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 beans[2] and liver[3]). 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 poly-isoprenoid 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-P_i-P_i-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 communication[6] and herein we report the results of the enzymatic studies.

β-D-GlcNAc-(1-4)-β-D-GlcNAc-(1)-P_i-P_i-R

2, 3, 4

Mannosyl transferases: GDP-Man → GDP

R = [dolichyl, $_{17-20}$] **2**; = [phytanyl, $_3$] **3**; = [lauryl] **4**

α-D-Man—(1-6)

β-D-Man-(1-4)-β-D-GlcNAc-(1-4)-β-D-GlcNAc-(1)-P_i-P_i-R

α-D-Man—(1-3)

1

Scheme 1 –P_i–P_i– = pyrophosphate

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-^{14}C]-mannose. At the end of the reaction lipid-containing products were extracted into $CHCl_3$–MeOH–H_2O (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

$(GlcNAc)_2$-P_i-P_i-R —(GDP-*Man → GDP; pig liver microsomal fraction)→ (*Man)-$(GlcNAc)_2$-P_i-P_i-R

Scheme 2 *Man = [U-^{14}C]-Man

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Table 1 Mannosyl incorporation

Entry	Acceptor lipid added	Detergent	A^d	B^e I	B^e II	B^e III
1	3	a	56 000	700	1000	800
2	3	b	46 000	700	1200	100
3	4	a	29 000	1020		
4	None	a	34 000	950		
5	5 [b]	a	49 000	1300		
6	6 [c]	a	76 000	650		3000

[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 $CHCl_3$–MeOH (1 : 1; 1 ml) was mixed with $MnCl_2$ (0.1 mol dm^{-3}; 20 μl), ethylenediaminetetraacetic acid (0.1 mol dm^{-3}); sufficient $CHCl_3$–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-^{14}C]-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-^{14}C]-mannose. The lipid-linked oligosaccharides were extracted with $CHCl_3$–MeOH–H_2O (10 : 10 : 3; 3 × 3 ml) and separated by gel-filtration chromatography (Sephadex LH-20, eluent $CHCl_3$–MeOH–H_2O, 12 : 6 : 1). Three distinct radiolabelled peaks (fractions *I*, *II* and *III* in Table 1) were obtained and the ^{14}C 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 dissaccharide **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 compound[10] 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-P_i-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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