

Part 3:
Synthetic combinatorial
OPA-cyclic peptide library

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1. Materials and methods

TentaGel® S NH₂ resin (particle size: 90 µm; loading capacity: 0.24 mmol/g) was purchased from Rapp Polymere GmbH (Tübingen, Germany). All commercially available amino acids and coupling reagents (purchased from Aldrich and GL Biochem) were used without further purification. All solvents in reagent grade (RCI) or HPLC grade (DUKSAN) were used without purification. Anhydrous dichloromethane (DCM) was freshly distilled from calcium hydride (CaH₂) before use. Analytical HPLC was performed on a Waters system equipped with a photodiode array detector (Waters 2996), using a Vydac 218TPTM C18 column (5 µm, 4.6 × 250 mm) at a flow rate of 0.6 mL/min. Waters UPLC H-class system equipped with an ACQUITY UPLC photodiode array detector and a Waters SQ Detector 2 mass spectrometer using a Waters ACQUITY BEH C18 column (1.7 µm, 130 Å, 2.1 × 50 mm) at a flow rate of 0.4 mL/min. Preparative HPLC was performed on a Waters system, using a Vydac 218TPTM C18 column (10 µm, 22 × 250 mm) at a flow rate of 10 mL/min or a Vydac 218TPTM C18 column (10 µm, 30 × 250 mm) at a flow rate of 20 mL/min. Mobile phases of HPLC used are as followed: Solvent A: 0.1% TFA (v/v) in acetonitrile; Solvent B: 0.1% TFA (v/v) in water. Mass analysis was performed with a Waters 3100 mass spectrometer. Tandem mass spectrometry was performed on Thermo Scientific™ Q Exactive™ Plus.

2. Materials and methods

2.1 Fmoc-based Solid Phase Peptide Synthesis (SPPS) and synthesis of the linker region

TentaGel S NH₂ resin (20 mg) was swollen in DCM/DMF 1:1 (1 mL) for 15 minutes then drained. After that, the resin was incubated in DMF for 15 minutes and drained. A

solution of Fmoc-Xaa-COOH (4.0 equiv. relative to resin loading capacity), HATU (4.0 equiv. relative to resin loading capacity) and DIEA (8.0 equiv. relative to resin capacity) dissolved in DMF was added to the resin and reacted for 1 hour. Then, the resin was washed with DMF (3 x 1 mL), DCM (3 x 1 mL) and DMF (3 x 1 mL). Next, the Fmoc group was removed by applying a deblock solution of 20% piperidine in DMF and reacted for 20 minutes. Finally, it was submitted to iterative peptide assembly until completion of the linker region H₂N-ABBFM-TentaGel resin.

2.2 Topological segregation utilizing the biphasic synthetic approach

To install the Fmoc group on the exterior layer of the beads, the resin was washed with DMF, followed by degassed ddH₂O, and soaked in ddH₂O overnight. The solution was then drained and immediately mixed with a solution of Fmoc-OSu (4 equiv.) and DIEA (12 equiv.) in 1:1 (v/v) DCM/diethyl ether co-solvent. The resin was shaken for 30 minutes, followed by washing with 1:1 DCM/diethyl ether co-solvent for three times and DMF for eight times.

Then, Alloc-Ala-OH was coupled to the interior of the beads. A solution of Alloc-Ala-OH (4.0 equiv. relative to resin loading capacity), HATU (4.0 equiv. relative to resin capacity) and DIEA (8.0 equiv. relative to resin capacity) dissolved in DMF was added to the resin and reacted for 1 hour, followed by washing with DMF (3 x 1 mL), DCM (3 x 1 mL) and DMF (3 x 1 mL). Next, the Fmoc group on the exterior layer of the resin was removed by the 20% piperidine in DMF deblock solution after 20 minutes treatment, followed by the addition of a solution of Fmoc-Pen(Trt)-OH (4.0 equiv. relative to resin loading capacity), HATU (4.0 equiv. relative to resin capacity) and DIEA (8.0 equiv. relative to resin capacity) dissolved in DMF. After 1 hour, the resin was washed with DMF (3 x 1 mL), DCM (3 x 1 mL) and DMF (3 x 1 mL). Then, the

alloc group on the interior layer of the bead was removed by adding $\text{Pd(PPh}_3)_4$ (0.5 equiv.) and PPh_3 (12.0 equiv.) in anhydrous DCM, under argon protection. After 4 hours, the resin was washed with DMF (3 x 1 mL), DCM (3 x 1 mL) and DMF (3 x 1 mL). The final step is Fmoc deprotection. The Fmoc group was removed upon 20% piperidine in DMF treatment. After 20 minutes, the resin was washed with DMF (3 x 1 mL), DCM (3 x 1 mL) and DMF (3 x 1 mL) to complete the topological segregation step.

Random region coupling

For the model peptide

The resin was subjected to iterative peptide assembly as described in procedure 3.10.2 Fmoc-based Solid Phase Peptide Synthesis (SPPS) and synthesis of the linker region. Fmoc-Phe-OH, Fmoc-Glu(OtBu)-OH, Fmoc-Leu-OH and Fmoc-Lys(Boc)-OH were coupled in sequential order. Then, the Fmoc group was removed by the 20% piperidine in DMF deblock solution. The peptide was then subjected to N-terminal acetylation by adding Ac_2O (8.0 equiv.) and DIEA (16.0 equiv.) in DMF and reacted for 1 hour. Finally, the bilayer model peptides with Ac-KLEFPenABBFM-TentaGel resin (on the exterior layer) and Ac-KLEFAABBFM-TentaGel resin (on the interior layer) were obtained.

For the combinatorial library with 16 random residues

The resin was split into sixteen equal portions and placed in sixteen peptide synthesis vessels. The solution of 16 random amino acids, Fmoc-Xaa-COOH (4.0 equiv. relative to resin loading capacity), HATU (4.0 equiv. relative to resin loading capacity) and DIEA (8.0 equiv. relative to resin capacity) dissolved in DMF was added to the resin

respectively and reacted for 1 hour. The resin was then washed with DMF (3 x 1 mL), DCM (3 x 1 mL) and DMF (3 x 1 mL) and combined back into a reaction vessel. After the coupling was completed, the resin was split into sixteen equal portions again for iterative peptide assembly. This step was repeated for n cycles for n random residues incorporated. Upon completion of the random region, the resin was washed, combined back into a single reaction vessel and the Fmoc group was removed for the coupling of the last amino acid Fmoc-Lys(Boc)-OH. Finally, the peptide was subjected to N-terminal acetylation by adding Ac₂O (8.0 equiv.) and DIEA (16.0 equiv.) in DMF and reacted for 1 hour to afford the desired combinatorial library.

2.3 Bromophenol blue test to visualize beads segregation

A stock solution of bromophenol blue 0.05% (w/v) in DMF was prepared. To perform the test, around 50 beads were placed in the microcentrifuge tube and five drops of the stock solution was added. The beads were then transferred onto the microscope slide and inspected under an upright microscope imaging system, Nikon DS-Ri2. Free amines reacted with bromophenol blue to produce a blue color.

2.4 Side-chain deprotection of the peptide library

A mixture solution of TFA/H₂O/TIPS/EDT (92.5%/2.5%/2.5%2.5%) was added to the resin-bound peptide, and the mixture was gently agitated for 3 hours. The resin was then washed extensively with DMF, DCM, 5% DIEA in DMF, 1:1 (v/v) DCM/diethyl ether, DMF and DCM.

2.5 Intramolecular OPA-thiol-amine reaction

Stock solution of OPA (1 mg / 100 μ L DMSO) was freshly prepared before use. 1^o-Thiol/3^o-Thiol and amine containing peptide (1.0 equiv.) was first dissolved in PBS

buffer pH 7.4, at a concentration of 0.5 mM. OPA (1.2 equiv.) was then added and reacted in an air atmosphere for 15 minutes at room temperature (rt). The crude reaction mixture was then purified by preparative reverse-phase HPLC.

2.6 On-resin intramolecular OPA cyclization

The resin was first washed extensively with degassed ddH₂O, PBS buffer pH 7.4 and incubated in the PBS buffer pH 7.4 for 15 minutes then drained. Next, PBS buffer pH 7.4 was added to the resin-bound unprotected peptides, at a concentration of 2 mM based on the resin loading. Followed by the addition of OPA (1.8 equiv.) and reacted for 30 min. The resin was then washed extensively with H₂O, DMF and DCM.

2.7 Peptide cleavage and sample preparation for tandem mass spectrometry

Each single bead selected from the library was treated with 20 µL of a CNBr solution (40 mg/mL in 70% TFA) in a microcentrifuge tube in the dark for 20 hours at room temperature. After evaporation of the solvent under vacuum, 200 µL of 50:50 (v/v) acetonitrile/water was added into each tube and vortexed for 30 seconds, followed by centrifugation for 10 minutes at 10,000 g to spin down and isolate the TentaGel beads. The peptide solution was carefully pipetted into another microcentrifuge tube. This process was repeated thrice to ensure all peptides cleaved from the beads were dissolved and transferred. The combined peptide solution was then lyophilized.

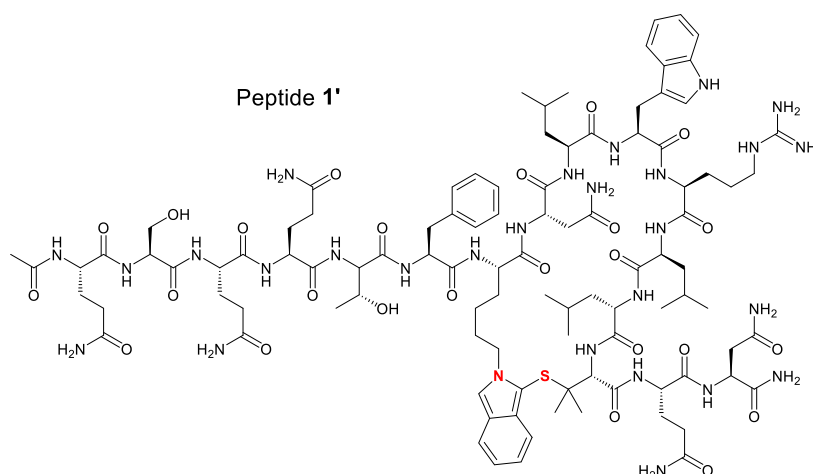
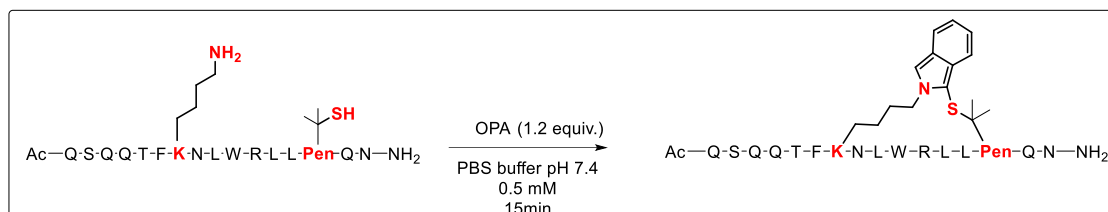
2.8 De novo peptide sequencing by PEAKS Studio

De novo peptide sequencing was performed on PEAKS Studio v10.6 using the raw files obtained from LC-MS/MS. The search parameters were set as 15 ppm precursor mass error and 0.02 Da fragment mass error. Three fixed modifications were applied,

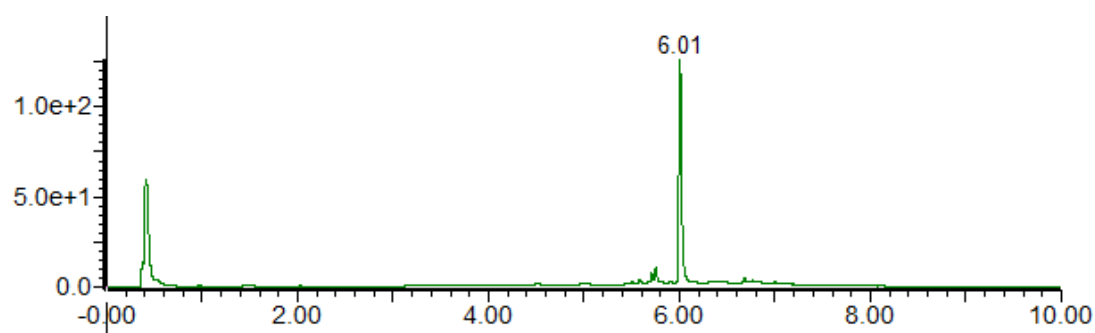
including fixed C-terminal homoserine lactone modification on methionine (-48.00 Da), fixed N-terminal OPA modification (+116.03 Da) and acetylation on Lys (+42.01 Da).

3. UPLC-Chromatogram and MS-Spectra

3.1 Cyclization of model peptide 1



The reaction was carried out at 0.0044 mmol scale according to general procedure 3.10.6. The crude reaction mixture was purified by preparative reverse-phase HPLC (15-80% CH₃CN/H₂O over 35 min) and lyophilized to afford peptide 1' (4.15 mg, 44.6% yield).



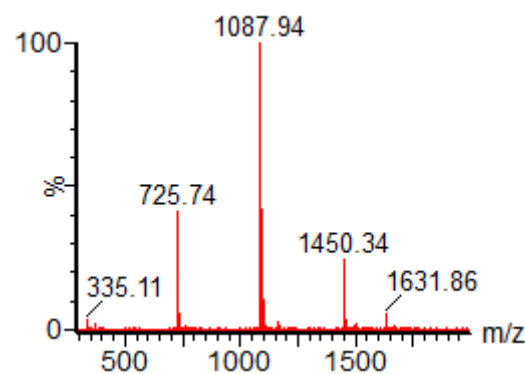


Figure S1: UV trace and corresponding MS trace from LC-MS analysis of the crude **Compound 1'**. Gradient: 5-95% ACN/H₂O with 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. ESI-MS calcd. for C₁₀₀H₁₄₈N₂₈O₂₅S = 2173.09; [M+2H]²⁺ m/z = 1087.54 found 1087.94; [M+3H]³⁺ m/z = 725.36 found 725.74; 2[M+2H]²⁺ m/z = 1451.48 found 1450.34.

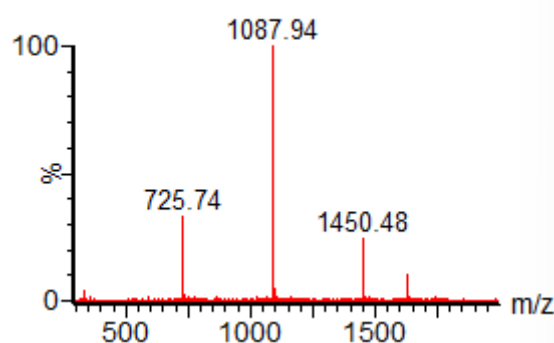
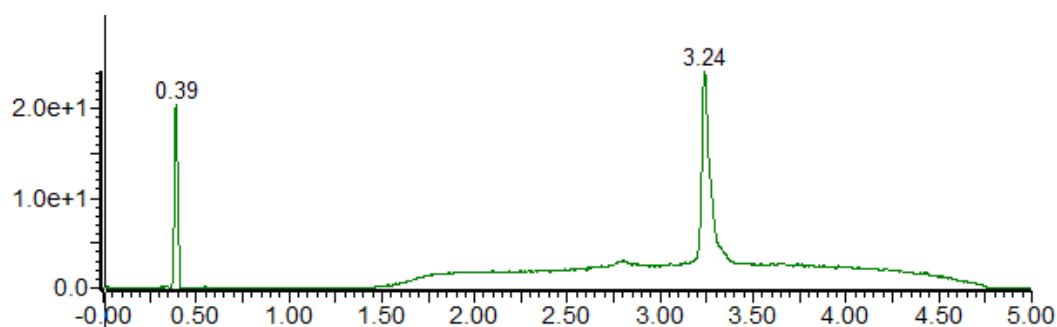
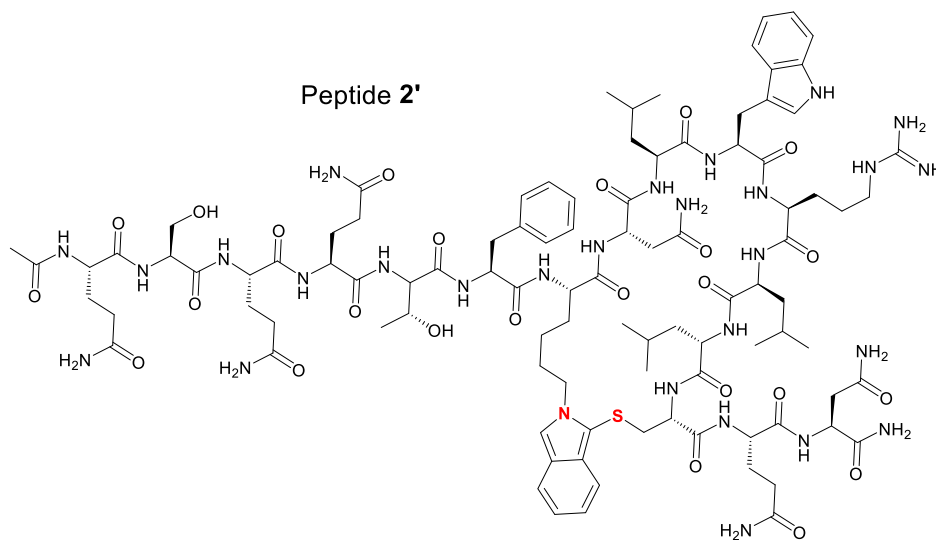
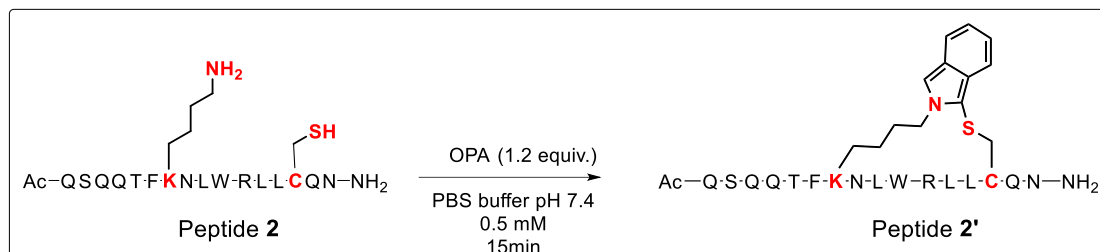


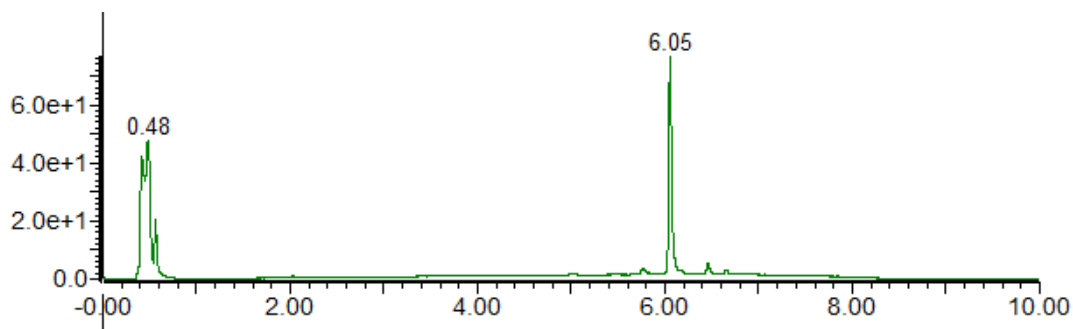
Figure S2: UV trace and corresponding MS trace from LC-MS analysis of the purified **Compound 1'**. Gradient: 5-95% ACN/H₂O with 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. ESI-MS calcd. for C₁₀₀H₁₄₈N₂₈O₂₅S = 2173.09; [M+2H]²⁺

$m/z = 1087.54$ found 1087.94 ; $[M+3H]^{3+}$ $m/z = 725.36$ found 725.74 ; $2[M+2H]^{2+}$ $m/z = 1451.48$ found 1450.48 .

3.2 Cyclization of model peptide 2



The reaction was carried out at 0.0049 mmol scale according to general procedure 3.10.6. The crude reaction mixture was purified by preparative reverse-phase HPLC (15-80% CH₃CN/H₂O over 35 min) and lyophilized to afford peptide 2' (5.18 mg, 49.3% yield).



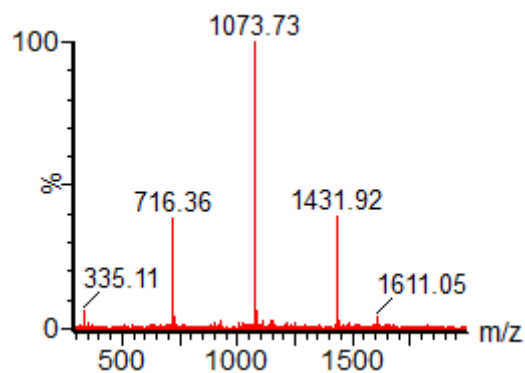


Figure S3: UV trace and corresponding MS trace from LC-MS analysis of the crude **Compound 2'**. Gradient: 5-95% ACN/H₂O with 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. ESI-MS calcd. for C₉₈H₁₄₄N₂₈O₂₅S = 2145.06; [M+2H]²⁺ m/z = 1073.53 found 1073.73; [M+3H]³⁺ m/z = 716.02 found 716.36; 2[M+3H]³⁺ m/z = 1432.04 found 1431.44.

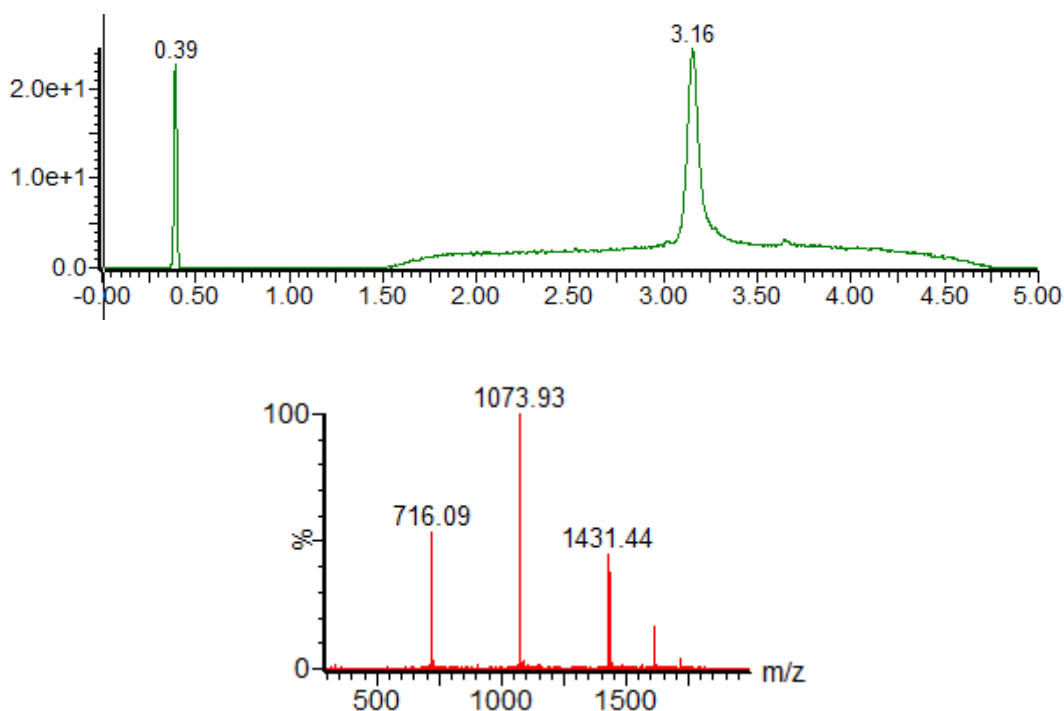


Figure S4: UV trace and corresponding MS trace from LC-MS analysis of the purified **Compound 2'**. Gradient: 5-95% ACN/H₂O with 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. ESI-MS calcd. for C₉₈H₁₄₄N₂₈O₂₅S = 2145.06; [M+2H]²⁺ m/z = 1073.53

found 1073.93; $[M+3H]^{3+}$ $m/z = 716.02$ found 716.09; $2[M+3H]^{3+}$ $m/z = 1432.04$ found 1431.44.

3.3 Stability test of peptides 1' and 2'

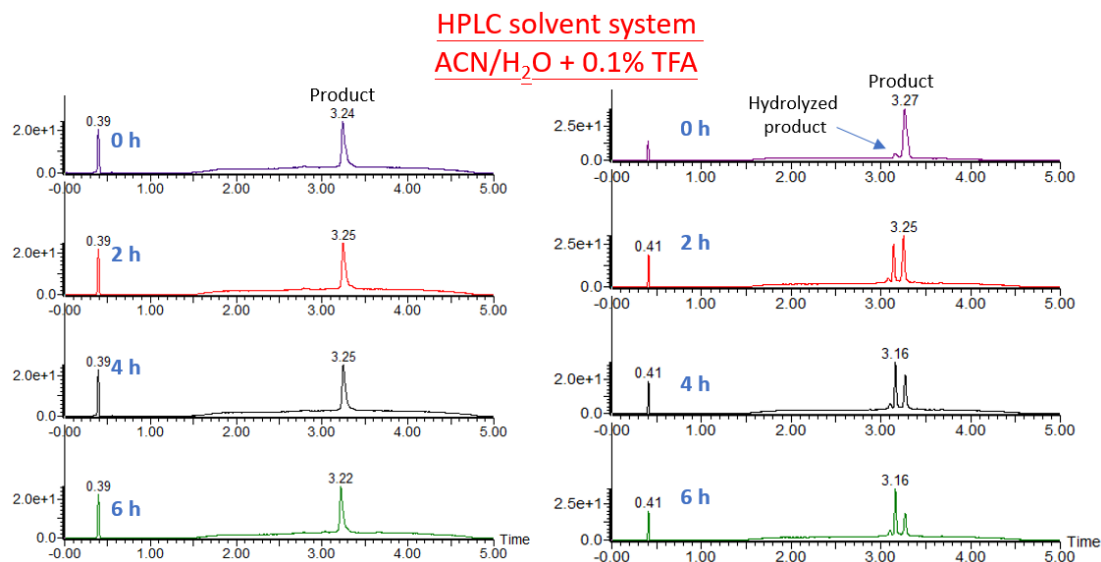


Figure S5: UV trace of the purified peptides 1' and 2' in the HPLC solvent system ACN/H₂O + 0.1% TFA, placed at room temperature and an air atmosphere. The compound was checked by LCMS after at 2, 4 and 6 hours. Gradient: 5-95% ACN/H₂O with 0.1% TFA over 5 min.

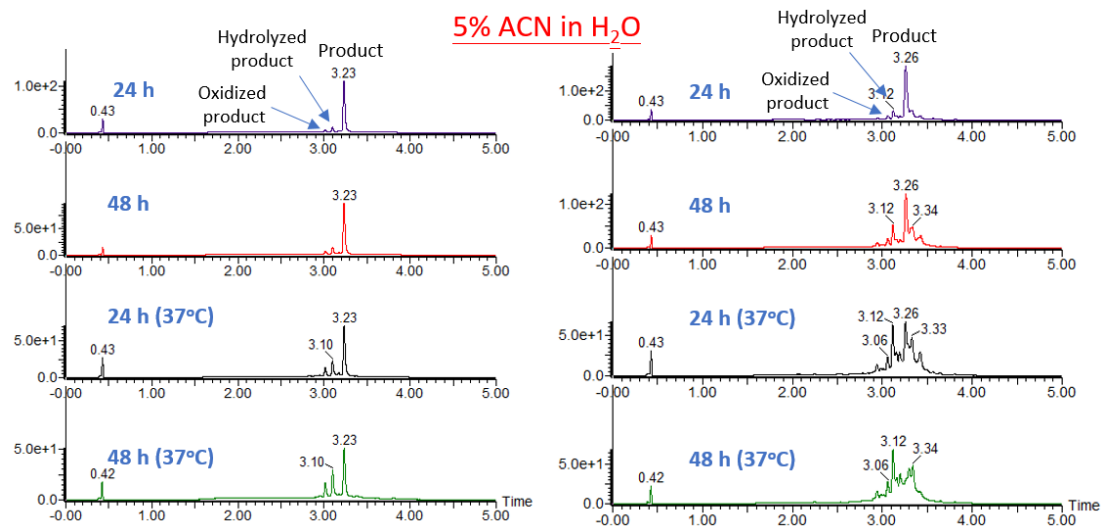


Figure S6: UV trace of the lyophilized peptides 1' and 2' in 5% ACN in H₂O, final

pH =5, placed at room temperature or 37°C as indicated above, at an air atmosphere.

The compound was checked by LCMS after 24 hours and 48 hours. Gradient: 5-95% ACN/H₂O with 0.1% TFA over 5 min, and 5-95% ACN/H₂O with 0.1% TFA over 10 min at a flow rate of 0.4 mL/min.

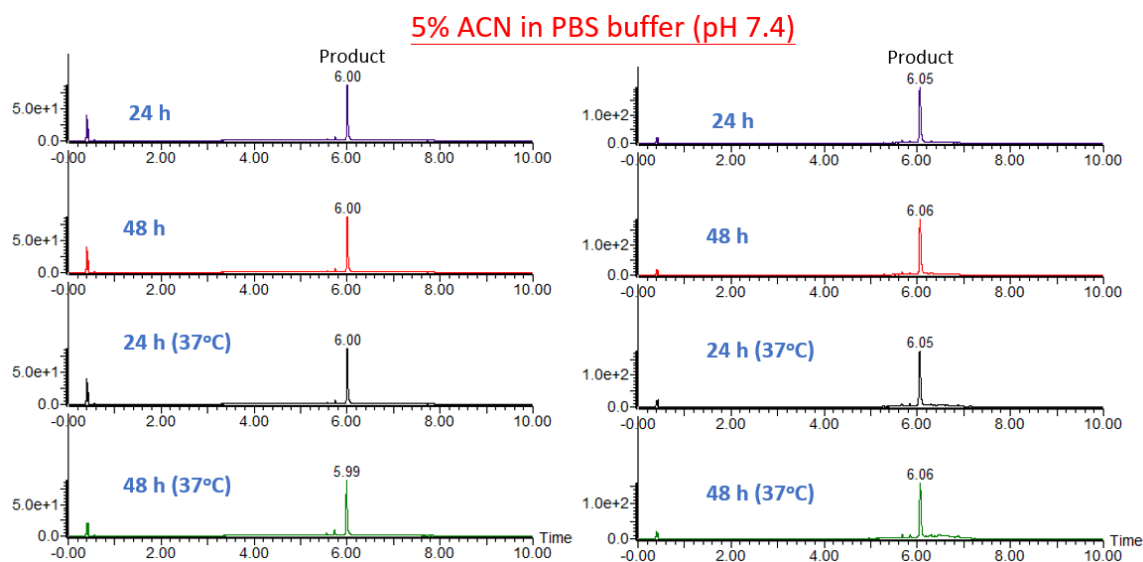
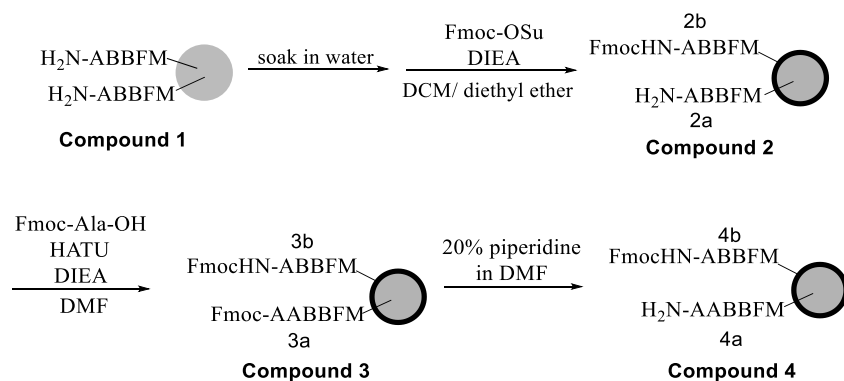


Figure S7: UV trace of the lyophilized peptides **1'** and **2'** in 5% ACN in PBS buffer, final pH =7.4, placed at room temperature or 37°C as indicated above, at an air atmosphere. The compound was checked by LCMS after 24 hours and 48 hours. Gradient: 5-95% ACN/H₂O with 0.1% TFA over 5 min, and 5-95% ACN/H₂O with 0.1% TFA over 10 min at a flow rate of 0.4 mL/min.

3.4 Topological segregation of beads



^a Equivalent of Fmoc-OSu used was listed below per condition. ^b Equivalent of DIEA used was 3-fold of Fmoc-OSu.

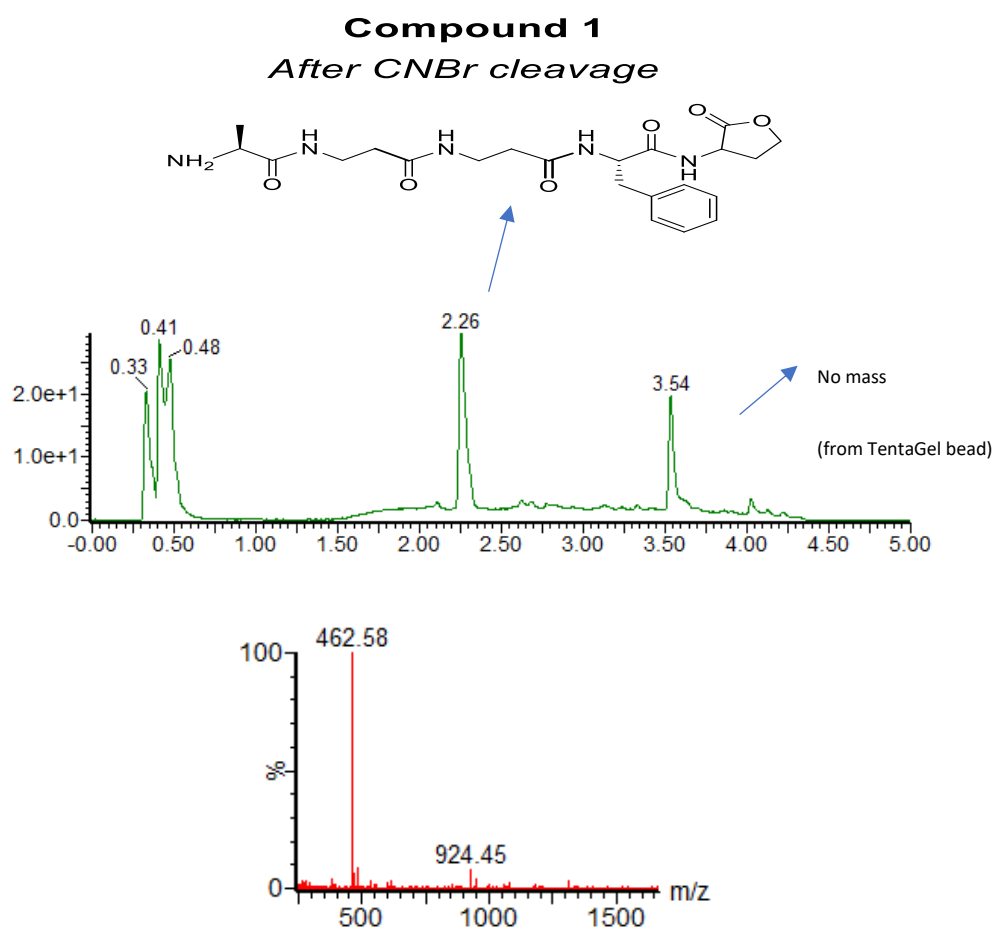


Figure S8: UV trace and corresponding MS trace from LC-MS analysis of the **Compound 1**. Gradient: 5-95% ACN/H₂O with 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. ESI-MS calcd. for C₂₂H₃₁N₅O₆ = 461.23; [M+H]⁺m/z = 462.23 found 462.58.

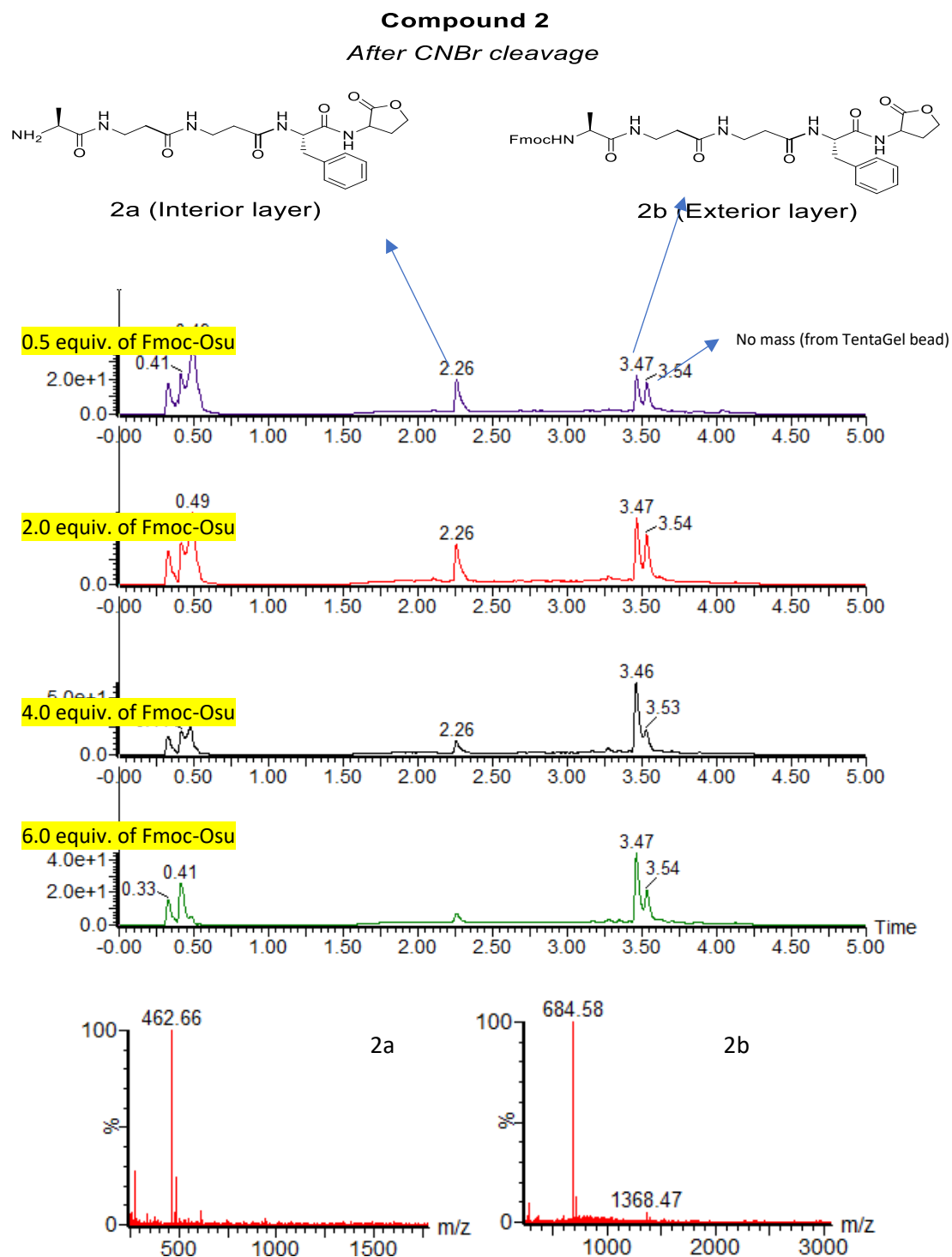
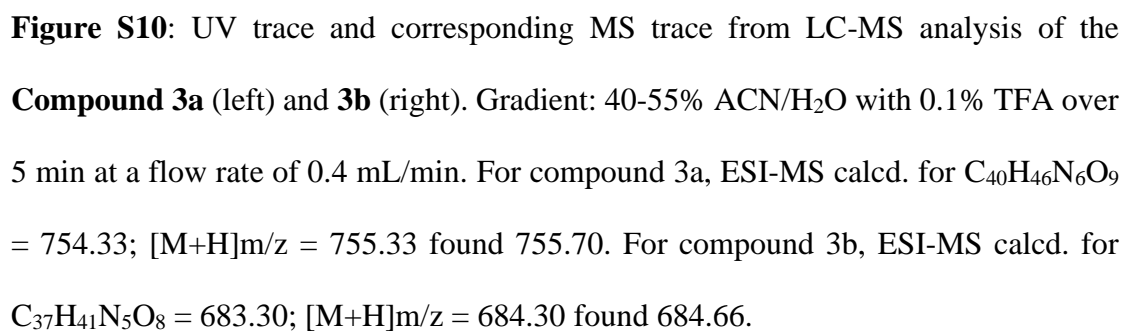


Figure S9: UV trace and corresponding MS trace from LC-MS analysis of the **Compound 2a** (left) and **2b** (right). Gradient: 5-95% ACN/H₂O with 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. For compound 2a, ESI-MS calcd. for C₂₂H₃₁N₅O₆ = 461.23; [M+H]⁺m/z = 462.23 found 462.66. For compound 2b, ESI-MS calcd. for C₃₇H₄₁N₅O₈ = 683.30; [M+H]⁺m/z = 684.30 found 684.58.

3a (Interior layer)

3b (Exterior layer)



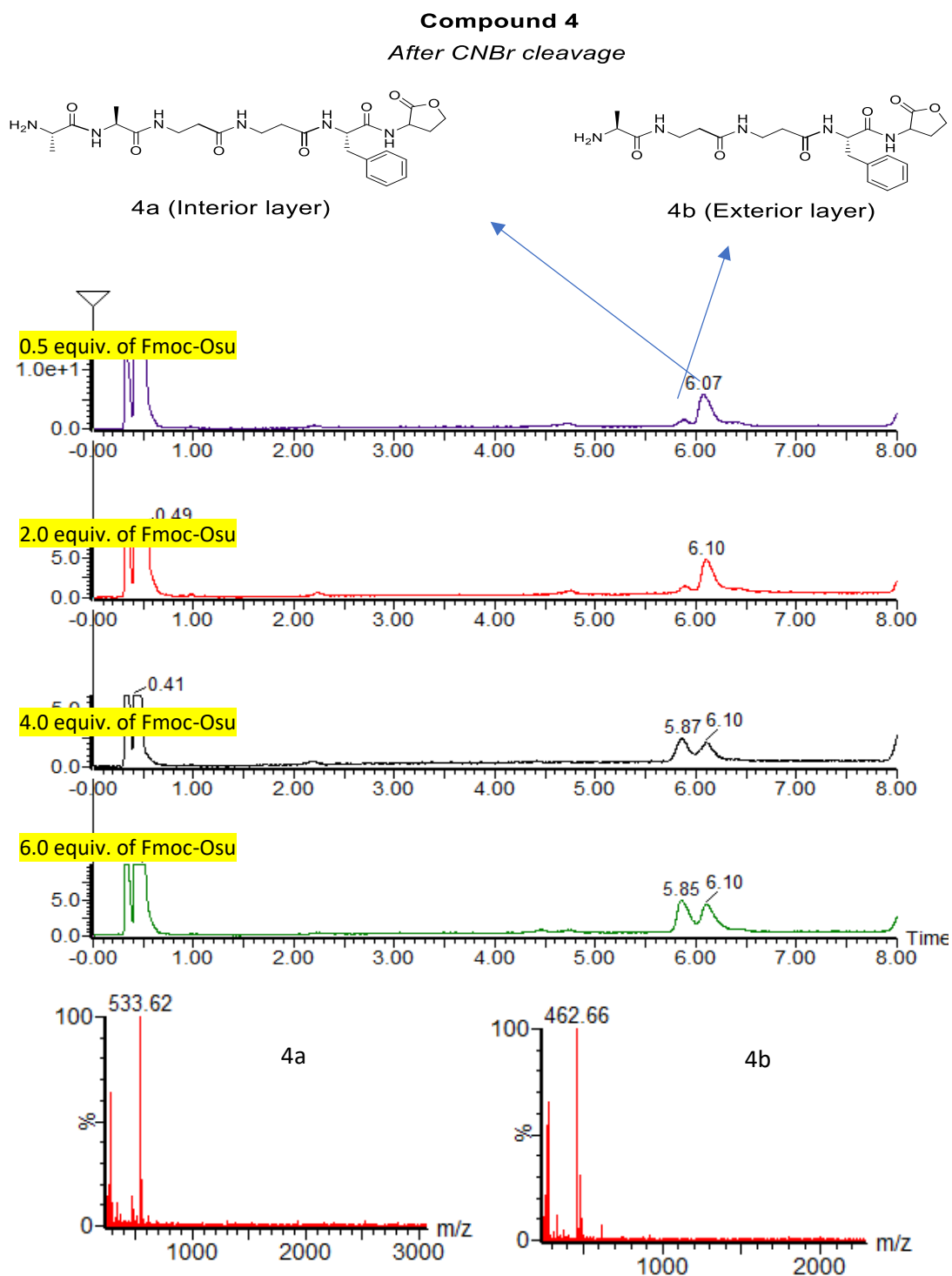
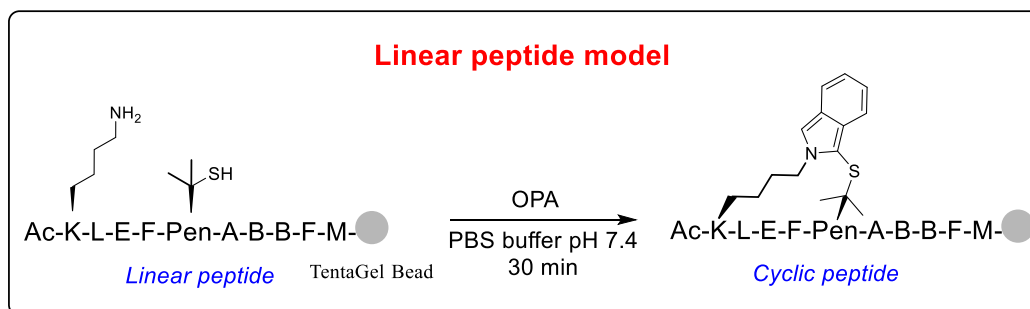


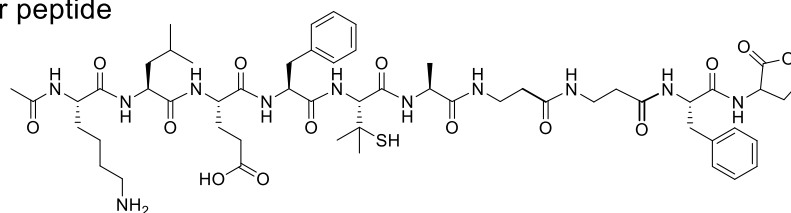
Figure S11: UV trace and corresponding MS trace from LC-MS analysis of the **Compound 4a** (top) and **4b** (bottom). Gradient: 15-30% ACN/H₂O with 0.1% TFA over 8 min at a flow rate of 0.4 mL/min. For compound 4a, ESI-MS calcd. for C₂₅H₃₆N₆O₇ = 532.26; [M+H]⁺m/z = 533.26 found 755.70. For compound 4b, ESI-MS calcd. for C₂₂H₃₁N₅O₆ = 461.23; [M+H]⁺m/z = 462.23 found 462.66.

3.5 Linear peptide model



After CNBr cleavage

Linear peptide



Cyclic peptide

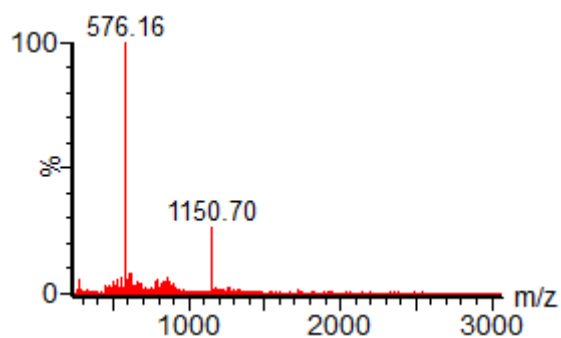
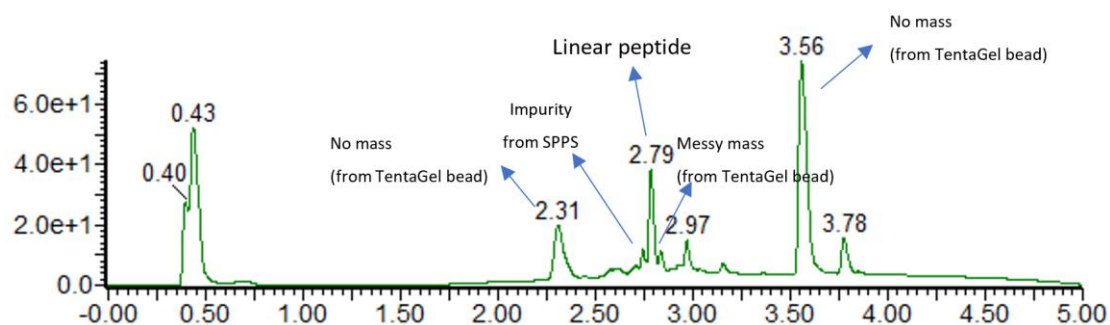
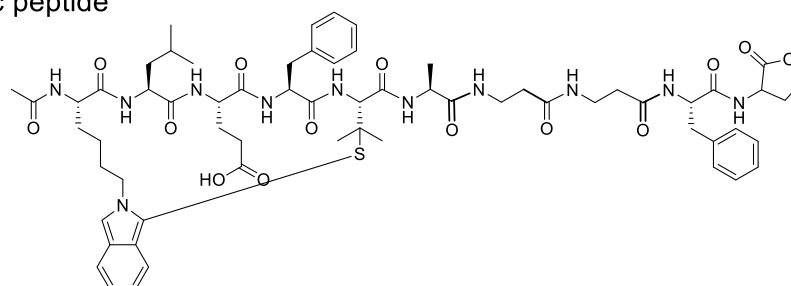


Figure S12: UV trace and corresponding MS trace from LC-MS analysis of the **Linear peptide**. Gradient: 5-95% ACN/H₂O with 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. ESI-MS calcd. for C₅₅H₈₁N₁₁O₁₄S = 1150.57; [M+H]⁺m/z = 1151.57 found 1150.70; [M+H]⁺m/z = 576.28, found 576.16.

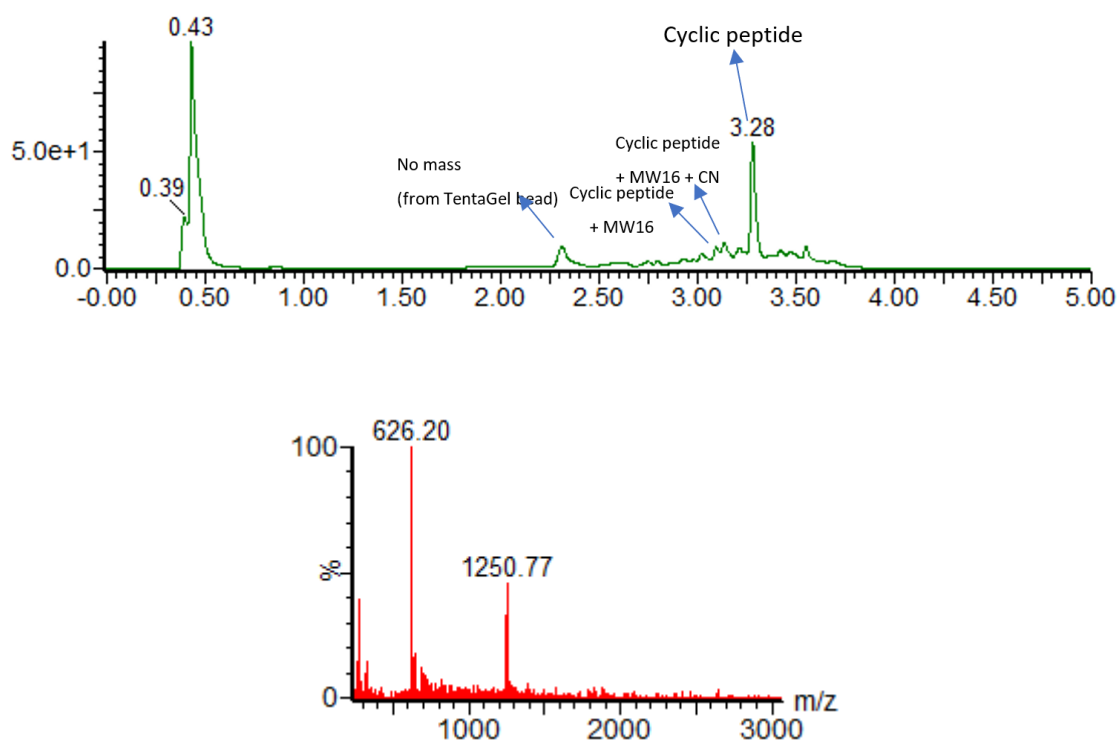
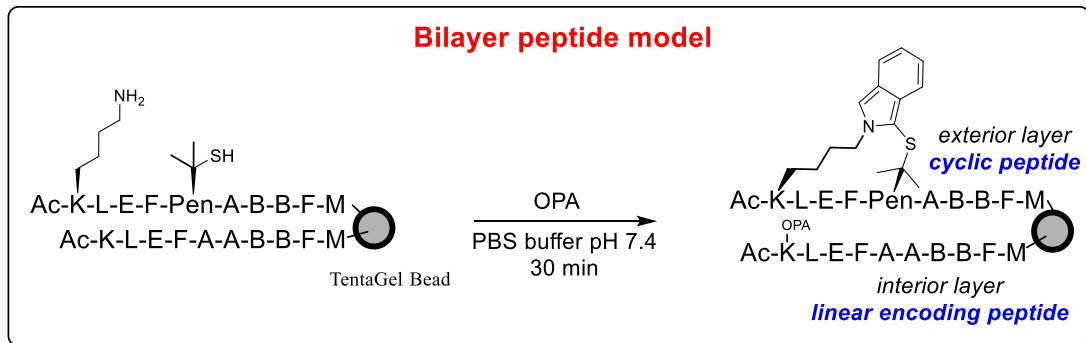


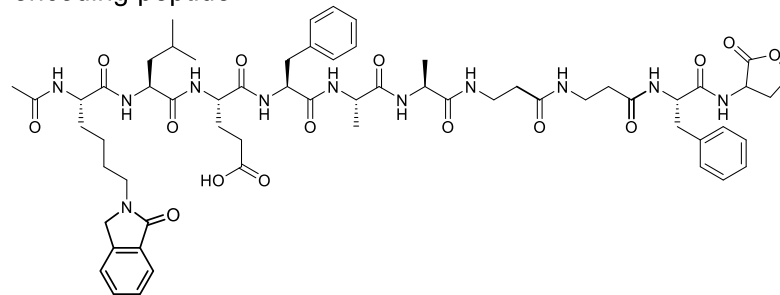
Figure S13: UV trace and corresponding MS trace from LC-MS analysis of the **Cyclic peptide**. Gradient: 5-95% ACN/H₂O with 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. ESI-MS calcd. for C₆₃H₈₃N₁₁O₁₄S = 1249.58; [M+H]⁺m/z = 1250.58 found 1250.77; [M+H]⁺m/z = 625.79, found 626.20.

3.6 Linear peptide model

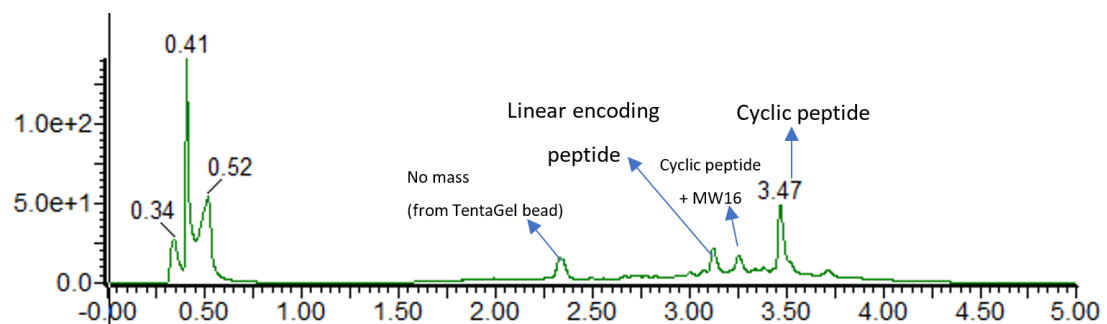
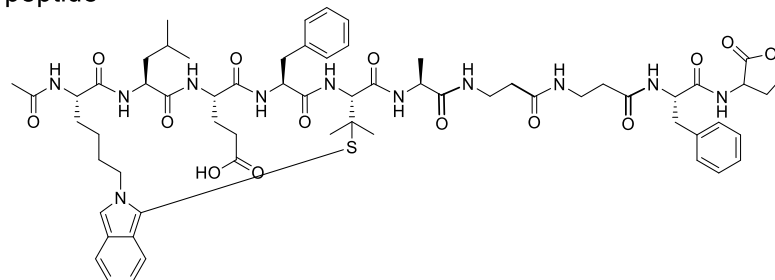


After CNBr cleavage

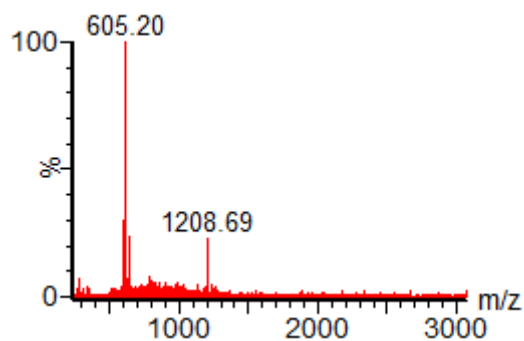
Linear encoding peptide



Cyclic peptide



Linear encoding peptide



Cyclic peptide

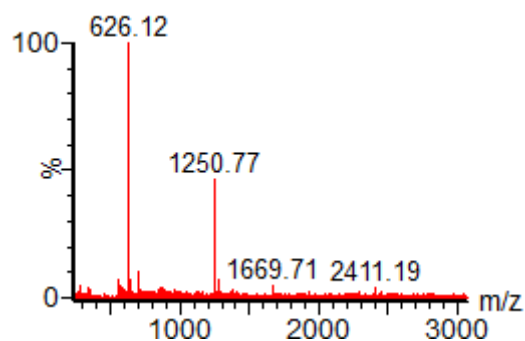


Figure S14: UV trace and corresponding MS trace from LC-MS analysis of the **Linear encoding peptide** (top) and **Cyclic peptide** (below). Gradient: 5-95% ACN/H₂O with 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. For the linear encoding peptide, ESI-MS calcd. for C₆₁H₈₁N₁₁O₁₅ = 1207.59; [M+H]⁺m/z = 11208.59 found 1208.69; [M+H]⁺m/z = 604.80, found 605.20. For the cyclic peptide, ESI-MS calcd. for C₆₃H₈₃N₁₁O₁₄S = 1249.58; [M+H]⁺m/z = 1250.58 found 1250.77; [M+H]⁺m/z = 625.79, found 626.12.