Synthetic Insect Antifreeze Peptides Modify Ice Crystal Growth Habit

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Contents

Materials and Methods. The resin, coupling reagents and all Fmoc amino acids were purchased from GL Biochem (Shanghai, China). All purchased amino acids were of the L configuration. Trifluoroacetic acid, piperidine, ethanedithiol and triisopropylsilane were obtained from Sigma-Aldrich.

Synthesis of Linear Antifreeze Peptides. The linear analogues of the *Dendroides Canadensis* Repeat (DCR) series of peptides were synthesized following standard Fmoc solid phase peptide synthesis protocols on Fmoc-amino acid preloaded Wang resin having a substitution level of 0.55 mmol/g. HCTU was used as the coupling reagent. In a typical 1 h coupling procedure, 4 equivalents of the Fmoc amino acid, 3.8 equivalents of the coupling reagent and 10 equivalents of N,N-Diisopropylethylamine (DIPEA) were used. Fmoc-protecting groups were removed using 20% piperidine in dimethylformamide (DMF). The final peptides were cleaved from the resin using a TFA cocktail mixture: (trifluoroaceticacid/H_2O/ethanedithiol/triisopropylsilane (95:2.5:2.5:1, v/v) for 3 h. The crude peptides were extracted with diethyl ether and purified using reversed-phase HPLC on GE Pharmacia AKTApurifier 10 system on a Phenomenex Luna C_{18} semi-preparative column (20 mm × 250 mm, 300 Å, 5-μm particle size) using gradient elutions with 0.1% TFA in water as solvent A and 0.1% TFA and 0.9% water in 99% acetonitrile as solvent B with UV detection at 214 nm (amide) and 280 nm (aromatic) at the flow rates shown in Table S1. The final purity of the peptides (> 98) was assessed by RP-HPLC on an analytical C_{18} column (4.6 mm × 250 mm, 300 Å, 5-μm particle size) using the same solvent system as above at flow rate of 1 mL per min. The identity of the peptides was established using electrospray ionization mass spectrometry (ESIMS) recorded on a Bruker micrOTOFQ mass spectrometer.

Reduction and Alkylation of Linear Antifreeze Peptide. In order to generate free SH groups, the peptide analogue was dissolved in 0.1 M ammonium bicarbonate containing dithiothreitol (DTT) (~10 fold molar excess) and held at 56 ºC for 2 h. The reduced disulfide

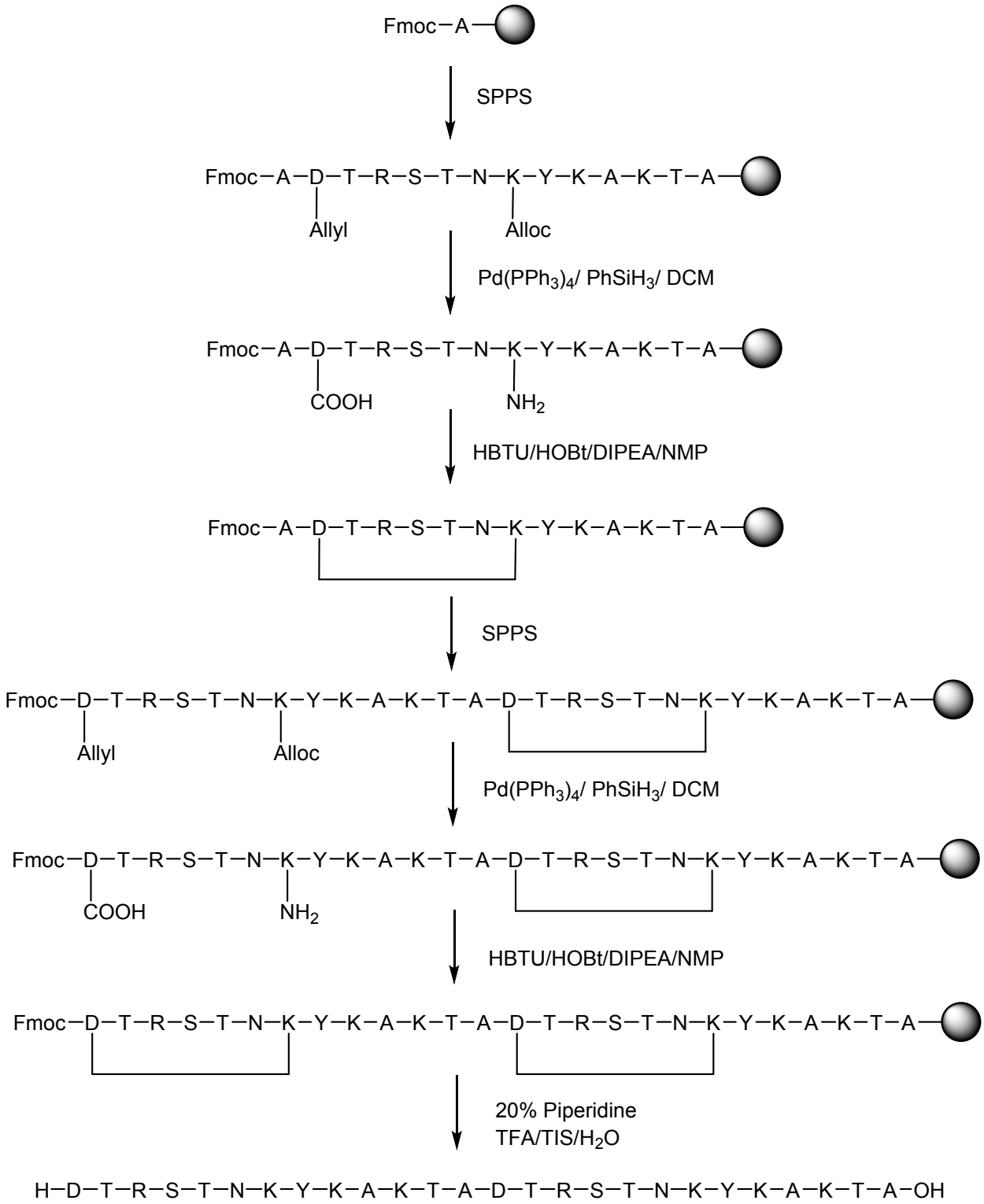
bonds were alkylated by incubation in iodoacetamide (~50 fold molar excess) at 37 ºC for 48 h. The reduced and alkylated peptides were precipitated using cold diethyl ether and recovered by freeze drying.

Synthesis of Cyclic Antifreeze Peptides. The cyclic analogues of the *Dendroides Canadensis* Repeat (DCR) series of peptides were synthesized following the standard Fmoc solid phase peptide synthesis technique mentioned above. The Lys and Asp side-chains at the cyclization points were protected using alloc and allyl protecting groups respectively. Selective deprotection of these two side-chains was achieved following a reported strategy with slight modifications (Scheme S1) as described below. After assembling the protected linear peptides up to the first cyclisation point, the peptide resin was washed with CH_2Cl_2 (3 × 2 min) under nitrogen atmosphere to which was added a solution of PhSiH3 (24 equiv.) in 2 mL of CH_2Cl_2 and the resin manually stirred for 2 min. Subsequently, a solution of Pd (PPh3)4 (0.25 equiv.) in 6 mL of CH_2Cl_2 was added as nitrogen was bubbled continuously through the resin and stirred for 30 min. The liquid was drained off and the peptide resin washed with CH_2Cl_2 (3 × 50 mL, 2 min), DMF (3 × 50 mL, 1 min) and again with CH_2Cl_2 (4 × 50 mL, 2 min) and the process of deprotection and washing repeated for a second time. Cyclization of the peptide via the deprotected side-chain carboxylic acid of Asp and amine of Lys was achieved using HBTU (6 equiv.), HOBt (6 equiv.), and DIPEA (12 equiv.) for 2 h in NMP. The coupling reagent was added as a dilute solution in 10 mL of the solvent for the cyclization reaction. This process was repeated until a negative Kaiser test resulted. After the first cyclic bond was formed, the synthesis of the linear peptide was continued to the second cyclization point and the procedure of selective side-chain deprotection and cyclization repeated as above.

Ice Crystal Growth Morphology Observation. The antifreeze peptides were dissolved in water at a concentration of 0.01 mol/L. Antifreeze activity measurement and direct

observation of ice crystal growth was performed using a Clifton nanoliter osmometer (Otago Osmometers, Dunedin, NZ). Calibration of the nanoliter freezing point osmometer was done using pure water and standard solutions of glucose and sodium chloride. A 20 nL drop of the peptide solution was inserted into a droplet of Cargille B immersion oil (heavy) in the cold-stage sample wells of the Clifton nanoliter osmometer using a micropipette attached to a Gilmont micrometer syringe. The peptide solution was rapidly frozen and then slowly warmed until a single ice crystal remained. The temperature was then slowly and carefully decreased in order to observe the growth of the crystal. Thermal hysteresis (TH) was measured as the difference in the freezing and melting points of the small ice crystal. The ice crystal images were digitally collected by a Nikon DS camera mounted on a microscope.

NMR Experiments. ^{1}H NMR spectra were recorded on a Bruker Avance III HD spectrometer equipped with a BBFO probe operating at a ^{1}H frequency of 500 MHz. Standard 5 mm NMR tubes were used. Samples were prepared in 50 mM Tris-D11 (Cortecnet, France) at pH 6.5 in 90% H_2O and 10% D_2O. Standard ^{1}H pulse sequence was used and water suppression was achieved with the excitation sculpting method. Unless otherwise stated, temperature was 300 K. Chemical shifts (δ) were expressed in parts per million, and DSS served as an internal standard (δ=0 ppm).



Scheme S1. Solid phase synthesis of cyclic AFP analogues using allyl and alloc strategy

Table S1: Physical Characteristics of peptides

Peptide	HPLC gradient (% solvent B; Time)	HPLC t_g (min) and % Purity	Molecular Formula	Calculated MW (Da)	Observed m/z
DCR13	1-50%; 30mins	13.26; 99.74 %	$C_{58}H_{99}N_{19}O_{20}S_2$	$[M+H]^+$1447.6674	$[M+H]^+$1447.04
DCR26	1-50%; 30mins	13.46; 99.05 %	$C_{116}H_{196}N_{38}O_{39}S_4$	$[M+2H]^{2+}$1438.6597	$[M+2H]^{2+}$1438.1721
DCR26 A	10-50%; 25mins	8.48; 96.93 %	$C_{116}H_{196}N_{38}O_{39}$	$[M+2H]^{2+}$1374.5397	$[M+2H]^{2+}$1374.2366
DCR26 cyclic	1-50%; 30mins	14.19; 98.18 %	$C_{124}H_{206}N_{40}O_{41}$	[M]2913.21	[M]2913.23
DCR39	10-30%; 30mins	13.16; 97.88 %	$C_{174}H_{293}N_{57}O_{58}S_6$	$[M+4H]^{4+}$1076.9949	$[M+4H]^{4+}$1076.5268
DCR39 A	1-50%; 30mins	14.14; 99.87 %	$C_{174}H_{293}N_{57}O_{58}$	$[M+3H]^{3+}$1371.5372	$[M+3H]^{3+}$1371.4051
DCR39 cyclic	1-50%; 30mins	13.34; 99.84 %	$C_{186}H_{308}N_{60}O_{61}$	$[M+3H]^{3+}$1454.6079	$[M+3H]^{3+}$1454.4399
DCR39 V	10-50%; 30mins	16.79; 97.22 %	$C_{183}H_{311}N_{57}O_{49}S_6$	$[M+3H]^{3+}$1429.7396	$[M+3H]^{3+}$1429.4148
DCR39 S	10-60%; 30mins	9.41; 99.73 %	$C_{165}H_{275}N_{57}O_{58}S_6$	$[M+3H]^{3+}$1393.5765	$[M+3H]^{3+}$1393.3168

Figure S1: Ice crystal morphology observed in 1M citrate with 0.01M of (a) DCR13, TH = 0 ºC (b) DCR26 A, TH = 0 ºC, (c) DCR39 A, TH = 0 ºC, (d) DCR39 (reduced and alkylated), TH = 0 ºC, (e) DCR39 V, TH = 0 ºC. N=5.

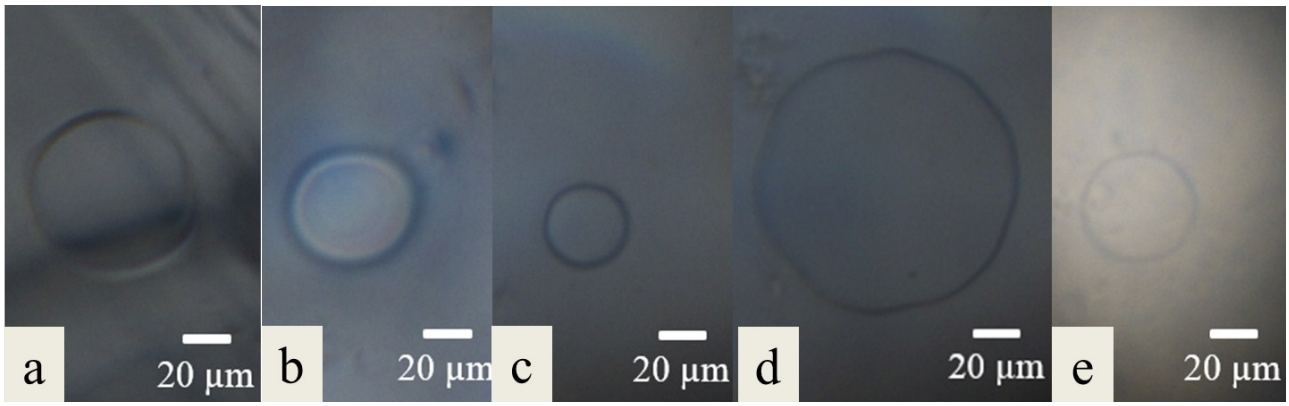
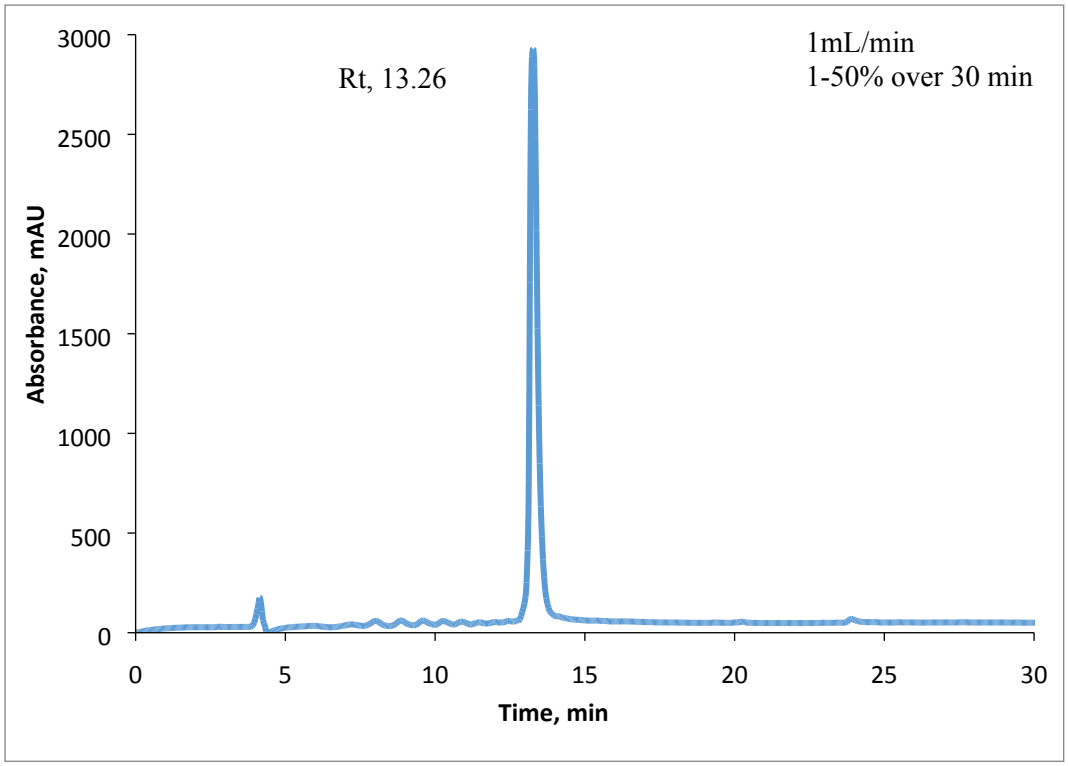
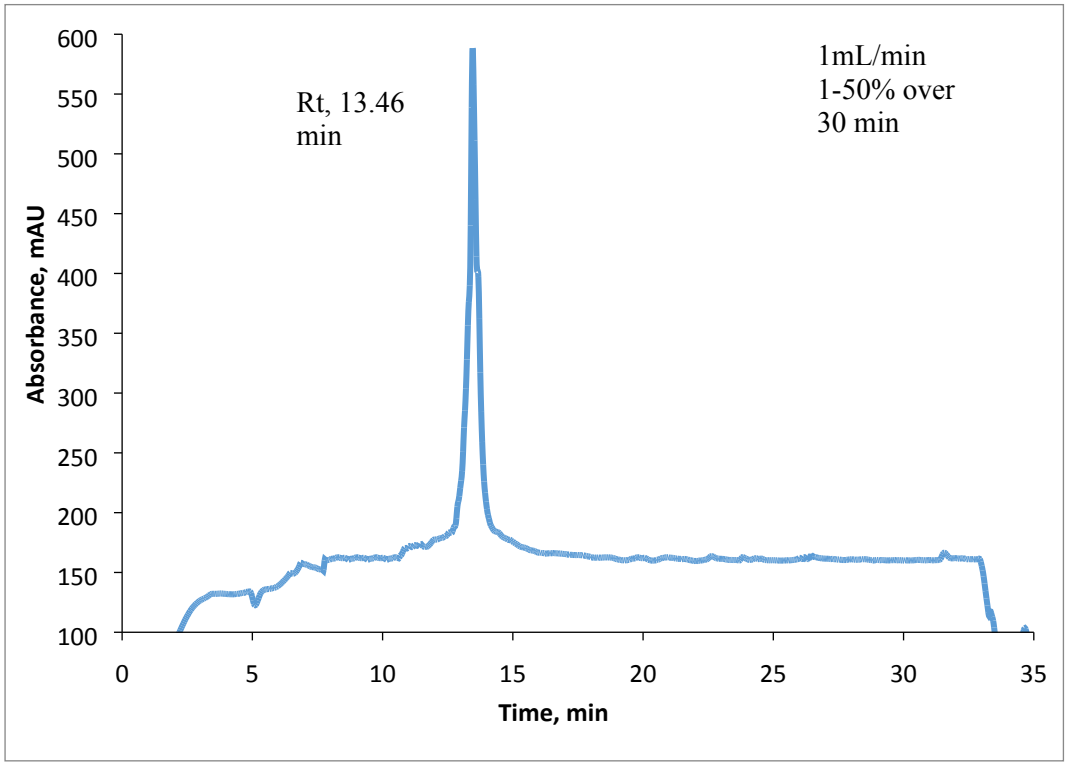


Figure S2: Analytical RP-HPLC traces (absorbance at 214 nm) and MS spectra of all peptides

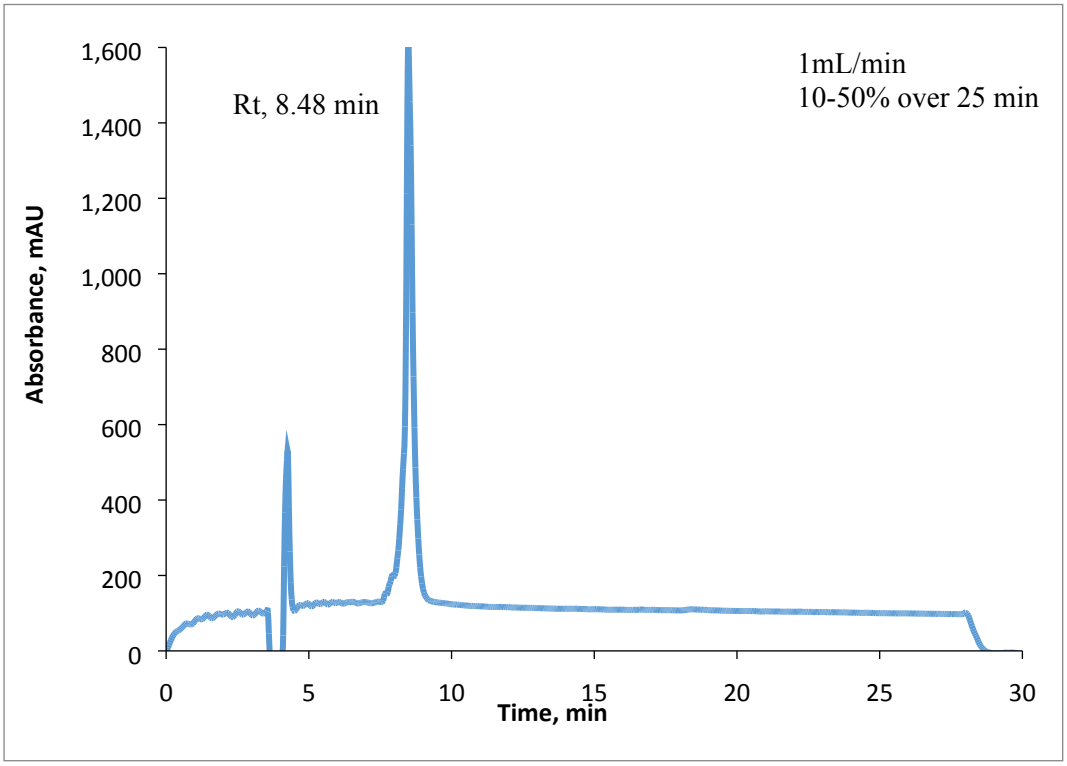
DCR13: CTRSTNCYKAKTA



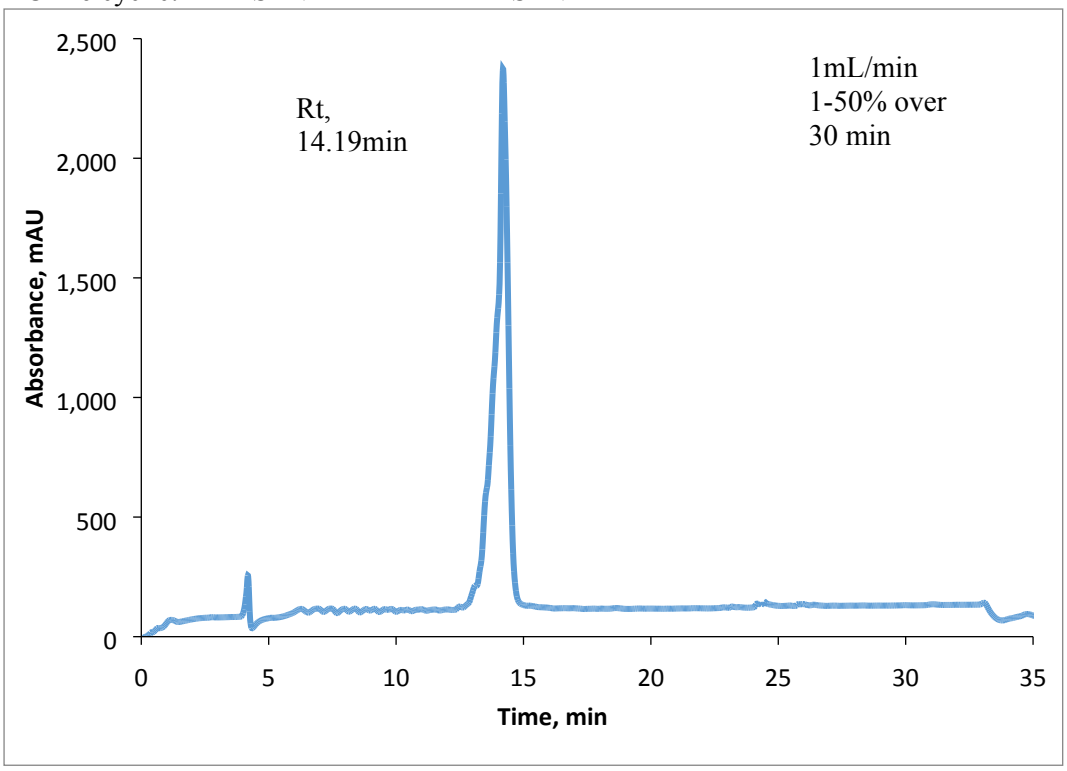
DCR26: CTRSTNCYKAKTACTRSTNCYKAKTA



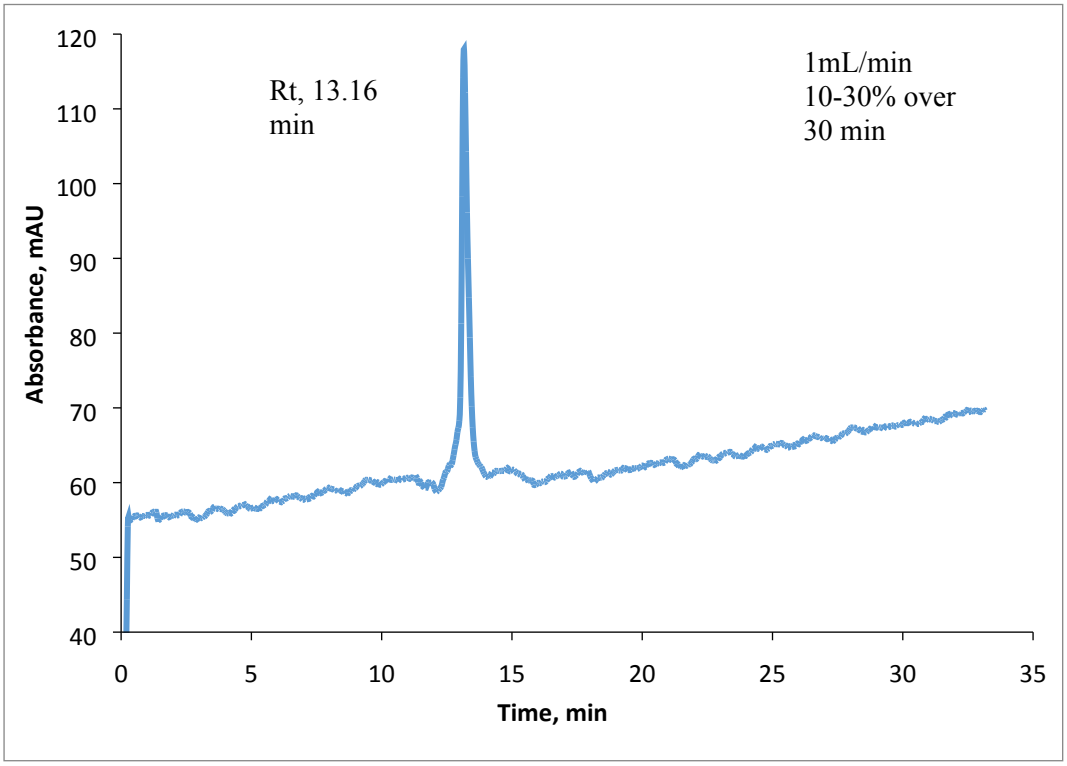
DCR26 A: ATRSTNAYKAKTAATRSTNAYKAKTA



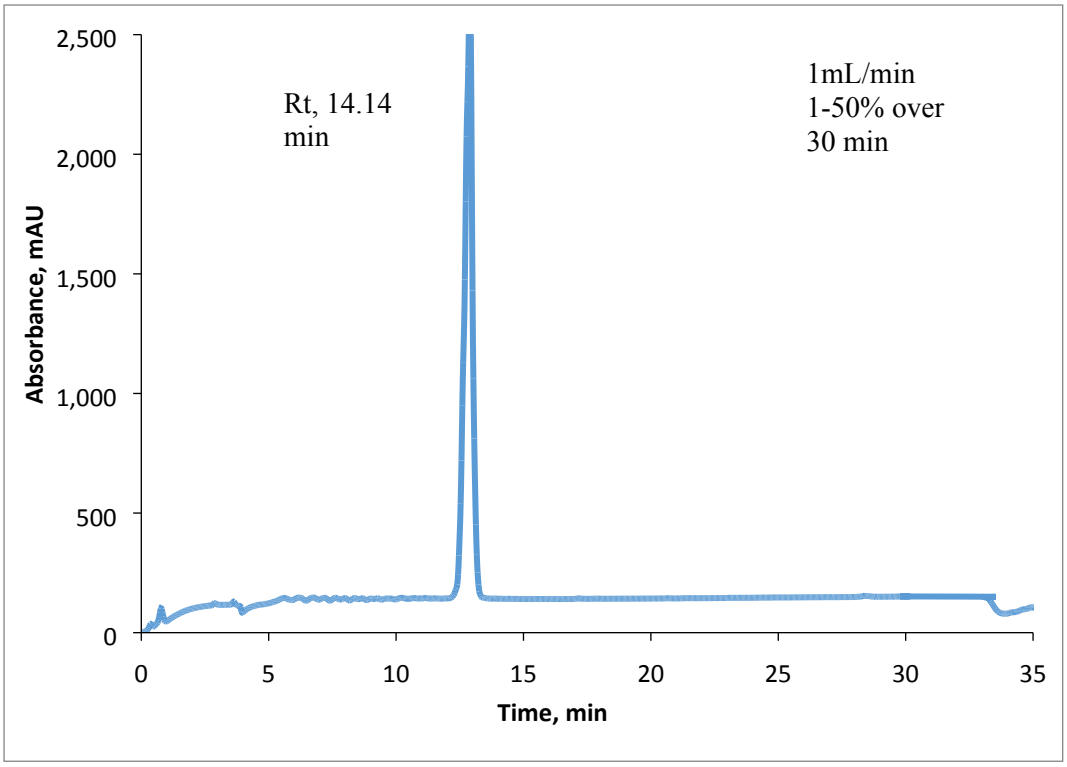
DCR26 cyclic: DTRSTNKYKAKTADTRSTNKYKAKTA



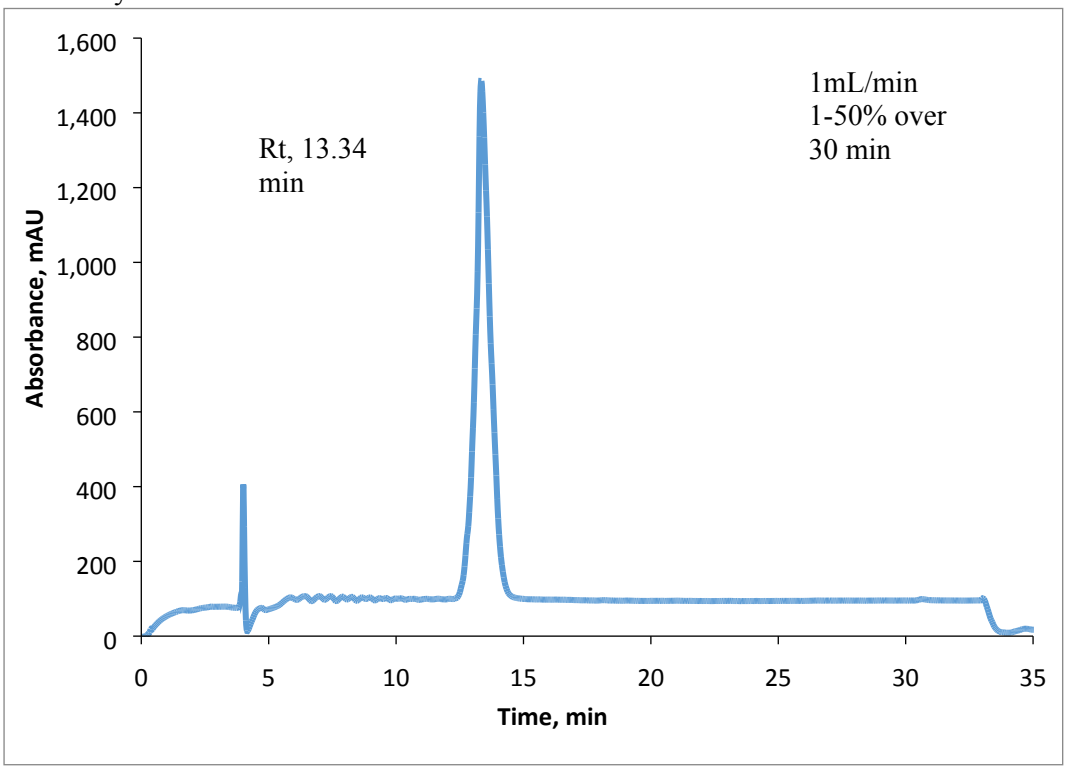
DCR39: CTRSTNCYKAKTACTRSTNCYKAKTACTRSTNCYKAKTA



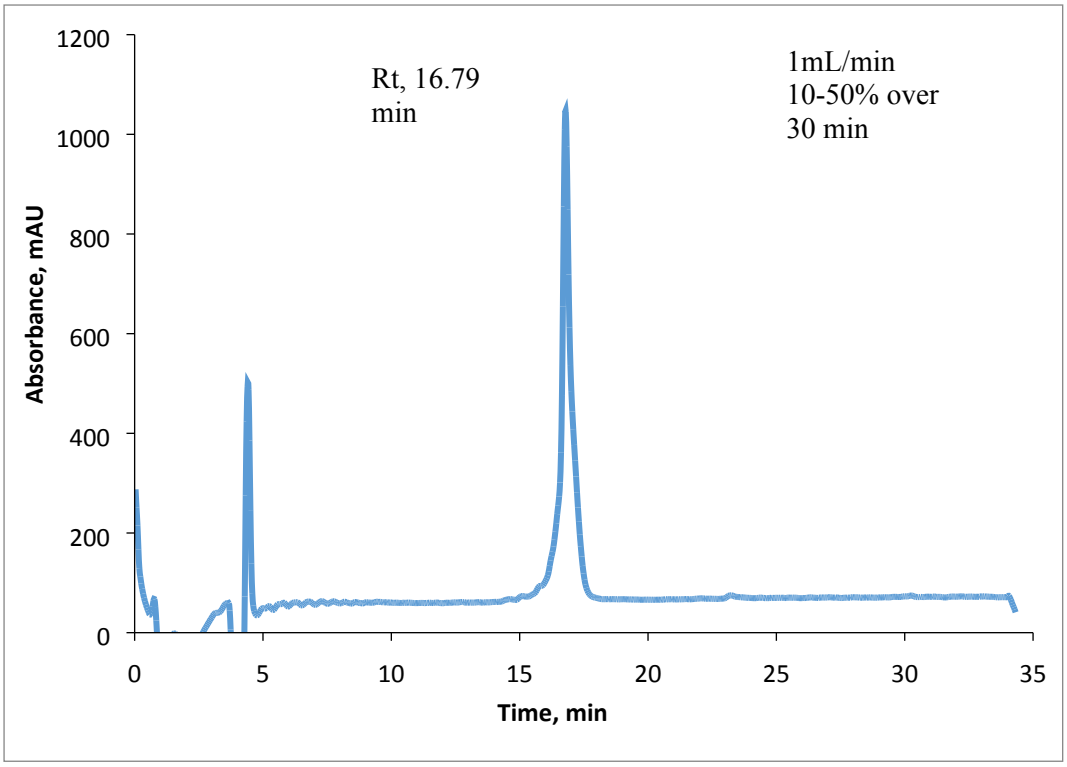
DCR39 A: ATRSTNAYKAKTAATRSTNAYKAKTAATRSTNAYKAKTA



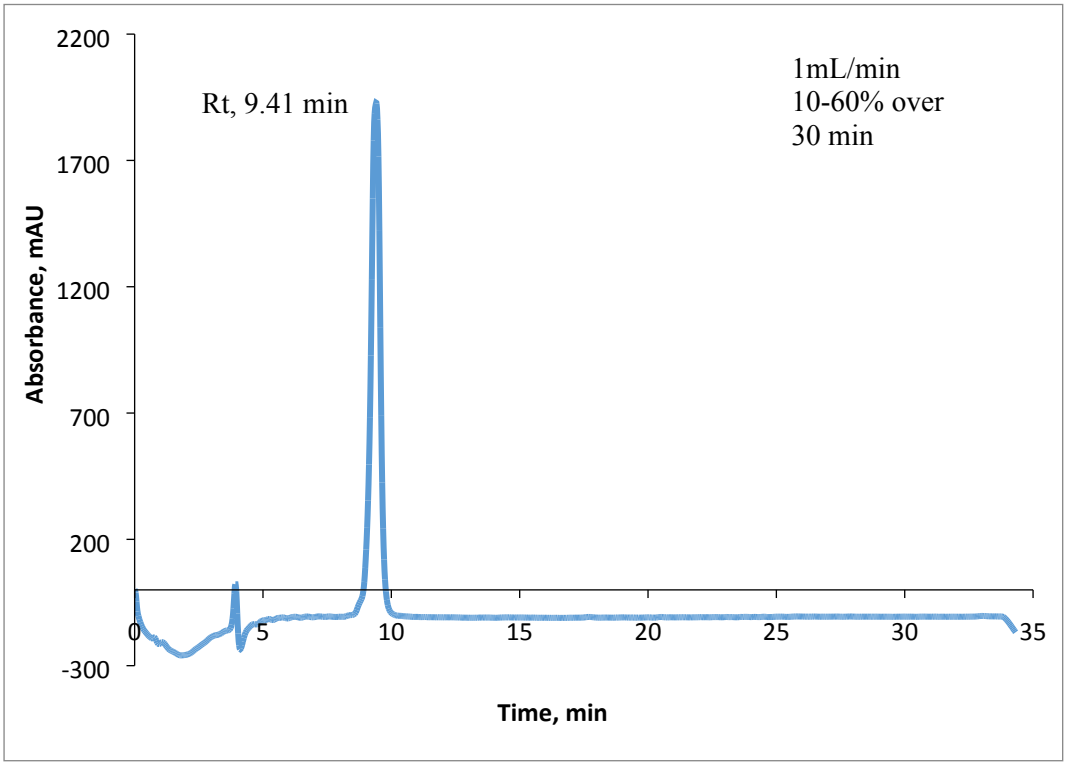
DCR39 cyclic: DTRSTNKYKAKTADTRSTNKYKAKTADTRSTNKYKAKTA



DCR39 V: CVRSVNCYKAKVACVRSVNCYKAKVACVRSVNCYKAKVA

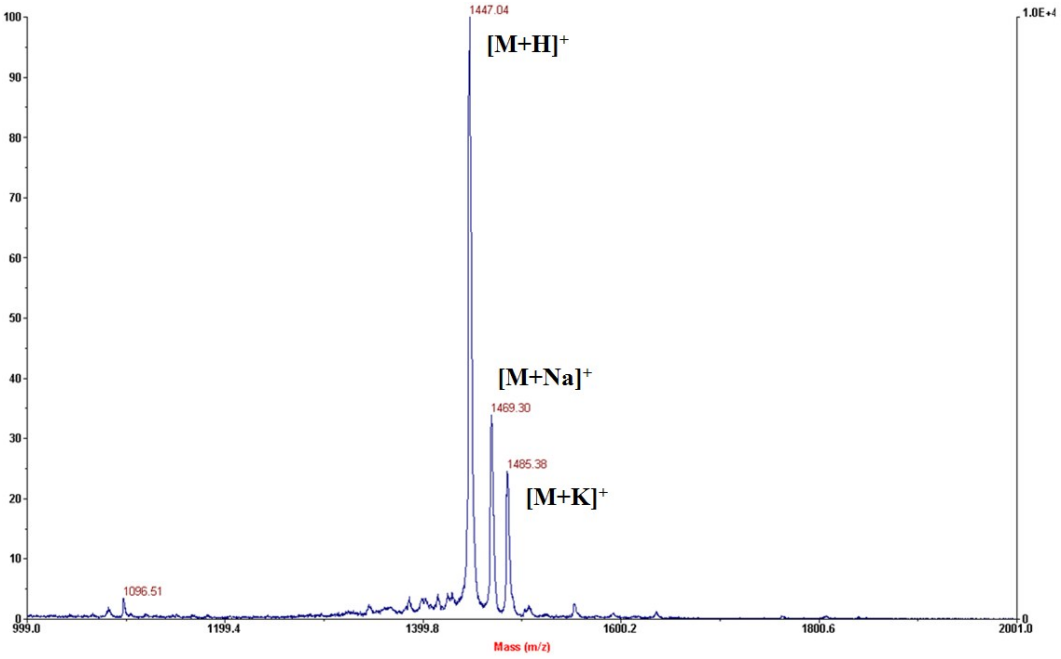


DCR39 S: CSRSSNCYKAKSACSRSSNCYKAKSACSRSSNCYKAKSA

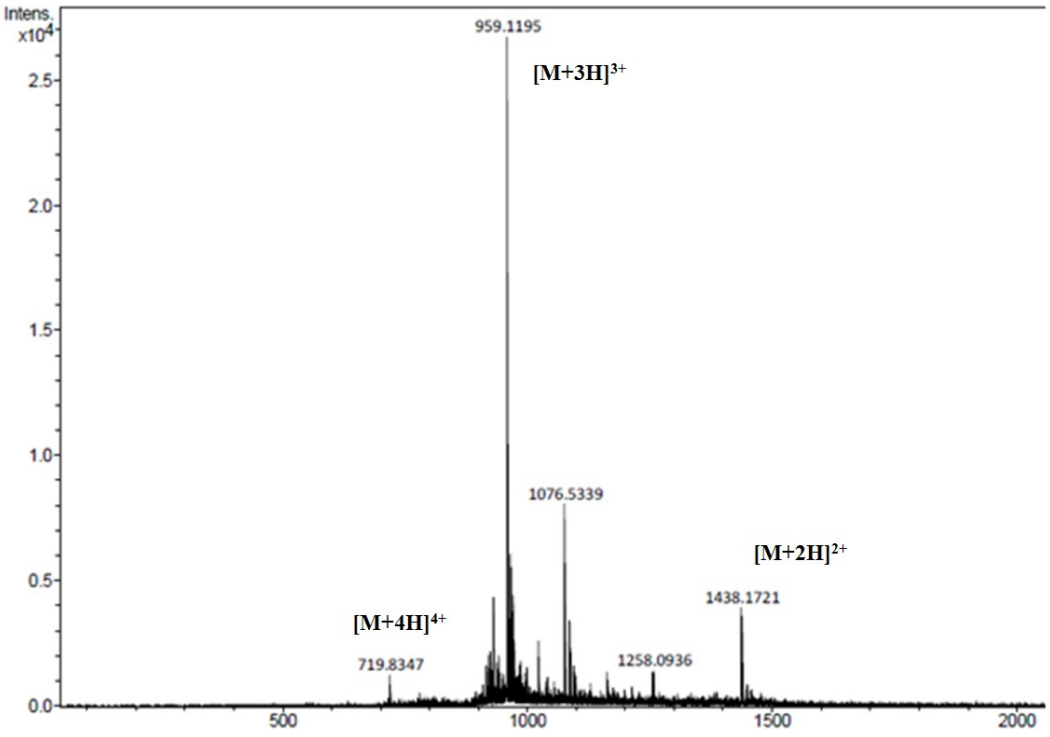


ESI-MS Spectra

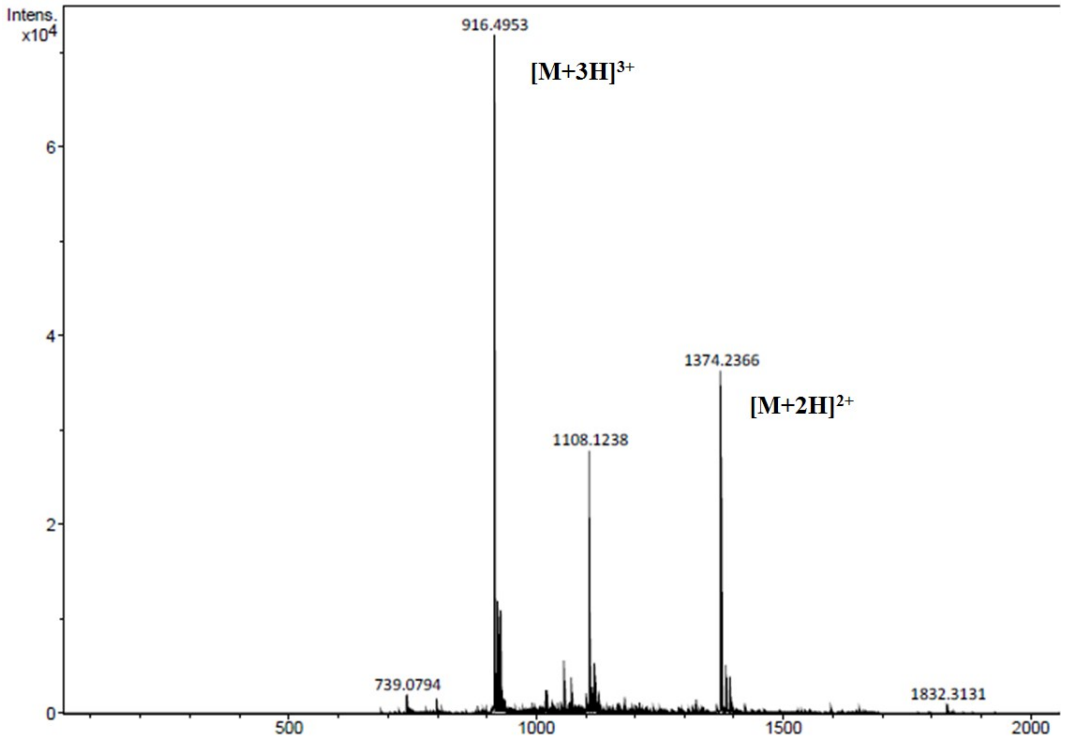
DCR13 MALDI-TOF MS $[M+H]^+$ = 1447.6674



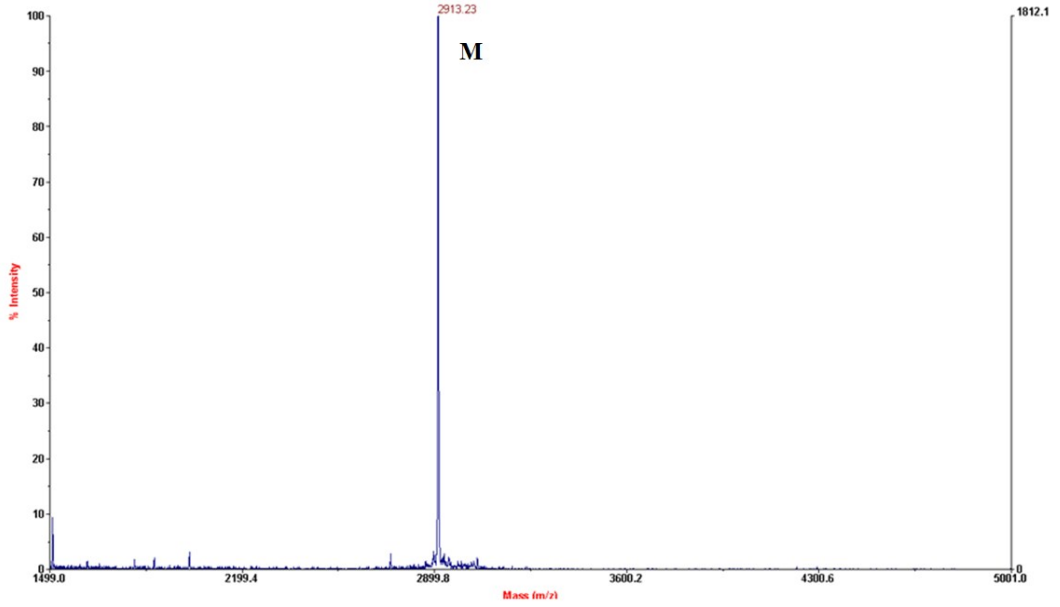
DCR26 ESI-MS $[M+2H]^{2+}$ = 1438.6597



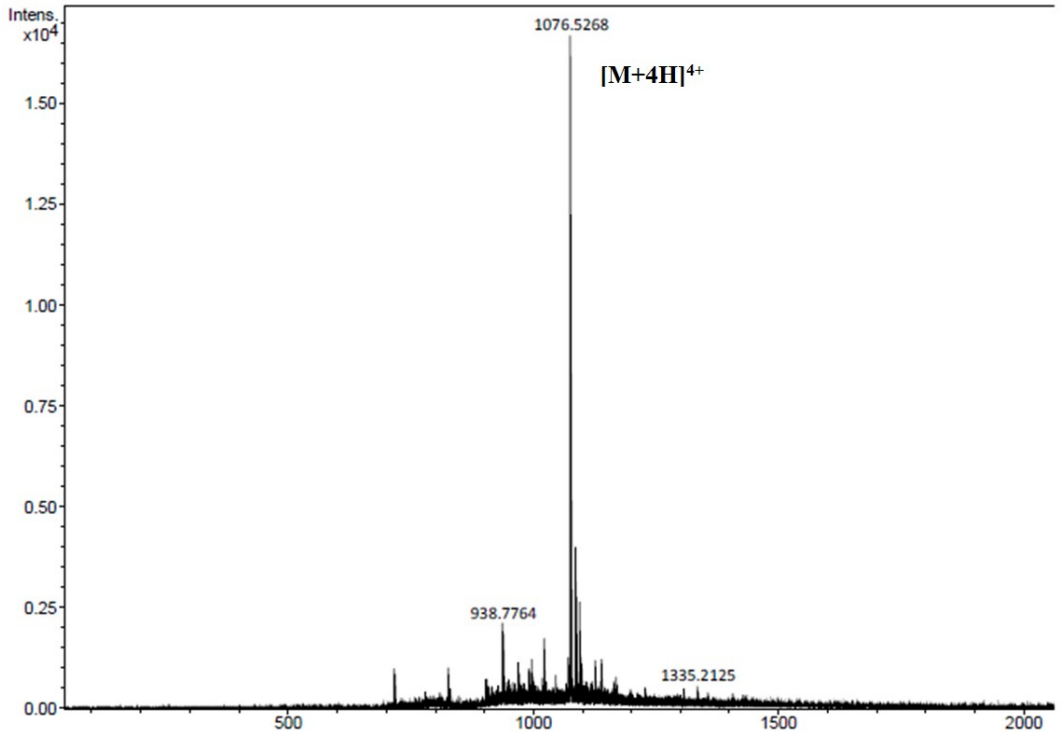
DCR26 A ESI-MS $[M+2H]^{2+}$ = 1374.5397



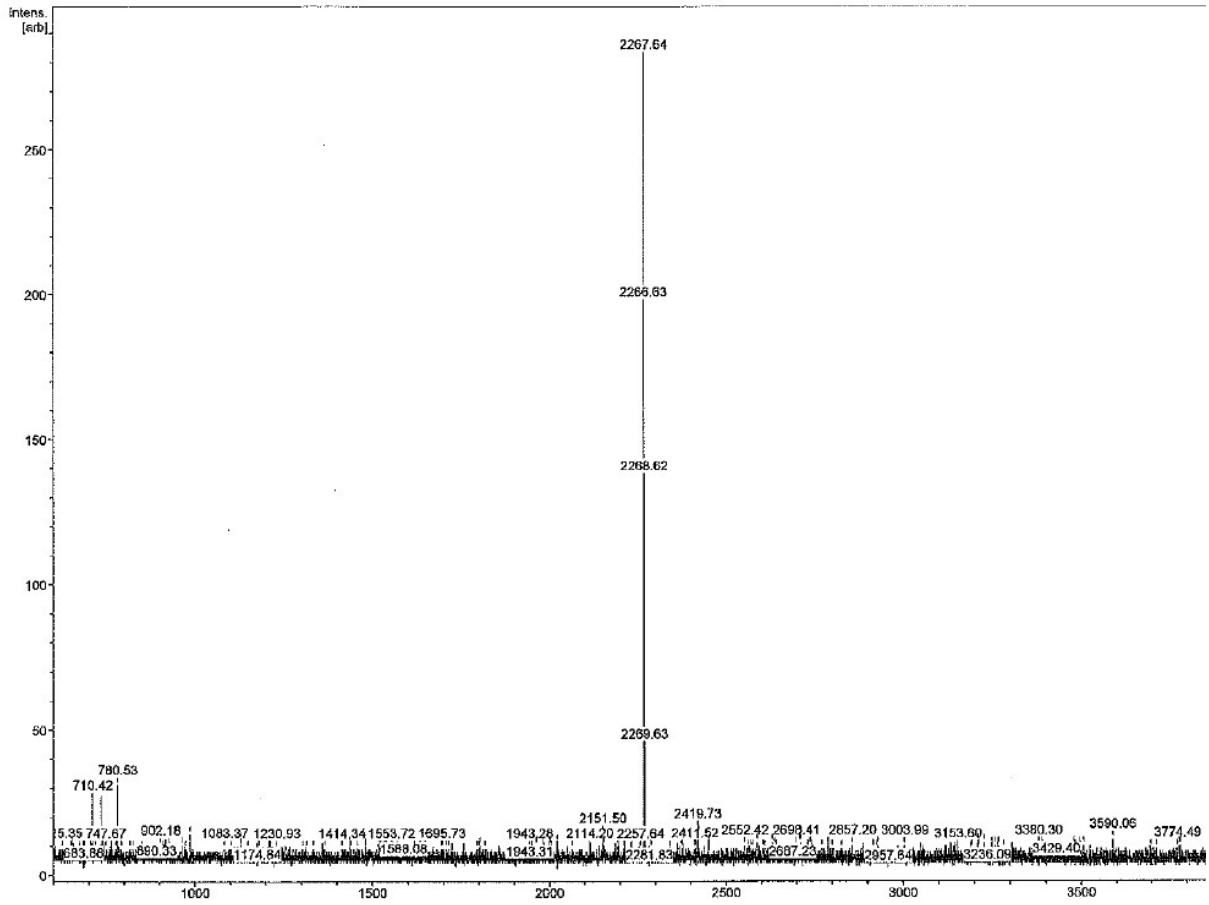
DCR26 cyclic MALDI-TOF MS (Exact mass = 2913.21)



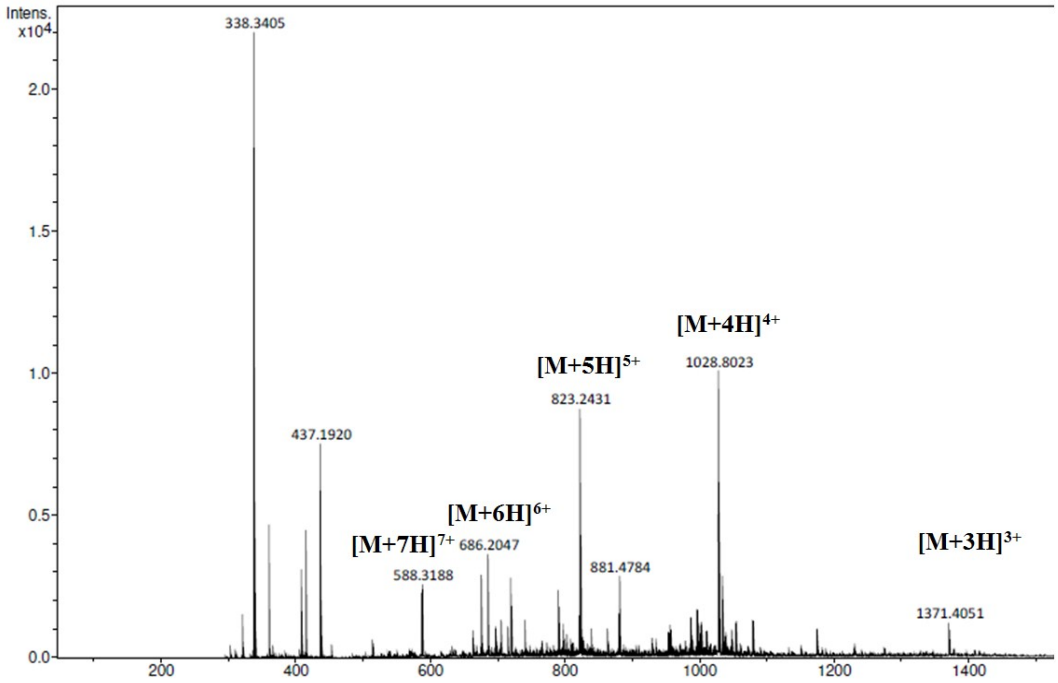
DCR39 ESI-MS $[M+4H]^{4+}$ = 1076.9949



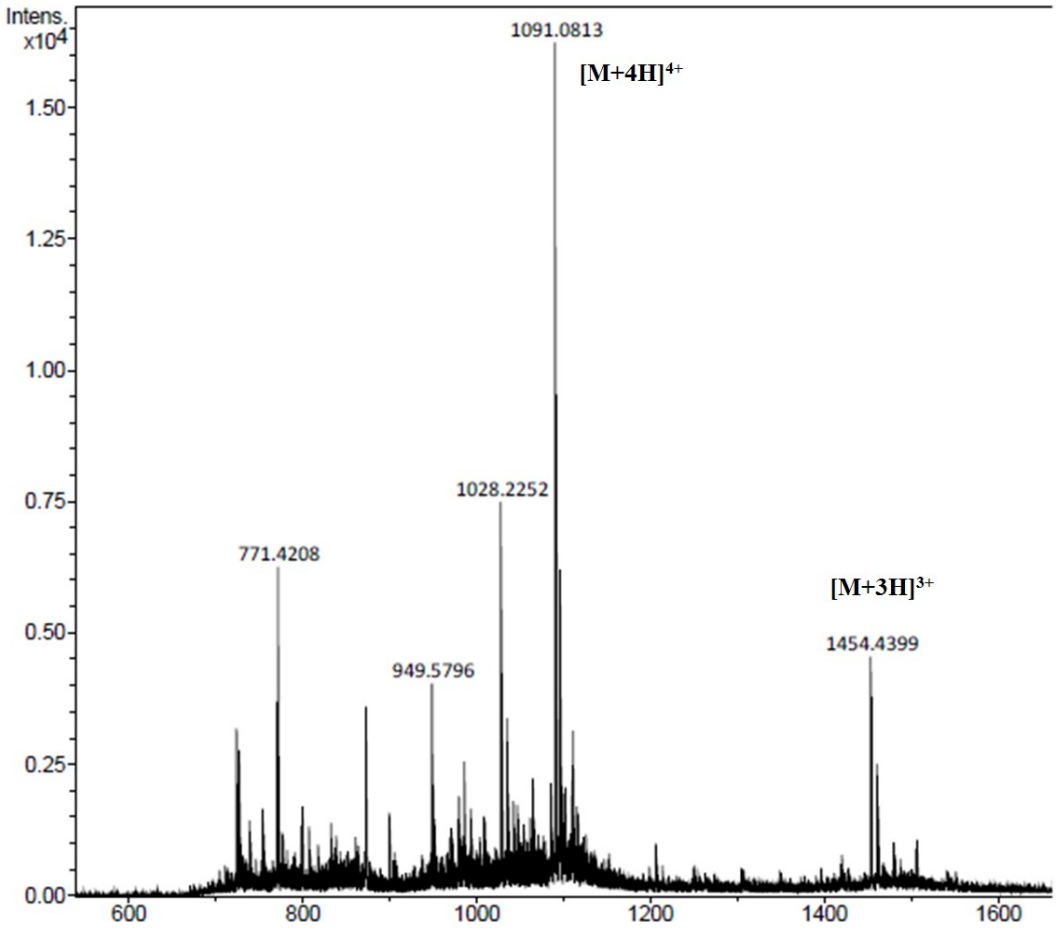
DCR39 (reduced and 4/6 cysteine alkylated) MALDI-TOF MS $[M+2H]^{2+}$ = 2267.0930



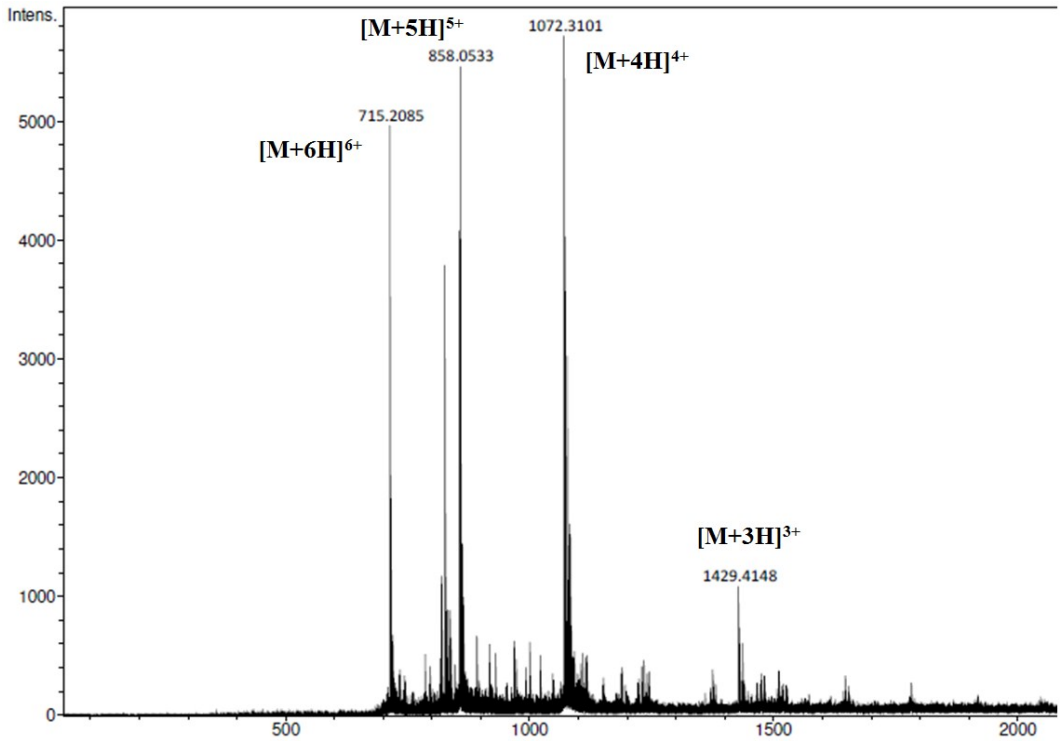
DCR39 A ESI-MS $[M+3H]^{3+}$ = 1371.5372



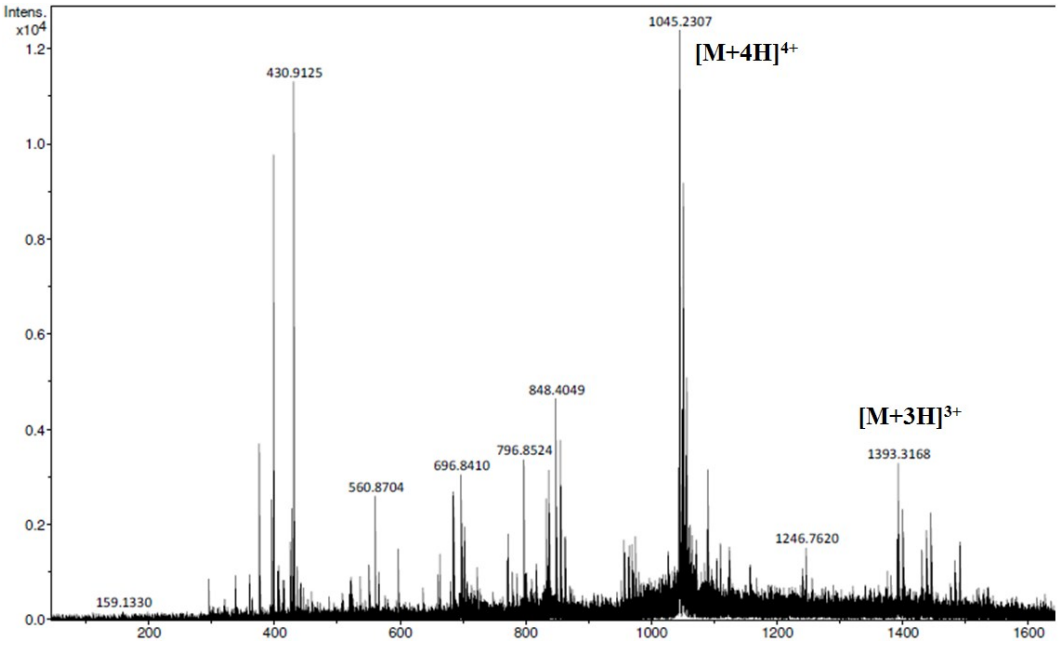
DCR39 cyclic ESI-MS $[M+3H]^{3+}$ = 1454.6079



DCR39 V ESI-MS $[M+3H]^{3+}$ = 1429.7396



DCR39 S ESI-MS $[M+3H]^{3+}$ = 1393.5765



DCR13: CTRSTNCYKAKTA

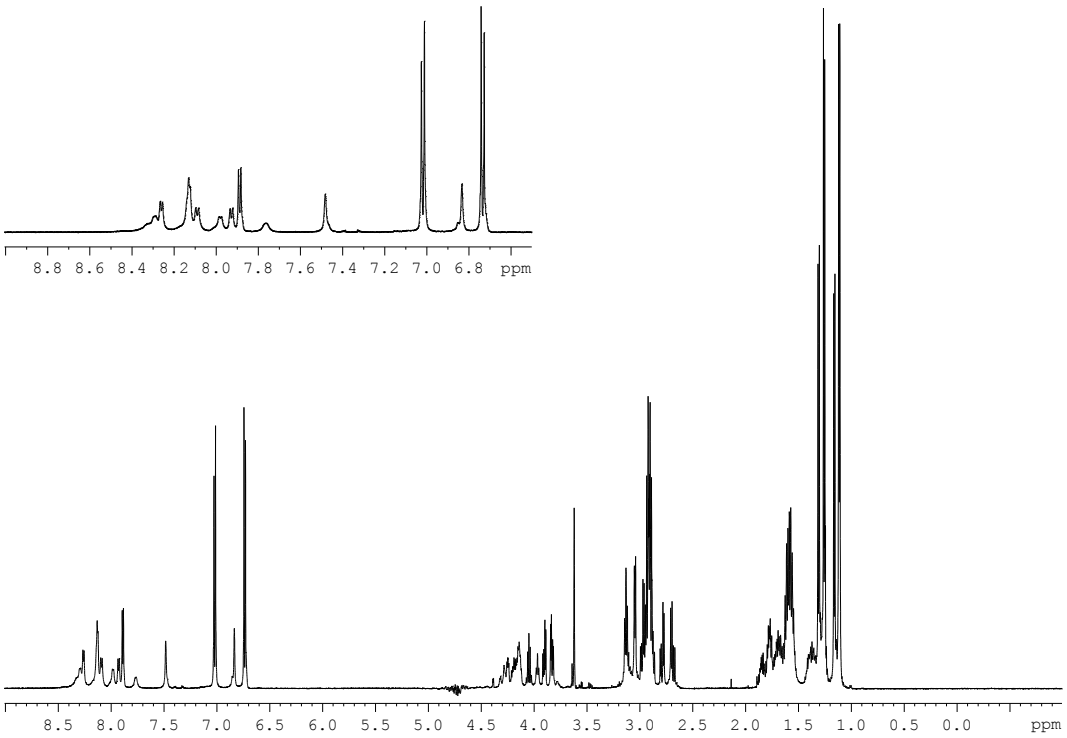


Figure S3: 500 MHz ^{1}H NMR spectrum of **DCR13** in 50 mM Tris-d_{11}, H_2O:D_2O (90:10) at 298K

DCR26: CTRSTNCYKAKTACTRSTNCYKAKTA

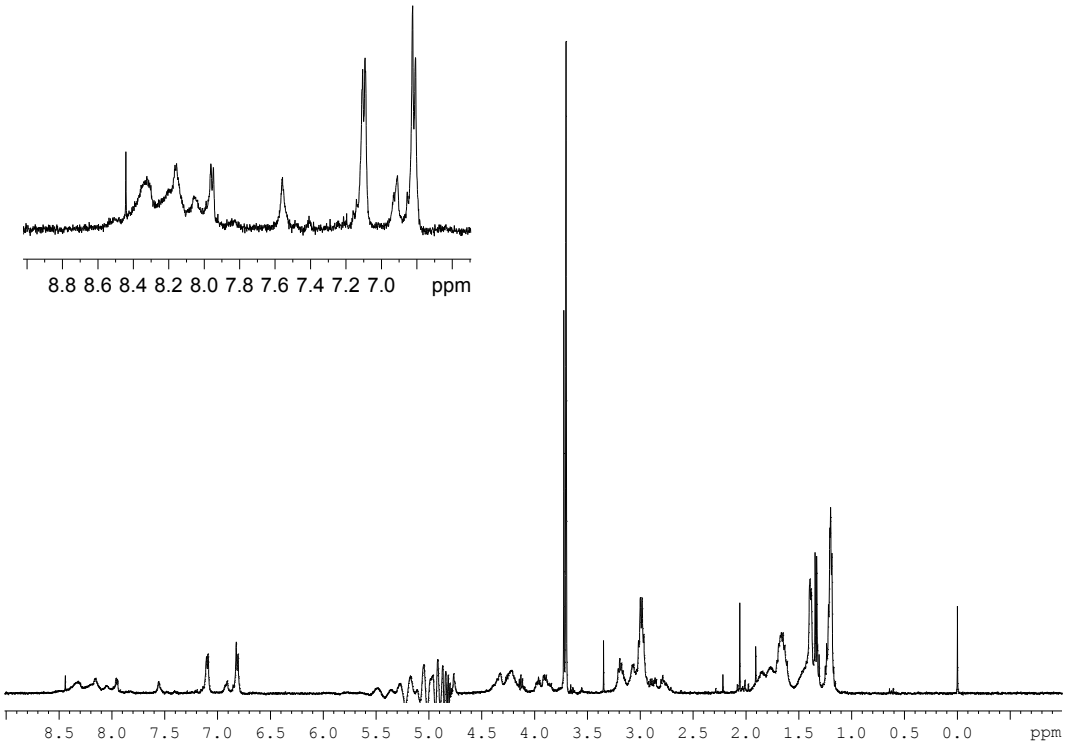


Figure S4: 500 MHz ^{1}H NMR spectrum of **DCR26** in 50mM Tris-d_{11}, H_2O:D_2O (90:10) at 300K

DCR26 A: ATRSTNAYKAKTAATRSTNAYKAKTA

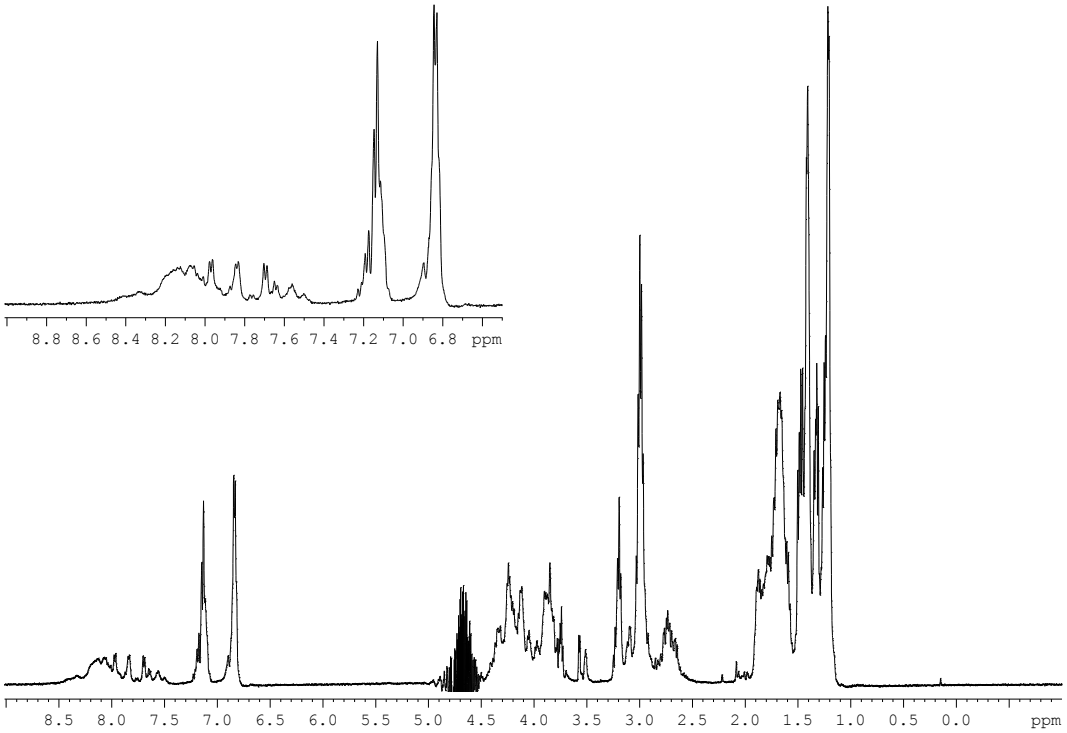


Figure S5: 500 MHz ^{1}H NMR spectrum of **DCR26 A** in H_2O:D_2O (90:10) at 300K

DCR26 cyclic: DTRSTNKYKAKTADTRSTNKYKAKTA

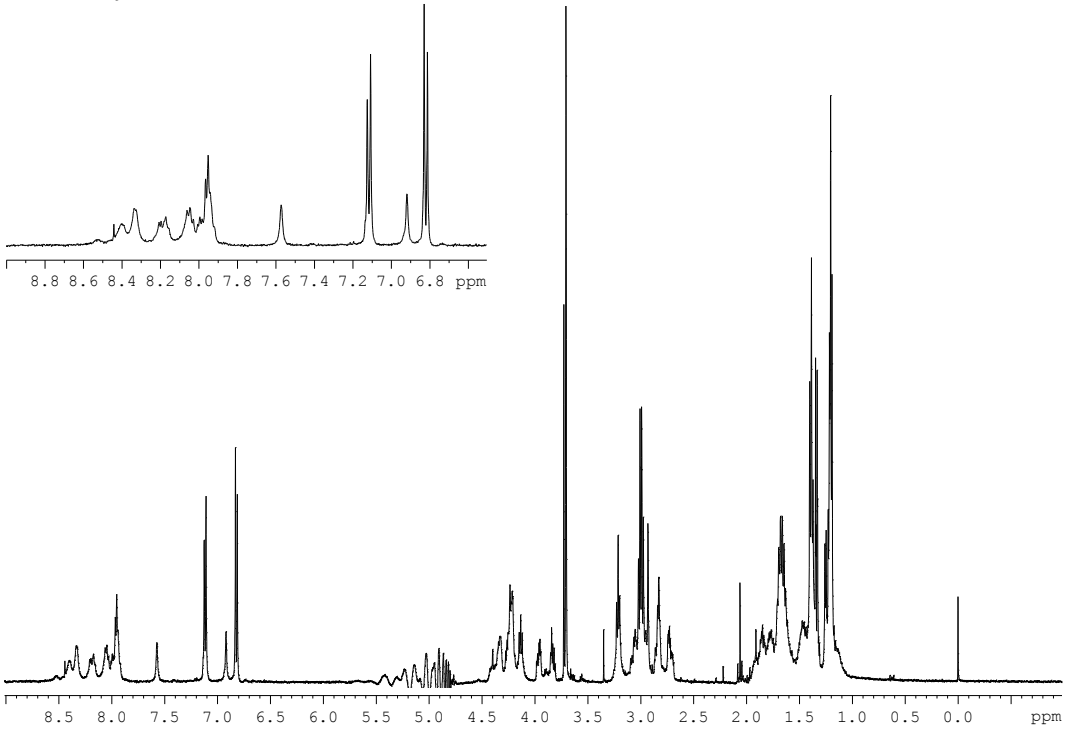


Figure S6: 500 MHz ^{1}H NMR spectrum of **DCR26 cyclic** in 50 mM Tris-d_{11}, H_2O:D_2O (90:10) at 300K

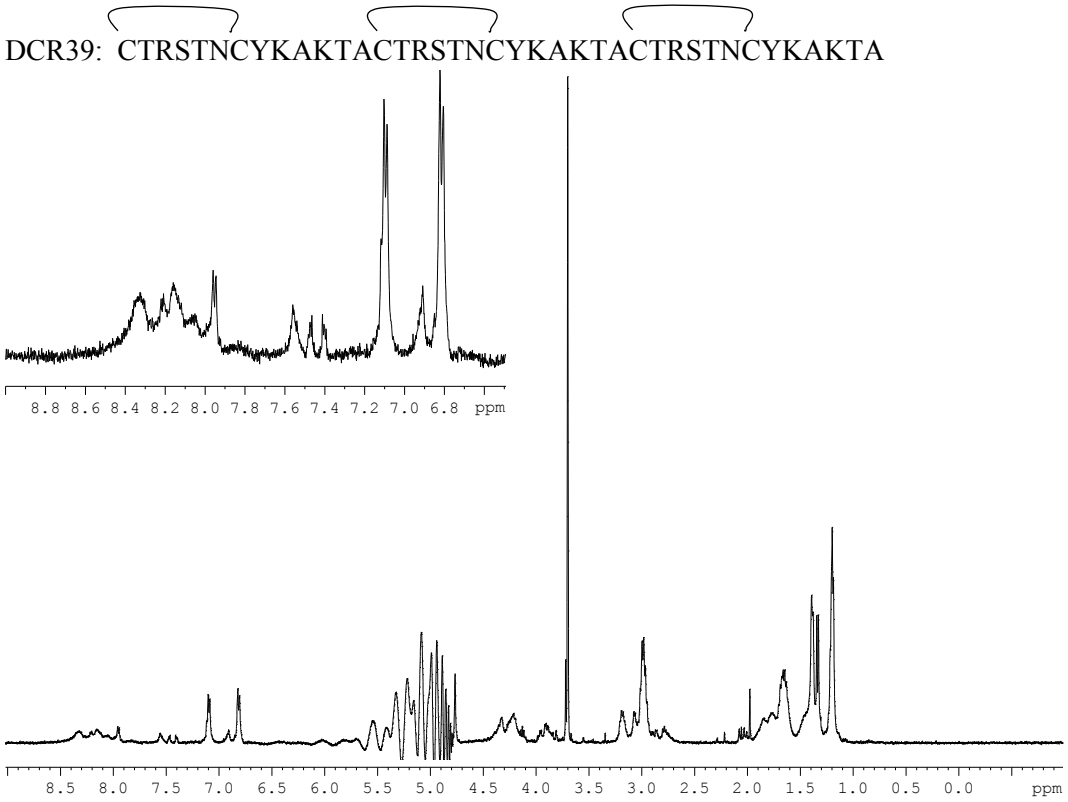


Figure S7: 500 MHz ^{1}H NMR spectrum of **DCR39** in 50 mM Tris-d_{11}, H_2O:D_2O (90:10) at 300K

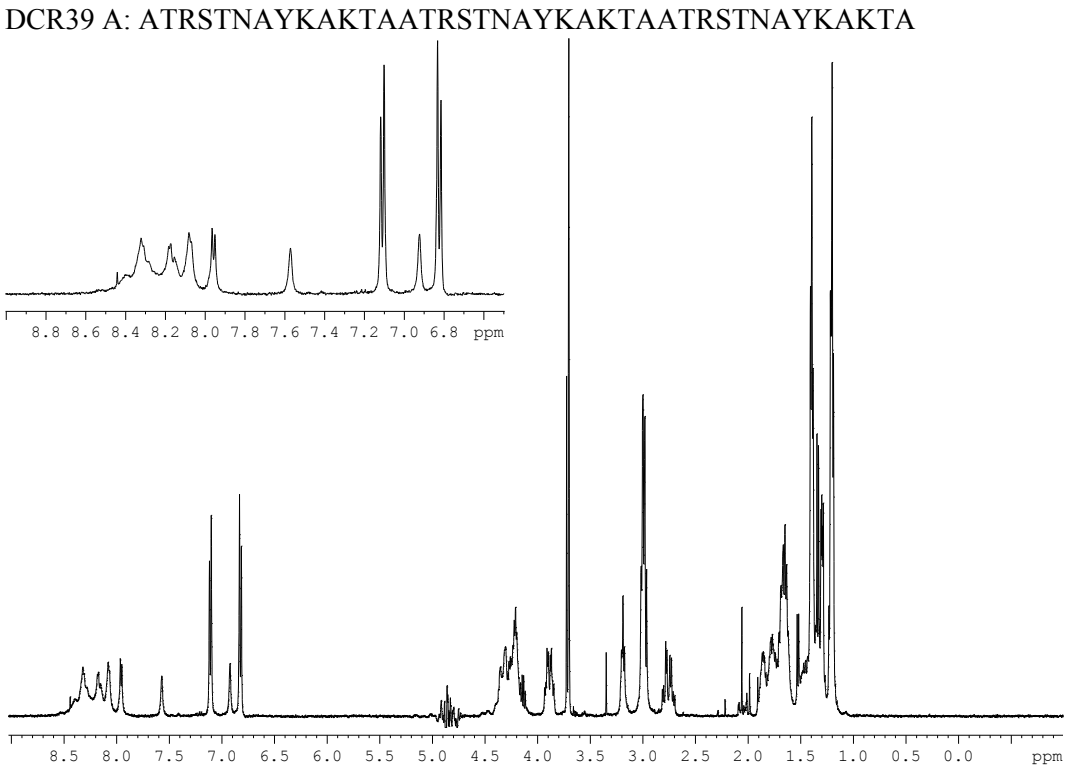


Figure S8: 500 MHz ^{1}H NMR spectrum of **DCR39 A** in 50 mM Tris-d_{11}, H_2O:D_2O (90:10) at 300K

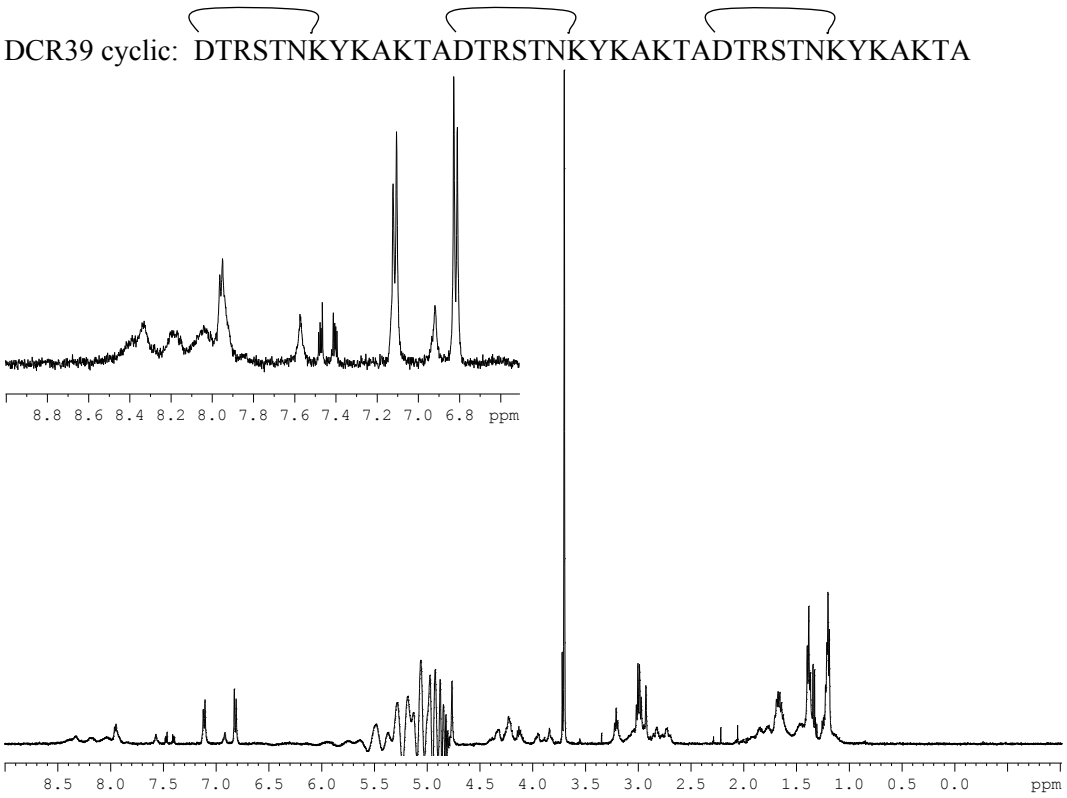


Figure S9: 500 MHz ^{1}H NMR spectrum of **DCR39 cyclic** in 50 mM Tris-d_{11}, H_2O:D_2O (90:10) at 300K

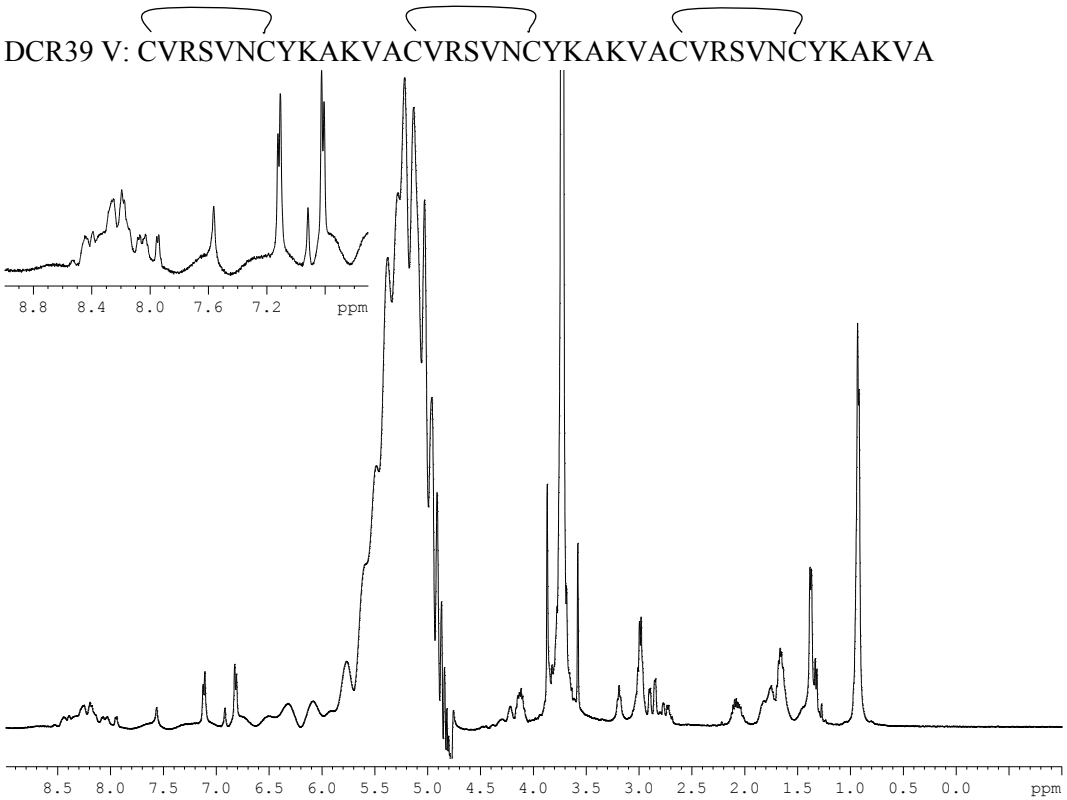


Figure S10: 500 MHz ^{1}H NMR spectrum of **DCR39 V** in 50 mM Tris-d_{11}, H_2O:D_2O (90:10) at 300K

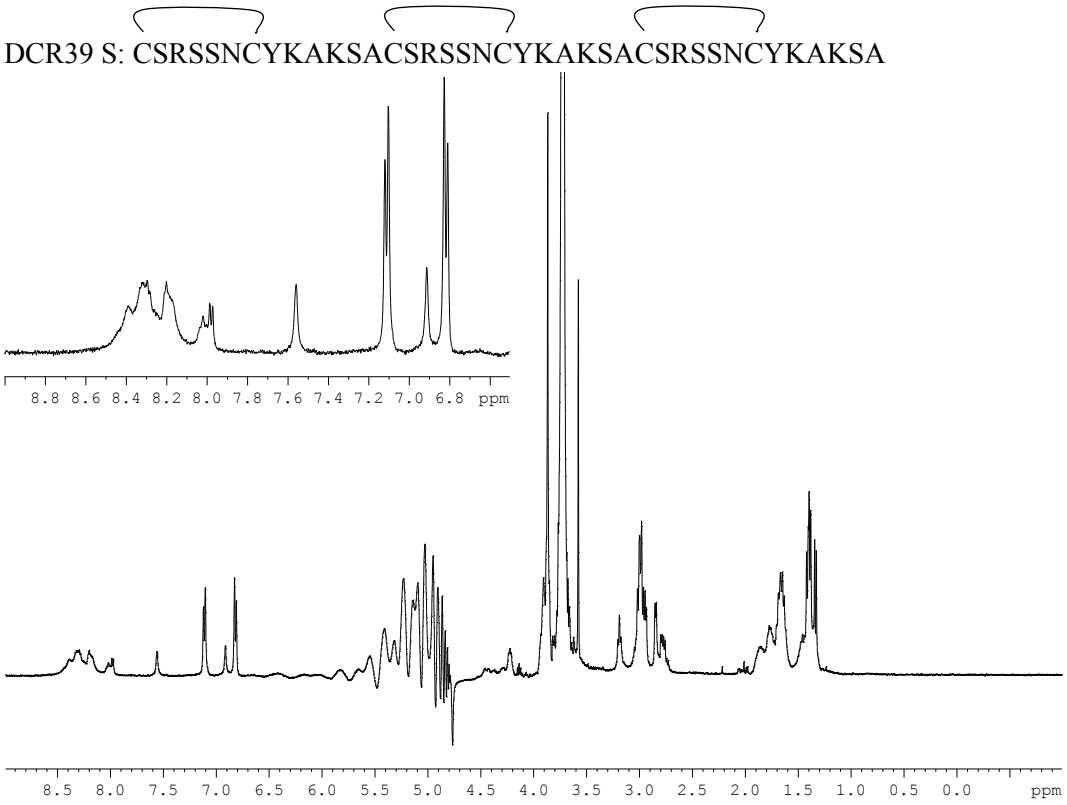


Figure S11: 500 MHz ^{1}H NMR spectrum of **DCR39 S** in 50 mM Tris-d_{11}, H_2O:D_2O (90:10) at 300K

Description of Movies

Water: below the equilibrium melting/freezing temperature, a slight decrease in temperature induced continuous crystals growth in all directions in a circular disk form (without faceting)

Citrate: below the equilibrium melting/freezing temperature, a slight decrease in temperature induced continuous crystals growth in all directions in a circular disk form (without faceting)

DCR39 Ala: below the equilibrium melting/freezing temperature, a slight decrease in temperature induced continuous crystals growth in all directions in a circular disk form (without faceting)

DCR39 Ala with citrate: below the equilibrium melting/freezing temperature, a slight decrease in temperature induced continuous crystals growth in all directions in a circular disk form (without faceting)

DCR39 Cyclic: below the equilibrium melting/freezing temperature, the crystal grows into a rice grain-shaped ice crystal and then stops growing until the freezing hysteresis point, after which the crystal grows rapidly.

DCR39 Cyclic with citrate: below the equilibrium melting/freezing temperature, the crystal grows into a small hexagonal shaped ice crystal, and then stops growing until the freezing hysteresis point, after which the crystal burst and grows rapidly.