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SYNTHESIS OF DOLICHYLPYROPHOSPHATE-LINKED OLIGOSACCHARIDES

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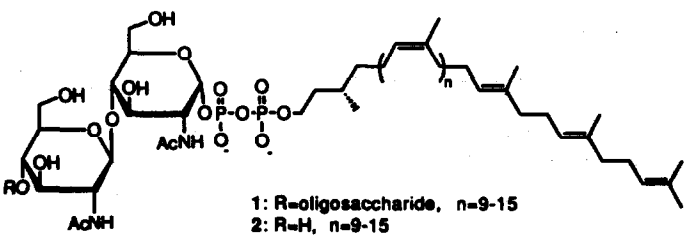
Summary: An efficient synthesis of radiolabelled P^1-di-N-acetyl-α-chitobiosyl P^2-dolichylpyrophosphate is described. The synthesis involves both chemical and enzymatic transformations.

Asparagine glycosylation represents the primary pathway by which glycoproteins are biosynthesized.[1] However, mechanistic studies of *oligosaccharyl transferase,* the membrane-associated enzyme responsible for asparagine glycosylation, have been hampered by the lack of a reliable source of the biochemical oligosaccharide donors utilized by this enzyme. The donors are characterized by a lipophilic moiety, dolichol, linked *via* a pyrophosphate bridge to the oligosaccharide as typified by **1**. While *in vivo*, the extended glycosyl moiety usually transferred to nascent polypeptides is comprised of a branched oligosaccharide containing two N-acetylglucosamine, nine mannose and three glucose units, it has been demonstrated that the enzyme can also transfer the chitobiose moiety from the simpler, truncated, substrate **2** in an *in vitro* system.[2] Thus, to facilitate further investigation of this system an optimal synthesis of **2** which also allows for the efficient incorporation of radiolabel into the saccharide portion is desirable.



1: R=oligosaccharide, n=9-15
2: R=H, n=9-15

Previously **2** has been prepared from dolichyl phosphate (DolP) and uridine diphosphate N-acetylglucosamine (UDPNAG) through the action of two microsomal enzymes responsible for the sequential transfer of N-acetyl glucosaminyl phosphate (Enzyme I) and N-acetylglucosamine (Enzyme II) to dolichyl phosphate (as illustrated below).[3,4] Utilization of this method for large scale preparation of lipid-linked substrate is problematic due to product inhibition of enzyme I by uridine monophosphate (UMP). In addition, the two enzymes require rather different optimum conditions for maximum efficiency. Thus, a "one-pot" biosynthetic transformation of dolichyl phosphate into **2** is not feasible on a significant scale.

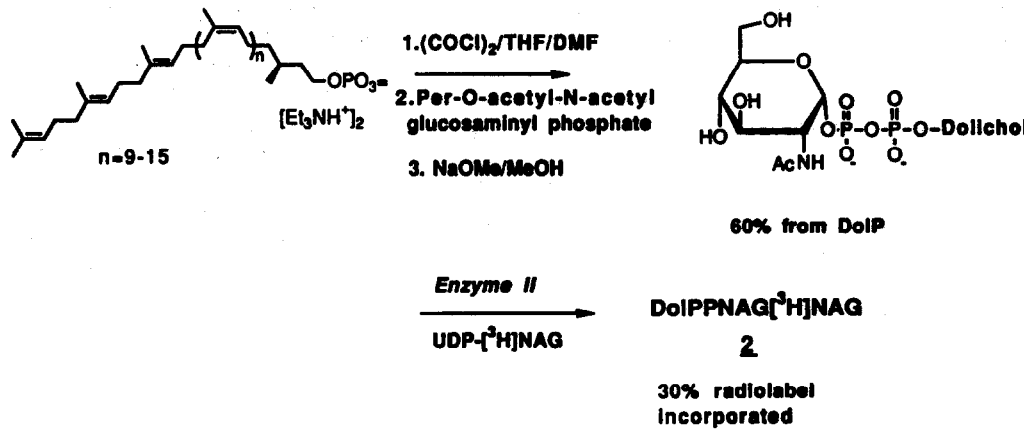
$$\text{DolP} + \text{UDPNAG} \xrightarrow{\textit{Enzyme I}} \text{DolPPNAG} + \text{UMP}$$

$$\text{DolPPNAG} + \text{UDPNAG} \xrightarrow{\textit{Enzyme II}} \text{DolPP(NAG)}_2 + \text{UDP}$$

Metabolite 2 has also been prepared synthetically on a very small scale by reaction of the diphenylpyrophosphate ester of dolichol with a peracetylated mono- or disaccharide phosphate to afford the N-acetyl glucosaminyl or chitobiosyl derivatives respectively.[5] Use of the pyrophosphate activation strategy is complicated by the need to remove excess diphenylphosphochloridate in an intermediate step in the coupling process. In addition this form of activation is rather weak and pyrophosphate coupling requires long reaction times.

The strategy that we have developed to facilitate production of radiolabelled P^1-di-N-acetyl-α-chitobiosyl P^2-dolichylpyrophosphate has involved improvement of the pyrophosphate coupling procedure through use of a highly activated dolichylphosphoryl dichloride and optimization of the glycosyl transfer step catalyzed by enzyme II using synthetic lipid-linked substrate. This is outlined in Scheme 1 and described in detail thereafter.

Scheme 1



Synthesis of P^1-2-Acetamido-2-deoxy-α-D-glucopyranosyl P^2-dolichylpyrophosphate

Dolichyl phosphate bis(triethylammonium) salt[6] (90mg, 0.058mmol) was azeotropically dried by three treatments with anhydrous toluene. The phosphate was then redissolved in 3mL anhydrous tetrahydrofuran (THF). In a separate flask was combined 3mL THF, oxalyl chloride (200μL, 2.1mmol) and dimethylformamide (5μL). The dolichyl phosphate solution was then added dropwise over 2h to the latter reaction mixture at room temperature. (A slow addition rate is essential in order to minimize formation of a di-dolichyl pyrophosphate side product.) After a stirring further 2h all volatiles were removed *in vacuo* (caution frothing) and the resultant residue concentrated several times with dry toluene to afford dolichylphosphoryl dichloride. The product was used immediately in the subsequent reaction.

2-Acetamido-3,4,6-tri-O-acetyl-2-deoxy-α-D-glucopyranosyl phosphate (pyridinium form)[7] (60mg, 0.1mmol) was dried by treatment with anhydrous pyridine, and subsequently redissolved in the same solvent (1mL). The pyridine solution of the glycosyl phosphate was then added to the preparation of dolichyl phosphoryl dichloride. The reaction mixture was stirred for approximately 1h at which time the pyrophosphate coupling reaction was complete as evidenced by TLC (R_f 0.55; 65:25:4 chloroform/methanol/water). The reaction mixture was then concentrated and redissolved in 10 mL acetone:water:triethylamine (88:10:2) and stirred vigorously for

2h to hydrolyze any remaining phosphoryl chloride. The crude reaction mixture was then concentrated and purified by flash silica chromatography, eluent 10:1 chloroform/methanol (0.5% water) followed by 5:1 chloroform/methanol (0.5% water) to afford 65mg (60% yield) pure product. Both ^{1}H and ^{13}C NMR spectra are dominated by signals due to the polyisoprenoid moiety, however the correct identity of the product has been established based on the following physical data. ^{1}H NMR ($CDCl_3/(CD_3)_2SO$, 500MHz) δ: 0.85(d,3H), 1.64(br s,~48), 2.0(br s,~64), 3.94(m,2H), 4.18(m,2H), 4.27(m,2H), 5.09(m, ~16H), 5.26(m,2H) 5.67(m,1H).[8] The ^{13}C NMR is consistent with the product.[9] Negative ion FAB mass spectrum (chloroform/3-nitrobenzyl alcohol matrix) MH^+ for several homologs of the bis pyridinium salt of the product observed: 1416(n=7), 1483(n=8), 1552(n=9), 1619(n=10), 1754(n=12), 1822(n=13), 1890(n=14).

The dolichylpyrophosphoryl ester was cleanly O-de-acetylated according to the literature procedure[5] with 3% anhydrous sodium methoxide in methanol and used without further purification.[10]

Biosynthesis of P^1-di-N-acetyl-α-chitobiosyl P^2-dolichylpyrophosphate:

Dolichyl pyrophosphoryl N-acetylglucosamine was converted to the corresponding disaccharide derivative by reaction with uridine diphosphate N-acetyl-D-glucosamine-6-^{3}H (specific activity 18.3Ci/mmol) in the presence of a freshly prepared porcine liver microsomal preparation[11] containing enzyme II. In a typical preparation resuspended microsomes (15μL, ~100mg/mL protein) were incubated with 55mM tris-maleate (pH 7.7), 0.5% sodium deoxycholate, 110mM β-mercaptoethanol, 170mM $MgCl_2$, 2mM DolPPNAG and 2μCi UDP-[^{3}H]NAG (~2.5μM) for 30 min at room temperature. At this time the reaction was quenched and extracted as described previously.[5] The crude product was purified by DEAE-cellulose anion exchange chromatography (acetate form)[12] and on silica gel G.[13] Incorporation of radioactivity was approximately 30%. The purified product has been characterized by acid catalyzed hydrolysis followed by gel filtration on a Biogel P-2 column calibrated with N-acetylglucosamine and chitobiose standards. In addition the semisynthetic material was identical in all respects when compared with authentic material made by the biosynthetic route from dolichyl phosphate and UDP[^{3}H]NAG. We anticipate that this step could also be optimized for reaction with unlabelled UDPNAG to afford a greater supply of synthetic **2**.

In conclusion, by this method we have been able to prepare as much as 20μCi of labelled dolPP$(NAG)_2$ which represents ample supply for extensive studies of the oligosaccharyl transferase. The general strategy described, involving a combination of synthetic and enzymatic transformations, should prove useful in providing substrates for investigation of the central biosynthetic pathway responsible for the production of glycoproteins.

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References and Notes:

1.H.A. Kaplan, J.K. Welply and W.J. Lennarz, *Biochim. Biophys. Acta.*, (1987) **906**, 161.
2.C.B. Sharma, L. Lehle and W. Tanner, *Eur. J. Biochem.*, (1981) **116**, 101.
3.N.H. Behrens and E. Tabora, *Methods in Enzymology*, (1978) **50**, 402.
4.J.J. Elting and W.J. Lennarz, *Methods in Enzymology*, (1982) **83**, 408.
5.C.D. Warren and R.W. Jeanloz, *Methods in Enzymology*, (1978) **50**,122. The synthetic work described by these authors was carried out on a very small scale *ca.* 6-10 mg. (0.005 mmol) of dolichyl phosphate due to the scarcity of the dihydropolyisoprenol dolichol. Our investigations have been facilitated by the increased availability of semisynthetic supplies of this lipid. See B. Imperiali and J. W. Zimmerman, *Tetrahedron Lett.* (1988) **29**, 5343 and S. Suzuki, F. Mori, T. Takigawa and K. Ibata, *Tetrahedron Lett.*, (1983) **24**, 5103.
6.L.L. Danilov and T. Chojnacki, *FEBS Lett.*, (1981) **131**, 310. This method has been used to prepare up to 1 gram of dolichyl phosphate in >80% yield. The only modification made to the procedure is in the purification which can be conveniently carried out utilizing flash silica gel chromatography, eluent 10:1 chloroform/methanol (0.5% water) followed by 5:1 chloroform/methanol (0.5% water).
7.A.Ya. Khorlin, S.E. Zurabyan and T.S. Antonenko, *Tetrahedron Lett.*, (1970) 4803.
8.In order to observe the signals due to the N-acetylglucosamine moiety, it is crucial to carry out the NMR spectrum at high dilution (<0.4mg/mL) since at higher concentrations the signals are broad and indistinguishable from baseline, potentially due to aggregation of the substrate. Even at this concentration, however, it is not possible to discern coupling constants due to residual line broadening which cannot be overcome.
9.The concentrations necessary to achieve a reasonable signal to noise ratio cause significant line broadening, however signals due to the peracetylglucosamine residue (60-75 and 165-170 ppm) are observed in the ^{13}C NMR spectrum.
10.Only small quantities of material are O-de-acetylated at a given time since the pyrophosphate in this form is significantly less stable even at -80°C.
11.The procedure used for microsome preparation is based on that described in reference 3. More complete procedural information on the microsomal enzymes will be published in a forthcoming paper.
12.Lipid pyrophosphoryl compounds are eluted with 10:10:3 chloroform/methanol/water (20mM ammonium acetate).
13.This column is needed to separate mono- and disaccharide derivates. DolPPNAG is eluted with 72:21:2 chloroform/methanol/water and dolPPNAG[^{3}H]NAG with 60:25:4 chloroform/methanol/water.

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