Pergamon

Bioorganic & Medicinal Chemistry, Vol. 2, No. 11, pp. 1243-1250, 1994

0968-0896/94 $7.00 + .00

0968-0896(94)00097-2

The Chemoenzymatic Synthesis of Neoglycolipids and Lipid-Linked Oligosaccharides Using Glycosyltransferases

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Abstract—The application of glycosyltransferases to the chemoenzymatic synthesis of neoglycosphingolipids and lipid-linked oligosaccharides allows the regio- and stereoselective formation of glycosidic bonds. In our laboratory galactosyl-, sialyl-, and fucosyltransferases have been used to assemble oligosaccharide headgroups directly on a sphingosine derivative without the need for any protection group strategies, including the Lewis^x antigen. In complementary studies on N-linked oligosaccharide biosynthesis, chemically phosphorylated dolichol analogues have been tested as substrates for Dol-P-Man synthetase. Also, the substrate recognition of the core β-1,4-mannosyltransferase from yeast has been investigated using a range of chitobiose derivatives as potential substrates.

Introduction

Glycolipids have been the targets for numerous chemical syntheses,[1] since, next to glycoproteins and proteoglycans, they are one of the most important classes of glycoconjugates in biological systems and are to date the only class which is accessible by total synthesis. The chemical synthesis of glycolipids generally uses the following strategy: (i) synthesis of the suitably protected and activated mono- or oligosaccharide headgroup; (ii) coupling to the lipid and (iii) final deprotection. Thus, the lipid moiety is introduced as late as possible to avoid the problems of generally low reactivity of lipid intermediates. The most challenging and complex part of the synthesis is the assembly of the oligosaccharide headgroup, especially in the case of large oligosaccharides.

Complementary to the chemical synthesis of oligosaccharides themselves, an enzymatic approach has recently been developed, which uses glycosyltransferases to form the glycosidic linkages in a highly stereo- and regioselective manner.[2] Although this method is currently restricted by the availability of suitable enzymes, where it can be applied, it offers enormous advantages in terms of ease, length and speed of synthesis. Such an enzymatic approach has already been applied by Paulson *et al.* to the synthesis of the glycosphingolipid GM_3.[3] However, with the given strategy of glycolipid synthesis, this required the use of protection groups after the enzymatic step, i.e. protection and activation of the enzymatically prepared oligosaccharide, followed by coupling and deprotection. The advantage that no protection groups are needed when using enzymes is thus partly lost. It would therefore be clearly advantageous if the oligosaccharide were assembled directly on the lipid as indeed occurs in most of the biosyntheses of glycolipids.

For such an approach, two types of glycosyltransferases could in principle be used: (i) those involved in the biosynthesis of glycolipids and (ii) 'soluble' glycosyltransferases which form the same linkage but normally act on non-lipid substrates. Since the repertoire of glycosyltransferases is still very limited, it would clearly be an advantage if the same transferases could be used for both soluble and lipid glycoconjugates. This would allow us to apply the chemoenzymatic syntheses of soluble bioactive oligosaccharides, of which many have been reported, directly to the synthesis of natural and non-natural (neo-) glycolipids. By studying the acceptor specificity of several glycosyltransferases we have shown that this is indeed possible, if a suitable aglycon lipid is chosen. This is illustrated for two types of glycosyltransferases: (i) soluble glycosyltransferases, which were used for glycosphingolipid synthesis and (ii) membrane associated mannosyltransferases which normally recognise lipid linked acceptors.

Results and Discussion

Chemoenzymatic synthesis of glycosphingolipids

In biological membranes, sphingolipids are a major class of lipids, whose backbone is sphingosine (**1**), which can be acylated with fatty acids at the 2-amino function to give ceramide (**2**). In glycosphingolipids, the C-1 hydroxyl group can be linked to glucose or galactose or to more complex oligosaccharides containing up to 20 monosaccharide residues. An important class of glycosphingolipids are gangliosides, which are negatively charged because they contain sialic acids, of which the most simple representative is GM_3 (**3**). Although very little is still understood about the function of glycolipids, they have been identified to

play key roles in biological processes such as cell adhesion,[4] embryogenesis,[5] signal transduction[6] and can be functional receptors for toxins.[7] Indeed, there are several preliminary reports suggesting that glycosphingolipids might find use in therapy, such as in treatment of Parkinson's disease[8] or spinal cord injuries.[9] Glycolipids also have great potential in the targetting of liposome delivery systems[10] for drugs, proteins or DNA.

Ceramides are often too hydrophobic to act as acceptors for soluble glycosyltransferases, in particular, when the sugar headgroups are small. Thus it was found that *N*-acetyl glucosaminyl ceramide (**4**) was not accepted by the β-1,4-galactosyltransferase from bovine milk,[11] nor was lactosyl ceramide (**5**) a substrate for either the soluble α-2,3- or the α-2,6-sialyltransferases from rat liver.[11]

An alternative strategy is to enzymatically build up the oligosaccharide headgroup on an aglycon, which can act as a precursor to ceramide and can be converted to ceramide without the need for further protection groups. An obvious choice is sphingosine (**1**) or 2-azido sphingosine, which can be chemically acylated, or reduced and acylated, respectively, to give the corresponding glyco-ceramide.

The first enzyme we investigated was the β-1,4-galactosyltransferase from bovine milk, which is commercially available, and found that both the 2-azido sphingosine and sphingosine glycosides **6** and **7** were excellent substrates and could give **8** and **9** in high yields (Scheme I).[12]

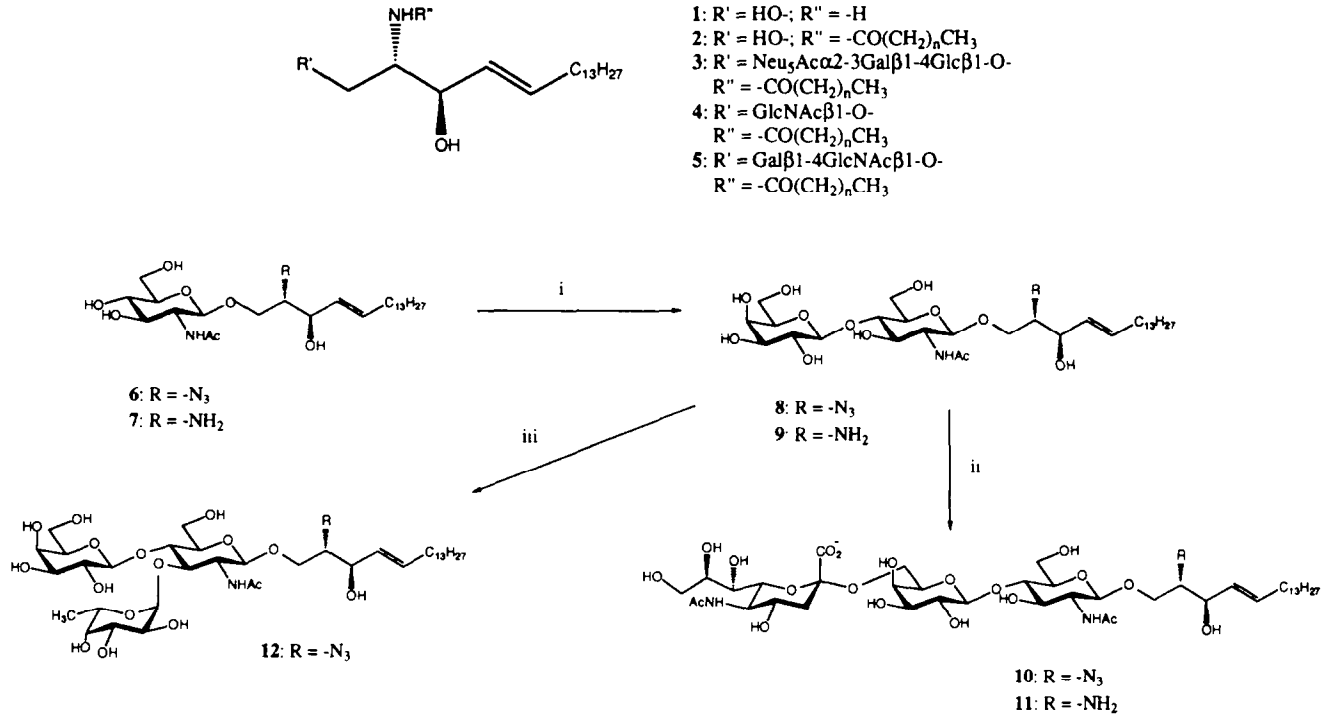
The products of the galactosylation (**8** and **9**) were then tested as substrates for the commercially available α-2,6-sialyltransferase, which again accepted both the 2-azido sphingosine and the sphingosine derivatives (Scheme I) leading to neoglycolipids **10** and **11** containing the sialyl-*N*-acetyl-lactosamine headgroup.

N-Acetyllactosamine itself is also a known substrate for mammalian α-1,3-fucosyltransferase, generating the Lewis^x epitope. Glycolipid **8** was therefore also tested with a soluble form of the mammalian fucosyltransferase VI.[13] It was shown to be as good an acceptor as *N*-acetyllactosamine itself and generated the neoglycolipid **12** which carries the Lewis^x epitope (Scheme I).

This novel synthetic route to glycolipids, which involves the chemical synthesis of a simple glycosphingoside, such as **6** or **7**, extension by soluble glycosyltransferases and further simple acylation of the lipid, is the shortest route to these compounds and utilises the characteristics of the glycosyltransferases to the best advantage. The examples given here show that the methodology is quite generally applicable to soluble glycosyltransferases. Indeed, it has recently been used by Danishefsky *et al.*[14] for the synthesis of GM_3 using as the key step the α-2,3-sialyltransferase from rat liver to sialylate lactosyl-2-azido-sphingosine which was then reduced and acylated.

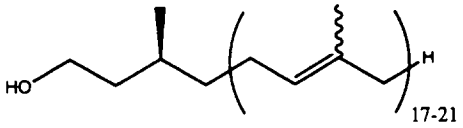
Enzymatic mannosylation of lipid phosphates

The N-linked oligosaccharides of glycoproteins are first biosynthesised on the isoprenoid lipid dolichol (**13**)



Scheme I. Chemoenzymatic synthesis of neoglycosphingolipids. *Reagents* : i. β-1,4-galactosyltransferase, UDP-Gal; ii. α-2,6-sialyltransferase, CMP-Neu-5-Ac; iii. α-1,3-fucosyltransferase VI, GDP-Fuc.

before transfer to the nascent protein chain.[15] The glycosyltransferases involved in this biosynthetic pathway are all membrane associated and act on lipid substrates. The extent of molecular recognition of the dolichol lipid by these transferases is as yet poorly understood. We are particularly interested in the mannosyltransferases of this pathway, which catalyse reactions that are difficult to perform chemically, such as the formation of the β-mannosyl linkage in the core pentasaccharide region of N-linked oligosaccharides. Since these transferases can be obtained from yeast and some of them have already been overexpressed in bacterial systems,[16] they should become available in large enough quantities to be used in synthetic studies of these important bioactive oligosaccharides. However, if dolichol really was required as an aglycon for substrate recognition, a chemoenzymatic synthetic approach to these oligosaccharide structures would be impractical, since dolichol is not readily available, heterogeneous and difficult to work with. Both, in order to understand lipid recognition by these enzymes and, with the ultimate goal of finding a simpler aglycon, we have therefore studied the substrate requirements of several enzymes involved in the N-linked oligosaccharide precursor biosynthesis.



13: *dolichol*

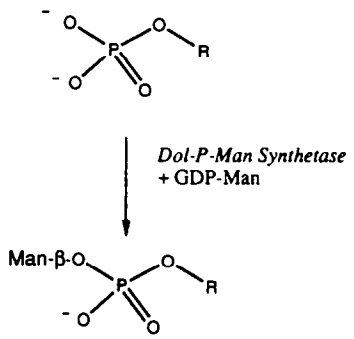
The first enzyme we investigated was Dol-P-Man synthetase due to its simple substrate. It catalyses the transfer of mannose from GDP-mannose to dolichyl phosphate (**14**) (Scheme II).[17] It had been reported by Clark and Villemez,[18] that this enzyme from mung beans can mannosylate phytanyl phosphate (**15**), a structurally much simpler lipid than dolichyl phosphate. We therefore synthesised phytanyl phosphate together with a series of phosphate esters (**16–18**) containing even smaller hydrophobic sidechains and found that phytanyl phosphate (**15**) was a good substrate for the Dol-P-Man synthetase from yeast or pig liver, whereas shorter branched phosphates **17** or phosphates carrying chains of similar length but with less branching (**16** and **18**), were less able or apparently unable to accept mannose (Scheme II).[17] Thus, even though a monobranched lipid phosphate (*S*-3-methyloctadecanyl phosphate) was concluded to have the minimal structure required for recognition by this enzyme, phytanyl phosphate was the preferred candidate for further investigation, since it was the better substrate.

Investigation of the specificity of β-1,4-mannosyltransferase

The knowledge gained from the studies on Dol-P-Man synthetase were applied to β-1,4-mannosyltransferase, which catalyses the formation of the core trisaccharide, Manβ1–4GlcNAcβ1–4GlcNAc-R from the corresponding chitobiosyl pyrophosphoryl lipid ($PPGn_2$). This chitobiosyl lipid (**28**) can be synthesised by a convergent synthesis from chitobiose octa-acetate (**19**), which is readily available by acetolysis from chitin.[19] We have now improved the overall yield of the synthesis of this acceptor to 50–60 % from chitobiose octa-acetate (Scheme III). Chitobiose octa-acetate (**19**) was 1-*O*-deacetylated and phosphorylated as previously described to give the dibenzylphosphate diester **24**. Hydrogenation of **24** gave the phosphate ester **25** which could then be coupled in high yield to phytanyl phosphate (**15**), using *N,N*-carbonyldiimidazole as the coupling reagent.[20] Final deprotection of the product **27** with sodium methoxide in methanol was quantitative.

The chitobiosyl phytanyl pyrophosphate (**28**) was found to be a good substrate for the recombinant β-1,4-mannosyltransferase[21] to give **29** with an apparent K_m value of 17 μM, which is close to that reported for the natural dolichyl linked substrate.[22]

To probe the structural requirement for the β-1,4-mannosyltransferase further, substrates lacking the pyrophosphate diester (phytanyl chitobioside **23**) or the lipid moiety (chitobiosyl phosphate **26**) were synthesised (Scheme III). Chitobiose hepta-acetate (**21**) was treated with base (lithium diisopropylamide) and alkylated with phytanyl triflate to give the protected phytanyl glycoside **23**. This was deprotected using sodium methoxide in methanol, yielding the lipid analogue **26**, which lacks the pyrophosphate bridge. Chitobiosyl phosphate **26** was prepared by the same deprotection procedure from **25**.



14: R = *dolichyl*

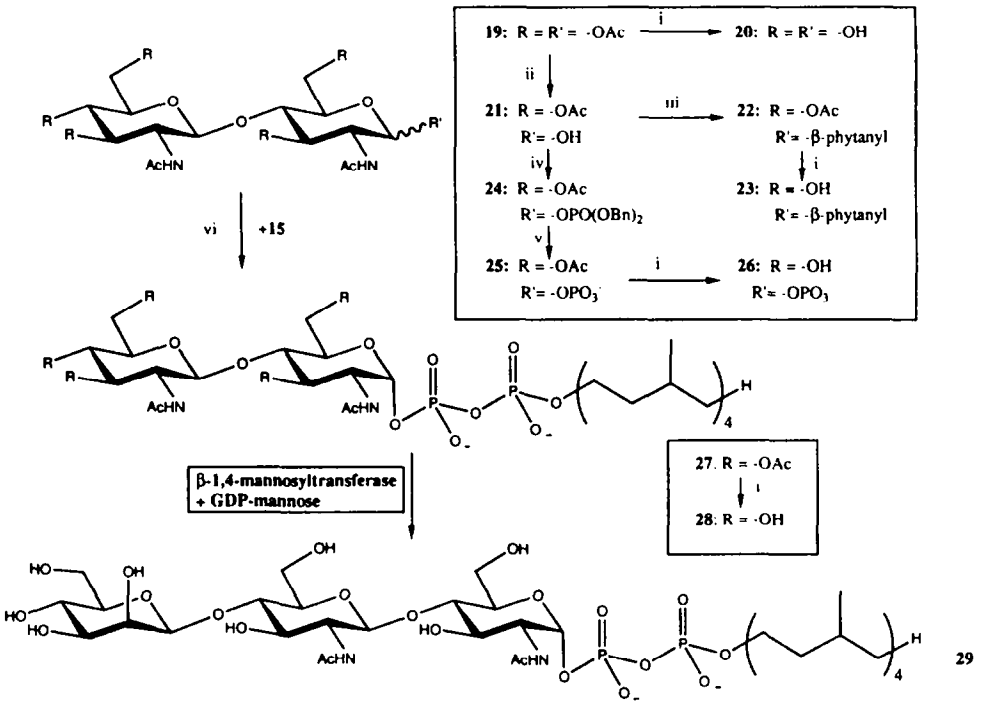
15: R = *phytanyl*

16: R = $C_{14}H_{29}$

17: R =

18: R = $-C_{14}H_{29}$

Scheme II. Enzymatic mannosylation of lipid phosphates.



Scheme III. Chemoenzymatic synthesis of a trisaccharyl pyrophosphate lipid and synthesis of substrate analogues of β-1,4-mannosyltransferase. *Reagents*: i. NaOMe, MeOH; ii. NH_2NH_2-HOAc, DMF, 50 °C; iii. LDA, THF; phytanyltriflate CH_2Cl_2; iv. LDA, THF; $(BnO)_2PO(OBn)_2$; v. H_2, Pd/C, MeOH; vi. carbonyldiimidazole; MeOH; phytanylphosphate (**15**).

Compounds **20**, **23** and **26** were compared with **28** as acceptors for the β-1,4-mannosyltransferase using radiolabelled GDP-mannose by determining the incorporation of radiolabeled mannose into lipid fractions. In initial experiments, yeast microsomes were used as the enzyme source for the comparison of the phytanyl glycoside **23** with $PPGn_2$ **28** (Table 1). It appeared that the phytanyl glycoside **23** did not function as a substrate for yeast mannosyltransferases in any significant way and did not inhibit mannosyl transfer to **28**.

Table 1. Incorporation of radiolabel into lipid fractions when substrate analogues **23** and **28** were incubated with radiolabelled GDP-[2-^{3}H]-mannose and yeast microsomes. The levels of incorporations are relative to the value obtained for **28**. Typical numbers of counts for these experiments were 10,000 dpm with background values of 700 dpm

Exogeneous Acceptor	Incorporation dpm (%)
none	6
28 (10μM)	100
23 (10μM)	7
23 (100μM)	8
28 (10μM) + **23** (10μM)	97

Subsequently, crude lysates of *E. coli* expressing the yeast β-1,4-mannosyltransferase (ALG1) gene and lacking the transmembrane domain[21] were used in inhibition studies with **20**, **23** and **26** against **28** as substrate. The results (Table 2) suggest that the phytanyl glycoside **23** may act as a poor inhibitor at concentrations in excess of $PPGn_2$ (**28**). These results, together with those indicating the phytanyl glycoside is not a substrate in any significant way for the yeast enzyme, suggest that β-1,4-mannosyltransferase requires a pyrophosphate bridge in the substrate. In addition, the results with chitobiose (**20**) and chitobiose phosphate (**26**), which appear not to inhibit incorporation of mannose into lipid, indicate the necessity of the lipid chain, even though a form of the enzyme lacking the transmembrane domain was active. The data suggest that $PPGn_2$ (**28**) is probably the most convenient analogue, having all the required features of the natural dolichyl linked substrate of β-1,4-mannosyltransferase.

Table 2. Incorporation of radiolabel into lipid fractions when **28** (10 μM) was incubated in the presence of other substrate analogues **20**, **23** and **26** with radiolabelled GDP-[2-^{3}H]-mannose and *E. coli* extracts containing the recombinant truncated β-mannosyltransferase. The levels of incorporations are relative to the value obtained for **28** only (= 100%). Typical numbers of counts for these experiments were 10,000 dpm with background values (no acceptor) of 200 dpm

+ Substrate Analogue	Incorporation dpm (%)
none	100
26 (10μM)	106
26 (100μM)	106
20 (10μM)	87
20 (50μM)	86
23 (10μM)	89
23 (50μM)	77

Conclusions

The oligosaccharide headgroups of neoglycosphingolipids can be synthesised using glycosyltransferases which *in vivo* are either involved in glycoprotein or glycolipid biosynthesis. These neoglycosphingolipids, rather than their natural glycosyl ceramides, were synthesised due to the limitations of substrate specificity of readily available glycosyltransferases and were then chemically converted to the natural ceramides. In contrast, with studies on the enzymes of N-linked oligosaccharide biosynthesis, flexibility in the requirements for the lipid moiety has been investigated so as to circumvent problems associated with utilising dolichyl-linked substrates in chemoenzymatic synthesis. In addition to this, an overexpressed form of the β-1,4-mannosyltransferase has been used to improve the synthesis of the core trisaccharide of N-linked glycans.

The advantages of glycosyltransferases lie not only in their regio- and stereoselectivity but also in their ability to glycosylate complex glycoconjugates, such as glycoproteins and, as shown here, glycolipids. This allows us to design different strategies from that of the chemical synthesis, where large aglycons can reduce the yields of the chemical transformations, especially in the formation of glycosidic bonds.

Experimental

3,7,11,15-Tetramethylhexadecanol (phytanol) and phytanyl phosphate (**15**) were prepared from phytol (Aldrich Chemical Company, Gillingham, U.K.) and 2-acetamido-4-*O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-3,6-di-*O*-acetyl-2-deoxy-D-glucopyranose (chitobiose hepta-acetate) (**21**) was prepared from commercially available chitobiose octa-acetate (Dextra Laboratories, Reading, U.K.) as described previously.[19] TLC was performed on Merck aluminium backed Silica Gel 60 F254 plates purchased from BDH (Poole, U.K.). All other reagents and chemicals were of the highest quality grade available.

2-Acetamido-4-O-(2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosyl)-3,6-di-O-acetyl-2-deoxy-D-glucopyranose 1-α-phosphate dibenzyl ester (hepta-acetyl chitobiose 1-α-phosphate dibenzyl ester)[20] *(24)*

To a stirred suspension of chitobiose hepta-acetate (**21**) (0.32 g, 0.505 mmol) in THF (24 mL), under a stream of argon, at –72 °C, was added a 2 M solution of lithium diisopropylamide in heptane:THF:ethylbenzene (1:1:1, v/v/v, 0.355 mL). The mixture was stirred at this temperature for 45 min, whereupon a solution of tetrabenzyl pyrophosphate (Fluka Chemicals Ltd, Gillingham, U.K.) (0.342 g, 0.634 mmol) in THF (10 mL) was added. The reaction was stirred for 4 h at this temperature and then allowed slowly to warm to room temperature. The solvents were removed under reduced pressure and the crude material was purified by flash chromatography to yield 0.361 g (80 %) of the desired compound. Mp 139–141 °C (lit.[23] 132–137 °C); ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.69–2.12 (21H, 7 × s, CH_3CO), 3.63 (1H, m, H-5'), 3.74 (1H, t, H-4), 3.80 (1H, q, H-2'), 3.96 (1H, m, H-5), 4.00 (1H, dd, H-6''), 4.12 (1H, dd, H-6), 4.27 (2H, m, H-6', H-2), 4.37 (1H, dd, H-6'''), 4.60 (1H, d, $J_{1'-2'}$ = 8.5 Hz, H-1'), 5.04 (1H, q, H-4'), 5.15 (1H, dd, H-3), 5.23 (1H, dd, H-3'), 5.64 (1H, d, $J_{1,2}$ = 3 Hz, H-1), 5.72 (1H, d, N–H), 6.04 (1H, d, N–H), 7.37 (10H, m, Ph); ^{13}C NMR (125 MHz, $CDCl_3$): δ 20.52 (3 × CH_3), 20.72 (2 × CH_3), 22.64 (CH_3), 23.03 (CH_3), 52.03 (CH_2), 55.02 (CH_2), 61.47 (CH_2), 61.82 (CH_2), 68.30 (CH), 69.80 (CH), 69.90 (CH), 70.19 (CH), 70.67 (CH), 71.90 (CH), 72.42 (CH), 75.49 (CH), 96.07 (CH), 101.01 (CH), 127.93 (2 × CH), 127.98 (2 × CH), 128.71 (2 × CH), 128.77 (2 × CH), 128.85 (2 × CH), 135.32 (2 × C), 169.22 (C=O), 170.22 (C=O), 170.37 (C=O), 170.54 (C=O), 170.68 (C=O), 170.79 (C=O), 171.01 (C=O); ^{31}P NMR (101 MHz, $CDCl_3$): δ –1.60; HRMS, m/z calcd for $C_{40}H_{51}N_2NaO_{19}P$ 917.2706, found 917.2721 $(M + Na)^+$.

P^1-Phytanyl-P^2-[2-acetamido-4-O-(2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosyl)-3,6-di-O-acetyl-2-deoxy-α-D-glucopyranosyl]-pyrophosphate (27)

A solution of hepta-acetyl chitobiose 1-α-phosphate dibenzyl ester (**24**) (0.200 g, 0.219 mmol) in a mixture of dry DCM (8 mL) and dry MeOH (12 mL) was hydrogenated in the presence of 5 % palladium on carbon (0.3 g). After 3 h, the mixture was filtered through Celite and the solution was concentrated to dryness. The residue was co-evaporated with toluene (3 × 3 mL) to give 0.15 g (96 %) of the phosphate monoester **25**. This crude phosphate monoester was dissolved in dry MeOH (5 mL) under argon. Pyridine (1 mL) was added and the solution was stirred for 5 min before being concentrated to dryness. The residue was redissolved in dry MeOH (5 mL) and dry triethylamine (2 mL) was added. This solution was stirred for 5 min before the solvents were removed under reduced pressure. The residue was then dried by co-evaporation with toluene (3 × 3 ml). The preformed triethylammonium salt of **25** was dissolved in dry DMF (8 mL) and a solution of *N,N'*-carbonyldiimidazole (0.183 g, 1.126 mmol) in dry DMF (7 mL) was added. The reaction was stirred for 4.5 h, under argon. After this time, MeOH (0.079 mL, 1.95 mmol) was added to quench any remaining carbonyldiimidazole. After stirring the solution for 30 min, a solution of phytanyl phosphate triethylammonium salt (**15**) (0.131 g, 0.273 mmol) in dry DCM (6 mL) was added and this mixture was left to stir for 45 h under argon. The solvents were then removed under reduced pressure and the residue was co-evaporated with toluene (3 × 3 mL). The crude material was purified by flash chromatography (eluant $CHCl_3$:MeOH:H_2O (80:20:0.5) to $CHCl_3$:MeOH:H_2O (60:40:0.5) to give 0.161 g (60 %) of the title compound **27** as a white solid. ^{1}H NMR (500 MHz, $CDCl_3$:CD_3OD, 1:1, v/v): δ 0.98–1.43 (36H, m, alkyl H), 1.47–1.70 (3H, m, alkyl H), 1.82–2.12 (21H, 7 × s, CH_3CO), 3.63 (1H, m, H-2'), 3.71 (1H, dd, H-5'), 3.84 (1H, t, H-4), 3.95 (2H, t, CH_2O - phytanyl), 4.01 (1H, dd, H-6''), 4.06 (1H, dd, H-6), 4.19 (2H, m, H-5, H-2),

4.38 (1H, dd, H-6'''), 4.50 (1H, obscured s, H-6'), 4.70 (1H, d, $J_{1',2'}$ = 8 Hz, H-1'), 4.95 (1H, t, H-4'), 5.26 (2H, m, H-3, H-3'), 5.50 (1H, d, $J_{1,2}$ = 3 Hz, H-1); ^{13}C NMR (125 MHz, $CDCl_3$:CD_3OD, 1:1, v/v): δ 19.56 (CH_3), 19.87 (CH_3), 19.99 (2 × CH_3), 20.60 (CH_3), 20.65 (CH_3), 20.73 (CH_3), 20.92 (CH_3), 20.98 (CH_3), 22.71 (CH_3), 22.86 (d, CH_3), 23.12 (CH_3), 24.92 (CH_2), 24.98 (CH_2), 25.29 (CH_2), 28.49 (CH), 29.82 (CH), 32.42 (CH), 33.34 (CH), 37.85 (CH_2), 37.99 (2 × d, 2 × CH_2), 38.10 (CH_2), 38.18 (CH_2), 39.95 (2 × CH_2), 52.64 (CH_2), 55.79 (CH_2), 62.63 (2 × CH), 65.61 (CH_2), 69.43 (CH), 70.24 (CH), 72.15 (2 × CH), 72.94 (CH), 76.15 (CH), 95.00 (CH), 101.24 (CH), 170.62 (C=O), 171.31 (C=O), 171.61 (C=O), 172.08 (C=O), 172.80 (C=O), 172.86 (C=O), 173.14 (C=O); ^{31}P NMR (101 MHz, $CDCl_3$:CD_3OD): δ −13.43, −15.98; HRMS, m/z calcd for $C_{46}H_{79}N_2O_{22}P_2$ 1073.4599, found 1073.4600 $(M+H)^-$.

P^1-Phytanyl-P^2-[2-acetamido-4-O-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-2-deoxy-α-D-glucopyranosyl]-pyrophosphate (triethylammonium salt) (28)

To a stirred solution of P^1-phytanyl-P^2-[2-acetamido-4-*O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-3,6-di-*O*-acetyl-2-deoxy-α-D-glucopyranosyl]-pyrophosphate (**27**) (2 mg, 1.57 mmol) in freshly distilled DCM (2 mL) and anhydrous MeOH (1 mL), under balloon pressure of argon, at 4 °C, was added dry Et_3N (0.25 mL). This solution was allowed to stir for 5 min before the solvents were removed under reduced pressure and the residue was dried by co-evaporation with toluene (3 × 2 mL). Subsequently, to a solution of this triethylammonium salt in dry DCM (2 mL) was added a freshly prepared 7 % (w/v) solution of sodium methoxide in MeOH (0.05 mL). After stirring for 1 h at this temperature, TLC [$CHCl_3$:MeOH:2M NH_4OH:H_2O 65:35:4:4 (v/v/v/v)] showed that the reaction was complete (R_f starting material = 0.43; R_f product = 0.11). The reaction was neutralised by the addition of Bio-Rad (Richmond, CA, U.S.A.) AG 50W-X8 ion exchange resin (H^+ form). The resin was removed by filtration and washed with MeOH. The combined filtrate and washings were then evaporated and the residue was dried by co-concentrations with toluene (3 × 2 mL). The solid obtained was redissolved in dry MeOH (2 mL) and dry DCM (1 mL) and kept under argon, at room temperature. Freshly distilled triethylamine (0.25 mL) was added and the mixture was stirred for 5 min. The solvents were removed under reduced pressure and the white solid was co-concentrated with toluene (3 × 2 mL) to give 1.7 mg (100 %) of the triethylammonium salt of the desired product **28**. ^{1}H NMR (500 MHz, D_2O): δ 0.81–1.66 (39H, complex overlapping m, alkyl H), 2.01 (3H, s, CH_3CO), 2.02 (3H, s, CH_3CO), 3.46–4.00 (14H, complex overlapping m, alkyl and disaccharide H), 4.51 (1H, d, $J_{1',2'}$ = 8.4 Hz, H-1'), 5.50 (1H, d, $J_{1,2}$ = 3.4 Hz, H-1); ^{31}P NMR (101 MHz, D_2O): δ −13.12 (d, J_{PP} = 15 Hz), −15.74 (d, J_{PP} = 15 Hz).

2-Acetamido-4-O-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-2-deoxy-D-glucopyranosyl 1-α-phosphate (triethylammonium salt) [N,N'-diacetyl chitobiose 1-α-phosphate, (triethylammonium salt)] (26)

Hepta-acetyl chitobiose 1-α-phosphate dibenzyl ester (0.090 g, 0.10 mmol), was hydrogenated in the presence of Pd/C (0.09 g) in MeOH (4 mL) and DCM (2 mL), as described above. After removal and subsequent washing of the catalyst, the combined filtrate and washings were concentrated to dryness and the residue was dried by co-concentration with toluene (3 × 2 mL) to give 0.072 g (100 %) of the phosphate monoester as a white solid. This solid was dissolved in MeOH (2 mL) and placed under argon. The solution was treated with Et_3N (0.025 mL) for 5 min, before the solvents were removed by rotary evaporation. The residue was then dried by co-evaporation with anhydrous toluene (3 × 2 mL). The triethylammonium salt of peracetylated chitobiose 1-α-phosphate (**25**) (0.072 g, 0.10 mmol) was dissolved in a freshly prepared 7 % (w/v) solution of sodium methoxide in MeOH (3 mL). The reaction was allowed to stir at room temperature for 2 h. The reaction solution was then neutralised by the addition of AG 50W-X8 (H^+) ion exchange resin. The resin was filtered off and washed with MeOH (15 mL). The combined filtrate and washings were concentrated to dryness and the residue was dried by co-evaporation with anhydrous toluene (3 × 2 mL). The residue was then dissolved in water (5 mL) and Et_3N (1 mL) was added. After stirring the reaction for 15 min, the solvents were removed by rotary evaporation and the residue was dried by co-concentration with anhydrous toluene (3 × 2 mL). The crude material was purified by reverse-phase flash chromatography (Sorbsil C200 Silica Gel RP18, obtained from BDH) with water as the eluant. Relevant fractions were combined and lyophilised to give 0.049 g (70 %) of the title compound **26** as a white solid. $[\alpha]^{25.5}_D$ +43° (c 0.45, MeOH:water, 1:1);[23] ^{1}H NMR (500 MHz, D_2O): δ 1.97 (3H, s, $COCH_3$), 1.99 (3H, s, $COCH_3$), 3.41 (2H, m), 3.50 (1H, dt), 3.59 (2H, m), 3.68 (2H, m), 3.74 (1H, dd), 3.86 (4H, m), 4.53 (1H, d, $J_{1',2'}$ = 8.5 Hz, H-1'), 5.31 (1H, dd, $J_{1,2}$ = 2.7 Hz, $J_{H,P}$ = 7.4 Hz, H-1); ^{13}C NMR (125 MHz, D_2O): δ 22.39 (CH_3), 22.51 (CH_3), 53.83 (d, CH), 56.01 (CH), 60.30 (CH_2), 60.93 (CH_2), 69.90 (CH), 70.09 (CH), 71.25 (CH), 73.86 (CH), 76.27 (CH), 79.71 (CH), 93.12 (CH), 101.74 (CH), 174.98 (C=O), 174.98 (C=O); ^{31}P NMR (101 MHz, D_2O): δ 0.70.

Phytanyl trifluoromethane sulphonate (phytanyl triflate)

To a stirred solution of triflic anhydride (0.705 g, 0.420 mL, 2.5 mmol) and phytanol (0.746 g, 2.5 mmol) in anhydrous DCM (2 mL), at 0 °C, under an argon atmosphere, was added a solution of pyridine (237 mg, 0.243 mL, 3.0 mmol) in DCM (6 mL). After 10 min, the reaction was diluted with DCM (50 mL) and washed with 0.5 M HCl (20 mL), saturated aqueous NaCl (20 mL) and water (20 mL). The organic phase was dried over anhydrous $MgSO_4$ and the solvent removed under

reduced pressure to obtain 1.041 g (97 %) of the crude product. ^{1}H NMR (200 MHz, $CDCl_3$): δ 0.74–2.10 (39H, m), 4.70 (2H, m).

2-Acetamido-4-O-(2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosyl)-3,6-di-O-acetyl-2-deoxy-β-D-glucopyran-ose 1-O-phytanol (22)

To a solution of chitobiose hepta-acetate (**21**) (0.25 g, 0.39 mmol, dried over P_2O_5 under vacuum for 18 h prior to use) in dry THF (5 mL) under an argon atmosphere, at −78 °C, was added a 2 M solution of lithium diisopropylamide in heptane:THF:ethylbenzene (1:1:1, v/v/v) (0.25 mL, 0.50 mmol). After stirring at this temperature for 1 h, a solution of phytanyl triflate (181 mg, 0.42 mmol) in dry DCM (5 mL) was added. The reaction mixture was then kept in the temperature range −78 °C to −30 °C (CO_2-acetone) for 48 h. After this time, the mixture was diluted with DCM (50 mL) and washed with saturated aqueous NaCl solution (50 mL). The organic phase was removed, dried over anhydrous $NaSO_4$ and then evaporated under reduced pressure. The desired product was purified by column chromatography (eluant DCM:MeOH, 10:1 and subsequently, $CHCl_3$:MeOH, 30:1) to give 0.256 g (55 %) of the pure glycoside **22**, as an oil. ^{1}H NMR (500 MHz, $CDCl_3$): δ 0.84–0.90 (15H, m, 5 × CH_3), 1.19–1.65 (24H, m), 1.94–2.15 (21H, m, 7 × CH_3CO), 3.44–3.50 (2H, m, CH_2O), 3.62–3.68 (2H, m, H-5, H-5'), 3.73 (1H, dd, $J_{4,3}$ = 8 Hz, $J_{4,5}$ = 8 Hz, H-4), 3.83–4.06 (6H, m), 4.47 (1H, d, $J_{1,2}$ = 8 Hz, H-1), 4.55 (1H, d, $J_{1',2'}$ = 8 Hz, H-1'), 5.05 (1H, dd, $J_{4',3'}$ = 10 Hz, $J_{4',5'}$ = 10 Hz, H-4'), 5.13 (1H, dd, $J_{3,4}$ = 8 Hz, $J_{3,2}$ = 8 Hz, H-3), 5.19 (1H, dd, $J_{3',4'}$ = 9 Hz, $J_{3',2'}$ = 9 Hz, H-3'), 5.68 (1H, d, J = 8 Hz, N-H), 5.98 (1H, J = 8 Hz, N-H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 19.68 (2 × CH_3), 20.57 (2 × CH_3), 20.87 (CH_3), 22.65 (2 × CH_3), 23.14 (3 × CH_3), 24.48 (2 × CH_3), 24.78 (2 × CH_2), 27.97 (2 × CH_2), 29.68 (2 × CH_2), 29.93 (2 × CH), 32.83 (2 × CH_2), 36.57 (2 × CH), 37.46 (3 × CH_2), 39.40 (CH), 53.51 (CH), 54.90 (CH), 61.94 (CH_2), 62.68 (CH_2), 68.12 (CH), 68.38 (CH), 72.03 (CH), 72.30 (CH), 73.00 (CH), 99.84 (CH), 100.98 (CH), 169.27 (C=O), 170.03 (C=O), 170.46 (3 × C=O), 170.78 (2 × C=O); HRMS, m/z calcd for $C_{46}H_{79}N_2O_{16}$ 915.5430, found 915.5529 $(M + H)^+$.

2-Acetamido-4-O-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-2-deoxy-β-D-glucopyranose 1-O-phytanol (23)

To a stirred solution of 14 % NaOMe in MeOH (2 mL), under an argon atmosphere, was added a solution of hepta-acetyl chitobiose 1-*O*-phytanol (**22**) (0.03 g, 0.03 mmol) in dry DCM (7 mL). At the end of 60 min, excess cation exchange resin (Dowex 50W-X8, H^+ form) was added and this mixture was stirred for 5 min. The resin was filtered off and washed with $CHCl_3$. The combined washings were concentrated under reduced pressure to give 0.024 g (100 %) of the title compound **23** as an oil. ^{1}H NMR (500 MHz, $CDCl_3$): δ 0.81–0.90 (15H, m, 5 × CH_3), 1.04–1.60 (24H, m), 1.95–2.00 (6H, 2 × s, CH_3CO), 3.30–4.07 (1H, m), 4.36 (1H, d, $J_{1,2}$ = 8 Hz, H-1), 4.45 (1H, d, $J_{1',2'}$ = 8 Hz, H-1'), 4.56–4.67 (3H, m).

Assay conditions

Crude yeast microsomes and pellets of recombinant *E. coli* were stored at −80 °C. After thawing and addition of DNase I and Triton X-100, mannosyltransferase activity was typically assayed at 37 °C by incubation of the cell lysate with final concentrations of 10 μM $PPGn_2$, (triethylammonium salt) (**28**), 20 μM GDP-[2-^{3}H]-mannose (0.2 μCi/mL), 1 % (v/v) Triton X-100 in 50 mM Tris–Cl, pH 7.5, 5 mM magnesium chloride, 10 mM 2-mercapto-ethanol (final volume of 1 mL). Incubations were performed in Reactivials with continuous stirring in a Pierce Reacti-Therm heating/stirring module. Aliquots (100 μL) were removed at time intervals and the reaction quenched by an equal volume of 1:1 (v/v) chloroform:methanol. The aqueous phase was removed after centrifugation, and the organic phase and particulate material at the interface were washed twice with one volume of water and one half-volume of 1:1 (v/v) chloroform:methanol. The chloroform phase was retained and the particulate material washed with 10:10:3 (v/v/v) chloroform:methanol:water. The organic phases were combined and the incorporation measured by scintillation counting (Ultima Gold scintillation fluid; LKB scintillation counter). After this procedure, minimal counts were retained in the pellet. Previous results have demonstrated by gel filtration chromatography and β-mannosidase digestion the synthesis of a β-mannosyl linkage attached to the oligosaccharide moiety of $PPGn_2$,[21] and have thus suggested that **29** had been formed.

Acknowledgements

We are grateful to the SERC for providing Grant support (to S.L.F.) and a Quota studentship (to L.R.). We thank Mrs E. McGuinness and Dr R. Aplin for recording NMR and mass spectra respectively and the SERC Mass Spectrometry Service Centre at the University College of Swansea for accurate mass measurements.

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(*Received in U.S.A.* 15 *February* 1994; *accepted* 16 *April* 1994)