

Part 2:
Guanidineadditive enabled intermolecular
ortho-phthalaldehyde-amine-thiol
three-component reactions for modular
constructions

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1. General remarks on materials and methods

All commercially available amino acids and coupling reagents (purchased from Aldrich and CS Bio) 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 (CaH_2) before use. Analytical HPLC was performed on a Waters system equipped with a photodiode array detector (Waters 2996), using a Vydac 218TPTM C18 column ($5\ \mu\text{m}$, $4.6 \times 250\ \text{mm}$) at a flow rate of $0.6\ \text{mL/min}$. 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 ($1.7\ \mu\text{m}$, $130\ \text{\AA}$, $2.1 \times 50\ \text{mm}$) at a flow rate of $0.4\ \text{mL/min}$. Preparative HPLC was performed on a Waters system, using a Vydac 218TPTM C18 column ($10\ \mu\text{m}$, $22 \times 250\ \text{mm}$) at a flow rate of $10\ \text{mL/min}$ or a Vydac 218TPTM C18 column ($10\ \mu\text{m}$, $30 \times 250\ \text{mm}$) at a flow rate of $20\ \text{mL/min}$. 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. ^1H and ^{13}C NMR spectra were recorded on Bruker Avance DRX 400 FT-NMR spectrometer at $400\ \text{MHz}$ for ^1H NMR and $100\ \text{MHz}$ for ^{13}C NMR and Bruker Avance DRX 500 FTNMR spectrometer at $500\ \text{MHz}$ for ^1H NMR and $125\ \text{MHz}$ for ^{13}C NMR at 298K . The spectra were processed using TopSpin software.

U87-MG cell line was a gift from Prof. Chi Ming Che (HKU). HEK-293T cell line was a gift from Dr. Cheung, Pak Hang Peter (CUHK). Dulbecco's Modified Eagle Medium (DMEM), foetal bovine serum (FBS) and penicillin-streptomycin (P/S) were purchased from Gibco, Life Technologies Inc. Recombinant anti-integrin beta 3 antibody [ERP17507] was purchased from Abcam. Anti-rabbit IgG, HRP-linked antibody was purchased from Cell Signaling Technology. Cell Counting Kit-8 (CCK-8) was purchased from Beyotime Biotechnology. Immobilon western chemiluminescent HRP substrate was purchased from Millipore.

2. General experimental procedures

2.1 Fmoc-based Solid Phase Peptide Synthesis (SPPS)

2-chloro-trityl resin ($100\ \text{mg}$) was swollen in anhydrous CH_2Cl_2 ($3\ \text{mL}$) for $15\ \text{minutes}$ then washed with CH_2Cl_2 ($6 \times 3\ \text{mL}$). A solution of Fmoc-Xaa-COOH ($4.0\ \text{equiv.}$

relative to resin loading capacity) and DIPEA (8.0 equiv. relative to resin capacity) in anhydrous CH_2Cl_2 was gently vortexed for 30 s. The solution was then added to the resin and was shaken at room temperature for 2 h to load the first amino acid. Then the resin was washed with DMF (5 x 3 mL) and CH_2Cl_2 (5 x 3 mL) and subsequently treated with a solution of $\text{CH}_2\text{Cl}_2/\text{CH}_3\text{OH}/\text{DIPEA}$ (17:2:1, v/v/v, 5 mL) for 1 h. The resin was then washed with DMF (5 mL x 3), CH_2Cl_2 (5 mL x 3), and DMF (5 mL x 3). Finally, it was submitted to iterative peptide assembly (Fmoc-SPPS). All proteinogenic amino acids and Fmoc-Pen(Trt)-OH were commercially available, while Fmoc-Lys-Pen-OH was obtained by synthesis (Supporting information). For the coupling step, a solution of Fmoc-AA-OH (4.0 equiv. according to the resin capacity), HATU (4.0 equiv. relative to resin capacity) and DIPEA (8.0 equiv. relative to resin capacity) in DMF was gently vortexed for 30 s. The solution was then added to the resin and was shaken at room temperature for 1 hour. After each coupling cycle, the resin was washed with DMF (5 x 3 mL) and CH_2Cl_2 (5 x 3 mL).

2.2 Mild acidic cleavage to obtain side-chain protected peptides

The 2-chlorotriptyl chloride resin-bound fully protected peptide with C-terminal Gly obtained according to general procedure 2.1 was subjected to mild acidic cleavage cocktail (5-10 mL) of $\text{CH}_2\text{Cl}_2/\text{AcOH}/\text{trifluoroethanol}$ (8/1/1, v/v/v), two times for 60 min each. Followed by filtration, the resulting cleavage solutions were combined and concentrated to give crude side-chain protected peptide with a free carboxylic acid at the C-terminus.

2.3 Head-to-tail solution phase cyclization

The side-chain protected peptide obtained according to general procedure 2.2 was dissolved in DMF/ CH_2Cl_2 1:3, at a concentration of 0.3 mM. HATU (1.1 equiv.) was then added, followed by DIPEA (3.0 equiv.). The reaction was stirred from 0°C to room temperature for overnight. The reaction mixture was then concentrated under *vacuo*, and directly subjected to global deprotection according to general procedure 2.5.

2.4 Global deprotection to obtain free linear peptides

A mixture solution of TFA/ $\text{H}_2\text{O}/\text{TIPS}$ (95%/2.5%/2.5%, cocktail A) was added linear peptide obtained according to general procedure 2.1 (6 mL cleavage cocktail/ 100 mg resin). The mixture was gently agitated for 2 h at room temperature. TFA was then

blown off and crude peptide was triturated with cold diethyl ether to give a white suspension. After centrifugation, the resulting white pellet was subjected to HPLC purification.

2.5 Global deprotection to obtain free cyclic peptides

A mixture solution of TFA/H₂O/TIPS (95%/2.5%/2.5%, cocktail A) was added to the crude cyclic peptide obtained according to general procedure 2.3, at a concentration of 10 mM. The mixture was gently agitated for 2 h at room temperature. TFA was then blown off and the oily residue was triturated with cold diethyl ether to give a white suspension. After centrifugation, the resulting white pellet was subjected to HPLC purification.

2.6 Preparation of PBS buffer containing guanidine Guanidine hydrochloride (1 M to 8 M) was added to the PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, and 1.8 mM KH₂PO₄). The pH of the solution was adjusted to 7.4 by the addition of 1 M NaOH solution.

2.7 Conditions screening and intermolecular OPA-thiol-amine reaction for peptide-peptide/drug conjugation

General remarks: Stock solution of OPA (1 mg / 100 μ L DMSO) was freshly prepared before use. For the OPA-peptide, the stock solution was prepared at 1 mg / 10 μ L DMSO. For the peptide-drug conjugates, components consisting of DM1 were freshly dissolved in 3% ACN, MeOH and DMSO (of the total reaction volume) respectively before use.

Conditions screening: The additives were first dissolved in the buffer and adjusted to the desired pH before use. The thiol-carrying peptide Ac-QSQQTFSNLWRLLCQN-NH₂ (1.0 equiv.) and OPA (1.2 equiv.) were dissolved in the buffer prepared. The amine-carrying peptide Ac-QSQQTFFKNLWRLLPQN-NH₂ (1.0 equiv.) was then added and reacted in an air atmosphere. The reactions were monitored by LCMS. For the details and observation of each condition tested, please refer to the manuscript (Table 1, entries 1-11).

Testing additive-free conditions reported by Chen for OPA-amine-amine reaction¹:

Three solvent systems were prepared, including MeOH with 2 equiv. DIPEA, MeOH/H₂O co-solvent with 2 equiv. DIPEA and MeOH/PB (pH 10.0). As excess amine was used in their conditions, the thiol-carrying peptide Ac-QSQQTFSNLWRLLCQN-NH₂ was added in 1.0 equiv. and 3.0 equiv. respectively. The thiol-carrying peptide (1.0 equiv. and 3.0 equiv. respectively) and OPA (1.05 equiv.) were dissolved in the solvent prepared, at a concentration of 1 mM. The amine-carrying peptide Ac-QSQQTFKNLWRLLPQN-NH₂ (1.0 equiv.) was then added and reacted in an air atmosphere for 1 h at room temperature (rt). The reactions were monitored by LCMS.

Conditions [A]: Thiol-containing peptide (1.0 equiv.) and OPA (1.2 equiv.) were first dissolved in PBS buffer pH 7.4, at a concentration of 2 mM. The amine-containing peptide (1.0 equiv.) was then added and reacted in an air atmosphere for 1 h at rt. The crude reaction mixture was then purified by preparative reverse-phase HPLC.

Conditions [B]: Thiol-containing peptide (1.0 equiv.) and OPA (1.2 equiv.) were first dissolved in PBS buffer pH 7.4 with guanidine at a concentration of 2 mM. (The concentration of guanidine applied for the peptide-peptide conjugate models was optimized on a case-by-case basis. It ranged from 3 M to 8 M, as specified below per entry.) The amine-containing peptide (1.0 equiv.) was then added and reacted in an air atmosphere for 1 h at rt. The crude reaction mixture was then purified by preparative reverse-phase HPLC.

Conditions [C]: DM1 (1.0 equiv.) and OPA (1.2 equiv.) were first dissolved in PBS buffer pH 7.4 with 1 M guanidine, at a final concentration of 0.5 mM. The c[RGDfK] or c[RGEfK] peptide (1.5 equiv.) was then added and reacted in an air atmosphere for 1 h at rt. The crude reaction mixture was then purified by preparative reverse-phase HPLC.

Conditions [D]: DM1 (1.0 equiv.) and OPA (1.2 equiv.) were first dissolved in PBS buffer pH 7.4 with 1 M guanidine, at a final concentration of 0.5 mM. The c[RGDfK] or c[RGEfK] peptide (1.5 equiv.) was then added and reacted in an air atmosphere for 1 h at rt. Afterwards, guanidine was added to the crude reaction mixture to achieve an 8 M guanidine buffer, followed by the addition of DMAC (2.0 equiv.) and reacted for another 1 h. The crude reaction mixture was purified by preparative reverse-phase HPLC.

Conditions [E]: c[RGDfPen] or c[RGEfPen] peptide (1.0 equiv.) and OPA (1.2 equiv.)

were first dissolved in PBS buffer pH 7.4 with 6 M guanidine, at a concentration of 5 mM. H₂N-CH₂CH₂-S-S-DM1 (1.0 equiv.) was then added and reacted in an air atmosphere for 3 h at rt. The crude reaction mixture was then purified by preparative reverse-phase HPLC.

Conditions [F]: c[RGDf(K-Pen)] (2.0 equiv.) and OPA-peptide (1.2 equiv.) were first dissolved in PBS buffer pH 7.4 with 6 M guanidine, at a concentration of 1 mM. H₂N-CH₂CH₂-S-S-DM1 (1.0 equiv.) was then added. The reaction was carried out at rt in an air atmosphere for 2 h. The crude reaction mixture was then purified by preparative reverse-phase HPLC.

Typical procedure for the OPA-amine-thiol conjugation: Taking **PPC-2** as an example, the thiol-containing fragment (2.02 mg, 0.0016 mmol, 1.0 equiv.) and OPA (0.26 mg, 26 μ L stock solution in DMSO, 0.0019 mmol, 1.2 equiv.) were first dissolved in PBS buffer pH 7.4 with 3 M guanidine, at a concentration of 2 mM (800 μ L). The amine-containing fragment (0.97 mg, 0.0016 mmol, 1.0 equiv.) was then added and reacted in an air atmosphere for 1 h at rt. The crude reaction mixture was then purified by preparative reverse-phase HPLC (H₂O and ACN with 0.1% TFA as eluents) to give **PPC-2** as a white powder after lyophilization (1.57 mg, 50% yield). The peptide-drug conjugates were constructed according to the similar procedure with details listed in conditions [C] to [F].

2.8 Cell culture

U87-MG and HEK-293T cells were cultured in DMEM supplemented with 10% FBS, and 100U/mL P/S in a humidified incubator with 5% carbon dioxide at 37°C.

2.9 Western blot analysis

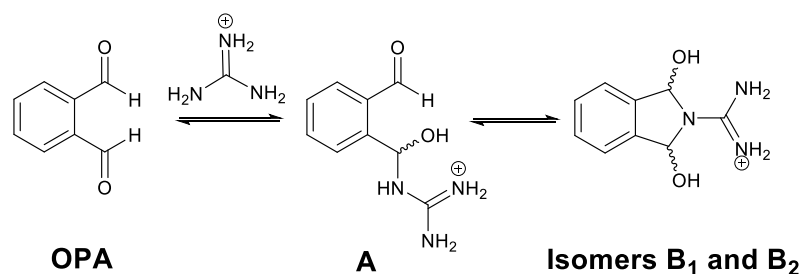
U87-MG and HEK-293T cells were washed with PBS buffer for two times and incubated with RIPA buffer for 20 minutes at 4°C, followed by centrifugation at 16000 g for 20 minutes. The supernatant was then loaded and separated by SDS-PAGE on 10% SDS-polyacrylamide gels before being transferred to PVDF membranes. The membranes were first incubated with Tris-buffered saline containing 0.1% (v/v) Tween-20 (TBST) with 5% (w/v) nonfat milk for 1 h to block the non-specific binding. Then, the membranes were washed with TBST for 3 times and incubated with recombinant anti-integrin β 3 antibody at 1:1000 dilution in 5% nonfat milk in TBST for overnight

at 4°C. The membranes were then incubated with Cell Signaling Technology, anti-rabbit IgG, HRP-linked antibody, at 1:10000 dilution in TBST for 1 h, developed using immobilon western chemiluminescent HRP substrate and imaged using ChemiDoc. The image was analyzed using the Image Lab Software. The intensity of beta-actin in each cell was set to 1.0 as an internal reference. The intensities of integrin beta 3 referenced to the beta-actin were shown in red.

2.10 *In vitro* cytotoxicity assay

Cells were plated in 96-well microplates at a density of 5×10^4 cells/well for 24 h for attachment, 90 μL of DMEM with 10% FBS and 100U/mL P/S was added to each well. PDCs 1-6 were first dissolved in DMSO respectively, at a concentration of 10 mM, followed by serial dilution in culture medium to achieve different concentrations as specified. For U87-MG cells, the concentration of compounds administered ranged from 1.37 to 27000 nM. While for HEK-293T cells, the concentration of compounds administered ranged from 0.03 to 1000 nM. The cells treated with the compounds were then incubated for 48 h. 10 μL of CCK-8 solution was then added to each well and incubated for 30 min. Absorbance was acquired at 450 nm using a spectrophotometer (Synergy HTX, Biotek). Three experiments were performed separately in triplicates and the data was processed using the GraphPad Prism (GraphPad Software, La Jolla, CA).

3. Mechanistic study



OPA (8.13mg, 0.0607 mmol) was added to PBS buffer pH 7.4 with 1 M guanidine at a concentration of 2 mM (30.34 mL), reacted for 15 min at rt. The crude reaction mixture was purified by preparative reverse-phase HPLC (2-20% $\text{CH}_3\text{CN}/\text{H}_2\text{O}$ over 35 min) and lyophilized to afford intermediates B₁ and B₂ as a mixture of two isomers (6.4 mg,

54% yield). ^1H NMR (500 MHz, DMSO) of isomer B₁ δ 7.69 (3H, bp), 7.45-7.52 (4H, m), 7.00-7.02 (2H, d, J = 9.98 Hz), 6.40-6.42 (2H, d, J = 9.98 Hz), ppm; ^1H NMR (500 MHz, DMSO) of isomer B₂ δ 7.69 (3H, bp), 7.45-7.52 (4H, m), 6.97-6.99 (2H, d, J = 9.27 Hz), 6.22-6.24 (2H, d, J = 9.63 Hz); ^{13}C NMR (125 MHz, DMSO), δ 159.7, 159.4, 156.2, 155.9, 138.7, 130.4, 130.2, 124.2, 118.8, 116.4, 84.0, 83.3 ppm. HRMS (ESI⁺) calcd. for C₉H₁₁N₃O₂ (+) [M+H]⁺ 194.0851, found 194.0824.

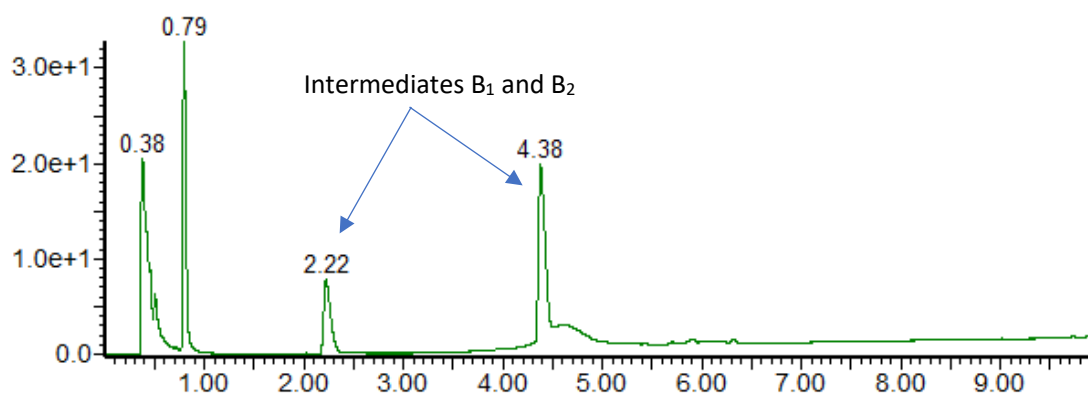


Figure S1: UV trace from LC-MS analysis of the crude reaction mixture. Gradient: 1-30% ACN/H₂O with 0.1% TFA over 10 min at a flow rate of 0.4 mL/min.

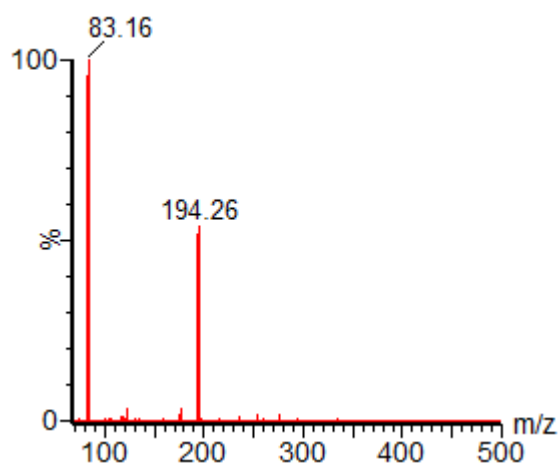
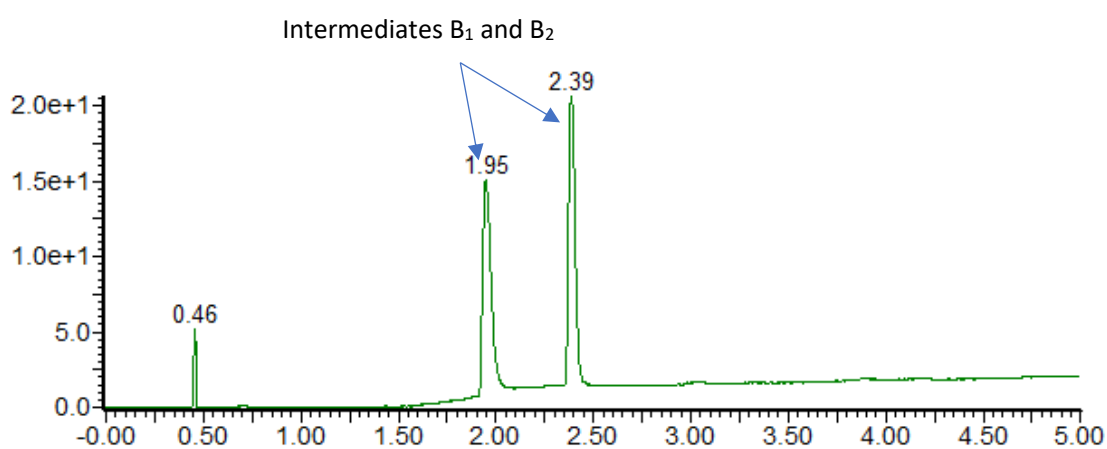
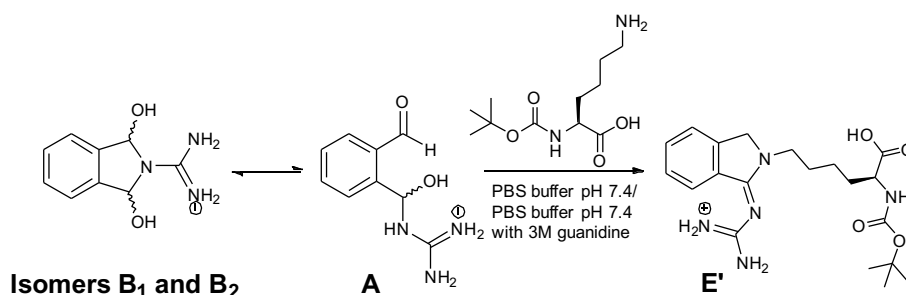


Figure S2: UV trace and corresponding MS trace from LC-MS analysis of the purified intermediates **B₁** and **B₂** as a mixture of two isomers. Gradient: 2-50% ACN/H₂O with 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. ESI-MS calcd. for C₉H₁₁N₃O₂ = 193.21; [M+H]⁺ m/z = 194.21, found 194.26.



No guanidine conditions:

Intermediates **B₁** and **B₂** (3.0 mg, 0.0155 mmol) were added to Boc-Lys-OH (4.8 mg, 0.0139 mmol) in PBS buffer pH 7.4 at a concentration of 2 mM (6.95 mL) and reacted for 30 min. The reaction was checked by LC-MS.

Guanidine-added condition:

Intermediates **B₁** and **B₂** (3.0 mg, 0.0155 mmol) were added to Boc-Lys-OH (4.8 mg, 0.0139 mmol) in PBS buffer pH 7.4 with 3 M guanidine, at a concentration of 2 mM (6.95 mL) and reacted for 30 min. The reaction was checked by LC-MS. The crude reaction mixture was then purified by preparative reverse-phase HPLC (15-70% CH₃CN/H₂O over 35 min) and lyophilized to afford compound **E'** (0.7 mg, 13% yield). This reaction was repeated to accumulate enough material for the NMR experiment. ¹H NMR (500 MHz, DMSO), δ 7.97 (1H, s), 7.74-7.76 (1H, d, J = 7.6 Hz), 7.64-7.70 (2H, m), 7.59 (1H, s), 7.52-7.55 (1H, d, J = 7.7 Hz), 7.06-7.98 (1H, d, J = 8.1 Hz), 6.94 (2H, s), 4.73 (2H, s), 3.87-3.88 (1H, m), 3.60-3.62 (2H, m), 1.59-1.72 (4H, m), 1.36 (9H, s & 2H, m) ppm; ¹³C NMR (125 MHz, DMSO), δ 174.7, 163.0, 156.2, 142.7, 132.1, 130.3, 128.4, 124.8, 124.2, 78.6, 53.7, 53.4, 44.4, 30.9, 28.6, 27.1, 23.4 ppm. HRMS (ESI⁺) calcd. for C₂₀H₂₉N₅O₄ (+) [M+H]⁺ 404.2220, found 404.2292.

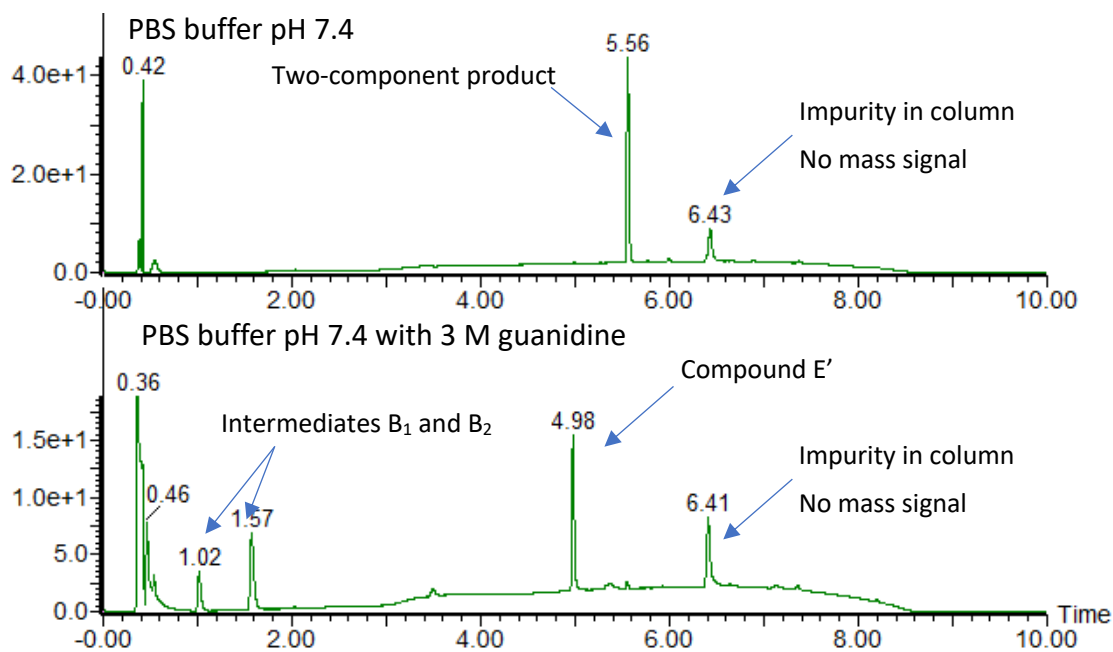


Figure S3: UV trace from LC-MS analysis of the crude reaction mixture. Gradient: 5-95% ACN/H₂O with 0.1% TFA over 10 min at a flow rate of 0.4 mL/min.

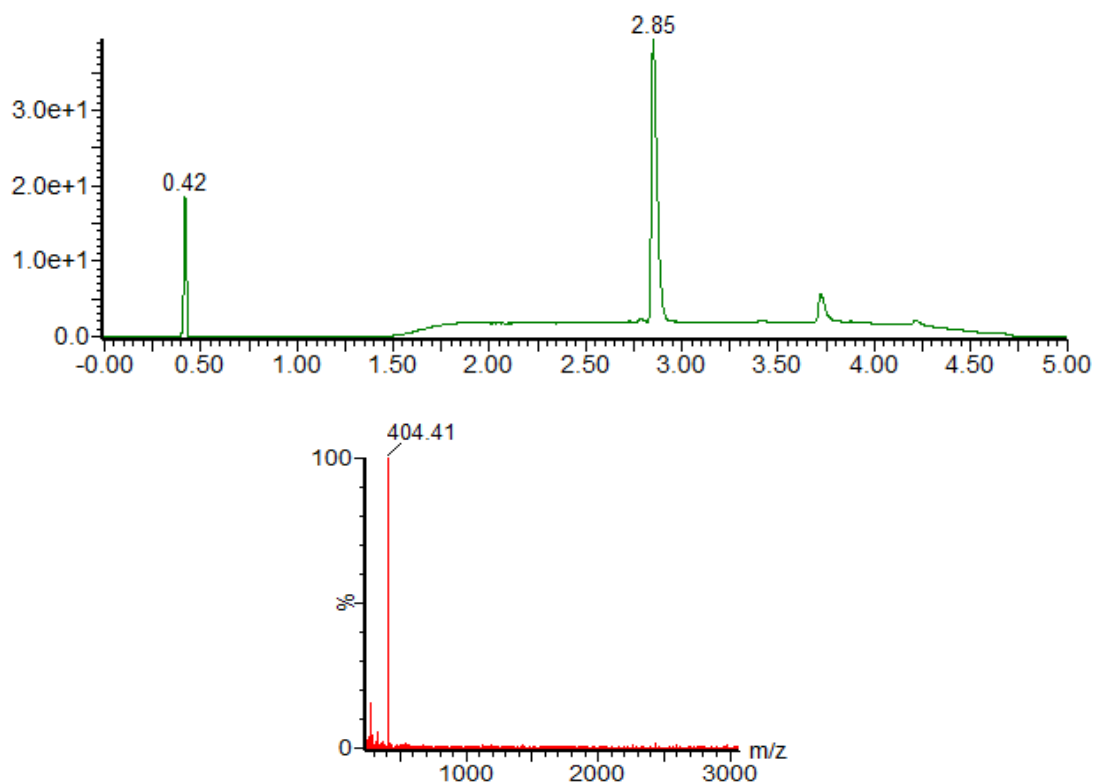


Figure S4: UV trace and corresponding MS trace from LC-MS analysis of the purified compound E'. Gradient: 5-95% ACN/H₂O with 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. ESI-MS calcd. for C₂₀H₂₉N₅O₄ = 403.48; [M+H]⁺ m/z = 404.48, found 404.41.

The guanidine adduct formation was only observed under high concentration (1 M-6 M) of guanidine added. No adduct was observed when OPA was mixed with the peptide substrate carrying the guanidium group (Arg) under low concentration (0.5 – 2 mM). Three parallel experiments were performed. The Arg-containing peptide Ac-AELRVTERARQ-NH₂ (0.12mg, 0.088 μ mol for each batch) was dissolved in PBS buffer pH 7.4 at a concentration of 0.5 mM, 1 mM and 2 mM respectively. OPA (0.014mg, 0.11 μ mol) was added to each peptide solution and reacted for 30 min at rt. The crude reaction mixture in each case was checked by LC-MS and no reaction was observed.

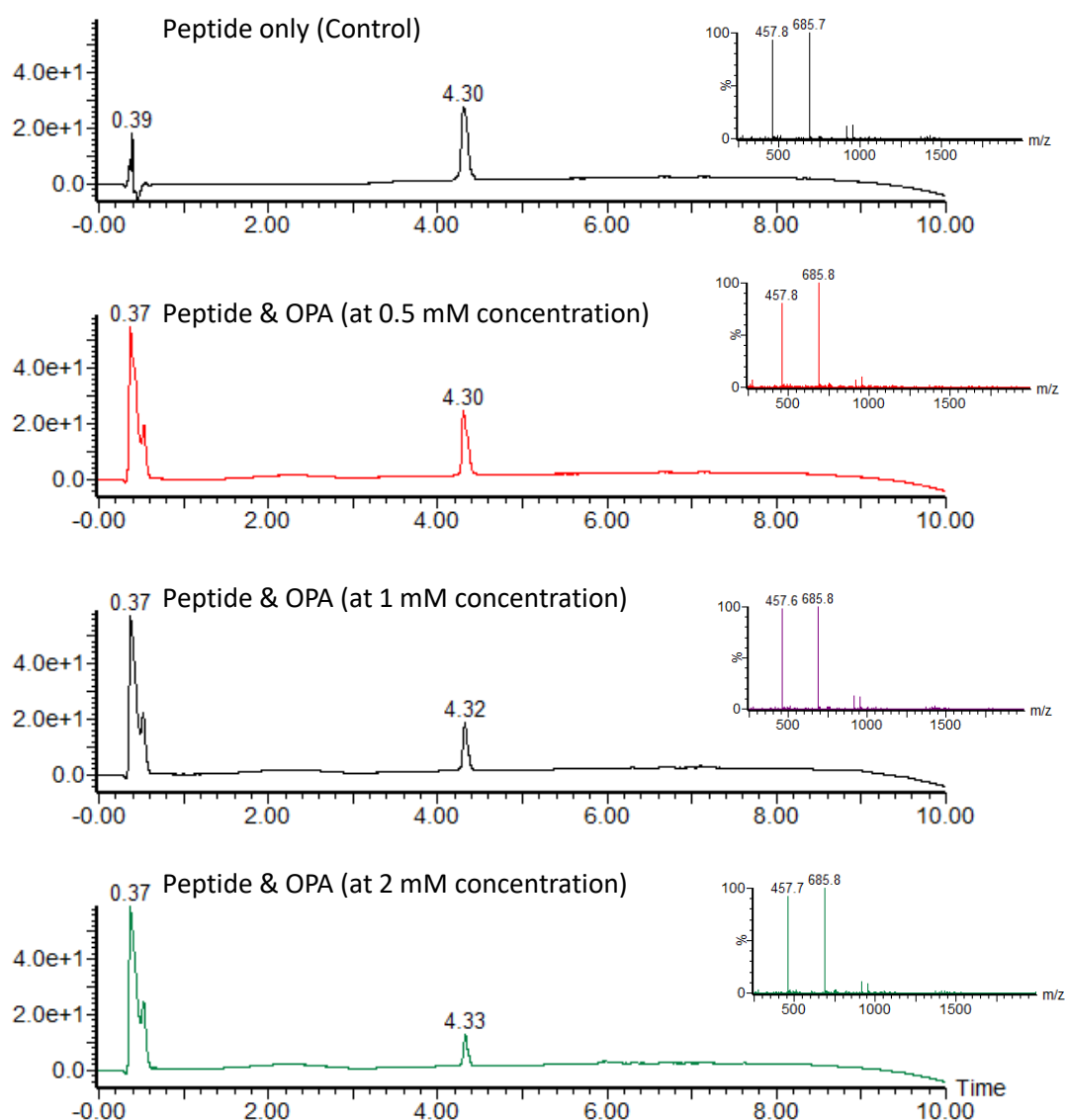
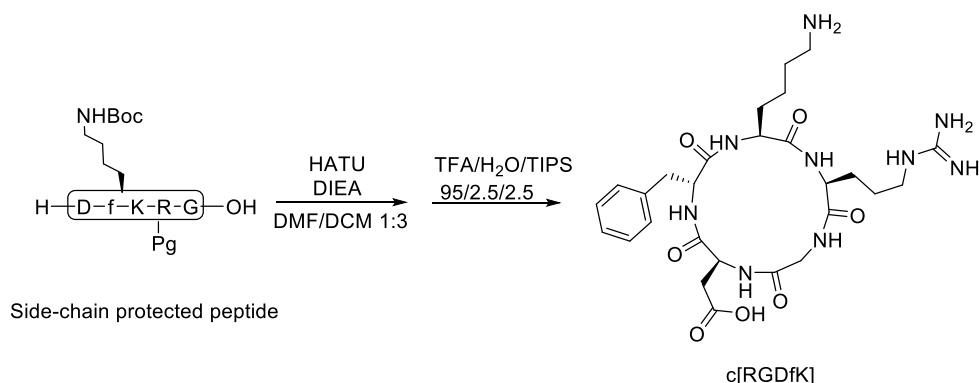


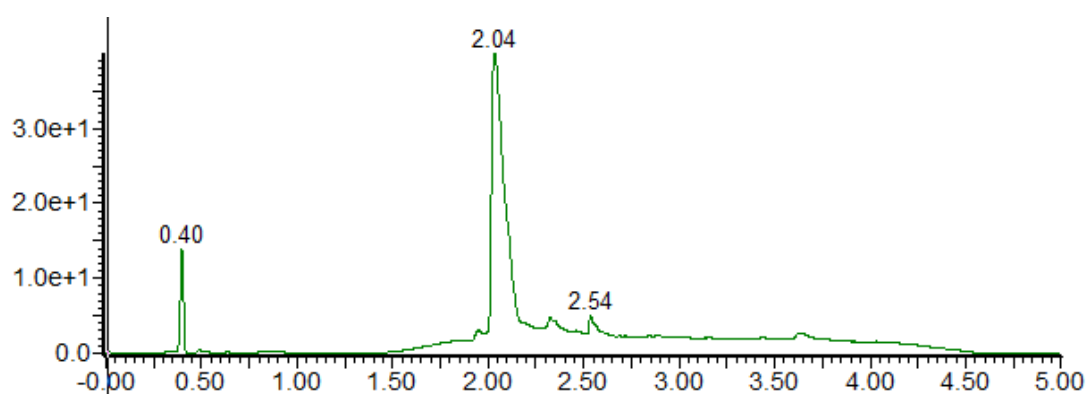
Figure S5: UV trace and corresponding MS trace from LC-MS analysis of the crude reaction mixture. Gradient: 5-95% ACN/H₂O with 0.1% TFA over 10 min at a flow rate of 0.4 mL/min.

4. Synthesis of building blocks

4.1 Synthesis of *c*[RGDfK] and *c*[RGEfK] peptide



The linear peptide H-DfKRG-OH was obtained via Fmoc-SPPS according to general procedure 2.1, and subjected to mild acidic cleavage according to general procedure 2.2 to afford the side-chain protected peptide (84 mg, 88% yield based on the resin loading). The side-chain protected peptide (84.0 mg, 0.088 mmol) was then subjected to head-to-tail solution phase cyclization according to general procedure 2.3. After the reaction was completed, the reaction mixture was concentrated under *vacuo*. The residue was then diluted with EtOAc (100 mL), washed with 1N HCl (50 mL), NaHCO₃ (50 mL x2) and brine (50 mL x2). The combined organic layer was dried over Na₂SO₄ and concentrated under *vacuo*. The crude cyclic peptide was then subjected to global deprotection according to general procedure 2.5. The resulting white pallet was purified by preparative reverse-phase HPLC (10-20% CH₃CN/H₂O over 35 min) and lyophilized to afford the *c*[RGDfK] peptide (23.4 mg, 44% yield).



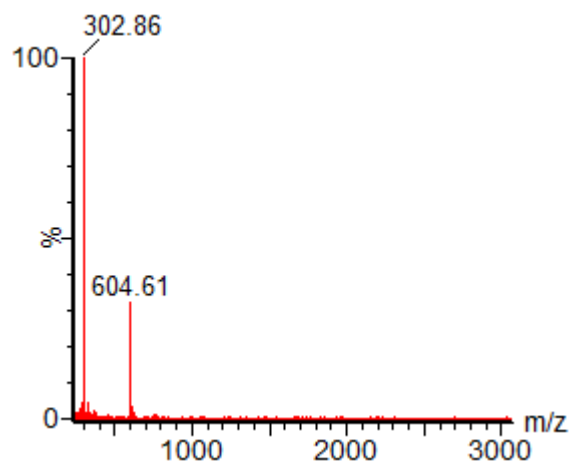


Figure S6: UV trace and corresponding MS trace from LC-MS analysis of the crude cyclization reaction mixture. Gradient: 5-95% ACN/H₂O with 0.1% TFA over 5 min at a flow rate of 0.4 mL/min.

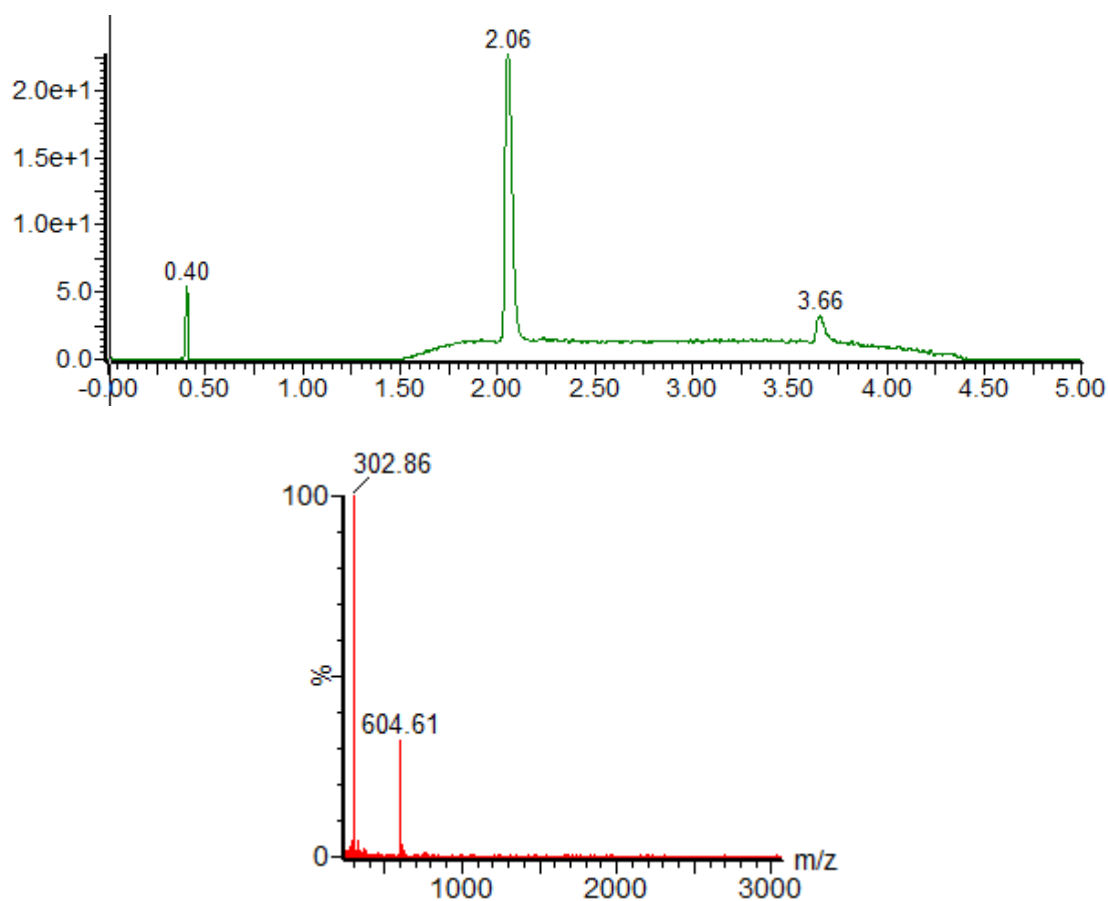
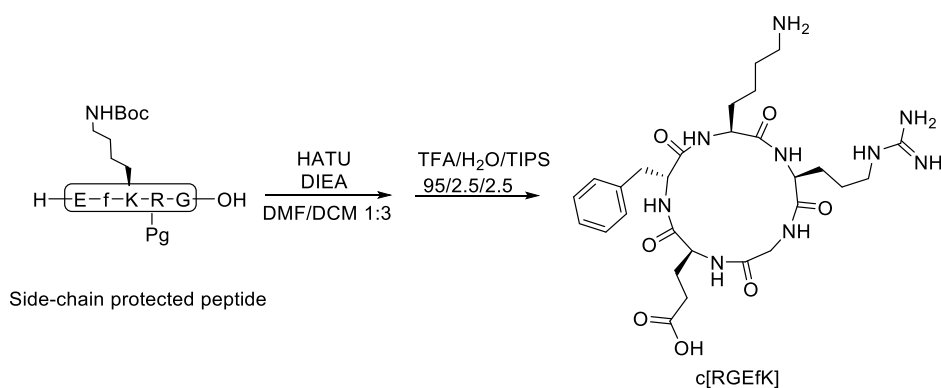


Figure S7: UV trace and corresponding MS trace from LC-MS analysis of the purified **c[RGDfK] peptide**. Gradient: 5-95% ACN/H₂O with 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. ESI-MS calcd. for C₂₇H₄₁N₉O₇ = 603.68; [M+H]⁺ m/z = 604.68, found 604.61.



c[RGEfK] peptide was synthesized according to the procedure above. The crude cyclic peptide was used for the OPA reaction.

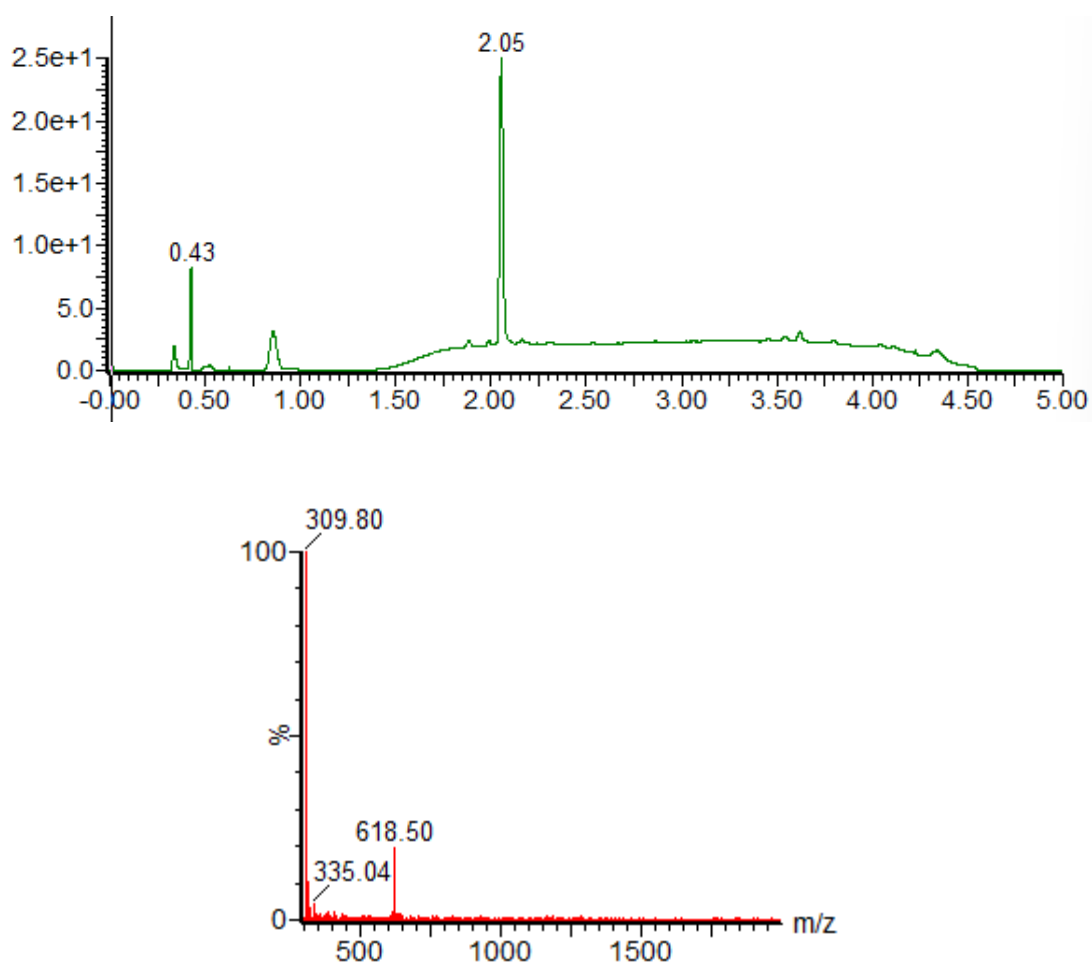
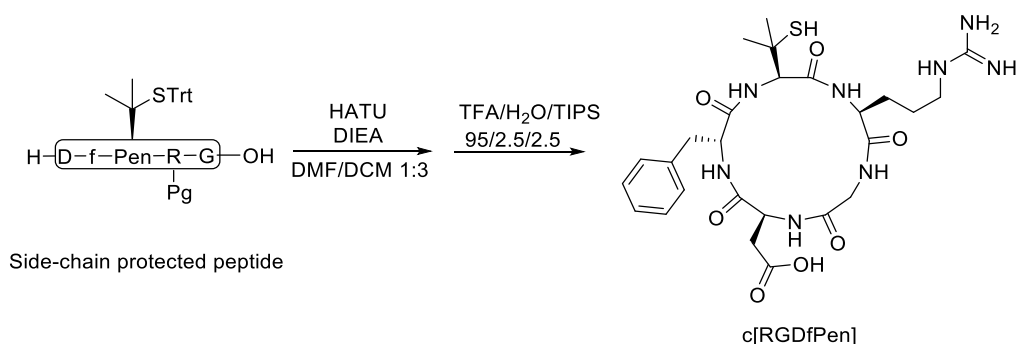
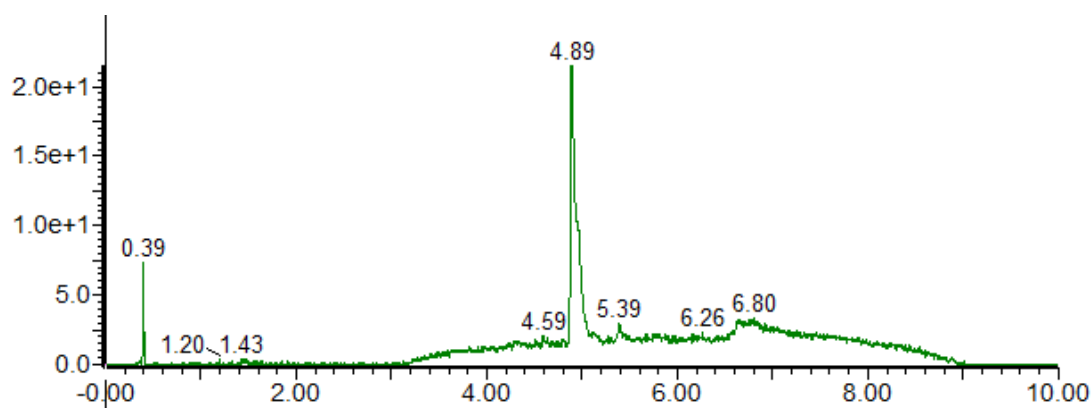


Figure S8: UV trace and corresponding MS trace from LC-MS analysis of the crude **c[RGEfK]** peptide. Gradient: 5-95% ACN/H₂O with 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. ESI-MS calcd. for C₂₈H₄₃N₉O₇ = 617.71; [M+H]⁺ m/z = 618.71, found 618.50.

4.2 Synthesis of *c*[RGDfPen] and *c*[RGEfPen] peptide



The linear peptide H-DfPenRG-OH was obtained via Fmoc-SPPS according to general procedure 2.1, and subjected to mild acidic cleavage according to general procedure 2.2 to afford the side-chain protected peptide (159 mg, 90% yield based on the resin loading). The side-chain protected peptide (74.5 mg, 0.065 mmol) was then subjected to head-to-tail solution phase cyclization according to general procedure 2.3. After the reaction was completed, the reaction mixture was concentrated under *vacuo*. The residue was then diluted with EtOAc (60 mL), washed with 1N HCl (30 mL), NaHCO₃ (30 mL x2) and brine (30 mL x2). The combined organic layer was then dried over Na₂SO₄ and concentrated under *vacuo*. The crude cyclic peptide was then subjected to global deprotection according to general procedure 2.5. The resulting white pallet was purified by preparative reverse-phase HPLC (15-50% CH₃CN/H₂O over 35 min) and lyophilized to afford the *c*[RGDfPen] peptide (18.4 mg, 46% yield).



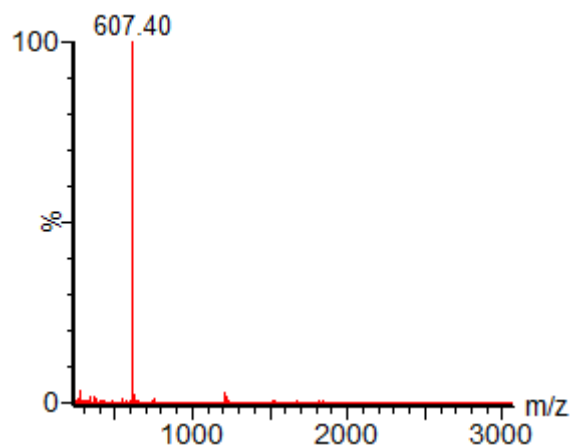


Figure S9: UV trace and corresponding MS trace from LC-MS analysis of the crude cyclization reaction mixture. Gradient: 5-95% ACN/H₂O with 0.1% TFA over 10 min at a flow rate of 0.4 mL/min.

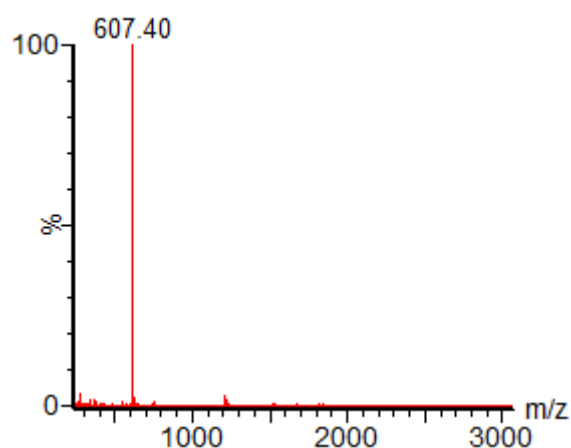
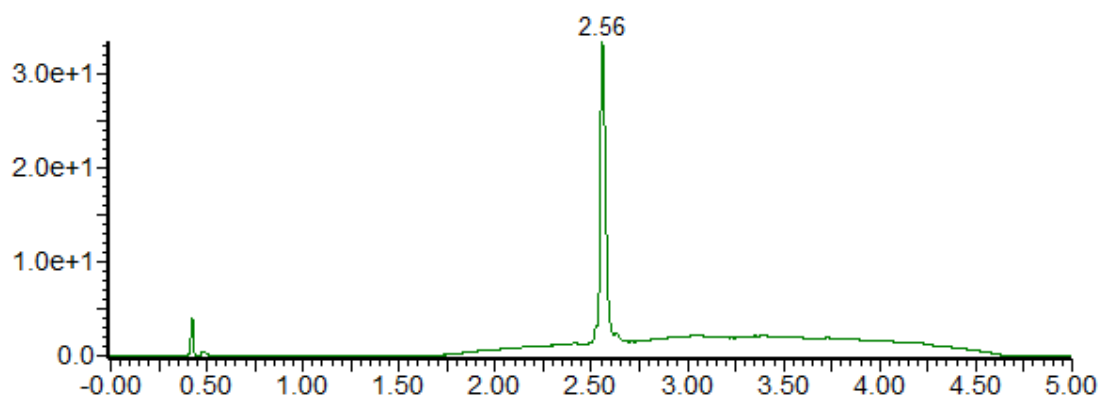
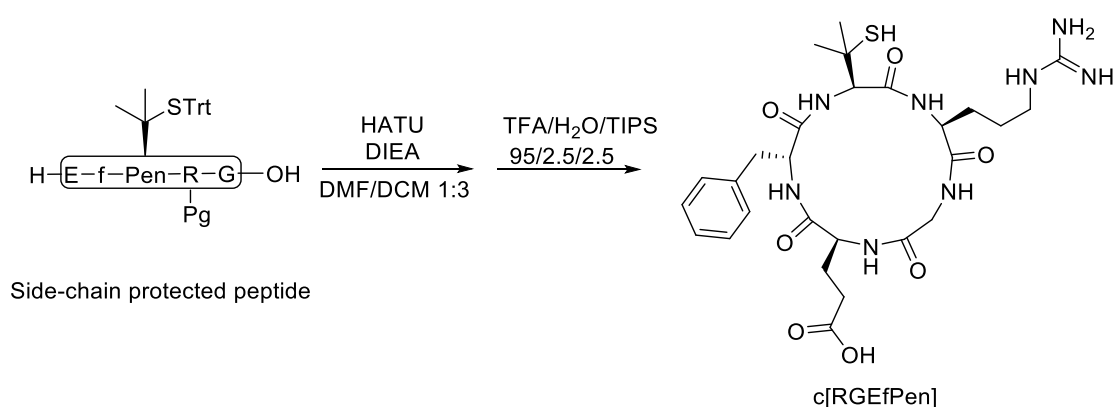


Figure S10: UV trace and corresponding MS trace from LC-MS analysis of the purified **c[RGDfPen]** peptide. Gradient: 5-95% ACN/H₂O with 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. ESI-MS calcd. for C₂₆H₃₈N₈O₇S = 606.70; [M+H]⁺ m/z = 607.70, found 607.40.



c[RGEfPen] peptide was synthesized according to the procedure above. The crude cyclic peptide was used for OPA reaction.

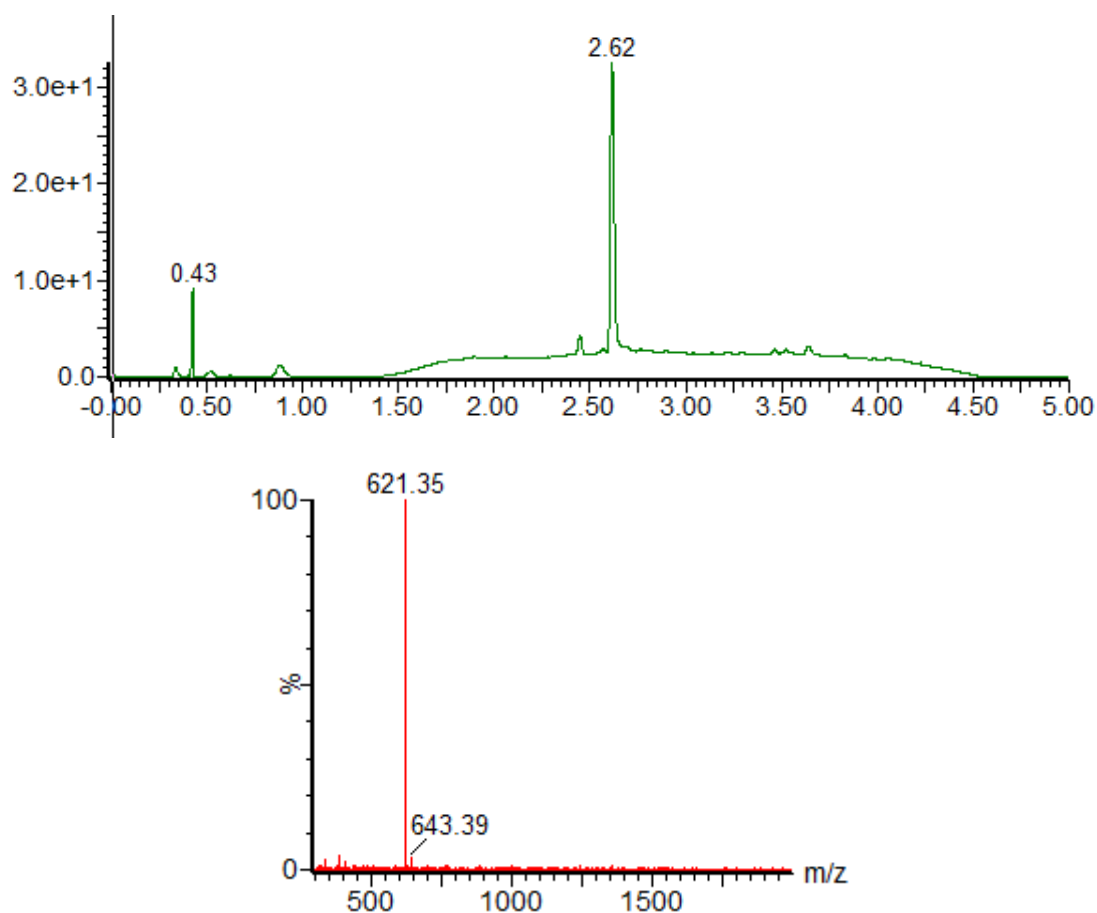
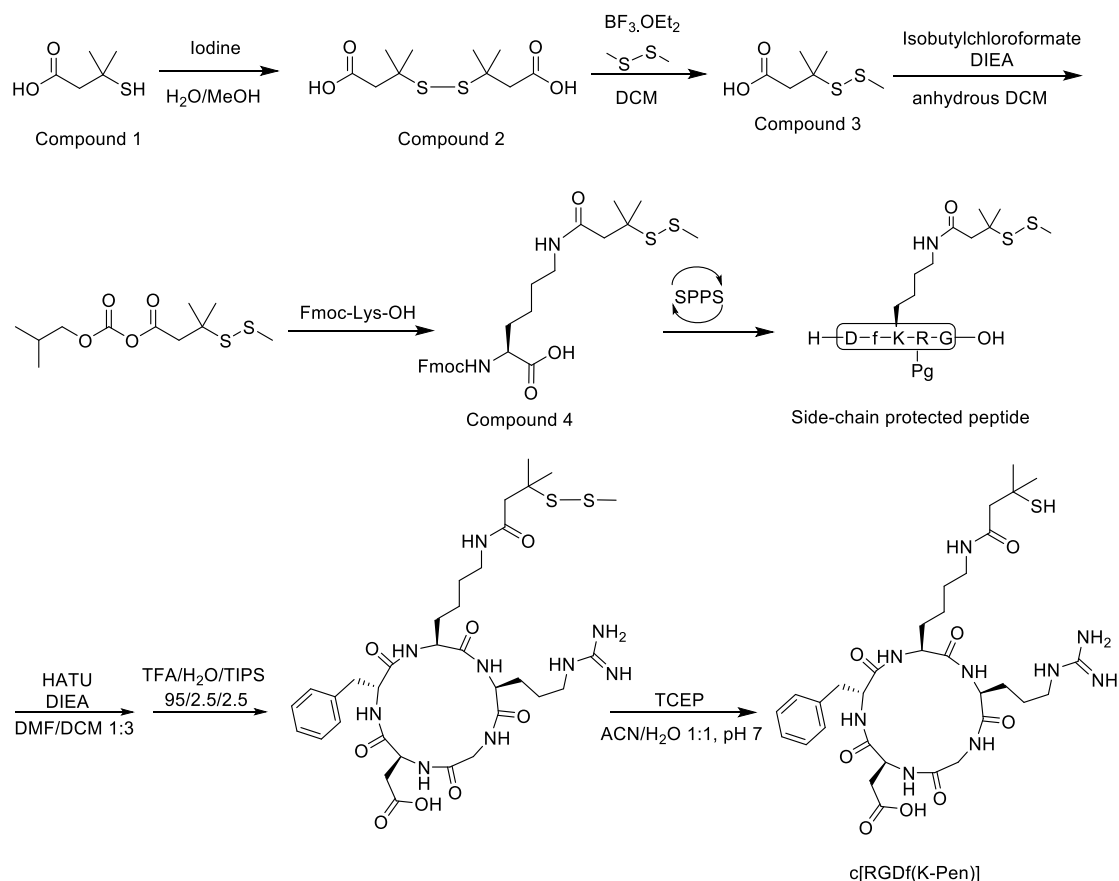


Figure S11: UV trace and corresponding MS trace from LC-MS analysis of the crude c[RGEfPen] peptide. Gradient: 5-95% ACN/H₂O with 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. ESI-MS calcd. for C₂₇H₄₀N₈O₇S = 620.73; [M+H]⁺ m/z = 621.73, found 621.35.

4.3 Synthesis of c[RGDf(K-Pen)] peptide



Compound **1** was synthesized according to a patent.² Compound **3** was synthesized with reference to a method reported previously.³ To a stirred, biphasic solution of compound **1** (200 mg, 1.49 mmol) in H₂O (960 μ L), a solution of iodine (221.2 mg, 0.88 mmol) in methanol (1318 μ L) was added dropwise. When the iodine colour persisted, the solution was diluted with EtOAc (50 mL) and washed with H₂O (50 mL). The aqueous layer was removed and extracted with an additional portion of EtOAc (50 mL). The combined organic phase was washed with brine (50 mL) and dried over Na₂SO₄, then concentrated under *vacuo* to afford the crude compound **2** (225 mg containing iodine).

To a stirred solution of the disulfide compound **2** (225 mg, 0.846 mmol) in CH₂Cl₂ (5.3 mL), methyldisulfide (1.5 mL, 16.92 mmol) was added, followed by BF₃OEt₂ (2.4 mL, 16.92 mmol). The reaction was stirred at room temperature for 4 h and quenched by the addition of NaHCO₃ (2.84 g, 33.81 mmol). The reaction mixture was then diluted with CH₂Cl₂ (20 mL), acidified by 1N HCl until pH = 1, and washed with brine (20 mL). The aqueous layer was removed and extracted with an additional portion of CH₂Cl₂ (20 mL). The combined organic phase was dried over Na₂SO₄, concentrated under *vacuo* and purified by silica gel chromatography (DCM/EtOAc = 50:0.5) to afford compound **3** as yellowish oil (138 mg, 91% yield). ¹H NMR (400 MHz, CDCl₃), δ 2.71 (2H, s), 2.46 (3H, s), 1.49 (6H, s). ¹³C NMR (100 MHz, CDCl₃), δ 177.13, 48.78, 45.87, 27.31, 25.31

ppm. HRMS (ESI⁺) calcd. for C₆H₁₂O₂S₂ (+) [M+Na]⁺ 203.0278, found 203.0292.

For the synthesis of Fmoc-K-Pen-OH, compound **3** (114 mg, 0.63 mmol) was first dissolved in 8 mL anhydrous CH₂Cl₂ under argon protection, in an ice bath. Isobutyl chloroformate (91 μ L, 0.69 mmol) was then added slowly to the solution, followed by the dropwise addition of DIPEA (496 μ L, 2.8 mmol). The reaction mixture was stirred for 1.5 h, from 0°C to room temperature. Fmoc-Lys-OH (296 mg, 0.76 mmol) was then added and reacted for 2 h. After the reaction was completed, the mixture was diluted with EtOAc (20 mL), washed with 1N HCl (5 mL x1) and brine (20 mL x2). The organic phase was dried over Na₂SO₄, concentrated under *vacuo* and purified by silica gel chromatography (n-hexane/EtOAc = 1:1, 0.5% AcOH) to afford compound **4** as white solid (207 mg, 62% yield). ¹H NMR (400 MHz, CDCl₃), δ 8.26 (1H, s), 7.73-7.76 (2H, d, J = 7.3 Hz), 7.60-7.63 (2H, t, J = 7.6 Hz), 7.37-7.41 (2H, t, J = 7.3 Hz), 7.28-7.32 (2H, t, J = 7.3 Hz), 6.34 (1H, s), 5.88-5.90 (1H, d, J = 8.1 Hz), 4.36-4.39 (2H, m), 4.19-4.23 (1H, t, J = 7.3 Hz), 3.23-3.25 (2H, m), 2.51 (2H, s), 2.44 (1H, s), 2.38 (2H, s), 1.91-1.93 (1H, m), 1.75-1.78 (1H, m), 1.51-1.54 (2H, m), 1.42 (6H, s), 1.29 (1H, s), 0.90-0.93 (1H, m) ppm; ¹³C NMR (100 MHz, CDCl₃), 174.9, 170.9, 156.3, 143.8, 143.6, 141.1, 127.6, 127.0, 125.1, 125.1, 119.9, 67.0, 53.5, 49.7, 48.3, 47.6, 47.0, 39.2, 31.8, 31.5, 28.7, 27.7, 25.2, 22.5, 22.3, 22.0, 14.0 ppm. HRMS (ESI⁺) calcd. for C₂₇H₃₄N₂O₅S₂ (+) [M+Na]⁺ 531.1909, found 531.1981.

The linear peptide H-Df(K-Pen)RG-OH was obtained via Fmoc-SPPS according to general procedure 2.1, and subjected to mild acidic cleavage according to general procedure 2.2 to afford the side-chain protected peptide (194 mg, 79% yield based on the resin loading). The side-chain protected peptide (194 mg, 0.18 mmol) was then subjected to head-to-tail solution phase cyclization according to general procedure 2.3. After the reaction was completed, the reaction mixture was concentrated under *vacuo* and subjected to global deprotection according to general procedure 2.5. Subsequently, the crude deprotected peptide was lyophilized. The crude peptide (107 mg, 77% yield) was aliquoted into batches. One batch (25 mg, 0.033 mmol) was dissolved in ACN/H₂O 1:1 with 82mM TCEP, pH = 7, at a concentration of 2.5 mM and reacted for 2 h. The crude reaction mixture was purified by preparative reverse-phase HPLC (15-50% CH₃CN/H₂O over 35 min) and lyophilized to afford c[RGDf(K-Pen)] (14.2 mg, 60% yield).

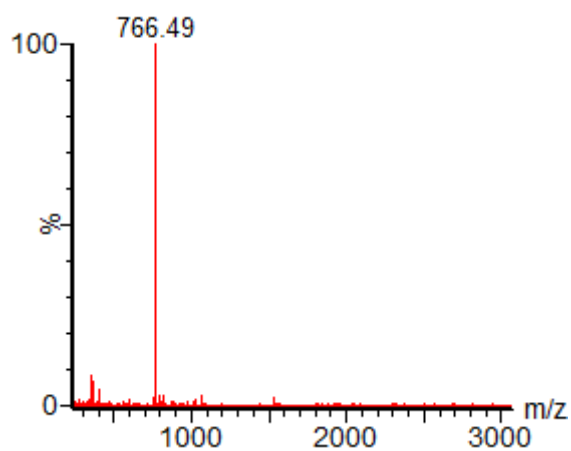
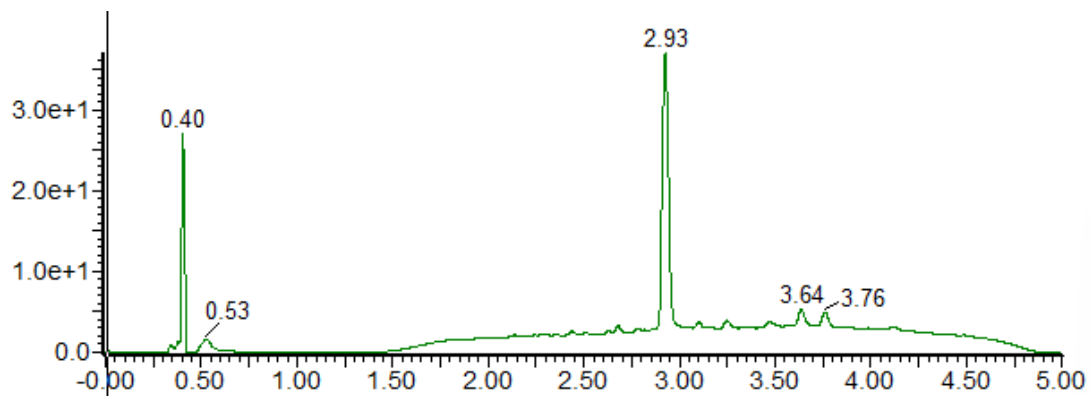
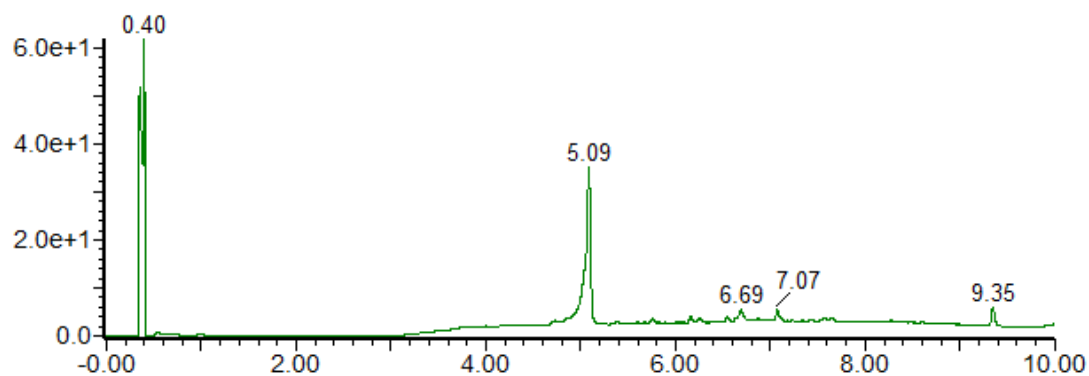


Figure S12: UV trace and corresponding MS trace from LC-MS analysis of the crude cyclization reaction mixture. Gradient: 5-95% ACN/H₂O with 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. ESI-MS calcd. for C₃₃H₅₁N₉O₈S₂ = 765.95; [M+H]⁺ m/z = 766.95, found 766.49.



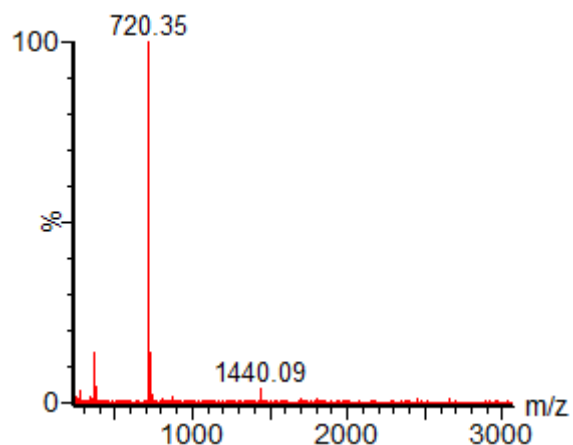


Figure S13: UV trace and corresponding MS trace from LC-MS analysis of the crude SMe deprotection reaction mixture. Gradient: 5-95% ACN/H₂O with 0.1% TFA over 10 min at a flow rate of 0.4 mL/min.

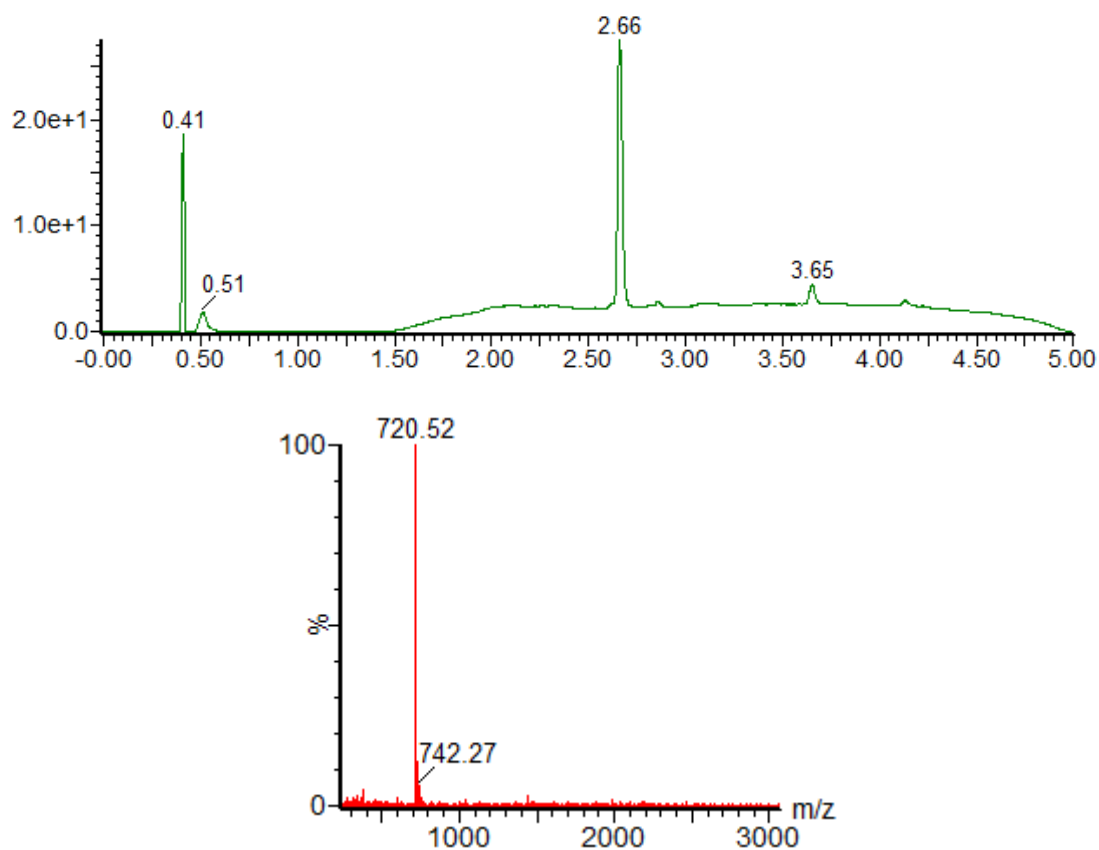
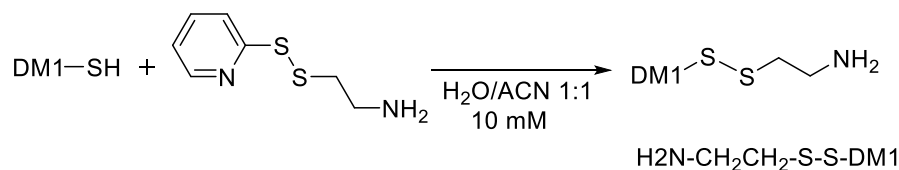


Figure S14: UV trace and corresponding MS trace from LC-MS analysis of the purified **c[RGDf(K-Pen)] peptide**. Gradient: 5-95% ACN/H₂O with 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. ESI-MS calcd. for C₃₂H₄₉N₉O₈S = 719.86; [M+H]⁺ m/z = 720.86, found 720.52.

4.4 Synthesis of $H_2N-CH_2CH_2-S-S-DM1$



2-aminoethyl-2-pyridyldisulfide was prepared according to the reported procedure.⁴ DM1 (66.0 mg, 0.0894 mmol) was dissolved in ACN/H₂O 1:1, at a concentration of 10 mM, followed by the addition of 2-aminoethyl-2-pyridyldisulfide (30.0 mg, 0.1613 mmol) and reacted for 2 h. The crude reaction mixture was purified by preparative reverse-phase HPLC (30-60% CH₃CN/H₂O over 35 min) and lyophilized to afford H₂N-CH₂CH₂-S-S-DM1 (51.3 mg, 71% yield).

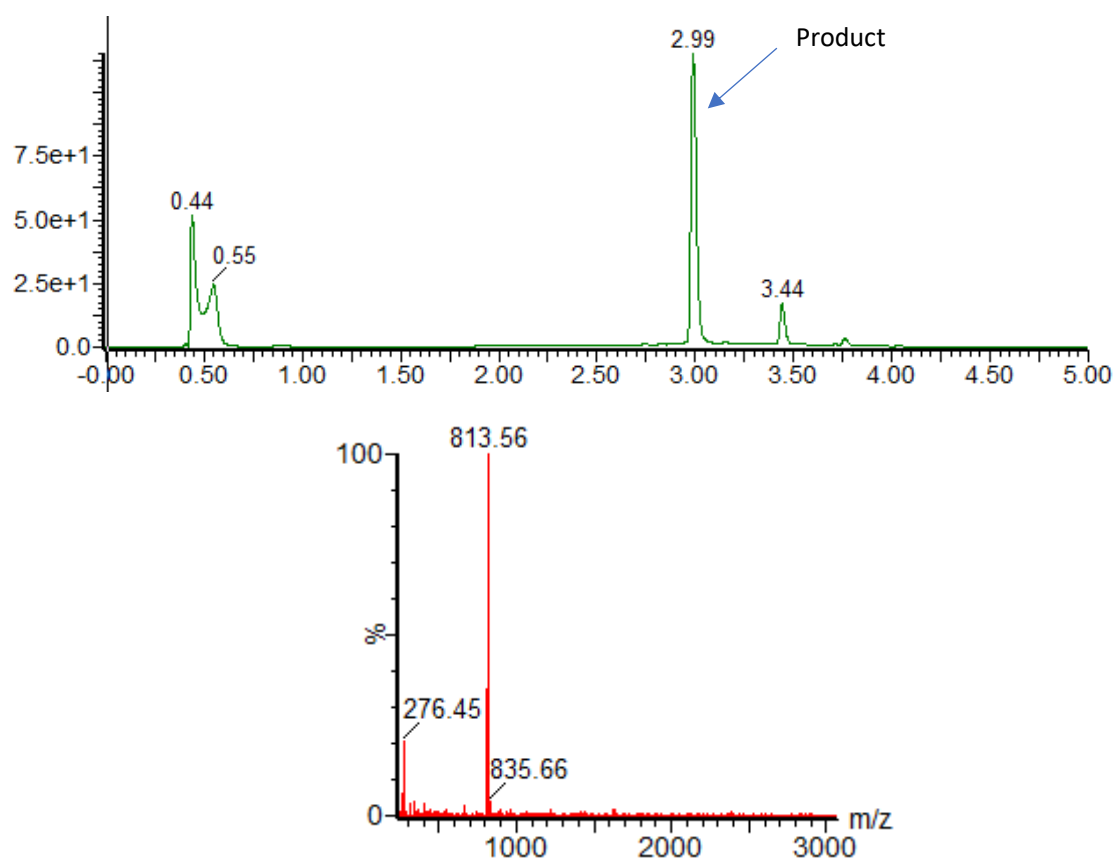


Figure S15: UV trace and corresponding MS trace from LC-MS analysis of the crude reaction mixture. Gradient: 5-95% ACN/H₂O with 0.1% TFA over 5 min at a flow rate of 0.4 mL/min.

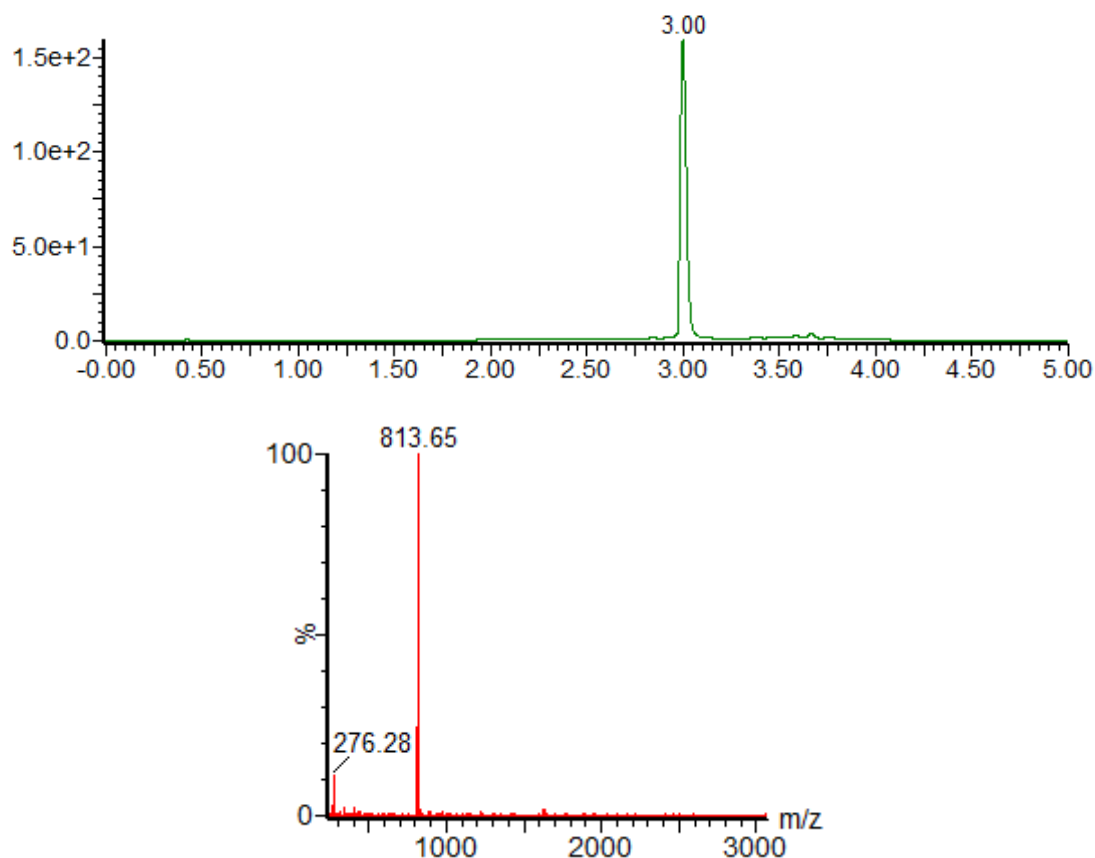
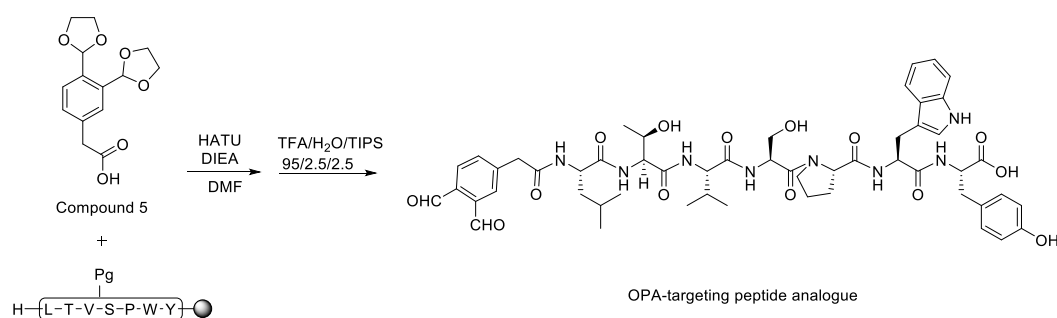


Figure S16: UV trace and corresponding MS trace from LC-MS analysis of the purified **H₂N-CH₂CH₂-S-S-DM1**. Gradient: 5-95% ACN/H₂O with 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. ESI-MS calcd. for C₃₇H₅₃ClN₄O₁₀S₂ = 812.29; [M+H]⁺ m/z = 813.29, found 813.65.

4.5 Synthesis of OPA-targeting peptide analogue



Compound **5** was prepared according to the synthetic route reported.^{5,6} The starting material was 3-(4-hydroxyphenyl)ethanoic acid instead of 3-(4-hydroxyphenyl)propanoic acid, while the stepwise procedures remained unchanged. The linear peptide was obtained via SPPS according to general procedure 2.1, followed by global deprotection according to general procedure 2.4. The crude peptide was

purified by preparative reverse-phase HPLC (25-45% CH₃CN/H₂O over 35 min) and lyophilized to afford the OPA-targeting peptide analogue (16.6 mg, 32% yield).

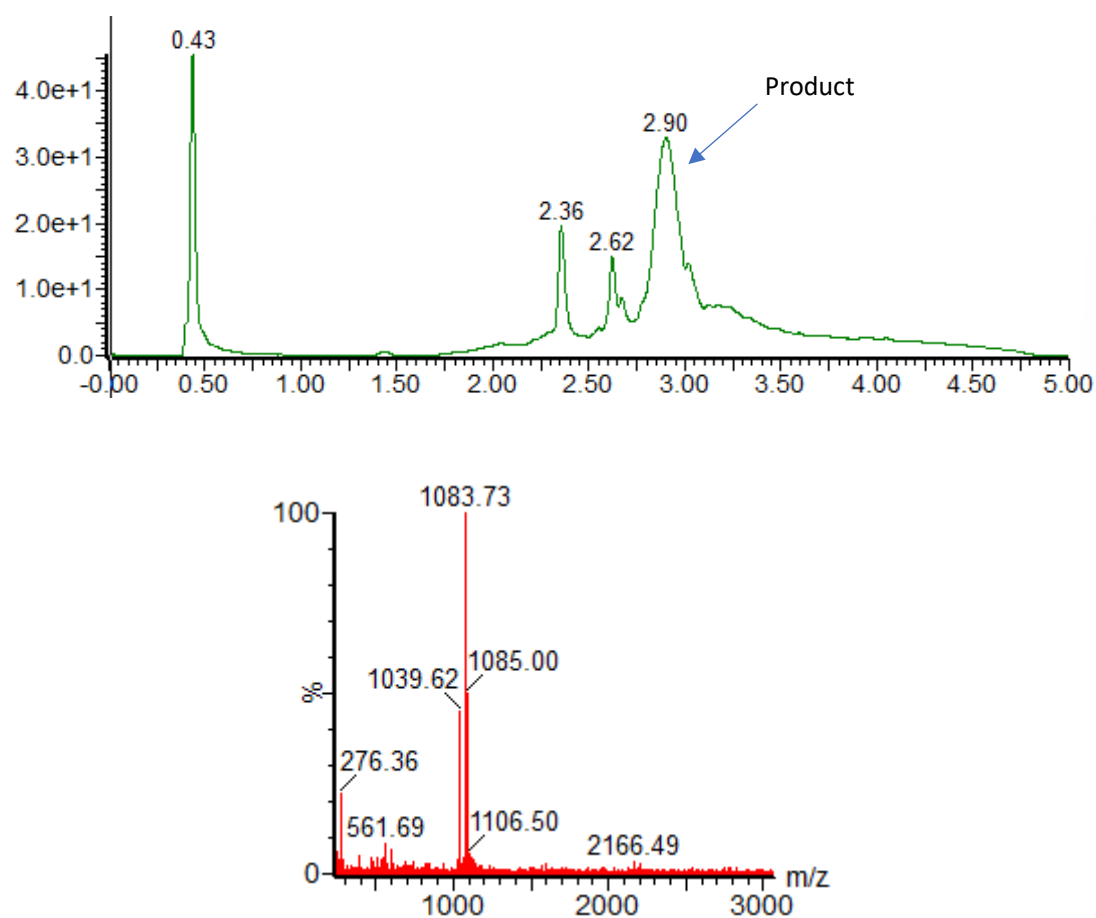
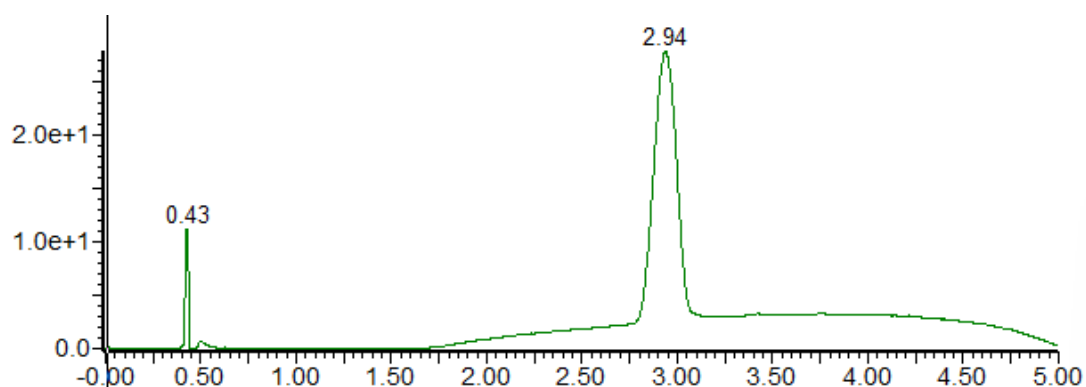


Figure S17: UV trace and corresponding MS trace from LC-MS analysis of the crude OPA-targeting peptide. Gradient: 5-95% ACN/H₂O with 0.1% TFA over 5 min at a flow rate of 0.4 mL/min.



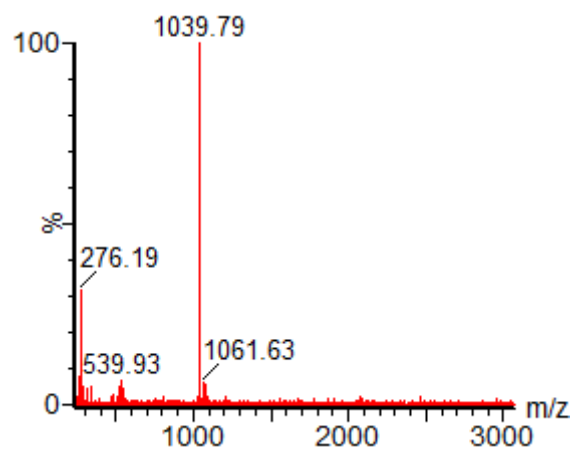


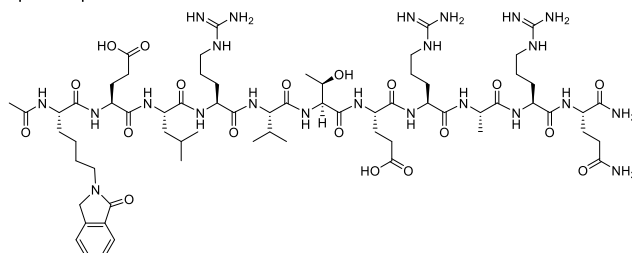
Figure S18: UV trace and corresponding MS trace from LC-MS analysis of the purified **OPA-targeting peptide analogue**. Gradient: 5-95% ACN/H₂O with 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. ESI-MS calcd. for C₅₃H₆₆N₈O₁₄ = 1038.47; [M+H]⁺ m/z = 1039.47, found 1039.79

5. UPLC-Chromatogram and MS-Spectrum

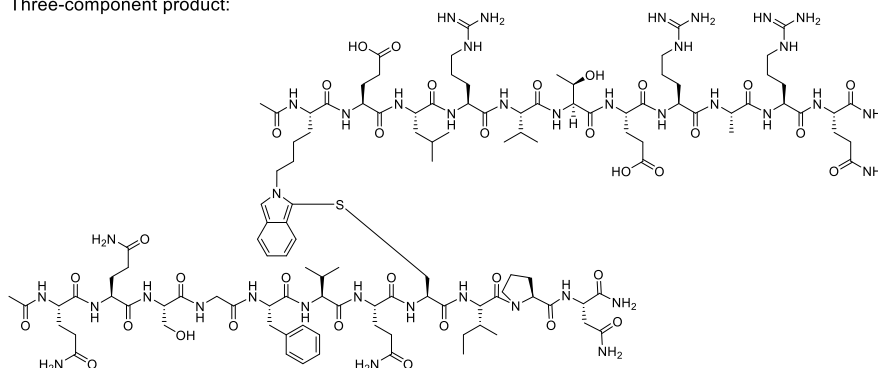
5.1 Synthesis of peptide-peptide conjugates (PPC-1 to PPC-12)

PPC-1 (Ac-QQSGFVQCIPN-NH₂ + Ac-KELRVTERARQ-NH₂)

Two-component product:



Three-component product:



No guanidine condition

The reaction was carried out at 0.0017 mmol scale according to general procedure 2.6, conditions [A]. The crude reaction mixture was purified by preparative reverse-phase HPLC (15-60% CH₃CN/H₂O over 35 min) and lyophilized to afford the two-component product (0.53 mg, 21% yield) and the three-component product (0.83 mg, 18% yield).

Guanidine-added condition

The reaction was carried out at 0.0013 mmol scale according to general procedure 2.6, conditions [B] with 3M guanidine. The crude reaction mixture was purified by preparative reverse-phase HPLC (15-60% CH₃CN/H₂O over 35 min) and lyophilized to afford the three-component product (1.54 mg, 43% yield).

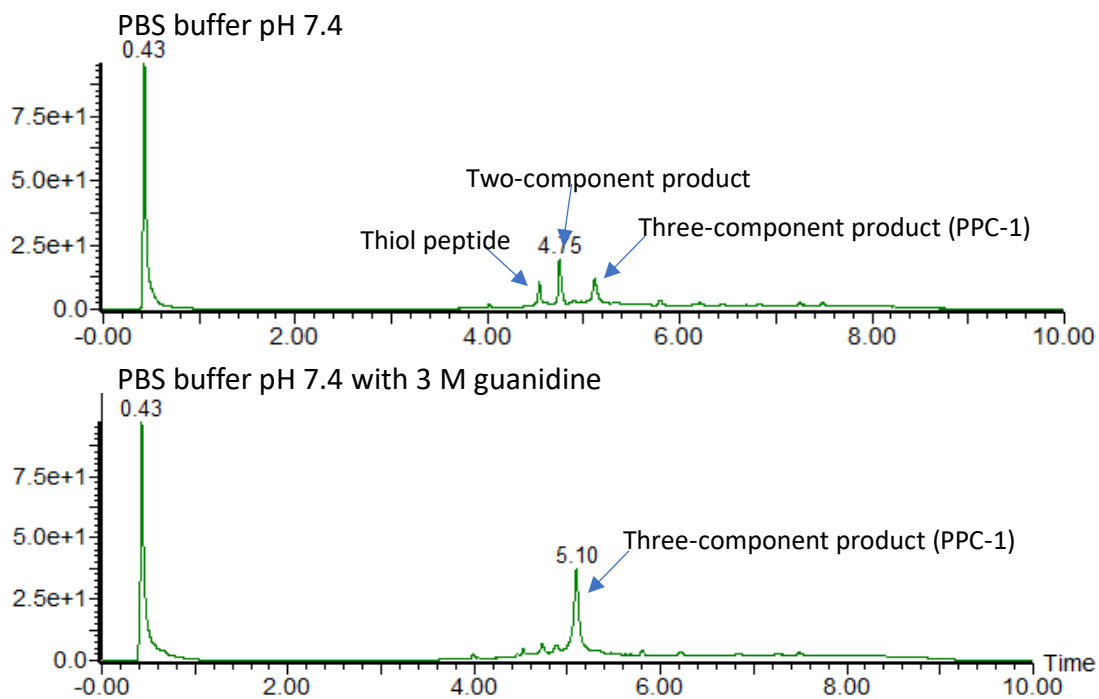


Figure S19: UV trace of the crude reaction mixture. Gradient: 5-95% ACN/H₂O with 0.1% TFA over 10 min at a flow rate of 0.4 mL/min.

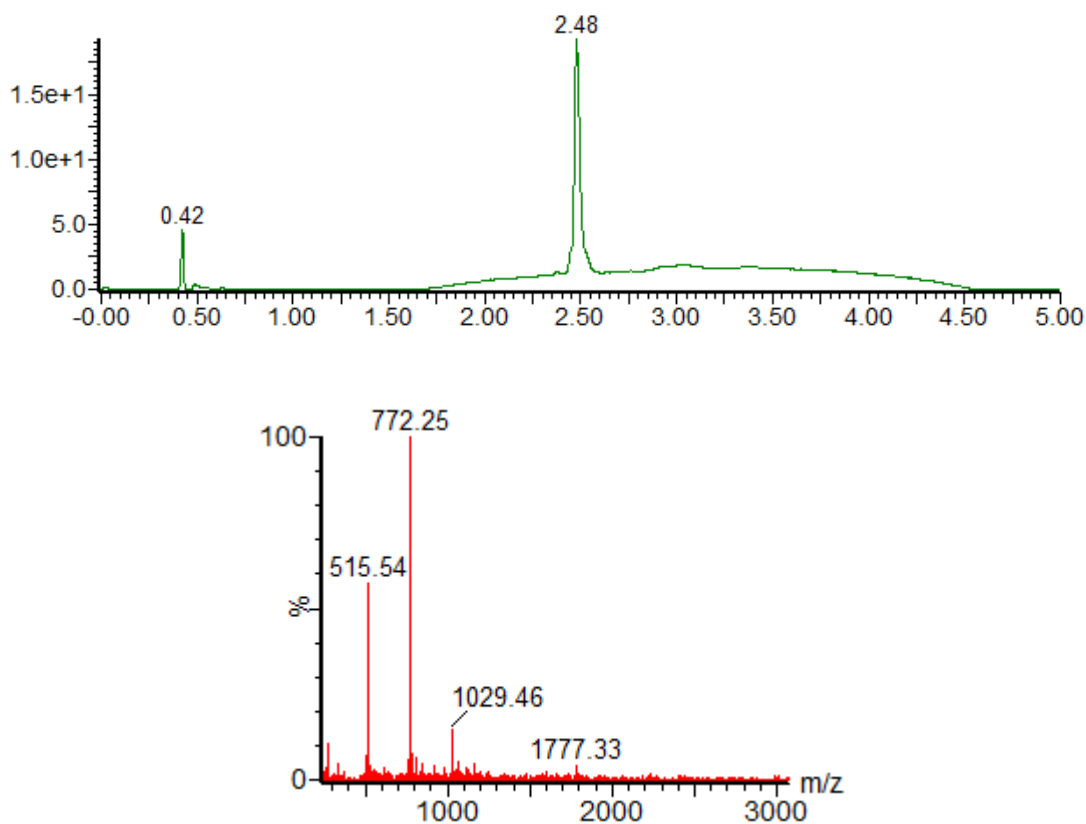


Figure S20: UV trace and corresponding MS trace from LC-MS analysis of the purified two-component product. Gradient: 5-95% ACN/H₂O with 0.1% TFA over 5 min at a

flow rate of 0.4 mL/min. ESI-MS calcd. for $C_{67}H_{111}N_{23}O_{19}$ = 1542.77; $[M+H]^{2+}$ m/z = 772.39, found 772.25; $[M+H]^{3+}$ m/z = 515.26, found 515.54.

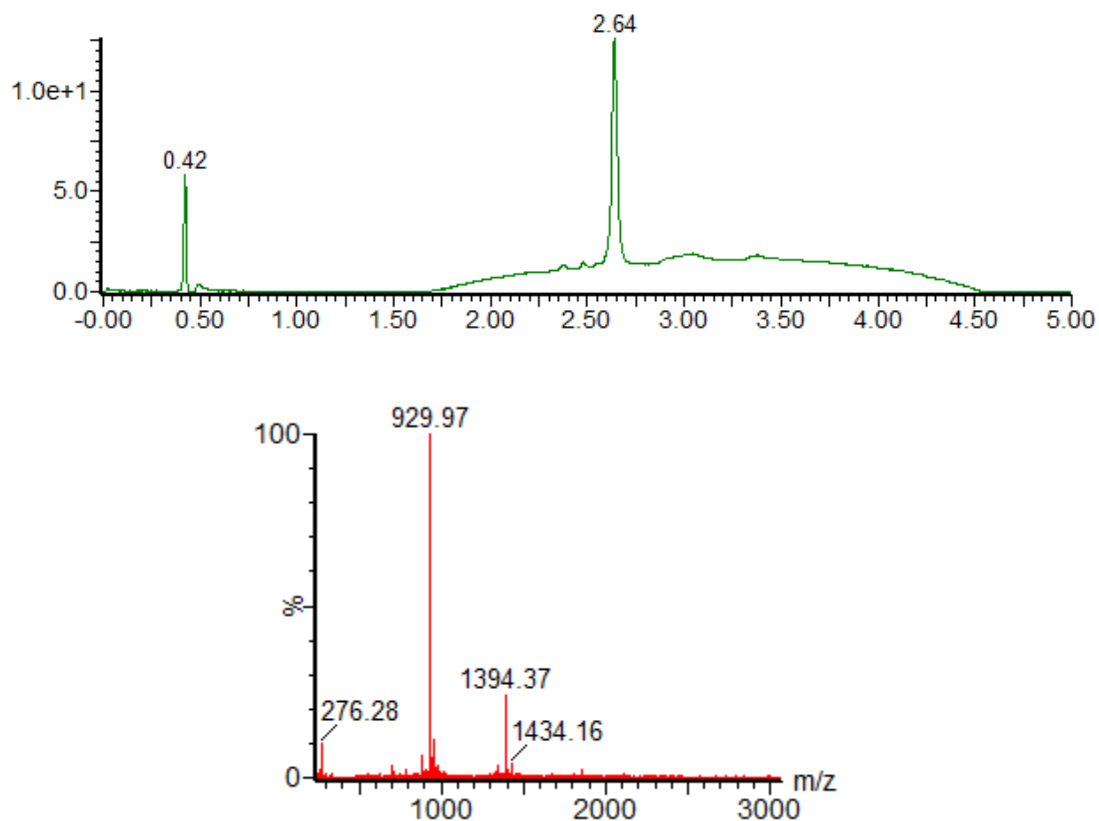
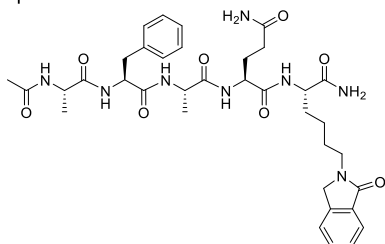


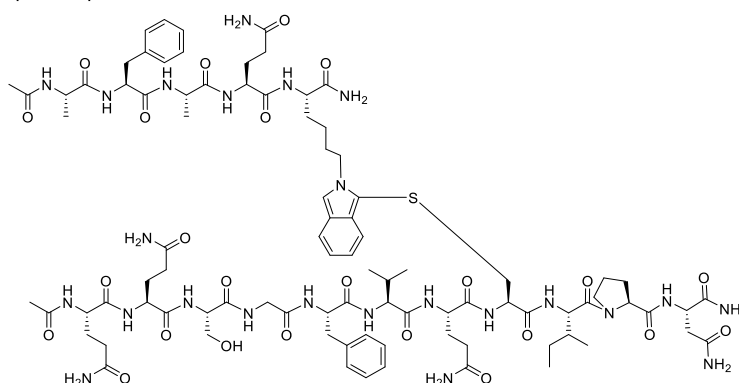
Figure S21: UV trace and corresponding MS trace from LC-MS analysis of the purified **PPC-1**. Gradient: 5-95% ACN/H₂O with 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. ESI-MS calcd. for $C_{121}H_{193}N_{39}O_{35}S$ = 2786.17; $[M+H]^{2+}$ m/z = 1394.09, found 1394.37; $[M+H]^{3+}$ m/z = 929.72, found 929.97.

PPC-2 (Ac-QQSGFVQCIPN-NH₂ + Ac-AFAQK-NH₂)

Two-component product:



Three-component product:



No guanidine condition

The reaction was carried out at 0.0017 mmol scale according to general procedure 2.6. conditions [A]. The crude reaction mixture was purified by preparative reverse-phase HPLC (15-60% CH₃CN/H₂O over 35 min) and lyophilized to afford the two-component product (0.33 mg, 26% yield) and the three-component product (0.85 mg, 25% yield).

Guanidine-added condition

The reaction was carried out at 0.0016 mmol scale according to general procedure 2.6, conditions [B] with 3M guanidine. The crude reaction mixture was purified by preparative reverse-phase HPLC (15-60% CH₃CN/H₂O over 35 min) and lyophilized to afford the three-component product (1.57 mg, 50% yield).

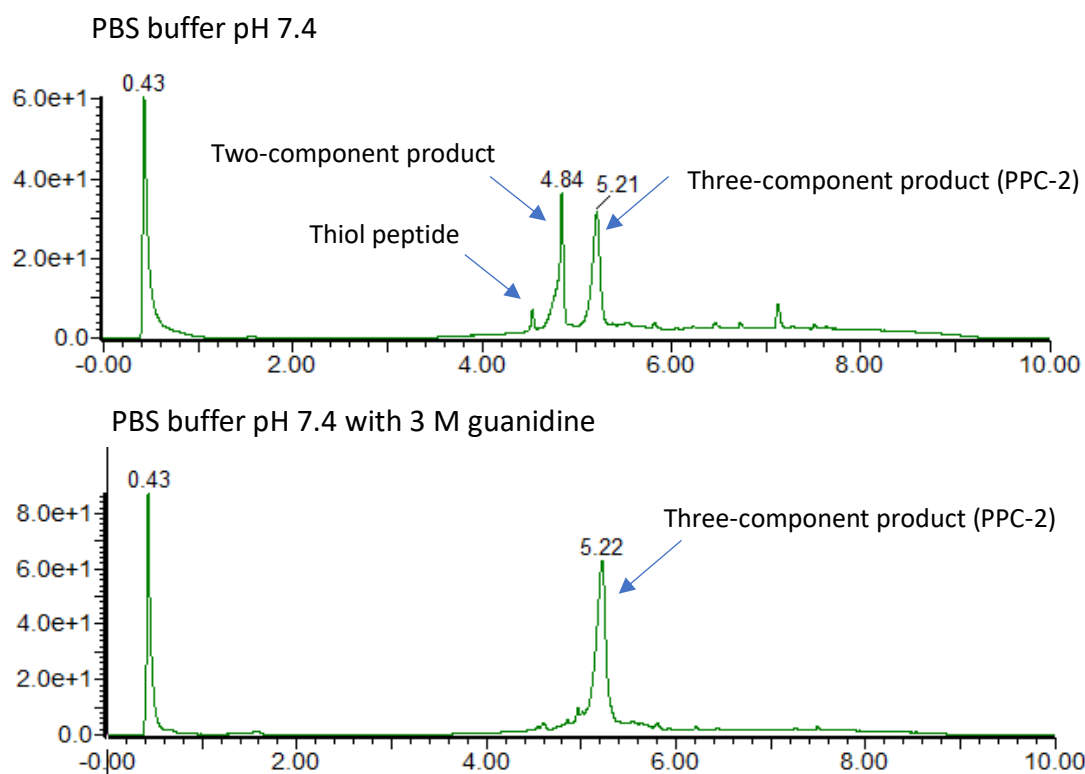


Figure S22: UV trace of the crude reaction mixture. Gradient: 5-95% ACN/H₂O with 0.1% TFA over 10 min at a flow rate of 0.4 mL/min.

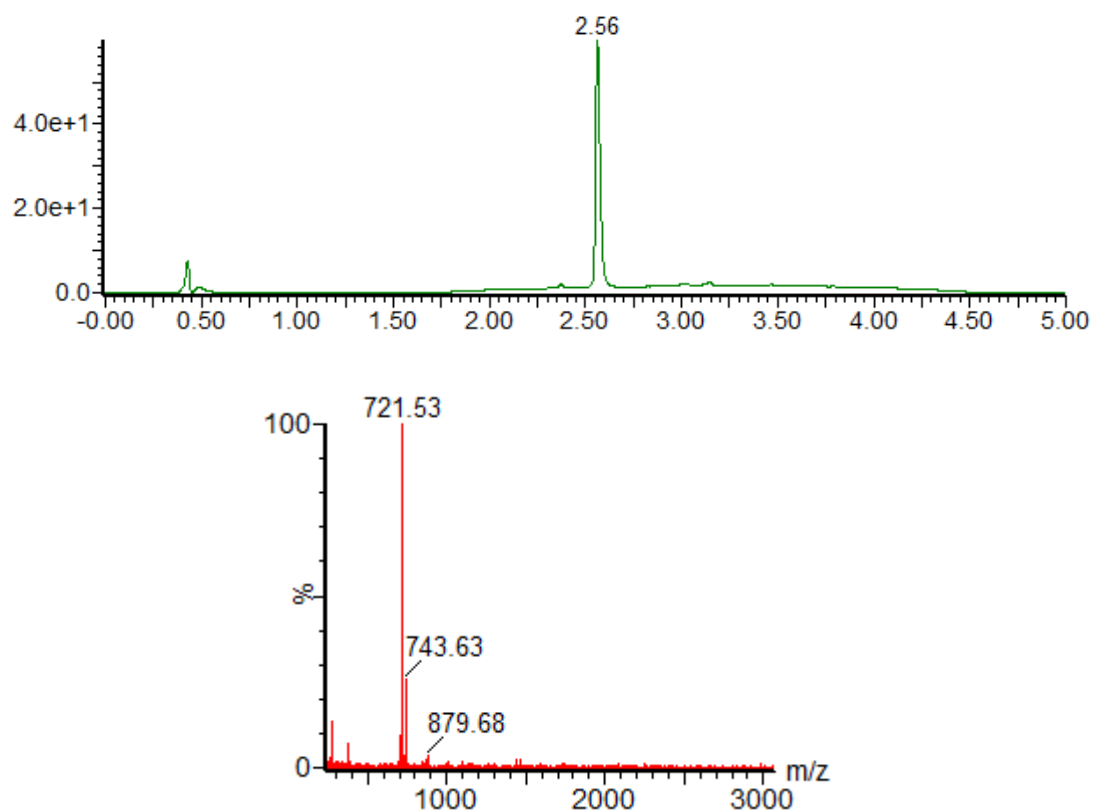


Figure S23: UV trace and corresponding MS trace from LC-MS analysis of the purified

two- component product. 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 C₃₆H₄₈N₈O₈ =720.83; [M+H]²⁺ m/z = 721.83, found 721.53.

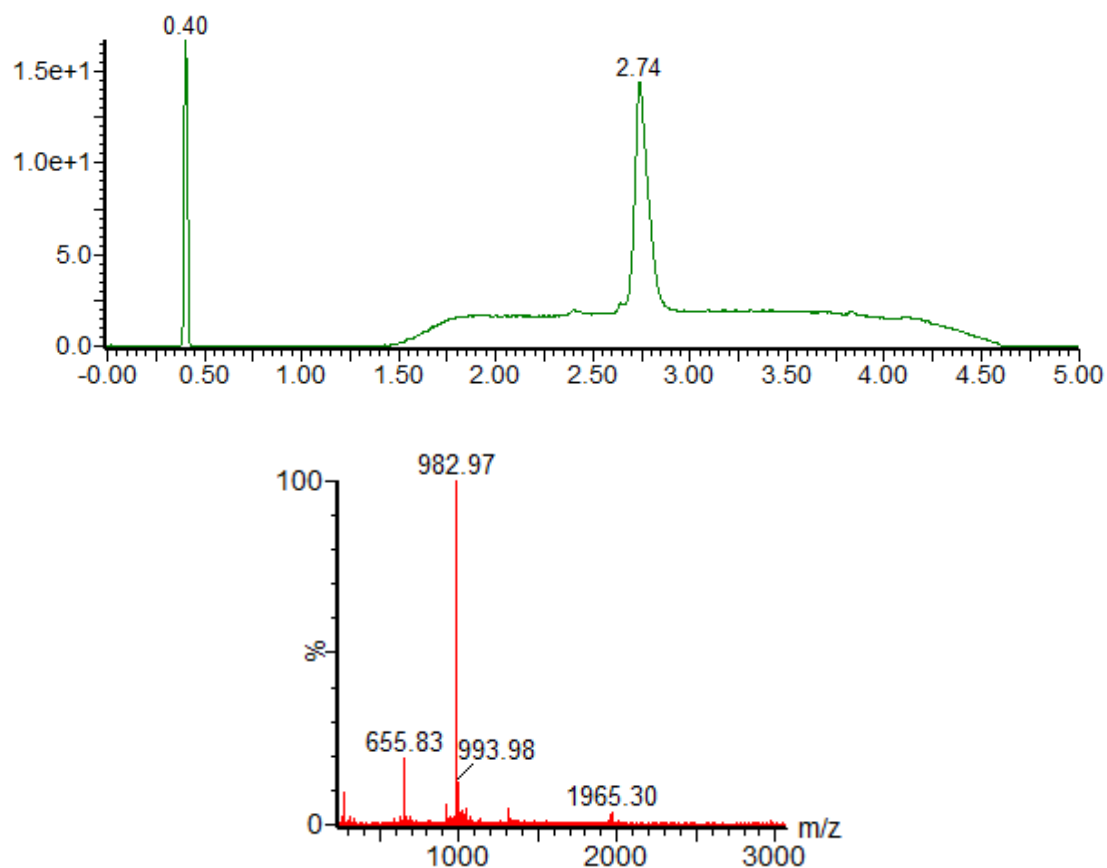
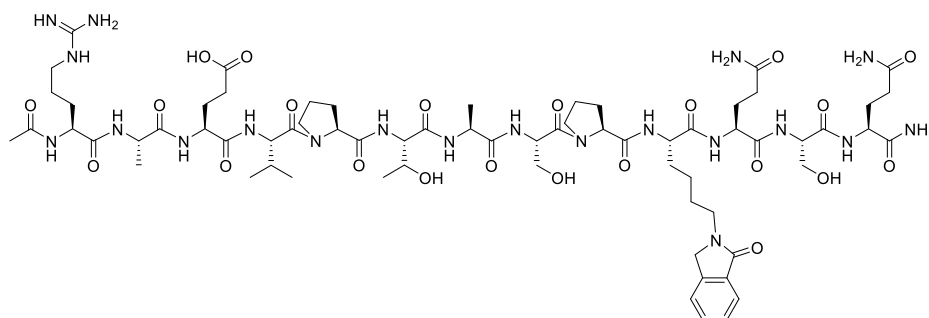


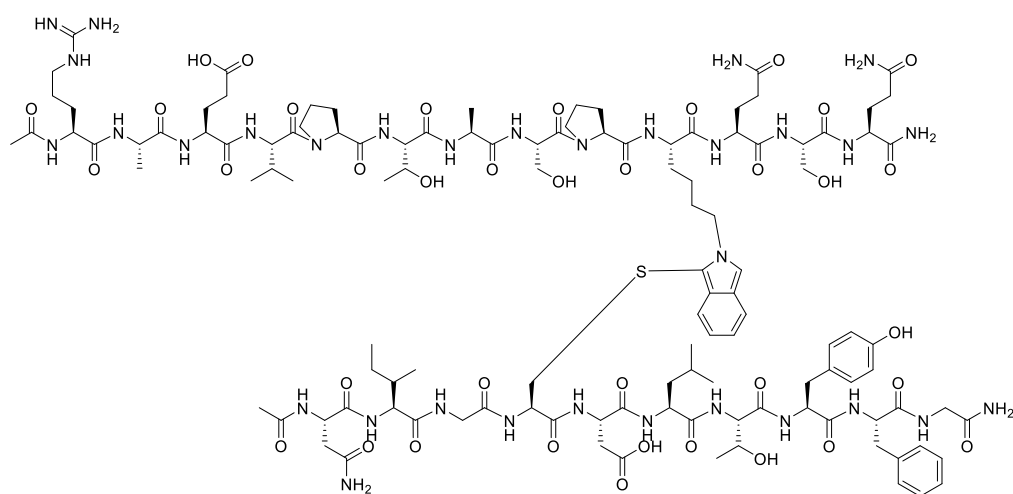
Figure S24: UV trace and corresponding MS trace from LC-MS analysis of the purified **PPC-2**. Gradient: 5-95% ACN/H₂O with 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. ESI-MS calcd. for C₉₀H₁₃₀N₂₄O₂₄S =1964.23; [M+H]⁺ m/z = 1965.23, found 1965.30; [M+H]²⁺ m/z = 983.11, found 982.97; [M+H]³⁺ m/z = 655.74, found 655.83.

PPC-3 (Ac-NIGCDLTYFG-NH₂ + Ac-RAEVPTASPKQSQ-HN₂)

Two-component product:



Three-component product:



No guanidine conditions

The reaction was carried out at 0.0015 mmol scale according to general procedure 2.6, conditions [A]. The crude reaction mixture was purified by preparative reverse-phase HPLC (15-60% CH₃CN/H₂O over 35 min) and lyophilized to afford the two-component product (0.4 mg, 16% yield) and the three-component product (1.3 mg, 32% yield).

Guanidine-added conditions

The reaction was carried out at 0.0015 mmol scale according to general procedure 2.6, conditions [B] with 6 M guanidine. The crude reaction mixture was purified by preparative reverse-phase HPLC (15-60% CH₃CN/H₂O over 35 min) and lyophilized to afford the three-component product (2.07 mg, 51% yield).

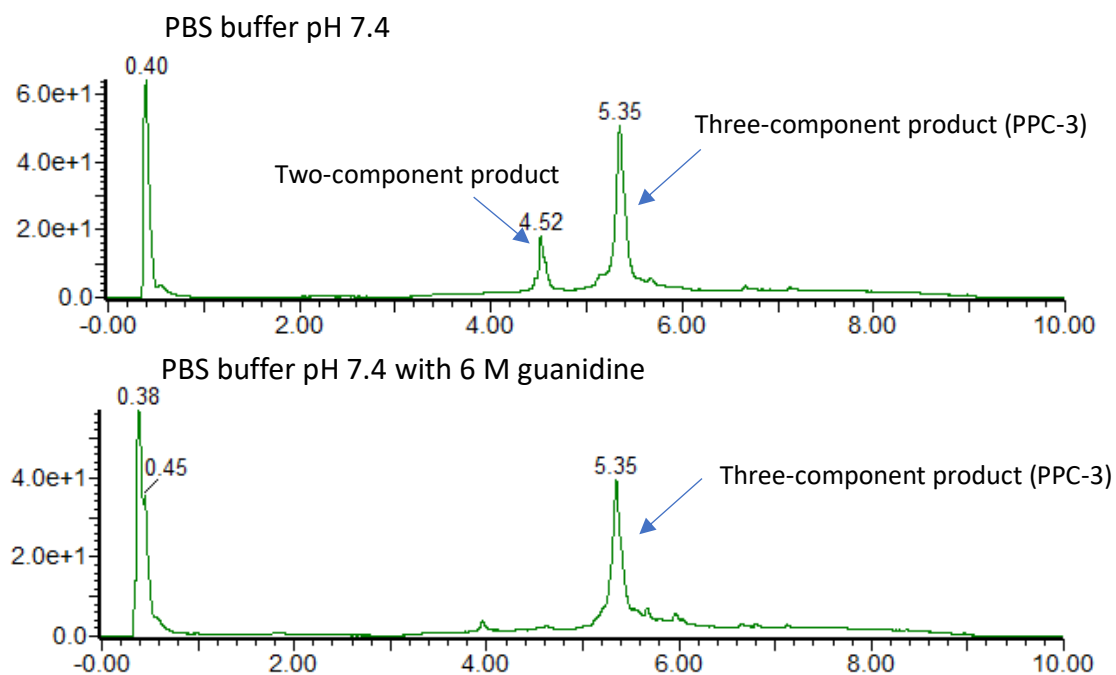


Figure S25: UV trace of the crude reaction mixture. Gradient: 5-95% ACN/H₂O with 0.1% TFA over 10 min at a flow rate of 0.4 mL/min.

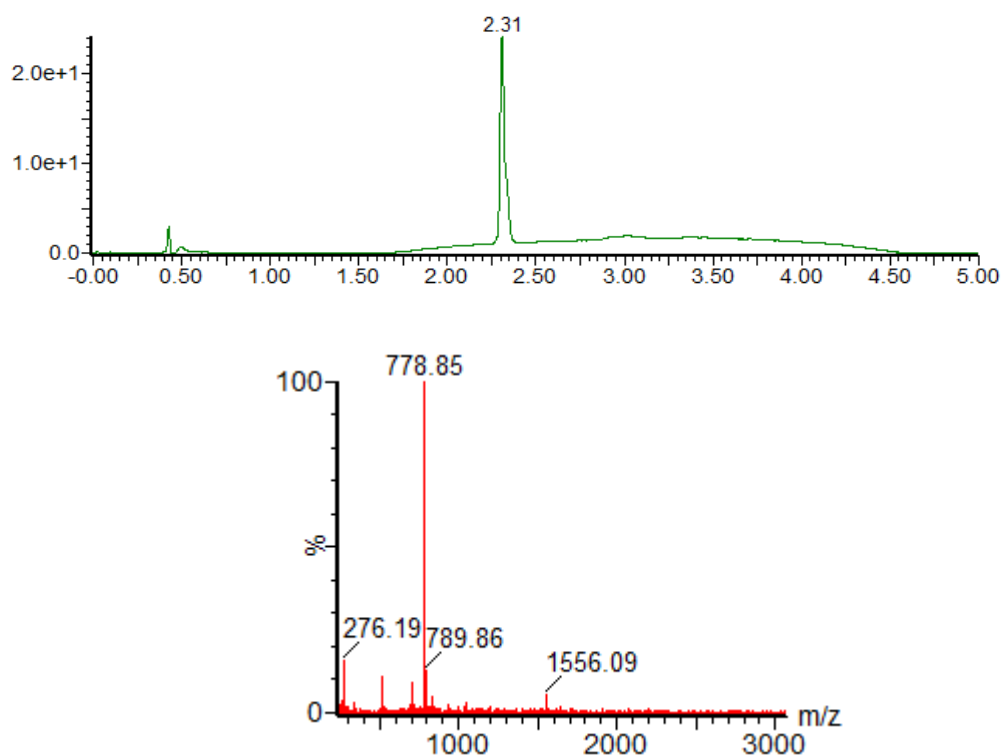


Figure S26: UV trace and corresponding MS trace from LC-MS analysis of the purified two-component product. Gradient: 5-95% ACN/H₂O with 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. ESI-MS calcd. for C₆₈H₁₀₆N₂₀O₂₂ = 1555.71; [M+H]⁺ m/z = 1556.71, found 1556.09; [M+H]²⁺ m/z = 778.86, found 778.85.

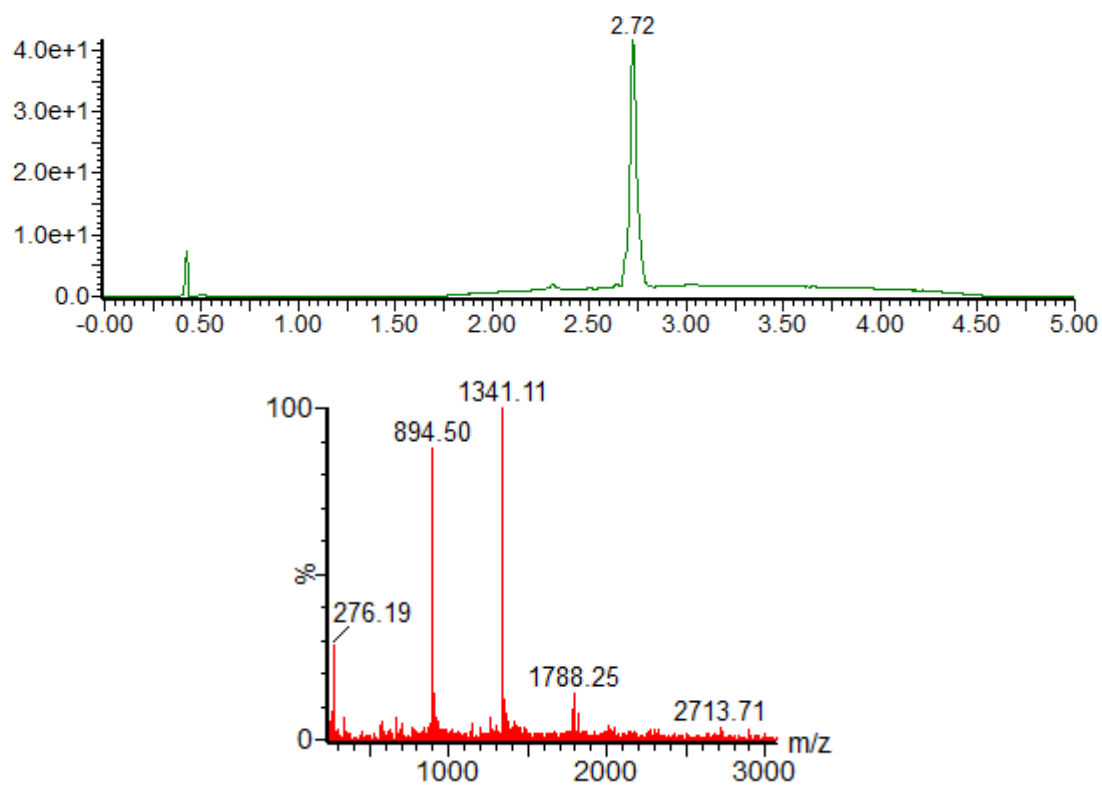


Figure S27: UV trace and corresponding MS trace from LC-MS analysis of the purified **PPC-3**. Gradient: 5-95% ACN/H₂O with 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. ESI-MS calcd. for C₁₁₉H₁₇₈N₃₂O₃₇S =2680.98; [M+H]²⁺ m/z =1341.49, found 1341.11; [M+H]³⁺ m/z =894.66, found 894.50.

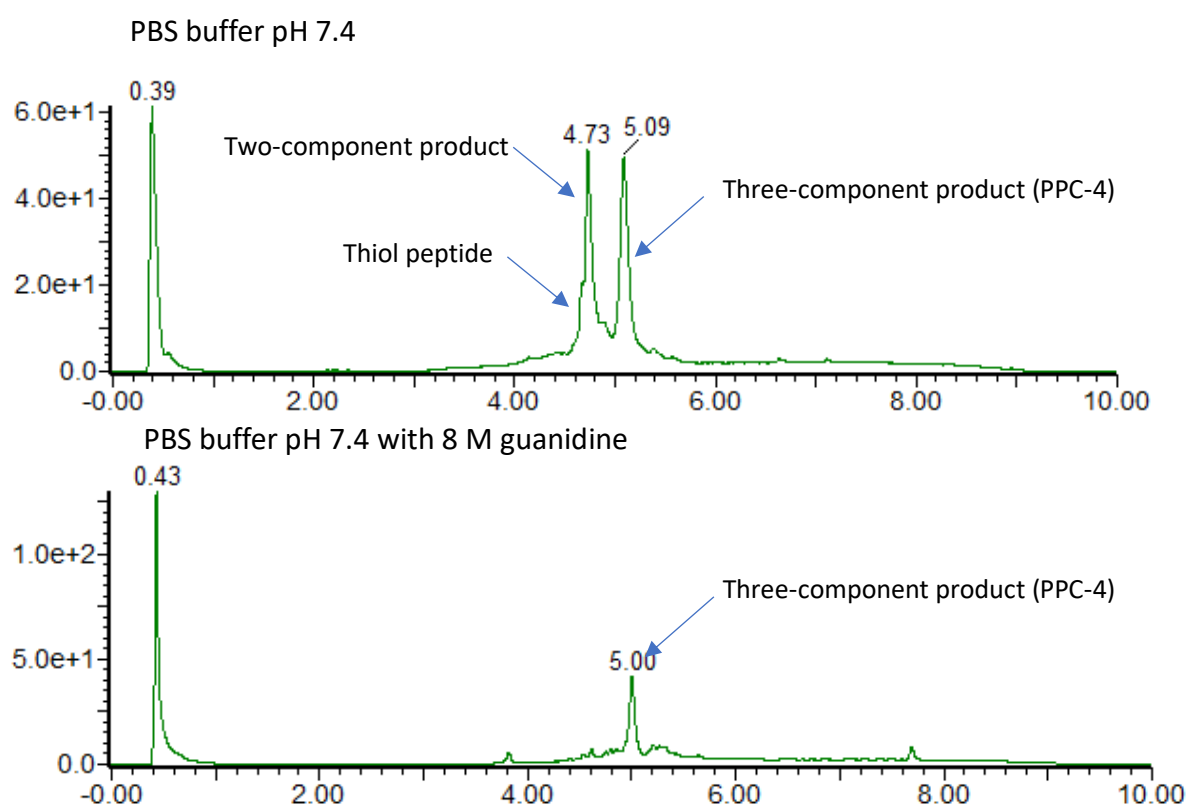
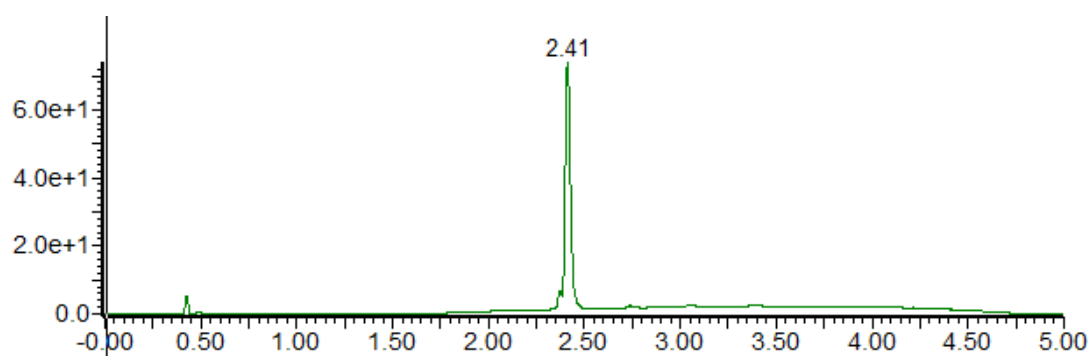


Figure S28: UV trace of the crude reaction mixture. Gradient: 5-95% ACN/H₂O with 0.1% TFA over 10 min at a flow rate of 0.4 mL/min.



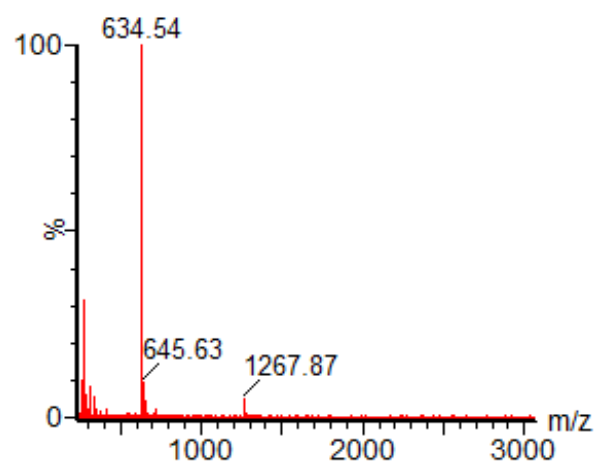


Figure S29: UV trace and corresponding MS trace from LC-MS analysis of the purified two-component product. Gradient: 5-95% ACN/H₂O with 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. ESI-MS calcd. for C₆₀H₈₁N₁₅O₁₆ = 1267.41; [M+H]⁺ m/z = 1268.41, found 1267.87; [M+H]²⁺ m/z = 634.71, found 634.54.

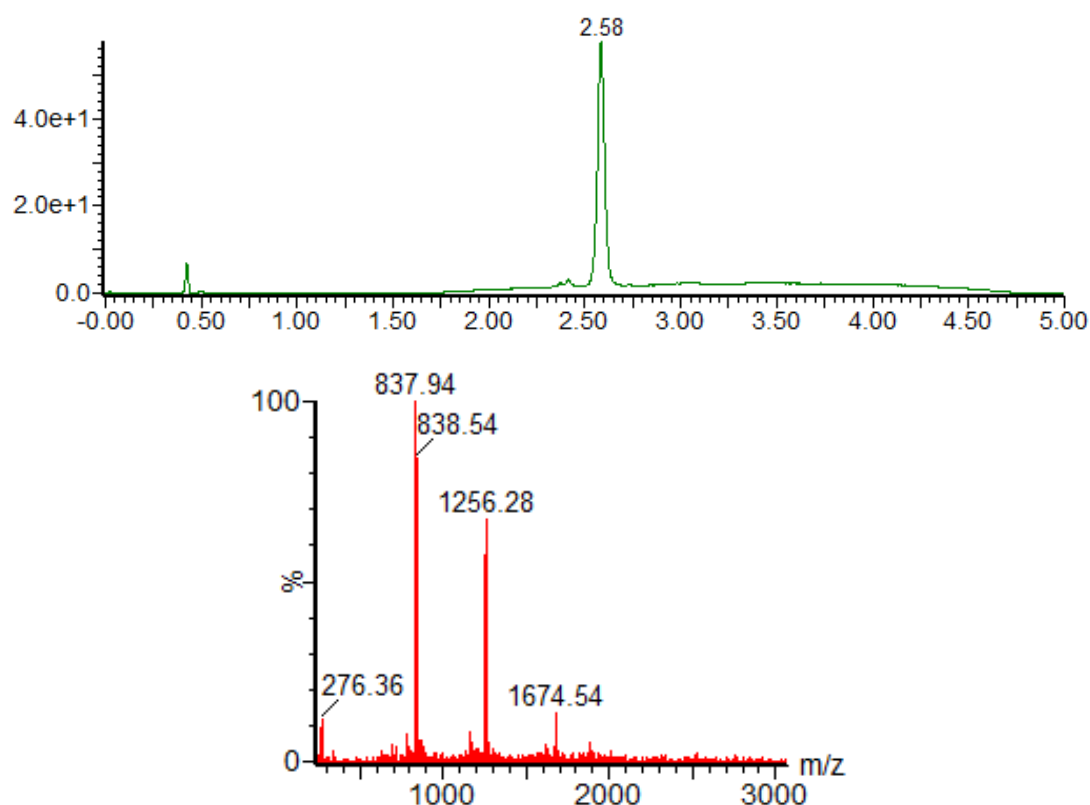
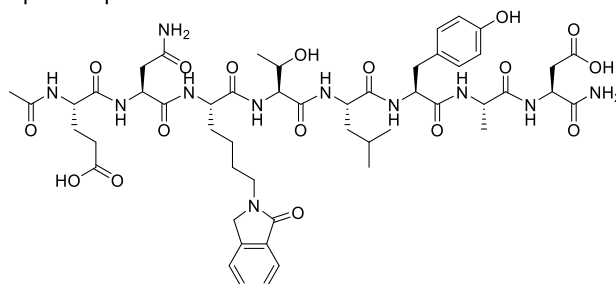


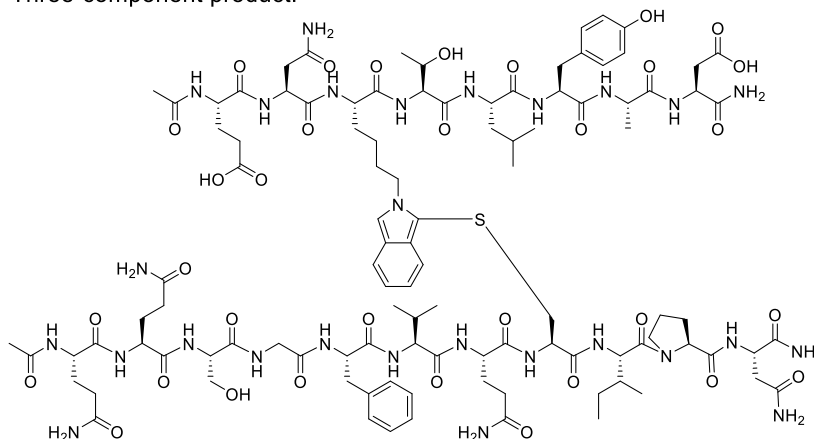
Figure S30: UV trace and corresponding MS trace from LC-MS analysis of the purified **PPC-4**. Gradient: 5-95% ACN/H₂O with 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. ESI-MS calcd. for C₁₁₄H₁₆₃N₃₁O₃₂S = 2511.80; [M+H]²⁺ m/z = 1256.90, found 1256.28; [M+H]³⁺ m/z = 838.27, found 837.94.

PPC-5 (Ac-QQSGFVQCIPN-NH₂ + Ac-ENKTLTYAD-NH₂)

Two-component product:



Three-component product:



No guanidine conditions

The reaction was carried out at 0.0015 mmol scale according to general procedure 2.6, conditions [A]. The crude reaction mixture was purified by preparative reverse-phase HPLC (15-60% CH₃CN/H₂O over 35 min) and lyophilized to afford the two-component product (0.72 mg, 42% yield) and the three-component product (0.13 mg, 3% yield).

Guanidine-added conditions

The reaction was carried out at 0.0019 mmol scale according to general procedure 2.6, conditions [B] with 3 M guanidine. The crude reaction mixture was purified by preparative reverse-phase HPLC (15-60% CH₃CN/H₂O over 35 min) and lyophilized to afford the three-component product (2.10 mg, 46% yield).

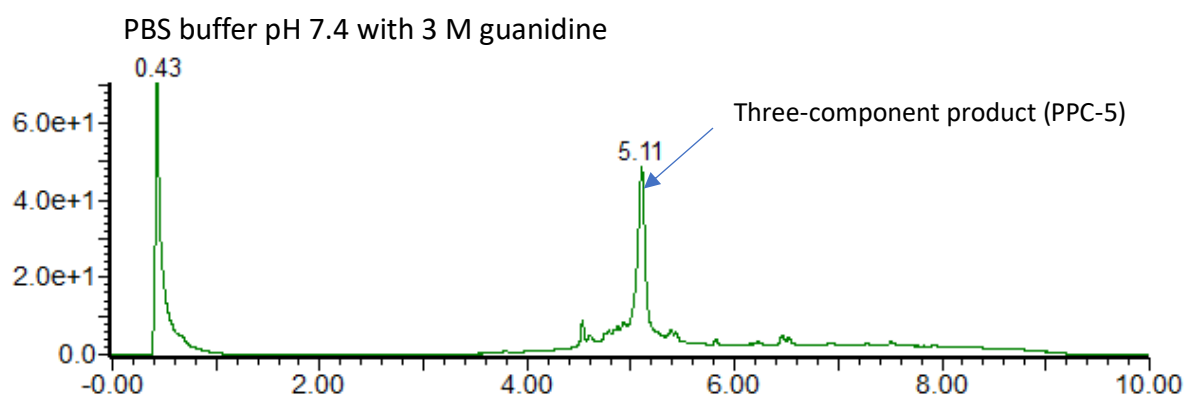
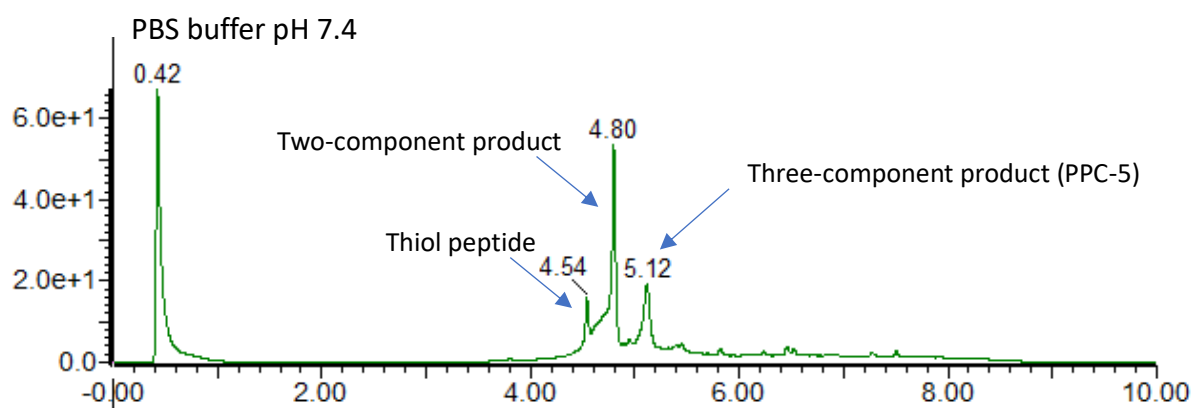
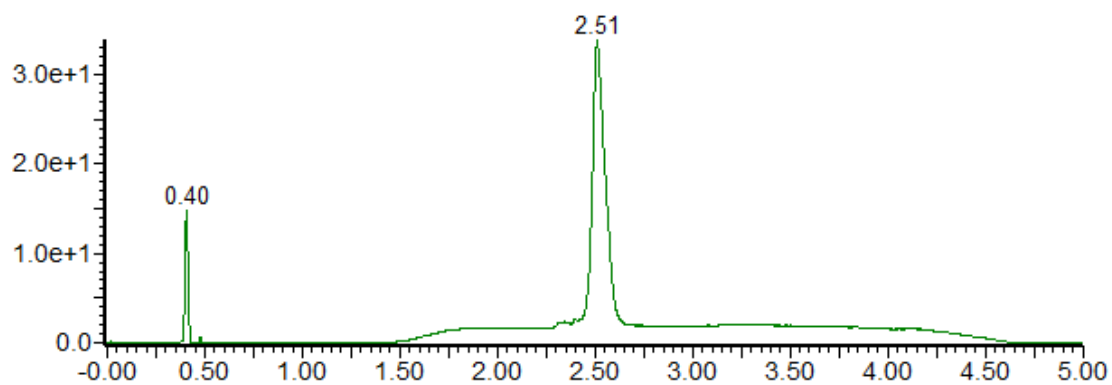


Figure S31: UV trace of the crude reaction mixture. Gradient: 5-95% ACN/H₂O with 0.1% TFA over 10 min at a flow rate of 0.4 mL/min.



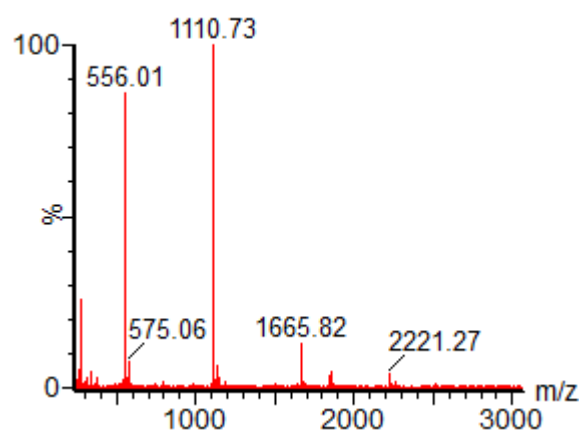


Figure S32: UV trace and corresponding MS trace from LC-MS analysis of the purified two-component product. Gradient: 5-95% ACN/H₂O with 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. ESI-MS calcd. for C₅₁H₇₁N₁₁O₁₇ = 1110.19; [M+H]⁺ m/z = 1111.19, found 1110.73; [M+H]²⁺ m/z = 556.09, found 556.01.

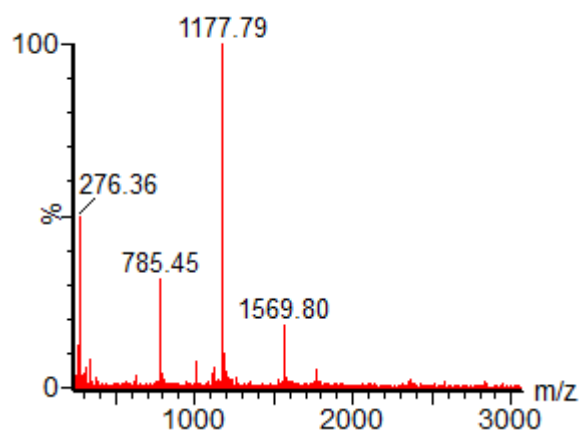
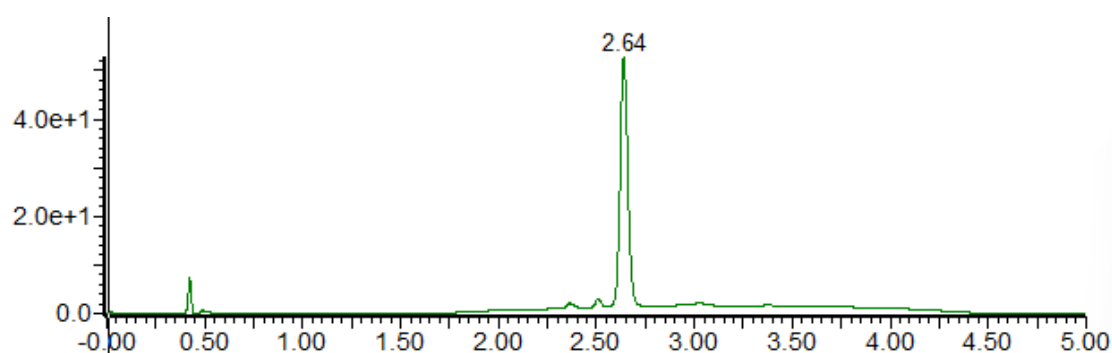
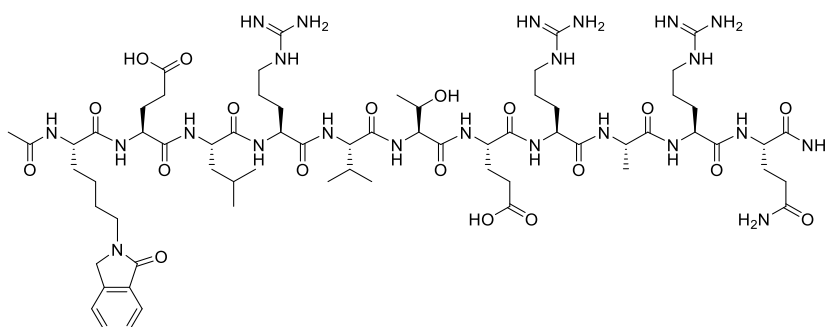


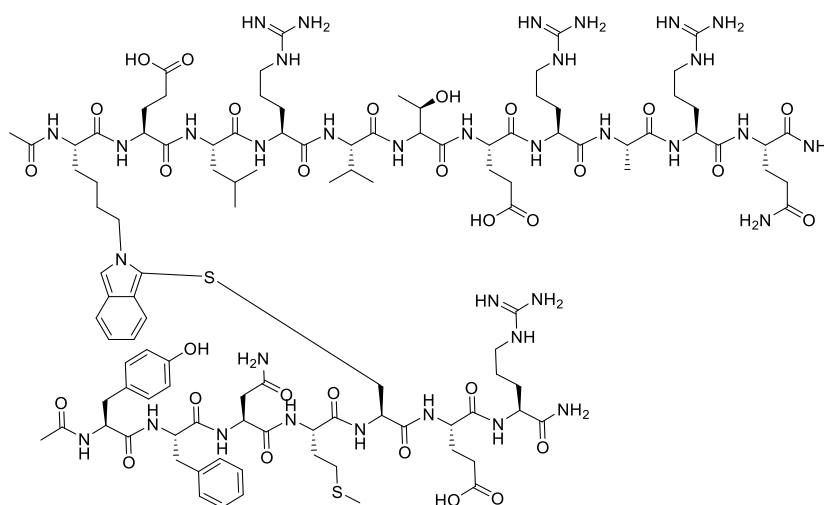
Figure S33: UV trace and corresponding MS trace from LC-MS analysis of the purified **PPC-5**. Gradient: 5-95% ACN/H₂O with 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. ESI-MS calcd. for C₁₀₅H₁₅₃N₂₇O₃₃S = 2353.60; [M+H]²⁺ m/z = 1177.80, found 1177.79; [M+H]³⁺ m/z = 785.53, found 785.45; 2[M+H]²⁺ m/z = 1571.07, found 1569.80.

PPC-6 (Ac-YFNM CER-NH₂ + Ac-KELRVTERARQ-NH₂)

Two-component product:



Three-component product:



No guanidine conditions

The reaction was carried out at 0.0016 mmol scale according to general procedure 2.6, conditions [A]. The crude reaction mixture was purified by preparative reverse-phase HPLC (20-45% CH₃CN/H₂O over 35 min) and lyophilized to afford the two-component product (0.30 mg, 12% yield) and the three-component product (1.10 mg, 27% yield).

Guanidine-added conditions

The reaction was carried out at 0.0013 mmol scale according to general procedure 2.6, conditions [B] with 3 M guanidine. The crude reaction mixture was purified by preparative reverse-phase HPLC (20-45% CH₃CN/H₂O over 35 min) and lyophilized to afford the three-component product (1.51 mg, 44% yield).

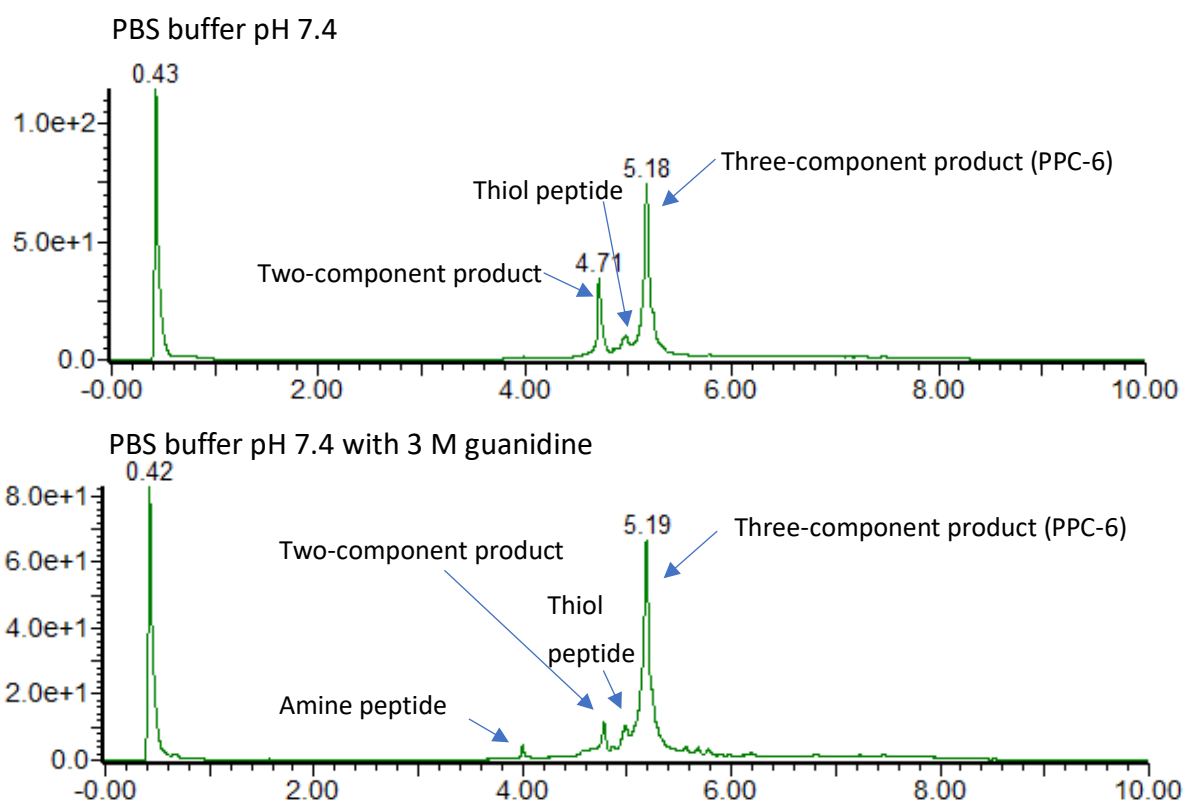


Figure S34: UV trace of the crude reaction mixture. Gradient: 5-95% ACN/H₂O with 0.1% TFA over 10 min at a flow rate of 0.4 mL/min.

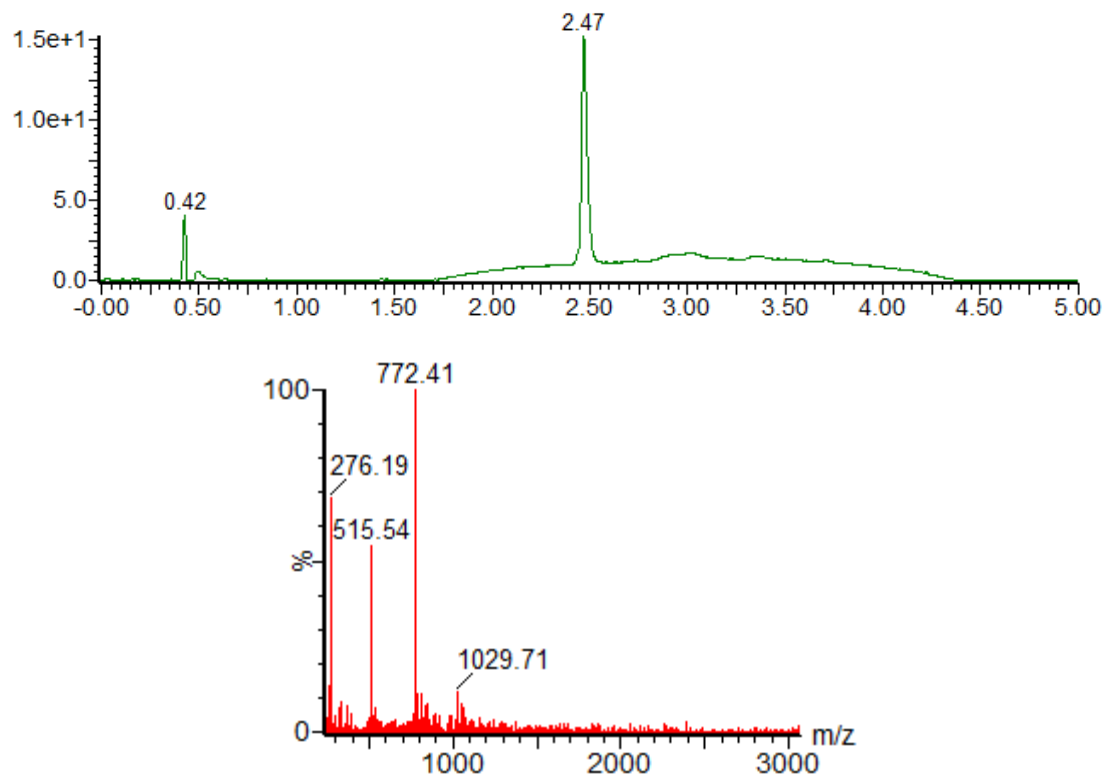


Figure S35: UV trace and corresponding MS trace from LC-MS analysis of the purified two-component product. Gradient: 5-95% ACN/H₂O with 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. ESI-MS calcd. for C₆₇H₁₁₁N₂₃O₁₉ =1542.77; [M+H]²⁺ m/z =772.39, found 772.41; [M+H]³⁺ m/z=515.26, found 515.54.

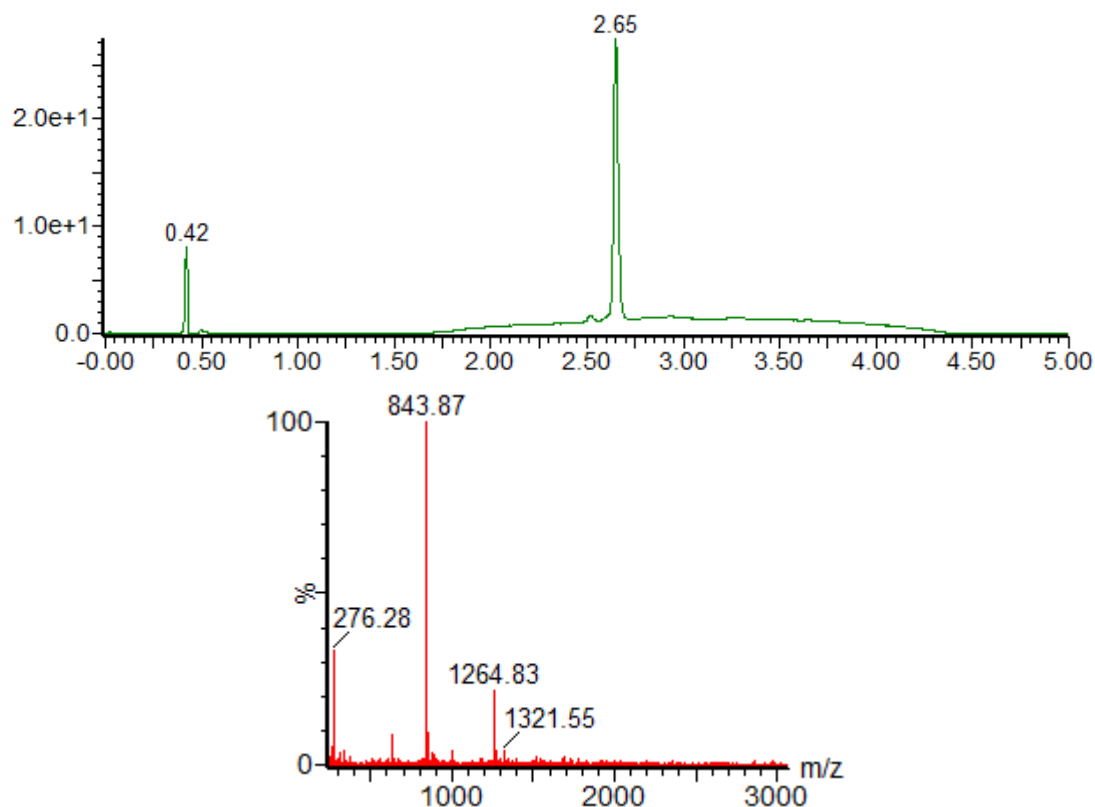
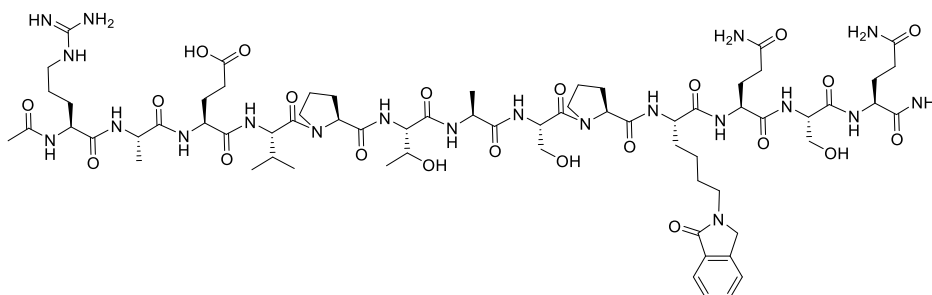


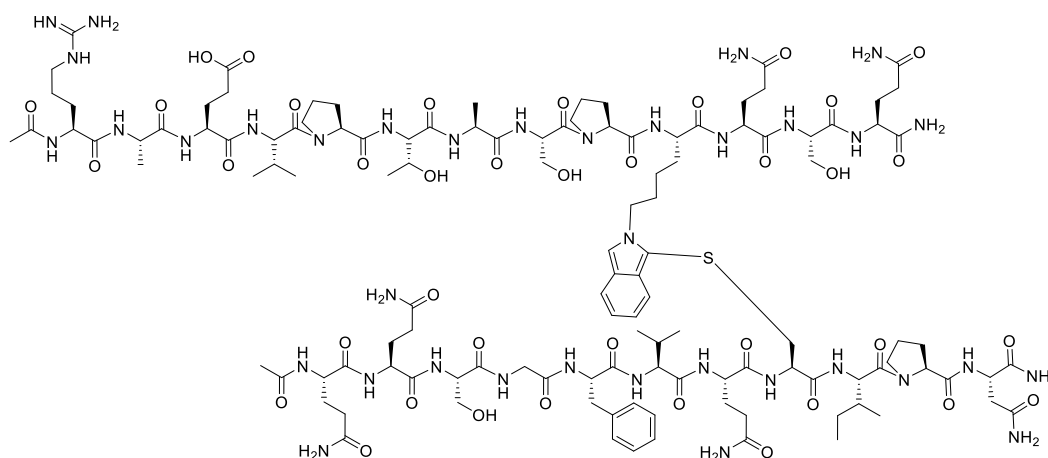
Figure S36: UV trace and corresponding MS trace from LC-MS analysis of the purified **PPC-6**. Gradient: 5-95% ACN/H₂O with 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. ESI-MS calcd. for C₁₁₀H₁₇₁N₃₅O₁₀S₂ =2527.91; [M+H]⁺ m/z =2527.91, found 1706.88; [M+H]²⁺ m/z =853.99, found 853.69.

PPC-7 (Ac-QQSGFVQCIPN-NH₂ + Ac-RAEVPTASPKQSQ-NH₂)

Two-component product:



Three-component product:



No guanidine conditions

The reaction was carried out at 0.0013 mmol scale according to general procedure 2.6, conditions [A]. The crude reaction mixture was purified by preparative reverse-phase HPLC (20-45% CH₃CN/H₂O over 35 min) and lyophilized to afford the two-component product (0.61 mg, 29% yield) and the three-component product (0.43 mg, 11% yield).

Guanidine-added conditions

The reaction was carried out at 0.0016 mmol scale according to general procedure 2.6, conditions [B] with 3 M guanidine. The crude reaction mixture was purified by preparative reverse-phase HPLC (20-45% CH₃CN/H₂O over 35 min) and lyophilized to afford the three-component product (2.21 mg, 48% yield).

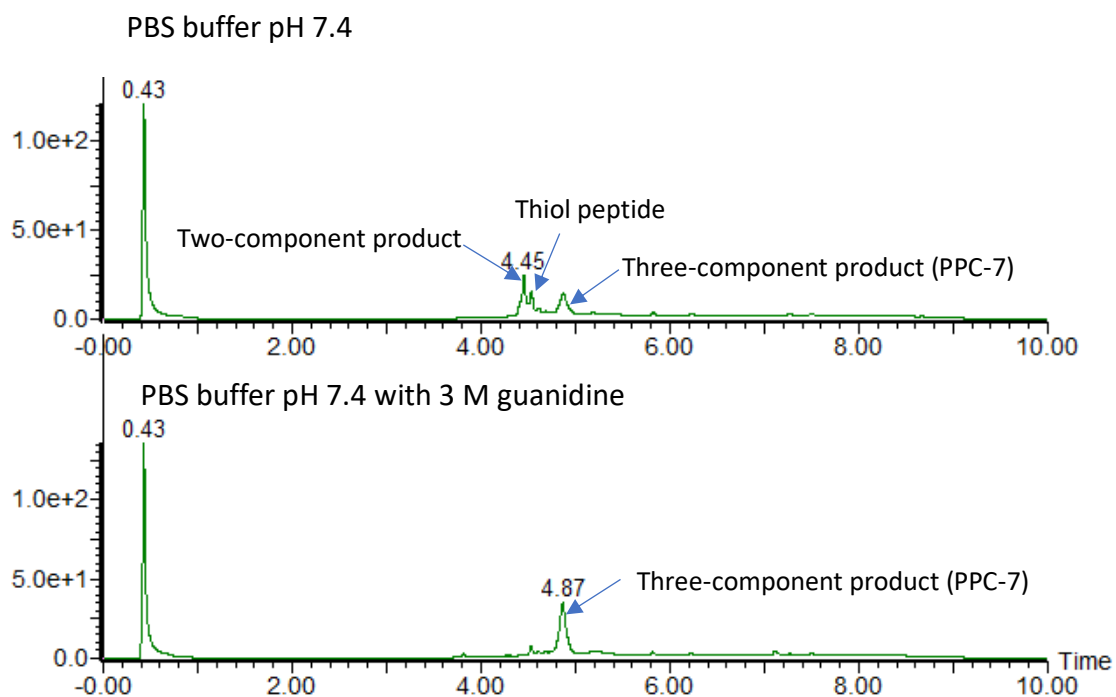


Figure S37: UV trace of the crude reaction mixture. Gradient: 5-95% ACN/H₂O with 0.1% TFA over 10 min at a flow rate of 0.4 mL/min.

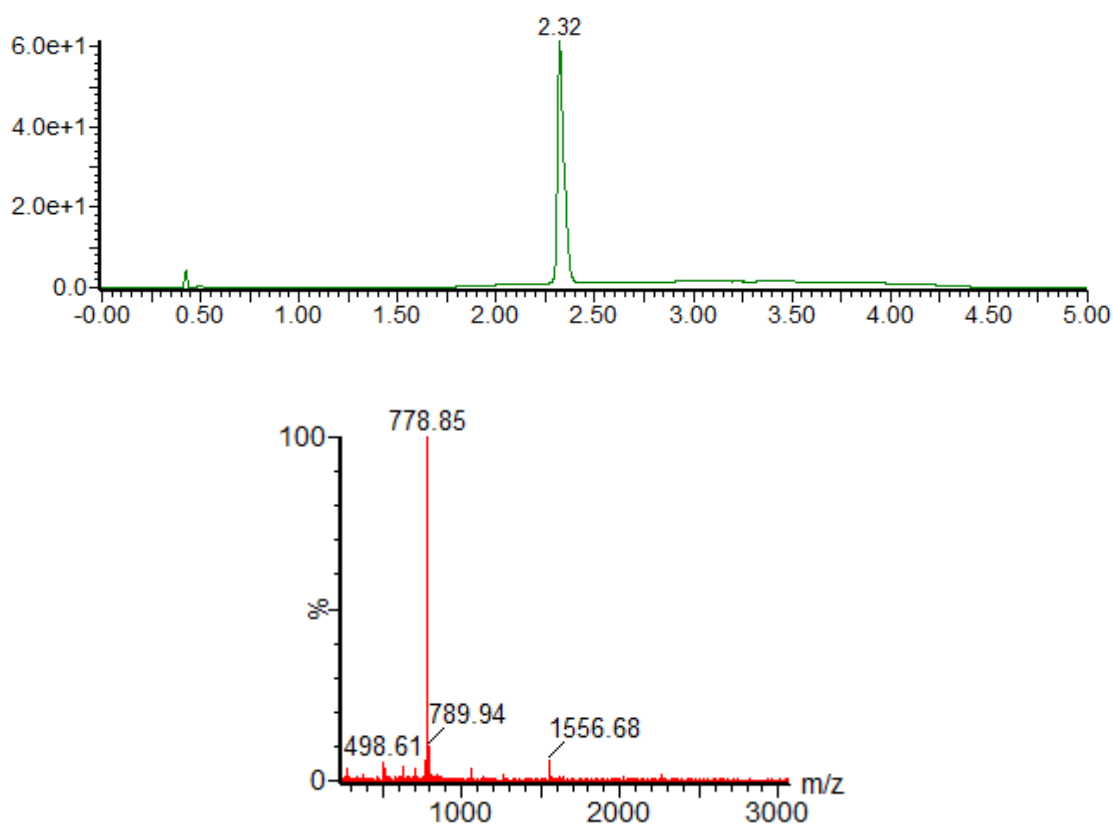


Figure S38: UV trace and corresponding MS trace from LC-MS analysis of the purified two-component product. Gradient: 5-95% ACN/H₂O with 0.1% TFA over 5 min at a

flow rate of 0.4 mL/min. ESI-MS calcd. for $C_{68}H_{106}N_{20}O_{22}$ = 1555.71; $[M+H]^+$ m/z = 1556.71, found 1556.68; $[M+H]^{2+}$ m/z = 778.86, found 778.85.

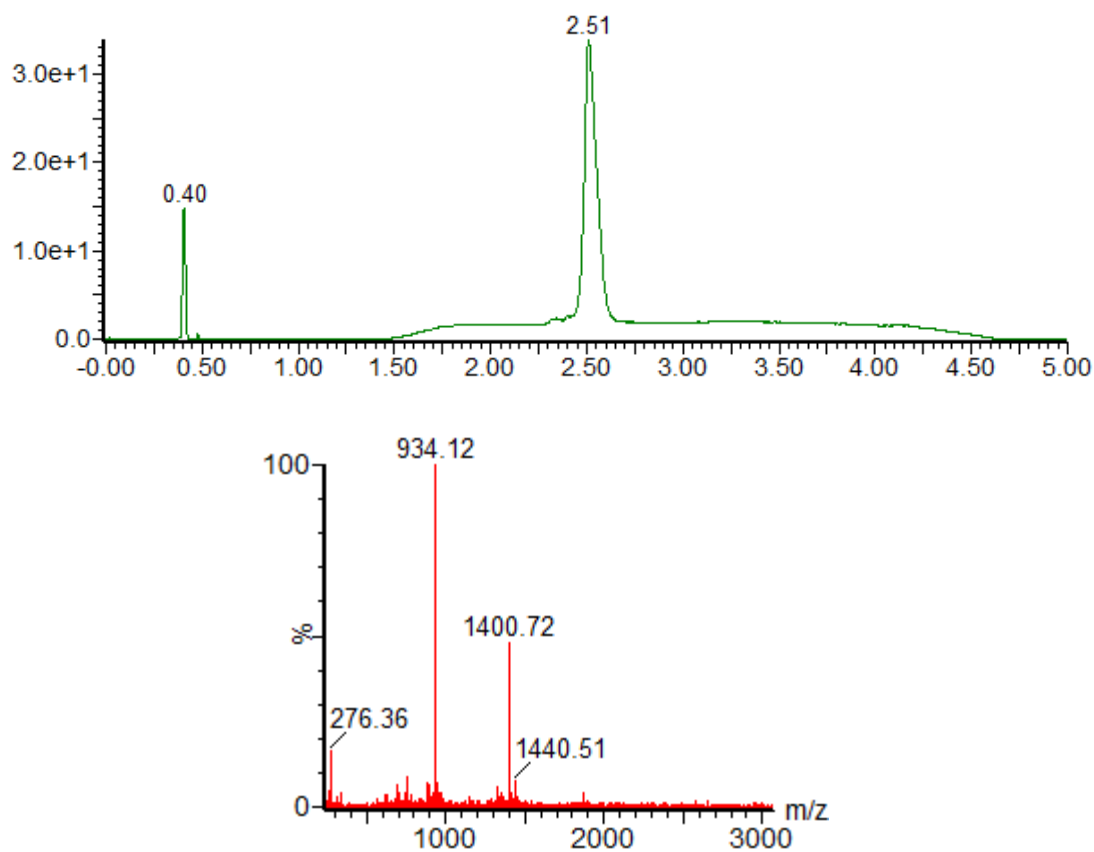
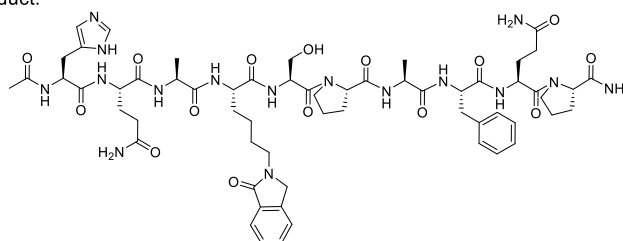


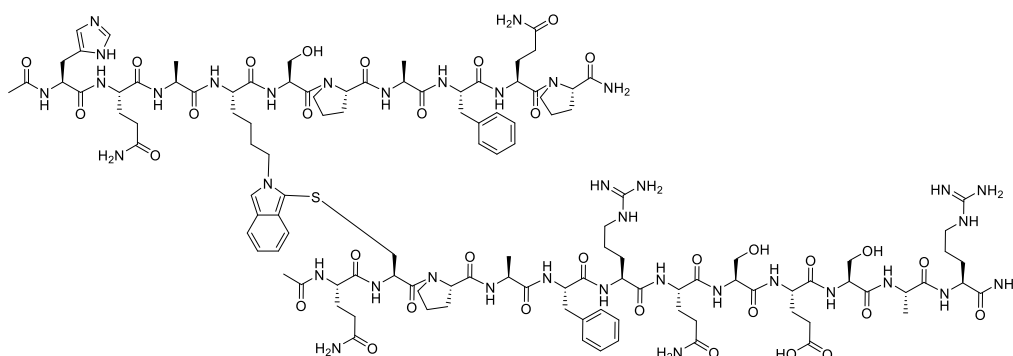
Figure S39: UV trace and corresponding MS trace from LC-MS analysis of the purified **PPC-7**. Gradient: 5-95% ACN/H₂O with 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. ESI-MS calcd. for $C_{122}H_{188}N_{36}O_{38}S$ = 2799.12; $[M+H]^{2+}$ m/z = 1400.56, found 1400.72; $[M+H]^{3+}$ m/z = 934.04, found 934.12.

PPC-8 (Ac-QCPAFRQSESAR-NH₂ + Ac-HQAKSPAFAQP-NH₂)

Two-component product:



Three-component product:



No guanidine conditions

The reaction was carried out at 0.0018 mmol scale according to general procedure 2.6, conditions [A]. The crude reaction mixture was purified by preparative reverse-phase HPLC (10-80% CH₃CN/H₂O over 35 min) and lyophilized to afford the two-component product (0.50 mg, 21% yield) and the three-component product (1.15 mg, 24% yield).

Guanidine-added conditions

The reaction was carried out at 0.0018 mmol scale according to general procedure 2.6, conditions [B] with 3 M guanidine. The crude reaction mixture was purified by preparative reverse-phase HPLC (10-80% CH₃CN/H₂O over 35 min) and lyophilized to afford the three-component product (2.50 mg, 52% yield).

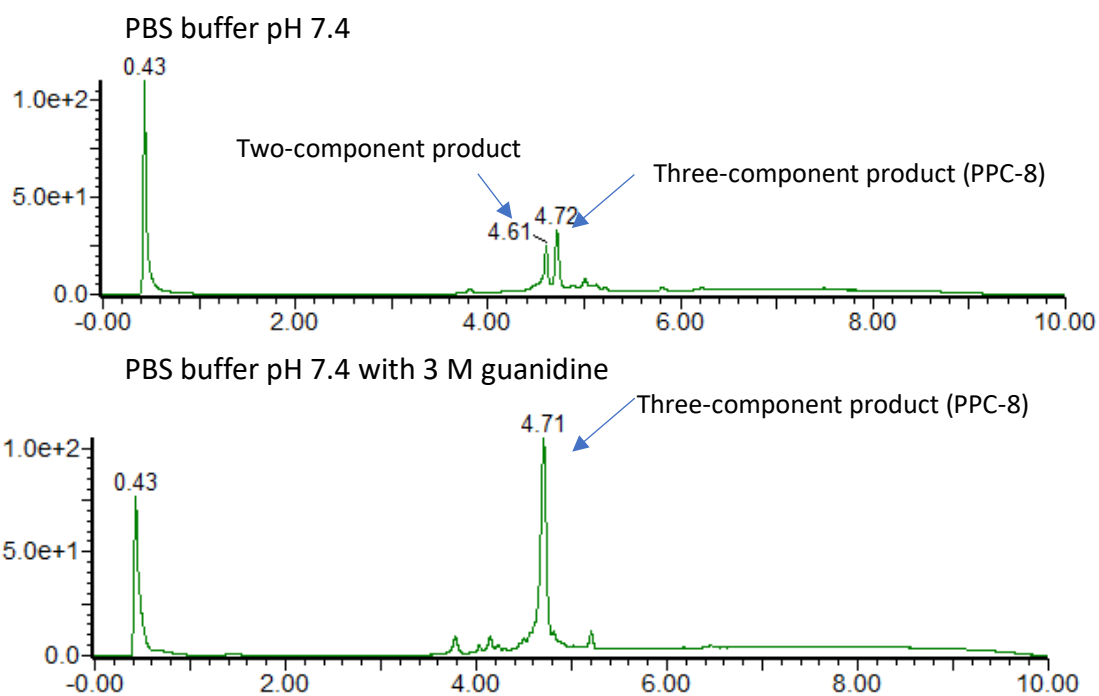


Figure S40: UV trace of the crude reaction mixture. Gradient: 5-95% ACN/H₂O with 0.1% TFA over 10 min at a flow rate of 0.4 mL/min.

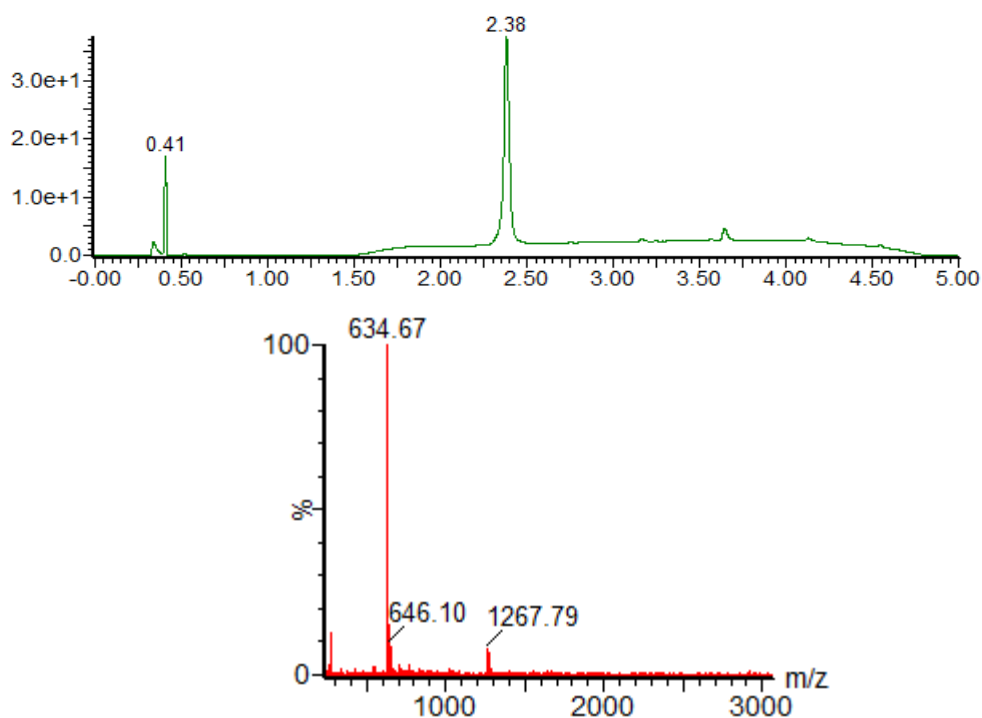


Figure S41: UV trace and corresponding MS trace from LC-MS analysis of the purified two-component product. Gradient: 5-95% ACN/H₂O with 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. ESI-MS calcd. for C₆₀H₈₂N₁₆O₁₅ = 1267.41; [M+H]⁺ m/z = 1268.41, found 1267.79; [M+H]²⁺ m/z = 634.71, found 634.67.

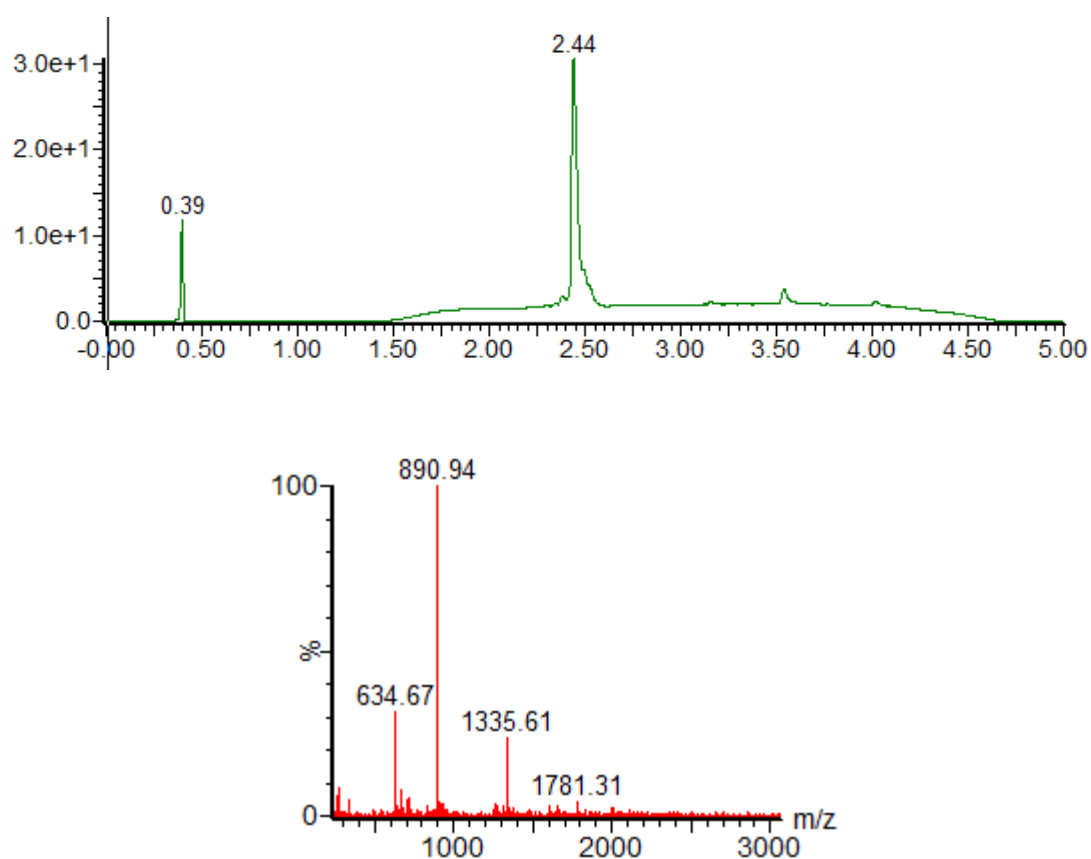
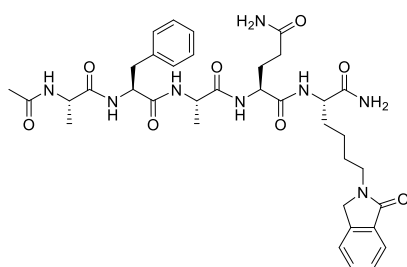


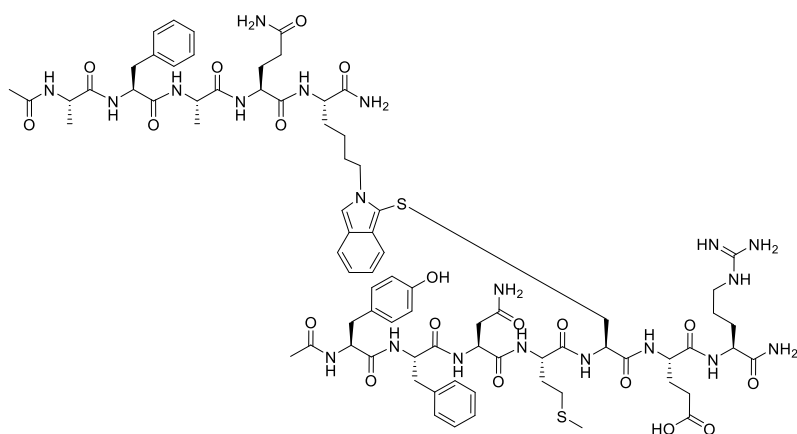
Figure S42: UV trace and corresponding MS trace from LC-MS analysis of the purified **PPC-8**. Gradient: 5-95% ACN/H₂O with 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. ESI-MS calcd. for C₁₁₈H₁₇₃N₃₇O₃₃S = 2669.97; [M+H]²⁺ m/z = 1335.99, found 1335.61; [M+H]³⁺ m/z = 890.99, found 890.94.

PPC-9 (Ac-YFNM CER-NH₂ + Ac-AFAQK-NH₂)

Two-component product:



Three-component product:



No guanidine conditions

The reaction was carried out at 0.0027 mmol scale according to general procedure 2.6, conditions [A]. The crude reaction mixture was purified by preparative reverse-phase HPLC (20-45% CH₃CN/H₂O over 35 min) and lyophilized to afford the two-component product (0.51 mg, 26% yield) and the three-component product (1.11 mg, 24% yield).

Guanidine-added conditions

The reaction was carried out at 0.0027 mmol scale according to general procedure 2.6, conditions [B] with 3 M guanidine. The crude reaction mixture was purified by preparative reverse-phase HPLC (20-45% CH₃CN/H₂O over 35 min) and lyophilized to afford the three-component product (2.21 mg, 47% yield).

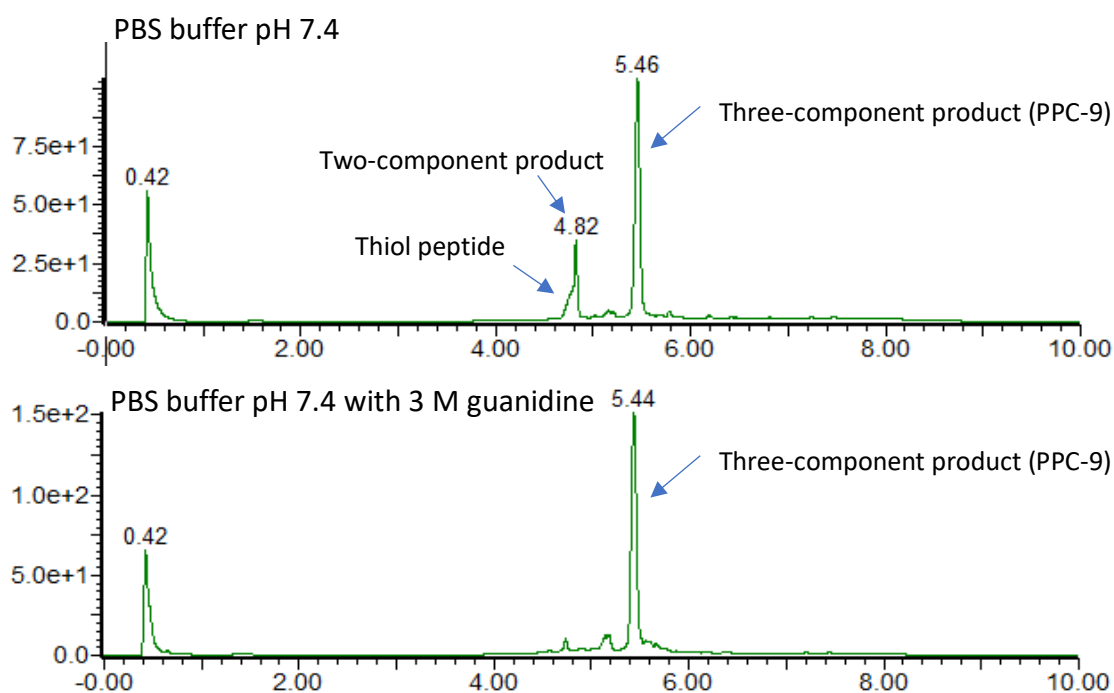


Figure S43: UV trace of the crude reaction mixture. Gradient: 5-95% ACN/H₂O with 0.1% TFA over 10 min at a flow rate of 0.4 mL/min.

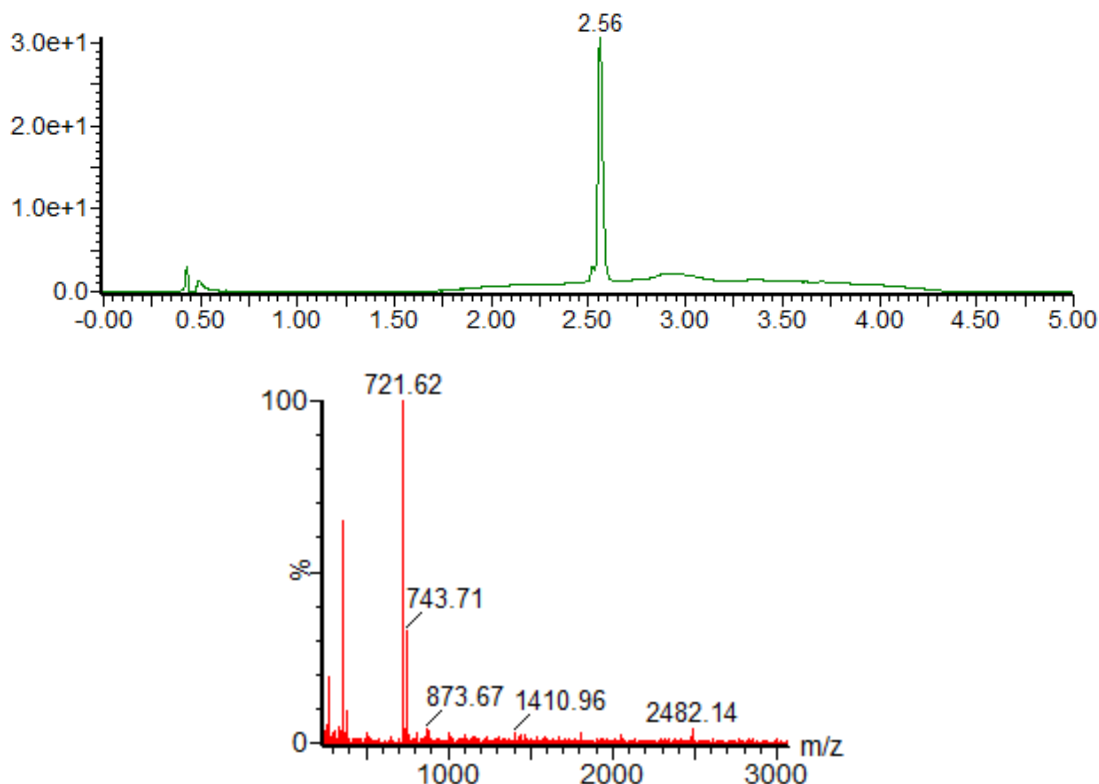


Figure S44: UV trace and corresponding MS trace from LC-MS analysis of the purified two-component product. Gradient: 5-95% ACN/H₂O with 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. ESI-MS calcd. for C₃₆H₄₈N₈O₈ = 720.83; [M+H]²⁺ m/z = 721.83, found 721.62.

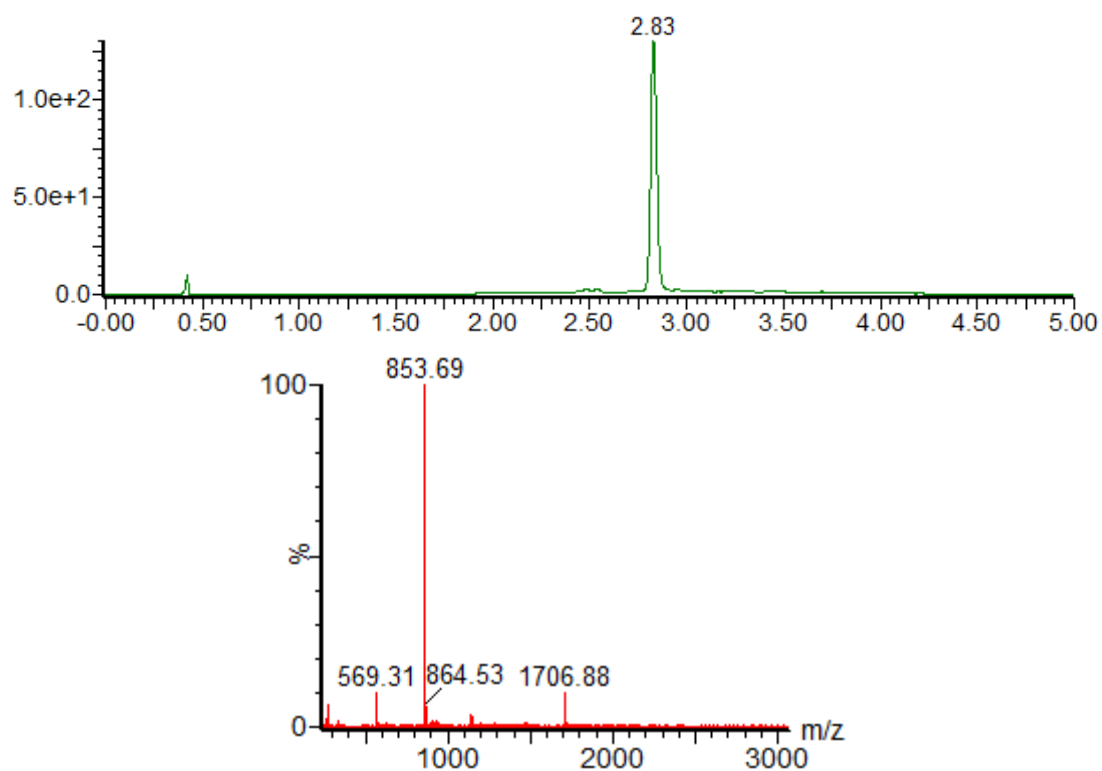
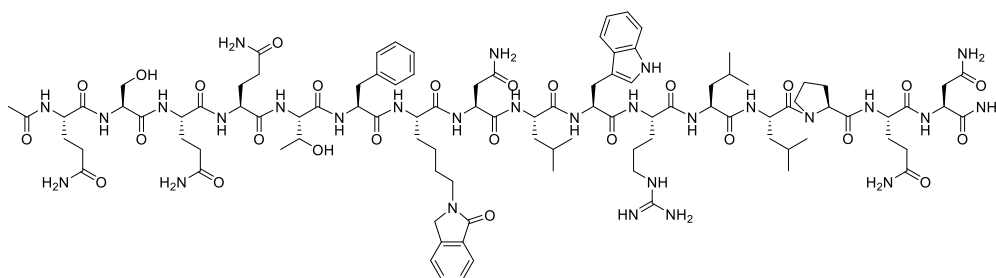


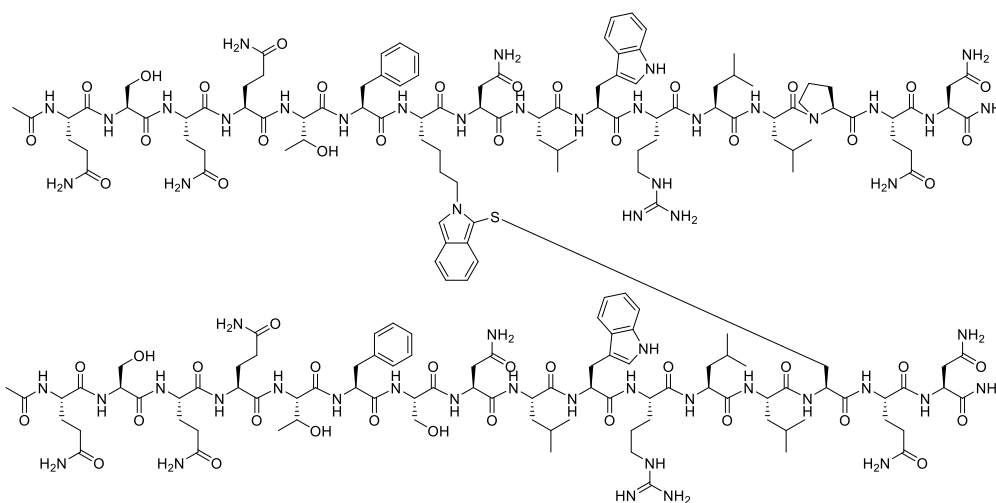
Figure S45: UV trace and corresponding MS trace from LC-MS analysis of the purified **PPC-9**. Gradient: 5-95% ACN/H₂O with 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. ESI-MS calcd. for C₇₉H₁₀₈N₂₀O₁₉S₂ = 1705.97; [M+H]⁺ m/z = 1706.97, found 1706.88; [M+H]²⁺ m/z = 853.99, found 853.69.

PPC-10 (Ac-QSQQTFSNLWRLLCQN-NH₂ + Ac-QSQQTFKNLWRLLPQN-NH₂)

Two-component product:



Three-component product:



No guanidine conditions

The reaction was carried out at 0.0013 mmol scale according to general procedure 2.6, conditions [A]. The crude reaction mixture was purified by preparative reverse-phase HPLC (10-80% CH₃CN/H₂O over 35 min) and lyophilized to afford the two-component product (0.74 mg, 27% yield) and the three-component product (0.86 mg, 16% yield).

Guanidine-added conditions

The reaction was carried out at 0.0013 mmol scale according to general procedure 2.6, conditions [B] with 3 M guanidine. The crude reaction mixture was purified by preparative reverse-phase HPLC (10-80% CH₃CN/H₂O over 35 min) and lyophilized to afford the three-component product (2.25 mg, 42% yield).

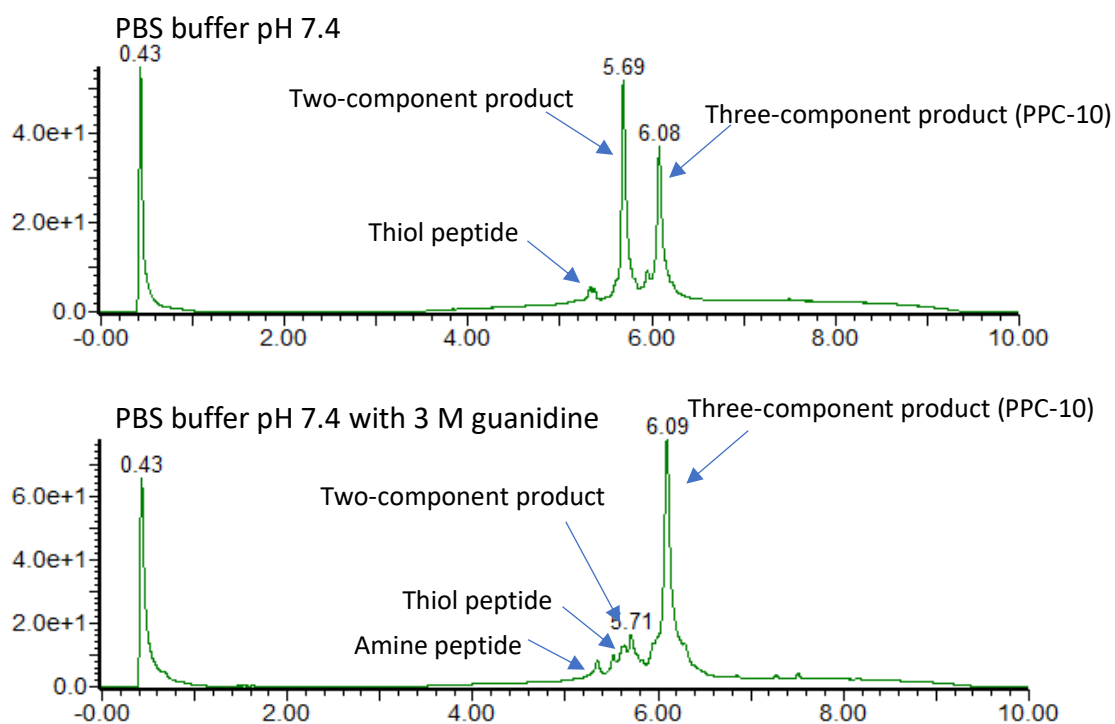


Figure S46: UV trace of the crude reaction mixture. Gradient: 5-95% ACN/H₂O with 0.1% TFA over 10 min at a flow rate of 0.4 mL/min.

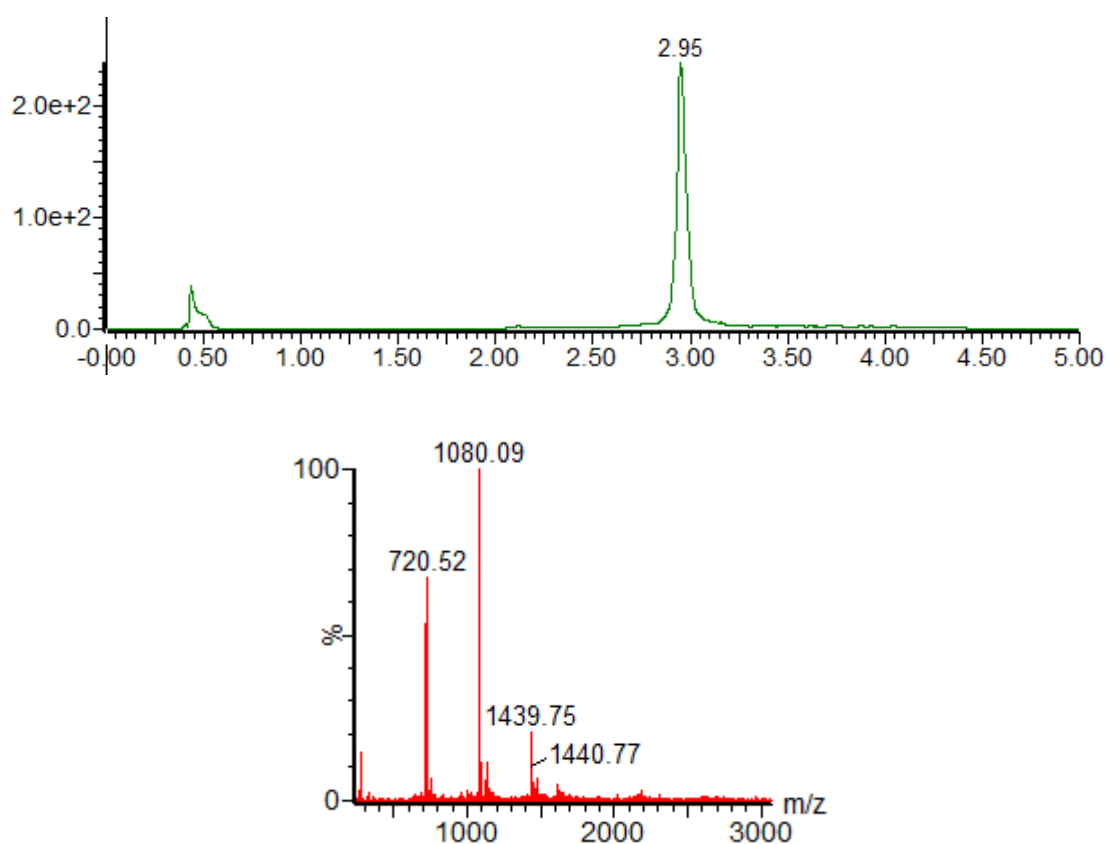


Figure S47: UV trace and corresponding MS trace from LC-MS analysis of the purified

two-component product. 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 C₁₀₀H₁₄₈N₂₈O₂₆ =2158.45; [M+H]²⁺ m/z =1080.22, found 1080.09; [M+H]³⁺ m/z =720.48, found 720.52.

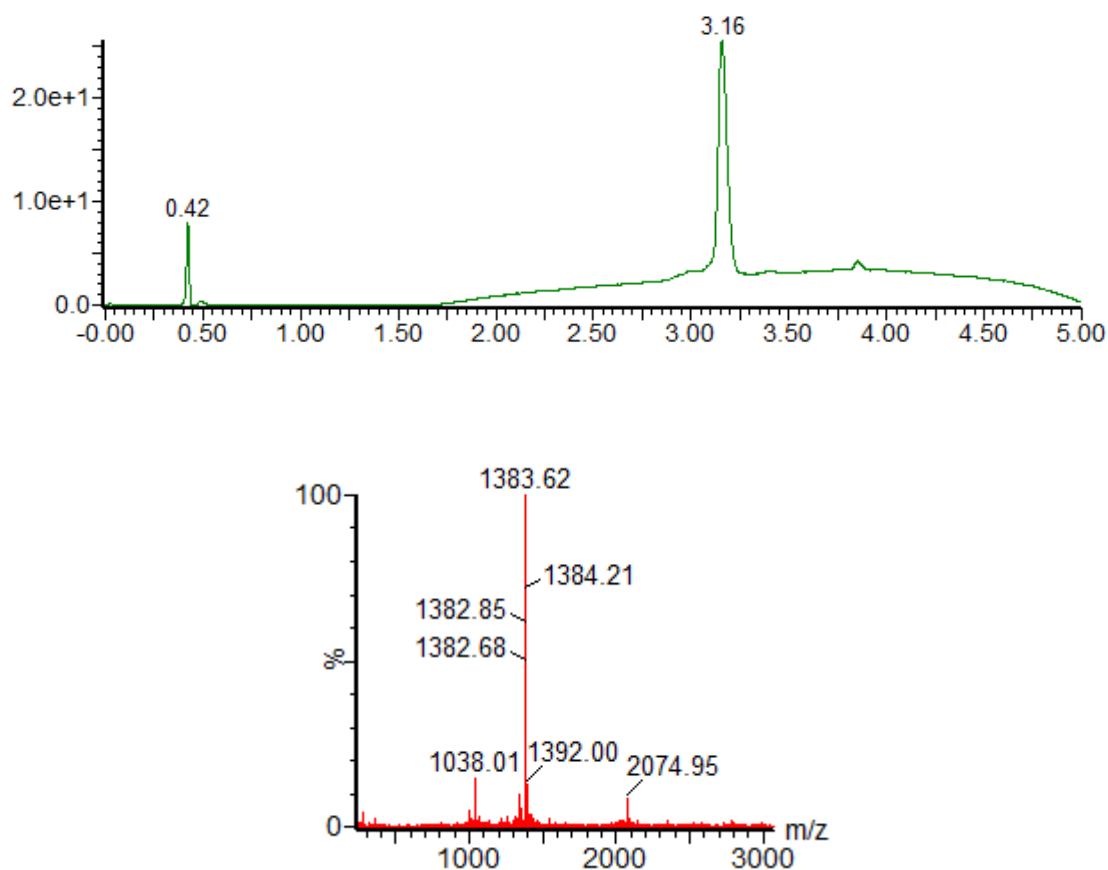




Figure S48: UV trace and corresponding MS trace from LC-MS analysis of the purified **PPC-10**. Gradient: 5-95% ACN/H₂O with 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. ESI-MS calcd. for C₁₈₇H₂₈₁N₅₅O₅₁S =4147.70; [M+H]²⁺ m/z =2074.85, found 2074.95; [M+H]³⁺ m/z =1383.57, found 1383.62; [M+H]⁴⁺ m/z =1037.93, found 1038.01.

PPC-11 (Ac-QCPAFRQSESAQ-NH₂ + Ac-C(StBu)AFAQK-NH₂)

[illegible]

SEC chromatogram of PPC-11 in PBS buffer pH 7.4 with 3 M guanidine. The x-axis represents elution volume from -0.00 to 10.00. The y-axis represents intensity from 0.0 to 8.0e+1. Two peaks are labeled: 0.36 and 5.67. A blue arrow points to the peak at 5.67, which is labeled "Three-component product (PPC-11)".



The chromatogram displays detector response over a 5.00-minute period. The y-axis represents intensity, with major ticks at 0.0, 2.0e+1, and 4.0e+1. Two peaks are identified: a small peak at 0.40 minutes and a large, sharp peak at 2.76 minutes. The baseline is relatively flat with minor noise.

Retention Time (min)	Approximate Intensity
0.40	1.0e+1
2.76	4.5e+1

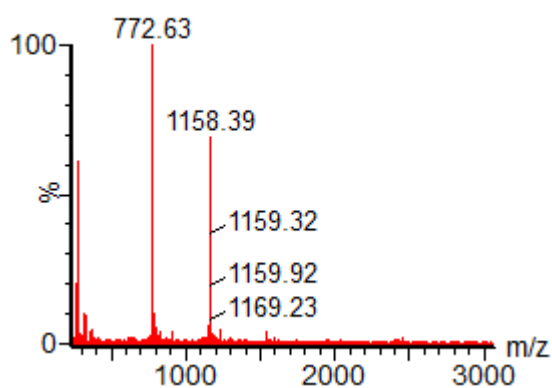
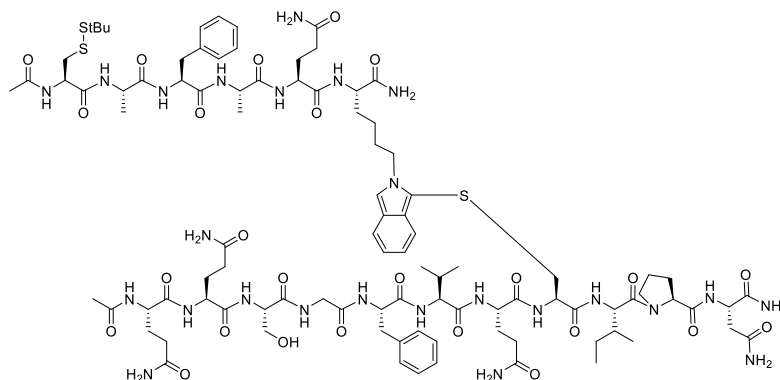


Figure S50: UV trace and corresponding MS trace from LC-MS analysis of the purified **PPC-11**. Gradient: 5-95% ACN/H₂O with 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. ESI-MS calcd. for C₁₀₁H₁₅₂N₃₀O₂₇S₃ = 2314.69; [M+H]²⁺ m/z = 1158.35, found 1158.39; [M+H]³⁺ m/z = 772.50, found 772.63.

PPC-12 (Ac-QQSGFVQCIPN-NH₂ + Ac-C(StBu)AFAQK-NH₂)

Three-component product:



The reaction was carried out at 0.0017 mmol scale according to general procedure 2.6, conditions [B] with 3 M guanidine. The crude reaction mixture was purified by preparative reverse-phase HPLC (10-80% CH₃CN/H₂O over 35 min) and lyophilized to afford the three-component product (1.70 mg, 47% yield).

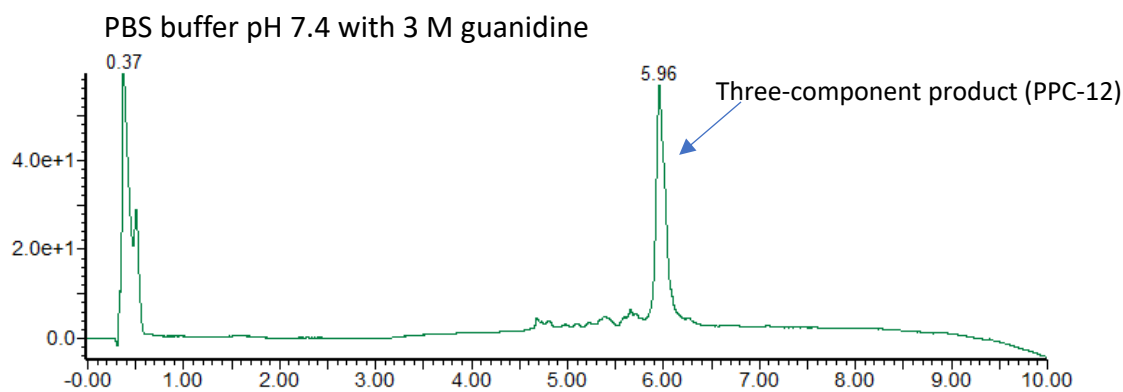


Figure S51: UV trace of the crude reaction mixture. Gradient: 5-95% ACN/H₂O with 0.1% TFA over 10 min at a flow rate of 0.4 mL/min.

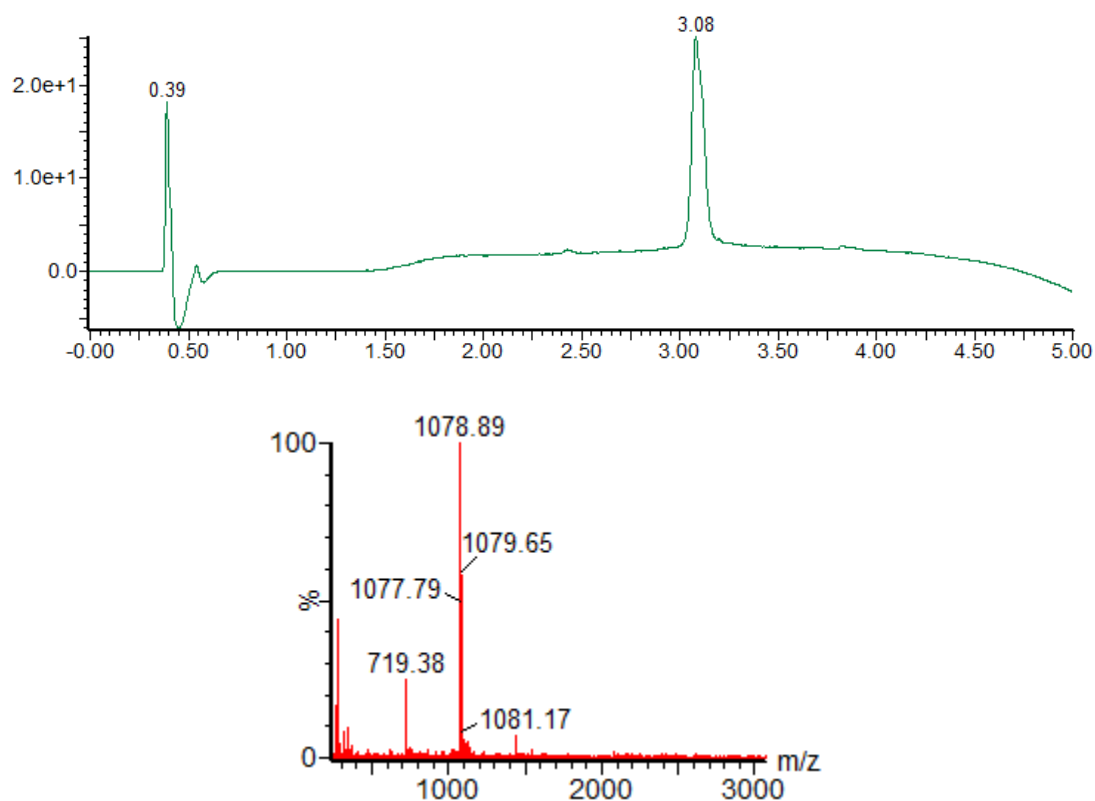
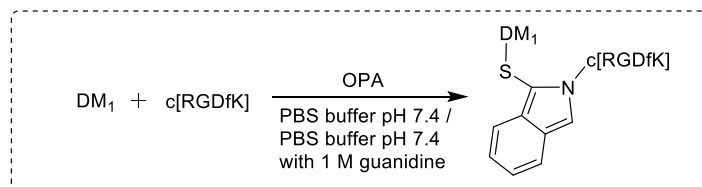


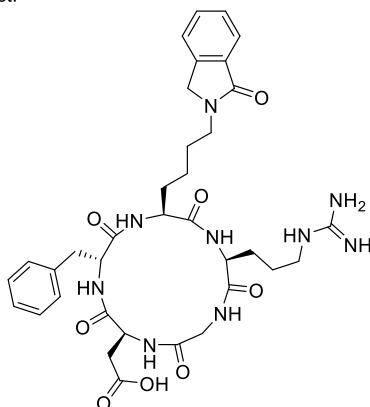
Figure S52: UV trace and corresponding MS trace from LC-MS analysis of the purified **PPC-12**. Gradient: 5-95% ACN/H₂O with 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. ESI-MS calcd. for C₉₇H₁₄₃N₂₅O₂₅S₃ = 2155.54; [M+H]²⁺ m/z = 1078.77, found 1078.89; [M+H]³⁺ m/z = 719.51, found 719.38.

5.2 Synthesis of peptide-drug conjugates PDC-1 to PDC-7

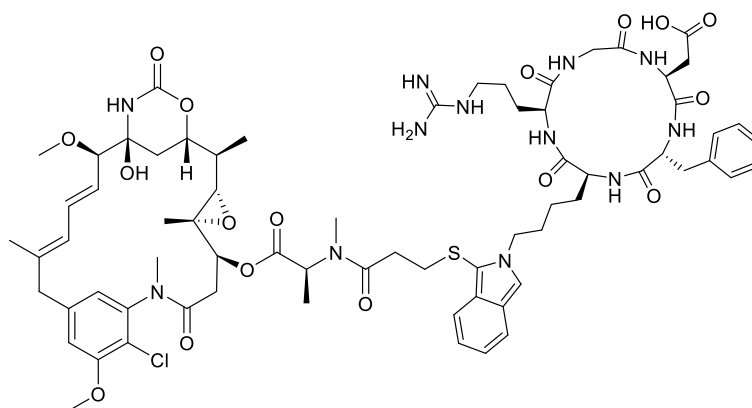
PDC-1 (DM1 + c[RGDfK])



Two-component product:



Three-component product:



Small scale reaction: No guanidine conditions

DM1 (0.5 mg, 0.0006 mmol, pre-dissolved in 3% ACN, MeOH and DMSO respectively) and OPA (0.09 mg, 0.0007 mmol, 9 μL stock solution in DMSO) was first added to PBS buffer pH 7.4, at a final concentration of 0.5 mM (1355 μL). Followed by the addition of c[RGDfK] peptide (0.54 mg, 0.0009 mmol) and reacted for 1 h.

Small scale reaction: Guanidine-added conditions

DM1 (0.5 mg, 0.0006 mmol, pre-dissolved in 3% ACN, MeOH and DMSO respectively) and OPA (0.09 mg, 0.0007 mmol, 9 μ L stock solution in DMSO) was first added to PBS buffer pH 7.4 with 3 M guanidine, at a final concentration of 0.5 mM (1355 μ L). Followed by the addition of c[RGDfK] peptide (0.54 mg, 0.0009 mmol) and reacted for 1 h.

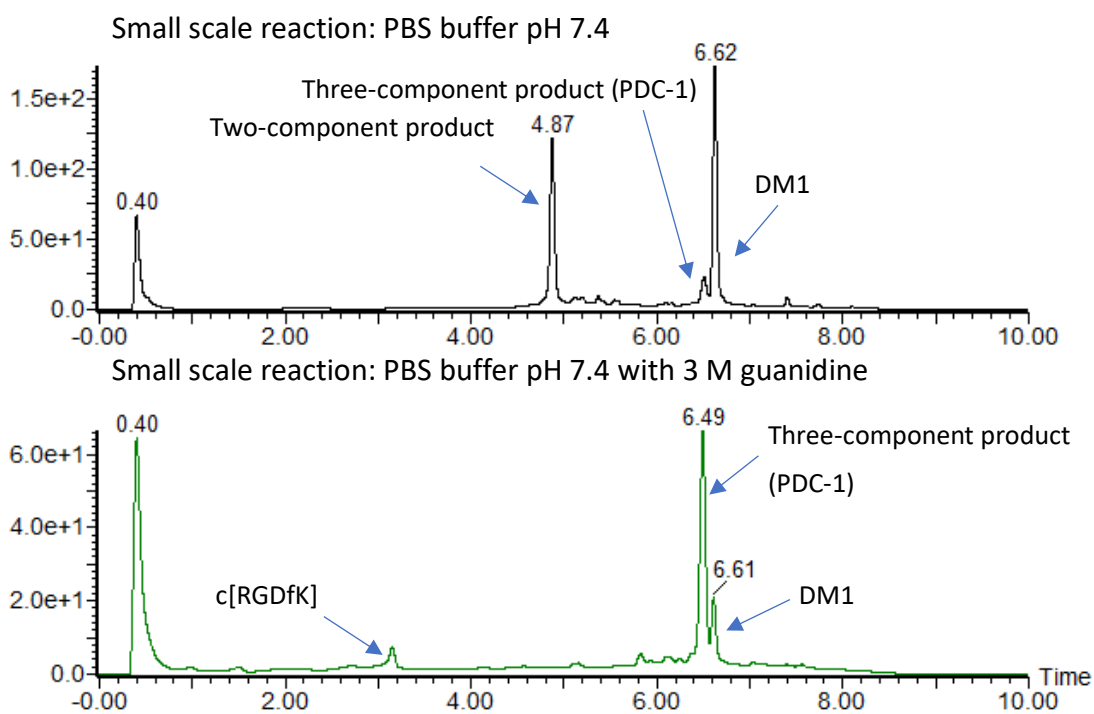


Figure S53: UV trace of the crude reaction mixture. Gradient: 5-95% ACN/H₂O with 0.1% TFA over 10 min at a flow rate of 0.4 mL/min.

Large scale reaction: The reaction was carried out at 0.0027 mmol scale according to general procedure 2.6, conditions [C]. The crude reaction mixture was purified by preparative reverse-phase HPLC (25-80% CH₃CN/H₂O over 35 min) and lyophilized to afford product (2.00 mg, 51% yield).

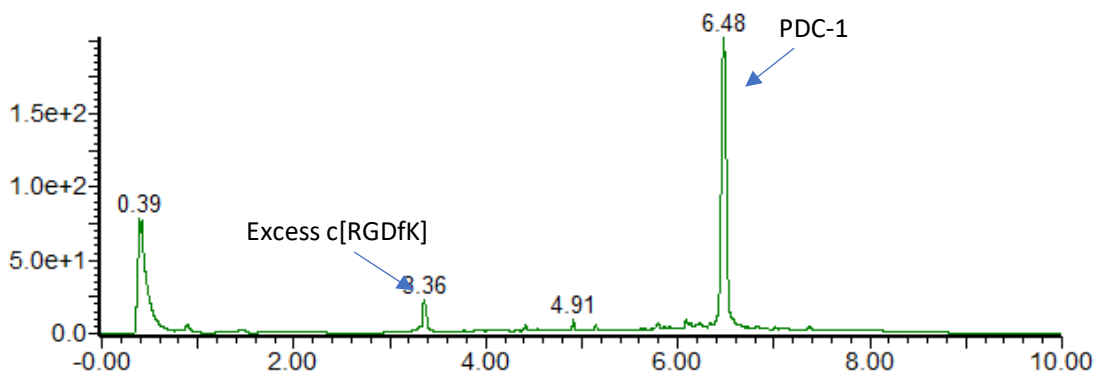


Figure S54: UV trace of the crude reaction mixture. Gradient: 5-95% ACN/H₂O with 0.1% TFA over 10 min at a flow rate of 0.4 mL/min.

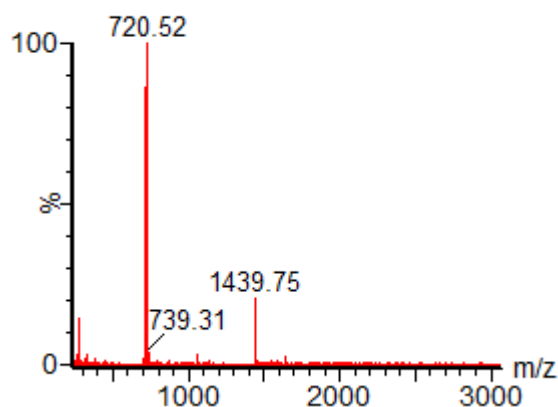
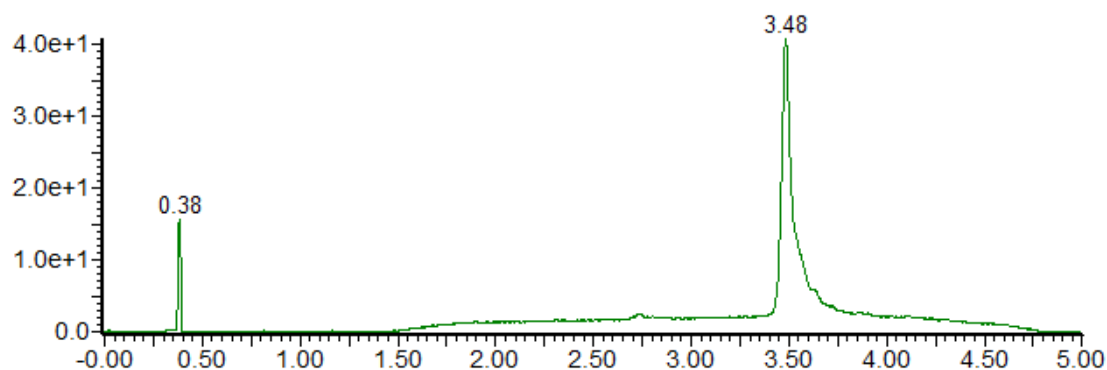
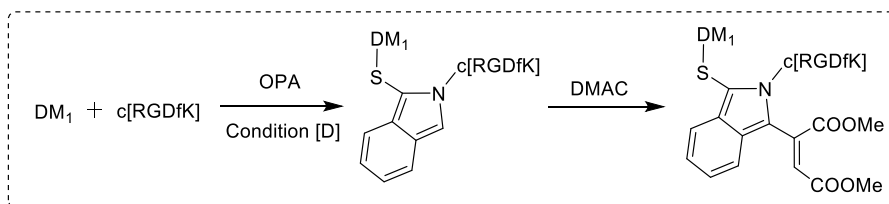
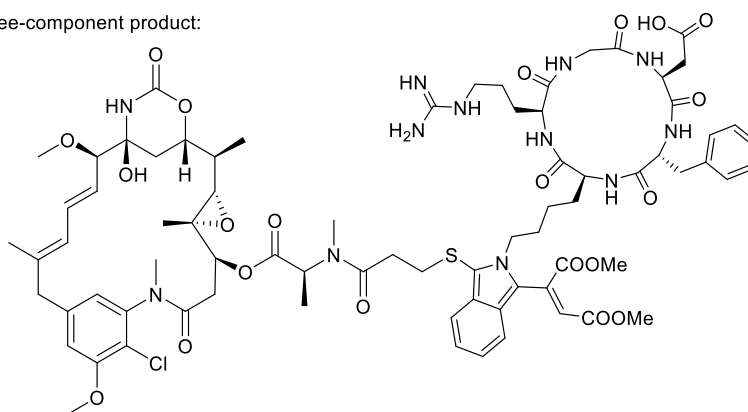


Figure S55: UV trace and corresponding MS trace from LC-MS analysis of the purified **PDC-1**. Gradient: 5-95% ACN/H₂O with 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. ESI-MS calcd. for C₇₀H₉₁ClN₁₂O₁₇S = 1438.60; [M+H]⁺ m/z = 1439.60, found 1439.75; [M+H]²⁺ m/z = 720.30, found 720.52.

PDC-2 (DM1 + c[RGDfK] + DMAC)



Three-component product:



The reaction was carried out at 0.0029 mmol scale according to general procedure 2.6, conditions [D]. The crude reaction mixture was purified by preparative reverse-phase HPLC (15-80% CH₃CN/H₂O over 35 min) and lyophilized to afford product (2.44 mg, 53% yield).

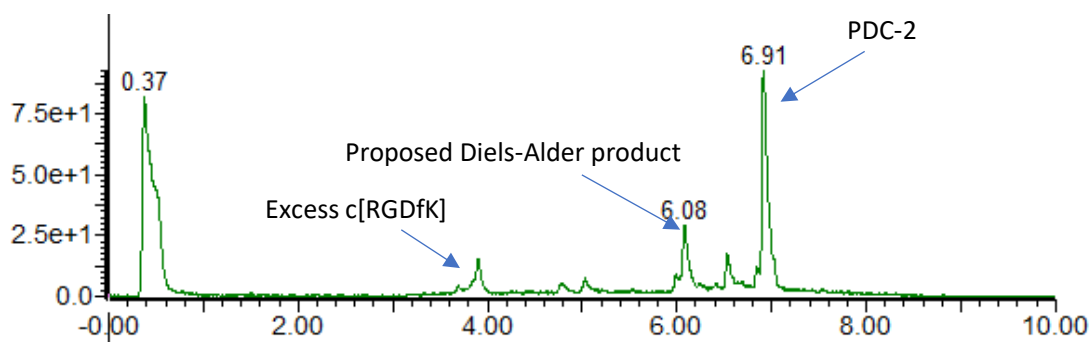


Figure S56: UV trace of the crude reaction mixture. Gradient: 5-95% ACN/H₂O with 0.1% TFA over 10 min at a flow rate of 0.4 mL/min.

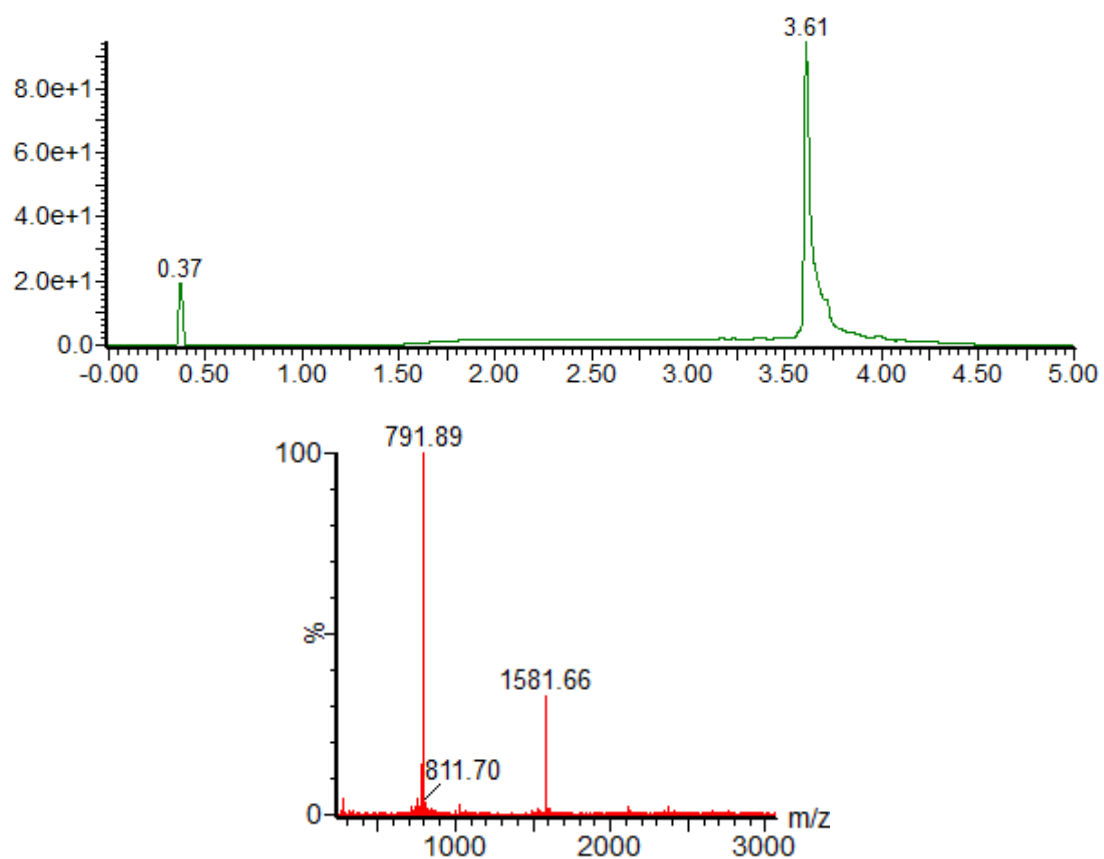
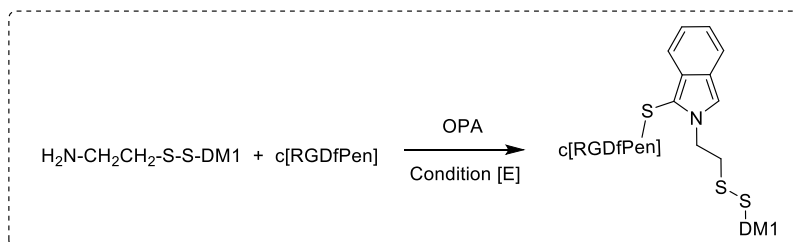
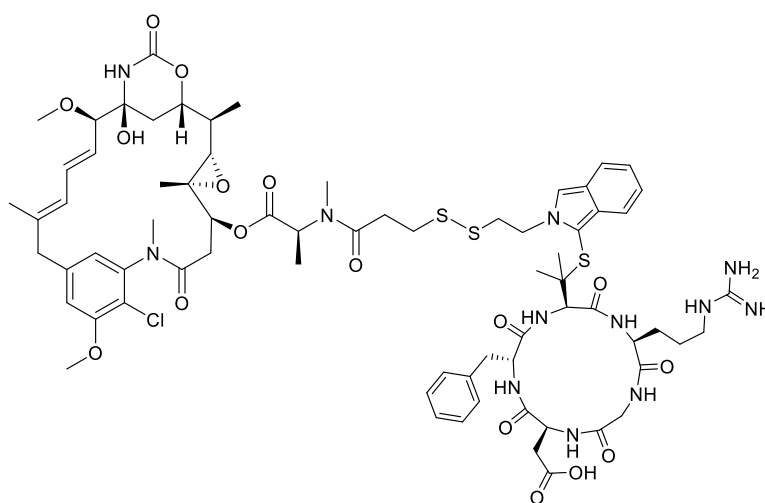


Figure S57: UV trace and corresponding MS trace from LC-MS analysis of the purified **PDC-2**. Gradient: 5-95% ACN/H₂O with 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. ESI-MS calcd. for C₇₆H₉₇ClN₁₂O₂₁S = 1580.63; [M+H]⁺ m/z = 1581.63, found 1581.66; [M+H]²⁺ m/z = 791.32, found 791.89.

PDC-3 (H₂N-CH₂CH₂-S-S-DM1 + c[RGDfPen])



Three-component product:



The reaction was carried out at 0.0021 mmol scale according to general procedure 2.6, conditions [E]. The crude reaction mixture was purified by preparative reverse-phase HPLC (25-80% CH₃CN/H₂O over 35 min) and lyophilized to afford product (1.44 mg, 46% yield).

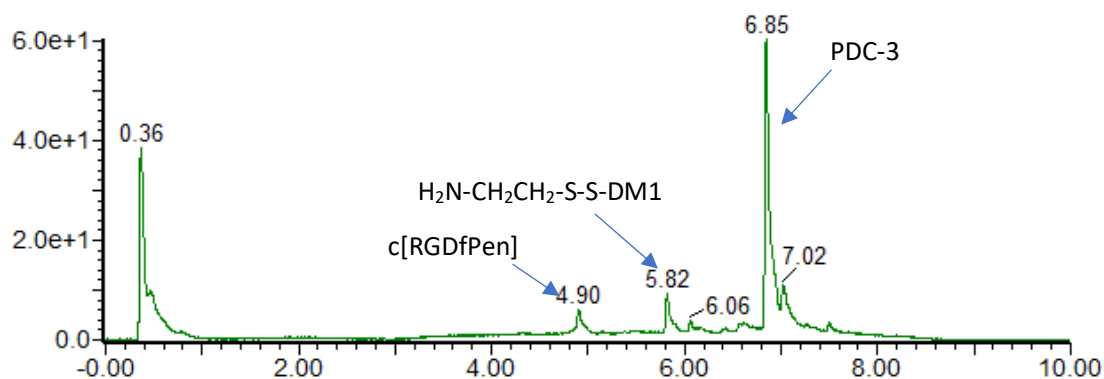


Figure S58: UV trace of the crude reaction mixture. Gradient: 5-95% ACN/H₂O with 0.1% TFA over 10 min at a flow rate of 0.4 mL/min.

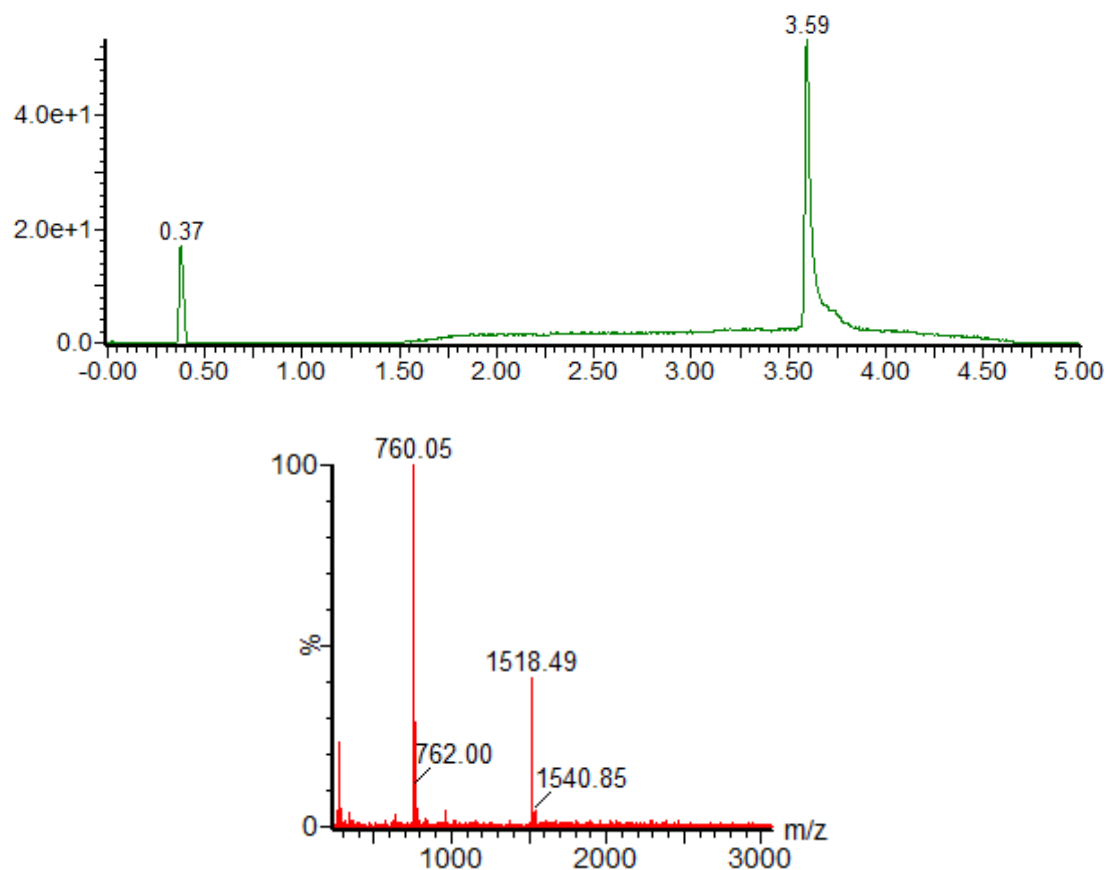
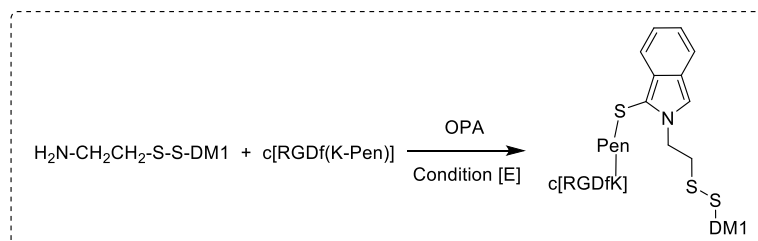
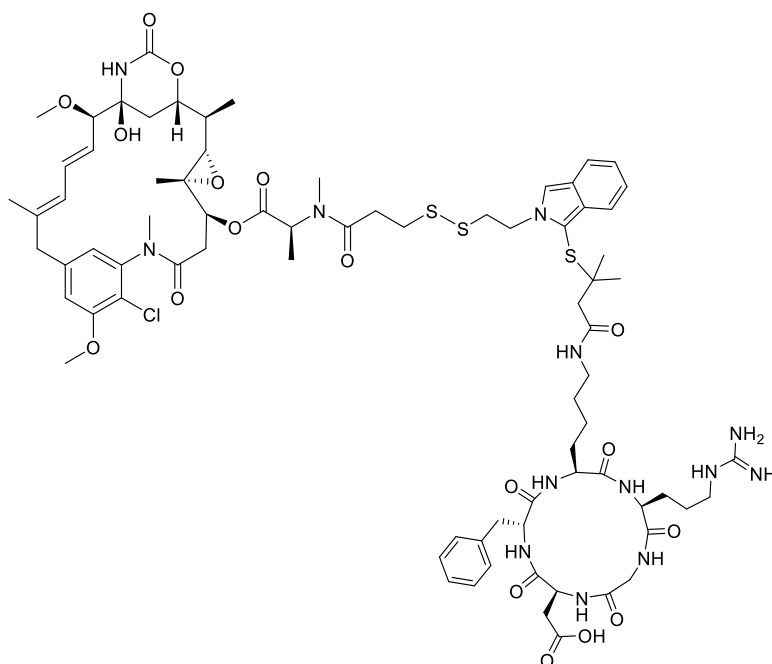


Figure S59: UV trace and corresponding MS trace from LC-MS analysis of the purified **PDC-3**. Gradient: 5-95% ACN/H₂O with 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. ESI-MS calcd. for C₇₁H₉₃ClN₁₂O₁₇S₃ = 1518.22; [M+H]⁺ m/z = 1519.22, found 1518.49; [M+H]²⁺ m/z = 760.11, found 760.05.

PDC-4 (H₂N-CH₂CH₂-S-S-DM1 + c[RGDf(K-Pen)])



Three-component product:



The reaction was carried out at 0.0024 mmol scale according to general procedure 2.6, conditions [E]. The crude reaction mixture was purified by preparative reverse-phase HPLC (25-80% CH₃CN/H₂O over 35 min) and lyophilized to afford product (1.8 mg, 46% yield).

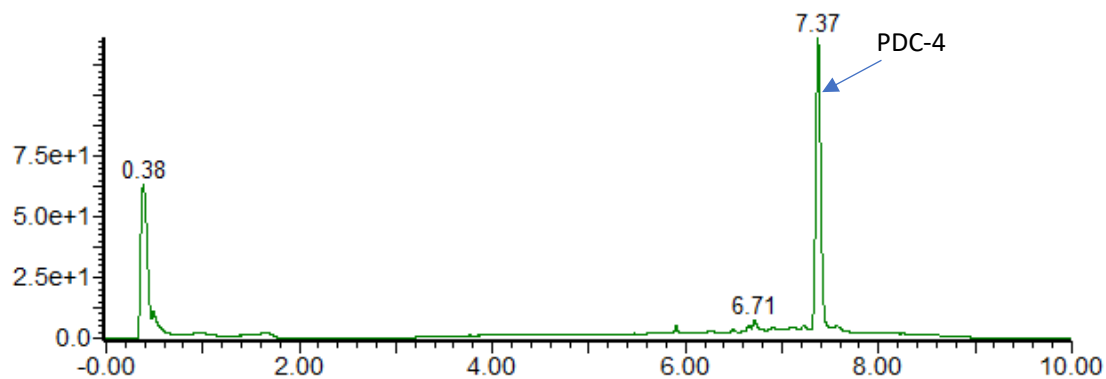


Figure S60: UV trace of the crude reaction mixture. Gradient: 5-95% ACN/H₂O with 0.1% TFA over 10 min at a flow rate of 0.4 mL/min.

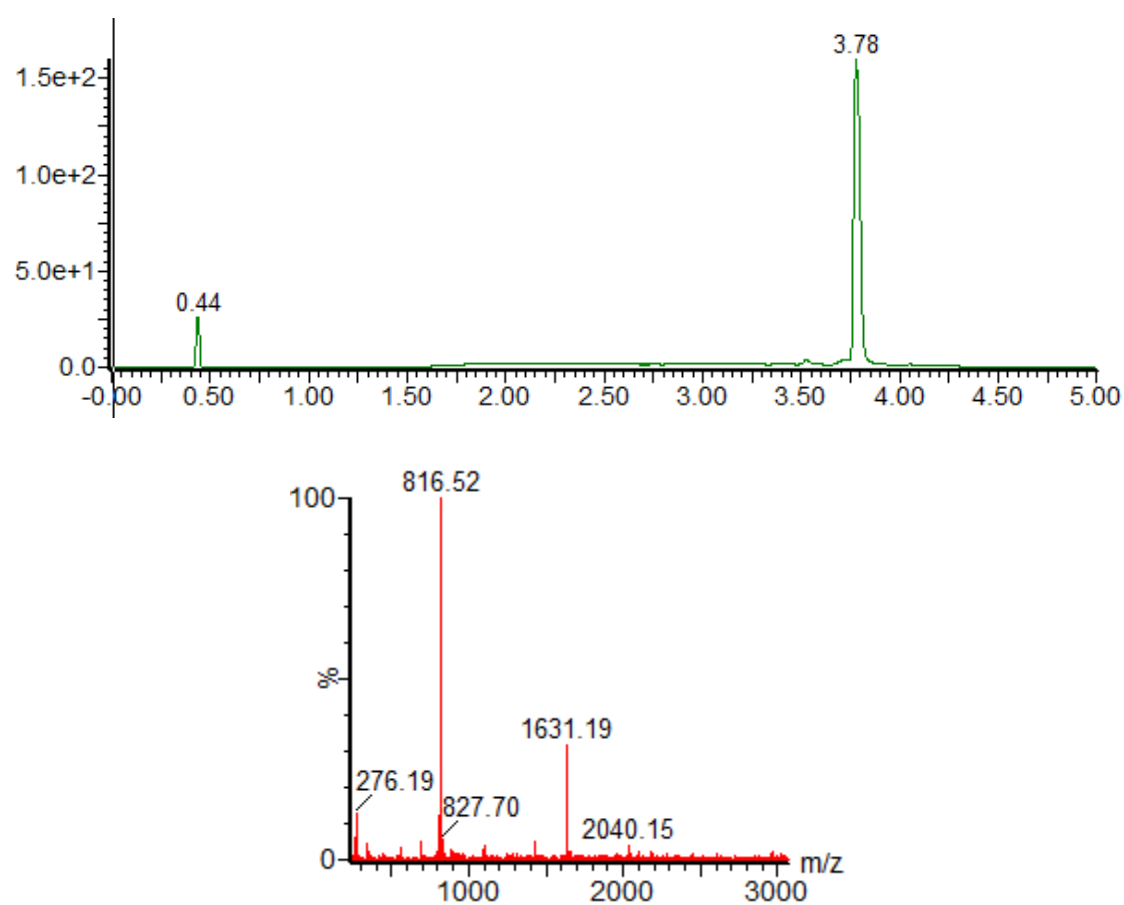
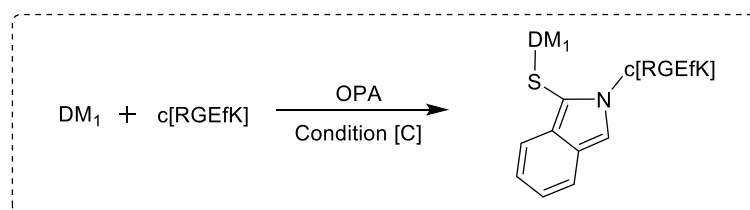
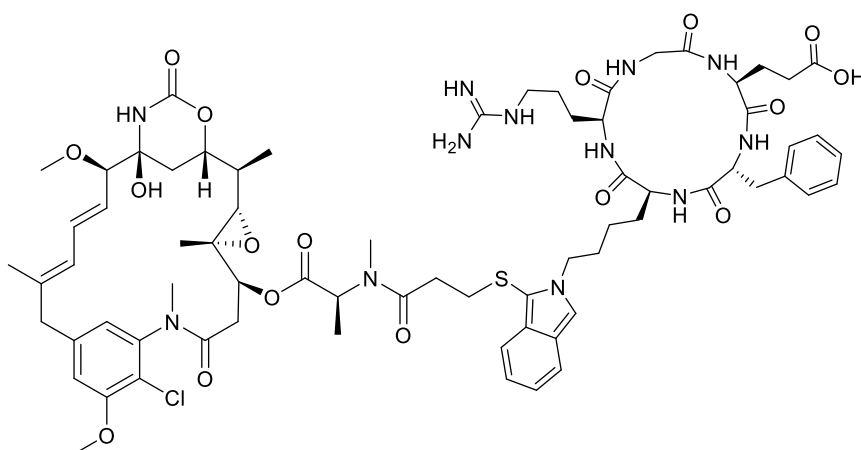


Figure S61: UV trace and corresponding MS trace from LC-MS analysis of the purified **PDC-4**. Gradient: 5-95% ACN/H₂O with 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. ESI-MS calcd. for C₇₇H₁₀₄ClN₁₃O₁₈S₃ = 1631.38; [M+H]⁺ m/z = 1632.38, found 1631.19; [M+H]²⁺ m/z = 816.69, found 816.52.

PDC-5 (H₂N-CH₂CH₂-S-S-DM1 + c[RGEfK])



Three-component product:



The reaction was carried out at 0.0081 mmol scale according to general procedure 2.6, conditions [C]. The crude reaction mixture was purified by preparative reverse-phase HPLC (25-80% CH₃CN/H₂O over 35 min) and lyophilized to afford product (5.61 mg, 48% yield).

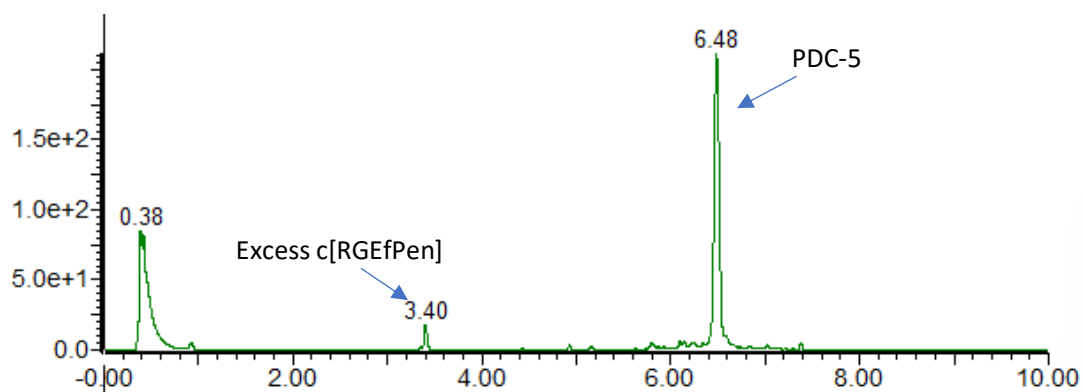


Figure S62: UV trace of the crude reaction mixture. Gradient: 5-95% ACN/H₂O with 0.1% TFA over 10 min at a flow rate of 0.4 mL/min.

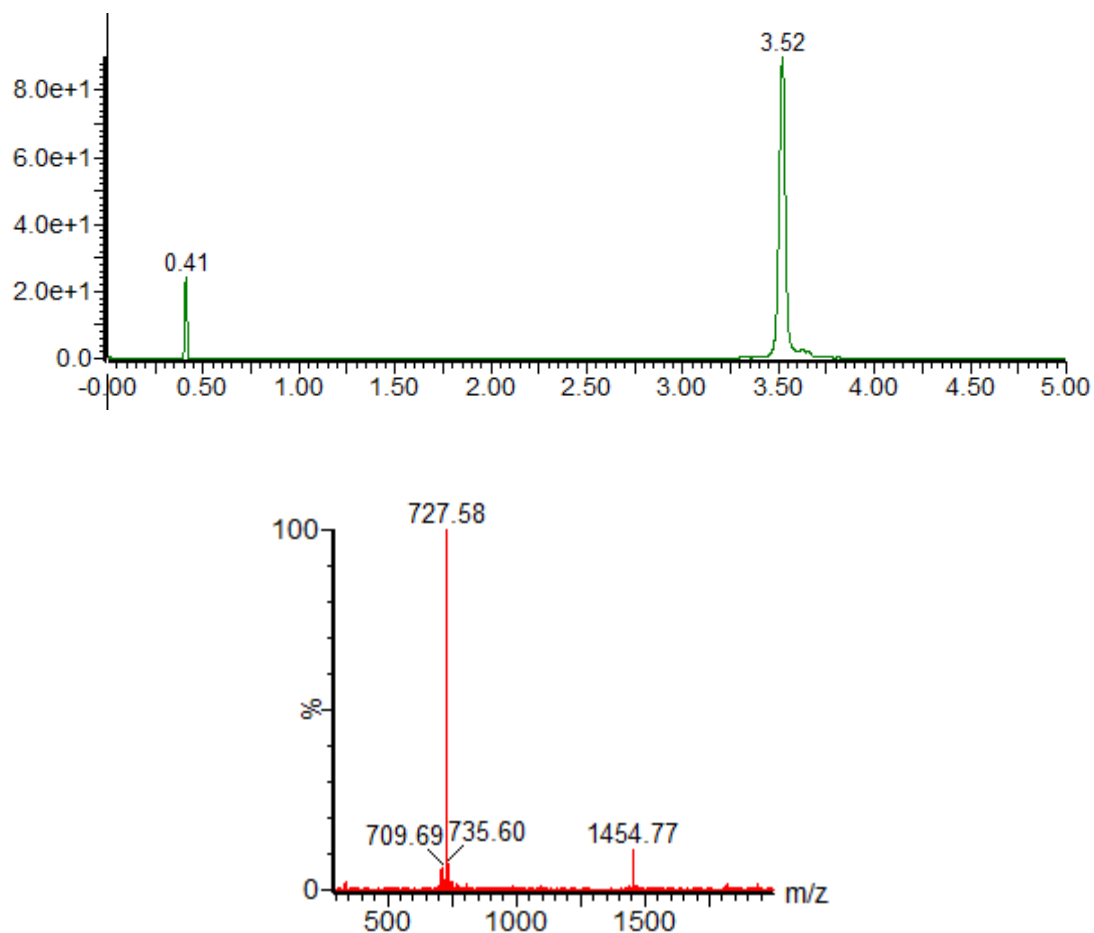
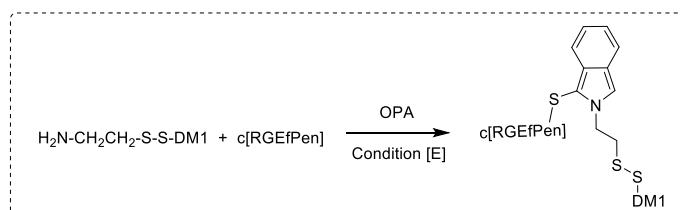
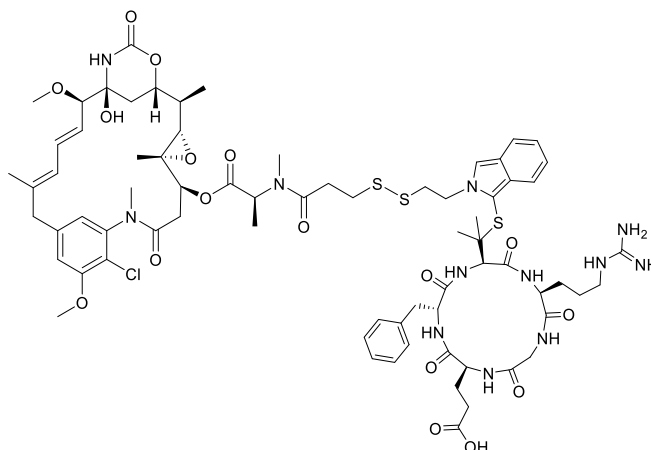


Figure S63: UV trace and corresponding MS trace from LC-MS analysis of the purified **PDC-5**. Gradient: 5-95% ACN/H₂O with 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. ESI-MS calcd. for C₇₁H₉₃ClN₁₂O₁₇S = 1454.10; [M+H]⁺ m/z = 1454.20, found 1454.77; [M+H]²⁺ m/z = 728.05, found 727.58.

PDC-6 (H₂N-CH₂CH₂-S-S-DM1 + c[RGEfPen])



Three-component product:



The reaction was carried out at 0.0046 mmol scale according to general procedure 2.6, conditions [E]. The crude reaction mixture was purified by preparative reverse-phase HPLC (25-80% CH₃CN/H₂O over 35 min) and lyophilized to afford product (2.76 mg, 40% yield).

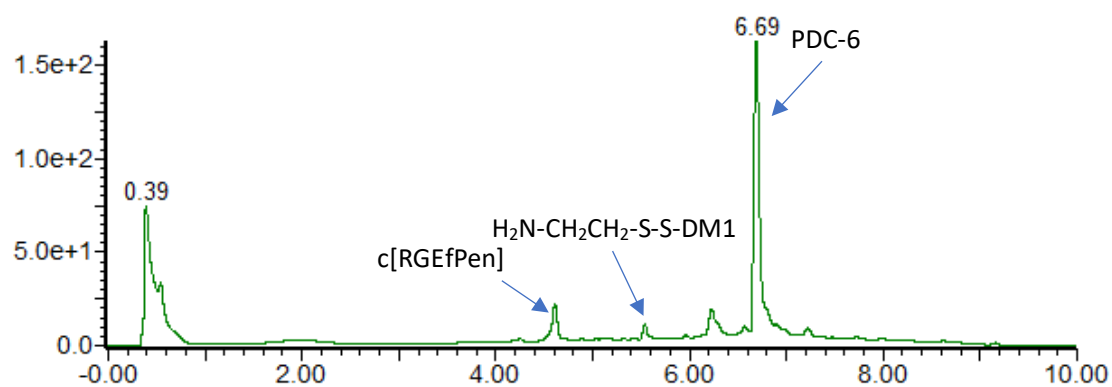


Figure S64: UV trace of the crude reaction mixture. Gradient: 5-95% ACN/H₂O with 0.1% TFA over 10 min at a flow rate of 0.4 mL/min.

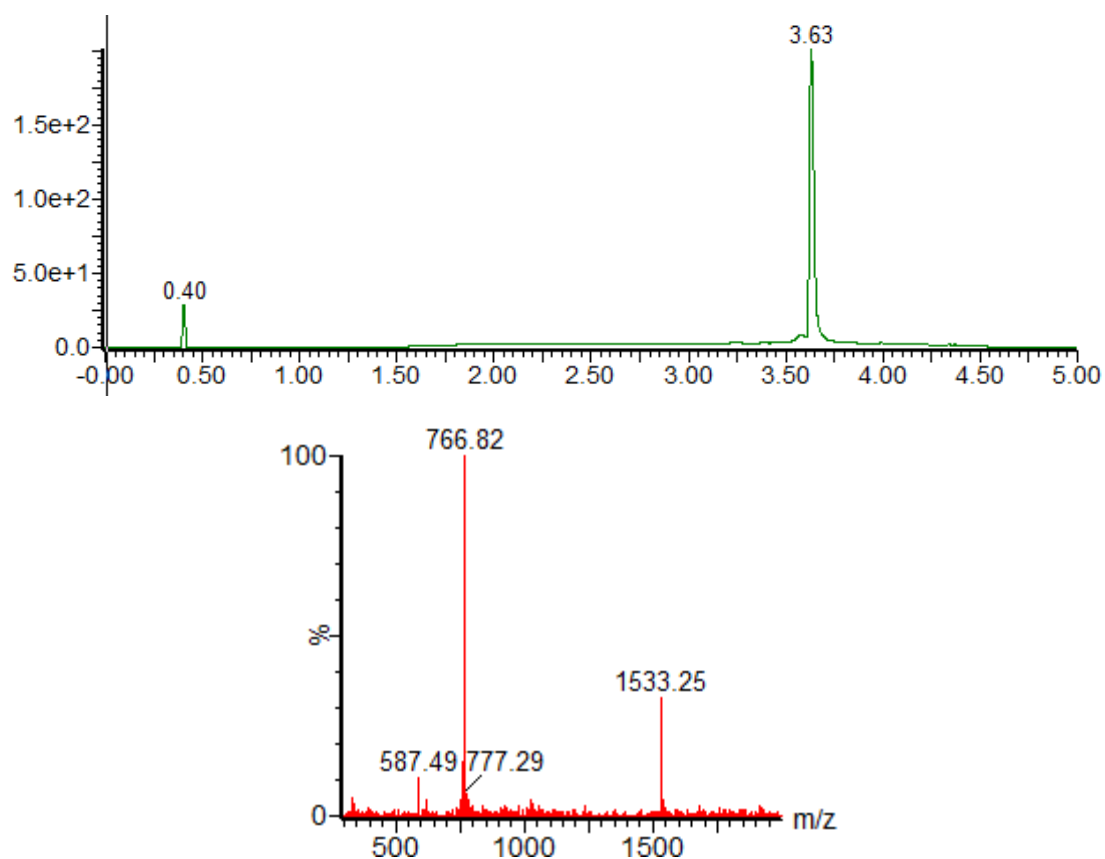
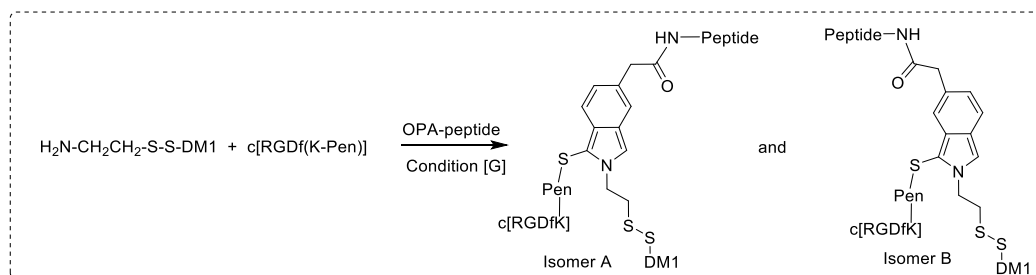
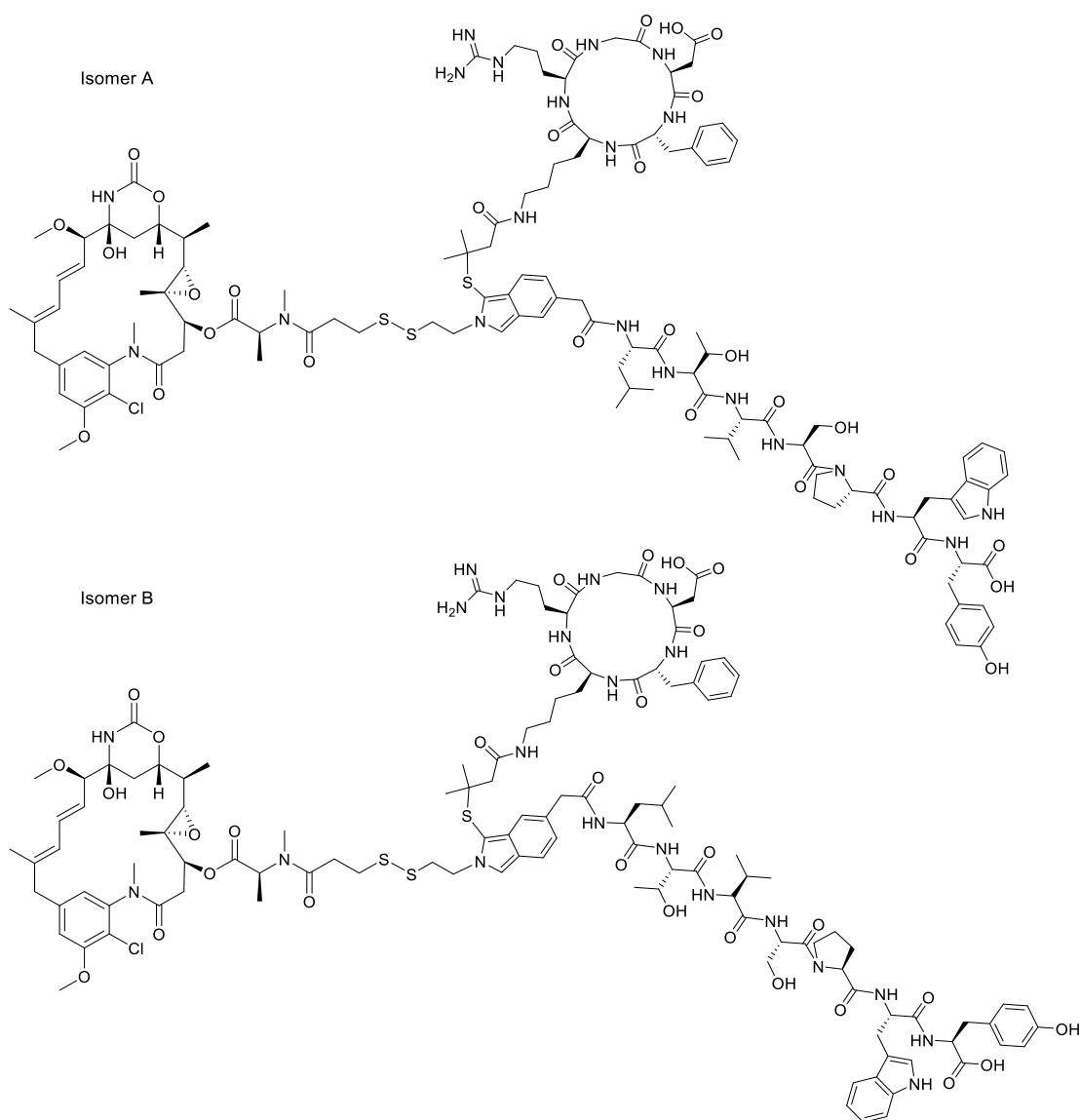


Figure S65: UV trace and corresponding MS trace from LC-MS analysis of the purified **PDC-6**. Gradient: 5-95% ACN/H₂O with 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. ESI-MS calcd. for C₇₂H₉₅ClN₁₂O₁₇S₃ = 1532.25; [M+H]⁺ m/z = 1533.25, found 1533.25; [M+H]²⁺ m/z = 767.13, found 766.82.

PDC-7 (H₂N-CH₂CH₂-S-S-DM1 + c[RGDf(K-Pen)] + OPA-peptide)



Three-component products:



The reaction was carried out at 0.0012 mmol scale according to general procedure 2.6, conditions [F]. The crude reaction mixture was purified by preparative reverse-phase HPLC (35-60% CH₃CN/H₂O over 35 min) and lyophilized to afford product as two isomers (0.62 mg + 0.76 mg, 46% yield).

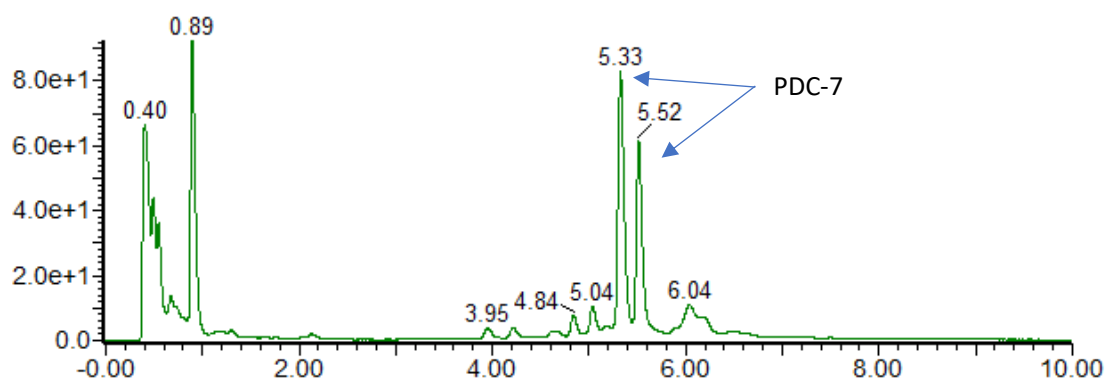
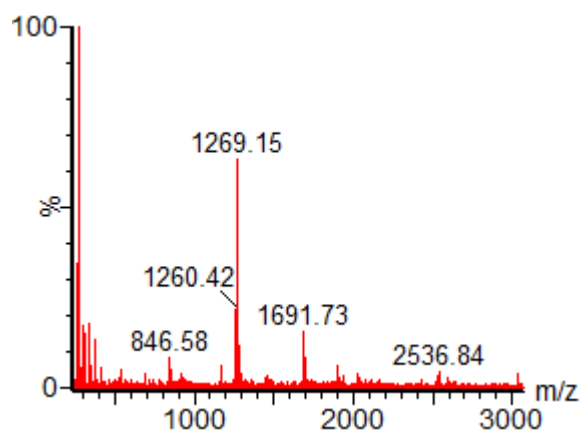
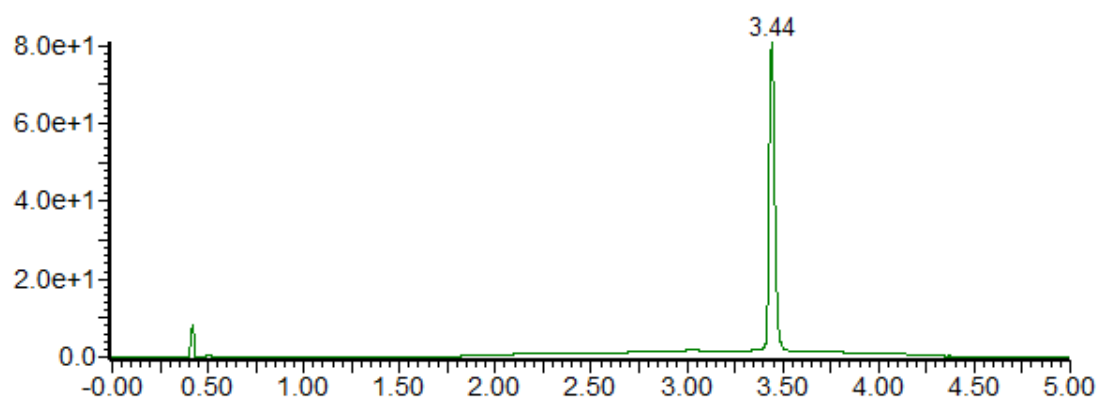


Figure S66: UV trace of the crude reaction mixture. Gradient: 35-60 % ACN/H₂O with 0.1% TFA over 10 min at a flow rate of 0.4 mL/min.



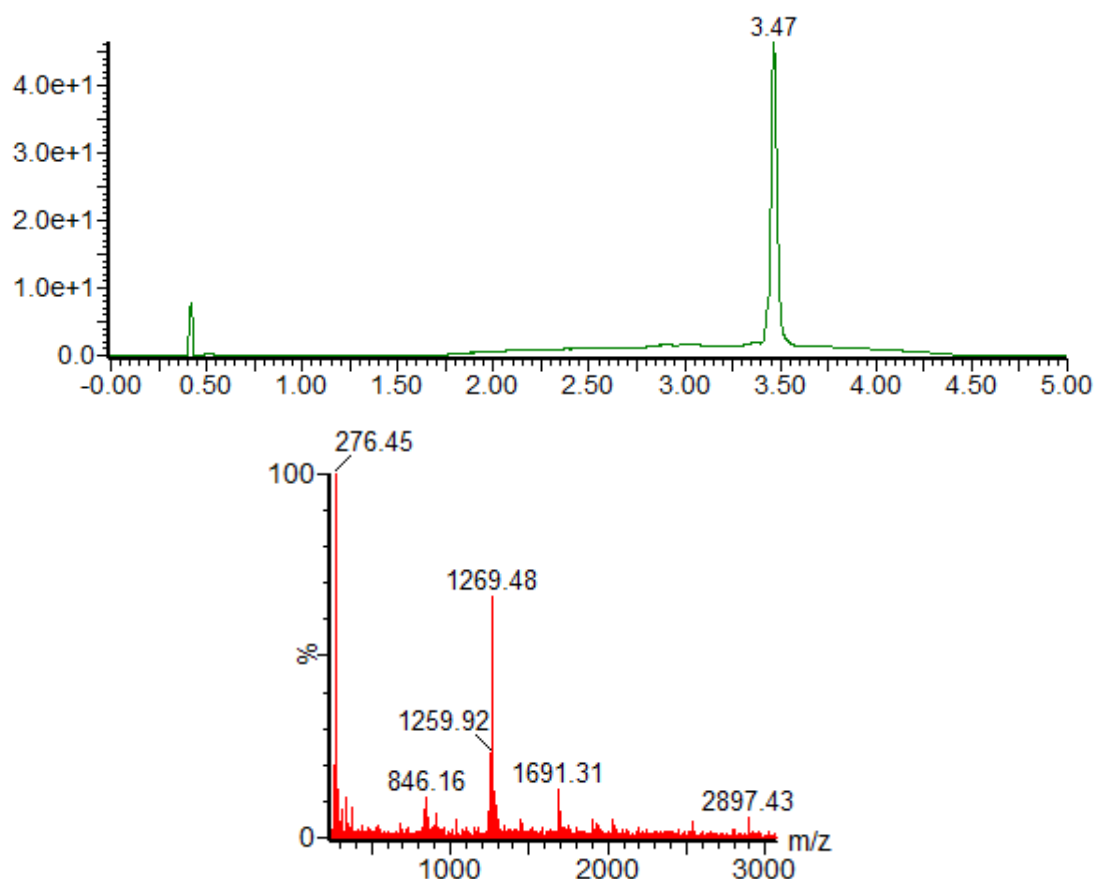


Figure S67: UV trace and corresponding MS trace from LC-MS analysis of the purified **PDC-7** as two isomers. Gradient: 5-95% ACN/H₂O with 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. ESI-MS calcd. for C₁₂₂H₁₆₄ClN₂₁O₃₀S₃ = 2536.40; [M+H]⁺ m/z = 2537.40, found 2536.84; [M+H]²⁺ m/z = 1269.20, found 1269.15 and 1269.48; 2[M+H]²⁺ m/z = 1691.93, found 1691.73 and 1691.31; [M+H]³⁺ m/z = 846.47, found 846.58 and 846.16.

6. Stability test

Stability test of PDC-2

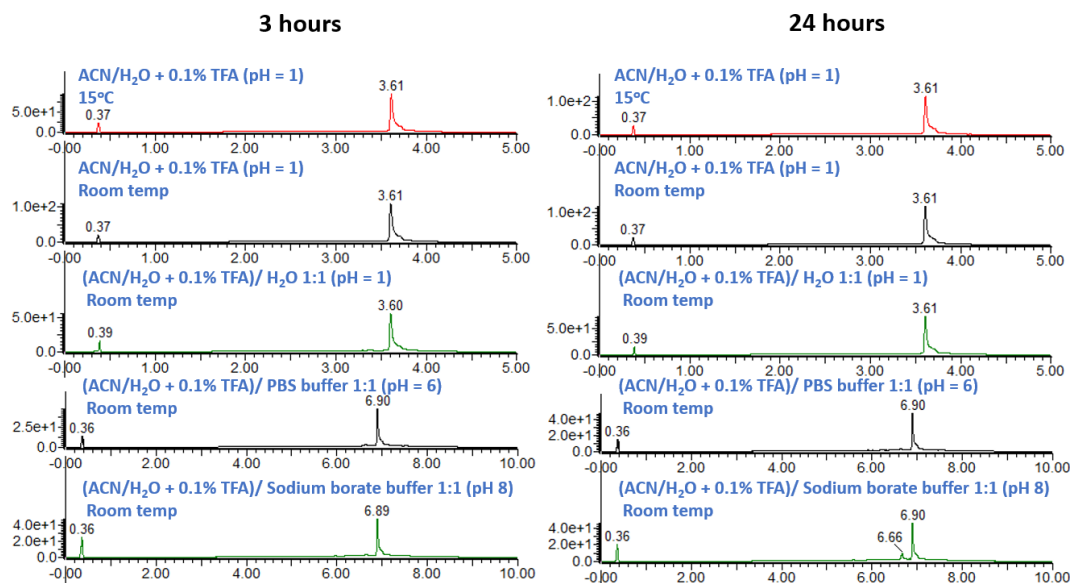


Figure S68: UV trace of the HPLC purified **PDC-2**. The purified compound in ACN/H₂O + 0.1% TFA was placed at 15°C, room temperature, 1:1 dilution with H₂O, PBS buffer pH 7.4 and sodium borate buffer pH 8.5 respectively. The compound was checked by LCMS after 3 h and 24 h. Gradient: 5-95% ACN/H₂O with 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.

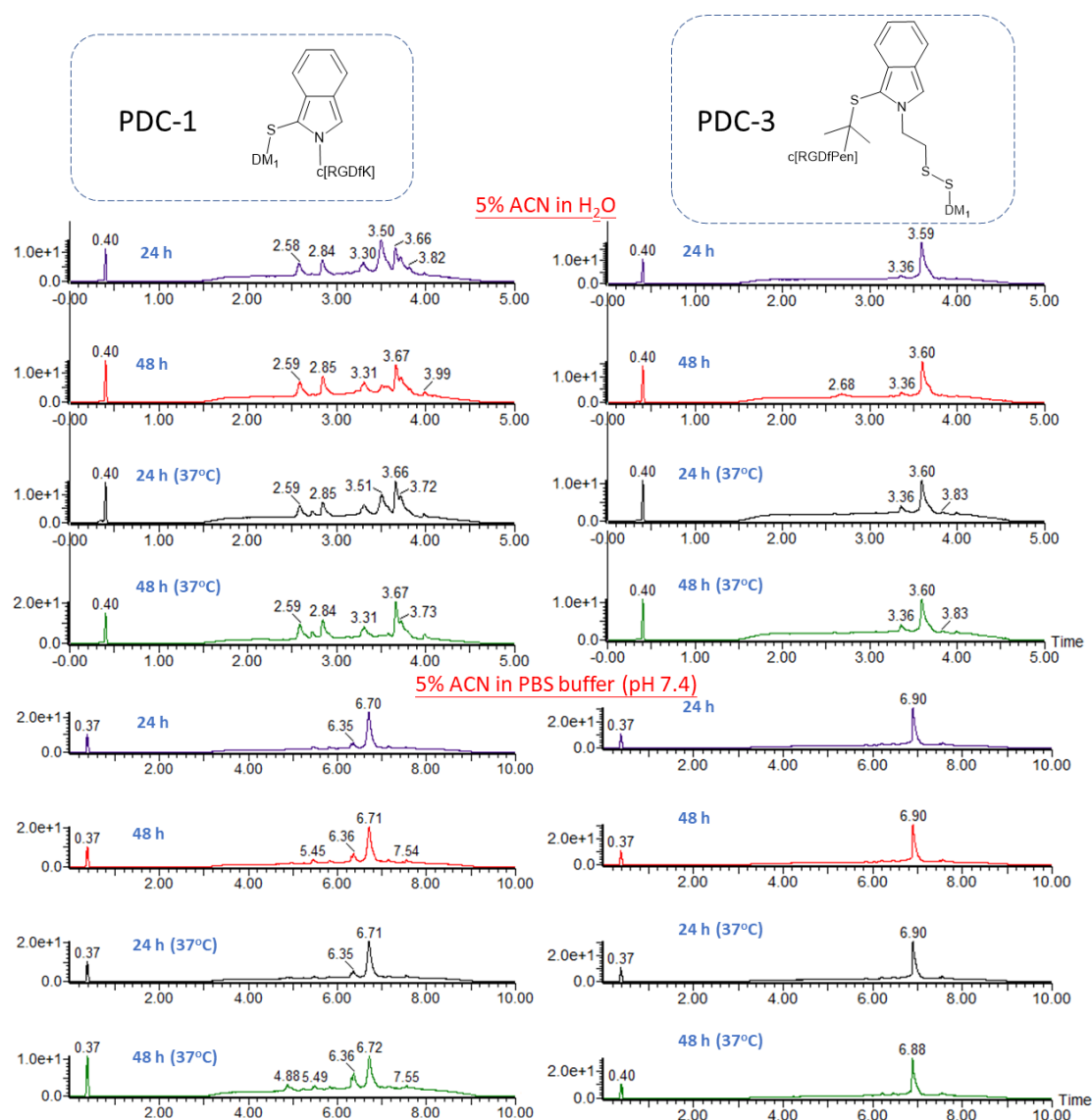
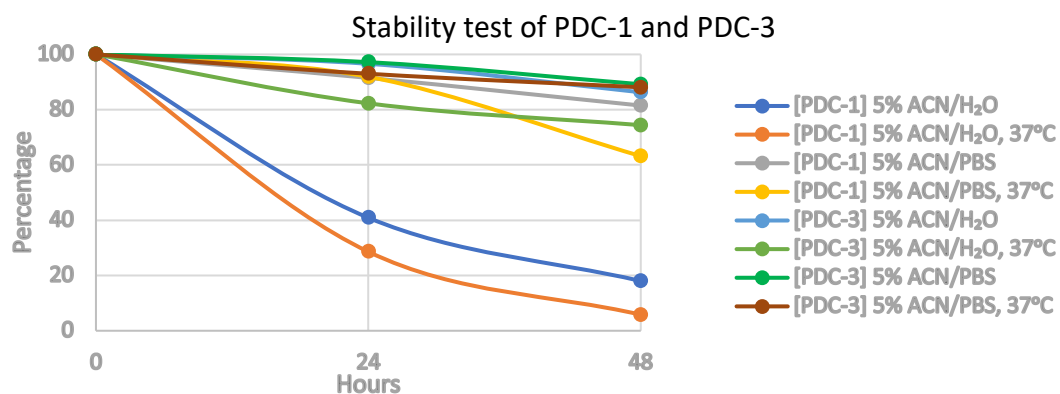


Figure S69: UV trace of the lyophilized **PDC-1** and **PDC-3** dissolved in 5% ACN in H₂O or 5% ACN in PBS buffer pH 7.4, at a concentration of 0.5 mM and placed at room temperature or 37°C as indicated above. The compound was checked by LCMS after 24 h and 48h. Gradient: 5-95% ACN/H₂O with 0.1% TFA over 5 min, and 5-95% ACN/H₂O with 0.1% TFA over 10 min at a flow rate of 0.4 mL/min.

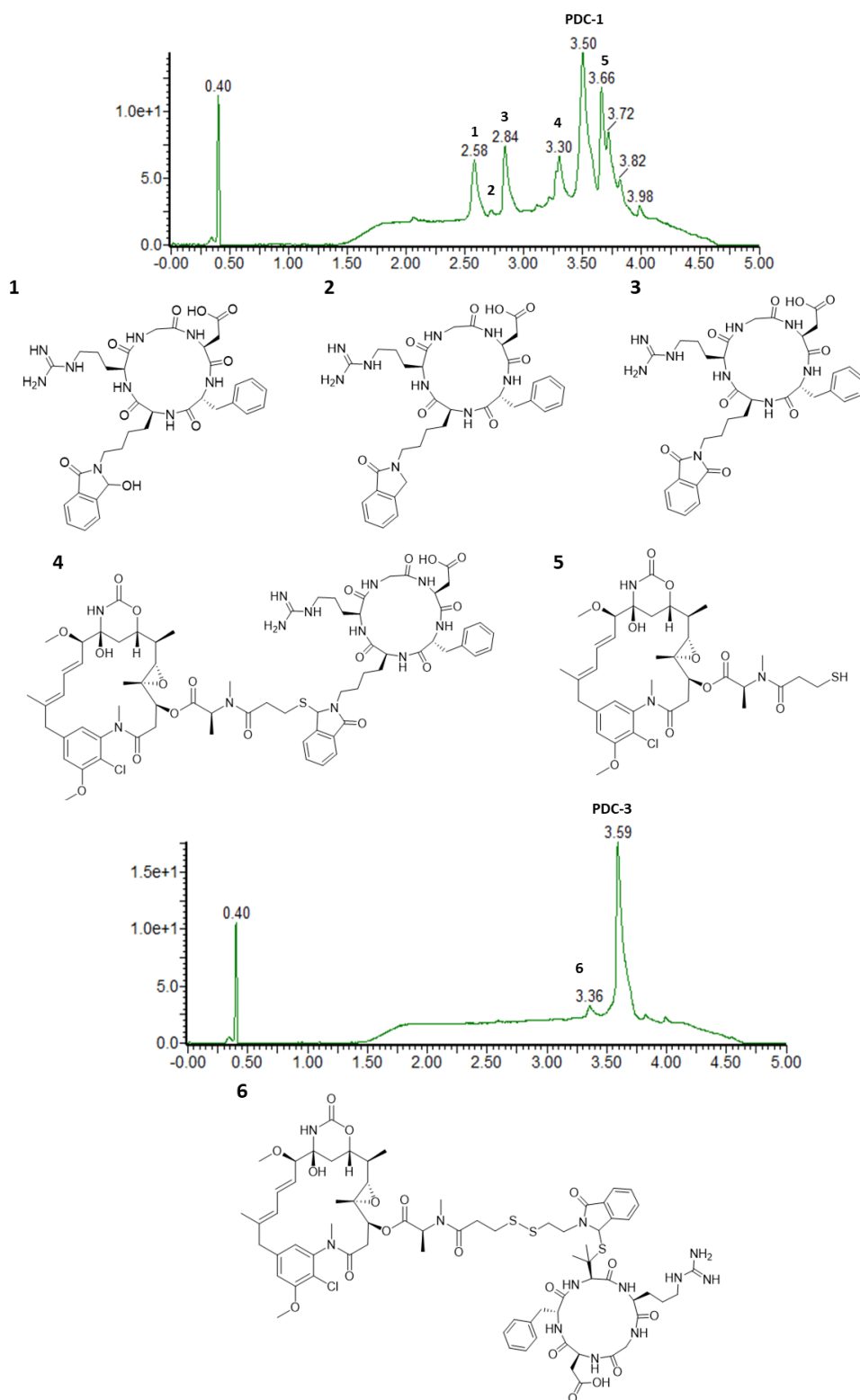
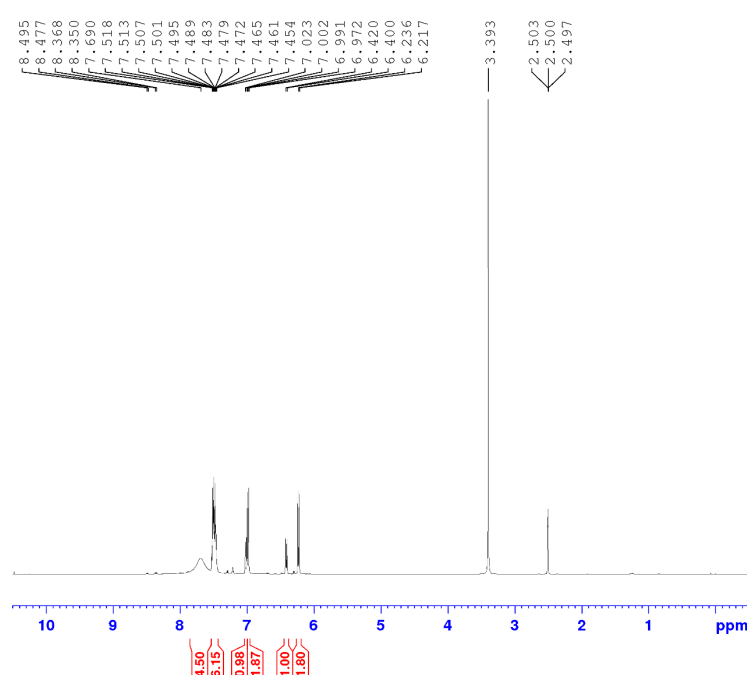
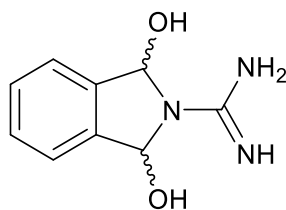


Figure S70: Proposed structures of the decomposed compounds. (Top) UV trace of **PDC-1** dissolved in 5% ACN in H₂O and placed for 24 h. (Bottom) UV trace of **PDC-3** dissolved in 5% ACN in H₂O and placed for 24 h. Gradient: 5-95% ACN/H₂O with 0.1% TFA over 5 min.

7. NMR spectrum

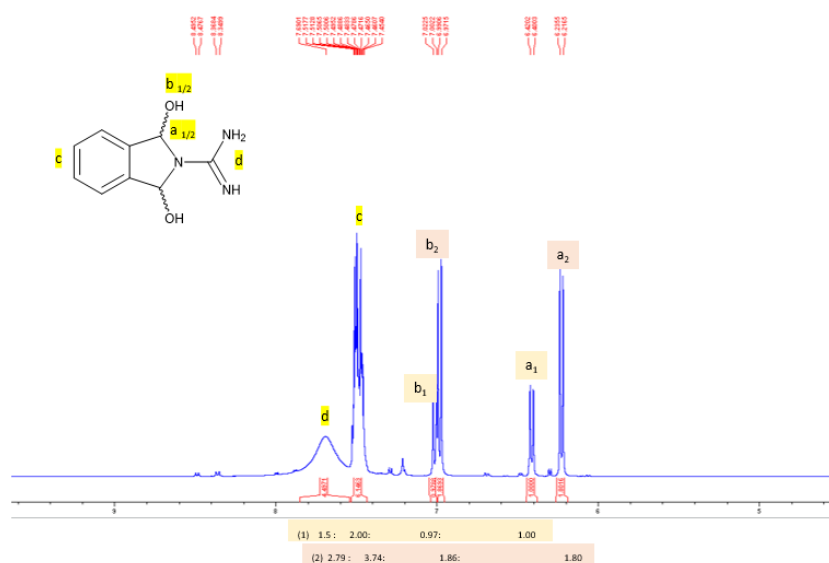
^1H spectrum of intermediates **B**₁ and **B**₂ as a set of isomers



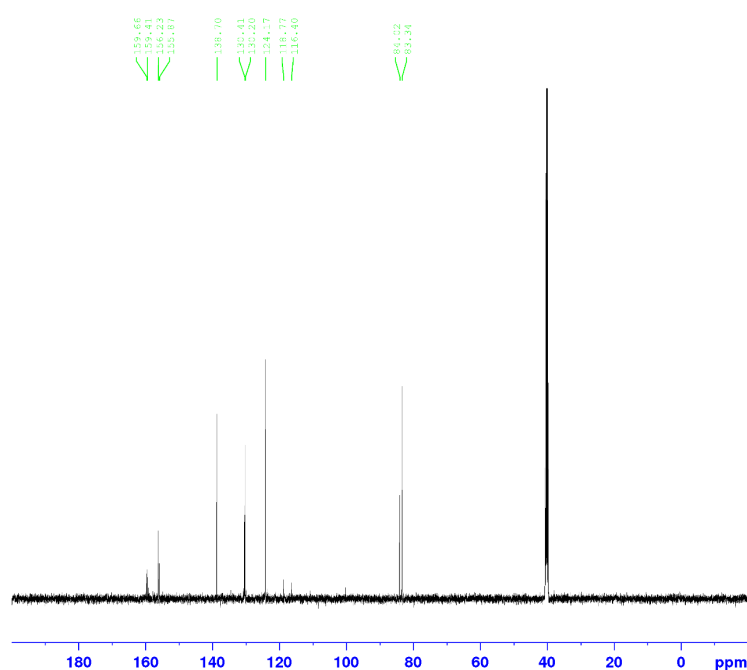
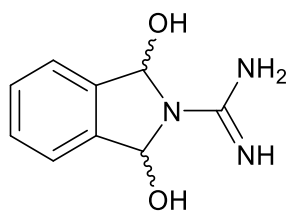
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RG 70.76
DW 50.000 usec
DE 6.50 usec
TE 295.9 K
D1 1.00000000 sec
TD0 1
SF01 500.1330883 MHz
NUC1 1H
P1 8.10 usec
PLWL 23.50000000 W

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GB 0
PC 1.00



^{13}C spectrum of intermediates **B**₁ and **B**₂ as a set of isomers

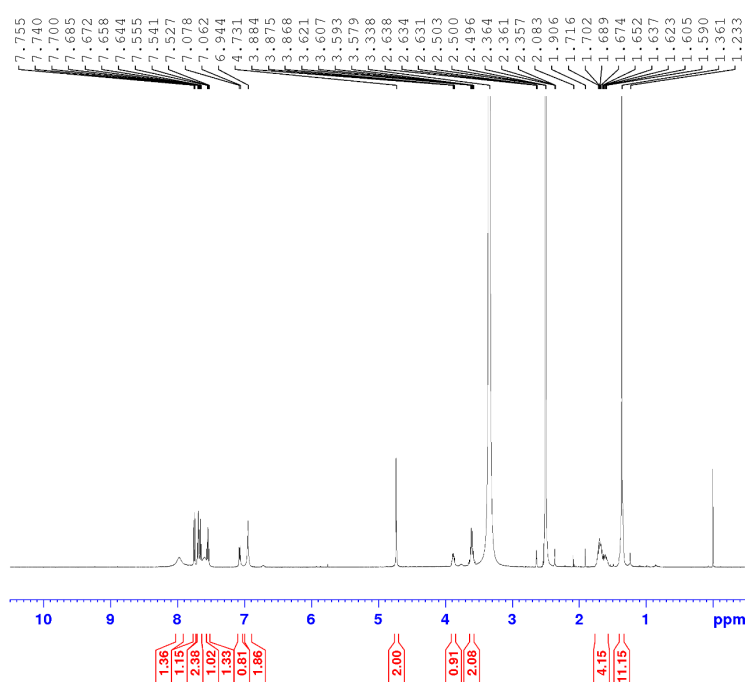
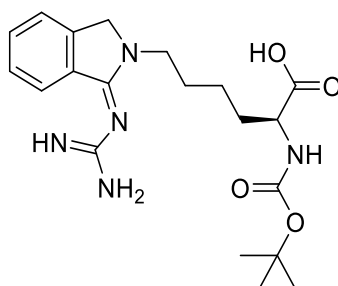


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NUC1 13C
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PLW1 81.29100037 W
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NUC2 1H
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¹H spectrum of compound E'

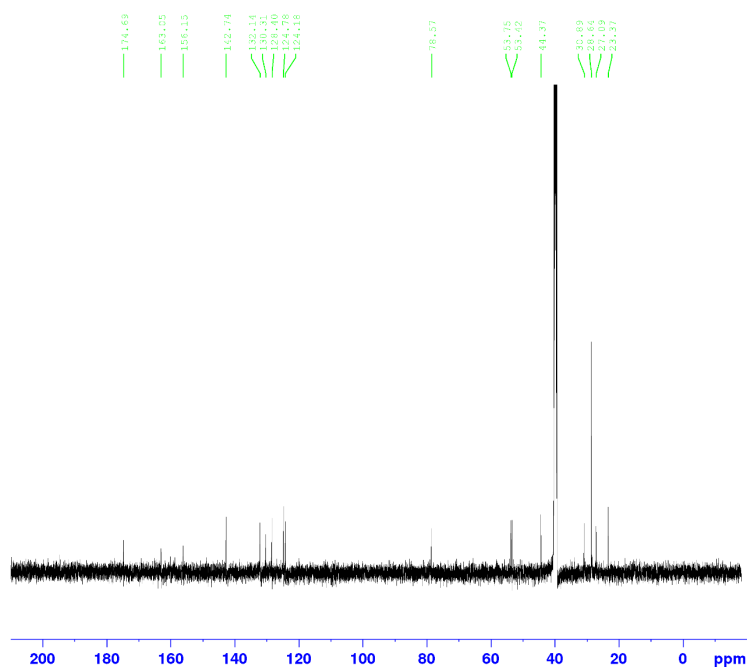
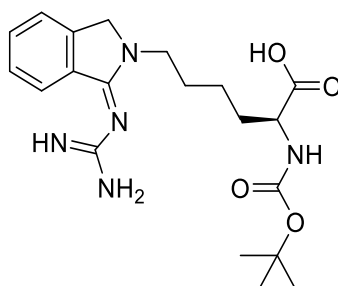


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¹³C spectrum of compound E'

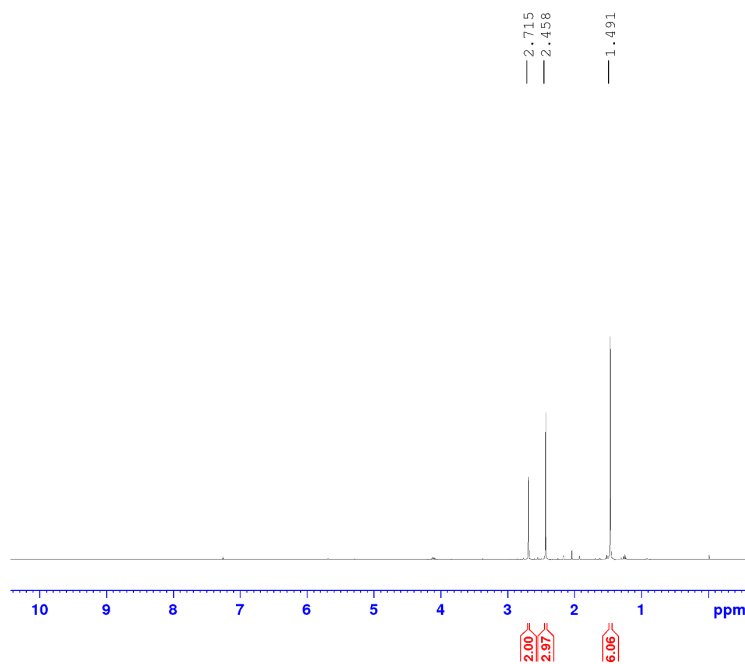
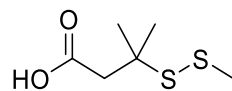


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¹H spectrum of compound 3

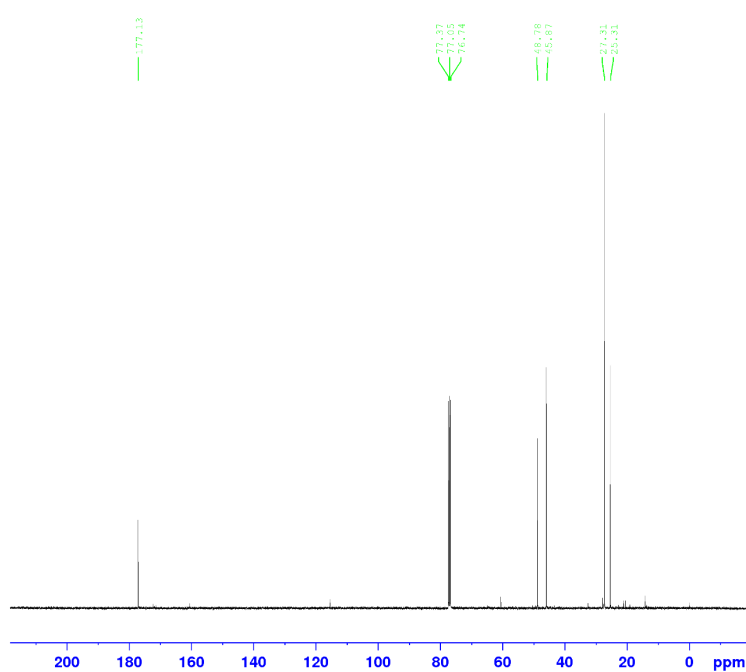
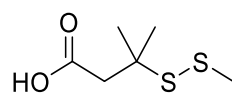


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¹³C spectrum of compound 3

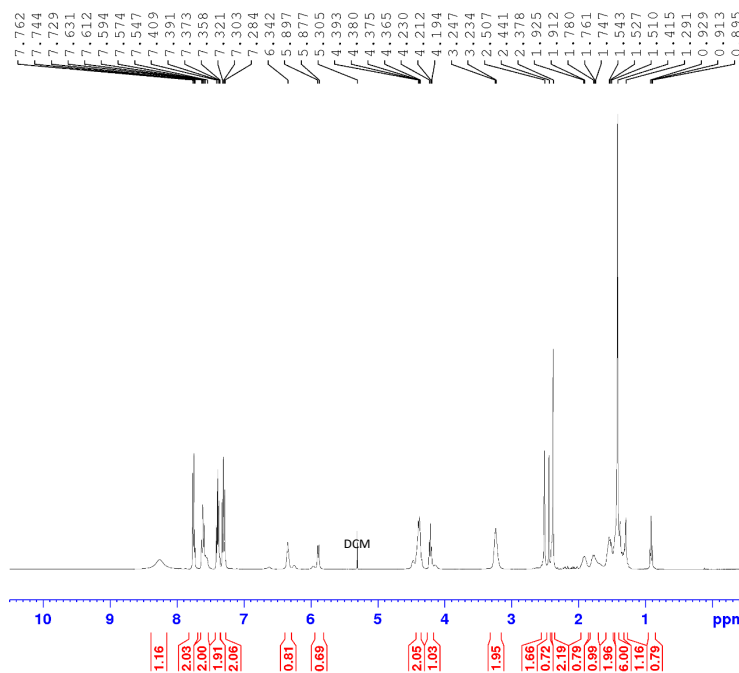
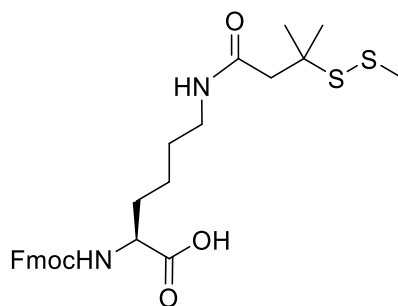


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NUC1 13C
P0 3.33 usec
P1 10.00 usec
PLW1 69.00000000 W
SFO2 400.1416006 MHz
NUC2 1H
CPDPRG2 waltz65
PCPD2 90.00 usec
PLW2 18.00000000 W
PLW12 0.25920001 W
PLW13 0.13038000 W

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GB 0
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¹H spectrum of compound 4

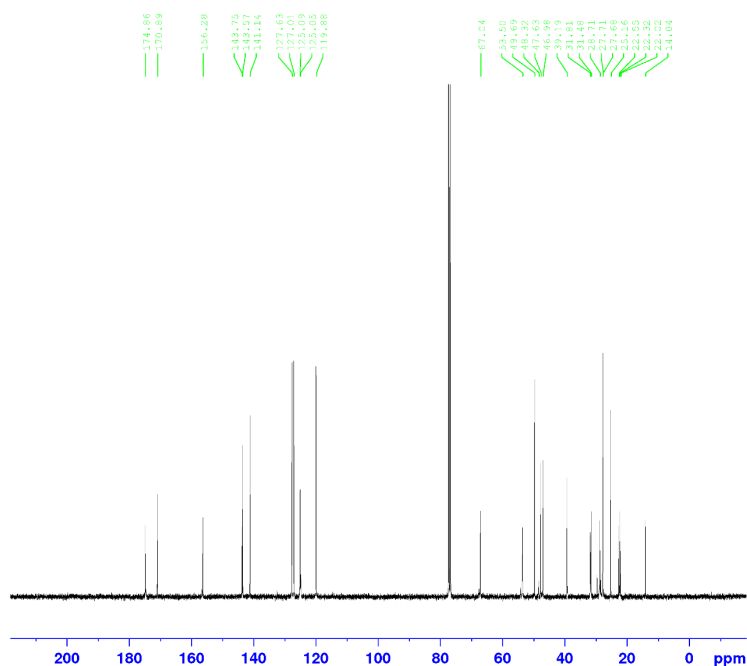
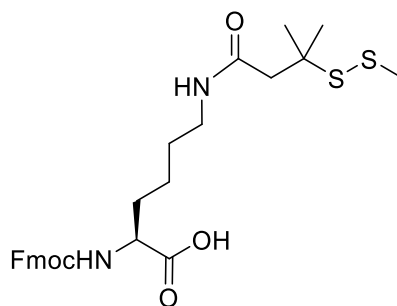


Current Data Parameters
NAME chp-4-90_DC
EXPNO 3
PROCNO 1

F2 - Acquisition Parameters
Date_ 20210917
Time 14.06 h
INSTRUM Avance
PROBHD Z116098_0868 (1
PULPROG zg30
TD 65536
SOLVENT CDCl3
NS 4
DS 2
SWH 8196.722 Hz
FIDRES 0.250144 Hz
AQ 3.3976959 sec
RG 41.6667
DW 61.000 usec
DE 13.42 usec
TE 296.2 K
D1 1.0000000 sec
TDC 1
SFO1 400.1424709 MHz
NUC1 1H
P0 3.60 usec
F1 10.80 usec
PL1 18.0000000 W

F2 - Processing parameters
SI 65536
SF 400.1400000 MHz
WDW EM
SSB 0
LB 0.30 Hz
GB 0
PC 1.00

¹³C spectrum of compound 4



Current Data Parameters
NAME chp-4-30_DC
EXPNO 2
PROCNO 1

F2 - Acquisition Parameters
Date_ 20210917
Time 13.19 h
INSTRUM Avance
PROBHD Z116098_C888 (
PULPROG zgpg30
TD 65536
SOLVENT CDCl3
NS 512
DS 4
SWH 23809.523 Hz
FIDRES 0.726609 Hz
AQ 1.3762550 sec
RG 101
DW 21.000 usec
DE 6.50 usec
TE 297.0 K
D1 2.00000000 sec
D11 0.03000000 sec
TD0 1
SF01 100.6253446 MHz
NUC1 13C
P0 3.33 usec
P1 10.00 usec
PLW1 69.00000000 W
SFO2 400.1416006 MHz
NUC2 1H
CPDPRG2 waltz16
PCPD2 90.00 usec
PLW2 18.00000000 W
PLW12 0.25920001 W
PLW13 0.13038000 W

F2 - Processing parameters
SI 32768
SF 100.6152968 MHz
WDW EM
SSB 0
LB 1.00 Hz
GB 0
PC 1.40

8. References

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