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Synthesis of Proteophosphoglycans of *Leishmania major* and *Leishmania mexicana*

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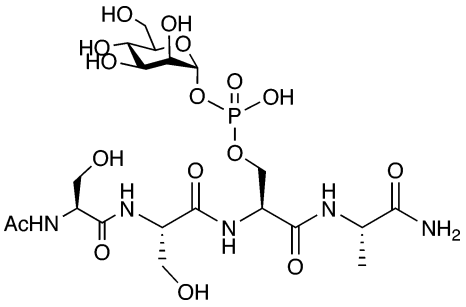

A novel approach for the synthesis of various fragments of proteophosphoglycans from *Leishmania major* and *Leishmania mexicana* proteophosphoglycans has been developed. These compounds have been obtained by coupling α-mannosyl and α-*N*-acetyl-glucosamine phosphoramidite derivatives with the serine hydroxyl of various amino acids and peptides to give, after oxidation with *tert*-BuOOH, phosphotriesters exclusively as α-anomers in good yield. The resulting compounds could be deblocked using conventional methods. Glycophosphorylation of preassembled and properly protected peptides was found to be more efficient for the preparation of proteophosphoglycan fragments than a building block approach strategy using a phosphoglycosylserine derivative.

Introduction

Most eukaryotic cellular proteins, with the exception of certain hormones and enzymes, are reliant on covalently attached sugar units to confer a broad range of important biological functions such as immunogenicity, solubility, cell−cell communication, protection from proteolytic attack, and induction and maintenance of the protein conformation in biologically active forms.[1−6] The vast majority of glycoproteins can be divided into two principal groups: the *N*-linked glycoproteins having an *N*-glycosidic linkage to the side chain of L-asparagine and the more diverse *O*-linked group, bearing an *O*-glycosidic linkage to L-serine, L-threonine, 4-hydroxy-L-proline, or L-tyrosine.

Recently, several new classes of glycoproteins have been identified including an intriguing group that contains oligosaccharides linked to serine or threonine moieties via a phosphodiester linkage. This new type of protein modification has been referred to as protein phosphoglycosylation.[7] The first reported example of a protein modified by a phosphoglycoside was an endopeptidase known as proteinase I, isolated from the slime mold *Dictyostelium discoideum*.[8] It was demonstrated that this glycoprotein contains a serine moiety modified by a phosphodiester linked to *N*-acetyl glucosamine (α-D-GlcNAc-1-PO_4-Ser). It has also previously been established that two other cysteine proteinases isolated from *D. discoideum* carry GlcNAc-1-PO_4 modifications.[9]

Protein phosphoglycosylation is the predominant type of protein glycosylation in the parasite Leishmania.[7,10] For example, a secreted acid phosphatase of *Leishmania mexicana* is modified by α-mannosidic phosphodiester linkages, which can either be monomeric or may consist

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of a series of neutral α(1−2)-linked oligomannanes or phosphorylated oligosaccharides composed of PO_4-6Galβ-(1−4)Man and PO_4-6[Glcβ(1−3)]Galβ(1−4)Man repeating units capped with a neutral mannosyl oligosaccharide.[11−13]

Phosphoproteoglycans are also major components of fibrous filaments produced by Leishmania promastigotes.[14] The purified fiber-forming compound of *L. major* has been characterized in detail, and these studies have revealed that half of the amino acids of the polypeptide chain are serine, most of which are modified by phosphoglycosylation. The extensive phosphoglycosylation confers proteinase resistance to the polypeptide chain and inhibits the formation of secondary structures. This leads to a threadlike appearance with filaments of 3−6 nm in diameter and up to 6 μm in length that form a gel-like mass. Several studies[15] have suggested that these phosphoproteoglycans are important for the colonization of the *Leishmania* parasites in sand flies and may be important to survive in a hostile environment.

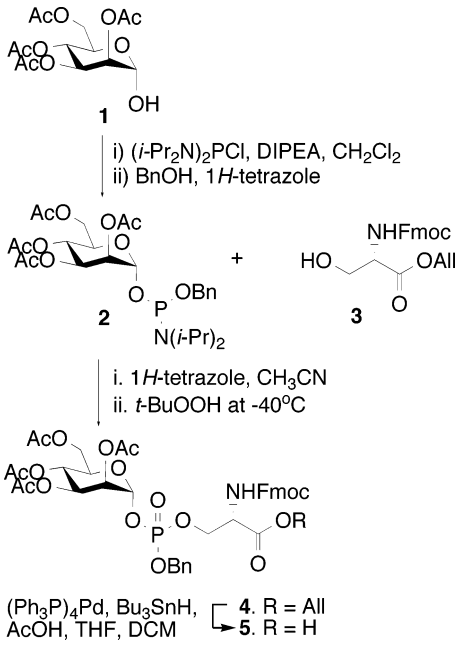
Phosphoglycosylation has also been observed in other micro-organisms. In *Trypanosoma cruzi*, rhamnose-, fucose-, xylose-, and galactose-containing phosphoglycans are linked to several proteins via phosphothreonine.[16] These components may be important for the development of the parasite in the insect stage. The recently identified GPI anchor of *Entamoeba histolytica* carries linear α(1−6) glucans linked to serine phosphate and is thought to be a virulent factor and vaccine candidate.[17] In mammals, as in other vertebrates, phosphoglycosylation appears to be absent, therefore implying that protein phosphoglycosylation is an attractive target for drug development.

To study the biological functions of protein phosphoglycosylation in detail, reasonable quantities of well-defined compounds are required. For the first time, we report a convenient approach for the synthesis of properly protected α-D-mannosyl and α-D-glucosamine phosphate serine derivatives,[18] which can be employed for the preparation of phosphoglycopeptides. It has been found that the most versatile approach for phosphoglycopeptide synthesis involves the coupling of a glycosyl phosphoramidite with the hydroxyl of the serine of preassembled and properly protected peptides.

Results and Discussion

It was envisaged that the preparation of glycosyl phosphate serine derivatives would be complicated by the inherent acid lability of anomeric phosphates and the possibility of base-mediated β-elimination of the serine phosphotri(di)ester linkage. Furthermore, due to unfavorable hydrogen bonding, the side-chain hydroxyl of carbamate-protected serine and threonine derivatives are of low nucleophilicity,[19−21] complicating the phosphorylation. In addition, anomeric phosphorylations are challenging due to the fact that these reactions can give a mixture of α/β-anomers.

SCHEME 1



Different synthetic methodologies for the synthesis of glycosyl phosphodiesters have been developed, including the phosphodiester,[22] phosphotriester,[23] phosphoramidite,[24] and hydrogen phosphonate (H-phosphonate)[25] methodologies. The first two methods entail the use of P(V), compounds which are rarely used due to long reaction times and low yields. On the other hand, the latter two methods have been widely used for the anomeric phosphorylation of saccharides.

Thus, 2,3,4,6-tetra-*O*-acetyl-D-mannose (**1**) was reacted with the commercially available bifunctional phosphitylating reagent *N,N*-diisopropyl chlorophosphoamidite in the presence of the hindered base diisopropylethylamine in dichloromethane (DCM) to afford an intermediate anomeric phosphoramidite, which was coupled with benzyl alcohol in the presence of 1*H*-tetrazole in acetonitrile to give **2** in 79% yield (Scheme 1). The latter derivatives could be further activated with 1*H*-tetrazole, and reaction with the hydroxyl of serine derivative **3** gave the expected phosphite, which was oxidized in situ with *t*-BuOOH at −40 °C to give phosphotriesters **4**. The allyl group of **4** was removed by using $(Ph_3P)_4Pd$, Bu_3SnH and AcOH to give the corresponding acid **5** in 82% yield.[26]

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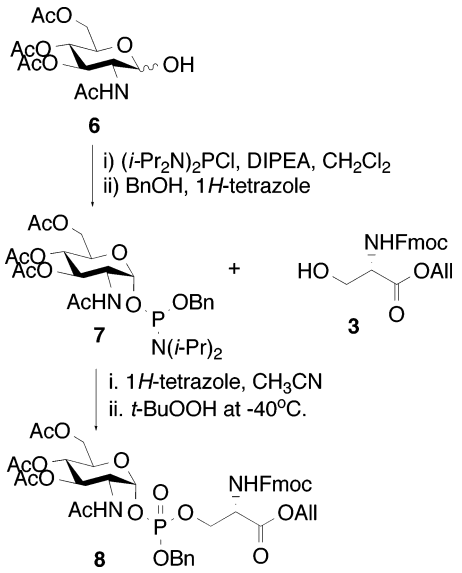
SCHEME 2

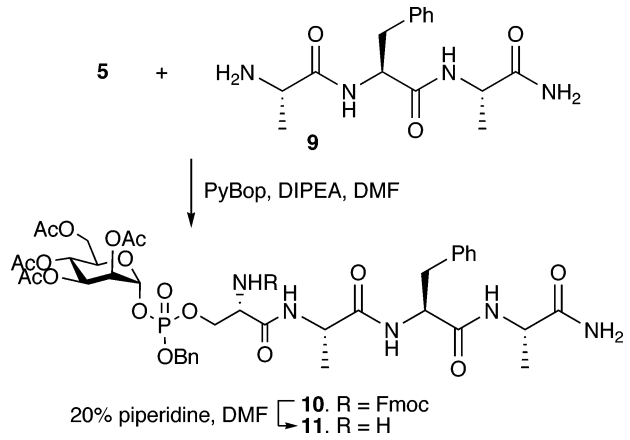


^{1}H NMR of **4** showed H-1 at ~5.6 ppm ($J_{H1,P}$ = 7.3 Hz) and deshielding of H-3 and H-5 by ~0.2 ppm compared to the same protons in hemiacetal **1** confirming the α-configuration of the phosphate.[27−29]

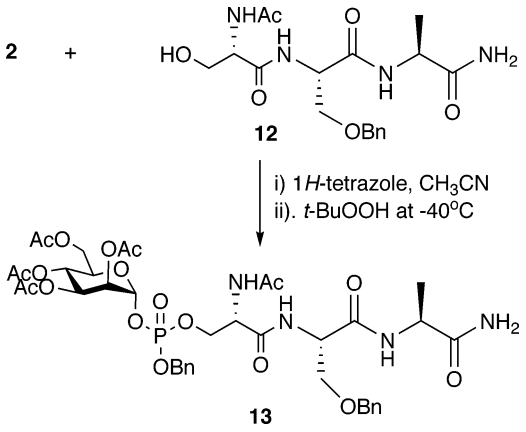
An identical reaction sequence was employed for the conversion of 2,3,4,6-tetra-*O*-acetylated-*N*-acetyl-glucosamine **6** into phosphotriester **8**. It was, however, observed that the intermediate phosphoramidite **7** was relatively labile and therefore was used immediately in the next reaction step (Scheme 2). The anomeric configuration of **8** was confirmed by $J_{H1,H2}$ = 3.4 Hz and $J_{H1,P}$ = 7.2 Hz.[27−29]

Having established a convenient procedure for the synthesis of glycosylphosphoamino acids, attention was focused on the use of these compounds for the preparation of glycosylphosphopeptides. In the first instance, a procedure was explored whereby phosphoglycosylated amino acid **5** was used as a building block in conventional stepwise peptide synthesis. Thus, coupling of mannosylphosphoserine derivative **5** with the tripeptide Ala-Phe-Ala-NH_2 (**9**) was carried out by using the powerful coupling reagent PyBOP/DIPEA to give, after purification by size-exclusion column chromatography over Sephadex LH-20, the glycosylated phosphopeptide **10** in 38% yield (Scheme 3). Unfortunately, removal of the Fmoc protecting group of **10** to give compound **11** using 20% piperidine in DMF led to a mixture of compounds including a cyclic phosphoamide derivative. The formation of the latter type of compound is common in phosphopeptide synthesis when phosphotriester-modified serine derivatives are employed in the stepwise peptide assembly.[30] It has been shown that this side reaction can be suppressed by employing phosphodiester derivatives of serine. Therefore, the benzyl-protecting group of **5** was removed by treatment with NaI, and the resulting phosphodiester

SCHEME 3



SCHEME 4



was used in a coupling with tripeptide **9**. Unfortunately, this procedure also led to a mixture of compounds.

Because of the disappointing results of the stepwise approach, a convergent strategy was pursued whereby a hydroxyl of a properly protected peptide is phosphitylated with compound **2**. In the first instance, a peptide was selected that contained a serine with a free hydroxyl at the N-terminus. The target tripeptide AcNH-Ser(OH)-Ser(OBn)-Ala-NH_2 (**12**) was synthesized using Rink amide resin as a solid support and Fmoc continuous-flow strategy on an Applied Biosystems automated peptide synthesizer. The hydroxyl of the terminal serine residue was protected with a *tert*-butyl group, while the hydroxyl group of the internal serine was protected as a benzyl ether. During the cleavage of this tripeptide from the Rink amide resin under acidic condition (95% TFA, 2.5% triisopropyl silane, 2.5% water), the *t*-Bu protecting group was selectively removed to give the desired tripeptide **12** which contained a free hydroxyl group. Coupling of this tripeptide **12** with phosphoramidite **2** was performed by using the mild acid 1*H*-tetrazole, followed by in situ oxidation with *t*-BuOOH at −40 °C to give the phosphoglycosylated tripeptide **13** in 69% yield (Scheme 4).

To synthesize a fragment of *Leishmania* proteophosphoglycan, the tetrapeptide AcNH-Ser(OBn)-Ser(OBn)-Ser(OH)-Ala-NH_2 (**14**) was synthesized using a similar strategy to that of peptide **12**. Coupling of the tetrapeptide **14** with phosphoramidite **2** was mediated by 1*H*-tetrazole, and the resulting phosphite was oxidized with *t*-BuOOH at −40 °C to give phosphotriester **15** in 65%

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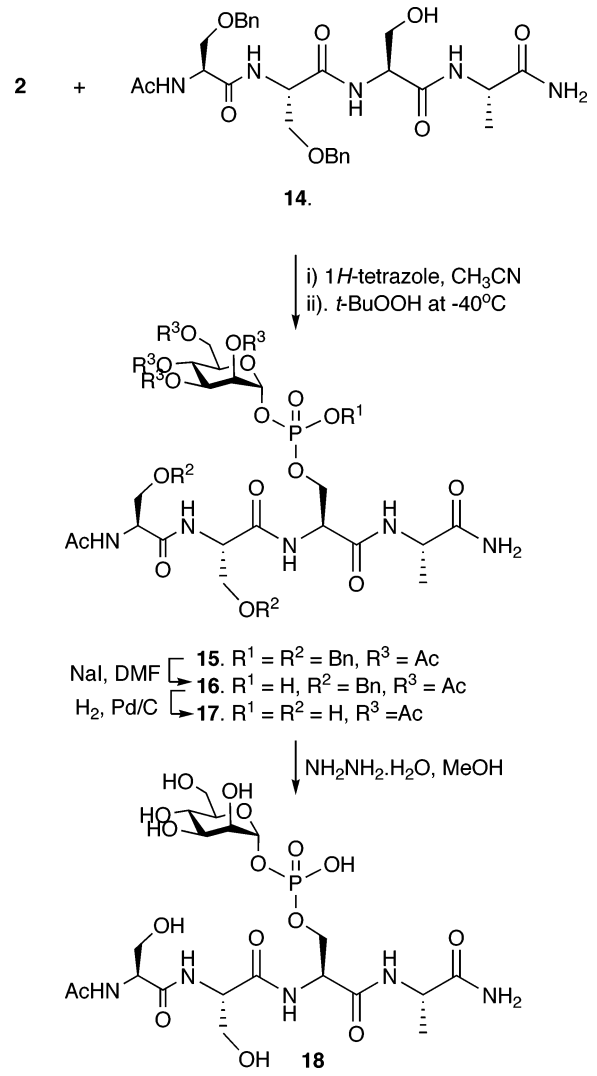
SCHEME 5



yield (Scheme 5). In the next step, compound **15** was treated with NaI to remove the benzyl ester of the phosphate to give the glycophosphopeptidediester **16** in 85% yield. The ^{1}H NMR spectrum of **16** showed the disappearance of the CH_2Ph peak at δ = 5.06 ppm. MALDI-TOF mass spectrometry showed a peak at *m/z* 1027.37 corresponding to the disodium salt of the glycophosphopeptidediester [(M + 2Na)]. Removal of the benzyl ethers of the serine residues was performed by hydrogenolysis over Pd−C to give the de-*O*-benzylated product **17** in a quantitative yield. This resulting compound was deacetylated using $NH_2NH_2{\cdot}H_2O$/MeOH to give the target glycosylphosphopeptide **18** in 76% yield.[31] The structural integrity of the final compound was confirmed by NMR spectroscopy (^{1}H-COSY, HSQC, TOCSY) and MALDI-TOF high-resolution mass spectrometry.

Conclusion

An efficient approach has been developed for the preparation of glycosylphosphopeptides using α-mannosyl and α-glucosamine phosphoramidites. These phosphoramidites could be coupled with hydroxyls of the serine moieties of various amino acid derivatives and peptides to give the corresponding phosphotriesters as exclusively α-anomers in good yield. Removal of the protecting groups furnished a glycosylphosphopeptide derived from *Leishmania major* and *Leishmania mexicana*. It has been found that glycophosphorylation of preassembled peptides is a more versatile methodology for phosphoglycopeptide synthesis than the use of glycosylphosphoserine derivatives in linear stepwise peptide assembly. By employment of orthogonally protected serine derivatives, regioselective phosphoglycosylation could be accomplished.

Experimental Section

(2,3,4,6-Tetra-*O*-acetyl-α-D-mannopyranosyl)benzyl *N,N*-diisopropyl phosphoramidite (2). Compound **1** (967 mg, 2.78 mmol) and bis-*N,N*-diisopropyl chlorophosphoramidite (704 mg, 2.63 mmol) were stirred in DCM (15 mL) at room temperature under an atmosphere of argon for 5 min. Diisopropylethylamine (4.84 mL) was added dropwise, and stirring was continued for 6 h. The solvent was evaporated under reduced pressure, and the residue was purified by silica gel column chromatography (eluent, hexane/EtOAc/Et_3N, 75/20/5) to give (2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl) bis-*N,N*-diisopropyl phosphoramidite as a colorless oil (1.14 g, 72%). R_f 0.20 (hexane/ EtOAc, 3/2); $[\alpha]_D$ +30.8 (*c* 3.41, $CHCl_3$). ^{1}H NMR (300 MHz; $CDCl_3$) δ_H: 5.40 (dd, 1H, H-3, J = 10.2, 3.0 Hz), 5.29 (t, 1H, H-4, J = 9.9 Hz), 5.17 (bs, 1H, H-2), 5.08 (d, 1H, H-1, J = 11.4 Hz), 4.28 (dd, 1H, H-6, J = 12.0, 4.8 Hz), 4.15−4.01 (m, 2H, H-5, H-6′), 3.65−3.40 (m, 4H, C*H* of isopropyl), 2.13, 2.06, 2.01, 1.96 (s, 12H, CH_3CO), 1.20−1.14 (m, 24H, CH_3 of isopropyl). ^{13}C NMR (75 MHz; $CDCl_3$) δ_C: 171.01, 170.37, 170.23, 170.01 (*C*OCH_3), 93.51 (d, C-1, J_{CP} = 80.7), 69.61 (C-2), 69.28 (C-3), 66.33 (C-4), 62.74 (C-5), 45.60, 45.42, 45.20, 45.03 (*C*H of isopropyl), 24.62, 24.56, 24.53, 24.51, 24.43, 24.36 (*C*H_3CO), 22.64, 22.60, 22.01, 21.98, 21.19, 20.98, 20.92 (*C*H_3 of isopropyl). ^{31}P NMR (121 MHz; $CDCl_3$) δ_P: 116.82. HR MALDI-TOF MS (*m/z*): calcd for $C_{26}H_{47}N_2O_{10}P$, 601.3012 (M + Na); found, 601.3001. (2,3,4,6-Tetra-*O*-acetyl-α-D-mannopyranosyl)bis-*N,N*-diisopropyl phosphoramidite (2.28 g, 3.94 mmol) and benzyl alcohol (367 μL, 3.54 mmol) in DCM (20 mL) were stirred for 5 min at room temperature under argon atmosphere. 1*H*-Tetrazole (5.82 mL, 1.97 mmol) was added dropwise and stirred for 4 h. The solvent was evaporated under reduced pressure. The residue was dissolved in diethyl ether and filtered, and the filtrate was concentrated under reduced pressure. The residue was chromatographed over silica (eluent, EtOAc/hexane/Et_3N, 28/70/2) to give compound **2** as an oil (1.87 g, 79%). R_f 0.14 (hexane/EtOAc, 3/2). $[\alpha]_D$ +34.1 (*c* 3.20, $CHCl_3$). ^{1}H NMR (300 MHz; $CDCl_3$) δ_H: 7.34−7.21 (m, 5H, Ar-*H*), 5.40−5.19 (m, 3H, H-3, H-2, H-4), 5.13 (bs, 1H, H-1), 4.78−4.56 (m, 2H, CH_2Ph), 4.20−3.85 (m, 3H, H-5, H-6, diastereomers), 3.82 (dd, 1H, H-6′, J = 7.5, 1.2 Hz), 3.66−3.44 (m, 2 H, C*H* of isopropyl), 2.14, 2.13, 2.06, 2.02, 2.01, 2.00, 1.97 (s, 12H, CH_3CO, diastereomers), 1.22, 1.19, 1.17, 1.15 (m, 12H, CH_3 of isopropyl). ^{13}C NMR (75 MHz; $CDCl_3$) δ_C: 170.93, 170.36, 170.21, 170.14, 169.97, 169.92 (*C*OCH_3), 128.59, 128.55, 127.70, 127.64, 127.22, 127.04 (Ar-*H*), 93.13 (d, C-1, J_{CP} = 70, 92 Hz), 70.9, 70.9, 69.5, 69.3, 69.2, 66.2, 66.2, 66.2, 66.0, 65.9, 62.7, 62.5 (C-2, C-3, C-4, C-5, C-6, C-6′), 44.0, 43.9, 43.8, 43.7 (*C*H of isopropyl), 24.8, 24.8, 24.7, 24.6 (*C*H_3-CO), 21.2, 20.9, 20.9 (*C*H_3 of isopropyl). ^{31}P NMR (121 MHz; $CDCl_3$) δ_P: 152.88, 149.42. HR MALDI-TOF MS (*m/z*): calcd for $C_{27}H_{40}NO_{11}P$, 608.2301 (M + Na); found, 608.2239. Anal. Calcd for $C_{27}H_{40}NO_{11}P$: C, 55.38; H, 6.89; N, 2.39; P, 5.29. Found: C, 55.31; H, 6.94; N, 2.34; P, 5.26.

Benzyl-{{[(*S*)-2-allyloxycarbonyl-2-(9-fluorenylmethoxycarbonylamino)] ethyl}-2,3,4,6-tetra-*O*-acetyl-α-D-manno-

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pyranosyl}phosphate (4). To a mixture of compounds **2** (100 mg, 0.17 mmol) and **3** (62.4 mg, 0.17 mmol) in acetonitrile (2 mL) was added 1*H*-tetrazole (0.72 mL, 3 wt % solution in acetonitrile). The mixture was stirred for 4 h at room temperature under an atmosphere of argon. TLC analysis showed the complete disappearance of the starting material. The reaction mixture was cooled (−40 °C), a solution of *t*-BuOOH in decane (77 μL, 5.5 M) was added dropwise, and stirring was continued at −40 °C for 3 h. The solvent was evaporated under reduced pressure, and the residue was purified by a size-exclusion column chromatograph over Sephadex LH-20 (eluent, CH_2Cl_2/MeOH, *v*/*v*, 1/1) to give compound **4** (102 mg, 73%) as a colorless syrup. R_f 0.22 (hexane/EtOAc, 2/3). $[\alpha]_D$ +22.3 (*c* 5.53, $CHCl_3$). ^{1}H NMR (300 MHz; CD_3OD) δ_H: 7.75 (d, 2H, Ar-*H*, *J* = 7.3 Hz), 7.61 (d, 2H, Ar-*H*, *J* = 6.8 Hz), 7.40−7.20 (m, 9H, Ar-*H*), 6.00−5.80 (m, 1H, $OCH_2CH{=}CH_2$), 5.62 (dd, 1H, H-1, $J_{1,2}$ = 1.5 Hz, $J_{1,P}$ = 7.3 Hz), 5.36−5.04 (m, 7H, H-3, H-2, H-4, OCH_2−CH=CH_2, OCH_2Ph), 4.66−4.55 (m, 3H, OCH_2CH=CH_2, α-C*H*), 4.48−3.91 (m, 8H, CH_2 of Fmoc, C*H* of Fmoc, OCH_2 of Ser, H-5, H-6, H-6′, diastereomers), 2.10−1.94 (s, 12H, CH_3-CO). ^{13}C NMR (75 MHz; DMSO-d_6) δ_C: 170.6, 170.2, 170.0, 169.6, 132.8, 118.7, 118.5, 95.6, 79.5, 70.6, 68.8, 68.7, 68.4, 67.4, 66.0, 65.3, 63.6, 62.1, 28.7, 21.2, 21.1, 21.0, 19.7. ^{31}P NMR (121 MHz; $CDCl_3$) δ_P: −3.19, −3.29 (diastereoisomers). Anal. Calcd for $C_{42}H_{46}NO_{17}P$: C, 58.13; H, 5.34; N, 1.61. Found: C, 57.96; H, 5.26; N, 1.61. HR MALDI-TOF MS (*m*/*z*): calcd for $C_{42}H_{46}NO_{15}P$, 890.2485 (M + Na); found, 890.2479.

Benzyl-{{[(*S*)-2-carboxy-2-(9-fluorenylmethoxycarbonylamino)]ethyl}-2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl}phosphate (5). To a stirred solution of **4** (40 mg, 0.04 mmol) in DCM (0.5 mL) was added a mixture of $Pd(PPh_3)_4$ (2 mg, 0.001 mmol), Bu_3SnH (24.7 μL, 0.09 mmol), and AcOH (6 μL, 0.11 mmol) in THF (0.5 mL). The reaction mixture was stirred at room temperature for 48 h, after which the solvent was removed by coevaporation with toluene under reduced pressure. The crude product was purified by silica gel column chromatography (eluent, MeOH/DCM, 5/95, *v*/*v*) to give **5** as a colorless syrup (23 mg, 82%). R_f 0.19 (MeOH/DCM, 5/95). ^{1}H NMR (300 MHz, CD_3OD) δ: 7.71 (d, 2H, Ar-*H*, *J* = 7.5 Hz), 7.60 (d, 2H, Ar-*H*, *J* = 7.2 Hz), 7.40−7.18 (m, 9H, Ar-*H*), 5.60, 5.56 (2xbd, 1H, H-1, *J* = 6.3, 5.7 Hz, diastereomers), 5.32−5.14 (m, 3H, H-2, H-3, H-4), 5.12−5.02 (m, 2H, OCH_2Ph), 4.50−3.88 (m, 9H, α-C*H*, C*H* of Fmoc, CH_2 of Fmoc, H-5, H-6, H-6′, OCH_2 of Ser), 2.04, 1.93, 1.92, 1.89 (s, 12 H, CH_3CO). ^{13}C NMR (75 MHz, DMSO-d_6) δ_C: 170.7, 170.6, 170.2, 170.2, 170.0, 169.9, 144.6, 144.5, 141.4, 136.5, 129.1, 128.9, 128.7, 128.5, 127.7, 125.8, 120.7, 95.2, 70.1, 69.8, 69.7, 69.6, 69.0, 68.8, 68.6, 68.5, 66.4, 65.3, 65.1, 62.0, 56.7, 47.3, 41.1, 40.8, 40.5, 40.2, 39.9, 39.7, 39.4, 21.2, 21.1, 21.0. ^{31}P NMR (121 MHz, CD_3OD) δ_P: −2.39, −2.43 (diastereomers). HR MALDI-TOF MS (*m*/*z*): calcd for $C_{39}H_{42}NO_{17}P$, 873.6712 (M + 2Na); found, 873.6701. Anal. Calcd for $C_{39}H_{42}NO_{17}P$: C, 56.59; H, 5.11; N, 1.69; P, 3.74. Found: C, 56.53; H, 5.09; N, 1.67; P, 3.75.

Benzyl-{{[(*S*)-2-allyloxycarbonyl-2-(9-fluorenylmethoxycarbonylamino)]ethyl}-3,4,6-tri-*O*-acetyl-2-acetamido-α-D-glucopyranosyl}phosphate (8). A solution of compound **6** (581 mg, 1.67 mmol) and bis-*N*,*N*-diisopropyl chlorophosphoramidite (424 mg, 1.59 mmol) in DCM (15 mL) was stirred at room temperature under argon for 5 min. Diisopropylethylamine (2.9 mL) was added dropwise, and stirring was continued for 2 days. Then, the reaction mixture was heated under reflux for 2 h after which the solvent was evaporated under reduced pressure. The residue was purified by silica gel column chromatography (eluent, hexane/EtOAc/Et_3N, 75/20/5, *v*/*v*/*v*) to give (3,4,6-tri-*O*-acetyl-2-acetamido-α-D-glucopyranosyl)bis-*N*,*N*-diisopropyl phosphoramidite as a colorless oil (432 mg, 48%). R_f 0.36 (hexane/EtOAc, 3/2). $[\alpha]_D$ +30.0 (*c* 3.9, $CHCl_3$). ^{1}H NMR (300 MHz; $CDCl_3$) δ_H: 5.57 (d, 1H, N*H*, *J* = 5.4 Hz), 5.24 (t, 1H, H-3, *J* = 6.0 Hz), 5.16−5.12 (m, 2H, H-1, H-4), 4.32−4.21 (m, 2H, H-2, H-5), 4.08−4.04 (m, 2H, H-6, H-6′), 3.56−3.48 (m, 4H, C*H* of isopropyl), 2.06, 2.01, 2.00, 1.91 (4s, 12H, CH_3CO), 1.90 (m, 24H, CH_3 of isopropyl). ^{13}C NMR (75 MHz, $CDCl_3$) δ_C: 171.5, 170.5, 169.6, 169.2, 92.4, 92.3, 71.2, 68.3, 67.9, 61.9, 61.4, 52.9, 52.9, 45.2, 45.1, 44.0, 44.9, 44.3, 44.2, 24.3, 24.2, 24.2, 24.1, 24.0, 23.5, 23.4, 23.0, 22.4, 22.4, 20.8, 20.5, 20.4, 20.4. ^{31}P NMR (121 MHz; CD_3OD) δ_P: 8.60. HR MALDI-TOF MS (*m*/*z*): calcd for $C_{26}H_{48}N_3O_9P$, 600.3128 (M + Na); found, 600.3112. (3,4,6-Tri-*O*-acetyl-2-acetamido-α-D-glucopyranosyl)bis-*N*,*N*-diisopropyl phosphoramidite (389 mg, 0.67 mmol) and benzyl alcohol (66 μL, 0.64 mmol) were stirred in DCM (4 mL) at room temperature under argon for 5 min. 1*H*-Tetrazole (1.8 mL, 0.64 mmol) was added dropwise, and stirring was continued for 2 days. The reaction mixture was refluxed for 2 h, and then the solvent was evaporated under reduced pressure. The residue was purified by silica gel column chromatography (eluent, hexane/EtOAc/Et_3N, 75/20/5, *v*/*v*/*v*) to give **7** as a colorless oil (157 mg, 42%). R_f 0.33 (hexane/EtOAc, 3/2). ^{1}H NMR (300 MHz; $CDCl_3$) δ_H: 5.25 (d, 1H, N*H*, *J* = 5.4 Hz), 5.40−4.85 (m, 3H, H-3, H-1, H-4), 4.80−4.45 (m, 2H, CH_2Ph), 4.40−4.00 (m, 2H, H-2, H-5, H-6), 3.87 (d, 1H, H-6′, *J* = 11.7 Hz), 3.80−3.60 (m, 2H, C*H* of isopropyl), 2.10−1.84 (s, 12H, CH_3CO), 1.30−1.15 (s, 12H, CH_3 of isopropyl). HR MALDI-TOF MS (*m*/*z*): calcd. for $C_{27}H_{41}N_2O_{10}P$, 607.2499 (M + Na); found, 607.2478. To a mixture of compounds **7** (88 mg, 0.15 mmol) and **3** (55 mg, 0.15 mmol) in DCM (4 mL) under an atmosphere of nitrogen was added 1*H*-tetrazole solution (1.32 mL, 3 wt % solution in acetonitrile). The reaction mixture was stirred for 12 h, cooled to −40 °C, *t*-BuOOH (90 μL, 6 M solution in decane) was added, and stirring was continued for 2 h. The solvent was removed under reduced pressure, and the residue was purified by silica gel column chromatography (eluent, EtOAc/Hexane, 4/1, *v*/*v*). The resulting product was further purified by size-exclusion column chromatography over Sephadex LH-20 (eluent, MeOH/DCM, 1/1, *v*/*v*) to give compound **8** (53 mg, 41%) as an oil. R_f 0.11 (hexane/EtOAc, 1/4). $[\alpha]_D$ 28.1 (*c* 0.15, CH_3OH). ^{1}H NMR (500 MHz; CD_3OD) δ_H: 7.73 (d, 2H, Ar-*H*, *J* = 9 Hz), 7.60 (d, 2H, Ar-*H*, *J* = 9 Hz), 7.40−7.18 (m, 9H, Ar-*H*), 5.85−5.75 (m, 1H, OCH_2CH = CH_2), 5.70−5.50 (dd, 1H, H-1, *J* = 3.4 Hz, diastereomers), 5.35−4.80 (m, 6H, H-3, H-4, POCH_2Ph, OCH_2−CH=CH_2), 4.70−3.60 (m, 12H, H-2, OCH_2CH=CH_2, α-C*H*, H-5, H-6, H-6′, OCH_2 of Ser, CH_2 of Fmoc, C*H* of Fmoc), 2.02−1.80 (s, 15H, CH_3CO). ^{13}C NMR (75 MHz; CD_3OD) δ_C: 173.4, 172.3, 171.8, 171.2, 170.4, 145.2, 145.2, 142.6, 133.1, 129.6, 129.4, 128.8, 128.8, 128.2, 126.3, 126.2, 120.9, 118.8, 102.9, 74.3, 72.9, 70.2, 69.9, 68.3, 67.2, 63.2, 57.2, 56.0, 56.0, 55.4, 22.7, 20.6, 20.6, 20.5. ^{31}P NMR (121 MHz; CD_3OD) δ_P: −3.05, −3.07 (diastereomers). HR MALDI-TOF MS (*m*/*z*): calcd for $C_{42}H_{47}N_2O_{16}P$, 889.2663 (M + 2Na); found, 889.2597. Anal. Calcd for $C_{42}H_{47}N_2O_{16}P$: C, 58.2; H, 5.47; N, 3.23; P, 3.57. Found: C, 58.19; H, 5.43; N, 3.21; P, 3.54.

NH_2AlaPheAlaCONH_2 (9). Peptide **9** was synthesized using Rink Amide Resin under standard automated Fmoc protocol. Removal of Fmoc was accomplished with a solution of NMP/Piperidine (80/20, *v*/*v*). After completion of the automated synthesis on a 0.25 mmol scale, the peptide resin was washed into a peptide synthesis vessel with methanol and dried under vacuum. This dry peptide resin was kept in DCM for 2 h and then treated with the cleavage cocktail of CF_3COOH/H_2O/triisopropylsilane (10 mL, 95/2.5/2.5, *v*/*v*/*v*) for 1 h. The resin was removed by filtration, the filtrate was concentrated under reduced pressure and cooled to 0 °C, and ice-cold *tert*-butyl methyl ether (15 mL) was added to give a thick, white suspension, which was transferred to a polypropylene conical tube. The tube was centrifuged and the ether decanted. This procedure was repeated twice to give compound **9** as a white precipitate (72 mg). ^{1}H NMR (300 MHz; CD_3OD) δ_H: 7.23−7.01 (m, 5H, Ar-*H*), 4.56−4.48 (m, 1H, α-C*H*), 4.23−4.01 (m, 1H, α-C*H*), 3.80−3.69 (m, 1H, α-C*H*), 3.04 (dd, 1H, CH*H*Ph of Phe, *J* = 14.9, 7.5 Hz), 2.82 (dd, 1H, C*H*HPh of Phe, *J* = 14.9, 9.4 Hz), 1.33 (d, 3H, CH_3 of Ala, *J* = 7.6 Hz), 1.20 (d, 3H, CH_3 of Ala, *J* = 7.5 Hz). ^{13}C NMR (75 MHz; CD_3OD) δ_C: 177.1, 172.9, 171.3, 138.2, 130.3, 129.6, 127.9, 56.3, 38.6, 18.3, 17.7.

Benzyl-{{[(*S*)-2-carbonyl-2-(9-fluorenylmethoxycarbonylamino)]ethyl}-2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl}phosphate-alanine-phenylalanine-alanine amide (10). To a stirred solution of **5** (23 mg, 0.03 mmol) in DMF (1 mL) was added PyBOP (14.1 mg, 0.03 mmol) and DIPEA (9.4 μL, 0.06 mmol) at room temperature under an atmosphere of argon. After the reaction mixture was stirred for 15 min, a solution of NH_2AlaPheAlaCONH$_2$ (**9**) (8.5 mg, 0.03 mmol) in DMF (0.5 mL) was added. Stirring of the reaction mixture was continued for 10 h, after which the solvents were removed under reduced pressure and the residue was purified by silica gel column chromatography (eluent, MeOH/DCM, 15/85, *v*/*v*) to give **10** as a white solid (12.7 mg, 38%). R_f 0.55 (eluent, MeOH/DCM, 10/90, *v*/*v*). $[\alpha]_D$ 22.1 (*c* 0.21, CH_3OH). ^{1}H NMR (300 MHz, CD_3OH) δ_H: 7.72 (d, 2H, Ar-*H*, *J* = 7.8 Hz), 7.59 (d, 2H, Ar-*H*, *J* = 7.2 Hz), 7.40−7.10 (m, 14H, Ar-*H*), 5.60 (dd, 1H, H-1, *J* = 12.0, 1.5 Hz), 5.30−5.13 (m, 3H, H-2, H-3, H-4), 5.08 (d, 2H, CH_2 of Bn, *J* = 9.6 Hz,), 4.54−3.98 (m, 12H, 4 α-C*H*, H-5, H-6, H-6′, CH_2 of Fmoc, C*H* of Fmoc, OCH_2 of Ser), 3.18−3.02 (m, 1H, C*H*H of Phe), 2.98−2.84 (m, 1H, C*H*H of Phe), 2.04, 2.03, 1.92, 1.91, 1.90, 1.88 (s, 12H, CH_3CO), 1.32−1.08 (m, 6H, 2xCH_3 of Ala). ^{13}C NMR (125 MHz; CD_3OD) δ_C: 177.3, 174.7, 173.0, 172.3, 172.2, 171.6, 171.4, 171.3, 171.3, 170.8, 158.5, 145.2, 142.6, 138.4, 136.8, 136.7, 130.3, 130.1, 129.9, 129.6, 129.4, 128.9, 128.2, 127.9, 126.2, 121.0, 97.1, 71.9, 71.9, 71.8, 71.7, 70.1, 70.0, 70.0, 69.9, 69.8, 69.8, 68.6, 68.5, 62.9, 62.8, 56.4, 56.2, 55.8, 51.1, 50.2, 49.6, 38.2, 20.6, 20.6, 20.5, 18.2, 17.6. ^{31}P NMR (121 MHz; CD_3OD) δ_P: −3.00, −3.15 (diastereomers). HR MALDI-TOF MS (*m*/*z*): calcd for $C_{54}H_{62}N_5O_{19}P$, 1161.3776 (M + 2Na); found, 1161.3718. Anal. Calcd for $C_{54}H_{62}N_5O_{19}P$: C, 58.11; H, 5.60; N, 6.28; P, 2.78. Found: C, 58.18; H, 5.58; N, 6.36; P, 2.73.

AcNHSerSer(OBn)AlaCONH$_2$ (12). Peptide **12** was synthesized using Rink Amide Resin under standard automated Fmoc protocol. Removal of Fmoc was accomplished with a solution of NMP/Piperidine (80/20, *v*/*v*). The following side chain protection schemes for Fmoc amino acids of Nova Biochem were employed: Ser(OBn), Ser(O^{t}Bu). Acetylation of the terminal NH_2 group was performed using Ac_2O/DIPEA/NMP (10/5/85, *v*/*v*/*v*). After completion of the automated synthesis on a 0.25 mmol scale, the resin was washed into a peptide synthesis vessel with methanol and dried under vacuum. This dry resin was kept in DCM for 2 h and then treated with the cleavage cocktail of CF_3COOH/H_2O/triisopropylsilane (10 mL, 95/2.5/2.5, *v*/*v*/*v*) for 1 h. The resin was removed by filtration, the filtrate was concentrated under reduced pressure and cooled to 0 °C, and ice-cold *tert*-butyl methyl ether (15 mL) was added to give a thick, white suspension, which was transferred to a polypropylene conical tube. The tube was centrifuged and the ether decanted. This procedure was repeated twice to give compound **12** as a white precipitate (91 mg). ^{1}H NMR (300 MHz, CD_3OD) δ_H: 7.28−7.18 (m, 5H, Ar-*H*), 4.80−4.64 (m, 4H, CH_2 of Bn, 2α-C*H*), 4.38−4.20 (m, 1H, α-C*H*), 3.85−3.62 (m, 4H, CH_2 of Ser), 1.92 (s, 3H, CH_3CO), 1.28 (d, 3H, CH_3 of Ala, *J* = 7.5 Hz). ^{13}C NMR (75 MHZ, CD_3OD) δ_C: 177.6, 173.6, 173.4, 171.8, 139.1, 129.4, 128.9, 128.9, 74.3, 70.2, 63.0, 56.6, 55.6, 50.4, 22.4, 17.9.

Benzyl-{{[(*S*)-carbonyl-2-(*N*-acetyl)]ethyl}-2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl}phosphate-serine(*O*-benzyl)-alanine-amide (13). To a mixture of compound **2** (58 mg, 0.10 mmol) and compound **12** (29 mg, 0.07 mmol) in DMF (1 mL) was added 1*H*-tetrazole (217 μL, 3 wt % solution in acetonitrile). The reaction mixture was stirred for 8 h at room temperature under an atmosphere of argon, after which it was cooled to −40 °C, *t*-BuOOH solution (50 μL, 5.5 M solution in decane) was added dropwise, and stirring was continued at −40 °C for 1.5 h. The solvents were evaporated under reduced pressure, and the residue was purified by size-exclusion column chromatography over Sephadex LH-20 (eluent, MeOH) to afford **13** as a colorless syrup (45 mg, 69%). R_f 0.53 (MeOH/DCM, 10/90, *v*/*v*). $[\alpha]_D$ 17.8 (*c* 0.24, CH_3OH). ^{1}H NMR (500 MHz; CD_3OD) δ_H: 8.30−8.10 (m, 3H, 3xN*H*), 7.42−7.18 (m, 10H, Ar-*H*), 5.62, 5.57 (2xdd, 1H, H-1, *J* = 6.5, 1.7 Hz, diastereomers), 5.32−5.14 (m, 3H, H-2, H-3, H-4), 5.14−5.07 (m, 2H, OP(=O)OCH_2Ph), 4.80−4.41 (m, 4H, 2 α-C*H*, OCH_2-Ph), 4.38−3.92 (m, 6H, α-C*H*, H-5, H-6, H-6′, OCH_2 of Ser), 3.77−3.62 (m, 2H, OCH_2 of Ser), 2.08, 1.98, 1.96, 1.95, 1.94, 1.92, 1.91 (s, 15H, CH_3CO), 1.28 (d, 3H, CH_3 of Ala, *J* = 7.3 Hz). ^{13}C NMR (75 MHz, CD_3OD) δ_C: 177.4, 173.6, 172.3, 172.2, 171.6, 171.50, 171.4, 171.4, 171.3, 170.7, 170.6, 138.9, 138.9, 136.7, 130.1, 129.9, 129.5, 129.5, 129.4, 129.0, 128.9, 97.0, 74.4, 71.9, 71.8, 71.7, 70.6, 70.4, 70.1, 70.0, 69.9, 69.8, 69.7, 68.4, 68.4, 66.2, 62.9, 62.8, 54.9, 54.7, 50.3, 22.5, 20.6, 20.6, 20.6, 20.5, 17.9. ^{31}P NMR (121 MHz; CD_3OD) δ_P: −3.31, −3.33 (diastereomers). HR MALDI-TOF MS (*m*/*z*): calcd for $C_{39}H_{51}N_4O_{18}P$, 917.2936 (M + Na); found, 917.3344. Anal. Calcd for $C_{39}H_{51}N_4O_{18}P$: C, 52.35; H, 5.74; N, 6.26; P, 3.46. Found: C, 52.31; H, 5.71; N, 6.24; P, 3.41.

AcNHSer(OBn)Ser(OBn)SerAlaCONH$_2$ (14). Peptide **14** was synthesized using a similar procedure as described for **12**. ^{1}H NMR (300 MHz, CD_3OD) δ_H: 7.26−7.16 (m, 10H, Ar-*H*), 4.60−4.40 (m, 6H, 2 CH_2 of Bn, 2 α-C*H*), 4.38−4.20 (m, 2H, 2 α-C*H*), 3.80−3.48 (m, 6H, 3 CH_2 of Ser), 1.98 (s, 3H, CH_3CO), 1.22 (d, 1H, CH_3 of Ala, *J* = 7.5 Hz). ^{13}C NMR (75 MHz, CD_3OD) δ_C: 177.7, 173.9, 172.8, 172.3, 171.9, 139.1, 129.4, 129.0, 128.9, 128.8, 74.3, 70.4, 70.3, 62.8, 57.1, 55.3, 55.2, 50.4, 22.4, 17.8.

Benzyl-{{[(*S*)-carbonyl-2-(*N*-(*N*-acetyl-serine-*O*-benzyl-serine-*O*-benzyl)]ethyl}-2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl}phosphate-alanine-amide (15). To a solution of compounds **2** (106 mg, 0.18 mmol) and **14** (27 mg, 0.05 mmol) in DMF (1.3 mL) was added 1*H*-tetrazole (401 μL, 3 wt % solution in acetonitrile). The reaction mixture was stirred at 65 °C for 3.5 h under an atmosphere of argon. The reaction mixture was cooled to −40 °C, *t*-BuOOH solution (60 mL, 5.5 M solution in decane) was added dropwise, and stirring was continued at −40 °C for 1.5 h. The solvents were evaporated under reduced pressure, and the residue was purified by size-exclusion column chromatography over Sephadex LH-20 (eluent, MeOH) to afford **15** as a colorless syrup (32 mg, 65%). R_f 0.47 (MeOH/DCM, 10/90, *v*/*v*). $[\alpha]_D$ 19.7 (*c* 0.25, CH_3OH). ^{1}H NMR (500 MHz; CD_3OD) δ_H: 7.40−7.15 (m, 15H, Ar-*H*), 5.60, 5.57 (2xdd, 1H, H-1, *J* = 6.1, 1.2 Hz; *J* = 5.3, 1.5 Hz, diastereomers), 5.28−5.13 (m, 3H, H-2, H-3, H-4), 5.12−5.02 (m, 2H, POCH_2Ph), 4.63−4.44 (m, 6H, 2 α-C*H*, 2 CH_2Ph), 4.40−3.88 (m, 7H, 2 α-C*H*, H-5, H-6, H-6′, OCH_2 of Ser), 3.82−3.62 (m, 4H, 2 OCH_2 of Ser), 2.06,1.96, 1.95, 1.92, 1.91, 1.90 (s, 15H, CH_3CO), 1.25, 1.24 (2xd, 3H, CH_3 of Ala, *J* = 7.0 Hz, diastereomers). ^{13}C NMR (75 MHz; CD_3OD) δ_C: 177.2, 173.8, 172.8, 172.3, 172.2, 171.6, 171.4, 171.4, 171.3, 169.6, 139.1, 130.0, 129.9, 129.6, 129.5, 129.4, 128.9, 128.8, 97.0, 74.3, 71.9, 71.8, 70.4, 70.2, 69.9, 69.8, 68.2, 66.2, 62.9, 62.8, 55.4, 55.2, 22.5, 20.7, 20.6, 18.1. ^{31}P NMR (121 MHz; CD_3OD) δ_P: −3.03, −3.04 (diastereomers). HR MALDI-TOF MS (*m*/*z*): calcd for $C_{49}H_{62}N_5O_{20}P$, 1094.3726 (M + 2Na); found, 1094.3394. Anal. Calcd for $C_{49}H_{62}N_5O_{20}P$: C, 54.9; H, 5.83; N, 6.53; P, 2.89. Found: C, 54.88; H, 5.79; N, 6.49; P, 2.86.

{{[(*S*)-Carbonyl-2-(*N*-(*N*-acetyl-serine-serine)]-ethyl}-α-D-mannopyranosyl}phosphate-alanine-amide (18). Compound **15** (5 mg, 0.004 mmol) was dissolved in DMF (0.6 mL) and stirred under an atmosphere of nitrogen for 5 min. NaI (1.4 mg, 0.009 mmol) was added, and stirring was continued for 31 h. Formation of diester was monitored using NMR spectroscopy which showed the disappearance of the POCH_2Ph peak at δ_H = 5.12−5.02 ppm. The solvent was removed under reduced pressure, and the crude product was purified using size-exclusion column chromatography over Sephadex LH-20 (eluent, MeOH) to give **16** in 85% yield. The phosphodiester derivative **16** was dissolved in MeOH (0.5 mL), and Pd−C (2 mg, 10 wt %) was added. The resulting suspension was placed under an atmosphere of H_2. The reaction mixture was stirred under H_2 atmosphere for 20 h. The progress of the reaction was monitored by ^{1}H NMR spectroscopy, which showed the disappearance of Ar-*H* peaks at δ_H =

7.40−7.15 ppm. The reaction mixture was filtered through a polytetrafluoroethylene 0.2 μM membrane filter to give the fully debenzylated phosphodiester derivative **17**. $NH_2NH_2 \cdot H_2O$ (0.24 mL) was added to a solution of phosphodiester derivative **17** in MeOH (1 mL), and the reaction mixture was stirred for 8 h. The solvent was removed under reduced pressure, and the crude product was purified by size-exclusion column chromatography over Sephadex LH-20 (eluent, MeOH) to give **18** (1.6 mg, 76%). $[\alpha]_D$ 27.1 (*c* 0.12, CH_3OH). ^1H NMR (500 MHz; CD_3OD) δ_H: 5.30 (dd, 1H, H-1, $J_{1,2}$ = 1.5 Hz, $J_{1,P}$ = 8 Hz), 4.46−4.44 (m, 1H, α-C*H*), 4.40−4.30 (m, 2H, α-C*H*), 4.24−4.14 (m, 3H, α-C*H*, OCH_2 of Ser), 4.06−3.96 (m, 2H, OCH_2 of Ser), 3.83−3.46 (m, 8H, H-2, H-3, H-4, OCH_2 of Ser, H-5, H-6, H-6′), 1.94 (s, 3H, CH_3CO), 1.35 (d, 1H, CH_3 of Ala, J = 7.0 Hz). ^{13}C NMR (125 MHz; CD_3OD) δ_C: 100.8, 75.8, 74.9, 71.3, 68.6, 68.2, 66.0, 65.9, 65.7, 65.6, 60.6, 59.5, 59.3, 53.7, 25.1, 20.7. ^{31}P NMR (202 MHz, D_2O) δ_P: −0.59. HR MALDI-TOF MS (*m/z*): calcd for $C_{20}H_{36}N_5O_{16}P$, 678.2095 [(M-1) + 2Na]; found, 678.2075. Anal. Calcd for $C_{20}H_{36}N_5O_{16}P$: C, 37.92; H, 5.73; N, 11.06; P, 4.89. Found: C, 37.89; H, 5.71; N, 11.15; P, 4.86.

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Supporting Information Available: ^1H, ^{31}P, and ^{13}C NMR spectra of all new compounds. This information is available free of charge via the Internet at http://pubs.acs.org.

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