*Carbohydrate Research*, 77 (1979) 61–70


# THE SYNTHESIS OF ANTIGENIC DETERMINANTS FOR YEAST D-MANNANS AND A LINEAR (1→6)-α-D-GLUCO-D-MANNAN, AND THEIR PROTEIN CONJUGATES

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(Received November 11th, 1978; accepted for publication, December 8th, 1978)

## ABSTRACT

2-*O*-Benzoyl-3,4,6-tri-*O*-benzyl-1-*O*-tosyl-D-mannopyranose and 2,3,4-tri-*O*-benzyl-6-*O*-(*N*-phenylcarbamoyl)-1-*O*-tosyl-D-glucopyranose were allowed to react with partially blocked 2-[4-(*p*-toluenesulfonamido)phenyl]ethyl α-D-manno- and -gluco-pyranosides. Disaccharides having α-D-Man*p*-(1→2)-α-D-Man*p*, α-D-Man*p*-(1→6)-α-D-Glc*p*, α-D-Man*p*-(1→6)-α-D-Man*p*, and α-D-Glc*p*-(1→6)-α-D-Man*p* structures, and a branched trisaccharide having the structure α-D-Man*p*-(1→2)-[α-D-Man*p*-(1→6)]-α-D-Man*p* were synthesized. The oligosaccharides were deblocked with sodium in liquid ammonia to give glycopyranosides having a free primary aromatic amine which were converted into isothiocyanate derivatives with thiophosgene. The functionalized oligosaccharides were then coupled to bovine serum albumin to give protein conjugates.

## INTRODUCTION

Goebels, Avery and associate[1–3] have shown that a synthetic antigen can be prepared by coupling a carbohydrate or oligosaccharide to a protein support and that animals immunized with these synthetic antigens produce antibodies with specificity for the carbohydrate moiety.

With the recent advances made in the stereoselective synthesis of oligosaccharides in high yields[4–7], complex oligosaccharides having a functional group that can be used for coupling to a protein have been synthesized[8,9].

The structures of several yeast mannans have been elucidated by both chemical[10] and physical ($^{13}$C-n.m.r.)[11] means. The immunological determinants of the yeast mannans have also been studied by inhibition reactions and by preparing artificial antigens with yeast oligosaccharides coupled to a protein support[12–15]. The yeast determinants were found to be located mainly on the side chains, which in many cases consist of (1→2)-linked α-D-mannopyranosyl units. The backbone sequence of the yeast mannans, which consists mainly of (1→6)-linked α-D-mannopyranosyl units, showed little cross-reactivity with yeast-mannan antisera.

To investigate the determinants of yeast mannans further, we have synthesized a series of 2-(4-aminophenyl)ethyl α-D-mannopyranoside oligosaccharides, which correspond to the backbone, side chain, and branch points of a typical yeast mannan, *viz.*, α-D-Man*p*-(1→6)-α-D-Man*p*, α-D-Man*p*-(1→2)-α-D-Man*p*, and α-D-Man*p*-(1→2)-[α-D-Man*p*-(1→6)]-α-D-Man*p*.

A linear α-(1→6)-linked D-gluco-D-mannan had previously been synthesized by Schuerch and associates[16,17], and had been shown by Richter[18] to contain both dextran and α-(1→6)-D-mannopyranan determinants. To investigate further the determinants of this synthetic heteropolysaccharide, we have also prepared a series of 2-(4-aminophenyl)ethyl disaccharides corresponding to sequences in the polymer, *viz.*, α-D-Glc*p*-(1→6)-α-D-Man*p*, α-D-Man*p*-(1→6)-α-D-Glc*p*, and α-D-Man*p*-(1→6)-α-D-Man*p*. The α-D-Glc*p*-(1→6)-α-D-Glc*p* disaccharide has been synthesized previously[9].

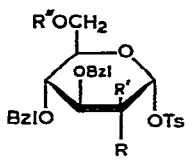
All of the oligosaccharides synthesized have been coupled to bovine serum albumin *via* an isothiocyanate derivative to give protein conjugates for use as artificial antigens.

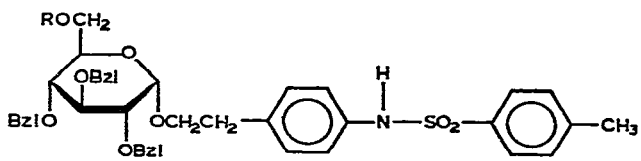
## RESULTS AND DISCUSSION

In our previous paper[9], it was shown that the 2-[4-(*p*-toluenesulfonamido)-phenyl]ethyl group could be the aglycon, since it is stable under all the reaction conditions for coupling and deblocking and can be converted into the 2-(4-amino-phenyl)ethyl group by reduction with sodium–liquid ammonia. Converting the primary aromatic amine to a diazonium salt allowed coupling of the haptens to protein, but the yield of the coupling was somewhat low. We have since found that the isothiocyanate coupling method described by Goldstein and associates[19] gives higher yields of oligosaccharides coupled to protein.

Our previous work[5,6] provided methods to synthesize in high yields and with high stereoselectivity both α-D-gluco- and α-D-manno-pyranosides. The synthesis of two of the starting glycopyranosides, **6** (ref. 6) and **4** (ref. 9), has been described previously, and these compounds were available for use.

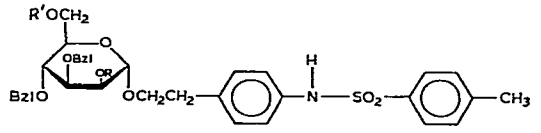
The α-D-mannopyranoside **6** was debenzoylated by transesterification with sodium methoxide in methanol. The resulting mono-ol **7** was coupled with **1** in dichloromethane to give the α-D-(1→2)-linked disaccharide **8**. Chromatography on



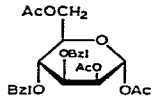

**1** R = H, R' = OBz, R" = Bzl
**2** R = OBzl, R' = H, R" = CONHPh
**3** R = H, R' = OAc, R" = Ac

**4** R = H
**5** R = 2,3,4,6-tetra-*O*-benzyl-α-D-mannopyranosyl



6 R = Bz,R' = Bzl
7 R = H,R' = Bzl
8 R = 2,3,4,6-tetra-*O*-benzyl-α-D-mannopyranosyl

9 R = R' = Ac
10 R = R' = H
11 R = R' = 2,3,4,6-tetra-*O*-benzyl-α-D-mannopyranosyl
12 R = Bz,R' = H
13 R = Bz,R' = 2,3,4,6-tetra-*O*-benzyl-α-D-mannopyranosyl
14 R = Bz,R' = 2,3,4-tri-*O*-benzyl-6-*O*-(*N*-phenylcarbamoyl)



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silica gel with chloroform gave a product, which by $^1$H-n.m.r. spectroscopy was shown to be a disaccharide.

The α-D-glucopyranoside **4** was similarly coupled with **1** in dichloromethane to give the α-D-(1→6)-linked disaccharide **5**. Purification by silica gel chromatography with chloroform gave a product which by $^1$H-n.m.r. was shown to be a disaccharide. The high optical rotation of **5** also indicated the presence of an α-D-glucopyranoside bond.

The remaining α-D-(1→6)-linked disaccharides **13** and **14**, and the trisaccharide **11** were synthesized starting from 1,2,6-tri-*O*-acetyl-3,4-di-*O*-benzyl-α-D-mannopyranose[20,21] (**15**). The triacetate **15** was converted into the corresponding α-D-mannopyranosyl chloride with hydrogen chloride in diethyl ether. The α-D-mannopyranosyl chloride was allowed to react with silver *p*-toluenesulfonate in acetonitrile under anhydrous conditions, as described previously[6]. The resulting 1-*O*-tosyl derivative **3** was dissolved in dichloromethane and added to 2-[4-(*p*-toluenesulfonamido)phenyl]ethanol[9] to give the α-D-mannopyranoside **9**. The stereoselectivity of the glycosidation reaction was shown by the presence of almost 100% of α anomer, as detected by only one C-1 signal at 97.9 p.p.m. in the $^{13}$C-n.m.r. of **9**. Compound **9** was deacylated by transesterification with sodium ethoxide in ethanol to give the diol **10**, which was coupled with 2.1 equiv. of **1** in dichloromethane. The resulting product was purified by chromatography, and the $^1$H-n.m.r. showed it to be the trisaccharide **11**.

To prepare the two α-D-(1→6)-linked disaccharides **13** and **14**, diol **10** had to be blocked selectively at C-2. This was accomplished by treating **10** first with trityl bromide to block O-6, and then with benzoyl chloride to block O-2. Detritylation of O-6 with hydrogen bromide in acetic acid gave **12**. Coupling of **12** with **1** in dichloromethane gave a product that, after purification by silica gel chromatography, was shown to be the disaccharide **13** by $^1$H-n.m.r., and coupling of **12** with the 1-*O*-tosyl-D-glucopyranosyl derivative **2** in diethyl ether gave the heterodisaccharide **14**; again $^1$H-n.m.r., after purification on silica gel, showed the product to be a disaccharide composed of glucose and mannose units.

TABLE I

PHYSICAL CONSTANTS AND $^{13}$C-N.M.R. CHEMICAL SHIFTS OF 2-[4-(*p*-TOLUENESULFONAMIDO)PHENYL]ETHYL OLIGOSACCHARIDES

| *Data* | *Compound* | | | | |
|---|---|---|---|---|---|
| | **5** | **8** | **11** | **13** | **14** |
| $[\alpha]_D^{25}$ (°) | +32.2 | +14.3 | +14.4 | +12.9 | +37.8 |
| *Anal.*[a] | | | | | |
| C | 72.41 | 72.41 | 72.47 | 71.63 | 71.17 |
| | 71.82 | 72.63 | 72.54 | 71.62 | 70.79 |
| H | 6.16 | 6.16 | 6.08 | 5.80 | 5.68 |
| | 6.26 | 6.33 | 6.16 | 5.93 | 5.95 |
| N | | | 0.82 | 0.93 | 1.90 |
| | | | 0.80 | 1.10 | 2.17 |
| S | 2.54 | 2.54 | | | |
| | 2.49 | 2.35 | | | |
| $^{13}$*C-Chemical shifts* ($\delta$) | | | | | |
| C-1 | 96.9 | 98.7 | 98.6 | 98.1[b] | 98.0[c] |
| C-1′ | 98.3 | 99.9 | 99.9 | 98.1[b] | 97.5 |
| C-1″ | | | 97.8 | | |
| C-2 | | 79.7 | 79.8 | 78.4 | 78.6 |
| C′-2 | 78.0 | 78.3 | 78.3 | 78.0 | |
| C″-2 | | | 78.3 | | |

[a]Upper line, calculated value; lower line, experimental value. [b]$^{13}$C-N.m.r. shows a single peak for both C-1 atoms. [c]$^{13}$C-N.m.r. shows ~5% $\beta$-D-glucopyranoside C-1 at 100.5 p.p.m.

The physical constants and elemental analyses for all the oligosaccharides synthesized are shown in Table I. In all the oligosaccharides containing only $\alpha$-D-mannopyranosyl residues, the specific rotations values are $\sim +13$ to $+14.5°$, and are independent of the number and position of the mannopyranosyl residues. The structures of the oligosaccharides were determined from their $^{13}$C-n.m.r. spectra (see Table I). Disaccharide **8** shows signals for two anomeric carbon atoms at 99.9 and 98.7 p.p.m., which were assigned to $\alpha$-C-1 of the nonreducing- and to $\alpha$-C-1 of the reducing-end unit, respectively. The assignments were based on the work of Rachaman *et al.*[6] and Gorin[11], which showed that the signal for $\alpha$-C-1 of a mannosyl residue linked to the phenethyl alcohol group is at 97.7 p.p.m. when O-2 is blocked with a benzoyl group, and that the signal for $\alpha$-C-1 linked to O-2 of a mannose unit is at ~100 p.p.m. Gorin[11] also showed, for the deblocked mannose-containing oligosaccharides, that the signal for C-1 of the nonreducing-end unit is downfield of the signal for C-1 of the reducing-end unit, if both are $\alpha$-linked. The downfield shift of the $\alpha$-C-1 signal of the reducing-end unit to 98.7 p.p.m. is due to the mannose unit linked to C-2. Gorin[11] reported that the signal for C-2 of a mannose unit which is linked to another $\alpha$-D-mannopyranosyl unit appears at 80.0 p.p.m., and Rachaman *et al.*[6]

reported that the signal for C-2 of a mannose unit linked to a benzoate group appears at 78.3 p.p.m. Thus, the signals at 79.9 and 78.5 p.p.m. were assigned to C-2 of the reducing-end unit and C-2 of the unit blocked with the benzoyl group, respectively.

The disaccharide **5** shows two signals for C-1 at 98.3 and 96.9 p.p.m., which were assigned to the α-C-1 of the mannose and glucose units, respectively. Again, the assignments agree with the previous assignments for mannose[6] and glucose[9]. A signal at 78.0 p.p.m. was assigned to C-2 of mannose, and the signals at 82.1 and 80.6 p.p.m. may be assigned to C-2 and C-3 of the glucose unit[22].

The trisaccharide **11** showed three single signals in the anomeric region, at 99.8, 98.6, and 97.8 p.p.m., which were assigned to α-C-1 of the mannosyl residue linked to O-2, to the α-C-1 of the reducing-end unit, and to α-C-1 of the mannosyl residue linked to O-6, respectively. In the C-2 region of the spectrum, two signals were observed at 79.8 and 78.3 p.p.m., the area of the latter peak being twice that of the former peak. The signal at 79.8 p.p.m. was assigned to C-2 of the reducing-end unit, and the signal at 78.3 to the two C-2 of the nonreducing-end units that were blocked with benzoate groups.

The disaccharide **13** showed one wide signal at 98.1 p.p.m. corresponding to both α-C-1. Gorin[11] reported for yeast mannans that the C-1 signals of the α-(1→6)-linked units are shifted upfield about 2 p.p.m., as compared to the C-1 signals of α-(1→2)-linked units. The shift observed for the C-1 signal of **13**, as compared to that of **8**, is almost 2 p.p.m. The C-1 signal of the reducing unit was also shifted slightly upfield. Two peaks at 78.4 and 78.0 p.p.m. were assigned to the C-2 of the reducing and nonreducing-end unit, respectively.

The glucose-mannose disaccharide **14** showed two anomeric signals at 98.0 and 97.5 p.p.m., which were assigned to α-C-1 of the mannose unit and α-C-1 of the glucose unit, respectively. The $^{13}$C-n.m.r. spectrum also showed a small peak (~5%) at 100.5 p.p.m. corresponding to β-C-1 of the glucose unit. As shown previously[5,9], the stereoselectivity of the glucoside-forming reaction is at best 95% of α anomer, and the small β-glucopyranoside peak at 100.5 p.p.m. was not unexpected.

In all of the $^{13}$C-n.m.r. spectra, the presence of any β-mannopyranoside C-1 could not be detected. The work of Rachaman *et al.*[6] has shown that the stereoselectivity of the α-mannoside-forming reactions gives >98% of α anomer. Since 5% of β-glucosidic linkages could be detected in the $^{13}$C-n.m.r. of **14** and no β-mannosidic linkage in any of the other $^{13}$C-n.m.r. spectra, it is assumed that the coupling reactions had a stereoselectivity giving >95% of α anomer.

The oligosaccharides were de-esterified and decarbanilated by transesterification with sodium ethoxide in ethanol. The remaining benzyl and *p*-toluenesulfonyl groups were removed by reductive cleavage with sodium in liquid ammonia. The resulting deblocked oligosaccharides having a 2-(4-aminophenyl)ethyl group as aglycon were coupled immediately to prevent oxidation of the primary, aromatic amine group. Reaction with an excess of thiophosgene[19] gave the fairly stable isothiocyanate derivative, which was used without isolation. Coupling of the isothiocyanate derivative

TABLE II

DATA ON THE PROTEIN–OLIGOSACCHARIDE CONJUGATES

| *Oligosaccharide* | *Amount coupled*[a] *(g)* | *Weight of conjugate*[b] *(g)* | *Proportion of carbohydrate* | |
|---|---|---|---|---|
| | | | *μg/mg of protein*[c] | *mol oligosaccharide/mol BSA*[d] |
| **5** | 1.6 | 1.22 | 128.6 | 22.1 |
| **8** | 1.2 | 1.24 | 122.5 | 23.3 |
| **11** | 1.7 | 1.27 | 227.0 | 35.3 |
| **13** | 0.8 | 1.04[e] | 122.6 | 25.2 |
| **14** | 1.3 | 1.30[f] | 128.0 | 26.5 |

[a]Fully blocked oligosaccharides. [b]Starting weight of BSA 1.0 g, unless otherwise indicated. [c]Determined by anthrone method. [d]Mol. wt. of BSA 65 000. [e]Initial wt. of BSA 0.9 g. [f]Initial wt. of BSA 1.1 g.

of the oligosaccharides with bovine serum albumin (BSA) at pH 9.0 gave the desired protein conjugates. The carbohydrate content of the protein conjugates was determined by the quantitative anthrone method[23] with mannose as standard for the conjugates of **8**, **11**, and **13**, and 1 : 1 (w/w) mannose–glucose for the conjugates of **5** and **14** (see results in Table II).

## EXPERIMENTAL

*General methods.* — $^1$H-N.m.r. spectra were recorded with a Varian A-60-A spectrometer and $^{13}$C-n.m.r. spectra with a Varian XL-100-15 spectrometer in pulsed Fourier-transform–proton-noise decoupled mode, on solutions in chloroform-*d* and with tetramethylsilane as internal standard. All chemical shifts of the $^{13}$C-spectra are given in p.p.m. units from the $Me_4Si$ signal. Optical rotations were determined with a Perkin–Elmer model 141 polarimeter equipped with jacketed 1-dm cells at 25°.

*Materials.* — Spectrograde dichloromethane, acetonitrile, and diethyl ether were dried and stored over calcium hydride. Silver *p*-toluenesulfonate (Eastman Chem. Co.) was dried in high vacuum before use. 2-[4-(*p*-Toluenesulfonamido)phenyl]-ethanol[9], 2,3,4-tri-*O*-benzyl-6-*O*-(*N*-phenylcarbamoyl)-1-*O*-tosyl-α-D-glucopyranose[5] (**2**), 1,2,6-tri-*O*-acetyl-3,4-di-*O*-benzyl-α-D-mannopyranose[20,21] (**15**), 2-*O*-benzoyl-3,4,6-tri-*O*-benzyl-1-*O*-tosyl-α-D-mannopyranose[6] (**1**), 2-[4-(*p*-toluenesulfonamido)-phenyl]ethyl 2-*O*-benzoyl-3,4,6-tri-*O*-benzyl-α-D-mannopyranoside[6] (**6**), and 2-[4-(*p*-toluenesulfonamido)phenyl]ethyl 2,3,4-tri-*O*-benzyl-α-D-glucopyranoside[9] (**4**) were prepared as described previously. Thiophosgene (Aldrich Chem. Co.) and bovine serum albumin (BSA) (Sigma Chem. Co.) were used as received.

*2-[4-(p-Toluenesulfonamido)phenyl]ethyl 3,4,6-tri-*O*-benzyl-α-D-mannopyranoside* (**7**). — To a solution of **6** (1.3 g) in absolute methanol was added a catalytic amount of sodium methoxide at room temperature. After the transesterification was

shown to be complete by t.l.c., the reaction mixture was neutralized with acetic acid and evaporated to a syrup. The product was extracted with dichloromethane. The organic phase was washed with water and aqueous sodium hydrogencarbonate, dried over magnesium sulfate, and evaporated to a syrup. Chromatography on silica gel (1.5 cm × 12 cm) with chloroform gave **7** as a syrup (1.0 g, 88%), $[\alpha]_D^{25}$ +47.1° (*c* 1, dichloromethane); $^1$H-n.m.r. showed loss of the benzoyl group and $^{13}$C-n.m.r. the presence of a single C-1α signal at 99.88 p.p.m.

*Anal.* Calc. for $C_{42}H_{45}NO_8S$: C, 69.69; H, 6.27; N, 1.94. Found: C, 69.34; H, 6.20; N, 1.93.

*2-[4-(*p*-Toluenesulfonamido)phenyl]ethyl 2-*O*-(2-*O*-benzoyl-3,4,6-tri-*O*-benzyl-*α*-*D*-mannopyranosyl)-3,4,6-tri-*O*-benzyl-*α*-*D*-mannopyranoside* (**8**). — A solution of **1** (0.96 g) in dry dichloromethane (4.0 mL) was added to **7** (0.98 g) under high vacuum by use of the technique described previously[6]. After 16 h at room temperature in the dark, the solution was diluted with dichloromethane and washed with aqueous sodium hydrogencarbonate and water. Evaporation of the dried (magnesium sulfate) solution gave a crude product which was chromatographed on silica gel (1.5 cm × 10 cm) with dichloromethane to remove the fast-moving impurities, and then with chloroform to give **8** as a single spot in t.l.c. (chloroform), 1.3 g (76%) (see Table I); $^1$H-n.m.r.: δ 8.3–6.9 (m, 44 H), 5.6 (s, 1 H), 5.2–3.0 (m, 27 H), 2.85 (broad t, 2 H), and 2.5 (s, 3 H).

*2-[4-(*p*-Toluenesulfonamido)phenyl]ethyl 6-*O*-(2-*O*-benzoyl-3,4,6-tri-*O*-benzyl-*α*-*D*-mannopyranosyl)-2,3,4-tri-*O*-benzyl-*α*-*D*-glucopyranoside* (**5**). — A solution of **1** (1.0 g) in dichloromethane (4.0 mL) was added to **4** (0.98 g) under high vacuum. The reaction mixture was stirred at room temperature in the dark for 16 h. After being processed as just described, the crude product was chromatographed on silica gel (1.5 cm × 10 cm) to give syrupy **5**, 1.67 g; $^1$H-n.m.r.: δ 8.3–7.3 (m, 44 H), 5.6 (s, 1 H), 5–3 (m, 3 H), 2.85 (m, 2 H), and 2.5 (s, 3 H).

*2-[4-(*p*-Toluenesulfonamido)phenyl]ethyl 3,4-di-*O*-benzyl-*α*-*D*-mannopyranoside* (**10**). — A solution of **15** (refs. 20, 22; 1.0 g) in dry diethyl ether (30 mL) was saturated at 0° with dry hydrogen chloride. The flask was tightly stoppered and kept at room temperature for 2 days. T.l.c. (1:1, v/v, ether–pet. ether) showed a single, fast-moving spot. Dry nitrogen was bubbled through the solution to remove most of the hydrogen chloride, and then the solution was evaporated to a syrup. This was dissolved in dichloromethane and the solution was washed with water, aqueous sodium hydrogencarbonate, and water, dried ($MgSO_4$), and evaporated to give syrupy *α*-D-mannopyranosyl chloride, $[\alpha]_D^{25}$ +55.1° (*c* 1, chloroform). This was converted into the 1-*O*-tosyl derivative **3** and treated with 2-[4-(*p*-toluenesulfonamido)phenyl]ethanol (0.58 g) as described previously[6]. The product was isolated by column chromatography on silica gel (chloroform) to give 1.2 g of **9**, $[\alpha]_D^{25}$ +26.8° (*c* 1, chloroform). The product was deacetylated with ethanol–sodium ethoxide to give syrupy **10** after chromatography on silica gel (diethyl ether), 0.88 g, $[\alpha]_D^{25}$ +38.0° (*c* 1, chloroform).

*Anal.* Calc. for $C_{39}H_{43}NO_{10}S$: C, 65.19; H, 6.03; N, 4.46. Found: C, 65.06; H, 6.02; N, 4.56.

*2-[4-(p-Toluenesulfonamido)phenyl]ethyl 2,6-di-O-(2-O-benzoyl-3,4,6-tri-O-benzyl-α-D-mannopyranosyl)-3,4-di-O-benzyl-α-D-mannopyranoside* (**11**). — A solution of **1** (2.5 g) in dichloromethane (4.0 mL) was added to **10** (1.0 g). After 16 h the reaction mixture was processed as described for **8**. Chromatography on silica gel (chloroform) gave the syrupy trisaccharide **11** (2.0 g, 74%); $^1$H-n.m.r.: $\delta$ 8.2–6.9 (m, 59 H), 5.6 (s, 2 H), 5.0–3.0 (m, 37 H), 2.8 (m, 2 H), and 2.5 (s, 3 H).

*2-[4-(p-Toluenesulfonamido)phenyl]ethyl 2-O-benzoyl-3,4-di-O-benzyl-α-D-mannopyranoside* (**12**). — To a solution of **10** (1.2 g) in dry pyridine (25 mL) was added trityl bromide (0.6 g). The mixture was stirred at room temperature until t.l.c. (9:1, v/v, chloroform–ethyl acetate) showed the reaction was complete (2 h). Benzoyl chloride (0.4 g) was added slowly and the mixture stirred overnight. Water (2 mL) was added, and after 2 h the solution was evaporated to remove most of the pyridine. Extraction with dichloromethane, washing with water, aqueous sodium hydrogencarbonate, dilute hydrochloric acid, and water, drying ($MgSO_4$), and evaporation gave the 2-*O*-benzoyl-6-*O*-trityl derivative of **10**. The crude product was dissolved in glacial acetic acid, cooled to 0°, and 30% hydrogen bromide in acetic acid (0.5 g) was added. Trityl bromide precipitated out within 1 min. The mixture was filtered and the filtrate poured into ice–water. Extraction with dichloromethane followed by washing with water, aqueous sodium hydrogencarbonate, and water, and then by evaporation, gave a syrup. On chromatography on silica gel (1.5 cm × 20 cm), dichloromethane eluted the trityl compounds, and then chloroform **12** to give, on evaporation, 1.1 g of syrup, pure by t.l.c. (chloroform), $[\alpha]_D^{25}$ +8.5° (*c* 1, chloroform); $^1$H-n.m.r.: $\delta$ 8.2–6.9 (m, 24 H), 5.6 (broad s, 1 H), 5.2–3.0 (m, 12 H), 2.85 (m, 2 H), 2.58 (s, 3 H), and 2.15 (s, exchangeable with $D_2O$, 1 H).

*Anal.* Calc. for $C_{44}H_{43}NO_9S$: C, 69.36; H, 5.69; N, 1.84. Found: C, 69.57; H, 5.49; N, 1.50.

*2-[4-(p-Toluenesulfonamido)phenyl]ethyl 2-O-benzoyl-6-O-(2-O-benzoyl-3,4,6-tri-O-benzyl-α-D-mannopyranosyl)-3,4-di-O-benzyl-α-D-mannopyranoside* (**13**). — A solution of **1** (0.6 g) in dichloromethane (4.0 ml) was added to **12** (0.5 g) under high vacuum. After being kept for 16 h at room temperature and in the dark, the solution was processed as described for **8**. Chromatography on silica gel (1.5 cm × 7 cm) with chloroform gave **13**, 0.82 g (93%); $^1$H-n.m.r.: $\delta$ 8.2–6.9 (m, 44 H), 5.5 (broad s, 2 H), 3.0–5.2 (m, 24 H), 2.8 (m, 2 H), and 2.5 (s, 3 H).

*2-[4-(p-Toluenesulfonamido)phenyl]ethyl 2-O-benzoyl-3,4-di-O-benzyl-6-O-[2,3,4-tri-O-benzyl-6-O-(N-phenylcarbamoyl)-α-D-glucopyranosyl]-α-D-mannopyranoside* (**14**). — A solution of **2** (1.0 g) in anhydrous diethyl ether (4.0 mL) was added to **12** (0.8 g) under high vacuum. The solution was kept for 16 h at room temperature in the dark and then processed as described for **8**. Chromatography on silica gel with chloroform gave **14** (1.3 g) as a syrup; $^1$H-n.m.r.: $\delta$ 8.2–6.9 (m, 45 H), 5.6 (s, 1 H), 5.2–3.0 (m, 25 H), 2.85 (m, 2 H), and 2.5 (s, 3 H).

*Preparation of the oligosaccharide–protein conjugates.* — A weighed portion of

the oligosaccharide (**5**, **8**, **11**, **13**, and **14**) was de-esterified with ethanol–sodium ethoxide. The reaction mixture was neutralized with acetic acid and evaporated to a syrup. The product was extracted with dichloromethane, washed with water and aqueous sodium hydrogencarbonate, dried ($Na_2SO_4$), and evaporated to a syrup. This was dissolved in dry toluene, and the solution added to liquid ammonia (150 mL). A calculated amount of sodium was added in small pieces until the solution remained blue for 1 h. Water was added dropwise to destroy the excess of sodium, and the ammonia evaporated off. The deblocked oligosaccharide was extracted with water, and the solution washed with chloroform and pet. ether, neutralized to pH 7 with dilute hydrochloric acid, and evaporated to dryness. The solid was dissolved in 80% ethanol–water (40 ml), and 3 equiv. of thiophosgene were 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 most of the alcohol removed in vacuo. Water was added and the solution concentrated to a volume of 5–10 mL. The aqueous solution of the isothiocyanate derivative was added dropwise to a stirred solution of BSA (1.0 g) in 0.15M sodium chloride (10 mL) at pH 9.0. The pH was maintained at 9.0 by the addition of small amounts of 0.1M sodium hydroxide. After 6 h at room temperature, the reaction mixture was refrigerated (2°) overnight. The pH was adjusted to 7.0, and the solution ultrafiltered 5 times through a PM-10 membrane (Amicon). The protein conjugate was then freeze-dried and analyzed for carbohydrate content by a quantitative anthrone method[23]; the results are shown in Table II.

ACKNOWLEDGMENTS

This investigation was supported by a research grant (1 RO1 AI 12509-01) from the National Institute of Allergy and Infectious Diseases, National Institutes of Health. The authors thank Dr. Eliezer Rachaman for his assistance.

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