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Supporting Information

A Facile Strategy for the Construction of A Phage Display Cyclic Peptide Library for the Selection of Functional Macrocycles

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‡ H. X. and L.W. B contributed equally.

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1. Materials and Methods

All Fmoc−protected amino acids, HBTU, and HOBT were purchased from CS Bio LTD. Rink Amide MBHA resin was purchased from Bide Pharm. Solid−phase reaction vessels were purchased from Genius Hi−Tech, Peptide synthesis was carried out on Peptide Synthesizer Division (CS Bio CO.). Trifluoro acetic acid (TFA) was purchased from ChemImpex Inc. Dichloromethane (DCM) and dimethylformamide (DMF)) were purchased from Aladdin. DMAP, Acetic anhydride, Pyridine were purchased from Merck Chemical. Peptides were purified by reverse phase HPLC using ZORBAX SB−C18 column (Agilent Technologies). Mass spectrometry data were recorded on a Thermo Scientific™ Q−Exactive LC−MS system. The affinity of the active molecules was characterized using Octet® R Series: Label−Free Biomolecular Interaction Analysis (Sartorius)

2. Solid−phase peptide synthesis

The peptides obtained via screening were synthesized using Rink Amide resin through Fmoc solid−phase peptide synthesis (SPPS). DMF was used to swell the rink amide resin with a loading capacity of 0.93 mmol/g for 1 h and the protection of the resin was removed by pyridine. Subsequently, the resin was washed by DMF (3 x 20 mL). Fmoc−Cys (Trt)−OH (3 equivalents) was pre−activated in DMF, following which HOBT (3.5 equivalents), HBTU (3.5 equivalents), and DIEA (3.5 equivalents) were added. Transferring the Fmoc−protected amino acid to the resin, and the mixture was stirred for 3 h. After draining and washing the resin, the next batch of Fmoc−Cys (Trt)−OH was added to the resin for an additional reaction over 3 h. DMF (5 x 10 mL) was subsequently used to wash the resin to remove the unreacted amino acids and catalysts. In order to determine the capping efficiency of amino acids, a very small amount of resin was transferred to the Ninhydrin in DMF and then heated for 5 min to observe the color change of the resin. Piperidine was used to remove amino acid F−moc protection for the next amino acid connection. 20 mL of piperidine solution was added to the resin and the reaction was stirred for 15 min, then the process was repeated. The procedure is repeated until the final amino acid is reacted. For the

synthesis of N−terminal acetylated peptides, anhydrous DCM: Pyridine: Acetic anhydride (2:1:1, v: v: v) with resin was applied for 1 h at room temperature.

3. Cleavage procedure

A mixture of 20 mL of trifluoroacetic acid (TFA), triisopropyl silane (TIS), and water (95:2.5:2.5) was transferred to the resin to produce free peptides. The reaction lasted for 3 h under agitation. Following treatment, the resin was removed via filtration, and the resulting crude products were precipitated with cold diethyl ether (Et_2O). 30 mL of ether was added to precipitate the peptides, which were then retained after centrifugation at 6000 rpm over 7 min. The resulting precipitated peptides underwent washing with ice−cold ether (20 mL, 15 mL, and 10 mL, respectively) and centrifugation at 6000 rpm for 7 min after each wash step. Purification of the final product to a high degree of purity (96–99%) was achieved through reverse−phase high−performance liquid chromatography (HPLC) using an Eplipse XDB−C18 column (99.4 mm × 250 mm, 5 µm) in an Agilent 1260 Infinity system. Peptides were purified with a gradient of acetonitrile (CH_3CN) and water (0.05% TFA) from 10:90 to 100:0, with a flow rate of 4 mL/min, and detection at 220 nm. Purity analysis of all peptides was conducted using an HPLC Agilent system with a ZORBAX SB−C18 column (4.6 mm × 250 mm, 5 µm) at a flow rate of 1 mL/min, employing a gradient of CH_3CN (solvent A): H_2O (0.05% TFA) (solvent B) from 10% to 90% A over 30 min. Additionally, the characterization of all peptides was confirmed by Thermo Scientific™ Q−Exactive LC−MS system.

4. Peptide cyclization with OPA

Phosphate−buffered saline (PBS 7.4) was used to dissolve purified linear peptides (1 equivalent) up to a final concentration of 1 mM for all experiments. In order to initiate the cyclization reaction, Phthalaldehyde (1.2 equivalents) was introduced to the PBS 7.4, which was reacted at room temperature for approximately 15 min. Following reaction completion, peptides underwent purification using CH_3CN: water (0.05% trifluoroacetic acid, TFA) (10:90 to 100:0) with a flow rate of 4 mL/min, and detection at 220 nm. Purity analysis of all cyclic peptides was conducted using an HPLC Agilent system with a ZORBAX SB−C18 column (4.6 mm × 250 mm, 5 µm) at a flow rate

of 1 mL/min. Additionally, the characterization of all peptides was confirmed by Thermo Scientific™ Q−Exactive LC−MS system.

5. The expression of the targets

The *Escherichia coli* strain BL21(DE3) was used to express Protein Tyrosine Phosphatase 1B (PTP1B) carrying a C−terminal His tag, NIMA−related kinase 7 (NEK7) with a N−terminal His tag and Homo Kelch−like ECH−associated protein 1 (hKeap1) with a N−terminal His tag, genes of which were inserted into the cytoplasmic expression plasmid pET22b−PTP1B. Taking PTPIB as an example, in order to initiate protein expression, a single colony was selected and inoculated in a small LB medium with 50 μg/mL kanamycin and incubated overnight at 37 °C, 220 rpm. The amplified bacterial solution was then added to 1 L medium for large−scale culture at 37 °C, 220 rpm until its OD_{600} reached about 0.8. β−D−1−thiogalactopyranoside (IPTG) was added to the solution to a final concentration of 0.5 mM and incubated at 18 °C for 14 h to express the target protein. Following incubation, the cells were harvested by centrifugation at 4000 × g for 20 min. The precipitate was then re−suspended using a lysis buffer (50 mM HEPES, 500 mM NaCl, 10 mM imidazole, and 10 mM PMSF). Centrifugation at 10,000 × g for 15 min removed insoluble debris from the lysis solution and Ni−NTA was used to purify protein from the supernatant. The purity of the protein was determined using SDS−PAGE. After desalting using a stored buffer (50 mM HEPES, 150 mM NaCl, 1 mM TCEP, and 10% glycerol), the protein was stored at −80 °C for further use.

6. Using OPA modifies linear peptide on the phage surface

A specific peptide sequence (KGGSC) was genetically inserted into the N−terminus of the phage coat protein III (pIII) through polymerase chain reaction (PCR) amplification of the Pbcomb3xga plasmid from Pbcomb3xss (Addgene) using the following primers:

GGS−fw:

5'− CTAGGGATCCAAGGGTTCTTCTTGCGGTGAAAACCTGTACTTCCAGG

GCGGCTCGGCCTC −3'

GGS−rv:

5'− CTAGGGATCCGCAACGACCGTCGATAGAACCACCGGCCGCCTGG −3'.

The resulting plasmids were introduced into *Escherichia coli* TG1 cells via electroporation and cultured initially in 1 mL of LB medium. The 1 mL of TG1 cells harboring the Pbcomb3xga_GGS plasmid were then expanded to 100 mL of 2xYT−medium (50 μg/mL ampicillin) and incubated at 37 °C with 220 rpm. Following incubation, the cultures were centrifuged, and the resulting phage−containing supernatant was transferred to new centrifuge bottles. Ice−cold polyethylene glycol (PEG)−NaCl solution was added to the supernatant, followed by a 30 min incubation on ice to precipitate the phages. Precipitated phages were pelleted by centrifugation, and the supernatant was carefully decanted. The centrifuge bottles were inverted on filter paper for 2 min to remove residual liquid. The phage pellet was resuspended in 10 mL of phosphate−buffered saline (PBS) at pH 7.4 and transferred to a 50 mL tube. Any remaining cell and phage debris were removed by centrifugation, and the resulting supernatant was carefully transferred to a new 50 mL Greiner tube. 40 μL factor Xa was introduced to the phage solution and incubated for 4 h at room temperature. Following digestion, tris (2−carboxyethyl) phosphine (TCEP) (1 mM) was added to the reaction mixture, which was then incubated at 37°C for 1 h. Blocked phages were obtained by treating with 6−hydroxy−1,3−benzothiazole−2−carbonitrile (CBT, 0.5 mM) at 25 °C for 2 h. Washes with ice−cold PBS were performed three times, with special attention to resuspending phages prone to filter accumulation. Phage

samples were incubated with *ortho*−phthalaldehyde (OPA) (300 μM) at 25 °C for 20 min, concentrated to 1 mL, and washed three times with PBS. TEV (50 μL) was added to the blocked phage solution and incubated for 8 h at room temperature. Samples were then concentrated using the centrifugal ultrafiltration tubing (Amicon® Ultra−15, 10 kDa), and the filtrate was collected for subsequent HPLC and mass spectrometry characterization.

7. Construction of Phage Library

We constructed the phagemid library by undergoing PCR to directly amplify the Pbcomb3xga plasmid from Pbcomb3xss (Addgene) using two primers:

L7MGA−F:

5'−CTAGGGATCCATCGACGGTCGTTGCGGTTCTAAGNNKNNKNNKNNKNN KNNKNNKTGCGGCGGCTCGGCCTC −3'

L7MGA−R:

5'−CTAGGGATCCACCGGCCGCCTGGGCCACG−3'.

The PCR product was digested with BamHI restriction enzyme, and the resulting fragments were ligated using T4 DNA ligase. DpnI was utilized to remove the template phagemid. Subsequently, the ligated plasmids were introduced into competent *E. coli* TG1 cells via electroporation. The transformed cells were then cultured in 1 mL LB medium and inoculated into 100 mL LB medium (50 μg/mL ampicillin). Upon reaching an optical density (OD_{600}) of 1.0, 50 mL of the bacterial culture was harvested, mixed with 30% glycerol, and stored at −80 °C to ensure viability. From this library, 20 clones were randomly selected for DNA sequencing, the results of which are provided in Supporting Figure S17. Notably, in all 20 designed clones, the insertion sites were randomized without any discernible bias towards specific codons.

8. Phage Library production and infectivity studies

The 1 mL of TG1 cells harboring the Pbcomb3xga_GGS phagemid were then expanded to 300 mL of 2xYT−medium (50 μg/mL ampicillin) and incubated at 37 °C with 220 rpm. After infection at 1 h with helper phage vcsm13, 1 mM IPTG was used to initiate peptide expression in phagemid and incubated at 37 °C, 220 rpm overnight. After expression, the cells were removed by centrifugation at 4 °C, 8000 rpm. After transferring the supernatant to a new centrifuge bottle, PEG−NaCl solution stored at 4 °C was added to precipitate the phage. After 4 h, the precipitated insoluble solid was separated by centrifugation at 4 °C, 9000 rpm for 20 min. After the supernatant solution was completely removed, 3 mL of PBS (pH 7.4) was added to the precipitate in two separate increments, followed by centrifugation to remove the remaining bacteria at 4 °C, 12000 rpm for 3 min. From the retained phage solution, 40 μL were separated and stored at 4 °C. A moderate amount of factor Xa was used to digest the bulk of the phage solution and incubate the phage solution for 4 h at room temperature. To reduce the phages, tris (2−carboxyethyl) phosphine (TCEP) was added to achieve a final concentration of 1 mM, followed by incubation at 37 °C for 1 h. Blocked phages were obtained by treating with 6−hydroxy−1,3−benzothiazole−2−carbonitrile (CBT) at a concentration of 0.5 mM for 2 h at 25 °C. Samples were concentrated to 1 mL using the centrifugal ultrafiltration tubing (Amicon® Ultra−15, 30 kDa). The blocked phages were then washed three times with 10 mL of PBS. After solution replacement, the remaining phage solution was transferred to new tubes and replenished to 3 mL using PBS with TCEP (1 mM) and then stored at 4 °C. The phages with different concentrations of OPA were incubated at 25 °C for 20 min. After the reaction, the samples were purified using a PEG−NaCl solution, and then the final 3 mL phage solution was placed in a 4 °C environment.

9. Infectivity studies

40 μL of the 'purified', 'TCEP 1 mM', 'CBT 0.5 mM', 'OPA 100 mM', 'OPA 300 mM', and 'OPA 500 mM' samples were stored for subsequent determination of phage infectivity following each step. Using the same method to prepare diluents at 10−fold of different samples. *Escherichia coli* TG1 cells with OD_{600} at 0.4−0.5 (100 μL) were mixed with diluents (10 μL) of 10^{-5}, 10^{-7}, and 10^{-9}, respectively, and incubated at 37 °C, 220 rpm for 1 h. Then the infected bacteria were successively coated on the solid medium with ampicillin. The number of infected colonies was counted after overnight culture.

10. Phage Panning

For the selection experiments, immobilizing the target proteins (PTP1B, NEK7, and hKeap1) with an N−terminal His_6 tag on plates at first. The protein solution diluted with the PBS buffer (100 μL, 4 μM) was placed in micropores and incubated at 4 °C overnight. A PBS buffer (200 μL) with 2% BSA was used to block the remaining blank area, and then the blocked micropores were washed three times by the PBS with 0.5% Tween−20. The negative selection step during screening is achieved by using phage library coated plates prior to the blocking step. Phages were added to the micropores coated with the target protein and incubated at room temperature for 1 h. The PBS with 0.5% Tween−20 was then applied to wash the micropores 10 times. Bound phages were incubated at room temperature for 15 min with 100 μL of elution buffer (0.2 M Glycine−HCl, pH 2.2, 1 mg/mL BSA). Transfer the eluent to the tubes containing a neutralizing buffer (15 μL, 1 M Tris−HCl, pH 9.0) and mix thoroughly. 10 μL of the retained phage solution was used to determine the phage titers as per the previous protocol. The remaining phage was used to amplify by infecting TG1. Each target was panned for four rounds. Subsequent sequencing was performed to determine the enrichment status after panning.

11. Bio Layer Interferometry (BLI) assay

Target proteins (PTP1B, NEK7, and hKeap1) were labeled with biotin in buffer solution. Ni−NTA (NTA) Biosensors and SA Biosensors were from Gator Bio.[1, 2] Prior to functionalization, sensors were immersed for 20 min in PBS 7.4. Subsequently, the biosensors were immersed for 600 s in protein solutions at 20 μM concentration and rinsed in buffer solution for 10 min. The PBS with 0.1% Tween−20 was applied to immobilize proteins. The same buffer was then used to wash away the unstable binding proteins, with an overall equilibrium time of 180 s. Ligand solutions with different concentration gradients were used to measure the binding curve, and each concentration determination is followed by a dissociation solution treatment. The protein−immobilized sensors were used to trap the peptide molecules by submerging the sensor into the peptide solution for 3−5 min. The dissociation solution was then used to dissociate the peptide molecules bound to the protein surface, and this process lasts 1−3 min. The sensor regeneration process was interspersed between the two detection cycles to ensure the normal binding of the analyte at the next concentration. Blank control was achieved by using a sensor without immobilizing protein to counteract non−specific binding of the analyte. For gaining the sensorgrams, a 1:1 model was applied to fit curves.

12. Molecular docking studies

LigPrep and Protein Preparation Wizard from the Schrödinger Suite release 2015–1 (LigPrep, Schrödinger, 2015) were applied to prepare the ligands and proteins, respectively.[3-5] The structures of PTP1B, NEK7, and hKeap1 were retrieved from the protein−ligand complexes (PDB codes: 1PXH, 6S75, and 2FLU). For the purpose of defining the docking pocket, the cubic box centered on the ligand was pre-pared with 25 Å. In these investigations, we employed the Induced Fit Docking protocol available from the Schrödinger suit. First, Glide was used to set out the docking of the ligand towards to the target with flexibility. The van der Waals radii (vdW) were set to 0.5 for reducing the probability of protein and ligand collision. The prime was used to

optimize the side chains of residues within 5 Å in poses. The docking procedure was initiated in the generated receptor conformation under extra precision. For the purpose of distinguishing the best result, the Induced Fit Docking (IFD) score was applied to rank the docking poses.

13. Molecular Dynamic Simulation

Desmond package of Schrodinger was utilized to simulate the complexes of ligand binding to protein. Molecular docking generated initial static protein–ligand complexes, while molecular dynamics (MD) simulations traced atom evolution over time, offering insights into ligand binding orientation in a physiological environment. The protein preparation wizard was used to optimized the complex, and the system builder tool was used to assembled the system. The simulation system was immersed in a TIP3P model within an orthorhombic box and employed the OPLS 2005 force field during the production run. Neutralization was accomplished by adding Na+ and Cl+ ions (0.15 M). Equilibration included 1 ns in the NVT ensemble (300 K, 1 atm) followed by an additional 1 ns in the NPT ensemble. Martyna−Tobias−Klein and Nose−Hoover thermostat were utilized to regulate temperature and pressure in system throughout the simulation, which lasted for 120 ns.

14. Supporting Figures

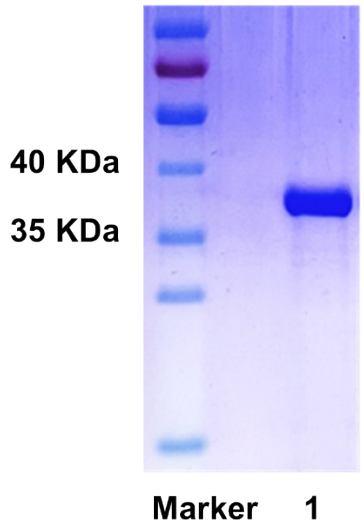

Figure S1. Identification map of PTP1B size and purity.

K	T	F	L	H	W	R	Y	C
K	T	F	L	H	W	R	Y	C
K	T	F	L	H	W	R	Y	C
K	T	F	L	H	W	R	Y	C
K	T	F	L	H	W	R	Y	C
K	T	F	L	H	W	R	Y	C
K	T	F	L	H	W	R	Y	C
K	L	F	L	H	W	A	R	C
K	L	F	L	H	W	A	R	C
K	L	F	L	H	W	A	R	C
K	L	F	L	H	W	A	R	C
K	T	L	L	S	H	W	F	C
K	T	L	L	S	H	W	F	C
K	L	R	F	H	W	A	Y	C
K	Y	A	I	H	R	Y	E	C

Figure S2. Sequencing results of 20 clones after four rounds of screening for the target protein PTP1B, five of which were wrong sequences.

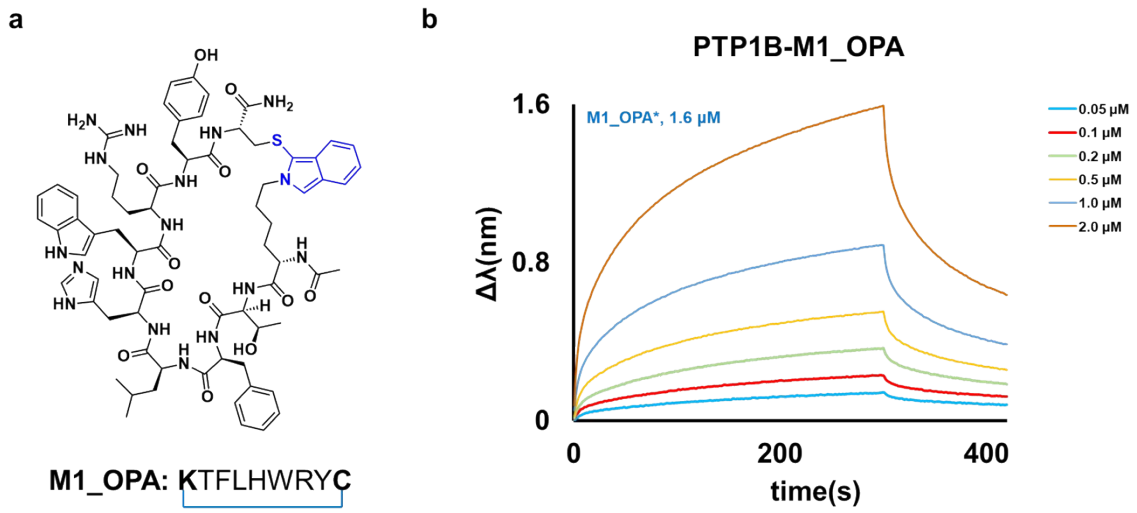

Figure S3. Identification of cyclic peptide binder of PTP1B. (a) Chemical structure of M1_OPA. (b) Bio−Layer Interferometry assay result of M1_OPA binding PTP1B from raw.

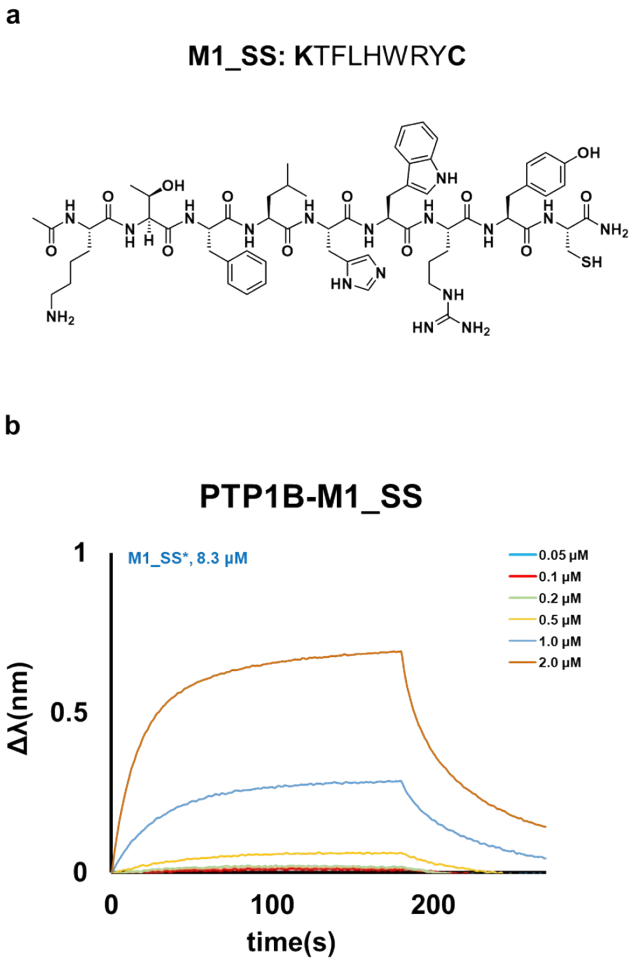

Figure S4. Identification of peptide binder of PTP1B. (a) Chemical structure of M1_SS. (b) Bio−Layer Interferometry assay result of M1_SS binding PTP1B from raw.

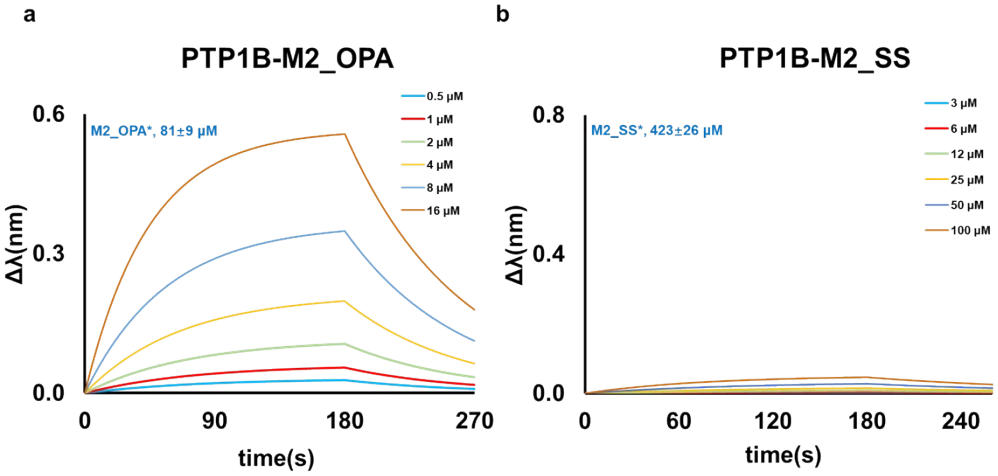

Figure S5. BLI assay result (fit). (a) M2_OPA binding PTP1B. (b) Hk2_SS binding PTP1B.

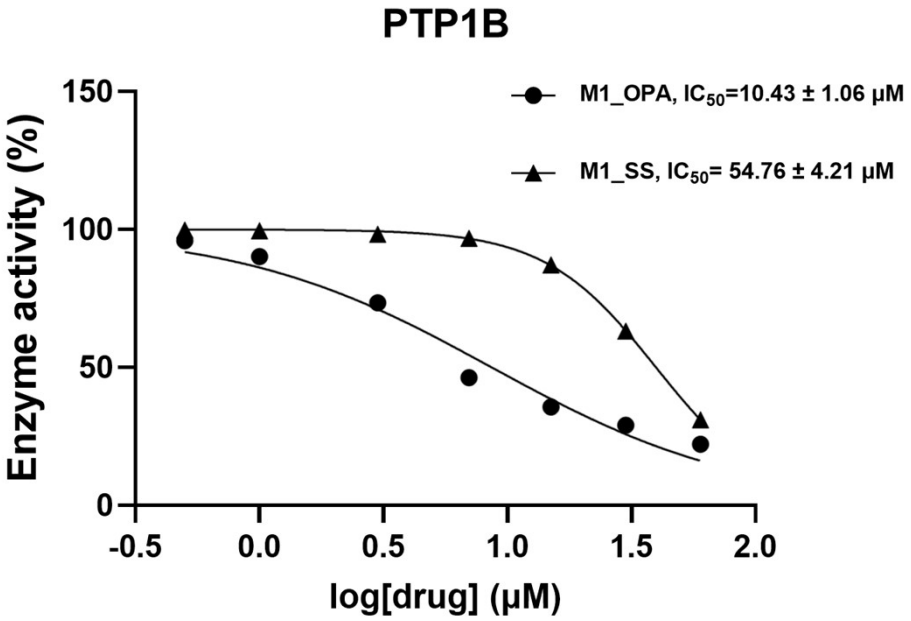

Figure S6. The results of half maximal inhibitory concentration (IC_{50}) for M1_OPA and M1_SS targeted PTP1B.

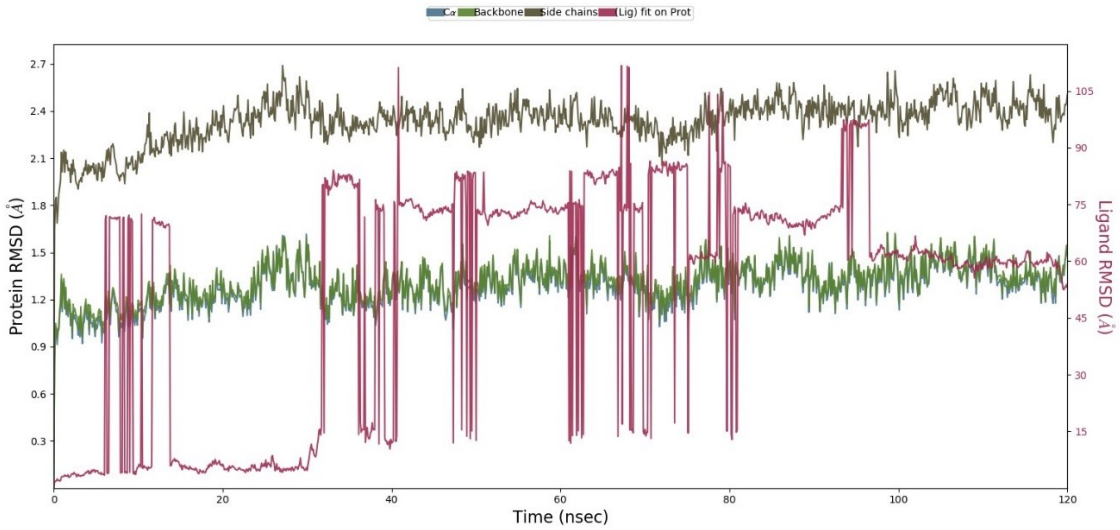

Figure S7. Residue wise root−mean−square deviation (RMSD) of the C−alpha atoms of PTP1B – M1_OPA Complex.

Protein RMSD: The above plot shows the RMSD evolution of a protein (left Y−axis). All protein frames are first aligned on the reference frame backbone, and then the RMSD is calculated based on the atom selection. Monitoring the RMSD of the protein can give insights into its structural conformation throughout the simulation. RMSD analysis can indicate if the simulation has equilibrated — its fluctuations towards the end of the simulation are around some thermal average structure. Changes of the order of 1−3 Å are perfectly acceptable for small, globular proteins. Changes much larger than that, however, indicate that the protein is undergoing a large conformational change during the simulation. It is also important that your simulation converges — the RMSD values stabilize around a fixed value.

Ligand RMSD: Ligand RMSD (right Y−axis) indicates how stable the ligand is with respect to the protein and its binding pocket. In the above plot, 'Lig fit Prot' shows the RMSD of a ligand when the protein−ligand complex is first aligned on the protein backbone of the reference and then the RMSD of the ligand heavy atoms is measured.

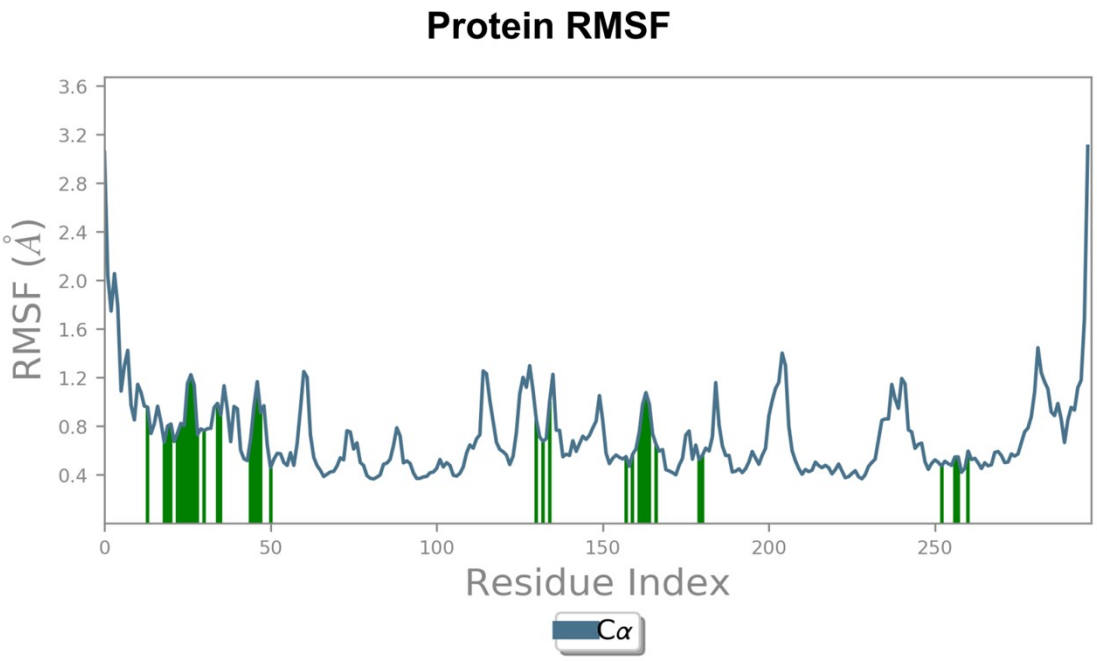

Figure S8. Root−mean−square fluctuations (RMSF) of the C−alpha atoms of PTP1B. The Root Mean Square Fluctuation (RMSF) is useful for characterizing local changes along the protein chain. **Ligand Contacts:** Protein residues that interact with the ligand are marked with green−colored vertical bars.

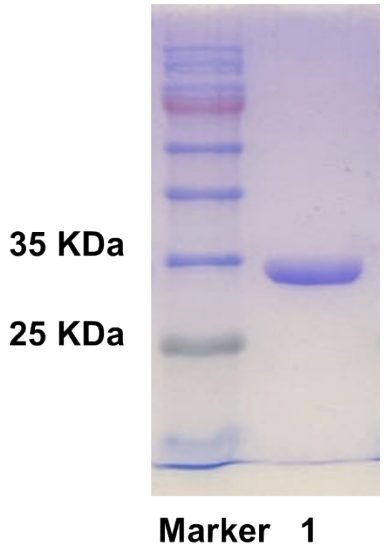

Figure S9. Identification map of NEK7 size and purity.

K	T	F	P	L	S	S	F	C
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K	T	F	P	L	S	S	F	C
K	T	F	P	L	S	S	F	C
K	T	F	P	L	S	S	F	C
K	T	F	P	L	S	S	F	C
K	T	F	P	L	S	S	F	C
K	T	F	P	L	S	S	F	C
K	T	F	P	L	S	S	F	C
K	T	F	P	L	S	S	F	C
K	T	F	P	L	S	S	F	C
K	T	F	P	L	S	S	F	C
K	W	L	I	A	Q	R	M	C
K	W	L	I	A	Q	R	M	C
K	W	L	I	A	Q	R	M	C
K	L	L	F	P	L	I	N	C
K	L	L	F	P	L	I	N	C

Figure S10. After four rounds of screening for the target protein NEK7, 20 clones were sequenced, three of which were wrong sequences.

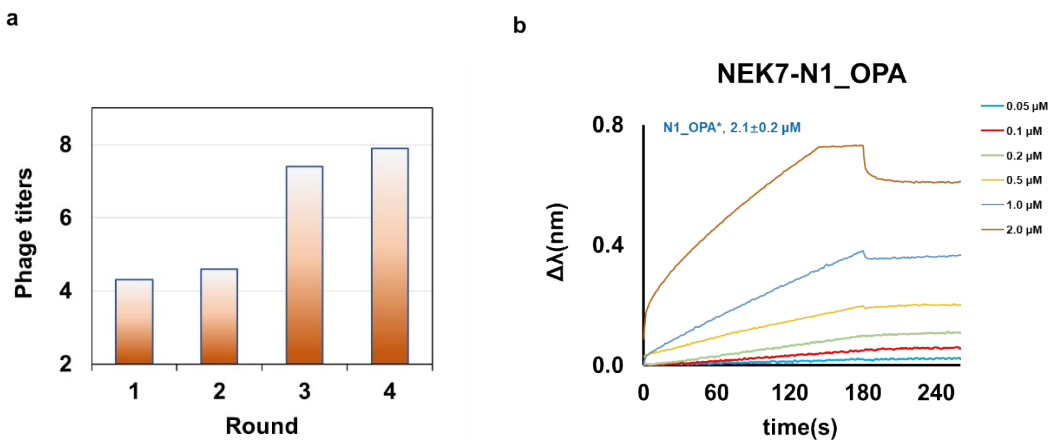

Figure S11. Phage selection of OPA cyclized phage library against NEK7 and identification of cyclic peptide binder of NEK7. (a) Phage titers corresponding to the number of retained phages after selection against NEK7 per selection round. An increase in phage titers for the selections over consecutive rounds is indicative of the amplification of binders. (b) Bio−Layer Interferometry assay result of N1_OPA binding NEK7 from raw.

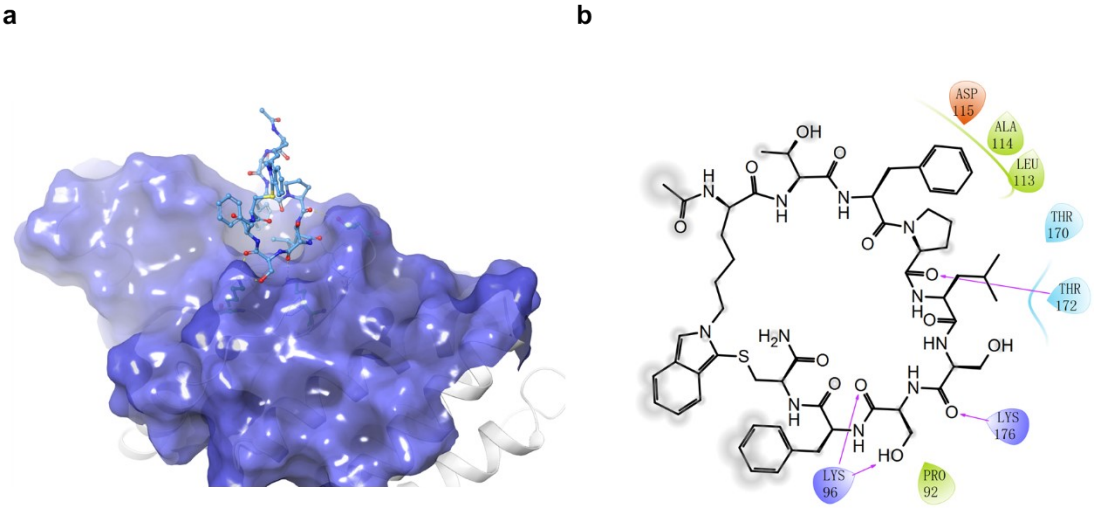

Figure S12. The molecular docking result of N1_OPA binding to the NEK7. (a) Binding interface in dimensional space. (b) Bonding interface in plane. N1_OPA produces salt bridges, hydrogen bonds, and conjugation interactions with the specific residues Lys 96, Lys 17, and Thr 172.

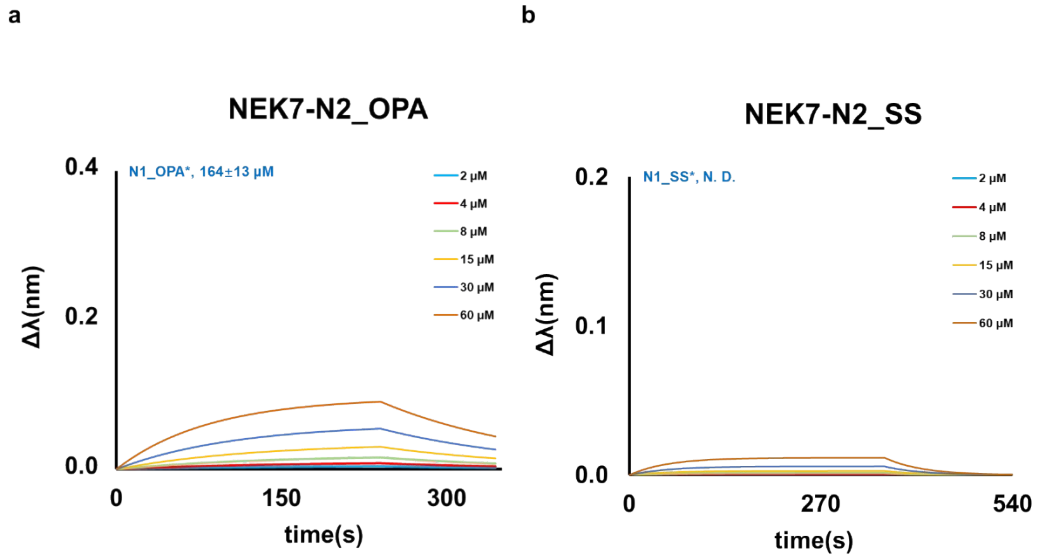

Figure S13. BLI assay result (fit). (a) N2_OPA binding NEK7. (b) N2_SS binding NEK7.

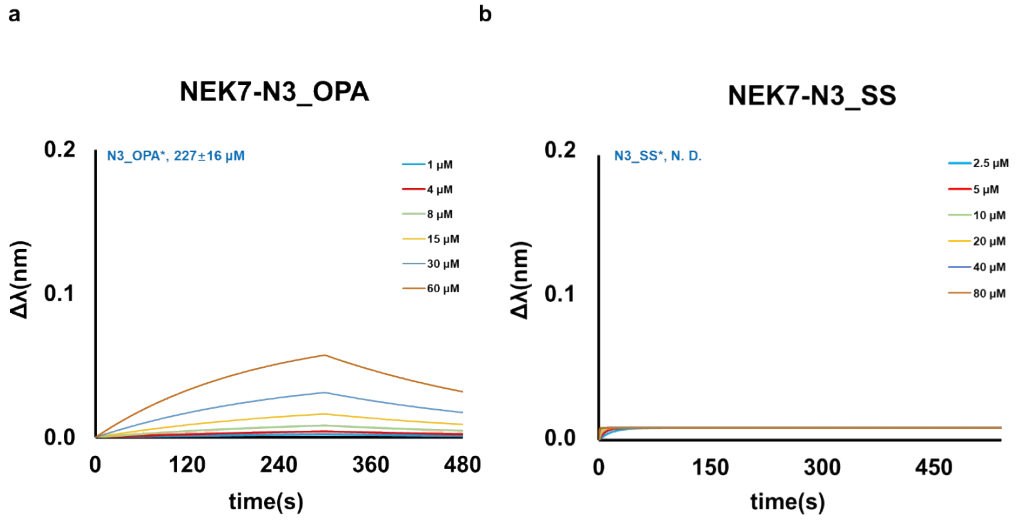

Figure S14. BLI assay result (fit). (a) N3_OPA binding NEK7. (b) N3_SS binding NEK7.

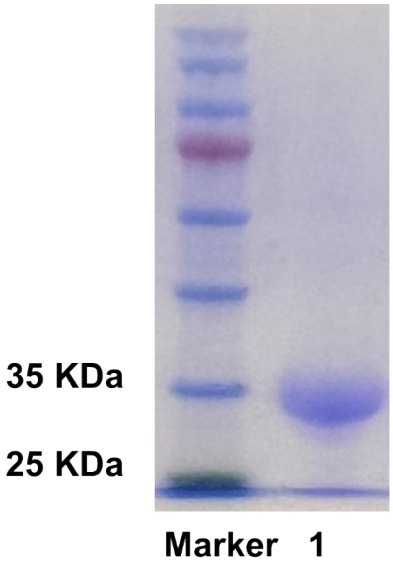

Figure S15. Identification map of hKeap1 size and purity.

K	Q	T	A	T	G	R	G	C
K	Q	T	A	T	G	R	G	C
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K	Q	T	A	T	G	R	G	C
K	Q	T	A	T	G	R	G	C
K	Q	T	A	T	G	R	G	C
K	Q	T	A	T	G	R	G	C
K	Q	T	A	T	G	R	G	C
K	Q	T	A	T	G	R	G	C
K	L	D	P	D	T	G	E	C
K	L	D	P	D	T	G	E	C
K	L	D	P	D	T	G	E	C
K	Q	T	A	T	G	E	G	C
K	Q	T	A	T	G	E	G	C
K	D	P	E	T	G	E	Y	C
K	L	D	S	E	T	G	E	C
K	L	D	S	E	T	G	E	C
K	Q	S	A	E	T	G	E	C

Figure S16. After four rounds of screening for the target protein hKeap1, 20 clones were sequenced, two of which were wrong sequences.

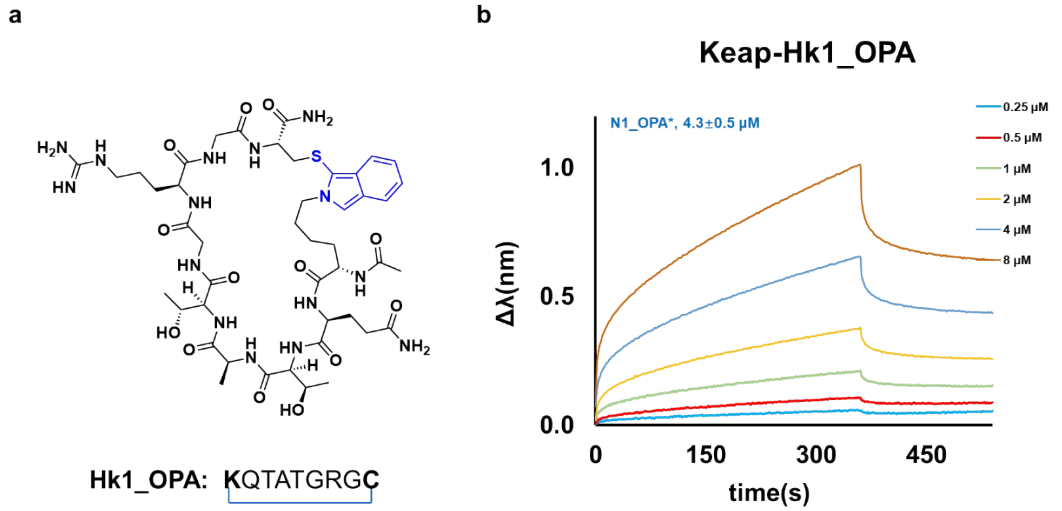


Figure S17. Identification of cyclic peptide binder of hKeap1. (a) Chemical structure of Hk1_OPA. (b) Bio−Layer Interferometry assay result of Hk1_OPA binding hKeap1 from raw.

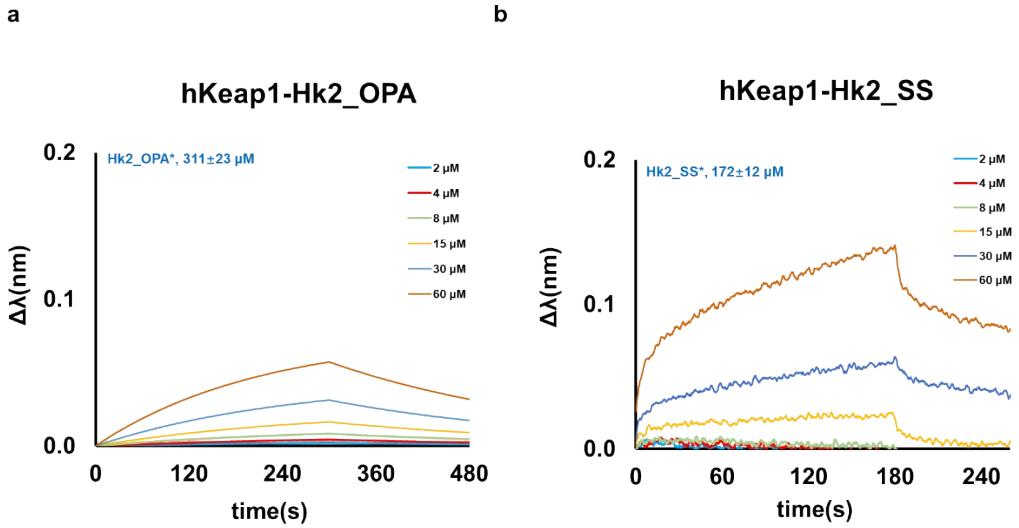


Figure S18. BLI assay result (raw). (a) Hk2_OPA binding hKeap1. (b) Hk2_SS binding hKeap1.

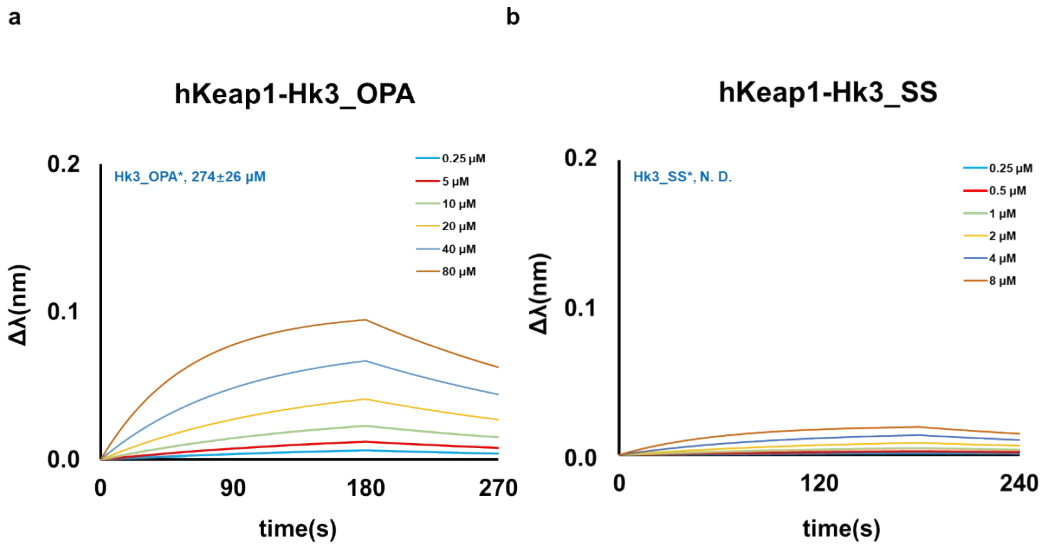


Figure S19. BLI assay result (raw). (a) Hk3_OPA binding hKeap1. (b) Hk3_SS binding hKeap1.

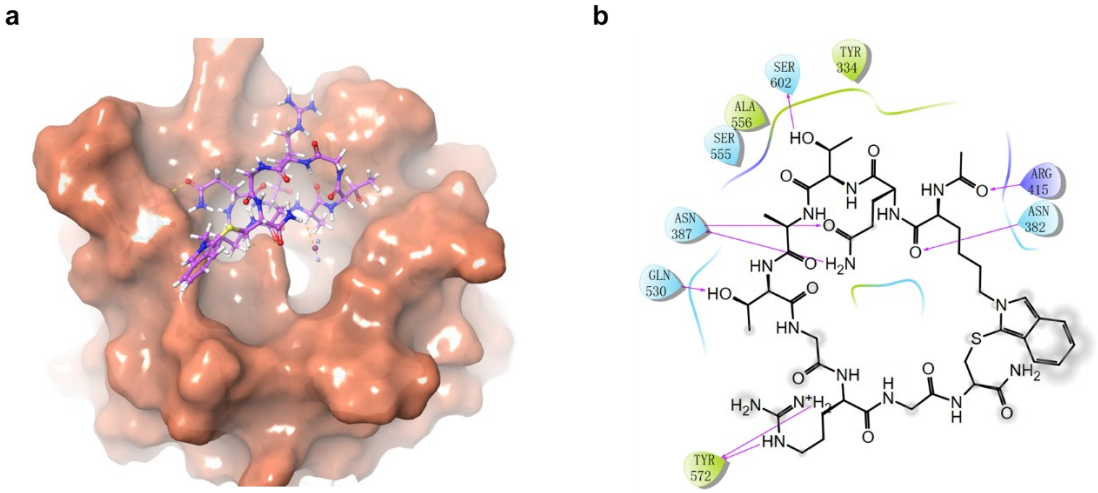


Figure S20. The molecular docking result of Hk1_OPA binding to the hKeap1. (a) Binding interface in dimensional space. (b) Bonding interface in plane. Hk1_OPA produces salt bridges and hydrogen bonds with the specific residues Asn 382, Asn 387, Arg 415, Gln 530 and Tyr 572 at Nrf2 binding site.

K	T	L	R	F	T	F	Y	C
K	I	S	S	L	T	M	W	C
K	V	Y	S	L	C	G	L	C
K	P	L	T	S	A	R	N	C
K	V	A	W	I	L	G	M	C
K	I	K	V	T	V	E	D	C
K	M	S	G	L	V	T	R	C
K	Q	S	C	R	Q	W	Y	C
K	S	Y	L	W	L	F	M	C
K	V	M	I	G	Y	R	C	C
K	F	L	S	I	S	T	H	C
K	I	I	N	L	L	V	P	C
K	R	F	D	S	S	P	F	C
K	M	D	F	I	T	L	P	C
K	F	F	F	P	H	F	L	C
K	P	K	M	C	V	S	P	C
K	Q	P	G	T	A	L	Y	C
K	S	S	R	S	V	L	H	C
K	V	F	H	L	D	L	F	C
K	P	T	F	Y	L	T	F	C

Figure S21. The peptide library was constructed and 20 random clones were sequenced.

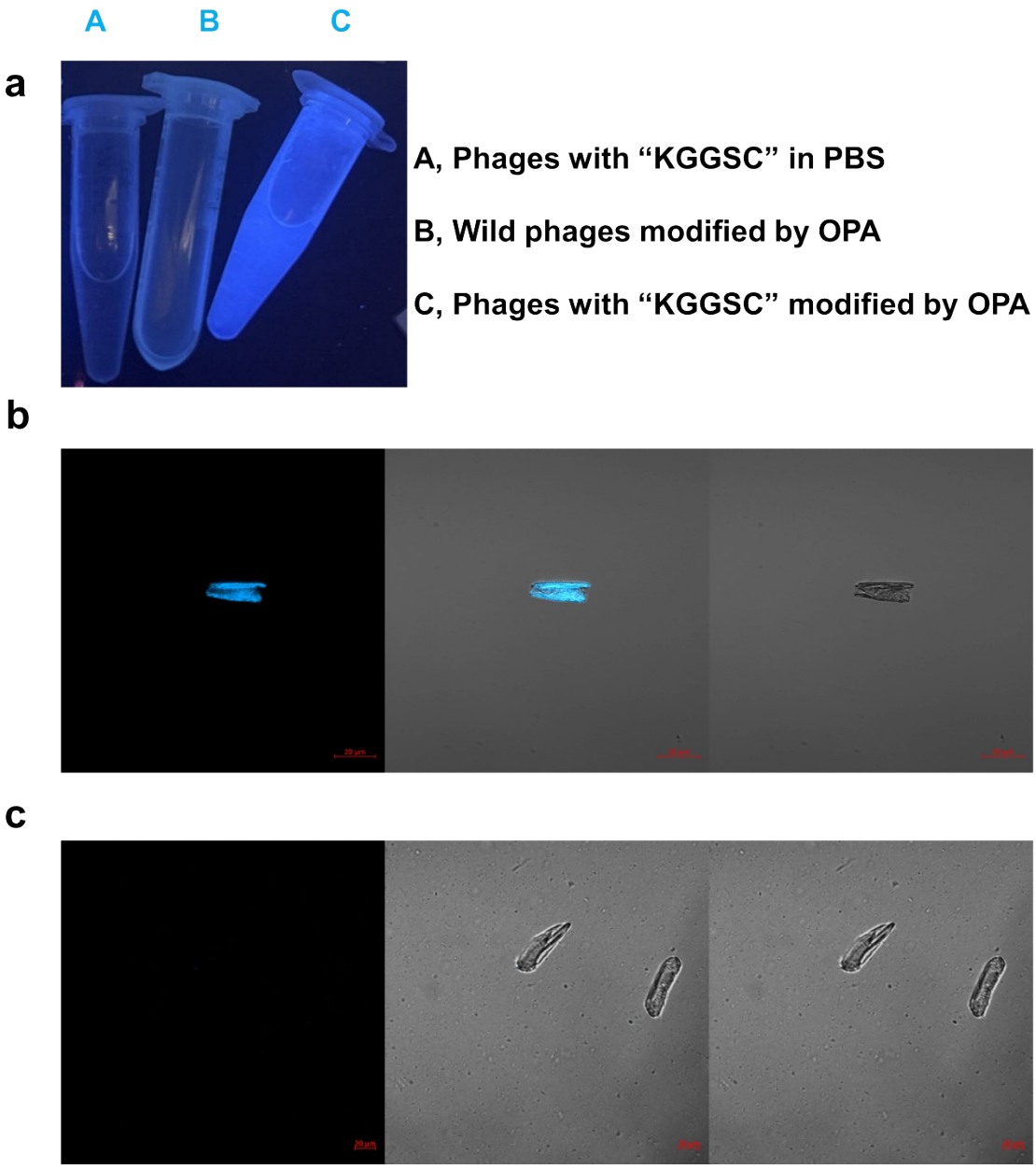


Figure S22. (a) the fluorescence of cyclic reaction production was observed with the naked eyes. High-resolution fluorescence images of b) the phages with “KGGSC” modified by OPA. c) the wild phages treated with OPA. Scale bar is 20 μm.

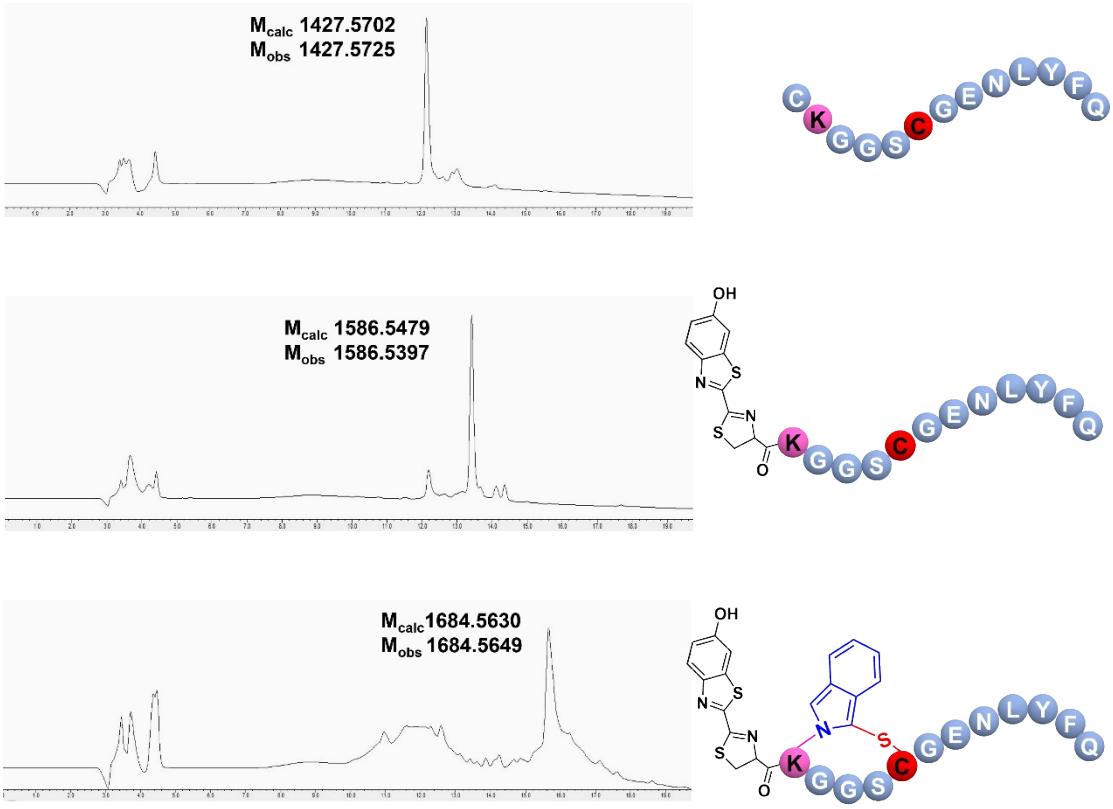
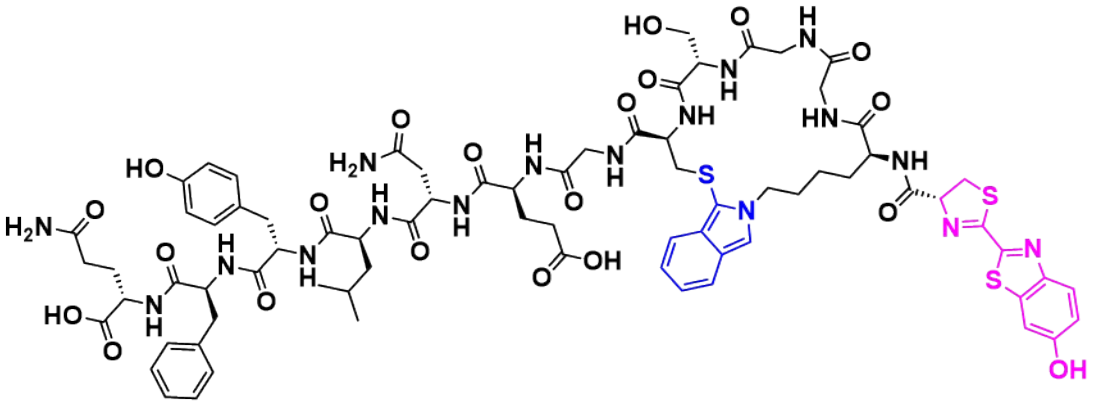


Figure S23. HPLC and MS results of multi-enzyme digestion products derived from controlled experiments with various modification stages in the cyclization strategy. To obtain more detailed modification effects, a large number of enzymatic digestion products derived from phasmid were enriched. Controlled trials have shown the practical conversion of phages with the modified strategy, in turn, the products digested by multi-enzyme without chemical modification, multi-enzyme digestion products with CBT blocking and multi-enzyme digestion products with OPA cyclization, which showed that the conversion rate with OPA modification was 82%.

15. High Performance Liquid Chromatography and Mass Spectrometry



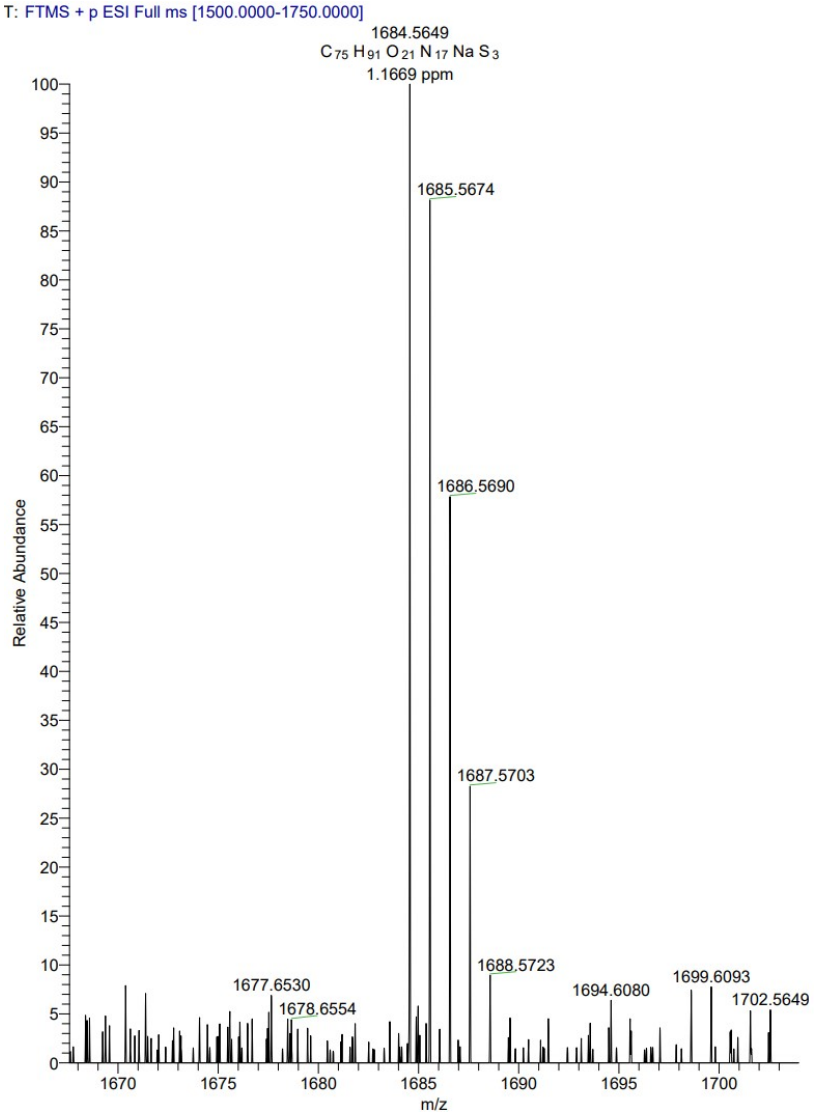


Figure S24. MS analysis of the cyclic '**KGGSC**'. ESI−MS calcd. for $\mathbf{C_{75}H_{91}N_{17}O_{21}S_3}$ [M + Na]$^+$ m/z =1684.5630, found 1684.5649.

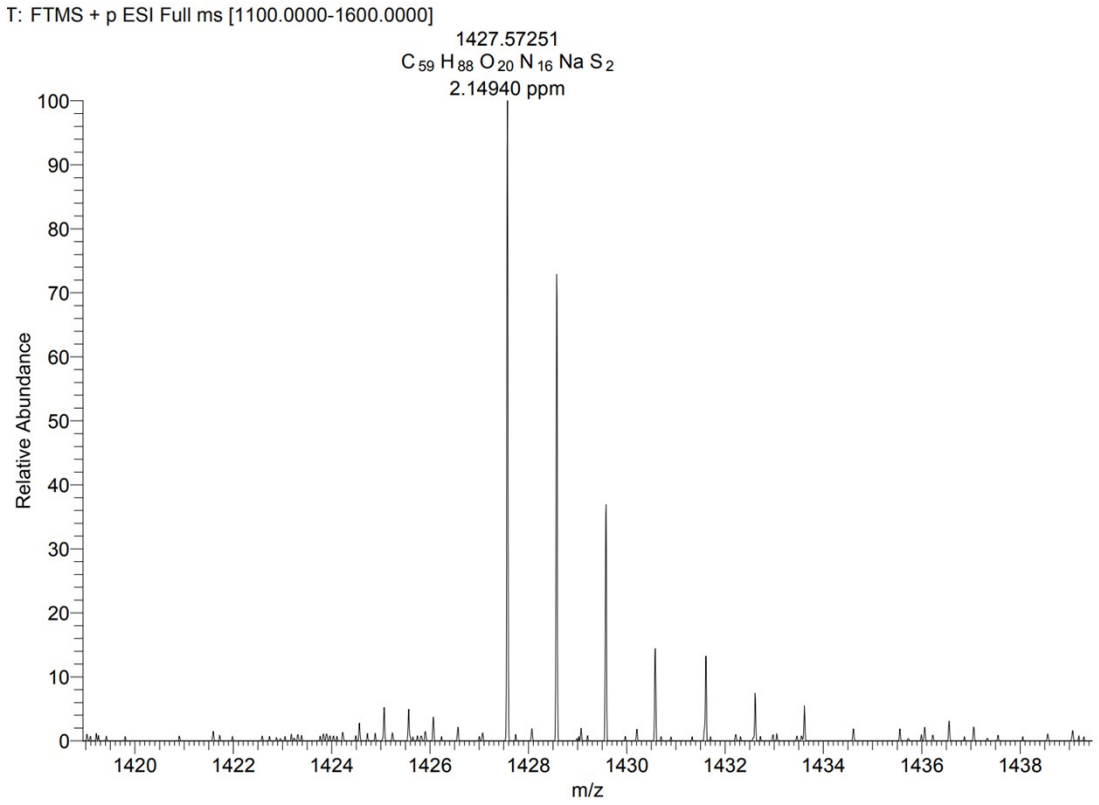


Figure S25. MS analysis of the 'KGGSC'. ESI−MS calcd. for **$C_{59}H_{88}N_{16}O_{20}S_2$** $[M + Na]^+$ m/z =1427.5702, found 1427.5725.

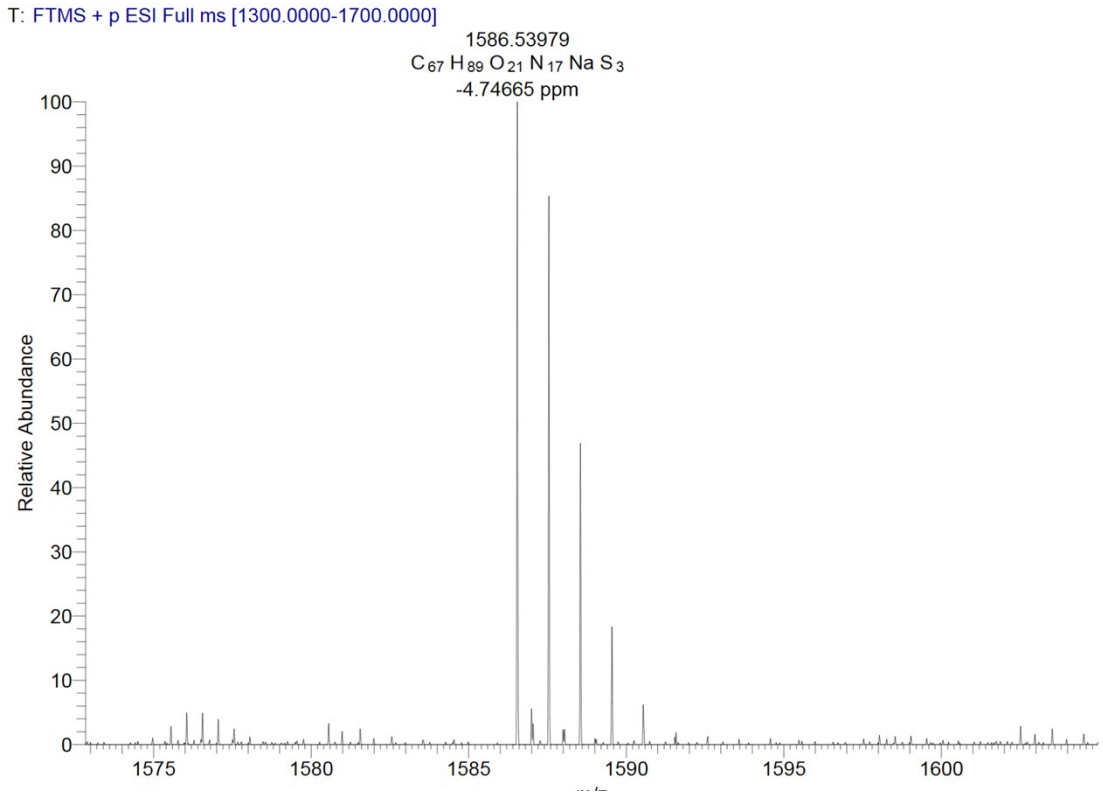
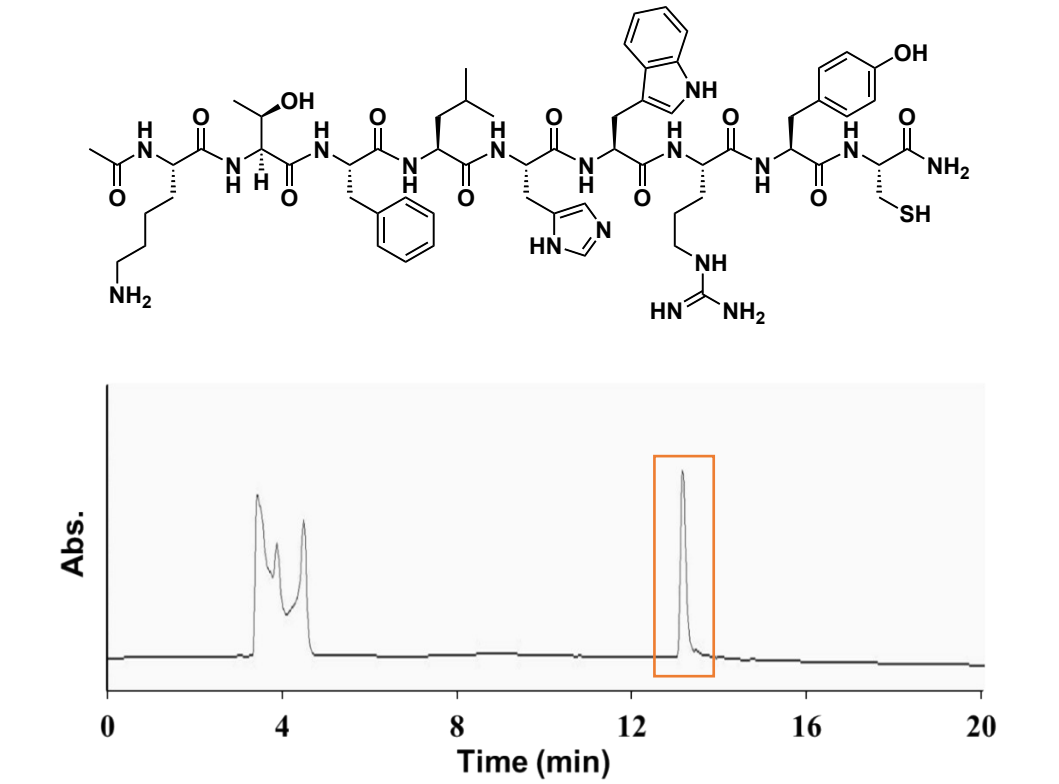


Figure S26. MS analysis of the 'KGGSC' with CBT blocking. ESI−MS calcd. for **$C_{67}H_{89}N_{17}O_{21}S_3$** $[M + Na]^+$ m/z =1586.5479, found 1586.5397.



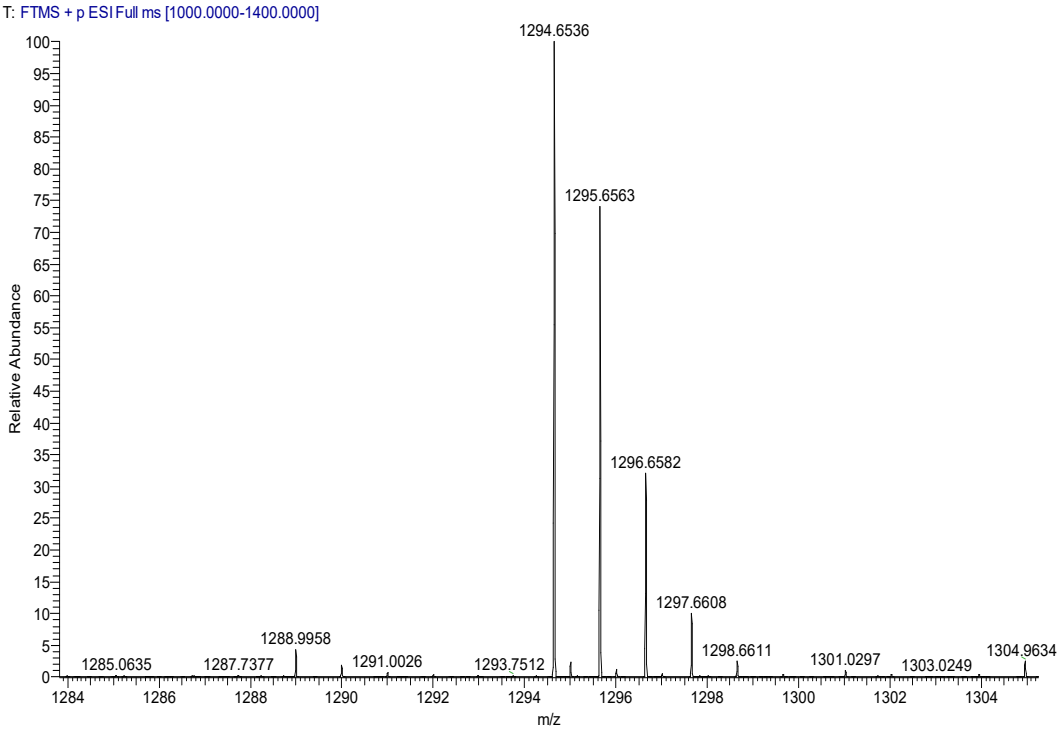
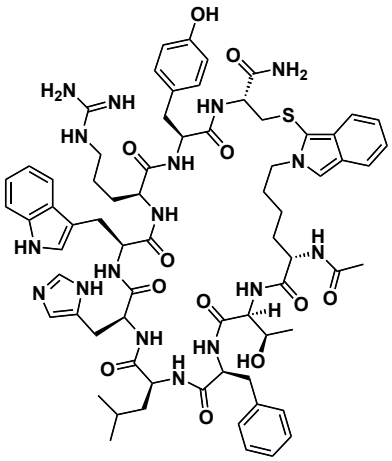
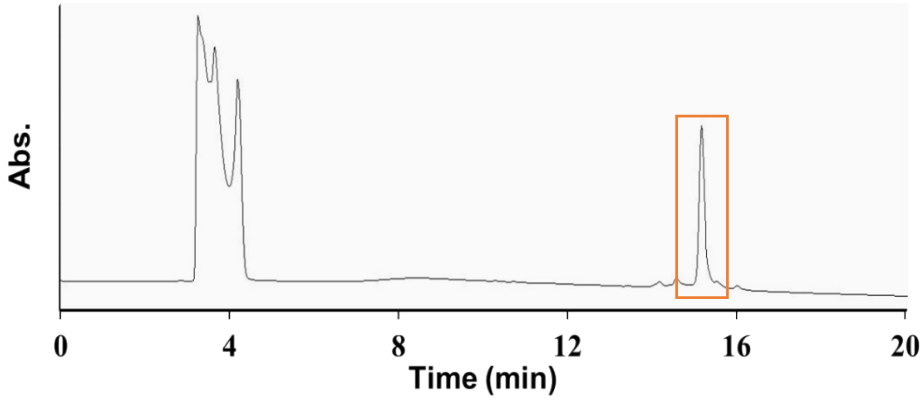


Figure S27. HPLC analysis of linear peptide **M1_SS** (AcNH−KTFLHWRYC−$CONH_2$). Gradient: 10%−100% acetonitrile with 0.1% TFA over 20 min at a flow rate of 1.0 mL/min. ESI−MS calcd. for $\mathbf{C_{62}H_{87}N_{17}O_{12}S}$ $[M+H]^+$ m/z =1294.6514, found 1294.6536.





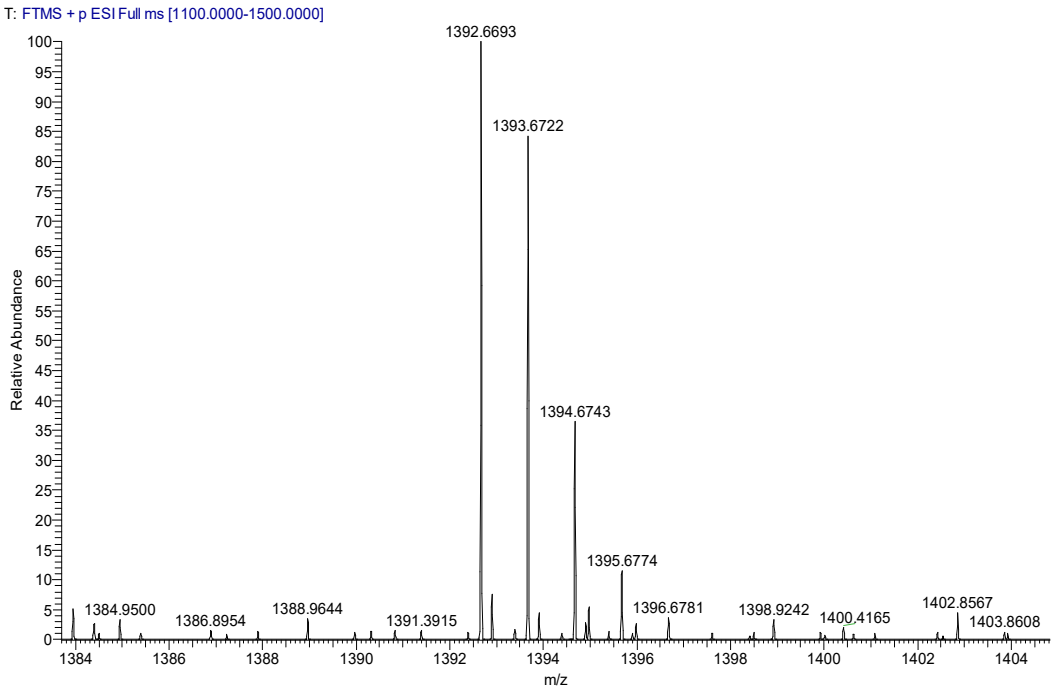
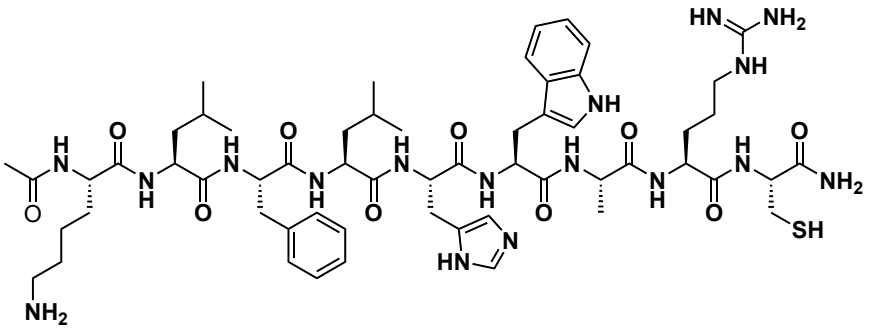
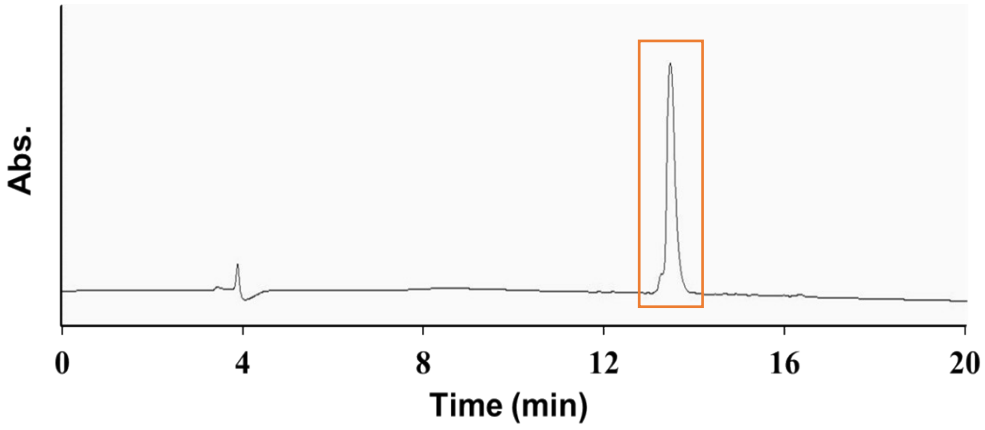


Figure S28. HPLC analysis of cyclic peptide **M1_OPA** (AcNH−KTFLHWRYC−$CONH_2$) formed 15 min after cyclization. Gradient: 10%−100% acetonitrile with 0.1% TFA over 20 min at a flow rate of 1.0 mL/min. ESI−MS calcd. for **$C_{70}H_{89}N_{17}O_{12}S$** $[M+H]^+$ m/z = 1392.6670, found 1392.6693.





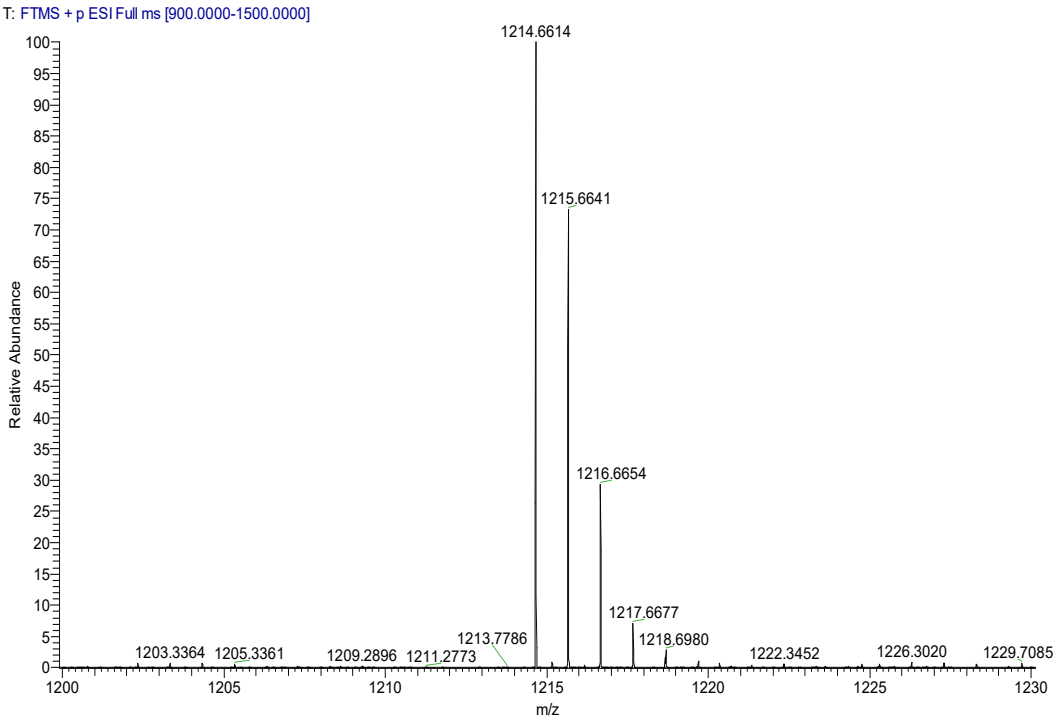
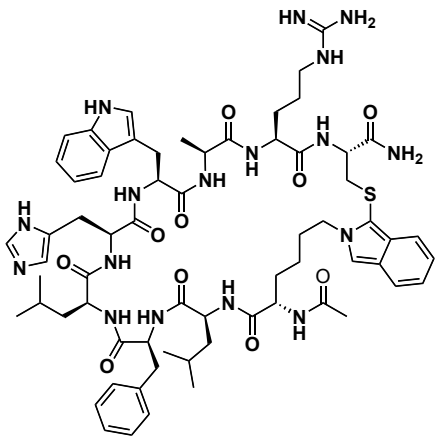
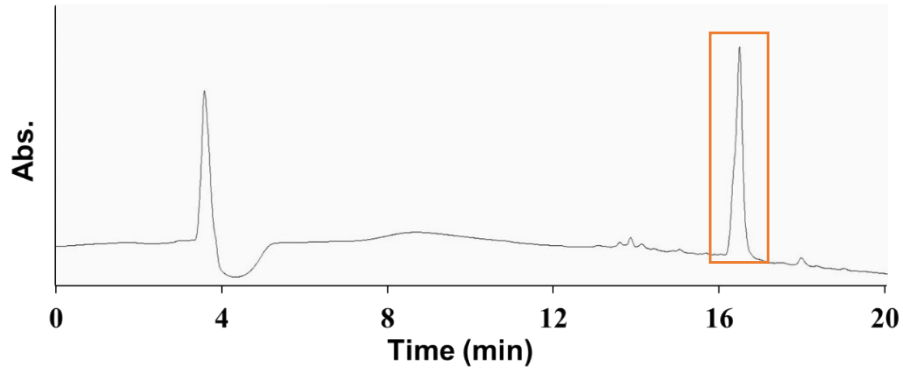


Figure S29. HPLC analysis of linear peptide **M2_SS** (AcNH−KLFLHWARC−$CONH_2$). Gradient: 10%−100% acetonitrile with 0.1% TFA over 20 min at a flow rate of 1.0 mL/min. ESI−MS calcd. for $\mathbf{C_{58}H_{87}N_{17}O_{10}S}$ $[M+H]^+$ m/z = 1214.6615, found 1214.6614.





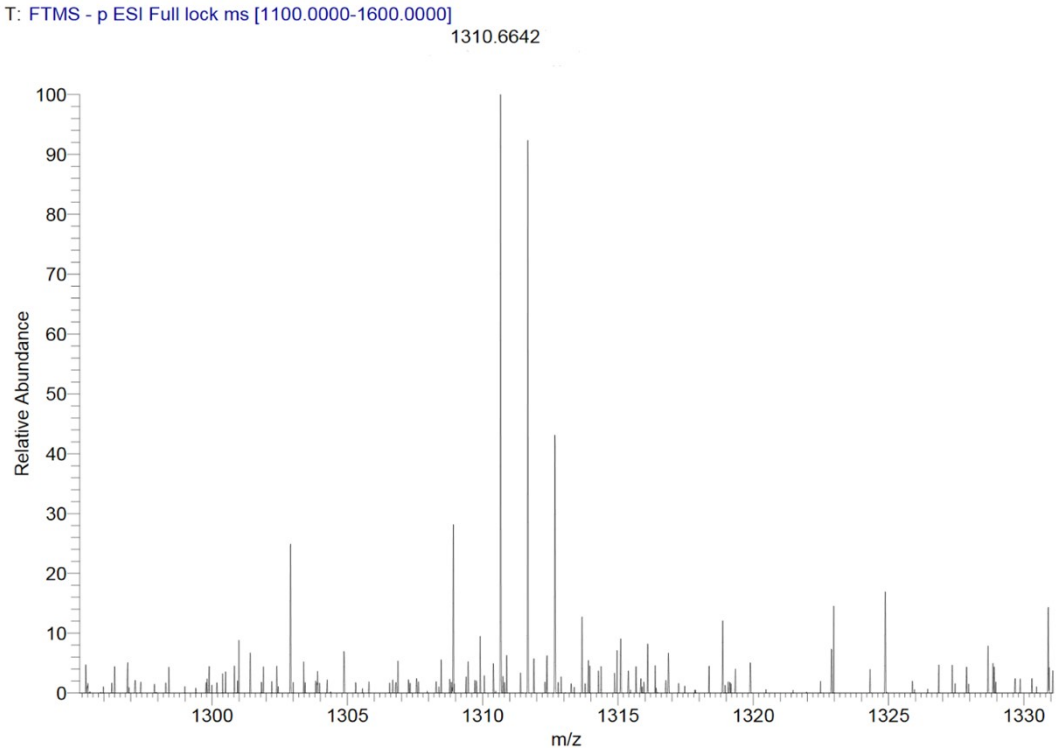
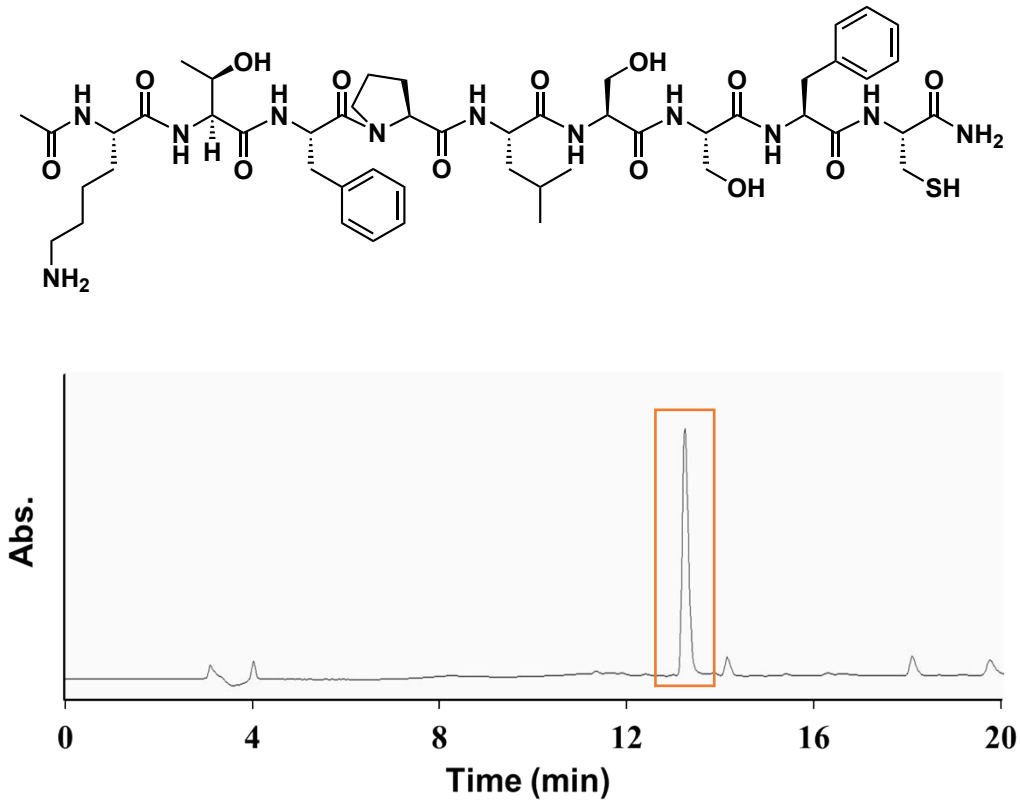


Figure S30. HPLC analysis of cyclic peptide **M2_OPA** (AcNH−KLFLHWARC−$CONH_2$) formed 15 min after cyclization. Gradient: 10%−100% acetonitrile with 0.1% TFA over 20 min at a flow rate of 1.0 mL/min. ESI−MS calcd. for **$C_{66}H_{89}N_{17}O_{10}S$** $[M-H]^-$ m/z = 1310.6626, found 1310.6642.



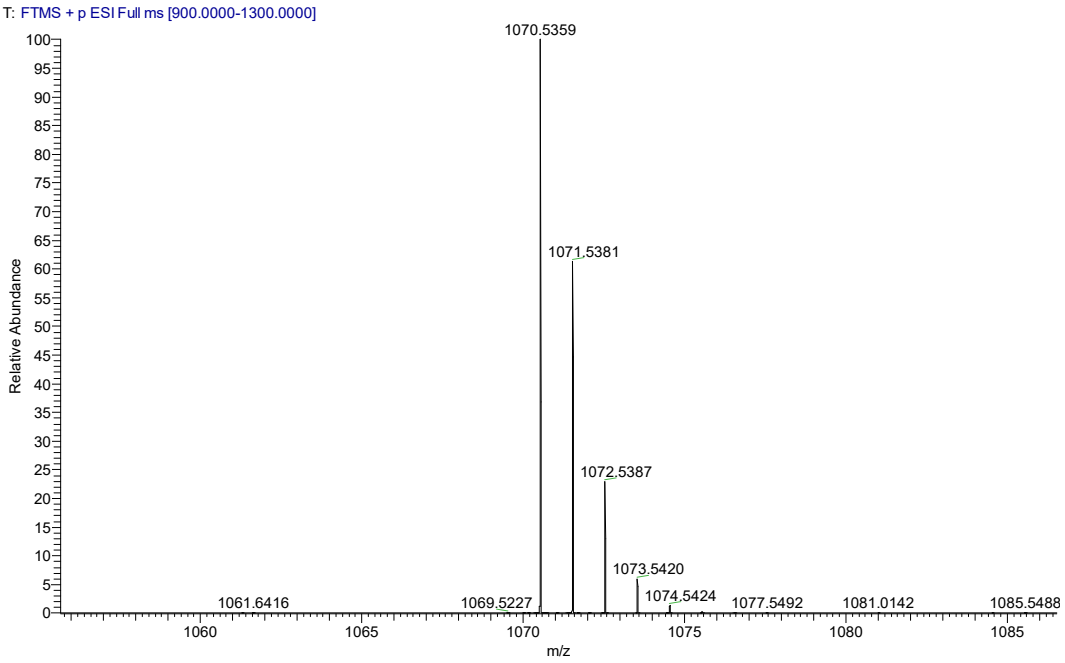
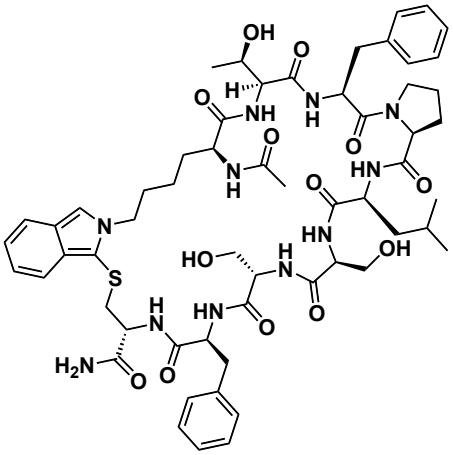
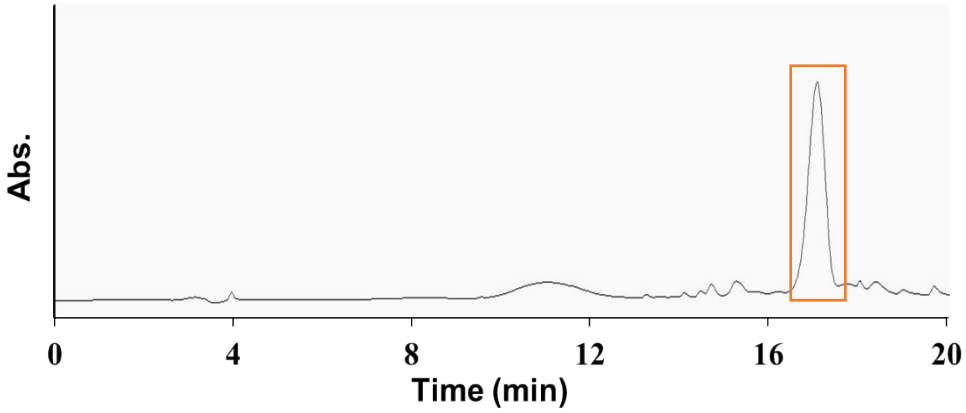


Figure S31. HPLC analysis of linear peptide **N1_SS** (AcNH−KTFPLSSFC−$CONH_2$). Gradient: 10%−100% acetonitrile with 0.1% TFA over 20 min at a flow rate of 1.0 mL/min. ESI−MS calcd. for **$C_{50}H_{75}N_{10}O_{14}S$** $[M+H]^+$ m/z = 1070.5339, found 1070.5359.





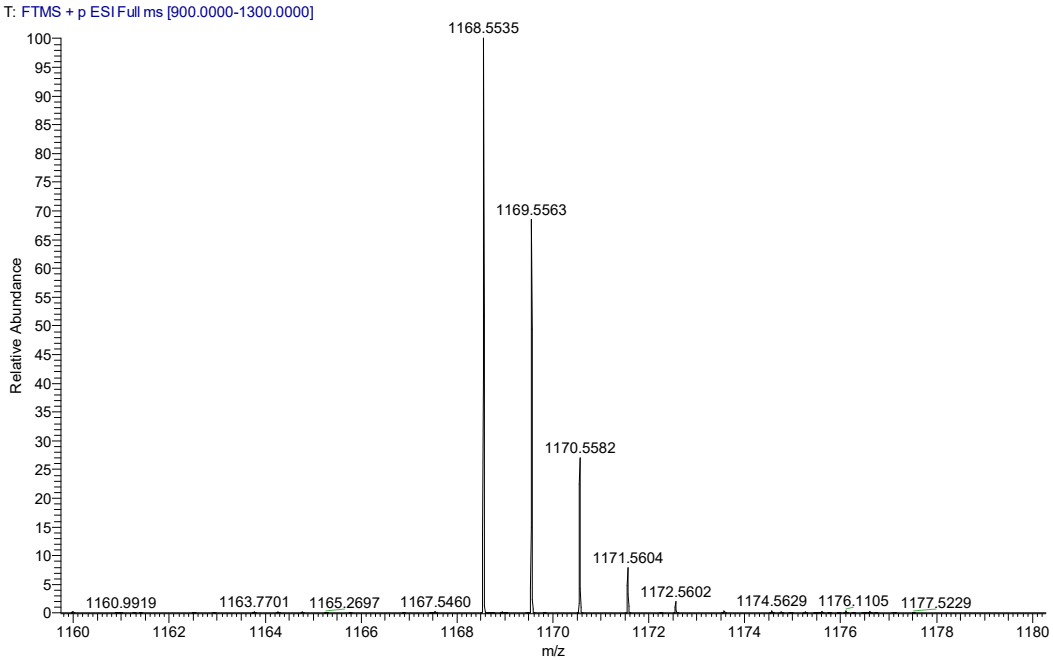
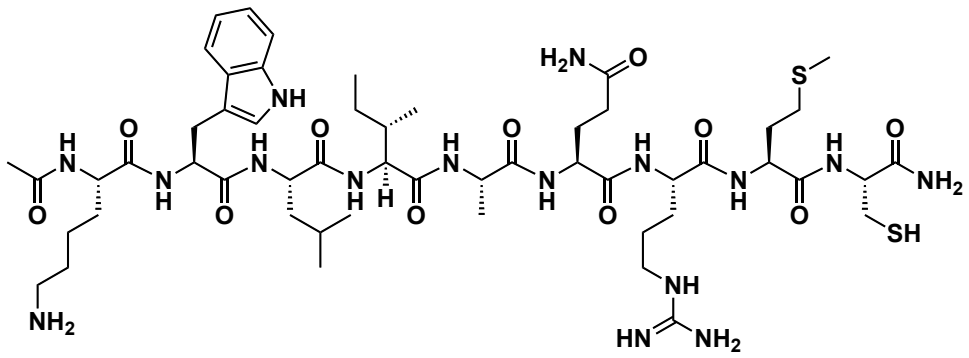
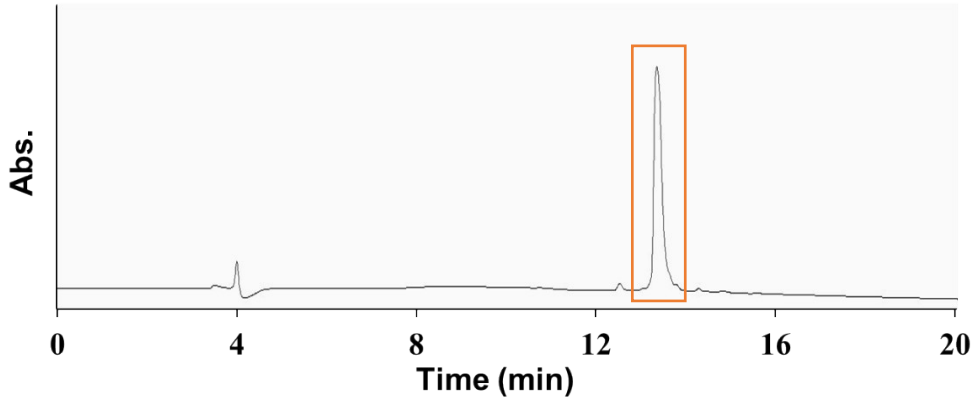


Figure S32. HPLC analysis of cyclic peptide **N1_OPA** (AcNH−KTFPLSSFC−$CONH_2$) formed 15 min after cyclization. Gradient: 10%−100% acetonitrile with 0.1% TFA over 20 min at a flow rate of 1.0 mL/min. ESI−MS calcd. for $\mathbf{C_{58}H_{77}N_{11}O_{13}S}$ $[M+H]^+$ m/z = 1168.5496, found 1168.5535.





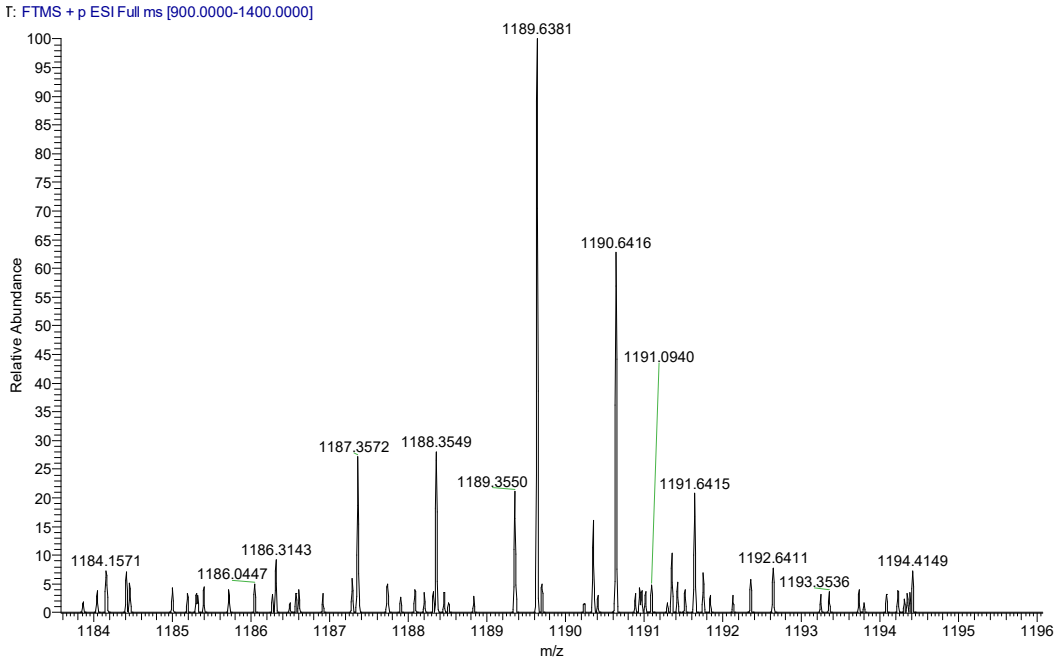
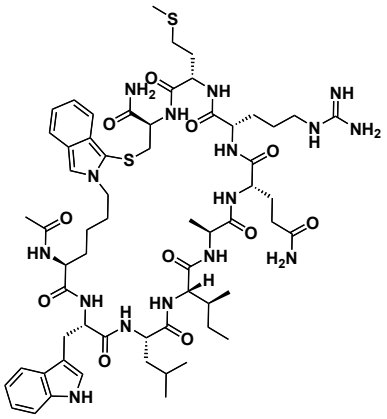
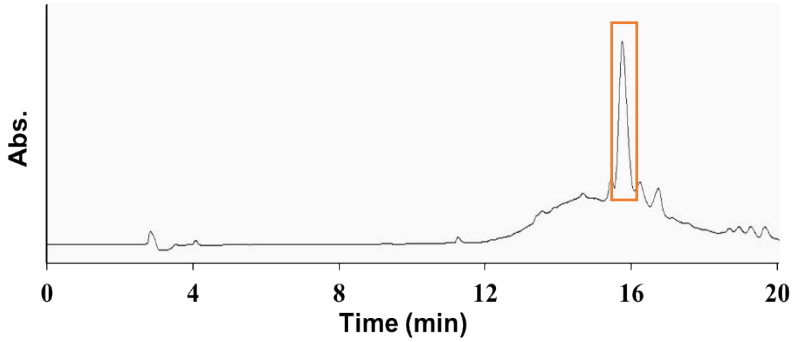


Figure S33. HPLC analysis of linear peptide **N2_SS** (AcNH−KWLIAQRMC−$CONH_2$). Gradient: 10%−100% acetonitrile with 0.1% TFA over 20 min at a flow rate of 1.0 mL/min. ESI−MS calcd. for $\mathbf{C_{53}H_{88}N_{16}O_{11}S_2}$ $[M+H]^+$ m/z = 1189.6333, found 1189.6381.





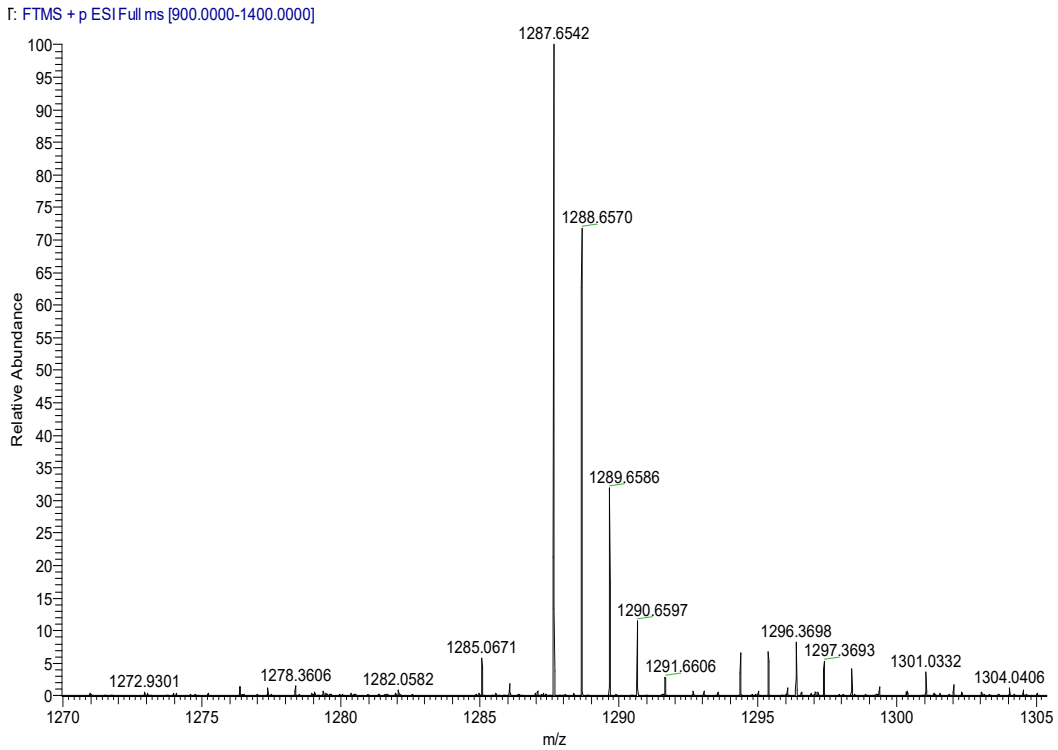


Figure S34. HPLC analysis of cyclic peptide **N2_OPA** (AcNH−KWLIAQRMC−$CONH_2$) formed 15 min after cyclization. Gradient: 10%−100% acetonitrile with 0.1% TFA over 20 min at a flow rate of 1.0 mL/min. ESI−MS calcd. for $\mathbf{C_{61}H_{90}N_{16}O_{11}S_2}$ $[M+H]^+$ m/z = 1287.6489, found 1287.6542.

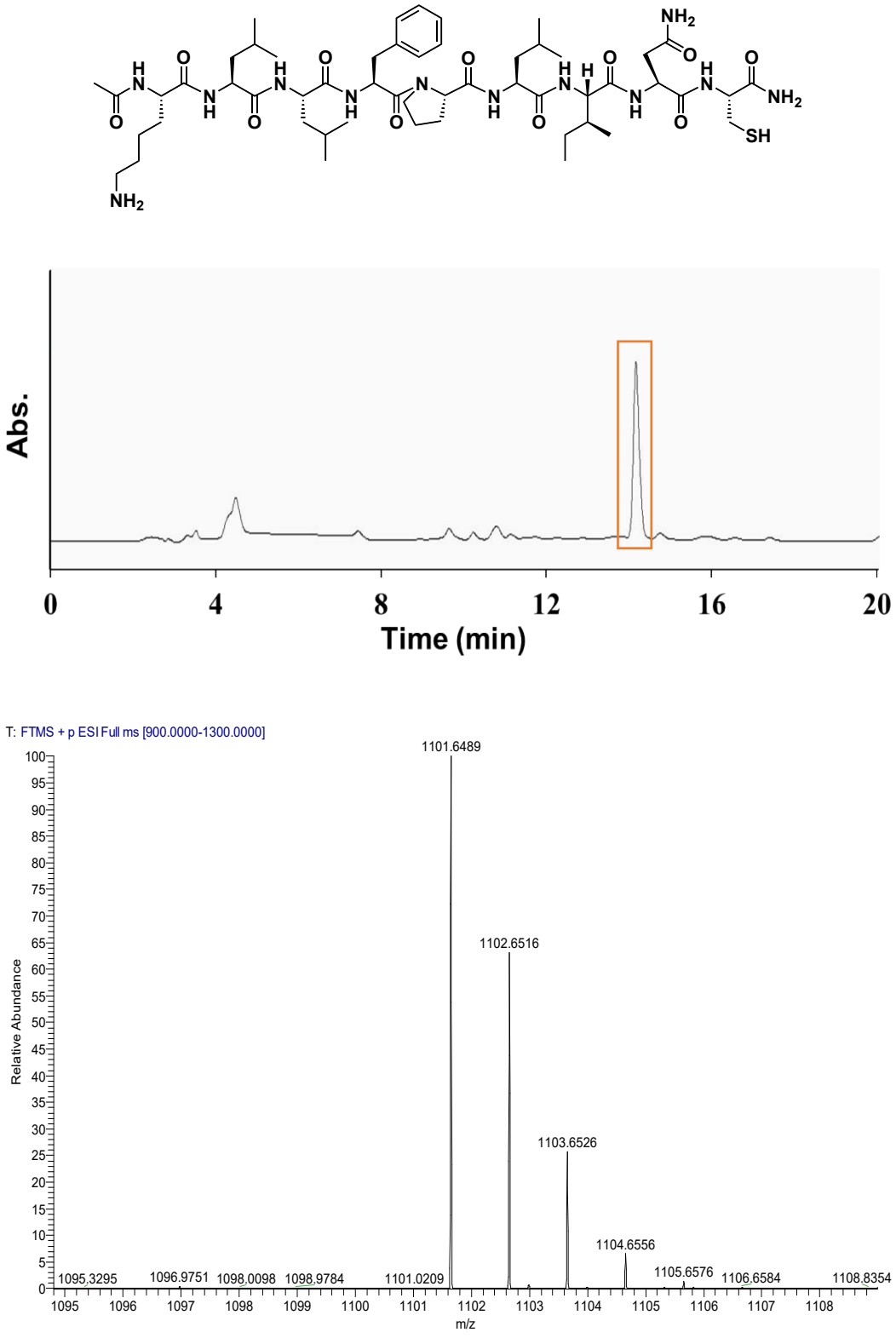
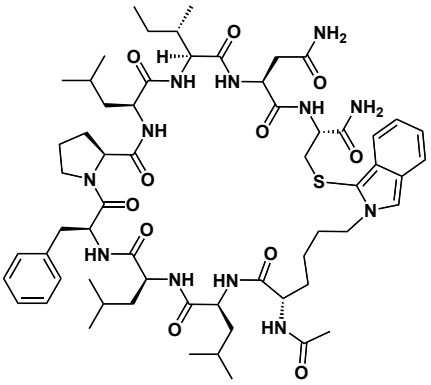
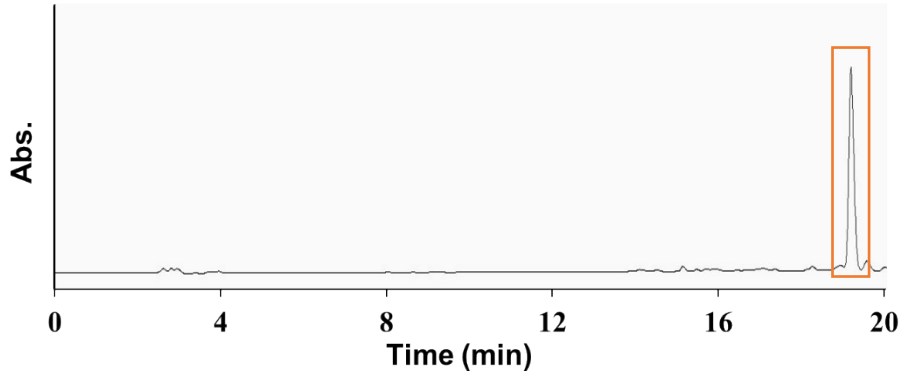


Figure S35. HPLC analysis of linear peptide **N3_SS** (AcNH−KLLFPLINC−$CONH_2$). Gradient: 10%−100% acetonitrile with 0.1% TFA over 20 min at a flow rate of 1.0 mL/min. ESI−MS calcd. for **$C_{53}H_{88}N_{12}O_{11}S$** $[M+H]^+$ m/z = 1101.6489, found 1101.6489.





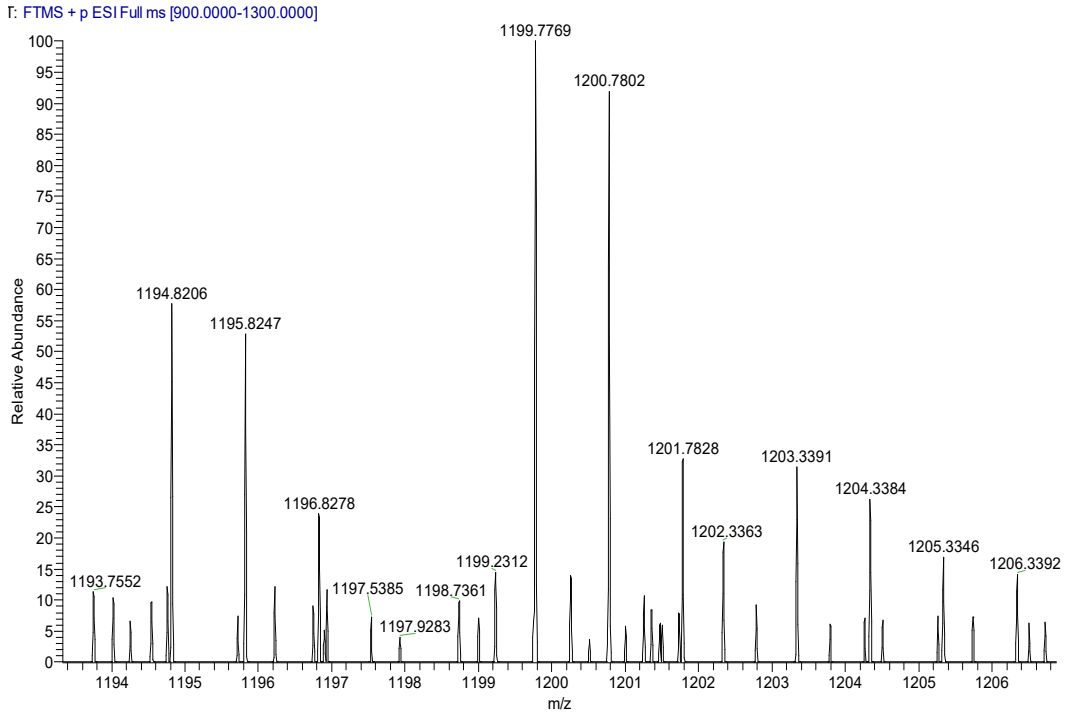
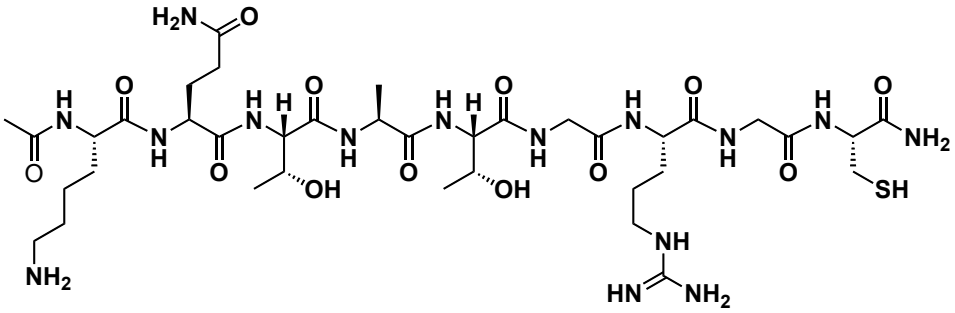
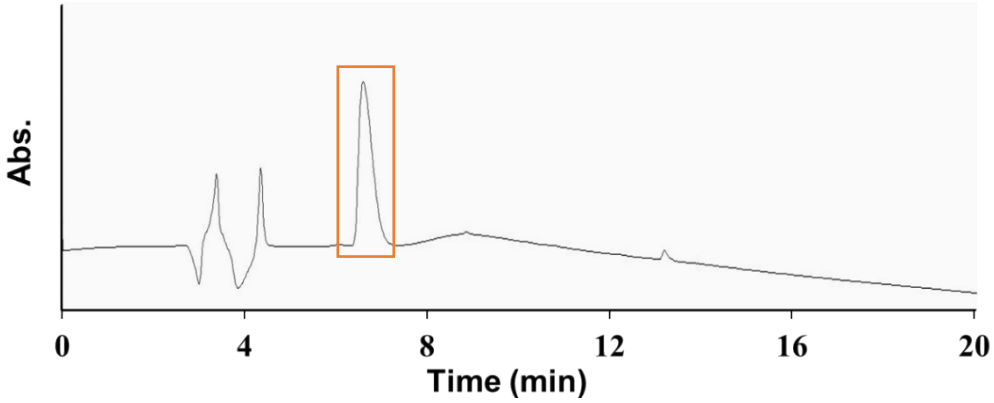


Figure S36. HPLC analysis of cyclic peptide **N3_OPA** (AcNH−KLLFPLINC−$CONH_2$) formed 15 min after cyclization. Gradient: 10%−100% acetonitrile with 0.1% TFA over 20 min at a flow rate of 1.0 mL/min. ESI−MS calcd. for $\mathbf{C_{53}H_{69}N_{11}O_{18}S}$ $[M+H]^+$ m/z = 1199.6645, found 1199.7769.





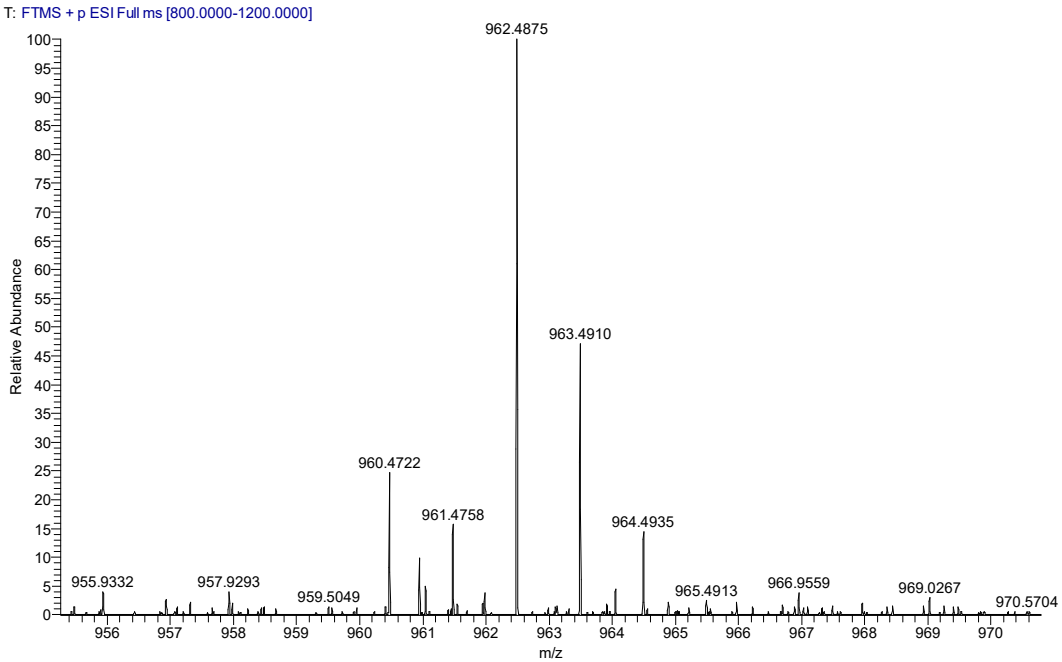
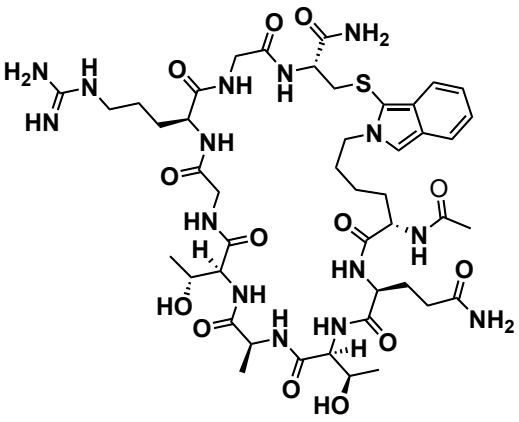
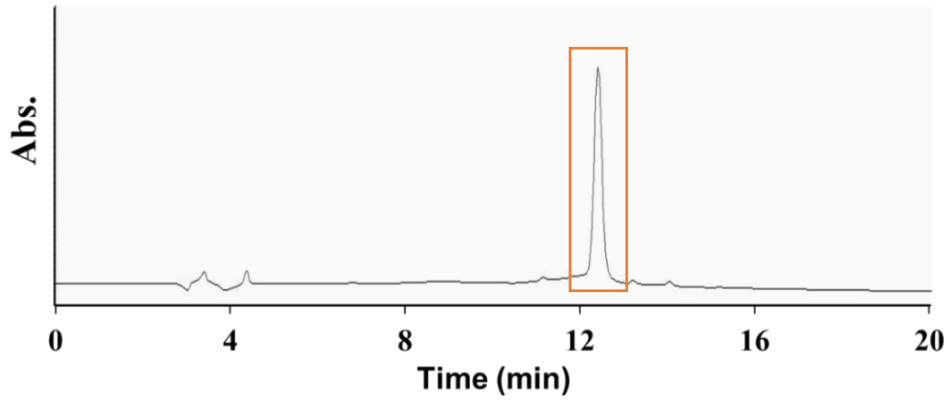


Figure S37. HPLC analysis of linear peptide **Hk1_SS** (AcNH−KQTATGRGC−$CONH_2$). Gradient: 10%−100% acetonitrile with 0.1% TFA over 20 min at a flow rate of 1.0 mL/min. ESI−MS calcd. for $\mathbf{C_{37}H_{67}N_{15}O_{13}S}$ $[M+H]^+$ m/z = 962.4836, found 962.4875.





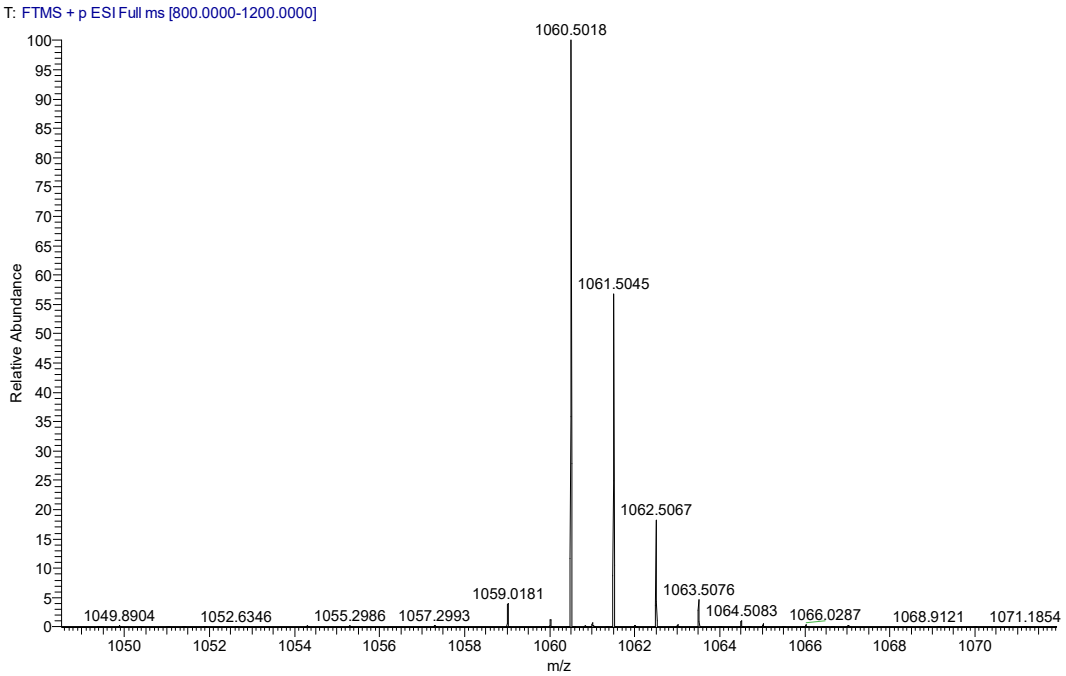
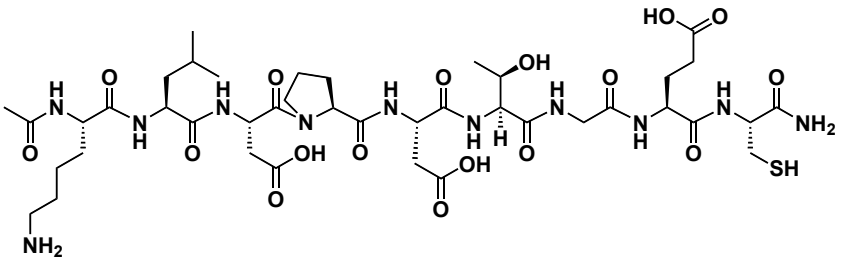
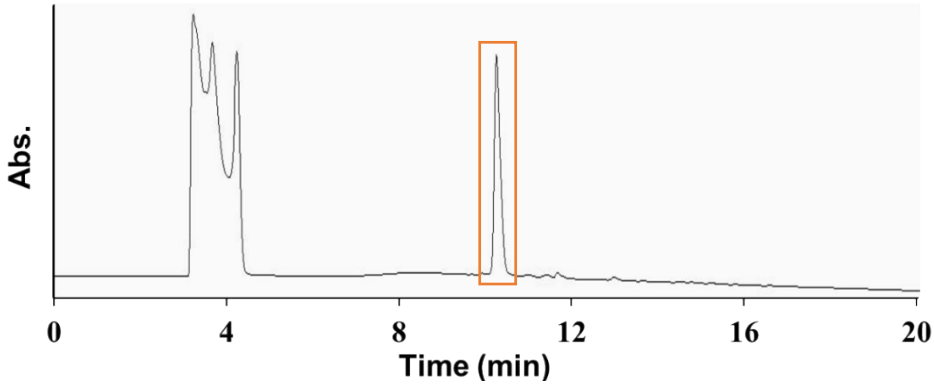


Figure S38. HPLC analysis of cyclic peptide **Hk1_OPA** (AcNH−KQTATGRGC−$CONH_2$) formed 15 min after cyclization. Gradient: 10%−100% acetonitrile with 0.1% TFA over 20 min at a flow rate of 1.0 mL/min. ESI−MS calcd. for **$C_{45}H_{69}N_{15}O_{13}S$** $[M+H]^+$ m/z = 1060.4993, found 1060.5018.





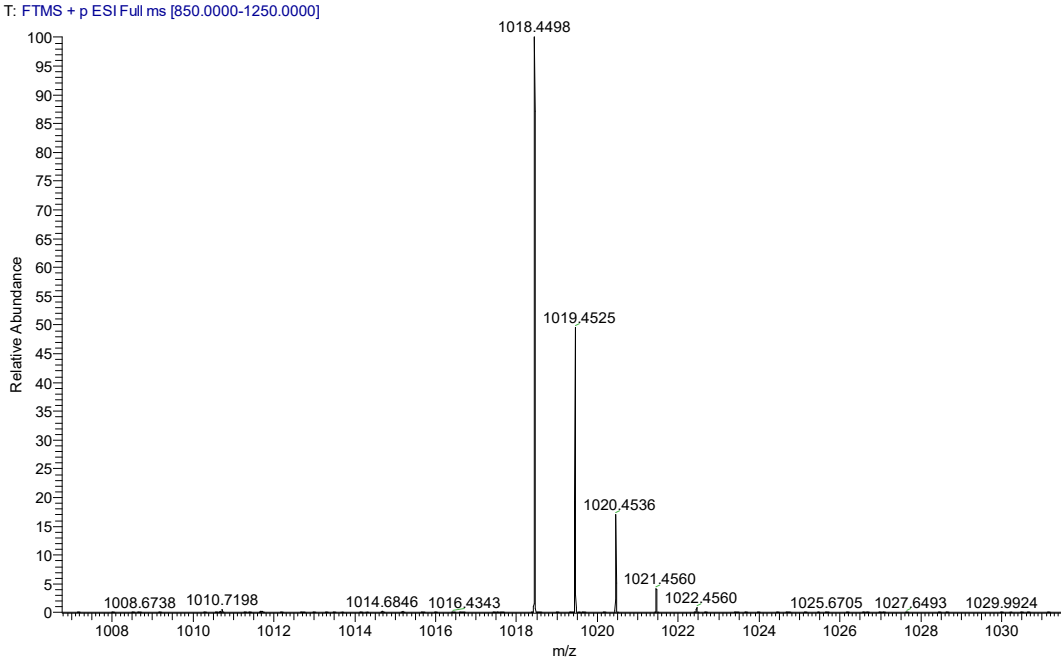
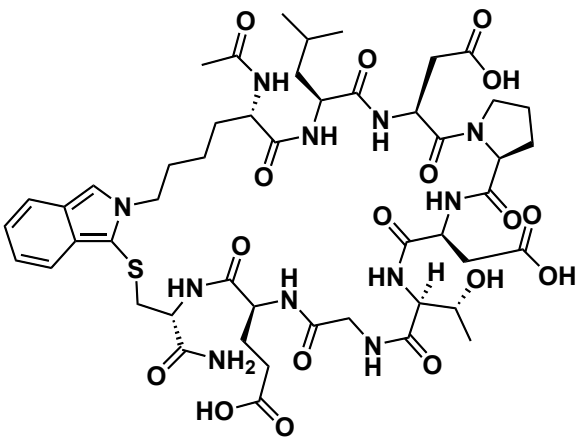
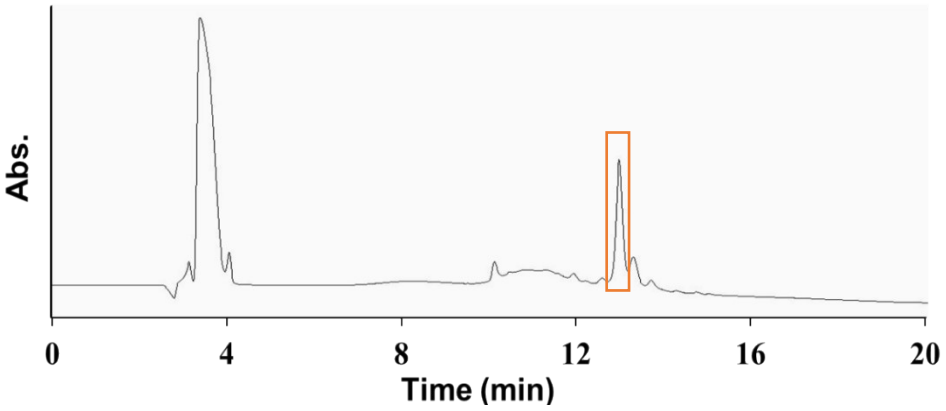


Figure S39. HPLC analysis of linear peptide **Hk2_SS** (AcNH−KLDPDTGEC−$CONH_2$). Gradient: 10%-100% acetonitrile with 0.1% TFA over 20 min at a flow rate of 1.0 mL/min. ESI−MS calcd. for $C_{41}H_{67}N_{11}O_{17}S$ $[M+H]^+$ m/z = 1018.4510, found 1018.4498.





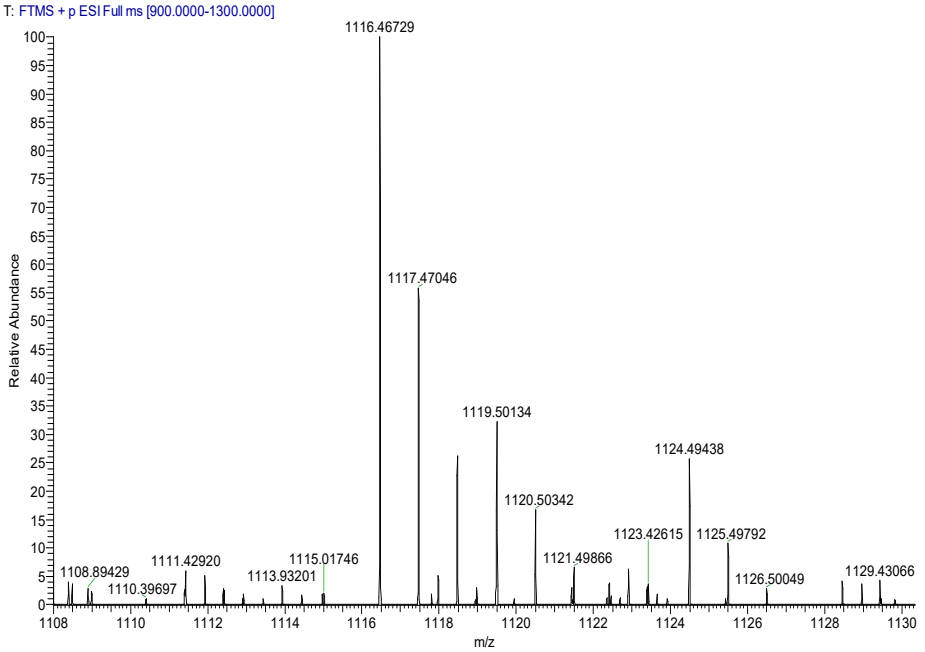
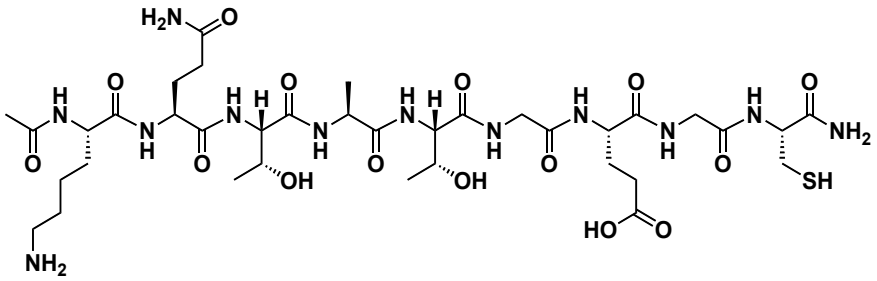
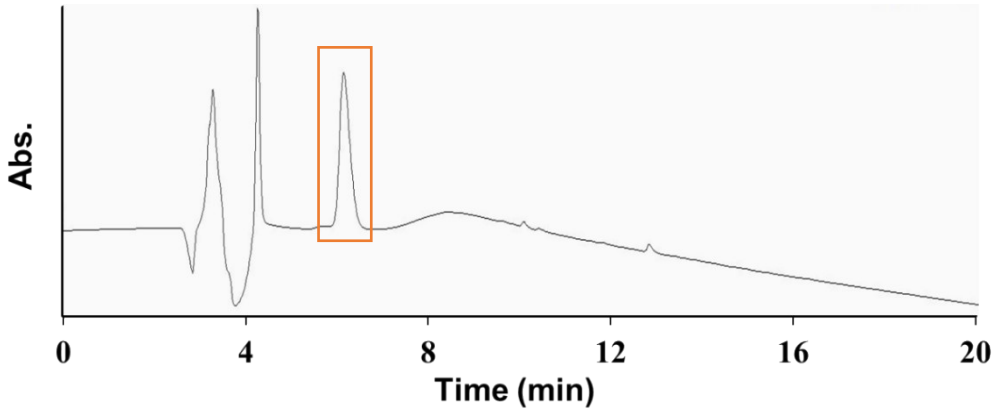


Figure S40. HPLC analysis of cyclic peptide **Hk2_OPA** (AcNH−KLDPDTGEC−$CONH_2$) formed 15 min after cyclization. Gradient: 10%−100% acetonitrile with 0.1% TFA over 20 min at a flow rate of 1.0 mL/min. ESI−MS calcd. for $\mathbf{C_{53}H_{69}N_{11}O_{18}S}$ $[M+H]^+$ m/z = 1116.4666, found 1116.46729.





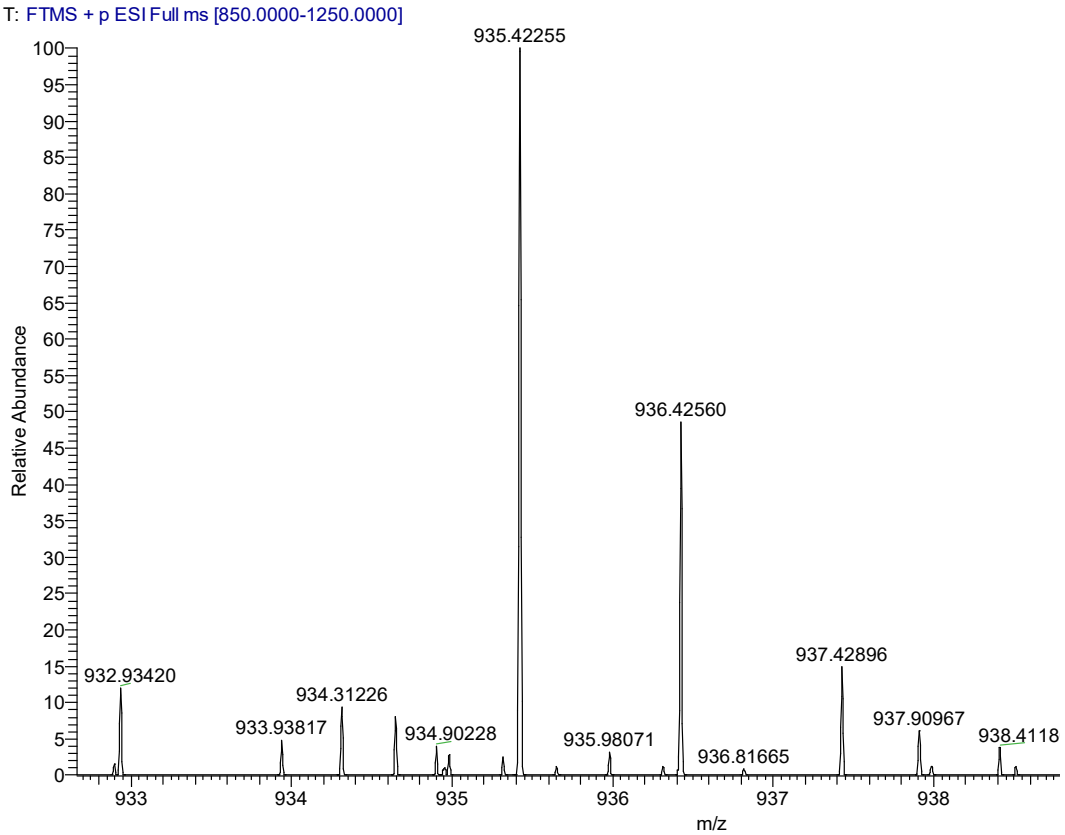
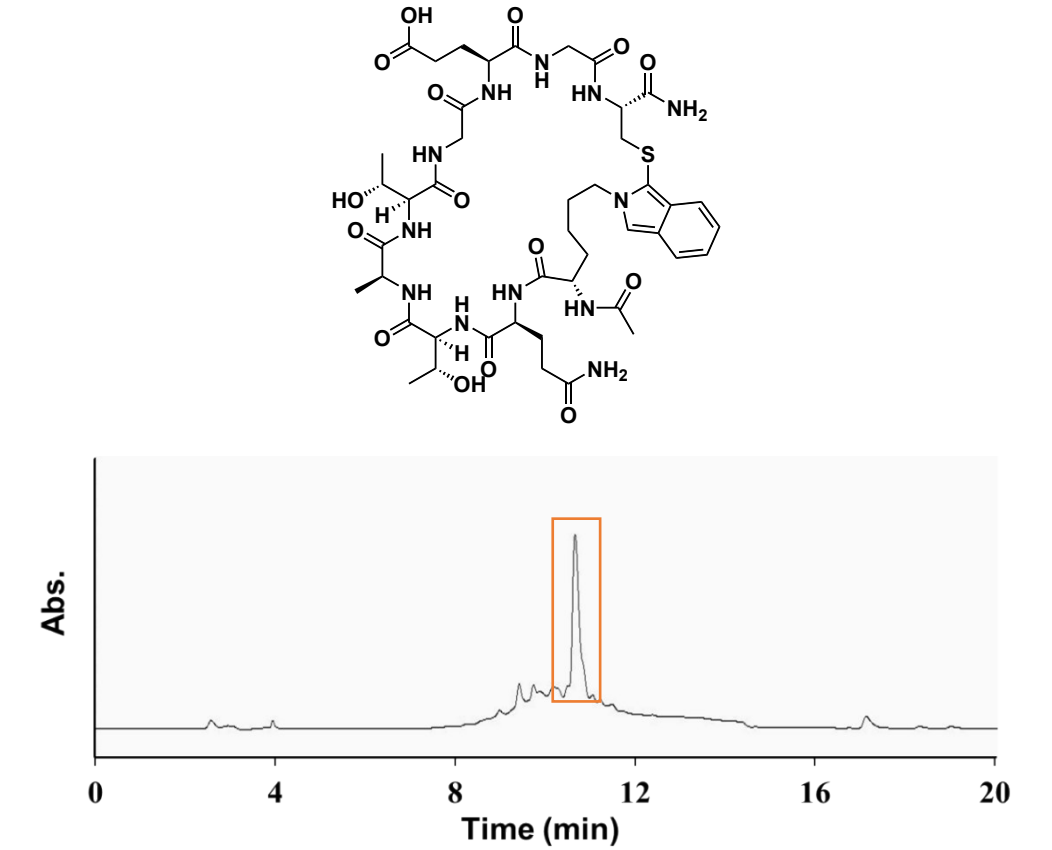


Figure S41. HPLC analysis of linear peptide **Hk3_SS** (AcNH−KQTATGEGC−$CONH_2$). Gradient: 10%−100% acetonitrile with 0.1% TFA over 20 min at a flow rate of 1.0 mL/min. ESI−MS calcd. for $\mathbf{C_{45}H_{67}N_{11}O_{18}S}$ $[M+H]^+$ m/z = 935.4251, found 935.42255.



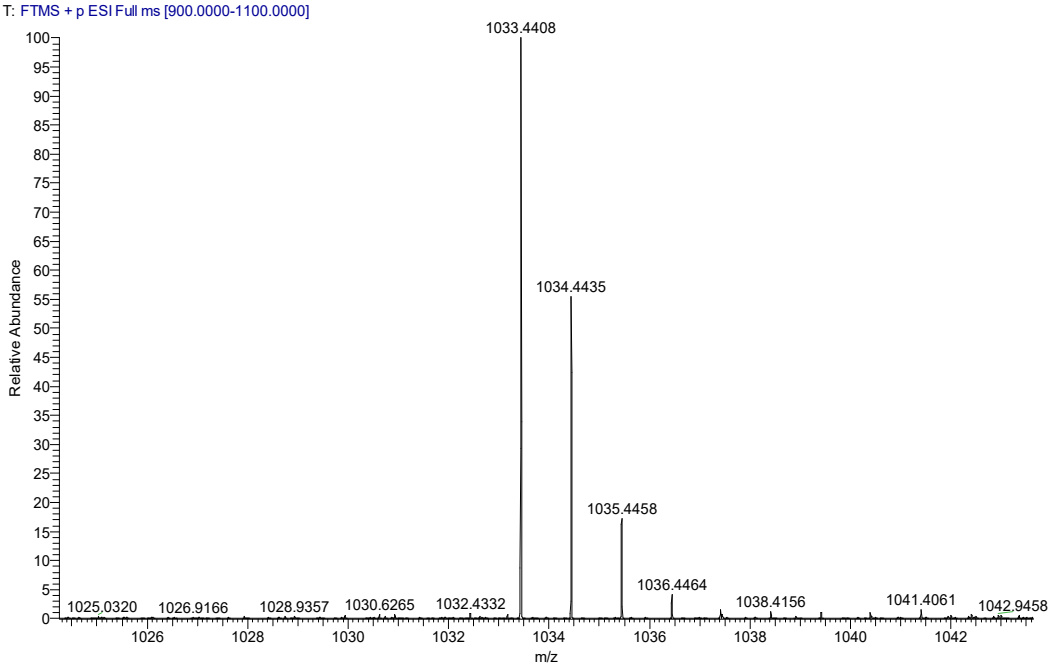


Figure S42. HPLC analysis of cyclic peptide **Hk3_OPA** (AcNH−KQTATGEGC−$CONH_2$) formed 15 min after cyclization. Gradient: 10%−100% acetonitrile with 0.1% TFA over 20 min at a flow rate of 1.0 mL/min. ESI−MS calcd. for **$C_{53}H_{69}N_{11}O_{18}S$** $[M+H]^+$ m/z = 1033.4408, found 1033.4408.

16. Primer List

Name	Sequence 5’ → 3’
hKeap−fw	CATGGCCATATGGGTTCTTCTCACCACCACCACCACCACTCTTCTGGTGA
hKeap−rv	CCTAGGCAAGCTTTTAGGTAACCGCAACACCAACACC
PTP1B−fw	CATGGCCATATGGAAATGGAAAAAGAATTCGAAC
PTP1B−rv	CCTAGGCAAGCTTTCAGTGGTGGTGGTGGTGGTGGTTGTGCGGTTC
NEK7−fw	TACGACCATATGATGGATGAGCAATCACAAGGAATGCAA
NEK7−rv	CCGCTCGAGGCTGCTTGCAGTGCATGCATGCATCCT
GGS−fw	CTAGGGATCCAAGGGTTCTTCTTGCGGTGAAAACCTGTACTTCCAGGGC GGCTCGGCCTC
GGS−rv	CTAGGGATCCGCAACGACCGTCGATAGAACCACCGGCCGCCTGG
L7MGA−fw	CTAGGGATCCATCGACGGTCGTTGCGGTTCTAAGNNKNNKNNKNNKNN KNNKNNKTGCGGCGGCTCGGCCTC
L7MGA−rv	CTAGGGATCCACCGGCCGCCTGGGCCACG

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