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Supplementary Information

Oxidation-guided and collision-induced linearization assists *de novo* sequencing of thioether macrocyclic peptides

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Materials and Methods

Abbreviations for chemical compounds.

DMSO, Dimethylsulfoxide; DMF, *N*,*N*-Dimethylformamide; Fmoc, 9-Fluorenylmethyloxycarbonyl; HATU, 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-Oxide Hexafluorophosphate; HOAt, 1-Hydroxy-7-azabenzotriazole; TFA, Trifluoroactic acid; DCM, Dichloromethane; DIPEA, *N*,*N*-Diisopropylethylamine; DIC, *N*,*N'*-Diisopropylcarbodiimide

General remarks for synthesis.

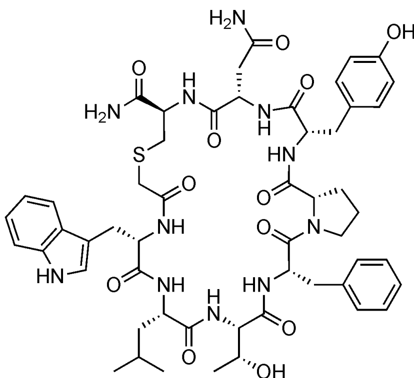
Chemicals and solvents used in this study were purchased from commercial suppliers and used without further purification. Preparative high performance liquid chromatography (HPLC) was performed on a Prominence HPLC system (Shimadzu) with a 5C_{18}-AR-II column (Nacalai tesque, 10 mm I.D.×150 mm, 34350-41). Analytical ultra-high performance liquid chromatography (UHPLC) was performed on a Nexera-i (Shimadzu) with a Shim-pack Velox C18 column (Shimadzu, 1.8 µm, 2.1 mm×50 mm, 227-32007-02). LC-MS/MS was performed on an ACQUITY™ Premier system (Waters Corp., Milford, MA, USA) coupled to a Waters Xevo™ G3 QTof mass spectrometer using an ACQUITY Premier Peptide BEH C18 Column (130Å, 1.7 µm, 2.1 x 100 mm) and controlled with the MassLynx™ 4.2 software.

General procedures for synthesis of thioether macrocyclic peptides.

The peptides were synthesized on RinkAmide-MBHA resin (#A00172, Watanabe Chemical Industries). Peptides were synthesized using an automated peptide synthesizer (Syro I, Biotage). RinkAmide resin (25 µmol) was swollen in DMF for 30 min. Fmoc deprotection was performed by shaking the resin with 20% piperidine/DMF for 3 min, then shaking the resin with 20% piperidine/DMF for 12 min. The resin was washed with DMF six times. Coupling reaction was performed by shaking the resin with Fmoc-protected amino acid (4 equiv.), HATU (4 equiv.), HOAt (4 equiv.), and DIPEA (8 equiv.) in 600 µL of DMF for 1 h at 75 °C. After the reaction, the resin was washed with DMF three times. The deprotection and coupling reaction were repeated until the N-terminal residue. After the deprotection of Fmoc group of the N-terminal residue, a DMF solution of chloroacetic acid (20 equiv., 1 M) and DIC (10 equiv., 0.5 M) were added to the resin and the reaction syringe was shaken for 30 min. After the reaction, the resin was washed with DMF and DCM three times each. The peptide was cleaved from the resin by shaking the resin with 95:2.5:2.5 TFA/triisopropylsilane/water for 2 h. After the solution was removed under the reduced pressure, the peptide was dissolved in 40% acetonitrile/water. DIPEA was added to the peptide solution until the pH becomes above 9. The solution was shaken for 30 min to let the peptides cyclize. After the cyclization, the peptides were lyophilized and dissolved in acetonitrile/water. The solution was filtered and subjected to purification on a reversed phase column using HPLC. The peptide solution obtained after HPLC purification was lyophilized. The lyophilized peptides were dissolved in DMSO. Purified products were analyzed on a reversed phase column using HPLC and ESI-TOF MS.

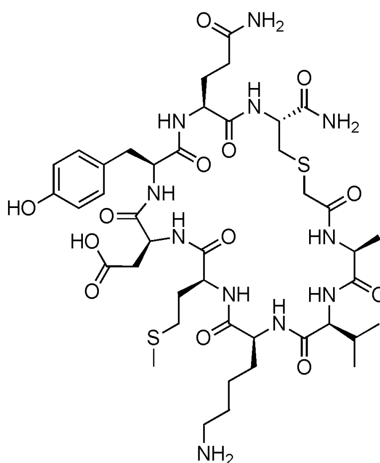
Synthesis of thioether macrocyclic peptides 1–3 and S1–S3.

Synthesis of 1.



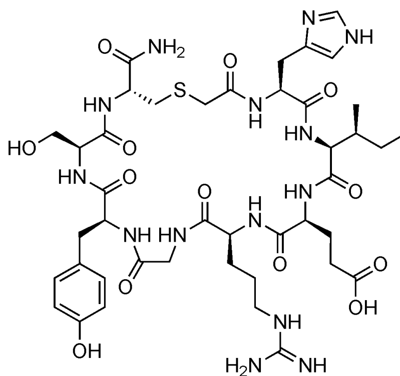
62.4 mg of RinkAmide-MBHA resin (0.41 mmol/g, 26 μmol) was used for synthesis. Yield: 9%. HRMS (ESI-TOF MS) m/z: $[M+H]^+$ Calcd for $C_{53}H_{68}N_{11}O_{12}S^+$ 1082.4764; Found 1082.4720.

Synthesis of S1.



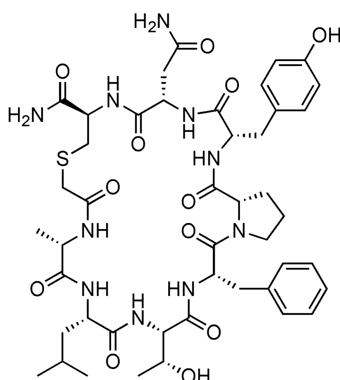
63.7 mg of RinkAmide-MBHA resin (0.41 mmol/g, 26 μmol) was used for synthesis. Yield: 20%. HRMS (ESI-TOF MS) m/z: $[M+H]^+$ Calcd for $C_{42}H_{66}N_{11}O_{13}S_2^+$ 996.4278; Found 996.4250.

Synthesis of S2.



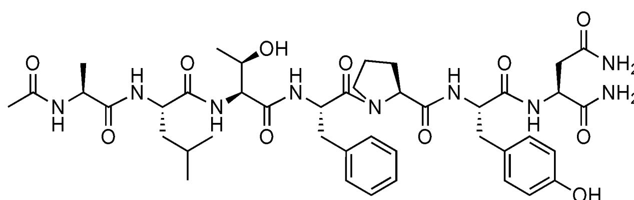
60.8 mg of RinkAmide-MBHA resin (0.41 mmol/g, 25 μmol) was used for synthesis. Yield: 13%. HRMS (ESI-TOF MS) m/z: $[M+H]^+$ Calcd for $C_{42}H_{63}N_{14}O_{13}S^+$ 1003.4414; Found 1003.4375.

Synthesis of S3.



58.4 mg of RinkAmide-MBHA resin (0.43 mmol/g, 25 μmol) was used for synthesis. Yield: 4%. HRMS (ESI-TOF MS) m/z: $[M+H]^+$ Calcd for $C_{45}H_{63}N_{10}O_{12}S^+$ 967.4342; Found 967.4318.

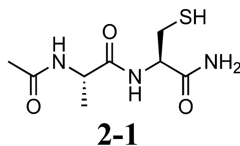
Synthesis of S3-linear.



62.9 mg of RinkAmide-MBHA resin (0.41 mmol/g, 26 μmol) was used for synthesis. Yield: 12%. HRMS (ESI-TOF MS) m/z: $[M+H]^+$ Calcd for $C_{42}H_{60}N_9O_{11}^+$ 866.4407; Found 866.4393.

Synthesis of thioether-containing linear peptide 2.

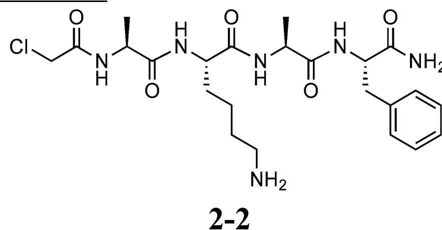
Step 1-1. Synthesis of peptide fragment **2-1**



2-1

Solid phase synthesis of the peptide fragment **2-1** was conducted using 241.2 mg of RinkAmide resin in the same manner with the synthesis of the thioether macrocyclic peptides. The synthesis was conducted manually. The N-terminal residue was kept protected with Fmoc group. After the synthesis, the resin was washed with DMF and DCM, three times each. The peptide was cleaved from the resin by shaking the resin with 1 mL of 2.5% water/2.5% DCM/95% TFA for 2 h at RT. The filtrate was collected in a 15 mL tube and evaporated. Et_2O was added to precipitate peptide, and Et_2O was removed by decantation.

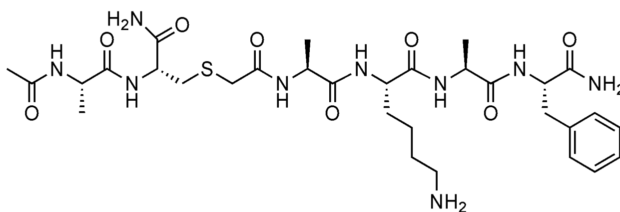
Step 1-2. Synthesis of peptide fragment **2-2**



2-2

Solid phase synthesis of the peptide fragment **2-2** was conducted using 59.0 mg of RinkAmide resin in the same manner with the synthesis of the thioether macrocyclic peptides. After chloroacetylation of the N-terminal residue, the resin was subjected to reaction with peptide fragment with **2-1**.

Step 2. Conjugating **2-1** and **2-2** via a thioether linkage to produce peptide **2**



2

The **2-2**-bound resin was incubated with Fmoc-AC-NH_2 (4 equiv.) and DIPEA (8 equiv.) in 600 μL of DMF for 1 h at room temperature. After the reaction, the resin was washed with DMF three times. Fmoc deprotection was performed by shaking the resin with 20% piperidine/DMF for 3 min; then shaking the resin with 20% piperidine/DMF for 12 min. A DMF solution of acetic anhydride (10 equiv., 0.5 M) and pyridine (20 equiv., 1 M) was added to the resin and the reaction vessel was shaken for 30 min. After the procedure, the resin was washed with DMF and DCM three times each. The precursor linear peptide was cleaved from the resin by shaking the resin with 1 mL of 2.5% water/2.5% DCM/95% TFA for 2 h at room temperature. The filtrate was collected in a 15 mL tube and evaporated. Et_2O was added to precipitate peptide, and Et_2O was removed by decantation. The peptide was dissolved in acetonitrile/water and purified on a reversed phase column using HPLC. The peptide solution obtained after HPLC purification was lyophilized. The lyophilized peptides were dissolved in DMSO. Purified products were analyzed on a reversed phase column using HPLC and ESI-TOF MS. Yield: 2%. HRMS (ESI-TOF MS) m/z: $[M+H]^+$ Calcd for $C_{31}H_{49}N_9O_8S^+$ 708.3498; Found 708.3535.

Oxidation reaction of thioether-containing peptides using H_2O_2.

40 μL of 15.3% (w/w) hydrogen peroxide in water was added to 160 μL of 12.5 μM peptide solution in 0.25% DMSO/water. Final concentrations of H_2O_2 and peptide are 1 M and 10 μM, respectively. The solution was incubated at 37 °C for 7 h.

Oxidation reaction of thioether-containing peptides using $NaIO_4$.

40 μL of 100 mM $NaIO_4$ in water was added to 160 μL of 12.5 μM peptide solution in 0.25% DMSO/water. Final concentrations of H_2O_2 and peptide are 20 mM and 10 μM, respectively. The solution was incubated at room temperature for 7 h.

Quantification of oxidation reaction efficiency

Peptide solution after oxidation was spined at 20,000×g for 30 min at 4 °C. 1 μL of the supernatant was injected to LC-MS and the elution was monitored with PDA at 220 nm.

LC-MS/MS analysis

After oxidation, peptide solution was filtered using a 0.45 μm filter. 1 μL of 10 μM peptide aqueous solution containing 0.1% DMSO was injected into the LC-MS/MS system. For MS/MS analysis, the collision energy was set to 20–60 eV and the measurement was conducted using data independent acquisition (MS^E). For the measurement of **2-oxidized** shown in Fig. 3, MSMS mode was performed instead of MS^E mode with the calculated mass of **2-oxidized** used as the selected input mass. During the measurements, continuous lockmass correction was conducted using leucine enkephalin. The obtained LC-MS/MS data was analyzed using the Peptide Mapping function of the Waters UNIFI™ software.

Supporting Figures & Tables

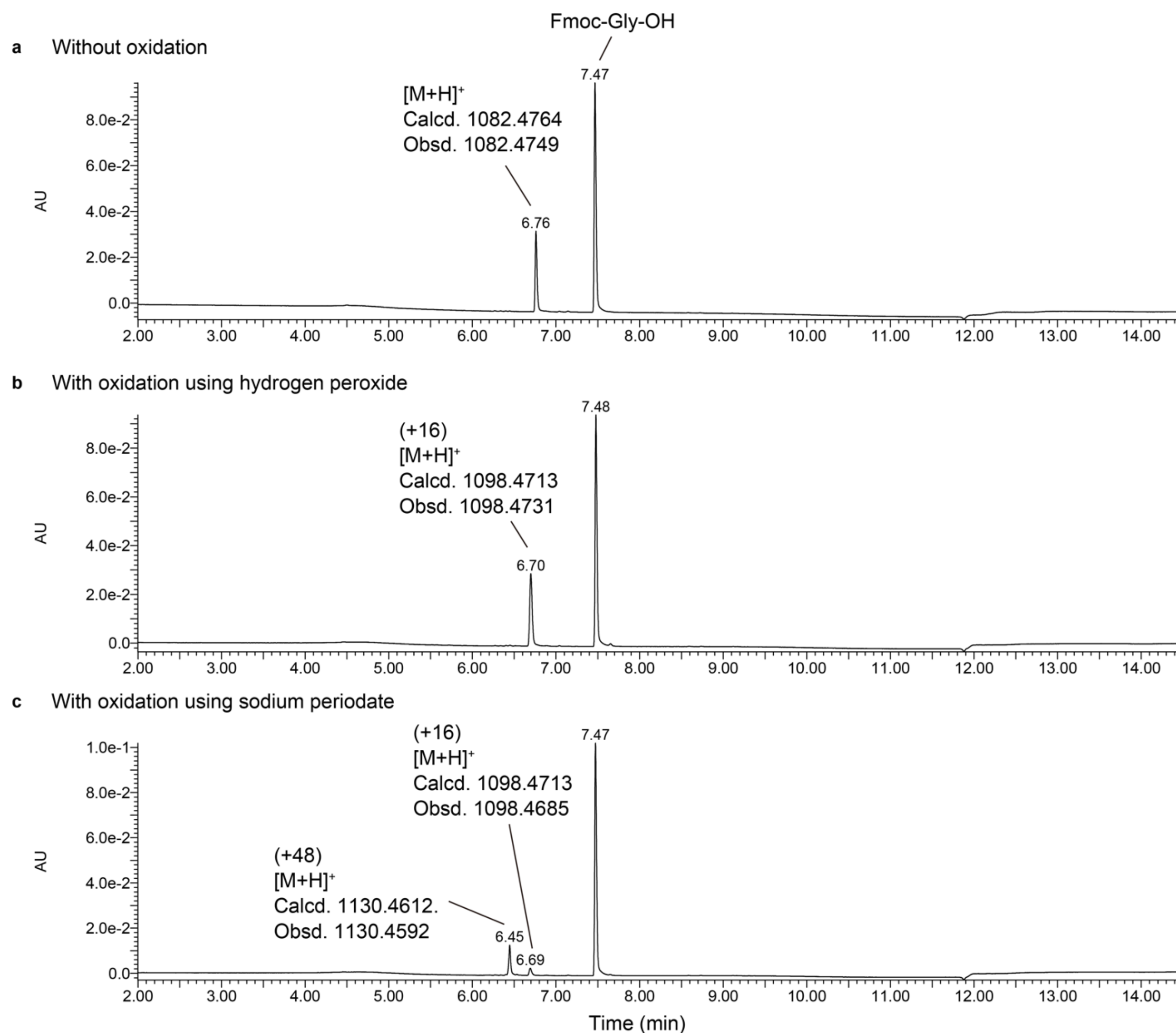
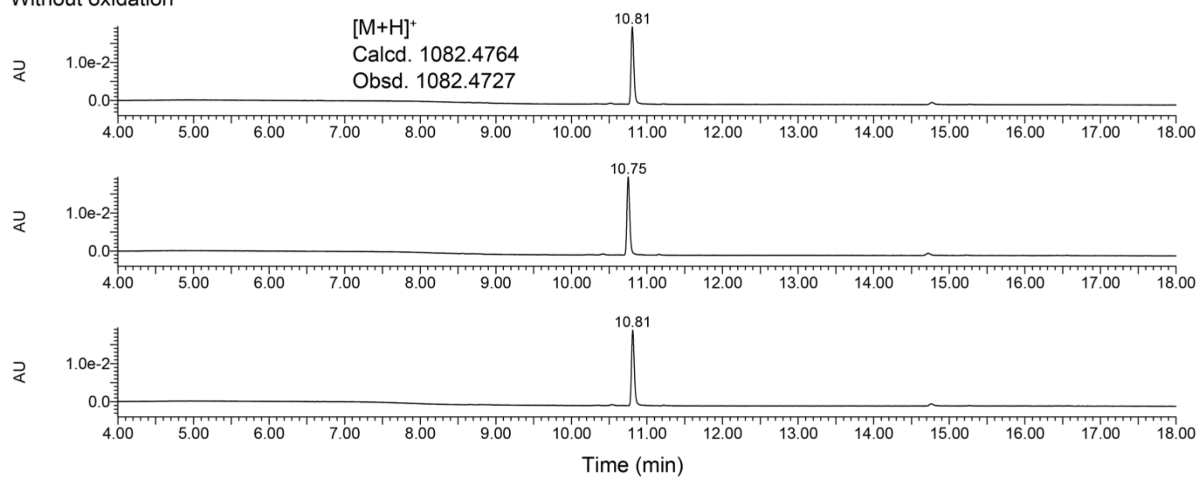


Fig. S1 UV chromatograms of **1** (a) without oxidation, (b) with oxidation using hydrogen peroxide (10 µM peptide with 1 M hydrogen peroxide, 7 h at 37 °C), and (c) with oxidation using sodium periodate (10 µM peptide with 20 mM sodium periodate, 7 h at 25 °C). The observed mass values are labeled above the corresponding peaks. Fmoc-Gly-OH was used as a reference.

1 (cyclo[WLTFPYNC])

a



b

With oxidation

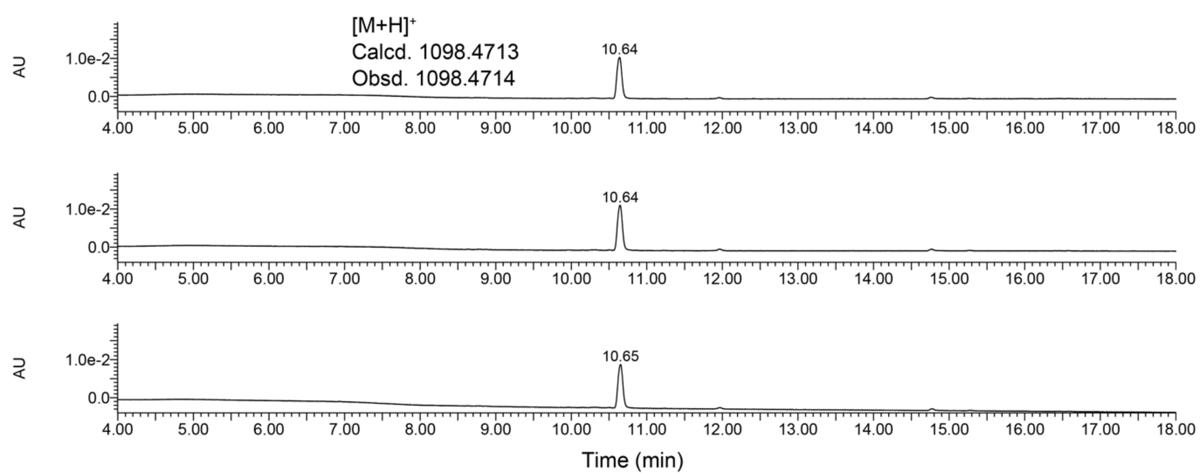
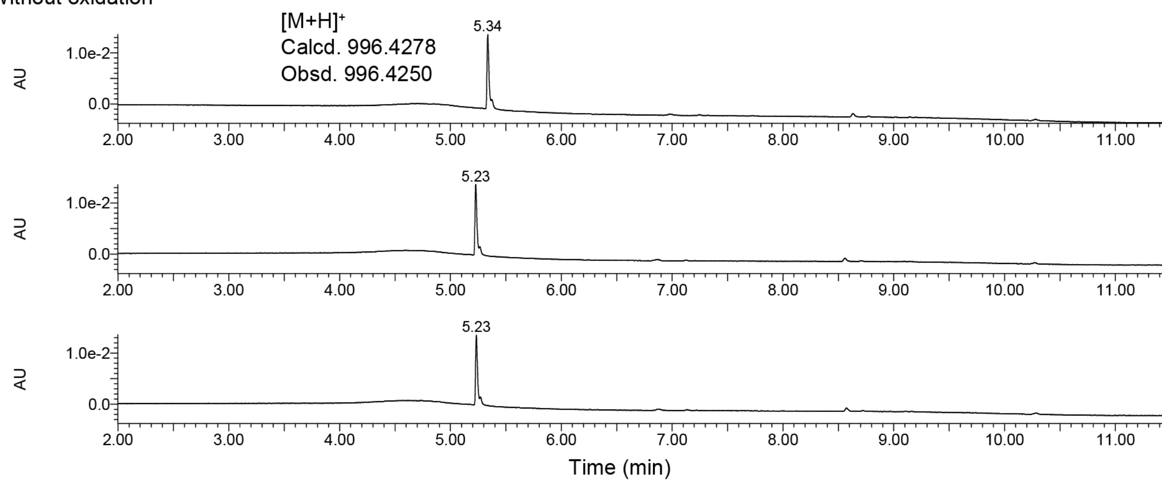


Fig. S2 UV chromatograms of **1** (a) without oxidation and (b) with oxidation. The experiment was conducted in triplicate.

S1 (cyclo[AVKMDYQC])

a

Without oxidation



b

With oxidation

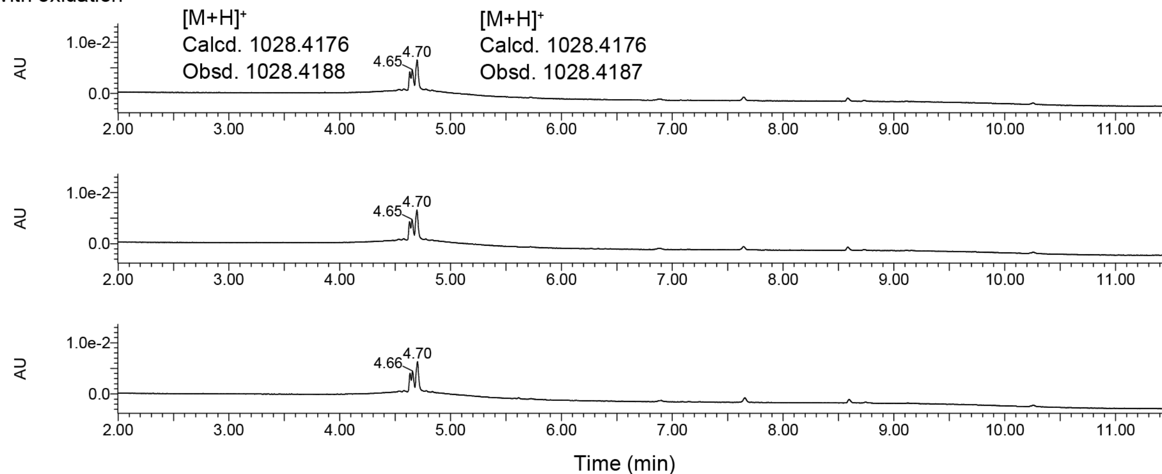
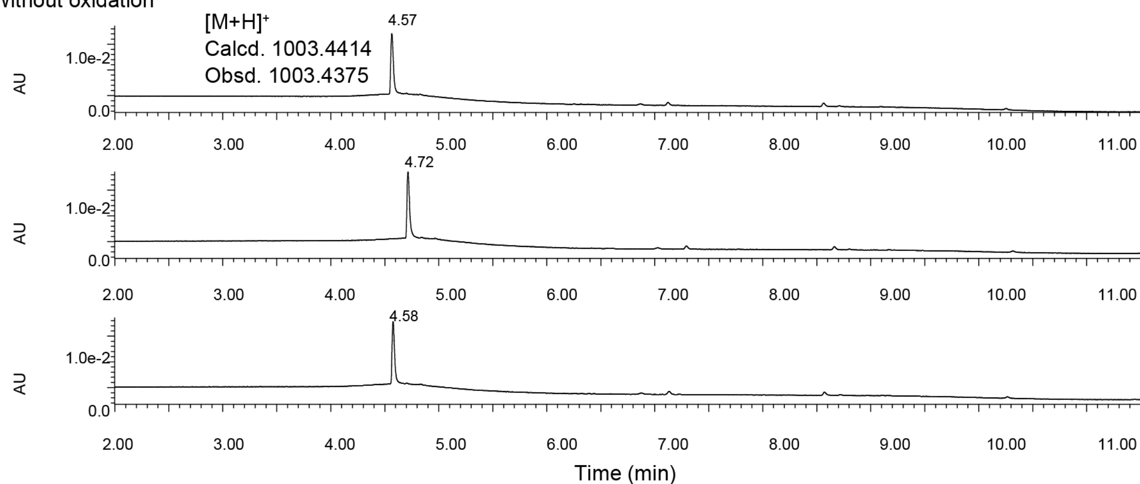


Fig. S3 UV chromatograms of **S1** (a) without oxidation and (b) with oxidation. The experiment was conducted in triplicate.

S2 (cyclo[HIERGYSC])



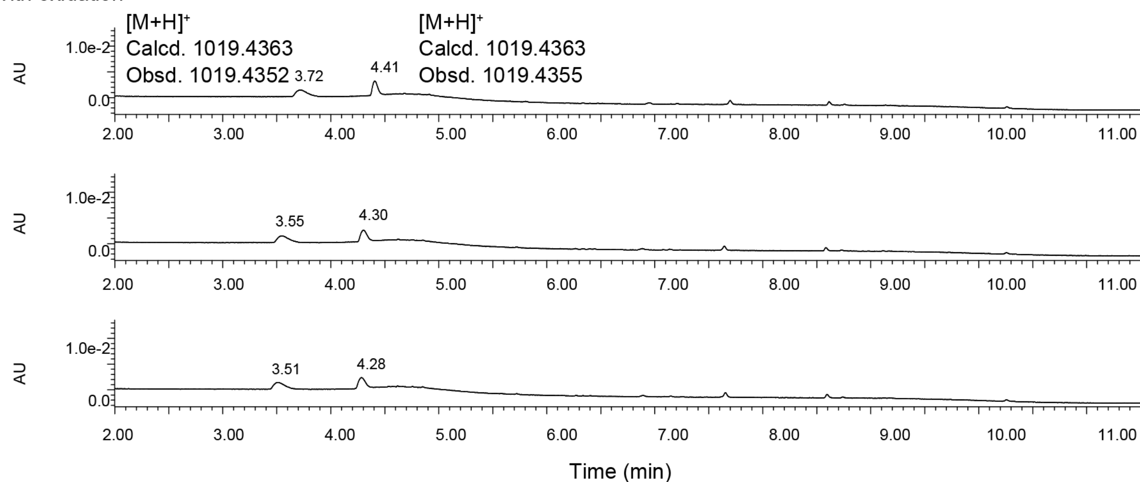
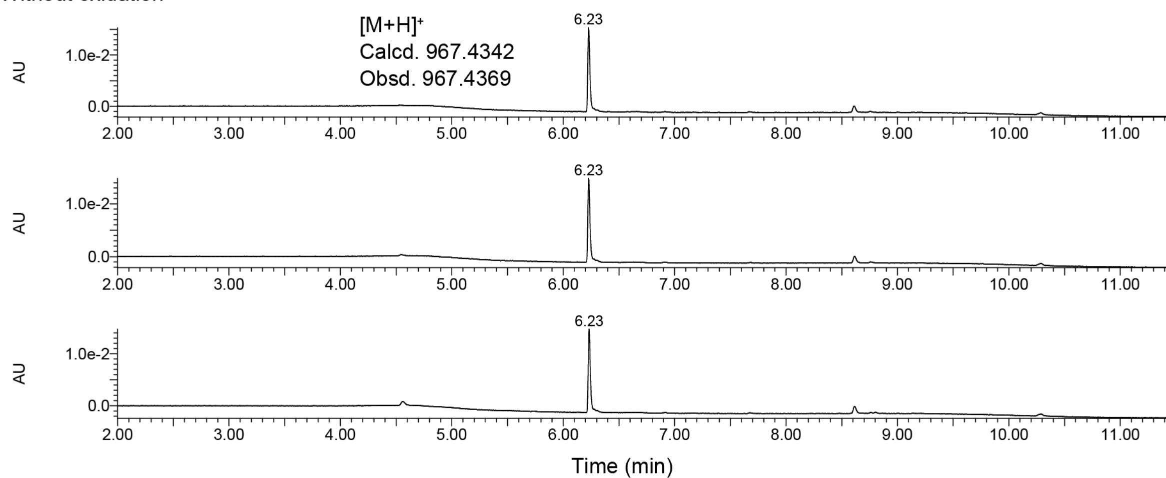


Fig. S4 UV chromatograms of **S2** (a) without oxidation and (b) with oxidation. The experiment was conducted in triplicate.

S3 (cyclo[ALTFPYNC])

a

Without oxidation



b

With oxidation

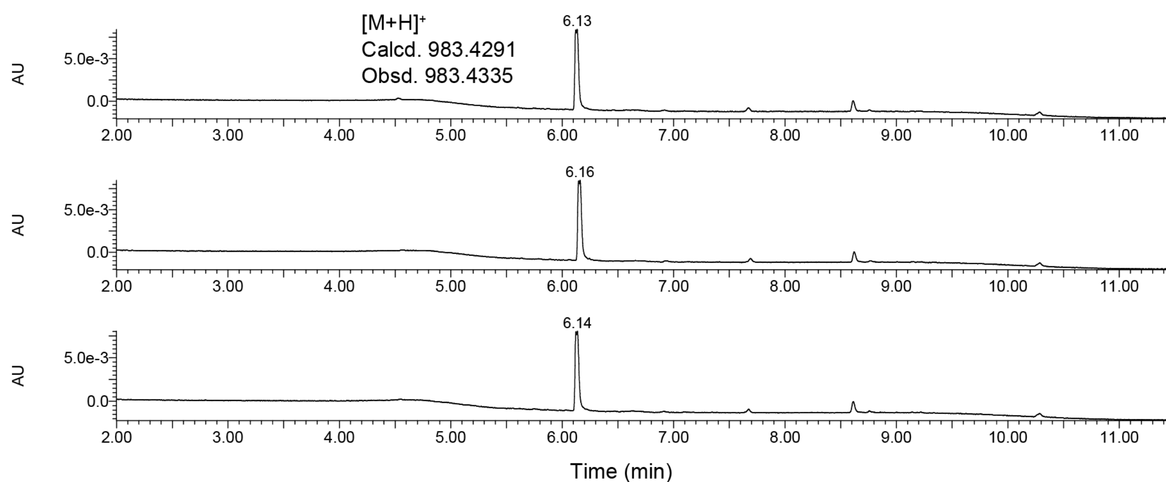
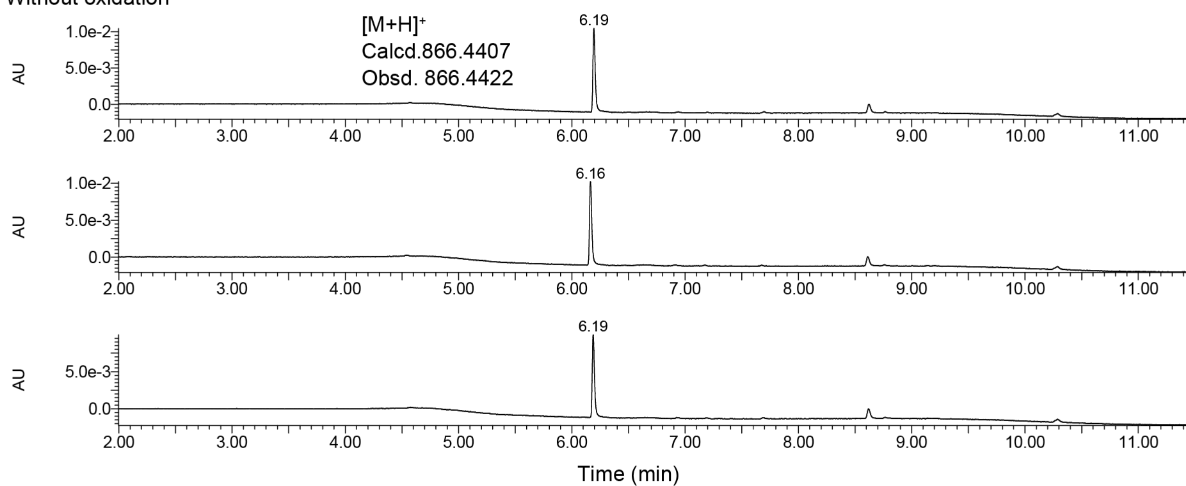


Fig. S5 UV chromatograms of **S3** (a) without oxidation and (b) with oxidation. The experiment was conducted in triplicate.

S3-linear (Ac-ALTFPYN-NH_2)

a

Without oxidation



b

With oxidation

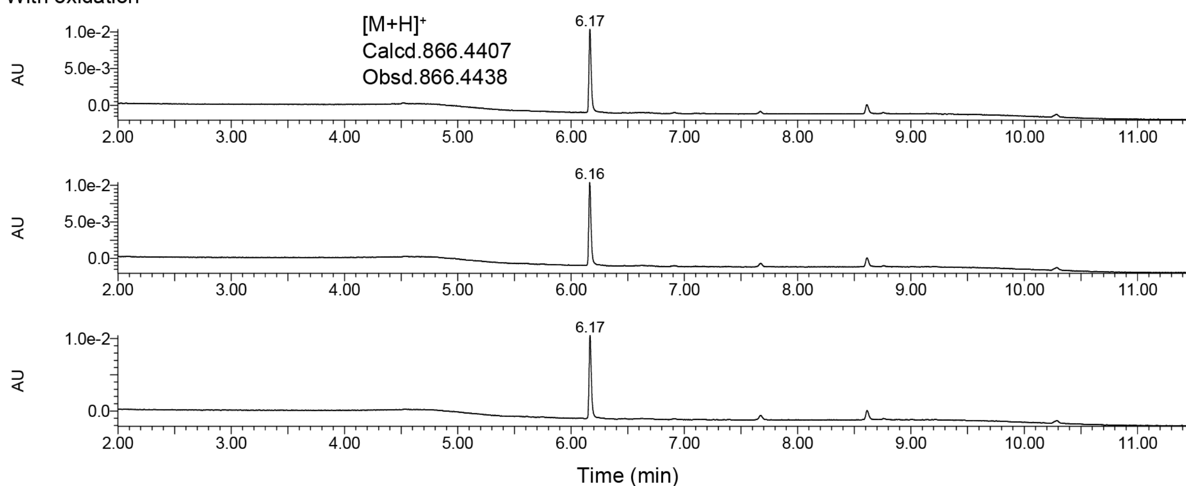


Fig. S6 UV chromatograms of **S3-linear** (a) without oxidation and (b) with oxidation. The experiment was conducted in triplicate.

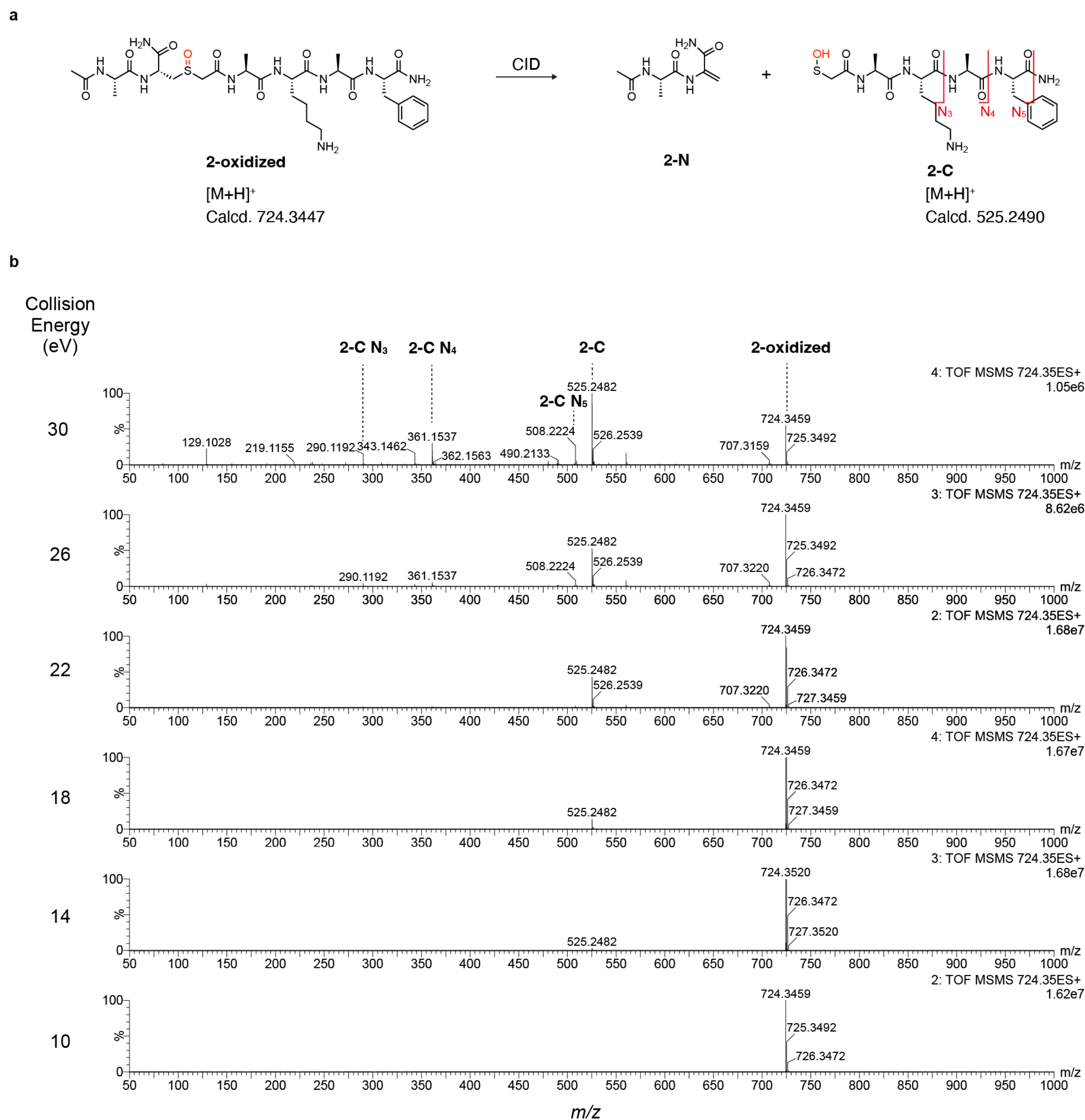


Fig. S7 (a) The scheme of CID of **2-oxidized**. (b) Collision energy dependency of the fragments generated from CID of **2-oxidized**.

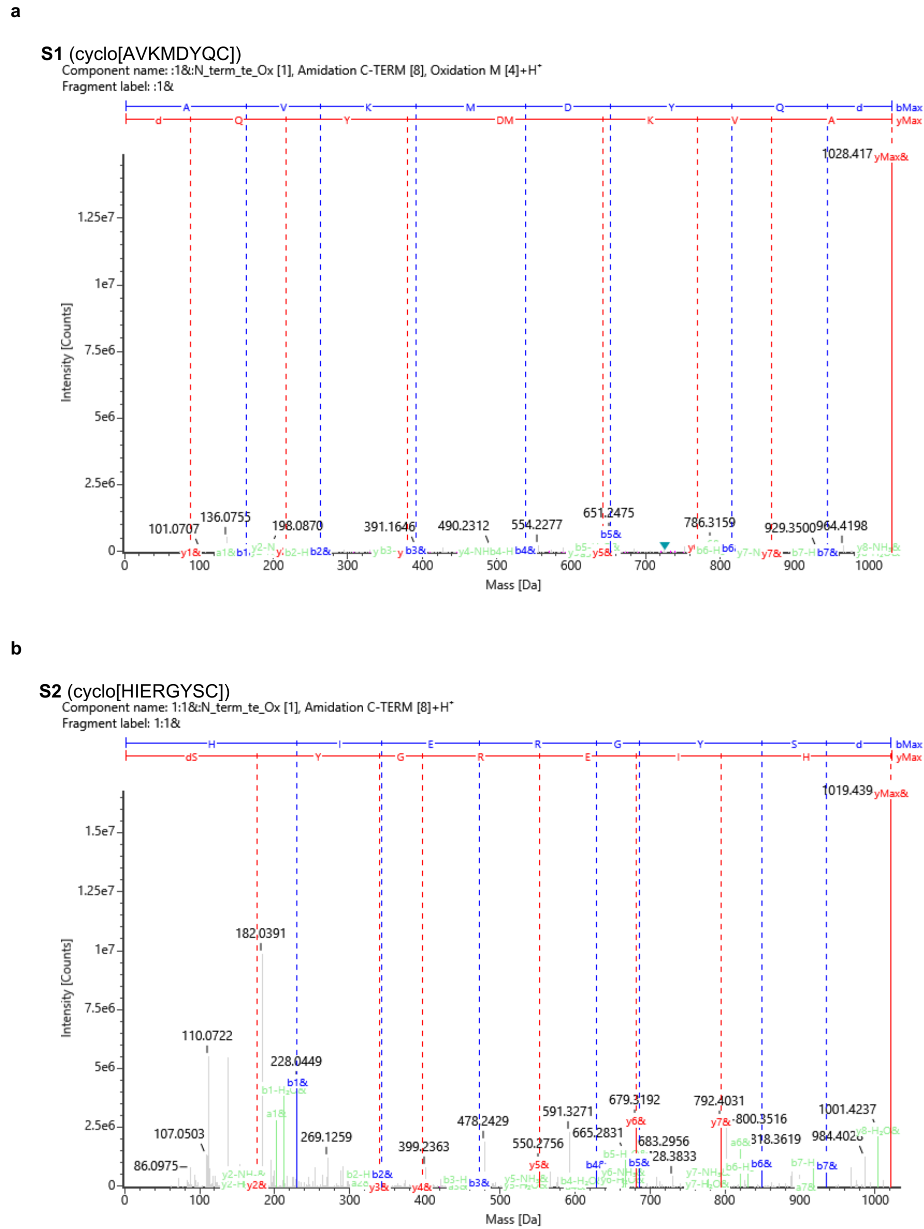
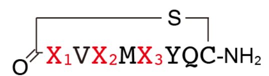


Fig. S8 MS/MS spectra of peptides (a) **S1** and (b) **S2**.

Table S1 Sequence identification of peptide **S1** from a virtual library.



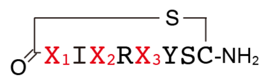
A virtual library of peptide **S1**

Assigned sequences (with the same parent mass value)

	Sequence	$[M+H]^+$ (Calculated)		
Correct sequence	AVKMDYQC			
Candidate[a]	Sequence	Mass error (Δ ppm)[b]	Matched fragments	Assigned intensity (%)
1	AVKMDYQC	–1.2	13 (93%)	62
2	AVDMKYQC	–1.2	11 (79%)	61
3	VVQMSYQC	–1.2	10 (71%)	60
6	KVAMDYQC	–1.2	9 (64%)	61
5	VVNMTYQC	–1.2	9 (64%)	59
7	VVSMQYQC	–1.2	9 (64%)	59
4	NVLMSYQC	–1.2	9 (64%)	57
8	QVVMSYQC	–1.2	8 (57%)	60
9	LVNMSYQC	–1.2	8 (57%)	60
10	KVGMEYQC	–1.2	8 (57%)	59
11	GVKMEYQC	–1.2	8 (57%)	59
12	VVTMNYQC	–1.2	8 (57%)	59
13	NVVMTYQC	–1.2	8 (57%)	59

[a]Only sequences corresponding to the strongest parent ion peak with more than 50% matched fragments are shown.
[b]Mass errors from the mass value of the correct sequence are shown.

Table S2 Sequence identification of peptide **S2** from a virtual library.



A virtual library of peptide **S2**

Assigned sequences (with the same parent mass value)

	Sequence	$[M+H]^+$ (Calculated)		
Correct sequence	HIERGYSC			
Candidate[a]	Sequence	Mass error (Δ ppm)[b]	Matched fragments	Assigned intensity (%)
1	HIERGYSC	–0.5	13 (93%)	36
2	EIHRGYSC	–0.5	10 (71%)	24
2	HIDRAYSC	–0.5	9 (64%)	34
3	HIGREYSC	–0.5	9 (64%)	33
4	HIARDYSC	–0.5	9 (64%)	33

[a]Only sequences corresponding to the strongest parent ion peak with more than 50% matched fragments are shown. Although two peaks with the same mass value were observed on the chromatogram, the peak with the higher intensity was recruited for the analysis.

[b]Mass errors from the mass value of the correct sequence are shown.

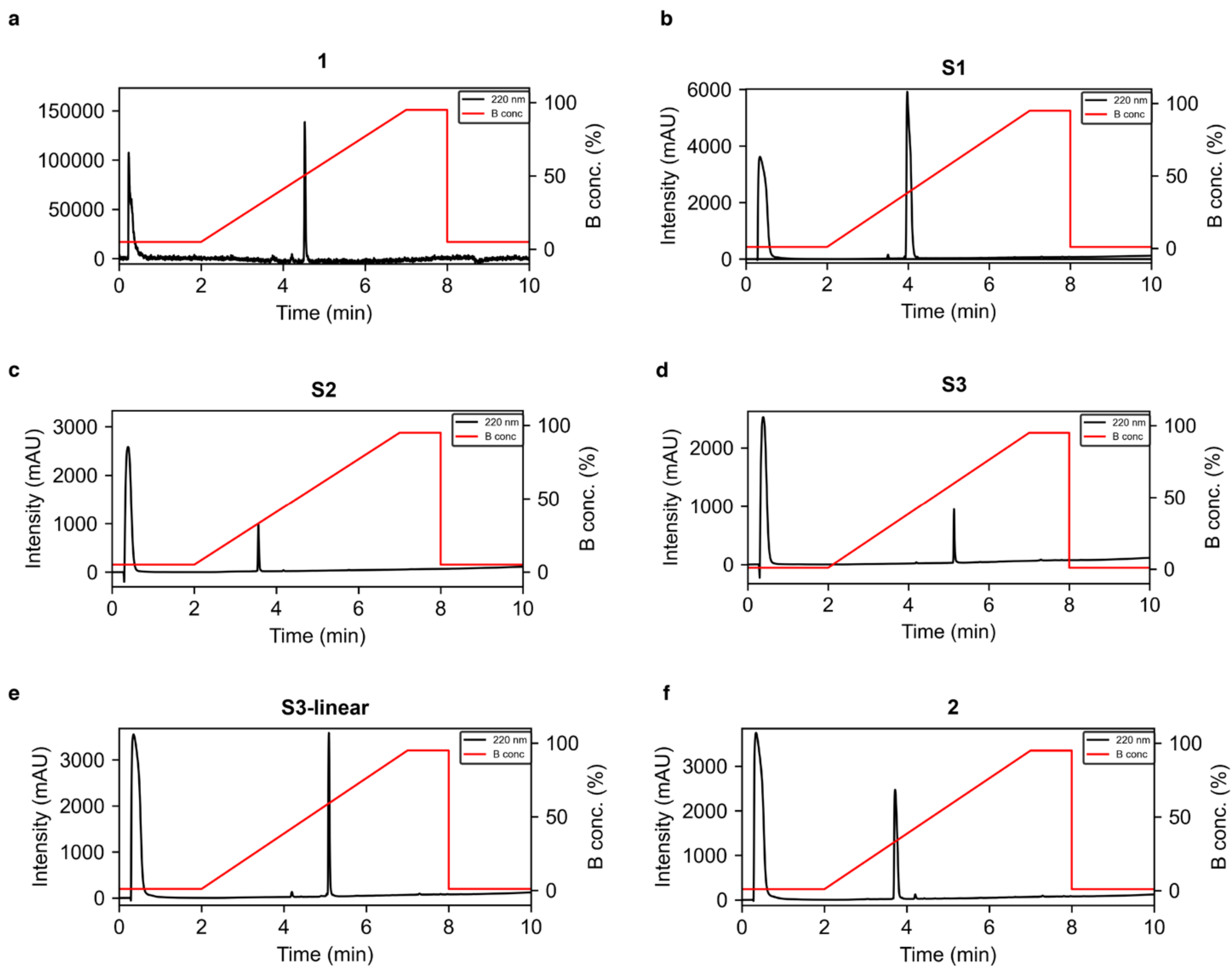


Fig. S9 LC chromatograms of the peptides used in this study after purification. Compounds were monitored at 220 nm.