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SYNTHESIS AND EVALUATION OF SYNTHETIC ANALOGUES OF DOLICHYL-P-P-CHITOBIOSE AS OLIGOSACCHARYLTRANSFERASE SUBSTRATES.

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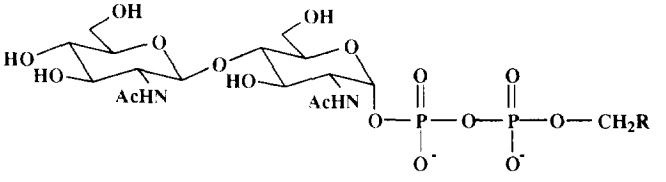
Abstract. Five analogues of dolichylpyrophosphorylchitobiose containing short, well defined lipid components were synthesized and evaluated as substrates for yeast oligosaccharyltransferase. All of the modified lipids led to a large decrease in enzyme-catalyzed glycosylation of the peptide substrate, $BzAsnLeuThrNH_2$, thus indicating a critical role for the polyisoprene, dolichol, in anchoring the oligosaccharide donor during catalysis.

In the course of research on the initial characterization of the enzyme oligosaccharyltransferase (OST, EC.2.4.1.119), we sought to synthesize a series of lipid pyrophosphoryl chitobioses (**1**). We have previously reported the synthesis of the dolichyl derivative, **1a**,[1] (Figure 1) and its use as an OST substrate for mechanistic studies.[2] Unfortunately, dolichol prepared as previously described[1,3] is derived from a mixture of naturally occurring polyprenols containing from 17-21 isoprene units (C_{85}-C_{105}). It was of interest to explore the use of



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simpler, more readily available lipids, with fewer possibilities for oligomeric or isomeric mixtures, as possible alternatives to the dolichyl moiety of **1a**. Previous research on the role of lipids in the reactions catalyzed by various enzymes of the "dolichyl phosphate cycle"[4,5] has led to the conclusion that shorter lipids, e.g., phytanyl,[6,7] and citronellyl,[8] can substitute for dolichol. In this paper we report the synthesis and evaluation of several analogues of **1a**, in which lipid portion, RCH_2-, is phytanyl (**1b**), dihydrofarnesyl (**1c**), dodecanyl (**1d**), and citronellyl (**1e**), as substrates for OST-catalyzed glycosylation of the peptide, Bz-Asn-Leu-Thr-NH_2.

Synthesis of the desired lipid derivatives, **1**, was carried out by the route outlined in Scheme 1. Phosphorylation of the parent alcohol to give **2** was effected either by reaction with $POCl_3$ followed by hydrolysis of the intermediate phosphorodichloridate (Method A),[1,9] or by reaction with di-*t*-butyl *N,N*-diethyl-phosphoramidate followed by oxidation of the intermediate phosphite and acid-mediated hydrolysis of the $P(OBu^t)$ esters (Method B).[6,10] As indicated in Table 1, yields of **2** were higher using Method A but isolation and purification was less time-consuming with Method B. Heptaacetyl-chitobiosyl-1-phosphate was prepared as

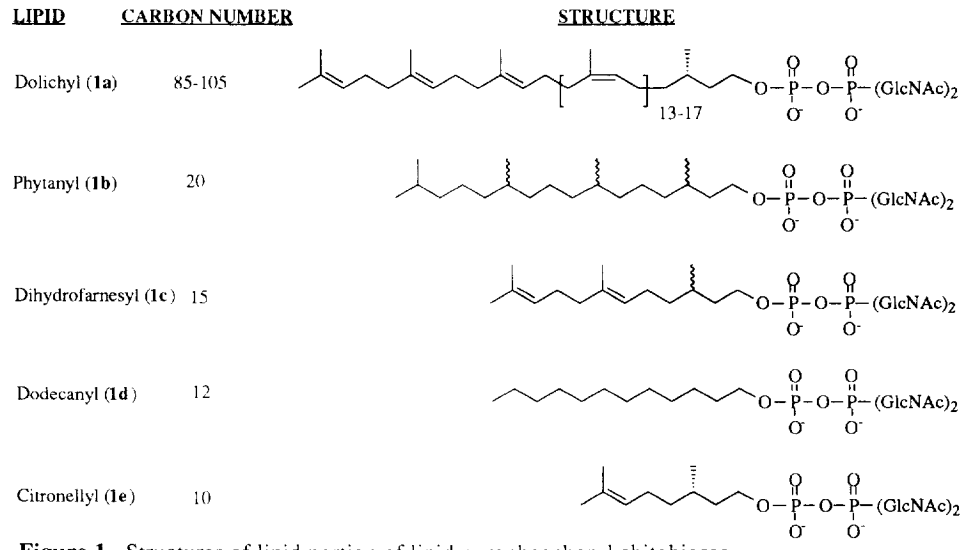


Figure 1. Structures of lipid portion of lipid pyrophosphoryl chitobioses.

previously described,[1] with the exception that dibenzylphosphorochloridate[11] was used in place of tetrabenzylpyrophosphate to phosphorylate the reducing sugar. Coupling of the lipid phosphates to peracetylated chitobiose-1-phosphate, mediated by carbonyldiimidazole,[1,12] followed by deacetylation gave the desired lipid pyrophosphoryl chitobioses (Table 1).

All newly synthesized compounds were fully characterized by NMR and MS. In particular, ^{1}H-NMR spectra of **3a-e** all show the same complex pattern attributable to the disaccharide portion (δ 3.5-6.0 ppm) and individual resonances were assigned by analysis of 500 MHz homonuclear COSY spectra. Compounds **1b** and **1d** were synthesized previously by Flitsch and co-workers using similar chemistry as that shown in Scheme 1;[6,7] spectral properties of **1b** and **1d** are in agreement with those provided in the literature.

Table 1. Summary of yields for reactions shown in Scheme 1.[a]

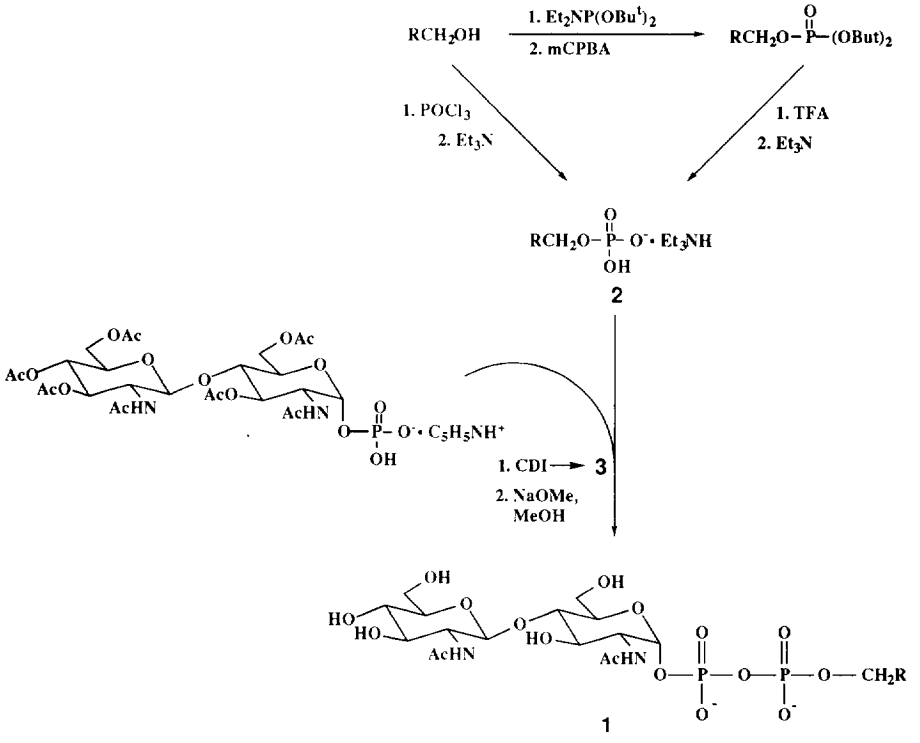
RCH_2–	Yield **2**, % [b]	Yield **3**, %	Yield **1**, %
Dolichyl (**1-3a**)	– [c]	51	100
Phytanyl (**1-3b**)	75 (B)	80	100
H_2-Farnesyl (**1-3c**)	97 (A)	77	100
Dodecanyl (**1-3d**)	88 (B)	55	100
Citronellyl (**1-3e**)	95 (A)	78	100

[a] All compounds were fully characterized by ^{1}H NMR, ^{13}C NMR, ^{31}P NMR, and FAB-MS. Spectral data are consistent with the structures indicated.

[b] Method A or B, See Experimental Section for details.

[c] Prepared as previously described using Method A.[1]

Scheme 1



The newly synthesized compounds, **1a**-**1e**, were assessed as OST substrates. The data, summarized in Figure 2, show that lipids of various degrees of saturation, chain length, stereochemical homogeneity, and containing up to 20 carbon atoms, are very poor substrates for this membrane-associated enzyme. The amount of glycopeptide formed using the dolichyl substrate, **1a**, was ca. 8 nmol and ca. 1.2 nmol for the membrane-bound and solubilized forms of OST, respectively. Flitsch et al. have shown that **1b** is a substrate for crude microsomal β-1,4-mannosyltransferase isolated from pig liver or yeast[6] or in lysates of *E. coli* expressing the *ALG1* gene of *S. Cerevisiae*.[7] In other research, it was shown that shorter polyisoprenes could function as substrates, albeit considerably less efficiently than the longer chain polyisoprenes, for several enzymes of the dolichyl phosphate cycle.[8,13] However, several reports suggest that the α-isoprene unit of a polyprenol must be reduced, as is found in dolichol, for substrate activity.[8,14-17] In one case where this question was addressed in intact cells, it was found that mutant Chinese hamster ovary cells with a defect in the conversion of polyprenols to dolichol synthesize less glycoproteins than the wild type, again suggesting a requirement for dolichol in glycoprotein biosynthesis.[18] The results presented in this Letter extend these studies to OST and indicate that this key enzyme in *N*-linked glycoprotein biosynthesis requires lipids containing more than 20 carbons for efficient glycosylation of peptides in vitro. The presence of multiple *cis*-isoprenes in **1a** vs. either a fully saturated lipid (**1b**, **1d**) or lipid containing a single *trans*-isoprene (**1c**) in the compounds studied in this research is worthy of note. It is possible that the *cis*-olefins are critical for anchoring the lipid portions of OST substrate **1** in the membrane of the endoplasmic reticulum. The synthesis of **1** containing one or more *cis*-isoprenes and their evaluation as OST

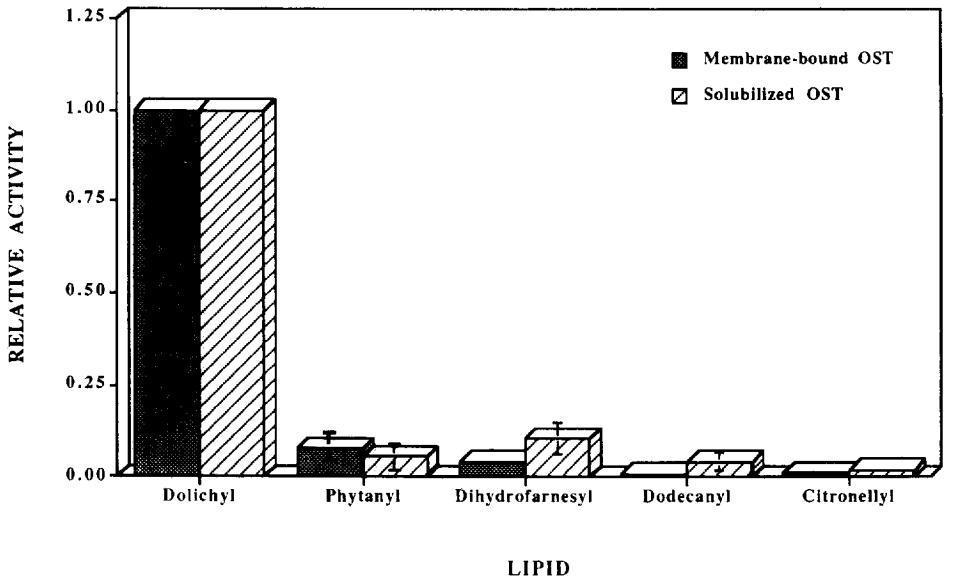


Figure 2. Activity of lipid pyrophosphoryl chitobioses in OST-catalyzed glycosylation. All data are relative to the dolichyl substrate (**1a**) for either form of OST.

substrates would be of considerable interest in further elucidating the structural requirements for the lipid portion of substrate **1**.

Experimental

Synthesis

1. Lipid phosphate (**2a-e**)

Method A (unsaturated lipids) Reaction of the lipid alcohol with $POCl_3$ was carried out as previously described.[9] After workup, the residue was dissolved in $CHCl_3$/MeOH (2:1) and purified by DEAE 53 anion exchange column chromatography eluted with a linear gradient of 0 - 0.1M NH_4OAc in $CHCl_3$/MeOH (2:1) . Fractions containing the desired product were pooled and lyophilized to give the phosphate as a white solid which was converted to the NEt_3 salt, obtained as a colorless oil.

Method B (saturated lipids) Reaction of the lipid alcohol with di-*t*-butyl-*N*,*N*-diethylphosphoramidate, followed by oxidation of the phosphite intermediate was carried out as previously described.[10] After workup, the product was purified by flash column chromatography on silica gel ($CHCl_3$/MeOH, 10:1, v:v) to give the protected lipid phosphate as a clear, colorless oil. Deprotection by TFA (8 equiv) in dry CH_2Cl_2 for 2 h at rt gave the monophosphate as a colorless oil. The residue was converted to a triethylamine salt by dissolving it in $CHCl_3$/MeOH/triethylamine (2:1:1) followed by removal of solvent.

2. Disaccharide phosphate Chitin was converted to peracetylated chitobiose followed by selective 1-O-deacetylation to give the reducing disaccharide.[1] Formation of the lithium salt (BuLi) and its reaction with

dibenzyl phosphorochloridate (3 equiv) gave pure dibenzyl protected sugar phosphate as a white solid after purification by flash column chromatography. Hydrogenation over 10% Pd-C at 30 psi for 3 h and subsequent workup with pyridine gave the desired phosphate as a white solid sufficiently pure for use in further transformations.

3. Acetyl-protected lipid pyrophosphate (**3a-e**) To a solution of disaccharide phosphate (1 equiv) in $CHCl_3$/MeOH (2:1) was added NBu_3 (1 equiv). After stirring for 5 min, solvent was removed under reduced pressure to give a white solid which was redissolved in dry DMF. To the solution was added carbonyl diimidazole (CDI, 4.2 equiv) in dry DMF. The mixture was allowed to stir for 4 h at rt when TLC showed complete consumption of starting material. Dry MeOH was then added to react with the excess CDI and the mixture was stirred for an additional 30 min. Lipid phosphate (0.9 equiv) in dry CH_2Cl_2 was added. After the mixture was stirred for 60 h at rt, the solvent was removed under reduced pressure, the residue dissolved in $CHCl_3$/MeOH (2:1), and the crude material was purified by DEAE 53 anion exchange column chromatography. The mixture was eluted with a linear gradient of 0 to 0.1 M NH_4OAc in $CHCl_3$/MeOH (2:1). Fractions containing the desired product were pooled and lyophilized to give the protected lipid pyrophosphodisaccharide as a white solid.

4. Lipid pyrophosphodisaccharides (**1a-e**) Methanolic NaOMe (1.6% w/v) containing 1.5 equiv. NaOMe per O-acetyl group was added to a stirred solution of acetyl-protected lipid disaccharide in dry CH_2Cl_2 at 0 °C. The resulting mixture was stirred for 40 min at this temp, following which excess Dowex 50X-8 (PyH^+) was added and the mixture was stirred for an additional 10 min at ambient temp. Dowex was removed by filtration and washed with MeOH. The combined filtrates were concentrated in vacuo to give the desired lipid disaccharide as a white solid in quantitative yield.

Enzymology

1. Microsomal P40 OST Assay: The assay mixture contained 0.05 M Tris, pH 7.5, 1% Triton X-100, 5 mM $MnCl_2$, 5% DMSO, 360 μM N^{α}-[^{14}C]Bz-Asn-Leu-Thr-NH_2 (Gibbs and Coward, manuscript in preparation), 660 μM Lipid-P-P-$(GlcNAc)_2$ (**1a-e**), and 875 μg of P40 microsomes[19] in a total volume of 100 μL. The assay was kept at rt for 2 h with constant shaking at 250 rpm and stopped by the addition of 3 mL of $CHCl_3$:CH_3OH (3:2, v/v). After incubating the samples on ice for 30 min the insoluble material was pelleted by centrifugation at 1000 x g for 15 min. The supernatant was removed and extracted with 1 mL of 4 mM $MgCl_2$ and the phases separated by centrifugation as described above. The upper aqueous phase was carefully removed, taken to dryness under vacuum (Savant Speed Vac), and analyzed for glycopeptide product by reversed phase HPLC (see below).

2. Solubilized P40 OST Assay: P40 microsomes were solubilized by a modification of the method described by Chalifour and Spiro.[20] Microsomes were diluted to a final protein concentration of 17 mg/mL into a buffer containing 0.05 M Tris, pH 7.5, 0.85% NP-40, 5 mM $MgCl_2$, and 5 mM $MnCl_2$ at 4 °C. Using a Dounce homogenizer (2-3 strokes every 5 min over a 20 min period), the microsomal P40 was solubilized and the latter form was isolated by ultracentrifugation at 158,000 x g for 1 h. The supernatant was removed carefully and either used immediately in the assay or frozen in liquid N_2 and stored at -80 °C.

The assay mixture contained 0.05 M Tris, pH 7.5, 5 mM $MnCl_2$, 0.2% Triton X-100, 1 mM egg phosphatidylcholine, 0.4% NP-40, 5% DMSO, 360 μM N^{α}-[^{14}C]-Bz-Asn-Leu-Thr-NH_2, 660 μM Lipid-P-P-$(GlcNA_c)_2$ (**1a-e**), and 370-400 μg solubilized P40 microsomes in a total volume of 100 μL. The

assay mixture was kept at rt with constant shaking at 250 rpm. After 2 h the assay was stopped by the addition of 3 mL of $CHCl_3$:CH_3OH (3:2, v/v) and worked up as described above.

3. HPLC Analysis of Glycopeptide Product. HPLC analysis of peptide substrates and glycopeptide products was performed on a Waters liquid chromatography system (6000A & 510 pumps, μBondpak C_{18} 300Å column (3.9 mm x 15 cm)) and monitored using a Waters 996 diode array spectrophotometer and Millenium Software. Samples taken to dryness were resuspended in water containing 0.1% TFA. The initial mobile phase was 15% CH_3CN and the product was isolated with a gradient of 15-30% acetonitrile over 15 min. Fractions (0.3 mL) were collected and analyzed for ^{14}C by scintillation counting. Routinely, >90% of the radioactivity applied to the column was recovered in the various fractions collected. The amount of glycopeptide formed was calculated from the radioactivity recovered using a specific radioactivity of 0.35 mCi/mmol for the labeled peptide (Gibbs and Coward, manuscript in preparation).

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