Head-to-tail cyclization of side chain-protected linear peptides to recapitulate genetically-encoded cyclized peptides

Samir Bouayad-Gervais, Daniel J. St-Cyr, Mathieu Courcelles, Éric Bonneil, Florence H. Gohard, Pierre Thibault, William C. Earnshaw and Mike Tyers

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I. General Procedures

Materials

Fluorenlymethyloxycarbonyl (Fmoc) \( N \)-protected amino acids were purchased from CEM Inc. (Matthews, NC). The following chemicals were obtained from Sigma Aldrich Inc. (St. Louis, MO): 3-(diethoxyphosphoryloxy)-1,2,3-benzotriazin-4(3H)-one (DEPBT), (7-azabenzotriazol-1-yl)-N,N,N’,N’-tetramethyluronium hexafluorophosphate (HATU), benzotriazole-1-yl-N,N,N’,N’-tetramethyluronium hexafluorophosphate (HBTU), dithiothreitol (DTT) and 2,2,2-trifluoroethanol (TFE). Tris(2-carboxyethyl)phosphine (TCEP) was obtained from Pierce Biotechnology (Rockford, IL). The following chemicals were obtained from Fischer Scientific Inc. (Waltham, MA): dimethyl formamide (DMF), dimethylsulfoxide (DMSO), acetonitrile (MeCN), and dichloromethane (CH\(_2\)Cl\(_2\)). Anhydrous DMF and CH\(_2\)Cl\(_2\) were prepared by treating the solvents with activated molecular sieves (4 Å, ca. 20% by volume) under nitrogen and waiting >2 d prior to use. Chloro-(2’-chloro)trityl polystyrene (2-Cl-Trt-Cl) resin was purchased from Rapp Polymere GmbH (Tübingen, Germany). The preloaded resin 7e [H-Lys(Boc)-2-Chlorotrityl resin, styrene with 1% divinylbenzene copolymer, 100-200 mesh, 0.54 mmol/g] was bought from GL Biochem Ltd. (Shanghai, China). Unless specified otherwise chemicals were used without further purification.

Manual peptide synthesis

During manual peptide elongation, the highly-reactive HATU amide bond coupling reagent was chosen to reduce reaction times and avoid resort to repeated couplings. To facilitate the optimal use of HATU under anhydrous conditions, resin for manual SPPS was loaded into disposable polypropylene syringes fitted with polypropylene frits (70 \( \mu \)m porosity) and needles rather than filter tubes.\(^1\) To remove adventitious water from resin-charged fritted syringes, dry solvents (DMF or CH\(_2\)Cl\(_2\)) under nitrogen were repeatedly (2–5 times) aspirated into and expelled from the syringe over 5–30 minutes.

Manual Trt resin loading procedure

2-ClTrt-Cl Resin (250 mg, 0.250 mmol, 1 equiv.) was loaded into a fritted syringe (6 mL capacity), washed twice with dry CH\(_2\)Cl\(_2\), and immersed in dry DMF under nitrogen. After 0.5 h, the liquid phase was expelled and the solid phase was treated with a mixture of Fmoc amino acid (0.3 mmol, 1.2 equiv.) and Hünig’s base (218 \( \mu \)L, 1.25 mmol, 5 equiv.) in CH\(_2\)Cl\(_2\) (2.5 mL). After mixing by repeated inversion at room temperature
for 2 h, the reaction medium was removed and the resin was washed multiple times with CH₂Cl₂/MeOH/Hünig’s base (17:2:1, 3x over 3 min), CH₂Cl₂ (3x over 3 min), DMF (2x over 2 min), and CH₂Cl₂ (2x over 2 min). The resulting 2-ClTrt resin bearing a single Fmoc amino acid was used directly in subsequent elongation reactions by assuming quantitative yield (0.25 mmol) for subsequent stoichiometry calculations.

**Manual peptide elongation procedure**

2-ClTrt resin bearing an N-terminal Fmoc amino acid residue (0.25 mmol nominal loading) was deprotected by treatment with two batches of piperidine (20%) in DMF (3-4 mL) over a total of 17 min (2 min, then 15 min) and the liquid phase was removed. The solid phase was washed multiple times with DMF (5x over 5 min) and CH₂Cl₂ (2x over 2 min) to afford a resin-bound free amine. The latter was elongated by treatment with a dry DMF (2.8–3 mL) solution of the subsequent Fmoc-amino acid (0.750 mmol, 3 equiv.), HATU (271 mg, 0.713 mmol, 2.85 equiv.), and Hünig’s base (258 µL, 1.48 mmol, 5.9 equiv.). After mixing by repeated inversion at room temperature for 45 min, the reaction medium was removed and the resin was washed multiple times with DMF (5x over 5 min) and CH₂Cl₂ (2x over 2 min). To confirm reaction completion, resin aliquots from before and after the acylation reaction were subjected to the Kaiser colorimetric test and compared. The deprotection/elongation sequence was appropriately iterated according to the targeted sequence to afford an Fmoc-peptidyl resin, which was deprotected by repeating the piperidine process to afford the final N-terminal free amine peptidyl resin 2. The latter was used directly in the TFE-mediated Trt resin cleavage procedure described below.

**Automated peptide synthesis**

Automated Solid Phase Peptide Synthesis (SPPS) was conducted on a CEM Liberty1 microwave synthesizer, which accelerates peptide synthesis on Rink amide and Wang resins by employing microwave heating to 75 °C during coupling/deprotection cycles. Microwave-accelerated peptide synthesis on 2-ClTrt-Cl resin preferentially occurs at 50 °C, which was modified as elaborated below to accommodate automated attachment of the first amino acid. During automated peptide synthesis, complications due to frit clogging were overcome by substituting resins featuring standard bead diameter ranges [100-200 mesh (74–149 µm)] with larger counterparts (125–160 µm or 250–315 µm).
Automated Trt resin loading procedure

A Liberty1 synthesizer was charged with 2-CITrt-Cl resin (250 mg, 1.0 mmol/g, 0.25 mmol), 35% Hünig’s base in DMF (1.5 mL, ca. 3.9 mmol, steps 1–2, Figure S1), and Fmoc amino acid in CH₂Cl₂ (3.75 mL, 0.2 M, 0.75 mmol, step 3). The mixture was heated to 50 °C by microwave irradiation for 30 min, the liquid phase was removed (step 4), and the solid phase was washed with DMF (20 mL total, steps 5–6). The coupling and wash sequence was repeated (steps 7–12), and the resulting Fmoc amino acid resin was treated with 35% Hünig’s base in DMF (4 mL, ca. 10 mmol, step 13) and MeOH (3.75 mL, 93 mmol, step 14). After mixing by argon bubbling for 10 min at room temperature (step 15), the liquid phase was removed and the resin was washed with DMF (10 mL, step 16). The capping sequence was repeated (steps 17–20) and the resin was washed with additional DMF (20 mL total, steps 21–22) to afford 2-CITrt resin bearing a single Fmoc amino acid. The latter was used directly in subsequent elongation reactions by assuming quantitative yield (0.25 mmol) for subsequent stoichiometry calculations.

Figure S1. Screenshot of the automated method used for loading Fmoc-amino acids onto 2-CITrt-Cl resin using a Liberty1 instrument.

| Operation            | Parameter                  | Volume | Drain | Cycles | Pause |
|----------------------|----------------------------|--------|-------|--------|-------|
| 1 Wash - Top         | Main Wash (DMF)            | 10     | ✓     | 1      |       |
| 2 Add Activator Base | DMEA                      | 1.5    |       | 1      |       |
| 3 Add Amino Acid     |                           | 3.75   |       | 1      |       |
| 4 Microwave Method   | Coupling 5OC Trt           |         | ✓     | 1      |       |
| 5 Wash - Top         | Main Wash (DMF)            | 10     | ✓     | 1      |       |
| 6 Wash - Bottom      | Main Wash (DMF)            | 10     | ✓     | 1      |       |
| 7 Add Activator Base | DME A                     | 1.5    |       | 1      |       |
| 8 Add Amino Acid     |                           | 3.75   |       | 1      |       |
| 9 Microwave Method   | Coupling 5OC Trt           |         | ✓     | 1      |       |
| 10 Wash - Top        | Main Wash (DMF)            | 10     | ✓     | 1      |       |
| 11 Wash - Bottom     | Main Wash (DMF)            | 10     | ✓     | 1      |       |
| 12 Wash - Top        | Main Wash (DMF)            | 10     | ✓     | 1      |       |
| 13 Add Activator Base| DMEA                      | 4      |       | 1      |       |
| 14 Add Custom Amino Acid | Position 23 (Ex3 - 3) | 3.75   |       | 1      |       |
| 15 Wait Scale        |                           | 600    |       | 1      |       |
| 16 Wash - Top        | Main Wash (DMF)            | 10     | ✓     | 1      |       |
| 17 Add Activator Base| DMEA                      | 4      |       | 1      |       |
| 18 Add Custom Amino Acid | Position 23 (Ex3 - 3) | 3.75   |       | 1      |       |
| 19 Wait Scale        |                           | 600    |       | 1      |       |
| 20 Wash - Top        | Main Wash (DMF)            | 10     | ✓     | 1      |       |
| 21 Wash - Bottom     | Main Wash (DMF)            | 10     | ✓     | 1      |       |
| 22 Wash - Top        | Main Wash (DMF)            | 10     | ✓     | 1      |       |
**Automated peptide elongation procedure.**

Resin-bound amino acid was achieved using the recommended precursors, reagents, and solvents, including DMF (peptide grade) solutions of Fmoc-amino acids (0.2 M), HBTU (0.5 M), Hüning base (35%), and piperidine (20%). The default microwave heating setting for the coupling was changed from 75 °C to 50 °C and the default 300 s coupling reaction time setting was changed to 1800 s. As final automated step, the resin was treated with piperidine (20%) in DMF, heated to 50 °C for 30 min, and washed with DMF to afford N-terminal free amine peptidyl resin 2. The latter was transferred from the synthesizer into a fritted syringe for direct use in the TFE-mediated resin cleavage procedure.

**TFE-mediated Trt resin cleavage procedure**

N-terminal free amine peptidyl resin 2 was treated with TFE (30%) in CH₂Cl₂ (12 mL), mixed by periodic inversion for 1 h at room temperature, and the liquid phase was collected. The cleavage was repeated using fresh TFE/CH₂Cl₂ mixture and the combined liquid phases were concentrated in vacuo. The resulting crude side-chain protected peptide 3 was employed directly in the linear peptide macrocyclization procedure.

**Peptide macrocyclization procedure**

A solution of NEt₃ (35 µL, 250 µmol, 10 equiv.) in CH₃CN (11.2 mL) was added to a DMSO (470 µL) solution of linear peptide 3 (25 µmol, 1 equiv.) and the resulting mixture was treated with DEPBT (18.6 mg, 62 µmol, 2.5 equiv.). After stirring at room temperature for 24 h, the reaction was quenched with AcOH (0.5–1 mL) and the volume was reduced in vacuo by ≥ 10 fold (to 1–2 mL). The resulting DMSO/CH₃CN solution of the crude was filtered (3 mm syringe filter, 0.2 µm pore size) and purified by preparatory HPLC to afford cyclic peptides 4, 5, or a 4/5 mixture dependent on sequence. Preparatory HPLC purifications were performed on an Agilent 1200 instrument or a Waters Inc. (Milford, MA) 2795 coupled to a 2996 diode array and micromass ZQ for UV and MS detection respectively. Cyclic peptides 4 were eluted using flow rates of 20 mL/min under the conditions detailed in Table S1. The collected fractions were concentrated in vacuo (2–5 Torr) at 50 °C, then concentrated to dryness with assistance from multiple azeotropic coevaporations with i-PrOH or 1,4-dioxane as necessary.
**Table S1. Methods used for preparatory HPLC purification.**

| # | Column | Eluent system (weak mixture “A”/ strong mixture “B”) | Gradient | Peak detection |
|---|---|---|---|---|
| A | Kinetex C18 100A AXIA 21.2 x 100 mm | [H₂O / MeOH / TFA (95 : 5 : 0.1)] / [MeOH / H₂O / TFA (95 : 5 : 0.1)] | 0-3 min, 70% “B” in “A”; 3-10 min, 70 to 100% “B”; 10-20 min 100% “B”. | UV: 220.16, 254.16. |
| B | Zorbax SB-C18 PrepHT 5 µm; 21.2 x 100 mm | [H₂O / MeOH / AcOH (95 : 5 : 0.1)] / [MeOH / H₂O / AcOH (95 : 5 : 0.1)] | 0-2 min, 20% “B” in “A”; 2-15 min, 20 to 100% “B”; 15-20 min, 100% “B”. | UV: 220.4 nm, 254.4 nm. or MS: |
| C | Zorbax SB-C18 PrepHT 5 µm; 21.2 x 100 mm | [H₂O / MeOH / TFA (95 : 5 : 0.05)] / [MeOH / H₂O / TFA (95 : 5 : 0.05)] | 0-2 min, 50% “B” in “A”; 2-10 min, 50 to 100% “B”; 10-15 min, 100% “B”. | UV: 220.4 nm, 254.4 nm. |
| D | Atlantis Prep. OBD, 5 µm: 30 x 100 mm | [H₂O / MeOH / HCO₂H (95 : 5 : 0.1)] / [MeOH / H₂O / HCO₂H (95 : 5 : 0.1)] | 0-15 min, 20 to 75% “B” in “A”. | MS, ESI+ |

**TFA-mediated deprotection procedure**

To avoid known complications associated with the deprotection of peptides containing Cys(Trt) and Trp(Boc) residues, the cleavage cocktail containing DTT as nucleophilic scavenger was employed. Among alternative thiols, DTT was selected due to reduced stench.

A heterogeneous mixture of DTT (~0.6 mg, 4 µmol), H₂O (30 µl), TFA (0.53 mL), and triisopropylsilane (12.1 µl, 59 µmol) was added to protected peptide 4, 5, or a 4/5 mixture (4 µmol) and stirred for 0.5 h at room temperature. The volatiles were removed in vacuo and an i-PrOH solution of the residue was purified by manual reversed-phase chromatography using a Sep-Pak C-18 3cc cartridge (Waters Inc., 37–55µm size) and 20% MeOH in H₂O as eluent when crude peptides were obtained in low quantities (< 7 mg). Otherwise, purification was conducted with DMSO solutions using preparative HPLC. The collected fractions were concentrated in vacuo (1–5 Torr) at 50 °C, then concentrated to dryness with assistance from multiple azeotropic coevaporations with i-PrOH or 1,4-dioxane. The residue was dissolved in dilute HCl(aq) (> 10 equiv.), and lyophilized to yield 1, 6, or a 1/6 mixture, typically as HCl salts, dependent on structure.

**Chromatographic characterization of peptidic products**

The purity and identity of synthetic intermediates 3–5 was established by a combination of Analytical High Performance Liquid Chromatography (analytical HPLC), HPLC-low resolution Mass Spectrometry (HPLC-MS), and HPLC-High Resolution Mass Spectrometry (HPLC-HRMS). Analytical HPLC was monitored by diode array UV detector and a 1260 infinity Evaporative Light Scattering (ELS) detector.
operating at 50 °C. Analytical HPLC-MS spectra were recorded on an Agilent Inc. (Santa Clara, CA) 1200 series HPLC coupled to a diode array UV detector and a 6120 Quadrupole low resolution mass spectrometer equipped with an Electrospray Ionization (ESI) source. HPLC-HRMS spectra were recorded using an Agilent Mass Selective Detector with Time-of-Flight analyzer (MSD-TOF, model 61969A). Analytes were eluted under the conditions listed in Table S2.

Cyclic peptides 1 and 6 were analyzed using a Dionex/Thermo UltiMate 3000 binary RSLCnano Ultra High Performance Liquid Chromatography (UHPLC) system coupled to a Q-Exactive MS operating under the chromatographic and spectrometric conditions detailed in Table S2 and Table S3. Purified cyclic peptides 1 harbouring Cys residues were prone to dimerization by disulfide bond formation, complicating mass spectrometry analyses, which was avoided by adding TCEP (0.5 mM final concentration) reductant to samples prior to injection.

Table S2. Methods used for analytical HPLC-MS, HPLC-ELS, and UHPLC-HRMS analysis.

| #  | Column               | Flow (mL/min) | Eluent system (weak mixture “A”)/strong mixture “B”) | Gradient | Peak detection                      |
|----|----------------------|---------------|------------------------------------------------------|----------|-------------------------------------|
| E  | Kinetex-C18, 2.6 µm; 3.0 x 30 mm | 1.50          | [H2O / MeOH / AcOH (95 : 5 : 0.1)] / [MeOH / H2O / AcOH (95 : 5 : 0.1)] | 0-0.5 min, 0% to 100% “B” in “A”; 0.5-2 min, 100% “B”. | UV: 220.4 nm, 254.4 nm. MS: ESI+, ESI- |
| F  | Kinetex-C18, 2.6 µm; 3.0 x 30 mm | 1.0           | [H2O/ MeOH/ HCO2H (95 : 5 : 0.1)] / [MeOH/ H2O/ HCO2H (95 : 5 : 0.1)] | 0 to ~7 min, 70 to 80% “B” in “A”; ~7 to 15 min, 80% “B”. | UV: 220.4 nm, 254.4 nm. MS: ESI+, ESI- |
| G  | Agilent Poroshell 120; EC-C18, 2.7 µm; 2.1 x 30 mm | 1.0           | [H2O / MeOH / AcOH (95 : 5 : 0.1)] / [MeOH / H2O / AcOH (95 : 5 : 0.1)] | 0-1.5 min, 0% to 100% “B” in “A”; 1.5-4 min, 100% “B”. | UV: 220.4 nm, 254.4 nm. MS: ESI+, ESI- |
| H  | Zorbax SB-Phenyl 3.5 µm; 4.6 x 30mm. | 1.0           | [H2O / MeOH / TFA (95 : 5 : 0.05)] / [MeOH / H2O / TFA (95 : 5 : 0.05)] | 0-1.5 min, 0% to 100% “B” in “A”; 1.5-4 min, 100% “B”. | UV: 220.8 nm, 254.8 nm. ELS: 50 °C |
| I  | Phenomenex Jupiter C18, 3µm, 300 Å, 15 cm x 150 µma | 600 nl/min | [H2O / HCO2H (99.8 :0.2)] / [MeCN / HCO2H (99.8 :0.2)] | 0-30 min, 50 to 99% “B” in “A”. | HRMS: ESI+ |
| J  | Phenomenex Jupiter C18, 3µm, 300 Å, 15 cm x 150 µma | 600 nl/min | [H2O / HCO2H (99.8 :0.2)] / [MeCN / HCO2H (99.8 :0.2)] | 0-70 min, 40 to 99% “B” in “A”. | HRMS: ESI+ |

a Trap column: Phenomenex Jupiter C18, 3µm, 300 A, 0.5 cm x 360 µm.
**Table S3.** MS and MS/MS Parameters used during UHPLC analysis.

| Parameter                              | Value               |
|----------------------------------------|---------------------|
| Instrument                             | Q-Exactive          |
| Run time                               | 30 or 70 min        |
| Spray voltage                          | + 3.5 kV            |
| MS1 scan range                         | 300-1500 m/z        |
| MS1 resolution                         | 70 000              |
| MS1 AGC target                         | 1e6                 |
| MS1 injection time                     | 100 ms              |
| MS2 resolution                         | 35 000              |
| MS2 AGC target                         | 5e5                 |
| MS2 injection time                     | 500 ms              |
| MS2 Isolation window                   | 2.0 m/z             |
| MS2 HCD - Normalized collision energy  | 27                  |
| MS2 intensity threshold                | 1e4                 |
| MS2 dynamic exclusion                  | 10 s                |
| MS2 inclusion list                     | yes                 |
II. Synthesis and characterization of cyclic peptides 1 and 6

Synthesis of cyclic RCTWA

Intermediate 3a: H-Arg(Pbf)-Cys(Trt)-Thr(t-Bu)-Trp(Boc)-Ala-OH

The automated Trt resin loading procedure was executed using a CH$_2$Cl$_2$ solution of Fmoc-Ala-OH (5 mL, 0.2 M, 1 mmol) and 2.75 mL of MeOH during resin capping to afford Fmoc-alaninyl Trt resin 7a. Continuation of SPPS by the automated peptide elongation procedure using a more dilute HBTU solution (0.5 M) and standard DMF solutions (0.2 M) of Fmoc-Trp(Boc)-OH, Fmoc-Thr(t-Bu)-OH, Fmoc-Cys(Trt)-OH, and Fmoc-Arg(Pbf)-OH as inputs ultimately afforded N-terminal free amine peptidyl resin 2a. The latter was subjected to the TFE-mediated Trt resin cleavage procedure to afford 144 mg (MW 1286.60, 112 µmol, 45% yield) of side chain-protected peptide 3a as an off-white powder. HPLC-MS characterization using method “E”; retention time: 1.51 min, crude purity: 53 %, MS (ESI$^+$) m/z: [M+H]$^+$ Calcd for C$_{68}$H$_{87}$N$_9$O$_{12}$S$_2$ 1286.6, found 1286.4
Intermediate 4a: cyclo-[Arg(Pbf)-Cys(Trt)-Thr(t-Bu)-Trp(Boc)-Ala]

Side chain-protected peptide 3a (32 mg, 25 µmol) was subjected to the peptide macrocyclization procedure using preparatory HPLC method “A” for purification to ultimately afford 5 mg (MW 1268.59, 3.9 µmol, 16% yield) protected cyclic peptide 4a as a white solid. HPLC-MS characterization using method “E”; retention time: 1.97 min, purity: 98 %, MS (ESI') m/z: [M+H–Boc]⁺ Calcd for C₆₅H₇₈N₁₀O₆S₂ 1168.5, found 1169.0.

Product 1a: cyclo-[Arg-Cys-Thr-Trp-Ala]
The TFA-mediated deprotection procedure was conducted on peptide 4a (5 mg, 3.9 µmol) using manual preparative HPLC-MS in conjunction with eluent system 20% MeOH in Water for purification to ultimately afford 2 mg (TFA salt FW: 731.7, 2.7 µmol, 69% yield) of cyclo-[argininyl-cysteinyl-threoninyl-tryptophanyl-alanine] (1a) as a solid. UHPLC-HRMS characterization using method “I”; retention time: 13.3 min, HRMS (ESI+) \( m/z \) [M+H]+ Caled for \( \text{C}_{27}\text{H}_{46}\text{N}_{9}\text{O}_{6}\text{S} \) 618.2817, found 618.2811. HCD MS/MS fragment count: Calcd for b/y ions 35, found 30.

Mass chromatogram of 1a (Total Ion Chromatogram, TIC)

MS expansion illustrating the isotopic profile of 1a. The resolution (R) and charge state (Z) of each peak is given.
**Attempted synthesis of cyclic RPTWA**

**Intermediate 3b: H-Arg(Pbf)-Pro-Thr(t-Bu)-Trp(Boc)-Ala-OH**

The manual Trt resin loading procedure was conducted using Fmoc-Ala-OH (93 mg, 0.3 mmol) as input to afford Fmoc-alaninyl Trt resin 7a. Subsequent execution of the manual peptide elongation procedure using Fmoc-Trp(Boc)-OH (395 mg, 0.75 mmol), Fmoc-Thr(Ot-Bu)-OH (298 mg, 0.750 mmol), Fmoc-Pro-OH (253 mg, 0.750 mmol), and Fmoc-Arg(Pbf)-OH (324 mg, 0.500 mmol) as inputs afforded peptidyl Trt resin free amine 2b. Half of the latter was subjected to the TFE-mediated Trt resin cleavage procedure to ultimately afford 111.7 mg (MW 1038.26, 107.6 µmol, 86% yield) of side-chain protected peptide 3b. HPLC-MS characterization using method “E”; retention time: 1.31 min, crude purity: 92 %, MS (ESI⁺) m/z: [M+H–Boc]⁺ Calcd for C₄₆H₆₈N₉O₁₀S 938.5, found 938.5.
Intermediate 5b: cyclo-[(Arg(Pfb)-Pro-Thr(t-Bu)-Trp(Boc)-Ala)₂]

Side chain-protected peptide 3b (20 mg, 19 µmol) was subjected to the peptide macrocyclization procedure using scaled amounts of NET₃ (26.9 µL, 193 µmol), CH₃CN (9.7 mL), DMSO (460 µL), and DEPBT (14.4 mg, 48 µmol). Preparatory HPLC method “A” for purification ultimately afforded 3.1 mg (MW 2040.49, 1.5 µmol, 16% yield) of cyclic dimeric side product 5b. HPLC-MS characterization using method “E”; retention time: 1.56 min, purity: >98 %, MS (ESI⁺) m/z: [M+2H]²⁺ Calcd for C₁₀₂H₁₄₈N₁₈O₂₂S₂ 1021.0, found 1021.7. HPLC-HRMS (ESI-TOF) m/z: [M+2H]²⁺ Calcd for C₁₀₂H₁₄₈N₁₈O₂₂S₂ 1020.5223, found 1020.5223.
Side-product 6b: cyclo-[(Arg-Pro-Thr-Trp-Ala)\textsubscript{2}]

The TFA-mediated deprotection procedure was conducted on peptide 5b (3 mg, 1.5 µmol) using scaled amounts of DTT (~0.2 mg, 1.5 µmol), H\textsubscript{2}O (11 µl), TFA (0.20 mL), and triisopropylsilane (4.5 µl, 22 µmol). Purification was achieved by manual reversed-phase chromatography to ultimately afford 1.2 mg (bis TFA salt FW: 1451.4, 0.827 µmol, 56% yield) of cyclo-[argininyl-prolinyl-threoninyl-tryptophanyl-alaninyl-argininyl-prolinyl-threoninyl-tryptophanyl-alanine] (6b) as a solid. UHPLC-HRMS characterization using method “I”; retention time: 13.6 min, HRMS (ESI\textsuperscript{+}) m/z: [M+H]\textsuperscript{+} Calcd for C\textsubscript{58}H\textsubscript{83}N\textsubscript{18}O\textsubscript{12} 1223.6432, found 1223.6421; [M+2H]\textsuperscript{2+} Calcd for C\textsubscript{58}H\textsubscript{84}N\textsubscript{18}O\textsubscript{12} 612.3253, found 612.3246. HCD MS/MS fragment count: Calcd for b/y ions 35, found 22.

Mass chromatogram of 6b (TIC)

MS expansion illustrating the isotopic profile of 6b in the singly-charged ion region
MS expansion illustrating the isotopic profile of 6b in the doubly-charged ion region

**Synthesis of cyclic RATWA**

**Intermediate 3c: H-Arg(Pbf)-Ala-Thr(t-Bu)-Trp(Boc)-Ala-OH**

The manual Trt resin loading procedure was conducted using Fmoc-Ala-OH (93 mg, 0.3 mmol) as input to afford Fmoc-alaninyl Trt resin 7a. Subsequent execution of the manual peptide elongation procedure using Fmoc-Trp(Boc)-OH (395 mg, 0.75 mmol), Fmoc-Thr(Ot-Bu)-OH (298 mg, 0.750 mmol), Fmoc-Ala-OH (233 mg, 0.750 mmol), and Fmoc-Arg(Pbf)-OH (324 mg, 0.500 mmol) as inputs afforded peptidyl Trt resin free amine 2c. Half of the latter was subjected to the TFE-mediated Trt resin cleavage procedure to ultimately afford 90 mg (MW 1012.22, 89 µmol, 71% yield) of side-chain protected peptide 3c. HPLC-MS
characterization using method “E”; retention time: 1.28 min, crude purity: 92 %, MS (ESI⁺) m/z: [M+H]⁺ Calcd for C₉₉H₇₂N₆O₁₂S 1012.5, found 1012.5.

**Intermediates 4c and 5c: cyclo-[Arg(Pbf)-Ala-Thr(t-Bu)-Trp(Boc)-Ala] with dimer**

Side chain-protected peptide 3c (26.3 mg, 26 µmol) was subjected to the peptide macrocyclization procedure using scaled amounts of NEt₃ (36 µL, 260 µmol), CH₂CN (12.5 mL), DMSO (1 mL, 7%), and DEPBT (19.4 mg, 65 µmol). Preparatory HPLC method “A” for purification ultimately afforded 7 mg (27% yield) of a mixture containing cyclic peptide 4c and dimeric side product 5c. HPLC-MS characterization using method “E”; retention time: 1.54 min, purity: >99 %, MS (ESI⁺) m/z: [4c+H]⁺ Calcd for C₉₉H₇₂N₆O₁₁S 994.5, found 994.5; and [5c+H]⁺ Calcd for C₉₇¹³CH₁₄₃N₁₃O₂₂S₂ 1990.0, found 1990.1; Calcd for C₉₈H₁₄₃N₁₈O₂₂S₂ 1989.0 found 1988.8. HRMS (ESI-TOF) m/z: [5c+H]⁺ Calcd for C₉₈H₁₄₃N₁₈O₂₂S₂ 1988.0061, found 1988.0060; [5c+Na]⁺ Calcd for C₉₈H₁₄₃N₁₈NaO₂₂S₂ 2009.9880, found 2009.9880. The 4c/5c ratio was ascertained from analysis of deprotected derivatives 1c/6c. Note that the high molecular weight of 5c causes the abundance of the ¹³C isotope to surpass its natural isotope.
Product 1c/ side-product 6c mixture: cyclo-[Arg-Ala-Thr-Trp-Ala] with dimer

The TFA-mediated deprotection procedure was conducted on the mixture containing cyclic peptide 4c and dimeric side product 5c (7 mg, 7 µmol) using scaled amounts of DTT (1 mg, 7 µmol), H₂O (53 µl), TFA (0.96 mL), and triisopropylsilane (21.6 µl, 105 µmol). The reaction time was adjusted to 1 h, and purification was achieved by manual reversed-phase chromatography to ultimately afford 3.2 mg (major component TFA salt FW: 699.7, 3 µmol, 65%) of a solid mixture containing cyclo-[argininyl-alaninyl-threoninyl-tryptophanyl-alanine] (1c) and cyclo-[argininyl-alaninyl-threoninyl-tryptophanyl-alaninyl-argininyl-alaninyl-threoninyl-tryptophanyl-alanine] (6c) in a ratio of 86:14. UHPLC-HRMS characterization using method “I”; retention time: 13.8 min, HRMS (ESI⁺) m/z: [1c+H]⁺ Calcd for C₂₇H₄₆N₉O₆ 586.3096, found 586.3164; [6c+H]⁺ Calcd for C₃₄H₇₉N₁₈O₁₂ 1171.6119, found 1171.6094. HCD MS/MS fragment count (1c): Calcd for b/y ions 35, found 30.

Mass chromatogram of the 1c/6c mixture (TIC)

Mass chromatogram after extracting 1c-ions
MS expansion illustrating the isotopic profile of 1c

Mass chromatogram after extracting 6c-ions

MS expansion illustrating the isotopic profile of 6c
**Synthesis of cyclic RCTCA**

**Intermediate 3d: H-Arg(Pbf)-Pro-Thr(t-Bu)-Cys(Trt)-Ala-OH**

The manual Trt resin loading procedure was conducted using Fmoc-Ala-OH (93 mg, 0.3 mmol) as input to afford Fmoc-alaninyl Trt resin 7a. Subsequent execution of the manual peptide elongation procedure using Fmoc-Cys(Trt)-OH (293 mg, 0.500 mmol), Fmoc-Thr(Ot-Bu)-OH (298 mg, 0.750 mmol), Fmoc-Cys(Trt)-OH (293 mg, 0.500 mmol), and Fmoc-Arg(Pbf)-OH (324 mg, 0.500 mmol) as inputs afforded peptidyl Trt resin free amine 2d. Half of the latter was subjected to the TFE-mediated Trt resin cleavage procedure to afford 144 mg (MW 1345.73, 107 µmol, 86% yield) of side-chain protected peptide 3d. HPLC-MS characterization using method “E”; retention time: 1.98 min, crude purity: 92 %, MS (ESI+) m/z: [M+H]+ Calcd for C_{74}H_{89}N_{8}O_{8}S_{3} 1345.6, found 1346.0.
Intermediate 4d and 5d: cyclo-[Arg(Pbf)-Cys(Trt)-Thr(t-Bu)-Cys(Trt)-Ala] with dimer

Side chain-protected peptide 3d (21.5 mg, 16 µmol) was subjected to the peptide macrocyclization procedure using scaled amounts of NEt₃ (22.3 µL, 260 µmol), CH₃CN (7.7 mL), DMSO (320 µL), and DEPBT (12 mg, 40 µmol). Preparatory HPLC method “A” for purification ultimately afforded 3.3 mg (16% yield) of a mixture containing cyclic peptide 4d and dimeric side product 5d. HPLC-MS characterization using method “E”; retention time: 1.80 min, MS (ESI⁺) m/z: [4d+H]⁺ Calcd for C₇₃H₇₅N₃O₅S₃ 1327.6, found 1327.2. HRMS (ESI-TOF) m/z: [4d+H]⁺ Calcd for C₇₃H₇₅N₃O₅S₃ 1327.5753, found 1327.5718. The 4d/5d ratio was ascertained from analysis of deprotected derivatives 1d/7d.
Product 1d/ side-product 6d mixture: cyclo-[Arg-Cys-Thr-Cys-Ala] with dimer

The TFA-mediated deprotection procedure was conducted on the mixture containing cyclic peptide 4d and dimeric side product 5d (3 mg, 2.3 µmol) using scaled amounts of DTT (~0.3 mg, 2.3 µmol), H₂O (17 µL), TFA (0.31 mL), and triisopropylsilane (6.9 µl, 34 µmol). Purification was achieved by manual reversed-phase chromatography to afford 0.7 mg (major component TFA salt FW: 648.68, 1.1 µmol, 48% yield) of a solid mixture containing cyclo-[argininyl-cysteinyl-threoninyl-cysteinyl-alanine] (1d) and cyclo-[argininyl-cysteinyl-threoninyl-cysteinyl-alanineyl-argininyl-cysteinyl-threoninyl-cysteinyl-alanine] (6d) in a ratio of 88:12. UHPLC-HRMS characterization using method “I”; retention time: 13.6 min, HRMS (ESI⁺) m/z: [1d+H]⁺ Calcd for C₁₉H₃₅N₈O₆S₂ 535.2115, found 535.2116; [6d+H]⁺ Calcd for C₃₆H₆₉N₁₆O₁₉S₄ 1069.4158, found 1069.4125. HCD MS/MS fragment count (1d): Calcd for b/y ions 35, found 32.

Mass chromatogram of the 1d/6d mixture (TIC)

Mass chromatogram after extracting 1d-ions
MS expansion illustrating the isotopic profile of 1d

Mass chromatogram after extracting 6d-ions

MS expansion illustrating the isotopic profile of 6d
Synthesis of cyclic RCTAA

Intermediate 3e: H-Arg(Pbf)-Cys(Trt)-Thr(t-Bu)-Ala-Ala-OH

The manual Trt resin loading procedure was conducted using Fmoc-Ala-OH (93 mg, 0.3 mmol) as input to afford Fmoc-alaninyl Trt resin 7a. Subsequent execution of the manual peptide elongation procedure using Fmoc-Ala-OH (233 mg, 0.750 mmol), Fmoc-Thr(t-Bu)-OH (298 mg, 0.750 mmol), Fmoc-Cys(Trt)-OH (293 mg, 0.500 mmol), and Fmoc-Arg(Pbf)-OH (324 mg, 0.500 mmol) as inputs afforded peptide resin free amine 2e. Half of the latter was subjected to the TFE-mediated Trt resin cleavage procedure to afford 126.6 mg (MW 1071.35, 118 µmol, 95% yield) of side-chain protected peptide 3e. HPLC-MS characterization using method “E”; retention time: 1.58 min, crude purity: 72 %, MS (ESI\(^+\)) \textit{m/z}: [M+H]\(^+\) Calcd for C\(_{35}H_{78}N_{8}O_{16}S_{2}\) 1071.5, found 1071.2.

Intermediate 4e: cyclo-[Arg(Pbf)-Cys(Trt)-Thr(t-Bu)-Ala-Ala]
Side chain-protected peptide 3e (22 mg, 21 µmol) was subjected to the peptide macrocyclization procedure using scaled amounts of NEt$_3$ (28.6 µL, 205 µmol), CH$_3$CN (9.3 mL), DMSO (933 µL), and DEPBT (15.4 mg, 51 µmol). Preparatory HPLC method “A” for purification ultimately afforded 5.1 mg (21% yield) protected cyclic peptide 4a. HPLC-MS characterization using method “F”; retention time: 1.60 min, purity: >99 %, MS (ESI$^+$) $m/z$: [M+H]$^+$ Calcd for C$_{55}$H$_{73}$N$_8$O$_9$S$_2$ 1053.5, found 1054.4. HRMS (ESI-TOF) $m/z$: [4e+H]$^+$ Calcd for C$_{55}$H$_{73}$N$_8$O$_9$S$_2$ 1053.4936, found 1054.4952; [4e+Na]$^+$ Calcd for C$_{55}$H$_{72}$NaN$_8$O$_9$S$_2$ 1057.4756, found 1075.4755; a trace of dimeric byproduct 5e was also identified: [5e+H]$^+$ Calcd for C$_{110}$H$_{145}$N$_{16}$O$_{18}$S$_4$ 2105.9834, found 2105.9866.

**Product 1e: cyclo-[Arg-Cys-Thr-Ala-Ala]**

The TFA-mediated deprotection procedure was conducted on peptide 4e (4.5 mg, 4.3 µmol) using scaled amounts of DTT (~0.6 mg, 4.3 µmol), H$_2$O (33 µL), TFA (0.58 mL), and triisopropylsilane (13.1 µL, 64 µmol). Purification was achieved by manual reversed-phase chromatography to afford 1.9 mg (TFA salt FW: 616.6, 3.1 µmol, 72% yield) of cyclo-[argininyl-cysteinyl-threoninyl-alaninyl-alanine] (1e) as a solid. UHPLC-HRMS characterization using method “I”; retention time: 13.6 min, HRMS (ESI$^+$) $m/z$: [M+H]$^+$ Calcd for C$_{19}$H$_{23}$N$_9$O$_5$S 503.2395, found 503.2390. HCD MS/MS fragment count: Calcd for b/y ions 35, found 32.
**Synthesis of cyclic WCKPIPT**

**Intermediate 3f: H-Trp(Boc)-Cys(Trt)-Lys(Boc)-Pro-Ile-Pro-Thr(O-tBu)-OH**

The automated Trt resin loading procedure was executed using a CH$_2$Cl$_2$ solution of Fmoc-Thr(t-Bu)-OH (5 mL, 0.2 M, 1 mmol) to afford Fmoc-threoninyl Trt resin 7b. Continuation of SPPS by the automated peptide elongation procedure using standard DMF solutions (5 mL, 0.2 M, 4 equiv.) of Fmoc-Pro-OH, Fmoc-Ile-OH, Fmoc-Pro-OH, Fmoc-Lys(Boc)-OH, Fmoc-Cys(Trt)-OH, and Fmoc-Trp(Boc)-OH as input.
ultimately afforded N-terminal free amine peptidyl resin 2f. The latter was subjected to the TFE-mediated Trt resin cleavage procedure using adjusted solvent (15 mL) and time (2x 1 h) quantities to afford 166 mg (FW 1342.68, 125 µmol, 50% yield) of side chain-protected peptide 3f. HPLC-MS characterization using method “G”; retention time: 2.36 min, crude purity: 95 %, MS (ESI⁺) m/z: [M+H]⁺ Calcd for C₇₃H₁₀₀N₅O₁₃S 1342.7, found 1342.7.

Intermediate 4f: cyclo-[Trp(Boc)-Cys(Trt)-Lys(Boc)-Pro-Ile-Pro-Thr(O-tBu)]

Side chain-protected peptide 3f (143 mg, 107 µmol) was subjected to the peptide macrocyclization procedure using scaled amounts of NEt₃ (178 µL, 1.28 mmol), CH₃CN (60 mL), DMSO (1-1.5 mL), and DEPB (80 mg, 266 µmol). Preparatory HPLC method “C” for purification, collecting the peak that eluted at 9.80 min, ultimately afforded 17.5 mg (MW 1324.67, 13.0 µmol, 12% yield) of protected cyclic peptide 4f. HPLC-MS characterization using method “G”; retention time: 3.41 min, purity: 98 %, MS (ESI⁺) m/z: [M+H–Boc]⁺ Calcd for C₆₈H₉₀N₇O₁₅S 1224.7, found 1224.6.

Product 1f: cyclo-[Trp-Cys-Lys-Pro-Ile-Pro-Thr]
The TFA-mediated deprotection procedure was conducted on peptide 4f (17.5 mg, 13 µmol) using scaled amounts of DTT (~1 mg, ~6.5 µmol), H₂O (55 µl), TFA (0.99 mL), and triisopropylsilane (54.1 µl, 264 µmol). Purification was achieved by preparative HPLC-MS using method “C” to ultimately afford 4.4 mg (HCl salt FW: 862.5, 3 µmol, 39% yield) of cyclo-[tryptophanyl-cysteinyl-lysyl-prolyl-isoleucyl-prolyl-threonyl] (1f) as a solid. UHPLC-HRMS characterization using method “I”; retention time: 13.6 min, HRMS (ESI⁺) m/z: [M+H]+ Calcd for C₄₀H₆₀N₀₉O₈S 826.4280, found 826.4285. HCD MS/MS fragment count: Calcd for b/y ions 77, found 56. Peak tailing in the UHPLC trace was attributed to the presence of slowly equilibrating conformational isomers.

Mass chromatogram of 1f (TIC)

Higher-energy Collisional Dissociation Tandem Mass Spectrometry (HCD MS/MS) fragmentation spectrum of 1f for sequence confirmation (For clarity, annotations for selected b and y peptide fragment ions are shown. Standard peptidic fragment ion nomenclature⁷ was adapted to cyclic peptide 1f using the illustrated arbitrary 1–7 numerical assignments for the isomeric ring-opening intermediates).
Synthesis of cyclic KCKPFKSI

Intermediate 3g: H-Lys(Boc)-Cys(Trt)-Lys(Boc)-Pro-Phe-Lys(Boc)-Ser(t-Bu)-Ile-OH

The automated Trt resin loading procedure was executed using a CH$_2$Cl$_2$ solution of Fmoc-Ile-OH (5 mL, 0.2 M, 1 mmol) to afford Fmoc-isoleucinyl Trt resin 7c. Continuation of SPPS by the automated peptide elongation procedure using standard DMF solutions (5 mL, 0.2 M, 4 equiv.) of Fmoc-Ser(t-Bu)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Phe-OH, Fmoc-Pro-OH, Fmoc-Lys(Boc)-OH, Fmoc-Cys(Trt)-OH, and Fmoc-Lys(Boc)-OH as inputs afforded N-terminal free amine peptidyl resin 2g. The latter was subjected to the TFE-mediated Trt resin cleavage procedure using an adjusted volume of TFE/CH$_2$Cl$_2$ solvent (20 mL) to ultimately afford 170 mg (MW 1548.97, 110 µmol, 44% yield) of side chain-protected peptide 3g. HPLC-
MS characterization using method “G”; retention time: 2.33 min, crude purity: 94 %, MS (ESI⁺) m/z: [M+H]⁺ Calcd for C₈₂H₁₂₂N₁₁O₁₆S 1548.9, found 1548.7.

**Intermediate 4g: cyclo-[Lys(Boc)-Cys(Trt)-Lys(Boc)-Pro-Phe-Lys(Boc)-Ser(t-Bu)-Ile]**

Side chain-protected peptide 3g (170 mg, 110 µmol, 1 equiv) was subjected to the peptide macrocyclization procedure using scaled amounts of NEt₃ (153 µL, 1.10 mmol), CH₃CN (100 mL), DMSO (1 mL), and DEPBT (65.7 mg, 220 µmol, 2 equiv). Preparatory HPLC method “B for purification ultimately afforded 36.5 mg (MW 1530.95, 23.8 µmol, 22% yield) of protected cyclic peptide 4g. HPLC-MS characterization using method “G”; retention time: 2.88 min, purity: 94 %, MS (ESI⁺) m/z: [M+H]⁺ Calcd for C₈₂H₁₂₀N₁₁O₁₅S 1530.9, found 1530.8.
The TFA-mediated deprotection procedure was conducted on peptide \(4g\) (18 mg, 12 \(\mu\)mol) using scaled amounts of DTT (~1 mg, ~6.5 \(\mu\)mol), \(H_2O\) (50 \(\mu\)l), TFA (0.88 mL), and triisopropylsilane (20 \(\mu\)l, 98 \(\mu\)mol). Purification was achieved by preparative HPLC-MS using method “D” to ultimately afford 5.5 mg (tris HCl salt FW: 1041.6, 5.3 \(\mu\)mol, 44% yield) of cyclo-[lysyl-cysteinyl-lysinyl-prolinyl-phenylalaninyl-lysyl-serinyl-isoleucine] \(1g\) as a solid. UHPLC-HRMS characterization using method “J”; retention time: 15.4 min, HRMS (ESI\(^{+}\)) \(m/z\): \([M + 2H]^{2+}\) Calcd for \(C_{44}H_{75}N_{11}O_{9}S\) 466.7730, found 466.7737. HCD MS/MS fragment count: Calcd for b/y ions 104, found 47.

Mass chromatogram of \(1g\) (TIC)

MS expansion illustrating the isotopic profile of \(1g\) in the doubly-charged ion region (singly-charged ion absent)
Synthesis of cyclic ELCPPPNNL

Intermediate 3h: H-Glu(t-Bu)-Leu-Cys(Trt)-Pro-Pro-Asn(Trt)-Leu-Leu-OH

The automated Trt resin loading procedure was executed using a CH$_2$Cl$_2$ solution of Fmoc-Leu-OH (5 mL, 0.2 M, 1 mmol) to afford Fmoc-leucinyl Trt resin 7d. Continuation of SPPS by the automated peptide elongation procedure using standard DMF solutions (5 mL, 0.2 M, 4 equiv.) of Fmoc-Leu-OH, Fmoc-Asn(Trt)-OH, Fmoc-Pro-OH, Fmoc-Pro-OH, Fmoc-Cys(Trt)-OH, Fmoc-Leu-OH, and Fmoc-Glu(t-Bu) as input afforded N-terminal free amine peptidyl resin 2h. The latter was subjected to the TFE-mediated Trt resin cleavage procedure using a scaled amount of TFE/ CH$_2$Cl$_2$ solvent (5 mL) to afford 168 mg (MW 1438.81, 117 µmol, 47% yield) of side chain-protected peptide 3h. HPLC-MS characterization using method “G”; retention time: 2.45 min, crude purity: >98 %, MS (ESI$^+$) m/z: [M+H]$^+$ Caled for C$_{82}$H$_{104}$N$_9$O$_{12}$S 1438.8, found 1439.2.
Intermediate 4h: cyclo-[Glu(O-tBu)-Leu-Cys(Trt)-Pro-Pro-Asn(Trt)-Leu-Leu]

Side chain-protected peptide 3h (168 mg, 117 µmol) was subjected to the peptide macrocyclization procedure using scaled amounts of NEt$_3$ (153 µL, 1.17 mmol), CH$_3$CN (55 mL), DMSO (2 mL), and DEPBT (69.9 mg, 234 µmol, 2 equiv). Preparatory HPLC method “B” for purification, collecting the peak that eluted at 17.99 min, ultimately afforded 44 mg (MW 1420.80, 31 µmol, 26% yield) of protected cyclic peptide 4h. HPLC-MS characterization using method “G”; retention time: 3.17 min, purity: >98%.

Product 1h: cyclo-[Glu-Leu-Cys-Pro-Pro-Asn-Leu-Leu]

The TFA-mediated deprotection procedure was conducted on peptide 4h (5 mg, 3.5 µmol), omitting the DTT and H$_2$O, using adjusted amounts of TFA (1 mL), triisopropylsilane (0.3 mL, 1.5 mmol), and 45 min as reaction time. Purification was achieved by manual reversed-phase chromatography using 50% MeOH in H$_2$O as eluent to ultimately afford 1 mg (FW: 880.1, 1 µmol, 32% yield) of cyclo-[glutamyl-leucinyl-
cysteinyl-prolinyl-prolinyl-aspariginyl-leucinyl-leucine] (1h) as a solid. UHPLC-HRMS characterization using method “J”; retention time: 63.9 min, HRMS (ESI+) m/z: [M+H]+ Calcd for C_{40}H_{66}N_{9}O_{11}S 880.4597, found 880.4603. HCD MS/MS fragment count: Calcd for b/y ions 104, found 82.

Mass chromatogram of 1h (XIC)

MS expansion illustrating the isotopic profile of 1h

Synthesis of cyclic TKPCPWI

Intermediate 3i: H-Thr(t-Bu)-Lys(Boc)-Pro-Cys(Trt)-Pro-Trp(Boc)-Ile-OH

The automated Trt resin loading procedure was executed using a CH2Cl2 solution of Fmoc-Ile-OH (5 mL, 0.2 M, 1 mmol) to afford Fmoc-isoleucinyl Trt resin 7c. Continuation of SPPS by the automated peptide elongation procedure using standard DMF solutions (5 mL, 0.2 M, 4 equiv.) of Fmoc-Trp(Boc)-OH, Fmoc-
Pro-OH, Fmoc-Cys(Trt)-OH, Fmoc-Pro-OH, Fmoc-Lys(Boc)-OH, and Fmoc-Thr(t-Bu)-OH as input afforded N-terminal free amine peptidyl resin 2i. The latter was subjected to the TFE-mediated Trt resin cleavage procedure using an adjusted volume of TFE/CH2Cl2 solvent (15 mL) to afford 148 mg (MW 1342.68, 110 µmol, 44% yield) of side chain-protected peptide 3i. HPLC-MS characterization using method “G”; retention time: 2.40 min, crude purity: 78 %, MS (ESI+) m/z: [M+H]+ Calcd for C73H100N9O13S 1342.7, found 1342.6.

**Intermediate 4i: cyclo-[Thr(t-Bu)-Lys(Boc)-Pro-Cys(Trt)-Pro-Trp(Boc)-Ile]**

A heterogeneous mixture of side chain-protected peptide 3i (16.4 mg, 12 µmol), CH3CN (19 mL), NEt3 (17 µL, 122 µmol), and DMF (1 mL) was treated with DEPBT (7.3 mg, 24 µmol, 2 equiv) and stirred at room temperature. After 24 h, the reaction was quenched with AcOH, the volume was reduced in vacuo to 1-2 mL, and the resulting DMF/CH3CN solution of the crude was purified by preparatory HPLC using method “C”. The fraction that eluted at 8.65 min was concentrated in vacuo (2-5 Torr) at 50 °C, then concentrated to dryness with assistance from multiple azeotropic coevaporations with i-PrOH to afford 4.6 mg (MW 1324.67, 3.5 µmol, 29% yield) of protected cyclic peptide 4i as a solid.
Product 1i: cyclo-[Thr-Lys-Pro-Cys-Pro-Trp-Ile]

The TFA-mediated deprotection procedure was conducted on peptide 4i (29.5 mg, 22 µmol), omitting the DTT, using adjusted amounts of H₂O (90 µL), TFA (1.66 mL), triisopropylsilane (90 µL, 445 µmol), and 30 min as reaction time. Purification was achieved by preparative HPLC-MS using method “D” to ultimately afford 7.9 mg (HCl salt FW: 862.48, 9.2 µmol, 41% yield) of cyclo-[threoninyl-leucinyl-prolinyl-cysteinyl-prolinyl-tryptophanyl-isoleucine] (1i) as a solid. UHPLC-HRMS characterization using method “I”; retention time: 14.5 min, HRMS (ESI⁺) m/z: [M+H]⁺ Calcd for C₄₀H₆₀N₀O₈S 826.4280, found 826.4257. HCD MS/MS fragment count: Calcd for b/y ions 77, found 56. Peak tailing in the UHPLC trace was attributed to the presence of slowly equilibrating conformational isomers.

Mass chromatogram of 1i (TIC)
**Synthesis of cyclic KSKPCIFK**

Intermediate 3j: H-Lys(Boc)-Ser(t-Bu)-Lys(Boc)-Pro-Cys(Trt)-Ile-Phe-Lys(Boc)-OH

Commercial H-Lys(Boc)-2-Cl-Trt Resin 7e (465 mg, 0.54 mmol/g 0.250 mmol) was employed in SPPS using the automated peptide elongation procedure with standard DMF solutions (5 mL, 0.2 M, 4 equiv.) of Fmoc-Phe-OH, Fmoc-Ile-OH, Fmoc-Cys(Trt)-OH, Fmoc-Pro-OH, Fmoc-Lys(Boc)-OH, Fmoc-Ser(t-Bu)-OH, and Fmoc-Lys(Boc)-OH as input to afford N-terminal free amine peptidyl resin 2j. The latter was subjected to the TFE-mediated Trt resin cleavage procedure using an adjusted volume of TFE/CH$_2$Cl$_2$ solvent (20 mL) to afford 231mg (MW 1548.97, 149 mmol, 60% yield) of side chain-protected peptide 3j. HPLC characterization using method “H”; retention time: 2.476 min, crude purity: 72%. HPLC-MS characterization using method “G”; retention time: 2.29 min, crude purity: 86%, MS (ESI$^+$) m/z: [M+H]$^+$ Calcd for C$_{82}$H$_{122}$N$_{11}$O$_{16}$S 1548.9, found 1548.7.
Intermediate 4j: cyclo-[Lys(Boc)-Ser(t-Bu)-Lys(Boc)-Pro-Cys(Trt)-Ile-Phe-Lys(Boc)]

Side chain-protected peptide 3j (180 mg, 116 µmol, 1 equiv) was subjected to the peptide macrocyclization procedure using adjusted amounts of NEt₃ (162 µL, 1.16 mmol, 1 equiv), CH₃CN (170 mL), DMSO (1.2 mL), and DEPBT (69.5 mg, 232 µmol, 2 equiv). Reaction quenching was achieved using a scaled amount of AcOH (17 mL) while preparatory HPLC purification was conducted according to method “E”. The fraction eluting at 8.90 min was collected to ultimately afford 64.5 mg (MW 1530.95, 42.1 µmol, 36% yield) of protected cyclic peptide 4j. MS (ESI⁺) m/z: [M+H–Boc]⁺ Calcd for C₇₇H₁₁₂N₁₁O₁₃S 1430.8, found 1430.8.

Product 1j: cyclo-[Lys-Ser-Lys-Pro-Cys-Ile-Phe-Lys]
The TFA-mediated deprotection procedure was conducted on peptide 4j (57 mg, 37 µmol) using scaled amounts of DTT (5.7 mg, 37 µmol), H₂O (0.29 mL), TFA (5.0 mL), and triisopropylsilane (114 µl, 558 µmol). Purification was achieved by preparative HPLC-MS using method “D” to ultimately afford 12.5 mg (tris HCl salt FW: 1041.57, 12.0 µmol, 32% yield) of cyclo-[lysinyl-serinyl-lysyl-prolinyl-cysteinyl-isoleucinyl-phenylalaninyl-lysine] (1j) as a solid. UHPLC-HRMS characterization using method “J”; retention time: 15.6 min, HRMS (ESI⁺) m/z: [M + 2H]²⁺ Calcd for C₄₄H₇₅N₁₁O₉S 466.7730, found 466.7730. HCD MS/MS fragment count: Calcd for b/y ions 104, found 61. Disulfide bond dimer 8j was also identified in the sample; retention time: 15.1 min.

Mass chromatogram of 1j (XIC)

40-99 ACN – 70 min
MS expansion illustrating the isotopic profile of 1j in the doubly-charged ion region (singly-charged ion absent)
**Synthesis of cyclic LEPLNPLC**

Intermediate 3k: H-Leu-Glu(t-Bu)-Pro-Leu-Asn(Trt)-Pro-Leu-Cys(Trt)-OH

The automated Trt resin loading procedure was executed using a CH$_2$Cl$_2$ solution of Fmoc-Cys(Trt)-OH (5 mL, 0.2 M, 1 mmol) to afford Fmoc-Cysteiny1 Trt resin 7f. Continuation of SPPS by the automated peptide elongation procedure using standard DMF solutions (5 mL, 0.2 M, 4 equiv.) of Fmoc-Leu-OH, Fmoc-Pro-OH, Fmoc-Asn(Trt)-OH, Fmoc-Leu-OH, Fmoc-Pro-OH, Fmoc-Asn(Trt)-OH, and Fmoc-Leu-OH as input afforded N-terminal free amine peptidyl resin 2k. The latter was subjected to the TFE-mediated Trt resin cleavage procedure to afford 155 mg (FW 1438.81, 108 µmol, 43% yield) of side chain-protected peptide 3k. HPLC-MS characterization using method “G”; retention time: 2.52 min, crude purity: 62 %, MS (ESI’): m/z: [M+H]$^+$ Calcd for C$_{82}$H$_{104}$N$_{9}$O$_{12}$S 1438.8, found 1438.6.

Intermediate 4k: cyclo-[Leu-Glu(t-Bu)-Pro-Asn(Trt)-Pro-Leu-Cys(Trt)]

Side chain-protected peptide 3k (155 mg, 108 µmol) was subjected to the peptide macrocyclization procedure using scaled amounts of NEt$_3$ (141 µL, 1.08 mmol), CH$_3$CN (50 mL), DMSO (2 mL), and DEPB (64.5 mg, 215 µmol, 2 equiv). Preparatory HPLC method “C” for purification ultimately afforded 36 mg (MW 1420.80, 25 µmol, 23% yield) of protected cyclic peptide 4k. HPLC-MS characterization using method “G”; retention time: 3.36 min, purity: >99 %. HPLC-MS, MS (ESI’): m/z: [M+H]$^+$ Calcd for C$_{82}$H$_{102}$N$_{9}$O$_{11}$S 1420.7, found 1420.6.
Product 1k: cyclo-[Leu-Glu-Pro-Leu-Asn-Pro-Leu-Cys]

The TFA-mediated deprotection procedure was conducted on peptide 4k (36 mg, 25 µmol) using scaled amounts of DTT (~1 mg, ~6.5 µmol), H₂O (0.1 mL), TFA (1.9 mL), and triisopropylsilane (104 µl, 507 µmol). Purification was achieved by preparative HPLC-MS using method “D” to ultimately afford 10.9 mg (MW 880.06, 12.4 µmol, 49% yield) of cyclo-[leucinyl-glutamyl-prolinyl-leucinyl-asparaginyl-prolinyl-leucinyl-cysteine] (1k) as a solid. UHPLC-HRMS characterization using method “I”; retention time: 14.0, 16.7 min, HRMS (ESI⁺) m/z: [M+H]⁺ Calcd for C₄₀H₆₆N₉O₁₁S 880.4597, found 880.4572. HCD MS/MS fragment count: Calcd for b/y ions 104, found 74. The presence of two broad peaks in UHPLC trace was attributed to the presence of slowly equilibrating conformational isomers.

Mass chromatogram of 1k (XIC)

MS expansion illustrating the isotopic profile of 1k
III. References

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