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THE SYNTHESIS OF α- AND β-(1→2)- AND -(1→3)-LINKED GLUCOPYRANOSE DISACCHARIDES AND THEIR PROTEIN CONJUGATES

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ABSTRACT

2,3,4-Tri-*O*-benzyl-6-*O*-(*N*-phenylcarbamoyl)-1-*O*-tosyl-D-glucopyranose and 3,4,6-tri-*O*-benzyl-2-*O*-*p*-nitrobenzoyl-1-*O*-tosyl-D-glucopyranose were allowed to react with partially blocked 2-[4-(*p*-toluenesulfonamido)phenyl]ethyl α- and β-D-glucopyranosides. Disaccharides having the structure α-D-Glc*p*-(1→2)-α-D-Glc*p*, α-D-Glc*p*-(1→3)-α-D-Glc*p*, β-D-Glc*p*-(1→2)-β-D-Glc*p*, and β-D-Glc*p*-(1→3)-α-D-Glc*p* were synthesized. The oligosaccharides were debenzylated with sodium in liquid ammonia to give disaccharides having a free primary aromatic amino group, which were converted into isothiocyanate derivatives and then coupled to various proteins to give the corresponding conjugates.

INTRODUCTION

In a previous paper[1], we described the synthesis of several trisaccharides as determinants for the branch points in natural dextrans and their protein conjugates. In a continuation of the study of the immunochemical determination of the structure of dextrans, we have prepared four glucopyranose disaccharides having both α and β-(1→2) and -(1→3)-linkages. The disaccharides were coupled to protein carriers to give conjugates that will be used as substrates to determine antigenic specificity, and as artificial antigens to produce specific antisera.

The β-linked disaccharides were prepared to check also whether antisera produced from the trisaccharide conjugates[1], which contained small amounts of β-linkages, had any specificity for β-linkages.

RESULTS AND DISCUSSION

As described in our previous papers[2–5], 2,3,4-tri-*O*-benzyl-6-*O*-(*N*-phenylcarbamoyl)-1-*O*-tosyl-D-glucopyranose (3) can be used to prepare α-linked oligosaccharides in high yields and with high stereospecificity, and the 2-[4-(*p*-toluenesulfonamido)phenyl]ethyl aglycon can be used to couple oligosaccharides to a protein support. We[6,7] and others[8] have also shown that 1,2-*trans* glycosides are formed in

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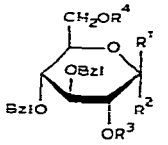
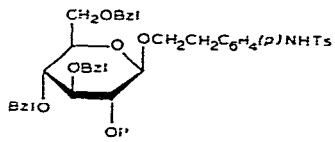
high yields and with very high stereospecificity when 1-*O*-sulfonyl-D-glucopyranose derivatives having an ester group at C-2 are coupled to aglycons. The high reactivity of the 1-*O*-sulfonyl derivatives permits coupling with aglycons which otherwise would react either in low yield or not at all with D-glycopyranosyl halides by conventional coupling-techniques.

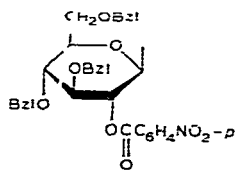


1 $R^1, R^3 = p\text{-}NO_2C_6H_4CO$, $R^2 = H$, $R^4 = Bzl$
2 $R^1 = H$, $R^2 = Cl$, $R^3 = p\text{-}NO_2C_6H_4CO$, $R^4 = Bzl$
3 $R^1 = H$, $R^2 = OTs$, $R^3 = Bzl$, $R^4 = PhNHCO$

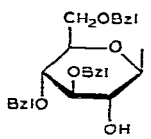


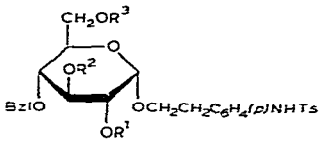
11 R = $p\text{-}NO_2C_6H_4CO$
12 R = H

13 R =



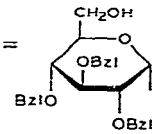
14 R =





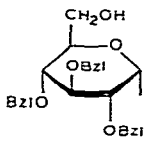
4 $R^1 = H, R^2, R^3 = Bzl$
5 $R^1 = Bzl, R^2 = Allyl, R^3 = PhNHCO$
6 $R^1 = Bzl, R^2 = H, R^3 = PhNHCO$

7 $R^1 =$



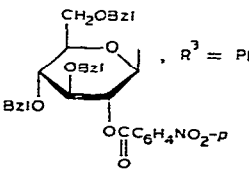
, $R^2, R^3 = Bzl$

8 $R^1 = Bzl, R^2 =$



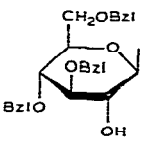
, $R^3 = H$

9 $R^1 = Bzl, R^2 =$



, $R^3 = PhNHCO$

10 $R^1 = Bzl, R^2 =$



, $R^3 = H$

We now find that 3,4,6-tri-*O*-benzyl-2-*O*-*p*-nitrobenzoyl-1-*O*-tosyl-D-glucopyranose gives high yields of β-D-glucopyranosides when coupled with alcohols in acetonitrile. The *p*-nitrobenzoyl derivative was used, as it was available and tends to give more-crystalline products than the benzoate. A systematic investigation of the coupling reaction using derivatives having different acyl groups at C-2 was not attempted, since the *p*-nitrobenzoyl compound also gave highly stereospecific products.

The first step in the syntheses was to prepare derivatives (**4**, **6**, and **12**) having free hydroxyl groups at either C-2 or C-3, the 2-[4-(*p*-toluenesulfonamido)phenyl]ethyl aglycon linked either α or β, and the remaining hydroxyl groups protected. Compound

4 was prepared by Fischer glycosylation of 3,4,6-tri-*O*-benzyl-D-glucopyranose[9] with 2-[4-(*p*-toluenesulfonamido)phenyl]ethanol in 50% yield. The product was separated on a column of silica gel (from the excess alcohol) and other impurities, and was crystallized from ether–petroleum ether.

The corresponding β-linked derivative **12** was also readily prepared by treating the glucopyranosyl chloride **2** with silver *p*-toluenesulfonate to give the 1-*O*-tosyl derivative, which gave the β-linked glucopyranoside **11** with high stereospecificity when coupled with 2-[4-(*p*-toluenesulfonamido)phenyl]ethanol in acetonitrile. De-*p*-nitrobenzoylation gave the alcohol **12** in good yield.

The derivative (**6**) having a free hydroxyl group on C-3 was obtained by de-allylation of **5**, which had been prepared previously[1]. Normally 1,4-diazabicyclo-[2,2,2]octane is added to the mixture to keep the pH above 7, thus preventing any cleavage of the propenyl group. In this instance, the base was omitted to prevent the possible hydrolysis of the C-6 carbanilate group. Slight cleavage of the propenyl group was detected, but since the next step (hydrolysis of the propenyl group) was conducted on the unpurified product, this presented no problem.

Methods for preparing a derivative having a free hydroxyl group at C-3 and a β-linked 2-[4-(*p*-toluenesulfonamido)phenyl]ethyl group proved to be rather lengthy, and so no attempt at its synthesis was tried. This compound would have been used to prepare a β-(1→3)-linked disaccharide in which all of the linkages are β. What effect the anomeric configuration of the linkage to the aglycon has on the specificity of antibodies produced against a disaccharide is unknown in this case. However, the β-(1→3)-linked disaccharide linked α to the aglycon may be a more appropriate antigen for a β-flaw in an α-linked sugar sequence than a completely β-linked disaccharide.

The α-linked disaccharides **7** and **8** were prepared by coupling **3** with **4** and **6**, respectively, followed by decarbanilation. It proved easier to separate the disaccharide from the by-products of the reaction after the carbanilate group had been removed. The ^{13}C-n.m.r. spectrum of **7** showed that the stereospecificity was ~95%. As the starting alcohol **4** was crystalline, the β-D linkages arose from the coupling reaction. The ^{13}C-n.m.r. spectrum of **8** showed >95% α-D linkages. As the starting alcohol **6** had ~5% of β-D linkages, the coupling reaction was >95% stereospecific for the α-D linkage.

The disaccharides **10** and **14** were prepared by first converting the chloride **2** into the 1-*O*-tosyl derivative and then coupling this derivative separately to the alcohols **6** and **12**, and de-esterifying the products. The ^{13}C spectrum of **10** showed C-1α at 96.3 p.p.m. and C-1′β at 105.4 p.p.m. The shifts are consistent with those observed for methyl α and β-D-glucopyranosides[10]. The ^{13}C spectrum of **14** showed peaks at 102.7 and 104.0 p.p.m., corresponding to C-1β and C-1′β. No peaks between 90–101 p.p.m. could be detected, indicating that the disaccharide is 100% β-D-linked. This is not surprising, as **13** was crystalline and any small proportion of α-linked compounds would have been removed on crystallization.

TABLE I

PHYSICAL CONSTANTS, YIELDS, AND ANALYSES OF DISACCHARIDE 2-[4-*p*-TOLUENESULFONAMIDO)PHENYL]-ETHYL GLYCOSIDES

Compound	*Yield (%)*	*M.p. (°C)*	$[\alpha]_D^{25a}$ *(degrees)*	^{13}C-*N.m.r. data (p.p.m.)*[b]		*Anal.*[c]		
				C-1	*C-1'*	*C*	*H*	*N*
7	55		+83.6	95.8	94.6	71.25	6.25	1.00
						71.66	6.36	1.21
8	58		+64.1	96.4	97.4	69.60	5.87	1.09
						69.84	6.33	1.31
9	68		+53.4	96.2	101.0	68.75	5.30	
						68.40	5.67	3.15
10	73	135–136	+45.5	96.3	105.4	71.08	6.36	0.93
						69.84	6.33	1.31
13	60	146.5–148	+3.7	102.1	100.1	69.61	5.93	1.65
						69.92	5.87	2.15
14	84		+10.9	102.7	104.0	71.41	6.50	0.90
						71.67	6.36	1.21

[a]Solution in chloroform (*c* 1). [b]In chloroform-*d*. [c]Upper line, found values; lower line, calc. values.

TABLE II

DATA FOR PROTEIN–OLIGOSACCHARIDE CONJUGATES

Reactants				*Products*	
Compound	*Wt. coupled*[a] *(mg)*	*Protein*[b]	*Wt. protein (mg)*	*Wt. conjugate (mg)*	*Glucose/protein*[c] *(μg/mg)*
7	160	BSA	303	367	136.3
	160	Polylysine	304	332	63.0
8	120	BSA	200	232	64.8
	120	Hemocyanin	200	210	45.4
10	90	BSA	200	196	37.9
	90	Polylysine	200	233.6	34.4
14	140	BSA	256	306.8	72.3
	140	Hemocyanin	256	275.4	62.0

[a]Completely blocked oligosaccharide. [b]BSA = bovine serum albumin. [c]Determined by anthrone assay.

The assignments of C-1 and C-1′ shown in Table I were made by comparison with the spectra of previously synthesized 2-[4-(*p*-toluenesulfonamido)phenyl]ethyl D-glucopyranosides and oligosaccharides[1,3,4], and with the spectra of **9**, **10**, **11**, **12**, **13**, and **14**.

The disaccharides **7**, **8**, **10**, and **14** were simultaneously debenzylated and detosylated by reductive cleavage with sodium in liquid ammonia. The deprotected

oligosaccharides were not isolated, but were immediately converted into the isothiocyanate derivatives[1] and these products coupled to a protein. Two different proteins were used for each disaccharide, so that the conjugates could be used as artificial antigens and as substrates to test the specificity of the antisera produced.

As the isothiocyanate group couples to the free amines of a protein, it was thought that poly-L-lysine, which has an abundance of free amino groups, would make a good carrier for the disaccharides. A simple and inexpensive way of producing a "poly-L-lysine" is to polymerize L-lysine thermally[11,12]. The crude polymerization product was dialyzed ten times by ultrafiltration with a PM-10 membrane (10,000 mol.wt. cut-off, Amicon Corp.) and then freeze-dried to give the polymer in 20% yield. Contrary to expectations, the yield in the coupling to poly-L-lysine proved to be no better than to bovine serum albumin or hemocyanin.

In the coupling reactions, the ratio of disaccharide derivative to protein used was about half the amount used in our previous work. The resulting carbohydrate content of the conjugates is lower than in previous couplings by ~50%, and indicates that the amount of carbohydrate in the conjugate is directly related to the starting sugar derivative:protein ratio. The amount of carbohydrate coupled should be sufficient for the conjugates to be used as substrates or artificial antigens.

EXPERIMENTAL

General methods. — ^{1}H-N.m.r. spectra were recorded with a Varian A-60A spectrometer with chloroform-*d* as solvent and tetramethylsilane (Me_4Si) as internal standard, and ^{13}C-n.m.r. spectra with 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. 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 a 76-mm immersion thermometer.

2-[4-(p-Toluenesulfonamido)phenyl]ethyl 3,4,6-tri-O-benzyl-α-D-glucopyranoside (**4**). — 3,4,6-Tri-*O*-benzyl-D-glucopyranose[9] (1.0 g) was dissolved in dry 1:1 (v/v) benzene–dimethoxyethane (50 mL). 2-[4-(*p*-Toluenesulfonamido)phenyl]-ethanol[4] (3.0 g) and dry ion-exchange resin (Dowex-1,H^+, 2.0 g) were added. The mixture was boiled under reflux using a Dean–Stark trap for 18 h, cooled, filtered and the residue washed with dimethoxyethane. The filtrate was evaporated to a solid and chromatographed on a column of silica gel [25 cm × 10 mm; 2:1 (v/v) hexane–ethyl acetate] to give **4** as a syrup that crystallized from ether–petroleum ether, 0.8 g (50%), m.p. 131–133°, $[\alpha]_D^{25}$ +71.9° (*c* 1, chloroform); ^{1}H-n.m.r. δ 6.9–7.8 (24 H, aromatic and N-H), 3.4–5.1 (15 H, ring, benzyl and CH_2), 2.8 (t, 2 H, CH_2), 2.3 (s, 3 H, CH_3), and 2.3–2.4 (b, 1 H, exchangeable with D_2O); ^{13}C-n.m.r. 98.5 (C-1α), 83.3 (C-3), 77.5, 75.2, 75.0, 73.6, 73.2, 70.8, 68.8, 68.6 (C-6), 35.4 (CH_2), and 21.5 (CH_3).

Anal. Calc. for $C_{42}H_{45}NO_8S$: C, 69.68; H, 6.22; N, 1.94. Found: C, 69.80; H, 6.13; N, 1.80.

*3,4,6-Tri-*O*-benzyl-1,2-di-*O*-p-nitrobenzoyl-*D*-glucopyranose* (**1**). — 3,4,6-Tri-*O*-benzyl-D-glucopyranose[9] (5.0 g) was dissolved in dry pyridine (50 mL) and *p*-nitrobenzoyl chloride (5.0 g) was added with stirring. The solution was kept overnight at room temperature and then poured into ice–water. The product was extracted with dichloromethane and the organic phase washed with water, aqueous sodium hydrogencarbonate, dilute hydrochloric acid and water, dried over magnesium sulfate, and evaporated to a solid. The crude product was crystallized from ether–petroleum ether to give **1**, 7.5 g (90%), m.p. 105–107°, $[\alpha]_D^{25}$ +23.5° (*c* 1, chloroform); ^{1}H-n.m.r. δ 7.0–8.3 (23 H, aromatic), 6.7 (d, 0.17 H, $J_{1,2}$ 3.5 Hz, H-1α), 6.5 (d, 0.83 H, $J_{1,2}$ 8.0 Hz, H-1β), 5.4–5.9 (m, 1 H, H-2), and 3.7–5.1 (m, 11 H, ring and benzyl).

Anal. Calc. for $C_{41}H_{36}N_2O_{12}$: C, 65.77; H, 4.85; N, 3.74. Found: C, 65.69; H, 4.90; N, 3.60.

*3,4,6-Tri-*O*-benzyl-2-*O*-p-nitrobenzoyl-α-D-glucopyranosyl chloride* (**2**). — Compound **1** (1.0 g) was dissolved in dry ether (40 mL) and the solution saturated at 0° with dry hydrogen chloride gas. The flask was tightly stoppered and kept for 24 h at room temperature. Nitrogen was bubbled through the solution to remove most of the excess of hydrogen chloride. The solvent was evaporated and the residue dissolved in dichloromethane. The precipitated *p*-nitrobenzoic acid was filtered off and the filtrate evaporated to a syrup that was sufficiently pure to be used in the coupling reactions; yield 0.8 g, $[\alpha]_D^{25}$ +148.9° (*c* 1, chloroform); ^{1}H-n.m.r. δ 7.0–8.3 (19 H, aromatic), 6.4 (d, 1 H, $J_{1,2}$ 3.5 Hz, H-1α), and 3.7–5.5 (m, 12 H, ring and benzyl).

*2-[4-(p-Toluenesulfonamido)phenyl]ethyl 3,4,6-tri-*O*-benzyl-2-*O*-p-nitrobenzoyl-β-D-glucopyranoside* (**11**). — To a solution of **2** (0.8 g) in dry acetonitrile (2.0 mL) was added silver *p*-toluenesulfonate (0.4 g). After 15 min at room temperature, the silver chloride was filtered off and the clear solution added to 2-[4-(*p*-toluenesulfonamido)phenyl]ethanol[4] (0.35 g). The mixture was kept for 24 h at room temperature and in the dark. The solution was diluted with dichloromethane, washed with aqueous sodium hydrogencarbonate and sodium thiosulfate, water, and saturated sodium chloride solution, dried (magnesium sulfate), and evaporated to a solid. Crystallization from ether–petroleum ether gave **11**; yield 1.25 g (74%), m.p. 160–161°, $[\alpha]_D^{25}$ +34.2° (*c* 1, chloroform); ^{1}H-n.m.r. δ 6.7–8.4 (28 H, aromatics and N-H), 3.3–5.5 (m, 15 H, ring, benzyl, and CH_2), 2.7 (t, 2 H, CH_2), and 2.35 (s, 3 H, CH_3).

Anal. Calc. for $C_{42}H_{48}N_2O_{11}S$: C, 67.41; H, 5.54; N, 3.21. Found: C, 67.26; H, 5.52; N, 2.99.

*2-[4-(p-Toluenesulfonamido)phenyl]ethyl 3,4,6-tri-*O*-benzyl-β-D-glucopyranoside* (**12**). — To a solution of **11** (2.0 g) in abs. ethanol (50 mL) was added sodium ethoxide (0.1 g). The mixture was stirred at room temperature until reaction was complete as shown by t.l.c. The base was made neutral with acetic acid and the solution evaporated to a syrup which crystallized from ether–petroleum ether to give **12**; yield 1.5 g (89%); m.p. 111.5–113°, $[\alpha]_D^{25}$ −4.9° (*c* 1, chloroform); ^{1}H-n.m.r. δ 6.5–7.8 (24 H, aromatics and N-H), 3.35–5.1 (m, 15 H, ring, benzyl and CH_2),

2.85 (t, 2 H, CH_2), 2.35 (s, 3 H, CH_3), and 2.7–3.1 (1 H, exchangeable with D_2O); ^{13}C-n.m.r. δ 103.0 (C-1β), 84.7 (C-3), 77.8, 75.4, 75.1, 74.9, 73.7, 70.4, 69.2 (C-6), 35.6 (CH_2), and 21.5 (CH_3).

Anal. Calc. for $C_{42}H_{45}NO_8S$: C, 69.68; H, 6.27; N, 1.94. Found: C, 69.62; H, 6.33; N, 1.70.

*2-[4-(p-Toluenesulfonamido)phenyl]ethyl 2,4-di-*O*-benzyl-6-*O*-(N-phenylcarbamoyl)-α-*D*-glucopyranoside* (**6**). — The 3-*O*-allyl derivative **5** (ref. 1) (0.50 g) was dissolved in 9:1 (v/v) ethanol–water (10 mL) and tris(triphenylphosphine)rhodium(I) chloride (50 mg) was added. The mixture was boiled under reflux overnight, filtered, and the filtrate evaporated to a syrup. The syrup was dissolved in ether and washed with water, aqueous sodium chloride at pH 2, and water, dried (magnesium sulfate), and evaporated to a syrup. The propenyl group was hydrolyzed by refluxing in 9:1 (v/v) acetone–water (20 mL), to which mercury(II) chloride (250 mg) and mercury(II) oxide had been added. T.l.c. showed complete cleavage of the propenyl group after 30 min. The mixture was filtered, and the filtrate evaporated to a syrup that was dissolved in ether. The organic phase was washed with aqueous potassium iodide and with water, dried (sodium sulfate), and evaporated to a syrup. Chromatography on silica gel (25 cm × 10 mm, 2:1 (v/v) hexane–ethyl acetate, gave **6** as a syrup, 0.34 g (72%); $[\alpha]_D^{25}$ +55.1° (*c* 1, chloroform); ^{1}H-n.m.r. δ 6.8–7.9 (m, 25 H, aromatic and 2N-H), 3.15–5.1 (m, 13 H, ring, benzyl and CH_2), 2.8 (t, 2 H, CH_2), 2.2 (s, 3 H, CH_3), and 2.4–3.1 (1 H, exchangeable with D_2O); ^{13}C-n.m.r. δ 96.8 (C-1α), 79.9 (C-2), 77.2, 74.5, 73.7, 72.9, 68.8, 68.8, 63.9 (C-6), 35.5 (CH_2), and 21.4 (CH_3).

Anal. Calc. for $C_{42}H_{45}N_2O_9S$: C, 67.00; H, 5.89; N, 3.72. Found: C, 66.63; H, 5.81; N, 3.58.

Syntheses of α-linked disaccharides **7** *and* **8**. — The 1-*O*-tosyl derivative **3** (1.1 equiv.) was coupled separately with the alcohols **4** and **6** (1.0 equiv.) in diethyl ether as described previously[2]. The isolation and decarbanilation was also performed as previously described[2–5]. Chromatography on silica gel [25 cm × 10 mm, 2:1 (v/v) hexane–ethyl acetate] gave the disaccharides **7** and **8**. Physical constants, yields, and analyses are reported in Table I.

Syntheses of β-linked disaccharides **9**, **10**, **13**, *and* **14**. — The glucopyranosyl chloride **2** was converted into the 1-*O*-tosyl derivative with silver *p*-toluenesulfonate in acetonitrile as described previously for the manno-[7] and galacto-pyranosyl[6] chlorides. Compound **3** (1.1 equiv.) was added separately to the alcohols **6** and **12** in acetonitrile and kept for 24 h at room temperature and in the dark. Isolation was the same as described for the galactopyranoside reactions[6]. Chromatography on silica gel [25 cm × 10 mm, 2:1 (v/v) hexane–ethyl acetate] gave the disaccharides **9** and **13**. Compound **13** could be crystallized from ether–petroleum ether. The disaccharide derivatives were deesterified with sodium ethoxide in ethanol at room temperature and the products isolated as described for **12**. Chromatography on silica gel [25 cm × 10 mm, 2:1 (v/v) hexane–ethyl acetate] gave **10** and **14**. Compound

10 could be crystallized from ether–petroleum ether. Physical constants, yields and analyses are given in Table I.

Synthses of the disaccharide–protein conjugates. — The disaccharides **7**, **8**, **10**, and **14** were debenzylated and detosylated with sodium in liquid ammonia, converted into the isothiocyanate derivatives, and coupled to proteins (bovine serum albumin, keyhole limpet hemocyanin, and thermally polymerized poly-L-lysine) as described previously[1,4,5]. The freeze-dried conjugates were analyzed for carbohydrate content by quantitative anthrone assay[13]. The results of the couplings and analyses are given in Table II.

ACKNOWLEDGMENT

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