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THE SYNTHESIS OF TRISACCHARIDE ANTIGENIC DETERMINANTS FOR THE BRANCH POINTS IN NATURAL DEXTRANS AND THEIR PROTEIN CONJUGATES

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ABSTRACT

Three *O*-allyl-di-*O*-benzyl-6-*O*-(*N*-phenylcarbamoyl)-1-*O*-tosyl-D-glucopyranose derivatives were coupled with 2-[4-(*p*-toluenesulfonamido)phenyl]ethanol to give the corresponding α-D-glucopyranosides. Decarbanilation and deallylation gave the corresponding 2,3-, 2,4-, and 3,4-di-*O*-benzyl-α-D-glucopyranosides. Reaction of the diols with two equivalents of 2,3,4-tri-*O*-benzyl-6-*O*-(*N*-phenylcarbamoyl)-1-*O*-tosyl-D-glucopyranose gave the branched trisaccharides having an α-D-glucopyranosyl group at O-6 and one at either O-2, O-3, or O-4. The oligosaccharides were deblocked with sodium in liquid ammonia to give the 2-(4-aminophenyl)ethyl α-D-glucosides, which were converted into the isothiocyanate derivatives with thiophosgene. The functionalized oligosaccharides were coupled to bovine serum albumin to give protein conjugates.

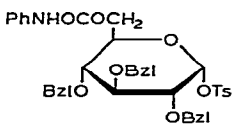
INTRODUCTION

The immunochemical characteristics and biochemical properties of natural dextrans are known to depend on the linkages and mode of branching of the D-glucose units. The chemical structures of dextran have been elucidated by chemical, physical, immunological, and immunochemical procedures[1]. Most of the immunochemical reactions involve either inhibition studies of antisera obtained by immunizing animals with a specific dextran, or from precipitation reactions between a dextran and an antiserum specific for a certain α-D-glucosyl linkage.

Herein, we extend the immunochemical determination of the structure of dextrans by preparing a series of branched trisaccharides having the structures α-D-Glc*p*-(1→6)-[α-D-Glc*p*-(1→2)]-α-D-Glc*p*, α-D-Glc*p*-(1→6)-[α-D-Glc*p*-(1→3)]-α-D-Glc*p*, and α-D-Glc*p*-(1→6)-[α-D-Glc*p*-(1→4)]-α-D-Glc*p* corresponding to the three possible branch points. The oligosaccharides were coupled to a protein support to give conjugates that can be used as artificial antigens to produce antisera specific for each of the branch points in dextrans.

RESULTS AND DISCUSSION

In our previous papers[2-4], we have shown that 2,3,4-tri-*O*-benzyl-6-*O*-(*N*-phenylcarbamoyl)-1-*O*-tosyl-D-glucopyranose (**1**) gave high yields of α-D-glycosides and that the 2-[4-(*p*-toluenesulfonamido)phenyl]ethyl aglycon is stable under all conditions for coupling and specific deblocking reactions, and could be converted into the 2-(4-aminophenyl)ethyl group by sodium in liquid ammonia. The three trisaccharide glycosides could, therefore, be prepared by synthesizing 2-[4-(*p*-toluenesulfonamido)phenyl]ethyl 2,3-, 2,4-, and 3,4-di-*O*-benzyl-α-D-glucopyranoside, treating each of them with two equivalents of **1**, and deblocking with sodium and liquid ammonia.



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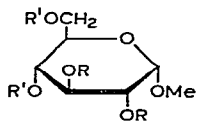
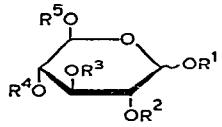
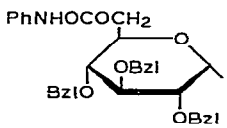


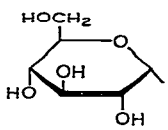


4 $R^1 = R^5 =$ CONHPh, $R^2 =$ All, $R^3 = R^4 =$ Bzl
5 $R^1 = R^5 =$ H, $R^2 = R^4 =$ Bzl, $R^3 =$ All
6 $R^1 = R^5 =$ CONHPh, $R^2 = R^4 =$ Bzl, $R^3 =$ All
7 $R^1 = CH_3$, $R^2 = R^3 =$ Bzl, $R^4 =$ All, $R^5 =$ H
8 $R^1 = R^5 =$ H, $R^2 = R^3 =$ Bzl, $R^4 =$ All
9 $R^1 = R^5 =$ CONHPh, $R^2 = R^3 =$ Bzl, $R^4 =$ Al

2 R = Bzl, R' =



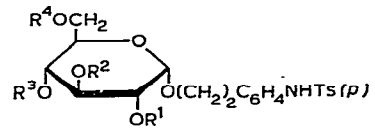
3 R = H, R' =



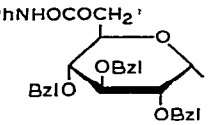
Methyl 2,3-di-*O*-benzyl-α-D-glucopyranoside was treated with 2 equiv. of **1** in diethyl ether as a model reaction to determine the yield and stereoselectivity of coupling two D-glucose units to one carbohydrate derivative. The yield of the trisaccharide **2** was high (83%), and the ^{13}C-n.m.r. spectrum of the deblocked oligosaccharide **3** showed three anomeric peaks at 101.0, 100.1, and 99.6 p.p.m. corresponding to α-C-1 of the reducing unit, and to the two α-C′-1 and -C″-1 linked to O-4 and O-6 of the reducing unit, respectively. The spectrum also showed two small peaks at 102.2 and 102.0 p.p.m. corresponding to the two β-C′-1 and -C″-1 linked to O-6 and O-4 of the reducing unit, respectively. The amount of β-D-(1→6) linkages was ~5% and of β-D-(1→4) linkages ~10%. Our previous papers[2,3] showed the stereoselectivity for an α-D linkage to be ~95% when the aglycon was a primary alcohol and ~90% when the aglycon was a secondary alcohol (cyclohexanol). Thus, it appears that the stereoselectivity and the yield are not affected by the number of D-glucose units being coupled.

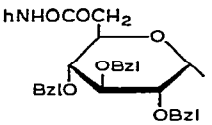
An attempt was made to remove the β-linked D-glucose residues from trisaccharide **3** by treatment with β-D-glucosidase emulsin, at 37° and pH 5.0. The

presence of free D-glucose was determined with a glucostat reagent (Worthington Biochemicals Corp., Freehold, NJ 07728) and by paper chromatography. After a period of 4 days, no free D-glucose could be detected and the ^{13}C-n.m.r. spectrum of the final product showed it to be identical to the starting material. No other attempts were made to remove the β-D-glucopyranosyl group from any of the oligosaccharides.



10 R^1 = All, R^2 = R^3 = Bzl, R^4 = CONHPh
11 R^1 = R^3 = Bzl, R^2 = All, R^4 = CONHPh
12 R^1 = R^2 = Bzl, R^3 = All, R^4 = CONHPh
13 R^1 = R^4 = H, R^2 = R^3 = Bzl
14 R^1 = R^3 = Bzl, R^2 = R^4 = H
15 R^1 = R^2 = Bzl, R^3 = R^4 = H

16 R^2 = R^3 = Bzl, R^1 = R^4 = 

17 R^1 = R^3 = Bzl, R^2 = R^4 = 

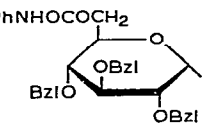
18 R^1 = R^2 = Bzl, R^3 = R^4 = 

TABLE I

PHYSICAL CONSTANTS, YIELDS, AND ANALYSES OF 2-[4-(*p*-TOLUENESULFONAMIDO)PHENYL]ETHYL α-D-GLUCOPYRANOSIDE DERIVATIVES

Compd.	*Yield (%)*	$[\alpha]_D^{25a}$ *(degrees)*	*Anal.*[b]			α-D *anom. (%)*	^{13}*C-n.m.r. data (p.p.m.)*	
			C	*H*	*N*		*α-C-1*	*β-C-1*
10[c]	77	+68.5	68.16	6.10	3.53	>98	97.02	
			68.18	6.05	3.24			
11	74	+56.5	68.16	6.10	3.53			
			68.41	6.18	3.77			
12	84	+30.4	68.16	6.10	3.53	>98	97.0	
			68.34	6.04	3.62			
13	72	+69.7	66.33	6.20	2.21			
			65.86	6.43	2.24			
14	78	+67.5	66.33	6.20	2.21	94[d]	96.4	103.3
			66.07	6.23	2.30			
15	74	+37.0	66.33	6.20	2.21	>98	96.8	
			66.11	6.42	2.13			

[a]Solution in chloroform (*c* 1). [b]Upper line, calc. value; lower line, found value. [c]Crystallized from ether–hexane, m.p. 96–98°. [d]Ratio of α- to β-D-peak heights.

To prepare the 2-[4-(*p*-toluenesulfonamido)phenyl]ethyl di-*O*-benzyl-α-D-glucopyranoside derivatives **13**, **14**, and **15**, three *O*-allyl-di-*O*-benzyl-1,6-di-*O*-(*N*-phenylcarbamoyl)-D-glucopyranose derivatives (**4**, **6**, and **9**) were synthesized. The dicarbanilate derivatives were converted into the α-D-glucopyranosyl chlorides with hydrogen chloride in dichloromethane, and then to the 1-*O*-tosyl derivatives with silver *p*-toluenesulfonate in acetonitrile. Reaction of 2-[4-(*p*-toluenesulfonamido)phenyl]ethanol with the 1-*O*-tosyl derivatives in diethyl ether gave the α-D-glucopyranosides **10**, **11**, and **12** in good yields and with high stereoselectivity (Table I).

The carbanilate groups were cleaved with sodium ethoxide in ethanol under reflux and the allyl groups were cleaved by rearrangement first to the propenyl ether with tris(triphenylphosphine)rhodium(I) chloride[5] in aqueous alcohol, followed by hydrolysis with mercuric bromide–mercuric oxide in aqueous acetone to give the diols **13**, **14**, and **15** (Table I).

The stereoselectivity of the coupling reactions was expected to be ~95% of α-D anomer, but for two of the compounds (**10** and **12**), the stereoselectivity was greater than 98% of α-D anomer. The region of the ^{13}C spectrum was expanded until the signal of α-C-1 was off the paper and still no β-C-1 signal could be detected. Noise peaks were generally 1% of the α-C-1 peaks. It appears that the substitution at O-2 and -4 of allyl groups for benzyl groups had a beneficial effect on the stereoselectivity in this reaction. Substitution of an allyl group at O-3 showed no effect on the stereoselectivity when compared to that of the 3-*O*-benzyl derivative[3].

The synthesis of **15** was attempted by preparing the 1,4,6-tri-*O*-(*N*-phenylcarbamoyl)-, -acetyl-, or -*p*-nitrobenzoyl-2,3-dibenzyl-D-glucopyranose derivatives. These were converted, *via* the α-D-glucopyranosyl chlorides, to 1-*O*-tosyl derivatives which were treated with 2-[4-(*p*-toluenesulfonamido)phenyl]ethanol. Deacylation with sodium ethoxide in ethanol gave the diol **15**. In every reaction, the yield of coupling was found to be very low (<30%), and the stereoselectivity was also found to be lower (~80% of α-D anomer). The 4,6-di-ester-1-*O*-tosyl-D-glucopyranose derivatives appear to react much more slowly than the corresponding 6-ester derivatives, and presumably anomerization of the 1-*O*-tosyl group also occurs, causing the aglycon to react with both the α-D and β-D anomers to give both β- and α-D-glycosides. In the case of the 4,6-di-*p*-nitrobenzoyl derivative, the reaction gave after processing a crystalline compound (from ether–pet. ether), which was shown by ^1H-n.m.r. to be 2,3-di-*O*-benzyl-4,6-di-*O*-*p*-nitrobenzoyl-1-*O*-tosyl-α-D-glucopyranose, m.p. 104–105° (dec.), $[\alpha]_D^{25}$ +85.0° (*c* 1, chloroform); ^1H-n.m.r.: δ 5.9 (d, $J_{1,2}$ 3.0 Hz). The fact that this compound survived the processing which involved washing with water and aqueous sodium hydrogencarbonate, attests to the stability and lower reactivity of these derivatives.

The diols **13**, **14**, and **15** were treated with 2 equiv. of **1** in diethyl ether as previously described[2–4]. The resulting trisaccharides **16**, **17**, and **18** (Table II) were shown by ^{13}C-n.m.r. to be mainly α-D-linked with only about 10% of β-D linkages present. Since two glycosidic bonds were formed in the reactions and since the starting diols, with the exception of **14**, had almost no β-D linkages, the stereoselectivity

TABLE II

PHYSICAL CONSTANTS, YIELDS, AND ANALYSES OF 2-[4-(*p*-TOLUENESULFONAMIDO)PHENYL]ETHYL BRANCHED TRISACCHARIDES

Compd.	*Yield (%)*	$[\alpha]_D^{25a}$ *(degrees)*	α-D *anom.*[b] *(%)*	^{13}C-*n.m.r. data (p.p.m.)*[c]			*Anal.*[d]		
				α-C-1	*α-C′-1→6*	*α-C″-1→ (2, 3, or 4)*	*C*	*H*	*N*
16	60	+77.0	92	95.8	96.9	94.4	71.22	6.09	2.42
							71.08	6.00	2.60
17	61	+81.8	>95	96.1	97.4[e]	97.4[e]	71.22	6.09	2.42
							70.98	6.03	2.34
18	67	+63.9	90	96.0	97.0	96.7	71.22	6.09	2.42
							71.38	6.04	2.68

[a]Solution in chloroform (*c* 1). [b]Determined from the ratio of the peak area of the β-C-1 to that of the α-C′-1→6 and α-C″-1→(2, 3, or 4). [c]β-C-1 at 103.7 p.p.m. [d]Upper line, calc. value; lower line, found value. [e]Single peak twice the height of the 96.1-p.p.m. peak.

was ~95% of α-D anomer for each glycoside-forming reaction at O-6 and ~90% of α-D anomer for the reactions at O-2 and O-4. Diol **14** contained ~6% of β-D anomer initially, and after the reaction less than 5% of β-D linkages could be detected, indicating that the coupling reactions at O-6 and O-3 gave 95% or more of α-D linkages. The high optical rotation (+81.8°) also confirms the lower proportion of β-D linkages.

The assignment of the α-C-1 of the reducing unit linked to the phenylethyl group and of the α-C-1 of the nonreducing unit linked to O-6 at 95.8–96.1 p.p.m. and 96.9–97.4 p.p.m., respectively, were made by comparison to the ^{13}C-n.m.r. spectra of 2-[4-(*p*-toluenesulfonamido)phenyl]ethyl isomaltooligosaccharides previously published[3]. The assignments of the α-C-1 atoms of the nonreducing units linked to O-2, O-3, and O-4 were made by comparison with the ^{13}C-n.m.r. spectra of several per-*O*-methyl methyl D-glucobiosides reported by Haverkamp *et al.*[6]. For **17**, it was fortunate that the α-C-1 atoms of both nonreducing units have the same chemical shift, since the spectrum of per-*O*-methylnigerose has not been reported. Small, broad peaks around 103.7 p.p.m. were assigned to the β-C-1 atoms, but the peaks were not sharp enough to make individual assignments. No other peaks were found in the anomeric region from 90 to 105 p.p.m., indicating that **16**, **17**, and **18** were uncontaminated trisaccharide derivatives.

The trisaccharide derivatives were deblocked by heating under reflux with sodium ethoxide in ethanol to remove the carbanilate groups, and then with sodium in liquid ammonia to remove the benzyl and *p*-tolylsulfonyl groups. The resulting 2-(4-aminophenyl)ethyl oligosaccharides were converted into the isothiocyanate derivatives and coupled to bovine serum albumin to give the protein conjugates, as previously described[4]. The extent of coupling was determined by carbohydrate analysis, and the results are shown in Table III. The lower-weight fraction of **16** coupled to BSA was the result of the smaller proportion of **16** used in the coupling.

TABLE III

DATA ON THE BOVINE SERUM ALBUMIN–OLIGOSACCHARIDE CONJUGATES

Compd.	*Wt. coupled*[a] *(g)*	*Yield of conjugate (g)*[b]	D-*Glucose content*[c] *(μg/mg)*	*Trisaccharide/ bovine serum albumin ratio*[d] *(mol/mol)*
16	0.6	0.93	64.7	8.6
17	1.0	1.11	136.1	19.5
18	1.8	0.98	130.0	18.5

[a]Completely blocked oligosaccharide. [b]Weight of initial bovine serum albumin was 0.8 g, except for **17**, which was 0.9 g. [c]Determined by anthrone assay. [d]Mol. wt. of 67 000 used for bovine serum albumin.

The amount of carbohydrate coupled to the protein should be sufficient for the conjugates to be used as artificial antigens to prepare antisera specific for the branch points in natural dextrans. The low levels of β-D linkages may not induce antibody formation. If minor amounts of antibodies against β-linked D-glucopyranosyl units were formed, they could be distinguished and, in any case, would not interfere with the immunological testing of dextrans, which have only α-D linkages.

EXPERIMENTAL

General methods. — ^{1}H-N.m.r. spectra were recorded on a Varian A-60-A spectrometer with chloroform-*d* as solvent and tetramethylsilane (Me_4Si) as internal standard, and ^{13}C-n.m.r. spectra on a Varian XL-100-15 in pulsed Fourier-transform–proton-noise decoupled mode with chloroform-*d* as the solvent and Me_4Si as internal standard; all chemical shifts are in p.p.m. units from the Me_4Si signal. Optical rotations were determined with a Perkin–Elmer Model 141 polarimeter with jacketed 1-dm cells at 25°. Melting points were determined with a "Meltemp" apparatus and 76-mm immersion thermometer.

*Methyl 2,3-di-*O*-benzyl-4,6-di-*O*-[2,3,4-tri-*O*-benzyl-6-*O*-(*N*-phenylcarbamoyl)-*D*-glucopyranosyl]-α-*D*-glucopyranoside* (**2**). — A solution of 2,3,4-tri-*O*-benzyl-6-*O*-(*N*-phenylcarbamoyl)-1-*O*-tosyl-D-glucopyranose[2] (**1**) (3.5 g) in dry diethyl ether (20 mL) was added to methyl 2,3-di-*O*-benzyl-α-D-glucopyranoside[7] (0.76 g) under high vacuum. The solution was kept overnight in the dark. Aqueous sodium hydrogencarbonate was added and the product extracted with dichloromethane. The organic phase was washed with water, and then dried ($MgSO_4$). Evaporation of the solution gave a crude product, which was purified on a silica gel column (25 × 1.5 cm) by elution with dichloromethane to give syrupy **2** containing 92–93% of α-D-glycopyranosyl linkages, (2.5 g, 83%), $[\alpha]_D^{25}$ +59.2° (*c* 1, chloroform); ^{1}H-n.m.r.: δ 97.7 (α-C-1), 96.9 (α-C′-1→6), and 96.5 (α-C″-1→4).

Anal. Calc. for $C_{89}H_{92}N_2O_{18}$: C, 72.34; H, 6.28; N, 1.90. Found: C, 72.20; H, 6.19; N, 1.80.

Methyl 4,6-di-O-(α-D-glucopyranosyl)-α-D-glucopyranoside (**3**). — The trisaccharide **2** (1.5 g) was decarbanilated with sodium ethoxide in ethanol for 3 h at reflux. The reaction mixture was neutralized with acetic acid and evaporated to a syrup. The product was extracted with dichloromethane, and the organic phase was washed with water, dried (Na_2SO_4), and evaporated to a syrup. Chromatography on silica gel (10 × 1.5 cm) with chloroform gave the decarbanilated trisaccharide (1.1 g), $[\alpha]_D^{25}$ +66.2° (chloroform). The compound was then debenzylated with sodium (0.25 g) in liquid ammonia (150 mL). The excess of sodium was decomposed with water, and the mixture evaporated to a white powder. Desalting on a Sephadex G-10 column gave the deblocked trisaccharide **3** in ~90% anomeric purity, $[\alpha]_D^{25}$ +136.5° (*c* 1, water).

Anal. Calc. for $C_{19}H_{34}O_{16} \cdot 3\,H_2O$: C, 39.86; H, 7.04. Found: C, 39.40; H, 7.00.

2-O-Allyl-3,4-di-O-benzyl-1,6-di-O-(N-phenylcarbamoyl)-β-D-glucopyranose (**4**). — 2-*O*-Allyl-3,4-di-*O*-benzyl-D-glucopyranose[8] (0.9 g) was dissolved in dry pyridine (25 mL) and phenyl isocyanate (0.6 mL) was added. The solution was heated to 100° and stirred until the reaction was complete (3 h), as shown by t.l.c. Evaporation of the mixture gave a syrup that crystallized from dichloromethane–pet. ether to give 0.84 g of **4**, m.p. 183–184°, $[\alpha]_D^{25}$ −4.4° (*c* 1, chloroform); ^{1}H-n.m.r.: δ 8.3–6.9 (22 H), 6.0–3.0 (16 H), and no 3.5-Hz doublet near δ 6.4.

Anal. Calc. for $C_{37}H_{38}N_2O_8$: C, 69.58; H, 6.00; N, 4.39. Found: C, 69.41; H, 6.01; N, 4.50.

3-O-Allyl-2,4-di-O-benzyl-D-glucopyranose (**5**). — 1,6-Anhydro-2,4-di-*O*-benzyl-β-D-glucopyranose[9] (2.0 g) was allylated in a mixture of dry tetrahydrofuran (50 mL), allyl chloride (20 mL), and sodium hydride (0.7 g, 50%, w/w, in mineral oil) for 2 h at reflux. The excess of sodium hydride was decomposed with methanol, and the solution evaporated to a syrup. The product was extracted with dichloromethane, washed with water, dried ($MgSO_4$), and evaporated to a syrup. The syrup was acetolyzed in acetic anhydride (20 mL) and sulfuric acid (1 drop) for 10 min at room temperature. The mixture was poured into water, stirred overnight, and the product extracted into dichloromethane. The organic phase was washed with water, aqueous sodium hydrogencarbonate, and water, dried ($MgSO_4$), and evaporated to a syrup. The crude diacetyl compound was hydrolyzed in 1,4-dioxane (50 mL) and M hydrochloric acid (20 mL) for 3 h at reflux. The solution was poured into water, and the product extracted in dichloromethane. The organic phase was washed with water, aqueous sodium hydrogencarbonate, and water, dried (Na_2SO_4), and evaporated to a syrup that crystallized from ether–pet. ether to give **5** (1.7 g), m.p. 67–69°, $[\alpha]_D^{25}$ +30.8° (*c* 1, chloroform); ^{1}H-n.m.r.: δ 7.5–6.9 (10 H), 6.2–3.2 (16 H), and 2.8–2.4 (2 H, exchangeable with D_2O).

Anal. Calc. for $C_{23}H_{28}O_6$: C, 68.98; H, 7.05. Found: C, 68.91; H, 7.08.

3-O-Allyl-2,4-di-O-benzyl-1,6-di-O-(N-phenylcarbamoyl)-β-D-glucopyranose (**6**). — To a solution of the diol **5** (1.7 g) in dichloromethane (30 mL) were added triethylamine (0.5 mL) and phenylisocyanate (1.3 g). The solution was heated for 2 h under reflux, and then evaporated to a syrup. The product (**6**) crystallized from

ether–pet. ether (1.24 g), m.p. 160–161°, $[\alpha]_D^{25}$ −15.2° (*c* 1, chloroform); ^{1}H-n.m.r.: δ 8.0–6.9 (22 H), 6.3–3.0 (16 H), and no 3.5-Hz doublet at or near δ 6.4. The mother liquors contained mostly the α-D anomer which failed to crystallize.

Anal. Calc. for $C_{37}H_{38}N_2O_8$: C, 69.58; H, 6.00; N, 4.39. Found: C, 69.76; H, 6.30; N, 4.60.

Methyl 4-O-allyl-2,3-di-O-benzyl-α-D-glucopyranoside (**7**). — Methyl 2,3-di-*O*-benzyl-α-D-glucopyranoside[7] (5.0 g) was tritylated with chlorotriphenylmethane (0.9 g) in pyridine (50 mL) for 4 h at 100°. The product was isolated as a crude syrup, which was allylated in a mixture of tetrahydrofuran (100 mL), allyl chloride (10 mL), and sodium hydride (1.0 g; 50%, w/w, in mineral oil) for 1 day at reflux. The excess of sodium hydride was decomposed with methanol, and the mixture evaporated to an oil. The product was extracted into dichloromethane, and the solution was washed with water, dried ($MgSO_4$), and evaporated to a syrup (~9 g). The crude product was detritylated with hydrogen bromide (1.5 g) in acetic acid (70 mL) for 5 min at room temperature. The mixture was filtered, and the filtrate poured into water. The product was extracted into dichloromethane, and the organic phase washed with water, aqueous sodium hydrogencarbonate, and water, dried ($MgSO_4$), and evaporated to a syrup. Column chromatography on silica gel (30 × 1.5 cm) with chloroform gave **7**, which crystallized from ether–hexane or hexane (3.6 g, 65%), m.p. 78–78.5°, $[\alpha]_D^{25}$ +40.1° (*c* 1, chloroform); ^{1}H-n.m.r.: δ 7.5–6.9 (10 H) and 6.3–3.0 (20 H, 1 H exchangeable with D_2O); lit.[10] m.p. 76–79°, $[\alpha]_D^{25}$ +39.2° (*c* 1, chloroform).

Anal. Calc. for $C_{24}H_{30}O_6$: C, 69.54; H, 7.30. Found: C, 69.49; H, 7.32.

4-O-Allyl-2,3-di-O-benzyl-D-glucopyranose (**8**). — Compound **7** (2.2 g) was dissolved in acetic anhydride (50 mL), and sulfuric acid (1 drop) was added. The reaction was monitored by ^{1}H-n.m.r. and stopped when the formation of an α-C-1 acetate was complete. The mixture was poured into water and stirred overnight. The product was extracted into dichloromethane, and the solution was washed with water, aqueous sodium hydrogencarbonate, water, and evaporated to a syrup. The crude diacetate derivative was deacetylated in 1,4-dioxane (50 mL) and M sulfuric acid (10 mL) for 48 h at 100°. The solution was neutralized with sodium hydrogencarbonate and evaporated to a syrup. The product was dissolved in dichloromethane, and the organic phase was washed with water, sodium hydrogencarbonate, water, dried (Na_2SO_4), and evaporated to a syrup. Chromatography on silica gel (10 × 1.5 cm) with ether gave the diol **8**, which crystallized from either ethanol–water or ether–pet. ether (1.2 g), m.p. 72–73.4°, $[\alpha]_D^{25}$ +46.6° (*c* 1.4, chloroform); ^{1}H-n.m.r.: δ 7.6–6.9 (10 H) and 6.3–2.5 (18 H, 2 H exchangeable with D_2O).

Anal. Calc. for $C_{23}H_{28}O_6$: C, 68.98; H, 7.05. Found: C, 68.97; H, 7.01.

4-O-Allyl-2,3-di-O-benzyl-1,6-di-O-(N-phenylcarbamoyl)-D-glucopyranose (**9**).— The diol **8** (0.9 g) was dissolved in chloroform (15 mL), and triethylamine (0.5 mL) and phenylisocyanate (1.0 mL) were added. The mixture was heated under reflux for 3 h, at which time t.l.c. showed the reaction to be complete. The mixture was evaporated to a syrup which crystallized from ether–pet. ether to give **9** (0.7 g),

m.p. 159–163°, $[\alpha]_D^{25}$ −19.3° (*c* 1, chloroform); ^{1}H-n.m.r.: δ 8.0–6.9 (22 H), 6.4 (d, $J_{1,2}$ 3.5 Hz, H-1 of α-D anomer), and 6.0–3.0 (16 H). The mother liquors gave additional product (0.37 g) which consisted of both α and β isomers, m.p. 155–157°, $[\alpha]_D^{25}$ −6.1 (*c* 1, $CHCl_3$).

Anal. Calc. for $C_{37}H_{38}N_2O_8$: C, 69.58; H, 6.00; N, 4.39. Found: C, 69.40; H, 6.30; N, 4.40.

2-[4-(p-Toluenesulfonamido)phenyl]ethyl mono-O-allyl-di-O-benzyl-6-O-(N-phenylcarbamoyl)-α-D-glucopyranosides (**10**, **11**, *and* **12**). — The 1,6-di-*O*-(*N*-phenylcarbamoyl) derivatives **4**, **6**, and **9** were converted separately to the α-D-glucopyranosyl chloride derivatives with hydrogen chloride in dichloromethane by use of the method previously described[2–4]. Each glucopyranosyl chloride derivative (1.5 mmol) was mixed with silver *p*-toluenesulfonate (1.7 mmol) in dry acetonitrile (5 mL) under high vacuum. After 10 min, the solvent was distilled off, and dry diethyl ether (5.0 mL) and dry dimethoxyethane (2 mL) was distilled in. The solution was then filtered to remove silver chloride and poured onto 2-[4-(*p*-toluenesulfonamido)phenyl]ethanol[3] (0.75 mmol). The mixture was kept overnight at room temperature, and then diluted with dichloromethane and washed with aqueous sodium hydrogencarbonate, water, dried ($MgSO_4$), and evaporated to a syrup. Chromatography on silica gel with chloroform gave the α-D-glucopyranosides (**10**, **11**, and **12**, respectively). Physical constants, yields, and analyses are reported in Table I.

2-[4-(p-Toluenesulfonamido)phenyl]ethyl di-O-benzyl-α-D-glucopyranosides (**13**, **14**, *and* **15**). — The α-D-glucopyranosides **10**, **11**, and **12** were decarbanilated separately with sodium ethoxide in ethanol for 3 h at reflux. The reaction mixtures were processed as described for **3**. Each decarbanilated product (1 mmol) was dissolved in 9:1 (v/v) ethanol–water (20 mL), and tris(triphenylphosphine)rhodium(I) chloride (60 mg) and 1,4-diazobicyclo[2.2.2.]octane (20 mg) was added. The mixture was heated overnight under reflux, filtered, and the filtrate evaporated to a syrup. The syrup was extracted with ether and the organic phase washed with water, aqueous sodium chloride at pH 2, and water, dried ($MgSO_4$), and evaporated to a syrup. The propenyl groups were removed by heating under reflux the crude products in 9:1 (v/v) acetone–water to which mercury(II) oxide (900 mg) and mercury(II) chloride (900 mg) had been added. T.l.c. showed complete cleavage of the propenyl groups after 30 min. The mixture was filtered, and the filtrate was extracted with ether. The organic phase was washed with aqueous potassium iodide and water, dried (Na_2SO_4), and evaporated to a syrup. Column chromatography on silica gel (10 × 1.5 cm) gave the diols as syrups. Physical constants, yields, and analyses are reported in Table I.

Synthesis of 2-[4-(p-toluenesulfonamido)phenyl]ethyl trisaccharide derivatives (**16**, **17**, *and* **18**). — The diols **13**, **14**, and **15** (1 mmol) were coupled separately with 2,3,4-tri-*O*-benzyl-6-*O*-(*N*-phenylcarbamoyl)-1-*O*-tosyl-α-D-glucopyranose (**1**) (2.2 mmol) in dry diethyl ether (5 mL) by use of the method previously described[2,3]. The processing was the same as that described for **2**. Chromatography on a silica gel column (10 × 1.5 cm) gave the purified, branched trisaccharides **16**, **17**, and **18**,

respectively. The physical constants, yields, and analyses are reported in Table II.

Synthesis of the trisaccharide–protein conjugates. — A weighed portion of the oligosaccharides **16**, **17**, and **18** was decarbanilated with sodium ethoxide in ethanol for 3 h under reflux. The processing was the same as described for **3**. The crude syrup was dissolved in dry toluene (15 mL) and added to liquid ammonia (100 mL). Sodium was added in small pieces until the solution remained blue for 1 h. Water was added dropwise to decompose the excess of sodium, and the solvents were evaporated off. The white solid was dissolved in water, and the solution was washed with chloroform and pet. ether. The solution was neutralized to pH 7 with dilute hydrochloric acid and evaporated to a solid. The deblocked oligosaccharide was dissolved in 4:1 (v/v) ethanol–water (25 mL), and thiophosgene (3 equiv.) was added dropwise. After 1.5 h, nitrogen was bubbled through the solution until most of the odor was removed. The solution was neutralized to pH 6 and evaporated to remove most of the alcohol. Water was added, and the solution concentrated to 5–10 mL. The aqueous solution was added dropwise to a weighed amount of bovine serum albumin in 0.15M sodium chloride (10 mL) (pH 9.0). The pH was maintained at 9.0 by addition of 0.1M sodium hydroxide. After 6 h at room temperature, the mixture was refrigerated (2°) overnight. The pH was adjusted to 7.0, and the solution was desalted (5 ×) by ultrafiltration on a PM-10 membrane (Amicon). The protein conjugates were freeze-dried and analyzed for carbohydrate content by quantitative anthrone assay[11]. The results are shown in Table III.

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REFERENCES

1 R. L. SIDEBOTHAM, *Adv. Carbohydr. Chem. Biochem.*, 30 (1974) 371–444.
2 R. EBY AND C. SCHUERCH, *Carbohydr. Res.*, 50 (1976) 203–214.
3 R. EBY, *Carbohydr. Res.*, 70 (1979) 75–82.
4 R. EBY AND C. SCHUERCH, *Carbohydr. Res.*, 77 (1979) 61–70.
5 R. GIGG AND C. D. WARREN, *J. Chem. Soc., C*, (1968) 1903–1911; P. A. GENT AND R. GIGG, *J. Chem. Soc., Chem. Commun.*, (1974) 277–278.
6 J. HAVERKAMP, M. J. A. DEBIE, AND J. F. G. VLIEGENTHART, *Carbohydr. Res.*, 37 (1974) 111–125.
7 D. J. BELL AND J. LORBER, *J. Chem. Soc.*, (1940) 453–455.
8 R. EBY, S. J. SONDHEIMER, AND C. SCHUERCH, *Carbohydr. Res.*, 73 (1979) 273–276.
9 G. ZEMPLÉN, Z. CSÜRÖS, AND S. ANGYAL, *Ber.*, 70 (1937) 1848–1856.
10 R. GIGG, *Am. Chem. Soc. Symp. Ser.*, 77 (1978) 44.
11 R. J. DIMLER, W. C. SCHAEFER, C. S. WISE, AND C. E. RIST, *Anal. Chem.*, (1952) 1411–1414.