A short Chemo-Enzymic Route to Glycosphingolipids using Soluble Glycosyl Transferases

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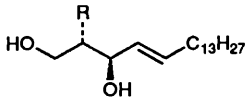
Two commercially available soluble glycosyl transferases, a β-1,4-galactosyl transferase from bovine milk and an α-2,6-sialyl transferase from rat liver, were used for the synthesis of several glycosphingolipids. The synthetic strategy involves the chemical synthesis of 2-azido- and 2-amino-sphingosine glycosides (**5**, **6**, **10** and **11**) and further glycosylation of their sugar headgroups to complex glycolipids. The azido- or amino-sphingosine lipid sidechain can then be further elaborated by reduction and/or acylation.

Glycosphingolipids are an important class of glycolipids in mammalian cells, which characteristically contain sphingosine as the lipid backbone. In ceramides, the sphingosine is *N*-acylated by a fatty acid sidechain of variable length. The diversity of glycosphingolipids in structure and biological function is mainly due to the complex oligosaccharide headgroups that are glycosidically linked to the primary hydroxy group of sphingosine.

Glycosphingolipids play key roles in many biological processes. They have been shown to be cell-surface receptors for viral and bacterial toxins,[1] regulators of cell proliferation by interacting with transmembrane signal transducers and to be mediators of cell-cell recognition events.[2] Gangliosides at the surface of leukocytes, which carry the Sialyl Lewis^x or Sialyl Lewis^a epitopes, are likely to play an important part in inflammatory responses[3] and have been shown to inhibit E-selectin binding to leukocytes.[4]



sphingosine R = NH_2
ceramide R = $NHCO[CH_2]_{14-22}Me$

Glycosphingolipids, and in particular gangliosides, have been the target of numerous chemical syntheses, which generally have been achieved by total synthesis of the oligosaccharide headgroup, selective coupling of suitably protected and activated oligosaccharides to the lipid, followed by final deprotection.[5] In recent years, the enzymic synthesis of oligosaccharides employing glycosyl transferases has emerged as an attractive alternative to the chemical synthesis.[6,7] This new methodology has also been applied to glycolipid synthesis by Paulson.[7] Thus, a chemo-enzymic synthesis of the ganglioside GM_3 has recently been reported, in which the enzymically synthesized oligosaccharide was selectively protected and activated to be coupled to the lipid moiety. A far more direct chemo-enzymic route to glycolipids is the direct glycosylation of lipids by glycosyl transferases, since it avoids protection and deprotection procedures as used by Paulson. Unfortunately, all the currently available glycosyl transferases are soluble, and have been reported not to recognise the hydrophobic ceramides as acceptor substrates, but only to accept hydrophilic substrates such as free sugars or glycopeptides/proteins as substrates. Indeed, lactosylceramide is not a substrate for the α-2,6-sialyl transferase from rat liver (EC 2.4.99.1). The only successful use of an enzymic glycosphingolipid synthesis has been reported on a solid support and with β-1,4-galactosyl transferase.[8]

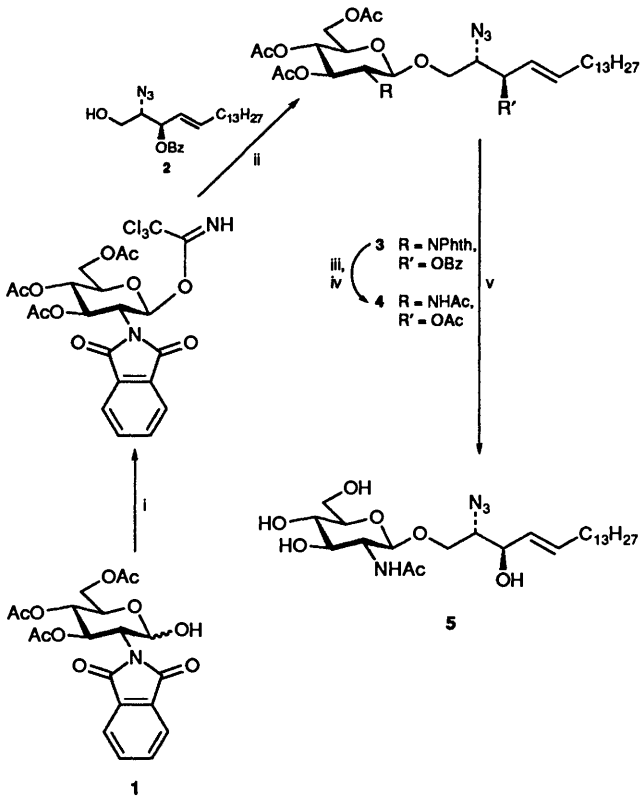
In a preliminary communication, we have published a chemo-enzymic route to glycosphingolipids in which the oligosaccharide headgroup is assembled when directly linked to 2-azidosphingosine.[9] We have shown that, unlike the lactosylceramide, which is very hydrophobic, the 2-azidosphingosine derivative is hydrophilic enough to allow it to be an acceptor substrate for a soluble galactosyl and a soluble sialyl transferase. The azido group can then be easily reduced and acylated without the need of further protection groups. The usefulness of our strategy has been demonstrated by Danishefsky[10] who used it more recently for the synthesis of the ganglioside GM_3. We report here further studies on the ability of soluble glycosyl transferases to accept glycosphingolipids as substrates, hence extending the scope of chemo-enzymic syntheses of oligosaccharide derivatives to glycolipids.

Results and Discussion

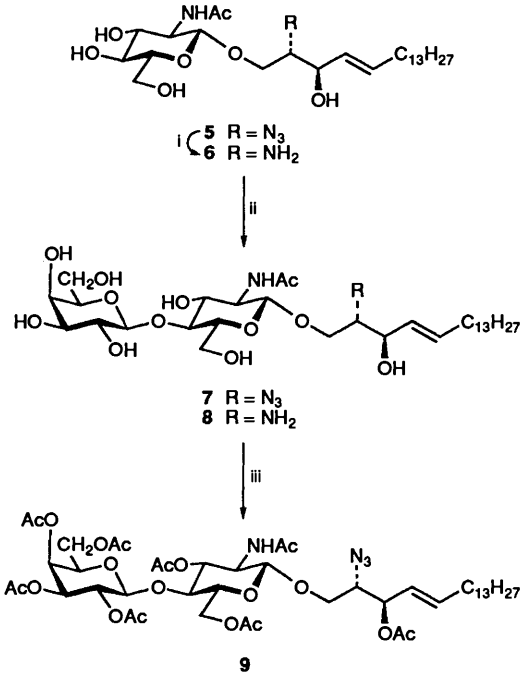
Chemical Synthesis of Acceptor Substrates.—The GlcNAc-2-azidosphingosine **5** was prepared in good yield (Scheme 1) from the suitably protected glucosamine **1**[11] by stereoselective coupling with 2-azido-3-*O*-benzoylsphingosine **2**[12] *via* the trichloroacetimidate method[13] in 94% yield to give compound **3**. The 2-phthaloyl protection group was removed by treatment with hydrazine hydrate in ethanol[14] followed by immediate acetylation to give compound **4** in 78% yield. During this step the benzoyl group of the sphingosine was exchanged for an acetyl group. Deprotection of the ester groups with sodium methoxide led to tetraol **5** in 99% yield.

The reagent that has been commonly used for the reduction of the azide group in azidosphingosines such as compound **5** is H_2S–pyridine–water.[13] We have used this method, but have found the mild procedure of using tin(II) chloride in methanol[15] to be superior for the synthesis of the sphingosine bases **6** and **8** (Scheme 2). Compound **6** could be purified by column chromatography on silica gel, although we found that the sphingosine compounds such as **6** were generally more difficult to purify and handle than the azidosphingosine glycosides such as compound **5**.

Incubation with β-1,4-Galactosyl Transferase.—Despite the poor solubility of compound **5** in the incubation medium, both glycosylsphingosines **5** and **6** proved to be good substrates for the β-1,4-galactosyl transferase from bovine milk (Scheme 2). We found that best yields were obtained when the acceptor **5** was sonicated for 15 min in sodium dimethylarsinate (sodium



Scheme 1 *Reagents and conditions:* i, NaH, CCl_3CN, CH_2Cl_2; ii, $Et_2O{\cdot}BF_3$, CH_2Cl_2, −20 °C; iii, $N_2H_4{\cdot}H_2O$, 95% EtOH, 70 °C; iv, Ac_2O, pyridine, 70 °C; v, NaOMe, MeOH



Scheme 2 *Reagents:* i, $SnCl_2$, MeOH; ii, β-1,4-galactosyl transferase, UDP-glucose, UDP-glucose 4-epimerase; iii, Ac_2O, pyridine

cacodylate) buffer before addition of the enzymes. For compound **6**, which appeared to be soluble in the incubation medium, sonication was not necessary. The expensive UDP-galactose was generated during the incubation from cheaper UDP-glucose by use of UDP-glucose 4-epimerase.[16,17] The *N*-acetyllactosamine derivatives **7** and **8** were both formed in 87% isolated yield after incubation for 18–22 h. The galactosyl β-1,4

Table 1 Amounts of radiolabel incorporated into glycolipid from CMP-*Neu-5-Ac

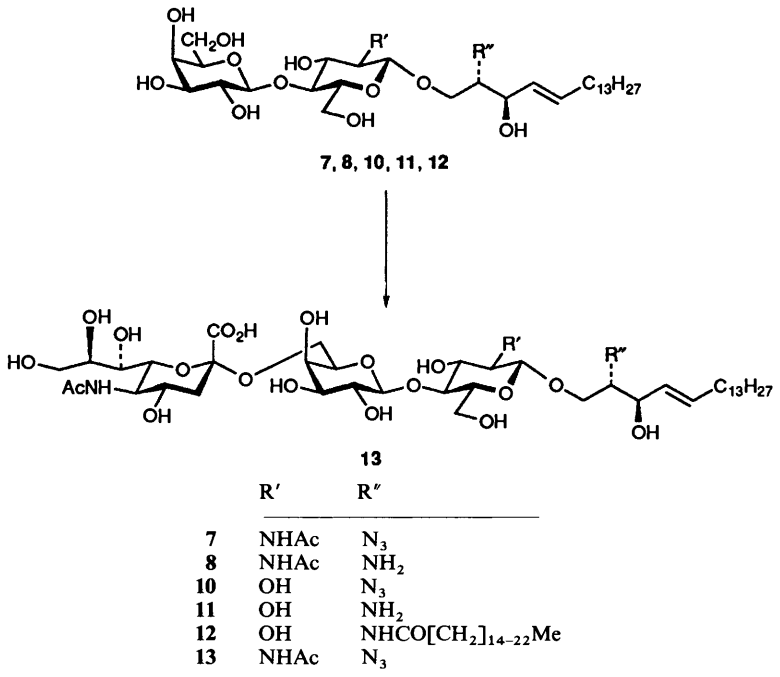
Acceptor substrate	Concentration of acceptor (mmol dm^{-3})	Incorporation of radiolabel (cpm) (±2 000 cpm)
7	6	18 300
	12	19 800
	50	37 000
	100	45 100
8	6	17 600
	12	21 500
	50	19 300
	100	16 300
10	6	2 000
	12	2 400
	50	6 000
	100	6 400
11	12	3 300
	100	3 300
12	43	2 000

linkage was confirmed by nuclear Overhauser enhancement (NOE) experiments on the peracetylated compound **9**. The acetylation allowed us to spread out the proton NMR signals for easier assignment. Thus, irradiation of the 1'-H doublet at δ 4.5 caused 3% enhancement of the 4-H signal and 3.5% of the 6-H^a signal. The latter enhancement would be expected since the 6-H^b signal is very close to that of 1'-H. Conversely, irradiation at δ 3.8 (4-H and OCH^aCN_3) led to 3.6% enhancement of 1'-H and 5.2% of OCH^bCN_3 signals.

Incubation with α-2,6-Sialyl Transferase.—The transfer of sialic acid from cytidine 5'-monophospho-*N*-acetylneuraminic acid (CMP-Neu-5-Ac) to the acceptor substrates was monitored by using radiolabelled CMP-*Neu-5-Ac (Scheme 3). Upon incorporation of *Neu-5-Ac into the acceptor, radioactivity was transferred to the lipid fraction, which could be extracted from the incubation mixture using a Sep-Pak C_{18} cartridge.[18] The cartridge was loaded, then washed with 0.2 mol dm^{-3} NaCl, and the radiolabelled lipids eluted with methanol and quantified by scintillation counting. To test the efficiency of separation by the Sep-Pak C_{18} cartridge, parallel control experiments with the same incubation mixtures but lacking enzyme were performed. The background level of binding to the C_{18} cartridge was always in the order of 600–1000 cpm.

Table 1 shows the results of these incubations of acceptor substrates **7**, **8**, **10**, **11** and **12**. Compounds **7** and **8** were good substrates for the α-2,6-sialyl transferase (2,6ST). The overall yield of conversion after 48 h was dependent on the lipid acceptor concentration. At lower substrate concentrations (6 mmol dm^{-3}) both substrates were converted in moderate yield (~20–30% based on CMP-Neu-5-Ac). For acceptor substrate **7** the yield of conversion (based on CMP-Neu-5-Ac, which was held constant at 6 mmol dm^{-3} for these experiments) increased to 60% when the acceptor concentration was increased to 100 mmol dm^{-3}. On the other hand, the overall yield of conversion with compound **8** did not seem to increase with higher substrate concentration. The yields of conversion, which we observe for our lipid substrates **7** and **8**, are comparable to those reported for soluble acceptors,[6] except that we have needed to use about three to five times as much of the commercial enzyme.

We have also studied the lactosylsphingosine derivatives **10** and **11**, which were synthesized as described by Schmidt and coworkers.[13] As expected, the overall yield of conversion was much lower than for the *N*-acetyllactosaminylglycosides **7** and **8**. It has been observed that, compared with *N*-acetyllactos-



	R′	R″
7	NHAc	N_3
8	NHAc	NH_2
10	OH	N_3
11	OH	NH_2
12	OH	$NHCO[CH_2]_{14-22}Me$
13	NHAc	N_3

Scheme 3 *Reagents:* α-2,6-sialyl transferase, CMP-*Neu-5-Ac

amine, lactose itself is a poor substrate for the sialyl transferase [K_m (Galβ1-4Glc) = 129 mmol dm^{-3}; K_m (Galβ1-4GlcNAc) = 1.62 mmol dm^{-3}; v_{max} (Galβ1-4Glc) = 0.3; v_{max}(Galβ1-4GlcNAc) = 0.93].[19] Accordingly, for the acceptor substrate **10**, incorporations of radioactivity which corresponded to yields of ~2–9% were measured. Substrate **11**, the spingosine base, was equally only sialylated in poor yield. It is interesting to note the results by Danishefsky and co-workers[10] who used a different transferase, a 2,3-sialyl transferase (2,3ST) from rat liver with substrate **10**. They found that compound **10** was a good substrate for 2,3ST. It has been reported[19] that, unlike the 2,6ST, the 2,3ST has similar affinity for lactose as for *N*-acetyllactosamine. Hence it appears that the ability of the sphingosine derivatives to act as acceptors for the sialyl transferases mirrors their affinity for the sugar headgroup.

For comparison, commercially available lactosylceramide **12** was tested in our assay. We could not measure any clear incorporation of label above background from CMP-*Neu-5-Ac into the lipid **12** at similar concentrations to that at which clear sialyl transfer to **10** was observed.

For further characterisation of a sialylation product the incubation of the 2-azidosphingosine glycoside **7** was scaled up. The product **13** could be isolated and purified by reverse-phase chromatography and was characterised by NMR spectroscopy and mass spectrometry. The proton NMR data suggested that the transfer of sialic acid to the galactose was regio- and stereo-specific. Particularly indicative for this are the 3″-H^e and 3″-H^a proton signals[17] which gave a clean doublet of doublets at δ 2.77 and a doublet of doublets which appears as a triplet at δ 1.67.

Conclusions.—We have shown that a variety of glycosphingolipids can be obtained by a short route involving the enzymic glycosylation of 2-azido- or 2-amino-sphingosine glycosides. The 2-amino group can then be selectively acylated by chemical means. We believe that this methodology is generally applicable to the enzymic synthesis of glycolipids, even with glycosyl transferases, which do not normally accept lipid substrates, such as the two described here. This strategy is useful for the synthesis of gangliosides.[10] As more glycosyl transferases become available for the synthesis of oligosaccharides, this strategy can give us also access to novel, non-natural glycolipids (neoglycolipids), which might have potentially useful applications, for example as ganglioside analogues, in liposome technology or in studying cell adhesion.[20]

Experimental

General.—Reactions were carried out in solvents distilled from standard drying agents; TLC was performed on aluminium sheets coated with silica gel 60F$_{254}$ (Merck; layer thickness 0.2 mm); the components were detected by heating the TLC plate after it had been sprayed with a solution of 5% sulfuric acid–5% anisaldehyde in ethanol; for the detection of amino derivatives a solution of 0.3% ninhydrin–3% acetic acid in butan-1-ol was used; silica gel C60 (Merck 40–60 μm) was used for flash chromatography, and Sorbsil C200 silica gel RP18 (40–60 μm, BDH) for reverse-phase chromatography; products of the radiolabelled incubations were isolated on Sep-Pak C$_{18}$ cartridges (Waters); HPLC was carried out on a Spherisorb ODS 2 (5 μm) column (10 mm internal diameter, 25 cm length) using a Waters automated gradient controller, two Waters 501 HPLC pumps and a Waters 486 tunable absorbance detector connected to a Waters 740 data module; scintillation counting was done with an LKB 1215 Rackbeta liquid scintillation counter; NMR spectra were recorded on a Bruker AM-500 MHz spectrometer using solvents as stated; coupling constants *J* are in Hz; IR spectra were recorded on a Perkin-Elmer 1750 spectrometer, and optical rotations ([α]$_D$-values are in 10^{-1} deg cm^2 g^{-1}) on a Perkin-Elmer 241 polarimeter; mass spectroscopy was carried out on VG Analytical Ltd, ZABIF or BIO-Q mass spectrometers using ammonia desorption chemical ionisation (DCI/NH$_3$), positive argon fast-atom bombardment (FAB) and electrospray (ES) as indicated; uridine 5′-diphosphoglucose (UDP-glucose), uridine 5′-diphosphoglucose 4-epimerase (EC 5.1.3.2), β-1,4-galactosyl transferase from bovine milk (EC 2.4.1.22) and ceramide β-lactoside were purchased from Sigma; α-2,6-sialyl transferase from rat liver (EC 2.4.99.1) and cytidine 5′-monophospho-*N*-acetylneuraminic acid (CMP-Neu-5-Ac) were supplied by Genzyme and Boehringer Mannheim; cytidine 5′-monophospho[^{14}C]sialic acid (74.6 μmol dm^{-3}, 10 μCi in 400

mm^3) was purchased from Amersham; calf intestinal alkaline phosphatase (CIAP) (EC 3.1.3.1) and bovine serum albumin (BSA) were obtained from Boehringer Mannheim. Petroleum spirit refers to the fraction boiling in the range 40–60 °C.

(2S,3R,4E)-2-*Azido*-3-*benzoyloxyoctadec*-4-*enyl* 3,4,6-*Tri*-O-*acetyl*-2-*deoxy*-2-*phthalimido*-β-D-*glucopyranoside* **3**.—A solution of *O*-(3,4,6-tri-*O*-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl) trichloroacetimidate (143 mg, 0.23 mmol) and (2*S*,3*R*,4*E*)-2-azido-3-benzoyloxyoctadec-4-en-1-ol (100 mg, 0.23 mmol) in dry dichloromethane (14 cm^3) was stirred at room temperature for 15 min in the presence of molecular sieves powder (4 Å) and was then cooled down to −20 °C. Boron trifluoride–diethyl ether (0.1 mol dm^{-3} solution in dichloromethane; 0.26 cm^3) was added and the reaction mixture was stirred for 10 min, diluted with petroleum spirit (20 cm^3) and washed with saturated aq. sodium hydrogen carbonate (10 cm^3). The organic fraction was filtered, dried over sodium sulfate, and concentrated under reduced pressure. The residue was chromatographed with ethyl acetate–petroleum spirit (1:3) (R_f 0.12) to give compound **3** as a crystalline solid (185 mg, 94%), m.p. 48 °C (Found: C, 63.6; H, 6.7; N, 6.5. $C_{45}H_{58}N_4O_{12}$ requires C, 63.8; H, 6.3; N, 6.6%); $[\alpha]_D^{21}$ −2 (*c* 2, $CHCl_3$); $\nu_{max}(CDCl_3)/cm^{-1}$ 2928, 2855 (CH), 2108 (N_3), 1780, 1745, 1719 (C=O) and 1174–1044 (C–O); δ_H(500 MHz; $CDCl_3$) 0.89 (3 H, t, *J* 7.0, $[CH_2]_{12}$*Me*), 1.23–1.34 (22 H, m, $[CH_2]_{11}$*Me*), 1.88 (3 H, s, Ac), 1.93–1.99 (2 H, m, CH=CHCH_2), 2.04 and 2.08 (6 H, 2 s, 2 × Ac), 3.62 (1 H, dd, *J* 4.6 and 9.9, OCH*H*CHN$_3$), 3.82–3.89 (3 H, m, 5-H, OC*H*HC*H*N$_3$), 4.17 (1 H, dd, *J* 2.4 and 12.3, 6-H^a), 4.28 (1 H, dd, *J* 4.7 and 12.3, 6-H^b), 4.38 (1 H, dd, *J* 8.5 and 10.7, 2-H), 5.19 (1 H, dd, *J* 9.1 and 10.1, 4-H), 5.42–5.49 (3 H, m, 1-H, C*H*=CHC$_{13}H_{27}$, C*H*OBz), 5.71–5.78 (1 H, m, CH=C*H*C$_{13}H_{27}$), 5.79 (1 H, dd, *J* 9.1 and 10.7, 3-H), 7.42–7.48 (2 H, m, OBz), 7.55–7.58 (1 H, m, OBz), 7.73–7.76 (2 H, m, Phth), 7.85–7.89 (2 H, m, Phth) and 7.99–8.01 (2 H, m, OBz); δ_C(125 MHz; $CDCl_3$) 14.03 (Me), 20.36, 20.54 and 20.60 (3 × CO*Me*), 22.64, 28.63, 29.10, 29.31, 29.35 and 29.54 (6 × CH_2), 29.62 (4 × CH_2), 31.89 and 32.25 (2 × CH_2), 54.46 (*C*HOBz), 61.97 (C-6), 63.31 (CN_3), 68.12 (OCH_2), 69.01, 70.76, 72.13 and 74.56 (4 × CH), 97.86 (C-1), 122.72 (CH), 123.62 (2 × CH), 128.37 (2 × CH), 129.70 (2 × CH), 129.99 (C), 131.51 (2 × C), 133.08 (CH), 134.23 (2 × CH), 138.82 (CH=), 164.89 (*C*OPh) and 169.35, 170.07 and 170.54 (3 × *C*OMe).

(2S,3R,4E)-3-*Acetoxy*-2-*azidooctadec*-4-*enyl* 2-*Acetamido*-3,4,6-*tri*-O-*acetyl*-2-*deoxy*-β-D-*glucopyranoside* **4**.—A solution of compound **3** (183 mg, 0.21 mmol) in 95% ethanol (7 cm^3) and hydrazine monohydrate (99%; 6.1 cm^3) was stirred for 45 min at 70–75 °C and the volatile products were removed under reduced pressure. The oily residue was redissolved three times in ethanol (2 cm^3) and concentrated under reduced pressure to give a powder of the deprotected compound, which was used without further purification in the following step.

The crude product was stirred in a mixture of pyridine (24 cm^3) and acetic acid (12 cm^3) at 70–80 °C for 1 h. The solution was concentrated under reduced pressure and the residue was chromatographed with ethyl acetate–petroleum spirit (2:3). The resulting oil was dissolved in methanol and water was added to precipitate compound **4**. The powder was collected by filtration, washed carefully with water, and dried *in vacuo* (118 mg, 78%), m.p. 77 °C (Found: C, 58.6; H, 8.0; N, 7.9. $C_{34}H_{56}N_4O_{11}$ requires C, 58.6; H, 8.1; N, 8.0%); R_f 0.5 [ethyl acetate–petroleum spirit (2:1)]; $[\alpha]_D^{21}$ −20 (*c* 1, $CHCl_3$); $\nu_{max}(CHCl_3)/cm^{-1}$ 2928 and 2856 (CH), 2104 (N_3), 1746 and 1686 (C=O) and 1046–1169 (C–O); δ_H(500 MHz; $CDCl_3$) 0.89 (3 H, t, *J* 7.0, $[CH_2]_{12}$*Me*), 1.23–1.40 (22 H, m, $[CH_2]_{11}$Me), 1.96, 2.03, 2.04, 2.08 and 2.09 (15 H, 5 s, 5 × Ac), 2.05–2.11 (2 H, m, CH=CHCH_2), 3.61 (1 H, dd, *J* 5.0 and 10.6, OC*H*HCHN$_3$), 3.67–3.75 (3 H, m, 5-H, N$_3$CH, 2-H), 3.85 (1 H, dd, *J* 6.6 and 10.6, OCH*H*CHN$_3$), 4.15 (1 H, dd, *J* 2.5 and 12.3, 6-H^a), 4.26 (1 H, dd, *J* 4.8 and 12.3, 6-H^b), 4.87 (1 H, d, *J* 8.2, 1-H), 5.06 (1 H, dd, *J* 9.6 and 9.6, 4-H), 5.33 (1 H, dd, *J* 4.7 and 8.1, HC=CHC*H*OAc), 5.40–5.46 (2 H, m, C*H*=CHCH$_2$, 3-H), 5.58 (1 H, d, *J* 8.3, NH) and 5.82 (1 H, dt, *J* 6.7 and 15.3, CH=C*H*CH$_2$); δ_C(125 MHz; $CDCl_3$) 13.98 (Me), 20.57 (3 × CO*Me*), 20.95 (CO*Me*), 22.60 (CH_2), 23.22 (NHCO*Me*), 28.74, 29.11, 29.28 and 29.37 (4 × CH_2), 29.60 (5 × CH_2), 31.86 and 32.28 (2 × CH_2), 55.17 (OAc*C*C=), 62.14 (C-6), 63.28 (CN_3), 67.54 (OCH_2), 68.88 (CH), 72.04 (2 × CH), 73.75 (CH), 99.88 (C-1), 123.16 and 138.41 (C=C), 169.31, 169.43, 170.30, 170.47 and 170.54 (5 × CO); *m*/*z* (DCI) 714 (MNH_4^+, 4%), 697 (MH^+, 11), 594 (10), 348 (9) and 330 (100).

(2S,3R,4E)-2-*Azido*-3-*hydroxyoctadec*-4-*enyl* 2-*Acetamido*-2-*deoxy*-β-D-*glucopyranoside* **5**.—A solution of compound **4** (408 mg, 0.586 mmol) in dry methanol (18 cm^3) was treated with 0.4 mol dm^{-3} sodium methoxide (0.58 cm^3, 0.232 mmol) and stirred at room temperature for 2.5 h, then was neutralised with ion-exchange resin [IR-120 (H^+)], filtered, and concentrated under reduced pressure, to give a powdery compound **5** (306 mg, 99%) which was recrystallised from MeOH, m.p. 154 °C (Found: C, 58.9; H, 9.3; N, 10.4. $C_{26}H_{48}N_4O_7$ requires C, 59.1; H, 9.1; N, 10.6%); R_f 0.65 [MeOH–$CHCl_3$–water (4:4:1)]; $[\alpha]_D^{25}$ −19 (*c* 1.8, MeOH); ν_{max}(KBr)/cm^{-1} 3335 (OH), 2920 and 2851 (CH), 2132 and 2095 (N_3), 1651 (C=O) and 1160–1036 (C–O); δ_H[500 MHz; $(CD_3)_2SO$] 0.86 (3 H, t, *J* 6.9, $[CH_2]_{12}$*Me*), 1.24–1.33 (22 H, m, $[CH_2]_{11}$Me), 1.80 (3 H, s, Ac), 1.98–2.02 (2 H, m, HC=CHCH_2), 3.08 (1 H, m, 4-H), 3.26–3.53 (5 H, m, N$_3$CH, 5-H, 6-H^a, 2- and 3-H), 3.64–3.70 (3 H, m, OCH_2CHN$_3$, 6-H^b), 4.01–4.04 (1 H, m, N$_3$CC*H*OH), 4.32 (1 H, d, *J* 8.4, 1-H), 4.49 (1 H, t, *J* 5.9, 6-OH), 4.88 (1 H, d, *J* 5.3, 3-OH), 4.96 (1 H, d, *J* 4.9, 4-OH), 5.22 (1 H, d, *J* 4.7, HC=CHCHO*H*), 5.42 (1 H, dd, *J* 15.4 and 7.0, C*H*=CHCH$_2$), 5.63 (1 H, dt, *J* 15.4 and 6.7, HC=C*H*CH$_2$) and 7.67 (1 H, d, *J* 8.8, NH); δ_C(125 MHz; CD_3OD) 14.38 (Me), 23.12 (CO*Me*), 23.69 (CH_2), 30.20 (2 × CH_2), 30.42 and 30.57 (2 × CH_2), 30.73 (5 × CH_2), 33.04 and 33.36 (2 × CH_2), 57.43 (*C*OHC=), 62.79 (C-6), 66.89 (CN_3), 69.51 (OCH_2), 72.19, 73.05, 76.01 and 78.08 (4 × CH), 102.48 (C-1), 129.93 and 135.70 (C=C) and 173.79 (CO); *m*/*z* (DCI) 529 (MH^+, 7%), 280 (43), 256 (45), 204 (87) and 102 (100).

(2S,3R,4E)-2-*Amino*-3-*hydroxyoctadec*-4-*enyl* 2-*Acetamido*-2-*deoxy*-β-D-*glucopyranoside* **6**.—A suspension of compound **5** (40 mg, 76 μmol) and tin(II) chloride dihydrate (25.6 mg, 113 μmol) in methanol (0.5 cm^3) was stirred at room temperature for 22 h and then evaporated under reduced pressure. The residue was chromatographed twice in methanol–chloroform–water (4:5:1) (R_f 0.45) to give compound **6** (26.1 mg, 69%), m.p. 140 °C (decomp.); $[\alpha]_D^{20}$ −27 (*c* 0.6, MeOH); ν_{max}(KBr)/cm^{-1} 3402 (OH), 2924 and 2853 (CH), 1653 (C=O) and 1159–1045 (C–O); δ_H[500 MHz; $(CD_3)_2SO$] 0.86 (3 H, t, *J* 6.9, $[CH_2]_{12}$*Me*), 1.24–1.37 (22 H, m, $[CH_2]_{11}$Me), 1.83 (3 H, s, Ac), 1.99–2.03 (2 H, m, HC=CHCH_2), 3.08 (1 H, dd, *J* 5.3 and 8.6, 4-H), 3.12–3.17 (2 H, m, 5-H, C*H*NH$_2$), 3.28–3.34 (3 H, m, 3-H, NH$_2$), 3.39–3.47 (2 H, m, 6-H^a, 2-H), 3.66–3.76 (3 H, m, 6-H^b, OCH_2CNH$_2$), 4.21–4.22 (1 H, m, HC=CHC*H*OH), 4.34 (1 H, d, *J* 8.4, 1-H), 4.60–4.61 (1 H, m, 6-OH), 5.00 (1 H, d, *J* 5.2, 3-OH), 5.06 (1 H, d, *J* 5.2, 4-OH), 5.44 (1 H, dd, *J* 6.4 and 15.4, *H*C=CHCH$_2$), 5.50 (1 H, d, *J* 4.3, HC=CHCHO*H*), 5.70 (1 H, dt, *J* 7.0 and 14.4, HC=C*H*CH$_2$) and 7.83 (1 H, d, *J* 8.7, N*H*Ac); δ_C(125 MHz; CD_3OD) 14.35 (Me), 23.04 (CO*Me*), 23.65 and 30.14 (2 × CH_2), 30.38 (2 × CH_2), 30.71 (6 × CH_2), 33.00 and 33.30 (2 × CH_2), 57.02 (*C*OHC=, CNH_2), 62.60 (C-6), 66.85 (OCH_2), 70.90, 72.16, 75.64 and 78.00 (4 × CH), 102.57

(C-1), 128.19 and 136.72 (C=C) and 174.36 (CO); *m/z* (ES^+) 503.5 (MH^+, 100%).

(2S,3R,4E)-2-*Azido*-3-*hydroxyoctadec*-4-*enyl* 2-*Acetamido*-2-*deoxy*-4-O-(β-D-*galactopyranosyl*)-β-D-*glucopyranoside* **7**.—Compound **5** (16.5 mg, 31 μmol) was sonicated for 15 min in sodium cacodylate buffer (pH 7.4) (50 mmol dm^{-3}, 0.776 cm^3) containing $MnCl_2$ (1.55 μmol) and NaN_3 (4.66 μmol). BSA (0.7 mg), CIAP (5.5 U), UDP-glucose (21.9 mg, 38 μmol), UDP-glucose 4-epimerase (1.9 U), and β-1,4-galactosyl transferase (487 mU) were added and the suspension was incubated at 37 °C for 18 h. The reaction mixture was diluted to 3 cm^3 with water and loaded on a short reverse-phase column (1.5 × 1 cm; packed in methanol and washed with water). The column was washed with water, and compound **7** was eluted with methanol. The organic fraction was concentrated under reduced pressure to give compound **7** as a powder (18.7 mg, 87%), m.p. 220 °C (decomp.); R_f 0.1 [$CHCl_3$–MeOH (3:1)]; $[\alpha]_D^{25}$ −18 (*c* 0.4, MeOH); ν_{max}(KBr)/cm^{-1} 3350 (OH), 2919 and 2851 (CH), 2132 and 2095 (N_3), 1651 (C=O) and 1135–1058 (C–O); δ_H[500 MHz; $(CD_3)_2SO$]0.86 (3 H, t, *J* 6.9, $[CH_2]_{12}Me$), 1.20–1.35 (22 H, m, $[CH_2]_{11}$Me), 1.79 (3 H, s, Ac), 1.98–2.02 (2 H, m, HC=CHCH_2), 3.24–3.79 (15 H, m, 2-, 2′-, 3-, 3′-, 4-, 4′-, 5-, 5′-H, 6-H^a, 6′-H^a, 6-H^b, 6′-H^b and CH_2CHN_3), 4.02 (1 H, dd, *J* 4.8 and 11.4, CH=CHC*H*OH), 4.21 (1 H, d, *J* 7.4, 1′-H), 4.37 (1 H, d, *J* 8.0, 1-H), 4.47 (1 H, d, *J* 4.5, OH), 4.56 (1 H, t, *J* 6.1, OH), 4.61–4.63 (2 H, m, 2 × HO), 4.74 (1 H, d, *J* 5.2, OH), 5.03 (1 H, d, *J* 4.5, OH), 5.21 (1 H, d, *J* 4.7, CH=CHCHO*H*), 5.42 (1 H, ddt, *J* 1.3, 7.0 and 15.4, C*H*=CHCH$_2$), 5.63 (1 H, dt, *J* 6.8 and 15.4, CH=C*H*CH$_2$) and 7.73 (1 H, d, *J* 8.6, NH); δ_C(125 MHz; CD_3OD) 14.31 (Me), 23.10 (CO*Me*), 23.62 (CH_2), 30.15 (2 × CH_2), 30.34 and 30.50 (2 × CH_2), 30.67 (5 × CH_2), 32.97 and 33.30 (2 × CH_2), 56.86 (*C*OHC=), 62.13 and 62.52 (C-6′, -6), 66.93 (CN_3), 69.55 (OCH_2), 70.36, 72.65, 73.07, 74.08, 74.95, 76.71, 77.16 and 81.33 (8 × CH), 102.49 (C-1), 105.18 (C-1′), 129.93 and 135.70 (C=C) and 173.57 (CO); *m/z* (ES^+) 713 (MNa^+, 100%) and 691 (MH^+, 60).

(2S,3R,4E)-2-*Amino*-3-*hydroxyoctadec*-4-*enyl* 2-*Acetamido*-2-*deoxy*-4-O-(β-D-*galactopyranosyl*)-β-D-*glucopyranoside* **8**.—Compound **6** (11.5 mg, 23 μmol) in sodium cacodylate buffer (50 mmol dm^{-3}, 0.576 cm^3) (pH 7.4) containing $MnCl_2$ (1.15 μmol), NaN_3 (3.46 μmol), BSA (0.5 mg), CIAP (4 U), UDP-glucose (15.9 mg, 27 μmol), UDP-glucose 4-epimerase (2.7 U) and β-1,4-galactosyl transferase (407 mU) was incubated at 37 °C. More galactosyl transferase (407 mU) was added after 4.5 h. After 22 h of incubation the gel obtained was loaded on a short reverse-phase column (1.5 × 1 cm; packed in methanol and washed with water) and the hydrophilic compounds were eluted with water. Compound **8** was eluted with methanol and the fraction was reduced in volume under reduced pressure to give compound **8** as a powder (13.2 mg, 87%). Part of the sample was further purified by chromatography with MeOH–$CHCl_3$–water (4:5:1) to give pure compound **8** (5.2 mg, 34%), m.p. 165 °C (decomp.); $[\alpha]_D^{20}$ −5 (*c* 0.3, MeOH); ν_{max}(KBr)/cm^{-1} 3421 (OH), 2922 and 2852 (CH), 1652 (C=O) 1160–1040 (C–O); δ_H[500 MHz; $(CD_3)_2SO$] 0.86 (3 H, t, *J* 6.9, $[CH_2]_{12}Me$), 1.20–1.36 (22 H, m, $[CH_2]_{11}$Me), 1.79 (3 H, s, Ac), 1.99 (2 H, dt, *J* 7.4 and 7.4, CH=CHCH_2), 2.74–2.75 (1 H, m, C*H*NH_2), 3.21–3.70 (15 H, m, 2-, 2′-, 3-, 3′-, 4-, 4′-, 5-, 5′-, 6-H^a, 6′-H^a, 6′-H^b and OCH_2CNH_2), 3.77 (1 H, dd, *J* 2.3 and 11.9, 6-H^b), 3.79–3.81 (1 H, m, CH=CHC*H*OH), 4.21 (1 H, d, *J* 7.7, 1′-H), 4.28 (1 H, d, *J* 7.9, 1-H), 5.42 (1 H, dd, *J* 6.5 and 15.4, C*H*=CHCH$_2$), 5.55 (1 H, dt, *J* 6.8 and 15.4, CH=C*H*CH$_2$) and 7.79 (1 H, d, *J* 8.3, N*H*Ac); *m/z* (ES^+) 665 (MH^+, 100%).

Synthesis of Compound **8** *by Reduction of Compound* **7**.—A suspension of compound **7** (17.5 mg, 25 μmol) in methanol (0.26 cm^3) was treated with tin(II) chloride dihydrate (8.6 mg, 38 μmol) and stirred at room temperature for 20 h. More tin(II) chloride (4.8 mg, 21 μmol) was added and the reaction mixture was stirred for another 5 h. The solution was neutralised with 1 mol dm^{-3} aq. sodium hydroxide and the resulting precipitate was removed by filtration through Celite. The filtrate was evaporated under reduced pressure to give crude compound **8** as a powder (15.6 mg, 92%), which contained a trace of starting material. R_f 0.33 [MeOH–$CHCl_3$–water (4:5:1)].

(2S,3R,4E)-3-*Acetoxy*-2-*azidooctadec*-4-*enyl* 2-*Acetamido*-3,6-*di*-O-*acetyl*-2-*deoxy*-4-O-(2,3,4,6-*tetra*-O-*acetyl*-β-D-*galactopyranosyl*)-β-D-*glucopyranoside* **9**.—A solution of compound **7** (18.5 mg, 27 μmol) in pyridine (1 cm^3) and acetic acid (0.5 cm^3) was stirred at room temperature for 6.5 h and then evaporated under reduced pressure. The residue was dissolved in toluene (2 cm^3) and the solution was evaporated. Chromatography with ethyl acetate–petroleum spirit (3:2) (R_f 0.13) led to compound **9** as a solid (18 mg, 68%) (Found: C, 55.9; H, 7.4; N, 5.4. $C_{46}H_{72}N_4O_{19}$ requires C, 56.1; H, 7.4; N, 5.7%); $[\alpha]_D^{20}$ −24 (*c* 1.1, $CHCl_3$); ν_{max}($CHCl_3$)/cm^{-1} 2928 and 2856 (CH), 2105 (N_3), 1750 and 1680 (C=O) and 1178–1030 (C–O); δ_H(500 MHz; $CDCl_3$) 0.88 (3 H, t, *J* 7.0, $[CH_2]_{12}Me$), 1.23–1.39 (22 H, m, $[CH_2]_{11}$Me), 1.98 (6 H, 2 s, 2 × Ac), 2.01–2.11 (2 H, m, CH=CHCH_2), 2.06 (6 H, 2 s, 2 × Ac), 2.07, 2.09, 2.12 and 2.16 (12 H, 4 s, 4 × Ac), 3.53 (1 H, dd, *J* 4.78 and 10.2, OC*H*HCHN$_3$), 3.66–3.70 (1 H, m, 5-H), 3.72–3.76 (1 H, m, CHN$_3$), 3.78–3.82 (2 H, m, OCH*H*CHN$_3$, 4-H), 3.89 (1 H, dd, *J* 6.8 and 6.8, 5′-H), 3.98 (1 H, ddd, *J* 6.9, 9.0 and 9.0, 2-H), 4.08–4.18 (3 H, m, 6-H^a, 6′-H^a, 6′-H^b), 4.51 (2 H, d, *J* 7.9, 1′-H and dd, *J* 3.0 and 11.9, 6-H^b), 4.58 (1 H, d, *J* 6.9, 1-H), 4.99 (1 H, dd, *J* 3.4 and 10.5, 3′-H), 5.11–5.16 (2 H, m, 3- and 2′-H), 5.31 (1 H, dd, *J* 4.6 and 8.0, CH=CHC*H*OAc), 5.37 (1 H, d, *J* 3.4, 4′-H), 5.42 (1 H, ddt, *J* 1.4, 8.0 and 15.4, C*H*=CHCH$_2$), 5.73 (1 H, d, *J* 9.1, NH) and 5.82 (1 H, dt, *J* 6.8 and 15.3, CH=C*H*CH$_2$); δ_C(125 MHz; $CDCl_3$) 14.01 (Me), 20.39 (CO*Me*), 20.51 (3 × CO*Me*), 20.56, 20.74 and 20.97 (3 × CO*Me*), 22.64 (CH_2), 23.14 (NHCO*Me*), 28.79, 29.16, 29.32 and 29.41 (4 × CH_2), 29.65 (5 × CH_2), 31.91 and 32.32 (2 × CH_2), 52.92 (CH), 60.95 and 62.55 (C-6′, -6), 63.45 and 66.85 (2 × CH), 67.77 (OCH_2), 69.32, 70.86, 71.04, 71.62, 72.92, 73.91 and 75.25 (7 × CH), 100.47 and 100.94 (C-1, -1′), 123.17 and 138.47 (C=C), 169.32, 169.41, 169.87, 169.98 and 170.04 (5 × CO) and 170.22 (3 × CO); *m/z* (FAB^+) 1008 (MNa^+, 28%), 43 ($COCH_3^+$, 100).

1-O-{2-*Acetamido*-1-O-[(2S,3R,4E)-2-*azido*-3-*hydroxyoctadec*-4-*enyl*]-2,4-*dideoxy*-β-D-*glucopyranos*-4-*yl*}-6-*deoxy*-β-D-*galactopyranos*-6-*yl* 5-*Acetamido*-3,5-*dideoxy*-α-D-glycero-D-galacto-*non*-2-*ulopyranosonic Acid* **13**.—Compound **7** (5.4 mg, 7.8 μmol) was sonicated for 15 min in sodium cacodylate buffer (12 mmol dm^{-3}, pH 7.4, 1.3 cm^3) containing 0.5% Triton CF-54. The suspension was incubated with BSA (1.3 mg), CIAP (3.2 U), CMP-Neu-5-Ac (5.3 mg, 8.6 μmol) and α-2,6-sialyl transferase (110 mU) at 37 °C for 42 h. More CMP-Neu-5-Ac (1 mg, 1.6 μmol) was added and the incubation was left at 37 °C for 25 h. The reaction mixture was loaded on a reverse-phase column (5 × 0.6 cm; packed in methanol and washed with water). The column was washed with water, the sialylated product **13** was eluted with methanol–water (8:2) and the unchanged acceptor **7** was eluted with methanol. After reduction of volume under reduced pressure, compound **13** was obtained as a powder, which was dissolved in methanol–10 mmol dm^{-3} NH_4HCO_3 (7:3) and purified by HPLC with methanol–10 mmol dm^{-3} NH_4HCO_3 (8:2; 2.5 cm^3 min^{-1}), t_R 8.4 min (1 mg, 13%), R_f 0.25 [methanol–chloroform–water (4:5:1)]; δ_H(500 MHz; CD_3OD) 0.90 (3 H, t, *J* 7.0, $[CH_2]_{12}Me$), 1.29–1.41 (22 H, m,

$[CH_2]_{11}Me$), 1.67 (1 H, dd, J 12.0 and 12.0, 3″-H^a), 1.98 and 1.99 (6 H, 2 s, 2 × Ac), 2.03–2.09 (2 H, m, CH=CHCH_2), 2.77 (1 H, dd, J 4.7 and 12.2, 3″-H^e), 4.32 (1 H, d, J 7.5, 1′-H), 4.64 (1 H, d, J 8.4, 1-H), 5.48 (1 H, ddt, J 1.3, 7.5 and 15.4, CH=CHCH$_2$) and 5.74 (1 H, dt, J 6.9 and 14.6, CH=CHCH$_2$); m/z (FAB$^+$) 1026 [$(MNa_2 - H)^+$, 100%] and 1004 (MNa^+, 40).

General Procedure for the Enzymic Sialylation using the α-2,6-Sialyl Transferase and Radiolabelled CMP-[^{14}C]Neu-5-Ac.—The acceptor substrate (6–100 mmol dm^{-3}) was sonicated for 15 min in sodium cacodylate buffer (12 mmol dm^{-3}, pH 7.4) containing Triton CF-54 (0.5%). The suspension was incubated in the presence of BSA (0.1%), CIAP (2.5 U cm^{-3}), α-2,6-sialyl transferase (92 mU cm^{-3}) and a mixture of CMP-Neu-5-Ac and CMP-[^{14}C]Neu-5-Ac (6 mmol dm^{-3}) for 48 h. The reaction mixture (0.024 cm^3) was diluted into aq. sodium chloride (0.2 mol dm^{-3}; 1 cm^3) and loaded onto a Sep-Pak C_{18} cartridge (pre-washed with methanol, then with water). The flow-through was re-loaded three times and the cartridge was washed with aq. sodium chloride (0.2 mol dm^{-3}; 10 cm^3) then with methanol (10 cm^3). The aqueous and organic fractions (1 cm^3) were mixed with the scintillation fluid (10 cm^3) and counted. Parallel to the incubations, control reactions were run containing the same components except sialyl transferase. These gave background values in the order of 600–1000 counts.

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References

1 K.-A. Karlsson, *Annu. Rev. Biochem.*, 1989, **58**, 309.
2 For a review see: S.-I. Hakomori, *J. Biol. Chem.*, 1990, **265**, 18713.
3 For a review see: T. A. Springer, *Nature (London)*, 1990, **346**, 425.
4 A. Takada, K. Ohmori, N. Takahishi, K. Tsuyuoka, A. Yago, K. Zenita, A. Hasegawa and R. Kannagi, *Biochem. Biophys. Res. Commun.*, 1991, **179**, 713.
5 For examples see: M. Numata, M. Sugimoto, Y. Ito and T. Ogawa, *Carbohydr. Res.*, 1990, **203**, 205; T. Murase, H. Ishida, M. Kiso and A. Hasegawa, *Carbohydr. Res.*, 1989, **188**, 71; M. Sugimoto and T. Ogawa, *Glycoconjugate J.*, 1985, **2**, 5.
6 For some examples see: (*a*) C. Auge, C. Gautheron and H. Pora, *Carbohydr. Res.*, 1989, **193**, 288; A. Lubineau, C. Auge and P. Francois, *Carbohydr. Res.*, 1992, **228**, 137; M. M. Palcic, L. D. Heerze, M. Pierce and O. Hindsgaul, *Glycoconjugate J.*, 1988, **5**, 49; (*b*) S. Sabesan and J. C. Paulson, *J. Am. Chem. Soc.*, 1986, **108**, 2068; (*c*) J. Thiem and W. Treder, *Angew. Chem., Int. Ed. Engl.*, 1986, **25**, C12; C. H. Wong, Y. Ichikawa, T. Krach, C. Gautheron-Le Narvor, D. P. Dumas and G. C. Look, *J. Am. Chem. Soc.*, 1991, **113**, 8137.
7 Y. Ito and J. C. Paulson, *J. Am. Chem. Soc.*, 1993, **115**, 1603.
8 U. Zehavi, M. Herchman, R. R. Schmidt and T. Baer, *Glycoconjugate J.*, 1990, **7**, 229.
9 B. Guilbert, T. H. Khan and S. L. Flitsch, *J. Chem. Soc., Chem. Commun.*, 1992, 1526.
10 K. K.-C. Liu and S. J. Danishefsky, *J. Am. Chem. Soc.*, 1993, **115**, 4933.
11 R. U. Lemieux, T. Takeda and B. Y. Chung, *ACS Symp. Ser.*, 1976, **39**, 90.
12 P. Zimmermann and R. R. Schmidt, *Liebigs Ann. Chem.*, 1988, 663.
13 P. Zimmermann, R. Bommer, T. Baer and R. R. Schmidt, *J. Carbohydr. Chem.*, 1988, **7**, 435.
14 G. Grundler and R. R. Schmidt, *Carbohydr. Res.*, 1985, **135**, 203.
15 S. N. Maiti, M. P. Singh and R. G. Micetich, *Tetrahedron Lett.*, 1986, **27**, 1423.
16 C.-H. Wong, S. L. Haynie and G. M. Whitesides, *J. Org. Chem.*, 1982, **47**, 5416.
17 C. Unverzagt, H. Kunz and J. C. Paulson, *J. Am. Chem. Soc.*, 1990, **112**, 9308. See also ref. 6(*b*).
18 L. J. Melkerson-Watson, K. Kanemitsu and C. C. Sweeley, *Glycoconjugate J.*, 1987, **4**, 7.
19 J. Weinstein, U. de Souza-e-Silva and J. C. Paulson, *J. Biol. Chem.*, 1982, **257**, 13845.
20 M. Larkin, T. J. Ahern, M. S. Stoll, M. Shaffer, D. Sako, J. O'Brien, C.-T. Yuen, A. M. Lawson, R. A. Childs, K. M. Barone, P. R. Langer-Safer, A. Hasegawa, M. Kiso, G. R. Larsen and T. Feizi, *J. Biol. Chem.*, 1992, **267**, 13661.

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