Chemoenzymatic Synthesis of a Glycosphingolipid

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The novel glycosphingolipid **7** is synthesised by two successive enzymatic glycosylation steps from **5** using a β(1-4)-galactosyl transferase and an α(2-6)-sialyl transferase, both of which are commercially available.

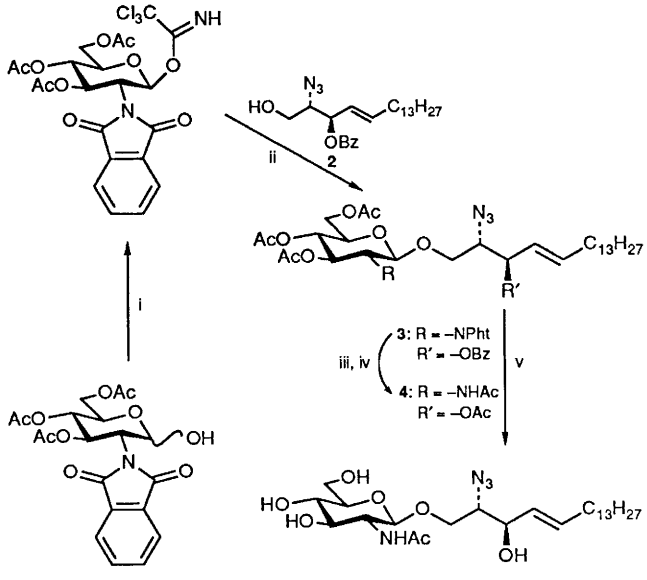
Gangliosides are glycosphingolipids located in the plasma membrane of mammalian cells. The oligosaccharide head-group, which contains the negatively charged sialic acid, is recognised as the binding site for enzymes, hormones, toxins, lectins, bacteria and viruses.[1]

Among the gangliosides, GM_3 [α-D-Neu-5-Ac-(2-3)-β-D-Gal-(1-4)-β-D-Glc-1-Ceramide] contains one of the simplest oligosaccharide sequences and is a biosynthetic precursor for many gangliosides.[2] It has been isolated from brain, human spleen and animal erythrocytes,[3] and is known to play a role in the differentiation of various types of cells[4] and in the regulation of cell growth by modulating growth factor receptors.[5,6]

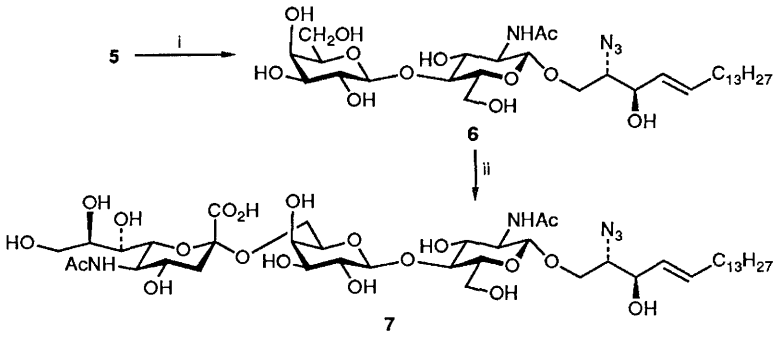
The chemical synthesis of GM_3 has been reported[7] but is lengthy since it requires extensive protection, deprotection and activation steps. An attractive alternative approach is the chemoenzymatic synthesis using glycosyl transferases which couple sugars in a regio- and stereo-selective manner without the need for protection groups. Glycosyl transferases have been used successfully by several groups[8–14] for the synthesis of oligosaccharides, glycopeptides and glycoproteins, but the synthesis of glycolipids has been little explored.

We report here the enzymic synthesis of **7**, a novel analogue of the ganglioside GM_3, in only two steps from the chemically synthesised *N*-acetyl glucosamine derivative **5** using a β(1-4)-galactosyl transferase for bovine milk and an α(2-6)-sialyl transferase from porcine liver.

The substrate **5** for the transferases was easily prepared (Scheme 1) from the suitably protected glucosamine **1**[15] by stereoselective coupling with 2-azido-3-*O*-benzoyl-sphingosine **2**[16] *via* the trichloroacetimidate method[17] in 90% yield to give **3**. The 2-phthaloyl protection group was removed by treatment with hydrazine hydrate in ethanol[18] followed by immediate acetylation to give **4** in 81% 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 **5** in 91% yield.



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 (Bz = benzoyl, Pht = Phthaloyl)



Scheme 2 *Reagents and conditions*: i, β(1-4)galactosyl transferase, UDP-glucose, UDP-glucose 4-epimerase; ii, α(2-6) sialyl transferase, CMP-Neu-5-Ac

Despite the poor solubility of **5** in the incubation medium, it proved to be a good substrate for the β(1-4)-galactosyl transferase from bovine milk (from Sigma). We found that best yields were obtained when **5** was sonicated for 15 min in the sodium cacodylate buffer before addition of the enzymes. The expensive uridine 5′-diphosphate (UDP)-galactose was generated during the incubation from UDP-glucose using UDP-glucose 4-epimerase[19]. The *N*-acetyl lactosamine derivative **6** was formed in 87% isolated yield after incubation for 18 h.†

Compound **6** was also a good substrate for the rat liver α(2-6) sialyl transferase (from Boehringer). This reaction was firstly investigated with a fast assay using radiolabelled cytidine 5′-monophosphate (CMP)-*Neu-5-Ac. Thus, upon incorporation of *Neu-5-Ac into **7**, radioactivity was transfered to the lipid fraction, which could be extracted from the incubation mixture using a Sep-Pak C18 cartridge.[20] The cartridge was then washed with 0.2 mol dm^{-3} NaCl and the radiolabelled lipids eluted with methanol and quantified by scintillation counting. The best yield we obtained was 40%. The reaction was also scaled up, compound **7** was isolated by reverse phase chromatography and characterised spectroscopically.‡

We had chosen the 2-azido sphingosine chain as the aglycon because we expected it to be less hydrophobic than the ceramide lipid sidechain of gangliosides. Thus lactosyl ceramide is known not to be an acceptor substrate for the type of sialyl transferase used here.[21] Furthermore, the conversion of compounds such as **7** to a ceramide derivative by reduction of the azido group using H_2S–pyridine–H_2O and acylation with an activated fatty acid can be achieved in high yield.[17]

† *Incubation conditions:*[19] Compound **5** (40 mmol dm^{-3}) was sonicated for 15 min in cacodylate buffer (2 mmol dm^{-3} $MnCl_2$, 6 mmol dm^{-3} NaN_3, 50 mmol dm^{-3} sodium cacodylate pH 7.4) and then incubated for 18 h at 37 °C after addition of bovine serum albumin (0.1%), calf intestinal alkaline phosphatase (7 U cm^{-3}), UDP-glucose 4-epimerase (1.76 U cm^{-3}), β(1-4) galactosyl transferase (0.36 U cm^{-3}) and UDP-glucose (49 mmol dm^{-3}). The product **6** was purified by reverse phase silica chromatography. *Selected spectroscopic data* for **6**: ^{1}H NMR (CD_3OD, 500 MHz) δ 0.89 [3H, t, *J* 7.0 Hz, $-(CH_2)_{11}CH_3$], 1.28–1.41 (22H, m, $-(CH_2)_{11}CH_3$), 1.97 (3H, s, $-COCH_3$), 4.38 [1H, d, *J* 7.5 Hz, C(1)H], 4.47 [1H, d, *J* 8.3 Hz, C′(1)H], 5.49 (1H, dd, *J* 7.6, 15.4 Hz, $-CH$=$CHCH_2-$), 5.74 (1H, dt, *J* 15.4 Hz, $-CH$=$CHCH_2-$).

‡ *Incubation conditions*: Compound **6** (6 mmol dm^{-3}), CMP-Neu-5-Ac (6.4 mmol dm^{-3}), Triton CF-54 (0.5%), bovine serum albumin (0.1%), calf intestine alkaline phosphatase (2.4 U cm^{-3}), NaN_3 (6 mmol dm^{-3}) and α(2-6) sialyl transferase (0.07 U cm^{-3}) were incubated in sodium cacodylate buffer pH 7.4 (12 mmol dm^{-3}) at 37 °C for 3 days. *Selected spectroscopic data* for **7**: ^{1}H NMR (500 MHz, CD_3OD), δ 0.90 [3H, t, *J* 7.0 Hz, $-(CH_2)_{12}CH_3$], 1.29–1.41 [22H, m, $-(CH_2)_{11}CH_3$], 1.67 (1H, t, *J* 12.0 Hz, H_{3a}), 1.98 and 1.99 (2 × 3H, 2s, 2 × $COCH_3$), 2.03–2.09 (2H, m, $-CH$=$CHCH_2-$), 2.77 (1H, q, *J* 4.7, 12 Hz, H_{3e}), 4.32 [1H, d, *J* 7.5 Hz, C(1)H], 4.64 [1H, d, *J* 8.4 Hz, C′(1)H], 5.48 (1H, ddt, *J* 1.3, 7.5, 15.4 Hz, $-CH$=$CHC_{13}H_{27}$), 5.74 (1H, dt, *J* 6.9, 14.6 Hz, $-CH$=$CHC_{13}H_{27}$). MS (FAB): $(M + Na)^+$ = 1004.

To our knowledge this is the first report of an enzymic synthesis of sialylated glycosphingolipids using this commercially available sialyl transferase from rat liver. The approach to glycolipid synthesis presented here is novel in that it assembles the oligosaccharide headgroup directly on the lipid. So far most synthetic strategies involve the coupling of the oligosaccharide to the lipid as the last step,[7] which requires extensive use of protection groups, even if the oligosaccharide had been synthesised enzymically. The extension of this chemoenzymatic method to more complex glycosphingolipids and their biological testing are currently in progress.

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