Substrate Specificity of the Glycosyl Donor for Oligosaccharyl Transferase

Vincent W.-F. Tai and Barbara Imperiali*

Department of Chemistry, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

imper@mit.edu

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Oligosaccharyl transferase (OT) catalyzes the co-translational transfer of a dolichol-linked tetradecasaccharide (Dol-PP-$GlcNAc_2Man_9Glc_3$, **1a**) to an asparagine side chain of a nascent polypeptide inside the lumen of the endoplasmic reticulum (ER). The glycosyl acceptor requires an Asn-Xaa-Thr/Ser sequon, where Xaa can be any natural amino acid except proline, for *N*-linked glycosylation to occur. To address the substrate specificity of the glycosyl donor, three unnatural dolichol-linked disaccharide analogues (Dol-PP-GlcNTFA-GlcNAc **1c**, Dol-PP-2DFGlc-GlcNAc **1d**, and Dol-PP-GlcNAc-Glc **1e**) were synthesized and evaluated as substrates or inhibitors for OT from yeast. The synthetic analogue Dol-PP-GlcNAc-Glc **1e**, with substitution in the distal sugar, was found to be a substrate (K_{mapp} = 26 μM) for OT. On the other hand, the analogues Dol-PP-GlcNTFA-GlcNAc **1c** (K_i = 154 μM) and Dol-PP-2DFGlc-GlcNAc **1d** (K_i = 252 μM), with variations in the proximal sugar, were inhibitors for OT. The dolichol-linked monosaccharide Dol-PP-GlcNAc **3** was found to be the minimum unit for glycosylation to occur.

Introduction

Asparagine-linked glycosylation is essential for structural and functional integrity of many eukaryotic proteins.[1−7] This glycosylation is mediated by the membrane-associated multimeric enzyme oligosaccharyl transferase (OT, EC 2.4.1.119)[†] in the lumen of the endoplasmic reticulum (ER). OT catalyzes the transfer of a dolichol-linked tetradecasaccharide (Dol-PP-$GlcNAc_2Man_9Glc_3$, **1a**) to an asparagine side chain within a nascent polypeptide chain as the peptide is translocated into the lumen of the ER (Scheme 1). The resulting β-linked glycopeptide **2a** is further processed by glycosidases and glycosyltransferases in the ER and Golgi apparatus to yield the mature glycan structures that include other glycosyl units such as galactose, fucose, and sialic acid. This cotranslational event is the first committed step in the biosynthesis of all *N*-linked protein glycoconjugates; therefore, an understanding of this reaction is critical.

It has been well established that the minimum recognition sequence for *N*-linked glycosylation is an Asn-Xaa-Thr/Ser triad where Xaa can be any amino acid except proline.[8,9] Both in vitro and in vivo data showed that peptides with threonine at the (i + 2) site are better substrates than the corresponding serine-containing peptides. Simple tripeptides with capped C- and N-termini can be used as substrates for in vitro OT studies. Several mechanisms have been proposed to explain the activation of the seemingly unreactive carboxamide of asparagine in *N*-linked glycosylation, and this topic has been reviewed.[3−5,7]

Dolichol-linked tetradecasaccharide **1a**, biosynthesized via the dolichol pathway, is the preferred substrate for OT both in vivo and in vitro. Exceptions occur in systems such as trypanosomatid protozoa where one or more of the glycosyltransferases are missing.[10] As a result of the poor availability of the full length substrate, most in vitro studies have been carried out with truncated glycosyl donors such as the chitobiosyl derivative, Dol-PP-$GlcNAc_2$, **1b**. Early studies by Sharma et al. showed that Dol-PP-$GlcNAc_2$Man was also a substrate, whereas no transfer was observed for Dol-PP-$GlcNAc_2Man_9$ and Dol-PP-GlcNAc **3**.[11] Contrary to this result, Bause and co-workers reported that Dol-PP-GlcNAc **3** was in fact a poor substrate for OT,[12] and this discrepancy will be addressed later (inter infra). Using yeast genetics techniques, Aebi et al. also showed that various truncated dolichol-linked oligosaccharides were substrates for OT.[13] In an attempt to study the chain length effect of the polyisoprene, Coward and co-workers showed that compounds with shorter isoprenoid chains or saturated alkyl chains attached to chitobiosyl diphosphate were poor substrates for OT.[14] The existence of the saturated isoprenoid unit

* Tel: (617) 253-1838. Fax: (617) 452-2419.

† Also known as dolichol-diphospho-oligosaccharide-protein glycosyl transferase, abbreviated both as OT and OST in the literature.

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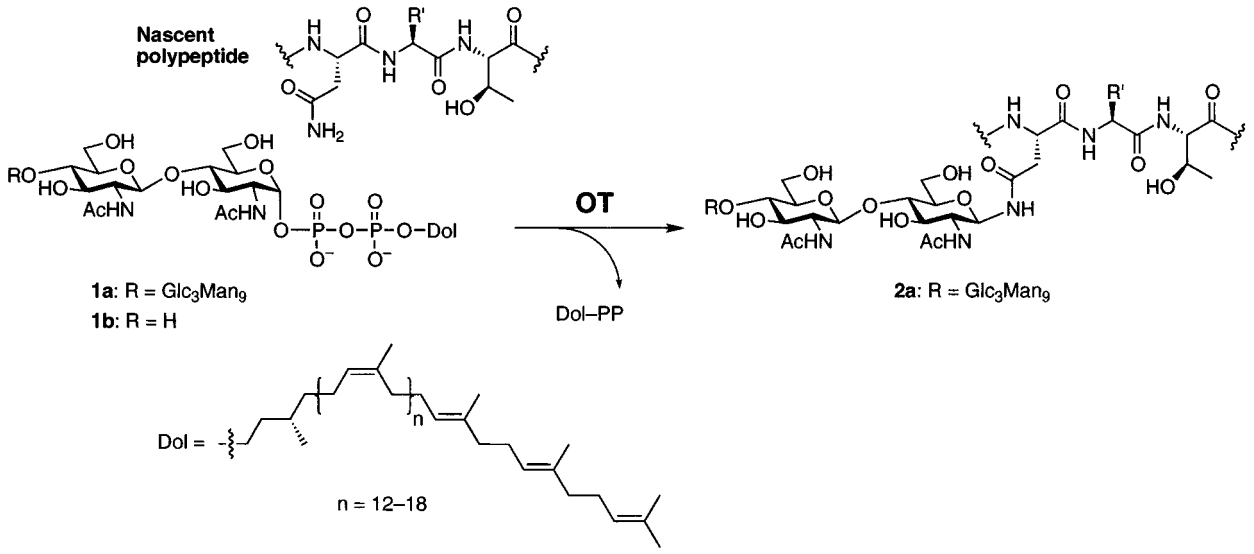
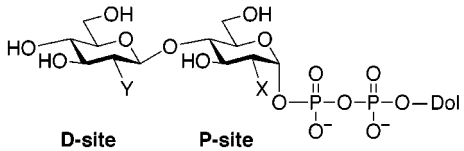
Scheme 1





	Y	X
1b	$NHCOCH_3$	$NHCOCH_3$
1c	$NHCOCH_3$	$NHCOCF_3$
1d	$NHCOCH_3$	F
1e	OH	$NHCOCH_3$

Figure 1. Structures of dolichol-linked disaccharides **1b**−**e**.

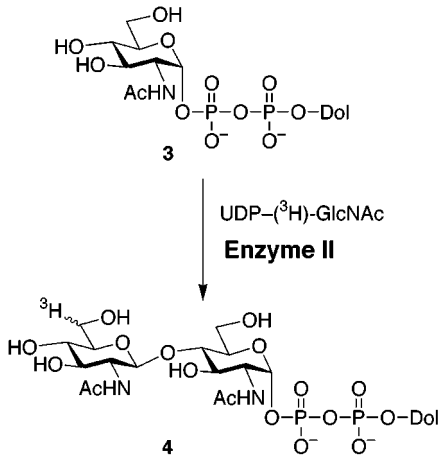
next to the pyrophosphate moiety as well as the presence of the *cis*-isoprenoid units still remains to be addressed.

Previous efforts have focused mainly on the study of the glycosyl acceptor; the investigation of the role of the glycosyl donor and its leaving group will help to better understand the complete process of *N*-linked glycosylation. The intriguing feature that *N*-acetylglucosamines (GlcNAc) constitute the first two saccharides in the glycosyl donor **1a** prompted us to study the role of the C-2 acetamido group in the first two GlcNAc units. Herein, the chemical synthesis of three unnatural dolichol-linked disaccharides are described, together with the evaluation of these compounds as substrates or inhibitors of yeast OT.

Results and Discussion

A substrate analogue approach was undertaken to examine the substrate specificity of the glycosyl donor. The trifluoroacetamido and 2-deoxy-2-fluoro derivatives **1c,1d** (Figure 1) were designed to address the importance of the C-2 acetamido group in the proximal saccharide site (P-site). Previously, the trifluoroacetamido analogue of UDP-GlcNAc was shown to be a substrate for "core-2" GlcNAc transferase.[15] On the other hand, substitution with an electronegative fluorine atom adjacent to the

Scheme 2



reaction center should result in significant destabilization of the oxocarbenium ion, thus inhibiting such a reaction pathway. Withers and co-workers have demonstrated that 2-deoxy-2-fluoroglycosides with good leaving groups at the C-1 position can be used as mechanism-based inhibitors of retaining β-glucosidases. In some cases, 2-deoxy-2-fluoroglycosyl-enzyme intermediates have been isolated and characterized.[16,17] So the study of analogue **1d** will be particularly interesting, both in terms of studying the mechanism of OT and trapping of any glycosyl-enzyme intermediate. Additionally, Dol-PP-GlcNAc-Glc **1e** was included to address the role of the acetamido group in the distal saccharide site (D-site).

Previously, radiolabeled Dol-PP-GlcNAc−(^{3}H)-GlcNAc **4** was prepared by an efficient chemoenzymatic synthesis of Dol-PP-GlcNAc **3** via Enzyme II from pig liver microsomes (Scheme 2).[18] However, this general approach failed to give sufficient amount of the unnatural derivatives **1c** and **1d**.[19] Our study showed that slight modifica-

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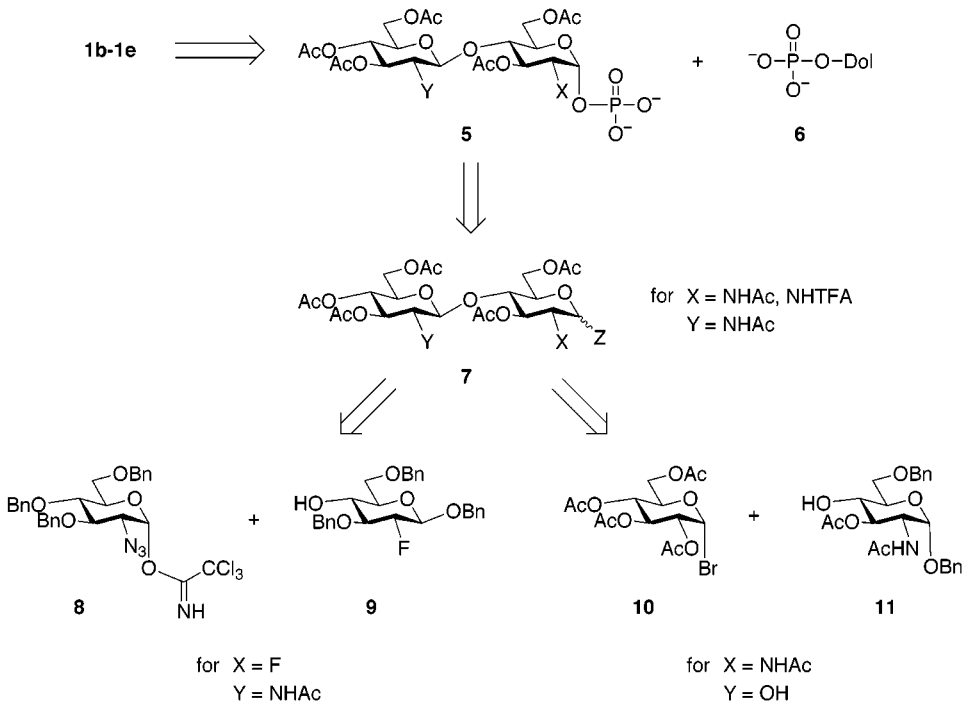
Scheme 3



tion of the acetamido group in **3** resulted in significant loss of Enzyme II activity, and therefore the C-2 acetamido group was proposed to be an important functional determinant for this transferase reaction. The failure of the chemoenzymatic approach prompted us to prepare compounds **1b**−**e** via chemical synthesis.

The preparation of dolichol-linked disaccharides **1b**−**e** followed a similar strategy as for the previous preparation of their monosaccharide counterparts.[19] The key step involves the condensation of α-linked disaccharide phosphates **5** with dolichol monophosphate **6** (Scheme 3). The α-phosphates **5** can in turn be prepared from phosphorylation of the lactol (for Z = OH in **7**), bromide (for Z = Br in **7**) or oxazoline (for X, Z = −N=C(CF_3)−O− in **7**). Commercially available chitobiose octaacetate was employed for the preparation of Dol-PP-GlcNAc-GlcNAc **1b** and Dol-PP-GlcNTFA-GlcNAc **1c**. The other unnatural disaccharides were assembled by two different routes to establish the β-1,4-linkages. The disaccharide in Dol-PP-2DFGlc-GlcNAc **1d** was prepared by glycosylation of the trichloroacetimidate Schmidt donor **8**[20] with glycosyl acceptor **9**. Glycosyl donor **8** was chosen because of its high reactivity and good stereoselectivity for the β-product.[20] On the other hand, glycosylation of commercially available bromoacetyl glucose **10** with the known acceptor **11**[21] afforded protected Glc-β-(1→4)-GlcNAc. This glycosylation has previously been communicated by O'Connor using a mixture of α- and β-benzyl glycosides **11**.[22] The in vitro substrate **1b** was prepared as described previously.[23]

Synthesis of Dol-PP-GlcNTFA-GlcNAc, 1c. Preparation of Dol-PP-GlcTFA-GlcNAc **1c** begins with the installation of the trifluoroacetamido group via the known amine hydrochloride **13**.[24] The amine derivative **13** can be readily prepared by the acyl migration of chitobiosyl chloride **12**[25] by heating in water/acetone (Scheme 4). Acylation of **13** with trifluoroacetic anhydride (TFAA)/pyridine gave 97% yield of **14**. The ^{1}H NMR spectrum of **14** looks similar to that of chitobiose octaacetate, while the ^{19}F NMR spectrum shows a singlet at −76.6 ppm corresponding to the newly incorporated trifluoroacetate group. Bromination of compound **14** followed by oxazoline ring formation with 2,6-lutidine gave oxazoline **15** in 61% over two steps.[19,26] Oxazoline **15** shows the correct mass at 693 (MNa^+) and H-1 appears as a doublet at 6.22 ppm ($J_{1,2}$ = 7.4 Hz) in the ^{1}H NMR spectrum. Ring opening of oxazoline **15** with dibenzyl phosphate **16** in refluxing 1,2-dichloroethane[26] gave solely α-dibenzyl phosphate **17** in 64% yield. The stereochemistry of **17** was confirmed to be α on the basis of the coupling constant of H-1−H-2 protons. H-1 appears as a doublet of doublets at 5.65 ppm ($J_{1,2}$ = 4 Hz, $J_{1,P}$ = 6 Hz). Catalytic hydrogenolysis of α-dibenzyl phosphate **17** with 10% palladium on charcoal afforded a quantitative yield of α-phosphate **18** isolated as its triethylamine salt. Dolichol monophosphate (Dol-P) **6** was prepared according to the Danilov modified Cramer procedure.[27] Activation of α-phosphate **18** with 1,1′-carbonyldiimidazole (CDI) followed by coupling with Dol-P **6** gave protected Dol-PP-GlcNTFA-GlcNAc **19** in 49% yield. Chemoselective deprotection of the acetates in **19** with guanidine/guanidinium nitrate solution[28] afforded Dol-PP-GlcNTFA-GlcNAc **1c** in 39% yield after silica gel chromatography.

Synthesis of Dol-PP-2DFGlc-GlcNAc, 1d. As illustrated in Scheme 5, glycosyl acceptor **9** was prepared

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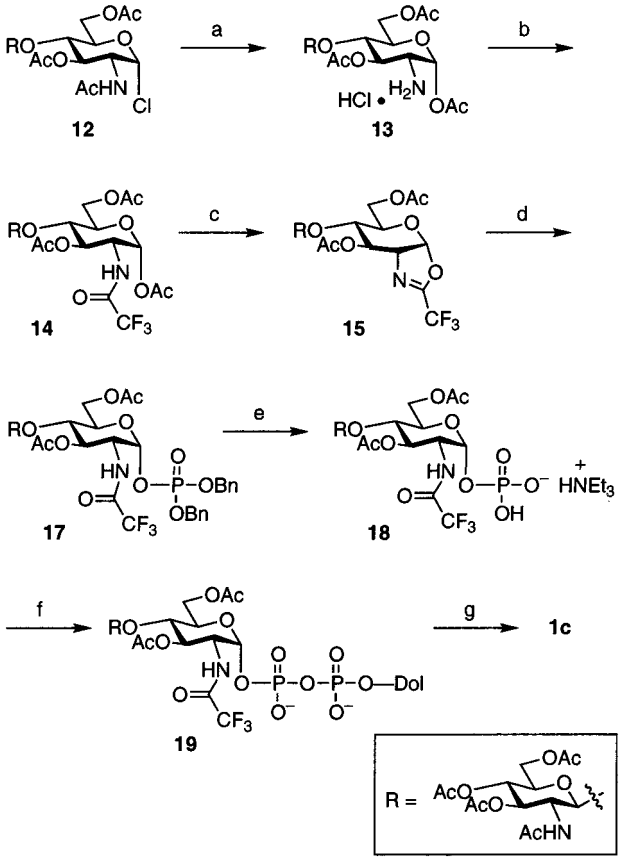
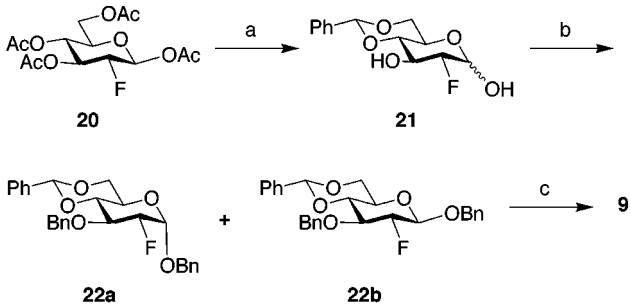
Scheme 4[a]



[a] Reagents and conditions: (a) H_2O, acetone, Δ (74%); (b) TFAA, pyridine, CH_2Cl_2, 0 °C (97%); (c) (1) 30% HBr/HOAc, CH_2Cl_2; (2) 2,6-lutidine, CH_3CN (61% for two steps); (d) dibenzyl phosphate **16**, 1,2-dichloroethane, Δ (64%); (e) H_2, 10% Pd/C, MeOH (quantitative); (f) (1) CDI, DMF; (2) Dol-P **6** (49%); (g) guanidine/guanidinium nitrate, CH_2Cl_2/MeOH (39%).

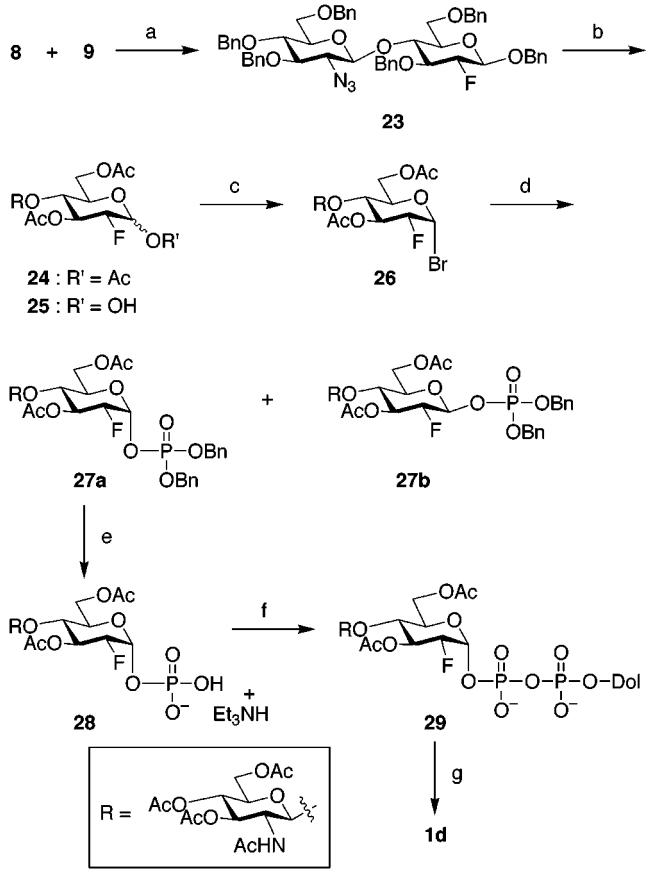
Scheme 5[a]



[a] Reagents and conditions: (a) (1) NaOMe, MeOH; (2) Ph-CH(OMe)$_2$, *p*-TsOH, DMF, 50 °C (76%, α:β = 1:1.8); (b) NaH, BnBr, DMF, 0 °C (85%); (c) Na(CN)BH_3, THF, HCl/Et_2O, 0 °C (87%).

from the known 2-deoxy-2-fluoro-1,3,4,6-β-D-glucose tetraacetate **20**.[29] The benzylidene moiety was readily installed by acidic benzylidenation of 2-deoxy-2-fluoro glucose to give 76% yield of the reducing sugar **21**. Benzylation of the free hydroxyl groups in **21** gave a separable mixture of α- and β-benzyl glucosides **22a** and **22b** in 85% combined yield (α:β = 1:1.8). Both anomers showed the correct mass at 451 (MH^+), and the stereochemistry was assigned on the basis of the H-1−H-2 coupling constants. The H-1 protons of **22a** and **22b** resonate at 5.15 ppm ($J_{1,2}$ = 4 Hz) and 4.67 ppm ($J_{1,2}$ = 7.6 Hz, $J_{1,F}$ = 4 Hz), respectively. For simplicity in the characterization of compounds at a later stage, the major anomer **22b** was chosen to carry on the synthesis. Reductive ring opening of β-anomer **22b** with sodium cyanoborohydride [Na(CN)BH_3] under acidic conditions (HCl/Et_2O)[30] gave the glycosyl acceptor **9** with good regioselectivity in 87% yield.

Scheme 6[a]



[a] Reagents and conditions: (a) BF_3·Et_2O, 4 Å molecular sieves, CH_2Cl_2, −78 → 5 °C (83%); (b) (1) H_2, Raney Ni, Ac_2O, EtOH; (2) H_2, 10% Pd/C, MeOH; (3) Ac_2O, pyridine (96%, α:β = 2:1); (c) 30% HBr/HOAc, CH_2Cl_2 (85%); (d) AgOTf, dibenzyl phosphate **16**, THF (60%, α:β = 1.6:1); (e) H_2, 10% Pd/C, MeOH (quantitative); (f) (1) CDI, DMF; (2) Dol-P **6** (36%); (g) NaOMe, CH_2Cl_2/MeOH (73%).

Glycosyl donor **8** was prepared according to the Schmidt procedure via azidonitration[31] of commercially available tri-*O*-benzyl-D-glucal followed by subsequent imidate formation with NaH/CCl_3CN.[20] Glycosylation of the 2-deoxy-2-fluoro derivative **9** with the Schmidt donor **8** under Lewis acidic conditions (BF_3·Et_2O) gave 83% yield of the perbenzylated disaccharide **23** (Scheme 6). The stereochemistry of the newly formed glycosidic bond was confirmed to be β ($J_{1',2'}$ = 7.6 Hz) by ^{1}H NMR analysis. The benzyl and azido groups in **23** were successively replaced by acetate groups to give disaccharide **24** in 96% yield as a mixture of α- and β-anomers (α:β ≈ 2:1). The mixture of acetates **24** was treated with 30% HBr in acetic acid to give exclusively α-bromide **26** in 85% isolated yield. Phosphorylation of α-bromide **26** with

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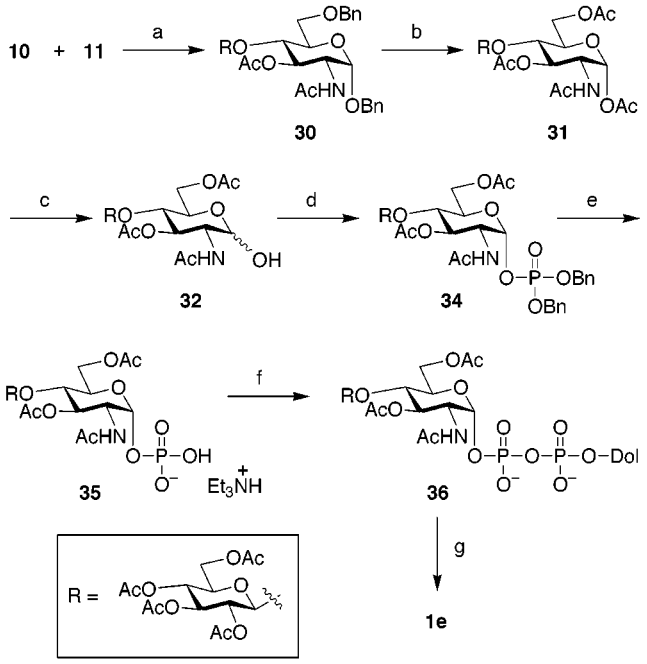
Scheme 7[a]



[a] Reagents and conditions: (a) AgOTf, CH_2Cl_2/toluene, −45 → 0 °C (84%); (b) (1) H_2, 10% Pd/C, EtOH; Ac_2O, DMAP (cat.), pyridine, CH_2Cl_2 (74%); (c) hydrazine monoacetate, DMF (84%); (d) (1) LiHMDS, THF, −68 °C; (2) tetrabenzyl pyrophosphate **33**, −68 → 0 °C (89%); (e) H_2, 10% Pd/C, MeOH (quantitative); (f) (1) CDI, DMF; (2) Dol-P **6** (59%); (g) NaOMe, CH_2Cl_2/MeOH (73%).

dibenzyl phosphate **16** using silver trifluoromethanesulfonate (AgOTf) as promoter gave 60% yield of a separable mixture of α- and *β*-dibenzyl phosphates **27a** and **27b** in the ratio of 1.6:1. The use of silver-promoted phosphorylation on bromide **26** was necessary since the preliminary attempts at phosphorylation of reducing sugar **25** failed as a result of the highly insoluble nature of this compound in solvents such as CH_2Cl_2, $^iPr_2O$, CH_3CN, and THF. Similarly, α-dibenzyl phosphate **27a** was hydrogenated to give α-phosphate **28,** which was further condensed with Dol-P **6** to give protected Dol-PP-2DFGlc-GlcNAc **29** in 36% yield. Deacetylation of **29** with sodium methoxide gave Dol-PP-2DFGlc-GlcNAc **1d**.

Synthesis of Dol-PP-GlcNAc-Glc, 1e. Synthesis of disaccharide **30** followed the preliminary account by O'Connor.[22] Glycosylation of commercially available bromoacetyl glucose **10** and readily available glycosyl acceptor **11**[21] with silver trifluoromethanesulfonate (AgOTf)[32] as promoter gave the desired disaccharide **30** in 84% yield (Scheme 7). The linkage of the newly formed glycosidic bond was assigned to be *β* on the basis of ^{1}H NMR analysis; H-1′ appeared as a doublet at 4.37 ppm with a coupling constant of 8.1 Hz for H-1′−H-2′. The benzyl groups were replaced with acetate groups by hydrogenolysis and subsequent acetylation to give 74% yield of peracetylated Glc-*β*-(1→4)-GlcNAc **31**. The α-anomer was found to be the major compound and could be crystallized from MeOH/pentane to afford pure α-**31**, mp 221−223 °C. Chemoselective deacetylation of the anomeric acetate in **31** by hydrazine monoacetate followed by phosphorylation of the reducing sugar with tetrabenzyl pyrophosphate {$[(BnO)_2P(O)]_2O$, **33**}[33,34] gave exclusively α-dibenzyl phosphate **34** in 75% yield over two steps. Similarly, removal of the benzyl protecting groups followed by condensation with Dol-P **6** gave protected Dol-PP-GlcNAc-Glc **36** in 59% yield. Deprotection of **36** gave Dol-PP-GlcNAc-Glc **1e** in 73% yield after silica gel chromatography.

In summary, three unnatural dolichol-linked disaccharides (**1c**−**e**) have been prepared from the condensation of activated sugar phosphates with Dol-P **6**. These analogues are stable in their acetate forms at −20 °C, and only small amounts of material are deprotected just prior to carrying out biological studies. Both the ^{1}H and ^{13}C NMR spectra of these compounds are dominated by signals from the isoprene units of dolichol. In general, these compounds are best characterized by electrospray ionization (ESI) mass spectroscopy (negative mode), which shows the typical Gaussian distribution pattern due to the dolichol isomers with variable number of isoprene units.

Biological Evaluation

The unnatural dolichol-linked disaccharides (**1c**−**e**) were first evaluated as glycosyl donor substrates for oligosaccharyl transferase from yeast (*Saccharomyces cerevisiae*). The standard OT assay, which uses radiolabeled Dol-PP-GlcNAc−(^{3}H)-GlcNAc **4** as substrate, is not applicable for the present study. This method cannot distinguish whether the unnatural analogues are substrates or inhibitors because both will compete with **4** and result in an apparent decrease in (^{3}H)-glycopeptide formation. So a modified assay method based on a radiolabeled peptide developed by Coward[35] was employed for this study. As illustrated in Scheme 8, the radiolabeled tetrapeptide Bz-Asn-Leu-Thr-Lys(^{3}H-Ac)-NH_2 **37r** was chosen as the acceptor substrate as a result of the ease of incorporating the tritium label at the final stage of synthesis. Upon quenching the enzymatic reaction, both the radiolabeled peptide substrate **37r** and the glycopeptide products partition into the aqueous phase after successive aqueous−organic extractions. The amount of product formed is quantified by scintillation counting after HPLC separation of the (^{3}H)-glycopeptide.

The tetrapeptide Bz-NLTK-NH_2 **42** was readily prepared by standard Fmoc-based solid-phase peptide synthesis (SPPS) (Scheme 9). Acetylation of Bz-NLTK-NH_2 **42** gave a quantitative yield of Bz-NLTK(Ac)-NH_2 **37**. Both tetrapeptides **42** and **37** gave the correct masses and displayed consistent ^{1}H NMR spectra. The radiolabeled tetrapeptide Bz-NLTK(^{3}H-Ac)-NH_2 **37r** was prepared by first treating **42** with 0.4 equiv of radioactive acetic anhydride [(^{3}H)-Ac_2O, 100 mCi/mmol] followed by capping with excess acetic anhydride. The specific activity of tetrapeptide Bz-NLTK(^{3}H-Ac)-NH_2 **37r** was found to be 29.3 μCi/μmol on the basis of the radioactivity from a single peak eluted from HPLC. The K_m for Bz-NLTK(Ac)-NH_2 **37** was determined to be 4.2 μM (∼6-fold better compared to the tripeptide Bz-NLT-NHMe).

Unnatural dolichol-linked saccharides were first examined for substrate activity with OT using radiolabeled

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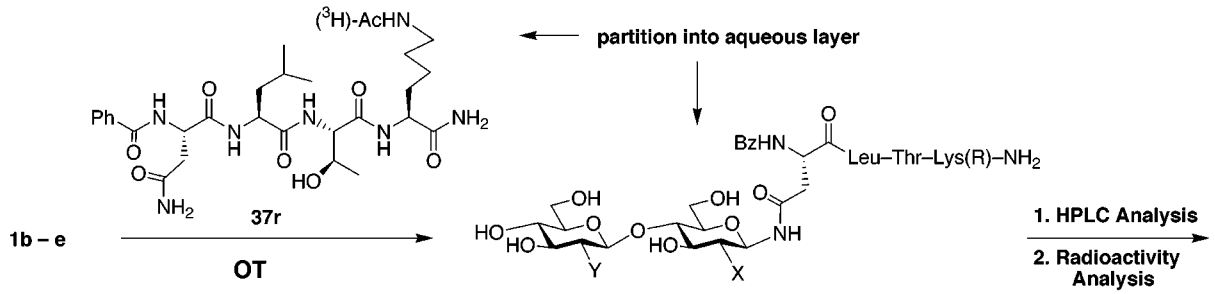
Scheme 8



	Y	X	R
38r	$NHCOCH_3$	$NHCOCH_3$	^3H-Ac
39r	$NHCOCH_3$	$NHCOCF_3$	^3H-Ac
40r	$NHCOCH_3$	F	^3H-Ac
41r	OH	$NHCOCH_3$	^3H-Ac

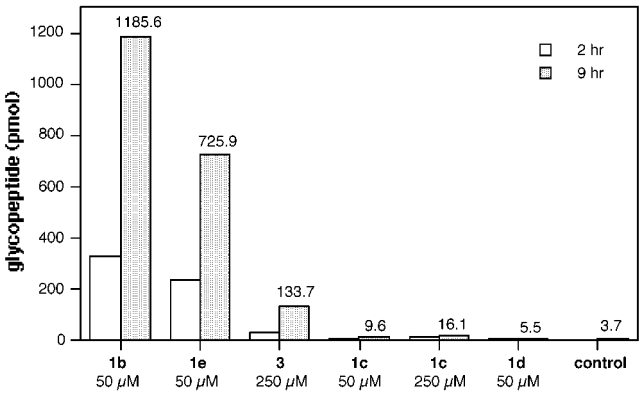
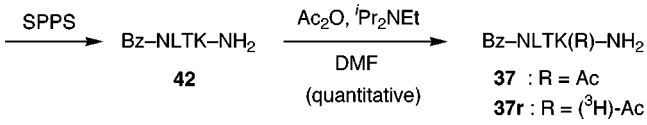


Figure 2. Glycopeptide formation over 2 and 9 h.

Scheme 9



Bz-Asn-Leu-Thr-Lys(^{3}H-Ac)-NH_2 **37r** as the acceptor. Reactions were carried out for 2 and 9 h followed by HPLC and radioactivity analyses. Generally, the peak corresponding to the newly formed glycopeptide was not directly observed by HPLC because of the sensitivity of the UV detector but was readily revealed by radioactivity monitoring. The differences in retention times (ca. 4 min) between peptide **37r** and glycopeptides allow easy and accurate quantification of products. Preliminary screening results of the unnatural analogues are shown in Figure 2. Dol-PP-GlcNAc-Glc **1e**, with a modification in the D-site, was found to be a substrate for OT with comparable activity to the in vitro substrate **1b**. Disaccharide analogues **1c** and **1d** (50 μM), with modifications in the P-site, showed no transferase activity over 2 h. However, a 5-fold increase in concentration of Dol-PP-GlcNTFA-GlcNAc **1c** resulted in 1.7-fold increase in glycopeptide formation over 9 h. Interestingly, the monosaccharide derivative Dol-PP-GlcNAc **3**[18] also showed product formation (11% relative to **1b**) by radioactivity analysis. These experiments were repeated with nonradiolabeled peptide acceptor **37** and fractions corresponding to the apparent glycopeptide (t_R = 19−20 min) were submitted for MALDI mass spectroscopy analysis. Glycopeptides Bz-Asp(GlcNAc-β-(1→4)-GlcNAc)-Leu-Thr-Lys(Ac)-NH_2 **38** and Bz-Asp(Glc-β-(1→4)-GlcNAc)-Leu-Thr-Lys(Ac)-NH_2 **41** displayed the correct masses at 1048.77 (MNa^+) and 1007.66 (MNa^+), respectively. Masses corresponding to glycopeptides **39** and **40** were not detected. Surprisingly, other than the expected masses (845.30, MNa^+; 860.98, MK^+) for Bz-Asp(GlcNAc)-Leu-Thr-Lys(Ac)-NH_2 **43**, a major peak corresponding to glycopeptide **38** was also observed when using Dol-PP-GlcNAc **3** as a substrate (**43**:**38** $\approx$ 1:2 based on peak intensity). This observation can be best explained by the presence of endogenous Enzyme II in the microsomal OT preparation, which biosynthesizes Dol-PP-GlcNAc-GlcNAc **1b** when **3** is at a high concentration. This is the first time that Dol-PP-GlcNAc **3** has been directly demonstrated to be a substrate for OT, which is in agreement with an earlier report by Bause[12] stating that **3** is a poor substrate (vide supra).

The kinetic parameters for substrates Dol-PP-GlcNAc-GlcNAc **1b** and Dol-PP-GlcNAc-Glc **1e** are shown in Table 1. Both the K_{mapp} and V_{maxapp} of the in vitro substrate **1b** are in accord with the literature values.[35] Interestingly, replacement of the acetamido group in the distal sugar gave 2.5-fold improvement in binding; on the other hand, V_{maxapp} was lowered by 3.2-fold. Overall, the unnatural substrate **1e** is 76% as effective (V_{maxapp}/K_{mapp}) as the in vitro substrate **1b**. Because of the complicated nature of the effects with Dol-PP-GlcNAc **3**, further analysis was not undertaken.

In contrast, analogues **1c** and **1d**, with a modified C-2 substituent in the proximal sugar, showed no apparent transferase activity. These analogues were further analyzed as inhibitors for OT. The sensitivity of our method allows kinetic studies using initial rates. Variable amounts of unnatural analogues were competed against constant amounts of the in vitro substrate **1b** (22 μM) and radiolabeled peptide **37r** (21 μM). The approximate K_i's were calculated as described by Segel.[36]

The estimated K_i's for analogues **1c** and **1d** are 2.4- to 3.9-fold the K_{mapp} of **1b**. Replacement of the C-2 acetamido group by the similarly sized trifluoroacetamido group reduces the binding to the enzyme; however, no significant transferase activity was observed. Further reduction in binding was noted by the smaller 2-deoxy-2-fluoro

(36) Segel, I. H. In *Enzyme Kinetics*; John Wiley & Sons: New York, 1975; pp 100−107.

Table 1. Kinetic Constants for Compounds 1b−e

compound	K_{mapp} (μM)	V_{maxapp} (pmol/min)	V_{maxapp}/K_{mapp} (rel)	K_i (μM)
Dol-PP-GlcNAc-GlcNAc **1b**	64.3	10.3	1	
Dol-PP-GlcNAc-Glc **1e**	26.0	3.2	0.76	
Dol-PP-GlcNTFA-GlcNAc **1c**				154 ± 14
Dol-PP-2DFGlc-GlcNAc **1d**				252 ± 71

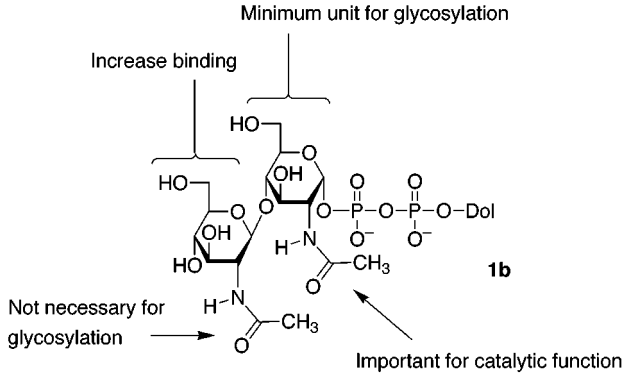


Figure 3. Substrate specificity of glycosyl donor for oligosaccharyl transferase.

derivative. Although the mode of inhibition was not determined in this study, it is most likely that these inhibitors act in a competitive fashion. The large experimental deviation of the K_i value for 2-deoxy-2-fluoro analogue **1d** suggests that the mode of inhibition may be more complicated.

The substrate specificity of glycosyl donor for yeast OT is summarized in Figure 3. A minimum requirement for glycosyl donors in OT catalysis is an *N*-acetylglucosamine (GlcNAc) unit as demonstrated by the glycopeptide **43** formed from Dol-PP-GlcNAc **3**. The efficiency of this transferase process can be improved by the addition of a second sugar. The requirement for an *N*-acetyl-glucosamine in the distal site is not absolute since substitution with a glucose unit (as in Dol-PP-GlcNAc-Glc **1e**) also results in comparable transferase activity. The presence of the distal sugar may simply assist the binding of the glycosyl donor. On the other hand, the acetamido group in the proximal sugar plays a significant role in OT catalysis. Minor changes in the acetamido group resulted in significant loss of activity as in the case of the trifluoroacetamido derivative **1c** or total loss of activity as in **1d**. The loss of transferase activity cannot be simply explained by the reduced binding (2.4- to 3.9-fold less than **1b**) of the unnatural analogues, the acetamido group is therefore proposed to be involved in the catalytic machinery. The acetamido group may interact with the enzyme through critical hydrogen bonding with the catalytic site. Additionally, the low transferase activity of **1c** may be explained by the strong electron-withdrawing effect of the trifluoromethyl group, rendering the adjacent carbonyl a less efficient hydrogen bond acceptor.

Experimental Section

Melting points were uncorrected. Optical rotations were measured with a Perkin-Elmer 241 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 INOVA 500, Bruker AM-500 (500.15 MHz for ^{1}H, 125 MHz for ^{13}C), or Varian Mercury 300 (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 Department of Chemistry, Massachusetts Institute of Technology, Cambridge, MA 02139, or UCR Mass Spectrometry Facility, Department of Chemistry, University of California, Riverside, CA 92521. All reactions were monitored by analytical thin-layer chromatography (TLC) on glass precoated with silica gel 60 F_{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 and used without further purification. Dol-PP-GlcNAc-GlcNAc **1b** was prepared according to Coward's procedure.[23] Stock solutions of dolichol-linked saccharides were quantified by the Barlett assay.[37,38]

General Procedure for Hydrogenolysis of Dibenzyl Phosphates 17, 27a, and 34. A solution of dibenzyl phosphate (0.16 mmol) and 10% Pd/C (20 mg) was stirred under H_2 in MeOH (8 mL) until no starting material remained as indicated by TLC. The catalyst was filtered through a pad of Celite and washed with MeOH (15 mL). Et_3N (1 mL) was added followed by evaporation of solvent in vacuo to give the mono(triethylammonium) salt of the phosphate.

General Procedure for Coupling of Sugar Phosphate with Dolichol Monophosphate.[23] 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 **6**[27] (tri-*n*-butylammonium form, 0.028 mmol) in CH_2Cl_2 (1 mL) was then added, and the mixture was allowed to stir for 3 d 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 combined and further purified by silica gel chromatography.

2-Acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl-(1→4)-1,3,6-tri-*O*-acetyl-2-deoxy-2-trifluoroacetamido-α-D-glucopyranose 14. To a solution of the amine hydrochloride **13**[24] (364.0 mg, 0.5424 mmol) in CH_2Cl_2 at 0 °C were added pyridine (219.4 μL, 2.712 mmol) and trifluoroacetic anhydride (TFAA) (114.9 μL, 0.8137 mmol). The mixture was allowed to stir at room temperature for 2 h and poured into a saturated aqueous solution of NH_4Cl (5 mL). The organic phase was separated, washed with brine (5 mL), dried (Na_2SO_4), and filtered. Concentration of the filtrate followed by crystallization from MeOH gave the desired product **14** (383.5 mg, 97%) as a white solid, mp > 250 °C; IR (neat) cm^{-1} 3295, 1745, 1667, 1558, 1370, 1227, 1157, 1033, 940, 754; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 1.97 (s, 3H, Ac), 2.01 (s, 3H, Ac), 2.02 (s, 3H, Ac), 2.06 (s, 3H, Ac), 2.08 (s, 3H, Ac), 2.17 (s, 3H, Ac), 2.21 (s, 3H, Ac), 3.63 (ddd, 1H, J = 2.3, 3.8, 9.8 Hz, H-5), 3.77 (t, 1H, J = 9.5 Hz, H-4), 3.91−3.96 (m, 2H, H-2′, H-5′), 4.02 (dd, 1H, J = 2.0, 12.5 Hz, H-6a), 4.19 (dd, 1H, J = 1, 12.2 Hz, H-6a′), 4.32 (m,

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1H, H-2), 4.39 (dd, 1H, J = 4.1, 12.5 Hz, H-6b), 4.47 (dd, 1H, J = 3.4, 12.2 Hz, H-6b′), 4.49 (d, 1H, J = 8.2 Hz, H-1′), 5.06 (t, 1H, J = 9.5 Hz, H-3′), 5.15 (dd, 1H, J = 9.5, 10.3 Hz, H-4′), 5.29 (dd, 1H, J = 9.1, 10.9 Hz, H-3), 5.88 (d, 1H, J = 9.1 Hz, N*H*), 6.18 (d, 1H, J = 3.6 Hz, H-1), 6.59 (d, 1H, J = 8.5 Hz, N*H*); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 20.33, 20.56, 20.59, 20.65, 20.91, 20.99, 23.19, 51.91, 54.44, 61.30, 61.61, 67.78, 70.06, 70.84, 71.98, 72.44, 75.66, 89.43, 101.78, 168.67, 169.28, 170.48, 170.54, 170.81, 171.24, 171.61; ^{19}F NMR ($CDCl_3$, 470.6 MHz) δ −76.6; $[\alpha]_D$ +16.8° (c = 0.7, $CHCl_3$); MS (FAB) 731 (MH^+); HRMS m/z calcd for $C_{28}H_{38}F_3N_2O_{17}$ 731.212258, found 731.211900.

2-Acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-*β*-D-glucopyranosyl-(1→4)-2-trifluoromethyl-(2-acetamido-3,6-di-*O*-acetyl-1,2-dideoxy-α-D-glucopyrano)-[2,1,*d*]-2-oxazoline 15. To a solution of compound **14** (352.0 mg, 0.4818 mmol) in CH_2Cl_2 (10 mL) was added a solution of hydrogen bromide in acetic acid (30%, 2.5 mL). The mixture was stirred at room temperature for 3 h. The hydrogen bromide was then blown off under N_2, and the remaining acetic acid was coevaporated twice with toluene. The crude bromide was stirred with 2,6-lutidine (112 μL, 0.9636 mmol) in CH_3CN (10 mL) at room temperature for 13 h. Concentration of the solvent followed by column chromatography [$CHCl_3$, MeOH (96:4 v/v)] gave oxazoline **15** (197.7 mg, 61%) as a white solid, mp 196−198 °C; IR (neat) cm^{-1} 3276, 1741, 1664, 1555, 1370, 1229, 1166, 1044, 922; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 1.93 (s, 3H, Ac), 2.01 (s, 3H, Ac), 2.02 (s, 3H, Ac), 2.08 (s, 3H, Ac), 2.11 (s, 3H, Ac), 2.12 (s, 3H, Ac), 3.44 (ddd, 1H, J = 2.6, 4.7, 9.0 Hz, H-5), 3.64 (brd, 1H, J = 9.2 Hz, H-4), 3.70−3.75 (m, 2H, H-2′, H-5′), 4.14 (dd, 1H, J = 2.5, 12.3 Hz, H-6a′), 4.19−4.26 (m, 3H, H-6a,b, H-6b′), 4.33 (brd, 1H, J = 7.2 Hz, H-2), 4.89 (dd, 1H, J = 8.4 Hz, H-1′), 5.07 (t, 1H, J = 9.5 Hz, H-3′), 5.32 (dd, 1H, J = 9.4, 10.4 Hz, H-4′), 5.71 (t, 1H, J = 1.1 Hz, H-3), 5.77 (d, 1H, J = 8.3 Hz, N*H*), 6.22 (d, 1H, J = 7.4 Hz, H-1); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 20.62, 20.67, 20.71, 20.76, 20.89, 23.14, 55.01, 62.81, 62.84, 64.46, 68.36, 68.87, 69.59, 71.93, 72.23, 76.34, 101.60, 101.91, 169.03, 169.40, 170.54, 170.66, 170.80, 170.91; $[\alpha]_D$ +0.7° (c = 1.2, $CHCl_3$); MS (FAB) 693 (MNa^+); HRMS m/z calcd for $C_{26}H_{33}F_3N_2O_{15}Na$ 693.173073, found 693.475600.

Dibenzyl 2-Acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-*β*-D-glucopyranosyl-(1→4)-3,6-di-*O*-acetyl-2-deoxy-2-trifluoroacetamido-α-D-glucopyranosyl Phosphate 17. A mixture of oxazoline **15** (178.3 mg, 0.2659 mmol) and dibenzyl phosphate **16** (111 mg, 0.3989 mmol) was heated in 1,2-dichloroethane at 80−90 °C for 5 h. The mixture was cooled and diluted with $CHCl_3$ (10 mL). The solution was washed successively with saturated aqueous $NaHCO_3$ solution (4 mL) and brine (4 mL). The combined organic extracts were dried (Na_2SO_4), filtered, and concentrated. Flash column chromatography [$CHCl_3$, MeOH (96:4 v/v)] of the crude product gave α-dibenzyl phosphate **17** (161.6 mg, 64%) as a white solid, mp 164 °C (decompose): IR (neat) cm^{-1} 3285, 1746, 1673, 1556, 1370, 1229, 1157, 1044, 958, 745, 700; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 1.92 (s, 3H, Ac), 1.99 (s, 3H, Ac), 2.01 (s, 3H, Ac), 2.02 (s, 3H, Ac), 2.06 (s, 3H, Ac), 2.09 (s, 3H, Ac), 3.61 (ddd, 1H, J = 2, 4, 10 Hz), 3.73 (t, 1H, J = 9.5 Hz), 3.78 (dt, 1H, J = 8.5, 10.5 Hz), 3.91 (dt, 1H, J = 2.5, 10.5 Hz), 3.99 (dd, 1H, J = 2.0, 12.5 Hz), 4.05 (dd, 1H, J = 2.0, 12.5 Hz), 4.27 (dd, 1H, J = 3.5, 12.5 Hz), 4.37 (dd, 1H, J = 4, 12 Hz), 4.56 (d, 1H, J = 8.0 Hz), 4.99−5.07 (m, 5H), 5.18 (dd, 1H, J = 9.5, 10.5 Hz), 5.21 (dd, 1H, J = 9.5, 10.5 Hz), 5.65 (dd, 1H, J = 4.0, 6.0 Hz), 5.80 (d, 1H, J = 9.0 Hz), 6.76 (d, 1H, J = 9.0 Hz), 7.29−7.37 (m, 10H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 20.02, 20.32, 20.56, 20.59, 20.63, 20.88, 23.16, 52.73, 52.76 (d, J = 7.5 Hz), 55.02, 61.10, 61.70, 68.04, 69.74, 70.14 (d, J = 5.5 Hz), 70.25 (d, J = 5.4 Hz), 70.74, 71.98, 72.28, 74.79, 75.18, 94.82 (d, J = 5.6 Hz), 101.11, 128.12, 128.15, 128.77, 128.81, 129.03, 134.96, 135.01, 169.33, 170.32, 170.47, 170.67, 170.97 (2×); $[\alpha]_D$ +32.1° (c = 1.1, $CHCl_3$); MS (FAB) 971 (MNa^+); HRMS m/z calcd for $C_{40}H_{48}F_3N_2O_{19}PNa$ 971.243871, found 971.246000.

Mono(triethylammonium) Salt of 2-Acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-*β*-D-glucopyranosyl-(1→4)-3,6-di-*O*-acetyl-2-deoxy-2-trifluoroacetamido-α-D-glucopyranosyl Phosphate 18. Hydrogenolysis of α-dibenzyl phosphate **17** (51.8 mg, 0.05460 mmol) gave α-phosphate **18** (51.7 mg, 100%) as the mono(triethylammonium) salt; IR (neat) cm^{-1} 3303, 1745, 1705, 1662, 1374, 1225, 1166, 1048, 967; ^{1}H NMR [$CDCl_3$, CD_3OD (2:1v/v), 500 MHz] δ 1.10 (t, 9H, J = 7.3 Hz), 1.67 (s, 3H, Ac), 1.80 (s, 3H, Ac), 1.81 (s, 3H, Ac), 1.81 (s, 3H, Ac), 1.88 (s, 3H, Ac), 1.92 (s, 3H, Ac), 2.87 (q, 6H, J = 7.3 Hz), 3.45 (dd, 1H, J = 8.5, 10.3 Hz), 3.52 (ddd, 1H, J = 2.2, 3.7, 10.0 Hz), 3.66 (t, 1H, J = 9.6 Hz), 3.81 (dd, 1H, J = 2.0, 12.4 Hz), 3.87 (dd, 1H, J = 3.5, 12.2 Hz), 3.97 (brd, 1H, J = 9.8 Hz), 4.06 (brd, 1H, J = 12.1 Hz), 4.22 (hidden under DOH signal), 4.28 (brd, 1H, J = 12.1 Hz), 4.54 (d, 1H, J = 8.3 Hz, H-1′), 4.80 (t, 1H, J = 9.5 Hz), 5.13 (dd, 1H, J = 9.4, 10.4 Hz), 5.15 (dd, 1H, J = 9.2, 10.5 Hz), 5.28 (dd, 1H, J = 3.1, 7.3 Hz, H-1); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 7.99, 19.92, 19.94, 19.99, 20.05, 22.23, 22.03, 45.41, 52.64 (d, J = 6.7 Hz), 54.74, 61.51, 68.16, 69.00, 70.87, 71.10, 71.86, 75.17, 92.55 (d, J = 5.3 Hz), 100.09, 169.62, 170.35, 170.43, 170.70, 171.13, 171.44; $[\alpha]_D$ +23.0° (c = 0.7, MeOH); MS (FAB) 767 (M^-); HRMS m/z calcd for $C_{26}H_{35}F_3N_2O_{19}P$ 767.152376, found 767.149500.

P^1-[2-Acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-*β*-D-glucopyranosyl-(1→4)-3,6-di-*O*-acetyl-2-deoxy-2-trifluoroacetamido-α-D-glucopyranosyl] P^2-Dolichyl Pyrophosphate 19. ^{19}F NMR [$CDCl_3$, CD_3OD (5:1 v/v) 282 MHz] δ −76.7; ^{31}P NMR [$CDCl_3$, CD_3OD (5:1 v/v), 121 MHz] δ −10.5, −12.7; MS (−ve ESI) 1003.3, 1037.4, 1071.4, 1105.4 (m/2 for n = 13−16).

P^1-[2-Acetamido-2-deoxy-*β*-D-glucopyranosyl-(1→4)-2-deoxy-2-trifluoroacetamido-α-D-glucopyranosyl] P^2-Dolichyl Pyrophosphate 1c. To a stirred solution of peracetylated Dol-PP-GlcNTFA-GlcNAc **19** (6.2 mg, 2.85 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 [$CHCl_3$, MeOH, H_2O (72:21:2 v/v)] gave Dol-PP-GlcNTFA-GlcNAc **1c** (2.3 mg, 39%); MS (−ve ESI) 1798.7, 1865.2, 1933.7, 2001.8 (M^- for n = 13−16). [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 (9:1 v/v, 10 mL).]

4,6-Di-*O*-benzylidene-2-deoxy-2-fluoro-α,*β*-D-glucopyranose 21. A solution of 2-deoxy-2-fluoro-1,3,4,6-β-D-glucose tetraacetate **20**[29] (2.50 g, 7.14 mmol) and sodium methoxide (193 mg, 3.57 mmol) was stirred in MeOH (50 mL) for 3 h followed by neutralization with Dowex 50 W-X8 (H^+) resin (1.7 g). The resin was filtered, and the solvent was removed in vacuo to give 2-deoxy-2-fluoroglucose (1.4497 g). A solution of the free sugar, benzaldehyde dimethyl acetal (1.18 mL, 7.85 mmol), and p-toluenesulfonic acid (136 mg) in DMF (1.5 mL) was heated at 50 °C in a water bath at 20 mmHg. After 7.5 h, $NaHCO_3$ (180 mg, 2.142 mmol) was added followed by the removal of solvent. To the crude product was added EtOAc/MeOH (50 mL) and $MgSO_4$. After 30 min, the suspension was filtered through a pad of silica gel topped with Celite. Concentration of the solvent followed by column chromatography [n-hex, EtOAc (1:1 v/v)] gave the desired compound **21** (1.4692 g, 76%) as a white solid: selected peaks for ^{1}H NMR ($CDCl_3$, 500 MHz) δ 4.95 (ddd, J = 3.7, 5.8, 9.8 Hz, H-1 for β-**21**), 5.47 (t, J = 3.7 Hz, H-1 for α-**21**); ^{19}F NMR ($CDCl_3$, 282 MHz) δ −200.91 (dd, J = 16, 52 Hz), −201.38 (dd, J = 14, 49 Hz); MS (FAB) 271 (MH^+); HRMS m/z calcd for $C_{24}H_{35}FNO_{15}$ 596.199073, found 596.200600.

1,3-Di-*O*-benzyl-4,6-di-*O*-benzylidene-2-deoxy-2-fluoro-α-D-glucopyranose 22a, and 1,3-Di-*O*-benzyl-4,6-di-*O*-benzylidene-2-deoxy-2-fluoro-*β*-D-glucopyranose 22b. To a stirred solution of reducing sugar **21** (200.0 mg, 0.7401 mmol) in DMF (3 mL) at 0 °C was added NaH (60%, 74.0 mg, 1.8501 mmol) in one portion. After 10 min, benzyl bromide (264 μL, 2.2202 mmol) was added, and the mixture was allowed to stir at 0 °C for 1 h. The mixture was diluted with EtOAc (50 mL) and water (25 mL). The aqueous phase was separated and extracted with EtOAc (2 × 25 mL). The combined organic extracts were washed with brine (25 mL), dried ($MgSO_4$),

filtered, and concentrated. Flash column chromatography [*n*-hex, EtOAc (9:1 v/v) followed by (7:1 v/v)] of the crude solid gave first β-glucoside **22b** (181.0 mg, 54%) followed by α-glucoside **22a** (101.8 mg, 31%) both as white solids. **Data for the α-glucoside 22a:** mp 119−121 °C; IR (neat) cm^{-1} 2915, 2865, 1453, 1370, 1352, 1178, 1162, 1135, 1085, 1067, 1054, 1027, 1017, 1000, 750, 732, 696, 660; ^1H NMR ($CDCl_3$, 500 MHz) δ 3.62 (dt, 1H, *J* = 0.9, 9.5 Hz, H-4), 3.73 (t, 1H, *J* = 10.4, H-6a), 3.93 (dt, 1H, *J* = 4.9, 10.1 Hz, H-5), 4.17 (dt, 1H, *J* = 9.5, 11.6 Hz, H-3), 4.24 (dd, 1H, *J* = 4.9, 10.4 Hz, H-6b), 4.53 (ddd, 1H, *J* = 4.0, 8.8, 49 Hz, H-2), 4.64 and 4.78 (ABq, 2H, *J* = 12.2 Hz, OC*H*$_2$Ph), 4.82 and 4.87 (ABq, 2H, *J* = 11.6 Hz, OC*H*$_2$-Ph), 5.56 (s, 1H, C*H*Ph), 7.26−7.50 (m, 15H, Ph); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 62.56, 68.79, 69.87, 74.73, 76.73 (d, *J* = 18 Hz), 81.09 (d, *J* = 8 Hz), 90.29 (d, *J* = 195 Hz), 96.19 (d, *J* = 20 Hz), 101.33, 126.00, 127.81, 127.90, 128.02, 128.22, 128.29, 128.49, 128.98, 136.65, 137.09, 138.23; ^{19}F NMR ($CDCl_3$, 282 MHz) δ −200.88 (dd, *J* = 13, 49 Hz); $[\alpha]_D$ +70.0° (*c* = 1.4, $CHCl_3$); MS (FAB) 451 (MH^+); HRMS *m*/*z* calcd for $C_{27}H_{28}FNO_5$ 451.192077, found 451.191800. **Data for the β-glucoside 22b:** mp 118−119 °C; IR (neat) cm^{-1} 2872, 1451, 1369, 1174, 1092, 1067, 1010, 744, 697; ^1H NMR ($CDCl_3$, 500 MHz) δ 3.44 (dt, 1H, *J* = 4.9, 10 Hz, H-5), 3.72 (t, 1H, *J* = 9.5 Hz, H-4), 3.82 (t, 1H, *J* = 10.5 Hz, H-6a), 3.84 (dt, 1H, *J* = 8.8, 15.3 Hz, H-3), 4.40 (dd, 1H, *J* = 4.9, 10.5 Hz, H-6b), 4.45 (dt, 1H, *J* = 7.9, 50 Hz, H-2), 4.67 (dd, 1H, *J* = 4.0, 7.6 Hz, H-1), 4.71 and 4.95 (ABq, 2H, *J* = 12 Hz, OC*H*$_2$Ph), 4.84 and 4.88 (ABq, 2H, *J* = 11.9 Hz, OC*H*$_2$Ph), 5.58 (s, 1H, C*H*Ph), 7.26−7.51 (m, 15H, Ph); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 66.17, 68.54, 71.16, 74.33, 78.84 (d, *J* = 18 Hz), 80.37 (d, *J* = 9 Hz), 92.84 (d, *J* = 188 Hz), 99.89 (d, *J* = 24 Hz), 101.30, 126.00, 127.75, 127.82, 127.92, 128.03, 128.25, 128.31, 128.49, 129.06, 136.54, 137.01, 137.89; ^{19}F NMR ($CDCl_3$, 282 MHz) δ −198.34 (dd, *J* = 14, 47 Hz); $[\alpha]_D$ −62.4° (*c* = 1, $CHCl_3$); MS (FAB) 451 (MH^+); HRMS *m*/*z* calcd for $C_{27}H_{28}FNO_5$ 451.192077, found 451.191400.

1,3,6-Tri-*O*-benzyl-2-deoxy-2-fluoro-β-D-glucopyranose 9. To a mixture of benzylidene **22b** (332.0 mg, 0.737 mmol) and sodium cyanoborohydride (695 mg, 11.05 mmol) in THF (15 mL) at 0 °C was added HCl/Et_2O solution until no bubble was observed. After 2 h, brine (25 mL) and Et_2O (100 mL) were added. The aqueous phase was separated and extracted with Et_2O (2 × 50 mL). The combined organic extracts were dried, filtered, and concentrated. Flash column chromatography [*n*-hex, EtOAc (3:1 v/v)] of the crude mixture gave glycosyl acceptor **9** (293.8 mg, 88%) as an oil; IR (neat) cm^{-1} 3467, 3030, 2871, 1497, 1454, 1365, 1209, 1124, 1074, 1005, 737, 698; ^1H NMR ($CDCl_3$, 500 MHz) δ 2.55 (d, 1H, *J* = 2.2 Hz, O*H*), 3.46 (ddd, 1H, *J* = 4.0, 5.2, 9.4 Hz, H-5), 3.56 (dt, 1H, *J* = 8.5, 14.6 Hz, H-3), 3.66 (dt, 1H, *J* = 2.2, 9.4 Hz, H-4), 3.72 (dd, 1H, *J* = 5.2, 10.5 Hz, H-6a), 3.78 (dd, 1H, *J* = 4.0, 10.5 Hz, H-6b), 4.40 (ddd, 1H, *J* = 7.6, 8.5, 51 Hz, H-2), 4.56 (dd, 1H, *J* = 3.1, 7.6 Hz, H-1), 4.62 and 4.57 (ABq, 2H, *J* = 12.2 Hz, OC*H*$_2$Ph), 4.69 (d, 2H, *J* = 11.6 Hz, OC*H*$_2$Ph), 4.92 (dd, 1H, *J* = 0.9, 11.5 Hz, OC*H*$_2$Ph), 4.93 (d, 1H, OC*H*$_2$Ph), 7.28−7.39 (m, 15H, OCH$_2$*Ph*); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 69.72, 70.68, 70.74, 73.64, 74.16, 74.46 (d, *J* = 2 Hz), 82.45 (d, *J* = 16 Hz), 92.86 (d, *J* = 187 Hz), 99.10 (d, *J* = 23 Hz), 127.67, 127.77, 127.88, 127.89, 127.99, 128.06, 128.43, 128.53, 136.78, 137.76, 137.91; ^{19}F NMR ($CDCl_3$, 282 MHz) δ −197.52 (dd, *J* = 14, 49 Hz); $[\alpha]_D$ −69.4° (*c* = 1, $CHCl_3$); MS (FAB) 453 (MH^+); HRMS *m*/*z* calcd for $C_{27}H_{30}FNO_5$ 453.207728, found 453.207200.

2-Azido-3,4,6-tri-*O*-benzyl-2-deoxy-β-D-glucopyranosyl-(1→4)-1,3,6-tri-*O*-benzyl-2-deoxy-2-fluoro-β-D-glucopyranose 23. To a stirred solution of compound **9** (135.4 mg, 0.2992 mmol), azido imidate **8** (260.0 mg, 0.4194 mmol), and 4 Å molecular sieves (500 mg) in CH_2Cl_2 (5 mL) at −78 °C was added $BF_3{\cdot}Et_2O$ (38 μL, 0.2992 mmol). The mixture was allowed to warm to 5 °C over 5 h, followed by the addition of Et_3N (0.5 mL). The mixture was filtered through a pad of Celite and washed with $CHCl_3$. Concentration of the solvent followed by column chromatography [*n*-hex, EtOAc (6:1 v/v) followed by (4.5:1 v/v)] gave the desired disaccharide **23** (227.2 mg, 83%) as an oil: IR (neat) cm^{-1} 2913, 2862, 2108, 1496, 1453, 1362, 1056, 1026, 733, 692; ^1H NMR ($CDCl_3$, 500 MHz) δ 3.18 (ddd, 1H, *J* = 1.8, 3.8, 9.8 Hz, H-5′), 3.28 (dd, 1H, *J* = 8.2, 9.8 Hz, H-4′), 3.32 (dd, 1H, *J* = 7.8, 9.8 Hz, H-2′), 3.48 (ddd, 1H, *J* = 1.8, 3.8, 9.9 Hz, H-5), 3.55 (dd, 1H, *J* = 4.0, 11 Hz, H-6a′), 3.61 (dd, 1H, *J* = 2.0, 11 Hz, H-6b′), 3.65 (dd, 1H, *J* = 8.5, 9.6 Hz, H-3′), 3.70 (dt, 1H, *J* = 8.7, 15.2 Hz, H-3), 3.81 (dd, 1H, *J* = 1.8, 11.1 Hz, H-6a), 3.88 (dd, 1H, *J* = 3.8, 11.1 Hz, H-6b), 4.02 (t, 1H, *J* = 9.4 Hz, H-4), 4.35 (d, 1H, *J* = 7.6 Hz, H-1′), 4.38 (dt, 1H, *J* = 8.4, 50.5 Hz, H-2), 4.39 and 4.43 (ABq, 2H, *J* = 12.2 Hz, OC*H*$_2$Ph), 4.53 (dd, 1H, *J* = 2.7, 7.8 Hz, H-1), 4.53 (d, 1H, *J* = 12.5 Hz), 4.55 (d, 1H, *J* = 11.0 Hz), 4.69 (d, 2H, *J* = 12.1 Hz), 4.76 and 4.90 (ABq, 2H, *J* = 11.9 Hz), 4.77 (d, 1H, *J* = 11.1 Hz), 4.79 and 4.83 (ABq, 2H, *J* = 10.8 Hz, OC*H*$_2$Ph), 4.94 (d, 1H, *J* = 12.2 Hz), 7.16−7.39 (m, 30H, OCH$_2$*Ph*); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 66.88, 68.01, 68.33, 70.65, 73.31, 73.44, 74.23, 74.74, 74.81, 75.03, 75.50, 76.03 (d, *J* = 8.1 Hz), 77.67, 81.07 (d, *J* = 17.3 Hz), 83.13, 92.37 (d, *J* = 183 Hz), 99.17 (d, *J* = 23.6 Hz), 100.84, 127.33, 127.47, 127.55, 127.62, 127.70, 127.77, 127.85, 127.90, 127.99, 128.09, 128.27, 128.40, 128.41, 128.46, 136.88, 137.80, 137.88, 127.92, 138.11, 138.55; $[\alpha]_D$ −27.7° (*c* = 1.1, $CHCl_3$); MS (FAB) 933 (MNa^+); HRMS *m*/*z* calcd for $C_{54}H_{56}FN_3O_9Na$ 932.389827, found 932.392900.

2-Acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl-(1→4)-1,3,6-tri-*O*-acetyl-2-deoxy-2-fluoro-α,β-D-glucopyranose 24. A solution of disaccharide **23** (190.0 mg, 0.2088 mmol) and a catalytic amount of Raney nickel was stirred under H_2 in acetic anhydride (3 mL) and EtOH (10 mL) for 6 h. The catalyst was filtered through a pad of Celite, and the solvent was removed under reduced pressure. The crude product was passed through a short plug of silica and washed with $CHCl_3$, MeOH (5:1 v/v) to give the acetamido compound. This compound was hydrogenated with 10% Pd/C (110 mg) in EtOH (25 mL) for 12 h. The catalyst was again filtered through Celite and washed with MeOH (20 mL). Concentration of the filtrate gave the free disaccharide (108.3 mg), which was acetylated with Ac_2O (2 mL) and DMAP (cat) in pyridine (8 mL) for 3 h. Concentration of the solvent followed by column chromatography [$CHCl_3$, MeOH (96:4 v/v)] gave the heptaacetate **24** (128.1 mg, 96%, α:β ≈ 2:1) as a white solid: IR (neat) cm^{-1} 1747, 1662, 1531, 1372, 1227, 1045; selected peaks for ^1H NMR ($CDCl_3$, 500 MHz) of α-anomer δ 6.35 (d, *J* = 4.0 Hz, H-1) and 4.64 (d, *J* = 8.2 Hz, H-1′); β-anomer δ 5.71 (dd, *J* = 3.1, 8.2 Hz, H-1) and 4.77 (d, *J* = 8.2 Hz, H-1′); MS (FAB) 660 (MNa^+); HRMS *m*/*z* calcd for $C_{26}H_{36}FNO_{16}Na$ 660.191581, found 660.190500.

2-Acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl-(1→4)-3,6-di-*O*-acetyl-2-deoxy-2-fluoro-α-D-glucopyranosyl Bromide 26. To a stirred solution of anomeric acetates **24** (120.0 mg, 0.1882 mmol) in CH_2Cl_2 at 0 °C was added a solution of hydrogen bromide in acetic acid (30%, 3 mL). The mixture was allowed to warm to room temperature and stirred for an extra 3.5 h. The solvent was then concentrated and coevaporated twice with toluene (4 mL). Flash column chromatography [$CHCl_3$, CH_3CN (2:1 v/v)] of the crude oil gave α-bromide **26** (105.0 mg, 85%) as a white solid, mp 210 ° C (decompose); IR (neat) cm^{-1} 1746, 1666, 1531, 1436, 1372, 1227, 1113, 1047, 903; ^1H NMR ($CDCl_3$, 500 MHz) δ 1.94 (s, 3H, Ac), 2.01 (s, 3H, Ac), 2.02 (s, 3H, Ac), 2.10 (s, 3H, Ac), 2.12 (s, 6H, 2 × Ac), 3.71 (ddd, 1H, *J* = 2.1, 5.0, 10.1 Hz, H-5′), 3.73 (dt, 1H, *J* = 8.4, 10.5 Hz, H-2′), 3.82 (t, 1H, *J* = 9.9 Hz, H-4), 4.07 (dd, 1H, *J* = 2.4, 12.4 Hz, H-6a′), 4.29 (dd, 1H, *J* = 4.3, 12.5 Hz, H-6a), 4.31 (dd, 1H, *J* = 5.0, 12.5 Hz, H-6b′), 4.36 (ddd, 1H, *J* = 2.0, 4.1, 10.4 Hz, H-5), 4.41 (dd, 1H, *J* = 2.0, 12.2 Hz, H-6b), 4.46 (ddd, 1H, *J* = 4.3, 9.2, 50 Hz, H-2), 4.88 (d, 1H, *J* = 8.4 Hz, H-1′), 5.02 (dd, 1H, *J* = 9.3, 10.1 Hz, H-3′), 5.33 (dd, 1H, *J* = 9.2, 10.5 Hz, H-4′), 5.63 (dt, 1H, *J* = 9.3, 11.6 Hz, H-3), 5.69 (d, 1H, *J* = 8.7 Hz, N*H*), 6.48 (d, 1H, *J* = 4.9 Hz, H-1); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 20.52, 20.54, 20.67, 20.74, 20.76, 23.15, 55.28, 61.35, 61.98, 68.38, 70.17 (d, *J* = 19 Hz), 71.97, 72.06, 73.12, 74.21 (d, *J* = 6.4 Hz), 85.52 (d, *J* = 25 Hz), 86.62 (d, *J* = 198 Hz), 99.23, 169.33, 169.88, 170.34, 170.42, 170.47; $[\alpha]_D$ +87.4° (*c* = 0.6, $CHCl_3$); MS (FAB) 680, 682 (MNa^+); HRMS *m*/*z* calcd for $C_{24}H_{33}BrFNO_{14}Na$ 680.096612, found 680.094500.

Dibenzyl 2-Acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl-(1→4)-3,6-di-*O*-acetyl-2-deoxy-2-fluoro-α-D-glucopyranosyl Phosphate 27a and Dibenzyl 2-Acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl-(1→4)-3,6-di-*O*-acetyl-2-deoxy-2-fluoro-β-D-glucopyranosyl Phosphate 27b. To a stirred solution of α-bromide **26** (28.5 mg, 0.04329 mmol) and dibenzyl phosphate **16** (24.1 mg, 0.08657 mmol) in THF (1.5 mL) at room temperature was added a solution of AgOTf (12.2 mg) in THF (94 μL). After 30 min, Et_3N (4 drops) was added, and the silver salt was filtered through a plug of glass wool. Concentration of the solvent followed by flash chromatography [$CHCl_3$, CH_3CN (3:2 v/v) followed by (1:1 v/v)] gave first α-dibenzyl phosphate **27a** (13.9 mg, 38%) followed by β-dibenzyl phosphate **27b** (8.5 mg, 23%), both as oil. **Data for α-dibenzyl phosphate 27a:** IR (neat) cm^{-1} 1747, 1667, 1369, 1227, 1154, 1044, 959, 907, 743, 697; ^1H NMR ($CDCl_3$, 500 MHz) δ 1.92 (s, 3H, Ac), 2.00 (s, 3H, Ac), 2.01 (s, 3H, Ac), 2.04(s, 3H, Ac), 2.07(s, 3H, Ac), 2.10(s, 3H, Ac), 3.63 (ddd, 1H, *J* = 2.1, 4.0, 10 Hz, H-5′), 3.65 (dt, 1H, *J* = 8.2, 10.4 Hz, H-2′), 3.70 (dd, 1H, *J* = 9.5, 9.8 Hz, H-4), 4.00 (dd, 1H, *J* = 2.1, 12.5 Hz, H-6a′), 4.03 (ddd, 1H, *J* = 1.8, 4.0, 9.8 Hz, H-5), 4.11 (dd, 1H, *J* = 1.8, 12.2 Hz, H-6a), 4.18 (dd, 1H, *J* = 4.0, 12.2 Hz, H-6b), 4.38 (dd, 1H, *J* = 4.0, 12.5 Hz, H-6b′), 4.49 (ddt, 1H, *J* = 3.7, 9.8, 49 Hz, H-2), 4.70 (d, 1H, *J* = 8.2 Hz, H-1′), 5.03 (dd, 1H, *J* = 9.3, 10 Hz, H-4′), 5.07−5.09 (m, 4H), 5.31 (dd, 1H, *J* = 9.3, 10.4 Hz, H-3′), 5.52 (dt, 1H, *J* = 9.5, 11.4 Hz, H-3), 5.78 (d, 1H, *J* = 8.2 Hz, N*H*), 5.93 (dd, 1H, *J* = 3.6, 6.7 Hz, H-1), 7.33−7.38 (m, 10H, Ph); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 20.59, 20.62, 20.66, 20.75, 20.83, 23.18, 55.30, 61.25, 61.66, 68.10, 69.50, 69.61 (d, *J* = 5.8 Hz), 69.86 (d, *J* = 4.6 Hz), 70.24, 71.79, 72.01, 74.93 (d, *J* = 6.9 Hz), 86.89 (dd, *J* = 8, 191 Hz), 93.50 (dd, *J* = 4, 22 Hz), 100.17, 127.90, 128.15, 128.64, 128.71, 128.73, 135.20 (d, *J* = 8 Hz), 135.32 (d, *J* = 8 Hz), 169.39, 169.75, 170.41, 170.52, 170.57, 170.81; ^{19}F NMR ($CDCl_3$, 282 MHz) δ −199.89 (dd, *J* = 12.1, 49 Hz); ^{31}P NMR ($CDCl_3$, 121 MHz) δ −2.08; $[\alpha]_D$ +5.8° (*c* = 1.0, $CHCl_3$); MS (FAB) 878 (MNa^+); HRMS *m/z* calcd for $C_{38}H_{47}FNO_{18}PNa$ 878.241249, found 878.243300. **Data for β-dibenzyl phosphate 27b:** IR (neat) cm^{-1} 1748, 1672, 1556, 1456, 1370, 1229, 1159, 1037, 892, 736, 692, 640; ^1H NMR ($CDCl_3$, 500 MHz) δ 1.93 (s, 3H, Ac), 2.01 (s, 3H, Ac), 2.02 (s, 3H, Ac), 2.03 (s, 3H, Ac), 2.09 (s, 3H, Ac), 2.12 (s, 3H, Ac), 3.63 (dt, 1H, *J* = 8.4, 10.5 Hz, H-2′), 3.68 (ddd, 1H, *J* = 2.3, 4.6, 10.1 Hz, H-5′), 3.76 (dd, 1H, *J* = 8.2, 10.1 Hz, H-4), 3.79 (ddd, 1H, *J* = 1.5, 4.5, 10.1 Hz, H-5), 4.03 (dd, 1H, *J* = 2.3, 12.6 Hz, H-6a′), 4.16 (dd, 1H, *J* = 4.5, 12.2 Hz, H-6a), 4.36 (ddd, 1H, *J* = 7.8, 9.2, 50.5 Hz, H-2), 4.36 (dd, 1H, *J* = 4.6, 12.5 Hz, H-6b′), 4.41 (dd, 1H, *J* = 1.5, 12.2 Hz, H-6b), 4.82 (d, 1H, *J* = 8.2, H-1′), 5.02 (dd, 1H, *J* = 9.3, 10.1 Hz, H-4′), 5.05−5.12 (m, 4H, OCH_2Ph), 5.33 (dt, 1H, *J* = 9.0, 14.2 Hz, H-3), 5.36 (dd, 1H, *J* = 9.3, 10.4 Hz, H-3′), 5.36 (dt, 1H, *J* = 2.7, 7.8 Hz, H-1), 5.70 (d, 1H, *J* = 8.7 Hz, N*H*), 7.31−7.37 (m, 10H, OCH_2Ph); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 20.58, 20.60, 20.65, 20.69, 20.77, 23.17, 55.40, 61.64, 61.80, 68.23, 69.53 (d, *J* = 5.8 Hz), 69.67 (d, *J* = 5.8 Hz), 71.61 (d, *J* = 20 Hz), 71.79, 71.83, 73.72, 75.12 (d, *J* = 6.9 Hz), 89.47 (dd, *J* = 9.2, 193 Hz), 95.75 (dd, *J* = 4.6, 24.2 Hz), 99.66, 127.87, 128.54, 128.56, 128.60, 128.65, 135.18 (d, *J* = 8 Hz), 135.31 (d, *J* = 8 Hz), 169.40, 169.73, 170.45, 170.50 (2 ×), 170.60; ^{19}F NMR ($CDCl_3$, 282 MHz) δ −199.16 (dd, *J* = 13.6, 50.3 Hz); ^{31}P NMR ($CDCl_3$, 121 MHz) δ −2.36; $[\alpha]_D$ +46.9° (*c* = 0.4, $CHCl_3$); MS (FAB) 878 (MNa^+); HRMS *m/z* calcd for $C_{38}H_{47}FNO_{18}PNa$ 878.241249, found 878.244200.

Mono(triethylammonium) Salt of 2-Acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl-(1→4)-3,6-di-*O*-acetyl-2-deoxy-2-fluoro-α-D-glucopyranosyl Phosphate 28. Hydrogenolysis of α-dibenzyl phosphate **27a** (25.2 mg, 0.0295 mmol) gave α-phosphate **28** (24.0 mg, quantitative) as a colorless film; IR (neat) cm^{-1} 1745, 1644, 1436, 1370, 1230, 1159, 1043, 959, 908, 841; ^1H NMR [($CDCl_3$, CD_3OD (2:1 v/v), 500 MHz] δ 1.05 (t, 9 H, *J* = 7.3 Hz, CH_2CH_3), 1.68 (s, 3H, Ac), 1.78 (s, 3H, Ac), 1.79 (s, 3H, Ac), 1.87 (s, 3H, Ac), 1.88 (s, 3H, Ac), 1.89 (s, 3H, Ac), 2.79 (q, 6H, *J* = 7.3 Hz, CH_2CH_3), 3.36 (dd, 1H, *J* = 8.4, 10.5 Hz, H-2′), 3.52 (ddd, 1H, *J* = 2.4, 4.0, 10.2 Hz, H-5′), 3.56 (t, 1H, *J* = 9.7 Hz, H-4), 3.82 (dd, 1H, *J* = 2.4, 12.5 Hz, H-6a′), 3.84 (dd, 1H, *J* = 3.7, 12.5 Hz, H-6a), 3.94 (ddd, 1H, *J* = 2.0, 3.7, 10.1 Hz, H-5), 4.20 (dddd, 1H, *J* = 2.4, 3.7, 9.6, 50 Hz, H-2), 4.22 (dd, 1H, *J* = 4.0, 12.4 Hz, H-6b′), 4.26 (dd, 1H, *J* = 2.0, 12.5 Hz, H-6b), 4.55 (d, 1H, *J* = 8.4 Hz, H-1′), 4.78 (dd, 1H, *J* = 9.3, 10.1 Hz, H-4′), 5.16 (dd, 1H, *J* = 9.3, 10.5 Hz, H-3′), 5.25 (dt, 1H, *J* = 9.5, 11.9 Hz, H-3), 5.49 (dd, 1H, *J* = 3.7, 6.9 Hz, H-1); ^{13}C NMR [($CDCl_3$, CD_3OD (2:1 v/v), 125 MHz] δ 7.90, 19.83, 19.91, 19.99, 20.12, 20.16, 21.93, 45.21, 54.81, 61.32, 61.48, 68.10, 68.30, 70.42 (d, *J* = 18.5 Hz), 70.98, 71.63, 74.75 (d, *J* = 6.3 Hz), 87.29 (dd, *J* = 8, 195 Hz), 91.19 (dd, *J* = 6, 22 Hz), 99.84, 169.60, 170.08, 170.32, 170.60, 171.00, 171.49; ^{19}F NMR [($CDCl_3$, CD_3OD (2:1 v/v) 282 MHz] δ −200.11 (dd, *J* = 12.1, 49 Hz); ^{31}P NMR ($CDCl_3$, 121 MHz) δ −1.02; $[\alpha]_D$ +39.8° (*c* = 0.6, $CHCl_3$); MS (−ve FAB) 674 ($M - H)^-$; HRMS *m/z* calcd for $C_{24}H_{34}FNO_{18}P$ 674.149755, found 674.148300.

P¹-[2-Acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl-(1→4)-3,6-di-*O*-acetyl-2-deoxy-2-fluoro-α-D-glucopyranosyl] P²-Dolichyl Pyrophosphate 29. ^{19}F NMR [$CDCl_3$, CD_3OD (5:1 v/v), 282 MHz] δ −201.5 (brd, *J* = 50.8 Hz); ^{31}P NMR [$CDCl_3$, CD_3OD (5:1 v/v), 202.5 MHz] −10.8, −13.2; MS (−ve ESI) 922.7, 956.8, 990.8, 1024.9, 1058.9, 1092.9, 1127.0 (*m/2* for *n* = 12−18).

P¹-[2-Acetamido-2-deoxy-β-D-glucopyranosyl-(1→4)-2-deoxy-2-fluoro-α-D-glucopyranosyl] P²-Dolichyl Pyrophosphate 1d. To a stirred solution of peracetylated Dol-PP-2DFGlc-GlcNAc **29** (4.4 mg, 2.11 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 solvent followed by column chromatography [$CHCl_3$, MeOH, HO (72:21:2 v/v) followed by (60:25:4 v/v)] gave Dol-PP-2DFGlc-GlcNAc **1d** (3.1 mg, 73%): ^{31}P NMR [$CDCl_3$, CD_3OD (2:1 v/v), 202.3 MHz] δ −11.1, −13.0; MS (−ve ESI) 852.5, 885.8, 919.8, 953.8, 987.9 (*m/2* for *n* = 13−17).

2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl-(1→4)-2-acetamido-3-*O*-acetyl-1,6-di-*O*-benzyl-2-deoxy-α-D-glucopyranose 30. To a solution of glycosyl acceptor **11**[21] (383.6 mg, 0.8649 mmol) and bromoacetyl glucose **10** (533.5 mg, 1.2974 mmol) in CH_2Cl_2/toluene (8 mL, 1:1 v/v) at −45 °C was added AgOTf (366.7 mg, 1.4271 mmol) in one portion. The mixture was allowed to warm to 0 °C over 4 h. Et_3N (1 mL) and CH_2Cl_2 (40 mL) were then added. The organic phase was washed with water (15 mL), a saturated aqueous solution of KI (15 mL), and brine (15 mL). The organic extracts were dried (Na_2SO_4) and filtered through a pad of silica gel topped with Celite. Concentration of the filtrate followed by flash chromatography [$CHCl_3$, EtOAc (3:1 v/v) followed by (2:1 v/v)] gave disaccharide **30** (563.1 mg, 84%) as a white solid: mp 150−152 °C (EtOH); IR (neat) cm^{-1} 1756, 1676, 1540, 1456, 1348, 1230, 1038, 903, 747, 699; ^1H NMR ($CDCl_3$, 500 MHz) δ 1.87 (s, 3H, Ac), 1.93 (s, 3H, Ac), 1.97 (s, 3H, Ac), 2.00 (s, 3H, Ac), 2.01 (s, 3H, Ac), 2.06 (s, 3H, Ac), 3.38 (ddd, 1H, *J* = 2, 4.3, 10 Hz, H-5′), 3.55 (dd, 1H, *J* = 1.3, 10.7 Hz, H-6a), 3.70 (brd, 1H, *J* = 10.5 Hz, H-5), 3.73 (dd, 1H, *J* = 2.7, 10.7 Hz, H-6b), 3.94 (dd, 1H, *J* = 9.5, 9.9 Hz, H-4), 3.97 (dd, 1H, *J* = 2, 12.4 Hz, H-6a′), 4.25 (ddd, 1H, *J* = 3.7, 9.7, 10.8 Hz, H-2), 4.32 (dd, 1H, *J* = 4.3, 12.4 Hz, H-6b′), 4.37 (d, 1H, *J* = 8.1 Hz, H-1′), 4.44 (d, 1H, *J* = 12 Hz, OCH*H*Ph), 4.50 (d, 1H, *J* = 12 Hz, OCH*H*Ph), 4.69 (d, 1H, *J* = 12 Hz, OCH*H*Ph), 4.79 (d, 1H, *J* = 12 Hz, OCH*H*Ph), 4.82 (dd, 1H, *J* = 8, 10 Hz, H-2′), 4.91 (d, 1H, *J* = 3.7 Hz, H-1), 4.95−4.99 (m, 2H, H-3′, H-4′), 5.13 (dd, 1H, *J* = 9.4, 10.7 Hz, H-3), 5.65 (d, 1H, *J* = 9.3 Hz, N*H*), 7.30−7.45 (m, 10H, OCH_2Ph); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 20.51, 20.53, 20.56, 20.59 (2 ×), 23.09, 51.97, 61.56, 67.05, 67.90, 70.10, 70.50, 71.12, 71.55, 71.60, 73.10, 73.67, 74.89, 96.75, 100.03, 128.07, 128.09, 128.16, 128.20, 128.55, 128.67, 136.80, 137.65, 168.79, 169.30, 169.90, 170.19, 170.48, 171.22; $[\alpha]_D$ +55.3° (*c* = 0.7, $CHCl_3$); MS (FAB) 796 (MNa^+); HRMS *m/z* calcd for $C_{33}H_{47}NO_{16}Na$ 796.279255, found 796.280900.

2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl-(1→4)-2-acetamido-1,3,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranose 31. Disaccharide **30** (171.1 mg, 0.2211 mmol) underwent hydrogenolysis and acetylation to give octaacetate **31** (119.2 mg,

91%) as a white solid, mp 221−223 °C (MeOH/pentane); IR (neat) cm^{-1} 1746, 1674, 1540, 1433, 1370, 1226, 1130, 1036, 943, 908, 755; ^1H NMR ($CDCl_3$, 500 MHz) δ 1.93 (s, 3H, Ac), 1.99 (s, 3H, Ac), 2.02 (s, 3H, Ac), 2.05 (s, 3H, Ac), 2.07 (s, 3H, Ac), 2.09 (s, 3H, Ac), 2.12 (s, 3H, Ac), 2.19 (s, 3H, Ac), 3.67 (ddd, 1H, J = 2.2, 4.1, 9.8 Hz, H-5′), 3.84 (dd, 1H, J = 8.4, 10 Hz, H-4), 3.88 (ddd, 1H, J = 1.7, 3.8, 10.5 Hz, H-5), 4.04 (dd, 1H, J = 2.2, 12.5 Hz, H-6a′), 4.11 (dd, 1H, J = 3.8, 12.2 Hz, H-6a), 4.35−4.40 (m, 2H, H-2, H-6b′), 4.42 (dd, 1H, J = 1.7, 12.2 Hz, H-6b), 4.53 (d, 1H, J = 7.9 Hz, H-1′), 4.94 (dd, 1H, J = 7.9, 9.1 Hz, H-2′), 5.08 (t, 1H, J = 9.5 Hz, H-4′), 5.15 (t, 1H, J = 9.3 Hz, H-3′), 5.20 (dd, 1H, J = 8.5, 11.5 Hz, H-3), 5.54 (d, 1H, J = 9.1 Hz, N*H*), 6.10 (d, 1H, J = 3.6 Hz, H-1); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 20.49 (2 ×), 20.55 (2 ×), 20.61, 20.76, 20.93, 23.00, 51.08, 61.44, 61.59, 67.76, 70.45, 70.59, 71.61, 71.99, 72.88, 75.86, 90.49, 100.94, 168.71, 169.19, 169.29, 170.04, 170.09, 170.23, 170.40, 171.39; $[\alpha]_D$ +47.7° (c = 1.3, $CHCl_3$); MS (FAB) 700 (MNa^+); HRMS m/z calcd for $C_{28}H_{39}NO_{18}Na$ 700.206484, found 700.206400.

2,3,4,6-Tetra-*O*-acetyl-*β*-D-glucopyranosyl-(1→4)-2-acetamido-3,6-di-*O*-acetyl-2-deoxy-α,*β*-D-glucopyranose 32. A solution of octaacetate **31** (61.9 mg, 0.09135 mmol) and hydrazine monoacetate (42 mg, 0.4568) was stirred in DMF (3 mL) for 4 days. The mixture was diluted with EtOAc (20 mL) and washed with brine (6 mL). The aqueous phase was separated and extracted with EtOAc (2 × 6 mL). The combined organic extracts were dried (Na_2SO_4) and filtered. Concentration of the filtrate followed by column chromatography [$CHCl_3$, MeOH (93:7 v/v)] gave compound **32** (48.9 mg, 84%) mainly as the α-anomer: MS (FAB) 636 (MH^+), 658 (MNa^+); HRMS m/z calcd for $C_{26}H_{38}NO_{17}$ 636.213974, found 636.211300.

Dibenzyl 2,3,4,6-Tetra-*O*-acetyl-*β*-D-glucopyranosyl-(1→4)-2-acetamido-3,6-di-*O*-acetyl-2-deoxy-α-D-glucopyranosyl Phosphate 34. To a solution of compound **32** (48.9 mg, 0.0769 mmol) in THF (2.5 mL) at −65 °C was added a solution of LiHMDS (1 M in hexanes, 100 μL, 0.1 mmol). The mixture was stirred at this temperature for 10 min followed by the slow addition of tetrabenzyl pyrophosphate **33** (53.9 mg, 0.1 mmol) in THF (0.8 mL). The mixture was allowed to warm to 0 °C and stirred for an extra 30 min. The mixture was diluted with Et_2O (25 mL) and washed with a saturated aqueous solution of $NaHCO_3$ (10 mL) and brine (10 mL). The organic phase was dried (Na_2SO_4), filtered, and concentrated. Flash column chromatography [$CHCl_3$, MeOH (97:3 v/v)] of the crude product gave α-dibenzyl phosphate **34** (61.6 mg, 89%) as a white film; IR (neat) cm^{-1} 1747, 1678, 1544, 1369, 1228, 1166, 1133, 1036, 955, 749, 699; ^1H NMR ($CDCl_3$, 500 MHz) δ 1.69 (s, 3H, Ac), 1.98 (s, 3H, Ac), 2.00 (s, 3H, Ac), 2.01 (s, 3H, Ac), 2.02 (s, 3H, Ac), 2.05 (s, 3H, Ac), 2.08 (s, 3H, Ac), 3.64 (ddd, 1H, J = 2.2, 4.0, 9.7 Hz, H-5′), 3.79 (t, 1H, J = 9.6 Hz, H-4), 3.90 (ddd, 1H, 2, 3.8, 10.5 Hz, H-5), 3.98 (dd, 1H, J = 3.8, 12.3 Hz, H-6a), 4.02 (dd, 1H, J = 2.0, 12.5 Hz, H-6a′), 4.26 (ddt, 1H, J = 3, 9, 10 Hz, H-2), 4.31 (dd, 1H, J = 2, 12.3 Hz, H-6b), 4.37 (dd, 1H, J = 4.2, 12.5 Hz, H-6b′), 4.49 (d, 1H, J = 7.9 Hz, H-1′), 4.92 (dd, 1H, J = 8.3, 8.7 Hz, H-2′), 5.01−5.18 (m, 7H, OC*H*$_2$Ph, H-3, H-3′, H-4′), 5.60 (d, 1H, J = 9.3 Hz, N*H*), 5.60 (dd, 1H, J = 3.3, 6.0 Hz, H-1), 7.32−7.47 (m, 10H, OCH$_2$*Ph*); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 20.47 (4 ×), 20.58, 20.68, 22.69, 51.77, 51.83, 61.09, 61.48, 67.68, 69.78, 69.82, 69.87, 69.90, 70.30, 71.55, 71.91, 72.90, 75.59, 96.01 (d, J = 6 Hz, C-1), 100.80, 127.97, 128.04, 128.71, 128.78, 128.88, 128.89, 135.17 (d, J = 6.5 Hz), 135.27 (d, J = 6.5 Hz), 168.98, 169.23, 170.04, 170.13, 170.24, 170.41, 170.77; ^{31}P NMR ($CDCl_3$, 162 MHz) δ −0.75; $[\alpha]_D$ 24.1° (c = 1.3, $CHCl_3$); MS (FAB) 918 (MNa^+); HRMS m/z calcd for $C_{40}H_{50}NO_{20}NaP$ 918.256152, found 918.254600.

Mono(triethylammonium) Salt of 2,3,4,6-Tetra-*O*-acetyl-*β*-D-glucopyranosyl-(1→4)-2-acetamido-3,6-di-*O*-acetyl-2-deoxy-α-D-glucopyranosyl Phosphate 35. Hydrogenolysis of α-dibenzyl phosphate **34** (25.8 mg, 0.0288 mmol) yielded α-phosphate **35** (25.8 mg, quantitative); IR (neat) cm^{-1} 3339, 2959, 1747, 1668, 1369, 1230, 1166, 1125, 1038, 962, 909, 753; ^1H NMR [$CDCl_3$, CD_3OD (2:1 v/v), 500 MHz] δ 1.12 [t, 9H, J = 7.3 Hz, N(CH$_2$C*H*$_3$)$_3$], 1.74 (s, 3H, Ac), 1.79 (s, 3H, Ac), 1.82 (s, 3H, Ac), 1.83 (s, 3H, Ac), 1.84 (s, 3H, Ac), 1.89 (s, 3H, Ac), 1.93 (s, 3H, Ac), 2.90 [q, 6H, J = 7.3 Hz, N(C*H*$_2$CH$_3$)$_3$], 3.53 (ddd, 1H, J = 2, 3.9, 10.3 Hz, H-5′), 3.65 (dd, 1H, J = 9.5, 9.7 Hz, H-4), 3.85 (dd, 1H, J = 1.8, 12.5 Hz, H-6a′), 3.89 (dd, 1H, J = 3.5, 12.1 Hz, H-6a), 3.93 (ddd, 1H, J = 1.8, 3.5, 9.7, H-5), 4.04 (brdt, 1H, J = 2.5, 10.7 Hz, H-2), 4.21 (dd, 1H, J = 3.9, 12.6 Hz, H-6b′), 4.37 (dd, 1H, J = 1.8, 11.6 Hz, H-6b), 4.38 (d, 1H, J = 8 Hz, H-1′), 4.71 (dd, 1H, J = 8, 9.3 Hz, H-2′), 4.87 (t, 1H, J = 9.7 Hz, H-4′), 4.96 (t, 1H, J = 9.4 Hz, H-3′), 5.04 (dd, 1H, J = 9.5, 10.4 Hz, H-3), 5.22 (dd, 1H, J = 3.3, 6.6 Hz, H-1); ^{13}C NMR [$CDCl_3$, CD_3OD (2:1 v/v), 125 MHz] δ 7.92, 19.84(2 ×), 19.95, 20.01, 20.17, 21.74, 29.19, 45.65, 51.60, 51.65, 61.13, 61.27, 67.61, 68.99, 70.92, 71.38, 72.80, 75.79, 93.38, 100.34, 169.12, 169.44, 170.15, 170.53, 170.62, 170.78, 171.60; ^{31}P NMR [$CDCl_3$, CD_3OD (2:1 v/v) 162 MHz] δ −0.08; $[\alpha]_D$ +42.9° (c = 0.8, $CHCl_3$); MS (−ve FAB) 714 (M^-); HRMS m/z calcd for $C_{26}H_{37}NO_{20}P$ 714.164657, found 714.164900.

P^1-[2,3,4,6-Tetra-*O*-acetyl-*β*-D-glucopyranosyl-(1→4)-2-acetamido-3,6-di-*O*-acetyl-2-deoxy-α-D-glucopyranosyl] P^2-Dolichyl Pyrophosphate 36. ^{31}P NMR [$CDCl_3$, CD_3OD (2:1 v/v), 162 MHz] δ −9.36, −11.50; MS (−ve ESI) 1954, 2022, 2090, 2158, 2227 (M^- for n = 13−17).

P^1- [*β*-D-Glucopyranosyl-(1→4)-2-acetamido-2-deoxy-α-D-glucopyranosyl] P^2-Dolichyl Pyrophosphate 1e. Similar to the deprotection of **29**, peracetylated Dol-PP-GlcNAc-Glc **36** (6.1 mg, 2.87 μmol) gave after column chromatography Dol-PP-GlcNAc-Glc **1e** (4.2 mg, 73%): ^{31}P NMR [$CDCl_3$, CD_3OD (2:1 v/v), 121 MHz] δ −11.4, −13.4; MS (−ve ESI) 850.8, 884.8, 918.8, 952.9, 987.0 ($m/2$ for n = 13−17).

Bz-Asn-Leu-Thr-Lys-NH$_2$·TFA 42. Tetrapeptide Bz-Asn-Leu-Thr-Lys-NH$_2$·TFA **42** was prepared by standard Fmoc-based solid-phase peptide synthesis (SPPS) on PAL-PEG-PS resins (0.175 mmol/g, 1.18 g). In situ activated esters were generated from the free acid derivatives using (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (PyBOP) chemistry. In general, coupling was carried out manually with Fmoc free acid (1.5 equiv), PyBOP (1.5 equiv), and di-*iso*-propylethylamine (1.5 equiv) in DMF (10 mL) inside a reaction vessel with shaking (Fisher orbital shaker) for 1 h. The resins were filtered through suction and washed successively with DMF (2 × 20 mL) and CH_2Cl_2 (20 mL). Fmoc deprotection was performed with 20% piperidine/DMF (10 mL, 3 × 5 min) followed by washing with DMF (5 × 20 mL). The peptides were deprotected and cleaved from the solid support by treatment with trifluoroacetic acid (TFA)/CH_2Cl_2/triisopropylsilane (TIPS)/water (47.5:47.5:2.5:2.5 v/v, 2 × 10 mL). The resin was filtered and washed with TFA/CH_2Cl_2 (50:50 v/v, 2 × 10 mL). The combined filtrates were concentrated and triturated with Et_2O (3 × 10 mL). The white pellet was dissolved in H_2O (15 mL) and 0.1% TFA/CH_3CN (100 μL) and coevaporated with toluene to give the desired tetrapeptide **42** (101.4 mg, 71%); ^1H NMR (d_6-DMSO, 500 MHz) δ 0.81 (d, 3H, J = 6.6 Hz, Leu-H$_\delta$), 0.85 (d, 3H, J = 6.5 Hz, Leu-H$_\delta$), 1.04 (d, 3H, J = 6.4 Hz, Thr-H$_\gamma$), 1.24−1.34 (m, 2H, Lys-H$_\gamma$), 1.45−1.75 (m, 9H), 2.58 (dd, 1H, J = 8.4, 15.3 Hz, Asn-H$_\beta$), 2.65 (dd, 1H, J = 5.6, 15.3 Hz, Asn-H$_\beta$), 2.75 (t, 2H, J = 7.5 Hz, Lys-H$_\epsilon$), 4.01 (m, 1H, Thr-H$_\beta$), 4.15 (dt, 1H, J = 4.9, 8.7 Hz, Lys-H$_\alpha$), 4.20 (dd, 1H, J = 4.4, 8.1 Hz, Thr-H$_\alpha$), 4.33 (brq, 1H, J = 8.2 Hz, Leu-H$_\alpha$), 4.75 (dt, 1H, J = 5.6, 7.9 Hz, Asn-H$_\alpha$), 5.00 (d, 1H, J = 5.6 Hz, Thr-O*H*), 7.02 (brs, 1H, CON*H*H), 7.15 (brs, 1H, CON*H*H), 7.25 (brs, 1H, CON*H*H), 7.42 (brs, 1H, CON*H*H), 7.48 (t, 2H, J = 7.8 Hz, *Ph*), 7.53−7.57 (m, 1H, *Ph*), 7.61 (brs, 3H, NH_3^+), 7.75 (d, 1H, J = 8.1 Hz, Leu-N*H*), 7.79 (d, 1H, J = 8.1 Hz, Lys-N*H*), 7.84 (dd, 1H, J = 1.5, 7.8 Hz, *Ph*), 8.14 (d, 1H, J = 8.1 Hz, Thr-N*H*), 8.64 (d, 1H, J = 7.5 Hz, Asn-N*H*); MS (ESI) 579 (MH^+).

Bz-Asn-Leu-Thr-Lys(Ac)-NH$_2$ 37. A solution of the tetrapeptide **42** (41.5 mg, 0.06 mmol), acetic anhydride (17.0 μL, 0.18 mmol,) and ^{i}Pr$_2$NEt (62.7 μL, 0.36 mmol) was stirred in DMF (4 mL) for 1.5 h. The solvent was blown down under N_2 overnight (0.5 mL remaining) and triturated with Et_2O/*n*-hex (1:1 v/v, 4 × 5 mL). The white pellet was dried under high vacuum to give a quantitative yield of the acetylated peptide **37** (42.5 mg); ^1H NMR (d_6-DMSO, 500 MHz) δ 0.81 (d, 3H, J = 6.6 Hz, Leu-H$_\delta$), 0.85 (d, 3H, J = 6.6 Hz, Leu-H$_\delta$), 1.03 (d, 3H, J = 6.3 Hz, Thr-H$_\gamma$), 1.2−1.7 (m, 9H), 1.77 (s, 3H, Ac),

2.58 (dd, 1H, J = 8.5, 15.3 Hz, Asn-H$_\beta$), 2.65 (d, 1H, J = 5.6, 15.3 Hz, Asn-H$_\beta$), 2.97 (q, 2H, J = 6.9 Hz, Lys-H$_\epsilon$), 4.01 (m, 1H, Thr-H$_\beta$), 4.12 (dt, 1H, J = 4.8, 8.5 Hz, Lys-H$_\alpha$), 4.19 (dd, 1H, J = 4.4, 8.2 Hz, Thr-H$_\alpha$), 4.32 (q, 1H, J = 7.5 Hz, Leu-H$_\alpha$), 4.76 (dt, 1H, J = 5.8, 8.1 Hz, Asn-H$_\alpha$), 4.95 (brd, 1H, Thr-O*H*), 7.02 (d, 1H, J = 1.5 Hz, CON*H*H), 7.10 (d, 1H, J = 1.5 Hz, CON*H*H), 7.23 (d, 1H, J = 1.7 Hz, CON*H*H), 7.41 (d, 1H, J = 1.7 Hz, CON*H*H), 7.48 (brt, 2H, J = 7.7 Hz, *Ph*), 7.53−7.56 (m, 1H, *Ph*), 7.70 (d, 1H, J = 8.0 Hz, Leu-N*H*), 7.79 (d, 1H, J = 8.3 Hz, Lys-N*H*), 7.80−7.85 (m, 3H, N*H*Ac, *Ph*), 8.15 (d, 1H, J = 7.9 Hz, Thr-N*H*), 8.61 (d, 1H, J = 7.6 Hz, Asn-N*H*); MS (ESI) 620 (MH^+), 643 (MNa^+).

Bz-Asn-Leu-Thr-Lys(^{3}H−Ac)-NH$_2$ 37r. To a solution of the tetrapeptide **42** (41.5 mg, 0.06 mmol) and ^{i}Pr$_2$NEt (71.4 μL, 0.41 mmol) in DMF (1 mL) in a 15 mL Oakridge tube was added tritium-labeled acetic anhydride [(^{3}H)-Ac$_2$O, 2.36 μL, 0.025 mmol, specific activity of 100 mCi/mmol from American Radiolabeled Chemicals Inc., St. Louis, MO]. After 2 h, the free amine was capped by treating with excess Ac$_2$O (17 μL, 0.18 mmol) for another 2 h. The reaction was monitored by TLC [EtOAc, ^{n}BuOH, AcOH, H$_2$O (3:1:1:1 v/v)]; R_f of peptide **42** = 0.26; R_f of peptide **37** = 0.51. The product was triturated with Et$_2$O/*n*-hex (1:1 v/v, 8 × 4 mL) to give radiolabeled tetrapeptide **37r** (29.3 mg, 79%).

OT Assay with Radiolabeled Peptide 37r. Yeast (*S. cerevisiae*) OT was purified to the solubilized membrane stage according to the Pathak protocol.[39] The final buffer solution contains 140 mM sucrose, 50 mM HEPES, pH 7.5, 10 mM MgCl$_2$, 0.1 mM AEBSF, 0.5 μg/mL pepstatin, and 0.5 μg/mL leupeptin. The assay buffer for OT assay consisted of 140 mM sucrose, 50 mM HEPES, pH 7.5, 1.2% Triton X-100, 15 mM MnCl$_2$, and 0.5 mg/mL phosphatidylcholine. Different amounts of dolichol-linked saccharides (**1b**−**e**, **3**) were aliquoted from chloroform/methanol stock solutions into eppendorf tubes, and the solvents were evaporated under a gentle stream of nitrogen. DMSO (10 μL) and assay buffer (final volume of 200 μL) were added and vortexed vigorously. The reaction was initiated by the addition of OT and the radiolabeled peptide **37r** (0.42 mM, 10 μL in DMSO, 29.3 μCi/μmol, final concentration of 21 μM ≈ 5 K_m). (Nonlabeled peptide **37** was used for the mass spectroscopy analysis of glycopeptides.) The mixture was shaken at 150 rpm for the specified time at room temperature followed by quenching with 6 mL of chloroform/methanol/4 mM MgCl$_2$ (3:2:1 v/v). The upper aqueous phase was removed and the lower phase reextracted twice with TUP {theoretical upper phase, chloroform/methanol/water/0.25 M MgCl$_2$ (12:192:186:2.69 v/v), 3 mL}. The combined aqueous phases were blown down under nitrogen for HPLC analysis. For competition assay of unnatural analogues **1c** and **1d**, a constant amount of Dol-PP-GlcNAc-GlcNAc **1b** (22 μM) was mixed with variable amounts of **1c** or **1d**.

HPLC Analysis of Glycopeptide. HPLC analysis of assay mixtures was carried out on a Beckman System Gold liquid chromatography system equipped with a C18 column (microsorb MV C18, 5 μm, 100 Å, 25 cm). Samples were resuspended in 0.1% TFA in water (0.8 mL) and centrifuged before injection onto the HPLC. Separation of glycopeptides and peptides were accomplished by elution with a gradient of 15−30% acetonitrile over 25 min. Fractions (0.5 mL) were collected for 15−30 min, mixed with Ecolite (5.5 mL), and counted on a Beckman LS-5000TD scintillation counter. The amount of glycopeptide formation can be calculated on the basis of the specific activity of radiolabeled peptide **37r** (29.3 mCi/mmol).

Product formation was shown to be linear over 2 h in accord with Coward's results. The use of long assay times allows enough radiolabeled glycopeptide to be detected. The detection limit of this assay is ca. 3 pmol (which corresponds to 200 dpm) and glycopeptide formation is generally within 10% of substrate. Possible product inhibition has previously been addressed and shown to be lacking. Michaelis−Menten parameters were obtained by Lineweaver−Burk reciprocal plots. The approximate K_i's were calculated as described by Segel[36] using eq 1,

$$K_i = \frac{[\mathrm{I}]\,(1 - \mathrm{i})}{\mathrm{i}\,(1 + \frac{[\mathrm{S}]}{K_m})} \qquad (1)$$

where i represents the fraction inhibition, [I] is the concentration of the inhibitor, and [S] is the concentration of in vitro substrate **1b**.

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Supporting Information Available: Graphs for kinetic analysis of tetrapeptide **37** and dolichol-linked saccharides **1b**−**e** and mass spectra of glycopeptides **38**, **39**, and **43**. This material is available free of charge via the Internet at http://pubs.acs.org.

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