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Synthetic, Structural, and Biosynthetic Studies of an Unusual Phospho-Glycopeptide Derived from α-Dystroglycan

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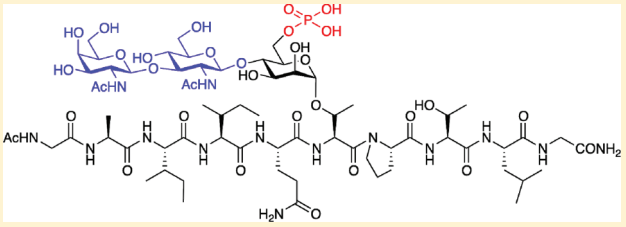
Supporting Information

ABSTRACT: Aberrant glycosylation of α-dystroglycan (α-DG) results in loss of interactions with the extracellular matrix and is central to the pathogenesis of several disorders. To examine protein glycosylation of α-DG, a facile synthetic approach has been developed for the preparation of unusual phosphorylated *O*-mannosyl glycopeptides derived from α-DG by a strategy in which properly protected phospho-mannosides are coupled with a Fmoc protected threonine derivative, followed by the use of the resulting derivatives in automated solid-phase glycopeptide synthesis using hyper-acid-sensitive Sieber amide resin. Synthetic efforts also provided a reduced phospho-trisaccharide, and the NMR data of this derivative confirmed the proper structural assignment of the unusual phospho-glycan structure. The glycopeptides made it possible to explore factors that regulate the elaboration of critical glycans. It was established that a glycopeptide having a 6-phospho-*O*-mannosyl residue is not an acceptor for action by the enzyme POMGnT1, which attaches $\beta(1,2)$-GlcNAc to *O*-mannosyl moietes, whereas the unphosphorylated derivate was readily extended by the enzyme. This finding implies a specific sequence of events in determining the structural fate of the *O*-glycan. It has also been found that the activity of POMGnT1 is dependent on the location of the acceptor site in the context of the underlying polypeptide/glycopeptide sequence. Conformational analysis by NMR has shown that the *O*-mannosyl modification does not exert major conformational effect on the peptide backbone. It is, however, proposed that these residues, introduced at the early stages of glycoprotein glycosylation, have an ability to regulate the loci of subsequent *O*-GalNAc additions, which do exert conformational effects. The studies show that through access to discrete glycopeptide structures, it is possible to reveal complex regulation of *O*-glycan processing on α-DG that has significant implications both for its normal post-translational maturation, and the mechanisms of the pathologies associated with hypoglycosylated α-DG.



INTRODUCTION

The glycoprotein α-dystroglycan[1] (α-DG) is a component of the dystrophin glycoprotein complex found prominently in the cell membranes of skeletal muscle, nerves, and brain.[2,3] It has received considerable attention because of its critical involvement in linking the cytoskeleton to the extracellular matrix. Although no mutations in the dystroglycan gene have been identified, defects in *O*-glycan modification of the central mucin-like region of α-DG have devastating effects on muscle fiber integrity and neuronal migration and result in muscular dystrophies (dystroglycanopathies).[2,4,5] α-DG is also the cellular receptor for arena viruses[6] and *Mycobacterium leprae*[7] and perturbations in α-DG processing have been associated with cancer progression.[8,9] There is evidence suggesting that *O*-glycans of α-DG are ligands for extracellular proteins[2,10,11] such as laminin, agrin, and perlecan in muscle and neurexin in brain. Aberrant glycosylation of α-DG results in loss of these interactions that is causal to the pathogenesis of several disorders.[2,3,5]

The *O*-linked glycans of α-DG are structurally diverse.[12,13] In addition to the common mucin modification initiated with *N*-acetyl-α-D-galactosamine (*O*-GalNAc) on the side chains of serine (Ser) or threonine (Thr), these side chains can, instead, be modified by α-*O*-mannosyl moieties (*O*-Man).[12−14] This type of protein modification was first described in yeast; however, it has been established that it is widespread in nature and has, for example, been found in plants, insects, and mammals. While there is evidence for the existence of other mammalian glycoproteins modified with *O*-Man,[15,16] α-DG is the only one specifically identified as having this modification. The amino acid sequence for the mucin-like region of α-DG is highly conserved across vertebrates, reinforcing its significance. The sites of *O*-glycosylation on α-DG have been mapped in unprecedented detail,[12,13] but the full characterization of this glycoprotein remains a considerable challenge because of the intrinsic heterogeneity associated with its glycosylation and the novel structures of the glycans that continue to emerge.

The mannosyltransferases POMT1 and POMT2, which are localized in the endoplasmic reticulum compartment, initiate the attachment of a mannosyl residue to Ser/Thr by employing dolichol phosphate-activated mannose (Dol-P-Man) as the glycosyl donor.[2,3] It is only after the *O*-mannosylated glycoprotein

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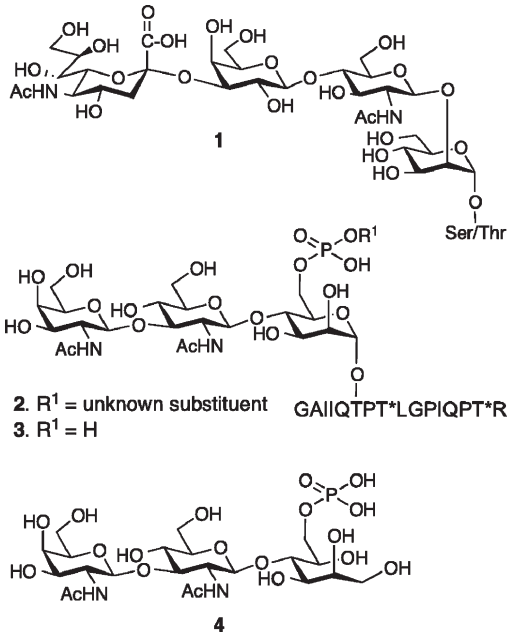


Figure 1. *O*-Mannosyl glycan **1** is a common structural motif of glycoproteins of muscle and brain tissue. The unusual phosphoglycopeptide **2** is derived from the mucin-like domain of α-DG. Phosphodiester extension of this compound is required for laminin binding. The identity of this extension (R^1) is unknown. The saccharide moiety of **3** was identified by NMR analysis of **4**, which was obtained by *β*-elimination followed by reduction of the anomeric center. Glycopeptide **3** can have additional *O*-mannosyl sites as indicated by T*.

undergoes translocation to the Golgi that it can be elongated by POMGnT1,[17,18] which attaches a *β*(1,2)-linked GlcNAc residue. The GlcNAc-*β*(1,2)-Man disaccharide can be further extended by mammalian glycosyltransferases, and the resulting structures are often variations of the tetrasaccharide NeuAcα(2−3)-Gal*β*-(1−4)-GlcNAc*β*(1−2)-Manα1-Ser/Thr (compound **1**, Figure 1) with different lengths and content (e.g., asialo and fucosides α1,3-linked to GlcNAc). Small quantities of branched *O*-mannosylated structures have been found in brain and testes tissue, and the biosynthesis of such compounds is mediated by *β*(1,6)-*N*-acetylglucosaminyltransferase Vb (GlcNAcT-Vb or GlcNAcT-IX) which adds a *β*(1,6)-linked GlcNAc residue to *O*-linked mannosides that also express *β*(1,2)-linked GlcNAc.[19,20]

Muscle-eye-brain disease (MEB), a form of muscular dystrophy, results when POMGnT1 activity is absent or compromised, which also abrogates IIH6 antibody binding that serves as a marker for functional α-DG.[2] This observation suggests that extension of the *O*-Man modification by a C2-linked GlcNAc is an important element for proper α-DG function. The specific mechanisms and glycan structures that impart functionality have yet to be elucidated.

Recently, Campbell and co-workers[21] isolated a highly unusual phosphorylated *O*-mannosyl glycopeptide from the mucin-like domain of α-DG (compound **2**, Figure 1). It was shown that a phosphodiester modification of this compound is required for laminin binding. Furthermore, patients with muscle-eye-brain disease and Fukuyama congenital muscular dystrophy, as well as mice with myodystrophy, were shown to have defects in post-phosphoryl modification that prevent mannosyl phosphatediester (**3**, R = H) formation.

Although the biological importance of the phosphorylated *O*-mannosyl glycopeptide is evident, there are major gaps in our understanding of its biosynthesis, the chemical nature of the phosphodiester extension, and regulation of the formation of the various classes of *O*-mannosylated structures. In this respect, compound **2**, isolated after tryptic digestion of α-DG, is the first identified mammalian structure that has a GlcNAc*β* (1−4)Man motif in a non-*N*-linked glycan. The enzyme that adds the *β*(1−4)GlcNAc residue has not been identified nor has the process that controls differential extension of an *O*-mannosyl residue with a 1,2- vs 1,4-linkage been uncovered. Furthermore, compound **2** is the first vertebrate instance of a nonglycosylphosphatidylinositol-anchored glycoprotein that is modified by a phosphodiester linkage. The molecular weights from gel mobility of α-DG produced either in the absence of POMGnT1 activity or after chemical removal of the distal phosphoester component are similar.[21] Furthermore, POMGnT1 knockout brain proteins lack extended *O*-Man structures and are not IIH6 reactive[16] providing definitive evidence for a regulatory role of POMGnT1 in the full elaboration of the laminin binding component. The identity of the distal ester component remains to be elucidated.

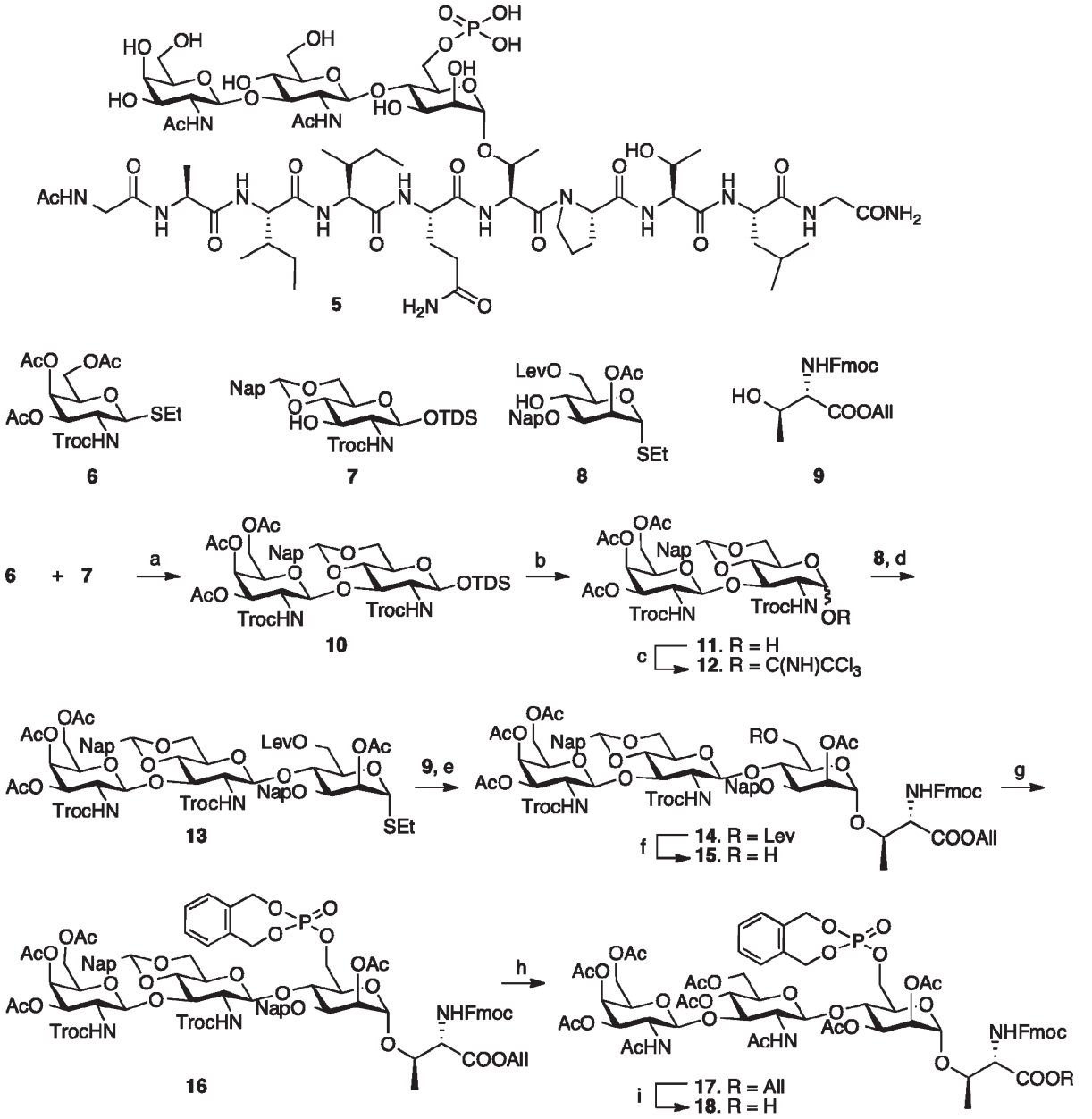
We anticipate that well-defined synthetic glycopeptides will offer critical probes for uncovering the biosynthetic pathway and enzymes involved in the assembly of compounds such as **2**, which includes the site where the novel laminin binding glycan is attached, as well as proximal threonine residues on which *O*-Man tetrasaccharides are found. To this end, we report here, for the first time, a facile synthetic approach for the preparation of phosphorylated *O*-mannosyl glycopeptides. Furthermore, relevant α-DG biosynthetic glycopeptides intermediates were prepared modified both with *O*-Man and *O*-Man-6-Phosphate (Man-6-P). These compounds made it possible to explore factors that regulate the elaboration of these critical glycans. The synthetic effort has also provided the reduced trisaccharide **4** and the NMR data of this synthetic compound corresponds the spectral features and assignments of the isolated compound earlier reported,[21] confirming the proper structural assignment of the unusual phospho-glycan.

RESULTS AND DISCUSSION

Chemical Synthesis of Glycopeptides. In general, glycopeptides are chemically synthesized by a strategy in which preformed glycosylated amino acids are used in stepwise solid-phase peptide synthesis (SPPS).[22] This approach requires, however, special care in the selection of protecting groups because *O*-glycosidic bonds can be hydrolyzed under acid conditions and furthermore can undergo *β*-elimination[23] upon treatment with moderately strong bases. It has been established that these problems can be circumvented by the use of acetyl esters for hydroxyl protection combined with N^{α}-9-fluorenylmethyloxycarbonyl (Fmoc) protected amino acids. Furthermore, solid-phase synthesis of phospho-peptides has been accomplished by employing the Fmoc-strategy and amino acid derivatives bearing a protected phospho-ester function. Thus, we envisaged that solid-phase synthesis using trisaccharide threonine **18** modified with stable phosphotriester and standard Fmoc protected amino acids in combination with appropriate activation reagents would provide a facile synthetic route to phosphorylated *O*-mannosyl peptide **5** (Scheme 1).

We designed a concise synthetic route for glycosylated amino acid **18** based on the following strategic considerations.

Scheme 1[a]

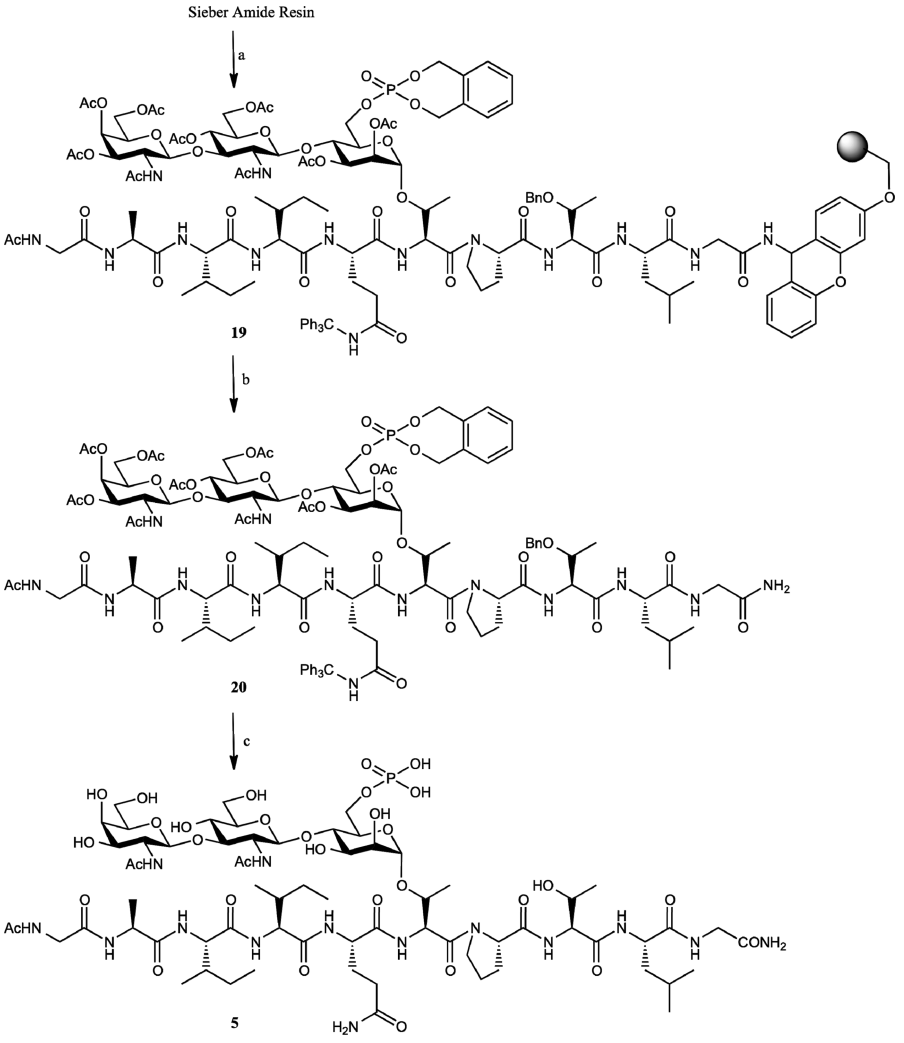


[a] Reagents and conditions: (a) NIS, TMSOTf, DCM, 0 °C (91%); (b) TBAF, THF/AcOH; (83%); (c) Cl_3CCN, Cs_2CO_3, DCM (86%); (d) TMSOTf, DCM, −35 °C (86%); (e) NIS, AgOTf, DCM, −20 °C (72%); (f) H_2NNH_2, HOAc, DCM/MeOH; (g) *N*,*N*-diethyl-1,5-dihyro-2,4,3-benzodioxaphosphepin-3-amine, 1*H*-tetrazole, DCM followed by *m*CPBA oxidation at −20 °C; (71%, 3 steps); (h) DDQ, DCM/H_2O then Zn, AcOH then Ac_2O, Py (56%, 3 steps); (i) $Pd(PPh_3)_4$, THF/H_2O (92%).

Trisaccharide **13**, which was assembled from building blocks **6**,[24] **7**, and **8**, was coupled with properly protected threonine **9**[25] to give amino acid modified trisaccharide **14**. We expected that the Lev ester[26] of **14** could be removed without affecting the other protecting groups providing an opportunity to install the phosphotriester. The late stage introduction of the anomeric amino acid and phosphotriester was expected to avoid difficulties due to the chemical labilities of these functionalities. Benzyl ethers were avoided as permanent protecting groups for the carbohydrate moiety because they may be difficult to remove after glycopeptide assembly,[27] and furthermore, catalytic hydrogenation to remove benzyl ethers of an Fmoc protected glycosylated amino acid is problematic because it will result in loss of the Fmoc protecting group. Therefore, we explored 2-methylnaphthyl (Nap) ethers[28] and acetals[29] as permanent protecting groups. These protecting groups are significantly more stable to acidic conditions compared to a *p*-methoxybenzyl ether and acetal but can readily be removed by oxidation with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ).[30,31]

Coupling of the thiogalactoside **6** with glycosyl acceptor **7** in the presence of NIS/TMSOTf[32] as the promoter provided the disaccharide **10** in a yield of 91% as only the β-anomer (Scheme 1). Removal of the anomeric dimethylthexylsilyl (TDS) ether of **10** was easily accomplished by treatment with tetra-*n*-

Scheme 2[a]



[a] Reagents and conditions: (a) standard Fmoc solid-phase peptide synthesis, HBTU/HOBt, DIPEA, DMF and manual coupling of **18**, HATU, DIPEA, DMF; (b) 1% TFA/DCM; (c) TFA/TIPS/DCM (5/5/90) followed by Pd/C, H_2, MeOH/DCM then 5% H_2NNH_2/MeOH.

butylammonium fluoride (TBAF) buffered with AcOH in THF, and the resulting hemiacetal **11** was immediately converted into the corresponding trichloroacetimidate **12** by reaction with trichloroacetonitrile in the presence of Cs_2CO_3. A TMSOTf-promoted glycosylation of trichloroacetimidate **12** with thiomannosyl acceptor **8** gave trisaccharide **13** in an excellent yield of 86%. In this coupling reaction, no aglycon transfer[33−35] of the thioethyl moiety of **8** was observed, and furthermore, only the β-anomer was formed due to neighboring group participation by the 2,2,2-trichloroethoxycarbamate (Troc) function. Compound **13** could be used as a glycosyl donor without a need for any manipulations and coupling with threonine glycosyl acceptor **9** in the presence of NIS/AgOTf[36] as the activator furnished glycosylated amino acid **14** in 72% yield. Phosphorylation of **14** was achieved by a three-step procedure involving selective removal of the Lev ester using hydrazine acetate to give alcohol **15**, which was reacted with *N*,*N*-diethyl-1,5-dihyro-2,4,3-benzodioxaphosphepin-3-amine[37] in the presence of 1*H*-tetrazole to give an intermediate phosphite ester which was oxidized in situ with *meta*-chloroperoxybenzoic acid (*m*CPBA) to give the phosphotriester **16**. Next, oxidative removal of the Nap ether and naphthalene acetal was easily accomplished by treatment with excess of DDQ to give an intermediate triol which was used without purification in the subsequent step which involved reductive removal of two Troc moieties using Zn/AcOH followed by acetylation of the amines and alcohols using acetic anhydride in pyridine provided compound **17**. Finally, deallylation of **17** using $Pd(PPh_3)_4$ as the catalyst furnished the corresponding glycosylated threonine **18**, which was employed for the assembly of the glycopeptide **5**. A full analysis of the ^1H and ^{13}C NMR (HMQC and HMBC) spectra of this glycan was carried out confirming the desired structure and linkages.

Hyper-acid-sensitive Sieber amide resin[38] was selected as the solid support for glycopeptides assembly, since release of the compound can be accomplished under mild conditions using 1% TFA in DCM. It was expected that these conditions would not interfere with the rather labile phosphotriester function. The assembly of the target glycopeptide **5** was performed on an automatic solid-phase peptide synthesizer using a standard Fmoc-based protocol using 2-(1*H*-benzotriazole-1-yl)-oxy-1,1,3,3-tetramethyl-uronium hexafluorophosphate (HBTU) and 1-hydroxybenzotriazole (HOBt)[39] as the activator system

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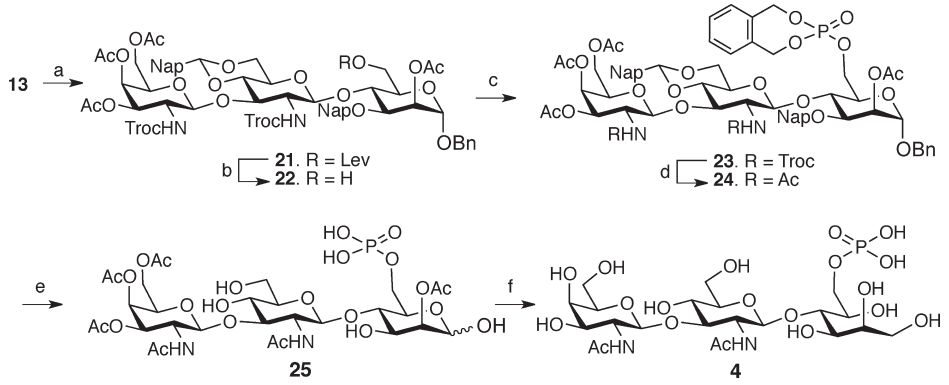
Scheme 3[a]



[a] Reagents and conditions: (a) BnOH, NIS, TMSOTf, DCM, −20 °C (58%); (b) H_2NNH_2, HOAc, DCM/MeOH; (c) *N*,*N*-diethyl-1,5-dihyro-2,4,3-benzodioxaphosphepin-3-amine, 1*H*-tetrazole, DCM followed by *m*CPBA, −20 °C; (65%, 3 steps); (d) Zn, Ac_2O/AcOH/THF, $CuSO_4$(aq) (71%); (e) $Pd(OH)_2$/C, H_2, MeOH/DCM (78%); (f) NaOMe/MeOH/DCM followed by $NaBH_4$, H_2O (75%, 2 steps).

(Scheme 2). The glycosylated threonine **18** was introduced manually using 2-(7-aza-1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU)[40] as the activator in the presence of the *N*,*N*-diisopropylethylamine (DIPEA) in DMF. Under these conditions, only low equivalents (2 equiv) of glycosylated amino acid were required and it was possible to recover some of the excess starting material (∼0.7 equiv). The fully assembled glycopeptide **19** was released from the resin by treatment in 1% TFA in DCM to give, after purification by size exclusion column chromatography (LH-20), the fully protected phosphoglycopeptide **20**. The trityl protecting group of the glutamine moiety was removed by treatment with a cocktail of trifluoroacetic acid (TFA)/ triisopropylsilane (TIPS)/DCM (5/5/90, v/v/v) and subsequent hydrogenolysis using Pd/C as the catalyst in a mixture of MeOH and DCM resulted in the removal of the phosphate and threonine side chain protecting groups and finally treatment with anhydrous hydrazine in MeOH resulted in the cleavage of the acetyl esters to give the target glycopeptide **5**.

Previously, the structure of the carbohydrate moiety of phosphoglycopeptide **3** was determined by NMR analysis of compound **4** (Figure 1), which was obtained by treatment of α-DG with aqueous NaOH (1 M) to release oligosaccharides followed by reduction with $NaBH_4$ and purification.[21] As outlined above, the structure of **3** is highly unusual and the presence of a β-GlcNAc moiety linked to the C-4 hydroxyl of a mannosyl threonine residue had never been observed before. In this respect, all previously identified mammalian *O*-mannosylated glycoproteins are modified by a GlcNAc moiety linked to C-2 of mannosyl serine or threonine. Thus, it was prudent to ascertain the chemical structure of **3** by chemically synthesizing compound **4** and comparing its spectroscopic characteristics with that of isolated material. For this purpose, thioglycoside **13** was converted into benzyl glycoside **21** by a NIS/TMSOTf mediated glycosylation with benzyl alcohol. Next, the Lev ester of **21** was removed under standard conditions, and the resulting alcohol phosphorylated by a two-step procedure entailing reaction with *N*,*N*-diethyl-1,5-dihyro-2,4,3-benzodioxaphosphepin-3-amine in the presence of 1*H*-tetrazole followed by in situ oxidation with *m*CPBA to give the phospho-trisaccharide **23**. The Troc moieties of **23** were converted into acetamido residues by treatment with Zn in a mixture of aqueous $CuSO_4$, Ac_2O, AcOH, and THF[41] to give phospho-trisaccharide **24**, which was subjected to hydrogenolysis over $Pd(OH)_2$/C in MeOH/DCM followed by deacetylation and finally reduction of the anomeric center with $NaBH_4$ to furnish the target compound **4** (Scheme 3) whose characterization by NMR is described below.

NMR Characterization of Trisaccharide 4 and Glycopeptide 5. NMR provides a powerful tool to characterize carbohydrate structures with several of its spectral parameters being very sensitive to features of the glycosidic linkage. In view of the novelty of the phospho-trisaccharide, we compared NMR parameters of the synthetic trisaccharide **4** with reported data to confirm the proposed structural identity.

The assignment of saccharide resonances of **4** and **5** started at the anomeric center of each monosaccharide residue as the coordinates of their proton and carbon shifts occur in distinct region of the 2-dimensional $^1H-^{13}C$ correlation spectrum. Homonuclear COSY and TOCSY experiments[42] were used to identify the proton-coupled spin systems of each of the monosaccharide residues. $^1H-^{13}C$ HMQC experiments,[42] which provide correlations between directly bonded protons and carbons, were employed to assign carbon resonances, similar to the strategy followed for the material isolated from natural sources.[21] Vicinal proton coupling constants between the H1 and H2 protons of each monosaccharide residue, 8.4 Hz for GalNAc and 8.3 Hz for GlcNAc, were used to establish the β anomeric configurations. HMBC experiments,[42] which can reveal long-range through-bond correlation, permitted identification of connections across glycosidic bonds. In this respect, cross peaks correlating C1 of GalNAc to H3 of GlcNAc and C3 of GlcNAc to H1 of GalNAc, as well as C4 of the Man-ol to H1 of GlcNAc and H4 of Man-ol to C1 of GlcNAc, were observed.

The resulting assignments of synthetic compounds **4** and **5** and the reported data for the reduced trisaccharide isolated from α-DG are summarized in Table 1. As can be seen, there is excellent agreement in chemical shifts of **4** and the previously isolated material confirming that α-DG is modified with a trisaccharide having an unusual GlcNAc-(1−4)-Man-6-P moiety. The small variations in chemical shifts of the A5 and A6 sites of **4** between those reported previously and here are likely due to small differences in the pH of the samples, which could have an impact on chemical shifts through minor changes in the average ionization state of the phosphate group. It was observed that the chemical shifts of the GlcNAc and GalNAc residues of the

Table 1. Chemical Shifts of Carbon and Proton Signals of Carbohydrate Moieties of Compound 4, the Corresponding Isolated Derivative, and Glycopeptide 5[a]

	synthetic trisaccharide **4**		Yoshida-Moriguchi et al.		glycopeptide **5**	
	H1	C13	H1	C13	H1	C13
A1	3.61, 3.86	65.85	3.63, 3.87	65.80	4.95	104.15
A2	3.90	73.18	3.92	73.10	3.85	72.37
A3	3.67	71.93	3.68	71.90	3.82	71.71
A4	4.00	78.76	4.02	78.70	3.90	79.17
A5	3.82	71.90	3.89	71.70	3.81	73.15
A6	3.87, 4.01	67.66	3.92, 4.06	68.30	3.98	65.32
B1	4.68	103.53	4.64	103.50	4.61	103.96
B2	3.83	57.14	3.85	57.11	3.86	56.98
B3	3.71	83.68	3.76	83.60	3.80	83.50
B4	3.47	71.87	3.49	71.80	3.52	73.38
B5	3.55	77.47	3.54	77.50	3.57	77.98
B6	3.69, 3.92	63.79	3.71, 3.94	63.80	3.69, 3.76	63.44
C1	4.48	104.29	4.49	104.20	4.50	104.20
C2	3.83	55.23	3.84	55.21	3.84	55.26
C3	3.72	73.18	3.74	73.20	3.73	73.25
C4	3.90	70.39	3.91	70.40	3.91	70.45
C5	3.69	77.70	3.69	77.70	3.68	77.69
C6	3.74	63.64	3.77	63.80	3.75	63.70

[a] $^{1}H-^{13}C$ HMQC NMR correlation spectra of compound **4** and the corresponding isolated derivative are presented in the Supporting Information (Figure SI 1). Letters refer to the sugar residues proceeding from the reducing end, *A* = Man, *B* = GlcNAc, and *C* = GalNAc, with the associated numbers referring to the carbon atom involved.

reduced trisaccharide **4** and the glycopeptide **5** were very similar, while the structural change associated with the cyclization of the mannitol in **4** to a mannosyl in **5** is, as expected, accompanied by some larger chemical shift changes. The insensitivity of the shifts of the peripheral sugars between the trisaccharide and the glycopeptide suggests at most limited interactions between these carbohydrate residues and the peptide backbone.

Electron micrograph images of the mucin-like central region of α-DG, which contains multiple *O*-Man and *O*-GalNAc residues,[43] indicate that it has an extended global conformation. This type of organization has been recognized for sites with *O*-GalNAc substitutions, and can be rationalized in terms of intramolecular interactions between the proximal sugar residue and the polypeptide backbone.[44] To establish whether an *O*-Man-6-P residue can affect the organization of the peptide backbone, the patterns of peptide amide shifts, which are known to be sensitive to backbone conformation, were analyzed for glycopeptide **5**, the similar unmodified peptide **26** (Ac-GAIIQTPTLG-NH_2), and *O*-GalNAc derivatized glycopeptide **27** (Ac-GAIIQT(α-GalNAc)PTLG-NH_2).

Amide proton resonances of the peptide and glycopeptides were observed in 90% H_2O/10% D_2O solutions. TOCSY spectra[42] with correlations originating from the amides were used to identify the type of amino acid associated with the individual amides. This experiment was complemented by ROESY[42] spectra that provide through-space correlations between the NH and the α proton of the preceding residue to establish the identity of the neighboring residue, thereby resolving ambiguities where there are multiple instances of the same

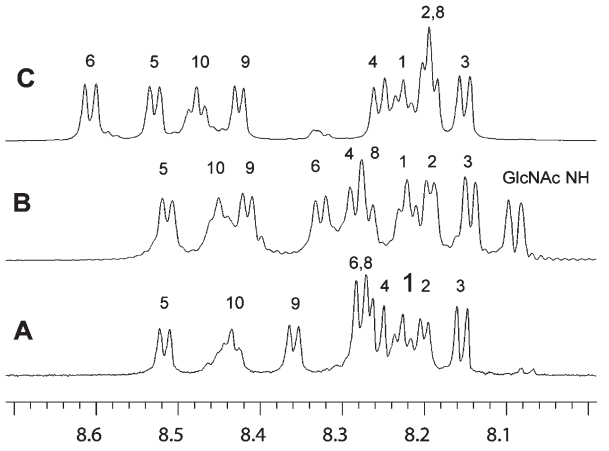


Figure 2. ^{1}H NMR spectra taken in 90% H_2O/10% D_2O of the amide protons of (A) Ac-GAIIQTPTLG-NH_2 (**26**), (B) this peptide with the phophorylated trisaccharide attached at T6 (**5**), and (C) this peptide with an α-linked GalNAc at T6 (**27**). Peak numbering by residue starting from N-termini, chemical shifts relative to DSS.

Table 2. Kinetic Parameters for the Modification of Glycopeptides 28−33 by POMGnT1

glycopeptide	K_m (mM)	V_{max} (nmol/h)
GAIIQT(Man)PTLG (**28**)	1.7	1.03
GAIIQT(Man-6-P)PTLG (**29**)	no reaction	-
GAIIQTPT(Man)LG (**30**)	no reaction	-
GAIIQT(Man)PT(Man)LG (**31**)	2.3	1.17
YVEPT(Man)AV (**32**)	4.0	0.52
RIRTT(Man)TSGVPR (**33**)	no reaction	-

amino acid. As can be seen in Figure 2, the patterns of peptide amide shifts of phosphoglycopeptide **5** (B) and peptide **26** (A) are very similar. The largest difference due to the presence of the *O*-Man-6-P modification was a downfield shifts of ∼0.04 ppm for amides of residue 6 (saccharide modified T) and residue 9, indicating the absence of significant peptide conformational differences between two compounds. Glycopeptide **27** (C), which is modified by an *O*-GalNAc moiety, exhibited more pronounced changes, and in particular, the amide proton of glycosylated T6 was shifted downfield by 0.35 ppm shift and T8 upfield by ∼0.07 ppm. These observations are consistent with structural stabilization of **27** that is expected to arise from hydrogen bonding interactions between the *O*-GalNAc amide proton and the peptide backbone as well as interactions between the methyl group of the acetamide moiety of *O*-GalNAc and the side chain of the amino acid in the *i*+2 position of mucin structures.[44]

The data are consistent with a model in which mannosyl modification does not exert major conformational effect on the peptide backbone.[45] These residues are, however, introduced at the early stages of glycoprotein glycosylation (ER-based process) and therefore have an ability to regulate the loci of subsequent *O*-GalNAc additions through the action of polypeptide *O*-GalNAc glycotransferases (Golgi-based process). Thus, by influencing the patterning of *O*-GalNAc moieties on α-DG, *O*-mannosylation will indirectly influence the protein backbone conformation, which in turn is expected to impact specific molecular recognition.

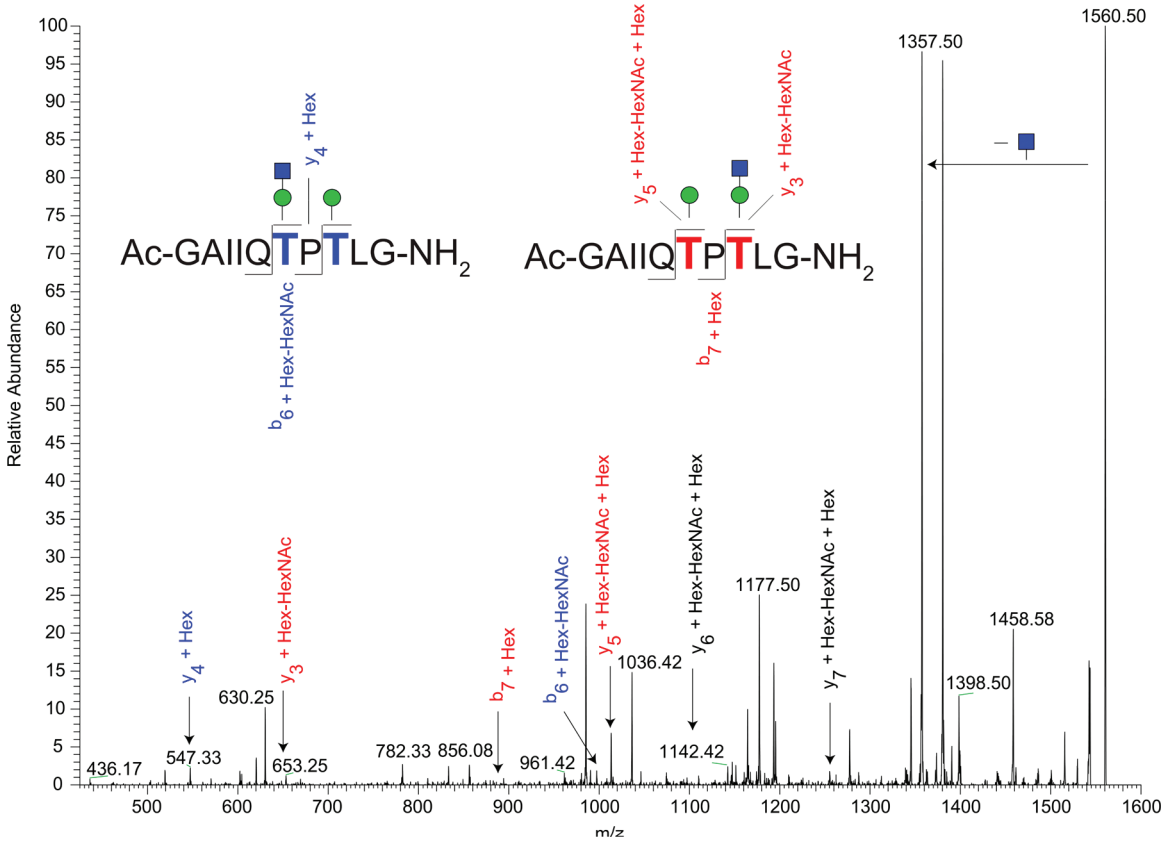


Figure 3. MS/MS fragmentation of **31** following incubation with recombinant POMGnT1 (m/z 1560) yielded a glycopeptide mixture containing one nonextended mannose and one GlcNAc-extended mannose as defined by accurate mass measurement in which one glycopeptide was extended at Thr379 while the other isobaric glycopeptide was extended at Thr381. The generated b and y fragment ions allowed for confident identification and assignment of the extended Thr residues. The b and y fragment ions shown in blue support the extension of Thr 379, while the b and y ions shown in red support the extension of O-mannosyl residue on Thr381. In particular, the y3+Hex-HexNAc defines Thr381 as a site of extension while b6+Hex-HexNAc define Thr-379 as being extended.

Enzymatic Studies. Recent studies have shown that, in addition of β-D-GlcNAc-(1−2)-α-D-Man-Thr motifs, α-DG is modified with an unusual β-D-GlcNAc-(1−4)-6-P-α-D-Man-Thr moiety in the core of a glycan that is functionally relevant.[21] Biosynthetic mechanisms that control elaboration α-D-Man-Thr into various classes of extended structures have yet to be defined. So far, the phosphorylated trisaccharide β-D-GalNAc-(1−3)-β-D-GlcNAc-(1−4)-6-O-P-α-D-Man has only been associated with a specific site of α-DG, namely, Thr379, though other loci have been suggested,[21] and it appears that this site is not modified by the more common tetrasaccharide structure (compound **1**, Figure 1). Thus, a specific mechanism must control 1,2- vs 1,4-extension of *O*-mannosyl residues in a site-specific manner. It is possible that phosphorylation of an *O*-mannosyl residue blocks the action of POMGnT1 thereby preventing the formation of (1−2)-linked oligosaccharides. Furthermore, it is conceivable that the underlying peptide influences the activity of POMGnT1, directing activity to specific positions.

To test these hypotheses, glycopeptides **28**−**31**, based on the sequence GAIIQTPTLG (amino acids 374−383 of full-length α-DG) were prepared, including those modified with the unusual phospho-trisaccharide (see Figure 1, compounds **2** and **3**). Thus, compounds **28** and **29** have an *O*-Man or *O*-Man-6-P on T6 (corresponding to Thr379 of α-DG), respectively. Compound **30** carries an *O*-Man residue on T8 and derivative **31** is bis-*O*-mannosylated at T-6 and T-8. In addition, compounds **32** and **33** were prepared which are derived from residues 411−416 and 479−489 of α-DG and have an *O*-Man moiety at one of the threonine residues. The apparent kinetic parameters for the addition of a β(1−2)-*O*-GlcNAc moiety to the glycopeptide acceptors **28**−**33** by recombinant POMGnT1 were determined following earlier reported assays.[17,18] As can be seen in Table 2, the mannosyl moiety of glycopeptide **28** was readily modified by POMGnT1, whereas the phophorylated derivative **29** was not an acceptor for the enzyme. Interestingly, the mannosyl moiety at T8 of glycopeptide **30** was also not an acceptor for POMGnT1, while the bis-*O*-mannosylated glycopeptide **31** exhibited similar kinetic parameters compared to monomannosylated derivative **28**. The transformed product of **31** was further examined by tandem mass spectrometry. The resulting MS/MS spectra revealed a mixture of two structurally similar glycopeptides in which either Thr379 or Thr381 was observed to be extended with a *O*-GlcNAc residue following incubation with the recombinant POMGnT1 enzyme (Figure 3). Based on the b and y fragment ions generated from the CID of m/z 1560, a confident assignment can be made confirming that one or the other of the *O*-mannosyl residues were extended with a GlcNAc. For example, the observation of b6 + Hex-HexNAc and y4 + Hex indicates that Thr379 is extended with GlcNAc in one glycoform. Likewise, the observation of b7 + Hex and y3 + Hex-HexNAc in the

same MS/MS spectra indicated that Thr381 is extended with a GlcNAc residue in the other. Although we were able to observe signature b and y ions indicating the presence of two peptides with a single Thr extended, we were unable to observe an instance in which both Thr residues were extended simultaneously. Compound **32** (411−416 of α-DG) exhibited glycosyl-accepting properties for POMGnT1; however, the Km was less favorable compared to those of compound **28**, and finally, compound **33** (479−489 of α-DG) was not modified by the enzyme.

Collectively, the data suggest a model in which regulation of modification of *O*-mannosyl residues by either a β(1,2)- or β(1,4)-*O*-GlcNAc moiety is controlled by phosphorylation of the C-6 position of an *O*-mannosyl residue at an early stage of the biosynthetic transformation. Although the more common *O*-mannosyl structures having a β-D-GlcNAc-(1−2)-α-D-Man linkage have not been observed at T379 of α-DG, experiments with model peptides **28** and **31** indicate that this site can readily be modified by POMGnT1. It is well-established that mannosylation of Ser and Thr moieties of α-DG by POMT1 and POMT2 takes place in the ER, whereas POMGnT1 appears to be located in the Golgi apparatus. Thus, it is conceivable that a site-specific phosphorylation of *O*-Man can direct its modification to a β-D-GlcNAc-(1−4)-6-P-α-D-Man moiety. Future identification of the kinase and GlcNAc-transferase responsible for the biosynthesis of the unusual phospho-trisaccharide will make it possible to confirm the model.

The data presented in Table 2 also indicate that the modification preference of *O*-Man residues by POMGnT1 is modulated by the peptide sequence and glycosylation status of neighboring sites. A particularly surprising finding was that compound **30**, which has an *O*-Man residue at T-8, was not modified by POMGnT1, whereas compound **31**, which has an *O*-Man at T-6 and T-8, was glycosylated by this enzyme at either site, but not both. While this complicates the quantitative analysis of the enzyme kinetic results, it points out that proximal glycosylation as well as peptide sequence can affect the locus of POMGnT1 activity. Further studies are required to provide a rationale for this observation.

CONCLUSIONS

Although it has been evident that the glycan structures on α-DG, particularly those associated with *O*-mannosyl linkages, are key elements in critical interaction with components of the extracellular matrix (ECM), only recently have structural features of compounds been revealed that directly interact with the ECM. The core structure has features distinct from the more abundant *O*-mannosyl glycan, which, although apparently not directly a ligand for the ECM, plays a role in the ultimate function of α-DG. The modification of the initiating *O*-mannosyl residues by a β(1−2)-GlcNAc or a β(1−4)-GlcNAc moiety raises intriguing questions regarding the regulation of biosynthetic pathways associated with these structures. This issue is all the more significant in light of the pathologies associated with aberrations in either of these glycans. Faced with the myriad difficulties of fully elucidating these aspects using cell-based studies, we have exploited chemical synthesis to provide appropriate well-defined models of intermediate forms of the post-translational pathway. The availability of these reagents has allowed us to pursue the experiments described, which has provided insights into glycan modifications of this glycoprotein.

The enzyme POMGnT1 was previously assumed to comprise the obligatory activity in extending the initial *O*-Man residue with a β(1−2)-GlcNAc residue. The recent elucidation of a novel *O*-mannosyl modification having a β(1−4) linked *O*-GlcNAc, as well as a 6-phosphate, suggests the existence of previously unappreciated enzymatic activities. While several questions remain to be answered, the observations reported here shed light on the biosynthesis of *O*-mannosylated structures of α-DG and the impact of POMGnT1 on determining the structural fate of the glycan. First, the chemical synthesis of an alditol analogue of the phospho-glycan confirmed the proper structural elucidation of the novel form of glycosylation. Evaluation of several glycopeptides indicates that when the relevant *O*-Man on residue T379 does not have a 6-phosphoryl modification; it is an appropriate substrate for POMGnT1, which, if modified by this enzyme, would direct the glycan to a different biosynthetic path. Furthermore, previous glycan mapping of α-DG suggests that residue T379 is not modified by the more common tetrasaccharide structure. These observations suggest that regulation by a temporal separation needs to exist between the activities associated with generating the Man-6-P structures and exposure to POMGnT1, and suggests that the former shows considerable specificity to this site in the sequence. The use of several possible *O*-man glycopeptide substrates for POMGnT1 also demonstrated that its activity is modulated by the underlying polypeptide sequence and the presence of glycans in the vicinity of a potential acceptor site. Conformational analysis by NMR of several glycopeptides showed that the *O*-mannosyl modification does not exert major conformational effect on the peptide backbone. These residues are, however, introduced at the early stages of glycoprotein glycosylation, and therefore have an ability to regulate the loci of subsequent *O*-GalNAc additions, which do exert conformational effects. Collectively, the results reveal additional levels of complexity in the regulation of *O*-glycan processing on α-DG and provide important new clues in searching for the enzyme activities associated with critical steps in the processing of α-DG and spatial localization of glycans. The synthetic phosphoglycopeptides presented here will provide further opportunities to explore the biosynthesis and biological properties of glycans of α-DG. In particular, we anticipate that they will make it possible to identify enzymes associated with phosphorylation and β(1−4)GlcNAcT activity and the chemical nature of the phosphodiester moiety.

EXPERIMENTAL PROCEDURES

Reagents and General Procedures. Reagents were obtained from commercial sources and used as purchased. Dichloromethane (DCM) was freshly distilled using standard procedures. Other organic solvents were purchased anhydrous and used without further purification. Unless otherwise noted, all reactions were carried out at room temperature in oven-dried glassware with magnetic stirring. Molecular sieves were flame-dried under high vacuum prior to use. Organic solutions were concentrated under diminished pressure with bath temperatures <40 °C. Flash column chromatography was carried out on silica gel G60 (Silicycle, 60−200 μm, 60 Å). Thin-layer chromatography (TLC) was carried out on Silica gel 60 F_{254} (EMD Chemicals Inc.) with detection by UV absorption (254 nm) were applicable, and by spraying with 20% sulfuric acid in ethanol followed by charring at ∼150 °C or by spraying with a solution of $(NH_4)_6Mo_7O_{24}.H_2O$ (25 g/L) in 10% sulfuric acid in ethanol followed by charring at ∼150 °C. ^1H and ^{13}C NMR spectra were recorded on a Varian

Inova-300 (300/75 MHz), a Varian Inova-500 (500/125 MHz), and a Varian Inova-600 (600/150 MHz) spectrometer equipped with Sun workstations. Multiplicities are quoted as singlet (s), broad singlet (br s), doublet (d), doublet of doublets (dd), triplet (t), or multiplet (m). Spectra were assigned using COSY, DEPT, and HSQC experiments. Signals marked with a superscript Roman numeral I were the reducing end, whereas II and III were the second sugar from the reducing end and the nonreducing end, respectively. All chemical shifts are quoted on the δ-scale in parts per million (ppm). Residual solvent signals were used as an internal reference. Reverse-phase HPLC was performed on an Aglient 1200 series system equipped with an autosampler, fraction-collector, UV-detector, and eclipse XDB-C18 column (5 μm, 4.6 × 250 mm or 9.4 × 250 mm) at a flow rate of 1.5 mL/min. Mass spectra were recorded on an Applied Biosystems 4800 MALDI-TOF proteomics analyzer. The matrix used was 2,5-dihydroxybenzoic acid (DHB) and Ultamark 1621 as the internal standard.

General Methods for Glycopeptide Synthesis. Glycopeptides derived from the GAIIQTPTLG sequence were synthesized by an automatic solid-phase peptide synthesizer (Applied Biosystems, ABI 433A) on Rink amide resin (79 mg, 0.05 mmol, loading: 0.63 mmol/g) or Sieber amide resin (70.4 mg, 0.05 mmol, loading: 0.71 mmol/g) via N^{α}-Fmoc-based approach. 2-(1*H*-Benzotriazole-1-yl)-oxy-1,1,3,3-tetramethyl-uronium hexafluorophosphate (HBTU)/1-hydroxybenzotriazole (HOBt) were employed as the coupling reagents. The coupling of glycosylated amino acids was performed manually using 2-(7-aza-1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU)/*N*,*N*-diisopropylethylamine (DIPEA) as the activating reagents. Upon completion of the manual coupling reaction as determined by the Kaiser test, resins were resubjected to the automatic synthesizer and completed the elongation.

Dimethylthexylsilyl *O*-[3,4,6-tri-*O*-Acetyl-2-deoxy-(2,2,2-trichloroethoxy)carbonylamino-β-D-galactopyranosyl]-(1→3)-2-deoxy-4,6-*O*-(2-naphthylidene)-2-(2,2,2-trichloroethoxy)-carbonylamino-β-D-glucopyranoside (10). A mixture of the glucosyl acceptor **7** (0.45 g, 0.66 mmol) and galactosyl donor **6** (0.38 g, 0.79 mmol) and 4 Å molecular sieves (1.3 g) in DCM (15 mL) was stirred under Ar for 1 h. The reaction was cooled (0 °C) and NIS (267 mg, 1.19 mmol) was added followed by the addition of TMSOTf (29 μL, 0.16 mmol). After stirring for 30 min, the reaction was quenched by the addition of Et_3N (0.5 mL). The mixture was filtered, and the filtrate (100 mL) was washed with 10% $Na_2S_2O_3$ (120 mL) and brine (100 mL). The organic phase was dried ($MgSO_4$) and filtered, and the filtrate was concentrated under reduced pressure. The resulting yellow oil was purified by flash chromatography over silica gel (EtOAc/hexanes, 2/5, v/v) to give **10** (0.66 g, 91%) as an amorphous white solid: R_f = 0.21 (EtOAc/hexanes, 1/3, v/v). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.92−7.46 (m, 7H), 5.69 (s, 1H, >C*H*Nap), 5.42 (br s, 1H, N*H*Troc^{I}), 5.25 (s, 1H, H-4^{II}), 5.13−5.03 (m, 3H, H-1^{I}, H-3^{II} and N*H*Troc^{II}), 4.79−4.56 (m, 5H, H-1^{II} and OC*H*$_2$CCl$_3^{I\text{ and }II}$), 4.38 (br s, 1H, H-3^{I}), 4.32 (dd, 1H, *J* = 4.8 and 10.3 Hz, H-6e^{I}), 4.09 (br s, 1H, H-6e^{II}), 3.85−3.71 (m, 5H, H-4^{I}, H-6a^{I}, H-2^{II}, H-5^{II} and H-6a^{II}), 3.51 (br s, 1H, H-5^{I}), 3.19 (br s, 1H, H-2^{I}), 2.09 (s, 3H), 1.88 (s, 3H), 1.82 (br s, 3H), 1.64−1.59 (m, 1H), 0.87−0.83 (m, 12H), 0.14 (s, 3H), 0.12 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 170.24, 154.00, 153.88, 134.60, 133.82, 132.93, 128.45, 128.39, 127.90, 126.67, 126.41, 125.71, 123.73, 101.64, 101.22 (C-1^{II}), 95.71, 95.56 (C-1^{I}), 95.45, 80.07 (C-4^{I}), 77.76 (C-3^{I}), 74.63, 74.43, 70.72 (C-5^{II}), 69.58 (C-3^{II}), 68.86 (C-6^{I}), 66.47 (C-4^{II}), 66.34 (C-5^{I}), 61.02 (C-6^{II}), 60.50 (C-2^{I}), 53.16 (C-2^{II}), 34.03, 24.91, 20.77, 20.60, 20.54, 20.14, 20.10, 18.68, 18.65, −1.79, −3.22. HR MALDI-TOF MS *m*/*z*: calcd for $C_{43}H_{56}Cl_6N_2O_{16}Si$ [M+Na]$^+$, 1117.1428; found 1117.1441.

***O*-[3,4,6-Tri-*O*-acetyl-2-deoxy-(2,2,2-trichloroethoxy)-carbonylamino-β-D-galactopyranosyl]-(1→3)-2-deoxy-4,6-*O*-(2-naphthylidene)-2-(2,2,2-trichloroethoxy)-carbonylamino-α-D-glucopyranosyl Trichloroacetimidate (12).** To a stirred and cooled (0 °C) solution of **10** (524 mg, 0.478 mmol) in a mixture of THF and AcOH (20/1, v/v, 7.2 mL) was added 1 M TBAF (1.43 mL, 1.43 mmol). After stirring at room temperature for 24 h, the reaction mixture was diluted with DCM (180 mL) and washed with saturated $NaHCO_3$ (2 × 170 mL) and brine (120 mL). The organic layer was dried ($MgSO_4$) and filtered, and the filtrate was concentrated under reduced pressure. The resulting yellow oil was purified by flash chromatography over silica gel (EtOAc/hexanes, 1/1, v/v) to give **11** as an inseparable mixture of α- and β-anomer (378 mg, 83%) as an amorphous white solid: R_f = 0.34 (EtOAc/hexanes, 1/1, v/v). HR MALDI-TOF MS *m*/*z*: calcd for $C_{35}H_{38}Cl_6N_2O_{16}$ [M+Na]$^+$, 975.0250; found 975.0255. To a stirred and cooled (0 °C) solution of α- and β-hemiacetals **11** (350 mg, 0.368 mmol) in DCM (5.5 mL) was added trichloroacetonitrile (3.68 mL, 3.68 mmol) followed by Cs_2CO_3 (120 mg, 0.368 mmol) under Ar. After stirring at room temperature for 2 h, the reaction mixture was filtered, and the filtrate was concentrated under reduced pressure. The resulting yellow oil was purified by flash chromatography over silica gel (EtOAc/hexanes, 1/2, v/v) to give **11** (347 mg, 86%) as an amorphous white solid: R_f = 0.19 (EtOAc/hexanes, 1/2, v/v). ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.73 (s, 1H, $Cl_3CC{=}NH$), 7.95−7.45 (m, 7H), 6.60 (br s, 1H, H-1^{I}), 5.74 (s, 1H, >C*H*Nap), 5.71 (d, 1H, *J* = 4.5 Hz, N*H*Troc^{I}), 5.45 (dd, *J* = 3 and 11.4 Hz, 1H, H-3^{II}), 5.29 (d, *J* = 2.7 Hz, 1H, H-4^{II}), 5.16 (d, *J* = 9 Hz, 1H, H-1^{II}), 5.07 (br s, 1H, N*H*Troc^{II}), 4.84−4.54 (m, 4H, OC*H*$_2$CCl$_3^{I\text{ and }II}$), 4.38 (dd, 1H, *J* = 4.7 and 10.4 Hz, H-6e^{I}), 4.29 (t, *J* = 9.8 Hz, 1H, H-3^{I}), 4.13−3.83 (m, 7H, H-2^{I}, H-4^{I}, H-5^{I}, H-6a^{I}, H-5^{II} and H-6a^{II} and H-6e^{II}), 3.51−3.42 (m, 1H, H-2^{II}), 21.5 (s, 3H), 1.88 (s, 3H), 1.76 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 170.13, 169.97, 169.91, 160.48, 154.51, 153.85, 134.02, 133.80, 133.00, 128.81, 128.45, 127.90, 127.02, 126.67, 125.86, 123.33, 102.45, 98.35 (C-1^{II}), 95.56 (C-1^{I}), 95.50, 95.06, 90.99, 80.07 (C-4^{I}), 74.68, 74.28, 72.19 (C-3^{I}), 71.01 (C-5^{II}), 66.73 (C-6^{I}), 68.31 (C-3^{II}), 66.35 (C-4^{II}), 65.85 (C-5^{I}), 60.68 (C-6^{II}), 54.73 (C-2^{I}), 52.40 (C-2^{II}), 20.81, 20.60, 20.36.

Ethyl *O*-[3,4,6-Tri-*O*-acetyl-2-deoxy-2-(2,2,2-trichloroethoxy)-carbonylamino-β-D-galacto-pyranosyl]-(1→3)-*O*-[2-deoxy-4,6-*O*-(2-naphthylidene)-2-(2,2,2-trichloroethoxy)carbonyl-amino-β-D-glucopyranosyl]-(1→4)-2-*O*-acetyl-6-*O*-levulinoyl-3-*O*-(2-methylnaphthyl)-1-thio-α-D-mannopyranoside (13). A mixture of the thioglycosyl acceptor **8** (250 mg, 0.50 mmol) and trichloroacetimidate **12** (367 mg, 0.33 mmol) and 4 Å molecular sieves (900 mg) in DCM (9 mL) was stirred under Ar for 1 h. The reaction was cooled (−35 °C) and TMSOTf (12 μL, 0.066 mmol) was added. After stirring at −30 °C for 1.5 h, the reaction was quenched by the addition of Et_3N (0.1 mL). The reaction mixture was filtered, and the filtrate was concentrated under reduced pressure. The resulting yellow oil was purified by flash chromatography over silica gel (EtOAc/hexanes, 1/1, v/v) to give **13** (372 mg, 86%) as an amorphous white solid: R_f = 0.26 (EtOAc/hexanes, 1/1, v/v). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.85−7.42 (m, 14H), 5.73 (br s, 1H, N*H*Troc^{II}), 5.52 (s, 1H, >C*H*Nap), 5.39 (s, 1H, H-2^{I}); 5.24−5.13 (m, 4H, H-1^{I}, H-3^{III}, H-4^{III} and N*H*Troc^{III}), 4.94−4.69 (m, 8H, H-1^{II}, H-1^{III}, OC*H*$_2$Nap and OC*H*$_2$CCl$_3^{II\text{ and }III}$), 4.57 (br s, 1H, H-6a^{I}), 4.25−4.16 (m, 3H, H-5^{I}, H-6e^{I} and H-3^{II}), 4.03−4.00 (m, 3H, H-4^{I}, H-6e^{II} and H-6e^{III}), 3.86 (dd, 1H, *J* = 3.5 and 9 Hz, H-3^{I}), 3.81 (br s, 1H, H-6a^{III}), 3.70−3.63 (m, 4H, H-2^{II}, H-4^{II}, H-2^{III} and H-5^{III}), 3.49 (t, 1H, *J* = 10.3 Hz, H-6a^{II}), 3.41 (br s, 1H, H-5^{II}), 2.93−2.52 (m, 6H), 2.25 (s, 3H), 2.15 (s, 3H), 2.07 (s, 3H), 1.85 (s, 3H), 1.77 (br s, 3H), 1.25 (t, 1H, *J* = 7.3 Hz). ^{13}C NMR (75 MHz, $CDCl_3$): δ 207.87, 172.92, 170.25, 170.22, 154.45, 154.05, 135.77, 134.58, 133.82, 133.37, 133.09, 132.92, 128.43, 128.34, 128.21, 128.01, 127.94, 127.87, 126.66, 126.39, 126.33, 126.01, 125.65, 125.55, 123.65, 101.54, 101.07 (C-1$^{II\text{ and }III}$), 96.01, 95.85, 82.78 (C-1^{I}), 80.22 (C-4^{II}), 78.42 (C-3^{II}), 77.44 (C-3^{I}), 76.81 (C-4^{I}), 76.00, 74.51, 72.44, 71.15 (C-2^{I}), 70.77 (C-5^{III}), 69.68 (C-5^{I} and C-3^{III}), 68.78 (C-6^{II}), 66.48 (C-4^{III}), 66.07

(C-5^{II}), 63.70 (C-6^{I}), 60.88 (C-6^{III}), 58.28 (C-2^{II}), 53.31 (C-2^{III}), 38.10, 30.19, 28.10, 25.69, 21.29, 20.77, 20.55, 20.52, 15.02. HR MALDI-TOF MS *m*/*z*: calcd for $C_{61}H_{68}Cl_6N_2O_{23}S$ $[M+Na]^+$, 1461.1962; found 1461.1974.

***N*$^{\alpha}$-(9-Fluorenylmethyloxycarbonyl)-*O*-{[3,4,6-tri-*O*-acetyl-2-deoxy-2-(2,2,2-trichloro-ethoxy)carbonylamino-β-D-galactopyranosyl]-(1→3)-*O*-[2-deoxy-4,6-*O*-(2-naphthylidene)-2-(2,2,2-trichloroethoxy)carbonylamino-β-D-glucopyranosyl]-(1→4)-2-*O*-acetyl-6-*O*-levulinoyl-3-*O*-(2-methylnaphthyl)-α-D-mannopyranosyl}-L-threonine allyl Ester (14).** A mixture of the threonine acceptor **9** (168 mg, 0.44 mmol) and glycosyl donor 13 (320 mg, 0.22 mmol) and 4 Å molecular sieves (700 mg) in DCM (7 mL) was stirred under Ar for 1 h. The reaction was cooled (−25 °C) and NIS (74 mg, 0.33 mmol) was added, followed by the addition of AgOTf (11 mg, 0.044 mmol) in toluene (0.44 mL). After stirring at −20 °C for 2 h, the reaction was quenched by the addition of pyridine (0.5 mL). The reaction mixture was filtered, and the filtrate (∼100 mL) was washed with 10% $Na_2S_2O_3$ (120 mL) and brine (100 mL). The organic phase was dried ($MgSO_4$) and filtered and the filtrate was concentrated under reduced pressure. The resulting yellow oil was purified by flash chromatography over silica gel (EtOAc/hexanes, 2/3→1/1, v/v) to give **14** (279 mg, 72%) as an amorphous white solid: R_f = 0.31 (EtOAc/hexanes, 1/1, v/v). ^{1}H NMR (500 MHz, $CDCl_3$): 7.86−7.31 (m, 22H), 5.91−5.83 (m, 1H, $OCH_2CH{=}CH_2$), 5.77 (br s, 1H, N*H*Troc^{II}), 5.53 (s, 1H, >C*H*Nap), 5.41 (d, *J* = 10 Hz, 1H, N*H*Fmoc), 5.33−5.11 (m, 6H, H-2^{I}, H-3^{III}, H-4^{III}, N*H*Troc^{III} and $OCH_2CH{=}CH_2$), 4.89−4.43 (m, 15H, H-1^{I}, H-6a^{I}, H-1^{II}, H-1^{III}, OCH_2Nap, $OCH_2CCl_3$$^{II\ and\ III}$, $OCH_2CH{=}CH_2$, CH_2CHFmoc and C*H*COOAll), 4.33−4.14 (m, 4H, H-6e^{I}, H-3^{II}, $OCHCH_3$ and CH_2CHFmoc), 4.01−3.91 (m, 6H, H-3^{I}, H-4^{I}, H-5^{I}, H-6e^{II} H-6a^{III} and H-6e^{III}), 3.68−3.65 (m, 4H, H-2^{II}, H-4^{II}, H-2^{III} and H-5^{III}), 3.50 (t, 1H, *J* = 9.8 Hz, H-6a^{II}), 3.37 (br s, 1H, H-5^{II}), 2.86−2.54 (m, 4H), 2.24 (s, 3H), 2.12 (s, 3H), 2.07 (s, 3H), 1.84 (s, 3H), 1.76 (br s, 3H), 1.26 (d, 3H, *J* = 6 Hz, $OCHCH_3$). ^{13}C NMR (75 MHz, $CDCl_3$): δ 207.87, 172.89, 170.23, 170.08, 169.92, 156.79, 154.51, 154.05, 144.02, 143.94, 141.49, 135.86, 134.53, 133.87, 133.43, 133.13, 132.95, 131.58, 128.46, 128.40, 128.29, 128.04, 127.98, 127.95, 127.90, 127.33, 126.72, 126.43, 126.08, 125.86, 125.69, 125.42, 125.38, 125.34, 123.66, 120.20, 119.70, 101.63, 101.07 (C-1^{I}), 99.60 (C-1$^{II\ and\ III}$), 96.07, 95.81, 80.28 (C-4^{II}), 78.31 (C-3^{II}), 77.43, 76.18 (C-3^{I}), 75.98 (C-4^{I}), 74.54, 72.36, 70.80 (C-5^{III}), 69.74 (C-5^{I}), 69.57 (C-3^{III} or C-4^{III}), 69.30 (C-3^{III} or C-4^{III}), 68.78 (C-6^{II}), 67.62, 66.73, 66.50 (C-2^{I}), 66.17 (C-5^{II}), 63.64 (C-6^{I}), 60.85 (C-6^{III}), 58.90, 58.42 (C-2^{II}), 53.34 (C-2^{III}), 47.35, 38.11, 30.22, 29.86, 28.08, 21.20, 20.79, 20.56, 20.55, 18.34. HR MALDI-TOF MS *m*/*z*: calcd for $C_{81}H_{85}Cl_6N_3O_{28}$ $[M+Na]^+$, 1780.3348; found 1780.3366.

***N*-(9-Fluorenylmethyloxycarbonyl)-*O*-{[3,4,6-tri-*O*-acetyl-2-deoxy-2-(2,2,2-trichloro-ethoxy)carbonylamino-β-D-galactopyranosyl]-(1→3)-*O*-[2-deoxy-4,6-*O*-(2-naphthylidene)-2-(2,2,2-trichloroethoxy)carbonylamino-β-D-glucopyranosyl]-(1→4)-2-*O*-acetyl-6-*O*-(1,5-dihydro-3-oxo-3λ^5-3*H*-2,4,3-benzodioxaphosphepin-3yl)-3-*O*-(2-methylnaphthyl)-α-D-mannopyranosyl}-L-threonine allyl ester (16).** To a stirred solution of **14** (245 mg, 0.139 mmol) in a mixture of MeOH and DCM (5 mL, 1/8, v/v) was added hydrazine acetate (26 mg, 0.278 mmol). After stirring for 1.5 h, the reaction mixture was diluted with DCM (50 mL) and washed with saturated $NaHCO_3$ (2 × 45 mL) and brine (40 mL). The organic phase was dried ($MgSO_4$) and filtered and the filtrate was concentrated under reduced pressure to afford crude **15** as an amorphous white solid. Compound **15** was dissolved in DCM (6 mL) and placed under an atmosphere of Ar, and subsequently *N*,*N*-diethyl-1,5-dihyro-2,4,3-benzodioxaphosphepin-3-amine (67 mg, 0.278 mmol) and 1*H*-tetrazole (3 wt % solution in CH_3CN, 1.6 mL, 0.556 mmol) were added. After stirring for 2 h, the reaction was cooled (−20 °C) and *m*-CPBA (77%, 124 mg, 0.556 mmol) was added. After stirring for 30 min, the reaction mixture was diluted with DCM (80 mL) and washed with saturated $NaHCO_3$ (2 × 70 mL) and brine (60 mL). The organic layer was dried ($MgSO_4$) and filtered and the filtrate was concentrated under reduced pressure. The resulting yellow oil was purified by flash chromatography over silica gel (EtOAc/hexanes, 2/3→1/1, v/v) to give **16** (182 mg, 71% over 3 steps) as an amorphous white solid: R_f = 0.41 (EtOAc/hexanes, 1/1, v/v). ^{1}H NMR (500 MHz, $CDCl_3$): 7.85−7.77 (m, 26H), 7.18 (d, *J* = 8.5 Hz, 1H, N*H*Troc^{II}), 5.89−5.81 (m, 1H, $OCH_2CH{=}CH_2$), 5.54 (s, 1H, >C*H*Nap), 5.42−5.06 (m, 12H, H-2^{I}, H-3^{III}, H-4^{III}, N*H*Fmoc, N*H*Troc^{III}, $OCH_2CH{=}CH_2$, two $POCH_2$ and one proton from $OCH_2CCl_3$$^{II\ or\ III}$), 4.87−4.53 (m, 11H, H-1^{I}, H-6a^{I}, H-1^{II}, H-1^{III}, OCH_2Nap and three protons from $OCH_2CCl_3$$^{II\ and\ III}$, $OCH_2CH{=}CH_2$), 4.44−4.41 (m, CH_2CHFmoc and C*H*COOAll), 4.31−4.23 (m, H-6e^{I}, $OCHCH_3$ and CH_2CHFmoc), 4.11−3.65 (m, 10H, H-3^{I}, H-4^{I}, H-5^{I}, H-2^{II}, H-3^{II}, H-4^{II}, H-6e^{II}, H-2^{III}, H-6a^{III} and H-6e^{III}), 3.57 (br s, 1H, H-5^{III}), 3.46 (t, 1H, *J* = 10 Hz, H-6a^{II}), 3.37 (br s, 1H, H-5^{II}), 2.11 (s, 3H), 2.05 (s, 3H), 1.84 (s, 3H), 1.74 (br s, 3H), 1.23 (d, 3H, *J* = 6 Hz, $OCHCH_3$). ^{13}C NMR (75 MHz, $CDCl_3$): δ 170.29, 170.14, 169.97, 169.77, 156.68, 154.70, 154.17, 143.89, 143.81, 141.38, 135.97, 135.05, 134.86, 134.51, 133.77, 133.32, 133.01, 132.85, 131.48, 129.68, 129.54, 129.32, 129.18, 129.11, 128.38, 128.34 128.07, 127.93, 127.87, 127.82, 127.25, 126.60, 126.32, 126.29, 125.92, 125.84, 125.66, 125.44, 125.28, 125.24, 123.64, 120.11, 119.53, 102.72 (C-1^{I}), 101.62, 101.32 (C-1^{II}), 99.34 (C-1^{III}), 96.43, 95.88, 80.46 (C-4^{II}), 79.05 (C-4^{I}), 77.44, 76.28 (C-3^{II}), 75.68 (C-3^{I}), 74.40, 74.27, 72.89, 70.68, 70.58 (C-5^{I}), 69.81 (C-5^{III}), 69.33 (C-3^{III} or C-4^{III}), 69.24 (C-3^{III} or C-4^{III}), 68.83, 68.74 (C-6^{II}), 67.54, 66.62 (C-2^{I}), 66.44 (C-5^{II}), 66.37 (C-6^{I}), 65.93, 60.93 (C-6^{III}), 58.65, 57.72 (C-2^{II}), 53.06 (C-2^{III}), 47.22, 29.75, 21.11, 20.69, 20.52, 20.44, 18.28. HR MALDI-TOF MS *m*/*z*: calcd for $C_{84}H_{86}Cl_6N_3O_{29}P$ $[M+Na]^+$, 1864.3113; found 1864.3128.

***N*-(9-Fluorenylmethyloxycarbonyl)-*O*-[(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-galactopyranosyl)-(1→3)-*O*-(2-acetamido-4,6-di-O-acetyl-2-deoxy-β-D-glucopyranosyl)-(1→4)-2,3-di-*O*-acetyl-6-*O*-(1,5-dihydro-3-oxo-3λ^5-3*H*-2,4,3-benzodioxaphosphepin-3yl)-α-D-mannopyranosyl]-L-threonine Allyl Ester (17).** To a stirred solution of **16** (100 mg, 0.054 mmol) in DCM (3 mL) and water (0.3 mL) was added 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) (123 mg, 0.54 mmol). After stirring vigorously in the dark for 16 h, the reaction mixture was diluted with DCM (60 mL) and washed with saturated $NaHCO_3$ (2 × 60 mL) and brine (45 mL). The organic phase was dried ($MgSO_4$) and filtered and the filtrate was concentrated under reduced pressure to afford a yellow oil. The crude product was dissolved in glacial acetic acid (2 mL), and zinc metal power (76 mg, 1.16 mmol) was added. After stirring for 1 h, the reaction mixture was filtered, and the filtrate was concentrated under reduced pressure to afford a yellow oil. The crude product was dissolved in pyridine (1 mL), and acetic anhydride (0.2 mL, 2.12 mmol) was added. After stirring for 12 h, the reaction mixture was diluted with DCM (50 mL) and washed with saturated $NaHCO_3$ (2 × 50 mL) and brine (40 mL). The organic phase was dried ($MgSO_4$) and filtered, and the filtrate was concentrated under reduced pressure. The resulting yellow oil was purified by flash chromatography over silica (MeOH/DCM, 1/40→1/30, v/v) to give **17** (43 mg, 56% over 3 steps) as an amorphous white solid: R_f = 0.26 (MeOH/DCM, 1/20, v/v). ^{1}H NMR (500 MHz, $CDCl_3$): 7.76−7.30 (m, 12H), 5.93−5.87 (m, 2H, N*H*Ac^{III} and $OCH_2CH{=}CH_2$), 5.62 (d, *J* = 9.5 Hz, N*H*Fmoc), 5.43 (t, 1H, *J* = 12 Hz, one proton from $POCH_2$), 5.34−5.10 (m, 9H, H-3^{I}, H-3^{III}, H-4^{III}, N*H*Ac^{II}, $OCH_2CH{=}CH_2$ and three protons from $POCH_2$), 5.02 (s, 1H, H-2^{I}), 4.92 (t, 1H, *J* = 8 Hz, H-4^{II}), 4.83−4.81 (m, 2H, H-1^{I} and H-1^{III}), 4.70−4.60 (m, 4H, H-1^{II}, H-6a^{I} and $OCH_2CH{=}CH_2$), 4.48−4.33 (m, 5H, H-6e^{III}, $OCHCH_3$, C*H*COOAll and CH_2CHFmoc), 4.27−4.20 (m, 2H, H-6e^{I} and CH_2CHFmoc), 4.12−3.85 (m, 8H, H-4^{I}, H-5^{I}, H-2^{II}, H-3^{II}, H-6a^{II}, H-6e^{II}, H-5^{III} and H-6a^{III}), 3.74−3.71 (m, 1H, H-5^{II}), 3.65−3.60 (m, 1H, H-2^{III}), 2.10 (s, 3H), 2.09 (s, 3H), 2.07 (s, 3H), 2.05

(s, 3H), 2.01 (s, 3H), 1.99 (s, 3H), 1.96 (s, 3H), 1.94 (s, 3H), 1.28 (d, 3H, J = 4.5 Hz, OCHCH_3). ^{13}C NMR (75 MHz, $CDCl_3$): δ 171.37, 171.21, 171.00, 170.59, 170.40, 170.35, 169.86, 169.76, 169.70, 156.87, 144.03, 143.96, 141.46, 135.01, 134.83, 131.58, 130.00, 129.84, 129.52, 129.40, 127.93, 125.48, 120.17, 119.74, 101.74 (C-1^{II}), 99.67 (C-1^{III}), 99.06 (C-1^{I}), 78.23 (C-3^{II}), 77.44, 74.13 (C-4^{I}), 72.27 (C-5^{II}), 70.83 (C-5^{III}), 70.71(C-5^{I}), 70.49 (C-2^{I}), 70.05, 69.62 (C-4^{II}), 69.54 (C-3^{III} or C-4^{III}), 68.94 (C-3^{III} or C-4^{III}), 67.84, 66.92, 66.83 (C-3^{I}), 65.34 (C-6^{I}), 62.76 (C-6^{III}), 61.16 (C-6^{II}), 58.69, 55.77 (C-2^{II}), 52.27 (C-2^{III}), 47.27, 29.89, 23.66, 23.52, 21.09, 21.03, 20.96, 20.86, 20.83, 18.31. HR MALDI-TOF MS m/z: calcd for $C_{66}H_{80}N_3O_{30}P$ $[M+Na]^+$, 1448.4462; found 1448.4477.

***N*-(9-Fluorenylmethyloxycarbonyl)-*O*-[(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-galactopyranosyl)-(1→3)-*O*-(2-acetamido-4,6-di-O-acetyl-2-deoxy-β-D-glucopyranosyl)-(1→4)-2,3-di-*O*-acetyl-6-*O*-(1,5-dihydro-3-oxo-3λ^5-3*H*-2,4,3-benzodioxaphosphepin-3yl)-α-D-mannopyranosyl]-L-threonine (18).** To a stirred solution of **17** (150 mg, 0.105 mmol) in THF (7 mL) and water (0.7 mL) was added tetrakis(triphenylphosphine)palladium(0) $[Pd(PPh_3)_4]$ (61 mg, 0.0525 mmol) under Ar. After stirring for 1 h, the reaction mixture was filtered through a short pad of silica gel and the filtrate was concentrated under reduced pressure. The resulting yellow oil was purified by flash chromatography over silica gel (MeOH/DCM, 1/20, v/v with 0.1% acetic acid) to give **18** (134 mg, 92%) as an amorphous white solid: R_f = 0.22 (MeOH/DCM, 1/20/, v/v with 0.1% acetic acid). ^{1}H NMR (500 MHz, acetone-d_6): 7.73−7.20 (m, 12H), 7.05 (d, J = 9 Hz, N*H*Fmoc), 6.80 (d, J = 8 Hz, N*H*Ac^{III}), 5.43−5.37 (m, 2H, one POCH_2), 5.28−5.25 (m, 1H, H-3^{III}), 5.17−4.99 (m, 6H, H-2^{I}, H-3^{I}, H-3^{III}, H-4^{III} and one POCH_2), 4.93 (d, J = 8 Hz, H-1^{III}), 4.90 (s, 1H, H-1^{I}), 4.73 (t, J = 9.5 Hz, H-4^{II}), 4.64 (d, J = 8 Hz, H-1^{II}), 4.43−4.36 (m, 3H, H-6a^{I}, H-6e^{I} and OC*H*CH$_3$), 4.27 (br s, 1H, C*H*COOAll), 4.23−3.82 (m, 11H, H-4^{I}, H-5^{I}, H-3^{II}, H-6a^{II}, H-6e^{II}, H-5^{III}, H-6a^{III}, H-6e^{III}, CH_2CHFmoc and CH$_2$C*H*Fmoc), 3.77−3.74 (m, 1H, H-2^{II}), 3.63−3.61 (m, 1H, H-5^{II}), 3.42−3.36 (m, 1H, H-2^{III}), 1.95 (br s, 6H), 1.92 (s, 6H), 1.91 (s, 3H), 1.90 (s, 3H), 1.85 (br s, 6H), 1.83 (s, 3H), 1.29 (d, 3H, J = 6.5 Hz, OCHCH_3). ^{13}C NMR (75 MHz, acetone-d_6): δ 171.13, 171.01, 170.84, 170.69, 170.65, 170.28, 170.14, 170.07, 169.98, 157.74, 145.13, 145.05, 142.11, 137.27, 137.08, 130.33, 130.22, 128.56, 128.04, 126.27, 120.82, 102.41 (C-1^{II}), 100.82 (C-1^{III}), 99.78 (C-1^{I}), 78.06 (C-4^{I}), 77.71, 74.34 (C-3^{II}), 72.51 (C-5^{II}), 71.34, 71.24, 71.06 (C-5^{I}), 70.61 (C-5^{III}), 70.49 (C-2^{I}), 70.16, 69.81 (C-3^{III}), 69.64 (C-3^{I}), 69.52 (C-4^{III}), 69.43, 67.75 (C-4^{II}), 67.44, 66.35 (C-6^{I}), 63.27 (C-6^{II}), 62.02 (C-6^{III}), 59.53, 56.38 (C-2^{II}), 52.89 (C-2^{III}), 48.03, 32.65, 23.76, 23.44, 23.39, 23.35, 21.10, 20.96, 20.77, 20.69, 20.63, 18.71, 14.38. HR MALDI-TOF MS m/z: calcd for $C_{63}H_{76}N_3O_{30}P$ $[M+Na]^+$, 1408.4149; found 1408.4158.

Ac-Gly-Ala-Ile-Ile-Gln-Thr[GalNAc-β-(1−3)-GlcNAc-β-(1−4)-Man(6-PO$_4$)-α-O]-Pro-Thr-Leu-Gly-NH$_2$ (5). According to the general method for glycopeptide synthesis, compound **5** was prepared using Sieber amide resin (70.4 mg, 0.05 mmol, loading: 0.71 mmol/g). The synthetic procedure is similar to that of glycopeptide **S8** except the glycosylated trisaccharide threonine **18** (138 mg, 0.1 mmol) was employed in the manual coupling step. The crude product was purified by reversed-phase HPLC on an analytical C-18 column using a gradient of 0→100% acetonitrile in H_2O (0.1% TFA) over a 50 min period to give, after lyophilization of the appropriate fractions, glycopeptide **5** (5.8 mg, 7%, based on initial loading of the resin) as an amorphous white solid. HR MALDI-TOF MS m/z: calcd for $C_{67}H_{115}N_{14}O_{32}P$ $[M+Na]^+$, 1681.7437; found 1681.7452.

Benzyl *O*-[3,4,6-Tri-*O*-acetyl-2-deoxy-2-(2,2,2-trichloroethoxy)-carbonylamino-β-D-galacto-pyranosyl]-(1→3)-*O*-[2-deoxy-4,6-*O*-(2-naphthylidene)-2-(2,2,2-trichloroethoxy)carbonyl-amino-β-D-glucopyranosyl]-(1→4)-2-*O*-acetyl-6-*O*-levulinoyl-3-*O*-(2-methylnaphthyl)-α-D-mannopyranoside (21). A mixture of the thioglycosyl **13** (108 mg, 0.0749 mmol) and benzyl alcohol (32 mg, 0.300 mmol) and 4 Å molecular sieves (200 mg) in DCM (2.5 mL) was stirred under an atmosphere of Ar for 1 h. The reaction was cooled (−20 °C) and NIS (25 mg, 0.11 mmol) was added followed by the addition of TMSOTf (2.7 μL, 0.015 mmol). After stirring for 40 min, the reaction was quenched by the addition of Et_3N (0.1 mL). The reaction mixture was filtered, and the filtrate (70 mL) was washed with 10% $Na_2S_2O_3$ (80 mL) and brine (60 mL). The organic phase was dried ($MgSO_4$) and filtered and the filtrate was concentrated under reduced pressure. The resulting yellow oil was purified by flash chromatography over silica gel (EtOAc/hexanes, 1/1, v/v) to give **21** (65 mg, 58%) as an amorphous white solid: R_f = 0.22 (EtOAc/hexanes, 1/1, v/v). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.84−7.26 (m, 19H), 5.72 (br s, 1H, N*H*Troc^{II}), 5.51 (s, 1H, >C*H*Nap), 5.36 (s, 1H, H-2^{I}), 5.22 (s, 1H, H-4^{III}), 5.15−5.13 (m, 2H, H-3^{III} and N*H*Troc^{III}), 4.86−4.46 (m, 12H, H-1^{I}, H-6a^{I}, H-1^{II}, H-1^{III}, OCH_2Ph, OCH_2Nap and OCH_2C-Cl$_3^{II\ and\ III}$), 4.18−3.99 (m, 6H, H-3^{I}, H-4^{I}, H-6e^{I}, H-3^{II}, H-6e^{II} and H-6e^{III}), 3.88−3.81 (m, 2H, H-5^{I} and H-6a^{III}), 3.67−3.63 (m, 4H, H-2^{II}, H-4^{II}, H-2^{III} and H-5^{III}), 3.48 (t, 1H, J = 10 Hz, H-6a^{II}), 3.37 (br s, 1H, H-5^{II}), 2.87−2.58 (m, 4H), 2.25 (s, 3H), 2.13 (s, 3H), 2.07 (s, 3H), 1.84 (s, 3H), 1.77 (br s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 207.91, 172.94, 170.32, 170.25, 154.47, 154.06, 136.60, 135.99, 134.59, 133.88, 133.43, 133.12, 132.96, 128.76, 128.48, 128.39, 128.33, 128.26, 128.05, 127.98, 127.91, 126.71, 126.43, 126.37, 126.04, 125.91, 125.70, 125.56, 123.70 101.62, 101.60 (C-1^{III}), 97.32 (C-1$^{I\ and\ II}$), 96.06, 80.22 (C-4^{I}), 78.37 (C-3^{II}), 76.32 (C-3^{I} and C-4^{I}), 75.80, 74.59, 72.33, 70.81 (C-5^{III}), 70.00 (C-3^{III}), 69.62 (C-2^{I}), 69.42 (C-5^{I}), 68.80 (C-6^{II}), 66.52 (C-4^{III}), 66.14 (C-5^{II}), 63.71 (C-6^{I}), 60.89 (C-6^{III}), 58.45 (C-2^{II}), 53.36 (C-2^{III}), 38.17, 30.24, 29.89, 28.17, 21.30, 20.82, 20.60. HR MALDI-TOF MS m/z: calcd for $C_{66}H_{70}Cl_6N_2O_{24}$ $[M+Na]^+$, 1507.2347; found 1507.2357.

Benzyl *O*-[3,4,6-Tri-*O*-acetyl-2-deoxy-2-(2,2,2-trichloroethoxy)carbonylamino-β-D-galactopyranosyl]-(1→3)-*O*-[2-deoxy-4,6-*O*-(2-naphthylidene)-2-(2,2,2-trichloroethoxy)-carbonyl-amino-β-D-glucopyranosyl]-(1→4)-2-*O*-acetyl-6-*O*-(1,5-dihydro-3-oxo-3λ^5-3*H*-2,4,3-benzodioxaphosphepin-3yl)-3-*O*-(2-methylnaphthyl)-α-D-mannopyranoside (23). To a stirred solution of **21** (60 mg, 0.0404 mmol) in a mixture of MeOH and DCM (2 mL, 1/8, v/v) was added hydrazine acetate (7.5 mg, 0.0808 mmol). After stirring for 1.5 h, the reaction mixture was diluted with DCM (40 mL) and washed with saturated $NaHCO_3$ (2 × 30 mL). The organic layer was dried ($MgSO_4$) and filtered, and the filtrate was concentrated under reduced pressure to afford crude 22 as an amorphous white solid. Compound **22** was dissolved in DCM (1.2 mL) and placed under an atmosphere of Ar, and subsequently, *N*,*N*-diethyl-1,5-dihyro-2,4,3-benzodioxaphosphepin-3-amine (19 mg, 0.0808 mmol) and 1*H*-tetrazole (3 wt % solution in CH_3CN, 0.47 mL, 0.16 mmol) were added. After stirring for 3 h, the reaction was cooled (−20 °C) and *m*-CPBA (77%, 28 mg, 0.16 mmol) was added. After stirring for 1 h, the reaction mixture was diluted with DCM (40 mL) and washed with saturated $NaHCO_3$ (2 × 30 mL) and brine (25 mL). The organic phase was dried ($MgSO_4$) and filtered, and the filtrate was concentrated under reduced pressure. The resulting yellow oil was purified by flash chromatography over silica gel (EtOAc/hexanes, 1/1→3/2, v/v) to give **23** (41 mg, 65% over 3 steps) as an amorphous white solid: R_f = 0.29 (EtOAc/hexanes, 3/2, v/v); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.89−7.27 (m, 23H), 7.11 (d, 1H, J = 9 Hz, N*H*Troc^{II}), 5.58 (s, 1H, >C*H*Nap), 5.46 (t, 1H, J = 12.3 Hz, one proton from POCH_2), 5.37−5.32 (m, 2H, H-2^{I} and one proton from POCH_2), 5.24−5.15 (m, 5H, H-3^{III}, H-4^{III}, two protons from POCH_2, one proton from OCH_2Ph), 5.03 (br s, 1H, N*H*Troc^{III}), 4.94−4.68 (m, 9H, H-1^{I}, H-6a^{I}, H-1^{II}, H-1^{III}, one proton from OCH_2Ph, and OCH_2CCl$_3^{II\ and\ III}$), 4.66−4.50 (m, 2H, OCH_2Nap), 4.25−4.23 (m, 1H, H-6e^{I}), 4.13−4.01 (m, 5H, H-3^{I}, H-4^{I}, H-3^{II}, H-6e^{II} and H-6e^{III}), 3.87−3.72 (m, 5H, H-5^{I}, H-2^{II}, H-4^{II}, H-2^{III} and H-6a^{III}), 3.62 (br s, 1H, H-5^{III}), 3.50 (t, 1H, J = 10.3 Hz,

H-6a^{II}), 3.40 (br s, 1H, H-5^{II}), 2.16 (s, 3H), 2.09 (s, 3H), 1.88 (s, 3H), 1.79 (br s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.35, 170.30, 170.21, 154.77, 154.14, 136.55, 136.14, 135.24, 135.01, 134.61, 133.91, 133.43, 133.11, 132.99, 129.75, 129.60, 129.41, 129.22, 128.78, 128.51, 128.47, 128.39, 128.18, 128.15, 128.05, 127.97, 127.92, 126.69, 126.42, 126.32, 125.98, 125.78, 125.64, 123.73, 102.69 (C-1^{II}), 101.77, 101.24 (C-1^{III}), 97.35 (C-1^{I}), 96.45, 95.93, 80.50 (C-4^{I}), 79.08 (C-3^{I} or C-4^{I}), 77.44 (C-3^{II}), 76.34 (C-3^{I} or C-4^{I}), 75.98, 74.46, 72.90, 70.73, 70.47 (C-5^{III}), 70.38(C-2^{I}), 70.09 (C-5^{I}), 69.92, 69.43(C-3^{III}), 69.35, 68.86 (C-6^{II}), 66.52 (C-5^{II}), 66.47 (C-6^{I}), 66.18 (C-4^{III}), 60.89 (C-6^{III}), 57.86 (C-2^{II}), 53.23 (C-2^{III}), 29.89, 21.31, 20.80, 20.60, 20.57. HR MALDI-TOF MS m/z: calcd for $C_{69}H_{71}Cl_6N_2O_{25}P$ $[M+Na]^+$, 1591.2112; found 1591.2131.

Benzyl *O*-(2-Acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-galactopyranosyl)-(1→3)-*O*-[2-acetamido-2-deoxy-4,6-*O*-(2-naphthylidene)-β-D-glucopyranosyl]-(1→4)-2-*O*-acetyl-6-*O*-(1,5-dihydro-3-oxo-3λ^5-3*H*-2,4,3-benzodioxaphosphepin-3yl)-3-*O*-(2-methylnaphthyl)-α-D-mannopyranoside (24). To a stirred solution of 23 (32 mg, 0.0204 mmol) in Ac_2O/AcOH/THF (4 mL, 1/2/3, v/v/v) was added zinc metal power (30 mg, 0.45 mmol) followed by saturated $CuSO_4$ (0.2 mL). After stirring for 30 min, the reaction mixture was filtered, and the filtrate was concentrated under reduced pressure. The resulting yellow oil was purified by flash chromatography over silica gel (MeOH/DCM, 1/25 v/v) to give 24 (20 mg, 71%) as an amorphous white solid: R_f = 0.30 (MeOH/DCM, 1/15, v/v). ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.85−7.25 (m, 23H), 5.74 (d, 1H, J = 7 Hz, N*H*Ac^{III}), 5.49 (s, 1H, >C*H*Nap), 5.38 (t, 1H, J = 10.5 Hz, one proton from POCH_2), 5.34−5.29 (m, 2H, H-2^{I} and one proton from POCH_2), 5.23−5.12 (m, 4H, H-3^{III}, H-4^{III}, POCH_2), 4.99 (d, 1H, J = 7 Hz, H-1^{II}), 4.87 (s, 1H, H-1^{I}), 4.83 (d, 1H, J = 7 Hz, H-1^{III}), 4.80 (s, 2H, OCH_2Ph), 4.60−4.50 (m, 2H, OCH_2Nap), 4.59 (t, 1H, J = 7 Hz, H-6a^{I}), 4.23−4.21 (m, 1H, H-6e^{I}), 4.15 (t, 1H, J = 7.8 Hz, H-3^{II}), 4.10 (t, 1H, J = 7.8 Hz, H-6a^{III}), 4.07−4.00 (m, 3H, H-3^{I}, H-4^{I} and H-6e^{III}), 3.95−3.83 (m, 5H, H-5^{I}, H-2^{II}, H-6e^{II}, H-2^{III} and H-6e^{III}), 3.67−3.64 (m, 2H, H-4^{II} and H-5^{III}), 3.45 (t, 1H, J = 10 Hz, H-6a^{II}), 3.37−3.33 (m, 1H, H-5^{II}), 2.08 (s, 6H), 2.06 (s, 3H), 1.90 (s, 3H), 1.87 (s, 3H), 1.82 (s, 3H). ^{13}C NMR (150 MHz, $CDCl_3$) δ 171.25, 170.97, 170.50, 170.44, 170.19, 136.78, 136.09, 135.27, 135.13, 134.82, 133.89, 133.50, 133.16, 133.04, 129.76, 129.67, 129.40, 129.27, 128.72, 128.49, 128.31, 128.22, 128.14, 128.07, 127.97, 127.90, 126.65, 126.39, 126.33, 125.97, 125.93, 125.82, 125.51, 123.82, 102.06 (C-1^{II}), 101.64, 100.17 (C-1^{III}), 97.51 (C-1^{I}), 80.12 (C-4^{I}), 77.68 (C-3^{II}), 76.24 (C-3^{I} or C-4^{I}), 75.72 (C-3^{I} or C-4^{I}), 72.61, 70.79 (C-5^{III}), 70.60 (C-5^{I}), 70.55, 70.52 (C-2^{I}), 70.18, 69.75, 69.35 (C-3^{III}), 69.30, 68.96, 68.92 (C-6^{II}), 66.85 (C-4^{III}), 66.21 (C-6^{I}), 66.06 (C-5^{II}), 61.36 (C-6^{III}), 57.02(C-2^{III}), 51.82 (C-2^{II}), 29.87, 23.68, 23.53, 21.19, 20.80, 20.74, 20.63. HR MALDI-TOF MS m/z: calcd for $C_{67}H_{73}N_2O_{23}P$ $[M+Na]^+$, 1327.4239; found 1327.4247.

***O*-(2-Acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-galactopyranosyl)-(1→3)-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1→4)-2-*O*-acetyl-6-*O*-phosphate-α/β-D-mannopyranose (25).** Compound **24** (10 mg, 7.67 μmol) was dissolved in a mixture of MeOH and DCM (1/1, v/v, 3 mL) and Pd(OH)$_2$/C (40 mg, 20 wt %, Degussa type) was added. The resulting mixture was placed under a hydrogen atmosphere (1 psi). After stirring for 24 h, the catalyst was filtered off and washed thoroughly with MeOH and DCM. The combined filtrates (∼50 mL) were concentrated under reduced pressure. The resulting yellow solid was purified by Bio-Gel P2 (MeOH/H_2O, 1/4, v/v) to give **25** (5.0 mg, 78%) as an amorphous white solid: ^{1}H NMR α-anomer (500 MHz, CD_3OD) δ 5.28 (d, 1H, J = 3.5 Hz, H-4^{III}), 5.02 (dd, 1H, J = 3.6 and 11 Hz, H-3^{III}), 4.96−4.94 (m, 2H, H-1^{I} and H-2^{I}), 4.66−4.62 (m, H-1^{II} and H-1^{III}), 4.15−4.09 (m, 4H), 3.91−3.81 (m, 6H), 3.67−3.53 (m, 3H), 3.44−3.41 (m, 1H), 3.36−3.32 (m, 1H), 2.06 (s, 3H), 2.03 (s, 3H), 2.00 (s, 3H), 1.96 (s, 3H), 1.88 (s, 3H), 1.86 (s, 3H). ^{13}C NMR (HSQC, 150 MHz, CD_3OD) δ 101.96 (C-1^{II}), 101.72 (C-1^{III}), 91.70 (C-1^{I}), 84.09, 77.45, 76.21, 72.64, 70.73, 70.62, 69.78, 69.64, 68.81, 67.59, 66.69, 63.06, 61.00, 57.73, 49.93, 19.27, 19.24, 19.17, 19.16.19.15. HR MALDI-TOF MS m/z: calcd for $C_{30}H_{47}N_2O_{23}P$ $[M+Na]+$, 857.2205; found 857.2200.

***O*-(2-Acetamido-2-deoxy-β-D-galactopyranosyl)-(1→3)-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1→4)-6-*O*-phosphate-D-mannitol (4).** To a stirred solution of **25** (5.0 mg, 6.0 μmol) in a mixture of MeOH and DCM (1/2, v/v, 1 mL) was added NaOMe in a methanolic solution (10 μL, 1.0 M, 10 μmol) and the resulting reaction mixture was stirred for 6 h. The reaction was then neutralized with 1% AcOH in MeOH until pH ∼7. Removal of solvents under reduced pressure afforded a white solid. The crude product was dissolved in H_2O (0.5 mL) and $NaBH_4$ (1.1 mg, 30 μmol) was added. After stirring for 2 h, the resulting mixture was concentrated under reduced pressure. The resulting yellow solid was purified by Bio-Gel P2 (H_2O) to give pure **4** (3.0 mg, 75% over 2 steps) as an amorphous white solid: ^{1}H NMR (600 MHz, D_2O) δ 4.68 (d, 1H, J = 8 Hz, H-1^{II}), 4.48 (d, 1H, J = 8 Hz, H-1^{III}), 4.01−3.92 (m, 3H), 3.90−3.74 (m, 9H), 3.72−3.59 (m, 6H), 3.56−3.52 (m, 1H), 3.50−3.45 (m, 1H), 2.02 (s, 3H), 1.91 (s, 3H). ^{13}C NMR (HMQC, 150 MHz, D_2O) δ 104.28 (C-1^{III}), 103.53 C-1^{II}), 83.68, 78.76, 77.70, 77.47, 73.18, 71.93, 71.90, 71.87, 70.39, 67.66, 65.85, 63.79, 63.64, 57.14, 55.23, 19.16.19.15. HR MALDI-TOF MS m/z: calcd for $C_{22}H_{41}N_2O_{19}P$ $[M+Na]+$, 691.1939; found 691.1947.

Structural and Conformational Analysis. NMR spectra were recorded on a Varian NMRS 600 MHz spectrometer with a 3 mm cold probe in D_2O or H_2O/D_2O, 9/1, v/v. Pulse sequences for double quantum filtered COSY (DQCOSY), total correlation spectroscopy (TOCSY), gradient heteronuclear multiple quantum coherence (gHMQC), and gradient heteronuclear multiple bond coherence (gHMBC) experiments, as provided in the *VnmrJ* software, were used. Data were processed and analyzed using the *VnmrJ* software and nmrPipe. The reported shifts were based on HMQC peak positions determined in nmrDraw. Shifts are referenced to the calculated position of 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) with the approach widely used in biomolecular NMR, using the position of the residual HDO signal as a secondary reference.

Enzyme Kinetics. A recombinant form of the soluble catalytic domain of POMGnT1 cloned into the plasmid pSecTAG2B was a gift of Prof. Huaiyu Hu. POMGnT1 was obtained from a culture of HEK293 cells stably transfected with the plasmid encoding an epitope tagged form of the human catalytic domain. The assays were carried out in solution of 6.25 mM $MnCl_2$, 80 mM MES buffer, pH 6.5, and 2 mM UDP-GlcNAc (10 μL) with 10^5 counts of tritium/assay tube. The glycopeptide concentration was varied up to 2 mM. The tubes were incubated for 6 h at 37 °C, after which the reaction was quenched with addition of water (1 mL). C_{18} SepPak material was prewash with ethanol (10 mL) and water (30 mL). The column was then washed with of water (60 mL), followed by elution of the glycopeptide with ethanol (1.5 mL). The latter fraction was collected and radioactivity counted to determine the amount of GlcNAc incorporated into the glycopeptides. Kinetic parameters were extracted from the data after fitting to the Michaelis−Menten equation using the enzyme kinetics module of the *Sigma Plot* software.

Analysis of O-Man Glycopeptides Following Enzymatic Extension by POMGnT1. Following separation by HPLC, the synthetic *O*-mannosyl glycopeptides that were extended with a GlcNAc residue following incubation with recombinant POMGnT1 were analyzed by direct infusion. Briefly, a solution of extended *O*-mannosyl glycopeptides (2 μL, 2 mM) was diluted into formic acid (20 μL, 0.1%). The glycopeptides were directly infused into a linear ion trap mass spectrometer (LTQ-XL, Thermo Scientific) at a flow rate of 0.4−0.6 μL/min and both full MS and MS/MS fragmentation spectra were acquired. From the acquired full MS scans, select m/z values were selected to undergo MS/MS fragmentation by collision-induced dissociation (CID). The MS/

MS fragmentation spectra were generated by applying 40% collision energy and collected over 30 s. The acquired MS/MS spectra were manually interpreted to determine which *O*-mannosyl residue had been extended with a GlcNAc residue. No signal was observed in the full MS spectra or trapped MS/MS spectra for a glycopeptide extended with two GlcNAc residues.

■ ASSOCIATED CONTENT

Supporting Information. Complete ref 11, ^{1}H and ^{13}C NMR spectra, and experimental procedures for the preparation of compounds **7** and **8** and glycopeptides **28**−**33**. This material is available free of charge via the Internet at http://pubs.acs.org.

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