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THE SYNTHESIS OF α-ISOMALTO-OLIGOSACCHARIDE DERIVATIVES AND THEIR PROTEIN CONJUGATES

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ABSTRACT

2-[4-(*p*-Toluenesulfonamido)phenyl]ethyl 2,3,4-tri-*O*-benzyl-α-D-glucopyranoside was condensed with 2,3,4-tri-*O*-benzyl-6-*O*-(*N*-phenylcarbamoyl)-1-*O*-tosyl-D-glucopyranose to give 2-[4-(*p*-toluenesulfonamido)phenyl]ethyl 2,3,4,2′,3′,4′-hexa-*O*-benzyl-6′-*O*-(*N*-phenylcarbamoyl)-α-isomaltoside. The disaccharide was decarbanilated in ethanol with sodium ethoxide. The sequence of coupling with the 1-*O*-tosyl-glucose derivative followed by decarbanilation was repeated to form the tri- and tetra-saccharide derivatives. The di-, tri-, and tetra-oligo-saccharides, were deblocked with sodium in liquid ammonia to give the 2-(4-aminophenyl)ethyl α-isomalto-oligosaccharides, which were diazotized with sodium nitrite in acid, and then coupled to bovine serum albumin and edestin to give the protein conjugates.

INTRODUCTION

The early work of Goebel and Avery[1–4] showed that a synthetic antigen could be made by coupling a carbohydrate to a protein support. It was also shown that antibodies generated by animals immunized with these synthetic antigens were specific for the carbohydrate structure attached to the protein, as well as to the protein and the aromatic portion of the aglycon used in coupling the saccharide to the protein. Many other synthetic antigens have been made since Goebel and Avery's original work, and most of these were made with naturally occurring mono- and oligosaccharides. Synthetic oligosaccharides were little used since most of the methods available were neither stereoselective nor gave high yields of product. It was not until recently that methods have been developed for the stereospecific synthesis of oligosaccharides in high yields. The new methods permit the synthesis of complex oligosaccharides having a functional group that can be used to couple the hapten to a protein.

The synthesis of an important synthetic antigen has been reported by Lemieux *et al.*[5]. By use of the halide ion-catalyzed glycosidation reaction developed by Lemieux and associates[6–8], a trisaccharide having the structure of the human Le^a blood-group determinant was synthesized. The oligosaccharide had as the aglycon an 8-alkoxy-

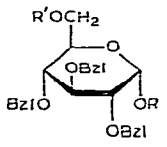
carbonyl-1-octanyl group which could be converted into the acyl azide and then coupled to a protein. When injected into animals, the synthetic antigen induced the formation of antibodies that cross-reacted with natural blood-group Le^a substance and agglutinated Le^a red blood cells.

We have recently reported the stepwise synthesis of a series of methyl α-isomalto-oligosaccharides derivatives and in particular of methyl α-isomaltopentaoside starting from a 1-*O*-tosyl-D-glucopyranose derivative[9]. The yields of couplings were generally high and the stereospecificity was about 95%. The present paper describes the synthesis of a series of α-isomalto-oligosaccharides derivatives having the 2-[4-(*p*-toluenesulfonamido)phenyl]ethyl group as aglycon. The oligosaccharides were deblocked to give 2-(4-aminophenyl)ethyl α-isomalto-oligosaccharides that were coupled to the proteins bovine serum albumin and edestin *via* a diazonium salt to form synthetic antigens.

RESULTS AND DISCUSSION

Many intermediates have been used to couple haptens to proteins. Among them are diazonium salts[1-4,10,11], isothiocyanates[12], aldonic acids[13], 1-(*m*-nitrophenyl)flavazoles[14], cyanogen bromide[15], and acyl azides[5,16]. The oldest and easiest method uses an aglycon with a primary aromatic amine group that can be converted into a diazonium salt, and then covalently coupled to a protein. Previously *p*-aminophenyl and *p*-aminobenzyl glycosides[1-4] were used; however, both of these aglycon groups would be cleaved either during the decarbanilation or debenzylation steps of the stepwise synthesis. The 2-(4-aminophenyl)ethyl group was chosen as it is both stable to strong base and to reduction by sodium in liquid ammonia. The amino group, however, had to be protected as it would participate in the coupling reactions.

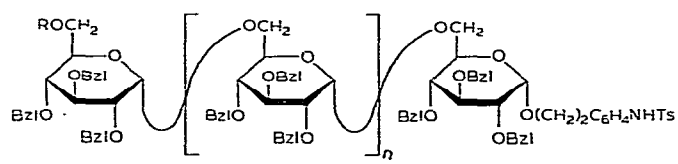
One possible way was to use the 2-(4-nitrophenyl)ethyl group and reduce the nitro group at the end of the coupling reactions into the free amine. Thus, the synthesis of the starting monosaccharide 2-(4-nitrophenyl)ethyl 2,3,4-tri-*O*-benzyl-α-D-glucopyranoside (**2**) was attempted by coupling 2-(4-nitrophenyl)ethanol with 2,3,4-tri-*O*-benzyl-6-*O*-(*N*-phenylcarbamoyl)-1-*O*-tosyl-D-glucopyranose (**1**) in diethyl ether.



1 R = Ts, R' = PhNHCO
2 R = $CH_2CH_2C_6H_4NO_2(p)$, R' = PhNHCO
3 R = (*p*)$TsNHC_6H_4(CH_2)_2$, R' = PhNHCO
4 R = (*p*)$TsNHC_6H_4(CH_2)_2$, R' = H

(*p*) $TsNHC_6H_4(CH_2)_2OH$

11



5 *n* = 0, R = OCNHPh
6 *n* = 0, R = H
7 *n* = 1, R = OCNHPh
8 *n* = 1, R = H
9 *n* = 2, R = OCNHPh
10 *n* = 2, R = H

The carbanilated α-D-glycoside formed was then decarbanilated in ethanol under reflux with sodium ethoxide to give a product that was shown by t.l.c. to be a very complex mixture. Obviously the presence of the aromatic nitro group caused many side reactions.

This was avoided by use of a suitably protected amine that was stable in the presence of the strong base and could be easily deblocked, preferably during the debenzylation step. Thus, 2-[4-(*p*-toluenesulfonamido)phenyl]ethanol (**11**) was synthesized and condensed with **1** in diethyl ether to give the α-D-glycoside, which was decarbanilated with sodium ethoxide in ethanol under reflux to give **4** with no loss of the *p*-toluenesulfonamido group or the formation of by-products.

The monosaccharide (**4**) was coupled with **1** as described previously[9], the processing of the reaction mixture being the same except that neutral alumina was used instead of silicic acid in order to avoid hydrolysis of the *p*-toluenesulfonamido group. The disaccharide **5** was decarbanilated to give a disaccharide with a free hydroxyl group at C-6′ (**6**). Repeating the coupling with **1** followed by decarbanilation gave the tri- and tetra-saccharides **8** and **10**, respectively.

The specific and molecular rotations increased steadily as the d.p. increased for the carbanilated and decarbanilated oligosaccharides. A plot of molecular rotation *vs.* d.p. was shown to be linear, suggesting that the products belong to a homologous series in which the glycosyl bonds are of the same configuration[17] or, as shown previously[9], that each coupling step has the same degree of stereoselectivity.

The ratio of the methyl protons of the *p*-toluenesulfonamido group (δ 2.3) to the total aromatic protons (δ 6.9–8.0) in the ^{1}H-n.m.r. spectrum gave the d.p. The spectrum also indicated the presence or absence of the *N*-phenylcarbamate group. The ^{13}C-n.m.r. spectrum of the benzylated oligosaccharides showed resonances at 96.2 to 97.2 p.p.m., corresponding to C-1 atoms in α configuration. As in the case of methyl α-isomalto-oligosaccharides[9], no resonances could be observed in the region between 99 to 110 p.p.m., which would be indicative of a β-D-glucosyl linkage. This lack of resonances at least shows that the coupling reactions are as stereoselective as those reported for the corresponding methyl α-isomaltooligosaccharides[9].

The di-, tri- and tetra-oligosaccharides **6**, **8** and **10** were debenzylated with sodium in liquid ammonia. During the reaction, the *p*-toluenesulfonamido group was also reductively cleaved to give the free, primary aromatic amine. Care was taken not to use an excess of sodium, which would have cleaved the aglycon group from the reducing end of the oligosaccharide. Aqueous solutions of the completely deblocked oligosaccharide decomposed very quickly at room temperature owing to oxidation of the free primary aromatic amine. Thus, the deblocked oligosaccharides were coupled to bovine serum albumin and edestin immediately after conversion to the diazonium salt by the method of Goebel and Avery[3]. The carbohydrate content of the conjugate (see Table I) showed a decrease in the bovine serum albumin series, which may be due in part to a decrease in the initial amount of carbohydrate used in the coupling reactions.

The stereoselectivity of the coupling reaction was estimated from the ^{13}C spectra

TABLE I

DATA ON PROTEIN-OLIGOSACCHARIDE CONJUGATES

Data	*Oligosaccharide*		
	6	**8**	**10**
Weight (g)[a]	1.53	1.55	1.9
Millimoles	1.32	0.97	0.94
Yield of conjugate (g)[b]			
Bovine serum albumin	1.05	1.00	0.958
Edestin	0.889	1.900[c]	0.888[c]
Proportion of carbohydrate (μg of glucose/mg of protein)			
Bovine serum albumin	22.5	16.0	10.7
Edestin	49.1	21.5	71.3

[a]Weight of fully benzylated oligosaccharide; each protein was coupled with one half of the amount shown; reaction time 24 h. [b]Starting from 1.0 g of the protein and after freeze-drying. [c]Reaction time 48 h.

of the debenzylated, and then acetylated oligosaccharides. These derivatives gave a better-resolved and enhanced ^{13}C-n.m.r. spectrum, which shows the presence of the signals of C-1 in the β configuration at 102.6 p.p.m. The percentage of β linkages was 5 and 7 for the di- and tri-saccharides, respectively.

The tetrasaccharide obtained by deblocking **10** was *N*-acetylated with acetic anhydride and methanol, and the product was isolated on a Sephadex G-10 column. The ^{13}C spectrum also showed a resonance at 102.5 p.p.m., indicating the presence of 6% of β linkage. Thus, the stereoselectively of the coupling reaction lies between 93% and 94% of α linkage.

Richter[18] and Cisar *et al.*[19] have shown that antidextran antibodies consist of a mixture having specificity for the terminal nonreducing group of dextran and for the nonterminal residues along the α-D-(1$\rightarrow$6)-linked chains. It has also been shown[19] that immunization of animals with isomaltotrionic acid coupled to bovine serum albumin produces antidextran antibodies that have a high specificity for the terminal nonreducing group of the dextran chain, as well as some antibodies that are specific for the nonterminal sites along the chain.

EXPERIMENTAL

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

constant temperature bath. Spectrograde dichloromethane, acetonitrile, and diethyl ether were dried and stored over calcium hydride.

*2-(4-Nitrophenyl)ethyl 2,3,4-tri-*O*-benzyl-6-*O*-(*N*-phenylcarbamoyl)-α-*D*-glucopyranoside* (**2**). — 2,3,4-Tri-*O*-benzyl-1,6-di-*O*-(*N*-phenylcarbamoyl)-α- or -β-D-glucopyranose[20] (4.8 g) was converted into **1** as described previously[9]. A solution of 2-(4-nitrophenyl)ethanol (Aldrich Chemical Co., Inc., Milwaukee, WI 53233; 1.0 g) in diethyl ether (20 ml) was added and the reactants were stirred in the dark for 16 h. Dichloromethane (40 ml) was added and the solution washed with saturated sodium hydrogencarbonate solution, water, and saturated sodium chloride solution. The organic phase was dried (magnesium sulfate) and evaporated to a syrup. The syrup was chromatographed on a short column of silicic acid (1.5 × 5 cm) with benzene as the eluting solvent. The purified product crystallized readily from diethyl ether–petroleum ether to give 3.9 g (90%) of **2**, m.p. 133–135°, $[\alpha]_D^{25}$ +49.8° (*c* 1, chloroform).

Anal. Calc. for $C_{42}H_{42}N_2O_9$: C, 70.18; H, 5.89; N, 3.90. Found: C, 69.98; H, 5.94; N, 3.87.

2-[4-(p-Toluenesulfonamido)phenyl]ethanol (**11**). — 2-(4-Nitrophenyl)ethanol (3.0 g) was dissolved in absolute ethanol (50 ml) and palladium-on-carbon (10%, 50 mg) was added. The flask was purged with hydrogen and the mixture stirred under 1 atm pressure of hydrogen until no more hydrogen was consumed. The mixture was filtered and the filtrate concentrated to give solid 2-(4-aminophenyl)ethanol, m.p. 104–107°. The crude product (2.4 g) was dried overnight in a vacuum oven at 35° and then dissolved in dry pyridine (50 ml). *p*-Toluenesulfonyl chloride (3.4 g) was added and the solution stirred for 16 h at room temperature. The reaction mixture was poured into water (200 ml) and then extracted with dichloromethane. The organic phase was washed with water, dilute hydrochloric acid, saturated sodium hydrogencarbonate, and water, dried (sodium sulfate), and evaporated. The solid residue was extracted several times with benzene under reflux and, upon cooling of the benzene extracts, **11** crystallized to give 4.5 g (86.5%), m.p. 126–127.5°; ^{1}H-n.m.r.: δ 9.0 (s broad, 1 H), 7.65 and 7.13 (d, 2 H, $J_{3,4}$ 9.0 Hz, H-3 and H-4), 6.98 (s, H-4), 4.4 (broad, 1 H), 3.75 and 2.62 (t, 2 H, $J_{1,2}$ 6 Hz, H-1, H-2), and 2.32 (s, 3 H).

*2-[4-(p-Toluenesulfonamido)phenyl]ethyl 2,3,4-tri-*O*-benzyl-α-*D*-glucopyranoside* (**4**). — 2,3,4-Tri-*O*-benzyl-1,6-di-*O*-(*N*-phenylcarbamoyl)-α-, or -β-D-glucopyranose[20] (8.6 g) was converted into **1** as described previously[9]. It was dissolved in a mixture of diethyl ether (50 ml) and dimethoxyethane (10 ml) and then added to **11** (2.91 g). The reactants were stirred at room temperature in the dark for 16 h. Dichloromethane (100 ml) was added and the solution washed with saturated sodium hydrogencarbonate solution, water, and saturated sodium chloride solution, dried (magnesium sulfate), and evaporated to a syrup. The syrup was chromatographed on a column of neutral alumina (2.0 × 30 cm) with dichloromethane as the eluting solvent. The product (**3**, 8.0 g) showed a single spot on t.l.c. but failed to crystallize. The crude material was then decarbanilated by heating a solution of the syrup in absolute ethanol (100 ml) containing sodium ethoxide (0.3 g of sodium) for 3 h under reflux.

The reaction mixture was concentrated to 10 ml, and water and dichloromethane were added. The organic phase was washed with dilute hydrochloric acid, saturated sodium hydrogencarbonate, and water, dried (sodium sulfate), and evaporated to a syrup. The syrup was dissolved in hot diethyl ether and **4** crystallized upon cooling (yield 4.5 g, 63%), m.p. 129.5–131° $[\alpha]_D^{25}$ +47.7° (*c* 1, chloroform). An additional 1.5 g of material was obtained by chromatography of the mother liquors on neutral alumina, followed by crystallization from diethyl ether–petroleum ether.

Anal. Calc. for $C_{42}H_{45}NO_8S$: C, 69.69; H, 6.27; N, 1.94. Found: C, 69.25; H, 6.21; N, 1.95.

2-[4-(p-Toluenesulfonamido)phenyl]ethyl 2,3,4-tri-O-benzyl-6-O-[2,3,4-tri-O-benzyl-6-O-(N-phenylcarbamoyl)-α-D-glucopyranosyl]-α-D-glucopyranoside (**5**).—This compound was prepared by coupling **4** (4.5 g) with **1** (4.8 g) in diethyl ether as described for the synthesis of the corresponding methyl isomaltoside derivative[9]. The processing of the reaction mixture was the same, except that the crude product was chromatographed on a neutral alumina column with benzene and dichloromethane as the eluting solvents. The disaccharide fraction was evaporated to a syrup (5.9 g, 75%). The product was homogeneous on t.l.c. silica gel (4:1, v/v, benzene–diethyl ether) but did not crystallize.

2-[4-(p-Toluenesulfonamido)phenyl]ethyl 2,3,4-tri-O-benzyl-6-O-(2,3,4-tri-O-benzyl-α-D-glucopyranosyl)-α-D-glucopyranoside (**6**). — Compound **5** (5.9 g) was dissolved in absolute ethanol (100 ml) containing sodium ethoxide (0.3 g of sodium). The solution was heated under reflux for 3 h and then concentrated to 10 ml. Water and dichloromethane were added, and the organic phase was washed with dilute hydrochloric acid, saturated sodium hydrogencarbonate solution, and water, dried (sodium sulfate), and evaporated to a syrup. The syrup was chromatographed on neutral alumina to remove ethyl carbanilate and the colored impurities. The disaccharide fraction, which was eluted with diethyl ether, was evaporated to give a syrup that was homogeneous on t.l.c. but did not crystallize (yield 4.7 g, 89%).

Preparation of higher oligosaccharides. — The tri- and tetra-oligo-saccharides were synthesized in the same manner as the disaccharide: the oligosaccharide having a free OH-6 was coupled with a slight excess of **1** in diethyl ether. The processing was essentially the same as just described, and the product was isolated after chromatography on a neutral alumina column to remove the monomeric impurity [mostly 2,3,4-tri-*O*-benzyl-6-*O*-(*N*-phenylcarbamoyl)-D-glucopyranose] and some colored impurities. None of the higher oligosaccharides were crystalline, and the yields and optical rotations were obtained from the purified syrups.

The *N*-phenylcarbamoyl group was removed by sodium ethoxide in ethanol under reflux. The decarbanilated products were purified on a neutral alumina column. The by-product, ethyl *N*-phenylcarbamate, was eluted with benzene or dichloromethane, and the decarbanilated oligosaccharide was eluted with diethyl ether. Again the products were not crystalline, and the yields and optical rotations were determined from the purified syrups. The yields and physical constants of each of the oligosaccharides are shown in Table II. The d.p. of each oligosaccharide was

TABLE II

PHYSICAL CONSTANTS AND ANALYTICAL DATA OF 2-[4(*p*-TOLUENESULFONAMIDO)PHENYL]ETHYL α-ISOMALTO-OLIGOSACCHARIDES

Compound	$[\alpha]_D^{25}$ (°)[a]	$[M]_D^{25} \times 10^{-2}$ (°)	*Anal.*[b] *C*	*H*	*N*	*Yield* (%)
2,3,4,2′,3′,4′-Hexa-*O*-benzyl-			71.56	6.16	2.20	75
6′-*O*-(*N*-phenylcarbamoyl)-	+53.8	686	71.31	6.20	2.11	
2,3,4,2′,3′,4′-Hexa-*O*-benzyl-	+54.4	629	71.69	6.37	1.21	89
			71.56	6.30	1.23	
2,3,4,2′,3′,4′,2″,3″,4″-Nona-*O*-benzyl-			72.43	6.26	1.64	85
6″-*O*-(*N*-phenylcarbamoyl)-	+64.1	1095	72.25	6.20	1.76	
2,3,4,2′,3′,4′,2″,3″,4″-Nona-*O*-benzyl-	+65.3	1038	72.57	6.41	0.88	80
			72.41	6.43	0.92	
2,3,4,2′,3′,4′,2″,3″,4″,2‴,3‴,4‴-Dodeca-			72.94	6.31	1.31	83
O-benzyl-6‴-*O*-(*N*-phenylcarbamoyl)-	+74.9	1603	72.81	6.08	1.20	
2,3,4,2′,3′,4′,2″,3″,4″,2‴,3‴,4‴-Dodeca-			73.08	6.43	0.69	75
O-benzyl-	+76.8	1552	73.13	6.44	0.65	

[a](*c* 1, chloroform). [b]Upper line, calculated value; lower line, experimental value.

calculated from the ratio of the aromatic protons to the methyl protons of the *p*-toluenesulfamido group (δ 2.3) in the ^{1}H-n.m.r. spectrum. The ^{13}C-n.m.r. spectrum of each of the oligosaccharides was recorded on samples of 200–300 mg in a 5-mm sample tube by use of a pulsed, Fourier-transform method with a 0.8-sec pulse delay.

Preparation of oligosaccharide–protein conjugates. — The decarbanilated oligosaccharide (1.5–1.9 g) was dissolved in a mixture of dry toluene (10 ml) and dry dimethoxyethane (5 ml). The solution was added to anhydrous liquid ammonia (100 ml). Sodium was added in small pieces to maintain the blue color of the mixture. When the blue color persisted for 1 h, the reaction was complete and the excess sodium was destroyed by the addition of ammonium chloride. The mixture was evaporated to dryness, and the residue dissolved in water (20 ml), washed with dichloromethane, and concentrated to a volume of 10 ml to remove any organic solvent. The aqueous solution was cooled to 0° and one equivalent of sodium nitrite (solid) was added. The solution was acidified (pH 4.0) and stirred for 30 min. Sodium nitrite was added, if necessary, until a slight excess of nitrous acid could be detected with starch iodine paper. The reaction mixture was then divided into 2 equal volumes, and added to a solution of bovine serum albumin (Sigma Chemical Co., St. Louis, MO 63178; 1 g) in water (15 ml) and to a solution of edestin (Sigma, 1 g) in water (15 ml), both at 0°. The pH of the protein–oligosaccharide solution was adjusted to 7.8 with sodium carbonate and maintained at this level for 24–28 h. During this time the solutions changed from a light yellow to a wine-red color. The bovine serum albumin–oligosaccharide conjugate was dialyzed against at least 5 changes of distilled water in a Diaflo ultrafiltration cell equipped with a PM-10 membrane and maintained at 2°. The solution was freeze-dried to give a brown solid. The edestin–oligosaccharide conjugate was dialyzed for 2 days in cellulose tubing against several changes of

distilled water at 2°. The mixture was then freeze-dried to give a brown powder. The carbohydrate content of the protein conjugates was determined by quantitative anthrone assay by the method of Rist et al.[21], after correcting for any absorbance due to the protein component. The yields and carbohydrate content of each of the protein conjugates are shown in Table I.

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