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The chemoenzymatic synthesis of the core trisaccharide of N-linked oligosaccharides using a recombinant β-mannosyltransferase

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Abstract

The chemical synthesis of the β-mannosyl linkage of N-glycans has presented a great challenge to synthetic carbohydrate chemists. We have therefore investigated the application of β-mannosyltransferases to the preparative synthesis N-linked core oligosaccharides. In this paper we report the chemoenzymatic synthesis of β-D-mannopyranosyl-(1 → 4)-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1 → 4)-2-acetamido-2-deoxy-α-D-glucopyranose on a preparative scale using a phytanyl-linked acceptor in the presence of a recombinant β-(1 → 4)-mannosyltransferase. © 1998 Elsevier Science Ltd.

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1. Introduction

The oligosaccharide side-chains of glycoproteins are ubiquitous in nature and can play many rôles in biological activity of the parent protein. [1] Although N-linked oligosaccharides are structurally diverse, almost all contain the conserved pentasaccharide core (**1**). Because core oligosaccharides are difficult to obtain from natural sources, there has been a lot of effort to synthesise them for studies of structure and function relationships. The chemical synthesis of the pentasaccharide (**1**) presents a particular challenge because it contains a β-(1 → 4) mannosidic linkage to *N*-acetylglucosamine. Several strategies have been used to form this linkage chemically: by epimerisation of β-D-glucosides via inversion at C-2, [2,3] or sequential oxidation–reduction steps at C-2, [4,5] by reaction of α-mannosyl donors in the presence of insoluble promoters, [6] and by intramolecular aglycon delivery. [7–12] More recently, β-mannosides have been synthesized using α-mannosyl sulfoxides, [13] 1,2-*O*-*cis*-stannylene acetals of mannose, [14] and by the selective reductive cleavage of glycosylidene acetals of mannose. [15,16] Although these are

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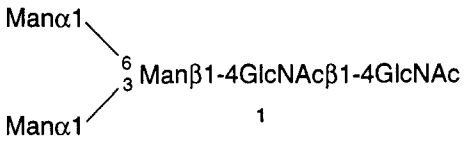
very elegant methods, they still require multistep protection and activation and leave scope for enzymatic methods. Thus, glycosidases have recently [17–19] been used for the synthesis of the β-mannosyl-linked trisaccharide (**2**). This method has the advantage of using simple starting materials, although a large excess of the expensive chitobiose acceptor is required and yields are low. As an alternative method, we have investigated the use of glycosyltransferases, in particular the β-mannosyltransferase [20–23] for the preparation of the core trisaccharide (**2**). The gene for this mannosyltransferase was isolated by Robbins and colleagues and was expressed at a low level in *Escherichia coli*. [24,25] The enzyme had several properties that made it unsuitable for immediate use in practical synthesis: its low availability in natural sources and its low stability, which makes isolation and purification difficult. The analysis of the polypeptide sequence suggested an N-terminal hydrophobic membrane anchor, and the need to complex lipid acceptors containing dolichol pyrophosphate aglycon groups. We have shown previously [26] that smaller more available lipids such as phytanol (**4**), which contains four isoprenoid units may be used in place of dolichol but that smaller alkyl glycosides or glycosyl phosphates are not accepted as substrates. [27] In this paper we report on the improved chemical synthesis of a phytanyl-linked acceptor substrate and its subsequent mannosylation using a recombinant β-mannosyltransferase to prepare the β-mannosyl-linked trisaccharide (**2**).



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Manβ1-4GlcNAcβ1-4GlcNAc

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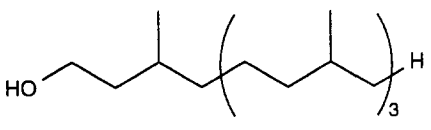

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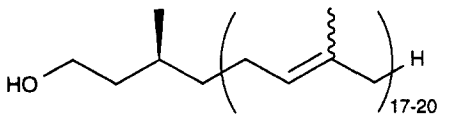

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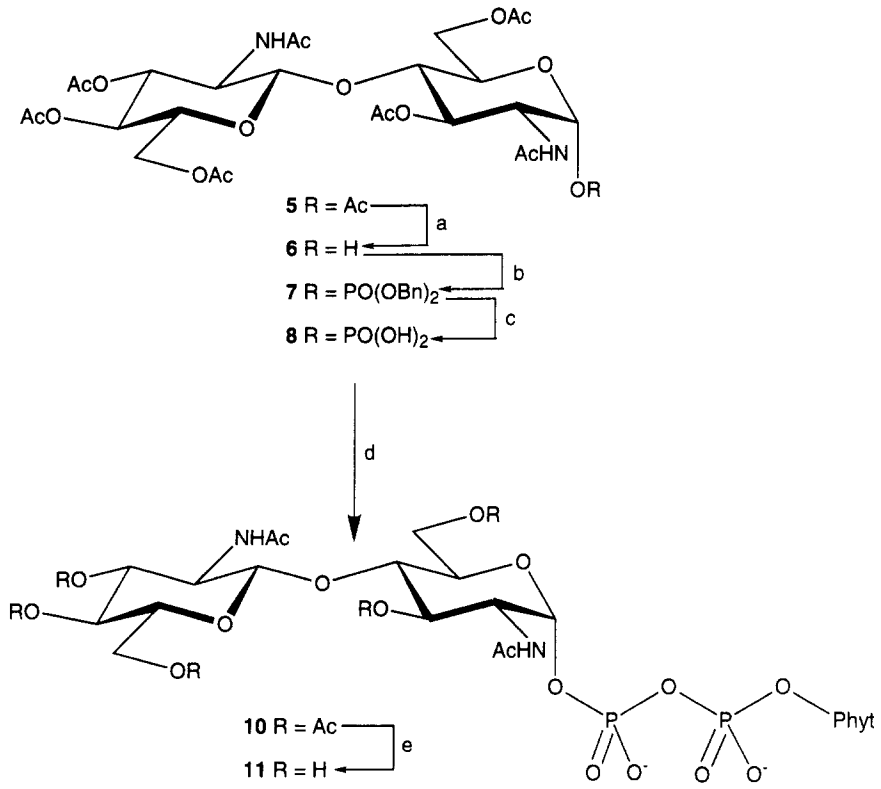
2. Results and discussion

Chemical synthesis of phytanyl-linked substrate acceptor (PPGn2) (**11**).—An efficient synthesis of the acceptor substrate in this case PPGn2, (**11**) is important for the application of glycosyltransferases. Several improvements have been made on the previously reported method, [27] which now allows efficient preparation from commercially available chitobiose octaacetate (**5**) as outlined in Scheme 1. The selective anomeric deacetylation of chitobiose octaacetate (**5**) could be markedly improved compared to previously reported [22] methods by using dimethylamine in Me_2NCHO to afford in quantitative yield the emiacetal disaccharide (**6**). This was treated with lithium diisopropylamide in tetrahydrofuran at −78 °C with the subsequent in situ addition of tetrabenzyl pyrophosphate to afford selectively the α-glycosyl phosphate dibenzyl ester (**7**) in 89% yield. Hydrogenolysis of the benzyl groups was achieved in the presence of 10% palladium on charcoal under an atmosphere of hydrogen gas to give heptaacetyl chitobiosyl phosphate (**8**) in 98% yield.

Conversion of phosphate (**8**) to its triethylammonium salt was followed by treatment with *N,N'*-carbonyl diimidazole in Me_2NCHO to form an intermediate imidazole-carbonyl phosphate anhydride. The addition of methanol to quench any excess *N,N'*-carbonyl diimidazole was followed by the addition of phytanyl phosphate (**9**) to afford after flash-column chromatography the phytanyl pyrophosphoryl chitobioside (**10**) in 77% yield. Saponification of the protected pyrophosphate derivative using sodium methoxide gave the acceptor substrate PPGn2 (**11**) in 97% yield. Thus, the overall yield of PPGn2 (**11**) from chitobiose was 65% compared to 45% reported previously, [27] which in yield is comparable to the synthesis of simple alkyl glycosides.

Construction of the β-mannosyltransferase expression vector.—The gene for the β-mannosyltransferase from yeast (*ALG1*) was kindly provided by Prof. P. Robbins [25]. Due to the poor expression of *ALG1* in the heterologous host (*E. coli*) and subsequent problems with the isolation and stability of the enzyme it was decided to modify the gene by deleting the hydrophobic N-terminal sequence of amino acids 2–35 to create *ALG1ΔTM*. [28] The addition of the myc-polypeptide (EQKLISEEDL) and a decahistidine sequence (His_{10}) to the N-terminus allowed the detection of the enzyme by Western blotting using the anti-Myc monoclonal antibody [29] and protein purification by metal



Scheme 1. The chemical synthesis of the substrate acceptor PPGn2 (**11**); reagents, conditions and yields: (a) dimethylamine in tetrahydrofuran, Me_2NCHO (100%); (b) lithium diisopropylamide, tetrahydrofuran; $O[PO(OBn)_2]_2$, −72°C (89%); (c) 10% palladium on carbon, hydrogen gas, MeOH, DCM (98%); (d) carbonyl diimidazole, Me_2NCHO; MeOH; phytanylphosphate (77%) (**9**); (e) NaOMe, MeOH (97%).

affinity chromatography. [30] Insertion of the new *His10mycALG1ΔTM* gene into the commercially available pET-16b overexpression vector (AMS Biotechnology, Whitney, Oxon, UK) generated the plasmid pLR36 shown in Fig. 1.

Preparation of immobilised β-mannosyltransferase enzyme.—A culture of *E. coli* (BL21(DE3)pLysS) cells harbouring the plasmid pLR36 yielded β-mannosyltransferase activity in cleared-cell lysates (obtained by freeze-thawing) of about 0.2 U/g of wet

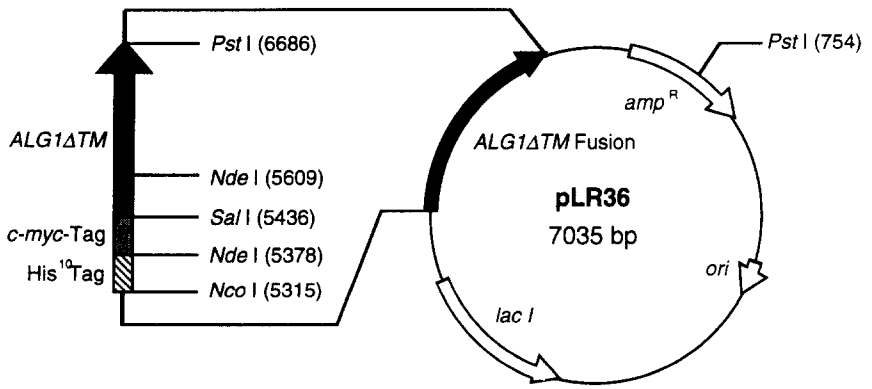
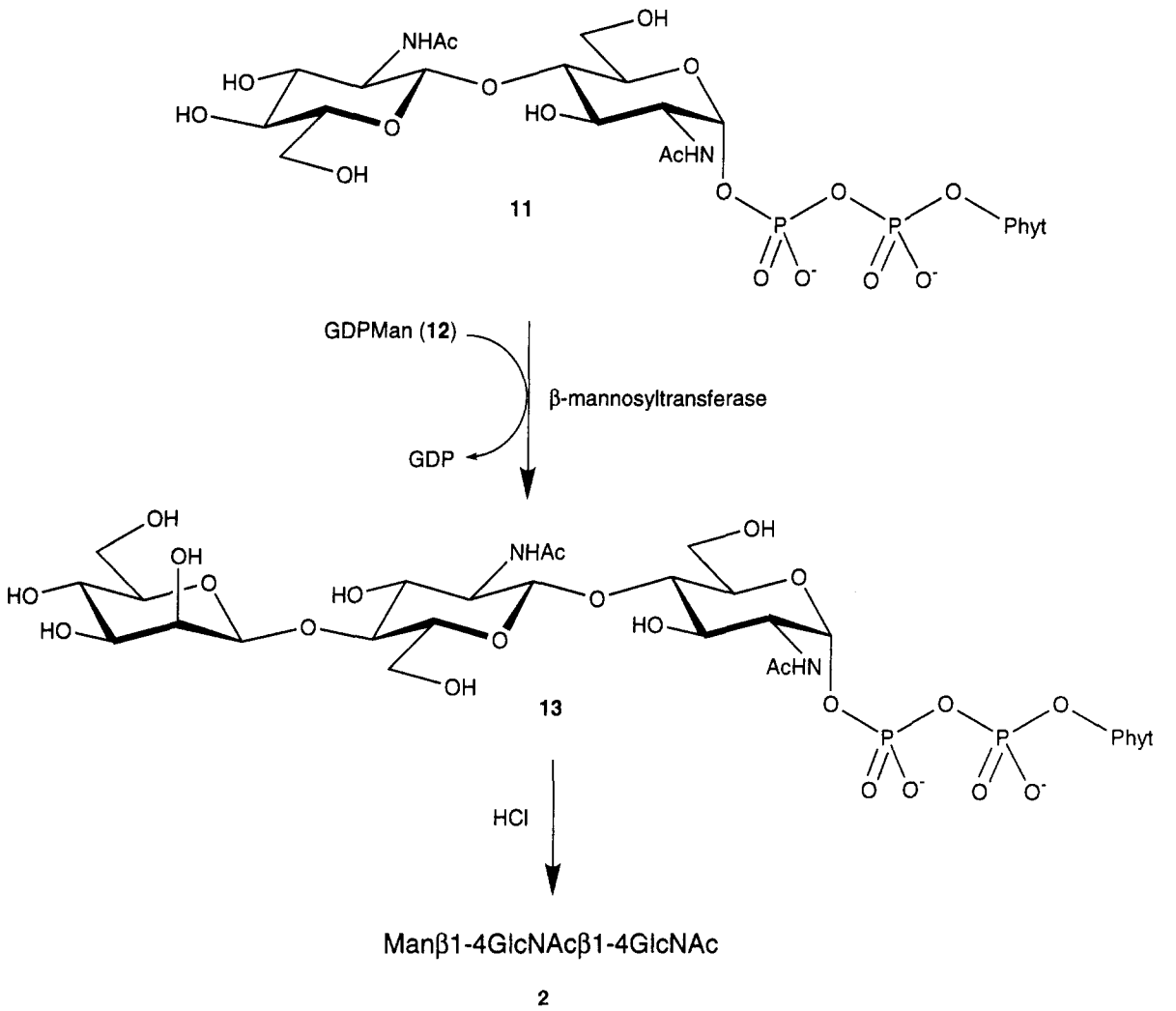


Fig. 1. The plasmid pLR36 containing the modified β-(1 → 4)-mannosyltransferase gene *His10mycALG1ΔTM* incorporated into the pET-16b overexpression vector.



Scheme 2. The formation of the mannose-linked trisaccharide (**2**) using immobilized β-(1-4)-mannosyltransferase.

cells. However, use of the β-mannosyltransferase for preparative synthesis at this stage was unsuitable, due to bacterial protein contaminants, which would make purification of the trisaccharide (**2**) difficult and cause a loss of transferase activity through the action of bacterial proteases.

Purification and immobilization the β-mannosyltransferase was achieved by passing the cleared cell lysate through a nickel (II) charged affinity column followed by washing to remove any impurities. The resulting functionally pure biocatalyst yielded a specific activity of about 35 mU per ml of resin and could be stored at 4 °C for several weeks without any significant loss of activity.

Synthesis of the β-mannosyl-linked trisaccharide (**2**).—The incubation of the phytanyl-linked acceptor substrate (**11**) with GDP-mannose (**12**) at 37 °C in the presence of the immobilised β-mannosyltransferase and alkaline phosphatase [31] gave the phytanyl-linked trisaccharide (**13**), which was subjected to acid hydrolysis without isolation (Scheme 2). Although alkaline phosphatase was used in large-scale mannosylations, its use in assays afforded no significant increase in GDP-mannose turnover. Extraction of the cleaved lipids with chloroform was followed by gel-permeation chromatography of the aqueous phase to give the β-mannosyl-linked trisaccharide (**2**). A yield of 80% was calculated by using radiolabelled GDP-mannose and comparing the combined counts in fractions eluting with (**2**) with those of unreacted mannose.

Characterisation of the β-mannosyl-linked trisaccharide (**2**) *by NMR*.—The ^{1}H NMR spectrum of compound (**2**) shown in Fig. 2 was identical to that of

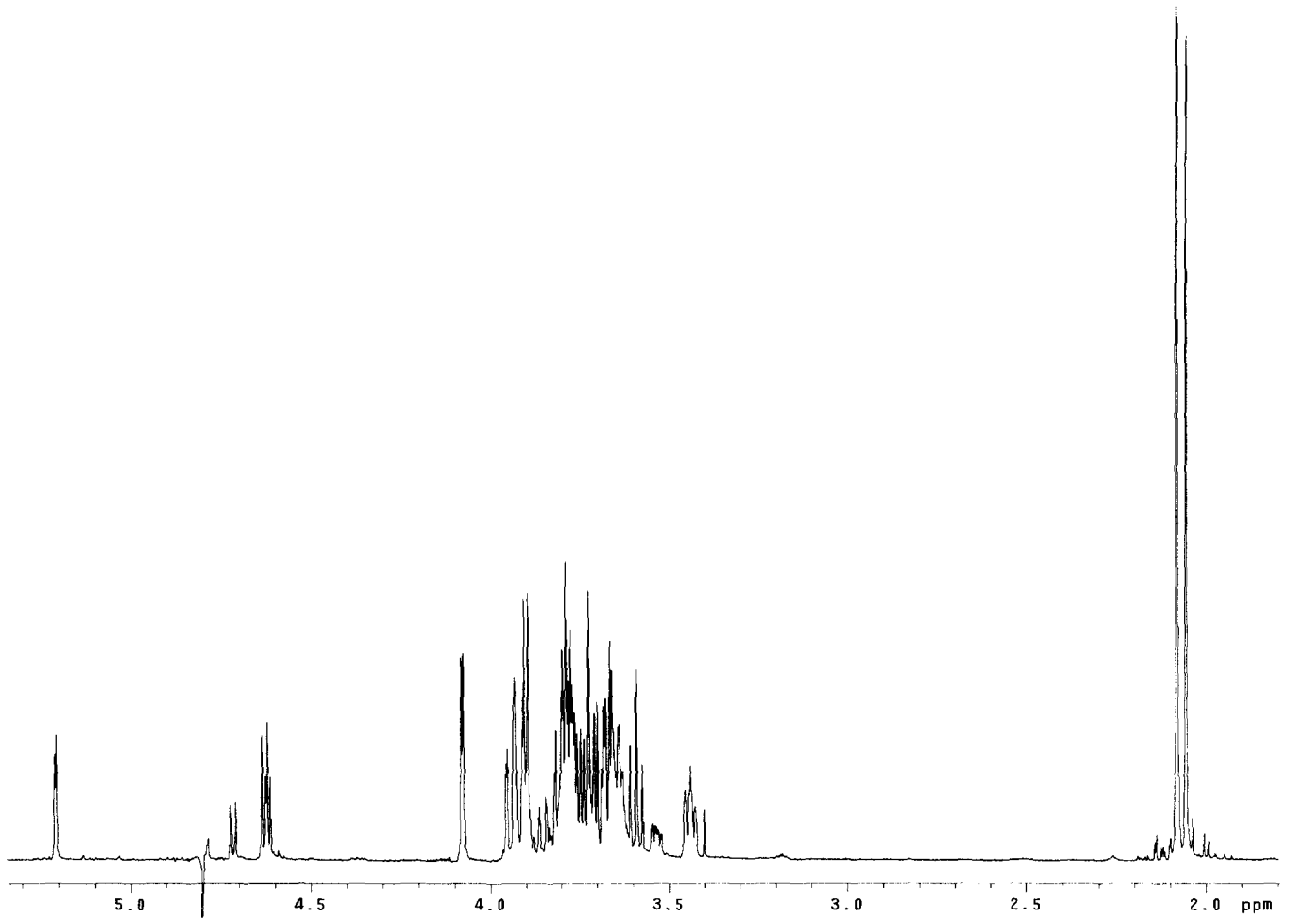


Fig. 2. The ^{1}H-NMR (600 MHz) spectrum of trisaccharide (**2**).

a sample of β-mannosyl-linked trisaccharide isolated from natural sources kindly provided by Dr. Tony Merry, Oxford Glycosciences, Oxford, UK. The ^{1}H NMR spectrum clearly revealed the β-mannosyl and the anomeric and protons. The reducing GlcNAc residue of compound (**2**) exists as a 2:1 mixture of α/β anomers resulting in the duplication of its signals and to a lesser extent those of the second GlcNAc. The overlapping signals of the two GlcNAc residues were assigned along with the carbon signals using HMQC NMR with the aid of published spectroscopic data of chitobiose [32] and of β-mannoside derivatives. [33]

Conclusions.—In conclusion the chemoenzymatic synthesis of β-D-mannopyranosyl-(1 → 4)-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1 → 4)-2-acetamido-2-deoxy-α-D-glucopyranose (**2**) was achieved both regio- and stereoselectively in good yield. The trisaccharide lipid (**13**) itself should be useful for providing biosynthetic intermediates for the studies of subsequent glycosyltransferases of the biosynthetic pathway of N-linked oligosaccharides [34] and for in vitro protein glycosylation [35,36].

3. Experimental

General methods.—NMR were carried out on Bruker WM 360 MHz and Varian INOVA 600 MHz machines. Melting points were recorded using Gallenkamp apparatus and are uncorrected. Optical rotations were obtained using an Optical Activity apparatus. Scintillation counting was carried out using a Packard Tri-Carb 2100TR scintillation analyzer. Chitobiose octaacetate was bought from Dextra Laboratories. GDP-[2-3H]mannose was bought from NEN Dupont. Other chemicals were obtained from Aldrich or Sigma.

Bacterial culture-growth media used were: LB (Luria Bertani) broth [1% w/v BACTO® tryptone, 1% w/v NaCl, 0.5% w/v BACTO® yeast extract, containing chloramphenicol (34 μg/mL) and carbenicillin (50 μg/mL)]; LB agar [1% w/v BACTO®

tryptone, 1% w/v NaCl, 0.5% w/v BACTO® yeast extract, 1.5% w/v agar, containing chloramphenicol (34 μg/mL) and carbenicillin (50 μg/mL)]; Fermentation medium [LB broth, containing chloramphenicol (34 mg/L), carbenicillin (17 mg/L) and ampicillin (30 mg/L)].

Buffers used were: Mannosyltransferase buffer [5 mM magnesium chloride, 50 mM Tris–HCl, 0.25% Triton X-100, pH 7.5]; Charge buffer [50 mM nickel sulfate]; Binding buffer [5 mM imidazole, 0.5 M NaCl, 20 mM Tris–HCl, 0.25% Triton X-100, pH 7.5].

2-Acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosyl-(1 → 4)-2-acetamido-3,6-di-O-acetyl-deoxy-D-glucopyranose (chitobiose heptaacetate) (**6**). —To a stirred and cooled (0 °C) solution of chitobiose octaacetate (1.0 g, 1.48 mmol) in Me_2NCHO (7.0 mL) was added a 2 M solution of dimethylamine (20 mmol) in tetrahydrofuran (10 mL), whereafter, stirring was continued for 1.5 h, TLC (9:1 chloroform/methanol) at that time showed that some starting material still remained. A further 10 mL of 2 M dimethylamine in tetrahydrofuran was added to the reaction which was left to stir at room temperature. After 19 h TLC revealed the formation of a single product. The solvents, excess reagent and dimethyl acetamide formed in the reaction were removed under reduced pressure and the residue was crystallised from dichloromethane and hexane to give the hemiacetal disaccharide (**6**) (0.935 g, 100%) as white crystals: mp 237 °C, lit. 245–247 °C [22]; $[\alpha]_D$ −30° (*c* 1, $CHCl_3$), lit. $[\alpha]_D^{25}$ −88° (*c* 1, $CHCl_3$) [22]; ^{1}H NMR (360 MHz) (2:1 $CDCl_3/CD_3OD$) (3:1 α/β ratio): δ 1.96–2.20 (21 H, 7 × $COCH_3$), 3.70 (m, H-5β), 3.80 (m, 2 H, H-4 and H-5′), 3.83 (dd, 1 H, $J_{1',2'}$ 8, $J_{2',3'}$ 10 Hz, H-2′), 3.90 (dd, $J_{1\beta,2\beta}$ 8, $J_{2\beta,3\beta}$ 10 Hz, H-2β), 4.09 (dd, 1 H, $J_{5',6a'}$ 2, $J_{6a',6b'}$ 12 Hz, H-6a′), 4.16 (m, H-5α, H-6a), 4.19 (dd, $J_{1\alpha,2\alpha}$ 4, $J_{2\alpha,3\alpha}$ 11 Hz, H-2α), 4.42–4.49 (m, 2 H, H-6b, H-6b′), 4.70 (d, $J_{1\beta,2\beta}$ 8 Hz, H-1β), 4.73 (d, $J_{1'\beta,2'\beta}$ 8 Hz, H-1′β), 4.74 (d, $J_{1'\alpha,2'\alpha}$ 8 Hz, H-1′α), 5.06 (dd, 1 H, $J_{3',4'}$ 9, $J_{4',5'}$ 10 Hz, H-4′), 5.13 (d, $J_{1\alpha,2\alpha}$ 4 Hz, H-1α), 5.30 (dd, $J_{2\beta,3\beta}$ 10, $J_{3\beta,4\beta}$ 9 Hz, H-3β), 5.32 (dd, 1 H, $J_{2',3'}$ 10, $J_{3',4'}$ 9 Hz, H-3′) and 5.35 (dd, $J_{2\alpha,3\alpha}$ 11, $J_{3\alpha,4\alpha}$ 9 Hz, H-3α).

2-Acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosyl-(1 → 4)-2-Acetamido-3,6-di-O-acetyl-2-deoxy-α-D-glucopyranosyl phosphate dibenzyl ester (heptaacetyl-chitobiosyl-1-α-phosphate dibenzyl ester) (**7**).—A cooled (−72 °C) and stirred suspension of the hemiacetal (**6**) (100 mg, 0.16 mmol) in anhydrous tetrahydrofuran (5.0 ml) was treated with a 2 M solution of lithium diisopropylamide (0.22 mmol) in 1:1:1 heptane/tetrahydrofuran/ethylbenzene (111 μL) for 45 min at −72 °C, whereafter, a solution of tetrabenzyl pyrophosphate (119 mg, 0.221 mmol) in tetrahydrofuran (2.0 mL) was added. Stirring was continued at −72 °C for 4 h, after which time the reaction mixture was slowly allowed to attain room temperature. The solvent and diisopropylamine formed in the reaction were removed under reduced pressure. Flash-column chromatography (1:24 methanol/chloroform as eluent) of the residue afforded the title compound (**7**) as a white solid (125 mg, 89%); $[\alpha]_D^{25}$ −15° (*c* 1.1, $CHCl_3$); ^{1}H NMR (360 MHz) ($CDCl_3$); δ 1.69, 1.86, 1.95, 1.96, 1.98, 1.99 and 2.02 (7 × s, 21 H, 7 × $COCH_3$), 3.60 (m, 1 H, H-5′), 3.70 (dd, 1 H, $J_{3,4}$ 9, $J_{4,5}$ 10 Hz, H-4), 3.77 (dd, 1 H, $J_{1',2'}$ 8, $J_{2',3'}$ 10 Hz, H-2′), 3.93 (m, 1 H, H-5), 3.97 (dd, 1 H, $J_{5',6a'} < 2$, $J_{6a',6b'}$ 12 Hz, H-6a′), 4.12 (d, 2 H, $J_{5,6}$ 2 Hz, H-6), 4.22 (m, 1 H, H-2), 4.32 (dd, 1 H, $J_{5',6b'}$ 4, $J_{6a',6b'}$ 12 Hz, H-6b′), 4.55 (d, 1 H, $J_{1',2'}$ 8 Hz, H-1′), 4.94–5.08 (m, 5 H, 2 × OCH_2Ph and H-4′), 5.10 (dd, 1 H, $J_{2,3}$ 11, $J_{3,4}$ 9 Hz, H-3), 5.16 (dd, 1 H, $J_{2',3'}$ 11, $J_{3',4'}$ 9 Hz, H-3′), 5.57 (dd, 1 H, $J_{1,2}$ 3, $J_{H\text{-}1,P}$ 6 Hz, H-1) and 7.26–7.48 (m, 10 H, 2 × Ph).

2-Acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosyl-(1 → 4)-2-acetamido-3,6-di-O-acetyl-deoxy-α-D-glucopyranosyl phosphate (heptaacetyl-chitobiosyl-1-α-phosphate) (**8**).—To compound (**7**) (128 mg, 0.143 mmol) in a 2:1 mixture of methanol and dichloromethane (6 mL) was added 10% palladium on charcoal (Merck) (144 mg) and the resulting suspension was vigorously stirred under a slight over pressure of hydrogen. TLC at 3 h showed that the reaction was complete, whereupon the reaction mixture was passed through a plug of Celite to remove the palladium/charcoal catalyst, which was washed with copious amounts of methanol to remove any remaining sugar. The combined filtrate and washings were evaporated to dryness under reduced pressure to afford the chitobiosyl phosphate (**8**) (100 mg, 98%) as a white solid, which was used in the next step without further purification.

P_1-*Phytanyl*-P_2-*[2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosyl-(1 → 4)-2-acetamido-3,6-di-O-acetyl-2-deoxy-α-D-glucopyranosyl]-pyrophosphate* (**10**).—A stirred solution of chitobiosyl phosphate (**8**) (300 mg, 0.42 mmol) in methanol (5 mL) was treated with triethylamine (1.5 mL) for 5 min, the reaction was concentrated and evaporated from toluene (5 mL) three times to ensure complete removal of triethylamine. The resulting triethylammonium salt of

compound (**8**) was taken up in anhydrous Me_2NCHO (6 mL) and treated with a solution of *N,N'*-carbonyl diimidazole (375 mg, 2.31 mmol) in anhydrous Me_2NCHO (6 mL) under an atmosphere of nitrogen. The reaction was stirred at room temperature for 4.5 h, whereafter methanol (160 μl, 3.93 mmol) was added to quench any unreacted *N,N'*-carbonyl diimidazole. After stirring for 30 min a solution of phytanyl phosphate (**9**) (240 mg, 0.50 mmol) in DCM (3 ml) was added and the reaction was left to stir at room temperature for 3 days; TLC (6.5:3.5:0.8 chloroform/methanol/1 M ammonium carbonate) showed the formation of a product and some remaining starting material. The reaction mixture was concentrated and evaporated from anhydrous toluene (10 ml) three times to remove traces of Me_2NCHO. Flash-column chromatography (80:20:4 chloroform/methanol/water) of the residue gave the pyrophosphate (**10**) (347 mg, 77%) as a white solid; ^{1}H NMR (360 MHz) (1:1 $CDCl_3/CD_3OD$): δ 0.85–1.80 (39 H, phytanyl) 2.00, 2.06 (6 H), 2.07, 2.12, 2.14 and 2.19 (6 × s, 21 H, 7 × $COCH_3$), 3.79 (m, 2 H, H-2 and H-5'), 3.95 (m, 1 H, H-4), 3.99–4.20 (m, 4 H, OCH_2, H-2' and H-6a'), 4.32 (m, 2 H, H-5 and H-6a), 4.50 (m, 2 H, H-6b and H-6b'), 4.79 (m, 1 H, H-1'), 5.04 (dd, 1 H, *J* 10 and 10 Hz, H-4'), 5.37 (m, 2 H, H-3 and H-3') and 5.61 (m, 1 H, H-1).

P_1-phytanyl-P_2-[2-acetamido-2-deoxy-β-D-glucopyranosyl-(1 → 4)-2-acetamido-2-deoxy-α-D-glucopyranosyl]-pyrophosphate (PPGn2) (**11**).—A cooled solution of the compound (**10**) (3.6 mg, 3.45 μmol) in a 1:4 mixture of anhydrous methanol/DCM (2.5 mL) was treated with freshly prepared 7% sodium methoxide in methanol (80 μL) for 30 min; TLC (6.5:3.5:0.8 chloroform/methanol/1 M ammonium carbonate) at that time showed the reaction to be complete. The reaction mixture was passed through a column of AG50W-X8 [hydrogen form] ion-exchange resin (0.5 mL), which was then washed with methanol (5 mL). The combined filtrate and washings were concentrated to give the deprotected compound (**11**) (2.9 mg) as a white solid in 97% yield; ^{1}H NMR (600 MHz) (10:10:3 $CDCl_3/CD_3OD/D_2O$): δ 0.80–1.70 (39 H, phytanyl) 2.00 and 2.02 (2 × s, 6H, 2 × $COCH_3$), 3.33–3.39 (m, 2 H, H-4', H-5'), 3.45 (dd, 1 H, $J_{2',3'}$ 10, $J_{3',4'}$ 10 Hz, H-3'), 3.54 (dd, 1 H, *J* 8 and 10 Hz, H-4), 3.58 (dd, 1 H, $J_{5,6a}$ 5, $J_{6a,6b}$ 13 Hz, H-6a), 3.65 (dd, 1 H, $J_{5',6a'}$ 6, $J_{6a',6b'}$, 13 Hz, H-6a'), 3.68 (dd, 1 H, $J_{1',2'}$ 8, $J_{2',3'}$ 10 Hz, H-2'), 3.78 (m, 1 H, H-6b), 3.84 (m, 1 H, H-5), 3.86 (dd, 1 H, $J_{5',6b'}$ 2, $J_{6a',6b'}$ 13 Hz, H-6b') and 3.90–3.99 (m, 4 H, OCH_2-, H-2 and H-3) 4.51 (d, 1 H, $J_{1',2'}$ 8 Hz, H-1'), 5.48 (dd, 1 H, $J_{1,2}$ 3, $J_{H\text{-}1,P}$ 7 Hz, H-1); ^{31}P NMR (242 mHz) (10:10:3 $CDCl_3/CD_3OD/D_2O$): δ −9.9 and −10.6 (2 × s, P-1 and P-2).

Growth of bacterial cultures.—E. coli (BL21(DE3)pLysS) cells were transformed with the plasmid pLR36 using the competent cell method [37] and plated out onto LB agar.

Incubation at 30 °C for 18 h yielded colonies of transformed cells, one of which was used to inoculate LB broth (10 mL), which was shaken at 250 rpm at 37 °C for 11 h. The resulting culture was used to inoculate LB broth (1 L) which was shaken at 250 rpm at 37 °C for 13 h (OD_{600} = 2.0), and then used as the starter culture for a 15 L fermentation, where the medium was LB broth. Induction (OD_{600} = 1.25) was achieved by the addition of isopropyl-1-thio-β-D-galactoside (IPTG) and after 3 h of induction time (OD_{600} = 1.9) the cells were harvested by centrifugation at 10,000 rpm at 4 °C for 10 min. The resulting cell pellets (76 g of wet cells) were stored at −80 °C until required.

Immobilization of the β-mannosyltransferase enzyme.—The above cells (4 g of wet cells) were rapidly thawed to promote lysis, whereafter mannosyltransferase buffer (40 ml) and about 5 crystals of deoxyribonuclease I (DNaseI) were added and the mixture kept on ice with occasional shaking until a uniform suspension was achieved. After centrifugation at 4500 rpm at 4 °C for 40 min the cleared-cell lysate was decanted into a fresh vessel and stored on ice until required.

The cleared-cell lysate (25 mL) was applied to a column containing His-Bind resin (10 ml), which was previously charged with distilled water (30 mL), charge buffer (50 mL) and binding buffer (30 mL). To ensure the maximum immobilisation of the β-mannosyltransferase the cleared-cell lysate was passed through the column again, which was subsequently washed with binding buffer (100 mL) and mannosyltransferase buffer (30 mL). The resulting immobilised β-mannosyltransferase was stored in mannosyltransferase buffer at 4 °C until required.

Mannopyranosyl-(1 → 4)-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1 → 4)-2-acetamido-2-deoxy-α-D-glucopyranose (Manβ-1,4GlcNAcβ-1,4GlcNAc) (**2**).—To the phytanyl-pyrophosphoryl chitobioside (**11**) (2.8 mg, 3.2 μmol) was added 50% (v/v) enzyme-charged resin in mannosyltransferase buffer (20 mL). The resulting suspension was shaken at 37 °C for 15 min, whereafter a solution of GDP-[2-^{3}H]mannose (**12**) (2.4 mg, 4.0 μmol, 0.8 μCi) in water (44 μL) was added with final concentrations being 160 μM

PPGn2 (**11**) and 200 μM GDP-mannose (**12**) (0.04 μCi/mL). The reaction was shaken at 100 rpm at 37 °C for 30 min, whereafter, alkaline phosphatase (8 U) was added; shaking was continued under those conditions for a further 1 h. The resin was separated from the aqueous fraction by vacuum filtration and washed with copious amounts of chloroform/methanol/water (10:10:3). The combined organic and aqueous phases were evaporated to dryness and the residue treated with 20 mM HCl (25 mL) at 100 °C for 1 h to release the free saccharides from their pyrophosphoryl derivatives. After extraction with an equal volume of chloroform, the aqueous phase was applied to a mixed resin column containing Chelex 100 [Na^+ form] (1 mL), Ag50W-X8 [H^+ form] (5 mL), AG3-X4A [OH^- form] (10 mL) and QAE-Sephadex (2ml) and the saccharides were eluted with distilled water (90 mL) and concentrated. The residue was taken up in distilled water (400 μL) and passed through a 0.2 μm filter before being subjected to Bio-Gel P4 gel-filtration chromatography. Fractions containing oligosacharides corresponding to a hydrodynamic volume of 5 glucose units were pooled and lyophilised to give the target compound (**2**) (1.5 mg) in a yield of 80% (calculated from the relative radioactivity of the pooled fractions of (**2**) and mannose); ^{1}H NMR (600 MHz) (D_2O): δ 2.08 and 2.10 (2 × s, 6 H, 2 × $COCH_3$), 3.44 (m, 1 H, H-5″), 3.53 (ddd, $J_{5\beta,6b\beta}$ 2, $J_{5\beta,6a\beta}$ 5, $J_{4\beta,5\beta}$ 10 Hz, H-5β), 3.59 (1H, dd, $J_{3'',4''}$ 10, $J_{4'',5''}$ 10 Hz, H-4″), 3.64 (m, 1 H, H-5′), 3.65 (m, H-4β), 3.66 (m, H-4α), 3.67 (m, H-6aβ), 3.67 (dd, 1 H, $J_{2'',3''}$ 3, $J_{3'',4''}$ 10 Hz, H-3″), 3.70 (m, H-6aα), 3.71 (m, H-3β), 3.72 (m, H-2β), 3.74 (dd, 1 H, $J_{5'',6a''}$ 7, $J_{6a'',6b''}$ 12 Hz, H-6a″), 3.78 (m, 2 H, H-4′ and H-6a′), 3.79 (m, H-2β' and H-3′), 3.81 (m, H-6bα and H-2α'), 3.85 (dd, $J_{5\beta,6b\beta}$ 2, $J_{6a\beta,6b\beta}$ 12 Hz, H-6bβ), 3.90 (m, H-2α), 3.91 (m, H-3α and H-5α), 3.92 (m, 1 H, H-6b′), 3.94 (dd, 1 H, $J_{5'',6b''}$ 2, $J_{6a'',6b''}$ 12 Hz, H-6b″), 4.08 (d, 1 H, $J_{2'',3''}$ 3 Hz, H-2″), 4.62 (d, $J_{1\beta',2\beta'}$ 8 Hz, H-1′β), 4.63 (d, $J_{1\beta',2\beta'}$ 8 Hz, H-1′α), 4.72 (d, $J_{1\beta,2\beta}$ 8 Hz, H-1β), 4.80 (s, 1 H, H-1″) and 5.21 (d, $J_{1\alpha,2\alpha}$ 3 Hz, H-1α); HMQC (150 MHz) (D_2O): δ (13C) 55.0 (C-2α), 56.4 (C-2′), 57.5 (C-2β), 61.5 (C-6 and C-6′), 62.4 (C-6″), 68.0 (C-4″), 70.6 (C-3α), 71.5 (C-5α), 72.0 (C-2″), 73.4 (C-3′), 73.7 (C-3β), 74.0 (C-3″), 75.9 (C-5β and C-5′), 77.7 (C-5″), 80.1 (C-4′), 80.6 (C-4β), 81.0 (C-4α), 92.0 (C-1α), 96.4 (C-1β), 101.4 (C-1″) and 102.8 (C-1′).

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