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Synthesis of Cyclic Antifreeze Glycopeptide and Glycopeptoids and Their Ice Recrystallization Inhibition Activity

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Until now, few groups reported the antifreeze activity of cyclic glycopeptides; however, the tedious synthetic procedure is not amenable to study the intensive structure activity relationship. A series of *N*-linked cyclic glycopeptoids and glycopeptide have been prepared to evaluate antifreeze activity as a function of peptide backbone cyclization and methyl stereochemical effect on the rigid Thr position. This study has combined the cyclization protocol with solid phase peptide synthesis and obtained significant quantities of homogeneous cyclic glycopeptide and glycopeptoids. Analysis of antifreeze activity revealed that our cyclic peptide demonstrated RI activity while cyclic glycopeptoids showed no RI activity. These results suggest that the subtle changes in conformation and Thr orientation dramatically influence RI activity of *N*-linked glycopeptoids

Key Words : Cyclic glycopeptide, Ice recrystallization inhibition, Solid phase synthesis, Cyclic glycopeptoid

Introduction

Antifreeze glycoproteins (AFGPs) are essential materials that allow organism to thrive in harsh conditions. Fish, bacteria and other organisms produce AFGPs which have the ability to inhibit the growth of ice, and thus allow them to survival in subzero temperature.[1] This unique property of AFGPs has attracted significant interest because of their potential medicinal and industrial applications.[2] Among AFGPs, AFGP 8 is well characterized in term of structure-activity relationship due to the lowest molecular weight (2.6 kDa) and it is consists of repeating tripeptide units, Alanyl-Alanyl-Threonyl (Ala-Ala-Thr)$_{n=4}$ units, connected with the disaccharide β-D-galactosyl-(1→3)-α-D-*N*-acetylgalactosamine through a glycosidic bond at the hydroxyl group of the threonine residue.[3] Many research groups have reported the synthesis and antifreeze activity of AFGP 8-related linear analogues.[4] Although considerable progress has been made using the synthetic linear AFGP 8 in the mechanistic studies, its application into medicinal and industrial field has been hindered by its limited stability and bioavailability. Therefore, cyclization is considered as a useful strategy to gain stability which eventually enhanced the bioavailability. Recently, Nishimura and his co-workers reported the first synthesis of cyclic AFGPs as pure glycoforms, but the key random polymerization step to mediate the cyclization was tedious and required the extensive time-consuming separation procedures.[4a] On the basis of our long-term involvement in the studies of glycopeptide synthesis,[5] we envisioned that the power of glycopeptide cyclization in combination with the solid phase peptide synthesis could overcome the daunting difficulties associated with isolating significant quantities of homogeneous cyclic glycopeptides. In this study, we report the synthesis of cyclic antifreeze glycopeptide and glycopeptoids by *N*-linked glycothreonine and the results of their ice recrystallizaion-inhibition (RI) activity.

Experimental

General. Materials were obtained from commercial suppliers and employed without further purification unless otherwise state. All reactions were carried out under argon or nitrogen atmosphere in oven-dried glassware. CH_2Cl_2 was distilled under N_2 from CaH_2 and toluene from sodium/benzophenone immediately before use. Anhydrous DMF and MeOH were purchased from Sigma Aldrich. Analytical TLC was performed on silica gel plate using UV light and/or anisaldehyde stain followed by heating. Column chromatography was performed on silica gel 60 (230-400 mesh). ^{1}H NMR spectra were obtained on a Bruker DRX 300 spectrometer using $CDCl_3$ and CD_3OD, and DMSO-d_6 as a solvent. Chemical shift were reported in parts per million (ppm, δ) relative to tetramethylsilane (δ 0.00). MS measurement was performed using an UltrafleXtreme MALDI time-of-flight (TOF)/TOF mass spectrometer equipped with a pulsed smartbeam II (355 nm Nd; YAG laser, repetition rate 1 kHz) in reflector mode (Bruker Daltonics) and α-cyano-4-hydroxycinnamic acid was used as a matrix. HPLC (Younglin, YL9100) was carried out on a reversed-phase column, which was eluted with H_2O and CH_3CN containing 0.05% TFA and

detected at 220 nm.

Synthesis of Peptoid Building Blocks.

Compound 2: To a solution of (*S*)-2-amino-1-propanol (**1**) (5.3 g, 70.56 mmol) in CH_2Cl_2 (150 mL) were added benzyl chloroformate (9.46 mL, 67.20 mmol) and DIEA (12.29 mL, 70.56 mmol) at 0 °C and stirred at room temperature for 15 h. The mixture was diluted with CH_2Cl_2 and washed with H_2O. The organic layer was dried (Na_2SO_4) and evaporated *in vacuo*. The residue was purified by silica gel column chromatography (hexanes/EtOAc; from 2:1 to 1:2) to provide the desired product **2** (11.8 g, 84%) as a white solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.32 (m, 5H), 7.01 (d, 1H, J = 7.7 Hz), 5.01 (s, 2H), 4.63 (t, 1H, J = 5.7 Hz), 3.52 (m, 1H), 3.35 (m, 1H), 3.20 (m, 1H), 1.03 (d, 3H, J = 6.7 Hz).

Compound 3: To a solution of **2** (11.5 g, 55.0 mmol) in CH_2Cl_2 (100 mL) were added a solution of trityl chloride (13.94 mL, 50.0 mmol) in CH_2Cl_2 (30 mL) and DIEA (9.58 mL, 55.0 mmol) at 0 °C and stirred at room temperature for 15 h. The mixture was diluted with CH_2Cl_2 and washed with H_2O. The organic layer was dried (Na_2SO_4) and evaporated *in vacuo*. The residue was purified by silica gel column chromatography (hexanes/EtOAc; from 5:1 to 3:1) to provide the desired product **3** (19.1 g, 85%) as a white solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.40-7.20 (m, 21H), 5.02 (s, 2H), 3.79 (m, 1H), 2.89 (br s, 2H), 1.09 (d, 3H, J = 6.8 Hz).

Compound 4: Compound **3** (4.10 g, 9.08 mmol) was dissolved in MeOH/CH_2Cl_2 (100 mL, 9:1) and 5% palladium on activated carbon (500 mg) was added. This mixture was stirred under H_2 gas balloon for 2 h. The Pd/C was filtered off from the suspension and the solution was evaporated *in vacuo*. The residue was dissolved in CH_2Cl_2 (30 mL) and *tert*-butyl bromoacetate (1.34 mL, 9.08 mmol) and DIEA (1.58 mL, 9.08 mmol) were added at 0 °C and stirred at room temperature for 15 h. The mixture was diluted with CH_2Cl_2 and washed with H_2O. The organic layer was dried (Na_2SO_4) and evaporated *in vacuo*. The residue was purified by silica gel column chromatography (hexanes/EtOAc; from 3:1 to 1:1) to provide the desired product **4** (3.17 g, 81%) as a colorless syrup. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.49-7.22 (m, 16H), 3.39 (d, 1H, J = 17.0 Hz), 3.26 (d, 1H, J = 17.0 Hz), 3.06 (d, 2H, J = 5.9 Hz), 2.92 (m, 1H), 1.50 (s, 9H), 1.04 (d, 3H, J = 6.4 Hz).

Compound 5: A solution of **4** (10 g, 23 mmol), Fmoc-L-Ala-OH (7.94 g, 25.5 mmol), DIC (3.95 mL, 25.5 mmol), HOBt (3.44 g, 25.49 mmol) and DIEA (4.44 mL, 25.49 mmol) in DMF (70 mL) were stirred at room temperature for 15 h. Solvent was removed *in vacuo* and the residue was partitioned between EtOAc and H_2O. The organic layer was dried (Na_2SO_4) and evaporated *in vacuo*. The residue was purified by silica gel column chromatography (hexanes/EtOAc; 3:1) to provide the desired product **5** (7.09 g, 42%) as a white solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.79 (d, 2H, J = 7.4 Hz), 7.66-7.62 (m, 2H), 7.44-7.17 (m, 19H), 6.16 (d, 1H, J = 6.8 Hz), 5.05 (m, 1H), 4.45-4.12 (m, 4H), 3.22 (d, 1H, J = 16.9 Hz), 3.11-3.07 (m, 2H), 1.46 (s, 9H), 1.44 (d, 3H, J = 6.9 Hz), 1.13 (d, 3H, J = 6.9 Hz); MS (MALDI-TOF) m/z = 747.3 $[M + Na]^+$, 763.3 $[M + K]^+$.

Compound 6: To a solution of **5** (6.96 g, 9.60 mmol) in MeOH (70 mL) was added TsOH·H_2O (2.19 g, 11.52 mmol) and stirred at room temperature for 4 h. Solvent was removed *in vacuo* and the residue was partitioned between EtOAc and H_2O. The organic layer was dried (Na_2SO_4) and evaporated in vacuo. The residue was purified by silica gel column chromatography (hexanes/EtOAc; from 1:1 to 1:2) to provide the desired product **6** (2.21 g, 48 %) as a white solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.77 (d, 2H, J = 7.5 Hz), 7.64-7.62 (m, 2H), 7.42-7.31 (m, 4H), 5.92 (d, 1H, J = 6.6 Hz), 4.90 (m, 1H), 4.37 (d, 2H, J = 4.4 Hz), 4.24 (m, 1H), 4.14 (m, 1H), 4.07 (d, 1H, J = 16.8 Hz), 3.58-3.55 (m, 2H), 3.37 (m, 1H), 1.51 (s, 9H), 1.39 (d, 3H, J = 6.9 Hz), 1.15 (d, 3H, J = 6.7 Hz); MS (MALDI-TOF) m/z = 504.6 $[M + Na-H]^+$, 520.6 $[M + K-H]^+$.

Compound 8: To a solution of **6** (2.03 g, 4.21 mmol) in toluene/CH_2Cl_2 (40 mL, 1:1) were added Ag_2CO_3 (3.48 g, 12.63 mmol) and 4 Å molecular sieve powder (3 g) and stirred at 0 °C for 30 min. Then $AgClO_4$ (873 mg) was added and stirred for another 20 min at room temperature. Subsequently, a solution of galactosyl bromide **7** (3.32 g, 8.42 mmol) in toluene/CH_2Cl_2 (20 mL, 1:1) was added slowly and stirred in the dark under argon at room temperature for 15 h. The mixture was diluted with CH_2Cl_2 and filtered through Celite. The filterate was washed with H_2O and saturated $NaHCO_3$ solution. The organic layer was dried (Na_2SO_4) and evaporated *in vacuo*. The residue was purified by silica gel column chromatography (hexanes/EtOAc; 1:1) to provide the desired α-product **8** (1.25 g, 37%) as a white solid (β-product was not identified by ^{1}H NMR). ^{1}H NMR (300 MHz, $CDCl_3$, major rotamer) δ 7.77 (d, 2H, J = 7.5 Hz), 7.64-7.60 (m, 2H), 7.44-7.31 (m, 4H), 5.84 (d, 1H, J = 7.5 Hz), 5.46 (m, 1H), 5.33 (m, 1H), 4.98 (d, 1H, J = 3.4 Hz), 4.83 (m, 1H), 4.47-4.06 (m, 8H), 3.90-3.51 (m, 4H), 2.14 (s, 3H), 2.08 (s, 3H), 2.03 (s, 3H), 1.48 (s, 9H), 1.45 (d, 3H, J = 6.9 Hz), 1.28 (d, 3H, J = 6.9 Hz); MS (MALDI-TOF) m/z = 818.2 $[M + Na]^+$, 834.2 $[M + K]^+$.

Compound 9: Compound **8** (1.17 g, 1.47 mmol) was dissolved in thioacetic acid/pyridine (9 mL, 2:1) and stirred at room temperature for 16 h. The mixture was evaporated *in vacuo* and the residue was purified by silica gel column chromatography (hexanes/acetone; 1:1) to provide the desired product **9** (1.08 g, 91%) as a white solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.77 (d, 2H, J = 7.3 Hz), 7.62 (d, 2H, J = 7.3 Hz), 7.43-7.30 (m, 4H), 7.19 (d, 1H, J = 8.9 Hz), 5.90 (d, 1H, J = 9.2 Hz), 5.35 (d, 1H, J = 3.0 Hz), 5.04 (m, 1H), 5.00 (d, 1H, J = 3.9 Hz), 4.89 (dd, 1H, J = 11.7, 3.2 Hz), 4.55 (m, 1H), 4.37-3.96 (m, 8H), 3.68 (t, 1H, J = 10.4 Hz), 3.49 (d, 1H, J = 16.8 Hz), 3.29 (dd, 1H, J = 10.4, 2.9 Hz), 2.12 (s, 3H), 2.09 (s, 3H), 1.97 (s, 3H), 1.72 (s, 3H), 1.49 (s, 9H), 1.43 (d, 3H, J = 7.0 Hz), 1.25 (d, 3H, J = 6.8 Hz); MS (MALDI-TOF) m/z = 834.3 $[M + Na]^+$, 850.3 $[M + K]^+$.

Compound 10 (Building Block 6): Compound **9** (990 mg, 1.22 mmol) was dissolved in TFA/H_2O (15 mL, 95:5) and stirred at room temperature for 2 h. The mixture was evaporated *in vacuo* and the residue was purified by silica gel column chromatography (CH_2Cl_2/MeOH; 10:1+0.5%

AcOH) to provide the desired product **10** (894 mg, 97%) as a white solid. ^{1}H NMR (300 MHz, DMSO-d_6, major rotamer) δ 8.34 (d, 1H, J = 8.0 Hz), 7.89 (d, 2H, J = 7.5 Hz), 7.73-7.69 (m, 2H), 7.61 (d, 1H , J = 8.0 Hz), 7.44-7.30 (m, 4H), 5.32 (br s, 1H), 4.99 (dd, 1H, J = 11.7, 3.2 Hz), 4.72 (d, 1H, J = 3.3 Hz), 4.64 (m, 1H), 4.30-3.90 (m, 9H), 3.66-3.54 (m, 3H), 2.10 (s, 3H), 2.01 (s, 3H), 1.89 (s, 3H), 1.81 (s, 3H), 1.21 (d, 3H, J = 6.6 Hz), 1.08 (d, 3H, J = 6.9 Hz); MS (MALDI-TOF) m/z = 778.2 [M + Na]$^+$.

Building Block 2: ^{1}H NMR (300 MHz, DMSO-d_6, major rotamer) δ 7.97 (br s, 1H), 7.90 (d, 2H, J = 7.5 Hz), 7.72 (d, 2H, J = 7.5 Hz), 7.63 (br s, 1H), 7.44-7.30 (m, 4H), 5.30 (d, 1H, J = 2.4 Hz), 4.99 (dd, 1H, J = 11.4, 3.0 Hz), 4.89 (d, 1H, J = 3.3 Hz), 4.43-3.45 (m, 14H), 2.11 (s, 3H), 1.99 (s, 3H), 1.87 (s, 3H), 1.83 (s, 3H), 1.14 (d, 3H, J = 6.3 Hz); MS (MALDI-TOF) m/z = 764.2 [M + Na]$^+$, 780.2 [M + K]$^+$.

Building Block 3: ^{1}H NMR (300 MHz, CD_3OD, major rotamer) δ 7.79 (d, 2H, J = 7.5 Hz), 7.67 (m, 2H), 7.40-7.29 (m, 4H), 5.38 (d, 1H, J = 2.4 Hz), 5.10 (dd, 1H, J = 11.5, 3.2 Hz), 5.01 (d, 1H, J = 3.5 Hz), 4.69 (m, 1H), 4.47-4.02 (m, 9H), 3.90 (d, 1H, J = 17.1 Hz), 3.52 (m, 1H), 3.37 (m, 1H), 2.16 (s, 3H), 2.00 (s, 3H), 1.97 (s, 3H), 1.93 (s, 3H), 1.33 (d, 3H, J = 7.2 Hz), 1.24 (d, 3H, J = 6.3 Hz); MS (MALDI-TOF) m/z = 777.9 [M + Na]$^+$, 794.0 [M + K]$^+$.

Building Block 4: ^{1}H NMR (300 MHz, CD_3OD, major rotamer) δ 7.79 (d, 2H, J = 7.5 Hz), 7.70 (t, 2H, J = 7.5 Hz), 7.40-7.30 (m, 4H), 5.45 (d, 1H, J = 2.7 Hz), 5.17 (dd, 1H, J = 11.7, 3.1 Hz), 5.06 (d, 1H, J = 3.8 Hz), 4.90 (m, 1H), 4.55-4.00 (m, 9H), 3.80 (d, 1H, J = 17.1 Hz), 3.75 (m, 1H), 3.35 (m, 1H), 2.12 (s, 3H), 2.01 (s, 3H), 1.97 (s, 3H), 1.77 (s, 3H), 1.40 (d, 3H, J = 7.0 Hz), 1.17 (d, 3H, J = 6.0 Hz); MS (MALDI-TOF) m/z = 778.0 [M + Na]$^+$, 794.0 [M + K]$^+$.

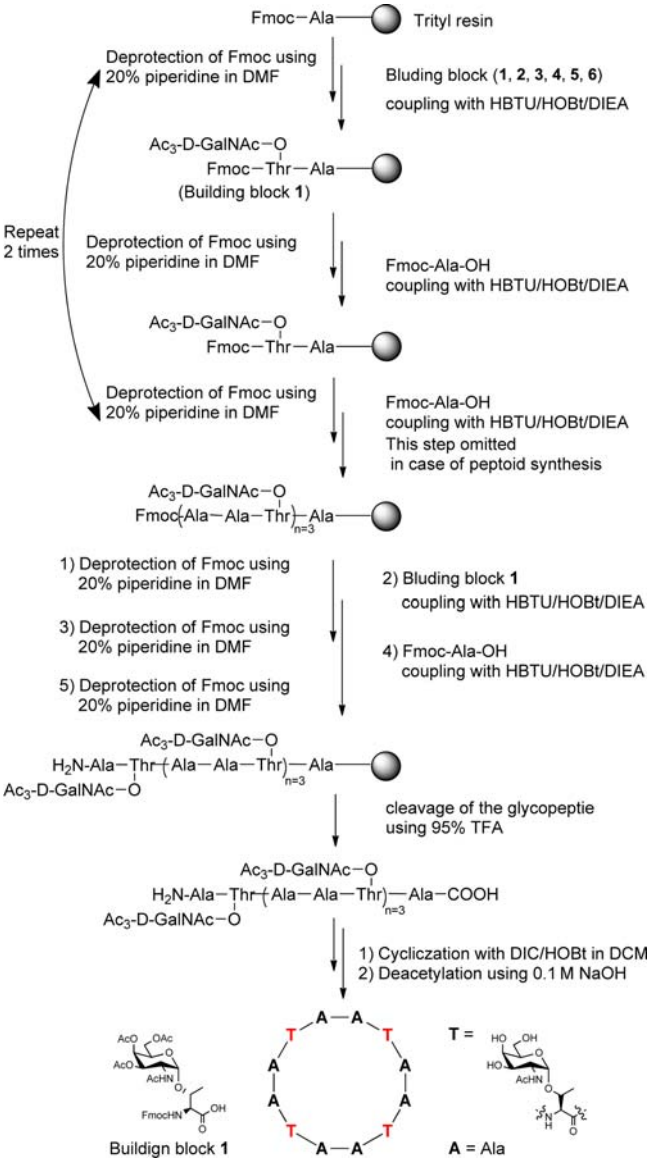
Building Block 5: ^{1}H NMR (300 MHz, DMSO-d_6, major rotamer) δ 8.03 (d, 1H, J = 8.6 Hz), 7.89 (d, 2H, J = 7.5 Hz), 7.75-7.70 (m, 2H), 7.47 (d, 1H, J = 7.4 Hz), 7.48-7.30 (m, 4H), 5.30 (d, 1H, J = 2.7 Hz), 4.97 (dd, 1H, J = 11.5, 3.0 Hz), 4.80 (d, 1H, J = 3.4 Hz), 4.49 (m, 1H), 4.31-3.94 (m, 9H), 3.60-3.41 (m, 3H), 2.09 (s, 3H), 1.97 (s, 3H), 1.84 (s, 3H), 1.81 (s, 3H), 1.21 (d, 3H, J = 6.6 Hz), 1.11 (d, 3H, J = 6.8 Hz); MS (MALDI-TOF) m/z = 778.2 [M + Na]$^+$.

Peptide Synthesis and Purification. Resin loaded with Fmoc-Ala-substituted trityl resin (0.56 mmol/g) was used as the support. The coupling of Fmoc-amino acids was performed in DMF with an equimolar mixture of *O*-(Benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU), 1-hydroxybenzotriazole (HOBT) and *N*,*N*-diisopropylethylamine (DIEA) as the coupling reagents. After coupling the last amino acid, the Fmoc-group was removed with 20% piperidine/DMF, and peptide-resin were cleaved with TFA/H_2O (95:5) for 2 h at room temperature. The crude peptides were evaoprated *in vacuo* and dissolved in H_2O/CH_3CN and purified by a reversed-phase high performance liquid chromatography (RP-HPLC) on a preparative (15 μm, 10 × 250 mm) C_{18} Bondapak column using a H_2O/CH_3CN gradient (30-70%/30 min) containing 0.05% TFA.

For cyclization, linear peptide AFGP1, AFGP2, AFGP3, AFGP4, AFGP5, AFGP6 (10 mg) was dissolved in CH_2Cl_2 (2 mL). The DIC/HOBt as promoters were added and the mixture was stirred at room temperature for 12 h. The progress of the cyclization reaction was checked by analytical C_{18} reverse-phase HPLC.

For deacetylation of the carbohydrate protecting group, the peptides were dissolved in 0.1 M NaOH and shaked mixtures for 1 min at room temperature. After complete deprotection, the solution was neutralized with acetic acid and concentrated *in vacuo*. The peptides were purified by RP-HPLC and identified by MALDI-TOF-Mass analysis.

Mass spectrum (MALDI-TOF): *cy*AFGP1, m/z 1950.9 [M+Na]$^+$, 1966.8 [M+K]$^+$; *cyp*AFGP2, m/z 1807.8 [M+Na]$^+$, 1823.8 [M+K]$^+$, *cyp*AFGP3; m/z 1863.9 [M+Na]$^+$, 1879.9 [M+K]$^+$, *cyp*AFGP 4; m/z 1863.9 [M+Na]$^+$, *cyp*AFGP 5; m/z 1863.9 [M+Na]$^+$, 1879.9 [M+K]$^+$, *cyp*AFGP 6; m/z 1863.9 [M+Na]$^+$, 1879.9 [M+K]$^+$.

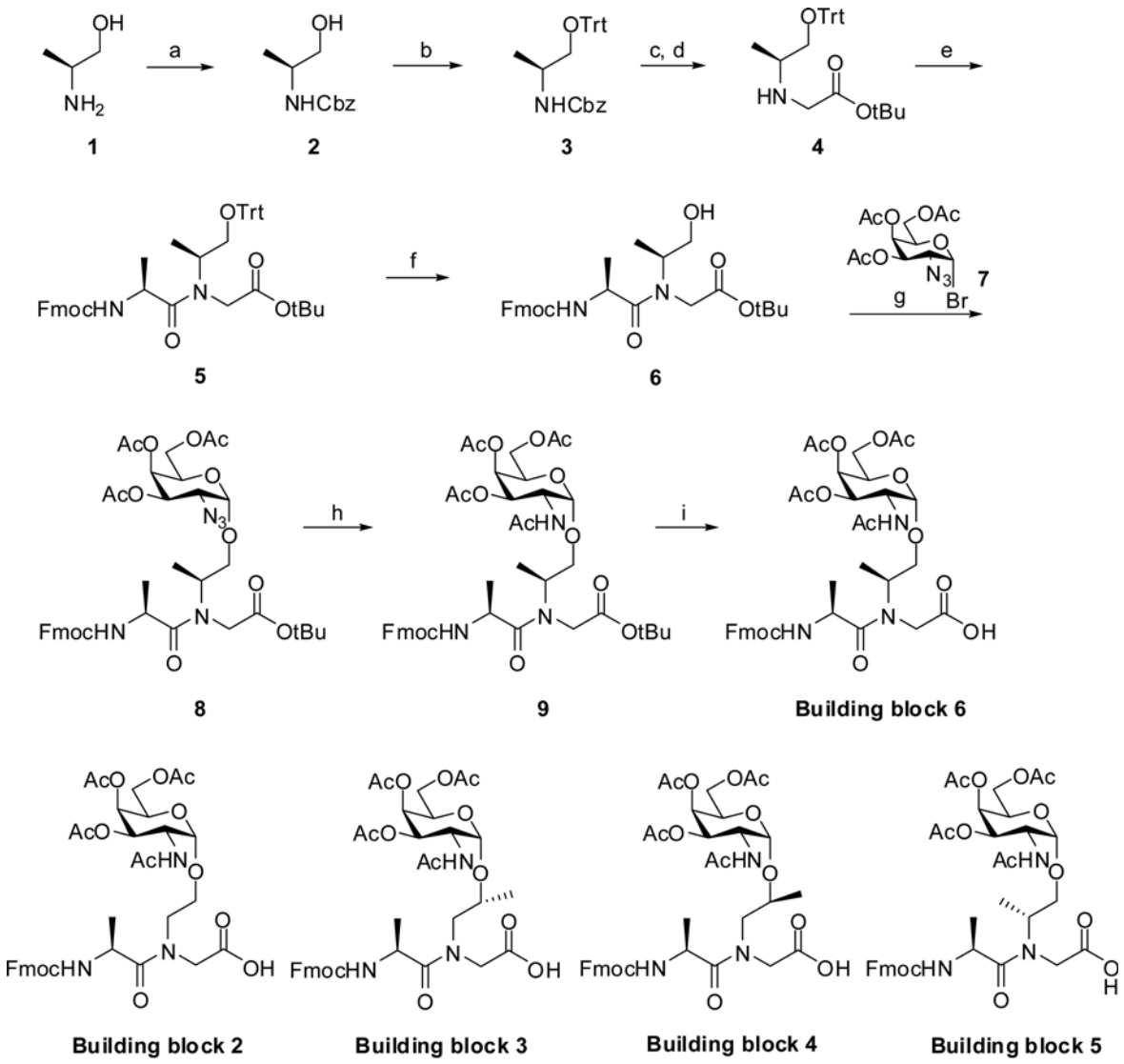


Scheme 1. The synthetic protocol of cyclic glycopeptide and glycopeptoides.

Ice Re-Crystallization Inhibition (RI) Activity Assay. All synthesized AFGPs were dissolved in distilled water and an equal volume of a 60% sucrose solution was mixed with AFGP solution. Total 4 μL of the mixed sample solution was sandwiched between two round 16 mm diameter cover slips. The sandwich droplet was pre-chilled to prevent frost on surface at 4 °C. After 1 min the sandwich droplet was transferred to a circulating cooling stage (THMS600 stage, Linkam Scientific Instruments) and the temperature was quickly lowered to −70 °C at a rate of 90 °C/min, and maintained for 1 min. The sandwich droplet was then warmed to −6 °C and incubated for 60 min to allow ice recrystallization. The sample was examined under a microscope (Olympus) and images were captured using a DP71 CCD camera (Olympus) every 5 min.

Results and Discussion

Scheme 1 shows a protocol designed for the cyclic glycopeptide (*cy*AFGP) and glycopeptoids (*cyp*AFGP) synthesis by standard Fmoc-based solid phase. In accordance with the recent report that states that the terminal galactose of disaccharide is not essential for the antifreeze activity, we simplify the synthesis by substituting the disaccharide β-D-galactosyl-(1→3)-α-D-*N*-acetylgalactosamine with α-D-*N*-acetylgalactosamine in our cyclic compounds.[4b] Under typical glycopeptide synthesis, the glycosylated Fmoc-Thr(Ac_3-α-D-GalNAc) (building block 1), prepared by the reported procedure,[5b] was incorporated on the Fmoc-Ala-Trityl resin after Fmoc deprotection using 20% piperidine in DMF in the presence of *O*-benzotriazole-*N,N,N′,N′*-tetramethyl-uronium-hexafluorophosphate (HBTU, 3 equiv.), 1-hydroxybenzotri-



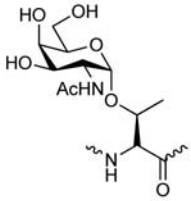
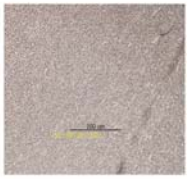
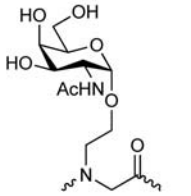
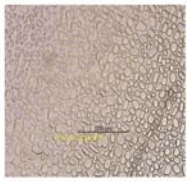
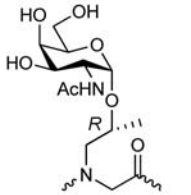
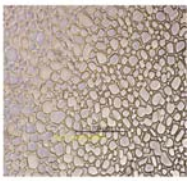
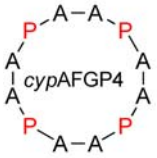
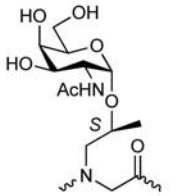
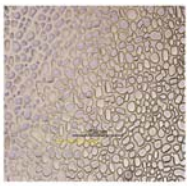
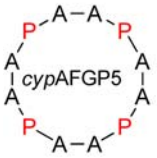
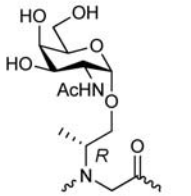
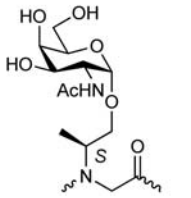
Scheme 2. Reagent and conditions: (a) Cbz-Cl, DIEA, DCM, rt, 15 h, 84% (b) trityl-Cl, DIEA, DCM, rt, 15 h, 85% (c) Pd/C (5 wt %), H_2, MeOH/CH_2Cl_2 (9:1), 1 atm, rt, 3 h (d) *tert*-butyl bromoacetate, DIEA, DCM, rt, 15 h, 81% over two steps (e) Fmoc-L-Ala-OH, DIC, HOBt, DIEA, DMF, rt, 15 h, 42% (f) TsOH·H_2O, MeOH, rt, 2 h, 48% (g) azido bromide **7**, Ag_2CO_3, $AgClO_4$, molecular sieve 4 Å, toluene/DCM (1:1), rt, 15 h, 37% (h) AcSH/pyridine (2:1), rt, 15 h, 91% (i) TFA:H_2O (95:5), rt, 2 h, 97%

azole (HOBt, 3 equiv.) and *N*,*N*-diisopropylethylamine (DIEA, 3 equiv.) as activating reagents for 2 h at room temperature. The remaining Ala amino acids were introduced by standard Fmoc-solid phase peptide chemistry. We repeated these procedures for the synthesis of linear acetylated glycopeptides and the resulting peptide was cleaved from the resin using the cleavage cocktail [trifluoroacetic acid-water (TFA-H_2O; 95:5)] for 2 h. Purification was performed *via* reverse phase-HPLC (RP-HPLC), eluting with a gradient 10-90% acetonitrile in water, both containing 0.05% (vol/vol) trifluoroacetic acid (TFA). After purification, the reaction mixture of linear glycopeptide was diluted with 2 mL of CH_2Cl_2 (DCM) and diisopropylcarbodiimide (DIC)/HOBt were added. Cyclization reaction proceeded smoothly at room temperature for 12 h and afforded a desired product. After the completion of the cyclization, the solution was evaporated *in vacuo*. The crude peptide was dissolved in H_2O/CH_3CN and loaded to a preparative RP-HPLC for purification, then lyophilized. Finally, for the deacetylation of sugar moieties, cyclic glycopeptide was then treated with 0.1 M NaOH in buffer stirred for 1 minute at room temperature, and the reaction was also monitored by RP-HPLC. The integrity of purified cyclic glycopeptide was determined by MALDI-TOF-Mass spectrometry, and the observed molecular mass was found to agree with calculated value. Cyclic glycopeptoids (*cyp*AFGP2, *cyp*AFGP3, *cyp*AFGP4, *cyp*AFGP5, *cyp*AFGP6) were synthesized by following the same route described above with building blocks **2**, **3**, **4**, **5** and **6** instead of building block **1** (Scheme 2).

The *cy*AFGP1 was assayed for ice recrystallization inhibition (RI) activity. Since the PP-helix conformation is favorable structure for AFGP activity,[6] we expect that *cy*AFGP1 does not show any RI effect because of its random structure. However, it was found that *cy*AFGP1 showed significant ice-recrystallization inhibitory effect compared to control PBS buffer condition (Table 1). This means that RI activity does not require the certain conformation such as PP-helix. Our result also demonstrated that the terminal galactose of disaccharide has no crucial effect on the RI activity even though rigidity is enhanced through the cyclization. According to this study, we opened the new possibility that the monosaccharide cyclic glycopeptide as pure AFGP material can be easily scaled up or modified to allow variants using Fmoc-based solid-phase peptide synthesis for the intensive structure-activity relationship investigation .

With this encouraging result, our research moved to investigate the structure-activity relationship using a diverse set of *N*-linked cyclic glycopeptoid analogues (*cyp*AFGP2, 3, 4, 5, 6) for the RI activity. Recently, we have developed a strategy for the efficient chemical synthesis of glycopeptoid using conventional Fmoc-based solid phase synthesis.[4a] Peptoids, or *N*-substituted oligoglycines are achiral peptide mimetics in which side chains are moved to the amide nitrogen atom of each glycine monomer rather the α-carbon atom of each amino acid. Peptoids were well known to introduce easily the structurally diverse analogues of AFGPs using hundreds of commercially available diverse primary amine at cheap cost.[7] We synthesized the several *cyp*AFGP analogues such as *cyp*AFGP2, *cyp*AFGP3, *cyp*AFGP4, *cyp*AFGP5, *cyp*AFGP6 using the building blocks **2**, **3**, **4**, **5** and **6**, respectively. Thus, the easy access to the *N*-linked glycosylated peptoid amino acids as building blocks was the

Table 1. Ice recrystallization inhibition activity of cyclic glycopeptide and glycopeptoides

Structure	Building block	Ice crystal size
PBS standard	without compound	
	T = 	
	P = 	
		
		
		
		

key to the success in the preparation of *cyp*AFGP analogues. For the synthesis of glycosylated peptoid amino acid (building block **6**), Fmoc-protected peptoid and azido bromide **7** were coupled (Scheme 2). Subsequently, the synthesis of glycosylated peptoid building block **6**, was started with the commercial available starting material as described below. Briefly, (*S*)- 2-amino-1-propanol (**1**) was treated with Cbz-Cl and Trityl-Cl under the basic condition to protect the primary amine and secondary alcohol, respectively. After standard hydrogenolysis of the Cbz group **3**, the resultant compound was treated with *tert*-butyl bromoacetate under the basic condition to give ester **4**. Then, Fmoc-L-Ala-OH was added with DIC/HOBt/DIEA as a coupling reagent and left to react overnight. Consequently, the synthesis of Fmoc-protected building block **6** was accomplished by the removal of trityl group of **5** using catalytic amount of *p*-toluene-sulfonic acid hydrate (overall yield 12% in 6 total steps). Next, the glycosylation of bromide **7** with Fmoc dipeptide derivative **6** and the reductive acetylation in the presence of neat thioacetic acid, produced glycopeptoid ester **9**. Finally, the deprotection of *tert*-butyl group using TFA:H_2O (95:5) yielded the peracetylated acid as building block **6** (overall yield 8% in total 9 steps).

Glycopeptoid building blocks **5** was also synthesized by following the same route as described above with the exception of using (*R*)-2-amino-1-propanol and the building block **2**, **3** and **4** were produced in a similar manner to that reported previously.[5a]

To see the stereo effect on ice recrystallization inhibition activity, we synthesized the cyclic glycopeptoids, *cyp*AFGP2, *cyp*AFGP3, *cyp*AFGP4, *cyp*AFGP5, and *cyp*AFGP6 by following same reaction (Scheme 1). Four compounds (*cyp*AFGP3, *cyp*AFGP4, *cyp*AFGP5, *cyp*AFGP6) having the (*R*) and (*S*) configured methyl groups at different position were assay for the RI activity. Unfortunately, all of them did not show any RI activity, including the *cyp*AFGP2 that lack the methyl branch (Table 1). This result may come from the enhanced flexibility of cyclic glycopeptoids through peptoid mimics which provides more flexibility in addition to the enzymatic stability than the natural amino acids. It means that the insertion of glycopeptoid residue into Thr position of peptide backbone destroyed the key interactions between the water lattice and AFGP.

Conclusion

In summary, our *cy*AFGP showed strong RI activity even though disaccharide was replaced with monosaccharide. This finding simplified the synthesis of AFGP analogues with the help of solid phase peptide synthesis and enable access to produce the cyclic analogues for the extensive structure-activity-relationship investigations. Since the *cyp*AFGP analogues did not show RI activity, flexibility is also an important factor to consider in designing the AFGP analogues.

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