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SYNTHETIC MUCIN FRAGMENTS: BENZYL 2-ACETAMIDO-6-*O*-(2-ACETAMIDO-2-DEOXY-β-D-GLUCOPYRANOSYL)-2-DEOXY-3-*O*-β-D-GALACTOPYRANOSYL-α-D-GALACTOPYRANOSIDE AND BENZYL 2-ACETAMIDO-6-*O*-(2-ACETAMIDO-2-DEOXY-β-D-GLUCOPYRANOSYL)-3-*O*-[6-*O*-(2-ACETAMIDO-2-DEOXY-β-D-GLUCOPYRANOSYL)-β-D-GALACTOPYRANOSYL]-2-DEOXY-α-D-GALACTOPYRANOSIDE*

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ABSTRACT

Glycosylation of benzyl 2-acetamido-4,6-*O*-benzylidene-2-deoxy-α-D-galactopyranoside with 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide, catalyzed by mercuric cyanide, afforded benzyl-2-acetamido-4,6-*O*-benzylidene-2-deoxy-3-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-α-D-galactopyranoside (**3**). *O*-Deacetylation of **3** gave benzyl 2-acetamido-4,6-*O*-benzylidene-2-deoxy-3-*O*-β-D-galactopyranosyl-α-D-galactopyranoside which, on acetalation with benzaldehyde–zinc chloride complex followed by acetylation of the resulting dibenzylidene acetal, gave the disaccharide diacetate (**7**). Cleavage of the acetal groups of **3** and **7** in hot, 80% aqueous acetic acid furnished, respectively, the disaccharide tetraacetate (**4**) and diacetate (**8**). *O*-Deacetylation of **8** in methanolic sodium methoxide gave the disaccharide (**9**). Condensation of **4** or **8** with 2-methyl-(3,4,6-tri-*O*-acetyl-1,2-dideoxy-α-D-glucopyrano)-[2,1-*d*]-2-oxazoline, followed by *O*-deacetylation, afforded the title tri- and tetra-saccharides, **12** and **14**, respectively. The structures of compounds **9**, **12**, and **14** were established by ^{13}C-n.m.r. spectroscopy.

INTRODUCTION

In many glycoproteins, particularly the mucins and blood-group substances, the oligosaccharide chains are *O*-glycosylically linked to a peptide, from 2-acetamido-2-deoxygalactose to serine, or threonine, or both. The structures of the carbohydrate moieties of various *O*-glycosylically linked glycoconjugates have been well documented[2]. It has been postulated that the biosynthesis of such glycopro-

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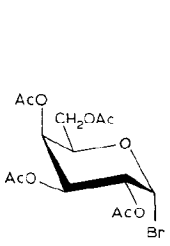
teins, which is catalyzed by glycosyltransferases, is accomplished in a stepwise manner by the addition, at each step, of a monosaccharide unit to the nonreducing terminus of the appropriate acceptor, or at a branch point[3]. It has been customary to employ acceptors derived from the parent mucin for the study of glycosyltransferases involved in the biosynthesis of such molecules. However, Schachter and co-workers[4] have recently demonstrated that synthetic benzyl 2-acetamido-2-deoxy-3-*O*-β-D-galactopyranosyl-α-D-galactopyranoside, and other related disaccharide derivatives having the general structure β-Gal-(1→3)-α-GalNAc-1→OR [R = C_6H_5, or $C_6H_4NO_2$-*o* or -*p*], act as acceptors for *N*-acetylglucosaminyltransferase (GlcNAc-T) present in canine, submaxillary-mucus gland, to give a trisaccharide. It was firmly established that the enzyme did not incorporate a GlcNAc residue at the terminal galactose group of the disaccharide unit β-Gal-(1→3)-GalNAc, to give β-GlcNAc-(1→3)-β-Gal-(1→3)-GalNAc or β-GlcNAc-(1→6)-β-Gal-(1→3)-GalNAc, commonly present as parts of the structures of various mucins. Recently, Wingert and Cheng[5] reported that GlcNAc-T present in the microsomal preparation of rabbit intestinal-epithelium also acts on the disaccharide β-Gal-(1→3)-GalNAc to give the same trisaccharide unit, β-Gal-(1→3)-[β-GlcNAc-(1→6)]-GalNAc. We now describe the chemical synthesis of the benzyl α-glycoside of this trisaccharide.

The fact that our synthetic trisaccharide acted as an acceptor for another GlcNAc-T present in rat-colonic mucosal-membranes[6] (to give a tetrasaccharide) enhanced our interest in the synthesis of the title tetrasaccharide. It was reasonable to assume that the enzyme catalyzed the incorporation of the GlcNAc residue at either O-3 or O-6 of the terminal galactose unit. It was thus desired to obtain the title tetrasaccharide as a reference compound for structural assignment of the biosynthetic,tetrasaccharide. However, it has now been established[7] that the tetrasaccharide obtained by the action of GlcNAc-T on the trisaccharide was, in fact, β-GlcNAc-(1→3)-β-Gal-(1→3)-β-GlcNAc-(1→6)-α-GalNAc-1→OBn, the synthesis of which has also been accomplished[8].

RESULTS AND DISCUSSION

Condensation of 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide (**1**) with benzyl 2-acetamido-4,6-*O*-benzylidene-2-deoxy-α-D-galactopyranoside (**2**) according to the procedure of Flowers and Shapiro[9], and purification of the crude reaction-product in a column of silica gel, afforded amorphous benzyl 2-acetamido-4,6-*O*-benzylidene-2-deoxy-3-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-α-D-galactopyranoside (**3**), which was sufficiently pure (t.l.c., solvent *A*) for further transformations. The ^{1}H-n.m.r. spectrum of **3** supported its overall structure; a ten-proton complex at δ 7.70–7.30 accounted for the benzyl and benzylidene group ring-protons, whereas the acetyl-group methyl protons resonated as distinct singlets at δ 2.14–1.96, and the benzylidene methine proton was observed as a singlet at δ 5.58.



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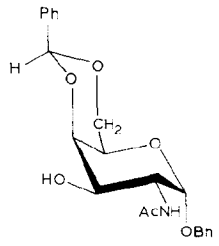

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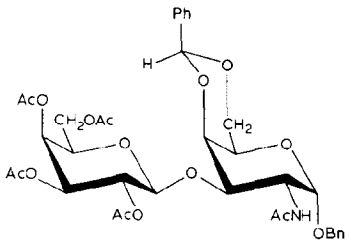

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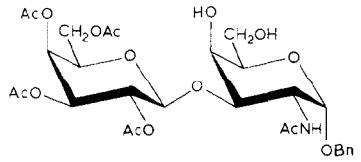

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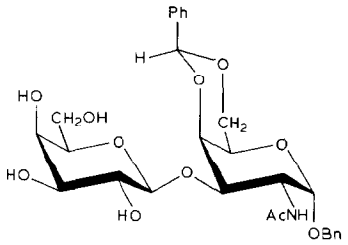


5

Cleavage of the benzylidene group of **3** in hot, 60% aqueous acetic acid furnished diol **4**, which was purified in a column of silica gel. The ^{1}H-n.m.r. spectrum of **4** supported its overall structure (see Experimental section).

O-Deacetylation of **3** in methanolic sodium methoxide, and purification of the crude product by column chromatography, gave, after crystallization from chloroform–ether, benzyl 2-acetamido-4,6-*O*-benzylidene-2-deoxy-3-*O*-β-D-galactopyranosyl-α-D-galactopyranoside (**5**). Treatment of **5** with benzaldehyde–zinc chloride complex[10] converted it into the dibenzylidene acetal **6**, the acetylation of which afforded the diacetate **7**. The ^{1}H-n.m.r. spectrum of **7** was in agreement with the structure expected (see Experimental section). Deacetalation of **7** in hot, 60% aqueous acetic acid gave diacetate **8**, the ^{1}H-n.m.r. spectrum of which was, also, in agreement with the structure assigned; the protons ascribed to the benzylidene groups in the spectrum of **7** were absent from that of **8**.

O-Deacetylation of **8** in methanolic sodium methoxide afforded the known[9] benzyl 2-acetamido-2-deoxy-3-*O*-β-D-galactopyranosyl-α-D-galactopyranoside (**9**). In the ^{13}C-n.m.r. spectrum of **9** (see Table I), the signals for C-1 and C-1′ occurred at 96.21 and 103.49 p.p.m., in accord with an α- and a β-glycosidic linkage, respectively. The signal for C-3 (75.66 p.p.m.) showed an expected downfield (8.47 p.p.m.) shift, by comparison to that in the spectrum of the parent benzyl 2-acetamido-2-deoxy-α-D-galactopyranoside, because of substitution at O-3.

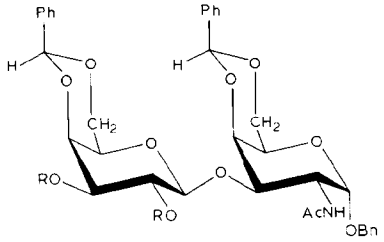
Condensation of diol **4** with 2-methyl-(3,4,6-tri-*O*-acetyl-1,2-dideoxy-α-D-glucopyrano)-[2,1-*d*]-2-oxazoline (**10**) in 1,2-dichloroethane, in the presence of *p*-toluenesulfonic acid, for 16 h at ~70°, and purification of the crude product in a column of silica gel, afforded the trisaccharide derivative **11**. A similar condensation of diacetate **8** with **10** gave the tetrasaccharide derivative **13**. *O*-Deacetylation

TABLE I

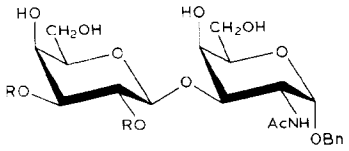
PROPOSED ^{13}C-N.M R CHEMICAL SHIFTS[a,b]

Compound	*C-1*	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*	*C-1'*	*C-2'*	*C-3'*	*C-4'*	*C-5'*	*C-6'*	*CH_3CO*	*OCH_3 or $CH_2C_6H_5$*
[c]	96.08	49.59	67.19	67.55	71.34	60.53	—	—	—	—	—	—	22.49	67.99
[d]	104.22	70.29	73.17	67.96	74.93	60.28	—	—	—	—	—	—	—	55.61
9	96.21	48.37	75.66	67.18	71.30	60.54	103.59	70.59	73.17	67.97	75.13	60.34	22.55	67.87

[a]In Me_2SO-d_6, with Me_4Si as the internal standard. [b]Carbonyl and aromatic carbon resonances are not shown. [c]Benzyl 2-acetamido-2-deoxy-α-D-galactopyranoside. [d]Methyl β-D-galactopyranoside.



6 R = H
7 R = Ac



8 R = Ac
9 R = H

TABLE II

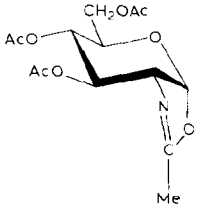
PROPOSED PARTIAL ASSIGNMENTS OF ^{13}C-N M R RESONANCES FOR TRISACCHARIDE **12** AND TETRASACCHARIDE **14**[a]

Residues	*Compound*	*C-1*	*C-2*	*C-3*	*C-4*	*C-6*	CH_3CO	$CH_2C_6H_5$
Benzyl α-GalNAc	**12**	96.07	48.27	76.75	67.62	—	22.54	67.62
β-Gal-(1→3)		103.43	70.55	—	—	60.41	—	—
β-GlcNAc-(1→6)		101.55	55.06	—	—	60.91	22.96	—
Benzyl α-GalNAc	**14**	96.07	48.27	76.75	67.62	—	22.55	67.62
β-Gal-(1→3)		103.39	70.52	—	—	—	—	—
β-GlcNAc-(1→6)-benzyl α-GalNAc		101.48	55.09	—	—	60.92	22.94	—
β-GlcNAc-(1→6)-β-Gal		100.86	55.30	—	—	62.69	22.94	—

[a]In Me_2SO-d_6, with Me_4Si as the internal standard.

of **11** and **13**, gave, respectively, the title saccharides **12** and **14**, the ^{13}C-n.m.r. spectra of which (see Table II) showed signals in support of the structures assigned.

Comments on the ^{13}C-n.m.r. assignments. — The assignments of the ^{13}C-n.m.r. resonances for compounds **12** and **14** were made by comparison of their spectra with each other, and with those assignments for the disaccharide **9**, for benzyl 2-acetamido-2-deoxy-α-D-galactopyranoside, and for methyl β-D-galactopyranoside, reported in Table I, as well as with those for methyl 2-acetamido-2-deoxy-β-D-glucopyranoside[11]. In the ^{13}C-n.m.r. spectrum of **12**, three resonances for anomeric carbon atoms, at δ 96.07, 101.55, and 103.43, were evidence of one α and two β configurations at the glycosidic linkages, which is consistent with the structure proposed. In the spectrum of **14**, an additional anomeric-carbon atom resonance was observed, at δ 100.86, indicating the presence of another 2-acetamido-2-deoxy-β-D-glucopyranosyl residue. The signal for C-3 in the spectra of both **12** and **14** occurred at 76.75 p.p.m., showing that O-3 was glycosylated, whereas one (or two) resonance(s) for C-6 was missing from the range normally observed for a carbon atom attached to an unsubstituted, primary hydroxyl group on a glycopyranose ring which was a clear indication that one (or two) O-6 atom(s) of **12** or **14**, respectively, was also a site of glycosylation.



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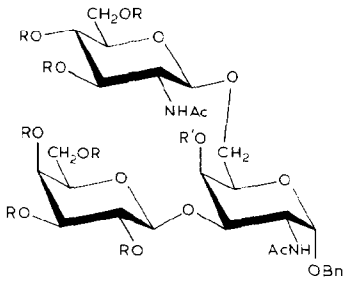
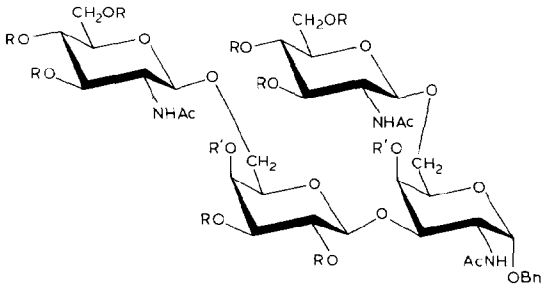


11 R' = Ac, R' = H
12 R = R' = H



13 R = Ac, R' = H
14 R = R' = H

EXPERIMENTAL

General methods. — These were the same as those already described[1], except that the following solvent systems (v/v) were used for chromatography: *A*, 4:1 chloroform–acetone; *B*, 9:1 chloroform–ethanol; *C*, 4:1 chloroform–methanol; and *D*, 15:1 chloroform–ethanol.

*Benzyl 2-acetamido-4,6-*O*-benzylidene-2-deoxy-3-*O*-(2,3,4,6-tetra-*O*-acetyl-β-*D*-galactopyranosyl)-α-*D*-galactopyranoside* (**3**). — A stirred solution of benzyl 2-acetamido-4,6-*O*-benzylidene-2-deoxy-α-D-galactopyranoside (**2**, 1.75 g) in 1:1 nitromethane–benzene (110 mL) was boiled until ~30 mL of the solvent mixture had distilled off. After cooling to room temperature, 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide (**1**, 1.58 g) and powdered mercuric cyanide (0.94 g) were added, and stirring was continued for a total of 72 h. After the customary processing, the crude product was subjected to column chromatography on silica gel, using 9:1 (v/v) chloroform–acetone as the eluant. On evaporation, the fractions corresponding to the product gave a solid material which was dissolved in a small volume of warm ethyl acetate. Addition of hexane caused the precipitation of **3** (1.84 g, 57.5%); amorphous, slightly contaminated (t.l.c., solvent *A*) with a

slower-migrating compound; $[\alpha]_D$ +97.4° (*c* 0.3, chloroform); ^{1}H-n.m.r. data ($CDCl_3$): δ 7.70–7.30 (complex, 10 H, aromatic), 5.58 (s, 1 H, C_6H_5CH), and 2.14, 2.04, 2.03, 1.99, and 1.96 (s, 15 H, 4 OAc and NAc).

*Benzyl 2-acetamido-2-deoxy-3-*O*-(2,3,4,6-tetra-*O*-acetyl-β-*D*-galactopyranosyl)-α-*D*-galactopyranoside* (**4**). — Compound **3** (1.82 g) in 60% aqueous acetic acid (125 mL) was stirred for 0.5 h at ~98°. T.l.c. (solvent *B*) then showed the presence of a major product, slower-migrating than **3**; traces of slower-migrating contaminants were also revealed by t.l.c. The acetic acid was evaporated under diminished pressure, the last traces being removed by co-evaporation with several portions of toluene, and the residue was purified in a column of silica gel by elution with 19:1 (v/v) chloroform–ethanol. The fractions corresponding to the debenzylidenated disaccharide were evaporated to dryness, and the residue (~1.34 g) was dissolved in a small volume of chloroform. Addition of ether–petroleum ether caused the precipitation of **4** (1.08 g, 67.5%); amorphous; $[\alpha]_D$ +118.2° (*c* 0.62, chloroform); ^{1}H-n.m.r. data ($CDCl_3$): δ 7.40 (s, 5 H, aromatic), and 2.17, 2.08, 2.05, 1.99, and 1.96 (s, 15 H, 4 OAc and NAc).

Anal. Calc. for $C_{29}H_{39}NO_{15}$: C, 54.28; H, 6.13; N, 2.18. Found: C, 54.01; H, 5.99; N, 2.05.

*Benzyl 2-acetamido-4,6-*O*-benzylidene-2-deoxy-3-*O*-β-*D*-galactopyranosyl-α-*D*-galactopyranoside* (**5**). — Compound **3** (3.0 g) was dissolved in methanol (25 mL), and treated with M sodium methoxide in methanol (1 mL). After being kept for 5 h at room temperature, the mixture was refrigerated overnight, the base neutralized by the addition of a few drops of glacial acetic acid, and the methanol evaporated. T.l.c. (solvent *C*) then showed the disappearance of **3** and the presence of a major product, slower-migrating than **3**; some slower-migrating contaminants were also revealed by t.l.c. The crude product was subjected to column chromatography on silica gel, using solvent *C* as the eluant. Evaporation of the fractions corresponding to the major product afforded, after crystallization from chloroform–ether, compound **5** (1.88 g, 81.4%); m.p. 216–218°, $[\alpha]_D$ +97.8° (*c* 0.51, chloroform).

Anal. Calc. for $C_{28}H_{35}NO_{11}$: C, 59.87; H, 6.29; N, 2.49. Found: C, 59.61; H, 6.29; N, 2.44.

*Benzyl 2-acetamido-4,6-*O*-benzylidene-3-*O*-(4,6-*O*-benzylidene-β-*D*-galactopyranosyl)-2-deoxy-α-*D*-galactopyranoside* (**6**). — Zinc chloride (1.85 g) was quickly added, with stirring, to benzaldehyde (6.7 mL), and stirring was continued for 0.5 h. Compound **5** (1.85 g) was then added, and the mixture was stirred overnight at room temperature. After the customary processing, the residue was dissolved in *N,N*-dimethylformamide. Addition of water caused the precipitation of the dibenzylidenated disaccharide **6** (1.76 g, 81.9%); $[\alpha]_D$ +135° (*c* 0.21, 1:1 chloroform–methanol).

*Benzyl 2-acetamido-4,6-*O*-benzylidene-2-deoxy-3-*O*-(2,3-di-*O*-acetyl-4,6-*O*-benzylidene-β-*D*-galactopyranosyl)-α-*D*-galactopyranoside* (**7**). — Compound **6** (1 g) was acetylated for 48 h at room temperature with acetic anhydride (20 mL) and

pyridine (30 mL). The acetic anhydride and pyridine were then evaporated under diminished pressure, the last traces being removed by co-evaporation with several portions of toluene, and the residue was dissolved in ethyl acetate. Addition of hexane caused the precipitation of **7** (1.05, 78.9%); amorphous; $[\alpha]_D$ +141.8° (*c* 0.66, chloroform); ^{1}H-n.m.r. data ($CDCl_3$): δ 7.70–7.20 (complex, 15 H, aromatic), 5.56 and 5.52 (s, 2 H, 2 C_6H_5CH), and 2.06, 2.02, and 1.92 (s, 9 H, 2 OAc and NAc).

Anal. Calc. for $C_{39}H_{43}NO_{13}$: C, 63.83; H, 5.92; N, 1.91. Found: C, 63.81; H, 5.90; N, 2.03.

*Benzyl 2-acetamido-2-deoxy-3-*O*-(2,3-di-*O*-acetyl-β-*D*-galactopyranosyl)-α-*D*-galactopyranoside* (**8**). — The dibenzylidene acetal **7** (1 g) in 60% aqueous acetic acid (75 mL) was heated for 40 min at ~98°. The acetic acid was evaporated, and several portions of toluene were added to, and evaporated from, the residue, which then crystallized from acetone–hexane to afford **8** (0.65 g, 85.5%); m.p. 235–237°, $[\alpha]_D$ +118.4° (*c* 0.5, methanol).

Anal. Calc. for $C_{25}H_{35}NO_{13}$: C, 53.85; H, 6.34; N, 2.51. Found: C, 53.68; H, 6.19; N, 2.71.

*Benzyl 2-acetamido-2-deoxy-3-*O*-β-*D*-galactopyranosyl-α-*D*-galactopyranoside* (**9**). — Compound **8** was *O*-deacetylated in methanolic sodium methoxide, to give disaccharide **9**; $[\alpha]_D$ +110.5° (*c* 0.5, 95% ethanol), lit.[9] $[\alpha]_D^{25}$ +108° (*c* 0.87, 95% ethanol); for ^{13}C-n.m.r. data, see Table I.

*Benzyl 2-acetamido-[6-*O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-*D*-glucopyranosyl)-3-*O*-(2,3,4,6-tetra-*O*-acetyl-β-*D*-galactopyranosyl)]-2-deoxy-α-*D*-galactopyranoside* (**11**). — A mixture of compound **4** (0.96 g), oxazoline **10** (1 g), and *p*-toluenesulfonic acid (38 mg) in 1,2-dichloroethane (20 mL), protected from moisture, was heated, with stirring, for 16 h at ~70°; additional amounts of **10** (0.5 g in 5 mL of 1,2-dichloroethane) and *p*-toluenesulfonic acid (19 mg in 5 mL of 1,2-dichloroethane) being added after 4 h. The mixture was cooled, the acid neutralized by the addition of a few drops of pyridine, and evaporated to dryness, to give a residue that was dissolved in chloroform. The solution was washed with water, concentrated to ~10 mL, and the concentrate applied to a column of silica gel. Elution with chloroform, followed by 6:1 (v/v) chloroform–acetone, removed some faster-migrating contaminants. On elution with 2:1 (v/v) chloroform–acetone, and evaporation of the fractions corresponding to the major product, attempted crystallization of the residue from ethyl acetate afforded **11** (1.1 g, 75.9%); amorphous; $[\alpha]_D$ +31.2° (*c* 1.7, chloroform).

Anal. Calc. for $C_{43}H_{58}N_2O_{23}$: C, 53.19; H, 6.02; N, 2.89. Found: C, 52.98; H, 6.12; N, 2.64.

*Benzyl 2-acetamido-6-*O*-(2-acetamido-2-deoxy-β-*D*-glucopyranosyl)-2-deoxy-3-*O*-β-*D*-galactopyranosyl-α-*D*-galactopyranoside* (**12**). — Compound **11** (0.55 g) in methanol (15 mL) was treated with Amberlyst A-26 (OH^-) anion-exchange resin (~150 mg), and the mixture was stirred for 6 h at room temperature. The thick, white precipitate that formed was dissolved in the minimal volume of water, the

resin filtered off, and washed with water, the filtrate and washings combined and evaporated, and the residue crystallized from ethanol, to furnish trisaccharide **12** (0.3 g, 76.9%); m.p. 254–257°; $[\alpha]_D$ +70° (*c* 0.6, water); for ^{13}C-n.m.r. data, see Table II.

Anal. Calc. for $C_{29}H_{44}N_2O_{16} \cdot 0.5\ H_2O$: C, 50.79; H, 6.62; N, 4.09. Found: C, 50.80; H, 6.63; N, 3.66.

Benzyl 2-acetamido-6-O-(2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosyl)-3-O-[6-O-(2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosyl)-2,3-di-O-acetyl-β-D-galactopyranosyl]-2-deoxy-α-D-galactopyranoside (**13**). — A mixture of compound **8** (0.56 g), oxazoline **10** (1 g), and *p*-toluenesulfonic acid (40 mg) in 1,2-dichloromethane (20 mL), protected from moisture, was heated, with stirring, for 16 h at ~70°, additional amounts of **10** (1 g in 10 mL of 1,2-dichloroethane) and *p*-toluenesulfonic acid (40 mg in 10 mL of 1,2-dichloroethane) being added after 4 h. After processing as described for **11**, t.l.c. (solvent *B*) showed the presence of a major product, faster-migrating than **8**; a trace of **8**, and some faster- and slower-migrating contaminants were also revealed by t.l.c. The crude product was dissolved in solvent *D*, and the solution applied to a column of silica gel. Elution with solvent *D* removed the faster-migrating contaminants. Elution with 7:1 (v/v) chloroform–ethanol, and evaporation of the fractions corresponding to the major product, afforded **13** (0.63 g, 51.6%); amorphous; $[\alpha]_D$ +31.2° (*c* 0.5, methanol).

Anal. Calc. for $C_{53}H_{73}N_3O_{29}$: C, 52.34; H, 6.05; N, 3.46. Found: C, 52.45; H, 6.16; N, 3.37.

Benzyl 2-acetamido-6-O-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-3-O-[6-O-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-β-D-galactopyranosyl]-2-deoxy-α-D-galactopyranoside (**14**). — The tetrasaccharide derivative **13** (0.3 g) was *O*-deacetylated with Amberlyst A-26 (OH^-) anion-exchange resin (~100 mg) in methanol (10 mL), as described for **11**, to give **12**. Crystallization from aqueous 2-propanol gave tetrasaccharide **14** (0.17 g, 77.3%); m.p. 213–216°, $[\alpha]_D$ +40.4° (*c* 0.5, water); for ^{13}C-n.m.r. data, see Table II.

Anal. Calc. for $C_{37}H_{57}N_3O_{21}$: C, 50.51; H, 6.53; N, 4.78. Found: C, 50.66; H, 6.69; N, 4.55.

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