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Efficient and versatile synthesis of mucin-like glycoprotein mimics

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Abstract—An efficient synthetic method of sequential glycopeptide-polymer by means of the combined chemical and enzymatic strategy was applied to the practical synthesis of some tandem repeating mucin-type glycoproteins. Versatility of the present synthetic strategy was demonstrated by elucidating some physicochemical and conformational analyses of these synthetic mucins. © 2002 Elsevier Science Ltd. All rights reserved.

1. Introduction

Carbohydrates of the glycoproteins are classified into two major classes as *N*-glycans and *O*-glycans according to chemical structures of the glycoside linkage between oligosaccharides and peptide main chains.[1] Proteoglycans and mucins are typical *O*-glycans showing a variety of specific functions as adhesive and signal molecules in cell–cell interactions.[2] These biological roles by *O*-glycans might be deeply dependent on the multivalent structures of carbohydrate branches bound to the peptide (protein) main chains. In the course of the studies on structure–function relationship of synthetic models for the glycoproteins,[3] our interest has been focused on a variety of tandem repeating peptide motifs bearing sugar chains found in naturally occurring mucins and proteoglycans.[4] We considered that the advent of the efficient synthetic strategy of these tandem repeating glycoproteins will be one of the key technologies to construct glycoclusters having both designated density and desired orientation of carbohydrate side chains.[5] These synthetic glycoproteins have been expected to become nice tools for the preparation of monoclonal antibody against essential carbohydrate–peptide epitopes and novel class of cell adhesive molecules in biomedical materials as well as for the investigation of structure–activity relationship of natural mucins. Here we would like to report a general concept and feasibility of the combined chemical and enzymatic synthesis of functional macromolecular mucins.

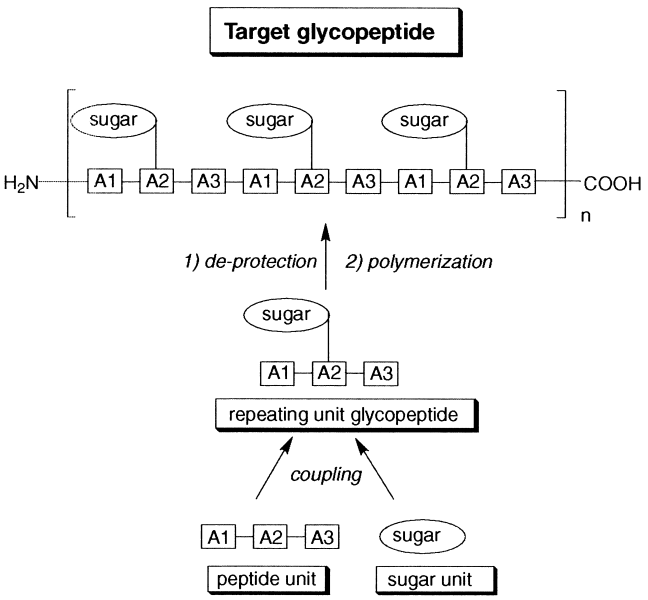

Figure 1. Synthetic strategy of sequential glycopeptides in this study.

Keywords: mucins; tandem-repeating glycoproteins; sialyl T antigenic glycoproteins; multivalency; cluster effect.

Abbreviations: AFGP, antifreeze glycoprotein; CMP-Neu5Ac, cytidine monophosphate-*N*-acetylneuramic acid; CD, circular dichroism; CSA, (±)-camphor-10-sulfonic acid; DAST, (diethylamino)sulfur trifluoride; DMF, *N*,*N*-dimethylformamide; DPPA, diphenylphosphorylazide; GalNAc, *N*-acetyl galactosamine; GPC, gel permeation chromatography; PPTS, pyridinium *p*-toluenesulfonate; TMSOTf, trimethylsiyl trifluoromethansulfonate.

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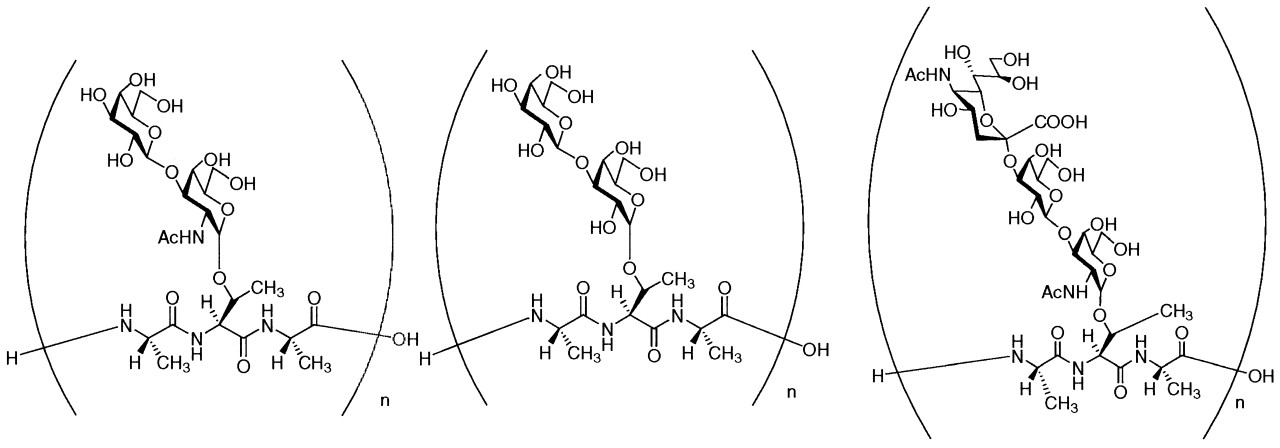


Figure 2. Glycopeptides synthesized in this study.

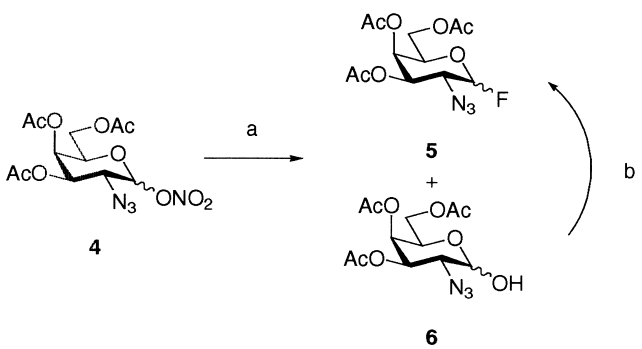
2. Results and discussion

Practical syntheses of mucin-type glycoprotein analogs were basically performed by utilizing direct polymerization of unprotected glycopeptide macromers in the presence of diphenylphosphorylazide (DPPA)[6,7] as a promotor (Fig. 1).[8] Since DPPA selectively activates a peptide carboxyl group even in the presence of unprotected hydroxyl groups on the complex carbohydrate moiety, glycopeptide units can be employed for the direct polymerization reactions without any protective groups at sugar hydroxyl groups.[9] Fig. 2 shows compounds synthesized in the present work and this method provided glycoproteins with molecular mass from 6600 to 11,650 Da, corresponding to 8–15-mers of the tripeptide repeating unit (Table 1). As shown in Schemes 1–3, a synthetic pathway of a typical mucin-like glycoprotein (**1**), also as a model for antifreeze glycoprotein (AFGP), involves an chemoselective synthesis[10] of the key glycotripeptide unit and its direct polymerization under a mild condition. In the present report, we have modified and improved the synthetic method previously reported. [8] To prevent from racemization or β-elimination reaction in case of *O*-deacylation of the intermediates under alkaline condition, acetyl groups were converted to benzyl protections that can be readily removed by mild hydrogenation. Moreover, a tripeptide unit (Ala-Ala-Thr) was changed to (Ala-Thr-Ala) in order to avoid the effect of the steric factor on the activity in the polymerization reactions at the terminal carboxyl groups.

Table 1. Average molecular weights of synthetic glycopeptides estimated from gel permeation chromatographical analysis

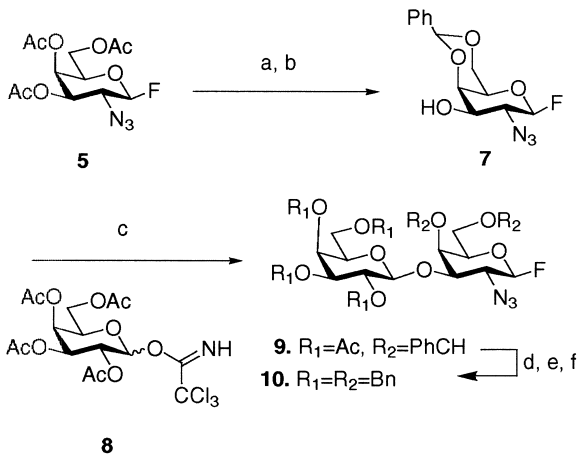
Compound	$\overline{Mn}$[a]	$\overline{Mw}$[b]
1	3950	6600
2	4100	5480
3	6370	11,650

Experiments were carried out with TOSOH TSKgel G3000PW$_{XL}$ using Pullulans (5.8, 12.2, 23.7, and 48.0 K) as standards.
[a] Number average molecular weight.
[b] Weight average molecular weight.

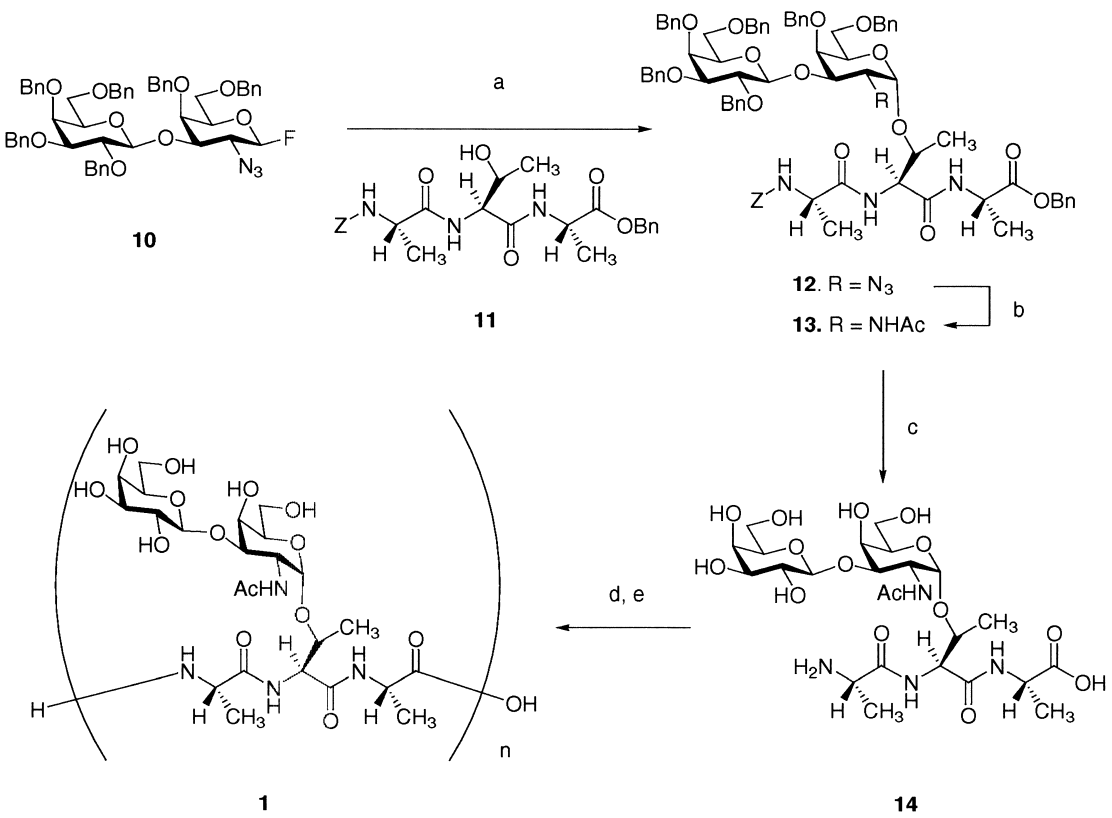


Scheme 1. *Reagents*: (a) Et_3N/3HF, Et_3N, CH_3CN, 50°C, 76% of **5** (α/β=2:3), 11% of **6**; (b) DAST, THF, 88% (α/β=1:4).

Schemes 1 and 2 indicate the synthetic route of the disaccharide moiety of glycopeptide repeating unit. Here, we employed glycosyl imidate **8** and glycosyl fluoride **7** as a convenient set of starting materials for a chemoselective glycosylation strategy. This greatly facilitated synthesis of a key glycotripeptide intermediate **14**. First, 3, 4, 6-tri-*O*-acetyl-2-azide-2-deoxy-α,β-D-galactopyranosyl nitrate **4** was prepared from D-galactose in four steps,[11] and the anomeric nitrate group was subsequently replaced by nucleophilic substitution with fluoride ion generated from Et_3N/3HF complex,[12] giving rise to the glycosyl fluoride **5** in 76% yield (α/β=2:3). At the same time, compound **6**,



Scheme 2. *Reagents*: (a) NaOMe, MeOH; (b) $PhCH(OMe)_2$, CSA, DMF, 40°C, 98% in two steps; (c) TMSOTf, MS 4 Å, CH_2Cl_2, −15°C, 87%; (d) NaOMe, MeOH; (e) CSA, MeOH; (f) BnBr, NaH, DMF, −15°C to rt, 81% from **9**.



Scheme 3. *Reagents*: (a) Cp_2ZrCl_2, $AgClO_4$, MS 4 Å, CH_2Cl_2, −20°C to rt, 63% (α/β=17:4); (b) AcSH, pyridine, 80%; (c) H_2 gas, Pd/C, DMF, H_2O, AcOH, 100%; (d) DPPA, Et_3N, DMF, 0°C to rt; (e) 25 mM NaOH, 100% in two steps.

which can be easily converted into **5** using DAST,[13] was obtained in 11% yield as a by-product. De-*O*-acetylation of **5** followed by benzylidenation furnished **7** having dual functions in 98% yield. Then, glycosyl fluoride **7** as an acceptor was coupled with galactosyl imidate **8** using trimethylsilyl trifluoromethansulfonate (TMSOTf) as a promoter,[14] to provide desired disaccharide **9** in 82% yield. Although both glycosyl imidate and glycosyl

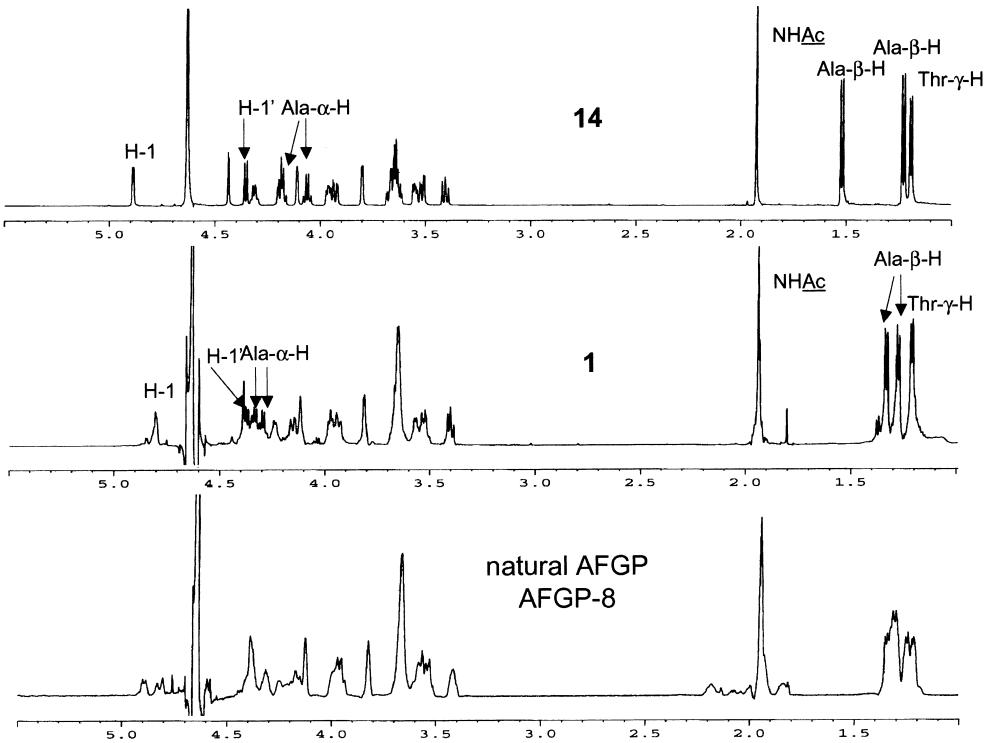


Figure 3. ^1H NMR spectra of compound **14**, compound **1**, and natural AFGP (AFGP-8).

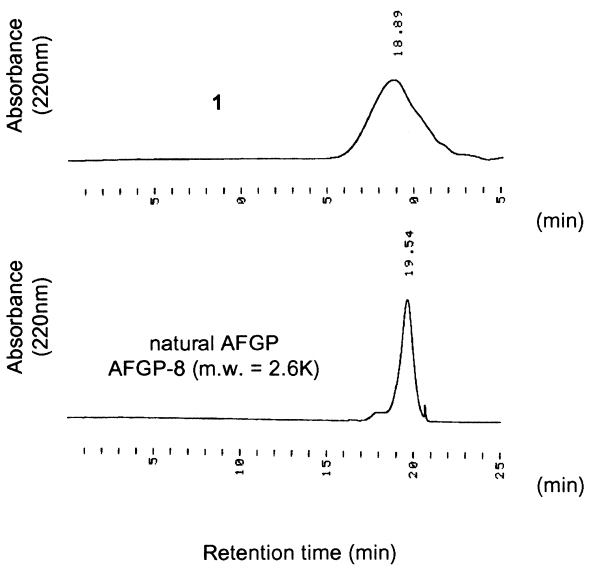
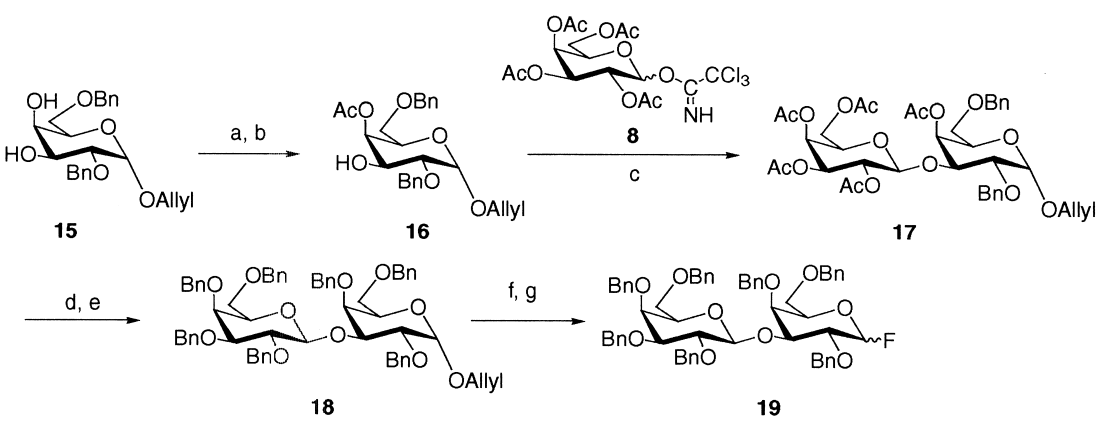


Figure 4. GPC elution profiles of a tandem repeating glycoprotein **1** and natural AFGP (AFGP-8, m.w.=2.6 K). TSKgel G3000PW$_{XL}$ was eluted with 0.1 M ammonium bicarbonate buffer (pH 7.6, flow rate=0.4 mL/min).

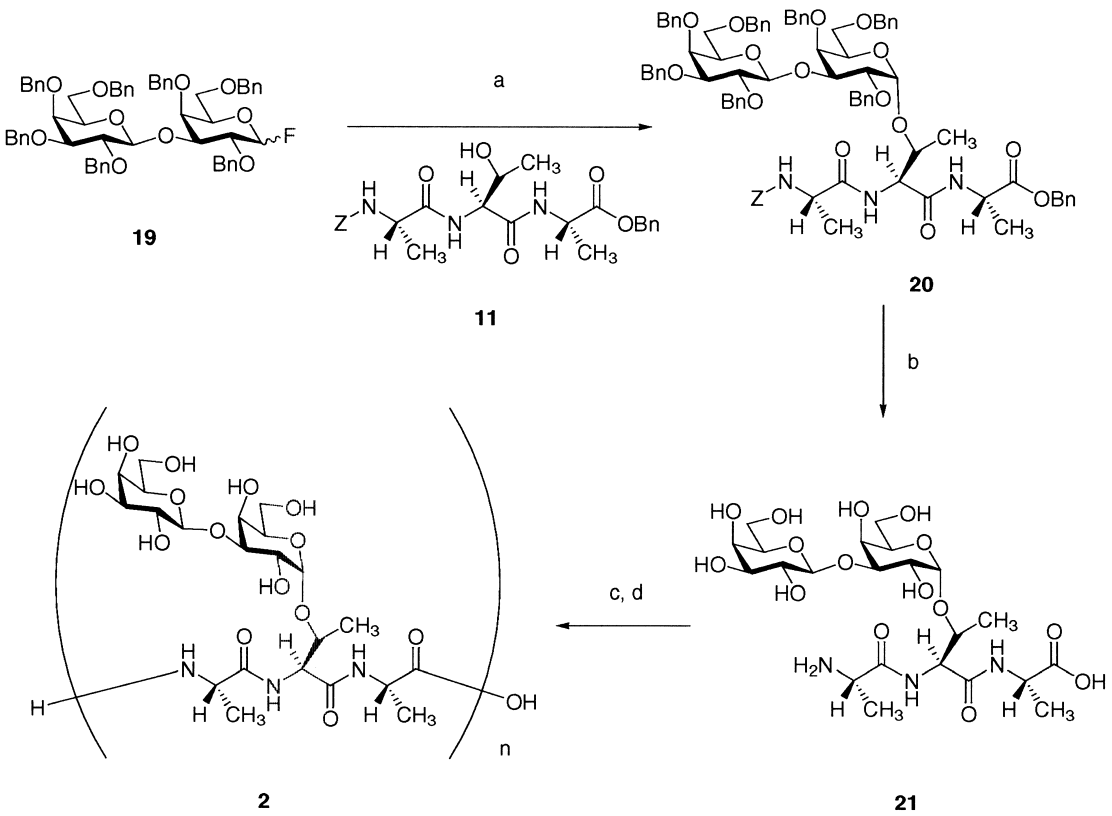
fluoride[15] are known to be activated by TMSOTf, we have succeeded in specifically activating glycosyl imidate by running the reaction at low temperature (−20°C) without any side reactions. Both *O*-acetyl groups and benzylidene group of the compound **9** were replaced with *O*-benzyl protections. These procedures were necessary to solve two serious problems: (a) the alkaline condition required for the removal of *O*-acetyl groups might cause base-catalyzed β-elimination of sugar moiety from peptide chain or base-catalyzed epimerization of amino acids moiety, (b) the benzylidene group became unstable in the presence of electron donating benzyl groups.[16] Thus, the intermediate **9** was converted into the per-*O*-benzylated glycosyl donor **10** in satisfactory yield (81% yield).

As shown in Scheme 3, disaccharidic donor **10** and a tripeptide **11** were coupled in the presence of Cp_2ZrCl_2/$AgClO_4$ (1:2)[17] in CH_2Cl_2 to give desired glycotripeptide **12** in 51% yield.[18] At the same time, 12% of β-isomer (**12β**) was yielded. Then, the azide group of **12** was converted into *N*-acetyl group by treating with thioacetic acid–pyridine[19] (80% yield) followed by quantitative hydrogenolytic removal of all protecting groups to afford a key macromonomer glycotripeptide **14**. Finally, polymerization of **14** was carried out in the presence of DPPA as a promoter and the mixture was further treated with 25 mM-NaOH.aq solution to quench the active azide generated at the C terminus of the polymer chains. After purification with gel permeation chromatography the desired polymer fractions, compound **1**, was obtained in 100% yield. The present method gave much improved polymerization yield in comparison with the previous method,[8] suggesting that reactivity of the glycotripeptide macromonomer seems to be enhanced by replacing the position of the threonin residue having sterically hindered disaccharide from Ala-Ala-Thr to Ala-Thr-Ala. It was clearly suggested from proton and ^{13}C NMR spectra that polymerization of the macromonomer glycotripeptide **14** proceeded smoothly with no side reaction to afford the macromolecular AFGP analog (^1H NMR spectra of compound **14**, a tandem repeating glycoprotein **1**, and natural AFGP are shown in Fig. 3). It was also demonstrated that the broad spectrum of the natural AFGP involves some typical signals at 1.9–2.3 ppm due to L-proline residues.[20] Compound **1** showed broader molecular weight distribution than that of natural AFGP and the weight average molecular weight (Mw) was estimated to be 6600 by gel permeation chromatography (Fig. 4).[21] In addition, intermediate **21** in which GalNAc residue of **14** was replaced with Gal residue was also synthesized by means of a conventional strategy and polymerized as indicated in Schemes 4 and 5.

The versatility and importance of the present synthetic strategy are evident because a variety of mucin-type glycoproteins[4] involving tandem-repeating peptide motifs such as RNA polymerase II, Muc families, and proteoglycans have been reported to exhibit significant biological functions as signals of cell adhesion. Moreover, enzymatic modification study strongly contributes to the efficient synthesis of further complicated mucin-type glycoproteins. Actually, it was demonstrated that 3-*O*-sialylation reaction by means of a commercially available sialyltransferase (recombinant rat 2,3-sialyltransferase) greatly facilitated the production of the sialylated glycoprotein (**3**) as shown in Scheme 6 and Fig. 5. It was suggested that both periodate–resorcinol method[22] (98%) and integration data of ^1H NMR spectrum (100%) exhibited quantitative sialylation reaction at *O*-3 position of the galactose residue by this enzyme. The sialylated glycoprotein (**3**) as well as enzymatically



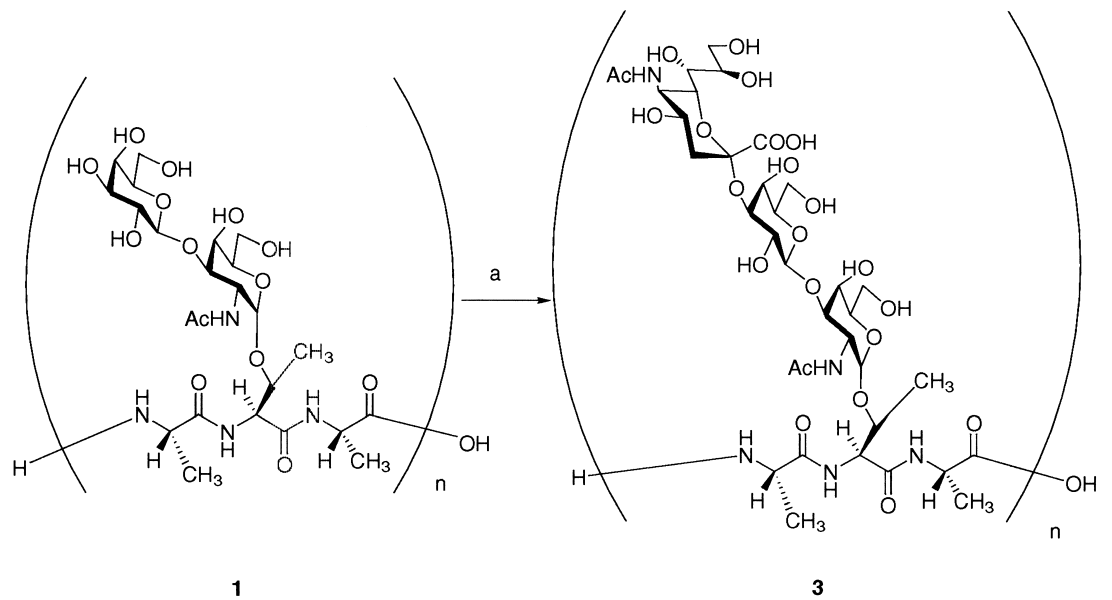
Scheme 4. *Reagents*: (a) $CH_3C(OCH_3)_3$, TsOH, CH_3CN; (b) 80% AcOH, 100% in two steps; (c) TMSOTf, CH_2Cl_2, −20°C, 84%; (d) NaOMe, MeOH; (e) BnBr, NaH, DMF, 0°C to rt, 80% in two steps; (f) $PdCl_2$, NaOAc, 90% AcOH; (g) DAST, THF, 66% in two steps (α/β=1:1).



Scheme 5. *Reagents*: (a) Cp_2ZrCl_2, $AgClO_4$, MS 4 Å, CH_2Cl_2, −20°C to rt, 58%; (b) H_2 gas, Pd/C, DMF, H_2O, AcOH, 62%; (c) DPPA, Et_3N, DMF, 0°C to rt; (d) 25 mM NaOH, 64% in two steps.

modified Tn-antigenic mucin mimics[23,24] may be valuable tools for investigating immunological and biological significance of oligosaccharide sequences of *O*-linked type glycoproteins.[25,26]

Since a tandem repeating glycoprotein **1** may be a simple model polymer of the naturally occurring AFGP,[27] we evaluated the inhibitory effect by **1** and analogs **2** and **3** on ice crystal growth. It is known that the natural AFGPs generate freezing point depression by adsorption to ice surfaces and consequent inhibition of further growth of ice crystals.[28] The formation of the characteristic bipyramidal ice crystals is one of the most important steps to inhibit the growth of ice crystal. Fig. 6 clearly showed that over a certain range of temperature (approximately from 0 to −0.5°C) both **1** (a) and natural AFGP (d) induced the formation of the typical hexagonal bipyramidal ice crystals. On the other hand, compound **2** (b) exhibited no significant



Scheme 6. *Reagents*: (a) CMP-Neu5Ac, α2,3-sialyltransferase, alkaline phosphatase, Triton CF-54, 50 mM sodium cacodyrate buffer (pH 7.0), 37°C, 93% (isolated yield).

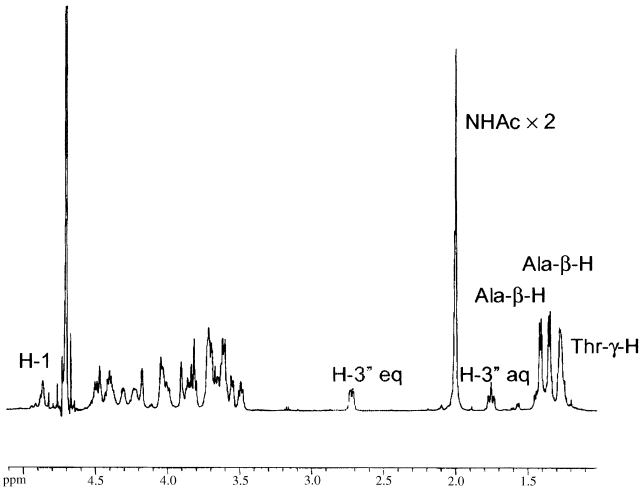


Figure 5. ^{1}H NMR spectrum of sialylated glycoprotein **3**.

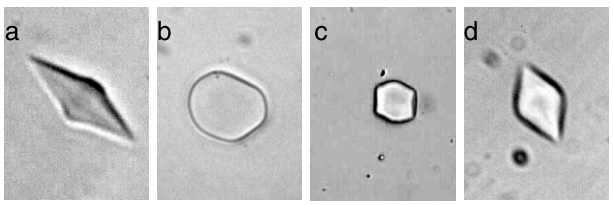


Figure 6. Ice crystal morphology in the presence of (a) a tandem repeating glycoprotein **1**, (b) Galβ1→3Gal analog **2** (c) sialylated glycoprotein **3**, (d) natural AFGP (AFGP-8) in water (10 mg/mL). Photos were taken at −0.2°C (a) and (d) or 0.0°C (b) and (c). The melting points of all solutions were 0.0°C.

inhibitory effect and sialylated glycoprotein **3** (c) showed no capacity to form the bipyramidal crystal. Moreover, the glycomacromonomer **14**, a repeating unit of the polymer, did not show any inhibitory effect on the growth of the ice (data not shown).

The CD spectra in the far-uv absorption region of synthetic glycoproteins in aqueous buffer at neutral pH are shown in Fig. 7. The CD patterns of **1** at 25°C clearly show a positive absorption at 218 nm in addition to a strong negative band at 195 nm, suggesting that this sequential glycoprotein con-

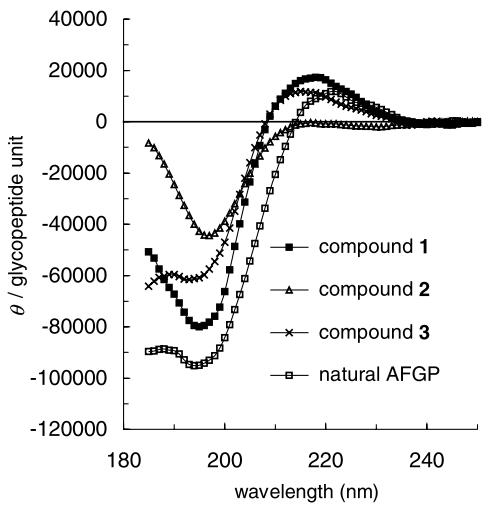


Figure 7. CD spectra of natural AFGP and its analogs. All curves were measured at 24°C in water (0.1 mg/mL).

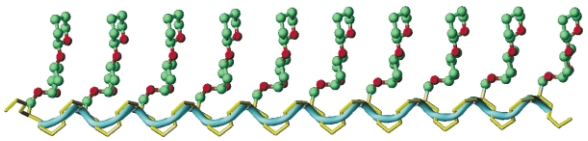


Figure 8. Plausible structure of compound **1** generated by SYBYL/advanced computation module.

tains an ordered helix structure different from typical α-helix or β-sheet such as a 3-fold left-handed helix structure.[29–32] As seen in the spectrum of the natural AFGP, compound **1** has quite similar secondary structure to that of natural AFGP. In addition, sialylated glycoprotein **3** which showed no inhibitory effect on the ice growth still retains the same secondary structure as that of natural AFGP. However, Galβ(1→3)Gal analog **2** exhibited typical CD spectrum found in the polypeptides composed of random coil structure. [32] It is likely that antifreeze activity by natural AFGP depends on the existence of this specific ordered helix as well as key molecular motifs to adsorb efficiently with ice lattice (Fig. 8). Further study of the antifreeze activity by using a series of analogs is under examination and the results will be reported in the nearest future.

In conclusion, a general synthetic strategy for the construction of tandem repeating polyglycopeptides was established by means of direct polymerization of the repeating glycopeptide unit as a macromonomer by DPPA without any protective groups. The versatility of the combined chemical and enzymatic synthesis of a biologically important mucin was also demonstrated by employing a recombinant rat α(2→3)sialyltransferase with synthetic glycoprotein **1** in the presence of CMP-Neu5Ac as a glycosyl donor substrate to give a sialylated glycoprotein **3** in a quantitative yield. The tandem repeating glycoproteins synthesized here were found to act as valuable macromolecular models for antifreeze glycoprotein.

3. Experimental

3.1. General

Thin-layer chromatography (TLC) was performed on Merck silica gel glass plates, 60F254; compounds were visualized by treatment with a solution of $(NH_4)_6Mo_7O_2{\cdot}4H_2O$ (20 g) and $Ce(SO_4)_2$ (0.4 g) in 10% sulfuric acid (400 mL) and heating at 150°C. Flash chromatography was performed on Kanto Chemical silica gel N60 (40–50 mm). NMR measurements were recorded at 27°C on a JEOL JNM-lambda-400 FT-NMR spectrometer (^{1}H (400 MHz)) or a BRUKER AVANCE 600 (^{1}H (600 MHz), ^{13}C (120 MHz)). FAB-mass spectra were obtained with a JEOL JMS-HX 110 mass spectrometer, using *m*-nitrobenzylalcohol (NBA) as matrix. All solvents were used as commercially received. The molecular weights of synthesized polyglycopeptides were estimated by gel permeation chromatography with a TOSOH-TSKgel G3000PW$_{XL}$ column (pullulans (5.8, 12.2, 23.7, and 48.0 K; Shodex Standard) were used as standards). Recombinant rat α-2,3-sialyltransferase and CMP-Neu5A were purchased from Calbiochem CO. Ltd. Calf intestine alkaline phosphatase were obtained from Sigma Co. Ltd. Natural AFGP was

obtained from A/F protein Inc. Circular dichroism (CD) spectra were measured in 1 mm path length quartz cells on a JASCO J-720 spectropolarimeter. Molecular simulation of AFGP was preliminarily performed by using SYBYL/advanced computation module.[33] Periodate-resorcinol method was carried out for the quantitative analysis of sialic acid content of **3** according to the method established by G. W. Jourdian et al.[22] The absorbance at 630 nm was observed with HITACHI U-2010 spectrophotometer.

The inhibitory effect on the ice crystal growth of synthetic polyglycopeptides and natural AFGPs were evaluated by observation of the ice crystal morphology using a Leica DMLB 100 photomicroscope equipped with a Linkam LK 600 temperature controller as described previously.[34] The compounds were dissolved in water (10 mg/mL), momentarily frozen (approximately −22°C), and warmed to 0°C on the sample stage of the optical microscope and create several ice nucleolus in the solution. This solution was then cooled at a rate of 0.07°C/min, and the crystal morphologies were monitored. The photos were taken at −0.2°C in the case the compound have ice crystal growth inhibition activity, or at 0.0°C for those did not have the activity.

3.1.1. 3,4,6-Tri-*O*-acetyl-2-azide-2-deoxy-D-galactopyranosyl fluoride (5). To a solution of **4** (2.92 g, 7.8 mmol) in CH_3CN (15 mL) was added Et_3N (0.75 mL) and Et_3N/3HF (3.1 mL) under nitrogen atmosphere and stirred at 50°C for 18 h. Then, MeOH (5.0 mL) was added to quench Et_3N/3HF, and the solvent was concentrated. The residue was diluted with $CHCl_3$, washed with water, $NaHCO_3$, brine, dried ($MgSO_4$), concentrated, and purified by flash column chromatography (toluene/EtOAc=4:1) to give **5** (1.97 g, 76%, α/β=2:3) and **6** (0.28 g, 11%). Compound **6** was easily converted into **5** using DAST. To a solution of **6** (2.66 g, 8.0 mmol) in THF (30 mL) was added DAST (1.3 mL, 8.2 mmol) at −20°C, and stirred at room temperature for 1 h. Then, the solution was cooled to 0°C, and MeOH was added to quench DAST. The solution was concentrated, and extracted with $CHCl_3$, washed with water, sat. $NaHCO_3$, brine, dried ($MgSO_4$), and concentrated. The crude residue was purified by flash column chromatography (toluene/EtOAc=7:1, then 5:1) to give **5** (2.37 g, 89%, α/β=1:4). In this procedure, α-isomer (minor product) could not be purified and β-isomer was used for the next step. **5β**: ^{1}H NMR (400 MHz, $CDCl_3$, δ): 5.36 (s, 1H, H-4), 5.13 (d, 1H, H-1, $J_{1,2}$=7.5 Hz, $J_{1,F}$=51.7 Hz), 4.86 (dd, 1H, H-3, $J_{2,3}$=10.9 Hz, $J_{3,4}$=3.2 Hz), 4.18 (dd, 2H, $J_{5,6a}$=$J_{5,6b}$=6.4 Hz, $J_{6a,6b}$=2.0 Hz, H-6a,b), 4.00 (dd, 1H, H-5), 3.82 (m, 1H, $J_{2,F}$=18.4 Hz, H-2), 2.17–2.06 (s×3, 9H, CH_3C=O×3); HRMS-FAB (*m*/*z*): $[M+H]^+$ calcd for $C_{12}H_{17}FN_3O_7$, 334.1051; found, 334.1076; elemental analysis calcd (%) for $C_{12}H_{16}FN_3O_7$: C, 43.25; H, 4.84; N, 12.61; found: C, 43.22; H, 4.70; N, 12.61.

3.1.2. 2-Azide-4,6-*O*-benzylidene-2-deoxy-D-galactopyranosyl fluoride (7). To a solution of **5β** (1.74 g, 5.22 mmol) in MeOH (20 mL) was added NaOMe (27 mg, 0.52 mmol) and stirred at room temperature for 1 h. Then, the solution was neutralized with Dowex 50W-X8 [H^+] resin, filtered, and concentrated. The concentrated mixture was dissolved in DMF (20 mL), and was added benzaldehyde dimethylacetal (1.57 mL, 10.4 mmol) and CSA (606 mg, 2.61 mmol). The solution was stirred at 50°C for 2 h, and neutralized with Et_3N (1.8 mL, 13 mmol), then concentrated. Flash column chromatography of the residue (hexane/EtOAc=4:1, then 2:1) yielded 1.51 g of **7** (98%) as colorless syrup. The physical data were in agreement with those reported by Ogawa et al. [35] Selected data of **7**; ^{1}H NMR (400 MHz, $CDCl_3$, δ): 5.59 (s, 1H, Ph-C*H*–O), 5.06 (d, 1H, $J_{1,2}$=7.5 Hz, $J_{1,F}$=55.2 Hz, H-1), 4.40 (dd, 1H, $J_{5,6a}$=1.6 Hz, $J_{6a,6b}$=12.8 Hz, H-6a), 4.10 (m, 1H, $J_{5,6b}$=1.8 Hz, H-6b), 3.75 (ddd, 1H, $J_{2,F}$=11.9 Hz, $J_{2,3}$=10.2 Hz, H-2), 2.58 (d, 1H, *J*=8.7 Hz, CHO*H*). (lit.[35]); ^{1}H NMR ($CDCl_3$, δ): 5.59 (s, 1H, Ph-C*H*–O), 5.06 (d, 1H, H-1, $J_{1,2}$=7.6 Hz, $J_{1,F}$=52.2 Hz), 4.39 (dd, 1H, $J_{5,6a}$=1.5 Hz, $J_{6a,6b}$=12.5 Hz, H-6a), 4.10 (m, 1H, $J_{5,6b}$=1.8 Hz, H-6b), 3.75 (ddd, 1H, $J_{2,F}$=11.9 Hz, $J_{2,3}$=10.4 Hz, H-2), 2.61 (d, 1H, *J*=8.2 Hz, CHO*H*).

3.1.3. 2,3,4,6-Tetra-*O*-acetyl-β-D-galactopyranosyl)-(1→3)-2-azide-4,6-*O*-benzylidene-2-deoxy-β-D-galactopyranosyl fluoride (9). A mixture of galactosyl imidate **8** (2.36 g, 4.8 mmol), glycosyl fluoride **7** (1.18 g, 4.0 mmol), and powdered molecular sieves 4 Å (1.0 g) in dry CH_2Cl_2 (15 mL) was cooled to −15°C under nitrogen atmosphere. Then, TMSOTf (77 mL, 0.40 mmol) in CH_2Cl_2 (0.5 mL) was added dropwisely, and the mixture was stirred at −15°C for 2 h, and then triethylamine (1.0 mL) was added to quench TMSOTf. The solution was filtered, concentrated, and purified by flash column chromatography (toluene/EtOAc=6:1 then 4:1) to give **9** (2.17 g, 87%). The physical data were in agreement with those reported by Ogawa et al.[36] Selected data of **9**; ^{1}H NMR (400 MHz, $CDCl_3$, δ): 5.50 (s, 1H, Ph-C*H*–O), 5.41 (d, 1H, $J_{3',4'}$=3.4 Hz, H-4′), 5.27 (dd, 1H, $J_{1',2'}$=8.0 Hz, $J_{3',4'}$=10.4 Hz, H-2′), 5.07 (dd, 1H, $J_{1,F}$=52.6 Hz, $J_{1,2}$=7.5 Hz, H-1), 4.80 (d, 1H, H-1′). (lit.[36]); ^{1}H NMR ($CDCl_3$, δ): 5.54 (s, 1H, Ph-C*H*–O), 5.41 (d, 1H, $J_{3',4'}$=3.3 Hz, H-4′), 5.28 (dd, 1H, $J_{1',2'}$=7.9 Hz, $J_{3',4'}$=10.3 Hz, H-2′), 5.08 (dd, 1H, $J_{1,F}$=52.5 Hz, $J_{1,2}$=7.9 Hz, H-1), 4.80 (d, 1H, H-1′).

3.1.4. 2,3,4,6-Tetra-*O*-benzyl-β-D-galactopyranosyl)-(1→3)-2-azide-4,6-di-*O*-benzyl-2-deoxy-β-D-galactopyranosyl fluoride (10). To a solution of **9** (200 mg, 2.08 mmol) in MeOH (6 mL) was added NaOMe (2 mg, 0.032 mmol), and stirred at room temperature for 1 h. The reaction mixture was then neutralized with Dowex 50W-X8 [H^+] resin, filtered, and concentrated. To a solution of residual amorphous in MeOH (6 mL) was added CSA (38 mg, 0.16 mmol) and stirred at room temperature for 2 h. Then, Et_3N (0.18 mL, 1.28 mmol) was added to quench the reaction, and concentrated. The residue was dissolved in DMF (6 mL), and cooled to −15°C, at which time 60% NaH (153 mg, 3.84 mmol) was added, and stirred at the same temperature for 30 min. Benzyl bromide (0.69 mL, 5.76 mmol) was added dropwisely, and the mixture was allowed to warm slowly to room temperature over 12 h. Then, methanol was added and the solution was evaporated. The residual syrup was extracted with $CHCl_3$, and the organic layer was washed with water, 1N HCl, sat. $NaHCO_3$, brine, dried ($MgSO_4$), and concentrated. The crude residue was purified by flash column chromatography (hexane/EtOAc=5:1, then 4:1) to give **10** (235 mg, 81%). **10**: ^{1}H NMR (400 MHz, $CDCl_3$, δ): 7.37–7.18 (m, 30H, aromatic), 5.02 (dd, 1H, $J_{1,2}$=7.4 Hz, $J_{1,F}$=52.6 Hz, H-1),

5.01–4.55 (m, 8H, Ph-C*H*–O), 4.63 (d, 1H, $J_{1',2'}$=7.6 Hz, H-1′), 4.56–4.34 (m, 4H, Ph-C*H*–O), 3.95–3.87 (m, 1H, H-2), 3.92 (dd, 1H, H-5′), 3.90 (d, 1H, $J_{3',4'}$=2.6 Hz, H-4′), 3.85 (dd, 1H, $J_{2',3'}$=9.6 Hz, H-2′), 3.64–3.53 (m, 7H, H-4, H-5, H-6a, H-6b, H-6′a, H-6′b), 3.52 (dd, 1H, $J_{3',4'}$=2.6 Hz, H-3′), 3.44 (dd, 1H, $J_{2,3}$=8.1 Hz, $J_{3,4}$=4.6 Hz, H-3); HRMS-FAB (*m*/*z*): $[M+Na]^+$ calcd for $C_{54}H_{56}FN_3O_9Na$, 932.3899; found, 932.3907; elemental analysis calcd (%) for $C_{54}H_{56}FN_3O_9$: C, 71.26; H, 6.21; N, 4.62; found: C, 71.02; 6.25; 4.52.

3.1.5. *N*-(Benzyloxycarbonyl)-L-alanyl-L-threonyl-L-alanine benzyl ester (11). To a stirred solution of Boc-Thr-OH (5.4 g, 24.6 mmol) and Ala-OBzl *p*-tosylate (9.9 g, 28.3 mmol) in DMF (50 mL) was added DPPA (6.1 mL, 28.3 mmol) in DMF (50 mL) at 0°C, followed by the dropwise addition of triethylamine (7.4 mL, 52.9 mmol) in DMF (50 mL). The solution was stirred at 0°C for 2 h, then warmed to room temperature and stirred for 22 h. The solution was concentrated, and extracted with EtOAc, washed with 5% citric acid, sat. $NaHCO_3$, brine, and dried ($MgSO_4$). Then the solution was evaporated, and the residue was purified by flash column chromatography (hexane/EtOAc=1:1 then 2:3) to give Boc-Thr-Ala-OBzl (8.2 g, 88%).

N-Protected dipeptide (Boc-Thr-Ala-OBzl, 3.3 g, 8.67 mmol) was dissolved in 4N HCl/dioxane (50 mL), and stirred at room temperature for 1 h, then concentrated to give colorless solid. To a stirred solution of the residual colorless solid and Z-Ala-OH (3.5 g, 7.9 mmol) in DMF (30 mL) was added DPPA (2.1 mL, 9.1 mmol) in DMF (30 mL) at 0°C, followed by the addition of triethylamine (2.5 mL, 18.2 mmol) in DMF (30 mL). The solution was stirred at 0°C for 2 h, then warmed to room temperature and stirred for 22 h. The solution was concentrated, and extracted with EtOAc, washed successively with 5% citric acid, sat. $NaHCO_3$, brine, and dried ($MgSO_4$), and concentrated. Crystallization of the residue from EtOAc giving **11** (3.6 g, 85%). **11**: mp 173°C; ^{1}H NMR (400 MHz, $CDCl_3$, δ): 7.38–7.26 (m, 10H, aromatic), 7.12 (d, 1H, *J*=7.5 Hz, Thr-N*H*), 7.03 (d, 1H, *J*=6.9 Hz, Ala-N*H*), 5.45 (d, 1H, *J*=6.8 Hz, Ala-N*H*), 5.17 (dd, 2H, Ph-CH_2–OH), 5.10 (s, 2H, Ph-CH_2–OH), 4.55 (m, 1H, *J*=7.3 Hz, Ala-α-H), 4.42 (dd, 1H, *J*=2.0 Hz, Thr-α-H), 4.31 (m, 2H, Thr-β-H, Ala-α-H), 1.40 (d, 3H, Ala-β-H), 1.39 d, 3H, Ala-β-H), 1.12 (d, 3H, *J*=6.2 Hz, Thr-γ-H); HRMS-FAB (*m*/*z*): $[M+H]^+$ calcd for $C_{25}H_{31}N_3O_7$, 486.2241; found, 486.2201; elemental analysis calcd (%) for $C_{25}H_{30}N_3O_7{\cdot}H_2O$: C, 59.63; H, 6.61; N, 8.34; found: C, 60.03; H, 6.42; N, 8.41.

3.1.6. *N*-(Benzyloxycarbonyl)-L-alanyl-*O*-[(2,3,4,6-tetra-*O*-benzyl-β-D-galactopyranosyl)-(1→3)-(2-azide-4,6-di-*O*-benzyl-2-deoxy-α-(12) and -β-D-galactopyranosyl)]-L-threonyl-L-alanine benzyl ester (12β). A mixture of Cp_2ZrCl_2 (1.13 g, 3.85 mmol), $AgClO_4$ (1.60 g, 7.70 mmol), **11** (2.57 g, 4.62 mmol), and powdered molecular sieves 4 Å (2.5 g) in dry CH_2Cl_2 (40 mL) was stirred at room temperature for 10 min under nitrogen atmosphere, then cooled to −20°C. A solution of **10** (1.40 g, 1.54 mmol) in dry CH_2Cl_2 (7 mL) was added, and the mixture was stirred at −20°C to room temperature for 24 h, then diluted with $CHCl_3$, filtered thorough celite. The filtrate was washed with sat. $NaHCO_3$, brine, dried ($MgSO_4$), and concentrated. The crude residue was purified by flash column chromatography (toluene/EtOAc=5:1, then 4:1) to give **12** (1.39 g, 51%) and its β-isomer **12β** (337 mg, 12%). **12**: ^{1}H NMR (400 MHz, $CDCl_3$, δ): 7.37–7.18 (m, 40H, aromatic), 7.34 (d, 1H, *J*=7.0 Hz, Ala-N*H*), 7.03 (d, 1H, *J*=6.7 Hz, Thr-N*H*), 5.26 (d, 1H, *J*=6.2 Hz, Ala-N*H*), 5.23 (d, 1H, $J_{1,2}$=3.8 Hz, H-1), 5.19–4.94 (m, 6H, Ph-CH_2–O), 4.77–4.29 (m, 13H, Ph-CH_2–O, Ala-α-H, Thr-α-H, Thr-β-H), 4.71 (d, 1H, $J_{1',2'}$=7.6 Hz, H-1′), 4.23 (m, 1H, Ala-α-H), 4.17 (dd, 1H, $J_{2,3}$=10.6 Hz, $J_{3,4}$=2.8 Hz, H-3), 4.13 (dd, 1H, H-4), 4.00 (dd, 1H, $J_{5',6'a}$=$J_{5',6'b}$=6.1 Hz, H-5′), 3.90 (d, 1H, $J_{3',4'}$=3.0 Hz, H-4′), 3.90–3.87 (m, 2H, $J_{2',3'}$=9.8 Hz, H-2, H-2′), 3.66–3.56 (m, 3H, H-5, H-6a, H-6b), 3.54–3.39 (m, 3H, $J_{6'a,6'b}$=9.6 Hz, H-3′, H-6′a, H-6′b), 1.40 (d, 3H, *J*=7.2 Hz, Ala-β-H), 1.37 (d, 3H, *J*=7.2 Hz, Ala-β-H), 1.11 (d, 3H, *J*=6.0 Hz, Thr-γ-H); ^{13}C NMR (120 MHz, $CDCl_3$, δ): 172.7, 172.5, 166.6, 139.3, 139.1, 138.8, 138.5, 138.2, 136.5, 135.8, 129.0, 128.9, 128.8, 128.7, 128.6, 128.5, 128.4, 128.3, 128.2, 128.0, 127.9, 127.8, 105.6, 99.5, 82.8, 79.8, 76.7, 75.6, 75.0, 74.8, 74.3, 74.2, 74.0, 73.8, 73.7, 73.5, 70.7, 69.8, 69.3, 67.5, 67.3, 61.2, 60.8, 56.4, 51.4, 48.7, 19.1, 17.1, 14.6; HRMS-FAB (*m*/*z*): $[M+Na]^+$ calcd for $C_{79}H_{86}N_6O_{16}Na$, 1397.5998; found, 1397.6050; elemental analysis calcd (%) for $C_{79}H_{86}N_6O_{16}$: C, 68.98; H, 6.30; N, 6.11; found: C, 68.88; H, 6.32; N, 5.94.

12β: ^{1}H NMR (400 MHz, $CDCl_3$, δ): 7.29–7.08 (m, 41H, aromatic, Ala-N*H*), 6.92 (d, 1H, *J*=6.6 Hz, Thr-N*H*), 5.24 (d, 1H, Ala-N*H*), 5.09–4.86 (m, 8H, Ph-CH_2–O), 4.72–4.63 (m, 4H, Thr-α-H, Ph-CH_2–O), 4.57 (d, 1H, $J_{1',2'}$=7.6 Hz, H-1′), 4.52–4.48 (m, 3H, H-1, Ph-CH_2–O), 4.35 (q, 1H, Ala-α-H), 4.32–4.28 (m, 3H, Ph-CH_2–O), 4.20–4.10 (br, 2H, Thr-β-H, Ala-α-H), 3.86 (d, 1H, $J_{3,4}$=2.8 Hz, H-4), 3.83 (d, 1H, $J_{3',4'}$=2.9 Hz, H-4′), 3.76 (dd, 1H, $J_{2',3'}$=9.5 Hz, H-2′), 3.75 (dd, 1H, H-2), 3.59 (dd, 1H, $J_{5,6a}$=6.2 Hz, $J_{5,6b}$=6.3 Hz, H-5), 3.54 (dd, 1H, $J_{5',6'a}$=4.9 Hz, $J_{5',6'b}$=5.4 Hz, H-5′), 3.51–3.46 (m, 4H, H-6a, H-6b, H-3′, H-6′a), 3.43 (dd, 1H, $J_{2,3}$=10.5 Hz, H-3), 3.38 (dd, 1H, H-6b), 1.25 (d, 3H, *J*=6.4 Hz, Ala-β-H), 1.18 (d, 3H, *J*=7.1 Hz, Ala-β-H), 1.08 (d, 1H, *J*=6.1 Hz, Thr-γ-H); ^{13}C NMR (120 MHz, $CDCl_3$, δ): 172.3, 172.2, 168.2, 139.3, 139.0, 138.9, 138.3, 138.2, 136.8, 136.6, 136.0, 128.9, 128.8, 128.7, 128.6, 128.5, 128.3, 128.1, 128.0, 127.9, 127.8, 105.4, 103.0, 82.4, 80.1, 79.9, 75.6, 75.4, 75.0, 75.0, 74.4, 74.1, 74.0, 73.7, 73.7, 73.6, 69.2, 68.9, 67.4, 67.3, 63.7, 55.8, 51.2, 48.9, 19.2, 17.9, 16.0; HRMS-FAB (*m*/*z*): $[M+H]^+$ calcd for $C_{79}H_{87}N_6O_{16}$, 1375.6179; found, 1375.6143; elemental analysis calcd (%) for $C_{79}H_{86}N_6O_{16}{\cdot}H_2O$: C, 68.18; H, 6.23; N, 6.04; found: C, 68.02; H, 6.24; N, 5.86.

3.1.7. *N*-(Benzyloxycarbonyl)-L-alanyl-*O*-[(2,3,4,6-tetra-*O*-benzyl-β-D-galactopyranosyl)-(1→3)-(2-acetamido-4,6-di-*O*-benzyl-2-deoxy-α-D-galactopyranosyl)]-L-threonyl-L-alanine benzyl ester (13). A solution of **13** (90 mg, 65.4 mmol) in AcSH (1 mL) and pyridine (0.5 mL) was stirred at room temperature for 20 h, and the residue was purified by flash column chromatography (run 1; hexane/EtOAc=5:1, then EtOAc, run 2; hexane/EtOAc=3:4) to give **13** (73 mg, 80%). **13**: ^{1}H NMR (400 MHz, $CDCl_3$, δ): 7.31–7.18 (m, 40H, aromatic), 7.10 (d, 1H, *J*=6.2 Hz, Ala-N*H*), 7.03 (d, 1H, Thr-N*H*), 6.44 (d, 1H, *J*=8.5 Hz,

CHN*H*C═O), 5.37 (d, 1H, *J*=7.2 Hz, Ala-N*H*), 5.23–4.90 (m, 7H, Ph-C*H*$_2$–O), 5.21 (d, 1H, $J_{1,2}$=3.0 Hz, H-1), 4.75–4.63 (m, 6H, $J_{1',2'}$=7.5 Hz, H-1′, H-2, Ph-C*H*$_2$–O), 4.55 (m, 1H, Ala-α-H), 4.53–4.23 (m, 8H, Ala-α-H, Thr-α-H, Thr-β-H, Ph-C*H*$_2$–O), 4.07 (s, 1H, H-4), 4.00 (dd, 1H, $J_{3',4'}$=2.4 Hz, H-3′), 3.98 (dd, 1H, H-5), 3.87 (d, 1H, H-4′), 3.84 (dd, 1H, $J_{2',3'}$=9.6 Hz, H-2′), 3.61–3.39 (m, 6H, H-5, H-6a, H-6b, H-3′, H-6′a, H-6′b), 1.63 (s, 3H, NHCOC*H*$_3$), 1.39 (d, 3H, *J*=6.9 Hz, Ala-β-H), 1.34 (d, 3H, *J*=7.0 Hz, Ala-β-H), 1.11 (d, 3H, *J*=6.4 Hz, Thr-γ-H); ^{13}C NMR (120 MHz, D_2O, δ): 173.5, 172.7, 171.3, 169.3, 139.4, 139.3, 138.8, 138.7, 138.2, 136.5, 135.4, 129.1, 129.0, 128.9, 128.8, 128.7, 128.6, 128.5, 128.4, 128.2, 128.0, 127.9, 127.8, 106.1, 99.0, 82.6, 79.8, 78.3, 76.4, 75.3, 75.0, 74.5, 74.2, 74.0, 73.7, 73.3, 70.8, 70.3, 69.2, 68.0, 67.5, 56.6, 51.1, 51.0, 50.4, 48.9, 18.6, 17.5, 14.5; HRMS-FAB (*m*/*z*): $[M+Na]^+$ calcd for $C_{81}H_{90}N_4O_{17}Na$, 1413.6199; found, 1413.6220; elemental analysis calcd (%) for $C_{81}H_{90}N_4O_{17}\cdot H_2O$: C, 69.02; H, 6.58; N, 3.97; found: C, 69.40; H, 6.82; N, 3.78.

3.1.8. L-Alanyl-*O*-[β-D-galactopyranosyl-(1→3)-2-acetamido-2-deoxy-α-D-galactopyranosyl]-L-threonyl-L-alanine (14). To a solution of **14** (15 mg, 0.012 mmol) in DMF (3 mL), acetic acid (1 mL), H_2O (1 mL) was added 10% Pd/C (100 mg), and stirred at room temperature for 48 h under H_2 gas atmosphere. Then, Pd/C was removed by filtration, and the solution was evaporated. The residue was purified by gel filtration chromatography (Sephadex G-10, water as eluent) to give **14** (7.2 mg, 100%). **14**: ^{1}H NMR (400 MHz, D_2O, δ): 4.88 (d, 1H, $J_{1,2}$=3.7 Hz, H-1), 4.43 (d, 1H, *J*=2.1 Hz, Thr-α-H), 4.33 (dd, *J*=2.1 Hz, Thr-β-H), 4.36 (d, 1H, $J_{1',2'}$=7.8 Hz, H-1′), 4.20 (dd, 1H, $J_{2,3}$=11.1 Hz, H-2), 4.16 (q, 1H, Ala-α-H), 4.11 (d, 1H, $J_{3,4}$=1.7 Hz, H-4), 4.05 (q, 1H, *J*=7.1 Hz, Ala-α-H), 3.97 (m, 1H, H-5), 3.94 (dd, 1H, H-3), 3.80 (d, 1H, $J_{3',4'}$=3.2 Hz, H-4′), 3.66 (m, 4H, H-6a, 6b, 6a′, 6b′), 3.97 (m, 1H, H-5′), 3.53 (dd, 1H, H-3′), 3.42 (dd, 1H, $J_{2',3'}$=9.8 Hz, H-2′), 1.91 (s, 3H, NHCOC*H*$_3$), 1.50 (d, 3H, *J*=7.0 Hz, Ala-β-H), 1.22 (d, 3H, Ala-β-H), 1.20 (d, 3H, Thr-γ-H); ^{13}C NMR (120 MHz, D_2O, δ): 179.2, 174.8, 171.8, 170.3, 105.2, 98.7, 77.6, 75.5, 75.0, 73.0, 71.6, 71.1, 69.3, 69.1, 61.7, 61.5, 57.9, 51.1, 49.4, 48.9, 22.8, 18.3, 18.3, 17.4; HRMS-FAB (*m*/*z*): $[M+H]^+$ calcd for $C_{24}H_{43}N_4O_{15}$, 627.2726; found, 627.2747.

3.1.9. Poly-[L-alanyl-*O*-(β-D-galactopyranosyl-(1→3)-2-acetamido-2-deoxy-α-D-galactopyranosyl)-L-threonyl-L-alanine] (1). To a stirred solution of **14** (20 mg, 0.032 mmol) in DMF (450 μL) was added 10% DPPA in DMF solution (90 μL, 0.042 mmol) at 0°C, followed by the addition of 10% triethylamine in DMF solution (100 μL, 0.073 mmol). The solution was stirred at 0°C for 2 h, then warmed to room temperature and stirred for 2 days. Then, the product in DMF was precipitated by addition of diethyl ether, and centrifuged. The crude product was then dis solved in 25 mM-NaOH aq. (1.0 mL) at 0°C, and stirred for 1h. Then, 0.1 M CH_3COOH aq. (25 μL) was added, and the mixture was subjected to the purification by gel filtration (Sephadex G-25, water as eluent) to give **1** (20 mg, 100%). The weight average molecular weight of **1** was estimated as 6600 by gel permeation chromatography. **1**: ^{1}H NMR (400 MHz, D_2O, δ): 4.88 (d, 1H, $J_{1,2}$=3.7 Hz, H-1), 4.37–4.12 (m, 7H, Thr-α-H, Thr-β-H, H-1′, Ala-α-H×2, H-2, H-4), 3.97–3.94 (m, 2H, H-5, H-3), 3.80 (d, 1H, $J_{3',4'}$=2.9 Hz, H-4′), 3.66 (m, 4H, H-6a, 6b, 6a′, 6b′), 3.97–3.53 (m, 2H, H-5′, H-3′), 3.42 (dd, 1H, $J_{1',2'}$=7.8 Hz, $J_{2',3'}$=9.8 Hz, H-2′), 1.92 (s, 3H, NHCOC*H*$_3$), 1.33 (m, 9H, Ala-β-H×2, Thr-γ-H); ^{13}C NMR (120 MHz, D_2O, δ): 176.0, 174.5, 174.2, 171.2, 105.1, 99.4, 77.8, 76.6, 75.3, 72.9, 71.4, 71.0, 69.2, 69.0, 61.7, 61.4, 57.5, 49.5, 49.0, 48.7, 22.7, 18.9, 17.6, 17.2.

3.1.10. Allyl 4-*O*-acetyl-2,6-di-*O*-benzyl-α-D-galactopyranoside (16). To a solution of **15**[37] (280 mg, 0.70 mmol) in CH_3CN (10 mL) was added $MeC(OMe)_3$ (270 μL) and a catalytic quantity of PPTS (10 mg).[38,39] After 10 min, a solution of 80% AcOH (18 mL) was added and stirred for 1.5 h. The solution was extracted with $CHCl_3$, washed with water, $NaHCO_3$, brine, dried ($MgSO_4$), concentrated, and purified by flash column chromatography (hexane/EtOAc=6:1, then 4:1) to give **16** (306 mg, 100%) as a white solid. **16**: ^{1}H NMR (600 MHz, $CDCl_3$, δ): 5.91 (m, 1H, CH_2–C*H*═CH_2), 5.45 (d, 1H, $J_{1,2}$=3.0 Hz, H-1), 5.31 (dd, 1H, CH═C*H*$_2$), 5,21 (dd, 1H, CH═C*H*$_2$), 4.92 (d, 1H, $J_{3,4}$=3.0 Hz, H-4), 4.55 (d, 1H, O–C*H*$_2$-Ph), 4.45 (d, 1H, O–C*H*$_2$-Ph), 4.20–4.14 (m, 2H, H-2, C*H*$_2$–CH═CH_2), 4.12 (dd, 1H, $J_{5,6a}$=$J_{5,6b}$=6.2 Hz, H-5), 3.95 (dd, 1H, C*H*$_2$–CH═CH_2), 3.71 (dd, 1H, $J_{2,3}$=9.9 Hz, H-3), 3.71 (s, 1H, 3-OH), 3.49 (dd, 2H, H-6a, H-6b); HRMS-FAB (*m*/*z*): $[M+H]^+$ calcd for $C_{25}H_{31}O_7$, 443.2070; found, 443,2085; elemental analysis calcd (%) for $C_{25}H_{30}O_7$: C, 67.79; H, 6.89; found: C, 67.86; H, 6.83.

3.1.11. Allyl *O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-(1→3)-4-*O*-acetyl-2,6-di-*O*- benzyl-α-D-galactopyranoside (17). A mixture of galactosyl imidate **8** (240 mg, 0.49 mmol), **16** (1.18 g, 4.0 mmol), and powdered molecular sieves 4 Å (500 mg) in dry CH_2Cl_2 (3 mL) was cooled to −15°C under nitrogen atmosphere. Then, TMSOTf (7.9 μL, 0.041 mmol) in CH_2Cl_2 (72 μL) was added dropwisely, and the mixture was stirred at −15°C for 2 h, and then triethylamine (1.0 mL) was added to quench TMSOTf. The solution was filtered, concentrated, and purified by flash column chromatography (toluene/EtOAc=6:1) to give **17** (305 mg, 96%). **17**: ^{1}H NMR (600 MHz, $CDCl_3$, δ): 7.36–7.26 (m, 10H, aromatic), 5.91 (ddd, 1H, CH_2–C*H*═CH_2), 5.44 (d, 1H, $J_{3,4}$=3.4 Hz, H-4), 5.34 (d, 1H, $J_{3',4'}$=3.4 Hz, H-4′), 5.31–5.20 (m, 2H, CH═C*H*$_2$), 5.17 (dd, 1H, $J_{1',2'}$=7.9 Hz, $J_{2',3'}$=10.5 Hz, H-2′), 4.97 (dd, 1H, H-3′), 4.83 (d, 1H, H-1′), 4.83 (d, 1H, $J_{1,2}$=3.9 Hz, H-1), 4.71 (d, 1H, O–C*H*$_2$-Ph), 4.52–4.45 (m, 3H, O–C*H*$_2$-Ph), 4.18 (dd, 1H, $J_{3,4}$=10.0 Hz, H-3), 4.15 (dd, 1H, O–C*H*$_2$–CH), 4.11–4.08 (m, 3H, H-6′a, H-6′b, H-5), 3.99 (dd, 1H, O–C*H*$_2$–CH), 3.84 (dd, 1H, $J_{5',6'}$=6.7 Hz, H-5′), 3.81 (dd, 1H, H-2), 3.50 (dd, 1H, H-6a), 3.44 (dd, 1H, H-6b), 2.16, 2.08, 2.06, 1.97, 1.94 (s×5, 3H×5, OA*c*); HRMS-FAB (*m*/*z*): $[M+H]^+$ calcd for $C_{39}H_{49}O_{16}$, 773.3020; found, 773.3043; elemental analysis calcd (%) for $C_{86}H_{93}N_3O_{17}\cdot 1/2H_2O$: C, 59.92; H 6.31; found: C, 59.83; H,6.17.

3.1.12. Allyl *O*-(2,3,4,6-tetra-*O*-benzyl-β-D-galactopyranosyl)-(1→3)-2,4,6-tri-*O*-benzyl-α-D-galactopyranoside (18). To a solution of **17** (305 mg, 0.40 mmol) in MeOH (10 mL) was added NaOMe (10 mg, 0.16 mmol),

and stirred at room temperature for 1 h. The reaction mixture was then neutralized with Dowex 50W-X8 [H^+] resin, filtered, and concentrated. To the residue was added DMF (10 mL), and cooled to −15°C, at which time 60% NaH in oil (104 mg, 2.6 mmol) was added, and stirred at the same temperature for 30 min. Benzyl bromide (362 μL, 3.0 mmol) was added dropwisely, and the mixture was allowed to warm slowly to room temperature over 12 h. Then, methanol was added and the solution was evaporated. The residual syrup was extracted with $CHCl_3$, and the organic layer was washed with water, brine, dried ($MgSO_4$), and concentrated. The crude residue was purified by flash column chromatography (hexane/EtOAc=8:1) to give **18** (276 mg, 67 %). **18**: ^{1}H NMR (600 MHz, $CDCl_3$, δ): 7.33–7.18 (m, 35H, aromatic), 5.88 (ddd, 1H, CH_2–C*H*=CH_2), 5.29–5.14 (m, 2H, CH=CH_2), 5.04–5.96 (m, 3H, Ph-CH_2–O), 4.86 (d, 1H, $J_{1',2'}$=7.7 Hz, H-1′), 4.83–4.60 (m, 7H, H-1, Ph-CH_2–O), 4.45–4.33 (m, 5H, Ph-CH_2–O), 4.23 (dd, 1H, $J_{2,3}$=10.1 Hz, $J_{3,4}$=3.0 Hz, H-3), 4.09 (dd, 1H, H-6a), 3.99 (dd, 1H, $J_{1,2}$=3.7 Hz, H-2), 3.99–3.93 (m, 4H, H-6b, O–CH_2–CH=CH_2, H-4′), 3.83 (dd, 1H, $J_{2',3'}$=9.4 Hz, H-2′), 3.66 (dd, 1H, $J_{5',6'}$=7.7 Hz, H-5′), 3.58–3.43 (m, 4H, H-6′a, H-3′, H-5, H-6′b); HRMS-FAB (*m*/*z*): $[M+Na]^+$ calcd for $C_{64}H_{68}O_{11}Na$, 1035.4660; found, 1035.4670.

3.1.13. 2,3,4,6-Tetra-*O*-benzyl-β-D-galactopyranosyl-(1→3)-2,4,6-tri-*O*-benzyl-α,β-D-galactopyranosyl fluoride (19). To a solution of **18** (250 mg, 0.25 mmol) in 90% AcOH was added $PdCl_2$ (88 mg, 0.49 mmol) and NaOAc (243 mg, 2.96 mmol), and stirred at room temperature for 18 h. The solution was diluted with $CHCl_3$, and the organic layer was washed with sat. $NaHCO_3$, brine, dried ($MgSO_4$), and concentrated. To a solution of the residue in THF (10 mL) was added DAST (39 μL, 0.30 mmol) at −20°C, and further stirred at room temperature for 1 h. Then, MeOH was added to quench DAST, and the solution was concentrated. The residue was extracted with $CHCl_3$, washed with sat. $NaHCO_3$, brine, dried ($MgSO_4$), concentrated, and purified by flash column chromatography (hexane/EtOAc=8:1) to give the anomeric mixture of **19** (160 mg, 66 %, α/β=1:1). This syrupy crude material was used for the next glycosidation reaction without further purification and characterization.

3.1.14. *N*-(Benzyloxycarbonyl)-L-alanyl-*O*-[(2,3,4,6-tetra-*O*-benzyl-β-D-galactopyranosyl)-(1→3)-(2,4,6-tri-*O*-benzyl-α-D-galactopyranosyl)]-L-threonyl-L-alanine benzyl ester (20). A mixture of Cp_2ZrCl_2 (42 mg, 1.44 mmol), $AgClO_4$ (60 mg, 2.88 mmol), **11** (87 mg, 1.80 mmol), and powdered molecular sieves 4 Å (1.0 g) in dry CH_2Cl_2 (8 mL) was stirred at room temperature for 2 h under nitrogen atmosphere, then cooled to −20°C. A solution of **19** (70 mg, 0.72 mmol) in dry CH_2Cl_2 (2 mL) was added, and the mixture was stirred at −20°C to room temperature for 72 h, then diluted with $CHCl_3$, filtered thorough celite. The filtrate was washed with sat. $NaHCO_3$, brine, dried ($MgSO_4$), and concentrated. The crude residue was purified by flash column chromatography (toluene/EtOAc=7:1, 6:1 then 4:1) to give **20** (60 mg, 58%). The trace amount of β-anomer was found in TLC analysis, but could not be isolated. **20**: ^{1}H NMR (600 MHz, $CDCl_3$, δ): 7.61 (d, 1H, *J*=7.3 Hz, Ala-N*H*), 7.35–7.12 (m, 45H, aromatic), 6.90 (d, 1H, *J*=4.3 Hz, Thr-N*H*), 5.31 (d, 1H, *J*=5.9 Hz, Ala-N*H*), 5.19 (d, 1H, $J_{1,2}$=3.7 Hz, H-1), 5.12–4.89 (m, 8H, Ph-CH_2–O), 4.79 (d, 1H, $J_{1',2'}$=7.7 Hz, H-1′), 4.75–4.57 (m, 5H, Ph-CH_2–O), 4.50–4.31 (m, 6H, Ph-CH_2–O, Thr-α-H), 4.30 (m, 1H, Ala-α-H), 4.23–4.20 (m, 2H, $J_{2,3}$=9.8 Hz, H-3, Ala-α-H), 4.07 (dd, 1H, H-2), 4.05–4.02 (m, 3H, H-4, Thr-β-H, H-5′), 3.94 (d, 1H, $J_{3',4'}$=2.5 Hz, H-4′), 3.88 (dd, 1H, $J_{2',3'}$=9.7 Hz, H-2′), 3.68 (dd, 1H, $J_{5,6}$=8.5 Hz, H-5), 3.57 (dd, 1H, H-6a), 3.52–3.44 (m, 4H, H-6b, H-5′, H-6′a, H-6′b), 1.37 (d, 3H, *J*=7.0 Hz, Ala-β-H), 0.95 (d, 3H, *J*=5.6 Hz, Thr-γ-H), 0.87 (d, 3H, *J*=7.3 Hz, Ala-β-H); ^{13}C NMR (120 MHz, $CDCl_3$, δ): 172.3, 171.5, 167.7, 138.9, 138.9, 138.7, 138.4, 138.2, 137.9, 137.4, 136.3, 135.5, 128.5–127.5, 82.6, 79.8, 77.5, 77.3, 77.2, 75.3, 74.8, 74.7, 74.6, 73.7, 73.6, 73.4, 73.3, 73.2, 72.8, 70.0, 69.2, 68.5, 66.7, 48.2, 19.1, 17.1, 15.1; HRMS-FAB (*m*/*z*): $[M+H]^+$ calcd for $C_{86}H_{94}N_3O_{17}$, 1440.6583; found, 1440.6620; elemental analysis calcd (%) for $C_{86}H_{93}N_3O_{17}$: C, 71.69; H 6.51; N, 2.69; found: C, 71.70; H, 6.51; N, 2.92.

3.1.15. L-Alanyl-*O*-[β-D-galactopyranosyl-(1→3)-α-D-galactopyranosyl]-L-threonyl-L-alanine (21). To a solution of **20** (50 mg, 0.035 mmol) in DMF (4 mL), acetic acid (300 μL), H_2O (1 mL) was added 10% Pd/C (300 mg), and stirred at room temperature for 72 h under H_2 gas atmosphere. Then, Pd/C was removed by filtration, and the solution was evaporated. The residue was purified by gel filtration chromatography (Sephadex G-10, water as eluent) to give **21** (12.6 mg, 62%). **21**: ^{1}H NMR (600 MHz, D_2O, δ): 5.04 (d, 1H, $J_{1,2}$=4.0 Hz, H-1), 4.55 (d, 1H, $J_{1',2'}$=7.7 Hz, H-1′), 4.48 (d, 1H, *J*=3.2 Hz, Thr-α-H), 4.31 (dd, 1H, Thr-β-H), 4.17 (d, 1H, $J_{3,4}$=2.9 Hz, H-4), 4.12 (q, 1H, *J*=7.1 Hz, Ala-α-H), 4.10 (q, 1H, *J*=7.2 Hz, Ala-α-H), 3.99 (dd, 1H, $J_{5,6a}$=7.2 Hz, $J_{5,6b}$=5.0 Hz, H-5), 3.96 (dd, 1H, $J_{2,3}$=10.3 Hz, H-3), 3.90 (dd, 1H, H-2), 3.86 (d, 1H, $J_{3',4'}$=3.3 Hz, H-4′), 3.72–3.59 (m, 6H, H-6a, H-6b, H-6a′, H-6b′, H-5′, H-3′), 3.54 (dd, 1H, H-2′), 1.55 (d, 3H, Ala-β-H), 1.29 (d, 3H, Ala-β-H), 1.24 (d, 3H, *J*=6.4 Hz, Thr-γ-H); ^{13}C NMR (120 MHz, D_2O, δ): 179.5, 171.5, 169.7, 104.4, 99.2, 79.1, 75.1, 74.3, 72.6, 71.1, 71.1, 69.3, 68.7, 67.4, 61.3, 61.1, 57.5, 51.2, 49.2, 17.7, 17.7, 16.8; HRMS-FAB (*m*/*z*): $[M+H]^+$ calcd for $C_{22}H_{40}N_3O_{15}$, 586.2454; found, 586.2458.

3.1.16. Poly-[L-alanyl-*O*-(β-D-galactopyranosyl-(1→3)-α-D-galactopyranosyl)-L-threonyl-L-alanine] (2). To a stirred solution of **21** (9 mg, 0.015 mmol) in DMF (150 μL) was added 10% DPPA in DMF solution (50 μL, 0.023 mmol) at 0°C, followed by the addition of 10% triethylamine in DMF solution (54 μL, 0.039 mmol). The solution was stirred at 0°C for 2 h, then warmed to room temperature and stirred for 2 days. Then, the product in DMF was precipitated by addition of diethyl ether, and centrifuged. The crude product was then dissolved in 25 mM NaOH aq. (2.0 mL) at 0°C, and stirred for 2 h. Then, 0.1 M CH_3COOH aq. (50 μL) was added, and the mixture was subjected to the purification by gel filtration (Sephadex G-25, water as eluent) to give **2** (5.8 mg, 64%). The weight average molecular weight of **2** was estimated as 5480 by gel permeation chromatography. **2**: ^{1}H NMR (600 MHz, D_2O, δ): 5.07 (br, 1H, H-1), 4.54 (d, 1H, $J_{1',2'}$=7.3 Hz, H-1′), 4.46 (br, 1H, Thr-α-H), 4.36–4.30 (br,

2H, Ala-α-H×2), 4.23 (br, 1H, Thr-β-H), 4.19 (br, 1H, H-4), 4.00–3.89 (m, 3H, H-5, H-3, H-2), 3.87 (br, 1H, H-4′), 3.71–3.53 (m, 6H, H-6a, H-6b, H-6a′, H-6b′, H-5′, H-3′), 3.55 (dd, 1H, H-2′), 1.37 (d, 3H, *J*=7.0 Hz, Ala-β-H), 1.34 (d, 3H, *J*=6.90 Hz, Ala-β-H), 1.22 (d, 3H, *J*=4.3 Hz, Thr-γ-H).

3.1.17. Poly-[L-alanyl-*O*-(5-acetamido-3,5-dideoxy-α-D-*glycero*-D-*galacto*-2-nonulopyranulosonic acid)-(2→3)-β-D-galactopyranosyl-(1→3)-(2-acetamido-2-deoxy-α-D-galactopyranosyl)]-L-threonyl-L-alanine] (sialylated glycoprotein, 3). To a solution of **1** (20 mg) in 50 mM sodium cacodyrate buffer (1.0 mL, pH 7.0, 0.1% Triton CF54) was added CMP-Neu5Ac (15.4 mg, 41 mmol), α2,3-sialyltransferase (50 mU), alkaline phosphatase (20 mU), and the mixture was incubated at 37°C overnight. Then the reaction mixture was taken in boiled water for 5 min, filtered with syringe filter (0.45 μm) and separated by gel filtration chromatography (Sephadex G-25, water as eluent), giving **3** (27.6 mg, 93% (98% from periodate–resorcinol method)). The weight average molecular weight of **3** was estimated as 11,650 by gel permeation chromatography. **3**: ^{1}H NMR (600 MHz, D_2O, δ): 4.82 (br, 1H, H-1), 4.43 (d, 1H, $J_{1',2'}$=7.7 Hz, H-1′), 4.41 (br, 1H, Thr-α-H), 4.34–4.31 (m, 2H, Ala-α-H×2), 4.25 (br, 1H, Thr-β-H), 4.17 (br, 1H, H-2), 4.11 (br, 1H, H-4), 3.98–3.92 (m, 2H, H-3, H-3′), 3.84 (br, 1H, H-4′), 3.81–3.48 (m, 13H, H-5, H-6a, H-6b, H-5′, H-6′a, H-6′b, H-4″, H-5″, H-6″, H-7″, H-8″, H-9″a, H-9″b), 3.42 (dd, 1H, H-2′), 2.66 (dd, 1H, $J_{3''eq,3''ax}$=12.0 Hz, $J_{3''eq,4''}$=3.7 Hz, H-3″eq), 1.92 (s, 6H, NHCOCH_3×2), 1.69 (dd, 1H, $J_{3''ax,4''}$=12.0 Hz, H-3″ax), 1.39 (d, 3H, *J*=7.1 Hz, Ala-β-H), 1.29 (d, 3H, *J*=6.9 Hz, Ala-β-H), 1.21 (d, 3H, Thr-γ-H), 1.33 (m, 9H, Ala-β-H×2, Thr-γ-H).

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References

1. Wittman, *Glycoscience: Chemistry and Chemicalbiology*; Fraser-Reid, B., Tatsuta, K., Thiem, J., Eds.; Springer: Berlin, 2001; Vol. 3, pp 2253–2287.
2. (a) Sharon, N.; Lis, H. In *Glycoscience: Status and Perspectives*; Gabius, H.-I., Gabius, S., Eds.; Chapman & Hall: Weinheim, 1997; pp 133–162. (b) Wittman, V. *Glycoscience: Chemistry and Chemical Biology*; Fraser-Reid, B., Tatsuta, K., Thiem, J., Eds.; Springer: Berlin, 2001; Vol. 3, pp 2289–2303.
3. Nishimura, S.-I.; Lee, Y. C. Synthetic Glycopolymers: New Tools for Glycobiology. In *Polysaccharides, Structural Diversity and Functional Versatility*; Dumitriu, S., Ed.; Marcel Dekker: New York, 1998; pp 523–537.
4. For example, see: Hanisch, F. G.; Muller, S. *Glycobiology* **2000**, *10*, 439–449.
5. Matsuura, K.; Hibino, M.; Yamada, Y.; Kobayashi, K. *J. Am. Chem. Soc.* **2001**, *123*, 357–358.
6. Shioiri, T.; Ninomiya, K.; Yamada, S. *J. Am. Chem. Soc.* **1972**, *94*, 6203–6205.
7. Nishi, N. T.; Naruse, K.; Hagiwara, B.; Nakajima, S. Tokura. *Makromol. Chem.* **1991**, *192*, 1799–1809.
8. Tsuda, T.; Nishimura, S.-I. *Chem. Commun.* **1996**, 2779–2780.
9. Recently, it was also suggested that DPPA can be applied for the blockwise synthesis of the glycopeptides with potent biological activity. See: Matsuda, M.; Nishimura, S.-I.; Nakajima, F.; Nishimura, T. *J. Med. Chem.* **2001**, *44*, 715–724.
10. Fraser-Reid, B.; Udodong, U. E.; Wu, Z.; Ottoson, H.; Merritt, J. R.; Rao, C. S.; Roberts, C.; Madsen, R. *Synlett* **1992**, 927–942.
11. Lemieux, R. U.; Ratcliffe, R. M. *Can. J. Chem.* **1979**, *57*, 1244–1251.
12. McClinton, M. A. *Aldarichim. Acta* **1995**, *28*, 31–35.
13. Posner, G. H.; Haines, S. R. *Tetrahedron Lett.* **1985**, *26*, 5–8.
14. Schmidt, R. R.; Kinzy, W. *Adv. Carbohydr. Chem. Biochem.* **1994**, *50*, 21.
15. Hashimoto, S.; Hayashi, M.; Noyori, R. *Tetrahedron Lett.* **1984**, *25*, 1379–1382.
16. For example, it was preliminarily suggested that glycosidation reaction using a disaccharidic donor having both *O*-benzyl and benzylidene protections reduces the yield of the coupling reaction with peptide due to the significant degree of de-*O*-benzylidenation. Interconversion from azide group into acetamido group at C-2 position in the presence of thioacetic acid also induced de-*O*-benzylidenation reaction.
17. Suzuki, K.; Maeta, H.; Matsumoto, T. *Tetrahedron Lett.* **1989**, *30*, 4853–4856.
18. Although the yield of this coupling reaction seems to be similar to that by the previous method, per-*O*-benzyl protection of carbohydrate moiety is apparently useful for further simple deprotection and polymerization steps.
19. Elofsson, M.; Salvador, L. A.; Kihlberg, J. *Tetrahedron* **1997**, *53*, 369–390.
20. Burcham, T. S.; Osuga, D. T.; Rao, B. N. N.; Bush, C. A.; Feeney, R. E. *J. Biol. Chem.* **1986**, *261*, 6384–6389.
21. Possibility of the epimerization in the DPPA-promoted polymerization of some amino acids or peptides was previously discussed by Nishi et al., see Ref. 7 and papers cited therein. It has been widely accepted that this method provides stereochemically pure polypeptides even in the presence of free hydroxyl groups due to L-serine or L-threonine residue.
22. Jourdian, G. W.; Dean, L.; Roseman, S. *J. Biol. Chem.* **1971**, *246*, 430–435.
23. Kono, M.; Tsuda, T.; Ogata, S.; Takashima, S.; Liu, H.; Hamamoto, T. S.; Itzkowitz, H.; Nishimura, S.-I.; Tsuji, S. *Biochem. Biophys. Res. Commun.* **2000**, *272*, 94–97.
24. Ikehara, Y.; Kojima, N.; Kurosawa, N.; Kudo, T.; Kono, M.; Nishihara, S.; Issiki, S.; Morozumi, K.; Itzkowitz, S.; Tsuda, T.; Nishimura, S.-I.; Tsuji, S.; Narimatsu, H. *Glycobiology* **1999**, *9*, 1213–1224.

25. Seitz, O.; Wong, C.-H. *J. Am. Chem. Soc.* **1997**, *119*, 8766–8776.
26. A general review for the chemoenzymatic carbohydrate synthesis, for example, Gijisen, H. J. M.; Qiao, L.; Fitz, W.; Wong, C.-H. *Chem. Rev.* **1996**, *96*, 443–473.
27. Yeh, Y.; Feeney, R. E. *Chem. Rev.* **1996**, *96*, 601–617.
28. Hew, C. L.; Yang, D. S. C. *Eur. J. Biochem.* **1992**, *203*, 33–42.
29. Franks, F.; Morris, E. R. *Biochim. Biophys. Acta* **1978**, *540*, 346–356.
30. Bush, C. A.; Feeney, R. E.; Osuga, D. T.; Ralapati, S.; Yeh, Y. *Int. J. Pept. Prot. Res.* **1981**, *17*, 125–129.
31. Bush, C. A.; Ralapati, S.; Matson, G. M.; Yamasaki, R. B.; Osuga, D. T.; Yeh, Y.; Feeney, R. E. *Arch. Biochem. Biophys.* **1984**, *232*, 624–631.
32. A general review of peptide conformational study by use of CD spectroscopy, see: Woody, R. W. Circular Dichroism of Peptides and Proteins. In *Circular Dichroism: Principles and Applications*; Nakanishi, K., Berova, N. R., Woody, W., Eds.; VCH: New York, 1994; pp 473–496.
33. Saunders, M.; Houk, K.; Wu, Y.-D.; Still, W. C.; Lipton, M.; Chang, G.; Guida, W. C. *J. Am. Chem. Soc.* **1990**, *112*, 1419–1427.
34. Miura, K.; Ohgiya, S.; Hoshino, T.; Nemoto, N.; Suetake, T.; Miura, A.; Spyracopoulos, L.; Kondo, H.; Tsuda, S. *J. Biol. Chem.* **2001**, *276*, 1304–1310.
35. Macindoe, W. M.; Ijima, H.; Nakahara, Y.; Ogawa, T. *Carbohydr. Res.* **1995**, *269*, 227–257.
36. Wang, Z.-G.; Zhang, X.-F.; Ito, Y.; Nakahara, Y.; Ogawa, T. *Carbohydr. Res.* **1996**, *295*, 25–39.
37. He, D.; Li, Z.; Cai, M. *Carbohydr. Res.* **1993**, *239*, 267–271.
38. Lemieux, R. U.; Driguez, H. *J. Am. Chem. Soc.* **1975**, *97*, 4069–4075.
39. Spijker, N. M.; Keuning, C. A.; Hooglugt, M.; Veeneman, G. H.; Boeckel, C. A. A. *Tetrahedron* **1996**, *52*, 5945–5960.