FlkO, a penicillin-binding protein-type thioesterase in cyclofaulknamycin biosynthesis

Kenichi Matsuda,* Rintaro Ichihara, and Toshiyuki Wakimoto*

Faculty of Pharmaceutical Sciences, Hokkaido University, Kita 12, Nishi 6, Kita-ku, Sapporo 060-0812, Japan

E-mail: kematsuda@pharm.hokudai.ac.jp

E-mail: wakimoto@pharm.hokudai.ac.jp

Table of Contents

Experimental Procedures

General remarks

^{1}H and ^{13}C NMR spectra were recorded on JEOL ECS 400 spectrometer (400 MHz for ^{1}H NMR). Chemical shifts were denoted in δ (ppm) relative to residual solvent peaks as internal standard (DMSO-d_6, ^{1}H δ 2.50, ^{13}C δ 39.5). Electrospray ionization mass spectrometry (ESI-MS) were performed by a Thermo Scientific Exactive mass spectrometer, an amaZon SL-NPC (Bruker Daltonics), and a Shimadzu LC-MS-2020 spectrometer. Optical rotations were recorded on a JASCO P-1030 polarimeter. MS/MS analysis were performed with amaZon SL-NPC (Bruker Daltonics) using helium gas with amplitude value 1.0 V. High-performance liquid chromatography (HPLC) experiments were performed with a Shimadzu HPLC system (LC-2050). All reagents were used as supplied unless otherwise stated. *Escherichia coli* DH5a was used as a host for general cloning.

Cultivation and metabolite analysis of *Streptomyces albidoflavus* NBRC12854

A single colony of *S. albidoflavus* NBRC12854 was inoculated in TSB medium and cultured at 30 °C for 2–4 days as seed culture. 1.0 ml of broth was transferred to 100 ml of K media (2.5% soluble starch, 1.5% soya flour, 0.2% dry yeast, 0.4% precipitated calcium carbonate, pH of the media was adjusted to 6.2) and cultivated at 30 °C for 4 days. Culture broth was extracted by 200 ml of acetone and debris were filtered off. After removing solvent, residues were dissolved into DMSO and analyzed by LC-MS. amaZon SL-NPC (positive mode) equipped with reversed phase column COSMOSIL $5C_{18}$-MS-II 2.0 mm I.D. × 150 mm. Column was eluted by H_2O/MeCN containing 0.05% TFA, with gradient mode from 2-90% for MeCN in 20 min. Flow rate was set at 0.2 mL/min.

Chemical synthesis

General SPPS procedure for peptide with ethylene glycol at *C*-terminus

Enzyme substrates used in this study were synthesized by the modified SPPS described previously[1] (step 1–8). Substrates used in this study were synthesized from 10 μmol of **S3** (Fmoc-D-aa-**S2**). Thus, the volumes of regents were described for the synthesis at a 10 μmol scale.

Step 1: 2-chlorotrityl resin (0.38 mmol) **S1** in Libra tube was swelled with DCM (dichloromethane) for 10 min, and then excess solvent was removed by filtration. To the resin was added a solution of ethylene glycol (EG) (1.50 mmol), and *i*-Pr_2NEt (250 μL, 1.50 mmol) in DCM (2.0 mL), and shaken for 2 h at 37 °C to give diol-2-chlorotrityl resins **S2**. To the resin was added a solution of Fmoc-amino acid (1.50 mmol), DIC (3.78 mmol) and DMAP (cat.) in DCM (2.0 mL) and stirred for 3 h at 37 °C to give Fmoc-aa-diol-2-chlorotrityl resins **S3**. Dried **S3** (5.0 mg) was added with 20% piperidine in DMF, and stirred for 1 h. The supernatant was diluted with DMF, and was subjected to UV measurement at 301 nm. Typically, loading rates for **S3** are ranging from 0.24-0.45 mmol g^{-1}.

Step 2: Fmoc group of the solid supported peptide was removed by using 360 μL of 20% piperidine/DMF solution. Reaction vessel was left for 10 min at room temperature. This step was repeated twice.

Step 3: The resin in the reaction vessel was washed with DMF (×3) and DCM (×3).

Step 4: To the solution of Fmoc amino acid (4 eq in 189 μL DMF) were added 30 μL of *N*,*N*'-diisopropylcarbodiimide (DIC, 0.50 M in DMF) 112.5 μL Oxyma Pure (0.50 M in DMF) and 28.5 μL NMP (*N*-Methyl-2-pyrrolidone). After 3 min of pre-activation, the mixture was injected to the reaction vessel. The resulting mixture was left for 15 min at room temperature. After washing the resin with 200 μL DCM, the coupling procedure was repeated, then solution was filtered off.

Step 5: 360 μL of 5% Ac_2O in DMF was added and the mixture was left for 5 min at room temperature.

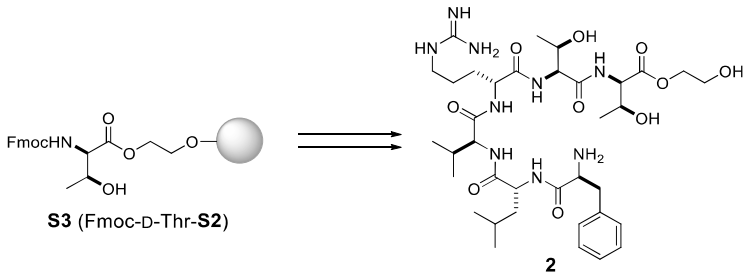
Step 6: The resin in the reaction vessel was washed with DMF (×3) and DCM (×3).

Fmoc amino acids were condensed onto the solid support by repeating Step 2–6.

Step 7: N-terminal Fmoc group was removed on solid support by the procedure in Step 2, then the peptide was cleaved from the solid support by adding deprotection cocktail TFA/H_2O/*i*-Pr_3SiH/DODT (3,6-dioxa-1,8-octanedithiol) = 92.5:2.5:2.5:2.5 (500 μL). The solid support was exposed to the deprotection cocktail for 3 hours and the solid support was filtered off.

Step 8: The filtrate was diluted with Et_2O (25 mL) and was chilled (–80 °C), then centrifuged with 3,500 × *g* for 10 min at 4 °C to afford crude peptide. The crude peptide was further purified by HPLC when necessary. The column (COSMOSIL 5C_{18}-MS-II 20 mm I.D. × 250 mm) was eluted by MeCN:H_2O (= 40:60) containing 0.05% TFA with flow rate at 10 ml/min to afford a pure peptide.

Synthesis of 2

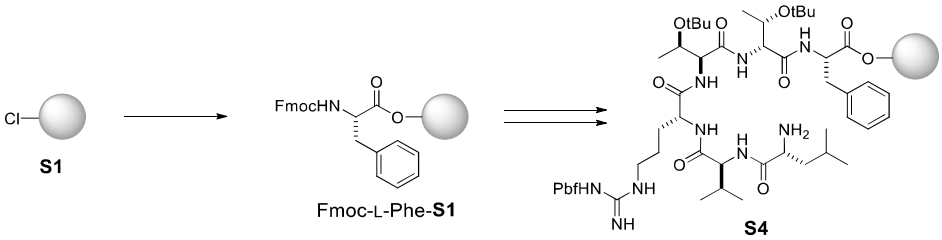


Fmoc-D-Thr-**S2** was synthesized by following the Step 1. Compound **2** was synthesized from 10 μmol of Fmoc-D-Thr-**S2** by following the step 1–8 described above. As a result, 15.8 mg of pure **2** (68.9% for 15 steps) was obtained as a while solid.

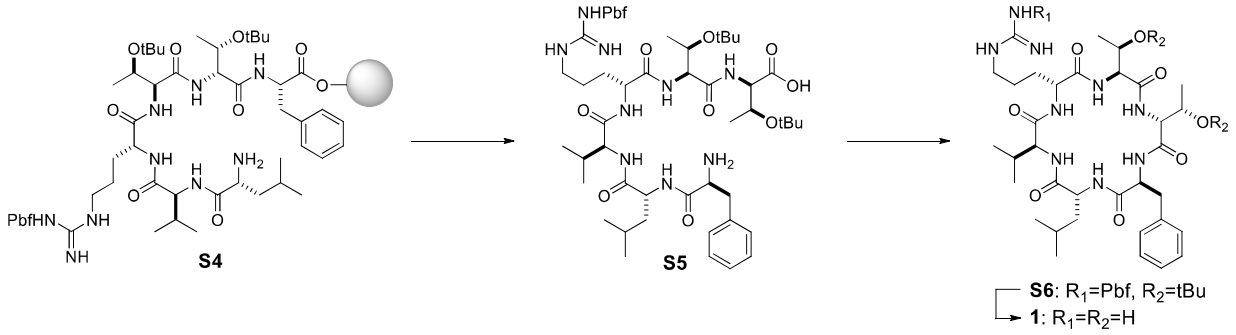
2: $[\alpha]_D^{18}$ +20. 66 (*c* 0.92, DMSO); ^{1}H-NMR (400 MHz, DMSO-d_6) δ 8.68 (s, 2H), 8.32 (d, *J* = 9.0 Hz, 1H), 8.00 (d, *J* = 9.0 Hz, 1H), 7.94 (d, *J* = 8.1 Hz, 1H), 7.20-7.29 (m, 10H), 5.13 (s, 2H), 4.88 (s, 1H), 4.23-4.43 (m, 4H), 4.11 (s, 2H),

4.01 (m, 2H), 3.93 (m, 1H), 3.53 (t, J = 5.2 Hz, 3H), 3.30 (s, 2H), 2.97-3.07 (m, 3H), 2.89 (m, 2H), 1.99 (m, 1H), 1.61 (m, 2H), 1.45 (m, 2H), 1.28 (m, 2H), 1.04 (d, J = 6.3 Hz, 3H), 0.99 (d, J = 6.3 Hz, 3H), 0.79 (d, J = 4.4 Hz, 3H), 0.78 (d, J = 4.4 Hz, 3H), 0.70 (d, J = 6.0 Hz, 3H), 0.66 (d, J = 6.0 Hz, 3H), ^{13}C-NMR (100 MHz, DMSO-d_6) δ 171.8, 171.6, 171.4, 170.6, 170.3, 168.0, 156.83, 135.2, 129.4 (2×CH), 128.5 (2×CH), 127.0, 66.8, 66.4, 66.2 (2×CH), 58.9 (3×CH), 58.2, 53.7, 51.2, 44.03, 40.4 (2×CH), 23.9, 23.1 (2×CH_3), 21.2, 20.0, 19.5 (2×CH_3), 19.4 (2×CH_3), 18.8; HRMS (ESI) calcd for $C_{36}H_{62}O_{10}N_9^+$ $[M+H]^+$ 780.46142, found 780.45879

Synthesis of 1



2-Chlorotrityl resin (**S1**) (15 mg, 0.019 mmol) in Libra tube was swollen with DCM for 30 min, and then excess solvent was removed by filtration. To the resin was added a solution of Fmoc-L-Phe-OH (29 mg, 0.076 mmol) and *i*-Pr_2NEt (12.6 μL, 0.076 mmol) in DCM (0.5 mL), and stirred for 1 h at 40°C. The reaction mixture was filtered, washed with DMF (× 3), DCM (× 3), methanol, and Ac_2O/DCM = 1:4, then dried under vacuum for 1 h to give Fmoc-L-Phe-**S1**. Fmoc-L-Phe-**S1** was swelled in DCM for 1 h, which was subjected to 5 cycles [Fmoc-D-Thr(tBu)-OH, Fmoc-L-Thr(tBu)-OH, Fmoc-D-Arg(Pbf)-OH, Fmoc-L-Val-OH, Fmoc-D-Leu-OH] of SPPS protocol described above (Step 2-6) to afford resin bound octapeptide **S4**.



To the peptide **S4** was added DCM/$(CF_3)_2$CHOH (= 80: 20) (2.0 mL), being stirred for 20 min, and the reaction product was filtered. Removal of solvent afforded peptide **S5**, which was used to the next reaction without further purification. **S5** was dissolved in DCM/DMF (= 9:1) (20 mL) and to the solution were added *i*-Pr_2NEt (9.4 μL, 0.057 mmol), HOAt (7.7 mg, 0.057 mmol) and PyBOP (30 mg, 0.057 mmol). After stirred overnight at 40 °C, the reaction mixture was extracted with DCM (× 3), washed with brine, dried over $MgSO_4$, and concentrated to afford crude peptide **S6**. To **S6** residue was added TFA/H_2O/*i*-Pr_3SiH/DODT = 92.5:2.5:2.5:2.5 (1 mL), being stirred for 180 min, and then the reaction mixture was filtered. The filtrate was diluted with Et_2O (12 mL) and was chilled (–80 °C), then centrifuged with 3,500 × *g* for 10 min at 4 °C to afford crude peptide **1**. The crude **1** was purified with reversed phase HPLC equipped with

COSMOSIL 5C_{18}-MS-II 10 mm I.D. × 250 mm. The column was eluted by MeOH:H_2O (= 55:45) containing 0.05% TFA with flow rate at 3.2 ml/min to afford a pure peptide **1** (3.8 mg, 28% for 15 steps) as a white solid.

1: $[\alpha]_D^{18}$ +12.47 (*c* 0.29, DMSO); ^{1}H-NMR (400 MHz, DMSO-d_6) δ 8.26 (m, 1H), 8.20 (m, 2H), 7.93 (m, 2H), 7.78 (m, 1H), 7.66 (m, 1H), 7.21 (m, 5H), 5.43 (m, 2H), 4.52 (m, 1H), 4.37 (m, 2H), 4.20 (m, 1H), 4.04 (m, 2H), 3.90 (m, 1H), 3.76 (m, 1H), 3.16 (m, 1H), 3.10 (m, 1H), 2.93 (m, 1H), 2.79 (m, 1H), 2.54 (m, 1H), 1.92 (m, 1H), 1.69 (m, 1H), 1.49 (m, 1H), 1.30 (m, 1H), 1.23 (m, 6H), 1.03 (m, 2H), 0.81 (m, 12H), ^{13}C-NMR (100 MHz, DMSO-d_6) δ 171.4, 171.1, 171.0, 170.1, 169.9, 169.7, 156.8, 137.6, 129.3 (2×CH), 128.0 (2×CH), 126.2, 66.4, 65.8, 63.1, 58.6, 57.9, 53.7, 51.7, 50.5, 48.6, 40.4, , 37.2, 29.9, 25.1, 24.0, 22.8, 22.1, 20.5, 20.0, 19.9, 19.3, 18.5

HRMS (ESI) calcd for $C_{34}H_{56}O_8N_9^+$ $[M+H]^+$ 718.42464, found 718.42293

Construction of expression plasmid and host

DNA fragment coding for FlkO with codons optimized for expression in *E. coli* was synthesized by Twist Bioscience, then inserted into NdeI/XhoI site of a modified pET28a(+), which has MBP-coding DNA fragment at the upstream of MCS, to generate expression plasmid pET-MBP-FlkO. The expression plasmid was introduced into *E. coli* BL21 (DE3) to give an expression host.

Preparation of recombinant enzymes

A single colony of *E. coli* host was inoculated into 10 mL of 2xYT media (1.6% Bacto tryptone, 1.0% Bacto yeast extract, 0.5% NaCl) containing 50 μg/ml kanamycin and was cultured at 37 °C for overnight as seed culture. 2.0 mL of cultural broth was transferred to 200 mL of 2xYT media containing 50 μg/ml kanamycin and cultured at 37 °C for three hours. The broth was cooled on ice and 0.1 mM of isopropyl β-D-thiogalactopyranoside (IPTG) was added to induce the expression of recombinant enzymes. *E. coli* was cultured at 16 °C for overnight. Cells were harvested by centrifugation with 4,000 × *g* for 15 min at 4 °C and washed with wash buffer (20 mM Tris-HCl pH8.0, 150 mM NaCl). Cells were resuspended into lysis buffer (20 mM Tris-HCl pH8.0, 150 mM NaCl, 20 mM imidazole pH8.0), then successively homogenized by sonication. Cell debris were precipitated by centrifugation with 20,630 × *g* for 20 min at 4 °C, then the supernatant was subjected to Ni-NTA His-Bind® resin (Merck Millipore), which was equilibrated by lysis buffer. The column was washed with additional lysis buffer, then eluted with elution buffer (20 mM Tris-HCl pH8.0, 150 mM NaCl, 500 mM imidazole pH8.0). Imidazole was removed by an Amicon Ultra 0.5ml filter (Merck Millipore). The concentrations of proteins were measured using a Bio-Rad protein assay kit (Bio-Rad). From 1 L of *E. coli* culture, 0.8 mg of MBP-FlkO was obtained.

***In vitro* enzymatic reactions**

Enzymatic cyclization was initiated by adding 20 μM of FlkO to 50 μL of reaction mixtures containing 20 mM Tris-HCl pH8.0 and 50 μM substrate peptides. Reaction mixtures were incubated at 30 ºC for 12 hours. Reaction mixtures were quenched by the addition of equal volume of 0.1% TFA, then centrifuged at 20,630 × *g* for 10 min. Resultant supernatants were subjected to HPLC analysis. Column elutes were monitored by UV absorption at 210 nm. The analytical conditions are listed in Table S1. Conversions (%) were calculated based on the area value of the cyclic products in the "+ FlkO" samples compared to the area value of the substrates in the "substrate standard" samples. The

"substrate standard" samples were prepared using an identical procedure to the "+ FlkO" sample, but without the addition of enzyme and incubation.

Kinetic analysis

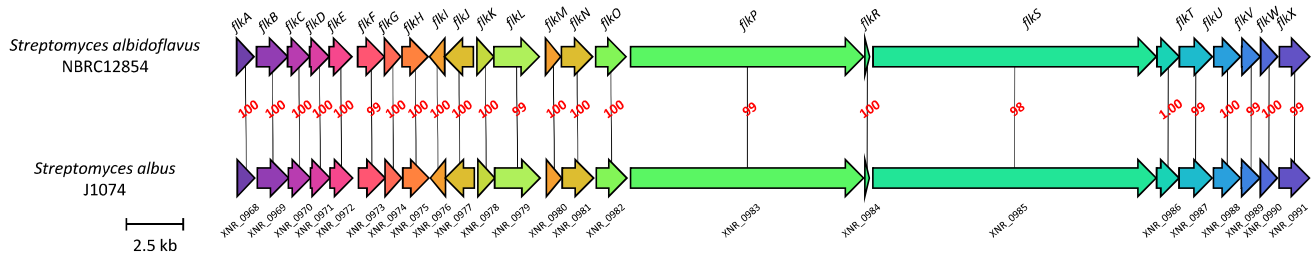
To determine the kinetic parameters of MBP-FlkO against **2**, 50 μL of reaction buffer containing 20 mM Tris-HCl (pH 8.0), 20 μM MBP-FlkO and 3.125–100 μM of **2** were incubated at 30 °C for 30 min. The reaction was quenched by adding 50 μL 0.1% TFA and centrifuged with 20,630 × g for 10 min at 4 °C. 15 μL of supernatant was subjected to HPLC operated in condition A (Table S1). The concentrations of cyclic peptide **1** were estimated using the experimentally determined extinction coefficient (ε 210) of **2**, assuming equal ε 210 values for both compounds. All reaction was carried out in triplicates. Kinetic parameters were estimated from the initial velocities using Michaelis-Menten equation and curve-fitting program Kaleidagraph.

Table S1. Conditions of HPLC analysis.

Condition	Description
A	Column: COSMOSIL 5C_{18}-MS-II 4.6 mm I.D. × 250 mm Mobile phases: A) H_2O + 0.05% TFA, B) MeOH + 0.05% TFA **Gradient: 40–70% for B in 30 min** Flow rate: 1.0 mL/min Injection volume: 15 μL
B	Column: COSMOSIL 5C_{18}-MS-II 4.6 mm I.D. × 250 mm Mobile phases: A) H_2O + 0.05% TFA, B) MeOH + 0.05% TFA **Gradient: 20–50% for B in 30 min** Flow rate: 1.0 mL/min Injection volume: 15 μL
C	Column: COSMOSIL 5C_{18}-MS-II 4.6 mm I.D. × 250 mm Mobile phases: A) H_2O + 0.05% TFA, B) MeOH + 0.05% TFA **Gradient: 10–40% for B in 30 min** Flow rate: 1.0 mL/min Injection volume: 15 μL
D	Column: COSMOSIL 2.5C_{18}-MS-II 2.0 mm I.D. × 100 mm Mobile phases: A) H_2O + 0.05% TFA, B) MeOH + 0.05% TFA **Gradient: 10–100% for B in 20 min** Flow rate: 0.3 mL/min Injection volume: 2 μL

Supplementary Figures

a.



b.

Gene	Protein ID	Description
flkA	BFM22936.1	DeoR/GlpR family DNA-binding transcription regulator
flkB	BFM22937.1	sugar ABC transporter substrate-binding protein
flkC	BFM22938.1	sugar ABC transporter permease
flkD	BFM22939.1	carbohydrate ABC transporter permease
flkE	BFM22940.1	zinc-dependent alcohol dehydrogenase family protein
flkF	BFM22941.1	glycosyltransferase 87 family protein
flkG	BFM22942.1	FkbM family methyltransferase
flkH	BFM22943.1	glycosyltransferase family 4 protein
flkI	BFM22944.1	response regulator transcription factor
flkJ	BFM22945.1	sensor histidine kinase
flkK	BFM22946.1	ABC transporter ATP-binding protein
flkL	BFM22947.1	hypothetical protein
flkM	BFM22948.1	response regulator transcription factor
flkN	BFM22949.1	hypothetical protein
flkO	BFM22950.1	serine hydrolase domain-containing protein
flkP	BFM22951.1	NRPS
flkR	BFM22952.1	MbtH family protein
flkS	BFM22953.1	NRPS
flkT	BFM22954.1	clavaminate synthase family protein
flkU	BFM22955.1	MFS transporter
flkV	BFM22956.1	cytochrome P450
flkW	BFM22957.1	hypothetical protein
flkX	BFM22958.1	alpha/beta fold hydrolase

Figure S1. Cyclofaulknamycin BGC (*flk*) in *S. albidoflavus* NBRC12854. **a.** Comparison of *flk* in strain NBRC12854 and J1074 (BGC0002358 in MIBiG database[2]). Locus tags are described for *flk* in J1074. Homologous genes are linked, and amino acid sequence identities (%) were described in red letters. Figure was generated by clinker.[3] **b.** Functional annotation and Protein Ids of *flk* genes in NBRC12854 strain. Genes detection and annotation was performed by dfast.[4] Sequence data of flk in *S. albidoflavus* NBRC12854 was deposited under following ID: LC816587

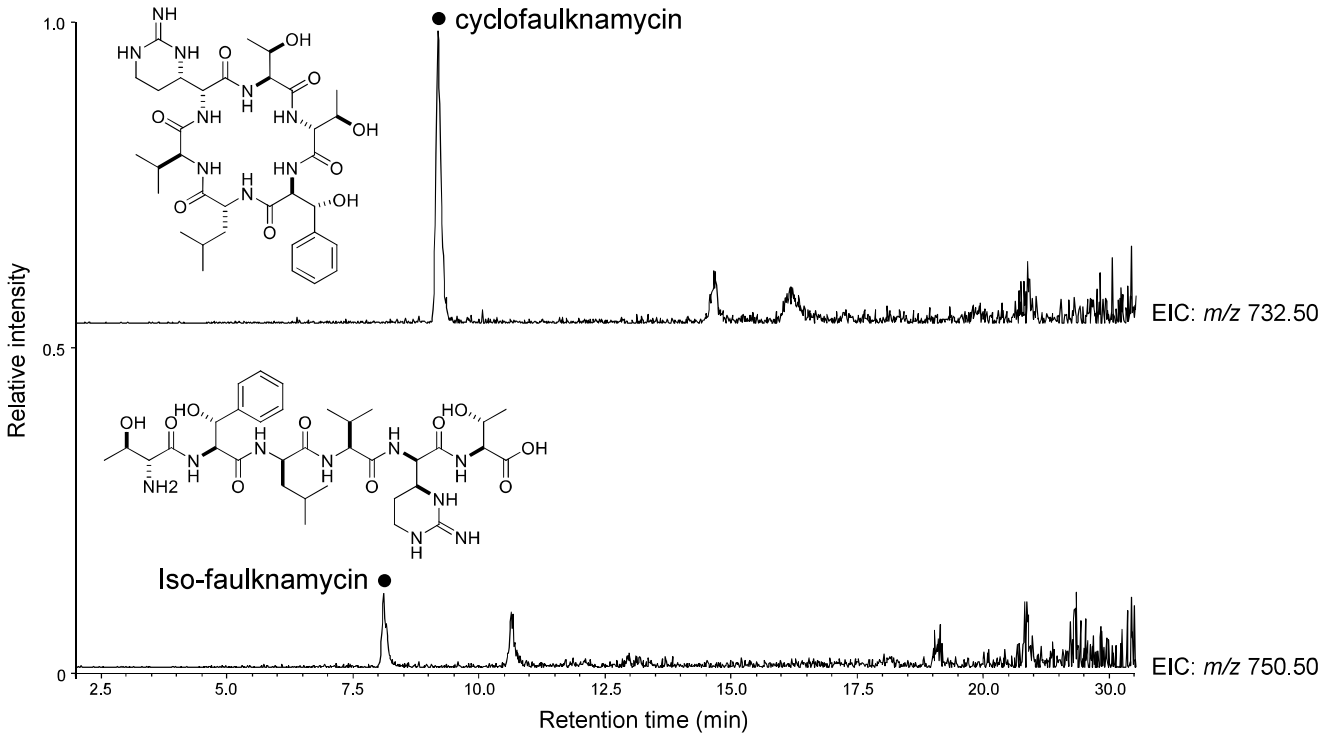


Figure S2. Production of cyclofaulknamycin and iso-faulknamycin by *S. albidoflavus* NBRC12854.

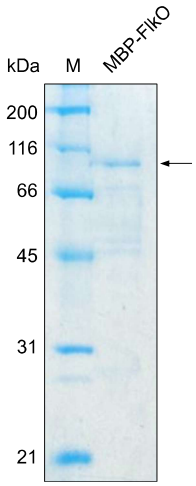


Figure S3. SDS-PAGE of MBP-FlkO. MBP-FlkO was obtained in approximately more than 90% purity. Theoretical molecular weight of MBP-FlkO is 93 kDa.

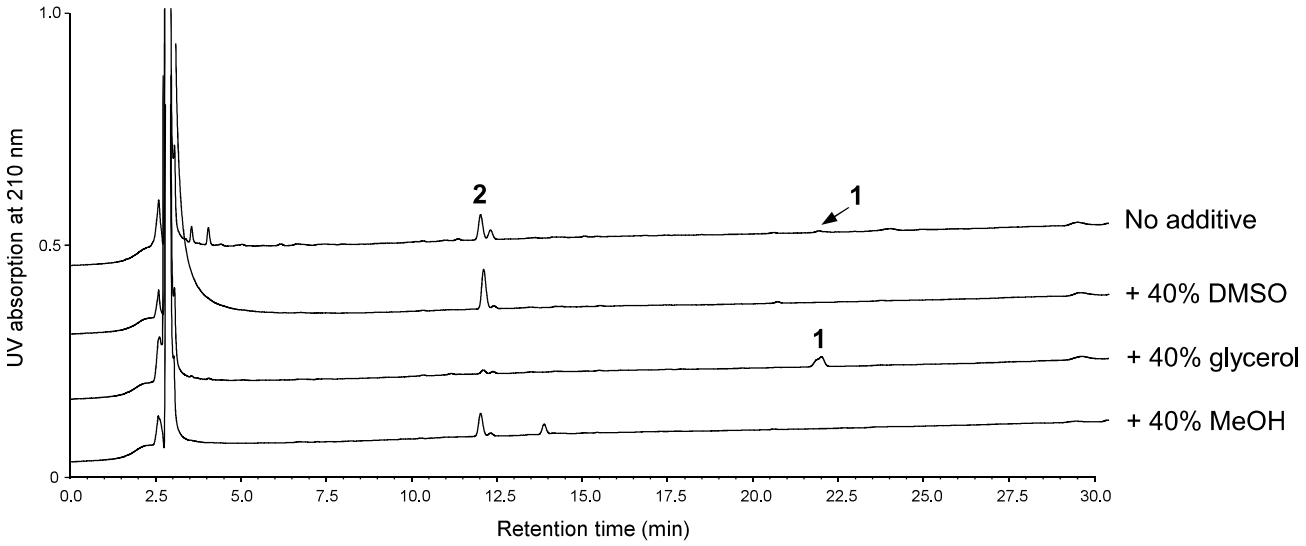


Figure S4. Effect of cosolvent in enzymatic reaction of FlkO. Samples were analyzed by HPLC condition A in Table S1. 50 μM of **2** was incubated with 20 μM of MBP-FlkO for 12 hours in the presence of 40% co-solvent; DMSO, glycerol, and MeOH. Amount of **1** was markedly enhanced by the addition of 40% glycerol.

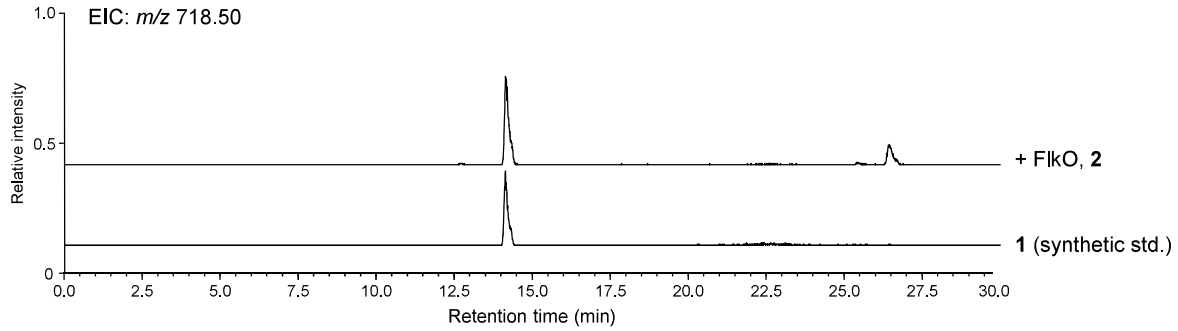


Figure S5. LC comparison of FlkO enzymatic reaction product and synthetic **1**. Samples were analyzed by LCMS2050 (Shimadzu) equipped with HPLC system. Samples were separated with reversed phase column (COSMOSIL 2.5C_{18}-MS-II 2.0 mm I.D. × 100 mm), using H_2O/MeOH + 0.05%TFA as mobile phases A and B. Column was eluted with gradient mode from 10-100% for mobile phase B in 20 min, with flow rate at 0.3 mL/min.

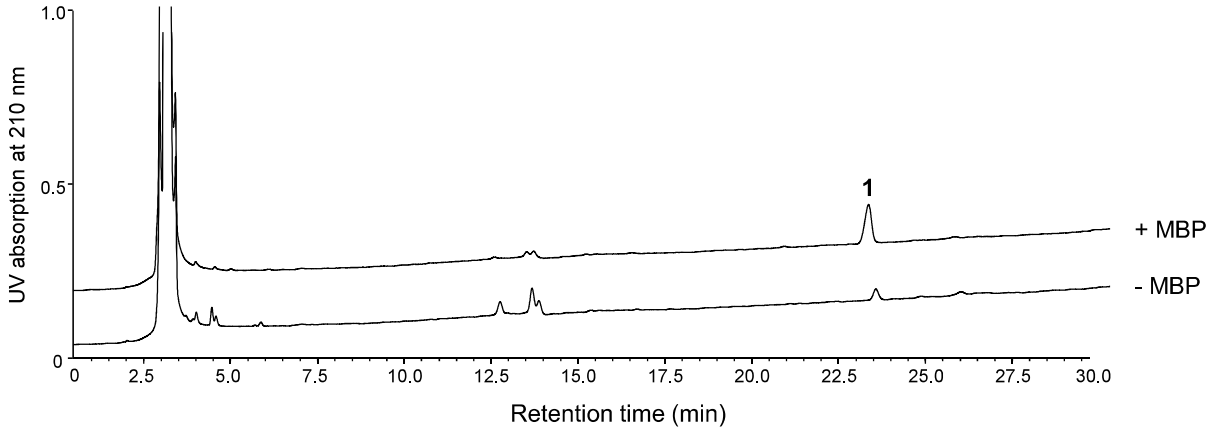


Figure S6. Effect of MBP-tag removal. Samples were analyzed by HPLC condition A in Table S1. **2** was incubated under the optimized reaction condition (incubation for 12 hours in the presence of 40% glycerol) with MBP-fused FlkO (+ MBP) and FlkO with the MBP tag removed (− MBP).

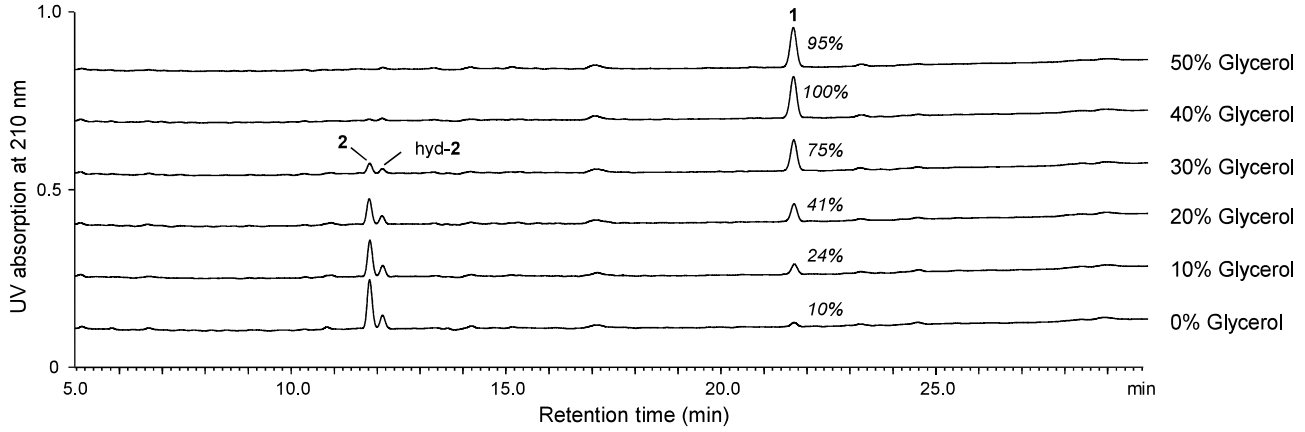


Figure S7. Effect of glycerol concentration on FlkO-mediated cyclization. Compound **2** (50 μM) was cyclized by 40 mol% of FlkO under glycerol content ranging from 10% to 50%. Reaction mixtures were incubated at 30 °C for 12 hours. The area value of **1** relative to that in the 40% glycerol sample was described alongside the peaks.

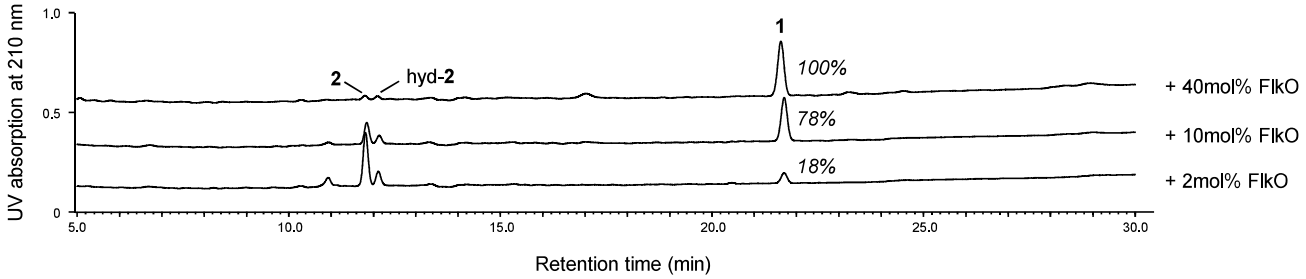


Figure S8. Substrate-enzyme concentration ratio. The cyclization reaction was performed with varying enzyme concentrations ranging from 2 μM–40 μM in the presence of 40% glycerol and 50 μM substrate. Reaction mixtures were incubated at 30 °C for 12 hours. The area value of **1** relative to that in the 40 mol% FlkO sample was described alongside the peaks.

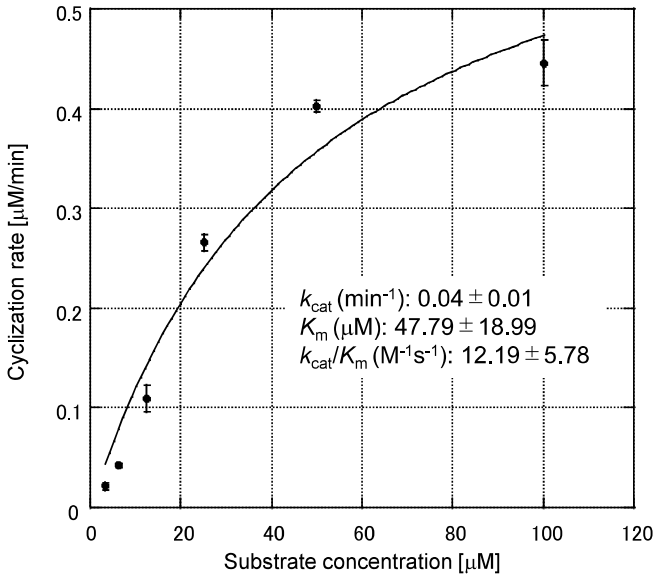


Figure S9. Michaelis-Menten plots of MBP-FlkO with **2** as a substrate. The plot is means of triplicate experiments.

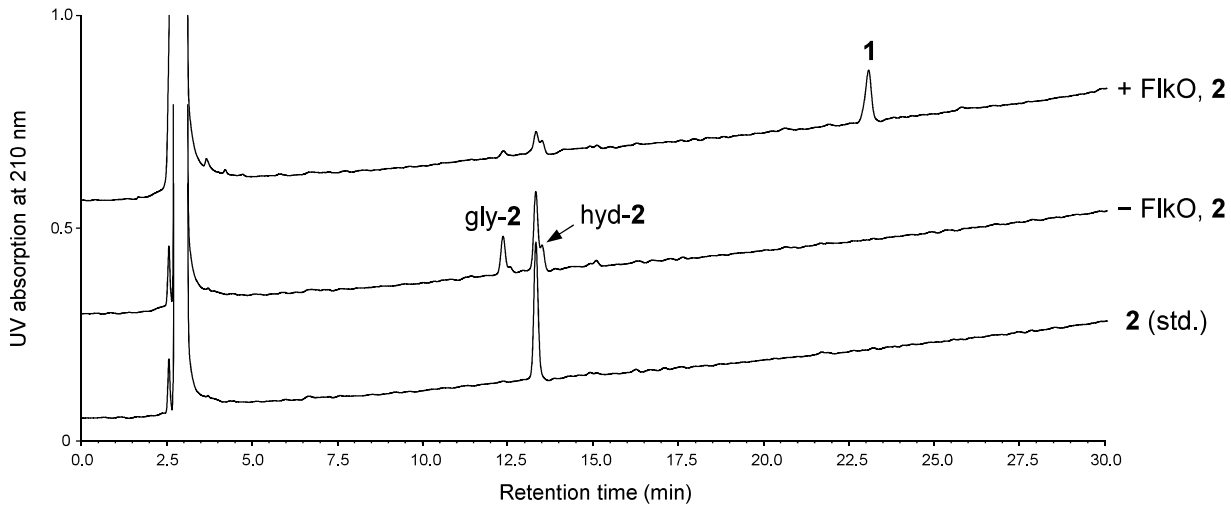


Figure S10. *In vitro* enzymatic reaction of FlkO using variant substrates **2**. Samples were analyzed by HPLC condition A in Table S1. This figure is the extended version of Figure 2. gly-**2**: glycerolysate of **2** (Fig. S39), hyd-**2**: hydrolysate of **2** (Fig. S40).

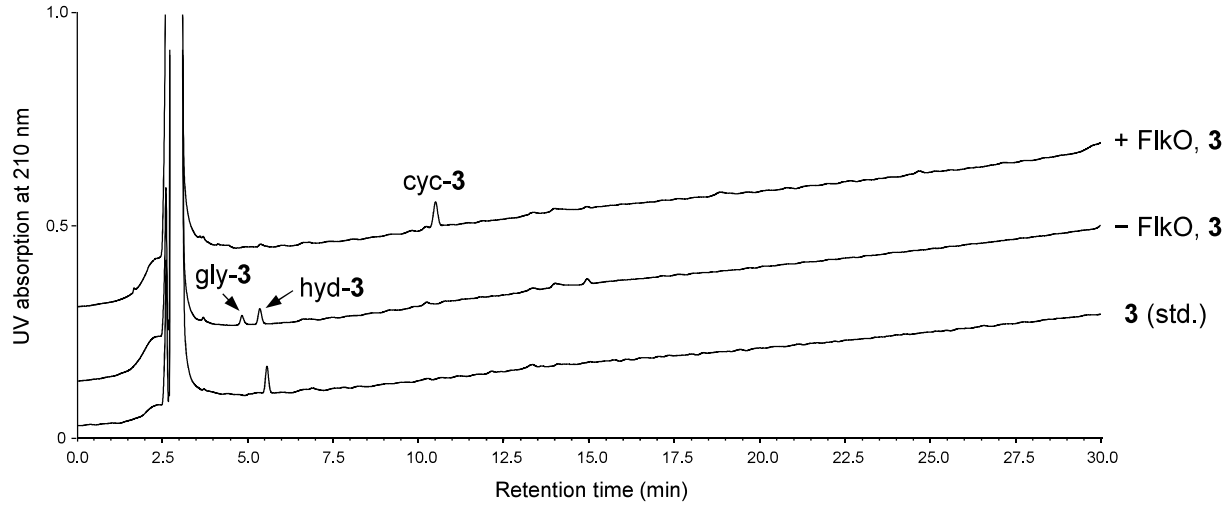


Figure S11. *In vitro* enzymatic reaction of FlkO using variant substrates **3**. Samples were analyzed by HPLC condition A in Table S1.

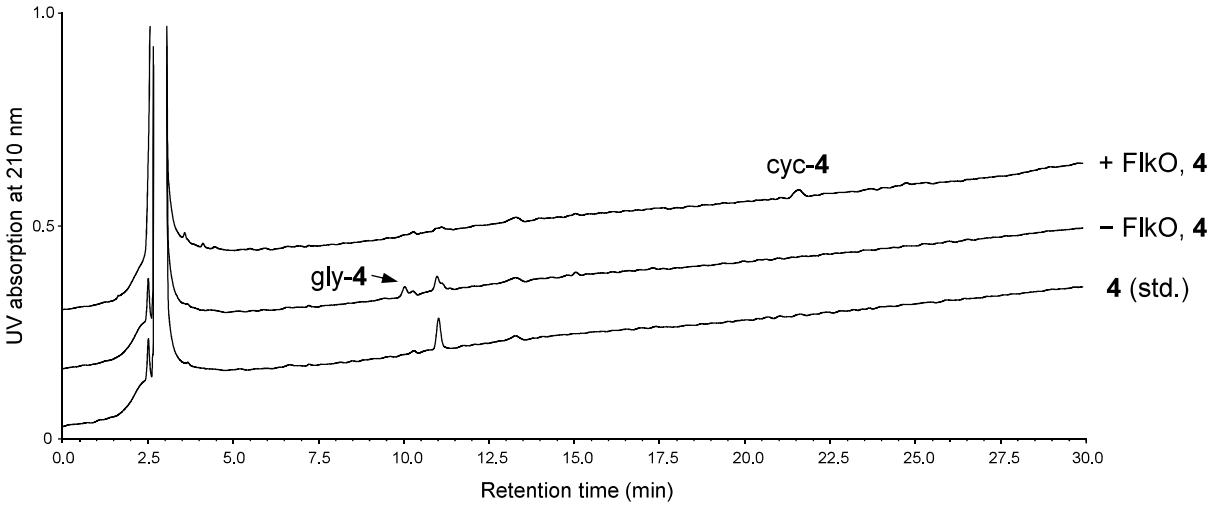


Figure S12. *In vitro* enzymatic reaction of FlkO using variant substrates **4**. Samples were analyzed by HPLC condition A in Table S1.

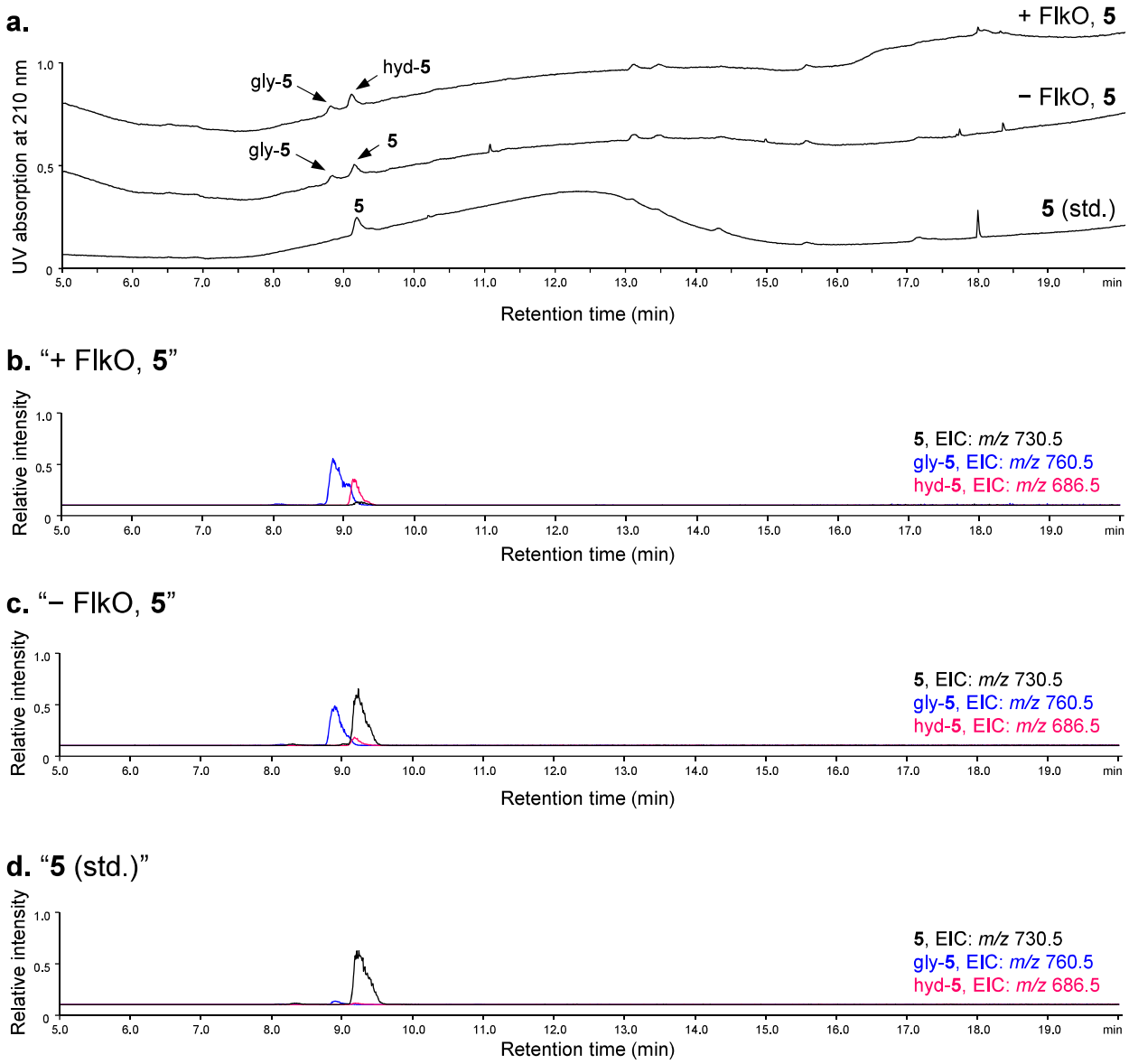


Figure S13. LC-MS analysis of *in vitro* enzymatic reaction of FlkO with variant substrates **5**. **a.** LC-UV chromatograms with absorption at 210 nm. Samples were analyzed by HPLC condition D in Table S1. **b-d.** Extracted ion chromatograms of **5** (black), glycerolysate of **5** (gly-**5**, blue), and hydrolysate of **5** (hyd-**5**, red). The result indicate that FlkO facilitates the hydrolysis of **5**.

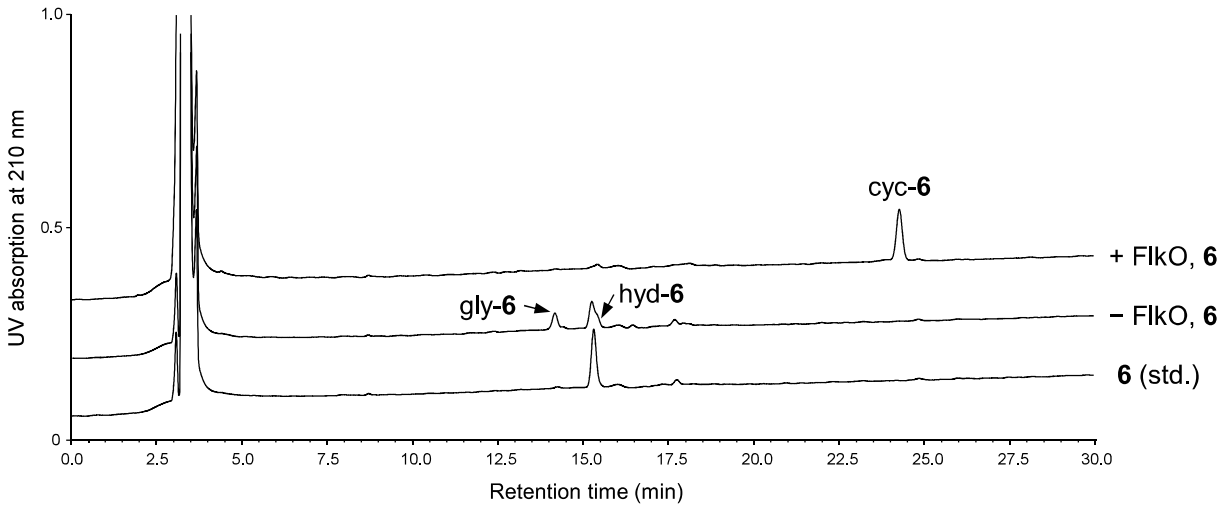


Figure S14. *In vitro* enzymatic reaction of FlkO using variant substrates **6**. Samples were analyzed by HPLC condition A in Table S1.

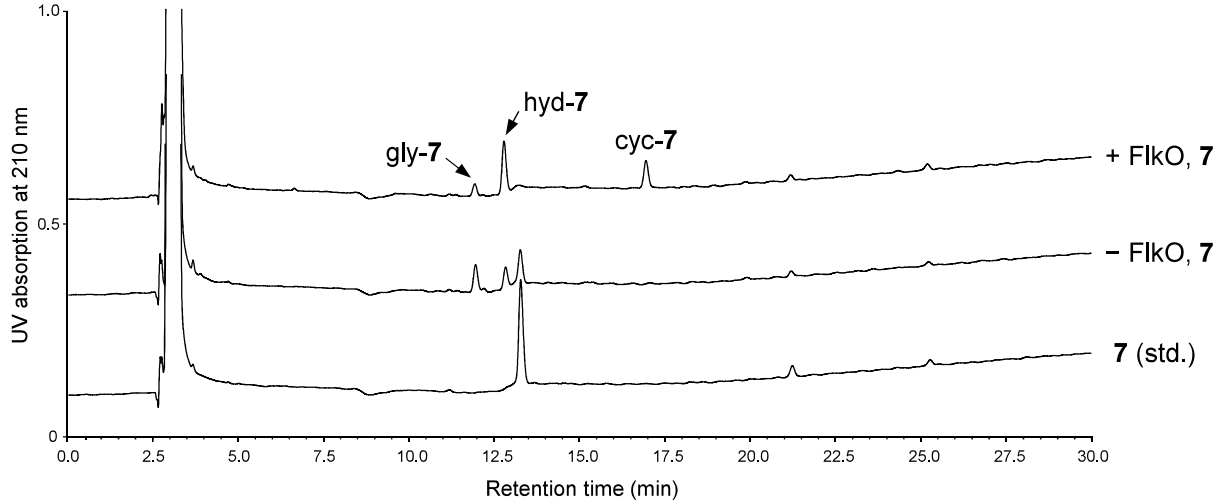


Figure S15. *In vitro* enzymatic reaction of FlkO using variant substrates **7**. Samples were analyzed by HPLC condition B in Table S1.

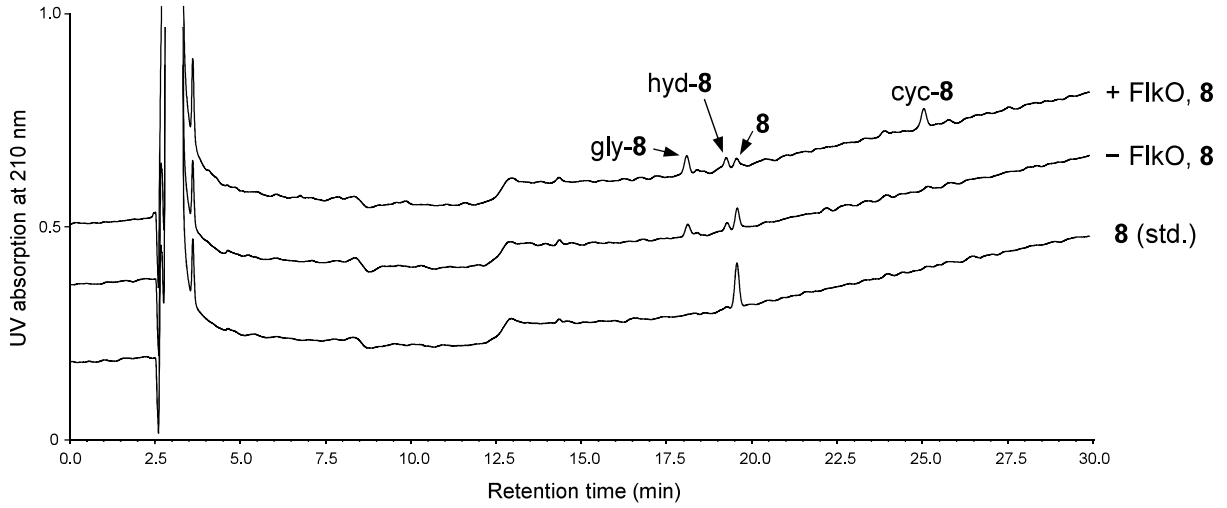


Figure S16. *In vitro* enzymatic reaction of FlkO using variant substrates **8**. Samples were analyzed by HPLC condition B in Table S1.

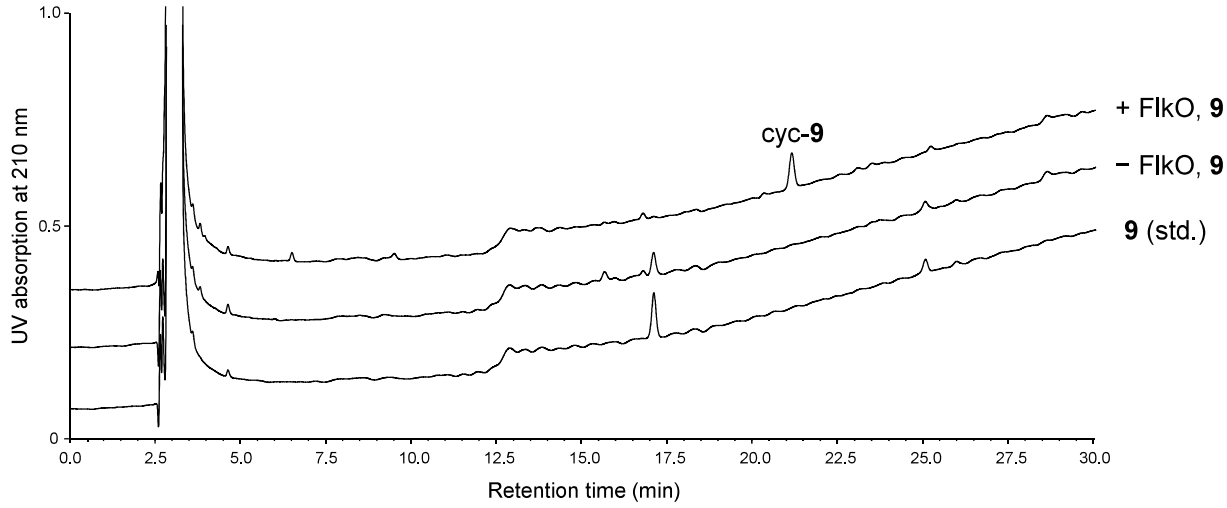


Figure S17. *In vitro* enzymatic reaction of FlkO using variant substrates **9**. Samples were analyzed by HPLC condition B in Table S1.

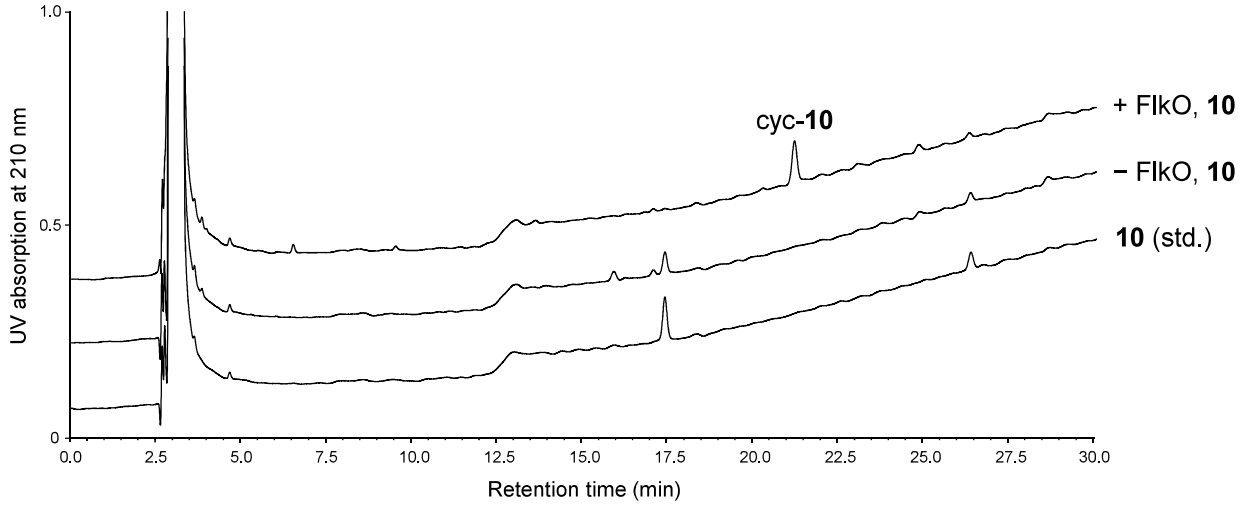


Figure S18. *In vitro* enzymatic reaction of FlkO using variant substrates **10**. Samples were analyzed by HPLC condition B in Table S1.

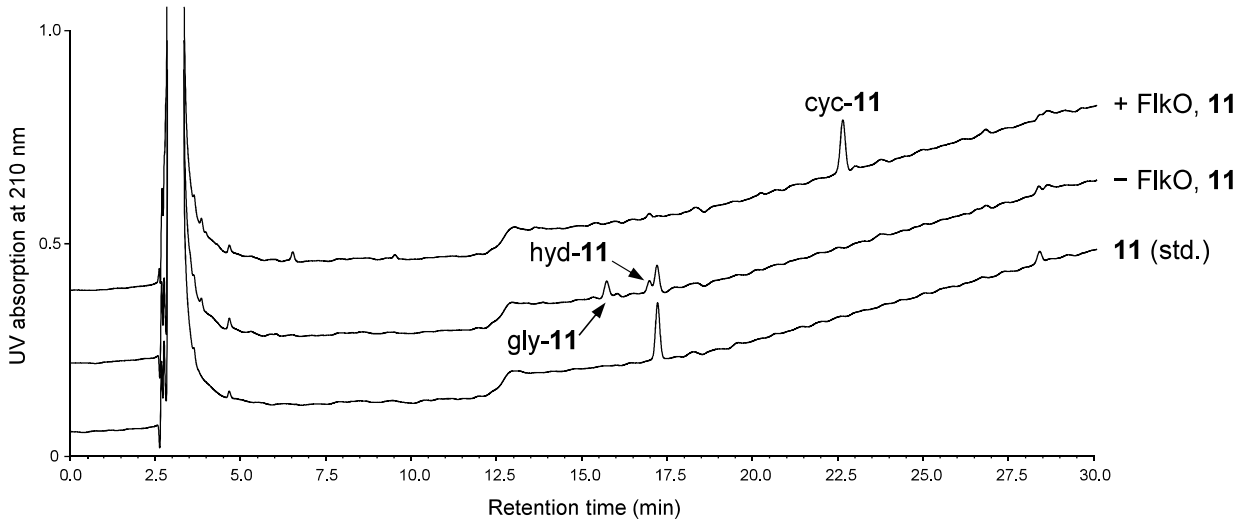


Figure S19. *In vitro* enzymatic reaction of FlkO using variant substrates **11**. Samples were analyzed by HPLC condition B in Table S1.

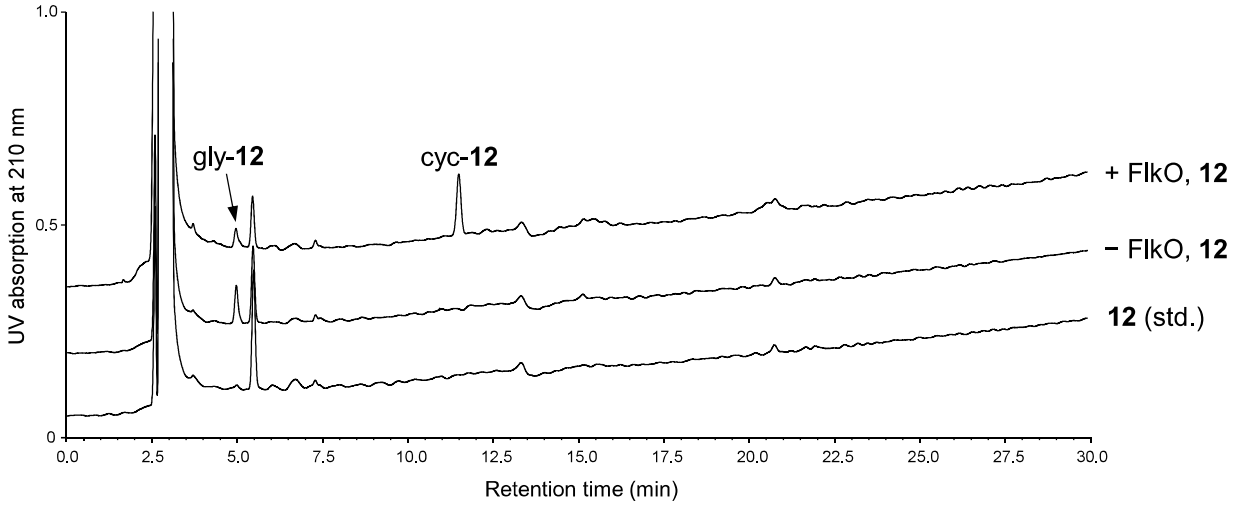


Figure S20. *In vitro* enzymatic reaction of FlkO using variant substrates **12**. Samples were analyzed by HPLC condition A in Table S1.

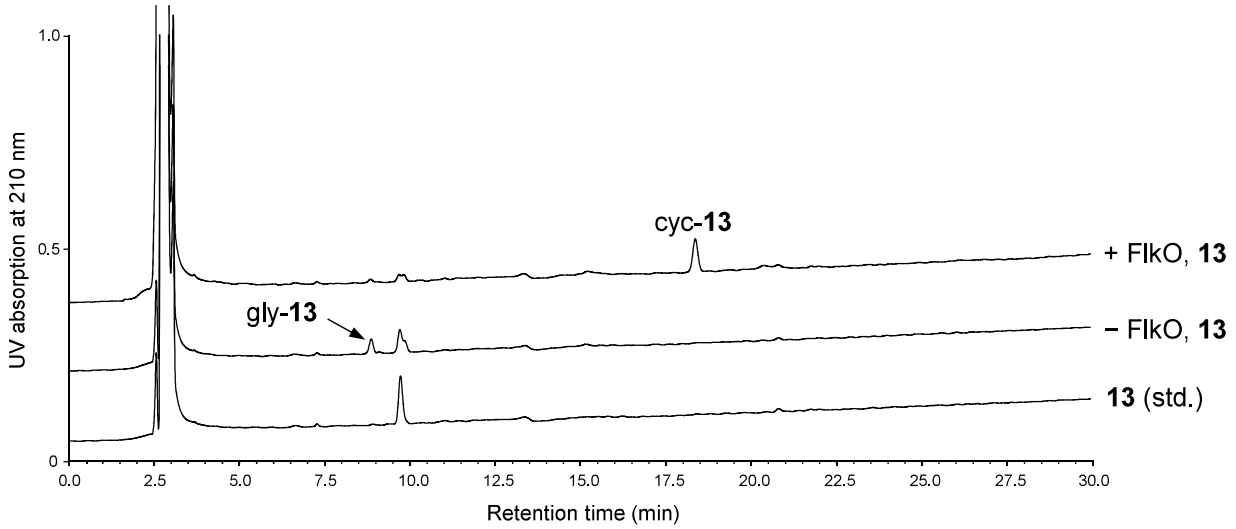


Figure S21. *In vitro* enzymatic reaction of FlkO using variant substrates **13**. Samples were analyzed by HPLC condition A in Table S1.

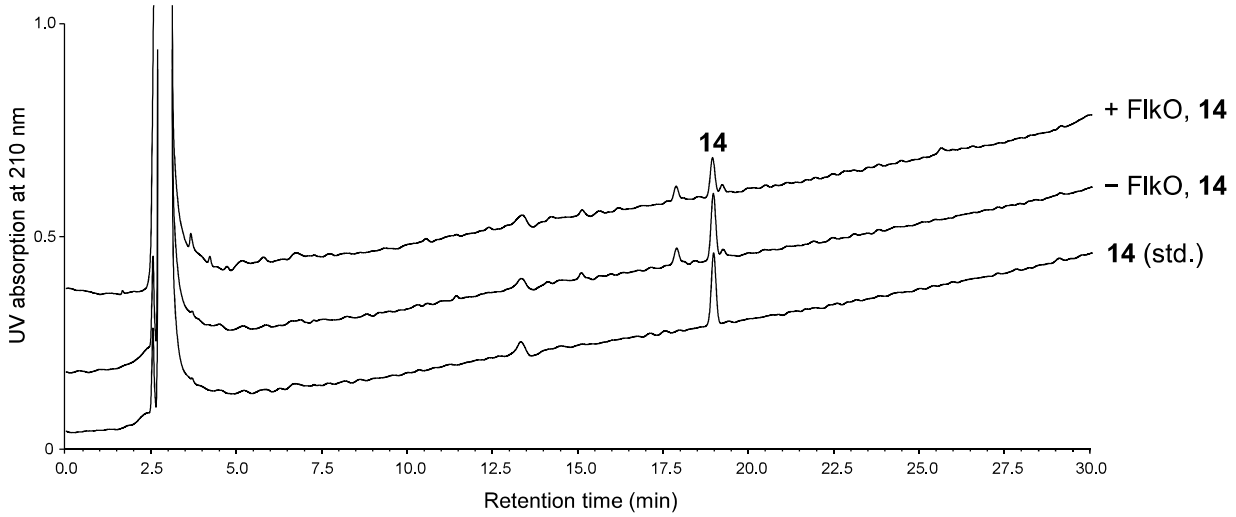


Figure S22. *In vitro* enzymatic reaction of FlkO using variant substrates **14**. Samples were analyzed by HPLC condition A in Table S1.

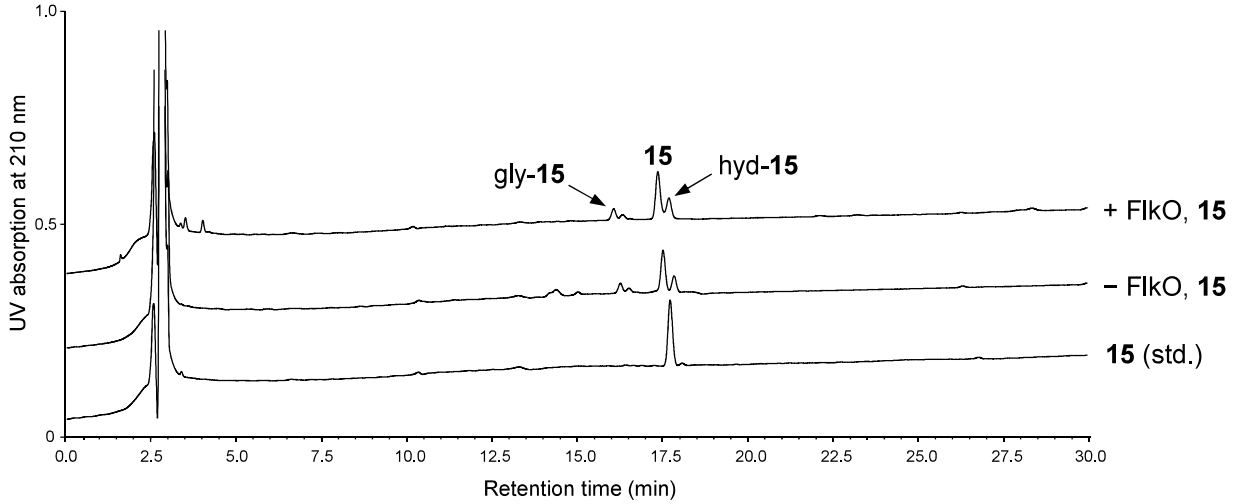


Figure S23. *In vitro* enzymatic reaction of FlkO using variant substrates **15**. Samples were analyzed by HPLC condition A in Table S1.

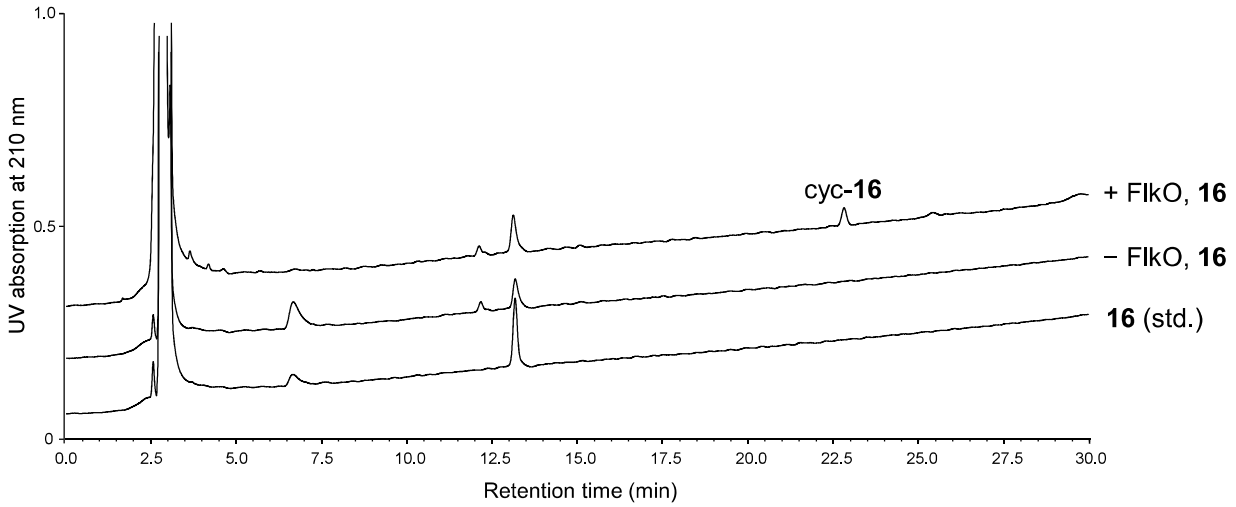


Figure S24. *In vitro* enzymatic reaction of FlkO using variant substrates **16**. Samples were analyzed by HPLC condition A in Table S1.

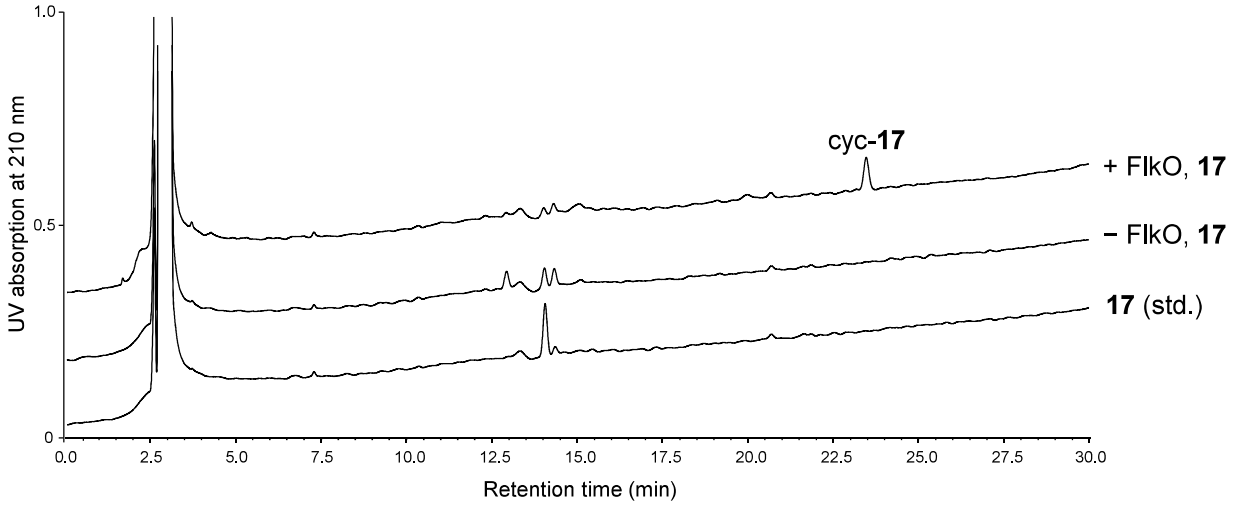


Figure S25. *In vitro* enzymatic reaction of FlkO using variant substrates **17**. Samples were analyzed by HPLC condition A in Table S1.

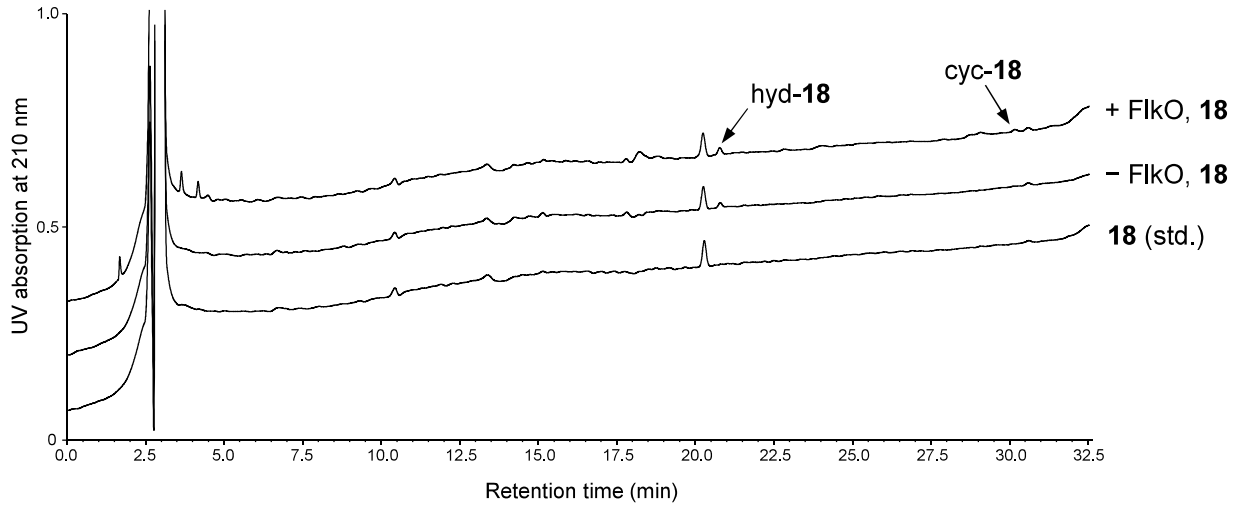


Figure S26. *In vitro* enzymatic reaction of FlkO using variant substrates **18**. Samples were analyzed by HPLC condition A in Table S1.

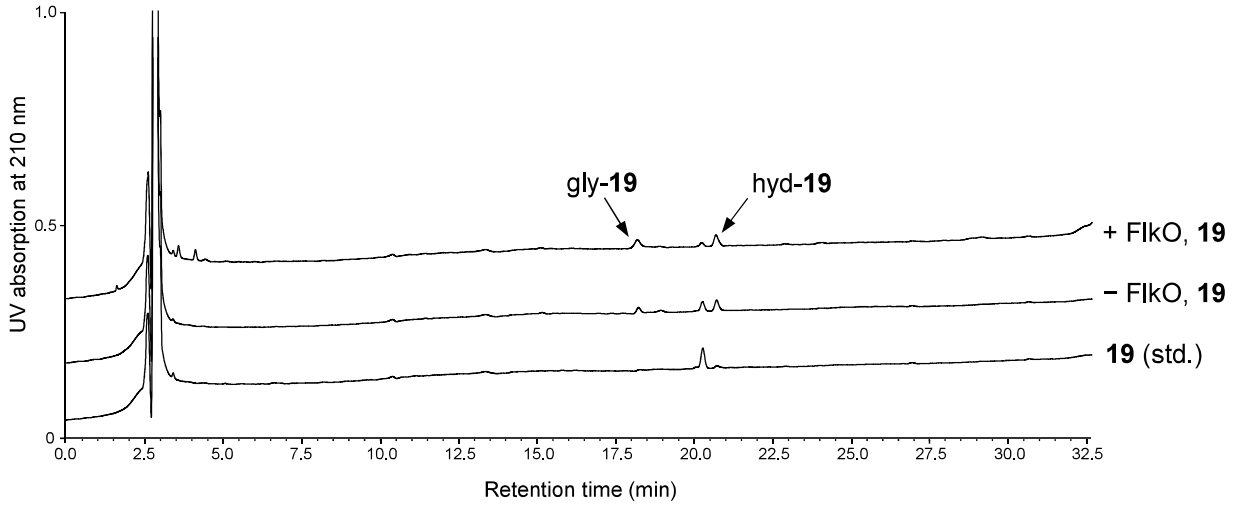


Figure S27. *In vitro* enzymatic reaction of FlkO using variant substrates **19**. Samples were analyzed by HPLC condition A in Table S1.

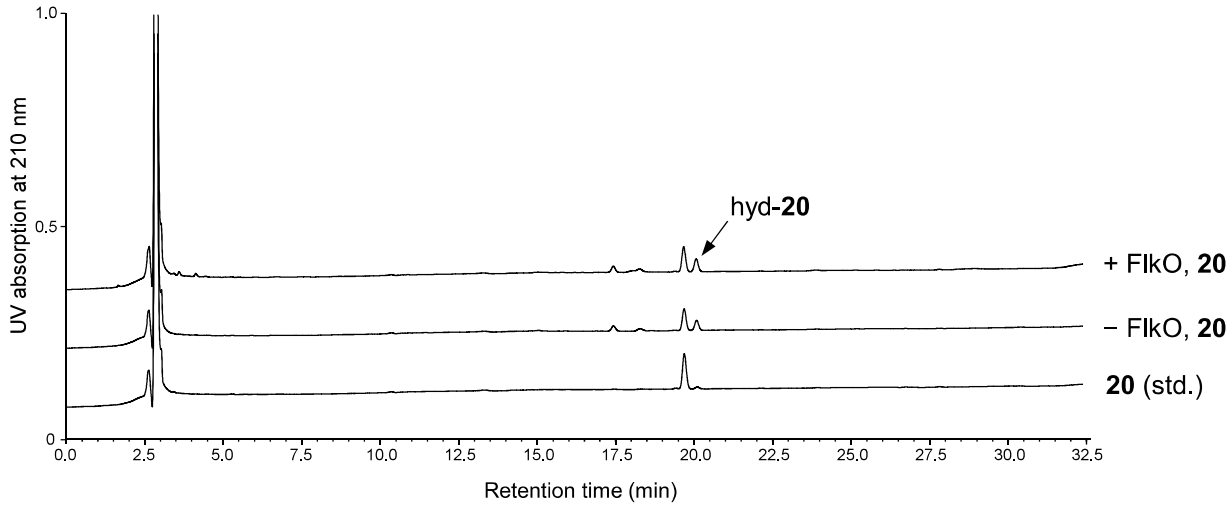


Figure S28. *In vitro* enzymatic reaction of FlkO using variant substrates **20**. Samples were analyzed by HPLC condition A in Table S1.

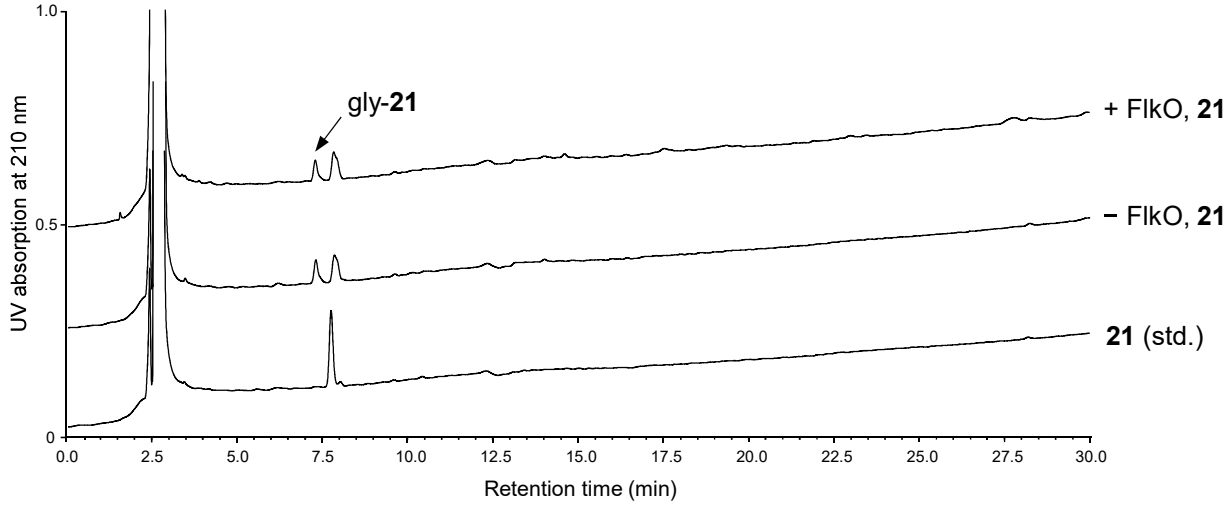


Figure S29. *In vitro* enzymatic reaction of FlkO using variant substrates **21**. Samples were analyzed by HPLC condition A in Table S1.

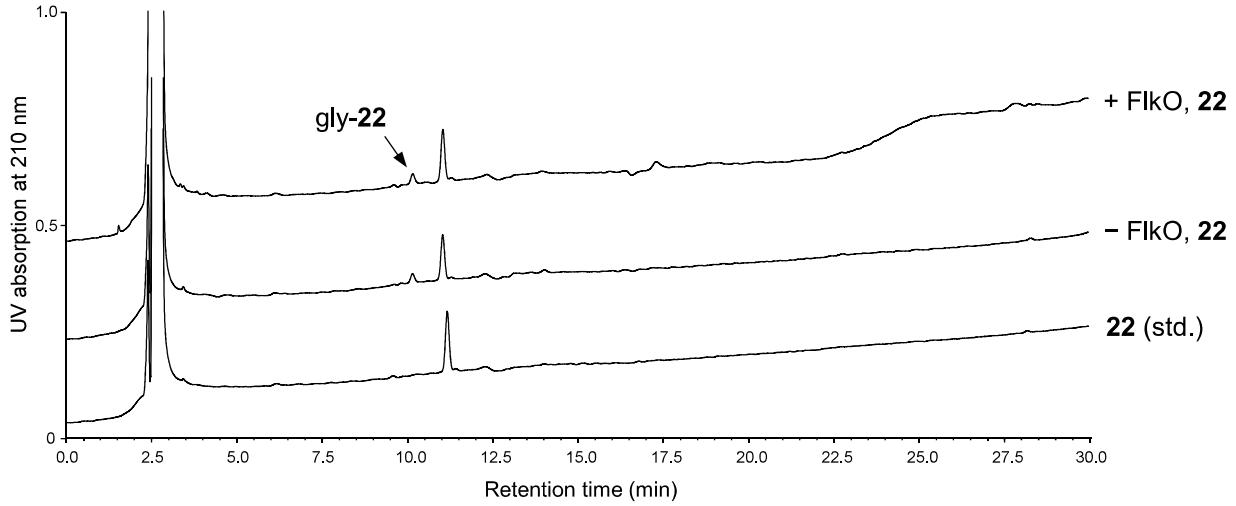


Figure S30. *In vitro* enzymatic reaction of FlkO using variant substrates **22**. Samples were analyzed by HPLC condition A in Table S1.

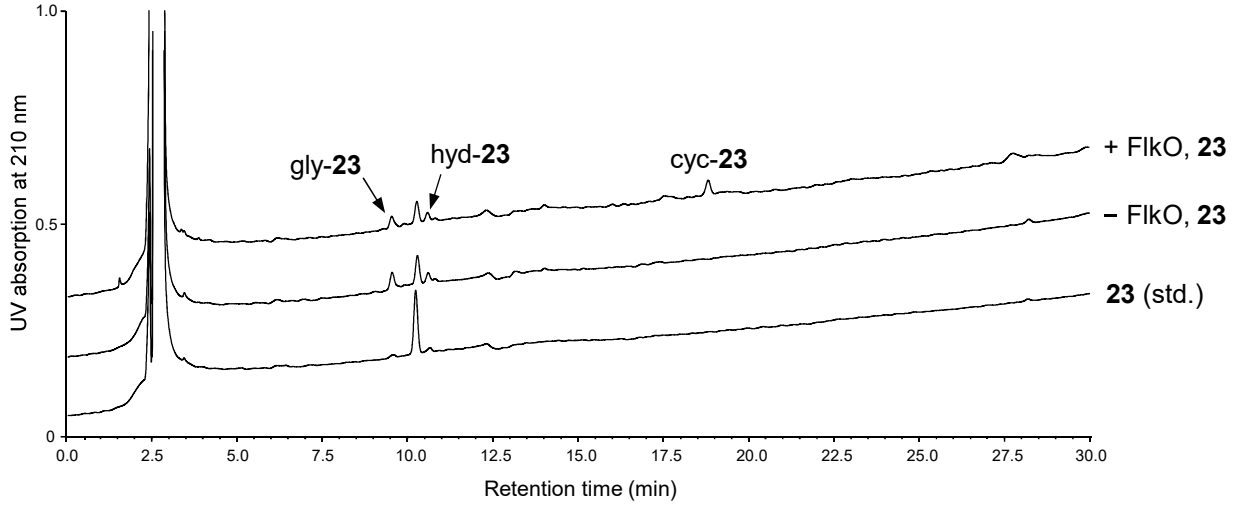


Figure S31. *In vitro* enzymatic reaction of FlkO using variant substrates **23**. Samples were analyzed by HPLC condition A in Table S1.

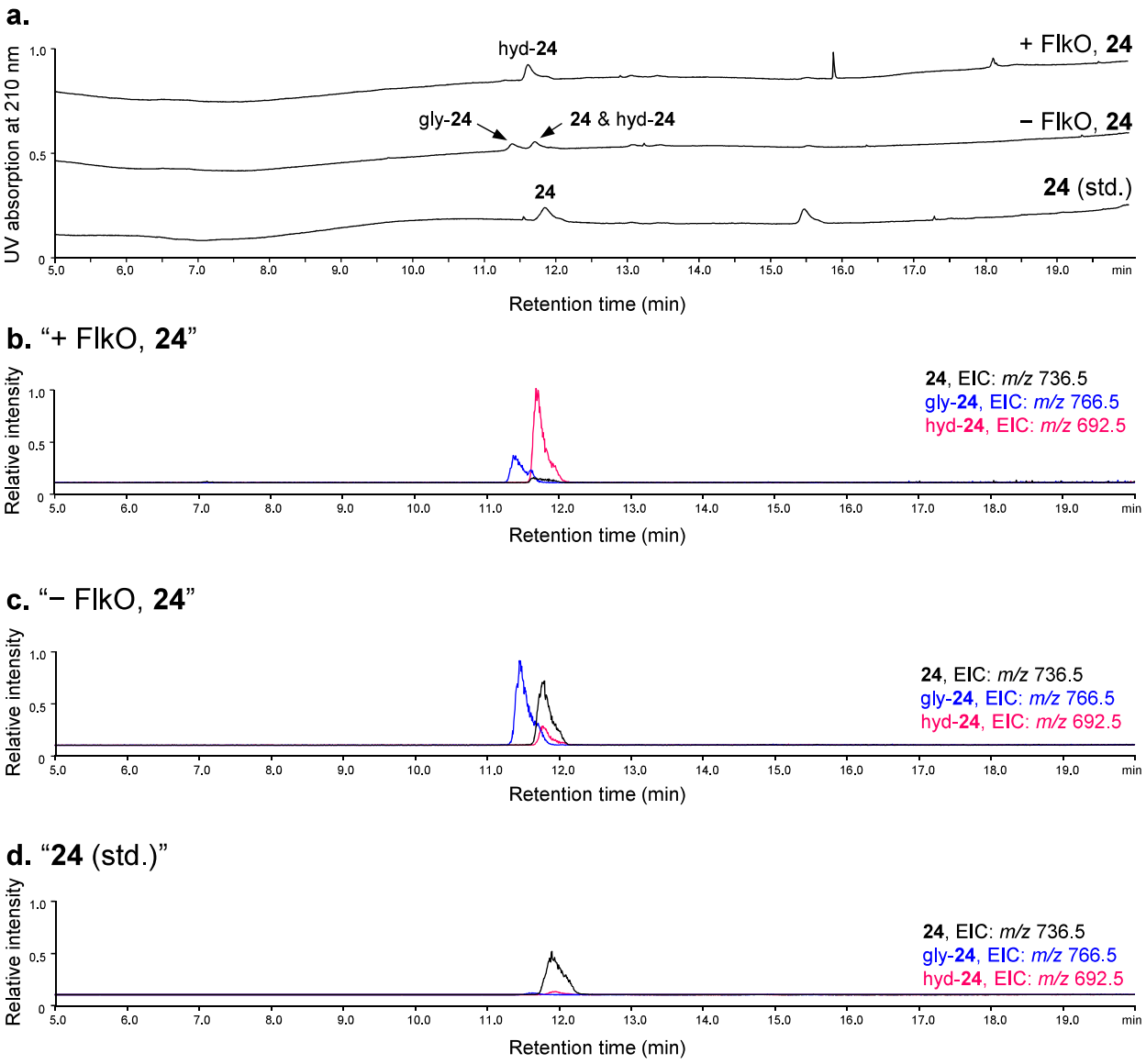


Figure S32. LC-MS analysis of *in vitro* enzymatic reaction of FlkO with variant substrates **24**. **a.** LC-UV chromatograms with absorption at 210 nm. Samples were analyzed by HPLC condition D in Table S1. **b-d.** Extracted ion chromatograms of **24** (black), glycerolysate of **24** (gly-**24**, blue), and hydrolysate of **24** (hyd-**24**, red). The result indicate that FlkO facilitates the hydrolysis of **24**.

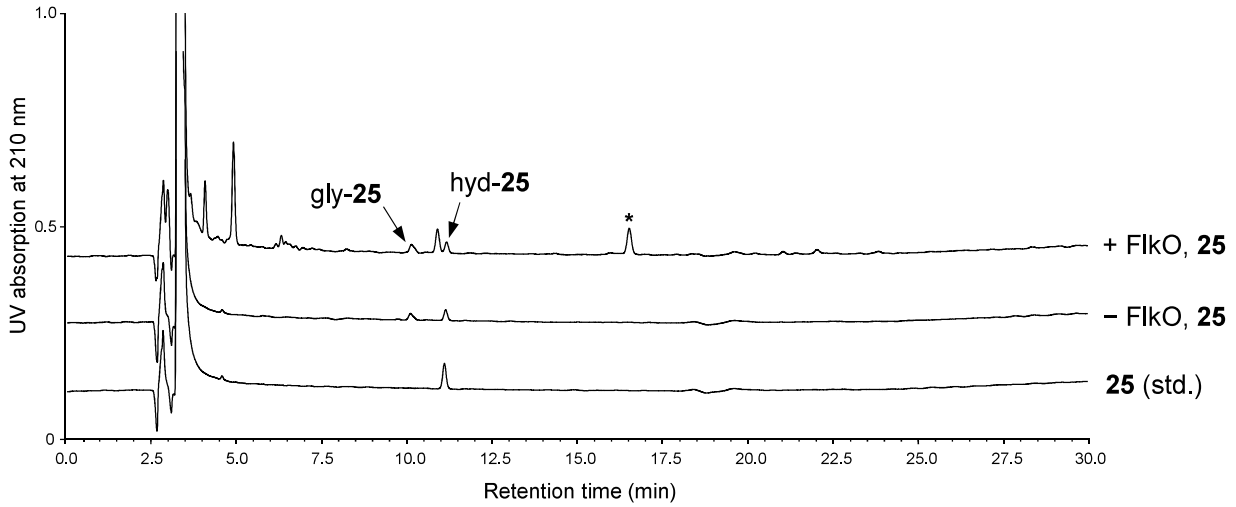


Figure S33. *In vitro* enzymatic reaction of FlkO using variant substrates **25**. Samples were analyzed by HPLC condition C in Table S1. *Impurity from enzyme solution.

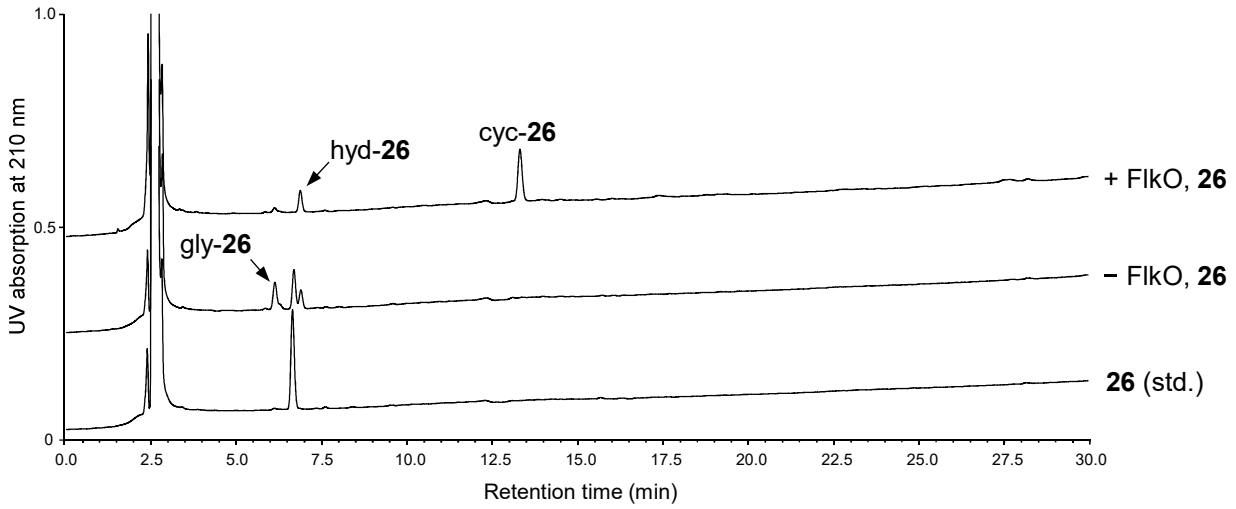


Figure S34. *In vitro* enzymatic reaction of FlkO using variant substrates **26**. Samples were analyzed by HPLC condition A in Table S1.

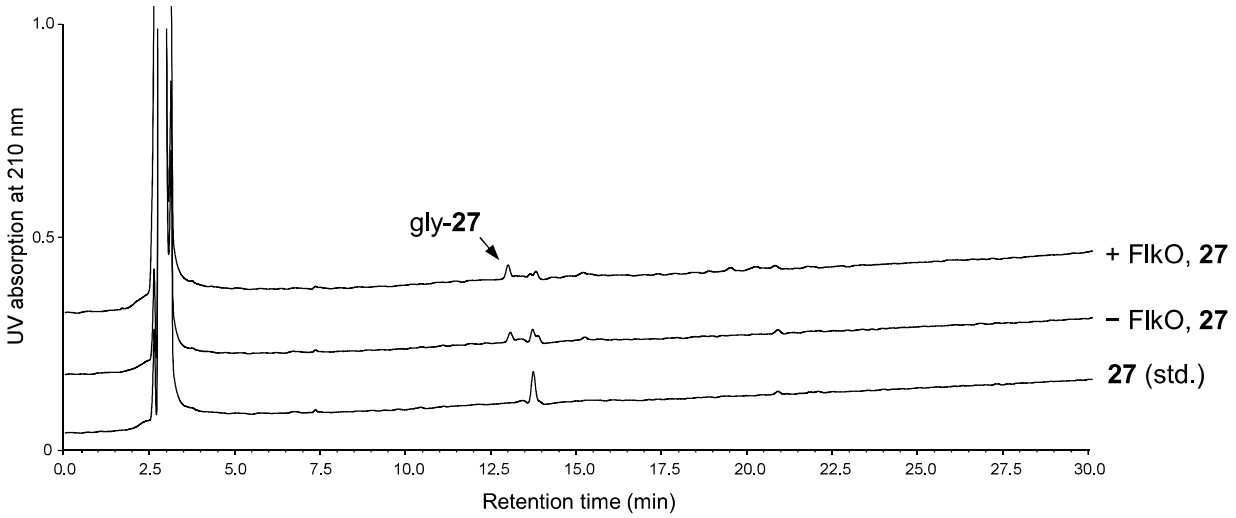


Figure S35. *In vitro* enzymatic reaction of FlkO using variant substrates **27**. Samples were analyzed by HPLC condition A in Table S1.

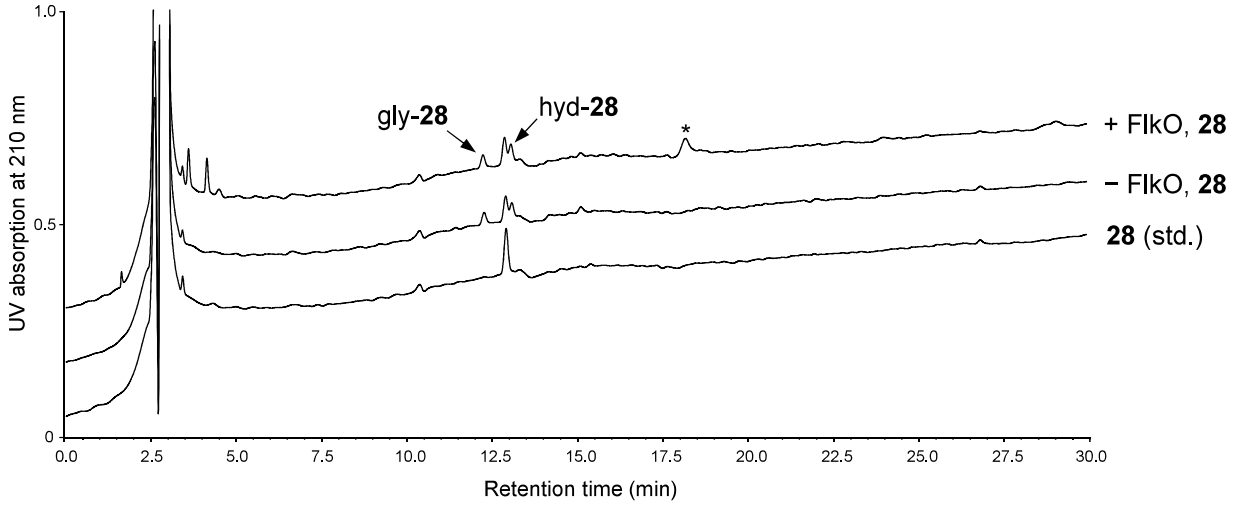


Figure S36. *In vitro* enzymatic reaction of FlkO using variant substrates **28**. Samples were analyzed by HPLC condition A in Table S1. *Impurity from enzyme solution.

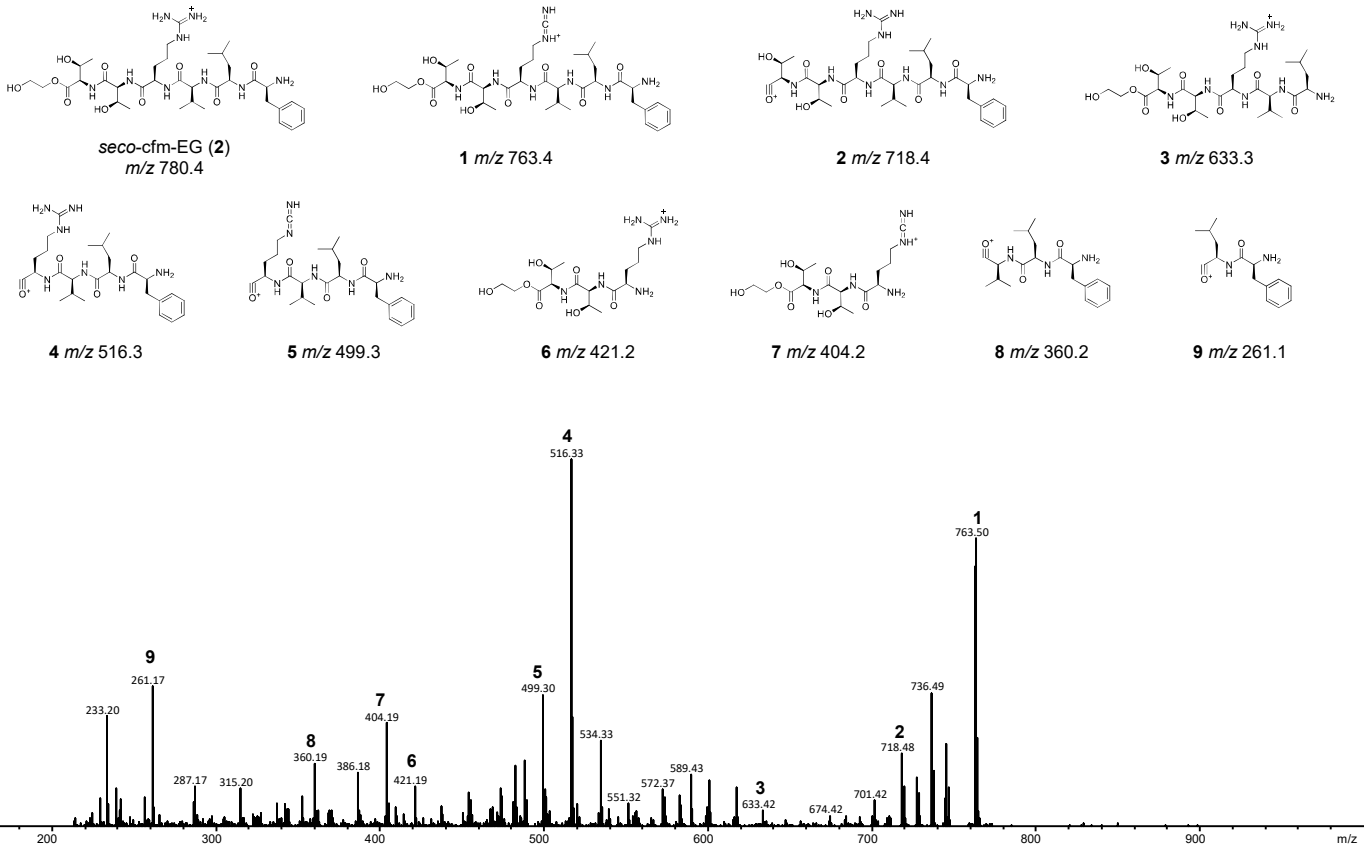


Figure S37. MS2 spectrum of *seco*-cfm-EG (**2**)

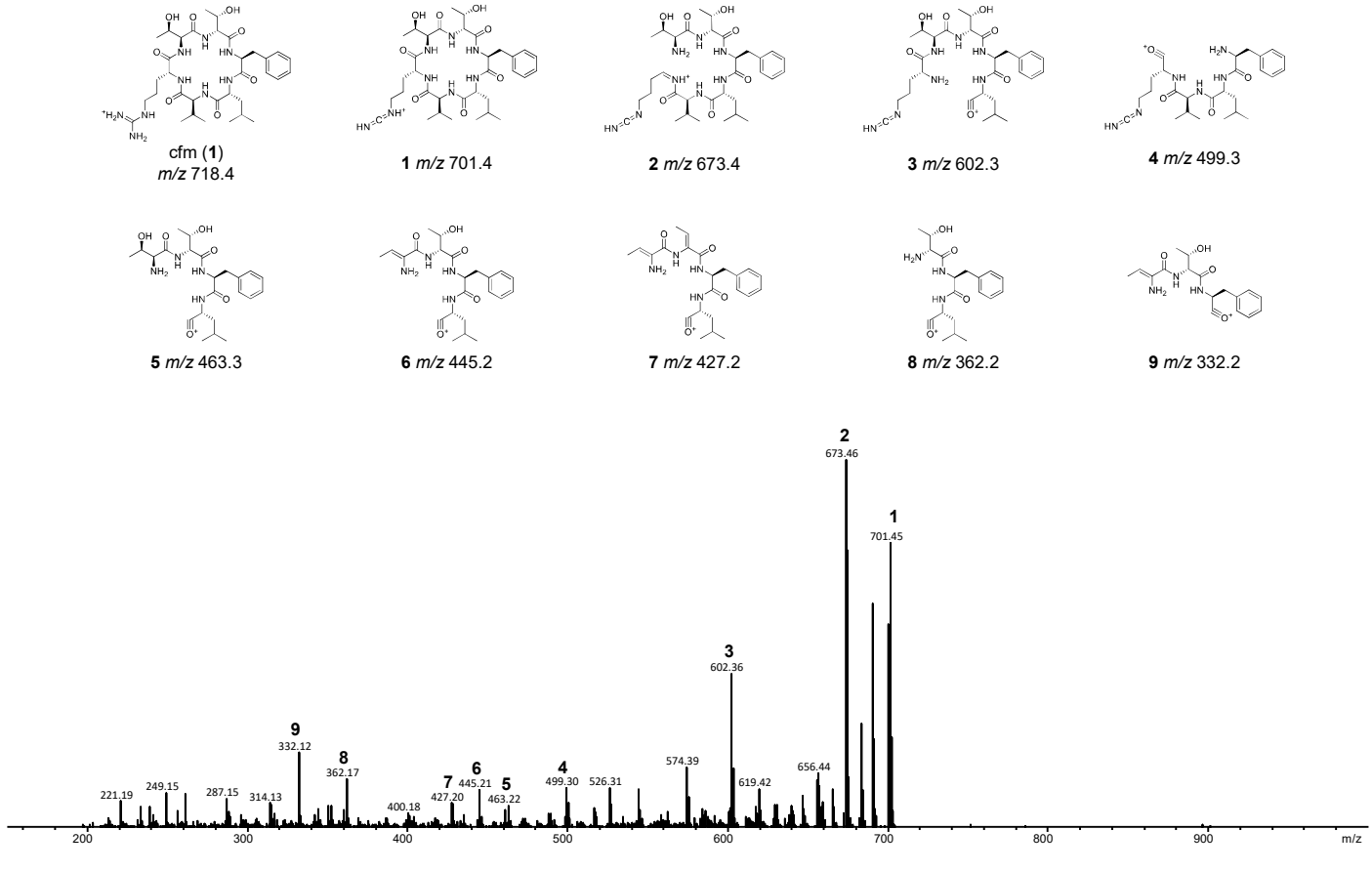


Figure S38. MS2 spectrum of cfm (**1**)

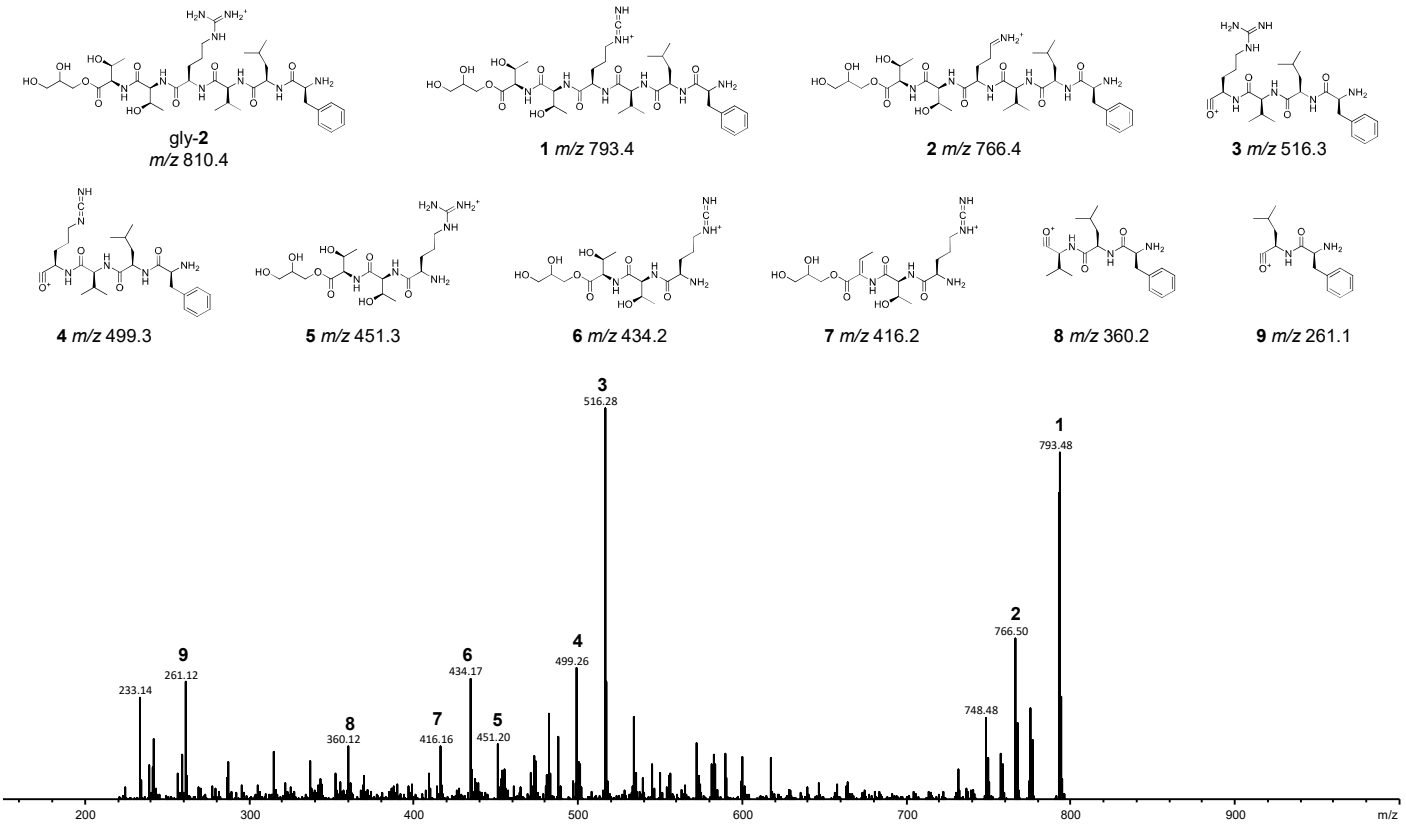


Figure S39. MS2 spectrum of glycerolysate of **2** (gly-**2**)

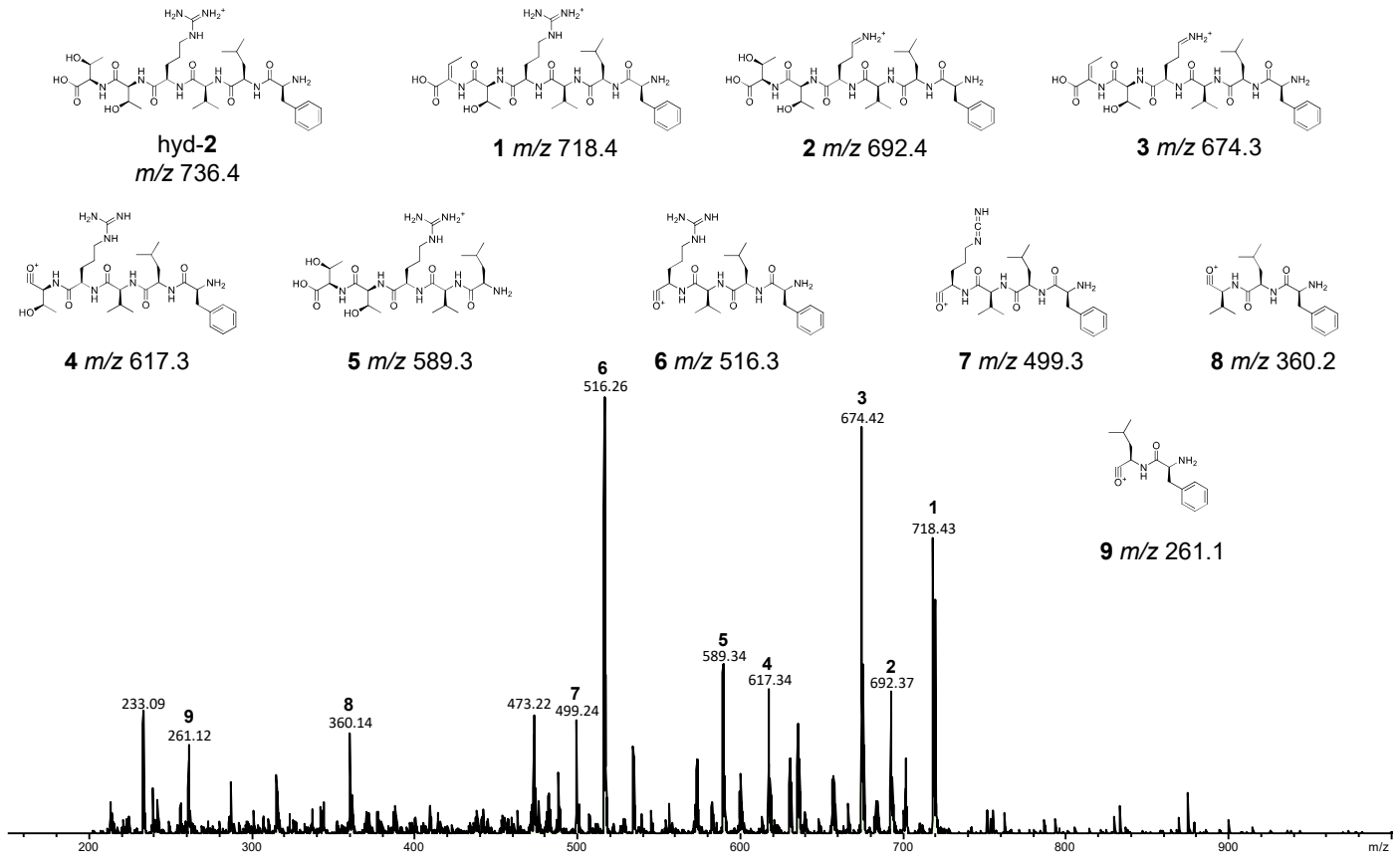


Figure S40. MS2 spectrum of hydrolysate of **2** (hyd-**2**)

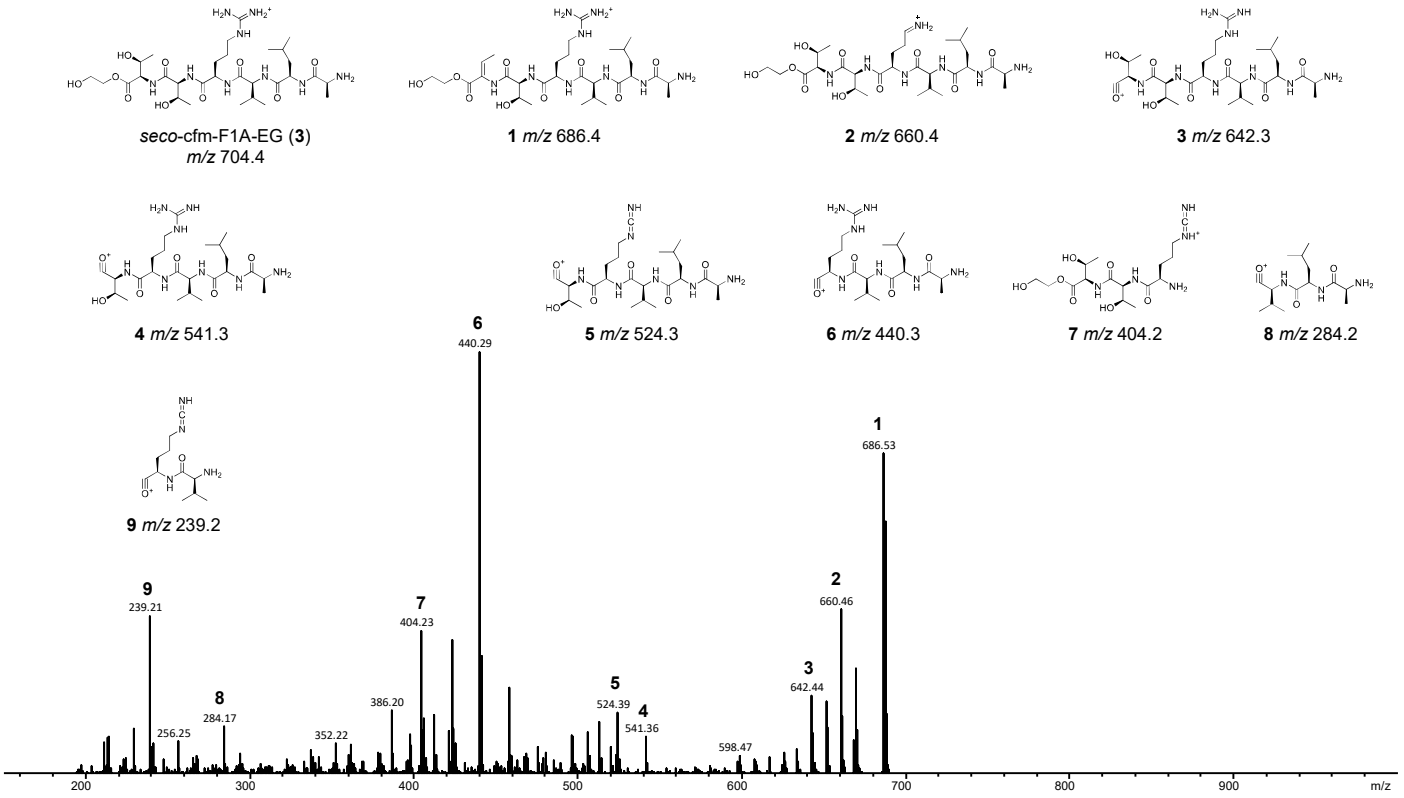


Figure S41. MS2 spectrum of *seco*-cfm-F1A-EG (**3**)

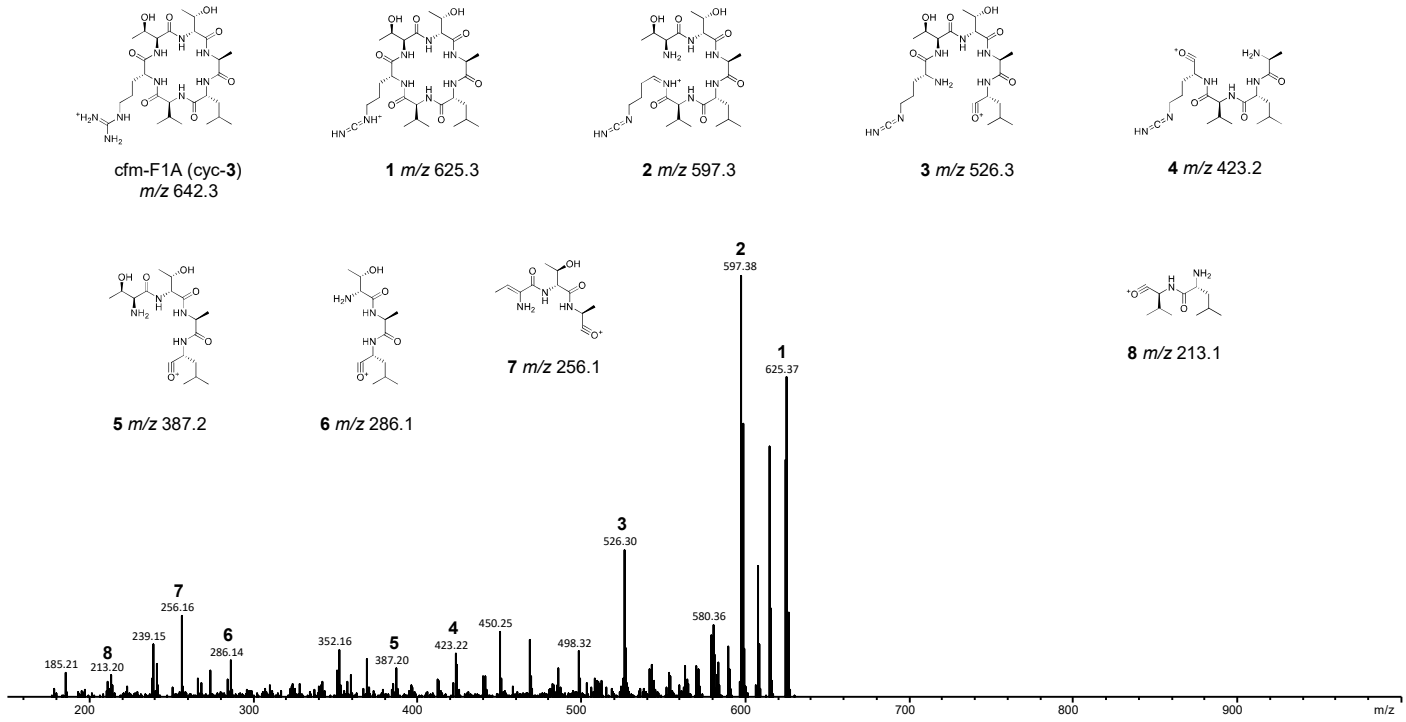


Figure S42. MS2 spectrum of cfm-F1A (cyc-**3**)

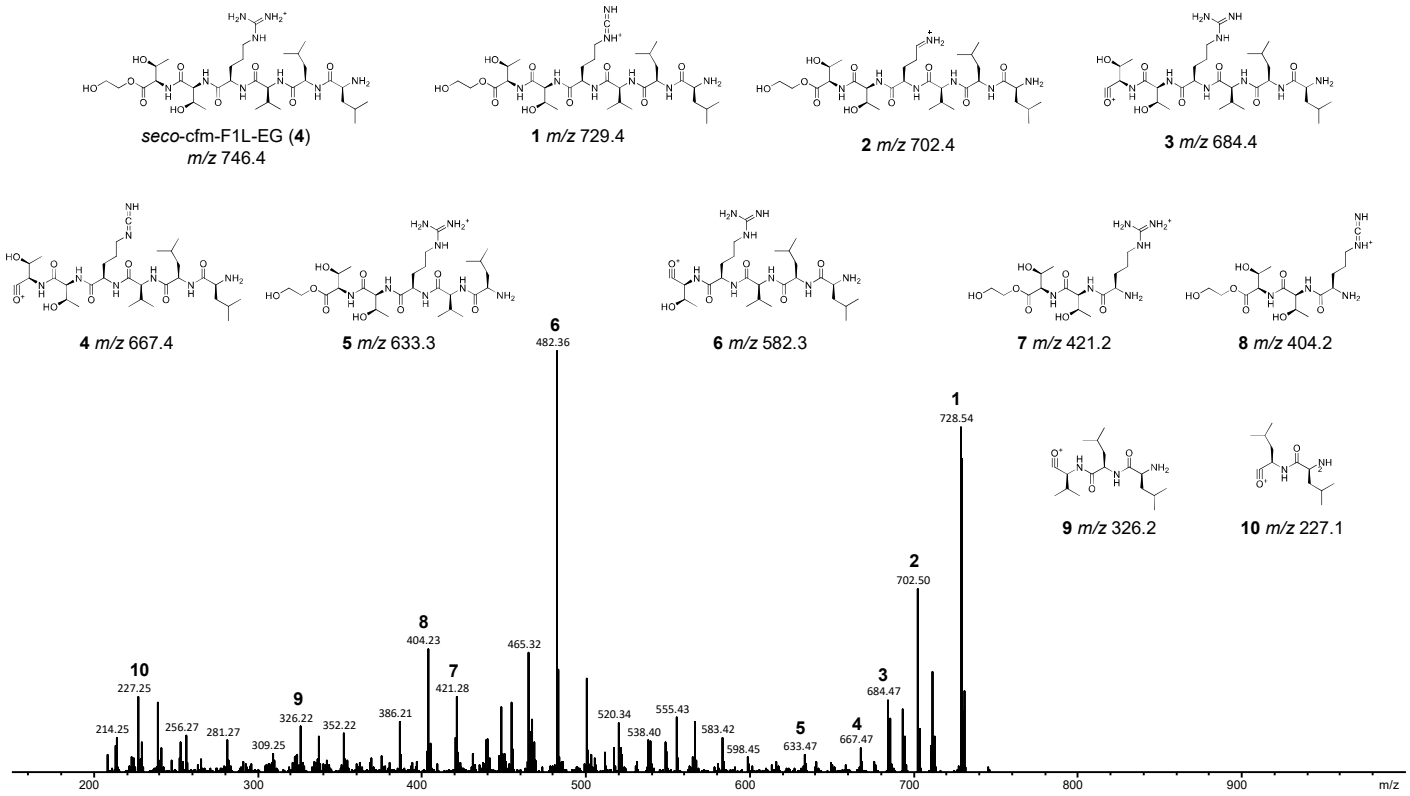


Figure S43. MS2 spectrum of *seco*-cfm-F1L-EG (**4**)

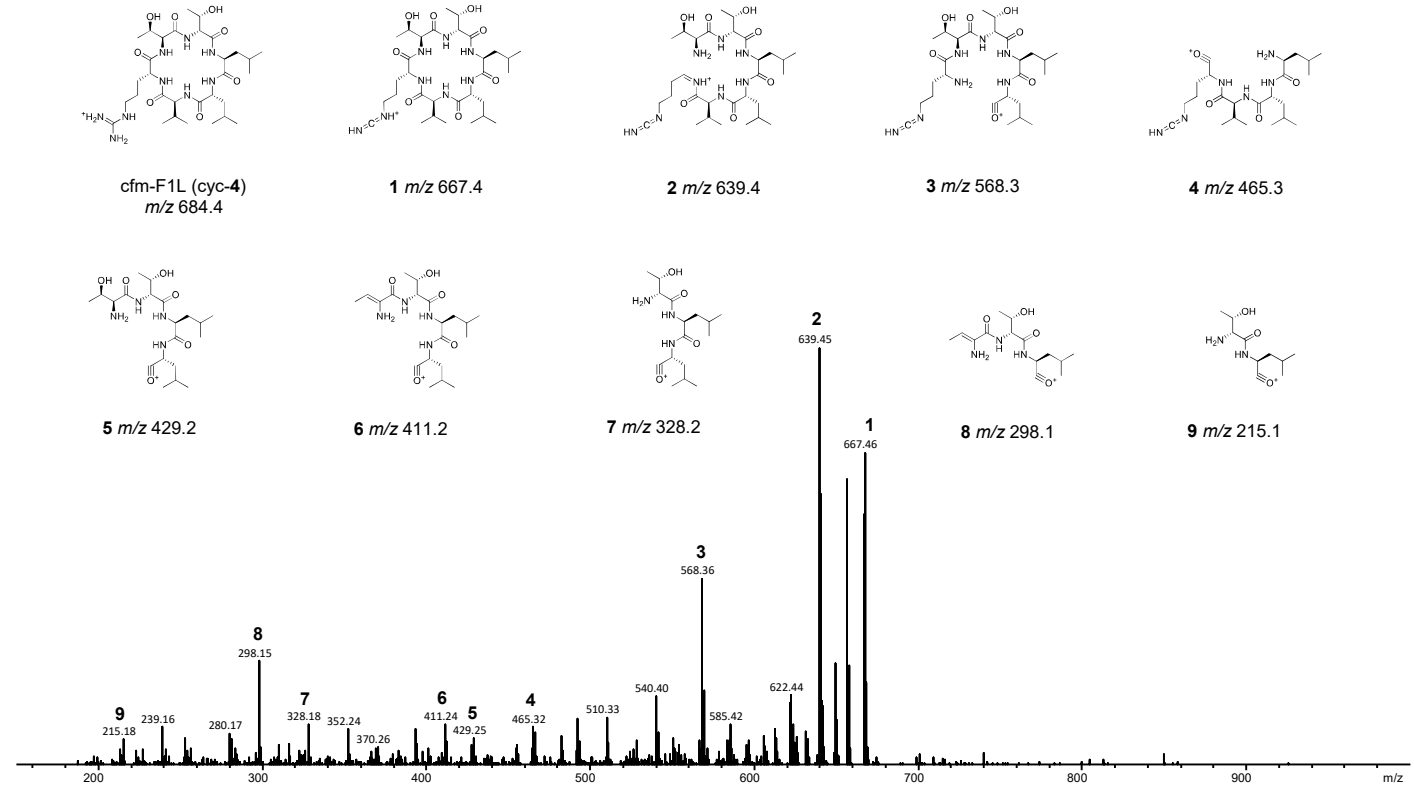


Figure S44. MS2 spectrum of cfm-F1L (cyc-**4**)

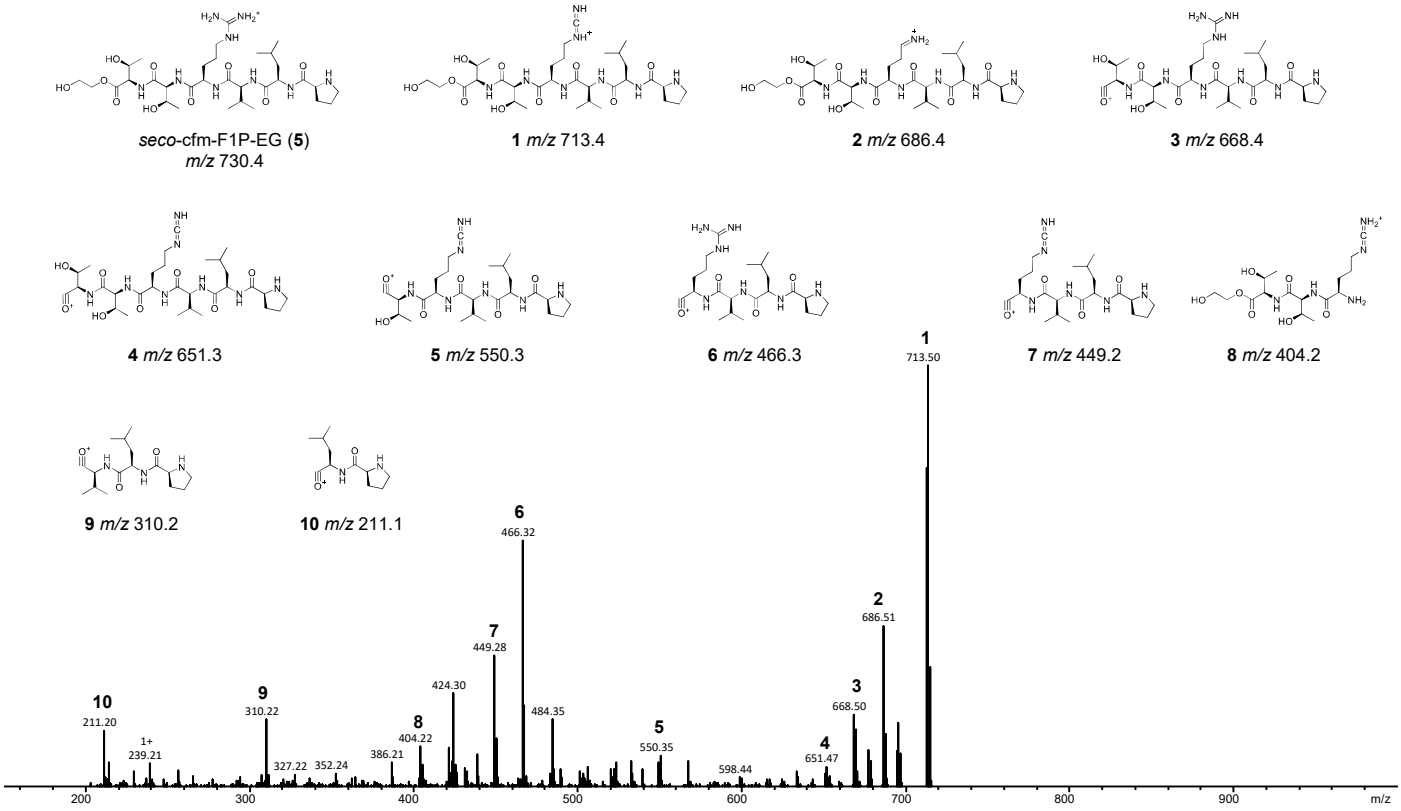


Figure S45. MS2 spectrum of *seco*-cfm-F1P-EG (**5**)

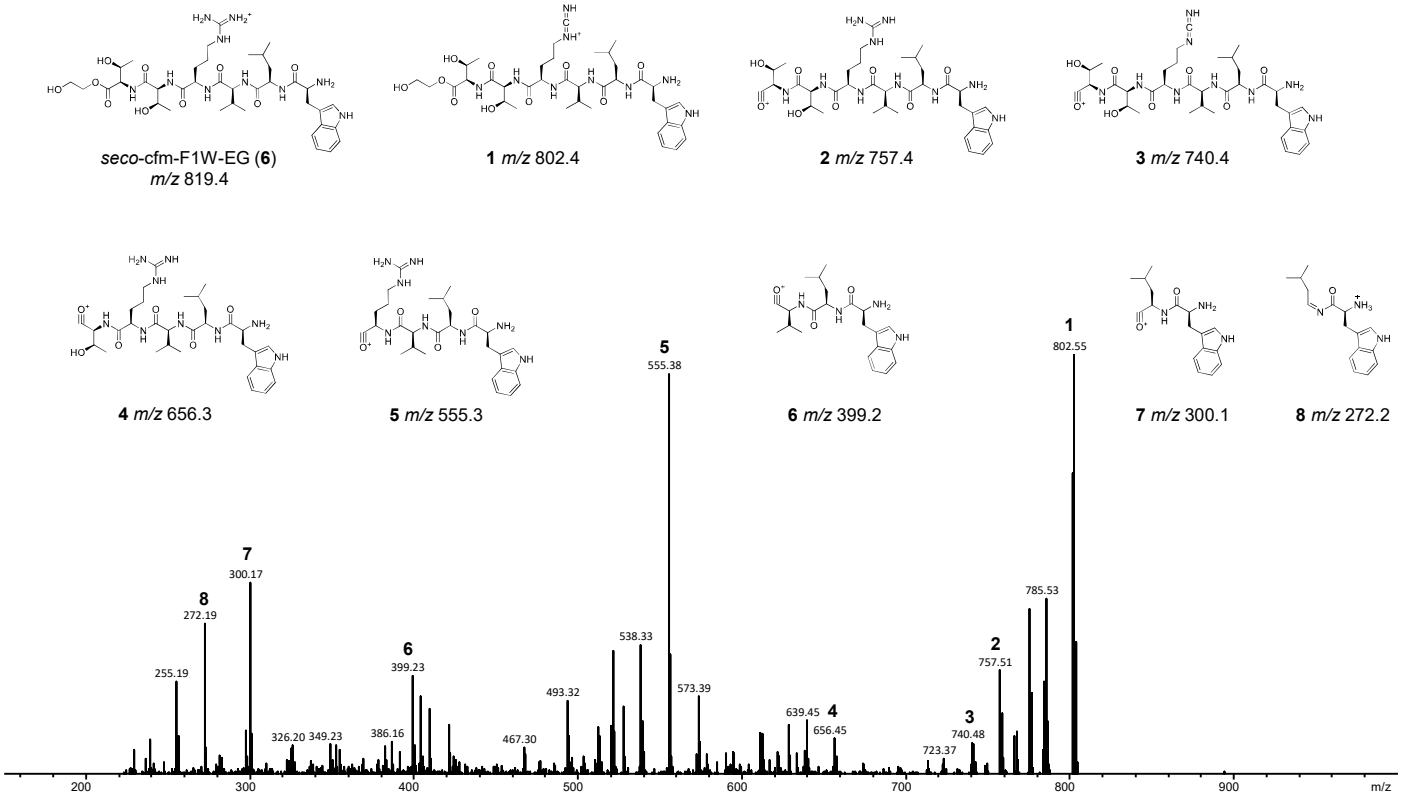


Figure S46. MS2 spectrum of *seco*-cfm-F1W-EG (**6**)

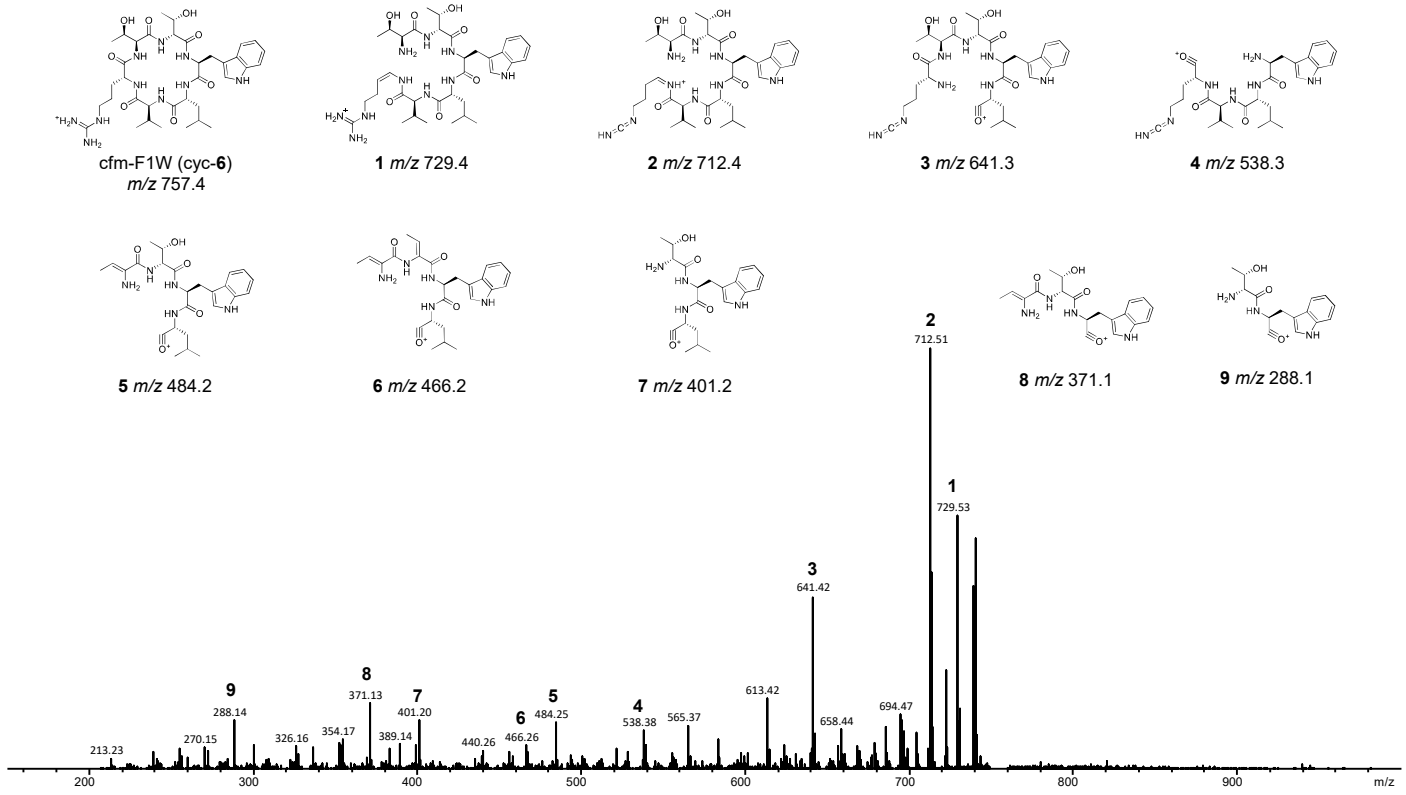


Figure S47. MS2 spectrum of cfm-F1W (cyc-**6**)

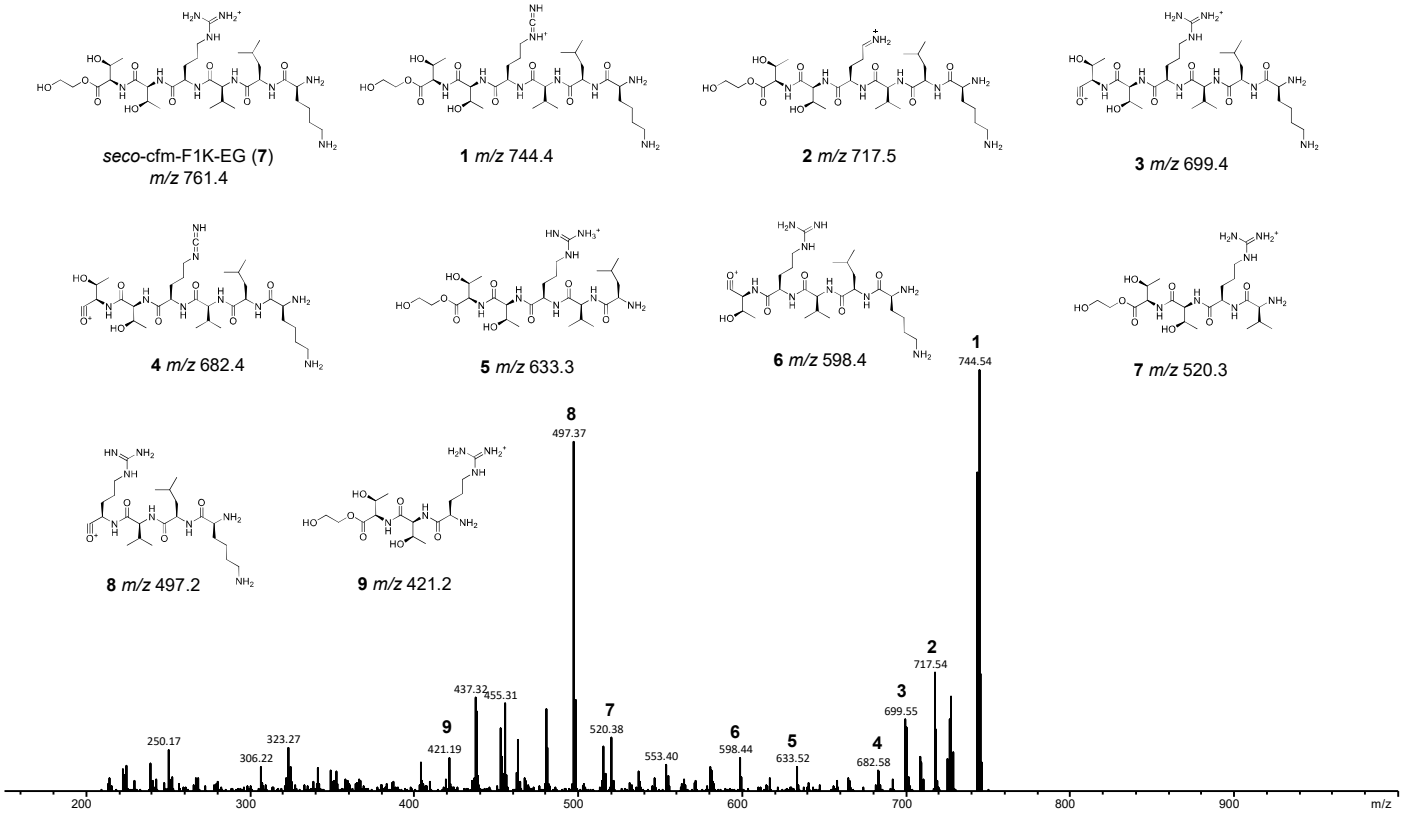


Figure S48. MS2 spectrum of *seco*-cfm-F1K-EG (**7**)

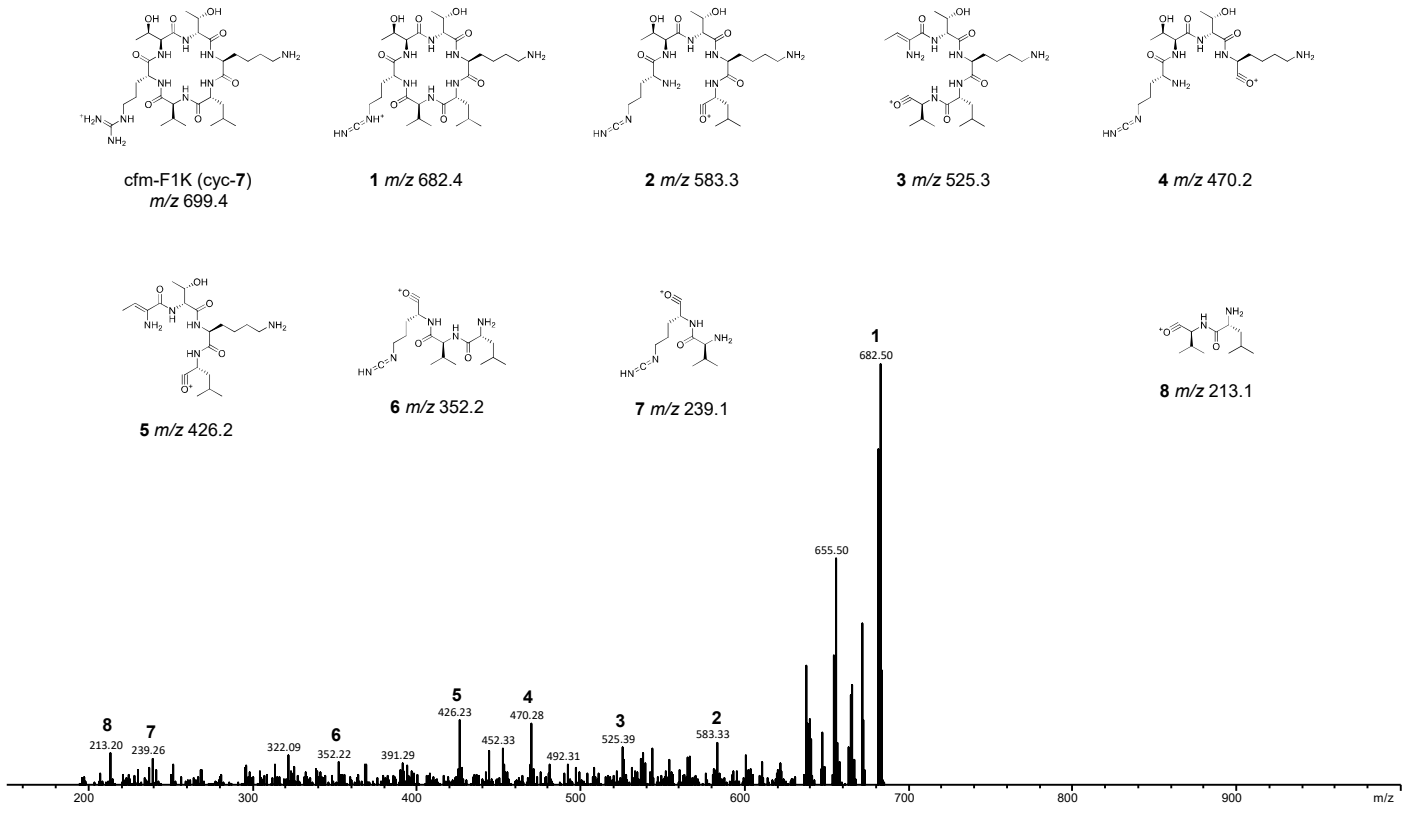


Figure S49. MS2 spectrum of cfm-F1K (cyc-**7**)

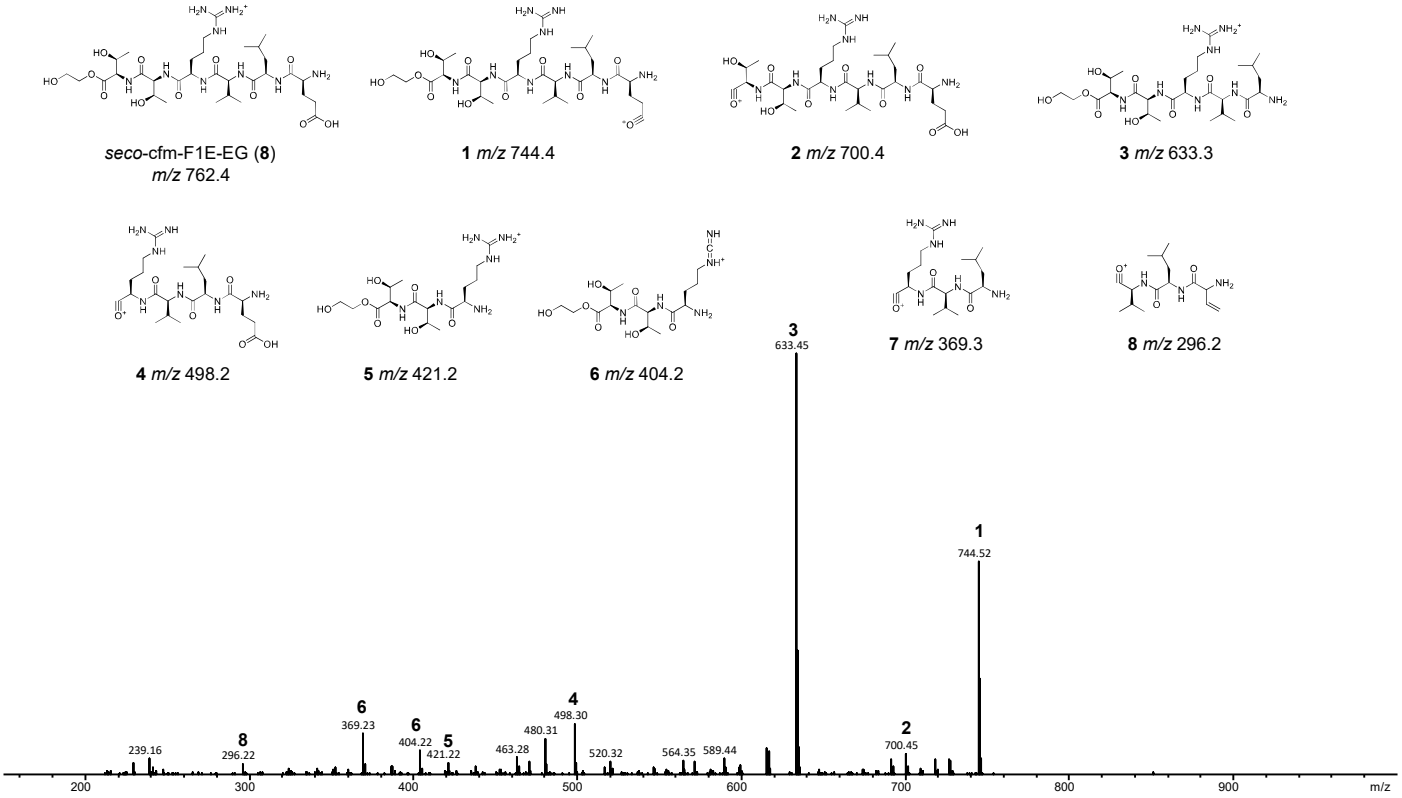


Figure S50. MS2 spectrum of *seco*-cfm-F1E-EG (**8**)

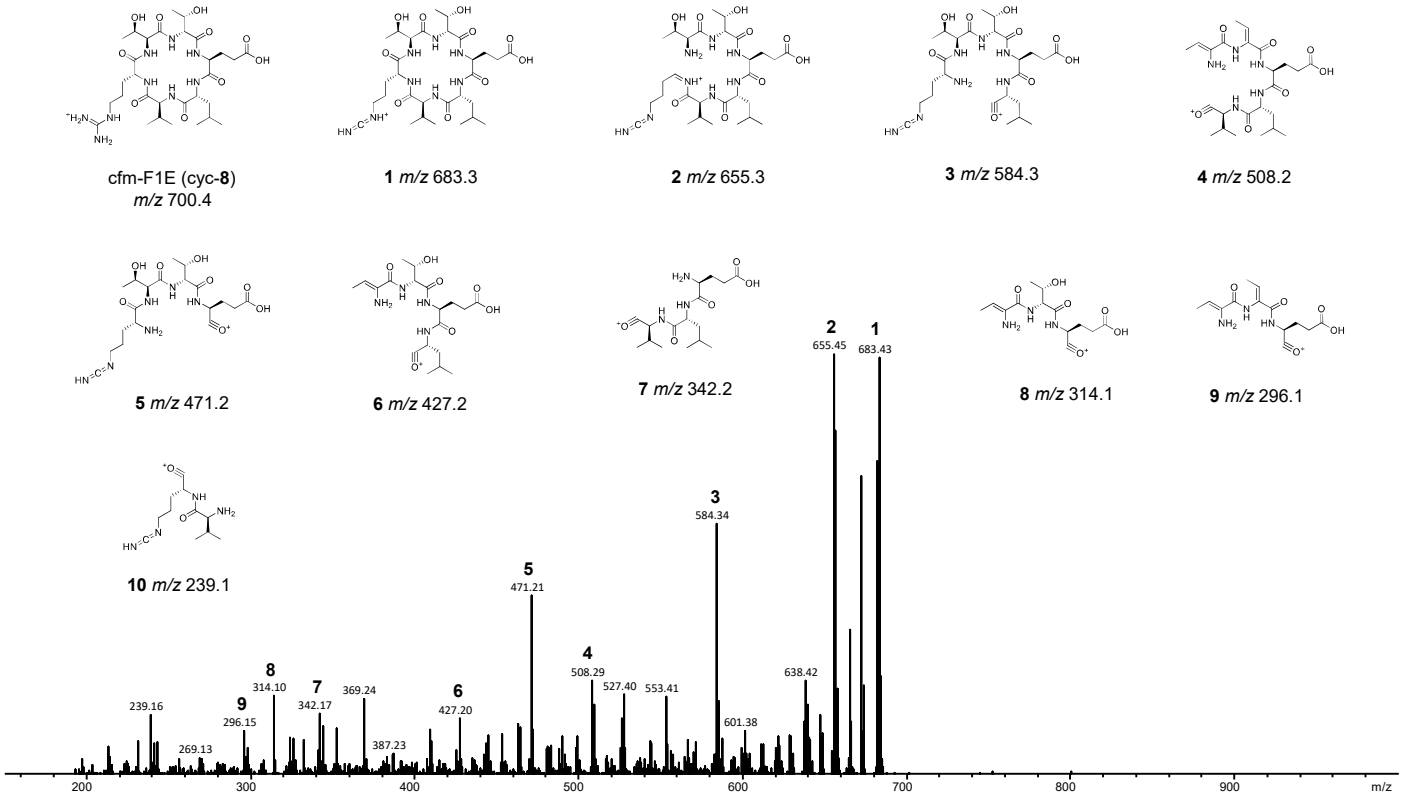


Figure S51. MS2 spectrum of cfm-F1E (cyc-**8**)

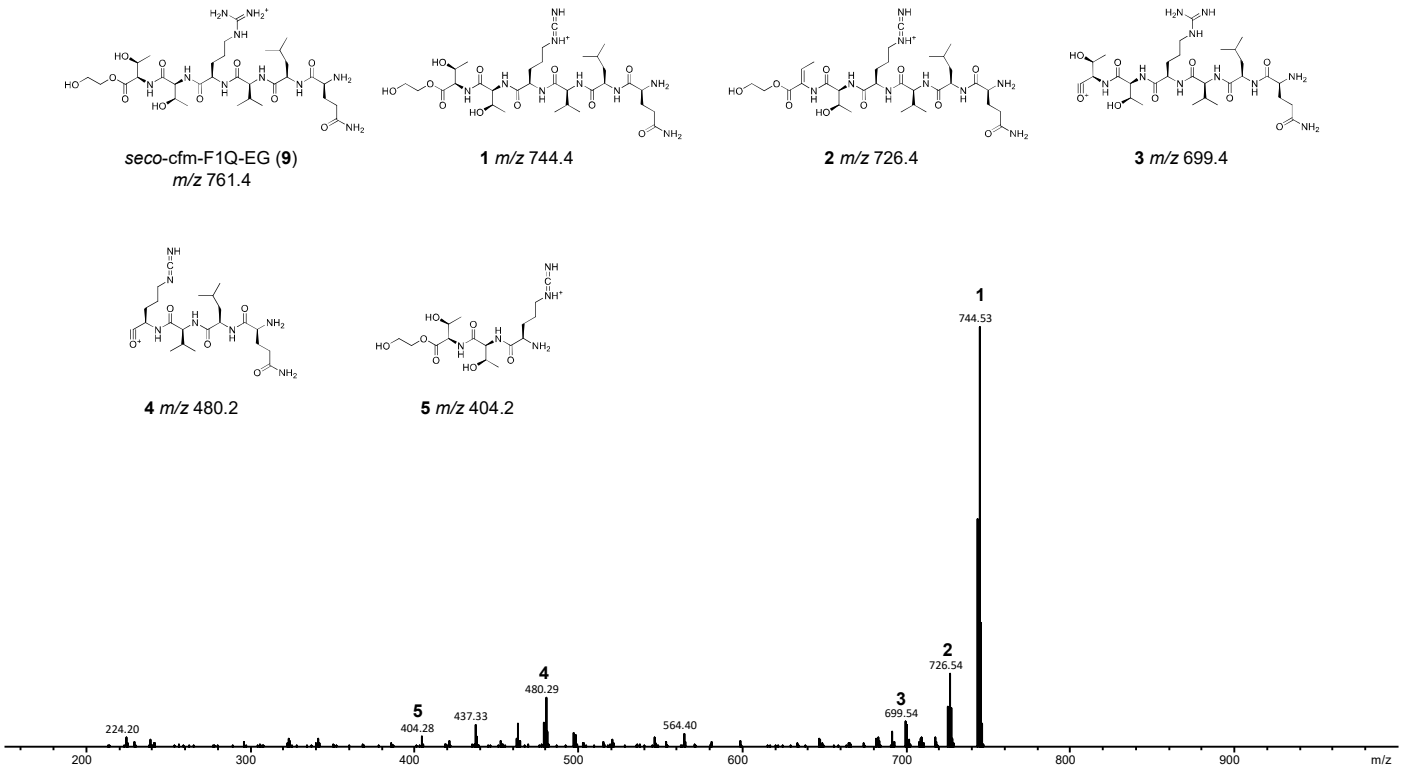


Figure S52. MS2 spectrum of *seco*-cfm-F1Q-EG (**9**)

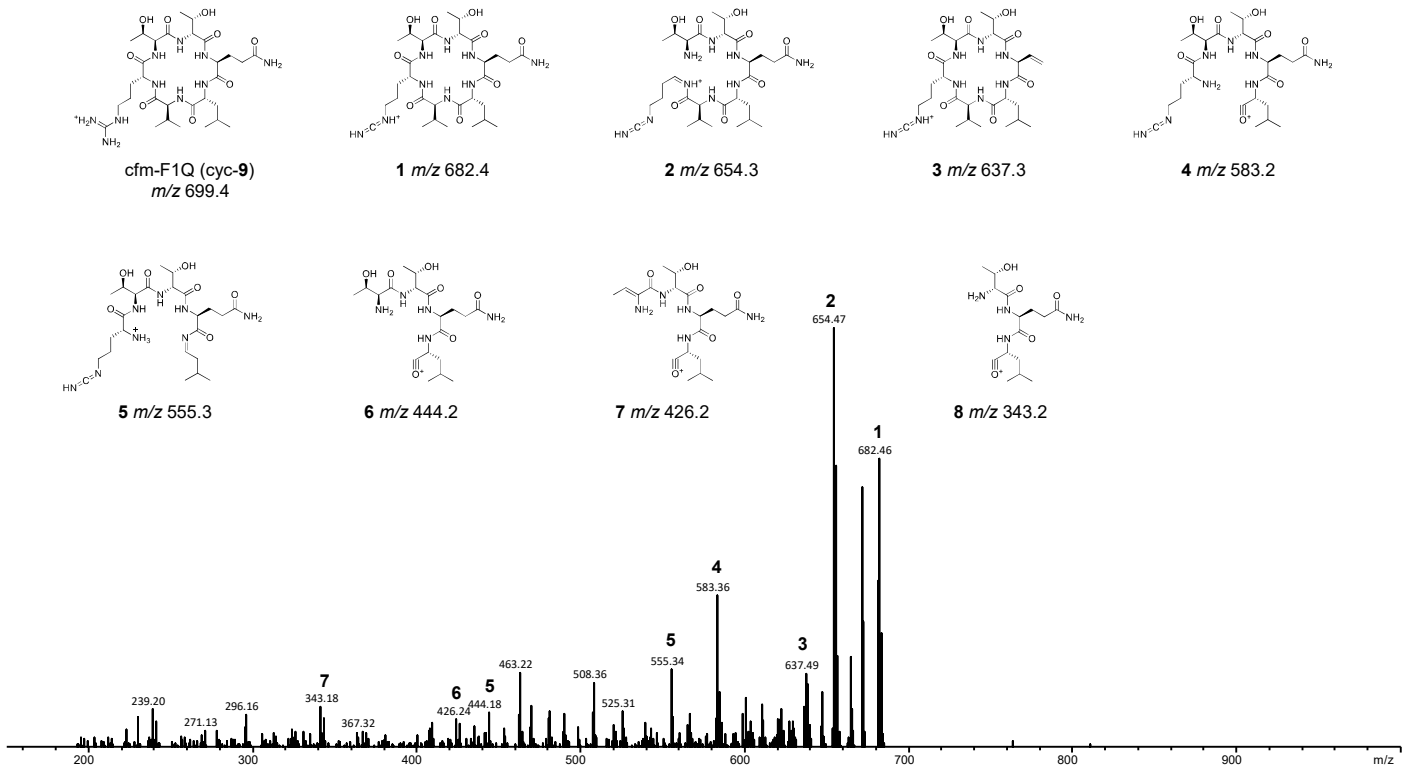


Figure S53. MS2 spectrum of cfm-F1Q (cyc-**9**)

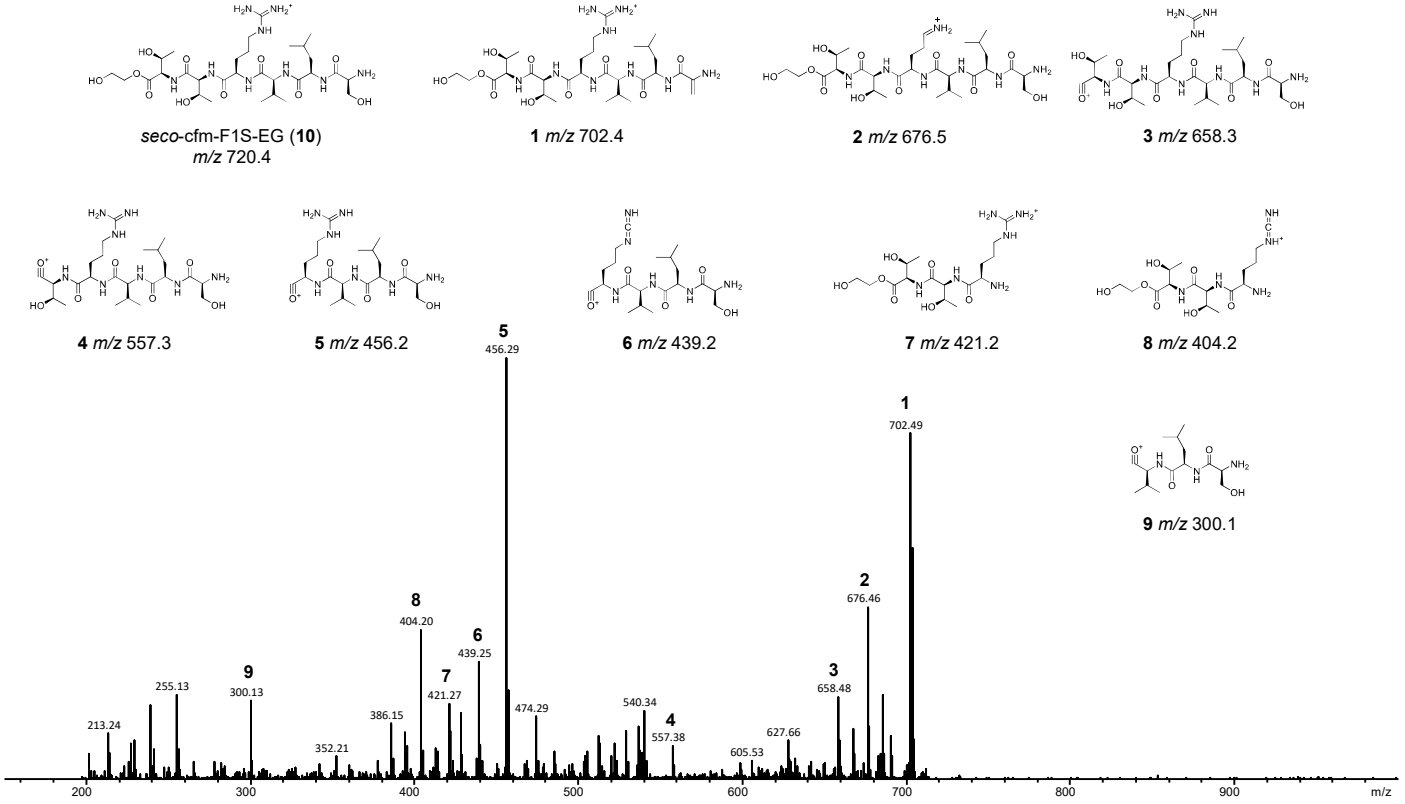


Figure S54. MS2 spectrum of *seco*-cfm-F1S-EG (**10**)

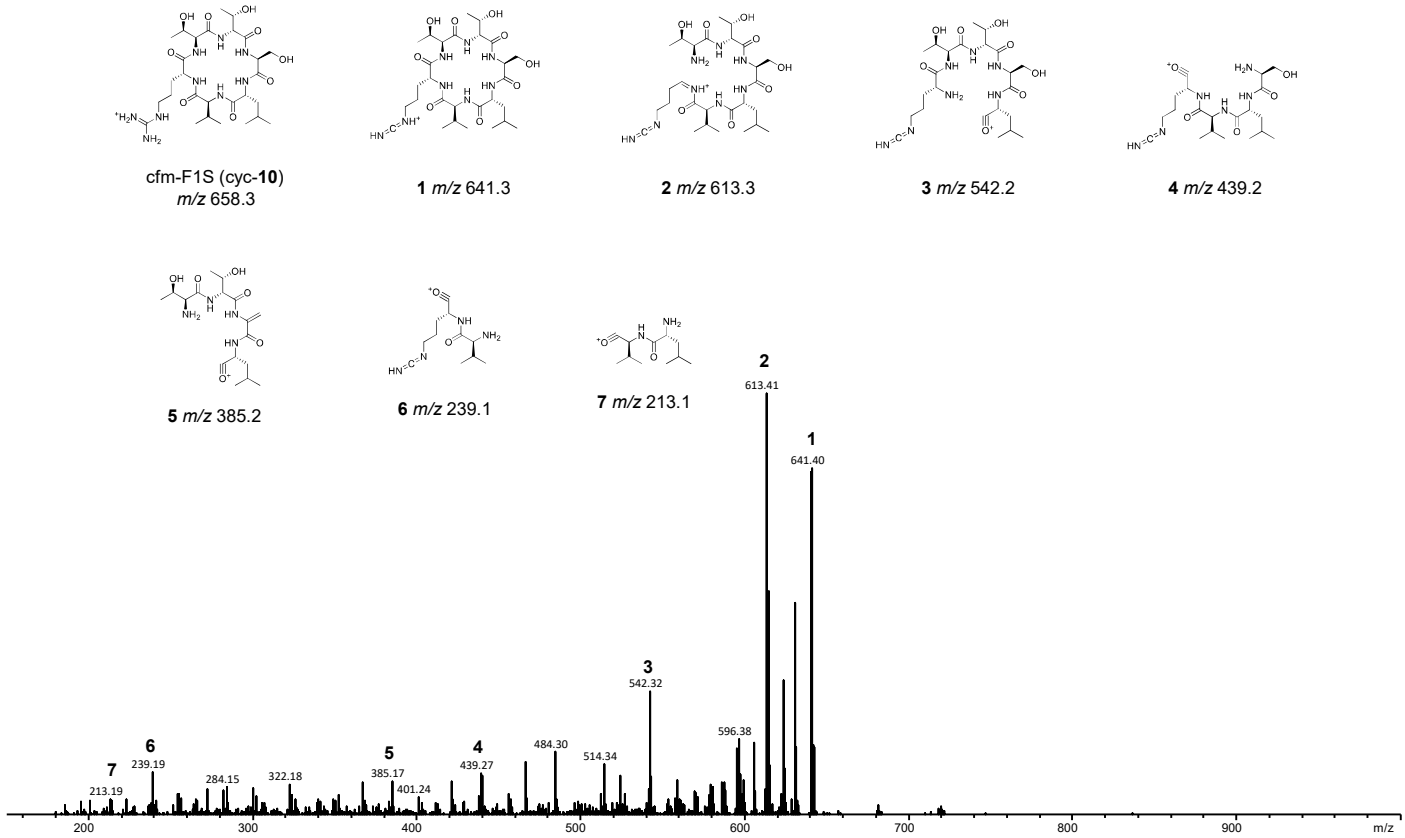


Figure S55. MS2 spectrum of cfm-F1S (cyc-**10**)

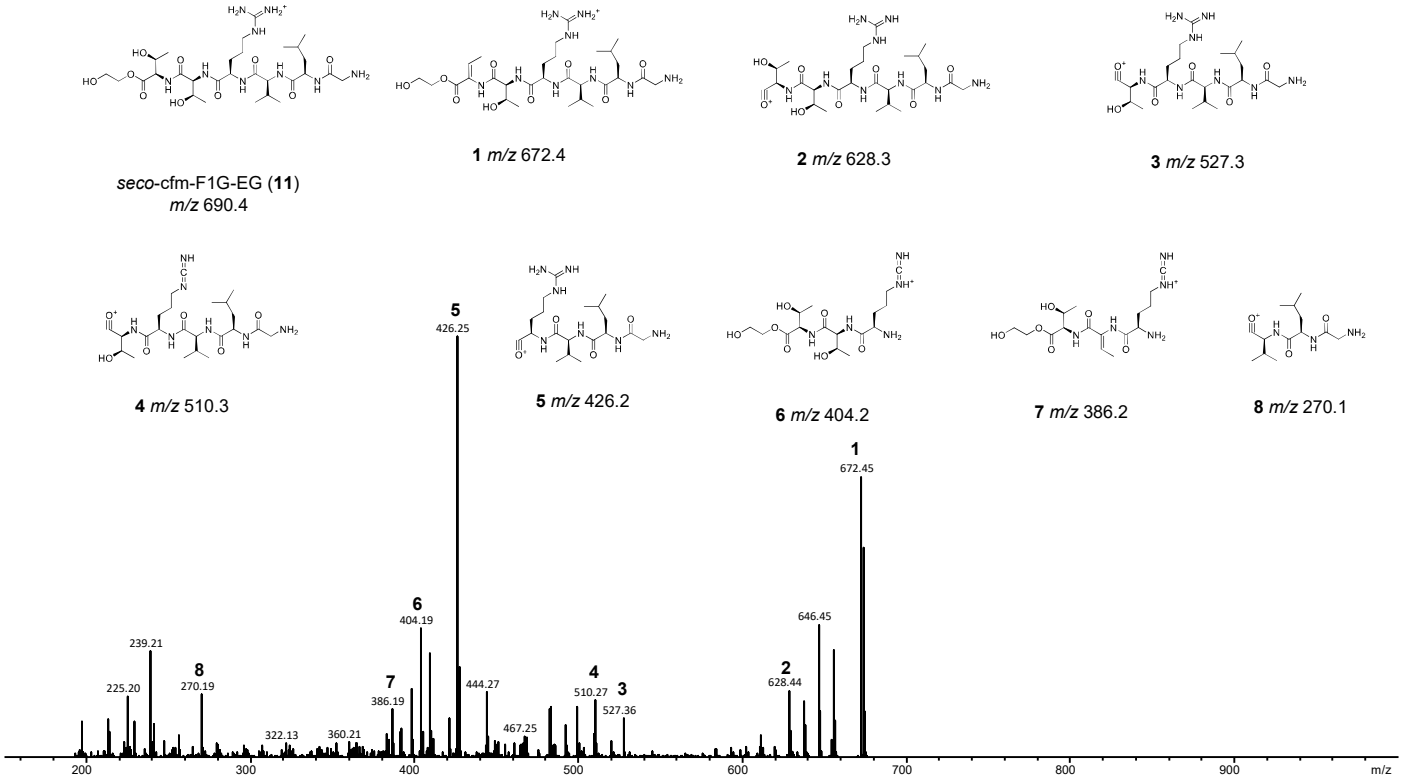


Figure S56. MS2 spectrum of *seco*-cfm-F1G-EG (**11**)

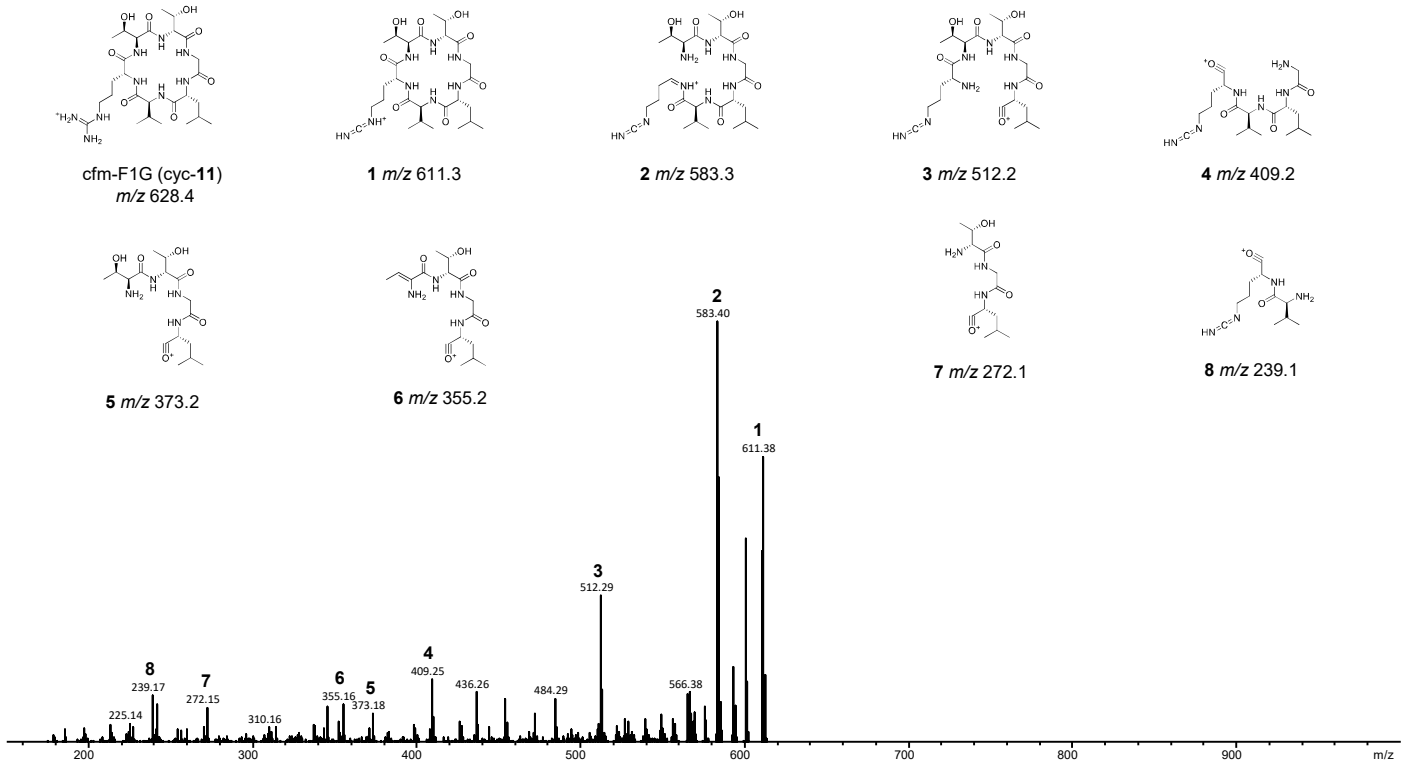


Figure S57. MS2 spectrum of cfm-F1G (cyc-**11**)

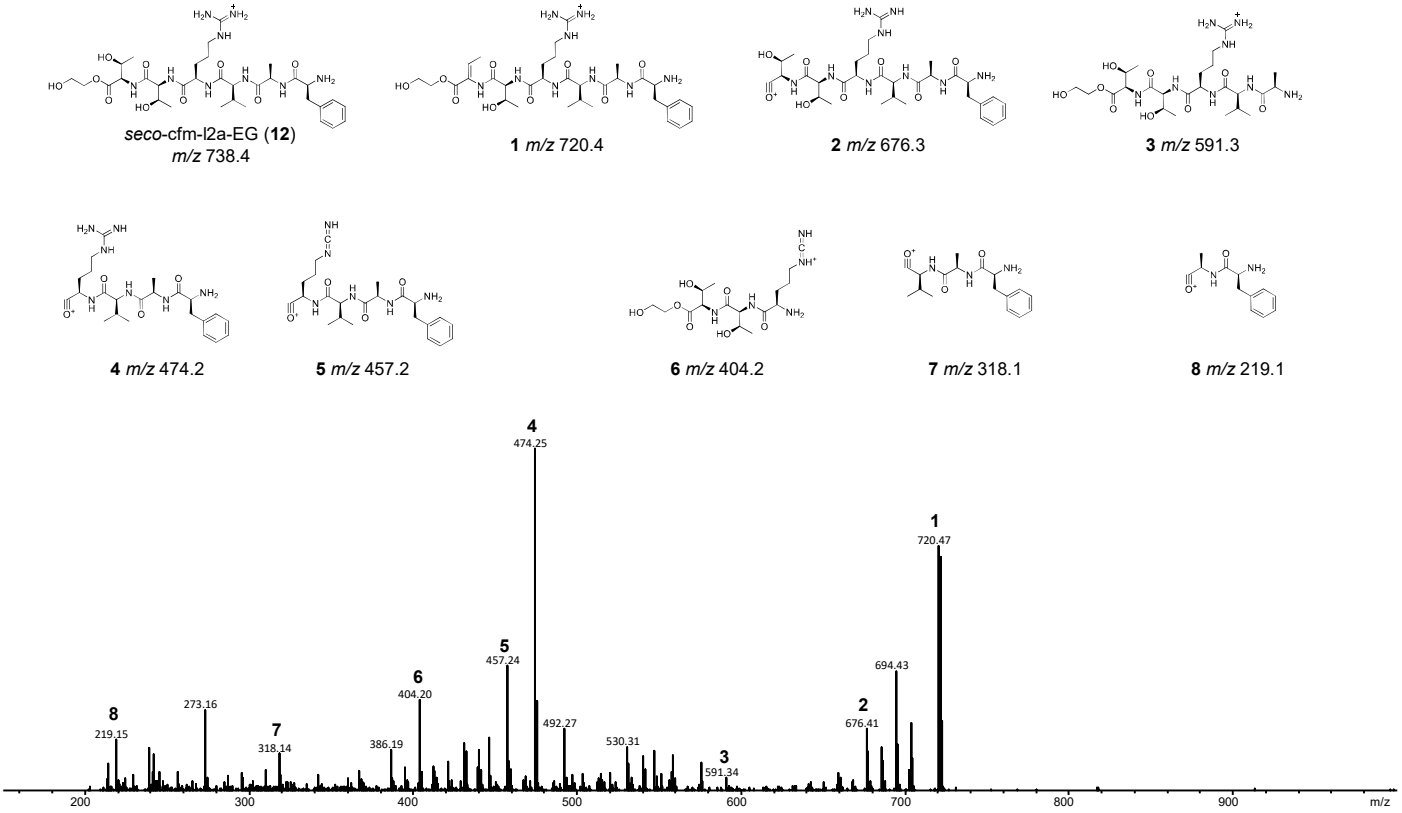


Figure S58. MS2 spectrum of *seco*-cfm-l2a-EG (**12**)

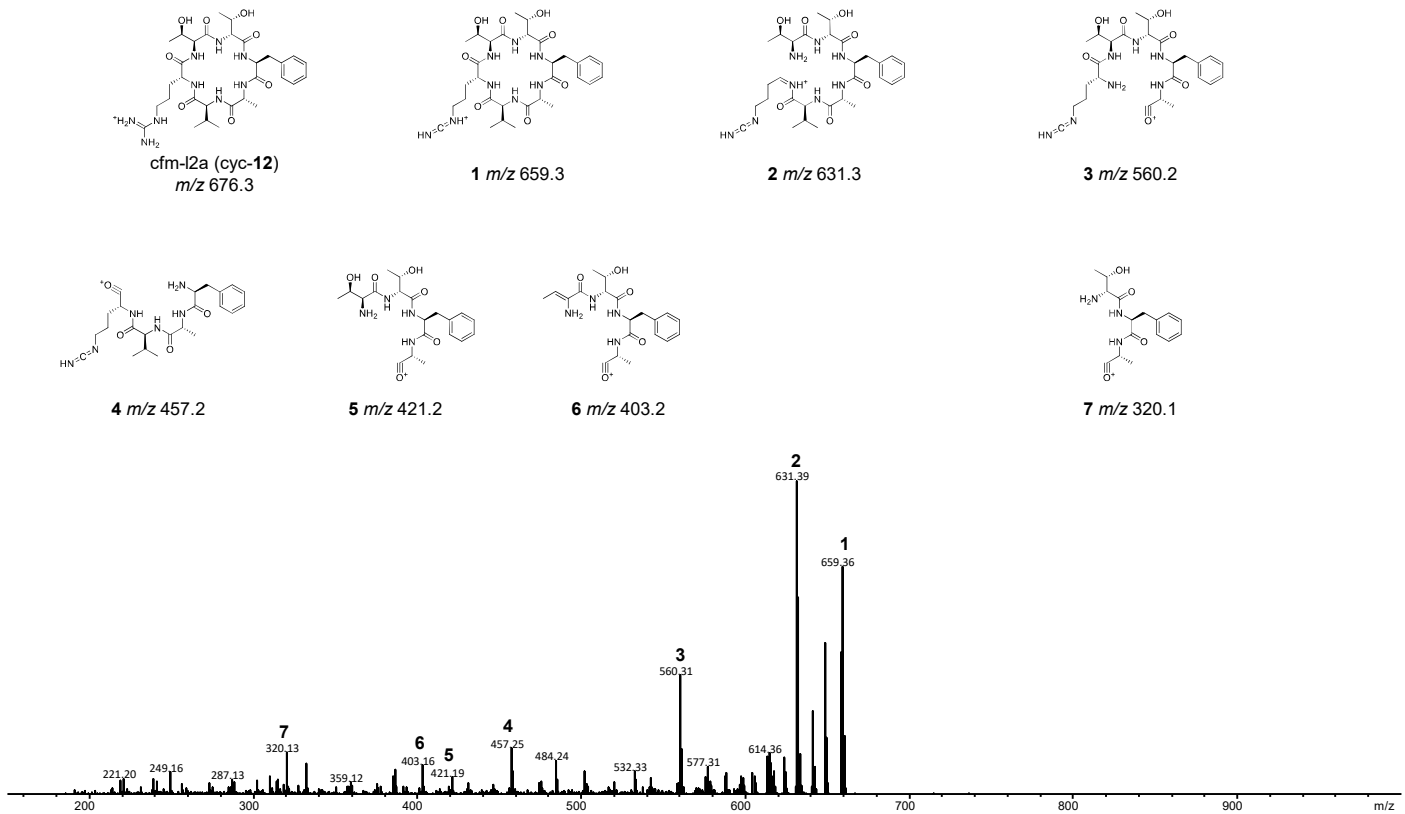


Figure S59. MS2 spectrum of cfm-l2a (cyc-**12**)

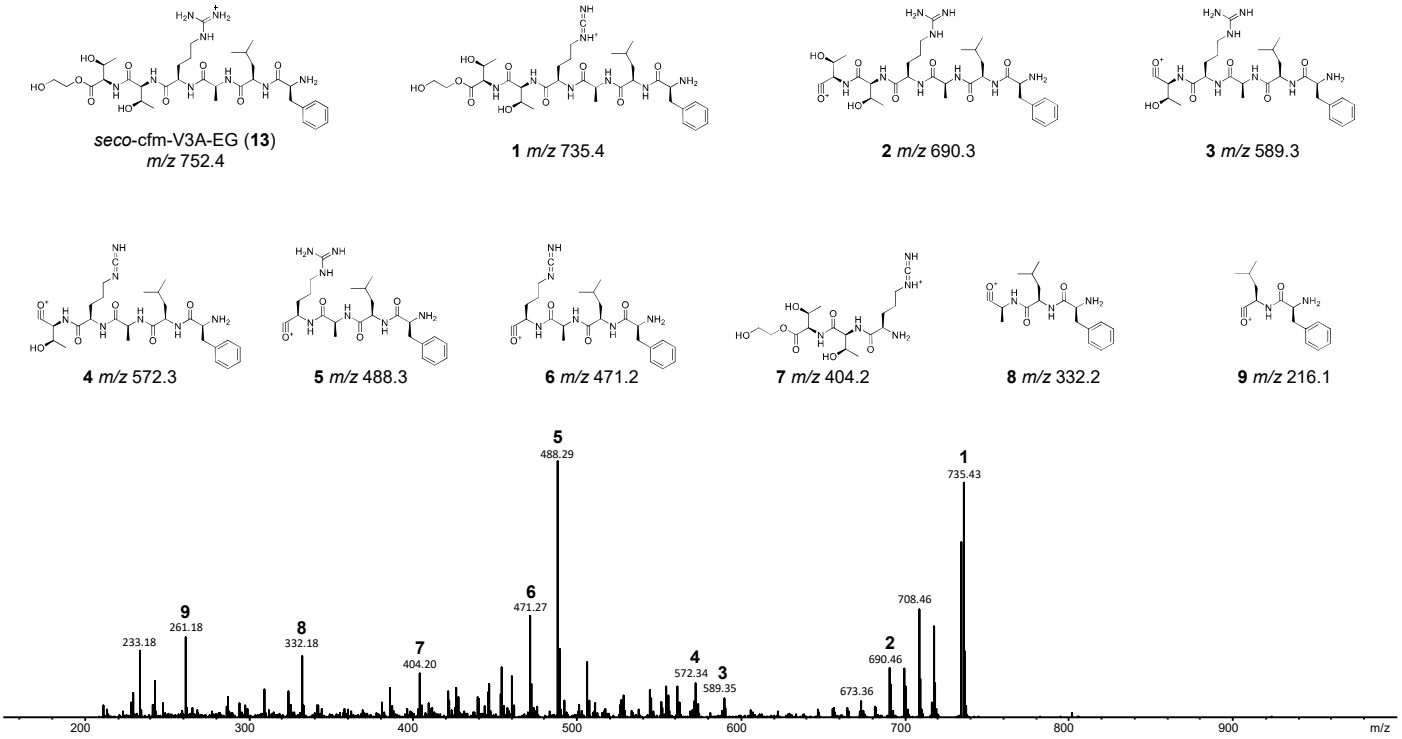


Figure S60. MS2 spectrum of *seco*-cfm-V3A-EG (**13**)

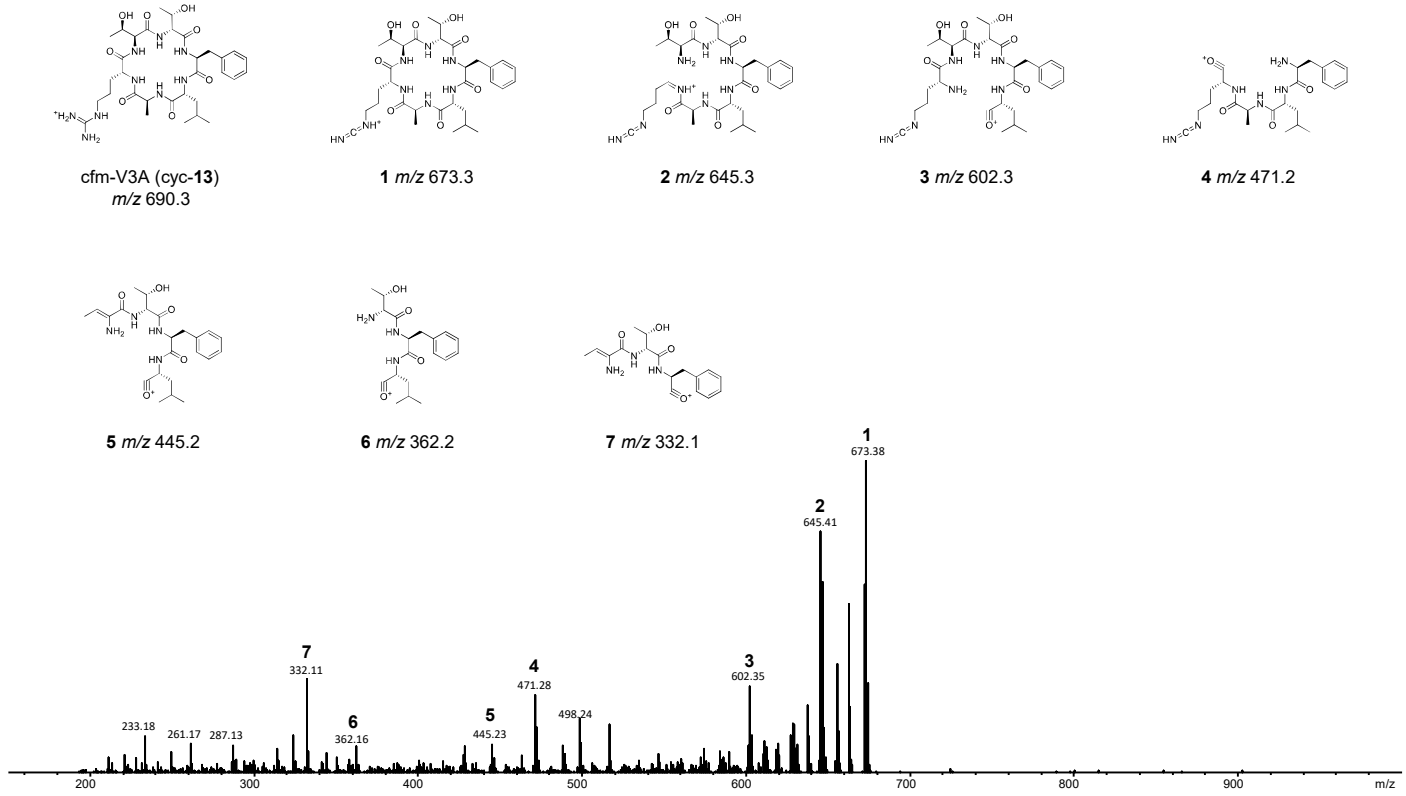


Figure S61. MS2 spectrum of cfm-V3A (cyc-**13**)

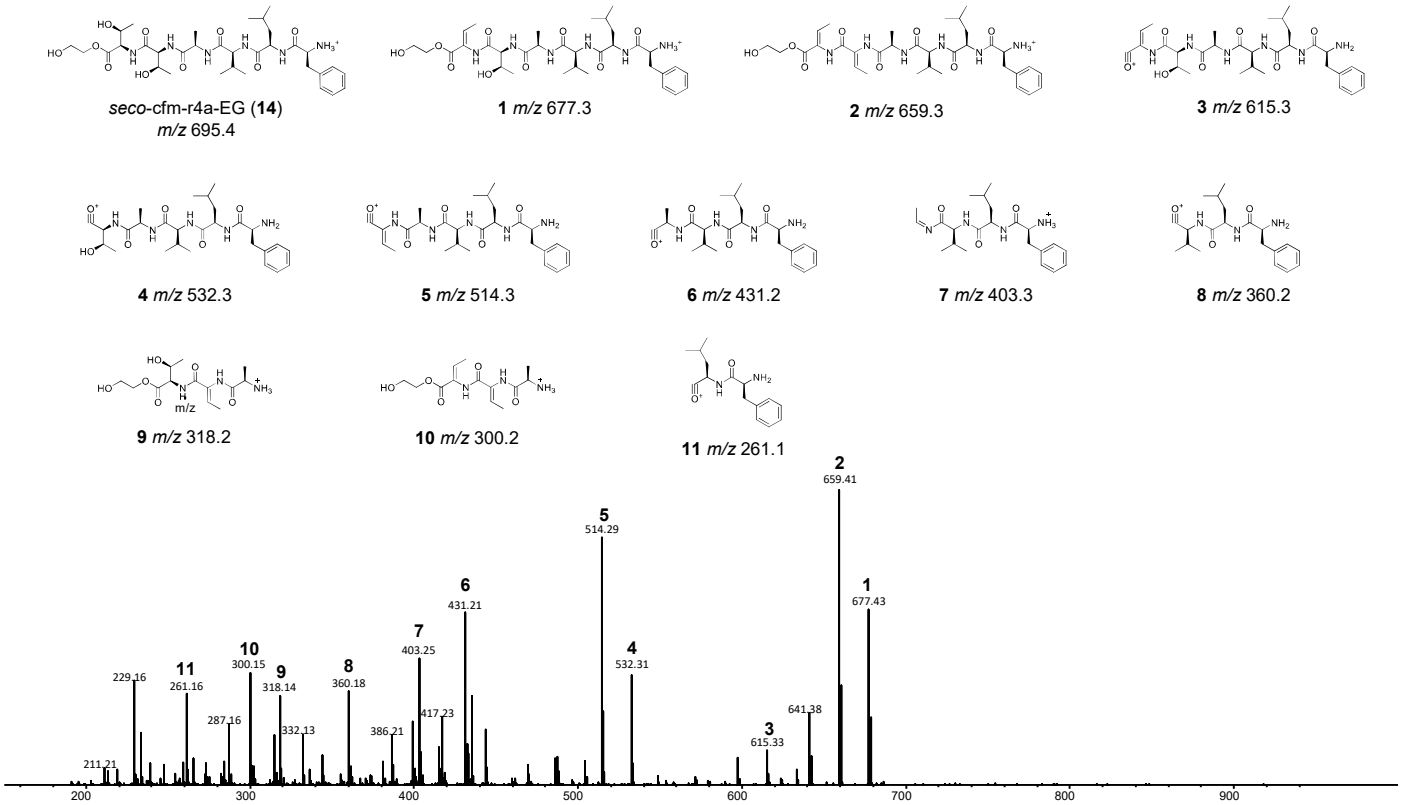


Figure S62. MS2 spectrum of *seco*-cfm-r4a-EG (**14**)

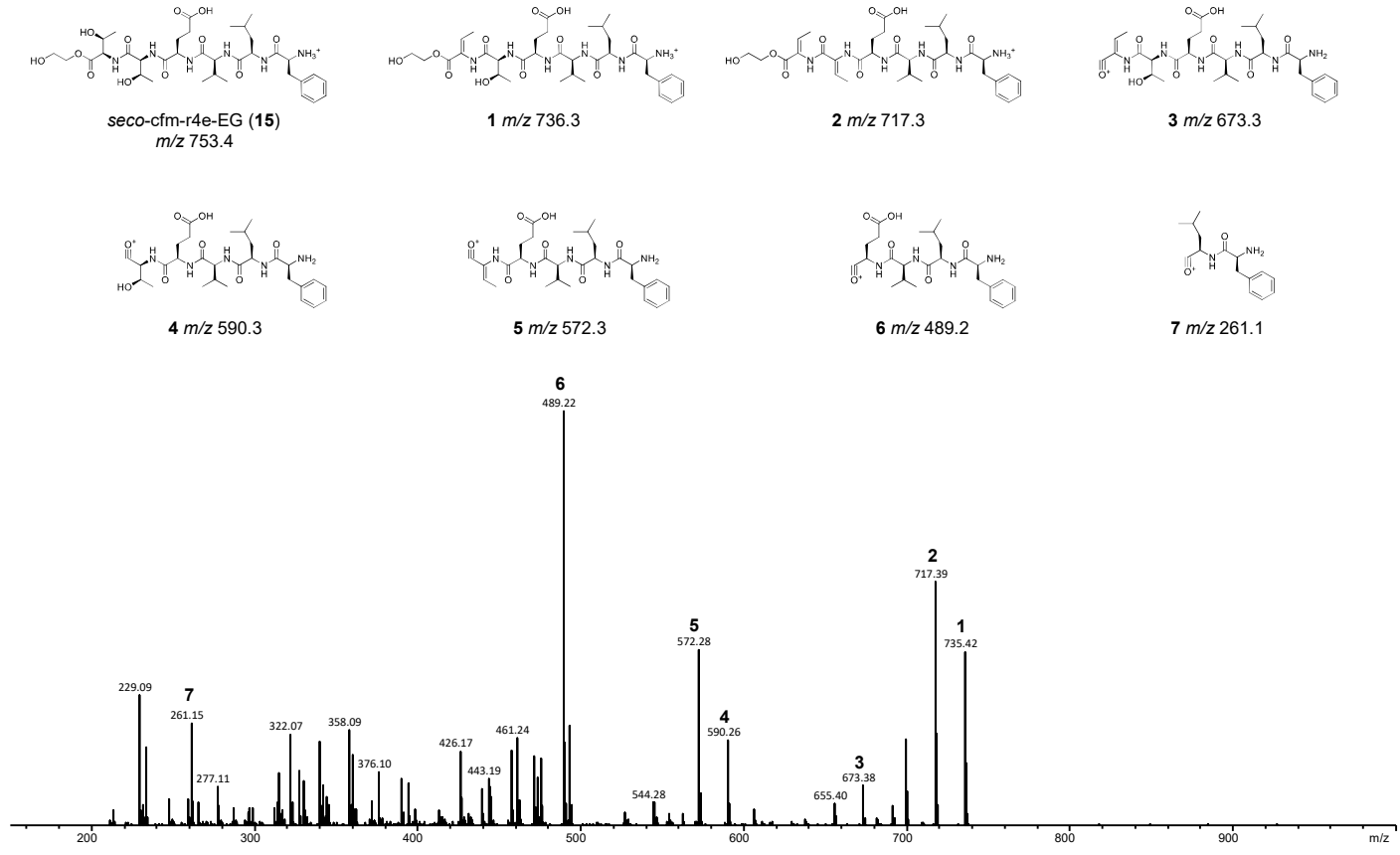


Figure S63. MS2 spectrum of *seco*-cfm-r4e-EG (**15**)

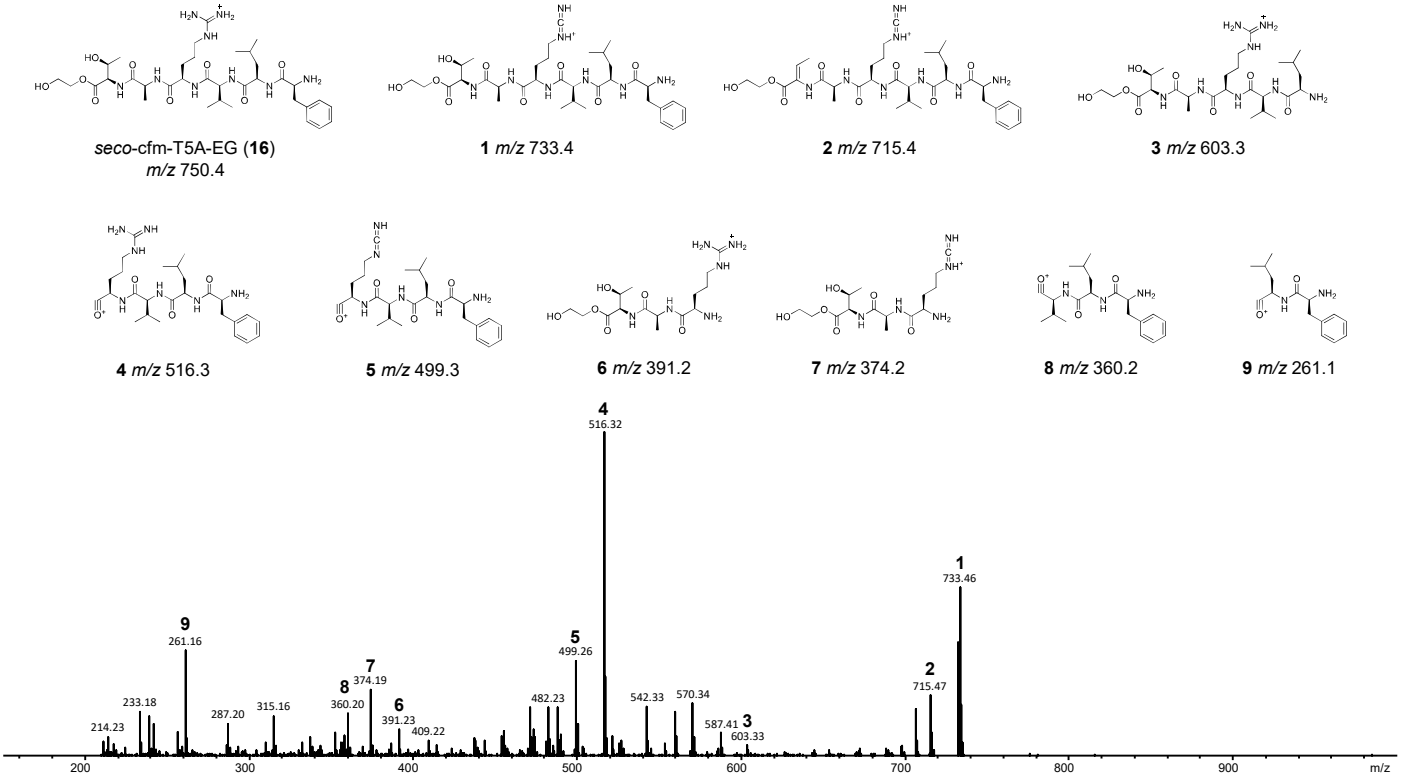


Figure S64. MS2 spectrum of *seco*-cfm-T5A-EG (**16**)

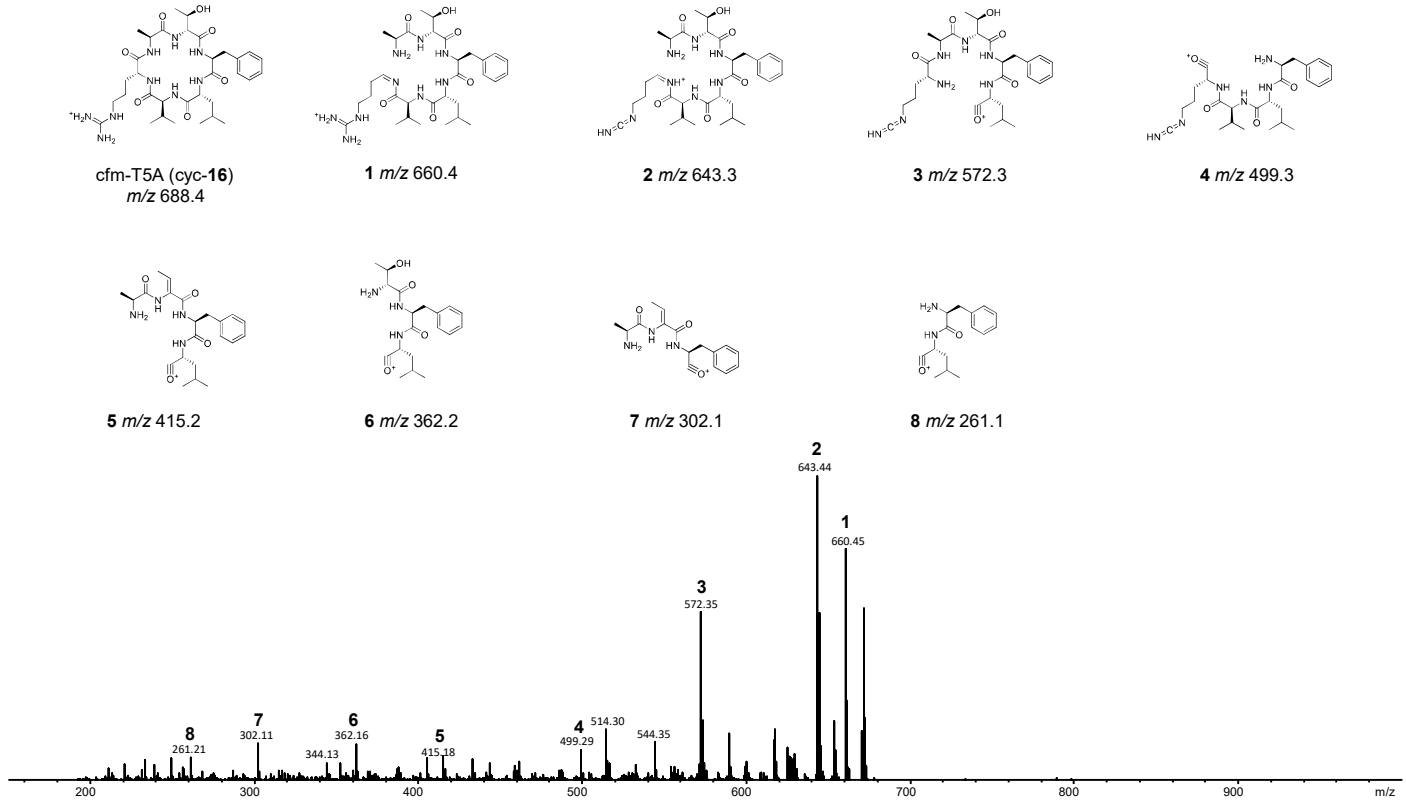


Figure S65. MS2 spectrum of cfm-T5A (cyc-**16**)

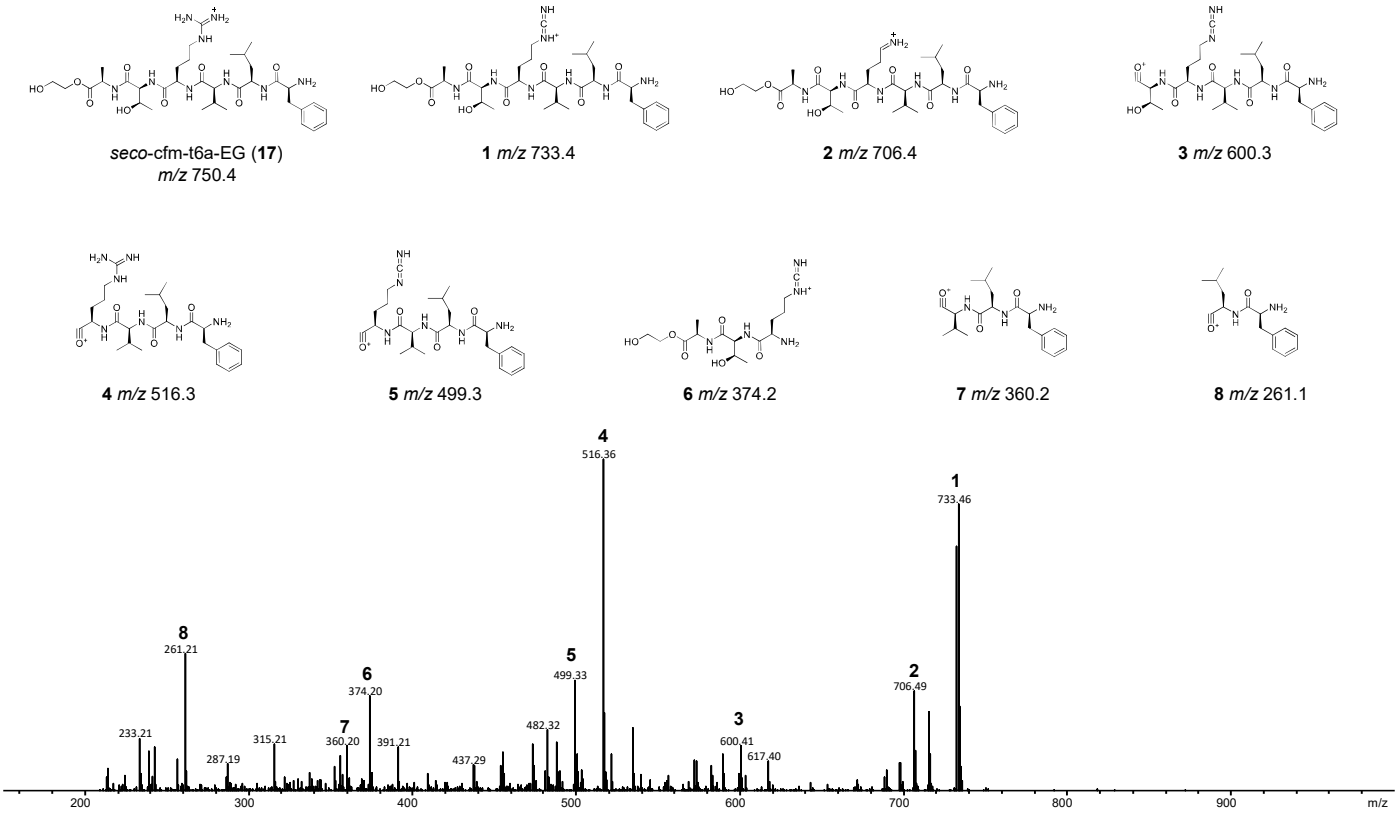


Figure S66. MS2 spectrum of *seco*-cfm-t6a-EG (**17**)

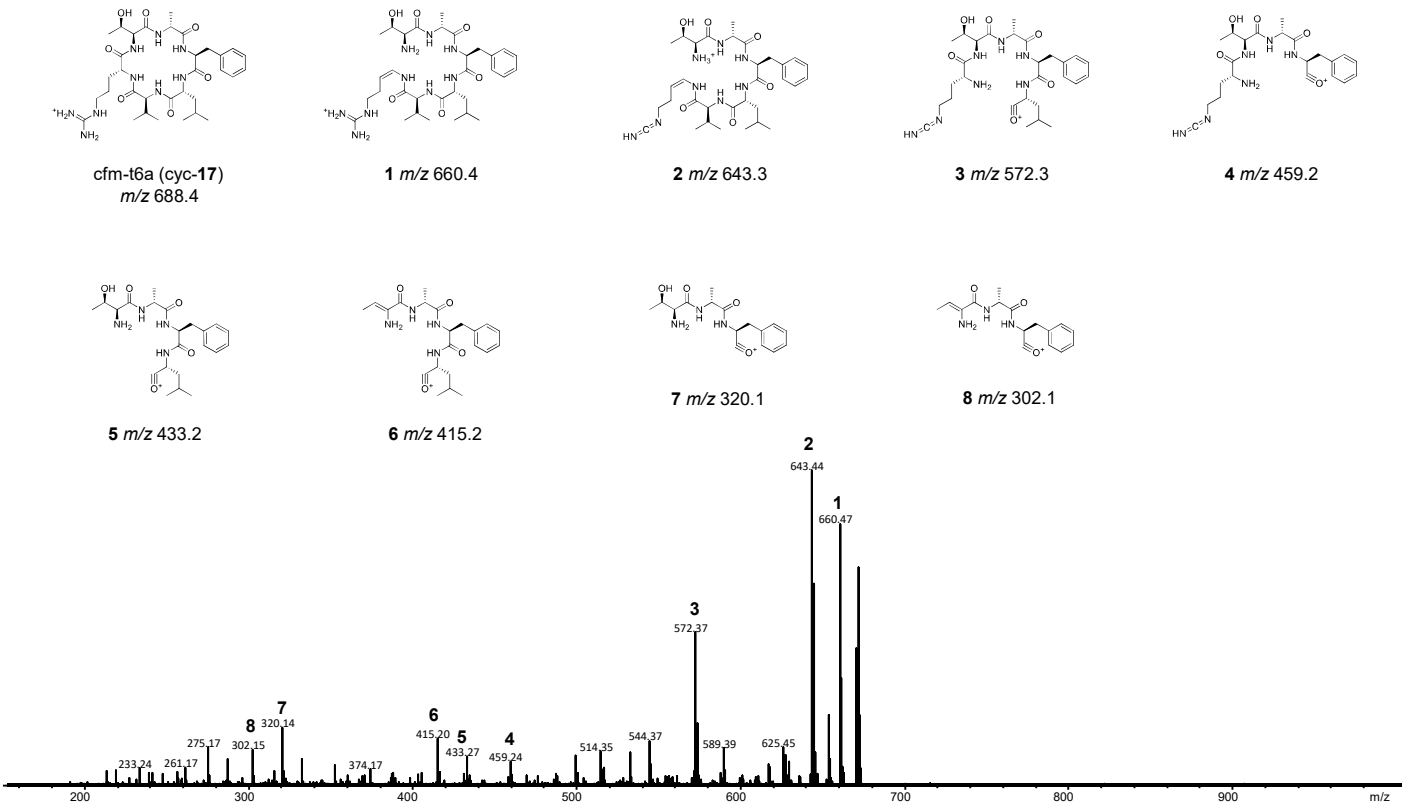


Figure S67. MS2 spectrum of cfm-t6a (cyc-**17**)

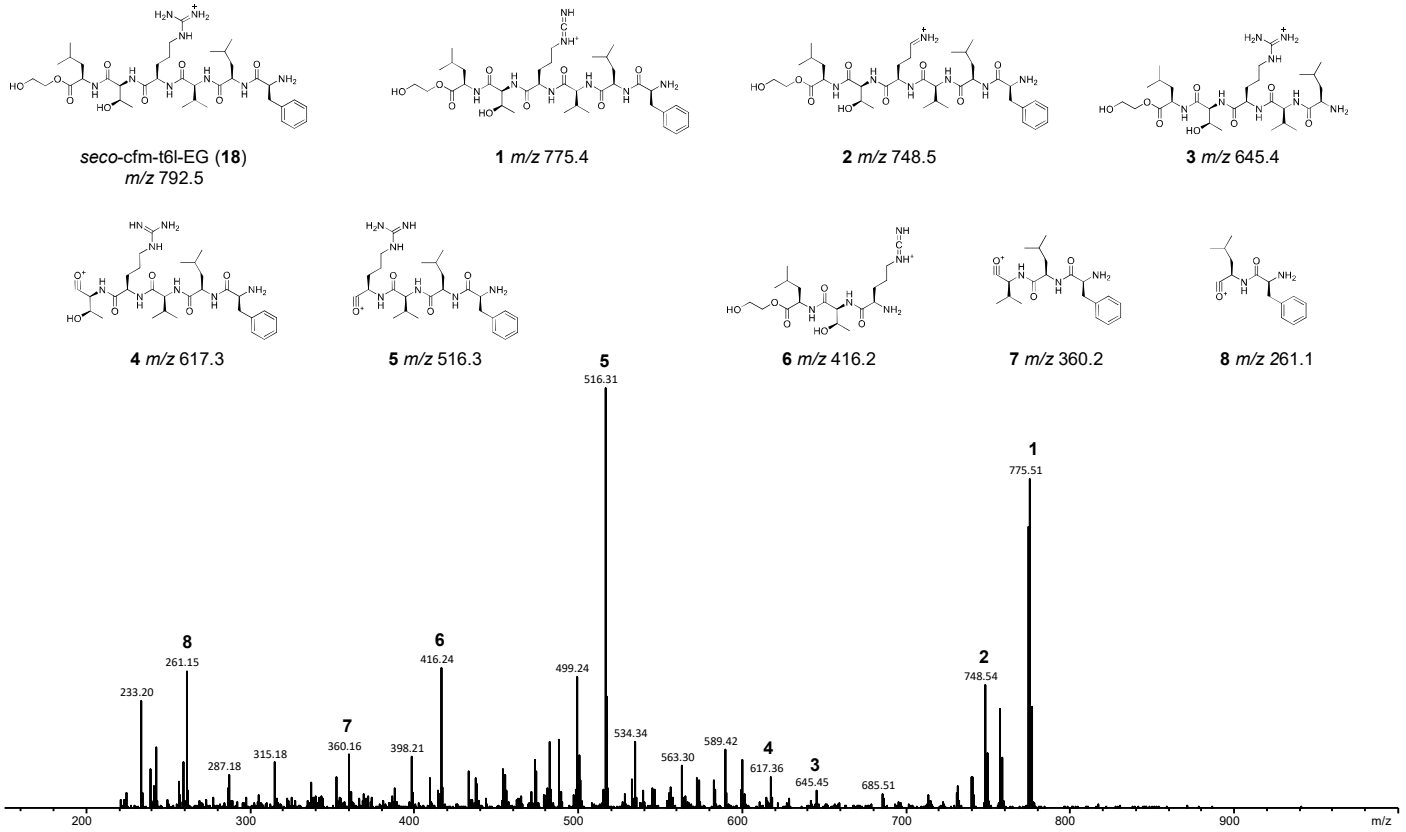


Figure S68. MS2 spectrum of *seco*-cfm-t6l-EG (**18**)

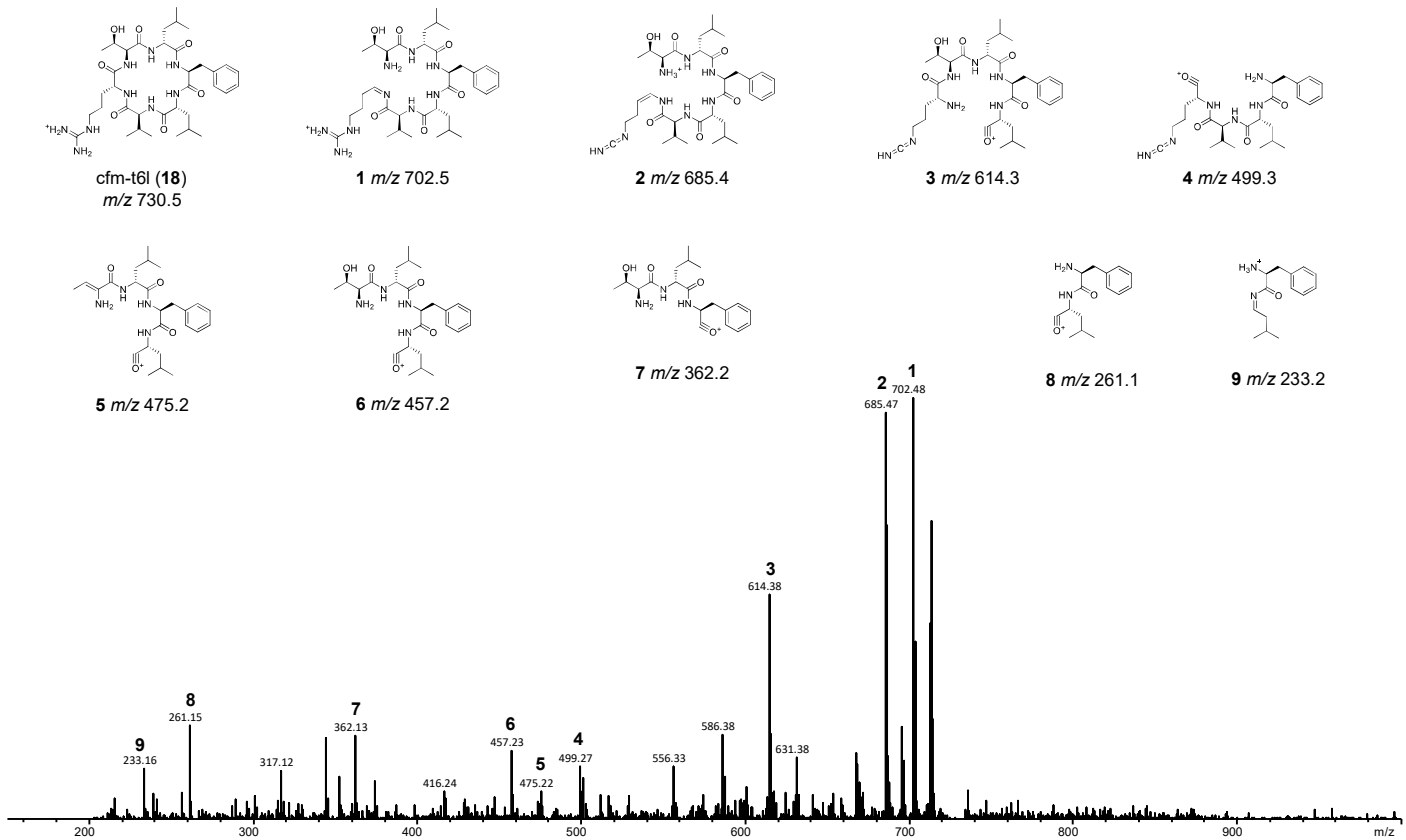


Figure S69. MS2 spectrum of cfm-t6l (cyc-**18**)

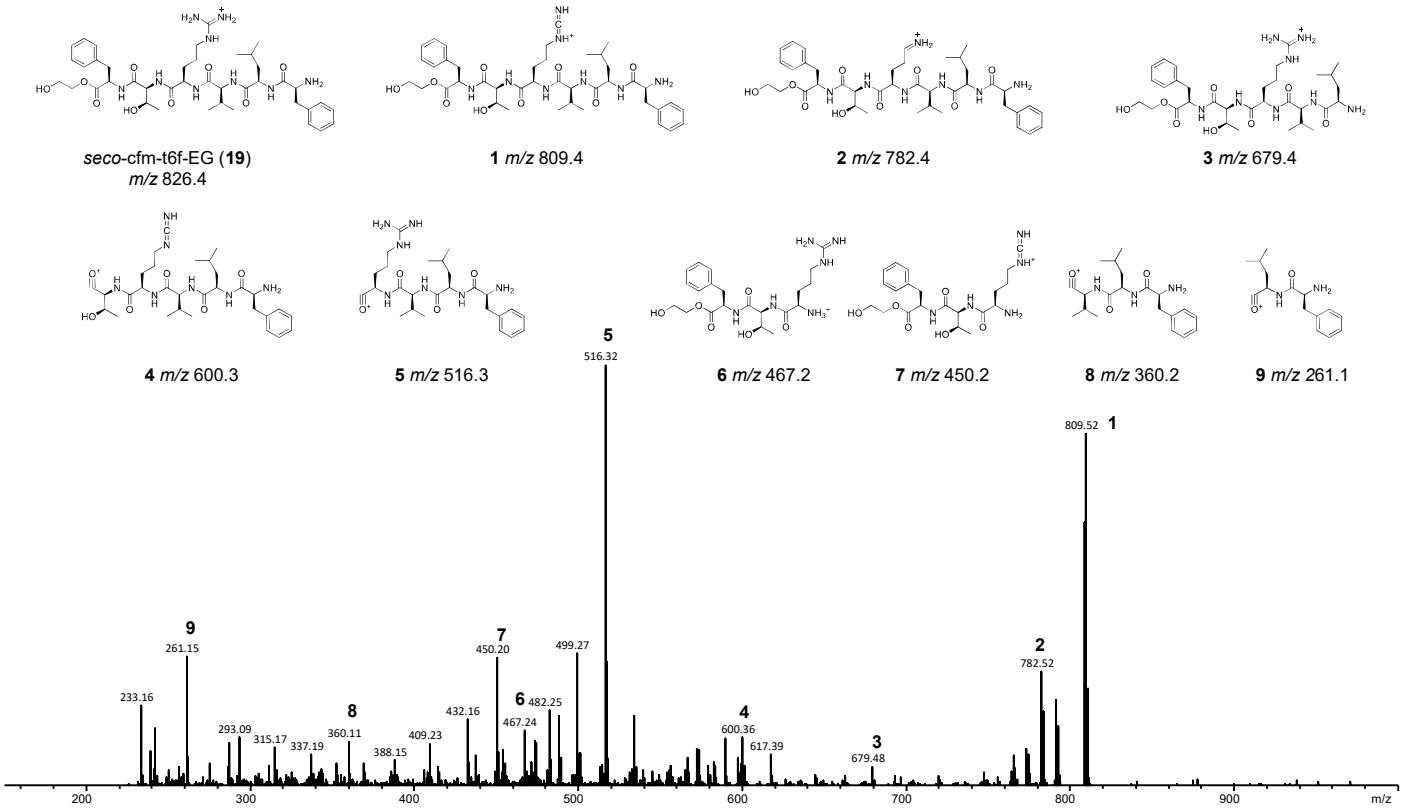


Figure S70. MS2 spectrum of *seco*-cfm-t6f-EG (**19**)

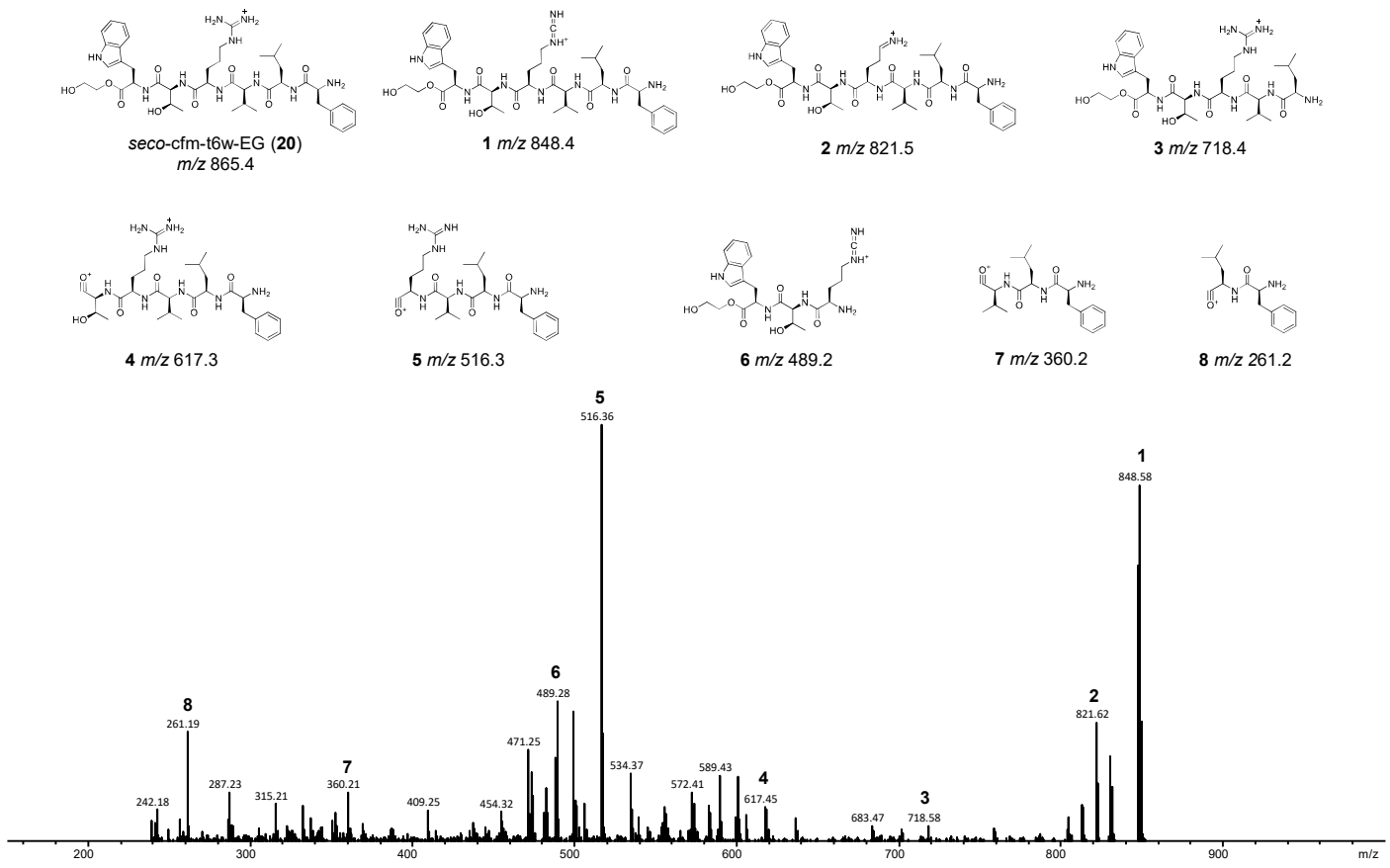


Figure S71. MS2 spectrum of *seco*-cfm-t6w-EG (**20**)

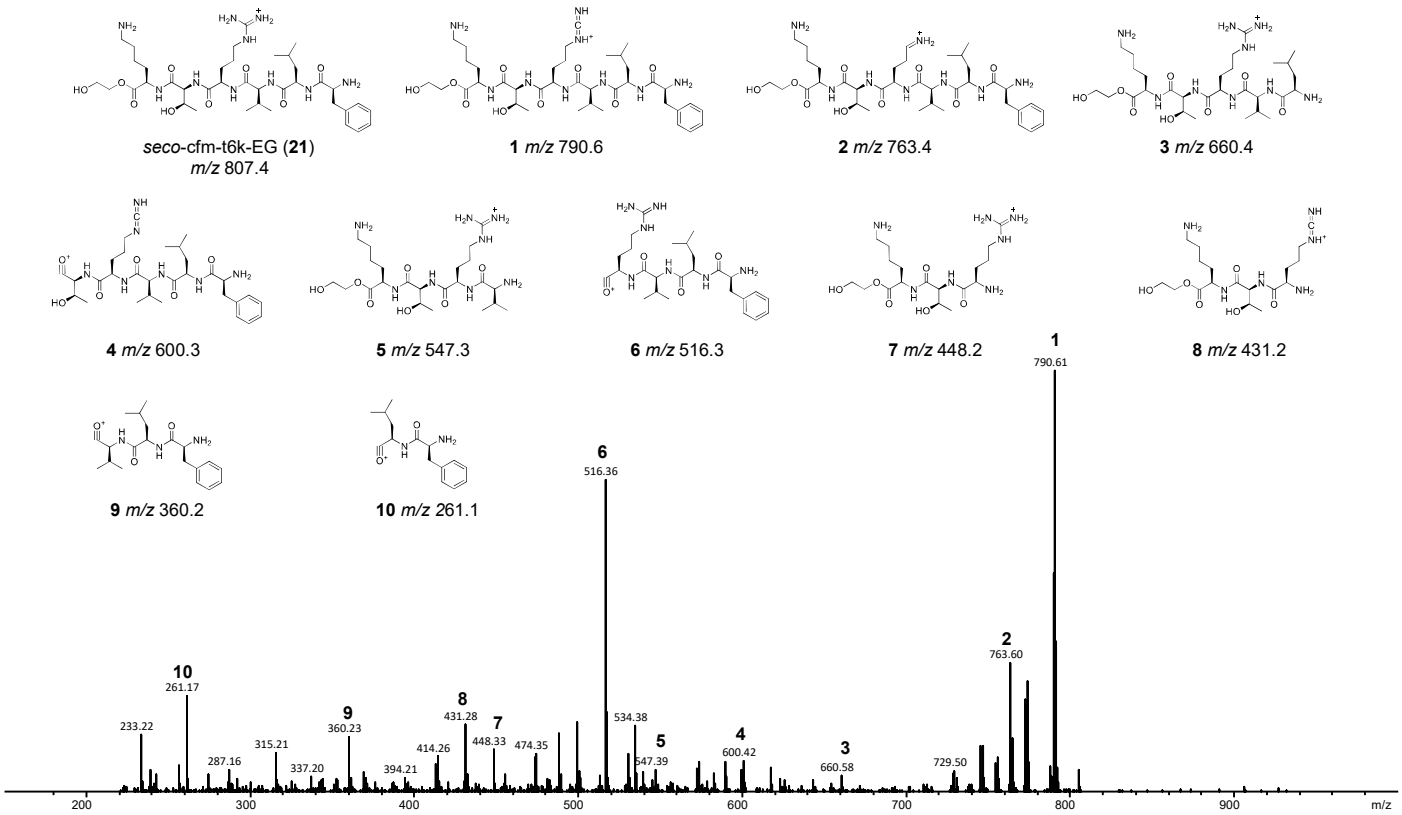


Figure S72. MS2 spectrum of *seco*-cfm-t6k-EG (**21**)

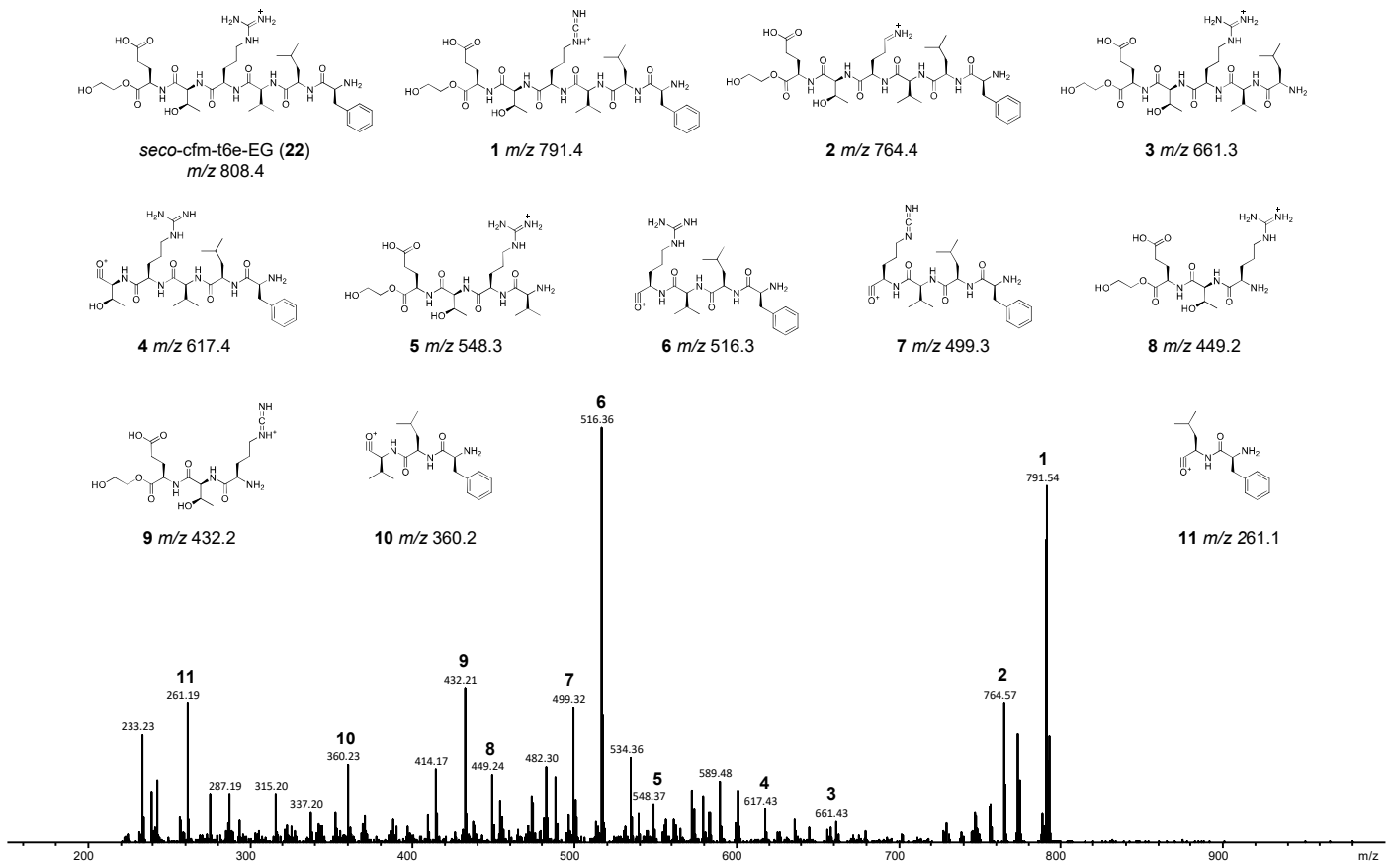


Figure S73. MS2 spectrum of *seco*-cfm-t6e-EG (**22**)

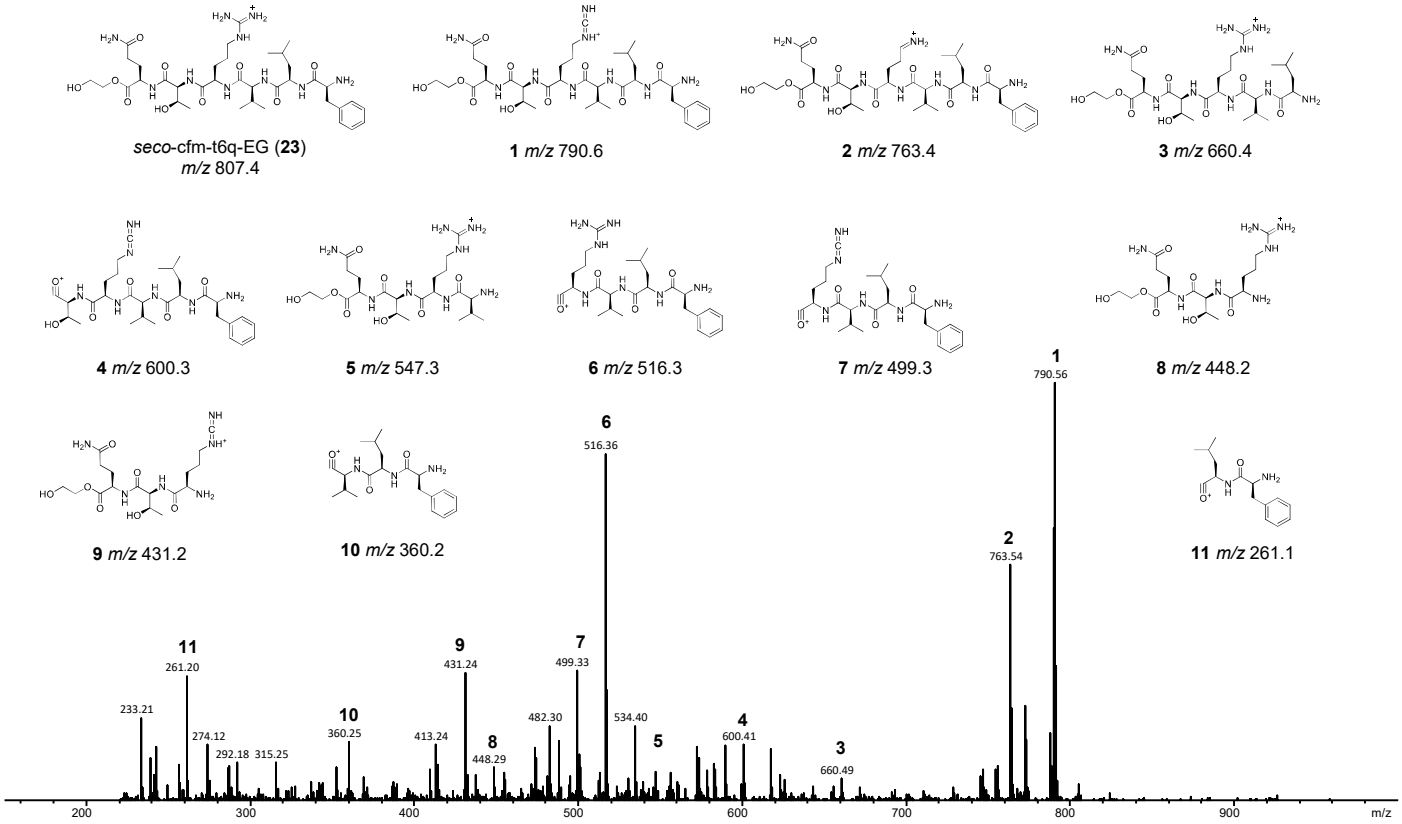


Figure S74. MS2 spectrum of *seco*-cfm-t6q-EG (**23**)

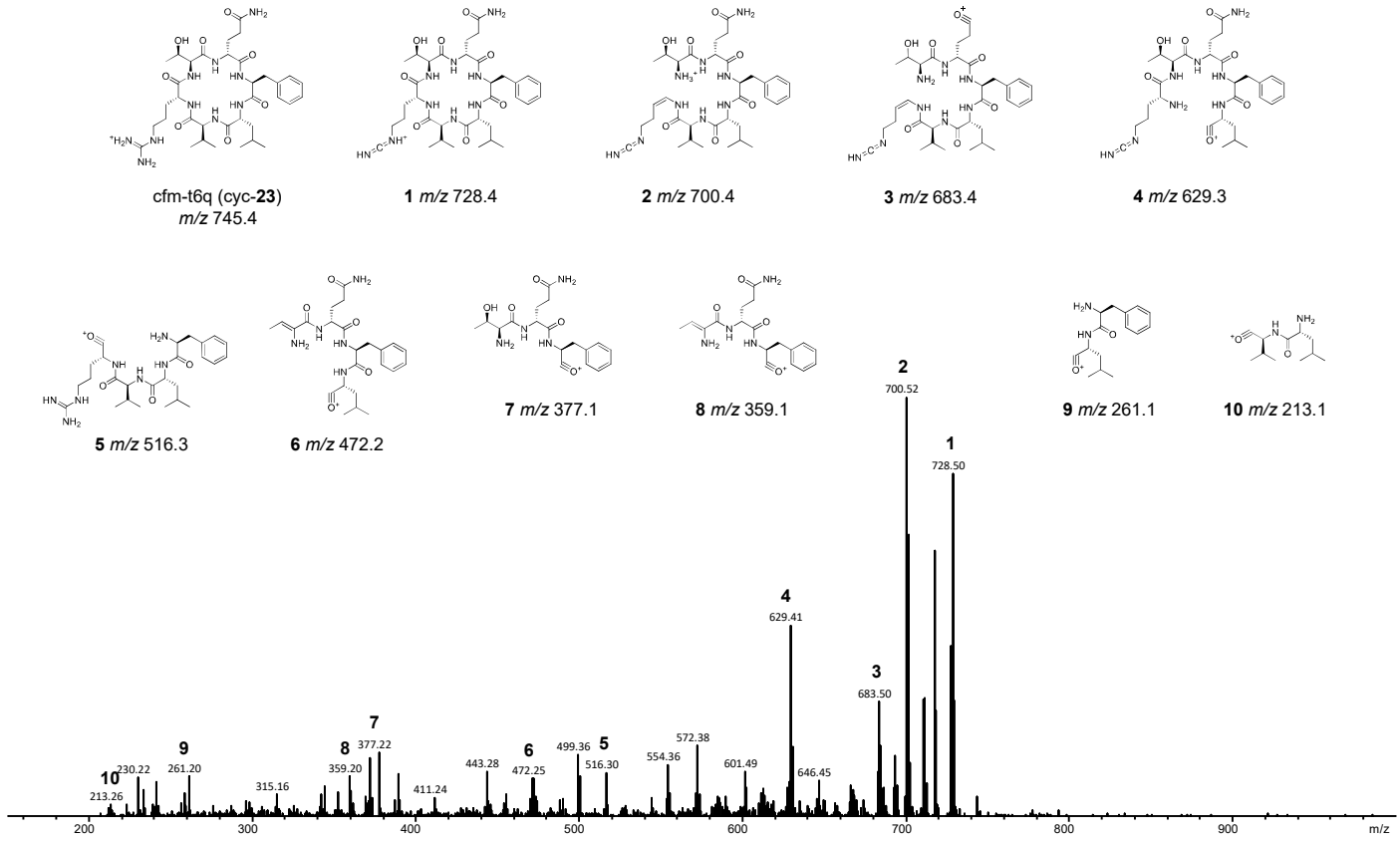


Figure S75. MS2 spectrum of cfm-t6q (cyc-**23**)

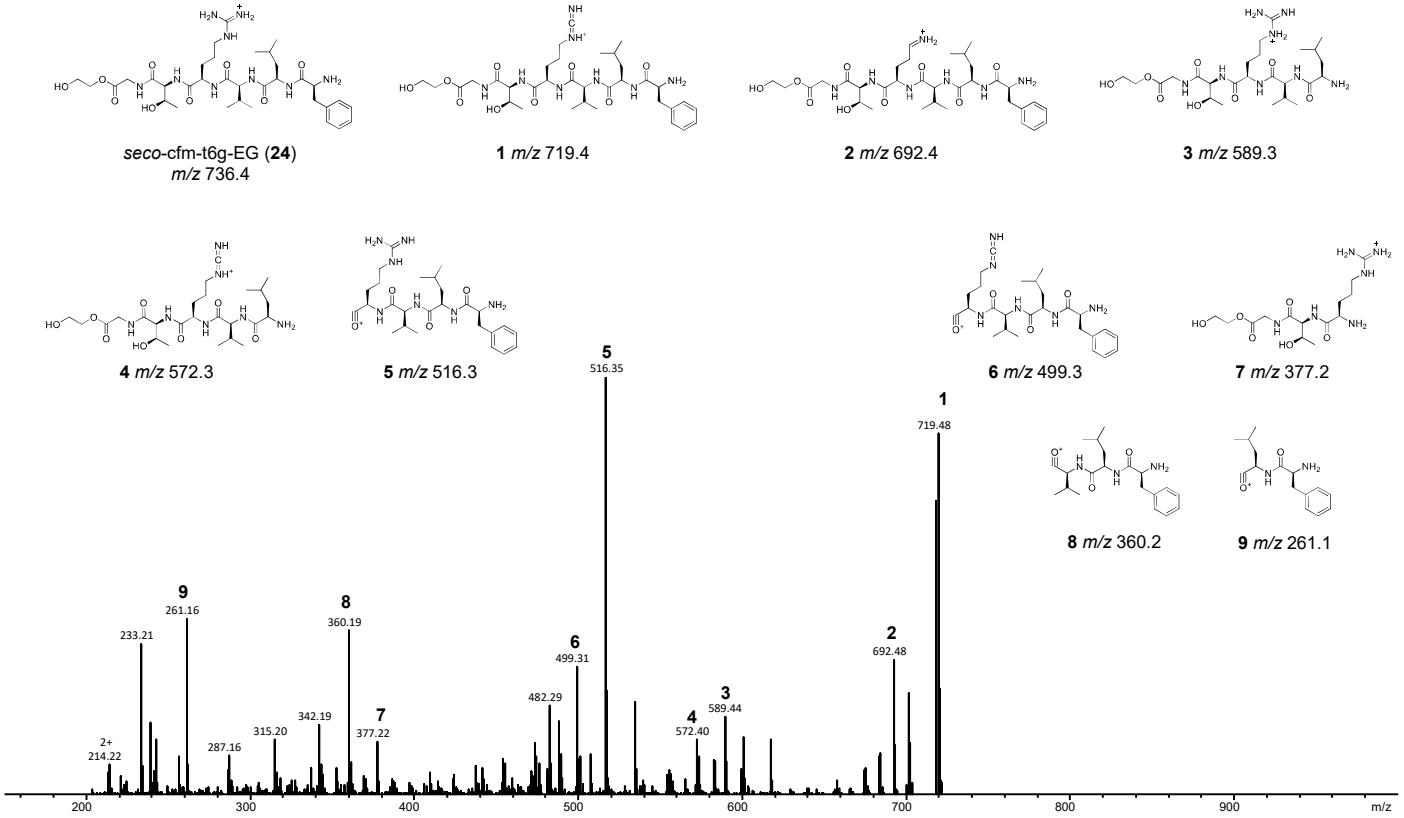


Figure S76. MS2 spectrum of *seco*-cfm-t6g-EG (**24**)

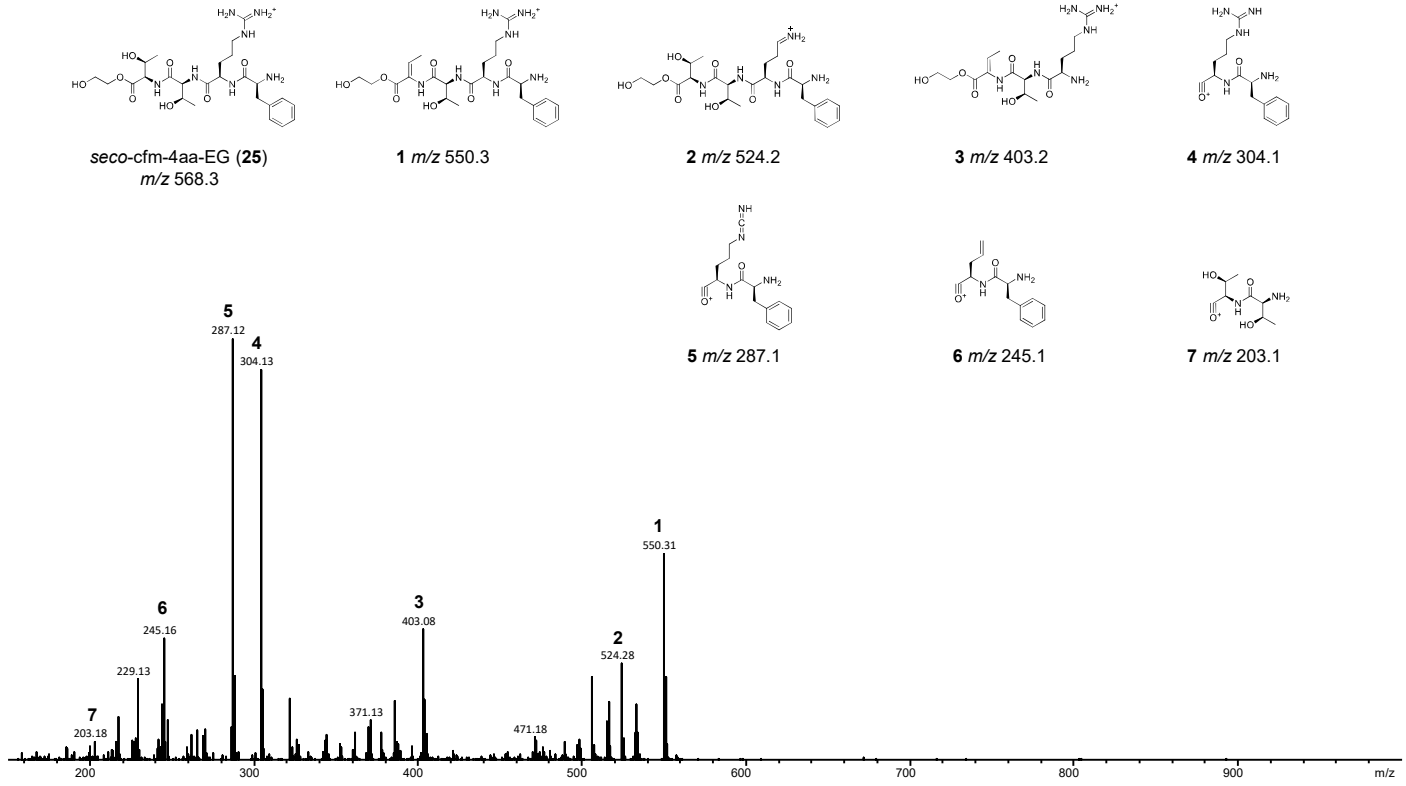


Figure S77. MS2 spectrum of *seco*-cfm-4aa-EG (**25**)

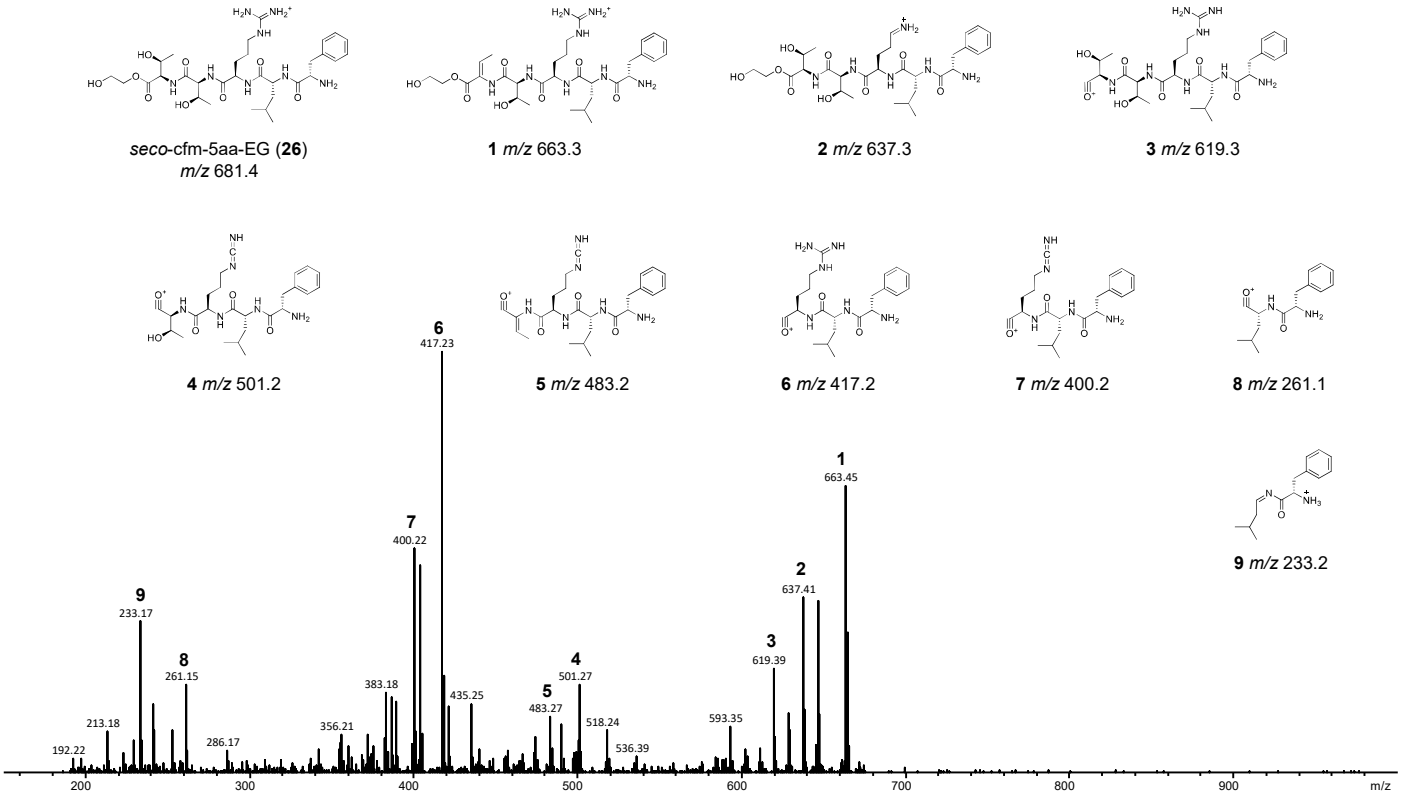


Figure S78. MS2 spectrum of *seco*-cfm-5aa-EG (**26**)

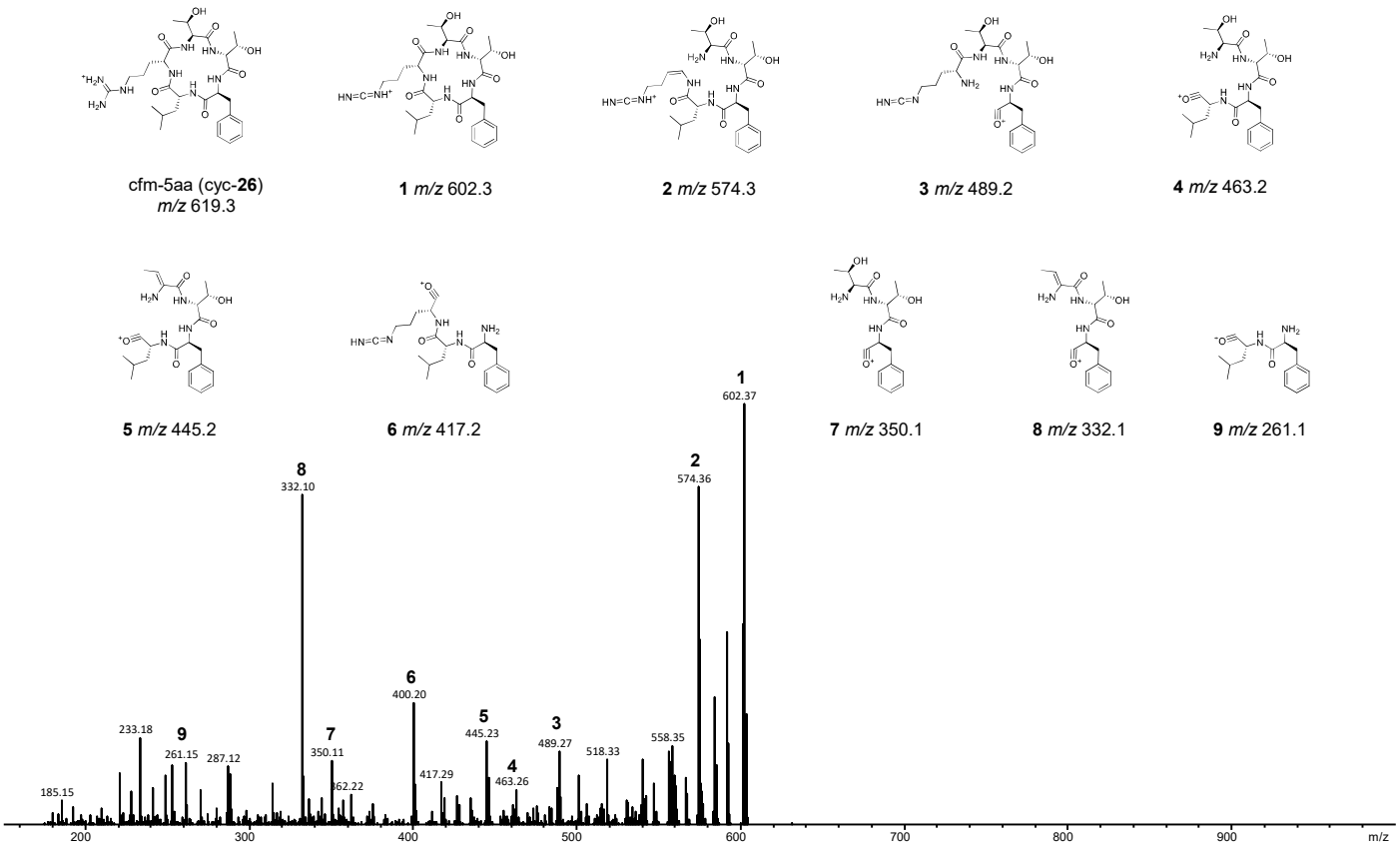


Figure S79. MS2 spectrum of cfm-5aa (cyc-**26**)

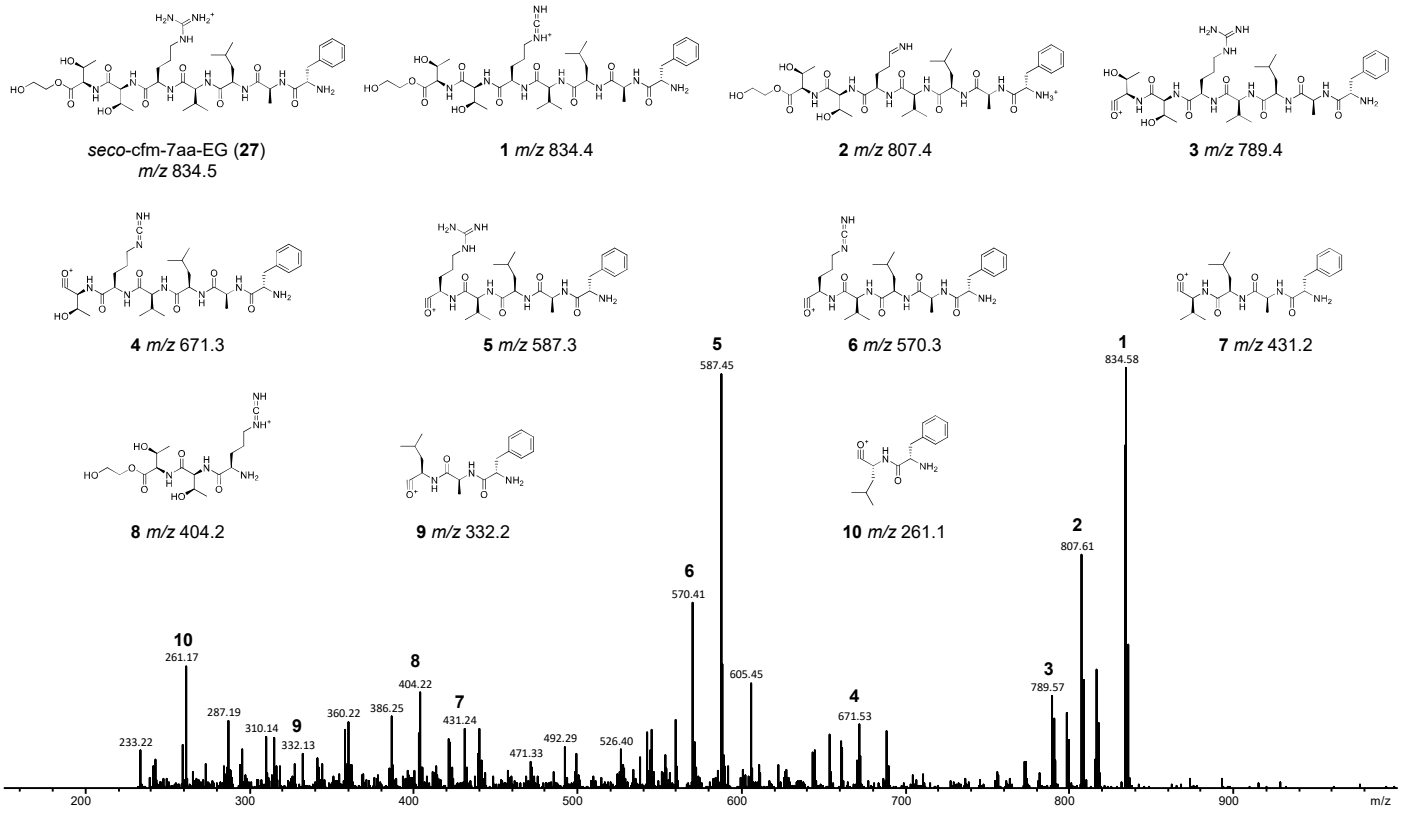


Figure S80. MS2 spectrum of *seco*-cfm-7aa-EG (**27**)

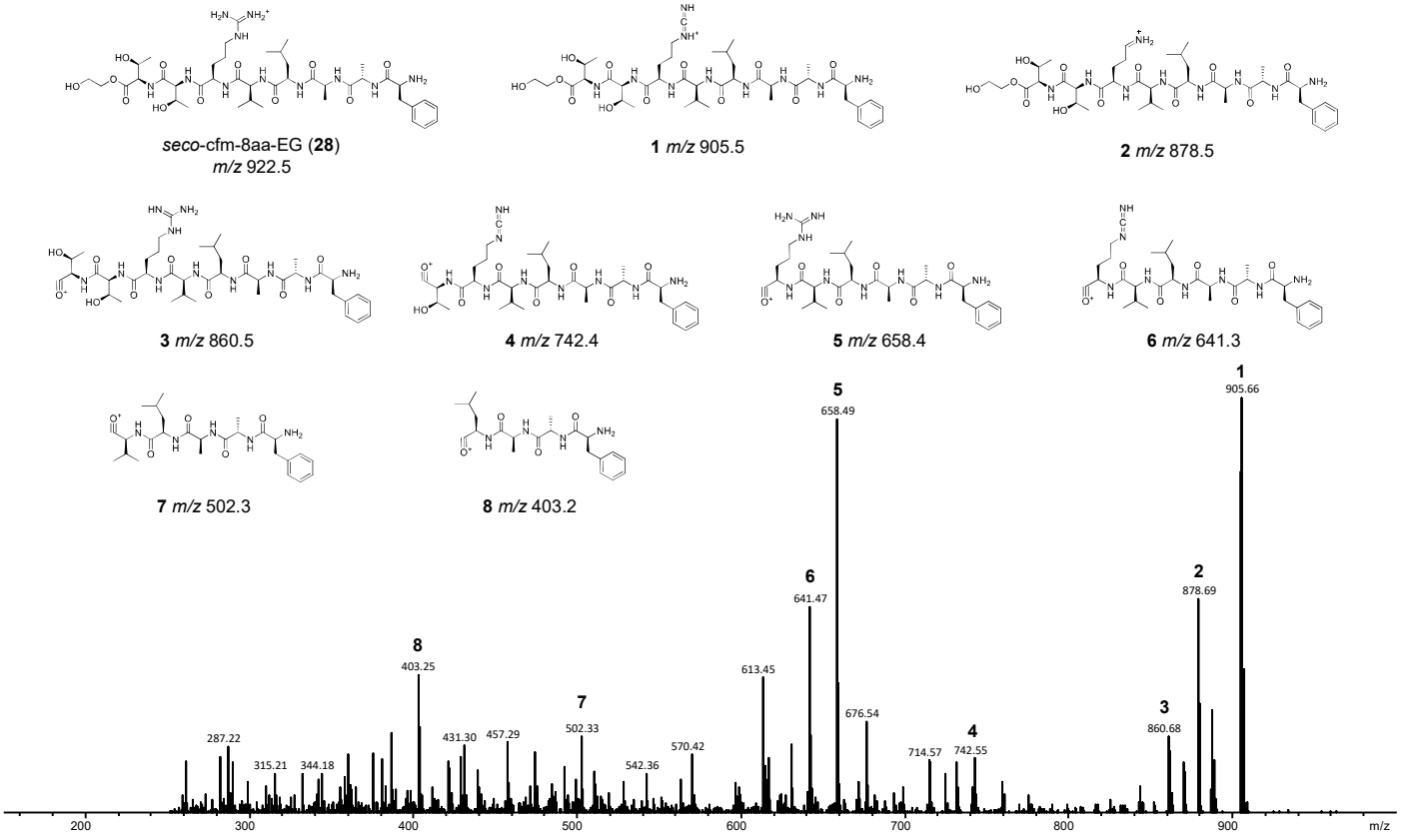


Figure S81. MS2 spectrum of *seco*-cfm-8aa-EG (**28**)

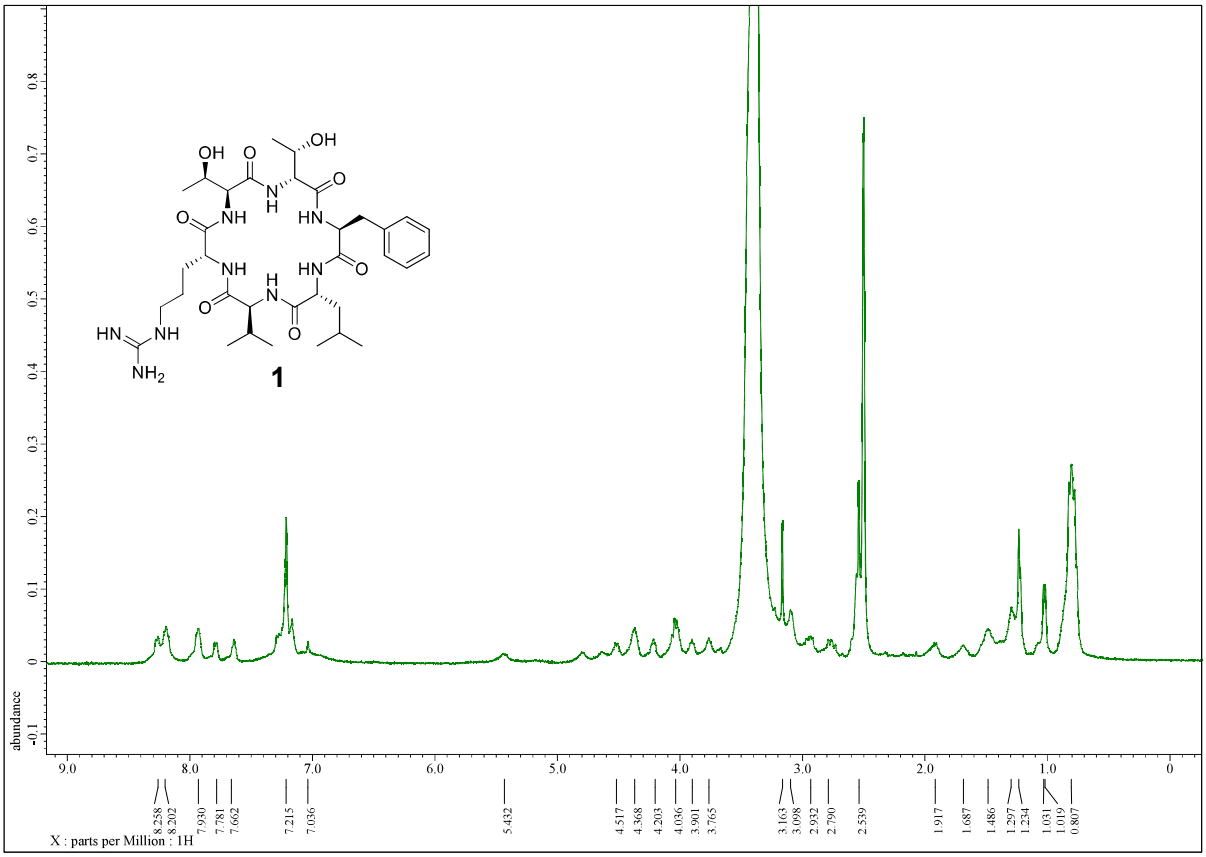


Figure S82. ^{1}H NMR spectrum of cfm (**1**) in DMSO-d_6 (400 MHz)

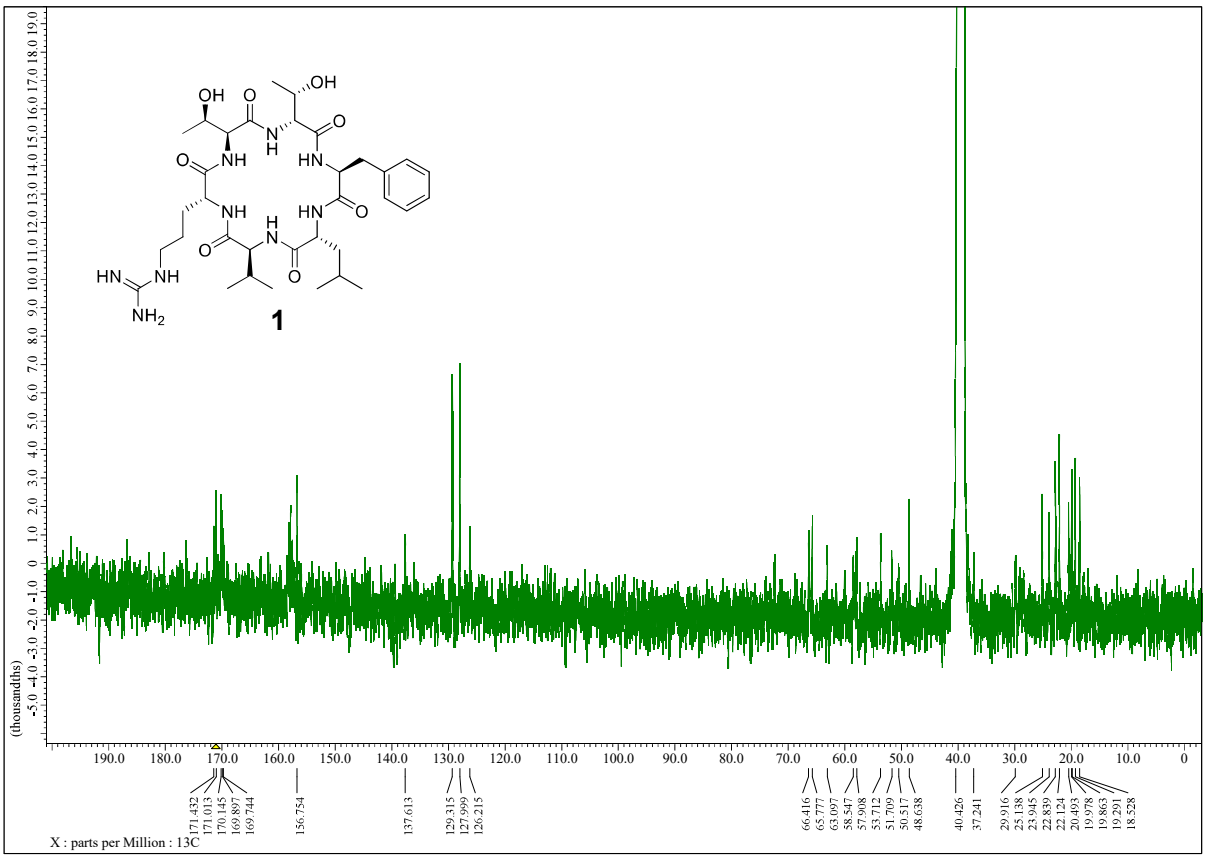


Figure S83. ^{13}C NMR spectrum of cfm (**1**) in DMSO-d_6 (100 MHz)

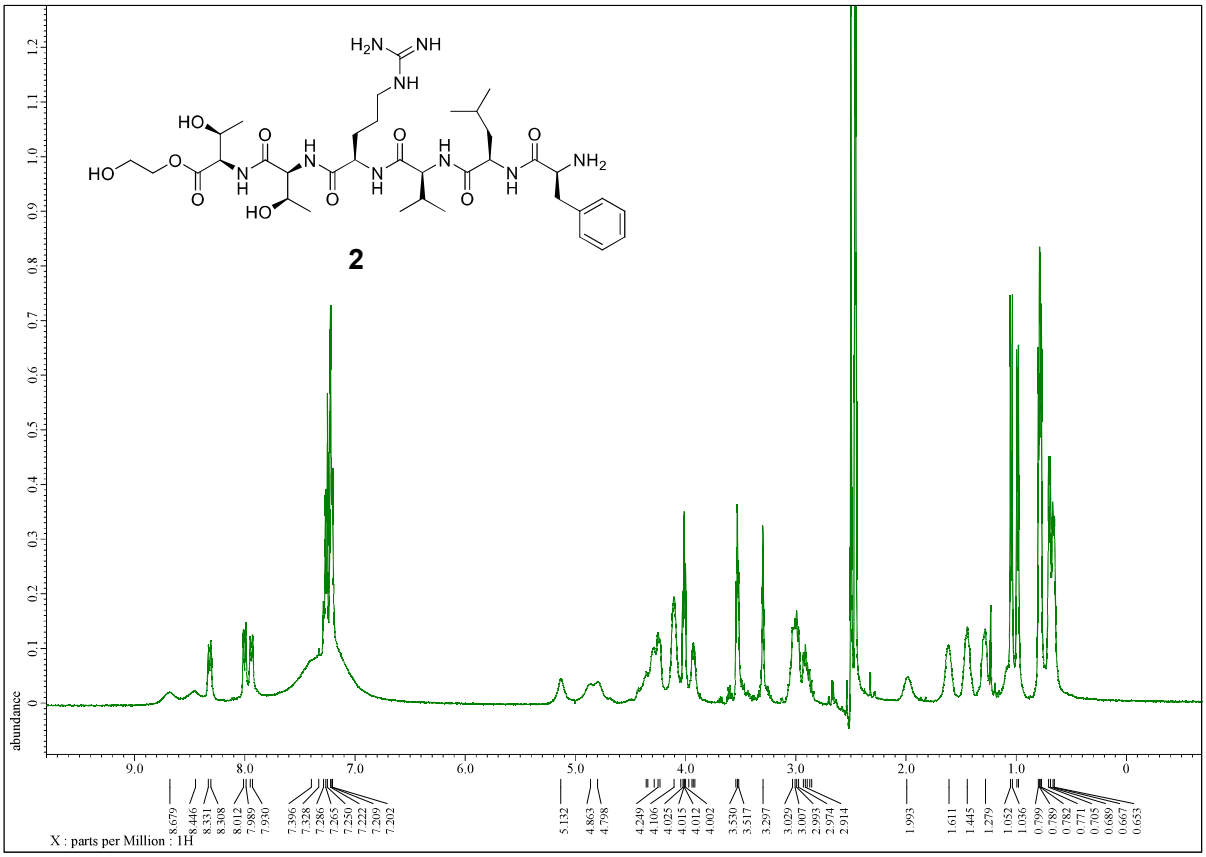


Figure S84. ^{1}H NMR spectrum of *seco*-cfm-EG (**2**) in DMSO-d_6 (400 MHz)

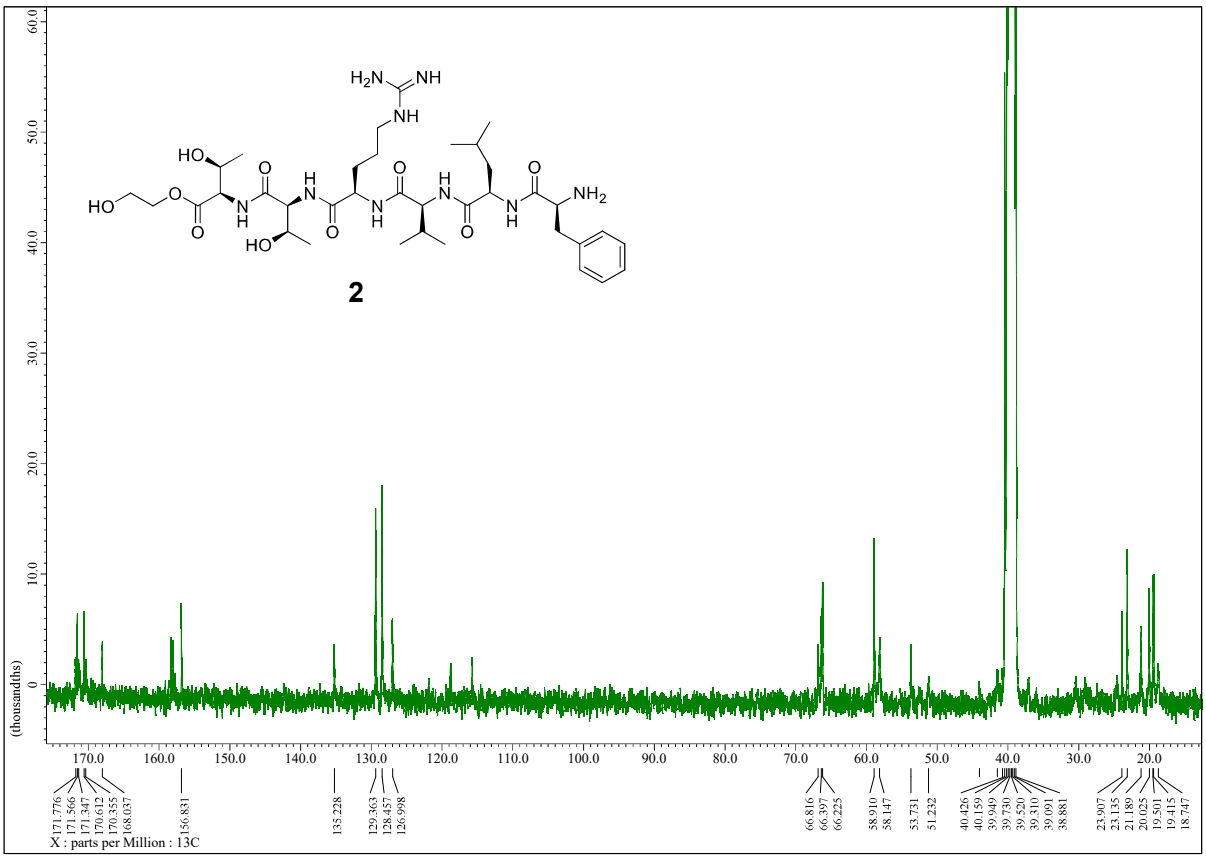


Figure S85. ^{13}C NMR spectrum of *seco*-cfm-EG (**2**) in DMSO-d_6 (100 MHz)

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