

Chemo-enzymatic Synthesis of a β -Mannosyl-containing Trisaccharide

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For the natural substrate GlcNAcGlcNAc-pyrophosphate-dolichyl **2**, replacement of dolichyl by phytanyl yields an analogue **3** that acts as an efficient acceptor substrate for a mannosyl transferase, whereas the corresponding lauryl compound **4** was found to be inactive.

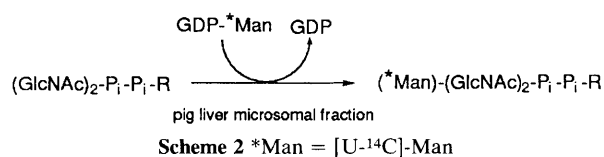
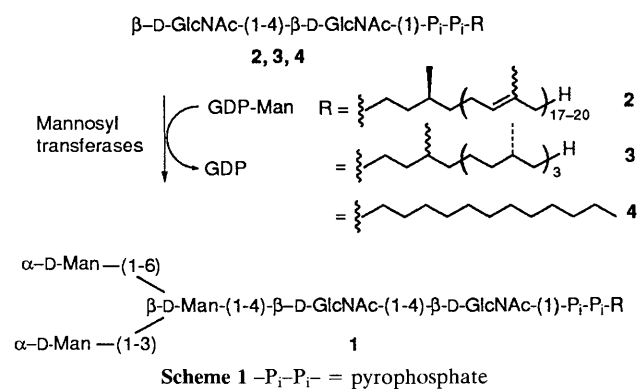
The enzymes of the biosynthetic pathway leading to N (asparagine)-linked oligosaccharides have been shown to occur in many different sources (*e.g.* yeast,¹ mung beans² and liver³). In all cases the initial stage involves synthesis of a core pentasaccharide **1** through addition of three mannosyl residues to a di-*N*-acetylglucosamine residue that is attached to a lipid **2** (R = dolichyl) (Scheme 1).⁴

The potential for using this pathway to prepare the pentasaccharide core by a chemo-enzymatic approach depends heavily on finding a replacement for the polyisoprenoid dolichol. Previous work has shown that the length

of the dolichyl lipid is important in the early stages of the biosynthesis (*e.g.* formation of GlcNAc-P₁-P₁-dolichyl).⁵ However, we reasoned that as the oligosaccharide chain becomes elongated, the specific nature of the lipid side chain may become less important. This led us to consider the phytanyl **3** and lauryl **4** analogues of **2** as candidate substrates for mannosyl transfer. An efficient synthesis of both **3** and **4** has been described in the preceding communication⁶ and herein we report the results of the enzymatic studies.

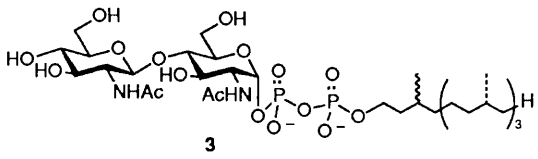
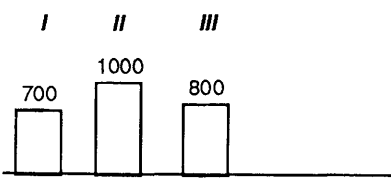
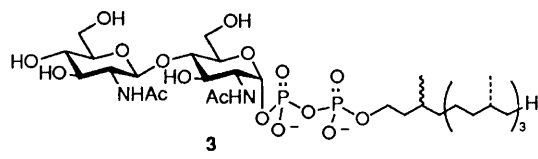
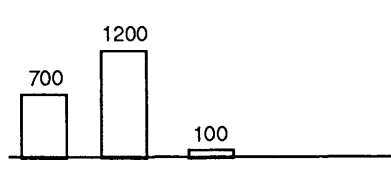
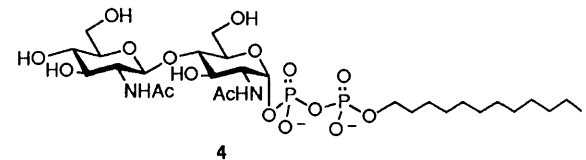
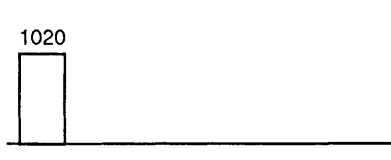

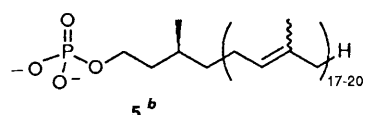
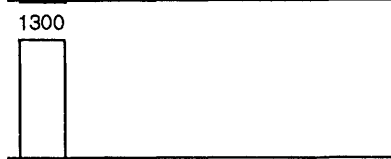
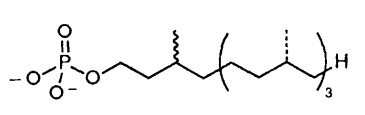
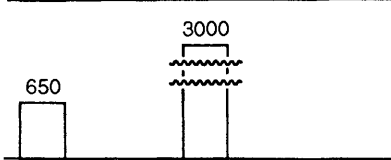
The method we chose to follow for incorporation of a mannosyl residue is shown in Scheme 2.⁷ The crude enzyme system containing the required mannosyl transferase was obtained from pig liver.³ Substrates were then incubated with this enzyme preparation in the presence of GDP-[U-¹⁴C]-mannose. At the end of the reaction lipid-containing products were extracted into CHCl₃-MeOH-H₂O (10:10:3) and the levels of incorporation of labelled mannose were then determined by scintillation counting. The addition of acceptor lipids **3**, **5** and **6** stimulated incorporation into these extractable lipids as shown in Table 1 (Column A), whereas compound **4** showed background levels of incorporation.

Efficient separation of any dolichyl, phytanyl and lauryl containing products was best carried out using gel filtration



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Table 1 Mannosyl incorporation

Entry	Acceptor lipid added	Detergent	A ^d	B ^e
1		a	56 000	
2		b	46 000	
3		a	29 000	
4	None	a	34 000	
5		a	49 000	
6		a	76 000	

^a a: Triton X-100; b: sodium deoxycholate. ^b Ref. 5. ^c Ref. 6. ^d * Man incorporated into lipid (counts min⁻¹). ^e Radioactivity of peaks from gel filtration column (counts min⁻¹).

chromatography on Sephadex LH-20.† Table 1 (Column B) shows the results obtained. With the phytanyl substrate **3** (entry 1) three separable radioactive peaks (*I*, *II* and *III*; *I* with the lowest and *III* the highest retention) were recovered from the gel filtration column whereas using the lauryl substrate **4** (entry 3) gave only a single peak that coeluted with fraction *I* from the phytanyl experiment. When the experiment with the phytanyl substrate **3** was repeated using the detergent sodium deoxycholate in place of Triton X-100 the level of fraction *III* was largely reduced (entry 2).

† The following protocol was used: the lipid acceptor (0.1 mg) dissolved in CHCl₃-MeOH (1:1; 1 ml) was mixed with MnCl₂ (0.1 mol dm⁻³; 20 μl), ethylenediaminetetraacetic acid (0.1 mol dm⁻³); sufficient CHCl₃-MeOH (1:1; ca. 1 ml) was added to obtain a one-phase system, and the solvent then removed *in vacuo*. The residue was redissolved in Tris-HCl buffer (20 mmol dm⁻³; pH 7.1; 400 μl) containing mercaptoethanol (50 μmol), either Triton X-100 (0.25 mg) or sodium deoxycholate (2.5 mg), the microsomal fraction from 0.7 g of pig liver,³ and GDP-[U-¹⁴C]-mannose (66 ng) and the mixture was incubated for 3 h at 30 °C. The reaction was terminated by boiling the incubation mixture at 100 °C for 90 s. Centrifugation of the mixture gave a pellet which was washed with distilled water (3 × 1 ml) to remove excess of GDP-[U-¹⁴C]-mannose. The lipid-linked oligosaccharides were extracted with CHCl₃-MeOH-H₂O (10:10:3; 3 × 3 ml) and separated by gel-filtration chromatography (Sephadex LH-20, eluent CHCl₃-MeOH-H₂O, 12:6:1). Three distinct radio-labelled peaks (fractions *I*, *II* and *III* in Table 1) were obtained and the ¹⁴C content was determined by scintillation counting.

The identity of fractions *I*, *II* and *III* was determined from control experiments. Thus when no exogenous lipid acceptor was added (entry 4) only fraction *I* was obtained, but the level of *I* increased when dolichyl phosphate **5** was added (entry 5). This suggests that fraction *I* in entries 1, 2, 3, 5 and 6 can be assigned to dolichyl-linked oligosaccharides (*M_r* larger than 2000) derived from endogenous dolichol present in the enzyme preparation. Addition of phytanyl phosphate **6** (entry 6) gave rise to a high level of fraction *III* only, and therefore this peak may be assigned to phytanyl-P_i-mannose (*M_r* = 505).⁸

These results suggested that fraction *II* arose from mannosyl transfer to the phytanyl-linked disaccharide **3** (*M_r* = 980). This was supported by isolation of fraction *II*, acid hydrolysis to remove the lipid side chain and pyrophosphate group,⁹ and analysis of the resulting oligosaccharide component by TLC (silica).¹⁰ The mobility of the oligosaccharide relative to standards was consistent with the trisaccharide structure *Man-GlcNAc-GlcNAc.¹⁰ Finally treatment of this trisaccharide with β-mannosidase resulted in the release of radioactive mannose, whereas α-mannosidase had no effect, in agreement with the expected presence of a β-mannoside linkage. This analysis of the oligosaccharide component of fraction *II* is in close agreement with that obtained from the corresponding dolichyl linked compound¹⁰ and demonstrates that phytanyl is indeed an effective mimic of dolichol in this reaction.

Hydrolysis of fraction *III* using the above procedure yielded only labelled mannose, confirming that *Man-P₁-phytanyl had been formed as reported before.¹¹

In conclusion, we have shown that the phytanyl containing acceptor **3** is a good substrate for the first mannosyl transferase involved in the synthesis of the pentasaccharide core. However, the lauryl analogue **4** was a poor mimic of the natural dolichyl substrate **2**. 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.

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