Electronic Supplementary Material (ESI) for Chemical Science.
This journal is © The Royal Society of Chemistry 2014

Discovery of potent inhibitors of human β-tryptase from pre-equilibrated dynamic combinatorial libraries

Qian-Qian Jiang, Wilhelm Sicking, Martin Ehlers and Carsten Schmuck*

Institut für Organische Chemie, Universität Duisburg-Essen, Universitätsstraße 7, 45141 Essen, Germany

Email address: carsten.schmuck@uni-due.de

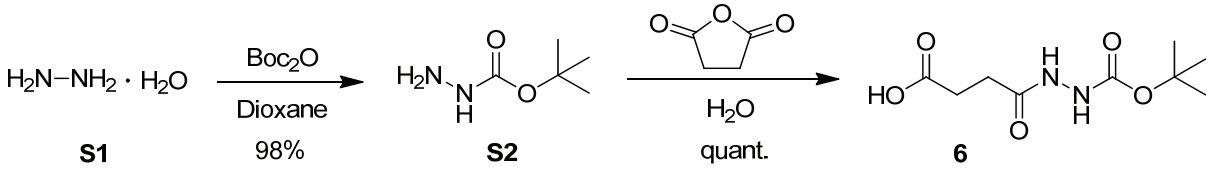
Table of Contents

1. Materials and Devices

All solvents were dried, distilled and stored under Argon before use. All reactions were carried out in oven dried glassware. All reagents were used as supplied from commercial sources unless otherwise stated. Standard SPPS were carried out with IKA KS 130 basic orbital shaker. Microwave assisted SPPS were performed in the CEM Discover Systems with Gas Addition Kit Accessory. Reversed-phase column chromatography was performed with an Armen Instrument Spot Flash Liquid Chromatography MPLC apparatus with RediSep C-18 reversed-phase column. The analytical "High Performance Liquid Chromatography" (HPLC) was done with Dionex HPLC apparatus: P680 pump, ASI-100 automated sample injector, UVD-340U UV detector, UltiMate 3000 Column Compartment. Commercially available HPLC grade solvents were used as eluents and solvent mixtures are reported in volume percent. Lyophilization was carried out in an Alpha 1-4 LD plus freeze drying apparatus from Christ. ^{1}H and ^{13}C NMR spectra in $CDCl_3$ or DMSO-d_6 were recorded with Bruker DMX 300, Bruker DRX 500 and Bruker Avance 700 spectrometer. The IR spectra were measured on a Jasco FT/IR-430 spectrometer with ATR attachment. High resolution ESI mass spectra were recorded with a Bruker BioTOF III spectrometer. Melting points were measured in open glass capillary tubes with a Büchi Melting Point B-540 instrument and are quoted uncorrected. A Varian Cary Eclipse Fluorescence spectrophotometer with microplate reader unit was used in the fluorescence assays. The pH values were measured with a Knick pH-Meter 766 Calimatic.

2. Synthesis Procedures

Synthesis of the Precursor 6



Synthesis of *tert*-butyl hydrazinecarboxylate (S2).

The general strategy employed here was adapted from the literature with some modifications.[1] Hydrazine monohydrate (**S1**) (12.5 g, 250 mmol) was dissolved in 50 mL dioxane at 0 ^{o}C. Then a cooled solution of Boc_2O (10.9 g, 50 mmol) in dioxane (100 mL) was added dropwise under vigorous stirring. The reaction mixture was stirred at room temperature for 12 h. Then the solvent was removed, the residue was dissolved in ethyl acetate and washed twice with brine. The organic phase was dried with $MgSO_4$ and the solvent evaporated under reduced

pressure to obtain compound **S2** as white crystals (6.49 g, 49 mmol, 98%). The product was used in the further synthesis without further purification. Mp: 37 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ [ppm] = 1.46 (s, 9 H, 3 × CH_3), 3.26 (s, 2 H, NH_2), 5.84 (s, 1H, NH). ^{13}C NMR (75 MHz, $CDCl_3$): δ [ppm] = 28.47 (CH_3), 80.65 (C_q), 158.17 (CO). FT-IR (ATR): $\tilde{\nu}$ [cm^{-1}] = 3277 (br), 3060 (br), 3275 (w), 2932 (w), 1635 (s), 1523 (s), 1394 (w), 1280 (m), 1253 (m), 1197 (m), 1047 (w), 952 (w). HRMS (ESI): m/z calculated for $C_5H_{12}N_2O_2Na^+$ $[M+Na]^+$: 155.0791; found: 155.0813.

Synthesis of 4-(*N'-tert*-butoxycarbonyl-hydrazino)-4-oxo-butyric acid (6).

The general strategy employed here was adapted from the literature with some modifications.[2] 1.0 g (10 mmol) of succinic anhydride and 1.32 g (10 mmol) of *tert*-butyl hydrazinecarboxylate were dissolved in 30 mL water and the reaction mixture was stirred at rt for 4 h. The reaction proceeded cleanly and full conversion to **6** which was confirmed by ESI-MS. The reaction mixture was lyophilized and **6** (2.32 g, 10 mmol, quant.) was used in SPPS without any further purification. Mp: 40 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ [ppm] = 1.46 (s, 9 H, 3 × CH_3), 2.53 (t, J = 6.7 Hz, 2 H, CH_2), 2.66-2.74 (m, 2 H, CH_2), 7.11 (s, 1H, NH), 8.42 (s, 1H, NH). ^{13}C NMR (75 MHz, $CDCl_3$): δ [ppm] = 28.27 (CH_3), 28.47 (CH_2), 29.06 (CH_2), 82.21 (C_q), 156.30 (CO), 172.65 (CO), 176.45 (CO). FT-IR (ATR): $\tilde{\nu}$ [cm^{-1}] = 3272 (br), 3053 (br), 2975 (w), 2931 (w), 1643 (s), 1524 (s), 1396 (w), 1252 (m), 1198 (m), 1046 (w), 954 (w). HRMS (ESI): m/z calculated for $C_9H_{15}N_2O_5^-$ $[M-H]^-$: 231.0986, found: 231.0938.

General procedure for the synthesis of peptide derived hydrazides

Peptide hydrazides **A–E** were synthesized from the corresponding peptide sequences, followed by the attachment of **6** using microwave-assisted solid phase peptide synthesis (SPPS).

General protocol for microwave-assisted solid phase peptide synthesis

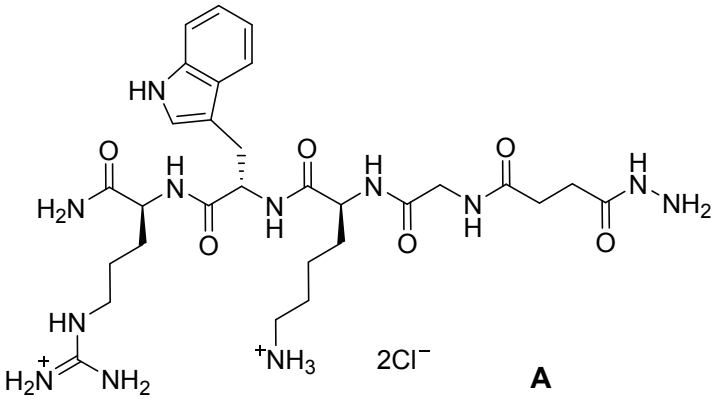
Fmoc deprotection: The Fmoc protecting group was removed by treatment with 20% piperidine/DMF for 1.5 min without and then 5 min under irradiation condition (20 W, T_{max} = 60 °C, ΔT = ± 5 °C). Then the resin was washed with DMF (6 × 5 mL) followed by a positive Kaiser test to confirm the cleavage of the Fmoc protecting group.

General coupling procedure: The amino acids were attached to the resin using microwave irradiation (20 W, T_{max} = 60 °C, ΔT = ± 5 °C) for 20 min under an argon atmosphere using PyBOP as the coupling reagent and DIPEA as the base in DMF. Then, the resin was washed

with DMF (3 × 5 mL) before the coupling and washing steps were repeated. A negative Kaiser test confirmed the successful attachment of the amino acids.

Cleavage from the Rink amide MBHA resin: Cleavage of the product from the resin was carried out without microwave irradiation. Therefore, the resin was transferred into Schlenk glass vessels equipped with a glass filter frit and then treated with a mixture of TFA/H_2O/triisopropylsilane (95:2.5:2.5). The suspension was shaken for 3 h at room temperature and was afterwards washed twice with TFA. The filtrates were combined and concentrated under vacuum to obtain an oily residue to which diethyl ether was added to precipitate the peptide. The resulting suspension was centrifuged and the supernatant solvent was decanted and the solid was washed once again with diethyl ether and centrifuged again. The obtained solid was dissolved in water and lyophilized to get the crude product.

Synthesis of hydrazide A.

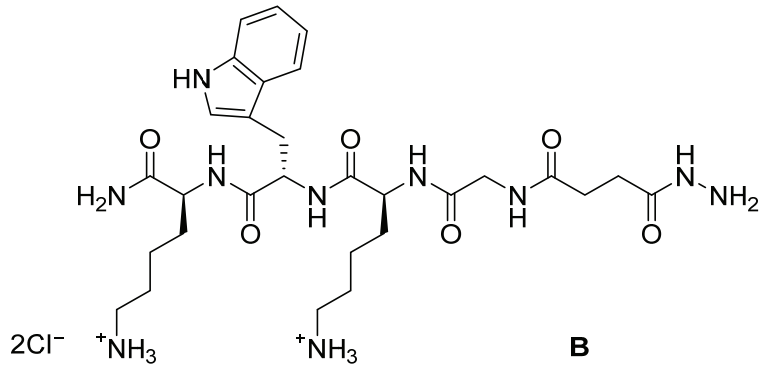


A

The synthesis was carried out in a microwave-transparent polyethylene column using a CEM Discover microwave apparatus according to the standard microwave-assisted SPPS procedure described above. Rink amide MBHA resin (200 mg, 0.84 mmol/g, 0.168 mmol, 1 eq) was swollen in DCM (10 mL) for 2 h. Then the Fmoc protecting group was removed by treatment with 20% piperidine in DMF. After a washing cycle with DMF, the first amino acid Fmoc-Arg(Pbf)-OH (327 mg, 0.504 mmol, 3 eq) was attached to the resin with the help of the coupling reagent PyBOP (262 mg, 0.504 mmol, 3 eq) and the base DIPEA (171 µL, 1.008 mmol, 6 eq) in DMF (5 mL) using microwave irradiation condition for 20 min under an argon atmosphere. The coupling was repeated and the resin was washed thoroughly. The Kaiser test showed a negative result. After Fmoc deprotection, Fmoc-Trp(Boc)-OH (265 mg, 0.504 mmol, 3 eq), Fmoc-Lys(Boc)-OH (236 mg, 0.504 mmol, 3 eq), Fmoc-Gly-OH (150 mg, 0.504 mmol, 3 eq) and **6** (117 mg, 0.504 mmol, 3 eq) were coupled successively in the same manner. Then the resin was thoroughly washed with DCM (3× 5 mL), MeOH (3× 5 mL), DCM (3× 5 mL) and dried under vacuum for 1 h. The cleavage of the product from the resin

was carried out without microwave irradiation using a mixture of TFA/H_2O/triisopropylsilane (95:2.5:2.5) according to the general procedure given above. The crude product was purified by RP18-MPLC using a gradient from 10% to 40% MeOH/H_2O + 0.1% TFA. Pure product was transformed into the hydrochloride salt by dissolving it in water, adding hydrochloric acid and subsequent lyophilization to provide **A** as a white solid (56 mg, 76.5 µmol, 46%, HPLC purity 96%). Mp: 134 °C. ^{1}H NMR (500 MHz, DMSO-d_6): δ [ppm] = 1.16-1.25 (m, 2 H, Lys-CH_2), 1.40-1.73 (m, 8 H, 2 × Lys-CH_2, 2 × Arg-CH_2), 2.46 (m, 4 H, 2 × CH_2), 2.68-2.72 (m, 2 H, Arg-CH_2), 2.99-3.19 (m, 6 H, Lys-CH_2, Trp-CH_2, NH_2), 3.70-3.71 (m, 2 H, Gly-CH_2), 4.16-4.20 (m, 2 H, Lys-CH, Arg-CH), 4.46-4.50 (m, 1 H, Trp-CH), 6.97 (t, J = 7.5 Hz, 1 H, Trp-CH_{ar}), 7.05 (t, J = 7.6 Hz, 1 H, Trp-CH_{ar}), 7.11 (s, 1 H, NH_2), 7.17 (s, 1 H, Trp-CH_{ar}), 7.31 (s, 1 H, NH_2), 7.33 (d, J = 8.1 Hz, 1 H, Trp-CH_{ar}), 7.57 (d, J = 7.8 Hz, 1 H, Trp-CH_{ar}),7.76 (t, J = 5.7 Hz, 1 H, NH), 7.89 (d, J = 8.0 Hz, 1 H, NH), 7.93 (brs, 3 H, Lys-NH_3^+), 8.09 (d, J = 7.6 Hz, 1 H, NH), 8.16 (d, J = 7.7 Hz, 1 H, NH), 8.26 (t, J = 5.7 Hz, 1 H, NH), 10.88 (s, 1 H, Trp-NH), 11.00 (s, 1 H, NH). ^{13}C NMR (125 MHz, DMSO-d_6): δ [ppm] = 22.10 (Lys-CH_2), 25.00 (Arg-CH_2), 26.52 (Lys-CH_2), 27.22 (Trp-CH_2), 28.11 (CH_2), 29.16 (Arg-CH_2), 29.55 (CH_2), 31.10 (Lys-CH_2), 38.54 (Lys-CH_2), 40.39 (Arg-CH_2), 42.17 (Gly-CH_2), 52.08 (Arg-CH), 52.71 (Lys-CH), 53.90 (Trp-CH), 109.94 (Trp-C_q), 111.37 (Trp-CH_{ar}), 118.33 (Trp-CH_{ar}), 118.41 (Trp-CH_{ar}), 120.91 (Trp-CH_{ar}), 123.74 (Trp-CH_{ar}), 127.26 (Trp-C_q), 136.07 (Trp-C_q), 156.92 (Gua-C_q), 169.25 (CO), 171.15 (CO), 171.35 (CO), 171.42 (CO), 171.65 (CO), 173.33 (CO). FT-IR (ATR): $\tilde{\nu}$ [cm^{-1}] = 3138 (br), 3045 (br), 2897 (br), 1637 (s), 1522 (s), 1403 (s), 1339 (w), 1230 (m), 1092 (s), 745 (m). HRMS (ESI): m/z calculated for $C_{29}H_{47}N_{12}O_6^+$ $[M+H]^+$: 659.3736; found: 659.3778.

Synthesis of hydrazide B

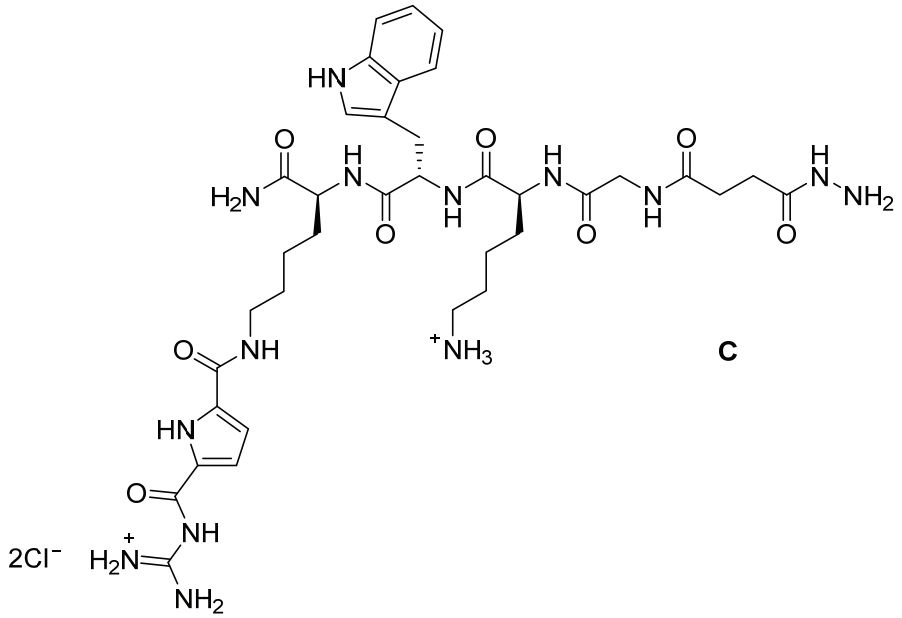


B

The synthesis of **B** was carried out accordingly on Rink amide MBHA resin (200 mg, 0.84 mmol/g, 0.168 mmol, 1 eq) following the above described microwave-assisted SPPS

procedure. As coupling reagent PyBOP (262 mg, 0.504 mmol, 3 eq) and as base DIPEA (171 µL, 1.008 mmol, 6 eq) in DMF (5 mL) were used during the coupling steps. After Fmoc deprotection, the amino acids Fmoc-Lys(Boc)-OH (236 mg, 0.504 mmol, 3 eq), Fmoc-Trp(Boc)-OH (265 mg, 0.504 mmol, 3 eq), Fmoc-Lys(Boc)-OH (236 mg, 0.504 mmol, 3 eq), Fmoc-Gly-OH (150 mg, 0.504 mmol, 3 eq) and **6** (117 mg, 0.504 mmol, 3 eq) were coupled successively. After the cleavage and purification steps, product **B** was obtained as a white solid (51 mg, 72.5µmol, 43 %, HPLC purity 97 %). Mp: 134 °C. ^{1}H NMR (500 MHz, DMSO-d_6): δ [ppm] = 1.17-1.29 (m, 4 H, 2 × Lys-CH_2), 1.48-1.67 (m, 8 H, 4 × Lys-CH_2), 2.40-2.47 (m, 4 H, 2 × CH_2), 2.67-2.76 (m, 4 H, 2 × Lys-CH_2), 3.00-3.19 (m, 2 H, Trp-CH_2), 3.71-3.72 (m, 2 H, Gly-CH_2), 4.14-4.21 (m, 2 H, 2 × Lys-CH), 4.46-4.50 (m, 1 H, Trp-CH), 6.97 (t, J = 7.5 Hz, 1 H, Trp-CH_{ar}), 7.04-7.08 (m, 2 H, Trp-CH_{ar}, NH_2), 7.18 (s, 1 H, Trp-CH_{ar}), 7.25 (s, 1 H, NH_2), 7.33 (d, J = 8.1 Hz, 1 H, Trp-CH_{ar}), 7.58 (d, J = 7.4 Hz, 1 H, Trp-CH_{ar}), 7.85 (d, J = 7.4 Hz, 1 H, NH), 8.00 (brs, 6 H, 2 × Lys-NH_3^+), 8.11 (d, J = 6.7 Hz, 1 H, NH), 8.20 (d, J = 8.2 Hz, 1 H, NH), 8.28 (brs, 1 H, NH), 10.91 (s, 1 H, Trp-NH), 11.06 (s, 1 H, NH). ^{13}C NMR (125 MHz, DMSO-d_6): δ [ppm] = 22.06 (Lys-CH_2), 22.10 (Lys-CH_2), 26.49 (Lys-CH_2), 26.53 (Lys-CH_2), 27.08 (Trp-CH_2), 28.08 (CH_2), 29.53 (CH_2), 31.10 (Lys-CH_2), 31.32 (Lys-CH_2), 38.45 (Lys-CH_2), 38.54 (Lys-CH_2), 42.15 (Gly-CH_2), 52.24 (Lys-CH), 52.68 (Lys-CH), 53.85 (Trp-CH), 109.94 (Trp-C_q), 111.31 (Trp-CH_{ar}), 118.25 (Trp-CH_{ar}), 118.38 (Trp-CH_{ar}), 120.84 (Trp-CH_{ar}), 123.70 (Trp-CH_{ar}), 127.22 (Trp-C_q), 136.03 (Trp-C_q), 169.19 (CO), 171.08 (CO), 171.19 (CO), 171.32 (CO), 171.65 (CO), 173.39 (CO). FT-IR (ATR): $\tilde{\nu}$ [cm^{-1}] = 3239 (br), 3038 (br), 2920 (br), 1642 (s), 1521 (s), 1339 (w), 1232 (m), 1160 (w), 1098 (w), 745 (m). HRMS (ESI): m/z calculated for $C_{29}H_{47}N_{10}O_6^+$ $[M+H]^+$: 631.3675; found: 631.3692.

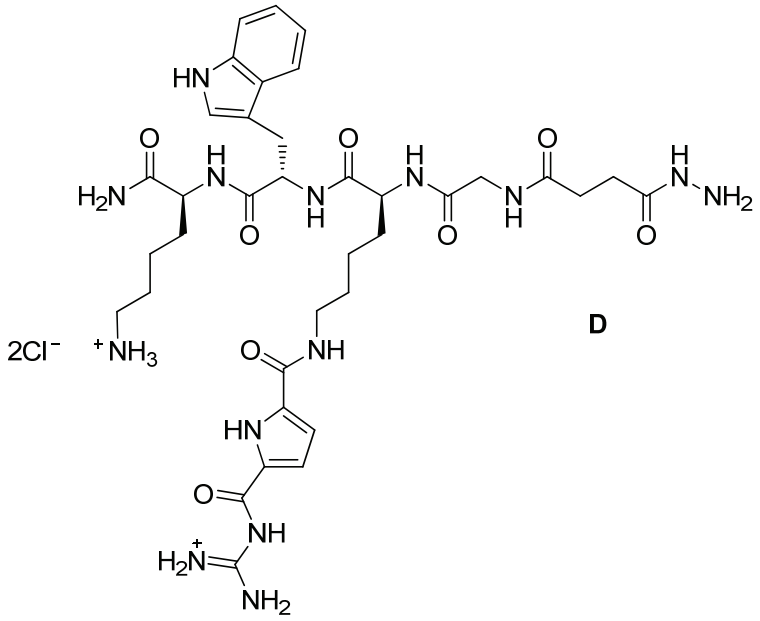
Synthesis of hydrazide C



The synthesis of **C** was carried out accordingly on Rink amide MBHA resin (200 mg, 0.84 mmol/g, 0.168 mmol, 1 eq) following the above described microwave-assisted SPPS procedure. PyBOP (262 mg, 0.504 mmol, 3 eq) and DIPEA (171 µL, 1.008 mmol, 6 eq) in DMF (5 mL) were used during the coupling steps. After Fmoc deprotection, the amino acids Fmoc-Lys(Alloc)-OH (228 mg, 0.504 mmol, 3 eq), Fmoc-Trp(Boc)-OH (265 mg, 0.504 mmol, 3 eq), Fmoc-Lys(Boc)-OH (236 mg, 0.504 mmol, 3 eq), Fmoc-Gly-OH (150 mg, 0.504 mmol, 3 eq) and **6** (117 mg, 0.504 mmol, 3 eq) were coupled successively. Then the Alloc protecting group was removed with $Pd(PPh_3)_4$ (19.4 mg, 0.0168 mmol, 0.1 eq) in the presence of $PhSiH_3$ (495 µL, 4.032 mmol, 24 eq) in DCM for 10 min at 20W microwave irradiation and a maximum temperature of 30 °C followed by washing with DCM (3× 5 mL) and DMF (3× 5 mL). Then the Boc-GCP group (200 mg, 0.504 mmol, 3 eq) was coupled following the same protocol. After the cleavage and purification steps, the product **C** was obtained as a white solid (52 mg, 59.0 µmol, 35 %, HPLC purity 94 %). Mp: 176 °C. ^{1}H NMR (500 MHz, DMSO-d_6): δ [ppm] = 1.20-1.33 (m, 4 H, 2 × Lys-CH_2), 1.46-1.69 (m, 8 H, 4 × Lys-CH_2), 2.37-2.47 (m, 4 H, 2 × CH_2), 2.69-2.72 (m, 2 H, Lys-CH_2), 2.97-3.02, 3.14-3.23 (m, 4 H, Lys-CH_2, Trp-CH_2), 3.69-3.71 (m, 2 H, Gly-CH_2), 4.15-4.22 (m, 2 H, 2 × Lys-CH), 4.47-4.52 (m, 1 H, Trp-CH), 6.87-6.88 (m, 1 H, GCP-CH_{ar}), 6.96 (t, J = 7.5 Hz, 1 H, Trp-CH_{ar}), 7.03-7.06 (m, 2 H, Trp-CH_{ar}, NH_2), 7.16 (s, 1 H, Trp-CH_{ar}), 7.25 (s, 1 H, NH_2), 7.32 (d, J = 8.8 Hz, 1 H, Trp-CH_{ar}), 7.54-7.57 (m, 2 H, GCP-CH_{ar}, Trp-CH_{ar}), 7.83 (d, J = 8.8 Hz,, 1 H, NH),7.93 (brs, 3 H, Lys-NH_3^+), 8.06 (d, J = 8.1 Hz,, 1 H, NH), 8.15 (d, J = 8.1 Hz,, 1 H, NH), 8.23 (brs, 1 H, NH), 8.50-8.68 (m, 5 H, 5 × NH), 10.87 (s, 1 H, Trp-NH), 11.03 (s, 1 H, NH), 12.06 (s, 1 H,

Gua-NH), 12.34 (s, 1 H, GCP-NH). ^{13}C NMR (125 MHz, DMSO-d_6): δ [ppm] = 22.05 (Lys-CH_2), 22.74 (Lys-CH_2), 26.50 (Lys-CH_2), 27.24 (Trp-CH_2), 28.06 (CH_2), 28.72 (Lys-CH_2), 29.50 (CH_2), 31.17 (Lys-CH_2), 31.77 (Lys-CH_2), 38.50 (Lys-CH_2), 38.73 (Lys-CH_2), 42.11 (Gly-CH_2), 52.41 (Lys-CH), 52.55 (Lys-CH), 53.77 (Trp-CH), 109.92 (Trp-C_q), 111.28 (Trp-CH_{ar}), 112.45 (GCP-CH_{ar}), 115.99 (GCP-CH_{ar}), 118.24 (Trp-CH_{ar}), 118.37 (Trp-CH_{ar}), 120.82 (Trp-CH_{ar}), 123.68 (Trp-CH_{ar}), 125.33 (GCP-C_q), 127.23 (Trp-C_q), 132.95 (GCP-Cq), 136.02 (Trp-C_q), 155.54 (Gua-C_q), 159.02 (CO), 159.64 (CO), 169.06 (CO), 171.09 (CO), 171.14 (CO), 171.29 (CO), 171.50 (CO), 173.50 (CO). FT-IR (ATR): $\tilde{\nu}$ [cm^{-1}] = 3188 (br), 3049 (br), 2926 (br), 1638 (s), 1523 (s), 1281 (s), 1195 (m), 813 (w), 745 (m). HRMS (ESI): m/z calculated for $C_{36}H_{53}N_{14}O_8^+$ $[M+H]^+$: 809.4165; found: 809.4177.

Synthesis of hydrazide D

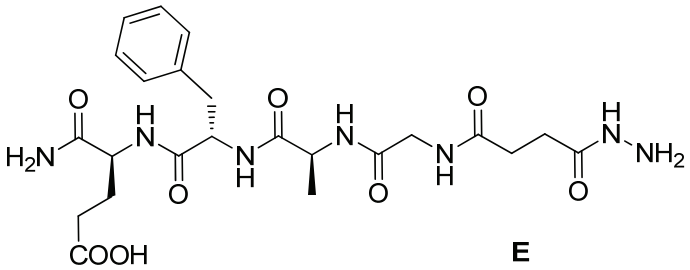


D

The synthesis of **D** was carried out accordingly on Rink amide MBHA resin (200 mg, 0.84 mmol/g, 0.168 mmol, 1 eq) following the above described microwave-assisted SPPS procedure. PyBOP (262 mg, 0.504 mmol, 3 eq) and DIPEA (171 µL, 1.008 mmol, 6 eq) in DMF (5 mL) were used during the coupling steps. After Fmoc deprotection, the amino acids Fmoc-Lys(Boc)-OH (236 mg, 0.504 mmol, 3 eq), Fmoc-Trp(Boc)-OH (265 mg, 0.504 mmol, 3 eq), Fmoc-Lys(Alloc)-OH (228 mg, 0.504 mmol, 3 eq), Fmoc-Gly-OH (150 mg, 0.504 mmol, 3 eq) and **6** (117 mg, 0.504 mmol, 3 eq) were coupled successively. After the removal of Alloc protecting group with $Pd(PPh_3)_4$ (19.4 mg, 0.0168 mmol, 0.1 eq) in the presence of $PhSiH_3$ (495 µL, 4.032 mmol, 24 eq) in DCM, the Boc-GCP group (200 mg, 0.504 mmol, 3 eq) was coupled. After the cleavage and purification steps, the product **D** was obtained as a white solid (53 mg, 60.1 µmol, 36 %, HPLC purity 94 %). Mp: 178 °C. ^{1}H NMR (500 MHz, DMSO-d_6): δ [ppm] = 1.18-1.29 (m, 4 H, 2 × Lys-CH_2), 1.42-1.70 (m, 8 H, 4 × Lys-CH_2), 2.36-2.47 (m, 4 H, 2 × CH_2), 2.71-2.76 (m, 2 H, Lys-CH_2), 2.99-3.04, 3.15-3.19 (m, 4 H, Lys-CH_2, Trp-CH_2), 3.71-3.72 (m, 2 H, Gly-CH_2), 4.13-4.21 (m, 2 H, 2 × Lys-CH), 4.46-4.50 (m, 1 H, Trp-CH), 6.87-6.88 (m, 1 H, GCP-CH_{ar}), 6.97 (t, J = 7.4 Hz, 1 H, Trp-CH_{ar}), 7.03-7.06 (m, 2 H, Trp-CH_{ar}, NH_2), 7.16 (s, 1 H, Trp-CH_{ar}), 7.21 (s, 1 H, NH_2), 7.32 (d, J = 8.2 Hz, 1 H, Trp-CH_{ar}), 7.54-7.58 (m, 2 H, GCP-CH_{ar}, Trp-CH_{ar}), 7.83 (d, J = 8.5 Hz,, 1 H, NH), 7.93 (brs, 3 H, Lys-NH_3^+), 8.09 (d, J = 7.9 Hz,, 1 H, NH), 8.13 (d, J = 7.8 Hz,, 1 H, NH), 8.24 (t, J = 6.2 Hz,, 1 H, NH), 8.49-8.68 (m, 5 H, 5 × NH), 10.87 (s, 1 H, Trp-NH), 11.01 (s, 1 H, NH), 12.07 (s, 1 H, Gua-NH), 12.33 (s, 1 H, GCP-NH). ^{13}C NMR (125 MHz, DMSO-d_6): δ [ppm] = 22.09 (Lys-CH_2), 22.79 (Lys-CH_2), 26.54 (Lys-CH_2), 27.07 (Trp-CH_2), 28.07 (CH_2), 28.74

(Lys-CH_2), 29.51 (CH_2), 31.29 (Lys-CH_2), 31.48 (Lys-CH_2), 38.58 (Lys-CH_2), 38.65 (Lys-CH_2), 42.08 (Gly-CH_2), 52.24 (Lys-CH), 52.87 (Lys-CH), 53.77 (Trp-CH), 109.93 (Trp-C_q), 111.28 (Trp-CH_{ar}), 112.42 (GCP-CH_{ar}), 116.00 (GCP-CH_{ar}), 118.23 (Trp-CH_{ar}), 118.37 (Trp-CH_{ar}), 120.83 (Trp-CH_{ar}), 123.67 (Trp-CH_{ar}), 125.33 (GCP-C_q), 127.24 (Trp-C_q), 132.96 (GCP-Cq), 136.02 (Trp-C_q), 155.53 (Gua-C_q), 159.01 (CO), 159.63 (CO), 169.17 (CO), 171.10 (CO), 171.15 (CO), 171.27 (CO), 171.81 (CO), 173.36 (CO). FT-IR (ATR): $\tilde{\nu}$ [cm^{-1}] = 3183 (br), 3044 (br), 2925 (br), 1638 (s), 1533 (s), 1281 (s), 1195 (m), 814 (w), 745 (m). HRMS (ESI): m/z calculated for $C_{36}H_{53}N_{14}O_8^+$ $[M+H]^+$: 809.4165; found: 809.4186.

Synthesis of hydrazide E



E

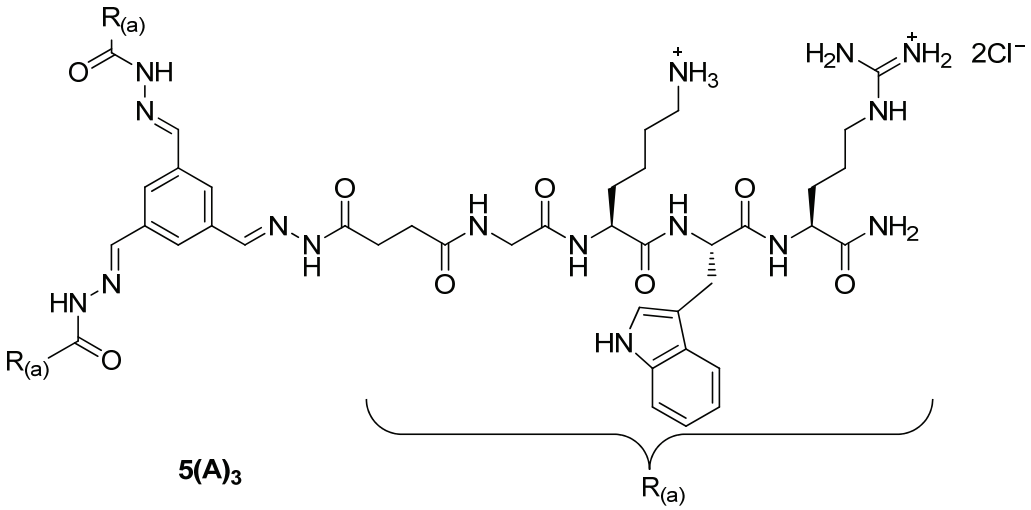
The synthesis of **E** was carried out accordingly on Rink amide MBHA resin (200 mg, 0.84 mmol/g, 0.168 mmol, 1 eq) following the above described microwave-assisted SPPS procedure. PyBOP (262 mg, 0.504 mmol, 3 eq) and DIPEA (171 µL, 1.008 mmol, 6 eq) in DMF (5 mL) were used during the coupling steps. After Fmoc deprotection, the amino acids Fmoc-Glu(O^tBu)-OH (214 mg, 0.504 mmol, 3 eq), Fmoc-Phe-OH (195 mg, 0.504 mmol, 3 eq), Fmoc-Ala-OH (157 mg, 0.504 mmol, 3 eq), Fmoc-Gly-OH (150 mg, 0.504 mmol, 3 eq) and **6** (117 mg, 0.504 mmol, 3 eq) were coupled successively. After the cleavage and purification steps, the product **E** was obtained as a white solid (31 mg, 57.9 µmol, 34 %, HPLC purity 92 %). Mp: 109 °C. ^{1}H NMR (500 MHz, DMSO-d_6): δ [ppm] = 1.10-1.18 (m, 3 H, Ala-CH_3), 1.70-1.83, 1.89-2.00 (m, 2 H, Glu-CH_2), 2.19-2.30 (m, 2 H, Glu-CH_2), 2.36-2.46 (m, 4 H, 2 × CH_2), 2.79-3.07 (m, 2 H, Phe-CH_2), 3.68-3.69 (m, 2 H, Gly-CH_2), 4.15-4.24 (m, 2 H, Glu-CH, Ala-CH), 4.41-4.50 (m, 1 H, Phe-CH), 7.09 (s, 1 H, NH_2), 7.15-7.19 (m, 2 H, 2 × Phe-CH_{ar}), 7.24-7.26 (m, 4 H, 3 × Phe-CH_{ar}, NH_2), 7.82-8.18 (m, 4 H, 4 × NH), 10.98 (s, 1 H, NH). ^{13}C NMR (125 MHz, DMSO-d_6): δ [ppm] = 18.03 (Ala-CH_3), 27.31 (Glu-CH_2), 28.03 (CH_2), 29.45 (CH_2), 30.04 (Glu-CH_2), 36.84 (Phe-CH_2), 42.01 (Gly-CH_2), 48.30 (Ala-CH), 51.80 (Glu-CH), 54.23 (Phe-CH), 126.28 (Phe-CH_{ar}), 128.03 (Phe-CH_{ar}), 128.08 (Phe-CH_{ar}), 129.18 (Phe-CH_{ar}), 129.22 (Phe-CH_{ar}), 137.80 (Phe-C_q), 170.73 (CO). 171.12 (CO), 171.19 (CO), 172.25 (CO), 172.88 (CO), 173.77 (CO), 173.94 (CO). FT-IR (ATR): $\tilde{\nu}$ [cm^{-1}]

= 3197 (br), 3044 (br), 2925 (br), 1640 (s), 1524 (s), 1407 (w), 1234 (m), 1197 (m), 1031 (w), 744 (m). HRMS (ESI): m/z calculated for $C_{23}H_{34}N_7O_8^+$ $[M+H]^+$: 536.2463; found: 536.2399.

General procedure for the synthesis of acyl hydrazones 5(A-E)$_3$, 3(A-C)$_2$, 3(AC).

The acyl hydrazones were synthesized from the peptide-derived hydrazides **A-E** and the corresponding aldehydes using a slightl excess of the hydrazides compared to the amounts of the aldehyde groups.

Synthesis of acyl hydrazone 5(A)$_3$

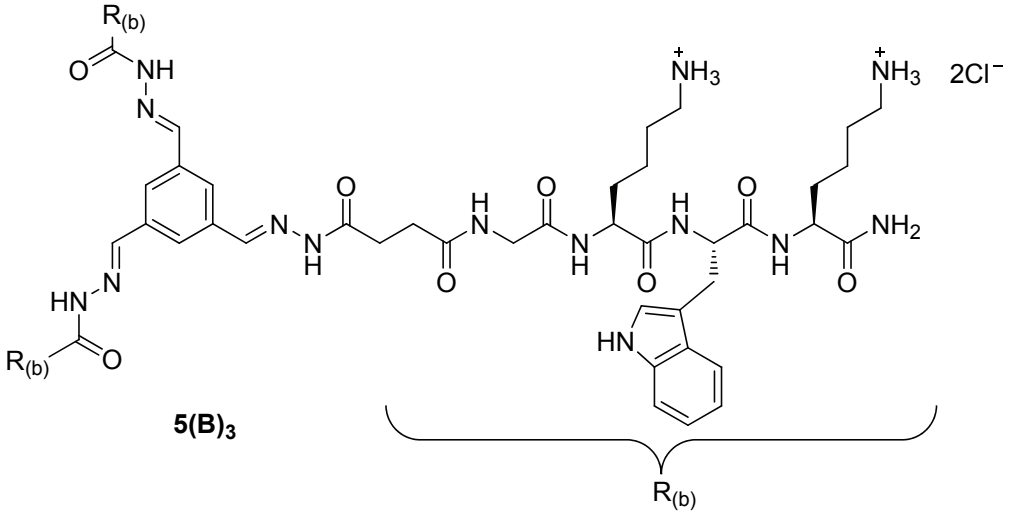


5(A)$_3$

Trialdehyde **5** (1.62 mg, 0.01 mmol, 1 eq) and **A** (25.6 mg, 0.035 mmol, 3.5 eq) were dissolved in 10 mL MeOH under argon. Then the reaction mixture was stirred under reflux overnight. After the solvent was removed, the crude product was purified by RP18-MPLC using appropriate conditions (MeOH/H_2O + 0.1% TFA). The pure product was transferred into the hydrochloride salt to obtain **5(A)$_3$** as a white solid (6 mg, 2.6 µmol, 26%, HPLC purity 93%). Mp: 215 ^oC (decomposition). ^1H NMR (500 MHz, DMSO-d_6): δ [ppm] = 1.19-1.24 (m, 6 H, 3 × Lys-CH_2), 1.44-1.72 (m, 24 H, 6 × Lys-CH_2, 6 × Arg-CH_2), 2.69-2.72 (m, 6 H, 3 × Lys-CH_2), 2.91-3.19 (m, 12 H, 3 × Arg-CH_2, 3 × Trp-CH_2), 3.73 (s, 6 H, 3 × Gly-CH_2), 4.16-4.20 (m, 6 H, 3 × Lys-CH, 3 × Arg-CH), 4.46-4.51 (m, 3 H, 3 × Trp-CH), 6.95 (q, J = 7.4 Hz, 3 H, 3 × Trp-CH_{ar}), 7.03 (q, J = 7.2 Hz, 3 H, 3 × Trp-CH_{ar}), 7.13 (s, 3 H, NH_2), 7.17 (s, 3 H, 3 × Trp-CH_{ar}), 7.27 (s, 3 H, NH_2), 7.31 (t, J = 8.6 Hz, 3 H, 3 × Trp-CH_{ar}), 7.55 (t, J = 7.5 Hz, 3 H, 3 × Trp-CH_{ar}), 7.72 (s, 3 H, 3 × NH), 7.88 (brs, 9 H, 3 × Lys-NH_3^+), 7.93-8.05 (m, 5 H, 5 × NH), 8.07-8.17 (m, 6 H, 3 × Phe-CH_{ar}, 3 × CH=N), 8.25-8.30 (m, 4 H, 4 × NH), 10.87 (s, 3 H, 3 × Trp-NH), 11.40 (t, J = 10.9 Hz, 1 H, NH), 11.75 (s, 1 H, NH). ^{13}C NMR (125 MHz, DMSO-d_6): δ [ppm] = 22.09 (Lys-CH_2), 24.96 (Arg-CH_2), 26.45 (Lys-CH_2), 27.12

(Trp-CH_2), 27.42 (CH_2), 29.09 (Arg-CH_2), 29.49 (CH_2), 30.03 (Lys-CH_2), 30.88 (Lys-CH_2), 38.47 (Lys-CH_2), 40.32 (Arg-CH_2), 43.93 (Gly-CH_2), 52.06 (Arg-CH), 52.80 (Lys-CH), 53.84 (Trp-CH), 109.94 (Trp-C_q), 111.34 (Trp-CH_{ar}), 118.23 (Trp-CH_{ar}), 118.31 (Trp-CH_{ar}), 120.86 (Trp-CH_{ar}), 123.68 (Trp-CH_{ar}), 126.01 (Phe-CH_{ar}), 127.24 (Trp-C_q), 135.36 (Phe-C_q), 136.07 (Trp-C_q), 142.01 (CH=N), 156.87 (Gua-C_q), 169.48 (CO), 171.23 (CO), 171.62 (CO), 171.92 (CO), 172.24 (CO), 173.20 (CO). FT-IR (ATR): $\tilde{\nu}$ [cm^{-1}] = 3189 (br), 3044 (br), 2926 (br), 1647 (s), 1522 (s), 1339 (w), 1237 (m), 1161 (w), 1098 (w), 1025 (w), 955 (w), 745 (m). HRMS (ESI): m/z calculated for $C_{96}H_{142}N_{36}O_{18}^{4+}$ $[M+4H]^{4+}$: 522.0327; found: 522.0328.

Synthesis of acyl hydrazone 5(B)$_3$

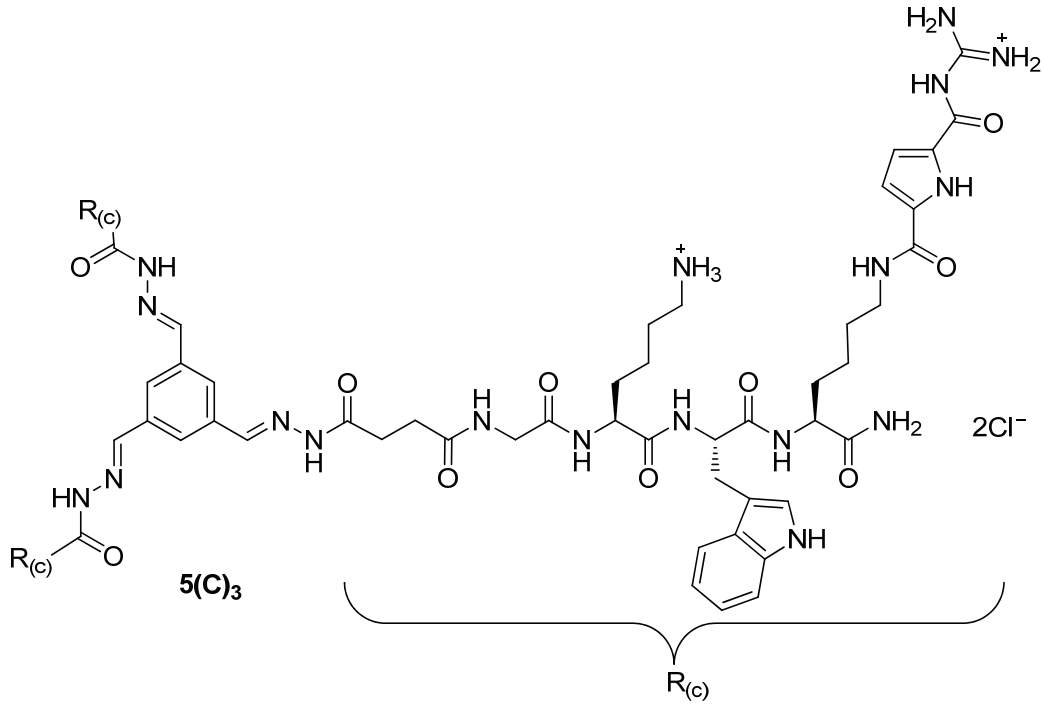


5(B)$_3$

The synthesis of compound **5(B)$_3$** was carried out using analogous conditions as described for compound **5(A)$_3$**. After purification by RP18-MPLC and lyophilization, **5(B)$_3$** was obtained as a white solid (5 mg, 2.3 µmol, 23%, HPLC purity 93%). Mp: 216 °C (decomposition). ^{1}H NMR (500 MHz, DMSO-d_6): δ [ppm] = 1.18-1.27 (m, 12 H, 6 × Lys-CH_2), 1.52-1.67 (m, 24 H, 12 × Lys-CH_2), 2.69-2.74 (m, 12 H, 6 × Lys-CH_2), 3.04-3.19 (m, 6 H, 3 × Trp-CH_2), 3.73 (s, 6 H, 3 × Gly-CH_2), 4.16 (s, 6 H, 6 × Lys-CH), 4.48 (s, 3 H, 3 × Trp-CH), 6.96 (t, J = 7.3 Hz, 3 H, 3 × Trp-CH_{ar}), 7.01-7.13 (m, 6 H, 3 × Trp-CH_{ar}, NH_2), 7.18-7.23 (m, 6 H, 3 × Trp-CH_{ar}, NH_2), 7.30-7.33 (m, 3 H, 3 × Trp-CH_{ar}), 7.56 (t, J = 8.0 Hz, 3 H, 3 × Trp-CH_{ar}), 7.81-7.88 (m, 4 H, 4 × NH), 7.87 (brs, 18 H, 6 × Lys-NH_3^+), 8.09-8.14 (m, 6 H, 3 × Phe-CH_{ar}, 3 × CH=N), 8.26-8.33 (m, 5 H, 5 × NH), 10.89 (s, 2 H, 2 × Trp-NH), 11.43 (s, 1 H, NH), 11.79 (s, 1 H, NH). ^{13}C NMR (125 MHz, DMSO-d_6): δ [ppm] = 22.09 (Lys-CH_2), 26.44 (Lys-CH_2), 26.53 (Lys-CH_2), 27.02 (Trp-CH_2), 30.88 (Lys-CH_2), 31.23 (Lys-CH_2), 38.45 (Lys-CH_2), 38.53 (Lys-CH_2), 43.90 (Gly-CH_2), 52.28 (Lys-CH), 52.80 (Lys-CH), 53.84 (Trp-CH), 109.93 (Trp-C_q), 111.26 (Trp-CH_{ar}), 118.21 (Trp-CH_{ar}), 118.30 (Trp-CH_{ar}), 120.82 (Trp-

CH_{ar}), 123.65 (Trp-CH_{ar}), 127.18 (Trp-C_q), 132.54 (Phe-CH_{ar}), 135.50 (Phe-C_q), 136.04 (Trp-C_q), 169.52 (CO), 171.18 (CO), 171.65 (CO), 173.39 (CO). FT-IR (ATR): $\tilde{\nu}$ [cm^{-1}] = 3239 (br), 3038 (br), 2919 (br), 1646 (s), 1522 (s), 1339 (w), 1235 (m), 1159 (w), 1102 (w), 1025 (w), 954 (w), 745 (m). HRMS (ESI): m/z calculated for $C_{96}H_{143}N_{30}O_{18}^{5+}$ $[M+5H]^{5+}$: 401.0239; found: 401.0228.

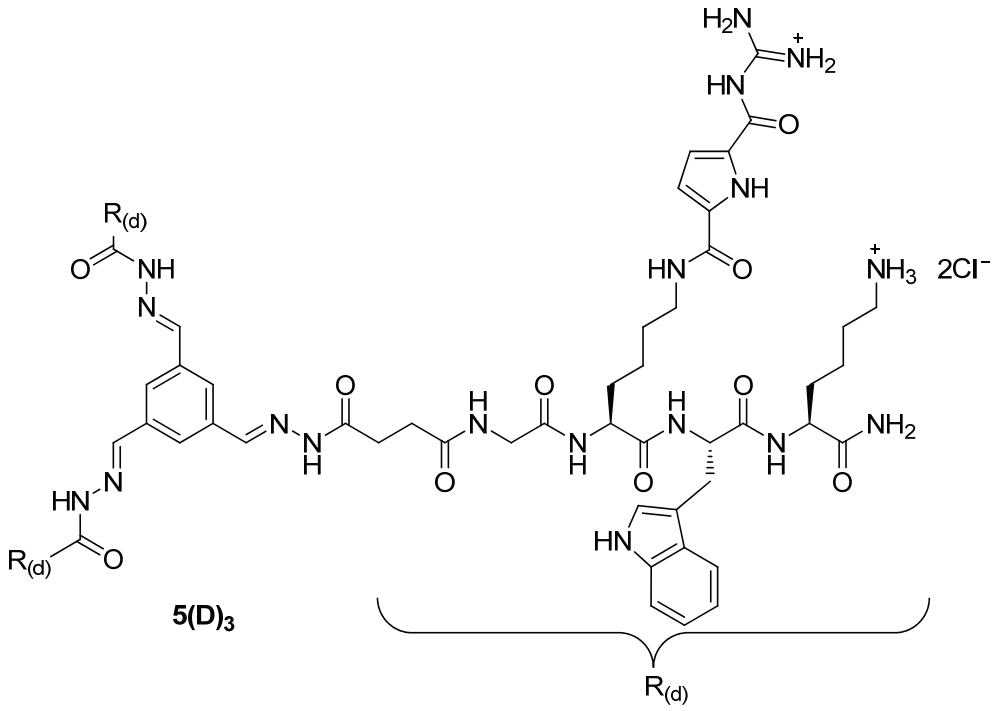
Synthesis of acyl hydrazone 5(C)$_3$



The synthesis of compound **5(C)$_3$** was carried out using analogous conditions as described for compound **5(A)$_3$**. After purification by RP18-MPLC and lyophilization, **5(C)$_3$** was obtained as a white solid (8 mg, 2.9 µmol, 29%, HPLC purity 95%). Mp: 229 °C (decomposition). ^{1}H NMR (700 MHz, DMSO-d_6): δ [ppm] = 1.19-1.33, 1.49-1.71 (m, 36 H, 18 × Lys-CH_2), 2.71-2.76 (m, 6 H, 3 × Lys-CH_2), 2.92-3.02 (m, 6 H, 3 × Trp-CH_2), 3.16-3.25 (m, 6 H, 3 × Lys-CH_2), 3.69-3.76 (m, 6 H, 3 × Gly-CH_2), 4.16-4.22 (m, 6 H, 6 × Lys-CH), 4.49-4.52 (m, 3 H, 3 × Trp-CH), 6.87 (s, 3 H, 3 × GCP-CH_{ar}), 6.94-6.97 (m, 3 H, 3 × Trp-CH_{ar}), 7.02-7.09 (m, 6 H, 3 × Trp-CH_{ar}, NH_2), 7.15-7.16 (m, 3 H, 3 × Trp-CH_{ar}), 7.23 (s, 3 H, NH_2), 7.30-7.33 (m, 3 H, 3 × Trp-CH_{ar}), 7.45 (s, 3 H, 3 × GCP-CH_{ar}), 7.55 (t, J = 8.7 Hz, 3 H, 3 × Trp-CH_{ar}), 7.80 (brs, 9 H, 3 × Lys-NH_3^+), 7.84-8.01 (m, 6 H, 6 × NH), 8.06-8.07 (m, 6 H, 6 × NH), 8.21-8.25 (m, 6 H, 3 × Phe-CH_{ar}, 3 × CH=N), 8.41-8.57 (m, 13 H, 13 × NH), 10.81, 10.82 (s, 3 H, 3 × Trp-NH), 11.37(t, J = 18.1 Hz, 1 H, NH), 11.66 (t, J = 9.5 Hz, 1 H, NH), 11.87 (s, 3 H, 3 × Gua-NH), 12.31 (s, 3 H, 3 × GCP-NH). ^{13}C NMR (175 MHz, DMSO-d_6): δ [ppm] = 22.09 (Lys-CH_2), 22.75 (Lys-CH_2), 26.50 (Lys-CH_2), 27.22 (Trp-CH_2), 28.73 (Lys-CH_2), 29.45 (CH_2),

30.02 (CH_2), 31.04 (Lys-CH_2), 31.73 (Lys-CH_2), 34.26 (Lys-CH_2), 38.60 (Lys-CH_2), 38.74 (Lys-CH_2), 42.29 (Gly-CH_2), 52.45 (Lys-CH), 52.56 (Lys-CH), 53.74 (Trp-CH), 109.89 (Trp-C_q), 111.27 (Trp-CH_{ar}), 112.36 (GCP-CH_{ar}), 115.89 (GCP-CH_{ar}), 118.23 (Trp-CH_{ar}), 118.33 (Trp-CH_{ar}), 120.82 (Trp-CH_{ar}), 123.64 (Trp-CH_{ar}), 125.33 (GCP-C_q), 127.21 (Trp-C_q), 132.91 (GCP-Cq), 135.31 (Phe-CH_{ar}), 135.48 (Phe-C_q), 136.00 (Trp-C_q), 144.78 (CH=N), 155.43 (Gua-C_q), 159.05 (CO), 159.62 (CO), 168.34 (CO), 169.23 (CO), 169.34 (CO), 171.14 (CO), 171.54 (CO), 171.92 (CO), 172.27 (CO), 173.54 (CO). FT-IR (ATR): $\tilde{\nu}$ [cm^{-1}] = 3272 (br), 3047 (br), 2940 (br), 1645 (s), 1541 (s), 1284 (s), 1199 (m), 815 (w), 746 (m). HRMS (ESI): m/z calculated for $C_{117}H_{160}N_{42}O_{24}^{4+}$ $[M+4H]^{4+}$: 634.5649; found: 634.5649.

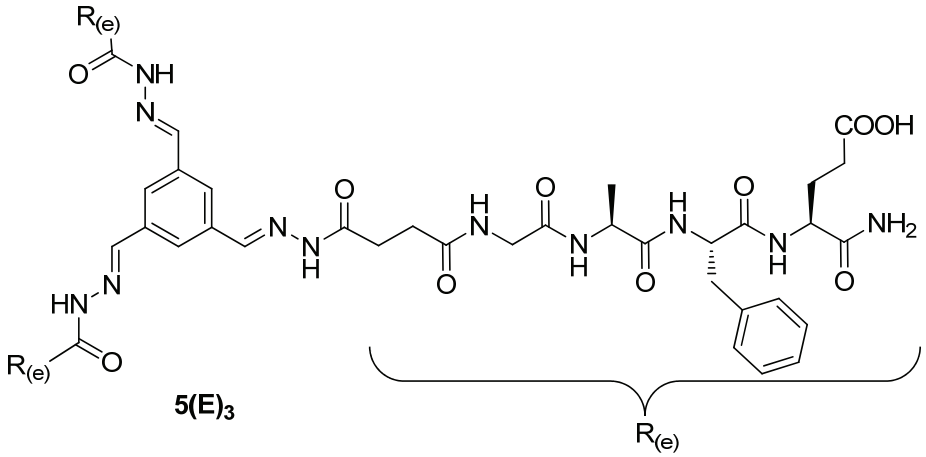
Synthesis of acyl hydrazone 5(D)$_3$



The synthesis of compound **5(D)$_3$** was carried out using analogous conditions as described for compound **5(A)$_3$**. After purification by RP18-MPLC and lyophilization, **5(D)$_3$** was obtained as a white solid (10 mg, 3.6 µmol, 36%, HPLC purity 95%). Mp: 231 °C (decomposition). ^{1}H NMR (500 MHz, DMSO-d_6): δ [ppm] = 1.19-1.29, 1.45-1.68 (m, 36 H, 18 × Lys-CH_2), 2.74-2.75 (m, 6 H, 3 × Lys-CH_2), 2.91-3.04 (m, 6 H, 3 × Trp-CH_2), 3.15-3.19 (m, 6 H, 3 × Lys-CH_2), 3.73 (s, 6 H, 3 × Gly-CH_2), 4.14-4.17 (m, 6 H, 6 × Lys-CH), 4.45-4.49 (m, 3 H, 3 × Trp-CH), 6.86 (s, 3 H, 3 × GCP-CH_{ar}), 6.92-6.97 (m, 3 H, 3 × Trp-CH_{ar}), 7.00-7.09 (m, 6 H, 3 × Trp-CH_{ar}, NH_2), 7.16-7.20 (m, 6 H, 3 × Trp-CH_{ar}, NH_2), 7.30 (t, J = 9.8 Hz, 3 H, 3 × Trp-CH_{ar}), 7.49 (s, 3 H, 3 × GCP-CH_{ar}), 7.55 (t, J = 9.1 Hz,, 3 H, 3 × Trp-CH_{ar}), 7.85 (brs, 9 H, 3 × Lys-NH_3^+), 7.79-7.80, 7.91-7.92 (m, 6 H, 6 × NH), 8.04-8.07 (m, 6 H, 6 × NH), 8.11-8.30

(m, 7 H, NH, 3 × Phe-CH_{ar}, 3 × CH=N), 8.43-8.62 (m, 15 H, 15 × NH), 10.81, 10.84 (s, 3 H, 3 × Trp-NH), 11.35 (t, J = 13.8 Hz, 1 H, NH), 11.67 (s, 1 H, NH), 11.97 (s, 3 H, 3 × Gua-NH), 12.30 (s, 3 H, 3 × GCP-NH). ^{13}C NMR (125 MHz, DMSO-d_6): δ [ppm] = 22.09 (Lys-CH_2), 22.83 (Lys-CH_2), 26.55 (Lys-CH_2), 27.01 (Trp-CH_2), 28.70 (Lys-CH_2), 29.47 (CH_2), 31.23 (Lys-CH_2), 38.61 (Lys-CH_2), 43.95 (Gly-CH_2), 52.26 (Lys-CH), 52.99 (Lys-CH), 53.77 (Trp-CH), 109.90 (Trp-C_q), 111.26 (Trp-CH_{ar}), 112.35 (GCP-CH_{ar}), 115.91 (GCP-CH_{ar}), 118.20 (Trp-CH_{ar}), 118.30 (Trp-CH_{ar}), 120.81 (Trp-CH_{ar}), 123.58 (Trp-CH_{ar}), 123.63 (Trp-CH_{ar}), 125.31 (GCP-C_q), 127.18 (Trp-C_q), 132.91 (GCP-Cq), 135.25 (Phe-CH_{ar}), 135.43 (Phe-C_q), 136.00 (Trp-C_q), 144.56 (CH=N), 155.45 (Gua-Cq), 158.97 (CO), 159.59 (CO), 168.26 (CO), 169.39 (CO), 171.15 (CO), 171.84 (CO), 173.35 (CO). FT-IR (ATR): $\tilde{\nu}$ [cm^{-1}] = 3232 (br), 3049 (br), 2921 (br), 1648 (s), 1541 (s), 1284 (m), 1252 (m), 1201 (w), 1098 (w), 1025 (w), 955 (w), 748 (m). HRMS (ESI): m/z calculated for $C_{117}H_{160}N_{42}O_{24}^{4+}$ $[M+4H]^{4+}$: 634.5649; found: 634.5650.

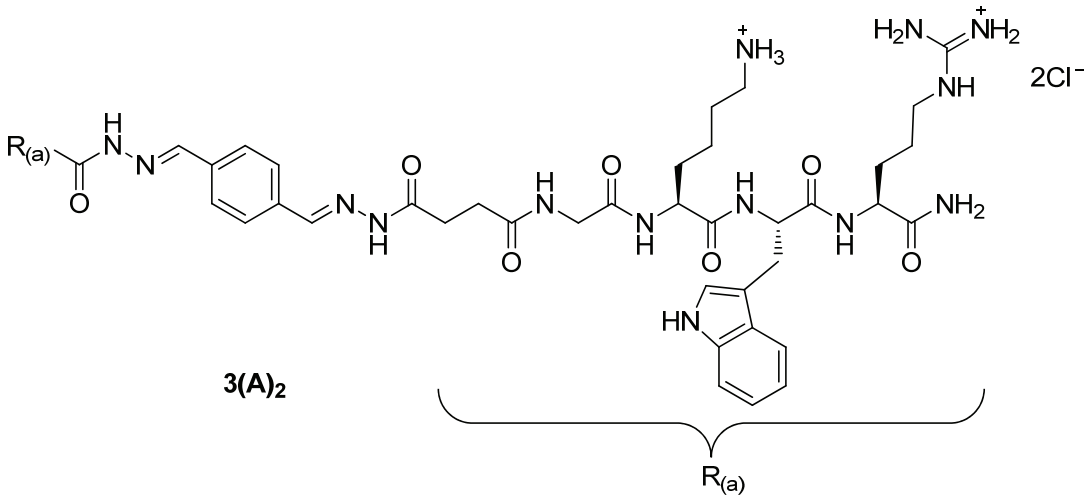
Synthesis of acyl hydrazone 5(E)$_3$



The synthesis of compound **5(E)$_3$** was carried out using analogous conditions as described for compound **5(A)$_3$**. After purification by RP18-MPLC and lyophilization, **5(E)$_3$** was obtained as a white solid (4 mg, 2.3 µmol, 23%, HPLC purity 90%). Mp: 149 °C (decomposition). ^{1}H NMR (500 MHz, DMSO-d_6): δ [ppm] = 1.14-1.19 (m, 9 H, 3 × Ala-CH_3), 1.72-1.78, 1.91-1.96 (m, 6 H, 3 × Glu-CH_2), 2.19-2.22 (m, 6 H, 3 × Glu-CH_2), 2.82-2.91 (m, 6 H, 3 × Phe-CH_2), 3.70 (s, 6 H, 3 × Gly-CH_2), 4.14-4.22 (m, 6 H, 3 × Glu-CH, 3 × Ala-CH), 4.42 (s, 3 H, 3 × Phe-CH), 7.09-7.26 (m, 21 H, 15 × Phe-CH_{ar}, 3 × NH_2), 7.78-8.23 (m, 18 H, 12 × NH, 3 × CH=N, 3 × CH_{ar}), 11.36 (t, J = 12.1 Hz, 1 H, NH) 11.56 (t, J = 10.5 Hz, 1 H, NH). ^{13}C NMR (125 MHz, DMSO-d_6): δ [ppm] = 17.73 (Ala-CH_3), 27.23 (Glu-CH_2), 29.47 (CH_2), 30.05 (Glu-CH_2), 34.32 (CH_2), 36.80 (Phe-CH_2), 44.21 (Gly-CH_2), 48.53 (Ala-CH), 51.84 (Glu-CH),

54.25 (Phe-CH), 126.26 (Phe-CH_{ar}), 126.28 (Phe-CH_{ar}), 128.07 (Phe-CH_{ar}), 128.09 (Phe-CH_{ar}), 129.09 (Phe-CH_{ar}), 129.13 (Phe-CH_{ar}), 137.76 (Phe-C_q), 137.79 (Phe-C_q), 169.21 (CO), 170.75 (CO), 172.31 (CO), 172.36 (CO), 172.90 (CO), 173.97 (CO). FT-IR (ATR): $\tilde{\nu}$ [cm^{-1}] = 3264 (br), 3060 (br), 2930 (br), 1636 (s), 1531 (s), 1407 (w), 1254 (m), 1198 (m), 1130 (m), 953 (w), 800 (w), 745 (m). HRMS (ESI): m/z calculated for $C_{78}H_{96}N_{21}O_{24}^{3-}$ [M-3H] $^{3-}$: 570.2318; found: 570.2401.

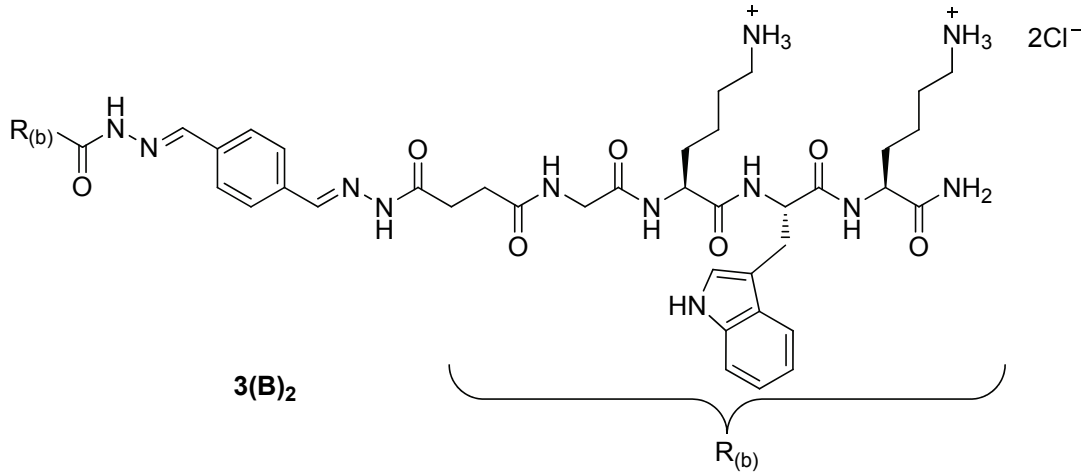
Synthesis of acyl hydrazone 3(A)$_2$



Dialdehyde **3** (1.34 mg, 0.01 mmol, 1 eq) and **A** (18.3 mg, 0.025 mmol, 2.5 eq) were dissolved in 10 mL MeOH under argon. Then the reaction mixture was refluxed overnight. After the solvent was removed, the crude product was purified by RP18-MPLC using appropriate conditions (MeOH/H_2O + 0.1% TFA). Pure product was transferred into hydrochloride salt to obtain **3(A)$_2$** as a white solid (6 mg, 3.8 µmol, 38%, HPLC purity 97%). Mp: 220 ^{o}C (decomposition). ^{1}H NMR (500 MHz, DMSO-d_6): δ [ppm] = 1.18-1.24 (m, 4 H, 2 × Lys-CH_2), 1.44-1.71 (m, 16 H, 4 × Lys-CH_2, 4 × Arg-CH_2), 2.63-2.74 (m, 4 H, 2 × Lys-CH_2), 2.89-3.20 (m, 8 H, 2 × Arg-CH_2, 2 × Trp-CH_2), 3.71-3.72 (m, 4 H, 2 × Gly-CH_2), 4.16-4.20 (m, 4 H, 2 × Lys-CH, 2 × Arg-CH), 4.46-4.51 (m, 2 H, 2 × Trp-CH), 6.94-6.98 (m, 2 H, 2 × Trp-CH_{ar}), 7.04 (t, J = 7.3 Hz, 2 H, 2 × Trp-CH_{ar}), 7.12 (s, 2 H, NH_2), 7.16 (s, 2 H, 2 × Trp-CH_{ar}), 7.25-7.26 (m, 2 H, NH_2), 7.31-7.33 (m, 2 H, 2 × Trp-CH_{ar}), 7.54-7.57 (m, 2 H, 2 × Trp-CH_{ar}), 7.62-7.70 (m, 6 H, 6 H, 4 × Phe-CH_{ar}, 2 × NH), 7.86 (brs, 6 H, 2 × Lys-NH_3^+), 8.00-8.17 (m, 6 H, 2 × CH=N, 4 × NH), 8.25-8.30 (m, 2 H, 2 × NH), 10.85 (s, 2 H, 2 × Trp-NH), 11.36 (s, 1 H, NH), 11.64 (s, 1 H, NH). ^{13}C NMR (125 MHz, DMSO-d_6): δ [ppm] = 22.08 (Lys-CH_2), 24.94 (Arg-CH_2), 26.48 (Lys-CH_2), 27.11 (Trp-CH_2), 27.50 (CH_2), 29.09 (Arg-CH_2), 30.88 (Lys-CH_2), 38.48 (Lys-CH_2), 40.34 (Arg-CH_2), 42.35 (Gly-CH_2), 52.06 (Arg-CH), 52.72 (Lys-CH), 53.82 (Trp-CH), 109.91 (Trp-C_q), 111.26 (Trp-CH_{ar}), 118.23

(Trp-CH_{ar}), 118.29 (Trp-CH_{ar}), 120.84 (Trp-CH_{ar}), 123.61 (Trp-CH_{ar}), 126.99 (Phe-CH_{ar}), 127.18 (Trp-C_q), 135.50 (Phe-C_q), 136.03 (Trp-C_q), 142.19 (CH=N), 156.80 (Gua-C_q), 169.57 (CO), 171.23 (CO), 171.62 (CO), 171.94 (CO), 172.29 (CO), 173.21 (CO), 173.63 (CO). FT-IR (ATR): $\tilde{\nu}$ [cm^{-1}] = 3171 (br), 3048 (br), 2925 (br), 1638 (s), 1523 (s), 1231 (w), 1167 (w), 1096 (w), 1024 (w), 952 (w), 745 (m). HRMS (ESI): m/z calculated for $C_{66}H_{97}N_{24}O_{12}^{3+}$ $[M+3H]^{3+}$: 472.5900; found: 472.5863.

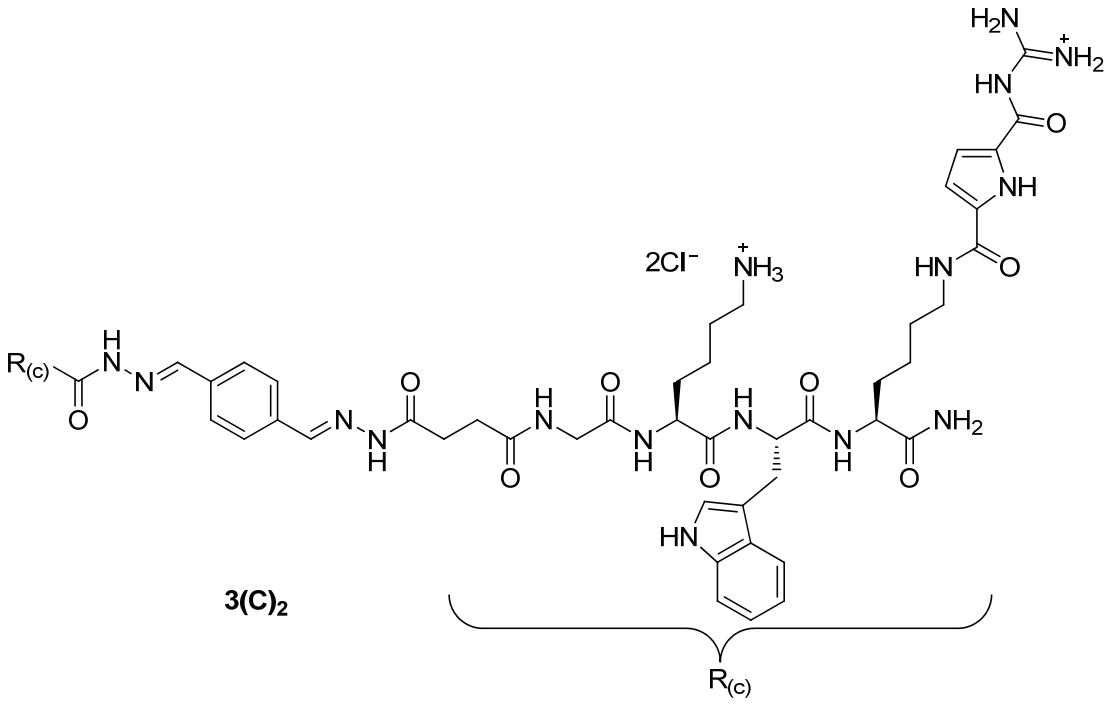
Synthesis of acyl hydrazone 3(B)$_2$



The synthesis of compound **3(B)$_2$** was carried out using analogous conditions as described for compound **3(A)$_2$**. After purification by RP18-MPLC and lyophilization, **3(B)$_2$** was obtained as a white solid (13 mg, 8.6 µmol, 86%, HPLC purity 97%). Mp: 182 °C (decomposition). ^{1}H NMR (500 MHz, DMSO-d_6): δ [ppm] = 1.17-1.29 (m, 8 H, 4 × Lys-CH_2), 1.48-1.70 (m, 16 H, 8 × Lys-CH_2), 2.69-2.74 (m, 8 H, 4 × Lys-CH_2), 3.04-3.09 (m, 4 H, 2 × Trp-CH_2), 3.72 (s, 4 H, 2 × Gly-CH_2), 4.12-4.21 (m, 4 H, 4 × Lys-CH), 4.47 (s, 2 H, 2 × Trp-CH), 6.94-6.99 (m, 2 H, 2 × Trp-CH_{ar}), 7.03-7.07 (m, 4 H, 2 × Trp-CH_{ar}, NH_2), 7.17-7.20 (m, 4 H, 2 × Trp-CH_{ar}, NH_2), 7.31-7.33 (m, 2 H, 2 × Trp-CH_{ar}), 7.54-7.57 (m, 2 H, 2 × Trp-CH_{ar}), 7.61 (s, 1 H, Phe-CH_{ar}), 7.66 (s, 1 H, Phe-CH_{ar}), 7.70 (s, 1 H, Phe-CH_{ar}), 7.78-7.82 (m, 2 H, 2 × NH), 7.89 (brs, 12 H, 4 × Lys-NH_3^+), 8.00-8.02 (m, 2 H, 2 × CH=N), 8.07-8.34 (m, 6 H, 6 × NH), 10.86 (s, 2 H, 2 × Trp-NH), 11.36 (s, 1 H, NH), 11.65 (s, 1 H, NH). ^{13}C NMR (125 MHz, DMSO-d_6): δ [ppm] = 22.11 (Lys-CH_2), 26.49 (Lys-CH_2), 26.56 (Lys-CH_2), 27.06 (Trp-CH_2), 27.49 (CH_2), 29.28 (CH_2), 30.02 (CH_2), 30.88 (Lys-CH_2), 31.27 (Lys-CH_2), 38.53 (Lys-CH_2), 38.60 (Lys-CH_2), 42.35 (Gly-CH_2), 52.28 (Lys-CH), 52.78 (Lys-CH), 53.80 (Trp-CH), 109.93 (Trp-C_q), 111.27 (Trp-CH_{ar}), 118.22 (Trp-CH_{ar}), 118.33 (Trp-CH_{ar}), 118.51 (Trp-CH_{ar}), 120.84 (Trp-CH_{ar}), 123.66 (Trp-CH_{ar}), 126.98 (Phe-CH_{ar}), 127.22 (Trp-C_q), 136.03 (Trp-C_q), 142.21 (CH=N), 169.37 (CO), 169.60 (CO), 171.17 (CO), 171.65 (CO), 171.94 (CO), 172.30 (CO),

173.37 (CO), 173.64 (CO). FT-IR (ATR): $\tilde{\nu}$ [cm^{-1}] = 3176 (br), 3047 (br), 2925 (br), 1637 (s), 1523 (s), 1340 (w), 1200 (m), 1174 (m), 1128 (m), 1026 (w), 954 (w), 835 (w), 798 (w), 744 (m). HRMS (ESI): m/z calculated for $C_{66}H_{97}N_{20}O_{12}^{3+}$ $[M+3H]^{3+}$: 453.9193; found: 453.9185.

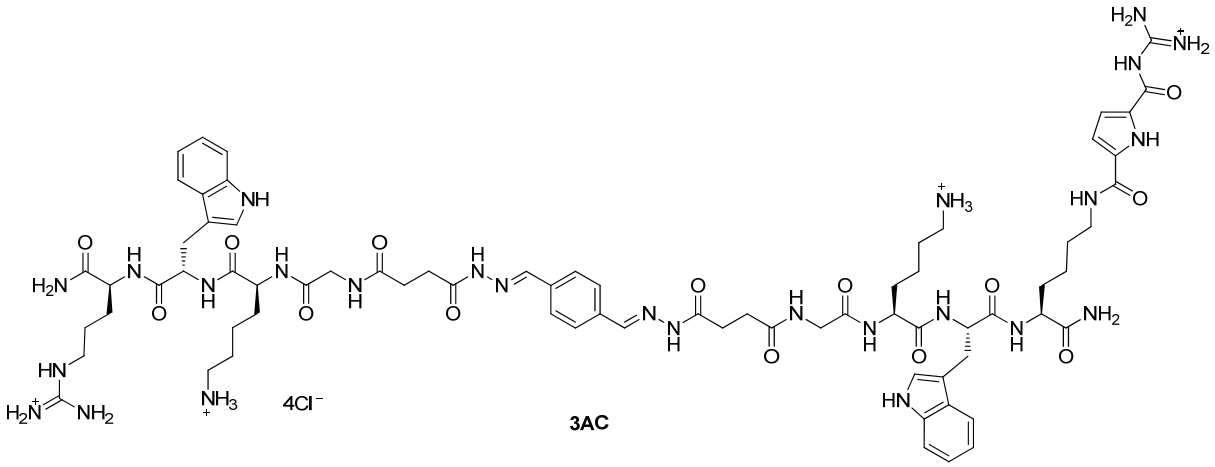
Synthesis of acyl hydrazone 3(C)$_2$



The synthesis of compound **3(C)$_2$** was carried out using analogous conditions as described for compound **3(A)$_2$**. After purification by RP18-MPLC and lyophilization, **3(C)$_2$** was obtained as a white solid (12 mg, 6.4 µmol, 64%, HPLC purity 91%). Mp: 214 ^{o}C (decomposition). ^{1}H NMR (500 MHz, DMSO-d_6): δ [ppm] = 1.19-1.33 (m, 8 H, 4 × Lys-CH$_2$), 1.47-1.68 (m, 16 H, 8 × Lys-CH$_2$), 2.69-2.71 (m, 4 H, 2 × Lys-CH$_2$), 2.89-3.04(m, 4 H, 2 × Trp-CH$_2$), 3.16-3.23 (m, 4 H, 2 × Lys-CH$_2$), 3.71 (s, 4 H, 2 × Gly-CH$_2$), 4.15-4.19 (m, 4 H, 4 × Lys-CH), 4.48-4.49 (m, 2 H, 2 × Trp-CH), 6.87 (s, 2 H, 2 × GCP-CH$_{ar}$), 6.95 (t, J = 7.5 Hz, 2 H, 2 × Trp-CH$_{ar}$), 7.02-7.06 (m, 4 H, 2 × Trp-CH$_{ar}$, NH$_2$), 7.16 (s, 2 H, 2 × Trp-CH$_{ar}$), 7.21-7.23 (m, 2 H, NH$_2$), 7.30-7.32 (m, 2 H, 2 × Trp-CH$_{ar}$), 7.51 (s, 2 H, 2 × GCP-CH$_{ar}$), 7.54-7.55 (m, 2 H, 2 × Trp-CH$_{ar}$), 7.60 (s, 1 H, Phe-CH$_{ar}$), 7.65 (s, 2 H, 2 × Phe-CH$_{ar}$), 7.69 (s, 1 H, Phe-CH$_{ar}$), 7.77-7.81 (m, 2 H, 2 × NH), 7.86 (brs, 6 H, 2 × Lys-NH$_3^+$), 7.99-8.18 (m, 6 H, 6 × NH), 8.26-8.30 (m, 2 H, 2 × CH=N), 8.46-8.64 (m, 8 H, 8 × NH), 10.84 (s, 2 H, 2 × Trp-NH), 11.35 (s, 1 H, NH), 11.63 (s, 1 H, NH), 12.00 (s, 2 H, 2 × Gua-NH), 12.32 (s, 2 H, 2 × GCP-NH). ^{13}C NMR (125 MHz, DMSO-d_6): δ [ppm] = 22.09 (Lys-CH$_2$), 22.75 (Lys-CH$_2$), 26.48 (Lys-CH$_2$), 27.19 (Lys-CH$_2$), 27.52 (Trp-CH$_2$), 28.71 (Lys-CH$_2$), 29.30 (CH$_2$), 30.03 (CH$_2$), 30.93 (Lys-

CH_2), 31.71 (Lys-CH_2), 38.54 (Lys-CH_2), 38.73 (Lys-CH_2), 42.25 (Gly-CH_2), 42.37 (Gly-CH_2), 52.46 (Lys-CH), 52.58 (Lys-CH), 52.69 (Lys-CH), 53.84 (Trp-CH), 109.93 (Trp-C_q), 111.26 (Trp-CH_{ar}), 112.41 (GCP-CH_{ar}), 115.95 (GCP-CH_{ar}), 118.22 (Trp-CH_{ar}), 118.30 (Trp-CH_{ar}), 120.80 (Trp-CH_{ar}), 123.62 (Trp-CH_{ar}), 125.31 (GCP-C_q), 126.96 (Phe-CH_{ar}), 127.21 (Trp-C_q), 132.93 (GCP-Cq), 135.28 (Phe-C_q), 136.01 (Trp-C_q), 142.17 (CH=N), 155.48 (Gua-C_q), 158.99 (CO), 159.62 (CO), 168.22 (CO), 169.23 (CO), 169.44 (CO), 171.14 (CO), 171.57 (CO), 171.90 (CO), 172.27 (CO), 173.49 (CO), 173.63 (CO). FT-IR (ATR): $\tilde{\nu}$ [cm^{-1}] = 3273 (br), 3055 (br), 2930 (br), 1643 (s), 1540 (s), 1282 (s), 1197 (m), 953 (w), 815 (w), 746 (m). HRMS (ESI): m/z calculated for $C_{80}H_{109}N_{28}O_{16}^{3+}$ $[M+3H]^{3+}$: 572.6187; found: 572.6192.

Synthesis of acyl hydrazone 3(AC)



3AC

Dialdehyde **3** (67 mg, 0.5 mmol, 10 eq) and **A** (36.6 mg, 0.05 mmol, 1 eq) were dissolved in 10 mL MeOH under argon. Then the reaction mixture was refluxed overnight. After the purification by RP18-MPLC and lyophilization, the intermediate with one free aldehyde group was obtained (HPLC purity 90%).[3] ^{1}H NMR (500 MHz, DMSO-d_6): δ [ppm] = 1.09-1.31, 1.39-1.72 (m, 10 H, 3 × Lys-CH_2, 2 × Arg-CH_2), 2.63-3.27 (m, 6 H, Arg-CH_2, Lys-CH_2, Trp-CH_2), 3.71-3.72 (m, 2 H, Gly-CH_2), 4.17-4.18 (m, 2 H, Lys-CH, Arg-CH), 4.46-4.51 (m, 1 H, Trp-CH), 6.94-6.96 (m, 1 H, Trp-CH_{ar}), 7.02-7.05 (m, 1 H, Trp-CH_{ar}), 7.11-7.14 (m, 2 H, NH_2, Trp-CH_{ar}), 7.23 (s, 1 H, NH_2), 7.30-7.32 (m, 1 H, Trp-CH_{ar}), 7.42-7.57 (m, 4 H, Trp-CH_{ar}, 3 × NH), 7.68 (brs, 3 H, Lys-NH_3^+), 7.82-8.05 (m, 8 H, 4 × Phe-CH_{ar}, 4 × NH), 8.12-8.21 (m, 2 H, CH=N, NH), 10.14 (s, 1 H, CHO), 10.79 (s, 1 H, Trp-NH), 11.48 (s, 1 H, NH). HRMS (ESI): m/z calculated for $C_{37}H_{52}N_{12}O_7^{2+}$ $[M+2H]^{2+}$: 338.2035; found: 338.2025.

Then the intermediate was reacted with **C** by mixing equimolar amounts in 10 mL MeOH under argon. The reaction mixture was refluxed overnight and the crude product was purified

by RP18-MPLC using appropriate conditions (MeOH/H_2O + 0.1% TFA). Pure product was transferred into the hydrochloride salt to give **3(AC)** as a white solid (3 mg, 1.8 μmol, 18%, HPLC purity 91%). Mp: 210 °C (decomposition). ^{1}H NMR (700 MHz, DMSO-d_6): δ [ppm] = 1.19-1.30 (m, 6 H, 3 × Lys-CH_2), 1.42-1.71 (m, 16 H, 6 × Lys-CH_2, 2 × Arg-CH_2), 2.69-2.71 (m, 4 H, 2 × Lys-CH_2), 2.91-3.22 (m, 8 H, Lys-CH_2, Arg-CH_2, 2 × Trp-CH_2), 3.72 (s, 4 H, 2 × Gly-CH_2), 4.15-4.23 (m, 4 H, 3 × Lys-CH, Arg-CH), 4.47-4.52 (m, 2 H, 2 × Trp-CH), 6.86 (s, 1 H, GCP-CH_{ar}), 6.95-6.98 (m, 2 H, 2 × Trp-CH_{ar}), 7.02-7.07 (m, 3 H, 2 × Trp-CH_{ar}, NH_2), 7.12 (s, 2 H, NH_2), 7.16-7.17 (m, 2 H, 2 × Trp-CH_{ar}), 7.20-7.26 (m, 2 H, NH_2), 7.31-7.34 (m, 2 H, 2 × Trp-CH_{ar}), 7.47 (s, 1 H, GCP-CH_{ar}), 7.54-7.56 (m, 2 H, 2 × Trp-CH_{ar}), 7.62 (s, 1 H, Phe-CH_{ar}), 7.67-7.71 (m, 3 H, 3 × Phe-CH_{ar}), 7.77-7.91 (m, 8 H, 2 × NH, 2 × Lys-NH_3^+), 7.95-8.19 (m, 6 H, 6 × NH), 8.24-8.33 (m, 2 H, 2 × CH=N), 8.42-8.64 (m, 5 H, 5 × NH), 10.83, (s, 1 H, Trp-NH), 10.85 (s, 1 H, Trp-NH), 11.35 (s, 1 H, NH), 11.62 (d, J = 19.7 Hz, 1 H, NH), 11.93 (s, 1 H, Gua-NH), 12.32 (s, 1 H, GCP-NH). ^{13}C NMR (175 MHz, DMSO-d_6): δ [ppm] = 22.07 (Lys-CH_2), 22.74 (Lys-CH_2), 24.93 (Arg-CH_2), 26.48 (Lys-CH_2), 27.14 (Trp-CH_2), 27.47 (CH_2), 28.71 (Lys-CH_2), 29.05 (Arg-CH_2), 29.27 (CH_2), 30.00 (CH_2), 30.94 (Lys-CH_2), 31.69 (Lys-CH_2), 38.50 (Lys-CH_2), 38.56 (Lys-CH_2), 38.70 (Lys-CH_2), 40.34 (Arg-CH_2), 42.36 (Gly-CH_2), 52.05 (Arg-CH), 52.43 (Lys-CH), 52.60 (Lys-CH), 52.76 (Lys-CH), 53.78 (Trp-CH), 109.92 (Trp-C_q), 111.27 (Trp-CH_{ar}), 112.33 (GCP-CH_{ar}), 115.90 (GCP-CH_{ar}), 118.21 (Trp-CH_{ar}), 118.30 (Trp-CH_{ar}), 120.82 (Trp-CH_{ar}), 123.61 (Trp-CH_{ar}), 125.31 (GCP-C_q), 126.96 (Phe-CH_{ar}), 127.22 (Trp-C_q), 129.91 (Phe-CH_{ar}), 132.99 (GCP-Cq), 135.26 (Phe-C_q), 136.03 (Trp-C_q), 142.17 (CH=N), 156.83 (Gua-C_q), 159.02 (CO), 168.22 (CO), 169.39 (CO), 169.58 (CO), 171.11 (CO), 171.23 (CO), 171.49 (CO), 171.54 (CO), 171.85 (CO), 172.24 (CO), 173.19 (CO), 173.45 (CO), 173.61 (CO). FT-IR (ATR): $\tilde{\nu}$ [cm^{-1}] = 3175 (br), 3048 (br), 2930 (br), 1637 (s), 1523 (s), 1232 (w), 1165 (w), 1095 (w), 1011 (w), 952 (w), 744 (m). HRMS (ESI): m/z calculated for $C_{73}H_{103}N_{26}O_{14}^{3+}$ $[M+3H]^{3+}$: 522.6044; found: 522.6066.

3. Generation of Dynamic Combinatorial Libraries

In order to determine the appropriate conditions for generating the DCLs, initial control experiments with one test library containing only one peptide-derived hydrazide **B** and one aldehyde **5** were performed in 100 mM sodium acetate buffer at pH 4.0, upon agitation overnight. The resulting mixture was evaluated by analytical HPLC and electrospray ionization mass spectroscopy (ESI-MS). As shown in Figure S1, the HPLC runs showed that

at these conditions, the hydrazide was formed as identified with ESI-MS. Thus, these conditions were used for the generation of DCLs.

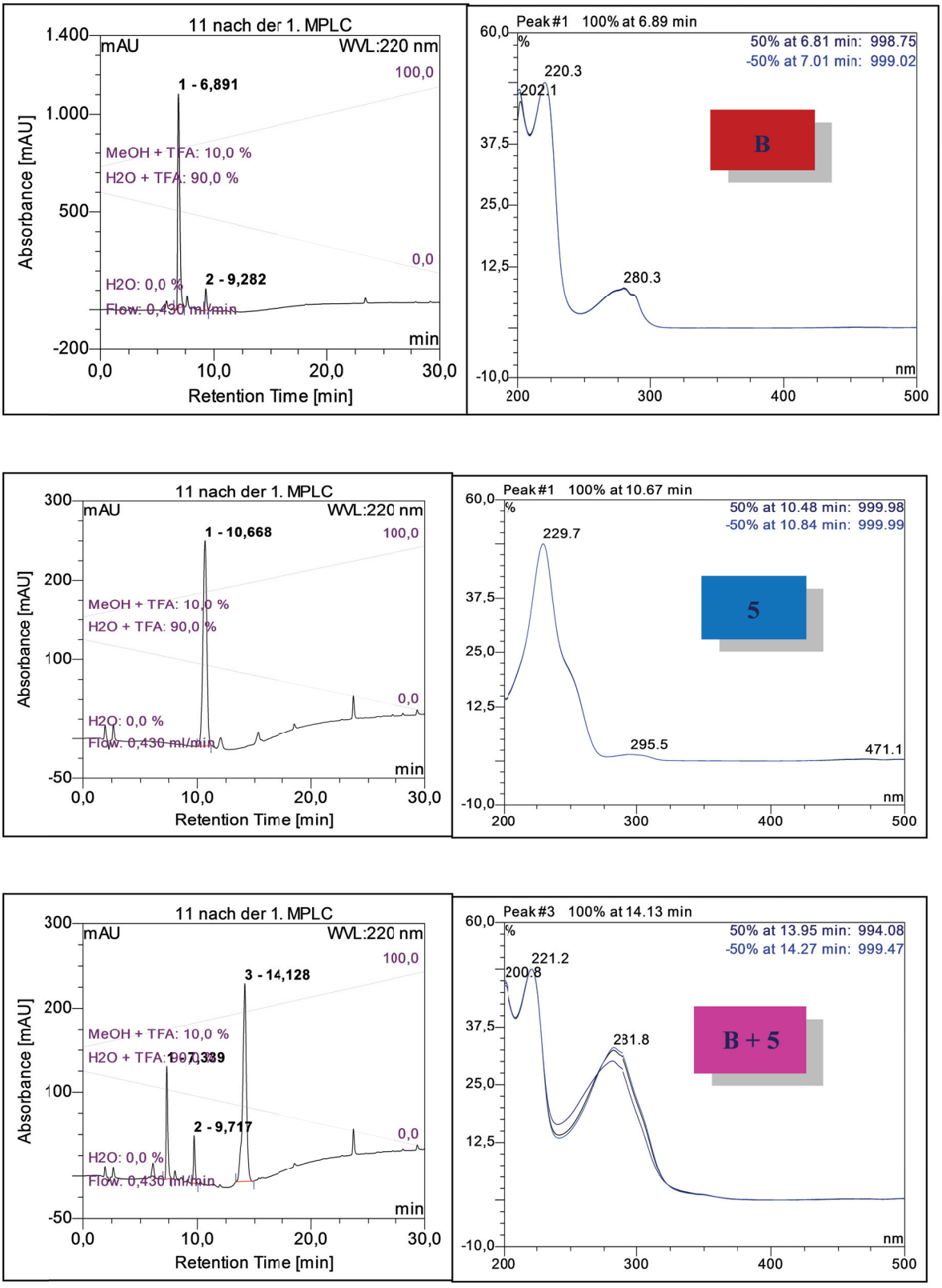


Figure S1. Analytical HPLC runs of peptide-derived hydrazide **B** (*top*), tri-aldehyde **5** (*middle*) and the resulting mixture with the formation of new acyl hydrazone (*bottom*).

Therefore, the dynamic libraries can be easily generated in sodium acetate buffer solutions at pH 4.0 containing 10% DMSO (v/v) to help dissolve the aromatic aldehydes and the acyl hydrazones, upon gently agitation for three days. The full library was prepared by mixing all the ten building blocks (five hydrazides and five aldehydes) together in an aqueous acetate buffer solution at room temperature. Similarily, 10 sub-libraries were generated using the same conditions by mixing all components except one specific building block, which was omitted and replaced by the blank acetate buffer solution containing 10% DMSO. Equilibration of all the libraries was carried out in the absence of the enzyme. The concentrations of the hydrazide groups or aldehyde groups of each building block were kept the same. For example, the stock solutions of hydrazides **A**-**E** (1 mM), aldehydes **1**-**4** (0.5 mM) and aldehyde **5** (0.33 mM) were prepared in sodium acetate buffer (100 mM, pH = 4.0, containing 10% DMSO, v/v). Then, the full library was generated by adding all the five hydrazides and five aldehydes (each 10 μL) to sodium acetate buffer (100 mM, pH = 4.0, containing 10% DMSO, v/v). The ten sub-libraries were synthesized under the same conditions except that the building block to be removed was exchanged by sodium acetate buffer (100 mM, pH = 4.0, containing 10% DMSO, v/v). The final concentration of the libraries amounted to 0.5 mM in total for both hydrazide and aldehyde groups. Together with a reference sample (acetate buffer containing 10% DMSO) without any building blocks, this series of 12 samples was sufficient to screen the complete 95-membered library (not counting partially formed species). All libraries were allowed to equilibrate at room temperature with shaking for 3 days and the resulting acyl hydrazones were subsequently tested for inhibitory activity in the presence of β-tryptase after adjusting the pH to 7.4. The second size-reduced library containing all the five aldehydes but only hydrazide **C**, as well as the corresponding six sub-libraries were generated and analyzed in the same way.

Reversibility of hydrazone formation

In order to test the reversibility of the hydrazine formation within the library, two acyl hydrazones **3(B)$_2$** and **3(C)$_2$** were mixed in acetate buffer (100 mM, pH = 4.0). The mixture was allowed to equilibrate at room temperature with shaking for 3 days. After the pH was adjusted to 7.0, the resulting acyl hydrazones were evaluated by analytical HPLC and ESI-MS. As shown in Figure S2, the HPLC runs showed that at this condition, a new compound was formed in the mixture. The ESI-MS verified that the new compound is the scrambling product, the acyl hydrazone **3(BC)** formed by exchange between **3(B)$_2$** and **3(C)$_2$**. Thus, under this condition (pH = 4.0) acyl hydrazone formation is reversible.

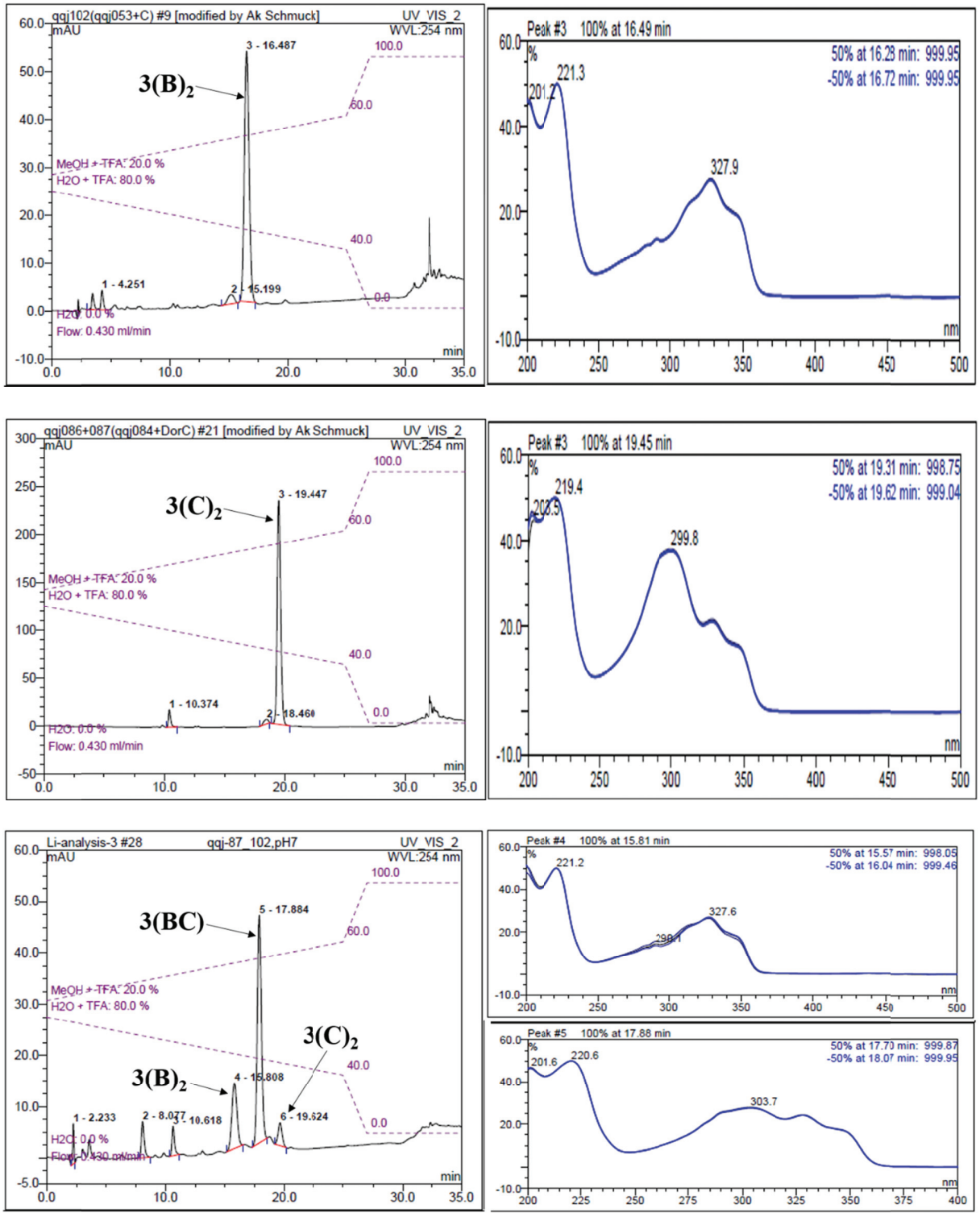


Figure S2. Analytical HPLC runs of acyl hydrazones **3(B)$_2$** (*top*), **3(C)$_2$** (*middle*) and the resulting mixture with the formation of new acyl hydrazone **3(BC)** (*bottom*).

4. Enzyme Assay

Enzyme inhibition assay

Inhibition experiments were performed in white 96 well microplate using heparin-stabilized human rhSkin β-tryptase in a high throughput assay using a fluorescence spectrophotometer. The assays were carried out in a 50 mM Tris-HCl buffer at pH 7.4, containing additional 50 μg/mL heparin, 0.02% Triton-X and 100 mM NaCl using the chromogenic substrate Tos-Gly-Pro-Arg-AMC. Upon cleavage by the enzyme a strong increase of fluorescence emission at 460 nm (380 nm excitation) occurs due to the release of the free 7-amino-4-methylcoumarin (AMC). The slope of the increasing fluorescence over time is related to the enzyme activity and can therefore be used to monitor the cleavage reaction. The enzyme was diluted in assay buffer and the final concentration of the enzyme was determined prior the tests in order to find appropriate concentrations to obtain a slope of the linear graph in the region between 15 and 30 in the absence of inhibitor.

In the enzyme screening measurement of DCLs, 10 μL of β-tryptase was added to 180 μL Tris-HCl buffer. This mixture was incubated with the different library mixtures (5 μL) and finally the substrate (5 μL, final concentration 50 μM) was added. The solutions were thoroughly mixed and the rate of hydrolysis of the AMC substrate was determined by monitoring the fluorescence change at 460 nm for 15 min. Due to the high concentration of the initial libraries (the total concentration of hydrazides is 0.5 mM in the full library), the DCLs were diluted 10 times with acetate buffer solution containing 10% DMSO accordingly for the enzyme assay in order to significantly discriminate the inhibitory potency among the different libraries. Thus, the total concentration of hydrazides in the full library was 50 μM, resulting in a final hydrazide concentration of 1.25 μM in the enzyme screening mixtures. An overall inhibition of approximately 60%-90% for the full library was obtained using these concentrations which ensures the optimal discrimination of the different libraries.

The enzyme inhibition assays were carried out in a final volume of 200 μL in assay buffer (165 μL) wherein the enzyme (10 μL) was incubated with various concentrations of inhibitors (20 μL). Finally the substrate (5 μL, final concentration 50 μM) was added. All the solutions were thoroughly mixed and the rate of hydrolysis of the AMC substrate was determined by monitoring the fluoresence change at 460 nm for 15 min. All the measurements were done in triplicate. To determine the half maximum inhibitory concentration (IC_{50}) and from this the absolute inhibition constant (K_i), a series with the following final concentrations were measured: 100 μM, 80 μM, 60 μM, 40 μM, 20 μM, 10 μM, 8 μM, 6 μM, 4 μM, 2 μM, 1 μM, 0.8 μM, 0.6 μM, 0.4 μM, 0.2 μM, 0.1 μM, 0.01 μM. Depending on the inhibition activity,

sometimes also larger and smaller concentrations had to be measured (1000-0.001 μM). The resulting data of the residual enzyme activity (in % relative to the buffer solution) were processed with the program GraFit by using the following 4-parameter equation to calculate the IC_{50} values:

$$y = \frac{Range}{1 + \left(\frac{x}{IC_{50}} \right)^{s}} + \text{Background}$$

In this equation, *x* is the concentration of the inhibitor, *y* is the enzyme activity, *Range* is the fitted uninhibited value minus the Background, and *s* is a slope factor. The equation assumes that *y* falls with increasing *x*.

Enzyme selectivity assay

Two other serine proteases Trypsin and *α*-Chymotrypsin were chosen to determine the enzyme selectivity of the inhibitors. The assays were performed in a 50 mM Tris-HCl buffer at pH 8.0, containing 5 mM EDTA and 100 mM NaCl. Chromogenic substrates Z-Phe-Arg-AMC and Suc-Leu-Tyr-AMC were used for Trypsin and Chymotrypsin, respectively. The kinetic assays were performed in a final volume of 200 μL by adding the enzyme (10 μL) to Tris-HCl buffer (pH 8.0, 165 μL), followed by the addition of inhibitor (20 μL) and finally the substrate (5 μL, final concentration 50 μM). All the solutions were thoroughly mixed and finally the increase of fluorescence activity was measured at 460 nm emission (380 nm excitation) over the time of 15 min.

Reversibility of tryptase inhibition

The first test on reversibility of β-tryptase inhibition was performed in a dialysis experiment. The Float-A-Lyzer G2 dialysis separation tubes (20 kDa) in which a mixture (total volume of 1000 μL) of assay buffer (Tris-HCl, 50 mM, pH 7.4, 750 μL), β-tryptase (50 μL, final concentration 0.5 nM) and inhibitor **5(A)$_3$** (200 μL, final concentration 20 μM) in buffer (containing 10% DMSO) or pure buffer (containing 10% DMSO, 200 μL) without inhibitor as the reference were contained. The dialysis tubes were floated vertically in the dialysate reservoir containing a stir bar, adjusting the stirring rate to form a gently rotating current. The samples were dialyzed at room temperature and with four complete buffer changes (every 12-24 h) over a period of 99 h. The enzymatic activity was determined 3, 18, 28, 43, 66 and 99 hours after starting the dialysis by taking a 100 μL sample from the dialysis tube, adding 95 μL assay buffer and the substrate (5 μL, final concentration 50 μM). The mixtures were

submitted to determine the residual activity of the enzyme using a fluorescence assay. The enzyme activity was restored slowly (see Figure S3), which indicated that the interaction between the enzyme and the inhibitor is in principle reversible.

In a second experiment to test the reversibility of enzyme inhibition, three different combinations were mixed in a final volume of 200 μL. In the first mixture, the blank sample, the enzyme (10 μL) was incubated with pure DMSO (20 μL) in assay buffer (155 μL) while in the second and the third mixtures, the enzyme (10 μL) was incubated with the inhibitor (20 μL, final concentration 10 μM) in assay buffer (155 μL). After the three mixtures were incubated for 5 minutes, blank assay buffer (10 μL) was added to the first and the second vials while heparin in buffer (10 μL, final concentration 1 mg/mL) was added to the third vial. All the mixtures were incubated for another 5 minutes, followed by the addition of substrate (5 μL, final concentration 50 μM) and the fluorescence readout. The recovery of the enzyme activity (relative to the first vial) in the third vial with heparin compared to the second vial with blank assay buffer indicates reversible binding of the inhibitor to the enzyme.[4] In this case, 16% recovery of the enzyme activity was observed.

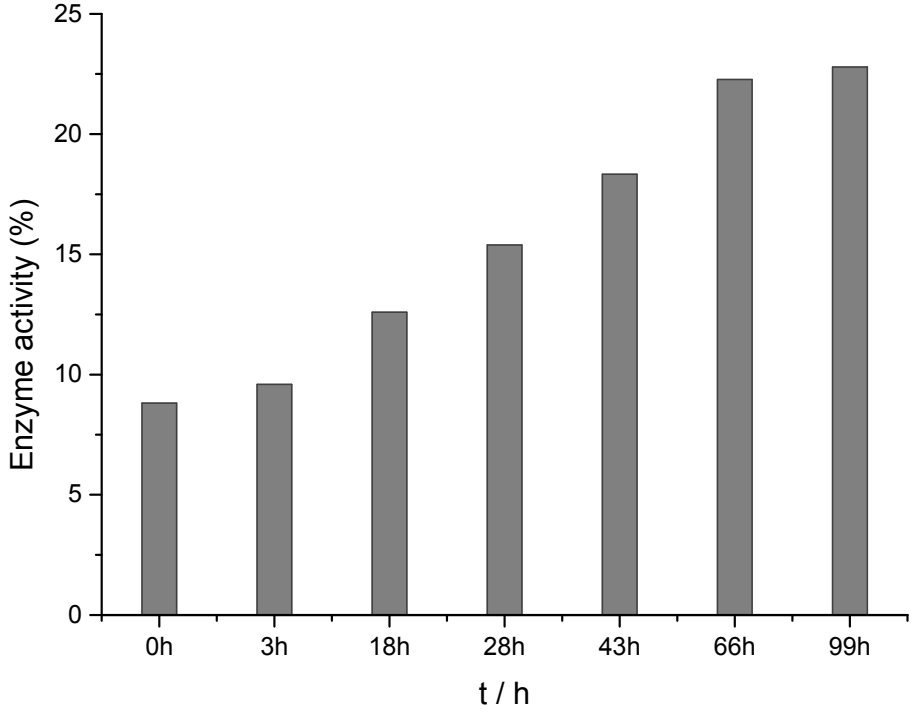


Figure S3. The recovery of the enzyme activity over time in the dialysis experiment.

5. Molecular Mechanics Calculations

Calculation of the Energy of $5(C)_3$ and $5(D)_3$ bound to β-tryptase - Generation of Figure 4

Software: Virtual studies were done using Schrodinger MacroModel and Glide software (Version 2012 Update 2). Ligand preparation was done by the following steps: After generation of the 2D structure, they were converted into a 3D model, which was minimized in the OPLS (optimized potentials for liquid simulations) 2005 forcefield using MacroModel with 1500 iterations and a convergence threshold of 0.05.

File preparation: Before starting the docking study on β-tryptase (pdb id: 1A0L), the pdb-file included ligand and water molecules, which were deleted. Afterwards the protein structure was prepared with the protein preparation wizard assigning bond orders, adding hydrogens and disulfides.

Glide calculations: After this basic preparation of ligand and protein structure files, Glide was used to generate receptor grid files of different surface areas of the central pore of β-tryptase to get detailed information about possible binding motifs. Before docking the three-armed ligands **$5(C)_3$** and **$5(D)_3$** were reduced to the central scaffold with just one arm. (Two arms were replaced by methyl groups.) These one-armed species were docked to all inner surface areas of the pore while the scaffold was fixed in the middle of the pore and the volume of the active site was restricted for the ligand. Using this technique the surface area was scanned for possible non-competitive binding sites. Ligand docking was performed in the extra precision mode with flexible polar groups of the protein and sampling of nitrogen inversions and ring conformations. Post-docking minimizations were performed using a rejecting threshold of 2.09 kJ/mol. After completing the preliminary scan with the single armed species, the three-armed ligands were assembled placing the additional arms in those positions which scored best during one armed surface scan. The resulting structures were minimized in the OPLS forcefield and afterwards treated with a conformational search to test the stability of the predicted conformation. The finally received total energies of the ligand-enzyme-complexes for **$5(C)_3$** and **$5(D)_3$** were -53717.2 and -53683.9 kJ/mol, respectively. The difference of these energies (-33.3 kJ/mol) is referred to as “relative interaction energy”.

Calculation of the Energy of $5(C)_3$ and $5(D)_3$ in the Unboud State

In order to obtain the energy-minimized structures of **$5(C)_3$** and **$5(D)_3$**, conformational searches were performed with 67000 and 76000 cycles, with the resulting energies of -4336.5 kJ/mol and -4372.0 kJ/mol, respectively. After 1000 cycles, the resulting minimum structures of **$5(C)_3$** and **$5(D)_3$** were already found several times with energies of -4330 kJ/mol and -4361 kJ/mol, respectively, which confirmed that the consequent calculations did not result in pronounced structure changes. The calculated structures are shown in Figure S4.

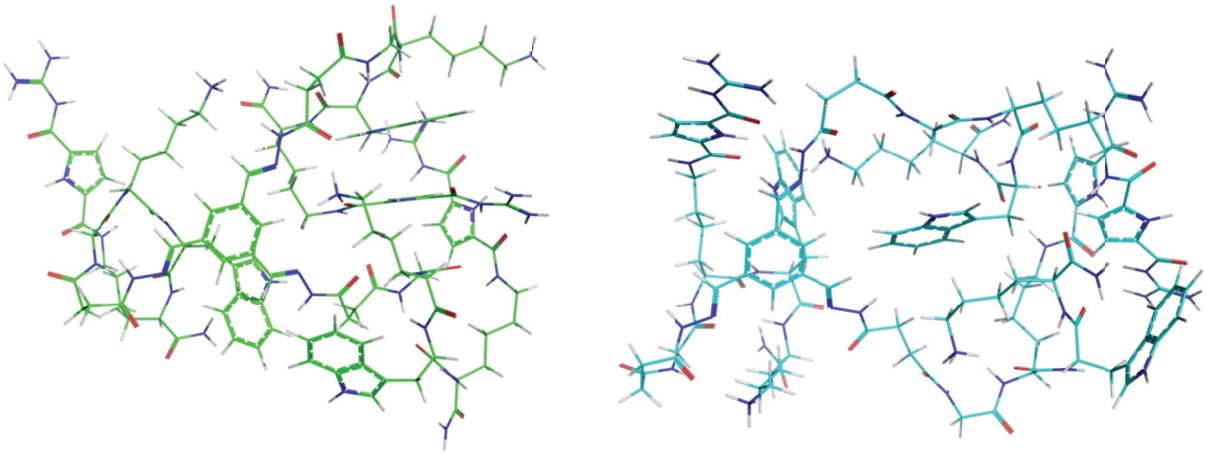
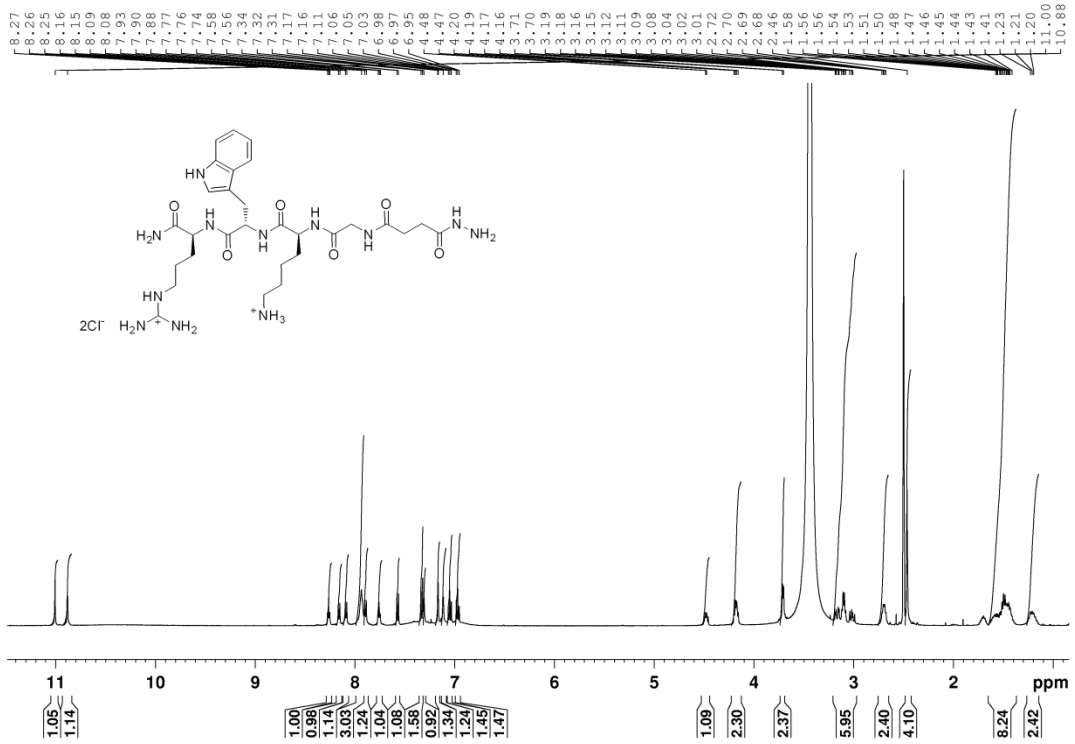


Figure S4. Calculated energy-minimized structures of **$5(C)_3$** (left) and **$5(D)_3$** (right). The structures are displayed as sticks with oxygen atoms colored red, nitrogen atoms colored blue, carbon atoms colored green and cyan, respectively. The minimal energies of **$5(C)_3$** and **$5(D)_3$** were found -4336.5 kJ/mol and -4372.0 kJ/mol after 67000 and 76000 calculation cycles, respectively.

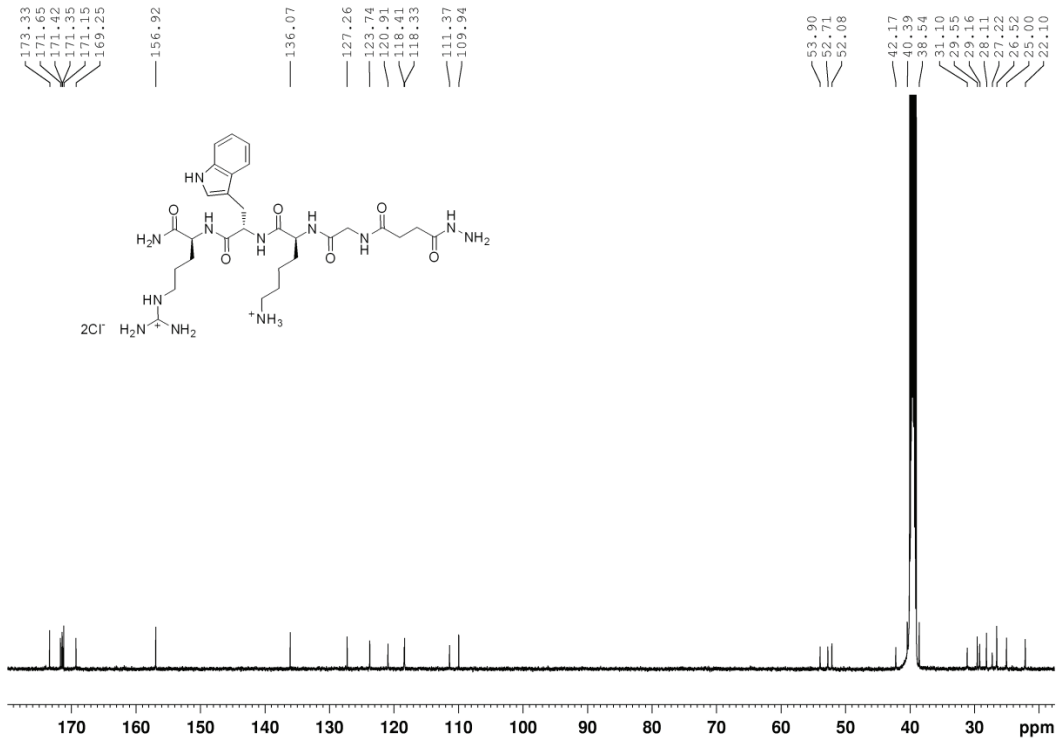
6. ^{1}H and ^{13}C NMR Spectra

Arg-Trp-Lys-Gly-butyric hydrazide (A)

^{1}H NMR (500 MHz, DMSO-d_6)

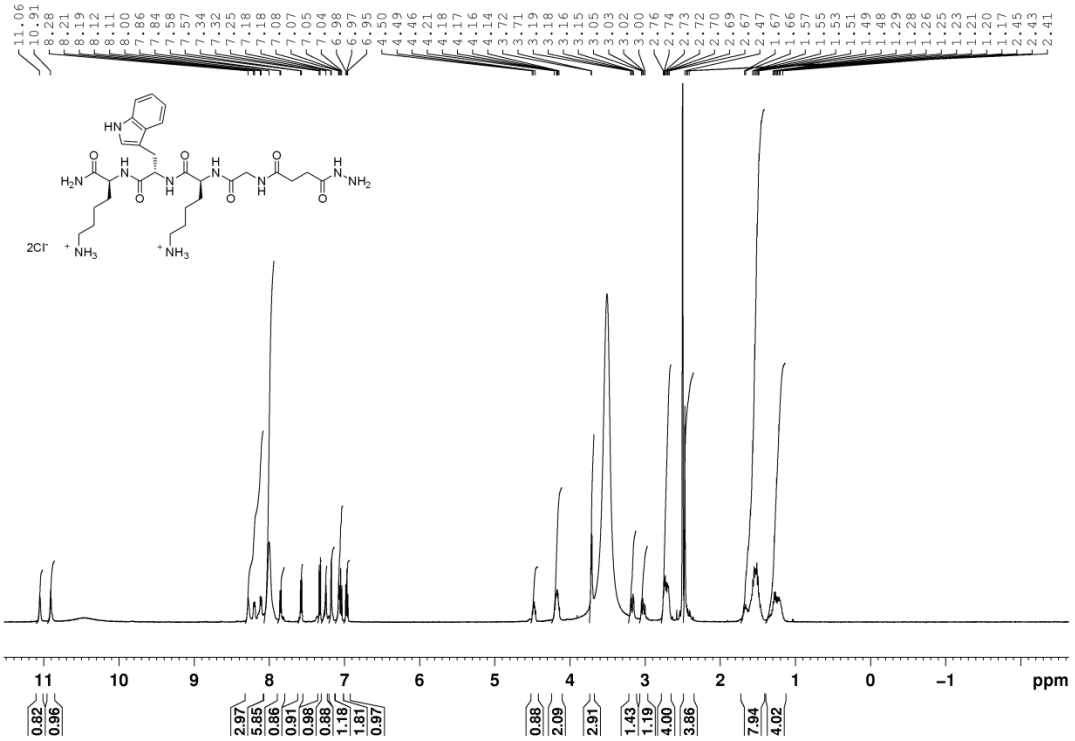


^{13}C NMR (125 MHz, DMSO-d_6)

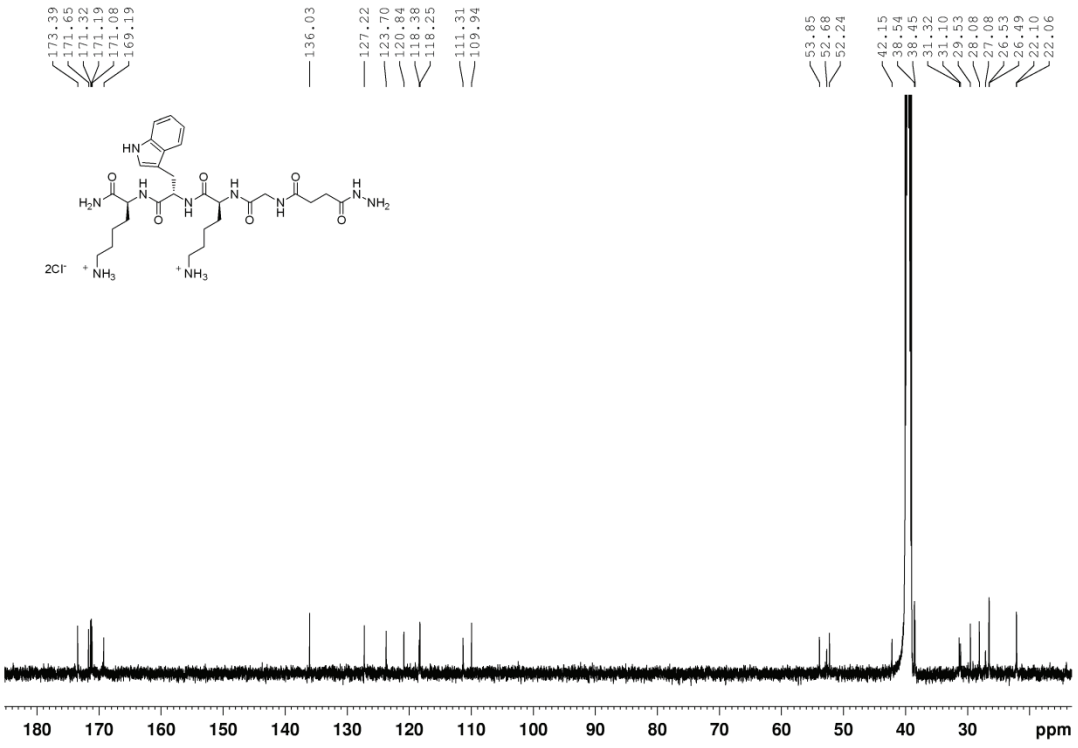


Lys-Trp-Lys-Gly-butyric hydrazide (B)

^{1}H NMR (500 MHz, DMSO-d_6)

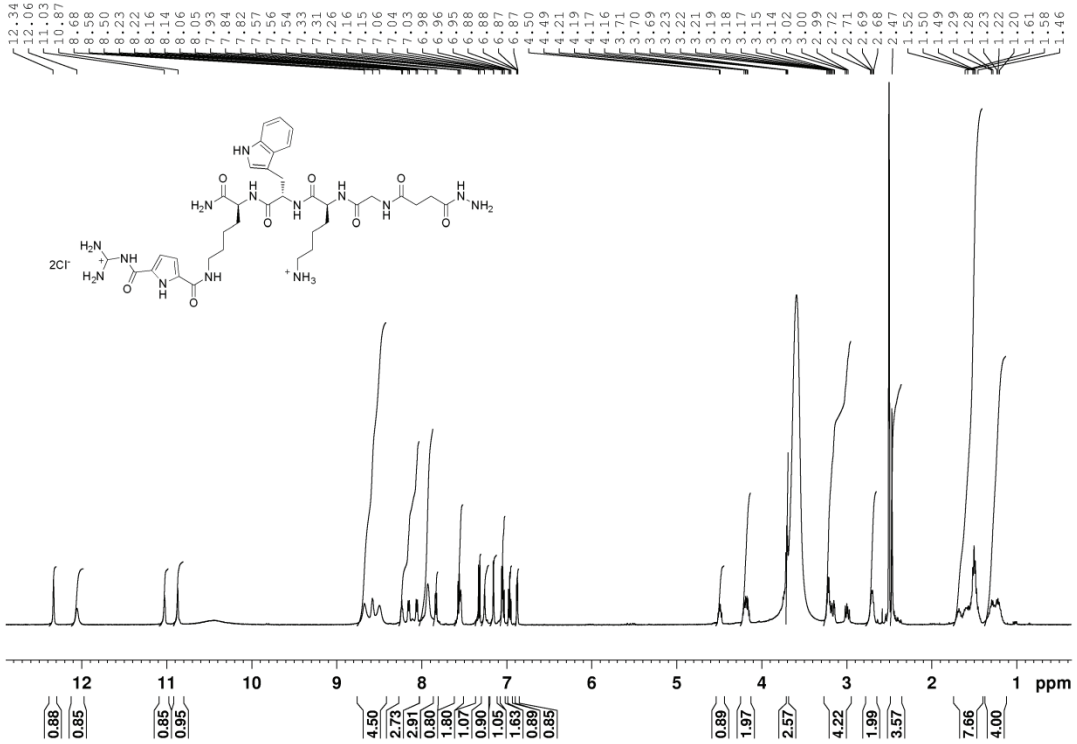


^{13}C NMR (125 MHz, DMSO-d_6)

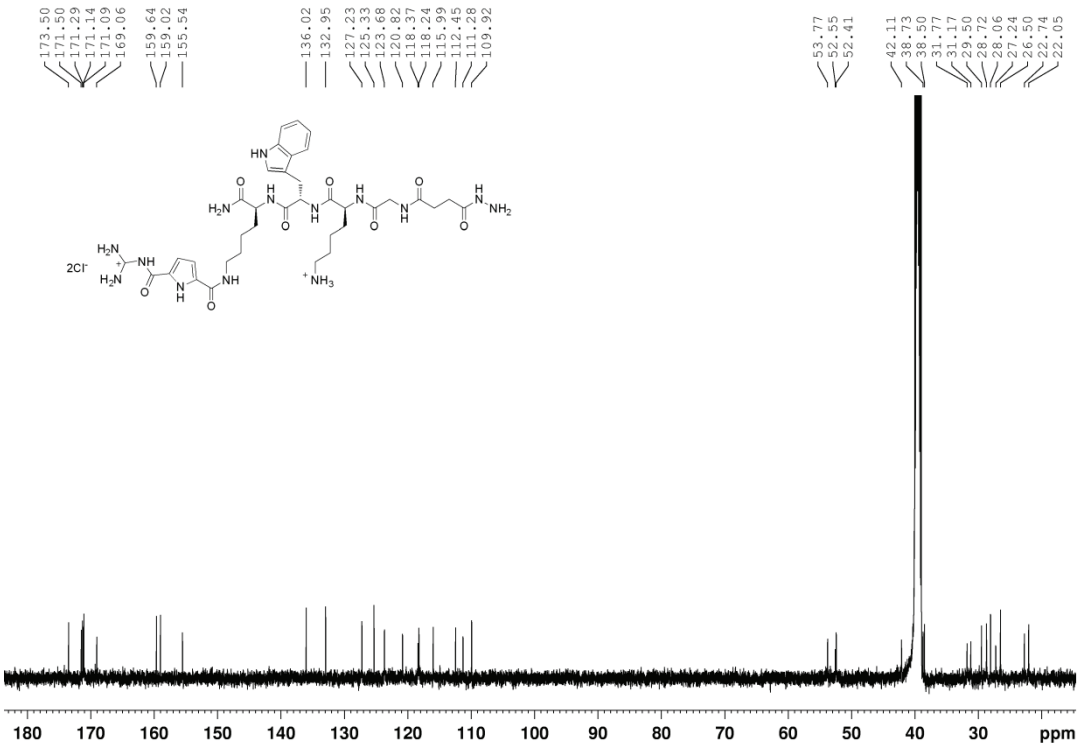


Lys(GCP)-Trp-Lys-Gly-butyric hydrazide (C)

^{1}H NMR (500 MHz, DMSO-d_6)

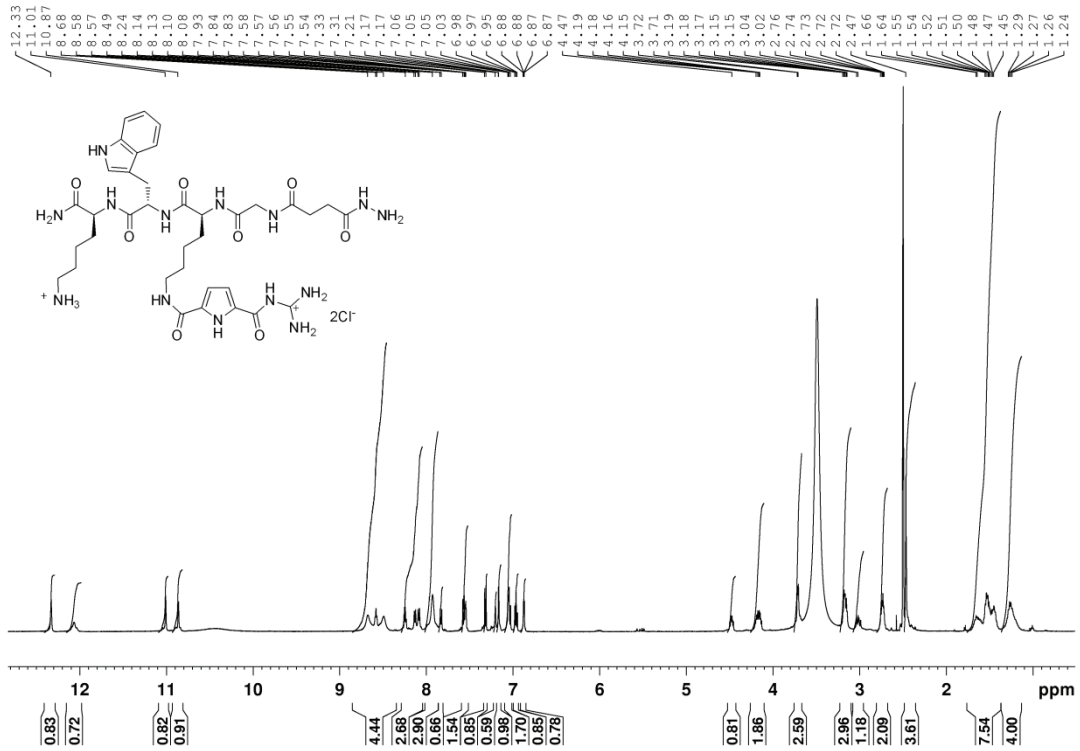


^{13}C NMR (125 MHz, DMSO-d_6)

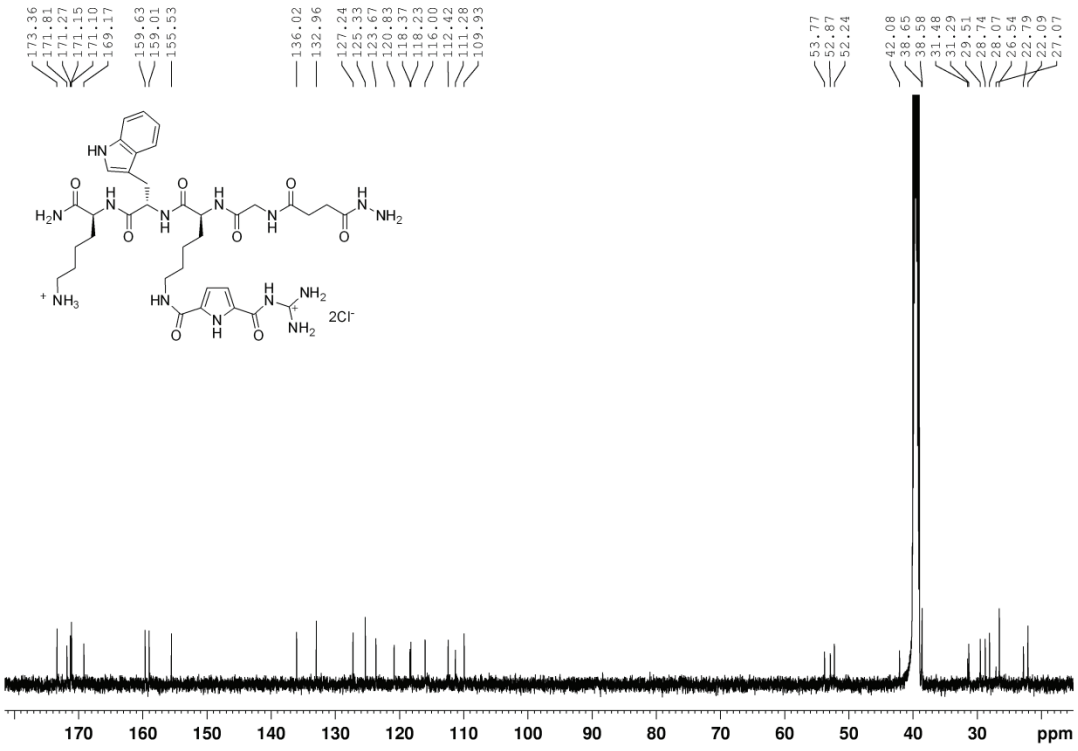


Lys-Trp-Lys(GCP)-Gly-butyric hydrazide (D)

^{1}H NMR (500 MHz, DMSO-d_6)

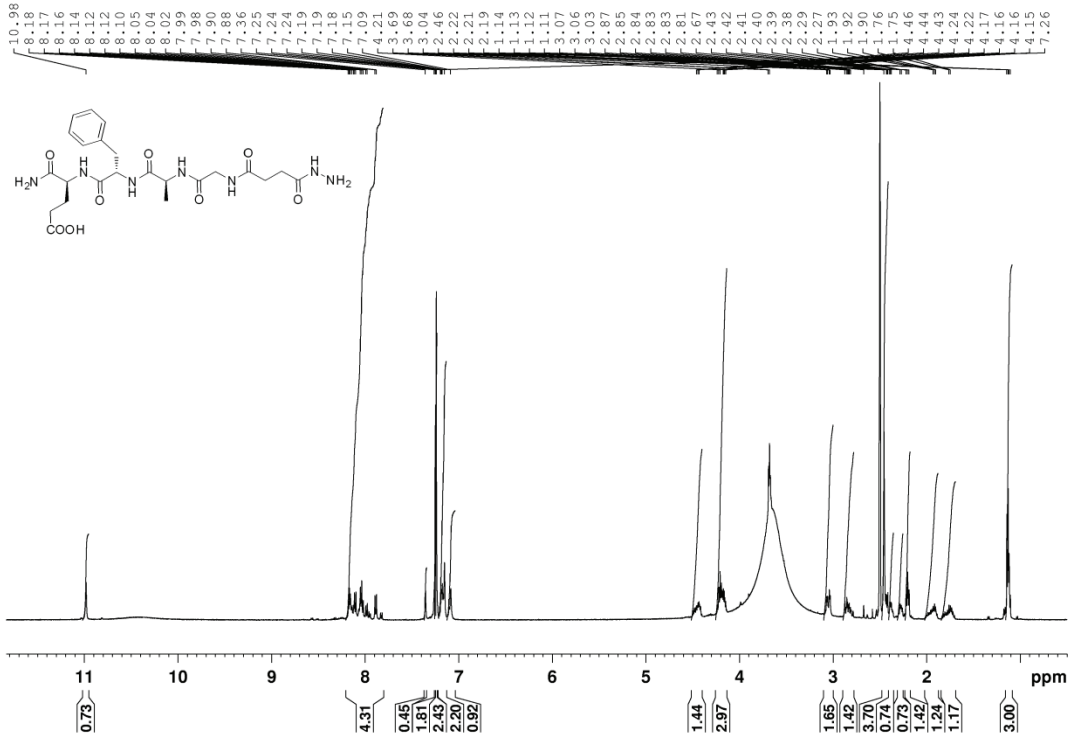


^{13}C NMR (125 MHz, DMSO-d_6)

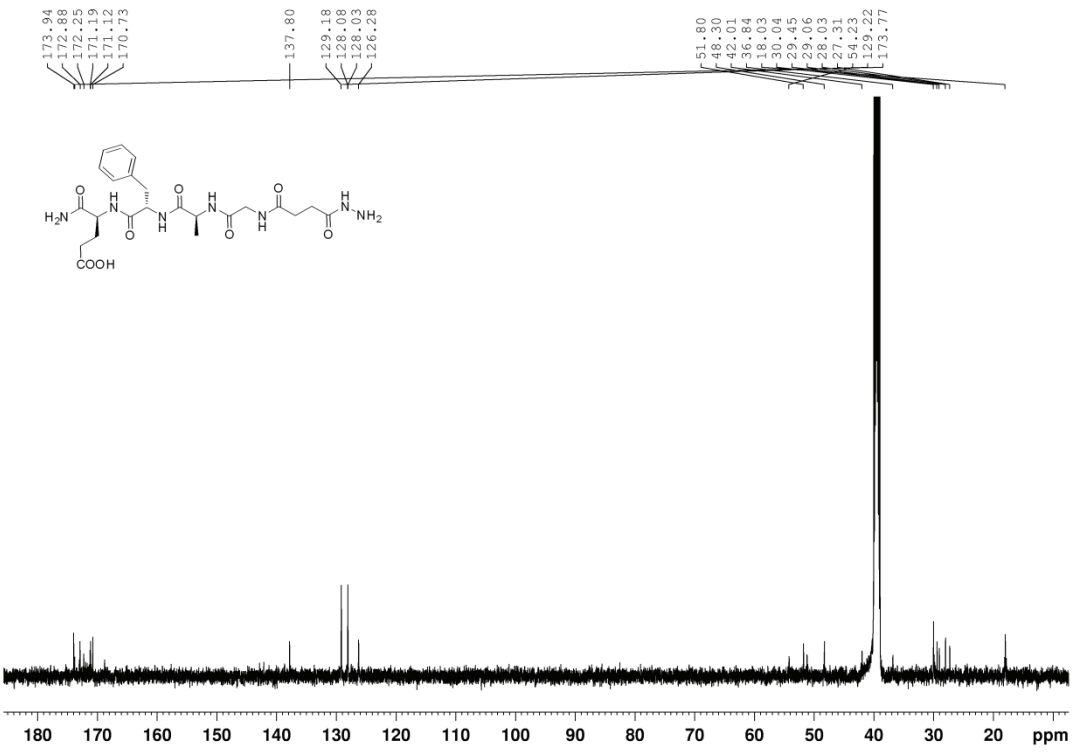


Glu-Phe-Ala-Gly-butyric hydrazide (E)

^{1}H NMR (500 MHz, DMSO-d_6)

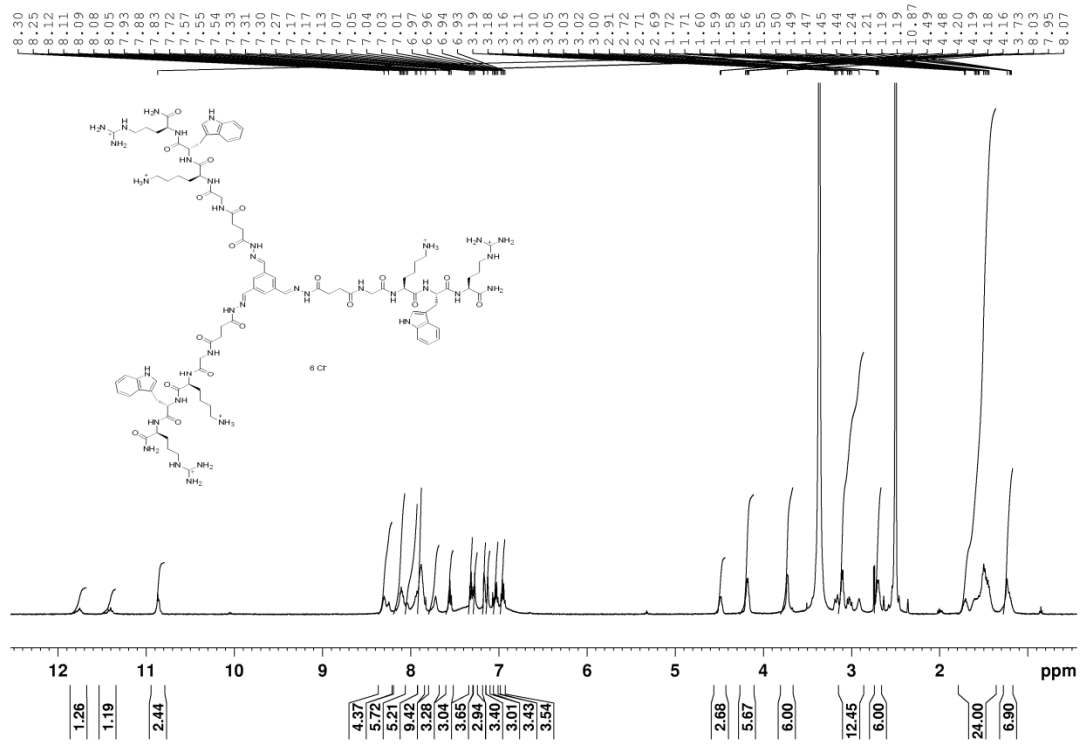


^{13}C NMR (125 MHz, DMSO-d_6)

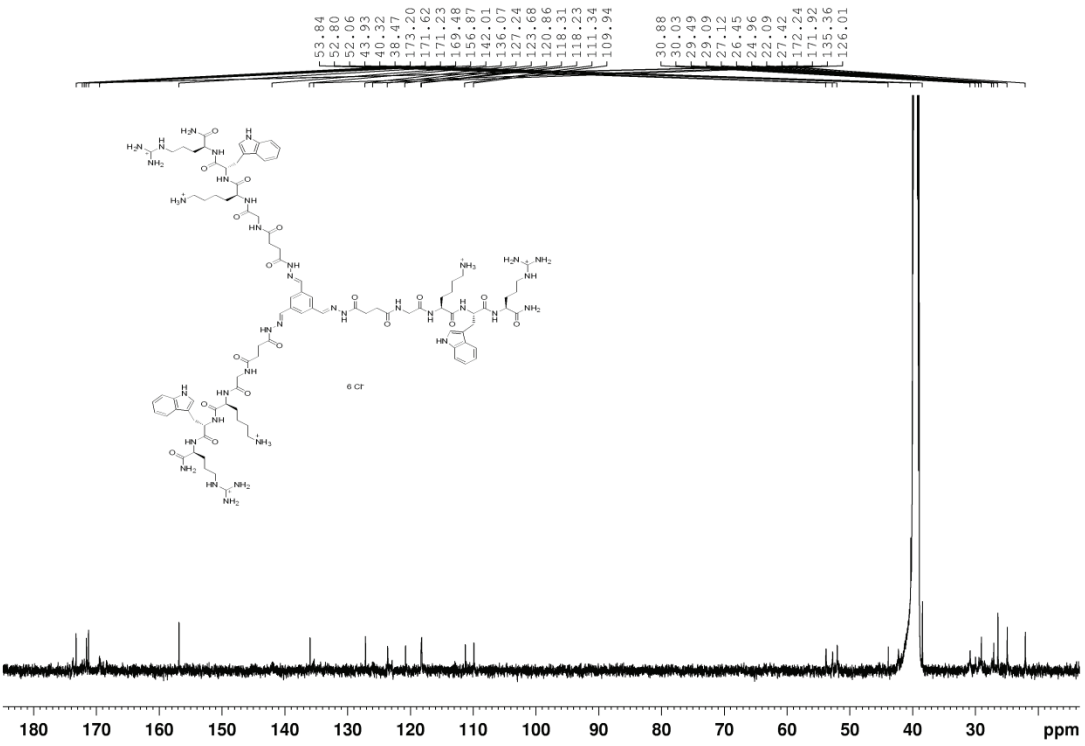


5(A)$_3$

^{1}H NMR (500 MHz, DMSO-*d*$_6$)

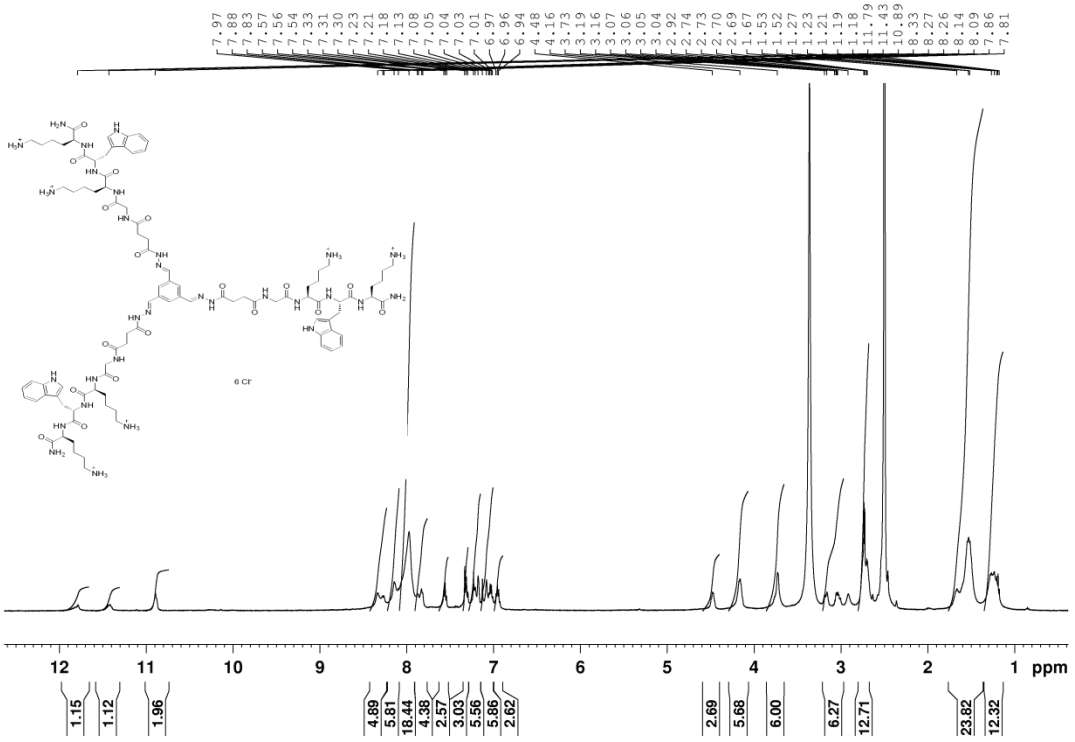


^{13}C NMR (125 MHz, DMSO-*d*$_6$)

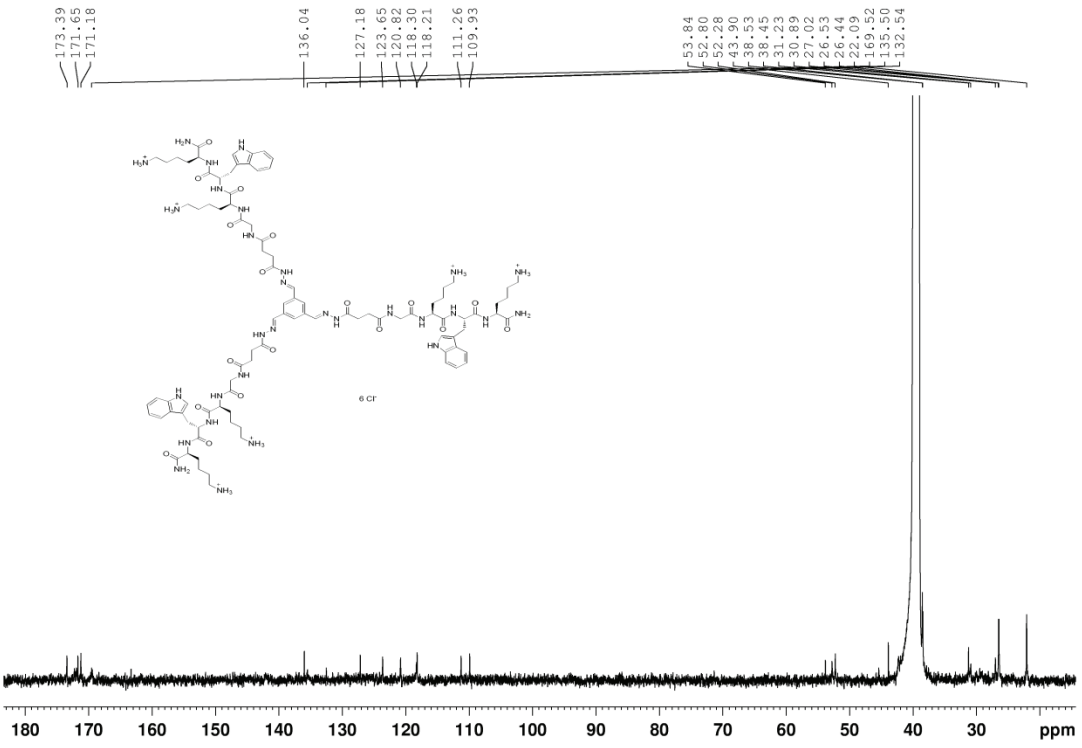


5(B)$_3$

^{1}H NMR (500 MHz, DMSO-*d*$_6$)

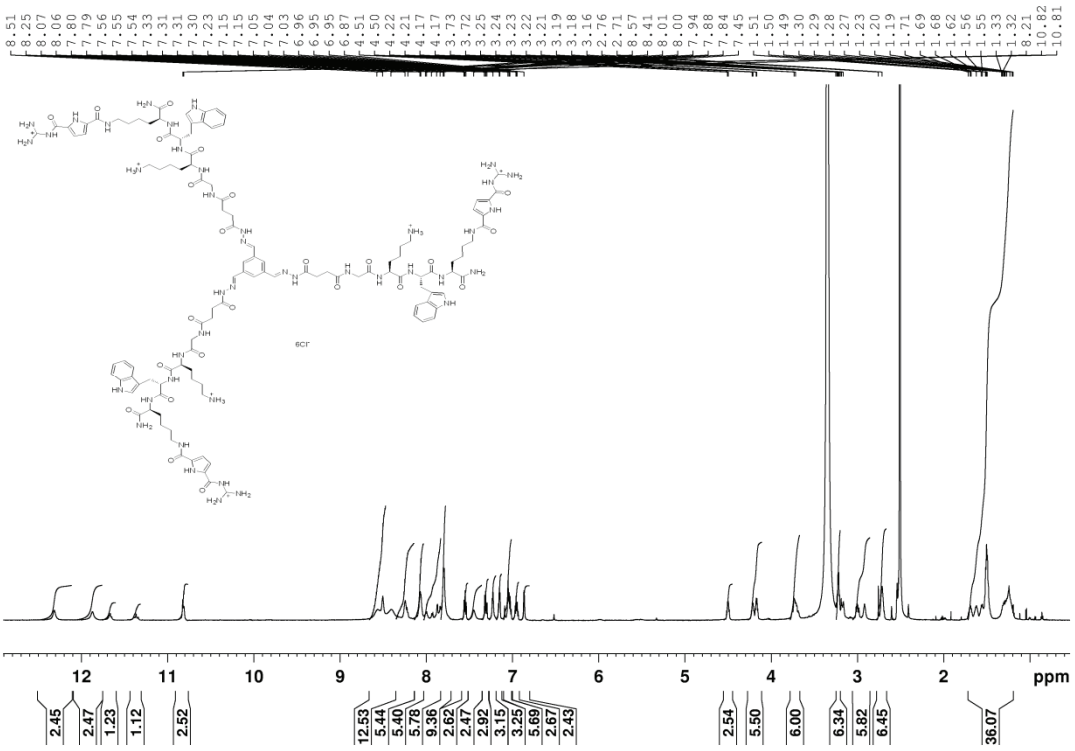


^{13}C NMR (125 MHz, DMSO-*d*$_6$)

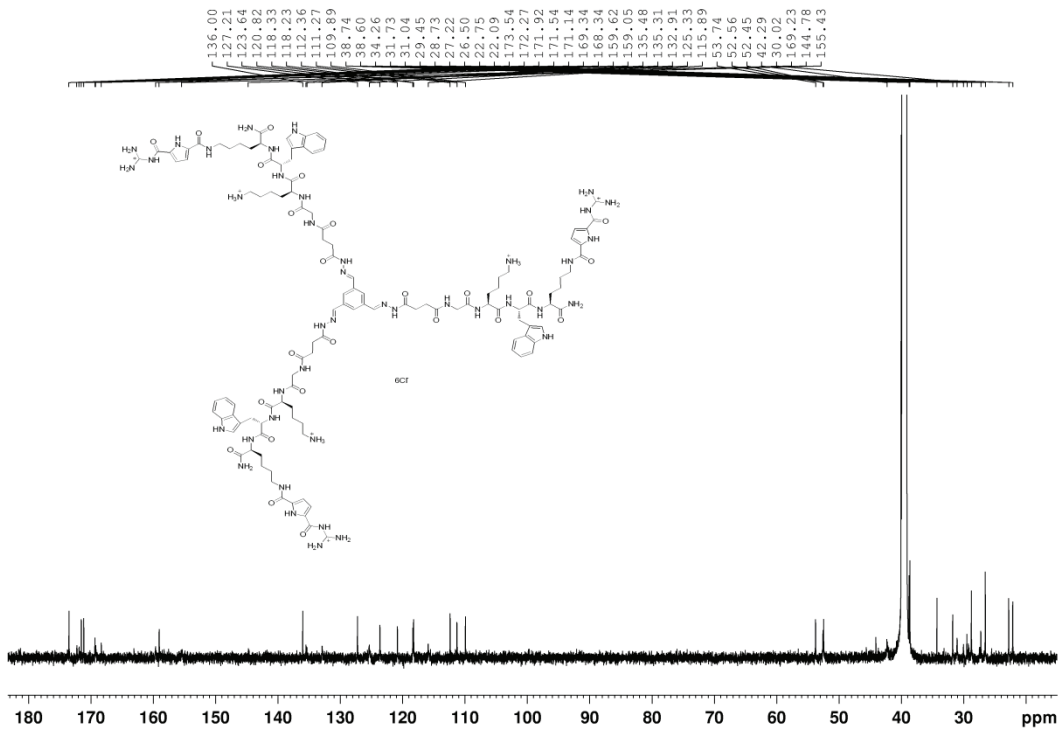


5(C)$_3$

^{1}H NMR (700 MHz, DMSO-d_6)

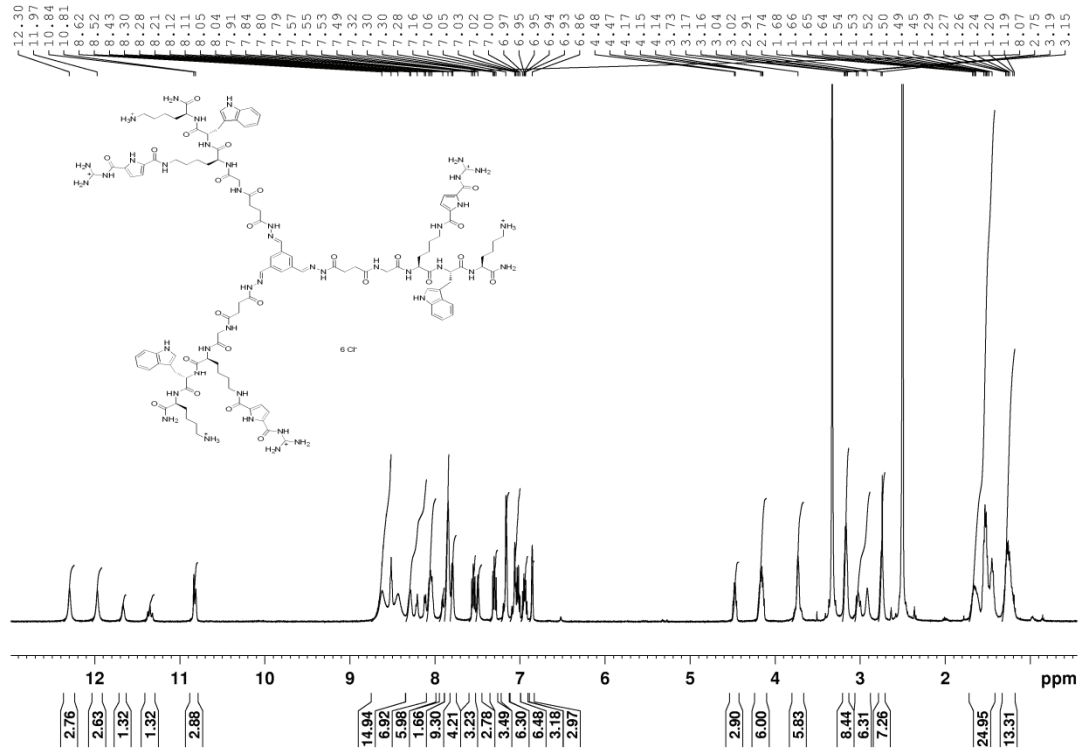


^{13}C NMR (175 MHz, DMSO-d_6)

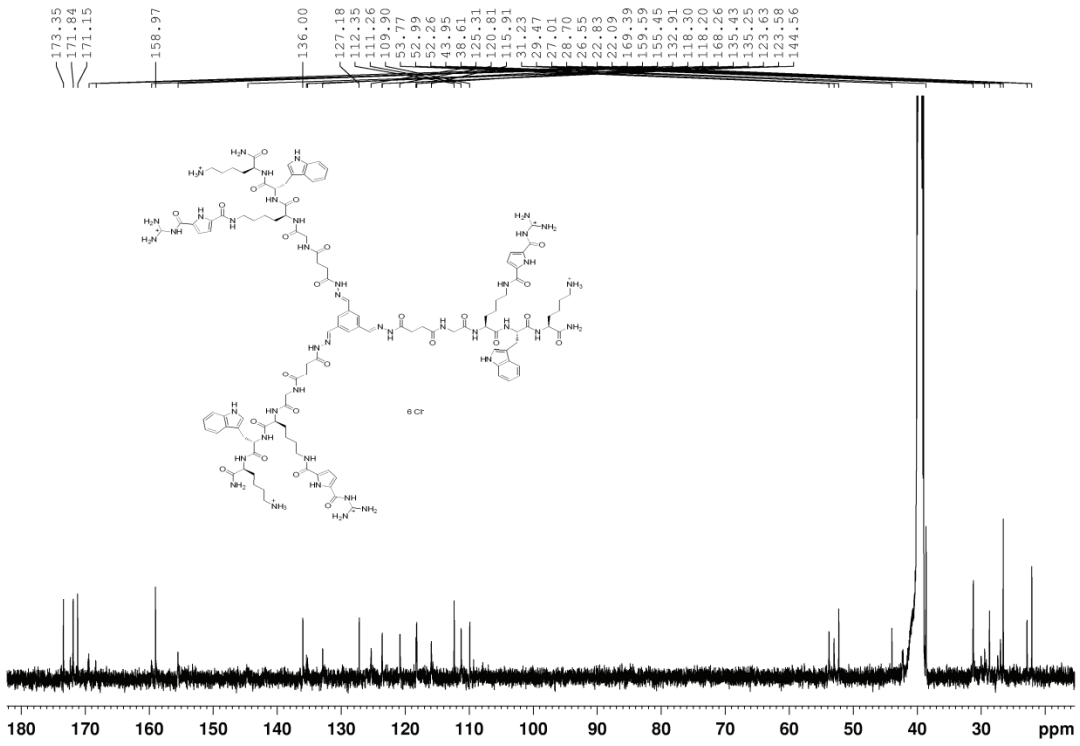


5(D)$_3$

^{1}H NMR (500 MHz, DMSO-*d*$_6$)

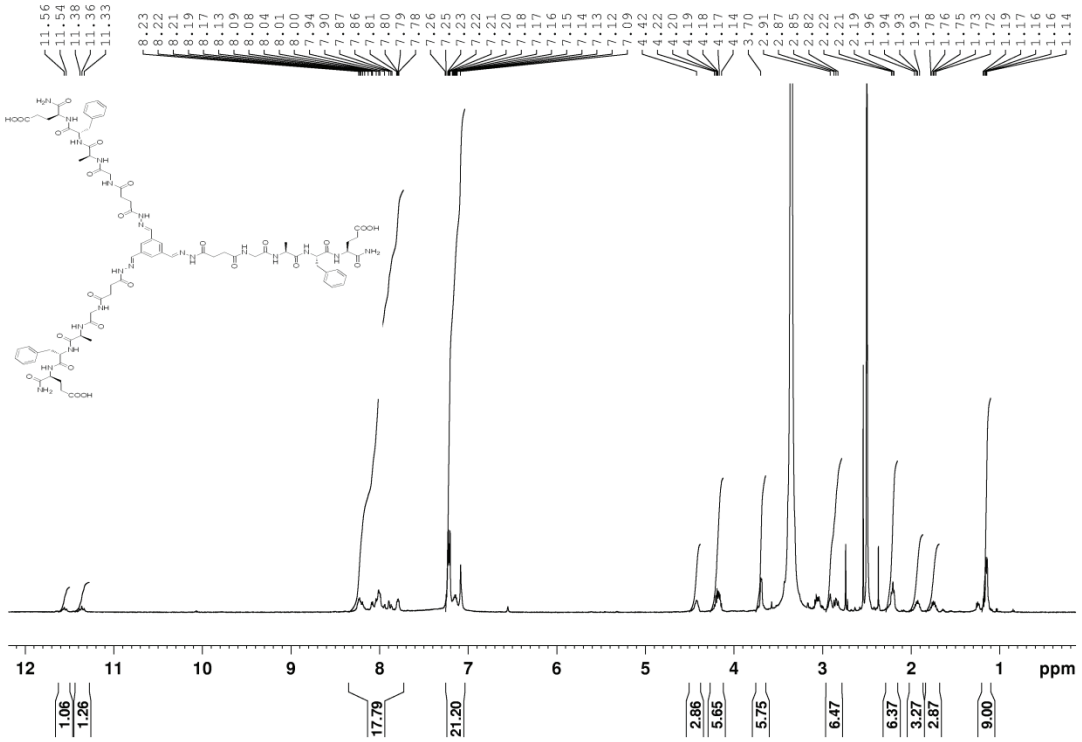


^{13}C NMR (125 MHz, DMSO-*d*$_6$)

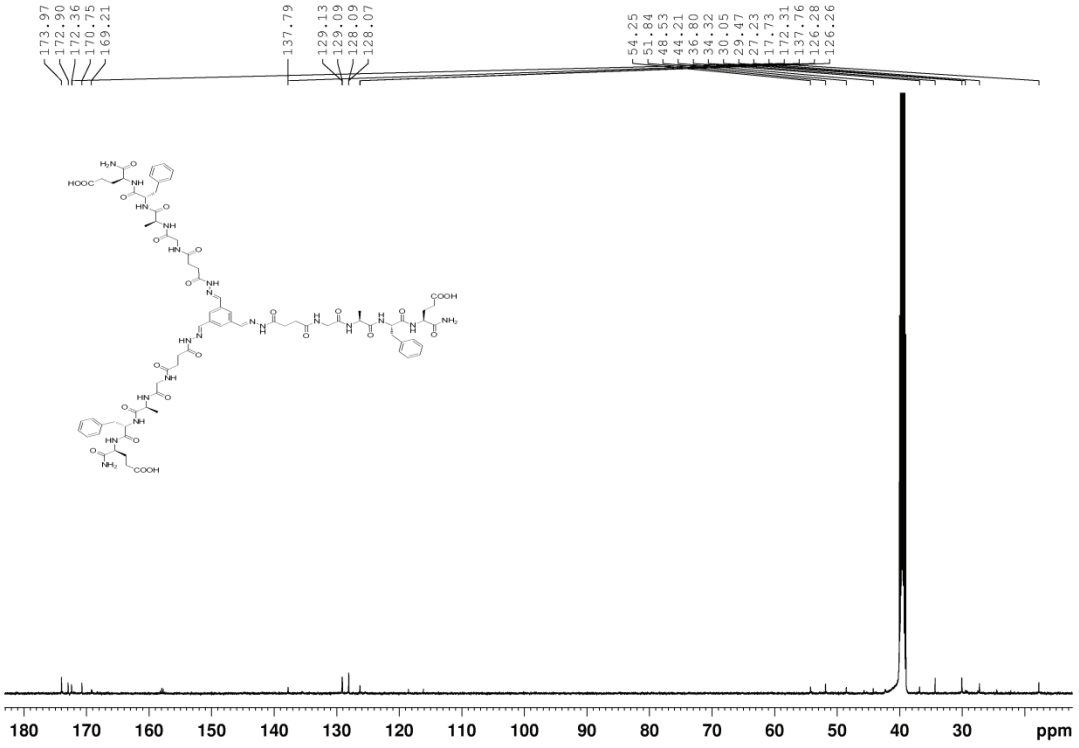


5(E)$_3$

^{1}H NMR (500 MHz, DMSO-*d*$_6$)

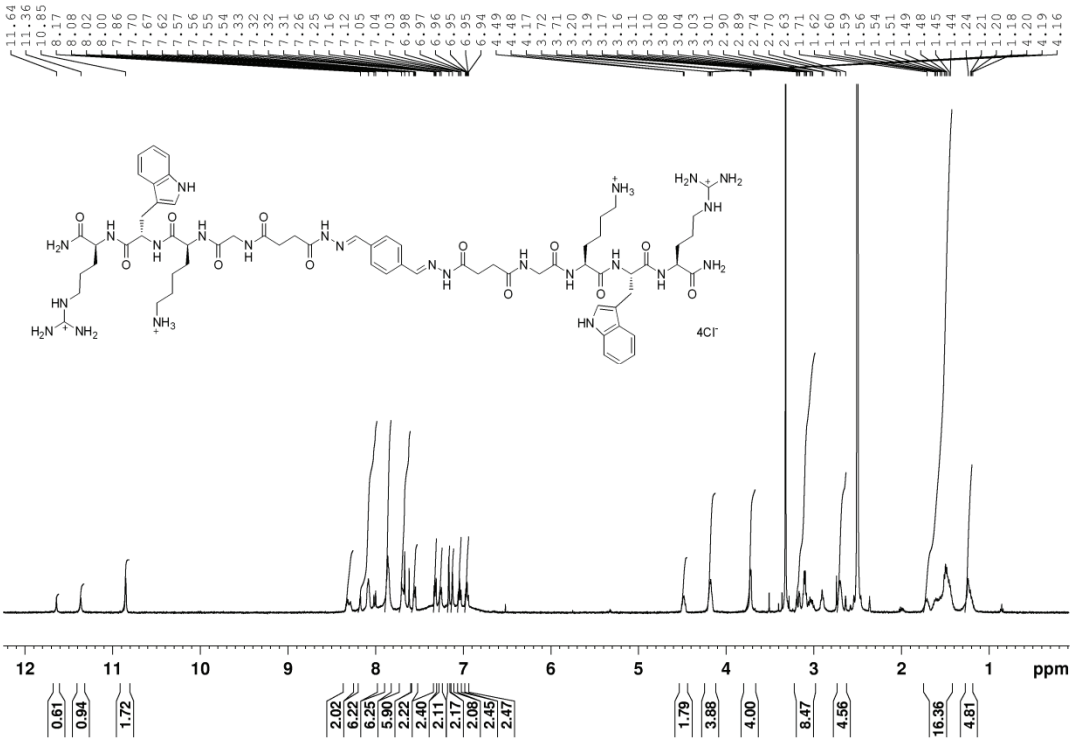


^{13}C NMR (125 MHz, DMSO-*d*$_6$)

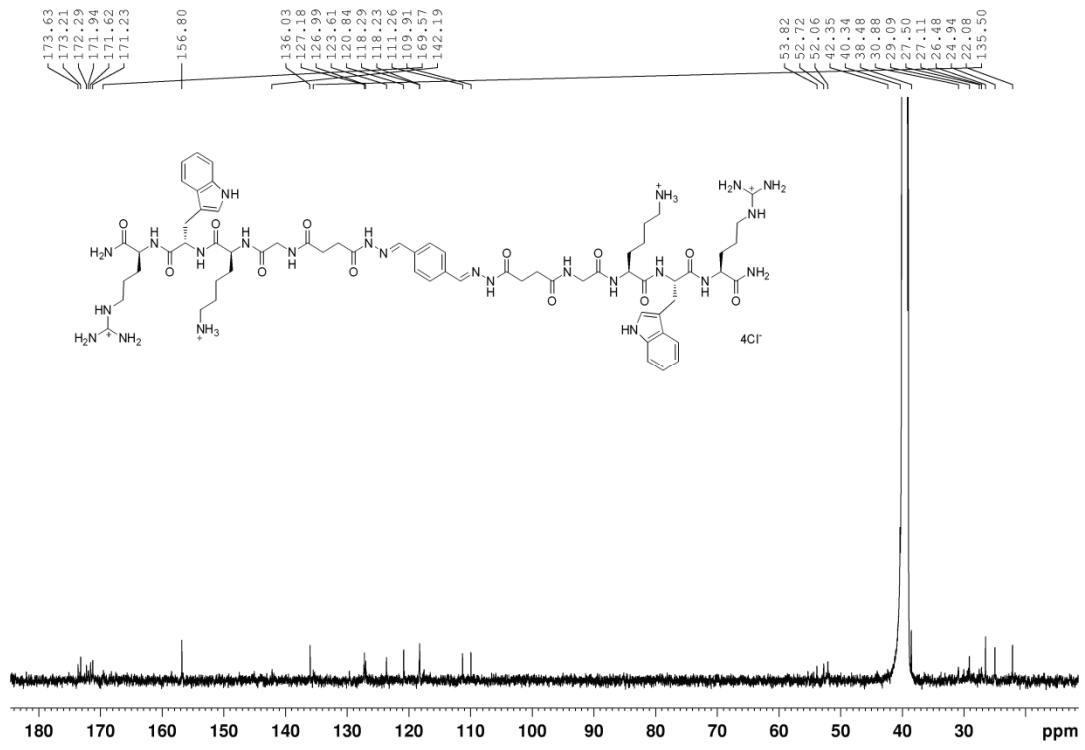


3(A)$_2$

^{1}H NMR (500 MHz, DMSO-d_6)

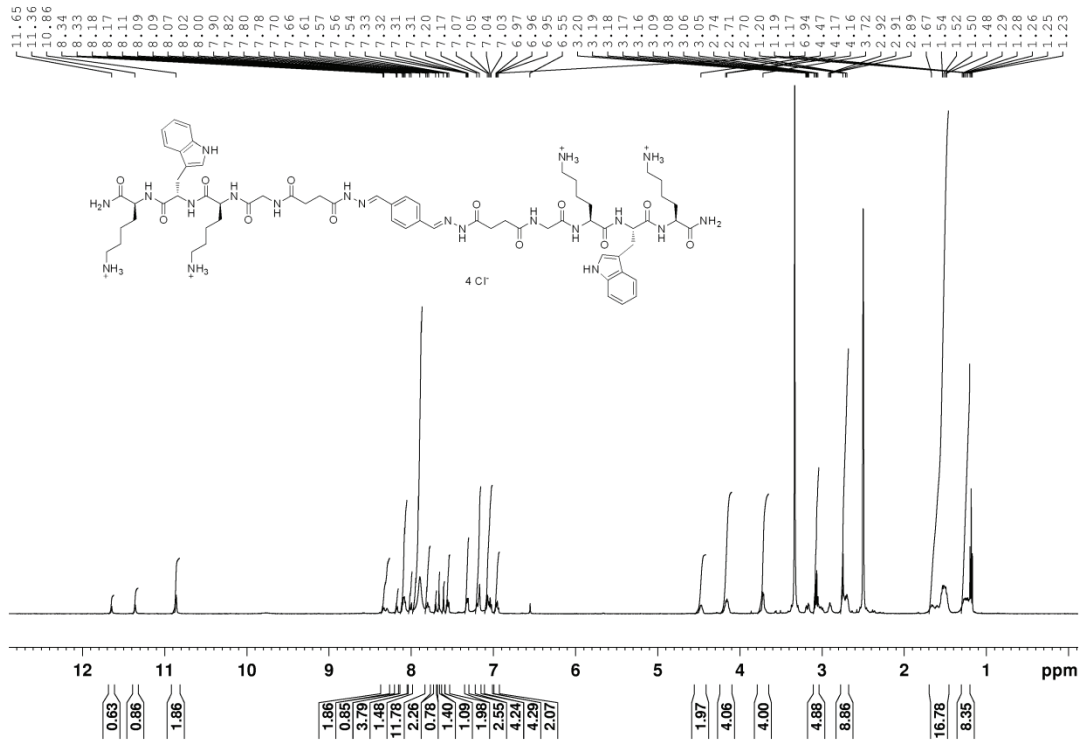


^{13}C NMR (125 MHz, DMSO-d_6)

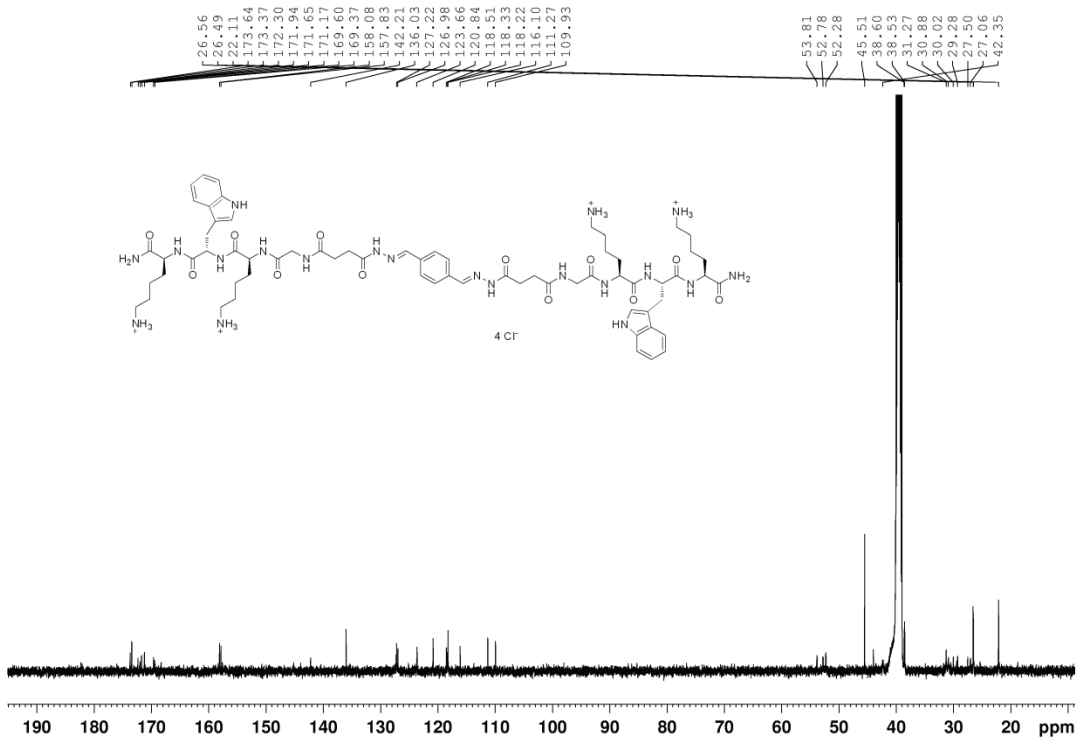


3(B)$_2$

^{1}H NMR (500 MHz, DMSO-*d*$_6$)

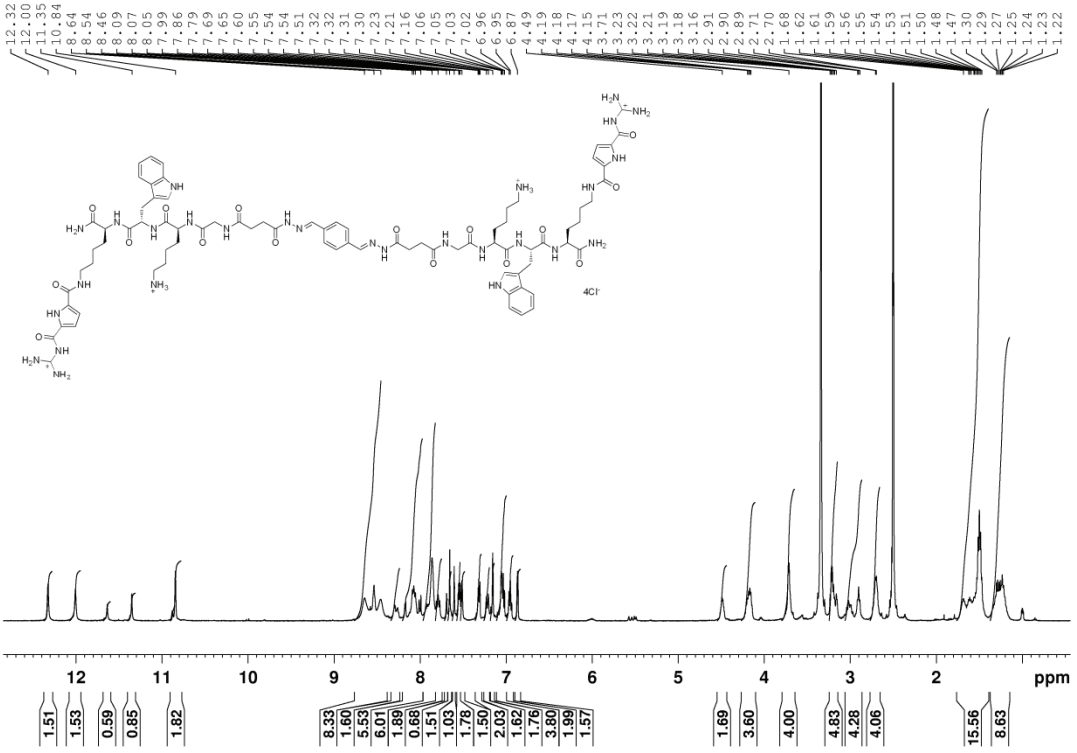


^{13}C NMR (125 MHz, DMSO-*d*$_6$)

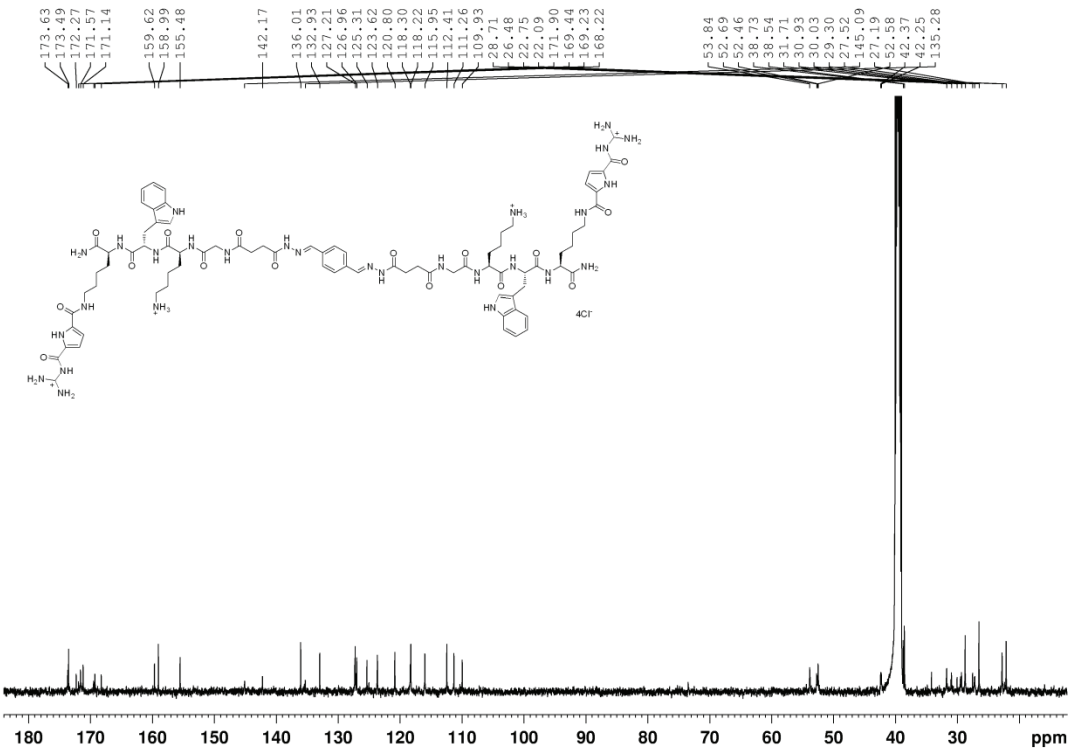


3(C)$_2$

^{1}H NMR (500 MHz, DMSO-*d*$_6$)

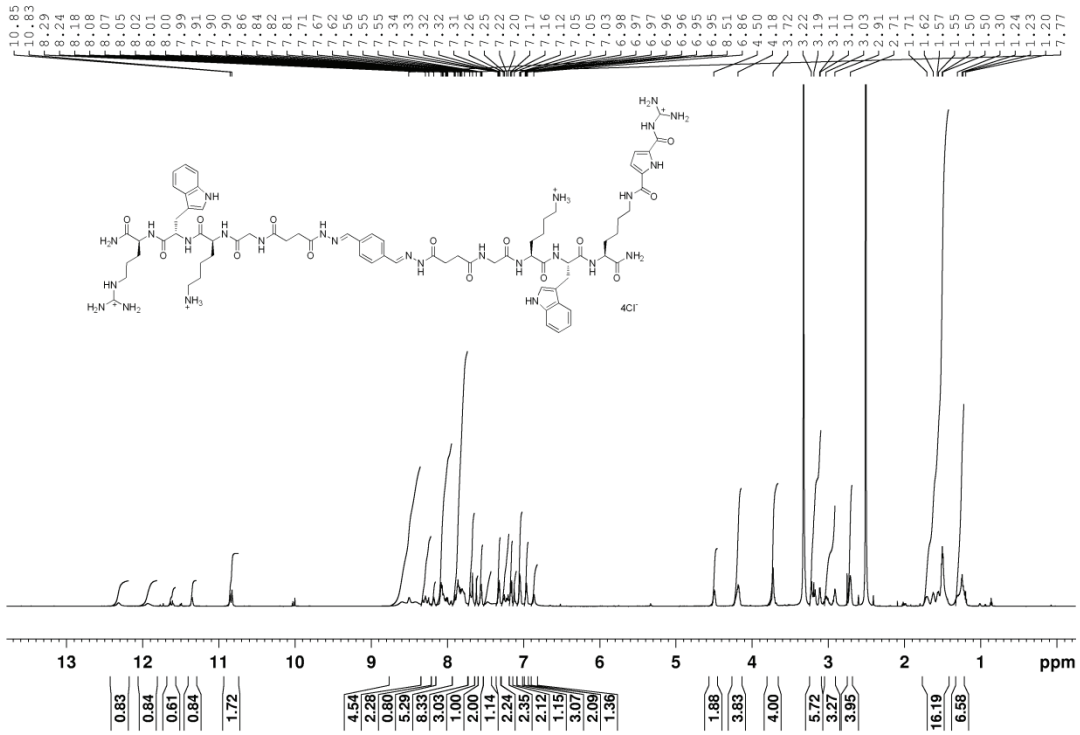


^{13}C NMR (125 MHz, DMSO-*d*$_6$)

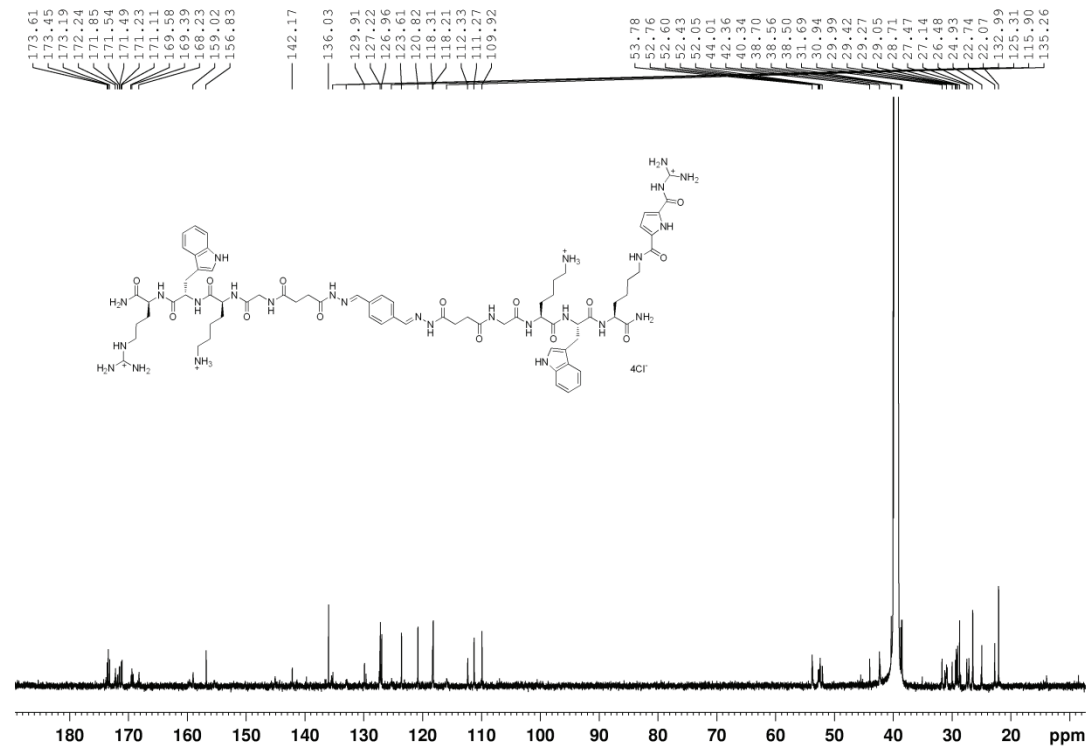


3(AC)

^{1}H NMR (700 MHz, DMSO-d_6)



^{13}C NMR (175 MHz, DMSO-d_6)



7. References

1 A. Bredihhin and U. Mäeorg, *Tetrahedron*, 2008, **64**, 6788–6793.

2 A. Dirksen, S. Dirksen, T. M. Hackeng and P. E. Dawson, *J. Am. Chem. Soc.*, 2006, **128**, 15602–15603.

3 Y. Benadie, M. Deysel, D. G. R. Siko, V. V. Roberts, S. Van Wyngaardt, S. T. Thanyani, G. Sekanka, A. M.C. Ten Bokum, L. A. Collett, J. Grooten, M. S. Baird and J. A. Verschoor, *Chem. Phys. Lipids*, 2008, **152**, 95-103.

4 A. Lundequist, M. A. Juliano, L. Juliano and G. Pejler, *Biochem. Pharmacol*., 2003, **65**, 1171–1180.