Synthetic Carbohydrate Vaccines Based on Tumour-Associated Antigens

Tatsushi Toyokuni† and Anil K. Singhal††

The Biomembrane Institute, 201 Elliott Avenue West, Seattle, Washington 98119, U.S.A.

and

†Department of Chemistry, University of Washington, Seattle, Washington 98195, U.S.A.

††Department of Pathobiology, University of Washington, Seattle, Washington 98195, U.S.A.

1 Introduction

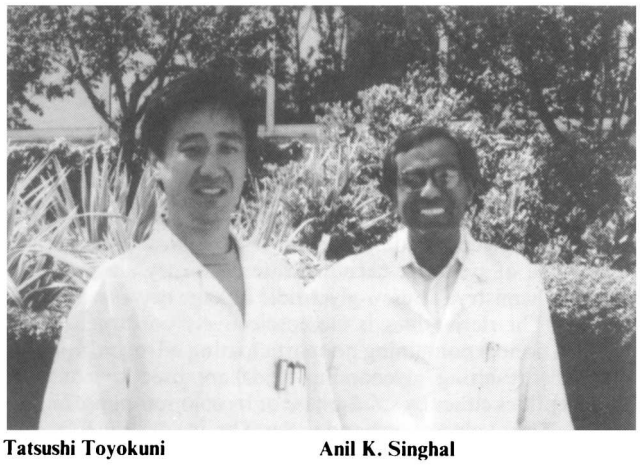
The use of cancer vaccines to induce an anti-cancer immune response is an attractive idea for the treatment of cancer patients. The idea derives from the success of vaccinations in controling viral infections. However, there are some conceptual differences between viral and cancer vaccines. Viral vaccines are used for prophylactic purposes, whereas cancer vaccines are aimed in most cases at a mode of treatment. Since immunity is, in general, highly specific and operates throughout the body, vaccination is a preferable alternative to less specific treatments such as chemotherapy and radiation therapy, particularly for treating farflung metastases.

Earlier studies on cancer vaccines utilized whole tumour cells or tumour cell extracts of uncertain composition. Although some evidence of efficacy was reported, most of these attempts were not successful owing to our poor understanding of the molecular nature of tumour antigens. Recent advances in monoclonal antibody (mAb) technology, coupled with the progress of synthetic and structural chemistry, have identified and characterized a number of tumour-associated antigens. These antigens are composed of carbohydrates and proteins (peptides). Thanks to great strides made by synthetic chemists, some of these antigens are now available in large amounts through chemical synthesis. This is important for vaccine development since some tumour-associated antigens, generally, are not expressed or are expressed in minimum quantities in cancer cells growing in tissue culture. In addition, there has been a rapid accumulation of knowledge of the molecular mechanisms involved in immune regulations. These advances in molecular biology, immunology, and chemistry offer great potential for the development of new and diverse cancer vaccines.

The ultimate goal of a cancer vaccine design is the generation of antigen-specific vaccines (active specific immunotherapy, ASI) by using chemically well-characterized synthetic antigens as immunogens. Synthetic antigens are, however, generally poor immunogens.[1] This shortcoming has been, to some extent, overcome by (i) constructing semi-synthetic vaccines, which are conjugates of synthetic antigens with immunogenic protein carriers, for the induction of T helper (Th) cell activity and an efficient antibody response; (ii) the use of potent immunological adjuvants capable of enhancing the immune response, including a cytotoxic T lymphocyte (CTL) response. Although the use of protein carriers is effective in the production of antibodies, these conjugates are ambiguous in chemical composition and structure. Furthermore, the only adjuvant authorized for use in human vaccines is aluminium salt (alum), which is not always effective, and the most commonly used adjuvant in laboratory animals, *viz.* Freund's complete adjuvant, is toxic for humans. It is, therefore, preferable to develop a chemically unambiguous system for human cancer vaccines. Thus, an ideal cancer vaccine should be a totally synthetic, low-molecular-weight vaccine which does not require either carriers or adjuvants. It is evident that the success of synthetic cancer vaccines relies heavily on the involvement of synthetic chemists.

This review is intended to give a brief overview of the recent progress in synthetic cancer vaccines based on tumour-associated carbohydrate antigens, especially the blood group related antigens of epithelial cancers. These include Tn (GalNAcα1→*O*-Ser/Thr), sialosyl Tn (sTn; NeuAcα2→6GalNAcα1→*O*-Ser/Thr), and T (Galβ1→3GalNAcα1→*O*-Ser/Thr) antigens (Figure 1). Examples are taken mainly from our ongoing project on the development of synthetic Tn (GalNAcα1→*O*-Ser) vaccines as well as those of others and sTn and T antigens. Throughout this review, emphasis will be placed on the important role of synthetic chemists in the cancer vaccine development. For more immunological and clinical aspects in cancer vaccine developments, the reader should refer to more specialized reviews.[2] The development of synthetic peptide vaccines has been reviewed recently.[3]

Tatsushi Toyokuni is head of the Organic Chemistry Department at The Biomembrane Institute and Affiliate Associate Professor of Chemistry at the University of Washington. He obtained his Ph.D. in chemistry under the supervision of T. Suami and S. Ogawa at Keio University, Japan, in 1982. He spent the next 4 years as a postdoctoral research associate with K. L. Rinehart at the University of Illinois at Urbana-Champaign. His current research interests include the design and synthesis of totally synthetic carbohydrate vaccines, glycosyltransferase inhibitors, and carbohydrate mimetics, as well as the development of solid-phase methodology for oligosaccharide synthesis.



Tatsushi Toyokuni **Anil K. Singhal**

Anil K. Singhal is head of the Department of Cancer Diagnostics and Therapeutics at The Biomembrane Institute and Affiliate Associate Professor of Pathobiology at the University of Washington. He obtained his Ph.D. from the Waksman Institute of Microbiology at Rutgers University in 1978. After positions at the Mount Sinai School of Medicine, Imre Co., and Pacific Northwest Research Foundation, he obtained his present position in 1986. His research interests include the study of cellular and humoral immune responses to tumour-associated antigens. It is hoped that these studies will be useful in designing an ideal 'cancer vaccine'.

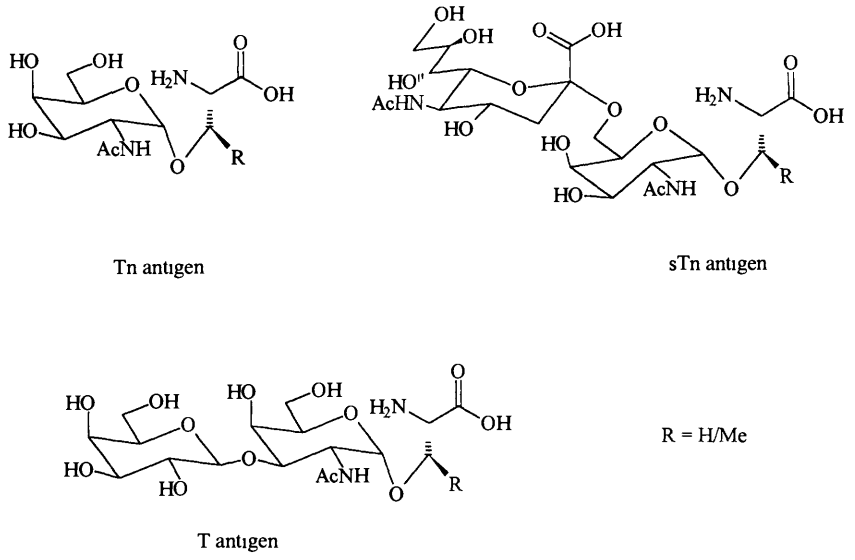


Figure 1 Structures of Tn, sTn, and T antigens

2 Tumour-Associated Carbohydrate Antigens

Cell-surface carbohydrates undergo dramatic changes as a consequence of malignant transformation The alteration results from either incomplete glycosylation or neoglycosylation by tumour cells leading to accumulation of precursors or neostructures Over the past decade studies with specific mAbs have identified a number of tumour-associated carbohydrate antigens (TACAs) expressed on glycolipids, glycoproteins, or both Many of these TACAs have been repeatedly reviewed [4] The TACAs, found on both glycolipids and glycoproteins, are comprised of common backbone structures (lacto-series structures) Galβ1→3GlcNAcβ1→3Gal (type 1) or Galβ1→4GlcNAcβ1→3Gal (type 2) These antigens are widely detected in the most common human cancers, including lung, gastrointestinal, breast, colorectal, liver, and pancreatic cancers The TACAs expressed exclusively on glycolipids are, on the other hand, characterized by ganglio- or globo-series structures Galβ1→3GlcNAcβ1→4Gal or GalNAcβ1→3Galα1→4Gal, respectively), and are abundantly present in specific types of human cancers, such as melanoma, Burkitt's lymphoma, neuroblastoma, and small-cell lung carcinoma The TACAs, identified exclusively on glycoproteins, are derived from the precursors of *O*-linked carbohydrate chains, which include Tn, sTn, and T antigens Among the large numbers of known TACAs, Tn and sTn antigens are the most specific to human cancers, and the most restrictive in their expression on normal cells and tissues Several epithelial tumours express these antigens

2.1 Tn, sTn, and T Antigens

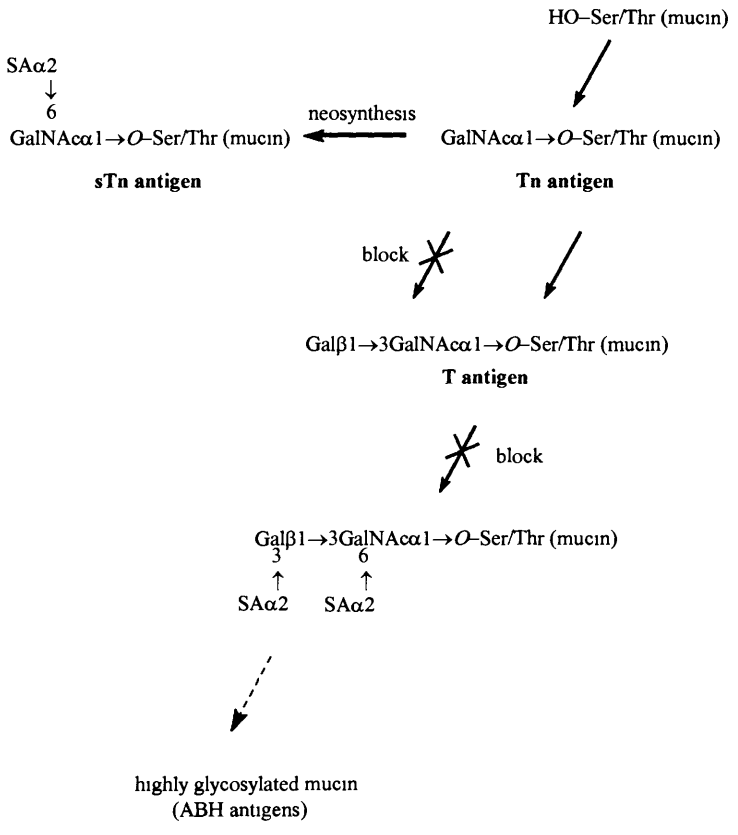
Tn, sTn, and T antigens are expressed in carcinoma-associated mucins Mucins are highly *O*-glycosylated (≈ 75% of total weight), high molecular weight glycoproteins expressed on endodermal epithelial cells, particularly those showing glandular secretory activity The Tn structure in normal cells is cryptic since it is further glycosylated to construct complex *O*-linked glycans on mucin-type glycoproteins, whereas in most human carcinomas this cryptic structure is exposed at the surface due to incomplete synthesis of carbohydrate chains (Scheme 1) The sTn structure is, on the other hand, formed by neoglycosylation, *i e* α(2→6) sialosylation, of Tn antigen Since sialosylation terminates further glycosylations, the resultant disaccharide antigen is accumulated on cancer cells Increased expression of Tn and sTn antigens has been correlated with tumour aggressiveness and poor prognosis in a number of epithelial tumours In addition to Tn and sTn antigens, T antigen - also known as the TF (Thomsen-Friedenreich) antigen - is often referred to as a tumour-associated blood group antigen T antigen, the immediate precursor of human blood group MN antigens, results from the addition of galactose to the Tn antigen Recent studies have indicated, however, that the T structure may not be tumour-associated since mAbs directed to Galβ1→3GalNAcβ1-R but not to Galβ1→3GalNAcα1-R react strongly with tumours [4] During the preparation of this manuscript, increased expression of T antigen in colonic adenocarcinoma has been reported [5] The true nature of tumour-associated T antigen is, however, still ambiguous Tn antigen is expressed in over 70% of breast, lung, colon, and stomach carcinomas Hence, the vaccine based on Tn antigen is of particular interest because of the potential usefulness in treating metastatic breast cancer

2.2 Immunogenicity of Tn Antigen

TACAs are generally not, or only weakly, immunogenic Tn antigen, as well as sTn and T antigens, however, displays strong immunogenicity We have demonstrated that immunization of mice with asialo ovine submaxillary mucin (A-OSM), which contains almost exclusively Tn antigen, provided protection against challenge with a highly invasive mouse mammary carcinoma, TA3-Ha, which expresses Tn antigen[6] (Figure 2) Furthermore A-OSM induced *in vitro* proliferation of CD4 + T lymphocytes (T helper cell, Th-cell) obtained from mice preimmunized with A-OSM or irradiated TA3-Ha cells (Figure 3) These findings indicated that Tn antigen on a protein back bone is capable of providing cellular immunity and protection against tumours in mice This is important in view of the recent notion that antigen-specific cellular immune responses are more effective than antibody responses for cancer treatment [2] Springer *et al* have reported recently that a Tn vaccine composed of O red blood cell-derived Tn antigen adsorbed onto $Ca_3(PO_4)_2$ and the typhoid vaccine prevents breast carcinoma recurrence [7]

3 Synthesis of Tn, sTn, and T Antigens

Several laboratories have synthesized Tn, sTn, and T antigens as well as their glycopeptides The synthesis requires the combined knowledge of synthetic carbohydrate chemistry and synthetic peptide chemistry Thus, α-glycosidic linkage between GalNAc and Ser/Thr derivatives is stereoselectively constructed with glycosyl donors containing nonparticipating nitrogen groups at C-2 The resulting glycoamino acids are used to construct glycopeptides either by solid-phase or by solution-phase methodology The *O*-glycosidic bond to Ser/Thr, in addition to its acid sensitivity, is also susceptible to cleavage by base-induced β-



Scheme 1 Accumulation of Tn, sTn, and T antigens in cancer

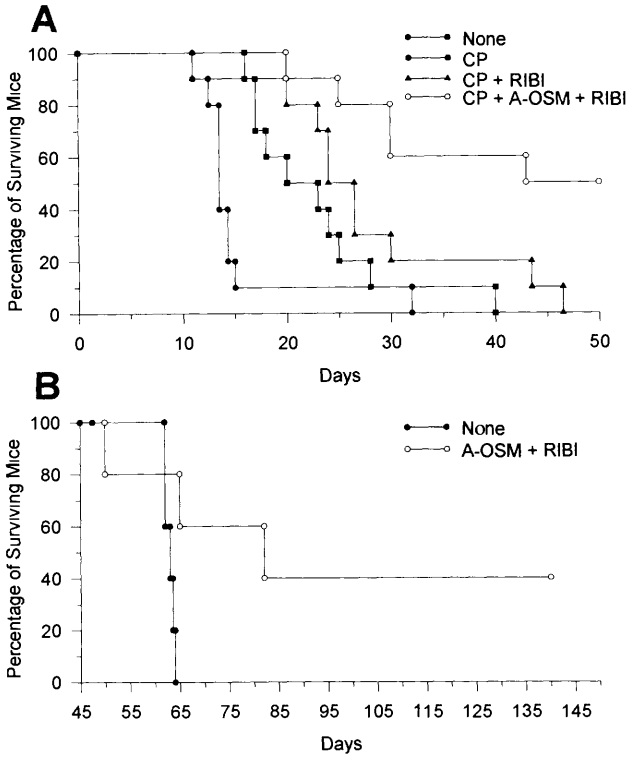


Figure 2 Survival of mice from challenge with TA3-Ha tumour cells (A) Mice were immunized subcutaneously (s c) with A-OSM or Ribi adjuvant (Ribi) alone on day − 7 and were then challenged with 7×10^2 TA3-Ha cells intraperitoneally (i p) on day 0 Mice received a single i p injection of cyclophosphamide (CP) on day 1 The animals were further immunized s c with different antigens on days 2, 5, 12, and 19 (B) Mice immunized with A-OSM, surviving from the initial challenge, were rechallenged with 2×10^3 cells i p on day 48 and survival was monitored

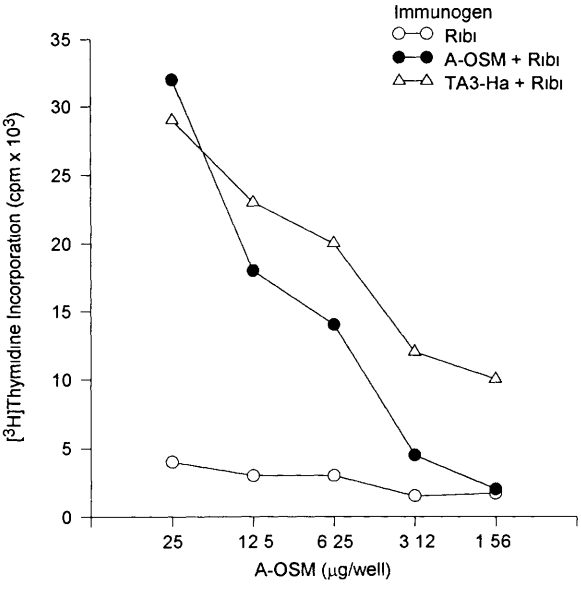
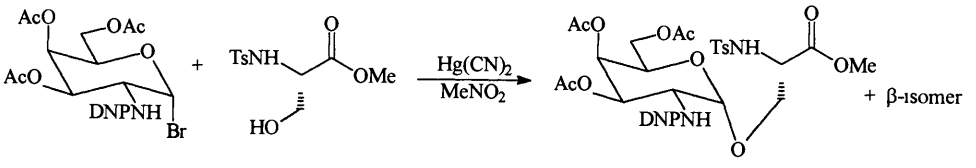


Figure 3 Lymphocyte proliferation in response to Tn-containing mucin, A-OSM Mice were immunized with A-OSM plus Ribi, irradiated TA3-Ha plus Ribi, or Ribi alone Lymph node lymphocyte were stimulated *in vitro* with different dosages of A-OSM Lymphocyte proliferation was measured by 24 h pulse with [^{3}H]thymidine after 72 h

elimination (leading to a dehydroamino acid) The selection of protecting groups, therefore, plays a major role in the successful synthesis of glycoamino acids and glycopeptides Synthesis of glycopeptides has been a subject of many reviews [8]

In spite of the striking development of gene techniques, genetic production of glycopeptides or glycoproteins is still limited This is due to the fact that the glycosylation process is controlled by glycosyltransferases without direct participation of the gene code (DNA or RNA) In addition, isolation of



DNP = 2,4-dinitrophenyl
Ts = *p*-toluenesulfonyl

Scheme 2

glycoamino acids and glycopeptides from natural sources is often troublesome because of the instability of the *O*-glycosidic bond to Ser/Thr, as described above, and biological microheterogeneities Consequently, chemical synthesis is the best way of supplying these compounds in a large amount

3.1 Stereoselective Formation of α-*O*-Glycosidic Linkage between GalNAc and Ser/Thr

The α-glycosidic linkage between GalNAc and the side-chain hydroxyl group of Ser was first achieved by the Konigs–Knorr condensation of the glycosyl bromide, in wich the 2,4-dinitrophenyl (DNP) group was used as a nonparticipating amino protection, with the Ser derivative[9] (Scheme 2) Since Paulsen and co-workers successfully utilized the azide group as a nonparticipating 2-substituent in the synthesis of the trisaccharide chain of blood group substance A (GalNAcα1→3Galβ1→3GlcNAc)[10] (Scheme 3), 2-azido-2-deoxy-galactose derivatives have become versatile glycosyl donors for the synthesis of α-glycosides of GalNAc In 1979, Lemieux and Ratcliffe introduced an efficient method for the preparation of 2-azido-2-deoxy-glycopyranoses by azidonitration of *O*-protected glycals[11] (Scheme 4) The nitrate adduct (1) is readily converted into various glycosly donors [12] For example, the anomeric nitrate group is directly displaced by halide ions or *O*-ethyldithiocarbonate to give glycosyl halides (2) or *S*-glycosides (3), respectively, while trichloroacetimidates (4) and fluorides (5) are prepared through hydrolysis of the anomeric nitrate Mild and neutral conditions for the anomeric denitration have been devised [13]

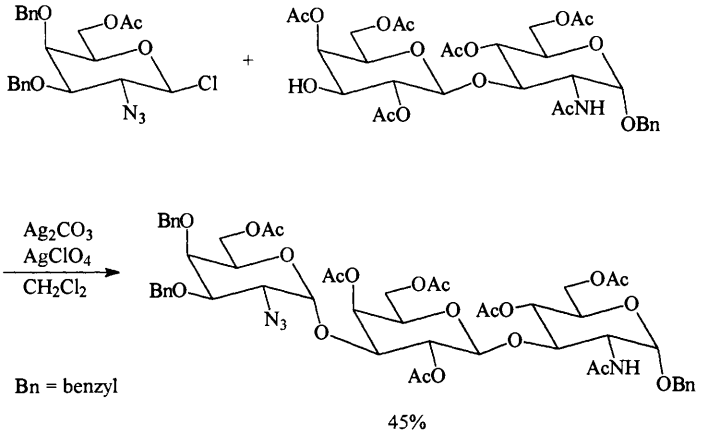
Recently, we have developed an alternative route to 2-azido-2-deoxy-glycopyranoses by electrophilic azidation of 2-deoxy-aldono-1,5-lactones[14] (Scheme 5) The reaction proceeds in one pot with high stereoselectivity The 2-deoxygalactono-1,5-lactone (6) gave 2-azido-2-deoxy-galactopyranose (7) in 80% yield, whereas 2-deoxyglucono-1,5-lactone (8) yielded 2-azido-2-deoxymannopyranose (9) in 65% yield Azidophenylselenylation of *O*-protected glycals has also proven to be effective for the preparation of 2-azido-2-deoxy glycopyranoses [15]

3.2 Tn, sTn, and T Antigens

We have prepared Tn antigen on a practical large-scale (50—100 mmol) by employing the trichloroacetimidate method[16] (Scheme 6) Glycosylation of the Ser derivative (11) (R = H) with glycosyl imidate (10) using trimethylsilyl trifluoromethanesulfonate (TMSOTf) as a promoter yielded a 5 1 mixture (72% yield) of the α- (12) and β-glycosides The α-glycoside predominates regardless of the anomeric configuration of (10) Stereoselectivity of the glycosylation with the Thr derivative (11) (R = Me) was reduced to 4 1 (α/β) owing to the bulky Me group Selective reduction of the azido group in (12) is effective with, for example, H_2S in aqueous pyridine, $NaBH_4$–$NiCl_2$,[17] or Lindlar catalyst [18] Direction converstion (N_3→NHAc) is also possible by treatment with thioacetic acid (AcSH) [19] After conversion of N_3 into NHAc, hydrogenolysis followed by re-protection of the amino group by *t*-butyloxycarbonyl (Boc) group afforded the key glycosyl amino acid (13), a useful building block for the synthesis of Tn vaccines

Starting from the properly protected Tn antigen or its precursor, sTn and T antigens have been synthesized by α(2→6) sialosylation[19] and β(1→3)galactosylation,[20] respectively (Schemes 7 and 8) These antigens have been alternatively prepared by condensation of the corresponding disaccharides with Ser/Thr derivatives (Schemes 9[18] and 10[17]) Based on the synthetic sTn antigen, it was determined that monoclonal antibodies B72 3 and MLS 102, raised against metastatic breast adenocarcinoma (for B72 3) and human colonic cancer cells (for MLS 102), recognize the sTn structure [21] In Scheme 7, the C-3β phenylthio derivative of sialic acid was used to secure α-selectivity of sialosylation by taking advantage of the directing effect of the phenylthio group The stereoselective α-glycosylation of sialic acid is of great interest among synthetic carbohydrate chemists and has been reviewed extensively [22]

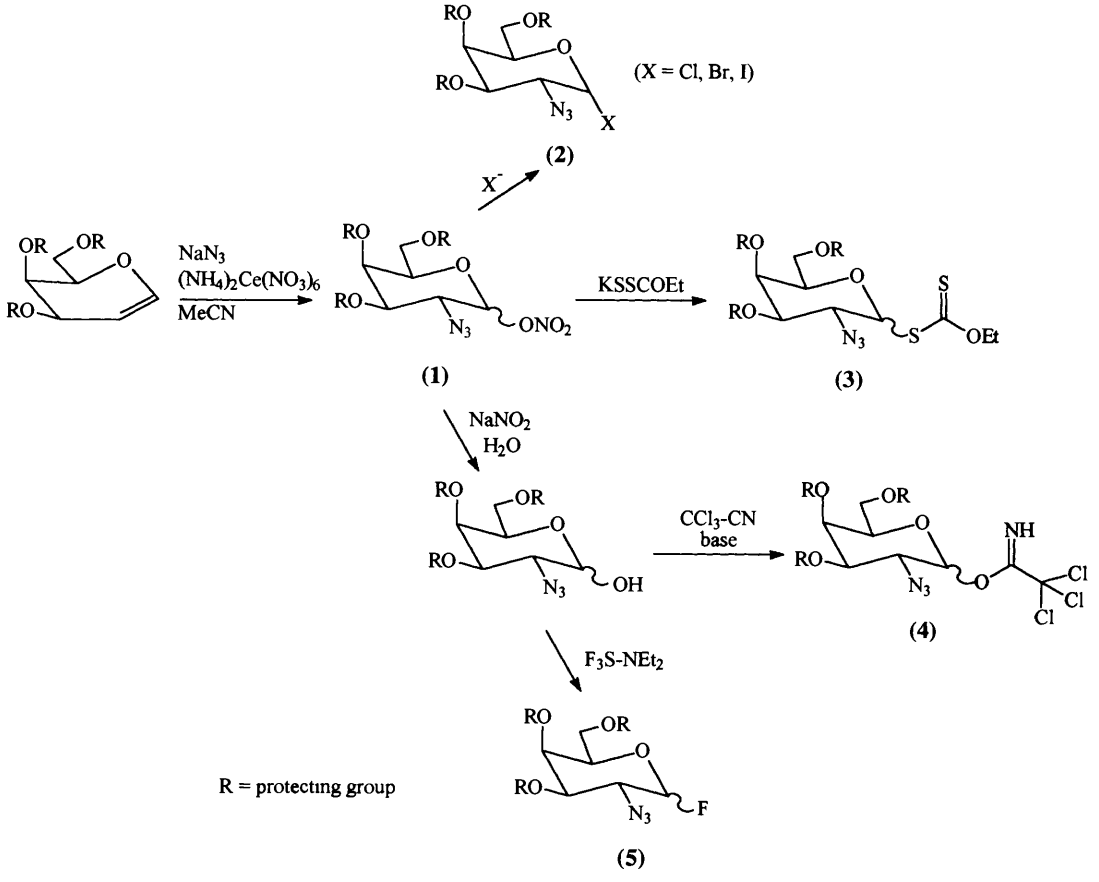
It is also reported that α-*N*-acetylgalactosaminidase from beef liver synthesizes Tn antigen from GalNAc and Ser under reverse hydrolysis condition [23]



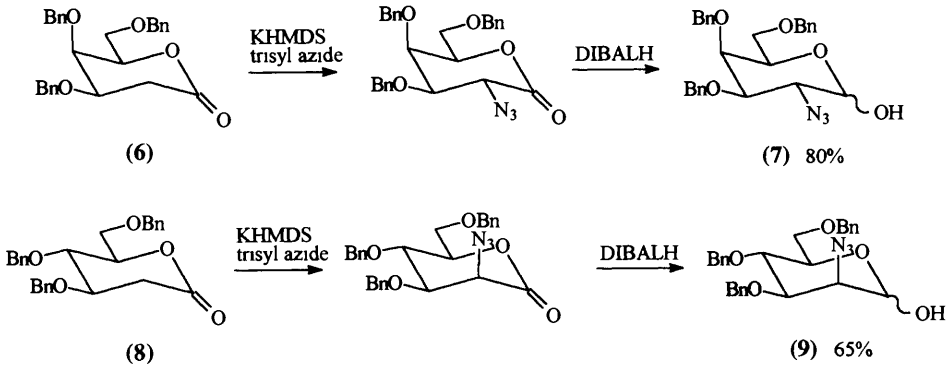
Bn = benzyl

45%

Scheme 3



Scheme 4



KHMDS = potassium bis(trimethylsilyl)amide
DIBALH = diisobutylaluminum hydride

Scheme 5

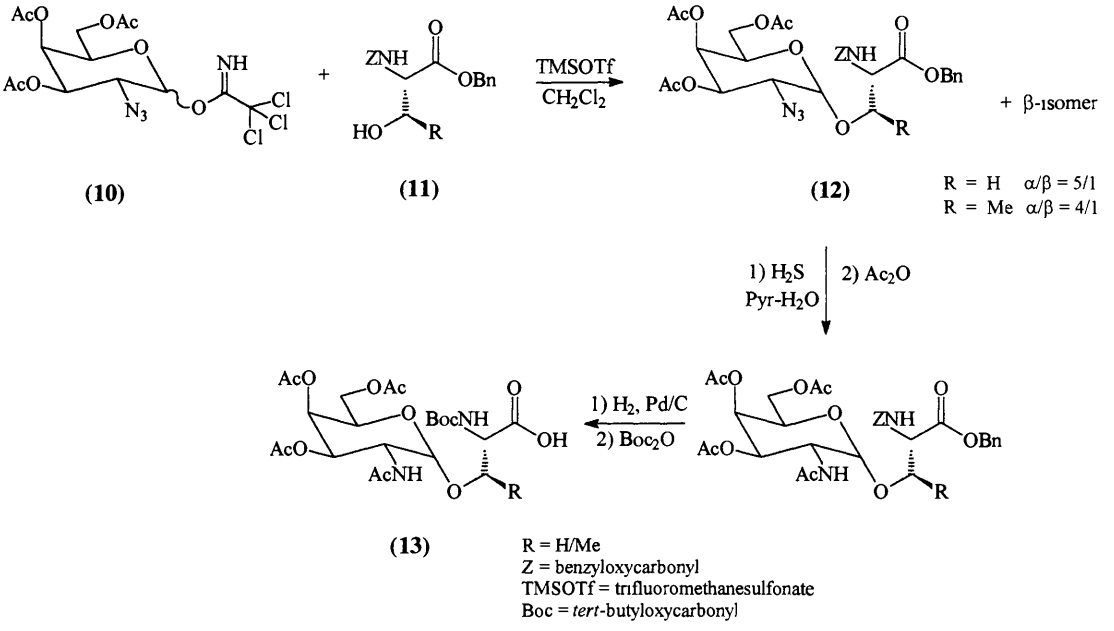
4 Amplification and Modification of Tn Antigen

It is suggested that antigenicity of melanoma cells might depend on the density of ganglioside GM_3 expressed on those cells [24] It is also reported that the minimum Tn antigen structure required for antibody binding is a trimeric structure but not a monomeric one [25] Therefore, assemblage of Tn antigen in a clustered fashion is of practical importance to induce efficient immune responses

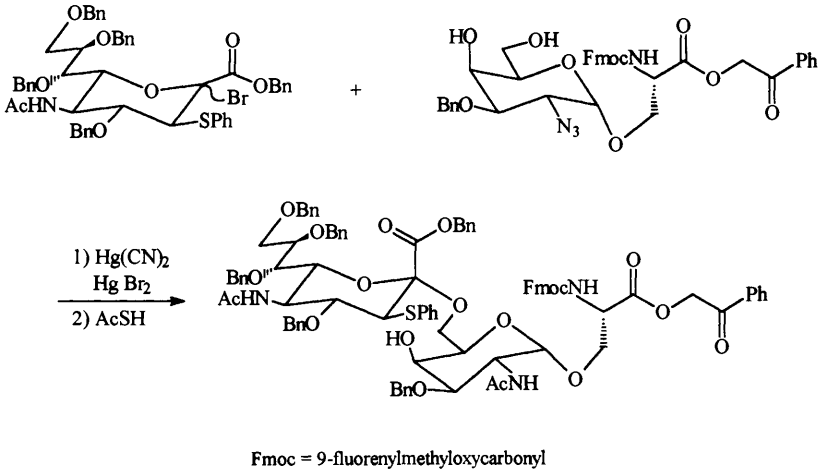
Several laboratories have succeeded in synthesizing carbohydrate-cluster structures found in mucin-type glycoproteins either by solid-phase or by solution-phase methodology [8]

4.1 Linear Amplification

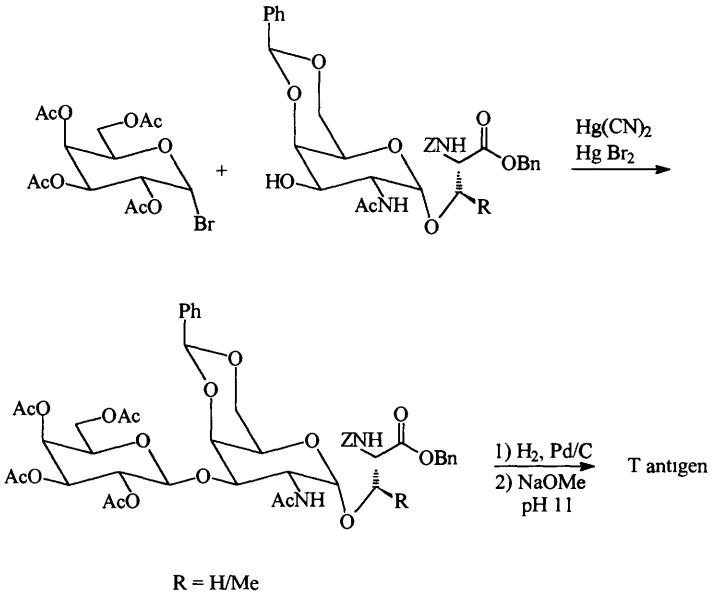
Starting from (13) (R = H), monomeric, dimeric, and trimeric Tn antigens with a spacer [(15), (17), and (19), respectively] were synthesized[16] (Scheme 11) A spacer was installed in order to preclude any steric interference between Tn antigen and a carrier molecule Coupling as well as introduction of a spacer was successful by the *N*-hydroxysuccinimide (NHS) ester method The coupling sequence consisted of (i) acidolysis to unmask the amino group, (ii) condensation with the pre-formed NHS ester (14) (for the trimers reiteratation of steps i and ii) (iii) conversion of the *N*-Boc into *N*-Ac group, and (iv) *O*-deacetylation The acid-labile glycosidic bond was resistant to the condition of



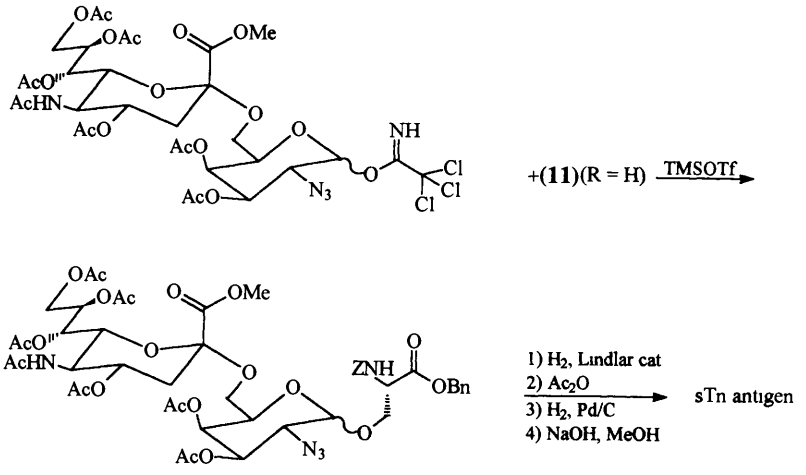
Scheme 6



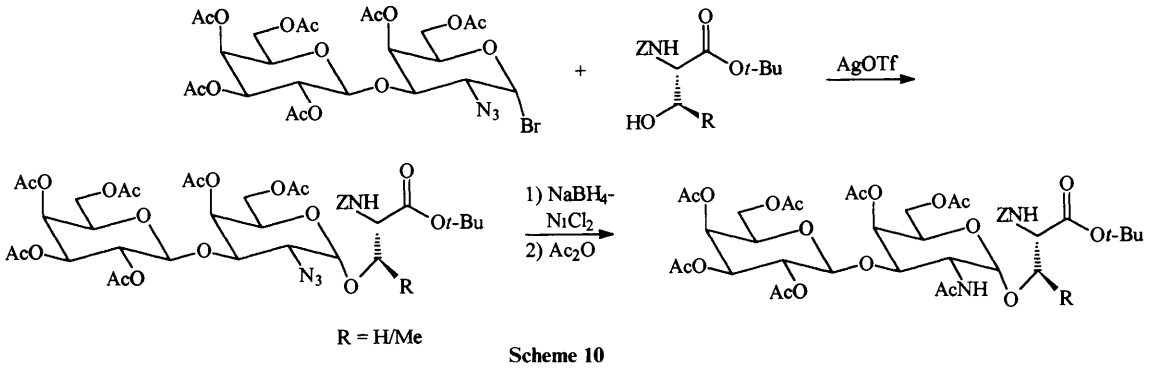
Scheme 7



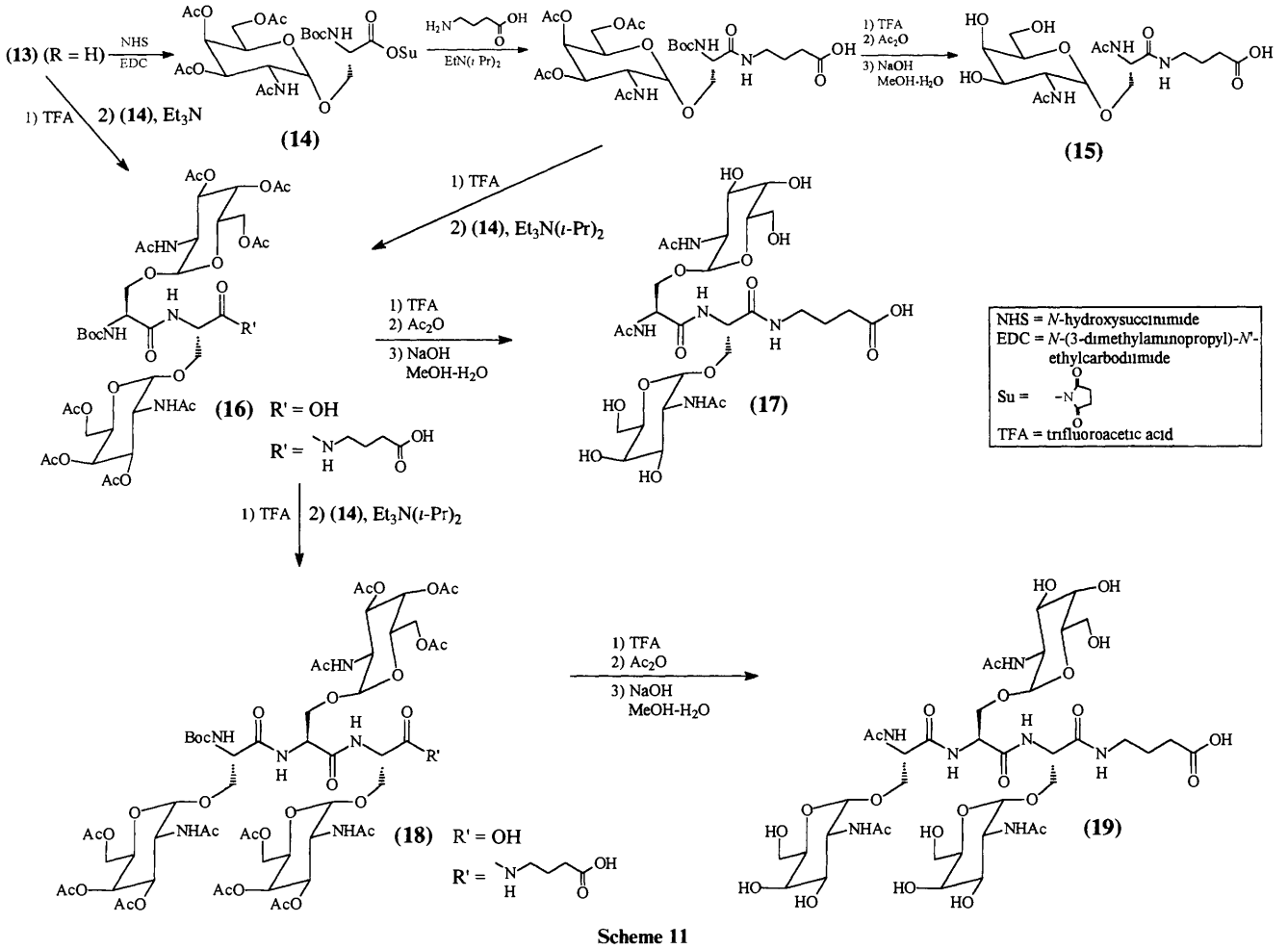
Scheme 8



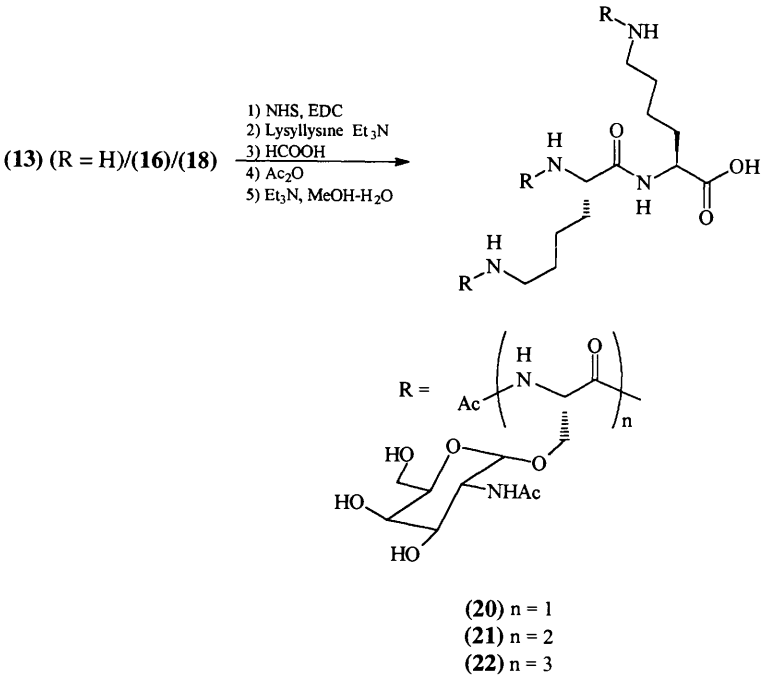
Scheme 9



Scheme 10



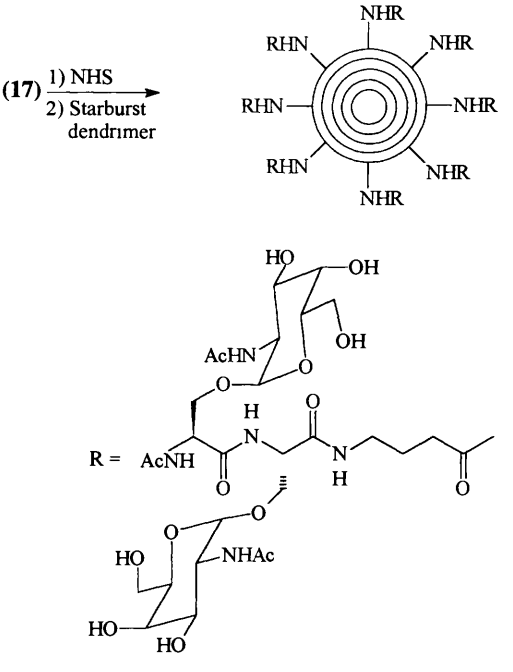
Scheme 11



(20) n = 1
(21) n = 2
(22) n = 3

Scheme 12

acidolysis (CF_3COOH, room temperature, 10 min) In addition no products from β-elimination or racemization were observed during the saponification (1M aqueous NaOH, MeOH-H_2O, 15 min) A similar coupling sequence was also used to prepare protected dimeric and trimeric Tn antigens[26] [(16) and (18), respectively]



(23)

Scheme 13

4.2 Three-dimensional Amplification

The oligomeric Tn antigens were assembled on a lysyllysine backbone constructing trivalent Tn conjugates [26] The three amino groups (one α- and two ε-amino groups) are utilized to attach three Tn-antigen clusters and one carboxyl group being available for conjugation with a carrier molecule [27] A similar system called MAPS (multiple antigen peptide system), developed by Tam, has been shown to be effective without a carrier protein for inducing strong immune responses against peptide antigens [28] The monomeric (13), dimeric (16), and trimeric Tn antigens (18) were first converted into their NHS esters and coupled to lysyllysine Sequential acidolysis, capping, and saponification furnished the corresponding trivalent conjugates (20), (21), and (22) (Scheme 12)

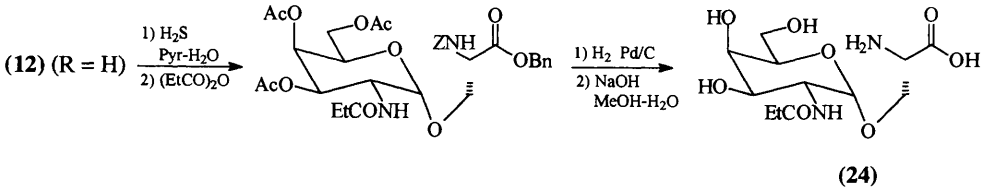
We have also assembled Tn antigen on a commercially available Starburst dendrimer, which is a spherical polymer constructed from methyl acrylate and ethylenediamine [16] Dimeric Tn antigen (17) was, after conversion into the NHS ester, incorporated to the 5th generation dendrimer (diameter 40 1 Å, the number of terminal amino groups 48 0) to give (24) (Scheme 13)

4.3 Modification of Tn Antigen

Successful improvement of the poor immunogenicity of the Group B meningococcal polysaccharide has been achieved by substituting *N*-propionyl for the *N*-acetyl groups in the polysaccharide [29] It is conceivable that the chemically modified antigen is recognized as more foreign by the immune system, resulting in the induction of a stronger immune response On a similar premise, the *N*-propionyl analogue of Tn antigen (24) was prepared by using propionic anhydride in lieu of Ac_2O (Scheme 14)

5 Semi-Synthetic Cancer Vaccines

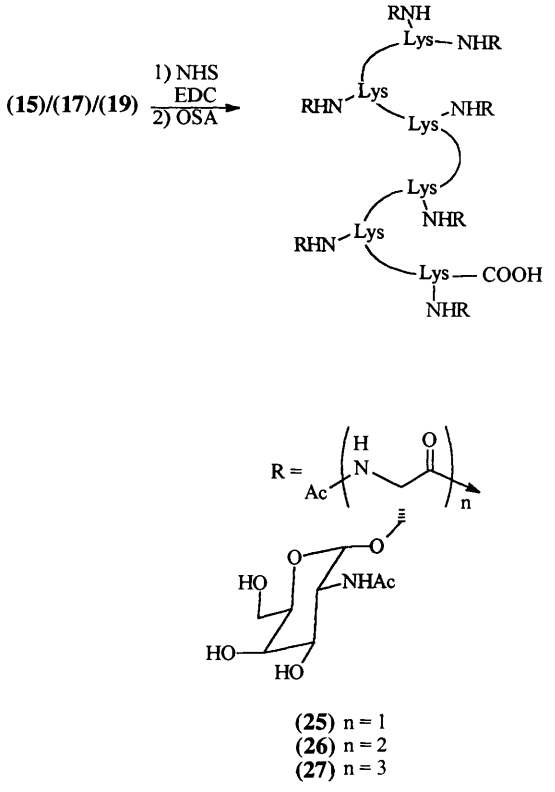
Synthetic carbohydrate antigens are coupled to immunogenic carrier proteins and administered with potent immunological adjuvants Semi-synthetic cancer vaccines composed of Tn, sTn, or T antigen covalently linked to keyhole limpet hemocyanin (KLH) and other carrier proteins have been actively investigated in several laboratories Although none of these conjugates has been fully characterized yet, the primary amino groups, including one *N*-teminal amino group and lysine ε-amino groups in the carrier protein are probable sites for the attachment of antigens These amino groups are unprotonated under ordinal coupling conditions (pH > 7)



Scheme 14

5.1 Semi-Synthetic Tn Vaccines

Monomeric, dimeric, and trimeric Tn antigens [(15), (17), and (19), respectively] were coupled to ovine serum albumin (OSA) by the NHS ester method[16] (Scheme 15) The coupling typically yielded 250 μg of Tn antigen per 1 mg of OSA ($Mr \approx 66000$, 66 lysine residue/mol) The conjugates (25)—(27) were examined for their ability to stimulate Tn specific immune responses in mice Sera from mice immunized with the conjugates were measured for the presence of antibody titers against A-OSM in an enzyme-linked immunosorbant assay (ELISA) The conjugates produced high-titre IgM responses against Tn antigen (Figure 4) In addition, the conjugates (26) and (27) induced measurable IgG anti-Tn antibody responses As expected, oligomeric Tn antigens (17) and (19) proved to be more effective in the production of anti-Tn antibodies than did the monomeric one (15) Interestingly, the dimeric Tn-Starburst dendrimer conjugate (23) did not generate anti-Tn antibodies in spite of the high density of Tn antigen Immunological evaluation of trivalent conjugates (20)—(22) are currently underway



Scheme 15

5.2 Semi-Synthetic sTn and T Vaccines

Longenecker and his associates have constructed a semi-synthetic T vaccine composed of synthetic T-disaccharide (Galβ-1→3GalNAcα1→) covalently attached to KLH through a spacer (T–KLH)[30] (Figure 5) T–KLH plus Ribi adjuvant is shown to induce IgM and IgG antibodies and delayed type hypersensitivity (DTH) in mice against mouse mammary adenocarcinoma expressing the T antigen In addition, this vaccine prolongs survival of mice bearing the same tumour Phase I clinical trials in patients bearing metastatic ovarian carcinoma have been carried out with T–KLH plus DETOX adjuvant resulting in the generation of high-titre specific anti-T IgG and IgM responses

They have also reported similar results with synthetic sTn and

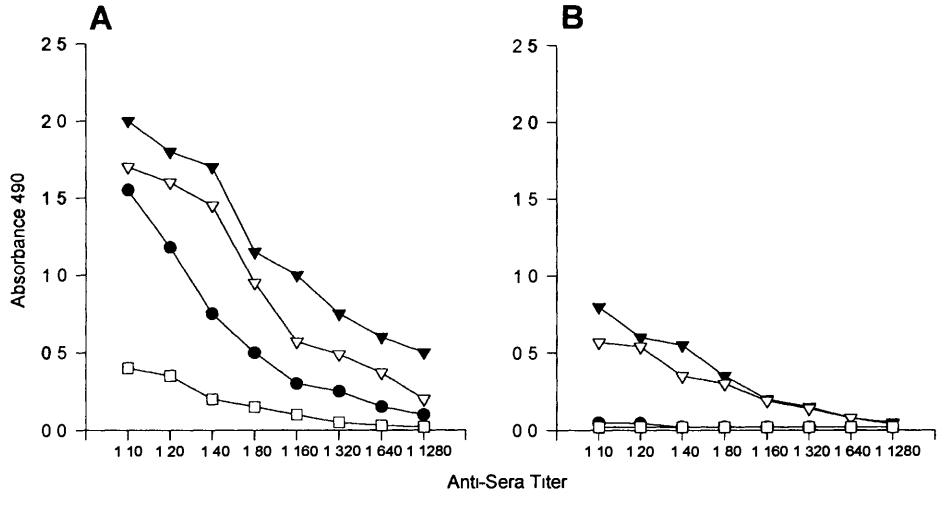
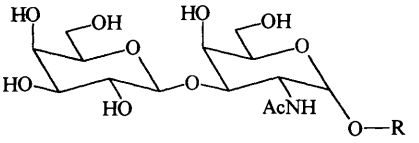
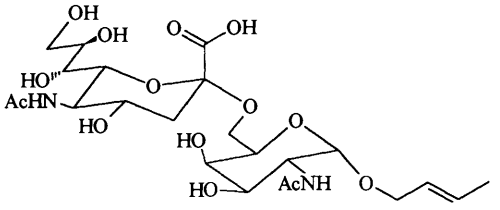


Figure 4 Serum anti-Tn IgM (A) and IgG (B) titres in mice immunized with either (25) (●), (26) (▽), (27) (▼), or (23) (□) All conjugates were suspended in Ribi adjuvant at a concentration of 0 5 mg/ml according to the manufacturer's instructions Mice were immunized twice (one week apart) with 100 μg of antigen s c at the base of the tail and at the neck Seven days after the second immunization, sera were titred against A-OSM in ELISA



R = a spacer

T-disaccharide



sTn-disaccharide

Figure 5 Structures of the T-disaccharide and sTn-disaccharide haptens.

KLH conjugate[31] (Figure 5). The α-crotyl derivative of sTn-disaccharide (NeuAcα2→6GalNAcα1→) was first synthesized and conjugated to KLH through the crotyl linker arm (sTn-KLH), and a semi-synthetic vaccine containing sTn-KLH in DETOX adjuvant was formulated. They find that breast cancer patients immunized with this vaccine produce IgG antibodies specific to sTn antigen and appear to have an improved survival rate.

These encouraging results with Tn–OSA, sTn-KLH, and T-KLH vaccines are significant since carbohydrate antigens are believed to react poorly with T cells and to stimulate B cells in the absence of any Th cell enlistment, producing only an IgM antibody response. T-cell stimulation by antigens is necessary in order to produce antigen-specific cellular immune responses, which are important for effective cancer treatment. There has been accumulating evidence supporting T-cell recognition by carbohydrates.[32] Cancer vaccines based on tumour-associated gangliosides, neuraminic-acid-containing glycosphingolipids, have recently shown some promise in the suppresion of tumour growth.[33] These include purified GM_2 [GalNAcβ1→4(NeuAcα2→3)Galβ1→4Glcβ1→Cer] coated on *Bacillus Calmette-Cuérin* (BCG) and G_{D3} (NeuAcα2→8NeuAcα2→3Galβ1→4Glcβ1→Cer)–KLH conjugate.

6 Totally Synthetic Tn Vaccine

Over the past decade, Jung *et al.* have developed totally synthetic peptide vaccines using the lipopeptide tripalmitoyl-*S*-glyceryl-cysteinylserine (P_3CS) as a combined carrier and adjuvant system.[34] The lipopeptide P_3CS is a highly potent B-cell and macrophage activator derived from the *N*-terminus of Braun's lipoprotein, the major outer-membrane protein in gram-negative bacteria. The disease-causing virus peptide and P_3CS conjugate without any carrier proteins or adjuvants has been shown to protect guinea pigs from foot-and-mouth disease.[35] Furthermore, it has been demonstrated that the influenza peptide and P_3CS conjugate can induce *in vivo* priming of virus-specific cytotoxic Ty lymphocytes (CTLs).[36] Very recently, the lipopeptide has been used to design a synthetic AIDS vaccine.[37] P_3CS is non-immunogenic and has no toxic side effects nor does it cause tissue damage in animals. In addition, its adjuvanticity is comparable to the classical Freund's adjuvant. It is speculated that lipopeptide P_3CS acts as a lipid anchor to mediate direct binding of the antigen to Ia molecules which then leads to Th-cell activation.[34]

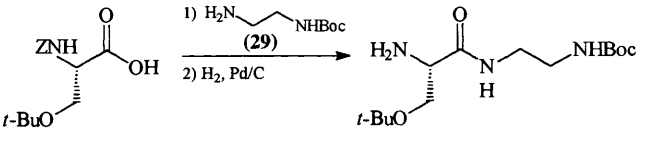
Besides P_3CS conjugates, other systems have been reported. Conjugates of luteinizing hormone releasing hormone (LH-RH) with *N*-acetyl-muramyl-L-alanyl-D-isoglutamine (MDP) have been shown to produce anti LH-RH antibodies and an immunological castration in mice.[38] It is also reported that conjugates of HIV-1-derived peptide with lauroyl-L-alanyl-D-glutamyl-L,L-2,6-diaminopimeloylglycine (Pimelautide, RP40639) and with lauroyl-L-alanyl-D-glutamyl-L,L-2,6-diaminopimelic acid (trimexautide, RP 56142) induce strong antibody and virus-specific CTL responses.[39] Conjugation of antigens with a combined carrier and adjuvant system appears to be a promising way of designing totally synthetic vaccines. It should be emphasized that these vaccine constructs are obtained by reproducible and chemically well-defined synthetic methods.

We have constructed a conjugate of dimeric Tn antigen with P_3CS.[16]

6.1 Synthesis

The diastereomeric mixture of the lipoamino acid (31) (P_3C-OH) (see Scheme 17) is commercially available (Boehringer Mannheim, Indianapolis, IN, USA), and is readily prepared in a large amount by the published method.[34] Although we used the diastereomeric mixture, P_3CS with *R* configuration at the asymmetric carbon of the glyceryl unit is reported to be immunologically more active than *S* isomer.[40]

An ethylamino spacer was introduced to the *C*-terminal of P_3CS to allow coupling with dimeric Tn antigen (17) (Scheme 16). Thus, Ser derivative (28) was converted into (30) by reaction with diaminoethane derivative (29) followed by hydrogenolysis. Amine (30) was joined to (31) by the 1-hydroxybenzotriazole (HOBt) method (Scheme 17). Acidolysis of the product to amine (32) with TFA, followed by coupling to (17), provided synthetic vaccine (33). As a control experiment, the serylserine–P_3CS conjugate (34) was also prepared in a similar manner.



(28) **(30)**

Scheme 16

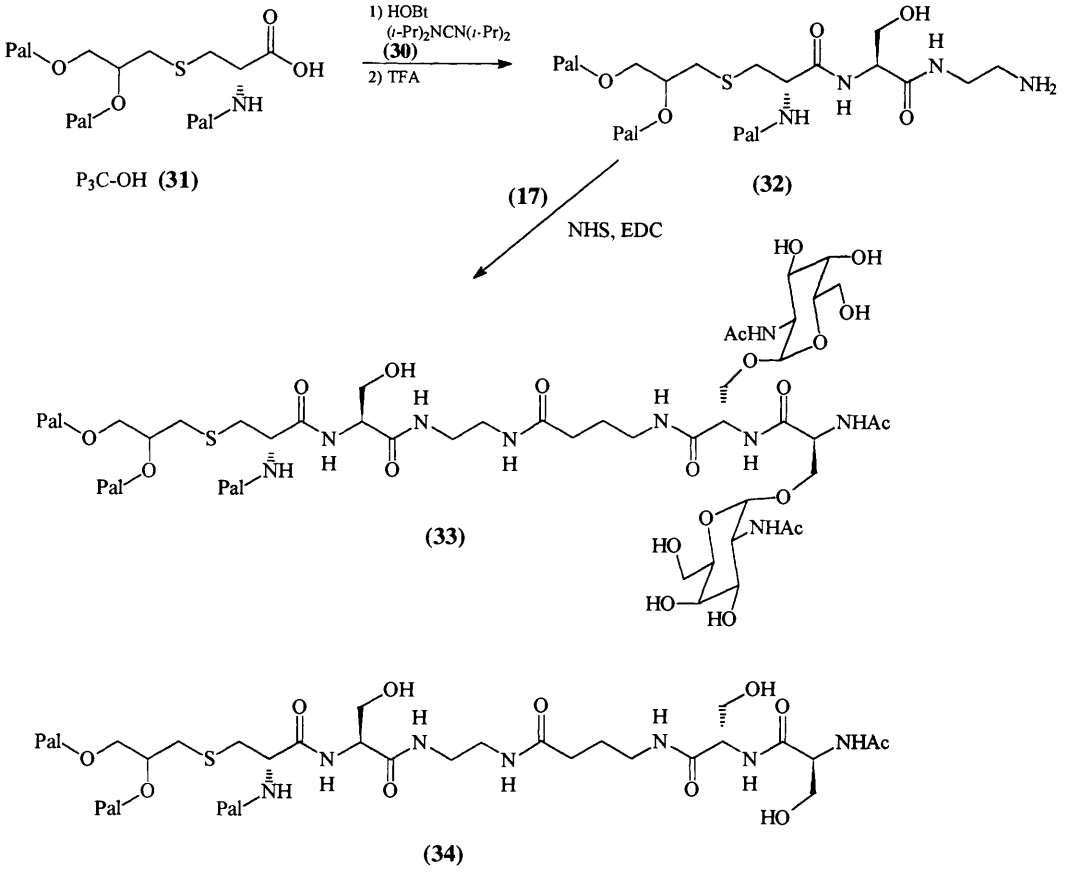
6.2 Immunological Evaluation

Synthetic Tn vaccine (33) elicited, without any protein carriers or additional adjuvants, Tn antigen specific immune responses (IgM and IgG) in mice[16] (Figure 6). This is the first example demonstrating that a synthetic, small carbohydrate antigen alone can induce antigen-specific immune responses.

Recently we have found that T cells from mice immunized with (33) exhibited antigen-specific stimulation. Furthermore, the synthetic vaccine provides protection against challenge by highly invasive TA3-Ha tumour cells in syngeneic mice (manuscript in preparation). Being a totally synthetic, low-molecular weight, and carrier-free immunogen, (33) could be a prototype of synthetic carbohydrate vaccines.

7 Conclusions

The confluence of chemistry and immunology has cultivated a new approach to vaccine development, *i.e.* a totally synthetic vaccine. One of the merits of synthetic vaccines lies in their structural simplicity, allowing ready accessibility, characterization, and manipulation. It is, therefore, possible to study the structure–activity relationship in order to design more effective vaccines. As the combined efforts of chemists and immunologists continue, success in creating totally synthetic vaccines might become a reality in the not too distant future.



Scheme 17

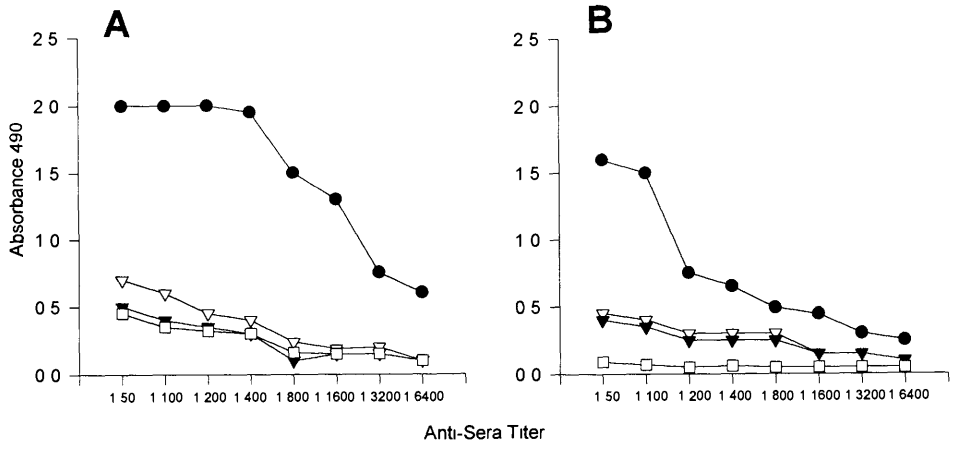


Figure 6 Serum anti-Tn IgM (A) and IgG (B) titres in mice immunized with either (33) (●), (32) (▽), (34) (▼), or Intralipid (□) All conjugates were dissolved in Intralipid phosphate-buffered saline (PBS) (1/1) at a concentration of 0 5 mg/ml Mice were immunized twice (one week apart) with 100 μg of antigen s c at the base of the tail and at the neck Seven days after the second immunization, sera were titred against A-OSM in ELISA

Acknowledgements We thank our valued colleagues, especially Dr Sen-itiroh Hakomori, for their advice and help We are also grateful to Ms Jennifer Stoeck for reading through this manuscript Our work described was supported by funds from the Biomembrane Institute

8 References

1 M J Francis, in 'Vaccines Recent Trends and Progress', ed G Gregoriadis, A C Allison, and G Poste, Plenum Press, New York, 1991, p 13

2 G D MacLean and B M Longenecker, *Can J Oncol*, 1994, **4**, 249, D M Pardoll, *Trends Pharmacol Sci*, 1993, **14**, 202, 'Specific Immunotherapy of Cancer with Vaccines', ed J -C Bystryn, S Ferrone, and P Livingston, The New York Academy of Sciences, New York, 1993

3 P T P Kaumaya, S Kobs-Conrad, A M DiGeorge, and V C Stevens, in 'Peptides', ed C Basava and G N Anantharamaiah, Birkhauser, Boston, 1994, p 133, A G Beck-Sickinger and G Jung, *Pharm Acta Helv*, 1993, **68**, 3, D N Levy and D B Weiner, *Biomed Appl Biotechnol*, 1993, **1**, 219

4 S Hakomori, *Curr Opin Immunol*, 1991, **3**, 646, A Singhal and S

Hakomori, *BioEssays*, 1990, **12**, 223, S Hakomori, *Adv Cancer Res*, 1989, **52**, 257
5 B J Campbell, I A Finnie, E F Hounsell, and J M Rhodes, *J Clin Invest*, 1995, **95**, 571
6 A Singhal, M Fohn, and S Hakomori, *Cancer Res*, 1991, **51**, 1406
7 G F Springer, P R Desai, H Tegtmeyer, B D Spencer, and E F Scanlon, *Ann N Y Acad Sci* 1993, **690**, 355
8 T Norberg, B Luning, and J Tejbrant, *Methods Enzymol*, 1994, **247**, 87, M Meldal, *Curr Opin Struct Biol*, 1994, **4**, 710, H G Garg, K Von Dem Bruch, and H Kunz, *Adv Carbohydr Chem Biochem*, 1994, **50**, 277, Y Nakahara, H Iijima, and T Ogawa, *ACS Symp Ser*, 1994, **560**, 249
9 R Kaifu and T Osawa, *Carbohydr Res*, 1977, **58**, 235
10 H Paulsen, C Kolar, and W Stenzel, *Chem Ber*, 1978, **111**, 2358 and 2370
11 R U Lemieux and R M Ratcliffe, *Can J Chem*, 1979, **57**, 1244
12 A Marra, F Gauffeny, and P Sinay, *Tetrahedron*, 1991, **47**, 5149, G Grundler and R R Schmidt, *Leibigs Ann Chem*, 1984, 1826, W Rosenbrook, Jr, D A Riley, and P A Lartey, *Tetrahedron Lett*, 1985, **26**, 3
13 T Toyokuni, S Cai, and B Dean, *Synthesis*, 1992, 1236, F Gauffeny, A Marra, L K S Shun, P Sinay, and C Tabeur, *Carbohydr Res*, 1991, **219**, 237
14 F -Y Dupradeau, S Hakomori, and T Toyokuni, *J Chem Soc Chem Commun*, 1995, 221
15 S Czernecki, E Ayadi, and D Randriamandimby, *J Chem Soc Chem Commun*, 1994, 35, F Sanoyo-Gonzalez, F G Calvo-Flores, P Garcia-Mendoza, F Hernandez-Mateo, J Isac-Garcia, and R Robles-Diaz, *J Org Chem*, 1993, **58**, 6122
16 T Toyokuni, S Hakomori, and A K Singhal, *Bioorg Med Chem*, 1994, **2**, 1119, T Toyokuni, B Dean, S Cai, D Boivin, S Hakomori, and A K Singhal, *J Am Chem Soc*, 1994, **116**, 395
17 V V Bencomo, J -C Jacquinet, and P Sinay, *Carbohydr Res*, 1982, **110**, C9
18 H Iijima and T Ogawa, *Carbohydr Res*, 1988, **172**, 183
19 Y Nakahara, H Iijima, S Shibayama, and T Ogawa, *Carbohydr Res*, 1991, **216**, 211
20 H Paulsen and J -P Holck, *Carbohydr Res*, 1982, **109**, 89
21 A Kurosaka, H Kitagawa, S Fukui, Y Numata, H Nakada, I Funakoshi, T Kawasaki, T Ogawa, H Iijima, and I Yamashina, *J Biol Chem*, 1988, **263**, 8724, T Kjeldsen, H Clausen, S Hirohashi, T Ogawa, H Iijima, and S Hakomori, *Cancer Res*, 1988, **48**, 2214
22 A Hasegawa, *ACS Symp Ser*, 1994, **560**, 184, K Okamoto and T Goto, *Tetrahedron*, 1990, **46**, 5835
23 E Johansson, L Hedbys, and P -O Larsson, *Enzyme Microb Technol*, 1991, **13**, 781
24 Y Harada, M Sakatsume, G A Nores, S Hakomori, and M Taniguchi, *Jpn J Cancer Res*, 1989, **80**, 988
25 H Nakada, Y Numata, M Inoue, N Tanaka, H Kitagawa, I Funakoshi, S Fukui, and I Yamashina, *J Biol Chem*, 1991, **266**, 12402
26 T Toyokuni, B Dean, and S Hakomori, *Tetrahedron Lett*, 1990, **31**, 2673
27 T Toyokuni and S Hakomori, *Methods Enzymol*, 1994, **247**, 325
28 J P Tam, in 'Vaccine Research and Developments', ed W C Koff and H R Six, Marcel Dekker, Inc, New York, 1992, Vol 1, p 51, J P Tam, *Proc Natl Acad Sci USA*, 1988, **85**, 5409
29 H J Jennings, R Roy, and A Gamian, *J Immunol*, 1986, **137**, 1708
30 P Y S Fung, M Madej, R R Koganty, and B M Longenecker, *Cancer Res*, 1990, **50**, 4308, G D MacLean, M B Bowen-Yacyshyn, J Samuel, A Meikle, G Stuart, J Nation, S Poppema, M Jerry, R Koganty, T Wong, and B M Longenecker, *J Immunother*, 1992, **11**, 292
31 G D MacLean, M Reddish, R R Koganty, T Wong, S Gandhi, M Smolenski, J Samuel, J M Nabholtz, and B M Longenecker, *Cancer Immunol Immunother*, 1993, **36**, 215, B M Longenecker, M Reddish, R Koganty, and G D MacLean, *Ann N Y Acad Sci*, 1993, **690**, 276
32 J S Haurum, G Arsequell, A C Lellouch, S Y C Wong, R A Dwek, A J McMichael, and T Elliott, *J Exp Med*, 1994, **180**, 739, E Michaelsson, V Malmstrom, S Reis, Å Engstrom, H Burkhardt, and R Holmdahl, *J Exp Med*, 1994, **180**, 745
33 D L Morton, M H Ravindranath, and R F Irie, *Prog Brain Res*, 1994, **101**, 251, P O Livingston, G Y Wong, S Adluri, Y Tao, M Padavan, R Parente, C Hanlon, M J Calves, F Helling, G Ritter, H F Oettgen, and L J A Old, *J Clin Oncol*, 1994, **12**, 1036, F Helling, A Shang, M Calves, S Zhang, S Ren, R K Yu, H F Ottgen, and P O Livingston, *Cancer Res*, 1994, **54**, 197
34 W G Bessler and G Jung, *Res Immunol*, 1992, **143**, 548, K -H Wiesmuller, W G Bessler, and G Jung, *Int J Pept Protein Res*, 1992, **40**, 255, P J F Pietrobon and P Kanda, in 'Vaccine Research and Developments', ed W C Koff and H R Six, Mercel Dekker, Inc, New York, 1992, Vol 1 p 3
35 K -H Wiesmuller, G Jung, and G Hess, *Vaccine*, 1989, **7**, 29
36 K Deres, H Schild, K -H Wiesmuller, G Jung and H -G Rammensee, *Nature*, 1989, **342**, 561
37 J -P Defoort, B Nardelli, W Huang, D D Ho, and J P Tam, *Proc Natl Acad Sci USA*, 1992, **89**, 3879
38 J M Bernard, H Gras-Masse, H Drobecq, A Tartar, P Lefrancier, A Hosmalin, C Carelli, and L Chedid, *Int J Pept Protein Res*, 1987, **29**, 455
39 B Deprez, H Gras-Masse, F Martinon, E Gomard, J -P Levy, and A Tartar, *J Med Chem*, 1995, **38**, 459
40 J Metzger, G Jung, W G Bessler, P Hoffmann, M Strecker, A Lieberknecht, and U Schmidt, *J Med Chem*, 1991, **34**, 1969