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Studies directed toward the synthesis of protein-bound GPI anchor

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Abstract—Mannobioside-linked phosphoethanolamine **10**, a prototype model of the GPI anchor, was synthesized via glycosidation of the monosaccharide donor and acceptor, and subsequent phosphorylation. In order to test the reactivity of the amino group involved in **10** against the activated amino acid esters, **10** was reacted with *N*-protected amino acid pentafluorophenyl esters in the presence of HOBt. The reactions gave the aminoacylated products in moderate yields. When Fmoc-Ser-OPfp **12** and Fmoc-Cys(SBu^t)-OPfp **14** were reacted with **10**, byproducts **19**, **20** and **21** derived from *N*- and *O*-acylation were produced. In contrast, reactions of **10** and *N*-protected amino acid thioesters were promoted with $AgNO_3$, HOSu, and DIEA to afford the coupling products without the undesired *O*-acylation. Peptidylation of **10** with the synthesized oligopeptide thioesters **24** and **27** was also successful under the segment coupling conditions of the peptide thioester method as well as those of the native chemical ligation. © 2003 Published by Elsevier Science Ltd.

1. Introduction

Glycosylphosphatidylinositol (GPI) membrane anchors are glycolipids, which anchor proteins in membranes to form specific membrane microdomains by associating with glycosphingolipids, cholesterol, and accessory proteins at the cell surface. In addition to the anchoring function, GPIs are thought to participate in transmembrane signaling.[1] The unique molecular architecture of GPI has prompted a number of synthetic challenges, and the first total synthesis was reported by Murakata and Ogawa in 1992.[2,3] The major subjects of those studies were concentrated on construction of the common pentasaccharide [α-D-Man-(1→2)-α-D-Man-(1→6)-α-D-GlcNH_2-(1→6)-D-myo-Ins] backbone, synthesis and strategic attachment of the branching oligosaccharides, and conjugation with the phospholipid moiety. However, little attention has been paid to the question of how to reconstruct the linkage between the protein C-terminal and the GPI anchor.

During the last decade, remarkable progress has been made in the chemical synthesis of proteins, where the thioester method[4] and the native chemical ligation[5] are of particular value for large-molecule construction. It is noteworthy that both methods now allow usage of the microbially expressed segments for the total or semisynthesis of proteins. The practical value of the native chemical ligation method has further been encouraged by combination with the protein-splicing strategy.[6] These modern peptide technologies are promising not only in protein engineering but also in synthesizing protein-bound GPIs. The synthesized protein-bound GPIs will become indispensable probes to gain more insight into the GPI-mediated signaling mechanism and also will be used as potent devices for cell surface engineering.

In this paper, we report our preliminary studies on the construction of the peptide-GPI linkage. Coupling reactions between some peptides and a mannobioside-linked phosphoethanolamine are demonstrated (Fig. 1).

2. Results and discussion

Our studies began with the preparation of known mannnobioside **7**[7] by an alternative route. Phenyl 1-thio-α-D-mannopyranoside **1** was regioselectively silylated with *t*-BuMe_2SiCl and imidazole. The resulting triol **2** was benzylated to give **3** (92%). NIS/TfOH-promoted glycosidation of **3** and **4**[8] afforded a mixture of α-glycoside **5** and β-glycoside **6** in 86% yield (**5**/**6**=15/4). The anomeric configurations were assigned from the $^1J_{CH}$ coupling constant. Compound **5** was desilylated with Bu_4NF in THF to give **7** (89%), to which a phosphoethanolamine moiety was introduced in good yield by reaction with amidite **8**[2] followed by oxidation. The resultant phosphotriester **9** was deprotected by treatment with $NaOCH_3$/CH_3OH–ether and then hydrogenated to give **10** in 97% yield.

Keywords: GPI anchor; mannobioside; peptide thioester; native chemical ligation; CD 52.

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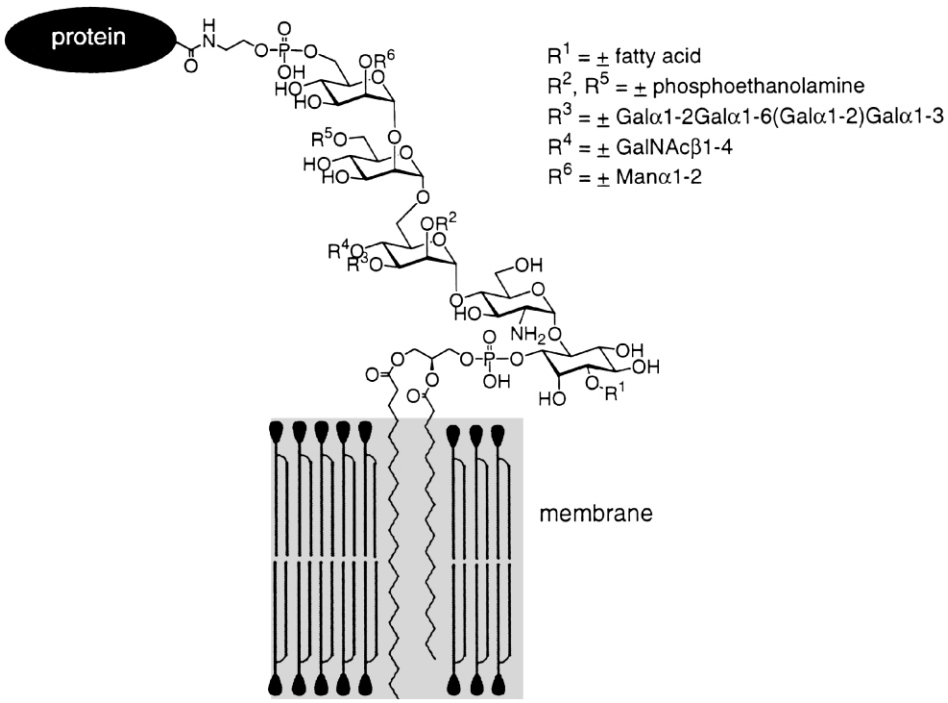


Figure 1. Structure of GPI anchors.

By employing compound **10** as a prototype of GPI, amide linkage-forming reaction on the ethanolamine terminus was tested. As the first model, a simple amino acid derivative, *N*-Fmoc alanine pentafluorophenyl ester (Fmoc-Ala-OPfp: 2 equiv.) **11**, was reacted with **10** in dry DMF in the presence of HOBt (2.2 equiv.) at room temperature. Compound **10** was consumed after overnight stirring, the reaction being monitored by reversed phase TLC (C-18) with iodine coloration. The water-soluble product was isolated by column chromatography on Sephadex LH-20 with H_2O/MeOH (1/1) and then by preparative HPLC with a reversed phase (C-8) column. The desired coupling product **15** was obtained in 64% yield and characterized by ^{1}H NMR and MALDI TOF MS. Coupling reactions with Fmoc-Ser-OPfp **12**, Fmoc-Thr-OPfp **13**, and Fmoc-Cys(SBu^t)-OPfp **14**, also proceeded to yield **16** (47%), **17** (65%), and **18** (61%), respectively. In addition to the desired products, di-acylated products were produced in substantial quantities in the preparation of **16** and **18**. HPLC profiles of the coupling products are exhibited in Figure 2. The byproducts **19** (4%), **20** (18%), and **21** (28%) most probably resulted from *N*- and *O*-acylation showed their molecular ions (+Na) at 1148.6, 1148.6, and 1356.0, respectively. It is to be noted that amino acid Pfp esters have often been utilized in solid-phase synthesis of the glycopeptides carrying unmasked oligosaccharides on account of the presumed poor *O*-acylation ability of the Pfp esters.[9]

In contrast, reaction of **10** with Fmoc amino acid thioesters exclusively produced the *N*-acylated compounds under the

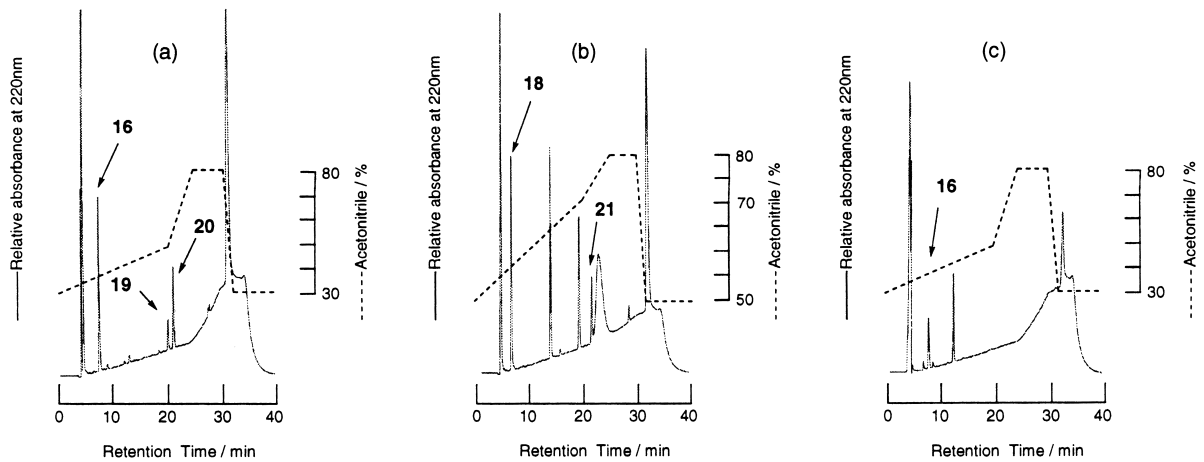
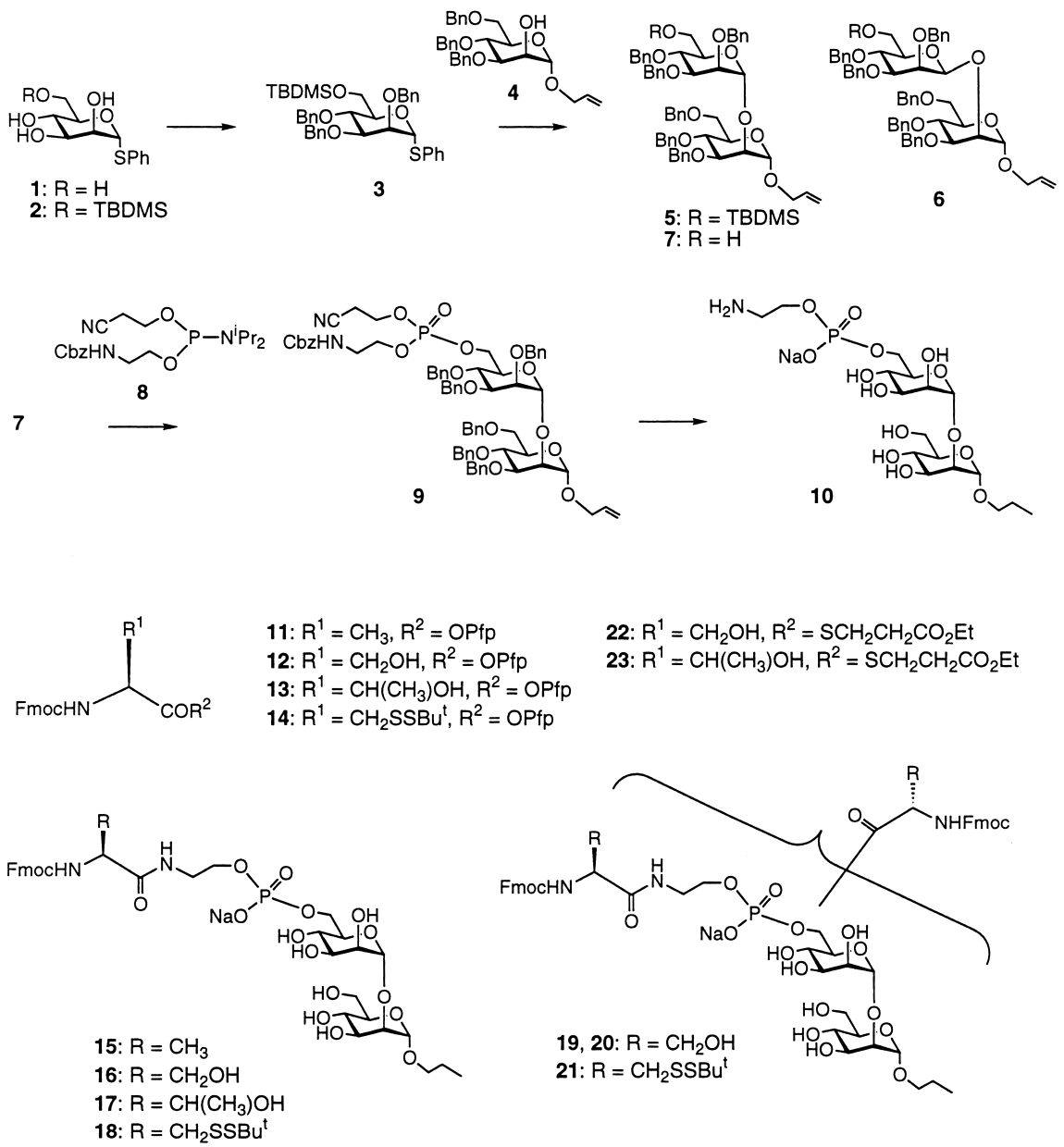


Figure 2. HPLC of the products from the reaction of **10** and **12** (a), the reaction of **10** and **14** (b), and the reaction of **10** and **22** (c). Column: Mightysil RP-8 (250×4.6 mm). Eluent A: distilled water containing 0.1% TFA, B: acetonitrile containing 0.1% TFA. Flow rate: 1 ml/min. In the chromatogram (b), the peaks at 13.2 min and at 18.7 min are Fmoc·Cys(SBu^t)·OH and Fmoc·Cys(SBu^t)·OMe probably generated during gel-permeation chromatography. In the chromatogram (c), the peak at 11.9 min corresponds to Fmoc·Ser·OH.



Scheme 1. Synthesis of mannobioside-linked phosphoethanolamine **10** and the aminoacylated products.

conditions reported for the peptide thioester method.[4] The reaction with Fmoc-Ser-$SCH_2CH_2CO_2C_2H_5$ **22**[10] (2 equiv.) smoothly proceeded in the presence of $AgNO_3$, *N*-hydroxysuccimide (HOSu), and diisopropylethylamine (DIEA) in DMF and completed within 2 h at room temperature. The reaction mixture was chromatographed on a gel-permeation column, and then product **16** was isolated in 76% yield by preparative HPLC. Under similar conditions, Fmoc-Thr-$SCH_2CH_2CO_2C_2H_5$ **23**[10] gave **17** in 76% yield. These results suggest that the amino group on the GPI molecule is as reactive as the peptide amine (Fig. 2 and Scheme 1).

With the successful results on the chemoselective *N*-acylation of the phosphoethanolamine moiety with thioesters, we next investigated the coupling reaction using a more complex oligopeptide. A dodecapeptide representing the peptide backbone of CD 52,[11] a GPI-anchored glycoprotein of lymphocytes, was chosen as a protein model.[12] The peptide thioester **24** was prepared on Rink amide MBHA resin by Fmoc-based solid-phase synthesis according to the reported protocol.[13] The thioester, however, was produced as a couple of diasteromers (**24a**/**24b**=13/7, 20% overall yield) probably generated by epimerization at the α carbon center of the C-terminal serine residue (Fig. 3(a)). An additional complication arose from the reaction of **10** (1.5 equiv.) with the separated diastereomer **24b**. When the reactants were treated with $AgNO_3$, HOSu, and DIEA in DMF, three products were generated. Two of them (**25a**,**25b**) showed the desired molecular ion peaks at 1761.34 and 1761.47, respectively, in their mass spectra, whereas the spectrum of the third one (**26**) exhibited *m*/*z* 1743.47 (Fig. 3(b)). Therefore, it seems likely that the former two (**25**) were diastereomers to each other and the latter (**26**) was derived from dehydration of the intermediate. Coupling reaction between **10** and another isomer of the thioester

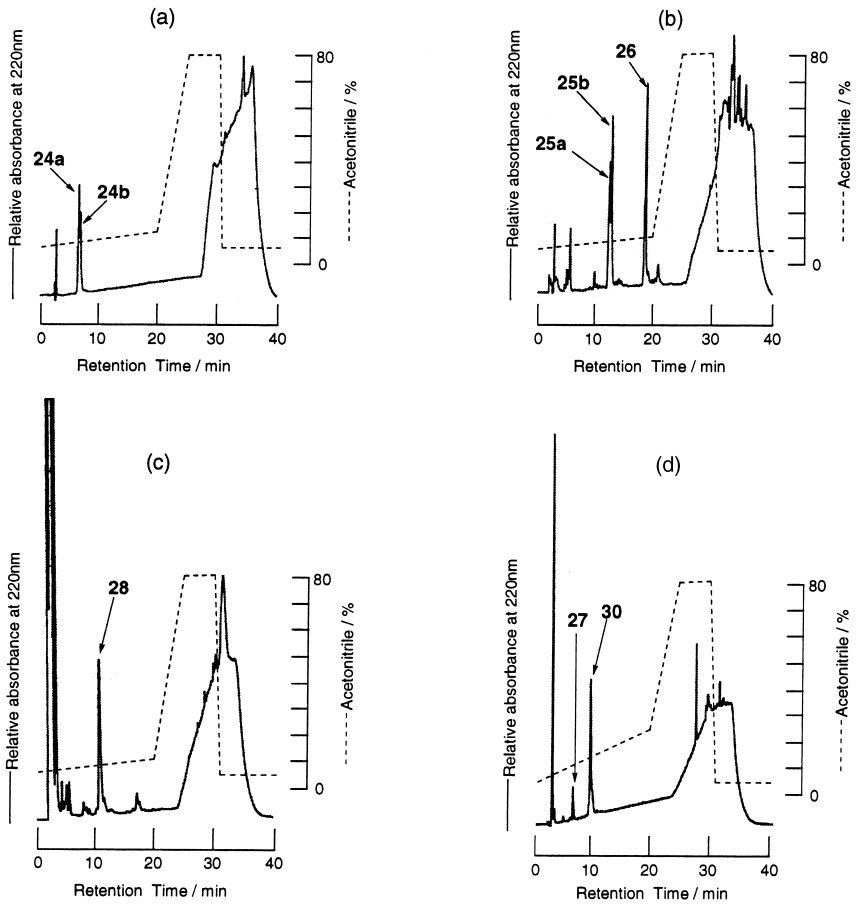


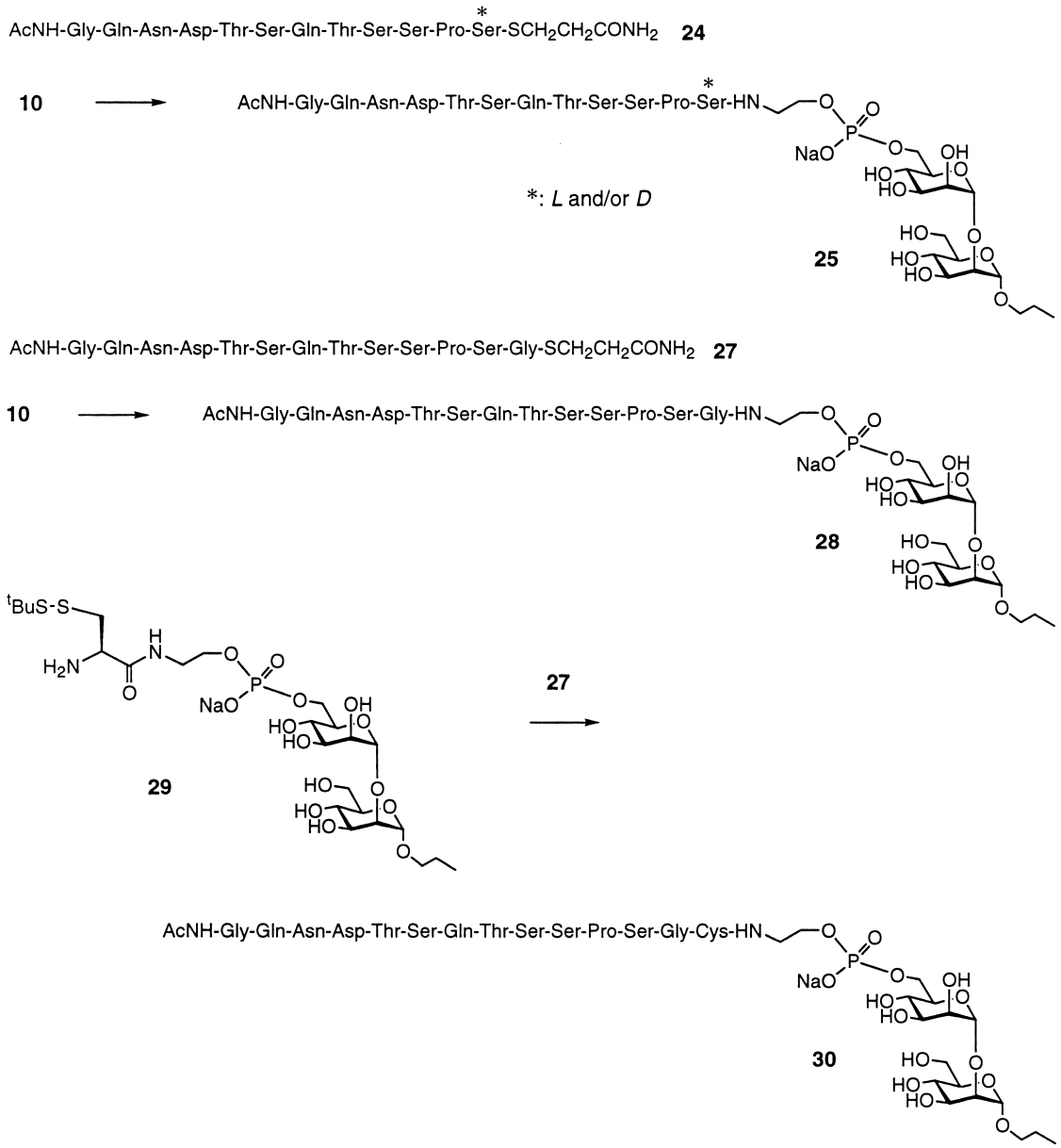
Figure 3. HPLC of the synthesized peptide thioesters **24a** and **24b** (a), the products from the reaction of **10** and **24b** (b), the products from the reaction of **10** and **27** (c), and the products from the reaction of **27** and **29** (d). Column: Mightysil RP-18 (150×4.6 mm). Eluent A: distilled water containing 0.1% TFA, B: acetonitrile containing 0.1% TFA. Flow rate: 1 ml/min.

(**24a**) was also carried out under the same conditions to yield a sole product but identical with the dehydrated molecule (**26**: *m*/*z* 1743.61).

In order to avoid the cumbersomeness arising from the labile serine thioester, a homologous peptide having C-terminal glycine thioester was synthesized. Starting with Fmoc-Gly-$SCH_2CH_2CO_2H$ and Rink amide MBHA resin, tridecapeptide thioester **27** was prepared in 12% overall yield. The thioester was allowed to react with **10** (1.5 equiv.) in the presence of the same promoters. The reaction was run for 3 days with monitoring by HPLC and MALDI TOF MS. The desired coupling product **28** was the major component in the reaction mixture at the stage of one-day running (Fig. 3(c)), and no further increase of the product in the mixture was observed. By gel permeation chromatography and subsequent preparative HPLC **28** was isolated in 34% yield.

In addition, we attempted to couple thioester **27** and a GPI anchor model under the conditions of the native chemical ligation.[5] The cystein-linked mannobiose **18** was *N*-deprotected with NaOMe/MeOH to **29** in 74% yield. Based on the expectation of in situ cleavage of the S–S linkage under the ligation conditions, the *S*-protected cysteinyl mannnobiose derivative was allowed to react with tridecapeptide **27**. A mixture of **27** and **29** (1/1) was stirred at room temperature with thiophenol (22 equiv.) in a phosphate buffer at pH 8.0. The desired coupling product **30** was slowly but certainly produced as thioester **27** was consumed. The progress of the reaction was readily monitored by HPLC and MALDI TOF MS. After 10 days running (Fig. 3(d)), the reaction was quenched by addition of AcOH and the product was isolated by HPLC. The compound **30** was obtained in 62% yield (78% based on the consumed **27**), while 20% of the starting thioester **27** remained unreacted. Elevated temperature (45–50°C) had little effect on acceleration of the total reaction. Further studies will be necessary to optimize the conditions (Scheme 2).

In conclusion, a synthetic strategy has been developed that allows reconstruction of the protein-GPI anchor linkage motif. Amino acid thioesters readily reacted with the mannnobiose-linked phosphoethanolamine group by activation with $AgNO_3$ and HOSu, whereas amino acid pentafluorophenyl esters resulted in not only *N*-amino acylation but additional *O*-amino acylation in some experiments. Peptide thioester involving the amino acid sequence of CD 52 was reactive with the phosphoethanolamine in the presence of $AgNO_3$ and HOSu to afford the coupling product in 34% yield. The coupling reaction designed in accordance with the native chemical ligation procedure was successful to give the desired product in higher yield. Although insertion of the Gly or Gly-Cys residue generated the non-native peptide sequence, such



Scheme 2. Synthesis of the peptidyl mannobiosides.

modification facilitated coupling of the peptide and the phosphoethnolamine. The chemistry described here will open the way for the total synthesis of the GPI-anchored proteins of biological significance. Studies on coupling reactions with a more complex mannoside carrying the glycero-phospholipid moiety are currently undertaken.

3. Experimental

3.1. General

Optical rotations were determined with a Jasco DIP-370 polarimeter for solutions in $CHCl_3$, unless noted otherwise. Column chromatography was performed on silica gel PSQ 100B (Fuji Silysia). TLC and HPTLC were performed on Silica Gel 60 F_{254} (E. Merck). ^1H and ^{13}C NMR spectra were recorded with a JEOL AL400 [^1H (400 MHz), ^{13}C (100 MHz)] spectrometer. Chemical shifts are expressed in ppm downfield from the signal for internal Me_4Si for solutions in $CDCl_3$. MALDI·TOF mass spectra were obtained with a Bruker AUTOFLEX-T spectrometer (2,5-dihydroxybenzoic acid was used as a matrix). High resolution Fab mass spectra were measured with JEOL JMS HX-110 spectrometer (2,5-dihydroxybenzoic acid was used as a matrix). All solid-phase reactions were performed at room temperature in the capped polypropylene test tubes with stirring on a vortex tube-mixer. HPLC was performed using Mightysil RP-8, RP-18 (4.6×150 mm for analysis and 10×250 mm for preparation, Kanto Chemical Co.). Amino acids were analyzed on a Hitachi L-8500 amino acid analyzer. Fmoc Rink amide MBHA resin was purchased from NOVA biochem.

3.1.1. Phenyl 6-*O*-*tert*-butyldimethylsilyl-1-thio-α-D-mannopyranoside 2. A mixture of **1** (500 mg, 1.84 mmol), *t*-$BuMe_2SiCl$ (332 mg, 2.20 mmol) and

imidazole (375 mg, 5.51 mmol) in dry DMF (3 ml) was stirred at 0°C for 10 min. The reaction was quenched by addition of ice-water, the mixture was extracted with $CHCl_3$, and the extract was washed with sat. $NaHCO_3$ and brine, dried (Na_2SO_4), and concentrated in vacuo. The residue was chromatographed on silica gel with toluene/EtOAc (1/4) to give **2** (512 mg, 72%). $[\alpha]_D$=+178.2° (*c* 1). ^{1}H NMR: δ 7.48–7.25 (m, 5H, Ar), 5.53 (s, 1H, H-1), 4.21 (s, 1H, H-2), 4.12–3.88 (m, 5H, H-3, H-4, H-5,H-6, H-6′), 3.79, 3.68, and 3.40 (3s, O*H*), 0.90 (s, 9H, *t*-Bu), 0.01 (s, 6H, Me_2). Anal. calcd for $C_{18}H_{30}O_5SSi{\cdot}0.3H_2O$: C, 55.15; H, 7.87; S, 8.18%. Found: C, 55.22; H, 7.77; S, 7.97%.

3.1.2. Phenyl 2,3,4-tri-*O*-benzyl-6-*O*-*tert*-butyldimethylsilyl-1-thio-α-D-mannopyranoside 3. To a stirred mixture of **2** (1.0 g, 2.6 mmol) and 60% NaH (0.47 g, 11.6 mmol) in dry DMF (10 ml) was added BnBr (1.38 ml, 11.6 mmol) at 0°C under Ar. The mixture was stirred at the temperature for 1 h before quenching the reaction with ice-water. The product was extracted with ether, washed with water and brine, dried (Na_2SO_4), and concentrated in vacuo. The residue was chromatographed on silica gel with toluene to afford **3** (1.57 g, 92%). $[\alpha]_D$=+69.4° (*c* 1). ^{1}H NMR: δ 7.45–7.15 (m, 20H, Ar), 5.56 (d, 1H, *J*=1.5 Hz, H-1), 4.95 (d, 1H, *J*=11.0 Hz, PhCH_2–), 4.63 (m, 5H, PhCH_2–), 4.10 (m, 1H, H-5), 3.99–3.88 (m, 5H, H-2, H-3, H-4, H-6, H-6′), 0.89 (s, 9H, *t*-Bu), 0.06 and 0.05 (2s, 6H, Me_2). Anal. calcd for $C_{39}H_{48}O_5SSi{\cdot}0.5H_2O$: C, 70.34; H, 7.42; S, 4.82%. Found: C, 70.30; H, 7.59; S, 4.82%.

3.1.3. Allyl *O*-(2,3,4-tri-*O*-benzyl-6-*O*-*tert*-butyldimethylsilyl-α-D-mannopyranosyl)-(1→2)-3,4,6-tri-*O*-benzyl-α-D-mannopyranoside 5 and allyl *O*-(2,3,4-tri-*O*-benzyl-6-*O*-*tert*-butyldimethylsilyl-β-D-mannopyranosyl)-(1→2)-3,4,6-tri-*O*-benzyl-α-D-mannopyranoside 6. A mixture of **3** (1.52 g, 2.3 mmol), **4** (0.95 g 1.9 mmol), NIS (0.71 g, 2.9 mmol), and dried molecular sieves 3 Å (5 g) in dry CH_2Cl_2 (50 ml) was stirred at 0°C under Ar for 15 min. TfOH (25.6 μl, 0.3 mmol) was added to the mixture, which was then stirred for 1 h before quenching with sat. $NaHCO_3$. The mixture was filtered through Celite, the filtrate was extracted with $CHCl_3$, and the extract was washed with 10% $Na_2S_2O_3$ and brine, dried (Na_2SO_4), and concentrated in vacuo. The crude product was chromatographed on silica gel with toluene/EtOAc (49/1) to give **5** (1.36 g, 68%) and **6** (0.36 g, 18%).

Compound **5**. $[\alpha]_D$=+18.3° (*c* 1). ^{1}H NMR: δ 7.33–7.15 (m, 30H, Ar), 5.87 (m, 1H, CH_2=C*H*–), 5.24 (s, 1H, H-1b), 5.23 (brd, 1H, *J*=17.0 Hz, CH_2=CH–), 5.15 (d, 1H, *J*=10.5 Hz, CH_2=CH–), 4.90 (d, 1H, *J*=11.0 Hz, PhCH_2–), 4.86 (s, 1H, H-1a), 1.61 (s, 9H, *t*-Bu), 0.08 and 0.07 (2s, 6H, Me_2). ^{13}C NMR: δ 98.2 ($^1J_{CH}$=171.7 Hz, C-1b), 98.6 ($^1J_{CH}$=170.8 Hz, C-1a). MALDI TOF MS: calcd for $C_{63}H_{76}O_{11}S$ i·Na; *m*/*z* 1059.51, found; 1059.94. Anal. calcd for $C_{63}H_{76}O_{11}Si$: C, 72.94; H, 7.38%. Found: C, 72.73; H, 7.41%.

Compound **6**. $[\alpha]_D$=−37.6° (*c* 1). ^{1}H NMR: δ 7.54–7.10 (m, 30H, Ar), 5.89 (m, 1H, CH_2=C*H*–), 5.28 (dd, 1H, *J*=1.5, 17.3 Hz, CH_2=CH–), 5.20 (brd, 1H, *J*=10.2 Hz, CH_2=CH–), 5.05 (brs, 1H, H-1a), 4.63 (brs, 1H, H-1b), 0.87 (s, 9H, *t*-Bu), 0.03 and 0.01 (2s, 6H, Me_2). ^{13}C NMR: δ 95.7 ($^1J_{CH}$=166.7 Hz, C-1a), 98.7 ($^1J_{CH}$=153.4 Hz, C-1b). MALDI TOF MS: calcd for $C_{63}H_{76}O_{11}Si{\cdot}Na$; *m*/*z* 1059.51, found; 1059.94. Anal. calcd for $C_{63}H_{76}O_{11}Si{\cdot}0.5H_2O$: C, 72.18; H, 7.40%. Found: C, 72.38; H, 7.28%.

3.1.4. Allyl *O*-(2,3,4-tri-*O*-benzyl-α-D-mannopyranosyl)-(1→2)-3,4,6-tri-*O*-benzyl-α-D-mannopyranoside 7. To a solution of **5** (500 mg, 0.48 mmol) in freshly distilled THF (10 ml) was added 1 M Bu_4NF/THF (1.4 ml, 1.4 mmol) at 0°C. The mixture was stirred at 0°C–room temperature overnight and concentrated in vacuo. The residue was dissolved in $CHCl_3$, washed with water and brine, dried (Na_2SO_4), and concentrated in vacuo. The crude product was chromatographed on silica gel with toluene/EtOAc (5/1) to give **7** (397 mg, 89%).

^{1}H NMR: δ 7.51–7.14 (m, 30H, Ar), 5.86 (m, 1H, CH_2=C*H*–), 5.24 (brd, *J*=17.1 Hz, CH_2=CH–), 5.17 (brd, 1H, *J*=10.2 Hz, CH_2=CH–), 5.16 (brs, 1H, H-1b), 4.87 (brs, 1H, H-1a). MALDI TOF MS: calcd for $C_{57}H_{62}O_{11}{\cdot}Na$; *m*/*z* 945.41, found; 945.70.

3.1.5. Allyl *O*-{2,3,4-tri-*O*-benzyl-6-*O*-[2-(*N*-benzyloxycarbonylamino)ethyl 2-cyanoethyl phosphonato]-α-D-mannopyranosyl}-(1→2)-3,4,6-tri-*O*-benzyl-α-D-mannopyranoside 9. A mixture of **7** (458 mg, 0.50 mmol), 1*H*-tetrazole (70 mg, 0.99 mmol), **8** (982 mg, 2.48 mmol), and dried molecular sieves 3 Å (2 g) in dry CH_3CN (20 ml) was stirred for 4 h under Ar. Then another equivalent of 1*H*-tetrazole (35 mg, 0.49 mmol) was added to the mixture, which was further stirred for 5 min. A solution of 5.5 M *t*-BuOOH/nonane (1 ml, 5.5 mmol) was added to the mixture. After stirring for 10 min, the reaction was quenched with sat. $NaHCO_3$, and the mixture was filtered through Celite. The product in the filtrate was extracted with CH_2Cl_2 and dried (Na_2SO_4), and concentrated in vacuo. The residue was chromatographed on silica gel with toluene/EtOAc (1/2) to afford **9** (553 mg, 90%) as a mixture of diastereomeric phosphotriesters. ^{1}H NMR: δ 7.32–7.18 (m, 30H, Ar), 5.86 (m, 1H, CH_2=C*H*–), 5.44 and 5.38 (2m, 1H, N*H*), 5.24 (brd, 1H, *J*=17.1 Hz, CH_2=CH–), 5.17 (brd, 1H, *J*=10.4 Hz, CH_2=CH–), 5.12 (brs, 1H, H-1b), 5.07 (brs, 2H, –CO_2CH_2Ph), 4.84 (brs, 1H, H-1a), 3.38 and 3.29 (2 m, 2H, ZNHCH_2–), 2.48 and 2.25 (2m, 2H, –CH_2CN). ^{13}C NMR: δ 98.0 (C-1a), 99.6 and 99.7 (C-1b). HRMS: calcd for $C_{70}H_{77}N_2O_{16}PNa$ $(M+Na)^+$; *m*/*z* 1255.4908, found; 1255.4905. MALDI TOF MS: calcd for $C_{70}H_{77}N_2O_{16}P{\cdot}Na$; *m*/*z* 1255.49, found; 1255.66.

3.1.6. *n*-Propyl *O*-[6-*O*-(2-aminoethyl phosphonato)-α-D-mannopyranosyl]-(1→2)-α-D-mannopyranoside 10. To a stirred solution of **9** (523 mg, 0.42 mmol) in anhydrous MeOH/Et_2O (1/1, 6 ml) was added 1 M NaOMe/MeOH till the mixture became basic. Then the mixture was stirred for 45 min, before being neutralized with Amberlite IR 120 (H^+), filtered, and concentrated in vacuo. The residue was chromatographed on silica gel with $CHCl_3$/MeOH (4/1) containing 1% Et_3N to afford de-cyanoethylated product as the triethylammonium salt (527 mg, 97%), $[\alpha]_D$=+13.6° (*c*, 1). MALDI TOF MS: calcd for $C_{67}H_{74}NO_{16}P{\cdot}Na$; *m*/*z* 1202.46, found; 1202.15. The product (513 mg, 0.40 mmol) was dissolved in 80% aq. MeOH (10 ml) with an additional distilled THF, and hydrogenated with $Pd(OH)_2$ (200 mg) for

3 days. The mixture was filtered through Celite, the filtrate was concentrated in vacuo, and the residue was passed through a column of Amberlite IR 120 (Na^+). The resulting solution was lyophilized to quantitatively give **10** (237 mg). $[\alpha]_D$=+38.3° (*c* 1, H_2O). ^1H NMR (as Et_3N salt, D_2O): δ 4.89 and 4.87 (2 brs, 2H, H-1a and H-1b), 1.45 (m, 2H, $-CH_2CH_2CH_3$), 0.76 (t, 3H, *J*=7.0 Hz, Me). ^{13}C NMR (D_2O): δ 98.9 and 103.0 (C-1a and C-1b). MALDI TOF MS: calcd for $C_{17}H_{34}NO_{14}P{\cdot}Na$; *m/z* 530.15, found; 529.65.

3.1.7. *n*-Propyl *O*-{6-*O*-[2-{[*N*-(9-fluorenylmethoxycarbonyl)-L-alanyl]amido}ethyl phosphonato]-α-D-mannopyranosyl}-(1→2)-α-D-mannopyranoside 15. A mixture of **10** (10 mg, 19 μmol), **11** (18 mg, 38 μmol), and HOBt (2.8 mg, 21 μmol) in dry DMF (2 ml) was stirred for 3.5 h at room temperature under Ar. Then an additional amount (2.8 mg) of HOBt was added to the mixture, which was further stirred overnight. The reaction mixture was concentrated in vacuo to give the residue, which was chromatographed on Sephadex LH 20 with 50% aq. MeOH. The product was purified by HPLC using an RP-8 column eluted with a gradient of 30–50%/0–20 min and 50–80%/20–25 min of CH_3CN in H_2O containing 0.1% TFA to afford **15** (9.9 mg, 64%). $[\alpha]_D$=+25.1° (*c* 0.5, MeOH). ^1H NMR (D_2O; *t*-BuOH, δ 1.24): δ 7.75 (brd, 2H, *J*=6.8 Hz, Ar), 7.58 (brd, 2H, *J*=6.4 Hz, Ar), 7.46–7.41 (m, 4H, Ar), 5.00 (brs, 2H, H-1a and H-1b), 1.50 (m, 2H, $-CH_2CH_2CH_3$), 1.08 (br, 1H, Ala-βH), 0.82 (t, 3H, *J*=7.3 Hz, Me). MALDI TOF MS: calcd for $C_{35}H_{49}N_2O_{17}P{\cdot}Na$; *m/z* 823.27, found; 823.58.

3.1.8. *n*-Propyl *O*-{6-*O*-[2-{[*N*-(9-fluorenylmethoxycarbonyl)-L-seryl]amido}ethyl phosphonato]-α-D-mannopyranosyl}-(1→2)-α-D-mannopyranoside 16. Reaction of **10** (10 mg, 19 μmol) and **12** (18.6 mg, 38 μmol) was performed in the presence of HOBt as described above for **15**. HPLC purification of the product afforded **16** (7.5 mg, 47%), **19** (0.8 mg, 4%) and **20** (4.0 mg, 18%).

Compound **16**. $[\alpha]_D$=+20.1° (*c* 0.4, H_2O). ^1H NMR (D_2O; *t*-BuOH, δ 1.24): δ 7.83 (brd, 2H, *J*=7.1 Hz, Ar), 7.65–7.60 (m, 2H, Ar), 7.44–7.38 (m, 4H, Ar), 4.84 (brs, 2H, H-1a and H-1b), 1.52 (m, 2H, $-CH_2CH_2CH_3$), 0.85 (t, 3H, *J*=7.2 Hz, Me). MALDI TOF MS: calcd for $C_{35}H_{49}N_2O_{18}P{\cdot}Na$; *m/z* 839.26, found; 839.07.

Compound **19**. ^1H NMR (CD_3OD): δ 7.79 (brd, 4H, *J*=7.6 Hz, Ar), 7.68 (m, 4H, Ar), 7.41–7.30 (m, 8H, Ar), 5.04 and 5.00 (2s, 2H, H-1a and H-1b), 1.58 (m, 2H, $-CH_2CH_2CH_3$), 0.93 (t, 3H, *J*=7.4 Hz, Me). MALDI TOF MS: calcd for $C_{53}H_{64}N_3O_{22}P{\cdot}Na$; *m/z* 1148.36, found; 1148.62.

Compound **20**. ^1H NMR (CD_3OD): δ 7.82 (m, 4H, Ar), 7.70 (brs, 4H, Ar), 7.43–7.35 (m, 8H, Ar), 5.07 and 5.04 (2 brs, 2H, H-1a and H-1b), 1.62 (m, 2H, $-CH_2CH_2CH_3$), 0.98 (t, 3H, *J*=7.3 Hz, Me). MALDI TOF MS: found; 1148.62.

3.1.9. *n*-Propyl *O*-{6-*O*-[2-{[*N*-(9-fluorenylmethoxycarbonyl)-L-threonyl]amido}ethyl phosphonato]-α-D-mannopyranosyl}-(1→2)-α-D-mannopyranoside 17. Reaction of **10** (10 mg, 19 μmol) and **12** (19.1 mg, 38 μmol) was performed in the presence of HOBt as described above for **15**. HPLC purification of the product afforded **17** (10.4 mg, 65%). $[\alpha]_D$=+20.9° (*c* 5, H_2O). ^1H NMR (D_2O; *t*-BuOH, δ 1.24): δ 7.72 (br, 2H, Ar), 7.60–7.55 (m, 2H, Ar), 7.34 (m, 4H, Ar), 5.00 (s, 2H, H-1a and H-1b), 1.50 (m, 2H, $-CH_2CH_2CH_3$), 1.06 (br, 3H, Thr-βH), 0.83 (brt, 3H, *J*=7.2 Hz, Me). MALDI TOF MS: calcd for $C_{36}H_{51}N_2O_{18}P{\cdot}Na$; *m/z* 853.28, found; 853.20.

3.1.10. *n*-Propyl *O*-{6-*O*-[2-{[*N*-(9-fluorenylmethoxycarbonyl)-*S*-(*tert*-butylthio)-L-cysteinyl]amido}ethyl phosphonato]-α-D-mannopyranosyl}-(1→2)-α-D-mannopyranoside 18. Reaction of **10** (10 mg, 19 μmol) and **13** (22.5 mg, 38 μmol) was performed in the presence of HOBt as described above for **15**. Gel permeation chromatography was done with MeOH. The product was purified by HPLC using an RP-8 column eluted with a gradient of 50–70%/0–20 min and 70–80%/20–25 min of CH_3CN in H_2O containing 0.1% TFA to afford **18** (10.9 mg, 61%) and **21** (7.1 mg, 28%).

Compound **18**. $[\alpha]_D$=-16.0° (*c* 1.3, H_2O). ^1H NMR (CD_3OD): δ 7.78 (d, 2H, *J*=7.6 Hz, Ar), 7.69 (d, 2H, *J*=7.3 Hz, Ar), 7.40–7.30 (m, 4H, Ar), 5.00 (d, 1H, *J*=1.4 Hz) and 4.98 (1s, 1H) (H-1a and H-1b), 4.45 (m, 1H, Cys-αH), 3.20 (dd, 1H, *J*=4.9, 13.4 Hz, Cys-βH), 2.99 (dd, 1H, *J*=8.8, 13.4 Hz, Cys-βH), 1.58 (m, 2H, $-CH_2CH_2CH_3$), 1.34 (s, 9H, *t*-Bu), 0.93 (t, 3H, *J*=7.3 Hz, Me). MALDI TOF MS: calcd for $C_{39}H_{57}N_2O_{17}PS_2{\cdot}Na$; *m/z* 943.27, found; 943.20.

Compound **21**. ^1H NMR (CD_3OD): δ 7.68 (brd, 4H, *J*=7.5 Hz, Ar), 7.57 (brd, *J*=7.3 Hz, Ar), 7.30–7.18 (m, 8H, Ar), 4.90 and 4.89 (2 brs, 2H, H-1a and H-1b), 1.47 (m, 2H, $-CH_2CH_2CH_3$), 1.23 (s, 18H, *t*-Bu), 0.82 (t, 3H, *J*=7.3 Hz, Me). MALDI TOF MS: calcd for $C_{61}H_{80}N_3O_{20}PS_4{\cdot}Na$; *m/z* 1356.39, found; 1356.00.

3.1.11. Reaction of 10 with 22 (alternative synthesis of 16). A mixture of **10** (10 mg, 19 μmol), **22** (16.7 mg, 38 μmol), HOSu (43.3 mg, 376 μmol), $AgNO_3$ (19.2 mg, 113 μmol), and DIEA (20 μl, 113 μmol) in dry DMF (2 ml) was stirred for 3 h in the dark at room temperature under Ar, and then concentrated n vacuo. The residue was dissolved in 50% aq. MeOH, and insoluble material was filtered off through Celite and a disk of membrane filter. The filtrate was concentrated in vacuo to give the residue, which was chromatographed on Sephadex LH 20 with 50% aq. MeOH. The product was purified by HPLC as described above to give **16** (12.1 mg, 76%).

3.1.12. Reaction of 10 with 23 (alternative synthesis of 17). Coupling reaction of **10** (10 mg, 19 μmol) and **23** (17.2 mg, 38 μmol) was performed in an analogous procedure as described above to produce **17** (12.2 mg, 76%).

3.1.13. *S*-(*N*-Acetyl-L-glycyl-L-glutaminyl-L-asparaginyl-L-α-aspartyl-L-threonyl-L-seryl-L-glutaminyl-L-threonyl-L-seryl-L-seryl-L-proryl-L-seryl)-3-thiopropionamide 24. A mixture of Fmoc-Ser(Bu^t)-OH (2.0 g, 5.2 mmol), HOSu (0.72 g, 6.3 mmol), and WSCI (1.6 g, 8.4 mmol) in dry DMF (20 ml) was stirred at 0°C–room temperature for 9 h. The mixture was concentrated in vacuo

to the residue, which was extracted with EtOAc, washed with 5% aq. $KHSO_4$ and brine, dried (Na_2SO_4), and concentrated in vacuo. The residue was dissolved in dry DMF (20 ml), and stirred with 3-mercaptopropionic acid (350 μl, 4.0 mmol) and DIEA (350 μl, 2.0 mmol) at room temperature for 8 h. The mixture was concentrated in vacuo, the residue was extracted with EtOAc, and the extract was washed with 5% aq. $KHSO_4$ and brine, dried (Na_2SO_4), and concentrated in vacuo. The crude product was chromatographed on C-18 silica gel with MeOH/H_2O (3/1) to give Fmoc-Ser(Bu^t)-$SCH_2CH_2CO_2H$ (2.0 g, 83%), which was used for the following solid-phase synthesis. All the solid-phase reactions were performed in a polypropylene tube equipped with a coarse filter and three-way stopcock by stirring with a vortex mixer.

Commercial Fmoc Rink amide MBHA resin (274 mg, 0.2 mmol) was stirred with 20% piperidine/NMP (5 ml) for 5 min. After filtration the resin was again stirred with 20% piperidine/NMP (5 ml) for 15 min to complete *N*-deprotection. The resin was washed several times with NMP (5 ml) and then stirred with Fmoc-Ser(Bu^t)-$SCH_2CH_2CO_2H$ (377 mg, 0.8 mmol), 1 M DCC/NMP (0.88 ml, 0.88 mmol), and 1 M HOBt/NMP (0.88 ml, 0.88 mmol) in NMP (5 ml) for 1 h. The mixture was filtered and the resultant resin was washed successively with NMP and MeOH/CH_2Cl_2 (1/1). The unreacted amino group on the resin was masked by acetylation with 10% Ac_2O–5% DIEA/NMP (5 ml). After washing with NMP, the resin was stirred with a mixture of 25% 1-methylpyrrolidine–2% hexamethylene imine–2% HOBt/NMP-DMSO (1/1, 5 ml)[13] for 3 min in order to selectively remove the Fmoc group. After filtration, the de-Fmoc procedure was again repeated but for 10 min to complete *N*-deprotection. The resin thoroughly washed with NMP was used for further peptide assembling with Fmoc-Pro-OH, Fmoc-Ser(Bu^t)-OH, Fmoc-Thr(Bu^t)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Asp(OBu^t)-OH, Fmoc-Asn(Trt)-OH, and Fmoc-Gly-OH. Fmoc amino acids (4 equiv.), DCC (4.4 equiv.), and HOBt (4.4 equiv.) were used for each coupling reaction. *N*-Deprotection with the above mixture (3 and 18 min) and acetyl capping procedures (5 min) were performed as described above. The dodecapeptide thus prepared was finally transformed into the N-terminally acetylated peptide and cleaved from the resin by treatment with reagent K (82.5% TFA, 2.5% ethaneditiol, 5% phenol, 5% thioanisole, 5% H_2O) for 2 h. The resin was filtered off, and the product was precipitated from the filtrate by adding Et_2O. The precipitate was washed with Et_2O, dissolved in aq. CH_3CN, and purified by HPLC with a reversed-phase (C-18) column eluted with a gradient of 7–14%/0–20 min of CH_3CN in H_2O containing 0.1% TFA to give **24a** and **24b** as two diastereomers in 13% (34 mg) and 7% (18 mg) overall yields, respectively. MALDI TOF MS: calcd for $C_{50}H_{80}N_{16}O_{25}S$·Na; *m*/*z* 1359.51, found; 1359.51 (for **24a**) and 1359.39 (for **24b**).

3.1.14. Coupling reaction of 10 and 24. A mixture of **10** (5.9 mg, 11 μmol), **24b** (10 mg, 38 μmol), HOSu (17 mg, 148 μmol), $AgNO_3$ (7.5 mg, 44 μmol), and DIEA (7.7 μl, 44 μmol) in dry DMF (2 ml) was stirred overnight in the dark at room temperature under Ar, and then concentrated in vacuo. The residue was dissolved in MeOH/H_2O (1/1), and filtered through a membrane filter and then through a C-18 cartridge. The crude product was chromatographed on Sephadex LH 20 with MeOH/H_2O (1/1), and further purified by HPLC on a C-18 column eluted with a gradient of 5–10%/0–20 min of CH_3CN in H_2O containing 0.1% TFA to give **25a** (1.1 mg, 8%), **25b** (2.5 mg, 19%). Compound **26** was also obtained in the less mobile fraction (20%).

On the other hand, reaction of **10** and **24a** performed in an analogous conditions exclusively gave **26**.

3.1.15. *S*-(*N*-Acetyl-L-glycyl-L-glutaminyl-L-asparaginyl-L-α-aspartyl-L-threonyl-L-seryl-L-glutaminyl-L-threonyl-L-seryl-L-seryl-L-proryl-L-seryl-L-glycyl)-3-thiopropionamide 27. Tridecapeptide thioester **27** was synthesized starting from Fmoc-Gly-OH in a similar manner as described for **24**. Purification of the product by reversed-phase HPLC with a gradient of 5–10%/0–30 min of CH_3CN in H_2O containing 0.1% TFA afforded **27** (12%). MALDI TOF MS: calcd for $C_{52}H_{83}N_{17}O_{26}S$·Na; *m*/*z* 1416.53, found; 1416.44.

3.1.16. *n*-Propyl *O*-[6-*O*-{2-[(*N*-acetyl-L-glycyl-L-glutaminyl-L-asparaginyl-L-α-aspartyl-L-threonyl-L-seryl-L-glutaminyl-L-threonyl-L-seryl-L-seryl-L-proryl-L-seryl-L-glycyl)amido]ethyl phosphonato}-α-D-mannopyranosyl]-(1→2)-α-D-mannopyranoside 28. Coupling reaction of **10** (5.6 mg, 11 μmol) and **27** (10 mg, 7 μmol) was performed by stirring with HOSu (16.3 mg, 142 μmol), $AgNO_3$ (7.2 mg, 43 μmol), and DIEA (7.4 μl, 43 μmol) in dry DMF (4 ml) for 3 days and worked up as described for **25**. Purification of the product by reversed-phase HPLC with elution of a gradient of 5–10%/0–20 min of CH_3CN in H_2O containing 0.1% TFA afforded **28** (4.4 mg, 34%). MALDI TOF MS: calcd for $C_{66}H_{110}N_{17}O_{39}P$·Na; *m*/*z* 1817.67, found; 1818.53.

3.1.17. *n*-Propyl *O*-{6-*O*-[2-{[*S*-(*tert*-butylthio)-L-cysteinyl]amido}ethyl phosphonato]-α-D-mannopyranosyl}-(1→2)-α-D-mannopyranoside 29. To a solution of **18** (36 mg, 38 μmol) in MeOH (2 ml) was added 1 M NaOMe/MeOH (378 μl, 378 μmol). The mixture was stirred for 2 h at room temperature and concentrated in vacuo. The residue was chromatographed on Sephadex LH 20 with MeOH/H_2O (1/1) and then on silica gel with $CHCl_3$/MeOH/H_2O (12/7/1) to give **29** (20 mg, 74%).$[\alpha]_D$=+32.9° (*c* 1, MeOH). ^{1}H NMR (CD_3OD): δ 5.00 (brs, 1H) and 4.98 (d, 1H, *J*=1.5 Hz) (H-1a and H-1b), 3.59 (brt, 1H, *J*=9.5 Hz) and 3.45 (dt, 1H, *J*=6.3, 9.5 Hz) (–OC*H*$_2$Et), 3.23 (dd, 1H, *J*=5.7, 14.1 Hz) and 3.06 (dd, 1H, *J*=7.8, 14.1 Hz) (Cys-βH), 1.60 (m, 2H, –$CH_2CH_2CH_3$), 1.37 (s, 9H, *t*-Bu), 0.95 (t, 3H, *J*=7.6 Hz, Me). ^{13}C NMR: δ 99.7 and 103.9 (C-1a and C-1b). HRMS: calcd for $C_{24}H_{47}N_2O_{15}PS_2$ $(M+H)^+$; *m*/*z* 699.2234, found; 699.2153. MALDI TOF MS: calcd for $C_{24}H_{46}N_2O_{15}PS_2$·Na; *m*/*z* 721.21, found; 721.46.

3.1.18. *n*-Propyl *O*-[6-*O*-{2-[(*N*-acetyl-L-glycyl-L-glutaminyl-L-asparaginyl-L-α-aspartyl-L-threonyl-L-seryl-L-glutaminyl-L-threonyl-L-seryl-L-seryl-L-proryl-L-seryl-L-glycyl-L-cysteinyl)amido]ethyl phosphonato}-α-D-mannopyranosyl]-(1→2)-α-D-mannopyranoside 30.

Compound **29** (2.6 mg, 3.6 μmol) was dissolved in minimum volume of a mixture (1/1) of MeOH/0.1 M phosphate buffer (pH 8.0). To the solution was added thioester **27** (5.0 mg, 3.6 μmol), and the mixture was diluted with the phosphate buffer (70 μl). Then thiophenol (2.4 μl, 23.4 μmol) was added. The mixture was stirred at room temperature on a vortex mixer for 2 days. Since more than half of starting **27** had remained as judged by HPLC analysis, the mixture was allowed to stir with an additional thiophenol (5.5 μl, 53.6 μmol) for further 8 days. Then the reaction was quenched by addition of AcOH (50 μl) and the mixture was centrifuged. The supernatant solution was submitted to the purification by HPLC on a C18 column eluted with a gradient of 5–25%/0–20 min of CH_3CN in H_2O containing 0.1% TFA, affording **30** (4.3 mg, 62%) in addition to the recovered **27** (1.0 mg). MALDI TOF MS: calcd for $C_{69}H_{115}N_{18}O_{40}PS{\cdot}Na$; *m/z* 1921.69, found; 1921.45.

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