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THE PREPARATION OF A PARTIALLY PROTECTED HEPTASACCHARIDE-ASPARAGINE INTERMEDIATE FOR GLYCOPEPTIDE SYNTHESIS*

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ABSTRACT

The heptasaccharide *O*-α-D-mannopyranosyl-(1→6)-*O*-[α-D-mannopyranosyl-(1→3)]-*O*-α-D-mannopyranosyl-(1→6)-*O*-[α-D-mannopyranosyl-(1→3)]-*O*-β-D-mannopyranosyl-(1→4)-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1→4)-2-acetamido-2-deoxy-D-glucopyranose, isolated from the urine of swainsonine-intoxicated sheep, was peracetylated and was converted into the glycosyl azide by three alternative procedures, the most successful of which was formation of peracetyl oxazoline by treatment with trimethylsilyl trifluoromethanesulfonate, followed by treatment with trimethylsilyl azide. Reduction of the glycosyl azide in the presence of Lindlar catalyst gave the glycosylamine derivative, which was coupled with 1-benzyl *N*-fluoren-9-ylmethoxycarbonyl-L-aspartate to yield a protected glycosylasparagine. The benzyl ester group was easily removed by hydrogenolysis to form an intermediate suitable for glycopeptide synthesis.

INTRODUCTION

In the *N*-glycoproteins, oligosaccharide and polypeptide components are linked by a residue of 2-acetamido-1-*N*-(L-aspart-4-oyl)-2-deoxy-β-D-glucopyranosylamine. Synthetic glycosylasparagine derivatives containing this linkage are important for several reasons. Firstly, the compounds are useful as exogenous substrates for the glycosidases active in *N*-glycoprotein "processing"[1]. Secondly, the compounds are needed for studies of the substrate-specificity of lysosomal α-D-

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mannosidase[2] and endo-*N*-acetyl-β-D-glucosaminidase[3] active in normal cells, or in humans and animals with α-mannosidosis[4,5]. Thirdly, glycosylasparagines may be incorporated into synthetic peptides to provide structures that are not naturally occurring, for studies of the intrinsic function of the carbohydrate chains in glycoproteins. We describe herein the synthesis of a selectively protected glycosylasparagine, in which the glycosyl residue is a heptasaccharide with a structure that occurs in many *N*-glycoproteins[6]. The synthetic product is a suitable starting material for glycopeptide synthesis and the method described is generally applicable to the attachment of oligosaccharides to compounds containing a carboxyl group, such as aspartic acid residues in glycopeptides.

RESULTS AND DISCUSSION

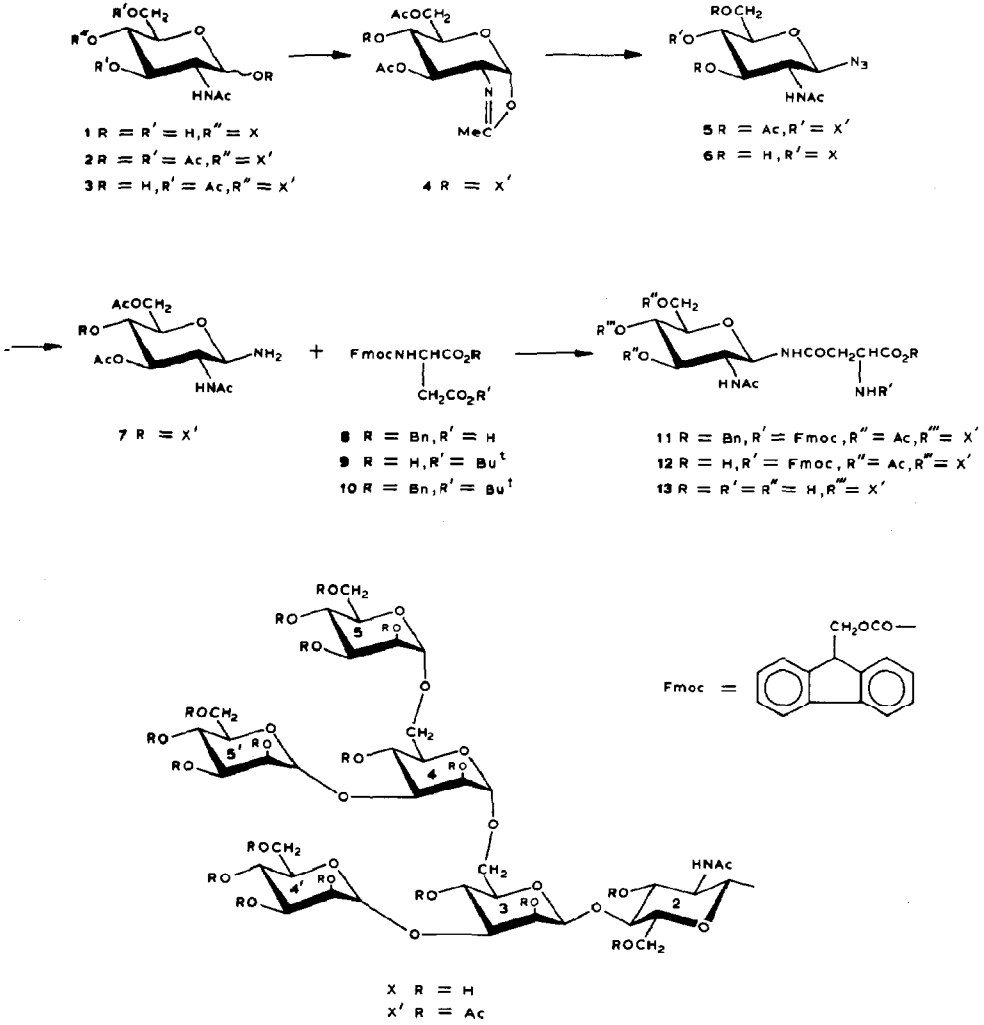
Oligosaccharides with structures corresponding to those found in the glycan chains of *N*-glycoproteins may be isolated from the tissues or body fluids of animals with α-mannosidosis[5,7,8]. For this study, the urine of sheep with swainsonine-induced α-mannosidosis was employed because the concentrations of tetra- and penta-D-mannosyl compounds were enhanced[9], compared with the levels of these oligosaccharides in the urine of animals with genetic α-mannosidosis. The heptasaccharide *O*-α-D-mannopyranosyl-(1→6)-*O*-[α-D-mannopyranosyl-(1→3)]-*O*-α-D-mannopyranosyl-(1→6)-*O*-[α-D-mannopyranosyl-(1→3)]-*O*-β-D-mannopyranosyl-(1→4)-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1→4)-2-acetamido-2-deoxy-D-glucopyranose (**1**) was chosen because it occurs in many "high mannose" glycoproteins[7] and because isomers of this structure do not occur in the urine, making chromatographic separations relatively simple[10].

The major synthetic problem involved in the conversion of the peracetyl heptasaccharide **2** into the glycosyl azide **5** *en route* to the glycosylamine derivative **7** and, thence, the aspartic acid conjugate **11**, is the "activation" of **2** for glycosylamine formation. This must be achieved without any cleavage or modification of inter-residue glycosidic linkages and, for **2**, this is a particular challenge because of the presence of two acid-labile α-D-(1→6) linkages. Three approaches were tried (for a short review of methods for glycosyl azide synthesis, see ref. 11). In the first, peracetyl heptasaccharide **2** was treated with hydrogen chloride gas, and the resulting glycosyl chloride, without isolation, was converted into the azide **5** by the action of lithium azide in *N,N,N′,N′,N″,N″*-hexamethylphosphoric triamide. The yield of **5** was low, primarily because of unwanted side effects of the hydrogen chloride on the 1→6 linkages in **2**, and, to a minor extent, on the linkage between the two 2-acetamido-2-deoxy-D-glucose residues. In the second approach, the intermediate glycosyl chloride was generated under neutral conditions, and converted *in situ* into the azide **5** by treatment with triethylammonium azide. This method involved the initial preparation of **3**, in which OH-1 is free. During the course of other studies[12] aimed at the synthesis of oligosaccharide phosphates, it had been noticed that when a peracetyl oxazoline was treated with dibenzyl phosphate under conditions that

were not completely anhydrous, a major side reaction occurred to yield the free OH-1 compound. This observation was utilized in the present study to obtain **3** in 77% yield from the heptasaccharide oxazoline **4**, after which conversion into the glycosyl chloride employed a mixture of carbon tetrachloride and tris(dimethylamino)phosphine[13]. The preparation, in high yield, of the peracetyl oxazoline **4** involved the treatment of the peracetyl compound **2** with trimethylsilyl trifluoromethanesulfonate. This has been developed in our laboratory as a general procedure for the high yield synthesis of oligosaccharide oxazolines without the occurrence of serious side reactions[14]. The third approach to the glycosyl azide **5** also employed the peracetyl oxazoline **4**, but in this case the conversion was direct, involving the treatment of **4** with trimethylsilyl azide[11] in the presence of stannic chloride and diisopropylethylamine. The structure of **5** was confirmed by *O*-deacetylation to **6**, followed by mass spectrometry and ^{1}H-n.m.r. spectroscopy. The n.m.r. data for **6** were consistent with a β-D configuration for the glucosyl azide residue, and this configuration was expected from the mechanism of formation of **5** by ring-opening of the oxazoline **4** with trimethylsilyl azide[11]. In the 500-MHz ^{1}H-n.m.r. spectrum of **5**, the H-1 resonance for the 2-acetamido-2-deoxy-D-glucopyranosyl azide residue appeared as a quartet at δ 4.79 instead of a doublet at δ 5.70 characteristic of the signal from this residue in glycosylasparagine compounds[15]. When the spectrum was recorded at 60°, the signal became a complex multiplet whereas, at 10°, it was simplified into a doublet. This behavior arises from "virtual coupling"[16] involving long-range interactions when resonances for H-2 and H-3 have similar chemical shifts, and it is typical of β-D-linked derivatives of di-*N*-acetylchitobiose.

The next problem in the formation of a glycosylasparagine from the heptasaccharide azide **5** was the reduction of the azido group to give glucosylamine **7**, and the immediate coupling of **7** with an appropriately protected aspartic acid derivative. It was essential that this coupling occurred before the highly reactive **7** could be consumed by side reactions, principally inter- or intra-molecular migration of an *O*-acetyl group to yield a synthetically useless 1-*N*-acetyl-2-acetamido-2-deoxy-D-glucopyranosylamine. The best method for the reduction of peracetyl azide **5** was a brief hydrogenation over Lindlar catalyst, but t.l.c. of the reaction mixture showed the presence of the 1-*N*-acetyl derivative as a minor product. The glucosylamine **7** gave a single spot on t.l.c., with no sign of the formation of an anomeric mixture. This was further confirmation that azide **5** has the β-D configuration, as Ogawa *et al.*[17] have shown that the reduction of α-D-glucosyl azides gives rise to an anomeric mixture of amines unless triethylamine is included in the hydrogenation mixture, whereas β-D-glucosyl azides give only β-D-glucosylamines.

The aspartic acid compound used for coupling with glucosylamine **7** was the 1-benzyl *N*-fluoren-9-ylmethoxycarbonyl derivative **8**. This compound was chosen so that the coupling product **11** would have the possibility of selective deprotection of either the carboxyl or the primary amino group, thus allowing eventual extension of the asparagine residue in two different directions to form synthetic glycopeptides.



Compound **8** was obtained from commercially available 4-*tert*-butyl *N*-fluoren-9-ylmethoxycarbonyl L-aspartate (**9**) by a sequence of 1-benzyl ester formation to give **10**, followed by acid-catalyzed hydrolysis of the *tert*-butyl group. The coupling of **7** with **8** was performed in the presence of diethyl cyanophosphonate, after which chromatographic purification gave a 50% yield of **11**. The 400-MHz ^{1}H-n.m.r. spectrum of **11** confirmed the presence of 22 acetyl groups, one fluorenylmethoxycarbonyl group, and one benzyl group.

For removal of the 1-benzyl group, **11** was subjected to brief catalytic hydrogenolysis to give **12**. A prolonged hydrogenation, or the use of high pressures, caused partial removal of the fluorenylmethoxycarbonyl group. Complete deprotection of **12** to yield the free glycosylasparagine **13** was readily achieved by treat-

ment with sodium methoxide in methanol; the structure was confirmed by mass spectrometry, which also showed that **13** was accompanied by small proportions of analogous compounds containing one or two *O*-acetyl groups that had resisted saponification.

EXPERIMENTAL

General methods. — Optical rotations were determined for solutions in 1-dm semimicro tubes with a Perkin–Elmer No. 141 polarimeter. ^{1}H-N.m.r. spectra were recorded at 60 MHz with a Varian T-60 spectrometer, and at 500 MHz with a Bruker WM-500 spectrometer at the Northeast Regional NSF-NMR Facility, Yale University, New Haven, CT, or at 400 MHz with a JEOL JNM-GX400 spectrometer at Meiji Seika Kaisha, Ltd., Tokyo, Japan. Mass spectra were recorded with a ZAB-SE instrument (V.G. Analytical, Manchester, U.K.) operated in the positive ion mode and employing a thioglycerol matrix. The cation-exchange resin used was AG 50W-X8 (200–400 mesh; Bio Rad Laboratories, Richmond, CA). Evaporations were conducted *in vacuo*, with the bath temperature kept below 30°. 1,2-Dichloroethane and *N,N*-dimethylformamide were dried by sequential treatment with 3A molecular sieve, and oxolane, tris(dimethylamino)phosphine, and *N,N,N',N'N'',N''*-hexamethylphosphoric triamide (HMPA) by sequential treatment with 4A molecular sieve. The microanalyses were performed by Galbraith Laboratories, Inc., Knoxville, TN.

Chromatographic methods. — T.l.c. was performed on precoated plates of Kieselgel 60 F254, 0.25-mm thick (E. Merck AG, Darmstadt, W. Germany); the plates supplied were cut to a length of 6-cm before use, but otherwise were used without pretreatment. Preparative t.l.c. was performed on precoated plates of Kieselgel 60 F254, 0.5-mm thick (Merck). The spray reagent was 1:1:18 anisaldehyde–sulfuric acid–ethanol[18], and the plates were heated to 125°. Column chromatography was performed on Kieselgel 60, 0.04–0.063 mm (230–400 mesh; Merck). Liquid chromatography was performed at ~7 MPa on a Model 5020 instrument (Varian Associates, Palo Alto, CA), equipped with a u.v. detector Model ERC 7210 (Erma Optical CO., Japan), and a printer–plotter integrator Model 3380A (Hewlett Packard, Avondale, PA). The column used was a 5-μm Hi-chrom reversible Amino Spherisorb (Regis Chemical Co., Morton Grove, IL), eluted with 7:3 acetonitrile–15mM KH_2PO_4 at a flow rate of 2 mL/min with detection at 195 nm. For all types of chromatography, solvent proportions are v/v.

Acetylation of **1**. — The heptasaccharide **1** (200 mg, 0.16 mmol), prepared from the urine of swainsonine-intoxicated sheep[5,8,10] without purification beyond the stage of preparative-layer chromatography, was acetylated as described previously for *O*-α-D-mannopyranosyl-(1→3)-*O*-β-D-mannopyranosyl-(1→4)-2-acetamido-2-deoxy-D-glucopyranose[12], to give *O*-(2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl)-(1→6)-*O*-[(2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl)-(1→3)]-*O*-(2,4-di-*O*-acetyl-α-D-mannopyranosyl)-(1→6)-*O*-[(2,3,4,6-tetra-*O*-acetyl-α-D-man-

nopyranosyl)-(1→3)]-*O*-(2,4-di-*O*-acetyl-β-D-mannopyranosyl)-(1→4)-*O*-(2-acetamido-3,6-di-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-(1→4)-2-acetamido-1,3,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranose (**2**) (268 mg), $[\alpha]_D^{20}$ +20° (*c* 0.22, chloroform); t.l.c. R_F (10:1 chloroform–methanol) 0.54; ^{1}H-n.m.r. (60 MHz, $CDCl_3$): δ6.10 (d, 1 H, $J_{1,2}$ 3.6 Hz, H-1), and 6.02 (d, 1 H, *J* 9.5 Hz, N*H*). This material was suitable for the next step without purification. The β-D anomer was not detectable by ^{1}H-n.m.r. spectroscopy or t.l.c.

O-*(2,3,4,6-Tetra-*O*-acetyl-α-*D*-mannopyranosyl)-(1→6)-*O-[*(2,3,4,6-tetra-*O*-acetyl-α-*D*-mannopyranosyl)-(1→3)]-*O*-(2,4-di-*O*-acetyl-α-*D*-mannopyranosyl)-(1→6)-*O-[*(2,3,4,6-tetra-*O*-acetyl-α-*D*-mannopyranosyl)-(1→3)]-*O*-(2,4-di-*O*-acetyl-β-*D*-mannopyranosyl)-(1→4)-*O*-(2-acetamido-3,6-di-*O*-acetyl-2-deoxy-β-*D*-glucopyranosyl)-(1→4)-2-methyl-(3,6-di-*O*-acetyl-1,2-dideoxy-α-*D*-glucopyrano)-[2,1-d]-2-oxazoline* (**4**) *and its hydrolysis*. — A solution of **2** (450 mg, 0.21 mmol; α-D-anomer according to 500 MHz ^{1}H-n.m.r. spectroscopy) in 1,2-dichloroethane (2 mL) was treated with trimethylsilyl trifluoromethanesulfonate (0.35 mmol; Aldrich Chemical Co., Milwaukee, WI), and the mixture stirred under anhydrous conditions for 40 h at room temperature, when t.l.c. (10:1 chloroform–methanol) showed complete conversion of **2** (R_F 0.54) into **4** (R_F 0.58). After the addition of triethylamine (0.02 mL) the mixture was applied directly to a column of silica gel (30 g) and **4** was eluted with 400:100:1 ethyl acetate–acetonitrile–triethylamine. The fractions containing **4** (t.l.c.) were combined and evaporated to yield 400 mg (90%), $[\alpha]_D^{19}$ +8° (*c* 0.9, chloroform); ^{1}H-n.m.r. (500 MHz, $CDCl_3$): δ 5.90 (d, 1 H, $J_{1,2}$ 7.5 Hz, H-1) and 5.92 (d, 1 H, *J* 9.5 Hz, N*H*).

Anal. Calc. for $C_{86}H_{116}N_2O_{55}\cdot 1.5N(C_2H_5)_3$: C, 51.63; H, 6.32; N, 2.22. Found: C, 51.78; H, 6.48; N, 1.84.

To confirm the identity of **4**, a sample was subjected to acid-catalyzed hydrolysis, reduction with sodium borohydride, and comparison by l.c. with an authentic sample of the alditol prepared from **1**, as described elsewhere for the peracetyl oxazoline derived from *O*-α-D-mannopyranosyl-(1→6)-*O*-β-D-mannopyranosyl-(1→4)-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1→4)-2-acetamido-2-deoxy-D-glucopyranose[19]. The l.c. trace showed a single peak corresponding to the alditol of **1**.

Compound **4** (103 mg, 0.05 mmol) was treated with a solution of dibenzyl phosphate (0.1 mmol, Aldrich) in 1,2-dichloromethane (1 mL) without any precautions to exclude water. The mixture was kept for 3 days at room temperature, then directly applied to three preparative t.l.c. plates which were developed with 10:1 chloroform–methanol. The band containing *O*-(2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl)-(1→6)-*O*-[(2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl)-(1→3)]-*O*-(2,4-di-*O*-acetyl-α-D-mannopyranosyl)-(1→6)-*O*-[(2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl)-(1→3)]-*O*-(2,4-di-*O*-acetyl-β-D-mannopyranosyl)-(1→4)-*O*-(2-acetamido-3,6-di-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-(1→4)-2-acetamido-3,6-di-*O*-acetyl-2-deoxy-D-glucopyranose (**3**) was located with the anisaldehyde reagent on a 0.2-cm strip cut from each plate, and extraction of the silica gel with 2:1

chloroform–methanol gave **3** (80 mg, 77%), $[\alpha]_D^{20}$ +3.9→+4.4° (*c* 0.87, chloroform); t.l.c. R_F (10:1 chloroform–methanol) 0.50.

O-(2,3,4,6-Tetra-O-acetyl-α-D-mannopyranosyl)-(1→6)-O-[(2,3,4,6-tetra-O-acetyl-α-D-mannopyranosyl)-(1→3)]-O-(2,4-di-O-acetyl-α-D-mannopyranosyl)-(1→6)-O-[(2,3,4,6-tetra-O-acetyl-α-D-mannopyranosyl)-(1→3)]-O-(2,4-di-O-acetyl-β-D-mannopyranosyl)-(1→4)-O-(2-acetamido-3,6-di-O-acetyl-2-deoxy-β-D-glucopyranosyl)-(1→4)-2-acetamido-3,6-di-O-acetyl-2-deoxy-β-D-glucopyranosyl azide (**5**). — (*a*). Into a solution of **2** (88 mg, 42 μmol) in 3:1 1,2-dichloroethane–diethyl ether (20 mL) was passed dry HCl gas for 15 min at −10° (needle and septum vial), and then the reaction vial was sealed and the mixture kept at room temperature for 66 h. Hydrogen chloride was removed by passage of N_2 gas, and the solvents were evaporated. The residue (glycosyl chloride derived from **2**) was treated with lithium azide (21 mg, 0.42 mmol) and HMPA, (2 mL) and the mixture stirred at room temperature overnight when t.l.c. (10:1 chloroform–methanol) showed the formation of **5**, R_F 0.52. After the addition of ethyl acetate (50 mL) the solution was washed with water (4 × 20 mL) to remove HMPA, and the organic phase concentrated to 0.5 mL, which was applied to three preparative t.l.c. plates. The plates were developed with 20:1 chloroform–methanol, and the band containing **5** located by u.v. irradiation. Extraction of the silica gel with 2:1 chloroform–methanol gave **5** (20 mg, 22%), $[\alpha]_D^{20}$ +3° (*c* 0.22, chloroform).

Anal. Calc. for $C_{86}H_{117}N_5O_{55}\cdot 2H_2O$: C, 48.34; H, 5.71; N, 3.28. Found: C, 48.40; H, 5.66; N, 2.95.

In order to characterize the product by mass spectrometry and ^{1}H-n.m.r., a sample of **5** was *O*-deacetylated by treatment with an excess of a 0.5% solution of sodium methoxide in dry methanol, and kept for 40 h at room temperature. The mixture was passed through a column (1 × 0.5 cm) of cation-exchange resin and evaporation gave **6**, $[\alpha]_D^{21}$ +32.5° (*c* 0.12, 1:1 methanol–water); ^{1}H-n.m.r. (500 MHz, D_2O): δ 5.098 (2 d, 2 H, H-1$^{4'}$,1$^{5'}$), 4.910 (d, 1 H, H-1^5), 4.874 (d, 1 H, H-1^4), 4.810 (s, 1 H, H-1^3), 4.790 [d (10°), m (60°), 1 H, H-1^1], and 4.598 [d, 1 H, H-1^2)]; m.s.: *m/z* 1283 (M + Na^+).

(*b*). To a solution of **3** (80 mg, 38.5 μmol) in 5:1 dichloromethane–carbon tetrachloride (0.6 mL) was added, at −60°, an 85mM solution of tris(dimethylamino)-phosphine in dichloromethane (0.7 mL). The mixture was stirred for 1 h at −60°, and a solution of triethylammonium azide [60 mg; prepared by treatment of an aqueous solution of sodium azide with 0.1M H_2SO_4, extraction of the product into diethyl ether, brief drying (P_2O_5), addition of a slight excess of triethylamine, decantation of the clear liquid, and co-evaporation with toluene] in tris(dimethylamino)phosphine (1 mL) was added. After the evaporation of dichloromethane and addition of ethyl acetate (80 mL), the solution was washed with water (4 × 50 mL) to remove tris(dimethylamino)phosphine. Most of the solvent was evaporated, and the residue was applied to three preparative t.l.c. plates, which were developed (a) with 10:1 chloroform–methanol, and (b) twice with 20:1 chloroform–methanol. The bands containing **5** and residual **3** were located by u.v. irradiation or by cutting a 0.2-cm strip from each plate and spraying with the anisal-

dehyde reagent. Extraction of the silica gel with 2:1 chloroform–methanol gave **5** (30 mg, 37%) and starting material **3** (30 mg). This product had the same properties as those of **5** prepared by method (*a*).

(*c*). Compound **4** (180 mg, 87.5 μmol) was treated at room temperature with a 0.2M solution of trimethylsilyl azide (1.5 mL) (Aldrich) in dichloromethane[11], followed by tin(IV) chloride (0.03 mL, 0.25 mmol) and diisopropylethylamine (0.03 mL, 0.17 mmol). The mixture was stirred for 86 h at 50°, when t.l.c. (10:1 chloroform–methanol) showed that **4** (R_F 0.55) had been completely transformed into **5** (R_F 0.52). The mixture was directly applied to a column of silica gel (30 g) which was eluted with 40:1 chloroform–methanol and then with 10:1 chloroform–methanol at a flow rate[20] of 1 mL/10 s. The latter elution gave **5** (78 mg, 42%) with the same properties as the products of methods (*a*) and (*b*). The fair yield and clean reaction according to t.l.c. suggests that loss of **5** occurred because of adsorption to tin compounds prior to chromatography.

Conversion of 4-tert-*butyl Fmoc-aspartate to its benzyl ester.* — A mixture of 4-*tert*-butyl *N*-fluoren-9-ylmethoxycarbonyl-L-aspartate (**9**; 410 mg, 1 mmol; Fluka Chemical corp., Ronkonkoma, NY), $NaHCO_3$ (170 mg, 2 mmol), benzyl bromide (0.6 mL, 5 mmol), and *N,N*-dimethylformamide (5 mL) was stirred at room temperature for 24 h. After the addition of ethyl acetate (20 mL), the organic layer was washed with water (4 × 20 mL), and evaporated to dryness. The residue was treated with hexane (20 mL) and the mixture stirred vigorously until precipitation of 1-benzyl 4-*tert*-butyl *N*-fluoren-9-ylmethoxycarbonyl-L-aspartate (**10**) was complete. Filtration and washing with hexane gave **10** (465 mg, 93%), t.l.c. (4:1 toluene–ethyl acetate) R_F 0.64; ^{1}H-n.m.r. (60 MHz, $CDCl_3$): δ 8.0–7.3 (m, 13 H, arom.), 5.15 (s, 2 H, PhCH_2), 4.45 (m, 2 H, CH_2–OCOHN–), 2.95 (m, 2 H, CH_2CO$_2$Bu^t), and 1.45 [s, 9 H, C(CH_3)$_3$].

Partial deprotection of **9**. — A mixture of **9** (400 mg, 0.8 mmol), anisole (1 mL), and trifluoroacetic acid (3 mL) was stirred for 1 h at room temperature. After evaporation of the trifluoroacetic acid, the residue was applied to a short column of silica gel (30 g). Elution with 1:4 toluene–ethyl acetate, and then with 2:1 chloroform–methanol, gave fractions that were evaporated to afford 1-benzyl *N*-fluoren-9-ylmethoxycarbonyl-L-aspartate (**8**) (250 mg, 70.4%), $[\alpha]_D^{21}$ +12° (*c* 1.84, chloroform); t.l.c. R_F (10:1 chloroform–methanol) 0.51; ^{1}H-n.m.r. (60 MHz, $CDCl_3$): δ 7.8–7.1 (m, 13 H, arom.), 5.15 (s, 2 H, PhCH_2), and 2.95 (m, 2 H, CH_2CO$_2$H).

Reduction of **5** *to the glucosylamine and coupling to partially protected aspartic acid* **8**. — To a solution of **5** (100 mg, 47.6 μmol) in methanol (4 mL) was added Lindlar catalyst (5% Pd-on-$CaCO_3$, poisoned with Pb, 250 mg; Aldrich) and the mixture was hydrogenated at 0.2 MPa for 35 min at room temperature. T.l.c. showed the conversion of **5**, [t.l.c. R_F (10:1 chloroform–methanol) 0.48] into **7** (R_F 0.21), and showed evidence of *O*- to *N*-acetyl migration, but no evidence of an anomeric mixture. The catalyst was filtered off and washed with methanol, and evaporation gave the amine **7** as a colorless oil.

To compound **7** was added **8** (42 mg, 95 μmol) and a 0.21M solution of diisopropylethylamine (125 μmol) in *N,N*-dimethylformamide (0.6 mL). After being cooled to −10°, the mixture was treated with a 0.22M solution of diethyl cyanophosphonate (110 μmol) (Aldrich) in *N,N*-dimethylformamide (0.5 mL). The red-colored mixture was stirred for 2.5 h at 5° when t.l.c. (10:1 chloroform–methanol) showed the conversion of **7** (R_F 0.21) into *O*-(2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl)-(1→6)-*O*-[(2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl)-(1→3)]-*O*-(2,4-di-*O*-acetyl-α-D-mannopyranosyl)-(1→6)-*O*-[(2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl)-(1→3)]-*O*-(2,4-di-*O*-acetyl-β-D-mannopyranosyl)-(1→4)-*O*-(2-acetamido-3,6-di-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-(1→4)-2-acetamido-3,6-di-*O*-acetyl-*N*-(-benzyl 1-*N*-fluoren-9-ylmethoxycarbonyl-L-aspart-4-oyl)-2-deoxy-β-D-glucopyranosylamine (**11**) (R_F 0.51). After the addition of glacial acetic acid (0.2 mL) and ethyl acetate (30 mL), the organic phase was separated and washed with water (5 × 10 mL). Evaporation of the solvent gave a residue that was applied to five preparative t.l.c. plates, developed with 10:1 chloroform–methanol. The band containing **11** was located by u.v. irradiation and by cutting a 0.5-cm strip from each plate and spraying with the anisaldehyde reagent. Extraction of the silica gel by stirring for 10 min with 2:1 chloroform–methanol, and then washing on a filter with 1:1 chloroform–methanol, followed by evaporation of the combined filtrates, gave **11** (60 mg, 50%), $[\alpha]_D^{21}$ +12.4° (*c* 3.0, chloroform); ^{1}H-n.m.r. (400 MHz, $CDCl_3$): δ 8.0–7.3 (m, 13 H, arom.) and 2.3–1.95 (s, 66 H, 20 OCOCH_3, 2 NHCOCH_3). Because t.l.c. showed a slight contamination of **11** with other compounds, elemental analysis was performed after removal of the benzyl ester group (see compound **12**).

O-(2,3,4,6-Tetra-O-acetyl-α-D-mannopyranosyl)-(1→6)-O-[(2,3,4,6-tetra-O-acetyl-α-D-mannopyranosyl)-(1→3)]-O-(2,4-di-O-acetyl-α-D-mannopyranosyl)-(1→6)-O-[(2,3,4,6-tetra-O-acetyl-α-D-mannopyranosyl)-(1→3)]-O-(2,4-di-O-acetyl-β-D-mannopyranosyl)-(1→4)-O-(2-acetamido-3,6-di-O-acetyl-2-deoxy-β-D-glucopyranosyl)-(1→4)-2-acetamido-3,6-di-O-acetyl-2-deoxy-1-N-(N-fluoren-9-ylmethoxycarbonyl-L-aspart-4-oyl)-β-D-glucopyranosylamine (**12**). — A solution of **11** (60 mg, 24 μmol) in oxolane (1.5 mL) was treated with 10% Pd–C (60 mg, Matheson, Coleman, and Bell, Norwood, OH) and hydrogenated at 0.1 MPa for 45 min at room temperature, when t.l.c. (10:1 chloroform–methanol) showed the conversion of **11** (R_F 0.51) into **12** (R_F 0.10). (Further hydrogenation, or the use of a higher pressure caused removal of the Fmoc group.) The catalyst was filtered off and washed with methanol. Evaporation gave an oil (50 mg) that was applied to three preparative t.l.c. plates which were developed with 45:8:8 toluene–methanol–glacial acetic acid. The band containing **12** was located by u.v. irradiation, and the product was extracted from the silica gel by stirring with 1:1 chloroform–methanol, filtration, and washing with 10:10:3 chloroform–methanol–water, to give **12** (46 mg, 80%), $[\alpha]_D^{20}$ +14° (*c* 0.58, chloroform); t.l.c. (45:8:8 toluene–methanol–glacial acetic acid) R_F 0.44; ^{1}H-n.m.r. (400 MHz, $CDCl_3$): δ 8.0–7.3 (m, 8 H, arom.) and 2.21–1.96 (s, 66 H, 20 OCOCH_3, 2 NHCOCH_3).

Anal. Calc. for $C_{105}H_{134}N_4O_{60}\cdot 0.5H_2O$: C, 50.39; H, 5.80; N, 2.23. Found: C, 50.07; H, 5.75; N, 2.17.

For characterization by mass spectrometry, a sample of **12** (25 mg) was dissolved in methanol (1.5 mL) and treated with an excess (pH paper) of a 0.25% solution of sodium methoxide in methanol. The mixture was kept overnight at room temperature, treated with water (2 mL), and washed with toluene (4 × 2 mL). The aqueous methanol layer was concentrated to 0.5 mL and passed through a column (2.5 × 0.5 cm) of cation-exchange resin. The resin was washed with 1:1 methanol–water, and the combined eluates were evaporated to give **13** (11 mg, 78.6%), $[\alpha]_D^{21}$ +36° (*c* 1.05, water); t.l.c. (1:2:1 butanol–methanol–water) R_F 0.10 (a single spot); m.s.: *m/z* 1350 ($M + H^+$) and 1372 ($M + Na^+$). The mass spectrum also showed that *O*-deacetylation of **12** was not complete, showing small peaks at *m/z* 1392 ($M + COCH_3 + H^+$) and 1434 ($M + 2\ COCH_3 + H^+$). A satisfactory ^{1}H-n.m.r. spectrum of **13** was not obtained owing to instrument problems.

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