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Substrate Specificity of *N*-Acetylglucosaminyl(diphosphodolichol) *N*-Acetylglucosaminyl Transferase, a Key Enzyme in the Dolichol Pathway

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Abstract—*N*-Acetylglucosaminyl(diphosphodolichol) *N*-acetylglucosaminyl transferase, also known as Enzyme II, is the second enzyme in the dolichol pathway. This pathway is responsible for the assembly of the tetradecasaccharide pyrophosphate dolichol, which is the substrate for oligosaccharyl transferase. In order to study the specificity of Enzyme II, four unnatural dolichol diphosphate monosaccharides were synthesized, with the C-2 acetamido group in the natural substrate Dol-PP-GlcNAc **1a** replaced by fluoro, ethoxy, trifluoroacetamido, and amino functionalities. These analogues **1b–e** were evaluated as glycosyl acceptors for Enzyme II, which catalyzes the formation of dolichol diphosphate chitobiose (Dol-PP-$GlcNAc_2$) from UDP-GlcNAc and Dol-PP-GlcNAc. Enzyme II from pig liver was found to be highly specific for its glycosyl acceptor and the acetamido group shown to be a key functional determinant for this glycosylation reaction.

Introduction

Asparagine-linked glycosylation, the first committed step in *N*-linked glycoprotein biosynthesis, is essential for structural and functional integrity of eukaryotic cells. This modification occurs cotranslationally with the transfer of a common tetradecasaccharide from dolichol diphosphate-$GlcNAc_2Man_9Glc_3$ to an asparagine residue of a nascent polypeptide chain. The tetradecasaccharide donor is assembled in a stepwise fashion via a series of glycosyltransferases along the dolichol pathway.[1] Enzyme II in the pathway (*N*-acetylglucosaminyl(diphosphodolichol) *N*-acetylglucosaminyl transferase, EC 2.4.1.141) catalyzes the transfer of a *N*-acetylglucosamine (GlcNAc) from UDP-GlcNAc to dolichol diphosphate *N*-acetylglucosamine (Dol-PP-GlcNAc). The resulting dolichol diphosphate chitobiose (Dol-PP-GlcNAc-β-1,4-GlcNAc) is further glycosylated to give Dol-PP-$GlcNAc_2Man_9Glc_3$, the glycosyl donor for *N*-linked glycosylation. In vitro, Dol-PP-GlcNAc2 is often used as a substrate for oligosaccharyl transferase (OT) due to the difficulty in isolation of the tetradecasaccharide donor. Radiolabeled Dol-PP-GlcNAc-(^{3}H)-GlcNAc **3a** was previously prepared by an efficient chemoenzymatic synthesis via Enzyme II (Fig. 1).[2] As part of our ongoing program on studies of OT, several dolichol-linked disaccharides with substitutions at the C-2 position (**3b–e**) are required. This prompted us to explore the possibility of preparing these derivatives **3b–e** via a chemoenzymatic approach employing Enzyme II. The study of the glycosyltransferases along the dolichol pathway is also interesting, as they may be useful for the preparation of modified oligosaccharides and glycoproteins.

Early studies on Enzyme II were focused mainly on the characterization of the product from this enzymatic reaction and the application of this enzyme to prepare Dol-PP-$GlcNAc_2$. Enzyme II from different sources such as yeast,[3–6] mung bean seedling,[7] rat[8,9] and pig livers[10,11] has been documented. Compared with other enzymes along the dolichol pathway such as Enzyme I (and OT), reports on the purification of this enzyme are scarce. To the best of our knowledge, only two reports concerning the purification and biochemical studies of Enzyme II have appeared.[3,7] Enzyme II activity from yeast and mung bean seedling was stimulated by the addition of divalent metals such as Mg^{2+} and Mn^{2+}. Furthermore, uridine nucleotides, especially UDP and UDP-Glc, were found to inhibit Enzyme II from mung bean seedlings.[7] Herein, the chemical synthesis of four unnatural dolichol-linked monosaccharides (**1b–1e**) are

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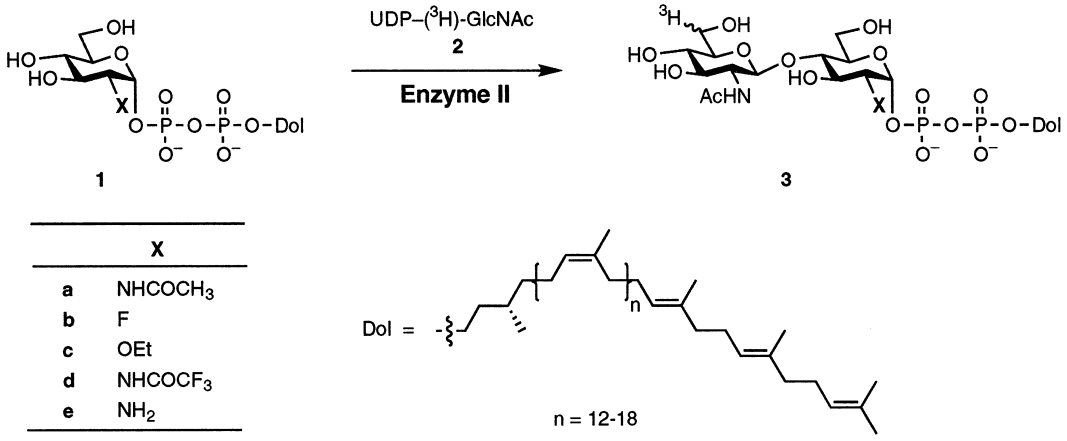


Figure 1. Enzymatic reaction catalyzed by Enzyme II.

described together with the evaluation of these compounds as substrates for Enzyme II from pig liver.

Results and Discussion

Planning

The key step for the preparation of dolichol diphosphate sugars involves the condensation of a protected α-glycosyl phosphate **4a–d** with dolichol monophosphate (Dol-P, **5**) (Fig. 2). Two strategies have been developed in the literature.[12–14] The first method involves the activation of Dol-P **5** via the mixed anhydride or the phosphoroimidazolidate followed by the nucleophilic attack of glycosyl phosphate.[13] Alternatively, α-glycosyl phosphate **4a–d** can be activated as the phosphoroimidazolidate followed by the attack of Dol-P **5**.[14] The second method was applied throughout these synthesis. Acetate protecting groups have been used exclusively for the sugar portion due to the special characteristic of the dolichol pyrophosphate moiety. Specifically, the presence of a large number of double bonds in dolichol does not allow the use of protecting groups that require hydrogenolysis for deprotection. Moreover, the acid labile pyrophosphate group also limits the use of protecting groups that require cleavage under acidic conditions. The α-glycosyl phosphate **4a–c** can be obtained by the phosphorylation of saccharides **6a–c** with electrophilic phosphorylating reagents or from oxazoline **7** with nucleophilic phosphorylating reagents.

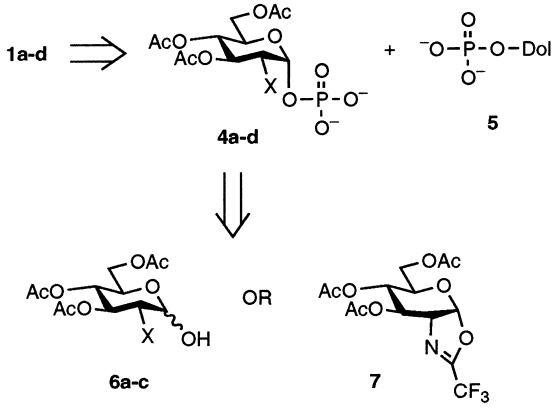
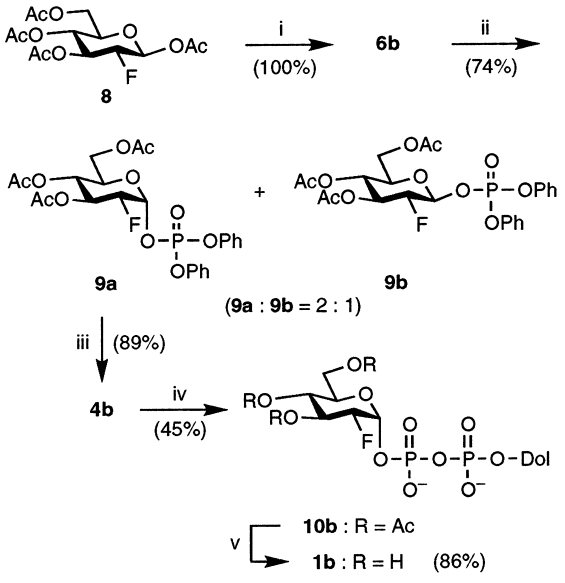


Figure 2. Retrosynthetic analysis of dolichol diphosphate monosaccharides.

Synthesis of Dol-PP-2DFGlc 1b

3,4,6-Tri-*O*-acetyl-2-deoxy-2-fluoro-α,β-D-glucopyranose **6b** was prepared in quantitative yield from chemoselective deacetylation of the known tetraacetate **8**[15–17] with ammonium carbonate in DMF (Scheme 1). Phosphorylation of reducing sugar **6b** with DMAP/diphenyl chlorophosphate[18] gave a separable mixture of anomeric diphenyl phosphates **9a** and **9b** in 74% yield (α/β = 2/1). The stereochemistry of the products was assigned based on their ^{1}H NMR signatures: H-1 of **9a** resonates as a doublet of doublets at 6.13 ppm ($J_{1,2}$ = 3.5 Hz, $J_{1,P}$ = 6.7 Hz). Interestingly, H-1 of **9b** shows an extra coupling with the C-2 fluorine atom at 5.50 ppm (dt, $J_{1,2} = J_{1,P}$ = 7.5 Hz, $J_{1,F}$ = 2.9 Hz). In



Scheme 1. Reagents and conditions: (i) $(NH_4)_2CO_3$, DMF; (ii) (1) DMAP, CH_2Cl_2, rt; (2) diphenyl chlorophosphate, −12 → 0 °C; (iii) H_2, Pt_2O, EtOH/EtOAc; (iv) (1) CDI, DMF; (2) Dol-P **5**; (v) NaOMe, CH_2Cl_2/MeOH.

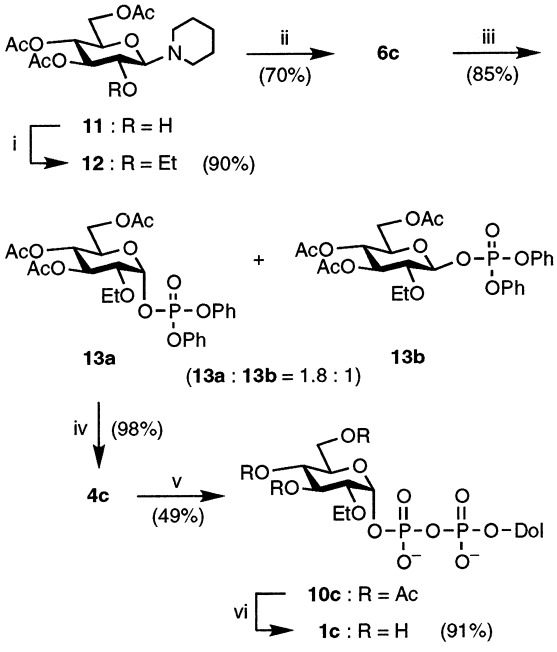
addition, the C-2 fluorine atom also coupled with the H-2 ($J_{2,F} \sim 50$ Hz), and H-3 ($J_{3,F} \sim 12$–14 Hz) protons. ^{31}P NMR shows the corresponding phosphate peaks at ca. −12 ppm for both compounds. Catalytic hydrogenolysis of α-diphenyl phosphate **9a** with platinum (IV) oxide gave 89% yield of α-phosphate **4b** isolated as the triethylamine salt. Dolichol monophosphate (Dol-P) **5** was prepared according to the Danilov modified Cramer procedure.[19] Activation of α-phosphate **4b** with 1,1′-carbonyldiimidazole (CDI) followed by coupling with Dol-P **5** gave the desired protected Dol-PP-2DFGlc **10b** in 45% yield. ^{31}P NMR of **10b** showed the corresponding peaks at −10.6 and −13.3 ppm, respectively. Removal of the acetate groups in **10b** gave the desired Dol-PP-2DFGlc **1b** in 86% yield.

Synthesis of Dol-PP-2OEtGlc 1c

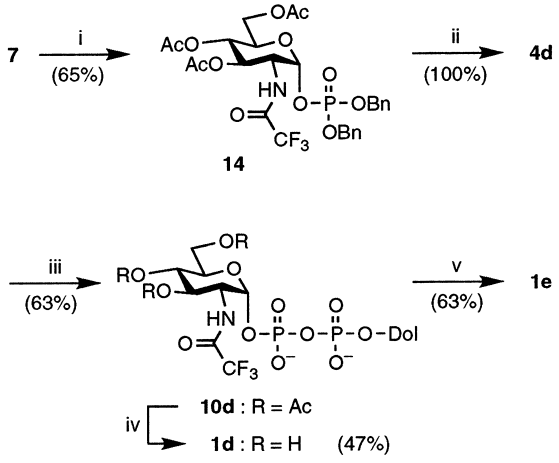
The preparation of Dol-PP-2OEtGlc **1c** followed a similar strategy to that of Dol-PP-2DFGlc **1b**. A protected glucose derivative with a free hydroxyl group at C-2 is required to incorporate the ethoxy functionality. However, methylation of 1,3,4,6-tetra-*O*-acetyl α-D-glucopyranose with Purdie's reagent (MeI, Ag_2O) was known to result in 1,2-acyl migration.[20,21] Glucosamine **11** has been shown earlier to give good yields for methylation and benzylation, and can be removed easily under acidic conditions.[22–24] The known *N*-(3,4,6-tri-*O*-acetyl-β-D-glucopyranosyl)-piperidine **11** was prepared from commercially available β-D-glucose pentaacetate according to the Hodge procedure.[22] Alkylation of compound **11** with ethyl iodide and freshly prepared silver oxide (Ag_2O)[25] gave 90% yield of the desired 2-*O*-ethyl glucose derivative **12** (Scheme 2). Removal of the piperidine group in **12** with acetic acid (HOAc)[23] gave reducing sugar **6c** in 70% yield as a mixture of α- and β-anomers (α/β ∼1/1 as determined by ^{1}H NMR in $CDCl_3$). Similar to the phosphorylation of fluorinated analogue **6b**, compound **6c** gave a mixture of α- and β-diphenyl phosphates **13a** and **13b** in 85% yield (α/β ∼1.8/1) employing the phosphorochloridate method (DMAP/diphenyl chlorophosphate). α-Diphenyl phosphate **13a** was readily transformed to the desired Dol-PP-2OEtGlc **1c** via the coupling of Dol-P **5** with α-phosphate **4c** followed by subsequent deprotection.

Synthesis of Dol-PP-GlcNTFA 1d and Dol-PP-GlcN 1e

3,4,6-Tri-*O*-acetyl-2-deoxy-2-trifluoroacetamido-α-D-glucopyranosyl phosphate **4d** has recently been prepared by two independent groups.[26] The α-phosphate **4d** was prepared using the phosphorochloridate method of the corresponding reducing sugar in 52% yield by the Tanner group.[26] This compound **4d** was further transformed to UDP-GlcNTFA and proven to be a substrate of 'core-2' GlcNAc transferase. On the other hand, Busca and Martin showed that both the α- and β-phosphates could be obtained in high yields from the ring opening of oxazoline **7** with dibenzyl phosphate under different conditions.[27] Following the Martin protocol, ring opening of the known oxazoline **7**[27,28] with dibenzyl phosphate in refluxing 1,2-dichloroethane gave the thermodynamic product **14** in 65% yield (Scheme 3).[27] The stereochemistry of **14** was confirmed to be α-based on the coupling constant of H-1–H-2 protons ($J_{1,2} = 3.3$ Hz) in the ^{1}H NMR spectrum. Catalytic hydrogenolysis of **14** with 10% palladium on charcoal gave quantitative yield of α-phosphate **4d**, which was then condensed with Dol-P **5** to give 63% yield of the protected Dol-PP-GlcNTFA **10d**. Chemoselective deprotection of the acetates in **10d** with guanidine/guanidinium nitrate solution[29] gave Dol-PP-GlcNTFA **1d** in 47% yield after silica gel chromatography. On the other hand, treatment of **10d** with lithium hydroxide (10 equiv) gave the amine derivative Dol-PP-GlcN **1e** in 63% yield.



Scheme 2. Reagents and conditions: (i) EtI, Ag_2O, PhH; (ii) AcOH, H_2O, acetone; (iii) (1) DMAP, CH_2Cl_2, rt; (2) diphenyl chlorophosphate, −10 °C → rt; (iv) H_2, Pt_2O, EtOH/EtOAc; (v) (1) CDI, DMF; (2) Dol-P **5**; (vi) NaOMe, CH_2Cl_2/MeOH.



Scheme 3. Reagents and conditions: (i) dibenzyl phosphate, 1,2-dichloroethane, Δ; (ii) H_2, 10% Pd/C, MeOH; (iii) (1) CDI, DMF; (2) Dol-P **5**; (iv) guanidine/guanidinium nitrate, CH_2Cl_2/MeOH; (v) LiOH, CH_2Cl_2/MeOH.

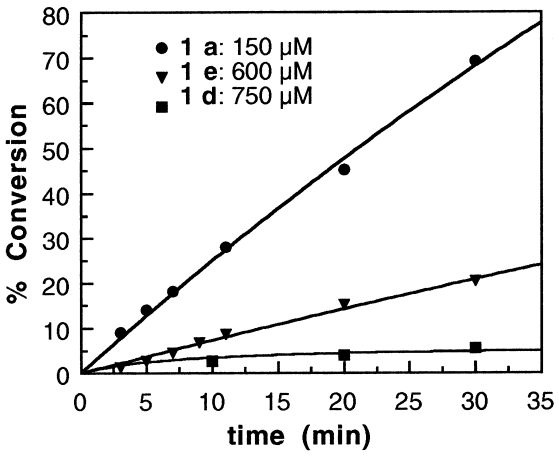


Figure 3. Percent conversion of **1a**, **1d**, and **1e**.

Biological evaluation

Unnatural dolichol diphosphate monosaccharides (**1b–e**) were evaluated as glycosyl acceptors for Enzyme II from partially purified pig liver microsomes using radiolabeled UDP-(^{3}H-GlcNAc) as the donor. The formation of dolichol-linked disaccharides was quantified by measuring the amount of tritium incorporated into the organic layer (unreacted UDP-(^{3}H-GlcNAc) partitions into the aqueous layer) after successive aqueous-organic extractions.[2] Unnatural analogues Dol-PP-2DFGlc **1b** and Dol-PP-2OEtGlc **1c** showed no transferase activity even at high concentrations and long reaction times.[30] On the other hand, the amine derivatives Dol-PP-GlcNTFA **1d** and Dol-PP-GlcN **1e** were shown to be extremely poor and moderate substrates for Enzyme II (Fig. 3), respectively. Even at higher concentrations of **1d** (1.5 mM) and **1e** (600 μM), only 13 and 41% conversions were observed after 14 h, respectively. Derivatives **1b–d** were further shown to be inhibitors when studied in competition with the natural substrate **1a**. As shown in Table 1, the percent conversions of Dol-PP-GlcNAc **1a** were reduced to 68–73% when equivalent amounts of the unnatural derivatives **1b–d** were added.

It is most likely that the unnatural analogues **1b–d** act as competitive inhibitors of Enzyme II by binding specifically to the Dol-PP-GlcNAc binding site, however detailed kinetic studies to confirm this have not been carried out at the current time due to difficulties associated with the discontinuous HPLC-based assay. It is surprising that even a slight modification at the C-2 position diminishes the activity of this enzyme. The electronic effects of the substituents at the C-2 position should induce minimal effects on the nucleophilicity of the C-4 hydroxyl group. The acetamido group is postulated to act as a functional determinant for this transferase reaction through highly specific hydrogen bondings of the N–H and C=O groups to the enzyme. Neither the 2-deoxy-2-fluoro analogue **1b** nor the 2-*O*-ethyl analogue **1c** function as substrates, perhaps due to the lack of the proposed essential acetamido group. The low transferase activity of the trifluoroacetamido analogue **1d** may be accounted for by the strong electronic effects of the trifluoromethyl group rendering the carbonyl a less efficient hydrogen bond acceptor. The fact that the amine analogue Dol-PP-GlcN **1e**, without a carbonyl group, is a better substrate than **1d** suggests that the minimal recognition element for this transferase reaction is an N–H unit. It is puzzling at this stage why the trifluoroacetamido analogue **1d** is such a poor substrate since the more acidic N–H proton should be a better hydrogen bond donor compared to an acetamido group. On the other hand, the product inhibition of Dol-PP-GlcNTFA-(^{3}H)-GlcNAc **3d** has not yet been examined. It is possible that **3d** inhibits the transferase reaction through a stronger hydrogen bonding via the more acidic proton of the trifluoroacetamido group. The availability of Dol-PP-GlcNTFA-GlcNAc will allow competition studies with the natural substrate **1a** which may give further insights to the present findings. The substrate specificity observed for Enzyme II is different from that of 'core-2' GlcNAc transferase reported by Tanner.[26] UDP-GlcNTFA was found to be a substrate for 'core-2' GlcNAc transferase but neither the 2-amino (UDP-GlcN) nor the 2-hydroxyl (UDP-Glc) derivatives were. In addition, the *N*-acetylgalactosamine derivative Dol-PP-GalNAc, with an axial hydroxyl group at C-4, was also not a substrate for Enzyme II.[31]

Table 1. Relative percent conversion of **1a** when competed with **1b–d**

Compounds[a]	Relative % conversion[b]
1a	1
1a+**1b**[c]	0.68
1a+**1c**	0.73
1a+**1d**	0.68

[a]150 μM of each compound and 5 μL of pig liver microsomes were used.
[b]Percent conversion for 10 min of reaction.
[c]10 μL of pig liver microsomes were used in this case.

Conclusion

In summary, Enzyme II utilizes highly specific glycosyl acceptors as substrates. Slight modifications remote from the reaction center result in loss of transferase activity. The acetamido group of the natural substrate Dol-PP-GlcNAc **1a** is proposed to function as a switch for this glycosylation through highly specific hydrogen bondings of the N–H and C=O moieties to complementary residues of the enzyme while the minimum recognition element is an N–H unit (Fig. 4).

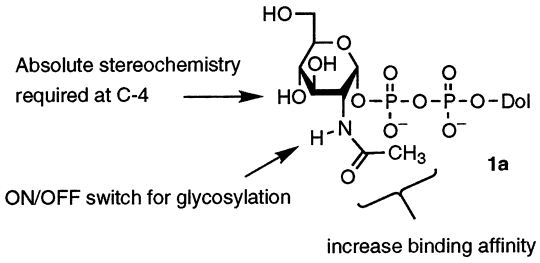


Figure 4. Substrate specificity of glycosyl acceptor for Enzyme II.

Experimental

General methods for synthesis

Melting points were uncorrected. Optical rotations were measured with a JASCO DIP-1000 automatic digital polarimeter operating at 589 nm at 25 °C and are reported in degrees. Concentration (*c*) is indicated as units of 10 mg/mL. IR spectra were recorded on a Perkin-Elmer 1600 FT-IR spectrometer. NMR spectra were measured on a Varian 500, Bruker AM-500 (500.15 MHz for ^{1}H, 125 MHz for ^{13}C), or General Electric QE300 (75 MHz for ^{13}C). All chemical shifts were recorded in ppm downfield from tetramethylsilane on the δ scale. ^{31}P NMR chemical shifts are reported in ppm relative to 85% phosphoric acid external standard. Multiplicities are reported in the following abbreviations: s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), dd (doublet of doublets), etc. Mass spectra were obtained at the Mass Spectrometry Laboratory, operated by the College of Chemistry, California Institute of Technology, Pasadena, CA 91125, USA or UCR Mass Spectrometry Facility, Department of Chemistry, University of California, Riverside, CA 92521, USA. All reactions were monitored by analytical thin-layer chromatography (TLC) on glass precoated with silica gel $60F_{254}$ (E. Merck) and compounds were visualized with 20% w/v dodecamolybdophosphoric acid in ethanol and subsequent heating. Phosphorus compounds were visualized with molybdenum blue spray reagent. All columns were packed wet with E. Merck silica gel 60 (230–400 mesh) as the stationary phase and eluted by flash chromatography. All solvents were reagent grade. Methylene chloride and triethylamine were distilled from calcium hydride. Tetrahydrofuran and toluene were distilled from sodium/benzophenone ketyl. Other reagents were purchased from commercial suppliers (Aldrich, Sigma) and used without further purification.

General procedure for phosphorylation. To a solution of the reducing sugar (0.42 mmol) in CH_2Cl_2 (2.5 mL) at room temperature was added DMAP (0.92 mmol) in one portion. The mixture was stirred for 15 min and cooled to −12 °C. Diphenyl chlorophosphate (0.92 mmol) was then added and the mixture was allowed to warm slowly to 0 °C over 2 h. The mixture was diluted with CH_2Cl_2 (80 mL) and washed successively with ice water (25 mL), aq HCl (1 N, 25 mL), and a saturated aqueous solution of $NaHCO_3$ (25 mL). The organic extracts were dried (Na_2SO_4), filtered, and concentrated. The anomers were then separated by flash column chromatography.

General procedure for removal of protecting groups on dialkyl phosphates. A solution of the protected phosphate (0.16 mmol) and Pt_2O (10 mol%, for diphenyl phosphate) (or 10% Pd/C (20 mg, for dibenzyl phosphate)) was stirred under H_2 in alcoholic solvent (8 mL) until no starting material as shown by TLC. The catalyst was filtered thorugh a pad of Celite and washed with EtOH (15 mL). Et_3N (1 mL) was added followed by the evaporation of solvent in vacuo to give the mono-(triethylammonium) salt of the phosphate.

General procedure for coupling of sugar phosphate with dolichol monophosphate.[14] To a solution of the sugar phosphate (0.04 mmol) in DMF (1 mL) at room temperature was added 1,1′-carbonyldiimidazole (CDI, 0.2 mmol) in one portion. After 2 h, MeOH (0.36 mmol) was added (to quench excess CDI) to the mixture and stirred for 30 min. Dolichol monophosphate **5**[19] (tri-*N*-butyl ammonium form, 0.028 mmol) in CH_2Cl_2 (1 mL) was then added and the mixture was allowed to stir for 3 days at room temperature. The crude mixture was concentrated followed by chromatography with DE-52 (acetate form, eluted with increasing concentration of NH_4OAc in $CHCl_3$, MeOH (2:1 v/v)). The products were concentrated and further purified by silica gel chromatography.

General procedure for deprotection of peracetylated dolichol-linked saccharides

Method A. To a stirred solution of peracetylated dolichol-linked saccharide (3 μmol) in CH_2Cl_2 (2.5 mL) at room temperature was added a solution of NaOMe (4 mg) in MeOH (0.5 mL). After 30 min, cation exchange resin (Dowex 50×8, pyridinium form, 200 mg) was added and the mixture was stirred for 10 min. The resin was then filtered and washed with $CHCl_3$/MeOH (2/1 v/v). Concentration of the solvent gave dolichol-linked saccharide in the pyridinium form.

Method B. To a stirred solution of peracetylated dolichol-linked saccharide (3 μmol) in CH_2Cl_2 (1.5 mL) at room temperature was added a solution of guanidine/guanidinium nitrate (1 mL). After 20 min, cation exchange resin (Dowex 50×8, pyridinium form, 200 mg) was added and the mixture was stirred for 10 min. The resin was then filtered and washed with $CHCl_3$/MeOH (2/1 v/v). Concentration of the solvent followed by column chromatography gave dolichol-linked saccharide. (Stock solution of the guanidine/guanidinium nitrate reagent was prepared by dissolving guanidinium nitrate (122.1 mg, 1 mmol) and sodium methoxide (10.8 mg, 0.2 mmol) in MeOH/CH_2Cl_2.)

Method C. To a stirred solution of peracetylated dolichol-linked saccharide (1.1 μmol) in CH_2Cl_2/MeOH (0.3 mL, 5/1 v/v) at room temperature was added aqueous lithium hydroxide solution (3 M, 11 μmol). After 20 min, the mixture was worked up as in method B.

3,4,6-Tri-*O*-acetyl-2-deoxy-2-fluoro-α,β-D-glucopyranose (6b). A solution of tetraacetate **8**[17] (356 mg, 1.02 mmol) and ammonium carbonate (977 mg, 10.2 mmol) was stirred in DMF (10 mL) at room temperature for 26 h. The mixture was diluted with EtOAc (80 mL) and washed with water (80 mL), and brine (80 mL). The organic layer was dried (Na_2SO_4), filtered, and concentrated to give compound **6b** (316.8 mg, quantitative) as a mixture of α- and β-anomers. MS (DCI, NH_3): 326 (MNH_4^+); HRMS: calcd for $C_{12}H_{21}FNO_8$: *m*/*z* 326.125120; found: 326.123990.

Diphenyl 3,4,6-tri-*O*-acetyl-2-deoxy-2-fluoro-α-D-glucopyranosyl phosphate (9a), diphenyl 3,4,6-tri-*O*-acetyl-2-

deoxy-2-fluoro-β-D-glucopyranosyl phosphate (9b). Compound **6b** (129.3 mg, 0.4195 mmol) gave after column chromatography (*n*-hex, EtOAc (3/2 v/v)) a mixture of α- and β-diphenyl phosphates (167.3 mg, 74%, α/β ~2/1). The two compounds were further separated by chromatography (Et_2O, *n*-hex (2/1 v/v)) to give α-diphenyl phosphate **9a** (94.9 mg, 42%) as an oil followed by β-diphenyl phosphate **9b** (39.8 mg, 18%) as a white solid, mp 73–75 °C. Data for α-diphenyl phosphate **9a**: IR (neat) cm^{-1} 1754, 1590, 1488, 1368, 1292, 1220, 1187, 1162, 1083, 1042, 962, 776, 689; ^1H NMR ($CDCl_3$, 500 MHz) δ 1.99 (s, 3H, Ac), 2.03 (s, 3H, Ac), 2.09 (s, 3H, Ac), 3.80 (dd, 1H, *J* = 3.9, 12.6 Hz, H-6a), 4.00 (ddd, 1H, *J* = 2.1, 3.9, 10.4 Hz, H-5), 4.15 (dd, 1H, *J* = 3.9, 12.6 Hz, H-6b), 4.61 (ddt, 1H, *J* = 3.5, 9.6, 48 Hz, H-2), 5.07 (t, 1H, *J* = 10 Hz, H-4), 5.55 (dt, 1H, *J* = 9.6, 11.8 Hz, H-3), 6.13 (dd, 1H, *J* = 3.5, 6.7 Hz, H-1), 7.20–7.39 (m, 10H, Ph); ^{13}C NMR ($CDCl_3$, 125 MHz) (δ 20.4, 20.5, 20.6, 60.8, 66.9 (d, *J* = 7 Hz, C-4), 69.5, 69.9 (d, *J* = 19 Hz, C-3), 86.4 (dd, *J* = 8, 196 Hz, C-2), 94.6 (dd, *J* = 5, 22 Hz, C-1), 120.0, 120.03, 120.3 (2×), 125.7 (2×), 129.8, 129.9, 150.1 (d, *J* = 7.5 Hz), 150.2 (d, *J* = 7 Hz), 169.3, 169.8, 170.3; ^{31}P NMR ($CDCl_3$, 162 MHz) δ −12.0; $[\alpha]_D$ +100.6° (*c* 0.8, $CHCl_3$); MS (FAB): 541 (MH^+), 563 (MNa^+); HRMS: calcd for $C_{24}H_{26}O_{11}FNaP$: *m*/*z* = 563.109448; found: 563.109000.

Data for β-diphenyl phosphate **9b**: IR (neat) cm^{-1} 1750, 1590, 1489, 1368, 1294, 1225, 1187, 1163, 1081, 1036, 1010, 957, 775, 689, 668; ^1H NMR ($CDCl_3$, 500 MHz) δ 2.03 (s, 3H, Ac), 2.04 (s, 3H, Ac), 2.07 (s, 3H, Ac), 3.82 (ddd, 1H, *J* = 2.1, 4.7, 10.1 Hz, H-5), 4.02 (dd, 1H, *J* = 2.1, 12.5 Hz, H-6a), 4.21 (dd, 1H, *J* = 4.7, 12.5 Hz, H-6b), 4.44 (ddd, 1H, *J* = 7.5, 9, 50 Hz, H-2), 5.07 (t, 1H, *J* = 9.7 Hz, H-4), 5.32 (dt, 1H, *J* = 9.2, 14.2 Hz, H-3), 5.50 (dt, 1H, *J* = 2.9, 7.5 Hz, H-1), 7.19–7.37 (m, 10H, Ph); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 20.5 (2×), 20.6, 61.3, 67.5 (d, *J* = 7 Hz, H-4), 72.2 (d, *J* = 19 Hz, H-3), 72.7, 89.0 (dd, *J* = 10, 192 Hz, H-2), 96.5 (dd, *J* = 5, 25 Hz, H-1), 120.1, 120.2, 120.3, 120.4, 125.7 (2×), 129.7, 129.8, 150.1 (d, *J* = 7 Hz), 150.2 (d, *J* = 7 Hz), 169.4, 169.7, 170.4; ^{19}F NMR ($CDCl_3$, 470 MHz) δ −200.4 (dd, *J* = 13.6, 50 Hz); ^{31}P NMR ($CDCl_3$, 162 MHz) δ −12.1; $[\alpha]_D$ +43.4° (*c* 1, $CHCl_3$); MS (FAB): 563 (MNa^+); HRMS: calcd for $C_{24}H_{26}O_{11}FNaP$: *m*/*z* 563.109448; found: 563.110600.

Mono(triethylammonium) salt of 3,4,6-tri-*O*-acetyl-2-deoxy-2-fluoro-α-D-glucopyranosyl phosphate (4b). α-Diphenyl phosphate **9a** (87.1 mg, 0.1612 mmol) underwent hydrogenolysis to give α-phosphate **4b** (70.1 mg, 89%) as the mono(triethylammonium) salt; IR (neat) cm^{-1} 3401, 2906, 2687, 1746, 1646, 1456, 1229, 1162, 1039, 957, 917, 836, 772; ^1H NMR ($CDCl_3$, 500 MHz) δ 1.27 (t, 9H, *J* = 7.2 Hz, $N(CH_2CH_3)_3$), 1.97 (s, 3H, Ac), 2.00 (s, 3H, Ac), 2.01 (s, 3H, Ac), 3.01 (q, 6H, *J* = 7.2 Hz, $N(CH_2CH_3)_3$), 4.11 (dd, 1H, *J* = 1.5, 12.5 Hz, H-6a), 4.24 (dd, 1H, *J* = 3.2, 12.5 Hz, H-6b), 4.34 (brd, 1H, *J* = 10 Hz, H-5), 4.44 (ddt, 1H, *J* = 3.4, 9.6, 50 Hz, H-2), 5.02 (t, 1H, *J* = 10 Hz, H-4), 5.50 (dt, 1H, *J* = 9.1, 11.5 Hz, H-3), 5.83 (dd, 1H, *J* = 3.4, 6.4 Hz, H-1); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 8.4, 20.5, 20.6, 20.7, 45.4, 61.5, 67.4, 67.8 (d, *J* = 7 Hz, C-4), 71.0 (d, *J* = 19 Hz, C-3), 87.5 (dd, *J* = 9, 193 Hz, C-2), 91.5 (dd, *J* = 5, 20 Hz), 169.6, 170.0, 170.7; ^{19}F NMR ($CDCl_3$, 470 MHz) δ −200.8 (dd, *J* = 12, 50 Hz); ^{31}P NMR ($CDCl_3$, 162 MHz) δ 0.6; $[\alpha]_D$ +83.3° (*c* 1, $CHCl_3$); MS (−ve FAB): 387 (M^-); HRMS: calcd for $C_{12}H_{17}O_{11}FP$: *m*/*z* 387.049253; found: 387.049400.

P^1-Dolichyl P^2-[3,4,6-tri-*O*-acetyl-2-deoxy-2-fluoro-α-D-glucopyranosyl] diphosphate (10b). ^{19}F NMR ($CDCl_3$, CD_3OD (5/1 v/v), 470 MHz) δ −201.5 (brd, *J* = 48 Hz); ^{31}P NMR ($CDCl_3$, CD_3OD (5/1 v/v), 162 MHz) δ −10.6, −13.3; MS (−ve ESI): 779.1, 813.2, 847.2, 881.3, 915.3, 949.3, 983.4 (*m*/2 for *n* = 12–18).

P^1-Dolichyl P^2-[2-deoxy-2-fluoro-α-D-glucopyranosyl] diphosphate (1b). MS (−ve ESI): 716.1, 750.2, 784.2, 818.2, 852.3, 885.8, 920.9 (*m*/2 for *n* = 12–18).

3,4,6-Tri-*O*-acetyl-1-deoxy-2-*O*-ethyl-1-piperidino-β-D-glucopyranose (12). A mixture of compound **11**[22] (730.6 mg, 1.9566 mmol), iodoethane (1.56 mL, 19.566 mmol) and silver oxide (680 mg, 2.935 mmol) was stirred vigorously in benzene (7.3 mL) for 1 day. The solid was filtered through a pad of Celite and washed with toluene (8 mL). The solvent was removed under high vacuum to give the 2-ethoxy compound **12** (751.1 mg, 90%) as a white solid, mp 99–101 °C; IR (neat) cm^{-1} 935, 1749, 1442, 1367, 1234, 1111, 1089, 1048, 993, 907, 861; ^1H NMR ($CDCl_3$, 500 MHz) δ 1.12 (t, 3H, *J* = 7.0 Hz, OCH_2CH_3), 1.45–1.60 (m, 6H, CH_2), 2.01 (s, 3H, Ac), 2.05 (s, 3H, Ac), 2.06 (s, 3H, Ac), 2.61 (m, 2H, NCH*H*), 2.86 (m, 2H, NC*H*H), 3.48 (t, 1H, *J* = 9.2 Hz, H-2), 3.48–3.53 (m, 2H, OCH*H*CH_3, H-5), 3.86 (dq, 1H, *J* = 7.0, 9.2 Hz, OCH*H*CH_3), 3.91 (d, 1H, *J* = 9.2 Hz, H-1), 4.06 (dd, 1H, *J* = 2.3, 12.0 Hz, H-6a), 4.21 (dd, 1H, *J* = 4.9, 12.0 Hz, H-6b), 4.89 (t, 1H, *J* = 9.7 Hz, H-3/H-4), 5.07 (t, 1H, *J* = 9.4 Hz, H-4/H-3); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 15.6, 20.6, 20.7 (2×), 24.6, 26.1, 49.1, 62.6, 67.2, 69.1, 72.7, 74.9, 75.4, 95.9, 169.8, 170.0, 170.6; $[\alpha]_D$ +27.1° (*c* 0.9, $CHCl_3$); MS (FAB): 402 (MH^+); HRMS: calcd for $C_{19}H_{32}NO_8$: *m*/*z* 402.212792; found: 402.211900.

3,4,6-Tri-*O*-acetyl-2-*O*-ethyl-α,β-D-glucopyranose (6c). To a solution of the 2-ethoxy compound **12** (494.9 mg, 1.2328 mmol) in acetone (15 mL) was added water (200 μL) and acetic acid (100 μL) at room temperature. The mixture was heated under reflux for 2 days. The solvent was removed under reduced pressure and the crude product was chromatographed (EtOAc, *n*-hex (1/1 v/v)) to give compound **6c** (290 mg, 70%); MS (FAB): 357 (MNa^+); HRMS: calcd for $C_{14}H_{22}O_9Na$ *m*/*z* 357.116152; found: 357.116300.

Diphenyl 3,4,6-tri-*O*-acetyl-2-*O*-ethyl-α-D-glucopyranosyl phosphate (13a) and diphenyl 3,4,6-tri-*O*-acetyl-2-*O*-ethyl-β-D-glucopyranosyl phosphate (13b). Phosphorylation of compound **6c** (219.2 mg, 0.6557 mmol) gave after column chromatography ($CHCl_3$, CH_3CN (15/1 v/v) followed by (10/1 v/v)) α-diphenyl phosphate **13a** (204.9 mg, 55%) and β-diphenyl phosphate **13b** (112.2 mg, 30%) both as oils. Data for α-diphenyl phosphate **13a**: IR (neat) cm^{-1} 2977, 1750, 1590, 1489, 1367, 1291,

1222, 1188, 1163, 1111, 1049, 1010, 959, 915, 776, 690; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 1.08 (t, 3H, J = 7.0 Hz, OCH_2CH_3), 1.99 (s, 3H, Ac), 2.00 (s, 3H, Ac), 2.06 (s, 3H, Ac), 3.48 (dq, 1H, J = 7.0, 9.2 Hz, $OCHHCH_3$), 3.55 (dt, 1H, J = 3.3, 9.9 Hz, H-2), 3.67 (dq, 1H, J = 7.0, 9.2 Hz, $OCHHCH_3$), 3.75 (dd, 1H, J = 2.1, 12.6 Hz, H-6a), 3.91 (ddd, 1H, J = 2.1, 3.9, 10.3 Hz, H-5), 4.14 (dd, 1H, J = 3.9, 12.6 Hz, H-6b), 5.04 (t, 1H, J = 9.9 Hz, H-3/H-4), 5.36 (t, 1H, J = 9.8 Hz, H-4/H-3), 6.05 (dd, 1H, J = 3.3, 6.9 Hz, H-1), 7.19–7.37 (m, 10H, Ph); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 15.2, 20.5 (2×), 20.7, 61.1, 67.0, 67.5, 69.5, 71.0, 76.8 (d, J = 7 Hz), 96.0 (d, J = 6 Hz), 120.0, 120.1 (2×), 120.6, 120.7 (2×), 125.5, 129.8, 129.9, 150.3, 150.4, 169.5, 170.0, 170.5; ^{31}P NMR ($CDCl_3$, 162 MHz) δ −11.8; $[\alpha]_D$ +98.7° (*c* 1.3, $CHCl_3$); MS (FAB): 589 (MNa^+); HRMS: calcd for $C_{26}H_{13}O_{12}NaP$: *m*/*z* 589.145085; found: 589.146200.

Data for β-diphenyl phosphate **13b**: IR (neat) cm^{-1} 2978, 1749, 1590, 1489, 1367, 1295, 1226, 1189, 1163, 1102, 1072, 1043, 1026, 1010, 956, 773, 690, 668; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 0.99 (t, 3H, J = 7.0 Hz, OCH_2CH_3), 2.01 (s, 3H, Ac), 2.02 (s, 3H, Ac), 2.04 (s, 3H, Ac), 3.42 (dd, 1H, J = 7.8, 9.0 Hz, H-2), 3.46 (dq, 1H, J = 7.0, 9.4 Hz, $OCHHCH_3$), 3.54 (dq, 1H, J = 7.0, 9.4 Hz, $OCHHCH_3$), 3.77 (ddd, 1H, J = 2.3, 4.7, 9.9 Hz, H-5), 3.99 (dd, 1H, J = 2.3, 12.4 Hz, H-6a), 4.21 (dd, 1H, J = 4.7, 12.4 Hz, H-6b), 5.04 (t, 1H, J = 9.5 Hz, H-3/H-4), 5.12 (t, 1H, J = 9.2 Hz, H-4/H-3), 5.35 (t, 1H, J = 7.2 Hz, H-1), 7.18–7.36 (m, 10H, Ph); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 15.3, 20.6 (3×), 61.6, 68.0, 68.3, 72.4, 73.7 (d, J = 2 Hz), 79.1 (d, J = 9 Hz), 99.1 (d, J = 6 Hz), 119.9, 120.0, 120.4 (2×), 125.5, 129.6, 129.7, 150.3 (d, J = 6 Hz), 150.4 (d, J = 6 Hz), 169.6, 169.8, 170.5; ^{31}P NMR ($CDCl_3$, 162 MHz) δ −12.1; $[\alpha]_D$ +29.1° (*c* 1.0, $CHCl_3$); MS (FAB): 589 (MNa^+); HRMS: calcd for $C_{26}H_{13}O_{12}NaP$: *m*/*z* 589.145085; found: 589.145400.

Mono(triethylammonium) salt of 3,4,6-tri-*O*-acetyl-2-*O*-ethyl-α-D-glucopyranosyl phosphate (4c). Hydrogenolysis of α-diphenyl phosphate **13** (89.1 mg, 0.1573 mmol) gave the mono(triethylammonium) salt of α-phosphate **4c** (79.5 mg, 98%); IR (neat) cm^{-1} 3402, 2980, 1747, 1451, 1368, 1234, 1166, 1107, 1044, 957, 921, 840; ^{1}H NMR ($CDCl_3$, CD_3OD (2/1 v/v) 500 MHz δ) 0.96 (t, 3H, J = 7.0 Hz, OCH_2CH_3), 1.14 (t, 9H, J = 7.3 Hz, $N(CH_2CH_3)$), 1.85 (s, 3H, Ac), 1.87 (s, 3H, Ac), 1.89 (s, 3H, Ac), 2.92 (dq, 6H, J = 3.3, 7.3 Hz, $N(CH_2CH_3)$), 3.30 (dt, 1H, J = 2.9, 10 Hz, H-2), 3.33 (dt, 1H, J = 7.0, 9.5 Hz, $OCHHCH_3$), 3.56 (dq, 1H, J = 7.0, 9.5 Hz, $OCHHCH_3$), 3.94 (brd, 1H, J = 10.5 Hz, H-6a), 4.05–4.09 (m, 2H, H-5, H-6b), 4.88 (t, 1H, J = 9.6 Hz, H-3/H-4), 5.15 (t, 1H, J = 9.7 Hz, H-4/H-3), 5.53 (dd, 1H, J = 3.3, 6.9 Hz, H-1); ^{13}C NMR ($CDCl_3$, CD_3OD (2/1 v/v) 75 MHz) δ 7.8, 17.8, 20.0, 20.1, 20.2, 45.2, 61.4, 66.1, 67.5, 67.9, 71.4, 77.2 (d, J = 8 Hz), 92.3 (d, J = 6 Hz), 169.8, 170.4, 171.0; ^{31}P NMR ($CDCl_3$, CD_3OD (2/1 v/v) 162 MHz) δ 0.5; $[\alpha]_D$ +79.2° (*c* 1.1, $CHCl_3$); MS (−ve FAB): 413 (M^-); HRMS: calcd for $C_{14}H_{22}O_{12}P$: *m*/*z* 413.084890; found: 413.085900.

P^1-Dolichyl P^2-[3,4,6-tri-*O*-acetyl-2-*O*-ethyl-α-D-glucopyranosyl] diphosphate (10c). ^{31}P NMR ($CDCl_3$, CD_3OD (5/1 v/v) 162 MHz) δ −10.0, −12.4; MS (−ve ESI): 1652.4, 1720.5, 1788.6, 1857.0, 1924.5 (M^- for n = 13–17).

P^1-Dolichyl P^2-[2-*O*-ethyl-α-D-glucopyranosyl] diphosphate (1c). MS (−ve ESI): 1526.1, 1594.2, 1662.3, 1730.7, 1798.2 (M^- for n = 13–17).

Mono(triethylammonium) salt of 3,4,6-tri-*O*-acetyl-2-deoxy-2-trifluoroacetamido-α-D-glucopyranosyl phosphate (4d).[27] Hydrogenolysis of α-dibenzyl phosphate **14**[27] (145.2 mg, 0.2195 mmol) gave α-phosphate **4d** (135.5 mg, quantitative) as a foam; IR (neat) cm^{-1} 3411, 2995, 1748, 1560, 1458, 1369, 1224, 1189, 1157, 1040, 962, 921, 840, 718; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 1.08 (t, 9H, J = 7.3 Hz, $N(CH_2CH_3)$), 1.75 (s, 3H, Ac), 1.79 (s, 3H, Ac), 1.85 (s, 3H, Ac), 2.87 (q, 6H, J = 7.3 Hz, $N(CH_2CH_3)$), 3.90 (dd, 1H, J = 2.0, 12.4 Hz, H-6a), 4.03 (dd, 1H, J = 3.4, 12.4 Hz, H-6b), 4.07 (dt, 1H, J = 2.9, 10.2 Hz, H-5), 4.14 (dt, 1H, J = 2.6, 10.5 Hz, H-2), 4.92 (t, 1H, J = 9.6 Hz, H-3/H-4), 5.19 (t, 1H, J = 9.6 Hz, H-4/H-3), 5.32 (dd, 1H, J = 3.3, 6.8 Hz, H-1); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 7.9, 19.8, 20.0, 20.1, 45.7, 52.3 (d, J = 7.5 Hz), 61.3, 67.9 (2×), 68.0, 70.6, 92.8, 157.9 (q, J = 37 Hz), 169.7, 170.4, 171.0; ^{31}P NMR ($CDCl_3$, 162 MHz) δ −0.9; $[\alpha]_D$ +33.9° (*c* 0.9, $CHCl_3$); MS (−ve FAB): 480 (M^-); HRMS: calcd for $C_{14}H_{18}NO_{12}F_3P$: *m*/*z* 480.051874; found: 480.051500.

P^1-Dolichyl P^2-[3,4,6-tri-*O*-acetyl-2-deoxy-2-trifluoroacetamido-α-D-glucopyranosyl] diphosphate (10d). ^{19}F NMR ($CDCl_3$, 470 MHz) δ −76.4; ^{31}P NMR ($CDCl_3$, 162 MHz) δ −10.1, −12.4; MS (−ve ESI): 825.7, 859.8, 893.8, 927.9, 961.9, 995.9, 1030.0 (*m*/2 for n = 12–18).

P^1-Dolichyl P^2-[2-deoxy-2-trifluoroacetamido-α-D-glucopyranosyl] diphosphate (1d). MS (−ve ESI): 1593.1, 1661.2, 1729.6, 1797.7, 1865.2 (M^- for n = 13–17).

P^1-Dolichyl P^2-[2-amino-α-D-glucopyranosyl] diphosphate (1e). MS (−ve ESI): 1498.5, 1566.6, 1634.6, 1702.7, 1736.7 (M^- for n = 13–17).

Biological methods

Crude porcine liver microsomes were prepared from fresh pig liver based on a protocol developed for rat liver.[32,33] Briefly, a 100 g sample of fresh pig liver typically affords 2.5–5 mL of a microsomal preparation (~100 mg/mL protein) that can be stored at −80 °C in the presence of 30% glycerol. This material can be used for several months without significant loss of transferase activity. The natural substrate Dol-PP-GlcNAc **1a** was prepared according to literature procedure.[2] Radiolabeled uridine diphosphate *N*-acetyl-D-glucosamine-6-^{3}H (UDP-(^{3}H)-GlcNAc **2**, specific activity of 60 Ci/mmol, concentration = 1 mCi/mL) was obtained from American Radiolabeled Chemicals, Inc., St. Louis, MO, USA.

Enzyme II assay

The assay buffer consisted of 50 mM Tris acetate, pH 7.0, 3 mM dithiothreitol (DTT), 5 mM $MgCl_2$, 0.25 M sucrose and 1% Nonidet P-40. Dolichol-linked mono-

saccharides (**1a**–**e**) were aliquoted from a chloroform/methanol stock solution into an eppendorf, and the solvent was evaporated under a stream of nitrogen. To this eppendorf was added UDP-(^{3}H)-GlcNAc (60 Ci/mmol for the study of Dol-PP-2DFGlc **1b**, and 0.6 Ci/mmol for the other analogues, final concentration = 0.167 μM) in ethanol/water, and the solvent was again evaporated under nitrogen. Assay buffer was added (final volume = 100 μL) to the eppendorf and vortexed vigorously. The reaction was initiated by the addition of freshly thawed pig liver microsomes (5 or 10 μL) and shaken at 180 rpm. Aliquots (10 μL) of the reaction mixture were quenched with 0.6 mL of chloroform/methanol/2.5 mM $MgCl_2$ (3/2/1 v/v) at different times. The upper aqueous phase was removed and the lower phase re-extracted twice with TUP (theoretical upper layer, chloroform/methanol/water 2.75/44/53.25 v/v, 100 μL). The combined aqueous phases were then mixed with Ecolite (5.5 mL) and counted on a Beckman LS-5000TD scintillation counter to give the amount of unreacted UDP-(3H)-GlcNAc. The organic layer was mixed with Betamax (5.5 mL) and counted as well. The percent conversion of dolichol-linked disaccharide was based on $[^3H_{org}/(^3H_{aq} + {}^3H_{org}) \times 100]$.

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