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FUNCTIONAL-GROUP MODIFICATIONS OF DEXTRAN FOR LINKAGE TO A DIAZONIUM GROUP. A POTENTIAL VEHICLE FOR TUMOUR TARGETING OF ANTINEOPLASTIC TRIAZENES*

A. VEYSI BAKI AND KEITH VAUGHAN†
Department of Chemistry, Saint Mary's University, Halifax, Nova Scotia B3H 3C3 (Canada)
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ABSTRACT

Several derivatives of dextran, containing a triazene side-chain, which are analogues of the antitumour agent DTIC (Dacarbazine), were prepared. The extent of triazene incorporation was measured by determination of the nitrogen content, and the presence of triazene groups was corroborated by u.v.- and n.m.r.-spectroscopic measurements. Some dextran-triazenes exhibited cytotoxic activity against M21 tumour cells *in vitro*.

INTRODUCTION

Most drugs used in the clinical treatment of human cancer have the disadvantage of producing severe toxicity to normal, as well as neoplastic, cells. The success of chemotherapy is often limited because of the lack of selectivity of the anti-cancer drug. A possible strategy for overcoming this problem is to bind the drug to a biological carrier, such as an antibody molecule having a specific affinity for the tumour cell; such "targeted" drugs would offer a distinct improvement in selectivity[1]. A commonly used technique for covalent linkage to the antibody is to bind the drug to a "spacer" molecule, such as dextran or polylysine, prior to covalent attachment of the drug-spacer conjugate to the antibody[2].

One of the synthetic drugs used clinically in cancer treatment is DTIC [dacarbazine; 5-(3,3-dimethyltriazeno)imidazole-4-carboxamide; NSC 45388], which is the single, most active agent for the treatment of human, malignant melanoma[3]. DTIC is only one of many dimethyltriazenes (Me_2N–N=NAr) having significant antitumour activity against experimental tumour models[4]. DTIC possesses some severe disadvantages as a clinical agent; for example, it is photosensitive and produces toxic material when photodegraded[5]. Most dimethyltriazenes are not water-soluble, and do not always lend themselves well to pharmacological studies under aqueous

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†To whom all correspondence should be addressed.

conditions. Thus, there are several reasons for attempting to link the antitumour triazene moiety to a carrier biopolymer: (a) such conjugates might be water soluble; (b) the conjugates should be photostable; and (c) increased selectivity might result. In this study, the modifications of the functional groups on a dextran backbone were investigated in order to allow coupling to appropriate diazonium groups and produce triazenodextrans (TRIADEX).

In devising the synthetic methods for this study, it was important to recognize that the triazene side-chain linked to dextran could reproduce the 3,3-dimethyltriazene structure itself, or could "mimic" the structure of the proposed metabolites of DTIC. Dimethyltriazenes (Me_2N–N=NAr) are presumed[6] to be metabolised by *N*-demethylation, *via* the methyloltriazene [$HOCH_2$(Me)N–N=NAr], to give the monomethyltriazene (MeNH–N=NAr). Thus, the dextran-triazenes have various structures, from the straightforward 3,3-dimethyltriazene (Dex–ArN=N–NMe_2) type, to the 3-monomethyl (Dex–Ar–N=N–NHMe), and 3-hydroxymethyl-3-methyl [Dex–Ar–N=N–N(Me)CH_2OH] types, in which the aryl group connects the triazene group to the dextran. In addition, an alternative mode of linkage, in which the triazene group links the dextran to the aryl nucleus, *e.g.*, Dex$O(CH_2)_n$–NH–N=NAr, may be viewed as a "reverse (monoalkyldextran)triazene".

RESULTS AND DISCUSSION

The first approach to incorporating aminoalkyl groups into the dextran side-chain (**1**) was attempted by nucleophilic displacement of the halogen from a 2-haloethylamine (**2**), followed by diazonium coupling to the amino group of the 2-amino-

$$\underset{\mathbf{1}}{\text{Dextran}} \overset{OH^-}{\rightleftharpoons} [\text{Dex-}O^-] + \underset{\mathbf{2}}{H_3\overset{+}{N}(CH_2)_2X} \rightarrow \underset{\mathbf{3}}{H_3\overset{+}{N}(CH_2)_2O\text{-Dex}}$$

(X = Cl or Br)

$$\text{Dex-}O(CH_2)_2NH\text{–}N{=}N\text{–Ar} \leftarrow H_2N(CH_2)_2O\text{-Dex}$$

4 (Ar = EtO_2C–C_6H_4)

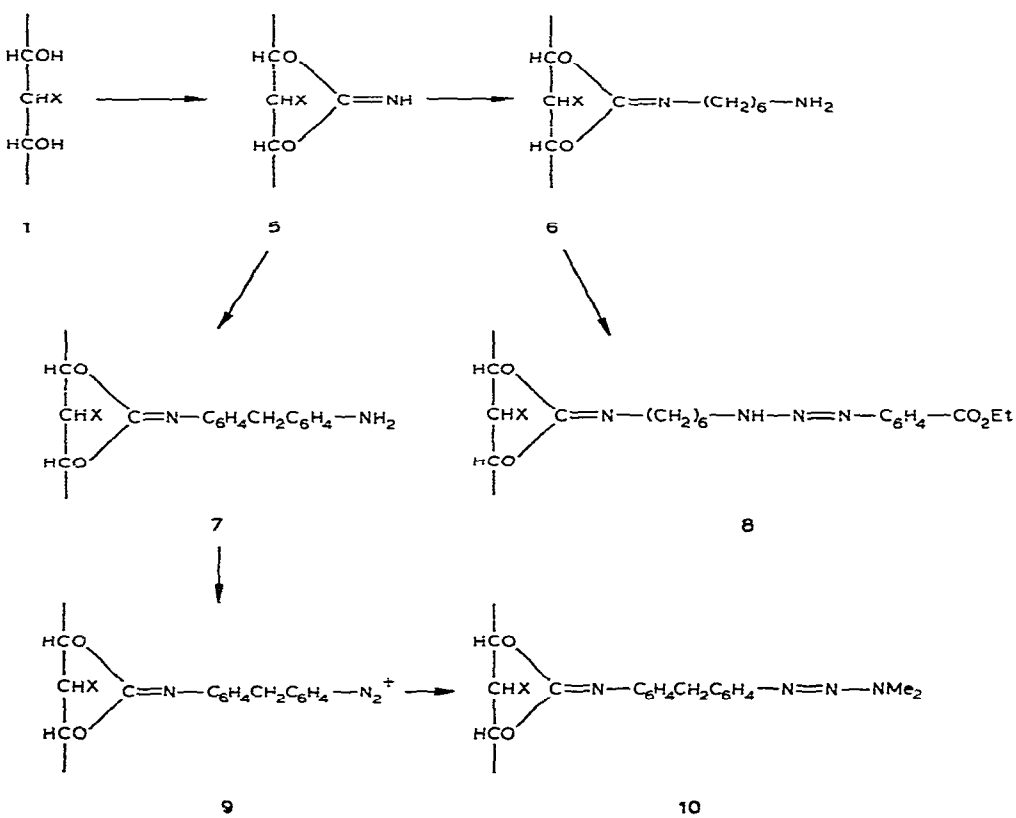
ethyldextran (**3**) to give the aryltriazenoalkyldextran **4**.

The alkylation of dextran by 2-bromoethylamine has been studied by Kuznetsova *et al.*[7]; the formation of short oligoethylenimine side-chains was observed under alkaline conditions that favour the intermolecular dimerisation or oligomerisation of the haloethylamine: Dex-$O(CH_2)_2NH_2$ + $X(CH_2)_2NH_3^+ \rightarrow$ Dex-$O(CH_2)_2NH(CH_2)_2NH_3^+$. Clearly, careful control of pH is necessary in order to accomplish the modification of **1** into **4**, and minimize oligomerisation.

Dextran was incubated in aqueous solution with 2-chloroethylamine hydrochloride at various levels of pH, from 6 to 10.4; the resulting solutions were dialysed and coupled with the diazonium ion from ethyl 4-aminobenzoate. The modified dextrans were isolated by dialysis, concentration, and precipitation. Most of these attempts resulted in zero nitrogen incorporation; however, at an optimum pH of

9.85, a triazenodextran having a nitrogen content of 0.23%, corresponding to a molar substitution ratio of 5:1 of triazene to dextran, was obtained by this method. The amount of nitrogen incorporation was not improved by use of bromoethylamine in place of chloroethylamine; evidently, the extent of aminoethyl incorporation is not influenced by the nature of the leaving group. An explanation of this result may be the relative acidity of the sugar hydroxyl (pKa ~ 16) and alkylammonium group, $R\text{-}NH_3^+$ (pKa ~ 10). It may be impossible to achieve the necessary concentration of ionized dextran, ($Dex\text{-}O^-$) without releasing the amino group of the haloethylamine, thus leading to oligomerisation. Although the incorporation of triazene groups by this method was low, the (aryltriazenoalkyl)dextran (**4**) displayed cytotoxic activity towards tumour cells in culture (see later discussion), and may be the promising triazenodextran for future antitumour evaluation.

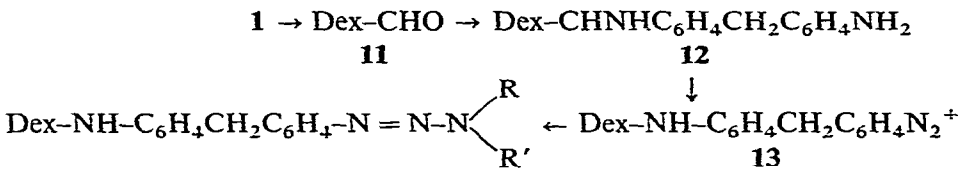
An alternative route to an aryltriazenodextran *via* a "dextran cycloimidate" was more successful. Dextran (**1**) was treated with cyanogen bromide, according to the method of Kuznetsova *et al.*[7], to yield a solution of the dextran carbonimidate (**5**), which was treated with 1,6-diaminohexane (in excess) to produce *O*-[(6-aminohexyl)amidino]dextran (**6**). The latter was not isolated but was used, *in situ*, after purification, to give the dextran (3-aryltriazenoalkyl)imidate (**8**), which had a



nitrogen content substantially higher than that of **4**; the molar substitution ratio of triazene to dextran in **8** was 21 : 1. However, this method was not entirely reproducible and much lower nitrogen incorporation was recorded in several duplications of the method. Evidence of triazene incorporation in these conjugates was provided by u.v.-spectroscopic measurement (see later Discussion).

The dextran cycloimidate **5** was also employed successfully in the preparation of a triazenodextran *via* an anilinodextran complex, by treatment with bis(*p*-aminophenyl)methane to afford the dextran anilinoimidate **7**. This method of preparing an anilinodextran has not been reported previously. Diazotisation of **7** and coupling of the resulting diazodextran **9** with dimethylamine afforded the (3,3-dimethyltriazenoanilino)dextran **10**, which had a high content of nitrogen (1.81%). The molar ratio of 3,3-dimethyltriazene to dextran was determined to be 34 : 1. This triazene derivative was also characterised by its u.v.–visible spectrum. However, the dextran cycloimidate method gave variable results with a nitrogen content as low as 0.44%.

The most consistent method for the preparation of a triazenodextran is a modification of the anilinodextran method reported by Pitha[8]. Dextran was oxidised by periodate to give an aldehyde derivative (**11**), which was condensed with bis(*p*-aminophenyl)methane; the resulting Schiff base was reduced with sodium cyanoborohydride to give the anilinodextran **12**. Diazotisation of **12** afforded the diazodextran **13**, which was then coupled with several primary or secondary amines to give the triazenodextrans **14**–**17**. Application of this method consistently gave triazenodextrans having a nitrogen content between 1 and 2%; in the coupling with dimethylamine, some batches of the (3,3-dimethyltriazeno)dextran (**14**) had more than 5% of nitrogen, which corresponds to a molar ratio of dimethyltriazene to dextran higher than 100 : 1. The method was also applied successfully to the preparation of the (3-monomethyltriazeno)dextran **15**, the methyl (3-hydroxy-3-triazeno)dextran **16**, and the [3,3-bis(chloroethyl)triazeno]dextran **17**. The nitrogen content of these dextrans varied between 1.38 and 1.65%, which corresponds to a molar substitution between 24 : 1 and 32 : 1.



14 R = R′ = Me
15 R = H, R′ = Me
16 R = CH_2OH, R′ = Me
17 R = R′ = $-CH_2CH_2Cl$

Not all of the attempted methods of triazene incorporation into dextran were successful. Attempts to condense 1,2-diaminoethane with the dextran aldehyde, obtained by periodate oxidation of dextran, followed by reduction of the Schiff base

TABLE I

ANALYTICAL DATA FOR COMPOUNDS 4, 8, 10, AND 14–17

Compound	*Nitrogen content (%)*[a]	*Molar substitution*[b]
4	0.23	5:1
8	1.05	21:1
10	1.81	34:1
14	1.14	21:1
	2.94	53:1
	5.74	105:1
15	1.38	24:1
16	1.65	31:1
17	1.44	32:1

[a]Error limits ±0.10%. [b]For calculation, see Experimental section.

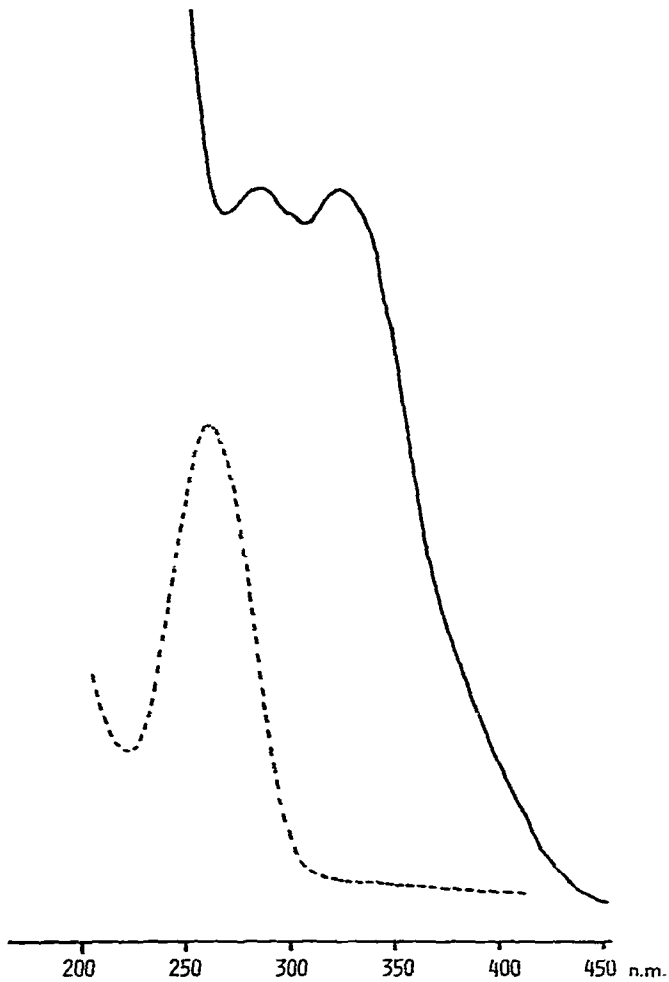


Fig. 1. U.v. spectrum of an aqueous solution of (dimethyltriazenoanilino)dextran (**10**) (———) and untreated dextran (**1**) (– – – –); the absorption maximum at 265 nm in the latter spectrum may be due to a protein impurity.

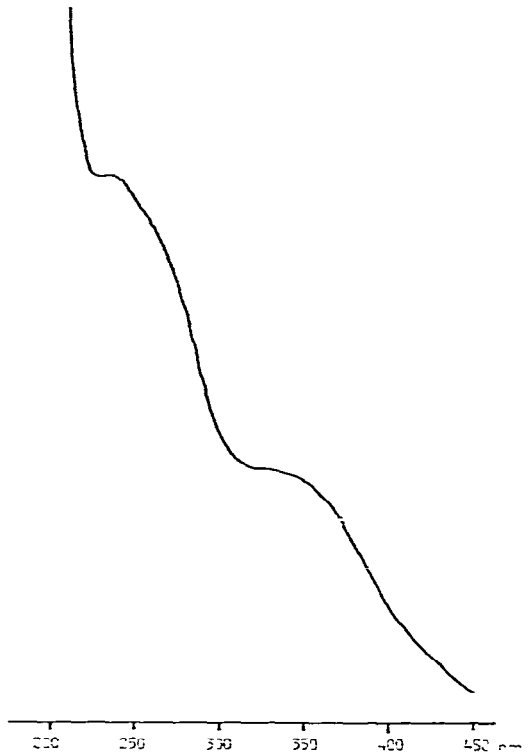


Fig. 2. U.v. spectrum of an aqueous solution of dextran (aryltriazenohexamethyleno)imidate (8).

and coupling of the intermediate aminodextran with diazonium ion, did not succeed. In most cases, the dextran could not be precipitated from the concentrated solution; in one case, a precipitate was obtained, but the nitrogen content was only 0.05%. An equally unsuccessful attempt was the reaction of dextran cycliccarbonate, prepared by the method of Kol'tsova *et al.*[9], with 1,2-diaminoethane, followed by coupling of the intermediate dextranamine with diazonium ion. The dextran failed to precipitate after concentration of the solution.

The nitrogen content of dextran derivatives was used to estimate the extent of triazene substitution (see Table I). As the presence of nitrogen does not necessarily imply that the nitrogen-containing side-chains are triazene groups, corroborating evidence for the presence of triazene groups was obtained as follows: U.v. absorption in the range 300–350 nm is a characteristic of all 1-aryl-3-alkyl- and 1-aryl-3,3-dialkyltriazenes; Figs. 1 and 2 show the u.v. spectra of aqueous solutions of dimethyl-

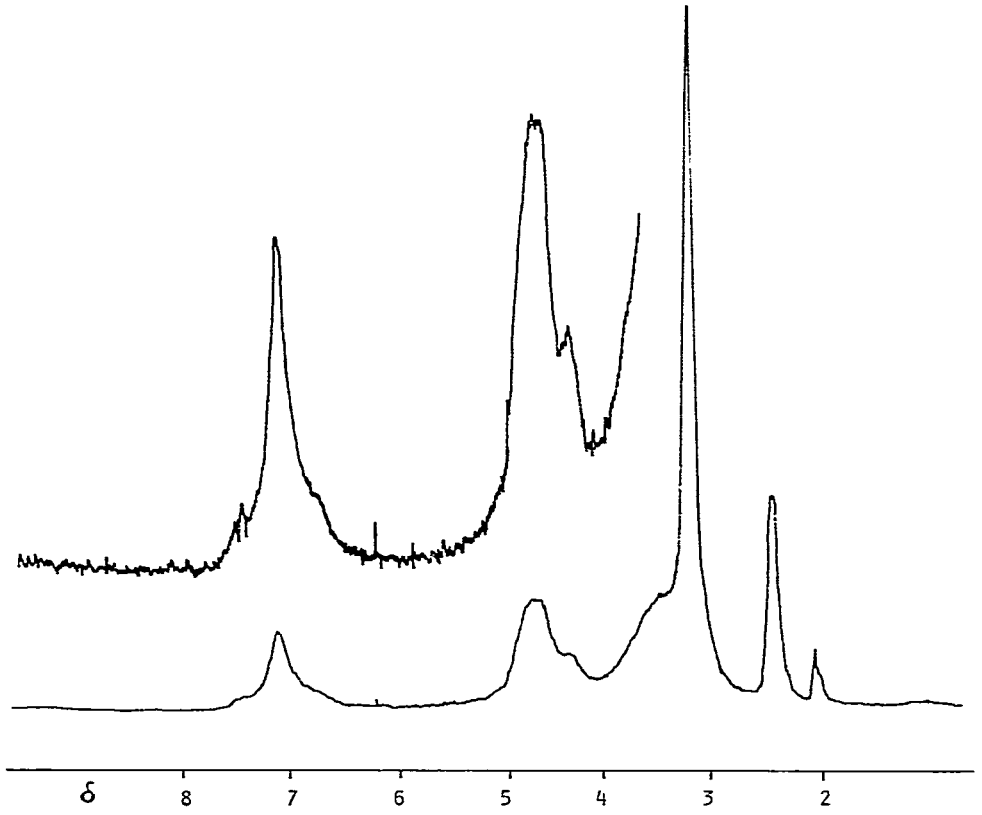


Fig. 3. ^{1}H-N.m.r. spectrum of a solution **14** in Me_2SO-d_6.

triazenoanilinodextran (**10**) and of dextran (aryltriazenohexamethyleno)imidate (**8**), respectively. Further evidence for the presence of triazene groups in the dextran derivatives was obtained by n.m.r. spectrometry. Surprisingly the triazenodextrans, which had been precipitated from an aqueous solution with acetone, did not redissolve readily in deuterium oxide, but the n.m.r. spectrum of a solution of (3,3-dimethyl-triazenoanilino)dextran (**14**) in dimethyl sulphoxide-d_6 could be obtained (Fig. 3); the presence of aryl groups is indicated by a broad signal at δ 7.15, and the sharp signal at δ 3.32 may arise from the *N*-methyl of the triazene group.

The potential of the triazenodextrans as cytotoxic agents was assessed by testing their *in vitro* activity against M21 melanoma tumour cells[10] growing in culture (full details will be published elsewhere). Several triazenodextrans showed cytotoxic activity against this cell line (see Fig. 4). A free, unbound triazene (MeNH–N=N–C_6H_4–CO_2Me) inhibited cell growth at a concentration of 78 g/L. The inhibition by untreated dextran was not significantly different from that of the control. The 3-(methyltriazenoanilino)dextran **15** did not inhibit cell growth at all, although the lack of cytotoxicity may be due to the low solubility of **15** in the cell-growth medium. Although the (3-aryltriazenoalkyl)dextran **4** had also low solubility and was tested

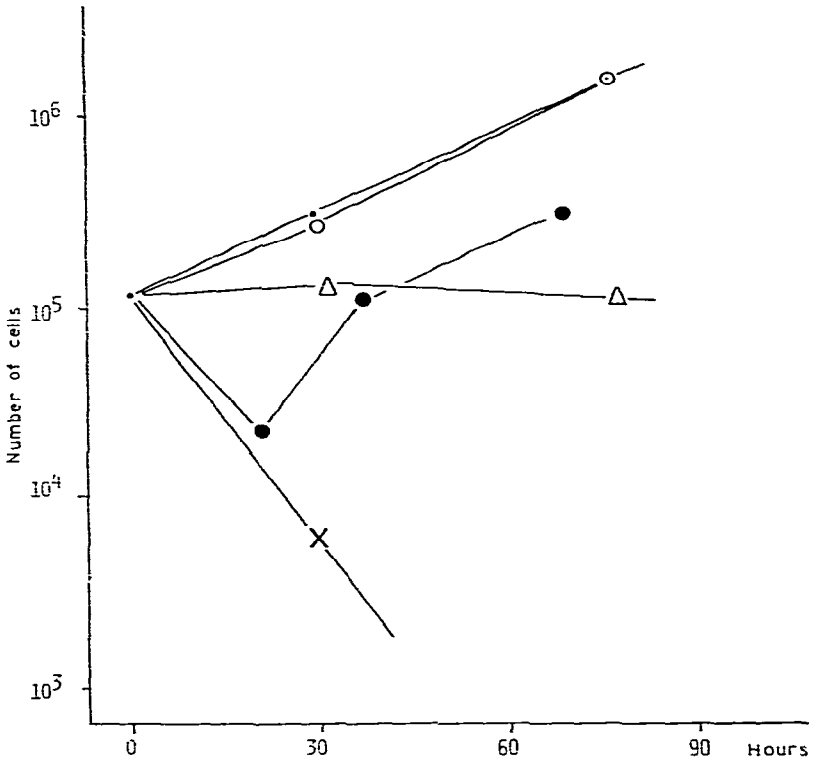


Fig. 4. Effect of triazenodextrans on the *in vitro* growth of M21 melanoma cells: (•) control; (○) (monomethyltriazenoanilino)dextran (15) (10 g/L); (●) dextran (aryltriazenohexamethylene)-imidate (8) (5 g/L); (×) (aryltriazenoalkyl)dextran (4) (10 g/L); and (△) MeNH–N=N–C_6H_4–CO_2Me (78 mg/L).

for cytotoxicity in suspension, its effect on cell growth was dramatic. To avoid the problems of solubility and redissolution, a sample of dextran (3-aryltriazenohexamethylene)imidate (8) was prepared, and the concentrated solution was used directly for treatment of M21 cells in culture. Significant inhibition of cell growth was observed with this compound.

The chemical modifications of dextran described herein afford a means to attach triazene groups having the necessary structural requirements for antitumour activity. The triazenodextrans do not appear to be photosensitive; samples have been kept in storage without protection from light for up to one year and show no sign of deterioration. However, the triazenodextrans may not offer the advantages of water solubility, once precipitated from solution. Some triazenodextrans exhibit significant *in vitro* cytotoxic activity against tumour cells growing in culture, and display distinct potential for evaluation as anti-tumour agents.

EXPERIMENTAL

Materials and methods. — T-40 Dextran (mol. wt. 37 000–43 000, was obtained from ICN Nutritional Biochemicals, Cleveland, OH 44128; chloroethylamine hydrochloride, cyanogen bromide, bis(*p*-aminophenyl)methane (recrystallised from ethanol before use), bis(chloroethylamine) hydrochloride, sodium cyanoborohydride, and aqueous dimethylamine from Aldrich Chemical Co., Inc. (Milwaukee, WI 53201); ethyl *p*-aminobenzoate from Eastman Kodak Co. (Rochester, NY 14650); 1,6-diaminohexane from Chebucto Enterprises, Halifax, N.S.; and aqueous methylamine (40%) from Fisher Scientific Co. (Pittsburgh, PA 15219).

Microanalyses were performed by the Canadian Microanalytical Laboratory, Vancouver, B.C. U.v. and visible spectra were recorded with a Varian Cary 219 spectrophotometer, and 80-MHz ^{1}H-n.m.r. spectra with a Varian CFT20 spectrometer.

General synthetic method. — The synthetic procedures described in detail hereafter incorporate several features designed to ensure that the triazenodextrans are free from nondextran-bound compounds. The general method is as follows: (a) Modification of the dextran by chemical treatment to attach a suitable side-chain; (b) dialysis of the modified dextran to remove unbound small molecules so that only dextrans are retained; (c) diazotisation and diazonium coupling with the modified dextran; (d) dialysis to remove unreacted small molecules or ions; (e) centrifugation or filtration, if necessary, to remove water insoluble material; (f) concentration of the aqueous solution to a small volume; (g) centrifugation if necessary; (h) treatment of the clear aqueous concentrate with acetone to precipitate the triazenodextran; (i) isolation if the solid triazenodextran by filtration, washing, and drying; and (j) analysis of the triazenodextran to ascertain the identity of side chains and the extent of substitution.

Determination of molar substitution from nitrogen content. — The extent of incorporation of triazene groups into the dextran, expressed as the molar ratio of triazene groups to dextran molecule, was calculated from the percentage of nitrogen content of the dextran and the molecular weight of each fragment. For example, for **4**, a ratio of one triazene side-chain per D-glucose residue corresponds to a nitrogen content of 11.0% (42/381); to a triazenodextran **4** having a nitrogen content of 1.1% corresponds a substitution ratio of one triazene group per ten D-glucose residues, which may alternatively be expressed as a molar substitution ratio of triazene to dextran of 24.8:1 for a dextran having an average mol. wt. of 40 000. The molar-substitution values reported in Table I have been calculated in this way, taking into account the variation in molecular weight of the triazene fragment.

(*A*) *Chloroethylamine method*: *(3-Aryltriazenoalkyl)dextran* (**4**). — To a solution of T-40 Dextran (0.5 g) in water (10 mL), cooled in ice to <5°, was added chloroethylamine hydrochloride (0.2 g) dissolved in water (3 mL). The solution was kept cold for 1 h, and the pH adjusted to 9.85 with sodium hydroxide solution. The mixture

was stirred at room temperature for 24 h, and the resulting solution dialysed against distilled water, with repeated changes, for 48 h to give an (aminoethyl)dextran.

Ethyl 4-aminobenzoate was diazotised in hydrochloric acid with sodium nitrite at 0–5° to produce a clear solution of the diazonium salt. An aliquot of this solution containing 4 mmol of the diazonium salt was added slowly with stirring to the precooled (aminoethyl)dextran solution and the pH of the mixture rose to 8.9. This mixture was stirred in the cold for 2 h and then filtered. The cloudy filtrate was centrifuged for 40 min at 12 000 r.p.m. at 0°. The clear supernatant solution was dialysed against distilled water for 24 h, and then concentrated in the dialysis chamber with a steady current of cold air. The triazenodextran (**4**) was precipitated by addition of acetone, filtered off, and dried under vacuum (yield 0.439 g).

Anal. Found: N, 0.23.

(*B*) *Cyanogen bromide–1,2-diaminohexane method*: *Dextran (3-aryltriazeno-hexamethylene)imidate* (**8**). — T-40 Dextran (1.0 g) was dissolved in water (125 mL), and the pH was adjusted to 10.5 with M sodium hydroxide. The solution was cooled to below 5°, cyanogen bromide (0.19 g) dissolved in acetonitrile (minimum volume) was added to the dextran solution slowly with constant stirring, and then the mixture was stirred cold for 30 min, during which time the pH was maintained at 10.5 with sodium hydroxide solution. A solution of 1,6-diaminohexane (0.82 g) in 2M hydrochloric acid (2.0 mL) was added to the cold dextran solution, whereupon the pH increased to 11 and was then adjusted to 9.05 by the addition of hydrochloric acid. The resulting mixture was stirred overnight in the cold, and then dialysed against distilled water until chloride ions could not be detected in the dialysate with silver nitrate solution (~30 h). The dialysed dextran solution was cooled to below 5°, and then treated with a solution of the ethyl 4-diazobenzoate. The pH was increased to 8.9 by the addition of solid sodium hydrogencarbonate. The cold mixture was stirred for 24 h, and then centrifuged for 90 min at 14 000 r.p.m. The clear supernatant solution was dialysed against water for 24 h, centrifuged again for 30 min at 14 000 r.p.m., concentrated as described for method (*A*), and precipitated with acetone. The triazenodextran was filtered off and dried (yield 0.36 g).

Anal. Found: N 1.05.

(*C*) *Cyanogen bromide–anilinodextran method*: *Anilinodextran 3,3-dimethyl-triazenoimidate* (**10**). — T-40 Dextran (1.0 g) was dissolved in water (100 mL), and the pH adjusted to 10.5 with M sodium hydroxide before cooling to below 5°. A solution of cyanogen bromide (0.19 g) in acetonitrile was added dropwise to the cold dextran solution, and the pH maintained at 10.5 with sodium hydroxide while the cold mixture was stirred for 30 min. A previously prepared solution of bis(*p*-aminophenyl)methane (1.23 g) in glycerol (10 mL) and acetic acid (2.0 mL) was slowly added to the dextran cycloimidate solution while the pH was maintained at 9.0. The mixture was stirred for 30 min at room temperature, and then centrifuged for 75 min at 14 000 r.p.m. at 0°. The clear supernatant solution was dialysed against water for 24 h, and then centrifuged again. The clear supernatant solution of anilino-dextran was treated with conc. hydrochloric acid (2 mL), cooled to 0°, and diazotised

with sodium nitrite (0.3 g) in water for 1.0 h. The solution of diazodextran was treated with 25% aqueous dimethylamine until the pH reached 8, and stirred cold for 75 min. The mixture was centrifuged cold for 30 min at 14 000 r.p.m., and the clear supernatant solution dialysed against water for two days. The mixture was centrifuged again, concentrated, and precipitated with acetone. The (3,3-dimethyltriazeno)dextran (**10**) was filtered off and dried in vacuum (yield 0.51 g).

Anal. Found: N, 1.81.

(*D*) *Periodate oxidation–anilinodextran method.* — A solution of T-40 dextran (5.0 g) in water (35 mL) was cooled to 0°, periodic acid (0.5 g) was added, and the solution stirred cold for 5 h, and then at room temperature overnight. The oxidised-dextran solution was treated with a previously prepared mixture of bis(*p*-aminophenyl)-methane (2.5 g) and sodium cyanoborohydride (1.0 g) in glycerol (25 mL) containing acetic acid (0.5 mL). The whole mixture was stirred at room temperature for two days. The resulting anilinodextran (**12**) was dialysed first against 20% acetic acid, and then against water. This solution was divided into two portions, one- and four-fifths. The latter portion was treated with conc. hydrochloric acid (6.0 mL), cooled to 0°, and then diazotised with sodium nitrite (0.41 g) for 1.5 h. The clear solution of diazodextran (**13**) was divided into four equal portions.

Anilinodextran (**12**). — The first portion (20%) of anilinodextran (**12**) solution was dialysed, concentrated, and precipitated to give **12**.

Anal. Found: N, 1.12.

(*3,3-Dimethyltriazeno*)*dextran* (**14**). — The first portion of **13** was treated with 25% aqueous dimethylamine at 0° until the pH reached 9.2, and then stirred cold for 1 h. The solution was then dialysed against water for two days, concentrated, and the dextran precipitated with acetone (yield 0.25 g).

Anal. Found: N, 1.14.

When this preparation was repeated, the yield of **14** was 0.39 g.

Anal. Found: N, 5.74.

(*3-Methyltriazeno*)*dextran* (**15**). — Treatment of the second portion of **13** with 40% aqueous methylamine to pH 9 gave **15** (yield 0.32 g).

Anal. Found: N, 1.38.

(*3-Hydroxymethyl-3-methyltriazeno*)*dextran* (**16**). — Treatment of the third portion of **13** with a premixed solution of 40% methylamine (0.02 mol) and 37% formaldehyde (0.20 mol) gave **16** (yield 0.31 g).

Anal. Found: N, 1.65.

[*3,3-Bis*(*chloroethyl*)*triazeno*]*dextran* (**17**). — Treatment of the fourth portion of **13** with bis(chloroethyl)amine hydrochloride (3.6 g) at pH 9.0 (saturated sodium carbonate) gave **17** (yield 0.255 g).

Anal. Found: N, 1.44.

Measurement of in vitro *activity of triazenodextrans against M21 melanoma cells.* — Tests of *in vitro* cytotoxicity were performed on M21 melanoma cells growing in monolayer culture. The growth medium consisted of a mixture of 90% (v/v) RPMI 1640 medium, 10% fetal-calf serum, and penicillin–streptomycin. Cells were

grown on circular (35 × 10 mm), Falcon™ 3001 tissue-culture dishes in an incubator at 37–38°, in a water-saturated, 5% carbon dioxide atmosphere. All cell counting was performed with a hemocytometer. M21 melanoma cells were detached from the surface of a "stock" flask by incubating the cells for 5 min at 37° in the presence of a 0.02% EDTA solution, and the cells so obtained were resuspended in growth medium to a density of 25 000–30 000 cells per mL. Aliquots (2.0 mL) of cells were added to each tissue-culture dish; two tissue-culture dishes were plated for each experimental point.

The triazenodextran was added to the growing cells in 0.10 mL of 20% (v/v) dimethyl sulphoxide in water. Controls consisted of dishes to which 0.10 mL of dimethyl sulphoxide (20%, v/v) was added to a final dimethyl sulphoxide concentration of 1%. *In vitro* cytotoxicity of a triazenodextran was assessed by counting the number of cells remaining attached to the surface of the culture dish. The number of attached cells was determined by first removing the medium and floating cells, then adding 1 mL of 0.02% EDTA solution to detach the cells, rinsing with phosphate-buffered saline (PBS), and centrifuging the suspension at 240*g* for 8 min. Cells were then resuspended in a suitable volume (0.1–2.0 mL) of PBS, and counted with a hemocytometer. Counts made on the same dish were required to agree within ±10%.

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