Supplementary Material (ESI) for Chemical Communications

One-Pot synthesis of cyclic antifreeze glycopeptides

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Reagents and general methods.

All commercially available solvents and reagents were used without further purification. Reactions were monitored by thin-layer chromatography (TLC) carried out on Merck silica gel glass plates, 60F254; compounds were visualized by treatment with a solution of 5% H_2SO_4 in MeOH and heating at 200°C. Flash chromatography was performed on Kanto Chemical silica gel N60 (40-50 mm). The AFGP monomer unit, L-alanyl-*O*-[β-D-galactopyranosyl-(1→3)-2-acetamide-2-deoxy-α-D-galactopyranosyl]-L-threonyl-L-alanine (**1**) was synthesized according to the method reported by our group (see, references cited in the main text). Linear AFGPs [syAFGPn ($2 \leq n \leq 4$)] were prepared and isolated according to the procedure described in our previous papers (references cited in the main text). NMR measurements were recorded at 27°C on BRUKER AVANCE 600 (^{1}H: 600 MHz, ^{13}C: 150 MHz) and BRUKER AVANCE 500 (^{1}H: 500 MHz, ^{13}C: 125 MHz), using residual undeuterated solvent as an internal reference. Circular dichroism (CD) spectra were measured in 1 mm path length quartz cells on a JASCO J-820 spectropolarimeter.

All mass measurements were performed using an Ultraflex TOF/TOF massspectrometer (Broker Daltonics GmbsH, Bremen, Germany) equipped with a reflector and controlled by the Flexcontrol 1.2 software package. Ions generated by a pulsed UV laser beam (nitrogen laser, λ = 337 nm, 5 Hz) were accelerated to a kinetic energy of 23.5 kV. External calibration of MALDI-mass spectra was carried out using singly charged monoisotopic peaks of a mixture of the human angiotensin II (*m*/*z* 1046.542), bombesin (*m*/*z* 1619.823), ACTH (*m*/*z* 2465.199), and somatostain 28 (*m*/*z* 3147.472). To achieve mass accuracy better than 60 ppm, internal calibration was carried out by doping the matrix solution with a mixture of the calibration peptides. Calibration of these mass spectra was performed automatically utilizing a customized macro command of the XMASS 5.1.2 NT software package. The macro

command was used for the calibration of the monoisotopic singly charged peaks of the abovementioned peptides. All of data processing and calculations were performed by Microsoft Excel and Synergy KaleidaGraph.

Synthesis of cyclic AFGPs (*cy*AFGP$_n$).

Cyclic analogs of AFGPs (*cy*AFGP$_n$ $2 \leq n \leq 4$) were synthesized by a protocol indicated in the main text (Scheme 1) and Fig. S1 by means of controlled cyclization reaction of the pre-formed small linear *sy*AFGP$_n$ ($2 \leq n \leq 4$). The polymerization of AFGP monomer **1** (50 mg, 0.08 mmol in 0.8 mL of DMF) was carried out by employing 0.8 equivalent molar of DPPA (130 μL of 10% solution in DMF, 0.06 mmol) and 1.5 equivalent molar of TEA (160 μL of 10% solution in DMF, 0.10 mmol), the polymerization gave a mixture of short chain lengths of linear AFGPs ranging from *sy*AFGP$_2$ to *sy*AFGP$_4$ as major products (Fig. S2). Subsequently the reaction mixture of *sy*AFGP$_n$ was diluted by 4.7 mL of DMF (total volume = 5.5 mL) and added with DPPA (75 μL of 10% solution in DMF, 0.5 equiv., 0.03mmol) and TEA (100 μL of 10% solution in DMF, 0.9 equiv., 0.07 mmol). Cyclization reaction proceeded smoothly at 25°C for 48 h. The product mixture was precipitated by addition of diethyl ether and centrifuged. The desired cyclic product was separated by passing through the ion-exchange short columns [Amberlite IRA96SB (OH^- form) and Amberlite IRC50(H^+ form)] (crude mixture; 28 mg, 59%). As shown in Figure 1 of the main text, ion peaks due to linear AFGPs could not be detected. ^{1}H-NMR data of *cy*AFGP$_n$ (600 MHz, D_2O, δ): 4.88 (d, 1 H, $J_{1,2}$ = 3.7 Hz, H-1), 4.47-4.20 (m, 7 H, Thr-α-H, Thr-β-H, H-1', Ala-α-H × 2, H-2, H-4), 4.04-4.01 (m, 2 H, H-5, H-3), 3.89 (d, 1 H, $J_{3',4'}$ = 2.9 Hz, H-4'), 3.66 (m, 4 H, H-6a, 6b, 6a', 6b'), 3.89-3.60 (m, 2 H, H-5', H-3'), 3.48 (dd, 1 H, $J_{1',2'}$ = 7.8 Hz, $J_{2',3'}$ = 9.8 Hz, H-2'), 2.03 (s, 3 H, NHCOCH_3), 1.35 (m, 9 H, Ala-β-H × 2, Thr-γ-H).

Preparative isolation of *cy*AFGPs.

The mixture of *cy*AFGP$_n$ were then subjected to the purification into homogeneous fractions of discrete size by means of recycling preparative gel permeation chromatography (GPC) (Shodex OHPak SB-2002.5 × 2, water as an eluent, 2ml/min.). The small *cy*AFGPs (*cy*AFGP$_n$ $2 \leq n \leq 4$) were successfully isolated by recycle GPC. As for high-molecular weight fractions (*cy*AFGP$_n$ $5 \leq n \leq 8$), it was difficult to isolate them due to the lower yield and overlapping (Fig. S4). **C2** (1.3 mg, 10%): ^{1}H-NMR (500 MHz, D_2O, δ): 4.81 (d, 1 H, $J_{1,2}$ = 3.2 Hz, H-1), 4.45-4.13 (m, 7 H, Thr-α-H, Thr-β-H, H-1', H-2, Ala-α-H × 2, H-4), 4.00-3.94 (m, 2 H, H-5, H-3), 3.82 (d, 1 H, $J_{3',4'}$ = 3.0 Hz, H-4'), 3.66 (m, 4 H, H-6a, 6b, 6a', 6b'), 3.59-3.53 (m, 2 H, H-5', 3'), 3.42 (dd, 1 H, $J_{2',3'}$ = 8.1 Hz, H-2'), 1.95 (s, 3 H, NHCOCH_3), 1.35 (d, 3 H, *J* = 7.0 Hz, Ala-β-H), 1.29 (d, 3 H, *J* = 7.1 Hz, Ala-β-H), 1.22 (d, 3 H, *J* = 5.0 Hz, Thr-γ-H); MALDI-TOF MS (*m*/*z*): $C_{48}H_{80}N_8O_{28}Na$ $[M+Na]^+$ calcd for 1239.50, found 1239.35. **C3** (1.83 mg, 14%): ^{1}H-NMR (500 MHz, D_2O, δ): 4.81 (d, 1 H, $J_{1,2}$ = 3.2 Hz, H-1), 4.46-4.13 (m, 7 H, Thr-α-H, Thr-β-H, H-1', H-2, Ala-α-H × 2, H-4), 4.00-3.94 (m, 2 H, H-5, H-3), 3.82 (s 1 H, H-4'), 3.66 (m, 4 H, H-6a, 6b, 6a', 6b'), 3.59-3.53 (m, 2 H, H-5', 3'), 3.41 (dd, 1 H, $J_{2',3'}$ = 8.4 Hz, H-2'), 1.95 (s, 3 H, NHCOCH_3), 1.35 (d, 3 H, *J* = 7.0 Hz, Ala-β-H), 1.29 (d, 3 H, *J* = 7.0 Hz, Ala-β-H), 1.23 (d, 3 H, *J* = 5.5 Hz, Thr-γ-H) ; MALDI-TOF MS (*m*/*z*): $C_{72}H_{120}N_{12}O_{42}Na$ $[M+Na]^+$ calcd for 1239.50, found 1239.35. **C4** (0.80 mg, 6%): ^{1}H-NMR (500 MHz, D_2O, δ): 4.81 (s, 1 H, H-1), 4.47-4.13 (m, 7 H, Thr-α-H, Thr-β-H, H-1', H-2, Ala-α-H × 2, H-4), 4.00-3.93 (m, 2 H, H-5, H-3), 3.82 (s, 1 H, $J_{3',4'}$ = 3.0 Hz, H-4'), 3.66 (m, 4 H, H-6a, 6b, 6a', 6b'), 3.59-3.53 (m, 2 H, H-5', 3'), 3.41 (dd, 1 H, $J_{2',3'}$ = 8.3 Hz, H-2'), 1.95 (s, 3 H, NHCOCH_3), 1.34 (d, 3 H, *J* = 6.6 Hz, Ala-β-H), 1.29 (d, 3 H, *J* = 6.6 Hz, Ala-β-H), 1.22 (d, 3 H, *J* = 5.4 Hz, Thr-γ-H) ; MALDI-TOF MS (*m*/*z*):

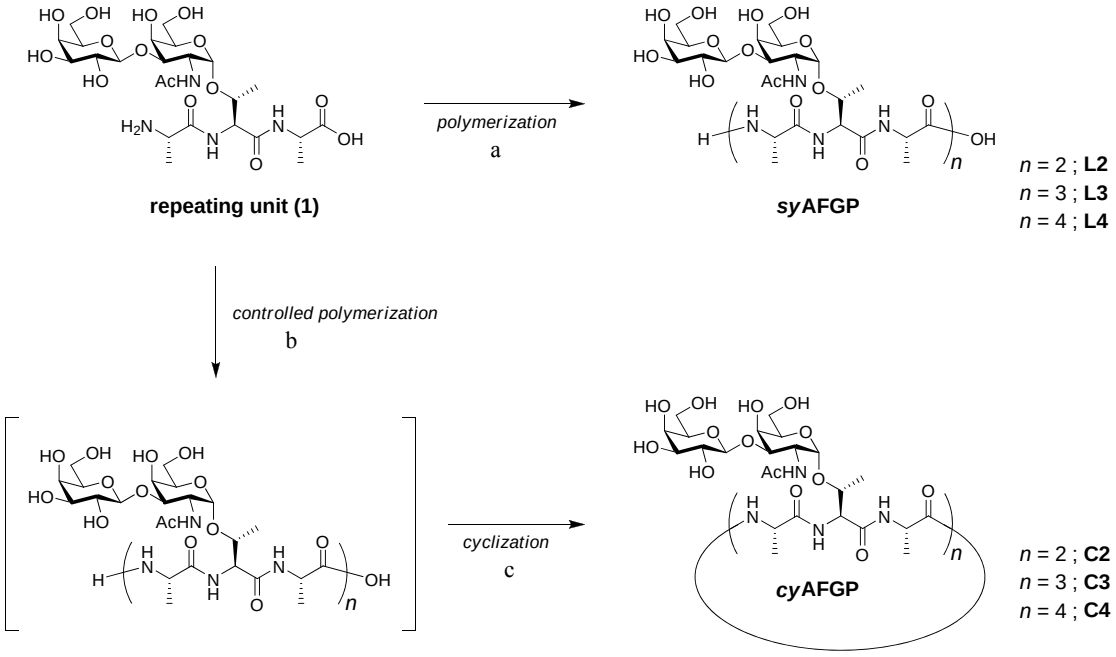
$C_{96}H_{160}N_{16}O_{56}Na$ $[M+Na]^+$ calcd for 1239.50, found 1239.35.

Measurement of antifreeze activity.

Thermal hysteresis (TH) activity was determined by the observation of an ice crystal in the sample solution by using in-house photomicroscope system (see, reference 5 cited in the main text and the following paper; M. Takamichi, Y. Nishimiya, A. Miura, S. Tsuda, *FEBS J*. 2007, **274**, 6469-6476). Compounds were dissolved in 0.1 M ammonium bicarbonate solution and a single ice crystal 10-25 mm in diameter was prepared in the solution. The temperature at which the crystal melts was measured as the melting point (T_m). Single ice crystal was prepared again and then cooled at a constant rate of 0.2 °C/min. The temperature at which the ice crystal initiates rapid and continuous growth was measured as the freezing point (T_f). The difference between T_f and T_m was determined as thermal hysteresis activity:

$$TH = T_m - T_f$$

Measurement of the T_f and T_m values was repeated three times, and the values were averaged. The ice crystal photo images were taken at approximately 0.1°C below the melting point.



	DPPA (equiv)	TEA (equiv)	DMF (mL)	temp	time (h)	yield (%)
a	1.2	2.2	0.5	0°C	12	78
b	0.8	1.5	0.5	0°C	12	–
c	0.5	0.9	4.5	r.t	48	59

Fig. S1 Synthetic scheme of the linear and cyclic AFGPs and the conditions of polycondensation reactions of glycopeptides.

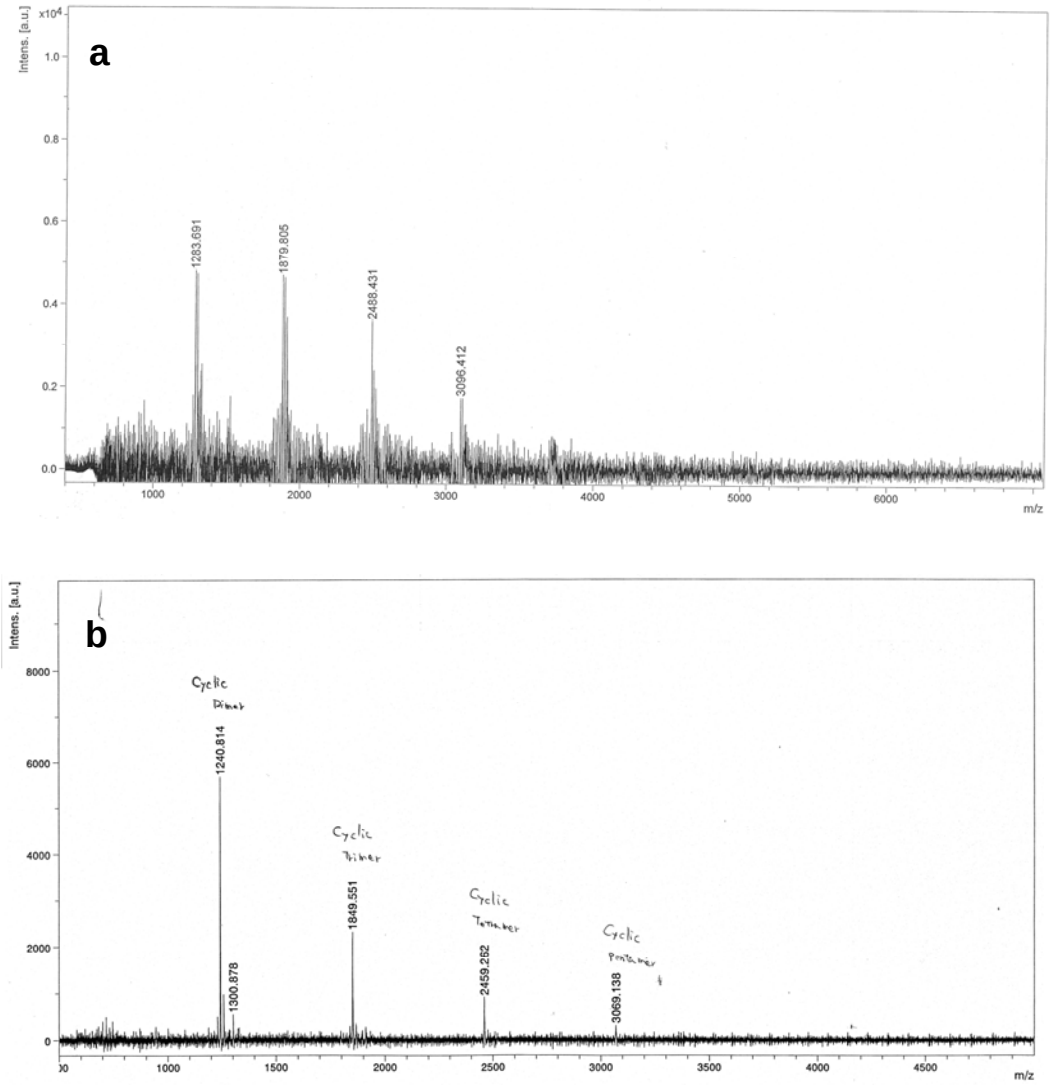


Fig. S2 MALDI-TOFMS spectra of AFGPs. a) reaction mixuture of polycondensation, b) cyclization products.

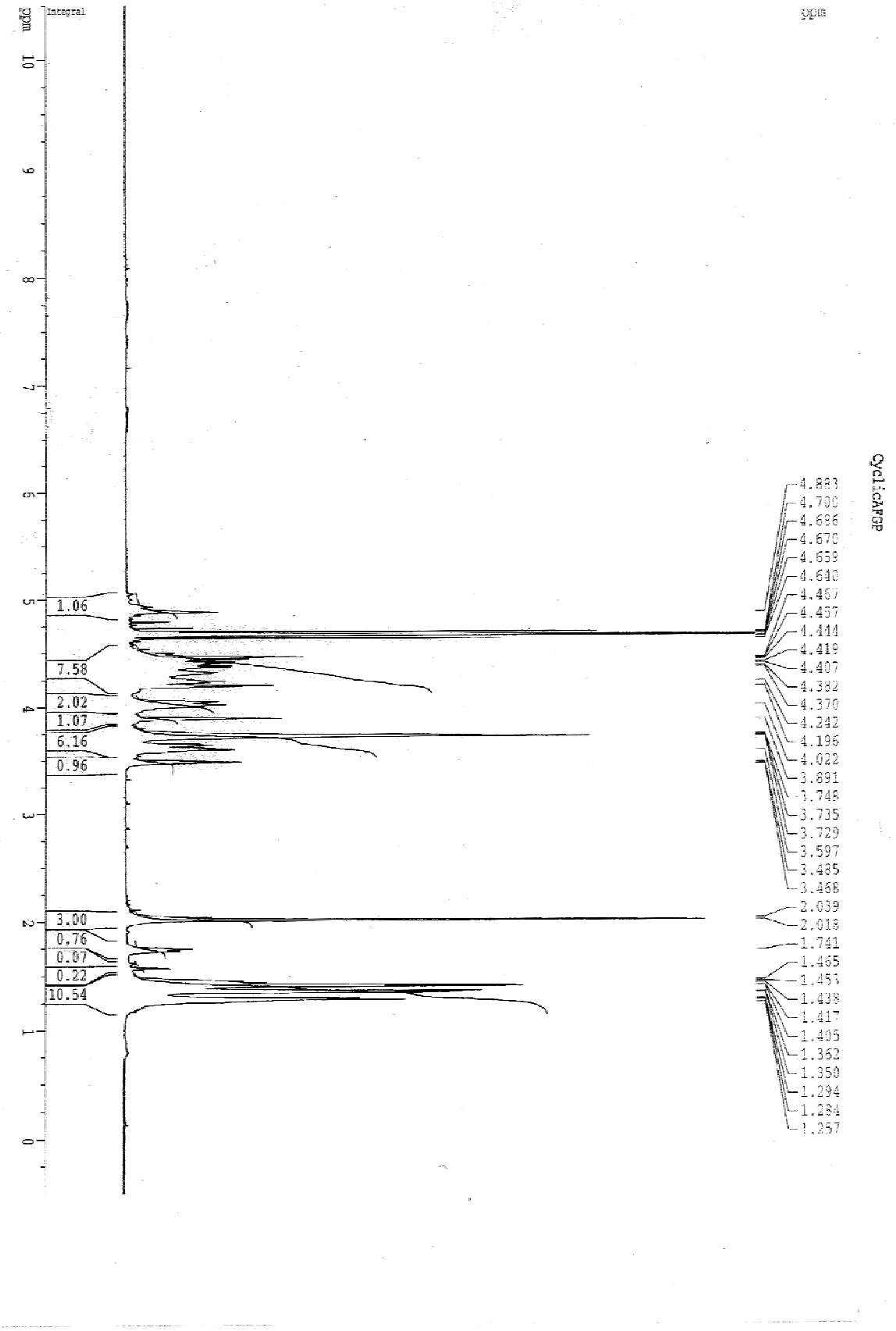


Fig. S3 ^{1}H-NMR spectra of *cy*AFGP$_n$ ($2 \leq n \leq 5$).

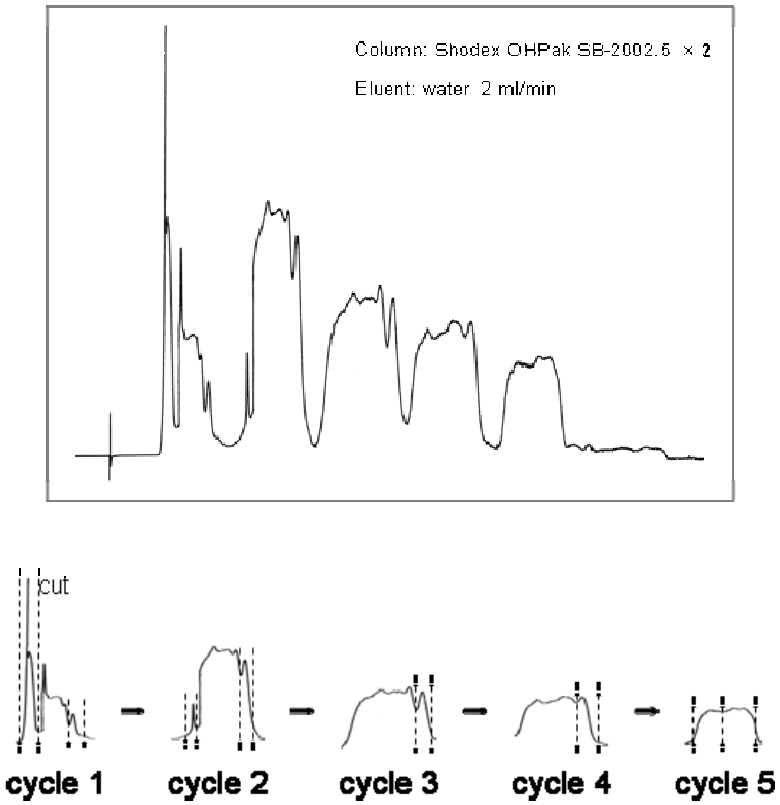


Fig. S4 Recycle GPC profile of *cy*AFGPs.

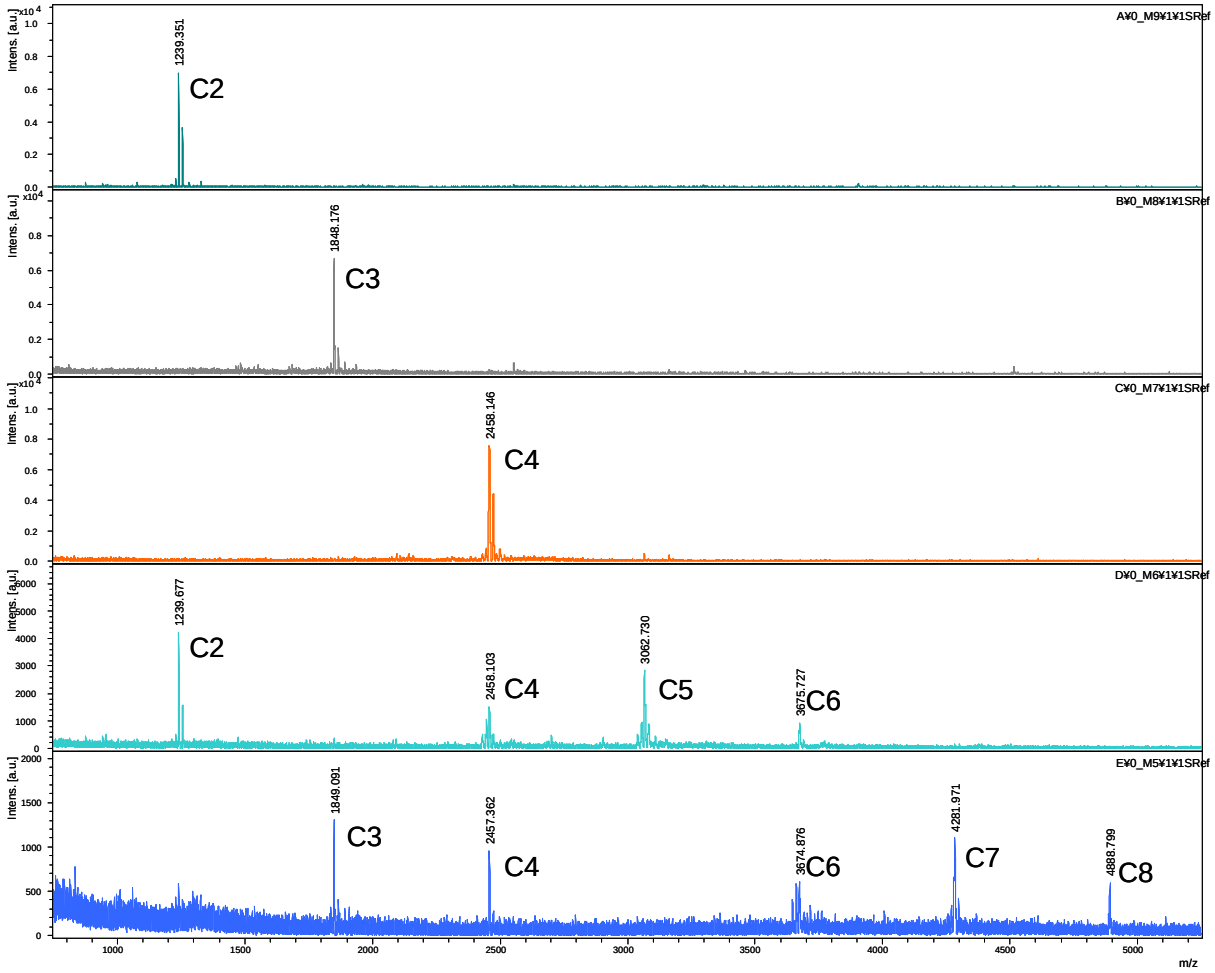


Fig. S4 MALDI-TOFMS spectra of typical fractions isolated by recycle GPC.

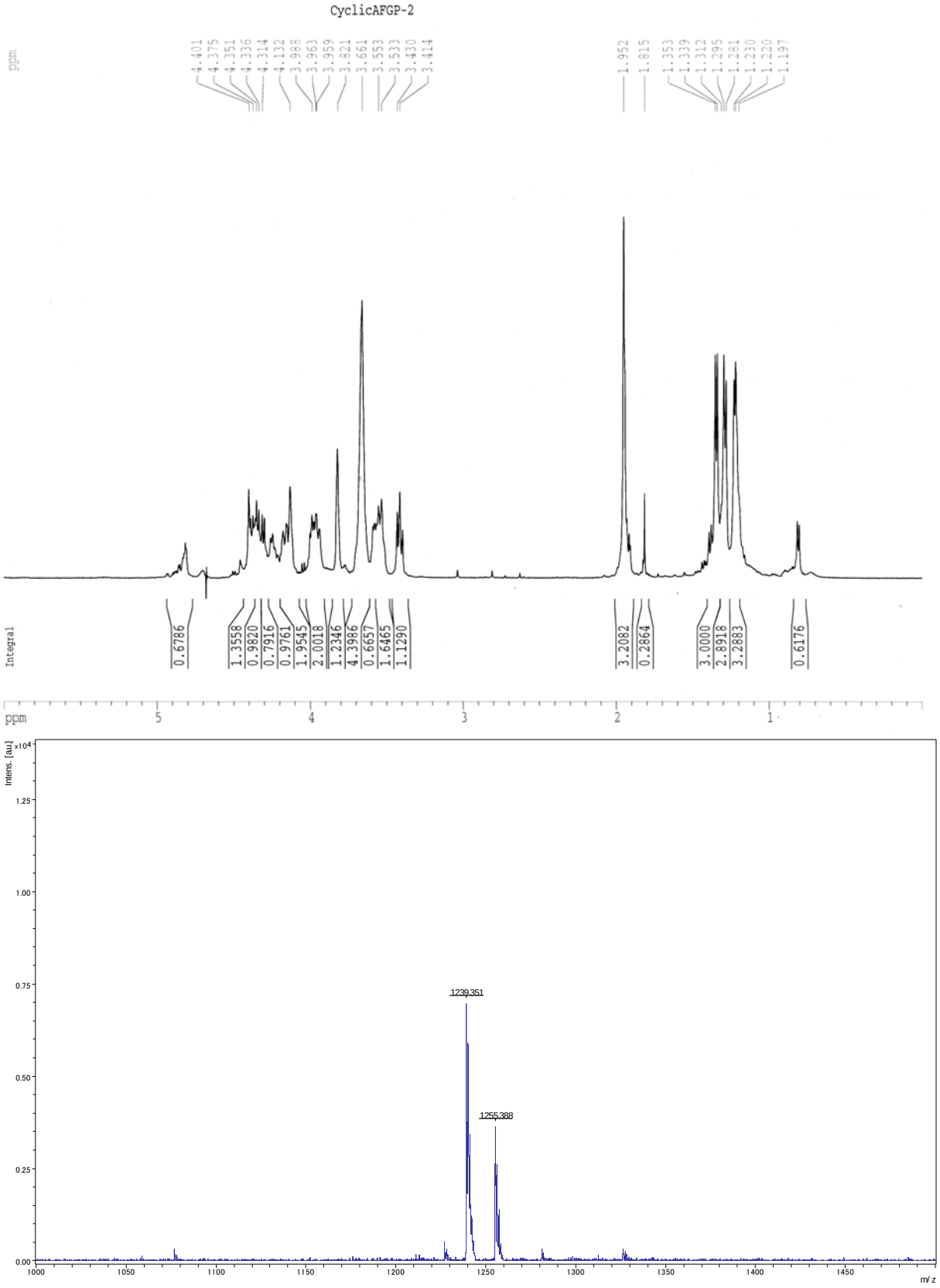


Fig. S6 ^{1}H-NMR and MALDI-TOFMS spectra of isolated **C2 (*cy*AFGP$_2$)**.

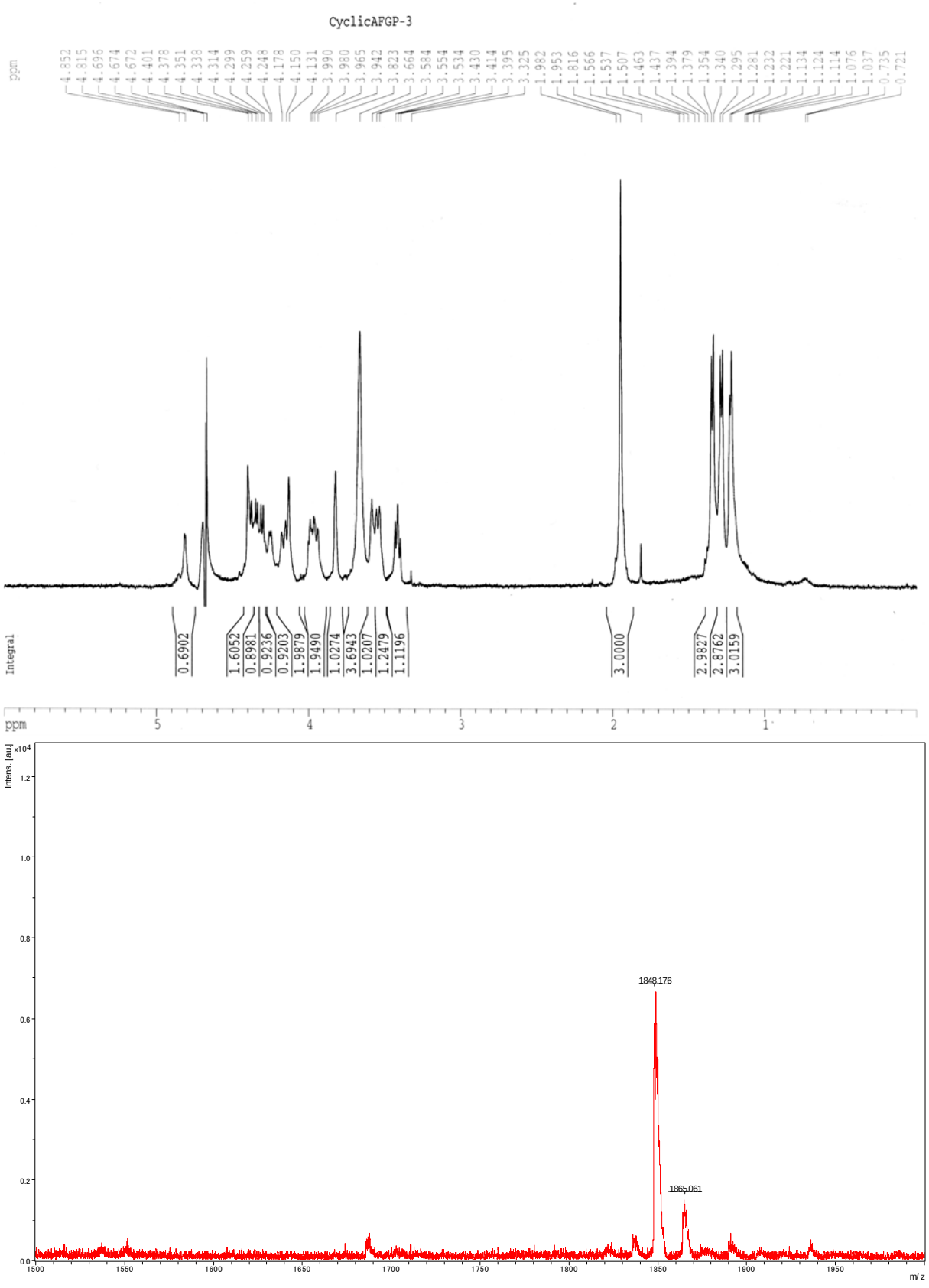


Fig. S7 ^{1}H-NMR and MALDI-TOFMS spectra of **C3 (*cy*AFGP$_3$)**.

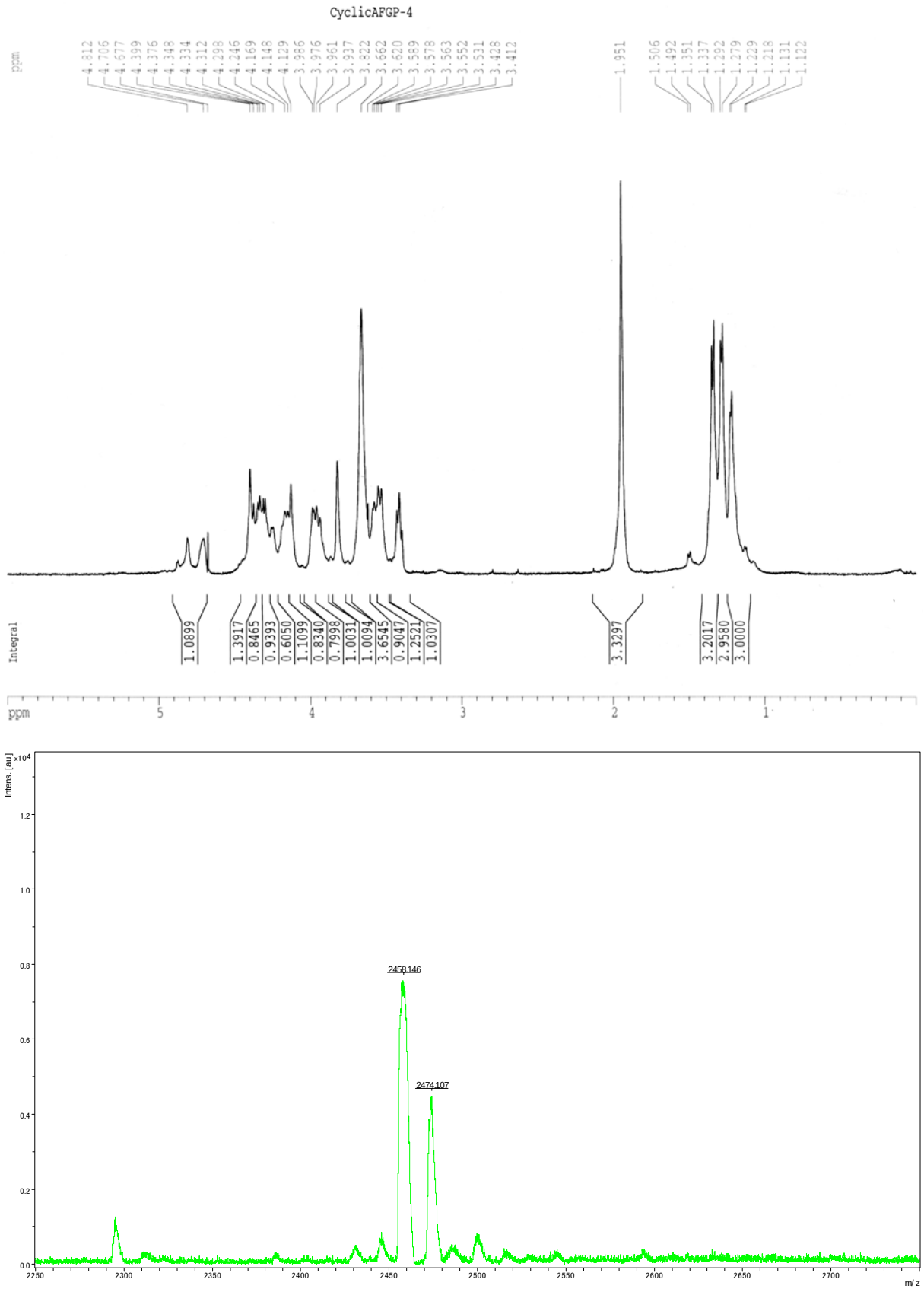


Fig. S8 ^{1}H-NMR and MALDI-TOFMS spectra of **C4 (*cy*AFGP$_4$)**.