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A spacer-modified disaccharide as a photoaffinity reagent for the acceptor-binding area of bovine (1 → 4)-β-D-galactosyltransferase: comparison of its acceptor properties with those of other 2-acetamido-2-deoxy-β-D-glucopyranosides

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ABSTRACT

The spacer-modified disaccharide 1,10-di-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-2-azi-1,10-decanediol (**10**) that mimics the biantennary core heptasaccharide of *N*-glycoproteins has been synthesised. Compound **10** is an excellent acceptor in galactosyltransferase-catalysed galactosylation by UDP-galactose, is superior (7–8-fold) to analogues that have only one GlcNAc unit, and is an efficient photoaffinity reagent for galactosyltransferase. In the presence of UDP-Gal, no photoaffinity labelling by **10** takes place, which agrees with the mechanism of galactosyltransferase action.

INTRODUCTION

One of the natural acceptors for β-D-galactosylation by galactosyltransferase and UDP-Gal is the biantennary core oligosaccharide **1** with the 3-linked antenna being galactosylated five times more rapidly than the 6-linked antenna[1]. Rao et al.[2] showed that the non-galactosylated acceptor **2** has a K_M one order of magnitude less than that of the monogalactosylated glycopeptide. The question as to whether the galactosyltransferase has two separate recognition and binding subsites for the terminal GlcNAc residues, one of which is also the site of galactosylation, could be answered by differential labelling of the subsites with a suitable photolabile ligand. The high specificity of transglycosylases, not only for the donor (usually a sugar diphosphonucleotide), but also for the acceptor (glycoprotein or glycolipid), is one reason why specific covalent modification of such enzymes by active-site-directed

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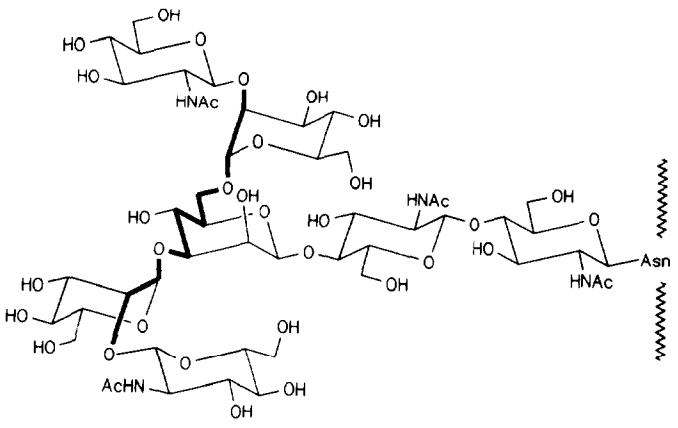
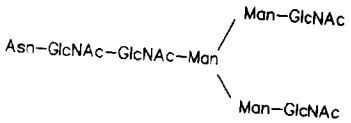


1

reagents is difficult. The problem of the total synthesis of a native donor or acceptor molecule that carries a photolabile group can be simplified by using spacer-modified oligosaccharides. Such compounds, where parts of the native oligosaccharide structure are replaced by corresponding flexible spacers, are recognised by glycanases[3], antibodies[4], or transportproteins[5]. The synthetic ligands for Gal/GalNAc receptors, described by Lee[6], are comparable to these spacer-modified oligosaccharides. A simple model (**3**) of the terminal pentasaccharide moiety of the oligosaccharidic biantennary core oligosaccharide (**1**) and its facile galactosylation by UDP-Gal and galactosyltransferase have been described[7]. Following the same concept of "shorthand synthesis", an asymmetrically placed diazirino group has been attached to the spacer in **3** to give **10**, which has potential for regiospecific covalent modification of the acceptor site of the enzyme.



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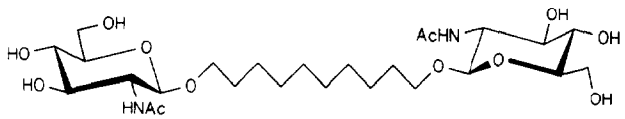

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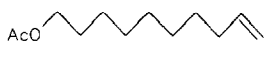
RESULTS AND DISCUSSION

Syntheses.—The shortest route between the anomeric oxygen atoms of the two terminal GlcNAc groups in **1** comprises 10 atoms and therefore a C_{10} spacer is required. In order to place a diazirino group on C-2 of the spacer, 10-acetoxy-1-decene (**4**) was epoxidised and hydrolysed to yield 10-acetoxy-1,2-decanediol (**5**), which could be converted selectively[8] into the 2-oxo derivative (**6**), the precursor for the preparation of 2-azi-1,10-decanediol (**7**). Compound **7** was di-*O*-glycosylated using 1,3,4,6-tetra-*O*-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranose[9] and $SnCl_4$ as catalyst. Deacylation[10] of the product **8**, reacetylation (→ **9**), and then *O*-deacetylation yielded the desired spacer-modified disaccharide **10**. For purposes of comparison, the 5-hydroxypentyl (**13**) and pentyl (**16**) glycosides of 2-acetamido-2-deoxy-β-D-glucopyranose were synthesised using the same glycosylation procedure (see Experimental). Enzymic galactosylation of **10** gave the mono- (**25a,b**) and di-*O*-galactosyl (**26**) derivatives; the isomers **25a** and **25b** were not separated.



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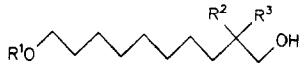
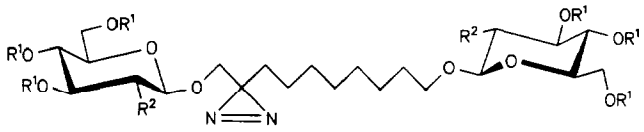


5 R^1 = Ac, R^2 = OH, R^3 = H

6 R^1 = Ac, R^2,R^3 = O

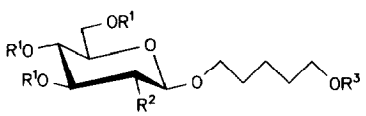
7 R^1 = H, R^2,R^3 = N=N



8 R^1 = Ac, R^2 = NPhth

9 R^1 = Ac, R^2 = HNAc

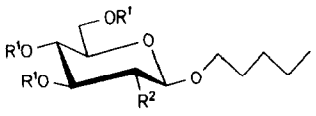
10 R^1 = H, R^2 = HNAc



11 R^1 = Ac, R^2 = NPhth, R^3 = H

12 R^1 = R^3 = Ac, R^2 = HNAc

13 R^1 = R^3 = H, R^2 = HNAc

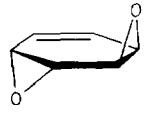


14 R^1 = Ac, R^2 = NPhth

15 R^1 = Ac, R^2 = HNAc

16 R^1 = H, R^2 = HNAc

In order to assess the effect of reducing the flexibility of the spacer on the rate of galactosylation, a spacer-modified disaccharide (**24a,b**), containing a less-flexible spacer, was synthesised. Thus, (±)-(3,4/5,6)-3,4:5,6-diepoxy-1-cyclohexene[11] (**17**) was treated with $NaOCH_2CH_2OH$ in ethylene glycol to give (±)-(1,3/2,4)-1,4-di-*O*-(2-hydroxyethyl)-5-cyclohexene-1,2,3,4-tetrol (**19**), which was purified by acetylation (→ **18**) and then deacetylation. Tritylation of the primary hydroxyl groups in **19** and then acetylation gave (±)-(1,3/2,4)-2,3-di-*O*-acetyl-1,4-di-*O*-(2-trityloxyethyl)-5-cyclohexene-1,2,3,4-tetrol (**20**). Detritylation of **20** using trifluoroacetic acid gave (±)-(1,3/2,4)-2,3-di-*O*-acetyl-1,4-di-*O*-(2-hydroxyethyl)-5-cyclohexene-1,2,3,4-tetrol (**21**) that was di-*O*-glycosylated using 3,4,6-tri-*O*-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl bromide[9] and silver trifluoromethanesulfonate as catalyst (the allyl ether groups in compound **20** were not stable in the presence of $SnCl_4$). The product, (±)-(1,3/2,4)-2,3-di-*O*-acetyl-1,4-di-*O*-[2-(3,4,6-tri-*O*-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranosyloxy)ethyl]-5-cyclohexene-1,2,3,4-tetrol (**22a,b**), was obtained in good yield. The additional chiral element introduced through the dihydroxycyclohexenyl moiety gives rise to two stereoisomers **22a** and **22b**, which were not separated, and only one isomer is depicted. Deacylation of **22a,b** with butylamine, reacetylation (→ **23a,b**), and then *O*-deacetylation yielded **24a,b**.



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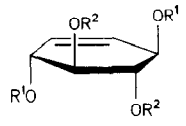
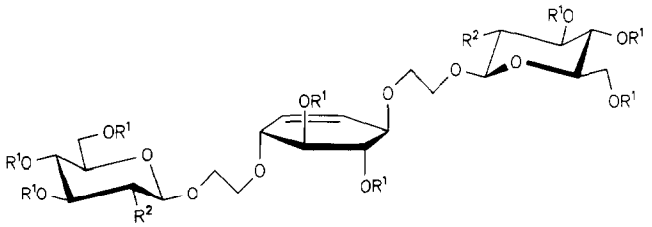


18 R^1 = $AcOCH_2CH_2$, R^2 = Ac
19 R^1 = $HOCH_2CH_2$, R^2 = H
20 R^1 = $TrOCH_2CH_2$, R^2 = Ac
21 R^1 = $HOCH_2CH_2$, R^2 = Ac



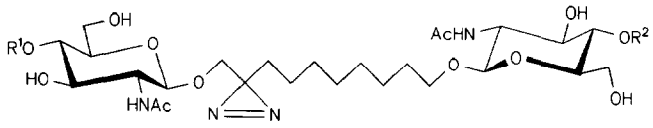
22a R^1 = Ac, R^2 = NPhth
23a R^1 = Ac R^2 = HNAc
24a R^1 = H, R^2 = HNAc

Galactosylation experiments.—The compounds in Table I were galactosylated by UDP-Gal and bovine (1 → 4)-β-D-galactosyltransferase. The relative rates were determined by competition between the acceptors and GlcNAc, using UDP-

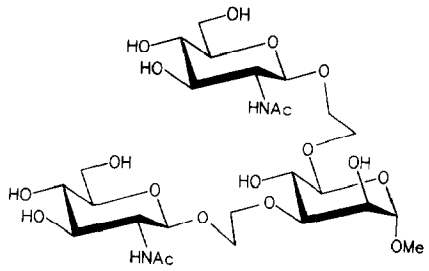
TABLE I
Relative rates of enzymic galactosylation

Relative rate	Compound	K_M [mmol]	Ref.
145	**3, 10**	0.20	
40	**25a**	0.20	
20	**16**		
18	**13**		
16	**24a**	0.28	
10	**27**	0.18	13
1	GlcNAc	8.3	16
–	**2**	0.25	2
–	Asn-GlcNAc-GlcNAc-Man(Man-GlcNAc-Gal)(Man-GlcNAc)	2.0	2

[^{14}C]Gal as the donor. Compound **10** was converted at a rate far higher than those of all of the other acceptors, including **13** and **16**, which have comparable lipophilicities[12]. The enhanced rate for **10**, especially in comparison with those of the monogalactosylated products **25a,b**, is possibly suggestive of a second GlcNAc-binding subsite. The introduction of a rigid element in the spacer, as in **24a,b**, also reduces the rate of galactosylation, as does the removal of the two α-D-Man*p* residues from the terminal pentasaccharide moiety of **1** to give **27**[13].



25a R^1 = β-D-Gal*p*, R^2 = H
25b R^1 = H, R^2 = β-D-Gal*p*
26 R^1 = R^2 = β-D-Gal*p*



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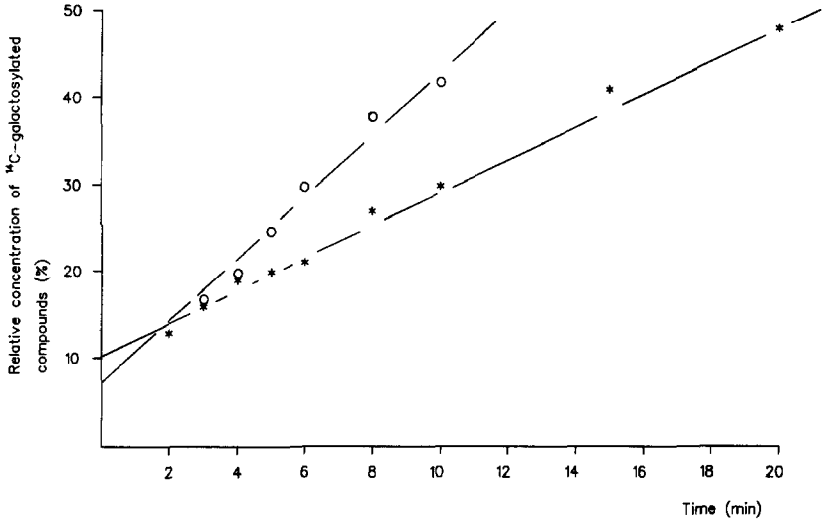

Fig. 1. Irreversible deactivation of β-D-galactosyltransferase by photoaffinity labelling with **10** (see Experimental). Mixtures prepared from 10-μL portions of buffer solutions were irradiated for 10 min at 350 nm and 25°, and the residual enzyme activity was measured by adding UDP-[^{14}C]Gal in buffer and determining the rate of [^{14}C]Gal transfer; *, enzyme + **10**; ○, enzyme + **10** + UDPGal.

Labelling experiments.—The half life of photolytic decay of **10** on irradiation in buffer with UV light of 350 nm was 3.5 min. Irradiation of the enzyme for 10 min under N_2 at ambient temperature caused no measurable loss of enzymic activity but, in the presence of **10**, there was ~ 40% irreversible deactivation (Fig. 1). The residual activity was determined by adding UDP-[^{14}C]Gal and measuring the relative rate of ^{14}C-galactosylation of the acceptors present in the mixture. The ^{14}C-galactosylated products and UDP-[^{14}C]Gal separated well in TLC (4:2:1

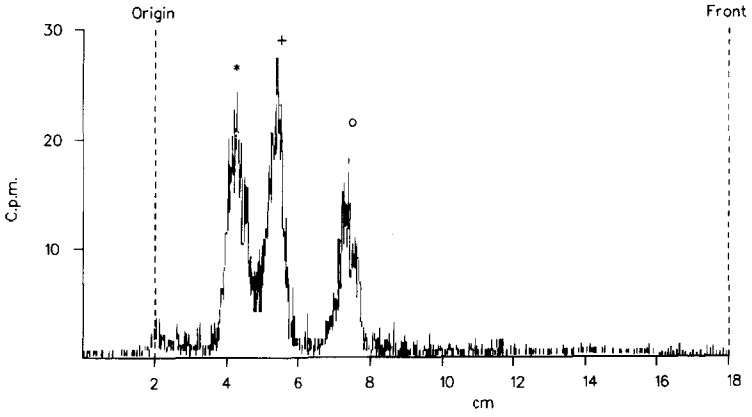

Fig. 2. TLC of ^{14}C-galactosylated products; *, UDP-[^{14}C]Gal; ○, **25a,b**; +, **26**.

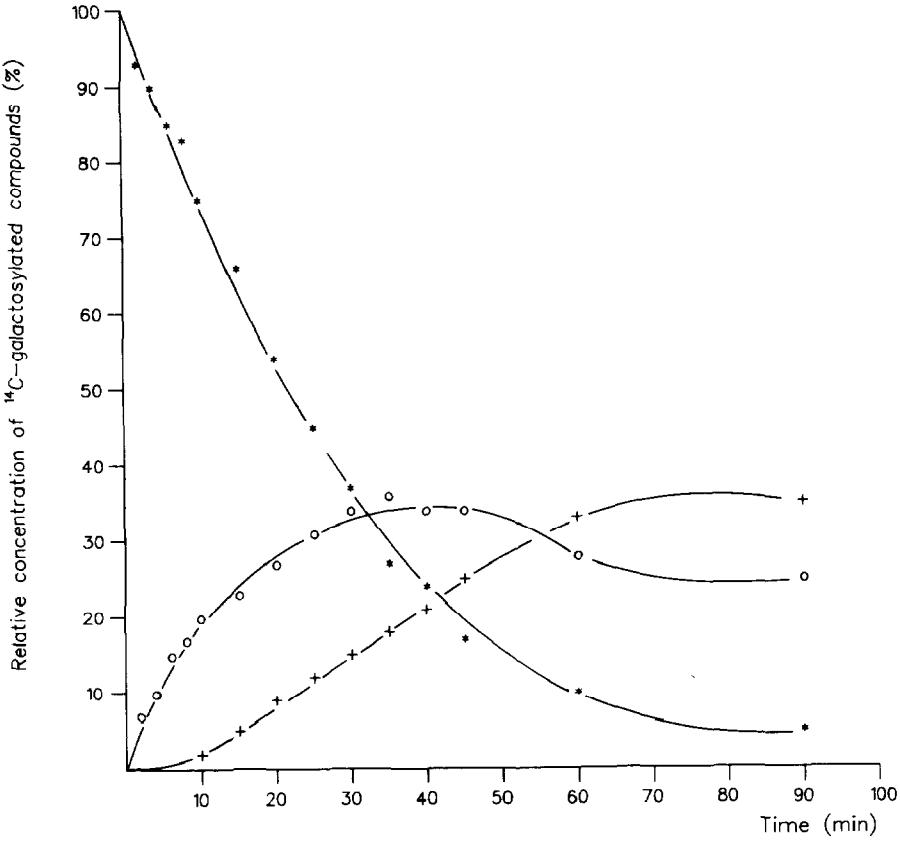

Fig. 3. The conversion of **10** into **25a,b** (○) and **26** (+) by enzymic galactosylation using UDP-[^{14}C]Gal (*).

EtOAc–MeOH–H_2O) as shown in Fig. 2, and the rates of formation are shown in Fig. 3.

In the presence of unlabelled UDP-Gal, the enzyme is protected and does not lose any activity (Fig. 1). This agrees well with the ordered transfer mechanism of the enzyme[14]. Fragmentation of the enzyme following inactivation with radiolabelled **10** should give a clue as to the presence of two separate binding subsites for GlcNAc, and this work is in progress.

EXPERIMENTAL

General.—All reactions were monitored by TLC on Silica Gel 60 F_{254} (Merck). Scanning for radioactivity after TLC was carried out with a Berthold TLC-Linear Analyzer LB 282. Column chromatography by the flash method[15] was performed on ICN-silica 32-63 (ICN Biomedicals). Size-exclusion chromatography was performed on a column (2.5 × 85 cm) of Bio-Gel P-2 (BioRad, −400 mesh) by elution

with water at 10 mL/h. Melting points were measured with a Büchi apparatus and are uncorrected. Optical rotations were obtained with a Schmidt & Haensch Polartronic I polarimeter. Photolysis of **10** was performed with a Rayonet-RPR-100-reactor equipped with 16 RPR 3500 A lamps. UV spectra were recorded with a Zeiss PMQ II spectrophotometer. ^{1}H NMR spectra (250 MHz) were recorded with a Bruker WM 250 spectrometer for solutions in $CDCl_3$ (internal Me_4Si). Elemental analyses were obtained with a Perkin–Elmer 240 analyser.

β-D-Galactosyltransferase (UDP-galactose : N-acetylglucosamine 4-β-D-galactosyltransferase; EC 2.4.1.22) from bovine milk (4 U/mg) was purchased from Sigma, UDP-Gal from Fluka, and UDP-[^{14}C]Gal (300 mCi/mmol) from Amersham-Buchler.

10-Acetoxy-1-decene (**4**).—A solution of 9-decen-1-ol (20 g, 128 mmol) in pyridine (160 mL) and acetic anhydride (60 mL) was stirred overnight at 25°, then concentrated in vacuo, and toluene was distilled several times from the residue which was filtered through silica gel (EtOAc). The solution was concentrated and the residue was distilled to yield **4** (22.6 g, 89%) as a colourless oil; R_F 0.6 (3 : 1 cyclohexane–EtOAc); bp 73–74°/0.01 torr. ^{1}H NMR data ($CDCl_3$): δ 1.31 (m, 10 H, 5 CH_2), 1.62 (m, 2 H, CH_2), 2.04 (m, 5 H, $CH_2CH{=}CH_2$ and OAc), 4.06 (t, 2 H, *J* 7 Hz, CH_2OAc), 4.92–5.01 (m, 2 H, $CH_2CH{=}CH_2$), 5.71–5.90 (m, 1 H, $CH{=}CH_2$).

Anal. Calcd for $C_{12}H_{22}O_2$: C, 72.68; H, 11.18. Found: C, 72.88; H, 11.34.

10-Acetoxy-1,2-decanediol (**5**).—Aqueous 30% H_2O_2 (14 mL) was dropped slowly into a stirred solution of **4** (10 g, 50 mmol) in formic acid (100 mL) at 0° and stirring was continued for 10 h at 25°. The solution was concentrated in vacuo to give the partially formed formyl esters (R_F 0.7, 0.53, 0.47; 1 : 2 cyclohexane–EtOAc) that were then dissolved in MeOH (150 mL), and the pH was adjusted to 7.5–8 by adding methanolic NH_3. The solution was stirred for 5 h at 25°, then concentrated in vacuo. Flash-column chromatography (1 : 2 cyclohexane–EtOAc) of the residue gave **5**, isolated as a colourless oil (10.7 g, 92%); R_F 0.16. ^{1}H NMR data ($CDCl_3$): δ 1.25–1.50 (m, 14 H, 7 CH_2), 1.62 (m, 2 H, CH_2), 2.06 (s, 3 H, OAc), 3.17 (s, 2 H, 2 OH), 3.42 (dd, 1 H, *J* 7 and 10.5 Hz, $CHOH$), 3.60–3.69 (m, 2 H, CH_2OH), 4.06 (t, 2 H, *J* 7 Hz, CH_2OAc).

Anal. Calcd for $C_{12}H_{24}O_4$: C, 62.07; H, 10.34. Found: C, 62.07; H, 10.59.

10-Acetoxy-1-hydroxy-2-decanone (**6**).—To a stirred mixture of **5** (5 g, 21.55 mmol) and acetic acid (15 mL) was added aq 15% NaOCl (20 mL, 32.3 mmol) dropwise during 20 min, and stirring was continued for 3 h. Water (100 mL) was added, the mixture was extracted with $CHCl_3$ (3 × 30 mL), and the extracts were combined, dried (Na_2SO_4), and concentrated in vacuo. Flash-column chromatography (3 : 1 cyclohexane–EtOAc) of the residue gave **6**, isolated as a colourless oil (4.5 g, 90.8%), R_F 0.5 (1 : 2 cyclohexane–EtOAc); bp 127–128°/0.01 torr. ^{1}H NMR data ($CDCl_3$): δ 1.34 (m, 8 H, 4 CH_2), 1.63 (m, 4 H, 2 CH_2), 2.06 (s, 3 H, OAc), 2.45 (t, 2 H, $J_{3,4}$ 7.5 Hz, H-3,3), 3.27 (s, 1 H, OH), 4.06 (t, 2 H, $J_{9,10}$ 6.8 Hz, H-10,10), 4.25 (s, 2 H, H-1,1).

Anal. Calcd for $C_{12}H_{22}O_4$: C, 62.60; H, 9.57. Found: C, 62.32; H, 9.70.

2-Azi-1,10-decanediol (**7**).—Dry NH_3 was condensed into a solution of **6** (5.0 g, 21.7 mmol) in dry MeOH (200 mL) at −30° until the volume increased by ~20%. A solution of hydroxylamine-*O*-sulfonic acid (4.2 g, 37.1 mmol) in dry MeOH (50 mL) was added dropwise, stirring at −30° was continued for 2 h, and the mixture was then allowed to attain 25° overnight. Only one product could be detected by TLC (R_F 0.09, 2:1 cyclohexane–EtOAc). The mixture was filtered, concentrated in vacuo to ~25 mL, then diluted with dry MeOH to 50 mL, and triethylamine (8 mL) was added. The diaziridine was oxidised by adding I_2 at 0° until the colour persisted for at least 30 min, and the mixture was then stirred at 25° for 30 min and concentrated in vacuo. A solution of the residue in CH_2Cl_2 was washed with satd aq Na_2SO_3 (2 × 100 mL) and water (100 mL), dried (Na_2SO_4), and concentrated in vacuo. The resulting yellow oil was *O*-deacetylated (Zemplén). Flash-column chromatography (2:1 cyclohexane–EtOAc) of the product gave **7** (3 g, 69%) isolated as a light-yellow oil; R_F 0.35; λ_{max} 345 nm (ϵ 56). ^{1}H NMR data ($CDCl_3$): δ 3.63 (t, 2 H, *J* 7 Hz, CH_2OH), 3.48 (s, 2 H, $HOCH_2CN_2$), 2.17 (m, 2 H, 2 OH), 1.56 (t, 2 H, CH_2), 1.42 (q, 2 H, CH_2), 1.27 (m, 8 H, 4 CH_2), 1.13 (m, 2 H, CH_2).

Anal. Calcd for $C_{10}H_{20}N_2O_2$: C, 59.97; H, 10.06; N, 13.98. Found: C, 59.34; H, 10.02; N, 14.27.

*2-Azi-1,10-di-*O*-(3,4,6-tri-*O*-acetyl-2-deoxy-2-phthalimido-β-*D*-glucopyranosyl)-1,10-decanediol* (**8**).—To a solution of 1,3,4,6-tetra-*O*-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranose[9] (7.16 g, 15 mmol) in CH_2Cl_2 (200 mL) was added $SnCl_4$ (1.08 mL, 9.2 mmol) under anhydrous conditions. The solution was stirred for 15 min at 25°, **7** (2.0 g, 5.0 mmol) was added, and stirring was continued for 6 h. Ice-cold water (200 mL) was added, the suspension was stirred at 25° until the organic layer cleared (20 min), the aqueous layer was extracted with CH_2Cl_2 (2 × 60 mL), and the combined organic layers were washed with satd aq $NaHCO_3$ (200 mL), dried (Na_2SO_4), and concentrated in vacuo. Flash-column chromatography (1:1 cyclohexane–EtOAc) of the residue yielded a colourless oil (4.2 g, 81.2%), which was crystallised from EtOH to give **8** (3.7 g, 71.5%); R_F 0.19 (1:1 cyclohexane–EtOAc); mp 141–142°, $[\alpha]_D^{23}$ + 19° (*c* 1, $CHCl_3$); λ_{max} 345 nm (ϵ 57). ^{1}H NMR data ($CDCl_3$): δ 0.74 (s, 4 H, 2 CH_2), 0.94 (m, 4 H, 2 CH_2), 1.08–1.44 (m, 6 H, 3 CH_2), 1.88 (s, 6 H, 2 OAc), 2.04 (s, 6 H, 2 OAc), 2.12 (s, 6 H, 2 OAc), 3.31 (d, 1 H, *J* 12 Hz, 0.5 OCH_2CN_2), 3.42 (dt, 1 H, *J* 6 Hz, 0.5 OCH_2), 3.52 (d, 1 H, *J* 12 Hz, 0.5 OCH_2CN_2), 3.77–3.94 (m, 3 H, $J_{4,5}$ 10.5, $J_{5,6a}$ 3, $J_{5,6b}$ 4.5 Hz, 0.5 OCH_2 and 2 H-5), 4.17 (ddd, 2 H, $J_{6a,6b}$ 12 Hz, 2 H-6a), 4.32 (m, 4 H, $J_{1,2}$ 8.7, $J_{2,3}$ 10.5 Hz, 2 H-2 and 2 H-6b), 5.18 (ddd, 2 H, $J_{3,4}$ 9 Hz, 2 H-4), 5.35 (dd, 2 H, 2 H-1), 5.81 (ddd, 2 H, 2 H-3), 7.76 (m, 4 H, aromatic), 7.86 (m, 4 H, aromatic).

Anal. Calcd for $C_{50}H_{58}N_4O_{20}$: C, 58.02; H, 5.6; N, 5.4. Found: C, 58.18; H, 5.54; N, 5.11.

*1,10-Di-*O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-*D*-glucopyranosyl)-2-azi-1,10-decanediol* (**9**).—To a suspension of **8** (1 g, 0.97 mmol) in 6:1 2-propanol–H_2O (56 mL) was added $NaBH_4$ (733 mg, 19.4 mmol) in 4 lots during 8 h. The mixture

was stirred for 24 h at 25° when one product (R_F 0.54) was detectable by TLC (7:2:1 EtOAc–MeOH–H_2O). The mixture was neutralised (pH 6) with glacial acetic acid, and the solution was boiled under reflux for 1–1.5 h, then concentrated in vacuo. Toluene (3 × 10 mL) was distilled from the residue, which was then acetylated in the usual way with acetic anhydride (10 mL) in pyridine (30 mL) for 16 h to give one product; R_F 0.32 (5:1 toluene–EtOH). Flash-column chromatography (10:1 toluene–EtOH) then afforded a colourless solid (700 mg), which was crystallised from EtOAc to give **9** (480 mg, 58%); mp 168°, $[\alpha]_D^{23}$ −14° (*c* 1, $CHCl_3$); λ_{max} 330 nm (ϵ 51). ^{1}H NMR data ($CDCl_3$): δ 1.08 (m, 2 H, CH_2), 1.25 (m, 8 H, 4 CH_2), 1.43 (m, 2 H, CH_2), 1.55 (m, 2 H, CH_2), 1.97 (s, 3 H, NAc), 1.98 (s, 3 H, NAc), 2.03 (m, 12 H, 4 OAc), 2.1 (s, 6 H, 2 OAc), 3.36 (d, 1 H, *J* 12 Hz, 0.5 OCH_2N_2), 3.47 (dt, 1 H, *J* 6 Hz, 0.5 OCH_2), 3.63 (d, 1 H, *J* 12 Hz, 0.5 OCH_2N_2), 3.72 (m, 2 H, $J_{4,5}$ 9.75, $J_{5,6a}$ 2.25, $J_{5,6b}$ 4.5 Hz, 2 H-5), 3.85 (m, 3 H, $J_{1,2}$ 8.7, $J_{2,3}$ 9.0, $J_{6a,6b}$ 12 Hz, 2 H-2 and 0.5 OCH_2), 4.13 (dd, 2 H, 2 H-6a), 4.27 (m, 2 H, 2 H-6b), 4.69 (dd, 2 H, 2 H-1), 5.06 (ddd, 2 H, $J_{3,4}$ 9.75 Hz, 2 H-4), 5.31 (ddd, 2 H, 2 H-3), 6.01 (d, 1 H, *H*NAc), 6.11 (d, 1 H, *H*NAc).

Anal. Calcd for $C_{38}H_{58}N_4O_{18}$: C, 53.09; H, 6.80; N, 6.60. Found: C, 52.75; H, 6.79; N, 6.05.

*1,10-Di-*O*-(2-acetamido-2-deoxy-β-*D*-glucopyranosyl)-2-azi-1,10-decanediol* (**10**). —To a solution of **9** (100 mg, 0.11 mmol) in dry MeOH (20 mL) at 25° was added methanolic M NaOMe (~ 4 drops). After 2 h, the reaction was complete, as shown by TLC; R_F 0.35 (7:2:1 EtOAc–MeOH–H_2O). The solution was filtered through silica gel, using MeOH, and the filtrate was concentrated in vacuo. Chromatography of the residue on Bio-Gel P-2 gave **10** (62 mg, 93%); $[\alpha]_D^{23}$ −29° (*c* 1, H_2O); λ_{max} 342 nm (ϵ 44); $t_{1/2}$ 3.5 min (decay on irradiation at 350 nm).

*5-Hydroxypentyl 3,4,6-tri-*O*-acetyl-2-deoxy-2-phthalimido-β-*D*-glucopyranoside* (**11**).—A solution of 1,3,4,6-tetra-*O*-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranose[9] (10 g, 21 mmol) and $SnCl_4$ (9.5 g, 80 mmol) in CH_2Cl_2 (500 mL) was treated with 1,5-pentanediol (4.4 g, 42 mmol), as described for **8**. Flash-column chromatography (1:2 cyclohexane–EtOAc) of the product yielded a colourless oil (7 g) which crystallised from EtOH to give **11** (5 g, 46%); R_F 0.18; mp 100°, $[\alpha]_D^{23}$ +21° (*c* 1.1, $CHCl_3$). ^{1}H NMR data ($CDCl_3$): δ 1.18 (m, 2 H, CH_2), 1.29–1.55 (m, 4 H, 2 CH_2), 1.88 (s, 3 H, OAc), 2.04 (s, 3 H, OAc), 2.13 (s, 3 H, OAc), 3.35 (t, 2 H, *J* 6 Hz, C*H*$_2$OH), 3.47 (dt, 1 H, *J* 6 and 9.75 Hz, 0.5 OCH_2), 3.86 (m, 2 H, $J_{4,5}$ 10.5, $J_{5,6a}$ 3, $J_{5,6b}$ 4.5 Hz, H-5 and 0.5 OCH_2), 4.18 (dd, 1 H, $J_{6a,6b}$ 12 Hz, H-6a), 4.33 (m, 2 H, $J_{1,2}$ 8.7, $J_{2,3}$ 10.5 Hz, H-2,6b), 5.18 (dd, 1 H, $J_{3,4}$ 9 Hz, H-4), 5.36 (d, 1 H, H-1), 5.8 (dd, 1 H, H-3), 7.76 (m, 2 H, aromatic), 7.87 (m, 2 H, aromatic).

Anal. Calcd. for $C_{25}H_{31}NO_{11}$: C, 57.88; H, 5.95; N, 2.69. Found: C, 57.32; H, 5.94; N, 2.56.

*5-Acetoxypentyl 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-*D*-glucopyranoside* (**12**). —To a solution of **11** (2 g, 3.8 mmol) in EtOH (100 mL) was added $BuNH_2$ (20 mL), the solution was boiled under reflux for 24 h and then concentrated in vacuo, and MeOH (2 × 50 mL) and then toluene (2 × 50 mL) were evaporated from the

residue. Pyridine (40 mL) and Ac_2O (20 mL) were added at 0°, and the mixture was left at 25° overnight. When the reaction was complete as shown by TLC [R_F 0.38 (5:1 toluene–EtOH)], the mixture was concentrated in vacuo. Flash-column chromatography (1:5 cyclohexane–EtOAc) of the residue gave **12**, isolated as a colourless oil (1.6 g, 88%); $[\alpha]_D^{23}$ −16° (*c* 1, $CHCl_3$). ^1H NMR data ($CDCl_3$): δ 1.42 (m, 2 H, CH_2), 1.63 (m, 4 H, 2 CH_2), 1.95 (s, 3 H, OAc), 2.03 (m, 9 H, 2 OAc and NAc), 2.09 (s, 3 H, OAc), 3.49 (dt, 1 H, *J* 9 Hz, 0.5 OCH_2), 3.72 (m, 1 H, $J_{4,5}$ 9.75, $J_{5,6a}$ 2.25, $J_{5,6b}$ 4.5 Hz, H-5), 3.87 (m, 2 H, $J_{1,2}$ 8.7, $J_{2,3}$ 9 Hz, 0.5 OCH_2 and H-2), 4.06 (t, 2 H, *J* 6.75 Hz, CH_2OAc), 4.13 (dd, 1 H, $J_{6a,6b}$ 12 Hz, H-6a), 4.28 (dd, 1 H, H-6b), 4.7 (d, 1 H, H-1), 5.07 (dd, 1 H, $J_{3,4}$ 9.75 Hz, H-4), 5.32 (dd, 1 H, H-3), 5.95 (d, 1 H, *H*NAc).

*5-Hydroxypentyl 2-acetamido-2-deoxy-β-*D*-glucopyranoside* (**13**).—Compound **12** (1.6 g, 3.36 mmol) was *O*-deacetylated as described for **10**. Flash-column chromatography (7:2:1 EtOAc–MeOH–H_2O) of the product yielded a colourless oil (1 g) which crystallised from EtOH to give **13** (600 mg, 58%); R_F 0.33; mp 123°; $[\alpha]_D^{23}$ −18° (*c* 1, H_2O).

Anal. Calcd for $C_{13}H_{25}NO_7$: C, 50.80; H, 8.2; N, 4.56. Found: C, 49.49; H, 8.21; N, 4.41.

*Pentyl 3,4,6-tri-*O*-acetyl-2-deoxy-2-phthalimido-β-*D*-glucopyranoside* (**14**).—To a solution of 1,3,4,6-tetra-*O*-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranose[9] (2 g, 4.12 mmol) in dry CH_2Cl_2 (50 mL) was added $SnCl_4$ (0.76 mL, 6.18 mmol) under anhydrous conditions. After 15 min, 1-pentanol (544 mg, 6.18 mmol) was added and the reaction was continued as described for **8**. Flash-column chromatography (2:1 cyclohexane–EtOAc) of the product yielded a colourless oil (2 g) which was crystallised from EtOH to give **14** (1.6 g, 76.8%); R_F 0.20; mp 93°; $[\alpha]_D^{23}$ +23° (*c* 1, $CHCl_3$). ^1H NMR data ($CDCl_3$): δ 0.59 (t, 3 H, CH_2CH_3), 1.05 (m, 4 H, 2 CH_2), 1.42 (m, 2 H, CH_2), 1.88 (s, 3 H, OAc), 1.04 (s, 3 H, OAc), 2.11 (s, 3 H, OAc), 3.43 (dt, 1 H, *J* 6.3 and 9.8 Hz, 0.5 OCH_2), 3.87 (m, 2 H, $J_{4,5}$ 10.5, $J_{5,6a}$ 3, $J_{5,6b}$ 4.5 Hz, H-5 and 0.5 OCH_2), 4.18 (dd, 1 H, $J_{6a,6b}$ 12 Hz, H-6a), 4.35 (m, 2 H, $J_{1,2}$ 8.7, $J_{2,3}$ 10.5 Hz, H-2,6b), 5.18 (dd, 1 H, $J_{3,4}$ 9 Hz, H-4), 5.36 (d, 1 H, H-1), 5.88 (dd, 1 H, H-3), 7.74 (m, 2 H, aromatic), 7.87 (m, 2 H, aromatic).

Anal. Calcd for $C_{25}H_{31}NO_{10}$: C, 59.39; H, 6.18; N, 2.77. Found: C, 59.19; H, 6.11; N, 2.71.

*Pentyl 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-*D*-glucopyranoside* (**15**).—To a solution of **14** (500 mg, 0.99 mmol) in EtOH (50 mL) was added $BuNH_2$, and the solution was treated as described for **12**. The product was crystallised from 1:2 cyclohexane–EtOAc to give **15** (250 mg, 64.6%); R_F 0.2; mp 132°; $[\alpha]_D^{23}$ −16° (*c* 1, $CHCl_3$). ^1H NMR data ($CDCl_3$): δ 0.89 (t, 3 H, CH_2CH_3), 1.31 (m, 4 H, 2 CH_2), 1.57 (m, 2 H, CH_2), 1.96 (s, 3 H, OAc), 2.04 (d, 6 H, OAc and NAc), 2.1 (s, 3 H, OAc), 3.48 (dt, 1 H, *J* 6.3 and 9.8 Hz, 0.5 OCH_2), 3.71 (m, 1 H, $J_{4,5}$ 9.75, $J_{5,6a}$ 2.25, $J_{5,6b}$ 4.5 Hz, H-5), 3.86 (m, 2 H, $J_{1,2}$ 8.7, $J_{2,3}$ 9.0, $J_{6a,6b}$ 12 Hz, H-2 and 0.5 OCH_2), 4.14 (dd, 1 H, H-6a), 4.28 (dd, 1 H, H-6b), 4.7 (d, 1 H, H-1), 5.07 (dd, 1 H, $J_{3,4}$ 9.75 Hz, H-4), 5.33 (dd, 1 H, H-3), 5.73 (d, 1 H, *H*NAc).

Anal. Calcd for $C_{19}H_{31}NO_9$: C, 54.67; H, 7.48; N, 3.35. Found: C, 54.31; H, 7.39; N, 3.28.

Pentyl 2-acetamido-2-deoxy-β-D-glucopyranoside (**16**).—A solution of **15** (150 mg, 0.39 mmol) in MeOH (20 mL) was *O*-deacetylated as described for **10**. Chromatography of the product on Bio-Gel P-2 yielded **16** as an amorphous solid (78 mg, 71%); R_F 0.14 (27:2:1 EtOAc–MeOH–H_2O); $[\alpha]_D^{23}$ −26° (*c* 1, H_2O).

(±)-(1,3 / 2,4)-1,4-Di-O-(2-acetoxyethyl)-2,3-di-O-acetyl-5-cyclohexene-1,2,3,4-tetrol (**18**).—Under anhydrous conditions, Na (1.66 g, 72 mmol) was added to ethylene glycol (40 mL) at 0° with stirring. When all the Na had reacted (~ 2 h), (±)-(3,4/5,6)-3,4:5,6-diepoxy-1-cyclohexene[11] (**17**; 4 g, 36.4 mmol) was added portionwise during 1 h. Stirring was continued at 50° and the reaction was monitored by TLC (7:2:1 EtOAc–MeOH–H_2O). After 1 h, **17** had reacted to give a product with R_F 0.42. The solution was concentrated and xylene was distilled several times from the residue which was acetylated in the usual manner with 2:1 pyridine–acetic anhydride (100 mL) for 6 h. Flash-column chromatography (1:1 cyclohexane–EtOAc) of the product gave syrupy **18** (10.4 g, 71%); R_F 0.53 (1:2 cyclohexane–EtOAc). ^{1}H NMR data ($CDCl_3$): δ 1.97 (s, 12 H, 4 OAc), 3.63 (m, 4 H, 2 CH_2), 4.06 (m, 4 H, 2 CH_2), 4.14 (dd, 2 H, *J* 5.25 and 3 Hz, H-3,6), 5.1 (dd, 2 H, *J* 5.25 and 3 Hz, H-4,5), 5.67 (s, 2 H, H-1,2).

(±)-(1,3 / 2,4)-1,4-Di-O-(2-hydroxyethyl)-5-cyclohexene-1,2,3,4-tetrol (**19**).—A solution of **18** (10.4 g, 25.9 mmol) in MeOH (100 mL) was *O*-deacetylated as described for **10**. Flash-column chromatography (7:2:1 EtOAc–MeOH–H_2O) of the product yielded **19** as a colourless oil (5.8 g, 95.6%), R_F 0.46.

(±)-(1,3 / 2,4)-2,3-Di-O-acetyl-1,4-di-O-(2-triphenylmethoxyethyl)-5-cyclohexene-1,2,3,4-tetrol (**20**).—To a solution of **19** (6 g, 24.7 mmol) in pyridine (100 mL) was added chlorotriphenylmethane (20.6 g, 73.9 mmol) at 0°. After 30 min, the temperature was allowed to increase to 25° and the solution was then stirred overnight at 50°. TLC (2:1 cyclohexane–EtOAc) then revealed that **19** had reacted completely to give a main product (R_F 0.23). Acetic anhydride (50 mL) was added, and the mixture was stirred for 6 h at 25°, poured into ice-cold water (1 L), and stirred for 2 h. The mixture was extracted with CH_2Cl_2 (3 × 200 mL), and the extracts were combined, washed with satd aq $NaHCO_3$ (50 mL), dried (Na_2SO_4), and concentrated. Flash-column chromatography (5:1 cyclohexane–EtOAc) of the residue gave a colourless oil (16.4 g) that crystallised from EtOH to yield **20** (13.8 g, 70%); R_F 0.54 (2:1 cyclohexane–EtOAc); mp 148°. ^{1}H NMR data ($CDCl_3$): δ 1.99 (s, 6 H, 2 OAc), 3.20 (m, 4 H, 2 CH_2), 3.67 (m, 4 H, 2 CH_2), 4.30 (dd, 2 H, *J* 3.75 and 8.25 Hz, H-3,6), 5.27 (dd, 2 H, *J* 3.75 and 8.25 Hz, H-4,5), 5.85 (s, 2 H, H-1,2), 7.18–7.46 (m, 30 H, 6 Ph).

Anal. Calcd for $C_{52}H_{50}O_8$: C, 77.78; H, 6.27. Found: C, 78.19; H, 6.24.

(±)-(1,3 / 2,4)-2,3-Di-O-acetyl-1,4-di-O-(2-hydroxyethyl)-5-cyclohexene-1,2,3,4-tetrol (**21**).—To a solution of **20** (4 g, 5.0 mmol) in $CHCl_3$ (25 mL) was added trifluoroacetic acid (0.5 mL), and the solution was stirred for 15 min at 25°, then concentrated. Flash-column chromatography (5:1 toluene–EtOH) of the residue

yielded a colourless oil (1.4 g, 88%) that was crystallised from EtOH–Et_2O to give **21**; R_F 0.30; mp 82°. ^1H NMR data ($CDCl_3$): δ 2.1 (6 H, 2 OAc), 2.35 (s, 2 H, 2 OH), 3.57 (m, 2 H, CH_2), 3.69 (m, 6 H, 3 CH_2), 4.24 (dd, 2 H, *J* 3 and 5.25 Hz, H-3,6), 5.2 (dd, 2 H, *J* 3 and 5.25 Hz, H-4,5), 5.83 (s, 2 H, H-1,2).

Anal. Calcd for $C_{14}H_{22}O_8$: C, 52.82; H, 6.96. Found: C, 52.67; H, 6.94.

*(±)-(1,3 / 2,4)-1,4-Di-*O*-[2-(3,4,6-tri-*O*-acetyl-2-deoxy-2-phthalimido-β-*D*-glucopyranosyloxy)ethyl]-5-cyclohexene-1,2,3,4-tetrol* (**22a,b**).—To a solution of **21** (600 mg, 1.89 mmol) in dry CH_2Cl_2 (100 mL) were added silver trifluoromethanesulfonate (1.46 mg, 5.67 mmol) and molecular sieves (4A), and the suspension was stirred for 1 h at 25°, then cooled to −20°. A solution of 3,4,6-tri-*O*-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl bromide[9] (2.8 g, 5.67 mmol) and *sym*-collidine (0.75 mL, 5.67 mmol) in dry CH_2Cl_2 (20 mL) was added dropwise. The mixture was stirred at −20° for 1 h, then left overnight at 25°, filtered, and concentrated in vacuo. Flash-column chromatography (1:2 cyclohexane–EtOAc) of the residue yielded a colourless oil (1.9 g, 88%) which was crystallised from 1:2 cyclohexane–EtOAc to give **22a,b** (507 mg); R_F 0.24; $[\alpha]_D^{23}$ +19° (*c* 1, $CHCl_3$). ^1H NMR data ($CDCl_3$): δ 1.88 (s, 6 H, 2 OAc), 1.98 (s, 6 H, 2 OAc), 2.04 (s, 6 H, 2 OAc), 2.11 (s, 6 H, 2 OAc), 3.46 (m, 4 H, 2 CH_2), 3.57 (m, 2 H, OCH_2), 3.74–3.95 (m, 6 H, $J_{4',5'}$ 10.5, $J_{5',6'a}$ 3, $J_{5',6'b}$ 4.5 Hz, 2 H-5′, OCH_2, and H-3,6), 4.18 (dd, 2 H, $J_{6'a,6'b}$ 12 Hz, 2 H-6′a), 4.32 (m, 4 H, $J_{1',2'}$ 8.7, $J_{2',3'}$ 10.5 Hz, 2 H-2′ and 2 H-6′b), 4.83 (ddd, 2 H, *J* 2.5 and 6 Hz, H-4,5), 5.17 (s, 1 H, H-1), 5.18 (dd, 2 H, $J_{3',4'}$ 9 Hz, 2 H-4′), 5.32 (s, 1 H, H-2), 5.40 (dd, 2 H, H-1′), 5.8 (ddd, 2 H, 2 H-3′), 7.76 (m, 4 H, aromatic), 7.87 (m, 4 H, aromatic).

Anal. Calcd for $C_{54}H_{60}N_2O_{26}$: C, 56.25; H, 5.24; N, 2.43. Found: C, 56.23; H, 5.26; N, 2.42.

*(±)-(1,3 / 2,4)-1,4-Di-*O*-[2-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-*D*-glucopyranosyloxy)ethyl]-5-cyclohexene-1,2,3,4-tetrol* (**23a,b**).—Compound **22a,b** (1.4 g, 1.2 mmol) was treated with $BuNH_2$ (20 mL) in EtOH (100 mL), as described for **12**. Flash-column chromatography (27:2:1 EtOAc–MeOH–H_2O) of the product yielded a colourless oil which was crystallised from EtOH to give **23a,b** (930 mg, 79.3%); R_F 0.27; mp 161°; $[\alpha]_D^{23}$ −19° (*c* 1, $CHCl_3$). ^1H NMR data ($CDCl_3$): δ 1.96 (s, 6 H, 2 OAc), 2.04 (s, 12 H, 2 OAc and 2 NAc), 2.1 (s, 12 H, 4 OAc), 3.67 (m, 9 H, $J_{4',5'}$ 9.75, $J_{5',6'a}$ 4.5, $J_{5',6'b}$ 2.25 Hz, 3 CH_2, 2 H-5′ and 0.5 OCH_2), 3.9 (m, 3 H, $J_{1',2'}$ 8.25, $J_{2',3'}$ 9 Hz, 2 H-2′ and 0.5 OCH_2), 4.13 (dt, 2 H, $J_{6'a,6'b}$ 12 Hz, 2 H-6′a), 4.18 (m, 2 H, 2 H-6′b), 4.28 (ddd, 2 H, H-3,6), 4.71 (d, 1 H, H-1′), 4.90 (d, 1 H, H-1′), 5.07 (ddd, 2 H, $J_{3',4'}$ 10 Hz, 2 H-4′), 5.16 (m, 2 H, H-4,5), 5.28 (dd, 1 H, H-3′), 5.5 (dd, 1 H, H-3′), 5.8 (d, 2 H, H-1,2), 6.07 (d, 1 H, N*H*Ac), 6.21 (d, 1 H, N*H*Ac).

Anal. Calcd for $C_{42}H_{60}N_2O_{24}$: C, 51.64; H, 6.2; N, 2.87. Found: C, 51.12; H, 6.11; N, 2.89.

*(±)-(1,3 / 2,4)-1,4-Di-*O*-[2-(2-acetamido-2-deoxy-β-*D*-glucopyranosyloxy)ethyl]-5-cyclohexene-1,2,3,4-tetrol* (**24a,b**).—A solution of **23a,b** (150 mg, 0.15 mmol) in MeOH (20 mL) was *O*-deacetylated as described for **10**. Flash-column chromatog-

raphy (4:2:1 EtOAc–MeOH–H_2O) of the product and then chromatography on Bio-Gel P-2 gave **24a,b**, isolated as an amorphous solid (90 mg, 93%); R_F 0.22 (3:2:1 EtOAc–MeOH–H_2O), $[\alpha]_D^{23}$ −32° (*c* 1, H_2O).

Galactosylation of **10** *by β-D-galactosyltransferase.*—(*a*) To a solution of **10** (0.6 mg, 1 μmol) and UDP-Gal (1.22 mg, 2 μmol) in 50 mM Tris-HCl (100 μL, pH 7.4, 10 mM $MnCl_2$, 150 mM NaCl) was added 85.6 μM UDP-[^{14}C]Gal (40 μL, 3.42 nmol). The reaction was started by adding β-D-galactosyltransferase (0.1 mg in 10 μL of the same buffer) at 37°. The reaction was monitored by TLC (4:2:1 EtOAc–MeOH–H_2O). The radioactive UDP-Gal (R_F 0.14), monogalactosylated **25a,b** (R_F 0.34), and digalactosylated **26** (R_F 0.21) separated well and could be determined quantitatively by scanning (see Figs. 2 and 3).

(*b*) To a solution of **10** (6.02 mg, 10 μmol) and UDP-Gal (12 mg, 20 μmol) in the above-mentioned buffer (1 mL), was added galactosyltransferase (0.1 mg) at 37°. The reaction was monitored by TLC as in (*a*). After 24 h, **25a,b**, **26**, and a little **10** were present. The solution was applied to a column (1 × 5 cm) of Sephadex G-25M and eluted with water to separate **10**, **25a,b**, and **26** from the enzyme, UDP, and UDP-Gal. Column chromatography on Bio-Gel P-2 then yielded **25a,b** (4.1 mg, 55%) and **26** (2.6 mg, 28%).

Competitive galactosylations.—Assays involved 5 mM of each competing acceptor in buffer (10 μL, 50 μmol), aq 85.6 μM UDP-[^{14}C]Gal (10 μL, 0.86 nmol), and buffer (10 μL). Each mixture was incubated with enzyme solution in buffer (10 μL, 10 μg/400 μL) at 37° and the reactions were monitored by TLC as described in (*a*) above. In one reaction (**25a,b** as acceptor), the product **26** could not be separated from lactosamine, and galactosylation was carried out in the presence of **10**. The relative rate in comparison with the galactosylation of GlcNAc was then calculated.

The concentrations of GlcNAc were 0.1 and 0.5 M when a competing acceptor was galactosylated at a rate much higher than that of GlcNAc, so that the rates of galactosylation could be determined with higher accuracy.

For optimal separation of galactosylated compounds, the following solvent mixtures were used: 6:4:3 1-BuOH–pyridine–H_2O for **10**, **13**, and **16** competing with 0.5 and 0.1 M GlcNAc, respectively; 4:2:1 EtOAc–MeOH–H_2O for **24a,b** competing with 0.5 M GlcNAc and **10** with **25a,b**.

Deactivation of β-D-galactosyltransferase by photoaffinity labelling with **10**.—Each assay, except the standard incubation mixture, was flushed with N_2 in order to remove O_2, and irradiated (350 nm) at 25° for 10 min. Irradiation of the enzyme for 10 min caused no measurable loss of the enzymic activity. No difference in the rate of galactosylation of **10** and the products of its irradiation could be detected. A standard incubation mixture containing enzyme in buffer (10 μL), 5 mM **10** (10 μL), bovine serum albumin (BSA) in buffer (10 μL, 1 mg/mL), and pure buffer (10 μL) was incubated with 85.6 μM UDP-[^{14}C]Gal (10 μL), and the rate of galactosylation was determined as described above. In a parallel experiment, the standard incubation mixture was irradiated as described above. UDP-[^{14}C]Gal was

added and the rate of galactosylation determined to be 60% of the standard (Fig. 2). To an assay containing enzyme in buffer (10 μL), 342 μM UDP-Gal (10 μL), and BSA in buffer (10 μL) was added 5 mM **10** (10 μL), and the mixture was irradiated for 10 min. After 30 min, UDP-[^{14}C]Gal was added at 35°. No loss of enzymic activity in comparison with the unirradiated standard could be detected.

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