Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry

A New Approach to Inhibit Human β-Tryptase by Protein Surface Binding of Four-Armed Peptide Ligands with Two Different Sets of Arms

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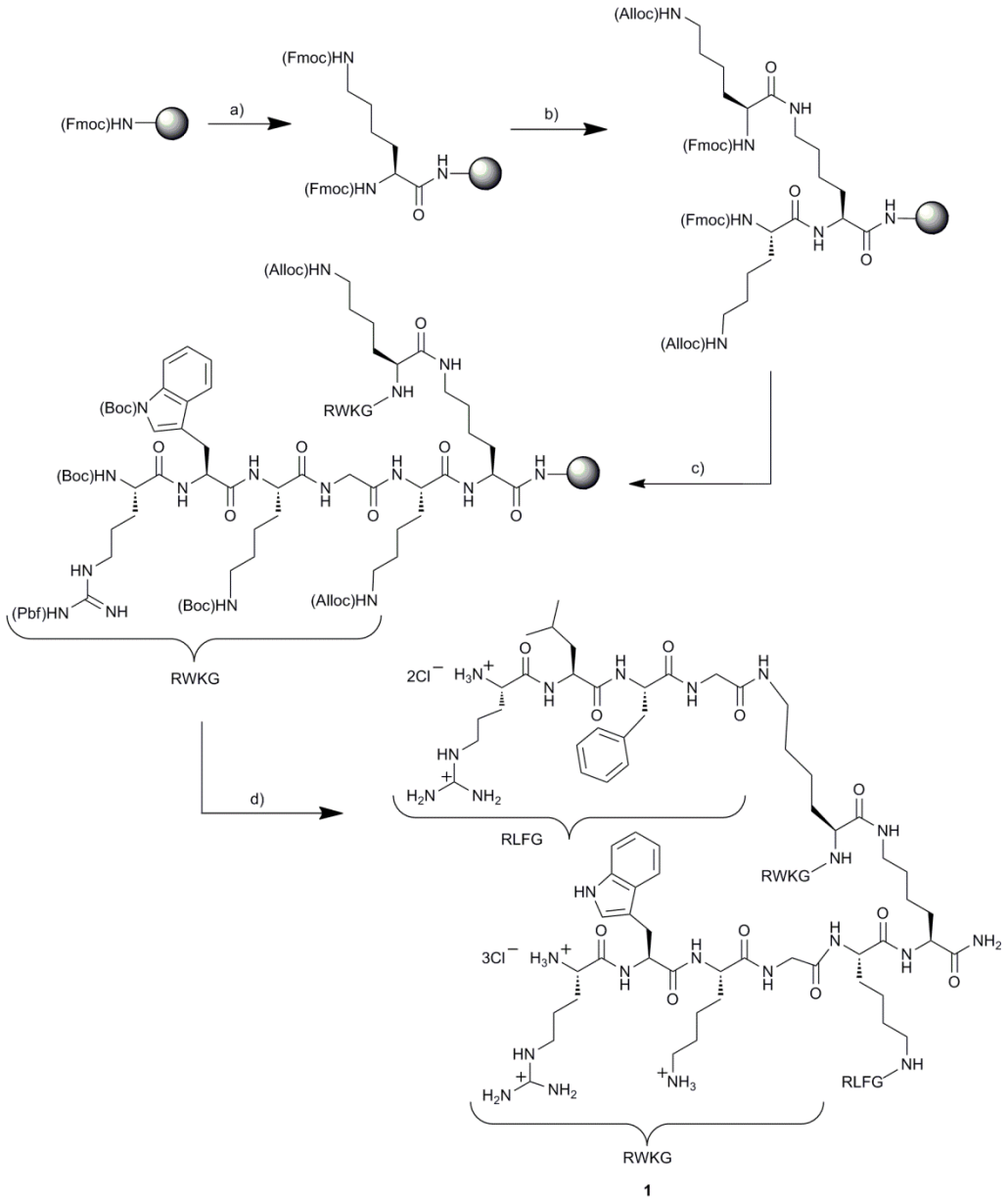
Table of contents

1. Materials and devices

All reactions were carried out in oven dried glassware at ambient temperature unless otherwise stated. All reagents were used as supplied from commercial sources unless otherwise stated. All solvents were dried and distilled and stored under Ar before use. ^{1}H and ^{13}C NMR spectra were recorded with Brucker DRX 500, Brucker DMX 600 and Brucker Avance 700 spectrometer. All IR spectra were measured on a Jasco FT/IR-430 with ATR attachment spectrometer. All high resolution ESI mass spectra were recorded with a Bruker BioTOF III spectrometer and MALDI-TOF mass spectra were measured by Bruker Daltonik AutoflexTOF II LRF 50. All melting points were measured in open glass capillary tubes with Büchi Melting Point B-540 and are quoted uncorrected. Varian Cary Eclipse Fluorescence spectrophotometer with microplate reader unit was used in the fluorescence assays. 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 were reported in volume percent. Lyophilization was carried out in an Alpha 1-4 LD plus freeze drying apparatus from Christ. The pH values were measured with Knick pH-Meter 766 Calimatic. 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.

2. Synthesis procedure

2.1 General synthesis procedure of 1-6 with two different sets of arms using SPPS and an orthogonal protecting group strategy



Scheme S1 Fmoc solid phase peptide synthesis of inhibitor **1**. a) i. 20 % piperidine/DMF, ii. Fmoc-Lys(Fmoc)-OH, PyBop, DIPEA; b) i. 20 % piperidine/DMF, ii. Fmoc-Lys(Alloc)-OH, PyBop, DIPEA; c) i. 20 % piperidine/DMF, ii. Fmoc-Gly-OH, PyBop, DIPEA, iii. rep of i. and ii. (3 times with Fmoc-Lys(Boc)-OH, Fmoc-Trp(Boc)-OH and Boc-Arg(Pbf)-OH); d) i. $Pd(Ph_3P)_4$, $PhSiH_3$, ii. Fmoc-Gly-OH, PyBop, DIPEA, iii. 20 % piperidine/DMF, iv. rep of ii. and iii. (3 times with Fmoc-Phe-OH, Fmoc-Leu-OH and Boc-Arg(Pbf)-OH), v. TFA/TIS/H_2O (95:2.5:2.5), vi. lyophilization from HCl/H_2O.

The synthesis of inhibitors **1-6** were carried out according to standard Fmoc solid phase peptide synthesis by using an orthogonal protecting strategy (Scheme S1, using inhibitor **1** as an example). Completion of each coupling and deprotecting step were monitored by a Kaiser

test. The synthesis started from Fmoc-protected Rink amide resin. The resin (100 mg, 0.99 mmol/g, 99.0 μmol, 1 equiv) was weighed out into a glass peptide synthesis vessel and allowed to swell in DCM (10 mL) for 1 h. Then, the Fmoc protection group was removed by agitation with 20% piperidine in DMF twice each for 20 min. After an intensive washing cycle with DMF (6 × 8 mL) the resin was allowed to react with Fmoc-Lys(Fmoc)-OH (147 mg, 248 μmol, 2.5 equiv) and PyBOP (129 mg, 248 μmol, 2.5 equiv) as coupling reagent in 4% DIPEA/DMF (8 mL) for 6 h to introduce the first branching. After washing with DMF (3 × 8 mL), the first coupling step was repeated with another 2.5 equivalents of the reactants to assure complete conversion of all accessible amino groups on the resin. Then, all Fmoc groups were removed under standard deprotection conditions and the resin was treated with the orthogonally protected amino acid Fmoc-Lys(Alloc)-OH (292 mg, 495 μmol, 5 equiv) and PyBOP (258 mg, 495 μmol, 5 equiv) as coupling reagent in 4% DIPEA/DMF (10 mL) for 6 h to obtain the orthogonally protected lysine scaffold with two sets of different arms. Then the Fmoc protection group was removed, followed by an intensive washing cycle with DMF (6 × 8 mL), the first two arms of tetrapeptide sequences were attached under standard conditions according to SPPS, each time with 5 equivalents of amino acid to ensure a complete coupling in all four positions: Fmoc-protected amino acid (495 μmol, 5 equiv) and PyBOP (258 mg, 495 μmol, 5 equiv) as coupling reagent in 4% DIPEA/DMF (10 mL). Then, the removal of the Alloc protection group was achieved with $Pd(PPh_3)_4$ (11.4 mg, 9.9 μmol, 0.1 equiv) in the presence of $PhSiH_3$ (292 μL, 2.38 mmol, 24 equiv) in DCM. After an intensive washing cycle with DCM (6 × 8 mL) and DMF (3 × 8 mL), the other two arms of tetrapeptide sequences were attached under standard conditions according to SPPS, each time with 5 equivalents of amino acid to ensure a complete coupling in all four positions: Fmoc-protected amino acid (495 μmol, 5 equiv) and PyBOP (258 mg, 495 μmol, 5 equiv) as coupling reagent in 4% DIPEA/DMF (10 mL). All the Kaiser tests showed quantitative reactions with protected or deprotected amines. After the final coupling and washing step with DMF (3 × 8 mL), the resin was thoroughly washed with DCM (3 × 8 mL), methanol (3 × 8 mL), and DCM (3 × 8 mL) again and dried under reduced pressure for one hour. Then the product was cleaved from the solid support by treatment with a mixture of 10 mL TFA/TIS/H_2O (95:2.5:2.5). The suspension was shaken for 3 hours and washed twice with TFA (each 5 mL). The filtrates were combined and concentrated under reduced pressure. Diethyl ether was added to the residue to precipitate the product. The solid was collected by centrifugation and washed again with diethyl ether. Crude peptide was purified by reversed phase medium-pressure liquid chromatography (RP18-MPLC) using appropriate conditions

(H_2O/MeOH + 0.05 % TFA). Pure product was transferred into hydrochloride salt by dissolving in water with hydrochloric acid and lyophilized. The compounds were obtained as colorless solids with > 95% purity according to analytical HPLC analysis.

(Arg-Trp-Lys-Gly)$_2$(Arg-Leu-Phe-Gly)$_2$(Lys)$_2$Lys-NH$_2$ (short form: (RWKG)$_2$(RLFG)$_2$) (1) (40 mg, 15.4 µmol, yield 16%). Mp: >250 °C (decomposed). FT-IR (ATR): $\tilde{\nu}$ [cm^{-1}] = 3254 (br), 2971 (br), 2923 (br), 1645 (s), 1521 (s), 1456 (m), 1242 (m), 1066 (m). ^{1}H NMR (500 MHz, DMSO-d_6): δ [ppm] = 0.83 (d, J = 6.3 Hz, 6 H, 2 × Leu-CH$_3$), 0.86 (d, J = 6.4 Hz, 6 H, 2 × Leu-CH$_3$), 1.16-1.80, 2.70-2.80 (m, 70 H, 20 × Lys-CH$_2$, 12 × Arg-CH$_2$, 2 × Leu-CH$_2$, 2 × Leu-CH), 2.85-3.03 (m, 8 H, 2 × Trp-CH$_2$, 2 × Phe-CH$_2$), 3.68-4.62 (m, 23 H, 4 × Gly-CH$_2$, 15 × CH), 6.97-8.88 (m, 78 H, 10 × Trp-CH, 10 × Phe-CH, 6 × NH_3^+, 22 × NH, 9 × NH$_2$), 10.93 (s, 2 H, 2 × Trp-NH). ^{13}C NMR (125 MHz, DMSO-d_6): δ [ppm] = 21.5, 22.0, 22.7, 23.0, 23.8, 23.9, 26.4, 27.4, 28.3, 28.7, 29.2, 31.1, 31.4, 31.9, 38.5, 40.7, 42.1 (48 C, 4 × Leu-CH$_3$, 20 × Lys-CH$_2$, 12 × Arg-CH$_2$, 2 × Leu-CH$_2$, 2 × Leu-CH, 2 × Phe-CH$_2$, 2 × Trp-CH$_2$, 4 × Gly-CH$_2$), 51.5, 52.6, 53.9, 64.1 (15 C, 15 × CH), 109.6, 111.3, 116.9, 118.2, 118.6, 120.9, 124.1, 124.9, 126.2, 127.2, 128.0, 129.2, 133.8, 136.1, 137.7 (28 C, 12 × Phe-C, 16 × Trp-C), 157.0 (4 C, 4 × Gua-Cq), 168.5, 171.2, 171.6, 171.8, 173.5 (19 C, 19 × CO-Cq). HRMS (ESI, MeOH): m/z calculated for $C_{114}H_{185}N_{39}O_{19}^{2+}$ $[M+2H]^{2+}$: 1202.7363; found: 1202.7422.

(Lys-Trp-Lys-Gly)$_2$(Lys-Leu-Phe-Gly)$_2$(Lys)$_2$Lys-NH$_2$ (short form: (KWKG)$_2$(KLFG)$_2$) (2) (26 mg, 9.8 µmol, yield 14%). Mp: >250 °C (decomposed). FT-IR (ATR): $\tilde{\nu}$ [cm^{-1}] = 3239 (br), 3053 (br), 2923 (br), 2871 (w), 1651 (s), 1539 (s), 1456 (m), 1114 (m), 1056 (m). ^{1}H NMR (500 MHz, DMSO-d_6): δ [ppm] = 0.82 (d, J = 6.5 Hz, 6 H, 2 × Leu-CH$_3$), 0.85 (d, J = 6.5 Hz, 6 H, 2 × Leu-CH$_3$), 1.18-1.76, 2.68-2.79 (m, 78 H, 36 × Lys-CH$_2$, 2 × Leu-CH$_2$, 2 × Leu-CH), 3.02-3.05 (m, 8 H, 2 × Trp-CH$_2$, 2 × Phe-CH$_2$), 3.69-4.64 (m, 23 H, 4 × Gly-CH$_2$, 15 × CH), 6.96-8.86 (m, 70 H, 10 × Trp-CH, 10 × Phe-CH, 10 × NH_3^+, 18 × NH, 1 × NH$_2$), 10.92 (s, 2 H, 2 × Trp-NH). ^{13}C NMR (125 MHz, DMSO-d_6): δ [ppm] = 20.9, 21.0, 21.6, 23.0, 24.0, 26.3, 26.4, 27.4, 28.7, 30.4, 31.2, 37.3, 38.3, 38.4, 38.5, 40.1, 40.2, 40.7, 42.1 (52 C, 4 × Leu-CH$_3$, 36 × Lys-CH$_2$, 2 × Leu-CH$_2$, 2 × Leu-CH, 2 × Phe-CH$_2$, 2 × Trp-CH$_2$, 4 × Gly-CH$_2$), 51.6, 51.8, 52.6, 54.0 (15 C, 15 × CH), 109.7, 111.3, 118.3, 118.6, 121.0, 124.1, 126.3, 127.2, 128.1, 129.3, 136.1, 137.7 (28 C, 12 × Phe-C, 16 × Trp-C), 168.4, 168.5, 168.7, 171.2, 171.3, 171.7 (19 C, 19 × CO-Cq). HRMS (ESI, MeOH): m/z calculated for $C_{114}H_{185}N_{31}O_{19}^{2+}$ $[M+2H]^{2+}$: 1146.2201; found: 1146.7158.

(Arg-Trp-Lys-Gly)$_2$(Lys-Leu-Phe-Gly)$_2$(Lys)$_2$Lys-NH$_2$ (short form: (RWKG)$_2$(KLFG)$_2$) (3) (22 mg, 8.1 µmol, yield 12%). Mp: >250 °C (decomposed). FT-IR (ATR): $\tilde{\nu}$ [cm^{-1}] = 3216 (br), 3053 (br), 2932 (br), 1646 (s), 1521 (s), 1457 (m), 1232 (m), 1085 (m). ^{1}H NMR (700 MHz, DMSO-d_6): δ [ppm] = 0.82 (d, J = 5.7 Hz, 6 H, 2 × Leu-CH$_3$), 0.85 (d, J = 5.7 Hz, 6 H, 2 × Leu-CH$_3$), 1.15-1.83, 2.68-2.86 (m, 74 H, 28 × Lys-CH$_2$, 6 × Arg-CH$_2$, 2 × Leu-CH$_2$, 2 × Leu-CH), 2.97-3.04 (m, 8 H, 2 × Trp-CH$_2$, 2 × Phe-CH$_2$), 3.78-4.61 (m, 23 H, 4 × Gly-CH$_2$, 15 × CH), 6.96-8.84 (m, 74 H, 10 × Trp-CH, 10 × Phe-CH, 8 × NH$_3^+$, 20 × NH, 5 × NH$_2$), 10.91 (s, 2 H, 2 × Trp-NH). ^{13}C NMR (175 MHz, DMSO-d_6): δ [ppm] = 20.9, 21.6, 22.0, 23.0, 23.8, 23.9, 26.2, 26.4, 27.4, 28.3, 28.7, 30.3, 31.1, 31.4, 37.2, 38.2, 38.5, 40.7, 42.0 (50 C, 4 × Leu-CH$_3$, 28 × Lys-CH$_2$, 6 × Arg-CH$_2$, 2 × Leu-CH$_2$, 2 × Leu-CH, 2 × Phe-CH$_2$, 2 × Trp-CH$_2$, 4 × Gly-CH$_2$), 51.4, 51.8, 52.7, 53.9 (15 C, 15 × CH), 109.6, 111.3, 118.2, 118.6, 120.9, 124.1, 126.3, 127.2, 128.0, 129.2, 136.1, 137.7 (28 C, 12 × Phe-C, 16 × Trp-C), 156.9 (2 C, 2 × Gua-Cq), 168.3, 168.4, 168.5, 168.6,168.8, 171.1, 171.2, 171.3, 171.5, 171.6, 171.8, 173.7 (19 C, 19 × CO-Cq). HRMS (ESI, MeOH): m/z calculated for C$_{114}$H$_{185}$N$_{35}$O$_{19}^{2+}$ [M+2H]$^{2+}$: 1174.7302; found: 1174.7286.

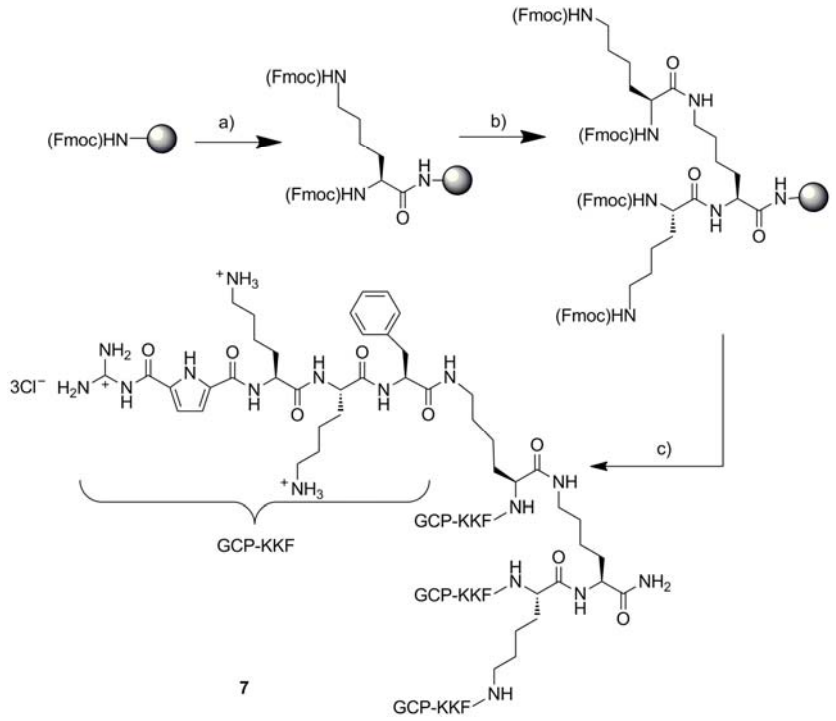
(Arg-Trp-Lys-Gly)$_2$(Lys-Trp-Lys-Gly)$_2$(Lys)$_2$Lys-NH$_2$ (short form: (RWKG)$_2$(KWKG)$_2$) (4) (32 mg, 11.1 µmol, yield 16%). Mp: >250 °C (decomposed). FT-IR (ATR): $\tilde{\nu}$ [cm^{-1}] = 3222 (br), 3053 (br), 2932 (br), 1645 (s), 1520 (s), 1456 (m), 1233 (m). ^{1}H NMR (700 MHz, DMSO-d_6): δ [ppm] = 1.29-1.78, 2.74-2.92 (m, 84 H, 36 × Lys-CH$_2$, 6 × Arg-CH$_2$), 3.00-3.03 (m, 8 H, 4 × Trp-CH$_2$), 3.67-4.61 (m, 23 H, 4 × Gly-CH$_2$, 15 × CH), 6.97-8.84 (m, 80 H, 20 × Trp-CH, 10 × NH$_3^+$, 20 × NH, 5 × NH$_2$), 10.91 (s, 4 H, 4 × Trp-NH). ^{13}C NMR (175 MHz, DMSO-d_6): δ [ppm] = 20.9, 22.1, 22.4, 22.9, 23.9, 26.3, 26.5, 27.5, 28.4, 28.8, 30.4, 30.8, 31.1, 38.3, 38.6, 40.0, 40.2 , 42.0 (50 C, 36 × Lys-CH$_2$, 6 × Arg-CH$_2$, 4 × Trp-CH$_2$, 4 × Gly-CH$_2$), 51.0, 51.6, 51.9, 52.7, 53.9, 54.0 (15 C, 15 × CH), 109.6, 111.3, 118.3, 118.7, 120.9, 124.2, 125.1, 127.1, 136.1 (32 C, 32 × Trp-C), 157.1 (2 C, 2 × Gua-Cq), 168.5, 168.6,168.7, 168.8, 171.2, 171.3, 171.4, 171.6, 171.8, 171.9, 173.7 (19 C, 19 × CO-Cq). HRMS (ESI, MeOH): m/z calculated for C$_{118}$H$_{189}$N$_{39}$O$_{19}^{2+}$ [M+2H]$^{2+}$: 1128.7519; found: 1128.7384.

(Arg-Trp-Lys-Gly)$_2$(Phe-Arg-Lys-Gly)$_2$(Lys)$_2$Lys-NH$_2$ (short form: (RWKG)$_2$(FRKG)$_2$) (5) (20 mg, 7.0 µmol, yield 10%). Mp: >250 °C (decomposed). FT-IR (ATR): $\tilde{\nu}$ [cm^{-1}] = 3209 (br), 3053 (br), 2932 (br), 2869 (w), 1644 (s), 1531 (s), 1455 (m), 1233 (m). ^{1}H NMR (500 MHz, DMSO-d_6): δ [ppm] = 1.19-1.77, 2.74-3.00 (m, 80 H, 28 × Lys-CH$_2$, 12 × Arg-CH$_2$), 3.10-3.15 (m, 8 H, 2 × Trp-CH$_2$, 2 × Phe-CH$_2$), 3.68-4.61 (m, 23 H, 4 × Gly-CH$_2$, 15 × CH), 6.96-8.95 (m, 84 H, 10 × Trp-CH, 10 × Phe-CH, 8 × NH$_3^+$, 22 × NH, 9 × NH$_2$), 10.92

(s, 2 H, 2 × Trp-NH). ^{13}C NMR (125 MHz, DMSO-d_6): δ [ppm] = 22.0, 22.1, 23.9, 24.8, 26.4, 27.4, 28.3, 28.7, 28.9, 30.9, 31.4, 36.8, 38.5, 40.3, 42.0 (48 C, 28 × Lys-CH_2, 12 × Arg-CH_2, 2 × Phe-CH_2, 2 × Trp-CH_2, 4 × Gly-CH_2), 51.5, 52.4, 52.7, 52.8, 53.3 (15 C, 15 × CH), 109.6, 111.3, 118.3, 118.6, 120.9, 124.1, 127.1, 127.2, 128.6, 129.7, 135.0, 136.1 (28 C, 12 × Phe-C, 16 × Trp-C), 157.1 (4 C, 4 × Gua-Cq), 168.0, 168.4, 168.6, 170.9, 171.2, 171.4, 171.7, 171.8, 173.1 (19 C, 19 × CO-Cq). HRMS (ESI, MeOH): m/z calculated for $C_{114}H_{187}N_{41}O_{19}^{2+}$ $[M+2H]^{2+}$: 1217.7472; found: 1217.7508.

(Arg-Trp-Lys-Gly)$_2$(GCP-Leu-Phe-Gly)$_2$(Lys)$_2$Lys-NH$_2$ (short form: (RWKG)$_2$(GCP-LFG)$_2$) (6) (26 mg, 9.5 µmol, yield 10%). Mp: 244-248 °C. FT-IR (ATR): $\tilde{\nu}$ [cm^{-1}] = 3242 (br), 3059 (br), 2933 (br), 2863 (w), 1645 (s), 1524 (s), 1238 (m). ^{1}H NMR (500 MHz, DMSO-d_6): δ [ppm] = 0.82 (d, J = 6.4 Hz, 6 H, 2 × Leu-CH_3), 0.87 (d, J = 6.4 Hz, 6 H, 2 × Leu-CH_3), 1.24-1.79, 2.75-3.01 (m, 58 H, 20 × Lys-CH_2, 6 × Arg-CH_2, 2 × Leu-CH_2, 2 × Leu-CH), 3.16-3.20 (m, 8 H, 2 × Trp-CH_2, 2 × Phe-CH_2), 3.77-4.63 (m, 21 H, 4 × Gly-CH_2, 13 × CH), 6.90-8.82 (m, 74 H, 10 × Trp-CH, 10 × Phe-CH, 4 × GCP-CH, 4 × NH_3^+, 20 × NH, 9 × NH_2), 10.90 (s, 2 H, 2 × Trp-NH), 12.09 (s, 2 H, 2 × Gua-NH), 12.49 (s, 2 H, 2 × GCP-NH). ^{13}C NMR (125 MHz, DMSO-d_6): δ [ppm] = 21.5, 22.0, 22.9, 23.8, 24.2, 26.4, 27.5, 28.3, 28.8, 31.1, 38.5, 40.1, 42.1 (42 C, 4 × Leu-CH_3, 20 × Lys-CH_2, 6 × Arg-CH_2, 2 × Leu-CH_2, 2 × Leu-CH, 2 × Phe-CH_2, 2 × Trp-CH_2, 4 × Gly-CH_2), 51.5, 52.6, 53.9, 54.0 (13 C, 13 × CH), 109.6, 111.3, 113.6, 115.8, 116.9, 118.2, 118.6, 120.9, 124.0, 125.6, 126.2, 127.2, 128.0, 129.2, 132.3, 136.1, 137.8 (36 C, 12 × Phe-C, 16 × Trp-C, 8 × GCP-C), 155.5, 157.0, 158.9, 159.7 (4 C, 4 × Gua-Cq), 168.3, 168.5, 171.1, 171.2, 171.3, 171.9, 172.0 (21 C, 21 × CO-Cq). HRMS (ESI, MeOH): m/z calculated for $C_{116}H_{174}N_{39}O_{21}^{3+}$ $[M+3H]^{3+}$: 816.7919; found: 816.7965.

2.2 General synthesis procedure of 7 and 8 with four identical arms terminating with the GCP-group using SPPS



Scheme S2 Fmoc solid phase peptide synthesis of inhibitor **7**. a) i. 20 % piperidine/DMF, ii. Fmoc-Lys(Fmoc)-OH, PyBop, DIPEA; b) i. 20 % piperidine/DMF, ii. Fmoc-Lys(Fmoc)-OH, PyBop, DIPEA; c) i. 20 % piperidine/DMF, ii. Fmoc-Phe-OH, PyBop, DIPEA, iii. rep of i. and ii. (twice with Fmoc-Lys(Boc)-OH, once with GCP), iv. TFA/TIS/H_2O (95:2.5:2.5), v. lyophilization with HCl/H_2O.

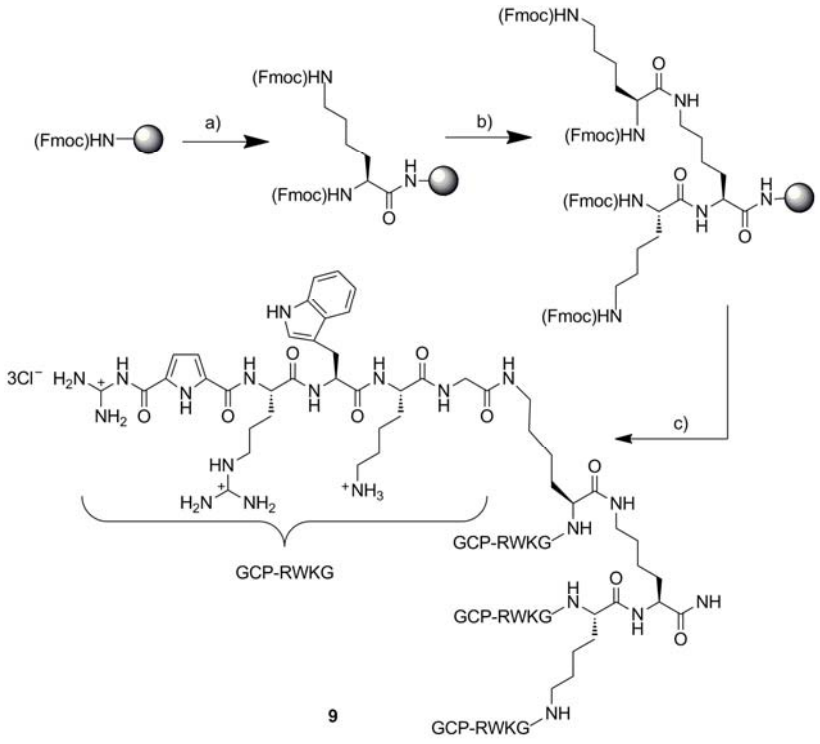
Inhibitor **7** and **8** were synthesized according to a standard Fmoc solid phase peptide synthesis (Scheme S2, using inhibitor **7** as an example). Rink amide resin (100 mg, 940 µmol/g, 94.0 µmol, 1 equiv) was prepared for the attachment of the first amino acid according to the general procedures as described above. Then the resin was allowed to react with Fmoc-Lys(Fmoc)-OH (139 mg, 235 µmol, 2.5 equiv) and PyBOP (122 mg, 235 µmol, 2.5 equiv) in DMF containing 3 % NMM (10 mL) for 20 h to introduce the first branching. Then, all Fmoc groups were removed under standard deprotection conditions by treating with 20 % piperidine in DMF and the resin was treated once more with Fmoc-Lys(Fmoc)-OH (278 mg, 470 µmol, 5 equiv) and PyBOP (245 mg, 470 µmol, 5 equiv) in DMF containing 3 % NMM (10 mL) for 20 h to result in the lysine scaffold with four identical branches. After removal of all the Fmoc group, the following three amino acids were attached under standard conditions for SPPS, each time with 10 equivalents of amino acid to ensure a complete coupling in all three positions: Fmoc-protected amino acid (940 µmol, 10 equiv), PyBOP (489 mg, 940 µmol, 10 equiv) in DMF (10 mL) containing 3 % NMM. The mixture was shaken for 20 h to ensure quantitative coupling. After the final Fmoc deprotection the attachment of the 5-guanidiniocarbonylpyrrole-2-carboxylic acid (GCP) was performed under related conditions: GCP (374 mg, 940 µmol, 10 equiv), PyBOP (489 mg, 940 µmol, 10 equiv) and DMF

containing 5 % NMM with a reaction time of 20 h. The last step was repeated to ensure quantitative coupling. After the final washing cycle with 3 × DCM, 3 × MeOH and 3 × DCM again, the resin was dried and the product was cleaved from the solid support according to the general procedure for the Rink amide resin as described above and further purified by RP18-HPLC ($MeOH/H_2O$ + 0.05 % TFA). Pure product was transferred into hydrochloride salt by dissolving in water with hydrochloric acid and lyophilized. The compounds were obtained as colorless solids with > 95% purity according to analytical HPLC analysis.

$(GCP\text{-}Lys\text{-}Lys\text{-}Phe)_4(Lys)_2Lys\text{-}NH_2$ (short form: $(GCP\text{-}KKF)_4$) (7) (94.5 mg, 29.5 µmol, yield 32%). Mp: 192 °C (decomposed). FT-IR (ATR): $\tilde{\nu}$ [cm^{-1}] = 3243 (br), 3046 (br), 2918 (br), 2087 (w), 1644 (s), 1526 (s), 1450 (m). ^{1}H NMR (600 MHz, DMSO-d_6): δ [ppm] = 1.11-1.82 (m, 64 H, 32 × Lys-CH_2), 2.63-3.15 (m, 32 H, 12 × Lys-CH_2, 4 × Phe-CH_2), 4.04-4.60 (m, 15 H, 15 × CH), 6.84-8.83 (m, 88 H, 20 × Phe-CH, 8 × GCP-CH, 18 × NH, 9 × NH_2, 8 × NH_3^+), 12.15 (s, 4 H, 4 × Gua-NH), 12.54 (s, 4 H, 4 × GCP-NH). ^{13}C NMR (150 MHz, DMSO-d_6): δ [ppm] = 22.1, 22.6, 22.7, 26.2, 26.4, 26.6, 28.3, 28.7, 28.8, 30.3, 31.1, 31.7, 31.9, 36.9, 37.1, 38.3, 38.6, 44.6 (48 C, 48 × CH_2), 51.7, 52.4, 52.5, 52.7, 52.8, 53.2, 53.5, 53.7, 53.9, 54.7 (m, 15 C, 15 × CH), 113.6, 115.8 (8 C, 8 × GCP-CH), 126.2, 126.4, 127.1, 128.0, 128.1, 128.4, 129.2, 129.5 (20 C, 20 × Phe-CH), 125.6, 132.3, 135.0, 137.6, 155.6, 159.0, 159.8, 167.6, 168.4, 170.6, 171.2, 171.6, 173.7 (39 C, 23 × CO-Cq, 16 × Cq). HRMS (MALDI, DHB/HCCA 1:1, MeOH, H_2O): m/z calculated for $C_{130}H_{200}Cl_4N_{43}O_{23}^+$ $[M+4HCl+H]^+$: 2875.0648; found: 2876.552

$(GCP\text{-}Trp\text{-}His\text{-}Arg)_4(Lys)_2Lys\text{-}NH_2$ (short form: $(GCP\text{-}WHR)_4$) (8) (57.1 mg, 16.5 µmol, yield 17%). Mp: 199 °C (decomposed). FT-IR (ATR): $\tilde{\nu}$ [cm^{-1}] = 3230 (br), 3140 (br), 1659 (s), 1540 (s), 1453 (m), 1275 (w), 1070 (m), 820 (w). ^{1}H NMR (600 MHz, DMSO-d_6): δ [ppm] = 1.39-1.74, 2.75-3.11 (m, 64 H, 12 × Lys-CH_2, 4 × Trp-CH_2, 4 × His-CH_2, 12 × Arg-CH_2), 4.03-4.39 (m, 15 H, 15 × CH), 6.75-9.14 (m, 96 H, 20 × Trp-CH, 8 × GCP-CH, 8 × His-CH, 26 × NH, 17 × NH_2), 12.15 (s, 4 H, 4 × Gua-NH), 12.55 (s, 4 H, 4 × GCP-NH), 14.46 (bs, 8H, 8 × His-NH). ^{13}C NMR (150 MHz, DMSO-d_6): δ [ppm] = 20.4, 20.9, 21.2, 21.7, 22.1, 22.7, 23.7, 24.2, 25.0, 26.2, 26.3, 28.2, 28.3, 28.7, 29.9, 30.3, 30.6, 31.5, 32.5, 38.2, 38.3, 41.2, 41.3, 41.5, 43.6, 44.5 (32 C, 32 × CH_2), 113.7, 115.8, 116.9, 117.7, 118.1, 133.6, 133.9, 134.2 (36 C, 8 × GCP-CH, 8 × His-CH, 20 × Trp-CH), 124.2, 125.8, 126.6, 126.9, 155.6, 157.0, 159.0, 159.6, 166.9, 168.2, 168.3, 171.3, 171.4, 173.1, 173.3, 173.9 (55 C, 23 × CO-Cq, 32 × Cq). HRMS (MALDI, DHB/HCCA 1:1, MeOH, H_2O): m/z calculated for $C_{138}H_{180}N_{59}O_{23}^+$ $[M+H]^+$: 3033.287; found: 3033.564.

2.3 General synthesis procedure of 9 and 10 with four identical arms terminating with the GCP-group using microwave-assisted SPPS



Scheme S3 Microwave assisted solid phase peptide synthesis of inhibitor **9**. a) i. 20 % piperidine/DMF, ii. Fmoc-Lys(Fmoc)-OH, PyBop, DIPEA; b) i. 20 % piperidine/DMF, ii. Fmoc-Lys(Fmoc)-OH, PyBop, DIPEA; c) i. 20 % piperidine/DMF, ii. Fmoc-Gly-OH, PyBop, DIPEA, iii. rep of i. and ii. (4 times with Fmoc-Lys(Boc)-OH, Fmoc-Trp(Boc)-OH, Fmoc-Arg(Pbf)-OH and GCP), iv. TFA/TIS/H_2O (95:2.5:2.5), v. lyophilization from HCl/H_2O.

Scheme S3 describes the synthesis of ligand **9**, and ligand **10** was synthesized accordingly. These two inhibitors were synthesized following by microwave-assisted solid phase peptide synthesis. The Rink amide MBHA resin (200 mg, 0.67 mmol/g, 0.134 mmol, 1 equiv) was allowed to swell in DCM (10 mL) for 1 h. Then, the Fmoc protection group was removed by treating with 20 % piperidine in DMF first for 1.5 min and then in a second treatment for 5 min (20 Watt, T_{max} = 60 °C, ΔT = ± 5 °C), after a washing step with DMF (6 × 5 mL), followed by a Kaiser test to confirm the completely formation of free amino functions. Then the first amino acid Fmoc-Lys(Fmoc)-OH (198 mg, 0.335 mmol, 2.5 equiv) was attached with the help of the coupling reagent PyBOP (175 mg, 0.335 mmol, 2.5 equiv) in 4% DIPEA/DMF (5 mL) for 20 min (20 Watt, T = 60 °C, ΔT = ± 5 °C). The first coupling was repeated and the resin were washed with DMF (3 × 5 mL), then the Kaiser test was performed to ensure the complete reaction. If the coupling was not quantitative the coupling step was repeated until the Kaiser test was showed a negative result. After removal of the Fmoc protecting group and the washing step with DMF (6 × 5 mL), Fmoc-Lys(Fmoc)-OH (396 mg, 0.67 mmol, 5 equiv)

was attached once again to the two branches by the help of the coupling reagent PyBOP (350 mg, 0.67 mmol, 5 equiv) in 4% DIPEA/DMF (5 mL) for 20 min to introduce the scaffold with four identical arms. Then the peptide sequence of the four arms were built by using similar procedure as described above, each time adding Fmoc protected amino acids or the artificial GCP group (1.34 mmol, 10 equiv), PyBOP (1.34 mmol, 10 equiv) in 4% DIPEA/DMF (5 mL). After the last coupling step and the final washing cycle with 3 × DCM, 3 × MeOH and 3 × DCM again, the resin was dried and the product was cleaved from the solid support according to the general procedure (TFA/TIS/H_2O = 95:2.5:2.5) for the Rink amide resin as described above. Crude peptide was purified by RP18-MPLC using appropriate conditions (H_2O/MeOH + 0.05 % TFA). Pure product was transferred into hydrochloride salt by dissolving in water with hydrochloric acid and lyophilized. The products were obtained as colorless solids with > 95% purity according to analytical HPLC analysis.

(GCP-Arg-Trp-Lys-Gly)$_4$(Lys)$_2$Lys-NH$_2$ (short form: (GCP-RWKG)$_4$) (9) (10 mg, 2.7 µmol, yield 2%). Mp: 245-250 °C. FT-IR (ATR): $\tilde{\nu}$ [cm^{-1}] = 3310 (br), 3055 (br), 2868 (w), 1643 (s), 1527 (s), 1470 (m). ^{1}H NMR (700 MHz, DMSO-d_6): δ [ppm] = 1.15-1.76, 2.95-3.11 (m, 80 H, 12 × Arg-CH_2, 28 × Lys-CH_2), 2.68-2.77 (m, 8 H, 4 × Trp-CH_2), 3.64-4.57 (m, 23 H, 15 × CH, 4 × Gly-CH_2), 6.91-8.70 (m, 100 H, 8 × GCP-CH, 20 × Trp-CH, 26 × NH, 17 × NH_2, 4 × NH_3^+), 10.82 (s, 4 H, 4 × Trp-NH), 11.97 (s, 4 H, 4 × Gua-NH), 12.52 (s, 4 H, 4 × GCP-NH). ^{13}C NMR (175 MHz, DMSO-d_6): δ [ppm] = 22.0, 22.1, 22.7, 22.9, 25.1, 26.5, 27.2, 28.7, 28.9, 31.1, 31.2, 38.5, 38.6, 40.4, 42.0 (48 C, 28 × Lys-CH_2, 12 × Arg-CH_2, 4 × Trp-CH_2, 4 × Gly-CH_2), 52.6, 52.7, 53.6 (15 C, 15 × CH), 109.8, 127.3, 136.0 (12 C, 12 × Trp-Cq), 111.3, 118.2, 120.8, 123.7 (s, 20 C, 20 × Trp-CH), 113.7, 115.8 (8 C, 8 × GCP-CH), 125.7, 132.2 (8 C, 8 × GCP-Cq), 155.5 (s, 4 C, 4 × GCP-Gua-Cq), 156.8 (4 C, 4 × Arg-Cq), 159.1, 159.8, 168.5, 171.4, 171.5, 171.7 (27 C, 27 × CO-Cq). HRMS (ESI, MeOH): m/z calculated for $C_{146}H_{215}N_{59}O_{27}^{4+}$ $[M+4H]^{4+}$: 806.9317; found: 806.9315.

(GCP-Arg-Trp-Lys)$_4$(Lys)$_2$Lys-NH$_2$ (short form: (GCP-RWK)$_4$) (10) (17 mg, 5.0 µmol, yield 4%). Mp: 230-233 °C. FT-IR (ATR): $\tilde{\nu}$ [cm^{-1}] = 3063 (br), 2939 (w), 1634 (s), 1538 (s), 1471 (m). ^{1}H NMR (500 MHz, DMSO-d_6): δ [ppm] = 1.18-1.77, 2.89-3.19 (m, 80 H, 12 × Arg-CH_2, 28 × Lys-CH_2), 2.68-2.78 (m, 8 H, 4 × Trp-CH_2), 4.12-4.59 (m, 15 H, 15 × CH), 6.89-8.75 (m, 96 H, 8 × GCP-CH, 20 × Trp-CH, 22 × NH, 17 × NH_2, 4 × NH_3^+), 10.86 (d, J = 15.1, 4 H, 4 × Trp-NH), 12.11 (s, 4 H, 4 × Gua-NH), 12.53 (s, 4 H, 4 × GCP-NH). ^{13}C NMR (125 MHz, DMSO-d_6): δ [ppm] =22.1, 22.2, 22.8, 22.9, 25.2, 26.5, 27.3, 28.8, 28.9, 31.5, 38.6, 40.1, 40.5 (44 C, 28 × Lys-CH_2, 12 × Arg-CH_2, 4 × Trp-CH_2), 52.5, 52.7, 53.6 (15 C, 15

× CH), 109.8, 127.3, 136.0 (12 C, 12 × Trp-Cq), 111.3, 118.2, 118.4, 120.8, 123.7 (20 C, 20 × Trp-CH), 113.8, 115.9 (8 C, 8 × GCP-CH), 125.7, 132.3 (8 C, 8 × GCP-Cq), 155.6 (4 C, 4 × GCP-Gua-Cq), 156.9 (4 C, 4 × Arg-Cq), 159.1, 159.8, 171.1, 171.3, 171.4 (23 C, 23 × CO-Cq). HRMS (ESI, MeOH): m/z calculated for $C_{138}H_{202}N_{55}O_{23}^{3+}$ $[M+3H]^{3+}$: 999.5446; found: 999.5459.

3. Enzyme assay

3.1 Enzyme kinetic measurements

In order to measure the kinetic parameters for the hydrolysis of the chromogenic substrate Tos-Gly-Pro-Arg-AMC by rhSkin β-tryptase, the kinetic assays were performed in white 96 well microplate in a 50 mM Tris-HCl buffer at pH 7.4, containing additional 50 μg/mL heparin (to stabilize the enzyme), 0.02 % Triton-X (to minimize the aggregation) and 100 mM NaCl. The enzyme was prepared in assay buffer and the final concentration of the enzyme used in the assay had to be determined prior the tests in order to find appropriate concentration to make the slope of the linear graph representing the product conversion over time between 15 and 30 in the absence of inhibitor. The stock solution of substrate was prepared in DMSO at a concentration of 2 mM. All the inhibitors were prepared as a stock solution of 1 mM in DMSO. The kinetic assays (at 25 ^{o}C) were carried out in a final volume of 200 μL in assay buffer (165 μL) wherein the enzyme (10 μL, final concentration 0.25 nM) was incubated with various concentrations of inhibitors (20 μL) and finally the substrate (5 μL, final concentration 50 μM) was added. All the solutions were thoroughly mixed and finally the increase of fluorescence activity was measured over the time of 15 min. The inhibitory activities (in %) of all the inhibitors was first determined four-fold at an inhibitor concentration of 100 μM. To determine the IC_{50} and K_i values, a series of the stock solution in DMSO resulting the following final concentrations: 100 μM, 80 μM, 60 μM, 40 μM, 20 μM, 10 μM, 8 μM, 6 μM, 4 μM, 2 μM, 1 μM, 0.1 μM were measured. Depending on the inhibition activity, sometimes bigger and smaller concentrations had to be prepared (1000-0.01 μM).

3.2 Enzyme selectivity assay

Trypsin and α-Chymotrypsin were chosen to check the enzyme selectivity. 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 α-Chymotrypsin respectively. The stock solution of substrate was prepared in DMSO at a concentration of 2 mM. All the inhibitors were prepared as a stock solution of 1 mM in DMSO. The kinetic assays (at 25 ^{o}C) were carried out in a final volume of 200 μL by

adding the enzyme (10 µL) to Tris-HCl buffer (pH 8.0, 165 µL) and then the addition of inhibitor (20 µL) and finally the substrate (5 µL, final concentration 50 µM) was added. 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.

3.3 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) which contained a mixture (total volume of 800 µL) of assay buffer (Tris-HCl, 50 mM, pH 7.4, 600 µL), β-tryptase (40 µL, final concentration 0.5 nM) and inhibitor **6** in DMSO (160 µL, final concentration 100 µM) or pure DMSO (160 µL) as the reference. The dialysis tubes were floated vertically in the dialysate reservoir containing a stirring bar to adjust the stirring rate to form a gentle rotating current. The samples were dialyzed at room temperature and with 4 complete buffer changes (every 12 h) over a period of 48 h. The tests were done from time to time by taking a 100 µL sample from the dialysis tube, adding 95 µL assay buffer and the substrate (5 µL, final concentration 50 µM) before the mixtures were submitted to a fluorescence assay to determine the residual activity of the enzyme. In the second experiment to test the reversibility of enzyme inhibition, the enzyme (10 µL) was incubated with the inhibitor (10 µL, final concentration 50 µM) or blank DMSO (10 µL) in 170 µL assay buffer (Tris-HCl, 50 mM, pH 7.4) for 5 minutes. Then in a parallel experiment, either heparin in buffer (5 µL, final concentration 0.5 mg/mL) or buffer alone (5 µL) was added to the vials. All the vials were mixed and incubated for another 5 minutes, followed by the addition of substrate (5 µL, final concentration 50 µM) and the fluorescence readout. The higher enzyme activity in the vial with heparin compared to the vial with buffer indicates a reversibility binding of the inhibitor to the enzyme.

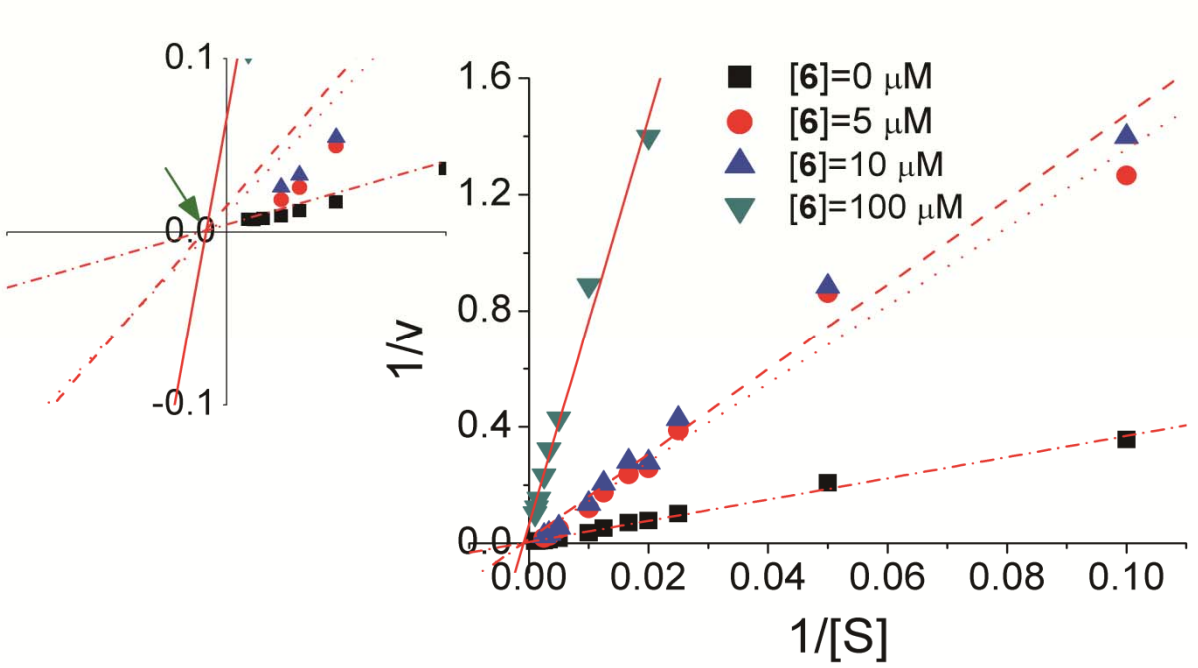


Fig. S1 Lineweaver-Burk plots of 1/v against 1/[S] for inhibitor **6**. The plots have a common intersection on the x-axis but not on the y-axis, which indicates a non-competitive inhibition.

3.4 Equation for calculating IC_{50} values

4-parameter equation:

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

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

4. Computational calculation details

Software of Schrödinger MacroModel Vers. 9.6 is used for the molecular modeling studies. The enzyme input is based on the reported crystal structure of β-tryptase from the RCSB Protein Data Bank (PDB code: 1A0L). All water molecules and substrates of the PDB crystal structure were removed from the binding pore. The calculation was performed based on the force field OPLS (optimized potentials for liquid simulations) 2005 choosing water as the solvent. The structure of the enzyme was fixed while the inhibitor was put as mobilized during the calculation. So the calculations began at the potential energy of the start conformation and by simulating new conformations a minimal potential was searched. The resulting structure was obtained from the result of 1000 calculation cycles.

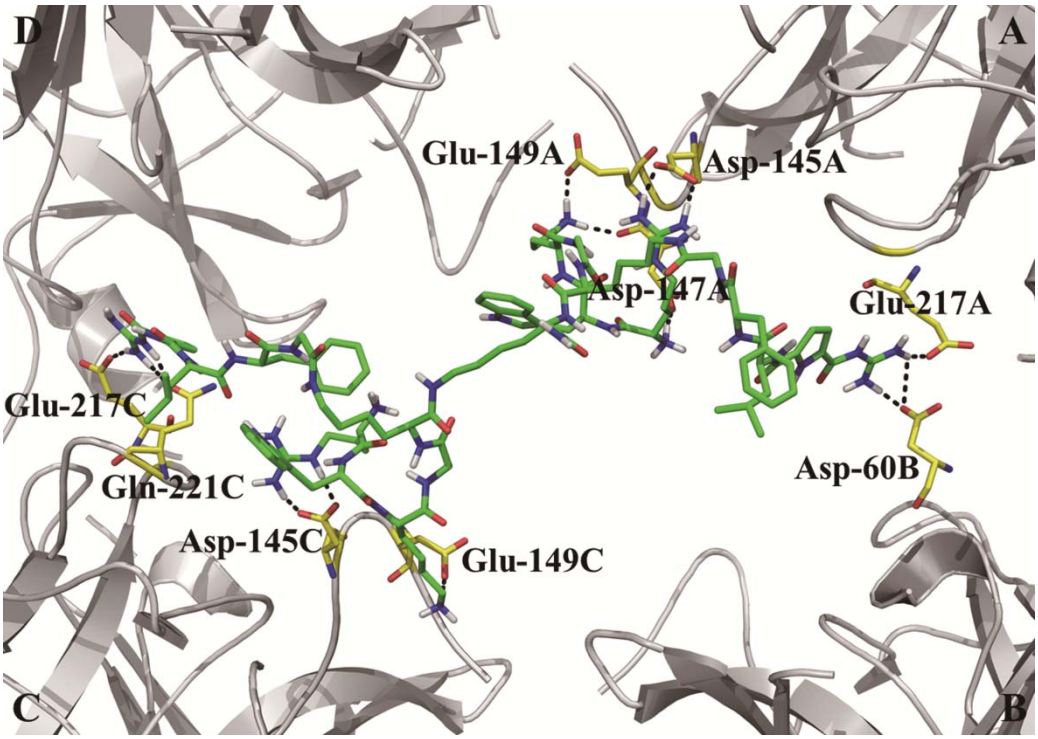
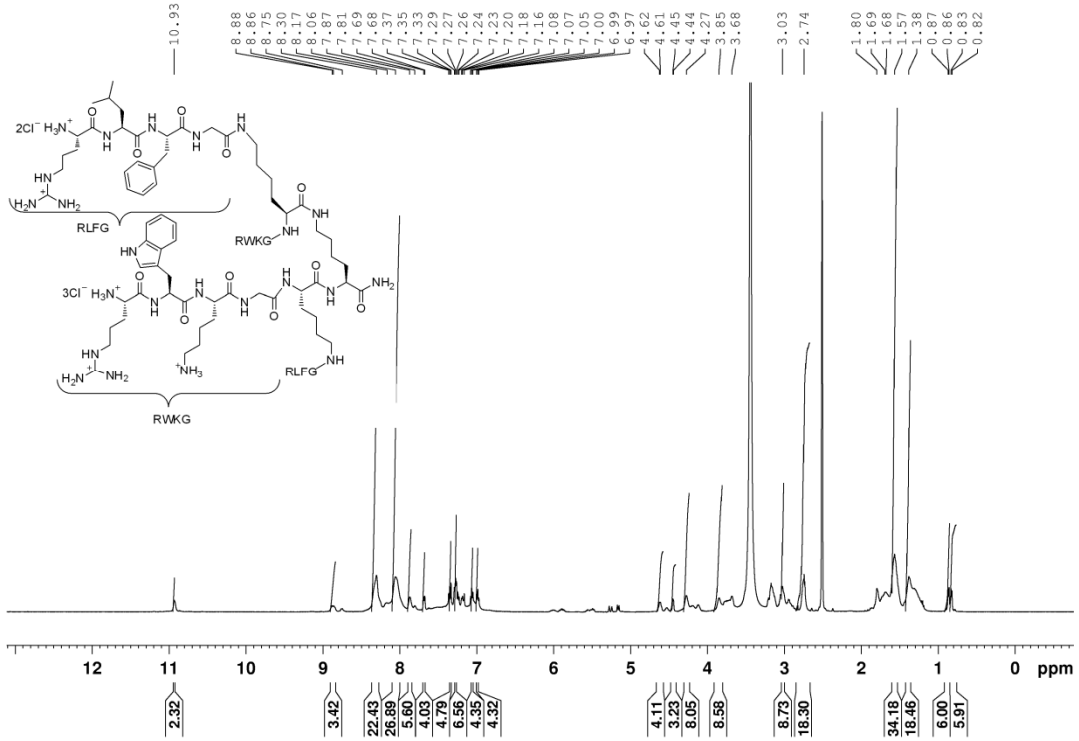


Fig. S2 Ribbon cartoon of the crystal structure of β-tryptase. Inhibitor **6** is shown as sticks with carbon atoms colored green, nitrogen atoms colored blue and oxygen atoms colored red. Non-polar hydrogen atoms have been removed for clarity. Key residues that interact with the inhibitor are displayed as sticks (carbon atoms colored yellow) and numbered according to the protein sequence and different monomers (four monomers are named A, B, C and D). Potential interactions in the enzyme-inhibitor complex are shown as black dashed lines.

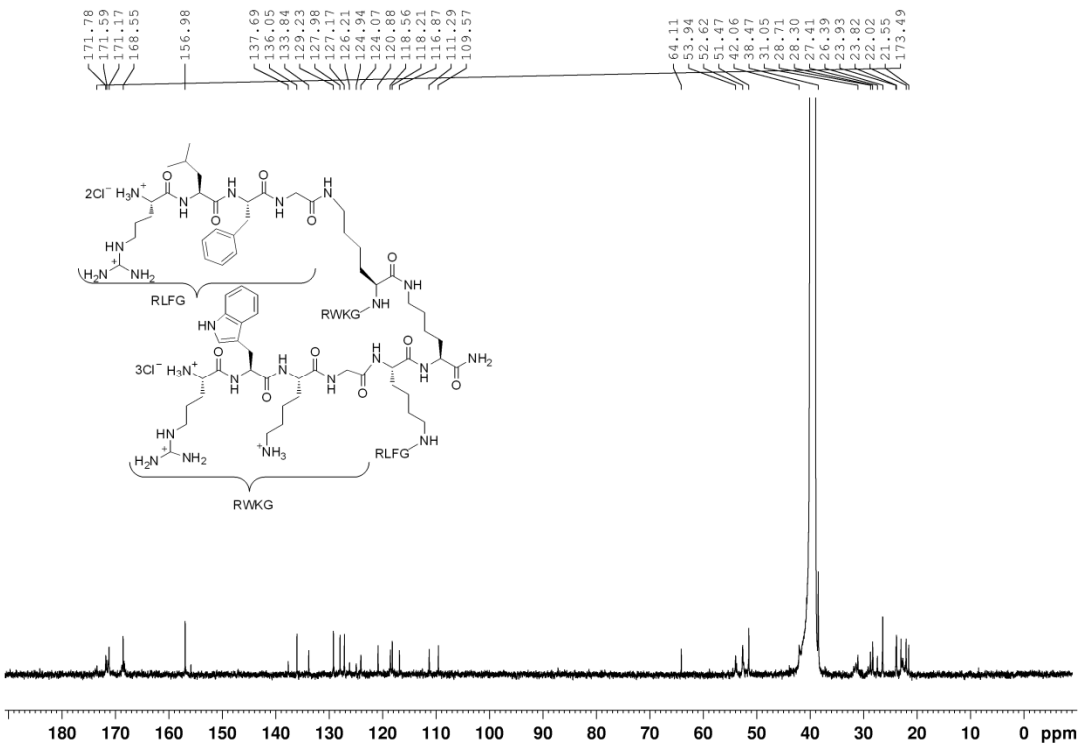
5. ^{1}H and ^{13}C NMR spectra of 1-10 in DMSO-d_6

$(RWKG)_2(RLFG)_2$ (1)

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

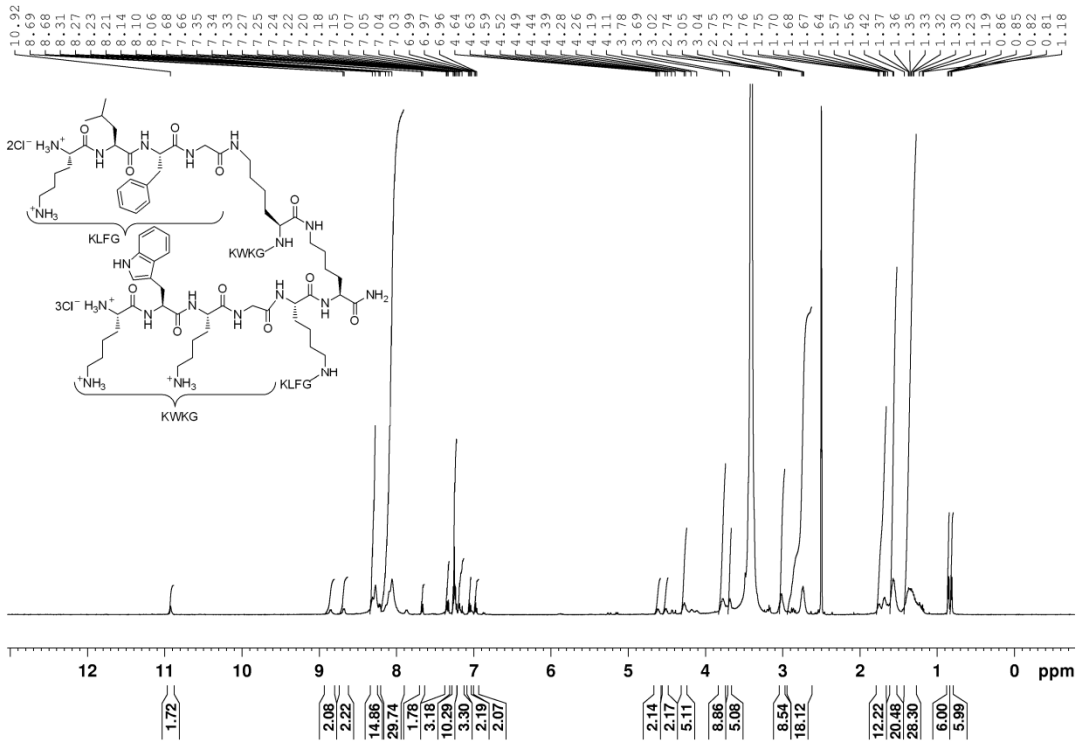


^{13}C NMR (125 MHz, DMSO-d_6)

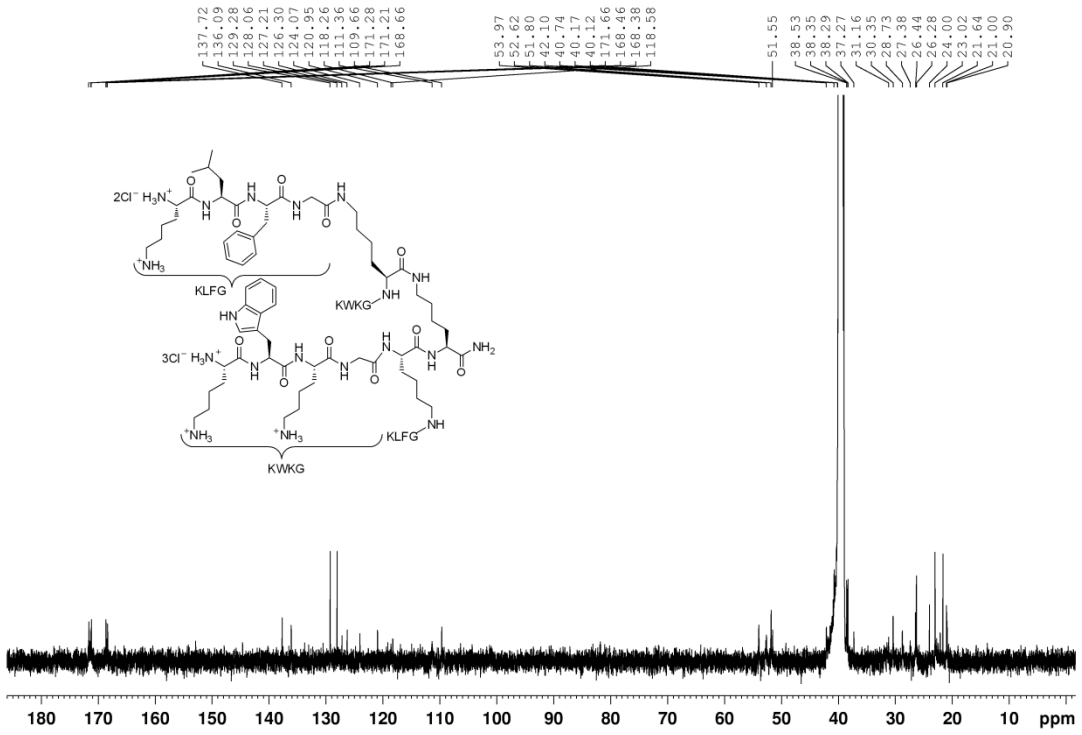


$(KWKG)_2(KLFG)_2$ (2)

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

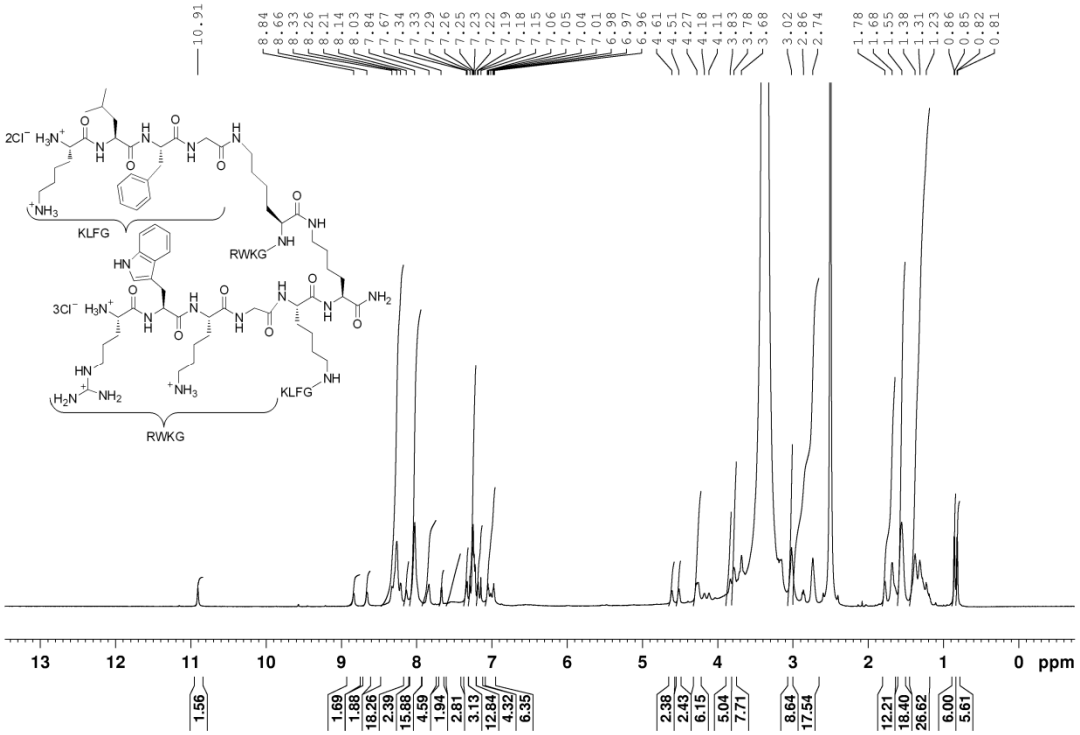


^{13}C NMR (125 MHz, DMSO-d_6)

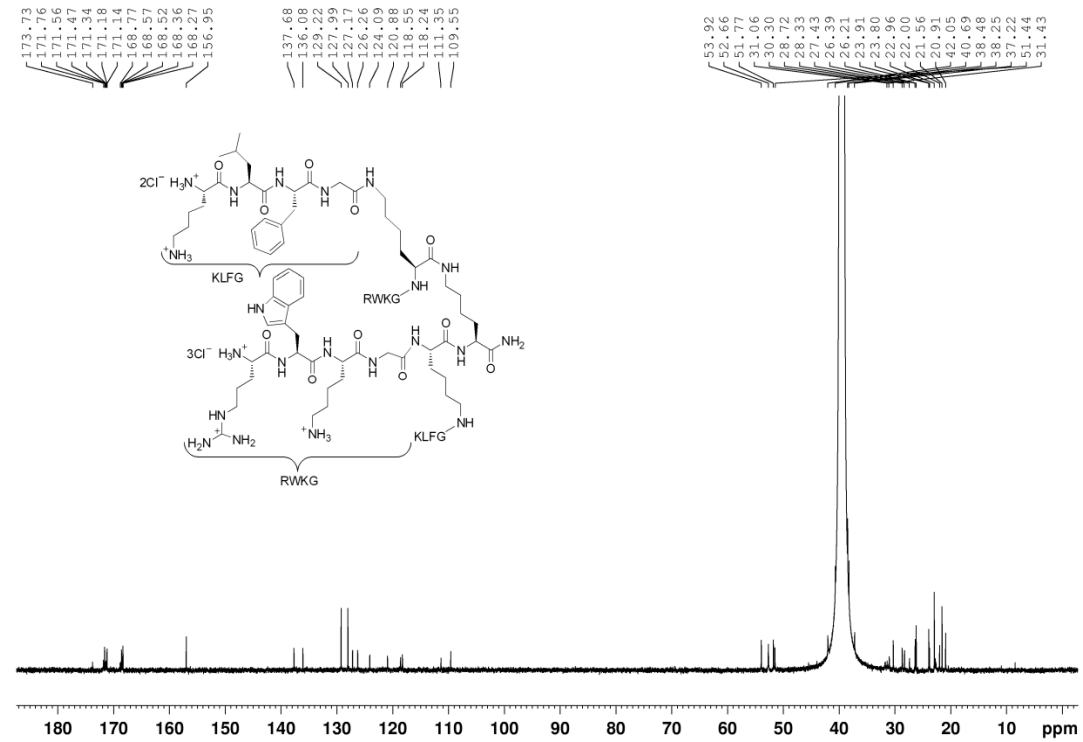


$(RWKG)_2(KLFG)_2$ (3)

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

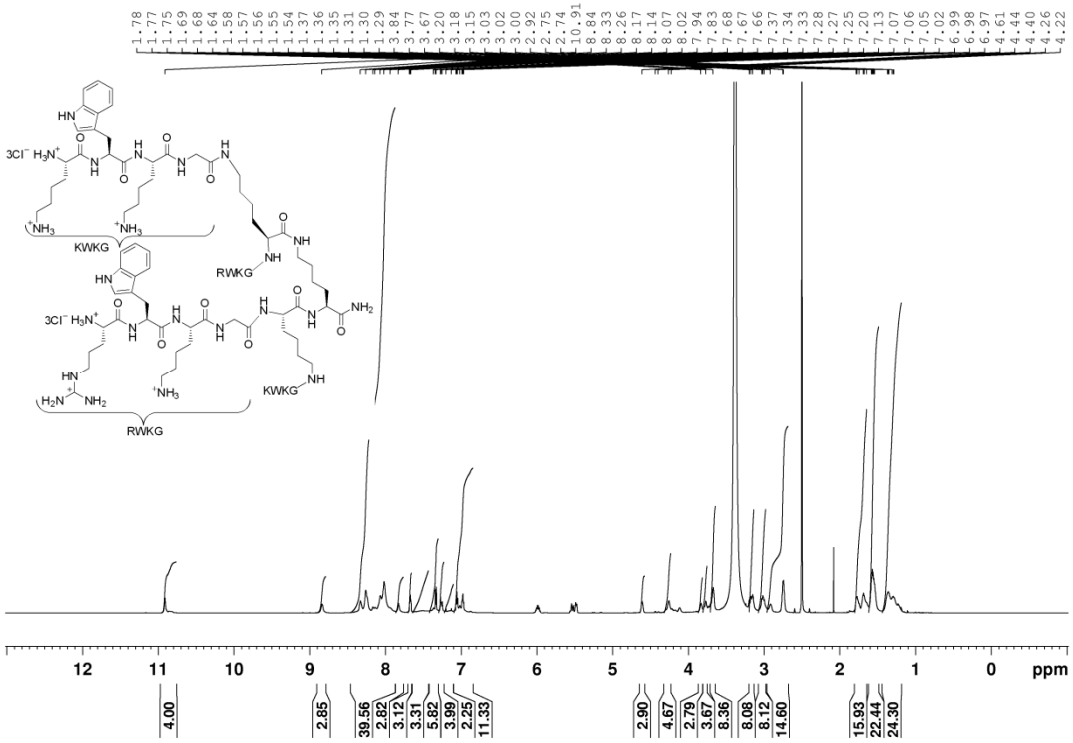


^{13}C NMR (175 MHz, DMSO-d_6)

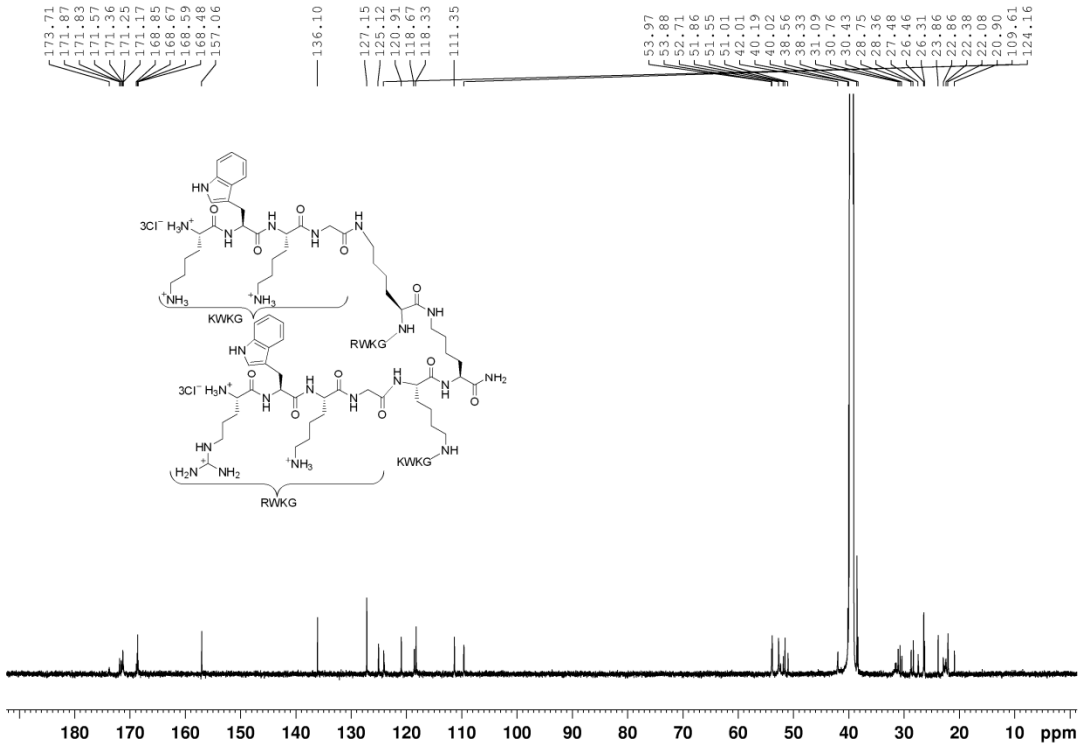


$(RWKG)_2(KWKG)_2$ (4)

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

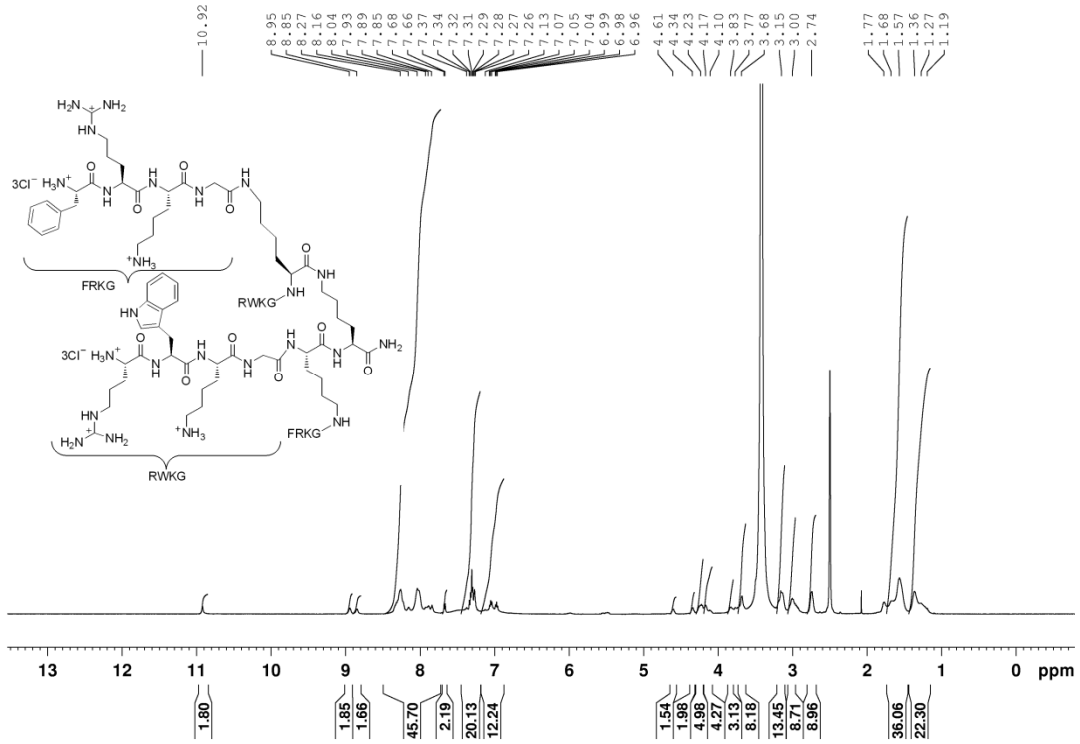


^{13}C NMR (175 MHz, DMSO-d_6)

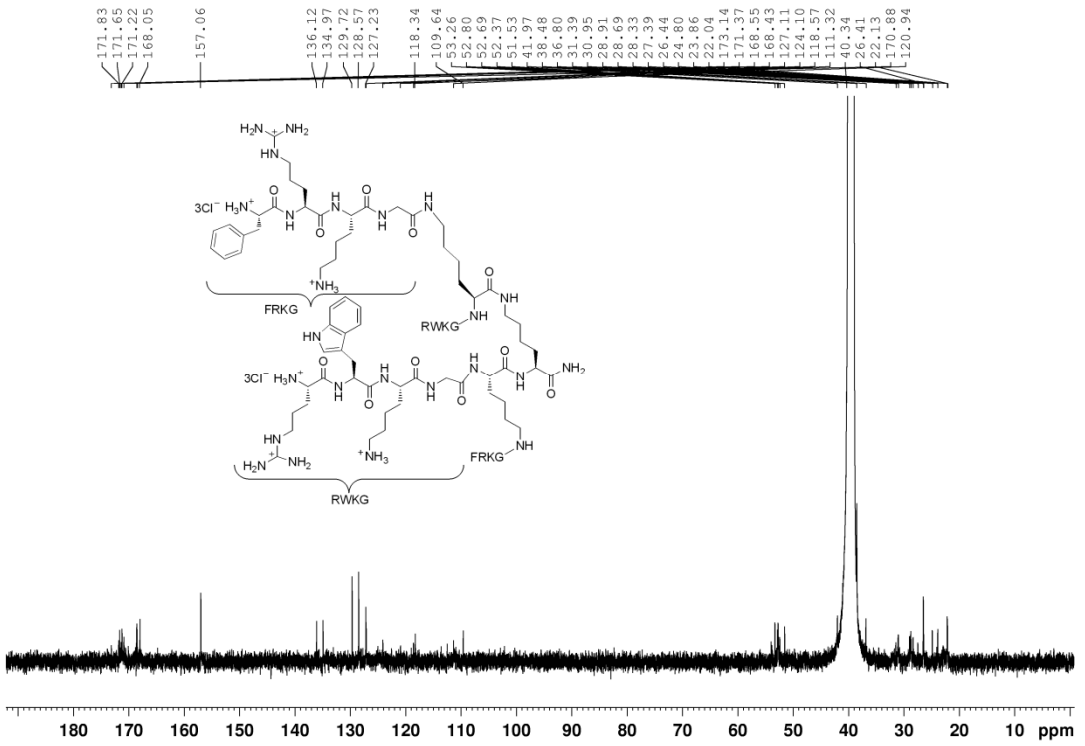


$(RWKG)_2(FRKG)_2$ (5)

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

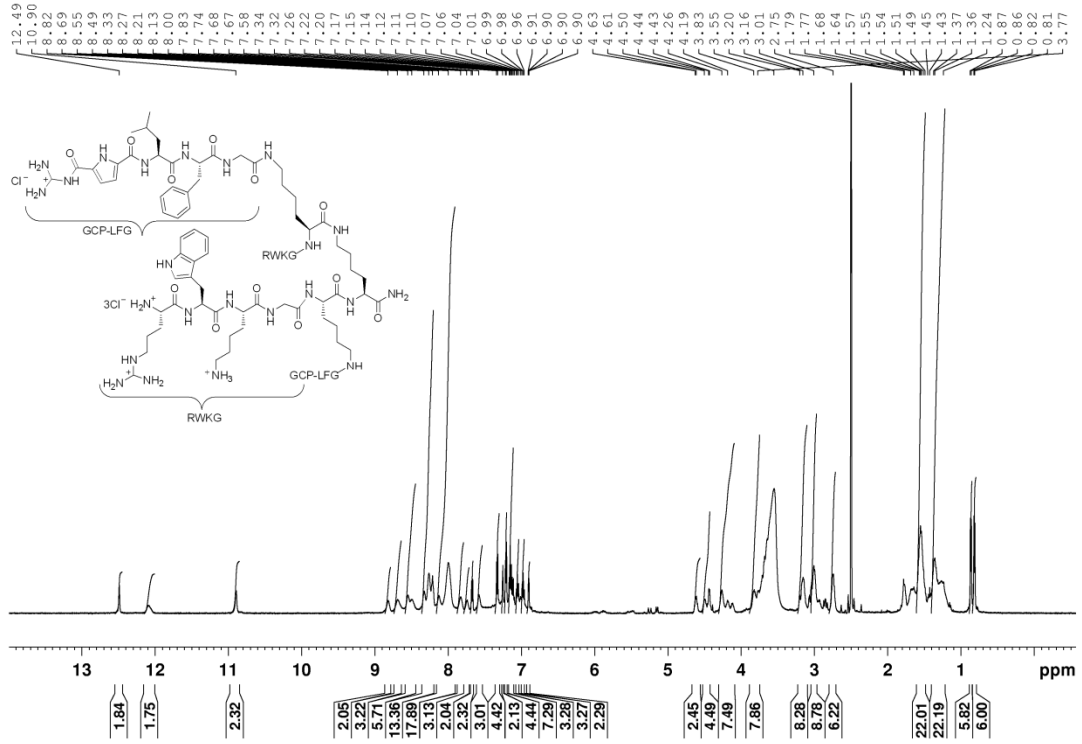


^{13}C NMR (125 MHz, DMSO-d_6)

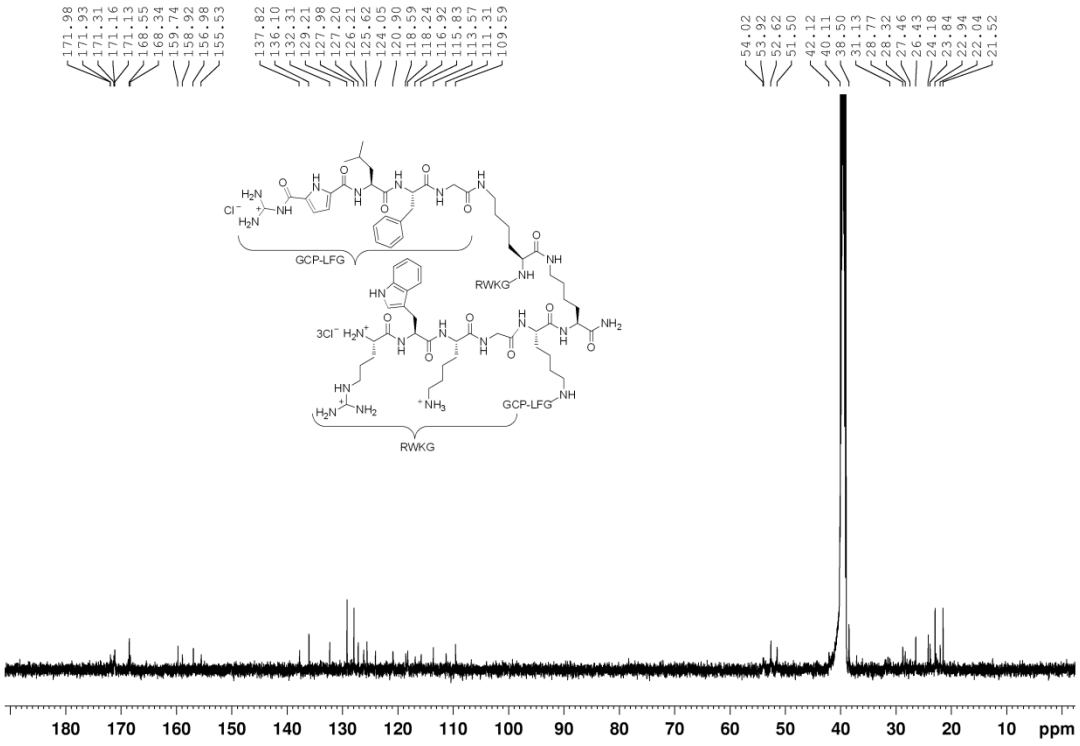


$(RWKG)_2(GCP\text{-}LFG)_2$ (6)

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

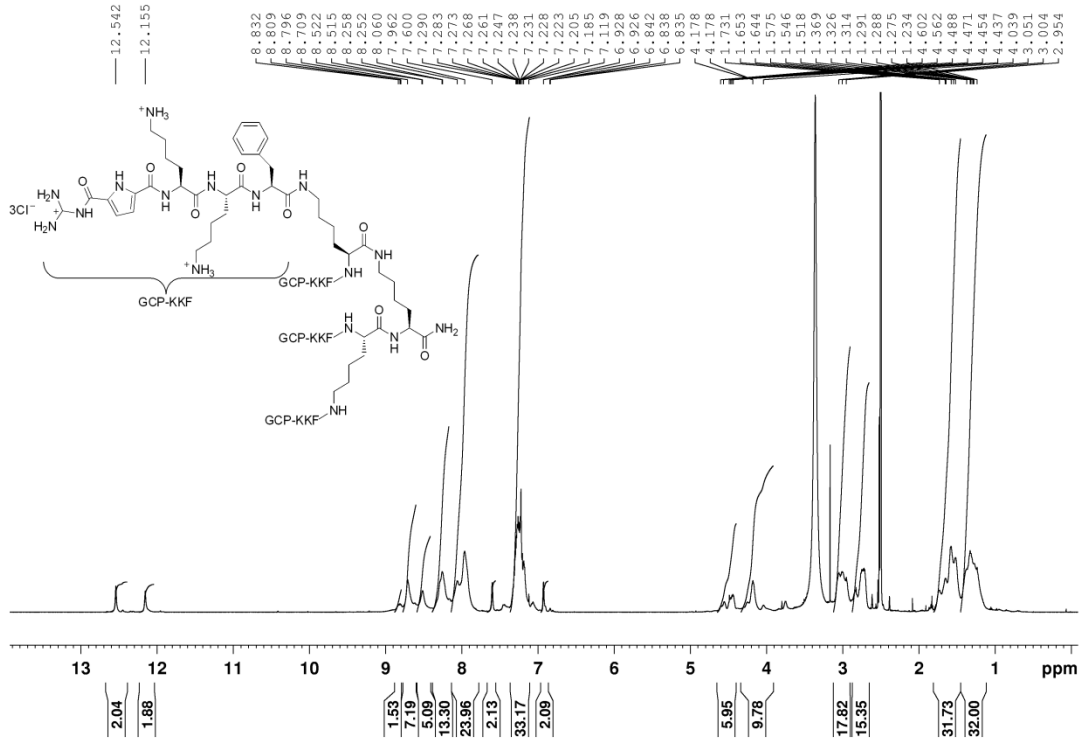


^{13}C NMR (125 MHz, DMSO-d_6)

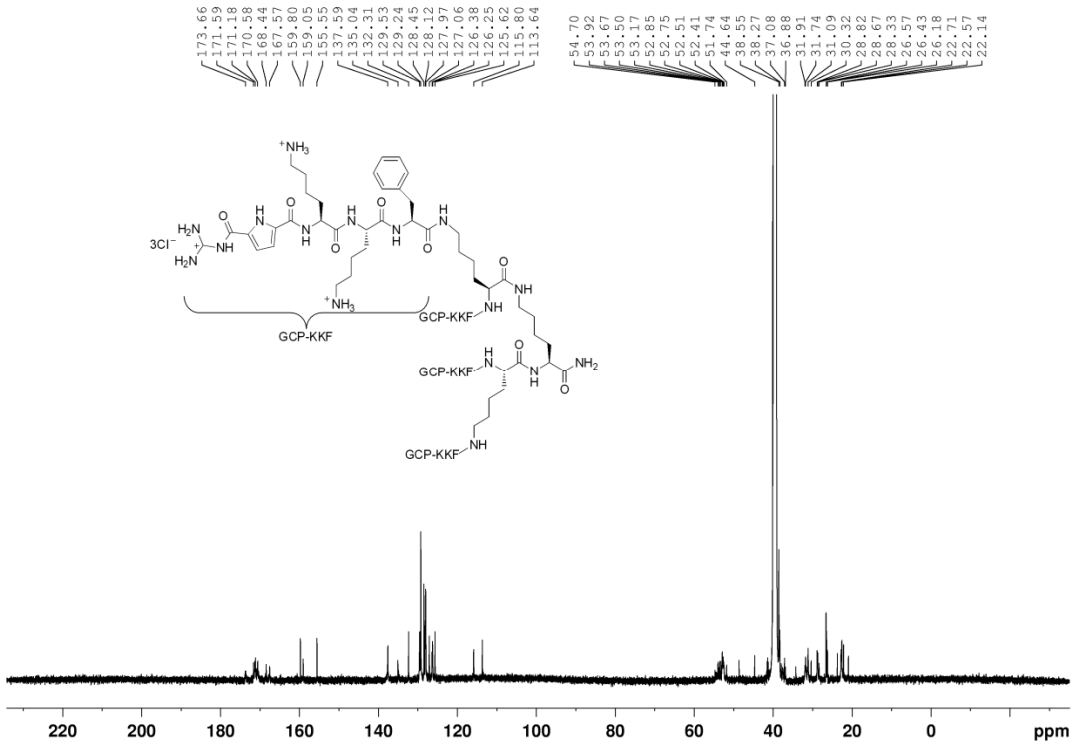


(GCP-KKF)$_4$ (7)

^{1}H NMR (600 MHz, DMSO-d_6)

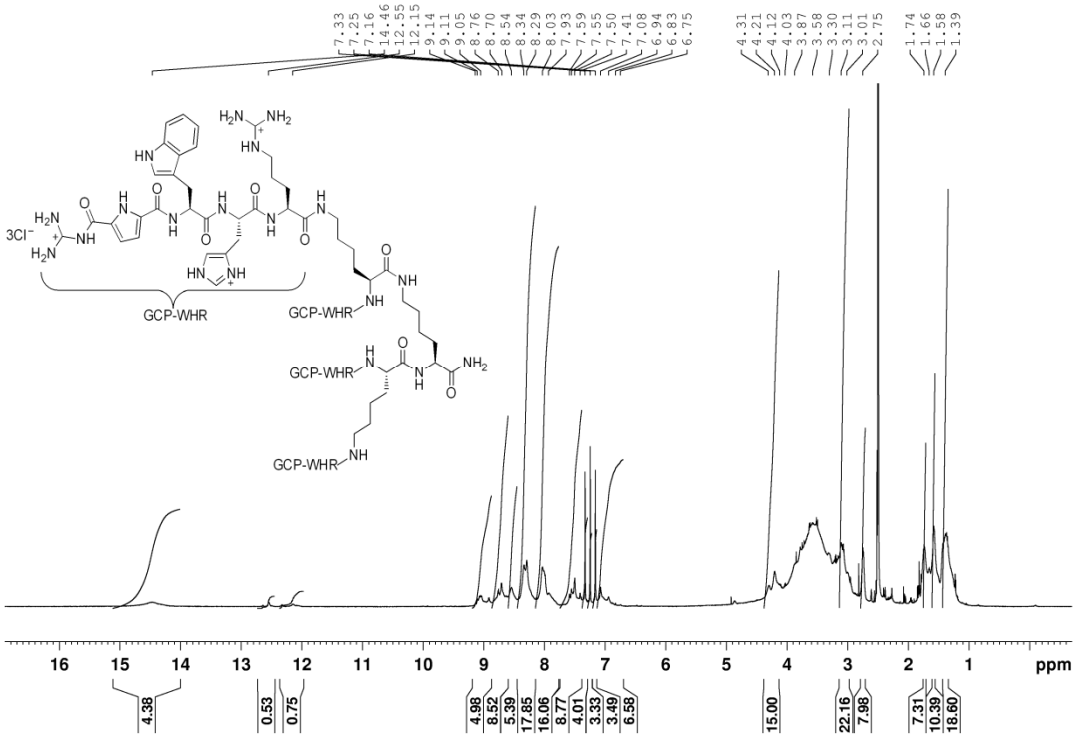


^{13}C NMR (150 MHz, DMSO-d_6)

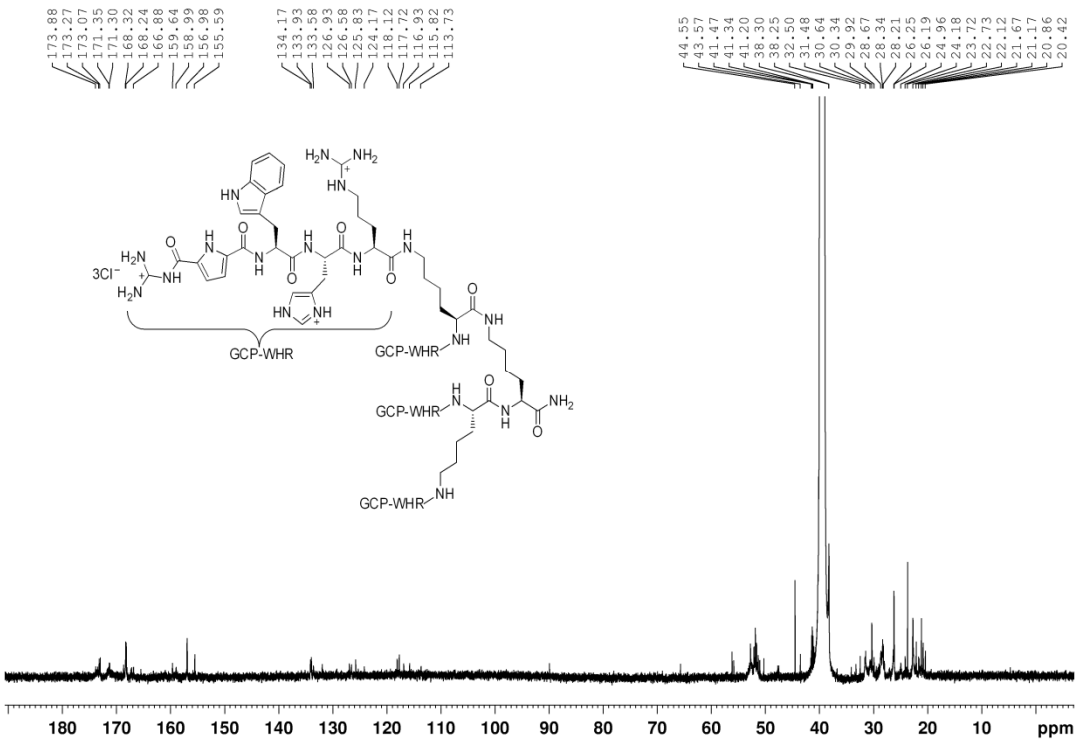


(GCP-WHR)$_4$ (8)

^{1}H NMR (600 MHz, DMSO-d_6)

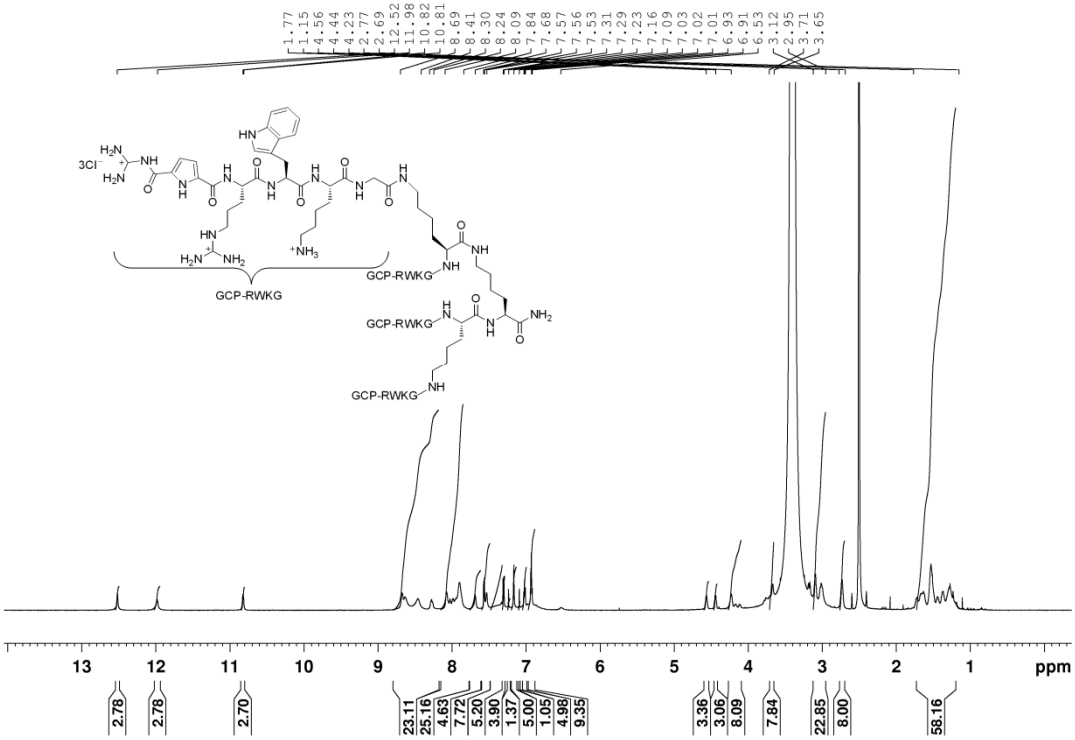


^{13}C NMR (150 MHz, DMSO-d_6)

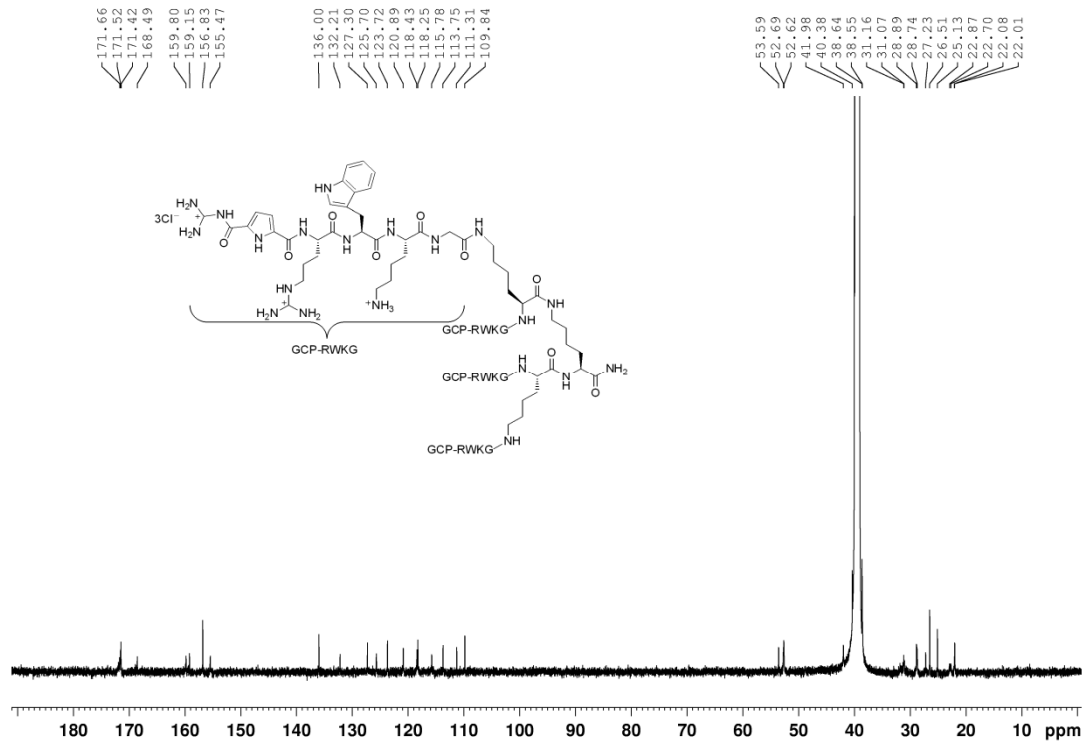


(GCP-RWKG)$_4$ (9)

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

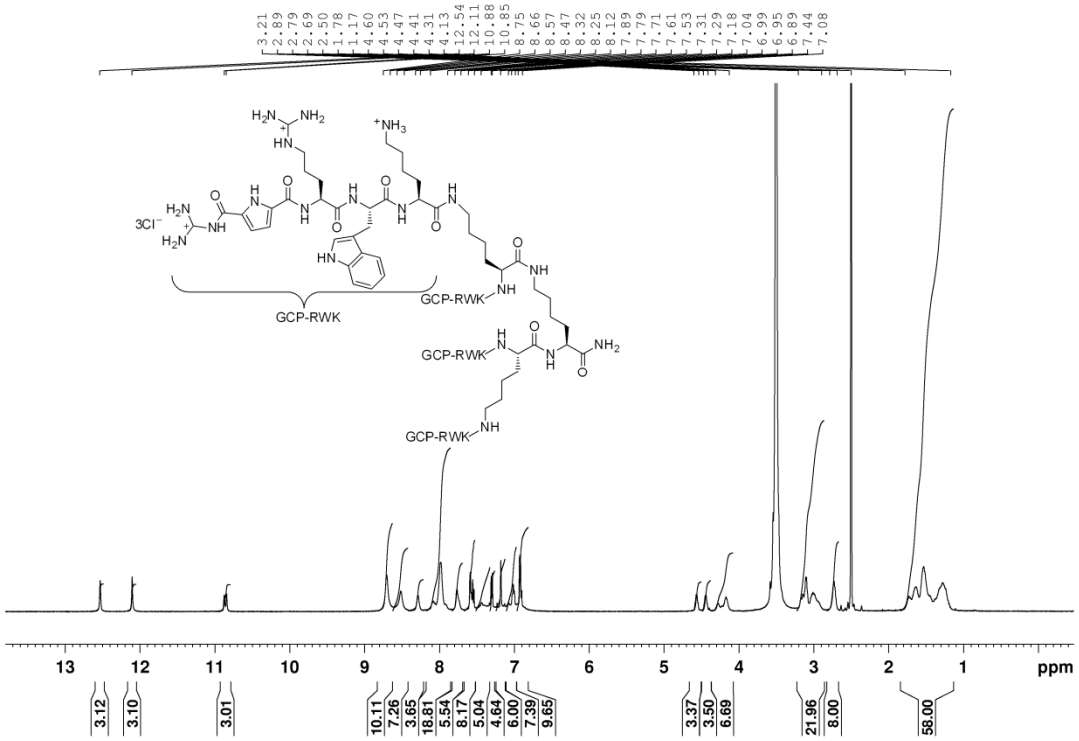


^{13}C NMR (175 MHz, DMSO-d_6)

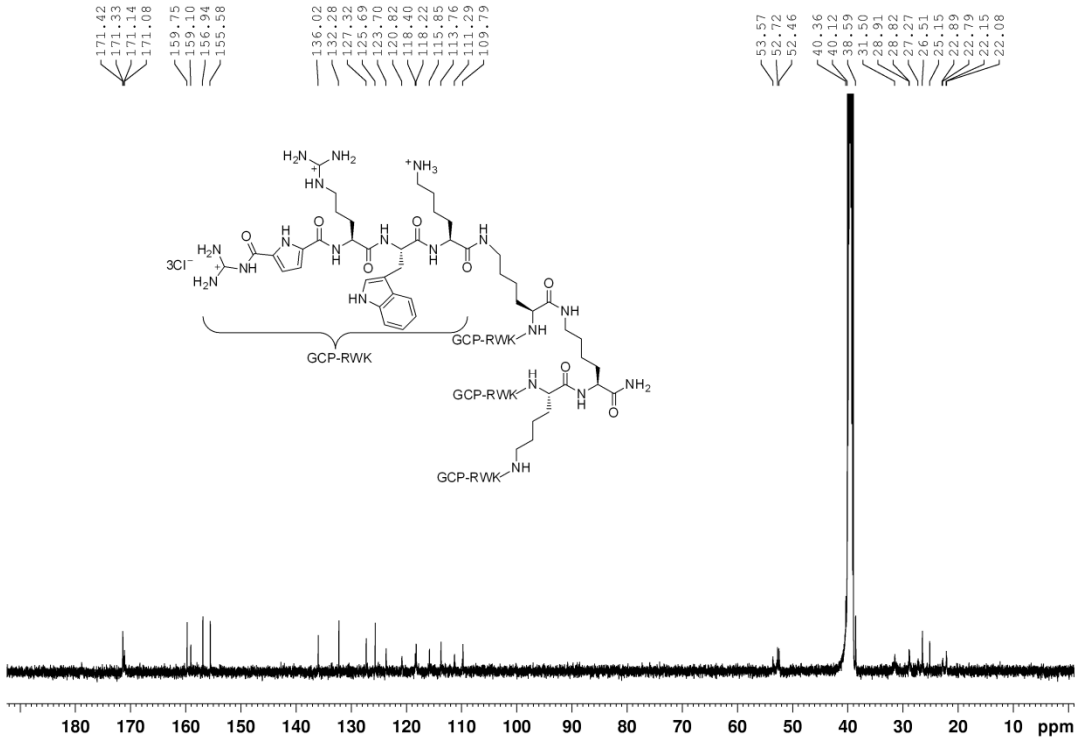


(GCP-RWK)$_4$ (10)

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



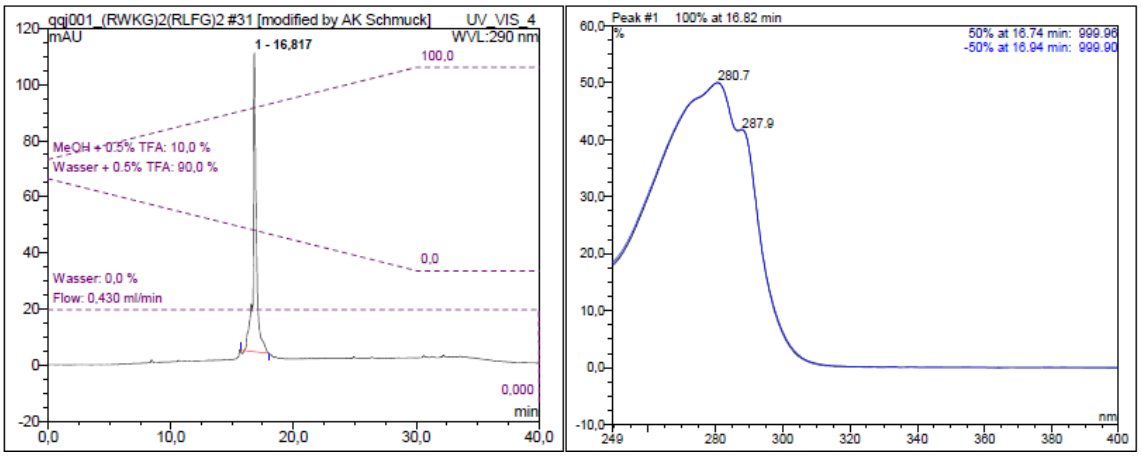
^{13}C NMR (125 MHz, DMSO-d_6)



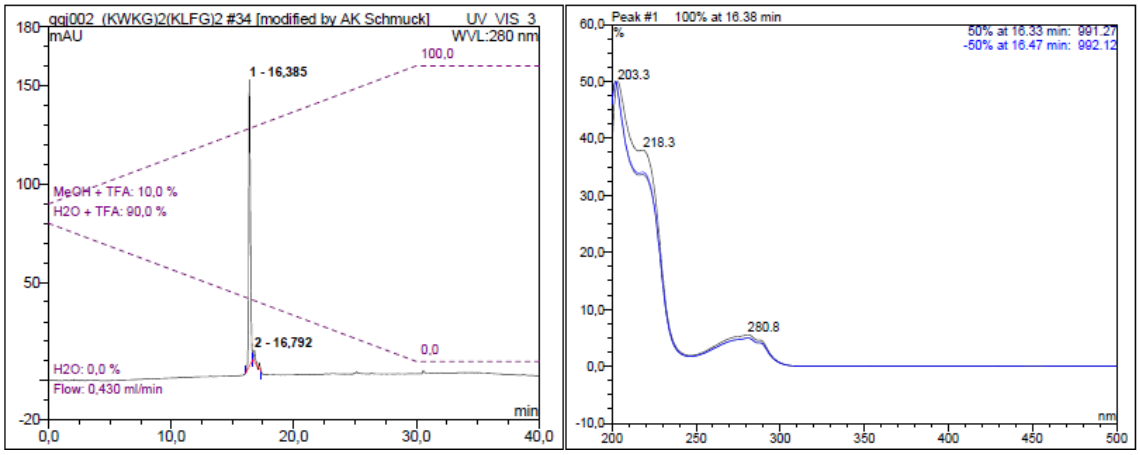
6. Analytical HPLC spectra of 1-10

Analytical HPLC runs were done on a YMC ODS-A column (15 cm × 3.0 mm, 5 µm) using gradient program with solvent mixtures MeOH/H_2O + TFA.

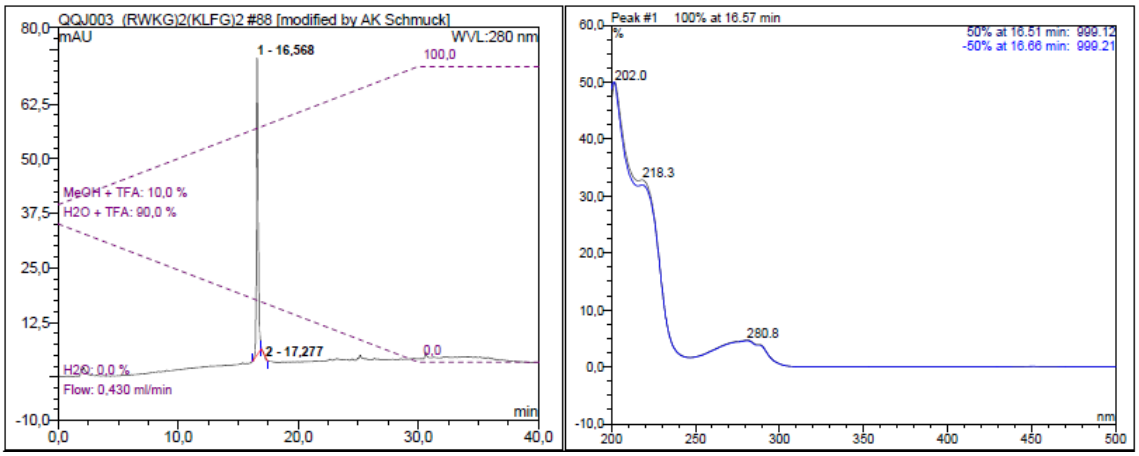
$(RWKG)_2(RLFG)_2$ (1) Retention time t_R = 16.8 min. Solvent: 10% to 100% MeOH/H_2O with 0.05% TFA.



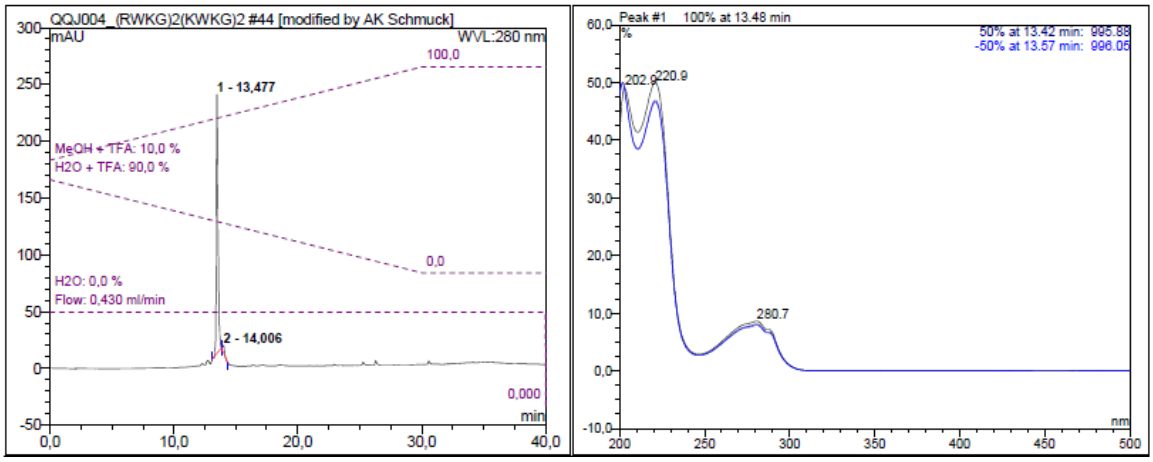
$(KWKG)_2(KLFG)_2$ (2) Retention time t_R = 16.4 min. Solvent: 10% to 100% MeOH/H_2O with 0.05% TFA.



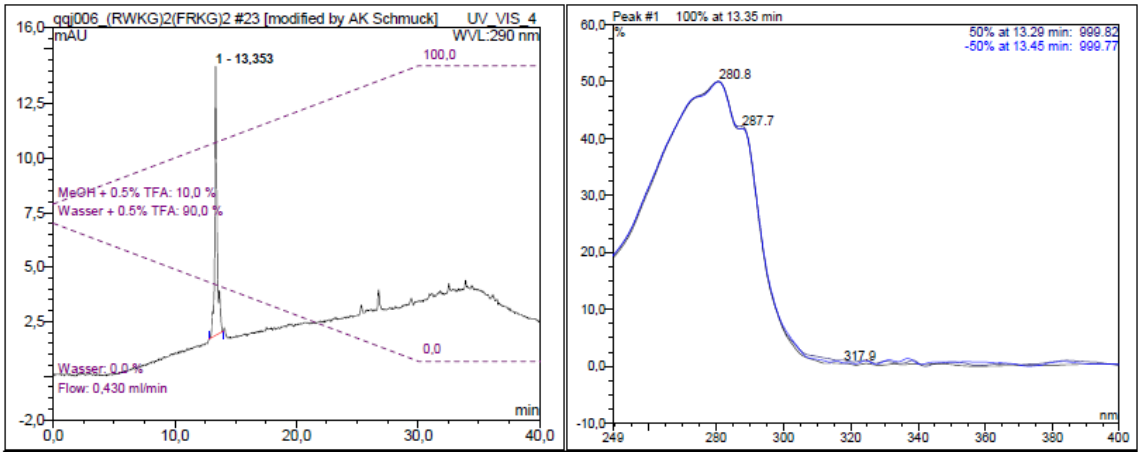
$(RWKG)_2(KLFG)_2$ (3) Retention time t_R = 16.6 min. Solvent: 10% to 100% MeOH/H_2O with 0.05% TFA.



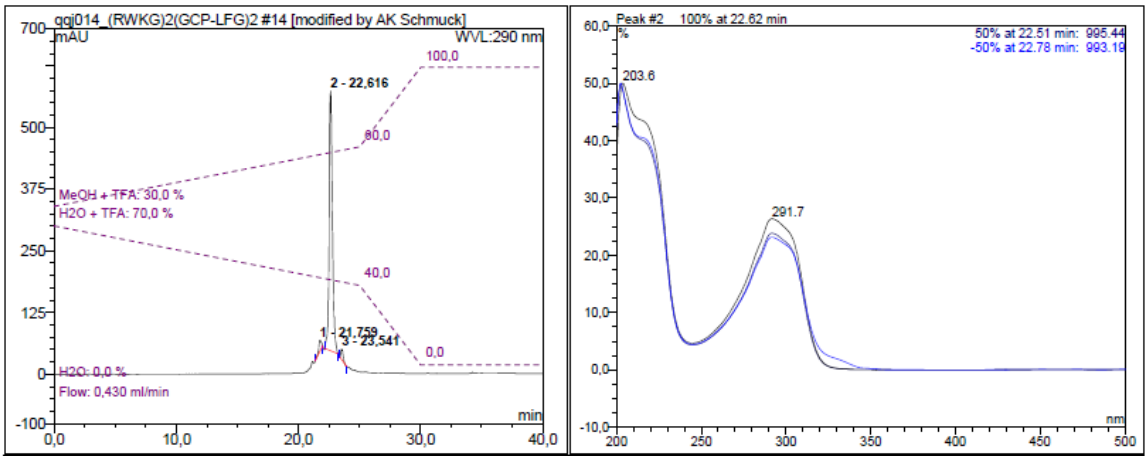
$(RWKG)_2(KWKG)_2$ (4) Retention time t_R = 13.5 min. Solvent: 10% to 100% MeOH/H_2O with 0.05% TFA.



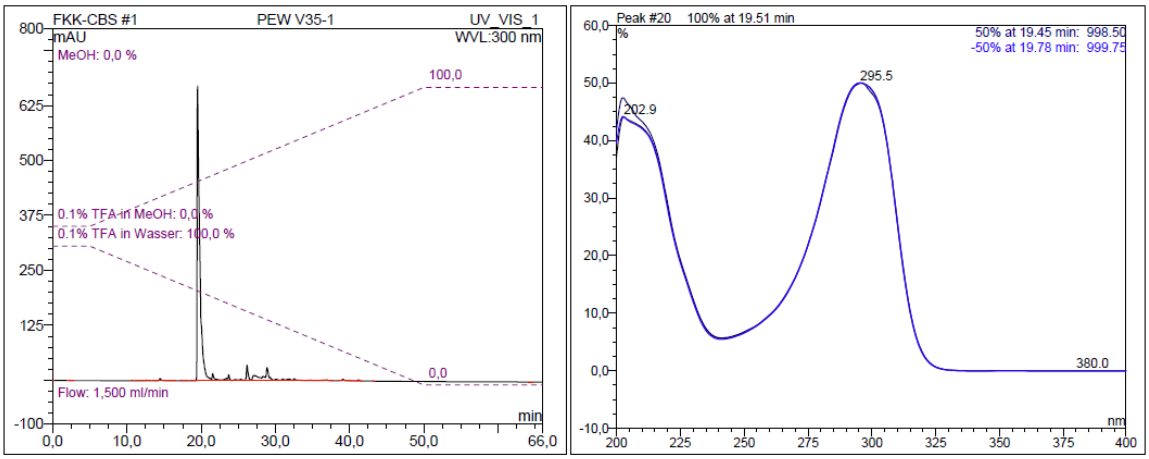
$(RWKG)_2(FRKG)_2$ (5) Retention time t_R = 13.4 min. Solvent: 10% to 100% MeOH/H_2O with 0.05% TFA.



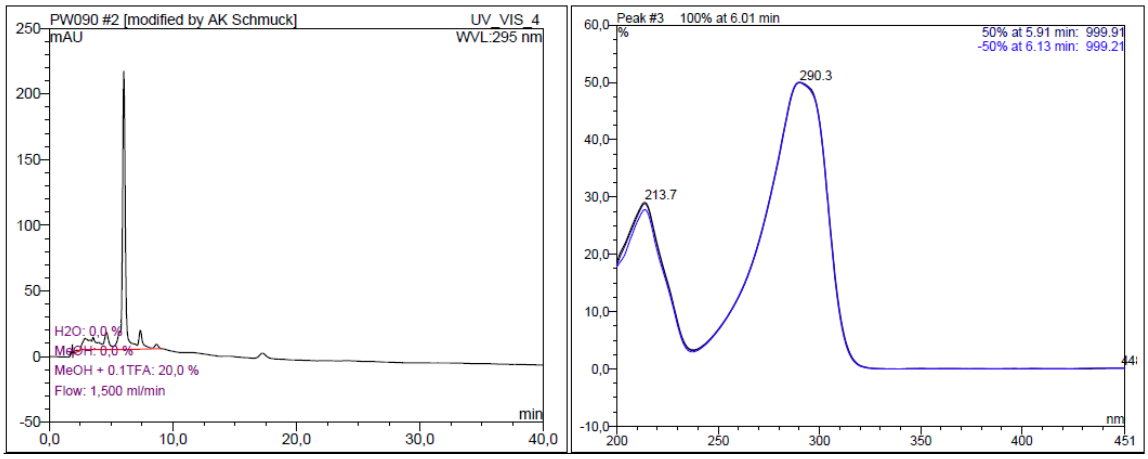
$(RWKG)_2(GCP\text{-}LFG)_2$ (6) Retention time t_R = 22.6 min. Solvent: 30% to 60% MeOH/H_2O with 0.05% TFA.



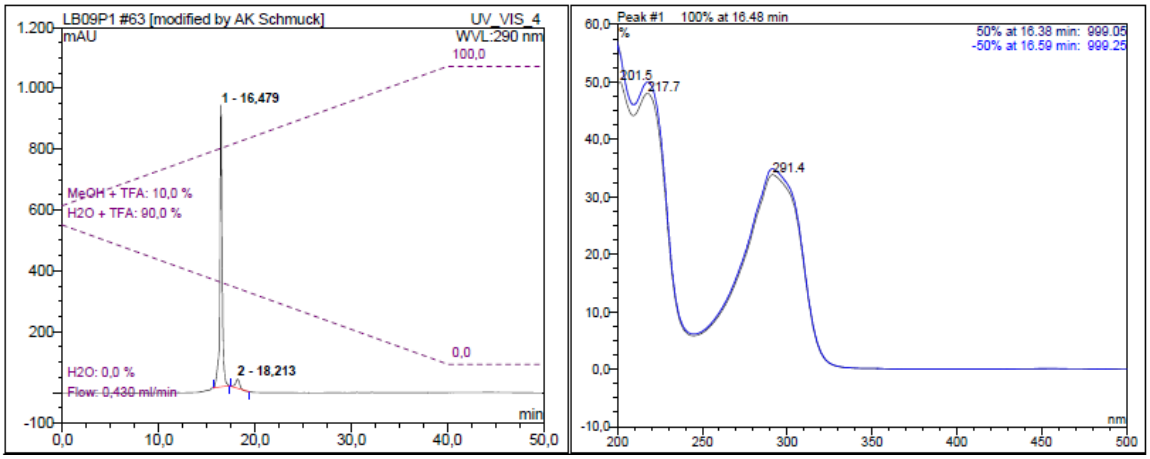
$(GCP\text{-}KKF)_4$ (7) Retention time t_R = 19.5 min. Solvent: 0% to 100% MeOH/H_2O with 0.1% TFA.



$(GCP\text{-}WHR)_4$ (8) Retention time t_R = 6.0 min. Solvent: 20% MeOH/H_2O with 0.1% TFA.



$(GCP\text{-}RWKG)_4$ (9) Retention time t_R = 16.5 min. Solvent: 10% to 100% MeOH/H_2O with 0.05% TFA.



$(GCP\text{-}RWK)_4$ (10) Retention time t_R = 15.9 min. Solvent: 10% to 100% MeOH/H_2O with 0.05% TFA.