Chemo-enzymatic Synthesis of a Lipid-linked Core Trisaccharide of *N*-Linked Glycoproteins[1]

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Two novel analogues of the mannosyl transferase acceptor substrate $(GlcNAc)_2$-P_i-P_i-dolichyl **2** have been prepared in which dolichyl is replaced by phytanyl, **4**, and lauryl, **5**; both compounds **4** and **5** were synthesized using readily available chitin as the disaccharide precursor. Compound **4** acts as an efficient acceptor substrate for mannosyl transferases from porcine liver and *Saccharomyces cerevisiae*.

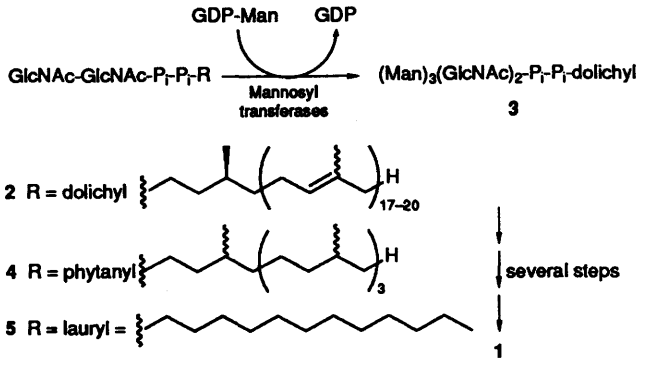
The oligosaccharide side-chains of glycoproteins are well known to control both the biodistribution and the biological properties of the parent protein or peptide.[2] Common to most glycoproteins containing asparagine-linked oligosaccharides is the pentasaccharide core structure **1**.

In view of the inherent problems associated with the chemical synthesis[3] of the pentasaccharide core, we have initiated a programme whose aim is to prepare it by a chemo-enzymatic approach. The use of glycosyl transferases for the synthesis of oligosaccharides is currently being explored since it offers an efficient method for controlling both the regio- and stereoselectivity of glycoside-bond formation.[4] However, the mannosyl transferases involved in the assembly of the core **1** have not been used to any significant extent owing to the complexity of the substrates involved (*vide infra*).

The biosynthesis of the pentasaccharide starts at the reducing end by sequential attachment of two *N*-acetylglucosamine residues to the polyisoprenoid dolichol through a pyrophosphate linkage to give compound **2** (Scheme 1). Subsequent mannosyl transfer is catalysed by various mannosyl transferases which use GDP (guanosine diphosphate)-mannose as the donor substrate to give the pentasaccharide **3**. After further modification, transfer of the oligosaccharide from the lipid anchor to the protein occurs, resulting in the asparagine-linked structure **1**.[5]

The enzymes of the biosynthetic pathway leading to *N*-(asparagine)-linked oligosaccharides have been shown to occur in many different organisms and tissues (*e.g.*, yeast,[5] mung beans[6] and liver[7]). The biosynthetic pathways in all are very similar, occurring *via* the same intermediate sugars and using the same sugar nucleotides as donor substrates.

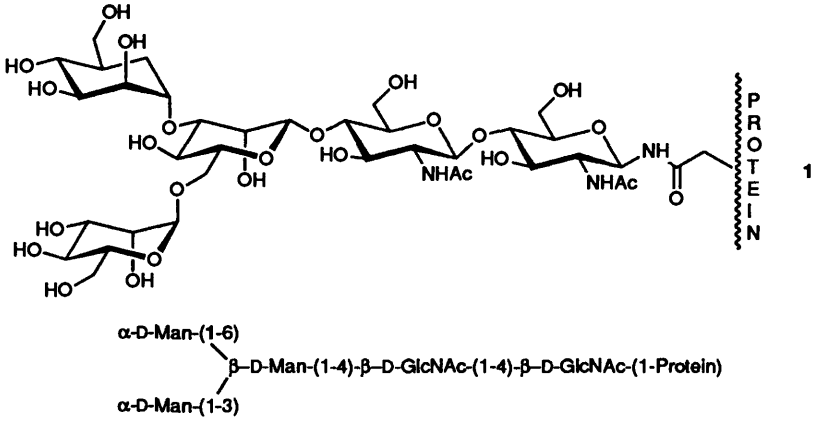
A major drawback to employing an enzymatic approach to



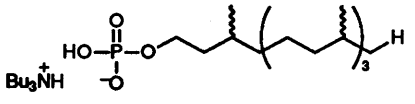
Scheme 1 P_i-P_i- = pyrophosphate

the synthesis of *N*-linked oligosaccharides has been the need for dolichyl-linked substrates. Dolichol must be isolated from pig's liver where it is present only in low abundance (5 kg of liver yields 100–200 mg of dolichol as a mixture of homologues)[8]. Very recently a semisynthetic preparation of dolichol has been described which has proven useful for the synthesis of radiolabelled intermediates for investigations into glycoprotein biosynthesis, but still produces dolichol on a relatively small scale.[9]

The question of which parts of the dolichol lipids are important in the initial stages of the oligosaccharide biosynthesis has been addressed by several groups. It was found that isoprenoid analogues, containing an unsaturated isoprene unit (allylic alcohol) instead of the saturated isoprene end in dolichol, were poor acceptors for hexose transferases.[10] The



influence of dolichol length on hexose transfer was also investigated and it was found that at least a decaprenol phosphate was needed for hexose transfer.[11] Parallel to these studies a report by Clark and Villemez has claimed that phytanyl phosphate **6**, which is a fully saturated tetraprenyl phosphate, can act as an acceptor for a mannosyl transferase from mung beans.[12] This transferase is presumably the mannosyl transferase involved in the synthesis of Man-P_i-dolichol, since Man-P_i-phytanol was formed in the reported assays. Phytanol seemed to be an excellent dolichol analogue since it is easily available in gram quantities from phytol by catalytic hydrogenation and contains the saturated isoprene unit of dolichol at the alcohol end. Although phytanyl phosphate is not a substrate for the first glycosyl transferase in the biosynthetic pathway (*N*-acetylglucosaminyl transferase), we reasoned that the requirement for dolichol would be less strict further along the biosynthetic pathway. In addition, the first disaccharide, GlcNAcβ1-4GlcNAc, is chemically easily accessible from chitin and could therefore be used as a chemically synthesized building block in a chemo-enzymatic approach to the pentasaccharide core.



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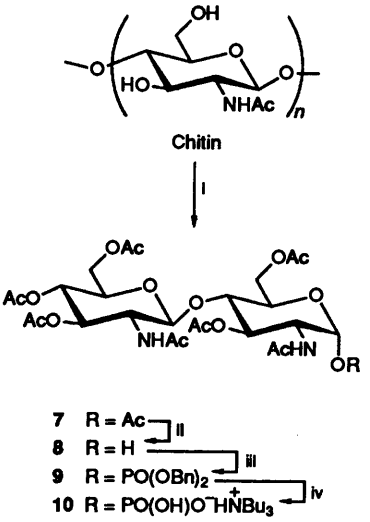
Results

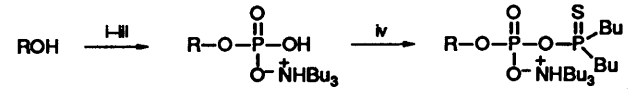
Chemical Synthesis of Lipid-linked Acceptors.—Based on these considerations we decided to synthesize the phytanyl-linked disaccharide **4** and the lauryl (dodecyl)-linked equivalent **5**. The dolichyl substrate **2**, of which compounds **4** and **5** are analogues, has previously been prepared during the pioneering studies of Jeanloz and Warren.[13] Initially we used their protocol to prepare compounds **4** and **5** but encountered several problems associated with scale-up and reproducibility and therefore devised an alternative route. Chitobiose octaacetate **7** was obtained by controlled acetolysis of chitin[14] and was selectively 1-*O*-deacetylated with hydrazine acetate to give compound **8** (82%) using the same conditions as described for monosaccharides.[15] Treatment of compound **8** with butyllithium (1.1 mol equiv., dry tetrahydrofuran (THF)–dimethylformamide (DMF) (1:1)] followed by dibenzyl phosphorochloridate[16] (2.5 mol equiv.) gave the required glycosyl phosphate[17] **9** exclusively as the α-anomer (90%). Catalytic hydrogenolysis of the benzyl protecting groups, followed by treatment with tributylamine (1 mol equiv.) gave the desired phosphate **10** in quantitative yield (Scheme 2).

The synthesis of disaccharide pyrophosphates **16** and **17** was achieved either *via* thiophosphoryl bromides **11** and **12** (method one)[18] or directly by *in situ* conversion of substrate **6** into the dichloride by reaction with oxalyl dichloride (method two).[19] Using method one, the corresponding activated phytanyl **11** and lauryl **12** phosphates required for coupling to the salt **10** were prepared as outlined in Scheme 3. Phytol (Aldrich, 97% pure) was catalytically hydrogenated with Pt/C to give phytanol **13** in 44% isolated yield as mixtures of epimers at C-3. Conversion of the alcohol **13** into the phytanyl phosphate **6** and of lauryl alcohol **14** into the lauryl phosphate **15** was achieved in 90% yield using the recently published phosphorylation procedure.[20] Treatment of the phosphate **6** with dibutyl(thiophosphoryl) bromide (1.1 mol equiv., THF, room temperature) yielded the required thiophosphoryl anhydride **11**.[18] A similar sequence of reactions furnished the corresponding lauryl compound **12** from lauryl alcohol **14**. Both products **11** and **12** were used directly for the next step without further purification.



7 R = Ac (ii)
8 R = H (iii)
9 R = $PO(OBn)_2$ (iv)
10 R = $PO(OH)O^-HNBu_3$

Scheme 2 *Reagents:* (and yields) i, Ac_2O, H_2SO_4 (11%); ii, NH_2NH_2·HOAc (82%); iii, BuLi (1.1 mol equiv.), $(BnO)_2POCl$ (90%); (iv) H_2/Pd, NBu_3 (1 mol equiv.) (>95%)

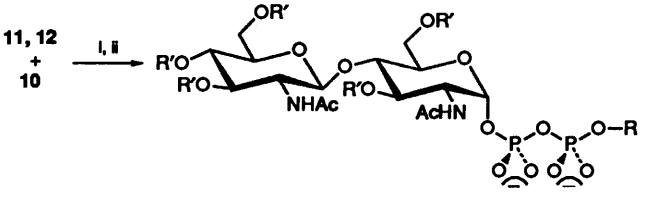


13, 14 **6, 15** **11, 12**

6, 11, 13, R = phytanyl
12, 14, 15 R = lauryl

Scheme 3 *Reagents:* i, $Et_2NP(OBu^t)_2$, 1*H*-tetrazole; ii, MCPBA; iii, TFA; NBu_3 (1 mol equiv.); iv, $BrP(S)Bu_2$

Individual coupling of anhydrides **11** and **12** with peracetylated chitobiose phosphate **10** (AgOAc then H_2S)[18] gave, after purification on silica [$CHCl_3$–MeOH (9:1)], the required protected diphosphates **16** (21%) and **17** (40%), respectively (Scheme 4). This procedure was initially used but proved to be inferior in yield to the second method using oxalyl dichloride, which was reported by Imperiali *et al.*[19] during the course of this work.



16 R = phytanyl, R′ = Ac
17 R = lauryl, R′ = Ac
4 R = phytanyl, R′ = H
5 R = lauryl, R′ = H

Scheme 4 *Reagents:* i, AgOAc, H_2S; ii, NaOMe, MeOH, NH_4Cl

Deprotection of compounds **16** and **17** (7% NaOMe–MeOH in CH_2Cl_2) gave **4** and **5** respectively (Scheme 4). Both the phytanyl **4** and lauryl **5** substrates gave spectroscopic data in full accord with their structures.

Assay of Acceptor Substrates for Mannosyl Transferases.—The method we chose to follow the incorporation of a mannosyl residue is shown in Scheme 5. The crude enzyme system containing the required mannosyl transferase was obtained either from porcine liver[8] or *Saccharomyces cerevisiae*. Substrates were then incubated with the enzyme preparation in the presence of GDP-[U-^{14}C]mannose. At the end of the reaction lipid-containing products were extracted into $CHCl_3$–MeOH–

Table 1 Incorporation of radioactivity from GDP-*Man[a] into solvent-extractable lipids

Compound	Radioactivity/cpm	
	Porcine liver	Yeast
18	49 000	90 000
6	76 000	187 000
4	56 000	71 000
4*	46 000	3 600
5	29 000	
Background	34 000	45 000

[a] *Man = [U-^{14}C]mannose.

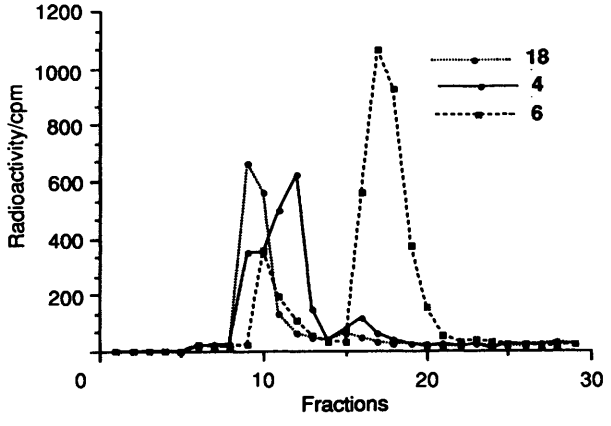


Fig. 1 Gel filtration chromatography (LH 20) profiles of the organic extracts (see Table 1) of porcine liver incubation mixtures containing substrates **18**, **4** and **6**

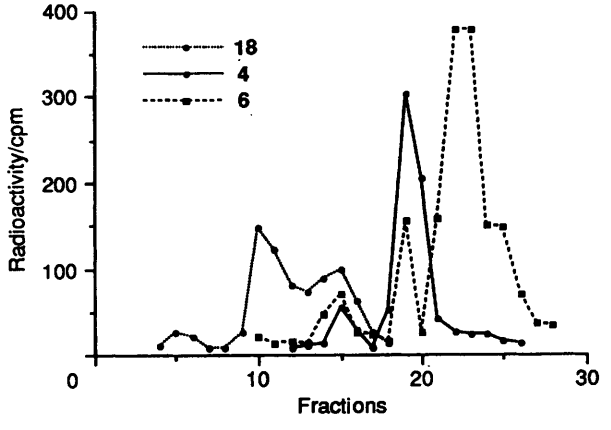
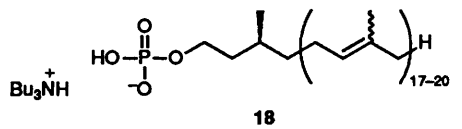


Fig. 2 Gel filtration chromatography (LH 20) profiles of the organic extracts (see Table 1) of yeast incubation mixtures containing substrates **18**, **4** and **6**

water (10:10:3) and the levels of incorporation of labelled mannose were then determined by scintillation counting. The addition of acceptor lipids **4**, **6** and dolichol phosphate **18** stimulated incorporation into these extractable lipids as shown in Table 1. Incorporation of the lauryl-linked acceptor analogue **5** was only tested with pig liver extracts and did not give rise to increased incorporation of radioactivity into extractable lipids.



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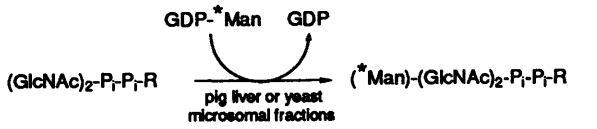
It is interesting to note that mannosyl transfer was dependent on the type of detergent used in incubation mixtures, as can be seen from Table 1. Thus, when Triton X-100 was used



Scheme 5 *Man = [U-^{14}C]mannose

(incubation **4**) mannosyl transfer into lipid occurred with both the pig liver and the yeast microsomal fractions. With sodium deoxycholate, however (incubation **4***), the transfer activity of the yeast extracts was greatly reduced whereas the porcine liver extracts gave incorporation of radioactive mannose into extractable lipids above background levels.

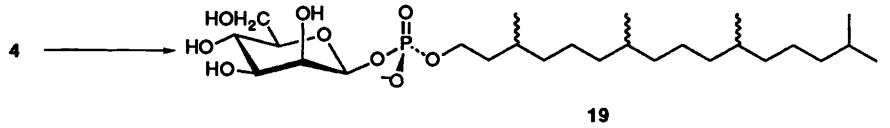
The lipid-containing extracts were further analysed by gel filtration chromatography using Sephadex LH-20. Fig. 1 and 2 show the elution profiles for the pig liver experiment and the yeast experiments, respectively. In both cases three peaks of distinct retention times could be separated and these will be referred to as high-, medium- and low-molecular-mass peaks in the following discussion. The first peak containing the lipids with largest molecular mass (MM) was the only one observed when no exogeneous lipids were added (data not shown) or when dolichol phosphate **18** was added. This suggests that this peak in the gelfiltration chromatogram can be assigned to dolichyl-linked oligosaccharides (MM > 2000) derived from endogenous dolichol present in the enzyme preparation or from exogeneous dolichol phosphate **18**. The third fraction, containing radioactive lipids with the lowest molecular mass, was the only peak observed when phytanol phosphate **6** was incubated with both the liver and the yeast microsomal fractions. It is interesting to note that the background reaction, *i.e.* incorporation of radioactivity into a high-molecular-mass endogeneous dolichol containing fraction, had been suppressed. This suggests that the phytanyl phosphate, which is added in excess, can successfully compete with endogeneous dolichol-acceptors in the mannosyl-transfer reaction. Radioactive lipids eluting with the second (medium MM) peak were observed only when the phytanyl pyrophosphate-linked disaccharide **4** was incubated both with the pig liver and the yeast enzyme.

These results suggested to us that phytanyl phosphate **6** was mannosylated to give Man-P_i-phytanyl (MM 505, low-MM peak) as reported for the mung bean system.[12] Acceptor **4** led to higher molecular mass species such as Man_x-GlcNAC-GlcNAc-P_i-P_i-phytanol (x × 1; MM 980) and hence would be expected to elute as the medium-MM peak.

The oligosaccharide parts of the radiolabelled lipids obtained from the yeast incubations were further analysed. The fractions which eluted with the medium- (for incubation of **4**) and low-MM peak (for incubation of **6**) of the LH20 column were treated with acid to hydrolyse the sugar phosphate linkages. The free oligosaccharides were separated by gel permeation chromatography on Biogel P4.[21] The elution profiles of the hydrolysis products are shown in Fig. 3. The retention times were compared with those of malto-oligosaccharides obtained by hydrolysis of dextran. We found that the hydrolysis product from the low-MM fraction co-eluted with the glucose monomer and the hydrolysis product of the medium-MM fraction co-eluted with the maltose pentamer Glc_5. It has been shown by Kobata *et al.*[21] that the trisaccharide Manβ1-4GlcNAcβ1-4GlcNAc has the same hydrodynamic volume as the glucose pentamer Glc_5. This suggests that *Man-GlcNAcβ1-4GlcNAc-Pi-Pi-phytanol had been formed as the predominant radiolabelled lipid in our incubation mixtures.

Discussion

The results shown in this paper suggest that two mannosyl transferases in crude extracts of pig liver and of yeast can accept



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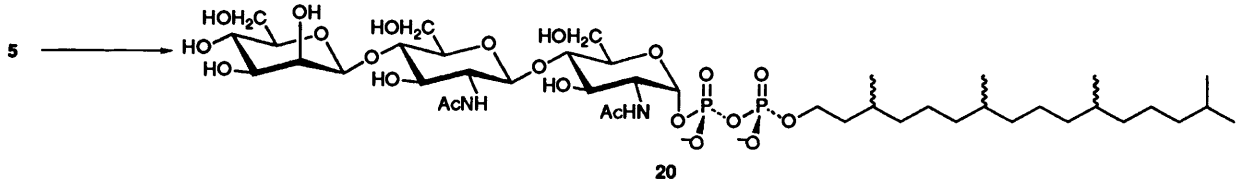
Scheme 6



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Scheme 7

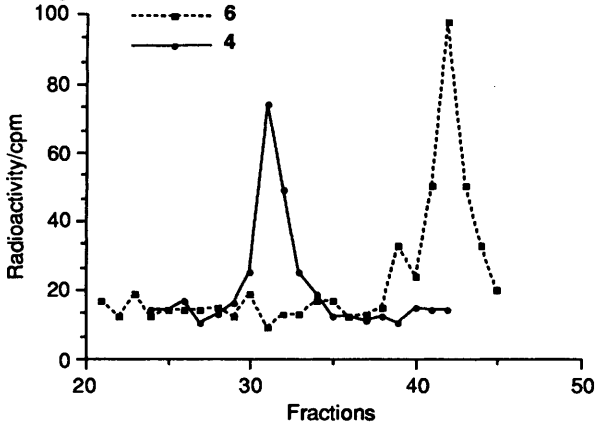

Fig. 3 Gel permeation chromatography (BioGel P4) elution profiles of the acid hydrolysates of yeast incubation mixtures containing acceptor substrates **5** and **6**

phytanol analogues of dolichol-linked acceptor substrates. The first mannosyl transferase would catalyse the reaction shown in Scheme 6, the formation of Man-P_i-phytanol **19**. It is likely that this mannosyl transferase is normally responsible for the synthesis of Man-P_i-dolichol, which is a mannosyl donor substrate further along the biosynthetic pathway of oligosaccharides. It has been generally accepted that the first five mannose residues along the biosynthetic pathway originate from GDP-mannose and that any mannoses which are added later originate from Man-P_i-dolichol.[5]. The incorporation of mannose into phytanol phosphate[12] and into retinol phosphate[22] have previously been reported in microsomal fractions.

The second mannosylation reaction we observed in our studies would be catalysed by the β(1-4)mannosyl transferase involved in core pentasaccharide biosynthesis as described in Scheme 7 to give the lipid-linked core trisaccharide **20**. The arguments in favour of this structure being formed are as follows: we have shown that GlcNAcβ1-4GlcNAc-P_i-P_i-phytanol is mannosylated and that hydrolysis of the sugar-lipid linkage generates an oligosaccharide that has the same hydrodynamic volume on a P4 sizing column as reported for Manβ1-4GlcNAcβ1-4GlcNAc.[21] It is surprising that only a trisaccharide derivative and not higher oligomers were detected. This could possibly be due to the fact that the acceptor substrate concentration for the next enzyme was much lower and thus further mannosylation could not compete with the first mannosylation under limiting concentrations of GDP-mannose. We are currently pursing further studies using lower acceptor and higher GDP-mannose concentrations.

The fact that the branched phytanol **13** but not the straight-chain dodecanol **14** can act as a substitute for dolichol in some steps of oligosaccharide biosynthesis could give us possible insight into one of the roles dolichol plays in this pathway. Both mannosyl transferases described here have been sequenced and cloned from yeast by Robbins and co-workers,[23] who have proposed that a conserved peptide sequence within the hydrophobic part of the polypeptide chain could act as a dolichol-binding site.[24] Based on the same homology such dolichol-binding sites have also been proposed for polysialyl transferases of *Escherichia coli*.[25] Our results indicate that if such a recognition site exists in the yeast mannosyltransferases it should be interacting with the first saturated isoprenoid unit of dolichol, since this is the only structural feature it shares with phytanol. We are currently studying these interesting interactions between lipids and proteins in further detail.

It will also be interesting to see whether phytanol is translocated through the membrane in the same manner proposed for dolichol in later stages of the biosynthetic pathway of *N*-linked oligosaccharides and whether the oligosaccharyl transferase which is responsible for transferring the oligosaccharide chain from dolichyl pyrophosphate to the nascent protein chain can use phytanyl pyrophosphate-linked oligosaccharides as substrates.[26]

Finally, we believe that the successful use of the phytanyl substrate opens up the possibility of preparing the pentasaccharide core by a combined chemical and enzymatic approach. Work concerned with the scale-up of these experiments is in progress.

Experimental

General.—All starting materials were obtained from commercial suppliers unless otherwise stated and used without further purification. Tetrahydrofuran (THF) and toluene were distilled from sodium–benzophenone ketyl prior to use. All other anhydrous solvents were distilled from the stated drying agents and stored under argon or nitrogen over activated 4Å molecular sieves; acetonitrile (CaH_2), 1,2-dichloroethane (P_2O_5), dichloromethane (DCM) (P_2O_5), dimethyl formamide (DMF) (CaH_2), ethanol (Mg), methanol (magnesium methoxide), pyridine (KOH), triethylamine (KOH). Light petroleum refers to the fraction of boiling range 40–60 °C. Light petroleum and ethyl acetate were distilled prior to use. Unless otherwise stated all reactions were carried out under an atmosphere of dry argon or nitrogen.

Flash chromatography was carried out on silica gel 60H (Merck 7385). TLC was performed on Merck 60F-254 (0.25 mm, Art. 5715) glass-backed silica gel plates. Visualisation was by UV fluorescence or treatment with an acidic solution of ethanolic *p*-anisaldehyde.

M.p.s were determined on an Electrothermal instrument and are uncorrected.

IR spectra were recorded on a Perkin-Elmer 881 grating IR spectrophotometer and are recorded as wavenumbers relative to a polystyrene standard.

Optical rotations were performed in $CHCl_3$ on a Thorn NPL

Automatic Polarimeter Type 243, and $[\alpha]_D$-values are given in units of 10^{-1} deg cm^2 g^{-1}.

^{1}H, ^{13}C and ^{31}P spectra were recorded by Dr. V. Sik (Chemistry Department, University of Exeter) on a Bruker AM250 spectrometer. Unless otherwise stated spectra are for solutions in $CDCl_3$. NMR data are expressed as δ-values, with coupling constants (J/Hz) and assignment as appropriate.

High-resolution mass spectra were recorded at the SERC mass spectrometry centre, Swansea, on a VG ZAB-E high-resolution instrument. Unless otherwise indicated they were measured by FAB (Fast Atom Bombardment), Xe, from a 3-nitrobenzyl alcohol matrix.

*2-Acetamido-*O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-*β*-*D*-glucopyranosyl)-(1-4)-1,3,6-tri-*O*-acetyl-2-deoxy-*α*-*D*-glucopyranose (Chitobiose Octaacetate)* **7**.[27]—Chitin (100 g) (obtained from Pfanstiehl Lab. Inc., 1219 Glenrock Ave, Waukegan, IL, USA) was placed in a 1 dm^3 conical flask and covered with conc. hydrochloric acid (750 cm^3). The mixture was then subjected to ultrasound (25 kHz) for 1 h at room temperature. The resulting suspension was poured into ice–water (1:1; 5 dm^3) and stored at 5 °C overnight. The solid was isolated by Buchner filtration, washed successively with water (1 dm^3), 2 mol dm^{-3} NaOH (1 dm^3), water (1 dm^3), acetone (1 dm^3) and diethyl ether (1 dm^3) and allowed to dry in air. Colloidal chitin (80 g) was thus obtained and used without further purification. Colloidal chitin (10 g) was added to ice-cold acetic anhydride (100 cm^3), conc. sulfuric acid (10 cm^3) was added, and the reaction mixture was allowed to warm to room temperature and stirred overnight. The reaction mixture was then heated at 55 °C for 6 h, poured into ice-cold aq. sodium acetate (20 g in 130 cm^3), and filtered. The filtrate was extracted with chloroform (3 × 200 cm^3). The combined extracts were washed successively with water (400 cm^3), ice-cold saturated aq. sodium hydrogen carbonate (400 cm^3) and water (400 cm^3), then dried over anhydrous sodium sulfate. The $CHCl_3$ layer was then filtered, and evaporated under reduced pressure, and the resulting solid was chromatographed on silica, eluent $CHCl_3$–MeOH (10:1) to yield a solid, which was recrystallised from MeOH to yield chitobiose octaacetate **7** (1.1 g, 11% by mass from purified chitin), m.p. 305–306 °C (lit.,[28] 304–305 °C); $[\alpha]_D^{25}$ +29.2 (*c* 1, $CHCl_3$); $\nu_{max}(CHCl_3)$/cm^{-1} 1507 (amide), 1678 (amide), 1745 (Ac, str), 2960 (C–H, str) and 3434 (N–H, str); δ_H 1.90–2.20 (24 H, m, Ac × 8), 3.60–4.24 (6 H, m), 4.30–4.48 (3 H, m), 4.50 (1 H, d, *J* 8), 5.00–5.26 (3 H, m), 5.65 (1 H, d, NH), 5.98 (1 H, d, NH) and 6.10 (1 H, d, *J* 3, 1′-H) (Found: MH$^+$, 677.2400. Calc. for $C_{28}H_{41}N_2O_{17}$: *m*/*z* 677.23934).

*2-Acetamido-*O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-*β*-*D*-glucopyranosyl)-(1-4)-3,6-di-*O*-acetyl-2-deoxy-*α*-*D*-glucopyranose (Chitobiose Heptaacetate)* **8**.[15]—Chitobiose octaacetate **7** (1.027 g, 1.52 mmol) and hydrazine acetate (350 mg, 4.2 mmol) were both dried over phosphorus pentaoxide *in vacuo* overnight. The reactants were subsequently stirred over activated 4Å molecular sieves in dry DMF (20 cm^3) at 50 °C for 2 h. The reaction mixture was then treated with ethyl acetate (60 cm^3) and stirred for a further 30 min at room temperature. Solvent was removed under reduced pressure by three co-evaporations with toluene. The resulting solid was chromatographed on silica, eluent CH_2Cl_2–MeOH (95:5) to give *chitobiose heptaacetate* **8** (790 mg, 82%), m.p. 245–247 °C; $[\alpha]_D^{24}$ −88.1 (*c* 1, $CHCl_3$); $\nu_{max}(CHCl_3)$/cm^{-1} 1036, 1239, 1371, 1526 (amide), 1669 (amide), 1740s (CO), 3003s (CH) and 3379s (NH); δ_H 2.00 (21 H, 7 s), 3.75 (2 H, m), 4.00–4.50 (8 H, m), 5.00–5.30 (4 H, m), 5.70 (1 H, t), 6.30 (1 H, d) and 7.20 (1 H, d); δ_C 20.40, 20.50, 20.60, 20.70, 20.90, 22.80, 23.00, 51.90, 54.20, 61.70, 62.50, 67.90, 68.20, 71.00, 71.90, 72.10, 91.70, 102.20 and 170.00 (7 × CO) (Found: MH$^+$, 635.2312. $C_{26}H_{39}N_2O_{16}$ requires *m*/*z* 635.2287).

*2-Acetamido-*O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-*β*-*D*-glucopyranosyl)-(1-4)-3,6-di-*O*-acetyl-2-deoxy-*α*-*D*-glucopyranosyl Dibenzyl Phosphate (Dibenzyl Chitobiosyl Phosphate)* **9**.[17]—To a solution of chitobiose heptaacetate **8** (21 mg, 0.033 mmol) in freshly distilled THF–dry DMF (1:1; 1 cm^3) at −70 °C (CO_2–acetone) was added 1.6 mol dm^{-3} BuLi (0.022 cm^3, 0.036 mmol). The resulting bright pink solution was stirred at −70 °C for 0.5 h after which freshly prepared dibenzyl phosphorochloridate (26 mg, 0.088 mmol) was added as a solution in freshly distilled THF (1 cm^3). The reaction mixture was allowed to warm to −60 °C and was stirred for 0.5 h. The solvent was evaporated off under reduced pressure, DMF being removed by three co-evaporations with toluene, and the residue was chromatographed on silica, eluent $CHCl_3$–MeOH (97:3) to yield dibenzyl chitobiosyl phosphate **9** (26.5 mg, 90%), m.p. 139–141 °C (lit.[13b] 132–137 °C); $\nu_{max}(CHCl_3)$/cm^{-1} 1028, 1195, 1367, 1416, 1510 (amide), 1676s (amide), 1742s (CO), 2858s (CH), 2923 and 3426s (NH); δ_H 1.95–2.20 (21 H, m, 7 × Ac), 3.60–4.45 (9 H, m), 4.60 (1 H, d), 5.05–5.40 (7 H, m), 5.65 (2 H, t), 5.90 (1 H, d, NH) and 7.40 (10 H, m); δ_C 20.49–23.06 (6 × Me), 51.97, 52.09, 55.02, 61.53, 61.65, 69.80 (d, 2 × CH_2OP), 66.3–72.46 (7 × CH), 75.52, 96.04, 96.14, 101.05, 126.90–128.90 (7 × CH) and 169.27–170.76 (7 × CO); δ_P − 1.53 [Found: (M + Na)$^+$ 917.2721. Calc. for $C_{40}H_{51}N_2NaO_{19}P$: *m*/*z* 917.2706].

3,7,11,15-Tetramethylhexadecan-1-ol (Phytanol) **13**.[12]—3,7,11,15-tetramethylhexadec-2-en-1-ol (phytol) (5.00 g, 16.86 mmol) was stirred in dry EtOH (50 cm^3) under hydrogen with 5% platinum on carbon (50 mg) as catalyst. After 2 h the reaction mixture was filtered through Celite and evaporated under reduced pressure. The oily residue was chromatographed on silica, eluent light petroleum–ethyl acetate (10:1) to yield phytanol **13** (2.19 g, 44%); ν_{max}(film)/cm^{-1} 1056, 1375, 1457, 1743, 2920br and 3300br; δ_H 0.87 (14 H, m), 1.00–1.46 (22 H, m), 1.56–1.74 (4 H, m) and 1.67 (2 H, m, CH_2O); δ_C 19.61, 19.67, 19.73, 22.59, 22.68, 24.37, 24.47, 24.78, 27.96, 29.56, 29.57, 32.77, 32.79, 37.30, 37.35, 37.40, 37.45, 37.48, 37.52, 39.38, 39.99, 40.07 and 61.18 [Found: M$^+$ (EI): 298.3232. Calc. for $C_{20}H_{42}O$: *M*, 298.3236].

Di-tert-butyl 3,7,11,15-Tetramethylhexadecyl Phosphate. (Di-tert-butyl Phytanyl Phosphate).[20]—Phytanol **13** (1.60 g, 5.4 mmol) and di-*tert*-butyl-*N*,*N*-diethylphosphoramidite (2.02 g, 8.1 mmol) were stirred under nitrogen in freshly distilled THF (13.5 cm^3). To this was added 1*H*-tetrazole (1.13 g, 16.2 mmol) and the reaction mixture was stirred for 15 min at room temperature. The reaction mixture was then cooled to −40 °C (CO_2–acetone) and *m*-chloroperbenzoic acid (MCPBA) (2.2 g, 12.7 mmol) as a solution in dry CH_2Cl_2 (17 cm^3) was added. The reaction mixture was then allowed to return to room temperature. After 10 min the reaction mixture was quenched by addition of aq. sodium sulphite (10%; 80 cm^3) and then extracted into CH_2Cl_2 (3 × 130 cm^3). The CH_2Cl_2 layer was then washed with saturated aq. sodium hydrogen carbonate (200 cm^3), the saturated aq. sodium hydrogen carbonate layer was re-extracted with CH_2Cl_2 (130 cm^3). The combined CH_2Cl_2 layers were then dried over magnesium sulfate and chromatographed on silica, eluent CH_2Cl_2–MeOH (100% to 90:10) to yield di-*tert*-butyl phytanyl phosphate (2.0 g, 90%); ν_{max}(film)/cm^{-1} 754, 1000br, 1173, 1263, 1368, 1461, 1635, 1740, 2850br and 3454br; δ_H 0.90 (15 H, m), 1.00–1.90 (44 H, m), 4.00 (2 H, dt); δ_C 19.34, 19.41, 19.58, 19.64, 19.71, 22.56 and 22.65 (7 × Me), 24.28, 24.42 and 24.75 (3 × CH_2), 27.93 and 29.31 (2 × CH), 29.81 and 29.88 (2 × Me), 32.75 (CH), 37.27, 37.37, 37.43, 39.40 and 65.30 (5 × CH_2), 77.20 (CH) and 81.70 and

81.80 (2 × C); δ_P −8.81 [Found: (2M + H)$^+$; 981.8380. Calc. for $C_{56}H_{119}O_8P_2$: *m/z*, 981.8349].

By the same method di-*tert*-butyl lauryl phosphate (73%); ν_{max}(film)/cm^{-1} 984, 1184br, 1370, 1458, 2855, 2924 and 3417br; δ_H 1.84 (3 H, t), 2.26 (18 H, s), 2.50 (18 H, s), 2.66 (2 H, m) and 3.92 (2 H, q); δ_C 14.02 (Me), 22.63, 25.61, 29.16, 29.29, 29.49, 29.53 and 29.58 (7 × CH_2), 29.85 (Me, d, *J* 4.3), 30.20, 30.32 and 31.87 (3 × CH_2), 66.85 (CH_2, d, *J* 6.5), 81.73 (C, d, *J* 7.2); δ_P −8.83.

Dibutylthiophosphinyl 3,7,11,15-*Tetramethylhexadecyl Hydrogen Phosphate* **11**.[18]—Di-*tert*-butyl phytanyl phosphate (500 mg, 1.01 mmol) was stirred in dry CH_2Cl_2 (4 cm^3) with trifluoroacetic acid (TFA) (0.63 cm^3, 8.2 mmol) under nitrogen for 2 h after which the reaction mixture was evaporated under reduced pressure. Following addition of tributyl amine (2.5 cm^3, 10.5 mmol) and $CHCl_3$–MeOH (2:1; 12 cm^3) the solvent was removed under reduced pressure and the oily residue was dried by three co-evaporations with toluene. This was subsequently stirred in freshly distilled THF (6 cm^3) over powdered activated 4Å molecular sieves at 30 °C with dibutylthiophosphoryl bromide (202 mg, 1.12 mmol) for 4 h. The reaction mixture was then filtered through Celite, evaporated under reduced pressure, and chromatographed on silica, eluent $CHCl_3$–MeOH (97:3) to yield dibutyl phytanyl thiopyrophosphoryl anhydride **11** (456 mg, 61%); ν_{max}($CHCl_3$)/cm^{-1} 932, 1061, 1091, 1141, 1203, 1379, 1463, 1672, 2489, 2874s, 2960 and 3348br; δ_H 0.80–1.80 (m), 2.00–2.25 (m), 2.79 (t) and 3.90 (m); δ_C 13.64, 19.37, 19.43, 19.59, 19.65, 20.29, 22.51, 22.60, 23.56, 23.84, 24.34, 24.41, 24.71, 24.90, 24.95, 26.67, 27.89, 29.59, 32.73, 32.76, 34.19, 35.27, 37.24, 37.33, 37.48, 39.31, 52.70, 64.48, 64.58 and 77.29; δ_P −8.93 (d, *J* 32.8) and 96.16 (d, *J* 32.7) [Found: (M + H)$^+$; 740.5909. Calc. for $C_{40}H_{88}NO_8P_2S$: *m/z* 740.5887].

By the same method *dibutyl lauryl thiopyrophosphoryl anhydride* **12** (55%); δ_H 0.90–1.00 (m), 1.10–1.55 (m), 1.55–1.80 (m), 2.15 (m), 3.00 (br s) and 3.95 (m); δ_C 13.34, 13.51, 13.88, 19.63, 19.91, 22.48, 23.40, 23.68, 24.76, 24.81, 25.04, 25.71, 29.15, 29.28, 29.48, 30.46, 30.58, 31.73, 33.98, 35.06, 51.93 and 66.29; δ_P −10.34 (d, *J* 33) and 97.55 (d, *J* 33) [Found: (M + H)$^+$, 628.4657. $C_{32}H_{72}NO_4P_2S$ requires *m/z* 628.4639].

2-*Acetamido*-O-(2-*acetamido*-3,4,6-*tri*-O-*acetyl*-2-*deoxy*-β-D-*glucopyranosyl*)-(1-4)-3,6-*di*-O-*acetyl*-2-*deoxy*-α-D-*glucopyranosyl Phytanyl Pyrophosphate* **16**.—*Method one*.[18] Dibenzyl chitobiosyl phosphate (104 mg, 0.15 mmol) was dried overnight *in vacuo* over P_2O_5 and was subsequently stirred in dry MeOH (10 cm^3) under hydrogen in the presence of catalytic 5% palladium on carbon (5 mg) for 2 h. The reaction mixture was filtered through Celite, tributyl amine (60 mg, 0.82 mmol) was added, and the reaction mixture was diluted with water (9 cm^3) and then washed with hexane (3 × 4 cm^3). The MeOH–water layer was co-evaporated with toluene (3 × 4 cm^3). To this was added phytanyl thioanhydride **11** (202.5 mg, 0.112 mmol), previously dried overnight *in vacuo* over P_2O_5; the mixture was then co-evaporated under reduced pressure with dry pyridine. The residue was then dissolved in dry pyridine (6 cm^3), silver(I) acetate (75 mg, 0.45 mmol), dried overnight *in vacuo* over P_2O_5, was added and the reaction mixture was stirred for 4 days in a desiccator over P_2O_5. The reaction was quenched by passage of hydrogen sulfide through the mixture for 5 min and the resulting solution was filtered through Celite, evaporated under reduced pressure, and chromatographed on silica, eluent $CHCl_3$–MeOH–water (65:25:4) to yield the title compound **16** (46 mg, 21%).

Method two.[19] Dibenzylchitobiosyl phosphate (35 mg, 0.051 mmol) was dried overnight *in vacuo* over P_2O_5 and subsequently stirred in dry MeOH (5 cm^3) under hydrogen in the presence of catalytic 5% palladium on carbon (5 mg) for 2 h. The reaction mixture was filtered through Celite and co-evaporated with dry pyridine (3 cm^3). The chitobiose phosphate was then dried *in vacuo* over P_2O_5 for 48 h.

Di-*tert*-butyl phytanyl phosphate (31.2 mg, 0.064 mmol) was stirred in dry CH_2Cl_2 (1 cm^3) with TFA (35 mm^3) for 2 h, tributylamine (160 cm^3, 0.66 mmol) was added and the mixture was co-evaporated with toluene (3 times). The phytanyl phosphate was dried *in vacuo* over P_2O_5 for 48 h.

A mixture of oxalyl dichloride (200 mm^3, 2.1 mmol) and dry DMF (5 mm^3) in freshly distilled THF (3 cm^3) was added dropwise, under nitrogen, during 2 h to a stirred solution of phytanyl phosphate in freshly distilled THF (3 cm^3). The reaction mixture was stirred for a further 2 h, then concentrated under reduced pressure and dried by three co-evaporations from toluene. To the residue was added a solution of the chitobiose phosphate in dry pyridine (1 cm^3) and the reaction mixture was stirred for 1.5 h, concentrated under reduced pressure, diluted with acetone–water–triethylamine (88:10:2; 10 cm^3) and stirred vigorously for 2 h. This mixture was then evaporated under reduced pressure and the residue was chromatographed on silica, eluent $CHCl_3$–MeOH–water (90:10:0.5 to 80:20:0.5) to yield the title compound **16** (33 mg, 45%); ν_{max}($CHCl_3$)/cm^{-1} 922, 1032, 1124, 1202, 1369, 1459, 1541, 1666, 1739, 2466, 2956 and 3301br; δ_H[$CDCl_3$–CD_3OD–D_2O (10:10:3)] 1.01–2.05 (39 H, complex overlapping m, phytanyl), 2.05, 2.11, 2.17, 2.19, 2.23, 2.27 and 2.33 (7 × 3 H, 7 s, 7 × Ac), 3.77–4.48 (10 H, complex overlapping m, disaccharide), 5.13–5.20 (1 H, m, disaccharide), 5.37–5.56 (2 H, m, disaccharide) and 5.67–5.75 (1 H, m, disaccharide); δ_C[125.7 MHz; $CDCl_3$–CD_3OD–D_2O (10:10:3)] 19.54, 19.97, 20.61, 20.73, 20.95, 22.84 and 22.90 (7 × Me), 24.93, 25.00 and 25.29 (3 × CH_2), 28.48, 30.28 and 33.35 (3 × CH), 37.85, 37.99, 38.10, 38.19 and 39.95 (5 × CH_2), 52.67 and 55.77 (2 × CH), 62.55, 62.59 and 65.68 (3 × CH_2), 69.38, 70.16, 72.14, 72.85, 76.05, 97.99 and 101.31 (7 × CH) and 170.60, 171.28, 171.57, 172.07, 172.60, 172.95 and 173.13 (7 × CO); δ_P −9.07 (d, J_{PP} 14) and −11.96 (d, J_{PP} 14).

2-*Acetamido*-O-(2-*acetamido*-3,4,6-*tri*-O-*acetyl*-2-*deoxy*-β-D-*glucopyranosyl*)-(1-4)-3,6-*di*-O-*acetyl*-2-*deoxy*-α-D-*glucopyranosyl Lauryl Pyrophosphate* **17**.—By method one, 40%; δ_H 0.57 (3 H, t, lauryl), 0.87–1.12 (18 H, complex m, lauryl), 1.22–1.48 (2 H, complex m, lauryl), 1.59, 1.67, 1.68, 1.69, 1.73, 1.77 and 1.84 (7 × 3 H, 7 s, Ac), 3.35 (1 H, m, disaccharide), 3.42–3.48 (1 H, m, disaccharide), 3.54–3.64 (3 H, m, disaccharide), 3.71–3.81 (2 H, m, disaccharide), 4.10 (1 H, m, disaccharide), 4.44 (1 H, d, *J* 9, disaccharide), 4.67 (1 H, m, disaccharide), 4.91–5.07 (2 H, m, disaccharide) and 5.16–5.20 (1 H, m, disaccharide); δ_C 13.62, 20.01, 20.08, 20.11, 20.31, 21.92 and 22.06 (7 × Me), 22.36, 25.42, 29.05, 29.21, 29.38 and 29.41 (6 × CH_2), 30.32 (CH_2, d, *J* 7.9), 31.63 (CH_2), 51.51 and 54.55 (2 × CH), 61.63 and 61.72 (2 × CH_2), 66.35 (CH_2OP, d, *J* 6), 68.44, 69.36, 71.17, 71.27, 72.04 and 75.11 (6 × CH), 93.96 (CH, d, *J* 5.9), 100.52 (CH), 169.89, 170.77, 170.95, 171.42, 171.86, 172.26 and 172.39 (7 × CO); δ_P −10.17 (d, J_{PP} 14) and −12.88 (d, J_{PP} 14).

2-*Acetamido*-O-(2-*acetamido*-2-*deoxy*-β-D-*glucopyranosyl*)-(1-4)-2-*deoxy*-α-D-*glucopyranosyl*-3,7,11,15-*Tetramethylhexadecyl Pyrophosphate* [(*GlcNAc*)$_2$-*P$_i$*-*P$_i$*-*phytanyl*] **4**.—To a solution of compound **16** (10 mg, 0.7 μmol) in dry CH_2Cl_2 (2.5 cm^3) was added 7% sodium methoxide in MeOH (50 mm^3, 0.07 μmol) and the reaction mixture was stirred for 2 h until completion of reaction as evidenced by TLC [($CHCl_3$–MeOH–water–ammonium hydroxide (2 mol dm^{-3}) (65:35:4:4), visualisation by naphthoresorcinol]. The reaction was then quenched by addition of MeOH (100 mm^3) and ammonium chloride [400 mm^3, 0.1 μmol as a solution of ammonium chloride (68 mg) in water (5 cm^3)], and the mixture was concentrated under reduced pressure then freeze-dried to give

compound **4**; δ_H (500 MHz; D_2O) 0.81–1.66 (39 H, complex overlapping m, phytanyl), 2.01 and 2.02 (2 × 3 H, 2 s, NHAc), 3.46–4.00 (14 H, complex overlapping m, phytanyl and disaccharide), 4.51 (1 H, d, J 8.4) and 5.50 (1 H, dd, J_{HH} 3.3, J_{HP} 7.1); δ_P (101.256 MHz) −13.12 (d, J_{PP} 15) and −15.74 (d, J_{PP} 15).

By the same method 2-acetamido *O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1-4)-2-deoxy-α-D-glucopyranosyl lauryl pyrophosphate **5**; δ_H (500 MHz; D_2O) 0.87 (3 H, t, J 7, lauryl), 1.24–1.40 (18 H, br m, lauryl), 1.59–1.68 (2 H, m, lauryl), 2.08 and 2.10 (2 × 3 H, 2 s, NHAc), 3.46–4.04 (14 H, complex overlapping m, lauryl and disaccharide), 4.64 (1 H, d, J 8.5) and 5.36 (1 H, dd, J_{HH} 3, J_{HP} 7.7); δ_P (101.256 MHz) −13.77 (d, J_{PP} 21) and −16.33 (d, J_{PP} 21).

Preparation of Microsomal Fractions from Porcine Liver.[7]—Fresh porcine liver (1 h after slaughter) was obtained from FMC Meat, Exeter and kept on ice for *ca.* 1 h. Portions (77 g) of the liver were minced with scissors and homogenised in 0.25 mol dm^{-3} sucrose: 50 mmol dm^{-3} Tris-HCl, pH 7.1 buffer (175 cm^3). The pink homogeneous suspension was centrifuged at 20 000 g for 10 min to give a thick brown pellet. The supernatant was then centrifuged for 5 h at 40 000 g. The pellet from the second centrifugation step was suspended in buffer (15 cm^3), divided into aliquots (1.3 cm^3), and frozen at −20 °C.

Preparation of Microsomal Fractions from Saccharomyces cerevisiae.—*S. cerevisiae* (X2180-A) was grown in 2% malt extract, 0.1% yeast extract in shake flasks at 27 °C for 40 h. The cells were collected by centrifugation (6000 g; 10 min), resuspended in buffer (50 mmol dm^{-3} Tris-HCl, pH 7.5, 5 mmol dm^{-3} magnesium chloride, 10 mmol dm^{-3} β-mercaptoethanol), washed twice by centriguation with buffer, and stored at −20 °C. Typically, wet cell paste (17.5 g) was resuspended in the same buffer and cells were disrupted by sonication (12 × 30 s bursts) at 0 °C. The cell extract was centrifuged (5000 g; 10 min; 4 °C) and the supernatant was recentrifuged (40 000 g; 2 h; 4 °C). The pellet was resuspended in the minimum of buffer and aliquoted into 4 × 0.5 cm^3 samples, which were frozen in liquid nitrogen and stored at −80 °C. The protein concentration was 4.6 mg/cm^3.

Typical Incubation of Acceptor Substrates.—The lipid acceptor (0.1 mg), dissolved in $CHCl_3$–MeOH (1:1; 1 cm^3), was mixed with $MnCl_2$ (0.1 mol dm^{-3}; 20 mm^3) and ethylenediaminetetraacetic acid (0.1 mol dm^{-3}; 5 mm^3); sufficient $CHCl_3$–MeOH (1:1; ~1 cm^3) was added to obtain a one-phase system, and the solvent was removed under reduced pressure. The residue was redissolved in Tris-HCl buffer (20 mmol dm^{-3}; pH 7.1; 400 mm^3) containing β-mercaptoethanol (50 μmol), either Triton X-100 (0.25 mg) or sodium deoxycholate (2.5 mg), the microsomal fraction [either from porcine liver (0.7 g) or 1.5 mg of yeast protein] and GDP-[U-^{14}C]mannose (66 mg) and this mixture was incubated for 3 h at 30 °C. The reaction was terminated by boiling of the incubation mixture at 100 °C for 90 s.

Analysis of Glycolipid Products.—Centrifugation of the boiled incubation mixture gave a pellet, which was washed with distilled water (3 × 1 cm^3) to remove GDP-[U-^{14}C]mannose. The lipid-linked oligosaccharides were extracted with $CHCl_3$–MeOH–water (10:10:3; 3 × 3 cm^3) and counted (Table 1). Three distinct peaks could be separated by gel filtration chromatography [Sephadex LH-20, 3 × 90 cm column, eluent $CHCl_3$–MeOH–water (12:6:1)]. Elution profiles are shown in Figs. 1 and 2.

Acid Hydrolysis of Glycolipid Products and Analysis of the Released Oligosaccharides by P4 Chromatography.—The pooled fractions from the LH-20 column were evaporated and the residues were redissolved in hydrochloric acid (1 cm^3; 0.02 mol dm^{-3} in 20% methanol) and heated in a boiling water-bath for 20 min. After cooling, the lipids were extracted with chloroform (2 × 1 cm^3) and final traces of chloroform were removed by blowing argon through the aqueous fractions. The oligosaccharides were then analysed on a Bio-Gel P4 gel filtration column (2.6 × 100 cm, Fig. 3).[21]

Acknowledgements

We thank the University of Essex for a Frank Southenden studentship (J. P. T.) and a grant from the Research Fund and the Royal Society for an equipment grant (to S. L. F.).

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Paper 2/01589G
Received 25*th March* 1992
Accepted 28*th April* 1992