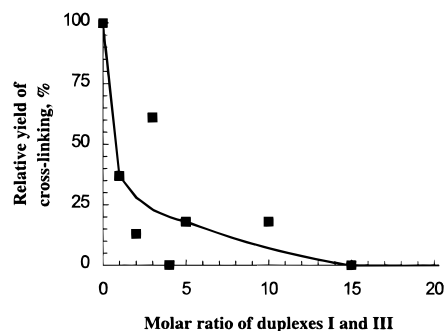


Figure 1. The dependence of relative cross-linking yields of *R.MvaI* to duplex I on the C_D ratio of duplexes I and III. C_D of duplex I is $1.8 \times 10^{-7} \text{ M}$. Cross-linking yield in the absence of duplex III was referred to as 100%.

Table 1. Cleavage of Duplexes I, II, and IV by *R.MvaI* at 37°C in Buffer X, Containing 10 mM MgCl_2 , for 1 h

duplex	strand	enzymatic cleavage, %
I	a	— ^a
	b	8
II	a	9; 51 ^b
	b	84
IV	a + b	88

^a Modified bond coincides with the scissile bond. ^b For 18 h.

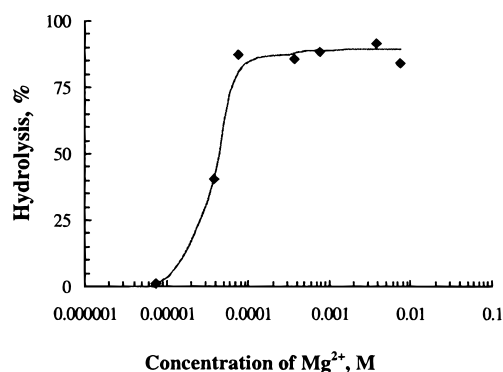


Figure 2. The dependence of duplex IV hydrolysis by *R.MvaI* on Mg^{2+} concentration. C_D of duplex IV is $3.5 \times 10^{-7} \text{ M}$. The Mg^{2+} concentration is adduced in the logarithmic scale.

modified strand of duplex I does not occur. It was also demonstrated in ref 8 that *R.MvaI* is capable of cutting certain single-stranded oligodeoxyribonucleotides, for example, strand IVa, having the same sequence as Ia, but not strand IVb (Ib). This eliminates the possibility that the weak cleavage of strand Ib in duplex I is due to the formation of an incomplete duplex by strand Ib alone.

When monosubstituted pyrophosphate internucleotide bond is located on the border between the recognition site and the flanking nucleotide sequence (duplex II), the hydrolysis of modified strand IIa at the Mg^{2+} concentration equal to 10 mM occurs in a time-dependent manner. The intact strand IIb is efficiently cleaved by the enzyme (Table 1).

At low Mg^{2+} concentrations (till $7.5 \mu\text{M}$), *R.MvaI* practically does not cleave duplex IV (Figure 2). At Mg^{2+} concentration equal to $37.5 \mu\text{M}$, the hydrolysis extent of duplex IV is equal to 20%. The following addition of Mg^{2+} ions causes the fast growth of enzymatic activity. At the Mg^{2+} concentration $75 \mu\text{M}$, duplex IV is completely digested by *R.MvaI*. The efficiency of hydrolysis does not depend on incubation time (1 and 18 h, data not shown). We demonstrated that Tris-HCl replacement for MeIm-HCl in the reaction buffer did not decrease *R.MvaI* enzymatic activity in cleaving duplex IV (data not

shown). The weak enzymatic hydrolysis of only strand Ib in duplex I made it impossible to investigate the influence of Mg^{2+} on the enzymatic cleavage of this duplex by R.MvaI. All the reactions with duplex IV, having the identical sequence as duplex I, were performed under the same conditions as cross-linking experiments, with C_D of duplex IV being in the same range as the C_D of the cross-linking reagent (350 nM for duplex IV and 180 nM for duplex I).

The necessity of the presence of Mg^{2+} in low concentration for the R.MvaI covalent attachment to duplex I and the hydrolysis studies is believed to show the following features of DNA recognition by R.MvaI:

(1) In the absence of Mg^{2+} ions, there are no nucleophilic amino acid residues in the proximity to the scissile bond modified in duplex I.

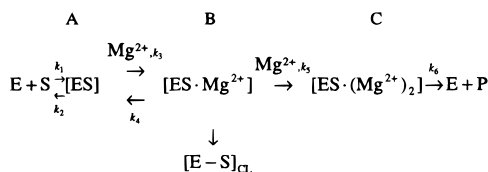
(2) The introduction of Mg^{2+} in an enzyme–substrate complex results in a conformational change of the enzyme. As a consequence of this transition, a nucleophilic amino acid residue is moved toward the scissile bond. This is demonstrated by the increase of the cross-linking extent of the enzyme to duplex I in these conditions.

(3) The Mg^{2+} concentration equal to $37.5 \mu M$ is not sufficient for the effective substrate hydrolysis (Figure 2), though Mg^{2+} ion is already incorporated into the enzyme–substrate complex as revealed by cross-linking studies. Hence, more than one Mg^{2+} ion is necessary for the R.MvaI catalytic act.

(4) With the further increase of Mg^{2+} concentration, the catalytically active complex is formed and the substrate is cleaved. In the case where duplex I is the substrate, the covalent attachment is inhibited due to enzymatic hydrolysis. This explains the reduction in the cross-linking extent of duplex I to R.MvaI at Mg^{2+} concentrations above $150 \mu M$.

(5) The ability of duplex II to modify R.MvaI demonstrates the contact of a nucleophilic group with the phosphate group adjacent to 5'-terminus of recognition sequence. The absence of the dependence of the cross-linking yield of duplex II to R.MvaI on Mg^{2+} concentration $150 \mu M$ confirms that the enzyme conformational change has a local character and occurs only in the surroundings of the scissile bond.

Thus, R.MvaI requires more than one Mg^{2+} ion for catalysis. The same feature was supposed in ref 9 for EcoRV restriction endonuclease and confirmed in ref 10. One can conclude that these enzymes share the same mode of action. From the data obtained, as well as taking into account (10), we proposed the following scheme of the enzymatic reaction together with the parallel cross-linking process:



where E is the enzyme, S is the general substrate or duplex I in the case of covalent attachment, and $[E-S]_{CL}$ is the product of attachment of R.MvaI to duplex I.

On the basis of this scheme, we calculated the theoretical dependence of R.MvaI cross-linking to duplex I on Mg^{2+} concentration and compared it with the experimental data.

To simplify the calculation, we assumed that the formation of the complex with two Mg^{2+} ions is irreversible since it undergoes quick substrate hydrolysis and

dissociation into the enzyme and products. If only complex B with one Mg^{2+} ion can be transformed into the cross-linked product, the cross-linking yield is proportional to the complex B concentration in the reaction mixture. Complex C can also retain the conformation favorable for cross-linking, but the degradation of this complex through substrate cleavage proceeds faster than the affinity modification of the enzyme. Using the assumption of steady-state concentrations of intermediate compounds as well as material balance for enzyme concentration, we get the system of equations:

$$\frac{d[A]}{dt} = k_1[E][S] - k_2[A] - k_3[A][Mg^{2+}] + k_4[B] = 0$$

$$\frac{d[B]}{dt} = k_3[A][Mg^{2+}] - k_5[B][Mg^{2+}] - k_4[B] = 0$$

$$\frac{d[C]}{dt} = k_5[B][Mg^{2+}] - k_6[C] = 0$$

$$[E] = [E_0] - [A] - [B] - [C]$$

To calculate the concentration of B we use the following assumptions: (1) the consumption of the complex B in covalent attachment is too small to influence its concentration and (2) analysis of the Mg^{2+} binding was performed in terms of the concentration of free Mg^{2+} ions. The concentration of free Mg^{2+} ions was obtained by the subtraction of the phosphate group concentration from the initial concentration of Mg^{2+} . We used an assumption that one phosphate group binds one cofactor ion. The concentration of the phosphate groups in the cross-linking reactions was equal to $4.5 \mu M$.

Since the concentrations of E and S are fixed, the concentration of B is adduced as a function of Mg^{2+} concentration. The final equation is

$$[B] = \frac{l[Mg^{2+}]}{m[Mg^{2+}]^2 + n[Mg^{2+}] + p}$$

where

$$l = k_1 k_3 k_6 [S] [E_0]$$

$$m = k_1 k_3 k_5 [S] + k_3 k_5 k_6$$

$$n = k_1 k_5 k_6 [S] + k_1 k_3 k_6 [S] + k_2 k_5 k_6$$

$$p = k_1 k_4 k_6 + k_2 k_4 k_6$$

Since we suppose that γ (the extent of affinity modification) is proportional to a concentration of B with the coefficient q determining the probability of formation of covalent conjugate, we get

$$\gamma = q \frac{l[Mg^{2+}]}{m[Mg^{2+}]^2 + n[Mg^{2+}] + p}$$

We picked the coefficients via computer simulations. The function obtained is in a good agreement with the experimental data (Figure 3).

The requirement of two Mg^{2+} ions for successful cleavage of substrate demonstrates the similarity of mechanisms of R.MvaI and EcoRV restriction enzyme, the later being extensively studied by different methods including kinetics, X-ray structure analysis, and stop flow technique. As proposed in ref 11 on the basis of the

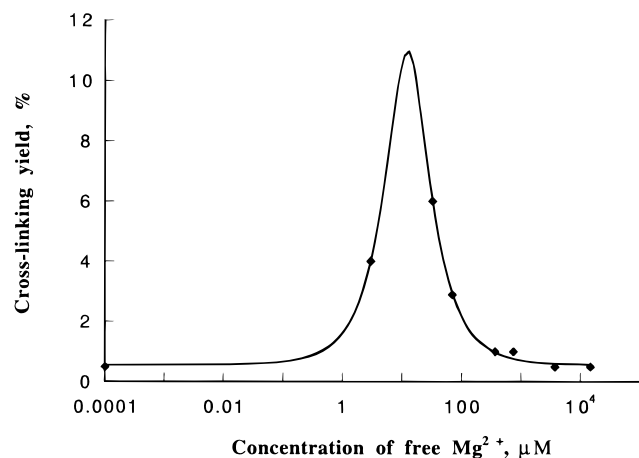


Figure 3. Theoretical dependence of covalent attachment of R.MvaI to duplex I on the Mg^{2+} concentration (solid line) in comparison with the experimental data (◆) (5).

kinetic order of enzymatic hydrolysis on Mg^{2+} concentration, R.EcoRV involves only one ion in catalysis. But as mentioned in ref 12, the kinetic equation for R.EcoRV would have the second order on Mg^{2+} concentration only in the case when both ions are attached with high cooperativity in one action. By kinetic means, it is possible to determine only the order of the slowest step of the process. One can conclude the possibility of successive binding of Mg^{2+} ions to sites with different affinity to cofactor.

In ref 12, on the basis of the previously obtained X-ray structure of the R.EcoRV complex with substrate, two Mg^{2+} -binding sites formed by carboxyl-groups of Asp90, Asp74, and Glu45 residues were proposed. Both Asp residues originate from consensus sequence common to a number of restriction endonucleases Pro-Asp...Asp-X-Lys, where X is the hydrophobic amino acid residue. Glu residue is in close contact with scissile phosphodiester bond only in the case of R.EcoRV compared to R.EcoRI, for which one Mg^{2+} ion was proved to be sufficient for DNA cleavage.

Modeling the crystal structure of R.EcoRV with substrate and one cofactor ion also allowed a confirmation of the binding site and the catalytic significance of the second Mg^{2+} ion (13).

Though the participation of two Mg^{2+} ions does not raise any doubts now, the location of the second cofactor binding site is still under discussion. EcoRV triple mutant Glu45Ala/Asp74Ala/Asp90Ala was obtained in ref 14, which retained the ability to discriminate cognate DNA only in the presence of Mg^{2+} being catalytically inactive. By cleavage experiments with phosphorothioate bond containing substrates as well as site-mutagenesis experiments, it was revealed that the second Mg^{2+} -binding site is probably formed by Tyr 129 residue and a phosphate group between dG and dA residues of the EcoRV recognition site (14). But Asp residues from the consensus sequence are still critical for cofactor and substrate binding.

In the R.MvaI amino acid sequence, the motif ⁴⁹Pro-⁵⁰Asp...¹¹⁷Asp-¹¹⁸Phe-¹¹⁹Lys exists, which can play an analogous role in Mg^{2+} binding.

Complex B can be a covalent one, being an intermediate compound during enzymatic hydrolysis. To test this possibility we used duplex IV containing the ^{32}P -label within the scissile bond. The mixtures of R.MvaI and duplex IV were incubated under various Mg^{2+} concentrations during 2.5 or 18 h and analyzed by SDS-PAGE.

No radioactive bands with mobility less than that of initial DNA were detected. So, we did not manage to reveal the covalent intermediate between R.MvaI and duplex IV in the course of enzymatic hydrolysis. The catalysis of phosphodiester bond hydrolysis possibly occurs without the formation of enzyme-DNA linkage, which is in accordance with the data of ref 15. As computer simulations showed, the appropriate coordination of cofactor ions could cause the catalysis of internucleotide bond cleavage (16).

The possibility of conformational changes for restriction endonucleases in the complexes with substrates is now discussed for a number of enzymes. By X-ray analysis, the existence of structural disturbances of substrate is revealed on the example of R.EcoRI and R.EcoRV complexes with DNA (17). The further conformational changes of these complexes are suggested under the influence of Mg^{2+} ions. It is particularly essential for R.EcoRV, since only in the presence of Mg^{2+} , the enzyme can distinguish its recognition site in DNAs (18). Analogous data were recently obtained for R.TaqI and R.PaeR7 (19). Retardation assays of R.EcoRV-substrate complexes demonstrated that Mg^{2+} ions introduced into these complexes form contacts with both protein and DNA. Changes in DNA-protein interface lead to close contact of active amino acid residue and the scissile bond (20). Mg^{2+} ions induce conformational changes not only in the enzyme but in DNA as well (21).

So, by means of covalent attachment of R.MvaI to activated substrate analogues, we established the conformational perturbation of the enzyme under the influence of a cofactor. At the first stage of the catalytic act the complex is formed with one Mg^{2+} ion, which is a structural precursor of the catalytic active complex. In this complex, the initial conformational changes occur, induced by the cofactor, and are completed by the subsequent addition of the second Mg^{2+} ion to form the full catalytic unit.

ACKNOWLEDGMENT

This work was supported by the Russian Foundation of Fundamental Investigation (Grant 9404-12649a). The authors are thankful to Molchanova Ya.V. for the help with mathematical simulations.

LITERATURE CITED

- (1) Gromova, E. S., Kubareva, E. A., Vinogradova, M. N., Oretskaya, T. S., and Shabarova, Z. A. (1991) Peculiarities of recognition of CC^A/TGG sequences in DNA by restriction endonucleases MvaI and EcoRII. *J. Mol. Recognit.* 4, 133-141.
- (2) Kuznetsova, S. A., Ivanovskaya, M. G., and Shabarova, Z. A. (1990) Chemical reactions within the double-stranded DNA. IX. Directed introduction of substituted pyrophosphate bonds in the DNA structure. *Bioorgan. Khim. (Russ.)* 16, 219-225.
- (3) Purmal, A. A., Shabarova, Z. A., and Gumport, R. I. (1992) A new affinity reagent for the specific, covalent attachment of DNA to active-site nucleophiles: application to the EcoRI and RsrI restriction and modification enzymes. *Nucleic Acids Res.* 20, 3713-3719.
- (4) Shabarova, Z. A., Sheflyan, G. Ya., Kuznetsova, S. A., Kubareva, E. A., Sysoev, O. N., Ivanovskaya, M. G., and Gromova, E. S. (1994) Affinity modification of restriction endonuclease EcoRII by DNA duplex containing monosubstituted pyrophosphate internucleotide bond. *Bioorgan. Khim. (Russ.)* 20, 413-419.
- (5) Sheflyan, G. Ya., Kubareva, E. A., Volkov, E. M., Oretskaya, T. S., Gromova, E. S., and Shabarova, Z. A. (1995) Chemical cross-linking of MvaI and EcoRII enzymes to DNA duplexes

- containing monosubstituted pyrophosphate internucleotide bond. *Gene* 157, 187–190.
- (6) Sheflyan, G. Y., Kubareva, E. A., Kuznetsova, S. A., Karyagina, A. S., Nikolskaya, I. I., Gromova, E. S., and Shabarova, Z. A. (1996) Cross-linking of *SsoII* restriction endonuclease to cognate and noncognate DNAs. *FEBS Lett.* 390, 307–310.
- (7) Volkov, E. M., Romanova, E. A., Krug, A., Oretskaya, T. S., and Potapov, V. K. (1988) Automated synthesis of oligodeoxyribonucleotides with terminal phosphate groups. *Bioorgan Khim. (Russ.)* 14, 1034–1039.
- (8) Kubareva, E. A., Pein, C. D., Gromova, E. S., Kuznezova, S. A., Tashlitzki, V. N., Cech D., and Shabarova, Z. A. (1988) The role of modifications in oligonucleotides in sequence recognition by *MvaI* restriction endonuclease. *Eur. J. Biochem.* 175, 615–618.
- (9) Winkler, F. K., Banner, D. W., Oefner, C., Tsernoglou, D., Brown, R. S., Heathmen, S. P., Bryan, R. K., Martin, P. D., Petratos, K., and Wilson, K. S. (1993) The crystal structure of *EcoRV* endonuclease and of its complexes with cognate and noncognate DNA fragments. *EMBO J.* 12, 1785–1795.
- (10) Vipond, I. B., Baldwin, G. S., and Halford, S. E. (1995) Divalent metal ions at the active sites of the *EcoRV* and *EcoRI* restriction endonucleases. *Biochemistry* 34, 697–704.
- (11) Jeltsch, A., Alves, J., Wolfes, H., Maass, G., and Pingoud, A. (1993) Substrate assisted catalysis in the cleavage of DNA by the restriction enzymes *EcoRI* and *EcoRV*. *Proc. Natl. Acad. Sci. U.S.A.* 90, 8499–8503.
- (12) Baldwin, G. S., Vipond, I. B., and Halford, S. E. (1995) Rapid reaction analysis of the catalytic cycle of the *EcoRV* restriction endonuclease. *Biochemistry* 34, 705–714.
- (13) Jeltsch, A., Maschke, H., Selent, U., Wenz, C., Kohler, E., Conolly, B. A., Thorogood, H., and Pingoud, A. (1995) DNA binding specificity of the *EcoRV* restriction endonuclease is increased by Mg^{2+} binding to a metal ion binding site distinct from the catalytic center of the enzyme. *Biochemistry* 34, 6239–6246.
- (14) Kostrewa, D., and Winkler, F. K. (1995) Mg^{2+} binding to the active site of *EcoRV* endonuclease: a crystallographic study of complexes with substrate and product DNA at 2 Å resolution. *Biochemistry* 34, 683–696.
- (15) Pingoud, A., Alves, J., and Geiger, R. (1993) Restriction enzymes. *Methods in Molecular Biology*, v. 16, *Enzymes of Molecular Biology* (Burrell, M. M., Ed.) pp 107–200, Humana Press Inc., Totowa, NJ.
- (16) Uchimaru, T., Uebasi, M., Tanabe, K., and Taira, K. (1993) Theoretical analyses of the role of Mg^{2+} ions in ribozyme reactions. *FASEB J.* 7, 137–142.
- (17) Winkler, F. K. (1992) Structure and function of restriction endonucleases. *Curr. Opin. Struct. Biol.* 2, 93–99.
- (18) Taylor, J. D., Badcoe, I. G., Clarke, A. R., and Halford, S. E. (1991) *EcoRV* restriction endonuclease binds all DNA sequences with equal affinity. *Biochemistry* 30, 8743–8753.
- (19) Zebala, J. A., Choi, J., and Barany, F. (1992) Characterization of steady state, single-turnover and binding kinetics of the *TaqI* restriction endonuclease. *J. Biol. Chem.* 267, 8097–8105.
- (20) Thielking, V., Selent, U., Kohler, E., Landgraf, A., Wolfes, H., Alves, J., and Pingoud, A. (1992) Mg^{2+} confers DNA binding specificity to the R-*EcoRV* restriction endonuclease. *Biochemistry* 31, 3728–3732.
- (21) Stover, T., Kohler, E., Fagin, U., Wende, W., Wolfes, H., and Pingoud, A. (1993) Determination of the DNA bend angle induced by the restriction endonuclease R-*EcoRV* in the presence of Mg^{2+} . *J. Biol. Chem.* 268, 8645–8650.

BC9800163

Lactose-Poly(ethylene Glycol)-Grafted Poly-L-Lysine as Hepatoma Cell-Targeted Gene Carrier

Young Hun Choi,[†] Feng Liu,[‡] Jong Sang Park,[†] and Sung Wan Kim^{*,‡}

Department of Chemistry, Seoul National University, Seoul, Korea, and Center for Controlled Chemical Delivery, Department of Pharmaceutics and Pharmaceutical Chemistry, University of Utah, Biomedical Polymers Research Building, Room 205, Salt Lake City, Utah 84112. Received February 10, 1998; Revised Manuscript Received August 17, 1998

To investigate the delivery of DNA into cells, lactose-poly(ethylene glycol)-grafted poly-L-lysine (Lac-PEG-PLL) polymers were synthesized as polymeric gene carriers. The new synthetic carriers, varying the substitution ratio of lactose-poly(ethylene glycol) (lactose-PEG), were characterized by NMR spectroscopy and size-exclusion chromatography. Electrophoretic mobility assay confirmed that the new gene carrier makes a complex with plasmid DNA. The attached poly(ethylene glycol) gives better solubility properties to gene/carrier complex. Transfection experiments showed that Lac-PEG-PLL efficiently delivers DNA to a hepatoma cell line *in vitro*; the best efficiency was achieved at a 1:3 weight ratio of DNA to carrier. As the lactose-PEG substitution content increased up to 30%, the transfection efficiency increased, which demonstrates that the lactose serves as a targeting moiety. No considerable cytotoxicity was observed due to Lac-PEG-PLL or its complex with DNA within the concentration range for this experiment. The use of chloroquine increased transfection efficiency that indicates the involvement of hydrolytic degradation of the system in lysosome. It is likely that plasmid DNA/Lac-PEG-PLL complexes enter the cells through a receptor-mediated endocytosis mechanism. These results show that Lac-PEG-PLL can form a complex with plasmid DNA and serve as an efficient gene delivery carrier with lower cytotoxicity compared to that of poly-L-lysine. Therefore, it is expected that our Lac-PEG-PLL carrier can be used as an *in vivo* gene delivery vector.

INTRODUCTION

Twenty-five years ago, Friedmann outlined prospects for human gene therapy (1). Since then, gene therapy has represented a new paradigm for therapy of human disease and for drug delivery (2). The implicit emphasis of the research so far has been on determining the safety of gene transfer procedures, sometimes with efficacy as a secondary goal (3). A major technical impediment to gene transfer is the lack of ideal gene delivery systems (3). If it were possible to deliver the gene to the appropriate specific cells in sufficient quantities, gene therapy would be efficacious. Currently, very few organs or cells can be specifically targeted for gene delivery. There are many established protocols for transferring genes into cells, including calcium phosphate precipitation, electroporation, particle bombardment, liposomal delivery, viral-vector delivery, and receptor-mediated gene delivery (4). Although all of these methods can be used for mammalian cultured cells, there are many difficulties in introducing genes into target cells *in vivo*.

Transfection methods using retroviral or adenoviral vectors (5) permit us to overcome some of these limitations. Retroviral vectors, in particular, have been used successfully for introducing exogenous genes into the genomes of actively dividing cells such that stable transformants are obtained (6). All the viral vectors use approaches that involve complementation of defective vectors by genes inserted into helper cell lines to generate

the transducing infectious agent. It is well-known that the host immune response to adenoviruses limits their use to a single administration. To address this limitation, fusion peptides of the influenza virus hemagglutinin have been employed to replace adenoviruses as endosomal lytic agents, but with limited success (7). However, despite their high transfection efficiency *in vitro*, inserting genes into the host cell's genome in this method depends on the viral infection pathway. Its application for human gene therapy introduces serious concerns about endogenous virus recombinations, oncogenic effects, and inflammatory or immunologic reactions (3, 8, 9). Such concerns have limited the use of viral vectors for human gene therapy (6).

On the other hand, nonviral gene delivery systems such as cationic liposomes (8) or synthetic gene carriers, such as poly-L-lysine (PLL) (10), are being widely sought as alternatives (11). There are several theoretical advantages of nonviral therapies including their relative safety and cost of manufacture (2). No significant toxicity has been encountered in preclinical toxicology studies using several cationic lipid:DNA complexes (12, 13) or in initial human studies (12, 14). Despite a theoretical concern about raising anti-DNA antibodies (15), there is no evidence for the formation of antibodies against DNA or antinuclear antibodies after administration of "naked DNA" or DNA formulated with lipids or proteins in clinical gene therapy studies (12, 14, 15). The major limitation of plasmid-based approaches has been that both the efficiency of gene delivery to several important somatic targets (*i.e.*, liver and lung) and *in vivo* gene expression level are lower using nonviral approaches than with viral vectors.

* Author to whom correspondence should be addressed. Tel: 1-801-581-6801. Fax: 1-801-581-7848.

[†] Seoul National University.

[‡] University of Utah.

It has been shown previously that the presence of polycations in culture medium can increase in vitro cellular uptake of a variety of substances, for example, albumin (16) and nucleic acids (17). These effects were shown to be dependent on size and concentration (16, 17). The enhancement was found to be most effective with polyornithines, but PLLs also demonstrated considerable activity. Laemmli (18) characterized that PLL makes condensates with DNA. Since then, PLL, modified with various substances, was used as a part of gene carrier (4, 19–27).

Recently, poly(ethylene glycol) (PEG)-grafted PLL (PEG-g-PLL) was synthesized in this laboratory, based on the idea that PLL modified with PEG showed high solubility, low cytotoxicity, and enhanced cell permeability. The synthesized PEG-g-PLL was shown to make a complex with plasmid DNA and do so with lower cytotoxicity and improved transfection efficiency to that of over PLL (28). In addition, Kabanov et al. (29) and Wolfert et al. (11) have developed A–B type block polycations as carriers for oligonucleotide and gene delivery. For these carriers, one hydrophilic polymer region (PEG) combines with one polycationic polymer region (PLL). This block copolymer forms a complex with DNA, maintaining a low cytotoxicity comparable to the cation alone but with high solubility.

In this paper, the grafted polymers were synthesized by introducing lactose group to the end of PEG first and covalently attaching the carboxylic acid group of lactose-PEG to the ϵ -amino group of PLL. The purpose of using lactose is to target hepatoma cells for gene delivery. The ratio of lactose-PEG and PLL can be adjusted by changing reaction conditions. The synthesized carrier, named Lac-PEG-PLL, formed a complex with pSV- β -gal plasmid DNA. PEG, grafted to PLL, resulted in better solubility and reduced cytotoxicity of the plasmid DNA/carrier complex. The gene transfection efficiency and cytotoxicity of the Lac-PEG-PLL system were investigated and compared to those of DNA complexed with PLL alone. Hep G2 cells were transfected specifically with plasmid DNA/Lac-PEG-PLL complex, indicating that lactose serves as a targeting moiety for a hepatoma cell line.

MATERIALS AND METHODS

Materials. Isobutyl chloroformate (IBCF), *N,N'*-dimethylformamide (DMF), triethylamine (TEA), and dimethyl sulfoxide (DMSO) were purchased from Aldrich (St. Louis, MO). PLL-hydrobromide (120 repeating units; MW = 25 000), *p*-aminophenyl- α -D-lactopyranoside, Concanavalin A (Con A)-Sephacrose column, methyl- α -D-mannopyranoside, *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid) (HEPES), ethidium bromide, chloroquine, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and *o*-nitrophenyl- β -D-galactopyranoside (ONPG) were purchased from Sigma (St. Louis, MO). PEG diacid (MW = 550) was bought from Fluka (Ronkonkoma, NY). Plasmid pSV- β -gal, 5X reporter Lysis buffer, and DNA size marker were purchased from Promega (Madison, WI). Hep G2 cells (a human liver carcinoma cell line, ATCC accession no. HB 8065), A7R5 cells (a rat smooth muscle cell line, ATCC accession no. CRL 1444), and NIH3T3 cells (a mouse embryonic cell line, ATCC accession no. CRL 1658) were purchased from ATCC (Rockville, MD). Fetal bovine serum (FBS), minimal essential medium (MEM), Dulbecco's minimal essential medium (DMEM), 0.25% trypsin, and 1 $\times$ phosphate-buffered saline (PBS) were purchased from Hyclone (Logan, UT). Lipofectin was purchased from GIBCO

BRL (Gaithersburg, MD). Bigger Prep DNA purification kit was purchased from 5'-3' Prime Inc. (Boulder, CO). Dialysis tubings (molecular weight cutoff of 1000 and 25 000) were purchased from Spectrum (Houston, TX).

Synthesis of Lac-PEG-PLL. The reaction scheme for synthesis of Lac-PEG-PLL is shown on Figure 1a. It consists of two reactions, synthesis of lactose-PEG diacid and grafting of lactose-PEG diacid to PLL. The lactose-PEG diacid is synthesized and purified according to a similar reported procedure (50). Six hundred milligrams (1.0 mmol) of PEG diacid (MW = 550) was dissolved in 1.5 mL of dried DMF containing 1.0 mmol of TEA in an ice–water bath under nitrogen. One millimole of IBCF dissolved in 1.0 mL of dried DMF was added dropwise, and the mixture was stirred for 15 min in an ice–water bath. IBCF served as the activator of carboxylic acid. Then *p*-aminophenyl- α -D-lactopyranoside (1.0 mmol) dissolved in 1.0 mL of DMF was added, and the mixture was stirred 30 min in an ice–water bath. It was stirred for additional 3 h at room temperature. The product was precipitated in an excess of dry ether (100 mL). Then the precipitate was dissolved in 20 mL of distilled water, dialyzed against distilled water (dialysis tubing with MWCO 1000), and freeze-dried. The product was purified by removing unreacted PEG diacid on a Con A-Sephacrose column. Forty milligrams of the lyophilized product was dissolved in 40 mL of 0.02 M Tris buffer (pH 7.4, 0.5 M NaCl, 3 mM CaCl₂, and 3 mM MnCl₂) and loaded on Con A-Sephacrose column at a flow rate of 0.10 mL/min. The column was eluted until absorbance at 280 nm dropped below 0.01, and then identical buffer containing 0.1 M methyl- α -D-mannopyranoside was applied. The specifically eluted peak of lactose-PEG and lactose-PEG-lactose mixture was detected by monitoring at 280 nm, collected, dialyzed against distilled water, and finally freeze-dried. The content of lactose was assayed by UV using *p*-aminophenyl- α -D-lactopyranoside as standard. The content of carboxylic acid was measured by titration. The mole ratio of lactose-PEG to lactose-PEG-lactose was about 2:1.

From the lactose-PEG and PLL, four different grafted copolymers Lac-PEG-PLLs with various mole ratios of lactose-PEG to ϵ -amino groups of PLL (mol/mol %) were further synthesized. As an example, the synthesis of Lac-PEG-PLL with 30% (mol/mol %) lactose-PEG is described. Lactose-PEG and lactose-PEG-lactose mixture (51.1 mg; containing 0.042 mmol of lactose-PEG) was dissolved in 0.5 mL of dried DMF under nitrogen in an ice–water bath. IBCF (0.036 mmol) dissolved in 0.5 mL of DMF was added to the above solution dropwise. The mixture was stirred 30 min in an ice–water bath, then added dropwise to 1 mL of dried DMSO containing 25 mg of PLL-HBr and 10 μ L of TEA which had been purged with nitrogen for 10 min in an ice–water bath, then stirred an additional 3 h at room temperature. The product was precipitated in 100 mL of dried ether, and the precipitate was dissolved in 10 mL of distilled water. It was dialyzed (dialysis tubing with MWCO 25 000) first against 0.01 M NaHCO₃ solution, second against 0.01 M HCl solution, and finally against distilled water. The dialyzed product was finally freeze-dried. The mole ratios of lactose-PEG to the amino group in PLL were calculated from ¹H NMR.

Size-Exclusion Chromatography Lac-PEG-PLL. The relative distribution of Lac-PEG covalently grafted to PLL was examined by size-exclusion chromatography (SEC) on a analytical Superose 12 column (10 mm $\times$ 30 cm) (Pharmacia AB) using FPLC. The column was eluted with 0.05 M PBS buffer (pH 7.3) containing 0.5 M NaCl at a flow rate 0.3 mL/min at 25 $^{\circ}$ C. A 200 μ L sample (2

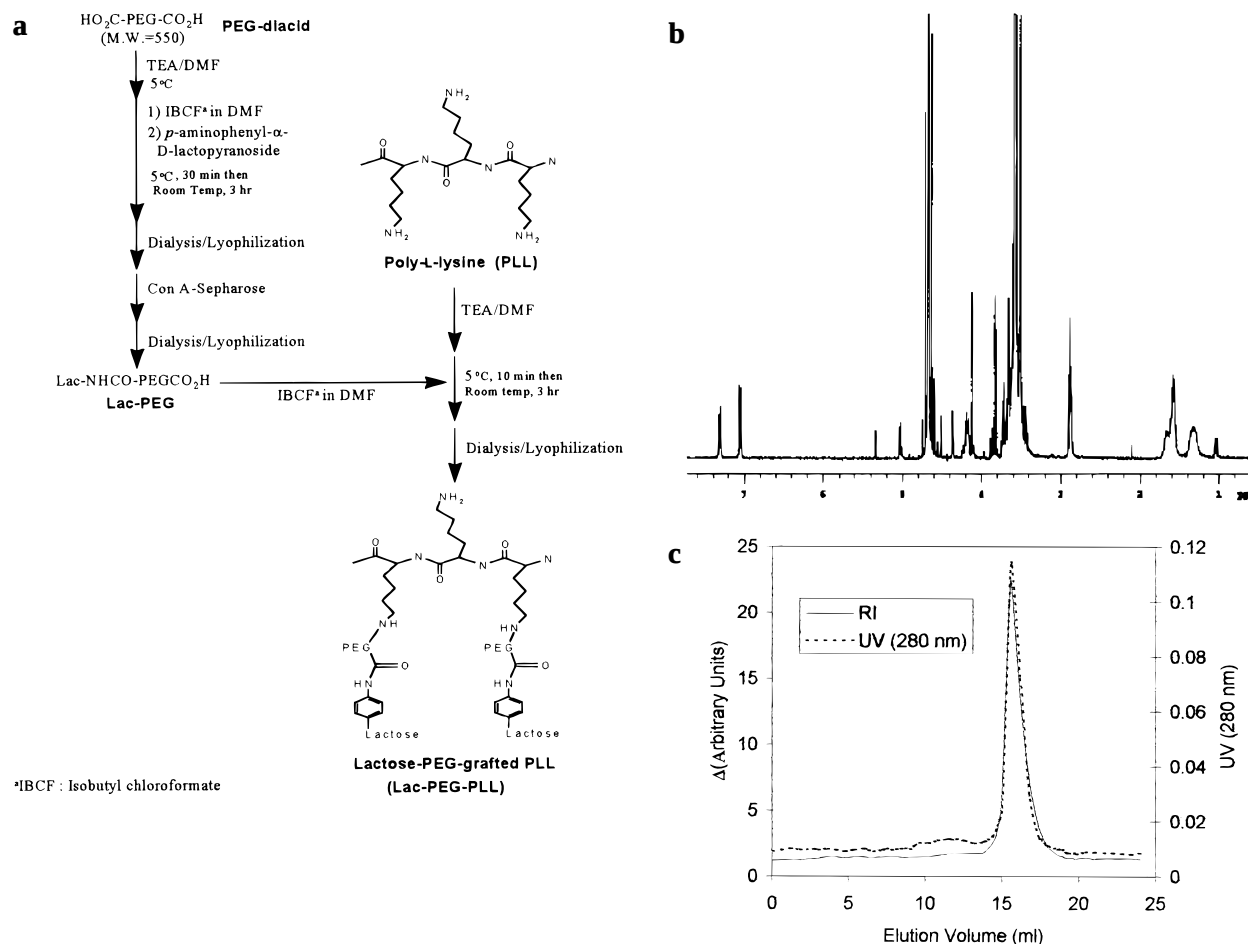


Figure 1. (a) Synthesis scheme of Lac-PEG-PLL. The ratio of lactose-PEG-attached amine group to total amine group was calculated from the peak intensities in the ¹H-NMR spectrum. (b) ¹H-NMR analysis of Lac-PEG-PLL. The ¹H-NMR spectrum of 30 mol % Lac-PEG-PLL was obtained in H₂O. The peak at about 3.5 ppm designates the existence of PEG. (c) SEC of Lac-PEG-PLL on an analytical Superose 12 column. The 30 mol % Lac-PEG-PLL was used. The column was equilibrated with PBS buffer (0.05 M, pH 7.3) containing 0.5 M of NaCl at a flow rate 0.3 mL/min. Sample (200 μ L, 2 mg/mL) was injected.

mg of Lac-PEG-PLL/mL) was injected and the refractive index (RI) and A_{280} of the elute were continuously monitored.

Synthesis of PEG-g-PLL. The procedure for synthesis of PEG-g-PLL was previously described (28).

Preparation of pSV- β -gal Plasmid DNA. The plasmid pSV- β -gal was prepared as previously reported (28).

Band Retardation Assay. Various amounts of Lac-PEG-PLL, ranging from 0.1 to 10 μ g, were added to 1 μ g of plasmid DNA in HEPES buffer (20 mM, 0.15 M NaCl, pH 7.4) and incubated for 30 min at room temperature. Gel electrophoresis sample buffer was added to each sample, and it was electrophoresed on 1% (w/v) agarose gel for 90 min at 100 V using the Easy-Cast Electrophoresis System (Owl Scientific Inc., Woburn, MA). TBE (45 mM Tris-Borate and 1 mM EDTA, pH 8.0) buffer was used as electrophoresis buffer. The gel was stained with ethidium bromide (0.5 μ g/mL) for 30 min and illuminated with an UV illuminator to show the location of the DNA. Lambda DNA, which was digested by *Hind*III, was used as a DNA size marker.

Solubility Test. The solubility of the carrier/plasmid DNA complex was determined by a method which was originally developed by Wadhwa et al. (4) with a slight modification. pSV- β -gal plasmid DNA and carrier were mixed in 1 mL of HEPES buffer (20 mM and 0.15 M NaCl, pH 7.4) with final concentration of 50 μ g/mL in 1.5 mL centrifuge tubes. After incubation for 30 min at

room temperature, the tubes were centrifuged for 5 min at 10 000 rpm. The supernatant was taken, and its absorbance at 260 nm was measured for the determining of the DNA remaining in solution. A DNA sample without carrier was used as a standard.

Cell Culture. Hep G2 cells were maintained in MEM medium supplemented with 1 mM sodium pyruvate and 10% FBS at 37 °C in a 5% CO₂ Napco incubator (Precision Scientific Inc., Chicago, IL). A7R5 cells were similarly maintained in DMEM medium supplemented with 10% FBS. NIH3T3 cells were likewise maintained in DMEM medium supplemented with 4.5 g/L glucose and 10% FBS. Cells were normally grown in 25 cm² (for Hep G2 cells) or 75 cm² (for the A7R5 and NIH3T3 cells) polystyrene tissue culture flasks (Becton Dickinson, Franklin Lakes, NJ), until they became approximately 80% confluent as assessed by light microscopy.

Transfection. In vitro transfection was performed as follows. For the transfection and cytotoxicity studies, Hep G2 cells were seeded at a density of 2×10^4 cells/each well in 96-well flat-bottomed microassay plates (Becton Dickinson, Franklin Lakes, NJ). The cells were incubated for 24 h before the addition of either the plasmid DNA/carrier complex or the carrier only. For A7R5 and NIH3T3 cells, the seeding concentration was 5×10^3 cells/well and 2×10^4 cells/well, respectively.

All reagents used in the transfection experiments were sterilized by filtering through 0.22 μ m polycarbonate

membrane filters. Plasmid pSV- β -gal/Lac-PEG-PLL complexes were prepared by mixing 1 μ g of pSV- β -gal and various amounts of carrier in 100 μ L of FBS-free cell culture medium and incubated for 30 min at room temperature. FBS and chloroquine were added at a final concentration of 10% (v/v) and 100 μ M, respectively. Medium from each well of the 96-well plate was replaced with 100 μ L of transfection mixture. The cells were then incubated for 4 h at 37 °C in a 5% CO₂ incubator. After 4 h, the transfection mixtures were removed and 100 μ L of fresh medium containing 10% FBS was added to each well. Cells were incubated for an additional 48 h at 37 °C.

Assay for Expressed β -Galactosidase. The β -galactosidase activity in transfected cells was determined spectrophotometrically at 405 nm using ONPG with a slight modification (30). Briefly, the growth medium was removed from the cells in 96-well plates to be assayed, and the cells were washed twice with 1 $\times$ PBS carefully to prevent adherent cell detachment. Cells were lysed by adding 60 μ L of 1X reporter lysis buffer (Promega, Madison, WI) to the cells and incubating for 30 min at room temperature, rocking the plate halfway through the incubation period, followed by scraping the cells. Sixty microliters of ONPG (1.33 mg/mL) in 2 $\times$ assay buffer (Promega, Madison, WI) was added to the lysed cells, and the cells were incubated at 37 °C for 4 h. The reaction was terminated by adding 150 μ L of 1 M sodium carbonate solution to each well and the absorbance at 405 nm was read with a Bio-Tek EL-311, the microplate reader (Bio-Tek Instrument Co., Winooski, VT) for the β -galactosidase activity.

Cell Viability Assay. Evaluation of cytotoxicity was performed as previously reported (28).

RESULTS

Synthesis of Gene Carriers. The synthesis of Lac-PEG-PLL consists of two reactions, synthesis of lactose-PEG diacid and synthesis of comb-shaped polymer Lac-PEG-PLL from lactose-PEG diacid and PLL. In the first reaction, IBCF and a carboxylic acid in PEG diacid formed a mixed anhydride, and a molecule of hydrochloric acid was released. TEA had a role as a base to neutralize hydrochloric acid which is formed during this reaction. The amino group in *p*-aminophenyl- α -D-lactopyranoside reacted with the carbonyl atom carbon on PEG-side, and an amide bond was formed between the sugar and PEG. After a 3.5 h reaction, the products were precipitated in an excess of ether. The precipitates were dissolved in distilled water and dialyzed using a dialysis membrane, 1000 MWCO, to remove unreacted *p*-aminophenyl- α -D-lactopyranoside. Con A-Sepharose column was used to remove unreacted PEG. Then lactose-PEG diacid was dissociated from the Con A-Sepharose column when 0.1 M methyl- α -D-mannopyranoside-containing buffer was applied. In that fraction, lactose-PEG-lactose, which means both carboxylic acid sides of PEG diacid had been modified to lactose moiety, was also eluted with lactose-PEG diacid, but no further purification was required because lactose-PEG-lactose will not take part in the second reaction. The content of carboxylic acid is determined by titration (50). Also, the lactose-PEG-lactose (MW = about 1400) was removed completely during the dialysis in the second reaction. In the second reaction, the carboxylic acid was activated by IBCF to form a mixed anhydride then the anhydride reacted with amino group on PLL.

The ¹H-NMR spectrum of 30 mol % Lac-PEG-PLL is shown on Figure 1b. The content of PEG was calculated

from the NMR spectrum by relating the PEG ($-\text{CH}_2-\text{CH}_2-$, s, 3.4–3.7 ppm) peak and side chain of PLL ($-\text{CH}_2\text{CH}_2\text{CH}_2-$, m, 1.1–1.8 ppm) peak. From this method, the lactose-PEG content ratios of the four carriers were verified as follows: 6, 12, 20, and 30 mol %, respectively.

The size exclusion chromatography data of Lac-PEG-PLL is shown in Figure 1c. From the peak number and peak shape, we can see that grafted polymer Lac-PEG-PLL is chromatographically homogeneous and that the RI and A₂₈₀ signal ratio was practically constant, which indicated that Lac-PEG was evenly distributed (conjugated) to PLL, and the Lac-PEG-PLL has certain molecular weight. Because the Lac-PEG-PLL is a grafted polymer and it is difficult to find suitable standard molecules, the molecular weight of the Lac-PEG-PLL is not calculated.

Physicochemical Characterization. Experiments were performed to investigate whether the synthesized Lac-PEG-PLL forms complexes with pSV- β -gal plasmid DNA. Figure 2a shows the result from the band retardation assay using Lac-PEG-PLL carrier for condensation with pSV- β -gal. A fixed amount of pSV- β -gal plasmid was titrated with increasing amounts of Lac-PEG-PLL resulting in complexes. The movement of pSV- β -gal (bands on lane 2) was retarded as the amount of Lac-PEG-PLL in a complex increased, suggesting that Lac-PEG-PLL forms a complex with pSV- β -gal and the negative charges of DNA were neutralized by the positive charges of Lac-PEG-PLL (lane 3–9). The pSV- β -gal/Lac-PEG-PLL complexes in lanes 6–9 did not enter the gel and show much weaker band in fluorescence intensity. It is most likely due to exclusion of ethidium bromide following formation of complex. Complete retardation was achieved at and above a 1:1 weight ratio of pSV- β -gal:Lac-PEG-PLL.

Plasmid DNA/carrier complexes were defined as soluble if centrifugation under the conditions selected failed to remove DNA from the supernatant. Figure 2b shows that complexation between pSV- β -gal and PLL at a final DNA concentration of 50 μ g/mL led to the formation of a fine precipitate which sedimented upon centrifugation at 10 000 rpm for 5 min. However, when the PEG-modified PLL was used to make complex with pSV- β -gal, nearly 98% of the DNA remained in the supernatant after centrifugation. The modification of the end of the PEG group with lactose moiety did not give more than a 10% decrease when compared to that of pSV- β -gal/PEG-*g*-PLL complexes. So the attached PEG groups on PLL prevent the pSV- β -gal/carrier complexes from making fine precipitates and becoming insoluble.

Transfection and Cytotoxicity. The transfection efficiency of the synthesized Lac-PEG-PLL was evaluated using in vitro Hep G2 cell line. Hep G2 cell, a hepatoma cell line, has a specific lactose receptor on its surface. For the transfection experiments, various pSV- β -gal/Lac-PEG-PLL complexes were formulated with a fixed amount of pSV- β -gal (10 μ g/mL) and increasing amounts of Lac-PEG-PLL (1–100 μ g/mL). The cell culture media without FBS was used as a mixing medium.

The Lac-PEG-PLL showed transfection ability into Hep G2 cells (Figure 3). Below 1:1 weight ratio of pSV- β -gal/Lac-PEG-PLL (0.3 or 0.5 μ g carriers), very weak or no transfection efficiency was observed regardless of using any lactosylated carrier. At 1:2 and 1:3 weight DNA/carrier ratios, those carriers with higher lactose quantity showed higher efficiency. The 30 mol % Lac-PEG-PLL was the best carrier among four lactosylated carriers at both of these ratios. This may be due to the targeting

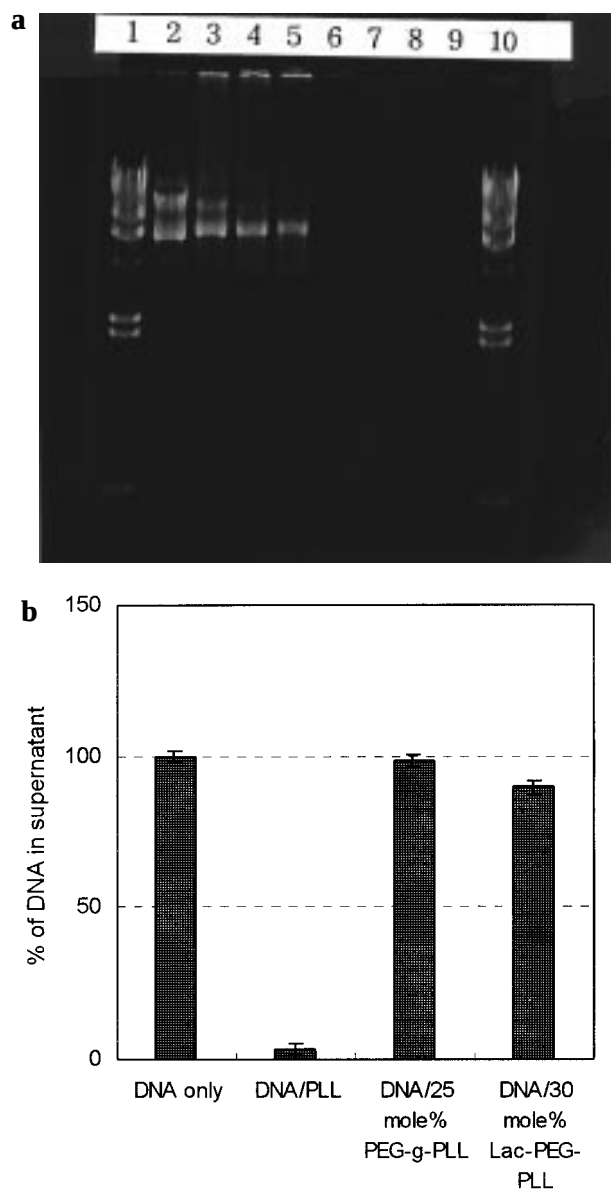


Figure 2. (a) Band retardation assay using Lac-PEG-PLL. Thirty mol % Lac-PEG-PLL was used. The band retardation showed that complete retardation was achieved at and above 1:1 weight ratio of plasmid DNA:Lac-PEG-PLL. The MWs of DNA size markers are 23 130, 9416, 6682, 4361, 2322, 2027, 564, and 265. Lanes 1 and 10: molecular weight marker. Lane 2: 1 μg of plasmid DNA. Lane 3: 1 μg of plasmid DNA + 0.1 μg of Lac-PEG-PLL. Lane 4: 1 μg of plasmid DNA + 0.3 μg of Lac-PEG-PLL. Lane 5: 1 μg of plasmid DNA + 0.5 μg of Lac-PEG-PLL. Lane 6: 1 μg of plasmid DNA + 1.0 μg of Lac-PEG-PLL. Lane 7: 1 μg of plasmid DNA + 3.0 μg of Lac-PEG-PLL. Lane 8: 1 μg of plasmid DNA + 5.0 μg of Lac-PEG-PLL. Lane 9: 1 μg of plasmid DNA + 10.0 μg of Lac-PEG-PLL. (b) Solubility test. Plasmid pSV-B-gal and carrier were mixed in 1 mL of HEPES buffer (20 mM, 0.15 M NaCl, pH 7.4) with final concentration of 50 μg/mL. After incubation for 30 min at room temperature, the tubes were centrifuged for 5 min at 10 000 rpm. The supernatant was taken and its absorbance at 260 nm was measured.

ability of lactose to Hep G2 cells, which reveals a rather different tendency from the case of PEG-*g*-PLL. As previously reported, 10 mol % PEG-*g*-PLL showed a higher efficiency than 25 mol % PEG-*g*-PLL (28). Using 30 mol % Lac-PEG-PLL carrier, the highest transfection efficiency was achieved at 1:3 weight ratio, but the 1:2 weight ratio revealed transfection of almost the same magnitude. It was shown that the higher the lactose-

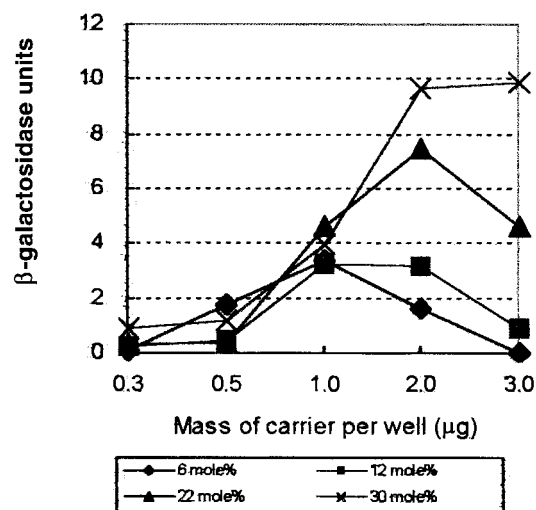


Figure 3. Optimization of transfection using different lactose-PEG substitution degree of Lac-PEG-PLL. "6 mol %" means 6 mol % Lac-PEG-PLL. The amount of each Lac-PEG-PLL was varied from 0.3 to 3.0 μg with 1 μg of pSV-B-gal in the presence of 10% FBS and 100 μM chloroquine. After subsequent culture for 48 h, the cells were lysed and assayed for β-galactosidase activity.

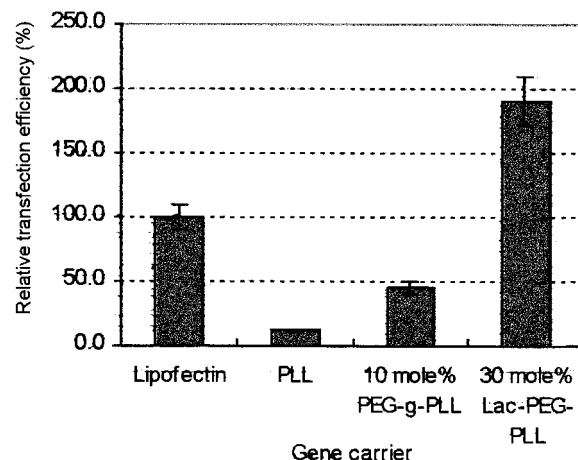


Figure 4. Comparison of transfection efficiencies of different polymeric gene carriers in Hep G2 cells. The concentration of gene carrier (except for lipofectin) and pSV-β-gal were 3 μg and 1 μg/well of 96-well plate, respectively (30 μg/mL and 10 μg/mL). The concentration of lipofectin was 1 μg/mL. Error bar shows standard deviation from six experiments.

PEG substitution degree in the carrier, the greater the quantity of carrier required to reach the highest transfection efficiency. When we used 6 mol % Lac-PEG-PLL, a 1:1 ratio complex showed the highest efficiency. In the case of 30 mol % Lac-PEG-PLL, 1:3 ratio complex showed the highest efficiency. This may be explained by the ability of the gene carrier to make complexes with pSV-β-gal. Higher substituted Lac-PEG-PLL has less free amine to interact with negative charge on DNA. Therefore, more carrier is needed to make complex with DNA to the same extent.

Figure 4 summarizes the results for the transfection using PLL and modified PLL. Lipofectin was used for the comparison of transfection efficiency. Among the 3 kinds of carrier—PLL, PEG-*g*-PLL, and Lac-PEG-PLL—and lipofectin, Lac-PEG-PLL showed the best efficiency for Hep G2 cells.

Cytotoxicity of the synthesized carriers for Hep G2 cells is shown in Figure 5. Hep G2 cells were incubated in the presence of 30 μg/mL of gene carrier for 4 h. The 30

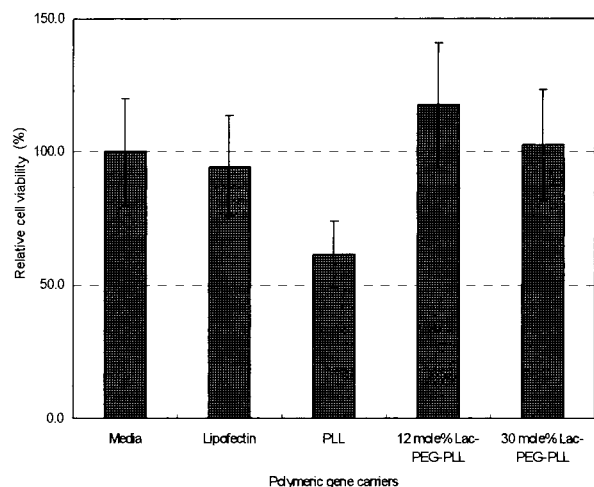


Figure 5. Comparison of cytotoxicity of various gene carriers in Hep G2 cells. The concentration of gene carrier was 3 μ g/well of 96-well plate (30 μ g/mL in cell culture medium). The gene carriers were incubated for 4 h with cells at 37 $^{\circ}$ C, then cell viability was determined using MTT assay method.

μ g/mL concentration was chosen because PEG-*g*-PLL and Lac-PEG-PLL showed the best transfection efficiency at that concentration. For Lac-PEG-PLL, no cytotoxicity was observed in Hep G2 cells, whereas PLL showed moderate to high cytotoxicity. The difference in lactose-PEG substitution ratio gave no significant change in cell viability. It was further supported by the phase contrast microscopic observation that the incubation of Lac-PEG-PLL with Hep G2 cells did not give any significant influence in cell viability. The incubation of PLL for 4 h with the cells transformed cell morphology, but PEG-*g*-PLL or Lac-PEG-PLL at the same concentration and incubation time did not significantly alter the morphology (data not shown).

To investigate the function of attached lactose moiety, the competition experiments with free sugars were performed. The pSV- β -gal/Lac-PEG-PLL complexes were formed and transfected into Hep G2 cells on the presence of free sugar for 4 h. After this, the transfection mixture including free sugar was removed and the cells were incubated for additional 48 h. Four sugars were used: galactose, glucose, lactose, and lactobionic acid. In the competition using free lactose, 0.5 mM lactose lowered the transfection efficiency up to 61.3% compared to that of no lactose (Figure 6a). Lactobionic acid was also used because of its greater solubility than lactose. Lactobionic acid showed a very close competition ability to that of lactose within the experimental range of lactose competition (0.01–0.05 mM). When the concentration of lactobionic acid was raised to 10 mM, the transfection was inhibited more than 80%. Over the concentration of 50 mM of lactobionic acid, almost no transfection activity was observed to Hep G2 cells, but the cell viability was also dramatically dropped. Below 10 mM concentration, free lactobionic acid did not give considerable cytotoxic effect to Hep G2 cells. Galactose and glucose also showed blockage of transfection derived from pSV- β -gal/Lac-PEG-PLL complex, though their blocking strengths were lower than that of lactose or lactobionic acid. It can be concluded from these observations that the pSV- β -gal/Lac-PEG-PLL complex is delivered to Hep G2 cells via a receptor-mediated endocytosis mechanism using lactose receptors existing on the cells.

We also tried to use Lac-PEG-PLL carrier as the gene delivery carrier to other cell lines. The cell lines that we used were A7R5 (a rat smooth muscle cell line) and

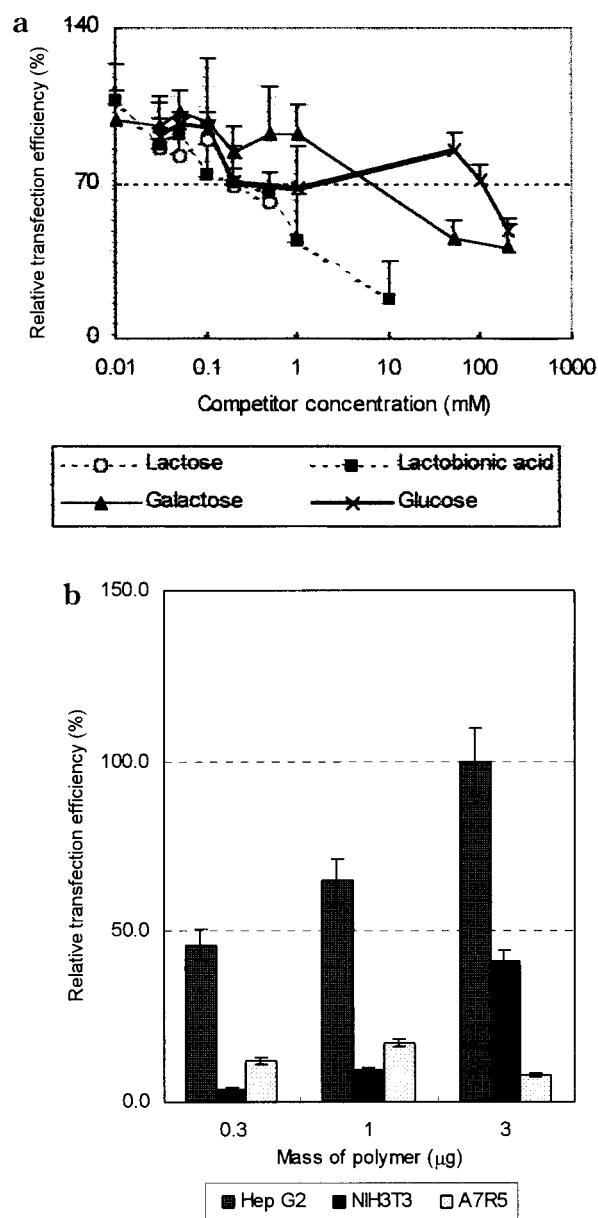


Figure 6. (a) Competition experiment with free sugar molecule. After making pSV- β -gal/Lac-PEG-PLL complex in medium, noted concentrations of each competitor were added, followed by incubation with Hep G2 cells for 4 h in the presence of 10% FBS and 100 μ M chloroquine. Error bar shows standard deviation from six experiments. (b) Comparison of transfection efficiency on various cell lines. ONPG β -galactosidase enzyme assay was used for expressed enzyme quantity. The amount of enzyme at 1:3 plasmid DNA/Lac-PEG-PLL ratio (w/w), on Hep G2 cells was regarded as 100%.

NIH3T3 (a mouse embryonic cell line). Neither cell line showed detectable or remarkable transfection results (Figure 6b), regardless of the degree of cytotoxic effect. This result suggests that the lactose serves as a targeting moiety for a specific gene delivery system.

Figure 7a shows the effect of chloroquine, an endosomal disrupting agent, to the transfection efficiency. In the absence of chloroquine, the transfection efficiency of pSV- β -gal/Lac-PEG-PLL complex on Hep G2 cells was as low as that obtained by PLL. As the concentration of chloroquine increased up to 100 μ M, the transfection efficiency from pSV- β -gal/Lac-PEG-PLL complex increased (Figure 7a). When 150 or 200 μ M chloroquine was used, the decrease in galactosidase activity was

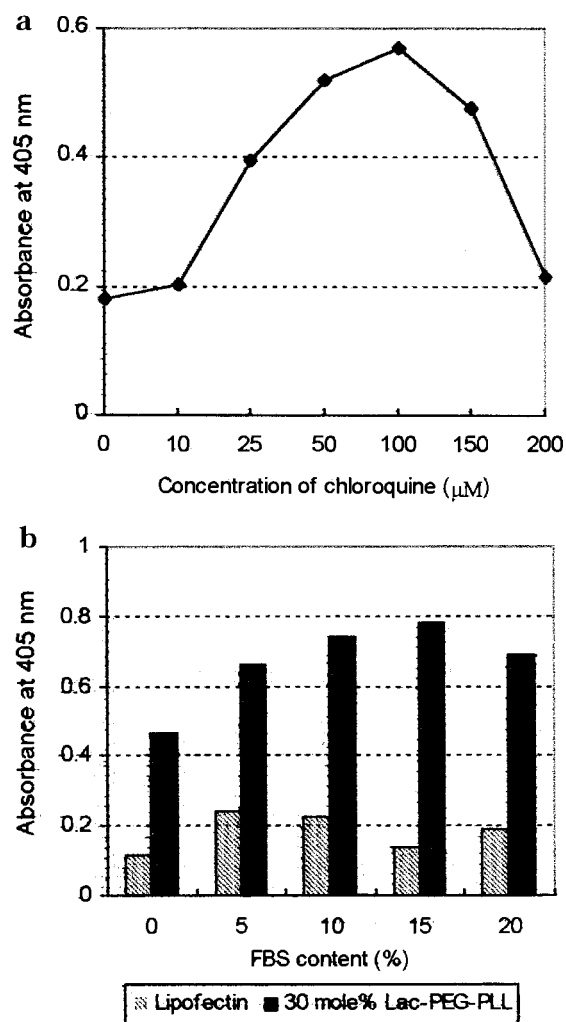


Figure 7. (a) Influence of chloroquine on gene transfection. Thirty mol % Lac-PEG-PLL was used as a gene carrier. The chloroquine at noted concentrations was added to the transfection mixture for 4 h, in the presence of 10% FBS, and the cells were incubated further for 48 h prior to assay for β -galactosidase. (b) Influence of FBS on gene transfection. Either lipofectin or 30 mol % Lac-PEG-PLL was used as a gene carrier. The FBS at noted concentrations was added to the transfection mixture for 4 h, in the presence of 100 μM of chloroquine, and the cells were incubated further for 48 h prior to assay for β -galactosidase.

observed. It is probable because large amounts of chloroquine were toxic to the cells and a morphological study showed granulated cells. From the results, it is likely that pSV- β -gal/Lac-PEG-PLL complexes enter the cells through an endocytosis mechanism.

The influence of FBS on transfection efficiency was investigated. Figure 7b shows that the presence of FBS in the transfection mixture increased transfection efficiency. The pSV- β -gal/Lac-PEG-PLL system showed about a 1.7-fold increased transfection efficiency on Hep G2 cells in the presence of 10% FBS. The best efficiency was achieved when 15% of FBS was included during the incubation of the transfection mixture with Hep G2 cells. It is not fully understood yet why transfection efficiency is decreased a little when 20% of FBS was used. It is possibly due to binding of the serum components to polycations (31). Lipofectin did not show any dependence of transfection efficiency on the concentration of FBS.

We also investigated the influence of other factors of in vitro transfection such as quantity of pSV- β -gal, incubation time with pSV- β -gal/carrier complex, and the

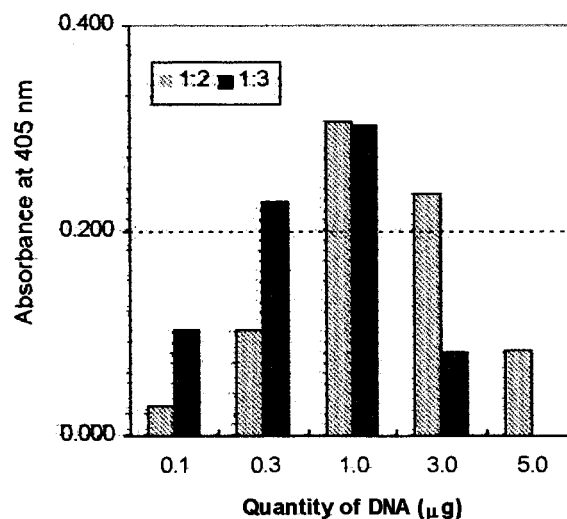


Figure 8. Optimization of pSV- β -gal quantity on transfection. Thirty mol % Lac-PEG-PLL was used. The amount of pSV- β -gal was varied from 0.1 to 5 μg with the same 1:2 or 1:3 ratio of pSV- β -gal to Lac-PEG-PLL, in the presence of 10% FBS and 100 μM chloroquine. After subsequent culture for 48 h, the cells were lysed and assayed for β -galactosidase activity.

time course of protein expression. Figure 8 shows the influence of DNA quantity. One microgram of pSV- β -gal is typically used for the transfection into cells which were grown in a well of 96-well microplate. It was already shown that at 1:2 and 1:3 ratio of pSV- β -gal/Lac-PEG-PLL complex the best transfection efficiency was achieved using 1 μg of pSV- β -gal. Therefore, we tried to optimize the quantity of pSV- β -gal per well. The results showed that the 1 μg of pSV- β -gal DNA is the best dose for a well of 96-well microplate on Hep G2 cells. It is very interesting that the pSV- β -gal/Lac-PEG-PLL complex at 1:3 ratio showed better efficiency than one at 1:2 ratio below 1 μg of pSV- β -gal dose, while the latter proved to be better over 1 μg of pSV- β -gal dose.

The optimal incubation time of the complex with Hep G2 cells was also determined (Figure 9a). It was shown that 4 h of incubation achieved the best transfection efficiency. The expression of β -galactosidase along the time course after transfection was also monitored up to 96 h (Figure 9b). The result showed that the expression of β -galactosidase in Hep G2 cells, which were transfected with pSV- β -gal/Lac-PEG-PLL complex, was maintained up to 96 h as a linear fashion. The synthesized Lac-PEG-PLL has ability to make complexes with DNA and showed enhanced transfection efficiency into Hep G2 cells.

DISCUSSION

Receptor-mediated gene delivery has its advantages and limitations (32). Its advantages for use in gene therapy are as follows. First, the gene delivery carrier can be designed and customized for a specific target receptor. Second, the DNA does not have to integrate into the host cell genome to be expressed. Third, the delivery system is theoretically unlimited by the size of the transgene. Finally, the technique does not involve the use of potentially infectious agents. There are also disadvantages which must be overcome before this procedure can be routinely used for human gene therapy. For example, the transgene is not integrated into the host cell chromosomes so that its expression is transient. Therefore, it will most likely be necessary to subject patients to multiple injections of a gene of interest. The

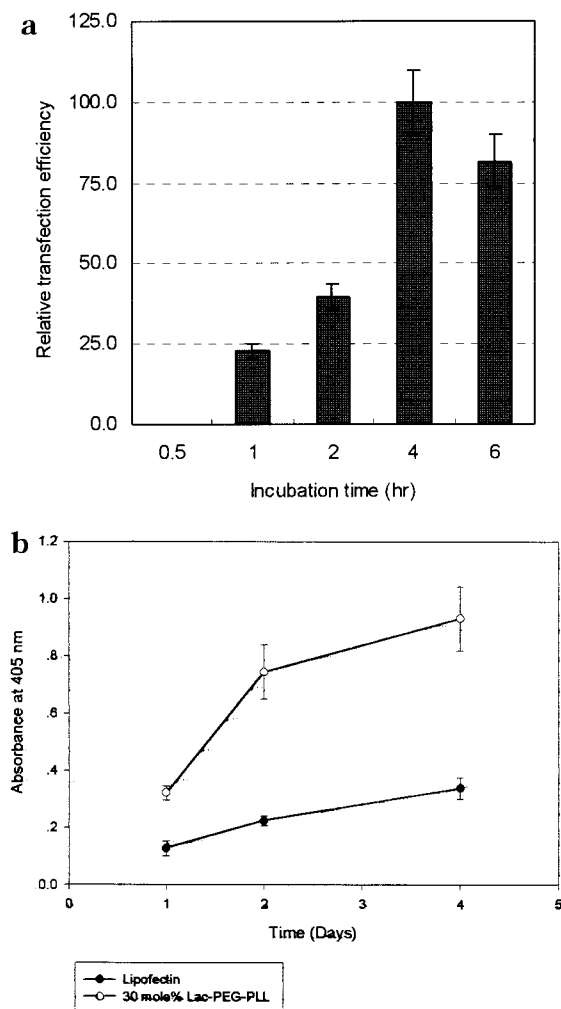


Figure 9. (a) Influence of the time of transfection. Thirty mol % Lac-PEG-PLL was used. The amount of pSV- β -gal was 1 μ g with the 1:3 ratio of pSV- β -gal to Lac-PEG-PLL. The transfection time was examined from 0.5 to 6 h in the presence of 10% FBS and 100 μ M chloroquine. After each time, the Hep G2 cells were incubated for 48 h, followed by β -galactosidase assay using ONPG. (b) Influence of the time of gene expression. Thirty mol % Lac-PEG-PLL was used as a gene carrier. The amount of pSV- β -gal was 1 μ g with the 1:3 ratio of pSV- β -gal to Lac-PEG-PLL. After 4 h incubation of pSV- β -gal/Lac-PEG-PLL with Hep G2 cells, the transfection medium was removed and the cells were incubated. At the noted times, the cells were lysed and assayed for β -galactosidase activity.

DNA-ligand complexes are difficult to prepare and, until recently, little was known about their structure–function relationship. Also there is only a fragmentary understanding of the biological process involved in the transfer of the transgene into the cell and its subsequent expression. These and other features of this system for gene therapy have recently been reviewed in detail (33).

PLL itself can be used as a DNA condensate (18), but the use of PLL as a gene delivery carrier has several disadvantages. First, its transfection efficiency is very low because PLL has no functional group except the amine group used in charge neutralization. Also due to the negative charges of the DNA phosphate backbone, an increase in the degree of charge neutralization of the DNA often results in extensive condensation and the separation of the DNA phase in the form of insoluble compact structures (32, 34, 35).

Lac-PEG-PLL was synthesized to serve as a nonviral vector. It was used to deliver the pSV- β -gal reporter gene

into Hep G2 cells with enhanced transfection efficiency, compared to that of PLL alone. Plasmid pSV- β -gal, which is a modification of pRSV- β -galactosidase vector, is often used as a reporter gene for monitoring gene expression in mammalian cells (36). The SV40 early promoter and enhancer drive the transcription of the *lacZ* gene in mammalian cells. β -Galactosidase is an excellent reporter enzyme (37) which can be assayed quickly and directly in cell extracts using a spectrophotometric assay.

It was shown that lactose-PEG-modified PLL has the ability to make a complex with pSV- β -gal from band retardation assay (Figure 2a). The shift of the pSV- β -gal/Lac-PEG-PLL band during electrophoresis depends on the pSV- β -gal:Lac-PEG-PLL ratio that alters the complex net charge as well as its size and density (9). The decrease in the electrophoretic mobility of pSV- β -gal/Lac-PEG-PLL complex was accompanied by the increase in the content of Lac-PEG-PLL in the system, and it is due to neutralization of the DNA negative charge by the carrier positive charge. Formation of a DNA–carrier complex was also observed in the dye displacement assay (data not shown).

As discussed earlier, one of the problems of PLL as a gene carrier is that complexation between PLL and DNA frequently results in formation of fine precipitates, limiting the concentration that can be used (38). In our experiment, complexation of pSV- β -gal with PLL in 20 mM HEPES (pH 7.4) with 0.15 M NaCl resulted in precipitates at 50 μ g/mL of pSV- β -gal. On the other hand, PEG-*g*-PLL and Lac-PEG-PLL maintained the solubility of pSV- β -gal when they made complexes with DNA at this concentration. Therefore, it is apparent that the attached PEG moiety makes pSV- β -gal/gene carrier complexes substantially more soluble.

The gene delivery ability of our synthesized carriers and its specificity were investigated. Hep G2 cells were chosen for specificity test because they have specific lactose receptors on their surface. The recognition of lactose is mediated by the lactose receptors on liver cell (39). They are used for removal of glycoproteins from the blood. Many newly synthesized glycoproteins, such as immunoglobulins and peptide hormones, contain carbohydrate units with terminal sialic acid and galactose residues. During hours or days, depending on the particular protein, terminal sialic acid residues are removed by sialylases on the surface of blood vessels. The exposed galactose residues of these trimmed proteins are detected by the asialoglycoprotein receptors in the plasma membranes of liver cells. The complex of the asialoglycoprotein and its receptor is then internalized into the liver cell by endocytosis process to remove the trimmed glycoprotein from the blood. We predicted that the lactose moiety on the carrier could serve as a targeting material for the hepatoma cell line.

Lac-PEG-PLL showed improved transfection efficiency over PLL and PEG-*g*-PLL. Among Lac-PEG-PLLs with different modified ratios, 30 mol % Lac-PEG-PLL accomplished the highest efficiency at 1:3 weight ratio to pSV- β -gal. It demonstrates that the lactose moiety on the end of PEG renders cell targeting ability to pSV- β -gal/Lac-PEG-PLL complexes. In our opinion, the fusogenic ability of PEG may serve as another factor for increase of transfection efficiency. It is supported by the fact that PEG is known to associate with the phospholipid headgroup of cell membranes, assisting modified proteins to penetrate into cell membranes (40).

The free sugar competition experiment of transfection using Lac-PEG-PLL emphasizes the probability that pSV- β -gal/Lac-PEG-PLL complexes are delivered into

Hep G2 cells by a receptor-mediated endocytosis mechanism. The increasing concentrations of lactose or lactobionic acid added to the transfection medium decreased the expression of β -galactosidase. Lactose (0.5 mM) and lactobionic acid (1 mM) caused 39.0 and 55.3% inhibition in β -galactosidase activity, respectively. In the case of other sugars, the higher concentration of galactose (50 mM) or glucose (200 mM) is needed to achieve more than 50% inhibition (54.8 and 51.0%, respectively). So lactose has 50-fold more specificity into Hep G2 cells than galactose. Aring et al. (41) found that lactosylated human serum albumin (HSA) strongly bound to the hepatic lectins, in contrast to galactosylated and glucosylated HSA. Also, it was reported that mammalian hepatic Gal/GalNAc receptors show preference for three terminal galactose residues in the triantennary ligand (42). It means that the spatial arrangement of the Gal residues is very important for optimal binding. Our Lac-PEG-PLL carrier contains multiple lactose groups in it, and may bind to the receptor with greater affinity compared with the monomeric sugars.

It was further supported that the lactose on Lac-PEG-PLL was served as targeting moiety by using different cell lines in transfection. In the A7R5 and NIH3T3 cell line, which do not have lactose receptors on their surface, the transfection efficiencies were much lower than that in Hep G2 cells. Wu and Wu (20) used an asialoglycoprotein-PLL conjugate for gene delivery and reported that all receptor (–) cells tested, regardless of the tissue source (SK-hep-1, hepatoma; IMR-90, fibroblasts; uterine smooth muscle cells), failed to undergo gene transformation under the conditions identical to those which were successful for Hep G2 [receptor (+)] cells. Their results further support our concept in which the cell specificity of this new delivery system for gene transformation is based on the presence of unique receptors on the target cells.

That the attached sugar on PEG-g-PLL can serve as a targeting moiety onto specific cells can bring up the other possibility for gene delivery. Membrane lectins are present at the surface of many normal cells as well as on various tumor cells, and the sugar specificity is dependent on the cell types (24). For example, mannose is recognized by macrophages, liver nonparenchymal cells, and some B cells; mannose-6-phosphate by monocytes; galactose by melanoma cells; glucose by colon carcinoma cells; and *N*-acetyllactosamine by T cells. Many ligands that bind to the target receptor can be used as targeting moieties for gene delivery (for reviews, see ref 43). Folate is indispensable for health, and therefore, its receptor is expressed on many cell types (44). In addition, this receptor is highly expressed on ovarian cancer cells (45). Several kinds of cancer cells are known to express the receptors highly for epidermal growth factor (EGF), low-density lipoprotein (LDL), and/or transferrin. Brain capillary endothelial cells express at least three types of receptors, transferrin, insulin, and insulin-like growth factor receptors, implying that these may be a suitable target for delivering gene to the brain (46). Fc and polymeric immunoglobulin receptors (pIgR) can be used for targeting the lung. In the lung, the Fc receptor is expressed on macrophages, the pIgR on epithelial cells (47).

The efficiency of gene transfection via receptor-mediated endocytosis can be augmented by the use of pharmacologic agents that disrupt the endocytic trafficking of DNA–ligand complexes (33). Chloroquine is known to induce a partial neutralization of acidic cell compartments, to reduce the fusion between endosomes and

lysosomes, to decrease intracellular degradation of the internalized plasmid by lysosomal enzymes, and to cause an increase in the volume of endocytic vesicles. In the presence of chloroquine, the DNA carried by Lac-PEG-PLL conjugates could escape degradation by endosome and lysosome hydrolases, allowing a correlative large number of DNA molecules to reach the cytosol and/or the nucleus (24). Moreover, chloroquine, which binds to DNA might also protect DNA molecules from nuclease degradation (25, 48). One hundred micromolar concentration of chloroquine was found to be optimal in transfecting Hep G2 cells using pSV- β -gal/Lac-PEG-PLL complexes. At higher concentration (150 and 200 μ M), the efficiency was lower in relation to a higher toxicity. It has not been definitely solved how and where DNA gets rid of the carrier chains in cell. Kabanov suggested that negatively charged membranes and/or polynucleotides in a cell may serve as components reacting with DNA/carrier complex and providing for DNA release as a result of carrier substitution (9).

Other factors of the in vitro transfection experiment were also optimized. The expression of β -galactosidase along the time course after transfection was also monitored up to 96 h (Figure 9b). The expression was maintained until 96 h. The maintenance of gene expression up to 96 h is an advantage of these synthesized gene carriers for transfection in cultured cells to provide a window for manipulation of transfected cells. As an opposite case, the transfected luciferase activity in human macrophage with mannosylated PLL/DNA was maximal after 24 h, then rapidly diminished to only 2% after 72 h (49).

In conclusion, the Lac-PEG-PLL polymers, with four ratios of lactose-PEG substitution, namely 6, 12, 22, and 30 mol %, were synthesized as gene carriers. Our synthetic comb-shaped polymers could make a complex with pSV- β -gal. The attached PEG made the complex soluble, while the complex between pSV- β -gal and PLL alone formed fine precipitate. The Lac-PEG-PLL carrier/DNA complex had more than a 10-fold increase in transfection efficiency when compared to that of a PLL complex on Hep G2 cells. This increase may be due to the cell-targeting effect of lactose moiety and partially from the fusogenic effect of PEG. The attached PEG group also allowed the Lac-PEG-PLL and the pSV- β -gal/Lac-PEG-PLL complex to be less cytotoxic than PLL. The lactose moiety specifically served as a targeting moiety for the transfection into Hep G2 cells, a human hepatoma cell line. This was indicated by two pieces of evidence. First, free lactose inhibited transfection at millimolar concentration. Second, very low transfection efficiency was monitored when other lactose receptor-deficient cell lines were used for transfection. The existence of FBS and chloroquine improved transfection, showing us that the transfection occurred via an endocytosis mechanism. Therefore, the application of these new carriers to animal studies in vivo has been concluded to be promising.

ACKNOWLEDGMENT

J.S.P. thanks Yon-Am Foundation, the Korean Ministry of Education, and Center for Molecular Catalysis (CMC) of Seoul National University supported by Korean Science Engineering Foundation for supporting his research.

LITERATURE CITED

- (1) Friedmann, T., and Roblin, R. (1972) Gene therapy for human genetic disease? *Science* 175, 949–955.

- (2) Ledley, F. D. (1996) Pharmaceutical approach to somatic gene therapy. *Pharm. Res.* 13, 1595–1614.
- (3) Ross, G., Erickson, R., Knorr, D., Motulsky, A. G., Parkman, R., Samulski, J., Straus, S. E., and Smith, B. R. (1996) Gene therapy in the United States: a five-year status report. *Hum. Gene Ther.* 7, 1781–1790.
- (4) Wadhwa, M. S., Knoell, D. L., Young, A. P., and Rice, K. G. (1995) Targeted gene delivery with a low molecular weight glycopeptide carrier. *Bioconjugate Chem.* 6, 283–291.
- (5) Rosenfeld, M. A., Siegfried, W., Yoshimura, K., Yoneyama, K., Fukayama, M., Stier, L. E., Paakko, P. K., Gilardi, P., Stratford-Perricaudet, L. D., Perricaudet, M., Jallat, S., Pavirani, A., Lecocq, J. P., and Crystal, R. G. (1991) Adenovirus-mediated transfer of a recombinant α_1 -antitrypsin gene to the lung epithelium in vivo. *Science* 252, 431–434.
- (6) Miller, D. G., Adam, M. A., and Miller, A. D. (1990) Gene transfer by retrovirus vectors occurs only in cells that are actively replicating at the time of infection. *Mol. Cell Biol.* 10, 4239–4242.
- (7) Gottschalk, S., Sparrow, J. T., Hauer, Mins, M. P., Leland, F. E., Woo, S. L. C., and Smith, L. C. (1996) A novel DNA-peptide complex for efficient gene transfer and expression in mammalian cells. *Gene Ther.* 3, 448–457.
- (8) Temin, H. M. (1990) Safety considerations in somatic gene therapy of human disease with retrovirus vectors. *Hum. Gene Ther.* 1, 111–123.
- (9) Kabanov, A. V., and Kabanov, V. A. (1995) DNA complexes with polycations for the delivery of genetic materials into cells. *Bioconjugate Chem.* 6, 7–20.
- (10) Farhood, H., Bottega, R., Epand, R. M., and Huang, L. (1992) Effect of cationic cholesterol derivatives on gene transfer and protein kinase C activity. *Biochim. Biophys. Acta* 1111, 239–246.
- (11) Wolfert, M. A., Schacht, E. H., Toncheva, V., Ulbrich, K., Nazarova, O., and Seymour, L. W. (1996) Characterization of vectors for gene therapy formed by self-assembly of DNA with synthetic block copolymers. *Hum. Gene Ther.* 7, 2123–2133.
- (12) Nabel, G. J., Nabel, E. G., Yang, Z. Y., Fox, B. A., Plautz, G. E., Gao, X., Huang, L., Shu, S., Gordon, D., and Chang, A. E. (1993) Direct gene transfer with DNA-liposome complexes in melanoma: expression, biologic activity, and lack of toxicity in humans. *Proc. Natl. Acad. Sci. U.S.A.* 90, 11307–11311.
- (13) Parker, S. E., Vahlsing, H. L., Serfilippi, L. M., Franklin, C. L., Doh, S. G., Gromkowski, S. H., Lew, D., Manthorpe, M., and Norman, J. (1995) Cancer gene therapy using plasmid DNA: safety evaluation in rodents and nonhuman primates. *Hum. Gene Ther.* 6, 575–590.
- (14) Nabel, E. G., Yang, Z. Y., Muller, D., Chang, A. E., Gao, X., Huang, L., Cho, K. J., and Nabel, G. J. (1994) Safety and toxicity of catheter gene delivery to the pulmonary vasculature in a patient with metastatic melanoma. *Hum. Gene Ther.* 5, 1089–1094.
- (15) Pisetsky, D. S. (1995) Immunologic consequences of nucleic acid therapy. *Antisense Res. Devel.* 5, 219–225.
- (16) Ryser, H. J.-P. (1967) A membrane effect of basic polymers dependent on molecular size. *Nature* 215, 934–936.
- (17) Farber, F. E., Melnick, J. L., and Butel, J. S. (1975) Optimal conditions for uptake of exogenous DNA by Chinese hamster lung cells deficient in hypoxanthine-guanine phosphoribosyl-transferase. *Biochim. Biophys. Acta* 390, 298–311.
- (18) Laemmli, U. K. (1975) Characterization of DNA condensates induced by poly(ethylene oxide) and polylysine. *Proc. Natl. Acad. Sci. U.S.A.* 72, 4288–4292.
- (19) Maruyama, A., Ishihara, T., Kim, J.-S., Kim, S. W., and Akaike, T. (1997) Nanoparticle DNA carrier with poly(L-lysine) grafted polysaccharide copolymer and poly(D, L-lactic acid). *Bioconjugate Chem.* 8, 735–742.
- (20) Wu, G. Y., and Wu, C. H. (1988) Evidence for targeted gene delivery to Hep G2 hepatoma cells in vitro. *Biochemistry* 27, 887–892.
- (21) Wu, G. Y., and Wu, C. H. (1988) Receptor-mediated gene delivery and expression in vivo. *J. Biol. Chem.* 263, 14621–14624.
- (22) Wagner, E., Plank, C., Zatloukal, K., Cotten, M., and Birnstiel, M. L. (1992) Influenza virus hemagglutinin HA-2 N-terminal fusogenic peptides augment gene transfer by transferrin-polylysine-DNA complexes: toward a synthetic virus-like gene-transfer vehicle. *Proc. Natl. Acad. Sci. U.S.A.* 89, 7934–7938.
- (23) Trubetskoy, V. S., Torchilin, V. P., Kennel, S. J., and Huang, L. (1992) Use of N-terminal modified poly(L-lysine)-antibody conjugate as a carrier for targeted gene delivery in mouse lung endothelial cells. *Bioconjugate Chem.* 3, 323–327.
- (24) Midoux, P., Mendes, C., Legrand, A., Raimond, J., Mayer, R., Monsigny, M., and Roche, A. C. (1993) Specific gene transfer mediated by lactosylated poly-L-lysine into hepatoma cells. *Nucleic Acids Res.* 21, 871–878.
- (25) Erbacher, P., Roche, A. C., Monsigny, M., and Midoux, P. (1995) Glycosylated polylysine/DNA complexes: gene transfer efficiency in relation with the size and the sugar substitution level of glycosylated polylysines and with the plasmid size. *Bioconjugate Chem.* 6, 401–410.
- (26) Kim, J.-S., Maruyama, A., Akaike, T., and Kim, S. W. (1997) In vitro gene expression on smooth muscle cells using a terplex delivery system. *J. Controlled Release* 47, 51–59.
- (27) Kim, J.-S., Maruyama, A., Akaike, T., and Kim, S. W. (1998) Terplex DNA delivery system as a gene carrier. *Pharm. Res.* 15, 117–122.
- (28) Choi, Y. H., Liu, F., Kim, J.-S., Choi, Y. K., Park, J. S., and Kim, S. W. (1998) Poly(ethylene glycol)-grafted poly-L-lysine as polymeric gene carrier. *J. Controlled Release* 54, 39–48.
- (29) Kabanov, A. V., Vinogradov, S. V., Suzdaltseva, Y. G., and Alakhov, V. Y. (1995) Water-soluble block polycations as carriers for oligonucleotide delivery. *Bioconjugate Chem.* 6, 639–643.
- (30) Promega Co. (1996) β -galactosidase enzyme assay system with reporter lysis buffer. Technical Bulletin, No. 097.
- (31) Wolfert, M. A., and Seymour, L. W. (1996) Atomic force microscopic analysis of the influence of the molecular weight of poly(L)lysine on the size of polyelectrolyte complexes formed with DNA. *Gene Ther.* 3, 269–273.
- (32) Perales, J. C., Grossmann, G. A., Molas, M., Liu, G., Ferkol, T., Harpst, J., Oda, H., and Hanson, R. W. (1997) Biochemical and functional characterization of DNA complexes capable of targeting genes to hepatocytes via the asialoglycoprotein receptor. *J. Biol. Chem.* 272, 7398–7407.
- (33) Perales, J. C., Ferkol, T., Molas, M., and Hanson, R. W. (1994) An evaluation of receptor-mediated gene transfer using synthetic DNA-ligand complexes. *Eur. J. Biochem.* 226, 255–266.
- (34) Olins, D. E., Olins, A. L., and von Hippel, O. H. (1967) Model nucleoprotein complexes: studies on the interaction of cationic homopolypeptides with DNA. *J. Mol. Biol.* 24, 157–176.
- (35) Shapiro, J. T., Leng, M., and Felsenfeld, G. (1969) Deoxyribonucleic acid-polylysine complexes. Structure and nucleotide specificity. *Biochemistry* 8, 3219–3232.
- (36) Promega Co. (1994) pSV- β -galactosidase control vector map and sequence. Promega Technical Bulletin TB094, 1.
- (37) Rosenthal, N. (1987) Identification of regulatory elements of cloned genes with functional assays. *Methods Enzymol.* 152, 704–720.
- (38) McKee, T. D., DeRome, M. E., Wu, G. Y., and Findeis, M. A. (1994) Preparation of asialoorosomucoid-polylysine conjugates. *Bioconjugate Chem.* 5, 306–311.
- (39) Stryer, L. (1988) Carbohydrates. *Biochemistry*, 3rd ed., p 345. W. H. Freeman and Company, New York.
- (40) Yamazaki, M., and Ito, T. (1990) Deformation and instability in membrane structure of phospholipid vesicles caused by osmophobic association: mechanical stress model for the mechanism of poly(ethylene glycol)-induced fusion. *Biochemistry* 29, 1309–1314.
- (41) Aring, J., Schlepper-Schaefer, J., Burkart, V., and Kolb, H. (1989) Nonenzymatically glycosylated serum albumin: interaction with galactose-specific liver lectins. *Biochim. Biophys. Acta* 1010, 140–144.
- (42) Lee, Y. C. (1989) Binding modes of mammalian hepatic Gal/GalNAc receptors. *Carbohydrate recognition in cellular function*, pp 80–95. Wiley, Chichester.

- (43) Kato, Y., and Sugiyama, Y. (1997) Targeted delivery of peptides, proteins, and genes by receptor-mediated endocytosis. *Crit. Rev. Ther. Drug Carrier Sys.* 14, 287–331.
- (44) Weitman, S. D., Lark, R. H., Coney, L. R., Fort, D. W., Fransca, V., Zurawski, Jr., V. R., and Kamen, B. A. (1992) Distribution of the folate receptor GP38 in normal and malignant cell lines and tissues. *Cancer Res.* 52, 3396–3401.
- (45) Campbell, I. G., Jones, T. A., Foulkes, W. D., and Trowsdale, J. (1991) Folate-binding protein is a marker for ovarian cancer. *Cancer Res.* 51, 5329–5338.
- (46) Ozawa, S., Ueda, M., Ando, N., Shimizu, N., and Abe, O. (1989) Prognostic significance of epidermal growth factor receptor in esophageal squamous cell carcinomas. *Cancer* 63, 2169–2173.
- (47) Ferkol, T., Kaetzel, C. S., and Davis, P. B. (1993) Gene transfer into respiratory epithelial cells by targeting the polymeric immunoglobulin receptor. *J. Clin. Invest.* 92, 2394–2400.
- (48) Luthman, H., and Magnusson, G. (1983) High efficiency polyoma DNA transfection of chloroquine treated cells. *Nucleic Acids Res.* 11, 1295–1308.
- (49) Erbacher, P., Bousser, M.-T., Raimond, J., Monsigny, M., Midoux, P., and Roche, A. C. (1996) Gene transfer by DNA/glycosylated polylysine complexes into human blood monocyte-derived macrophages. *Hum. Gene Ther.* 7, 721–729.
- (50) Liu, F., Song, F. C., Mix, Baudys, M., and Kim, S. W. (1997) Glucose-induced release of glycosyl poly(ethylene glycol) insulin bond to a soluble conjugated of concanavalin A. *Bioconjugate Chem.* 8 (5), 664–672.

BC980017V

Water-Soluble Conjugate of Double-Stranded DNA and Poly(*N*-isopropylacrylamide) for One-Pot Affinity Precipitation Separation of DNA-Binding Proteins

Daisuke Umeno, Masafumi Kawasaki, and Mizuo Maeda*

Department of Materials Physics and Chemistry, Graduate School of Engineering, Kyushu University, 6-10-1, Hakozaki, Higashi-ku, Fukuoka 812-8581, Japan. Received February 17, 1998;
Revised Manuscript Received June 4, 1998

Poly(*N*-isopropylacrylamide) having a terminal psoralen group was synthesized and covalently bound to plasmid pBR 322 DNA through a photochemical reaction. The resulting conjugate exhibited temperature-responsive precipitation due to the aggregation of poly(*N*-isopropylacrylamide) chains when heated above 31 °C. This system was used for the one-pot separation of restriction endonuclease *EcoRI*.

INTRODUCTION

Bioaffinity reactions using single- or double-stranded DNA have been widely used for the detection and purification of biomolecules. Double-stranded (ds) DNA is extensively employed as an affinity ligand (1, 2) for the separation and purification of DNA-binding proteins, such as transcription factors, and immobilized onto solid supports such as sepharose beads (3), synthetic polymer resins (4), and silica gels (5). These approaches, however, have fundamental disadvantages resulting from the insolubilization of DNA ligands. The vibrational and rotational motions of biomolecules immobilized on solid surfaces are restricted, and in most cases, these restrictions reduce the affinity of the ligands for the target molecules as compared with free DNA (6). In addition, solid–liquid heterogeneous reactions are generally slower than solution reactions due to restricted diffusion. Therefore, it is preferable that DNA ligands be immobilized on a water-soluble polymeric supports, enabling the homogeneous binding of the target molecules. However, there must exist a strategy for the collection of the molecules captured by the DNA ligands on the water-soluble carriers.

Poly(*N*-isopropylacrylamide) (polyNIPAAm) is one of the candidates which have both of the water solubility and collectable property: it has a phase transition between water-soluble and -insoluble forms in response to small temperature changes around 31 °C (7) and has recently been successfully utilized for the separation of biomolecules such as antibodies and enzymes (8, 9). In these systems, the binding of ligands and target molecules takes place in homogeneous solution (capturing mode) and the resulting complex is collected upon heat-induced precipitation (collection mode). This strategy is also applicable to the separation of DNA-binding proteins providing there exists a method for the conjugation of DNA with polyNIPAAm.

We have studied a method for the synthesis of soluble conjugates of ds DNA and synthetic polymers (10–12)

and recently found that psoralen derivatives are quite suitable for the stable conjugation between them (13). Psoralens are well-known DNA intercalators which cross-link double-stranded DNA when irradiated with a UV light (14). In previous work (15, 16), vinyl groups were introduced into dsDNA in two steps: a vinyl derivative of psoralen was photochemically bound to the ds DNA, then the resultant DNA-macromonomer was copolymerized with acrylamide in water. This resulted in the covalent grafting of polyacrylamide chains to ds DNA. The conjugate between calf thymus DNA and poly(*N*-isopropylacrylamide) was successfully applied to the separation of DNA-binding dyes (17). In the present study, we synthesized polyNIPAAm having a 4,5',8-trimethylpsoralen (trioxalen) moiety at its terminus. The psoralen-terminated polyNIPAAm was then photochemically bound to DNA. The resulting DNA–polyNIPAAm conjugate was applied to the one-pot affinity separation of restriction endonuclease *EcoRI*.

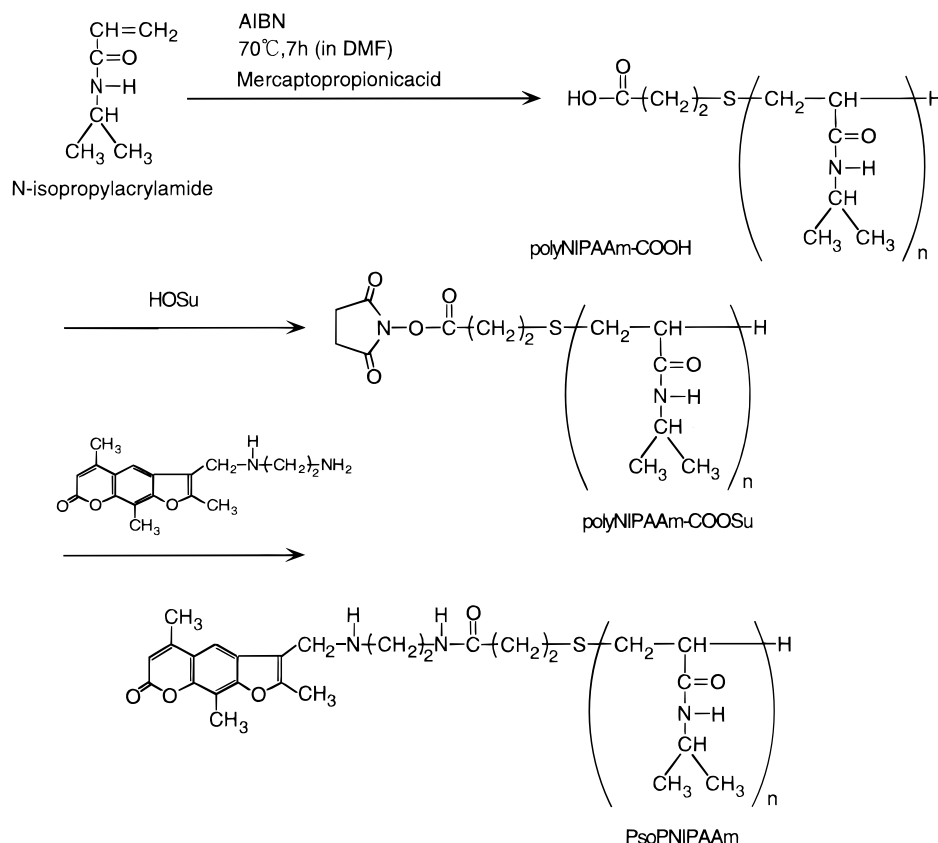
EXPERIMENTAL PROCEDURES

Materials. *N*-Isopropylacrylamide (NIPAAm) was obtained from Tokyo Kasei Kogyo and recrystallized from a mixture of benzene and hexane. *N,N'*-Azobisisobutyronitrile (AIBN) was purchased from Kishida Chemicals. Trioxalen (4,5',8-trimethylpsoralen) and 3-mercaptopropionic acid (MPA) were obtained from Aldrich. pBR322 DNA was purchased from Fermentas. *HindIII* and *EcoRI* were purchased from Boehringer Mannheim. Other reagents and solvents were obtained commercially and used without further purification.

Synthesis of Carboxyl-Terminated PolyNIPAAm (PolyNIPAAm-COOH). Semitelechelic polyNIPAAm possessing a carboxyl end group was synthesized by the method described by Takei et al. (18). Briefly, it was prepared by radical polymerization of NIPAAm in dimethylformamide (DMF) using AIBN as initiator and MPA as a chain-transfer agent. The polymer was purified by repeated reprecipitation from DMF-diethyl ether. The molecular weight of the resulting polymer was determined by acid–base titration of the terminal carboxyl group.

The phase-transition temperature (cloud point) of aqueous solutions of the polymer was monitored at 500

* Author to whom correspondence should be addressed.
Phone: +81-92-642-3606. Fax: +81-92-642-3611. E-mail: maedatcm@mbox.nc.kyushu-u.ac.jp.

Scheme 1. Synthetic Route for PsoPNIPAAm**Table 1. Analytical Data for PsoPNIPAAm and Precursors**

	number-averaged molecular weight	transition temperature (°C)
polyNIPAAm-COOH	5400 ^a	31.3
polyNIPAAm-COOSu	6700 ^b	
PsoPNIPAAm	6000 ^c	30.8

^a Calculated by NaOH titration of the terminal COOH group.^b Calculated from the UV absorption of the succinimidyl group at 260 nm ($\epsilon = 9700$). ^c Calculated by UV absorption of the psoralen moiety at 300 nm ($\epsilon = 11\,000$).

nm with temperature raised at the rate of 0.4 °C/min using a spectrophotometer (Hitachi, U-3210). The temperature at which the polymer solution exhibited 10% light transmittance was defined as the cloud point.

Activation of the Terminal Carboxyl Group of PolyNIPAAm. The carboxyl group at the polymer terminus was esterified using *N*-hydroxysuccinimide and dicyclohexylcarbodiimide (DCC) in a molar ratio of 1.0:1.2:1.2 in dry DMF at ca. 25 °C for 24 h. The reaction mixture was concentrated in vacuo and poured into diethyl ether to precipitate the product. The activated polymer (polyNIPAAm-COOSu) was purified by repeated reprecipitation from DMF-diethyl ether. Ester formation was confirmed by UV spectrometry (19).

Psoralen-Terminated PolyNIPAAm (PsoPNIPAAm). 4'-[[*N*-(2-Aminoethyl)amino]methyl]-4,5',8-trimethylpsoralen was synthesized according to a method described by Lee et al. (20). The amino derivative of psoralen (0.07 g, 235 μ mol) and the activated polyNIPAAm (polyNIPAAm-COOSu; 0.5 g, 94 μ mol) were dissolved in dry DMF (total volume, 10 mL). After stirring for 12 h at ca. 25 °C, the solution was concentrated in vacuo and poured into diethyl ether. The precipitate was collected

and washed with 100 mL of diethyl ether. The white powder was dried in vacuo. The polymer thus obtained was dissolved in pure water and its concentration was determined from the UV absorption due to the psoralen moiety (300 nm).

Photoimmobilization of PsoPNIPAAm on Plasmid pBR 322 DNA. Plasmid pBR322 DNA was linearized using *Hind*III and purified by phenol extraction and ethanol precipitation, then resuspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.4).

To a solution of linearized pBR 322 (615 μ M in base pair, bp) in a micro test tube (Eppendorf, 1.5 mL) was added a TE solution of PsoPNIPAAm. The total volume was then adjusted to 30 μ L to give a final DNA concentration of 62 μ M bp. The concentration of PsoPNIPAAm was varied from 50 nM to 10 μ M (in strand). Each solution was cooled in an ice bath and irradiated for 10 min (ca. 30 mW/cm²) using a 500 W ultrahigh pressure Hg lamp equipped with a high-pass filter (Toshiba, UV-31) for 10 min.

To the resultant mixture was added 9 μ L of aqueous NaOH (1.2 M) in order to make the DNA denatured, and then was combined with 9 μ L of gel-loading solution (glycerin:water, 1:1, v/v), quickly loaded to a 1.0% agarose gel, and electrophoresis was performed at 7 V/cm for 1 h in TAE buffer (80 mM Tris-acetate, 1 mM EDTA, pH 7.6). Gel electrophoresis was performed at 10 °C since polyNIPAAm and its conjugates precipitate from aqueous glycerin (25 v/v %) at ambient temperature (12). After electrophoresis, DNA in the gel was stained by ethidium bromide (Figure 1).

Thermal Response of the DNA-PolyNIPAAm Conjugate. To a solution of λ DNA (48 502 bp) was added PsoPNIPAAm from the stock (100 μ M in strand) to give a final concentration of 58 μ M bp DNA. The concentration of PsoPNIPAAm was varied from 0 to 50

μM (in strand). Samples were irradiated with UV light as described above, and the thermally induced precipitation of the resulting conjugates between λDNA and polyNIPAAm was examined. To the conjugate solution (100 μL) was added 20 μL of aqueous solution of poly(*N*-isopropylacrylamide) (10 w/v%; 1.7 w/v % in total) and the mixture was then incubated at 40 $^{\circ}\text{C}$ for 3 min and centrifuged (15 000 rpm) at the temperature for 3 min to precipitate the DNA–polyNIPAAm conjugate. After centrifugation, the supernatant was collected and subjected to gel electrophoresis (0.7% agarose, at 7 V/cm for 2 h) in TBE (80 mM Tris-borate, 2.5 mM EDTA, pH 8.0) buffer. After the gel was stained with ethidium bromide, the band due to the conjugate was evaluated using scanning densitometry to estimate the amount of unprecipitated conjugate.

Precipitation Separation of *EcoRI*. DNA–polyNIPAAm conjugates were synthesized by the photochemical reaction between pBR 322 (linearized using *BsmI*, 60 μM bp) and PsoPNIPAAm (20, 40, and 60 μM) in micro test tubes (30 μL in total) as described above. As a reference sample, a mixture of pBR 322 (*BsmI* digest, 58 μM bp) and PsoPNIPAAm (60 μM), which had not been irradiated with UV light, was also prepared. To these samples were then added *EcoRI* (60 units) and polyNIPAAm (1.0 w/v %). The solutions (total volume, 60 μL) were incubated at ca. 25 $^{\circ}\text{C}$ for 1 h then heated up to 40 $^{\circ}\text{C}$ in a water bath and centrifuged for 1 min at 40 $^{\circ}\text{C}$ (15 000 rpm). The supernatant (30 μL) was taken from each sample and combined with DNA (pBR 322/*BsmI* digest, 0.6 μg). DNA was not added to the supernatant of the reference sample (the mixture of the native DNA and polyNIPAAm), because the DNA in this precedent mixture remained in solution even after the heat and precipitation procedure unless it was conjugated to polyNIPAAm. These solutions were mixed with buffer (total volume, 40 μL ; 5.0 mM Tris-HCl, 10 mM NaCl, 1.0 mM MgCl_2 , 0.1 mM dithioerythritol, pH 7.5), and after incubation for 30 min at 37 $^{\circ}\text{C}$, samples were subjected to gel electrophoresis (1.0% agarose, at 7 V/cm for 1 h) in TBE buffer (Figure 3).

Sequence-Dependent Affinity Separation of *EcoRI*. DNA conjugates with and without *EcoRI* binding site were prepared. The former was synthesized by the photoreaction between PsoPNIPAAm (60 μL) and pBR 322 (61 μM bp) which had been linearized using *StyI*, while the latter was synthesized by the photoreaction between PsoPNIPAAm (60 μM) and pBR 322 (61 μM bp) linearized using *EcoRI*. A mixture of PsoPNIPAAm (60 μM) and pBR 322 (61 μM bp, linearized using *StyI*) was also prepared. Each sample was mixed with *EcoRI* (60 units) and polyNIPAAm (1.0 w/v %) and incubated for 2 h at 4 $^{\circ}\text{C}$, followed by the heating and precipitation procedure. Thirty microliters of each supernatant was collected, mixed with DNA (0.6 μg of pBR 322 linearized using *StyI*), and endonuclease reaction was allowed to proceed for 1 h upon the addition of Mg^{2+} (1 mM). The resultant solution was subjected to gel electrophoresis (1.0 % agarose, at 7 V/cm for 1 h) in TBE buffer (Figure 4).

RESULTS AND DISCUSSION

Psoralen-Terminated PolyNIPAAm. The synthesis of psoralen-terminated poly(*N*-isopropylacrylamide) (PsoPNIPAAm) is shown in Scheme 1. The number-averaged molecular weight of polyNIPAAm was determined by NaOH titration of terminal carboxyl group and the calculated value for the polyNIPAAm-COOH was about

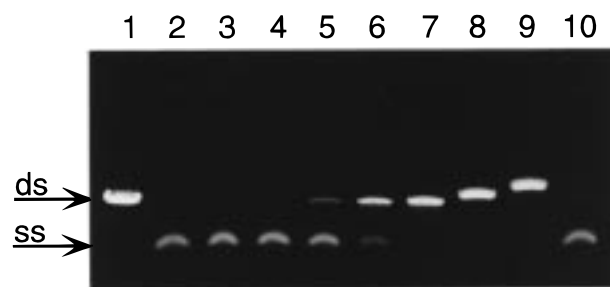


Figure 1. Gel electrophoresis (1.0 % agarose) of linear pBR 322 (62 μM in bp) which was irradiated with UV light in the presence or absence of PsoPNIPAAm. Samples from lane 2 to 10 were treated with NaOH (0.3 M) before loading. All samples were loaded on a nondenaturing gel. Lanes 1 and 2, control DNA; lane 3, [PsoPNIPAAm] = 0; lane 4 [PsoPNIPAAm] = 50 nM; lane 5 [PsoPNIPAAm] = 100 nM; lane 6 [PsoPNIPAAm] = 500 nM; lane 7 [PsoPNIPAAm] = 1 μM ; lane 8 [PsoPNIPAAm] = 5 μM ; lane 9 [PsoPNIPAAm] = 10 μM ; lane 10, [PsoPNIPAAm] = 10 μM , but no UV irradiation.

5400 (ca. 50 mer), which is in good agreement with the previous report (4500) (18). In each step of the synthesis, the nature of end groups was determined and the apparent molecular weights were estimated (Table 1). No significant differences were observed among the three molecular weights. Slight difference should be ascribed to the different methods of evaluation and to the fact that each reaction involved a reprecipitation which could change the molecular weight distribution.

PolyNIPAAm is a well known polymer which exhibits a phase transition (7): when an aqueous solution of polyNIPAAm is gradually heated, it abruptly precipitates at 31 $^{\circ}\text{C}$. The transition temperatures of the polymers prepared in this study were found to be all about 31 $^{\circ}\text{C}$ and the transition between soluble and insoluble form was fully reversible.

Photo-Induced Conjugation of PsoPNIPAAm to pBR 322. Psoralens are known to intercalate into DNA double strands in dark and form covalent bonds at their 3,4 and 4',5' double bonds with pyrimidines upon near-UV (~ 365 nm) irradiation (14). If both sites of the psoralen are reacted, an interstrand DNA cross-linking takes place to make both strands connected to each other.

Photoreactivity of the psoralen moiety which had been introduced to the terminus of polyNIPAAm was examined for pBR 322 plasmid DNA according to the literature (21). Figure 1 shows the electrophoretic migration profiles of linearized pBR 322 (*EcoRI* digest) after the photoreaction with PsoPNIPAAm and further treatment with 0.3 M NaOH. The alkali treatment denatures double-stranded DNA to give single strands, which are characterized by faster electrophoretic migration and the weaker band intensity (lane 2). The alkali denaturation also yielded the single strands from DNA which was irradiated with UV light (lane 3). On the contrary, there was observed significant amount of DNA which ran as ds form even after the alkali-treatment for DNAs which were mixed with PsoPNIPAAm and irradiated with UV light (lanes 4–9). Increasing amount of PsoPNIPAAm fed at the photoreaction resulted in increasing ratio of ds form DNA. When a certain amount of PsoPNIPAAm (1 μM for the 60 μM bp DNA) was used, 100% of DNA behaved as dsDNA (lanes 7–9), while even the highest concentration of PsoPNIPAAm produced no crosslinking in the absence of UV light (lane 10). This is because DNA double strands were cross-linked by the psoralen moiety of PsoPNIPAAm when irradiated with UV light, so that they were not separate to single strands even upon alkali treatment. Therefore, the cross-linked DNA quickly

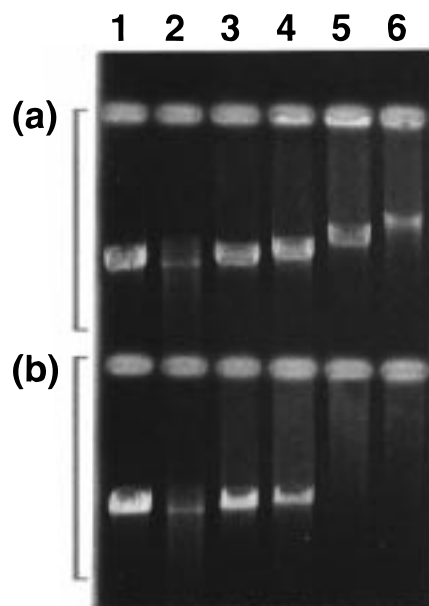


Figure 2. Gel electrophoresis (0.7 % agarose) of DNA-polyNIPAAm conjugates before (a) and after (b) centrifugation at 40 °C. The conjugate of polyNIPAAm and λ DNA (58 μ M bp) was heated and centrifuged in the presence of 1.7 wt% of NIPAAm homopolymer. Lane 1, DNA alone (control); lane 2, [PsoPNIPAAm] = 0 μ M; lane 3, [PsoPNIPAAm] = 2 μ M; lane 4, [PsoPNIPAAm] = 5 μ M; lane 5, [PsoPNIPAAm] = 10 μ M; lane 6, [PsoPNIPAAm] = 20 μ M.

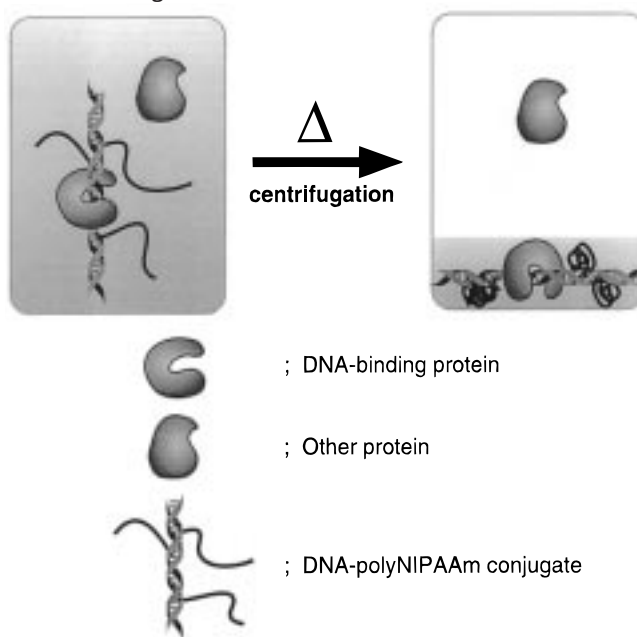
renatured to the ds form in the buffer (pH 7.6) used for gel electrophoresis and ran as the ds form, while non-cross-linked DNA remained single strands and ran with greater mobility. Thus, it was clearly shown that polyNIPAAm was successfully immobilized on the DNA strands with the aid of the terminal psoralen group when irradiated by UV light.

There was observed significant retardation in bands corresponding to the ds DNA which was reacted with PsoPNIPAAm, depending on the amount of PsoPNIPAAm used in the photochemical reaction (lanes 5–9). This result should reconfirm the modification of DNA with PsoPNIPAAm, since the conjugation should make the whole molecular size larger.

The gradual retardation dependently on the amount of PsoPNIPAAm (lanes 5–9) indicates that one can regulate the degree of modification with polyNIPAAm just by changing the amount of PsoPNIPAAm used in the photoreaction. We also found that the degree of modification can be regulated by changing reaction period, i.e., time period of UV irradiation (data not shown).

Temperature-Responsive Precipitation of the Conjugates. In order to take advantage of it for the precipitation separation, the concentration of polyNIPAAm was adjusted to 1.7 wt % by adding NIPAAm homopolymer because we found that a certain concentration of ca. 1.0 wt % is required to ensure reproducible precipitation. The conjugate solution was heated to 40 °C and centrifuged (15 000 rpm) at that temperature to give a white precipitate. The supernatant was carefully collected and subjected to gel electrophoresis. Figure 2 shows the electrophoretic profiles of the conjugate solution (upper lanes) and those of the supernatant after centrifugation at 40 °C (lower lanes). At the upper lanes of 3–6 are observed bands of DNA conjugates with less mobility than the control DNA (lane 1). Similarly to Figure 1, the degree of retardation increased with the amount of

Scheme 2. Affinity Precipitation Separation of DNA-Binding Substances

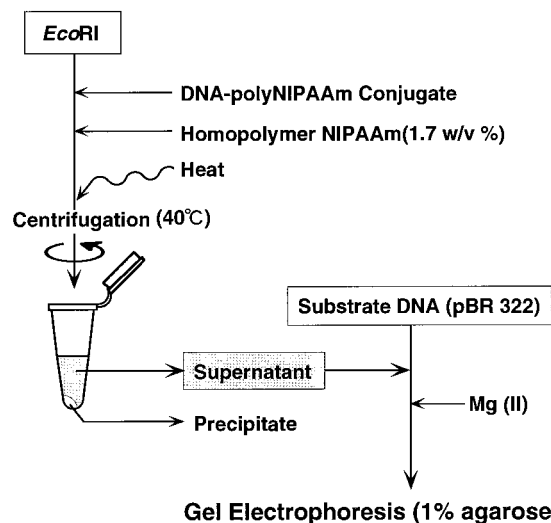


PsoPNIPAAm used in the photoinduced conjugation reaction. After heating and centrifugation, these profiles changed dramatically (lower lanes). The bands due to the DNA conjugate dramatically faded as the amount of polyNIPAAm used in the photochemical reaction was increased. At a certain level (lanes 5 and 6), almost 100% of the conjugate disappeared in the supernatant, due to precipitation. In contrast, the unirradiated mixture of DNA and PsoPNIPAAm did not show any change (lane 2). Samples containing relatively small amounts of PsoPNIPAAm did not show such diminution (lanes 3 and 4). These results indicate that the DNA conjugates acquired thermo-sensitivity derived from the polyNIPAAm chains. We assumed that the polyNIPAAm chains attached to DNA turned to be insoluble above 32 °C and the side chains and polyNIPAAm in bulk condensed via hydrophobic interaction to form a new phase separated from aqueous system, resulting in the distribution (extraction) of DNA conjugated with polyNIPAAm into the newly emerged phase.

Affinity Precipitation Separation of *Eco*RI. The conjugate was applied to the precipitation separation of a DNA-binding protein (Scheme 2). The restriction endonuclease *Eco*RI recognizes the 5'-GAATTC-3' unique sequence in pBR 322 (linearized using *B*smI; 4363 bp) to give two fragments of 3008 and 1355 bp.

The protocol is summarized in Scheme 3. The conjugate between pBR322 and PsoPNIPAAm was added to a buffer solution containing *Eco*RI. It should be noted that the *Eco*RI solution was free from magnesium ion in this stage so that DNA scission should not take place (22). It is reported that *Eco*RI bind tightly ($K_d \approx 10^{10}$) to its recognition site even in the absence of magnesium ion (23). The mixture was then centrifuged (15 000 rpm) at 4 °C to give a white precipitate. The supernatant was carefully collected and the endonuclease activity of any remaining *Eco*RI was evaluated. The results are shown in Figure 3. As seen in lane 1, the supernatant retained its *Eco*RI activity after the centrifugation (at 40 °C) in the presence of DNA and PsoPNIPAAm (no UV irradiation). In contrast, the *Eco*RI activity in the supernatant dramatically decreased after centrifugation when the pBR 322-polyNIPAAm conjugate was present (lanes

Scheme 3. Procedure for the precipitation separation of *Eco*RI and for the evaluation of precipitation/separation efficiency^a



^a DNA-polyNIPAAm conjugates were added to *Eco*RI (60 units) and the concentration of polyNIPAAm was adjusted to 1 wt %. The mixtures were heated at 40 °C and centrifuged to yield a white precipitate. The amount of *Eco*RI remaining in the supernatant was evaluated by its endonuclease activity.

2–4). The conjugate thus seems to be responsible for the decrease in the amount of *Eco*RI in the supernatant. These decrease in activity is proportional to the concentration of PsoPNIPAAm used in the modification of the DNA, reflecting the difference in the efficiency of precipitation of the conjugates. These results strongly corroborate the affinity separation system illustrated in Scheme 2.

To ascertain that the DNA conjugate functions as a sequence-dependent affinity separator for *Eco*RI, two types of conjugates were prepared and used for the precipitation separation of *Eco*RI: a conjugate between PsoPNIPAAm and pBR 322 digested with *Sty*I and a conjugate between PsoPNIPAAm and pBR 322 digested with *Eco*RI. The conjugates differ only in that the former has an *Eco*RI-binding site on its strand, while the latter does not. An aqueous solution of restriction endonuclease *Eco*RI (60 units), conjugate, and NIPAAm homopolymer (1.0 w/v % in total) were incubated, and then the mixture was heated to 40 °C and centrifuged at that temperature. The supernatant was collected and its *Eco*RI activity was determined.

The result is shown in Figure 4. Supernatant retained a certain level of *Eco*RI activity to give two fragments (1371 and 2992 bp) when the DNA-polyNIPAAm conjugate without the *Eco*RI-binding site was used as a separation agent (lane 4). The enzymatic activity was almost the same as that for the simple mixture of DNA and PsoPNIPAAm (no UV irradiation, lane 2) in the supernatant after the heating and precipitation. This indicates that the DNA-polyNIPAAm conjugate without an *Eco*RI binding site failed to complex with *Eco*RI. On the other hand, there was no sign of *Eco*RI activity in the supernatant when the DNA-polyNIPAAm conjugate having an *Eco*RI site was used (lane 3). These results indicate that ds DNA bearing a recognition site for *Eco*RI acts as an affinity ligand for *Eco*RI and that the presence of this site is exclusively responsible for the separation of *Eco*RI.

It has been clearly demonstrated that *Eco*RI was captured by the conjugate bearing its recognition site and

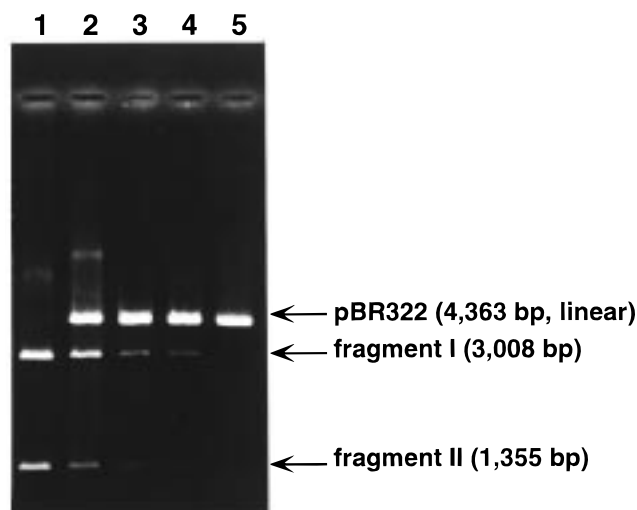


Figure 3. Precipitation separation of *Eco*RI using DNA-polyNIPAAm conjugates. *Eco*RI solution (60 units) and DNA-polyNIPAAm conjugates were mixed and the concentration of polyNIPAAm was adjusted to 1.0 w/v %. The mixtures were then heated at 40 °C and centrifuged. To a fixed volume of the supernatant, substrate DNA (pBR 322/*Bsm*I digest) and the reaction buffer were added, followed by incubation for 30 min at 37 °C. The solution was then subjected to gel electrophoresis. Lane 1, mixture of DNA (61 μM) and PsoPNIPAAm (60 μM); lanes 2–4, conjugates between DNA (61 μM) and polyNIPAAm (lane 2, 20 μM; lane 3, 40 μM; lane 4, 60 μM). Lane 5 is a control (*Bsm*I digest of pBR 322).

precipitated from aqueous solution as illustrated in Scheme 2. We have observed endonuclease activity in the precipitated fractions, but the quantitative evaluation of the recovery was not performed. The separation capacity of this system also remains to be clarified. Such information could be obtained if this system were used on a larger scale and could be much refined if the DNA sequence of the conjugate were properly designed. At present, plasmid DNA with a tandem array of *Eco*RI sites is being developed for further investigations of this separation system.

There remains an important question before applying this system to the practical uses: how could we recover the proteins isolated by this system? We assumed that it would be possible if the precipitation fraction was dissolved in fresh water and a certain concentration of salt was added. According to the literature (23), the interaction between *Eco*RI and its recognition sequence becomes drastically weak by increasing NaCl concentration, and *Eco*RI bound to the sepharose column carrying its recognition sequence could be easily eluted by adding ca. 0.3 M of KCl (24) or by adding 1.5 M of NaCl (25) to mobile phase. On the other hand, the transition temperature of polyNIPAAm is known to decrease with increasing salt concentration (7). According to our observation, it becomes as low as 15 °C at the NaCl concentration of 1.5 M and becomes insoluble at room temperature. Taking advantages of these two facts, *Eco*RI collected by our system would be separated from DNA-polyNIPAAm conjugate upon addition of NaCl (1.5 M) and isolated as supernatant fraction at room temperature. At present, however, we have no data supporting this conjecture.

We have demonstrated the affinity precipitation separation of DNA-binding molecules using a DNA-polyNIPAAm conjugate. This conjugate may provide a rapid and widely applicable separation technique, since the complexation with the target takes place under homogeneous conditions and the separation is accomplished

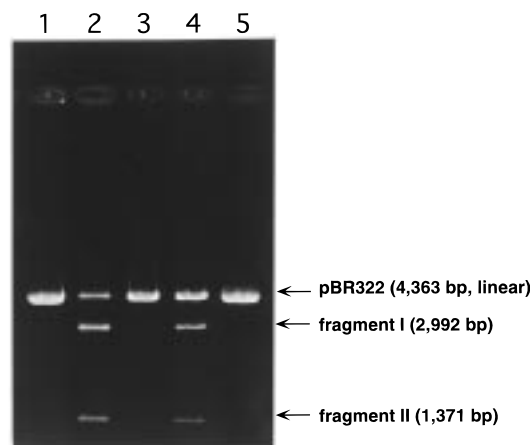


Figure 4. Sequence-dependent affinity separation of *EcoRI*. *EcoRI* (60 units), polyNIPAAm (1.0 w/v %), and conjugates were mixed and incubated for 2 h at 4 °C, then subjected to the heat and precipitation procedure. Supernatant was collected from each sample and the enzymatic activity of any residual *EcoRI* was evaluated. To a substrate DNA (0.6 μg of pBR 322 linearized using *StyI*) was added 30 μL of the supernatant, and the endonuclease reaction was performed for an hour upon adding Mg^{2+} (1.0 mM). The resultant solution was subjected to gel electrophoresis. Lanes 1 and 5, control DNA (pBR 322); Lane 2, the mixture of pBR 322 (62 μM) digested using *StyI* and PsoPNIPAAm (60 μM); lane 3, the conjugate between PsoPNIPAAm (60 μM) and pBR 322 digested using *StyI*; lane 4, conjugate between PsoPNIPAAm (60 μM) and pBR 322 digested using *EcoRI*.

in a single reaction vessel. The present method allows us to use double-stranded DNA as an affinity ligand, so that one may collect any ions, molecules, or macromolecules which bind to the DNA.

ACKNOWLEDGMENT

We thank Dr. Scott J. McNiven (Research Center for Advanced Science and Technology, the University of Tokyo) for helpful discussion. This work was supported in part by a Grant-in-Aid for Scientific Research from Ministry of Education, Science, Sports and Culture of Japan. Financial support by the General Sekiyu Research and Development Encouragement and Assistance Foundation is also acknowledged.

LITERATURE CITED

- (1) Kadonaga, J. T. (1991) Purification of Sequence-Specific Binding Proteins by DNA Affinity Chromatography. *Methods Enzymol.* 208, 10–23.
- (2) Jarrett, H. W. (1993) Affinity Chromatography with Nucleic Acid Polymers. *J. Chromatogr.* 618, 315–339.
- (3) Kadonaga, J. T., and Tjian, R. (1986) Affinity Purification of Sequence-Specific DNA Binding Proteins. *Proc. Natl. Acad. Sci. U.S.A.* 83, 5889–5893.
- (4) Kawaguchi, H., Asai, A., Ohtsuka, Y., Watanabe, H., Wada, T., and Handa, H. (1989) Purification of DNA-Binding Transcription Factors by Their Selective Adsorption on the Affinity Latex Particles. *Nucleic Acids Res.* 17, 6229–6240.
- (5) Goss, T. A., Bard, M., and Jarrett, H. W. (1991) High-Performance Affinity Chromatography of Messenger RNA. *J. Chromatogr.* 588, 157–164.
- (6) Bunemann, H. (1982) Immobilization of Denatured DNA to Macroporous Supports: II. Steric and Kinetic Parameter of Heterogeneous Hybridization Reactions. *Nucleic Acids Res.* 10, 7181–7196.
- (7) Schild, H. G. (1992) Poly(*N*-isopropylacrylamide): Experiment, Theory and Application. *Prog. Polym. Sci.* 17, 163–249.
- (8) Chen, J. P., Yang, H. J., and Hoffman, A. S. (1990) Polymer-Protein Conjugates. II. Affinity Precipitation Separation of Human Immunoglobulin by a Poly(*N*-isopropylacrylamide)-Protein A Conjugate. *Biomaterials* 11, 631–634.
- (9) Takei, Y. G., Matsukata, M., Aoki, T., Sanui, K., Ogata, N., Kikuchi, A., Sakurai, Y., and Okano, T. (1994) Temperature-Responsive Bioconjugates. 3. Antibody-Poly(*N*-isopropylacrylamide) Conjugates for Temperature-Modulated Precipitations and Affinity Bioseparations. *Bioconjugate Chem.* 5, 577–582.
- (10) Maeda, M., Hirai, A., and Takagi, M. (1989) Gel Electrophoretic Demonstration of Pseudo-Grafting of Polyacrylamide onto λ Phage DNA. *Chem. Lett.* 1989, 1831–1834.
- (11) Maeda, M., Hirai, A., and Takagi, M. (1991) Pseudo-Grafting of Polyacrylamide onto *HindIII* Restriction Fragments of λ DNA. *Reactive Polym.* 15, 103–109.
- (12) Maeda, M., Nishimura, C., Inenaga, A., and Takagi, M. (1993) Modification of DNA with Poly(*N*-isopropylacrylamide) for Thermally Induced Affinity Separation. *Reactive Polym.* 21, 27–35.
- (13) Kashiwagi, S., Ohmori, K., Maeda, M., and Takagi, M. (1992) Efficient Coupling of DNA on a Silica Support with Psoralen Derivatives as Anchoring Agents for High-Performance Liquid Affinity Chromatography. *Anal. Sci.* 8, 261–263.
- (14) Cimino, D. G., Gamper, H. B., Isaacs, S. T., and Hearst, J. E. (1985) Psoralens as Photoactive Probes of Nucleic Acid Structure and Function: Organic Chemistry, Photochemistry, and Biochemistry. *Ann. Rev. Biochem.* 54, 1151–1193.
- (15) Maeda, M., Nishimura, C., Umeno, D., and Takagi, M. (1994) Psoralen-Containing Vinyl Monomer for Conjugation of Double-Helical DNA with Vinyl Polymers. *Bioconjugate Chem.* 5, 527–531.
- (16) Maeda, M., Umeno, D., Nishimura, C., and Takagi, M. (1996) Conjugation of DNA with Functional Vinyl Polymers by Using Vinyl Derivative of Psoralen as a Linker. In *Hydrogels and Biodegradable Polymers for Bioapplications* (R. M. Ottenbrite, S. J. Huang, and K. Park, Eds.) pp 197–208. American Chemical Society, Washington, DC.
- (17) Umeno, D. and Maeda, M. (1997) Poly(*N*-isopropylacrylamide) Carrying Double-Stranded DNA for Affinity Separation of Genotoxins. *Anal. Sci.* 13, 553–556.
- (18) Takei, Y. G., Aoki, T., Sanui, K., Ogata, N., Okano, T., and Sakurai, Y. (1993) Temperature-Responsive Bioconjugates. 1. Synthesis of Temperature-Responsive Oligomers with Reactive End Groups and Their Coupling to Biomolecules. *Bioconjugate Chem.* 4, 42–46.
- (19) Miron, T. and Wilchek, M. (1982) A Spectrophotometric Assay for Soluble and Immobilized *N*-Hydroxysuccinimide Esters. *Anal. Biochem.* 126, 433–435.
- (20) Lee, B. L., Murakami, A., Blakes, K. R., Lin, S.-B., and Miller, P. S. (1988) Interaction of Psoralen-Derivatized Oligodeoxyribonucleoside Methylphosphonates with Single-Stranded DNA. *Biochemistry* 27, 3197–3203.
- (21) Saffran, W. A., Welsh, J. T., Knobler, R. M., Gasparro, F. P., Cantor, C. R., and Edelson, R. L. (1988) Preparation and Characterization of Biotinylated Psoralen. *Nucleic Acids Res.* 16, 7221–7231.
- (22) Halford, S. E. and Johnson, N. P. (1980) The *EcoRI* Restriction Endonuclease with Bacteriophage Lambda DNA. Equilibrium Binding Studies. *Biochem. J.* 191, 593–604.
- (23) Terry, B. J., Jack, W. E., Rubin, R. A., and Modrich, P. (1983) Thermodynamic Parameters Governing Interaction of *EcoRI* Endonuclease with Specific and Nonspecific DNA Sequences. *J. Biol. Chem.* 258, 9820–9825.
- (24) Vlatakis, G., and Bouritos, V. (1991) Sequence-Specific DNA Affinity Chromatography: Application to the Purification of *EcoRI* and *SphI*. *Anal. Biochem.* 195, 352–357.
- (25) Blanks, R. and McLaughlin, W. (1991) An Oligonucleotide Affinity Column for the Isolation of Sequence-Specific DNA Binding Proteins. *Nucleic Acids Res.* 19, 10283–10299.

In Vitro Enzymatic Biotinylation of Recombinant Fab Fragments through a Peptide Acceptor Tail

Petri Saviranta,* Tapio Haavisto, Pekka Rappu, Matti Karp, and Timo Lövgren

Department of Biotechnology, University of Turku, Tykistökatu 6, FIN-20520 Turku, Finland.
Received February 19, 1998; Revised Manuscript Received July 2, 1998

We describe the site-specific enzymatic biotinylation of recombinant anti-estradiol Fab fragments through a 13 amino acid acceptor peptide translationally fused to the C-terminus of the Fd chain. The Fab-peptide fusion proteins were secreted to the periplasm of *Escherichia coli*, purified, and biotinylated in vitro using biotin ligase, biotin, and ATP. The *E. coli* biotin ligase (the BirA protein) was produced as a novel N-terminal fusion protein with glutathione S-transferase (GST) and purified in one step from bacterial cell lysate using a Glutathione Sepharose affinity column. The purified fusion protein worked as such (without cleavage of the GST part) for the in vitro biotinylation of the Fab fragments. After the removal of nonbiotinylated Fab fragments by monomeric avidin chromatography, the overall yield of biotinylated Fab was 40%. The site-specifically biotinylated Fab fragments (BioFab) were tested in streptavidin-coated microtitration wells, to which they were shown to bind linearly with respect to the amount of BioFab added, specifically as indicated by biotin inhibition, and tightly with a half-life of several days. Moreover, the enzymatic BioFab exhibited uniform antigen binding affinity unlike the same recombinant Fab fragments biotinylated through random chemical conjugation to surface lysines. Finally, the BioFab demonstrated its potential as a well-behaving immunoassay reagent in a model competitive assay for estradiol.

INTRODUCTION

The biotin–avidin/streptavidin system is used in a variety of biotechnological and diagnostic applications owing to the exceptionally high affinity of this noncovalent interaction between a protein and a small organic molecule (Wilchek and Bayer, 1990a). In in vitro diagnostics, streptavidin-coated tubes, microtitration wells, or spherical particles are used as universal surfaces for the capture of biotinylated antibodies in immunoassays of various formats (Diamandis and Christopoulos, 1991). The biotinylation of antibodies can be done by several well-established chemical methods in which an activated biotin derivative is conjugated to the protein surface residues (most often lysine) to the hinge region cysteines or to a carbohydrate moiety (Bayer and Wilchek, 1990; Diamandis and Christopoulos, 1991; Hermanson, 1996). The most widely used biotin derivative, biotinyl-*N*-hydroxysuccinimide ester (BNHS),¹ reacts mainly with the ϵ -amino groups of surface lysines and, under optimal conditions, generally causes no serious damage to the antigen binding activity (Bayer and Wilchek, 1990). Mikkala et al. (1993) synthesized an isothiocyanate derivative of biotin (BITC), which is, like BNHS, prima-

rily reactive with amino groups. The BITC reagent has since been used in our laboratory for the biotinylation of a number of different purified mAbs and has proven to be a viable complement to BNHS and other commercially available biotin reagents.

The recent advances in antibody engineering (for a review, see Hayden et al., 1997) and the availability of bacterially produced recombinant Fab fragments has led to a situation in which increasingly more antibodies will be biotinylated in the Fab form. As the Fab fragment is only one-third of the size of the whole immunoglobulin, the chemical biotinylation at surface residues even at low degrees of conjugation will more often result in the destruction or modification of the antigen-binding site. The recombinant Fab fragments come with an advantage that they can be modified by protein engineering. For example, new functionalities useful in specific applications can be added to the carboxyl ends of the constant domains while leaving the variable regions intact (Rappley, 1995). Therefore, we sought for a protein engineering solution to aid in the safe biotinylation of recombinant Fab fragments.

In the living cells, a few proteins (depending on the organism) are naturally biotinylated through the covalent linkage of biotin to a unique lysine residue (Wood, 1977). This posttranslational modification is catalyzed by a biotin ligase and involves the formation of an amide bond between the carboxyl group of biotin and the ϵ -amino group of the lysine residue (Figure 4A; Shenoy and Wood, 1988). In *Escherichia coli*, the only biotinylated protein is the biotin carboxyl carrier protein (BCCP), a subunit of the acetyl-CoA carboxylase. The C-terminal 84–88 amino acids of BCCP form an independent domain that can be fused to recombinant proteins and biotinylated in vivo by the *E. coli* endogenous biotin ligase (Cronan, 1990; Li and Cronan, 1992). Although the biotinylation

* Author to whom correspondence should be addressed. Tel: 358-2-333 8083. Fax: 358-2-333 8050. E-mail: Petri.saviranta@utu.fi.

¹ Abbreviations: apo-Fab, nonbiotinylated form of the Fab-biotin acceptor peptide fusion protein; BioFab, Fab fragment that is biotinylated through the 13 amino acid peptide tail; BITC, biotin isothiocyanate; BirA, the bifunctional biotin-holocarboxylase synthetase/biotin operon repressor protein of *Escherichia coli*; BNHS, biotinyl-*N*-hydroxysuccinimide ester; BSA, bovine serum albumin; dNTP, deoxynucleoside triphosphate; GST, glutathione S-transferase of *Schistosoma japonicum*; IPTG, isopropyl β -D-thiogalactopyranoside; OD₆₀₀, optical density at the wavelength of 600 nm.

takes place in the cytoplasm, also fusions of the biotinyl domain with periplasmic proteins such as alkaline phosphatase and β -lactamase can be biotinylated to some extent (Reed and Cronan, 1991). The biotinylation of secreted fusion proteins can be enhanced by slowing down the secretory process (using *sec⁻* strains) so as to win extra time for the action of the biotin ligase and/or by coexpressing the biotin ligase so as to increase the biotinylation rate. (Reed and Cronan, 1991; Jander et al., 1996). However, we did not want to compromise the secretion of the Fab fragments and, therefore, considered an alternative approach to achieve higher biotinylation yields. We chose to perform enzymatic biotinylation *in vitro* after the production of the recombinant Fab fusion protein, which enabled us to control the biotinylation conditions independently of the various cellular factors. Furthermore, to possibly improve the secretion and periplasmic stability of the fusion protein, we aimed at minimizing the size of the fusion partner. Schatz (1993) used an elegant selection strategy to find novel peptide substrates for the biotin ligase from plasmid-encoded random peptide libraries. He defined a consensus sequence of 13 amino acids, containing an internal lysine residue, sufficient for *in vivo* biotinylation in *E. coli*. We here describe the construction of a Fab fusion vector in which the coding sequence for such a biotin acceptor peptide is inserted at the C-terminus of the Fd chain.

The biotin ligase (biotin holoenzyme synthetase) of *E. coli* is a bifunctional protein coded by the *birA* gene (Barker and Campbell, 1981). In addition to being biotin ligase, the BirA protein acts as the repressor of the *bio* operon responsible for biotin biosynthesis (Cronan, 1989). The BirA protein can be overexpressed in *E. coli* up to 1000-fold with apparently no toxic effects (Buonocristiani and Otsuka, 1988) thus facilitating the production of large amounts of biotin ligase for *in vitro* studies. To simplify the enzyme purification, we constructed a fusion protein between BirA and the glutathione S-transferase (GST) of *Schistosoma japonicum* (Smith and Johnson, 1988), which allowed us to use Glutathione-Sepharose for the one step purification of the enzyme from crude cell lysate. In this study, we show that the purified fusion protein is capable of *in vitro* biotinylation of the 13 amino acid peptide tail in the presence of the substrates biotin and ATP.

Recently, we cloned and expressed in *E. coli* the recombinant Fab fragment of the monoclonal anti-estradiol antibody 57-2 (Pajunen et al., 1997). In this paper, we describe the construction of the biotin acceptor peptide fusion protein of Fab 57-2 as well as the *in vitro* biotinylation of the apo-Fab using the GST-BirA fusion protein. The immunochemical properties of the enzymatically biotinylated Fab fragment relevant to immunoassay applications will be characterized and discussed. Finally, the functionality of the novel biotinylated Fab fragment is demonstrated by setting up a competitive estradiol immunoassay.

MATERIALS AND METHODS

Strains and Plasmids. *E. coli* MC1061 [cI⁺, Δ (*ara*, *leu*)7697, Δ *lacX*74, *galU*⁻, *galK*⁻, *hsr*⁻, *hsm*⁺, *strA*, *araD*139; Casadaban and Cohen, 1980] was used for DNA cloning and for the overproduction of the GST-BirA fusion protein. The strain XL-1 Blue [*recA*1, *endA*1, *gyrA*96, *thi*-1, *hsdR*17, *supE*44, *relA*1, *lac* (F' *proAB*, *lacI*^q Δ M15, Tn10 (tet^r)), Stratagene] was used for Fab expression. The plasmid pJMR1 containing the *birA* gene from the *E. coli* biotin biosynthetic operon was kindly provided by

Dr. Anthony J. Otsuka. The plasmid pGEX-1 λ T with the glutathione S-transferase gene of *Schistosoma japonicum* was purchased from Pharmacia Biotech (Uppsala, Sweden).

Construction of Biotin Acceptor Peptide:Fab Genetic Fusion. The plasmid p57-2 (Pajunen et al., 1997) was used as the vector for the insertion of the biotin acceptor peptide coding sequence. The vector was digested with the restriction enzymes *Spe*I and *Nhe*I and dephosphorylated with calf intestinal phosphatase (Pharmacia) followed by agarose gel purification of the 4.6 kb fragment. The insert was prepared from two partially complementary (15 nucleotide overlap at the 3'-ends) synthetic oligonucleotides: the forward 43-mer 5'-GTAC ACT AGT GG C GGT GGC CCA GGT GGC CTG GTT TCT ATC TT-3' and the reverse 53-mer 5'-CTAG GCT AGC TTA GTG CCA TTC GAT TTT CTG AGC TTC GAA GAT AGA AAC CAG-3'. The forward and reverse oligonucleotides (50 pmol each) were annealed together by heating to 90 °C for 5 min and slowly cooling to room temperature. The single-stranded ends were filled in with the Klenow fragment of DNA polymerase I (Boehringer Mannheim) for 15 min at room temperature in the presence of 1 mM dNTPs. The double-stranded 80-mer was digested with *Spe*I and *Nhe*I (the recognition sites are underlined in the oligonucleotide sequences above) followed by agarose gel purification with the Glass Milk procedure (Bio101). The purified fragment was ligated to the vector with T4 DNA ligase and electroporated into *E. coli* MC1061 cells. The presence of the insert was tested from plasmid minipreps by digestion with *Sfi*I, and the correct coding sequence was verified by nucleotide sequencing over the whole insert region. The plasmid construct (p572Bio) was later transferred to the strain XL-1 Blue for Fab production. An otherwise identical construct but lacking the biotin tail was produced for the chemical biotinylation experiments (see below) by self-ligating the compatible cohesive ends of the *Spe*I-*Nhe*I digested vector (the open reading frame ends right after the *Nhe*I site at the tandem stop codons TAG TAA).

Construction of the GST-BirA Genetic Fusion. The plasmid pGEX-1 λ T was used as the vector for the construction of the GST-BirA genetic fusion. The vector was linearized by digestion with *Eco*RI followed by filling in the cohesive ends with the Klenow fragment of DNA polymerase I for 15 min in the presence of 1 mM dNTPs. The *birA* gene was cut from the plasmid pJMR1 by double digestion with *Bsp*HI and *Xba*I and was made blunt ended similarly as the vector. The insert and the vector were gel purified, blunt ligated with T4 DNA ligase, and electroporated into MC1061 cells. The orientation of the insert as well as the integrity of the joints in the resulting plasmid were checked by test digestions with the appropriate restriction enzymes (in the correct construct all the restriction sites were regenerated as a result of the ligation of the blunt-ended fragments).

Overproduction and Purification of the GST-BirA Fusion Protein. *E. coli* MC1061 strain containing the plasmid pGEX-BirA was inoculated in two 1-L shake flasks each containing 250 mL of 2 $\times$ YT (16 g/L Bacto tryptone, 10 g/L Yeast extract, and 5 g/L NaCl) supplemented with 0.2% glucose and 100 mg/mL ampicillin. The cells were grown at 30 °C at 240 rpm until the OD₆₀₀ of 1 was reached. The fusion protein expression was induced by the addition of 0.1 mM IPTG, after which the cells were grown for an additional 6 h and harvested by centrifugation. The cell pellet was resuspended in 15 mL of PBS buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na₂-

HPO₄, and 1.8 mM KH₂PO₄, pH 7.4) containing 5% glycerol, 0.1 mM DTT, and 10 mM MgCl₂. The cells were disrupted with the French press (Aminco Corp.) after which DNaseI was added into the lysate (final concentration 1 µg/mL) and the cell debris was removed by centrifugation. Triton X-100 was added to a final concentration of 1% (v/v), and the extract was filtered with a 0.45 µm filter.

GST-BirA fusion protein was purified from the cell extract by affinity chromatography through Glutathione Sepharose 4B (Pharmacia Biotech). The column (bed volume 5 mL) was equilibrated with PBS buffer containing 5% glycerol, 0.1 mM DTT, 10 mM MgCl₂, and 1% Triton X-100. The sample (15 mL) was loaded on the column at a flow rate of 0.5 mL/min, followed by washing with 10 vol of the equilibration buffer except that the concentration of Triton X-100 was reduced to 0.01%. The purified product was eluted with 10 mM reduced glutathione in 50 mM Tris-HCl, pH 8.0, containing 5% glycerol and 10 mM MgCl₂. The pooled fractions were concentrated, and the protein concentration was determined by Bradford's protein determination assay (Bradford, 1976) using BSA as a standard. The purified GST-BirA protein was divided into aliquots and stored at -20 °C in a buffer containing 52% glycerol, 50 mM NaCl, 24 mM Tris-HCl, pH 8.0, 4.7 mM reduced glutathione, and 4.7 mM MgCl₂.

Fab Production and Purification. A fresh colony of *E. coli* XL-1 Blue cells containing the plasmid p572Bio was inoculated in 5 mL of SB medium (30 g/L tryptone, 20 g/L Yeast extract, and 10 g/L MOPS, pH 7.0) supplemented with glucose (0.2%), ampicillin (100 µg/mL) and tetracycline (10 µg/mL) and grown at 300 rpm, 37 °C until the OD₆₀₀ of approximately 0.5 was reached. One milliliter of the preculture was inoculated in 50 mL of SB medium supplemented with 0.05% glucose, 50 µg/mL ampicillin, 5 µg/mL tetracycline, and 100 µM IPTG and shaken at 240 rpm and 30 °C for 14 h. The cells were collected by centrifugation, resuspended in 10 mL of TBS buffer (50 mM Tris-HCl, pH 7.5, and 150 mM NaCl), and disrupted by sonication (model Labsonic U fitted with the needle probe 40T, B. Braun, Germany). The lysate was clarified by centrifugation (20000g, 4 °C, 15 min), the supernatant was recovered and stored frozen (-20 °C) until the purification. The Fab was purified from the clarified lysate with a prepacked 1 mL HiTrap Protein G column (Pharmacia Biotech) according to the manufacturer's instructions. Briefly, the thawed lysate was filtered through a 0.22 µm filter and loaded on the column which was preequilibrated with TBS buffer. After washing with 5 column volumes of TBS, the Fab was eluted with 100 mM glycine-HCl buffer, pH 2.5, containing 0.01% Tween20, at a flow rate of 0.5 mL/min. Fractions (1 mL) were collected in Eppendorf tubes that contained 30 µL of neutralization buffer (2 M Tris, pH 9.0, and 0.33% BSA). After the determination of the immunoreactive Fab concentrations, the peak fractions were pooled and stored at +4 °C.

Immunoassay Reagents and Equipment. All immunochemical measurements were performed using the DELFIA technology (Wallac, Finland), which is based on the time-resolved measurement of fluorescent lanthanide chelates formed in the enhancement solution after the lanthanide ion has been dissociated from the original, nonfluorescent chelate conjugated to the biomolecule. The following DELFIA assay reagents and equipment were used: (rabbit) anti-mouse IgG-coated microtitration strips, streptavidin-coated microtitration strips, europium-labeled streptavidin, Assay buffer, Washing solution,

Enhancement solution, and Plate Fluorometer (model 1232). Two europium-labeled estradiol derivatives, conjugated either from C4 or C6 of the steroid skeleton, were used as immunoassay tracers. The position 6 conjugate (estradiol-6-carboxymethyl oxime conjugated to an europium chelate; Pajunen et al., 1997) was kindly provided by H. Mikola while the position 4 conjugate (unpublished material) was obtained from N. Meltola.

Determination of Immunoreactive and Biotinylated Fab Fragments. Immunoreactive Fab concentrations were determined with a microtitration plate assay using europium-labeled estradiol (C4 conjugate) as the detection marker. Purified, nontailed recombinant Fab 57-2 (Pajunen et al., 1997) was used as the calibration standard. All dilutions were made in the Assay buffer. Samples and standards (200 µL) were added to pre-washed anti-mouse IgG-coated microtitration strips, shaken for 60 min at room temperature, followed by four washes after which 200 µL of europium-labeled estradiol (2 nM) was added to each well. The incubation was continued for 60 min, after which the strips were washed four times and Enhancement solution (200 µL) was added. The time-resolved fluorescence signal was measured with the Plate Fluorometer, controlled by the MultiCalc immunoassay program (1224-310) running on a personal computer. The program automatically produced a spline-fitted standard curve and calculated the unknown Fab concentrations.

The detection of biotinylated Fab fragments was performed as above, except that Eu-labeled streptavidin at 100 ng/mL instead of Eu-labeled estradiol was used. The degree of biotinylation was estimated by comparing the signals obtained from the same sample detected with either Eu-labeled estradiol or Eu-labeled streptavidin.

Enzymatic Biotinylation. The Fab sample (3 mL) was changed into the biotinylation buffer (100 mM potassium phosphate, 12 mM MgCl₂, and 0.06% Tween20) using a Centricon-30 concentrator (Amicon) with two cycles of concentration and dilution. Buffer-exchanged Fab (50 µg) was used for a 500 µL biotinylation reaction which contained 10 mM ATP, 100 µM D-biotin, 1 µg/mL pyrophosphatase, and 100 µg/mL of the GST-BirA fusion protein in the biotinylation buffer. The reaction was kept at 37 °C for 4 h, after which the unreacted biotin was removed by gel filtration using a prepacked Sephadex G-25 column (NAP-5, Pharmacia Biotech). The biotinylated and unbiotinylated Fab fragments were separated using monomeric avidin agarose (the Softlink resin, Promega) according to the manufacturer's instructions. Briefly, 1 mL of the Softlink resin was packed into a disposable minicolumn, regenerated with 10% acetic acid and equilibrated with TBS-Tween (TBS, pH 7.5, containing 0.05% Tween20). The Fab sample was passed through the column at a flow rate of 0.5 mL/min, followed by washing with 5 mL of TBS-Tween, after which the biotinylated Fab was eluted with 5 mM D-biotin in TBS-Tween supplemented with 0.01% BSA. Fractions (0.5–1.0 mL) were collected and analyzed for the immunoreactive Fab concentrations. The peak fractions eluted with biotin were pooled, and the free biotin was removed by repeated concentration with Centricon-30 followed by dilution with TBS-Tween. After seven cycles of concentration/dilution, the final volume was 0.3 mL and the estimated residual free biotin concentration was 0.3 µM.

Chemical Biotinylation with Biotin Isothiocyanate. Biotin isothiocyanate (BITC) was obtained as a gift from V.-M. Mikkala. BITC is reactive mainly with the primary amino groups in the proteins (Mikkala et al., 1993) and is used the same way as the commonly used

N-hydroxysuccinimide derivatives of biotin. The chemistry of the biotinylation reaction is described in Figure 4B. The recombinant Fab 57-2 fragments used for the chemical biotinylation were produced using an expression vector that did not contain the biotin acceptor tail at the end of the Fd chain. Purified Fab, originally stored in 10 mM MES, pH 6.0, was buffer exchanged into 0.9% NaCl using a NAP5 column (Pharmacia Biotech). The biotinylation was performed in a reaction volume of 100 μ L containing 100 μ g (2 nmol) of Fab, either 30 or 50 nmol of freshly dissolved BITC, and 50 mM sodium carbonate buffer (pH 9.8). The BITC reagent was used in either 15- or 25-fold molar excess, known from previous experience to be near optimal for Fab fragments. For a number of reasons, the conditions used do not lead to the stoichiometric conjugation of biotin and therefore we prefer to state the molar reagent concentrations (300 and 500 μ M) instead of the reagent excess per se. The biotinylation reaction was incubated at room temperature for 4 h after which the unreacted BITC was separated by passage through two buffer exchange columns (NAP5, followed by NAP10, Pharmacia Biotech), equilibrated with TSA (50 mM Tris-HCl, pH 7.75, 150 mM NaCl, and 0.02% NaN_3). After the buffer exchange, BSA (0.1%) and NaN_3 (0.05%) were added and the sample was filtered through a 0.22 μ m filter. The biotinylated fraction (>90%) was purified using the monomeric avidin resin similarly as described for the purification of the enzymatically biotinylated Fab.

Immunochemical Characterization of Biotinylated Fab Fragments. The biotinylated Fab fragments were tested in streptavidin-coated microtitration strips for binding capacity, biotin inhibition, binding stability, and antigen affinity constant. All incubations were done at room temperature, the strips covered with an adhesive tape. All dilutions were made in the Assay buffer.

To determine the *binding capacity* of the streptavidin-coated wells for BioFab, a series of BioFab samples (0.03–60 ng/200 μ L) was added to the wells and shaken for 60 min at room temperature, followed by four washes. The bound BioFab was then detected with europium-labeled estradiol (10 nM C6 conjugate/200 μ L) which was shaken for 30 min, followed by four washes. Finally, the europium content in the wells was determined by adding 200 μ L of Enhancement solution, shaking for 30 min to develop the fluorescent chelates, and measuring the europium signals with the Plate Fluorometer.

In the *biotin inhibition* test, the samples (200 μ L) contained fixed amounts of Fab (5.4 ng) and europium-labeled estradiol (2 nM C6 conjugate), while each sample contained a different concentration of biotin (0–10 μ M). The premixed samples were added to the streptavidin-coated wells, shaken for 60 min, and washed four times. Since the europium-labeled estradiol was already present from the beginning, there was no need for a separate tracer incubation for the detection of the bound BioFab. The europium signals were measured as above.

The *binding stability* test was started by preattaching BioFab to the streptavidin-coated microtitration wells (5.4 ng/200 μ L, 120 min), after which further binding and rebinding was stopped by the addition of 20 μ L of 33 μ M biotin (final concentration 3 μ M). The incubation was continued for varying times (15 min–24 h), followed by the removal of unbound/dissociated BioFab with four washes. The bound BioFab was detected with europium-labeled estradiol (2 nM C6 conjugate, 30 min, four washes); the europium signals were measured as described above.

In the *affinity constant* determination, the BioFab samples (2 ng/200 μ L) were preattached to the streptavidin-coated wells for 120 min, followed by four washes. Europium-labeled estradiol (200 μ L) (C4 tracer) was added at varying concentrations (0.08–8 nM) to each well and incubated for 2 h, followed by the removal of the unbound tracer with four washes. Finally, the bound tracer concentrations were determined by the measurement of the europium signals as before.

Calculation of Affinities. The affinities (K_d constants) were calculated with the KELL RADLIG program version 5.0.2 (Biosoft, Cambridge, U.K.). The analysis was performed with the hot saturation mode; binding data were input as molar concentrations; free ligand was calculated from specific bound. The multisite saturation models (1, 2, or 3 binding sites) were fitted with the $1/Y^2$ weighting; the fits were statistically compared using a built-in approximate F test included in the RADLIG program. In essence, the test determined whether there was a significant decrease in the weighted sum of squared deviations, given the increase in the model parameters when assuming more binding sites.

Competitive Immunoassay for Estradiol. BioFab (2 ng in 200 μ L of assay buffer) was preattached to streptavidin-coated microtitration wells for 60 min followed by four washes. Estradiol standards (0–100 nM) were prepared directly in the tracer solution (0.2 nM C6 conjugate in assay buffer). Each standard (200 μ L) (i.e., mixture of estradiol and tracer) was added to three replicate wells, incubated for 60 min, and washed four times, after which the measurement of the bound tracer was performed as above. The MultiCalc immunoassay program was used for the data management and the following calculations. A four-parameter logistic fitting algorithm was used to produce the standard curve and the associated parameters: ED_{50} , i.e., the concentration corresponding to the half-maximal signal and the slope factor, i.e., the measure of the steepness of the sigmoidal curve. The estradiol concentrations in the individual wells were back-calculated from the fitted standard curve using the measured signals, after which the coefficient of variation (CV%) was calculated for each set of triplicate wells.

RESULTS

Design of the Fab-Biotin Acceptor Peptide Fusion Protein. To produce the Fab-biotin acceptor peptide fusion protein, we constructed the plasmid p572Bio (Figure 1). The Fd and light chain genes of the original anti-estradiol mAb 57-2 (Pajunen et al., 1997) were previously cloned into the phage display vector pComb3 (Barbas et al., 1991), where the Fd chain was fused to the carboxyl-terminal domain of the gene III protein of the filamentous phage M13. We excised the gene III fragment using the unique flanking restriction sites *SpeI* and *NheI* and replaced it by a synthetic DNA cassette containing the coding sequence for a 13 amino acid biotinylation signal, adapted from Schatz (1993). To increase the availability of the biotin acceptor peptide for the BirA protein, we inserted a flexible linker of eight amino acids between the peptide and the carboxyl end of the Fd chain. The cassette was constructed from two overlapping synthetic oligonucleotides which also included the restriction sites *SpeI* and *NheI* for cloning into the pComb3 vector and an internal unique *SfiI* site for the checking of the presence of the insert. The nucleotide sequence of the cassette is shown in the upper part of Figure 1.

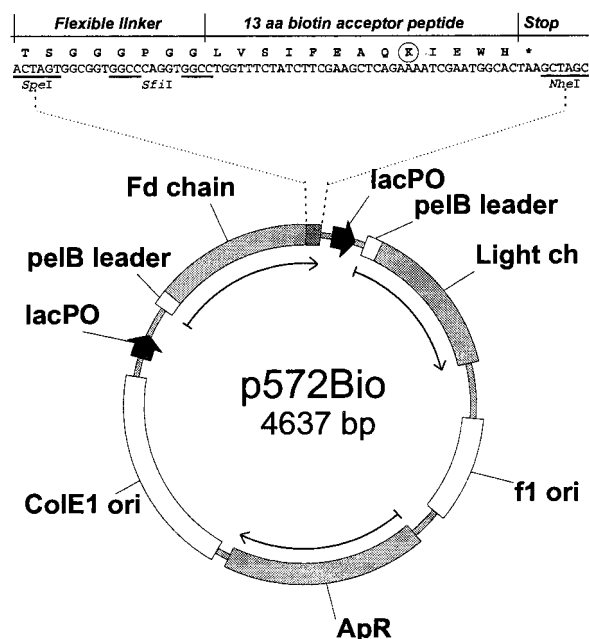


Figure 1. Map of the plasmid p572Bio. The expression of the heavy (Fd) and the light chains is controlled by the *lac* promoter-operator (*lacPO*) in front of each gene. *pelB* signal sequences, fused to the amino termini of each chain, direct the synthesized polypeptides to the periplasmic space. The biotin acceptor peptide coding region is cloned in-frame to the carboxyl terminus of the Fd chain which ends right before the *SpeI* site. The nucleotide sequence between the *SpeI* and *NheI* sites as well the translated amino acid sequence are shown above the plasmid map. The biotinylated lysine residue is indicated by a circle. Labels: ColE1 ori, the region containing the plasmid origin of replication; f1 ori, the region containing a filamentous phage origin of replication; ApR, ampicillin resistance (beta-lactamase) gene.

Construction, Expression and Purification of the GST-BirA Fusion Protein. The translational fusion between the GST and the BirA protein was carried out by cloning the *birA* gene of *E. coli* to the GST gene fusion vector pGEX-1 λ T containing the gene for the glutathione S-transferase of *S. japonicum* (Smith and Johnson, 1988). In the resulting plasmid pGEX-BirA, the GST coding region, followed by a thrombin cleavage site, is fused in-frame to the first methionine codon of the full-length *birA* gene (Figure 2). The overexpression of the fusion protein was achieved by IPTG induction of the strong *tac* promoter, resulting in the accumulation of approximately 10 mg/L of the fusion protein in the cells. Comparison of the SDS-PAGE samples of the whole cell lysate before and after centrifugation suggests that the fraction of insoluble protein was less than 30% of the total recombinant protein produced (Figure 3, lanes 2–3).

The GST-BirA protein was purified from the clarified cell lysate by one-step affinity chromatography using a glutathione Sepharose 4B column. The purification procedure was highly efficient as judged by the SDS-PAGE of the samples taken before the column, from the flow-through fraction and from the eluted material (Figure 3, lanes 3–5). The eluted fusion protein (lane 5) appeared to be over 90% pure, migrated roughly according to the expected size (62 kDa), and showed no signs of proteolytic degradation into truncated fragments such as the mere GST part (~26 kDa). The final yield of the purified GST-BirA from the 500 mL shake flask culture was 3.5 mg.

Purification and Biotinylation of apo-Fab 57-2. The total production level of apo-Fab in a shake flask

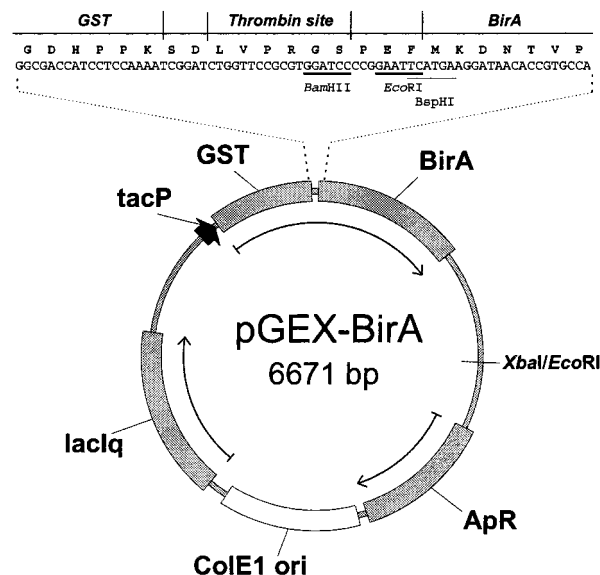


Figure 2. Map of the plasmid pGEX-BirA. A *BspHI*-*XbaI* fragment containing the *birA* gene was excised from the plasmid pJMR1 (a gift from A. Otsuka) and cloned into the *EcoRI* site of the vector pGEX-1 λ T. The cohesive ends of the insert and the linearized vector were filled in with the Klenow fragment of DNA polymerase I and ligated as blunt-ended fragments. The ligation of the insert in the correct orientation resulted in the regeneration of all the original restriction sites. The GST-BirA fusion gene is controlled by the strong *tac* promoter (*tacP*) which is in turn repressed by the plasmid-encoded *lac* repressor (*lacIq*). Fusion protein expression is induced by IPTG which derepresses the *tac* promoter. The joining region from the end of the glutathione S-transferase gene to the start of the *birA* gene is shown above the plasmid map. The thrombin site is indicated above the amino acid translation. For other labels, see legend to Figure 1.

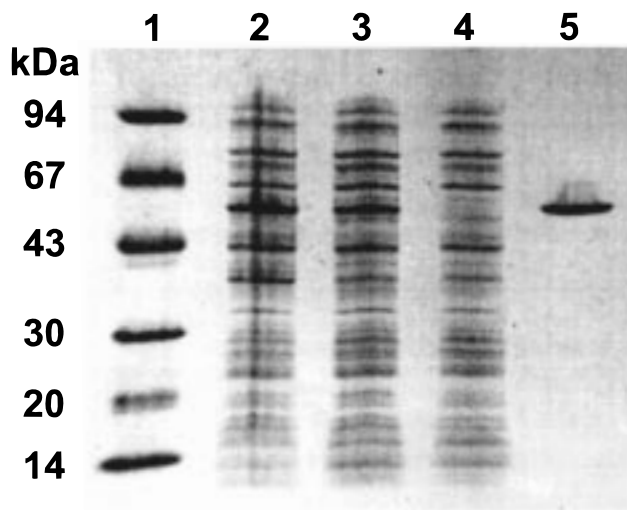


Figure 3. SDS-PAGE analysis of the purification of the GST-BirA fusion protein. Samples were diluted in PBS, mixed with equal volume of 2 $\times$ reducing sample buffer and heated to 95 $^{\circ}$ C for 5 min. After a brief centrifugation, 1 μ L of each sample was loaded on a PhastGel 8–25% polyacrylamide gel which was run with SDS buffer strips using the PhastSystem electrophoresis apparatus (Pharmacia Biotech). Samples: molecular weight markers (1); cell lysate before (2) and after (3) the removal of insoluble material by centrifugation; flow through fraction (4) and eluate (5) of the glutathione Sepharose 4B chromatography.

culture after overnight induction at 30 $^{\circ}$ C was 800 μ g/L, of which 80% remained in the periplasm. The cells were disrupted by sonication after which the apo-Fab was purified from the total cell lysate by protein G chromatography. This simple miniscale purification procedure

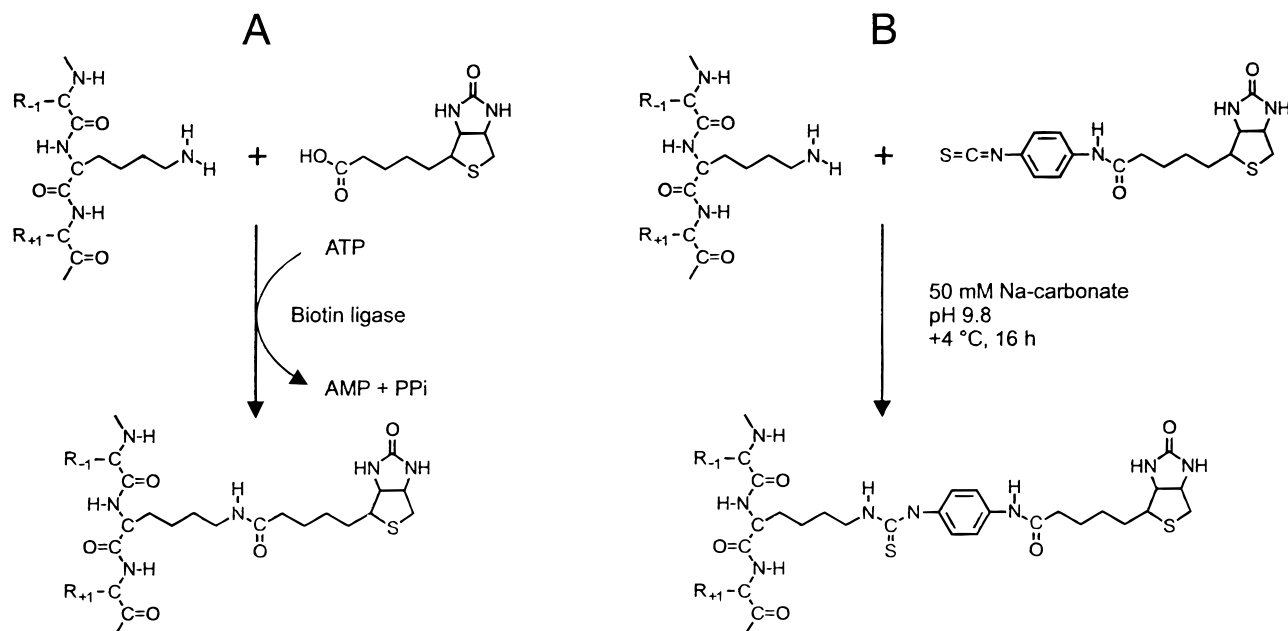


Figure 4. Protein biotinylation via the ϵ -amino group of a lysyl residue. A three-residue fragment of a larger polypeptide chain is shown with the lysyl residue in the middle. R_{-1} and R_{+1} denote the side chains of the residues preceding and following the lysyl residue. (A) The enzymatic biotinylation reaction catalyzed by the biotin ligase. The reaction proceeds through a biotinyl-AMP intermediate from which the biotinyl moiety is transferred to the acceptor protein. (B) The chemical biotinylation reaction using BITC. The nucleophilic amine of the lysyl residue attacks the central, electrophilic carbon of the isothiocyanate group. An electron shift and a proton loss create a stable thiourea bond.

Table 1. Purification and Biotinylation of the apo-Fab

procedural step	volume (mL)	Fab concn ($\mu\text{g/mL}$)	Fab mass (μg)	yield (%) ^a step-wise	overall
initial purification					
clarified lysate	10	21	207	100	100
after freeze and thaw	10	16	160	77	77
protein G pool	3	26	79	50	38
biotinylation ^b					
reaction mixture	0.5	100	50	100	100
after buffer exchange	1.1	48	53	106	106
monomeric avidin column ^c					
flow-through + washes	5.5	5.3	29	55	58
eluted fractions (pool)	2.5	6.2	16	29	31
total out			45	85	90
concentrated pool	0.3	64	19	123	38

^a Percentage of Fab remaining after each step, expressed with respect to the preceding step (stepwise yield) as well as with respect to the starting point of the procedure (overall yield). ^b The complete biotinylation procedure consisted of the enzymatic reaction, buffer exchange with NAP5 column, and separation of biotinylated and nonbiotinylated Fab with monomeric avidin column and the final concentration of the pooled fractions with a Centricon 30 concentrator. ^c All the buffer-exchanged material (1.1 mL) was loaded on the monomeric avidin column.

gave a relatively low overall yield (38%), since there were losses both in the intermediate frozen storage of the clarified lysate as well as in the protein G chromatography (Table 1). No optimization of the purification method was attempted.

GST-BirA fusion protein was used to biotinylate 50 μg of the purified apo-Fab. After the biotinylation reaction and a buffer change to remove free biotin, the biotinylated and nonbiotinylated Fab fragments were separated with monomeric avidin chromatography (SoftLink resin); the bound Fab fragments were eluted from the column with 5 mM free biotin. All fractions (flow-through, wash as well as elution) were recovered and subjected for the determination of immunoreactive Fab concentrations (Table 1). The total sum of the collected fractions was 85% of the input, consisting of 55% in the flow-through

and wash fractions (representing the apparently nonbiotinylated Fab fragments) and 29% in those fractions eluted with biotin (representing the biotinylated Fab fragments). Thus, the degree of biotinylation could be estimated to be about $29/85 \approx 34\%$. The fact that apparently 15% of the input Fab was not recovered at all may be partly explained by minor underestimation of the individual fraction volumes. After the pooling and final concentration steps, we had 19 μg of purified BioFab, which constituted 38% of the initial 50 μg used for the biotinylation procedure. Thus the net efficiency of the whole procedure was estimated to be 38% (bottom line of Table 1).

The degree of biotinylation was also estimated by another method in which the Fab samples were first immobilized on secondary antibody-coated wells and then detected either with europium-labeled streptavidin or with europium-labeled estradiol. For each sample, the ratio of the signals obtained with europium-labeled streptavidin versus europium-labeled estradiol was taken as an indicator of the degree of biotinylation. The analysis of the Fab samples before and after the enzymatic reaction as well as after the final purification is described in Table 2. Assuming that the signal ratio of the purified BioFab (4.68) represents a fully biotinylated population, the efficiency of the biotinylation reaction was calculated to be 42%, agreeing well with the previous estimate based on the separation of the biotinylated and nonbiotinylated forms with the monomeric avidin resin (above). The degree of biotinylation in vivo (i.e., before the enzymatic in vitro reaction) was approximately 2%.

Binding of BioFab to Streptavidin-Coated Wells.

The interaction of the enzymatically biotinylated Fab 57-2 with coated streptavidin was characterized in terms of the binding capacity, specificity, and stability. To study the binding capacity, varying amounts of BioFab (0.003–160 ng) were incubated in the streptavidin-coated wells. After washes, the bound BioFab was detected using europium-labeled estradiol as the tracer. The

Table 2. Degree of Biotinylation^a

process stage	streptavidin signal (CPS)	estradiol signal (CPS)	ratio ^b	proportion ^c biotinylated
purified apo-Fab	5460	54 398	0.10	2%
after enzyme reaction	129 680	65 403	1.98	42%
purified BioFab	422 192	90 294	4.68	100%

^a Fab samples were captured to anti-mouse IgG coated microtitration strips and detected with either europium-labeled streptavidin or europium-labeled estradiol. The streptavidin signal is proportional to the amount of biotinylated fragments in the sample while the estradiol signal is proportional to the amount of Fab fragments containing intact binding sites. ^b The ratio of the signals obtained with labeled streptavidin and estradiol. ^c The extent of biotinylation was estimated by comparing the streptavidin/estradiol signal ratio at each stage with that of the purified BioFab. It was assumed that the BioFab fragments purified with the monomeric avidin resin were completely biotinylated.

dependence of the measured europium signals on the amount of BioFab added is shown in Figure 5A. The response stays virtually linear for almost 5 orders of magnitude, indicating that the binding of the BioFab is not dependent on its own concentration. No saturation of the streptavidin surface could be demonstrated within the range of BioFab concentrations tested; therefore, the binding capacity is at least 160 ng/well.

As a control for the specificity of the attachment of BioFab to the streptavidin-coated wells, we tested whether the binding could be inhibited by free biotin. Figure 5B shows how the BioFab binding decreased rapidly as the free biotin concentration went from 40 to 100 nM, indicating that BioFab and biotin competed for the same binding sites. Consequently, the observed binding of the BioFab to the surface was not caused by general non-specific forces such as the stickiness of the protein. The inhibition experiment also revealed that 3 μ M biotin concentration blocked practically all BioFab binding. Consequently, this concentration was used in the stability test (below) to prevent rebinding of dissociated BioFab fragments.

The stability of the complex between BioFab and the coated streptavidin in the microtitration well was studied by incubating the preformed complexes in 3 μ M biotin solution for varying times, followed by washes and detection of the bound BioFab with labeled antigen. Although 3 μ M biotin effectively blocked the binding of BioFab when added simultaneously (Figure 5B), it could not displace BioFab already bound to streptavidin (Figure 5C). In the time period of 24 h, less than 10% of the bound BioFab dissociated from streptavidin, indicating that the half-life of the complex must be several days.

Affinity Comparison of Enzymatically and Chemically Biotinylated Fab Fragments. To study whether the enzymatic and chemical biotinylation methods differed in their effects on the antigen-binding affinity, we made a binding site titration using labeled antigen. Biotinylated Fab fragments were immobilized on streptavidin-coated wells, after which varying concentrations of europium-labeled estradiol (C4 conjugate) were added and allowed to equilibrate between the bound and free states. Figure 6A shows that the binding sites were efficiently titrated as the signal levels of the bound labeled antigen were approaching saturation at the higher total concentrations. To determine the affinity constants, the data were initially plotted as bound/free vs bound (the Scatchard's plot) so as to calculate the K_d s from the slopes of the plots by linear regression (Figure 6B). However, the plots for the chemically biotinylated Fab fragments were clearly curved, suggesting the pres-

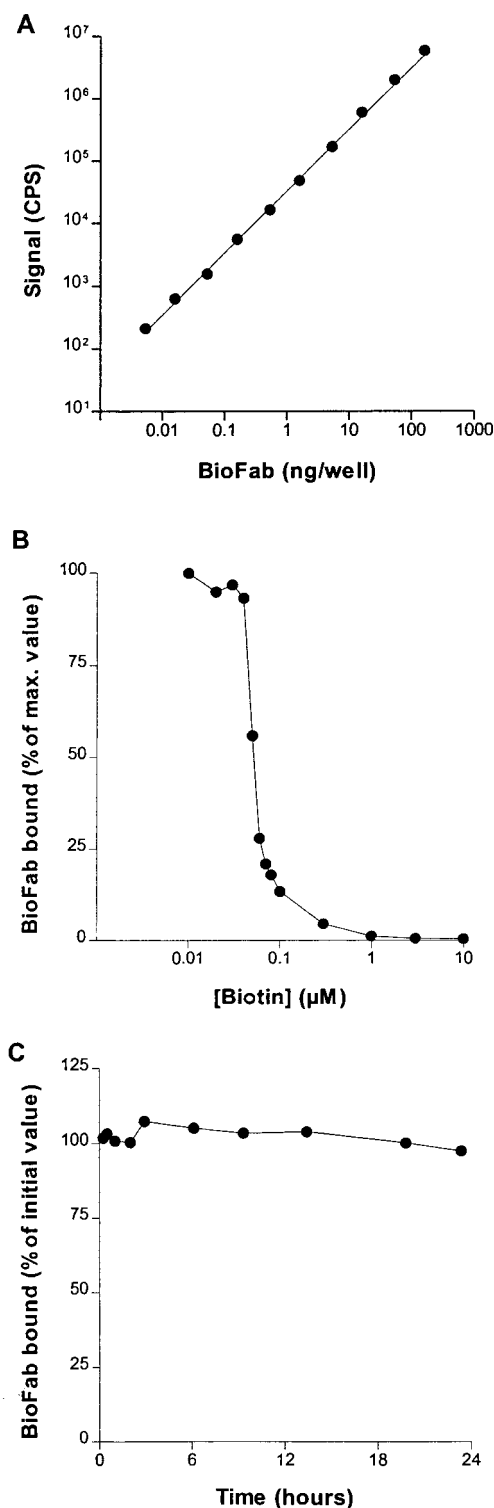


Figure 5. Testing the binding of BioFab to streptavidin-coated microtitration wells. The amount of BioFab bound to the wells was detected with europium-labeled antigen (C6 conjugate of estradiol). Each symbol in the plots represents the average of two replicate wells. (A) Capacity. The signal of the bound labeled antigen is linearly dependent on the amount of BioFab added throughout the tested range. (B) Specificity. BioFab binding is inhibited by increasing concentrations of free biotin. (C) Stability. The dissociation of bound BioFab from the coated streptavidin was monitored for 24 h. Excess free biotin was used to block the rebinding of dissociated BioFab.

ence of two or more subpopulations of binders with different affinity constants. Therefore, the binding data were nonlinearly fitted to different models involving one

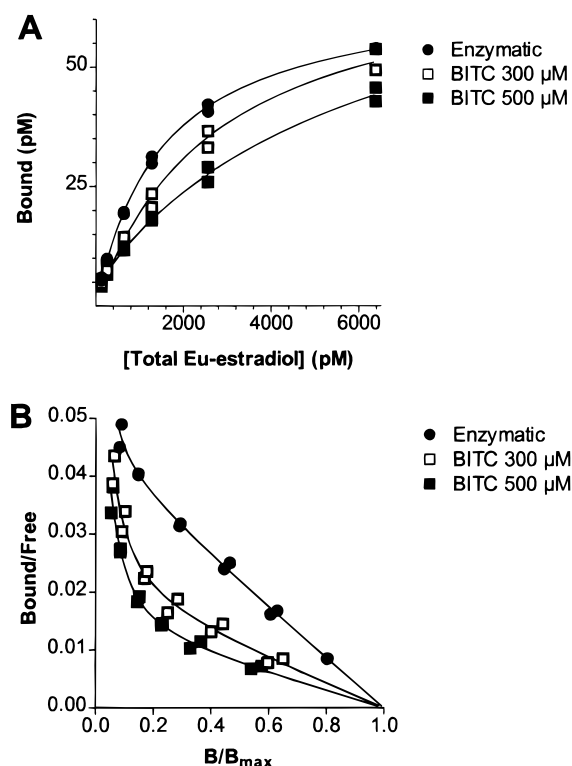


Figure 6. Affinity determination of the enzymatically and chemically biotinylated Fab fragments. Biotinylated Fab fragments were immobilized on streptavidin-coated wells, after which the binding sites were titrated with europium-labeled estradiol (C4 conjugate). (A) The bound concentration (pM) is plotted against the total added concentration of europium-labeled estradiol. The replicates (two parallel wells) are plotted separately. (B) Scatchard plot presentation of the same data. As an exception to the conventional presentation (bound/free vs bound), the x values were normalized to the form $B/B_{\max}$. This way, all the curves cross the x-axis at the same point and can be more readily compared. (The absolute $B_{\max}$ values are shown in Table 3). The curves drawn through the data points were fitted according to the model involving two binding sites.

ligand and either one, two, or three binders. Statistical comparison between the fits (performed by the RADLIG fitting program) supported the model involving two classes of binders in each case, although the exact nature of the minor classes could not be accurately defined. Realizing that the two-binder model only gave the statistically most plausible fit for the available binding data, we concentrate on the comparison of the major binder classes within that model (Table 3). While the major class always represented more than 90% of the total binding site population, it can be seen that the enzymatically biotinylated Fab fragments were the most uniform (98.6% major binder). The dissociation constants (K_d) of the chemically biotinylated Fab fragments were 2.6–3.6-fold higher than that of the enzymatically biotinylated Fab (Table 3), suggesting that the random attachment of biotin lead in most cases to the decrease of antigen-binding affinity.

Competitive Immunoassay for Estradiol. To demonstrate the usage of the BioFab as an immunoassay reagent, we set up a competitive estradiol immunoassay in streptavidin-coated microtitration strips, using europium-labeled estradiol (C6 conjugate) as the tracer. The B_0 signal (corresponding to zero estradiol concentration) was approximately 51 000 cps whereas the signals obtained with estradiol standards between 1 and 100 nM decreased from 44 800 down to 4300, covering practically the whole measurable concentration range (Figure 7).

Table 3. Affinities for Europium-Labeled Estradiol^a

biotinylation method ^b	percentage of major binder ^c	$B_{\max}$ (pM $\pm$ SD)	K_d (nM $\pm$ SD)
enzymatic	98.6%	66.0 $\pm$ 1.5	1.57 $\pm$ 0.16
BITC 300 μ M	94.5%	78.2 $\pm$ 9.5	4.11 $\pm$ 1.34
BITC 500 μ M	93.5%	74.2 $\pm$ 9.4	5.70 $\pm$ 1.62

^a The affinities (K_d constants) of the biotinylated Fab fragments for europium-labeled estradiol were calculated from the data obtained in the hot saturation experiment shown in Figure 6. ^b The Fab fragments were biotinylated either enzymatically (using the GST-BirA fusion protein as the biotin ligase) or chemically (using either 300 or 500 μ M BITC reagent). ^c The binding data were initially fitted into models involving either one, two or three binding site subpopulations. The two site model was the statistically most plausible one in each case, even when the second sites were present at very low proportions. However, the binding parameters of the minor sites could not be accurately defined and were omitted from the table.

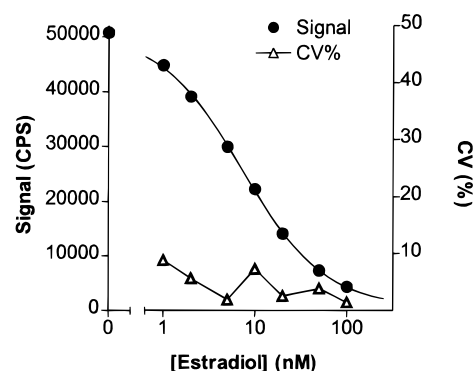


Figure 7. Competitive immunoassay for estradiol. The assay was performed in streptavidin-coated microtitration plates to which purified, enzymatically biotinylated anti-estradiol Fab fragments (BioFab) were precaptured in a separate incubation. The assay incubations (60 min) contained a fixed concentration of tracer (0.2 nM europium-labeled C6 conjugate of estradiol) and a varying concentration (0–100 nM) of estradiol; the x-axis refers to the final estradiol concentrations in the wells. After washes, the bound tracer was measured with time-resolved fluorescence as described in materials and methods. Filled circles indicate the average signal (CPS, left y-axis) obtained from the measurement of three replicate wells for each estradiol concentration. The MultiCalc program was used for curve fitting as well as for the reverse calculation of estradiol concentrations from the measured signals for the individual wells. The coefficient of variation (CV%, right y-axis) of the calculated estradiol concentrations for each set of triplicate wells is indicated by open triangles.

The europium signals were fitted with a sigmoidal dose–response curve model (the four-parameter logistic equation) producing a standard curve with an ED_{50} of 6.7 nM and a slope factor of -0.94 . When the estradiol concentrations of the standards were reverse calculated from the fitted curve using the measured signals, the coefficient of variation (CV%) of the concentrations remained less than 10% throughout the range (Figure 7), indicating that the assay is technically reliable and could be applied to the measurement unknown estradiol concentrations.

DISCUSSION

We describe the site-specific enzymatic biotinylation of recombinant anti-estradiol Fab fragments through a 13 amino acid acceptor peptide translationally fused at the C-terminus of the Fd chain. To our knowledge, this is the first report on the in vitro application of the biotin ligase to biotinylate antibodies. Moreover, we describe the usage of a novel fusion protein (GST-BirA) as the biotinylating enzyme.

The production of correctly folded recombinant Fab fragments in *E. coli* requires that they be secreted to the periplasm where the oxidative conditions allow for the formation of the necessary disulfide bridges (Plückthun and Skerra, 1989). Because efficient secretion is not ideally compatible with in vivo biotinylation (Reed and Cronan, 1991; Jander et al., 1996), we decided not to try to optimize the intracellular conditions but rather perform the biotinylation in vitro. Nevertheless, we observed a low (2%) basal level of biotinylation in the protein G purified apo-Fab fragments (Table 2). A similar figure was reported by Weiss et al. (1994) in a study of secreted recombinant Fab fragments to which a 101 amino acid C-terminal fragment of the *E. coli* BCCP was fused at the C-terminus of the Fd chain. Relying on the activity of the endogenous biotin ligase, they initially achieved an in vivo biotinylation level of 3%, although they reported a 5-fold enhancement of the basic level on the addition of biotin to the growth medium. The basal biotinylation level observed by us and by Weiss et al. (1994) may represent the fraction of fusion proteins that are biotinylated in the cytoplasm before they are secreted (Reed and Cronan, 1991); possibly some biotinylation occurs also when cytoplasmic biotin ligase enters the periplasm as a result of partial cell lysis typical of recombinant Fab expression. Furthermore, here we used a shortcut (sonication) instead of osmotic shock for the extraction of the periplasmic Fab fragments and, thus in effect, mixed the cytoplasm and periplasm for a while before the protein G purification. The biotinylation in the cell lysate could indeed be an effective alternative to in vivo biotinylation, as shown by Chapman-Smith et al. (1994). They achieved only limited biotinylation of the overexpressed BCCP biotin acceptor domain in vivo despite the coexpression of the biotin ligase. However, the biotinylation was taken to completion in the cell lysate on addition of ATP and biotin.

The minimum size for the C-terminal fragment of BCCP that can be biotinylated as a fusion protein is about 84 residues (Li and Cronan, 1992). Compared to this, the 13 residue peptides described by Schatz (1993) seem to bring a definite advantage for recombinant fusion protein applications at least when the size of the tail is critical. Other parameters important for the general utility of these fusion tails include their efficiency as biotin ligase substrates, compatibility with secretion, and possible susceptibility to proteolysis. The biotinylated lysine of BCCP is located in a highly conserved hairpin β -turn (Athappilly and Hendrickson, 1995), and the cloned acceptor proteins from various bacterial as well as eucaryotic sources act as substrates for *E. coli* biotin ligase (Cronan, 1990). However, the consensus sequence of the 13 amino acid peptides differs considerably from the natural acceptors, indicating that different constraints and perhaps a different mechanism of recognition apply to the small acceptor peptides (Schatz, 1993). Despite this, the peptides have been shown to be quantitatively biotinylated in vivo, both as N- and C-terminal fusions, provided that biotin ligase is coexpressed (Tsao et al., 1996). The periplasmic secretion of the 13 amino acid biotin acceptor peptides as fusion proteins has not been attempted before. Our results indicate that the peptides fused at the C-terminus of the Fd chain are compatible with membrane translocation which is a prerequisite for the assembly of functional Fab fragments. We have constructed several similar Fab-acceptor peptide fusion proteins, and the production levels vary between 30 and 100% of that of the nonfusion versions, depending on the particular Fab fragment (M. Vehni-

äinen, unpublished data). There are large inherent differences in the folding and stability of different recombinant Fab fragments in the bacterial periplasm (Knappik and Plückthun, 1995), which may be reflected in the differing effects of the fusion tail on the production levels. The fact that more than half of the purified apo-Fab 57-2 could not be biotinylated suggests that the tail was not always accessible for the biotin ligase or it was proteolytically degraded. The biotinylation degree did not improve with the use of more enzyme or prolonging the incubation time, nor in attempts to rebiotinylate the nonbiotinylated fraction (data not shown). Further studies will be needed to clarify whether the incomplete biotinylation was caused by proteolytic cleavage of the tail or by some conformation-dependent mechanism.

To facilitate the purification of the biotin ligase, we cloned the *birA* gene into the GST fusion vector pGEX-1 λ T (Smith and Johnson, 1988). The fusion of glutathione S-transferase to the amino terminus of BirA was considered a minor risk, especially because there was an option to remove the fusion partner by thrombin cleavage at a site in the joining linker (Figure 2). On the basis of genetic data as well as sequence comparisons, Buoncrisiani et al. (1986) suggested that the N-terminal part of the BirA protein contains a helix-turn-helix motif responsible for DNA binding, while the central part contains the catalytic activities. In the crystal structure of BirA (Wilson et al., 1992), there is only a loose connection between the N-terminal domain (residues 1–60) and the central domain (residues 68–269), suggesting that the fusion of GST to the amino-terminus of BirA would not affect the catalytic activity. However, the catalytic and repressor functions are actually intimately connected. The biotin ligase activity consists of two steps: the formation of biotinyl-5'-AMP from biotin and ATP and the transfer of the activated biotin moiety to the acceptor protein (BCCP). If all acceptor proteins are already biotinylated, the biotinyl-5'-AMP remains bound to BirA and acts as the corepressor of biotin biosynthesis (Cronan, 1989). Not only is biotinyl-5'-AMP required for the binding of BirA to the *bio* operator but, in turn, also the presence of the DNA-binding domain is important for the binding of biotinyl-5'-AMP to the catalytic domain. In a study of a truncation mutant of BirA lacking the N-terminal DNA-binding domain, Xu and Beckett (1996) found that the affinities for biotin and biotinyl-5'-AMP were 100- and 1000-fold decreased, respectively, although the maximal catalytic rates for the formation of biotinyl-5'-AMP and the transfer of biotin to BCCP were unchanged. These results implied that the N-terminal fusion of GST to BirA could, in an unfortunate case, affect the enzymatic activity. However, the fusion protein worked reasonably well for our purposes, so we did not attempt removing the GST part by taking advantage of the thrombin cleavage site. Biochemical studies are needed in the future to assess the possible effect of the GST fusion as well as to determine the catalytic parameters of the BirA biotin ligase for the peptide substrates.

The enzymatically biotinylated Fab fragments were characterized for their interaction with surface-bound streptavidin in order to assess their potential usefulness as immunoassay reagents. First, the binding of BioFab to streptavidin-coated wells was found to be linear in a wide concentration range (almost 5 decades), indicating that the Fab fragments bind independently of each other. The binding was also specific as demonstrated by the blocking of binding by submicromolar concentrations of biotin. The stability of the complex between BioFab and the coated streptavidin was a concern as it is known that

some biotinylated macromolecules show considerably weaker binding to streptavidin than does biotin (Vincent and Samuel, 1993). We tested the binding stability by measuring the time-dependent dissociation of Bio-Fab from streptavidin in the presence of a vast excess of free biotin to block the rebinding. The dissociation rate was extremely low, less than 10% in 24 h, which should be adequate for most immunoassay applications. Moreover, typical assay conditions would allow the rebinding of dissociated BioFab and thus would show even higher apparent binding stability. In conclusion, the above results indicate that the fusion peptide tail presents the biotin moiety in a favorable position for streptavidin binding.

The determination of the antigen-binding affinities of one enzymatically biotinylated and two chemically biotinylated Fab fragments indicated that the enzymatic biotinylation results in a more uniform binder population. Moreover, comparison of the major binders shows that the dissociation constants (K_d) of the chemically biotinylated Fabs are 2.6–3.6 times higher than that of the enzymatically biotinylated one (Table 3). These differences may be explained by the location of the biotin moiety on the surface of the Fab fragment. In the chemical procedure, in principle all surface lysines are targets for conjugation, although some may be preferred over the others. The outcome of the chemical biotinylation is therefore dependent on the content and distribution of surface lysines. The conjugation of a lysine residue at or near the antigen binding site can have an effect on the affinity either directly by interfering with the antibody–antigen interaction or indirectly by steering the immobilization of the Fab in an unfavorable orientation. The disadvantages of the chemical conjugation process can often be reduced by careful optimization of the conditions, aiming at one biotin per Fab. Even then, it depends on the primary structure of the Fab whether the single biotinylated lysine is preferentially found in the antigen binding site, elsewhere in the variable domains or in the constant domains. Ultimately, it is possible to engineer the primary structure of the binding site in order to decrease its sensitivity to chemical labeling, as shown in a recent study in which two lysine residues in the CDR loops of a recombinant immunotoxin were changed into arginines (Benhar et al., 1994). As this technique is by no means trivial or universally applicable, there will remain an increasing number of recombinant Fab (as well as single chain Fv) fragments that cannot be labeled with amine-reactive chemistries. Our results show that the enzymatic biotinylation through a peptide acceptor tail can produce a homogeneous, well-behaving binder population when the chemical biotinylation through amino groups fails to do so.

Finally, we demonstrated that the enzymatically biotinylated Fab fragments work as reagents in a competitive estradiol immunoassay. The standard curve (Figure 7) followed nicely the four-parameter logistic model and had a slope factor close to -1 which again indicated that the antigen and the tracer reacted with a single binder population. Although the assay is technically reliable (CV% was less than 10% throughout the measuring range), it is not yet useful for the measurement of clinical samples because the affinity of the antibody 57-2 for estradiol is not high enough. However, we are currently modifying its binding properties by random mutagenesis and phage display (Saviranta et al., 1998) and expect to use the mutated version for the assay of clinical samples. Furthermore, the site-specifically biotinylated Fab fragments should be ideal for the novel miniaturized assay

formats, such as single microparticle-based assays (Lövgren et al., 1997) where maximally efficient usage of the coated surface is required.

ACKNOWLEDGMENT

The financial support from State Technology Development Centre of Finland (TEKES) is gratefully acknowledged. We thank H. Mikola and N. Meltola for delivering the europium-labeled estradiol conjugates. M. Pajunen, M. Vehniäinen, and P. Nurmikko are thanked for help in DNA cloning, protein purification, and chemical biotinylation, respectively.

LITERATURE CITED

- Athappilly, F. K., and Hendrickson, W. A. (1995) Structure of the biotinyl domain of acetyl-coenzyme A carboxylase determined by MAD phasing. *Structure* 3, 1407–1419.
- Barbas, C. F., III, Kang, A. S., Lerner, R. A., and Benkovic, S. J. (1991) Assembly of combinatorial antibody libraries on phage surfaces: the gene III site. *Proc. Natl. Acad. Sci. U.S.A.* 88, 7978–82.
- Barker, D. F., and Campbell, A. M. (1981) Genetic and biochemical characterization of the *birA* gene and its product: evidence for a direct role of biotin holoenzyme synthetase in repression of the biotin operon in *Escherichia coli*. *J. Mol. Biol.* 146, 469–492.
- Bayer, E. A., and Wilchek, M. (1990) Protein biotinylation. *Methods Enzymol.* 184, 138–166.
- Benhar, I., Brinkmann, U., Webber, K. O., and Pastan, I. (1994) Mutations of two lysine residues in the CDR loops of a recombinant immunotoxin that reduce its sensitivity to chemical derivatization. *Bioconjugate Chem.* 4, 321–326.
- Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72, 248–254.
- Buoncristiani, M. R., and Otsuka, A. J. (1988) Overproduction and rapid purification of the biotin operon repressor from *Escherichia coli*. *J. Biol. Chem.* 263, 1013–1016.
- Buoncristiani, M. R., Howard, P. K., and Otsuka, A. J. (1986) DNA-binding and enzymatic domains of the bifunctional biotin operon repressor (BirA) of *Escherichia coli*. *Gene* 44, 255–261.
- Casadaban, M. J., and Cohen, S. N. (1980) Analysis of gene control signals by DNA fusion and cloning in *Escherichia coli*. *J. Mol. Biol.* 138, 179–207.
- Chapman-Smith, A., Turner, D. L., Cronan, J. E., Jr., Morris, T. W., and Wallace, J. C. (1994) Expression, biotinylation and purification of a biotin-domain peptide from the biotin carboxy carrier protein of *Escherichia coli* acetyl-CoA carboxylase. *Biochem. J.* 302, 881–887.
- Cronan J. E., Jr. (1989) The *E. coli* bio operon: transcriptional repression by an essential protein modification enzyme. *Cell* 58, 427–429.
- Cronan, J. E., Jr. (1990) Biotinylation of proteins in vivo. A posttranslational modification to label, purify and study proteins. *J. Biol. Chem.* 265, 10327–10333.
- Diamandis, E. P., and Christopoulos, T. K. (1991) The Biotin-(strept)avidin system: principles and applications in biotechnology. *Clin. Chem.* 37, 625–636.
- Hayden, M. S., Gilliland, L. K., and Ledbetter J. A. (1997) Antibody engineering. *Curr. Opin. Immunol.* 9, 201–212.
- Hermanson, G. T. (1996) *Bioconjugate techniques*, Academic Press, Inc., San Diego, CA.
- Jander, G., Cronan, J. E., and Beckwith, J. (1996) Biotinylation in vivo as a sensitive indicator of protein secretion and membrane protein insertion. *J. Bacteriol.* 178, 3049–3058.
- Knappik, A., and Plückthun, A. (1995) Engineered turns of a recombinant antibody improve its in vivo folding. *Protein Eng.* 8, 81–89.
- Li, S. J., and Cronan, J. E., Jr. (1992) The gene encoding the biotin carboxylase subunit of *Escherichia coli* acetyl-CoA carboxylase. *J. Biol. Chem.* 267, 855–863.
- Lövgren, T., Heinonen, P., Lehtinen, P., Hakala, H., Heinola, J., Harju, R., Takalo, H., Mikkala, V. M., Schmid, R.,

- Lönnberg, H., Pettersson, K., and Iltiä, A. (1997) Sensitive bioaffinity assays with individual microparticles and time-resolved fluorometry. *Clin. Chem.* 43, 1937–1943.
- Mukkala, V.-M., Hänninen, E., and Hemmilä, I. (1993) Synthesis of two biotin isothiocyanate derivatives. *Proceedings of 8th European Symposium on Organic Chemistry*, p 422, Sitges, Spain.
- Pajunen, M., Saviranta, P., Jauria, P., Karp, M., Pettersson, K., Mäntsälä, P., and Lövgren, T. (1997) Cloning, sequencing, expression, and characterization of three anti-estradiol-17 β Fab fragments. *Biochim. Biophys. Acta* 1351, 192–202.
- Plückthun, A., and Skerra, A. (1989) Expression of functional antibody Fv and Fab fragments in *Escherichia coli*. *Methods Enzymol.* 178, 497–515.
- Rapley R. (1995) The biotechnology and applications of antibody engineering. *Mol. Biotechnol.* 3, 139–154.
- Reed, K. E., and Cronan, J. E., Jr. (1991) *Escherichia coli* exports previously folded and biotinylated protein domains. *J. Biol. Chem.* 266, 11425–11428.
- Saviranta, P., Pajunen, M., Jauria, P., Karp, M., Pettersson, K., Mäntsälä, P., and Lövgren, T. (1998) Engineering the steroid-specificity of an anti-17 β -estradiol Fab by random mutagenesis and competitive phage panning. *Protein Eng.* 11, 143–152.
- Schatz, P. J. (1993) Use of peptide libraries to map the substrate specificity of a peptide-modifying enzyme: A 13 amino acids consensus peptide specifies biotinylation in *Escherichia coli*. *BioTechnology* 11, 1138–1143.
- Shenoy, B. C., and Wood, H. G. (1988) Purification and properties of the synthetase catalyzing the biotinylation of the apobunit of transcarboxylase from *Propionibacterium shermanii*. *FASEB J.* 12, 2396–2401.
- Smith, D. B., and Johnson, K. S. (1988) Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione S-transferase. *Gene* 67, 31–40.
- Tsao, K.-L., DeBarbieri, B., Michel, H., and Waugh, D. S. (1996) A versatile plasmid expression vector for the production of biotinylated proteins by site-specific, enzymatic modification in *Escherichia coli*. *Gene* 169, 59–64.
- Vincent, P., and Samuel, D. (1993) A comparison of the binding of biotin and biotinylated macromolecular ligands to an anti-biotin monoclonal antibody and to streptavidin. *J. Immunol. Methods* 165, 177–182.
- Weiss, E., Chatellier, J., and Orfanoudakis, G. (1994) In vivo biotinylated recombinant antibodies: Construction, characterization, and application of a bifunctional Fab-BCCP fusion protein produced in *Escherichia coli*. *Protein Expression Purif.* 5, 509–517.
- Wilchek, M., and Bayer, E. A. (1990a) Introduction to avidin–biotin technology. *Methods. Enzymol.* 184, 5–13.
- Wilchek, M., and Bayer, E. A. (1990b) Biotin-containing reagents. *Methods Enzymol.* 184, 123–138.
- Wilson, K. P., Shewchuk, L. M., Brennan, R. G., Otsuka, A. J., and Matthews, B. W. (1992) *Escherichia coli* biotin holoenzyme synthetase/bio repressor crystal structure delineates the biotin- and DNA-binding domains. *Proc. Natl. Acad. Sci. U.S.A.* 89, 9257–9261.
- Wood, H. G., Barden, R. E. (1977) Biotin enzymes. *Annu. Rev. Biochem.* 46, 385–413.
- Xu, Y. And Beckett, D. (1996) Evidence for interdomain interaction in the *Escherichia coli* repressor of the biotin biosynthesis from studies of an N-terminal domain deletion mutant. *Biochemistry* 35, 1783–1792.

BC9800217

Comparison of Recombinant Immunotoxins against Le^Y Antigen Expressing Tumor Cells: Influence of Affinity, Size, and Stability

Tapan K. Bera and Ira Pastan*

Laboratory of Molecular Biology, DBS, National Cancer Institute, National Institutes of Health, Building 37, Room 4E16, 37 Convent Drive MSC 4255, Bethesda, Maryland 20892-4255. Received March 6, 1998; Revised Manuscript Received August 11, 1998

Monoclonal antibody B3 (MAb B3) reacts with many epithelial cancers. It recognizes a carbohydrate antigen (Le^y) which is expressed in a variety of solid tumors including breast and colon. We have used the Fab portion of MAb B3 and a portion of the constant domain of human IgG1 to make recombinant immunotoxins of different compositions. The toxin component employed is a truncated form of *Pseudomonas* exotoxin (PE38). The light chain or Fd of the antibody was cloned from hybridoma RNA and fused to PE38. Immunotoxin (IT) was then expressed in *Escherichia coli* as a fusion protein and refolded with either the Fd or the light chain. We have also made B3(Fab) immunotoxins of different sizes ranging 85–140 kDa, by introducing different portions of the constant domain of human IgG1 at the junction of Fd and PE38 fusion site. We compared the properties of the resulting immunotoxins with existing anti-Le^y immunotoxins side by side. All recombinant Fab-immunotoxins made in this study were cytotoxic to antigen-positive cancer cell lines. However, in contrast to the B3(scFv) immunotoxin, the B3(Fab) immunotoxins are very stable, retaining 90% of their activity after 24 h of incubation in human serum albumin at 37 °C. A pharmacokinetics study with these immunotoxin molecules showed a longer survival in the circulation of mice compared to the smaller Fv immunotoxins. The smaller size of the Fab immunotoxins compared to B3Lys-PE38 and the increased $T_{1/2}$ value compared to B3(scFv)-PE38 and B3(dsFv)-PE38 make these recombinant immunotoxins alternative therapeutic agents to treat Le^y antigen positive cancers.

INTRODUCTION

Monoclonal antibodies (MAb) which recognize antigens on cancer cells are now being used for targeted therapy for the delivery of toxins, radioisotopes or drugs (Vitetta et al., 1987; Mulshine et al., 1991; Pastan et al., 1995; Brinkmann and Pastan, 1995). To produce an immunotoxin, the antibody is linked to a toxin made by bacteria or plants. Immunotoxins of this type are being developed for the treatment of cancer and other diseases including acquired immune deficiency syndrome (AIDS) and immunological disorders (Vitetta, 1990). In our laboratory we have used mutant forms of *Pseudomonas* exotoxin A (PE) to produce immunotoxins (Pastan and Fitzgerald, 1991). PE is a three domain protein, each with a different function. Domain I is responsible for cell binding, domain II for the translocation across the cell membrane, and domain III for the ADP-ribosylation activity of the toxin (Hwang et al., 1987). Using recombinant DNA technology, we have deleted domain I of PE to make a 38 kDa fragment (PE38) that retains the full ADP-ribosylation activity of the toxin (Brinkmann et al., 1991; Pai et al., 1991).

Immunotoxin B3Lys-PE38 (LMB1) is composed of MAb B3 chemically linked to PE38 (Pai et al., 1991). Single-chain Fv immunotoxins are recombinant fusion proteins composed of a cell-targeting moiety and the cytotoxic domain of the toxin molecule (Chaudhary et al., 1989). In B3(Fv)-PE38, the Fv portions of the monoclonal antibody B3 were cloned from the B3 hybridoma, fused to PE38 and expressed as a single molecule in *Escheri-*

chia coli (Brinkmann et al., 1991). Using this approach, a variety of other recombinant single-chain immunotoxins have been made. B3(Fv)-PE38 and B3Lys-PE38 are currently being examined for the treatment of Le^y-positive solid tumors. A phase I study was conducted with B3Lys-PE38 immunotoxin for 38 patients with solid tumors who failed conventional therapy and whose tumors expressed the Le^y antigen. Objective antitumor activity was observed in 5 patients, 18 had stable disease, and 15 progressed. A complete remission was observed in a patient with metastatic breast cancer to supraclavicular nodes. A greater than 75% tumor reduction and resolution of all clinical symptoms lasting for more than 6 months were observed in a colon cancer patient with extensive retroperitoneal and cervical metastasis (Pai et al., 1996). Anti-Tac(Fv)-PE38 is currently being evaluated for the treatment of leukemia (Kreitman et al., 1992). Although single-chain immunotoxins are relatively easy to make, they have some limitations. One is that frequently these molecules are unstable, probably due to the fact that the hydrophobic residues in the Fv fragments which are covered by the constant region of the whole antibody get exposed, and as a result, they have a tendency to aggregate. This problem has been solved by generating an Fv molecule in which the V_H and V_L fragments are connected by a disulfide bond (Brinkmann et al., 1993; Reiter et al., 1994a–c). The disulfide-stabilized Fv (dsFv) containing immunotoxins is very stable at 37 °C, effectively killing target cells and promoting tumor regressions. Depending on the antibody, the relative affinity of the dsFv molecule for antigen can be increased, unchanged, or reduced compared with the single chain Fv (Reiter et al., 1994a–c). Another

* Corresponding author. Phone: (301) 496-4797. Fax: (301) 402-1344.

limitation for both scFv and dsFv-containing immunotoxins is that their half-lives in the circulation of mice and monkeys are short with a $T_{1/2}$ ranging 20–60 min (Reiter et al., 1994c). In contrast, the half-life of the chemical conjugate of whole antibody is about 10 h in mice (Pai et al., 1991) and 9 h in humans (Pai et al., 1996). The plasma half-life of the immunotoxins constructed from the chemical conjugation between whole antibody and the toxin is mainly contributed by the antibody because the half-life of the toxin molecule is very short (Brinkmann and Pastan, 1995).

The Fab fragments of antibodies composed of Fd and light chain linked by a disulfide bond are very stable. These have been used to make chemical conjugates with several different toxins and also recombinant immunotoxins (Reiter et al., 1997; Kreitman et al., 1994). In this study, we have made an Fab immunotoxin with MAb B3 and combined it with different portions of the constant domain of human IgG1 and PE38. Our goal was to determine if we could make immunotoxin molecules of different sizes containing one or two PE38 domains and to study the properties of these molecules.

MATERIALS AND METHODS

Cloning of Fd and Light Chain of MAb B3. The Fd and light chain of MAb B3 were PCR-amplified from reverse transcribed hybridoma mRNA using primers designed from the published B3(Fv) sequences and from the sequences of the constant region of heavy and light chain. PCR primers used in this study are

T30	CAT ATG GAG GTG AAG CTG GTG GAA TCT
T31	TGA AGC TTC ACC ACA ATC CCT GGG CAC
T32	GG AAT TCA TTA ACA ATC CCT GGG CAC
T38	CAT ATG GAT GTT CTG ATG ACT CAA
T34	TGA AGC TTC ACA CTC ATT CCT GTT GAA GCT
T35	GG AAT TCA TTA ACA CTC ATT CCT GTT GAA GCT
T47	AGC CTC CAC CAA GGG CCC ATC G
T48	TCA TTT ACC CGG AGA CAG GGA G
T49	TCC ATC TCT TAA GCT TCA CCT GAG CTC CTG GGG GGA CCG
T50	CCC ACG GGT CCA AGC TTT GGC TTT GGA GAT GGT TTT CTC G
T51	AAC CTC TGT AAA GCT TCG CAG CCC CGA GAA CCA CAG
T52	CGG CCG TCG CAA AGC TTT ACC CGG AGA CAG GGA GAG

The 5' primers (T30 and T38) used to amplify Fd or light-chain fragments were designed from B3(Fv) sequence. The 3' primers (T31 and T32) for Fd fragment were designed from the nucleotide sequence of the constant region of heavy-chain subgroup III(A) to which B3 IgG belongs. Similarly, the 3' primers (T34 and T35) for light chain were designed from the sequence of the constant region of κ chain. *NdeI*–*HindIII* or *NdeI*–*EcoRI* sites were introduced to clone the fragment into the expression vector to express either antibody-immunotoxin

fusion protein (pTKB1.5.1 and pTKB3.1.1) or antibody fragment alone (pTKB2.1.1 and pTKB4.1.7), respectively.

Isolation of Constant Region of Human IgG1 from Spleen cDNA. Polymerase-chain-reaction (PCR)-ready human spleen cDNA was purchased from Clontech (Palo Alto, CA), and the constant region of human IgG1 was isolated by PCR. Primers T47 and T48 were designed from the published sequences (Ellison, 1982) and were used to amplify the constant region of human IgG1. The amplified fragment was cloned into TA vector (Invitrogen, Carlsbad, CA), the sequence verified by DNA sequencing. The cDNA clone was then used as a template to amplify CH2, CH3, or CH2–CH3 region by primer pairs T49/T50, T51/T52 or T49/T52, respectively.

Cloning of Various Constant Regions of Human IgG1 into Expression Vector pTKB1.5.1. Restriction site *HindIII* was generated by PCR on both sides of the fragment to be inserted between the Fd and the PE38 portions of plasmid pTKB1.5.1. To construct the plasmids expressing either CH2, CH3, or both CH2 and CH3 fragment, pTKB1.5.1 plasmid was linearized with *HindIII* and then ligated with *HindIII* restricted DNA fragments that contain either CH2, CH3, or both. The correct inframe insertion of the fragments in the resulting plasmids pTKB12.1.7, pTKB13.1.4, and pTKB15.1.3 were checked first by DNA sequencing and finally by determining the sizes of the fusion protein expressed in *E. coli*.

Protein Expression and Purification. *E. coli* BL21 (λ DE3) was transformed with different plasmids and the protein production was induced by adding 10 mM IPTG as described earlier (Brinkmann et al., 1991). All of the fusion proteins were accumulated as insoluble inclusion bodies (IBs) in *E. coli*. IBs were solubilized and refolded by the procedure described earlier (Buchner et al., 1992). Briefly, the purified IBs were dissolved in solubilization buffer (6 M guanidine-HCl, 0.1 M Tris-HCl, and 2 mM EDTA). After the protein concentration was determined, equimolar amounts of Fd or light chains and their PE38 fusion proteins were reduced by the addition of 0.3 M dithioerythritol (DTT) for 4 h at room temperature and then refolded for 48 h at 10 °C in a refolding buffer described earlier (Buchner et al., 1992). Properly folded protein was then chromatographically purified using first ion exchange (Q-Sepharose and MonoQ; Pharmacia) and finally size-exclusion chromatography (TSK G3000 SW, Toso HAAS) using the procedure described earlier (Buchner et al., 1992).

Cytotoxicity Assay. The cytotoxicity of different immunotoxins was determined by measuring the inhibition of protein synthesis in different antigen-positive and -negative cancer cell lines by using a method described earlier (Chaudhary et al., 1989; Batra et al., 1990). All assays were performed in 96 well plates, each well containing 1.6×10^4 cells in 200 μ L of RPMI medium containing either 10% (A431, LnCap, CRL1739, and Hut102) or 5% (MCF7) fetal bovine serum. Cytotoxicity assays were performed with different immunotoxins for 2 h, and then fresh medium was added to each well after removing the toxin. The incubation continued for 16 h before [³H]leucine was added.

Assay of Blood Levels of B3(Fab)-PE38 Immunotoxin in Mice. Female BALB/c mice were injected in the tail vein with 10 μ g of immunotoxin. Blood samples were drawn at various time intervals and the level of the active immunotoxin was measured by the cytotoxicity assay using A431 cells. The A431 cells were incubated with the diluted serum samples and their ability to inhibit protein synthesis was measured. A standard curve was made with each pure immunotoxin. The data

were analyzed by an exponential curve fitting program RSTRIP (Version 5, MicroMath Scientific Software, Salt Lake City, UT).

Stability Assay. The stability of B3(Fab)-PE38-1 and B3(Fab)-PE38-2 immunotoxin was determined by incubating the proteins at 10 μ g/mL at 37 °C in human serum albumin. Active immunotoxin remaining at different times was determined by a cytotoxicity assay on A431 cells.

Binding Assay. B3-antigen-positive A431 cells were used for a binding competition assay. B3 antibody, radioiodinated with Bolton–Hunter reagent (New England Nuclear, Boston, MA), was used as a tracer in the competition binding assay. Equal counts (50 000 cpm) of labeled B3 antibody with increasing amounts of competitors (cold B3 antibody or immunotoxin) were combined in RPMI, 1% BSA, 50 mM BES, pH 7, and 0.04% NaN_3 and added to the cells after nonspecific sites on the cells were blocked. After 2 h of incubation with gentle rocking at 4 °C, unbound counts were washed off and the cells were lysed with 0.5% SDS. The bound radioactivity was assessed in a Beckman 5500B γ counter (Beckman Instruments, Fullerton, CA).

RESULTS

The primary goal of this study was to make recombinant immunotoxins of different sizes and compositions using the Fab fragment of MAb B3 and different portions of the human IgG1 constant region and to compare their cytotoxicity, pharmacokinetics, and binding affinity with the chemical conjugate B3-LysPE38 ($M_r \approx 195$ kDa) and two other recombinant immunotoxins B3(Fv)-PE38 (M_r 63 kDa) and B3(dsFv)-PE38 (M_r 63 kDa) in which the Fv portion was fused to PE38.

Plasmid Construction and Production of Recombinant Immunotoxin. The cDNA fragments encoding Fd and light chain of monoclonal antibody B3 were obtained by reverse transcriptase reaction of the mRNA from the B3 hybridoma cell line (Pastan et al., 1991), using the PCR primer pair as described in Materials and Methods. PCR-ready human spleen cDNA was purchased from Clontech and used to isolate the constant region of human IgG1 using the primer pairs described in Materials and Methods. The CH2 and CH3 regions of the IgG1 were then isolated separately from the constant region using the specific primer pairs as described in Materials and Methods.

Plasmid Construction. To produce the recombinant B3(Fab)-PE38 molecule, we constructed four expression plasmids, pTKB2.1.1, pTKB4.1.7, pTKB1.5.1, and pTKB3.1.1. (Figure 1). The first two plasmids were constructed to produce the Fd and light chain of MAb B3, respectively. The third and fourth plasmids were constructed to generate the Fd-PE38 and LC-PE38 fusion proteins. A polypeptide connector (C3, ASGGPE) was inserted between the antibody molecule and the toxin (Brinkmann et al., 1991).

To produce recombinant immunotoxins of different sizes, we constructed three plasmids by introducing CH2, CH3, and CH2-CH3 fragments of human IgG1 into the plasmid pTKB1.5.1. Schematic pictures of the plasmids used in this study are shown in Figure 1.

Protein Expression, Refolding, and Purification of Immunotoxins. Plasmids encoding different components of B3(Fab)-PE38 immunotoxins were expressed separately in *E. coli* BL21 (λ DE3) cultures as described previously (Buchner et al., 1992). All recombinant proteins accumulated in large amounts as insoluble

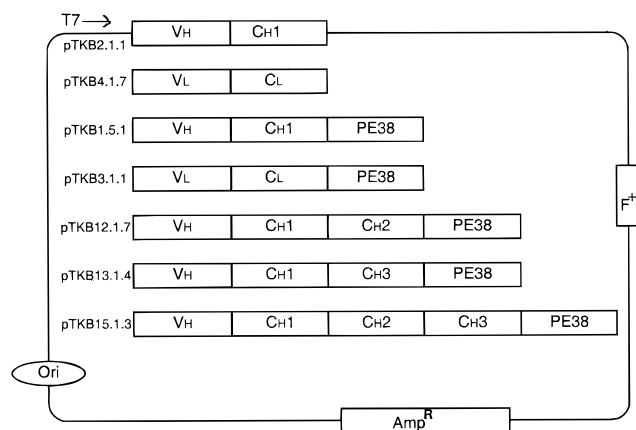


Figure 1. Plasmids for expression of the components of B3 Fab immunotoxin. The plasmid pTKB2.1.1 and pTKB4.1.7 code for the Fd and light chain of the MAb B3, respectively. The expression plasmid pTKB1.5.1 encodes Fd-PE38 fusion protein and plasmid pTKB3.1.1 codes for the fusion protein of B3 light chain and PE38. The plasmids pTKB12.1.7, pTKB13.1.4, and pTKB15.1.3 were generated from the parental plasmid pTKB1.5.1 by introducing CH2, CH3, and CH2-CH3 fragment of human IgG1 between the Fd and PE38 and encode Fd-CH2-PE38, Fd-CH3-PE38 and Fd-CH2-CH3-PE38 fusion protein, respectively.

intracellular inclusion bodies (IBs). IBs containing different components were used to produce different immunotoxins. Soluble recombinant immunotoxins were recovered using a purification scheme that included ion exchange and size-exclusion chromatography previously established for purification of single-chain immunotoxins (Buchner et al., 1992).

The scheme we used to make different immunotoxin molecules is shown in Figure 2, with the summary of the expected molecular mass and the yield of each purified protein. B3(Fab)-PE38-1 was made by refolding Fd-PE38 and light chain. B3(Fab)-PE38-2 was made by refolding light chain-PE38 and B3 Fd. Both B3(Fab)-PE38-1 and B3(Fab)-PE38-2 could be produced as monomers with final yields from purification of about 2.5%. The purity of those molecules after purification is shown in Figure 3. B3(Fab)-PE38-3 did not refold into a soluble monomeric protein, perhaps because of the inhibition of the disulfide bond formation between Fd and light chain by the PE38 portion of each component.

B3(Fab)-PE38-4 and B3(Fab)-PE38-6 also could be produced as pure proteins with final yields of about 2.5%. The purity of those molecules after refolding and subsequent purification is shown in Figure 3. B3(Fab)-PE38-5 and B3(Fab)-PE38-7 immunotoxins refolded very poorly. After size exclusion chromatography, the final yield for B3(Fab)-PE38-5 and B3(Fab)-PE38-7 was only about 0.1%.

Specific Cytotoxicity of B3(Fab)-PE38 Recombinant Immunotoxin. PE38 is a truncated form of *Pseudomonas* exotoxin, and the fusion proteins of antibody fragments with PE38 are cytotoxic to cells that specifically bind and internalize the fusion protein. Since the degree of cytotoxicity to a given antigen depends primarily on binding affinity, cytotoxicity assays of immunotoxin fusion proteins can thus serve as an accurate test system for specificity as well as for the relative affinity of engineered antibody fragments (Brinkmann and Pastan, 1995). To test whether all of the six immunotoxins made retain not only the affinity but also specificity, we analyzed the activity of these recombinant immunotoxins using several B3 antigen-positive and

B3Fab-PE38 Immunotoxins	Composition	Molecular weight in Da	% yield
B3Fab-PE38-1	V _H CH ₁ PE38	86,000	2.5
	V _L CL		
B3Fab-PE38-2	V _L CL PE38	86,000	2.5
	V _H CH ₁		
B3Fab-PE38-3	V _H CH ₁ PE38	124,000	No
	V _L CL PE38		
B3Fab-PE38-4	V _H CH ₁ CH ₂ PE38	96,000	2.5
	V _L CL		
B3Fab-PE38-5	V _H CH ₁ CH ₃ PE38	96,000	0.1
	V _L CL		
B3Fab-PE38-6	V _H CH ₁ CH ₂ PE38	134,000	2.5
	V _L CL PE38		
B3Fab-PE38-7	V _H CH ₁ CH ₂ CH ₃ PE38	106,000	0.1
	V _L CL		

Figure 2. Design of B3 Fab immunotoxin of different sizes. Linear composition of B3 Fab-PE38 immunotoxin of different sizes. The positions of the cystein residue forming the disulfied bond between Fd and light chain are Cys²³⁵ of Fd and Cys²¹⁴ of light chain. The calculated molecular mass and the corresponding % yield of each immunotoxin is indicated.

-negative cancer cell lines. For convenience, protein synthesis inhibition was measured. This assay has shown a strong correlation with cell death (Brinkmann et al., 1991). The antigen-positive cell lines used for protein synthesis inhibition assays were A431, MCF-7, LnCAP, and CRL1739. All of the immunotoxins generated were very cytotoxic to B3 antigen positive cell lines but were nontoxic for the antigen negative HUT102 cell line. The IC₅₀ values of individual immunotoxin molecules toward different cell lines are shown in Table 1. In 20 h cytotoxicity assays, both B3(Fab)-PE38-1 and B3(Fab)-PE38-2 were very cytotoxic for A431 cells (IC₅₀, 0.6 ng/mL), CRL 1739 cells (IC₅₀, 0.4 ng/mL), and MCF-7 cells (IC₅₀, 1.0 ng/mL). All of the three cancer cell lines express large amounts of B3 antigen on the surface. The prostate cancer cell line LnCAP, which expresses a moderate amount of the B3 antigen, was also sensitive to these immunotoxin molecules with an IC₅₀ of about 10 ng/mL. Except for B3(Fab)-PE38-5, the other three immunotoxins showed more or less similar IC₅₀ values

ranging 1–3 ng/mL for the A431 and MCF cell lines (Table 1). B3(Fab)-PE38-5 was about 10-fold more cytotoxic to all of the B3 antigen positive cell lines tested, compared to B3(Fab)-PE38-4, B3(Fab)-PE38-6, and B3(Fab)-PE38-7 molecules. The adult T-cell leukemia cell line HUT102, which does not express B3 antigen, was not sensitive to any of the above immunotoxin molecules but very sensitive to anti-Tac(scFv)-PE38 immunotoxin directed to IL2 receptor (Chaudhary et al., 1989).

B3(Fv)-PE38 immunotoxin is very unstable at 37 °C, however, the molecule is very cytotoxic to B3 antigen-positive cancer cell line. B3(Fv)-PE38 loses much of its activity after 2 h at 37 °C (Reiter et al., 1994c), even so its IC₅₀ value is similar to the IC₅₀ value of B3(dsFv)-PE38 in a 20 h cytotoxicity assay. This is because B3(Fv)-PE38 has a higher affinity for the Le^y antigen than B3(dsFv)-PE38 (Reiter et al., 1994c). In this study, we determined the cytotoxicity of B3 (Fab) immunotoxin at 37 °C for 2 h and compared the values with B3(Fv)-PE38 and B3(dsFv)-PE38.

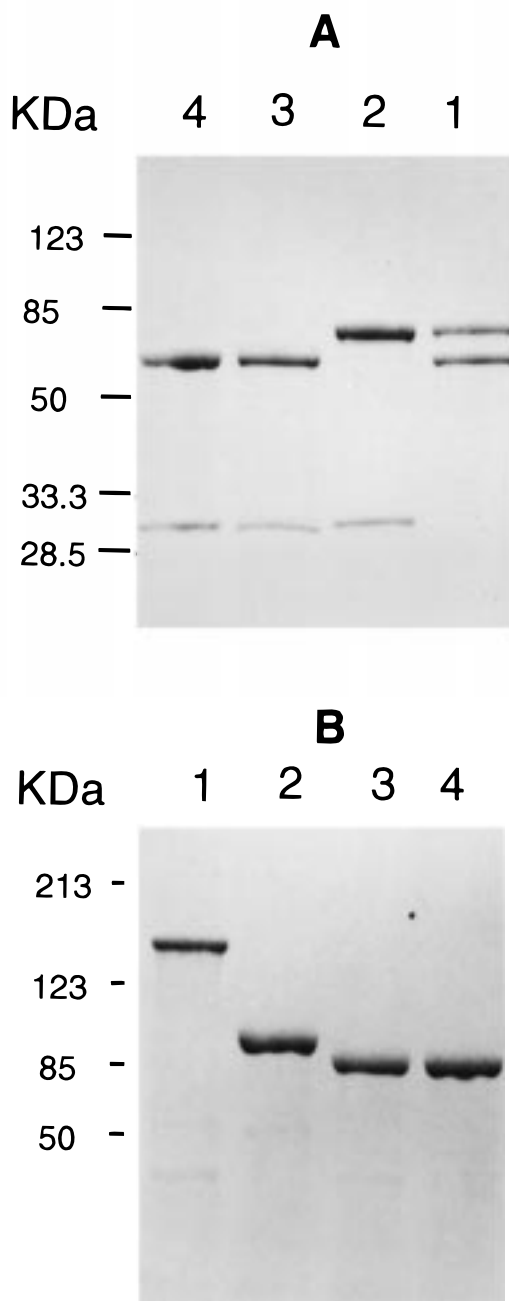


Figure 3. SDS-PAGE analysis of purified recombinant B3 Fab-PE38 immunotoxin. Coomassie blue stained SDS PAGE of (A) reduced, and (B) nonreduced samples of B3(Fab)-PE38 immunotoxin; lane 1, B3(Fab)-PE38-6; lane 2, B3(Fab)-PE38-4; lane 3, B3(Fab)-PE38-2; lane 4, B3(Fab)-PE38-1.

In 2 h cytotoxicity assays, the immunotoxins were washed away after 2 h of incubation, the cells were incubated for additional 16 h, and protein synthesis inhibition was measured after 18 h as described in Materials and Methods. The IC_{50} values of individual immunotoxin molecules for different cell lines in 2 h cytotoxicity assays are summarized in Table 2. Both B3(Fab)-PE38-1 and B3(Fab)-PE38-2 molecules were cytotoxic to all B3 antigen-positive cell lines tested. They were less active than B3(Fv)-PE38 but more active than B3(dsFv)-PE38. This difference in activity can be explained by the fact that the affinity of the B3(Fab)-PE38 immunotoxin is higher than the B3(dsFv)-PE38 but lower than B3(Fv)-PE38, and therefore in 2 h, the binding of the B3(Fab)-PE38 immunotoxin to the target cells was

Table 1. Specific Cytotoxicity of B3(Fab)-Immunotoxins

immunotoxins	cell lines, B3 antigen				
	A431 (+++)	MCF7 (+++)	LnCap (+)	CRL1739 (+)	HUT102 (-)
LMB1	2.0	1.2	2.8	1.5	>1000
LMB7	0.5	2.0	20	0.32	>1000
B3dsFv	2.5	5.5	60	4.5	>1000
B3Fab1	0.6	0.9	11	0.4	>1000
B3Fab2	0.55	1.0	10	0.4	>1000
B3Fab4	1.0	1.0	18		>1000
B3Fab5	0.1	0.15	2.5		>1000
B3Fab6	1.8	3.5	150		>1000
B3Fab7	1.5	2.5	45		>1000
anti-Tac(scFv)- PE38					0.05

Table 2. Specific Cytotoxicity of B3(Fab)-Immunotoxins; 2 h Assay^a

immunotoxins	cell lines, B3 antigen				
	A431 (+++)	MCF7 (+++)	LnCap (+)	CRL1739 (+)	HUT102 (-)
LMB1	5.2	10	5.0	2.5	>1000
LMB7	0.4	3.5	35	0.8	>1000
B3dsFv	40	7.5	275	30	>1000
B3Fab1	4.5	5.5	42	6.5	>1000
B3Fab2	5.0	5.5	60	4.5	>1000

^a Cytotoxicity assays were performed by measuring incorporation of tritiated leucine into cellular proteins as described in the Materials and Methods section. IC_{50} is the concentration that caused 50% inhibition of protein synthesis after incubation with immunotoxin. Antigen expression (+++); very high (+); present (-); absent.

Table 3. Stability of B3(Fab) Immunotoxins in Human Serum Albumin at 37 °C^a

time (h)	% activity remaining				
	B3dsFv	B3Fab-1	B3Fab-2	B3Fab4	B3Fab6
0	100	100	100	100	100
2	96	93	91	100	100
6	80	90	91	90	100
24	75	82	91	90	87

^a Different immunotoxins at a concentration of 10 μ g/mL were incubated with 0.2% human serum albumin for the times shown at 37 °C and then assayed for cytotoxic activity on A431 cells.

higher than the B3(dsFv)-PE38 and lower than the B3(Fv)-PE38 (Reiter et al., 1994c).

Stability of B3(Fab)-PE38 Immunotoxin. The recombinant Fv or Fab fragments of antibody are widely used in diagnosis and therapy of cancer in the form of radiolabeled or toxin fusion protein. For such applications, it is important that these therapeutic molecules are stable at 37 °C in human circulation so they will retain activity for a long time after injection into patients. Therefore, we examined the stability of B3(Fab)-PE38-1, B3(Fab)-PE38-2, B3(Fab)-PE38-4, and B3(Fab)-PE38-6 by incubating the purified proteins at 37 °C. The immunotoxins, at a concentration of 10 μ g/mL, were incubated at 37 °C for different periods of time in phosphate-buffered saline containing 0.2% human serum albumin. The remaining activity of the incubated immunotoxins was determined by protein synthesis inhibition assays using B3 antigen positive A431 cell lines. The data in Table 3 shows that all of the molecules were very stable at 37 °C retaining 80–90% of initial activity for at least 24 h when incubated in human serum albumin.

Pharmacokinetics of B3(Fab)-PE38 Recombinant Immunotoxin. We next determined the pharmacokinetics of B3(Fab)-PE38-1 and B3(Fab)-PE38-4 immunotoxin in mice. Each immunotoxin was injected i.v. with

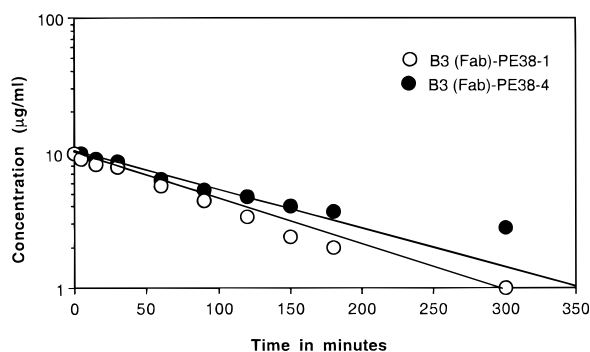


Figure 4. Blood levels of B3(Fab)-PE38 immunotoxin in mice. Female BALB/c mice were injected i.v. with 10 µg of B3(Fab)-PE38-1 or B3(Fab)-PE38-4. The level of immunotoxin in blood samples drawn at different times was measured by protein synthesis inhibition assay on A431 cells as described in Materials and Methods. Results are average of three animals for each time point. Open circle, blood levels of B3(Fab)-PE38-1; Close circle, blood levels of B3(Fab)-PE38-4.

Table 4. Relative Binding Affinity (IC₅₀) of B3 (Fab)-Immunotoxins^a

analyte	tracer	IC ₅₀ (nM)	cell line
B3 IgG	B3 IgG	85	A431
B3-Lys-PE38	B3 IgG	2500	A431
B3 Fab-PE38-1	B3 IgG	3000	A431
B3 Fab-PE38-2	B3 IgG	3500	A431

^a The affinities of different immunotoxins were determined by competition binding analyses to cells expressing B3 (Le^y) antigen. The antigen positive cell line used was A431. The relative binding affinities were calculated from the concentration of competitor which caused 50% inhibition of the binding of ¹²⁵I-B3 IgG to the Le^y-expressing cell line.

a single dose of 10 µg administered to a series of mice. Blood was drawn at different time points after the injection and assayed for immunotoxin activity by measuring its ability to inhibit protein synthesis on A431 cells as described in Materials and Methods. In Figure 4 the plasma clearance curve of B3(Fab)-PE38-1 and B3(Fab)-PE38-4 immunotoxin was shown. The *T*_{1/2} for B3(Fab)-PE38-1 and B3(Fab)-PE38-4 molecules was found to be 104 and 121 min, respectively.

Relative Binding of B3(Fab)-PE38 Immunotoxin.

The relative binding affinities of B3(Fab)-PE38-1 and B3(Fab)-PE38-2 immunotoxins were determined by competition binding assay on B3 antigen expressing A431 cells. Various amounts of B3(Fab)-PE38-1, B3(Fab)-PE38-2, B3Lys-PE38, or B3 IgG were used to compete for the binding of radiolabeled B3 IgG to the B3 antigen positive A431 cells. Table 4 summarizes the relative binding of B3(Fab)-PE38 immunotoxins. B3(Fab)-PE38-1, B3(Fab)-PE38-2, and B3Lys-PE38 are equipotent competitors with ¹²⁵I-labeled B3 IgG for antigen sites on cultured A431 cells. The IC₅₀ was between 2 and 5 µM. Both B3(Fab)-PE38-1 and B3(Fab)-PE38-2 competed approximately 30-fold less well than the whole B3 IgG antibody (IC₅₀ ≈ 85 nM). Since B3(Fab)-PE38-1 and B3(Fab)-PE38-2 are both monovalent, a decrease in apparent binding affinity is to be expected.

DISCUSSION

In this study, we have made recombinant immunotoxins of different sizes and compositions using the Fab fragment of the B3 antibody, a truncated version of *Pseudomonas* exotoxin (PE38), and different portions of the human IgG1 constant region. We have successfully made recombinant immunotoxins with molecular masses of 86 000, 96 000, and 134 000 kDa.

B3(Fab)-PE38-1 and B3(Fab)-PE38-2, both have molecular masses of 86 000 kDa. In the case of B3(Fab)-PE38-1, the Fd fragment of monoclonal antibody B3 was fused to PE38 and disulfide bonded with B3 light chain. The Fd fragment of B3 antibody, in B3(Fab)-PE38-2, was linked with light chain-PE38 fusion molecules by a disulfide bond. Both recombinant immunotoxin molecules refolded well and could be purified as active monomers. There is no difference between B3(Fab)-PE38-1 and B3(Fab)-PE38-2 molecules with regard to cytotoxicity toward antigen positive cancer cell lines, stability of the molecule, and total yield after refolding and subsequent purification steps.

In a previous report (Choe et al., 1994), the B3(Fab) was made from the fusion of the Fv fragment of MAb B3 and the constant region of Fd and the V_L of MAb MAK33 (Buckel et al., 1987). To make the immunotoxin, the resulting Fd or V_L chain was fused to PE38^M and then refolded with the corresponding V_L or Fd chain. In PE38^M, two of the lysine residues in domain III were mutated to glutamine and lysine 613 was deleted (Choe et al., 1994). In this study, we have used the natural constant region of the Fd and the V_L of MAb B3 to make the Fab. These fragments were isolated using mRNA from the B3 hybridoma. Although the C_κ chain sequence for antibody MAK33 is identical to that of B3 antibody, there is an alanine (Kabat position 113) instead of serine at the FR4 region of the V_H domain of the B3 antibody. Interestingly, the immunotoxins made using the natural B3 Fab fragment were about 4–5-fold more active than the previously made Fab immunotoxin on B3 antigen-positive cell line. At the same time, the final yield of the properly folded immunotoxin was severalfold less than the previous report. Whether the mutations in the PE38^M domain and the serine to alanine substitution at the FR4 region of the heavy chain have any effect on the yield and the activity of the immunotoxin molecules are not clear.

The components used to prepare B3(Fab)-PE38-3 were B3(Fd)-PE38 and the light-chain PE38 fusion protein. This molecule aggregated during the refolding procedure and purified monomer could not be produced. Western blot analysis of the material obtained after the ion-exchange chromatography purification step indicated the presence of nondisulfide bonded individual components in the mixture (data not shown). It is possible that the two PE38 portions interacted and prevented association of Fd with V_L. It is possible that the C3 linker used to fuse Fd or light chain to the PE38 molecule, which is only six amino acids long, is too short and caused the PE38 domains to interact and prevent folding and disulfide bond formation between Fd and the light chain. This view is supported by the fact that the monomeric form of B3(Fab)-PE38-6 could be produced with a yield of 2.5%. In B3(Fab)-PE38-6, the CH2 fragment of the human IgG1 was introduced between the Fd and PE38 and probably served as a flexible linker and thus prevented destructive interaction of the PE38 portions of the Fd and light chain.

For B3(Fab)-PE38-5 and B3(Fab)-PE38-7, we were not able to make highly purified monomeric molecules and the final yield was very low (0.1%). Both molecules contain the CH3 domain of human IgG1. In B3(Fab)-PE38-5, the CH3 domain was inserted between Fd and PE38. The B3(Fab)-PE38-7 molecule has the CH2 and CH3 domains of human IgG1. The CH3 portion of the IgG1 has a tendency to form a homodimer (Ridgway et al., 1996), and probably, that region is responsible for making noncovalent dimer with those immunotoxin molecules. B3(Fab)-PE38-4, which contained a CH2

Table 5. Summary of the Properties of Recombinant Immunotoxins against Le^y Antigen

immunotoxin	size (kDa)	IC ₅₀ on A431 cells (ng/mL)	binding (μM)	stability	T _{1/2} in circulation of mice	ref
B3Lys-PE38	190	2.0	2.5	Stable	10 h	present study, Pai (1991)
B3Fab-PE38-6	134	1.8	ND ^a	Stable	ND	present study
B3Fab-PE38-4	96	1.0	ND	Stable	2 h	present study
B3Fab-PE38-2	86	0.55	3.5	Stable	ND	present study
B3Fab-PE38-1	86	0.6	3.0	Stable	2 h	present study
B3scFv-PE38	65	0.5	1.3	Unstable	30 min	present study, Reiter (1994c)
B3dsFv-PE38	65	2.5	32	Stable	30 min	present study, Reiter (1994c)

^a ND; not determined.

instead of a CH3 domain and is the same size as B3-(Fab)-PE38-5, can be prepared on good yield as a monomer. These data support our interpretation that CH3 causes dimerization.

Cytotoxicity of B3(Fab)-PE38 Recombinant Immunotoxins. All of the six B3(Fab)-PE38 immunotoxins were tested for their cytotoxicity on several B3 antigen-positive cell lines and one negative cancer cell line. The molecules were very cytotoxic to B3 antigen-positive cell lines and nontoxic to the antigen-negative cell line. In 20 h cytotoxicity assays, both B3(Fab)-PE38-1 and B3-(Fab)-PE38-2 gave IC₅₀ values very similar to B3(Fv)-PE38, which were lower than the values for B3Lys-PE38 and B3(dsFv)-PE38 for most of the B3 antigen-positive cell lines tested (Table 1). But in 2 h cytotoxicity assays, the IC₅₀ values for B3(Fab)-PE38-1 and B3(Fab)-PE38-2 were closer to B3Lys-PE38, which were higher than the values for B3(Fv)-PE38 (Table 2). The IC₅₀ values for B3(dsFv)-PE38 in 2 h cytotoxicity assays were much higher than the values for B3(Fab)-PE38-1 and B3(Fab)-PE38-2 molecules for all of the B3 antigen-positive cell lines. The cytotoxicity data for the B3(Fab)-PE38 immunotoxins in 2 and 20 h assays are directly correlated with their relative binding affinity to B3 antigen and also the stability of the molecule at 37 °C. B3(Fv)-PE38 is very unstable and loses much of its activity by 2 h. Nevertheless, it has a very low IC₅₀ as the result of its higher affinity to B3 antigen and there is no difference in IC₅₀ values between 2 and 20 h assay. B3(dsFv)-PE38, on the other hand, had very poor IC₅₀ in a 2 h assay because of its poor binding to B3 antigen; but due to its extreme stability at 37 °C, it can kill the B3 antigen-positive cells as efficiently as B3(Fv)-PE38 in 20 h assays. B3(Fab) immunotoxins are also very stable at 37 °C, and their binding affinity is much better than B3(dsFv)-PE38. As a result, B3(Fab) immunotoxins had the same IC₅₀ values as B3(Fv)-PE38 in 20 h cytotoxicity assays and much better IC₅₀ than B3(dsFv)-PE38 in 2 h assays.

Except B3(Fab)-PE38-5, the other three immunotoxins had similar IC₅₀ values which are closer to the values of B3Lys-PE38. B3(Fab)-PE38-5 was about 10-fold more cytotoxic to all B3 antigen-positive cell lines tested, compared to B3(Fab)-PE38-4, B3(Fab)-PE38-6, and B3-(Fab)-PE38-7. Increased cytotoxicity of B3(Fab)-PE38-5 immunotoxin to the B3 antigen-positive cells could be due to its dimer formation and activity as a bivalent molecule. Surprisingly, B3(Fab)-PE38-6 which contains two PE38 molecules did not show increased cytotoxic activity to B3 antigen-positive cells.

Stability Compensates for Loss of Affinity. The stability of immunotoxin, as for all other therapeutic drugs, is an important factor. B3(Fv)-PE38 is very unstable when incubated at 37 °C and loses most of its activity within 2 h. We determined the stability of B3-(Fab)-PE38 immunotoxins by incubating the molecules at 37 °C. All molecules are very stable at least up to 24 h at 37 °C as are B3(dsFv)-PE38 molecules reported

earlier (Reiter et al., 1994c). The relative binding affinity of B3(Fab)-PE38 molecule was determined by radioactive binding and competition assay. Both B3(Fab)-PE38-1 and B3(Fab)-PE38-4 immunotoxins competed approximately 30-fold less than the whole B3 IgG antibody (IC₅₀ ≈ 85 nM). The lower affinity of these molecules toward B3 antigen compared to B3(Fv)-PE38 is compensated by the fact that all B3(Fab)-immunotoxins are very stable at 37 °C. As a result, the cytotoxic effect of those molecules is very similar to B3(Fv)-PE38 immunotoxin.

Increased Half-Life of B3(Fab)-PE38 Immunotoxin. Both B3(Fv)-PE38 and B3(dsFv)-PE38 have very short half-lives in mouse serum. The rate of elimination of these molecules from the mouse blood is very similar, with a T_{1/2} of 20–23 min (Reiter et al., 1994c). On the other hand, the chemical conjugate of the B3 antibody with PE38 (LMB-1) has a longer survival time in mouse blood with a T_{1/2} of about 10 h (Pai et al., 1991). We determined the pharmacokinetics of B3(Fab)-PE38-1 and B3(Fab)-PE38-4 immunotoxins in mice. The T_{1/2} for B3-(Fab)-PE38-1 and B3(Fab)-PE38-4 molecules was found to be 104 and 121 min, respectively, which are much higher than B3(Fv)-PE38 and B3(dsFv)-PE38 (Reiter et al., 1994c) but lower than B3Lys-PE38 (Pai et al., 1991). The increased T_{1/2} values of B3(Fab)-PE38-1 and B3(Fab)-PE38-4 immunotoxin molecules compared to B3-(Fv)-PE38 and B3(dsFv)-PE38 could be due to their increased size or to the CH1 and CL portion of the molecules or both. The CH2 domain insertion in B3-(Fab)-PE38-4 molecule does not have any significant effect on T_{1/2} value of the molecule in mouse blood.

Conclusion. We have made B3 immunotoxins of different compositions and compare their properties with other existing anti Le^y immunotoxins. The side by side comparison of the properties of these molecules is summarized in Table 5. We have successfully made recombinant immunotoxin molecules of sizes 86 000, 96 000, and 134 000 kDa. All molecules are very cytotoxic to B3 antigen-positive cancer cell lines. We are able to increase the retention time of the immunotoxin molecule in mouse blood by introducing the constant region of human IgG1. We believe that the increased T_{1/2} value compared to B3-(Fv)-PE38 and smaller size compared to B3 lys-PE38 will make these immunotoxin molecules potential alternative therapeutic agents to treat cancer patients with Le^y antigen positive cancer soon.

ACKNOWLEDGMENT

We thank V. Fogg and I. Margulies for cell culture, P. Chowdhury, U. Brinkmann, M. Essand, B. Jwang, and M. Gallo for carefully reading the manuscript, and A. Jackson and Jennie Evans for editorial assistance.

LITERATURE CITED

- (1) Batra, J. K., FitzGerald, D., Gately, M., Chaudhary, V. K., and Pastan, I. (1990) Anti-Tac(Fv)-PE40: A single-chain

- antibody Pseudomonas fusion protein directed at interleukin 2 receptor bearing cells. *J. Biol. Chem.* 265, 15198–15202.
- (2) Bird, R. E., Hardman, K. D., Jacobson, J. W., Johnson, S., Kaufman, B. M., Lee, S. M., Lee, T., Pope, S. H., Riordan, G. S., and Whitlow, M. (1988) Single-chain antigen-binding proteins. *Science* 242, 423–426.
- (3) Brinkmann, U., and Pastan, I. (1995) Recombinant immunotoxins: from basic research to cancer therapy. *Methods* 8, 143–156.
- (4) Brinkmann, U., Pai, L. H., FitzGerald, D. J., Willingham, M., and Pastan, I. (1991) B3(Fv)-PE38KDEL, a single-chain immunotoxin that causes complete regression of a human carcinoma in mice. *Proc. Natl. Acad. Sci. U.S.A.* 88, 8616–8620.
- (5) Brinkmann, U., Reiter, Y., Jung, S. H., Lee, B., and Pastan, I. (1993) A recombinant immunotoxin containing a disulfide-stabilized Fv fragment. *Proc. Natl. Acad. Sci. U.S.A.* 90, 7538–7542.
- (6) Buchner, J., Pastan, I., and Brinkmann, U. (1992) A method to increase the yield of properly folded recombinant fusion proteins: single-chain immunotoxins from renaturation of bacterial inclusion bodies. *Anal. Biochem.* 205, 263–270.
- (7) Buckel, P., Huenbner-Parajsz, C., Mattes, R., Lenz, H., Haug, H., and Beaucamp, K. (1987) Cloning and nucleotide sequence of heavy- and light-chain cDNAs from a creatine-kinase-specific monoclonal antibody. *Gene* 51, 13–19.
- (8) Chaudhary, V. K., Queen, C., Junghans, R. P., Waldmann, T. A., FitzGerald, D. J., and Pastan, I. (1989) A recombinant immunotoxin consisting of two antibody variable domains fused to Pseudomonas exotoxin. *Nature* 339, 394–397.
- (9) Choe, M., Webber, K., and Pastan, I. (1994) B3(Fab)-PE38M: A recombinant Immunotoxin in which a mutant form of Pseudomonas exotoxin is fused to the Fab fragment of monoclonal antibody B3. *Cancer Res.* 54, 3460–3467.
- (10) Ellison, J. W., Berson, B. J., and Hood, L. H. (1982) The nucleotide sequence of a human immunoglobulin C γ 1 gene. *Nucleic Acids Res.* 10, 4071–4079.
- (11) Huston, J. S., Levinson, D., Mudgett-Hunter, M., Tai, M. S., Novotny, J., Margolies, M. N., Ridge, R. J., Bruccoleri, R. E., Haber, E., Crea, R., and Oppermann, H. (1988) Protein engineering of antibody binding sites: recovery of specific activity in an anti-digoxin single-chain Fv analogue produced in *Escherichia coli*. *Proc. Natl. Acad. Sci. U.S.A.* 16, 5879–5883.
- (12) Hwang, J., FitzGerald, D. J., Adhya, S., and Pastan, I. (1987) Functional domains of Pseudomonas exotoxin identified by deletion analysis of the gene expressed in *E. coli*. *Cell* 48, 129–136.
- (13) Kreitman, R. J., Chaudhary, V. K., Kozak, R. W., FitzGerald, D. J., Waldman, T. A., and Pastan, I. (1992) Recombinant toxins containing the variable domains of the anti-Tac monoclonal antibody to the interleukin-2 receptor kill malignant cells from patients with chronic lymphocytic leukemia. *Blood* 80 (9), 2344–52.
- (14) Kreitman, R. J., Chang, C. N., Hudson, D. V., Queen, C., Bailon, P., and Pastan, I. (1994) Anti-Tac (Fab)-PE40, a recombinant double-chain immunotoxin which kills interleukin-2 receptor-bearing cells and induces complete remission in an in vivo tumor model. *Int. J. Cancer* 57, 1–9.
- (15) Mulshine, J. L., Magnani, J. L., and Linnoila, R. I. (1991) Applications of monoclonal antibodies in treatment of solid tumors. *Principles of Biotherapy* (V. De Vita, S. Hellman, and S. Rosenberg, Eds.) J. B. Lippincott, Philadelphia, PA.
- (16) Pai, L. H., Batra, J. K., FitzGerald, D. J., Willingham, M. C., and Pastan, I. (1991) Antitumor activities of immunotoxin made of monoclonal antibody B3 and various forms of Pseudomonas exotoxin. *Proc. Natl. Acad. Sci. U.S.A.* 88, 3358–3362.
- (17) Pai, L. H., Wittes, R., Setser, A., Willingham, M. C., and Pastan, I. (1996) Treatment of advanced solid tumors with immunotoxin LMB-1: an antibody linked to Pseudomonas exotoxin. *Nat. Med.* 2, 350–353.
- (18) Pastan, I., and FitzGerald, D. (1991) Recombinant toxins for cancer treatment. *Science* 254, 1173–1177.
- (19) Pastan, I., Lovelace, E. T., Gallo, M. G., Rutherford, A. V., Magnani, J. L., and Willingham, M. C. (1991) Characterization of monoclonal antibodies B1 and B3 that react with mucinous adenocarcinomas. *Cancer Res.* 51, 3781–3787.
- (20) Pastan, I., Pai, L. H., Brinkmann, U., and FitzGerald, D. J. (1995) Recombinant toxins: New therapeutic agents for cancer. *Ann. N. Y. Acad. Sci.* 758, 345–354.
- (21) Reiter, Y., Brinkmann, U., Jung, S.-H., Lee, B., Kasprzyk, P. G., King, C. R., Pastan, I. (1994a) Improved binding and antitumor activity of a recombinant anti-erbB2 immunotoxin by disulfide-stabilization of the Fv fragment. *J. Biol. Chem.* 269, 18327–18331.
- (22) Reiter, Y., Brinkmann, U., Jung, S.-H., Lee, B., and Pastan, I. (1994b) Engineering disulfide bonds into conserved framework regions of Fv fragments: Recombinant immunotoxins containing disulfide-stabilized Fv with improved biochemical characteristics. *Protein Eng.* 7, 697–704.
- (23) Reiter, Y., Pai, L. H., Brinkmann, U., Wang, Q.-C., and Pastan, I. (1994c) Antitumor activity and pharmacokinetics in mice of a recombinant immunotoxin containing a disulfide-stabilized Fv fragment. *Cancer Res.* 54, 2714–2718.
- (24) Reiter, Y., Di Carlo, A., Fugger, L., Engberg, J., and Pastan, I. (1997) Peptide-specific killing of antigen-presenting cells by a recombinant antibody-toxin fusion protein targeted to major histocompatibility complex/peptide class I complexes with T-cell receptor-like specificity. *Proc. Natl. Acad. Sci. U.S.A.* 94, 4631–4636.
- (25) Ridgway, J. B. B., Presta, L. G., and Carter, P. (1996) Knobs-into-holes engineering of antibody CH3 domains for heavy chains dimerization. *Protein Eng.* 9, 617–621.
- (26) Vitetta, E. S., Fulton, R. J., May, R. D., Till, M., and Uhr, J. M. (1987) Redesigning nature's poisons to create antitumor reagents. *Science* 238, 1098–1104.
- (27) Vitetta, E. S. (1990) Immunotoxins: new therapeutic reagents for autoimmunity, cancer, and AIDS. *J. Clin. Immunol.* 10, 15S–18S.

BC9800280

Cross-Linking of Protein Subunits by 1,3,5-Triacryloyl-hexahydro-s-triazine

Gervydas Dienys,[‡] Jolanta Sereikaitė,^{*,§} Gintaras Gavėnas,^{§,||} Rimantas Kvederas,[§] and Vladas-Algirdas Bumelis[§]

Faculty of Chemistry, Vilnius University, Lithuania, and Institute of Biotechnology, Graičiūno 8, Vilnius 2028, Lithuania. Received March 18, 1998; Revised Manuscript Received July 2, 1998

Six difunctional and trifunctional derivatives of acrylamide were synthesized and investigated as potential protein lysine residue cross-linking agents. 1,3,5-Triacryloyl-hexahydro-s-triazine (TAT) was considered the best. The rate constants for the reactions of TAT with model nucleophiles in water solution at 25 °C were with the glycine anion amino group, $7.69 \times 10^{-3} \text{ M}^{-1} \text{ s}^{-1}$; with the anionic form of the *N*-acetyl-L-cysteine thiol group, $5.54 \text{ M}^{-1} \text{ s}^{-1}$; and with the *N*α-acetyl-L-histidine imidazole ring, $1.19 \times 10^{-5} \text{ M}^{-1} \text{ s}^{-1}$ (at pH 9.0). The kinetics of modification of amino groups by TAT were studied for several proteins: α₁-casein, bovine serum albumin, recombinant human growth hormone, recombinant human interferons-α2b, and -γ. The results indicate that if proteins are associated into oligomeric structures in water, their subunits are effectively cross-linked by TAT.

Activated ethylenes of the general formula $\text{CH}_2=\text{CH}-\text{X}$ (X = CN, COOR, CONH₂, COR, NO₂, SO₂R, etc.) can be used in the chemical modification of protein nucleophilic functional groups. Acrylonitrile ($\text{CH}_2=\text{CH}-\text{CN}$) is considered the principal representative of the group (1, 2). Systematic investigation of interactions between acrylonitrile and amino acids and peptides or model compounds have previously been reported by Friedman and his collaborators (2–7), who demonstrated that of the nucleophilic groups of proteins thiol groups are modified most readily. Amino groups are less reactive by 2–3 orders of magnitude, and the imidazole groups of histidine are still less reactive (8). Other functional groups of proteins are not modified.

In addition, thiol groups (7, 9, 10) and amino groups (11) of proteins have been demonstrated to be successfully modified by acrylamide ($\text{CH}_2=\text{CH}-\text{CONH}_2$), though the reaction is slower (approximately by a factor of 10) than that with acrylonitrile. As a result, β-carbamidoethyl groups ($\text{CH}_2\text{CH}_2\text{CONH}_2$) are introduced into the protein molecule. These groups closely resemble glutamine residues. Neither protein electric charge nor pI is altered, nor is solubility decreased, a common result after modification by acrylonitrile or alkylating agents (11). Acrylamide therefore is seen as an agent for the “delicate” modification of proteins. Finally, there are also publications on the modification of proteins by several other activated ethylenes: methylacrylate (2, 7), 4-vinylpyridine (2, 12), and alkylvinylsulphones (2, 13, 14).

A large variety of *N*-substituted acrylamides can be synthesized easily (15). Reactivity of their ethylenic bonds in nucleophile-driven addition reactions varies considerably depending on the nature of *N*-substituents (16). We therefore investigated several bifunctional and

trifunctional derivatives of acrylamide as potential cross-linking agents for protein lysine residues. 1,3,5-Triacryloyl-hexahydro-s-triazine (TAT)¹ was found to be a very promising agent because of its high activity and stability in water solutions.

MATERIALS AND METHODS

Chemicals. Human serum albumin from Calbiochem, bovine serum albumin, myoglobin, acrylamide, *N,N'*-diacryloyl-diaminomethane, *N*-acetyl-L-cysteine, dithiothreitol, and 2,4,6-trinitrobenzene sulfonic acid from Serva, α₁-casein from Sigma, and *N*α-acetyl-L-histidine from Reachim (Russia) were used. Recombinant human interferon-α2b (IFN-α2b), recombinant human interferon-γ (IFN-γ), recombinant tumor necrosis factor α (TNFα), and recombinant human growth hormone (hGH) were products of Biofa AB (Lithuania). Protein markers for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) from Sigma, Pharmacia, and Bio-Rad were used.

¹H-NMR Analysis. ¹H-NMR spectra were recorded on a Hitachi 22 spectrometer, operating at 90 MHz, using tetramethylsilane as the internal standard.

Synthesis of Cross-Linking Agents. *N,N'*-Diacryloyl-diamines (Table 1, nos. 3–5). Acryloylchloride (8 mL, 0.10 mol) was slowly added with stirring to a mixture of diamine (0.05 mol), anhydrous sodium acetate (9 g, 0.11 mol), chloroform (50 mL), and hydroquinone (0.05 g) at 10 °C. The reaction mixture was stirred for 2 h at room temperature and then was filtered. The filtrate was left overnight at 4 °C. A white precipitate was collected and recrystallized from chloroform or hexane.

***N,N'*-Diacryloyl-1,2-diaminoethane.** Yield, 5 g (60%); mp, 145 °C [lit. (17) mp, 144.5–145 °C]. ¹H-NMR (CD₃-

* Author to whom correspondence should be addressed. Phone: (370 2) 642514. Fax: (370 2) 642624. E-mail: Sereik@ibt.lt.

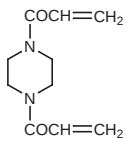
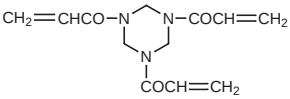
[‡] Vilnius University.

[§] Institute of Biotechnology.

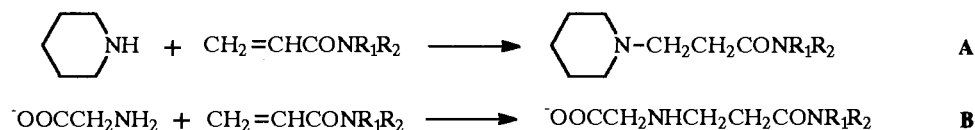
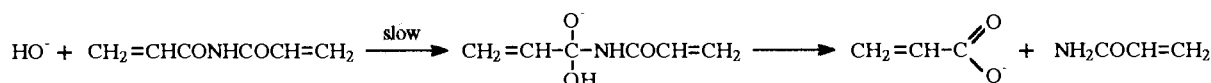
^{||} Present address: BIOK Ltd., P.O. Box 2546, Vilnius, Lithuania.

¹ Abbreviations: TAT, 1,3,5-triacryloyl-hexahydro-s-triazine; IFN-γ, recombinant human interferon-γ; IFN-α2b, recombinant human interferon-α2b; hGH, recombinant human growth hormone; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; BSA, bovine serum albumin; TNFα, recombinant tumor necrosis factor α.

Table 1. Rate Constants k ($M^{-1} s^{-1}$) for Reactions of Potential Cross-Linking Agents with Model N-Nucleophiles in Water Solution at 25 °C

	derivative of acrylamide	structure	N-nucleophile	
			 $k \times 10^3$ ^a	$NH_2CH_2COO^-$ $k \times 10^5$ ^a
1	acrylamide	$CH_2=CHCONH_2$	34.00 ± 3.5^b	38.30 ± 2.2^b
2	<i>N,N</i> -diacryloyl-diaminomethane	$CH_2=CHCONHCH_2NHCOCH=CH_2$	16.66 ± 1.43	32.54 ± 2.59
3	<i>N,N</i> -diacryloyl-1,2-diaminoethane	$CH_2=CHCONHCH_2CH_2NHCOCH=CH_2$	5.06 ± 0.50	7.53 ± 0.58
4	<i>N,N'</i> -diacryloyl-1,3-diaminopropane	$CH_2=CHCONH(CH_2)_3NHCOCH=CH_2$	2.79 ± 0.15	5.52 ± 0.54
5	<i>N,N'</i> -diacryloyl-piperazine		29.07 ± 2.37	63.10 ± 2.75
6	1,3,5-triacryloyl-hexahydro-s-triazine		752.00 ± 46.67	769.00 ± 47.33
7	diacrylimide	$CH_2=CHCONHCOCH=CH_2$	6123 ± 201	14590 ± 1300

^a Rate constants for one electrophilic group ($CH_2=CHCO-$) are given. ^b Standard deviations are given.

Scheme 1**Scheme 2**

SOCD₃): δ 3.43 (m, 4H, CH_2CH_2), 5.67 (q), 6.76 (m, 6H, $CH=CH_2$), 8.40 (br s, 2H, NH).

***N,N'*-Diacryloyl-1,3-diaminopropane.** Yield, 7.9 g (87%); mp, 109–110 °C. ¹H-NMR (CD₃SOCD₃): δ 1.88 (m, 2H, CH_2), 3.35 [m, 4H, (NCH_2)₂], 5.76 (q), 6.30 (q, 6H, $CH=CH_2$), 8.43 (br s, 2H, NH). Anal. Calcd for C₉H₁₄N₂O₂: C, 59.32; H, 7.74; N, 15.37. Found: C, 59.09; H, 7.85; N, 15.16.

***N,N'*-Diacryloyl-piperazine.** Yield, 6.3 g (65%); mp, 91–92 °C [lit. (18) mp, 98 °C]. ¹H-NMR (CD₃SOCD₃): δ 3.76 (s, 8H, piperazine ring), 5.91 (q), 6.31 (q), 7.04 (q, 6H, $CH=CH_2$).

1,3,5-Triacryloyl-hexahydro-s-triazine (TAT, Table 1, no. 6). TAT was prepared as described by T. L. Gresham and T. R. Steadman (19) and recrystallized from water. TAT polymerized when heated and no definite melting point could be identified. ¹H-NMR (CDCl₃): δ 5.39 (s, 6H, CH_2), 5.80 (q), 6.32 (q), 6.78 (q, 9H, $CH=CH_2$).

Diacrylimide (Table 1, no. 7). Diacrylimide was prepared as previously described (20). The product was purified by column chromatography on silica gel using ethyl acetate/chloroform (1/1, v/v) as the eluent. Diacrylimide was crystallized from water: mp, 179 °C [lit. (20) mp, 180 °C]. ¹H-NMR (CDCl₃): δ 5.82 (q), 6.42 (q), 6.81 (q, 6H, $CH=CH_2$).

Kinetic Measurements. The kinetics of reactions shown in Scheme 1 were followed spectrophotometrically in thermostated cell at 255 nm for unsaturated amides no. 1–5 and 7 (Table 1) and at 260 nm for TAT (no. 6). The extinction coefficients of amides no. 1–5 and 7 at 255 nm were 134, 1260, 1680, 1470, 8340, and 2530 M⁻¹ cm⁻¹, respectively, and that of TAT at 260 nm was 3120 M⁻¹ cm⁻¹. The optical density of the piperidine and glycine

at 255 and 260 nm was negligible. The extinction coefficient of piperidine at 255 nm was 1.46 M⁻¹ cm⁻¹. UV-absorption spectra of addition products were shifted toward the short-wave side with respect to the spectra of initial unsaturated amides. From 6 to 10 kinetic runs were carried out for each reaction. Molar concentration of the product at the time $t(x)$ was calculated by the equation

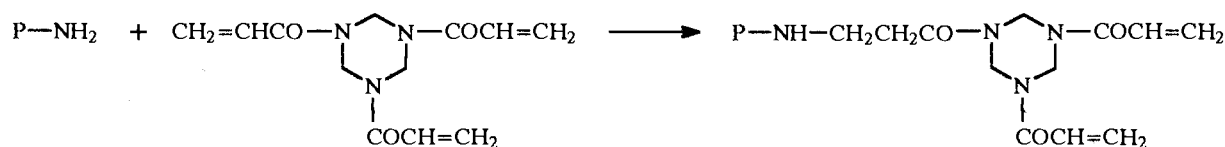
$$x = a(A_0 - A/A_0 - A_{inf})$$

where A is the absorbance at the time t , A_0 is the initial absorbance and A_{inf} is the final absorbance, when the reaction is completed, a is the initial concentration of N-substituted acrylamide.

The kinetics of the reactions shown in Scheme 1B were studied in alkaline solutions at several pH values and rate constants were calculated for anion $NH_2CH_2COO^-$ as the nucleophile. The kinetics of alkaline hydrolysis of diacrylimide were also investigated spectrophotometrically at pH values between 10.05 and 13.6.

The kinetics of modification of protein amino groups (Scheme 3, Table 2) were followed by measuring the concentration of free amino groups in aliquots of the reaction mixture every 30 min over a period of 3–4 h, using the 2,4,6-trinitrobenzene sulfonic acid method (21) as described by Jonušienė et al. (22). Three kinetic runs were carried out for each reaction. Second-order rate constants were calculated from the reaction rate values obtained by the method of cubic smoothing splines (23) at the initial moment and after modification of 50% of the amino groups.

The kinetics of the reaction of TAT with *N*-acetyl-L-cysteine thiol groups were followed spectrophotometri-

Scheme 3^a

^a P represents a protein molecule.

Table 2. Rate Constants^a and Half-Lives of Protein Amino Groups Modification by TAT at pH 9.2 and 30 °C

protein	$C_{\text{TAT}0}$ (mM)	$k \times 10^3$ ^b (M ⁻¹ s ⁻¹)	$k_1 \times 10^3$ ^c (M ⁻¹ s ⁻¹)	half-life ^d (h)
α_1 -casein	2.75	8.58 ± 0.43^e	6.66 ± 0.71^e	3.19 ± 0.27^e
BSA	3.24	9.15 ± 0.80	4.19 ± 0.18	3.21 ± 0.22
hGH	3.69	7.36 ± 0.58	6.15 ± 0.58	2.73 ± 0.29
IFN- α 2b	8.13	9.36 ± 0.88	8.06 ± 0.38	0.88 ± 0.05
IFN- γ	8.13	15.11 ± 3.49	8.13 ± 1.62	0.69 ± 0.15

^a Rate constants for one electrophilic group (CH₂=CHCO-) of TAT are given. ^b Rate constants calculated at the initial time. ^c Rate constants calculated after modification of 50% of the amino groups. ^d Time required for modification of 50% of the amino groups. ^e Standard deviations are given.

cally in a thermostated cell, at 275 nm. Six kinetic runs were carried out for each experiment. The second-order rate constants for the *N*-acetyl-L-cysteine dianionic form were calculated from initial rate values at 6.5, 10.5, and 14.5 °C: $2.67 \pm 0.08 \text{ M}^{-1} \text{ s}^{-1}$, $3.26 \pm 0.22 \text{ M}^{-1} \text{ s}^{-1}$ and $3.69 \pm 0.51 \text{ M}^{-1} \text{ s}^{-1}$, respectively. The rate constant at 25 °C was calculated using an Arrhenius plot, $5.54 \text{ M}^{-1} \text{ s}^{-1}$.

The kinetics of the reaction of TAT with the imidazole ring of *N*-acetyl-L-histidine at pH 9.0 was also followed spectrophotometrically in a thermostated cell at 275 nm, under pseudo-first-order conditions (150-fold excess of *N*-acetyl-L-histidine). Three kinetic runs were carried out for each experiment. The second-order rate constants calculated at 60, 70, and 80 °C were $(2.32 \pm 0.39) \times 10^{-4} \text{ M}^{-1} \text{ s}^{-1}$, $(3.98 \pm 0.56) \times 10^{-4} \text{ M}^{-1} \text{ s}^{-1}$, and $(9.31 \pm 0.33) \times 10^{-4} \text{ M}^{-1} \text{ s}^{-1}$, respectively. The rate constant at 25 °C was calculated using an Arrhenius plot, $1.19 \times 10^{-5} \text{ M}^{-1} \text{ s}^{-1}$.

Cross-Linking of Oligomeric Proteins by TAT.

Protein solutions were prepared in 0.05 M borate buffer, pH 9.2, such that the initial concentration of amino groups was between 0.06 and 0.59 mM. TAT was added in 4.5–67-fold molar excess with respect to the concentration of amino groups in the protein solution. The reaction mixture was incubated at 30 °C. Aliquots were withdrawn over the course of the reaction, and the reaction was stopped by the addition of excess cysteine or dithiothreitol. Samples of the reaction mixture were dialyzed and analyzed by SDS–PAGE for identification of cross-linked protein forms.

SDS–PAGE. SDS–PAGE was carried out on the Mighty Small electrophoresis unit (Hoefer Scientific Instruments) according to the method of Laemmli (24). The acrylamide concentrations used in the gel were 12.5% (for hGH, TNF α), 15% (for INF- γ , myoglobin), and 7.5% (for human serum albumin). Proteins were stained with Coomassie Brilliant Blue R-250 and the gels were scanned at 633 nm using the Dual-Wavelength TLC scanner (CS 930, Shimadzu).

RESULTS AND DISCUSSION

Investigation of Cross-Linking Agents. Six difunctional and trifunctional derivatives of acrylamide were synthesized. Their activity in nucleophilic addition reactions was evaluated by measuring the kinetics of the

reactions with low molecular weight model N-nucleophiles—piperidine and the anion of glycine (Scheme 1). The rate constants measured are given in Table 1. For comparison, acrylamide is included together with its polifunctional derivatives.

N,N'-Diacryloyl-diaminoalkanes (Table 1, nos. 2–4) were found to be less reactive than acrylamide. They are of little interest as cross-linking agents, however, because their rate of modification is too low for practical purposes. The half-life of the most reactive protein amino groups at 25 °C is over 6 h if a concentration of acrylamide of 0.1 M is used (11).

Diacrylimide was the most active of the tested compounds (Table 1, no. 7). It is not, however, stable in alkaline solutions. It is hydrolyzed to form acrylamide and acrylate (25). Diacrylimide is a weak acid, and in alkaline solution it ionizes partly to form the anion (CH₂=CHCO)₂N⁻; a process which needs to be taken into account when the kinetics of its hydrolysis and nucleophilic addition of amines are studied. We measured the diacrylimide ionization constant ($pK_a = 10.9$) using the spectrophotometric method (26) and investigated the kinetics of its alkaline hydrolysis. The kinetic data are consistent with the mechanism including, as the slow step, the attack of diacrylimide unionized molecule by an hydroxide anion (Scheme 2). The rate constant of the reaction described under Scheme 2 at 25 °C in water solution was $4.35 \text{ M}^{-1} \text{ s}^{-1}$, a value similar to or slightly higher than those for the hydrolysis of saturated imides: diacetimide ($k = 0.88 \text{ M}^{-1} \text{ s}^{-1}$), succinimide ($k = 3.14 \text{ M}^{-1} \text{ s}^{-1}$) (25). At pH 9.2, the half-life of diacrylimide is approximately 3 h, at pH 10, approximately 30 min. Diacrylimide may therefore be of interest, under conditions where temporary cross-linking is required.

We found that the best cross-linking agent from the compounds listed in Table 1 was TAT. Its activity was conveniently high and its stability in water was remarkable. No change was identified after storing TAT in buffer, pH 9.2, over the course of 1 week at room temperature. Its three active groups make it an especially effective cross-linking agent.

In order to characterize the reactivity of TAT, the kinetics of its reactions with two other model nucleophiles *N*-acetyl-L-cysteine and *N*-acetyl-L-histidine were investigated. The rate constant of the anionic form of *N*-acetyl-L-cysteine (a model of thiol groups of proteins) was $5.54 \text{ M}^{-1} \text{ s}^{-1}$ at 25 °C in water, a value which exceeded, by nearly three orders of magnitude, the rate constant of the glycine anion amino group (Table 1). Such an activity ratio is supported by the literature (4). The histidine residue imidazole ring is not as active a nucleophile as the amino group. The rate constant of the reaction between *N*-acetyl-L-histidine and TAT at 25 °C and pH 9.0 was $1.19 \times 10^{-5} \text{ M}^{-1} \text{ s}^{-1}$. Consequently, it is clear that, if there are no free thiol groups, TAT is quite a specific modifying agent for protein amino groups.

Modification and Cross-Linking of Proteins. The first step in the interaction between TAT and a protein molecule is generally the addition of an amino group to one of the acryloyl groups (Scheme 3). If free thiol groups

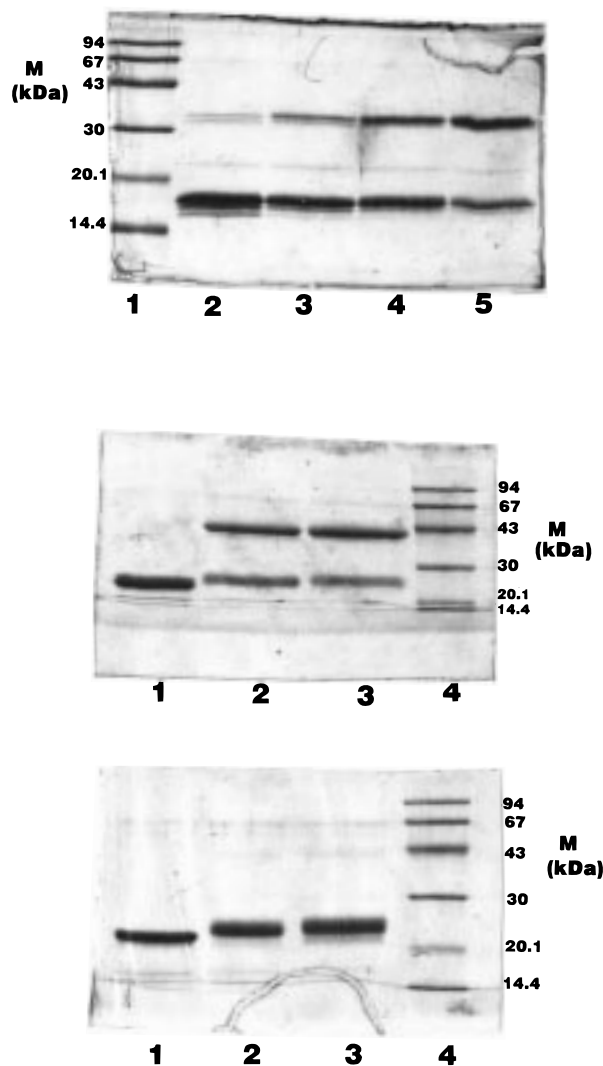


Figure 1. (Top) SDS-PAGE (15%) of IFN- γ samples. Lane 1, protein markers; lane 2, unmodified IFN- γ ; lanes 3-5, samples taken at 15, 30, and 60 min. Concentration of IFN- γ , 0.16 mg/mL; initial concentration of amino groups, 0.18 mM; concentration of TAT, 1.0 mM. (Bottom) SDS-PAGE (12.5%) of hGH samples modified in the absence of Zn^{2+} . Lane 1, unmodified hGH; lanes 2 and 3, samples taken at 60 and 120 min; lane 4, protein markers. Concentration of hGH, 0.75 mg/mL; initial concentration of amino groups, 0.39 mM; concentration of TAT, 4.5 mM. (Middle) SDS-PAGE (12.5%) of hGH samples modified in the presence of Zn^{2+} . Lane 1, unmodified hGH; lanes 2 and 3, samples taken at 60 and 120 min; lane 4, protein markers. Concentrations of hGH, amino groups and of TAT were the same as those in the bottom panel. Molar ratio of Zn^{2+} to hGH in the reaction mixture was 2:1.

are present, though, their reaction precedes that of the amino groups. Intra- and intersubunit cross-linking of amino groups then follows.

We therefore studied the kinetics of the TAT-induced modification of the amino groups of several proteins (Table 2). It was found that the rate constants decreased during the addition reaction. Solubility of TAT in water is limited (approximately 40 mM at room temperature). Half of the amino groups are modified in less than 15 min in the presence of such a concentration of TAT at 30 °C and pH 9.2. We therefore used mixed solvents water-methanol and water-ethanol to promote the modification rate in some cases (27).

Cross-linking investigations were carried out with several proteins, both monomeric and oligomeric forms. The course of cross-linking was followed by SDS-

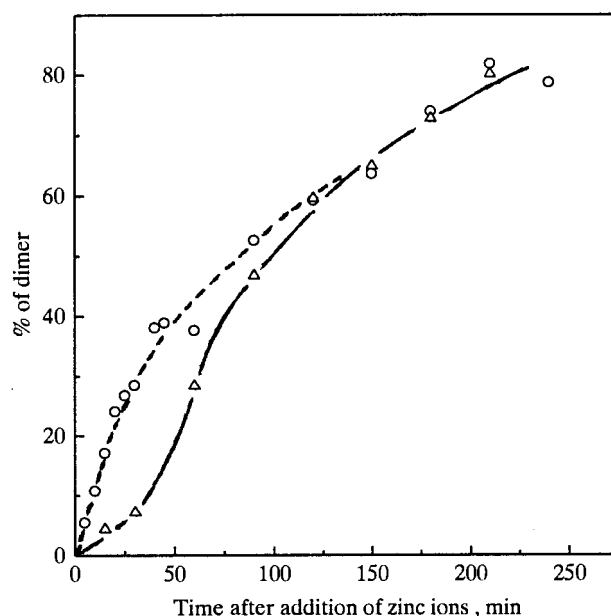


Figure 2. Kinetics of hGH cross-linking by TAT (0.05 M borate buffer, pH 9.2, 30 °C; initial concentrations: hGH, 0.3 mg/mL, 14 μM ; TAT, 1.8 mM). Experiment A (Δ), Zn^{2+} (28 μM) was added together with TAT; experiment B ($\circ$), Zn^{2+} (28 μM) was added after 25% of amino groups were modified by TAT.

electrophoresis of samples of reaction mixtures. Most experiments were performed at pH 9.2 and 30 °C using a 4.5–14-fold excess of TAT compared with the concentration of amino groups present. At pH 8.0, though, using the maximum possible concentration of TAT (approx. 40 mM) and leaving the reaction mixture overnight at 30 °C practically complete cross-linking could also be achieved.

At the protein concentrations used (0.5–2.0 mg/mL), no cross-linking was registered for proteins which were monomeric in water (see, for example, Figure 1, bottom). But after modification by TAT, electrophoretic bands of proteins were slightly displaced and became broader. The change was greatest for the proteins having the greater number of Lys residues (human serum albumin, myoglobin). Proteins, that are dimeric in water [for example, IFN- γ (28)], were transformed by TAT into covalently bonded dimers, which were not separated during SDS-electrophoresis (Figure 1, top).

It is known that Zn^{2+} ions induce the dimerization of hGH (29), an observation confirmed by our measurements of the action of TAT. In the presence of Zn^{2+} , cross-linked dimers were formed (Figure 1, middle), but no dimers were detected in the absence of Zn^{2+} (Figure 1, bottom). The kinetics of hGH cross-linking are shown in Figure 2. If Zn^{2+} ions were added after amino groups had been already partly modified by TAT (Figure 2, experiment B), formation of cross-linked dimers occurred more rapidly, where Zn^{2+} and TAT were added together (Figure 2, experiment A). Apparently, the cross-linking step is faster than the addition of amino groups to TAT. If experiments were continued for long enough (24 h), the yield of dimer in both cases approached 100%.

TNF α is known as a trimeric protein in water (30). The time course of its cross-linking by TAT was also investigated (Figure 3). Polymerization was evident after 30 min, when the dimeric form was clear, its presence was maximal after 90 min, but at longer time intervals the trimeric form developed to become predominant, though two bands of molecular weight higher than the dimeric form were identifiable. Most likely, both repre-

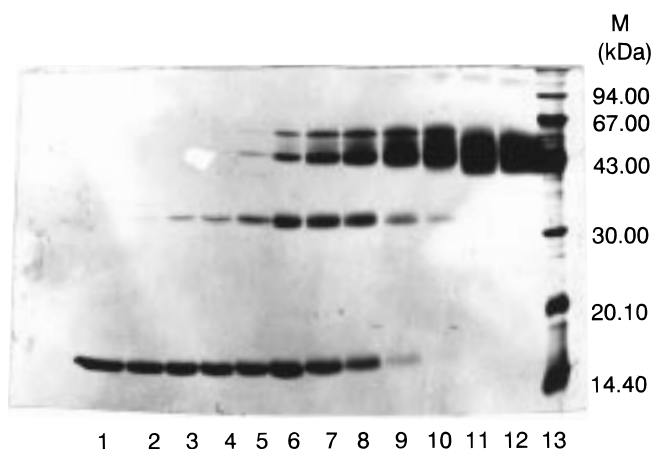


Figure 3. SDS-PAGE (12.5%) of TNF α samples. Lane 1, unmodified TNF α ; lane 2–12, samples taken at 15, 30, 45, 60, 90, 120, 180, 240, 480 min, 24, and 48 h; lane 13, protein markers. Concentration of TNF α , 0.15 mg/mL; initial concentration of amino groups, 0.06 mM; concentration of TAT, 4 mM.

sent cross-linked trimers (31). A very weak band of a molecular weight corresponding to that of a hexamer appeared also at long time intervals. X-ray studies indicate the assembly of two trimers of TNF α to make a hexameric unit in the crystal form (32). Dimerization of trimers may also occur to some extent in solution.

Cross-linking of protein amino groups by TAT is a very simple and rapid way to establish whether protein oligomers are formed in solution. The high stability of TAT in water promotes its utility. It would be very difficult to conduct experiments similar to those shown in Figures 2 and 3 with any unstable cross-linking agent, for example, dimethyl suberimide.

ACKNOWLEDGMENT

We thank Biofa AB for the gift of hGH, INF- α 2b, INF- γ , and TNF α .

LITERATURE CITED

- Means, G. E., and Feeney, R. E. (1971) *Acrylonitrile. Chemical Modification of Proteins*, pp 114–117, Holden-Day Inc., San Francisco.
- Friedman, M., and Wall, J. S. (1966) Additive linear free-energy relationships in reaction kinetics of amino groups with α,β -unsaturated compounds. *J. Org. Chem.* 31, 2888–2894.
- Friedman, M., and Wall, J. S. (1964) Application of a Hammet-Taft relation kinetics of alkylation of amino acids and peptide model compounds with acrylonitrile. *J. Am. Chem. Soc.* 86, 3735–3741.
- Friedman, M., Wall, J. S., and Cavins, J. F. (1965) Relative nucleophilic reactivities of amino groups and mercaptide ions in addition reactions with α,β -unsaturated compounds. *J. Am. Chem. Soc.* 87, 3672–3682.
- Friedman, M. (1967) Solvent effects in reactions of amino groups in amino acids, peptides and proteins with α,β -unsaturated compounds. *J. Am. Chem. Soc.* 89, 4709–4713.
- Cavins, J. F., and Friedman, M. (1967) New amino acids derived from reactions of epsilon - amino groups in proteins with alpha, beta-unsaturated compounds. *Biochemistry* 6, 3766–3770.
- Cavins, J. F., and Friedman, M. (1968) Specific modification of protein sulphhydryl groups with α,β -unsaturated compounds. *J. Biol. Chem.* 243, 3357–3360.
- Bosshard, H. R., Jorgensen, K. U., and Humbel, R. E. (1969) Preparation and properties of cyanoethylated insulin. An insulin derivative with blocked amino- and imidazole-groups. *Eur. J. Biochem.* 9, 353–362.
- Edwards, P. M. (1975) Neurotoxicity of acrylamide and its analogues and effects of these analogues and other agents on acrylamide neuropathy. *Br. J. Ind. Med.* 32, 31–38.
- Brune, D. C. (1992) Alkylation of cysteine with acrylamide for protein sequence analysis. *Anal. Biochem.* 207, 285–290.
- Danilevičiūtė, M., Adomėnienė, O., and Dienys, G. (1981) Kinetics of interaction of acrylamide with amino groups of some proteins. *Org. React.* 18, 217–223.
- Friedman, M., Krull, L. H., and Cavins, J. F. (1970) The chromatographic determination of cystine and cysteine residues in proteins as 5- β -(4-pyridylethyl)cysteine. *J. Biol. Chem.* 245, 3868–3871.
- Masri, M. S., and Friedman, M. (1988) Protein reactions with methyl and ethyl vinyl sulfones. *J. Protein Chem.* 7, 49–54.
- Morpurgo, M., Veronese, F. M., Kachensky D., and Harris, J. M. (1996) Preparation and characterization of poly(ethylene glycol) vinyl sulfone. *Bioconjugate Chem.* 7, 363–368.
- Beckwith, A. L. J. (1970) Synthesis of Amides. *The Chemistry of Amides* (J. Zabicky, Ed.) pp 73–186, Interscience Publishers, London.
- Gavėnas, G., Dienys, G., and Adomėnienė, O. (1984) Kinetics of nucleophilic addition of piperidine and glycine to N-substituted and N,N-disubstituted acrylamides. *Org. React.* 21, 295–303.
- Hirose, K., Ihashi, Y., and Yoshizawa, M. (1966) Infrared spectra of poly(methylenebisacrylamide). *Kogyo Kagaku Zasshi* 69, 240–244.
- Danusso, F. (1967) Sintesi di poli-amidi-ammine lineari ad elevato peso molecolare. Nota II. Polimeri da diammine disecundari a bisacrilamidi diverse. *Chim. Ind.* 49, 453–457.
- Gresham, T. L., and Steadman, T. R. (1949) Triazines from formaldehyde and nitriles. *J. Am. Chem. Soc.* 71, 1872–1873.
- Merger, F. (1968) Reaktionen des harnstoffs mit acrylsäure und methacrylsäurechlorid. *Chem. Ber.* 101, 2419–2425.
- Satake, K., Okuyama, T., Ohashi, M., and Shinoda, T. (1960) The spectrophotometric determination of amine, amino acid and peptide with 2,4,6-trinitrobenzene-l-sulfonic acid. *J. Biochem.* 47, 654–657.
- Jonušienė, Z., Dienys, G., and Steponavičius, J. (1988) Determination of amino groups in proteins and peptides with 2,4,6-trinitrobenzene sulfonic acid. *Zh. Anal. Khim.* 43, 536–540.
- Dagys, R., Pauliukonis, A., Kazlauskas, D., Mankevičius, M., and Simutis, R. (1986) Determination of initial velocities of enzymic reactions from progress curves. *Biochem. J.* 237, 821–825.
- Laemmli, U. K. (1970) Laemmli cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680–685.
- Edward, J. T., and Terry, K. A. (1957) The hydrolysis of amides and related compounds. Part IV. Diacetylamine and succinimide in aqueous alkali. *J. Chem. Soc.* 3527–3530.
- Albert, A., and Serjeant, E. P. (1962) *Ionization Constants of Acids and Bases*, John Wiley, New York.
- Dienys, G., Strazdienė, V., and Sereikaitė, J. (1996) The effect of solvent on the rate of addition of α_1 -casein amino groups and model nucleophiles to 1,3,5-triacryloyl-hexahydro-s-triazine. *Org. React.* 30, 21–23.
- Ealick, S. E., Cook, W. J., Vijai-Kumar, S., Carson, M., Nagabhushan, T. L., Trotta, P. P., and Bugg, C. E. (1991) Three-dimensional structure of recombinant human interferon- γ . *Science* 252, 698–702.
- Cunningham, B. C., Mulkerrin, M. G., and Wells, J. A. (1991) Dimerization of human growth hormone by zinc. *Science* 253, 545–548.
- Van Ostade, X., Tavernier, J., and Fiers, W. (1994) Structure-activity studies of human tumor necrosis factors. *Protein Eng.* 7, 5–22.
- Horiguchi, T., Miwa, Y., and Shigesada, K. (1997) The quaternary geometry of transcription termination factor rho: assignment by chemical cross-linking. *J. Mol. Biol.* 269, 514–528.
- Brookhaven Protein Data Bank filename 2TUN.

BC9800318

Delivery of Antisense Oligonucleotides Using HPMA Polymer: Synthesis of A Thiol Polymer and Its Conjugation to Water-Soluble Molecules

Laixin Wang, Jakob Kristensen, and Duane E. Ruffner*

University of Utah Department of Pharmaceutics and Pharmaceutical Chemistry 421 Wakara Way, Suite 318 Salt Lake City, Utah 84108. Received March 19, 1998; Revised Manuscript Received July 21, 1998

Here, we report the synthesis and characterization of an HPMA-based polymer for use as a carrier for the delivery of water soluble drugs. The polymer contains active-sulfhydryl groups for coupling of ligands through a disulfide linkage. The polymer can also be prepared containing pendant amino groups in addition to the active-sulfhydryl moiety. This allows the use of different chemistries to conjugate a variety of ligands to the polymer. We demonstrate that a sulfhydryl-terminated antisense oligonucleotide can be efficiently and rapidly conjugated to the polymer. The polymer–oligonucleotide conjugate is efficiently taken up by cultured cells.

INTRODUCTION

Antisense oligonucleotides have shown great potential as therapeutic agents in several areas including anti-cancer and antiviral chemotherapy (1). One limitation to their successful development as therapeutics is their poor uptake by mammalian cells. Many approaches to increase uptake efficiency have been examined, but none have achieved wide scale acceptance (see ref 2 for review). Development of a better delivery system is still being explored worldwide to take antisense to clinical usage.

Water-soluble macromolecular carrier systems have shown great potential in drug delivery due to their diversity in physicochemical and biological properties which can improve the chemical stability and pharmacokinetics of the conjugated drug (see ref 3 for review). One promising macromolecular carrier is the synthetic *N*-(2-hydroxypropyl)methacrylamide (HPMA) polymer. The polymer alone exhibits little or no binding to cell surfaces in a number of test systems [rat visceral yolk sacs (4) and hepatocytes (5)], exhibits little immunogenicity (6, 7), and is captured by cells at a rate consistent with fluid-phase pinocytosis. The pinocytotic uptake of soluble HPMA copolymers by cultured cells was dependent on the molecular mass (M_r) of polymers. Usually, the higher the M_r , the lower the rate of cell capture (8). But some cultured cells, such as rat intestinal sacs, showed an increased rate of pinocytic capture with increasing polymer M_r , which is proposed to be due to adsorptive-pinocytosis (8).

The M_r of HPMA copolymers also influences in vivo behavior, such as the rate of excretion and pharmacological activity (9). The blood-clearance of HPMA homopolymer preparations ($M_r = 28$ –91 kDa) has been studied following intravenous administration to rabbits. It was shown that polymers of higher M_r had a slower rate of clearance from the blood stream, and the limiting molecular-mass threshold for glomerular filtration was determined to be 45 kDa (9). Therefore, the size alone

of the HPMA carrier can provide a variable element to optimize the drug delivery system for a specific therapeutic purpose.

N-(2-Hydroxypropyl)methacrylamide polymer (HPMA) has shown great potential as a carrier for targeted delivery and controlled release of small molecule pharmaceuticals (10–15). Successful targeted delivery has been achieved using a variety of targeting moieties, including carbohydrate residues [galactosamine (16) and fucosylamine (11)], proteins [hormones or transferrin (12)], and antibodies (12, 17, 18). For controlled release, the drug molecules have been conjugated to HPMA polymer through peptide bonds using a reactive *p*-nitrophenyl ester comonomer. Since *p*-nitrophenyl ester is relatively unstable in aqueous solutions (19), this reaction usually needs to be performed in organic solvents in order to obtain high yields. In aqueous solution, hydrolysis of the active ester is expected to compete for the desired aminolysis, especially when the amino groups are carried by large molecules, such as peptides, antisense oligonucleotides, and proteins. Therefore, coupling efficiency in aqueous solution is expected to be low for these molecules. Additionally, coupling through a peptide linkage requires proteolytic degradation for release of the drug. This requires a lysosomal route of uptake which can place biopharmaceuticals such as peptides and nucleic acids in the presence of degradative enzymes.

Here, we describe the synthesis and characterization of an active thiol-containing HPMA copolymer. Coupling to the polymer of water-soluble molecules, such as an antisense oligodeoxynucleotide, occurs rapidly and in high yield. Since the drug and polymer are conjugated through a disulfide bond, the conjugate is expected to be stable in the bloodstream but cleaved intracellularly by thiols such as glutathione and/or redox enzymes. In preliminary experiments, we find that an antisense-oligonucleotide–polymer conjugate is efficiently internalized by cultured cells.

MATERIALS AND METHODS

Chemicals and instruments. *N*-(2-Hydroxypropyl)-methacrylamide (HPMA), *N*-(3-aminopropyl)methacryl-

* Author to whom correspondence should be addressed.
E-mail: duane.ruffner@m.cc.utah.edu. Phone: (801) 585-5093.
Fax: (801) 585-3614.

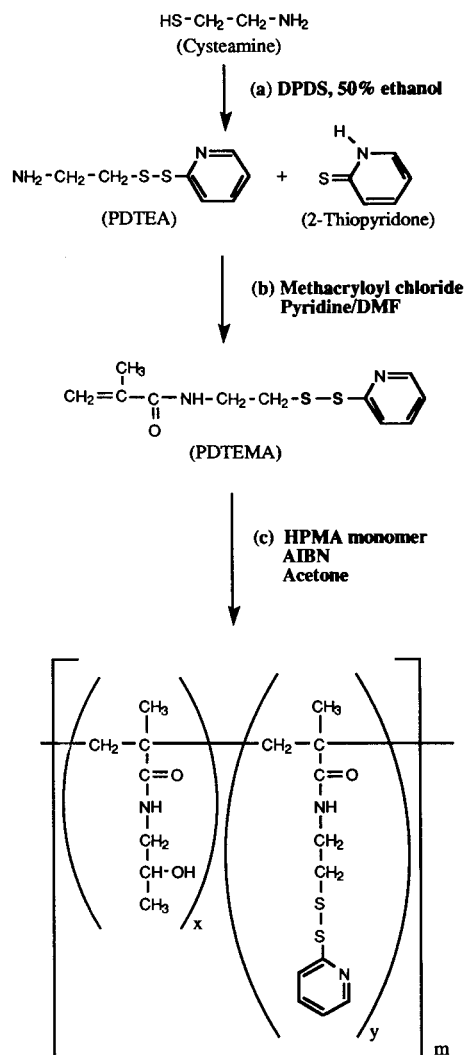


Figure 1. Reaction scheme for synthesis of *N*-[2-(2-pyridyldithio)ethyl] methacrylamide (PDTEMA) and its incorporation into HPMA copolymer.

amide (APMA) hydrochloride, 2,2'-azo-bis(isobutyronitrile) (AIBN), and methacryloyl chloride were purchased from Polyscience Inc. 2-Aldrithiol (DPDS) was from Aldrich, and cysteamine and Dulbecco's modified eagle's medium (DMEM) nutrient mixture were purchased from Sigma. HeLa S3 cells (human epithelial carcinoma cells) were from American Type Culture Collection. Other chemicals or reagents were from Aldrich or Sigma. NMR spectra were recorded on a Bruker AC200 NMR spectrometer. Mass spectra were obtained on a Thermabeam Mass detector. UV spectroscopy was performed using a Varian Cary 3E spectrophotometer. Buffer A (McIlvaine's citric acid-phosphate buffer, pH 3.0–8.0) was prepared by combining 0.5 M citric acid and 0.5 M disodium phosphate in a suitable ratio to give the desired pH. Buffer B (Sorensen's glycine II buffer, pH 9.0–13.0) was prepared by combining suitable volumes of 0.5 M glycine in 0.5 M NaCl with 0.5 M NaOH.

Sulfhydryl peptide (Figure 5, pAntp-SH) and disulfide modified oligonucleotide (Figure 5, RSS-OSH) were synthesized by the University of Utah DNA/Peptide core facility. Peptide was purified by HPLC and stored at -70°C in the elution buffer containing approximately 1% TFA. The oligodeoxynucleotide phosphorothioate was synthesized on an Applied Biosystems 394 automated DNA synthesizer on a 1.0 μmol scale using standard

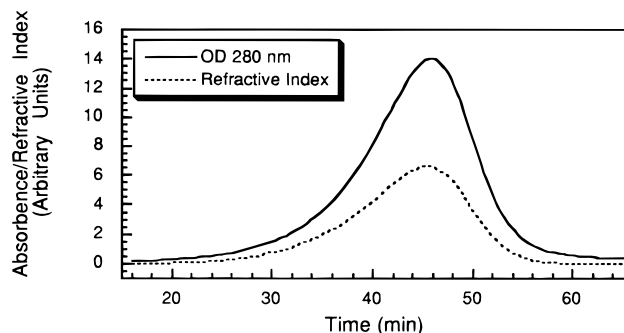


Figure 2. FPLC chromatogram of copolymer A eluted from a gel permeation column. UV absorbance was measured at 280 nm (solid line). The refractive index is indicated by the dotted line. Column: Pharmacia HR10/30. Buffer: pH 7.3, PBS. Flow rate: 0.4 mL/min. Chart speed: 0.5 cm/min.

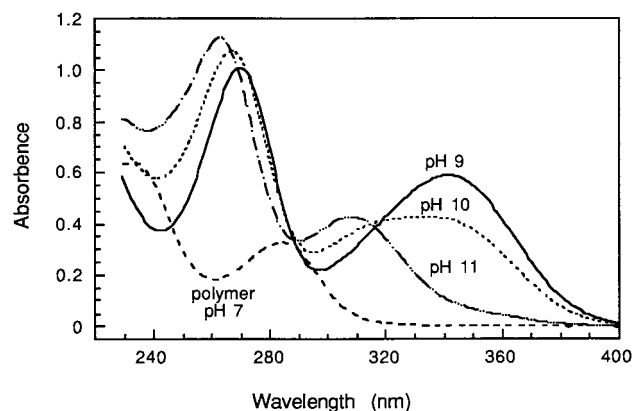


Figure 3. UV spectrum of copolymer A in 0.1 M, pH 7.0, phosphate buffer and 2-thiopyridone in 0.1 M buffer B at pH 9, 10, or 11, as indicated.

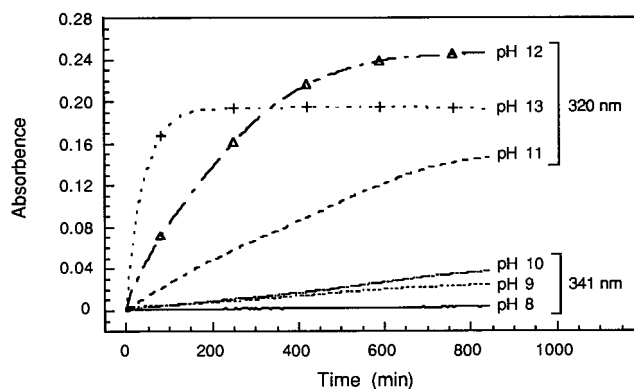


Figure 4. Kinetics of the hydrolysis of copolymer A at pH 8–13 as monitored by the absorbance at 320 or 341 nm.

phosphoramidite chemistry, except that 3H-1,2-benzodithiol-3-one 1,1-dioxide replaced aqueous iodine in the oxidation step (20). For the oligodeoxynucleotide synthesis, most reagents were from Applied Biosystems Inc. except Spacer 9, thiol-modifier C6 SS, and fluorescein phosphoramidite, which were from Glen Research. The oligonucleotides were purified using 20% PAGE and recovered by electroelution followed by ethanol precipitation.

Synthesis of *N*-[2-(2-Pyridyldithio)ethyl] Methacrylamide (PDTEMA). DPDS (5.135 g, 23 mmol) was dissolved in 60.0 mL of 95% EtOH in a 250 mL round-bottom flask. A solution of 1.223 g of cysteamine (16 mmol) in 60.0 mL of deionized water (pH approximately 8.5) was added dropwise over 10 min to the DPDS

Cys-Arg-Gln-Ile-Lys-Ile-Trp-Phe-Gln-Asn-Arg-Arg-Met-Lys-Trp-Lys-Lys

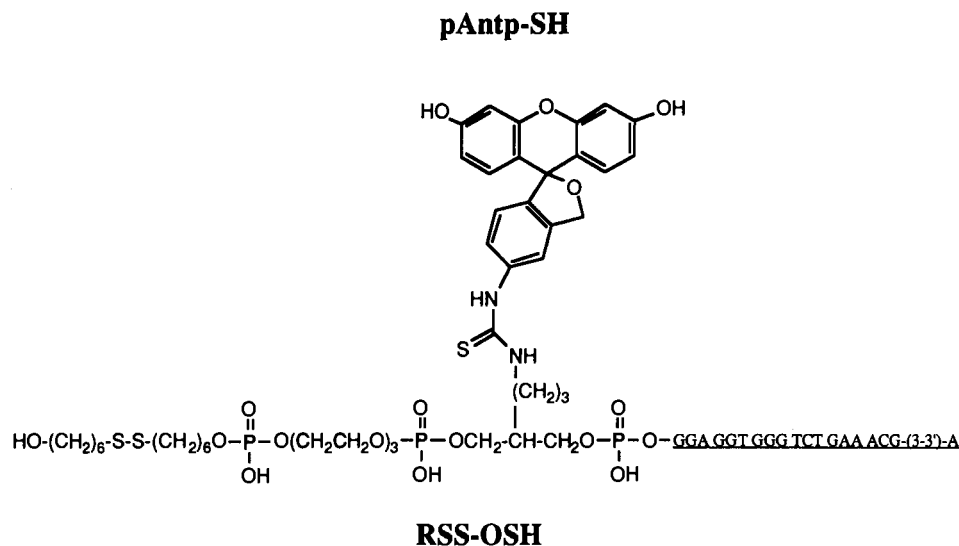


Figure 5. Chemical structure of sulfhydryl pAntp peptide (pAntp-SH) and fluorescein labeled oligonucleotide (RSS-OSH). The underlined portion of RSS-OSH indicates the sequence of the oligonucleotide which contains phosphorothioate linkages.

solution with stirring at room temperature. After incubation for 1.5 h at room temperature, the solvent was removed by rotary evaporation at 35 °C. The product was obtained as a syrup which was dried by coevaporation 3 times with 6 mL of anhydrous pyridine.

The dried crude product was dissolved in a mixture of 25.0 mL of anhydrous DMF and 50 mL of anhydrous pyridine. N₂ was bubbled through for 5 min to remove oxygen. A magnetic stirrer was added, and the bottle was sealed tightly with a rubber stopper. Methacryloyl chloride (3.10 mL, 32 mmol) was added slowly over 5 min using a syringe. After stirring the solution for 2 h at room temperature, the reaction was quenched by adding 400 mL of saturated NaHCO₃ and extracted twice with 400 mL of ethyl acetate. The combined organic phase was washed with 2 × 100 mL of deionized water and dried using anhydrous MgSO₄. The clear solution was evaporated to dryness at 35 °C by rotary evaporation. The crude product was dissolved in CHCl₃ and absorbed to about 10 mL of silica gel, which was then applied to a short silica gel column (12.5 × 5.5 cm, packed with hexane). The column was eluted with a hexane:EtOAc concentration gradient (5 to 40% ethyl acetate). The fractions were monitored by TLC, and the fractions containing the main product were combined and dried by rotary evaporation. The product was confirmed by ¹H NMR and mass spectral analysis. The yield was 2.9 g or 70% for the two steps. ¹H NMR (CDCl₃, 200 MHz): δ (ppm) 7.0–8.6 (m, 5H, Ar-H and -NH), 5.809 (s, 1H, one of =CH₂), 5.361 (s, 1H, one of =CH₂), 3.603 (m, 2H, -CH₂-NR), 2.960 (t, 2H, -S-CH₂-), 2.002 (d, 3H, -CH₃, the split of this peak is due to the tautomerization of the adjacent double bond). MS (FAB), *m/e* 255 (M + 1).

Synthesis of Pyridine Disulfide Modified HPMA Copolymer. HPMA, PDTEMA, and AIBA were dissolved in acetone (12.5 wt % monomers, 0.6 wt % AIBN). Insoluble impurities were removed by filtration or centrifugation. The solution was bubbled with N₂ for 5 min to remove the dissolved oxygen and the reaction vessel was closed tightly. The reaction was placed in a 50–55 °C water bath for 24 h. The white precipitate formed was recovered by filtration and was dried in a vacuum desiccator. The crude product was dissolved in a small

volume of MeOH (approximately 1 g of polymer in 4 mL of MeOH), then precipitated by slowly adding excess acetone. The MeOH/acetone precipitation was performed twice. After the last precipitation, the polymer was dried in a desiccator until the weight was constant, and the polymer was then stored at –20 °C. The mole percentage of pyridine ring in each polymer was quantified by the UV absorption at 281 nm using an extinction coefficient of 4900 in pH 7.0 aqueous solution (21–23).

Synthesis of Amino and Pyridine Disulfide Modified HPMA Copolymer. HPMA, PDTEMA, APMA, and AIBN were dissolved in methanol (12.5 wt % monomers, 0.6 wt % AIBN). The solution was bubbled with N₂ for 5 min, and the reaction vessel was sealed. The tube was placed in a 50–55 °C water bath for 24 h. Insoluble impurities were removed by filtration or centrifugation. Excess acetone was added to the clear solution to precipitate the copolymer product. The white product was purified by reprecipitation and stored as described above.

Hydrolysis of Pyridyl Disulfide Modified HPMA Copolymer. The kinetics of hydrolysis were measured using a dual beam spectrophotometer. Sample and blank cuvettes contained 0.1 M buffer A or B, depending on the desired pH. To the sample cuvette was added polymer A to a final concentration of 0.23 mg/mL. After addition, the absorbance at 320 nm (pH equal to or greater than 11) or 341 nm (pH equal to or less than 10) was measured at 2 min intervals for 14 h at room temperature.

Kinetics of the Reaction between Polymer A and Sulfhydryl Peptide. A sample and blank cuvette were filled with 0.1 M buffer A, and polymer A was added to the sample cuvette to a final concentration of 0.23 mg/mL. pAntp-SH (in 0.1% TFA) was added to both cuvettes to a final concentration of 0.50 mM, and the absorbance was measured at 341 nm every 0.5 min for 50 min. The free sulfhydryl in pAntp-SH was quantified using Ellman's reagent (24).

Kinetics of the Conjugation between the HPMA Copolymer and Oligonucleotides. Ten nanomoles of disulfide modified oligonucleotide (RSS-OSH) was treated with 0.2 M DTT in 100 μL of 0.1 M pH 9.0 buffer B for 2 h. Excess DTT was removed by ethyl acetate extraction (3 × 100 μL). The oligonucleotide was precipitated by

Table 1. Analysis of Pyridyl Disulfide Modified HPMA Copolymers

copolymer	mole % PDTEMA		conversion %	M_r (number)	M_r (weight)	polydispersity
	feed	polymer				
A	11.8	8.3	82	19 504	38 001	1.95
B	5.4	3.7	81	27 672	82 533	2.98
C	2.6	1.6	73	28 987	80 020	2.76
D	1.8	1.2	79	28 851	75 373	2.62

Table 2. Analysis of HPMA Copolymer Containing Pyridyl Disulfide and Amino Groups^a

polymer	mole %				conversion %		M_r (number)	M_r (weight)	polydispersity
	feed		polymer						
	NH ₂	Py	NH ₂	Py	NH ₂	Py			
E	1.01	1.41	1.02	1.13	100	80.1	23 640	36 980	1.56
F	0.95	7.3	0.90	5.5	95	75.3	15 640	23 840	1.52
G	5.8	1.2	5.0	0.87	86.2	72.5	8 430	10 540	1.25

^a The amino groups were quantified using a ninhydrin method calibrated using standard glycine solution (47).

adjusting the solution to 0.3 M NaOAc and adding 2.6 vol of ethanol. The mixture was placed at -20°C for 1 h and centrifuged for 25 min at 13000*g*. The oligonucleotide pellet was dissolved in 80 μL of deionized water, and 10 μL of 1.0 M buffer and 20 nmol (in pyridine ring) of copolymer A were added. The reaction was incubated at room temperature. Ten microliters of the reaction mixture was removed at different times, stopped, and immediately loaded into a running 20% PAGE. The gel was illuminated with UV light for fluorescence visualization. To confirm that oligonucleotide was covalently conjugated to HPMA polymer in the reaction product, 10 μL of the 30 min reaction mixtures was treated with 0.1 M DTT for 30 min at room temperature and loaded on the gel together with the 60 min reaction sample.

Preparation of Oligonucleotide–Polymer Conjugate. One hundred nanomoles of disulfide modified oligonucleotide (RSS-OSH) was treated with 0.2 M DTT in 300 μL of 0.1 M, pH 9.0, buffer B for 2 h. Excess DTT was removed by ethyl acetate extraction ($3 \times 300 \mu\text{L}$), and the oligonucleotide was precipitated by adjusting the solution to 0.3 M NaOAc and adding 2.6 vol of ethanol. The mixture was placed at -20°C for 1 h and centrifuged for 25 min at 13000*g*. The oligonucleotide pellet was dissolved in 100 μL of deionized water, and 200 nmol (in pyridine ring) of active HPMA copolymer and 30 μL of, pH 9.0, buffer B were added. The mixture was brought to a final volume of 300 μL with deionized water and incubated at room temperature for 2 h. The pH of the reaction mixture was adjusted to about 7 using 1.0 M HCl to inhibit disulfide exchange. L-Cysteine was added to the reaction mixture to a final concentration of 1.0 mM and incubated at room temperature for 10 min to destroy the excess pyridyl disulfide bonds in the copolymer. At pH 7, L-cysteine reacts rapidly and completely with all unreacted pyridyl disulfide linkages (data not shown). The product was purified by passing the reaction mixture through a short G-50 column. The reactions were characterized using 20% PAGE. Eighty percent of the oligonucleotide was conjugated to the HPMA polymer as indicated by its inability to enter the gel. The conjugation product was concentrated to a small volume using a Microcon-30 filter.

Preparation of Oligonucleotide–Peptide–Polymer Conjugate. The oligonucleotide–peptide–polymer conjugate was prepared as for the oligonucleotide–polymer conjugate, except that 200 nmol (in pyridine ring) of HPMA copolymer was first reacted for 10 min at room temperature with 20 nmol of pAntp-SH peptide in 0.1 M, pH 9.0, buffer B. Subsequently, the oligonucle-

otide was added. The product was worked up as described above.

RESULTS AND DISCUSSION

Synthesis of Pyridyl Disulfide Modified HPMA Copolymers. *N*-[2-(2-Pyridyldithio)ethyl methacrylamide (PDTEMA) was synthesized through a two step, one-pot reaction, as illustrated in Figure 1. Cysteamine reacted completely with DPDS in an ethanol/water mixture at room temperature in less than 1 h. Although 2-(2-pyridyldithio)ethylamine (PDTEA) has reportedly been crystallized from benzene (25), we used the dried crude product directly in the second step. Subsequently, PDTEMA was purified by silica gel chromatography in an overall yield of greater than 70%. The structure was confirmed by UV, ^1H NMR, and MS.

PDTEMA comonomer was incorporated into HPMA polymer by precipitation radical polymerization with HPMA monomer in acetone at $50\text{--}55^{\circ}\text{C}$ (26, 27). Copolymers varying in the percentage of active pyridyl disulfide groups were synthesized. The mole percent of pyridine ring in each polymer was quantified by the UV absorption at 281 nm using a molar extinction coefficient of 4900 at pH 7.0 in aqueous solution (21–23). The percent conversion of PDTEMA in all the syntheses was similar (Table 1), indicating that the pyridyl group of PDTEMA does not significantly affect its incorporation into the HPMA copolymer. This also suggests that there was no significant degradation of the active pyridyl disulfide moiety during the polymerization reactions. Since the structure of the comonomers is very similar, in terms of the active double bond, the pyridyl disulfide moieties are expected to be distributed homogeneously in the polymer molecules. This is confirmed by the overlapping UV detection and fraction index detection curves in the chromatogram of the gel permeation chromatography of the polymer (Figure 2). This indicates that the percentage of pyridyl groups is similar throughout the full size range of molecules within the same polymer batch.

Amine/pyridyl disulfide containing polymers could also be synthesized by the inclusion of APMA in the polymerization reaction (Table 2). Since APMA is supplied as a hydrochloride salt, and is insoluble in acetone, APMA-containing reactions were performed in methanol solutions. The ability to incorporate both groups into the polymer allows conjugation of different moieties to the polymer using different chemistries.

Aqueous Stability and Reactivity of Pyridyl Disulfide Side Chains in the Copolymer. The UV

absorbance spectra of the 2-pyridyl disulfide containing HPMA polymer and the PDTEMA monomer are similar to that for 2,2'-dipyridyl disulfide (DPDS) (21). DPDS has a maximum absorption at 284 nm, which is substantially different from the UV spectrum of 2-thiopyridone under the same conditions (Figure 3). 2-Thiopyridone has a maximum absorption at 341 nm, and this is relatively constant below a pH of 8 (21). Between pH 9 and 11 the spectrum changes drastically (Figure 3). This is likely the result of the ionization of the nitrogen proton which has a pK_a of approximately 10 and the presence of the 2-pyridine-thiolate ion at pH higher than 10. Since the absorbance at 320 nm is relatively high and constant for 2-thiopyridone and at a minimum for the pyridyl disulfide group, the absorbance at 320 nm was used to monitor release of 2-thiopyridone from the polymer at pH greater than 10. For pH equal to or less than 10 the absorption maximum at 341 nm was used to monitor the release of 2-thiopyridone from the copolymer.

The pyridyl disulfide bond is very stable in deionized water (pH 6.5), and there is no detectable release of 2-thiopyridone after storage for 2 months at -20°C in a 20 mM (in pyridine ring) polymer stock solution. The kinetics of hydrolysis/aminolysis were analyzed at room temperature using 0.1 M glycine/NaOH buffers over a wide range of pH values (Figure 4). The stability of the pyridine disulfide bond in the copolymer decreases with increasing pH in aqueous solution. There was no detectable degradation of the active disulfide bond within 14 h in 0.1 M, pH 8.0, citric acid/NaOH buffer. Incubation for the same time at pH 9.0 and 10.0, in 0.1 M glycine/NaOH buffered solution, released approximately 11 and 17% of the active pyridine group, respectively. Above a pH of 10 the degradation increased significantly with a half-life of hydrolysis and/or aminolysis of approximately 25 min in pH 13 glycine buffer. These results indicate that pyridyl disulfide modified HPMA copolymer can be safely stored and/or further derivatized in aqueous solutions of pH equal to or less than 8. If necessary, the excess active pyridyl disulfide bond can be quickly destroyed by increasing the pH to 13 or higher.

The reactivity of the pyridyl disulfide activated HPMA copolymers with thiol groups was first examined using the model compounds L-cysteine and 2-mercaptoethanol. Similar to DPDS, SPDP, and other small molecule active pyridyl disulfide compounds, the active pyridyl group in HPMA copolymer can be replaced by L-cysteine or 2-mercaptoethanol in aqueous solutions in a wide range of pH values (data not shown). At neutral pH, the reaction is rapid and complete, and disulfide exchange is suppressed.

Characterization of the Conjugation Reaction of HPMA Copolymer with Peptide and/or Oligonucleotide. To examine the reactivity of the activated polymer with larger bioactive molecules, a moderate sized peptide (pAntp-SH, FW = 2407) was first examined (Figure 5). pAntp-SH is 16 amino acids long and possesses a cysteine residue at its amino terminus. pAntp-SH is derived from the homeodomain of Antennapedia. pAntp was chosen since it has been shown to translocate across biological membranes in an energy independent and nonendocytotic manner (28). When conjugated to a bioactive peptide or antisense oligonucleotide, pAntp has been demonstrated to enhance uptake and activity of these molecules (28, 29). Similar to cysteine and 2-mercaptoethanol, pAntp-SH can readily react with pyridyl disulfide HPMA copolymer in aqueous solutions. As seen in Figure 6, the reaction goes to completion within minutes in a mildly basic solution. The rate of reaction decreases with

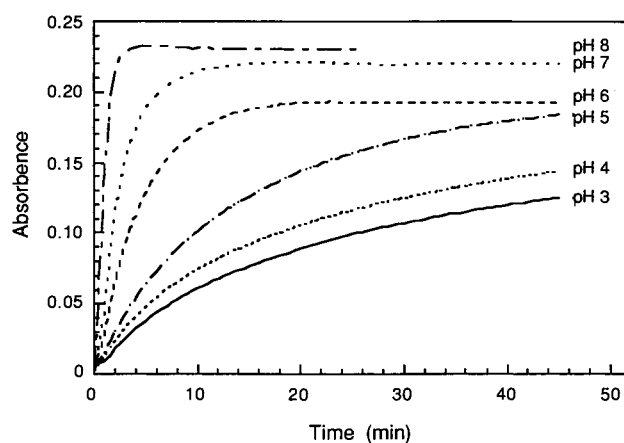


Figure 6. Kinetics of the conjugation reaction between pAntp-SH and copolymer A at pH 3–8 as monitored by the absorbance at 341 nm.

decreasing pH, but even in acidic solution the rate of reaction is relatively high. The half-life of reaction is less than 50 min at pH 3.

It is believed that the protonation of the pyridine ring nitrogen makes the pyridyl group a good leaving group in pyridyl disulfide mediated thiol-disulfide exchange reactions. This is in contrast to thiol-disulfide exchange reactions between aliphatic thiols and aliphatic disulfides, where the thiolate ion, rather than the thiol, is the reactive species in the nucleophilic displacement reaction. Since the pK_a of most aliphatic thiols is between 7 and 9, little or no reaction occurs in acidic solutions, owing to the low thiolate ion concentration.

An oligonucleotide targeted to the 3' splice junction of the second intron of the HIV tat gene was selected for examination of the conjugation of an antisense oligonucleotide to the polymer (Figure 5, RSS-OSH, FW = 7130). This oligonucleotide was chosen since, in an examination of 20 different targets on HIV, this target was one of the more effective ones at inhibiting viral replication (30). To increase the resistance of the oligonucleotide to nucleolytic degradation, all phosphodiester linkages were substituted with phosphorothioates, and a 3'-3' inverted cap was added to the 3'-end during chemical synthesis (31, 32). A fluorescein group was added to the 5'-end, followed by a linker and a disulfide group.

The disulfide group on the oligonucleotide was reduced to produce a free thiol using DTT. Subsequently, the oligonucleotide was allowed to react with the pyridyl disulfide modified copolymer. Since the oligonucleotide has a strong UV absorbance between 200 and 300 nm, UV absorbance could not be used to monitor the course of the reaction. Instead gel electrophoresis was used. We examined the kinetics of conjugation of RSS-OSH with the 2-pyridyl disulfide modified polymer at pH 7.0–10 (Figure 7). The reaction was allowed to proceed for up to 20 h, and aliquots were removed at various times, combined with an equal volume of gel loading dye, and immediately loaded onto a denaturing 20% polyacrylamide gel. Since the polymer is a large neutral molecule, the polymer-oligonucleotide conjugate barely migrates into the gel, while the mobility of the free oligonucleotide is very fast. The different apparent mobility of the free oligonucleotide, at various times, is due to the different times in which each sample was loaded on the gel. As seen in Figure 7, the conjugation was inefficient until the pH of the solution reached 8.0. When the pH was 7.0, less than half of the oligonucleotide coupled to the

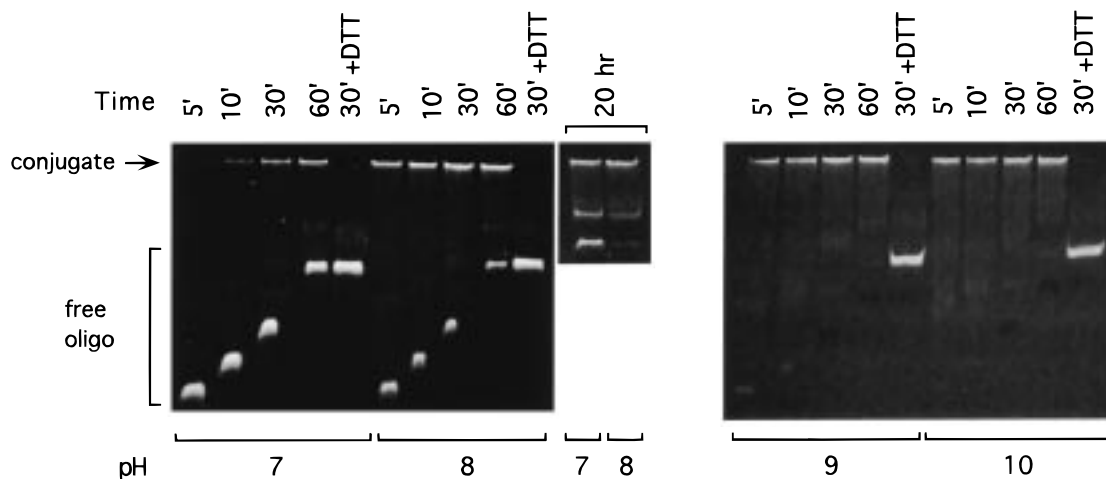


Figure 7. Kinetics of the conjugation of oligonucleotide to polymer C at different pHs as indicated. Aliquots of the reaction mixtures were removed and run into denaturing 20% polyacrylamide gels immediately at the times indicated. For the pH 7.0 and 8.0 reactions, 20 h samples are also shown. The control reactions were treated with 0.1 mM DTT for 30 min.

polymer after incubation for 20 h (Figure 7). However, the half-life of the conjugation was reduced to about 10 and 5 min, respectively, when the reaction was performed at pH 8.0 or 9.0. The reaction was 50% complete in minutes when performed at pH 10.0.

Preparative Conjugation of HPMA Copolymer with Peptide and/or Oligonucleotide. All preparative conjugation reactions were conducted in pH 9.0 buffer in the presence of excess pyridyl disulfide groups of the HPMA copolymer. For peptide–oligonucleotide conjugates, the peptide was conjugated first followed by the oligonucleotide. After completion of the conjugation, the pH of the reaction mixture was adjusted to about 7, and excess pyridyl disulfide groups were inactivated by addition of L-cysteine to a final concentration of 1.0 mM. The conjugation products could be easily separated from the free oligonucleotide and peptide as a result of the increased size of the polymer conjugate. The conjugate was characterized by PAGE, and conjugation was confirmed by release of the oligonucleotide after treatment with DTT (data not shown). The UV spectrum of the oligonucleotide–HPMA polymer is exactly the same as the free oligonucleotide, while there was a small change in the shape of the spectrum for the pAntp peptide–oligonucleotide conjugate (Figure 8). The ratio of the optical densities at 260 and 280 nm was significantly higher for the oligonucleotide–peptide–HPMA conjugate (1.77) than for the oligonucleotide–HPMA conjugate (1.51), indicating the presence of peptide in the former terplex conjugate. The structure of the oligonucleotide–peptide–HPMA terplex conjugate was further confirmed by DTT reduction. An orange precipitate formed after the addition of 0.2 M DTT in pH 9.0 buffer to the terplex conjugate. The precipitate is likely a polyelectrolyte complex between oligonucleotide and peptide resulting from electrostatic interaction of the 21 negative charges on the oligonucleotide and the 7–10 positive charges on the peptide (4 from lysines, $pK_a = 10.69$; and 3–6 from arginines, $pK_{a1} = 12.48$ and $pK_{a2} = 8.99$). This was confirmed by the immediate formation of an orange precipitate on combining equal amounts of free oligonucleotide and peptide in pH 9.0 buffer.

Cell Uptake of the HPMA–Oligonucleotide Conjugate. The uptake, by mammalian cells, of the oligonucleotide–HPMA conjugate was examined using the HeLa S3 cell line. Cells were precultured in DMEM medium containing 10% fetal bovine serum on sterilized

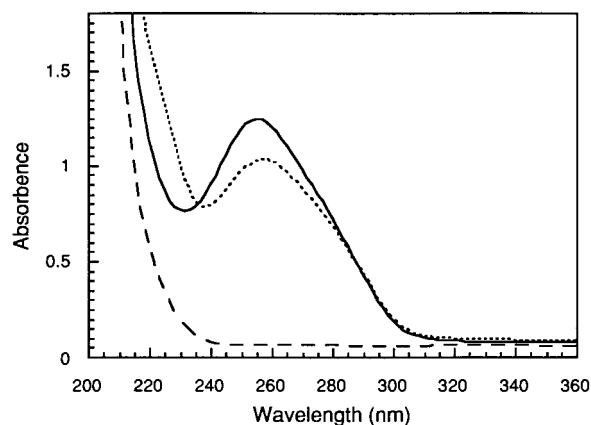


Figure 8. UV spectrum of oligonucleotide–HPMA conjugate (solid line), oligonucleotide–peptide–HPMA conjugate (dotted line), and HPMA copolymer (dashed line). The HPMA copolymer sample was prepared from the same pyridyl disulfide modified HPMA copolymer but was treated with 1.0 mM L-cysteine to release the active pyridyl groups. The polymer was purified away from the released thiopyridone by G-50 chromatography.

cover slips. The cells were plated to give approximately 10% coverage in 2–3 days. The media was removed, and the cells were washed with fresh media. The media was replaced and the free oligonucleotide or the oligonucleotide polymer conjugate was added to a final oligonucleotide concentration of 5 μ M in oligonucleotide. After incubation for 5 h, the cells were rinsed with PBS three times, fixed using acetone, and analyzed by confocal microscopy.

Uptake of the polymer–oligonucleotide conjugate is mildly enhanced relative to the free oligonucleotide (Figure 9). Most of the fluorescence of the oligonucleotide is present in endosomes as is typically observed for endocytotic uptake of antisense oligonucleotides (33) or HPMA polymers (8). To confirm an endocytotic mode of uptake, the experiments were repeated, except that incubation in the presence of oligonucleotide or oligonucleotide–polymer conjugate was performed at 4 $^{\circ}$ C. In this case, little or no internalized fluorescence was observed, indicative of a lack of significant uptake.

Since the oligonucleotide has a 3' inverted cap and is composed of phosphorothioate linkages, it is expected to be stable toward nuclease degradation. In fact, for the free oligonucleotide, it remained 90% intact after 24 h

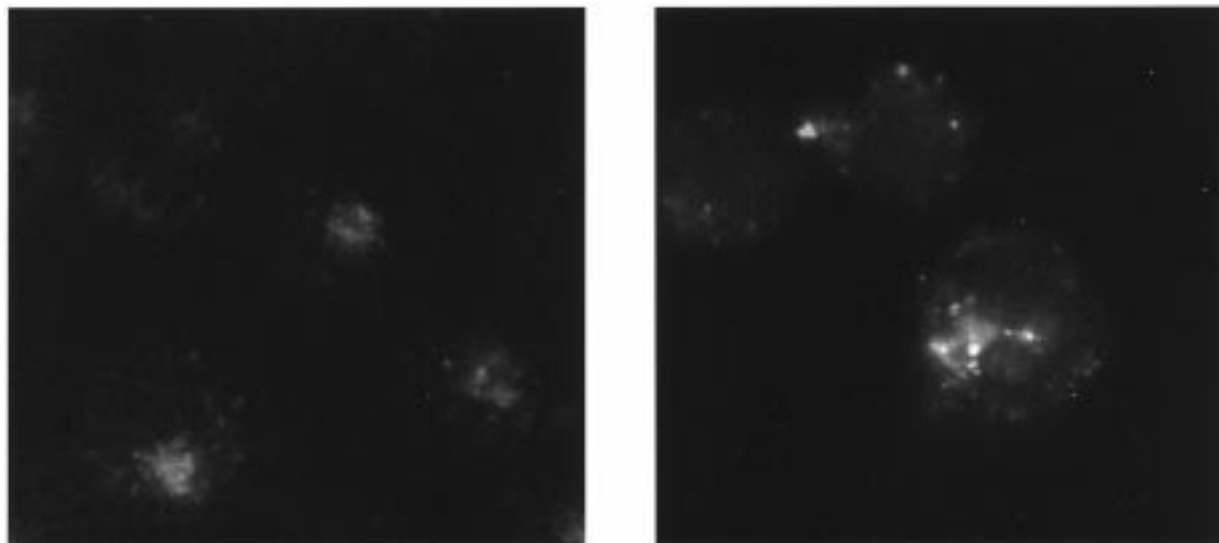


Figure 9. Cell uptake as determined by confocal microscopy. HeLa S3 cells were pre-cultured in DMEM medium containing 10% fetal bovine serum on 1.5 cm diameter cover slips for 2–3 days, such that about 10% of the cover slip was covered by cells. The media was replaced and the free oligonucleotide OSH (left panel) or the oligonucleotide-HPMA conjugate (right panel) was added to a final oligonucleotide concentration of 5 μ M. After 5 h incubation in a CO₂ incubator, the cells were rinsed with PBS three times and fixed using acetone. The coverslips were fixed to slides using nail polish and observed through a 100 $\times$ oil immersion objective on a Nikon Diaphot 200 confocal microscope equipped with an OmNichrome argon laser 150. Pictures were taken with a digital camera attached to the microscope and integrated five times.

at 37 °C in 50% calf serum (data not shown). Stability should be further enhanced for the conjugated oligonucleotide as its 5' end is blocked by coupling to the polymer. It is therefore likely that the internalized oligonucleotide remains primarily intact.

These results provide preliminary evidence to support the value of this novel polymer for delivery of antisense oligonucleotides. The effectiveness of this delivery system can be further enhanced in a variety of ways. First, in the uptake studies, the polymer was only loaded at a low level, approximately one oligonucleotide per molecule. Since the polymer contains 6–20 active thiols, depending on the mole % of PDTEMA in the polymer feed, higher loading will likely lead to further enhancement of uptake. Second, release of the oligonucleotide from endosomal compartments would certainly improve antisense efficacy. The coupling of a lysogenic agent, such as a fusogenic peptide, to the polymer could aid in this regard (34–36). A fusogenic peptide could be coupled to the polymer using the activated sulfhydryl. Alternatively, the use of our APMA/PDTEMA copolymer could allow different chemistries to be used for the oligonucleotide and lysogenic agent. Finally, targeted delivery would also enhance efficacy. This could be accomplished by coupling a targeting moiety through the activated thiol, or alternatively, in the case of our APMA/PDTEMA copolymer, through the amino groups. The use of both a targeting moiety and a lysogenic agent would likely further enhance efficacy.

Activity could likely also be enhanced by internalization of the conjugate by nonendocytotic means. This would prevent exposure of the oligonucleotide to the degradative enzymes of the lysosome. Additionally, direct cytoplasmic uptake would likely lead to rapid nuclear accumulation of the oligonucleotide (37, 38). It has been shown that a short peptide (pAntp) from the Antennapedia homeodomain protein can translocate across biological membranes in an energy-independent, nonendocytotic manner. Uptake of peptides and oligonucleotides has been reported to be increased greater than 100-fold when coupled to pAntp (29). In our hands,

this has proven ineffective for oligonucleotides. When we coupled our anti-HIV oligonucleotide directly to pAntp, we produced a conjugate that was only soluble at pH 13 and greater. We believe that this is due to the formation of an intramolecular polyelectrolyte complex. This could result from interaction of the approximately 10 positive charges on pAntp and the 20 negative charges on the oligonucleotide at neutral pH, as discussed above. To solve the solubility problem, we coupled pAntp to our polymer and subsequently conjugated the oligonucleotide. While this solved the solubility problem, we saw no alteration in the amount of uptake or the intracellular distribution of the oligonucleotide–pAntp–polymer conjugate. Again, this is likely the result of electrostatic association of peptide and oligonucleotide. This could prevent electrostatic interaction of the peptide with the cell membrane, which may be essential to its correct functioning. Additionally, this would likely prevent proper folding of the peptide into a structure necessary for translocation. It therefore seems unlikely that pAntp can be used successfully for translocation of negatively charged oligonucleotides, unless a delivery system can be developed that allows the peptide and oligonucleotide to be physically separated.

2-Pyridyl disulfide homogeneously modified HPMA copolymer was synthesized by copolymerization of PDTEMA and HPMA. To our knowledge, this is the easiest and probably the only way, to date, to synthesize homogeneous thiol or active thiol polymers. Most of the available thiol-polymers have been synthesized by derivatizing pendant hydroxyl or other functional groups on various hydrophilic polymers (20, 39–41).

The pyridyl disulfide activated copolymer is stable in aqueous solutions of pH lower than 8.0 and can readily react with thiol containing molecules in aqueous solutions of a wide range of pH. Consequently, it provides a very mild and efficient way to conjugate water-soluble molecules to HPMA polymer. Coupling of a drug through a disulfide linkages provides other advantages as well. Disulfide linkages have been shown to be stable in the blood stream so the drug will remain with its carrier until

it reaches its site of action (42–44). Subsequently, the drug can be released by disulfide exchange with intracellular thiols such as glutathione, or alternatively by the action of redox enzymes. Since drug release is not dependent on any enzymatic activity, the route of uptake, endocytotic or nonendocytotic, should have little effect on release. This can be very important since enzyme-dependent release likely requires endocytotic uptake and lysosomal targeting. This places the drug, an oligonucleotide in our case, in contact with degradative enzymes. The use of a fusogenic peptide on the drug carrier could limit lysosomal degradation. However, for other delivery systems that require enzymatic release of the drug, inclusion of a fusogenic peptide could also prevent drug release.

Our PDTEMA/HPMA copolymer has many attributes that make it a valuable alternative for delivery of water-soluble drugs such as antisense oligonucleotides. The utility of this polymer is further enhanced by the ability to incorporate pendant amino groups as well. It is also possible that *p*-nitrophenol or *N*-succinimidyl active ester groups could also be introduced during polymerization. This allows great flexibility in the design of an effective drug delivery system. The drug, a targeting moiety, a fusogenic peptide, and any other desirable functionality can be coupled using a variety of chemistries. For the PDTEMA/HPMA copolymer alone, there are different chemistries that can be employed. Aside from the obvious coupling of thiol-containing molecules through the activated pyridyl disulfide, one can reduce the pyridyl disulfide linkage (22) and couple directly through the free sulfhydryl group. In this way nondegradable linkages can be produced by reaction with maleimido (45, 46) or iodoacetal derivatized ligands.

ACKNOWLEDGMENT

We are greatly indebted to Pavla Kopeckova and Jindrich Kopecek of the CCCD at the University of Utah for their general help in synthesis and characterization of HPMA copolymers, and for suggestions on this manuscript. We thank Michael Pierce and Peter Mayer for technical assistance. We appreciate Dr. Robert Schackmann of the University of Utah DNA/Peptide core facility for synthesis of oligonucleotide and peptide. The core facility is supported by a grant from the NCI, CA42014. This work was supported by an NIH grant to D.E.R., 5R29AI34278.

LITERATURE CITED

- (1) Agrawal, S. (1996) *Antisense Therapeutics* (S. Agrawal, Ed.) Humana Press, Totowa.
- (2) Clarenc, J. P., Degols, G., Leonetti, J. P., Milhaud, P., and Lebleu, B. (1993) Delivery of antisense oligonucleotides by poly(L-lysine) conjugation and liposome encapsulation. *Anti-Cancer Drug Des.* 8, 81–94.
- (3) Takakura, Y., and Hashida, M. (1996) Macromolecular carrier systems for targeted drug delivery: pharmacokinetic consideration on biodistribution. *Pharm. Res.* 13 (6), 820–31.
- (4) Duncan, R., Cable, H. C., Rejmanova, P., Kopecek, J., and Lloyd, J. B. (1984) Tyrosinamide residues enhance pinocytic capture of *N*-(2-hydroxypropyl)methacrylamide copolymers. *Biochim. Biophys. Acta* 799 (1), 1–8.
- (5) Duncan, R., Lloyd, J. B., Rejmanova, P., and Kopecek, J. (1985) Methods of targeting *N*-(2-hydroxypropyl)methacrylamide copolymers to particular cell types. *Makromol. Chem.* 9, 3–11.
- (6) Rihova, B., Kopecek, J., Ulbrich, K., Pospisil, M., and Mancal, P. (1984) Effect of the chemical structure of *N*-(2-hydroxypropyl)methacrylamide copolymers on their ability to induce antibody formation in inbred strains of mice. *Biomaterials* 5 (3), 143–8.
- (7) Rihova, B., Kopecek, J., Ulbrich, K., and Chytrý, V. (1985) Immunogenicity of *N*-(2-hydroxypropyl)methacrylamide copolymers. *Makromol. Chem.* 9, 13–24.
- (8) Cartledge, S. A., Duncan, R., and Lloyd, J. B. (1986) Soluble, cross-linked *N*-(2-hydroxypropyl)methacrylamide copolymers as potential drug carriers 1. pinocytosis by rat visceral yolk sacs and rat intestine cultured in vitro. Effect of molecular weight on uptake and intracellular degradation. *J. Controlled Rel.* 3, 55–66.
- (9) Seymour, L. W., Duncan, R., Strohm, J., and Kopecek, J. (1987) Effect of molecular weight (Mw) of *N*-(2-hydroxypropyl)methacrylamide copolymers on body distribution and rate of excretion after subcutaneous, intraperitoneal, and intravenous administration to rats. *J. Biomed. Mater. Res.* 21 (11), 1341–58.
- (10) Rihova, B., and Kopecek, J. (1985) Biological properties of targetable poly [*N*-(2-hydroxypropyl)methacrylamide]-antibody conjugates. *J. Controlled Rel.* 2, 289–310.
- (11) Duncan, R., Kopeckova, P., Strohm, J., Hume, I. C., Lloyd, J. B., and Kopecek, J. (1988) Anticancer agents coupled to *N*-(2-hydroxypropyl)methacrylamide copolymers. II. Evaluation of daunomycin conjugates in vivo against L1210 leukemia. *Br. J. Cancer* 57 (2), 147–56.
- (12) Flanagan, P. A., Kopeckova, P., Kopecek, J., and Duncan, R. (1989) Evaluation of protein-*N*-(2-hydroxypropyl)methacrylamide copolymer conjugates as targetable drug carriers. 1. Binding, pinocytic uptake and intracellular distribution of transferrin and anti-transferrin receptor antibody conjugates. *Biochim. Biophys. Acta* 993 (1), 83–91.
- (13) Kopecek, J., Rejmanova, P., Strohm, J., Ulbrich, K., Rihova, B., and Chytrý, V. (1991) *Synthetic Polymeric Drugs*, United States Patent 5,037,883, Československá Akademie Věd.
- (14) Putnam, D., and Kopecek, J. (1995) Enantioselective release of 5-fluorouracil from *N*-(2-hydroxypropyl)methacrylamide-based copolymers via lysosomal enzymes. *Bioconjugate Chem.* 6(4), 483–92.
- (15) Peterson, C. M., Lu, J. M., Sun, Y., Peterson, C. A., Shiah, J. G., Straight, R. C., and Kopecek, J. (1996) Combination chemotherapy and photodynamic therapy with *N*-(2-hydroxypropyl)methacrylamide copolymer-bound anticancer drugs inhibit human ovarian carcinoma heterotransplanted in nude mice. *Cancer Res.* 56 (17), 3980–5.
- (16) Seymour, L. W., Ulbrich, K., Wedge, S. R., Hume, I. C., Strohm, J., and Duncan, R. (1991) *N*-(2-hydroxypropyl)methacrylamide copolymers targeted to the hepatocyte galactose-receptor: pharmacokinetics in DBA2 mice. *Br. J. Cancer* 63 (6), 859–66.
- (17) Rihova, B., Kopecek, J., Kopeckova Rejmanova, P., Strohm, J., Plocova, D., and Semoradova, H. (1986) Bioaffinity therapy with antibodies and drugs bound to soluble synthetic polymers. *J. Chromatogr.* 376, 221–33.
- (18) Rihova, B., Kopeckova, P., Strohm, J., Rossmann, P., Vetricka, V., and Kopecek, J. (1988) Antibody-directed affinity therapy applied to the immune system: in vivo effectiveness and limited toxicity of daunomycin conjugated to HPMA copolymers and targeting antibody. *Clin. Immunol. Immunopathol.* 46(1), 100–14.
- (19) Hosoda, H., Fukuda, K., and Gotoh, Y. (1991) Enzyme labeling in steroid enzyme immunoassays. Comparison of the *p*-nitrophenyl ester and *N*-succinimidyl ester methods. *Chem. Pharm. Bull.* 39(9), 2373–7.
- (20) Barker, S. A., and Gray, C. J. (1974) *Bonding of Enzymes, Enzyme Derivatives and Other Biologically Active Molecules to Polymeric Materials*, in United States Patent 3,846,306, Koch-Light Laboratories Limited, Buckinghamshire, England.
- (21) Grassetti, D. R., and Murray, J. J. F. (1967) Determination of sulfhydryl groups with 2,2'- or 4,4'-dithiodipyridine. *Arch. Biochem. Biophys.* 119, 41–49.
- (22) Carlsson, J., Drevin, H., and Axen, R. (1978) Protein thiolation reversible protein-protein conjugation: *N*-succinimidyl 3-(2-pyridyldithio)propionate, a new heterobifunctional reagent. *Biochem. J.* 173, 723–737.

- (23) Zecherle, G. N., Oleinikov, A., and Traut, R. R. (1992) The proximity of the C-terminal domain of Escherichia coli ribosomal protein L7/L12 to L10 determined by cysteine site-directed mutagenesis and protein-protein cross-linking. *J. Biol. Chem.* 267 (9), 5889–5896.
- (24) Ellman, G. L. (1959) Tissue sulfhydryl groups. *Arch. Biochem. Biophys.* 82, 70–77.
- (25) Ekrami, H. M., Kennedy, A. R., and Shen, W. C. (1995) Water-soluble fatty acid derivatives as acylating agents for reversible lipidization of polypeptides. *FEBS Lett.* 371 (3), 283–6.
- (26) Drobniak, J., Kopecek, J., Labsky, J., Exner, R. P. J., Saudek, V., and Kalal, J. (1976) Enzymatic cleavage of side chains of synthetic water-soluble polymers. *Makromol. Chem.* 177, 2833–2848.
- (27) Kopecek, J. (1977) Reactive copolymers of N-(2-Hydroxypropyl)methacrylamide with N-methacryloylated derivatives of L-leucine and L-phenylalanine. *Makromol. Chem.* 178, 2169–2183.
- (28) Derossi, D., Joliot, A. H., Chassaing, G., and Prochiantz, A. (1994) The third helix of the antennapedia homeodomain translocates through biological membranes. *J. Biol. Chem.* 269 (14), 10444–10450.
- (29) Troy, C. M., Derossi, D., Prochiantz, A., Greene, L. A., and Shelanski, M. (1996) Downregulation of Cu/Zn superoxide dismutase leads to cell death via the nitric oxide-peroxynitrite pathway. *J. Neurosci.* 16 (1), 253–261.
- (30) Goodchild, J., Agrawal, S., Civeira, M. P., Sarin, P. S., Sun, D., and Zamecnik, P. C. (1988) Inhibition of human immunodeficiency virus replication by antisense oligodeoxynucleotides [published erratum appears in *Proc. Natl. Acad. Sci. U.S.A.* (1989) 86 (5), 1504]. *Proc. Natl. Acad. Sci. U.S.A.* 85 (15), 5507–11.
- (31) Ortigao, J. F. R., Rosch, H., Selter, H., Lorenz, A. F., Montenarh, M., and Seliger, H. (1992) Antisense effect of oligodeoxynucleotides with inverted terminal internucleotidic linkages: a minimal modification protecting against nucleolytic degradation. *Antisense Res. Dev.* 2, 129–146.
- (32) Beigelman, L., McSwiggen, J. A., Draper, K. G., Gonzalez, C., Jensen, K., Karpeisky, A. M., Modak, A. S., Matulic Adamic, J., DiRenzo, A. B., Haeberli, P., et al. (1995). Chemical modification of hammerhead ribozymes. Catalytic activity and nuclease resistance. *J. Biol. Chem.* 270 (43), 25702–8.
- (33) Vlassov, V. V., Balakireva, L. A., and Yakubov, L. A. (1994) Transport of oligonucleotides across natural and model membranes. *Biochim. Biophys. Acta* 1197, 95–108.
- (34) Wagner, E., Plank, C., Zatloukal, K., Cotten, M., and Birnstiel, M. L. (1992) Influenza virus hemagglutinin HA-2 N-terminal fusogenic peptides augment gene transfer by transferrin-polylysine-DNA complexes: toward a synthetic virus-like gene-transfer vehicle. *Proc. Natl. Acad. Sci. U.S.A.* 89 (17), 7934–8.
- (35) Bongartz, J. P., Aubertin, A. M., Milhaud, P. G., Lebleu, B. (1994) Improved biological activity of antisense oligonucleotides conjugated to a fusogenic peptide. *Nucleic Acids Res.* 22 (22), 4681–8.
- (36) Plank, C., Oberhauser, B., Mechtler, K., Koch, C., and Wagner, E. (1994) The influence of endosome-disruptive peptides on gene transfer using synthetic virus-like gene transfer systems. *J. Biol. Chem.* 269 (17), 12918–24.
- (37) Chin, D. J., Green, G. A., Zon, G., Szoka, F. J., and Straubinger, R. M. (1990) Rapid nuclear accumulation of injected oligodeoxyribonucleotides. *New Biol.* 2(12), 1091–100.
- (38) Leonetti, J. P., Mechti, N., Degols, G., Gagnor, C., and Lebleu, B. (1991) Intracellular distribution of microinjected antisense oligonucleotides. *Proc. Natl. Acad. Sci. U.S.A.* 88(7), 2702–6.
- (39) Inman, J. K., and Dintzis, H. M. (1969) The derivatization of cross-linked polyacrylamide beads. controlled introduction of function groups for the preparation of special-purpose, biochemical adsorbents. *Biochemistry* 8 (8), 4074–4082.
- (40) Ngo, T. T. (1989) *Preparation of polymeric thiol gels for covalent bonding of biologically active ligands*, United States Patent 4,886,755, Bioprobe International, Inc., Tustin, CA.
- (41) Woghiren, C., Sharma, B., and Stein, S. (1993) Protected thiol-poly(ethylene glycol): a new activated polymer for reversible protein modification. *Bioconjugate Chem.* 4 (5), 314–318.
- (42) Feener, E. P., Shen, W. C., and Ryser, H. J. (1990) Cleavage of disulfide bonds in endocytosed macromolecules. A processing not associated with lysosomes or endosomes. *J. Biol. Chem.* 265 (31), 18780–5.
- (43) McIntyre, G. D., Scott, C. F., Jr., Ritz, J., Blattler, W. A., and Lambert, J. M. (1994) Preparation and characterization of interleukin-2-gelatin conjugates made using different cross-linking reagents. *Bioconjugate Chem.* 5(1), 88–97.
- (44) Bickel, U., Kang, Y. S., and Pardridge, W. M. (1995) In vivo cleavability of a disulfide-based chimeric opioid peptide in rat brain. *Bioconjugate Chem.* 6(2), 211–8.
- (45) Marrian, D. H. (1949) The reaction of substituted maleimides with thiols. *J. Chem. Soc.* 1515.
- (46) Peeters, J. M., Hazendonk, T. G., Beuvery, E. C., and Tesser, G. I. (1989) Comparison of four biofunctional reagents for coupling peptides to proteins and the effect of the three moieties on the immunogenicity of the conjugates. *J. Immunol. Methods* 120, 133–143.
- (47) Doi, E., Shibata, D., and Matoba, T. (1981) Modified colorimetric ninhydrin methods for peptide assay. *Anal. Biochem.* 118, 173–184.

BC980034K

Iodopyridine-for-Iodobenzene Substitution for Use with Low Molecular Weight Radiopharmaceuticals: Application to *m*-Iodobenzylguanidine

Ganesan Vaidyanathan,* Michael R. Zalutsky, and Timothy R. DeGrado

Department of Radiology, Duke University Medical Center, P.O. Box 3808, Durham, North Carolina 27710.
Received March 19, 1998; Revised Manuscript Received August 14, 1998

Substituting a pyridine ring for a benzene ring in the acylation agent *N*-succinimidyl 3-iodobenzoate has resulted in a useful approach for the radiohalogenation of monoclonal antibodies, peptides, and labeled biotin conjugates. It was hypothesized that such a substitution in *m*-iodobenzylguanidine (MIBG), a radiotracer used in the detection and treatment of neuroendocrine tumors, might result in an analogue with more rapid normal tissue clearance, thereby facilitating its use for tumor therapy. For the preparation of this analogue, 3-guanidinomethyl-5-iodopyridine (GMIP; **9b**), the silicon precursor **4** was synthesized starting from 5-bromonicotinic acid. Attempts to convert **4** to **9b** under various conditions were not successful. Radioiodinated **9b** could be prepared by the iododestannylation of the tin precursor **8** in 65–70% radiochemical yield. A number of in vitro, in vivo, and ex vivo studies showed that pyridine-for-benzene substitution in MIBG yielded a compound that no longer was taken up by the uptake-1 pathway.

INTRODUCTION

An important consideration in the design of radiopharmaceuticals for oncology is the achievement of maximum tumor-to-normal tissue ratios in a time frame compatible with the physical half-life of the radionuclide. One way of accomplishing this is to reduce the background activity in normal tissues. In many cases, normal tissue clearance is governed in large part by the lipophilicity of the radiolabeled agent.

Reducing the lipophilicity of a tracer generally accelerates its clearance from normal tissues. This fact has been made use of in the development of acylation agents for the radioiodination of mAbs (Garg et al., 1991). Using *N*-succinimidyl 5-iodopyridine-3-carboxylate (SIPC),¹ instead of the prototypical acylation agent *N*-succinimidyl 3-iodobenzoate (SIB), mAbs could be radioiodinated without any change in their stability, immunoreactivity, and affinity (Garg et al., 1993; Reist et al., 1996). Moreover, from a comparative biodistribution of the potential catabolites in normal mice, it was found that iodonicotinic acid cleared generally faster than iodobenzoic acid from most organs. Similarly, preliminary results have suggested that peptides labeled with SIPC are less lipophilic than those labeled using SIB (Garg et al., 1992). The utility of SIPC for the synthesis of radioiodinated biotin conjugates has been recently demonstrated (Foulon et al., 1997). A faster clearance of

activity from normal tissues was seen with the biotin conjugate radioiodinated with SIPC in comparison to that radioiodinated with SIB. In all of the above cases, an agent containing a pyridine backbone was appended to proteins or peptides or coupled at a site known to be of little importance to biological activity of the final tracer. Encouraged by the fact that substitution of pyridine moiety for that of benzene in the above cases resulted in faster tissue clearance with no other adverse effects, we wished to determine the effect of such a substitution in a low molecular weight molecule.

The radiopharmaceutical MIBG was chosen for these studies. Originally, MIBG was pursued based on an extensive structure–activity study of a series of ring and side-chain-modified arylalkyl guanidines (Wieland, 1986). Although a contrary view exists (Jaques et al., 1987; Smets et al., 1990), it has been suggested that the guanidino moiety (not the aromatic ring) is more important for the biological activity of MIBG (Smets et al., 1988; 1990). However, an MIBG analogue with a fluorine substituent in its aromatic ring (FIBG) actually had a higher uptake and retention than MIBG in SK–N–SH human neuroblastoma cells in vitro (Vaidyanathan et al., 1997). FIBG also had a higher resistance, compared to MIBG, toward in vivo deiodination.

In the structure–activity study mentioned above, no compound was evaluated in which the radioiodine-containing benzene ring was replaced with a heterocyclic ring. Heterocyclic phenylpiperazine derivatives as potential alternatives to MIBG for adrenomedullary and myocardial imaging have been reported (Hanson, 1982, 1983), and recently, some of these agents were evaluated in SK–N–SH and SK–N–BE(2c) human neuroblastoma cells in vitro (Babich et al., 1996). A preliminary evaluation of iodonicotinic acid as a brain and/or adrenomedullary imaging agent has also been reported (Chan et al., 1983).

We hypothesize that by replacing the benzene ring of MIBG with a pyridine, its lipophilicity will be reduced, thereby resulting in faster clearance from normal tissues.

* Author to whom correspondence should be addressed.
Phone: (919)684-7811. Fax: (919)684-7122. E-mail: ganesan.v@duke.edu.

¹ Abbreviations: ADV, apparent distribution volume; CPP, 1-carboxamdino(4-iodophenyl)piperazine; DEAD, diethyl azodicarboxylate; FIBG, 4-fluoro-3-iodobenzylguanidine; GMIP, 3-guanidinomethyl-5-iodopyridine; IPP, 4-(3-iodophenyl)piperazine; MIBG, *m*-iodobenzylguanidine; NCS, *N*-chlorosuccinimide; NE, norepinephrine; SIB, *N*-succinimidyl 3-iodobenzoate; SIPC, *N*-succinimidyl 5-iodopyridine-3-carboxylate; TBHP, *tert*-butylhydroperoxide; TFA, trifluoroacetic acid.

If this structural change does not affect the compound's tumor uptake, tumor-to-normal tissue ratios higher than that for the parent compound might be obtained. To investigate this possibility, the pyridine analogue 3-guanidinomethyl-5-iodopyridine (GMIP) was prepared and evaluated by *in vitro*, *in vivo*, and *ex vivo* methods. Unfortunately, the results indicated that this small structural change yielded a compound with poor specificity for the norepinephrine transporter for which MIBG is a substrate.

MATERIALS AND METHODS

General. All chemicals were purchased from Aldrich unless otherwise noted. Sodium [^{125} I]iodide and sodium [^{131} I]iodide with specific activities of 2200 and 1200 Ci/mmol, respectively, were obtained from DuPont-New England Nuclear. Unlabeled MIBG and radioiodinated MIBG (no-carrier-added) were prepared as reported (Vaidyanathan et al., 1993).

Melting points were determined on a Fisher-Johns or on a Haake Buchler melting point apparatus and were uncorrected. NMR spectra were obtained with a General Electric Midfield GN-300 spectrometer. Mass spectra were obtained on a Hewlett-Packard GC/MS/DS model HP-5988A instrument or on JEOL SX-102 high-resolution mass spectrometer. High-performance liquid chromatography was conducted using one of the following systems: (1) a Beckman System (System Gold) equipped with a model 126 programmable solvent module, a model 168 diode array detector, and a model 170 radioisotope detector which was interfaced using a model 406 analog interface module; (2) two LKB model 2150 pumps, an LKB model 2152 control system, an LKB model 2138 fixed-wavelength UV-detector, and a Beckman model 170 radioisotope detector. Peak analysis in the latter was performed using a Nelson Analytical software package on an IBM computer. For reversed-phase HPLC, a Waters 3.9 $\times$ 300 mm μ -Bondapak C18 (10 μ) column or a 3.9 $\times$ 300 mm Bondclone 10 (10 μ) C-18 column (Phenomenex, Torrance, CA) was eluted with 98:2 $\text{NH}_4\text{H}_2\text{PO}_4$ (0.2M):THF. For normal-phase HPLC, an Alltech Partisil 10 μ column (250 $\times$ 4.6 mm) was eluted with a mixture of acetic acid, ethyl acetate, and hexane of varying compositions. Analytical TLC was performed on aluminum-backed sheets (Silica gel 60 F₂₅₄), and normal-phase column chromatography was performed using Silica gel 60, both obtained from EM Science (Gibbstown, NJ). For column chromatography, factors such as silica gel amount and column diameter were kept in accordance with literature protocols (Still et al., 1978). In most cases, quartz columns were used facilitating visualization of the bands. In these cases, the silica gel was mixed with zinc silicate phosphor (Sigma) at a ratio of 99:1 (w/w). Fractions were collected using a Gilson model 203 micro fraction collector (Middleton, WI) or an ISCO Foxy 200 fraction collector (Lincoln, NE) and identified by TLC. In some cases, an ISCO UA-6 UV-vis detector was placed between the column outlet and the fraction collector to identify fractions. Preparative thick layer chromatography was performed using 20 $\times$ 20 cm, 1000 μ plates (Whatman, Clifton, NJ). Before applying the sample, the plates were run in ethyl acetate to clean the plates of any adsorbed impurities.

Cells and Culture Conditions. The human neuroblastoma cell line SK-N-SH (uptake-1 positive; Biedler et al., 1973) was purchased from American Type Culture Collection (Rockville, MD). The incubation medium (JRH Biosciences, Lenexa, KS) was made by mixing 440 mL

of RPMI 1640, 50 mL of Serum Plus, 5 mL of penicillin-G/streptomycin (5000 units of penicillin and 5000 μ g of streptomycin in 1 mL of 0.85% saline) and 5 mL of glutamine (200 mM in saline). The cells were grown at 37 $^\circ\text{C}$ in a humidified incubator containing 5% CO_2 . Cell viability was evaluated prior to each binding experiment by trypan blue dye (Kaltenbach et al., 1958) and was 95–98% for all studies.

5-(Trimethylsilyl)nicotinic Acid (2). A solution of 5-bromonicotinic acid (**1**; 2.02 g; 10 mmol) in 60–70 mL of freshly distilled THF under argon was cooled to -78°C in a dry ice/acetone bath. To this, butyllithium in hexanes (13–14 mL of 1.6 M; 22 mmol) was added dropwise via a syringe over a period of 20–25 min. After stirring the mixture at -78°C for 30 min under argon, chlorotrimethylsilane (3.2 mL; 25 mmol) was added via a syringe over a period of 10–15 min. The reaction mixture was allowed to warm gradually to room temperature and left stirring at room-temperature overnight. The THF was removed from the reaction mixture by rotary evaporation, and the residual oil was partitioned between water (50 mL) and ethyl acetate (50 mL). The aqueous layer was extracted with 3 $\times$ 50 mL of ethyl acetate and the pooled ethyl acetate layer was washed with brine (25 mL), dried with anhydrous sodium sulfate, and evaporated. Silica gel chromatography of this crude product using 1:4 ethanol:chloroform yielded 335 mg (17%) of **2** as an oil. An analytical sample was obtained by preparative TLC using 3:7 ethanol:chloroform and subsequent recrystallization from 4:1 chloroform:hexane mixture: mp 175 $^\circ\text{C}$. TLC: R_f (3:7 ethanol:chloroform) = 0.47. HPLC: μ -Bondapak C18 column; flow rate 1.5 mL/min; t_R = 22 min (see Supporting Information for the chromatogram). ^1H NMR (CDCl_3): δ 0.37 (s, 9H), 8.59 (s, 1H), 8.89 (s, 1H), 9.32 (s, 1H). ^{13}C NMR (CDCl_3): δ -1.4, 127.1, 136.3, 144.0, 149.6, 155.0, 168.3. MS (CI): m/z 196, 178, 152. HRMS (EI^+) calcd for $\text{C}_9\text{H}_{13}\text{NO}_2\text{Si}$ (M^+): 195.0716. Found: 195.0715. Anal. Calcd for $\text{C}_9\text{H}_{13}\text{NO}_2\text{Si} \cdot \frac{1}{2}\text{H}_2\text{O}$: C, 52.86; H, 6.85; N, 6.85. Found: C, 53.28; H, 6.59; N, 6.97.

3-Hydroxymethyl-5-(trimethylsilyl)pyridine (3). To a mixture of **2** (1.91 g; 9.8 mmol) and trimethyl borate (6.7 mL; 59 mmol) in 15 mL of dry THF under argon was added dropwise borane methyl sulfide (2.2 mL; 23 mmol) via a syringe. The mixture was stirred at room temperature for 3–4 h, and at the end of this period, methanol (3 mL) was added. After evaporating the THF and methanol, the residual oil was partitioned between water (50 mL) and ethyl acetate (50 mL). The aqueous layer was further extracted with 3 $\times$ 50 mL of ethyl acetate. The pooled ethyl acetate layer was washed with 1 N NaOH (10 mL) and brine (25 mL) in that order, and then dried with anhydrous sodium sulfate. Silica gel chromatography of the residual oil using 1:1 ethyl acetate:hexane yielded 258 mg (15%) of a crystalline solid (**3**). An analytical sample was obtained by two consecutive preparative TLCs using the solvent systems used for analytical TLC (see below): mp 117–119 $^\circ\text{C}$. TLC: R_f (2:3 ethyl acetate:hexane) = 0.25; (1:19 ethyl acetate:chloroform) = 0.15. Normal-phase HPLC: 0.2:35:65 acetic acid:ethyl acetate:hexane; flow rate 1 mL/min; t_R = 10.7 min (see Supporting Information for the chromatogram). ^1H NMR (CDCl_3): δ 0.34 (s, 9H), 4.80 (s, 2H), 7.98 (s, 1H), 8.52 (s, 1H), 8.55 (s, 1H). ^{13}C NMR (CDCl_3): δ -1.6, 61.7, 137.8, 138.3, 142.2, 145.7, 149.9. MS (CI): m/z 182, 164. HRMS (FAB^+) calcd for $\text{C}_9\text{H}_{16}\text{NOSi}$ (MH^+): 182.1001. Found: 182.0997. Anal. Calcd for $\text{C}_9\text{H}_{15}\text{NOSi} \cdot \text{H}_2\text{O}$: C, 54.19; H, 8.59; N, 7.03. Found: C, 54.65; H, 9.31; N, 7.01.

3-[*N*¹,*N*²-Bis(*tert*-butyloxycarbonyl)guanidinomethyl]-5-(trimethylsilyl)pyridine (4**).** Compound **3** (258 mg; 1.4 mmol), 700 mg (2.7 mmol) of triphenylphosphine, and 735 mg (2.84 mmol) of [*N*¹,*N*²-bis(*tert*-butyloxycarbonyl)guanidine (**5**; prepared according to Dodd and Kozikowski, 1994)] were dissolved in 10 mL of dry THF in an argon-purged and dried flask. Diisopropyl azodicarboxylate (0.43 mL; 3 mmol) was added via a syringe over a period of 25 min to this stirred solution at room temperature. The reaction mixture was stirred at room temperature for another 3–4 h. Evaporation of THF from the reaction mixture resulted in a highly viscous oil. This oil was redissolved in 50 mL of ethyl acetate, and 10 g of silica gel was added to it. Evaporation of ethyl acetate allowed uniform adsorption of the oil onto the silica gel which was then packed on the top of a silica gel column. Chromatography using 1:9 ethyl acetate:hexane yielded 141 mg (24%) of **4** as a white crystalline solid: mp 140–142 °C. TLC: R_f (1:4 ethyl acetate:hexane) = 0.31. Normal-phase HPLC: 0.2:20:80 acetic acid:ethyl acetate:hexane, flow rate 1 mL/min, t_R = 6.9 min (see Supporting Information for the chromatogram). ¹H NMR (CDCl₃): δ 0.33 (s, 9H), 1.50 (s, 9H), 1.54 (s, 9H), 5.12 (s, 2H), 8.33 (s, 1H), 8.46 (s, 1H), 8.62 (s, 1H), 9.20 (br s, 2H). ¹³C NMR (CDCl₃): −1.5, 28.1, 28.2, 44.6, 79.2, 85.6, 135.3, 137.9, 145.2, 147.7, 149.8, 154.2, 160.1, 163.2 (for ¹³C NMR of similar compounds see Yong et al., 1997). HRMS (FAB⁺) calcd for C₂₀H₃₅N₄O₄Si (MH⁺): 423.2428. Found: 423.2423. Anal. Calcd for C₂₀H₃₄N₄O₄·Si·1/2H₂O: C, 55.61; N, 8.11; O, 12.97. Found: C, 55.66; H, 8.17; N, 12.98.

3-Bromo-5-hydroxymethylpyridine (6**).** This compound was prepared from **1** (10 g; 50 mmol) using a procedure similar to that used for the preparation of **3** to yield 2.63 g (28%) of an oil. TLC: R_f (1:4 ethyl acetate:hexane) = 0.32. ¹H NMR (CDCl₃): δ 2.60 (br s, 1H), 4.74 (s, 2H), 7.90 (s, 1H), 8.47 (s, 1H), 8.58 (s, 1H). MS (CI): m/z 188 and 190. Anal. Calcd for C₆H₆BrNO: C, 38.33; H, 3.22; N, 7.45. Found: C, 38.16; H, 3.35; N, 7.31.

3-Bromo-5-[*N*¹,*N*²-bis(*tert*-butyloxycarbonyl)guanidinomethyl]pyridine (7**).** This compound was prepared using the same procedure described for the preparation of **4**. Briefly, to a solution of **6** (300 mg; 1.6 mmol), triphenylphosphine (615 mg; 2.34 mmol) and **5** (646 mg; 2.50 mmol) in 10 mL of dry THF was added dropwise 0.3 mL (1.92 mmol) of DEAD. An equivalent amount of diisopropyl azodicarboxylate can be substituted for DEAD. After stirring for 3–4 h at room temperature, the THF was evaporated and the residual oil was adsorbed onto silica gel as described before. Chromatography using 3:17 ethyl acetate:hexane yielded 680 mg (100%) of **7** as a waxy solid: mp 94–97 °C. TLC: R_f (1:3 ethyl acetate:hexane) = 0.25. ¹H NMR (CDCl₃) δ 1.44 (s, 9H), 1.53 (s, 9H), 5.13 (s, 2H), 7.93 (s, 1H), 8.51 (s, 1H), 8.56 (s, 1H), 9.35 (br s, 2H). MS (FAB⁺): m/z 429 and 431, 373 and 375, 317 and 319, 229 and 231. Anal. calcd for C₁₇H₂₅BrN₄O₄: C, 47.56; H, 5.87; N, 13.05. Found: C, 47.87; H, 6.28; N, 13.22.

3-[*N*¹,*N*²-Bis(*tert*-butyloxycarbonyl)guanidinomethyl]-5-(trimethylstannyl)pyridine (8**).** A mixture of **7** (940 mg; 2.5 mmol), hexamethylditin (3.5 g; 11 mmol), and bis(triphenylphosphine)palladium dichloride (355 mg; 0.5 mmol) in 45 mL of dioxane was refluxed for 2 h under argon. The dioxane was removed from the dark reaction mixture by rotary evaporation and the residue (oil plus black particles) was taken up in ethyl acetate (50 mL). This mixture was filtered through silica gel (20 g) using a frit funnel. The silica gel was washed with liberal amounts of ethyl acetate and the ethyl acetate was

evaporated off from the filtrate. The yellow oily residue was subjected to silica gel chromatography. The column was packed and eluted initially with chloroform. After the excess tin reagent was eluted off, the solvent was gradually changed to 10% ethyl acetate in chloroform. Evaporation of solvents from pooled fractions yielded 500 mg (40%) of **8** as an oil. An analytical sample was obtained as a white crystalline solid by preparative TLC using 1:3 ethyl acetate:hexane. mp 103–104 °C. TLC: R_f (7:13 ethyl acetate:hexane) = 0.28. ¹H NMR (CDCl₃): δ 0.34 (s, 9H), 1.43 (s, 9H), 1.50 (s, 9H), 5.12 (s, 2H), 7.90 (s, 1H), 8.53 (m, 2H), 9.30 (br s, 1H), 9.43 (br s, 1H). MS (FAB⁺): m/z cluster peaks at 515, 459, 415, 315. HRMS- (FAB⁺) calcd for C₂₀H₃₅N₄O₄Sn (MH⁺) 513.1680. Found 513.1689. Anal. Calcd for C₂₀H₃₄N₄O₄Sn: C, 46.81; H, 6.68; N, 10.92. Found: C, 46.74; H, 6.61; N, 10.77.

3-Guanidinomethyl-5-iodopyridine (9b**).** A mixture of **8** (392 mg; 0.8 mmol) and iodine (520 mg; 2 mmol) in 15 mL of acetic acid was heated at 70 °C for 15–20 min in a closed flask. At the end of this period, 20 mL of trifluoroacetic acid was added and heated for another 5–10 min. The solvents and excess iodine were removed at 35–40 °C using a rotary evaporator under aspirator vacuum. The dark residual solid was dissolved in 5 mL of water and a solution of saturated sodium bisulfite (0.3 mL) was added. This solution was extracted with 3 × 5 mL of ethyl acetate to remove nonpolar impurities. The aqueous layer was evaporated, and the residual solid was taken up in 25 mL of warm methanol. The undissolved inorganic material was filtered, and ether (50 mL) was added with stirring to the filtrate. The resultant granular precipitate was filtered and washed with ether to yield 80 mg (25%) of **9b**. An analytical sample was obtained by recrystallization from water: mp 248–250 °C (decomposing). TLC (NaBr impregnated plate; Bluhm and Li, 1998): R_f (1:4 methanol:dichloromethane) = 0.34. HPLC: μ -Bondapak C18 column; flow rate 1.0 mL/min; t_R = 13.6 min (see Supporting Information for the chromatogram). ¹H NMR (CD₃OD): δ 4.42 (s, 2H), 8.18 (s, 1H), 8.48 (s, 1H), 8.76 (s, 1H). ¹³C NMR (CD₃-SOCD₃): 41.1, 94.3, 135.2, 143.0, 147.3, 154.3, 156.7. MS (FAB⁺): m/z 277, 260, 235. HRMS (FAB⁺) calcd for C₇H₁₀IN₄ (MH⁺): 276.9950. Found: 276.9945. Anal. Calcd for C₇H₉IN₄·HI: C, 20.84; H, 2.50; N, 13.90. Found: C, 20.83; H, 2.96; N, 14.04.

Radioiodination of **8.** A solution of **8** (0.5 μ mol in 5 μ L) in chloroform was taken in a 1/2-dram vial and the chloroform was evaporated with an argon stream. Iodine-125 or ¹³¹I (0.2–2 mCi) in 1–2 μ L of 0.1 N NaOH was added to the vial followed by NCS in acetic acid (0.4 mg in 20 μ L). The mixture was heated at 70 °C for 10–12 min in an oil bath. Trifluoroacetic acid (20 μ L) was added and the mixture was heated again for 5 min. Most of the solvent was removed by evaporation with a stream of argon, and the residue was redissolved in 40 μ L of methanol. The product was isolated by reversed-phase HPLC. For HPLC, a 3.9 × 300 mm Bondclone 10 (10 μ) C-18 (Phenomenex, Torrance, CA) column was eluted with 98:2 ammonium dihydrogen phosphate (0.2 M):THF at a flow rate of 1 mL/min. Under these conditions, the product (**9b**) had a retention time of 17–18 min. The radiochemical yield was in the range 65–70%. No coeluting mass peak was detected by HPLC. The activity from HPLC fractions was concentrated using a C18 solid-phase cartridge as described for MIBG and other benzylguanidine derivatives (Vaidyanathan and Zalutsky, 1993; Vaidyanathan et al., 1997).

Lipophilicity Measurements. Octanol/buffer partition coefficients of MIBG and GMIP were determined in a paired-label format at two different pH values. This was performed following a reported method (Vaidyanathan et al., 1994) except that phosphate buffers of pH 7.4 and 4.7 (measured using a pH meter) were used instead of water. Lipophilicity was also determined by calculating the log k' values from HPLC data. For this a Waters μ Bondapak C18 column was eluted with 80:20 ammonium dihydrogen phosphate (0.2 M):THF at a flow rate of 1 mL/min.

In Vitro Assessment of 9b. SK-N-SH cells were plated in 6-well plates at an initial density of 5×10^5 cells/well in 3 mL of medium and incubated for 24 h. The medium was removed by aspiration, and 100 nCi each of [^{131} I]MIBG and [^{125} I]GMIP in 3 mL of medium was added to each well and incubated for 2 h at 37 °C. To determine the effect of DMI and ouabain, the initial medium was removed and the cells were incubated with 3 mL each of 1.5 μ M DMI and 1 mM ouabain, respectively, for 30 min. The medium was removed, and tracers were added as above and incubated for 2 h at 37 °C. To determine the effect of temperature, the original medium was removed, and fresh medium precooled to 4 °C was added followed by tracers (100 nCi each) in 10 μ L of the medium and then incubated for 2 h at 4 °C. Finally, the effects of norepinephrine and MIBG were ascertained by removing the original medium and coincubating the cells with the tracers (100 nCi in 10 μ L each) and 3 mL each of 50 μ M NE and 10 μ M MIBG, respectively, at 37 °C for 2 h.

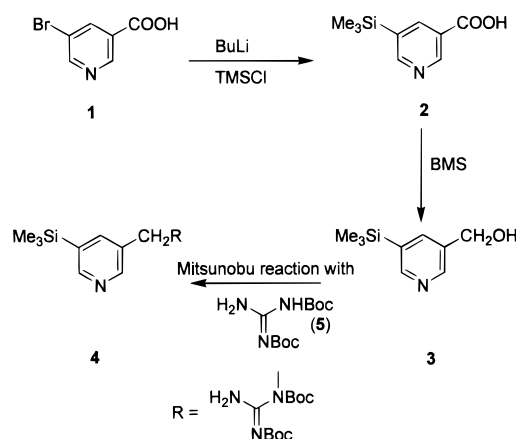
At the end of 2 h incubation period, cells were solubilized by incubation with 500 μ L of 0.5 N NaOH for 30 min at room temperature and then removed with cotton swabs. The cell-bound activity was counted along with input standards using a dual-channel γ counter. Each set of experiments was performed in six replicates. The entire experiment was conducted twice.

Paired-Label Biodistribution of [^{131} I]MIBG and [^{125} I]GMIP in Normal Mice. Male BALB/c mice weighing about 25 g were utilized. Tissue uptake of [^{125} I]GMIP was compared with that of [^{131} I]MIBG at 1, 4, and 24 h postinjection by administering via the tail vein 6 μ Ci of the former and 4 μ Ci of the latter in 100 μ L of phosphate-buffered saline. Groups of five mice were used for each time point. The mice were killed by an overdose of halothane. Tissues of interest were isolated, washed, blot-dried, and counted for ^{131} I and ^{125} I using a dual-label program in a γ counter. A 5 or 10% aliquot of the injected activity was also counted so that the percentage of injected dose per organ or per gram of tissue could be computed.

Isolated Rat Heart Studies. Perfusion of isolated working rat hearts was performed as described previously (DeGrado et al., 1995). The perfusion protocol consisted of a 15 min stabilization period, a 4 min radiotracer wash-in (pulse) period and a 56 min wash-out period in succession. Before each perfusion, the radiotracer perfusion circuit was filled with approximately 500 mL of perfusion medium containing 30–40 μ Ci/L [^{131} I]GMIP. Specific extraneuronal uptake (uptake-2) into myocytes was pharmacologically blocked in a group of hearts with 0.8 μ M *N*-(9-fluorenyl)-*N*-methyl- β -chloroethylamine hydrochloride (SKF550, SmithKline-Beecham, King of Prussia, PA; Iversen et al., 1972).

At the end of perfusion, radioactivity in the heart was measured and normalized to the radioactivity concentration of the wash-in medium and heart mass to obtain myocardial retention in units of apparent distribution

Scheme 1



volume [ADV (mL/g)] as previously described (DeGrado et al., 1995). Iodine-131 radioactivity in the rat heart was externally monitored using two lead-collimated 5×5 cm NaI(Tl) scintillation probes. Counts were sampled every second and stored on an IBM PC/AT computer. The total count rate in the heart [Y_i (counts/s)] was calculated as the sum of the count rates for the two detectors corrected for background and radioactive decay. The resultant time-activity curve was normalized by the concentration of radioactivity in the wash-in perfusate and the mass of the heart to give ADV (mL/g) at each sample point. This was accomplished by multiplication of each Y_i by a calibration factor (f_c):

$$ADV_i \text{ (mL/g)} = Y_i f_c \quad (1)$$

where f_c is given by

$$f_c \left(\frac{\text{mL/g}}{\text{counts/s}} \right) = \frac{ADV_e}{Y_e} \quad (2)$$

ADV_e is the apparent distribution volume measured at the end of perfusion and Y_e is the average total count rate over the last 10 s of the experiment.

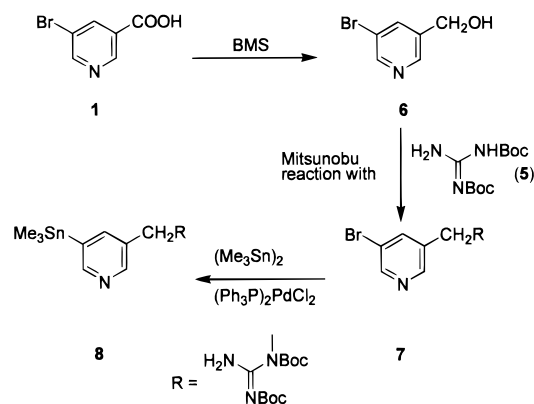
Indices of cellular accumulation and clearance of [^{131} I]-GMIP were derived from each of the myocardial uptake curves. The uptake rate, K_i (mL/min/g), was estimated from the linear portion of the curve over the interval of 2–3 min. In this isolated heart preparation, coronary flow is supraphysiologic (typically 11–12 mL/g/min), allowing rapid equilibration of tracer in extracellular spaces within 2 min (DeGrado et al., 1995). Therefore, K_i indicates the accumulation rate of [^{131} I]GMIP in cellular spaces. The clearance kinetics were described by the multiexponential model: $y = A_1 \exp[-E_1(t - t')]$ + $A_2 \exp[-E_2(t - t')]$, where t' = time of commencement of washout period. Estimates of the constants A_1 , A_2 , E_1 , and E_2 were obtained by a weighted nonlinear least-squares fitting procedure. Data are expressed as means $\pm$ standard error. The t -test (two-tailed) for unpaired samples was used to compare means.

RESULTS AND DISCUSSION

Due to the highly basic nature of guanidine functionality, it was anticipated that strong acidic conditions would be necessary to perform the halodemetalation reaction. Since a tin derivative may undergo proteodestannylation under strong acidic conditions, initial attempts were made to prepare a silicon precursor. The compound targeted was 3-[N^1, N^2 -bis(*tert*-butyloxycarbonyl)guani-

Table 1. Paired-label Uptake of [¹²⁵I]GMIP and [¹³¹I]MIBG in Normal Mice

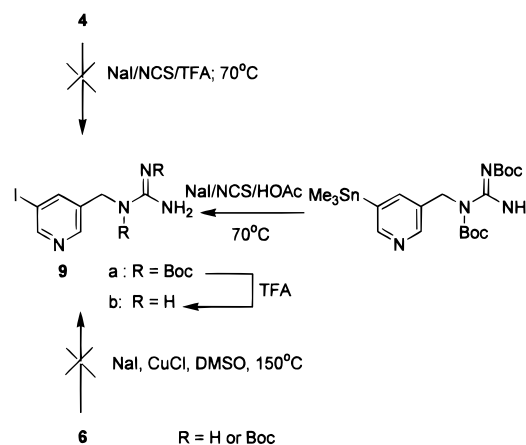
tissue	percent injected dose per gram ^a					
	1 h		4 h		24 h	
	[¹²⁵ I]GMIP	[¹³¹ I]MIBG	[¹²⁵ I]GMIP	[¹³¹ I]MIBG	[¹²⁵ I]GMIP	[¹³¹ I]MIBG
liver	7.73 ± 1.66	8.43 ± 0.56	2.27 ± 0.22	4.53 ± 0.33	0.08 ± 0.01	0.68 ± 0.10
spleen	3.37 ± 0.32	4.42 ± 0.39	2.39 ± 0.46	3.95 ± 0.52	0.11 ± 0.02	1.35 ± 0.21
lungs	3.95 ± 0.66	9.99 ± 2.19	1.60 ± 0.50	3.93 ± 0.83	0.06 ± 0.01	0.93 ± 0.16
heart	9.41 ± 1.99	19.82 ± 2.38	4.88 ± 1.42	15.24 ± 2.36	0.16 ± 0.01	3.30 ± 0.32
kidneys	1.89 ± 0.16	2.97 ± 0.15	1.01 ± 0.12	1.90 ± 0.14	0.07 ± 0.01	0.61 ± 0.08
stomach	3.72 ± 0.26	5.09 ± 0.55	1.61 ± 0.20	2.81 ± 0.59	0.32 ± 0.06	1.38 ± 0.18
sm. int.	8.31 ± 0.95	8.00 ± 0.73	2.30 ± 0.30	3.85 ± 0.45	0.20 ± 0.04	1.27 ± 0.24
lg. int.	5.03 ± 0.64	3.77 ± 0.45	5.48 ± 0.84	4.72 ± 0.44	0.29 ± 0.04	1.26 ± 0.21
thyroid	2.09 ± 0.23	3.00 ± 0.51	1.18 ± 0.45	3.09 ± 1.09	0.29 ± 0.09	3.79 ± 0.76
muscle	1.52 ± 0.19	1.91 ± 0.18	0.82 ± 0.15	1.59 ± 0.29	0.02 ± 0.00	0.34 ± 0.09
blood	0.41 ± 0.04	0.74 ± 0.04	0.16 ± 0.01	0.37 ± 0.03	0.01 ± 0.00	0.08 ± 0.01
adrenals	10.17 ± 4.32	19.32 ± 6.98	9.01 ± 7.32	15.11 ± 9.66	2.09 ± 1.05	7.51 ± 3.72
bone	0.92 ± 0.10	1.30 ± 0.10	0.47 ± 0.12	0.85 ± 0.13	0.01 ± 0.00	0.18 ± 0.04
brain	0.11 ± 0.10	0.23 ± 0.24	0.06 ± 0.07	0.15 ± 0.14	0.00 ± 0.00	0.04 ± 0.03

^a Normalized to a 25 g mouse; mean ± SD (n = 5).**Scheme 2**

dinomethyl]-5-(trimethylsilyl)pyridine (**4**). A number of approaches were made for the synthesis of this compound; the successful one is depicted in Scheme 1. Butyllithium-mediated silylation of 5-bromonicotinic acid gave **2**, which was reduced with borane methyl sulfide (Lane et al., 1974) to **3**. Treatment of this alcohol with *N*¹,*N*²-bis(*tert*-butoxycarbonyl)guanidine (**5**) under Mitsunobu conditions (Dodd and Kozikowski, 1994) gave the target silicon precursor. A number of attempts to iododesilylate **4** using various oxidizing agents (NCS, TBHP, etc.) and media (TFA, concentrated HCl, methanol, and combinations thereof) were unsuccessful even at 100 °C; 3-guanidinomethyl-5-trimethylsilylpyridine was isolated in almost quantitative yields. Attempts to convert **7** to **9** by copper-mediated exchange (Moerlin, 1990) also were unsuccessful.

Having failed to prepare GMIP by these two routes, a tin precursor was sought. The synthetic method used to prepare this compound, 3-[*N*¹,*N*²-bis(*tert*-butoxycarbonyl)guanidinomethyl]-5-(trimethylstannyl)pyridine (**8**) is shown in Scheme 2. First, 5-bromonicotinic acid was reduced with borane methyl sulfide to the corresponding alcohol **6**. This alcohol was converted to the guanidine derivative **7** by treatment with **5** under Mitsunobu conditions. Finally, **7** was converted to **8** by palladium catalyst-mediated coupling with hexamethylditin. Treatment of **8** with iodine in acetic acid at 70 °C did yield **9b**. The precursor **8** could also be radioiodinated using NCS in acetic acid to give [¹²⁵I]GMIP in about 70% radiochemical yield.

The octanol/buffer partition coefficient of GMIP, determined from a paired-label experiment, was more than 10-fold lower than that of MIBG. For example, the values using phosphate buffer of pH 7.4 were 0.17 ± 0.01

Scheme 3

and 2.83 ± 0.35, respectively. The log *k'* values calculated for MIBG and GMIP were 0.30 and -0.40, respectively. These results indicate that GMIP, as expected, has much lower lipophilicity than MIBG.

Results from a paired-label tissue distribution of [¹²⁵I]-GMIP and [¹³¹I]MIBG in normal mice are shown in Table 1. As expected, GMIP cleared much faster than MIBG from all tissues. Interestingly, [¹²⁵I]GMIP was less susceptible to *in vivo* deiodination because its thyroid uptake, especially at 24 h, was much lower than that for [¹³¹I]MIBG. This may reflect a greater difference in the chemical structure of GMIP, compared to MIBG, to that of monoiodotyrosine, one of the natural substrates for deiodinases. Heart (9.41 ± 1.99 vs 19.82 ± 2.38% ID/g) and adrenal (10.17 ± 4.32 vs 19.32 ± 6.98% ID/g) uptake of [¹²⁵I]GMIP at 1 h was about 50% lower than [¹³¹I]-MIBG, and at later time points, the difference in uptake of [¹²⁵I]GMIP and [¹³¹I]MIBG was even more apparent. The piperazine derivative, 1-carboxamidino(4-[¹²⁵I]iodophenyl)piperazine ([¹²⁵I]CPP) has myocardial and adrenal uptake similar to that for MIBG (Hanson, 1982). Another heterocycle-containing compound, 4-(3-[¹²⁵I]iodophenyl)piperazine ([¹²⁵I]IPP), had myocardial uptake 50% or lower than that for MIBG (Babich et al., 1996). The latter piperazine compound, however, was shown to have high affinity for the norepinephrine transporter in SK-N-SH and SK-N-BE(2c) cells *in vitro*. Provided GMIP is sequestered specifically in neuroendocrine tumors, its lower normal tissue uptake may result in tumor-to-normal tissue ratios higher than that of MIBG.

To investigate this possibility, [¹²⁵I]GMIP was evaluated *in vitro*, along with [¹³¹I]MIBG, using SK-N-SH human neuroblastoma cells which take up MIBG specif-

Table 2. Tracer Kinetic Parameters of [¹³¹I]GMIP in Isolated Rat Heart

condition ^a	uptake rate K_i (mL/g/min)	biexponential clearance parameters ($y = A_1e^{-E_1t} + A_2e^{-E_2t}$)			
		A_1 (mL/g)	E_1 (min ⁻¹)	A_2 (mL/g)	E_2 (min ⁻¹)
control	0.43 ± 0.03	1.00 ± 0.11	0.138 ± 0.038	1.25 ± 0.28	0.029 ± 0.014
uptake-2 blockade (0.8 μM SKF550)	0.52 ± 0.09	1.46 ± 0.50	0.108 ± 0.015	0.61 ± 0.08	0.013 ± 0.002

^a For each condition, $n = 3$.

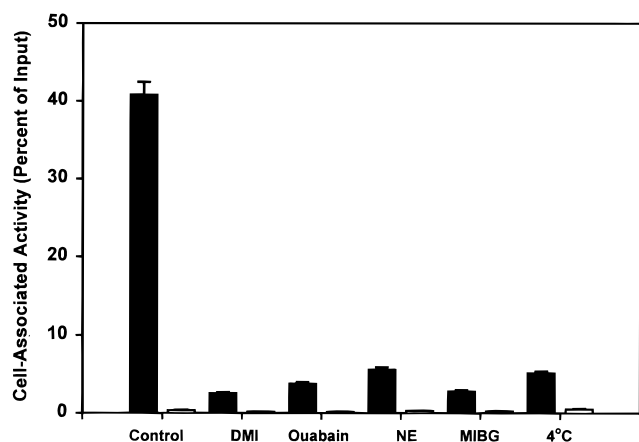


Figure 1. Paired-label uptake of [¹²⁵I]GMIP (□) and [¹³¹I]MIBG (■) by SK-N-SH human neuroblastoma cells in vitro: Influence of various blocking agents and conditions.

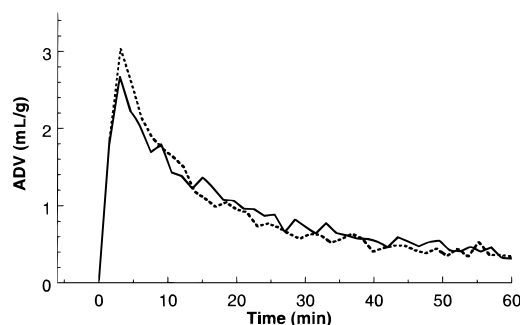


Figure 2. Representative time-activity curves of GMIP in control (solid) and uptake-2 blocked (dashed) perfused rat hearts. Uptake-2 blocked condition used 0.8 μM SKF550 in the perfusion medium. The uptake and clearance patterns were nearly identical in both conditions, showing negligible sensitivity of the kinetics to inhibition of the uptake-2 (extraneuronal) catecholamine transporter.

ically via the uptake-1 mechanism (Buck et al., 1985; Smets et al., 1989; Vaidyanathan et al., 1997). The results from this experiment showed that MIBG was taken up specifically by these cells. About 40% of input [¹³¹I]MIBG was incorporated into the cells, and this uptake could be effectively impeded by various agents and conditions that block the uptake-1 mechanism (Figure 1). In contrast, less than 1% of the input activity of [¹²⁵I]GMIP was cell-bound even in the absence of uptake-1 inhibitors. This suggests that the benzene ring of MIBG is a *sine qua non* for its recognition by the norepinephrine uptake-1 transporter, which also probably accounts for the lower uptake of GMIP in mouse heart and adrenals. To explore the possibility that GMIP could be useful as a tracer of specific extraneuronal transport of catecholamines (uptake-2; Iversen et al., 1972), experiments were carried out in an isolated rat heart model. Figure 2 shows representative time-activity curves for GMIP in isolated rat hearts perfused in control conditions and with uptake-2 blockade by 0.8 μM SKF550. The uptake and clearance parameters for GMIP in control and uptake-2 blocked conditions are shown in

Table 2. The uptake rate of GMIP was 0.43 ± 0.03 mL/g/min in control conditions and was not significantly different in uptake-2 blocked hearts. Clearance was biphasic. In control hearts, roughly one-half of the radioactivity cleared with a clearance half-time of 5 min. The other half showed a clearance half-life of 24 min. Thus, after 60 min, approximately 90% of the radioactivity had cleared from the heart. No significant differences were observed in the clearance kinetic parameters in uptake-2 blocked hearts versus control (Table 2). These results indicate minimal retention of GMIP in myocardial tissue, consistent with the lack of specific cellular uptake of the tracer. Indeed, the kinetic properties of GMIP are very similar to those previously observed for MIBG in the same isolated rat heart model with blockade of both neuronal (uptake-1) and extraneuronal (uptake-2) transporters (DeGrado et al., 1995).

In conclusion, a synthetic method was developed for the preparation of GMIP, a pyridine analogue of MIBG. In vitro, in vivo, and ex vivo experiments demonstrated that substitution of the benzene ring in MIBG with a pyridine ring resulted in its total loss of specificity for norepinephrine transporter. This may not be entirely due to the more polar nature of GMIP over MIBG as derivatives of MIBG with polar substituents such as -OH and -NH₂ on its benzene ring exhibited similar biological properties as MIBG itself (Wieland, 1986). Thus, although antibodies, peptides, and biotin iodopyridine conjugates can be synthesized with excellent biological properties, iodopyridine-for-iodobenzene substitution was not successful in MIBG.

ACKNOWLEDGMENT

This work was supported by Grants CA 74817, CA 60066, and HL 54882 from the National Institutes of Health. The authors thank Shuyan Wang, Donna Affleck, Jean Li, Susan Slade, and Phil Welsh for their excellent technical assistance.

SUPPORTING INFORMATION AVAILABLE

HPLC chromatograms of compounds **2**, **3**, **4**, and **9b** (4 pages). Ordering information is given on any current masthead page.

LITERATURE CITED

- Babich, J. W., Graham, W. A., and Fischman, A. J. (1996) Evaluation of phenylpiperazines as targeting agents for neuroblastoma. *Br. J. Cancer* 74, 917–924.
- Biedler, J. N., Helson, L., and Spengler, B. A. (1973) Morphology and growth, tumorigenicity, and cytogenetics of human neuroblastoma cells in continuous culture. *Cancer Res.* 33, 2643–2652.
- Bluhm, L. H., and Li, T. (1998) Chromatographic purification of quaternary ammonium and pyridinium compounds on normal phase silica gel. *Tetrahedron Lett.* 39, 3623–3626.
- Buck, J., Bruchelt, G., Girgert, R., Treuner, J., and Niethammer, D. (1985) Specific uptake of m-[¹²⁵I]iodobenzylguanidine in the human neuroblastoma cell line SK-N-SH. *Cancer Res.* 45, 6366–6370.
- Chan, S. M., Basmadjian, G. P., Marten, D. F., Sadek, S., Magarian, R. A., Grunder, J. R., and Ice, R. D. (1983)

- Synthesis and biodistribution of radioiodinated 5-iodonicotine. *J. Nuclear Med.* 24, P116 (Abstract).
- DeGrado, T. R., Zalutsky, M. R., and Vaidyanathan, G. (1995) Uptake mechanisms of meta-iodobenzylguanidine. *Nuclear Med. Biol.* 22, 1–12.
- Dodd, D. S., and Kozikowski, A. P. (1994) Conversion of alcohols to protected guanidines using the Mitsunobu protocol. *Tetrahedron Lett.* 35, 977–980.
- Foulon, C. F., Alston, K. L., and Zalutsky, M. R. (1997) Synthesis and preliminary biological evaluation of (3-iodobenzoyl)-norbiotinamide and ((5-iodo-3-pyridinyl)carbonyl)norbiotinamide: Two radioiodinated biotin conjugates with improved stability. *Bioconjugate Chem.* 8, 179–186.
- Garg, S., Garg, P. K., and Zalutsky, M. R. (1991) *N*-Succinimidyl 5-(trialkylstannyl)-3-pyridinecarboxylate: A new class of reagents for protein radioiodination. *Bioconjugate Chem.* 2, 50–56.
- Garg, S., Garg, P. K., and Zalutsky, M. R. (1992) Acylation agents for the radiohalogenation of peptides. *J. Labeled Compd. Radiopharm.* 32, 212 (Abstract).
- Garg, S., Garg, P. K., Zhao, X.-G., Friedman, H. S., Bigner, D. D., and Zalutsky, M. R. (1993) Radioiodination of a monoclonal antibody using *N*-succinimidyl 5-iodo-3-pyridinecarboxylate. *Nuclear Med. Biol.* 20, 835–842.
- Hanson, R. N. (1982) Radioiodinated 1-carboxamidino-4-phenylpiperazine: A potential adrenal and myocardial imaging radiopharmaceutical. *Int. J. Appl. Radiat. Isot.* 33, 629–632.
- Hanson, R. N. (1983) Preparation and evaluation of radioiodinated phenylpiperazines as adrenomedullary imaging agents. *Int. J. Nucl. Med. Biol.* 10, 219–222.
- Iversen, L. L., Salt, P. J., and Wilson, H. A. (1972) Inhibition of catecholamine uptake in the isolated rat heart by haloalkylamines related to phenoxybenzamine. *Br. J. Pharm.* 46, 647–657.
- Jaques, S., Jr., Tobes, M. C., and Sisson, J. C. (1987) Sodium dependency of uptake of norepinephrine and *m*-iodobenzylguanidine into cultured human pheochromocytoma cells: evidence for uptake-one. *Cancer Res.* 47, 3920–3928.
- Kaltenbach, J. P., Kaltenbach, M. H., and Lyons, W. B. (1958) Nigrosin as a dye for differentiating live and dead ascites cells. *Exp. Cell Res.* 15, 112–117.
- Lane, C. F., Myatt, H. L., Daniels, J., and Hopps, H. B. (1974) Organic synthesis using borane-methyl sulfide. II. Reduction of aromatic carboxylic acids in the presence of trimethyl borate. *J. Org. Chem.* 39, 3052–3054.
- Moerlein, S. M. (1990) Regiospecific aromatic radioiodination via no-carrier-added copper(I)chloride-assisted iododebromination. *Radiochim. Acta* 50, 55–61.
- Reist, C. J., Garg, P. K., Alston, K. L., Bigner, D. D., and Zalutsky, M. R. (1996) Radioiodination of internalizing monoclonal antibodies using *N*-succinimidyl 5-iodo-3-pyridinecarboxylate. *Cancer Res.* 56, 4970–4977.
- Smets, L. A., Bout, B., and Wisse, J. (1988) Cytotoxic and antitumor effects of the norepinephrine analogue meta-iodobenzylguanidine (MIBG). *Cancer Chemother. Pharmacol.* 21, 9–13.
- Smets, L. A., Loesberg, C., Janssen, M., Metwally, E. A., and Huiskamp, R. (1989) Active uptake and extravesicular storage of *m*-iodobenzylguanidine in human neuroblastoma SK-N-SH cells. *Cancer Res.* 49, 2941–2944.
- Smets, L. A., Janssen, M., Metwally, E., and Loesberg, C. (1990) Extracellular storage of the neuron blocking agent meta-iodobenzylguanidine (MIBG) in human neuroblastoma cells. *Biochem. Pharmacol.* 39, 1959–1964.
- Still, C. W., Kahn, M., and Mitra, A. (1978) Rapid chromatographic technique for preparative separations with moderate resolution. *J. Org. Chem.* 43, 2923–2925.
- Vaidyanathan, G., and Zalutsky, M. R. (1993) No-carrier-added synthesis of meta-[¹³¹I]iodobenzylguanidine. *Appl. Radiat. Isot.* 44, 621–628.
- Vaidyanathan, G., Affleck, D. J., and Zalutsky, M. R. (1994) 4-[¹⁸F]Fluoro-3-iodobenzylguanidine: A potential MIBG analogue for positron emission tomography. *J. Med. Chem.* 37, 3655–3662.
- Vaidyanathan, G., Zhao, X.-G., Strickland, D. K., and Zalutsky, M. R. (1997) No-carrier-added 4-fluoro-3-[¹³¹I]iodobenzylguanidine: Evaluation of an MIBG analogue. *J. Nuclear Med.* 38, 330–334.
- Wieland, D. M. (1986) Radiopharmaceutical Design: The adrenal medulla and its diseases. In *Radiopharmaceuticals: Progress and Clinical Perspectives* (A. R. Fritzberg, Ed.) Vol. 1, pp 117–153, CRC Press, Inc., Boca Raton, FL.
- Yong, Y. F., Kowalski, J. A., and Lipton, M. A. (1997) Facile and efficient guanylation of amines using thioureas and Mukaiyama's reagent. *J. Org. Chem.* 62, 1540–1542.

BC980037X

Protein and Peptide Labeling with (Cyclopentadienyl)tricarbonyl Rhenium and Technetium

Todd W. Spradau and John A. Katzenellenbogen*

Department of Chemistry, University of Illinois, 461 Roger Adams Laboratory, Box 37-5, 600 South Mathews Avenue, Urbana, Illinois 61801. Received April 13, 1998; Revised Manuscript Received July 24, 1998

A method for labeling proteins and peptides with (methoxycarbonyl cyclopentadienyl)tricarbonyl rhenium and technetium is described. The precursors used for this labeling are conveniently produced from perrhenate and pertechnetate, respectively, using a double ligand transfer reaction. For labeling the lysine residues of the model protein bovine serum albumin, the technetium methyl ester was saponified and then transformed into its N-hydroxysuccinimidyl ester. For the labeling of the model peptides leucine enkephalin, substance P, oxytocin, and the tumor imaging/therapy candidate octreotide, the rhenium methyl ester was saponified and activated using either 1-hydroxybenzotriazole or 1-hydroxy-7-azabenzotriazole. The activation and peptide-coupling reactions were followed using reversed-phase (C_{18}) HPLC and yields averaged approximately 70%.

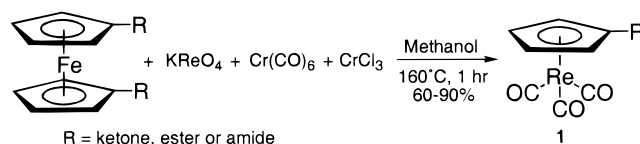
INTRODUCTION

We recently reported a novel double ligand transfer (DLT) reaction that permits the rapid synthesis of substituted (cyclopentadienyl)tricarbonylrhenium (CpTR) complex **1** from $KReO_4$ (Scheme 1) (1). The chemical stability and the ease of Cp-ring derivatization of these compounds make them attractive candidates for the preparation of technetium and rhenium-based radiopharmaceuticals, agents that are widely used in diagnostic imaging (^{99m}Tc) and radiotherapy (^{186}Re , ^{188}Re) (2–5). Complexes derived from **1** have not as yet been exploited for such applications, however, because they are difficult to prepare from ReO_4^- , the only available chemical form of ^{186}Re and ^{188}Re (6–8). ^{99m}Tc is also available only as $^{99m}TcO_4^-$, and as a result, most rhenium and technetium radiopharmaceuticals are inorganic complexes with the metals in a +5 oxidation state. If the scope of the DLT reaction could be extended to include the synthesis of the technetium analogues of the rhenium complex **1** [i.e., (cyclopentadienyl)tricarbonyl technetium (CpTT) complexes], a variety of new organometallic radiopharmaceuticals could be developed.

One simple method which has been widely used for the creation of new radiopharmaceuticals involves the attachment of the radioisotope to a protein (antibody) or peptide that is known to localize in a desired region of the body (9, 10). The resulting adducts can then be used for imaging and/or therapy of that region if their biodistribution characteristics remain favorable. The goal of this study was to apply the DLT reaction to prepare precursors suitable for attaching CpTR and CpTT complexes to proteins and peptides.

The many procedures that have been developed for incorporating technetium and rhenium into proteins and peptides can be grouped into three categories: (a) direct labeling, (b) indirect labeling, and (c) the preformed chelate approach. Each method has been used successfully in a variety of applications, but each has drawbacks and limitations (11).

Scheme 1. Double Ligand Transfer (DLT) Reaction



In the direct-labeling approach, which is the simplest of the three, the sulfhydryl groups of the protein itself are exploited as chelation sites for the metal, and labeling is accomplished simply by incubation of the protein with ReO_4^- or TcO_4^- and a reducing agent (usually stannous ion) or by transchelation of the prereduced metal onto the protein from a donor ligand (12). The stability and incorporation level of the label can sometimes be enhanced by increasing the number of free thiols of the native protein by reducing disulfide bonds or by adding exogenous sulfhydryl groups by prior derivatization of the protein (13). Such procedures, however, can alter the structure and, consequently, the biological properties of the protein or peptide (14). Furthermore, the direct-labeling approach is often plagued by ambiguities regarding the sites of attachment and oxidation states of the bound metals, as well as by instability of the label (13–17).

Indirect labeling involves the attachment of strong chelators to the protein or peptide prior to direct labeling. A wide variety of tightly binding chelates have also been attached to proteins and peptides and labeled successfully [e.g., hydrazino nicotinamide (HYNIC) (18, 19), diaminodithiol (DADT) (20), bis(aminoethanethiol) (BAT) (21), and short peptide sequences (22–24)]. However, even though these strategies often result in increased complexation yields and label stability, the shortcomings of the direct-labeling strategies (site of attachment and oxidation state ambiguity, label instability) still remain.

The preformed chelate approach seeks to eliminate the drawbacks associated with direct and indirect labeling. By this approach, the radiometal is first securely bound inside a chelate system, and then, in a subsequent step, the derivatized metal is firmly attached to the protein or peptide. The resulting product possesses a label that

* Author to whom correspondence should be addressed. Phone: (217) 333-6310. Fax: (217) 333-7325. E-mail: jkatzene@uiuc.edu.

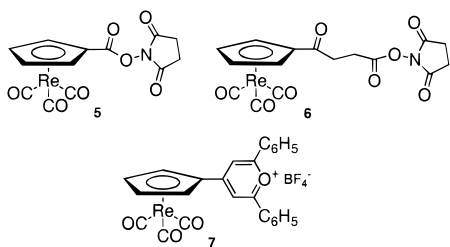
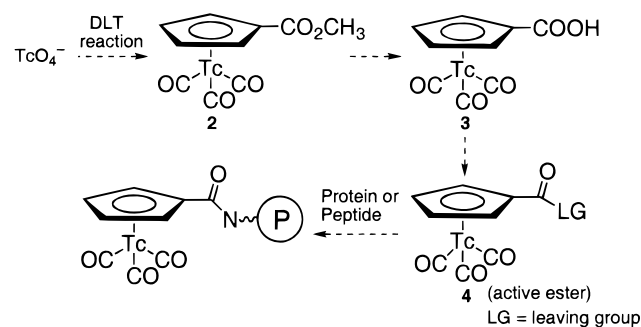


Figure 1. Reagents used to couple CpTR to proteins.

Scheme 2. General Strategy for Protein or Peptide Labeling with the Cyclopentadienyl Tricarbonyl Technetium (CpTT) Unit



is chemically well-defined and stable, but this approach comes at the cost of additional synthetic steps. Some "bifunctional" chelates that have been successfully employed in this strategy include tetradentate N_2S_2 (25, 26) and N_3S (27, 28) system, and boronic acid adducts of technetium dioximes (BATOs) (29).

The labeling strategy we have pursued is outlined in Scheme 2. The key step is the formation of methyl ester **2** from pertechnetate via the double ligand transfer (DLT) reaction. The subsequent steps involve simple modifications of the Cp ring side chain to make it suitable for coupling to the amino groups of proteins and peptides. Thus, this method can be classified as a preformed chelate approach, with active ester **4** being an organometallic analogue of the inorganic bifunctional chelates mentioned above.

Most proteins and peptides labeled with technetium or rhenium contain *inorganic* derivatives of the metals, but a few examples of *organometallic* variations exist. Alberto and co-workers reported the conjugation of the $Re(CO)_3$ fragment to proteins in a direct-labeling approach using $[Re(OH_2)_3(CO)_3]^+$ as the donor molecule (30), and, in an investigation similar to our own, Jaouen and co-workers described the coupling of CpTR to proteins using a preformed chelate approach with the active esters **5** and **6** (Figure 1) (31). In a subsequent study, pyridium salt **7** was used as well (32). Our study expands upon this earlier work.

RESULTS

Protein Labeling. The first step in the execution of our approach was to demonstrate that the DLT reaction could be extended from perrhenate to pertechnetate. Table 1 shows that the transformation did in fact work just as well with technetium as it did with rhenium. Notably, the desired methyl ester **2** could be prepared in very high yield.

As shown in Scheme 3, the methyl ester of **2** could be rapidly and quantitatively saponified in a mixture of 1:1 NaOH (2M):dioxane to provide carboxylic acid **3**. When cosolvents other than dioxane (e.g., THF, acetonitrile, and methanol) were tried, longer times (15–120 min) were

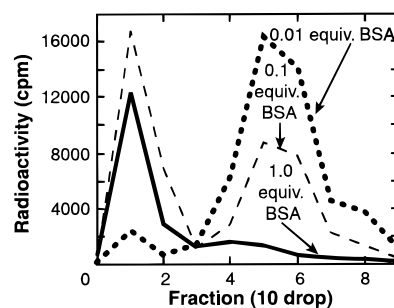
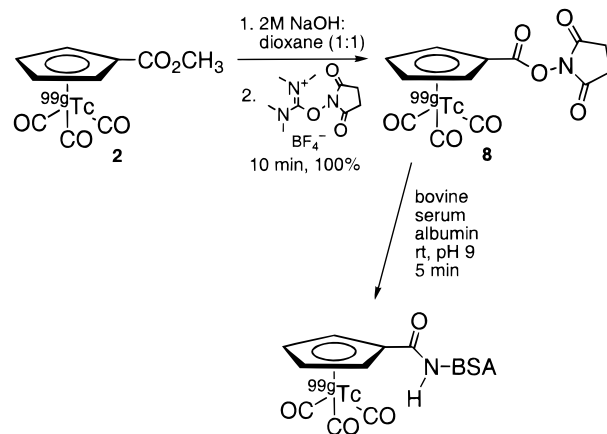


Figure 2. Incorporation of $[^{99m}Tc]CpTT$ into bovine serum albumin (BSA). Sephadex G-25 size exclusion chromatographic analysis of the labeling process. $[^{99m}Tc]CpTT$ -labeled BSA elutes in the void volume (fraction 1). Unreacted active ester **8** or hydrolysis product **3** elutes in fractions 4–8. Further details are given in the Experimental Section.

Table 1. Extension of the Double Ligand Transfer (DLT) Reaction from Rhenium to $[^{99m}Tc]$ Technetium

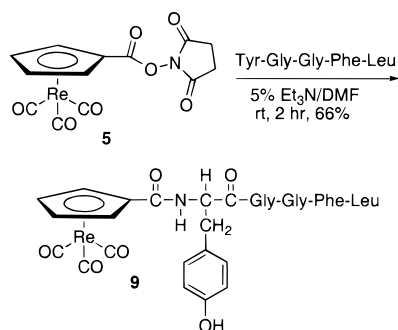
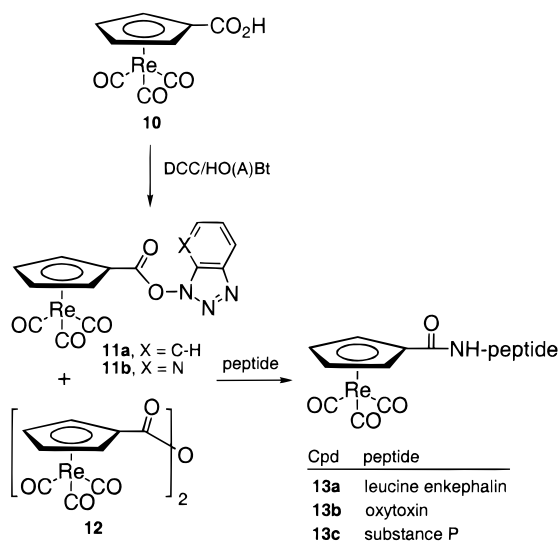
R	R'	% yield
$COCH_3$	H	73
$COCH_3$	$COCH_3$	81
CO_2CH_3	CO_2CH_3	89

Scheme 3. Demonstration of Protein Labeling with $[^{99m}Tc]CpTT$, Using Bovine Serum Albumin



required for the reaction to go to completion. The acid was then treated with a uronium salt described by Knorr and co-workers to provide the N-hydroxysuccinimide (NHS) ester **8** (33). We chose N-hydroxysuccinimide as the activating group for our protein coupling experiments because of the stability of the resultant ester in aqueous solution and its high reactivity toward the ϵ -amino groups of lysine residues (34).

Coupling experiments were performed with the model protein bovine serum albumin (BSA), which contains 59 lysine residues and one N-terminal amino group for a total of 60 possible covalent attachment sites. The reactions were found to be rapid (complete in less than 5 min), with yields that varied depending on the relative amount of BSA to active ester **8** that was used. Figure 2 is a graphical representation of the elution profiles obtained upon size-exclusion column chromatographic separation (Sephadex G-25) of the products from three reactions with varying ratios of BSA to **8**. The void volume of the column, which contained the labeled and unlabeled proteins, comprised the first 20–25 drops

Scheme 4. Labeling of Leucine Enkephalin with CpTT Using an NHS Ester**Scheme 5. Peptide Labeling with CpTT Using Esters Activated by HOBt or HABt**

collected from the 5 cm column, while the small molecules included within the pores of the gel [i.e., unreacted **8** and **3** (from ester hydrolysis)], began to elute at approximately 40–45 drops. As the amount of BSA was increased from 0.01 to 1.0 equiv of active ester (**8**), the incorporation yields increased from 5% to greater than 70%. Since radiolabeling experiments using ^{99m}Tc, ¹⁸⁶Re, and ¹⁸⁸Re are typically done in the presence of a large excess of protein, the potential for the tracer-level labeling of proteins by this approach appears to be great.

Peptide Labeling. The strategy outlined in Scheme 2 was also adopted for the labeling of selected peptides, with two changes from the protein-labeling protocol. First, rhenium was used instead of technetium, and second, activating groups other than NHS were employed. Scheme 4 shows that the conjugation of the CpTR NHS ester **5** to the model peptide leucine enkephalin was much slower than the corresponding reaction of the corresponding CpTT NHS ester **8** with BSA, most likely because of a relative increase in steric hindrance near the N-terminal amino group of the peptide compared to that of the numerous and more accessible ϵ -amino groups of the lysine residues of the protein.

Reaction sequences with short-lived radioisotopes need to be as rapid as possible to maximize the specific activities of the final products. For this reason, we investigated alternative activating agents, bases, and solvents for the coupling reactions of rhenium carboxylate **10** (Scheme 5) with the model peptides leucine enkephalin (Tyr-Gly-Gly-Phe-Leu), substance P (Arg-Pro-Lys-Pro-

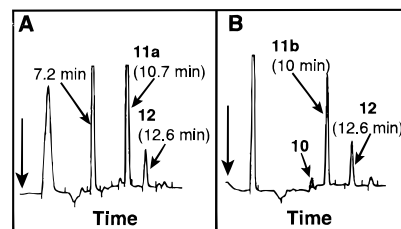


Figure 3. HPLC traces of the reaction of carboxylic acid **10** with HOBt (panel A) and HABt (panel B). The structures of compounds **10**, **11a**, **11b**, and **12** are given in Scheme 5. The vertical arrow indicates the time of injection. HPLC conditions are given in the Experimental Section.

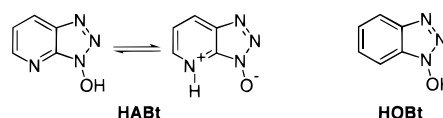


Figure 4. Structures of HABt and HOBt.

Gln-Gln-Phe-Phe-Gly-Leu-Met-NH₂), and oxytocin (Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH₂, disulfide bridge: 1–6). Leucine enkephalin was included because of its simple structure and affordability, substance P because it possesses a lysine residue and thus two potential sites for labeling, and oxytocin because of its intramolecular disulfide bond. The disulfide bond was important to us because the fourth peptide we chose for this study, octreotide, also contains a cystine bridge and is, moreover, an attractive candidate for radiolabeling with Tc-99m (35, 36).

Leucine Enkephalin, Oxytocin, and Substance P.

We found that the related reagents 1-hydroxybenzotriazole (HOBt) and 1-hydroxy-7-azabenzotriazole (HABt) could both be used to provide intermediates that were capable of coupling with the model peptides (Scheme 5). The activation/coupling sequences were complete in less than 20 min for each of the peptides studied, and both steps could be followed using reversed-phase (C₁₈) HPLC.

Interestingly, the reaction of acid **10** with HOBt in the presence of DCC produced three distinct intermediates that were visible by HPLC analysis, each of which disappeared upon addition of peptide, whereas DCC/HABt produced two intermediates (Figure 3). The product that was produced by both reagents, which eluted at 12.6 min under these conditions (Figure 3), was tentatively assigned structure **12**, a reactive anhydride dimer of the starting carboxylic acid **10**. The compound that eluted at 10 min in the DCC/HABt reaction was most likely the HABt adduct **11b** (panel A), while that at 10.7 min in the DCC/HOBt reaction is probably its slightly more hydrophobic analogue **11a** (panel B). The polar compound at 7.2 min may be a condensation product of acid **10** with DCC (or its urea), and its absence from the HABt reaction can be explained by the higher reactivity (nucleophilicity) of HABt vs HOBt toward this compound, due to its zwitterionic tautomer (Figure 4). The activation reactions were complete within 1 min in CH₂Cl₂, and the active intermediates produced were stable for at least 24 h in that solvent. When the same reactions were performed in DMF, no intermediates could be detected by HPLC, and starting acid **10** was the only peak observed (8.4 min; see a trace of **10** in Figure 3, panel B).

Representative traces of the reactions of the active intermediates (Figure 3) with each of the three model peptides are shown in Figure 5. The peptides themselves eluted at approximately 5.9 min in the solvent gradient

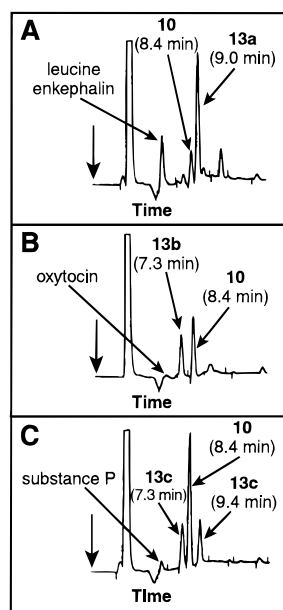


Figure 5. HPLC traces of the reactions of CpTR with leucine enkephalin (panel A), oxytocin (panel B), and substance P (panel C). The structures of compounds **10** and **13a–c** are shown in Scheme 5. The vertical arrow indicates the time of injection. HPLC conditions are given in the Experimental Section.

used, and varying amounts of the unreacted peptides can be seen in each trace. Yields averaged 70% for each reaction, based upon integration of the peaks corresponding to the coupled products **13a**, **13b**, and **13c** (Scheme 5), which appeared at 9.0 and 7.3 min in the leucine enkephalin and oxytocin reactions, respectively (Figure 5, panels A and B), and at 7.8 and 9.4 min for the two conjugates obtained in the reaction with substance P (Figure 5, panel C). The CpTR derivative of leucine enkephalin (**13a**) was isolated and characterized by ^1H NMR and FAB mass spectrometry.

A peak at 8.4 min corresponding to starting acid **10** was a prominent feature in all of the HPLC traces (Figure 5) and indicates that there is a competition between amide bond formation with the peptide and hydrolysis of the activated intermediates in the coupling reactions. Although the intermediates were stable in pure CH_2Cl_2 , their hydrolysis was found to be catalyzed by the base used in the reaction, with the rate of hydrolysis increasing in the order diisopropylethylamine < triethylamine << (dimethylamino)pyridine (DMAP). The yields of the labeled peptides produced in the presence of those bases decreased in the same order, and diisopropylethylamine, the most sterically hindered base that engendered the highest yields, was thereafter used in all of the reactions. Solvents other than methylene chloride, such as acetonitrile and DMF, were unsuitable for the preparation of the active esters; we found that if the active esters were first prepared in CH_2Cl_2 , then DMF could subsequently be added to form a mixed solvent system in which the active intermediates were moderately stable. The addition of DMF was important for the coupling reactions with all of the peptides except leucine enkephalin, because while the latter reacted rapidly in CH_2Cl_2 , the others were insoluble, and therefore unreactive, in that solvent.

Octreotide Labeling. Octreotide (**13a**, Figure 6) interested us because of its known affinity for a wide range of somatostatin receptor-containing neuroendocrine tumors, including those of the lung, pancreas, pituitary, breast, and prostate (37). If labeled with

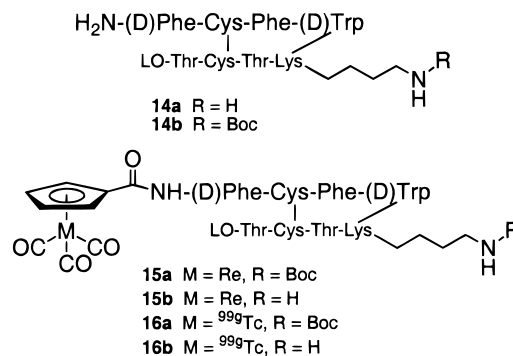


Figure 6. Structures of unprotected and Boc protected octreotide (**14a** and **14b**) and their CpTR and CpTT labeled derivatives **15a, b** and **16a, b**.

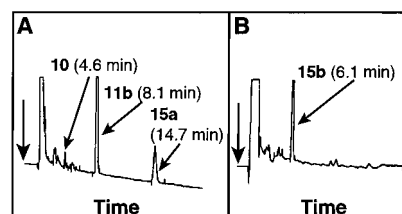


Figure 7. HPLC traces of reaction for the formation of Boc-protected CpTR-labeled octreotide **15a** (panel A) and from CpTR-labeled octreotide **15b** (panel B). The structures of compounds **10**, **11b**, **15a**, and **15b** are shown in Scheme 5 and Figure 6. The vertical arrow indicates the time of injection. HPLC conditions are given in the Experimental Section.

Tc-99m or Re-186/Re-188, it could potentially be used to detect and/or treat cancer in those tissues. Furthermore, it is an exceptionally challenging target for Tc/Re labeling, since its essential disulfide linkage is sensitive to the reducing conditions generally employed in labeling with those metals (38).

Like oxytocin and substance P, octreotide was insoluble in CH_2Cl_2 , and DMF was therefore required to promote its conjugation reactions. These reactions were typically performed by adding a methylene chloride solution of the freshly prepared active ester **11b** to a vial containing solid, Boc-protected octreotide (**14b**). After removal of the CH_2Cl_2 under a nitrogen stream, DMF and diisopropylethylamine were added to initiate the coupling. Again, all attempts to do this sequence from start to finish in DMF were unsuccessful.

Figure 7 shows representative reversed-phase (C_{18}) HPLC traces for the formation of the CpTR-N-terminally labeled Boc-octreotide **15a** (panel A) and its deprotection to CpTR-octreotide **15b** (panel B). Starting acid **10** and its active ester **11b** were visible at 4.6 and 8.1 min, respectively, in this gradient (panel A), while the Boc-octreotide (**14b**), which eluted at 6.2 min, had already been completely consumed. The coupled product (**15a**, 14.7 min) was isolated and characterized by its MALDI and FAB mass spectra, and its yield typically ranged 70–80%. The final, deprotected product (**15b**, 6.1 min) (panel B) was generated in 95% trifluoroacetic acid, and it also was isolated and identified by its MALDI, FAB (Figure 8), and high-resolution FAB mass spectra.

The same reaction sequence was finally repeated with technetium-99g-labeled acid **3** to provide the corresponding Boc-protected (**16a**) and deprotected (**16b**) [$^{99\text{Tc}}$]-CpTT-octreotide conjugates, which also were characterized by their FAB mass spectra. To our knowledge, these syntheses represent the first successful preparations of Re and Tc-99g-labeled octreotide, although Tc-99m-labeled octreotide derivatives have been reported (38–

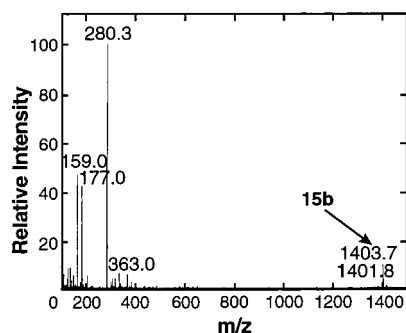


Figure 8. FAB mass spectrum of the CpTR-octreotide conjugate **15b**. The peaks at m/z 1401.8 and 1403.7 correspond to the $(M + Na)^+$ ion with ^{185}Re and ^{187}Re , respectively.

40). The *in vivo* properties of those compounds were not ideal, however. The results of our own Tc-99m labeling of octreotide using the chemistry described in this report and the tissue distribution of this radiopharmaceutical will be reported elsewhere (41).

CONCLUSION

A double ligand transfer reaction that was previously developed for the rapid synthesis of substituted (cyclopentadienyl)tricarbonylrhenium complexes from KReO_4 was found to be effective for the preparation of the corresponding technetium compounds from $\text{NH}_4^{99g}\text{TcO}_4$ as well. One of the products of this reaction, namely the methyl ester substituted derivative **2**, was found to be a useful precursor for the ^{99g}Tc labeling of protein lysine residues. The protocol included transformation of methyl ester **2** into NHS ester **8**, and then treatment with the model protein bovine serum albumin in pH 9 buffer. These reactions were complete in less than 5 min, and incorporation yields varied depending upon the relative amounts of BSA to active ester used, but exceeded 70% in the reactions performed with 1 equiv of each coupling partner.

In like manner, the model peptides leucine enkephalin, substance P, and oxytocin were labeled using the HOBt and/or HABt active ester derivatives of rhenium acid **10**. The activation and peptide-coupling reactions were followed using reversed-phase (C_{18}) HPLC, and it was found that although DMF was required as a solvent in the couplings of two of the three peptides, it was an unsuitable medium for the activation step, which worked best in CH_2Cl_2 .

Finally, the octapeptide octreotide, which is an attractive candidate for radiolabeling with ^{99m}Tc , ^{186}Re , and/or ^{188}Re , was labeled for the first time with nonradioactive rhenium and Tc-99g, using the reaction protocol optimized for the model peptides. It is hoped that the chemistry described in this paper will prove useful for the preparation of a variety of ^{99m}Tc , ^{186}Re , and ^{188}Re -labeled proteins and peptides, including octreotide.

EXPERIMENTAL SECTION

General Comments. The following reagents were purchased from the commercial sources indicated and used as received: acetylferrocene, 1,1'-diacetylferrocene, *O*-(*N*-succinimidyl)-*N,N,N',N'*-tetramethyluronium tetrafluoroborate, 1-hydroxybenzotriazole, 1-hydroxy-7-azabenzotriazole, dicyclohexylcarbodiimide, pH 9 buffer (Hydriion), from Aldrich; chromium hexacarbonyl from Alfa; chromium(III) chloride from Strem; ammonium pertechnetate from Alan Davison, MIT; and bovine serum albumin, leucine enkephalin, substance P, oxytocin, and

Sephadex G-25 superfine gel from Sigma. Diisopropylethylamine and dimethylformamide were purchased from Aldrich and distilled from CaH_2 and MgSO_4 , respectively. Boc-protected octreotide was provided by M. J. Welch, Washington University. 1,1'-Bis(carbomethoxy)ferrocene was prepared as described elsewhere (1).

General Procedure for Double Ligand Transfer

Reactions. Cautionary Note. Pressure tubes were placed within a specially made solid aluminum block containing holes drilled deep enough to admit the tubes to about three-fourths of their height and wide enough to allow room for the addition of some mineral oil to ensure good thermal contact. The tubes and aluminum base were covered with a matching hollow aluminum screw cap, equipped with a small hole aligned with one drilled in the base (to the same depth as those for the pressure tubes) to hold a metal thermometer. This containment device minimized the potential danger of explosions during heating, allowed for the efficient stirring of the reactions, and enabled the reaction temperature to be monitored readily. All manipulation of sealed reaction tubes during and after the reactions were done with suitable protective equipment: blast shield, full-face visor, heavy gloves. Details of the procedure are described elsewhere (1). Briefly, $\text{NH}_4^{99g}\text{TcO}_4$ (0.0106 g, 0.0586 mmol), 1,1'-bis(methoxycarbonyl)ferrocene (0.1034 g, 0.342 mmol), $\text{Cr}(\text{CO})_6$ (0.0923 g, 0.419 mmol), and CrCl_3 (0.0249 g, 0.157 mmol) were combined in a 4 mL pressure tube containing a magnetic stir bar. Dry methanol (1 mL) was added, and the tube was sealed, heated to 165 °C over the course of an hour, and then cooled to -78 °C. The reaction mixture was transferred to a 15 mL disposable vial with CH_2Cl_2 and the solvent was evaporated under a stream of N_2 at 30 °C. The mixture was redissolved in a minimum of CH_2Cl_2 and purified by flash column chromatography (1:1 hexane:benzene) to give 0.0159 g (89%) of tricarbonyl(methoxycarbonylcyclopentadienyl)technetium (**2**). ^1H NMR (200 MHz, CDCl_3) δ ppm: 5.96 [t, J = 2.38 Hz, 2H, -CO-Cp- $\text{H}_2(\alpha)$], 5.27 [t, J = 2.16 Hz, 2H, -CO-Cp- $\text{H}_2(\beta)$], 3.79 (s, 3H, CpCOOCH_3). MS (EI): m/z 306 (M^+). Anal. Calcd for $\text{C}_{10}\text{H}_7\text{O}_5\text{Tc}$: C, 39.23; H, 2.30. Found: C, 39.35; H, 2.27.

Tricarbonyl(acetylcyclopentadienyl)technetium. The reactions were performed according to the general procedure and purified by flash column chromatography (4:1 hexane:ethyl acetate) to give 0.040 g (81%) of tricarbonyl(acetylcyclopentadienyl)technetium from diacetylferrocene, and 0.033 g (73%) from acetylferrocene. ^1H NMR (500 MHz, CDCl_3) δ ppm: 5.94 [t, J = 2.31 Hz, 2H, -CO-Cp- $\text{H}_2(\alpha)$], 5.31 [t, J = 2.30 Hz, 2H, -CO-Cp- $\text{H}_2(\beta)$], 2.33 (s, 3H, CpCOCH_3). ^{13}C NMR (125 MHz, CDCl_3) δ ppm: 193.36 (COCH_3), 90.50, 87.12 (C_5H_4), 90.31 (C ipso), 29.68 ($\text{Tc}(\text{CO})_3$), 26.53 (COCH_3). MS (EI): m/z 290 (M^+).

General Procedure for the Labeling of Bovine Serum Albumin

Serum Albumin. To a stirred solution of tricarbonyl(methoxycarbonylcyclopentadienyl)technetium (**2**) (1.4 mg, 0.0046 mmol) in dioxane (0.5 mL) was added an aqueous solution of NaOH (2 M, 0.5 mL). After 5 min, concentrated HCl was added dropwise until the reaction was acidic to litmus paper. The solvent was evaporated at 40 °C under a gentle stream of nitrogen, and the solid residue was redissolved in DMF (400 μL). Triethylamine (2 μL , 0.014 mmol) and *O*-(*N*-succinimidyl)-*N,N,N',N'*-tetramethyluronium tetrafluoroborate (5 mg, 0.017 mmol) were added, and the solution was stirred for 5 min. The reaction mixture, containing NHS active ester **8**, was then added to a solution of bovine serum albumin (38

mg, 0.000 55 mmol) in pH 9 buffer (6 mL), and the reaction vial was further rinsed with DMF (200 μ L) and pH 9 buffer (1.4 mL) to provide a final solution of 7.5% DMF in the pH 9 buffer. After stirring for 10 min, 10 μ L of the reaction mixture was purified by passing it through 5 cm of Sephadex G-25 Superfine gel in a disposable Pasteur pipet and eluting with pH 9 buffer. Sixty drops of eluent was collected in 10-drop fractions, with the first two fractions (20 drops) roughly corresponding to the column void volume as determined by the elution profile of Blue Dextran. The amount of radioactivity in each fraction was determined by scintillation counting of the fractions in solutions of Aquasol (12 mL) scintillation fluid. An incorporation yield of at least 50% was determined by comparing the total activity in all fractions to the activity in the first three fractions.

Reactions performed according to the above procedure showed an incorporation yield of 5% from 1.34 mg (0.004 38 mmol) tricarbonyl(methoxycarbonylcyclopentadienyl)technetium and 3.81 mg (0.0552 μ mol) BSA and at least 70% from 1.16 mg (0.0038 mmol) tricarbonyl(methoxycarbonyl cyclopentadienyl)technetium and 309 mg (0.00447 mmol) BSA. A graphical representation of the elution profiles is shown in Figure 2.

Analytical Methods. Reversed-phase (C_{18}) HPLC analyses were performed using a Vydac C_{18} column (4.6 mm $\times$ 25 cm) at a flow rate of 1.5 mL/min. Gradients were performed with mobile phases A (0.1% TFA in H_2O) and B [0.1% TFA in acetonitrile/0.1% TFA in H_2O (10:1)] under conditions described in the individual reactions below. UV detection was performed at either 254 or 280 nm.

General Peptide Labeling Procedure. To a stirred solution of tricarbonyl(methoxycarbonyl cyclopentadienyl)rhenium (0.25 mg, 0.06 μ mol) in dioxane (0.5 mL) was added an aqueous solution of NaOH (2 M, 0.5 mL). After 5 min, concentrated HCl was added dropwise until the reaction was acidic to litmus paper. The solvent was evaporated at 40 $^{\circ}$ C under a gentle stream of nitrogen, and the solid residue was redissolved in CH_2Cl_2 (300 μ L). 1,3-Dicyclohexylcarbodiimide (0.5 mg, 2.4 μ mol) and 1-hydroxybenzotriazole (0.9 mg, 6.7 μ mol) were added, and the solution was stirred for 5 min. Reversed-phase (C_{18}) HPLC analyses were performed using the following gradient: 20% B for 1 min, 20% B to 30% B in 1 min, 30% B for 1 min, 30% B to 50% B in 2 min, and 50% B to 60% B in 10 min, which showed that the activation step to form the HOBt ester **11a** was complete [see Figure 3 (panel A)]. Leucine enkephalin (0.7 mg, 1.1 μ mol) and diisopropylethylamine (1 μ L) were added, and the reaction was stirred for 20 min, at which time RP-HPLC indicated the formation of CpTR/leucine enkephalin coupled product (rt = 9.0 min) in a yield of 70% [Figure 5 (panel A)]. The peak at 9.0 min, corresponding to the CpTR-labeled leucine enkephalin **13a**, was collected and concentrated in vacuo. Comparison of the ^1H NMR (300 MHz, $CDCl_3$) of the isolated product with leucine enkephalin revealed the following new peaks (δ ppm): 6.26 and 6.11 [d, J = 1.58 Hz, 2H, $-CO-Cp-H_2(\alpha)$], 5.54 and 5.51 [d, J = 1.95 Hz, 2H, $-CO-Cp-H_2(\beta)$]. MS (FAB): m/z 940 ($M + Na$) $^+$, 962 ($M + 2Na$) $^+$.

CpTR Labeling of Oxytocin. The reaction was performed according to the general procedure, with two changes: (a) 1-hydroxy-7-azabenzotriazole (1 mg, 7.4 μ mol) was used instead of 1-hydroxybenzotriazole (HOBt) (see Figure 3, Panel B for an HPLC trace of the HABT active ester **11b**), and (b) DMF (50 μ L) was added to the activated ester at the same time as the diisopropylethylamine (1 μ L) and oxytocin (0.5 mL, 0.47 μ mol). The

reaction was analyzed by RP-HPLC with the gradient of the general procedure, which revealed a new peak attributed to N-terminally CpTR-labeled oxytocin **13b** [70%, Figure 5 (panel B)] from tricarbonyl(methoxycarbonylcyclopentadienyl)rhenium (0.25 mg, 0.66 μ mol).

CpTR Labeling of Substance P. The reaction was performed according to the general procedure, with one change: DMF (50 μ L) was added to the activated ester at the same time as the diisopropylethylamine (1 μ L) and substance P (0.6 mg, 0.4 μ mol). The reaction was analyzed by RP-HPLC using the gradient of the general procedure, which revealed two new peaks of equal intensity attributed to N-terminally and lysine CpTR-labeled substance P **13c** [75%, Figure 5 (panel C)] from tricarbonyl(methoxycarbonylcyclopentadienyl)rhenium (0.25 mg, 0.66 μ mol).

CpTR Labeling of Boc-Octreotide. The reaction was performed according to the general procedure, with the following changes: (a) 1-hydroxy-7-azabenzotriazole (1 mg, 7.4 μ mol) was used instead of 1-hydroxybenzotriazole (HOBt), and (b) a methylene chloride solution of the newly formed active ester **11b** was added to a vial containing solid Boc-octreotide **14b** (150 μ g, 0.1 μ mol), and the solvent was evaporated at 40 $^{\circ}$ C under a gentle stream of nitrogen. DMF (100 μ L) and diisopropylethylamine (1 μ L) were added, and the reaction was stirred for 20 min. The reaction was analyzed by RP-HPLC using a gradient of 50% B to 70% B over 20 min. A new peak attributed to N-terminally CpTR-labeled Boc-octreotide **15a** [80%, Figure 7 (panel A)] was obtained from the original tricarbonyl(methoxycarbonylcyclopentadienyl)rhenium (0.125 mg, 0.33 μ mol). The peak was collected and concentrated in vacuo. MS (MALDI): m/z 1503 ($M + Na$) $^+$.

Deprotection of CpTR-Boc-Octreotide. CpTR-Boc-octreotide **15a** ($\leq$ 150 μ g, 0.1 μ mol) was dissolved in a 95% solution of trifluoroacetic acid in H_2O (100 μ L) and stirred for 2 min. The reaction was then quenched with water (100 μ L) and analyzed by RP-HPLC, again using a gradient of 50% B to 70% B in 20 min. A new peak attributed to N-terminally CpTR-labeled octreotide **15b** [90%, Figure 7 (panel B)] was collected and concentrated in vacuo. HR-FAB-MS m/z calcd for $C_{58}H_{69}N_{10}O_{14}S_2NaRe$: 1403.389151. Found: 1403.391400 [Δ -1.6 ppm, ($M + Na$) $^+$].

CpTT Labeling of Boc-Octreotide. The reaction was performed according to the general procedure, with the following changes: (a) tricarbonyl(methoxycarbonylcyclopentadienyl)technetium (15 μ g, 0.05 μ mol) was used instead of tricarbonyl(methoxycarbonylcyclopentadienyl)rhenium as the starting material; (b) 1-hydroxy-7-azabenzotriazole (0.1 mg, 0.7 μ mol) was used instead of 1-hydroxybenzotriazole (HOBt); and (c) a methylene chloride solution of the newly formed active ester **11b** was added to a vial containing solid Boc-octreotide **14b** (50 μ g, 0.03 μ mol), and the solvent was evaporated at 40 $^{\circ}$ C under a gentle stream of nitrogen. DMF (100 μ L) and diisopropylethylamine (1 μ L) were added, and the reaction was stirred for 30 min. The reaction was analyzed by RP-HPLC using a gradient of 45% B to 65% B in 20 min. A new peak attributed to N-terminally CpTT-labeled Boc-octreotide **16a** (80%, 14.6 min) was collected and concentrated in vacuo. MS (FAB): m/z 1414 ($M + Na$) $^+$, 1431 ($M + K$) $^+$.

Deprotection of CpTT-Boc-Octreotide. CpTT-Boc-octreotide **16a** ($\leq$ 50 μ g, 0.03 μ mol) was dissolved in a 95% solution of trifluoroacetic acid in H_2O (100 μ L) and stirred for 2 min. The reaction was then quenched with water (100 μ L) and analyzed by RP-HPLC, again using

a gradient of 45% B to 65% B in 20 min. A new peak attributed to N-terminally CpTT-labeled octreotide **16b** (90%, 10.0 min) was collected and concentrated in vacuo. MS (MALDI): m/z 1294 ($M + H$)⁺.

ACKNOWLEDGMENT

We are grateful for the support of this research through grants from the National Institutes of Health (PHS 5R01 CA25836) and the Department of Energy (DE FG02 86ER60401). We are grateful to A. Davison and M. J. Welch for providing us with research materials and to C. J. Anderson and M. Lanahan for providing Boc-octreotide.

LITERATURE CITED

- (1) Spradau, T. W., and Katzenellenbogen, J. A. (1998) Preparation of cyclopentadienyltricarbonylrhenium complexes using a double ligand transfer reaction. *Organometallics* (in press).
- (2) Schwochau, K. (1994) Technetium Radiopharmaceuticals—Fundamentals, Synthesis, Structure, and Development. *Angew. Chem., Int. Ed. Engl.* 33, 2258–2267.
- (3) Lisic, E. C., Mirzadeh, S., and Knapp, F. F., Jr. (1993) Synthesis of carrier-free rhenium-188(V)DMSA using triphenyl phosphine as a facile reducing agent. *J. Labeled Compd. Radiopharm.* 33, 65–75.
- (4) John, E., Thakur, M. L., DeFulvio, J., McDevitt, M. R., and Damjanov, I. (1993) Rhenium-186-Labeled Monoclonal Antibodies for Radioimmunotherapy: Preparation and Evaluation. *J. Nucl. Med.* 34, 260–267.
- (5) Goldenberg, D. M., and Griffiths, G. L. (1992) Radioimmunotherapy of Cancer: Arming the Missiles. *J. Nucl. Med.* 33, 1110–1112.
- (6) Crocker, L. S., Gould, G. L., and Heinekey, D. M. (1988) Improved synthesis of carbonylrhenium. *J. Organomet. Chem.* 342, 243–244.
- (7) Calderazzo, F., Mazzi, U., Pampaloni, G., Poli, R., Tisato, F., and Zanazzi, P. F. (1989) Reduction of Ammonium Pertechnetate and Ammonium Perrhenate with CO: Synthesis of $M_2(CO)_{10}$ ($M = \text{TC, RE}$) and crystal and molecular structure of the trinuclear cyano-bridged derivative $\text{Re}_3(\text{CN})_3(\text{CO})_{12}$. *Gazz. Chim. Ital.* 119, 241–247.
- (8) Knight Castro, H. H., Meetsma, A., Teuben, J. H., Vaalburg, W., Panek, K., and Ensing, G. (1991) Synthesis, reactions and structure of $\text{Cp}^*\text{Tc}(\text{CO})_3$ derivatives. *J. Organomet. Chem.* 410, 63–71.
- (9) Griffiths, G. L., Goldenberg, D. M., Jones, A. L., and Hansen, H. J. (1992) Radiolabeling of Monoclonal Antibodies and fragments with technetium and rhenium. *Bioconjugate Chem.* 3, 91–99.
- (10) Eckelman, W. C. (1995) Radiolabeling with technetium-99m to study high-capacity and low-capacity biochemical systems. *Eur. J. Nucl. Med.* 22, 249–263.
- (11) Liu, S., Edwards, D. S., and Barrett, J. A. (1997) ^{99m}Tc labeling of highly potent small peptides. *Bioconjugate Chem.* 8, 621–636.
- (12) Rhodes, B. A. (1991) Direct labeling of proteins with Tc-99m. *Nucl. Med. Biol.* 18, 667–676.
- (13) Eckelman, W. C., and Steigman, J. (1991) Direct labeling with Tc-99m. *Nucl. Med. Biol.* 18, 3–7.
- (14) Hnatowich, D. J., Mardirosian, G., Ruszkowski, M., Fogarasi, M., Virzi, F., and Winnard, J., P. (1993) Directly and indirectly technetium-99m-labeled antibodies—A comparison of in vitro and animal in vivo properties. *J. Nucl. Med.* 34, 109–119.
- (15) Garron, J. Y., Moinereau, M., Pasqualini, R., and Saccavini, J. C. (1991) Direct Tc-99m labeling of monoclonal antibodies: radiolabeling and in vitro stability. *Nucl. Med. Biol.* 18, 695–703.
- (16) Hnatowich, D. J., Virzi, F., Winnard, P., Fogarasi, M., and Ruszkowski, M. (1994) Investigations of ascorbate for direct labeling of antibodies with Tc-99m. *J. Nucl. Med.* 35, 127–134.
- (17) Gooden, C. S. R., Snook, D. E., Maraveyas, A., Rowlinson-Busza, G., Peters, A. M., and Epenetos, A. A. (1995) Direct Tc-99m labeling of three anticancer monoclonal antibodies: stability, pharmacokinetics and imaging. *J. Nucl. Med.* 36, 842–849.
- (18) Larsen, S. K., Solomon, H. F., Caldwell, G., and Abrams, M. J. (1995) Tc-99m Tricine: A useful precursor complex for the radiolabeling of hydrazinonicotinate protein conjugates. *Bioconjugate Chem.* 6, 635–638.
- (19) Liu, S., Edwards, D. S., Looby, R. J., Harris, A. R., Poirier, M. J., Barrett, J. A., Heminway, S. J., and Carroll, T. R. (1996) Labeling a hydrazino nicotinamide-modified cyclic IIb/IIIa receptor antagonist with Tc-99m using aminocarboxylates as coligands. *Bioconjugate Chem.* 7, 63–71.
- (20) Baidoo, K., E., Scheffel, U., and Lever, S. Z. (1990) Tc-99m labeling of proteins: Initial evaluation of a novel diaminedithiol bifunctional chelating agent. *Cancer Res.* 50 (Suppl), 799s–803s.
- (21) Eisenhut, M., Lehmann, W. d., Becker, W., Behr, T., Elser, H., Strittmatter, W., Steinstrasser, A., Baum, R. P., Valerius, T., Repp, R., and Deo, Y. (1996) Bifunctional NHS–BAT ester for antibody conjugation and stable technetium-99m labeling: Conjugation chemistry, immunoreactivity and kit formulation. *J. Nucl. Med.* 37, 362–370.
- (22) Knight, L. C., Radcliffe, R., Maurer, A. H., Rodwell, J. D., and Alvarez, V. L. (1994) Thrombus imaging with Tc-99m synthetic peptides based upon the binding domain of a monoclonal antibody to activated platelets. *J. Nucl. Med.* 35, 282–288.
- (23) Ram, S., and Buchsbaum, D. J. (1994) A peptide-based bifunctional chelating agent for Tc-99m and Re-186 labeling of monoclonal antibodies. *Cancer* 73 (Suppl) 769–773.
- (24) Pearson, D. A., Lister-Jones, J., McBride, W. J., Wilson, D. M., Martel, L. J., Civitello, E. R., Taylor, J. E., Moyer, B. R., and Dean, R. T. (1996) Somatostatin receptor-binding peptides labeled with technetium-99m: Chemistry and initial biological studies. *J. Med. Chem.* 39, 1361–1371.
- (25) Kasina, S., Rao, T. N., Srinivasan, A., Sanderson, J. A., Fitzner, J. N., Reno, J. M., Beaumier, P. L., and Fritzberg, A. R. (1991) Development and biologic evaluation of a kit for preformed chelate Tc-99m radiolabeling of an antibody Fab fragment using a diamide dimercaptate chelating agent. *J. Nucl. Med.* 32, 1445–1451.
- (26) Giblin, M. F., Jurisson, S. S., and Quinn, T. P. (1997) Synthesis and characterization of rhenium-complexed α -melanotropin analogues. *Bioconjugate Chem.* 8, 347–353.
- (27) Liu, S., Edwards, D. S., Looby, R. J., Poirier, M. J., Rajopadhye, M., Bourque, J. P., and Carroll, T. R. (1996) Labeling cyclic glycoprotein IIb/IIIa receptor antagonists with Tc-99m by the preformed chelate approach: Effects of chelators on properties of Tc-99m-chelator-peptide conjugates. *Bioconjugate Chem.* 7, 196–202.
- (28) van Gog, F. B., Visser, G. W. M., Klok, R., van der Schors, R., Snow, G. B., and van Dongen, G. A. M. S. (1996) Monoclonal antibodies labeled with Re-186 using the MAG3 chelate: Relationship between the number of chelated groups and biodistribution characteristics. *J. Nucl. Med.* 37, 352–362.
- (29) Linder, K. E., Wen, M. D., Nowotnik, D. P., Malley, M. F., Gougoutas, J. Z., Nunn, A. D., and Eckelman, W. C. (1991) Technetium labeling of monoclonal antibodies with functionalized BATOs. 1. $\text{TcCl}(\text{DMG})_3\text{PITC}$. *Bioconjugate Chem.* 2, 160–170.
- (30) Alberto, R., Schubiger, P. A., Schibli, R., and Ubram, U. (1995) Organometallic rhenium complexes for the labeling of proteins. *J. Nucl. Med.* 36, 91P.
- (31) Salmain, M., Gunn, M., Gorfti, A., Top, S., and Jaouen, G. (1993) Labeling of proteins by organometallic complexes of rhenium (I). Synthesis and biological activity of the conjugates. *Bioconjugate Chem.* 4, 425–433.
- (32) Salmain, M., Malisza, K. L., Top, S., Jaouen, G., Sénéchal-Tocquer, M.-C., Sénéchal, D., and Caro, B. (1994) [n5-Cyclopentadienyl]metal tricarbonyl pyrylium salts: Novel reagents for the specific conjugation of proteins with transition organometallic labels. *Bioconjugate Chem.* 5, 655–659.

- (33) Knorr, R., Trzeciak, A., Bannwarth, W., and Gillesen, D. (1989) New coupling reagents in peptide chemistry. *Tetrahedron Lett.* 30, 1927–1930.
- (34) Bannwarth, W., Schmidt, D., Stallard, R. L., Hornung, C., Knorr, R., and Müller, F. (1988) Bathophenanthroline-ruthenium(II) complexes as nonradioactive labels for oligonucleotides which can be measured by time-resolved fluorescence techniques. *Helv. Chim. Acta* 71, 2085–2099.
- (35) Reubi, J. C., Lamberts, S. J. W., and Krenning, E. (1995) Receptor imaging of human diseases using radiolabeled peptides. *J. Receptor Signal Transduction Res.* 15, 379–392.
- (36) Kvols, L. K. (1993) Somatostatin-receptor imaging of human malignancies: A new era in the localization, staging, and treatment of tumors. *Gastroenterology* 105, 1909–1911.
- (37) Vallabhajosula, S., Moyer, B. R., Lister-Jones, J., McBride, B. J., Lipszyc, H., Lee, H., Bastidas, D., and Dean, R. T. (1996) Preclinical evaluation of technetium-99m-labeled somatostatin receptor-binding peptides. *J. Nucl. Med.* 37, 1016–1022.
- (38) Maina, T., Stolz, B., Albert, R., Bruns, C., Koch, P., and Mäcke, H. (1994) Synthesis, radiochemistry and biological evaluation of a new somatostatin analogue (SDZ 219–387) labeled with technetium-99m. *Eur. J. Nucl. Med.* 21, 437–444.
- (39) Kolan, H., Li, J., and Thakur, M. L. (1996) Sandostatin labeled with Tc-99m: In vitro stability, in vivo validity and comparison with In-111-DTPA-octreotide. *Pept. Res.* 9, 144–150.
- (40) Zamora, P. O., Stratesteffan, M., Guhlke, S., Sass, K. S., Cardillo, A., Bender, H., and Biersack, H. J. (1996) Radiotracer binding to brain microsomes determined by thin-layer chromatography. *Nucl. Med. Biol.* 23, 61–67.
- (41) Spradau, T. W., Edwards, W. B., Anderson, C. J., Welch, M. J., and Katzenellenbogen, J. A. Synthesis and biological evaluation of Tc-99m-cyclopentadienyltricarbonyltechnetium-labeled octreotide. *Nucl. Med. Biol.* (in press).

BC980043T

⁹⁰Yttrium-Labeled Complementarity-Determining-Region-Grafted Monoclonal Antibodies for Radioimmunotherapy: Radiolabeling and Animal Biodistribution Studies[†]

Serengulam V. Govindan,[‡] Lisa B. Shih,[‡] David M. Goldenberg,[§] Robert M. Sharkey,[§] Habibe Karacay,[‡] Joseph E. Donnelly,[‡] Michele J. Losman,[‡] Hans J. Hansen,[‡] and Gary L. Griffiths^{*,‡}

Immunomedics, Inc., 300 American Road, Morris Plains, New Jersey 07950, and Garden State Cancer Center, Belleville, New Jersey 07109. Received April 21, 1998; Revised Manuscript Received July 21, 1998

⁹⁰Yttrium-labeled monoclonal antibodies (mAbs) are likely to be important to radioimmunotherapy (RAIT) of a variety of cancers. The goal of this study was to select and evaluate a form of [⁹⁰Y]mAb suitable for RAIT and determine conditions for high-yield, reproducible radiolabelings. ⁹⁰Y-Labelings, at 2–40 mCi levels, of cdr-grafted versions of anti-B-cell lymphoma (hLL2) and anti-CEA (hIMMU-14) mAbs were optimized to >90% incorporations using the macrocyclic chelator DOTA as the metal carrier. In vitro challenge assays, the stability of mAbs labeled with [⁹⁰Y]DOTA was better than that of the corresponding [⁹⁰Y]benzyl-DTPA conjugates. The retention of [⁹⁰Y]DOTA-hLL2 on Raji tumor cells in vitro was similar to that of the same mAb labeled with [⁹⁰Y]benzyl-DTPA and was about twice as much as with [¹²⁵I]hLL2, indicating residualization of metalated mAb. Both [⁹⁰Y]-hLL2 conjugates, prepared using DOTA and Bz-DTPA, had similar maximum tolerated doses of 125 μ Ci in BALB/c mice and showed no discernible chelator-induced immune responses. Animal biodistribution studies in nude mice bearing Ramos human B-cell lymphoma xenografts revealed similar tumor and tissue uptake over a 10 day period, with the exception of bone uptake which was up to 50% lower for [⁸⁸Y]DOTA-hLL2 compared to [⁸⁸Y]Bz-DTPA-hLL2 at time points beyond 24 h. With [⁹⁰Y]DOTA-hLL2 fragments, in vivo animal tumor dosimetries were inferior to those for the IgG, and kidney uptake was relatively high even with D-lysine administration. The ability of [¹¹¹In]-DOTA-hLL2 to accurately predict [⁹⁰Y]DOTA-hLL2 biodistribution was established. These preclinical findings demonstrate that [⁹⁰Y]DOTA-(CDR-grafted) mAbs are suitable for examination in clinical RAIT.

INTRODUCTION

RAIT¹ using radiolabeled mAbs has shown considerable promise against radiosensitive cancers such as NHL (1–6). Its role as a treatment against solid tumors has

yet to be clearly defined, but clinical and preclinical results obtained using unlabeled mAbs (7), radiolabeled mAbs alone (8), and radiolabeled mAbs along with chemotherapy (9) suggest that useful roles will also be identified in the treatment of at least some solid tumors. For adequate testing of RAIT and RAIT-related treatment modalities, high-quality radiolabeled mAbs must be made available.

As far as the antibody part of any radioimmunoconjugate is concerned, because of the induction of human anti-mouse antibody (HAMA), particularly in patients treated with multiple doses of murine antibodies (10), CDR-grafted (humanized) mAbs (hmAbs), comprising over 90% human sequence homology, are preferred over murine or chimerized versions of mAbs. Two such hmAbs were prepared in our laboratories using the same human framework, and each was described in detail with regard to its binding properties compared to the original murine versions. They are hIMMU-14 (anti-carcinoembryonic antigen mAb, anti-CEA) (11) and hLL2 (anti-lymphoma mAb, anti-CD22) (12).

¹³¹Iodine has been the most widely used nuclide for RAIT studies, but its principal 364 keV, 81% abundant, γ emission and 8 day half-life will engender substantial levels of nonspecific toxic radioactivity being received by the recipient. Indeed, it has been estimated that up to one-half to two-thirds of the energy deposited from ¹³¹I-labeled antibodies is due to penetrating γ emissions (13). The use of fragments (14) and antibody constructs (15) have been proposed to improve the therapeutic ratios

[†] Presented in part at the 43rd Annual meeting of the Society of Nuclear Medicine, Denver, CO, June 1996 (abstract numbers 238 and 664) and at the 45th Annual meeting of the Society of Nuclear Medicine, Toronto, Ontario, Canada, June 1998 (abstract number 411).

* Address correspondence to this author. Phone: 201-605-8200. Fax: 973-605-1103.

[‡] Immunomedics, Inc.

[§] Garden State Cancer Center.

¹ Abbreviations: ABS, 50 mM sodium acetate-150 mM sodium chloride-1% HSA, pH 6, buffer; anti-id, anti-idiotypic; aq, aqueous; BSA, bovine serum albumin; CDR, complementarity-determining region; Bz-DTPA, benzyl DTPA; CEA, carcinoembryonic antigen; cpm, counts per minute; DOTA, 1,4,7,10-tetraazacyclododecane-*N,N',N'',N'''*-tetraacetic acid; DTPA, diethylenetriaminepentaacetic acid; EDC, 1-ethyl-(3,3-dimethylamino)propyl carbodiimide; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; hmAbs, humanized mAbs; HRP, horseradish peroxidase; HSA, human serum albumin; % ID/g, percent injected dose per gram of tissue; ITC-Bz-DTPA, isothiocyanatobenzyl DTPA; ITLC, instant thin-layer chromatography; mAbs, monoclonal antibodies; MSR, molar substitution ratio; MTD, maximum tolerated dose; MW, molecular weight; NHL, non-Hodgkin's lymphoma; PBS, sodium phosphate buffer containing 150 mM sodium chloride; RAIT, radioimmunotherapy; SA, specific activity; SE-HPLC, size-exclusion HPLC; sulfo-NHS, *N*-hydroxysulfosuccinimide.

obtained with ^{131}I -based RAIT, although both approaches involve the adoption of radioimmunoconjugates with somewhat reduced tumor uptake, even when such fragments are chemically cross-linked to enhance stability (16). Another aspect to consider is the fact that certain mAbs are internalized, and when radioiodinated versions of these mAbs are used, radioiodine is not retained substantially in tumor after intracellular metabolism (17, 18).

Of the available nuclides that can be reasonably considered as alternatives to ^{131}I for RAIT, the pure β -particle-emitting ^{90}Y is one of the most promising. Its lack of γ emissions will be advantageous in terms of target-localized radiation as well as general radiation safety. Its half-life could be suited for use with a $\text{F}(\text{ab}')_2$ fragment, but like all radiometals used with antibody fragments, ^{90}Y tends to accumulate in kidney postinjection (19). Pretreatment with basic amino acids (20, 21) will reduce renal accretion substantially in animals and to some degree in humans, but there is still a significant portion of the injected fragment-associated-radioactivity retained.

Among the bifunctional chelates useful for ^{90}Y labeling of proteins, those based on the macrocycle DOTA produce the most stable complexes (22). However, a number of articles have pointed out the difficulties of obtaining reproducibly high yields of ^{90}Y -DOTA-mAb conjugates (22–24), and for this reason, bifunctional chelates based on Bz-DTPA and its derivatives are still in widespread use (25). In addition, there have been reports of immune responses to both types of chelate in previously published works (26, 27), although use of a syngeneic mAb with either chelate has been predicted to be minimally immunogenic (28).

The goal of this work was to choose a ^{90}Y chelator and a form of CDR-grafted mAb (intact or fragment) in ^{90}Y -mAb for eventual clinical application. For mAb labelings with radioyttrium, we set out to choose between DOTA and Bz-DTPA as preferred metal chelator on the basis of comparative *in vitro* and *in vivo* behavior of ^{90}Y -DOTA-mAb and the corresponding ^{90}Y -Bz-DTPA-mAb conjugates. The optimal conditions for the preparation and radiometal labelings of mAb-Bz-DTPA conjugates were already established (29). Optimization of mAb-DOTA conjugate preparation and radiolabeling was examined in this study. DOTA conjugation and ^{90}Y radiolabelings of two CDR-grafted mAbs were examined, and their *in vitro* stabilities and tumor cell bindings were compared with those of the corresponding ^{90}Y -Bz-DTPA-mAb conjugates. The ^{88}Y -labeled DOTA and Bz-DTPA conjugates of a CDR-grafted mAb, hLL2, were also compared in *in vivo* animal studies, in terms of biodistribution in tumor-bearing mice, immune responses to chelates, and maximum tolerated dose in normal mice. Finally, we determined whether an ^{111}In -mAb agent was predictive of the corresponding ^{90}Y -mAb biodistribution (30) and whether ^{90}Y -labeled fragments, used with D-lysine administration, might be superior to a comparably labeled IgG, using a humanized mAb.

EXPERIMENTAL PROCEDURES

General Procedures. Intact and fragment forms of mAbs used in this study were obtained from Immunomedics' antibody production laboratories. DOTA was purchased from Parish Chemical Co. (Orem, UT). All other chemicals, of high-purity grades, were obtained from commercial sources and used without further purification. Yttrium-90 chloride was purchased from

Pacific Northwest National Laboratory (Richland, WA) and, during the initial phase of the work, from NEN Life Science Products (Boston, MA). Indium-111 chloride was from NEN, while yttrium-88 chloride was from Los Alamos National Laboratory (Los Alamos, NM). HPLC analyses were performed on a Bio-Sil SEC-250 analytical column connected to a guard column (Bio-Rad Laboratories, Hercules, CA) and fitted with in-line UV and radiomatic detectors using 0.2 M sodium phosphate buffer, pH 6.8, as mobile phase at a flow rate of 1 mL/min. Cell lines were obtained from the American Type Culture Collection (Rockville, MD), and were routinely tested for mycoplasma by the Mycotect assay (Life Technologies, Gaithersburg, MD).

Preparation of DOTA Conjugates. A procedure based on the work of Lewis et al. (23) was adopted and modified. Briefly, antibodies were rendered metal-free, and exchanged with conjugation buffer in a sequence of dialyses or diafiltrations with 20 mM DTPA/0.25 M ammonium acetate, pH 7.0, 0.25 M ammonium acetate, pH 7.0, and 0.1 M potassium phosphate-0.1 M sodium bicarbonate, pH 8.5 buffers. Activated DOTA was prepared (4 °C, 45 min) using DOTA, sodium bicarbonate, sulfo-NHS, and EDC at 10:30:10:1 molar ratios. Conjugation was carried out by adding a 90-fold molar excess of activated DOTA to IgG, or a 30–50-fold molar excess to mAb fragments, adjusting pH to be in the 8.1–8.3 range, and incubating for 18 h at 4 °C. Purification was achieved by a series of dialyses or diafiltrations with 20 mM DTPA/0.25 M ammonium acetate, pH 7.0, and then with 0.25 M ammonium acetate, pH 5.4. The final conjugates, suitably concentrated, were filtered through 0.22 μm filters and stored under sterile conditions. A published procedure (31) involving conjugate labeling with a known excess of indium acetate, spiked with ^{111}In , was used to determine DOTA MSRs. Variations in conjugation chemistry examined included coupling pH (7.5, 8.0, and 8.5) and molar excess of activated DOTA to mAb used (40, 100, or 500). Preparation of Bz-DTPA-hLL2 conjugate was based on the procedure described previously (29).

Comparison of Affinities of DOTA-Conjugated and Unmodified mAbs. Competitive binding assays were carried out using Raji cells (a human Burkitt lymphoma cell line) and CEA as the source of antigens for hLL2 and hIMMU-14, respectively. For hLL2, a 96-well filtration plate fitted with a microporous (0.45 μm pore) hydrophilic membrane assembly (Millipore, Milford, MA) was used to incubate a 0.1 mL suspension of Raji cells (~200 000) with a fixed concentration of ^{125}I murine LL2 (~500 000 cpm) and varying concentrations (0.1–100 $\mu\text{g}/\text{mL}$) of competitors (murine LL2 control or DOTA conjugate of hLL2). After gently shaking the assembly for 2.5 h at ambient temperature, the well contents were drained by vacuum, and the wells were washed three times with 0.2 mL of 10 mM PBS, pH 7.4, and 1%BSA. Membrane-bound residual ^{125}I activity was determined in a γ counter, and the readings were corrected for nonspecific membrane binding. The latter was determined using a control consisting of ^{125}I mLL2 in the same final volume but without the antigen. Duplicate measurements were made, and the average residual bindings were plotted as a function of competitor concentrations. For hIMMU-14 substrates, an IMMU-14 blocking assay was performed as follows. ELISA plates were coated with CEA (Calbiochem, San Diego, CA) at a concentration of 2.5 $\mu\text{g}/\text{mL}$ and incubated for 1 h at 20 °C. HRP-conjugated murine IMMU-14, at a concentration of 200 ng/mL, was mixed with varying concentrations

of murine IMMU-14 (control) or hIMMU-14-DOTA conjugate, added to CEA-coated ELISA plates, and incubated for 1 h at 20 °C. CEA binding of HRP-conjugated IMMU-14 was determined with a substrate solution containing 0.0125% of H₂O₂ and 0.004 M *O*-phenylenediamine dihydrochloride. Each measurement was the mean of duplicate experiments.

Radiolabelings. In optimizing [⁹⁰Y]mAb yields, the DOTA MSR, pH (5–6 range), incubation time (up to 2.5 h), use of ascorbic acid as a radioprotectant, and the effect of trace metal contaminants in commercial ⁹⁰Y were examined. In an optimized radiolabeling, the DOTA conjugate, at a concentration of 3–10 mg/mL, was added to yttrium-90 acetate (obtained by buffering yttrium-90 chloride with 0.25 M ammonium acetate pH 5.4), so as to achieve a specific activity in the range 1–5 mCi/mg. Freshly prepared aq ascorbic acid (0.25 g of ascorbic acid dissolved in 1 mL of water) was added to the labeling mixture at 27 μL/mL of the labeling volume and heated at 45 °C (bath) for 2 h. The final ascorbic acid concentration was ~6 mg/mL. The solution was made 10 mM in DTPA using aq 0.1 M DTPA (pH 6–7), and heated for 20–30 min at the same temperature. The radiolabeled mAb was diluted to ~1 mCi/mL using ABS buffer. ¹¹¹In and ⁸⁸Y radiolabelings were carried out in a similar fashion, except the specific activities of [⁸⁸Y]mAbs were ~0.1 mCi/mg. Radiolabel incorporations in the latter categories were usually 90% or greater, but yields of less than 90% were observed in some ⁸⁸Y labelings. For this reason, the radiolabeled mAbs for *in vivo* studies were routinely purified by centrifuged-size-exclusion column purification using Sephadex G50/80 equilibrated in 0.1 M sodium acetate, pH 6.5. These conjugates were at least 95% pure by ITLC and HPLC; the remainder was present as EDTA- or DTPA-bound isotope.

In Vitro Stability. Purified [⁹⁰Y]DOTA-mAb (~16–25 μCi, in ~4–6 μL) was added to 0.55 mL of fresh human serum, which was made 0.02% in sodium azide, and incubated at 37 °C. The substrate concentration was 170 nM for hLL2 and 143 nM for hIMMU-14. In a similar fashion, radiolabeled preparations (~14–18 μCi, in ~3–6 μL) were added to 0.55 mL of 1 mM DTPA/1% HSA in 25 mM ammonium acetate/150 mM saline, pH 6.4, and incubated at 37 °C. The DTPA in the latter medium was in 10325-fold and 6244-fold molar excess with respect to hLL2 and hIMMU-14, respectively. Aliquots of serum and DTPA incubates were analyzed, on size-exclusion analytical HPLC, over a 10 day period. Serum aliquots were briefly challenged with DTPA (1 mM) prior to HPLC analyses. Analyses consisted of determining the percent of radioactivity associated with the antibody. This was accomplished by complexing the antibody with a 100-fold molar excess of either an anti-idiotopic antibody, WN (32), for hLL2, or CEA (for hIMMU-14), either of which converted radiolabeled antibody to a higher MW component (hLL2:WN complex or hIMMU-14:CEA complex), appearing as an earlier eluting peak on SE-HPLC. This method separated, by HPLC retention time, [⁹⁰Y]DOTA-mAb from serum proteins such as albumin and transferrin. Loss of label to serum proteins, as well as loss of label as free metal ion (analyzed as [⁹⁰Y]DTPA), were then determined from integrations of all HPLC peaks. In a separate experiment, [⁹⁰Y]DOTA-hLL2 was compared with [⁹⁰Y]Bz-DTPA-hLL2 for stability in serum and 1 mM DTPA, over an 11 day period at 37 °C, using 5–7 μg of radiolabeled conjugate and 0.55 mL of serum or DTPA, as described above.

In Vitro Retention of [⁹⁰Y]hLL2 on Lymphoma Cells. For these studies, a DOTA conjugate, as well as a Bz-DTPA conjugate derived from ITC-Bz-DTPA, of hLL2-IgG were radiolabeled at over 90% incorporation. The radiolabeled conjugates were made 10 mM in DTPA (for DOTA conjugate) or EDTA (for Bz-DTPA conjugate) to chelate unbound radiometal. ¹²⁵I-Labeled hLL2 (or [¹²⁵I]mLL2) was prepared using a chloramine-T procedure (33). Raji cells (American Type Culture collection, Rockville, MD) were cultured in Dulbecco's Eagle Medium (Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum, 5% horse serum, and 1% pyruvate, penicillin/streptomycin and glutamine, in a 5% CO₂ atmosphere. Cells were allowed to grow to confluence. For binding studies, cells (5 × 10⁵ to 1 × 10⁷) in cell culture media were treated with ~10–50 μCi of [⁹⁰Y]mAb conjugate (5–25 μg) and incubated at 37 °C for 2 h. The cells were separated from supernatant by centrifugation at 1500 rpm for 6 min, washed three times with cold media, resuspended in media, and incubated at 37 °C. At periodic intervals over 40 h, aliquots were withdrawn, centrifuged to separate cells from the supernatant, and the radioactivity associated with each was counted using appropriate windows set for ⁹⁰Y and ¹²⁵I. The percent of cell-associated radioactivity was thus calculated for each time point. In general, measurements reflect the mean of triplicate samples. To determine the percent of cell-associated radioactivity internalized into tumor cells, aliquots of cell suspension at various time points (as described in the above experiment) were first chilled at 4 °C and then centrifuged. The cell pellet was then treated with 0.1 M sodium acetate/0.1 M glycine at pH 3 for 30 min at 40 °C, then spun down and separated prior to counting. In these experiments, [¹²⁵I]hLL2 was used as a comparative substrate and [⁹⁰Y]IMMU-14 was used as nonspecific control.

Animal Experiments. All animal experiments were carried out under approved IACUC protocols. For hLL2 conjugates, 3–4 week old nude mice (Taconic, Germantown, NY) were quarantined for 2 weeks, and then injected s.c. with 5 × 10⁶ Ramos tumor cells grown in tissue culture. Tumor growth was visible after a further 2 week period, and animal experiments were started immediately thereafter. For biodistribution studies, 1–2 μCi of [⁸⁸Y]mAb or 10–20 μCi of [¹¹¹In]mAb was used for each tumor-bearing mouse. One experiment involved comparing [⁸⁸Y]DOTA-hLL2-IgG (SA, 0.06 μCi/μg; 2.3% aggregation; protein dose per animal, 17.4 μg) with [⁸⁸Y]-Bz-DTPA-hLL2-IgG (SA, 0.08 μCi/μg; 0% aggregation; protein dose per animal, 14.2 μg). A second experiment was a paired-label experiment involving targeting of [⁸⁸Y]DOTA-hLL2 (SA, 0.08 μCi/μg; 1.19 μCi/animal) and [¹¹¹In]DOTA-hLL2 (SA, 1.1 μCi/μg, 16.4 μCi/animal), with a total protein dose of 15 μg/animal. Corrections for the backscatter of ⁸⁸Y into the ¹¹¹In window were made in determining ¹¹¹In biodistributions. A third experiment consisted of two groups of animals administered with [⁸⁸Y]DOTA-hLL2 F(ab')₂ or [⁸⁸Y]DOTA-hLL2 Fab', with D-lysine administration (40 mg/mouse) given intraperitoneally 15 min before and 1, 2, and 3 h after the i.v. injection of radiolabeled conjugates. Each animal in this experiment received a protein dose of 21.3 μg [F(ab')₂ fragment] or 20.7 μg [Fab' fragment] and 1.0 μCi of ⁸⁸Y activity. A fourth experiment was the repetition of radiolabeled fragment biodistributions, as before, but without D-lysine administration. Necropsy was performed at 1, 3, 5, 7, and 10 days after radiolabeled IgG injection or at 4, 24, and 48 h after radiolabeled fragment

injection. At the indicated times, animals were anesthetized with sodium pentobarbital, bled by cardiac puncture, and then sacrificed. Tissues were weighed and the radioactivity was determined in a γ counter using appropriate windows. In each instance, counting standards were included for the determination of total injected radioactivity. The data were then calculated as a percentage per gram of the total injected activity.

For MTD experiments, 11 mg each of hLL2-DOTA-IgG and hLL2-Bz-DTPA-IgG were radiolabeled with 30 mCi of ^{90}Y activity. The radiochemical purity was 94% for the DOTA conjugate and 99% for the Bz-DTPA conjugate. Unbound ^{90}Y was present as [^{90}Y]DTPA in the DOTA conjugate and as [^{90}Y]EDTA in the Bz-DTPA conjugate. In each case, the radiolabeled mAb was diluted serially with ABS buffer to obtain the desired microcurie amount in a final volume of 0.15 mL. Radioactivity contained in the injectate volume in each case was determined by counting suitably diluted samples in a β -counter. Eighty-four normal BALB/c mice were sorted into six groups of seven animals, with animals in each group given 125, 150, 175, 200, 225, or 250 μCi of [^{90}Y]DOTA-hLL2 or [^{90}Y]Bz-DTPA-hLL2. Animal weights and mortality, postinjection of radiolabeled conjugates, were recorded over time.

To examine the immunogenicity of DOTA and Bz-DTPA, forty-eight normal BALB/c mice were divided into four groups of 12 animals each. Animals in each group received either murine LL2-IgG or LL2 F(ab')₂ conjugates of DOTA (containing 3–4 chelates/mAb) or Bz-DTPA (containing 1–2 chelates/mAb), administered i.v. at 33 $\mu\text{g}/\text{mouse}$. After 3 weeks, four mice in each group were anesthetized, bled by cardiac puncture, and sacrificed by cervical dislocation. Serum samples were collected and stored frozen for subsequent analyses. The remaining mice in each group were readministered the respective substrates at the same dose of 33 $\mu\text{g}/\text{mouse}$. After a further 3 week period, again, four mice from each group were taken and serum samples collected and stored, while remaining mice in each group received a third dose of the conjugates. These mice were taken in turn after an additional 3 week period. Serum samples (50 μL) from individual mice were incubated with 100 ng (10 μL) of [^{125}I]mLL2-Bz-DTPA and [^{125}I]mLL2-DOTA, in a total volume made up to 100 μL with 5% HSA, at 37 $^{\circ}\text{C}$ for 1 h. The samples were then analyzed, by SE-HPLC, for their ability to form immune complexes with added radiolabeled conjugates.

RESULTS AND DISCUSSION

Development of suitable bifunctional chelating agents for ^{90}Y has evolved from using DTPA cyclic anhydride (34), to backbone-substituted bifunctional DTPAs (Bz-DTPA, MX-DTPA, and cyclohexyl-DTPA) (35, 36) and to chelates based on the macrocyclic chelating agent DOTA (37). The exceptional kinetic inertness of DOTA-type chelates has been documented (38). Such stability portends well for RAIT applications, as any dissociation of ^{90}Y from mAb-chelate conjugates can result in a portion of this radioactivity being bound in cortical bone, which could increase the level of myelotoxicity associated with these agents. The routine use of DOTA-mAb conjugates has been hampered by required lengthy syntheses of DOTA bifunctional chelates and subsequent difficulties in ^{90}Y radiolabeling of DOTA-mAb conjugates. Lewis et al. (23) addressed these problems by using in situ-generated monoactivated DOTA for mAb conjugation, which obviated a long synthesis, and by employing a

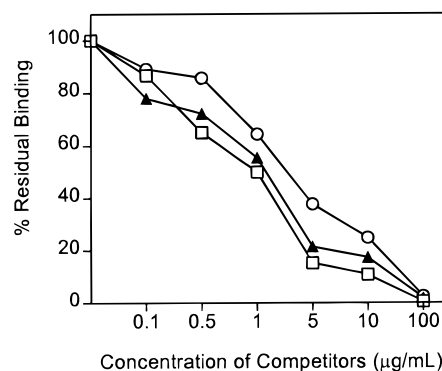


Figure 1. Comparisons of immunoreactivities of hLL2-DOTA conjugates with that of unmodified antibody, determined in competitive binding assays using Raji cells as the source of antigen. The competitive binding of hLL2 conjugate with a DOTA MSR of 3.7 ($\blacktriangle$) is similar to that of unmodified hLL2 ($\square$). The conjugate with a DOTA MSR of 6.7 ($\circ$) exhibits a somewhat reduced competitive binding.

higher temperature ^{90}Y -labeling of a mAb-DOTA conjugate. By conducting the radiolabelings at 43 $^{\circ}\text{C}$, a 59% ^{90}Y incorporation was obtained for a cT84.66-DOTA mAb conjugate.

We have optimized this methodology for our own humanized DOTA-mAbs, using a 90–100-fold molar excess of activated DOTA for IgGs and a 40–50-fold molar excess of activated DOTA for F(ab')₂ and Fab' fragments. The DOTA:mAb MSRs for our conjugates were determined by the method of Meares et al. (31) using ^{111}In -spiked indium. At a given molar excess of DOTA-NHS used in conjugations, variations of pH in the 7.5–8.5 range did not affect the final DOTA MSR. The MSR was influenced by the molar excess of activated DOTA used in conjugations. At pH 8.0–8.3, a 100-fold molar excess of DOTA gave a substitution ratio of 3.7–3.9 for hLL2, while a 500-fold excess of DOTA gave a MSR of 9.0. With hIMMU-14, typically 4–5 DOTAs were introduced under the same conditions, using 100-fold excess of monoactivated DOTA. For hLL2-F(ab')₂ and hLL2 Fab', DOTA substitutions of 3.6 and 1.5, respectively, were obtained using a 40–50-fold molar excess of activated DOTA. By comparison, the average number of Bz-DTPA groups on hLL2, in the hLL2-Bz-DTPA conjugate, was 1.5.

Competitive Binding Assays. The effect of MSR on immunoreactivity was examined for conjugates of hLL2 and hIMMU-14 by competitive binding assays on Raji cells and CEA, respectively. Figure 1 shows competitive binding to Raji cells of hLL2 (control) and two hLL2-DOTA conjugates differing in the DOTA MSR. Figure 2 shows results from an ELISA assay involving residual binding, to CEA, of HRP-conjugated IMMU-14 as a function of competitor concentration against either hIMMU-14-DOTA substituted at a 4.4:1 ratio or murine IMMU-14 as control. For the hLL2 conjugate, the binding profiles for a conjugate with 3.7 DOTA is similar to that of unmodified hLL2, whereas a DOTA substitution of 6.7 leads to a somewhat reduced competitive inhibition (Figure 1). The CEA-blocking experiment (Figure 2) showed that a hIMMU-14-IgG-DOTA conjugate, with a DOTA MSR of 4.4, had a target binding profile identical to that of control IMMU-14. For a murine IMMU-14-DOTA conjugate containing 6.8 DOTAs, competitive binding to CEA was similar to that of unsubstituted IMMU-14 (not shown). While the limit of DOTA substitution beyond which a reduction in immunoreactivity is observed is seen to be antibody dependent,

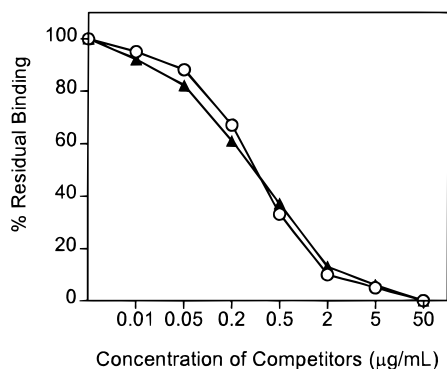


Figure 2. Comparison of immunoreactivity of hIMMU-14-DOTA conjugate with DOTA MSR of 4.4 (▲) with that of unmodified IMMU-14 (○), determined in a CEA-blocking assay. The competitive binding profiles of the two are very similar.

Table 1. Representative Incorporations of ⁹⁰Y into IgG-DOTA Conjugates

conjugate	mCi	specific activity (mCi/mg)	incorporation (%) by HPLC	
			IgG	aggregate
hIMMU-14-IgG	2.3 ^a	4.6	94.7	5.0
	2.8 ^a	2.8	93.6	5.2
	4.0 ^a	4.7	93.2	3.6
	4.9 ^a	3.3	91.3	6.5
	13.0 ^b	3.4	94.3 ^c	0.7
	22.0 ^b	2.2	99.5 ^d	0
hLL2-IgG	23.5 ^b	2.1	90.0	1.0
	30.0 ^b	2.7	94.4 ^e	1.6
	32.2 ^b	2.9	99.4 ^f	0
	45.0 ^b	4.1	98.6 ^g	0

^a Ascorbic acid was not used. ^b Ascorbic acid was used at a concentration of ~6 mg/mL, in these labelings. ^c ITLC incorporations of 96.3% were observed in these findings. ^d ITLC incorporations of 97.0% were observed in these findings. ^e ITLC incorporations of 94.5% were observed in these findings. ^f ITLC incorporations of 97.9% were observed in these findings. ^g ITLC incorporations of 97.6% were observed in these findings.

apparently even in mAbs having the same human framework, we conclude that DOTA substitution in the 3–5 range would generally be well tolerated.

⁹⁰Y Radiolabelings. Maximizing the DOTA:mAb substitution ratios, consistent with the preservation of tumor targeting properties, provided the best opportunity for near-quantitative ⁹⁰Y incorporations under optimized labeling conditions. Optimal radiolabeling conditions involved adding the desired radioactivity of yttrium-90 chloride buffered with 0.25 M ammonium acetate, pH 5.4) to the conjugate containing aq ascorbic acid (~6 mg/mL) and incubating at 45 °C for 2 h. The final conjugate concentration was 3–12 mg/mL (the range of concentrations examined), and the pH was approximately in the 5.0–5.4 range. Table 1 shows typical yields for ⁹⁰Y labelings of hIMMU-14- and hLL2-DOTA, using 2.3–45 mCi of ⁹⁰Y. Initially, ascorbic acid was not used during radiolabeling, but we found that addition of ascorbic acid during labeling significantly reduced aggregation (Table 1). During the course of our work, Chakrabarti et al. (39) reported on the use of ascorbic acid as a radioprotectant during ⁹⁰Y labelings. These workers found that the immunoreactivity of a 100 μg sample of a ¹²⁵I-labeled murine mAb was reduced by about 65–90% in the presence of 320–640 Gy doses of [⁹⁰Y]DTPA (1.1–2.2 mCi), but the immunoreactivity was completely restored when the same experiment was conducted in the presence of ~0.2–11 mg/mL concentration of ascorbic acid.

Incorporation of ⁹⁰Y into DOTA-mAb conjugates was

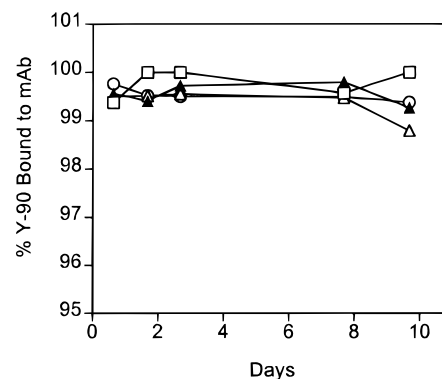


Figure 3. In vitro stabilities of [⁹⁰Y]DOTA-hIMMU-14 in serum (□) and in 1 mM DTPA/1% HSA (Δ) and of [⁹⁰Y]DOTA-hLL2 in serum (▲) and in 1 mM DTPA/1% HSA (○). The y-axis scale is displayed in a narrow range of 95–101%.

found to be affected by trace metal contaminants in the yttrium-90 chloride used. Stimmel et al. (24) have studied the role of ferrous, zinc, and calcium ions on the efficiency of ⁹⁰Y incorporation into free macrocyclic and acyclic ⁹⁰Y chelators. Their quantitative analyses showed that ⁹⁰Y chelation is most sensitive to the presence of trace levels of zinc and, to a lesser extent, ferrous and calcium ions. We found the same connection between ⁹⁰Y yields into DOTA-mAb conjugates and trace metal contaminants in ⁹⁰Y shipments, with >10–50 ppm levels of contaminating trace metals, particularly zinc, being detrimental.

In Vitro Stability of [⁹⁰Y]hLL2-DOTA and hIMMU-14-DOTA. The stability of radiolabeled conjugates to serum and to DTPA challenge was examined using substrate concentrations of 140–170 nM in serum and DTPA/conjugate molar ratios of ~6000–10000:1. Incubations were kept at 37 °C for 10 days. At regular intervals, aliquots were analyzed by SE-HPLC. To separate radiolabeled IgG from other serum proteins, as well as from HSA in DTPA-challenge assays, aliquots of challenged material were complexed with CEA (in the case of hIMMU-14) or WN (anti-id mAb to hLL2; ref 32) prior to HPLC analyses. As seen from Figure 3, the percentage of ⁹⁰Y associated with antibody was >98% over this time period, which affirmed the kinetic stability of these [⁹⁰Y]DOTA-mAb conjugates. In a separate experiment, 0, 0, and 0.4% of the radioactivity (analyzed as the [⁹⁰Y]DTPA by HPLC) were found to be dissociated from the [⁹⁰Y]DOTA-hLL2 conjugate when incubated in serum at 37 °C and analyzed after 18 h, 5 d, and 11 d, respectively. Under the same conditions, 3.0, 3.6, and 10.7% of the radioactivity (analyzed as the [⁹⁰Y]EDTA by HPLC) were found dissociated from the [⁹⁰Y]Bz-DTPA-hLL2 conjugate after serum challenge. When incubated in 1 mM DTPA under the same conditions and analyzed over the same period of time, 0, 1, and 0.3% of the radioactivity of the DOTA conjugate and 1.6, 5.2, and 8.7% of the radioactivity of the Bz-DTPA conjugate of hLL2, respectively, were found unbound.

In Vitro Binding Studies of Radiolabeled hLL2 Conjugates. In vitro binding studies were carried out using ⁹⁰Y-labeled murine and humanized LL2 mAbs, along with the respective mAbs radioiodinated by the chloramine-T method (33), to compare binding, retention, turnover, and metabolism by Raji tumor cells (40). At time points out to 40 h, a higher percentage of the [⁹⁰Y]-hLL2 (12–70% higher, increasing with time), compared to radioiodinated hLL2, remained associated with the cells (Figure 4A). A small reduction in the percentage of retained activity was observed for [⁹⁰Y]DOTA versus [⁹⁰Y]Bz-DTPA conjugates of hLL2 (Figure 4A) in some

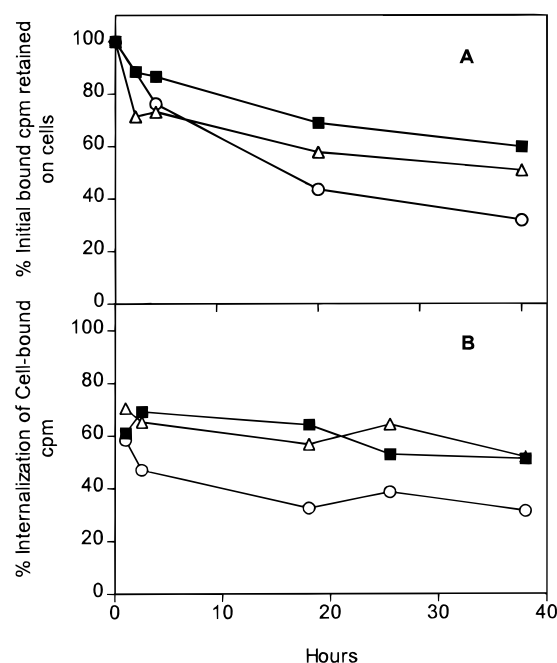


Figure 4. In vitro cell bindings of ^{90}Y -[Bz-DTPA]-hLL2 (■), ^{90}Y -DOTA-hLL2 (Δ) and ^{125}I -hLL2 (○) on Raji cells. (A) Percent of initially bound radioactivity still retained on cells at various times, and (B) percent of cell-bound radioactivity internalized into tumor cells at various times.

earlier experiments, but this difference was not noticed subsequently when the radiolabeling procedure included the use of ascorbic acid as radioprotectant (not shown). Percent of cell-bound mAb internalized at various times is shown in Figure 4B. At later time points, cells treated with ^{90}Y -DOTA-hLL2 and ^{90}Y -Bz-DTPA-hLL2 had between 50 and 60% of nonremovable cell-bound radioactivity, while those treated with ^{125}I -hLL2 had between 30 and 40% nonremovable activity. In a cell-binding experiment using ^{90}Y -Bz-DTPA-hLL2, the SE-HPLC of cell-culture supernatant showed a peak eluting with a retention time of 13.4 min, indicating that ^{90}Y was released from cells in a low MW form. This retention time was similar to that of ^{90}Y -DTPA, although the SE-HPLC would not distinguish it from ^{90}Y -DTPA-lysine adduct. Lysine adducts of metal chelates had been inferred to be the end products of antibody-chelate catabolism in the lysosomes (41). The bindings of ^{90}Y -

DOTA-hLL2 and ^{90}Y -Bz-DTPA-hLL2 were similar when continuously exposed to Raji, Daudi, or Ramos cell lines, as were the bindings observed for each of these conjugates, in the Raji cell line, using ~30 mCi level radiolabeling preparations (not shown). These findings implied that higher temperature DOTA conjugate labeling with high-energy ^{90}Y did not impair its cell-binding ability. Other experiments involving divalent fragments of murine and humanized LL2 showed similar trends of higher binding for ^{90}Y versus ^{125}I -labeled conjugates, while nonspecific controls (^{90}Y -Bz-DTPA-murIMMU-14 and other IMMU-14 derivatives) did not bind to Raji cells (not shown). This is the first extensive study involving radiolabeled conjugates of intact and fragment forms of hmAb, including comparisons with the murine counterparts and using three different cell lines (40). The results obtained are similar to in vitro tumor cell binding data reported for murine LL2 labeled with either a residualizing form of radioiodine or ^{111}In (42). Internalizations observed in Raji cell binding of ^{90}Y -hLL2 conjugates suggest that they should be preferred agents compared to iodinated hLL2 in targeting B-cell lymphoma.

In Vivo Experiments. For biodistributions involving radioyttrium, conjugates labeled with γ -emitting ^{88}Y were used to render determination of tissue-bound radioactivity more accurate. Tumor targeting and biodistribution of ^{88}Y -DOTA-hLL2 and ^{88}Y -Bz-DTPA-hLL2 were compared in s.c. Ramos tumor xenograft-bearing nude mice. Table 2 shows comparative biodistribution out to 10 days, which is 3.75 times ^{90}Y physical half-life. ^{88}Y -DOTA-hLL2 had 2.4, 1.8, 1.6, 1.4, and 1.0% injected dose/g (% ID/g) uptake in bone at 1, 3, 5, 7, and 10 days postinjection and the ^{88}Y -Bz-DTPA-hLL2 conjugate had 2.8, 3.4, 3.4, 2.7, and 3.6% ID/g in bone at the same times. As discussed by DeNardo et al. (43), these differences may be particularly significant when one considers clinical RAIT since human marrow volume is much larger than that of mouse, and the differences seen in these animals may be reflective of a larger toxicity difference in a clinical situation. Dosimetrically (44), per millicurie numbers derived from these data were 10 564, 8274, 6866, and 23 921 for ^{90}Y -DOTA-hLL2-IgG and 9901, 9579, 7403, and 27 124 for ^{90}Y -Bz-DTPA-hLL2 for blood, liver, bone, and Ramos tumor, respectively.

A second experiment compared biodistribution of ^{111}In -DOTA-hLL2 to ^{88}Y -DOTA-hLL2 (Table 3). This was a paired-label experiment which maintained identi-

Table 2. Biodistributions of ^{88}Y -DOTA-hLL2 (A) and ^{88}Y -Bz-DTPA-hLL2 (B) in Nude Mice Bearing s.c. Ramos Human Tumor Xenografts (Weight in Grams) at Times Indicated

		percent injected dose per gram $\pm$ SD, $n = 4-5$				
tissue		24 h	72 h	120 h	168 h	240 h
Ramos tumor (g)	A	16.0 $\pm$ 4.5 (0.5 $\pm$ 0.2)	27.6 $\pm$ 5.1 (0.3 $\pm$ 0.1)	18.5 $\pm$ 3.8 (0.7 $\pm$ 0.4)	15.5 $\pm$ 5.6 (0.8 $\pm$ 0.5)	12.6 $\pm$ 17.2 (1.6 $\pm$ 1.0)
	B	19.0 $\pm$ 3.4 (0.2 $\pm$ 0.1)	25.6 $\pm$ 3.9 (0.4 $\pm$ 0.1)	26.3 $\pm$ 15.9 (0.9 $\pm$ 0.5)	16.4 $\pm$ 5.5 (0.8 $\pm$ 0.3)	11.4 $\pm$ 10.1 (1.9 $\pm$ 1.3)
liver	A	9.6 $\pm$ 2.0	6.4 $\pm$ 0.9	6.3 $\pm$ 2.8	3.9 $\pm$ 0.6	2.4 $\pm$ 0.6
	B	8.3 $\pm$ 1.1	8.8 $\pm$ 1.3	7.2 $\pm$ 2.6	6.0 $\pm$ 1.3	4.8 $\pm$ 1.2
spleen	A	5.9 $\pm$ 1.6	4.4 $\pm$ 0.9	4.9 $\pm$ 1.0	4.2 $\pm$ 0.5	3.4 $\pm$ 1.1
	B	6.4 $\pm$ 0.9	6.6 $\pm$ 1.2	5.5 $\pm$ 1.5	4.7 $\pm$ 1.0	4.0 $\pm$ 1.4
kidney	A	5.9 $\pm$ 1.7	4.6 $\pm$ 0.7	3.6 $\pm$ 0.3	3.3 $\pm$ 0.5	2.0 $\pm$ 0.4
	B	6.0 $\pm$ 0.9	6.5 $\pm$ 0.8	4.6 $\pm$ 1.3	4.1 $\pm$ 0.4	2.6 $\pm$ 0.6
lungs	A	6.4 $\pm$ 1.4	5.2 $\pm$ 0.4	3.8 $\pm$ 0.5	2.9 $\pm$ 0.6	1.6 $\pm$ 0.6
	B	6.7 $\pm$ 1.1	5.2 $\pm$ 1.4	4.0 $\pm$ 0.5	3.6 $\pm$ 0.4	2.4 $\pm$ 1.1
blood	A	11.4 $\pm$ 3.1	7.4 $\pm$ 4.3	5.5 $\pm$ 1.4	5.2 $\pm$ 1.1	2.5 $\pm$ 0.9
	B	12.7 $\pm$ 1.2	9.3 $\pm$ 1.3	4.2 $\pm$ 0.9	4.2 $\pm$ 0.6	2.2 $\pm$ 1.6
bone	A	2.4 $\pm$ 0.8	1.8 $\pm$ 0.7	1.6 $\pm$ 0.3	1.4 $\pm$ 0.3	1.0 $\pm$ 0.4
	B	2.8 $\pm$ 0.1	3.4 $\pm$ 0.8	3.4 $\pm$ 0.6	2.7 $\pm$ 0.2	3.6 $\pm$ 0.6
washed bone	A	1.7 $\pm$ 0.3	1.7 $\pm$ 0.7	1.0 $\pm$ 0.2	1.1 $\pm$ 0.3	0.6 $\pm$ 0.2
	B	1.7 $\pm$ 0.2	2.3 $\pm$ 0.7	3.0 $\pm$ 0.7	2.7 $\pm$ 0.7	2.7 $\pm$ 0.6

Table 3. Biodistributions of [⁸⁸Y]DOTA-hLL2 (A) and [¹¹¹In]DOTA-hLL2 (B) in Nude Mice Bearing s.c Ramos Human Tumor Xenografts (Weight in Grams) at Times Indicated

tissue		percent injected dose per gram $\pm$ SD, $n = 4-5$				
		24 h	72 h	120 h	168 h	240 h
Ramos tumor (g)	A	17.8 $\pm$ 3.7 (0.2 $\pm$ 0.1)	17.6 $\pm$ 6.1 (0.2 $\pm$ 0.1)	18.9 $\pm$ 12.6 (0.2 $\pm$ 0.1)	11.0 $\pm$ 6.0 (0.4 $\pm$ 0.2)	7.7 $\pm$ 4.9 (1.0 $\pm$ 1.3)
	B	16.7 $\pm$ 3.2 (0.2 $\pm$ 0.1)	17.3 $\pm$ 6.6 (0.2 $\pm$ 0.1)	18.2 $\pm$ 11.4 (0.2 $\pm$ 0.1)	11.0 $\pm$ 5.7 (0.4 $\pm$ 0.2)	7.3 $\pm$ 4.4 (1.0 $\pm$ 1.3)
liver	A	7.7 $\pm$ 2.1	6.0 $\pm$ 1.2	4.1 $\pm$ 1.3	3.7 $\pm$ 0.7	2.8 $\pm$ 0.9
	B	6.2 $\pm$ 1.4	5.7 $\pm$ 1.1	4.0 $\pm$ 1.2	3.8 $\pm$ 0.7	2.7 $\pm$ 0.9
spleen	A	5.3 $\pm$ 1.0	4.7 $\pm$ 0.7	3.5 $\pm$ 1.0	3.9 $\pm$ 0.3	3.2 $\pm$ 1.4
	B	5.1 $\pm$ 1.0	4.3 $\pm$ 0.7	3.3 $\pm$ 0.9	3.5 $\pm$ 0.3	2.6 $\pm$ 1.4
kidney	A	3.6 $\pm$ 0.6	3.4 $\pm$ 0.8	2.0 $\pm$ 0.4	2.5 $\pm$ 0.5	1.5 $\pm$ 0.5
	B	3.8 $\pm$ 0.6	3.4 $\pm$ 0.7	2.0 $\pm$ 0.4	2.4 $\pm$ 0.5	1.3 $\pm$ 0.5
lungs	A	5.5 $\pm$ 0.6	4.8 $\pm$ 0.8	3.1 $\pm$ 0.6	2.8 $\pm$ 0.5	2.4 $\pm$ 1.2
	B	5.5 $\pm$ 0.5	4.6 $\pm$ 0.9	3.0 $\pm$ 0.7	2.8 $\pm$ 0.5	2.1 $\pm$ 1.0
blood	A	11.7 $\pm$ 1.8	9.3 $\pm$ 1.3	5.0 $\pm$ 1.5	5.7 $\pm$ 1.3	3.2 $\pm$ 1.7
	B	11.3 $\pm$ 1.6	8.9 $\pm$ 1.2	4.9 $\pm$ 1.4	5.6 $\pm$ 1.3	3.0 $\pm$ 1.6
small intestine	A	1.4 $\pm$ 0.1	1.1 $\pm$ 0.2	0.6 $\pm$ 0.1	0.8 $\pm$ 0.2	0.5 $\pm$ 0.3
	B	1.3 $\pm$ 0.1	1.1 $\pm$ 0.1	0.6 $\pm$ 0.1	0.7 $\pm$ 0.2	0.5 $\pm$ 0.3
large intestine	A	2.3 $\pm$ 1.1	1.2 $\pm$ 0.2	0.8 $\pm$ 0.3	0.7 $\pm$ 0.1	0.6 $\pm$ 0.3
	B	2.1 $\pm$ 1.0	1.2 $\pm$ 0.2	0.7 $\pm$ 0.3	0.7 $\pm$ 0.1	0.5 $\pm$ 0.3
bone	A	2.3 $\pm$ 0.4	2.5 $\pm$ 0.8	1.4 $\pm$ 0.4	1.5 $\pm$ 0.1	1.4 $\pm$ 0.7
	B	2.1 $\pm$ 0.3	2.1 $\pm$ 0.9	1.1 $\pm$ 0.3	1.3 $\pm$ 0.2	1.0 $\pm$ 0.7

Table 4. Biodistributions of [⁸⁸Y]DOTA-hLL2-F(ab')₂ with Administration of 4 $\times$ 2 mg of D-Lysine per Gram of Animal Weight (A) and without D-Lysine Administration (B) in Nude Mice Bearing s.c. Ramos Human Tumor Xenografts (Weight in Grams) at Times Indicated

tissue		percent injected dose per gram $\pm$ SD, $n = 4$		
		4 h	24 h	48 h
Ramos tumor (g)	A	2.5 $\pm$ 0.7 (0.2 $\pm$ 0.1)	2.6 $\pm$ 0.7 (0.3 $\pm$ 0.1)	1.7 $\pm$ 0.3 (0.23 $\pm$ 0.21)
	B	2.0 $\pm$ 0.8 (0.19 $\pm$ 0.15)	2.7 $\pm$ 0.8 (0.3 $\pm$ 0.1)	3.1 $\pm$ 1.0 (0.13 $\pm$ 0.07)
liver	A	8.7 $\pm$ 1.4	5.8 $\pm$ 0.8	5.2 $\pm$ 1.4
	B	7.7 $\pm$ 3.2	8.0 $\pm$ 0.9	7.7 $\pm$ 1.1
spleen	A	5.3 $\pm$ 0.8	4.9 $\pm$ 0.6	4.8 $\pm$ 1.3
	B	6.4 $\pm$ 3.1	6.9 $\pm$ 2.1	13.0 $\pm$ 4.1
kidney	A	19.9 $\pm$ 4.6	39.3 $\pm$ 4.3	28.4 $\pm$ 6.2
	B	69.9 $\pm$ 27.6	73.7 $\pm$ 6.9	76.0 $\pm$ 18.4
lungs	A	4.9 $\pm$ 0.8	1.1 $\pm$ 0.2	1.2 $\pm$ 0.8
	B	3.4 $\pm$ 1.0	1.4 $\pm$ 0.1	1.3 $\pm$ 0.3
blood	A	10.2 $\pm$ 5.6	0.5 $\pm$ 0.1	0.4 $\pm$ 0.1
	B	5.6 $\pm$ 1.2	0.4 $\pm$ 0.0	0.4 $\pm$ 0.1
bone	A	2.5 $\pm$ 0.3	1.7 $\pm$ 0.4	1.2 $\pm$ 0.3
	B	2.9 $\pm$ 1.5	2.7 $\pm$ 0.6	5.9 $\pm$ 1.9

cal animal and tumor weight conditions for both the labels. The ¹¹¹In radioconjugate very accurately predicted the blood clearance of the ⁸⁸Y conjugate with 11.3, 8.9, 4.9, 5.6, and 3.0% ID/g and 11.7, 9.3, 5.0, 5.7 and 3.2% ID/g at 1, 3, 5, 7, and 10 days postinjection, for the ¹¹¹In and ⁸⁸Y conjugates, respectively. As can be seen, the percent injected dose per gram and standard deviations for all other organs, particularly bone, at all time points were also remarkably similar. This finding is important in that at least one other study, with other mAb conjugates, has suggested that ¹¹¹In is not predictive of ⁹⁰Y biodistribution (45).

Tables 4 and 5 show animal data for [⁸⁸Y]DOTA-hLL2-F(ab')₂ and [⁸⁸Y]DOTA-hLL2-Fab', respectively, and in each case, biodistributions were determined with and without D-lysine administration. With radiometalated fragments, kidney uptake of the radiometal is a serious problem, and Behr et al. (20) and others (21) have shown that kidney radioactivity levels can be reduced by administering basic amino acids, such as lysine, with D-lysine recommended (20). Lysine dose selection and timing are in themselves problematic, as discussed by DePalatis et al. (21), in that timing of the lysine admin-

Table 5. Biodistributions of [⁸⁸Y]DOTA-hLL2-Fab' with Administration of 4 $\times$ 2 mg of D-Lysine per Gram of Animal Weight (A) and without D-Lysine Administration (B) in Nude Mice Bearing s.c. Ramos Human Tumor Xenografts

tissue		percent injected dose per gram $\pm$ SD, $n = 4$		
		4 h	24 h	48 h
Ramos tumor (g)	A	1.9 $\pm$ 0.9 (0.3 $\pm$ 0.3)	1.6 $\pm$ 0.2 (0.2 $\pm$ 0.1)	1.8 $\pm$ 0.7 (0.1 $\pm$ 0.03)
	B	1.9 $\pm$ 1.2 (0.2 $\pm$ 0.1)	2.1 $\pm$ 0.4 (0.2 $\pm$ 0.1)	1.1 $\pm$ 0.2 (0.3 $\pm$ 0.1)
liver	A	2.5 $\pm$ 0.5	1.8 $\pm$ 0.2	1.4 $\pm$ 0.2
	B	1.8 $\pm$ 0.8	2.1 $\pm$ 0.4	2.0 $\pm$ 0.4
spleen	A	1.3 $\pm$ 0.3	1.6 $\pm$ 0.2	1.1 $\pm$ 0.3
	B	2.0 $\pm$ 1.2	2.8 $\pm$ 0.5	2.1 $\pm$ 0.7
kidney	A	19.9 $\pm$ 11.5	29.1 $\pm$ 11.8	15.9 $\pm$ 4.8
	B	104 $\pm$ 24.7	105 $\pm$ 15.2	81.4 $\pm$ 4.4
lungs	A	1.5 $\pm$ 0.4	0.6 $\pm$ 0.1	0.4 $\pm$ 0.1
	B	1.6 $\pm$ 0.1	1.0 $\pm$ 0.2	3.1 $\pm$ 4.6
blood	A	2.0 $\pm$ 0.4	0.3 $\pm$ 0.02	0.2 $\pm$ 0.01
	B	1.4 $\pm$ 0.3	0.3 $\pm$ 0.03	0.2 $\pm$ 0.03
bone	A	0.8 $\pm$ 0.2	0.6 $\pm$ 0.3	0.4 $\pm$ 0.1
	B	1.0 $\pm$ 0.6	1.4 $\pm$ 0.5	1.0 $\pm$ 0.5

istration for Fab' and F(ab')₂ species is expected to be different. In our work, kidney levels decreased up to 5-fold in the case of the [⁸⁸Y]Fab' and somewhat less in the case of F(ab')₂, although kidney percent injected dose per gram then rose sharply between 4 and 24 h time points, from 19.89 to 29.11% ID/g for the Fab' and 19.93 to 39.31% ID/g for the F(ab')₂. The tendency to increased kidney percent injected dose per gram seems indicative of a need to continuously administer D-lysine beyond 3 h postinjection of the labels. For clinical applications, these D-lysine levels appear impractical. Even with lysine administration, dosimetry numbers remained adverse for both fragments at 1200, 5443, 17 129, and 1162 rads/mCi for [⁹⁰Y]DOTA-F(ab')₂ and 1536, 1497, 10 959, and 350 rads/mCi for [⁹⁰Y]DOTA-Fab' for Ramos tumor, liver, kidney and blood, respectively.

A MTD study was performed with ⁹⁰Y-labeled DOTA- and Bz-DTPA-IgG conjugates in normal BALB/c mice, using six increasing doses in the range 125–250 μ Ci. The MTD for both reagents was identical since all animals survived at a dose of 125 μ Ci, while with each reagent, three of seven died at the 150 μ Ci dose level. Two points are of significance here. First, these data differ from that

of DeNardo et al. (43) using radiolabeled Lym-1 antibody in that they found a significantly higher toxicity for their mAb when labeled with DTPA versus DOTA chelates. Second, the microcurie MTD amount of [^{90}Y]mAb using hLL2 in nude mice is much less than reported with other mAbs, which are usually in the 250 $\mu\text{Ci}/\text{animal}$ range (43).

The occurrence of immune response to DOTA chelate was first noticed (26) in the form of serum sickness in some patients in a phase I/II trial of i.p. therapy of ovarian cancer using a [^{90}Y]DOTA conjugate of murine mAb HMFG1 [[^{90}Y]2-IT-(s)-BAD-HMFG1]. In a detailed study of this phenomenon, Watanabe et al. (28) found that the carrier protein of the immunoconjugate played a significant role in eliciting anti-chelate responses. They found that when a rabbit IgG conjugate of the same bifunctional DOTA, in the form of ^{111}In -labeled conjugate, was injected into rabbits, no hapten immunogenicity was observed even when using a protein dose equivalent of 233 mg in 70 kg patient and a chelate/protein substitution ratio of 4.5:1. However, their study found the corresponding mouse IgG conjugate to be immunogenic when administered in rabbits. Watanabe et al. (28) concluded that the induction of chelate immunogenicity is observed when a conjugate of a foreign protein is administered, but not when the conjugate of same species mAb is used, and they further predicted that conjugates of humanized antibodies would be considerably less antigenic in humans than the murine forms.

In an experiment designed to test whether DOTA and/or Bz-DTPA chelators are immunogenic, we repeatedly (up to three times) injected BALB/c mice with conjugates using the respective murine LL2-IgG and murine-LL2 F(ab')₂ and tested serum for the presence of anti-chelate mAbs. There was no evidence of immune complex formation by HPLC after the addition of serum from these mice to trace amounts of radiolabeled mAbs. In terms of sensitivity, 100 ng of iodinated probe would be sufficient to form a 1:1 immune complex with anti-chelate-mAb entity, if present. We concluded that no immune responses were found, in mice, to murine mAb conjugate of either chelate, given three times over 9 weeks at protein doses equivalent to 100 mg/70 kg in humans. This finding supports the contention of Watanabe et al. (28) that chelates are substantially more immunogenic when attached to foreign antibodies compared to syngeneic immunoglobulins. It is encouraging that no immune response is seen in mice with murine mAb substituted with as much as four DOTA chelates per IgG.

CONCLUSIONS

We have optimized a procedure for simple DOTA conjugation of CDR-grafted monoclonal antibodies, leading to consistently >90% ^{90}Y incorporation in conjugate radiolabelings. Factors governing DOTA molar substitution ratios and optimized labelings were addressed. [^{88}Y]DOTA-hLL2 was more stable to challenge and had substantially lower bone uptake in Ramos lymphoma-bearing animals than [^{88}Y]Bz-DTPA-hLL2. In other properties such as cell binding and retention, MTD, and induction of anti-chelate response in animals, the reagents appeared to be similar. Whether DOTA conjugates will prove more useful than Bz-DTPA conjugates for ^{90}Y RAIT in humans can only be answered by a direct comparison of both in patients with similar disease burdens. It is our intention to develop [^{90}Y]DOTA-hLL2

and [^{90}Y]DOTA-hIMMU-14 for clinical RAIT applications against NHL and CEA-expressing solid tumors, respectively, due to their better stabilities, evidenced by lower bone uptake, in these preclinical studies.

ACKNOWLEDGMENT

We are grateful to Ms. Rosarito Aninipot for expert technical assistance. This work was supported in part by U.S. Public Health Service (PHS) Outstanding Investigator Grant CA 39841 from the National Institutes of Health (D.M.G.) and Grant CA 66348 under the SBIR program of the NIH (G.L.G.).

LITERATURE CITED

- (1) Corcoran, M. C., Eary, J. F., Bernstein, I., and Press, O. W. (1997) Radioimmunotherapy strategies for Non-Hodgkin's Lymphoma. *Ann. Oncol. (Suppl. 1)* 8, 133–138.
- (2) Kaminski, M. S., Zasadny, K. R., Francis, I. R., Fenner, M. C., Ross, C. W., Milik, A. W., Estes, J., Tuck, M., Regan, D., Fisher, S., Glenn, S. D., and Wahl, R. (1996) Iodine-131-anti-B1 radioimmunotherapy for B-cell lymphoma. *J. Clin. Oncol.* 14, 1974–1981.
- (3) Knox, S. J., Goris, M. L., Trisler, K., Negrin, R., Davis, T., Liles, T.-M., Grillo-López, A., Chinn, P., Varns, C., Ning, S.-C., Fowler, S., Deb, N., Becker, M., Marquez, C., and Levy, R. (1996) Yttrium-90-labeled anti-CD20 monoclonal antibody therapy of recurrent B-cell lymphoma. *Clin. Cancer Res.* 2, 457–470.
- (4) Press, O. W., Eary, J. F., Applebaum, F. R., Martin, P. J., Nelp, W. B., Glenn, S., Fisher, D. R., Porter, B., Matthews, D. C., Gooley, T., and Bernstein, I. D. (1995) Phase II trial of ^{131}I -B1 (anti-CD20) antibody therapy with autologous stem cell transplantation for relapsed B cell lymphomas. *Lancet* 346, 336–340.
- (5) DeNardo, G. L., Lamborn, K. R., Denardo, S. J., Goldstein, D. S., Dolber-Smith, E. G., Kroger, L. A., Larkin, E. C., and Shen, S. (1995) Prognostic factors for radioimmunotherapy in patients with B-lymphocytic malignancies. *Cancer Res.* 55, 5893s–5898s.
- (6) Juweid, M., Sharkey, R. M., Markowitz, A., Behr, T., Swayne, L. C., Dunn, R., Hansen, H. J., Shevitz, J., Leung, S.-O., Rubin, A. D., Herskovic, T., Hanley, D., and Goldenberg, D. M. (1995) Treatment of non-Hodgkin's lymphoma with radiolabeled murine, chimeric or humanized LL2, an anti-CD22 monoclonal antibody. *Cancer Res.* 55, 5899s–5907s.
- (7) Riethmüller, G., Schneider-Gädick, E., Schlimok, G., Schmieg, W., Raab, R., Höffken, K., Gruber, R., Pichlmaier, H., Hirche, H., Pichlmaier, R., Buggisch, P., and Witte, J.; German Cancer Aid 17–1A study group (1994) Randomized trial of monoclonal antibody for adjuvant therapy of resected Dukes C colorectal carcinoma. *Lancet* 343, 1177–1183.
- (8) Blumenthal, R. B., Sharkey, R. M., Natale, A. M., Kashi, R., Wong, G., and Goldenberg, D. M. (1994) Comparison of equitoxic radioimmunotherapy and chemotherapy in the treatment of human colonic cancer xenografts. *Cancer Res.* 54, 142–151.
- (9) Tschemelitsch, J., Barendswaard, E., Williams, Jr., C., Yao, T.-J., Cohen, A. M., Old, L. J., and Welt, S. (1997) Enhanced antitumor activity of combination radioimmunotherapy (^{131}I -labeled monoclonal antibody A33) with chemotherapy (fluorouracil). *Cancer Res.* 57, 2181–2186.
- (10) Khazaeli, M. B., Conry, R. M., and LoBuglio, A. F. (1994) Human immune response to monoclonal antibodies. *J. Immunother.* 15, 42–52.
- (11) Sharkey, R. M., Juweid, M., Shevitz, J., Behr, T., Dunn, R., Swayne, L. C., Wong, G. Y., Blumentahl, R. D., Griffiths, G. L., Siegal, J. A., Leung, S.-O., Hansen, H. J., and Goldenberg, D. M. (1995) Evaluation of a complementarity-determining region-grafted (humanized) anti-carcinoembryonic antigen monoclonal antibody in preclinical and clinical studies. *Cancer Res.* 55, 5935s–5945s.
- (12) Leung, S.-O., Goldenberg, D. M., Dion, A. S., Pellegrini, M. C., Shevitz, J., Shih, L. B., and Hansen, H. J. (1995) Construction and characterization of a humanized, internal-

- izing, B-cell (CD22)-specific, leukemia/lymphoma antibody, LL2. *Mol. Immunol.* 32, 1413–1427.
- (13) Schubiger, P. A., Alberto, R., and Smith, A. (1996) Vehicles, chelators and radionuclides: Choosing the 'building blocks' of an effective therapeutic radioimmunoconjugate. *Bioconjugate Chem.* 7, 165–179.
- (14) Buchegger, F., Pelegrin, A., Delaloye, B., Bischof-Delaloye, A., and Mach, J. P. (1990) Iodine-131-labeled MAbs F(ab')₂ fragments are more efficient and less toxic than intact anti-CEA antibodies in radioimmunotherapy of large human colon carcinoma grafted in nude mice. *J. Nucl. Med.* 31, 1035–1044.
- (15) Kobayashi, H., Yoo, T. M., Drumm, D., Kim, M.-K., Sun, B.-F., Le, N., Webber, K. O., Pastan, I., Waldmann, T. A., Paik, C. H., and Carrasquillo, J. A. (1997) Improved biodistribution of ¹²⁵I-labeled anti-Tac disulfide-stabilized Fv fragment by blocking its binding to the α subunit of the interleukin 2 receptor in the circulation with preinjected humanized anti-Tac IgG. *Cancer Res.* 57, 1955–1961.
- (16) King, D. A., Turner, A., Farnsworth, A. P. H., Adair, J. R., Owens, R. J., Pedley, R. B., Baldock, D., Proudfoot, K. A., Lawson, A. D. G., Beeley, N. R. A., Millar, K., Millican, T. A., Boyce, B. A., Antinow, P., Mountain, A., Begent, R. H. J., Shochat, D., and Yarranton, G. T. (1994) Improved tumor targeting with chemically cross-linked recombinant antibody fragments. *Cancer Res.* 54, 6176–6185.
- (17) Sharkey, R. M., Behr, T., Mattes, M. J., Stein, R., Griffiths, G. L., Shih, L. B., Hansen, H. J., Blumenthal, R. B., Dunn, R. M., Juweid, M. E., and Goldenberg, D. M. (1997) Advantage of residualizing radiolabels for an internalizing antibody against the B-cell lymphoma antigen, CD22. *Cancer Immunol. Immunother.* 44, 179–188.
- (18) Press, O. W., Shan, D., Howell-Clark, J., Eary, J., Appelbaum, F. R., Matthews, D., King, D. J., Haines, A. M. R., Hamann, P., Hinman, L., Shochat, D., and Bernstein, I. D. (1996) Comparative metabolism and retention of iodine-125, yttrium-90, and indium-111 radioimmunoconjugates by cancer cells. *Cancer Res.* 56, 2123–2129.
- (19) Sharkey, R. M., Motta-Hennessy, C., Pawlyk, D., Siegal, J. A., and Goldenberg, D. M. (1990) Biodistribution and radiation dose estimates for yttrium- and iodine-labeled monoclonal antibody IgG and fragments in nude mice bearing human colonic tumor xenografts. *Cancer Res.* 50, 2330–2336.
- (20) Behr, T., Sharkey, R. M., Juweid, M., Blumenthal, R. D., Dunn, R. M., Griffiths, G. L., Bair, H.-J., Wolf, F. G., Becker, W. S., and Goldenberg, D. M. (1995) Reduction in the renal uptake of radiolabeled monoclonal antibody fragments by cationic amino acids and their derivatives. *Cancer Res.* 55, 3825–3834.
- (21) DePalatis, L. R., Frazier, K. A., Cheng, R. C., and Kotite, N. J. (1995) Lysine reduces renal accumulation of radioactivity associated with injection of the [¹⁷⁷Lu]- α -[2-(4-aminophenyl)ethyl]-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid-CC49 Fab radioimmunoconjugate. *Cancer Res.* 55, 5288–5295.
- (22) Li, M., Meares, C. F., Zhong, G.-R., Miers, L., Xiong, C.-Y., DeNardo, S. J. (1994) Labeling monoclonal antibodies with ⁹⁰yttrium- and ¹¹¹indium-DOTA chelates: A simple and efficient method. *Bioconjugate Chem.* 5, 101–104.
- (23) Lewis, M. R., Raubitschek, A., and Shively, J. E. (1994) A facile, water-soluble method for the modification of proteins with DOTA. Use of elevated temperature and optimized pH to achieve high specific activity and high chelate stability in radiolabeled conjugates. *Bioconjugate Chem.* 5, 565–576.
- (24) Stimmel, J. B., Stockstill, M. E., and Kull, F. C., Jr. (1995) Yttrium-90 chelation properties of tetraazatetraacetic acid macrocycles, diethylenetriaminepentaacetic acid analogues, and a novel terpyridine acyclic chelator. *Bioconjugate Chem.* 6, 219–225.
- (25) DeNardo, S. J., Kramer, E. L., O'Donnell, R. T., Richman, C. M., Salako, Q. A., Shen, S., Noz, M., Glenn, S. D., Ceriano, R. L., and DeNardo, G. L. (1997) Radioimmunotherapy for breast cancer using indium-111/yttrium-90 BrE-3: Results of a phase I clinical trial. *J. Nucl. Med.* 38, 1180–1185.
- (26) Kosmas, C., Snook, D., Gooden, C. S., Courtenay-Luck, C. S., McCall, M. J., Meares, C. F., and Epenetos, A. A. (1992) Development of humoral immune responses against a macrocyclic chelating agent (DOTA) in cancer patients receiving radioimmunoconjugates for imaging and therapy. *Cancer Res.* 52, 904–911.
- (27) Kosmas, C., Maraveyas, A., Gooden, C. S., Snook, D., and Epenetos, A. A. (1995) Anti-chelate antibodies after intraperitoneal yttrium-90 labeled monoclonal antibody immunoconjugates for ovarian cancer therapy. *J. Nucl. Med.* 36, 746–753.
- (28) Watanabe, N., Goodwin, D. A., Meares, C. F., McTigue, M., Chaovapong, W., Ransone, C. McK., Renn, O. (1994) Immunogenicity in rabbits and mice of an antibody-chelate conjugate: Comparison of (S) and (R) macrocyclic enantiomers and an acyclic chelating agent. *Cancer Res.* 54, 1049–1054.
- (29) Sharkey, R. M., Motta-Hennessy, C., Gansow, O. A., Brechbiel, M. W., Fand, I., Griffiths, G. L., Jones, A. L., and Goldenberg, D. M. (1990) Selection of a DTPA chelate conjugate for monoclonal antibody targeting to a human colonic tumor in nude mice. *Int. J. Cancer* 46, 79–85.
- (30) Lechner, P. K., Akabani, G., Colcher, D., Harrison, K. A., Hawkins, W. G., Eckblade, M., Baranowska-Kortylewicz, J., Augustine, S. C., Wisecarver, J., and Tempero, M. A. (1997) Patient-specific dosimetry of indium-111- and yttrium-90-labeled monoclonal antibody CC49. *J. Nucl. Med.* 38, 512–516.
- (31) Meares, C. F., McCall, M. J., Reardan, D. T., Goodwin, D. A., Diamanti, C. I., and McTigue, M. (1984) Conjugation of antibodies with bifunctional chelating agents: Isothiocyanate and bromoacetamide reagents, methods of analysis, and subsequent addition of metal ions. *Anal. Biochem.* 142, 68–78.
- (32) Losman, M. J., Leung, S. O., Shih, L. B., Shevitz, J., Shukla, R., Haraga, L., Goldenberg, D. M., and Hansen, H. J. (1995) Development and evaluation of the specificity of a rat monoclonal anti-idiotypic antibody, WN, to an anti-B-cell lymphoma monoclonal antibody, LL2. *Cancer Res.* 55, 5978s–5982s.
- (33) Greenwood, F., and Hunter, W. (1963) The preparation of ¹³¹I-labeled human growth hormone of high specific radioactivity. *Biochem. J.* 89, 114–123.
- (34) Hnatowich, D. J., Childs, R. L., Lantaigne, D., and Najafi, A. (1983) The preparation of DTPA-coupled antibodies radiolabeled with metallic radionuclides: an improved method. *J. Immunol. Methods* 65, 147–157.
- (35) Brechbiel, M. W., and Gansow, O. A. (1991) Backbone-substituted DTPA ligands for ⁹⁰Y radioimmunotherapy. *Bioconjugate Chem.* 2, 187–194.
- (36) Kozak, R. W., Raubitschek, A., Mirzadeh, S., Brechbiel, M. W., Junghans, R., Gansow, O. A., and Waldmann, T. A. (1989) Nature of the bifunctional chelating agent used for radioimmunotherapy with yttrium-90 monoclonal antibodies: Critical factors in determining in vivo survival and organ toxicity. *Cancer Res.* 49, 2639–2644.
- (37) Moi, M. K., Meares, C. F., and DeNardo, S. J. (1988) The peptide way to macrocyclic bifunctional chelating agents: Synthesis of 2-(p-nitrobenzyl)-1,4,7,10-tetraazacyclododecane-N,N',N''-tetraacetic acid and study of its yttrium(III) complex. *J. Am. Chem. Soc.* 110, 6266–6267.
- (38) Li, M., and Meares, C. F. (1993) Synthesis, metal chelate stability studies and enzyme digestion of a peptide-linked DOTA derivative and its corresponding radiolabeled immunoconjugates. *Bioconjugate Chem.* 4, 275–283.
- (39) Chakrabarti, M. C., Le, N., Paik, C. H., De Graff, W. G., and Carrasquillo, J. A. (1996) Prevention of radiolysis of monoclonal antibody during labeling. *J. Nucl. Med.* 37, 1384–1388.
- (40) Griffiths, G. L., Goldenberg, D. M., Donnelly, J., Karacay, H., Govindan, S. V., Shih, L., and Hansen, H. J. (1996) Enhanced retention of radionuclides by B-lymphoma cells using a yttrium-90-labeled, cdr-grafted, internalizing monoclonal antibody. *J. Nucl. Med.* 37, 151 (abstract).
- (41) Franano, F. N., Edwards, W. B., Welch, M. J., and Duncan, J. R. (1994) Metabolism of receptor targeted ¹¹¹In-DTPA-glycoproteins: Identification of ¹¹¹In-DTPA- ϵ -lysine as the

- primary metabolic and excretory product. *Nucl. Med. Biol.* 8, 1023–1034.
- (42) Mattes, M. J., Shih, L. B., Govindan, S. V., Sharkey, R. M., Ong, G. L., Xuan, H., and Goldenberg, D. M. (1997) The advantage of residualizing radiolabels for targeting B-cell lymphomas with a radiolabeled anti-CD22 monoclonal antibody. *Int. J. Cancer* 71, 429–435.
- (43) DeNardo, G. L., Kroger, L. A., DeNardo, S. J., Miers, L. A., Salako, Q., Kukis, D. L., Fand, I., Shen, S., Renn, O., and Meares, C. F. (1994) Comparative toxicity studies of yttrium-90 MX-DTPA and 2-IT-BAD conjugated monoclonal antibody (BrE-3). *Cancer* 73, 1012–1022.
- (44) Siegel, J. A., and Stabin, M. G. (1988) Absorbed fractions for electrons and beta particles in small spheres. *J. Nucl. Med.* 29, 803.
- (45) Carrasquillo, J. A., White, J. D., Paik, C. H., Le, N., Rotman, M., Goldman, C. K., Brechbiel, M. W., Gansow, O. A., Top, L. E., Reynolds, J. C., Nelson, D. L., and Waldmann, T. A. (1996) Biodistribution of Indium-111 vs Yttrium-90 labeled anti-TAC monoclonal antibody. *J. Nucl. Med.* 37, 234P (abstract).

BC980040G

Cholesterol Phosphate Derivatives: Synthesis and Incorporation into a Phosphatase and Calcium-Sensitive Triggered Release Liposome

S. C. Davis and F. C. Szoka Jr.*

Department of Biopharmaceutical Sciences and Pharmaceutical Chemistry, School of Pharmacy, University of California, San Francisco, California 94143-0446. Received May 6, 1998;
Revised Manuscript Received August 27, 1998

A series of cholesterol derivatives that position a phosphate monoester at increasing distance from the sterol ring system was synthesized, and their utility as a triggered release liposome tested. Stable anionic liposomes consisting of the novel cholesterol phosphate derivatives and dioleoylphosphatidylethanolamine (DOPE) can be induced to collapse upon phosphatase-catalyzed removal of the phosphate group. Control liposomes containing DOPE and cholesterol phosphate or phosphatidic acid, which are not phosphatase substrates, do not undergo phosphatase-mediated collapse. The phosphatase-sensitive liposomes also collapse in the presence of calcium. The precise concentration of calcium that induces the collapse is controlled by the structure of the cholesterol phosphate derivative. Plasmid DNA encoding luciferase, encapsulated in the cholesterol derivative/DOPE liposomes, transfected cells in vitro. The level of transfection is dependent upon the cholesterol derivative and is mediated by both a calcium-independent and a calcium-dependent pathway; however, the involvement of phosphatase in the latter mechanism is not yet resolved. The transfection efficiency is between 10^6 and 10^7 of luciferase activity in relative light units per milligram of protein, which is similar to transfection values reported using other triggered release liposomes.

INTRODUCTION

One approach to the delivery of drugs and DNA to the cytoplasm of cells is to use triggered release liposomes (1, 2). Such liposomes are stable under normal physiological conditions but release their contents upon receipt of a defined stimulus (1). This allows the controlled delivery of the liposome contents to the site of the stimulus, either intracellularly or at defined sites in the body.

Triggered release liposomes rely on the ability of lipids to interconvert among different phases in aqueous media. The two phases most commonly utilized in triggered release liposomes are the lamellar and the inverted hexagonal phase. When the lipids prefer the lamellar phase, liposomes are formed which are capable of encapsulating exogenous material. When the lipids form an inverted hexagonal phase, no vesicle structure is possible.

A commonly employed lipid for triggered release compositions is dioleoylphosphatidylethanolamine (DOPE)¹ which prefers the inverted hexagonal phase under physiological conditions (3) but can be stabilized in the lamellar phase by the addition of at least 25 mole percent of a charged colipid. Removal of the charge from

the colipid is sufficient to induce the phase change. Several different strategies have been used for the controlled removal of charge from the surface of a phosphatidylethanolamine/colipid bilayer, including pH change [e.g., cholesterol hemisuccinate, CHEMS (4) and oleic acid (5)], enzyme catalysis [e.g., phospholipase C or acetylcholine esterase (6)], and light [cleavage of plasmalogen vinyl ethers (7)].

We hypothesized that lipids with a doubly negatively charged phosphate monoester headgroup (similar to phosphatidic acids) would stabilize phosphatidylethanolamine bilayers. Two mechanisms for the triggered release of the liposomes could then be envisaged, enzymatic and ionic. Phosphatase enzymes which catalyze the hydrolysis of phosphate monoesters could remove the phosphate and its corresponding charge from the bilayer surface, thus allowing collapse to the hexagonal phase and release of the liposome contents. Two examples of this class of enzymes are alkaline phosphatase (8, 9), which is a widely specific, highly active enzyme found in serum and on the cell membrane, and acid phosphatase, which is found intracellularly in endosomes and lysosomes (10). Alkaline phosphatase has long been utilized in the area of pro-drugs to convert inactive phosphate derivatives into the active compounds (11). Recently, this enzyme has been shown to inactivate endotoxin by removing the phosphate from this lipopolysaccharide (12). This suggested that alkaline phosphatase would be capable of hydrolyzing phosphate from a lipid derivative. Second, divalent cations such as calcium are well-known to cause aggregation and fusion (13) of anionic liposomes, and in this phosphate containing system, the calcium effect may trigger the collapse to the hexagonal phase (14).

* Address correspondence to this author. Fax: (415) 476 0688. Telephone: (415) 476 3895. E-mail: szoka@cgl.ucsf.edu.

¹ Abbreviations: DOPE, dioleoylphosphatidylethanolamine; PE, phosphatidylethanolamine; HEPES, (hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; HBS, HEPES buffered saline; ANTS, 8-aminonaphthalene-1,2,3-trisulfonic acid; DPX, *p*-xylenebis(pyridinium)bromide; CHEMS, cholesterol hemisuccinate; REV, reversed-phase evaporation vesicles; RLU, relative light units; I. U., international unit; sat, saturated aqueous solution; POPG, palmitoyl-*sn*-3-phosphatidylglycerol.

We show here that novel cholesterol phosphate derivatives can be incorporated into PE liposomes, confer calcium and phosphatase sensitivity, and can be used to trigger the release of liposome contents.

EXPERIMENTAL SECTION

General. All purchased chemicals were of ACS grade or better and were used without further purification. Solvents for HPLC analysis were obtained as HPLC grade and were filtered (0.2 μ M) and degassed before use. NMR spectra were recorded on a GE 300 MHz instrument and chemical shifts are expressed as parts per million using as internal standard, tetramethylsilane (δ = 0.0 ppm) for ^1H NMR and CDCl_3 (δ = 77.0 ppm) for ^{13}C NMR. Optical rotation measurements were taken on a Perkin-Elmer 241 Polarimeter using the sodium line. IR measurements were taken on a Nicolet Impact 400 as a film on a 3M Type 62 disposable IR card. Calf intestinal alkaline phosphatase and DNase 1 were purchased from Boehringer Mannheim. Acid phosphatase (Sweet potato) was purchased from Sigma (St. Louis, MO).

Cholest-5-en-3 β -tosylate (1). To a solution of cholesterol (5 g, 12.9 mmol) in anhydrous pyridine (70 mL) was added *p*-toluenesulfonyl chloride (5 g, 26 mmol), and the mixture was stirred for 16 h under an inert atmosphere at room temperature. The solvent was filtered off, and the white mass washed with pyridine (50 mL) and water (100 mL). Recrystallization from chloroform/methanol gave the product as white crystals (6.5 g, 12 mmol, 93%). R_f = 0.8 (CHCl_3). ^1H (300 MHz, CDCl_3): δ 0.65 (3H, s); 0.85–1.57 (33H, m); 1.78–2.01 (6H, m); 2.23–2.29 (2H, m); 2.44 (3H, s); 4.28–4.33 (1H, m); 5.29 (1H, d, J = 4.5 Hz); 7.32 (2H, d, J = 8 Hz); 7.79 (2H, d, J = 8 Hz). ^{13}C (75 MHz, CDCl_3): δ 11.77, 18.66, 19.11, 21.59, 22.52, 22.78, 23.77, 24.20, 27.96, 28.16, 28.58, 31.70, 31.81, 35.71, 36.12, 36.30, 36.83, 38.84, 39.46, 39.61, 42.25, 49.86, 56.05, 56.59, 82.15, 123.68, 127.58, 129.69, 134.65, 138.79, 144.34. LSIMS: m/z 369 ($\text{M} - \text{tosylate}$, 100%).

Cholest-5-en-3 β -oxyethan-2-ol (2a). To a suspension of cholest-5-en-3 β -tosylate (**1**) (500 mg, 0.9 mmol) in anhydrous dioxane (9 mL) was added ethylene glycol (1 g, 23.4 mmol), and the mixture was stirred under reflux for 4 h in an inert atmosphere. The solution was cooled, and the solvent removed in vacuo. The white residue was partitioned between diethyl ether (20 mL) and water (20 mL), and the organic layer was washed sequentially with sat NaHCO_3 (2 $\times$ 10 mL), water (10 mL) and sat brine (10 mL). The solution was dried over magnesium sulfate, and the solvent removed in vacuo. Recrystallization of the residue from chloroform/methanol gave the product as white crystals (350 mg, 0.81 mmol, 88%). Mp 97–98 $^\circ\text{C}$. Anal ($\text{C}_{29}\text{H}_{50}\text{O}_2$) C, H. R_f = 0.3 (CHCl_3). α_D^{20} (c = 0.55, CHCl_3) -31.6° [lit. -30.95° (15)]. IR (film): 3369 (m, br, O–H), 2930 (st, C–H), 2866 (st, C–H), 1466 (m, C=C), 1380 (m). ^1H (300 MHz, CDCl_3): δ 0.69 (3H, s); 0.86–1.57 (33H, m); 1.78–2.04 (6H, m); 2.19–2.22 (2H, m); 2.35–2.37 (1H, m); 3.17–3.21 (1H, m); 3.60 (2H, t, J = 4.5 Hz); 3.73 (2H, t, J = 4.5 Hz); 5.36 (1H, d, J = 4.5 Hz). ^{13}C (75 MHz, CDCl_3): δ 11.84, 18.70, 19.35, 21.06, 22.54, 22.79, 23.82, 24.27, 28.00, 28.21, 28.41, 31.57, 31.90, 35.77, 36.18, 36.85, 37.17, 39.10, 39.51, 39.78, 42.31, 50.18, 56.16, 56.76, 62.08, 68.92, 121.71, 140.71. EI^+ : m/z 429 ($\text{M} - \text{H}^+$, 12%); 369 (100%). EI–HRMS $\text{C}_{29}\text{H}_{50}\text{O}_2$: Calcd, 430.3811; found, 430.3800.

Cholest-5-en-3 β -oxyhexan-6-ol (2b). This compound was prepared similarly to cholest-5-en-3 β -oxyethan-2-ol

(**2a**) with 1,6-hexanediol to give a white powder that was recrystallized from chloroform/methanol (810 mg, 1.66 mmol, 83%). Anal. ($\text{C}_{33}\text{H}_{58}\text{O}_2$) C, H. R_f = 0.3 (CHCl_3). α_D^{20} (c = 1.25, CHCl_3) -26.9° . IR (film): 3300 (m, br, O–H), 2928 (st, C–H), 2860 (st, C–H), 1463 (m, C=C), 1371 (m). ^1H (300 MHz, CDCl_3): δ 0.65 (3H, s); 0.82–1.53 (41H, m); 1.74–2.00 (6H, m); 2.10–2.19 (1H, m); 2.29–2.33 (1H, m); 3.05–3.10 (1H, m); 3.42 (2H, t, J = 6.5 Hz); 3.57 (2H, t, J = 6.5 Hz); 5.30 (1H, d, J = 4.5 Hz). ^{13}C (75 MHz, CDCl_3): δ 11.94, 18.71, 19.39, 21.04, 22.61, 22.78, 23.94, 24.56, 25.60, 26.04, 27.93, 28.27, 28.60, 28.70, 30.13, 32.11, 32.71, 35.62, 35.76, 36.90, 37.66, 39.38, 39.50, 42.31, 50.18, 56.14, 56.78, 62.96, 67.97, 120.64, 141.12. $^+\text{LSIMS}$: m/z 486 (9%, M^+); 369 (100%). EI–HRMS $\text{C}_{33}\text{H}_{58}\text{O}_2$: calcd, 486.4437; found, 486.4423.

Cholest-5-en-3 β -oxypent-3-oxa-an-5-ol (2c). This compound was prepared similarly to cholest-5-en-3 β -oxyethan-2-ol (**2a**) with diethylene glycol to give an oil that was purified on silica eluting with chloroform to give a gum (830 mg, 1.75 mmol, 87%). Anal. ($\text{C}_{31}\text{H}_{54}\text{O}_3$) C, H. R_f = 0.15 (CHCl_3). α_D^{20} (c = 1.06, CHCl_3) -28.5° . IR (film): 3407 (m, br, O–H), 2929 (st, C–H), 2861 (st, C–H), 1462 (m, C=C), 1366 (m). ^1H (300 MHz, CDCl_3): δ 0.63 (3H, s); 0.81–1.55 (32H, m); 1.76–1.99 (6H, m); 2.17–2.21 (1H, m); 2.30–2.37 (1H, m); 3.15–3.28 (2H, m); 3.53–3.69 (8H, m); 5.29 (1H, d, J = 4.5 Hz). ^{13}C (75 MHz, CDCl_3): δ 11.77, 18.64, 19.29, 20.99, 22.48, 22.74, 23.77, 24.20, 27.91, 28.15, 28.23, 31.83, 35.70, 36.11, 36.76, 37.12, 38.89, 39.43, 39.70, 42.25, 50.09, 56.10, 56.68, 51.70, 67.32, 70.67, 72.51, 79.52, 121.60, 140.63. $^+\text{LSIMS}$: m/z 473 (10%, $\text{M} - \text{H}$); 369 (100%). EI–HRMS $\text{C}_{31}\text{H}_{54}\text{O}_3$: calcd, 474.4073; found, 474.4059.

Cholest-5-en-3 β -oxy-oct-3,6-oxa-an-8-ol (2d). This compound was prepared similarly to cholest-5-en-3 β -oxyethan-2-ol (**2a**) with triethylene glycol to give an oil that was purified on silica eluting with chloroform to give a gum (450 mg, 0.87 mmol, 94%). R_f = 0.15 (CHCl_3). α_D^{20} (c = 0.69, CHCl_3) -29.3° [lit. -25.38° (15)]. IR (film): 3425 (m, br, O–H), 2932 (st, C–H), 2859 (st, C–H), 1460 (m, C=C), 1376 (m). ^1H (300 MHz, CDCl_3): δ 0.85–1.60 (33H, m); 1.79–2.06 (6H, m); 2.18–2.29 (1H, m); 2.37–2.41 (1H, m); 3.16–3.23 (1H, m); 3.60–3.78 (12H, m); 5.37 (1H, d, J = 4.5 Hz). ^{13}C (75 MHz, CDCl_3): δ 11.76, 18.63, 19.27, 20.97, 22.47, 22.72, 23.74, 24.19, 27.90, 28.14, 28.18, 31.79, 31.84, 35.69, 36.10, 36.75, 37.11, 38.87, 39.42, 39.69, 42.21, 50.08, 56.07, 56.67, 61.57, 66.96, 67.08, 70.18, 70.47, 70.71, 72.58, 79.47, 121.52, 140.71. EI^+ : m/z 517 ($\text{M} - \text{H}^+$, 7%); 383 (21%); 369 (100%). EI–HRMS $\text{C}_{33}\text{H}_{58}\text{O}_4$: calcd, 518.4335; found, 518.4315.

Cholest-5-en-3 β -oxyethane-2-phosphate (3a). To a stirred solution of cholest-5-en-3 β -oxyethan-2-ol (**2a**) (86 mg, 0.2 mmol) in anhydrous chloroform (0.5 mL) was added a solution of tetra-*n*-butylammonium phosphate (340 mg, 1 mmol) in chloroform (0.5 mL) immediately followed by addition of trichloroacetonitrile (120 μL , 1.2 mmol). The solution was stirred under an inert atmosphere for 15 min and the solvent was removed in vacuo. The residue was dissolved in water saturated *n*-butanol (5 mL), and the solution washed sequentially with *n*-butanol-saturated water (2 $\times$ 2 mL), *n*-butanol-saturated 1 M HCl (2 $\times$ 2 mL), and sat brine (2 mL). The organic phase was dried over magnesium sulfate and filtered, and the solvent removed in vacuo. The residue was dissolved in chloroform (2 mL), and sodium iodide (150 mg, 1 mmol) in acetone (2 mL) was added. After 15 min, the precipitate was separated by centrifugation, the solvent decanted, and the precipitate washed with chloroform (2 $\times$ 2 mL) to yield the product as the sodium

salt (80 mg, 0.14 mmol, 72%). (Cholesterol C₂ phosphate) $R_f = 0.35$ (CHCl₃/MeOH/H₂O 65/25/4). IR (film): 2924 (st, C-H), 2844 (st, C-H), 2365 (st, PO-H), 2340 (st, PO-H), 1650 (m), 1457 (m, C=C). ¹H (300 MHz, CDCl₃/MeOD/*d*₅-pyridine/DCI 10/2/1/1): (16): δ 0.70 (3H, s), 0.83–1.60 (33H, m); 1.83–2.05 (6H, m); 2.16–2.18 (1H, m); 2.33–2.38 (1H, m); 3.20–3.33 (1H, m); 3.74 (2H, t, $J = 4.5$ Hz); 4.17 (2H, t, $J = 4.5$ Hz), 5.36–5.38 (1H, m). ¹³C (75 MHz, CDCl₃/MeOD/*d*₅-pyridine/DCI 10/2/1/1): δ 11.17, 18.03, 18.61, 20.46, 21.80, 22.04, 23.19, 23.65, 27.35, 27.59, 31.31, 35.16, 35.58, 36.18, 36.51, 38.26, 38.90, 39.19, 41.74, 49.62, 55.60, 56.18, 65.81, 66.52, 79.19, 121.39, 139.77. ⁻LSIMS: m/z 509 (M - H⁻, 100%).

Cholest-5-en-3 β -oxyhexane-6-phosphate (3b). This compound was prepared similarly to cholest-5-en-3 β -oxyethane-2-phosphate (3a) to yield a white powder (75 mg, 0.13 mmol, 66%). $R_f = 0.35$ (CHCl₃/MeOH/H₂O 65/25/4). IR (film): 2930 (st, C-H), 2856 (st, C-H), 2359 (st, PO-H), 2327 (st, PO-H), 1656 (m), 1451 (m, C=C). ¹H (300 MHz, CDCl₃/MeOD/*d*₅-pyridine/DCI 10/2/1/1): δ 0.72 (3H, s), 0.78–1.69 (33H, m); 1.84–2.07 (6H, m); 2.19–2.24 (1H, m); 2.34–2.37 (1H, m); 3.17–3.19 (1H, m); 3.46–3.52 (2H, m); 3.99–4.09 (2H, m), 5.38 (1H, d, $J = 4$ Hz). ¹³C (75 MHz, CDCl₃/MeOD/*d*₅-pyridine/DCI 10/2/1/1): δ 10.77, 17.66, 18.25, 20.01, 21.41, 21.67, 22.66, 23.21, 23.90, 24.29, 24.70, 26.85, 27.14, 30.86, 34.66, 35.08, 35.78, 36.18, 38.08, 38.40, 38.72, 41.26, 49.14, 55.08, 55.70, 66.90, 78.05, 120.57. ⁻ESI: m/z 565 (M - H⁻, 26%).

Cholest-5-en-3 β -oxypent-3-oxa-ane-5-phosphate (3c). This compound was prepared similarly to cholest-5-en-3 β -oxyethane-2-phosphate (3a) to yield a white powder (55 mg, 0.14 mmol, 50%). $R_f = 0.35$ (CHCl₃/MeOH/H₂O 65/25/4). IR (film): 2929 (st, C-H), 2856 (st, C-H), 2359 (st, PO-H), 2342 (st, PO-H), 1662 (m), 1463 (m, C=C). ¹H (300 MHz, CDCl₃/MeOD/*d*₅-pyridine/DCI 10/2/1/1): δ 0.68 (3H, s); 0.68–1.57 (33H, m); 1.77–2.06 (6H, m); 2.16–2.24 (1H, m); 2.31–2.37 (1H, m); 3.16–3.20 (1H, m); 3.64–3.71 (8H, m); 4.08–4.12 (2H, m); 5.33 (1H, d, $J = 4$ Hz). ¹³C (75 MHz, CDCl₃/MeOD/*d*₅-pyridine/DCI 10/2/1/1): δ 11.43, 18.29, 18.41, 18.89, 20.64, 22.09, 22.33, 23.40, 23.85, 27.54, 27.79, 31.48, 35.25, 35.76, 36.38, 36.72, 38.24, 38.52, 39.07, 41.90, 49.56, 49.75, 55.76, 56.33, 64.60, 66.64, 70.16, 79.21, 121.43, 140.11. ⁻LSIMS: m/z 554 (M - H⁻, 28%).

Cholest-5-en-3 β -oxy-oct-3,6-oxa-ane-8-phosphate (3d). This compound was prepared similarly to cholest-5-en-3 β -oxyethane-2-phosphate (3a) to yield a white powder (65 mg, 0.11 mmol, 55%). Cholesterol triethylene phosphase $R_f = 0.35$ (CHCl₃/MeOH/H₂O 65/25/4). IR (film): 2932 (st, C-H), 2861 (st, C-H), 2361 (st, PO-H), 2338 (st, PO-H), 1647 (m), 1456 (m, C=C). ¹H (300 MHz, CDCl₃/MeOD/*d*₅-pyridine/DCI 10/2/1/1): δ 0.68 (3H, s), 0.86–1.60 (33H, m); 1.77–2.04 (6H, m); 2.12–2.19 (1H, m); 2.31–2.36 (1H, m); 3.14–3.19 (1H, m); 3.61–3.70 (12H, m); 4.12–4.15 (2H, m), 5.33 (1H, d, $J = 5$ Hz). ¹³C (75 MHz, CDCl₃/MeOD/*d*₅-pyridine/DCI 10/2/1/1): δ 11.48, 18.33, 18.96, 20.68, 22.17, 22.43, 23.45, 23.91, 27.63, 27.86, 31.50, 35.41, 35.81, 36.44, 36.75, 38.56, 39.13, 39.37, 41.93, 49.33, 49.77, 55.77, 56.36, 65.35, 66.67, 69.35, 70.27, 76.22, 79.02, 79.44, 121.39. ⁻LSIMS: m/z 597 (M - H⁻, 38%).

Leakage Studies. A premixed solution of cholesterol and DOPE (5/4 molar ratio) was evaporated to a lipid film, and the film was exposed to high vacuum (<150 mTorr) for at least 2 h before use. The lipids were hydrated in 20 mM HEPES, 12.5 mM ANTS, and 45 mM DPX at pH 7.0 with vortexing and brief sonication (17). The liposomes thus formed were then extruded through

a hand held extrusion device (Avestin, Ottawa, Canada) with a 0.2 μ m polycarbonate membrane (Nucleopore Corp., Pleasanton, CA). The liposomes were applied to a Sephadex G50 gel filtration column (1 $\times$ 20 cm) to remove any unencapsulated dye. The liposome concentration was quantified using the phosphorus assay of Bartlett (18). The dequenching of ANTS upon liposome leakage was measured according to Ellens et al. (17). The fluorescence arising from excitation at 360 nm was measured through a Schott GG 435 nm cutoff filter (50% transmittance at 435 nm). Leakage experiments were performed at room temperature in triplicate for each condition, and each cuvette (Polymethacrylate Fluorimeter cuvette, Sigma, St. Louis, MO) was stirred and analyzed separately. The initial fluorescence of the intact liposomes at time 0 was measured and set as F_0 , or 0% leakage. The fluorescence for each cuvette was measured at the times indicated after addition of the enzyme. At the end of the time course F_{100} , 100% leakage was defined by the fluorescence arising from addition of the detergent, dodecyl octaethylene glycol monoether (C12E8) (Calbiochem, La Jolla, CA). The percent leakage at time t was determined for each cuvette using the formula $(F_t - F_0 / F_{100} - F_0)100$, and then the results were combined. Leakage experiments with calcium were performed sequentially with timepoints measured every second, and the percentage of F_{100} observed at the plateau point was calculated using the same formula as before. Lipid-mixing experiments were performed according to the method of Struck et al. (19). NBD-PE (0.5 mol %) and 0.5 mol % Rhodamine-PE were incorporated into cholesterol C2 phosphate (3a)/DOPE liposomes, and these were mixed with unlabeled liposomes (9-fold excess). Lipid mixing was followed using the increase of fluorescence at 535 nm upon addition of calcium. Values in the figures are the mean $\pm$ standard deviation from three determinations.

Identification of the Products of Reaction. One micromole of cholesterol C2 phosphate (3a)/DOPE (4/5) liposomes in HEPES buffered saline (10 mM HEPES and 150 mM NaCl, pH 7.4) (10 mL) was incubated with 0, 20, and 100 I. U. of alkaline phosphatase in triplicate. The samples were placed in sealed tubes and mixed by slow rotation ($\sim$ 50 rpm) for 24 h at room temperature and the incubation stopped by freezing and lyophilization. The residue was redissolved in water (1 mL), and the lipids were extracted using the Bligh and Dyer protocol (20) and dried down separately for HPLC analysis. The aqueous phase was lyophilized, taken up in water (300 μ L) and the phosphate concentration measured using a modification of the method of Drueckes et al. (21). Thirty microliters of this solution was mixed with the assay mixture (125 μ L) and incubated at 30 $^{\circ}$ C for 10 min, and the absorbance measured at 630 nm. To form the assay mixture, ammonium molybdate (10 mM final) was dissolved in a solution of zinc acetate (15 mM) at pH 5.0. This was mixed with a 10% ascorbate solution, pH 5.0, and a solution of sodium dodecyl sulfate (200 mg/mL) in a 40:10:1 ratio. The assay mixture was used immediately.

The lipids isolated in the Bligh and Dyer extraction were dissolved in chloroform/methanol (2/1, 200 μ L) and separated on a Hewlett-Packard 1090 HPLC system with a Partisil ODS3 column (5 μ m, 4.5 mm $\times$ 250 mm) using methanol (1 mL/min) as the mobile phase and UV detection at 215 nm. The cholesterol C2 alcohol (2a) was identified by coinjection with synthetic material and quantified via a standard curve of 2a.

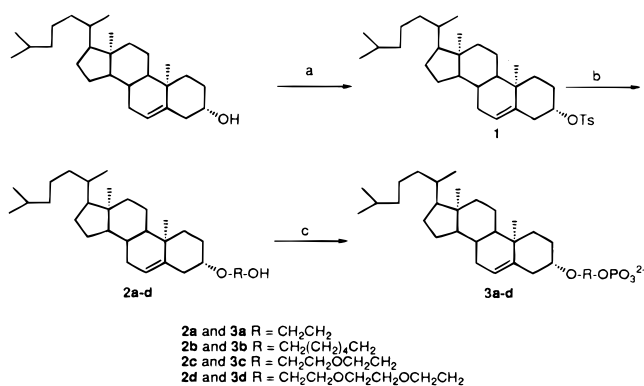
Encapsulation of Plasmid DNA in Liposomes. A cholesterol phosphate derivative/DOPE (4/5) lipid film (20 μmol) was dissolved in a mixture of diethyl ether and 1,1,2-trichloro-2,2,1-trifluoroethane (57:43, 3 mL), and 10 mM HEPES, 5% glucose buffer at pH 7.4 (0.5 mL) was added. The mixture was vigorously sonicated to form an emulsion to which plasmid DNA (375 μg) (pLC0785.112, a generous gift of GeneMedicine Inc.) in 10 mM HEPES and 5% glucose, pH 7.4 (0.5 mL), was added followed by further brief sonication. The emulsion was then dried down to the gel phase, which was broken under reduced pressure to yield liposomes, of 300 nm z-average diameter as determined by dynamic light scattering (Coulter Electronics Inc., Hialeah, FL). Using the multimodel algorithms, these liposomes were purified on a discontinuous Ficoll gradient to remove excess DNA (22). DNA encapsulation efficiency was measured after determining the amount of lipid phosphate and DNA in the fraction containing the liposomes. The DNA concentration was measured using the Hoescht dye 33258 (Polysciences, Inc., Warrington, PA) and a DNA fluorometer (TKO100, Hoefer Scientific Instruments, San Francisco, CA). The liposomal phosphate was quantified using the assay of Bartlett (18) for the total phosphate and subtracting the amount of phosphate contributed by the associated DNA.

DNase Protection Assay. Plasmid DNA (1 μg), either encapsulated in liposomes or free, was dissolved in HBS (10 μL) and DNase was added and the mixture incubated at room temperature for 16 h. The samples were then purified by a Bligh and Dyer extraction and the DNA separated by electrophoresis on a 0.8% agarose gel with ethidium bromide staining.

Transfection of Monkey Kidney Fibroblast Cells. CV-1 cells were plated in a 96 well plate at 2×10^4 cells/well in 100 μL of growth medium consisting of DME-H21 with 10% fetal calf serum and antibiotics (100 units/mL penicillin and 100 $\mu\text{g}/\text{mL}$ streptomycin). The cells were incubated for 19 h at 37 $^{\circ}\text{C}$ in a 95% air, 5% CO_2 atmosphere. The growth medium was then removed, the cells were washed with serum free medium, and serum free medium was added (150 μL). The transfection system was then added in 50 μL of 10 mM HEPES and 5% glucose, pH 7.4, buffer, and the cells were incubated in this medium for 3 h. Subsequently, the medium was replaced with complete growth medium, and the cells were incubated for a further 36 h. The medium was removed, and the cells were washed with phosphate-buffered saline and assayed for luciferase activity and total cell protein (Bio-Rad BCA protein assay). For the phosphatase inhibitor experiments, inorganic phosphate (10 mM final) and L-*p*-bromotetramisole (1 mM final) (Sigma, St. Louis, MO) were added to the serum-free medium before addition of the transfection system. Nonencapsulated DNA/liposomes were prepared by mixing 100 nmol of cholesterol phosphate derivative/DOPE (4/5) liposomes with 1 μg of pLC 0785.112 (GeneMedicine Inc.) in 50 μL of buffer before addition to the cells.

Transfections in low-calcium media were performed utilizing calcium/magnesium-free Hanks-buffered salt solution, supplemented with nonessential amino acids, antibiotics, and glutamine (2 mM) in place of serum free media. The calcium concentration noted was added to the media from a stock solution of CaCl_2 immediately before use. Where noted, the salt wash was performed, immediately after aspiration of the transfection medium, with 1 M NaCl and 20 mM HEPES buffer, pH 7.4, followed by a media wash and incubation in growth medium for a further 36 h.

Scheme 1. Synthesis of Cholesterol Phosphate Derivatives^a



^a (a) *p*-Toluene sulfonyl chloride, pyridine, ambient, 16 h, quant. (b) HO-R-OH, 1,4-dioxane, reflux, 4 h, 88%. (c) (i) Tetra-*n*-butylammonium phosphate, trichloroacetonitrile, 15 min. (ii) NaI, 15 min, 50–70% (two steps).

RESULTS

Synthesis. Our approach to the synthesis of a phosphatase-sensitive lipid employed cholesterol as the lipophilic anchor due to the ability of cholesterol to promote the lamellar to hexagonal transition in PE membranes (23) and for synthetic ease. Since the structural requirements of phosphatase enzymes using lipids as substrates are not known, we sought a versatile synthesis amenable to the production of a series of lipids (Scheme 1). $\text{S}_{\text{N}}1$ displacement of tosylate from the 3- β position of cholesterol with ethylene glycol had been shown to be an efficient method for derivatizing cholesterol (15), and this displacement proceeds smoothly for a wide variety of diols. Phosphorylation of the prepared diols was performed via the method of Danilov et al. (24) and the tetra-*n*-butylammonium salt was smoothly transformed into the sodium salt with sodium iodide.

Liposome Formation. To determine which lipids would be most appropriate to use in a triggered release system a variety of cholesterol alcohol derivatives (eight in all) (25) were synthesized. These were then tested for the ability to stabilize a DOPE bilayer. At pH 7.4, none of the derivatives formed liposomes with DOPE in a 4/5 cholesterol alcohol/DOPE ratio. At pH 9.0, DOPE is sufficiently charged to support a bilayer in the presence of cholesterol. Inclusion of the cholesterol alcohol derivatives in place of cholesterol showed that only those lipids with hydrophilic linkers or hydrophobic linkers of six carbons or less formed liposomes with DOPE. All the formulations collapsed upon reduction of the pH to 7.4, after protonation of the DOPE (25). This suggested that phosphatase-mediated removal of the phosphate groups from the cholesterol phosphate derivative/DOPE liposomes would initiate collapse of the liposomes with concomitant release of the contents. This initial screen of linker structure against liposome stability allowed us to identify a number of lipids to study in greater detail. The selected lipids were structurally distinguished by a short linker (3a), the longest stable hydrophobic linker (3b), a hydrophilic linker (3c), and the longest linker tested (3d), respectively (Scheme 1).

Sensitivity to Phosphatase. Liposomes were formed from mixtures of PE with three cholesterol phosphate derivatives (3a, b, d). The sensitivity of the phosphate derivatives to alkaline phosphatase was first measured by the leakage of encapsulated ANTS/DPX (17). Exposure of the liposomes to alkaline phosphatase resulted in the leakage of contents from all three compositions

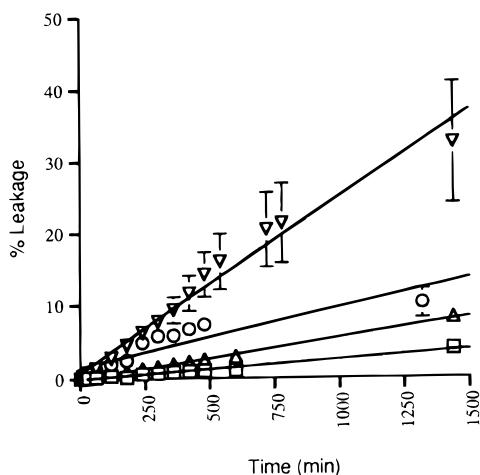


Figure 1. Effect of alkaline phosphatase upon the stability of liposomes encapsulating ANTS/DPX. (□) Cholesterol triethylene phosphate (3d)/DOPE in HBS (cholesterol C2 phosphate (3a)/DOPE and cholesterol C6 phosphate (3b)/DOPE showed the same leakage profile); (▽) cholesterol C2 phosphate (3a)/DOPE with 10 I. U. alkaline phosphatase; (○) cholesterol C6 phosphate (3b)/DOPE with 10 I. U. alkaline phosphatase; (△) cholesterol triethylene phosphate (3d)/DOPE with 10 I. U. alkaline phosphatase.

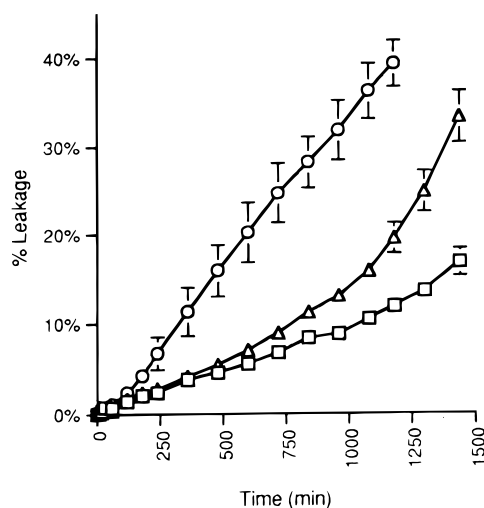


Figure 2. Dose response of cholesterol C2 phosphate (3a)/DOPE liposomes to alkaline phosphatase. (□) Cholesterol C2 phosphate (3a)/DOPE in HBS; (△) cholesterol C2 phosphate (3a)/DOPE with 1 I. U. alkaline phosphatase; (○) cholesterol C2 phosphate (3a)/DOPE with 20 I. U. alkaline phosphatase.

(Figure 1). In contrast, liposomes formulated with cholesterol phosphate or egg phosphatidate with DOPE showed no leakage under these conditions, showing that they were not phosphatase sensitive. The cholesterol C2 phosphate (3a)/DOPE liposomes were most sensitive to alkaline phosphatase, and further experiments were performed with this system.

The leakage of dye from the liposomes in the presence of alkaline phosphatase exhibited two phases, a lag phase followed by a more rapid phase. Exposure of cholesterol C2 phosphate (3a)/DOPE liposomes to increasing amounts of enzyme lead to an increased rate of release from the liposome (Figure 2). The lag phase before liposome leakage was also observed to be sensitive to the amount of enzyme activity present in the assay. Inactivation of the enzyme by heat treatment completely blocked the enzyme-mediated leakage of dye from the liposome (Figure 3). The leakage of dye from the liposomes was

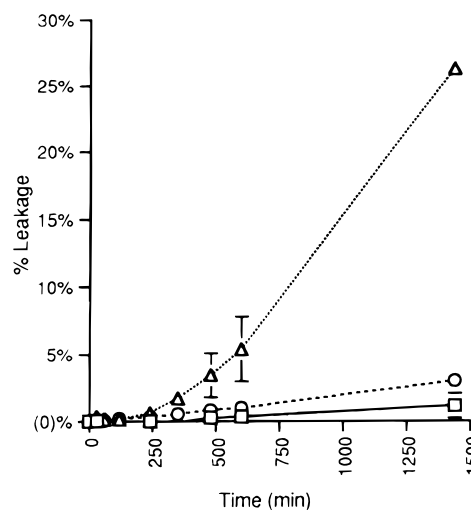


Figure 3. Effect of heat inactivation upon the alkaline phosphatase mediated leakage. (□) Cholesterol C2 phosphate (3a)/DOPE in HBS; (△) cholesterol C2 phosphate (3a)/DOPE with 10 I. U. alkaline phosphatase; (○) cholesterol C2 phosphate (3a)/DOPE with heat inactivated alkaline phosphatase.

Table 1. Stoichiometry of Alkaline Phosphatase Catalyzed Liposome Destruction

incubation conditions	cholesterol C2 alcohol (nmol)	inorganic phosphate (nmol)
no alkaline phosphatase	not detected	30 ± 15
20 units of alkaline phosphatase	190 ± 10	190 ± 40
100 units of alkaline phosphatase	180 ± 15	160 ± 30

thus shown to be dependent upon and sensitive to the presence of active alkaline phosphatase.

The expected products of the reaction of a phosphatase with cholesterol C2 phosphate (3a) are the cholesterol C2 alcohol (2a) and inorganic phosphate. To detect the products of any reaction between the liposomes and alkaline phosphatase, a large scale (1 μmol) incubation of the cholesterol C2 phosphate (3a)/DOPE liposomes with the enzyme was performed. Due to problems with product inhibition of the enzyme, it was necessary to perform the incubation at the same concentration as the leakage experiments (0.1 mM). After 24 h, the reaction was frozen and the mixture lyophilized. The residue was rehydrated in a small volume of water, and the lipids were separated by a Bligh and Dyer extraction (20).

HPLC separation of the lipids isolated in the extraction allowed identification and quantification of the lipids formed in the reaction. The lipids were isolated utilizing the method described by New (26) for the quantification of α-tocopherol with cholesterol as a standard. Under these conditions, cholesterol C2 alcohol (2a) eluted at 15 min, 2 min after cholesterol eluted. Analysis of the lipids isolated from the extraction showed the presence of a peak eluting at 15 min only in the reactions containing alkaline phosphatase. Coinjection of the reaction products with cholesterol C2 alcohol (2a) resulted in enhancement of this peak. To determine the amount of cholesterol C2 alcohol (2a) produced, a standard curve of 2a was set up and a linear relationship between the area under the peak and the amount of material injected was shown. From this curve, 190 ± 10 nmol of 2a was formed in the reaction with alkaline phosphatase (Table 1).

Identification and quantification of inorganic phosphate in the aqueous phase was performed at pH 5.0 to avoid any contamination from lipids that remained in the aqueous phase. The presence of zinc in the assay mixture

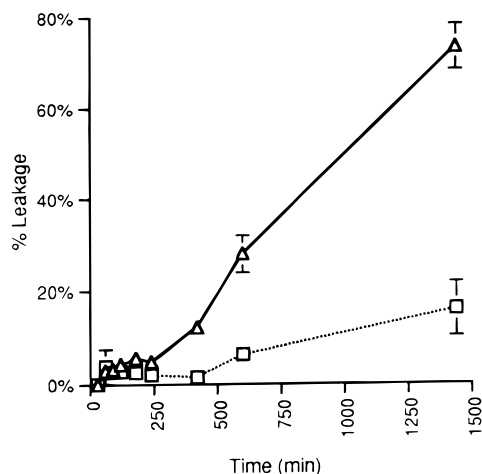


Figure 4. Effect of acid phosphatase upon cholesterol C2 phosphate (**3a**)/DOPE liposomes. (□) Cholesterol C2 phosphate (**3a**)/DOPE in acetate buffered saline, pH 5.0; (Δ) cholesterol C2 phosphate (**3a**)/DOPE with 2 I. U. acid phosphatase.

allows the formation of the reduced phosphomolybdate complex under mild conditions (21). Inorganic phosphate formation was shown to be dependent upon the presence of alkaline phosphatase. In the presence of alkaline phosphatase, 190 ± 40 nmol of inorganic phosphate are formed after 24 h (Table 1). In the absence of enzyme only, background levels of phosphate were detected in the assay. Under these conditions, only 40% of the initial cholesterol C2 phosphate (**3a**) was hydrolyzed by the enzyme. This was ascribed to product inhibition by inorganic phosphate, which at $20 \mu\text{M}$ concentration was severalfold higher than its K_i for the enzyme.

To test the general nature of the reaction observed with alkaline phosphatase, a second physiologically relevant isozyme, an acid phosphatase (10), was tested. Incubation of the cholesterol C2 phosphate (**3a**)/DOPE liposomes at pH 5, the optimal pH for this enzyme, highlighted the stability of this liposome composition. In the presence of acid phosphatase, the leakage of dye from the liposomes exhibited two phases, a lag phase followed by a more rapid phase (Figure 4). The extent of leakage observed for this isozyme was significantly higher than that observed with the alkaline phosphatase.

Sensitivity to Calcium. The second mechanism for triggering a phase change in an anionic liposome is the use of divalent cations. Addition of 0.4 mM calcium to cholesterol C2 phosphate (**3a**)/DOPE liposomes causes complete leakage of the liposome contents and the formation of large flocculate aggregates. The lipid mixing assay of Struck et al. (19) gave a transition threshold for this effect of $150 \mu\text{M}$ calcium (data not shown).

The ability of calcium to aggregate and fuse liposomes is thought to be mediated by the dehydration of the bilayer surface (27). Accordingly, a number of cholesterol phosphate derivatives (**3b–d**) in which the linker moiety was longer and more hydrophilic than the cholesterol C2 phosphate (**3a**) was formed into liposomes with DOPE and tested for their calcium sensitivity. The sensitivity to Ca^{2+} was reduced by increasing the hydrophilicity and length of the linker between the cholesterol moiety and the phosphate group (Table 2). The triethylene glycol phosphate (**3d**) is stable up to 1 mM Ca^{2+} , which is greater than physiological levels in some tissues in vivo (28). Intriguingly, the cholesterol C2 phosphate (**3a**)/DOPE liposomes are much more sensitive than the parent molecule in the series, cholesterol phosphate, and then the diacyl glycerol lipid, egg phosphatidate.

Table 2. Effect of Calcium upon the Stability of Cholesterol Phosphate Derivative/DOPE Liposomes

liposome formulation	200 μM Ca^{2+}	500 μM Ca^{2+}	1 mM Ca^{2+}	2 mM Ca^{2+}
cholesterol C2 phosphate (3a)/DOPE	+	++	++	++
cholesterol C6 phosphate (3b)/DOPE	—	+	++	++
cholesterol diethylene phosphate (3c)/DOPE	—	—	+	++
cholesterol triethylene phosphate (3d)/DOPE	—	—	+	++
cholesterol phosphate/DOPE	—	+	++	++
egg phosphatidate/DOPE	—	—	—	+

^a The liposomes were exposed to the indicated calcium concentration and the rate of dye leakage and increase of turbidity was measured. (—) No detectable effect. (+) Leakage of $<20\%$. (++) leakage and aggregation of $>60\%$.

Encapsulation of DNA. Encapsulation of DNA in such negatively charged liposomes was potentially a problem but was readily achieved utilizing a modification of the REV methodology (29). The liposomes were purified on a discontinuous Ficoll gradient to remove excess DNA. In a typical procedure, the final liposome concentration was 18 nmol of lipid and $0.2 \mu\text{g}$ of DNA/ μL of solution. This corresponds to a 29% encapsulation efficiency for DNA.

The DNA was shown to be intact and encapsulated within the liposomes using a DNase protection assay. Liposomes encapsulating DNA were exposed to DNase for 16 h at room temperature and the lipids were then extracted. Plasmid DNA was completely degraded under these conditions (lane 7), whereas the encapsulated DNA was not degraded (lane 2) even in the presence of 10% serum (lane 4, the material at the wells for lanes 3 and 4 is serum derived) (Figure 5). The DNA also gave the same mobility profile as the initial plasmid DNA, indicating that it had not been sheared in the encapsulation process. Indeed, the encapsulated DNA can be extracted via the Bligh and Dyer protocol (20) and complexed with the heat-activated dendrimer (30). This complex is completely transfection competent (data not shown), confirming the agarose gel data that the DNA structure was not damaged in the encapsulation protocol.

Transfection Studies. The ability of the cholesterol C2 phosphate (**3a**)/DOPE liposomes to transfect CV-1 cells in vitro is shown in Figure 6. The transfection efficiency observed was intermediate in level between the degraded cationic polyamidamine dendrimer (29), which was used as a positive control, and naked DNA as shown.

Transfection experiments in the presence of phosphatase inhibitors such as inorganic phosphate (31) and *L-p*-bromotetramisole (32) showed that the ability of the liposome system to transfect CV-1 cells was not affected by inhibition of phosphatase. At the levels of inhibitor included in the transfection experiments, no phosphatase activity was observed. At 1 mM , *L-p*-bromotetramisole mediated some cellular toxicity which reduced the transfection levels for all transfecting agents. The relative transfection levels were unaffected by the treatments, however, indicating that another mechanism is at work (Figure 6).

The ability of the liposomes to mediate transfection in the absence of encapsulation is shown in Figure 7. The cells were exposed to DNA and cholesterol C2 phosphate (**3a**)/DOPE liposomes with the DNA either inside or outside of the liposome. The transfection efficiency was the same in both cases, whereas naked DNA did not transfect in the absence of lipid. The flocculate lipid/DNA aggregate formed upon exposure to the calcium in the

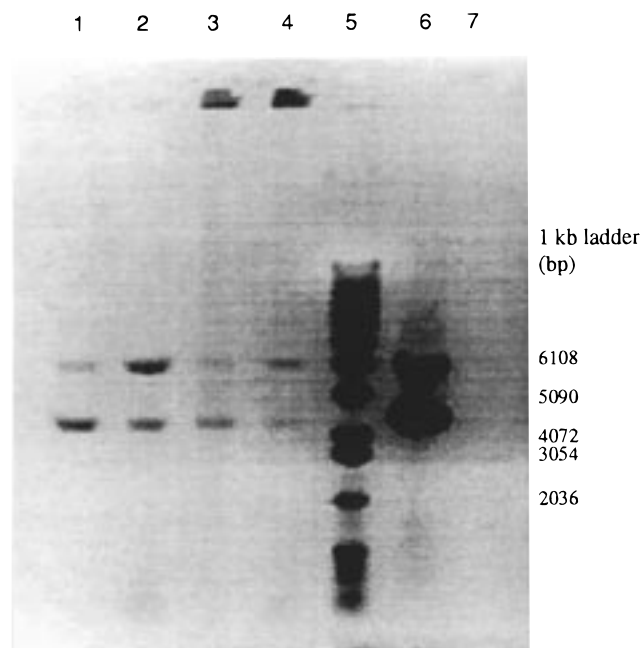


Figure 5. DNase protection assay with cholesterol C2 phosphate (**3a**)/DOPE liposomes: The plasmid DNA was extracted from the liposome after incubation and electrophoresed on an 0.8% agarose gel with ethidium bromide staining. Lane 1; cholesterol C2 phosphate (**3a**)/DOPE liposomes in HBS. Lane 2; cholesterol C2 phosphate (**3a**)/DOPE liposomes in HBS + 10 I. U. DNase 1. Lane 3; cholesterol C2 phosphate (**3a**)/DOPE liposomes in 10% serum in HBS. Lane 4; cholesterol C2 phosphate (**3a**)/DOPE liposomes in 10% serum + 10 I. U. DNase 1. Lane 5; 1 kb DNA ladder. Lane 6; plasmid DNA in HBS. Lane 7; plasmid DNA in HBS + 10 I. U. DNase 1.

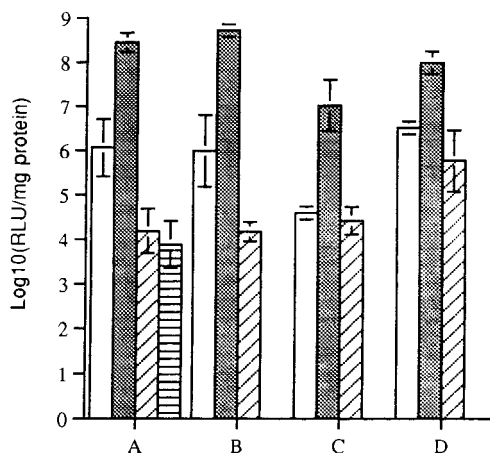


Figure 6. Effect of phosphatase inhibitors upon the transfection of CV-1 cells with a luciferase encoding plasmid. Transfection with cholesterol C2 phosphate (**3a**)/DOPE liposomes (0.5 μ g of encapsulated DNA/well) (open bars); cationic dendrimer (0.6 μ g DNA/well) (dark bars); plasmid DNA (0.5 μ g of DNA/well) (angled stripes); untreated cells (horizontal stripes). Condition A; No additions to serum free medium. Condition B; 50 nmol of inorganic phosphate added to medium. Condition C; 1 mM L-p-bromotetramisole added to medium. Condition D; 10 mM inorganic phosphate added to medium.

culture medium must therefore mediate the transfection. DNA encapsulated by the more calcium stable cholesterol triethylene phosphate (**3d**)/DOPE liposomes caused efficient transfection, whereas the mixed liposome/DNA suspension resulted in significantly reduced levels of transfection. These liposomes do not flocculate on addition to media but are active in transfection, indicating that a different mechanism of transfection is occurring

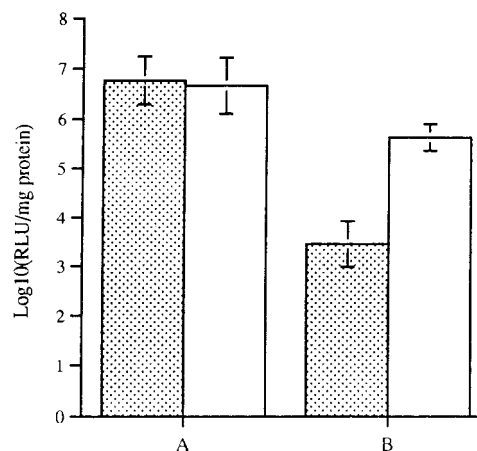


Figure 7. Effect of encapsulation of the plasmid on transfection efficiency. Transfection with liposomes premixed with plasmid DNA (1 μ g of DNA/well) (dotted bars). Transfection with liposomes encapsulating plasmid (1 μ g of DNA/well) (open bars). Condition A; cholesterol C2 phosphate (**3a**)/DOPE liposomes. Condition B; cholesterol triethylene phosphate (**3d**)/DOPE liposomes.

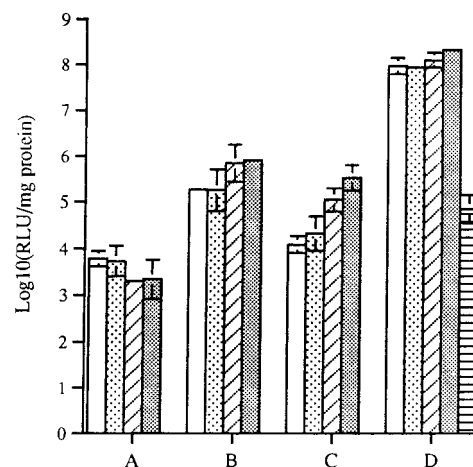


Figure 8. Effect of calcium upon the transfection of CV-1 cells with a luciferase encoding plasmid: Transfection in the presence of 0 mM Ca^{2+} (open bars); 0.1 mM Ca^{2+} (dotted bars); 0.5 mM Ca^{2+} (angled stripes); 1.36 mM Ca^{2+} (dark bars); 0 mM Ca^{2+} with salt wash (horizontal stripes). Condition A; naked DNA (0.5 μ g of DNA/well). Condition B; cholesterol triethylene phosphate (**3d**)/DOPE liposomes (0.5 μ g of encapsulated DNA/well). Condition C; cholesterol C2 phosphate (**3a**)/DOPE liposomes (0.5 μ g of encapsulated DNA/well). Condition D; cationic dendrimer (0.6 μ g DNA/well).

in this case, from that observed with the cholesterol C2 phosphate (**3a**)/DOPE system. We have tested anionic liposome formulations prepared from naturally occurring lipids, such as POPG/DOPE, cholesterol phosphate/DOPE or egg phosphatidate/DOPE, and these *do not* transfect when they are simply mixed with DNA.

Transfection of cells in vitro with reduced levels of calcium in the media was performed using calcium/magnesium-free Hanks-buffered salt solutions supplemented with an energy source (glutamine) and the required level of calcium. Cell viability was unaffected by this treatment in untransfected controls. The cholesterol C2 phosphate (**3a**)/DOPE liposomes were shown to be sensitive to the calcium concentration (Figure 8), showing only background levels of transfection in the absence of added calcium. Cholesterol triethylene phosphate (**3d**)/DOPE liposomes were less affected by the calcium concentration and were still active in transfection in the absence of any

added divalent cations. The cationic dendrimer was unaffected by the calcium concentration unless the surface-bound polyplex was removed by salt wash before addition of calcium-containing media.

DISCUSSION

Nonviral gene therapy remains a relatively inefficient process that could benefit from improved approaches for gene delivery. We hypothesized that a phosphatase-triggered release lipid composition could sequester DNA to prevent degradation until the target site is reached. The DNA would be released when triggered by the relatively high concentration of phosphatase isozymes associated with the cell surface and lysozymes (8, 28). A triggered release liposome sensitive to this activity may therefore provide an efficient method for the transfection of cells or delivery of drugs.

To test this hypothesis, we synthesized a series of novel phosphate monoester derivatives of cholesterol. The synthesis allowed a large number of different lipids to be constructed from cholesterol tosylate (Scheme 1), a stable crystalline solid which has been used for over 60 years as an intermediate for the derivatization of cholesterol at the 3- β position (33). The stereochemistry of the incoming nucleophile is defined by the cholesterol geometry, and in the absence of ionic promoters, the 3- β isomer is formed exclusively. Phosphorylation of the alcohol and counterion exchange provided the desired cholesterol phosphate derivatives. The cholesterol phosphate derivatives (**3a–d**) support a bilayer with DOPE at neutral pH whereas the expected products of phosphatase action, the cholesterol alcohol derivatives (**2a–d**), do not (25).

Alkaline phosphatase, a triggering enzyme, is associated with the extracellular matrix of cells and is known to be elevated in many disease states such as cancer (8). This enzyme has a pH optimum *in vitro* of 10.5, but acts upon polyanionic substrates at physiological pH (12). Moreover, the enzyme dephosphorylates and inactivates lipopolysaccharide (bacterial endotoxin) at the lipid–water interface of the micelle surface and should be capable of hydrolyzing the appropriate cholesterol phosphate derivatives in a bilayer. Liposomes encapsulating a fluorophore were prepared from a number of cholesterol phosphate derivatives formulated with DOPE and used to examine liposome leakage in the presence of the triggering enzyme. Exposure of these liposomes to physiologically relevant levels (28) of alkaline phosphatase led to a time-dependent leakage of the aqueous contents (Figure 1). Importantly, the naturally occurring lipids, cholesterol phosphate, and egg phosphatidate, were resistant to phosphatase-mediated leakage under these conditions. As the cholesterol C2 phosphate (**3a**)/DOPE system was significantly more sensitive to the presence of the enzyme than the other cholesterol derivatives, it was decided to test the activity of alkaline phosphatase more rigorously upon this system.

The proposed catalytic route of liposome destruction is based on the sequential removal of phosphate from individual lipid molecules. Little dye release is expected until a critical point of cholesterol hydrolysis is reached. Once there is insufficient cholesterol phosphate remaining in the bilayer to stabilize the lamellar phase, the liposomes will collapse to form the hexagonal phase with concomitant release of their contents. This observed lag phase is also sensitive to the enzyme concentration (Figure 2)—the greater the enzyme concentration the shorter the lag phase. Inactivation of the enzyme by heat

treatment (Figure 3) clearly indicates that an active enzyme, rather than a nonspecific protein–liposome interaction, must catalyze the transformation. This release profile may provide an advantage for *in vivo* delivery applications; the slow release of the lag phase might counteract the fast release observed in the burst phase with most controlled release systems (34).

The catalytic destruction of the liposomes is dependent upon the hydrolysis of the cholesterol C2 phosphate (**3a**) to the cholesterol C2 alcohol (**2a**) and inorganic phosphate. The expected products were isolated, identified, and quantified; inorganic phosphate and cholesterol C2 alcohol are produced in a 1:1 molar ratio (Table 1). This is consistent with the stoichiometry of the reaction of alkaline phosphatase with its substrate. Acid phosphatase also mediated the leakage contents from the cholesterol C2 phosphate (**3a**)/DOPE liposomes at pH 5.0 and was significantly more effective than alkaline phosphatase. The leakage profile induced by acid phosphatase was very similar to that observed with alkaline phosphatase supporting a catalytic mechanism of liposome collapse.

Divalent cations have been extensively used to trigger a phase change in anionic liposomes. Calcium, and to a lesser extent, magnesium and zinc are known to interact with anionic lipids such as phosphatidic acid (35), phosphatidylserine (36), and CHEMS (14), leading to phase separation and aggregation of liposomes. Depending upon the lipid composition, the calcium concentrations found in cell culture and *in vivo* are sufficient to mediate this transition.

Calcium is a very efficient trigger for the cholesterol C2 phosphate (**3a**)/DOPE liposomes and caused complete leakage of liposome contents at the comparatively low concentration of 0.4 mM. The threshold value at which calcium begins to show effects is 150 μ M for this liposome composition. *In vivo* administration would expose the liposomes to higher calcium concentrations, so we examined if calcium sensitivity was dependent upon the cholesterol phosphate derivative (**3a–d**). The structure of the linker has a pronounced effect upon the calcium sensitivity as shown in Table 2. Adding a linker initially slightly increases the sensitivity of DOPE liposomes to the effects of calcium [compare cholesterol phosphate to cholesterol C2 phosphate (**3a**)] then decreases the sensitivity, presumably because the phosphate group is positioned further from the bilayer surface. Incorporation of oxygens into the linker region improves the calcium stability still further, most likely because the linker is more hydrophilic. Liposomes formulated with the most hydrophilic derivative (**3d**) were largely unperturbed by physiological levels of calcium (28). Thus, the cholesterol phosphate derivative/DOPE liposomes form a triggered release system which is sensitive to two separate triggering mechanisms: phosphatase isozymes and calcium.

The utility of this system for delivery purposes was evaluated using gene transfer. Transfection with DNA encapsulated in the cholesterol C2 phosphate (**3a**)/DOPE liposome was tested using kidney fibroblast cells (CV-1) and the luciferase reporter gene. The levels of luciferase activity detected after transfection compare favorably with the CHEMS/DOPE pH-triggered release system (22) at 10⁷ RLU/mg of protein. This is 3–4 orders of magnitude above background, although lower than the cationic heat activated dendrimer (30).

To test whether the transfection was entirely or partially mediated by phosphatase activity, transfections were performed in the presence of known phosphatase

inhibitors. Inorganic phosphate competitively inhibits phosphatase isozymes with a micromolar K_i (31). With 10 mM phosphate in the transfection medium, no phosphatase activity was observed with the cells using *p*-nitrophenyl phosphate as the marker. The second inhibitor *L*-*p*-bromotetramisole (32) is a specific inhibitor of alkaline phosphatases, and complete inhibition of phosphatase activity is observed at the concentration used. The ability of the C2 system to transfect in the absence of alkaline phosphatase activity means that this isozyme is not the dominant mechanism in the transfection process but does not rule out the involvement of acid phosphatase.

Calcium was known (*vide supra*) to cause the aggregation of the liposomes at the concentration found in cell culture media (1.36 mM), and flocculate particles were observed in the transfection medium. The interaction of calcium with the negatively charged liposomes, causing aggregation and fusion of the liposomes, could mediate the transfection. Moreover, calcium phosphate precipitation is a widely used method for the transfection of cells in culture, though at much higher concentrations of calcium and phosphate (37). Addition of the same amount of inorganic phosphate as was found in the liposome experiments (50 nmol of P_i) to naked DNA or to the cholesterol C2 phosphate (**3a**)/DOPE liposomes had no effect upon the transfection efficiency (Figure 6). Therefore, a direct effect of released phosphate via a DNA/calcium phosphate precipitation does not mediate the transfection. However, the liposomes may provide a multivalent surface that could form a coprecipitate with DNA in the presence of calcium to mediate the transfection. Formation of this coprecipitate would not require the DNA to be encapsulated in the liposome before addition of the calcium as long as it could be recruited into the aggregate. Indeed, if cholesterol C2 phosphate (**3a**)/DOPE liposomes are mixed with DNA at the same concentrations used in the encapsulated transfection experiments, the transfection efficiency is identical.

The ability of anionic liposomes to form cochleates in the presence of divalent cations has long been recognized (38). More recently, this has been used to encapsulate macromolecules such as proteins and DNA inside the cochleate (39). These cochleates have been proposed for use as an oral DNA vaccine which is absorbed by the lower intestine before slowly releasing the trapped immunogen, thus, initiating a strong immune response (40). The cholesterol C2 phosphate (**3a**)/DOPE liposomes readily formed cochleate-like structures upon the addition of calcium to the suspension. Fluorescent DNA was incorporated into the precipitate even without preencapsulation, and addition of EDTA lead to partial release and partial encapsulation of the DNA in giant liposomes (data not shown). In the case of the cholesterol C2 phosphate (**3a**)/DOPE system, the coprecipitate appears to mediate the transfection of cells in culture.

The more calcium stable cholesterol triethylene phosphate (**3d**)/DOPE liposomes do not form flocculates in the culture medium. DNA encapsulated in the liposomes, however, is capable of transfecting cells in culture (Figure 7) which implies that the formation of visible aggregates is not essential for transfection with this liposome composition. Plasmid DNA mixed with empty liposomes of this composition is incapable of transfection. Indeed, only the cholesterol C2 phosphate (**3a**)/DOPE liposomes have shown this remarkable activity to date.

Removal of calcium from the transfection medium prevents endocytosis and will stop the calcium-mediated transfection of cells observed with CV-1 cells. The ability

of cholesterol C2 phosphate (**3a**)/DOPE liposomes to mediate transfection is dependent upon the presence of calcium in the medium (Figure 8). In the absence of the calcium required to form the flocculate, the cholesterol C2 phosphate (**3a**)/DOPE liposome did not transfer DNA into cells. The cationic dendrimer-mediated transfection is also unaffected by the calcium concentration in the culture medium. The cationic polyplex, as opposed to the anionic liposomes, would be expected to bind to the cell surface electrostatically and undergo endocytosis once calcium was returned to the system. Removal of surface-bound polyplex with a salt wash prior to the readministration of calcium containing media significantly reduced transfection.

Transfection mediated by the cholesterol triethylene phosphate (**3d**)/DOPE liposomes are less affected by the absence of calcium in the media. Thus, the phosphatase-sensitive cholesterol triethylene phosphate (**3d**)/DOPE liposomes do not mediate transfection exclusively through a calcium mediated mechanism.

The ability to modulate the calcium sensitivity of the triggered release liposomes and to utilize other release mechanisms should allow liposome formulations to be designed for specific tasks based upon the site of action and desired route of administration. For example, the extremely high levels (~50 units/mL) of acid phosphatase present in the prostate gland (28) suggests that this system may be an efficient agent for disease states of this gland. Alternatively, by utilizing the antibody-directed enzyme prodrug therapy (ADEPT) approach (41), very high concentrations of the activating enzyme, such as alkaline phosphatase (42), can be directed to tumors. The passively circulating cholesterol phosphate derivative/DOPE liposomes would then be specifically degraded at the site of the tumor, targeting the transfection or drug delivery to the desired site of action.

CONCLUSION

We have proposed and synthesized a novel class of triggered release liposomes. These conditionally stable liposomes collapse in the presence of phosphatase isozymes or calcium which results in the release of the liposome contents. The sensitivity of the liposomes to each triggering mechanism can be modulated by changing the structure of the cholesterol phosphate derivative. This allows the triggered release liposome to be tailored to give the desired balance of sensitivity for a particular purpose and site of action. DNA encapsulated in these liposomes is capable of transfecting CV-1 cells *in vitro* at levels competitive with other triggered release systems. The transfection is mediated by calcium for certain formulations, but other transfection mechanisms start to play a role in the less calcium-sensitive cholesterol triethylene phosphate (**3d**)/DOPE formulations.

ACKNOWLEDGMENT

This work was supported by NIH grants GM 26691, DK 46052, and DK 47766. Dr. Szoka has a financial interest in and serves as a consultant to GeneMedicine, Inc., a biotechnology company developing gene medicines. We thank A. Vlassov for the translation of ref 24, X. Guo for proofreading the manuscript, and L. S. Uyechi for the generous gift of the fluorescent DNA.

LITERATURE CITED

- (1) Gerasimov, O. V., Rui, Y., and Thompson, D. H. (1996) Triggered release from liposomes mediated by physically and chemically induced phase transitions. *Vesicles* (Rosoff M., Ed.) pp 681–740, Marcel Dekker, Inc., New York.

- (2) Chu, C.-J., and Szoka, F. C. (1994) pH-Sensitive Liposomes. *J. Liposome Res.* 4, 361–395.
- (3) Marsh, D. (Ed.) (1990) *CRC Handbook of Lipid Bilayers*, pp 128–130, 266–267, CRC Press, Boca Raton, FL.
- (4) Chu, C.-J., Dijkstra, J., Lai, M.-Z., Hong, K., and Szoka, F. C. (1990) Efficiency of cytoplasmic delivery by pH-sensitive liposomes to cells in culture. *Pharm. Res.* 7, 824–834.
- (5) Düzgünes, N., Straubinger, R. M., Baldwin, P. A., Friend, D. S., and Papahadjopoulos, D. (1985) Proton-induced membrane fusion of oleic acid-phosphatidylethanolamine liposomes. *Biochemistry* 24, 3091–3098.
- (6) Menger, F. M., and Johnston, D. E. (1991) Specific enzyme-induced decapsulation. *J. Am. Chem. Soc.* 113, 5467–5468.
- (7) Anderson, V. C., and Thompson, D. H. (1992) Triggered release of hydrophilic agents from plasmalogen liposomes using visible light or acid. *Biochim. Biophys. Acta* 1109, 33–42.
- (8) Millán, J. L., and Fishman, W. H. (1995) Biology of human alkaline phosphatases with special reference to cancer. *Crit. Rev. Clin. Lab. Sci.* 32, 1–39.
- (9) Simopoulos, T. T., and Jencks, W. P. (1994) Alkaline phosphatase is an almost perfect enzyme. *Biochemistry* 33, 10375–10380.
- (10) deDuke, C., Pressman, B. C., Gianetto, R., Wattiaux, R., and Appelmans, F. (1955) Tissue fractionation studies. 6. Intracellular distribution patterns of enzymes in rat-liver tissue. *Biochem. J.* 60, 605–617.
- (11) Banerjee, P. K., and Amidon, G. L. (1985) Design of drugs based on enzyme–substrate specificity. *Design of prodrugs* (Bundgaard H., Ed.) pp 93–135, Elsevier, Amsterdam.
- (12) Poelstra, K., Bakker, W. W., Klok, P. A., Hardonk, M. J., and Meijer, D. K. F. (1997) A physiologic function for alkaline phosphatase: Endotoxin detoxification. *Lab. Invest.* 76, 319–327.
- (13) Sundler, R., and Papahadjopoulos, D. (1981) Control of membrane fusion by phospholipid headgroups I. Phosphatidate/phosphatidylinositol specificity. *Biochim. Biophys. Acta* 649, 743–750.
- (14) Lai, M.-Z., Vail, W. J., and Szoka, F. C. (1985) Acid- and calcium-induced structural changes in phosphatidylethanolamine membranes stabilized by cholesteryl hemisuccinate. *Biochemistry* 24, 1654–1661.
- (15) Patel, K. R., Li, M. P., Schuh, J. R., and Baldeschweiler, J. D. (1985) Modification of vesicle surfaces with amphiphilic sterols. Effect on permeability and in vivo tissue distribution. *Biochim. Biophys. Acta* 814, 256–264.
- (16) Wang, Y., and Hollingsworth, R. I. (1995) A solvent system for the high-resolution proton nuclear magnetic resonance spectroscopy of membrane lipids. *Anal. Biochem.* 225, 242–251.
- (17) Ellens, H., Bentz, J., and Szoka, F. C. (1985) H^+ - and Ca^{2+} -induced fusion and destabilization of liposomes. *Biochemistry* 24, 3099–3106.
- (18) Bartlett, G. R. (1959) Phosphorus assay in column chromatography. *J. Biol. Chem.* 234, 466–468.
- (19) Struck, D. K., Hoekstra, D., and Pagano, R. E. (1981) Use of resonance energy transfer to monitor membrane fusion. *Biochemistry* 20, 4093–4099.
- (20) Bligh, E. G., and Dyer, W. J. (1959) A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* 37, 911–917.
- (21) Drueckes, P., Schinzel, R., and Palm, D. (1995) Photometric microtitre assay of inorganic phosphate in the presence of acid-labile organic phosphates. *Anal. Biochem.* 230, 173–177.
- (22) Legendre, J.-Y., and Szoka, F. C. (1992) Delivery of plasmid DNA into mammalian cell lines using pH-sensitive liposomes: comparison with cationic liposomes. *Pharm. Res.* 9, 1235–1242.
- (23) Cullis, P. R., and De Kruijff, B. (1979) Lipid polymorphism and the functional roles of lipids in biological membranes. *Biochim. Biophys. Acta* 559, 399–420.
- (24) Danilov, L. L., Maltsev, S. D., and Shibaev, V. N. (1990) Phosphorylation of nonacosanol and cholesterol with tetra-*n*-butylammonium dihydrogen phosphate and trichloroacetonitrile. *Bioorg. Khim.* 16, 1002–1003.
- (25) Davis S. C., and Szoka F. C. (1998) Conditionally Stable Liposomes for Gene Delivery. *Intelligent materials and novel concepts for controlled release technologies* (Dinh S. M., and DeNuzzio J. Eds.) ACS Symposium Series, American Chemical Society, Washington, DC (in press).
- (26) New, R. R. C. (Ed.) (1990) *Liposomes a practical approach*, pp 124–125 IRL Press, Oxford.
- (27) Sundler, R., Düzgünes, N., and Papahadjopoulos, D. (1981) Control of membrane fusion by phospholipid headgroups II. The role of phosphatidylethanolamine in mixtures with phosphatidate and phosphatidylinositol. *Biochim. Biophys. Acta* 649, 751–758.
- (28) Tietz, N. W. (Ed.) (1995) *Clinical Guide to Laboratory Tests*, W. B. Saunders and Co., Philadelphia PA.
- (29) Szoka, F., and Papahadjopoulos, D. (1978) Procedure for preparation of liposomes with large internal aqueous space and high capture by reverse-phase evaporation. *Proc. Natl. Acad. Sci. U.S.A.* 75, 4194–4198.
- (30) Tang, M. X., Redemann, C. T., and Szoka, F. C. (1996) In vitro gene delivery by degraded polyamidoamine dendrimers. *Bioconjugate Chem.* 7, 703–714.
- (31) Snyder, S. L., and Wilson, I. B. (1972) Phosphoramidic acids. A new class of nonspecific substrates for alkaline phosphatase from *Escherichia coli*. *Biochemistry* 11, 1616–1623.
- (32) Metaye, T., Mettey, Y., Lehuède, J., Vierfond, J. M., and Lalegerie, P. (1998) Comparative inhibition of human alkaline phosphatase and diamine oxidase by bromo-levamisole, cimetidine and various derivatives. *Biochem. Pharmacol.* 37, 4263–4268.
- (33) Wallis, E. S., Fernholz, E., and Gephart, F. T. (1937) Molecular rearrangements in the sterols. I. The action of anhydrous potassium acetate on cholesteryl *p*-toluenesulfonate in acetic acid solution. *J. Am. Chem. Soc.* 59, 137–140.
- (34) Langer, R. S., and Peppas, N. A. (1981) Present and future applications of biomaterials in controlled drug delivery systems. *Biomaterials* 2, 201–214.
- (35) Leventis, R., Gagné, J., Fuller, N., Rand, R. P., and Silvius, J. R. (1986) Divalent cation induced fusion and lipid lateral segregation in phosphatidylcholine-phosphatidic acid vesicles. *Biochemistry* 25, 6978–6987.
- (36) Brock, T. G., Nagaprakash, K., Margolis, D. I., and Smolen, J. E. (1994) Modeling degranulation with liposomes: effect of lipid composition on membrane fusion. *J. Membr. Biol.* 141, 139–148.
- (37) Jordan, M., Schallhorn, A., and Wurm, F. M. (1996) Transfecting mammalian cells: optimization of critical parameters affecting calcium-phosphate precipitate formation. *Nucleic Acids Res.* 24, 596–601.
- (38) Papahadjopoulos, D., Vail, W. J., Jacobson, K., and Poste, G. (1975) Cochleate lipid cylinders: formation by fusion of unilamellar lipid vesicles. *Biochim. Biophys. Acta* 394, 483–491.
- (39) Gould-Fogerite, S., Mazurkiewicz, J. E., Raska, K., Voelkerding, K., Lehman, J. M., and Mannino, R. J. (1989) Chimera-mediated gene transfer in vitro and in vivo. *Gene* 84, 429–438.
- (40) Gould-Fogerite, S., and Mannino, R. J. (1996) Mucosal and systemic immunization using cochleate and liposome vaccines. *J. Liposome Res.* 6, 357–379.
- (41) Niculescu-Duvaz, I., and Springer, C. J. (1997) Antibody-directed enzyme prodrug therapy (ADEPT): A review. *Adv. Drug Del. Rev.* 26, 151–172.
- (42) Wallace, P. M., and Senter, P. D. (1991) In vitro and in vivo activities of monoclonal antibody-alkaline phosphatase conjugates in combination with phenol mustard phosphate. *Bioconjugate Chem.* 2, 349–352.

BC980047Y

Functionalized Semitelechelic Poly[*N*-(2-hydroxypropyl)methacrylamide] for Protein Modification

Zheng-Rong Lu,[†] Pavla Kopečková,^{†,‡} Zhuchun Wu,[§] and Jindřich Kopeček^{*,†,‡}

Departments of Pharmaceutics and Pharmaceutical Chemistry/CCCD and of Bioengineering, Mass Spectrometry Facility, University of Utah, Salt Lake City, Utah 84112. Received May 12, 1998; Revised Manuscript Received July 17, 1998

Semitelechelic poly[*N*-(2-hydroxypropyl)methacrylamide]s (ST-PHPMA) with different functional end groups, namely carboxyl, methyl ester, hydrazide, and amino groups, were prepared by chain transfer free-radical polymerization. 2,2'-Azobisisobutyronitrile (AIBN) was used as an initiator and 3-mercaptopropionic acid, methyl 3-mercaptopropionate, 3-mercaptopropionic hydrazide, and 2-mercaptoethylamine were used as chain-transfer agents. The semitelechelic polymers have been characterized by end-group analysis, size-exclusion chromatography (SEC), and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). The effects of the concentrations of the mercaptans and the initiator on the molecular weight of the polymers have been investigated. The higher the concentration of mercaptan, the lower the molecular weight of ST-PHPMA. The concentration of initiator did not have a significant effect on the molecular weight of the semitelechelic polymers. The end groups of the ST polymers can be readily transformed by polymeranalogous reactions. A model protein, α -chymotrypsin, has been modified with ST-PHPMA-CONHNH₂ and ST-PHPMA-COOSu and the conjugates characterized by MALDI-TOF MS. The activity of modified chymotrypsins toward a high molecular weight substrate, P-Gly-Leu-Phe-Nap (where P is the HPMA copolymer backbone, and Nap is *p*-nitroanilide), was slightly lower than the activity of the native enzyme. The cleavage of a low molecular weight substrate, Z-Gly-Leu-Phe-Nap, by modified chymotrypsins was dependent on their structure. Whereas the activity of the amino group modified chymotrypsins was higher than that of the native enzyme, the activity of carboxyl-modified chymotrypsins was lower than that of the native enzyme. In summary, the data seem to indicate that ST-PHPMA is an effective protein-modifying agent.

INTRODUCTION

Semitelechelic (ST)¹ polymers are linear macromolecules containing a functional group at one end of the polymer chain. The functional group can be used for conjugating or grafting the polymer chains to other molecular species or surfaces without causing cross-

linking. For example, the covalent attachment of methoxy poly(ethylene glycol) (mPEG) to therapeutic proteins increases their resistance to proteolysis, reduces their antigenicity, and prolongs their intravascular half-life (1–3). The attachment of PEG to biomaterial surfaces results in protein repulsion as a result of PEG's low interfacial free energy with water, hydrophilicity, high surface mobility, and steric stabilization effects (4). Covalent conjugation of PEG with hydrophobic anticancer drugs results in more soluble and more easily formulated and delivered drugs (5).

Numerous ST polymers of different structures have been evaluated and many possess effects similar to PEG when conjugated to proteins. Structural characteristics of ST polymers, such as the molecular weight (6), linearity, or branching (7), chemical structure (8–15), and degree of substitution (16) are decisive for the properties of the conjugates. A suitable route for the synthesis of ST polymers is radical polymerization in the presence of chain-transfer agents. Okano et al. have shown that mercaptans are effective at introducing functional groups to the ends of macromolecules and regulating the molecular weight via chain-transfer reactions (12, 17).

N-(2-Hydroxypropyl)methacrylamide (HPMA) copolymers are water-soluble biocompatible polymers used in anticancer drug delivery (reviewed in ref 18). HPMA copolymers containing reactive groups at side-chain termini were used for the modification of trypsin (19), chymotrypsin (19, 20), and acetylcholinesterase (21). The attachment via side-chain termini most probably resulted

* To whom correspondence should be addressed. Phone: (+801) 581 7211. Fax: (+801) 581 7848. E-mail: Jindrich.Kopecek@m.cc.utah.edu.

[†] Departments of Pharmaceutics and Pharmaceutical Chemistry/CCCD.

[‡] Department of Bioengineering.

[§] Mass Spectrometry Facility.

¹ Abbreviations: AIBN, 2,2'-azobisisobutyronitrile; DCC, dicyclohexyl carbodiimide; DCU, dicyclohexyl urea; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; DP_{n(end)}, number average degree of polymerization determined by end group analysis; HPMA, *N*-(2-hydroxypropyl)methacrylamide; IBN, radical formed by decomposition of AIBN; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; M_n , number average molecular weight; $M_{n(end)}$, number average molecular weight determined by end group analysis; M_w , weight average molecular weight; MWCO, molecular weight cut off; Nap, *p*-nitroanilide; HOSu, *N*-hydroxysuccinimide; ONp, *p*-nitrophenoxyl; P, HPMA copolymer backbone; PBS, phosphate-buffered saline; PEG, poly(ethylene glycol); PHPMA, polyHPMA; SEC, size-exclusion chromatography; ST, semitelechelic; TNBS, trinitrobenzene sulfonic acid; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; Tris/CaCl₂ buffer, 80 mM Tris + 20 mM CaCl₂, pH 8.0; Z, benzyloxycarbonyl.

in multiple point attachment of the macromolecules to the protein. Nevertheless, a dramatic increase in the acetylcholinesterase survival in the blood stream of mice (21) and in the thermostability of modified enzymes (19, 21) was observed when compared to the native proteins.

The multipoint attachment of the polymers to protein has the disadvantage that it may cause unexpected cross-linking. The synthesis of semitelechelic poly[*N*-(2-hydroxypropyl)methacrylamide] (ST-PHPMA) permits one-point attachment of HPMA macromolecules to surfaces and proteins, and avoids the possible cross-linking caused by multipoint attachment. Its covalent attachment to nanospheres resulted in surfaces with decreased biorecognizability (10). It appears that ST-PHPMA has potential as a protein or biomaterial surface-modifying agent. Consequently, this study is devoted to the synthesis of ST-PHPMA containing different functional groups, namely amino, carboxyl, *N*-hydroxysuccinimide ester, methyl ester, and hydrazide groups. These groups were introduced at one end of the polymer chains using functionalized mercaptans as chain-transfer agents or by polymeranalogous reactions of semitelechelic intermediates. The functionality and the molecular weight of ST-PHPMA were characterized by end-group determination and size exclusion chromatography (SEC).

Carboxyl and amino group directed modifications of a model protein, α -chymotrypsin, with ST-PHPMA were performed. The impact of chemical modification on the enzymatic activity of ST-PHPMA-substituted chymotrypsins was determined using low and high molecular weight substrates. The macromolecules and conjugates have been characterized by classical methods (SEC, UV, protein content, and functional group determination) and by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) (22).

EXPERIMENTAL PROCEDURES

Chemicals. Phe and Leu were of L-configuration. HPMA (23) and the substrates Z-Gly-Leu-Phe-Nap (16) and HPMA copolymer attached Gly-Leu-Phe-Nap, P-Gly-Leu-Phe-Nap (16) were prepared as described. α -Chymotrypsin from bovine pancreas was from Sigma, St. Louis, MO. 2,2'-Azobisisobutyronitrile (AIBN) was purified by recrystallization from ethanol. Methyl 3-mercaptopropionate, 3-mercaptopropionic acid, and 2-mercaptoethylamine (Aldrich, Milwaukee, WI) were used without further purification. Solvents and all other reagents were of reagent grade or better.

Synthesis of 3-Mercaptopropionic Hydrazide. Methyl 3-mercaptopropionate (10 g, 83 mmol) was added dropwise to an excess of hydrazine monohydrate (10 g, 200 mmol) dissolved in 30 mL of methanol, and the reaction mixture was stirred at rt for 72 h. Methanol was removed under vacuum and the product separated by chromatography on a silica gel column eluted with methanol/diethyl ether (1:4). The fraction containing the product was collected and the solvent removed by rotoevaporation. The oily product, 3-mercaptopropionic hydrazide, was dried under vacuum; yield 8.3 g, (83%); R_f = 0.62 (methanol/acetonitrile, 1:4). ^1H NMR (CDCl_3 , ppm): 1.57 (t, 1H, SH), 2.43 (t, 2H, COCH_2), 2.76 (m, 2H, CH_2SH), 3.96 (sh, 2H, NH_2), 7.55 (sh, 1H, NH). MS (scan ES^+ , m/z): 121.6 ($\text{M}^+ + 1$).

Synthesis of Semitelechelic Polymers. Semitelechelic HPMA polymers were prepared by free-radical solution polymerization in methanol using functionalized mercaptans as chain-transfer agents (17) and AIBN as the initiator. A typical synthesis is described below using

Table 1. Synthesis and Characterization of ST-PHPMA-COOSu^a Polymers

code	original ST-PHPMA- COOH		ratio [DCC]/ [COOH]	ratio [HOSu]/ [COOH]	yield of polymer (%)	yield ^b of conversion COOH to COOSu
	M_n (SEC) ^c	$M_{n(\text{end})}$ ^d				
1	5400	5510	5	5	86	50 ^e
2	5400	5510	17	20	76	77 ^b
3	4580	3800	14	14	54	80 ^e
4	4340	3740	20	20	90	94 ^b
5	3440	2680	20	20	89	75 ^e

^a HOSu, *N*-hydroxysuccinimide. ^b Yield determined after hydrolysis from released *N*-hydroxysuccinimide. ^c M_n , the number average molecular weight determined from size-exclusion chromatography. ^d M_n , the number average molecular weight determined from titration of COOH end groups. ^e Yield determined after polymeranalogous reaction with glycyl *p*-nitroanilide using $\epsilon = 1.3 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ in H_2O .

as an example the synthesis of a ST-PHPMA-COOH polymer. All polymers were isolated by precipitation as described below, except the ST-PHPMA-CONHNH₂ polymers which were dialyzed against water using a membrane with molecular weight cut off (MWCO) 500 and isolated by freeze-drying.

Synthesis of ST-PHPMA-COOH. HPMA (1.0 g, 7.0 mmol), 3-mercaptopropionic acid (22 mg, 0.21 mmol), and AIBN (50 mg, 0.3 mmol) were dissolved in 10 mL of methanol. The solution was bubbled with nitrogen and sealed in an ampule. The reaction mixture was stirred at 50 °C for 24 h. After polymerization, the volume of the reaction mixture was reduced, the polymer was isolated by precipitation into diethyl ether, filtered, and washed with diethyl ether. The product was purified by reprecipitation three times. The weight-average (M_w) and number-average (M_n) molecular weights and polydispersity were determined by size-exclusion chromatography (SEC) on a Superdex 75 HR 10/30 column calibrated with ST-PHPMA samples of narrow polydispersity ($M_w/M_n < 1.1$; molecular weight of calibration samples were determined by MALDI-TOF MS) using a Pharmacia FPLC system (PBS buffer, pH 7.3); $M_n = 5410$, $M_w = 7530$, $M_w/M_n = 1.4$. The content of end groups determined by titration was 0.181 mmol/g. The number-average molecular weight [$M_{n(\text{end})}$] calculated from the content of end groups was 5510; the ratio of molecular weights obtained from SEC and end group determination, $M_n/M_{n(\text{end})} = 0.98$.

Fractionation of ST-PHPMA Polymers. The fractionation of ST-PHPMA polymers was performed by SEC on a Superdex 75 HR 16/60 column (PBS buffer, pH 7.3). For example, ST-PHPMA-NH₂ in PBS buffer was applied on the column, and the polymer fractions were collected at different elution time intervals. The polymer fractions were desalted by dialysis, lyophilized, and characterized by MALDI-TOF MS and SEC on a Superdex 75 HR 10/30 column (PBS buffer, pH 7.3) using a Pharmacia FPLC system equipped with a refractive index detector.

Polymeranalogous Transformations. *Synthesis of ST-PHPMA-COOSu.* ST-PHPMA-COOSu polymers were synthesized by the reaction of ST-PHPMA-COOH and *N*-hydroxysuccinimide in the presence of dicyclohexylcarbodiimide (DCC) in anhydrous DMF. Polymers with different properties were obtained under different synthetic conditions, and the yield and degree of the modification are reported in Table 1. The procedure will be demonstrated by the synthesis of polymer 3. ST-PHPMA-COOH [$M_{n(\text{end})} = 3800$; 280 mg, 0.073 mmol] and *N*-hydroxysuccinimide (120 mg, 1.0 mmol) were dissolved

Table 2. Characterization of ST-PPHMA-CONHNH₂ Prepared by Hydrazinolysis of ST-PPHMA-COOCH₃

code	ST-PPHMA-COOCH ₃				hydrazine (mmol)	ST-PPHMA-CONHNH ₂					
	$M_{n(\text{end})}^a$	M_n	M_w	mg		yield of polymer (%)	$M_{n(\text{end})}^b$	M_n	M_w	M_w/M_n^c	$M_n/M_{n(\text{end})}$
6	2000	2510	3160	500	10	46	2250	3020	4237	1.47	1.34
7	3000	2890	3780	500	10	40	2800	3610	5270	1.46	1.29
8	2900	3480	6340	700	15	58	3330	4830	7930	1.64	1.45
9	4200	4690	8830	700	10	65	5100	6070	10700	1.74	1.19

^a Number average molecular weight estimated from proton NMR. ^b Number average molecular weight determined by modified TNBS assay. ^c Data from size exclusion chromatography.

in 5 mL of anhydrous DMF and cooled in an ice–water bath. DCC (200 mg, 1.0 mmol) in 3 mL of DMF was added dropwise with stirring. The mixture was kept at 4 °C for 48 h, and at rt for an additional 12 h. The volume of the reaction mixture was reduced under vacuum, dicyclohexyl urea (DCU) was removed by filtration, and the polymer solution was precipitated by dropping it into an excess of diethyl ether. The product (**3**) was purified by reprecipitation three times (methanol solution to diethyl ether) and dried under vacuum. Yield: 150 mg, 54%. The amount of active ester groups was determined by detecting the released *N*-hydroxy-succinimide anion after addition of an excess of 1-amino-2-propanol; $\epsilon_{260\text{ nm}} = 8500\text{ M}^{-1}\text{ cm}^{-1}$ (24). The yield of active ester formation was 80%, based on the COOH content determined by titration.

Synthesis of ST-PPHMA-CONHNH₂ by Hydrazinolysis of ST-PPHMA-COOCH₃. An alternate route of ST-PPHMA-CONHNH₂ synthesis was the hydrazinolysis of ST-PPHMA-COOCH₃. The ST-PPHMA-COOCH₃ polymers used for hydrazinolysis, the yield and properties of ST-PPHMA-CONHNH₂ are reported in Table 2. The synthesis of polymer **8** (Table 2) is shown as an example. ST-PPHMA-COOCH₃ (0.7 g, 0.2 mmol) was dissolved in 5 mL of methanol and added dropwise to a 20 mL methanol solution of hydrazine monohydrate (0.75 g, 15 mmol) with stirring. The reaction mixture was refluxed for 5 h and concentrated by rotoevaporation. The product was isolated by precipitation into an excess of diethyl ether, dialyzed against deionized water (tubing, MWCO 500), and lyophilized; yield 0.41 g, 58%. The weight-average (M_w), number-average (M_n) molecular weights, and polydispersity were determined by SEC on a Superdex 75 column calibrated with fractions of PPHMA (PBS buffer, pH 7.3) using a Pharmacia FPLC system, $M_n = 4830$, $M_w = 7930$, $M_w/M_n = 1.64$. The content of hydrazide end groups was determined by the modified TNBS assay (0.300 mmol/g) (25). The number-average molecular weight [$M_{n(\text{end})}$] based on the content of end groups was 3330, $M_n/M_{n(\text{end})} = 1.45$.

Methods for End Group Determination. *Determination of COOH Groups.* ST-PPHMA-COOH samples were titrated with sodium hydroxide standard solution using an ABU80 autoburet and PHM84 research pH meter as monitor.

Determination of COOCH₃ Groups by Hydrolysis. ST-PPHMA-COOCH₃ samples (<50 mg) were dissolved in standard KOH solution (excess). The solution was kept at 50 °C for 2 h to hydrolyze all COOCH₃ groups, followed by back-titration of residual KOH by HCl.

Determination of COOCH₃ Groups by ¹H NMR. The proton NMR spectra were recorded on a Bruker NR200 FTNMR spectrometer using D₂O as the solvent. The integration of the peaks of the protons of the methyl ester group ($\delta = 3.58\text{ ppm}$) and the proton on C-2 ($\delta = 3.8\text{ ppm}$) of the 1-amino-2-propanol residue were used to estimate the content of methyl ester and the number average molecular weight, $M_{n(\text{end})}$.

Determination of Hydrazide Groups by TNBS Assay. ST-PPHMA-NHNH₂ was dissolved in 0.1 M sodium tetraborate, pH 9.3. The polymer solution (1.0–2.0 mg; 1.0 mL) was mixed with 25 μL of 0.03 M TNBS aqueous solution in a cuvette, vortexed, and kept at room temperature for 30 min. Absorbance was read at 500 nm (25). NH₂NHCOOC₂H₅ was used as a calibration standard.

*Determination of COOSu Groups by Polymeranalogous Reaction with Glycine *p*-Nitroanilide.* Example: ST-PPHMA-COOSu (**2**, Table 1; 5.31 mg, $1.0 \times 10^{-3}\text{ mmol}$) and (excess) glycine *p*-nitroanilide (5.0 mg, 0.026 mmol) were dissolved in 0.5 mL of DMF and kept at rt for 1 h and the polymer purified on a Sephadex G-25 column (PD-10, Pharmacia). The content of polymer bound *p*-nitroanilide was determined by UV spectrophotometry at 315 nm ($\epsilon = 1.3 \times 10^4\text{ M}^{-1}\text{ cm}^{-1}$ in water). The content of COOSu end groups was 77% of theory.

Determination of Amino Groups. The amino groups were determined by ninhydrin assay. Glycine was used for the calibration.

Modification of Chymotrypsin with ST-PPHMA. Nine conjugates of chymotrypsin with ST-PPHMA were synthesized. Conjugates **I–IV** were synthesized by amino group directed modification whereas conjugates **V to IX** were synthesized by carboxyl-directed modification. The synthesis and characterization of the conjugates are described below.

Amino Group-Directed Modification of Chymotrypsin with ST-PPHMA-COOSu. *Conjugate I.* ST-PPHMA-COOSu (**2**, Table 1) [200 mg, 0.036 mmol, based on $M_{n(\text{end})}$] and α -chymotrypsin (30 mg; $1.2 \times 10^{-3}\text{ mmol}$) were dissolved in 6 mL of 20 mM CaCl₂ aqueous solution [molar ratio of chymotrypsin to polymer, based on $M_{n(\text{end})}$ was 1:30]. The pH of the reaction mixture was maintained at 7.0–7.5 for 1 h at 4 °C by adding dilute NaOH solution, raised to 8.0 and maintained for 30 min. The pH was then adjusted with dilute HCl to 3.0 for storage. The unbound polymer was removed by ultrafiltration with a Centricon-30 concentrator (membrane MWCO = 30 000). The ultrafiltration was repeated 5 times by adding fresh 20 mM CaCl₂ solution (pH 3.0) each time. The degree of conjugation was 22% as determined by the TNBS assay (26) calibrated with chymotrypsin. The conjugate was characterized with MALDI-TOF MS, and the M_n and M_w of the conjugate were 44 900 and 46 600 Da, respectively. The molecular weights of the conjugate ($M_n = 46\text{ 300}$; $M_w = 54\text{ 000}$) were also determined by SEC on a Superose 6 HR 10/30 column with a light-scattering detector (MiniDawn, Wyatt) after the conjugate was desalted and lyophilized. The conjugate contained three to four ST-PPHMA chains per protein molecule.

Conjugate II. The reaction of ST-PPHMA-COOSu (**4**; Table 1) with chymotrypsin was performed as described for conjugate **I** with the exception that the molar ratio of chymotrypsin to the polymer was 1:40 and pH of the reaction mixture was maintained at 8.5. The degree of the conjugation was 43% as determined by the TNBS

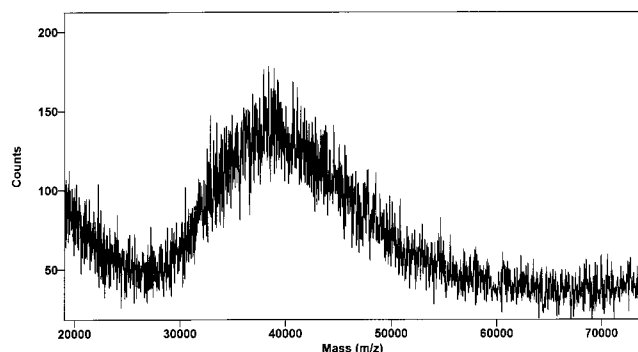


Figure 1. The MALDI-TOF mass spectrum of amino group modified chymotrypsin conjugate (**III**). ST-PHPMA-COOSu (**5**; Table 1) prepared from ST-PHPMA-COOH (**11**, Table 5, Figure 6) was used for the conjugation. The M_n and M_w of the fraction calculated from the MS were 40 860 and 41 690, respectively. Average six polymer chains were attached to each enzyme molecule.

assay. The M_n and M_w of the conjugate as determined by MALDI-TOF MS were 49 400 and 51 000 Da, respectively. The conjugate contained six to seven ST-PHPMA chains per protein molecule.

Conjugate III. The reaction of ST-PHPMA-COOSu (**5**; Table 1) with chymotrypsin was performed as described for conjugate **I**. The degree of conjugation was 34% as determined by the TNBS assay. The M_n and M_w of the conjugate from MALDI-TOF MS (Figure 1) were 40 900 and 41 700 Da, respectively. The conjugate contained six ST-PHPMA chains per protein molecule.

Conjugate IV. The reaction of ST-PHPMA-COOSu (**5**; Table 1) with chymotrypsin was performed as described for conjugate **III** with the exception that the molar ratio of α -chymotrypsin and polymer was 1:17 based on $M_{n(\text{end})}$. The degree of conjugation was 25% as determined by the TNBS assay. The M_n and M_w of the conjugate from MALDI-TOF MS were 36 100 and 37 800 Da, respectively. The conjugate contained four ST-PHPMA chains per protein molecule.

Carboxyl-Directed Modification of Chymotrypsin with ST-PHPMA-CONHNH₂. **Conjugate V.** ST-PHPMA-CONHNH₂ [$M_{n(\text{end})}$ = 2490; M_n = 3380, M_w = 4750] [300 mg, 0.12 mmol based on $M_{n(\text{end})}$] and α -chymotrypsin (25 mg, 1.0×10^{-3} mmol) were dissolved in 5 mL of 20 mM CaCl₂ aqueous solution, pH 5.0. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (38 mg, 0.2 mmol) was added to the solution, and the reaction mixture stirred at 4 °C for 12 h. The pH of the reaction mixture was maintained at 4.5–5.0 by adjusting with diluted HCl. After the reaction was completed, the pH of the reaction mixture was adjusted to 3.0 with diluted HCl and the unbound polymer removed by ultrafiltration with a Centricon-30 concentrator (membrane MWCO = 30 000). The ultrafiltration was repeated 5 times after adding fresh 20 mM CaCl₂ solution (pH 3.0) each time. The enzyme conjugate was characterized by SEC on a Superdex 75 HR 10/30 column (PBS buffer, pH 7.3) using a Pharmacia FPLC system. The result indicated that almost all free polymer was removed. In addition, the conjugate was characterized with MALDI-TOF MS (Figure 2); the M_n and M_w of the conjugate were 46 600 and 47 800 Da, respectively. The conjugate contained eight to nine ST-PHPMA chains per protein molecule.

Conjugate VI. The conjugate was synthesized by the reaction of α -chymotrypsin with a narrow fraction of ST-PHPMA-CONHNH₂. The ST-PHPMA-CONHNH₂ fraction collected from SEC on a Superdex 75 HR16/60 column was lyophilized. The salts were removed by

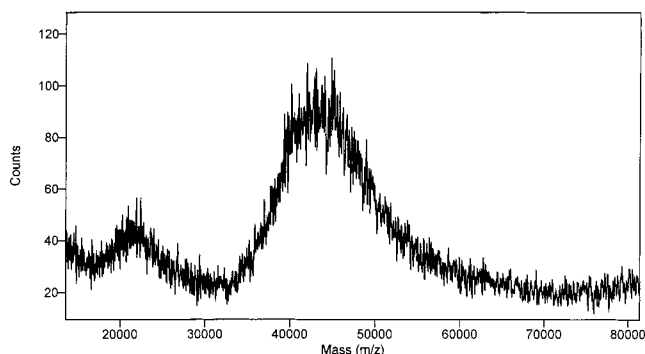


Figure 2. The MALDI-TOF mass spectrum of carboxyl group modified chymotrypsin conjugate (**V**). ST-PHPMA-CONHNH₂ [$M_{n(\text{end})}$ = 2490; M_n = 3380, M_w = 4750] was used for the conjugation. The peak around m/z = 45 000 is the single charged conjugate, the small peak around m/z = 22 000 is the double charged conjugate. The M_n and M_w of the conjugate calculated from the MALDI-TOF MS were 46 600 and 47 800 Da, respectively. There are eight to nine polymer chains attached to each enzyme molecule.

extracting the polymer with ethanol. The content of hydrazide groups was determined by TNBS assay, which was used for calculating the stoichiometry of the conjugation reaction. The ST-PHPMA-CONHNH₂ fraction (M_n = 1300, M_w = 1400; 22 μ mol), α -chymotrypsin (10 mg, 0.4 μ mol), and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (40 mg, 0.2 mmol) were dissolved in 2.0 mL of pH 4.5 water. The reaction mixture was stirred at 4 °C for 12 h and the pH of the reaction mixture maintained at 4.5–5.0. The conjugate was separated from the free polymer by SEC on a Superose 6 HR 10/30 column, concentrated and desalted by centrifuge ultrafiltration with a Centricon-10 concentrator (MWCO = 10 000). The conjugate was characterized by MALDI-TOF MS (Figure 3), and the average M_n and M_w of the conjugate as calculated from the MS were 36 650 and 36 800, respectively. Approximately nine ST-PHPMA chains were attached to one protein molecule.

The conjugates **VII**, **VIII**, and **IX** were prepared by the conjugation of chymotrypsin with ST-PHPMA-CONHNH₂ polymer fractions with different molecular weight. The conjugation reactions were carried out according to the procedure for preparation of conjugate **VI**. All of the conjugates were characterized by MALDI-TOF MS. The molarity of different reactants, the molecular weights of the fractions and the molecular weights of the conjugates calculated from the MALDI-TOF MS are reported in Table 3.

Chymotryptic Activity. α -Chymotrypsin contained 65% of active protein as determined by titration of active sites with Z-Tyr-ONp (27). The stock solutions of native and modified α -chymotrypsins were prepared in 1 mM HCl containing 20 mM CaCl₂, pH 3.0. Their concentrations were determined spectrophotometrically at 280 nm using ϵ = 5.2×10^4 M⁻¹ cm⁻¹. A low molecular weight substrate, Z-Gly-Leu-Phe-NAP and a high molecular weight substrate, P-Gly-Leu-Phe-NAP (HPMA copolymer containing 3.4 mol % of -Gly-Leu-Phe-NAP side chains) were used for the evaluation of enzyme activities. The stock solution of Z-Gly-Leu-Phe-NAP was prepared by dissolving it in the smallest amount of DMSO possible and then diluting with Tris/CaCl₂ buffer (80 mM Tris + 20 mM CaCl₂, pH 8.0). The final concentration of DMSO in the reaction mixture was less than 5% (v/v). The stock solution of P-Gly-Leu-Phe-NAP was prepared directly in the Tris/CaCl₂ buffer. The concentration of NAP in the

Table 3. Characterization of Carboxyl Group Modified Chymotrypsin Conjugates with ST-PHPMA-CONHNH₂ Polymer Fractions

ST-PHPMA-CONHNH ₂ fractions				chymotrypsin (μ mol)	EDC (mmol)	Chymotrypsin conjugates			
M_n^a	M_w	M_w/M_n	μ mol			code	M_n^a	M_w	N_p^b
1300	1400	1.08	22	0.4	0.2	VI	36 650	36 800	8.8
1350	1430	1.06	29	0.2	0.1	VII	37 800	38 000	9.3
2100	2200	1.05	20	0.2	0.1	VIII	38 900	39 300	6.5
3200	3370	1.05	9.2	0.4	0.2	IX	45 600	46 200	6.4

^a The average molecular weights of the polymer fractions and polymer–chymotrypsin conjugates were calculated from MALDI-TOF MS. ^b The average number of polymer chains on each chymotrypsin molecule.

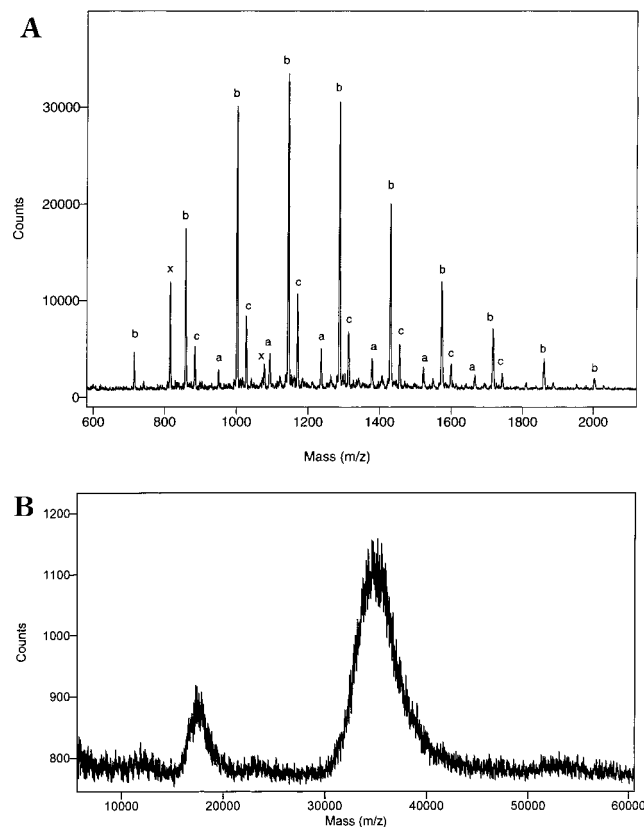


Figure 3. (A) The MALDI-TOF mass spectrum of ST-PHPMA-CONHNH₂ with narrow distribution of molecular weights obtained by SEC fractionation. Peak series **a** represent the peaks of polymer chains with initiator end groups; peak series **b** represent the peaks of semitelechelic polymer chains with hydrazide end groups; peak series **c** represent the peaks of polymer chains with unknown structure, probably formed by the side reaction during the polymerization. Peaks **x** are peaks related to the matrix used for analysis. The M_n and M_w of the ST-PHPMA-CONHNH₂ fraction calculated from the MS were 1300 and 1400, respectively. (B) The MALDI-TOF mass spectrum of the chymotrypsin conjugate (**VI**) with the ST-PHPMA-CONHNH₂ fraction shown above. The peak around $m/z = 35\,000$ is the single charged conjugate, and the small peak around $m/z = 17\,500$ is the double charged conjugate. The M_n and M_w of the conjugate were 36 650 and 36 800, respectively. Average nine (8.8) polymer chains were attached to each enzyme molecule.

stock solutions was determined by UV spectrophotometry (315 nm, $\epsilon = 13\,000\text{ M}^{-1}\text{ cm}^{-1}$).

Cleavage of *p*-Nitroanilide Substrates. The kinetics of enzymatic cleavage of the substrates was performed at 37 °C and the *p*-nitroanilide release monitored at 410 nm using a Lambda 9 Perkin-Elmer spectrophotometer. The concentration of enzyme was $2.16 \times 10^{-7}\text{ M}$; the concentration of substrates was (8.0×10^{-6}) – $(9.0 \times 10^{-5})\text{ M}$ for Z-Gly-Leu-Phe-NAP, and (8×10^{-5}) – $(1 \times 10^{-3})\text{ M}$ for P-Gly-Leu-Phe-NAP. Each set of kinetic measurements was performed with eight different substrate concentra-

tions. The initial rates were calculated using $\epsilon = 8800\text{ M}^{-1}\text{ cm}^{-1}$. The kinetic parameters were calculated from Lineweaver–Burk plots of the initial rates of release of *p*-nitroaniline.

MALDI-TOF Mass Spectrometric Analysis. All ST-PHPMA polymers and α -chymotrypsin polymer conjugates were dissolved in 50% aqueous acetonitrile solution. 2-(4-Hydroxyphenylazo)benzoic acid (HABA) was used as the matrix for polymer samples, while sinapinic acid [SA; *trans*-3-(4-hydroxy-3,5-dimethoxyphenyl)acrylic acid] was used for the α -chymotrypsin polymer conjugates. The cationizing reagent, sodium chloride, was added to the matrixes to facilitate the ionization process in the analysis of polymers. All spectra were collected on a MALDI-TOF mass spectrometer (Voyager-DE STR Biospectrometry Workstation, PerSeptive Biosystems, Inc., Framingham, MA). The mass spectrometer is equipped with a nitrogen laser with a wavelength of 337 nm. The instrument was operated at a 20 kV accelerating voltage with the use of delayed extraction and the low-mass gate. The low-mass gate was set to cut out the matrix ions. The delayed time was varied to achieve the highest mass resolution. All spectra were obtained in linear mode with the instrument calibrated externally using peptides or proteins, such as angiotensin, insulin, and myoglobin, in the same solution and matrixes as used for sample analysis.

RESULTS AND DISCUSSION

Synthesis of ST-PHPMA Polymers. ST-PHPMA polymers with amino, carboxy, hydrazo, and methyl ester groups were synthesized by free radical chain-transfer polymerization using alkyl mercaptans, 2-mercaptoethylamine, 3-mercaptopropionic acid, 3-mercaptopropionic hydrazide, and methyl 3-mercaptopropionate as chain transfer agents, respectively (Scheme 1). The polymerization was carried out in methanol with AIBN as an initiator in the presence of alkyl mercaptans. The course of the polymerization was investigated systematically by variation of the concentration of mercaptans with constant concentrations of HPMA and AIBN or by variation of the concentration of AIBN with constant concentrations of HPMA and mercaptan. The change of the molar ratio of AIBN to HPMA did not have a significant effect on the chain length of the polymers when polymerization proceeded in the presence of chain-transfer agents (data not shown). However, the molecular weights of the semitelechelic polymers were strongly affected by the molar ratio of the chain-transfer agent and HPMA. The molecular weight decreased with increasing concentration ratio of the chain transfer agent and HPMA, Figure 4.

The reciprocal of the number average polymerization degree $[1/DP_{n(\text{end})}]$, determined by end group assays, was plotted against the molar ratio of chain transfer agent and HPMA ($[S]/[M]$). The linear relation of $1/DP_{n(\text{end})}$ and $[S]/[M]$ observed for all four chain-transfer agents used

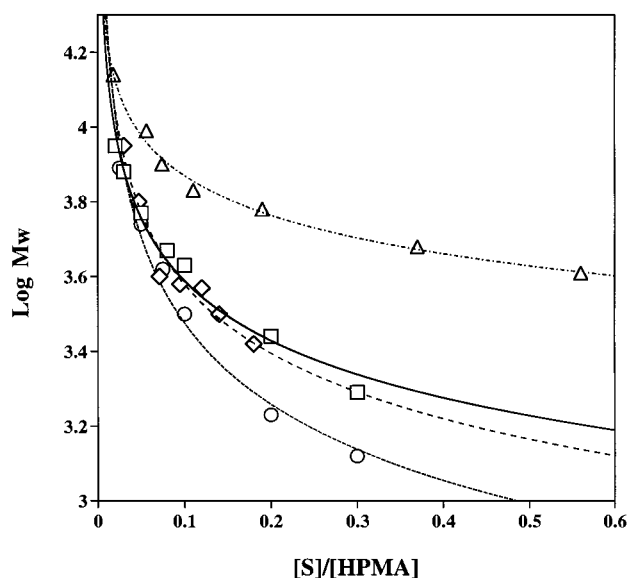
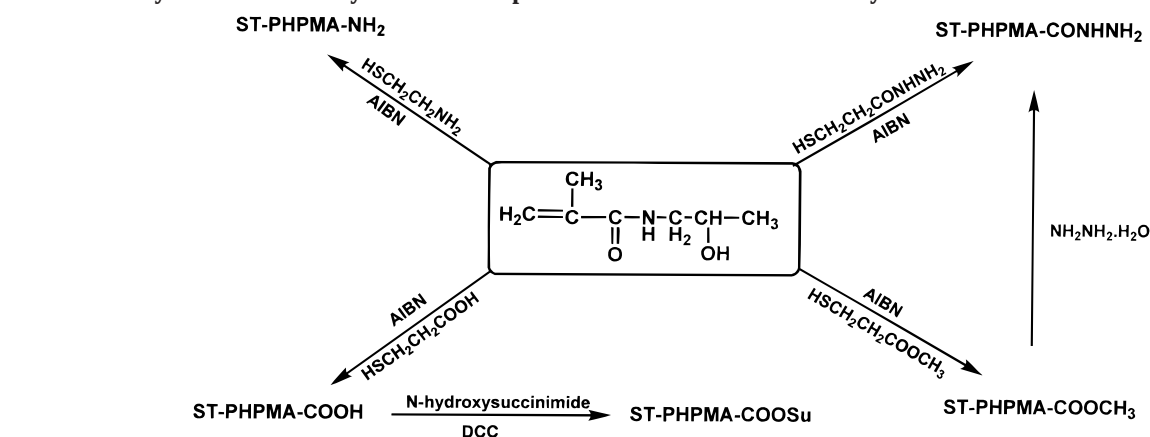
Scheme 1. Synthetic Pathways for the Preparation of Semitelechelic PolyHPMA

Figure 4. The effect of molar ratio of chain transfer agents [2-mercaptoethylamine (Δ), methyl 3-mercaptopropionate ($\square$), 3-mercaptopropionic acid ($\diamond$) and 3-mercaptopropionic hydrazide ($\circ$)] to HPMA ($[S]/[HPMA]$) on the molecular weight of functionalized ST-PHPMA. The weight average molecular weights of the polymers, determined by SEC on a Superdex 75 HR10/30 column are shown here.

Table 4. Chain Transfer Constants (C_s) of Mercaptans Used for the Synthesis of ST-PHPMA Polymers

mercaptan	C_s
$HSCH_2CH_2NH_2$	0.08
$HSCH_2CH_2COOCH_3$	0.32
$HSCH_2CH_2COOH$	0.38
$HSCH_2CH_2CONHNH_2$	0.8

fits well with the Mayo equation (28):

$$1/DP_{n(end)} = 1/DP_{n,0} + C_s[S]/[M]$$

where $DP_{n(end)}$ and $DP_{n,0}$ are the number-average degrees of the polymerization in the presence and in the absence of the chain-transfer agent S; C_s is the chain-transfer constant, and $C_s = k_{tr}/k_p$, where k_{tr} is the rate constant for chain transfer and k_p is the rate constant for propagation. The chain-transfer constants of 2-mercaptoethylamine, 3-mercaptopropionic acid, methyl 3-mercaptopropionate and 3-mercaptopropionic hydrazide are listed in Table 4. 3-Mercaptopropionic hydrazide has the highest C_s , and was the most efficient chain-transfer agent among the four mercaptans, while 2-mercaptoethylamine has

the lowest C_s and was the least efficient chain-transfer agent. This is reflected in the molecular weights obtained; the lower the C_s , the higher the molecular weight of the ST-PHPMA at a constant ratio $[S]/[HPMA]$ (Figure 4).

Polymeranalogous Transformations. Semitelechelic polymers with active ester groups ST-PHPMA-COOSu were synthesized by the functional transformation reaction of ST-PHPMA-COOH with a large excess of *N*-hydroxysuccinimide and DCC (Scheme 1). This reaction is also called polymeranalogous transformation, because the main structure of the polymer is not changed. The results of the conversion of COOH into COOSu groups are summarized in Table 1. The ST-PHPMA-COOSu reacted quantitatively with an excess of glycine-*p*-nitroanilide, which was used for the determination of the content of the active ester end groups. The molecular weight of ST-PHPMA after polymeranalogous esterification did not change, confirming our assumption that secondary OH groups of HPMA monomer units were not reactive under the experimental conditions used. ST-PHPMA-CONHNH₂ was synthesized by the reaction of ST-PHPMA-COOCH₃ with an excess of hydrazine in high conversion of the functional groups (Table 2). The molecular weight of the ST-PHPMA-CONHNH₂ was higher than that of the polymer precursor, most probably due to the loss of low molecular weight polymer fractions during the dialysis used for purification. The proton NMR of the products showed that all methyl ester groups were converted to hydrazide groups. ST-PHPMA-CONHNH₂ can readily react with acetone to form ST-PHPMA with a hydrazone end group, which was used to distinguish ST-PHPMA-CONHNH₂ from ST-PHPMA-COOCH₃ in the MALDI-TOF mass spectroscopy, because both end groups have almost the same mass.

Characterization of the ST-PHPMA Polymers. The molecular weights of the semitelechelic polymers were estimated by SEC. The average molecular weights of the narrow ST-PHPMA fractions ($M_w/M_n < 1.1$) calculated from MALDI-TOF mass spectra have been used for the calibration of the SEC column. The end-group content of ST-PHPMA was determined by different chemical assays. The amino groups in ST-PHPMA-NH₂ were determined by ninhydrin and TNBS assays. The carboxyl groups of ST-PHPMA-COOH were determined by titration. The methyl ester groups in ST-PHPMA-COOCH₃ were determined by proton NMR and by hydrolysis with excess KOH followed by titration of the remaining KOH with HCl. The hydrazide end groups in ST-PHPMA-CONHNH₂ were determined by TNBS assay, or by the spectrophotometric measurement of

Table 5. Comparison of Molecular Weights Obtained from SEC and MALDI-TOF Mass Spectrometry

polymer or conjugate	SEC			MALDI-TOF MS		
	M_n	M_w	M_w/M_n	M_n	M_w	M_w/M_n
ST-PHPMA-NH ₂ (fraction A) ^a	2040	2100	1.04	1900	1970	1.03
ST-PHPMA-NH ₂ (fraction B) ^a	9840	10100	1.02	9460	9650	1.03
conjugate (III)	46 300 ^b	54 000 ^b	1.17	44 900	46 600	1.04
ST-PHPMA-CONHNH ₂ (10)	2890	4930	1.71	2080	2830	1.36
ST-PHPMA-COOH (11)	3440	4220	1.23	2410	3050	1.26
ST-PHPMA-COOCH ₃ (12)	2890	3780	1.31	2400	3160	1.32
ST-PHPMA-NH ₂ (13)	4570	7490	1.64	2740	4590	1.65

^a **A** and **B** are fractions of polymer **13** prepared by size-exclusion chromatography on a preparative Superdex 75 column. ^b Determined by SEC on a Superose 6 HR 10/30 column with MiniDawn light scattering detector; the molecular weights of all other polymers were determined on a Superdex 75 HR 10/30 column calibrated with fractions of PHPMA of narrow polydispersity.

p-nitrobenzaldehyde hydrazone after reacting the polymer with *p*-nitrobenzaldehyde. The functionalities of the polymers determined by different assays used for the same functional group were in good agreement. The number-average molecular weight $M_{n(\text{end})}$ of the polymers was also calculated from the content of the functional end groups. However, it has been noticed that the $M_{n(\text{end})}$ of the ST polymers prepared with a low molar ratio of mercaptan to HPMA (used to prepared high molecular ST polymers) was higher than the M_n determined by SEC, because more polymer chains with initiator end groups were produced when the concentration of chain-transfer agent was lower.

The ST-PHPMA polymers were also characterized by MALDI-TOF mass spectrometry. MALDI-TOF MS is an important new tool for the characterization of proteins and synthetic polymers. It can accurately determine the molecular weight of a protein and a polymer chain. The precise measurement of the molecular weights of polymer chains by MALDI-TOF MS enables the determination of repeating units and end-group compositions (29, 30). The classic SEC can only provide the molecular weight distribution, which requires calibration with standard materials of similar structure to calculate the molecular weight, and the effect of column broadening in the SEC contributes to an increased value of the M_w/M_n observed (31). However, for synthetic polymers with a broad molecular weight distribution, MALDI-TOF MS could not provide the accurate molecular weight distribution, and the peaks corresponding to higher masses are underestimated in the spectra (32, 33). Published data on polymers of different structures indicate that MALDI-TOF MS analysis results in lower values of molecular weights for the polymers with high polydispersity ($PD > 1.1$) when compared to SEC. There were attempts to improve the analysis of MALDI-TOF MS spectra of polydisperse samples (32); however, the best agreement between data from MALDI-TOF MS and traditional methods was obtained for polymer samples with a narrow polydispersity (31, 34). The analyses of ST-PHPMA polymers here also show that there is a good agreement of the average molecular weights between MALDI-TOF MS and SEC for polymers with a narrow polydispersity ($PD < 1.1$), and the average molecular weight of the polymers with high polydispersity ($PD > 1.2$) determined by the MS was lower than those determined by SEC.

Table 5 lists the average molecular weights determined by SEC, MALDI-TOF MS for ST-PHPMA polymer fractions **A** and **B**, polymer–chymotrypsin conjugate (**III**), and some ST-PHPMA polymers. For polymer fractions (**A** and **B**) with a narrow polydispersity ($M_w/M_n < 1.1$), which were fractionated by SEC from polymer **13** (Table 5), both the number-average and weight-average molecular weights from the MS were only slightly lower than those from SEC, and the difference between two methods

was less than 7%. The molecular weights of the chymotrypsin–polymer conjugates determined by MALDI-TOF MS also agreed well with those determined by SEC equipped with an on-line light-scattering detector (MiniDawn, Wyatt). The difference between the number-average molecular weights (3%) was smaller than the difference between the weight-average molecular weights (14%). However, for polymers **10–13** with higher polydispersity ($PD > 1.2$), the molecular weights from MALDI-TOF MS were considerably lower than those obtained from SEC (using a column calibrated with fractions), because MALDI-TOF MS underestimated the contribution from higher molecular weight polymer chains (32). The difference between the molecular weights obtained from the two methods increased with increasing polydispersity.

Although MALDI-TOF MS could not give the true shape of the molecular weight distribution for ST-PHPMA polymers with high polydispersity (33), the accurate determination of the molecular weight of individual polymer chains may provide very important information on the chemical structure of the end groups and the composition of the individual macromolecules of semitelechelic polymers (29, 30).

Figure 5 shows the MALDI-TOF mass spectrum of ST-PHPMA-CONHNH₂ (**10**; Table 5). The mass spectrum clearly showed the presence of HPMA polymer chains with different end groups in the polymer mixture. The peak-to-peak increment for the polymer chains with identical end groups was 143.2, the molar mass of HPMA. The peak series **a** at $m/z = (n \times 143.2) + 23.0$ (Na^+) + 68.1 + 1.0 (n is the number of repeat units; mass of initiator residue $\text{Me}_2[\text{CN}]\text{C} = 68.1$) corresponds to the polymer chains initiated by isobutyronitrile (IBN) radicals and then terminated by a proton from the chain transfer agent. The sodium ion was used to cationize the polymer chains. The peak series **b** at $m/z = (n \times 143.2) + 23.0$ (Na^+) + 120.2 (molar mass of 3-mercaptopropionic hydrazide = 120.2 Da) represents the semitelechelic polymer chains $\text{H}-(\text{HPMA})_n\text{-SCH}_2\text{CH}_2\text{CONHNH}_2$. There was an unknown series **c** at $m/z = (n \times 143.2) + 23.0$ (Na^+) + 146.0 in the spectrum of ST-PHPMA-CONHNH₂, whose origin is not known. One might suspect that these macromolecules are products of side reactions during polymerization.

Figure 6 shows the MALDI-TOF mass spectrum of ST-PHPMA-COOH (**11**; Table 5) which was used, after esterification to ST-PHPMA-COOSu, for the synthesis of conjugate **III**. The spectrum contains the peak series **a** which corresponds to the polymer chains initiated by IBN radicals and then terminated by a proton from the chain-transfer agent (similar to that shown in the spectrum in Figure 5). The peak series **d** at $m/z = (n \times 143.2) + 23.0$ (Na^+) + 106.1 (molar mass of mercaptopropionic acid = 106.1) and **e** at $m/z = (n \times 143.2) + 23.0$ (Na^+) + 128.1

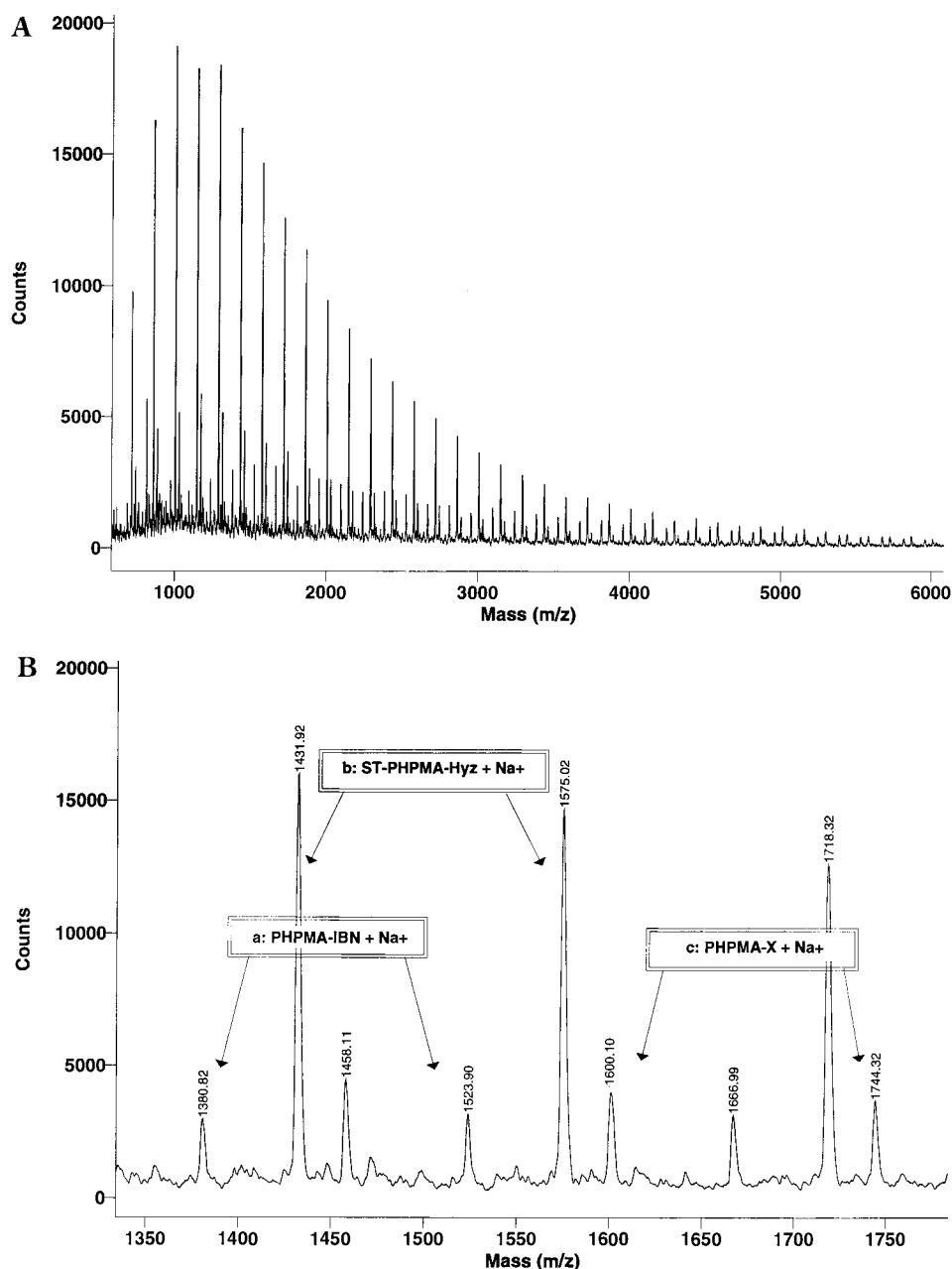


Figure 5. The MALDI-TOF mass spectrum (A) of ST-PHPMA-CONHNH₂ (**10**; Table 5) and its expanded spectrum (B) in the region of 1300–1800 Da. IBN is the isobutyronitrile radical, formed by decomposition of AIBN; Hyz is the hydrazide end group; X is an unknown end group.

(molar mass of sodium mercaptopropionate = 128.1) represents the peaks of the semitelechelic polymer chains of H-(HPMA)_n-SCH₂CH₂COOH and H-(HPMA)_n-SCH₂-CH₂COONa, respectively. The latter peak series is due to the replacement of the proton in the carboxyl group by Na⁺ during the analysis by MALDI-TOF MS.

It appears that the polymerization of HPMA in the presence of potent chain-transfer agents permits the synthesis of semitelechelic macromolecules. The polymerization method is based on the assumption that the reaction of the chain-transfer agent occurs with primary radicals formed by the decomposition of AIBN. However, this takes place only when a high excess of the chain-transfer agent is present. The participation of the chain-transfer reaction with growing polymer chains results in macromolecules containing the initiator residues at one end of the macromolecule and a saturated chain end, formed by hydrogen atom transfer, at the other (see peak series **a** in Figures 5 and 6). Moreover, for a given

composition of the polymerization mixture, the probability of the reaction of chain-transfer agent with primary radicals will decrease with time (conversion). It is important to note, that from the point of view of protein modification, macromolecules having the structure **a** are nonreactive, inert components of ST-PHPMA.

Modification of Chymotrypsin with ST-PHPMA Polymers. Both carboxyl and amino groups can be used for the modification of proteins with semitelechelic polymers. The selection of the modification mode may be important for the biological activity of the protein-polymer conjugate. For example, Sakam and Pardridge (35) have shown that carboxyl-directed pegylation of brain-derived neurotrophic factor preserved the biological activity of the conjugate, whereas the amino group directed modification did not. Pettit et al. (36) have shown that amino group directed pegylation of interleukin-15 alters the biological activity of the conjugate.

There are 17 carboxyl groups and 17 amino groups in

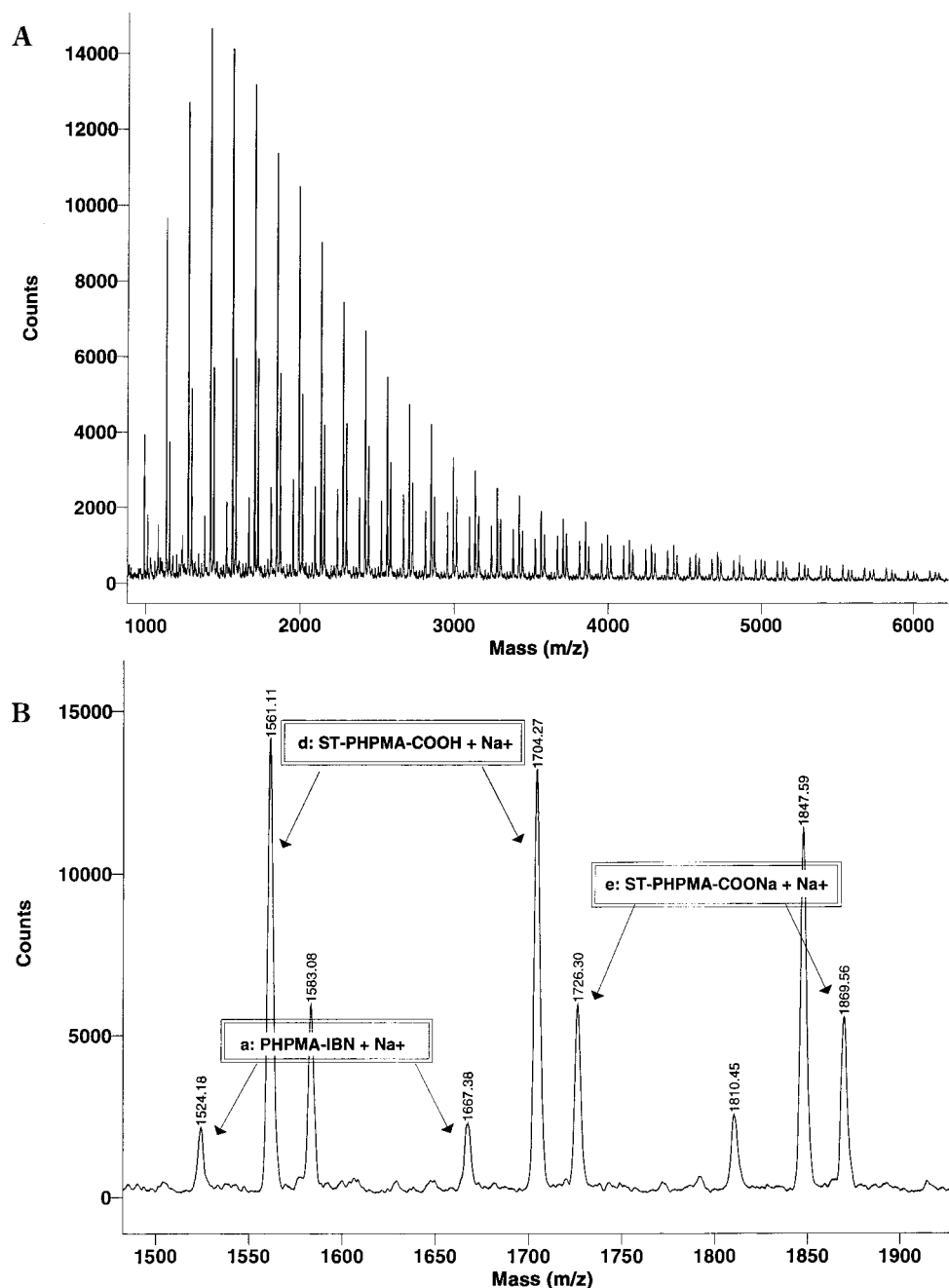


Figure 6. The MALDI-TOF mass spectrum (A) of ST-PHPMA-COOH (**11**; Table 5) and its expanded spectrum (B) in the region of 1500–1900. IBN is the isobutyronitrile radical, formed by decomposition of AIBN.

α -chymotrypsin. ST-PHPMA-CONHNH₂ and ST-PHPMA-COOSu were used for the carboxyl and amino group directed modification of chymotrypsin, respectively. The amino-directed modification of proteins with polymers containing active ester groups is the most frequently used method. The carboxyl group directed modification with polymers containing hydrazo groups is a new method, which was first introduced by Zalipsky (3, 25, 37). The difference of the pK_a of hydrazide ($pK_a \approx 3.0$) and the amino groups of proteins ($pK_a = 6.8$ – 8.0 for α -amino, 10.4–11.1 for ϵ -amino of lysine) makes it possible to react the hydrazo groups with the carboxyl groups of proteins in the presence of water soluble carbodiimide at mildly acidic pH, while the amino groups on the same proteins remain deactivated due to protonation.

Conjugates **I**, **II**, **III**, and **IV** have been prepared by the reaction of the amino groups on α -chymotrypsin with ST-PHPMA-COOSu nos. **2**, **4**, **5**, and **5** listed in Table 2,

respectively. This is a relatively easy reaction. To achieve a high yield of conjugation, a proper pH of the reaction mixture has to be chosen based on the pH dependence of the rate constants of hydrolysis and aminolysis. All the conjugates were characterized by MALDI-TOF MS, and Figure 1 shows the MALDI-TOF mass spectrum of conjugate **III**. The average molecular weights of the conjugates calculated from the MS are listed in Table 6.

Conjugate **V** has been prepared by the reaction of chymotrypsin with a large excess of ST-PHPMA-CONHNH₂; the molar ratio of chymotrypsin to polymer was about 1:120. The conjugation reaction was carried out at pH 4.5–5.0 in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), and the polymer chains were attached to carboxyl groups of chymotrypsin via hydrazide bonds. Since carboxyl groups were consumed by conjugation, dilute HCl was added to

Table 6. Enzymatic Activity of Polymer Modified α -Chymotrypsins. Kinetics of Cleavage of Low Molecular Weight and Polymeric *p*-Nitroanilides

conjugate	polymer $M_{n(\text{end})}^a$	conjugate M_n^b	conjugate M_w^b	conjugation degree (%) ^c	bond mode ^d	kinetic parameters		
							Z-GLF-NaP	P-GLF-NaP
native chymotrypsin						k_{cat} (s ⁻¹)	0.83	0.94
						K_M (M)	3.9×10^{-4}	1.3×10^{-3}
						k_{cat}/K_M (M ⁻¹ s ⁻¹)	2140	720
I	5510	44 900	46 600	21(22)	P-CONH-E	k_{cat} (s ⁻¹)	0.24	0.91
						K_M (M)	5.5×10^{-5}	2.2×10^{-3}
						k_{cat}/K_M (M ⁻¹ s ⁻¹)	4400	410
II	3740	49 400	51 000	38(43)	P-CONH-E	k_{cat} (s ⁻¹)	0.27	0.34
						K_M (M)	6.5×10^{-5}	5.7×10^{-4}
						k_{cat}/K_M (M ⁻¹ s ⁻¹)	4200	600
III	2680	40 900	41 700	35(34)	P-CONH-E	k_{cat} (s ⁻¹)	0.21	0.65
						K_M (M)	5.0×10^{-5}	1.75×10^{-4}
						k_{cat}/K_M (M ⁻¹ s ⁻¹)	4200	370
IV	2680	36 100	37 800	25(25)	P-CONH-E	k_{cat} (s ⁻¹)	0.18	0.55
						K_M (M)	4.5×10^{-5}	1.1×10^{-3}
						k_{cat}/K_M (M ⁻¹ s ⁻¹)	4000	480
V	2490	46 600	47 800	48	P-NHNHCO-E	k_{cat} (s ⁻¹)	0.34	0.64
						K_M (M)	3.0×10^{-4}	1.6×10^{-3}
						k_{cat}/K_M (M ⁻¹ s ⁻¹)	1100	410

^a Number average molecular weight determined by end group analysis. ^b Determined by MALDI-TOF MS. ^c The conjugation degree was calculated from M_n of the conjugate and the $M_{n(\text{end})}$ of the polymers; the data in the parentheses were obtained from TNBS assay. ^d P represents polymer, and E the enzyme.

maintain the pH at 4.5–5.0. The MALDI-TOF mass spectrum of conjugate **V** (Figure 2) showed a broad peak of the conjugate because of the random conjugation of macromolecules with different molecular weights to the enzyme. The main peak around $m/z = 45\,000$ reflects the single charged conjugate, whereas the small peak around $m/z = 22\,000$ corresponds to the double charged conjugate. The average molecular weights calculated from the MS are listed in Table 6.

Chymotrypsin was also modified with narrow fractions of ST-PHPMA-CONHNH₂, and conjugates **VI**, **VII**, **VIII**, and **IX** were prepared by conjugation with the polymer fractions (Table 3). In the preparation of conjugates **VI** and **IX**, the polymer enzyme molar ratio used was lower than that used in the preparation of conjugates **V**, **VII**, and **VIII**, and a larger excess of the coupling agent EDC was used to achieve a high conjugation degree. The results indicated that the larger excess of EDC was helpful when the polymer enzyme molar ratio was lower. The molar ratio in the preparation of conjugate **IX** was only 23:1, but the conjugation degree obtained was the same as that of conjugate **VIII**, which was prepared with the molar ratio of 100:1. All the conjugates prepared with the polymer fractions were characterized by SEC and MALDI-TOF MS, and the mass spectrum of conjugate **VI** is shown in Figure 3. The calculated average molecular weights of the conjugates and the average number of polymer chains attached to each chymotrypsin molecule were calculated from the MS and reported in Table 3. As expected, conjugates prepared from the polymer fractions possessed a more uniform structure than those prepared from unfractionated semitelechelic macromolecules. The MALDI-TOF MS of the conjugates also indicated that there was no cross-linking of proteins under the mildly acidic reaction conditions used, and no peak of protein–protein conjugate was found in the spectrum (see Figure 3 as an example). The molecular mass of the protein–protein conjugate would be about 50 000 Da (molar mass of α -chymotrypsin is 25 240 Da) if there was cross-linking between protein molecules. The molecular mass of most of the conjugates is well below 50 000 Da. Even when a larger excess of EDC and a lower polymer enzyme molar ratio was used, no evidence of protein–protein cross-linking was shown in the SEC and MALDI-TOF MS of the conjugate **IX**. The number

average and weight average molecular weights of the conjugate were 45 600 and 46 200, lower than the molecular weight of a possible protein–protein conjugate.

It seems that about 10 is the maximum number of ST-PHPMA polymer chains which can be attached to one chymotrypsin molecule. It appeared, however, that the molecular weight of the ST-PHPMA played a role. The results in Table 3 indicated that the molecular weight of the polymer fractions affected the conjugation degree of the conjugates. Lower molecular weight polymer fractions produced conjugates with a higher conjugation degree, because the smaller spherical size of the lower molecular weight fraction possessed a lower sterical exclusion effect. The influence of the properties of the ST-PHPMA on the distribution of molecular weights of the α -chymotrypsin conjugates was demonstrated by MALDI-TOF mass spectra (compare Figure 3A with Figures 5 and 6).

The conjugation degree of all conjugates was calculated from the M_n of the conjugates and the $M_{n(\text{end})}$ of the polymers (Table 6). The conjugation degrees of the amino group directed conjugates were also determined by the TNBS assay. The conjugation degrees obtained from both methods were in good agreement. Depending on the conjugation conditions and on the structure of the ST-PHPMA, there were about 3–9 polymer chains attached to each enzyme molecule. The number of chains attached depended on the ratio of the enzyme to the polymer in the reaction mixture as well as on the molecular weight of the ST-PHPMA used. A higher concentration ratio of polymer to enzyme gave conjugates with a higher conjugation degree; see, for example, conjugates **III** and **IV**. For conjugates **I** and **III**, the same reaction conditions and the same molar ratios were used; ST-PHPMA **5**, with a lower molecular weight, gave a higher conjugation degree in the conjugate **III** when compared to ST-PHPMA **2** and conjugate **I**. In conjugates **VI** and **VII**, the relatively high conjugation degree (~50%) was achieved when a high molar ratio of polymer to enzyme, and a low molecular weight narrow fraction of ST-PHPMA-CONHNH₂ was used in the conjugation. When the polymer enzyme molar ratio was low in the carboxyl directed modification, a much larger excess of coupling agent was necessary to achieve a relatively high conjugation degree.

The conjugation degree of ST-PHPMA with chymotrypsin was lower (about 9 vs 14 chains) than that of the chymotrypsin conjugates with PEG-SC (16). This might be attributed to the solution structure difference between the two polymers. PHPMA has a random coil structure in aqueous solution, while PEG possesses a more extended one. The coiled PHPMA may cover more surface of the enzyme, and the steric effect may prevent more PHPMA macromolecules from attaching to the enzyme. One may hypothesize that PHPMA may cover a larger area of the protein than the extended macromolecules per modifying chain of a particular molecular weight. The impact of different shapes of macromolecules on the protein repulsion characteristics of a modified biomolecule or biomaterial surface needs careful evaluation.

Activity of ST-PHPMA-Chymotrypsin Conjugates. The activity of ST-PHPMA-chymotrypsin conjugates for enzymatically catalyzed hydrolysis of peptide substrates was investigated. Native α -chymotrypsin was used as control; Z-Gly-Leu-Phe-NAP and P-Gly-Leu-Phe-NAP were the substrates. The Michealis-Menten kinetic constants of the enzymatic cleavage reaction of the substrates were calculated from Lineweaver-Burk plots of the initial rates of the release of *p*-nitroaniline and are listed in Table 6. Both the chymotrypsin conjugates and the native chymotrypsin were active for the cleavage of the substrates. All conjugates and the native enzyme exhibited a high activity for Z-Gly-Leu-Phe-NAP and a lower activity for P-Gly-Leu-Phe-NAP. The steric hindrance of the polymer chain of P-Gly-Leu-Phe-NAP renders the formation of the enzyme-polymer substrate complex more difficult, resulting in lower turnover rates, in agreement with previously published data (20).

For the cleavage of Z-Gly-Leu-Phe-NAP, amino group modified conjugates **I**, **II**, **III**, and **IV** showed higher reactivities than the native enzyme, similar to literature data (11, 16). Carboxyl group modified conjugate **V** showed a lower activity toward Z-Gly-Leu-Phe-NAP than the native enzyme. This indicated that the type of bond between the enzyme and the polymers affects the activity of the conjugates, at least for the low molecular weight substrate, as observed by modification of other proteins (35, 36). It is known that a carboxyl group of an aspartic acid was involved in the active site of chymotrypsin (38). In addition, the conjugation of a carboxyl group in the vicinity of the enzyme active site might cause the decrease of the activity of the carboxyl group-modified chymotrypsin conjugates. The activity of chymotrypsin-ST-PHPMA conjugates to catalyze the hydrolysis of the polymer-bound substrate, P-Gly-Leu-Phe-NAP, was similar and lower than the native enzyme with small variations. Conjugate **II** possessed a slightly higher activity than the average for all the conjugates. It appears that the conjugation degree and the molecular weight of the polymers did not have a pronounced effect on the activity of chymotrypsin-ST-PHPMA conjugates toward P-Gly-Leu-Phe-NAP. These data seem to indicate that the sterical hindrance that occurs upon the formation of the enzyme-polymer substrate complex is the leading cause of the decreased potential for enzyme-substrate formation.

ACKNOWLEDGMENT

The research was supported in part by Amgen, Inc., Thousand Oaks, CA.

LITERATURE CITED

- (1) Abuchowski, A., Kazo, G. M., Verhoest, C. R., van Es, T., Kafkewitz, D., Nucci, M. L., Viau, A. T., and Davis, F. F. (1984) Cancer therapy with chemically modified enzymes. I. Antitumor properties of poly(ethylene glycol) asparaginase conjugates. *Cancer Biochem. Biophys.* 7, 175-186.
- (2) Gaertner, H. F., and Offord, R. E. (1996) Site-specific attachment of functionalized poly(ethylene glycol) to the amino terminus of proteins. *Bioconjugate Chem.* 7, 38-44.
- (3) Zalipsky, S. (1995) Functionalized poly(ethylene glycol) for preparation of biologically relevant conjugates. *Bioconjugate Chem.* 6, 150-165.
- (4) Lee, J. H., Kopečková, P., Kopeček, J., and Andrade, J. D. (1990) Surface properties of copolymers of alkyl methacrylates with methoxy (poly(ethylene oxide)) methacrylates and their application as protein-resistant coatings. *Biomaterials* 11, 455-464.
- (5) Greenwald, R. B., Gilbert, C. W., Pendri, A., Conover, C. D., Xia, J., and Martinez, A. (1996) Drug delivery systems: Water soluble taxol 2'-poly(ethylene glycol) ester prodrugs—design and in vivo effectiveness. *J. Med. Chem.* 39, 424-431.
- (6) Bergström, K., Osterberg, E., Holmberg, K., Hoffman, A. S., Schuman, T. P., Kozłowski, A., and Harris, M. (1994) Effects of branching and molecular weight of surface-bound poly(ethylene oxide) on protein rejection. *J. Biomater. Sci. Polym. ed.* 6, 123-132.
- (7) Veronese, F. M., Monfardini, C., Caliceti, P., Schiavon, O., Scrawen, M. D., and Beer, D. (1996) Improvement of pharmacokinetic, immunological and stability properties of asparaginase by conjugation to linear and branched monomethoxy poly(ethylene glycol). *J. Controlled Relat.* 40, 199-209.
- (8) Sartore, L., Ranucci, E., Ferruti, P., Caliceti, P., Schiavon, O., and Veronese, F. M. (1994) Low molecular weight end-functionalized poly(N-vinylpyrrolidinone) for the modification of polypeptide aminogroups. *J. Bioactive Compatible Polym.* 9, 411-427.
- (9) Ranucci, E., Spagnoli, G., Sartore, L., and Ferruti, P. (1994) Synthesis and molecular weight characterization of low molecular weight end-functionalized poly(4-acryloylmorpholine). *Macromol. Chem. Phys.* 195, 3469-3479.
- (10) Kamei, S., and Kopeček, J. (1995) Prolonged blood circulation in rats of nanospheres surface-modified with semitelechelic poly[N-(2-hydroxypropyl)methacrylamide]. *Pharm. Res.* 12, 663-668.
- (11) Ulbrich, K., and Oupický, D. (1997) poly[N-(2-hydroxypropyl)methacrylamide] conjugates of biologically active proteins. *Eighth International Symposium on Recent Advances in Drug Delivery Systems*, pp 215-218, Salt Lake City, UT, February 24-27.
- (12) Takei, Y. G., Aoki, T., Sanui, K., Ogata, N., Okano, T., and Sakurai, Y. (1993) Temperature-responsive bioconjugates. 1. Synthesis of temperature-responsive oligomers with reactive end groups and their coupling to biomolecules. *Bioconjugate Chem.* 4, 42-46.
- (13) Matsukata, M., Aoki, T., Sanui, K., Ogata, N., Kikuchi, A., Sakurai, Y., and Okano, T. (1996) Effect of molecular architecture of poly(N-isopropylacrylamide)-trypsin conjugates on their solution and enzymatic properties. *Bioconjugate Chem.* 7, 96-101.
- (14) Topchieva, I. N., Efremova, N. V., Khvorov, N. V., and Magretova, N. N. (1995) Synthesis and physicochemical properties of protein conjugates with water-soluble poly(alkylene oxides). *Bioconjugate Chem.* 6, 380-388.
- (15) Zalipsky, S., Hansen, C. B., Oaks, J. M., and Allen, T. M. (1996) Evaluation of blood clearance rates and biodistribution of poly(2-oxazoline)-grafted liposomes. *J. Pharm. Sci.* 85, 133-137.
- (16) Chiu, H.-C., Zalipsky, S., Kopečková, P., and Kopeček, J. (1993) Enzymatic activity of chymotrypsin and its poly(ethylene glycol) conjugates toward low and high molecular weight substrates. *Bioconjugate Chem.* 4, 290-295.
- (17) Takei, Y. G., Matsukata, M., Aoki, T., Sanui, K., Ogata, N., Kikuchi, A., Sakurai, Y., and Okano, T. (1994) Temperature-responsive bioconjugates. 3. Antibody-poly(N-isopropyl

- ylacrylamide) conjugates for temperature-modulated precipitations and affinity bioseparations. *Bioconjugate Chem.* 5, 577–582.
- (18) Putnam, D., and Kopeček, J. (1995) Polymer conjugates with anticancer activity. *Adv. Polym. Sci.* 122, 55–123.
- (19) Chytrý, V., Kopeček, J., Sikk, P., Sinijärv, R., and Aaviksaar, A. (1982) A convenient model system for the study of the influence of water-soluble polymer carriers on the interaction between proteins. *Makromol. Chem. Rapid Commun.* 3, 11–15.
- (20) Kopeček, J., Rejmanová, P., and Chytrý, V. (1981) Polymers containing enzymatically degradable bonds. 1. Chymotrypsin catalyzed hydrolysis of *p*-nitroanilides of phenylalanine and tyrosine attached to side-chains of copolymers of N-(2-hydroxypropyl)methacrylamide, *Makromol. Chem.* 182, 799–809.
- (21) Lääne, A., Aaviksaar, A., Haga, M., Chytrý, V., and Kopeček, J. (1985) Preparation of polymer-modified enzymes of prolonged circulation times. Poly[N-(2-hydroxypropyl)-methacrylamide-bound acetylcholinesterase. *Makromol. Chem. Suppl.* 9, 35–42.
- (22) Chaudhary, A. K., Critchley, G., Diaf, A., Beckman, E. J., and Russell, A. J. (1996) Characterization of synthetic polymers using Matrix-Assisted Laser Desorption/Ionization-Time-of-Flight mass spectrometry. *Macromolecules* 29, 2213–2221.
- (23) Kopeček, J., and Bažilová, H. (1973) Poly[N-(2-hydroxypropyl)methacrylamide]. 1. Radical polymerization and copolymerization. *Eur. Polym. J.* 9, 7–14.
- (24) Zalipsky, S., Seltzer, R., and Menon-Rudolph, S. (1992) Evaluation of a new reagent for covalent attachment of poly(ethylene glycol) to proteins. *Biotechnology Appl. Biochem.* 15, 100–114.
- (25) Zalipsky, S., and Menon-Rudolph, S. (1997) Hydrazide derivatives of poly(ethylene glycol) and their bioconjugates. in *Poly(ethylene glycol) Chemistry and Biological Applications* (J. M. Harris, and S. Zalipsky, Eds.) 318–340, ACS, Washington, DC.
- (26) Snyder, S. L., and Sobocinski, P. Z. (1975) An improved 2,4,6-trinitrobenzenesulfonic acid method for the determination of amines. *Anal. Biochem.* 64, 284–288.
- (27) Kézdy, F. J., and Kaiser, E. T. (1970) Principles of active site titration of proteolytic enzymes. *Methods Enzymol.* 19, 3–20.
- (28) Heitz, W. (1989) Telechelics by radical polymerization reactions. in *Telechelic Polymers: Synthesis and Applications* (E. J. Goethals, Ed.) pp 61–94, CRC Press, Boca Raton, FL.
- (29) Nagasaki, Y., Ogawa, R., Yamamoto, S., Kato, M., and Kataoka K. (1997) Synthesis of heterotelechelic poly(ethylene glycol) macromonomers. Preparation of poly(ethylene glycol) possessing a methacryloyl group at one end and a formyl group at the other end. *Macromolecules* 30, 6489–6493.
- (30) Schadler, V., Spickermann, J., Rader, H. J., and Wiesner U. (1996) Synthesis and characterization of α,ω -macrozwitterionic block copolymers of styrene and isoprene. *Macromolecules* 29, 4865–4870.
- (31) Guttman, C. M., Blair W. R., and Danis P. O. (1997) Mass spectroscopy and SEC of SRM 1487, a low molecular weight poly(methyl methacrylate) standard. *J. Polym. Sci., Part B* 35, 2409–2419.
- (32) Vitalini, D., Mineo, P., and Scamporrìo, E. (1997) Further application of a procedure for molecular weight distribution measurement of polydisperse polymers from their matrix-assisted desorption/ionization time-of-flight mass spectra. *Macromolecules* 30, 5285–5289.
- (33) Axelson, J., Scrivener, E., Haddleton, D. M., and Derrick, P. J. (1996) Mass discrimination effects in an ion detector and other causes for shifts in polymer mass distribution measured by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Macromolecules* 29, 8875–8882.
- (34) Rader, H. J., Spickermann, J., and Mullen K. (1995) MALDI-TOF mass spectrometry in polymer analytics, 1. Monitoring the polymer-analogous sulfonation reaction of polystyrene. *Macromol. Chem. Phys.* 196, 3967–3978.
- (35) Sakane, T., and Pardridge, W. M. (1997) Carboxyl-directed pegylation of brain-derived neurotrophic factor markedly reduces clearance with minimal loss of biologic activity. *Pharm. Res.* 14, 1085–1091.
- (36) Pettit, D. K., Bonnert, T. P., Eisenman, J., Srinivasan, S., Paxton, R., Beers, C., Lynch, D., Miller, B., Grabstein, K. H., and Gombotz, W. (1997) Structure–function studies of interleukin 15 using site-specific mutagenesis, poly(ethylene glycol) conjugation, and homology modeling. *J. Biol Chem.* 272, 2312–2318.
- (37) Zalipsky, S., Lee, C., and Menon-Rudolph, S. (1992) Hydrazine containing conjugates of polypeptides and glycopeptides with polymers. *PCT Int. Appl. WO* 92 16555.
- (38) Voet, D., and Voet J. G. (1995) *Biochemistry* (2nd ed.) pp 371–410, John Wiley & Sons, Inc.

BC980058R

Self-Assembly of Polyamine–Poly(ethylene glycol) Copolymers with Phosphorothioate Oligonucleotides

Serguei V. Vinogradov, Tatiana K. Bronich, and Alexander V. Kabanov*

Department of Pharmaceutical Sciences, University of Nebraska Medical Center, 600 South 42nd Street, Omaha, Nebraska 68198. Received May 21, 1998; Revised Manuscript Received August 7, 1998

The cationic copolymers for DNA delivery were synthesized by conjugating poly(ethylene glycol) (PEG) and polyamines: polyspermine (PSP) and polyethyleneimine (PEI). These molecules spontaneously form electrostatic complexes with a model 24-mer phosphorothioate oligonucleotide, T₂₄ (PS-ODN). The copolymer complexes are water soluble. This is a marked contrast with the complexes formed by nonmodified PSP and PEI, which immediately precipitate out of solution. The potentiometric titration study suggests that the amino groups of the copolymers form a cooperative system of salt bonds with the thiophosphate groups of the PS-ODN. The PEG-PEI complexes are stable at physiological pH and ionic strengths. The PEG-PSP complexes are less stable in the presence of the low molecular mass electrolytes compared to the PEG-PEI complexes. The dynamic light scattering and transmission electron microscopy demonstrate that the complex particles are small—ca. 12 nm for PEG-PSP and ca. 32 nm for PEG-PEI. They can be lyophilized and redissolved or stored in solution for up to several months without changing size. The study suggests that as a result of formulation with the PEG-PEI the interactions of PS-ODNs with serum proteins (using the example of bovine serum albumin) are decreased and PS-ODN is protected against nuclease degradation. The simplicity of preparation and long shelf life make these systems attractive as potential pharmaceutical formulations for oligonucleotides.

INTRODUCTION

Chemically modified oligonucleotides (ODNs), such as antisense phosphorothioates (PS-ODNs), are promising candidates for clinical use (1, 2). These compounds show much higher resistance to exonucleases than unmodified phosphodiester ODNs (PO-ODNs). However, there is a growing body of evidence suggesting that nonspecific interactions with plasma proteins, liver and renal uptake, and degradation can significantly decrease the performance of PS-ODNs after systemic administration (3–5).

In efforts to improve the efficacy of gene therapies a number of delivery systems have been investigated (see, for review, ref 6). The most sustaining and promising attempts in this area were made with cationic lipids and polycations (7–10). These types of cationic carriers all bind spontaneously with negatively charged ODN molecules. In a number of cases, these carriers were shown to enhance stability of ODNs and their uptake into the cells in vitro. However, in vivo performance of these systems with ODNs appears to be much more problematic (11). A major problem with most cationic carriers is that their complexes with polynucleotides are often poorly soluble and tend to aggregate in aqueous solutions (8, 12).

A promising approach for overcoming the solubility problem has recently been proposed (13–17). It is based on the use of a new type of cationic carrier that has block (or graft) copolymer architecture and contains polycation and water-soluble nonionic segments. The complexes formed between polynucleotides and these carriers remain in solution due to the effect of the nonionic chains

that are soluble in water. Several systems have been recently described including poly(ethylene glycol)–polyspermine (PEG-PSP) (13), poly(ethylene glycol)–polylysine (PEG-PLL) (14, 15), dextran–PLL (16), poly-*N*-(2-hydroxypropyl)methacrylamide–poly(trimethylammonioethyl methacrylate chloride) (14), and some other copolymers (17). The use of PEG-PSP with modified PO-ODNs increased antiviral activity of antisense ODNs in vitro (13). It was also used successfully with PO-ODNs to transfect primary neurons (18) and with PS-ODNs to modulate gene expression in the rat retina in vivo (19).

Despite the recent use of the cationic copolymers for ODN delivery, little information is presently available about self-assembly and structural characterization of the copolymer and ODN species. Several papers have recently described the complexes of the double-stranded DNA with polycations (8, 13, 20–22). Short single-stranded ODNs represent one of the most difficult polynucleotide types for complexation and delivering with polycations. Indeed, the lengths of antisense ODNs lie in the range of ca. 6–30 bases, which limits the number of the negatively charged groups available for the cooperative binding with the polycation. Further, the effects of the single-stranded DNA vs double-stranded DNA as well as the effects of the backbone modification in PS-ODNs can significantly contribute to the binding with polycations. These problems have a general character, probably, contributing to relatively low success in using the cationic systems “tailored” for gene transfer for the ODN delivery (11). This work investigates complexes of PS-ODNs with PEG-PSP and PEG-polyethyleneimine (PEG-PEI) copolymers. It reports physicochemical characterization, solution behavior, and stability of these complexes and characterizes the effects of cationic co-

* Author to whom correspondence should be addressed.
E-mail: akabanov@mail.unmc.edu.

polymers on the binding of PS-ODNs with serum proteins and nuclease resistance of PS-ODNs.

MATERIALS AND METHODS

Polycations. PEI (MW 2000) was purchased from Sigma-Aldrich (St. Louis, MO). PSP (MW 500) was synthesized by polycondensation of *N*-(3-aminopropyl)-1,3-propanediamine and 1,4-dibromobutane using the previously described procedure (13).

Synthesis of Activated PEG. PEG (MW 8000) obtained from Aldrich (St. Louis, MO) was modified by one terminal hydroxyl group using with 4,4'-dimethoxytrityl (DMT) chloride (Sigma, St. Louis, MO). Briefly, 16 g (2 mmol) of PEG was dried by coevaporation in vacuo with anhydrous pyridine (3×50 mL) and dissolved in 100 mL of the solvent. After that, 0.34 g (1 mmol) of 4,4'-dimethoxytrityl chloride in 10 mL of anhydrous pyridine was added to this solution dropwise for 30 min and the mixture was left under stirring overnight at 25 °C. Monotritylated polymers were separated from fast-moving bis-substituted byproducts and nonreacted initial compounds by preparative column chromatography on Silicagel (Selecto Scientific, Norcross, GA), particle size 32–63 μ m, using a stepwise elution by methanol in dichloromethane. Fractions containing mono DMT-substituted PEG (DMT-PEG) were collected and solvents were removed in vacuo. DMT-PEG was obtained with 40% yield.

On the second stage, the free terminal hydroxy group of the DMT-PEG was activated for subsequent conjugation with polycations. 4 g (0.5 mmol) of the DMT-PEG were dried by coevaporation with anhydrous acetonitrile (3×20 mL) and then reacted with 0.51 g (3 mmol) of 1,1'-carbonyldiimidazole (Aldrich, St. Louis, MO) in 20 mL of anhydrous acetonitrile. The reaction mixture was agitated for 3 h at 40 °C and then diluted with water (25 mL). The solution obtained was dialyzed twice against 50% ethanol using Membra-Cel MD-25-03.5 (Applied Technologies Group, Chicago, IL), and the solvent was then removed in vacuo. The activated DMT-PEG (slightly yellow solid) was obtained with ca. 95% yield.

Conjugation of Activated PEG with PSP. Activated DMT-PEG (2.5 g, 0.3 mmol) was reacted with 1 g (1.4 mmol) of PSP in 20 mL of acetonitrile for 24 h at 25 °C. The reaction mixture was twice dialyzed against 50% ethanol, and the solvent was removed in vacuo. The DMT groups at the end of PEG chains were removed by treating the conjugates with 80% acetic acid (40 min at 25 °C). After solvent removing in vacuo, the remaining oily products were dissolved in 10 mL of methanol and precipitated into the cold ethyl ether (250 mL). The precipitates were washed twice with ether and dried in vacuo. The yield of the product was 1.9 g.

Conjugation of Activated PEG with PEI. Activated PEG (10 g, 1.2 mmol) was reacted with 7.2 g (3.6 mmol) of PEI in 80 mL of methanol for 24 h at 25 °C. The reaction mixture was twice dialyzed against 50% ethanol, and the DMT groups were removed by treating the conjugates with 80% acetic acid as described above. The remaining products were dissolved in 40 mL of methanol and precipitated into the cold ethyl ether (1000 mL). The precipitates were washed twice with ether and dried in vacuo. The yield of the product was 8.1 g.

Characterization of the Cationic Copolymers. The molecular masses of the synthesized copolymers were determined by static light scattering. The ratio of PEG and polyamine segments in the copolymers was deter-

mined from $^1\text{H-NMR}$ spectra using integral values obtained for the $-\text{CH}_2\text{CH}_2\text{O}-$ protons of PEG and $-\text{CH}_2\text{NH}-$ protons of polyamines. The total content of the nitrogen is 0.48 $\mu\text{mol/mg}$ for PEG-PSP and 2.2 $\mu\text{mol/mg}$ for PEG-PEI as determined by elemental microanalysis. The ratio of primary, secondary, and tertiary amino groups was determined from $^1\text{H-NMR}$ spectra. The concentration of the free primary amino groups was measured also by fluorescamine titration (23) using 1,6-diaminohexane (Sigma, St. Louis, MO) as a standard. The amount of ionizable groups in polycations and the degrees of ionization at a given pH were determined by potentiometric titration.

ODNs. 24-mer PS-ODNs (T_{24}) were synthesized on a 381 Applied Biosystems DNA synthesizer at a 10 μmol scale using the standard phosphoramidite chemistry and manufacturer's protocols. For fluorescent labeling, the 5'-Aminomodifier C6 (Glen Research, Sterling, VA) was introduced in PS-ODNs and the amino group containing PS-ODNs were then modified by FITC (Sigma, St. Louis, MO) using a standard protocol. The FITC-PS-ODNs were purified by reversed-phase HPLC using preparative Vydac C18, 10 μm column (9×250 mm) (Sigma, St. Louis, MO) and gradient elution with acetonitrile-triethylammonium acetate buffer, pH 7.5.

Formation of the Complexes and Their HPLC Analysis. The complexes were formed by simple mixing of the 0.1–0.5 mM solutions of PS-ODN and cationic copolymers and allowed to equilibrate for 60 min at 37 °C. After that, the solutions were filtered through 0.22 μm filter and analyzed by gel permeation chromatography (GPC) at 37 °C using Shimadzu LC-10AT system and SigmaChrom GFC-100 column (30 cm $\times$ 7.5 mm; 0.5 mL/min flow rate).

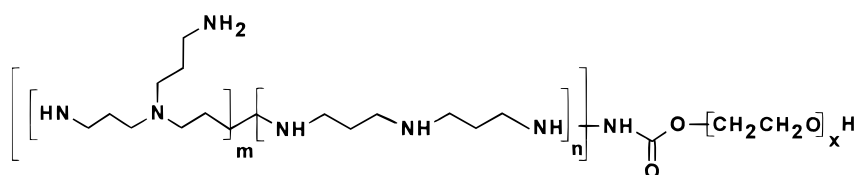
Static Light Scattering. Light-scattering experiments were performed using DAWN multiangle laser photometer (Wyatt Technology, Santa Barbara, CA) equipped with He-Ne laser (632.8 nm) and operating at 15 angles (26–137°). Minimum of four concentrations of polymers or complexes in 0.05 M NaCl were used for weight average molecular weight determination. The specific refractive index increments (dn/dc) were measured using Wyatt/Optilab 903 interferometric refractometer at 632.8 nm. Prior to the dn/dc measurements, the samples were dialyzed for at least 1 day against in 0.05 M NaCl using the membrane with the 3500 Da cutoff.

ζ -Potential and Sizing Measurements. ζ -Potential measurements were performed at 25 °C using "ZetaPlus" Zeta Potential Analyzer (Brookhaven Instrument Co.) equipped with a 15 mV solid-state laser operated at a wavelength of 635 nm. Effective hydrodynamic diameter was measured by photon correlation spectroscopy using the same instrument equipped with the Multi Angle option. The sizing measurements were performed at 25 °C at the angle of 90°.

Fluorescence Studies. Fluorescence measurements were carried out with the complexes containing 5 μM FITC-PS-ODN using Shimadzu P5000 spectrofluorophotometer ($\lambda_{\text{ex}} = 488$ nm, $\lambda_{\text{em}} = 520$ nm).

Electron Microscopy. The samples for the transmission electron microscopy (TEM) studies were prepared using negative staining with 1% uranyl acetate. Briefly, a drop of the sample solution was allowed to settle on a Formvar precoated grid for 1 min, the excess sample was wicked away with filter paper and a drop of staining solution was allowed to contact the sample for 1 min. The samples were analyzed using a Hitachi H-7000 electron microscope.

a. PEG-PSP



b. PEG-PEI

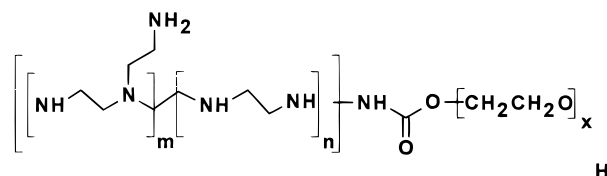


Figure 1. Cationic copolymers: (a) PEG–PSP and (b) PEG–PEI.

Protein-Binding and Nuclease Resistance Studies

For protein-binding study, 20–400 μM PS–ODN or their complexes were mixed with 2.5% BSA (Sigma, St. Louis, MO) in PBS, pH 7.4. The mixtures were incubated for either 2 or 16 h at 25 $^{\circ}\text{C}$ and then analyzed by GPC using Progel-TSK G3000 SW_{XL} column (7.8 mm $\times$ 30 cm) and sizing measurements as described above. For nuclease resistance studies, 100 μL samples of 25 μM free PS–ODNs or their complexes were treated with 20 μL (0.024 units) of snake venom phosphodiesterase (phosphodiesterase I from *Crotalus adamanteus*) (Sigma, St. Louis, MO) at 37 $^{\circ}\text{C}$. The samples were collected at 2, 6, and 18 h, and the reaction mixtures were analyzed by GPC as described above.

RESULTS AND DISCUSSION

Cationic Copolymers. Cationic copolymers were synthesized by conjugating PSP and PEI with PEG (Figure 1). According to NMR data, PEG–PSP and PEG–PEI contained an average of 1.4 and 1.7 PEG segments/polycation chain, respectively. This is in a reasonable agreement with the molecular weights of these copolymers determined by light scattering: 8900 for PEG–PSP and 16 600 for PEG–PEI.

The polyamine fragments of the synthesized copolymers are randomly branched, i.e., contain primary, secondary, and tertiary amino groups. The ratio of these groups determined by NMR is 1:1.7:1 for PEG–PSP and 1.3:1.2:1 for PEG–PEI. The basic properties of the PEG–PSP and PEG–PEI copolymers were characterized by potentiometric titration. As it is seen in the Figure 2, the potentiometric titration curves for each copolymer can be divided into two distinct regions corresponding to two different types of amino groups. The ratio of these groups determined from the titration curves is 1:1.8 for PEG–PSP and 1.5:1.3 for PEG–PEI. This is in a good agreement with the NMR data for the ratio between primary and secondary amino groups. Furthermore, the concentrations of the amino groups corresponding to the first regions of the potentiometric titration curves (Figure 2) equal the concentration of the primary amino groups determined by fluorescamine titration (data not shown). Therefore, potentiometric titration data reveal the primary and secondary amino groups. The amount of titrated groups of both types is less than the total amount of amino groups determined by the elemental analysis,

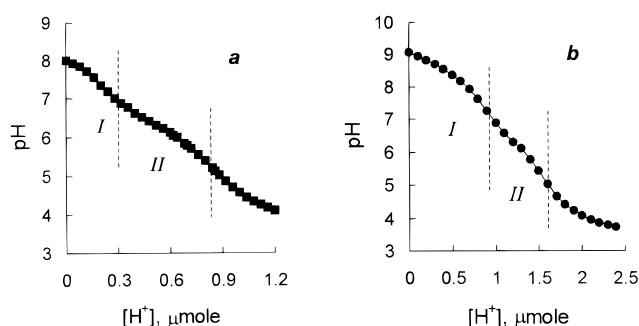
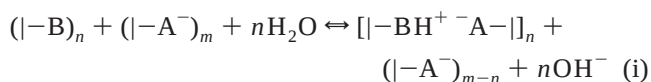


Figure 2. Potentiometric titration curves for the cationic copolymers titrated by HCl in 0.1 M NaCl: (a) PEG–PSP, polymer concentration 1 mg/mL, 0.002 M HCl; (b) PEG–PEI, polymer concentration 0.58 mg/mL, and 0.01 M HCl. Two regions corresponding to two different types of titrated amino groups are shown by dashed lines.

i.e., part of amino groups does not protonate. This part is ca. 27% for PEG–PSP and 32% for PEG–PEI, which is consistent with the content of the tertiary amino groups in these copolymers as determined by NMR. Therefore, it appears that the tertiary amino groups do not participate in the ion-exchange reactions under the conditions studied.

Polyion Coupling Reaction. The formation and solution behavior of the polyelectrolyte complexes of the nucleic acids are very similar to those of complexes formed by synthetic polyions (24, 25). These complexes are spontaneously produced as a result of the polyion coupling reaction after mixing of nucleic acid and polycation solutions (8). The stability of such system critically depends on the number of the salt bonds between interacting polyions. Normally, it takes at least 6–10 salt bonds to form a cooperative system (25). With a fixed polyion length, a charge density is a parameter determining possible number of electrostatic bonds in the complex. Therefore, with weak polybases, such as PSP and PEI, the polyion coupling reactions strongly depend on pH.

The polyion coupling reaction involving a weak polybase represents the ion-exchange process as shown by the following scheme:



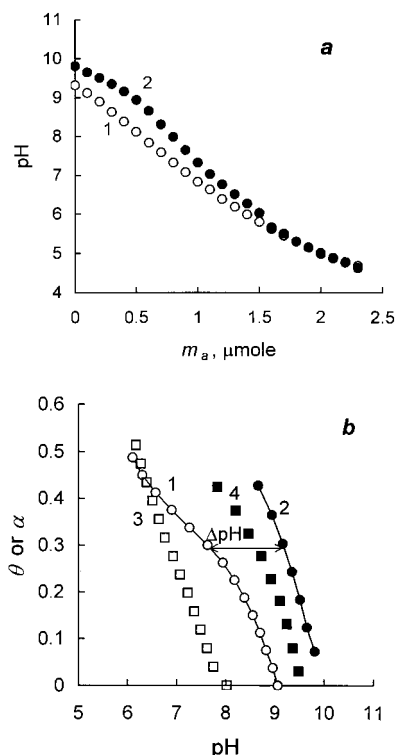


Figure 3. (a) Potentiometric titration curves for PEG-PEI (1, $\circ$) and for the mixture of PEG-PEI with PS-ODN (2, $\bullet$). The value reported on the x-axis is the number of moles of the added HCl. The concentration of copolymer is 0.6 mg/mL. (b) pH dependencies of the degree of conversion (θ) in the polyion coupling reactions ($\bullet$, $\blacksquare$) and degree of ionization (α) of polybase samples ($\circ$, $\square$) for the following systems: PEG-PEI (curve 1, $\circ$); PEG-PEI and PS-ODN (curve 2, $\bullet$); PEG-PSP (curve 3, $\square$); PEG-PSP and PS-ODN (curve 4, $\blacksquare$). The pH-shift, ΔpH , between the θ -pH and α -pH dependencies is shown for the PEG-PEI and PS-ODN complex as an example.

where B is a functional group of the weak polybase and A^- is the charged group of the polyacid. The formation of the complex is accompanied by the release of the hydroxyl ions, which can be determined by the potentiometric titration. For example, Figure 3a presents the potentiometric titration curves for PEG-PEI alone (curve 1) and in the presence of PS-ODN (curve 2). Curve 2 is shifted toward higher pH compared to curve 1. This shift is a result of the hydroxyl ion release and indicates the formation of the complex between the polycation and PS-ODN.

Degree of conversion, θ , in the reaction between polycation chains and PS-ODN can be defined as the ratio of the current number of salt bonds formed by the polyions to their maximal number. This parameter can be determined from the titration curves assuming that all acid is consumed for ionization of the amino groups of the copolymer, which then form ionic pairs with the PS-ODN. It was previously demonstrated that, in the presence of a strong polyacid, practically all ionized units of the weak polybase participate in the formation of ionic pairs and therefore θ is approximated as follows (24):

$$\theta = (m_a/V + [\text{OH}^-] - \sqrt{K_b C_0})/C_0 \quad (1)$$

where m_a is the number of moles of the added acid, V is the current volume of the reaction system, K_b is the characteristic ionization constant of the polybase, and C_0 is the base-molar concentration of the polybase.

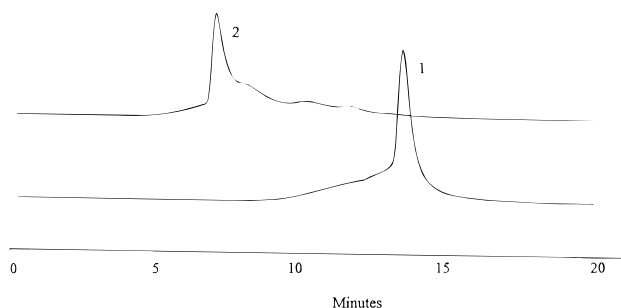


Figure 4. Chromatography profiles of the (1) free PS-ODN and (2) its complex with PEG-PEI ($Z = 1$). Conditions: SigmaChrom GFC-100 column (7.5 mm $\times$ 30 cm), flow rate 0.5 mL/min; temperature 37 $^\circ\text{C}$; mobile phase PBS, pH 7.4, PS-ODN concentration in samples is 10 μM .

Figure 3b presents the θ -pH dependencies for mixtures of PS-ODN with PEG-PSP and PEG-PEI (filled symbols). (To ensure that all amino groups of the copolymers form ionic pairs with the oligonucleotide units, the titration experiment was conducted at the PS-ODN excess.) Also the copolymers were titrated in the absence of PS-ODN to determine the degrees of ionization, α . The corresponding α -pH curves are presented in Figure 3a for comparison (empty symbols).

As it is seen in the Figure 3b, θ increases sharply in a narrow range of pH. It is noteworthy that for both copolymers the θ -pH curves are shifted in the alkaline area compared to the respective α -pH curves in the absence of PS-ODN. These shifts are indicative of the cooperative electrostatic interaction between the polyamine chains and PS-ODN that results in stabilization of the ionized form of the polyamines. The pH difference, ΔpH , between θ -pH and α -pH curves at each $\theta = \alpha$ is a differential measure of free energy of cooperative stabilization of the polyelectrolyte complexes (see Appendix). Overall, these studies suggest that PEG-PSP and PEG-PEI form stable polyelectrolyte complexes with PS-ODN in a wide range of pH.

Composition of the Mixture. At a given pH, the number of the salt bonds formed between the polyions depends on the ratio of the concentrations of the ionic groups of these polyions. This ratio is termed "the composition of the mixture", or "charge ratio", Z . In this work, Z is determined as the ratio of the concentration of ionizable amino groups of the copolymer and the concentration of the thiophosphate groups of PS-ODN. As a result of induced ionization of PSP and PEI fragments of the copolymers in the presence of PS-ODN, practically all ionizable amino groups participate in the formation of the salt bonds at pH 7.4.

Formation of the Soluble Complexes and Their Characterization by GPC. Simple mixing of the solutions of cationic copolymers and PS-ODN forms the complexes. Immediately after mixing, these systems are optically transparent. This is a marked contrast with the complexes formed by nonmodified PSP and PEI, which immediately precipitate out of solution. PEG-PSP- and PEG-PEI-based complexes did not precipitate and were optically transparent during the observation times (up to months).

The soluble complexes were further characterized by GPC. Typical chromatographic profiles are shown in Figure 4. The PS-ODN alone is eluted as a single peak. In the presence of the PEG-PEI, a peak with a lower retention time compared to the free PS-ODN is observed, which evidently corresponds to the complex. The portion of PS-ODN incorporated into the complex depends on

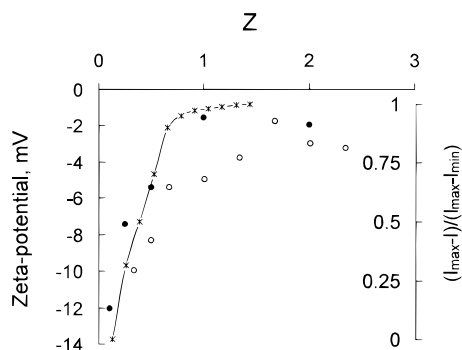


Figure 5. ζ -Potential of the complexes of PS-ODN with (●) PEG-PSP and (○) PEG-PEI, and (*) normalized fluorescence intensity, $(I_{\max} - I)/(I_{\max} - I_{\min})$, of FITC-PS-ODN complex with PEG-PEI as a function of the composition of the mixture. Phosphate buffer, 10 mM, pH 7.4, 22.5 °C.

the concentration of the cationic copolymer (data not shown). Complete incorporation of PS-ODN in the complex is observed at the charge equivalency, i.e., $Z = 1$. Similar behavior was also observed for PEG-PSP and PS-ODN mixtures in 20 mM phosphate buffer (data not shown). In this case, however, the retention time for the PEG-PSP complex was closer to the free PS-ODN retention time, so that the peaks for the complex and free oligonucleotide overlapped.

The chromatography analysis was used to evaluate the stability of the complexes at various ionic strengths. These studies suggest that PEG-PSP complexes are the least stable in the phosphate buffered saline (PBS), pH 7.4. These complexes are observed in PBS only at relatively high concentrations—above 100 μ M/PS-ODN chain in the injected samples. (Dilution of the samples during chromatography is ca. 100–200-fold.) At lower concentrations (e.g., 10 μ M in the injected sample), the complexes dissociate. However, in the phosphate buffer at lower ionic strengths (less than 0.1 M NaCl), the PEG-PSP and PS-ODN complexes are formed at all concentrations examined (minimal sample concentration was 10 μ M).

ζ -Potential Measurements. Soluble complexes from cationic copolymers and PS-ODNs are characterized using the laser microelectrophoresis technique. Figure 5 presents the ζ -potential of the PEG-PSP- and PEG-PEI-based complexes as a function of Z . At $0 < Z < 0.5$, the ζ -potential sharply increases with elevating the copolymer concentration. With the compositions approximating Z values of 0.7–1 the ζ -potential is leveled off at small negative values, ca. –2 mV for PEG-PSP and –4 to –2 mV for PEG-PEI, indicating that the particles are practically electroneutral. Since no changes in ζ -potential are observed at $Z > 1$ (at least up to $Z = 3$), the polycation excess does not incorporate into the complex.

Fluorescence Detection of the Complex Formation. Figure 5 also presents the results of the fluorescence measurements with FITC-PS-ODN in the presence of PEG-PEI. Addition of the copolymer results in the shift of the maximum of the UV absorption of fluorescent tag in FITC-PS-ODN from 494 to 503 nm. Simultaneously, the fluorescence emission at 520 nm (excitation 488 nm) is decreased. The results of the fluorescence measurements are presented as a function of normalized fluorescence, $(I_{\max} - I)/(I_{\max} - I_{\min})$, on Z (Figure 5). The trend of this curve is similar to that of the corresponding ζ -potential curve. The saturation is observed at ca. $Z = 0.7$ –1. It appears that all FITC-

Table 1. Effective Diameters of the Complexes of PS-ODN and Cationic Copolymers at Different Time Intervals after Preparation^a

cationic copolymer	effective diameter (nm)			
	30 min	24 h	1 week	3 months
PEG-PSP	11.8	11.6	12.0	n.d.
PEG-PEI	32.0	30.0	32.0	34.0

^a Complexes were prepared at $Z = 1$ in PBS, pH 7.4 (PEG-PEI complex), or 10 mM phosphate buffer, pH 7.4 (PEG-PSP complex). Solutions were stored at 4 °C for indicated time intervals. Prior to the measurements solutions were equilibrated for at least 2 h at room temperature.

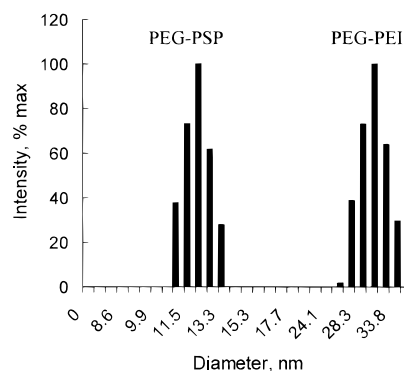


Figure 6. Size histograms for the PS-ODN complexes with PEG-PSP and PEG-PEI.

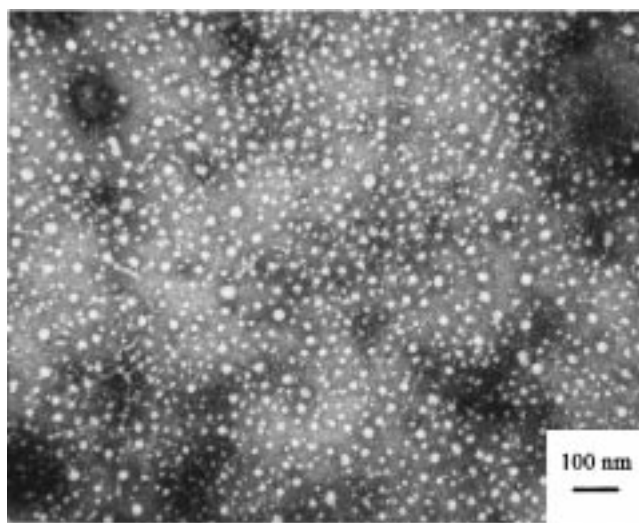


Figure 7. TEM micrograph of the complex between PS-ODN and PEG-PEI.

PS-ODN molecules bind to the copolymer at these compositions.

Size and Molecular Mass Determination. Table 1 presents the effective diameters of the complexes formed by PS-ODN and cationic copolymers measured at various time intervals after preparation of these systems. PEG-PSP and PEG-PEI complexes have average diameters of 12 and 32 nm, respectively. As it is seen from Figure 6, the particles are characterized by the relatively narrow distribution of the size. No size changes are observed after storing these systems in solution for very long periods (at least three months in the case of PEG-PEI complex) (Table 1). Further, sizes did not change after lyophilizing these complexes, storing them for several months, and then redissolving in buffer (data not shown).

Figure 7 presents typical microphotographs obtained for PS-ODN and PEG-PEI complexes with the cationic

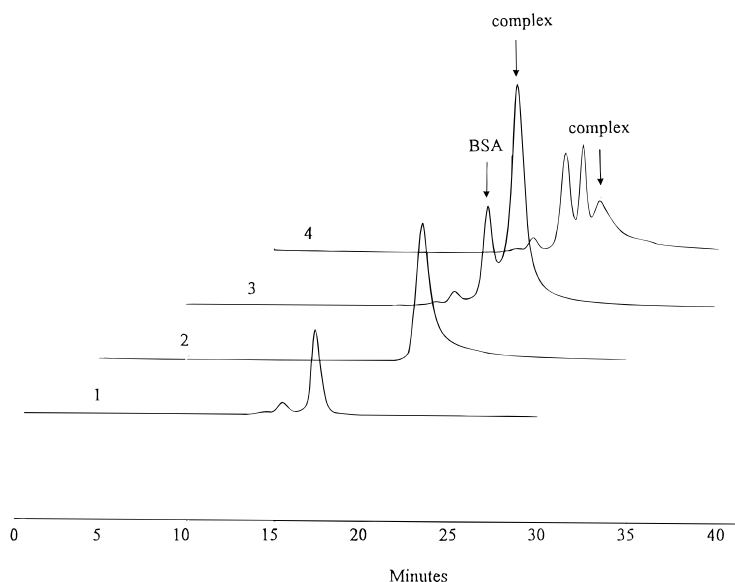


Figure 8. Interaction of PS-ODN/PEG-PEI complex ($Z = 1$) with BSA. The chromatography profiles are shown for (1) 2.5% solution of BSA in PBS, (2) complex in PBS, (3) complex in 2.5% BSA after 2 h incubation, and (4) complex in 2.5% BSA after 16 h incubation. Conditions: Progel-TSK G3000 SW_{XL} column (7.8 mm $\times$ 30 cm), flow rate 0.5 mL/min; temperature 37 $^{\circ}$ C; mobile phase PBS, pH 7.4, PS-ODN concentration in samples is 10 μ M.

copolymers. They reveal spherical particles with narrow size distributions. Similar type particles with the diameters ca. 3 times smaller are formed by the PEG-PSP complexes (data not shown).

The molecular weight of particles formed by PS-ODN and PEG-PEI at $Z = 1$ is ca. 590 000 as determined by static light scattering. These particles contain an average of 24 PS-ODN and 23 PEG-PEI molecules as was estimated assuming that the complex was electroneutral. The "driving forces" for self-assembly of such structures are hydrophobic interactions of the neutralized polyion chains, which form a core of a micelle-like particle. The particle hydrophilic shell is evidently formed by the PEO chains, which stabilize the particles in the aqueous media.

Interactions with Serum Proteins and Resistance to Nuclease Degradation. Addition of 0.5% bovine serum albumin (BSA) does not change the size of the PEG-PEI and PS-ODN complex ($Z = 1$) for several days at 37 $^{\circ}$ C, PBS, pH 7.4. In contrast, large aggregates (113 nm) are rapidly formed (within minutes) when BSA is added to the free copolymer. This result suggests that while BSA binds to the cationic copolymer alone, it does not readily substitute the PS-ODN in the complex. The BSA effects are further characterized in the GPC experiment. The retention time and amounts of the PEG-PEI and PS-ODN complex do not change in the presence of 2.5% BSA for 2 h (Figure 8). Also, BSA elution profiles are not affected in the presence of the complex during this time. However, after longer time intervals (16 h), the destruction of the complex is observed as determined from the changes in the chromatography profiles (Figure 8). This experiment suggests that the interactions of PS-ODN with serum proteins are decreased as a result of oligonucleotide formulation with the PEG-PEI.

Figure 9 shows the results of the study of nuclease degradation of free PS-ODN and its complex with PEG-PEI by phosphodiesterase I from *Crotalus adamanteus*. Following an 18 h incubation with the enzyme, the free PS-ODN was degraded by over 80%. In contrast, over 70% of PS-ODN incorporated in the complex with PEG-PEI remained undegraded at this time point. Therefore, incorporation of the PS-ODN into the complex with

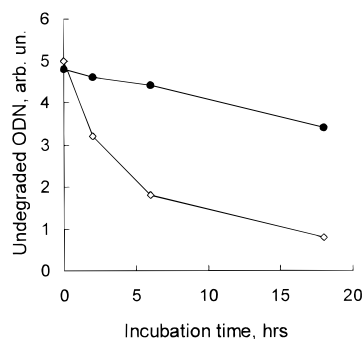


Figure 9. Degradation of ($\diamond$) free PS-ODN and ($\bullet$) PS-ODN in the complex with PEG-PEI, $Z = 1$ in the presence of phosphodiesterase I from *Crotalus adamanteus*. (0.1 mg of phosphodiesterase in 30 μ L of PBS, pH 7.4, 37 $^{\circ}$ C).

cationic copolymer stabilizes the oligonucleotide against nuclease degradation. The stabilization against the nuclease evidently results from masking the PS-ODN chain by the polycation fragments in the core of the complex particles. Similar effect was previously reported for the plasmid DNA bound to poly(4-vinylpyridinium) salts (26). In the previous report (26), the complete stabilization of the plasmid against enzymatic digestion was observed at $Z = 1$. However, the electroneutral complexes of the DNA with poly(4-vinylpyridinium) salts were aggregated. In contrast, the systems described in this work remain both stable to nuclease degradation and soluble due to the effect of the PEG segments.

Core-Shell Model for the Complexes of Polyamine-PEG Copolymers with PS-ODNs. The studies on the solution behavior and transfection activity of the polyelectrolyte complexes formed between polycations and plasmid DNA were reviewed recently in ref 27. It was shown that under certain conditions these systems produce small (under 100 nm) "core-shell" particles with the insoluble core from neutralized polyions and shell from positively charged polycation chains stabilizing these particles in aqueous solution. Incidentally, formation of the core-shell particles coincided with the conditions for the maximal transfection activity in vitro, suggesting that these particles represent the transfecting

species formed in the DNA and polycation mixtures (27). Among other factors necessary for the formation of such particles, the polycation excess is needed to produce soluble cationic corona. Also, the increased length of the polycation favors the formation of the corona at the polycation excess.

In contrast to plasmid and other high molecular mass DNA, antisense oligonucleotides represent one of the most difficult polynucleotide types for complexation and delivering with polycations. Indeed, the lengths of antisense oligonucleotides lie in the range 6–30 bases approximately (28). Hence, with short oligonucleotides, the number of phosphate charges may be insufficient to provide for cooperative binding to polycation. Due to the same reason, the polycation chain cannot be shorter than that of oligonucleotide. Thus, soluble polyelectrolyte complexes using polycation homopolymers can be produced only with sufficiently long polycations, and these complexes are always positively charged. Indeed, some earlier work on PEI and oligonucleotide transfection (29–31) used the polycation excess and high molecular mass PEI (50–800 kDa). Although these studies did not specifically characterize solution stability of the complexes, these systems may be soluble due to the lyophilizing effects of the charged sites in the PEI chain that are not neutralized by the oligonucleotide. The use of cationic copolymers instead of the homopolymer polycations offers different possibility for preparing soluble “core-shell” complexes of oligonucleotides. In the systems reported in refs 13–19 and in this study, the hydrophobic core is formed by the neutralized polyions while nonionic water-soluble chains such as PEG form hydrophilic shell. As a result, soluble uncharged complexes can be produced which are stabilized in solution by PEG corona. Such systems may have advantage compared to cationic core-shell structure for certain *in vivo* applications due to potentially lower toxicity (19) and decreased interactions with serum proteins (27). Also, the use of the inert hydrophilic corona may provide for better control of pharmacokinetics and biodistribution parameters compared to cationic particles. Indeed, PEG-modified polymeric micelles are currently developed for drug delivery having prolonged half-life in blood and decreased interactions with reticuloendothelial system (32, 33). Furthermore, the PEG corona can be modified with vector molecules, such as antibodies, to enable addressed delivery of the polymer micelle in the body (34).

CONCLUSION

This paper reports the studies on a new family of cationic carriers for ODNs. These carriers include conjugates of PEG with polycation segments: PSP and PEI. Aqueous solubility of the complexes formed by PEG–PSP and PEG–PEI copolymers is an apparent advantage compared to the complexes of homopolymer polycations (8) and cationic lipids (12) with PS–ODN that aggregate. The neutralized polyamine segments and PS–ODN chains self-assemble into a hydrophobic core surrounded by the PEG segments, determining the aqueous solubility of these systems. The complexes are small – ca. 12 nm for PEG–PSP and ca. 32 nm PEG–PEI and have relatively narrow size distribution. Small size of the DNA complexes (below 100 nm) and their aggregation stability were shown to be essential for high transfection efficacy with the polycations (8, 20). The small size and stability of the systems described in this work probably contributed to the efficient ODN delivery with PEG–PSP reported *in vitro* (13, 18) and *in vivo* (19).

PEG–PSP complexes appear to be less stable in the presence of the low molecular mass electrolytes when compared to PEG–PEI complexes. This probably results from lower charge density of PSP segments compared to PEI segments and/or some differences in their structure conformity with respect to PS–ODN chains. On the basis of high stability of PEG–PEI complexes in physiological conditions, we further focus on PEG–PEI as potential PS–ODN delivery systems. The simplicity of preparation and long shelf life makes these systems very attractive pharmaceutical formulations for PS–ODNs. Decreased interactions of PS–ODNs with serum proteins (using the example of BSA) and protection of PS–ODNs against nuclease degradation are further evidence of the potential benefits of these systems in delivery of PS–ODNs.

ACKNOWLEDGMENT

The National Science Foundation (BES-9712657) and Nebraska Research Initiative supported this work. Supratek Pharma Inc. sponsored the synthesis and characterization of the cationic copolymers. Dr. Kabanov has a financial interest in and serves as a consultant to this company. M-H-W Laboratories (Phoenix, AZ) did elemental analysis. TEM studies were performed at the Central Microscopy Research Facility, University of Iowa (Iowa City, IA). Core laboratories at the University of Nebraska Medical Center (Omaha, NE) carried out the NMR measurements and PS–ODN synthesis.

APPENDIX

The dependence of ionization equilibrium of the weak polybase in the absence of the PS–ODN is described by the following equation:

$$\frac{[\text{OH}^-]'\alpha}{1-\alpha} = K_b'(\alpha) = e^{-\Delta G'(\alpha)} \quad (2)$$

and in the presence of PS–ODN by the following equation:

$$\frac{[\text{OH}^-]''\alpha}{1-\alpha} = K_b''(\alpha) = e^{-\Delta G''(\alpha)/RT} \quad (3)$$

where α is the ionization degree, $K_b'(\alpha)$ and $K_b''(\alpha)$ are the apparent ionization constants, and $\Delta G'(\alpha)$ and $\Delta G''(\alpha)$ are the free energies of the ionization in the absence and presence of PS–ODN, respectively. Dividing eq 2 by eq 3 and then taking the logarithm of the resultant equation yields the following:

$$-\Delta\text{pH}(\alpha) = \frac{\Delta\Delta G_{st}(\alpha)}{2.303RT} \quad (4)$$

where $\Delta\text{pH}(\alpha)$ is the pH difference between θ –pH curve of the complexes and α –pH curves at each $\theta = \alpha$, and $\Delta\Delta G_{st}(\alpha)$ is the difference of free energies of ionization of polybase in the presence and absence of the DNA: $\Delta\Delta G_{st}(\alpha) = \Delta G''(\alpha) - \Delta G'(\alpha)$, R is the universal gas constant, and T is the absolute temperature. The $\Delta\Delta G_{st}(\alpha)$ value is a *differential* characteristic of the impact of DNA in ionization of polybase.

LITERATURE CITED

- (1) Brysch, W., and Schlingensiepen, K. H. (1994) Design and application of antisense oligonucleotides in cell culture, *in vivo*, and as therapeutic agents. *Cell. Mol. Neurobiol.* 14, 557–568.

- (2) Akhtar, S., and Agrawal S. (1997) In vivo studies with antisense oligonucleotides. *Trends Pharmacol. Sci.* 18, 12–18.
- (3) Qian, M., Chen, S. H., Von Hofe, E., and Gallo J. M. (1997) Pharmacokinetics and tissue distribution of a DNA-methyltransferase antisense (MT-AS) oligonucleotide and its catabolites in tumor-bearing nude mice. *J. Pharmacol. Exp. Ther.* 282, 663–670.
- (4) Bijsterbosch, M. K., Manoharan, M., Rump, E. T., De Vreuh, R. L., van Veghel, R., Tivel, K. L., Biessen, E. A., Bennett, C. F., Cook, P. D., and van Berkel, T. J. (1997) In vivo fate of phosphorothioate antisense oligodeoxynucleotides: predominant uptake by scavenger receptors on endothelial liver cells. *Nucleic Acids Res.* 25, 3290–3296.
- (5) Temsamani, J., Roskey, A., Chaix, C., and Agrawal, S. (1997) In vivo metabolic profile of a phosphorothioate oligodeoxyribonucleotide. *Antisense Nucleic Acid Drug Dev.* 7, 159–165.
- (6) Kabanov, A. V., Felgner, P. L., and Seymour, L. W., Eds. (1998) *Self-assembling complexes for gene delivery. From laboratory to clinical trial*, John Wiley & Sons, New York.
- (7) Behr, J. P. (1994) Gene transfer with synthetic cationic amphiphiles: prospects for gene therapy. *Bioconjugate Chem.* 5, 382–389.
- (8) Kabanov, A. V., and Kabanov, V. A. (1995) DNA complexes with polycations for the delivery of genetic material into cells. *Bioconjugate Chem.* 6, 7–20.
- (9) Lee, R. J., and Huang, L. (1997) Lipidic vector systems for gene transfer. *Crit. Rev. Ther. Drug Carrier Sys.* 14, 173–206.
- (10) Mahato, R. I., Takakura, Y., Hashida, M. (1997) Development of targeted delivery systems for nucleic acid drugs. *J. Drug Targeting* 4, 337–357.
- (11) Gewirtz, A. M., Stein, C. A., and Glazer, P. M. (1996) Facilitating oligonucleotide delivery: helping antisense deliver on its promise. *Proc. Natl. Acad. Sci. U.S.A.* 93, 3161–3163.
- (12) Jaaskelainen, I., Monkkonen, J., and Urtti, A. (1994) Oligonucleotide cationic liposome interactions. A physicochemical study. *Biochim. Biophys. Acta* 1195, 115–123.
- (13) Kabanov, A. V., Vinogradov, S. V., Suzdaltseva, Yu. G., and Alakhov, V. Yu. (1995) Water-soluble block polycations for oligonucleotide delivery. *Bioconjugate Chem.* 6, 639–643.
- (14) Wolfert, M. A., Schaht, E. H., Tonceva, V., Ulbrich, K., Nazarova, O., and Seymour, L. W. (1996) Characterization of vector for gene therapy formed by self-assembly of DNA with synthetic block copolymers. *Hum. Genet. Ther.* 7, 2123–2133.
- (15) Kataoka, K., Togawa, H., Harada, A., Yasugi, K., Matsumoto, T., and Katayose, S. (1996) Spontaneous formation of polyion complex micelles with narrow distribution from antisense oligonucleotide and cationic copolymer in physiological saline. *Macromolecules* 29, 8556–8557.
- (16) Maruyama, A., Katoh, M., Ishihara, T., and Akaike, T. (1997) Comb-type polycations effectively stabilize DNA triplex. *Bioconjugate Chem.* 3, 3–6.
- (17) Kabanov, A. V., Alakhov, V. Yu., and Vinogradov SV. Polynucleotide compositions. U.S. patent 5,656,611.
- (18) Mundigl, O., Ochoa, G.-C., Slepnev, V. I., Kabanov, A. V., and DeCamilli, P. (1998) The role of amphiphysin in neurite outgrowth. *J. Neurosci.* 18, 93–103.
- (19) Roy, S., Kabanov, A., Vinogradov, S., and Zhang, K. (1997) In vivo transfer of antisense oligonucleotides into rat retina using block polycation. *Invest. Ophthalmol. Vis. Sci.* 38, 2086.
- (20) Tang, M. X., and Szoka, F. C. (1997) The influence of polymer structure on the interactions of cationic polymers with DNA and the morphology of the resulting complexes. *Gene Ther.* 4, 823–832.
- (21) Katayose, S., and Kataoka, K. (1997) Water-soluble polyion complex associates of DNA and poly(ethylene glycol)-poly(L-lysine) block copolymer. *Bioconjugate Chem.* 8, 702–707.
- (22) Maruyama, A., Watanabe, H., Ferdous, A., Katoh, M., Ishihara, T., and Akaike, T. (1998) Characterization of interpolyelectrolyte complexes between double-stranded DNA and polylysine comb-type copolymers having hydrophilic side chains. *Bioconjugate Chem.* 9, 292–299.
- (23) Undenfriend, S., Stein, S., Bohlen, P., Dairman, W., Leimgruber, W., Weigle, M. (1972) Fluorescamine: a reagent for assay of amino acids, peptides, proteins, and primary amines in the picomole range. *Science* 178, 871–872.
- (24) Kabanov, V. A. (1994) Physicochemical basis and the prospects of using soluble interpolyelectrolyte complexes (Review). *Vysokomol. Soed. (in Russian)* 36, 183–197 (Engl. ed.: *Polym. Sci., Ser. A* 35, 143–156).
- (25) Papisov, I. M., and Litmanovich, A. A. (1988) Molecular “recognition” in interpolymer interactions and matrix polymerization. *Adv. Polym. Sci.* 90, 139–179.
- (26) Kabanov, A. V., Astafieva, I. V., Chikindas, M. L., Rosenblatt, G. F., Kiselev, V. I., Severin, E. S., and Kabanov, V. A. (1991) DNA interpolyelectrolyte complexes as a tool for efficient cell transformation. *Biopolymers* 31, 1437–1443.
- (27) Kabanov, A. V., Szoka, F. C., and Seymour, L. W. (1998) Interpolyelectrolyte complexes for gene delivery: polymer aspects of transfection activity. in *Self-Assembling Complexes for Gene Delivery. From Laboratory to Clinical Trial* (A. V. Kabanov, L. W. Seymour, and P. Felgner, Eds.) pp 197–218, John Wiley, New York.
- (28) Helene, C. (1991) Rational design of sequence-specific oncogene inhibitors based on antisense and antigene oligonucleotides. *Eur. J. Cancer* 27, 1466–1471.
- (29) Boussif, O., Lezoualch, F., Zanta, M. A., Mergny, M. D., Scherman, D., Demeneix, B., and Behr, J. P. (1995) A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine. *Proc. Natl. Acad. Sci. U.S.A.* 92, 7297–7301.
- (30) Lambert, R. C., Maulet, Y., Dupont, J.-L., Mykita, S., Craig, P., Volsen, S., and Fetz, A. (1996) Polyethyleneimine-mediated DNA transfection of peripheral and central neurons in primary culture: probing Ca^{2+} channel structure and function with antisense oligonucleotides. *Mol. Cel. Neurosci.* 7, 239–246.
- (31) Kren, B. T., Bandyopadhyay, P., and Steer, C. J. (1988) In vivo site-directed mutagenesis of the factor IX gene by chimeric RNA/DNA oligonucleotides. *Nat. Med.* 4, 285–290.
- (32) Cammas, S., and Kataoka, K. (1996) Site specific drug-carriers: polymeric micelles as high potential vehicles for biologically active molecules. in *Solvents and Self-Organization of Polymers* (S. E. Webber, Ed.) pp 83–113, Kluwer Academic Publishers, Netherlands.
- (33) Alakhov, V. Yu., and Kabanov, A. V. (1998) Block copolymeric biotransport carriers as versatile vehicles for drug delivery. *Exp. Opin. Invest. Drugs* 7, 1–21.

BC980048Q

Biotin Reagents for Antibody Pretargeting. 3. Synthesis, Radioiodination, and Evaluation of Biotinylated Starburst Dendrimers

D. Scott Wilbur,^{*,†} Pradip M. Pathare,[†] Donald K. Hamlin,[†] Kent R. Buhler,[‡] and Robert L. Vessella[‡]

Departments of Radiation Oncology, 2121 North 35th Street, and Urology, University of Washington, Seattle, Washington 98195. Received May 26, 1998; Revised Manuscript Received September 10, 1998

We are investigating the hypothesis that biotin multimers can be used with streptavidin and monoclonal antibody conjugates in cancer pretargeting protocols to provide a method of increasing the amount of radioactivity bound on cancer cells in patients. As part of that investigation, a series of biotinylated Starburst dendrimers (BSBDs) have been prepared and evaluated in vitro and in vivo. In this study, a new biotinidase-stabilized, water-solubilizing biotinylation reagent was prepared and reacted with Starburst (PAMAM) dendrimers, generations 0, 1, 2, 3, and 4. The reaction conditions employed resulted in perbiotinylation of generation 0 (four biotin moieties conjugated), generation 1 (eight biotin moieties conjugated), generation 2 (16 biotin moieties conjugated), and generation 3 (32 biotin moieties conjugated). With generation 4, incomplete biotinylation was achieved resulting in the largest portion of that BSBD having 51 biotin moieties (of 64 possible) conjugated. The ability of each BSBD to cross-link streptavidin (SAv) was examined in an in vitro assay. In that assay, an assessment was made of the quantity of [¹²⁵I]SAv bound with polystyrene-bound SAv after treatment with the synthesized BSBDs. All BSBDs cross-linked the polystyrene-bound SAv with [¹²⁵I]SAv; however, the amount of [¹²⁵I]SAv bound varied with the different BSBDs. Roughly 1 equiv of [¹²⁵I]-SAv was bound when Starburst dendrimers containing three or four biotin moieties (generation 0) were used. Two equivalents were bound with BSBD generation 1, and 4 equiv were bound with BSBDs generations 2, 3, and 4. To assess the distribution of BSBDs generations 0, 1, and 2 in mice (at 4 h postinjection), a method was developed for radioiodinating them using the NHS ester of *p*-[¹²⁵I]-iodobenzoate ([¹²⁵I]PIB). It was found that the radioiodinated BSBDs had low blood concentrations (i.e., 0.13–0.20% ID/g) at the 4 h time point. In fact, most tissues examined had low concentrations of biotinylated dendrimers, except kidney and liver. Kidney had the highest concentration of [¹²⁵I]-labeled BSBDs, and its concentration increased with increasing size and charge of dendrimer (e.g., 8–48% ID/g). On the basis of the increased radioactivity observed in the in vitro assay and the rapid clearance from blood in mice, additional in vivo studies with perbiotinylated Starburst dendrimer, generation 2, are planned.

INTRODUCTION

Reagents containing biotin, avidin, or streptavidin and tumor-specific monoclonal antibodies (mAbs),¹ in combination with a variety of radionuclides, are under investigation in an approach to cancer therapy that is termed "pretargeting" (1–3). The pretargeting approach is being investigated as a means of circumventing some of the inherent problems associated with directly radiolabeled

mAbs in therapy protocols (4–6). Importantly, in the pretargeting approach, the tumor selective targeting of mAbs is retained, but the delivery of the radionuclide is relegated to another molecule, which in many investigations is either a radiolabeled biotin derivative or a radiolabeled avidin or streptavidin molecule (7–17). The selection of these reagents for the pretargeting approach has been made because of the very high binding avidity of biotin with avidin (18, 19) and streptavidin (20). Tumor localization of the radiolabeled biotin or (strept)avidin is achieved by binding with an antibody conjugate that has been previously localized on tumor cells. Because avidin and streptavidin are composed of four identical subunits, four biotin molecules can bind each protein molecule. This tetrameric binding nature of avidin and streptavidin permits their use in various combinations of mAbs/biotin/(strept)avidin, which are administered in "2-step" or "3-step" pretargeting protocols (6, 21). The introduction of multiple "steps" in tumor targeting provides additional variables for optimizing the pharmacokinetics and distributions of the therapeutic radionuclide not previously obtainable with directly labeled mAbs (5).

In addition to improving the pharmacokinetics and distribution of the therapeutic radionuclide, the use of

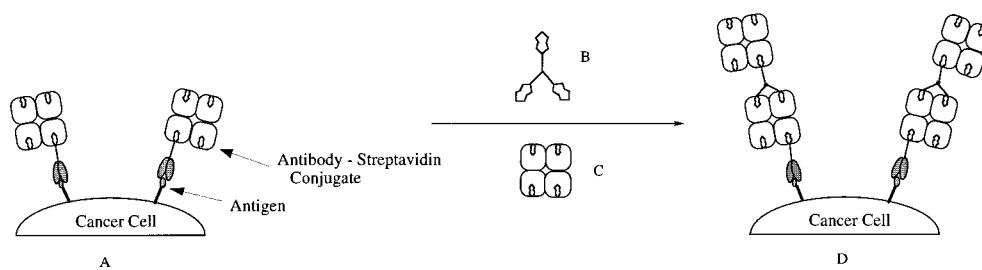
* Author to whom correspondence should be addressed. Phone: 206-685-3085. Fax: 206-685-9630. E-mail: dswilbur@u.washington.edu.

[†] Department of Radiation Oncology.

[‡] Department of Urology.

¹ Abbreviations: BSA, bovine serum albumin; BSBD(s), biotinylated Starburst dendrimer(s); ChT, chloramine-T; cpm, counts per minute; EDC, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride; G = 0, 1, 2, 3, or 4, Starburst dendrimer generation 0, 1, 2, 3, or 4; IBz-Trimer, iodobenzoyl-biotin trimer; MALDI-TOF MS, matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry; NCS, *N*-chlorosuccinimide; PBS, phosphate-buffered saline; r-SAv, recombinant streptavidin; rt, room temperature; (strept)avidin, denotes avidin or streptavidin; SAv, streptavidin; SBD(s), Starburst dendrimer(s); TFP, tetrafluorophenyl; TFP-OH, tetrafluorophenol; TsCl, *p*-toluenesulfonyl chloride.

I: Biotin Trimer or Tetramer



II: Biotin Multimer - Starburst Dendrimer Based

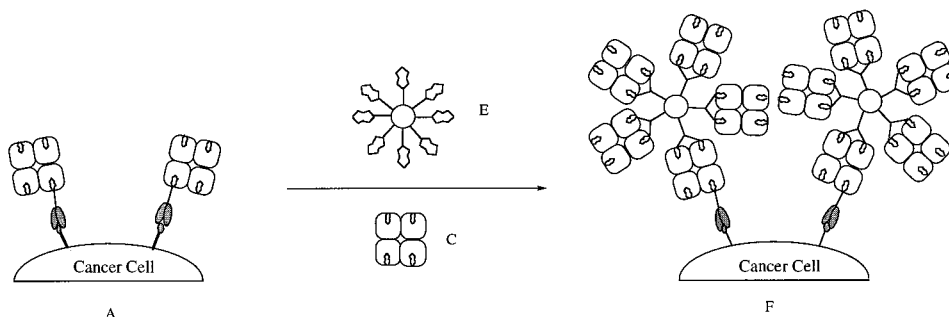


Figure 1. Schematic representation of biotin multimers cross-linking (strept)avidin with mAb-(S)Av conjugates bound to cancer cell antigens. Amplification (doubling) of the number of binding sites is provided in reaction sequence I by cross-linking tumor bound (strept)avidin with a biotin trimer (B) or biotin tetramer. In reaction I, either the biotin trimer or (strept)avidin molecule can be used as a carrier of the radionuclide. With Starburst dendrimer based biotin multimers (E) more than one (strept)avidin molecule (C) may be added in a single administration as depicted in reaction sequence II. In reaction II, amplification is obtained by having the radiolabel on the (strept)avidin molecule.

biotin/(strept)avidin-based reagents in pretargeting may provide a method for increasing the amount of radionuclide bound to cancer cells *in vivo* (3, 7, 15). If the number of binding sites for either radiolabeled biotin or radiolabeled (strept)avidin is increased, then it may be possible for more radioactivity to be bound to each cancer cell. One possible method for increasing the biotin/(strept)avidin-binding sites on cancer cells is to cross-link (strept)avidin with molecules that contain more than two biotin moieties as depicted in Figure 1. For example, a radiolabeled biotin trimer (B in Figure 1) could be used in a multistep protocol that alternates administration of that reagent with (strept)avidin (C in Figure 1) (22). In such a protocol, it might be possible to regenerate an equivalent number of new binding sites (biotin moieties) as there are bound mAbs with each administration cycle. If this occurred, the repetitive cycles could provide a doubling, tripling, and quadrupling of the total amount of radioactivity on the cancer cells in sequential steps. Such a protocol is under investigation in our research group, but it is not an ideal protocol for clinical adaptation as it involves multiple administrations of biotin/(strept)avidin reagents [and blood clearing agents/methods (23–28)].

Our desire to maximize the amount of radioactivity deposited on tumor cells in as few steps as possible led to consideration of molecules that contain multiple biotin moieties for cross-linking of streptavidin. Unlike biotin trimers, the use of molecules which contain multiple (i.e., more than four) biotin moieties could lead to the addition of two or more streptavidin molecules in a single step. Although there are many polymeric molecules that could be used as carriers of multiple biotin moieties,² poly-amidoamines (PAMAM) Starburst dendrimers (29–31) were particularly attractive candidates as they are small

spherical compounds, and a variety of sizes (generations)³ is available from commercial sources. We hypothesized that the number of streptavidin molecules bound to an mAb-streptavidin conjugate (F in Figure 1) might be significantly increased in a single step using the biotinylated Starburst dendrimers (E in Figure 1). As an initial evaluation of that hypothesis, an investigation of the synthesis, radioiodination, *in vitro* binding, and *in vivo* distribution of biotinylated Starburst dendrimers (BSBDs) has been conducted. In the investigation, a new biotinylation reagent was prepared in which the linking moiety provides increased water solubility and also provides resistance to degradation by the enzyme biotinidase (32–34). Biotinylation of five Starburst dendrimers (SBDs) containing a large variation in the number of biotin moieties per molecule was synthesized. Subsequently, the number of streptavidin (Sav) molecules that can bind with the biotinylated dendrimers was determined by an *in vitro* assay using streptavidin-coated wells. On the basis of the results of the *in vitro* studies, three radioiodinated biotinylated dendrimers were prepared. The radiolabeled biotinylated Starburst dendrimers were administered to athymic mice, and their distributions *in vivo* were determined at 4 h postinjection. The results of our investigation are described herein.

² For example, polybiotinylated aminodextrans are commercially available from Molecular Probes (Eugene, OR).

³ Starburst Dendrimers of increasing sizes are given the terminology of “generations” as they are built up from “shells”. The generations (abbreviated G) used are 0 (G = 0; four terminal amines); 1 (G = 1; eight terminal amines); 2 (G = 2; 16 terminal amines); 3 (G = 3; 32 terminal amines); and 4 (G = 4; 64 terminal amines). Information on the Starburst dendrimers used in this study is provided in Table 1. SBD nomenclature and chemistry are provided in reviews by Tomalia (29–31).

EXPERIMENTAL PROCEDURES

General. Chemicals purchased from commercial sources were of analytical grade or better and were used without further purification. Solvents for HPLC analysis were obtained as HPLC grade and were filtered (0.2 μ m) prior to use. Starburst Dendrimers, d-Biotin; triethylene glycol, **1**; *tert*-butyl acrylate, **2**; methylamine, 2,3,5,6-tetrafluorophenol; chloramine-T; and most other chemicals were purchased from Aldrich Chemical Co. (Milwaukee, WI). Recombinant streptavidin (r-SAv) was obtained as previously described (35). BSA was Fraction V (catalog no. 160069) obtained from ICN (Costa Mesa, CA).⁴ Silica gel chromatography was conducted with 70–230 mesh 60 Å silica gel (Aldrich Chemical Co.). Sephadex G-25 (NAP-10) columns were obtained from Pharmacia Biotech AB (Uppsala, Sweden). Avidin and Reacti-Bind streptavidin-coated polystyrene 96 well plates were obtained from Pierce (Rockford, IL). Melting points were obtained in open capillary tubes on a Mel-Temp II apparatus with a Fluke 51 K/J electronic thermometer and are uncorrected. ¹H-NMR data, MS data, and HPLC chromatography were used to assess compound identity and purity (data provided in the Supporting Information).

Spectral Analyses. ¹H NMR spectra were obtained on either a Bruker AC-200 (200 MHz) or a Bruker AC-500 (500 MHz) instrument. Proton chemical shifts are expressed as parts per million using tetramethylsilane as the internal standard (δ = 0.0 ppm). IR data were obtained on a Perkin-Elmer 1420 infrared spectrophotometer. Mass spectral data for the compounds of low molecular weight (i.e., <1000) were obtained on a VG Analytical (Manchester, England) VG-70SEQ mass spectrometer with associated 11250J Data System using fast atom bombardment (FAB⁺) at 8 keV in a matrix of MeOH/DMIX (Thioglycerol/DMSO/TFAA, 90/9/1) or 3NBA (3-nitrobenzyl alcohol). Mass spectral data for the high molecular weight biotinylated Starburst dendrimers were obtained with MALDI-TOF mass spectrometry conducted on a PerSeptive Biosystems Voyager-Elite EL time-of-flight mass spectrometer. In-source metastable ion decay MALDI-TOF mass spectra were produced using DE linear TOF-MS at an ion source bias potential of 25 kV and a 200 ns pulsed ion extraction delay time.

Chromatography. HPLC separations of biotin dendrimers were obtained on a Hewlett-Packard quaternary 1050 gradient pumping system with a variable wavelength UV detector (254 nm) and a Vares ELSD MKIII evaporative light-scattering detector. Analysis of the HPLC data were conducted on a Hewlett-Packard HPLC ChemStation software. All reactions were monitored by HPLC. Reversed-phase HPLC chromatography was carried out using an Alltech Altima C-18 column (5 μ m, 250 mm $\times$ 4.6 mm) with a gradient solvent system at a flow rate of 1 mL/min. The gradient mixture was composed of MeOH (solvent A) and 1% aqueous HOAc (solvent B). Starting with 40% MeOH, the initial solvent mixture was held for 2 min, increased to 100% MeOH over the next 10 min, then held at 100% MeOH for 5 min. Retention times (t_R) under these conditions for biotin conjugates

were (**3**) 10.3 min; (**4**) 13.3 min; (**5**) 5.2 min; (**7**) 11.6 min; (**8**) 8.1 min; (**9**) 12.4 min; (**11**) 8.6 min; (**13**) 7.5 min; (**15**) 7.1 min; (**17**) 7.6 min; (**19**) 7.6 min; (**21a**) 13.5 min; (**21b**) 9.8 min.

HPLC separations of radioiodinated biotin derivatives were conducted using a gradient system which consisted of two Beckman model 110B pumps, a Beckman 420 controller, a Beckman model 153 UV detector (254 nm), and a Beckman model 170 radioisotope detector. The separations were obtained using HPLC conditions described above, except water was used in the place of 1% aqueous HOAc. Retention times under these conditions were ([¹²⁵I]**21b**) 12.1 min; ([¹²⁵I]**22**) 11.4 min; ([¹²⁵I]**23**) 10.2 min.

Radioactive Materials. Na[¹²⁵I]I was purchased from NEN/Dupont (Billerica, MA) as high pH, high concentration solutions in 0.1 N NaOH. *N*-Succinimidyl *p*-[¹²⁵I]iodobenzoate ([¹²⁵I]PIB) was prepared as previously described (36). Measurement of ¹²⁵I was accomplished on the Capintec CRC-15R or a Capintec CRC-6A Radioisotope Calibrator. Radioactive samples were counted in a LOGIC Model 111B Gamma Counter (Abbott Laboratories, Chicago, IL).

12-Hydroxy-4,7,10-trioxadodecanoic Acid *tert*-Butyl Ester, 3. This compound was prepared from triethyleneglycol, **1**, and *tert*-butyl acrylate, **2**, as previously described (37).

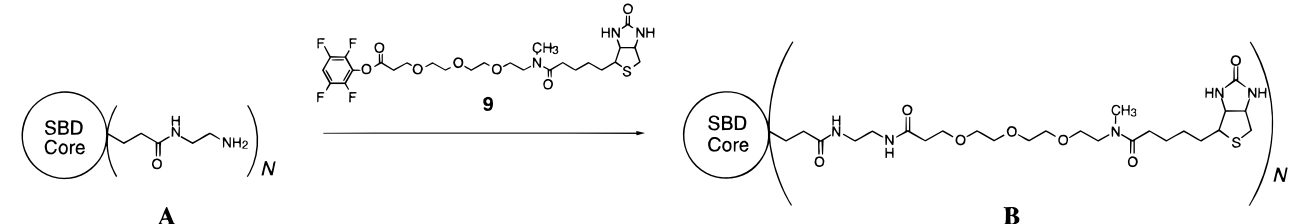
13-O-*p*-Tosyl-4,7,10,13-tetraoxadodecanoic Acid *tert*-Butyl Ester, 4. To a solution of **3** (15 g, 54 mmol) in 100 mL pyridine at 0 °C was added *p*-toluenesulfonyl chloride (20.6 g, 108 mmol). The reaction mixture was stirred at 0 °C for 4 h, poured into ice–H₂O, and the aqueous layer was extracted with CH₂Cl₂ (3 $\times$ 125 mL). The combined organic layers were washed with 2% HOAc and H₂O, and then dried over anhydrous Na₂SO₄. The solvent was removed under vacuum to yield a colorless oil. The oil was dried under vacuum to give 22 g (96%) of **4**. ¹H NMR (CDCl₃): δ 1.42 (s, 9H), 2.48 (s, 3H), 2.5 (t, 2H), 3.5–3.8 (m, 13H), 4.2 (m, 2H), 7.3 (d, 2H), 7.8 (d, 2H). IR (Nujol, cm^{−1}): 3420, 1720, 1630, 1590, 1450, 1360, 1250, 920. HRMS calcd for C₂₀H₃₂O₈SnA (M + Na): 455.1716. Found: 455.1707.

12-N-Methylamino-4,7,10-trioxadodecanoic Acid *tert*-Butyl Ester, 5. To a solution of **4** (11 g, 25.3 mmol) in THF (10 mL) was added a 2 M solution of methylamine in THF (176 mL, 352 mmol). The reaction was stirred at room temperature for 40 h. The solvent was removed under vacuum. The residue was extracted with chloroform (3 $\times$ 100 mL). The combined chloroform extracts were washed with (2 $\times$ 50 mL) H₂O and dried over anhydrous Na₂SO₄, and the CHCl₃ was removed under vacuum. The product was loaded on a silica column. The column was first eluted with ethyl acetate and then with varying percentages of ethyl acetate:methanol until 100% MeOH was eluted. The fractions containing product were reduced in volume, and the isolated product was dried under vacuum to yield 3 g (40%) of **5** as an oil. ¹H NMR (CDCl₃): δ 1.4 (s, 9H), 2.38 (s, 3H), 2.42 (t, 2H), 2.7 (m, 2H), 3.5–3.8 (m, 13H). IR (Nujol, cm^{−1}): 3320, 1720, 1550, 1450, 1360, 1250, 1100, 940. HRMS calcd for C₁₄H₃₀NO₅ (M + H)⁺: 292.2124. Found: 292.2116.

Biotin Tetrafluorophenyl Ester, 6. This compound was prepared by reaction of biotin with 2,3,5,6-tetrafluorophenyl trifluoroacetate as previously described (15).

12-N-Biotinyl-(*N*-methyl)amino-4,7,10-trioxadodecanoic Acid *tert*-Butyl Ester, 7. To a solution of **5** (0.12 g, 0.412 mmol) in 25 mL of DMF was added Et₃N (60 μ L, 0.412 mmol), followed by **6** (0.161 g, 0.412 mmol).

⁴ The BSA used in these experiments is stated to be "more highly purified than our traditional True Cohn Fraction V powder" by its manufacturer, ICN. It has been noted by manufacturers of in vitro assay kits using biotin/streptavidin that cell culture media such as BSA often contain free biotin. Although we did not measure the quantity of biotin in the BSA used, its concentration must be very low because the results obtained in the in vitro binding assay were very nearly the same with or without its use (unreported results).

Table 1. Generalized Reaction for Perbiotinylation of Starburst Dendrimers (SBD) Using Biotinylation Reagent 9


starting dendrimer A	MW (amu)	SBD core ^a	biotin moieties ^b <i>N</i>	product B	MW (amu)	yield (%)	distance ^c (Å)
10 ; SBD, <i>G</i> = 0	517	R ₂ NCH ₂ CH ₂ NR ₂ ^d	4	11	2289	49	55
12 ; SBD, <i>G</i> = 1	1430	SBD, <i>G</i> = 0	8	13	4978	57	71
14 ; SBD, <i>G</i> = 2	3256	SBD, <i>G</i> = 1	16	15	10 353	48	87
16 ; SBD, <i>G</i> = 3	6909	SBD, <i>G</i> = 2	32	17	21 103	81	104
18 ; SBD, <i>G</i> = 4	14 214	SBD, <i>G</i> = 3	64	19	42 602 ^e	74	122

^a The starburst dendrimer core for each generation (*G*) is composed of the previous generation, except the terminal primary amines are substituted with two of the groups shown in parentheses in structure **A**. ^b This is the maximum number that can be attached under normal conjugation conditions. ^c The distances cited are those between two biotin carboxylate carbon atoms that are the farthest apart when fully linearized. For binding purposes, this distance can be considered the diameter of the biotinylated SBD as the biotin moieties are buried within pockets on the (strept)avidin molecule. ^d R is shown in parentheses for generalized structure **A** above. ^e See discussion on product characterization of this dendrimer in the Results. The perbiotinylation product was not obtained under the reaction conditions employed in this study.

The reaction was stirred at room temperature for 4 h, and the solvent was removed under vacuum. The residue was extracted with CHCl₃ (300 mL). The CHCl₃ solution was washed with saturated aqueous NaHCO₃ (3 × 25 mL), then H₂O (2 × 25 mL), and was dried over anhydrous Na₂SO₄. The CHCl₃ was removed under vacuum to yield 0.21 g (98%) of **7** as an oil. ¹H NMR (CDCl₃): δ 1.4 (s, 10H), 1.6–1.8 (m, 4H), 2.3 (t, *J* = 4.25 Hz, 3.9 Hz, 1H), 2.4 (t, *J* = 3.75 Hz, 3.6 Hz, 1H), 2.5 (t, *J* = 3.5 Hz, 3.4 Hz, 2H), 2.8 (d, *J* = 6.75 Hz, 1H), 2.9–3.0 (m, 4H), 3.1 (s, 2H), 3.2 (bs, 1H), 3.45–3.7 (m, 13H), 4.35 (s, 1H), 4.55 (s, 1H), 5.7 (d, *J* = 7.5 Hz, 1H), 6.1 (d, *J* = 10.5 Hz, 1H). IR (Nujol, cm⁻¹): 3260, 3060, 1780, 1690, 1520, 1480, 1260, 1080, 930. HRMS calcd for C₂₄H₄₄N₃O₇S (M + H)⁺: 518.2900. Found: 518.2923.

12-*N*-Biotinyl-(*N*-methyl)amino-4,7,10-trioxadodecanoic Acid Tetrafluorophenyl Ester, **9.** To a solution of **7** (0.21 g, 0.406 mmol) in 10 mL CH₂Cl₂ was added CF₃CO₂H (1 mL). This mixture was stirred at room temperature for 5 h. The mixture was reduced in volume under vacuum, and the residue, **8**, was dissolved in 20 mL of CH₃CN. Tetrafluorophenol (0.083 g, 0.5 mmol) was added, followed by EDC (0.096 g, 0.5 mmol). The reaction mixture was stirred at room temperature for 16 h, and the solvent was removed under vacuum. The residue was extracted with CHCl₃ (300 mL), washed with saturated aqueous NaHCO₃ (3 × 25 mL), then H₂O (2 × 25 mL), and dried over anhydrous Na₂SO₄. The solvent was removed under vacuum to yield 0.23 g (93%) of **9** as an oil. ¹H NMR (CDCl₃): δ 1.4 (s, 2H), 1.5–1.7 (m, 4H), 2.3 (m, 2H), 2.65 (d, *J* = 6.25 Hz, 1H), 2.8–2.9 (m, 4H), 3.0 (s, 2H), 3.1 (s, 1H), 3.4–3.6 (m, 13H), 3.8 (s, 2H), 4.35 (s, 1H), 4.45 (s, 1H), 6.0 (d, *J* = 8.25 Hz, 1H), 6.3 (d, *J* = 6.75 Hz, 1H), 6.95 (m, 1H). IR (Nujol, cm⁻¹): 3260, 3060, 1780, 1690, 1520, 1480, 1260, 1080, 930. HRMS calcd for C₂₆H₃₅N₃O₇F₄S (M + H)⁺: 610.2210. Found: 610.2216.

Biotinylation of Starburst Dendrimers Generation 0, **11.** A 20% MeOH solution of Starburst dendrimer, generation 0, **10** (0.54 mmol, 1.64 mL), was reduced in volume and then dried under vacuum (50 micron) for 2 h. The resulting residue was dissolved in 10 mL of DMF, and Et₃N (0.378 mmol, 55 μL) was added. To that solution was added a solution of **9** (0.378 mmol, 0.230 g) in DMF (10 mL). The reaction mixture was stirred at room temperature for 48 h, and the DMF was removed under vacuum. The residue was dissolved in

water (100 mL) and was washed with chloroform (2 × 25 mL). The aqueous layer was lyophilized to yield 0.084 g (49%) of tetrabiotin derivative, **11**, as a tacky white solid. ¹H NMR (MeOH): δ 1.65 (m, 10H), 1.7–2.0 (m, 19H), 2.5–2.65 (m, 25H), 2.85–3.0 (m, 14H), 3.1 (s, 10H), 3.3 (s, 7H), 3.4–3.5 (m, 21H), 3.7–3.95 (m, 66H), 4.5 (m, 4H), 4.7 (m, 4H). IR (Nujol, cm⁻¹): 3160, 2720, 1450, 1370, 1250, 1160, 960. Mass calcd for C₁₀₂H₁₈₀N₂₂O₂₈S₄: 2290.91. Found: 2290.89.

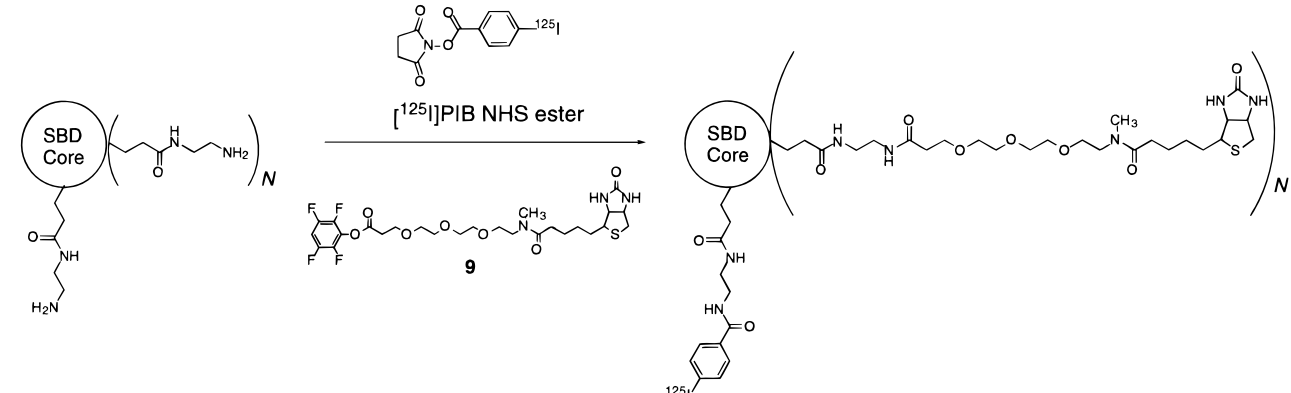
Biotinylation of Starburst Dendrimers, Generations 1, 2, 3, and 4. The solvent was removed from the Starburst Dendrimer (generation 1, **12**, 0.14 mmol; generation 2, **14**, 0.075 mmol; generation 3, **16**, 0.035 mmol; generation 4, **18**, 0.018 mmol), and the residue was dried under vacuum for 2 h, then it was dissolved in 10 mL of DMF. To this solution was added Et₃N (0.12 mmol, 157 μL), followed by the biotinylation reagent, **9** (1.12 mmol, 0.682 g). The reaction mixture was stirred at room temperature for 5 days. The DMF was removed under vacuum and the residue was dissolved in H₂O (100 mL). The aqueous solution was washed with CHCl₃ (2 × 25 mL), and then lyophilized to give the crude product. The crude product was purified by silica column chromatography, first eluting with MeOH, then with a MeOH/CHCl₃/H₂O mixture. Yields and information on the products are provided below and in Table 1.

Generation 1, **13.** A (5/5/1) MeOH/CHCl₃/H₂O elution mixture was used to obtain 0.4 g (57%) of product as a tacky white solid. ¹H NMR (MeOH): δ 1.4–1.8 (m, 48H), 2.3–2.6 (m, 68H), 2.7–2.85 (m, 32H), 2.95 (m, 20H), 3.1 (s, 12H), 3.15–3.3 (m, 76H), 3.5–3.8 (m, 120H), 4.3 (m, 8H), 4.5 (m, 8H). MS (MALDI-TOF): *m/e* 4975.

Generation 2, **15.** A (14/14/1) MeOH/CHCl₃/H₂O elution mixture was used to obtain 0.95 g (48%) of a tacky white solid. ¹H NMR (MeOH): δ 1.4–1.8 (m, 96H), 2.3–2.5 (m, 120H), 2.52–2.85 (m, 108H), 2.95 (m, 40H), 3.1 (s, 24H), 3.15–3.3 (m, 150H), 3.5–3.8 (m, 246H), 4.3 (m, 16H), 4.5 (m, 16H). MS (MALDI-TOF): *m/e* 10 353.

Generation 3, **17.** A (30/60/5/1) MeOH/CHCl₃/H₂O/Et₃N elution mixture was used to obtain 0.6 g (81%) of a tacky white solid. ¹H NMR (MeOH): δ 1.4–1.8 (m, 200H), 2.3–2.95 (m, 586H), 3.1 (s, 48H), 3.15–3.3 (m, 262H), 3.5–3.8 (m, 504H), 4.3 (m, 32H), 4.5 (m, 32H). MS (MALDI-TOF): *m/e* 21 110.

Generation 4, **19.** A (30/60/5/1) MeOH/CHCl₃/H₂O/Et₃N elution mixture was used to obtain 0.56 g (74%) of

Table 2. Generalized Reaction of Starburst Dendrimers (SBD) with [¹²⁵I]PIB NHS Ester and Biotinylation Reagent 9


starting dendrimer ^a A	MW (amu)	reaction time (days)	biotin moieties ^b <i>N</i>	product B	MW ^c (amu)	radiochemical yield (%)
10 ; SBD, <i>G</i> = 0	517	3	3	[¹²⁵ I] 21b	2077	54
12 ; SBD, <i>G</i> = 1	1430	3	7	[¹²⁵ I] 22	4765	28
14 ; SBD, <i>G</i> = 2	3256	3	15	[¹²⁵ I] 23	10 140	40

^a The starburst dendrimer core for each generation (*G*) is composed of the previous generation, except the terminal primary amines are substituted with two of the groups shown in parentheses in structure **A**. ^b This is the maximum number that can be attached under normal conjugation reaction conditions. ^c The molecular weight has been calculated with the predominant iodine nuclide, iodine-127.

a tacky white solid. ¹H NMR (MeOH): δ 1.4–1.8 (m, 426H), 2.3–2.95 (m, 1148H), 3.1 (s, 104H), 3.15–3.3 (m, 502H), 3.5–3.8 (m, 1052H), 4.3 (m, 64H), 4.5 (m, 64H). MS (MALDI-TOF): *m/e* 36887.

***p*-Tri-*n*-butylstannylbenzoate Tetrafluorophenyl Ester, 20a, and *p*-Iodobenzoate Tetrafluorophenyl Ester, 20b.** These compounds were prepared as previously described (32).

***p*-Iodobenzoyl-Biotin Trimer, 21b.** A 20% MeOH solution of Starburst dendrimer, generation 0, **10** (0.54 mmol, 1.64 mL) was reduced in volume under vacuum. The resulting residue was dissolved in 10 mL of DMF, and Et₃N (0.378 mmol, 55 mL) was added. To that solution, **20b** (0.5 mmol, 0.198 g) in 30 mL of DMF was added dropwise over a period of 4 h. Then, a solution of **9** (2.16 mmol, 1.33 g) in DMF was added (10 mL). The reaction was stirred at room temperature for 16 h, and the solvent was removed under vacuum. The residue was extracted with CHCl₃ (300 mL) and washed with saturated aqueous NaHCO₃ (3 × 25 mL) and then H₂O (2 × 25 mL). The CHCl₃ solution was dried over anhydrous Na₂SO₄. The CHCl₃ was reduced in volume, silica gel (0.5–1 g) was added, and the remaining CHCl₃ was evaporated. The residue was placed on a silica column. Column elution began with EtOAc/1% Et₃N. Subsequent elution used increasing quantities of MeOH in a EtOAc/MeOH/1% Et₃N mixture until 100% MeOH was eluted. The fractions containing desired product were reduced in volume under vacuum to yield 0.15 g (14%) of **21b** as an oil. ¹H NMR (MeOH): δ 1.4–1.9 (m, 20H), 2.3–3.9 (m, 120H), 4.35 (s, 3H), 4.5 (s, 3H), 7.6 (s, 2H), 7.9 (s, 2H). IR (Nujol, cm⁻¹): 3180, 2720, 1450, 1370, 1300, 1140, 960. Mass calcd for C₈₉H₁₅₀N₁₉O₂₃S₃I: 2076. Found: 2096 (M + Na).

***p*-Tributylstannylbenzoyl-Biotin Trimer, 21a.** This compound was prepared as described for **21b**, except **20a** was used and the reaction was allowed to proceed for 48 h at room temperature. A yield of 0.26 g (22%) of **21a** (an oil) was obtained. ¹H NMR (MeOH): δ 0.8–1.8 (m, 47H), 2.3–3.8 (m, 120H), 4.3 (m, 3H), 4.5 (m, 3H), 7.6 (m, 2H), 7.8 (m, 2H). IR (Nujol, cm⁻¹): 3180, 2720, 1450, 1370, 1300, 1140, 960. Mass calcd for C₁₀₁H₁₇₇N₁₉O₂₃S₃-Sn: 2239. Found: 2260 (M + Na).

Preparation of [¹²⁵I]21b from 21a. To 100 μL of a 1 mg/mL solution of **21a** in 5% HOAc was added a mixture containing 2–3 μCi Na[¹²⁵I] and 0.2–0.8 equiv of NaI in H₂O (1 mg/mL solution). To this mixture was added 10 μL of a 2 mg/mL solution of *N*-chlorosuccinimide in MeOH. After 5 min at room temperature, 10 μL of a sodium metabisulfite solution (2 mg/mL in H₂O) was added to quench the reaction. Radiochemical yields were determined by HPLC to be 32–56%.

General Procedure for Radioiodination of Biotinylated Starburst Dendrimers using [¹²⁵I]PIB to prepare [¹²⁵I]21b, [¹²⁵I]22, and [¹²⁵I]23. An aliquot (3.2–4.1 μL) of the SBD (*G* = 0, 1, or 2) in MeOH that contained the desired quantity (0.5–0.6 mg) was evaporated and dried under vacuum for 2 h. The residue was placed under Ar atmosphere and dissolved in 25 μL of anhydrous DMF. To this solution was added 0.2 equiv (18–87 μg, 1 mg/mL solution) of [¹²⁵I]PIB reagent in DMF, then 5 μL of TEA was added. The reaction was allowed to proceed for 1 h at room temperature before 5.5 (*G* = 0), 9.5 (*G* = 1), or 17.5 (*G* = 2) equiv of a 100 mg/mL solution of **9** in DMF was added. The reaction mixture was then stirred at room temperature for 3 days, and the radiolabeled BSBDs were purified by HPLC. Radiochemical yields are provided in Table 2.

Radioiodination of r-SAv. Radioiodination of r-SAv was conducted using chloramine-T and Na[¹²⁵I] as reported previously [15,38]. The radioiodinated r-SAv was purified on a NAP-10 column, eluting with PBS to give a radiochemical yield of 76% and a specific activity of 0.39 μCi/μg.

r-[¹²⁵I]SAv Binding in SAv-Coated Wells. A Reacti-Bind Streptavidin Coated Polystyrene Strip Plate was washed 2 times with 100 mM PBS/0.1% BSA⁴ (100 μL/well) prior to use. To lane one (12 wells) was added 100 pmol of biotin in 100 μL of PBS. To lanes 2–8 was added 100 pmol of biotin trimers (**29** and **21b**) or perbiotinylated Starburst dendrimers (*G* = 0, **11**; *G* = 1, **13**; *G* = 2, **15**; *G* = 3, **17**; *G* = 4, **19**) to be tested in 100 μL of PBS. The plate was then incubated at room temperature for 15 min. All the wells were emptied and rinsed with 100 μL of PBS/0.1% BSA. The PBS was removed and 25 pmol of r-[¹²⁵I]SAv (specific activity of 0.39 μCi/μg) was added

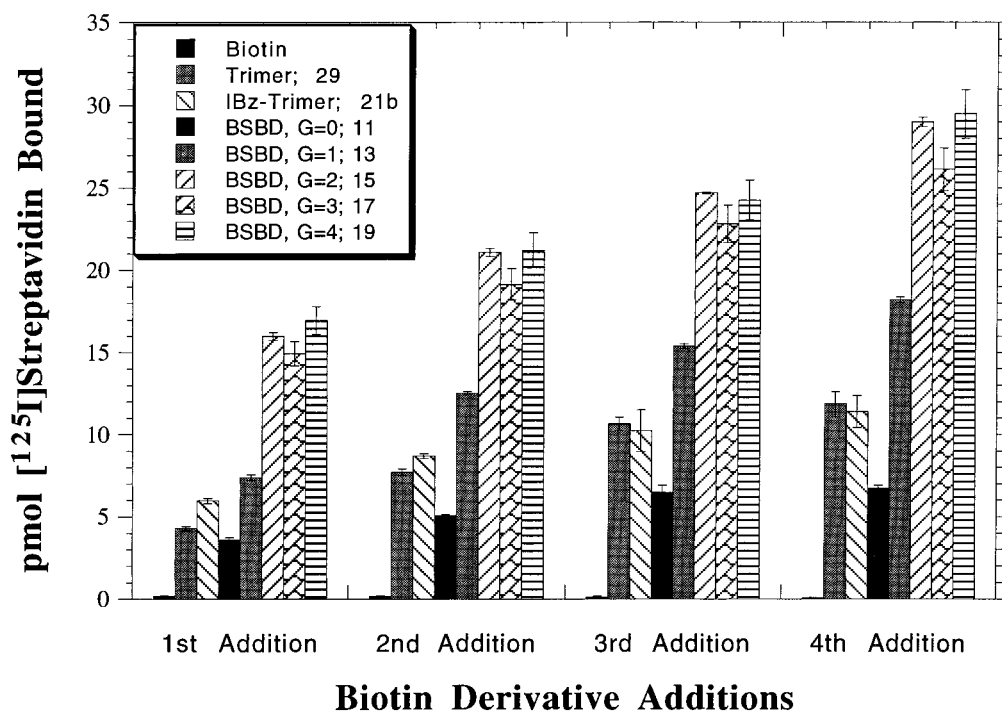


Figure 2. Graph of data obtained for cross-linking of polystyrene bound SAV and r-[¹²⁵I]SAV with biotinylated dendrimers and biotin trimer at room temperature for 15 min. Data were obtained from triplicate runs and are plotted as average $\pm$ standard deviation. The experiment was conducted as follows. An excess of biotin derivative was added, rinsed with PBS containing 1% BSA, then an excess of [¹²⁵I]SAV was added, incubated for 15 min, and rinsed with PBS containing 1% BSA. This procedure was repeated four times, with the only change being the number of additions of the biotin derivative (i.e., all wells received four additions of [¹²⁵I]SAV). When 1, 2, or 3 additions of biotin derivative were made, 100 μ L of PBS was added in the place of biotin derivative to keep the number of additions/rinses constant. All additions and rinses were made in a volume of 100 μ L. Determining the quantity of radioactivity in each well and the specific activity of the [¹²⁵I]SAV permitted calculation of the picomoles bound.

to each well in 100 μ L of PBS. The plate was incubated at room temperature for 15 min. The nonbound r-[¹²⁵I]-SAV was removed, and all wells were washed with 100 μ L of PBS/0.1% BSA. After removal of the wash PBS solution, the first three wells of each lane were again filled with 100 μ L of PBS/0.1% BSA. The remaining wells were filled with 100 μ L of their respective biotin trimer or biotinylated Starburst dendrimer (as in the first step). The plate was incubated for 15 min at room temperature, and all wells were rinsed with PBS/0.1% BSA as before. Another 100 μ L of the r-[¹²⁵I]SAV was added to all wells and incubated for 15 min at room temperature. After removal of the unbound r-SAV by rinsing the wells, 100 μ L of PBS was added to the first six wells of each lane. The remaining six wells in each lane were filled with 100 μ L of their respective biotin compound and again incubated for 15 min at room temperature. After removing the liquid in the wells and washing with PBS, 100 μ L of the r-[¹²⁵I]SAV was again added to each well followed by 15 min of incubation. The [¹²⁵I]SAV was removed, and the wells were washed with PBS/0.1% BSA. The first nine wells in each lane were then filled with 100 μ L of PBS, and the remaining three wells in each lane were filled with 100 μ L of their respective biotin compound. After incubating and washing, 100 μ L of r-[¹²⁵I]SAV was again added to each well. After incubating for 15 min at room temperature, each well was washed with 100 μ L of PBS/0.1% BSA and all liquid was removed. The wells were then separated and each well placed in a γ counter and counted. Results of the r-[¹²⁵I]SAV binding are shown in Figure 2.

Biodistributions of Radioiodinated Starburst Dendrimers Generations 0, [¹²⁵I]21b; 1, [¹²⁵I]22; and 2, [¹²⁵I]23. Animal care and handling was as previously described (15). Radiolabeled BSBDs ([¹²⁵I]21b, [¹²⁵I]22,

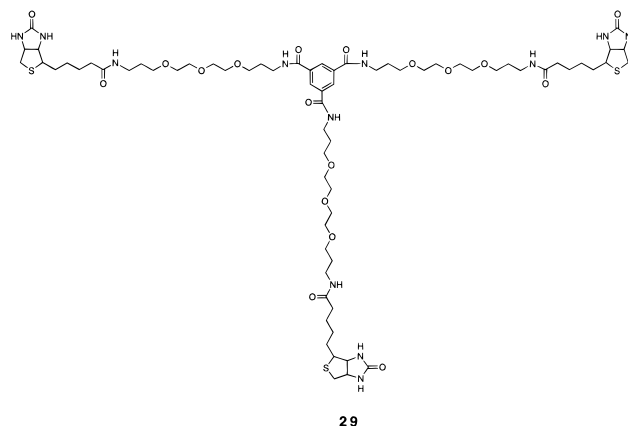


Figure 3. Structure of biotin trimer, **29**, used as control in the *in vitro* assay. Its synthesis and evaluation of binding in the same binding assay have been previously described (22).

and [¹²⁵I]23) were administered to mice in a total volume of approximately 100 μ L via the lateral tail vein. The actual amount of injectate administered to each animal was determined by weighing the administering syringe pre- and postinjection. The animals were sacrificed at 4 h postinjection, and muscle, lung, kidney, spleen, liver, intestine, neck (including thyroid), and stomach were excised. Blood samples were obtained by cardiac puncture immediately before sacrifice. Urine samples were obtained by syringe bladder tap at the time the tissues were excised. Excised tissues were blotted free of blood, weighed, and counted. Calculation of the percent injected dose (%ID) and percent injected dose per gram (% ID/g) in the tissues was based on internal standards containing 1 μ L of the injected dose. Total blood volume was estimated to be 8% of body weight for the calculations

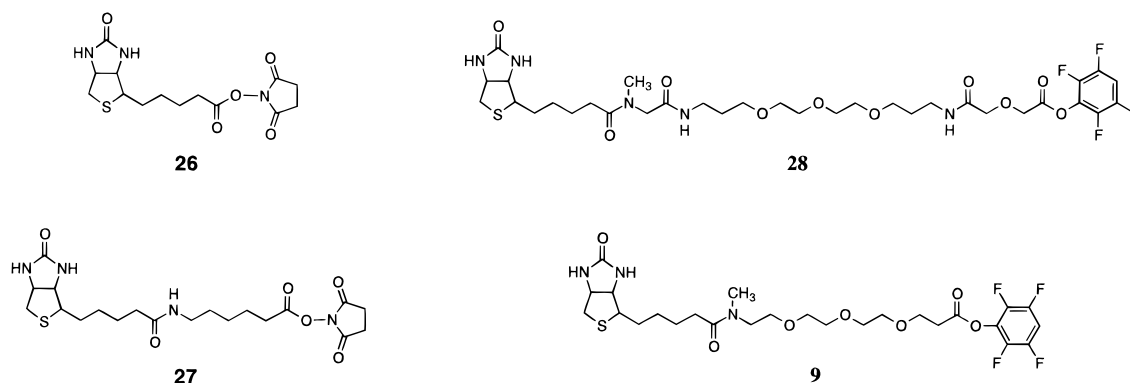


Figure 4. Amine reactive biotinylation reagents.

Table 3. Distribution of Radioactivity for [^{125} I]BSBDs in Athymic Mice^a

tissues	[^{125} I] 21b ^b	[^{125} I] 22 ^c	[^{125} I] 23 ^d
blood	0.20 $\pm$ 0.04	0.13 $\pm$ 0.02	0.20 $\pm$ 0.03
muscle	0.04 $\pm$ 0.00	0.04 $\pm$ 0.00	0.08 $\pm$ 0.03
lung	0.26 $\pm$ 0.05	0.18 $\pm$ 0.01	0.62 $\pm$ 0.19
kidney	8.28 $\pm$ 2.92	16.95 $\pm$ 2.82	47.64 $\pm$ 7.08
spleen	0.26 $\pm$ 0.11	0.20 $\pm$ 0.04	0.70 $\pm$ 0.07
liver	1.50 $\pm$ 0.10	1.79 $\pm$ 0.22	7.06 $\pm$ 1.23
intestine	3.77 $\pm$ 3.65	0.74 $\pm$ 0.66	1.34 $\pm$ 1.93
urine ^e	33.39 $\pm$ 36.85	9.70 $\pm$ 10.25	6.87 $\pm$ 2.10
neck	0.23 $\pm$ 0.04	0.20 $\pm$ 0.23	0.43 $\pm$ 0.09
stomach	0.25 $\pm$ 0.08	0.14 $\pm$ 0.03	0.47 $\pm$ 0.11

^a Values shown are average % injected dose/gram $\pm$ standard deviation; sacrifice was 4 h postinjection; data were obtained for $n = 5$ mice per group; average animal weight, 24.61 $\pm$ 2.28 g; all injections were made in approximately 100 μ L of 0.9% sterile saline. ^b Injectate for each animal had 10 μ Ci/284 μ g of [^{125} I]**21b**. ^c Injectate for each animal had 10 μ Ci/70 μ g of [^{125} I]**22**. ^d Injectate for each animal had 10 μ Ci/59 μ g of [^{125} I]**23**. ^e Urine was collected by syringe bladder tap after sacrifice.

(39). A spreadsheet program in Excel (Microsoft Corp., Redmond, WA) was used to perform the calculations. Data on the average animal weight per group, specific activity of each preparation, and quantities of [^{125} I]-labeled BSBDs administered are provided in the caption below the biodistribution data in Table 3.

RESULTS

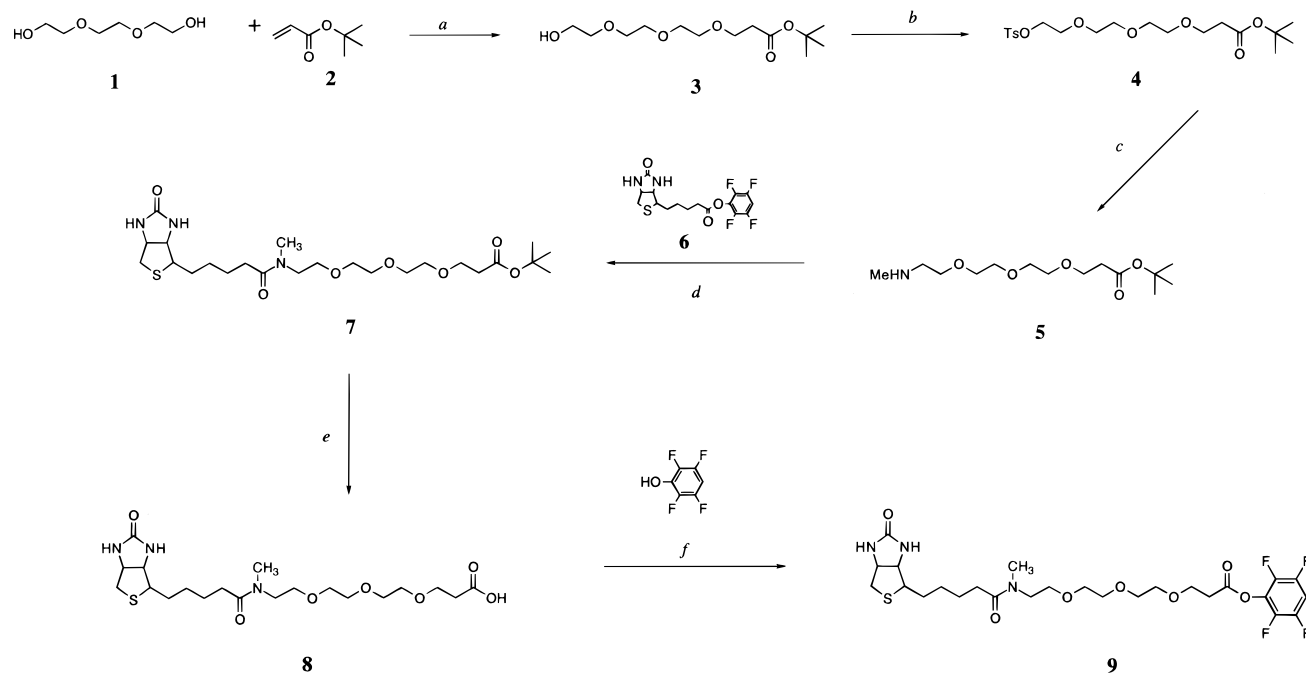
Preparation of the Biotinylation Reagent, 9. The synthetic approach to obtain **9** is shown in Scheme 1. A key step in the synthesis, the preparation of 12-hydroxy-4,7,10-trioxadecanoic acid *tert*-butyl ester, **3**, was previously described by Seitz and Kuntz (37). Tosylation of **3** provided **4** in high yield, but the displacement of the tosylate group with methylamine was problematic, resulting in low (e.g., 40%) isolated yields of **5**. However, conjugation of **5** with biotin TFP ester, **6**, was facile, as was the carboxylate deprotection and reesterification steps. Since most reaction steps are high yielding, an overall yield of 30% was obtained for the biotinylation reagent, **9**, from triethylene glycol.

Preparation and Characterization of Biotinylated Starburst Dendrimers. Five Starburst dendrimers (SBDs) were biotinylated with **9**. The SBDs, **10** ($G = 0$),³ **12** ($G = 1$), **14** ($G = 2$), **16** ($G = 3$), and **18** ($G = 4$) were obtained as 10–20% (by wt) solutions in MeOH. After removing the MeOH under vacuum, the SBDs were reacted with an excess of the biotinylation reagent **9** in DMF at room temperature to prepare the perbiotinylated dendrimers **11**, **13**, **15**, **17**, and **19**, respectively. Biotinylation of SBDs **10** and **12** are shown in Schemes 2 and 3, and a generalized scheme depicting biotinylation of all

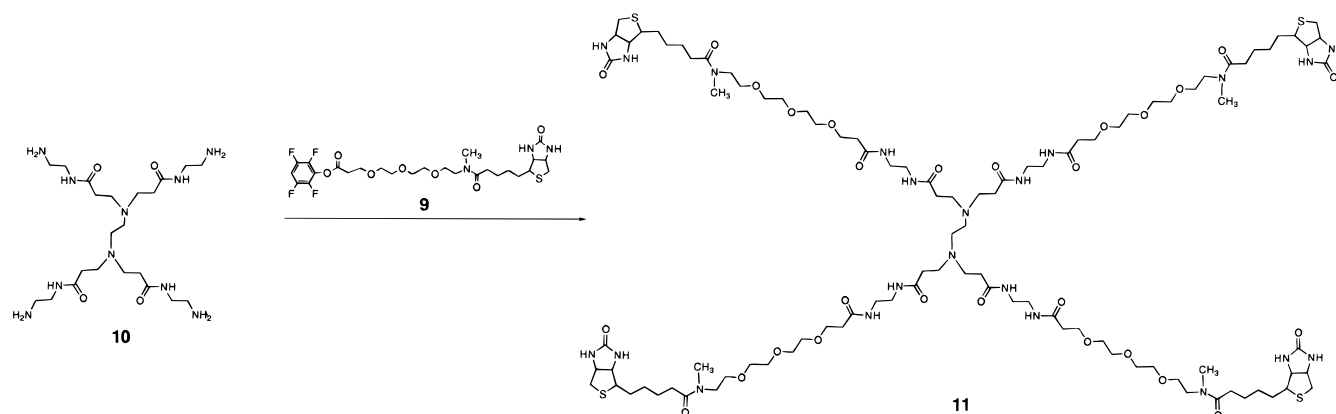
of the dendrimers studied is shown in Table 1. The crude products were purified by silica gel column chromatography. Reversed-phase HPLC chromatograms of the purified biotinylated dendrimers are provided in the Supporting Information. Proton NMR gave additional proof of the purity of the biotinylated dendrimers through integration of the biotin ring juncture protons ($\delta = 4.3$ and 4.5 ppm) relative to the integration of other proton peaks. Mass spectra data confirmed the identity of the compounds prepared. All of the SBDs, except SBD **18** ($G = 4$; 64 terminal amines), were perbiotinylated under the reaction conditions studied. The expected mass for perbiotinylated **18** is 42 602 whereas the MALDI-TOF mass spectra indicated that the mass of highest abundance was 36 887. Since the mass of the acylation portion of the biotinylation reagent **9** is 445, it appears that the largest portion of **19** has added 51 biotin moieties, not 64. It is likely that longer reaction times and/or elevated reaction temperatures could provide more biotin moieties attached to **19**. However, the results obtained in our studies suggest that perbiotinylation would not make a difference in the number of streptavidin molecules it could bind.

Preparation and Characterization of Radioiodinated Biotinylated Starburst Dendrimers. Radioiodinated derivatives of the BSBDs were prepared to evaluate their in vivo distribution. Our radioiodination studies began by preparing the *p*-tri-*n*-butylstannylbenzoate derivative of the smallest SBD ($G = 0$). Syntheses of the tris-biotinylated stannylbenzoyl-SBD, **21a**, and the corresponding iodinated derivative, **21b**, were accomplished as shown in Scheme 4. Synthesis of **21a** was accomplished by reaction of 1 equiv of the tetrafluorophenyl ester of *p*-tri-*n*-butylstannylbenzoate, **20a**, with Starburst dendrimer **10**, followed by reaction with an excess (4 equiv) of the biotinylation reagent **9**. The iodinated derivative, **21b**, was prepared in a similar manner. Difficulties in purification of **21a** and **21b** from the mixture of compounds produced resulted in low isolated yields of pure compounds. Radioiodination of purified **21a** was conducted in MeOH/5% HOAc at room temperature using *N*-chlorosuccinimide (NCS) as the oxidant. The radioiodination yields were also low, being only 32–56% by HPLC analysis (not isolated). The low yields obtained prompted us to investigate another method for radioiodination of the BSBDs.

As an alternative method of radioiodination, the NHS ester of *p*-[^{125} I]iodobenzoate ([^{125} I]PIB) (**36**) was reacted with SBDs **10**, **12**, and **14**. To ensure that most SBDs only reacted with one molecule of [^{125} I]PIB, the reactions were conducted with 0.2 equiv. The conjugation of [^{125} I]-PIB was conducted in DMF/Et₃N for 1 h at room temperature, then an excess of the biotinylation reagent

Scheme 1. Synthesis of Water Solubilized, Biotinidase Stabilized, Biotinylation Reagent, 9

^a THF, rt, 20 h, 1 M HCl, 86%. ^b Pyridine, 0 °C, TsCl, 4 h, 96%. ^c MeNH₂, THF, rt, 40 h; 40%. ^d DMF, Et₃N, **6**, rt, 4 h, 98%. ^e CF₃CO₂H, CH₂Cl₂, rt, 5 h. ^f CH₃CN, TFP-OH, EDC, rt, 16 h, 93%.

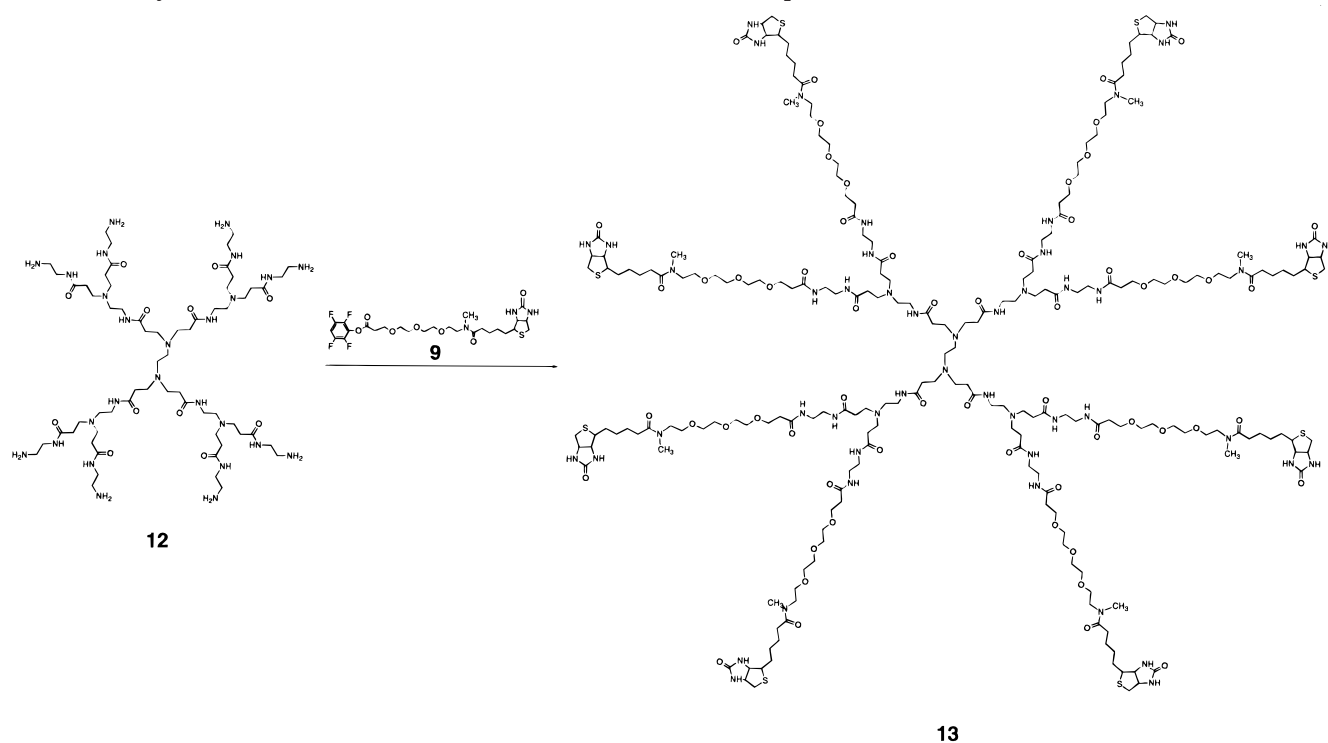
Scheme 2. Reaction of Starburst Dendrimer Generation 0 to Prepare Biotin Tetramer, 11

^a DMF, Et₃N, **9**, rt, 48 h, 49%.

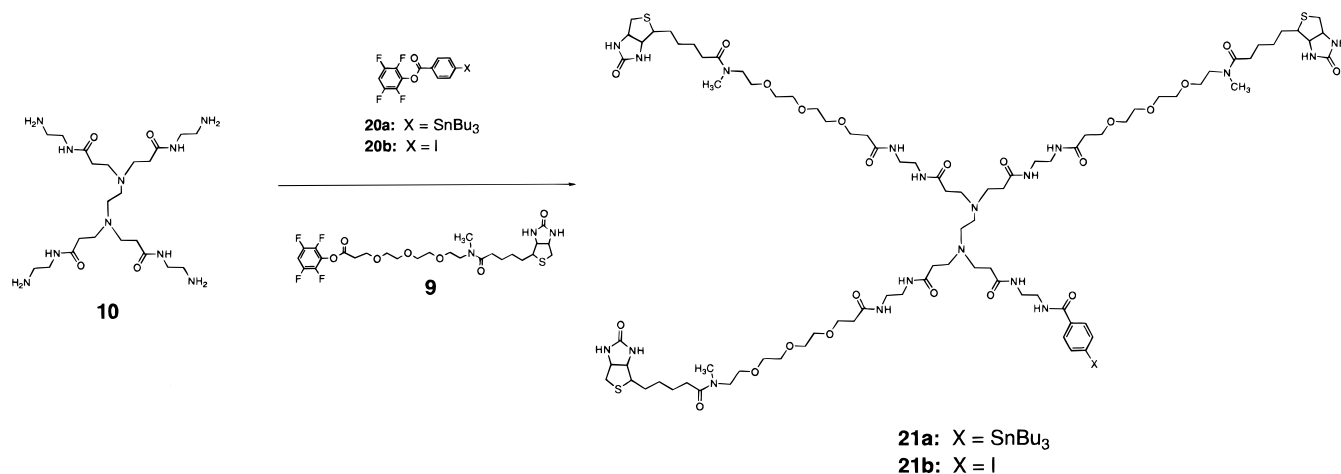
9 was added and allowed to react for an additional 3 days at room temperature. A general scheme for the radioiodinations and the radiochemical yields obtained is shown in Table 2. The radioiodinated products were isolated from the HPLC effluent and reduced in volume to provide an aqueous solution that was used directly in the *in vivo* distribution studies.

Biotinylated Dendrimer Cross-Linking of r-SAV with Immobilized SAV. The quantity (pmol) of [¹²⁵I]-SAV that binds with immobilized BSBD was determined in an *in vitro* binding assay. The assay involved binding the BSBD to streptavidin that is immobilized in polystyrene wells (in a "strip" plate), followed by binding r-[¹²⁵I]SAV to the resulting exposed biotin moieties. Four cycles of reagent additions were used to determine the increase of r-[¹²⁵I]SAV obtained after each addition. Excess reagents were used in each addition to saturate the binding capacity. The excess BSBD or [¹²⁵I]SAV was removed by rinsing the wells with PBS containing 0.1% BSA. Nonspecific binding of r-[¹²⁵I]SAV was minimized by addition of 0.1% BSA.⁴ Only one rinse was used after each addition due to concerns about removing polystyrene

bound SAV with multiple rinses. All wells received four additions of [¹²⁵I]SAV, but the number of additions of the biotin reagent to each well varied from 1 to 4. To ensure consistency, the wells that did not receive the biotin reagent got an equal volume of PBS added in its place. The incubation conditions for binding BSBDs and [¹²⁵I]-SAV were limited to 15 min at room temperature. These conditions were chosen as no difference in the quantity bound was noted when the incubations were conducted at 37 °C or when they were conducted over a 30 min period (unreported studies). Triplicate wells were used for each reagent so the variance in wells could be averaged. The quantity of r-[¹²⁵I]SAV which bound with immobilized BSBDs (**11**, **13**, **15**, **17**, **19**), biotin trimers (**21b**, **29**), and biotin (control) is shown in Figure 2. It is interesting to note that the quantity of [¹²⁵I]SAV bound for BSBD **13** is double (7.9 ± 0.2 pmol) that of the smaller BSBD **11** (3.6 ± 0.2 pmol) and is one-half of the larger BSBD **15** (16.0 ± 0.2 pmol). Another interesting observation is that the amount of [¹²⁵I]SAV bound is higher for the biotin trimers, **21b** and **29**, than it is for the biotin tetramer, **11**. It is tempting to believe that this difference

Scheme 3. Synthesis of Starburst Dendrimer Generation 1 to Prepare Biotin Octamer, 15

^a DMF, Et₃N, **9**, rt, 5 d, 57%.

Scheme 4. Synthesis of Stannylbenzoyl- and Iodobenzoyl-Biotin Trimers, 21a and 21b.

^a DMF, Et₃N, **20a** or **20b**, rt 4 h; **9**, rt, 16 h (or 48 h), 22% (**21a**), 14% (**21b**).

is due to branching as previously proposed (22), but that has not been proven in these studies. Perhaps the most interesting, and surprising, data was that no additional binding of [¹²⁵I]SAv, over that observed for BSBD **15** (16 biotin moieties), was obtained when BSBD **17** (32 biotin moieties) and BSBD **19** (up to 64 biotin moieties) were evaluated.

In Vivo Distribution of Radioiodinated Dendrimers. Biodistributions of radioiodinated BSBDs, [¹²⁵I]**21b** (three biotins), [¹²⁵I]**22** (seven biotins), and [¹²⁵I]**23** (15 biotins) were evaluated in athymic mice at 4 h postinjection. The biodistributions of the larger BSBDs, *G* = 3 and 4, were not evaluated because no increase in *r*-[¹²⁵I]-SAv binding had been noted over the smaller BSBDs in the *in vitro* study. For the comparative evaluation, a minimum number of mice (5 per compound tested) were used. The data obtained is provided in Table 3. The radioiodinated tribiotin compound, [¹²⁵I]**21b**, appears to be rapidly cleared from the blood via both renal and

hepato-biliary excretion. Similar tissue distributions were found for the larger biotinylated dendrimers, [¹²⁵I]**22** and [¹²⁵I]**23**. The most striking difference observed in the biodistributions of the three biotinylated dendrimers was that the kidney concentration (or retention) was increased significantly with increasing size and positive charge.

DISCUSSION

A focus of our tumor pretargeting studies has been to develop biotinylated reagents that will increase the amount of radioactivity associated with mAb targeted cancer cells in patients (15, 22). We hypothesized that it may be possible to increase the quantity of radiolabeled molecules attached to cancer cells by using the tetrameric biotin-binding nature of avidin or streptavidin to provide additional binding sites through cross-linking on targeted cells. This concept is depicted in Figure 1. Our initial evaluation of the concept was directed at biotin dimers

and trimers (22). The reason for choosing those compounds was that we believe that when they are used to carry the radionuclide, it will be important to keep the molecular weight low to obtain good tumor penetration and rapid blood clearance, preferably by renal excretion. Although those studies are still ongoing, we have expanded our investigation to include larger biotinylated molecules as well. This expansion in the scope of our studies has been undertaken as concerns have been raised about the multiple injections envisioned in therapeutic protocols with biotin trimers. Thus, optimization of the amount of radioactivity bound to cancer cells may have to be conducted in a single injection. A further consideration in the inclusion of biotinylated dendrimers in our studies came from the fact that the use of SAV as a carrier of the radionuclide is now practical due to our finding that kidney localization of SAV may be significantly decreased (e.g., from 29 to 2% injected dose/gram) by lowering its pI to below 4 (35). This latter point is important as compounds containing multiple biotin moieties could provide an increased number of binding sites for radiolabeled avidin or SAV.

In this investigation, we set out to synthesize a series of polybiotin compounds, determine the number of streptavidin molecules each could bind to a surface, and gain some information on biodistributions of the polybiotinylated compounds. Our initial task was to prepare the targeted polybiotin molecules. After evaluating the many possible approaches to making polybiotin compounds, we were attracted to amine containing Starburst dendrimers as core molecules for the targeted compounds. The SBDs were attractive as they are compact spherical molecules and a variety of sizes are commercially available. It seemed that the spherical shape of the SBDs might accommodate binding of a number of SAV molecules in a compact structure. A schematic representation of biotinylated SBDs is shown in Figure 1, panel II.

A key consideration in the development of biotinylated SBDs was the biotinylation reagent to be used. Although there are several amine reactive biotinylation reagents available from commercial sources, a new biotinylation reagent was designed and synthesized for biotinylation of SBDs. One reason for synthesizing the new biotinylation reagent was that commercially available biotinylation reagents, such as the *N*-hydroxysuccinimidyl (NHS) ester of biotin, **26** (Table 4) (40), or the NHS ester of aminocaproate linked biotin, **27** (41), could be degraded by the action of the serum enzyme biotinidase (42–45). Another consideration was the fact that most commercially available biotinylation reagents have low water solubility,⁵ making their use questionable for the preparation of compounds containing multiple biotin moieties. Although we have previously prepared another water solubilized, biotinidase stabilized amine reactive biotinylation reagent, **28** (46), its molecular weight (i.e., 617 g/mol for each conjugated moiety) made it unattractive for preparation of molecules that contain multiple biotin moieties. The new biotinylation reagent, **9**, has a lower mass (i.e., 445 g/mol for the conjugated moiety) than the previously prepared reagent, **28**, but it is similar in structure as it contains water solubilizing ether functionalities and a *N*-methyl functionality for stabilization toward biotinidase cleavage (32, 34). Synthesis of the new biotinylation reagent was readily accomplished and

biotinylation of the SBDs was facile. Low yields were obtained for the polybiotin compounds as only the fractions that contained relatively narrow peaks on reversed-phase HPLC were retained after purification by column chromatography. We felt that this would provide the most homogeneous biotinylated compounds for study. A method of radioiodinating the BSBDs was also developed so that their *in vivo* distribution could be evaluated. Radioiodination was accomplished by reacting substoichiometric quantities of the amine reactive *p*-[¹²⁵I]-iodobenzoate NHS ester ([¹²⁵I]PIB) prior to reaction with the biotinylation reagent, **9**. The low stoichiometric quantity of [¹²⁵I]PIB was used to ensure that only one iodobenzoate was incorporated in the radiolabeled polybiotin compounds.

Once the BSBDs had been prepared, an assessment of the number of [¹²⁵I]SAV molecules that bound each of the BSBDs was made. To accomplish this, we used an assay in which unlabeled BSBDs were bound with immobilized SAV in polystyrene wells, then radioiodinated SAV was bound to the exposed biotin moieties. In the studies, the picomole quantity of [¹²⁵I]SAV bound after saturating the biotin-binding sites with unlabeled BSBDs was measured. Those values, shown graphically in Figure 2, were obtained directly from the radioactivity measurements and the specific activity of the added [¹²⁵I]-SAV. However, to obtain an estimate of the average number of [¹²⁵I]SAV molecules bound with each BSBD (after one addition), an assumption had to be made. That assumption was that the picomole quantities required for 1 equiv of [¹²⁵I]SAV binding was equal to the amount of [¹²⁵I]SAV bound with the biotin tetramer, **11**. Our rationale for using this assumption was that the value obtained (3.6 ± 0.2 pmol) was very close to that previously obtained for biotin trimers (22) in the same assay system, and it seems most likely that **11** would bind with 1 molecule of r-[¹²⁵I]SAV after binding with immobilized SAV. If one uses the 3.6 pmol value for each equivalent of r-[¹²⁵I]SAV bound, the number of equivalents of [¹²⁵I]-SAV bound with the BSBDs can be readily estimated. By definition, BSBD **11** ($G = 0$), which has four biotin moieties, bound with 1 equiv of r-[¹²⁵I]SAV and 1 equiv of polymer bound SAV. In the BSBD **13** ($G = 1$), the number of biotin moieties is 8. If two of the biotin moieties on **13** are bound to the immobilized SAV, it might be estimated that three additional r-[¹²⁵I]SAV molecules could bind (assuming two biotin moieties bound each SAV molecule). Instead, a doubling was seen, binding 2 equiv of [¹²⁵I]SAV. Similarly, with the BSBD **15**, which has 16 total biotin moieties, 4 equiv of [¹²⁵I]SAV bound in addition to binding with SAV on the plate. No additional increase in the number of [¹²⁵I]SAV molecules bound was found with BSBD **17** (32 biotin moieties) or BSBD **19** (up to 64 biotin moieties when perbiotinylation).

The smaller number of r-[¹²⁵I]SAV molecules bound to the SAV-coated plates than had been predicted, based on the number of biotin moieties (pairs) available, might be a result of steric hindrance to addition of more SAV molecules about the SAV-coated BSBDs. This same explanation may account for the lack of increased binding observed for **17** and **19** over that observed for the smaller BSBDs. However, one cannot rule out the possibility that, as the BSBD molecules get larger, fewer of them bind with the immobilized SAV. Fewer molecules of the larger BSBDs might bind if (1) more than one immobilized SAV bound with each BSBD molecule and/or (2) if steric hindrance from the bound BSBD molecules precluded binding of more [¹²⁵I]SAV molecules. Another consideration is that after reaching a certain size, more

⁵ Sulfo-*N*-hydroxysuccinimidyl esters are sometimes used to provide water soluble biotinylation reagents, but after loss of sulfosuccinimide in the conjugation reaction, the biotin moiety has a high lipophilicity.

than two biotin moieties might bind with each SAV molecule. Through computer modeling,⁶ we have estimated that it would take a linker of >60 Å length (biotin carbonyl carbon to second biotin carbonyl carbon) to bind at opposite faces of SAV. The estimated through bond distances between any two biotin moieties in the SBDs $G = 1-4$ (Table 1) appear to be adequate for more than two biotin moieties to bind with a single SAV molecule. Irrespective of the explanation for the results obtained, there appears to be no advantages for our application to use BSBDs larger than generation 2.

Even though a smaller number of SAV molecules were attached to the immobilized BSBDs than might be predicted, substantial amplification of the amount of radioactivity bound to the SAV-coated plates was obtained. We have previously shown that in a single addition, cross-linking with biotin trimers in the same assay system resulted in 4–6 pmol of [¹²⁵I]SAV bound/well. Further, it was demonstrated that a doubling of that quantity (10–12 pmol) of [¹²⁵I]SAV was obtained in four alternating cycles of reagent additions (22). Those results were reproduced in this study with biotin trimer **29** and iodobenzoyl-biotin trimer **21b** (Figure 2). However, much larger quantities (16–30 pmol) of [¹²⁵I]SAV were bound when biotinylated SBDs **15**, **17**, and **19** were used. Indeed, with one addition of the biotin multimers **15**, **17**, or **19**, there was a 300% increase of [¹²⁵I]SAV bound over that of a single addition of the biotin trimers **29** or **21b**, and a 40+% greater quantity bound than obtained previously with four alternating administrations of the biotin trimers. The *in vitro* results support the potential for amplification of tumor targeting of radioactivity using the biotin multimers and radiolabeled SAV.

As our ultimate goal is to use the BSBDs for *in vivo* application to tumor pretargeting of radionuclides for cancer therapy, it is important to evaluate the *in vivo* distribution and pharmacokinetics of the BSBDs. We have previously shown that a variety of biotin trimers cross-linked SAV in the coated wells (22), but we had not conducted *in vivo* distribution studies with the biotin trimers. As we are interested in the development of radiolabeled biotin trimers as carriers of radionuclides, [¹²⁵I]**21b** was included in the *in vivo* evaluation. The BSBD [¹²⁵I]**23**, which has 15 biotin moieties, appeared to be the best candidate for *in vivo* amplification of binding sites, but the intermediate size of BSBD [¹²⁵I]**22** made its *in vivo* distribution of interest as well. Although it is envisioned that the perbiotinylated SBDs $G = 1$ or 2 will not be radiolabeled for *in vivo* use, it was necessary to radiolabel them to assess their biodistribution. To utilize the data obtained, it must be assumed that the radioiodinated versions of the biotinylated dendrimers, [¹²⁵I]**22** and [¹²⁵I]**23**, will have distribution similar to the perbiotinylated SBDs **13** and **15** (respectively).

In this preliminary investigation, we felt that it would be adequate to obtain comparative information about the tissue distribution of the three BSBDs ([¹²⁵I]**21b**, [¹²⁵I]**22**, and [¹²⁵I]**23**) in athymic mice⁷ at one time point (i.e., 4 h postinjection). The limited biodistribution study was designed to determine if additional, more detailed dis-

tributions were warranted. The results of the investigation are shown in Table 3. Interestingly, the concentrations of radiolabeled BSBDs in blood at 4 h postinjection are nearly the same. Differences in distribution between radiolabeled BSBDs are minor except for the kidney and liver concentrations. In previous distributions of radioiodinated biotin monomers in mice, blood clearance was similar at 4 h to that found for [¹²⁵I]**21b**, but quite high intestine concentrations were observed (unreported results). In this investigation, intestinal concentration of radioactivity was not particularly high for the biotin trimer [¹²⁵I]**21b**, but the kidney concentration is troubling. The high kidney concentration is problematic for a carrier of radioactivity, so chemical modification of the biotin trimer is being investigated to make it pass through the kidney more rapidly. Contrary to the biotin trimer, the high concentrations of [¹²⁵I]**22** and [¹²⁵I]**23** in the kidney and liver at 4 h postinjection may not present a problem for application to cross-linking SAV on cancer cells, as they will not be used as carriers of radionuclides.

In Summary. In this study, Starburst dendrimers, **10** ($G = 0$; four amines), **12** ($G = 1$; 8 amines), **14** ($G = 2$; 16 amines), **16** ($G = 3$; 32 amines), and **18** ($G = 4$; 64 amines), were reacted with a new water solubilized, biotinidase stabilized biotinylation reagent, **9**, to prepare polybiotinylated dendrimers for *in vivo* application. Data from an *in vitro* binding assay indicates that the biotinylated Starburst dendrimer **11**, which has four biotin moieties, binds one ¹²⁵I-labeled SAV molecule in addition to a SAV molecule bound to the polystyrene well. Biotinylated Starburst dendrimer **13**, which has eight biotin moieties, binds two additional [¹²⁵I]SAV molecules, and **15**, which has 16 biotin moieties, binds four additional [¹²⁵I]SAV molecules. Larger biotinylated Starburst dendrimers **17** and **19** were similar to **15** in that they only bound four additional SAV molecules. Biodistributions of radioiodinated polybiotin dendrimers in mice indicated that they were cleared from blood by 4 h, but that relatively high kidney and liver concentrations were noted at that time. A substantial increase in kidney localization or retention was noted as the polybiotin compounds increased in size and charge. The results presented herein suggest that further investigation of the concept of amplifying the amount of radiolabeled SAV that will bind to a tumor through the use of BSBDs is warranted. Animal studies to investigate this are planned.

ACKNOWLEDGMENT

We thank Dr. Ross Lawrence (Department of Medicinal Chemistry, University of Washington) and Santosh Kumar (Department of Biochemistry, University of Washington) for efforts in obtaining mass spectral data. We thank Dr. Patrick Stayton and Dr. Richard To for providing r-SAV used in the studies, and for their helpful comments on the manuscript. We are grateful for the financial support provided by the Department of Energy, Medical Applications and Biophysical Research Division, Office of Health and Environmental Research under Grant DE-FG06-95ER62029, and the Richard M. Lucas Foundation.

Supporting Information Available: Complete structures for the biotinylated dendrimers are provided along with HPLC chromatograms, NMR spectra, and mass spectra of all new compounds (**4**, **5**, **7**, **9**, **11**, **13**, **15**, **17**, **19**, **21a**, and **21b**). An HPLC chromatogram of biotin derivative **8**, which was not isolated, is also included (41

⁶ Measurements of distances in biotin derivatives were obtained from the computer program ChemDraw3D (Cambridge-Soft Corporation, Cambridge, MA) after structural and energy minimization of fully extended, planar conformations of the compounds.

⁷ Athymic mice (BALB/c nu/nu) were used in this investigation so that the results can be compared with planned studies involving human tumor xenografts in athymic mice.

pages). Ordering information is given on any current masthead page.

LITERATURE CITED

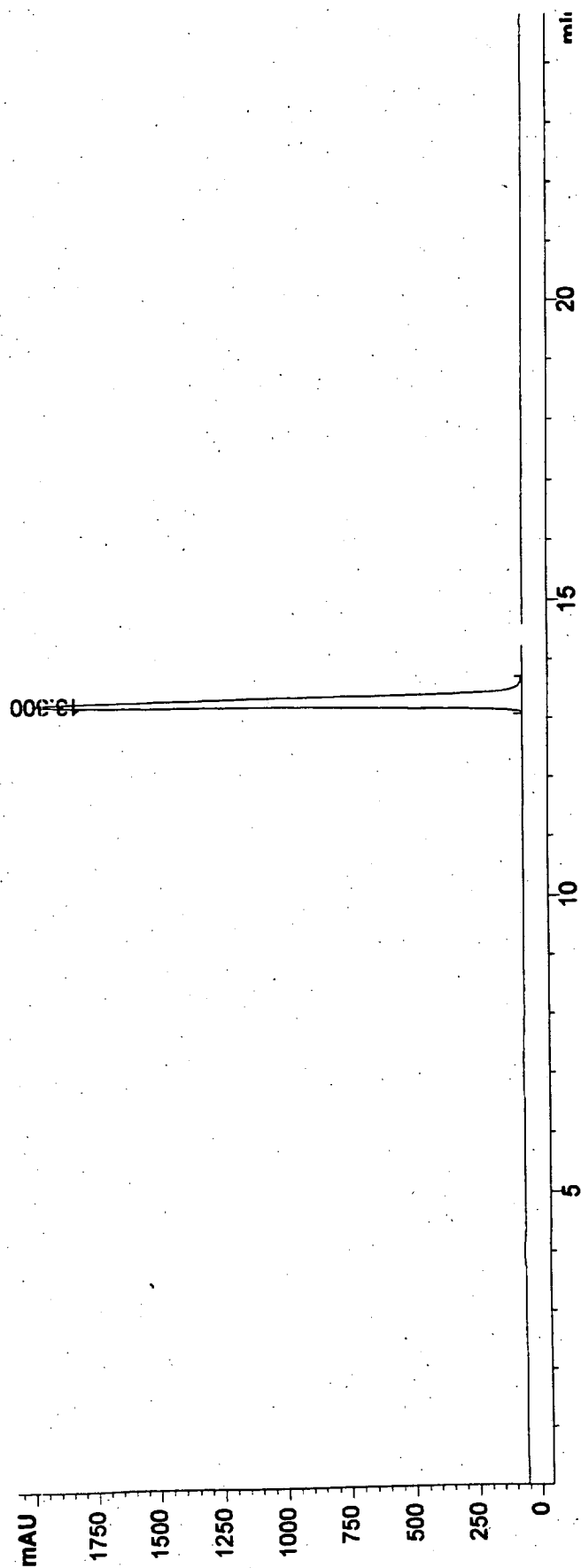
- (1) Goodwin, D. A., Meares, C. F., McTigue, M., and David, G. S. (1986) Monoclonal Antibody Hapten Radiopharmaceutical Delivery. *Nucl. Med. Commun.* 7, 569–580.
- (2) Hnatowich, D. J., Virzi, F., and Rusckowski, M. (1987) Investigations of Avidin and Biotin for Imaging Applications. *J. Nucl. Med.* 28, 1294–1302.
- (3) Paganelli, G., Riva, P., Deleide, G., Clivio, A., Chiolerio, F., Scassellati, G. A., Malcovati, M., and Siccardi, A. G. (1988) In Vivo Labeling of Biotinylated Monoclonal Antibodies by Radioactive Avidin: A Strategy to Increase Tumor Radiolocalization. *Int. J. Cancer* 2, 121–125.
- (4) Goodwin, D. A. (1995) Tumor Pretargeting: Almost the Bottom Line. *J. Nucl. Med.* 36, 876–879.
- (5) Goodwin, D. A., and Meares, C. F. (1997) Pretargeting: General Principles. *Cancer (Suppl.)* 80, 2675–2680.
- (6) Stoldt, H. S., Aftab, F., Chinol, M., Paganelli, G., Luca, F., Testori, A., and Geraghty, J. G. (1997) Pretargeting Strategies for Radio-immunoguided Tumour Localisation and Therapy. *Eur. J. Cancer* 33, 186–192.
- (7) Kassib, A. I., Jones, P. L., Matalka, K. Z., and Adelstein, S. J. (1996) Antibody-Dependent Signal Amplification in Tumor Xenografts after Pretreatment with Biotinylated Monoclonal Antibody and Avidin or Streptavidin. *J. Nucl. Med.* 37, 343–352.
- (8) Paganelli, G., Pervez, S., Siccardi, A. G., Rowlinson, G., Deleide, G., Chiolerio, F., Malcovati, M., Scassellati, G. A., and Epenetos, A. A. (1990) Intraperitoneal Radio-Localization of Tumors Pre-Targeted by Biotinylated Monoclonal Antibodies. *Int. J. Cancer* 45, 1184–1189.
- (9) Kalofonos, H. P., Rusckowski, M., Siebecker, D. A., Sivola-penko, G. B., Snook, D., Lavender, J. P., Epenetos, A. A., and Hnatowich, D. J. (1990) Imaging of Tumor in Patients with Indium-111-Labeled Biotin and Streptavidin-Conjugated Antibodies: Preliminary Communication. *J. Nucl. Med.* 31, 1791–1796.
- (10) del Rosario, R. B., and Wahl, R. L. (1993) Biotinylated Iodo-Polylysine for Pretargeted Radiation Delivery. *J. Nucl. Med.* 34, 1147–1151.
- (11) Khawli, L. A., Alauddin, M. M., Miller, G. K., and Epstein, A. L. (1993) Improved Immunotargeting of Tumors with Biotinylated Monoclonal Antibodies and Radiolabeled Streptavidin. *Antibody, Immunoconjugates, Radiopharm.* 6, 13–27.
- (12) Rowlinson-Busza, G., Hnatowich, D. J., Rusckowski, M., Snook, D., and Epenetos, A. A. (1993) Xenograft Localization Using Pretargeted Streptavidin-conjugated Monoclonal Antibody and ¹¹¹In-Labeled Biotin. *Antibody, Immunoconjugates, Radiopharm.* 6, 97–109.
- (13) Saga, T., Weinstein, J. N., Jeong, J. M., Heya, T., Lee, J. T., Le, N., Paik, C. H., Sung, C., and Neumann, R. D. (1994) Two-Step Targeting of Experimental Lung Metastases with Biotinylated Antibody and Radiolabeled Streptavidin. *Cancer Res.* 54, 2160–2165.
- (14) Ngai, W. M., Reilly, R. M., Polihronis, J., and Shtiz, B. (1995) In Vitro and In Vivo Evaluation of Streptavidin Immunoconjugates of the Second Generation TAG-72 Monoclonal Antibody CC49. *Nucl. Med. Biol.* 22, 77–86.
- (15) Wilbur, D. S., Hamlin, D. K., Vessella, R. L., Stray, J., Buhler, K. R., Stayton, P., Klumb, L., Pathare, P. M., and Weerawarna, S. A. (1996) Antibody Fragments in Tumor Pretargeting. Evaluation of Biotinylated Fab' Co-localization with Recombinant Streptavidin and Avidin. *Bioconjugate Chem.* 7, 689–702.
- (16) Alvarez-Diez, T. M., Polihronis, J., and Reilly, R. M. (1996) Pretargeted Tumour Imaging with Streptavidin Immunoconjugates of Monoclonal Antibody CC49 and ¹¹¹In-DTPA-Biotin. *Nucl. Med. Biol.* 23, 459–466.
- (17) Sharkey, R. M., Karacay, H., Griffiths, G. L., Behr, T. M., Blumenthal, R. D., Mattes, M. J., Hansen, H. J., and Goldenberg, D. M. (1997) Development of a Streptavidin-Anti-Carcinoembryonic Antigen Antibody, Radiolabeled Biotin Pretargeting Method for Radioimmunotherapy of Colon Cancer. Studies in a Human Colon Cancer Xenograft Model. *Bioconjugate Chem.* 8, 595–604.
- (18) Green, N. M. (1963) Avidin. *Biochem. J.* 89, 609–620.
- (19) Green, N. M. (1975) Avidin. *Adv. Protein Chem.* 29, 85–133.
- (20) Green, N. M. (1990) Avidin and Streptavidin. *Methods Enzymol.* 184, 51–67.
- (21) Paganelli, G., Malcovati, M., and Fazio, F. (1991) Monoclonal Antibody pretargeting techniques for tumour localization: the avidin-biotin system. *Nucl. Med. Commun.* 12, 211–234.
- (22) Wilbur, D. S., Pathare, P. M., Hamlin, D. K., and Weerawarna, S. A. (1997) Biotin Reagents for Antibody Pretargeting. 2. Synthesis and in Vitro Evaluation of Biotin Dimers and Trimers for Cross-Linking of Streptavidin. *Bioconjugate Chem.* 8, 819–832.
- (23) Klivanov, A. L., Martynov, A. V., Slinkin, M. A., Sakharov, I. Y., Smirnov, M. D., Muzykantov, V. R., Danilov, S. M., and Torchilin, V. P. (1988) Blood Clearance of Radiolabeled Antibody: Enhancement by Lactosamination and Treatment with Biotin-Avidin or Anti-Mouse IgG Antibodies. *J. Nucl. Med.* 29, 1951–1956.
- (24) Sinitsyn, V. V., Mamontova, A. G., Checkneva, Y. Y. S., Alexander, A., and Domogatsky, S. P. (1989) Rapid Blood Clearance of Biotinylated IgG After Infusion of Avidin. *J. Nucl. Med.* 30, 66–69.
- (25) Marshall, D., Pedley, R. B., Boden, J. A., Boden, R., and Begent, R. H. J. (1994) Clearance of Circulating Radio-Antibodies Using Streptavidin or Second Antibodies in a Xenograft Model. *Br. J. Cancer* 69, 502–507.
- (26) Kobayashi, H., Sakahara, H., Hosono, M., Yao, Z.-S., Toyama, S., Endo, K., and Konishi, J. (1994) Improved Clearance of Radiolabeled Biotinylated Monoclonal Antibody Following the Infusion of Avidin as a "Chase" without Decreased Accumulation in the Target Tumor. *J. Nucl. Med.* 35, 1677–1684.
- (27) Marshall, D., Pedley, R. B., Melton, R. G., Boden, J. A., Boden, R., and Begent, R. H. J. (1995) Galactosylated Streptavidin for Improved Clearance of Biotinylated Intact and F(ab')₂ Fragments of an Anti-Tumour Antibody. *Br. J. Cancer* 71, 18–24.
- (28) Rosebrough, S. F., and Hashmi, M. (1996) Galactose-Modified Streptavidin-GC4 Antifibrin Monoclonal Antibody Conjugates: Application for Two-Step Thrombus/Embolus Imaging. *J. Pharmacol. Exp. Ther.* 276, 770–775.
- (29) Tomalia, D. A., Naylor, A. M., and Goddard, W. A., III (1990) Starburst Dendrimers: Molecular-Level Control of Size, Shape, Surface Chemistry, Topology, and Flexibility from Atoms to Macroscopic Matter. *Angew. Chem., Int. Ed. Engl.* 29, 138–175.
- (30) Tomalia, D. A. (1993) Starburst™/Cascade Dendrimers: Fundamental Building Blocks for a New Nanoscopic Chemistry Set. *Aldrichim. Acta* 26, 91–101.
- (31) Tomalia, D. A., and Durst, H. D. (1993) Genealogically Directed Synthesis: Starburst/Cascade Dendrimers and Hyperbranched Structures. *Topics Curr. Chem.* 165, 193–313.
- (32) Wilbur, D. S., Hamlin, D. K., Pathare, P. M., and Weerawarna, S. A. (1997) Biotin Reagents for Antibody Pretargeting. Synthesis, Radioiodination and In Vitro Evaluation of Water Soluble, Biotinidase Resistant Biotin Derivatives. *Bioconjugate Chem.* 8, 572–584.
- (33) Rosebrough, S. F. (1993) Plasma Stability and Pharmacokinetics of Radiolabeled Deferoxamine-Biotin Derivatives. *J. Pharmacol. Exp. Ther.* 265, 408–415.
- (34) Axworthy, D. B., Theodore, L. J., Gustavson, L. M., and Reno, J. M. (1997) Biotinidase-Resistant Biotin-DOTA Conjugates. United States Patent Number 5,608,060.
- (35) Wilbur, D. S., Hamlin, D. K., Buhler, K. R., Pathare, P. M., Vessella, R. L., Stayton, P. S., and To, R. (1998) Streptavidin in Antibody Pretargeting. 2. Evaluation of Methods to Decrease Localization of Streptavidin to Kidney while Retaining its Tumor Binding Capacity. *Bioconjugate Chem.* 9, 322–330.
- (36) Wilbur, D. S., Hadley, S. W., Hylarides, M. D., Abrams, P. G., Beaumier, P. A., Morgan, A. C., Reno, J. M., and Fritzberg, A. R. (1989) Development of a Stable Radioiodinating Reagent

- to Label Monoclonal Antibodies for Radiotherapy of Cancer. *J. Nucl. Med.* 30, 216–226.
- (37) Seitz, O., and Kunz, H. (1997) HYCRON, an Allylic Anchor for High-Efficiency Solid-Phase Synthesis of Protected Peptides and Glycopeptides. *J. Org. Chem.* 62, 813–826.
- (38) Wilbur, D. S., Stayton, P. S., To, R., Buhler, K. R., Klumb, L. A., Hamlin, D. K., Stray, J. E., and Vessella, R. L. (1998) Streptavidin in Antibody Pretargeting. Comparison of a Recombinant Streptavidin with Two Streptavidin Mutant Proteins and Two Commercially Available Streptavidin Proteins. *Bioconjugate Chem.* 9, 100–107.
- (39) Fritzberg, A. R., Beaumier, P. L., Bottino, B. J., and Reno, J. M. (1994) Approaches to improved antibody- and peptide-mediated targeting for imaging and therapy of cancer. *J. Controlled Release* 28, 167–173.
- (40) Wilchek, M., and Bayer, E. A. (1990) Biotin-Containing Reagents. *Methods Enzymol.* 184, 123–138.
- (41) Bayer, E. A., and Wilchek, M. (1990) Protein Biotinylation. *Methods Enzymol.* 184, 138–161.
- (42) Hymes, J., Fleischhauer, K., and Wolf, B. (1997) Biotinidase in Serum and Tissues. *Methods Enzymol.* 279, 422–434.
- (43) Pispas, J. (1965) Animal Biotinidase. *Ann. Med. Exp. Biol. Fenniae* 43, 3–40.
- (44) Wolf, B., Hymes, J., and Heard, G. S. (1990) Biotinidase. *Methods Enzymol.* 184, 103–111.
- (45) Chauhan, J., Ebrahim, H., Bhullar, R. P., and Dakshinamurti, K. (1985) Human Serum Biotinidase. *Ann. N. Y. Acad. Sci.* 447, 386–388.
- (46) Wilbur, D. S., Pathare, P. M., Hamlin, D. K., Stayton, P. S., To, R., Klumb, L., Buhler, K. R., and Vessella, R. L. (1998) Development of New Reagents for Pretargeting. *Genet. Anal.: Biomol. Eng.* (submitted for publication).

BC980055E

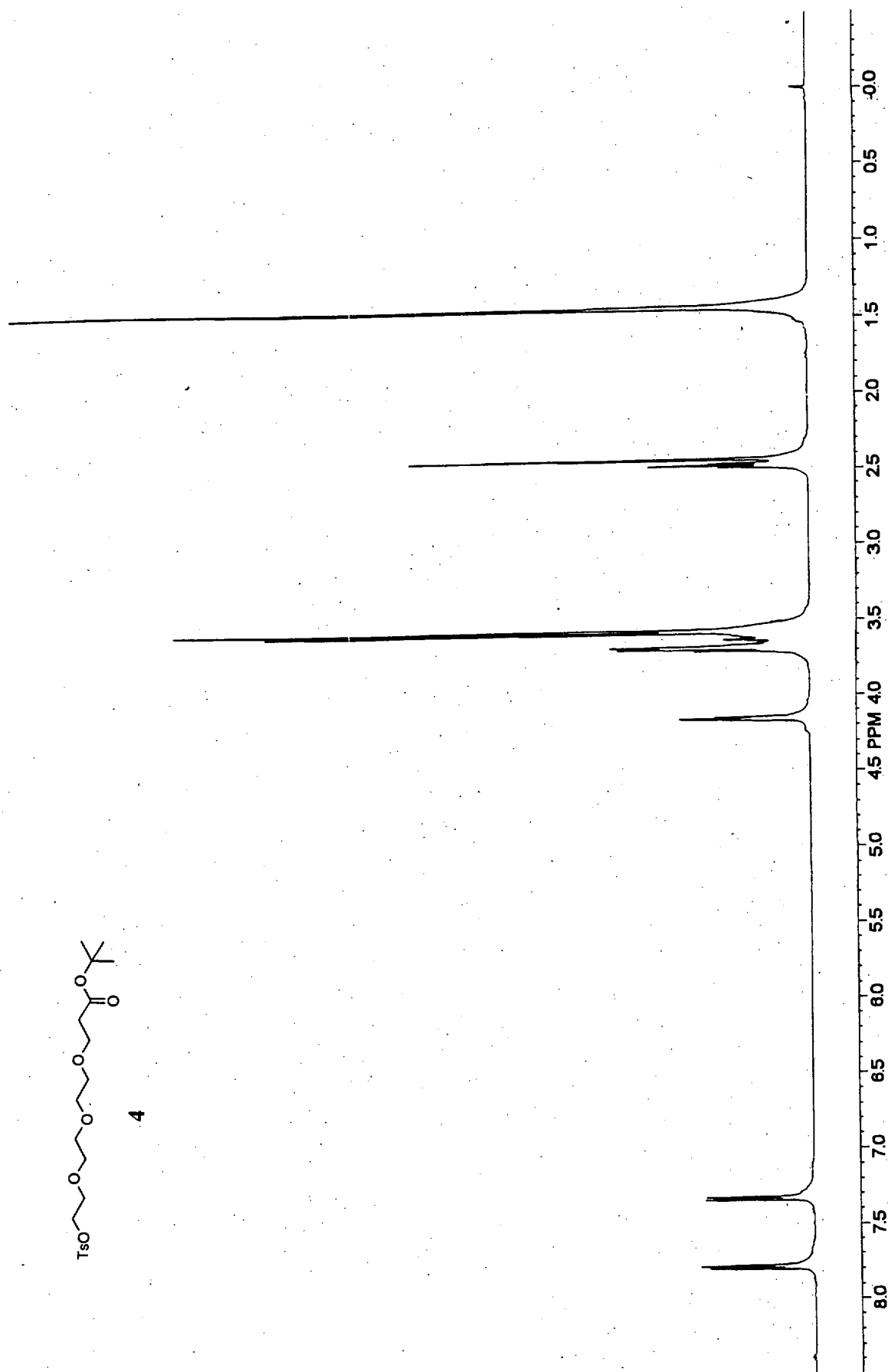


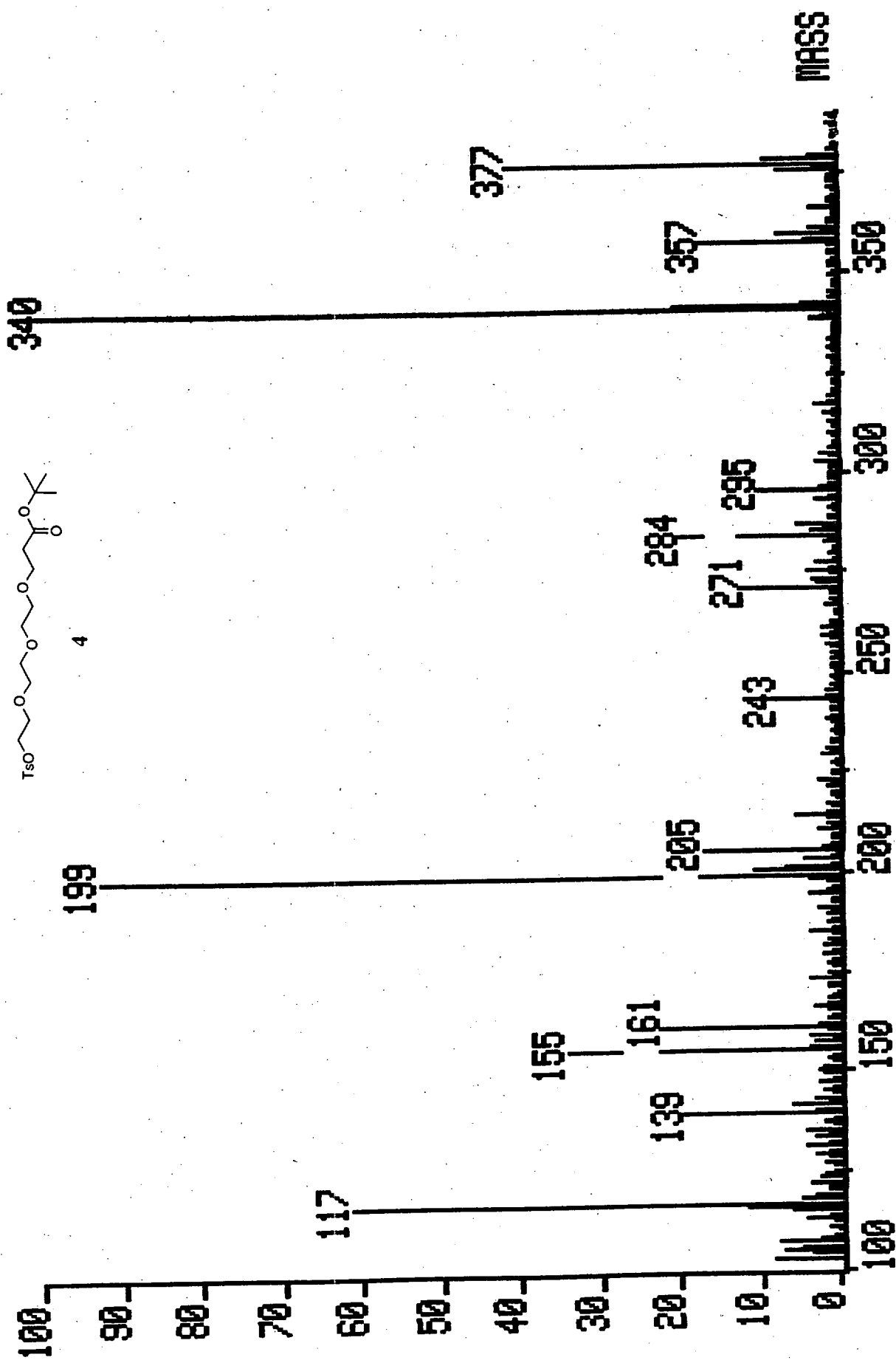
4

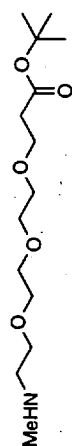




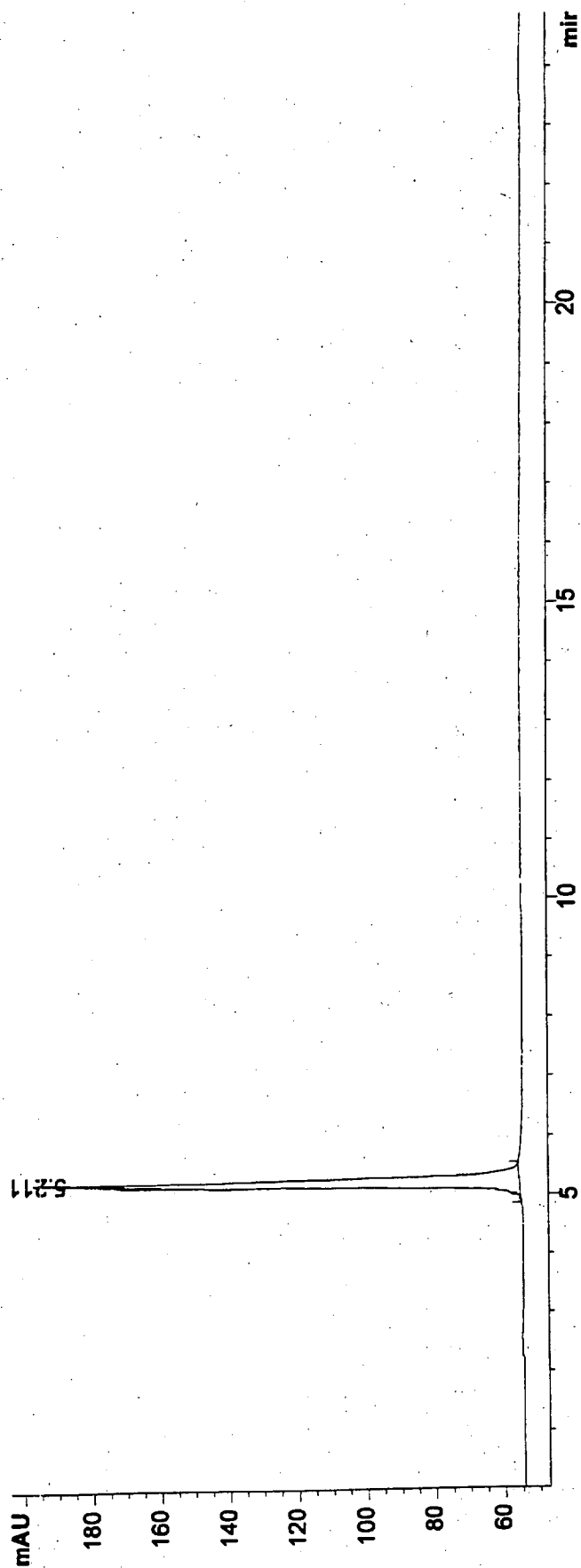
4

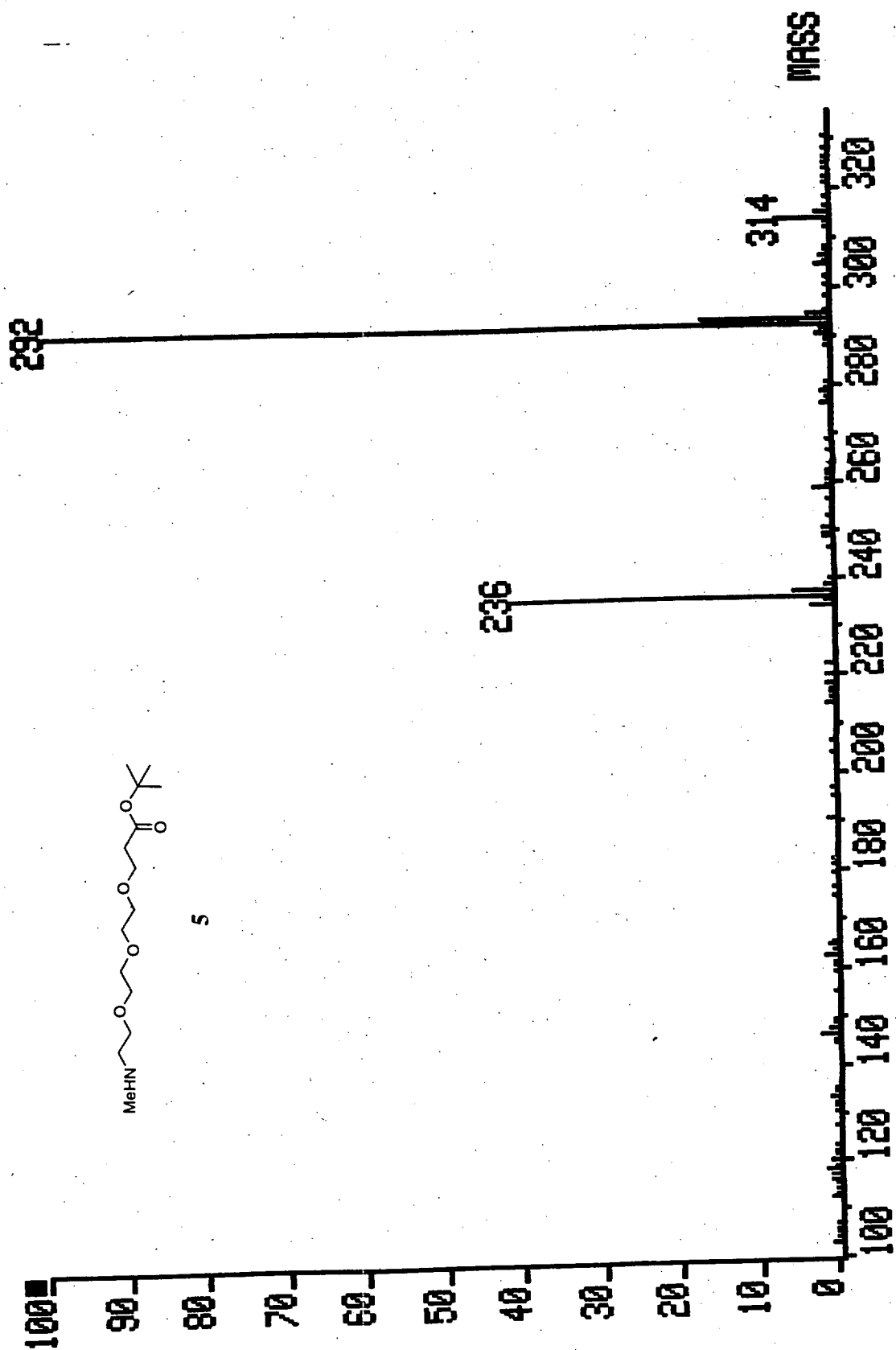


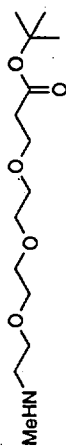




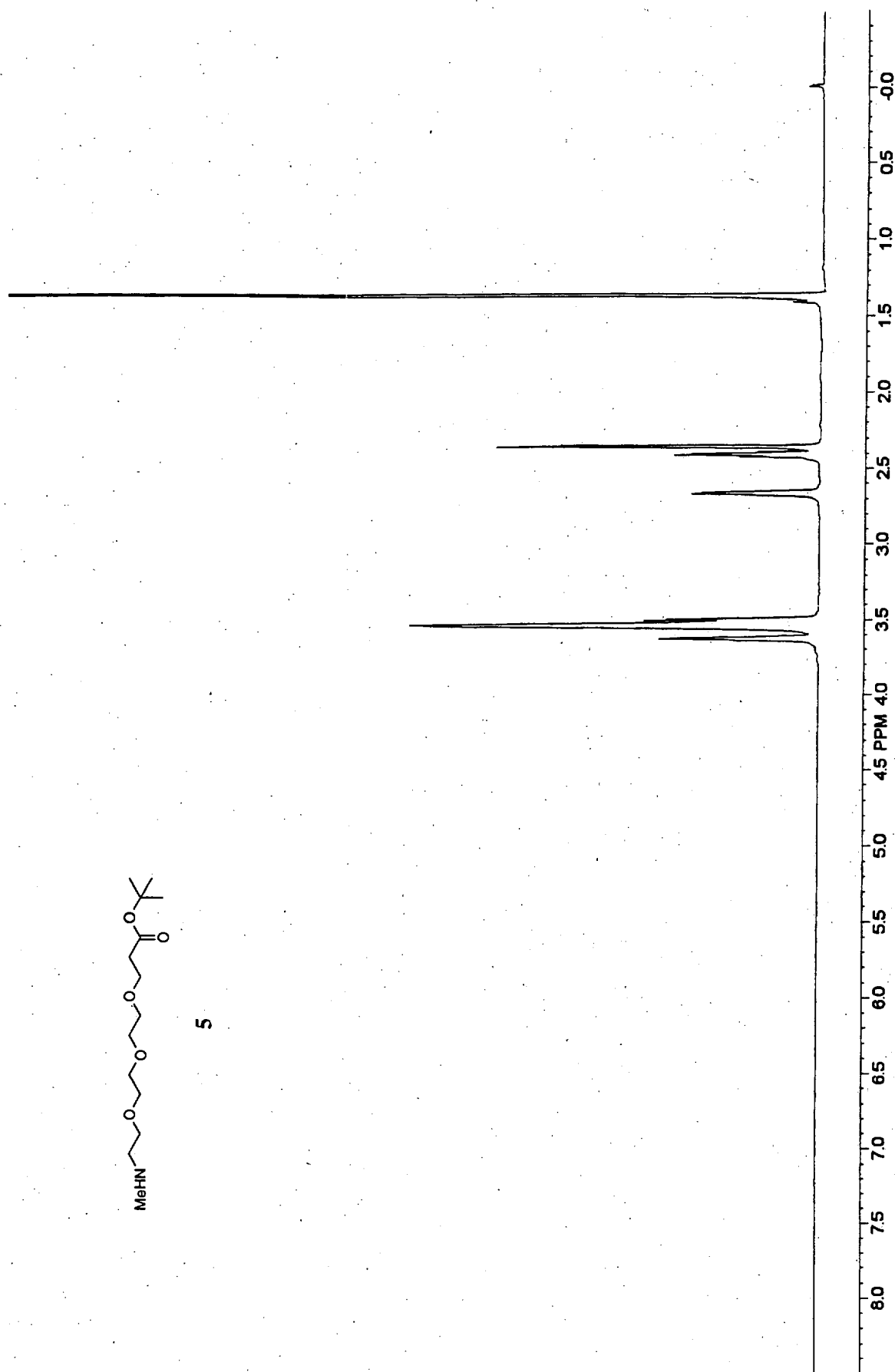
5

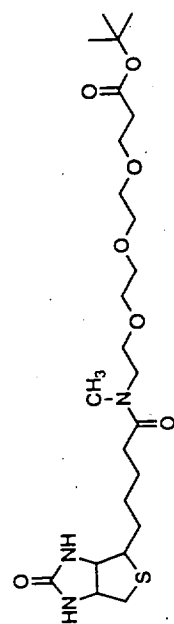




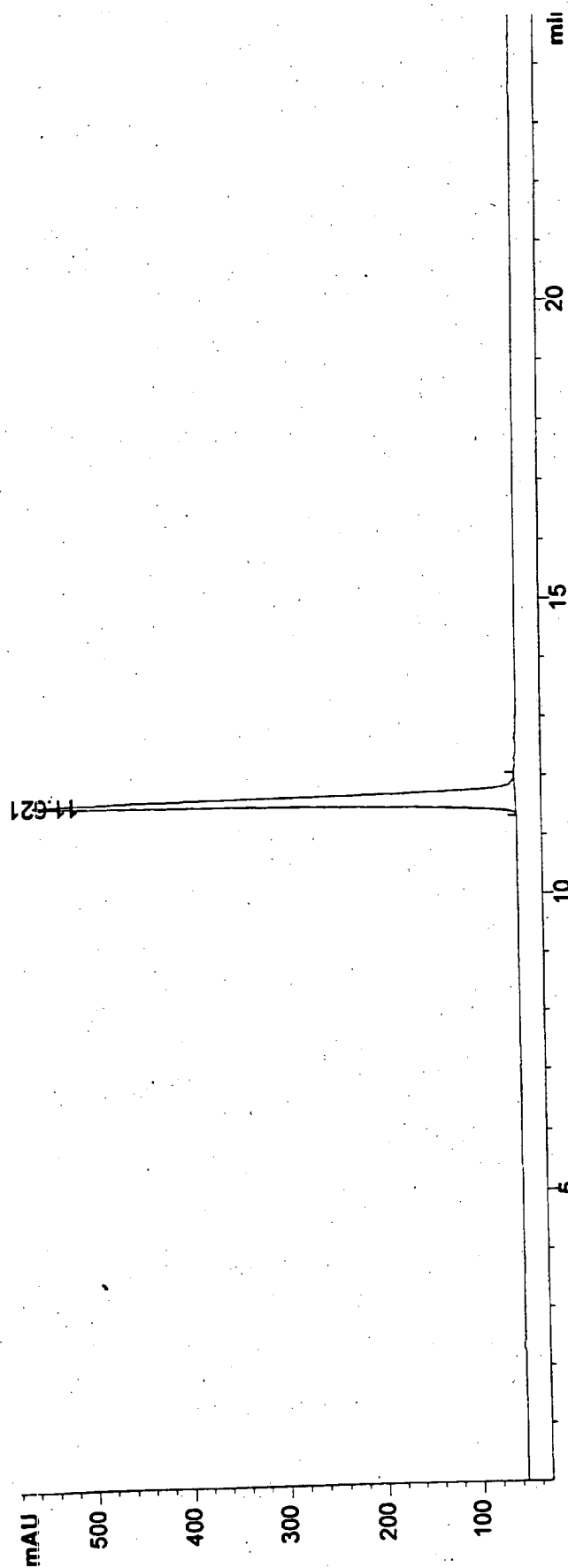


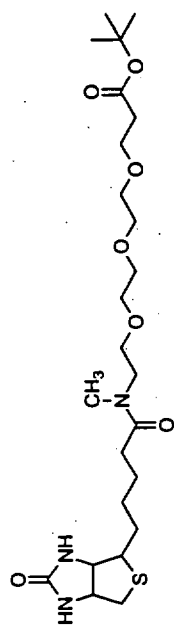
5



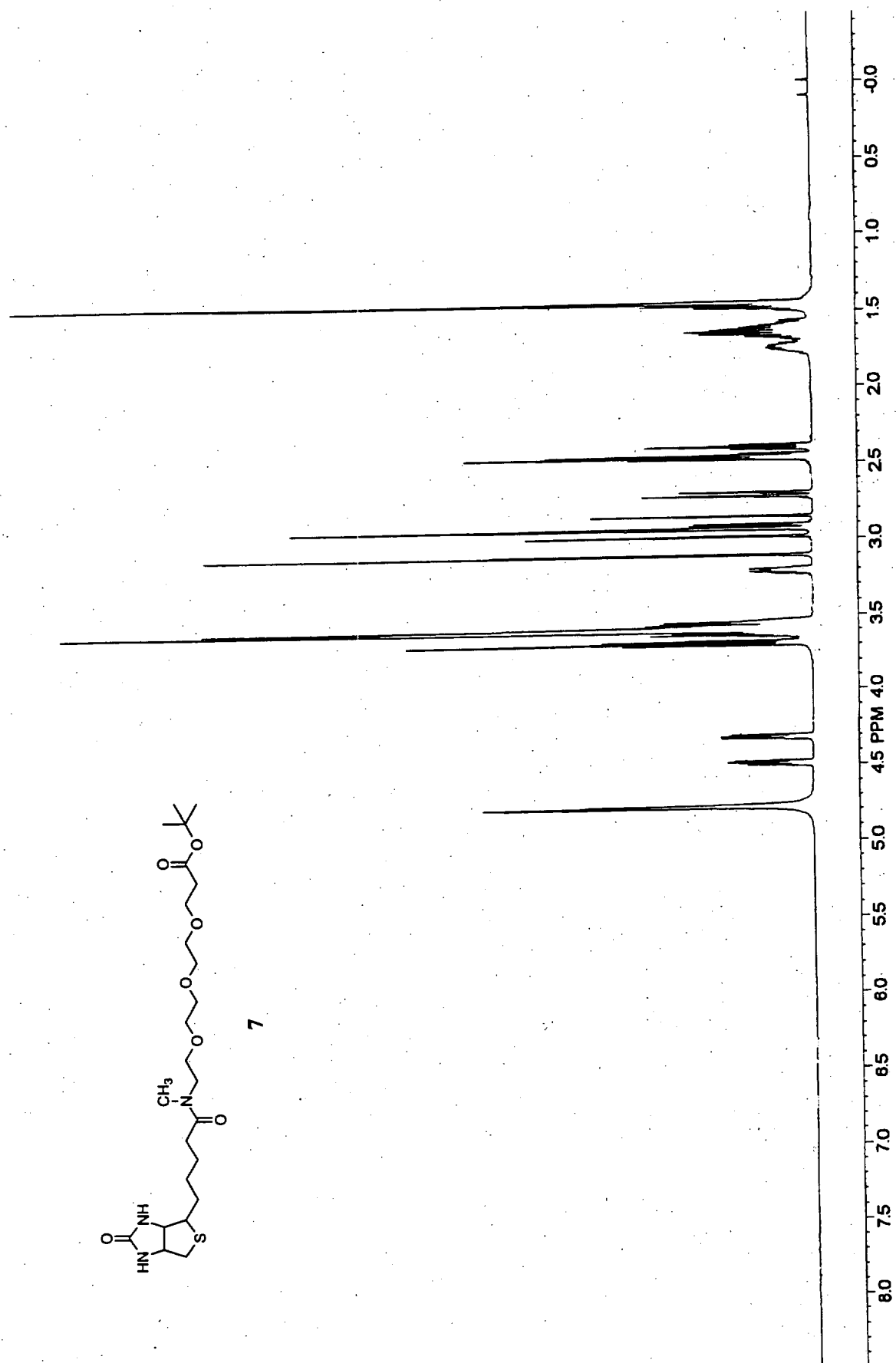


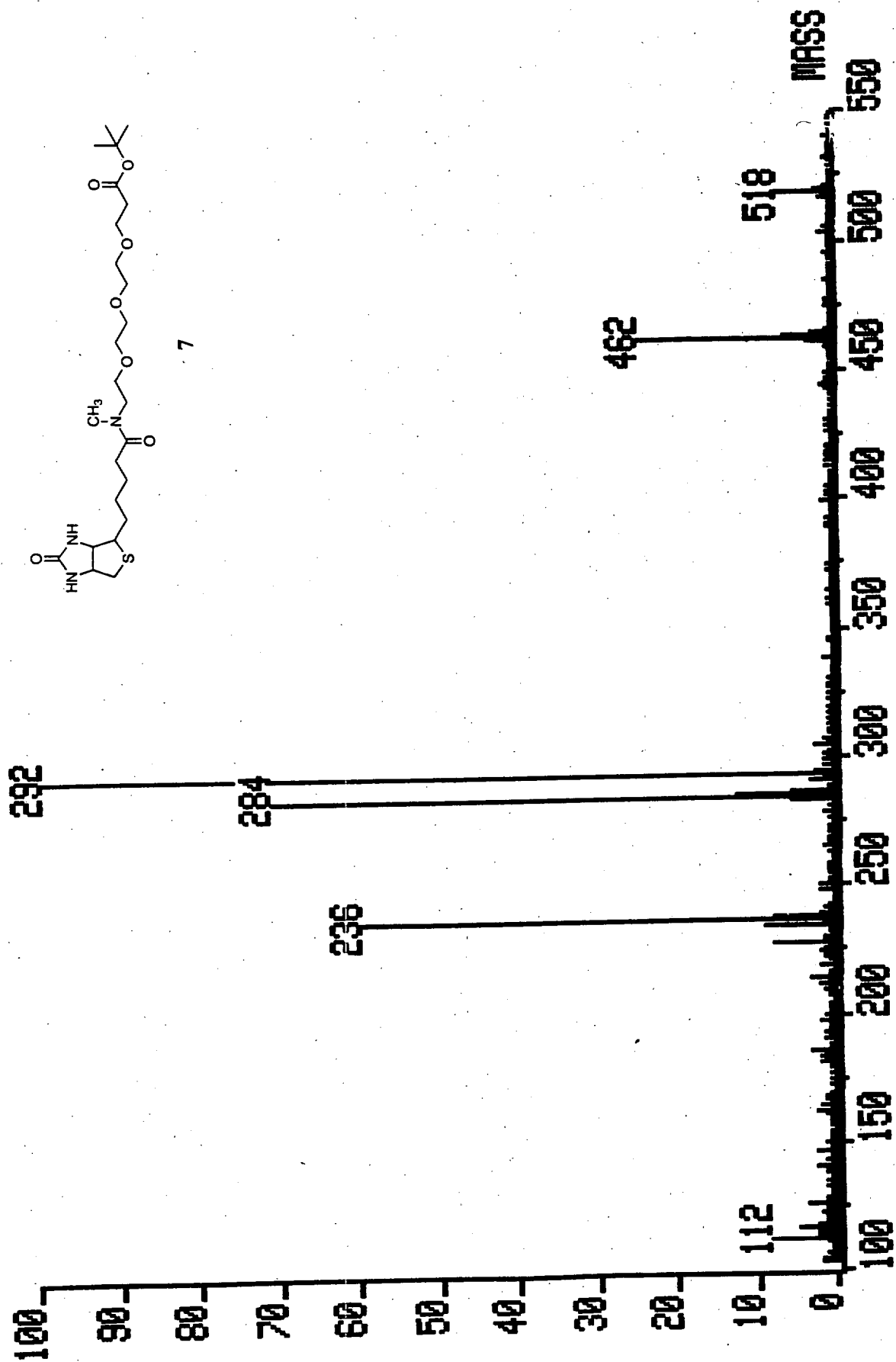
7



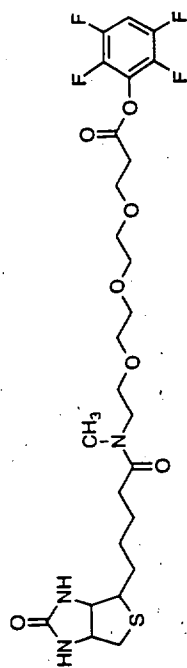


7

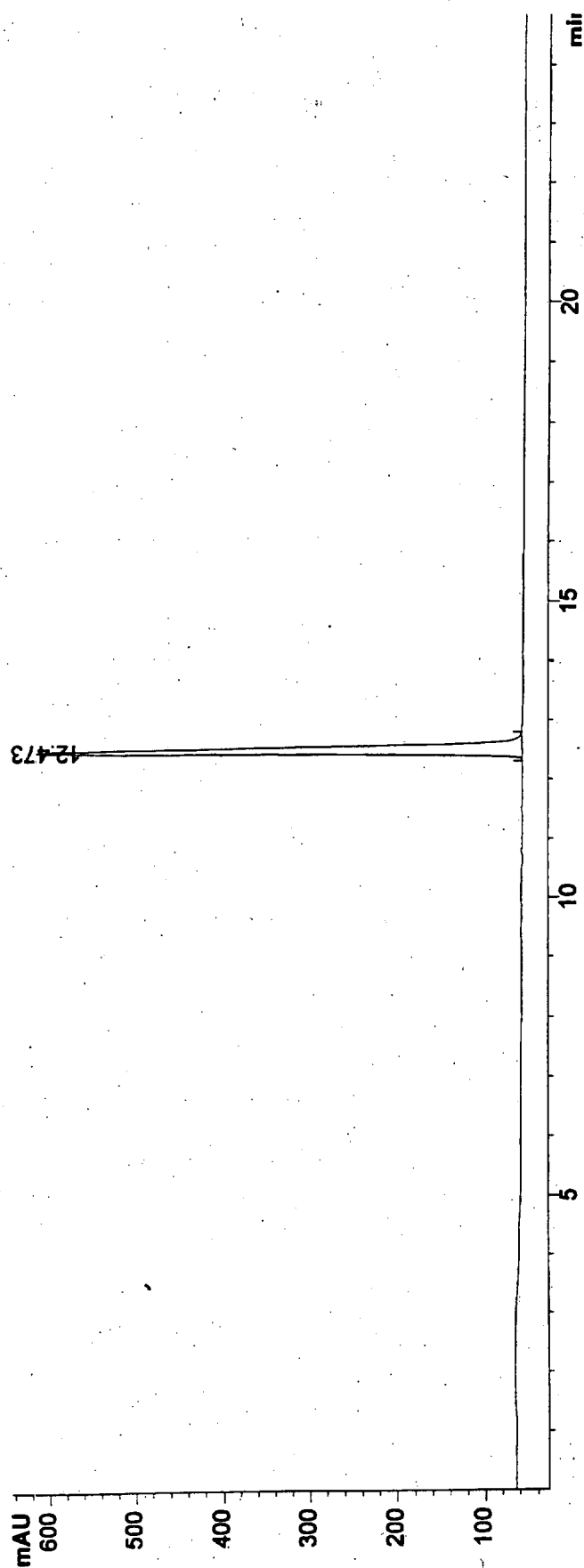


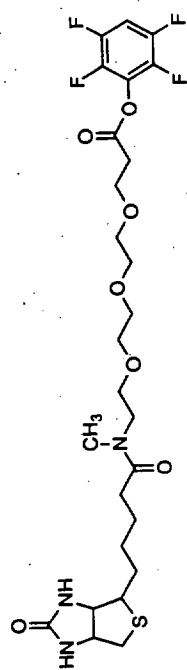


The chromatogram displays a single, sharp, and prominent peak at a retention time of 8.418 minutes. The y-axis represents the detector response in mAU (milliabsorbance units), ranging from 0 to 250. The x-axis represents the retention time in minutes (min), ranging from 0 to 25. The peak is well-resolved and reaches a maximum height of approximately 250 mAU.

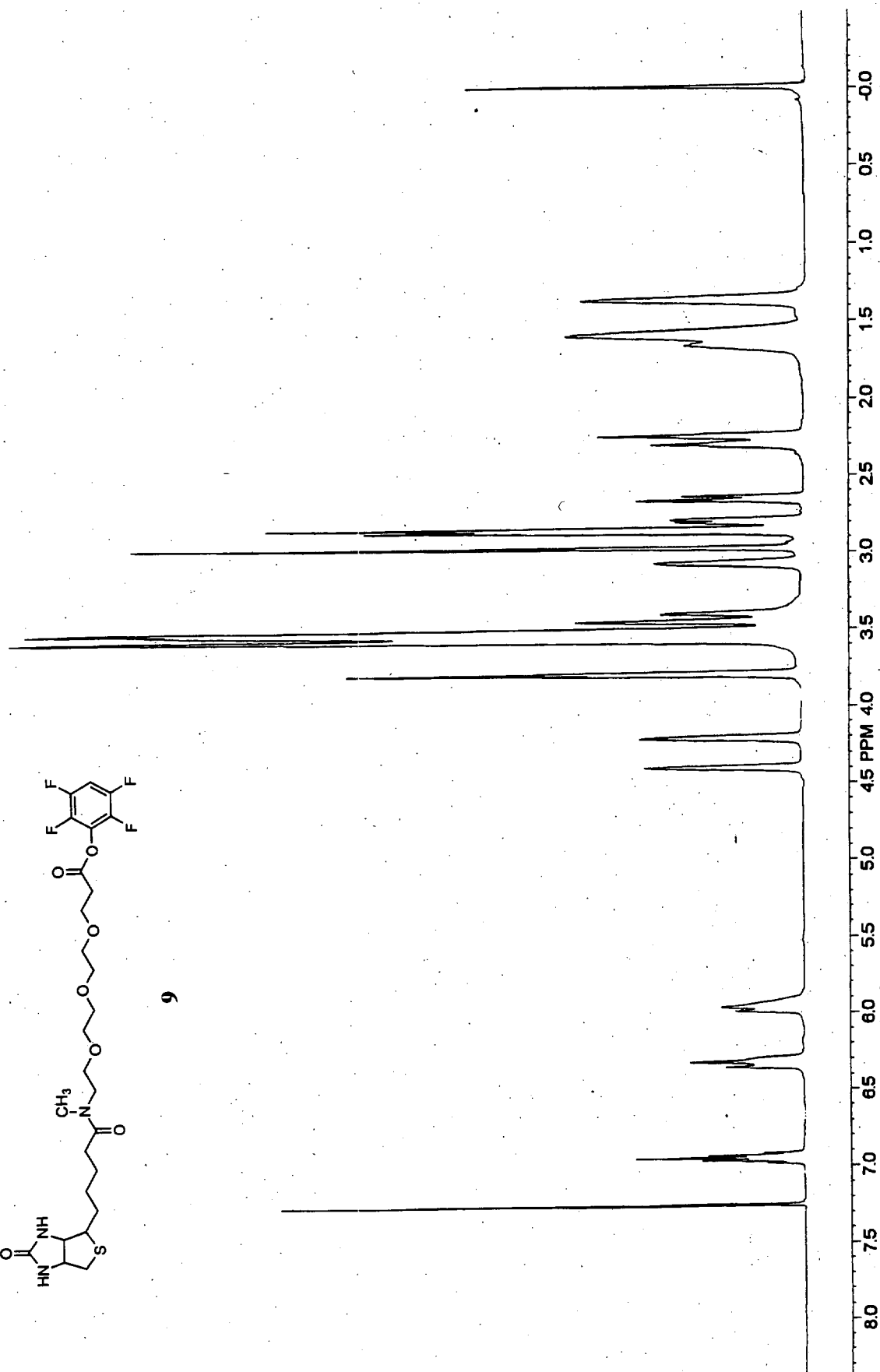


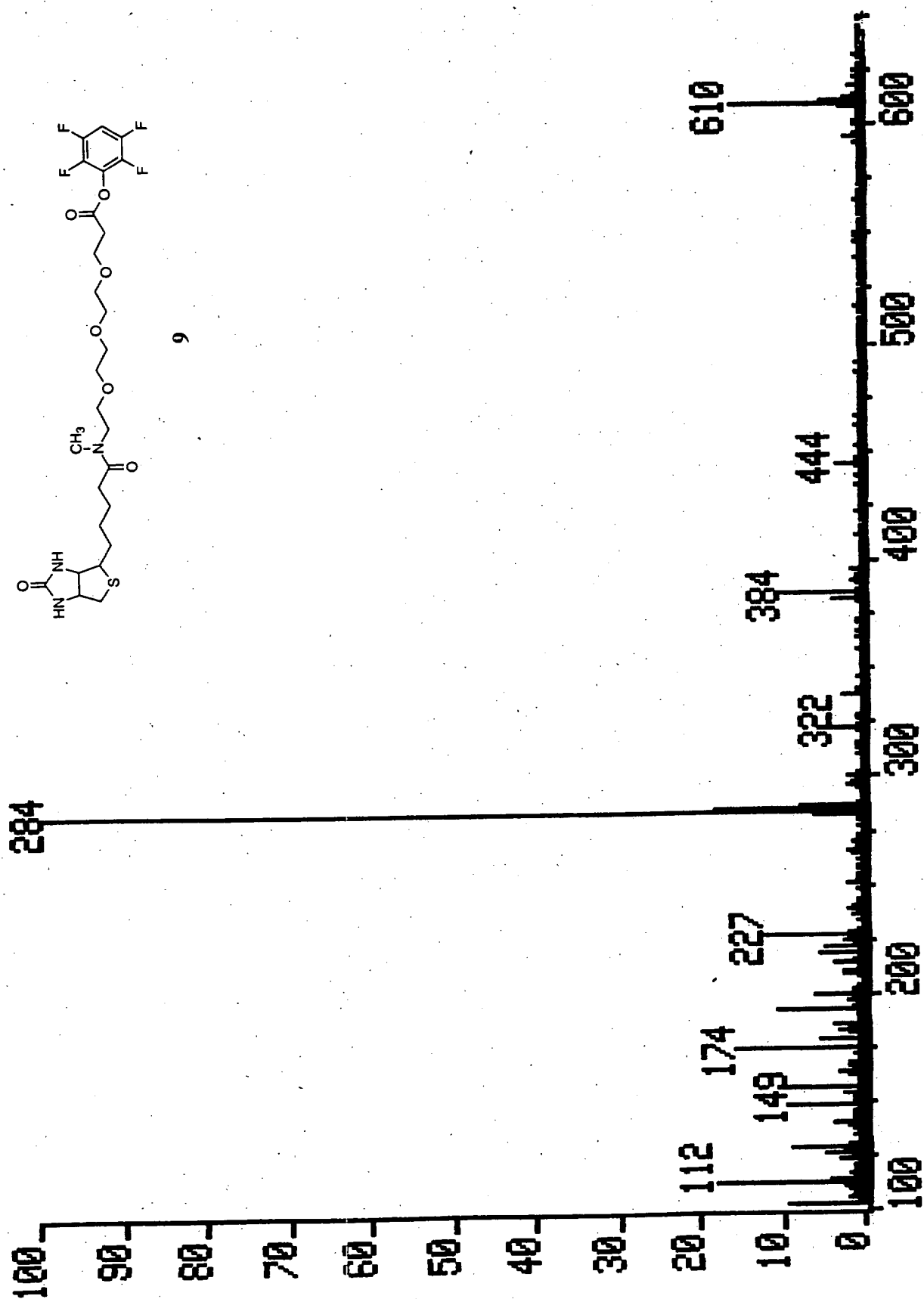
9

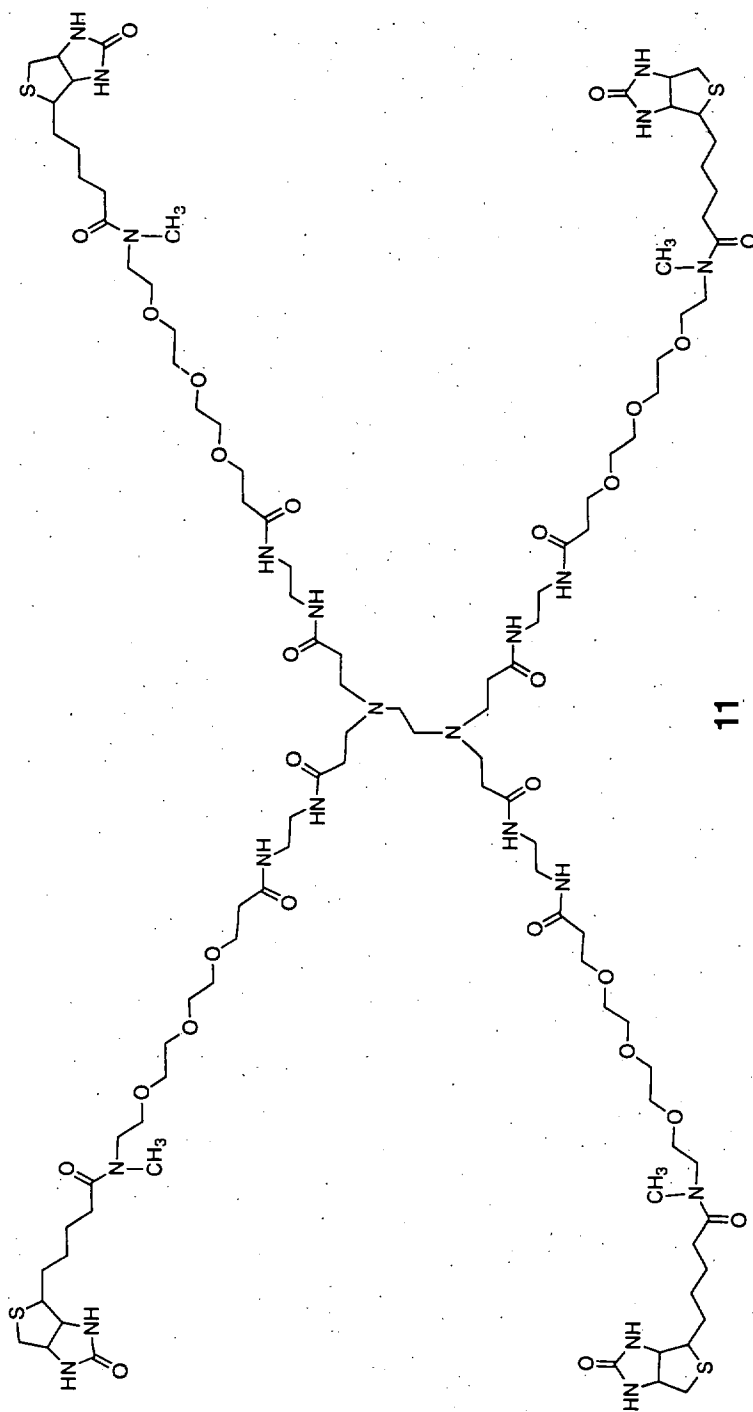




9

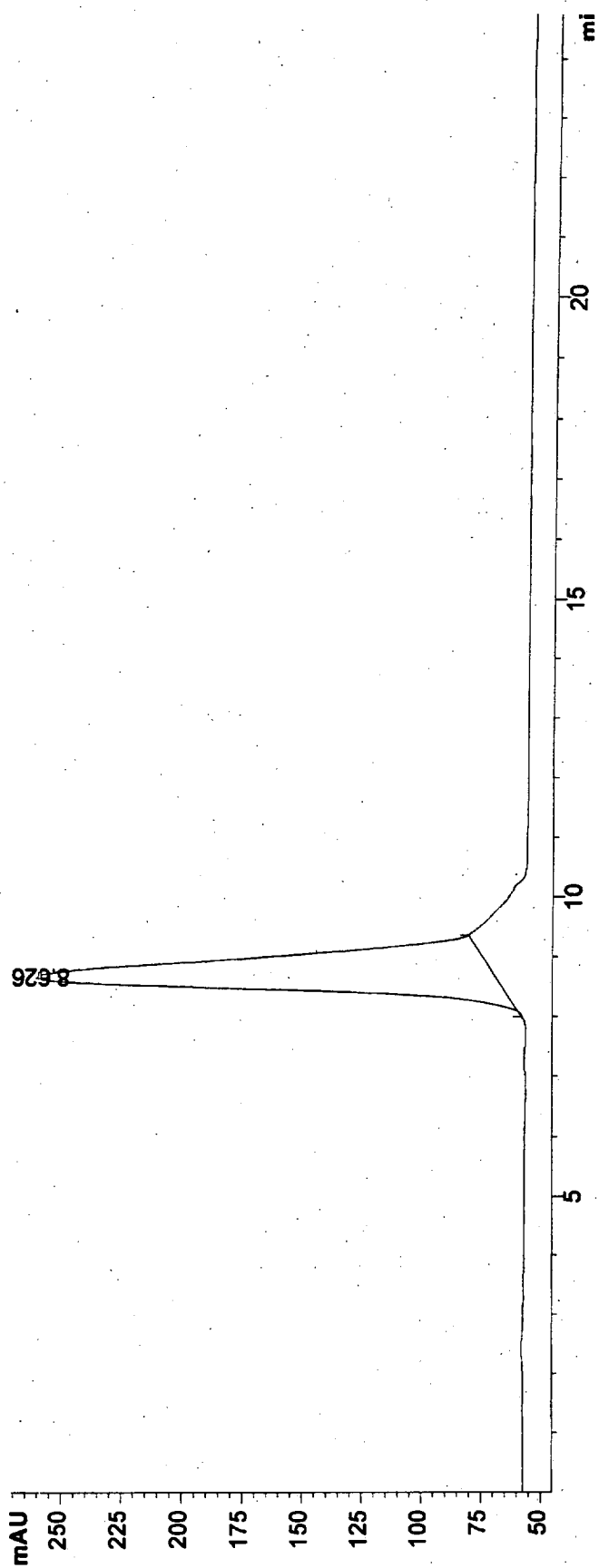


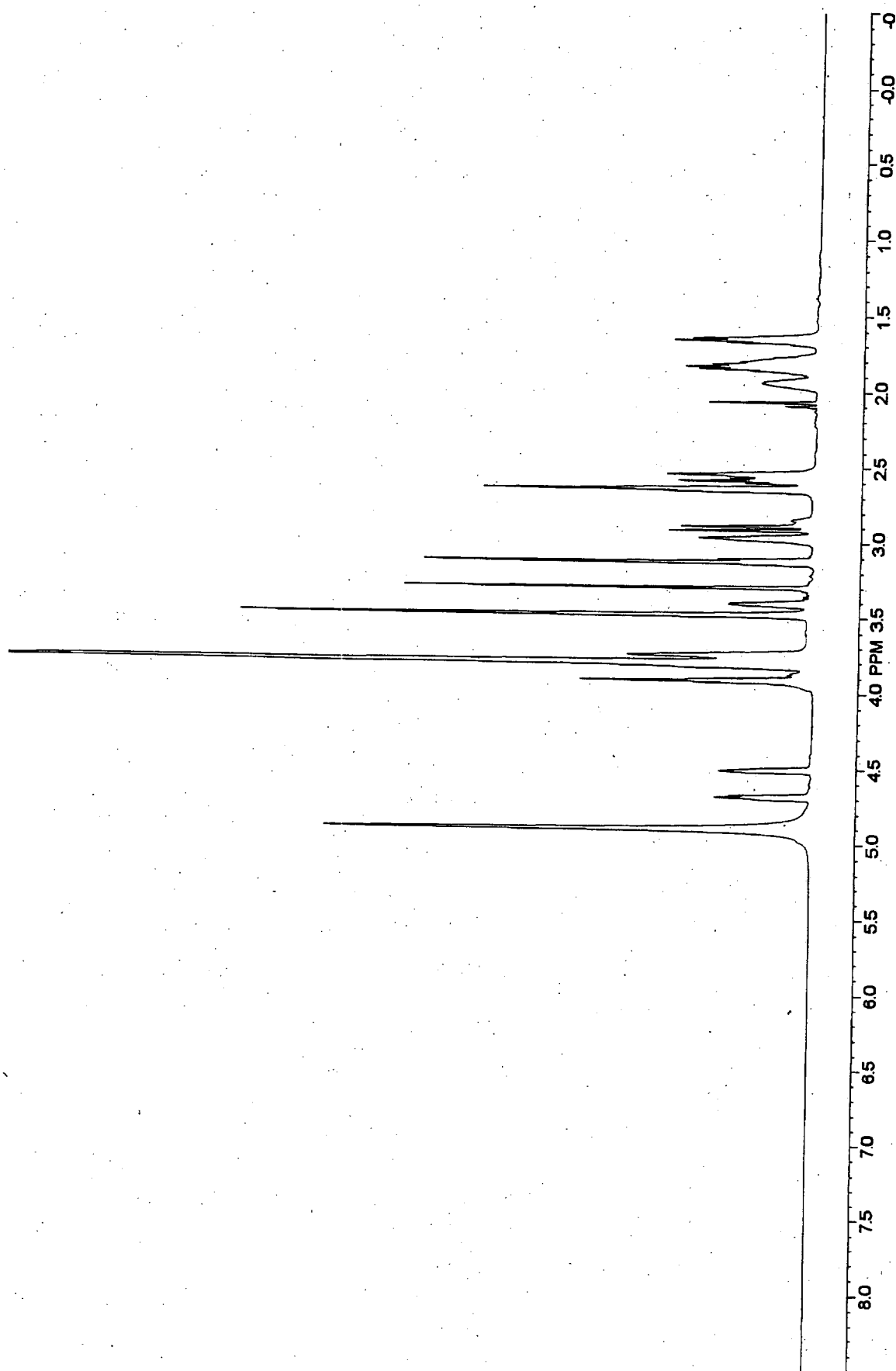




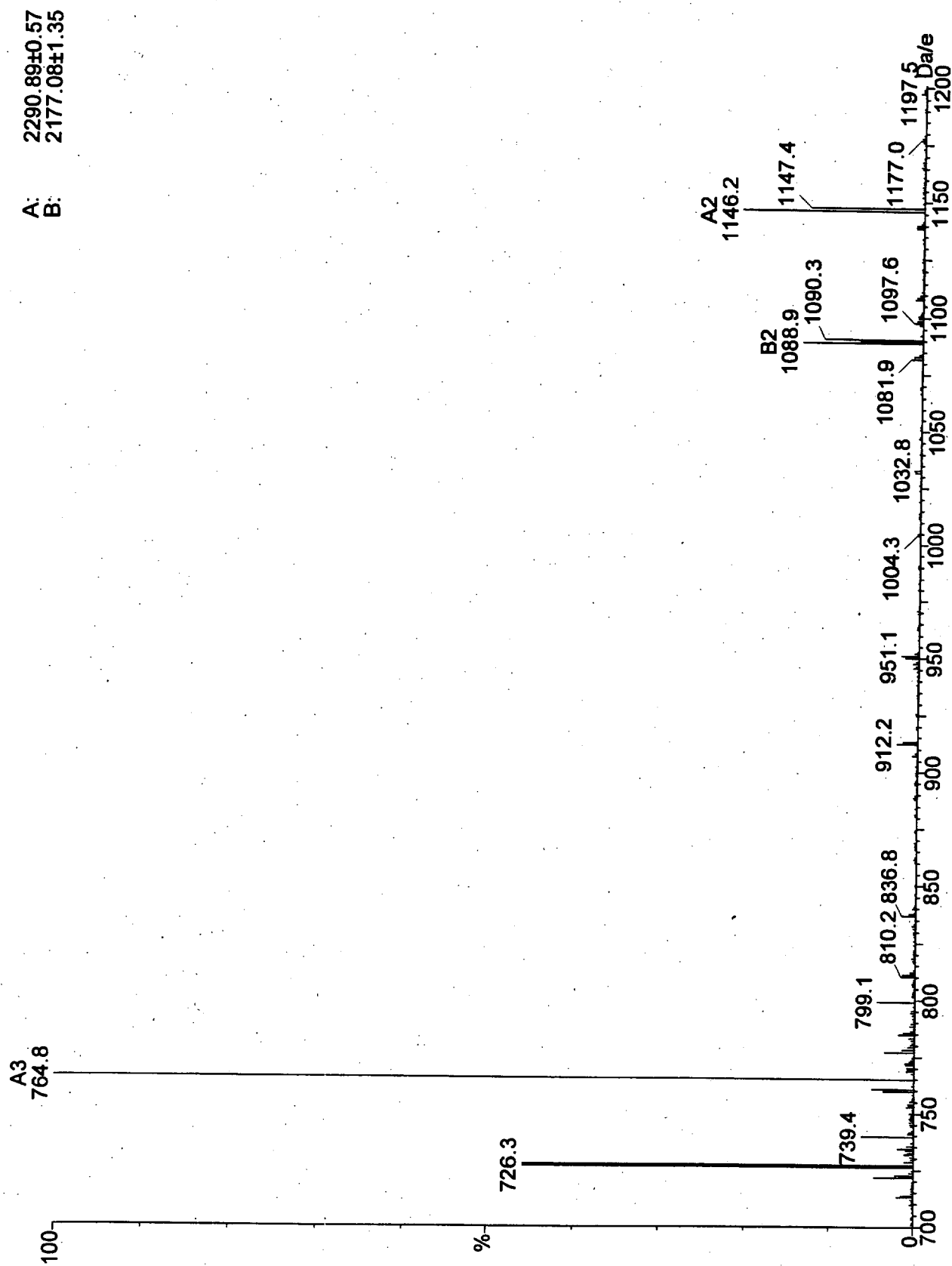
11

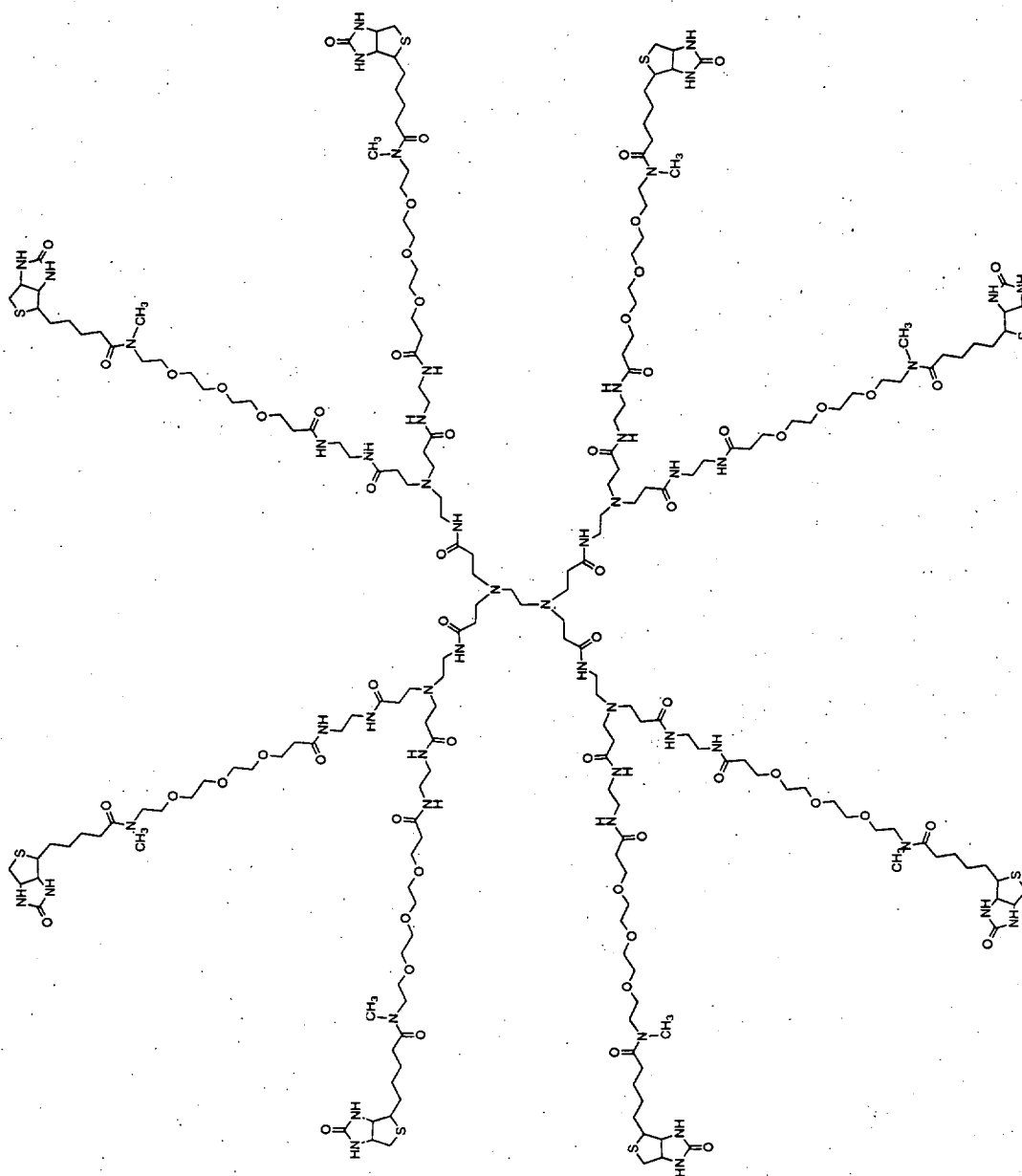
C₁₀₂H₁₈₀N₂₂O₂₈S₄
Exact Mass: 2289.22
Mol. Wt.: 2290.91





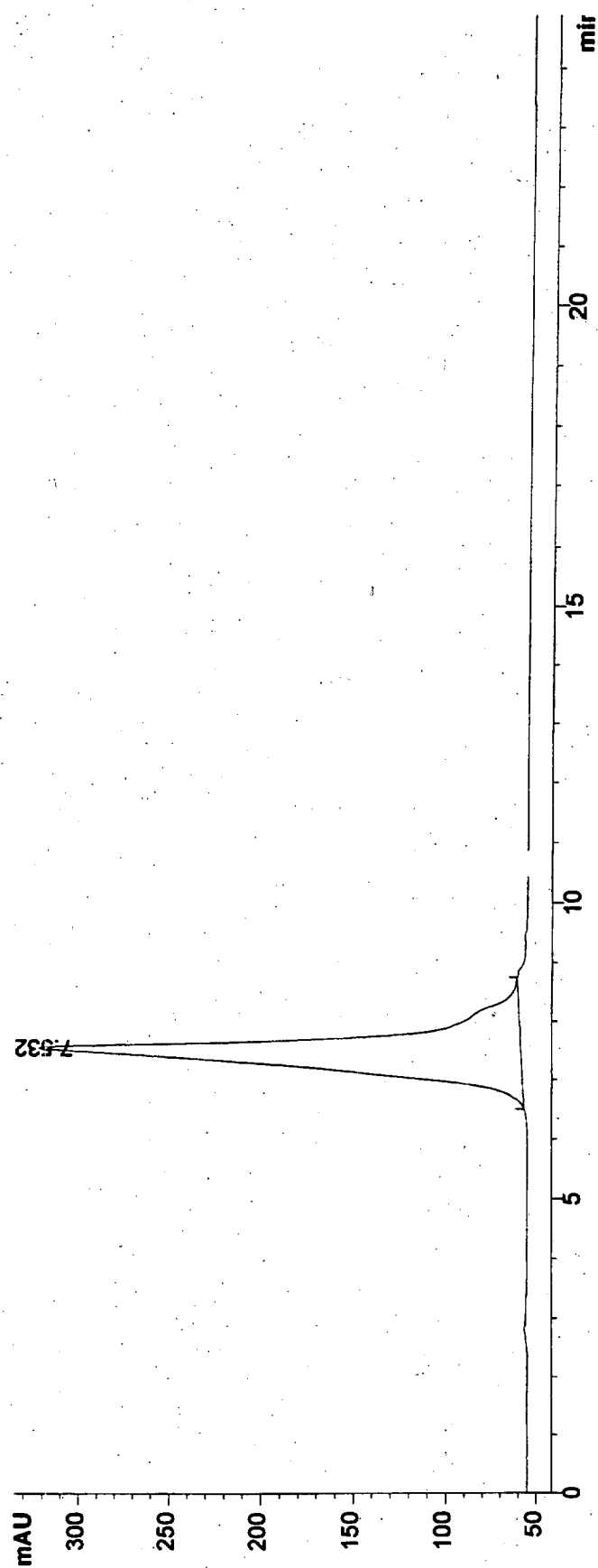
11



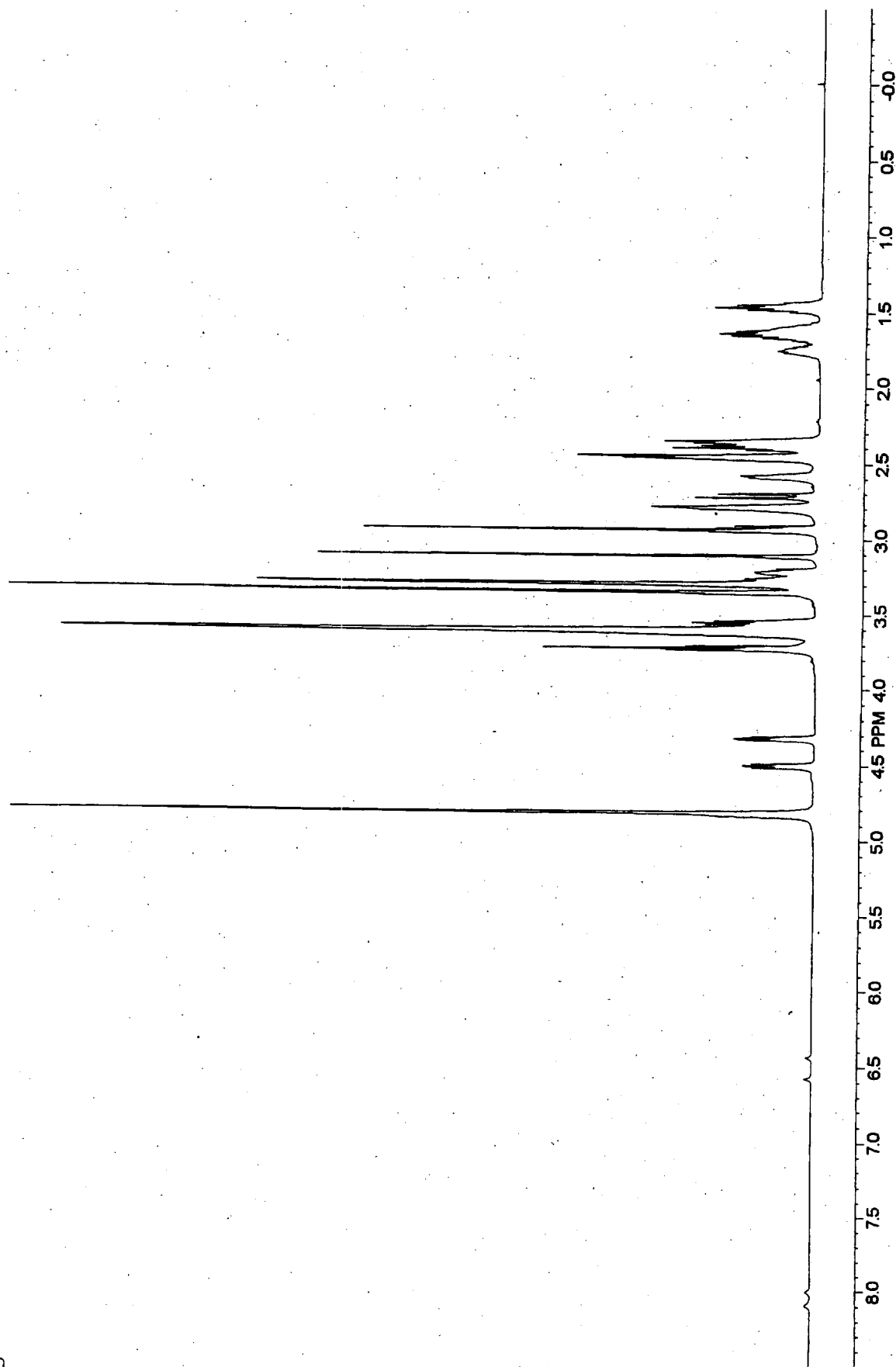


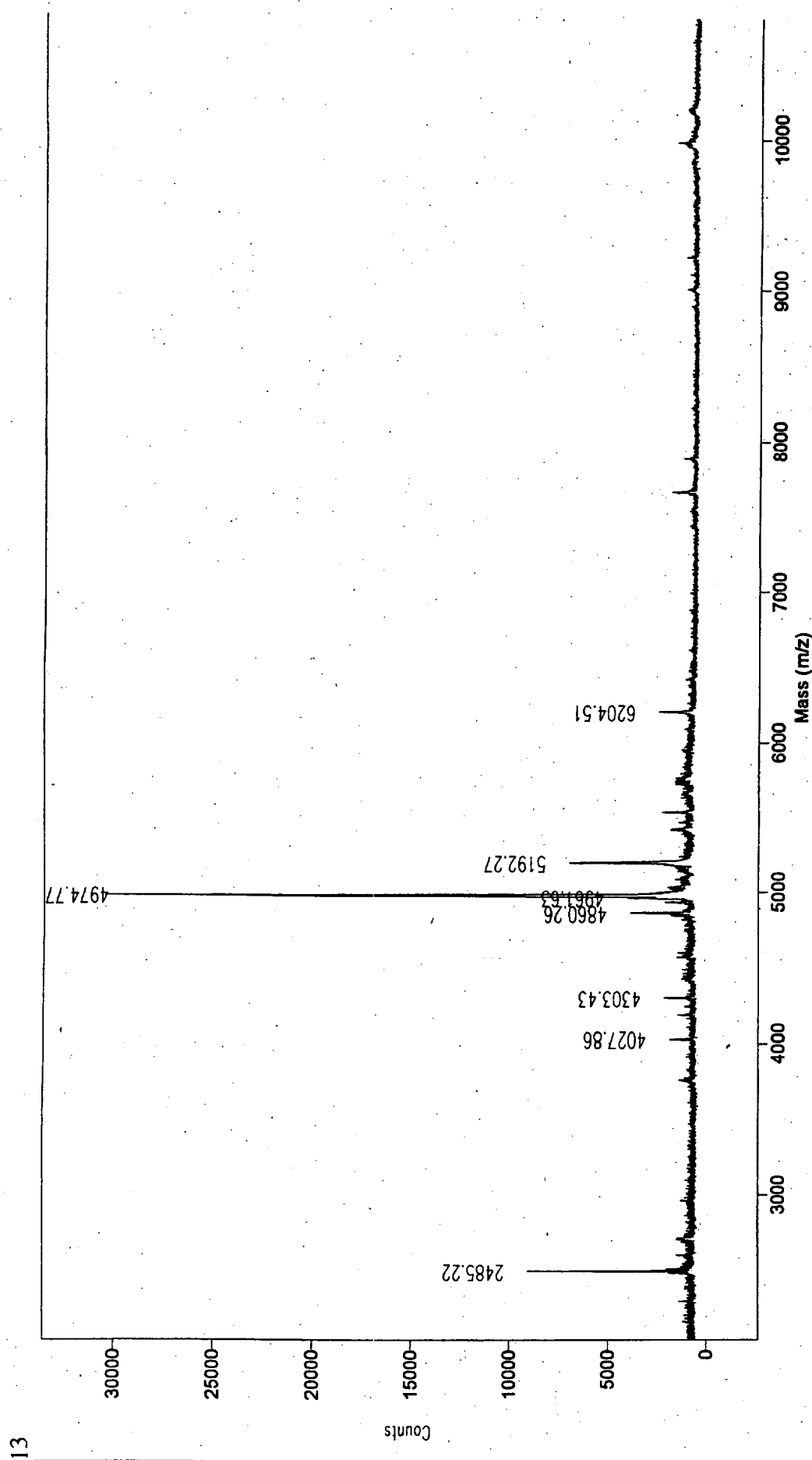
13

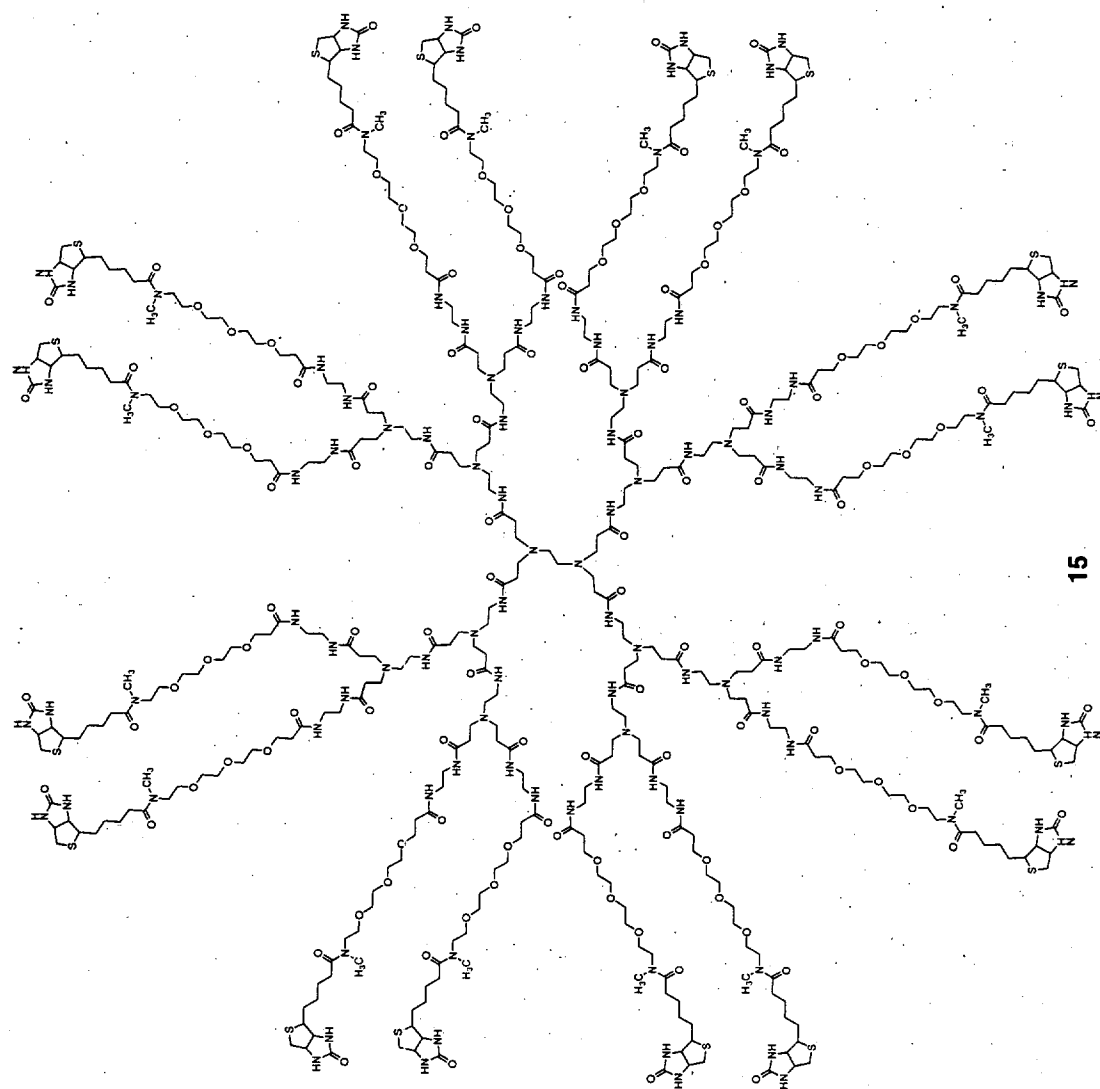
C₂₂₂H₃₉₂N₅₀O₆₀S₈
Exact Mass: 4974.69
Mol. Wt.: 4978.32



13



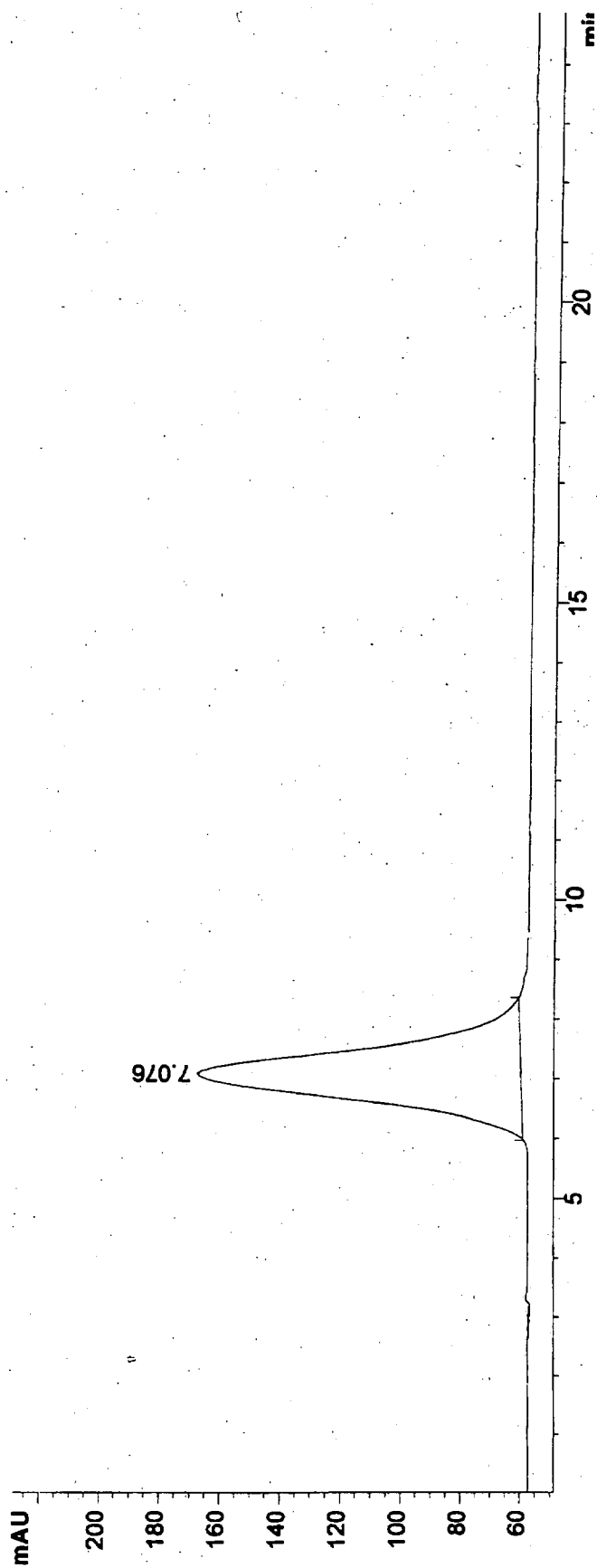


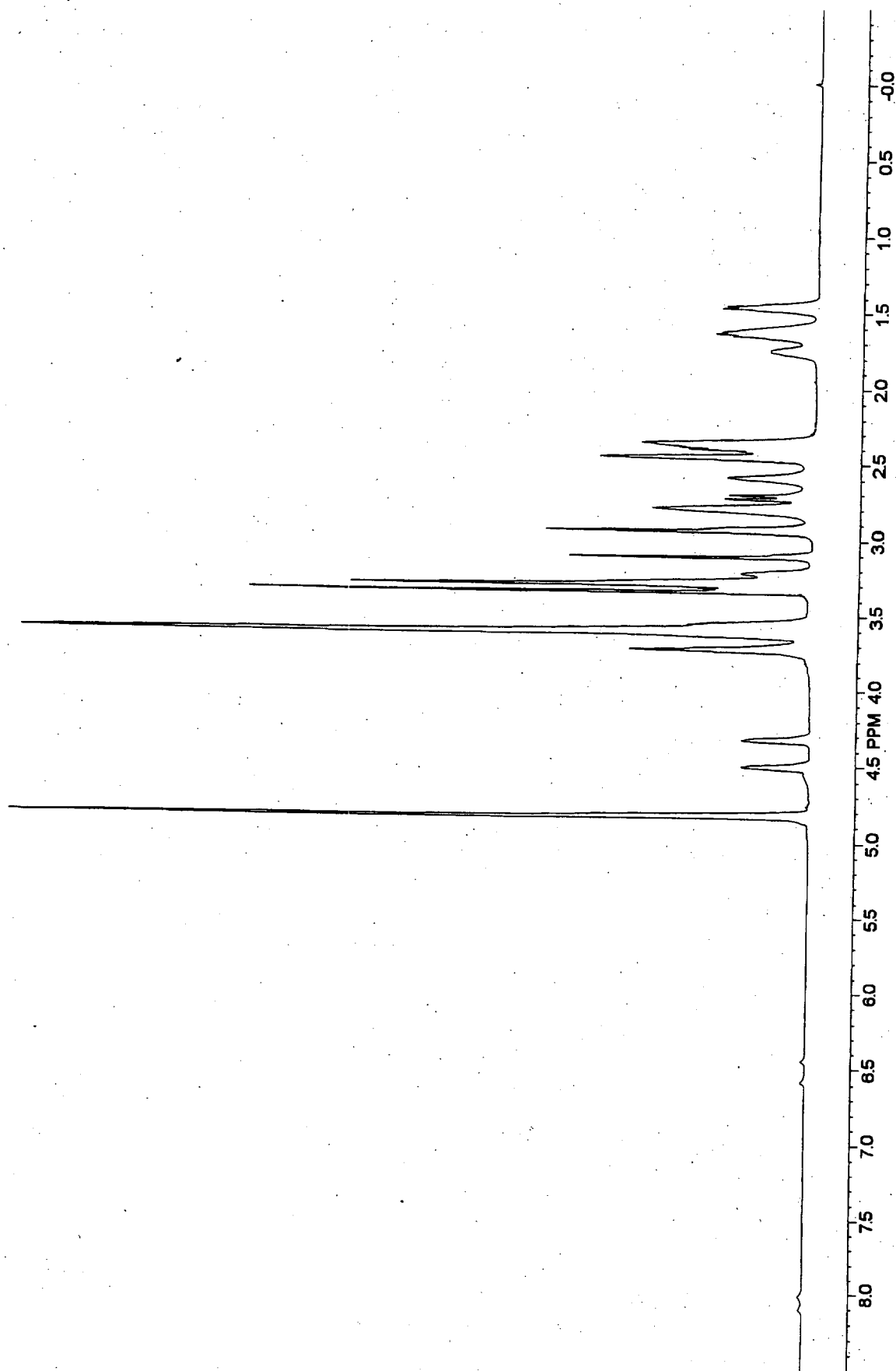


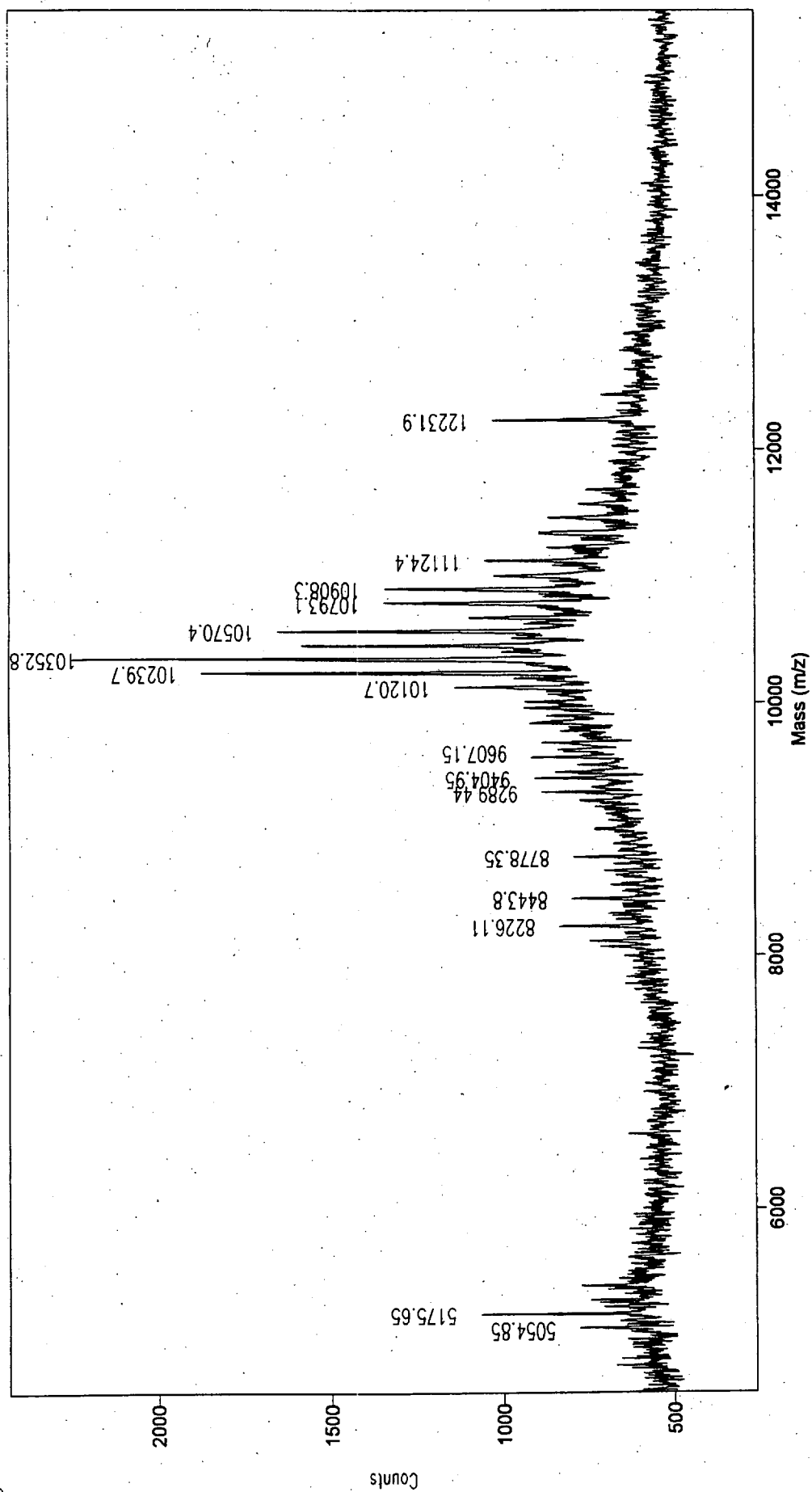
15

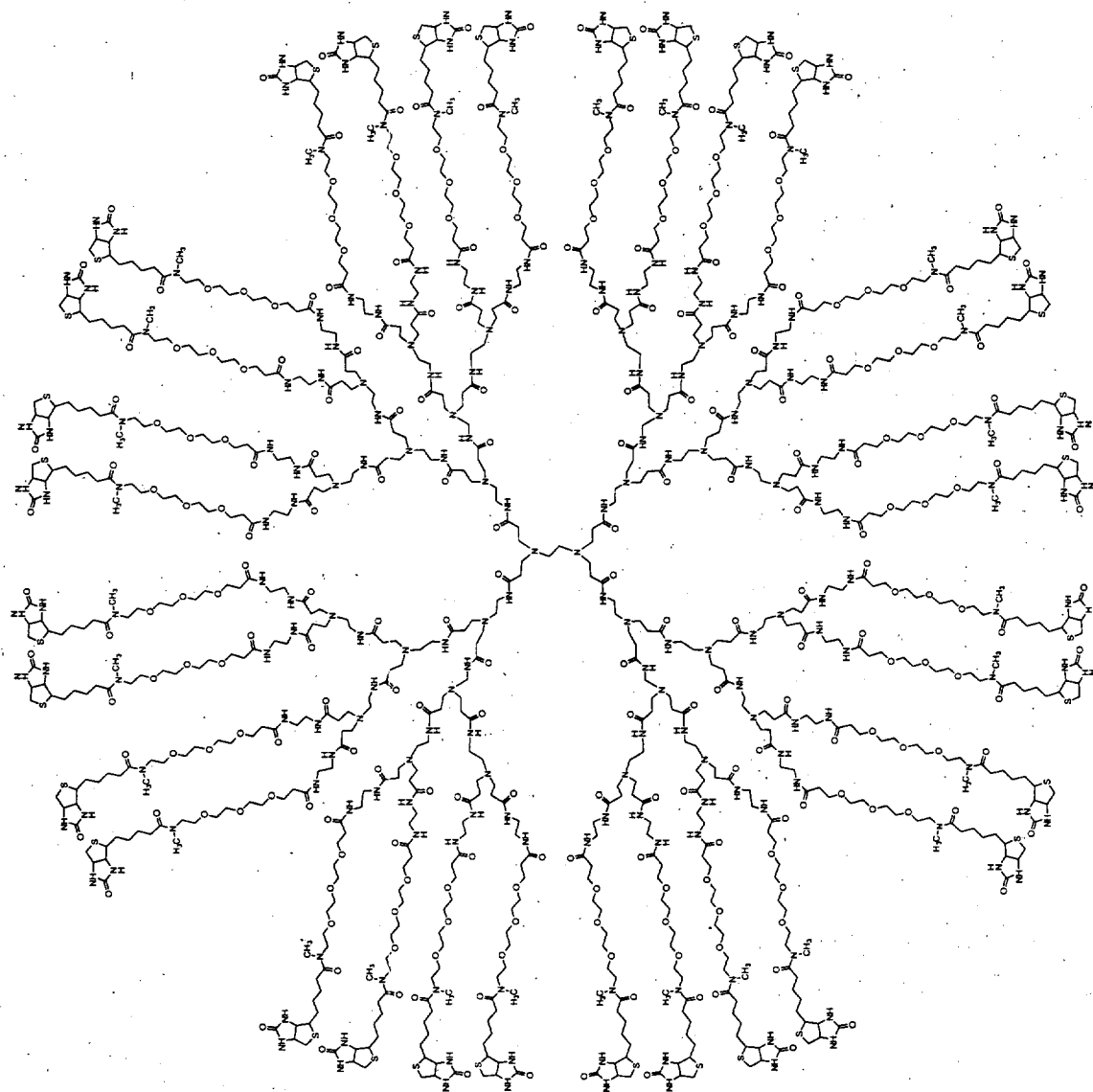
$C_{482}H_{816}N_{108}O_{123}S_{16}$
Exact Mass: 10345.63
Mol. Wt.: 10353.12

15



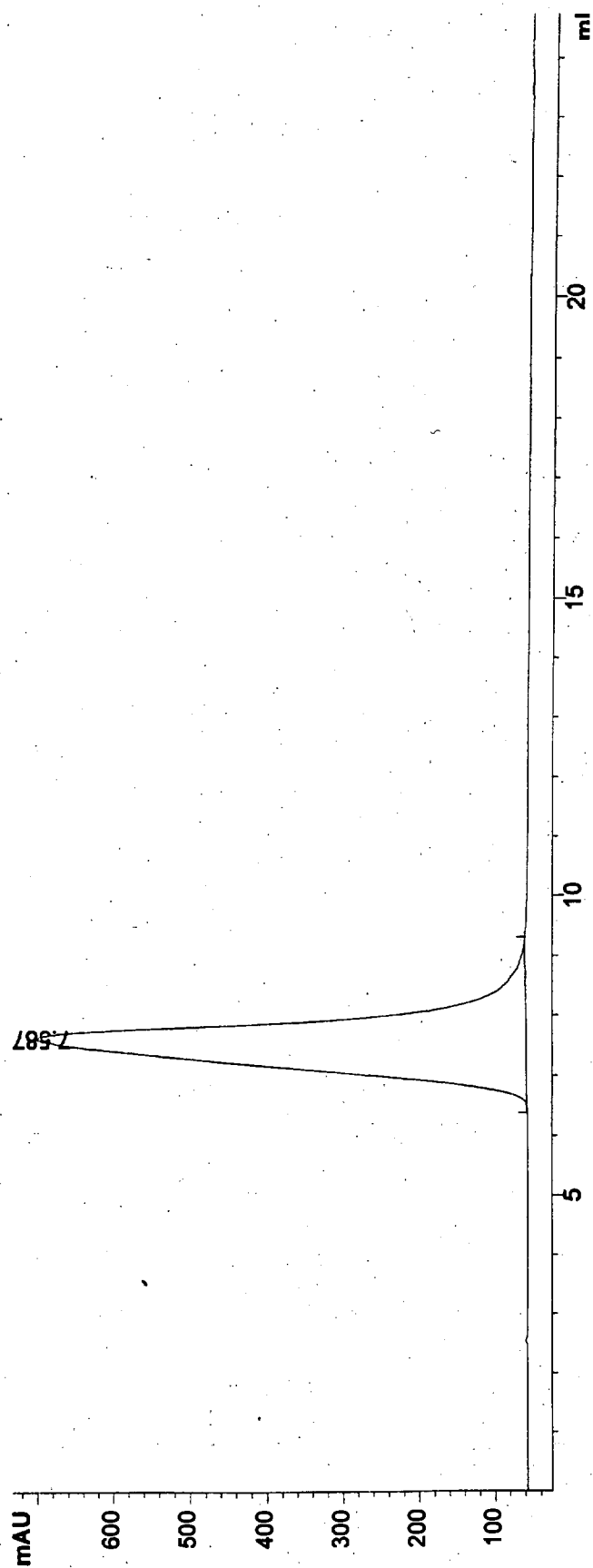


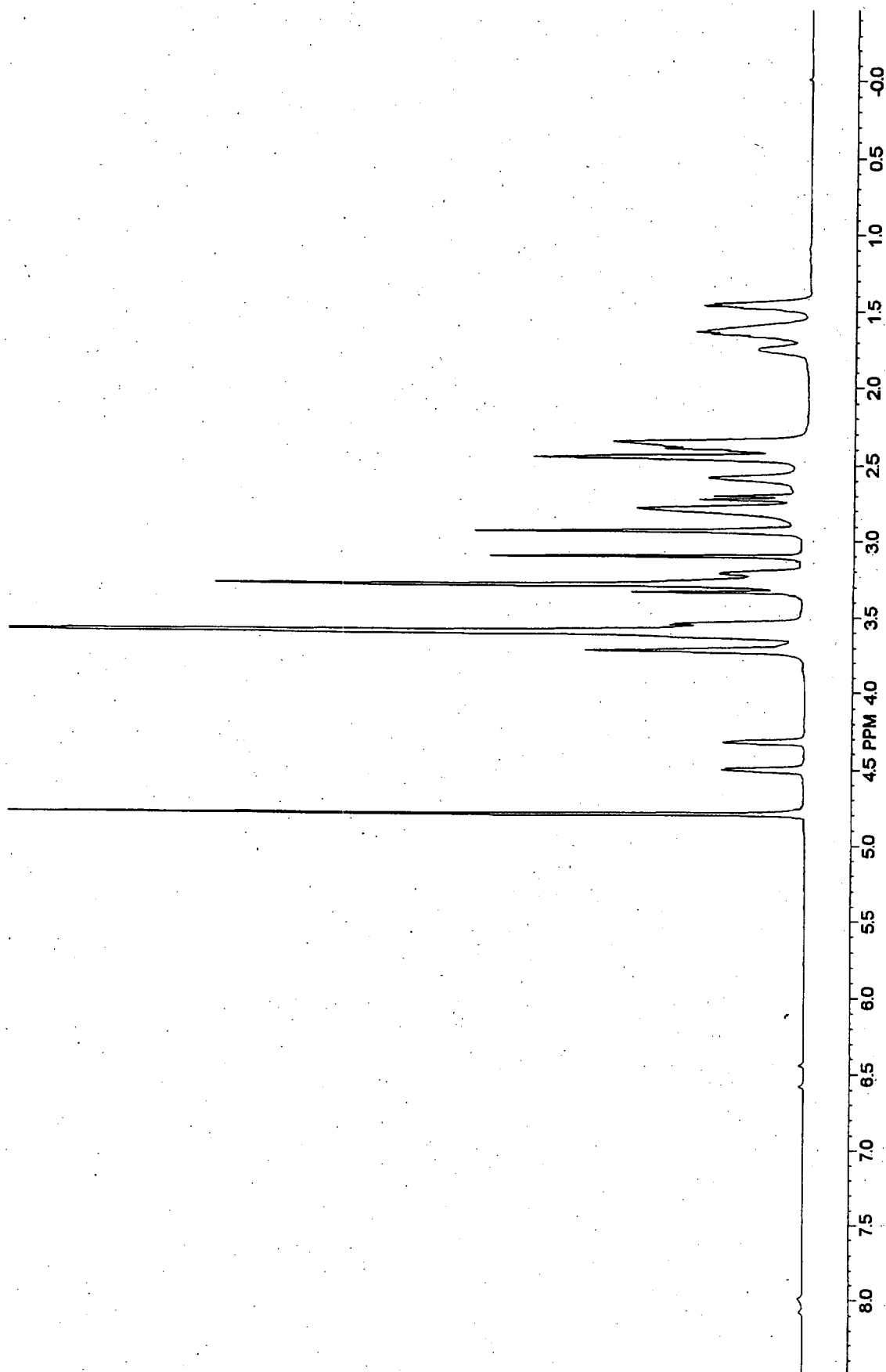


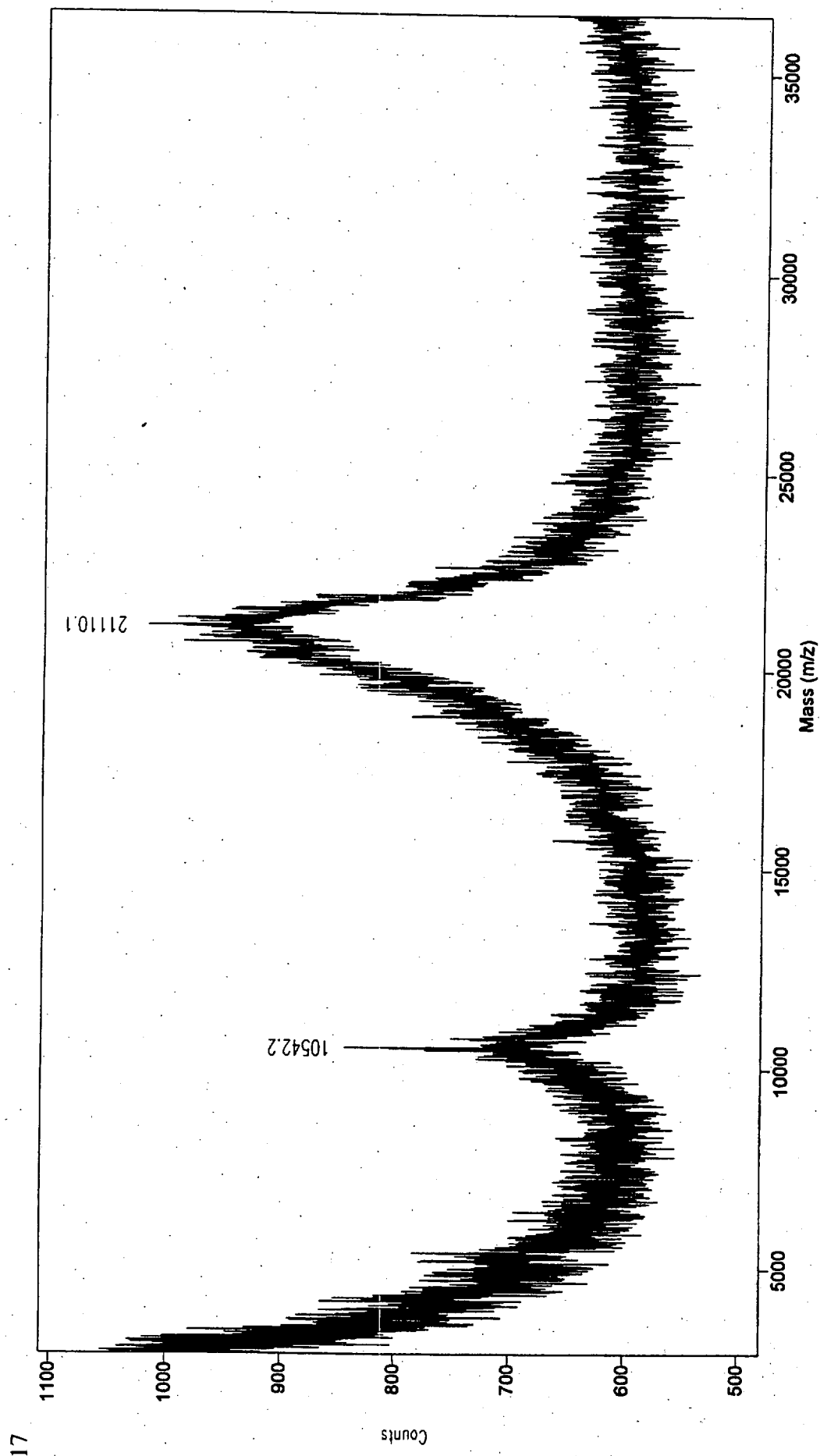


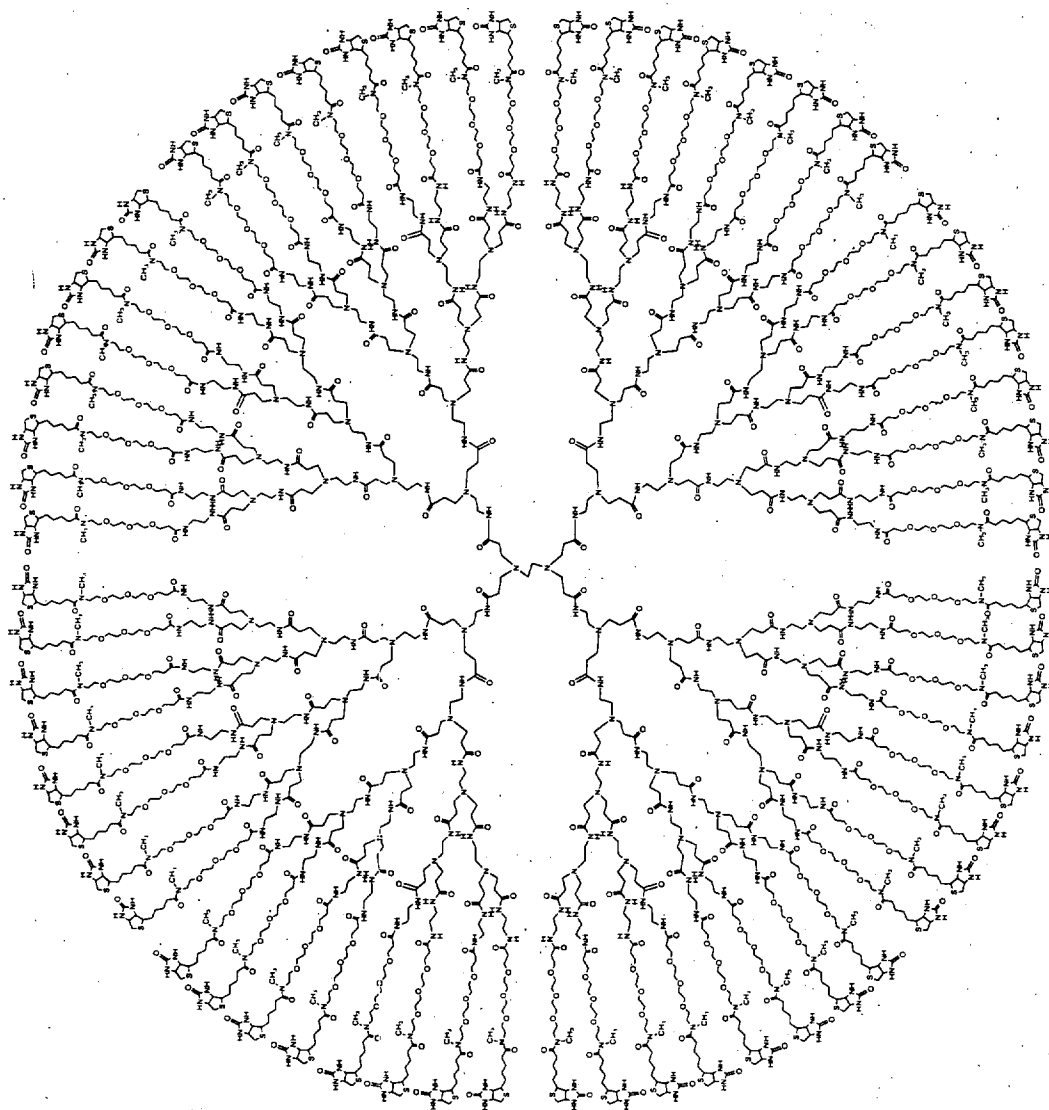
17

C₉₃₈H₁₆₅₆N₂₁₈O₂₅₂S₃₂
Exact Mass: 21031.45
Mol. Wt.: 21046.63



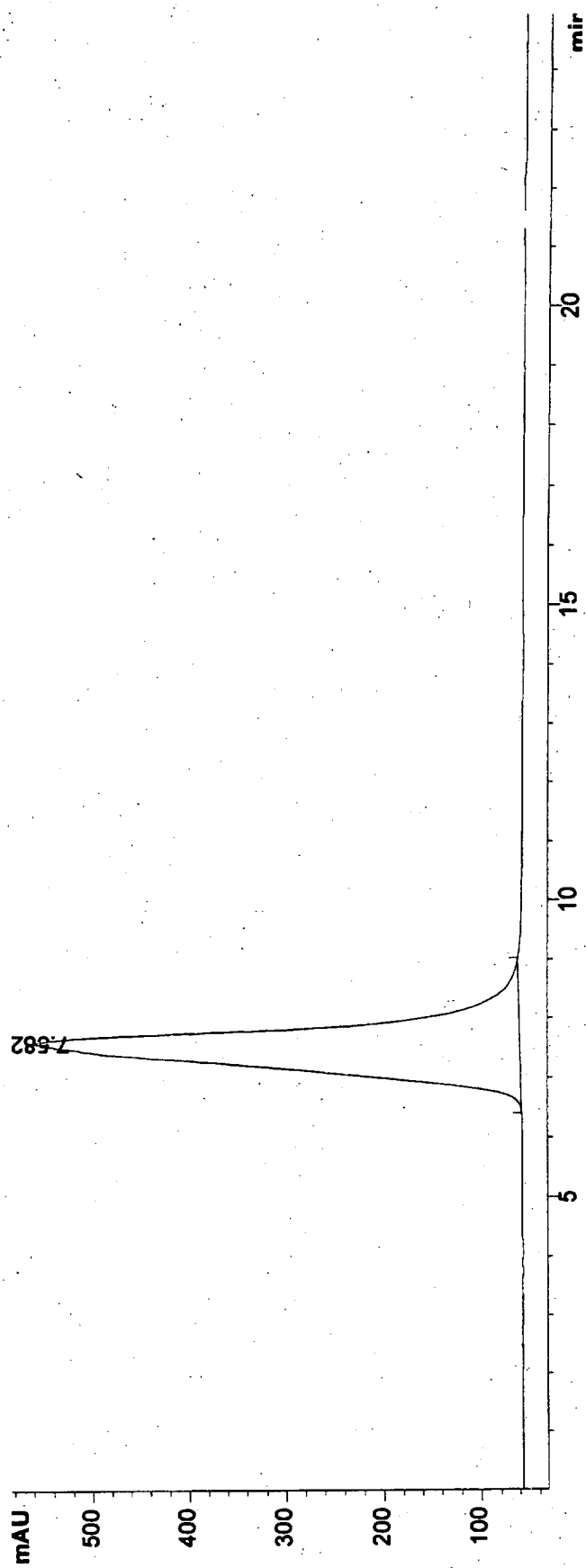


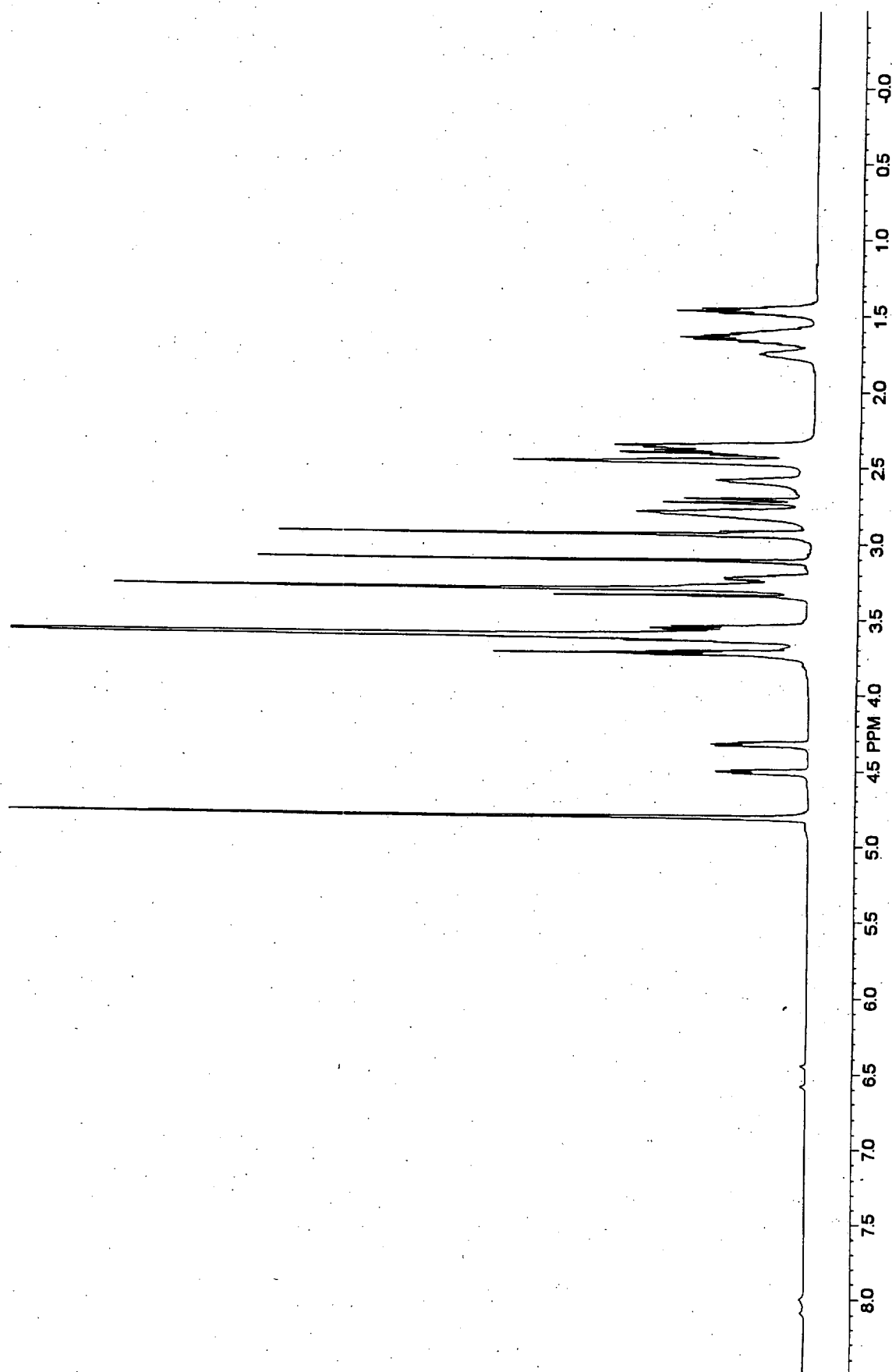


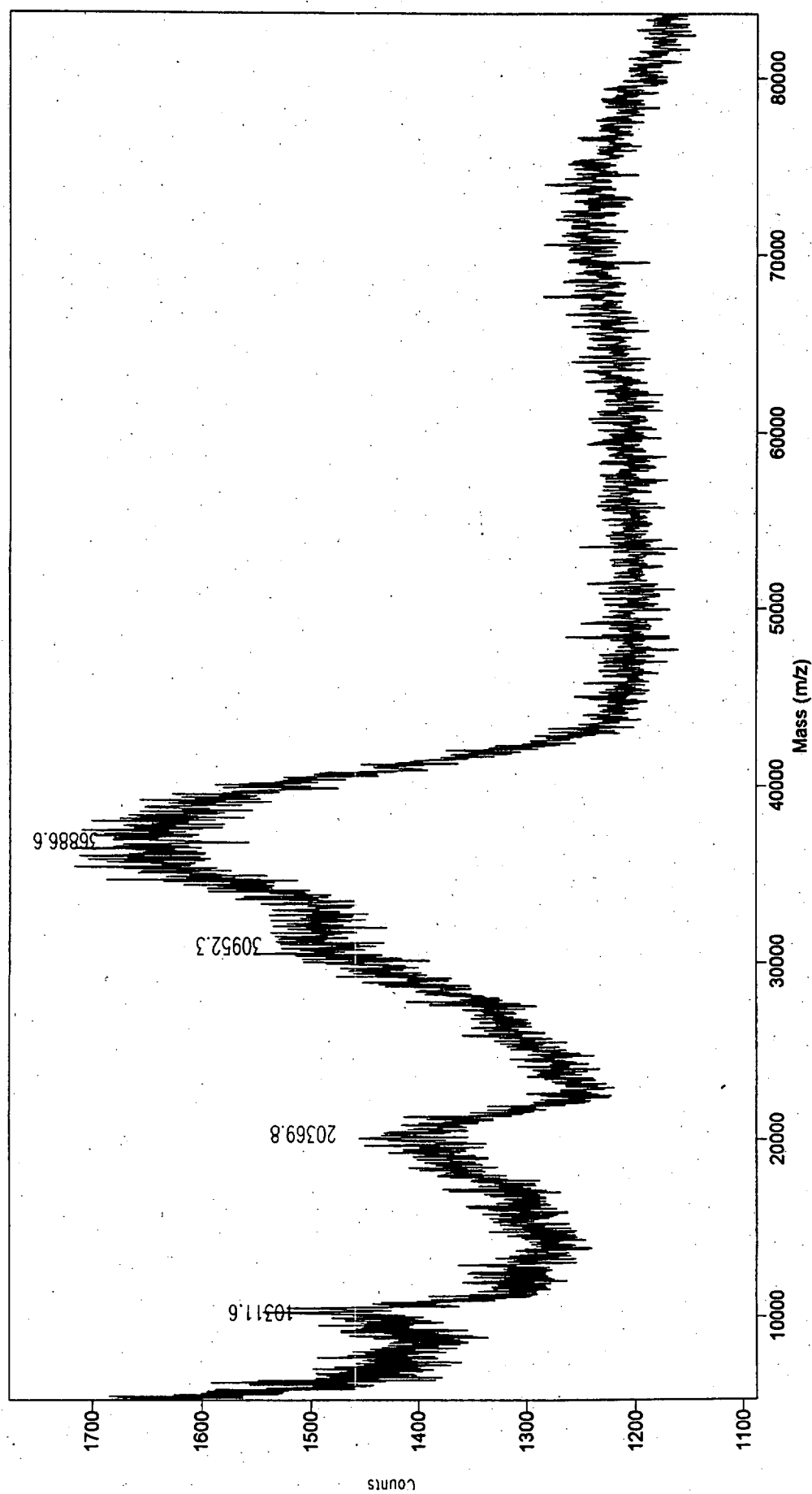


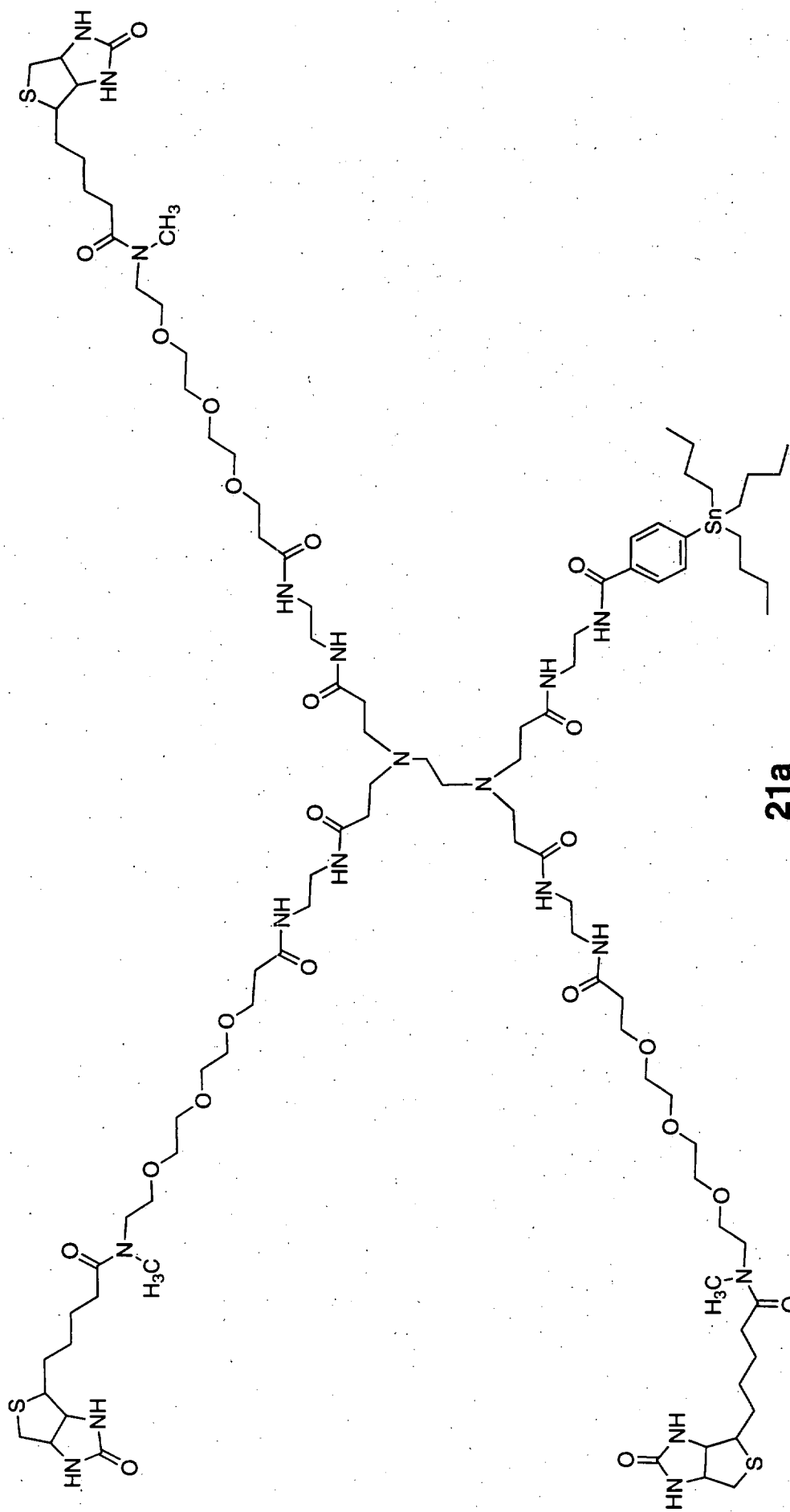
19

$C_{192}H_{360}N_{44}O_{58}S_4$
Exact Mass: 42571.28
Mol. Wt.: 42601.96





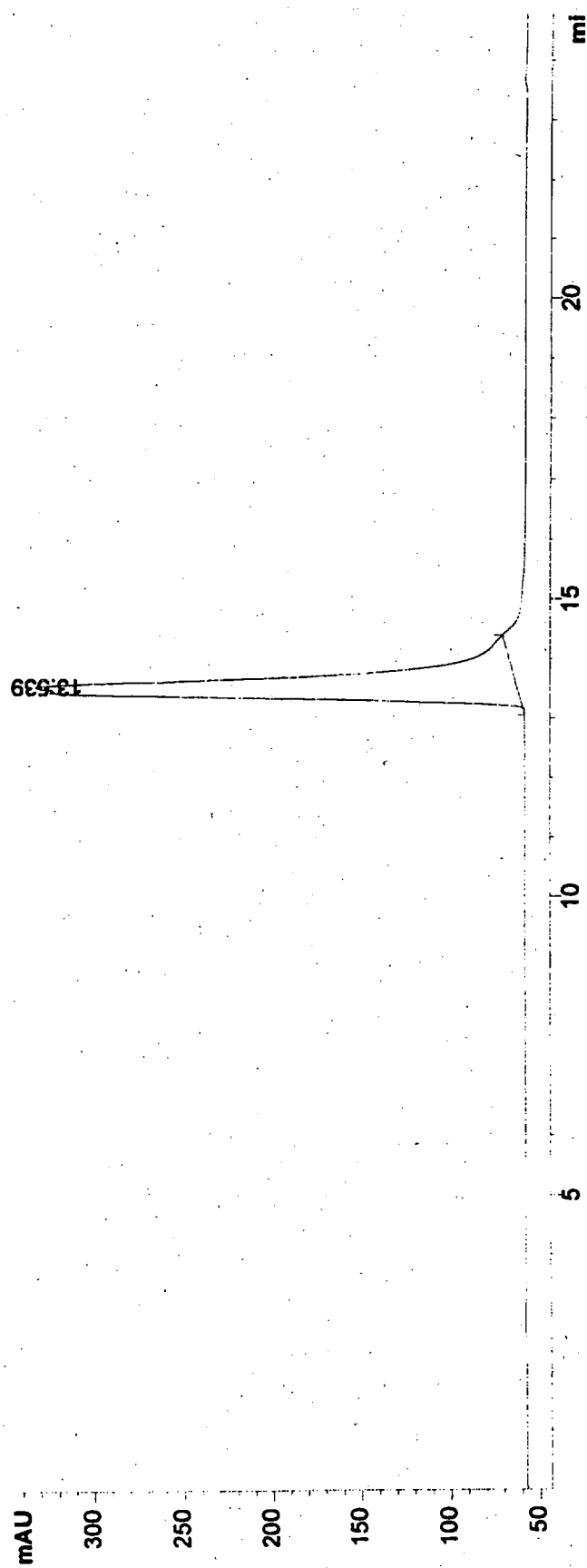




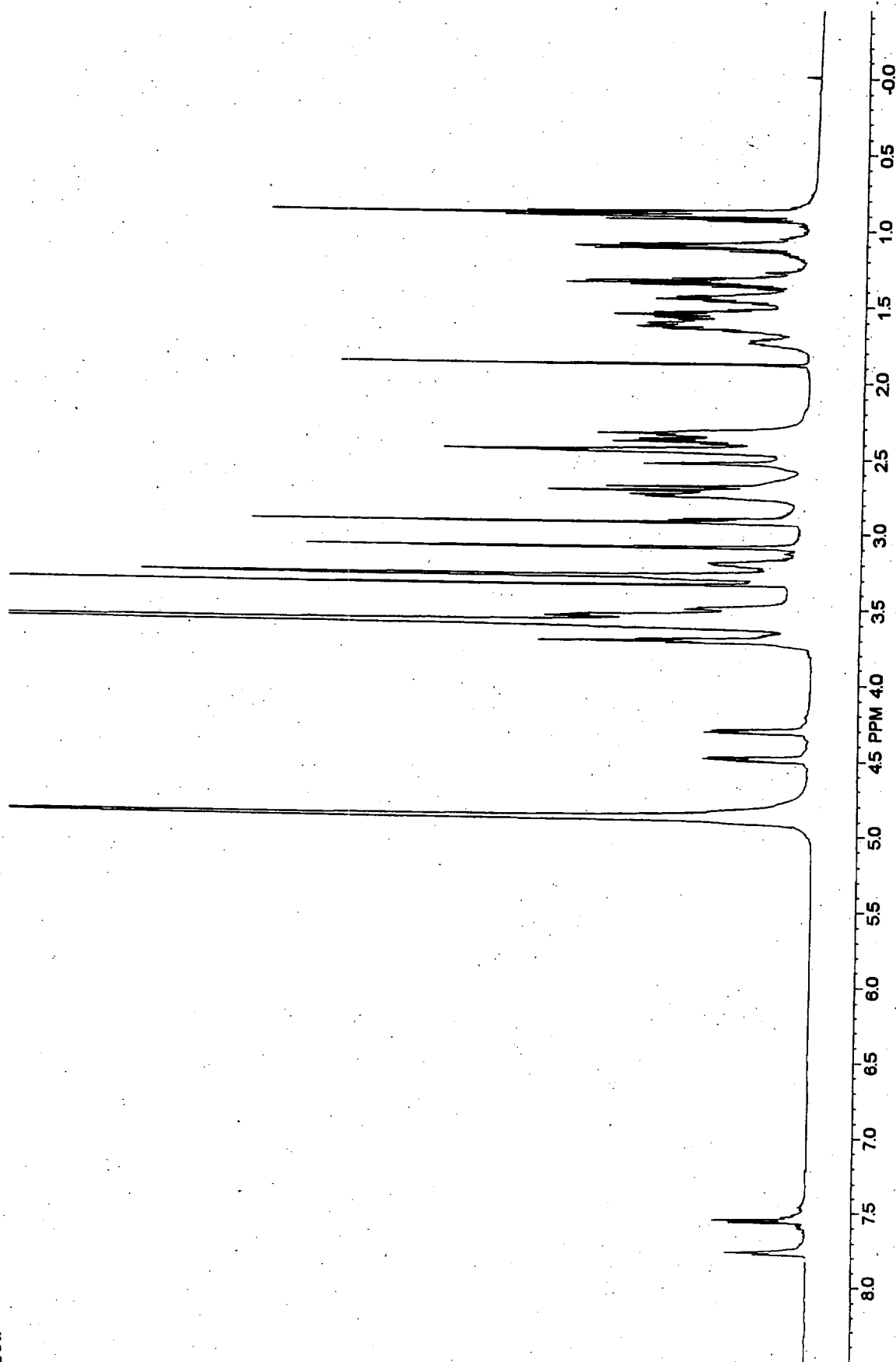
21a

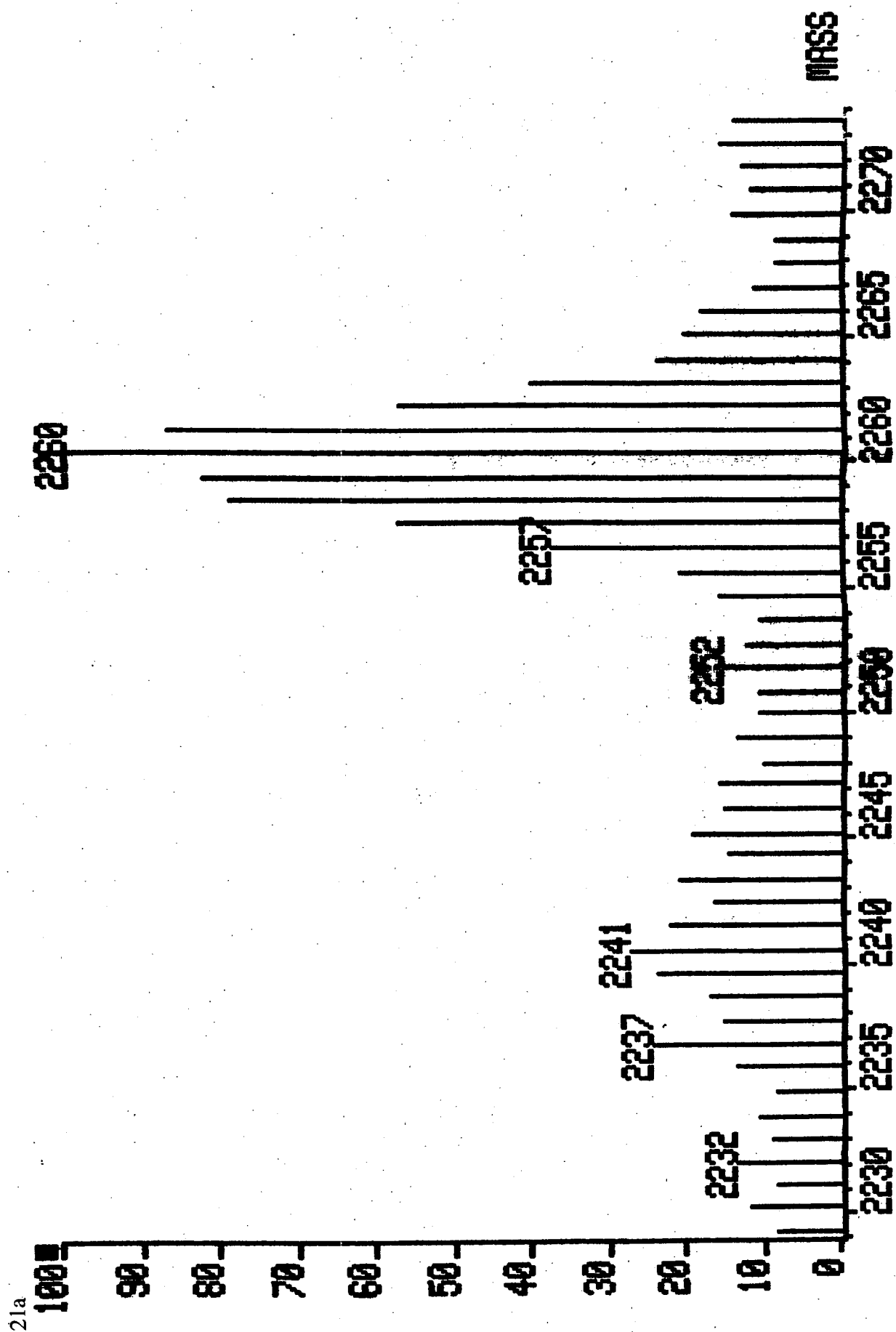
$C_{101}H_{177}N_{19}O_{23}S_3Sn$
Exact Mass: 2238.14
Mol. Wt.: 2240.49

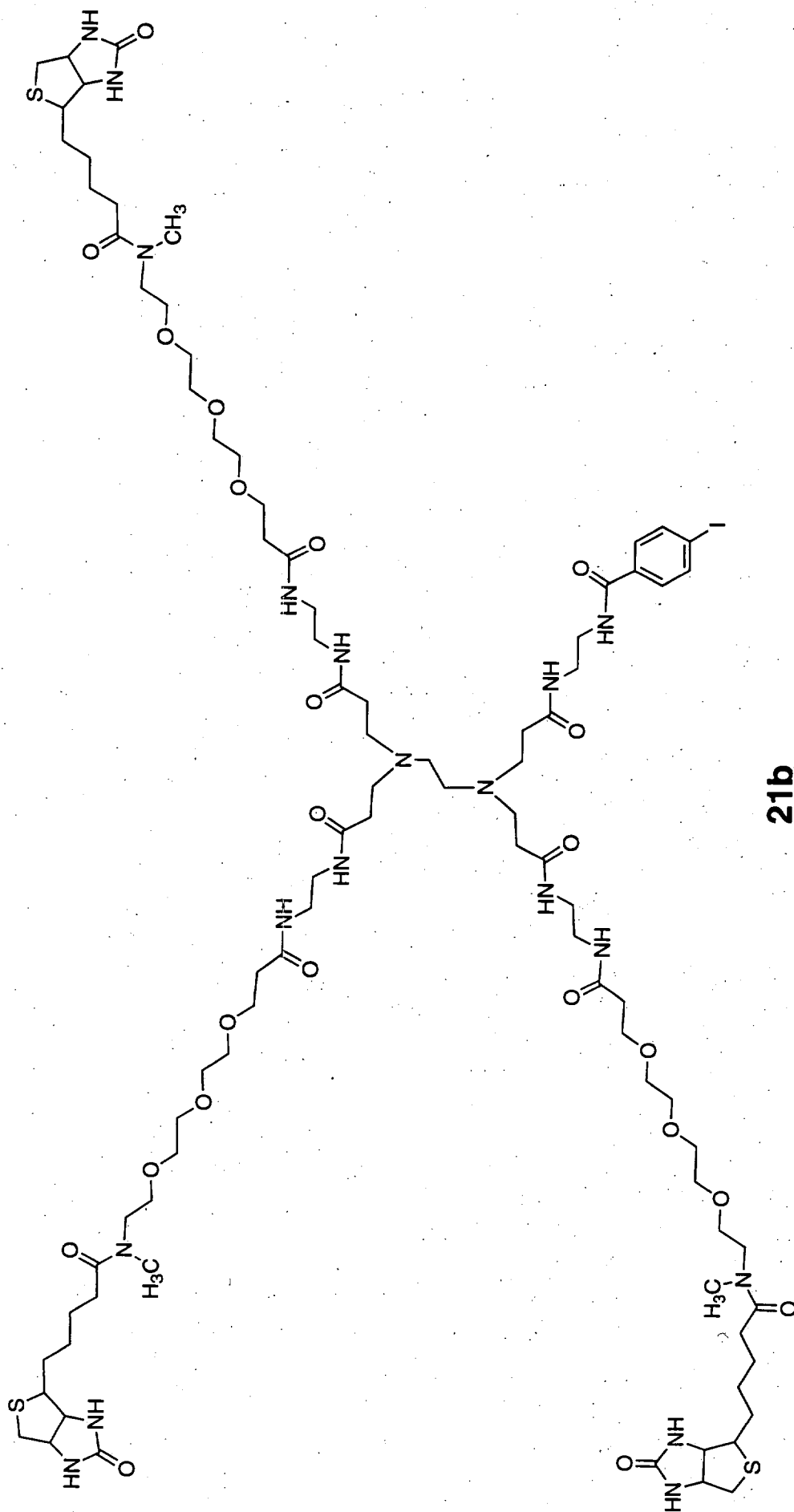
21a



21a



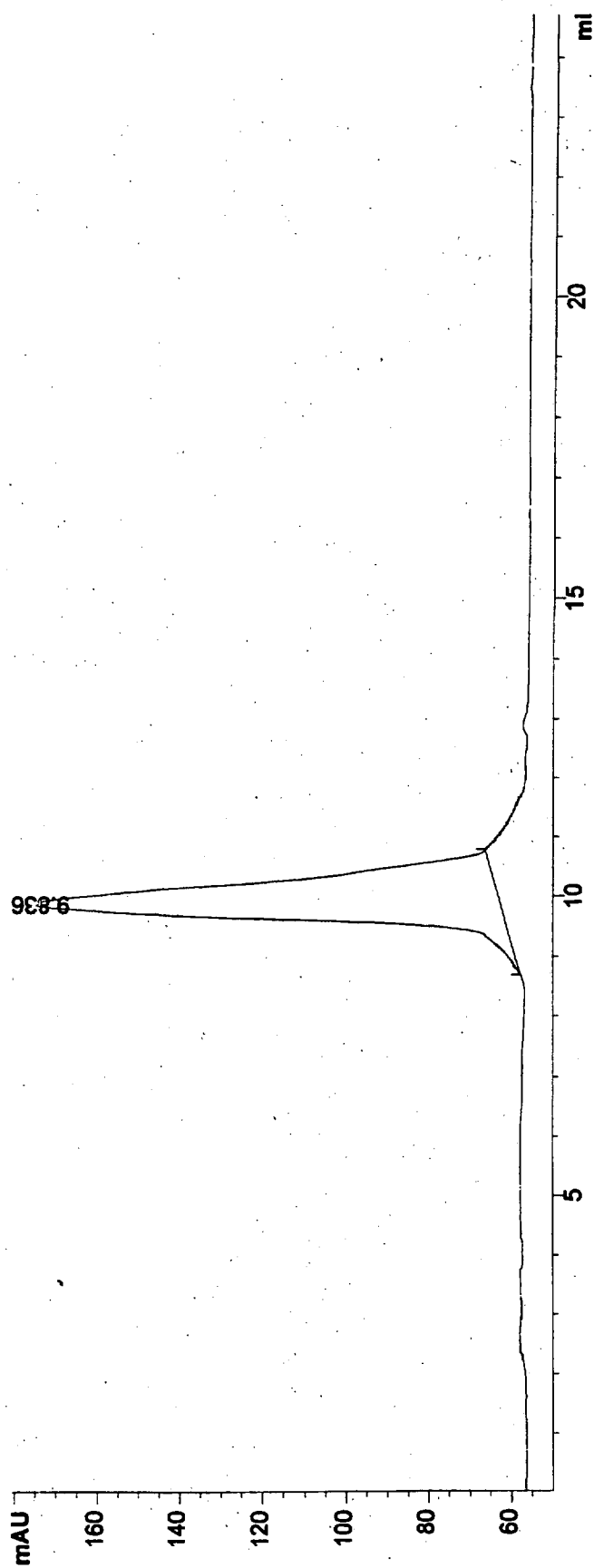




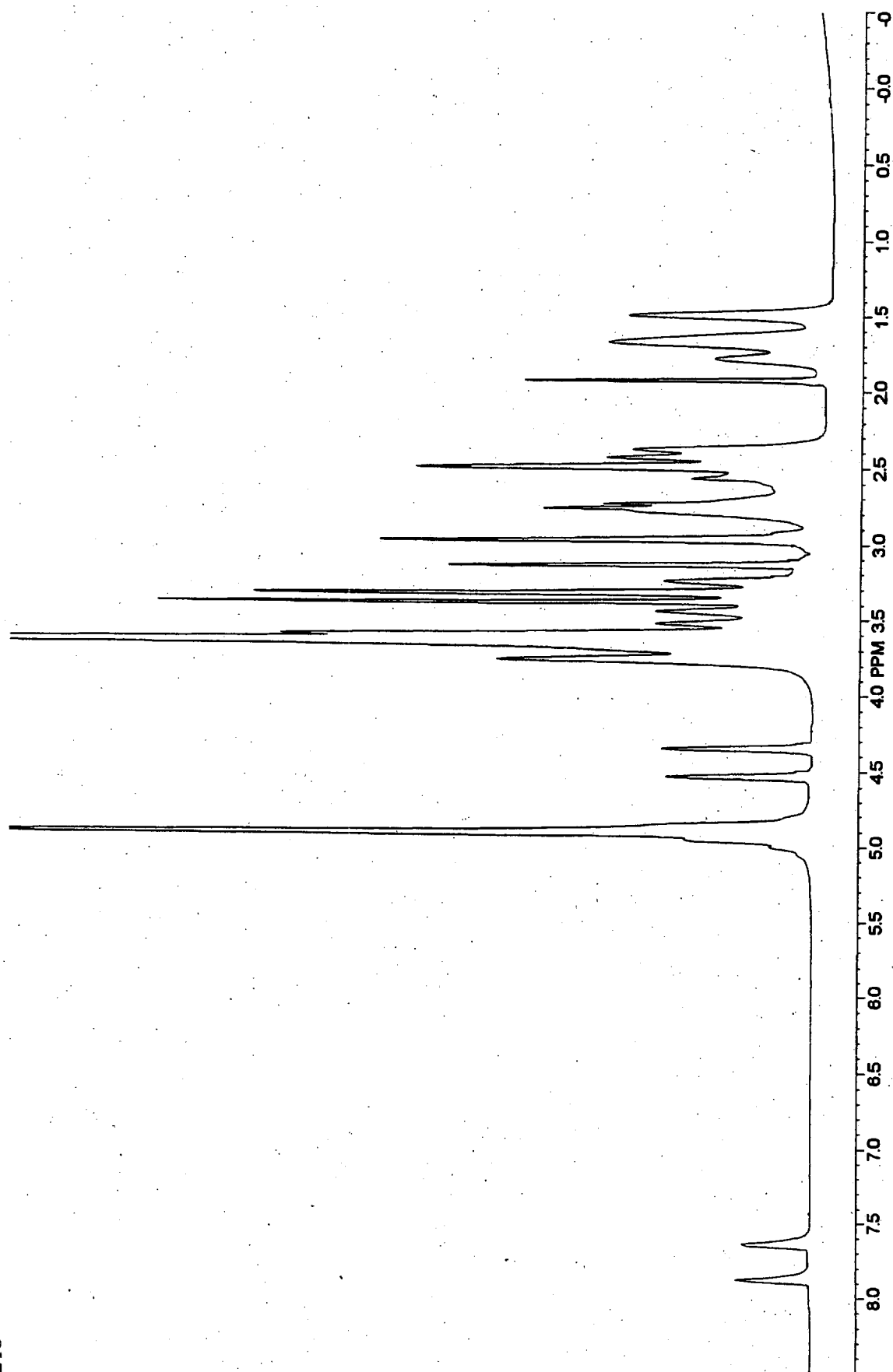
21b

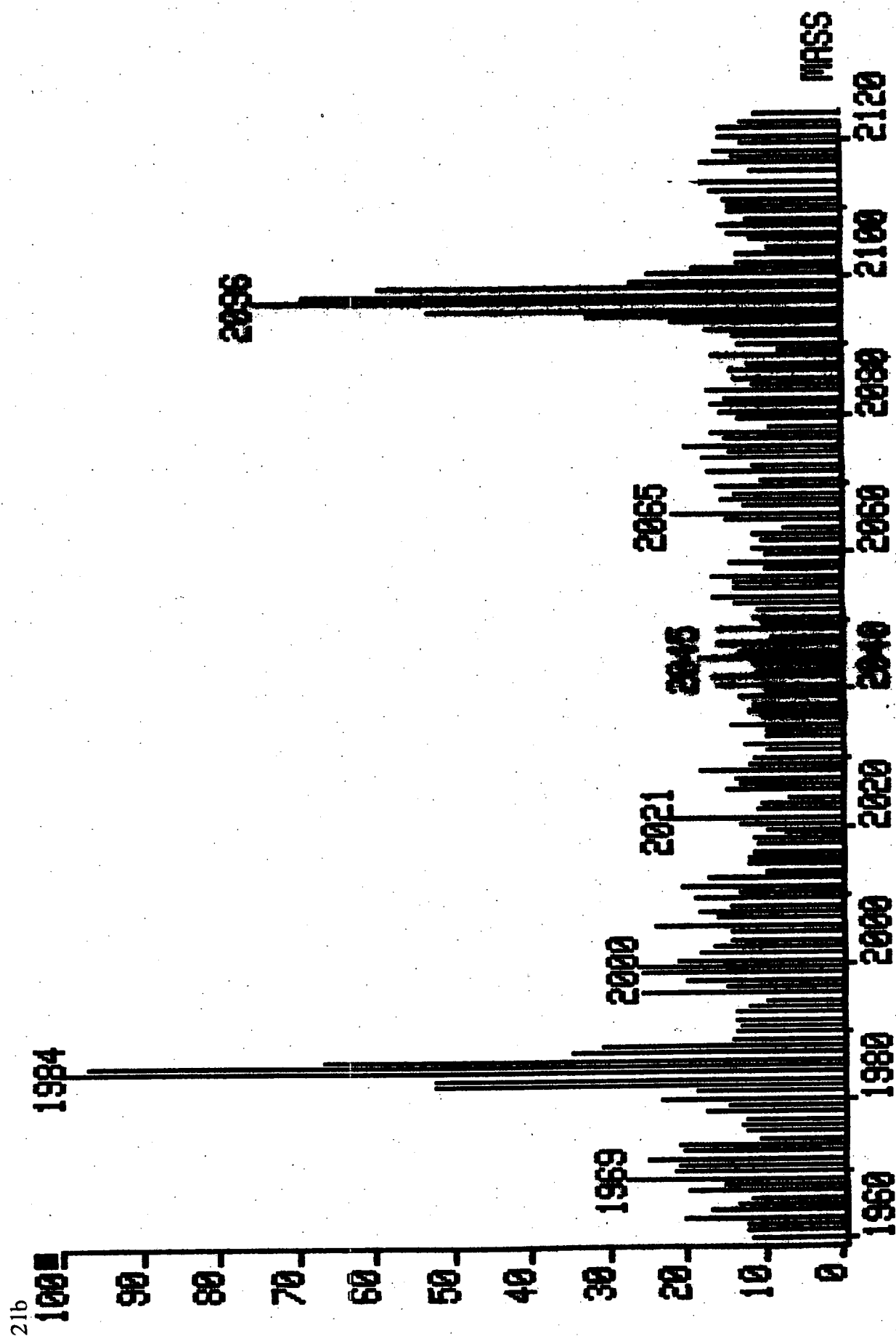
$C_{89}H_{150}N_{19}O_{23}S_3$
Exact Mass: 2075.93
Mol. Wt.: 2077.36

21b



21b





Tailored Hydrophobic Cavities in Oligonucleotide–Steroid Conjugates

Robert L. Letsinger* and Surendrakumar Chaturvedi†

Department of Chemistry and Department of Biochemistry, Molecular Biology, and Cell Biology, Northwestern University, Evanston, Illinois 60208. Received June 5, 1998; Revised Manuscript Received August 11, 1998

Hydrophobic pockets can be generated readily in aqueous solution by hybridization of oligonucleotide conjugates containing one or two androstane units inserted into each strand by short phosphoryl linkers. Both double- and triple-stranded complexes formed by the conjugates are stabilized by adding to the solution a water-soluble hydrophobic substrate, 3,17-diaminoandrostane dihydrochloride, that can bind in the pocket. This substrate has no effect on the dissociation of unmodified oligonucleotides, and 1,10-diaminodecane dihydrochloride has no effect on dissociation of complexes of these steroid conjugates under the same conditions. This system provides a new means for selectively modulating and triggering hybridization of oligonucleotide conjugates. Cetyltrimethylammonium bromide strongly enhances the stability of complexes of the steroid conjugates; however, it also leads to precipitation of complexes of unmodified oligonucleotides.

INTRODUCTION

Hydrophobic cavities play an important role in binding substrates to enzymes and receptor proteins in an aqueous environment. Technique for generating such cavities synthetically can be useful in creating new catalysts and assembly systems. However, the construction of hydrophobic-binding cavities, even for small molecules, can be challenging since it entails creation of a relatively large, defined structure that is soluble in water yet possesses hydrophobic surfaces suitably positioned in space to envelop the substrate. One approach to synthesizing enzymes mimics has been to append catalytically active functional groups to natural cyclodextrins that serve as binding sites for aromatic esters (1, 2). Cavities have also been created de novo by assembling appropriate peptides into four-helix bundles (3). We show here that complexes derived from appropriately designed oligonucleotide–steroid conjugates can function as entities with hydrophobic pockets that interact selectively with a small molecule substrate in aqueous solution. Distinctive features of the system are the simplicity of synthesis and the fact that the hydrophobic pockets that are generated also provide a new means for selectively modulating and triggering hybridization of oligonucleotide derivatives, namely, by addition of a low concentration of a hydrophobic binding agent targeted to the pocket.

RESULTS AND DISCUSSION

Duplex Assembly. Initial experiments were carried out with **1a** + **2a**, a duplex comprising two oligonucleotide conjugates with androstane units inserted into the chains via phosphoryl groups (Scheme 1). This system was selected since molecular models indicated that hybridization of the oligonucleotide segments would bring the steroid groups into proximity but at a distance

unfavorable for direct hydrophobic interaction. Oligomers **1a** and **2a** and the related conjugates indicated in Scheme 1 were synthesized by conventional solid support methodology using nucleoside phosphoramidite reagents along with intermediate **5** to introduce the androstane units. Compound **5** was prepared from epiandrosterone by successive treatment with dimethoxytrityl chloride, reduction of the 17-ketone function to the alcohol, and phosphitylation with 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite.

Thermal dissociation data for the duplex formed from **1a** + **2a** in fact support the view that contact between the two steroid units in the fully hybridized double stranded complex is minimal at best. The dissociation temperature (T_m) of the complex is only 17 °C (Figure 1), a value near that expected for independent dissociation of the 5-bp arms. This value contrasts markedly with data for systems in which organic groups on separate strands of a duplex are aligned by linkers that enable the organic groups to come into contact. Thus, the T_m for duplex **1b** + **2b** is 39 °C (4, 5) and unusually high dissociation temperatures have been observed for oligonucleotides conjugated to cholesterol groups than overlap in a duplex (6, 7). Melting curves exhibiting a hyperchromic change at 330 nm for the stilbenedicarboxamide band that parallels the change at 260 nm for dissociation of the oligonucleotide strands provided direct evidence that the stilbenedicarboxamide units were in contact in **1b** + **2b** (4, 5).

If the aligned androstane groups in **1a** + **2a** indeed form a hydrophobic pocket in the aqueous solution, one might expect that filling the pocket with a hydrophobic substrate would stabilize the duplex. As a test of this idea, we examined the effect of 3,17-diaminoandrostane dihydrochloride, **6**, on the dissociation of complex **1a** + **2a**. Compound **6** has a well-defined, large hydrophobic surface and contains two well separated cationic sites that confer water solubility, disfavor formation of micelles, and facilitate binding to the anionic oligonucleotide complex. Melting curves for duplex **1a** + **2a** carried out in the absence and in the presence of low concentrations

* To whom correspondence should be addressed. E-mail: r-letsinger@chem.nwu.edu. Phone: 847-491-7674. Fax: 847-491-7713.

† Current address: PE-Applied Biosystems, 850 Lincoln Center Drive, Foster City, CA 94404.

Scheme 1

1. 5' d-GCTCA-X-AGTCT 3. 5' d-TTTTTTTTTT-X-TTTTTTTTTT
 2. 5' d-AGACT-X-TGAGC 4. 5' d-AAAAAAAAAA-X-AAAAAAAAAA

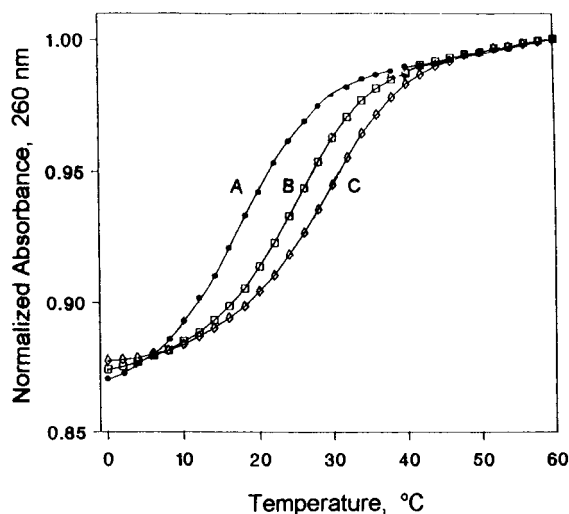
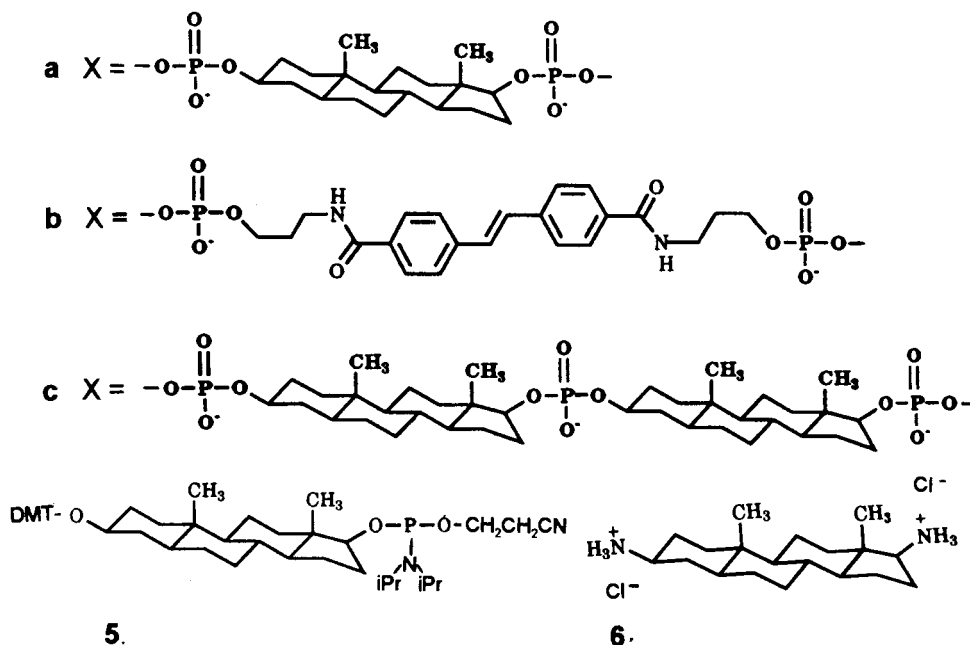


Figure 1. Thermal dissociation curves for complexes formed from **1a** + **2a** ($5 \mu M$ each) in 0.1 M NaCl, 10 mM Tris·HCl, pH 7.0, in the presence of A, no additive; B, compound **6** (1×10^{-4} M); and C, compound **6** (5×10^{-4} M).

of **6** are given in Figure 1. The results demonstrate that **6** does interact with and stabilize duplex **1a** + **2a**. For a solution 5×10^{-4} M in **6**, the increase in T_m is $\sim 10^\circ C$.

Control experiments carried out under comparable conditions showed that 1,10-diaminodecane dihydrochloride, a dicationic species with a relatively small hydrophobic surface, has no detectable effect on the dissociation of **1a** + **2a** and that substrate **6** has no detectable effect on dissociation of either **1b** + **2b** or 5' d-GCTGAAGTCT/3' d-CGACTTCAGA, duplexes which lack a hydrophobic pocket. We therefore conclude that the enhancement in thermal stability depends on both a hydrophobic pocket in the duplex and a water soluble substrate possessing a large hydrophobic surface.

A striking effect was observed when cetyltrimethylammonium bromide (CTAB) was added to the solution containing **1a** + **2a**. At a concentration of 5×10^{-4} M,

which is above the critical micelle concentration, it increased the T_m value for dissociation of the duplex by $34^\circ C$ (from 17 to $51^\circ C$). Below the critical micelle concentration, it was ineffective. These results suggest that the aligned steroid units in the duplex interact with the surfactant molecules to form micellar type structures in which the hydrophobic cavities are filled with hydrocarbon tails of the surfactant. A noteworthy feature is that the duplex formed by the conjugates did not precipitate in the presence of the CTAB micelles, even at $0^\circ C$. As previously reported, CTAB readily precipitates DNA and RNA from solution (8, 9). Also, we found that the oligonucleotides 5' d-GCTGAAGTCT/3' d-CGACTTCAGA precipitate when CTAB is added under the conditions used in the experiment with **1a** + **2a**. The cationic surfactant CTAB, therefore, is particularly effective as an additive for stabilizing the duplex formed by hybridization of the steroid-oligonucleotide conjugates; however, applications involving homogeneous systems containing unmodified oligonucleotides in addition to the modified oligonucleotides can be limited by the insolubility of the unmodified oligonucleotide-surfactant complexes.

Triplex Assembly. We then ask whether compound **6** might be exploited in enhancing stability or triggering formation of triple-stranded complexes possessing hydrophobic pockets. This question was examined with oligothymidylate and oligodeoxyadenylate conjugates **3a** and **4a**. Dissociation curves for complexes obtained using 1/1 and 2/1 dT/dA ratios in the presence and in the absence of substrate **6** are presented in Figure 2. It may be noted that the pattern for the duplex (curves C and D) is similar to that obtained for **1a** + **2a**, i.e., addition of **6** leads to an increase in T_m of several degrees. The melting curve for the mixture with a 2:1 dT/dA ratio in absence of **6** (curve A) exhibits a single break, corresponding to dissociation of the duplex, and significantly reduced normalized hyperchromicity, indicating that the second equivalent of the oligothymidylate conjugate is not bound to the duplex under the conditions of the

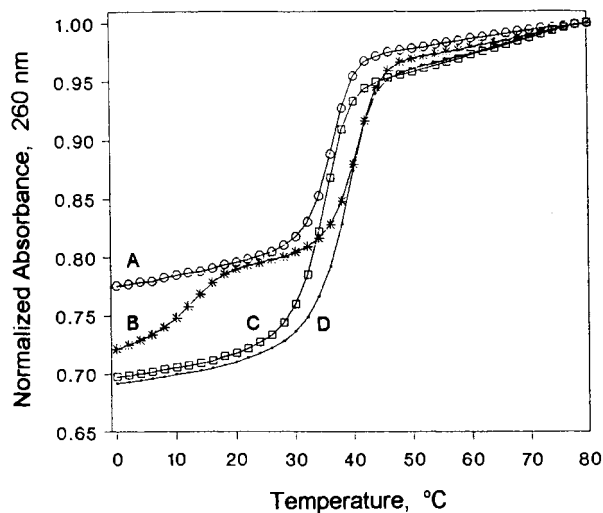


Figure 2. Dissociation curves for complexes of oligothymidylate conjugate **3a** + oligodeoxyadenylate conjugate **4a** in 0.1 M NaCl and 10 mM Tris·HCl, pH 7.0: A, **3a** (10 μ M) + **4a** (5 μ M); B, **3a** (10 μ M) + **4a** (5 μ M) + **6** (1×10^{-4} M); C, **3a** (5 μ M) + **4a** (5 μ M); D, **3a** (5 μ M) + **4a** (5 μ M) + **6** (1×10^{-4} M).

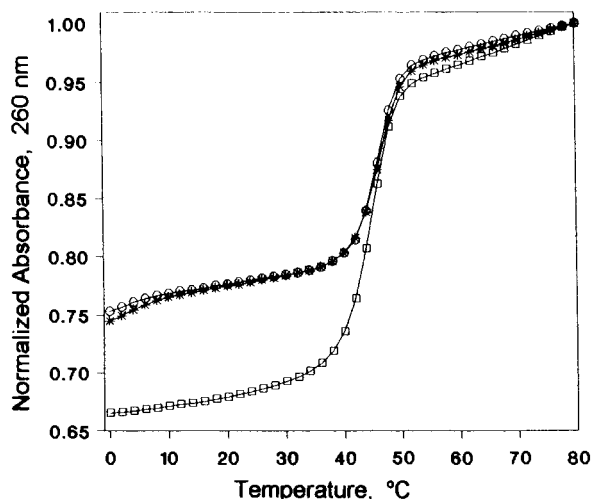


Figure 3. Dissociation curves for complexes of dT₂₀ + dA₂₀ in 0.1 M NaCl, 10 mM Tris·HCl, pH 7.0: circles, dT₂₀ (10 μ M) + dA₂₀ (5 μ M); stars, dT₂₀ (10 μ M) + dA₂₀ (5 μ M) + **6** (1×10^{-4} M); squares, dT₂₀ (5 μ M) + dA₂₀ (5 μ M).

experiment (0.1 M NaCl). In contrast, the 2:1 dT/dA mixture in the presence of **6** (1×10^{-4} M) exhibits not only an enhanced T_m for dissociation of the double-stranded complex but also a break in the curve at a lower temperature corresponding to dissociation of a third strand from a triple-stranded complex. As shown in Figure 3, **6** has no effect on complexes of comparable oligonucleotides lacking the hydrophobic sites. We conclude that **6** both interacts with the hydrophobic cavity in the double-stranded **3a** + **4a** complex and stimulates formation of a triple stranded complex in which the three androstane units linked within the conjugates are aligned in a structure stabilized by an interaction with **6**.

Double Pockets. Oligonucleotide conjugates containing multiple, contiguous phosphoryl–steroid bridges can be readily generated by repetitive condensations of phosphoramidite **5** at the proper stage in the oligomer synthesis. As representatives of this class of compounds, we prepared and examined the hybridization properties of conjugates **1c**, **2c**, **3c**, and **4c**. Interestingly, introduction of the second phosphoryl–androstane group leads to significant stabilization of the duplexes. For the mixed

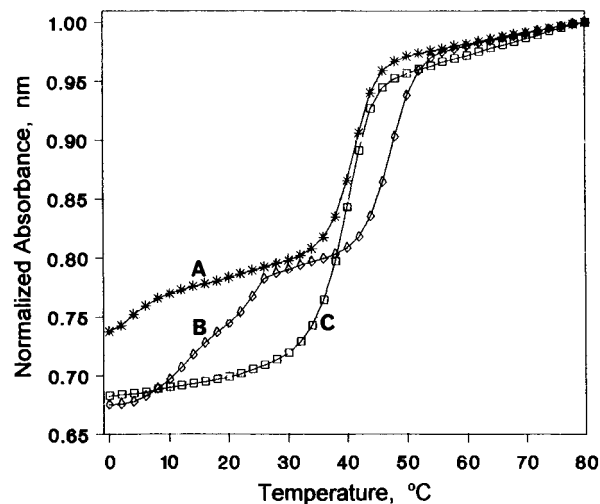


Figure 4. Dissociation curves for complexes of **3c** + **4c** in 0.1 M NaCl, Tris·HCl, pH 7.0: A, **3c** (10 μ M) + **4c** (5 μ M); B, **3c** (10 μ M) + **4c** (5 μ M) + **6** (1×10^{-4} M); C, **3a** (5 μ M) + **4c** (5 μ M).

base oligomers, **1c** + **2c**, the enhancement in T_m relative to that for **1a** + **2a** is 13 $^{\circ}$ C (T_m values, 30 and 17 $^{\circ}$ C for the **c** and **a** duplexes, respectively) and for the dT/dA compounds, **3c** + **4c**, the enhancement relative to **3a** + **3b** is 5 $^{\circ}$ C (T_m values, 41 and 36 $^{\circ}$ C, respectively). Addition of substrate **6** (1×10^{-4} M) further increases the stability of these duplexes (enhancement in T_m for **1c** + **2c**, 10 $^{\circ}$ C; for **3c** + **4c**, 6 $^{\circ}$ C). Our interpretation is that alignment of contiguous phosphoryl–androstane bridges in this system permits some, but not complete, contact between the hydrophobic units. This interaction stabilizes the duplex relative to that containing the single pocket (series **a**) even though the nonnucleotide bridge is longer and contains additional flexible linkages. Interaction with compound **6** then leads to additional hydrophobic contacts that further stabilize the complex.

The pattern for the melting curves for the double pocket 2dT/dA system (Figure 4) is similar to that for the single pocket 2dT/dA system (Figure 2) except that the complexes formed by the double pocket oligomers are somewhat more stable. A break at 10 $^{\circ}$ C in curve A (Figure 4) demonstrates that some triple-stranded complex forms below 10 $^{\circ}$ C even in absence of **6**; the break at 26 $^{\circ}$ C in curve B shows that addition of **6** further stabilizes the triple stranded complex, and the low absorbance at 0 $^{\circ}$ C in B indicates that formation of the triple stranded complex is essentially complete at 0 $^{\circ}$ C when **6** is present.

SUMMARY

These oligonucleotide–androstane derivatives may be considered prototypes for a family of conjugates that self-assemble in aqueous media to form hydrophobic cavities that can be used to take up appropriate substrates from solution. Two motifs were investigated. In one, a single androstane unit was inserted into the backbone of each member of a pair of complementary oligonucleotides such that hybridization would align the hydrophobic groups in proximity but at a distance unfavorable for direct hydrophobic interaction. Thermal dissociation experiments showed that the steroid inserts in these conjugates strongly destabilized the oligonucleotide duplex, as expected for a structure in which hydrophobic interaction between the bridging elements was weak and that addition of 3,17-diaminoandrostane dihydrochloride, a

water-soluble steroid derivative that could fit into the cavity formed by the androstane units, stabilized the duplex structure. In the second motif, a pair of androstane units linked by a phosphoryl group served as the insert. Complexes formed by these conjugates were somewhat more stable than those of the first type, yet the tandem bridges in the complexes still functioned as pockets that interacted with 3,17-diaminoandrostane dihydrochloride in solution. This additive was also effective in inducing formation of triple-stranded complexes in which three inserts of each type were aligned. The hydrophobic groups in these complexes may function both as a binding sites for appropriate substrates and as a control site for triggering selective enhancement in stability of a given segment of an oligonucleotide assembly. An attractive extension of the chemistry would be to utilize two conjugate strands to generate a binding site for a substrate together with a third strand that would bind to the duplex and align a functional group in position to catalyze a reaction of the bound substrate.

EXPERIMENTAL PROCEDURES

General Methods. Reagent grade chemicals were used throughout. Pyridine, 4-(dimethylamino)pyridine, and CH₃CN were dried over CaH₂. THF was freshly distilled over sodium and benzophenone prior to use. 3, 17-Diaminoandrostane was prepared as described (10) and was characterized by NMR, IR, and FAB MS. FAB mass spectra were obtained on a VG-70-250 SE mass spectrometer. ¹H and ³¹P NMR spectra were recorded on a Varian XL-300 MHz spectrometer using CDCl₃ as solvent. Chemical shifts are reported relative to TMS and 85% H₃PO₄ in parts per million. Elemental analyses were carried out by the Searle Laboratory and Oneida Research Services, Inc.

Melting curves were determined using a Perkin-Elmer Lambda 2 UV spectrometer equipped with a Peltier 2 temperature programmer for automatically increasing the temperature at the rate of 1 °C/min. The solutions were 5 μM in each oligonucleotide, 0.10 M in NaCl, and 10 mM in Tris·HCl (pH 7.0). Experiments with the hydrophobic additives (**6** and CTAB) were carried out by mixing the additive with the oligonucleotide complexes at room temperature, cooling slowly to 0 °C (~15 min), then monitoring the absorbance at 260 nm as the temperature was increased at the rate of 1 °C/min.

3β-(4,4'-Dimethoxytrityloxy)-5-α-androstan-17-one. 5-α-Androstan-3β-ol-17-one (2.9 g, 0.5 mmol) and 4-(dimethylamino)pyridine (61 mg, 0.5 mmol) were stirred with Et₃N (126 mL) for 15 min; then, dimethoxytrityl chloride (5.08 g, 15 mmol) was added, and the mixture was stirred at room temperature for 1.5 h. TLC indicated that the reaction of the steroid was complete. The mixture was poured into CH₂Cl₂ (100 mL), washed with 5% aqueous NaHCO₃, dried over Na₂SO₄, and evaporated under reduced pressure. Purification by column chromatography on silica gel with CH₂Cl₂/Et₃N/MeOH (98/1/1, v/v/v) then afforded 4.6 g (76%) of the title compound. ¹H NMR (CDCl₃): δ 0.79 (s, 3H, -CH₃), 0.82 (s, 3H, -CH₃), 0.5–2.5 (m, 20H), 3.35 (s, 1H, -C₃H), 3.8 (s, 6H, -OCH₃), 6.8–7.5 (m, 13 H, DMT). FAB MS: calcd M, 592.8; found, 593.3. Analysis calcd for C₄₀H₄₈O₄: C, 81.04; H, 8.16. Found: C, 80.71; H, 8.69.

3β-(4,4'-Dimethoxytrityloxy)-5-α-androstan-17-ol. NaBH₄ (5.2 g) was added with stirring to 3β-(4,4'-dimethoxytrityloxy)-5-α-androstan-17-one (4 g) in 150 mL of 1:1 THF/MeOH over a period of 45 min while maintaining the temperature <20 °C. After stirring for 14 h,

the mixture was poured into CH₂Cl₂, washed repeatedly with water, dried over Na₂SO₄, and evaporated. Column chromatography on silica gel using CH₂Cl₂/Et₃N/MeOH (98/1/1, v/v/v) afforded 3.5 g (88% yield) of the title compound. ¹H NMR (CDCl₃): 0.7 (s, 3H, -CH₃), 0.8 (s, 3H, -CH₃), 0.5–2.1 (m, 20H), 3.35 (s, 1H, -C₃H), 3.6 (m, 1H, -C₁₇H), 3.8 (s, 6H, -OCH₃), 5.8–7.5 (m, 13 H, DMT). FAB MS: calcd M, 594.8; found 594.4. Analysis calcd for C₄₀H₅₀O₄: C, 80.77; H, 8.47. Found: C, 80.28; H, 8.10.

2-Cyanoethyl N,N-Diisopropylphosphoramidite Derivative of 3β-(4,4'-Dimethoxytrityloxy)-5-α-androstan-17-ol (5). 2-Cyanoethyl N,N-diisopropylchlorophosphoramidite (0.42 mmol) was added with stirring under a nitrogen atmosphere to 3β-(4,4'-dimethoxytrityloxy)-5-α-androstan-17-ol (0.34 mmol) and diisopropylethylamine (1.00 mmol) in CH₂Cl₂ (3 mL). After 1 h, the reaction of the alcohol was complete as indicated by TLC. The mixture was diluted with CH₂Cl₂, washed twice with brine (50 mL each), dried (Na₂SO₄), and concentrated. Chromatography on a 250 mm silica gel plate (10 × 25 cm) using EtOAc:hexane:Et₃N (10/10/3 by volume) gave the desired product, **5** (0.26 g, 95%), as a white solid ³¹P NMR: 147.47 and 148.39 ppm.

Oligonucleotide Conjugates. These compounds were synthesized on an Expedite DNA Synthesizer using commercial phosphoramidite reagents and intermediate **5**. Couplings with **5** proceeded smoothly in >95% yield as judged by the dimethoxytrityl assay. The DMT-protected oligomers were isolated by reversed-phase (RP) HPLC on a Hypersil ODS column (4.6 × 200 mm, 5 μ particle size) with a 1%/min gradient of CH₃CN in Et₃NH⁺OAc⁻ buffer, pH 7.0. The products were lyophilized, detritylated (80% aqueous acetic acid, 30 min, followed by washing with ether and lyophilization), and purified by RP-HPLC. The elution times for **1a**, **2a**, **3a**, **4a**, **1c**, **2c**, **3c**, and **4c** were 20.5, 20.7, 21.2, 21.7, 26.5, 27.0, 26.9, and 27.5 min, respectively. These times are somewhat longer than those for the corresponding underivatized oligonucleotides (18.9, 19.1, 19.5, and 19.7 min for d-GCTCAAGTCT, d-AGACTTCAGC, dT₂₀, and dA₂₀, respectively), reflecting the presence of the hydrophobic inserts. The conjugates were also characterized by polyacrylamide gel electrophoresis carried out with nondenaturing cross-linked polyacrylamide gel (15% bisacrylamide) purchased from ICN, CA. The migration distances relative to bromophenol blue for oligomers **1a**, **2a**, **3a**, **4a**, **1c**, **2c**, **3c**, and **4c** were 1.03, 1.13, 0.65, 0.70, 0.83, 0.94, 0.58, and 0.65, respectively. For comparison, the relative migration distances for d-GCTCAAGTCT, d-AGACTTCAGC, dT₂₀, and dA₂₀ were 1.06, 1.17, 0.78, and 0.91, respectively.

ACKNOWLEDGMENT

This research was supported by a grant from the National Institute of General Medical Sciences (GM 10265). Conjugates **1b** and **2b** were provided by Dr. T. Wu.

LITERATURE CITED

- (1) Bender, M., and Komiyama, M. (1978) *Cyclodextrin Chemistry*, Springer-Verlag, New York.
- (2) Zhang, B., and Breslow, R. (1997) Ester hydrolysis by a catalytic cyclodextrin dimer enzyme mimic with a metallo-bipyridyl linking group. *J. Am. Chem. Soc.* 119, 1676–1681.
- (3) Chroma, C. T., Lear, J. D., Nelson, M. J., Dutton, P. L., Robertson, D. E., and DeGrado, W. F. (1994) Design of a heme-binding four-helix bundle. *J. Am. Chem. Soc.* 116, 856–865.

- (4) Letsinger, R. L., and Wu, T. (1994) Control of excimer emission and photochemistry of stilbene units by oligonucleotide hybridization. *J. Am. Chem. Soc.* **116**, 811–812.
- (5) Lewis, F. D., Wu, T., Burch, E. L., Bassani, D. M., Yang, J.-S., Schneider, S., Jäger, W., and Letsinger, R. L. (1995) Hybrid oligonucleotides containing stilbene units. Excimer fluorescence and photodimerization. *J. Am. Chem. Soc.* **117**, 8785–8792.
- (6) Letsinger, R. L., Chaturvedi, S. K., Farooqui, F., and Salunkhe, M. (1993) Use of hydrophobic substituents in controlling self-assembly of oligonucleotides. *J. Am. Chem. Soc.* **115**, 7535–7536.
- (7) Gryaznov, S. M., and Lloyd, D. H. (1993) Modulation of oligonucleotide duplex and triplex stability via hydrophobic interactions. *Nucleic Acids Res.* **21**, 5909–5915.
- (8) Trewaras, A. (1967) A new method for counting labeled nucleic acids by liquid scintillation. *Anal. Biochem.* **21**, 324–329.
- (9) Sibatani, A. (1970) Precipitation and counting of minute quantities of labeled nucleic acids as cetyltrimethylammonium salts. *Anal. Biochem.* **33**, 279–285.
- (10) Dodgson, D. P., and Haworth, R. D. (1952) Some basic steroid derivatives. *J. Chem. Soc.* 67–71.

BC980060I

Synthesis of Oligodeoxynucleotides Containing N^4 -Mercaptoethylcytosine and Their Use in the Preparation of Oligonucleotide–Peptide Conjugates Carrying *c-myc* Tag-Sequence

Dirk Gottschling, Hartmut Seliger,[†] Gema Tarrasón,[‡] Jaume Piulats,[‡] and Ramon Eritja*

European Molecular Biology Laboratory, Meyerhofstrasse 1, D-69117 Heidelberg, Germany, Universität Ulm, Sektion Polymere, Albert-Einstein-Allee 11, D-89081 Ulm, Germany, and Merck Farma-Química, Laboratori de BioInvestigació, C/Casp 108, 08010 Barcelona, Spain. Received June 26, 1998; Revised Manuscript Received August 17, 1998

The preparation and properties of oligodeoxynucleotides containing mercaptoethyl groups at position N-4 of cytosine are described. The resulting thiol-oligodeoxynucleotides were reacted with a maleimido-peptide carrying the *c-myc* tag-sequence. The peptide-oligonucleotide conjugate is specifically recognized by an anti *c-myc* monoclonal antibody, thus constituting a labeling system with sensitivity similar to other existing methods of nonradioactive labeling.

INTRODUCTION

Oligonucleotides carrying thiol groups are useful intermediates for different applications including (a) introduction of biotin, fluorescent compounds, and other nonradioactive labels (Beaucage and Iyer, 1993; Connolly, 1985); (b) preparation of conformationally restricted oligonucleotides (Ferentz et al., 1993; Glick, 1991; Osborne, 1997); (c) immobilization of oligonucleotides to solid supports such as affinity columns (Blanks and McLaughlin, 1988) and gold particles (Elghanian et al., 1997; Mirkin et al., 1996); and (d) preparation of oligonucleotide-peptide conjugates (Corey, 1995; Eritja, 1991; Zuckermann and Schultz, 1988).

In most of the cases, the thiol groups are introduced at the 5'- and 3'-ends in order to maintain the hybridization properties of oligonucleotides (Beaucage and Iyer, 1993; Connolly, 1985). But also thiol groups have been introduced at the phosphate bond (Fidanza and McLaughlin, 1992), at the 2'-position (Gundlach et al., 1997), and at the nucleobases (Goodwin and Glick, 1993; Osborne, 1997). We are interested in introducing peptide sequences in oligonucleotides as a nonradioactive labeling system. The great diversity of antibodies raised against peptide sequences and the many possibilities with regard to the labeling of such antibodies, leads us to consider oligo-tag-peptide conjugates as alternative labeling systems with great potential. Not only can such conjugates be used in hybridization assays but also different tag sequences can be introduced in the four nucleobases, which can be recognized by differently labeled antibodies, and used as sequencing tools. To test these possibilities, oligonucleotides carrying the *c-myc* tag-sequence have been prepared (Figure 1). For the preparation of the oligonucleotide-peptide conjugates, the protocol based on the reactivity of the thiol group was used (Eritja et al., 1991). In this method, a thiol group is introduced on the

oligonucleotide that will react with a maleimido-peptide carrying the *c-myc* tag-epitope. For this purpose, a derivative of 2'-deoxycytidine carrying the mercaptoethyl group at position N-4 has been developed (Figures 1 and 2). The synthesis of oligonucleotides carrying N^4 -mercaptoethyl-dC have been previously described (MacMillan and Verdine, 1991; Wolfe and Verdine, 1993) using a 2'-deoxyuridine derivative with the 2,4,6-trimethylphenyl group at position 4, which is reacted with cystamine at the end of the synthesis. This method needs postsynthetic steps and could produce some unwanted side reactions (MacMillan and Verdine, 1991). In the present article, a protected derivative of N^4 -mercaptoethyl-2'-deoxycytidine is described. This derivative is compatible with the standard phosphoramidite methodology and yields oligonucleotides carrying mercaptoethylcytosine residues using standard deprotection conditions. These oligonucleotides have been used for the preparation of an oligonucleotide-peptide conjugate carrying the *c-myc* tag-sequence. The conjugate is recognized by an anti-*c-myc* monoclonal antibody, and detection is achieved through interaction with a peroxidase conjugated antibody and a chemiluminiscent substrate.

EXPERIMENTAL SECTION

Materials and Methods. All ^1H -, ^{31}P -NMR, and ^{13}C -NMR spectra were recorded on a Bruker AM 250 spectrometer. Chemical shifts are reported in parts per million (δ) relative to an internal standard. High-performance liquid chromatography (HPLC) was performed either on a Waters chromatograph (Millipore) or on a BioCAD Sprint chromatograph (PerSeptive Biosystems). The products were purified by using one of the following columns: Nucleosil 120 C18 (250 $\times$ 4 mm) or PRP-1 (Hamilton, 305 $\times$ 7 mm). Buffer A was 5% acetonitrile in 0.1 M aqueous triethylammonium acetate (TEAA) (pH = 6.5). Buffer B was 70% acetonitrile in 0.1 M aqueous TEAA (pH = 6.5). A 30 min gradient from 0 to 60% B was used. Column chromatography was performed on Kieselgel (35–70 μm) (SDS) using solvent systems indicated in the text. Analytical thin-layer chromatography (TLC) was performed on aluminum foil supported silica gel 60 F₂₅₄ (Merck). For immunodetec-

* Author to whom correspondence should be addressed at European Molecular Biology Laboratory. Phone: 49-6221-387210. Fax: 49-6221-387306. Email: eritja@embl-heidelberg.de.

[†] Universität Ulm.

[‡] Merck Farma-Química.

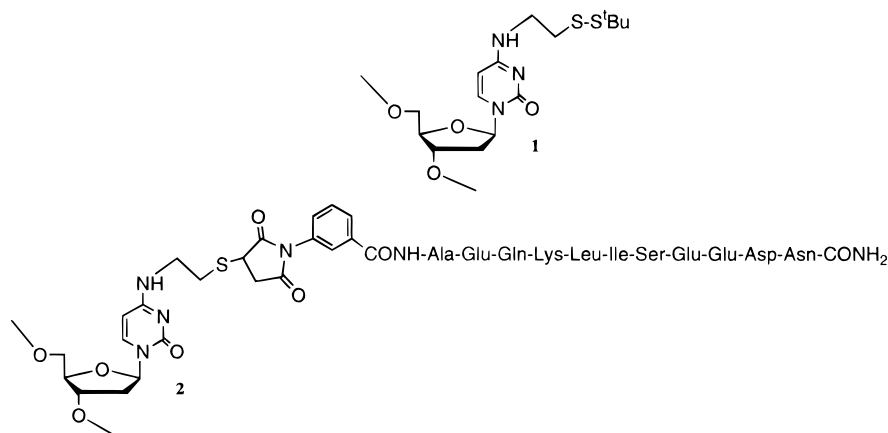


Figure 1. Chemical structures of N^4 -(*tert*-butylthioethyl)-dC (**1**) and N^4 -(peptide)-dC conjugate (**2**) prepared in this work.

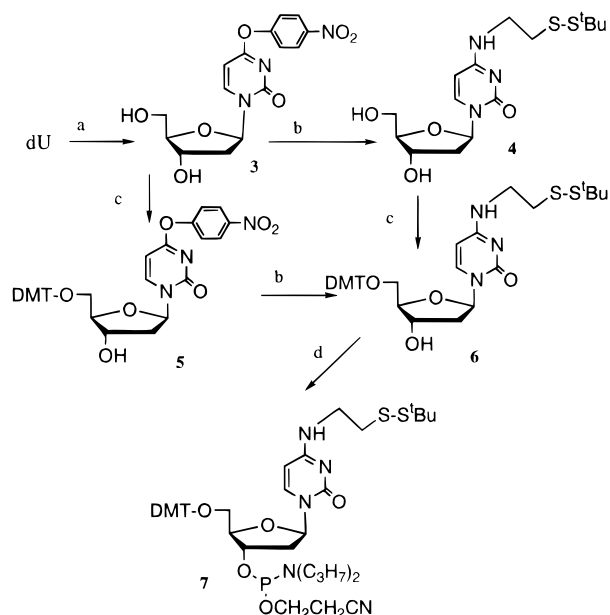


Figure 2. Synthesis of the N^4 -(*tert*-butylthioethyl)-dC phosphoramidite derivative. (a) Hexamethyldisilazane followed by mesitylenesulfonyl chloride, Et_3N , DMAP and *p*-nitrophenol. (b) 2-Aminoethyl-*tert*-butyl disulfide. (c) Dimethoxytrityl chloride, pyridine. (d) 2-Cyanoethoxy-*N,N*-diisopropylamino chlorophosphine.

tion of oligonucleotide-peptide conjugate, samples were blotted with a dot-blot manifold (GIBCO, BRL) on a nitrocellulose membrane 0.2 μm (Bio-Rad). Immunodetection was carried out with supernatant from a murine hybridoma anti-*c-myc* (clone 9E10, 24.6 $\mu\text{g/mL}$; Evan, 1985) and a Goat anti-mouse peroxidase-conjugated secondary antibody (IgG + IgM, Jackson Immunoresearch Laboratories), and finally developed with ECL (Amersham). Washes of the membrane between antibody incubations were performed in a phosphate-buffered saline containing high-salt concentration (0.69 g of $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 1.98 g of Na_2HPO_4 , 58.44 g of NaCl) and 0.1% Tween-20 (Merck).

Chemicals. Nucleosides were purchased from Pharma Waldhof GmbH (Germany). All chemicals for the synthesis of the modified nucleosides were obtained from Aldrich, Fluka (Switzerland), Merck (Germany), and Sigma. Anhydrous solvents were obtained from Merck (Germany), SDS (France), PerSeptive Biosystems, and Romil (U.K.). All chemicals for the peptide synthesis were purchased from Novabiochem (Switzerland) except 3-maleimidobenzoic acid *N*-hydroxysuccinimide ester

(Bachem), 1-hydroxybenzotriazole (HOBt), trifluoroacetic acid (TFA), and piperidine that were purchased from Aldrich. All chemicals for the oligonucleotide synthesis were purchased either from Perkin-Elmer-Applied Biosystems or PerSeptive Biosystems. Phosphoramidites protected with more labile *tert*-butylphenoxyacetyl groups (PerSeptive Biosystems) were used. Phosphodiesterase (from *Crotalus durissus*) and alkaline phosphatase were obtained from Boehringer Mannheim (Germany).

2-Aminoethyl-*tert*-butyl Disulfide. Di-*tert*-butyl 1-(*tert*-butylthio)-1,2-hydrazinedicarboxylate (Wünsch et al., 1982) (25 g, 78 mmol) and triethylamine (5.4 mL, 39 mmol) were dissolved in anhydrous dimethylformamide (DMF) (70 mL) under argon. A solution of cysteamine hydrochloride (4.43 g, 39 mmol) and dry DMF (150 mL) was added dropwise to the stirred solution. After stirring 24 h at room temperature, a white solid was precipitating. Dry DMF (200 mL) was added to obtain a nearly clear solution. Triethylamine was added until a white solid was precipitating again. The reaction mixture was stirred at room temperature for several hours. The solution was filtered and evaporated under reduced pressure. Wet diethyl ether (20 mL) was added to the residue and filtered. The white solid was collected and dried in a desiccator. Afterward, this white solid was dissolved five times in wet diethyl ether (5×10 mL), stirred for several minutes, and filtered. The desired product (3.0 g, 46%) was obtained as a white solid. TLC (methanol/dichloromethane 10:90) $R_f = 0.17$; ^1H NMR, δ_{H} (CDCl_3): 1.36 (s, 9H), 3.07 (t, 2H), 3.40 (t, 2H), 8.30 (s, 2H). ^{13}C NMR, δ_{C} (CDCl_3): 30.03, 35.91, 38.86, 48.42; FAB⁺MS 166 $[\text{M} + \text{H}]^+$ ($\text{M} = 165.1$ g/mol calculated for $\text{C}_6\text{H}_{15}\text{NS}_2$). Anal calcd for $\text{C}_6\text{H}_{15}\text{NS}_2$: C, 43.59; H, 9.15; N, 8.47; S 38.79. Found: C, 43.26; H, 9.11; N, 8.32; S, 38.65.

4-(*p*-Nitrophenoxy)-1-(β -D-2-deoxyribofuranosyl)-pyrimidin-2(1H)-one (3). 2'-Deoxyuridine (7.3 g, 32 mmol) was dried by evaporation of anhydrous DMF under reduced pressure, and the residue was dissolved in anhydrous DMF (150 mL) under argon. Hexamethyldisilazane (13.8 mL, 64 mmol) was added with a syringe to the stirred solution with exclusion of moisture. After 30 min of magnetic stirring at room temperature, the solution was evaporated under reduced pressure. The residue was dissolved three times in toluene (3×10 mL) and concentrated to dryness. The residue was dissolved in a small amount of dichloromethane. Triethylamine (24.1 mL, 172.2 mmol) and 4-(dimethylamino)pyridine (DMAP) (0.94 mg, 7.7 mmol) were added. A solution of mesitylenesulfonyl chloride (10.1 g, 46.4 mmol) in dichloromethane (20 mL) was added dropwise with exclusion

of moisture. After 30 min of magnetic stirring at room temperature 1,4-diazabicyclo[2.2.2]octane (0.69 g, 6.1 mmol) and a solution of 4-nitrophenol (8.6 mg, 62.3 mmol) in tetrahydrofuran (20 mL) was added to the red reaction mixture. The reaction mixture was stirred overnight at room temperature. After removing the solvent under reduced pressure, the residue was dissolved in dichloromethane (150 mL), washed with 5% aqueous sodium hydrogen carbonate (150 mL), and washed with saturated NaCl (150 mL), and the organic phase was dried with sodium sulfate. The solvent was removed under reduced pressure. Acetic acid:methanol (1:4 v/v, 96 mL) was added to the residue, and the resulting solution was stirred for 10 h at room temperature. The white solid was collected. To increase the yield, the solution was reduced to 25 mL, and diethyl ether (45 mL) was added dropwise. After 3 h of stirring, the white solid was collected. The solids were combined and washed three times with dichloromethane (20 mL). The desired product (5.2 g, 47%) was obtained as a white solid. TLC (10% methanol in dichloromethane) $R_f = 0.4$; physical and spectroscopic data were in agreement with previously published data (Miah et al., 1997). FAB-MS 349 $[M]^-$ ($M = 349.3$ g/mol calculated for $C_{15}H_{15}N_3O_7$).

2'-Deoxy-N⁴-(tert-butylthioethyl)cytidine (4). 2-Aminoethyl-tert-butyl disulfide (2.95 g, 18 mmol) was dissolved in a 40 °C warm solution of dry DMF (150 mL) and triethylamine (2.5 mL, 18 mmol). After addition of compound **3** (2.5 g, 7.16 mmol), the mixture was stirred for 14 h at 50 °C. The solvent was removed under reduced pressure, and the residue was dissolved two times in toluene (2×30 mL) and concentrated to dryness. The residue was dissolved in a small amount of dichloromethane and purified by chromatography on silica gel. The product was eluted with a gradient of methanol from 0 to 15% in dichloromethane. ¹H-NMR spectroscopy showed that the product was contaminated with 2-aminoethyl-tert-butyl disulfide. To purify the product a column with a 10-fold excess (by mass) of Dowex-50WX4 resin was used. First, the resin was washed with methanol until the solvent was colorless. After, the residue was dissolved in a small amount of methanol and the solution was applied on the column. The Dowex (50WX4) resin was washed several times with methanol. The product was eluted with a 5% triethylamine solution in methanol. The pure compound was obtained as white, oily foam (1.48 g, 54%). TLC (10% methanol in dichloromethane) $R_f = 0.18$. ¹H NMR, δ_H (CD_3OD): 1.32 (d, 9H), 2.15 (m, 1H), 2.32 (m, 1H), 2.91 (t, 2H), 3.65 (t, 2H), 3.74 (m, 2H), 3.91 (m, 1H), 4.34 (m, 1H), 5.84 (d, 1H), 6.25 (t, 1H), 7.89 (d, 1H). ¹³C NMR, δ_C (CD_3OD): 30.29, 39.93, 40.88, 42.03, 49, 62.86, 72.10, 87.56, 88.83, 97.04, 141.32, 158.65, 165.62. FAB-MS 376 $[M + H]^+$ ($M = 375.3$ g/mol calculated for $C_{15}H_{25}N_3O_4S_2$). Anal calcd for $C_{15}H_{25}N_3O_4S_2$: C, 47.98; H, 6.71; N, 11.19; S, 17.08. Found: C, 47.67; H, 6.52; N, 11.32; S, 17.13.

The silica gel purification step can be avoided. In this case, after Dowex purification, ¹H NMR of the product shows a very small amount of impurities in the final product which are not present when the silica gel purification is performed.

4-(p-Nitrophenoxy)-1-[5-O-(4,4'-dimethoxytriphenylmethyl)- β -D-2-deoxyribofuranosyl]pyrimidin-2(1H)-one (5). Compound **3** (2.25 g, 6.44 mmol) was dried by evaporation of anhydrous pyridine in vacuo and then dissolved in dry pyridine (26 mL) under argon. Triethylamine (2.13 mL, 15.3 mmol) and 4,4'-dimethoxytriphenylmethyl chloride (2.62 g, 7.74 mmol) were added with exclusion of moisture. The reaction mixture was

stirred overnight at room temperature. At this time, the reaction was not complete as judged by TLC. As a consequence more 4,4'-dimethoxytriphenylmethyl chloride was added (2×0.44 g, 2×1.3 mmol). After each addition the reaction mixture was allowed to stir at room temperature for 2 h. Afterward the reaction was quenched with methanol (1 mL). The solvent was removed in vacuo at room temperature, and the residue was dissolved in chloroform (100 mL). The solution was washed with 5% aqueous sodium hydrogen carbonate (100 mL) and with saturated aqueous sodium chloride (100 mL). After drying the organic phase with sodium sulfate, the solvent was evaporated under reduced pressure at room temperature. The residue was dissolved two times in toluene (2×10 mL) and evaporated to dryness. The residue was dissolved in a small amount of dichloromethane and purified by chromatography on silica gel. The column was packed with silica gel using a 1% triethylamine solution in dichloromethane. The product was eluted with a gradient of methanol from 0 to 2% in dichloromethane. The pure compound was obtained as yellow foam (2.76 g, 65.8%). TLC (5% methanol in dichloromethane) $R_f = 0.72$ (TLC plates were preincubated with triethylamine). ¹H NMR, δ_H ($CDCl_3$): 2.26 (m, 1H), 2.61 (m, 1H), 3.44 (dq, 2H), 3.80 (s, 6H), 4.08 (m, 1H), 4.52 (m, 1H), 5.82 (d, 1H), 6.21 (t, 1H), 6.82–7.41 (m, 15H), 8.23 (m, 2H), 8.34 (d, 1H). ¹³C NMR, δ_C ($CDCl_3$): 41.99, 55.29, 62.51, 70.67, 86.34, 87.06, 87.16, 94.75, 113.35, 122.74, 125.36, 127.18, 128.05, 128.17, 130.12, 135.29, 135.41, 144.34, 145.27, 145.27, 155.01, 156.38, 158.76, 170.48. FAB-MS 650 $[M - H]^-$ ($M = 651.6$ g/mol calculated for $C_{36}H_{33}O_9N_3$). Anal calcd for $C_{36}H_{33}O_9N_3$: C, 66.35; H, 5.10; N, 6.45. Found: C, 66.12; H, 5.25; N 6.27.

2'-Deoxy-5'-O-(4,4'-dimethoxytriphenylmethyl)-N⁴-(tert-butylthioethyl)-cytidine (6). **Starting from Compound 5.** 2-Aminoethyl-tert-butyl disulfide (0.51 g, 3.08 mmol) was dissolved in a 40 °C warm solution of dry DMF (26 mL) and triethylamine (0.43 mL, 3.08 mmol) under argon. Compound **5** (1 g, 1.53 mmol) was added with exclusion of moisture. The reaction mixture was stirred for 22 h at 45 °C. After this time, TLC (2% methanol in dichloromethane, TLC plates were preincubated with 1% triethylamine) showed no complete reaction. As a consequence, more 2-aminoethyl-tert-butyl disulfide (0.14 g, 0.85 mmol) was added and the solution was stirred for 7 h at 45 °C. After removing the solvent under reduced pressure, the residue was dissolved two times in toluene (2×10 mL) and concentrated to dryness. The residue was dissolved in chloroform (70 mL), washed with 5% aqueous sodium hydrogen carbonate (70 mL) and washed two times with saturated NaCl (70 mL). The organic phase was dried with sodium sulfate, filtered, and concentrated to dryness. The residue was dissolved in a small amount of dichloromethane and purified by chromatography on silica gel. The column was packed with silica gel using a 1% solution of triethylamine in dichloromethane. The product was eluted by a gradient of methanol from 0 to 2% in dichloromethane. The pure product was a colorless oil (0.55 g, 53%): TLC (5% methanol in dichloromethane) $R_f = 0.57$ (TLC plates were preincubated with triethylamine). ¹H NMR, δ_H ($CDCl_3$): 1.32 (s, 9H), 2.22 and 2.59 (m, 2H), 2.87 (m, 2H), 3.39 (m, 2H), 3.79 (m, 8H), 4.07 (m, 1H), 4.51 (m, 1H), 5.35 (d, 1H), 5.44 (s, 1H), 6.34 (t, 1H), 6.81–7.41 (m, 13 H), 7.81 (d, 1H). ¹³C NMR, δ_C ($CDCl_3$): 29.93, 39.07, 39.07, 41.99, 48.22, 55.26, 63.05, 71.32, 85.76,

86.11, 86.73, 94.78, 113.24, 126.97, 127.93, 128.17, 130.10, 135.54, 135.62, 140.24, 144.53, 156.16, 158.59, 163.58.

Starting from Compound 4. 2'-Deoxy-N⁴-(*tert*-butyldithioethyl)cytidine (**4**) (0.58 g, 1.54 mmol) was reacted with 4,4'-dimethoxytriphenylmethyl chloride (0.66 g, 1.95 mmol) in dry pyridine (2.5 mL) under argon. After 4 h of stirring at room temperature, the reaction mixture was quenched with methanol (1 mL), and the mixture was treated as described for compound **5** giving the desired product as colorless oil (0.6 g, 57%). The purified product has the same chromatographic and spectroscopic properties as the product described above. In this route, it is possible to observe the formation of a small percentage of a compound with two DMT groups: one at the 5'-OH and the other at position N⁴. This side product is easily separated during silica gel purification.

2'-Deoxy-5'-O-(4,4'-dimethoxytriphenylmethyl)-N⁴-(*tert*-butyldithioethyl)cytidine-3'-O-(2-cyanoethyl N,N-diisopropylphosphoramidite) (7). Compound **6** (0.32 g, 0.47 mmol) was dried by evaporation of anhydrous acetonitrile in vacuo, and the residue was dissolved in dry acetonitrile (10 mL) under argon. N,N-Diisopropylethylamine (0.17 mL, 0.95 mmol) was added with exclusion of moisture. The solution was cooled on ice and 2-cyanoethoxy N,N-diisopropylaminochlorophosphine (0.16 mL, 0.72 mmol) was added dropwise with a syringe over a period of 2 min. Afterward, the solution was stirred at 0 °C for 5 min and at room temperature for 1 h. After this time, TLC (ethyl acetate/hexane 1/1 with 5% triethylamine, TLC plates were preincubated with triethylamine) showed complete reaction. The solvent was removed in vacuo and the residue was dissolved in dichloromethane (20 mL). The solution was washed with 1 M aqueous sodium hydrogen carbonate (50 mL) and afterward with saturated NaCl (50 mL). After drying the organic phase with sodium sulfate, the solvent was removed in vacuo. The residue was dissolved in a solution (1 mL) of ethyl acetate/hexane (2/1) with 5% triethylamine and purified by chromatography on silica gel. The product was eluted with ethyl acetate/hexane (2/1) with 5% triethylamine. The pure product was obtained as white foam (0.23 g, 57%); TLC (ethyl acetate/hexane 2/1 with 5% triethylamine) *R_f* = 0.49 and 0.36 (TLC plates were preincubated with triethylamine). ¹H NMR, δ_{H} (CDCl₃): 1.0–1.7 (3 d, 12H), 1.32 (s, 9H), 2.20 (m, 1H), 2.40 and 2.60 (2 t, 2H, two diastereoisomers), 2.62 (m, 1H), 2.88 (t, 2H), 3.40 and 3.48 (m, 2H), 3.6–3.8 (m, 9H), 4.09 (m, 1H), 4.60 (m, 1H), 5.30 (m, 2H), 6.32 (m, 1H), 6.81–7.41 (m, 13H), 7.80 and 7.90 (2d, 1H, two diastereoisomers). ³¹P NMR, δ_{P} (CH₂Cl₂; concentric external D₂O lock): 145.62 ppm. Anal. Calcd for C₄₅H₆₀N₅O₇S₂P: C, 61.55; H, 6.89; N, 7.98; S, 7.30. Found: C, 61.16; H, 6.95; N, 7.58; S, 6.94.

Synthesis of the Peptide Containing the c-myc Tag Sequence. The peptide *m*-maleimido-benzoyl-Ala-Glu-Gln-Lys-Leu-Ile-Ser-Glu-Glu-Asp-Leu-Asn was synthesized manually by using a Rink-Amide support (0.4 g, functionalization 0.56 mmol/g) and Fmoc strategy on a 0.2 mmol scale. The Fmoc protecting group was removed by treating the resin with a 1/1 solution of piperidine/DMF for 2 and 8 min with a solution of 20% piperidine in DMF. Afterward, the resin was washed four times with DMF. A mixture of 4 equiv of Fmoc-protected amino acid (0.9 mmol), 4 equiv of N-hydroxybenzotriazole (0.121 g, 0.9 mmol), 4 equiv of 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) (0.34 μ L, 0.9 mmol), 8 equiv of N-ethyl-diisopropylamine (312 μ L, 1.8 mmol), and a small

amount of DMF was preactivated for 5 min and afterward added to the resin. Fmoc-Asn, Fmoc-Asp(*t*Bu) and Fmoc-Gln were used as pentafluorophenyl esters. In this case, 6 equiv of activated ester (1.34 mmol) and 6 equiv of HOBT (0.182 g, 1.34 mmol) were dissolved in a small amount of DMF and added directly to the resin. After 25 min, the completion of the coupling reaction was checked with the ninhydrin test (Sarin et al., 1981). If the ninhydrin test was positive, the reaction mixture was incubated for further 30 min. If the ninhydrin test was still positive after this time, the coupling reaction was repeated. To block unreacted amino groups, a mixture of acetic anhydride (1 mL), diisopropylethylamine (1.7 mL) and DMF (15.3 mL) was added for 15 min.

If the reaction was complete, the resin was washed three times with DMF, and the next cycle of deprotection and addition followed. At the last step, 6 equiv of 3-maleimidobenzoic acid N-hydroxysuccinimide ester (MBS) and 6 equiv of HOBT in a small amount of DMF were added. The maleimido-peptide was deprotected and released from the resin by treatment with TFA containing 5% of water for 4 h at room temperature. The resin was filtered and washed several times with water. The combined solutions were evaporated under reduced pressure. The residue was treated three times in diethyl ether and concentrated to dryness to eliminate the excess of TFA. MS (MALDI-TOF): 1587.2 [M + H]⁺ (M = 1585.5 g/mol calculated for C₆₉H₁₀₄N₁₈O₂₅).

Synthesis and Purification of Oligonucleotides.

The oligonucleotides were synthesized either on an ABI 394 DNA-synthesizer or on a PerSeptive Expedite 8900 synthesizer using the protocols recommended by the supplier. The standard β -cyanoethyl-protected phosphoramidite method was used. Phosphoramidites protected with *tert*-butylphenoxyacetyl groups (PerSeptive Biosystems) were used. The coupling efficiency of the modified phosphoramidite (**7**) was 98.2–99.8% (as measured by the absorbance of the DMT group). The oligonucleotides were removed from the solid support by treatment with concentrated ammonia for 6 h at 55 °C. Under these conditions, all base protecting groups were also cleaved. The crude oligonucleotides were purified with OPC cartridges (Cruachem, Glasgow, Great Britain). The following oligonucleotide sequences (Fàbrega, 1995) were synthesized on a 1 μ mol scale using the DMTr-on method: (O1) 5'-d(TAG AGG 1TC CAT TGC)-3', MS (MALDI-TOF) [M] = 4716.1 (M = 4715.5 g/mol calculated for C₁₅₂H₁₉₇N₅₅O₈₉P₁₄S₂); (O2) 5'-d(TAG AGG 1TC 1AT TGC)-3', MS (MALDI-TOF) [M] = 4864.6 (M = 4863.6 g/mol calculated for C₁₅₈H₂₀₉N₅₅O₈₉P₁₄S₄); (O3) 5'-d(TAG AGG CT1 1AT TGC)-3', MS (MALDI-TOF) 4868.1 [M + H]⁺ (M = 4863.6 g/mol calculated for C₁₅₈H₂₀₉N₅₅O₈₉-P₁₄S₄).

All oligonucleotides were characterized by mass spectrometry and enzymatic digestion.

Synthesis and Purification of the Oligonucleotide–Peptide Conjugate. To prevent side reactions, the oligonucleotide and the maleimido-peptide were cleaved from the solid support just before use. For the same reason, the maleimido-peptide was used without further purification. For the preparation of the oligonucleotide–peptide conjugate, an oligonucleotide (24 OD units at 260 nm) with the sequence 5'-d(TAG AGG 1TC CAT TGC)-3' was used. After purification with OPC cartridges, the oligonucleotide was treated with a solution (1 mL) of 0.1 M dithiothreitol (DTT) in concentrated ammonia at 55 °C overnight. Under these conditions, the *tert*-butylthiol protecting group was removed. The solution was concentrated to dryness. The residue was

dissolved in sterile water. DTT was removed by using a NAP-10 column (Pharmacia). The oligonucleotide was eluted with 1.5 mL of sterile water. This solution was collected directly in buffer A (500 mL), which contained a 10-fold excess of the maleimido-peptide. To adjust the solution to pH = 6.5, some drops of 1 M aqueous TEAA solution (pH = 6.5) were added. After 10 h at room temperature, the reaction mixture was concentrated to dryness, dissolved in sterile water, and purified with HPLC. Yield: 3 OD units at 260 nm (12%). MS (MALDI-TOF): 6214 [M + H]⁺ (M = 6213.0 g/mol calculated for C₂₁₇H₂₉₃N₇₃O₁₁₄P₁₄S). The oligonucleotide-peptide conjugate was also characterized by enzymatic digestion.

Melting Studies. Melting studies were performed in an aqueous solution (1 mL) of 0.15 M NaCl, 50 mM Tris-HCl, pH 7.5. Pentadecamer duplexes were made by mixing equimolar amounts (0.5 OD units at 260 nm) of two complementary pentadecamer strands. Duplexes were annealed by slow cooling from 80 °C to room temperature. UV-absorption and melting curves of the oligonucleotides were measured at 260 nm in 1 cm path-length cells on a Varian Cary 13 UV spectrometer.

Enzymatic Digestion. All oligonucleotides and oligonucleotide-peptide conjugates were subjected to enzymatic digestion. Pure product (0.5 OD) was incubated with 92 μ L of H₂O, 5 μ L of 1 M aqueous Tris-HCl solution (pH 8.0), 1 μ L of 1 M MgCl₂ solution, 1 μ L of phosphodiesterase solution, and 1 μ L of alkaline phosphatase solution for 8 h at 37 °C. Afterward, 15 μ L of this reaction mixture were analyzed with HPLC. All peaks in the HPLC diagram were automatically integrated. The retention time of 2'-deoxy-N⁴-(tert-butylthioethyl)cytidine was about 28.2 min. The retention time of the peptide containing nucleoside was about 18.7 min (see HPLC conditions in material and methods).

Immunodetection of the Oligonucleotide-Peptide Conjugate. Serial dilutions (from 5 μ g) of the oligonucleotide-peptide conjugate dissolved in water, were applied to a nylon or a nitrocellulose membrane (0.2 mm, Bio-Rad) using a manifold attached to a suction device. After drying at 70 °C for 30 min, the membrane was UV cross-linked for 2.5 min. The membrane was blocked with PBS containing 1% of Tween-20 for 1 h at room temperature, and incubated with culture supernatant from anti-*c-myc* murine hybridoma clone 9E10 (24.6 mg/mL) (Evans, 1985) for 1 h at room temperature. After washing twice with PBS containing 0.1% of Tween-20 for 5 min, the membrane was incubated with a horseradish peroxidase-conjugated goat anti-mouse antibody (Jackson, 0.9 mg/mL) at a 1:20000 dilution for 1 h. Finally, after several washes with PBS containing Tween-20, the membrane was developed in ECL and exposed to X-ray film.

RESULTS AND DISCUSSION

Synthesis of Monomers for Oligonucleotide Synthesis. The synthesis of the protected phosphoramidite derivative of N⁴-mercaptoethyl-2'-deoxycytidine is illustrated in Figure 2. The preparation of compound **3** was previously described (Miah et al., 1997), but in our hands, the desired product was difficult to isolate from the large excess of *p*-nitrophenol used in the reaction. For this reason, the following changes were introduced: transient protection of alcohol functions was performed with hexamethyldisilazane. Activation of position 4 of dU was done with mesitylenesulfonyl chloride, DMAP, triethylamine instead of phosphoryl chloride, and 1,2,4-

triazole. Further a smaller excess of *p*-nitrophenol was used. The desired product (**3**) was isolated pure without silica gel purification in a 47% yield. Displacement of the *p*-nitrophenyl group with protected 2-mercaptoethylamine gave protected N⁴-mercaptoethyl-dC (compound **4**). The *S*-tert-butylthio group (Goodwin and Glick, 1993; Gundlach et al., 1997; Osborne, 1997; Wünsch et al., 1982) was selected for the protection of the thiol group of mercaptoethylcytosine. Other groups such as the *S*-trityl (Connolly, 1985; Sproat et al., 1987), acetyl (Kuijpers and van Boeckel, 1993), benzoyl (Glick, 1991), and *S*-2,4-dinitrophenylethyl (de la Torre et al., 1993) have previously been used for the synthesis of oligonucleotides containing thiol groups. The *S*-tert-butylthio group is stable to oligonucleotide synthesis conditions and easily removed during or after standard synthesis conditions (Goodwin and Glick, 1993; Gundlach et al., 1997; Osborne, 1997) and for these reasons was selected. Protection of the 5'-OH of compound **2** with the dimethoxytrityl (DMT) group was accompanied with a small percentage of reaction at position N⁴, but the desired DMT-derivative (**6**) was isolated in good yields (57%). Alternatively, compound **3** was protected with the DMT group (66% yield) followed by displacement of the *p*-nitrophenyl group with *tert*-butylthio-2-mercaptoethylamine (53% yield) giving compound **6** in slightly better yields. Phosphitylation of compound **6** gave the desired phosphoramidite **7** (47% yield).

Preparation of Oligonucleotide-Peptide Conjugates. Pentadecamer sequences (O1) 5'-d(TAGAGG1TCCATTGC)-3', (O2) 5'-d(TAGAGG1TC1ATTGC)-3', and (O3) 5'-d(TAG AGG CT11AT TGC)-3' were prepared using phosphoramidite **7** and the phosphoramidites of the natural bases protected with *tert*-butylphenoxyacetyl groups. Coupling efficiencies of the modified phosphoramidite were of the same order as commercially available phosphoramidites (98–99%). Standard ammonia deprotection conditions (concentrated ammonia for 6 h at 55 °C) gave *tert*-butylthio protected oligonucleotides that were purified using standard protocols. Purified oligonucleotides were characterized by mass spectrometry and by enzymatic digestion followed by HPLC analysis of the nucleosides (see Figure 3). Removal of the *tert*-butylthio group was performed with dithiothreitol (DTT). Several conditions were tested including aqueous solutions at different pH and DTT concentrations. In general, at neutral pH, the removal of the protecting group was slow, but in alkaline solutions the removal was faster. For this reason, a 0.1 M DTT solution in concentrated ammonia was used. The reaction was always complete after an overnight treatment at 55 °C. These are the same conditions that were used for the removal of the protecting groups after oligonucleotide synthesis. Although the removal of the *tert*-butylthio group could be performed at the same time as the removal of the other protecting groups by adding DTT to the ammonia solution, we preferred to perform both reactions separately. In this way, oligonucleotides containing protected mercaptoethyl groups can be purified, characterized, and stored without inter- and intramolecular disulfide formation. Before use, the *tert*-butylthio protected oligonucleotide was deprotected with DTT, and the excess of DTT was eliminated by a Sephadex column (NAP 10). Thus, a solution of oligonucleotide with the free thiol group was obtained.

The peptide sequence maleimido-Ala-Glu-Gln-Lys-Leu-Ile-Ser-Glu-Glu-Asp-Leu-Asn carrying the *c-myc* tag-sequence, an extra alanine residue, and a maleimido group at the N-terminus was assembled on a Rink amide

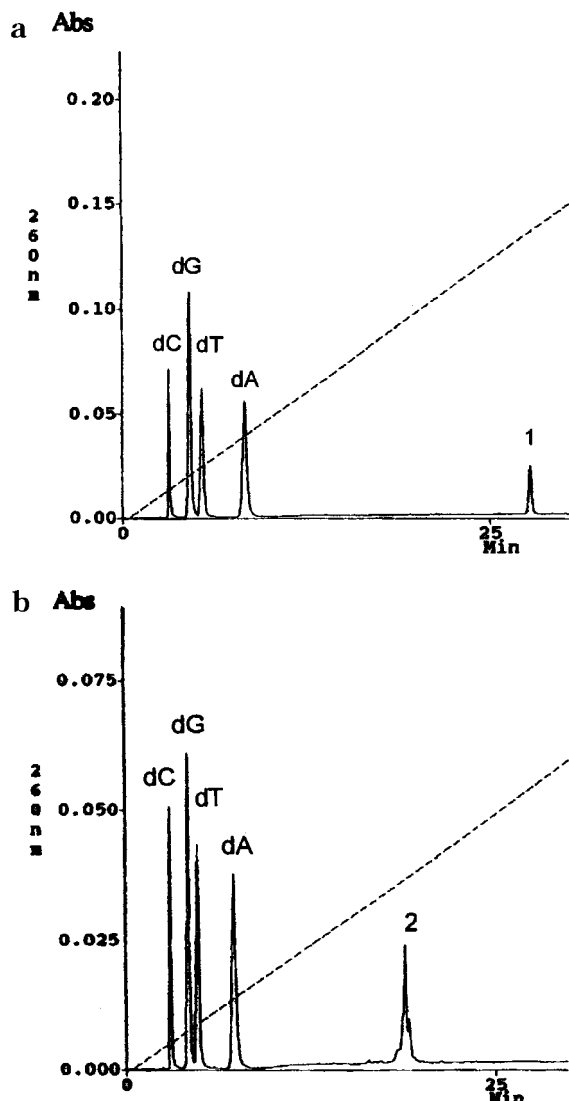


Figure 3. Enzyme digestion of (a) purified 15-mer $5'$ TAG AGG 1TC CAT TGC $3'$ and (b) purified oligonucleotide-peptide conjugate.

polystyrene support (Rink, 1987) using Fmoc-chemistry as described (Eritja et al., 1991). To prevent side reactions between amino acids lateral chains and the maleimido group (Eritja et al., 1991), the maleimido peptide was cleaved from the support just before use. An aliquot of peptide support was deprotected with trifluoroacetic acid (TFA) containing 5% of water as scavenger and the product was analyzed by HPLC. A major peak was obtained which had the expected molecular mass.

Conjugation of maleimido-peptide with thiol containing oligonucleotide was performed at pH 6.5, overnight at room temperature with 10-times excess of freshly deprotected maleimido-peptide over free thiol oligonucleotide O1 (Eritja et al., 1991). The slightly acidic pH was selected in order to protonate amino groups of the peptide and avoid possible side reactions with the maleimido group. One major oligonucleotide product was obtained that was isolated by reversed-phase HPLC. The oligonucleotide-peptide conjugate was characterized by mass spectrometry and enzymatic digestion.

Properties of Oligonucleotide-Peptide Conjugate. Hybridization of oligonucleotides carrying N^4 -tert-butylthioethyl-dC and oligonucleotide-peptide conjugate to their complementary DNA sequence was evaluated spectrophotometrically. The melting temperatures (T_m)

Table 1. Melting Temperatures^a (°C) of Duplexes Containing N^4 -(tert-Butylthioethyl)-dC (1) and Peptide-Oligonucleotide Conjugate (2)

Duplexes containing one N^4 -(tert-butylthioethyl)-dC (1) and N^4 -(peptide)-dC conjugate (2)		
$5'$ TAGAGGXTCCATTGC $3'$		
$3'$ ATCTCCYAGGTAACG $5'$		
X	Y	T_m (°C)
C	G	60.9
T	A	55.5
1	G	53.4
2	G	50.8
Duplexes containing two N^4 -(tert-butylthioethyl)-dC (1)		
$5'$ TAGAGG1TC1ATTGC $3'$		
$3'$ ATCTCCGAGGTAACG $5'$ ^b		
$5'$ TAGAGGCT11ATTGC $3'$		
$3'$ ATCTCCGAGGTAACG $5'$ ^c		

^a 0.15 M NaCl and 0.05 N Tris-HCl buffer, pH 7.5. ^b T_m = 48.9 °C. ^c T_m = 48.8 °C.

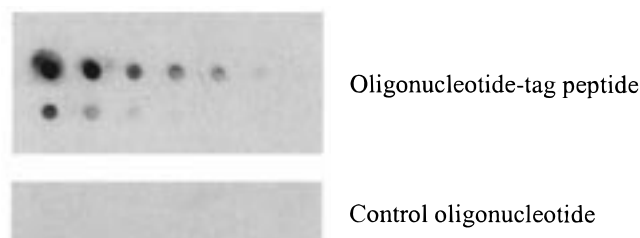


Figure 4. Dot-Blot analysis of oligonucleotide-peptide conjugate. Serial dilutions (from 5 μ g) of the oligonucleotide-peptide conjugate and an oligonucleotide control (without peptide) were applied on a nitrocellulose membrane, incubated with anti-c-myc murine hybridoma clone 9E10 followed by a horseradish peroxidase-conjugated goat anti-mouse antibody and developed with ECL system (Amersham) as described in the Experimental Section. First line: 1/2 dilutions from 5000 ng; 5000, 2500, 1250, 600, 300, 150, and 75 ng of oligonucleotide. Second line: 1/2 dilutions from 500 ng; 500, 250, 125, 60, 30, 15, and 7 ng of oligonucleotide.

for the various duplexes are shown in Table 1. These data show a decrease for the modified oligonucleotides on the ability to form duplexes. One protected mercaptoethyl-dC residue introduces on average a destabilization of 6–7 °C/residue and the c-myc sequence a destabilization of approximately 10 °C. The decrease on melting temperatures is assigned to the utilization of one of the hydrogen bonding amino groups of cytosine (N^4) for connecting to the peptide sequence. A similar destabilization has been described for oligonucleotides carrying a N^4 -cystamine-C derivative (MacMillan and Verdine, 1991). The destabilization effect of the peptide on the oligonucleotide is judged as relatively weak, taking into account the small size of the oligonucleotide (15 bases), the small size of the mercaptoethyl linker between oligonucleotide and peptide, and the high carboxylic acid (4 out of 13 residues) content of the c-myc sequence. These results indicate that oligonucleotides conjugated to peptide sequences may be used as specific hybridization probes.

Nonradioactive detection of oligonucleotide-peptide conjugate was assayed through immobilization on a dot-blot. Serial dilutions of the oligonucleotide-peptide conjugate (from 5 μ g) were applied to a membrane, either nylon or nitrocellulose using a manifold attached to a suction device. After immobilization of the oligonucleotides, the samples were incubated with an anti-c-myc monoclonal antibody (Evan, 1985), followed by incubation with a secondary antibody conjugated to horseradish

peroxidase, and developed with a chemiluminescent substrate (ECL, Amersham). Figure 4 shows the autoradiography of the detection, of both an oligonucleotide carrying the c-myc tag peptide and a control oligonucleotide without peptide. Positive dots were obtained for the oligonucleotide-peptide conjugate, the sensitivity of the detection being approximately 50 ng (8 pmol), while no signal was obtained for control oligonucleotide.

In conclusion, we have shown that oligonucleotides carrying mercaptoethyl-dC residues were easily prepared using phosphoramidite 7. These oligonucleotides are useful intermediates to obtain oligonucleotide-peptide conjugates such as oligonucleotides carrying peptide-tag-sequences that can be immunoreactive. Oligonucleotide-peptide conjugates carrying the c-myc tag-sequence are able to hybridize with their complementary sequences, maintaining their immunoreactivity. The possibility of using the large diversity of available antibodies and their many different labeling possibilities makes oligonucleotide-tag-peptide conjugates a valuable tool in multiple hybridization experiments.

ACKNOWLEDGMENT

We thank M. Wiersma for valuable suggestions and Dr. O. Massó for his generous gift of culture supernatants of anti-c-myc murine hybridoma clone 9E10. We also thank Dr. M. Mann and his group for mass spectroscopic measurements.

LITERATURE CITED

- Beaucage, S. L., and Iyer, R. P. (1993) The functionalization of oligonucleotides via phosphoramidite derivatives. *Tetrahedron* 49, 1925-1963.
- Blanks, R., and McLaughlin, L. W. (1988) An oligodeoxynucleotide affinity column for the isolation of sequence specific DNA binding proteins. *Nucleic Acids Res.* 16, 10283-10299.
- Connolly, B. A. (1985) Chemical synthesis of oligonucleotides containing a free sulphydryl group and subsequent attachment of thiol specific probes. *Nucleic Acids Res.* 13, 4485-4502.
- Corey D. R. (1995) 48000-fold Acceleration of hybridization by chemically modified oligonucleotides. *J. Am. Chem. Soc.* 117, 9373-9374.
- de la Torre, B. G., Aviñó, A., Escarceller, M., Royo, M., Albericio, F., and Eritja, R. (1993) Use of a base-labile protected derivative of 6-mercaptohexanol for the preparation of oligonucleotides containing a thiol group at the 5'-end. *Nucleosides Nucleotides* 12, 993-1005.
- Elghanian, R., Storhoff, J. J., Mucic, R. C., Letsinger, R. L., and Mirkin, C. A. (1997) Selective colorimetric detection of polynucleotides based on the distance-dependent optical properties of gold nanoparticles. *Science* 277, 1078-1081.
- Eritja, R., Pons, A., Escarceller, M., Giralt, E., and Albericio, F. (1991) Synthesis of defined peptide-oligonucleotide hybrids containing a nuclear transport signal sequence. *Tetrahedron* 47, 4113-4120.
- Evan, G. I., Lewis, G. K., Ramsay, G., and Bishop, J. M. (1985) Isolation of monoclonal antibodies specific for human c-myc proto-oncogene product. *Mol. Cell. Biol.* 5, 3610-3616.
- Fàbrega, C., Eritja, R., Sinha, N. D., Dosanjh, M. K., and Singer, B. (1995) Synthesis and properties of oligonucleotides containing the mutagenic base O4-benzylthymidine. *Bioorg. Med. Chem.* 3, 101-108.
- Ferentz, A. E., Keating, T. A., and Verdine, G. L. (1993) Synthesis and characterization of disulfide cross-linked oligonucleotides. *J. Am. Chem. Soc.* 115, 9006-9014.
- Fidanza, J. A., and McLaughlin, L. W. (1992) Use of a thiol tether for the site-specific attachment of reporter groups to DNA. *J. Org. Chem.* 57, 2340-2346.
- Glick, G. D. (1991) Synthesis of a conformationally restricted DNA hairpin. *J. Org. Chem.* 56, 6746-6747.
- Goodwin, J. T., and Glick, G. D. (1993) Incorporation of alkylthiol chains at C-5 of deoxyuridine. *Tetrahedron Lett.* 34, 5549-5552.
- Gundlach, C. W., Ryder, T. R., and Glick, G. D. (1997) Synthesis of guanosine analogues bearing pendant alkylthiol tethers. *Tetrahedron Lett.* 38, 4039-4042.
- Kuijpers, W. H. A., and van Boeckel, C. A. A. (1993) A new strategy for the solid-phase synthesis of 5'-thiolated oligodeoxynucleotides. *Tetrahedron* 49, 10931-10944.
- MacMillan, A. M., and Verdine, G. L. (1991) Engineering tethered DNA molecules by the convertible nucleoside approach. *Tetrahedron* 47, 2603-2616.
- Miah, A., Reese, C. B., and Song, Q. (1997) Convenient intermediates for the preparation of C-4 modified derivatives of pyrimidine nucleosides. *Nucleosides Nucleotides* 16, 53-65.
- Mirkin, C. A., Letsinger, R. L., Mucic, R. C., and Storhoff, J. J. (1996) A DNA-based method for rational assembling nanoparticles into macroscopic materials. *Nature* 382, 607-609.
- Osborne, S. E., Cain, R. J., and Glick, G. D. (1997) Structure and dynamics of disulfide cross-linked DNA triple helices. *J. Am. Chem. Soc.* 119, 1171-1182.
- Rink H. (1987) Solid-phase synthesis of protected peptide fragments using a trialkoxydiphenyl-methylester resin *Tetrahedron Lett.* 28, 3787.
- Sarin, U.K., Kent, S. B. H., Tam, J. P., and Merrifield, R. B. (1981) Quantitative Monitoring of solid-phase peptide synthesis by the Ninhydrin reaction. *Anal. Biochem.* 117, 147-157.
- Sproat, B. S., Beijer, B., Rider, P., and Neuner, P. (1987) The synthesis of protected 5'-mercapto-2', 5'-dideoxyribonucleoside-3'-O-phosphoramidites; uses of 5'-mercapto-oligodeoxynucleotides. *Nucleic Acids Res.* 15, 4837-4848.
- Wolfe, S. A., and Verdine, G. L. (1993) Ratcheting torsional stress in duplex DNA. *J. Am. Chem. Soc.* 115, 12585-12586.
- Wünsch, E., Moroder, L., and Romani, S. (1982) 1-(tert-Butylthio)-1,2-hydrazinedicarboxylic acid derivatives. *Hope-Seyler's Z. Physiol. Chem.* 363, 1461-1464.
- Zuckermann, R. N., and Schultz, P. G. (1988) A hybrid sequence-selective ribonuclease S. *J. Am. Chem. Soc.* 110, 6592-6594.

BC9800740

TECHNICAL NOTES

Acid-Polyacrylamide Gel Electrophoresis of Lysozyme–Estrone Glucuronide Conjugates

C. Mark Smales, David F. Elgar, Christopher H. Moore, and Leonard F. Blackwell*

Department of Chemistry, Massey University, Private Bag 11 222, Palmerston North, New Zealand.

Received October 25, 1997; Revised Manuscript Received February 26, 1998

Acid-polyacrylamide gel electrophoresis (acid-PAGE) was used for analysis of lysozyme–estrone glucuronide conjugates. The resolution of the system allowed the identification of individual conjugate families which differed only in the position of acylation or in the number of estrone glucuronide units. Acid-PAGE was a good alternative to denaturing cation-exchange chromatography for the analysis, separation, and small-scale purification of lysozyme–estrone glucuronide conjugates. It revealed the true order of the relative degree of positive charge on the lysozyme–estrone glucuronide conjugates.

INTRODUCTION

The Ovarian Monitor home assay system (1–3) for the definition of the fertile period of the menstrual cycle utilizes a homogeneous enzyme immunoassay (4) to measure urinary levels of estrone glucuronide (E1G) and pregnanediol glucuronide (PdG). These urinary metabolites of ovarian estradiol and progesterone are reliable markers for the beginning (5) and end (6) of ovarian activity and, hence, are closely related to the length of the fertile period (2). The success of a homogeneous enzyme immunoassay for this purpose depends on the ability to separate lysozyme–steroid glucuronide conjugates from unreacted lysozyme and the availability of an analytical method to monitor the purity of the conjugate products. We have previously published such an analytical method involving the use of Mono-S cation-exchange chromatography in 7 M urea (7), which successfully separates lysozyme–estrone glucuronide (E1G) conjugates on the basis of their relative degrees of positive charge. This analytical procedure can also be used preparatively; however, it involves the denaturation of the enzyme in 7 M urea solutions. The urea is also damaging to the FPLC system used for the analysis, hence it is desirable to develop an alternative analytical system which gives the same resolution of the individual lysozyme–steroid glucuronide conjugate families but which utilizes nondenaturing conditions. Such an analytical system also may be used to check on the overall structural integrity of the conjugates following urea denaturation.

Use of the active ester coupling procedure gave rise to six distinct conjugate families and an unreacted lysozyme peak (8). The six conjugate families were labeled E1–E6 based on their order of elution from the Mono S cation-exchange column (7). Fraction E6 eluted first and E1 eluted next to lysozyme. The other fractions, E2–E5,

eluted in the order of their overall degrees of positive charge. It would be expected on this basis that the electrophoretic mobilities would also differ for the different conjugate families. Both pH 8.9 polyacrylamide gel electrophoresis (PAGE) (9, 10) and acid-polyacrylamide gel electrophoresis (acid-PAGE) (11) have been used for the analysis of modified lysozymes and native lysozyme, respectively. Both of these methods have the potential to separate the different conjugate families on the basis of their charge differences. So far however, no studies with lysozyme–steroid glucuronide conjugates have been reported. Thus, we have examined the electrophoretic separation of the lysozyme–estrone glucuronide families under acidic, nondenaturing conditions.

EXPERIMENTAL PROCEDURES

All reagents were of analytical grade or better. Cytochrome *c* (from bovine heart) was purchased from Sigma Chemical Co. (St. Louis, MO). Minigels were run on a Mini-Protean II dual slab system while larger gels were run on a Protean II xi system, both from Bio-Rad laboratories (Richmond, CA). The synthesis of 17- α -oestra-1,3,5- β -yl-D-glucopyranosiduronic acid (E1G) was essentially according to Conrow and Bernstein (12) as previously reported (7). Conjugation of lysozyme with E1G by the active ester coupling method (8) and analysis of the resulting E1G–lysozyme conjugates by Mono-S cation-exchange chromatography in 7 M urea at pH 6.0 were undertaken as described previously (7). All urea was of analytical grade, and solutions were prepared immediately prior to use and stored at 4 °C. Analysis of the E1G–lysozyme conjugates by SDS–PAGE was undertaken at pH 8.30 using standard electrophoresis procedures (13). High pH nondenaturing PAGE was undertaken at pH 8.9 using the system of Habeeb and Atassi (9, 10).

E1G–lysozyme conjugates were also analyzed by acid-PAGE at room temperature on continuous acrylamide slab gels at pH 4.08 with reverse polarity (lower chamber cathode). The reservoir buffer in both upper and lower chambers consisted of glycine (28.1 g), glacial acetic acid

* To whom correspondence should be addressed. Phone: 64-6-350-4783. Fax: 64-6-350-5682. E-mail: L.F.Blackwell@massey.ac.nz.

(3.06 mL), and Milli-Q water as required to adjust the volume to 1 L at pH 4.08 as described by Hrkal (14). This buffer was freshly prepared before use.

Both minigels and larger gels were prepared and run. Minigels were prepared using commercially available Bio-Rad 40% acrylamide solution as follows: acrylamide (3.75 mL) to give a 15% final solution was mixed with pH 4.08 reservoir buffer (6.25 mL). After degassing, *N,N,N',N'*-tetramethylethylenediamine (15 μ L) and ammonium persulfate (100 μ L of a freshly prepared 50 mg/mL solution) were added and mixed before the gel was poured. For larger gels (160 $\times$ 200 mm), a 40% acrylamide solution was prepared and the gels were mixed in the same ratio's as for the minigels. Mini gels were prerun at 200 V for 1.5 h or until a constant current was obtained, while the larger gels were prerun for 3.5 h. Following prerunning, both the upper and lower chambers were refilled with fresh reservoir buffer. The conjugate samples were then loaded in reservoir buffer which contained 20% glycerol. The gel was run with one lane containing cytochrome *c* so that its bright orange color could be used as a tracking agent. Electrophoresis was undertaken at constant voltage (200 V) with the usual current reversed so that the polarity was from the anode (upper chamber) to the cathode (lower chamber) until the cytochrome *c* marker reached the bottom of the gel. All gels were stained with a 0.1% Coomassie Blue solution.

RESULTS AND DISCUSSION

SDS-PAGE analysis of the E1G-lysozyme conjugates showed no evidence of resolution between the conjugate families and lysozyme, hence there were no significant size differences between the different conjugate families and lysozyme. A nondenaturing system was therefore investigated for the separation of the E1G-lysozyme conjugates as it has previously been shown that nonurea, high pH PAGE (pH 8.9, 0.043 M Tris, and 0.046 M glycine) can be used to give distinct bands when analyzing lysozyme derivatives, with good results (9, 10). However, when the E1G-lysozyme conjugates were analyzed by this system, distinct bands were not observed and there was little visible separation between the collection of individual conjugate families and lysozyme. Habeeb and Atassi (10) have suggested that the laddering effect they reported when analyzing lysozyme derivatives (acetylated, succinylated, maleylated, and guanidinated lysozyme derivatives) was due to esterification of lysozyme and not to amino group modification. This could explain why the E1G-lysozyme conjugates did not exhibit the laddering effect since the stable conjugate derivatives are modified at lysine residues only. A similar system was therefore used but at low pH (pH 4.08) where most of the ionizable groups in the protein molecule would be protonated, and hence the more positively charged derivatives would migrate down the gel toward the negative electrode.

Under these conditions (pH 4.08), nondenaturing acid-PAGE analysis of the estrone glucuronide-lysozyme conjugates prepared by the active ester method (7) gave seven distinct conjugate populations (Figure 1) as expected from the results obtained previously with the Mono-S cation-exchange column in the presence of denaturing 7 M urea solutions (Figure 2) (7). Positive identification of the acid-PAGE bands with those from the 7 M urea cation-exchange column (7) was achieved by diffusing each one from the gel and running it through the Mono-S 7 M urea system. The presence of estrone

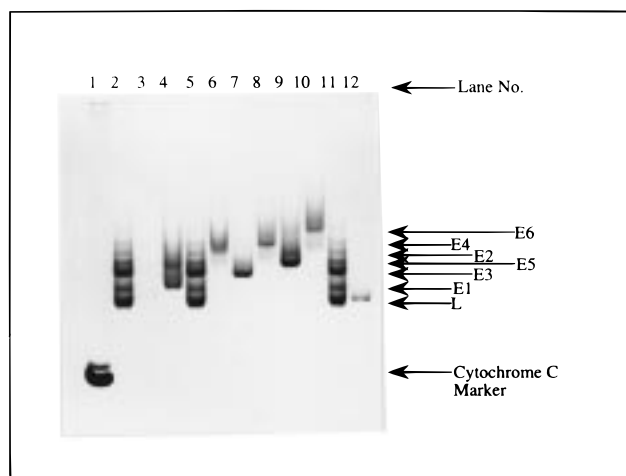


Figure 1. Analysis of E1G-lysozyme active ester conjugates by acid-PAGE as described in the text. Cytochrome *c* was run as a marker protein (lane 1) while the E1G-lysozyme active ester conjugation reaction mixture was run in lanes 2, 5, and 11. The individual conjugate peaks, as collected off the Mono-S column (Figure 2) were also analyzed by acid-PAGE and can be seen in lanes 4 (E1), 6 (E2), 7 (E3), 8 (E4), 9 (E5), 10 (E6), and 12 (lysozyme).

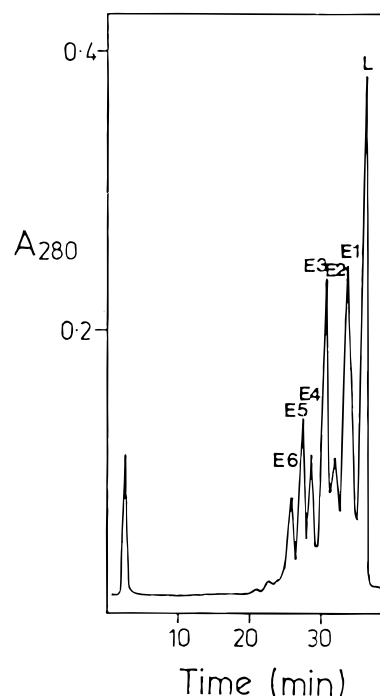


Figure 2. E1G-lysozyme active ester conjugation reaction mixture in 7 M urea on a Mono-S cation-exchange HR 5/5 column. Conditions as described in ref 7.

glucuronide in all of these bands, apart from the fastest moving (unmodified lysozyme) which was free of estrone glucuronide, was confirmed by homogeneous immunoassay using an Ovarian Monitor (1-3) as previously described (7). Alternatively, each conjugate fraction and unreacted lysozyme was collected from the Mono-S 7 M urea column and run on the acid-PAGE system (Figure 1) for positive identification. The distance each conjugate travels on the acid-PAGE system should decrease in the order $L(0) > E1(1) > E2(1-1.5) > E3(1) > E4(2) > E5(2) > E6(3)$, this being the order of decreasing positive charge as deduced from the relative elution positions on the Mono-S 7 M urea column (Figure 2) and the increasing degree of acylation (shown by the numbers in

brackets) as determined by the trinitrobenzenesulfonic acid method (7). However, despite the fact that the same number of conjugate bands was observed, their order of separation by acid-PAGE was not a simple correspondence between the degree of lysine substitution and the distance traveled toward the negative electrode. Experimentally, unmodified lysozyme being the most positively charged moved furthest down the gel as expected while the triacylated conjugate E6 (7) moved the shortest distance (Figure 1) being the least positively charged, also as expected. Native, unmodified lysozyme was shown to run in the same position as the experimentally unmodified lysozyme (data not shown). Conjugate E1 ran next to lysozyme (L) but moved as two bands, one migrating to the expected position (the furthest of any conjugate) and one which did not migrate as far (Figure 1), consistent with the presence of two closely related conjugates within this conjugate family. Supporting evidence that this is so can be seen also in the Mono-S 7 M urea analysis where a slight shoulder is observed on the trailing edge of the E1 peak (Figure 2).

However, the order of migration of the remaining conjugates (E2–E5) by acid-PAGE analysis was unexpected being $E3 > E5 > E2 > E4$; that is, conjugates E3 and E5 both had a higher degree of positive charge than indicated from their chromatographic behavior. This difference in apparent charge is unlikely to be due to denaturation or conformational changes in the presence of the concentrated urea solutions used during cation-exchange chromatography as lysozyme crystals grown in the presence of high urea concentrations (up to 9 M) are essentially isomorphous with the structure of native crystals (15). Also, in the presence of urea, the crystal structure of hen egg white lysozyme shows no evidence of carbamylation of the free amino groups (15) ruling out this possibility as a variable affecting the separation of the conjugates. Although there may be an increased hydrophobic interaction between the conjugates and the polyacrylamide matrix compared with the unmodified lysozyme, this factor alone cannot account for the observed separation and resolution of the estrone glucuronide–lysozyme conjugates by acid-PAGE. If hydrophobic interactions between the E1G moieties and the polyacrylamide were the sole determining factor, then those conjugates with the same level of acylation would be expected to run in the same position. This is obviously not the case as conjugates E1–E3 (all with E1G substitution levels of 1–1.5) are clearly separated from each other as are conjugates E4–E5 (both with E1G substitution levels of 2).

Urea does have a pronounced effect on the high-performance cation-exchange chromatography behavior of hen egg white lysozyme, significantly reducing its retention time over that observed in the absence of urea (16). Although this result is expected on the basis of the increase in solvent dielectric constant, it is also possible that it may be due, in part, to the specific binding of a urea molecule. For example, it has been reported that a urea molecule hydrogen bonds to lysine residue 116 of the hen egg white lysozyme structure (15), and this would be expected to reduce the apparent positive charge on the enzyme under these conditions. Thus, in the urea cation-exchange system, the contribution of lysine residue 116 to the overall positive electrostatic field of lysozyme may be underestimated for some conjugates.

The difference in the observed migration orders between the two analytical procedures can therefore be explained if it is assumed that conjugate families E2 and E4 are acylated with estrone glucuronide at lysine

residue 116 but conjugate families E3 and E5 are not. Hence, conjugate families E3 and E5 will elute from a cation-exchange column in the presence of 7 M urea earlier than expected on the basis of their true formal charges due to the masking effect of a urea molecule hydrogen binding to lysine residue 116. On the other hand, the elution positions of conjugate families E2 and E4 would not be effected by urea if lysine residue 116 is acylated. When the urea effect is absent as for the nondenaturing acid-PAGE system, lysine residue 116 can now exert its full effect on the positive field of these two conjugates (E3 and E5) and both migrate further than their counterparts E2 and E4. On this explanation, conjugates E1 and E6 must also be acylated at lysine residue 116. The true order of the overall positive charge for the lysozyme conjugates is, therefore, $L > E1 > E3 > E5 > E2 > E4 > E6$.

Whatever the correct explanation of the elution behavior, nondenaturing acid-PAGE can be used to assess, analyze, and purify small amounts of individual lysozyme–estrone glucuronide conjugates. The advantage of the system is that it is rapid, simple to perform, gives excellent resolution between the different E1G–lysozyme conjugate families, and avoids the use of urea. A major hurdle in analyzing protein conjugates is that often there is very little chemical, or size, difference between the unconjugated and conjugated protein products. It may therefore be possible to use the acid-PAGE system presented here for use with other conjugate systems which utilize proteins with high isoelectric points ($I.E.P > 6.0$), and for other modification studies (chemical and site directed mutagenesis) whereby lysozyme is used as a model protein.

LITERATURE CITED

- (1) Brown, J. B., Blackwell, L. F., Cox, R. I., Holmes, J. M., and Smith, M. A. (1988) Chemical and Homogeneous Enzyme Immunoassay Methods for the Measurement of Estrogens and Pregnanediol and their Glucuronides in Urine. *Prog. Clin. Biol. Res.* 285, 119–138.
- (2) Brown, J. B., Blackwell, L. F., Holmes, J., and Smyth, K. (1989) New Assays for Identifying the Fertile Period. *Int. J. Gynecol. Obstet. Suppl.* 1, 111–122.
- (3) Brown, J. B., Holmes, J., and Barker, G. (1991) Use of the Home Ovarian Monitor in Pregnancy Avoidance. *Am. J. Obstet. Gynecol.* 165, 2008–2011.
- (4) Rubenstein, K. E., Schneider, R. S., and Ullman, E. F. (1972) Homogeneous Enzyme Immunoassay. A New Immunological Technique *Biochim. Biophys. Res. Commun.* 47, 846–851.
- (5) Adlercreutz, H., Brown, J., Collins, W., Goebelsman, U., Kellie, A., Campbell, H., Spieler, J., and Braissand, G. (1982) The Measurement of Urinary Steroid Glucuronides as Indices of the Fertile Period in Women. *J. Steroid Biochem.* 17, 695–702.
- (6) Blackwell, L. F., Cooke, D., and Brown, J. B. (1998) Definition of the Potentially Fertile Period from Urinary Steroid Excretion Rates. Part II. A Threshold Value for Pregnanediol Glucuronide as a Marker for the End of the Potentially Fertile Period in the Human Menstrual Cycle. *Steroids* 63, 5–13.
- (7) Smales, C. M., Cooke, D., and Blackwell, L. F. (1994) Use of Ion-Exchange and Hydrophobic-Interaction Chromatography for the Rapid Purification of Lysozyme–Estrone Glucuronide Conjugates. *J. Chromatogr., B* 662, 3–14.
- (8) Rajkowski, K. M., and Cittanova, N. (1981) The Efficiency of Different Coupling Procedures for the Linkage of Oestradiol-16 α -Glucuronide, Oestrone-3-glucuronide and Pregnanediol-3 α -Glucuronide to Four Different Enzymes. *J. Steroid Biochem.* 14, 861–866.
- (9) Habeeb, A. F. S. A., and Atassi, M. Z. (1970) Enzymic and Immunochemical Properties of Lysozyme. Evaluation of

- Several Amino Group Reversible Blocking Reagents. *Biochemistry* 9, 4939–4944.
- (10) Habeeb, A. F. S. A., and Atassi, M. Z. (1971) Enzymic and Immunochemical Properties of Lysozyme-V. Derivatives Modified at Lysine Residues by Guanidination, Acetylation, Succinylation or Maleylation. *Immunochemistry* 8, 1047–1059.
- (11) Lockey, T. D., and Ourth, D. D. (1996) Purification and Characterisation of Lysozyme from Hemolymph of *Heliothis virescens* Larvae. *Biochem. Biophys. Res. Commun.* 220, 502–508.
- (12) Conrow, R. B., and Bernstein, S. (1971) Steroid Conjugates. VI. An Improved Koenigs-Knorr Synthesis of Aryl Glucuronides using Cadmium Carbonate, a New and Effective Catalyst. *J. Org. Chem.* 36, 863–870.
- (13) Laemmli, U. K. (1970) Cleavage of Structural Proteins During the Assembly of the Head of Bacteriophage T4. *Nature* 227, 680–685.
- (14) Hrkal, Z. (1979) Gel-Type Techniques. *Journal of Chromatogr. Library Electrophoresis—A Survey of Techniques and Applications Part A: Techniques* (Z. Deyl, Ed.) Vol. 18, p 121, Elsevier Scientific Publishing Company, Amsterdam.
- (15) Pike, A. S. C., and Ancharya, K. R. (1994) A Structural Basis for the Interaction of Urea with Lysozyme. *Protein Sci.* 3, 706–710.
- (16) Parente, E. S., and Wetlaufer, D. B. (1984) Influence of Urea on the High-Performance Cation-Exchange Chromatography of Hen Egg white. Lysozyme. *J. Chromatogr.* 288, 389–398.

BC9701914

Convenient Polymer-Supported Synthetic Route to Heterobifunctional Polyethylene Glycols

Thierry Bettinger, Jean-Serge Remy, Patrick Erbacher, and Jean-Paul Behr*

Laboratoire de Chimie Génétique associé CNRS/Université Louis Pasteur de Strasbourg, UMR 7514, Faculté de Pharmacie, BP 24, 67401 Illkirch Cedex, France. Received April 12, 1998; Revised Manuscript Received June 22, 1998

Conventional synthesis of heterobifunctional poly(ethylene glycol) derivatives, especially of medium size, is a rather tedious task. A straightforward solid-phase methodology has been developed that is illustrated here by the synthesis of α -pyridyldithio- ω -hydroxy-poly(ethylene glycol)₆₀₀. This derivative was prepared from resin-bound PEG₆₀₀ with a global yield of 65% for 6 individual steps, i.e., with an average yield of 93%/step. Intermediate purification steps simply consisted of resin washing. Progress of each reaction toward completion could conveniently be monitored by ¹³C NMR of the resin-bound PEG derivatives. This example highlights both the versatility and efficiency of combining polymer-supported synthesis with direct ¹³C-NMR characterization of the intermediate compounds.

INTRODUCTION

Poly(ethylene glycol) (PEG)¹ conjugation chemistry has been developed recently as a consequence of the potential therapeutic interest of pegylated drugs (Herman et al., 1995; Zalipsky, 1995). Indeed it is now demonstrated that PEG-modified biological molecules can benefit from extended plasma lifetimes, from reduced uptake by the reticuloendothelial system (Allen, 1994; Papahadjopoulos et al., 1991; Woodle and Lasic, 1992) and more generally from a decrease of the undesired consequences of electrostatic and van der Waals interactions. Following the synthesis of PEG–protein conjugates, new developments have emerged such as nonimmunogenic drugs and nucleic acid vectors. One of the future directions toward nonviral gene therapy is the use of PEG-grafted synthetic vectors as long circulating carriers for receptor-mediated gene delivery (Blume et al., 1993; Maruyama et al., 1995). In this context, heterobifunctional poly(ethylene glycol) linkers are required that allow attachment of ligands to one extremity of PEG-grafted synthetic vectors.

Protein modification with PEG does not require true heterobifunctional PEG derivatives. Methoxy-PEG (mPEG), carrying an inert ether end group, was used to make the chemical processing easier (Greenwald et al., 1996; Herman et al., 1995; Zalipsky, 1995). The chemistry of heterobifunctional PEG derivatives is not well developed, reaction yields are far from being quantitative, hence, cumbersome polymer purification techniques have to be used (Zalipsky, 1993). Most published procedures use classical derivatization chemistry in homogeneous

solution (sometimes even in potentially reactive aqueous medium), and often the functionalized PEG cannot be fully separated from the starting polymer (Zalipsky et al., 1994). Another possibility is to choose suitable initiator and/or terminator reagents for the polymerization of ethylene oxide (Yokoyama et al., 1992). However, recent work showed that such strategies may be obscured by diol impurities (Nagasaki et al., 1997), and the polymerization process still remains difficult to control (ethylene oxide is toxic and explosive).

In another register, poly(ethylene glycol) is also being widely used in solid-phase synthesis (Bayer, 1991). Indeed, improvement of polymeric supports for peptide synthesis has led to using PEG as a good solvent-swelling linker between the inert resin and the solid-supported reaction center. The PEG linker mobility is high (Bayer, 1990) and peptide yields are good, showing it to be possible to combine the advantages of a solid support with those of the liquid-phase methodology.

According to this background, we decided to develop a new strategy for heterobifunctionalization of poly(ethylene glycol) based on solid-phase chemistry. A major aspect of PEG functionalization lies in the proper characterization of intermediate and final products. We used gel-phase ¹³C NMR which allows convenient and nondestructive monitoring of reaction progress directly on the resin (Bayer, 1990). This is possible because of the rather long ¹³C relaxation times which are a consequence of the high mobility of the fully solvated PEG (Bayer, 1990). The method is sensitive and allows us to accurately determine the end point of each reaction step (among others, the terminal carbons of PEG usually exhibit characteristic chemical shifts).

To demonstrate the interest of this strategy, we have prepared a low molecular weight PEG derivative (MW 600), which is known to be difficult to purify by size exclusion chromatography or diethyl ether precipitation. Such small PEGs can act as very effective molecular scale filters for preventing binding to underlying liposome or particle surfaces (Needham et al., 1997). PEG was functionalized with a 2-pyridyldithio residue which is widely used for specific coupling with thiol groups (Carlsson et al., 1978; Woghiren et al., 1993) since coupling

* Author to whom correspondence should be addressed. Phone: 33(0)388 676983. Fax: 33(0)388 678891. E-mail: behr@pharma.u-strasbg.fr.

¹Abbreviations: PEG₆₀₀, poly(ethylene glycol) MW 600; HO-PEG₆₀₀-SSPy α -hydroxy- ω -pyridyldithio-poly(ethylene glycol)₆₀₀; HO-PEG₆₀₀-COOH, α -hydroxy- ω -carboxy-poly(ethylene glycol)₆₀₀; CH₃OH, methanol; CH₂Cl₂, dichloromethane; DMF, *N,N*-dimethylformamide; THF, tetrahydrofuran; Et₃N, *N,N,N,N*-triethylamine; TFA, trifluoroacetic acid; DCC, *N,N'*-dicyclohexylcarbodiimide; DCU, dicyclohexylurea; DTT, 1,4-dithio-L-threitol; NHS, *N*-hydroxysuccinimide; Tos-Cl, toluene-4-sulfonyl chloride; TLC, thin layer chromatography.

Table 1. ^{13}C -Nuclear Magnetic Resonance Peaks of Polymer-Grafted Poly(ethylene Glycol) Derivatives

structure	(δ) ^a (ppm)
resin-PEG-O-CH ₂ CH ₂ -OTos	69.3, 68.7, 145, 129.7, 128.1, 130.1, 21.8
resin-PEG-O-CH ₂ CH ₂ -S(C=S)O-CH ₂ CH ₃ ^b	69.8, 35.2, nd, 68.7, 13.7
resin-PEG-O-CH ₂ CH ₂ -SH	72.6, 24
resin-PEG-O-CH ₂ CH ₂ -S-Pyridine ^c	68.7, 38.2, nd, 119.4, 136.9, 120.4, 149.2

^a The polymer-bound derivative (150 mg) was added to CDCl₃ (0.5 mL). Chemical shifts were normalized relative to CDCl₃ = 77.0 ppm. The large poly(ethylene glycol) backbone peak was at 70.3 ± 0.3 ppm. Chemical shifts are listed according to the structures shown in Figure 1. ^b The SC=S resonance was not observed. ^c The SCNCH resonance was not observed.

releases a chromophore which allows straightforward reaction monitoring. We further activated the HO-PEG₆₀₀-SSPy as the *N*-hydroxysuccinimide carbonate derivative (Zalipsky et al., 1992); coupling of this compound with an amine to give a carbamate led to higher yields than classical amide bond formation.

EXPERIMENTAL PROCEDURES

General. Aminomethylated polystyrene-1% divinylbenzene was purchased from Novabiochem (Meudon, France). Toluene-4-sulfonyl-chloride (TsCl), potassium *O*-ethyl dithiocarbonate, propylamine, and poly(ethylene glycol) (MW 600) were from Fluka (St Quentin Fallavier, France). 2,2'-Dipyridyl disulfide was obtained from Lancaster (Strasbourg, France). Thin layer chromatography (TLC) was performed on Kieselgel 60 F₂₅₄ plates (Merck). Preparative chromatography was carried out on Chromatotron 8924 (Harrison Research). PEG was revealed by TLC by the Dragendorff assay (Thoma et al., 1964). The Kaiser qualitative ninhydrin test was carried out specifically as described elsewhere (Sarin et al., 1981). As a reaction vessel, we used a glass column ended with a join connection and a glass frit (porosity n°2) on the upper and lower end, respectively. This allowed us to stir the resin on a rotary evaporator and to wash the resin after each reaction step. Solid-phase reactions were carried out at room temperature. After each step, the resin was stirred (20 min) and rinsed successively with CH₂Cl₂ (5 × 50 mL) and CH₃OH (5 × 50 mL). The resin was dried in vacuo to allow analytical analysis (5 mg was taken for IR analysis and 150 mg for ^{13}C NMR). ^1H - and ^{13}C -NMR spectra were recorded on a Bruker DPX300 Avance instrument at 300 MHz (scan number = 1000 with classical acquisition parameters). Infrared spectra (IR, KBr pellet) were recorded on a spectrophotometer FT-IR 1600 (Perkin-Elmer).

Synthesis of Crude α -Hydroxy- ω -Carboxy Poly(ethylene Glycol) (HO-PEG₆₀₀-COOH) (1). Poly(ethylene glycol) (28 g, 46.7 mmol, 600 g/mol) was dissolved in 40 mL of dry CH₂Cl₂. To this solution was added 10 mL of dry THF containing diglycolic anhydride (1.02 g, 8.8 mmol) and pyridine (720 μL , 9 mmol). After stirring overnight under argon at room temperature, the reaction was completed as indicated by TLC (CH₂Cl₂/MeOH/Et₃N 90:10:1) (no anhydride left and detection of a single polar PEG-compound). The monoacid derivative of poly(ethylene glycol)₆₀₀ was used without purification.

PEG₆₀₀ Grafting to the Resin via Amide Bond Formation (2). The above HO-PEG₆₀₀-COOH solution (ca. 8.8 mmol) was activated with 1.2 molar equivalent of *N*-hydroxysuccinimide (1.22 g, 10.6 mmol) and *N,N'*-dicyclohexylcarbodiimide (2.19 g, 10.6 mmol). The solution was stirred for 2 h at room temperature under argon. The dicyclohexylurea (DCU) suspension was filtered off. Aminomethylated poly(styrene-co-1% divinylbenzene) resin (1.1 g, 1.2 mmol of amino group) was poured into the glass column (see General) with the activated carboxylic acid solution. The column was connected to a rotary

evaporator and stirred for 2 days at room temperature. The solvent was then filtered off and the resin beads were washed as described above. Two additional washes with DMF/CH₃OH (10/3) were necessary to remove the remaining DCU as checked by IR. Complete amino group capping was indicated by a negative Kaiser test. The resin was dried in vacuo for IR and ^{13}C -NMR characterization. At this stage, the resin did not stick anymore to the glass wall. IR spectrum of the resin showed the characteristic absorption band of PEG ether backbone (1100 cm⁻¹) and absorption bands at 1750 and 1680 cm⁻¹ for the ester and amide bond, respectively (C=O sym stretch).

Reaction of Polymer-Bound α -Hydroxy-Poly(ethylene Glycol)₆₀₀ with Tosyl Chloride (3). Twenty-fold molar excess of toluene-4-sulfonyl chloride (4.6 g, 24 mmol) and 3.34 mL of Et₃N (24 mmol) in 15 mL of CH₂Cl₂ were added slowly to the resin suspended in 30 mL of CH₂Cl₂. The mixture was stirred overnight at room temperature while the resin turned brown-yellow. The solvent was filtered off and the resin was washed as described above. The resin became white again after these washes. The IR spectrum showed the characteristic sulfonyl group absorption at 1177 cm⁻¹ (SO₂, sym stretch) and did not show any residual hydroxyl absorption (3200–3600 cm⁻¹) of the starting polymer. See ^{13}C -NMR data in Table 1.

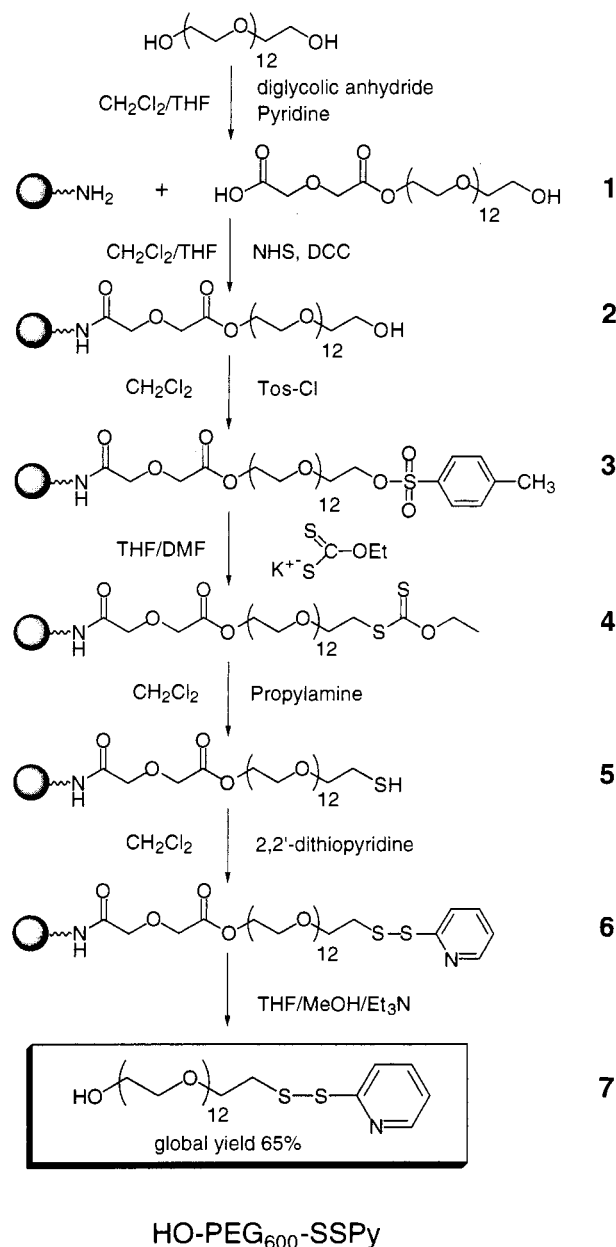
Reaction of the Polymer-Bound α -Tosyl-*O*-Poly(ethylene glycol)₆₀₀ with Potassium *O*-Ethyl Dithiocarbonate (4). Potassium *O*-ethyl dithiocarbonate (212 mg, 1.32 mmol) in 25 mL of THF/DMF (50/1) was added to the polymer. The mixture was stirred for 4 h at room temperature. The solvent was filtered off, and the resin was washed. IR spectrum of the resin confirmed the disappearance of the tosyl group (1177 cm⁻¹). See ^{13}C -NMR data in Table 1.

Synthesis of Polymer-Bound α -Thiol-poly(ethylene Glycol)₆₀₀ (5). Thirty-fold molar excess of propylamine (3 mL, 36 mmol) in 25 mL of CH₂Cl₂ was added to the column and stirred for 1 h. The solvent was filtered off and the resin was washed as above. ^{13}C NMR confirmed that no disulfide bond formation (PEG-S-S-PEG) had occurred. See ^{13}C -NMR data in Table 1.

Activation of Polymer-Bound α -Thiol-poly(ethylene Glycol)₆₀₀ with 2,2'-Dipyridyl Disulfide (6). Five fold molar excess of 2,2'-dipyridyl-disulfide (1.32 g, 6 mmol) in 30 mL of CH₂Cl₂ was added to the column and stirred overnight at room temperature. The resin turned yellow. The solvent was filtered off, and the resin was washed while turning white. Again, no disulfide bond formation had occurred as indicated by ^{13}C NMR. See ^{13}C -NMR data in Table 1.

Cleavage of α -Pyridyldithio- ω -hydroxy-poly(ethylene Glycol)₆₀₀ from the Solid Support (7). A mixture of 30 mL of THF/CH₃OH/Et₃N (1/1/1) was added to the resin and stirred for 3 days at room temperature. The solution was filtered and the resin was washed as usual. The combined organic fractions were concentrated

Scheme 1



in vacuo, and Et₃N was removed by rough chromatography on SiO₂ with CH₂Cl₂/CH₃OH (20/1) as eluent. A brown oil was recovered (550 mg, 0.78 mmol), which was identified by TLC and characterized by ¹H- and ¹³C-NMR spectroscopy. The global yield was 65% as determined by quantitative released of 2-thiopyridone upon reduction with DTT (Carlsson et al., 1978).

$R_f = 0.45$ (CH₂Cl₂/CH₃OH 10/1). ¹H NMR (CDCl₃): δ 3.01 (t, SSCH₂CH₂O, 2H), 3.54 (s, PEG, 48H), 3.66 (t, SSCH₂CH₂O, 2H), 7.17 (m, SCCHCH, 1H), 7.75 (m, SCCHCHCH, 2H), 8.46 (m, SCNCH, 1H). ¹³C NMR (CDCl₃): δ 38.4 (SSCH₂CH₂), 61.7 (OCH₂CH₂OH), 68.9 (SSCH₂CH₂), 70.5 (CH₂CH₂O of PEG backbone), 72.5 (OCH₂CH₂OH), 119.6 (SCCHCH), 120.6 (SCCHCHCH), 137.1 (SCCHCH) 149.5 (SCNCH).

RESULTS AND DISCUSSION

Stepwise conversion of PEG₆₀₀ to the thiol-activated HO-PEG₆₀₀-SSPy (7) was carried out according to Scheme 1. To avoid a tedious PEG monofunctionalization/isolation procedure, diglycolic anhydride was reacted with a

large excess of PEG. In such conditions, the only carboxylic acid formed should be the compound of interest. This compound can be selectively activated and grafted to the resin via amide bond formation. Resin washing should remove all other compounds. In practice, HO-PEG₆₀₀-COOH (1) was activated with *N*-hydroxysuccinimide (NHS) and *N,N'*-dicyclohexylcarbodiimide (DCC). The reaction mixture was filtered in order to remove most of the dicyclohexylurea (DCU) formed. After PEG grafting onto the resin, IR spectroscopy revealed some residual DCU sticking to the resin (broad absorption band at 1800–1600 cm⁻¹). The solid support was therefore further washed with DMF/CH₃OH (7/3). An extended reaction time was required for PEG grafting to the resin, which could be explained by the waxy constitution of PEG solution at this concentration (1 M). Complete reaction was confirmed by a negative Kaiser test. IR spectroscopy revealed the characteristic absorption bands for PEG (CH₂-O-CH₂, ether 1100 cm⁻¹) as well as for the connecting ester and amide functions (1750 and 1680 cm⁻¹, respectively, C=O sym stretch).

The glycolic ester bond being labile, thiol group formation with strong nucleophiles such as alcoholates (Woghiren et al., 1993) had to be avoided. Thiol derivatization was achieved by a modification of the protocol reported by Zalipsky (Zalipsky et al., 1987) which used milder conditions compatible with an ester linkage. The primary hydroxyl group of PEG was activated with tosyl chloride in standard conditions, and the tosylated resin (3) showed an IR spectrum with the expected strong absorption at 1177 cm⁻¹ (SO₂). The ¹³C-NMR spectrum contained the characteristic tosyl group resonances at 21.8 ppm and between 128 and 145 ppm, which correspond to the methyl and aromatic ring carbon atoms, respectively (Figure 1). The broad signals around 130 and 40 ppm were due to the phenyl and aminoethyl groups of the solid support. At this stage, the activated resin-grafted PEG is easy to handle as solid beads and is stable upon long-term storage (at least 4 months at -20 °C). It may be regarded as a convenient starting material for the preparation of a variety of PEGs. Tosyl-PEG₆₀₀ was reacted with a stoichiometric amount of potassium *O*-ethyl dithiocarbonate to give the diester 4. Preliminary experiments using excess reagent were shown by ¹³C NMR and IR to lead to complete cleavage of the PEG from the resin. Compound 4 could not be characterized by IR spectroscopy. However, ¹³C-NMR spectrum confirmed complete removal of tosyl group (21.8 ppm and 128–145 ppm) and the appearance of two new resonances at 13.7 ppm (O-CH₂-CH₃) and 35.2 ppm (O-CH₂-CH₂-S) (Figure 1).

The thiocarbonate was aminolyzed, but instead of glycine, we used propylamine to remain in organic solution and avoid disulfide bond formation. Several attempts to cleave the thiocarbonate in mild basic solution (<0.1 M propylamine, 1 h) led to uncomplete aminolysis as revealed by ¹³C NMR (4 peaks at 72.6, 35.2, 24, and 13.7 ppm). Complete conversion into the thiol-PEG derivative 5 required a high concentration of propylamine (1.2 M). Strictly anhydrous condition (CH₂Cl₂) prevented cleavage of the functionalized PEG from the resin. ¹³C NMR showed clean conversion to the thiol 5 (24 and 72.6 ppm) without oxidation into the disulfide (no resonance at ca. 36 ppm) (Figure 1). The thiol group was activated as the 2-pyridyldithio derivative 6. The conversion was complete as again indicated by ¹³C NMR (Figure 1).

Finally, the functionalized PEG₆₀₀ was cleaved off from the resin with a mildly basic polar solvent (THF/CH₃-

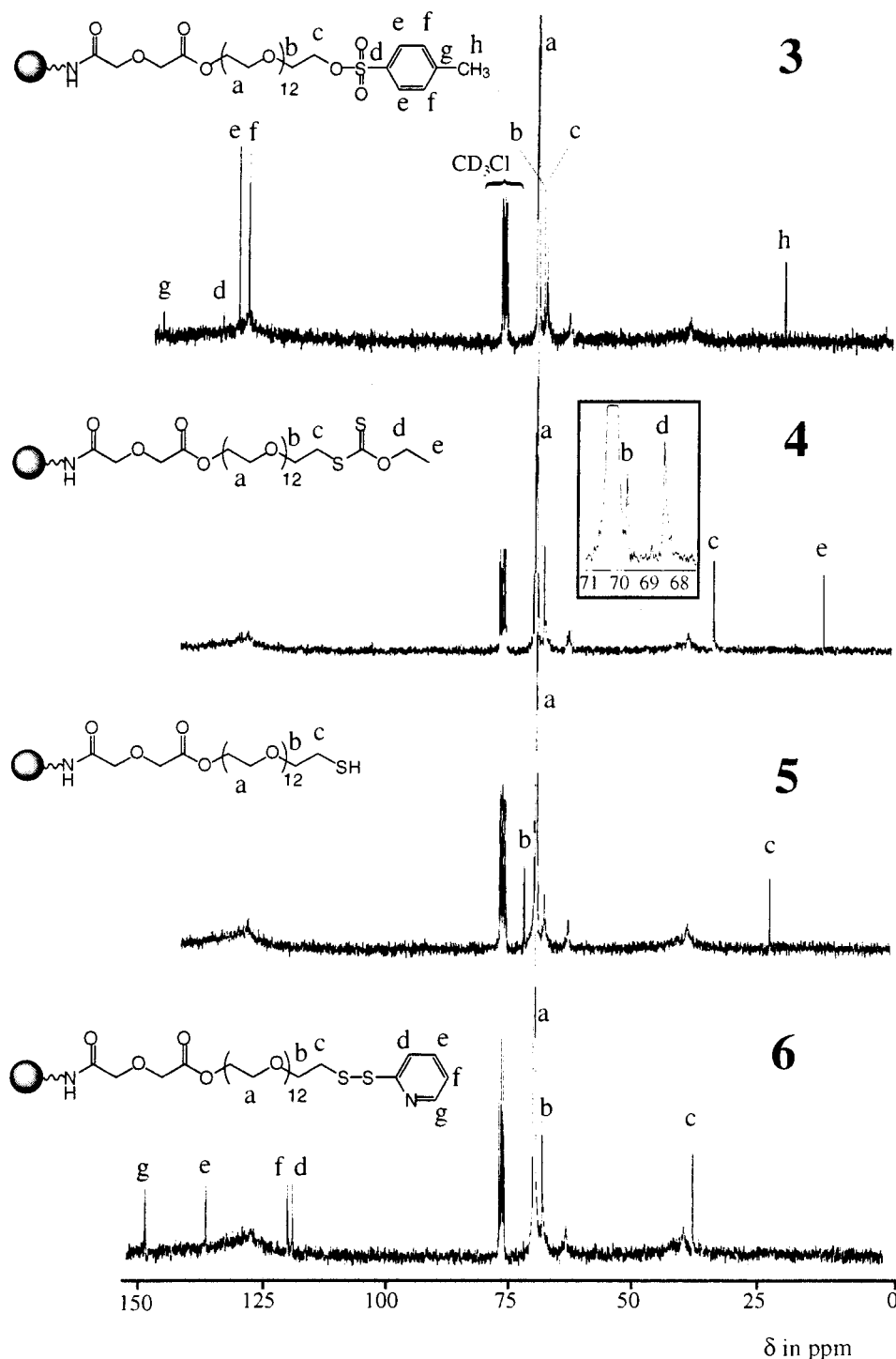


Figure 1. ^{13}C -NMR spectrum (300 MHz) of polymer-bound PEGs in CDCl_3 . Inset for **4** is an enlargement of the major peak.

$\text{OH}/\text{Et}_3\text{N}$). Acidic conditions ($\text{TFA}/\text{CH}_2\text{Cl}_2$) were avoided because a protonated pyridine residue would be a good leaving group, too. Examination of the remaining resin by IR and ^{13}C -NMR spectroscopy indicated that all material had been cleaved off. The global yield for the conversion of resin-bound poly(ethylene glycol) $_{600}$ -OH to α -pyridyldithio- ω -hydroxy-poly(ethylene glycol) $_{600}$ **7** was 65% (i.e., 93% per step) as determined by quantitative release of 2-thiopyridone upon reduction with excess 1-4-dithio-L-threitol (Carlsson et al., 1978).

In summary, we describe here a new, fast, and efficient route to heterobifunctionalized poly(ethylene glycol) derivatives using a solid-phase methodology and employing readily available and inexpensive PEG-diol of any length

as a starting material. Chemistry was combined with solid-phase ^{13}C NMR to optimize and determine the end point of each step. The multistep synthesis described here outlines a general approach valid for any functionalized poly(ethylene glycol) derivative.

ACKNOWLEDGMENT

We warmly thank Dr. Alain Wagner for advice concerning NMR of resin-bound molecules. This work was supported by the Association Française contre les Myopathies, Association Française de Lutte contre la Mucoviscidose, Association pour la Recherche contre le Cancer and Ligue Nationale contre le Cancer.

LITERATURE CITED

- Allen, T. M. (1994) The use of glycolipids and hydrophilic polymers in avoiding rapid uptake of liposomes by the mononuclear phagocyte system. *Adv. Drug Delivery Rev.* 13, 285–309.
- Bayer, E. (1991) Towards the chemical synthesis of proteins. *Angew. Chem., Int. Ed. Engl.* 30, 113–129.
- Bayer, E., Albert, K., Willisch, H., Rapp, W., and Hemmasi, B. (1990) ^{13}C NMR relaxation times of tripeptide methyl ester and its polymer-bound analogues. *Macromolecules* 23, 1937.
- Blume, G., Cevc, G., Crommelin, M. D. J. A., Bakkerwoudenberg, I. A. J. M., Kluft, C., and Storm, G. (1993) Specific targeting with poly(ethylene glycol)-modified liposomes—coupling of homing devices to the ends of the polymeric chains combines effective target binding with long circulation times. *Biochim. et Biophys. Acta* 1149, 180–184.
- Carlsson, J., Drevin, H., and Axen, R. (1978) Protein thiolation and reversible protein–protein conjugation: N–Succinimidyl 3-(2-pyridyldithio)propionate, a new heterofunctional reagent. *Biochem. J.* 173, 723–737.
- Greenwald, R. B., Pendri, A., Martinez, A., Gilbert, C., and Bradley, P. (1996) PEG thiazolidine-2-thione, a novel reagent for facile protein modification: conjugation of bovine hemoglobin. *Bioconjugate Chem.* 7, 638–641.
- Harris, J. M. (1992) In *Polyethylene glycol Chemistry* (J. M. Harris, Ed.) Chapter 1, Plenum Press, New York.
- Herman, S., Hoofman, G., and Schacht, E. (1995) Polyethylene glycol with reactive endgroups: I. Modification of Protein. *Journal Bioact. Comput. Polym.* 10, 145–187.
- Maruyama, K., Takizawa, T., Yuda, T., Kennel, S. J., Huang, L., and Iwatsuru, M. (1995) Targetability of novel immunoliposomes modified with amphipathic polyethylene glycols conjugated at their distal terminals to monoclonal antibodies. *Biochim. Biophys. Acta* 1234, 74–80.
- Nagasaki, Y., Iijima, M., Kato, M., and Kataoka, K. (1995) Primary amino-terminated heterofunctional poly(ethylene oxide). Facile synthesis of poly(ethylene oxide) with primary amino group at one end and a hydroxyl group at the other end. *Bioconjugate Chem.* 6, 702–704.
- Needham D., Stoicheva N., and Zhelev D. V. (1997) Exchange of monooleylphosphatidylcholine as monomer and micelle with membranes containing polyethylene glycol-lipid. *Biophys. J.* 73, 2615–2629.
- Papahadjopoulos, D., Allen, T. M., Gabizon, A., Mayhew, E., Matthey, K., Huang, S. K., Lee, K. D., Woodle, M. C., Lasic, D. D., Redemann, C., and Martin, F. J. (1991) Sterically stabilized liposomes — Improvements in pharmacokinetics and antitumor therapeutic efficacy. *Proc. Natl. Acad. Sci. U.S.A.* 88, 11460–11464.
- Sarin, V. K., Kent, S. B., Tam, J. P., and Merrifield, R. B. (1981) Quantitative monitoring of solid-phase peptide synthesis by the ninhydrin reaction. *Anal. Biochem.* 117, 147–157.
- Thoma, K., Rombach, R., and Ullmann, E. (1964) *Sci. Pharm.* 32, 216.
- Woghiren, C., Sharma, B., and Stein, S. (1993) Protected thiol-polyethylene glycol: a new activated polymer for reversible protein modification. *Bioconjugate Chem.* 4, 314–318.
- Woodle, M. C., and Lasic, D. D. (1992) Sterically stabilized liposomes. *Biochim. Biophys. Acta* 1113, 171–199.
- Yokowama, M., Okano, T., Sakurai, Y., Kikuchi, A., Ohsako, N., Nagasaki, Y., and Kataoka, K. (1992) Synthesis of poly(ethylene oxide) with heterobifunctional reactive groups at its terminals by anionic initiator. *Bioconjugate Chem.* 3, 275–276.
- Zalipsky, S. (1993) Synthesis of an end-group functionalized polyethylene glycol-lipid conjugate for preparation of polymer-grafted liposomes. *Bioconjugate Chem.* 4, 296–299.
- Zalipsky, S. (1995) Functionalized polyethylene glycol for preparation of biologically relevant conjugates. *Bioconjugate Chem.* 6, 150–165.
- Zalipsky, S., Alberico, F., Slomczynska, U., and Barany, G. (1987) A convenient general method for synthesis of N α - or N ω -dithiasuccinoyl (Dts) amino acids and dipeptides: application of polyethylene glycol as a carrier for functional purification. *Int. J. Pept. Protein Res.* 30, 740–783.
- Zalipsky, S., Seltzer, R., and Menonrudolph, S. (1992) Evaluation of a new reagent for covalent attachment of polyethylene glycol to proteins. *Biotechnol. Appl. Biochem.* 15, 100–114.
- Zalipsky, S., Chang, J. M., Albericio, F., and Barany, G. (1994) Preparation and application of polyethylene glycol-polystyrene graft resin supports for solid-phase peptide synthesis. *React. Polym.* 22, 243–258.

BC980039H

CORRECTIONS

Volume 9, Number 5, September/October 1998.

Kohji Ohno and Hiromi Kitano*

CATALYTIC PROPERTIES OF GALACTOSE OXIDASE TO LIPOSOME-FORMING AMPHIPHILES WHICH HAVE MANY PENDENT GALACTOSE RESIDUES

Page 552. The y-axis of Figure 4a should appear as follows.

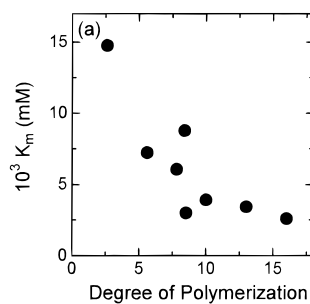


Figure 4.

BC9801981

10.1021/bc9801981

09/26/1998