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Enzymatic synthesis of β-mannosyl phosphates on solid support†

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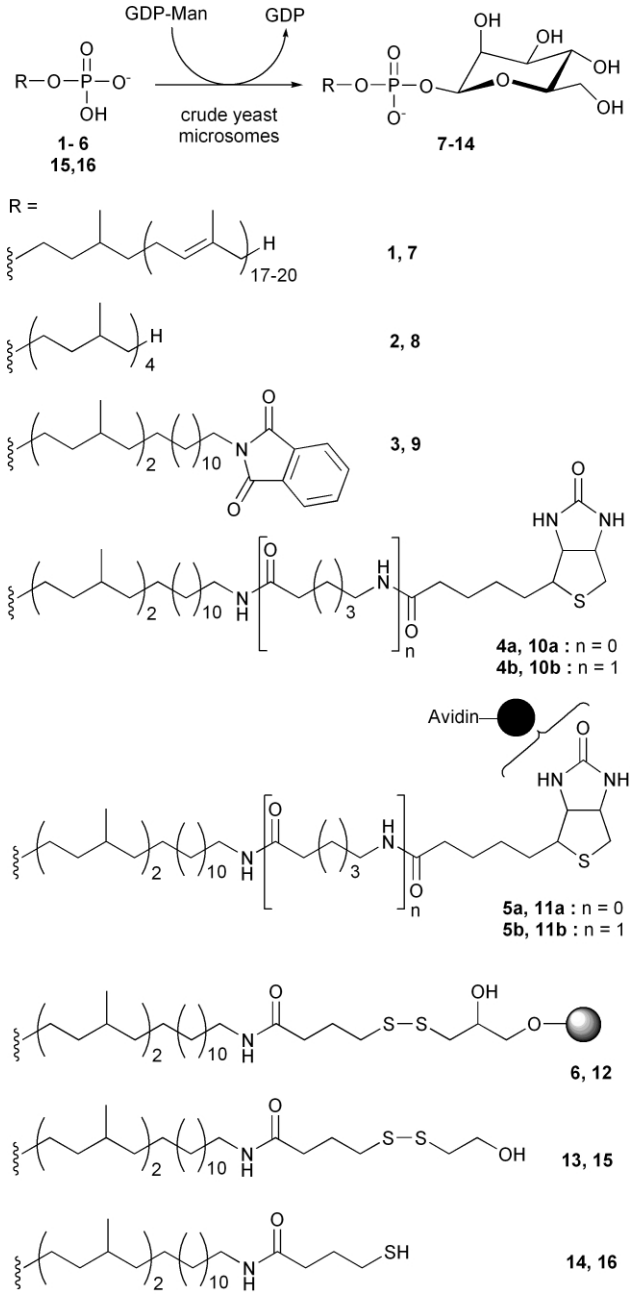
Synthetic bifunctional analogues 4a,b and 14 of dolichol phosphate 1 were attached to solid support and were shown to be substrates for Dol-P-Man synthase.

Dolichol phosphate **1** is a member of a class of polyisoprenoid phosphates that are linked to intermediates of glycoconjugate biosynthesis, notably that of *N*-glycans and bacterial cell surface polysaccharides.[1] Consequently, many of the glycosyltransferases involved in these biosynthetic processes require conjugation of substrate to polyisoprenoids for recognition. An example of such a class of glycosyltransferase is the Dol-P-Man synthase, which catalyses the stereoselective β-mannosylation of dolichol phosphate **1** to yield mannosyl dolichol phosphate diester **7** (Scheme 1).[2] Mannosyl phosphate **7** is the essential cofactor in several biosynthetic pathways including that of glycoproteins (*N*- and *O*-glycan) and glycosyl phosphatidylinositol anchors.

Polyisoprenoid dependent glycosyltransferases are of interest because they are linked to congenital disorders involving defective *N*-glycosylation of proteins.[3] They are also important targets for novel antibiotics (when involved in bacterial cell wall polysaccharide synthesis[4]) and they can be used as catalysts for the synthesis of *N*-glycans.[5] For all these applications a closer understanding of the role of the polyisoprenoid structure in substrate recognition by the transferases is important. Thus, we have found previously[6] that a minimal length polyisoprenoid needs to be conjugated to the substrate for Dol-P-Man synthase recognition, but that the much shorter and fully saturated polyisoprenoid phytanyl phosphate **2** was a comparable substrate to dolichol phosphate **1** for both the Dol-P-Man synthase and for subsequent mannosyltransferases involved in *N*-glycan biosynthesis.[5,6] The present paper reports the evaluation of bifunctionalised polyisoprenoid analogues and describes the first glycosylation catalysed by this class of glycosyltransferases on solid support.

ω-Substituted 3,7-dimethyl eicosyl phosphates **3**–**6** were chosen as the target structures based on minimal lipid size requirements defined by previous studies.[6g] Mixtures of epimers at the 3 and 7 position were synthesised, since stereochemical preference at position 3 had been shown previously to be only small.[7] Three ω-substituents were investigated: the *N*-phthalimide **3** was a synthetic intermediate of **4**, **5** and **6** and was tested first to establish tolerance for an amide/imide at the ω-position. The ability to tolerate conjugation to biotin as in **4a**,**b** would show that useful biochemical labels are tolerated[8] and would allow us to immobilise the substrate on avidin columns using the well-established high affinity between biotin and avidin. Finally, substrate **6** is covalently linked to sepharose and would establish if the enzyme can react on solid support. The disulfide link not only allows a straightforward attachment of the phospholipid linker to the solid support (in yields of 33–56%) but also a mild way of cleavage of the enzymatic product.[9] The synthesis of all these substrates **3**–**6** will be reported elsewhere.

Compounds **3**, **4a** and **4b** were tested first as substrates for Dol-P-Man synthase by incubation with radiolabelled GDP-mannose and crude microsomal fractions of *Saccharomyces cerevisiae*.‡ The soluble acceptor lipid **3** compared well to phytanyl phosphate as a substrate (62% of activity compared to



Scheme 1 β-Mannosylation of polyisoprenoid phosphates; **5a**, **5b**, **11a** and **11b** were immobilised on avidin linked to agarose and **6** and **12** were immobilised on sepharose.

† Electronic supplementary information (ESI) available: experimental. See http://www.rsc.org/suppdata/cc/b2/b206451k/

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phytanyl phosphate[6c]). Thus, polar groups appear to be tolerated at the ω-position. The soluble biotinylated compounds **4a** and **4b** were less good with relative activities of 17 and 11%, but were nevertheless clearly recognised and mannosylated by the enzyme.

The Dol-P-Man synthase was then tested for activity against the solid-supported substrates **5a**,**b** and **6** using the same assays as before, measuring incorporation of radioactivity from GDP-^{14}C-mannose into lipid.[6g] Incorporation of mannose into lipid could be monitored either by directly counting radioactivity on solid support or after cleavage off the support with biotin (for **5a** and **5b**) or mercaptoethanol (for **6**). All solid-supported compounds **5a**, **5b** and **6** appeared to be substrates for the Dol-P-Man synthase, since a significant amount of the radiolabelled starting material was converted to product (1190 cpm for **5a** and 970 cpm for **5b**; 3120 cpm for **6**; blank (no lipid) gave 65 cpm).

A more detailed analysis was carried out on the incubation products of **6** using LC-MS. After mercaptoethanol cleavage of the incubation products, **13** and **14** are clearly detectable by LC-MS next to starting materials **15** and **16** (Fig. 1).

These results demonstrate that bifunctional lipids **3**–**6** are substrates for the Dol-P-Man synthase present in the crude microsomes of yeast. Enzymatic mannosylation of phosphate lipids should be a useful route to Dol-P-Man substrate analogues, since the β-mannosyl phosphate linkage is particularly difficult to synthesise.[10] In addition, we have shown for the first time that a dolichol dependent glycosyltransferase can recognise substrates on solid support. These findings open up potential applications in enzyme-catalysed solid phase synthesis of *N*-glycans.

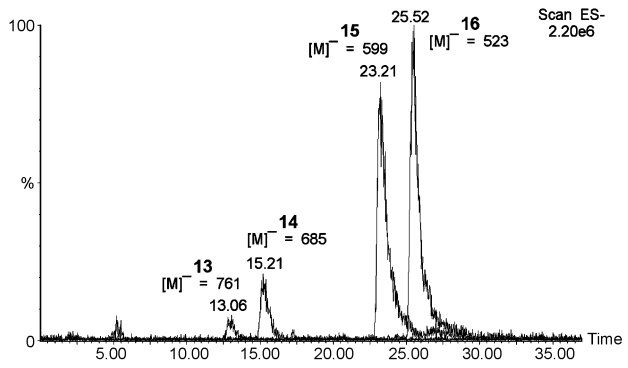

Fig. 1 Elution profiles (LC-MS; C18 reversed phase column; 150 × 2.00 mm 3 μm; water–acetonitrile gradient from 50:50 to 5:95; electrospray ionisation; negative ionisation) of **13**, **14**, **15** and **16**.

Finally, it is interesting to note a practical advantage of solid phase enzymatic synthesis: the product isolation consists simply of washing the resin and can thus tolerate the use of crude enzyme preparations such as the microsomal fractions in the present study. Since purification of glycosyltransferases, in particular of the membrane associated dolichol dependent transferase is difficult, solid phase synthesis is attractive.

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Notes and references

‡ Yeast microsomes were prepared as described before.[6g]

A typical enzymatic assay: acceptor lipids **2**, **3**, **4a** and **4b** (100 μM) were incubated, each with crude microsomes (1–2 mg), GDP-mannose (20 μM), GDP-[U-^{14}C]mannose (0.1 μCi mL^{-1}) and buffer (50 mM Tris-HCl, 5 mM $MgCl_2$, 10 mM mercaptoethanol, 0.5% Triton-X-100 (v/v), pH 7.5, 1 mL) at 37 °C for 1 h. Aliquots (100 μL) were removed and added to 100 μL of $CHCl_3$–MeOH (1:1, v/v). The aqueous phase was removed after centrifugation. The organic phase was washed twice with water and incorporation of radioactivity into organic soluble material was measured by scintillation counting.

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