Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry.

Incrementally increasing the length of a peptide backbone: effect on macrocyclisation efficiency

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SUPPORTING INFORMATION

Contents:

1. General Methods

All solvents were purchased in the highest available quality and used as supplied. *N*,*N*-Dimethylformamide was stored over molecular sieves prior to synthesis. Water was collected from a Millipore filtration system. Peptide synthesis was carried out in sinter-fitted polypropylene syringes purchased from Torviq, USA. Melting points were determined using a Mel-Temp II device. IR spectra were recorded using a Nicolet FTIR Avatar 380 spectrometer. Optical rotations were measured using a Perkin Elmer model 341 polarimeter (λ = 589 nm; l = 1 dm and c expressed in grams per 100 mL). NMR spectra were obtained at 298 K using a Bruker Avance III 300, 400 or 600 MHz instrument. Where necessary, resonances were assigned using 2D NMR experiments including COSY, NOESY, HMBC and HSQC. High-resolution mass spectra were recorded at the Bioanalytical Mass Spectrometry Facility (UNSW) using an Orbitrap LTQ XL ion trap MS in positive ion mode using an electrospray ionisation (ESI) source. Analytical LCMS was performed using a Shimadzu LC-20AD pump and SPD-M20A PDA detector connected to an LCMS-2010 EV mass spectrometer and a Shimadzu SIL-20A auto sampler. The column was a GRACE VisionHT C18 column (150 mm x 2.1 mm ID). Preparative reversed phase HPLC was performed using a Shimadzu LC20-AD instrument equipped with a SPDM20A PDA detector (254 nm) and GRACE column (150 mm × 22 nm ID). Water containing 0.1% formic acid was used as eluent A and acetonitrile containing 0.1% formic acid was used as eluent B for both analytical and preparative HPLC.

2. Synthetic procedures and characterisation of intermediates

General procedure A: Preparation of resin for SPPS

Wang resin (100–200 mesh) pre-loaded with Fmoc-leucine (0.64 mmol.g^{-1} resin loading) was agitated in DCM for 1 h followed by washing with DMF (3 × 1 min).

General procedure B: Fmoc deprotection

The resin was agitated in a solution of 10% piperidine in DMF (2 × 4 min), then drained and washed with DMF (3 × 1 min), DCM (3 × 1 min) and DMF (5 × 1 min). The deprotection solutions were combined and diluted with 10% piperidine / DMF as appropriate, and the absorbance of the piperidine-fulvene adduct was measured at 301 nm against 10% piperidine / DMF as reference. The resin loading was determined by calculating the concentration of the piperidine-fulvene adduct (ε = 7800 M^{-1} cm^{-1}) in the deprotection solution.

General procedure C: Peptide coupling

A solution was prepared of the appropriate Fmoc-protected amino acid (3 equiv. relative to resin loading) and HBTU (2.9 equiv. relative to resin loading) in minimal DMF. To this solution was added DIPEA (6 equiv. relative to resin loading) and the mixture was immediately added to the resin and agitated for 1.5 h. The resin was drained and washed with DMF (3 × 1 min), DCM (3 × 1 min) and DMF (5 × 1 min).

General procedure D: Cleavage

After the last Fmoc deprotection, the resin was washed with DCM (5 × 1 min) and dried *in vacuo*. The dried resin was agitated in a solution of 90:2.5:2.5 TFA/TIS/H_2O for 2 h. The resin was drained and washed with TFA (2 × 1 min). The combined cleavage solutions were concentrated by evaporation under a stream of N_2 to give a clear glassy solid. Diethyl ether was added and the supernatant decanted

(4×). The residue was dried *in vacuo* to afford the crude linear peptide, which was purified by reversed-phase HPLC eluting with 5–100% acetonitrile / water.

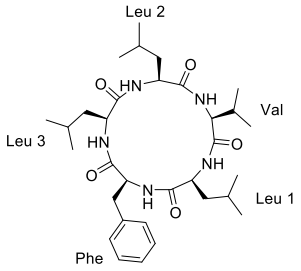
General procedure E: Cyclisation

The appropriate linear precursor peptide was dissolved in minimal DMF. A solution of DMTMM.BF_4 (3 equiv. relative to peptide) in DMF was added *via* cannula to give a final peptide concentration of 5 mM. Immediately, DIPEA (3.6 equiv. relative to peptide) was added. At regular time intervals thereafter, a 4 μL aliquot was withdrawn from the reaction mixture, diluted to 80 μL by addition of 1% TFA/MeCN, and directly analysed by LC-MS. When the cyclisation reaction was complete, the reaction mixture was concentrated and the crude product was purified by preparative HPLC. The appropriate fraction was freeze-dried to yield the target cyclic peptide.

General procedure F: Competition reaction

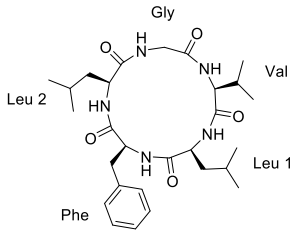
The appropriate linear precursor peptide and PheOMe (10 equiv. relative to peptide) were dissolved in minimal DMF. A solution of DMTMM.BF_4 (3 equiv. relative to peptide) in DMF was added *via* cannula to give a final peptide concentration of 5 mM. Immediately, DIPEA (10 equiv. relative to peptide) was added. After 3 hours, an aliquot (4 μL) was withdrawn from the reaction mixture, diluted to 80 μL by addition of 0.1% TFA/MeCN, and directly analysed by LCMS. The reaction mixture was concentrated and subjected to preparative HPLC in order to obtain a sufficient quantity of the linear hexapeptide for characterisation.

Cyclic peptide 2



The title compound was synthesised according to a literature procedure [*Chem. Commun*.2013, 49, 6430] on 0.025 mmol scale and the final reaction concentration was 0.5 mM. The crude reaction mixture was purified by preparative HPLC and the product was obtained as a white powder after freeze drying (1.4 mg, 9.6%); **m.p.** >200 °C; **[α]$_D$** +65 (*c* 0.016, DMF); **IR** (neat) ν_{max} (cm^{-1}) 3290, 2954, 1647, 1538, 1453, 1384, 1147; **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.64 (d, *J* = 7.0 Hz, 1H), 7.30–7.14 (m, 5H), 6.92 (bs, 1H), 6.53 (bs, 1H), 4.62 (dd, *J* = 14.9, 7.6 Hz, 1H), 4.26–4.17 (m, 3H), 3.89 (dd, *J* = 15.2, 6.8 Hz, 1H), 3.15 (dd, *J* = 14.0, 6.5 Hz, 1H), 2.94 (dd, *J* = 13.9, 8.5 Hz, 1H), 2.30–2.17 (m, 2H), 2.1 (m, 1H), 2.06–1.98 (m, 2H), 1.90–1.83 (m, 2H), 1.45–1.30 (m, 5H) 0.97–0.77 (m, 24H); data in accordance with literature values. **MS** (ESI, + ve) *m/z* 586 (MH^+, 100%); **HRMS** (ESI, +ve) $C_{32}H_{51}N_5NaO_5^+$ [MNa^+] requires *m/z* 608.3782, found 608.3779, (ESI, +ve) $C_{32}H_{52}N_5O_5^+$ [MH^+] requires *m/z* 586.3963, found 586.3964.

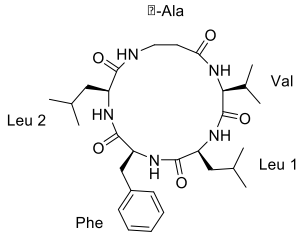
Cyclic peptide 3



The title compound was synthesised from compound **8** according to General Procedure E on 0.028 mmol scale. The product was obtained as white fluffy solid (6.2 mg, 43% yield); **m.p.** > 200 °C; **[α]$_D$** –133 (*c* 0.075, DMF); **IR** (neat) ν_{max} (cm^{-1}) 3310, 2955, 1651,1523, 1275, 1079; **^{1}H NMR** (600 MHz, DMSO-d_6) δ 8.34 (t, *J* = 5.5 Hz, 1H, Gly NH), 8.26 (d, *J* = 7.1 Hz, 1H, Phe NH), 8.11 (d, *J* = 7.9 Hz, 1H, Leu#2 NH), 7.96 (d, *J* = 8.1 Hz 1H, Val NH), 7.76 (d, *J* = 8.4 Hz 1H, Leu#1 NH), 7.27–

7.18 (m, 5H, Phe ArH), 4.21–4.16 (m, 2H, Leu#2 and Phe αH), 4.13 (dd, *J* = 15.7, 8.9 Hz, Leu#1 αH), 3.99 (dd, *J* = 14.4, 5.6 Hz, 1H, Gly αH), 3.89 (dd, *J* = 15.4, 7.9 Hz, 1H, Val αH), 3.43 (dd, *J* = 14.4, 5.6 Hz, 1H, Gly αH), 3.08 (dd, *J* = 13.6, 9.3 Hz, 1H, Phe βH), 3.02 (dd, *J* = 13.6, 6.6 Hz, 1H, Phe βH′), 2.00 (m, 1H, Val βH), 1.57 (m, 1H, Leu#2 βH), 1.54–1.42 (m, 3H, Leu#2 βH′, Leu#1 βH, Leu#1 γH), 1.36 (m, 1H Leu#1 βH′), 1.31 (m, 1H Leu#2 γH). 0.88–0.79 (m, 18H, Leu#1, 2 δH, Val γH); **^{13}C {^{1}H} NMR** (600 MHz, DMSO-*d*$_6$) δ 172.1 (Leu#2 C=O), 171.2 (Leu#2 C=O), 170.7 (Phe C=O), 170.3 (Val C=O), 169.5 (Gly C=O), 137.5 (Phe Ar C1), 129.0 (Phe Ar C2/C3), 128.1 (Phe Ar C4/C5), 126.4 (Phe Ar C6), 60.1 (Val αC), 56.9 (Phe αC), 55.7 (Leu#1 αC), 52.5 (Leu#2 αC), 43.2 (Gly αC), 40.4 (Leu#2 βC), 39.8 (Leu#1 βC), 36.4 (Phe βC), 29.5 (Val βC), 24.4 (Leu#1 γC), 24.3 (Leu#2 γC), 22.9 (Leu#1 δC or δC′ or Leu#2 δC or δC′), 22.5 (Leu#1 δC or δC′ or Leu#2 δC or δC′), 21.9 (Leu#1 δC or δC′ or Leu#2 δC or δC′), 21.7 (Leu#1 δC or δC′ or Leu#2 δC or δC′), 19.17 (Val γC or γC′), 18.16 (Val γC or γC′); **MS** (ESI, + ve) *m/z* 530 (MH^+, 100%); **HRMS** (ESI, +ve) $C_{28}H_{43}N_5NaO_5^+$ [MNa^+] requires *m/z* 552.3156, found 552.3152.

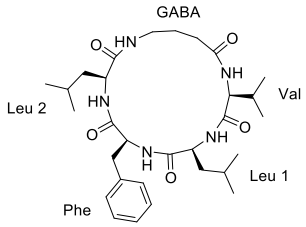
Cyclic peptide 4



Compound **4** was synthesised from compound **9** according to General Procedure E on 0.034 mmol scale. The product was obtained as a white fluffy solid (15.7 mg, 84% yield); **m.p.** > 200 °C; **[α]$_D$** –73 (*c* 0.041, DMF); **IR** (neat) ν_{max} (cm^{-1}) 3418, 3356, 3280, 2948, 1651, 1512, 1254; **^{1}H NMR** (600 MHz, DMSO-*d*$_6$) δ 8.43 (d, *J* = 7.4 Hz, 1H, Leu#1 NH), 8.11 (d, *J* = 6.4 Hz, 1H, Phe NH), 8.07 (d, *J* = 7.9 Hz, 1H, Leu#2 NH), 7.73 (d, *J* = 7.9 Hz, 1H, Val NH), 7.28–7.18 (m, 6H, Phe Ar H and β-Ala NH), 4.13 (dd, *J* = 14.9, 7.6 Hz, 1H, Phe αH), 4.04 (m, 1H, Leu#2 αH), 3.93 (dd, *J* = 13.5, 6.3 Hz, 1H Val αH), 3.81 (t, *J* = 11.2, 1H, Leu#1 αH), 3.53 (m, 1H, β-Ala βH), 3.09 (ddd, *J* = 20.4, 13.9, 7.6 Hz, 2H, Phe βH and βH′), 2.99 (m, 1H, β-Ala βH′), 2.46 (m, 1H, β-Ala αH), 2.14 (m, 1H, β-Ala αH′), 1.97 (m, 1H, Val βH), 1.69–1.56 (m, 3H, Leu#2 βH and βH′ and Leu#1 βH), 1.48–1.37 (m, 2H, Leu#2 γH and Leu#1 γH), 1.36–1.31 (m, 1H, Leu#1 βH′), 0.87–0.78 (m, 18H, Leu#1 δH, Leu#2 δH

and Val γH); **^{13}C {^{1}H} NMR** (600 MHz, DMSO-d_6) δ 172.0 (Leu#1 C=O), 171.6 (Val C=O), 171.57 (β-Ala C=O), 171.2 (Leu#2 C=O), 170.2 (Phe C=O), 138.1 (Phe Ar C1), 129.0 (Phe Ar C2/C3), 128.1 (Phe Ar C4/C5), 126.3 (Phe Ar C6), 59.3 (Val αC), 56.6 (Phe αC), 52.6 (Leu#1 αC), 51.7 (Leu#2 αC), 39.3 (Leu#2 βC), 39.0 (Leu#1 βC), 35.8 (Phe βC), 35.7 (β-Ala βC), 34.8 (β-Ala αC), 29.2 (Val βC), 24.2 (Leu#1 γC), 24.0 (Leu#2 γC), 23.2 (Leu#1 δC or δC′ or Leu#2 δC or δC′), 23.1 (Leu#1 δC or δC′ or Leu#2 δC or δC′), 21.4 (Leu#1 δC or δC′ or Leu#2 δC or δC′), 21.2 (Leu#1 δC or δC′ or Leu#2 δC or δC′), 19.1 (Val γC or γC′), 18.2 (Val γC or γC′); **MS** (ESI, + ve) *m/z* 544 (MH^+, 100%), 566 (MNa^+, 10%); **HRMS** (ESI, +ve) $C_{29}H_{45}N_5NaO_5^+$ [MNa^+] requires *m/z* 566.3313, found 566.3318.

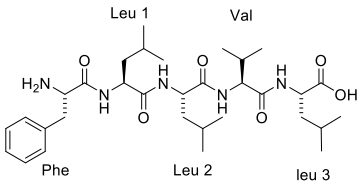
Cyclic peptide 5



The title compound was synthesised from compound **10** according to General Procedure E on 0.016 mmol scale. The product was obtained as a white fluffy solid (5.7 mg, 64% yield); **m.p.** > 200 °C; **[α]$_D$** –92 (*c* 0.087, DMF); **IR** (neat) ν_{max} (cm^{-1}) 3272, 2954, 1708, 1650, 1521, 1450, 1391; **^{1}H NMR** (600 MHz, DMSO-d_6) δ 8.23 (d, *J* = 6.9 Hz, 1H, Leu#1 NH), 8.14 (s, 1H, Phe NH), 8.08 (d, *J* = 7.4Hz, 1H, Leu#2 NH), 7.90 (d, *J* = 6.6 Hz, 1H, Val NH), 7.28–7.1 (m, 5H, Phe ArH), 7.12 (t, *J* = 4.8 Hz, 1H, GABA NH), 4.07 (dd, *J* = 14.5,7.2 Hz, 1H, Phe αH), 3.99–3.91 (m, 3H, Leu#2, Val and Leu#1 αH), 3.22–3.17 (m, 3H, GABA γH and Phe βH and βH′), 2.88 (m, 1H, GABA γH′), 2.31 (m, 1H, GABA αH), 2.14 (m, 1H, GABA αH′), 1.98 (m,1H, Val βH), 1.76–1.68 (m, 2H, GABA βH and βH′), 1.56–1.49 (m, 3H, Leu#2 βH and βH′ and γH), 1.4–1.34 (m, 3H, Leu#1 βH and βH′ and γH), 0.87–0.77 (m, 18H, Leu#1 δH and Leu#2 δH and Val γH); **^{13}C {^{1}H} NMR** (600 MHz, DMSO-d_6) δ 172.7 (GABA C=O), 171.8 (Leu#1 C=O), 171.7 (Leu#2 C=O), 171.3 (Val C=O), 170.4 (Phe C=O), 138.3 (Phe Ar C1), 129.1 (Phe Ar C2/C3), 128.1 (Phe Ar C4/C5), 126.2 (Phe Ar C6), 59.4 (Val αC), 56.4 (Phe αC), 52.3 (Leu#2 αC), 52.1 (Leu#1 αC), 39.8 (Leu#2 βC), 38.9 (Leu#1 βC), 38.3 (GABA γC), 35.1 (Phe βC), 32.2 (GABA αC), 29.7 (VAL βC), 24.2 (Leu#1 γC), 24.1 (Leu#2 γC), 23.3 (GABA βC), 23.2 (Leu#1 δC or δC′ or Leu#2 δC or δC′), 22.9 (Leu#1 δC or δC′ or Leu#2 δC or δC′),

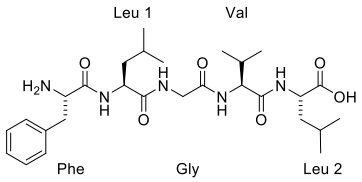
21.5 (Leu#1 δC or δC′ or Leu#2 δC or δC′), 21.0 (Leu#1 δC or δC′ or Leu#2 δC or δC′), 19.1 (Val γC or γC′), 18.2 (Val γC or γC′); **MS** (ESI, + ve) *m/z* 558 (MH^+, 100%), 580 (MNa^+, 10%); **HRMS** (ESI, +ve) $C_{30}H_{47}N_5NaO_5^+$ [MNa^+] requires *m/z* 580.3469, found 580.3468.

Linear peptide 7



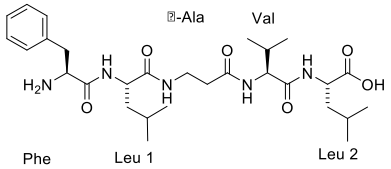
The title compound was prepared by SPPS according to General Procedures A–D on 0.1 mmol scale. The product was obtained as a flaky off-white solid (75 mg, 100%); **m.p.** > 200 °C; **$[\alpha]_D$** –9.8 (*c* 0.102, DMF); **IR** (neat) ν_{max} (cm^{-1}) 3262, 3082, 2955, 1621, 1542, 1416, 1200, 1136; **^{1}H NMR** (300 MHz, DMSO-d_6) δ 8.65 (d, *J* = 8.3 Hz, 1H, Leu#1 NH), 8.28 (d, *J* = 8.1 Hz, 1H, Leu#2 or Leu #3 NH), 8.12 (d, *J* = 7.9 Hz, 1H, Leu#2 or Leu #3 NH), 7.65 (d, *J* = 9.2 Hz 1H, Val NH), 7.33–7.23 (m, 5H, Phe ArH), 4.45–4.32 (m, 2H, Leu#1, 2 or 3 αH), 4.24–4.14 (m, 2H, Leu#2 or 3 and Val αH), 4.05(dd, *J* = 7.0, 5.0 Hz, 1H, Phe αH), 3.09 (dd, *J* = 14.2, 4.8 Hz, 1H, Phe βH), 2.90 (dd, *J* = 14.2, 7.6 Hz, 1H, Phe βH′), 1.94 (m, 1H, Val βH), 1.68–1.40 (m, 9H, Leu#1,2 and 3 6β and 3γH), 0.96–0.72 (m, 24H, Leu#1 δH, Leu#2 δH, Leu#3 δH, Val γH); **^{13}C {^{1}H} NMR** (75 MHz, DMSO-d_6) δ 173.8 (Leu#3 C=O), 171.4 (Leu#2 C=O), 171.2 (Leu#1 C=O), 170.7 (Val C=O), 167.6 (Phe C=O), 134.7 (Phe Ar C1), 129.6 (Phe Ar C2/C3), 128.5 (Phe Ar C4/C5), 127.1 (Phe Ar C6), 57.2 (Val αC), 53.1 (Phe αC), 51.2 (Leu#1 or Leu#2 αC), 51.0 (Leu#1 or Leu#2 αC), 50.0 (Leu#3 αC), 41.3 (Leu#1 or Leu#2 or Leu#3 βC), 40.4 (Leu #1 or Leu#2,or Leu#3 βC), 39.2 (Leu #1 or Leu#2 or Leu#3 βC), 37.0 (Phe βC), 30.9 (Val βC), 24.24 (Leu#1 or Leu#2 or Leu#3 γC), 24.2 (Leu#1 or Leu#2 or Leu#3 γC), 24.0 (Leu# or Leu#2 or Leu#3 γC), 23.0 (Leu#1 δC or δC′), 22.9 (Leu#2 δC or δC′ or Leu#3 δC or δC′), 21.8 (Leu#2 δC or δC′ or Leu#3 δC or δC′), 21.7 (Leu#2 δC or δC′ or Leu#3 δC or δC′), 21.2 (Leu#2 δC or δC′ or Leu#3 δC or δC′), 19.1 (Val γC or Val γC′), 17.9 (Val γC or Val γC′), [1 x Leu#1 δC overlapping or obscured]; **MS** (ESI, + ve) *m/z* 604 (MH^+, 100%), **HRMS** (ESI, +ve) $C_{32}H_{54}N_5O_6^+$ [MH^+] requires *m/z* 604.4069, found 604.4078.

Linear peptide 8



The title compound was prepared by SPPS according to General Procedurse A–D on 0.2 mmol scale. The product was obtained as an off-white flaky solid (132 mg, 100%); **m.p.** 179 °C; **[α]$_D$** –47 (*c* 0.086, DMF); **IR** (neat) ν_{max} (cm^{-1}) 3287, 3067, 2958, 2874, 1694, 1618, 1517, 1196, 1184, 1147; **^{1}H NMR** (400 MHz, DMSO-*d*$_6$) δ 8.67 (d, *J* = 8.4 Hz, 1H, Leu#1 NH), 8.24–8.22 (m, 2H, Gly and Leu#2 NH), 8.14 (s, 2H, Phe NH), 7.74 (d, *J* = 9.0 Hz, 1H, Val NH), 7.33–7.23 (m, 5H, Phe ArH), 4.39 (dd, *J* = 15.1, 8.1 Hz 1H, Leu#1 αH), 4.26 (dd, *J* =9.0, 6.6 1H, Val αH), 4.18 (m, 1H, Gly αH), 4.06 (s, 1H, Phe αH), 3.78–3.5 (m, 2H Gly αH and Leu#2 αH), 3.10 (dd, *J* = 14.1, 5.1 Hz 1H, Phe βH), 2.91 (dd, *J* = 14.1, 8.0 Hz 1H, Phe βH′), 1.97 (m, 1H, Val βH), 1.66–1.44 (m, 6H, Leu#1 βH and γH and Leu#2 β and γH), 0.90–0.81 (m, 18H, Leu#1 δH and Leu#2 δH and Val γH). **^{13}C {^{1}H} NMR** (400 MHz, DMSO-*d*$_6$) δ 173.9 (Gly C=O), 171.6 (Leu#1 C=O), 170.9 (Val C=O), 168.3 (Leu#2 C=O), 167.8 (Phe C=O), 134.8 (Phe Ar C1), 129.6 (Phe Ar C2/C3), 128.6 (Phe Ar C4/C5), 127.2 (Phe Ar C6), 56.9 (Val αC), 53.3 (Phe αC), 51.2 (Leu#1 αC), 50.3 (Gly αC), 41.9 (Leu#2 αC), 41.3 (Leu#1 βC), 40.4 (Leu #2 βC) 37.0 (Phe βC), 31.1 (Val βC), 24.3 (Leu#2 γC), 24.0 (Leu#1 γC), 23.0 (Leu#2 δC or δC′), 22.8 (Leu#1 δC or δC′), 21.7 (Leu#2 δC or δC′), 21.3 (Leu#1 δC or δC′), 19.1 (Val γC or Val γC′), 17.9 (Val γC or Val γC′); **MS** (ESI, + ve) *m/z* 548 (MH$^+$, 100%); **HRMS** (ESI, +ve) $C_{28}H_{46}N_5O_6^+$ [MH$^+$] requires *m/z* 548.3443, found 548.3441.

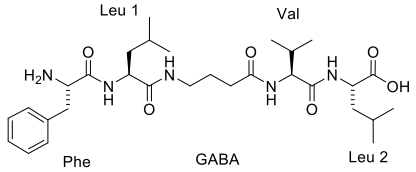
Linear peptide 9



The title compound was prepared by SPPS according to General Procedures A–D on 0.2 mmol scale. The product was obtained as an off-white flaky solid (129 mg, 95%); **m.p.** 175 °C; **[α]$_D$** –94 (*c* 0.032, DMF); **IR** (neat) ν_{max} (cm^{-1}) 3279, 3079, 2959, 2872, 1642, 1550, 1469, 1388, 1205, 1203, 1140;

^{1}H NMR (600 MHz, DMSO-d_6) δ 8.58 (d, J = 8.3 Hz, 1H, Leu#1 NH), 8.13 (d, J = 7.7 Hz, 1H, Leu#2 NH), 8.10 (s, 2H, Phe NH), 8.04 (t, J = 5.6 Hz, 1H, β-Ala NH), 7.89 (d, J = 8.9 Hz, 1H, Val NH), 7.32–7.22 (m, 5H, Phe ArH), 4.31 (dd, J = 14.9, 8.5 Hz 1H, Leu#1 αH), 4.22–4.17 (m, 2H, Val αH and Leu#2 αH), 4.04 (m, 1H, Phe αH), 3.27 (m, 1H, β-Ala βH), 3.18 (m, 1H, β-Ala βH′), 3.09 (dd, J = 14.2, 5.5 Hz 1H, Phe βH), 2.92 (dd, J = 14.1, 8 Hz 1H, Phe βH′), 2.38–2.29 (m, 2H, β-Ala αH and αH′), 1.96 (m, 1H, Val βH), 1.63 (m, 1H, Leu#2 γH), 1.58–1.46 (m, 3H, Leu#1 γH and Leu#2 βH and βH′), 1.44–1.39 (m, 2H, Leu#1 βH and βH′), 0.88–0.81 (m, 18H, Leu#1 δH and Leu#2 δH and Val γH); **^{13}C {^{1}H} NMR** (600 MHz, DMSO-d_6) δ 174.1 (Leu#2 C=O), 171.4 (Val C=O), 171.3 (Leu#1 C=O), 170.5 (β-Ala C=O), 167.7 (Phe C=O), 134.8 (Phe Ar C1), 129.7 (Phe Ar C2/C3), 128.7 (Phe Ar C4/C5), 127.3 (Phe Ar C6), 57.4 (Val αC), 53.3 (Phe αC), 51.3 (Leu#1 αC), 50.4 (Leu#2 αC), 41.5 (Leu#1 βC), 40.2 (Leu#2 βC), 37.1 (Phe βC), 35.6 (β-Ala βC), 35.1 (β-Ala αC), 30.7 (Val βC), 24.4 (Leu#2 γC), 24.2 (Leu#1 γC), 23.0 (Leu#2 δC or δC′), 22.9 (Leu#1 δC or δC′), 21.9 (Leu#2 δC or δC′), 21.4 (Leu#1 δC or δC′), 19.3 (Val γC or Val γC′), 18.2 (Val γC or Val γC′); **MS** (ESI, + ve) *m/z* 562 (MH^+, 100%); **HRMS** (ESI, +ve) $C_{29}H_{48}N_5O_6^+$ [MH^+] requires *m/z* 562.3599, found 562.3596.

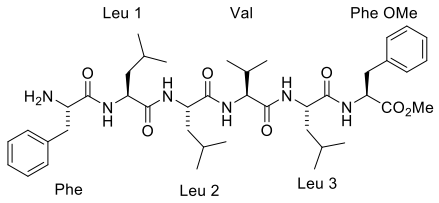
Linear peptide 10



The title compound was prepared by SPPS according to General Procedures A–D on 0.2 mmol scale. The product was obtained as an off-white flaky solid (128 mg, 93%); **m.p.** 127 °C; **[α]$_D$** –11 (*c* 0.175, DMF); **IR** (neat) ν_{max} (cm^{-1}) 3285, 3090, 2960, 2873, 1633, 1551, 1468, 1386, 1203, 1184, 1139; **^{1}H NMR** (600 MHz, DMSO-d_6) δ 8.33 (d, J = 8.4 Hz, 1H, Leu#1 NH), 7.95 (t, J = 5.6 Hz, 1H, GABA NH), 7.91 (d, J = 7.41 1H, Leu#2 NH), 7.85 (d, J = 9.1 Hz, 1H, Val NH), 7.28–7.19 (m, 5H, Phe ArH), 4.27 (m, 1H, Leu#1 αH), 4.18–4.11 (m, 2H, Val and Leu# 2 αH), 3.67 (s, 1H, Phe αH), 3.07–3.03 (m, 1H, GABA γH), 3.01–2.96 (m, 2H, GABA γH′ and Phe βH), 2.75 (m, 1H, Phe βH′), 2.16 (t, J = 7.4 Hz, 2H, GABA αH and αH′), 1.98 (m, 1H, Val βH), 1.66–1.56 (m, 3H, Lue#1 or 2 γH and GABA βH and βH′), 1.53–1.47 (m, 3H, Leu#1 or 2 γH Leu#2 βH and βH′), 1.45–1.41 (m, 2H, Leu#1 βH and βH′), 0.88–0.82 (m, 18H, Leu#1 δH and Leu#2 δH and Val γH). **^{13}C {^{1}H} NMR** (150 MHz, DMSO-d_6) δ 174.3 (Leu#2 C=O), 171.9 (GABA C=O), 171.8 (Phe C=O), 171.5 (Leu#1 C=O), 170.9 (Val C=O),

137.4 (Phe Ar C1), 129.4 (Phe Ar C2/C3), 128.2 (Phe Ar C4/C5), 126.4 (Phe Ar C6), 57.5 (Val αC), 55.1 (Phe αC), 51.1 (Leu#1 αC), 50.7 (Leu#2 αC), 41.5 (Leu#1 βC), 40.5 (Leu#2 βC), δ 38.1 (GABA γC), 34.3 (Phe βC), 32.5 (GABA αC), 30.3 (VAL βC), 25.4 (GABA βC), 24.3 (Leu#2 γC), 24.1(Leu#1 γC), 22.9 (Leu#2 δC or δC′), 22.8 (Leu#1 δC or δC′), 21.8 (Leu#2 δC or δC′), 21.6 (Leu#1 δC or δC′), 19.3 (Val γC or Val γC′), 18.1 (Val γC or Val γC′); **MS** (ESI, + ve) *m/z* 576 (MH^+, 100%); **HRMS** (ESI, +ve) $C_{30}H_{50}N_5O_6^+$ [MH^+] requires *m/z* 576.3756, found 576.3750.

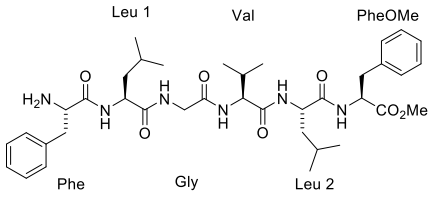
Linear peptide 11



The title compound was prepared from compound **7** according to General Procedure F on 0.022 mmol scale. The product was obtained as white powder (5 mg, 29.5%); **m.p.** > 200 °C; **[α]$_D$** –44 (*c* 0.023, DMF); **IR** (neat) ν_{max} (cm^{-1}) 3267, 3064, 2953, 1742, 1627, 1539, 1453, 1212; **^{1}H NMR** (600 MHz, DMSO-d_6) δ 8.22 (d, *J* = 7.6 Hz, 1H, Phe#2 NH), 8.11 (d, *J* = 8.1 Hz, 1H, Leu#2 NH), 8.01 (d, *J* = 7.7 Hz, 1H, Leu#1 NH), 7.87 (d, *J* = 8.4 Hz 1H, Leu#3 NH), 7.68 (d, *J* = 8.7 Hz 1H, Val NH), 7.28–7.18 (m, 10H, Phe#1 and Phe#2 ArH), 4.47 (ddd, *J* = 13.8, 8.5, 5.9 Hz 1H, Phe#2 αH), 4.38–4.29 (m, 3H, Leu#1 and Leu#2 and Leu#3 αH), 4.11 (dd, *J* = 8.7, 7.2 Hz, 1H, Val αH), 3.55 (s, 3H, OCH_3), 3.43 (m, 1H, Phe#1 αH), 3.01 (dd, *J* = 13.9, 5.9 Hz, 1H, Phe#2 βH), 2.95 (dd, *J* = 6.6, 1.9 Hz, 1H, Phe#2 βH′), 2.92 (dd, *J* = 6.3, 1.8 Hz, 1H, Phe#1 βH), 2.61 (dd, *J* = 13.5, 8.4 Hz, 1H, Phe#1 βH′), 1.89 (m, 1H, Val βH), 1.60–1.46 (m, 3H, Leu#1 and Leu#3 and Leu#2 γH), 1.45–1.39 (m, 4H, Leu#1 and Leu# 2 βH and βH′), 1.38–1.32 (m, 2H, Leu#3 βH and βH′), 0.87–0.76 (m, 24H, Leu#1 δH and Leu#2 δH and Leu#3 δH and Val γH); **^{13}C {^{1}H} NMR** (600 MHz, DMSO-d_6) δ 173.8 (Phe#1 C=O), 171.8 (Leu#3 C=O), 171.7 (Leu#1 C=O), 171.6 (Phe#2 C=O), 171.5 (Lue#2 C=O), 170.3 (Val C=O), 138.6 (Phe#1 Ar C1), 137.0 (Phe#2 Ar C1), 129.4 (Phe#1 Ar C2/C3), 128.9 (Phe#2 Ar C2/C3), 128.2 (Phe#1 Ar C4/C5), 128.0 (Phe#2 Ar C4/C5), 126.5 (Phe#1 Ar C6), 126.1 (Phe#2 Ar C6), 57.5 (Val αC), 55.8 (Phe#1 αC), 53.3 (Phe#2 αC), 51.8 (OCH_3), 51.0 (Leu#2 αC), 50.6 (Leu#1 αC), 50.5 (Leu#3 αC), 41.5 (Leu#2 βC), 40.9 (Leu#3 βC), 40.7 (Phe#1 βC), 40.5 (Leu#1 βC), 36.5 (Phe#2 βC), 30.5 (Val βC), 24.1 (Leu#1 γC), 24.06 (Leu#3 γC), 23.9 (Leu#2 γC), 23.1 (Leu#1 δC), 22.99 (Leu#3 δC), 22.94 (Leu#2 δC), 21.9 (Leu#1 δC′), 21.7 (Leu#3 δC′), 21.6 (Leu#2 δC′), 19.1 (Val γC or γC′), 18.1 (Val γC or γC′); **MS** (ESI, + ve) *m/z* 765 (MH^+, 100%), **HRMS** (ESI, +ve)

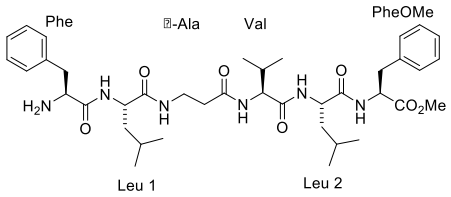
$C_{42}H_{64}N_6NaO_7^+$ [MH^+] requires *m/z* 787.4729, found 787.4712, (ESI, +ve) $C_{42}H_{65}N_6O_7^+$ [MH^+] requires *m/z* 765.4909, found 765.4905.

Linear peptide 12



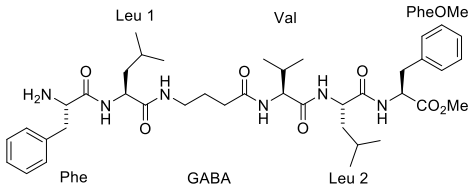
The title compound was prepared from compound **8** according to General Procedure F on 0.06 mmol scale. The product was obtained as white powder (3.6 mg, 8.5%); **m.p.** >200 °C; **[α]$_D$** –174 (*c* 0.012, DMF); **IR** (neat) ν_{max} (cm^{-1}) 3271, 2953, 1741, 1627, 1540, 1437, 1215; **^{1}H NMR** (600 MHz, DMSO-d_6) δ 8.22–8.19 (m, 2H, Phe#2 and Gly NH), 8.01 (bs, 1H, Leu#1 NH), 7.95 (d, *J* = 8.3 Hz, 1H, Leu#2 NH), 7.70 (d, *J* = 8.7 Hz, 1H, Val NH), 7.27–7.18 (m, 10H, Phe#1 and 2 ArH), 4.45 (ddd, *J* = 14.1, 7.8, 6.2 Hz 1H, Phe#2 αH), 4.33–4.27 (m, 2H, Leu#2 and Leu#1 αH), 4.16 (dd, *J* = 6.6, 6.6 Hz 1H, Val αH), 3.75–3.68 (m, 2H, Gly αH), 3.54 (s, 3H, OCH_3), 3.43 (m, 1H Phe#1 αH), 3.0 (dd, *J* = 13.9, 5.9 Hz 1H, Phe#2 βH or βH′), 2.96–2.92 (m, 2H, Phe#1 and Phe#2 βH or βH′), 2.63 (dd, *J* = 13.5, 8.3 Hz 1H, Phe#1 βH or βH′), 1.94 (m, 1H, Val βH), 1.57–1.48 (m, 2H, Leu#1 and Leu#2 γH), 1.45–1.43 (m, 2H Leu#1 βH and βH′), 1.40–1.37 (m, 2H Leu#2 βH and βH′), 0.86–0.76 (m, 18H, Leu#1 δH and Leu#2 δH and Val γH); **^{13}C {^{1}H} NMR** (400 MHz, DMSO-d_6) δ 174.3 (Phe#1 C=O), 172.4 (Leu#1 C=O), 172.0 (Leu#2 C=O), 171.8 (Phe#2 C=O), 170.5 (Val C=O), 168.75 (Gly C=O), 138.7 (Phe#1 Ar C1), 137.1 (Phe#2 Ar C1), 129.4 (Phe#1 Ar C2/C3), 129.0 (Phe#2 Ar C2/C3), 128.3 (Phe#1 Ar C4/C5), 128.1 (Phe#2 Ar C4/C5), 126.6 (Phe#1 Ar C6), 126.1 (Phe#2 Ar C6), 57.5 (Val αC), 56.1 (Phe#1 αC), 53.4 (Phe#2 αC), 51.8 (OCH_3), 50.8 (Leu#1 or Leu#2 αC), 50.7 (Leu#1 or Leu#2 αC), 42.1 (Gly αC), 41.3 (Leu#1 βC), 40.9 (Leu#2 βC), 40.6 (Phe#1 βC), 36.5 (Phe#2 βC), 30.6 (Val βC), 24.08 (Leu#1 or Leu#2 γC), 24.06 (Leu#1 or Leu#2 γC), 23.1 (Leu#1 or Leue#2 δC), 22.9 (Leu#1 or Leu#2 δC), 21.74 (Leu#1 or Lue#2 δC′), 21.72 (Leu#1 or Leu#2 δC′), 19.2 (Val γC or γC′), 18.0 (Val γC or γC′); **MS** (ESI, + ve) *m/z* 709 (MH^+, 100%); **HRMS** (ESI, +ve) $C_{38}H_{56}N_6NaO_7^+$ [MH^+] requires *m/z* 731.4103, found 731.4089, (ESI, +ve) $C_{38}H_{57}N_6O_7^+$ [MH^+] requires *m/z* 709.4283, found 709.4280.

Linear peptide 13



The title compound was prepared from compound **9** according to General Procedure F on 0.04 mmol scale. The product was obtained as white powder (11 mg, 37.5%); **m.p.** >200 °C; **[α]$_D$** +77 (*c* 0.013, DMF); **IR** (neat) ν_{max} (cm^{-1}) 3284, 2954, 1738, 1630, 1537, 1442, 1216; **^{1}H NMR** (600 MHz, DMSO-*d*$_6$) δ 8.26 (d, *J* = 7.5 Hz, 1H, Phe#2 NH), 7.98–7.96 (m, 2H, β-Ala and Leu#1 NH), 7.90–7.86 (m, 2H, Val and Leu#2 NH), 7.27–7.18 (m, 10H, Phe#1 and 2 ArH), 4.46 (dd, *J* = 14.2, 7.9 Hz, 1H, Phe#2 αH), 4.33 (dd, *J* = 15.2, 8.4 Hz, 1H, Leu#2 αH), 4.26 (dd, *J* = 14.9, 7.9 Hz, 1H, Leu#1 αH), 4.12 (dd, *J* = 8.4, 6.9 Hz, 1H, Val αH), 3.34 (s, 3H, OCH$_3$), 3.44 (dd, *J* = 7.8, 4.6 Hz, 1H Phe#1 αH), 3.27–3016 (m, 2H β-Ala βH), 3.0 (dd, *J* = 14.0, 6.0 Hz, 1H, Phe#2 βH or βH′), 2.96–2.92 (m, 2H, Phe#1 and Phe#2 βH or βH′), 2.63 (dd, *J* = 13.5, 8.3 Hz 1H, Phe#1 βH or βH′), 2.36–2.28 (m, 2H β-Ala αH), 1.92 (m, 1H, Val βH), 1.54 (m, 1H, Leu#2 γH), 1.46 (m, 2H Leu#1 γH), 1.42–1.35 (m, 4H, Leu#1 and Leu#2 βH and βH′), 0.86–0.77 (m, 18H, Leu#1 δH and Leu#2 δH and Val γH). **^{13}C {^{1}H} NMR** (400 MHz, DMSO-*d*$_6$) δ 173.7 (Phe#1 C=O), 172.0 (Leu#2 C=O), 171.8 (Leu#1 C=O), 171.7 (Phe#2 C=O), 170.7 (Val C=O), 170.5 (Gly C=O), 138.5 (Phe#1 Ar C1), 137.0 (Phe#2 Ar C1), 129.4 (Phe#1 Ar C2/C3), 129.0 (Phe#2 Ar C2/C3), 128.2 (Phe#1 Ar C4/C5), 128.1 (Phe#2 Ar C4/C5), 126.5 (Phe#1 Ar C6), 126.1 (Phe#2 Ar C6), 57.8 (Val αC), 55.9 (Phe#1 αC), 53.4 (Phe#2 αC), 51.7 (OCH$_3$), 50.6 (Leu#1 or 2 αC), 41.6 (Leu#1 βC), 40.9 (Leu#2 βC), 40.5 (Phe#1 βC), 36.5 (Phe#2 βC), 35.4 (β-Ala βC), 35.0 (β-Ala αC), 30.2 (Val βC), 24.1 (Leu#1 or Leu#2 γC), 24.0 (Leu#1 or Lue#2 γC), 23.0 (Leu#1 or Leu#2 δC), 22.9 (Leu#1 or Leu#2 δC), 21.8 (Leu#1 or Leu#2 δC′), 21.6 (Leu#1 or Leu#2 δC′), 19.2 (Val γC or γC′), 18.1 (Val γC or γC′), [1 x Leu#1 or 2 αC overlapping or obscured]; **MS** (ESI, + ve) *m/z* 723 (MH$^+$, 100%); **HRMS** (ESI, +ve) $C_{39}H_{58}N_6NaO_7^+$ [MH$^+$] requires *m/z* 745.4259, found 745.4242, (ESI, +ve) $C_{39}H_{59}N_6O_7^+$ [MH$^+$] requires *m/z* 723.4440, found 723.4436.

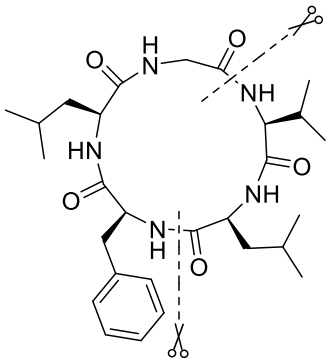
Linear peptide 14



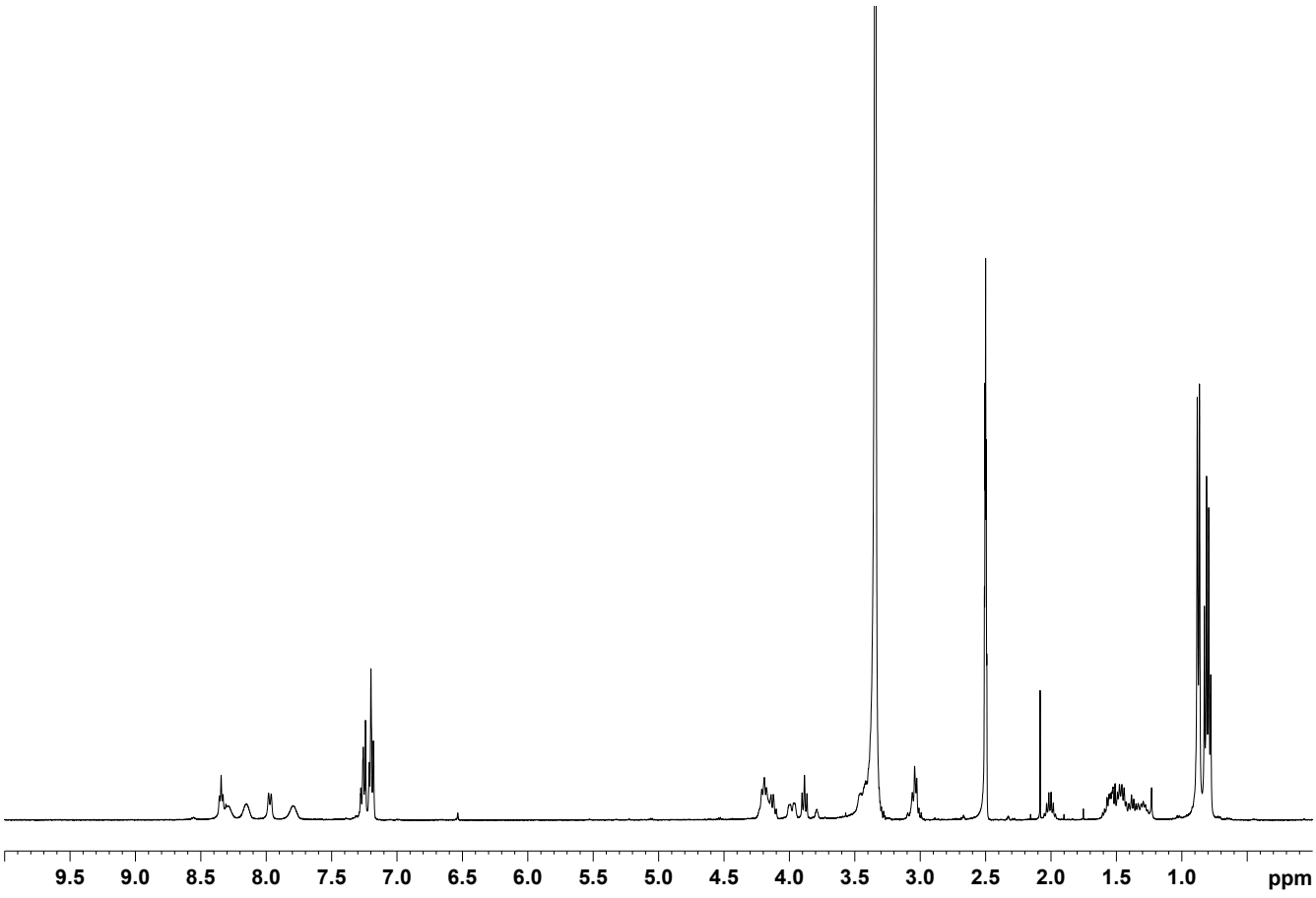
The title compound was prepared from compound **10** according to General Procedure F on 0.04 mmol scale. The product was obtained as white powder (5.9 mg, 22%); **m.p.** 175 °C; **$[\alpha]_D$** +47 (*c* 0.022, DMF); **IR** (neat) ν_{max} (cm^{-1}) 3283, 2953, 1736, 1630, 1539, 1440, 1216; **^{1}H NMR** (600 MHz, DMSO-d_6) δ 8.22 (d, *J* = 7.5 Hz, 1H, Phe#2 NH), 7.95 (d, *J* = 7.7 Hz, 1H, Leu#1 NH), 7.93–7.89 (m, 2H, GABA and Leu#2 NH), 7.83 (d, *J* = 8.7 Hz, 1H, Val NH), 7.27–7.18 (m, 10H, Phe#1 and Phe#2 ArH), 4.46 (ddd, *J* = 14.1, 8.1, 6.1 Hz, 1H, Phe#2 αH), 4.32 (ddd, *J* = 15.0, 8.7, 6.4 Hz, 1H, Leu#2 αH), 4.26 (dd, *J* = 15.1, 7.6 Hz, 1H, Leu#1 αH), 4.12 (dd, *J* = 8.7, 7.1 Hz, 1H, Val αH), 3.54 (s, 3H, OCH_3), 3.44 (bs, 1H Phe#1 αH), 3.05–3.01 (m, 2H, GABA γH and γH′), 3.0–2.96 (m, 1H, Phe#2 βH or βH′), 2.96–2.92 (m, 2H, Phe#1 and Phe#2 βH or βH′), 2.64 (m, 1H, Phe#1 βH or βH′), 2.18–2.08 (m, 2H GABA αH and αH′), 1.92 (m, 1H, Val βH), 1.61–1.59 (m, 2H, GABA βH and βH′), 1.53 (m, 1H Leu#2 γH), 1.46 (m, 1H Leu#1 γH), 1.41–1.39 (m, 2H, Leu#1 βH and βH′),1.38–1.34 (m, 2H, Leu#2 βH and βH′), 0.86–0.78 (m, 18H, Leu#1 and Leu#2 δH and Val γH); **^{13}C {^{1}H} NMR** (400 MHz, DMSO-d_6) δ 178.4 (Phe#1 C=O), 172.1 (GABA C=O), 172.0 (Leu#2 C=O), 172.0 (Leu#2 C=O), 171.9 (Phe#2 C=O), 171.7 (Leu#1 C=O), 170.8 (Val C=O), 138.5 (Phe#1 Ar C1), 137.0 (Phe#2 Ar C1), 129.4 (Phe#1 Ar C2/C3), 129.0 (Phe#2 Ar C2/C3), 128.2 (Phe#1 Ar C4/C5), 128.1 (Phe#2 Ar C4/C5), 126.5 (Phe#1 Ar C6), 126.1 (Phe#2 Ar C6), 57.7 (Val αC), 56.0 (Phe#1 αC), 53.3 (Phe#2 αC), 51.7 (OCH_3), 50.7 (Leu#1 or Leu#2 αC), 50.6 (Leu#1 or Leu#2 αC), 41.6 (Leu#1 βC), 40.9 (Leu#2 βC), 40.3 (Phe#1 βC), 38.1 (GABA γC), 36.5 (Phe#2 βC), 32.6 (GABA αC), 30.3 (Val βC), 25.6 (GABA βC), 24.1 (Leu#1 γC), 24.0 (Leu#2 γC), 23.0 (Leu#1 or Leu#2 δC or δC′), 22.9 (Leu#1 or Leu#2 δC or δC′), 21.9 (Leu#1 or Leu#2 δC or δC′), 21.6 (Leu#1 or Leu#2 δC or δC′), 19.2 (Val γC or γC′), 18.1 (Val γC or γC′); **MS** (ESI, + ve) *m/z* 737 (MH^+, 100%); **HRMS** (ESI, +ve) $C_{40}H_{60}N_6NaO_7^+$ [MNa^+] requires *m/z* 759.4416, found 759.4396, (ESI, +ve) $C_{40}H_{61}N_6O_7^+$ [MH^+] requires *m/z* 737.4596, found 737.4590.

3. Confirmation of no epimerization during cyclisation

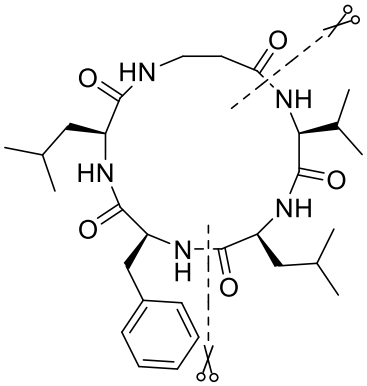
Cyclic peptide 3



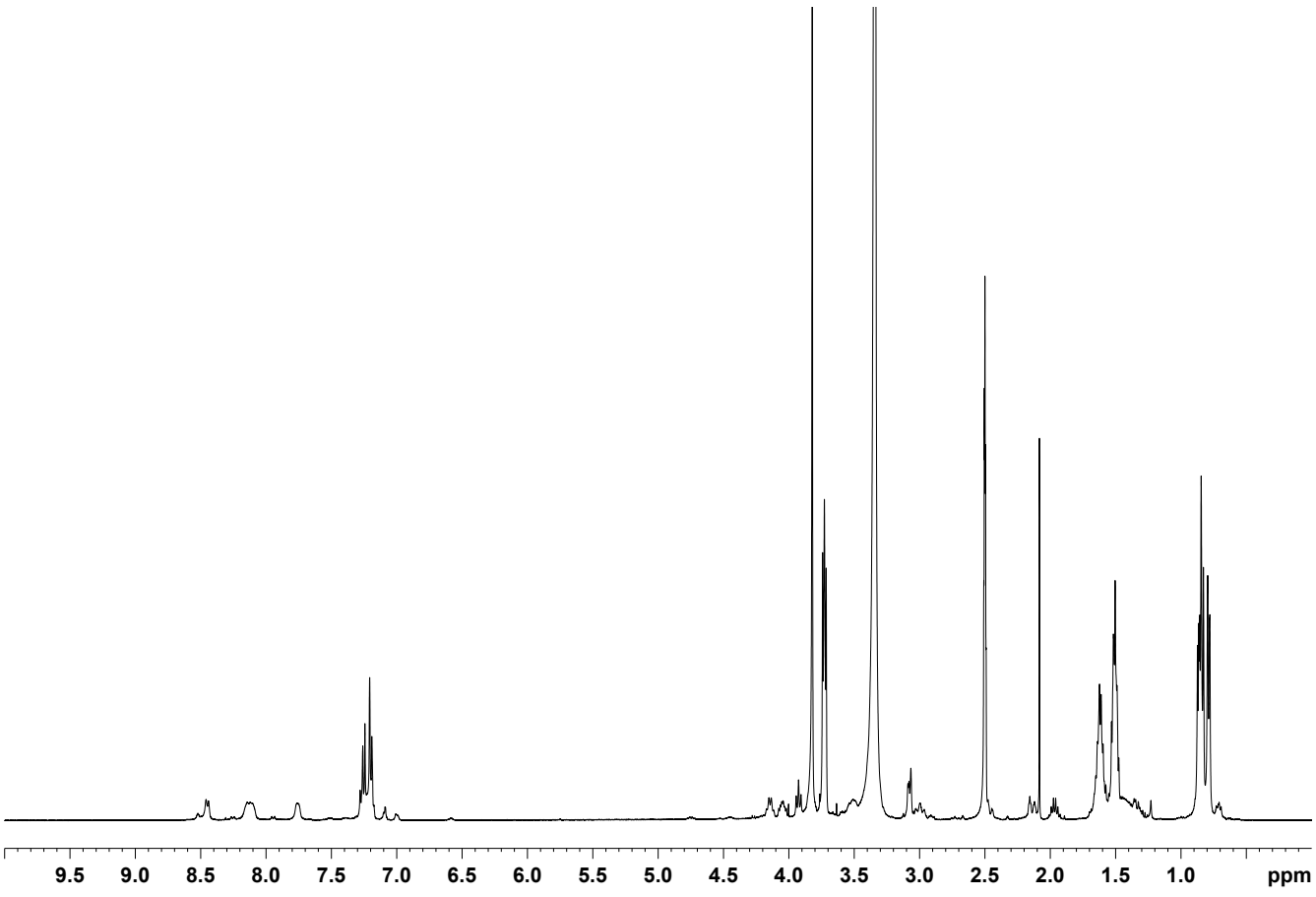
Cyclic peptide **3** was synthesized twice with the cyclisation points indicated in the structure. The two batches of cyclic peptide were combined in approximately 1:1 ratio and ^{1}H NMR spectrum was recorded (see below). Since only one compound is present, no epimerization had occurred during the first cylisation.



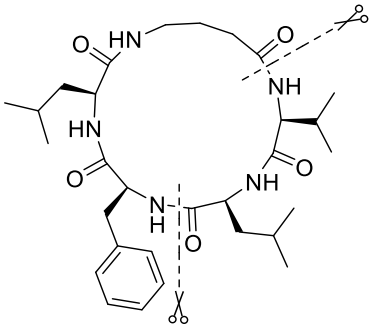
Cyclic peptide 4



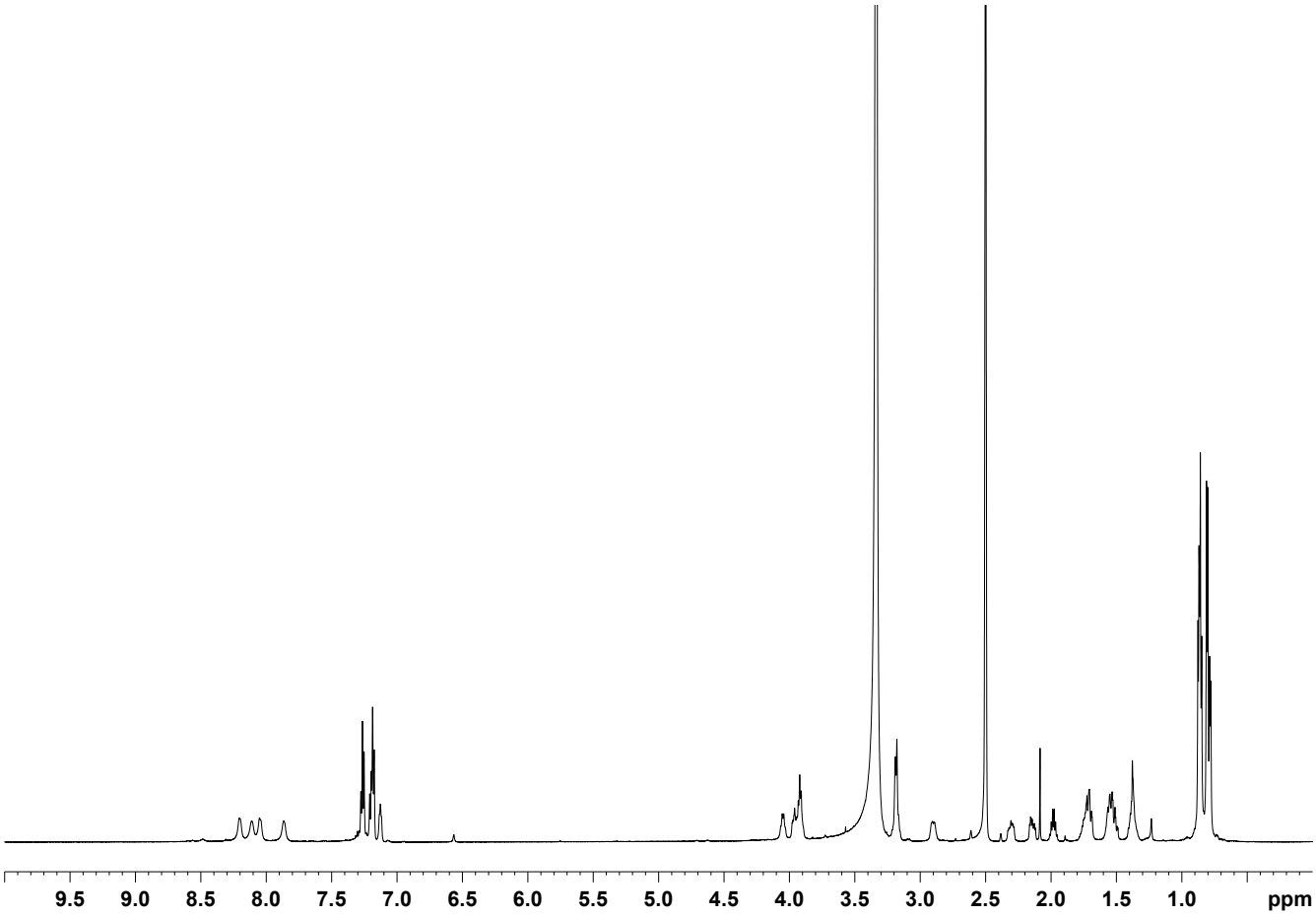
Cyclic peptide **4** was synthesized twice with the cyclisation points indicated in the structure. The two batches of cyclic peptide were combined in approximately 1:1 ratio and ^{1}H NMR spectrum was recorded (see below). Since only one compound is present, no epimerization had occurred during the first cylisation.



Cyclic peptide 5

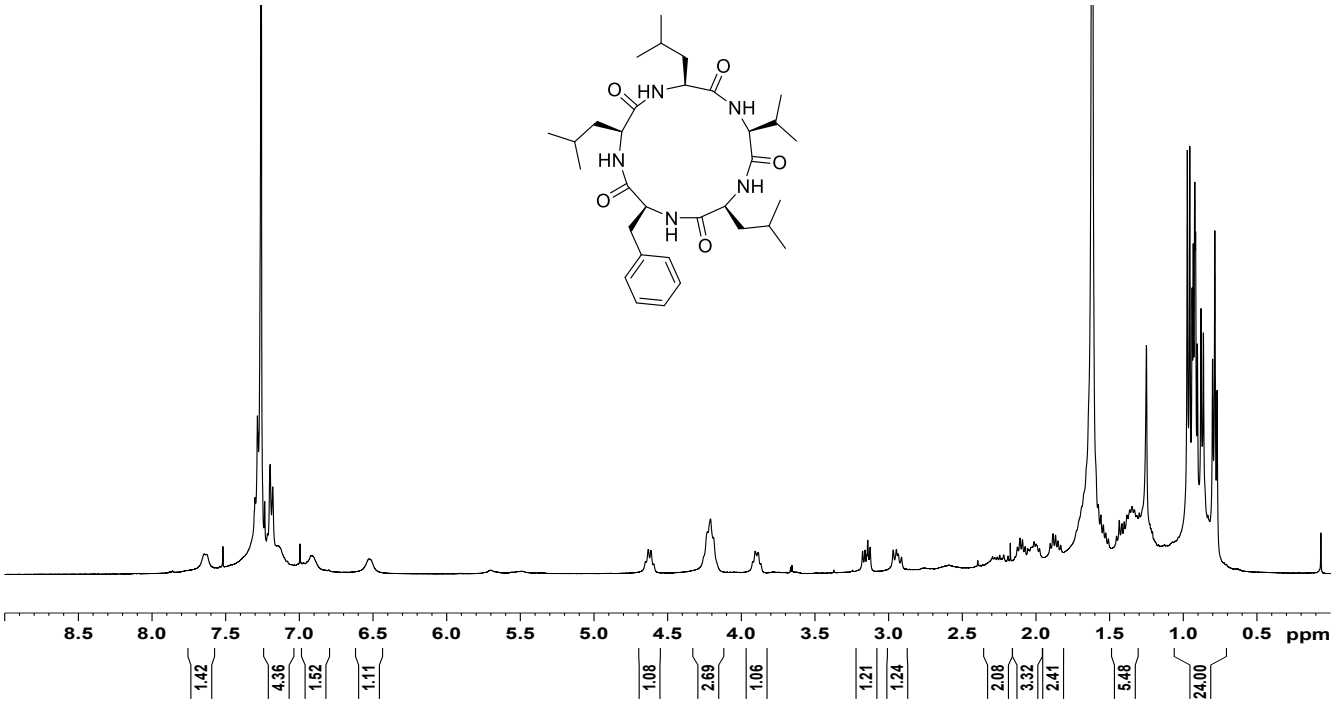


Cyclic peptide **5** was synthesized twice with the cyclisation points indicated in the structure. The two batches of cyclic peptide were combined in approximately 1:1 ratio and ^{1}H NMR spectrum was recorded (see below). Since only one compound is present, no epimerization had occurred during the first cylisation.

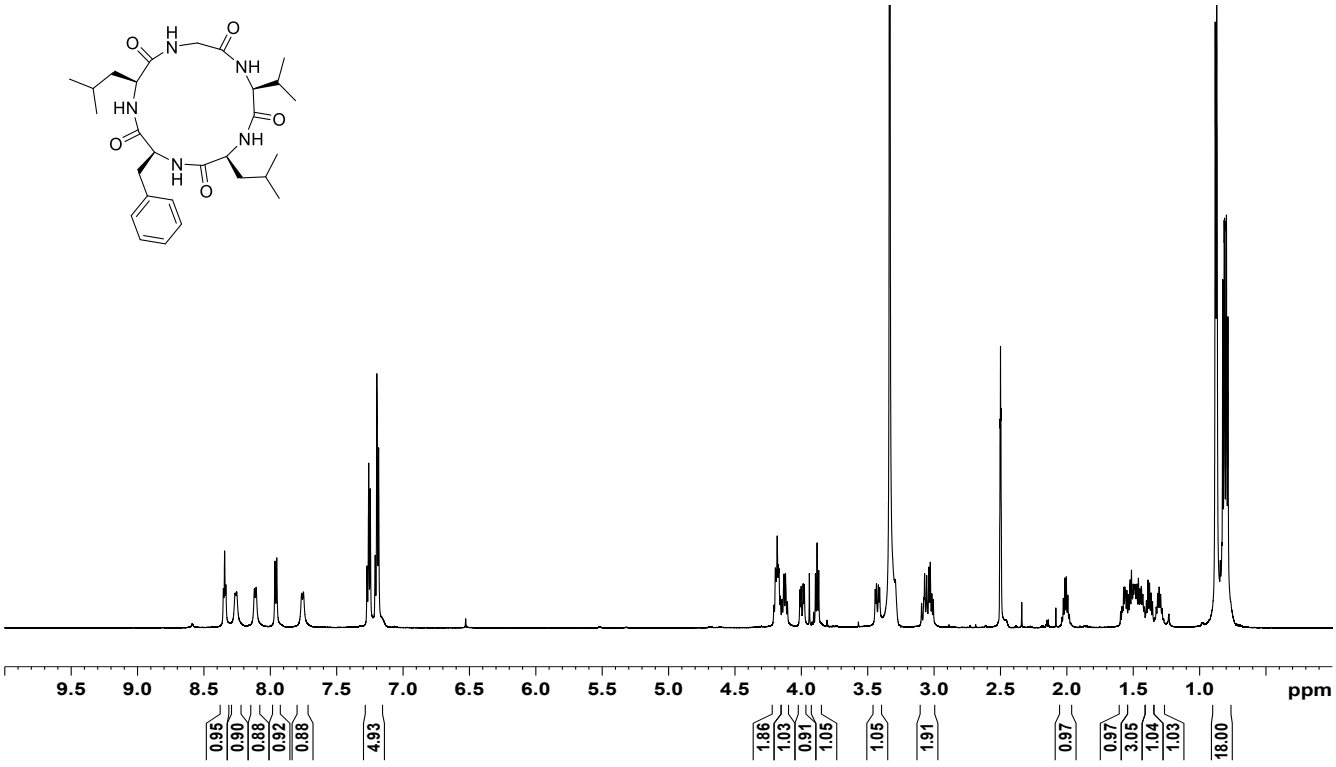


4. NMR spectra of novel compounds

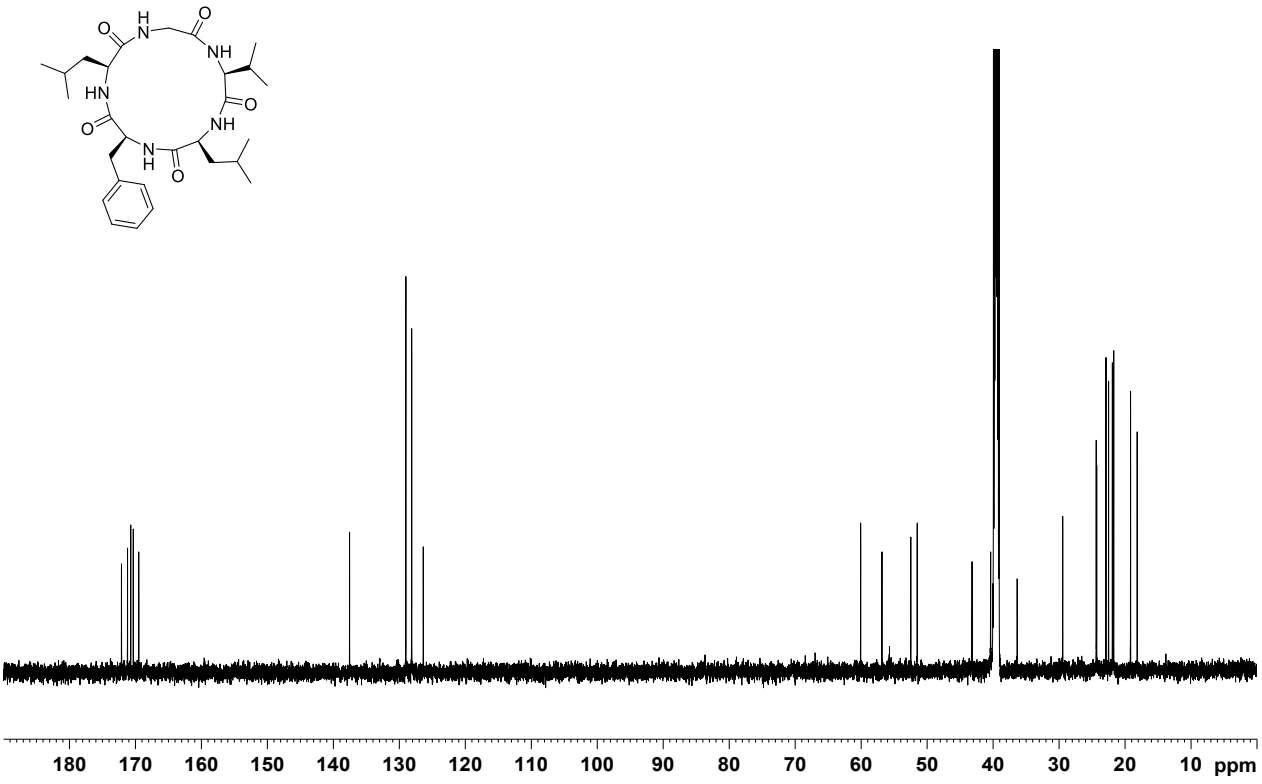
^{1}H NMR (400 MHz, $CDCl_3$) of **2**



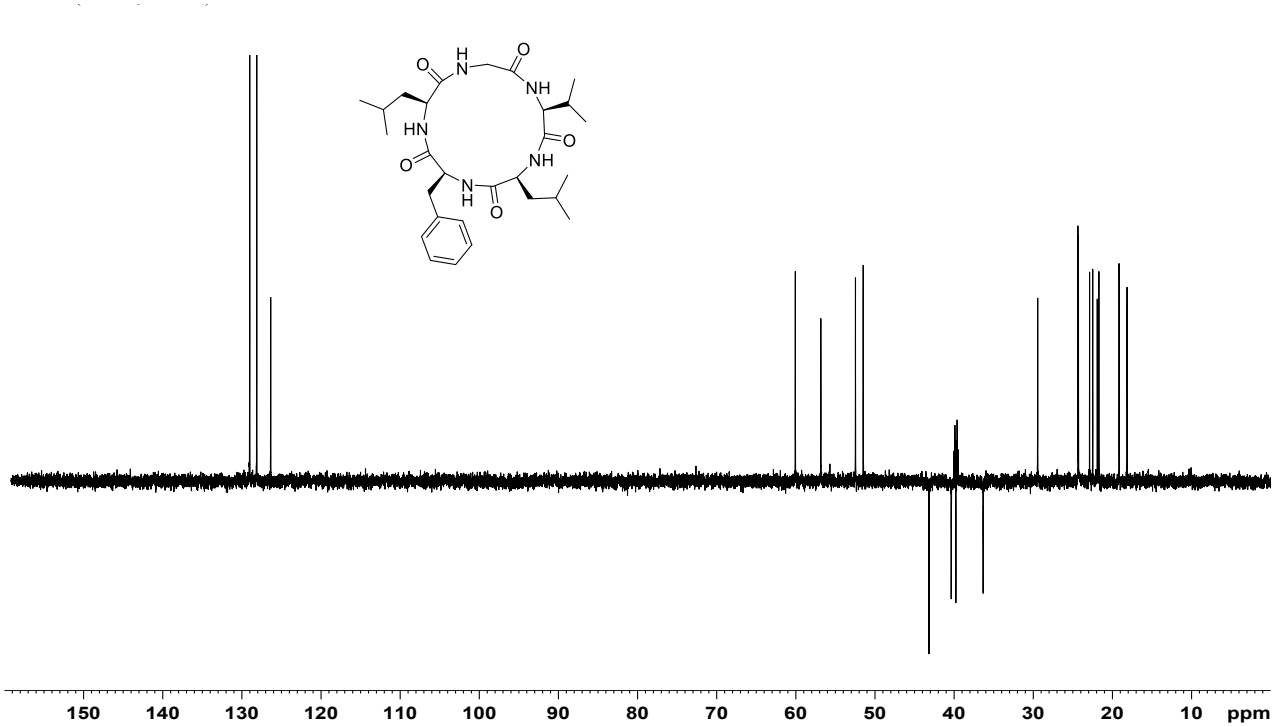
^{1}H NMR (600 MHz, DMSO-d_6) of **3**



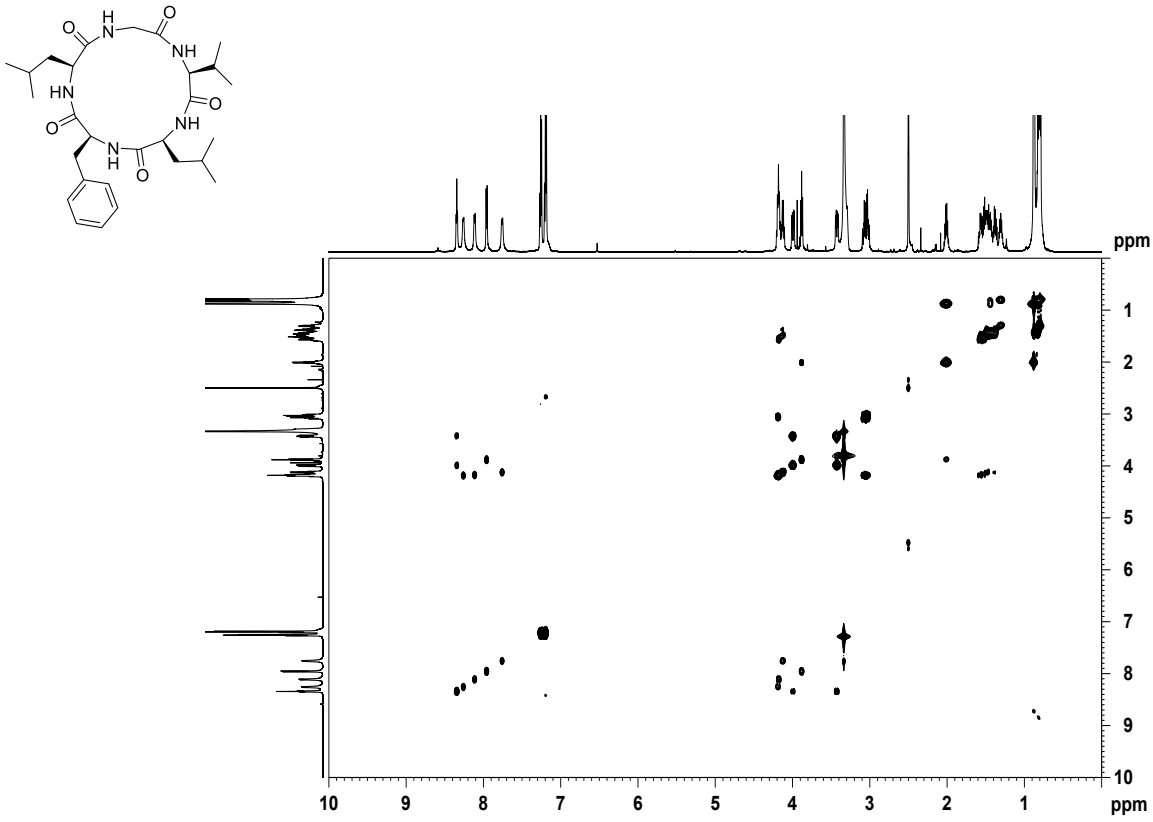
^{13}C NMR (600 MHz, DMSO-d_6) of **3**



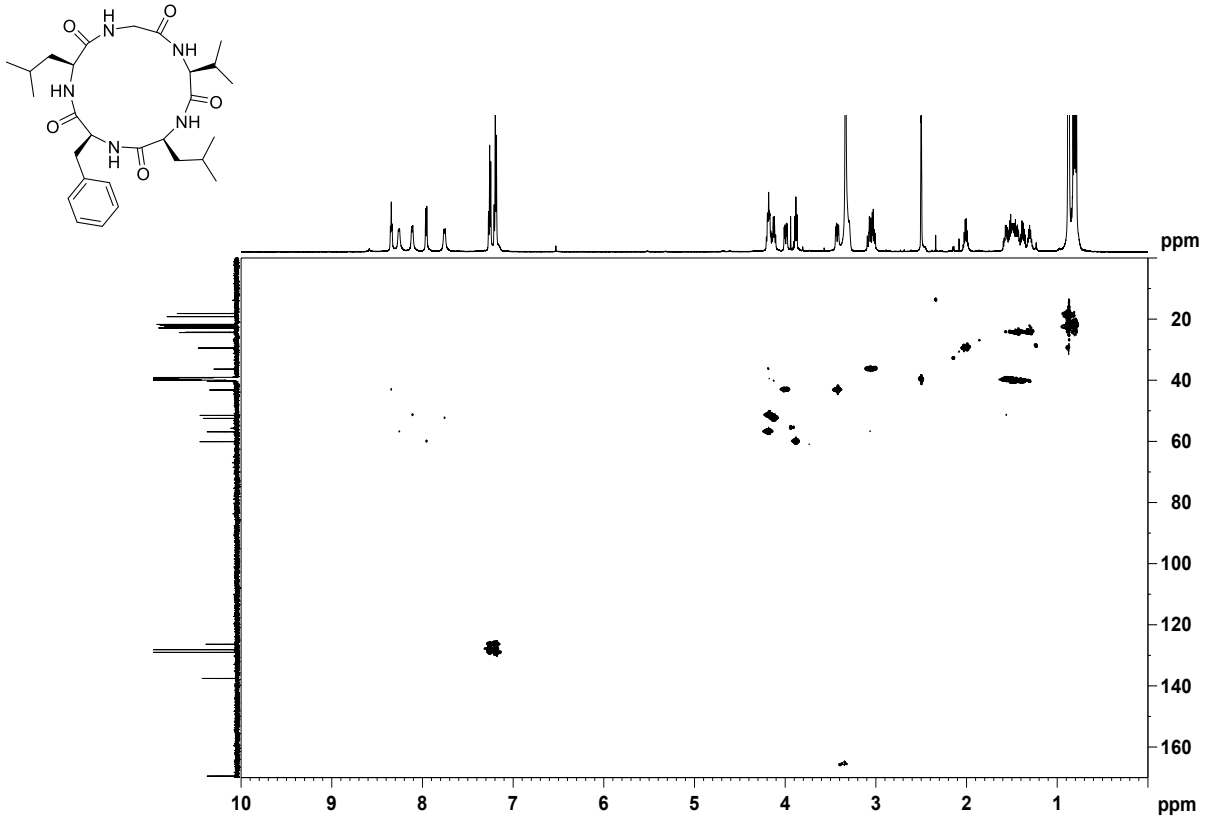
^{13}C DEPT (600 MHz, DMSO-d_6) of **3**



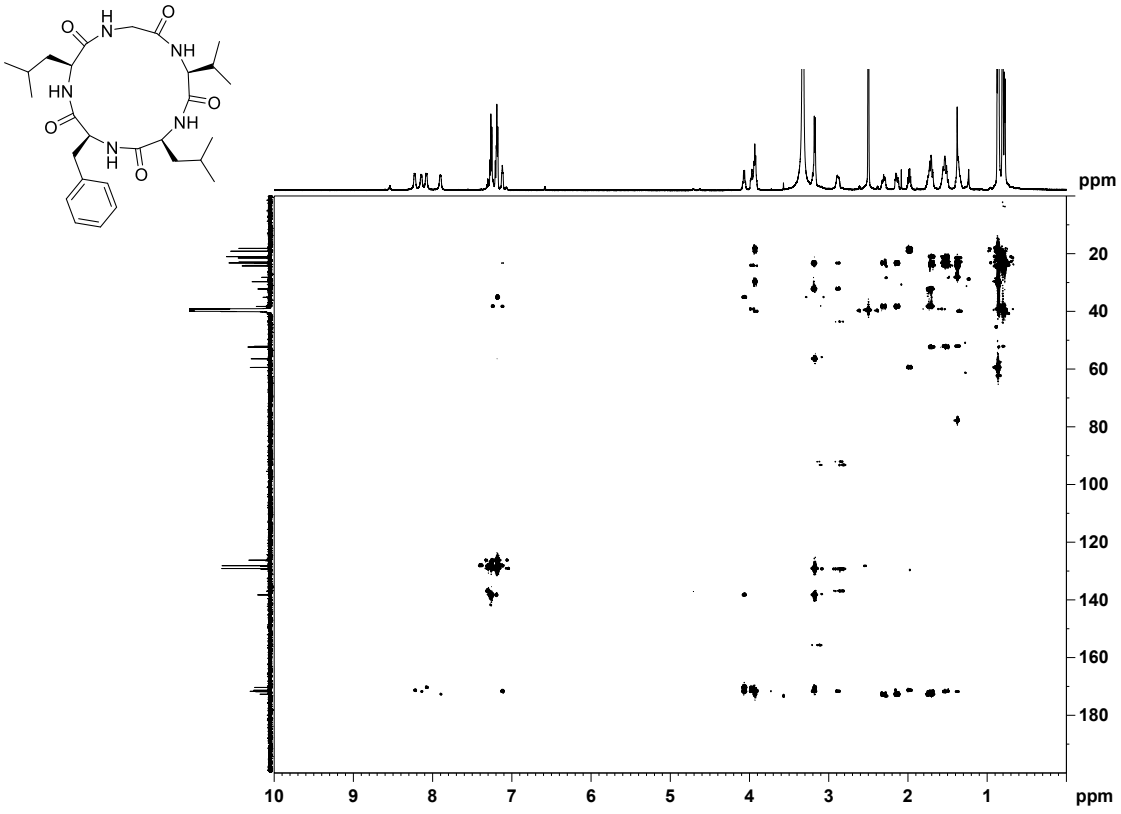
^{1}H-^{1}H COSY (600 MHz, DMSO-d_6) of **3**



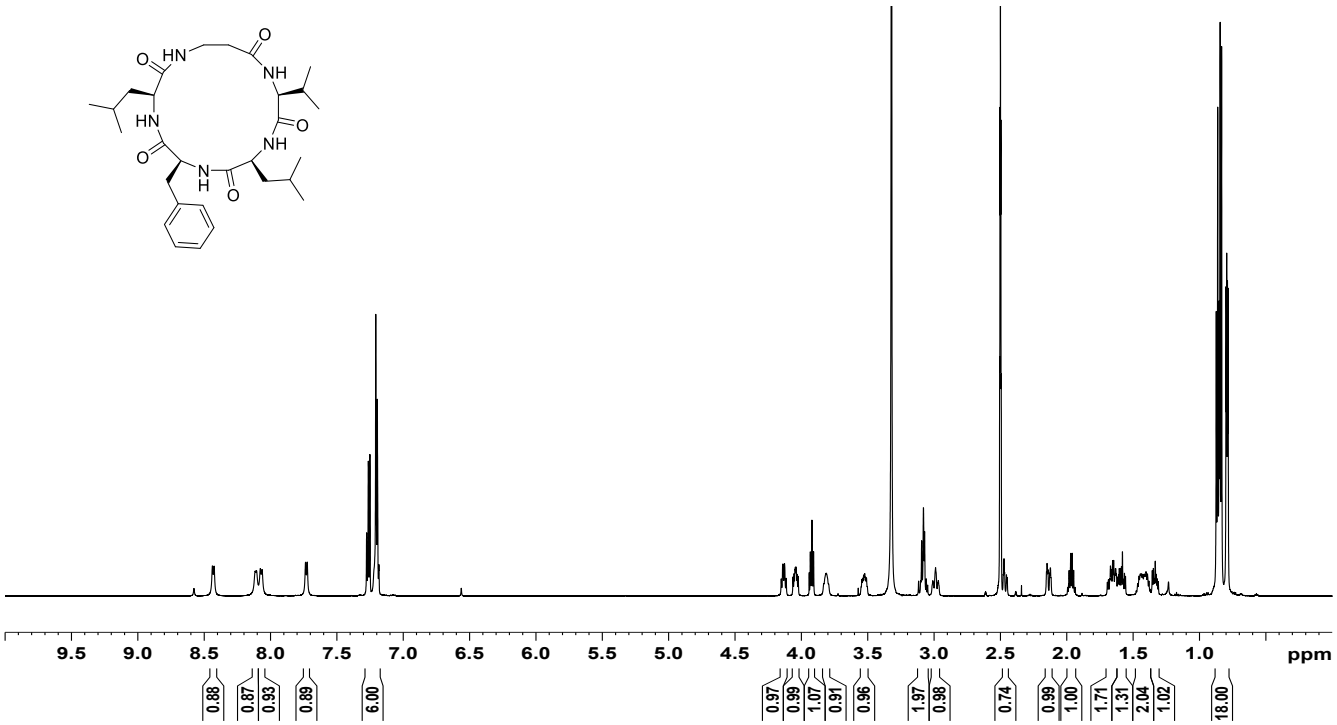
^{1}H-^{13}C HSQC (600 MHz, DMSO-d_6) of **3**



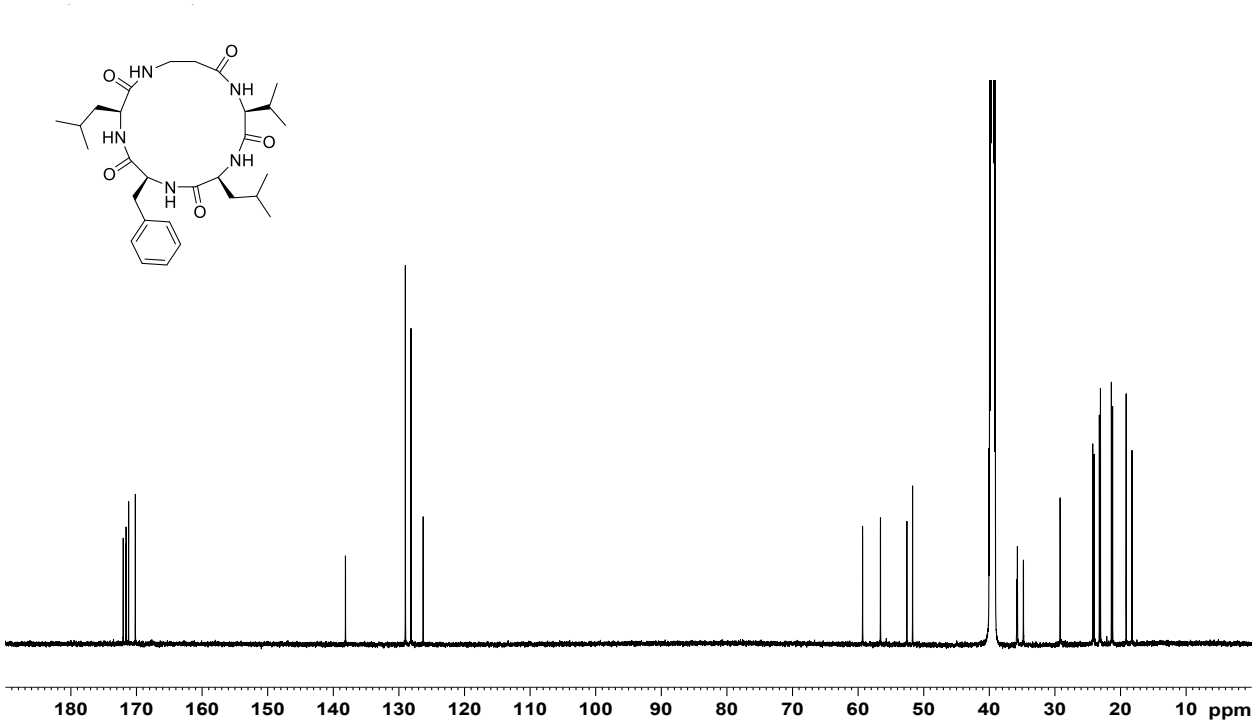
^{1}H-^{13}C HMBC (600 MHz, DMSO-d_6) of **3**



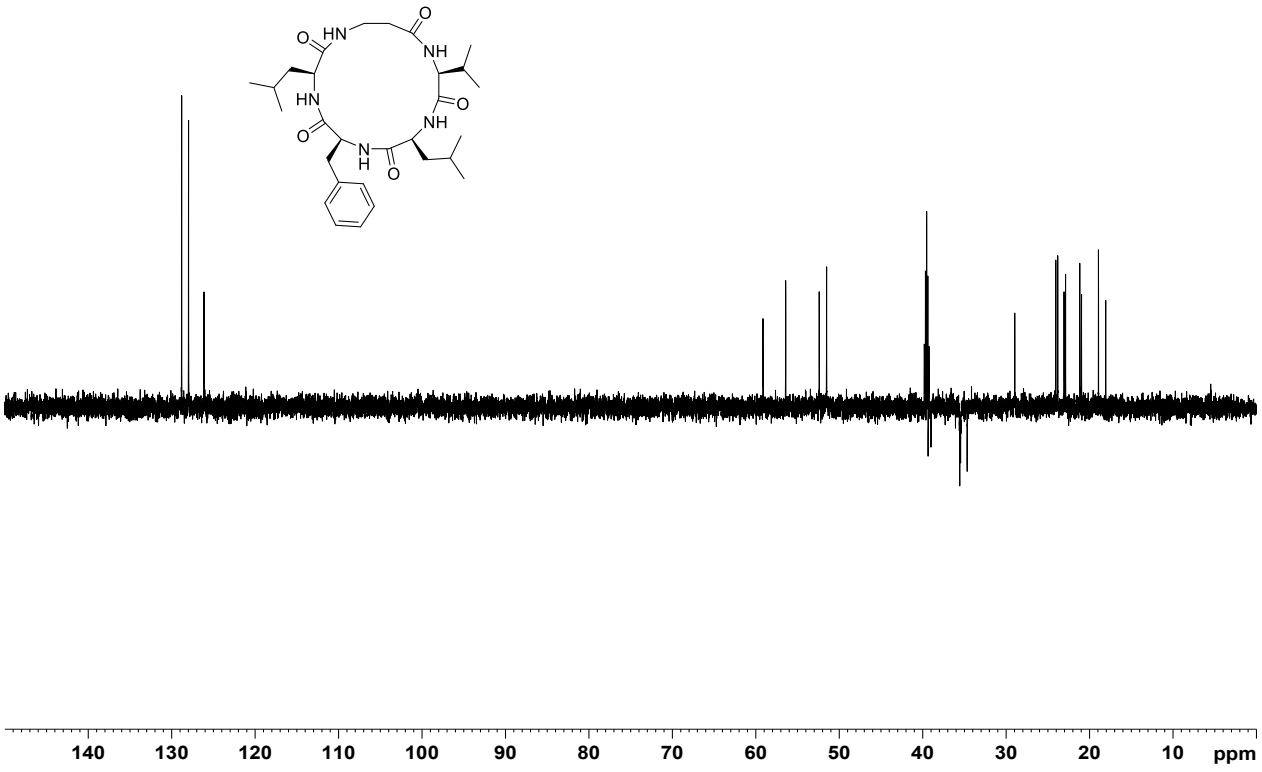
^{1}H NMR (600 MHz, DMSO-d_6) of **4**



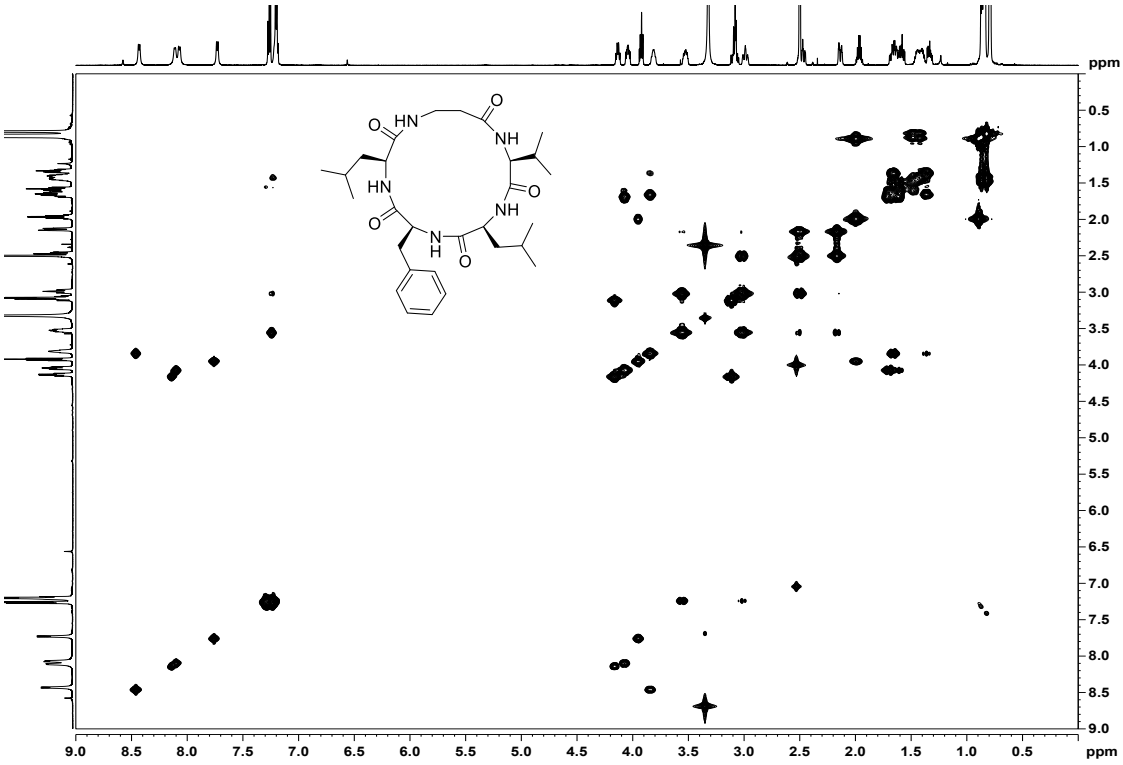
^{13}C NMR (150 MHz, DMSO-d_6) of **4**



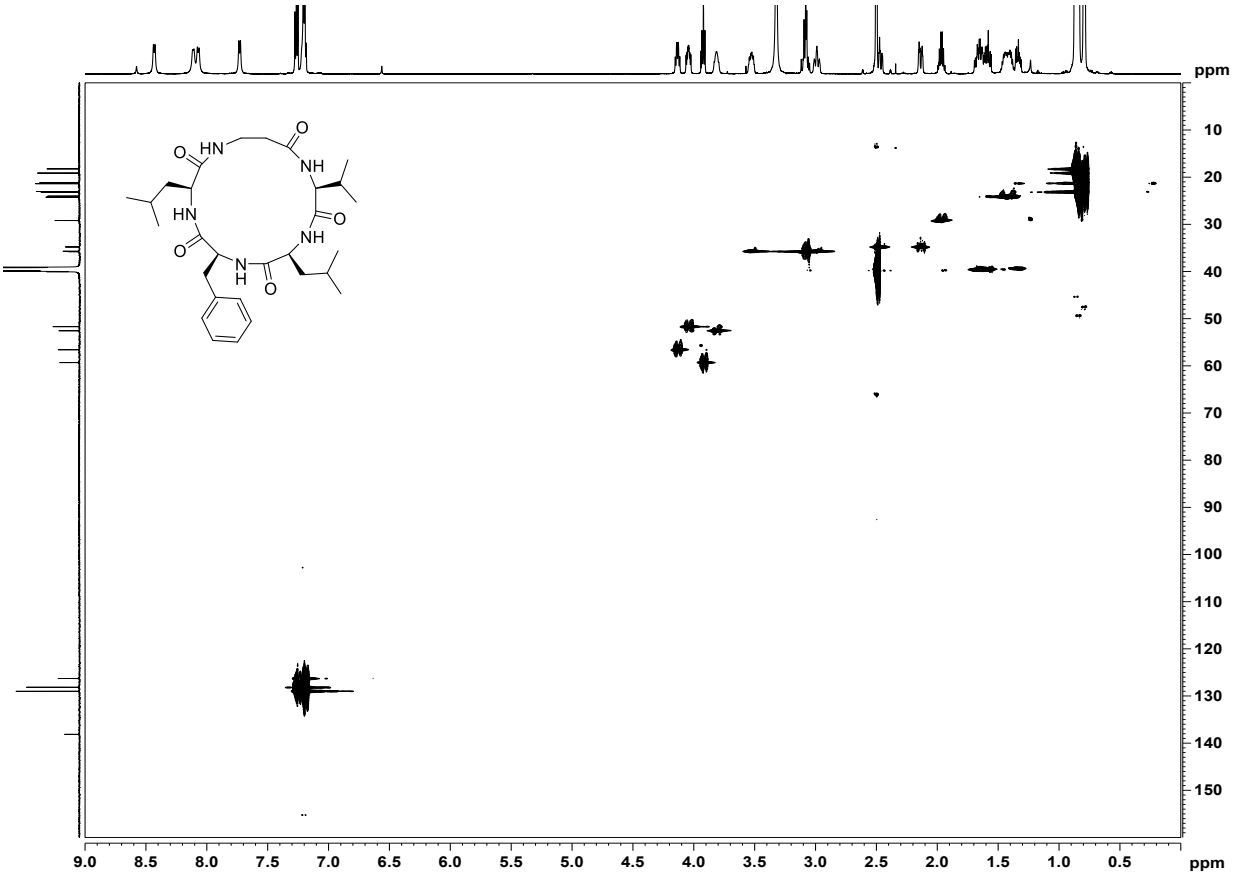
^{13}C DEPT (150 MHz, DMSO-d_6) of **4**



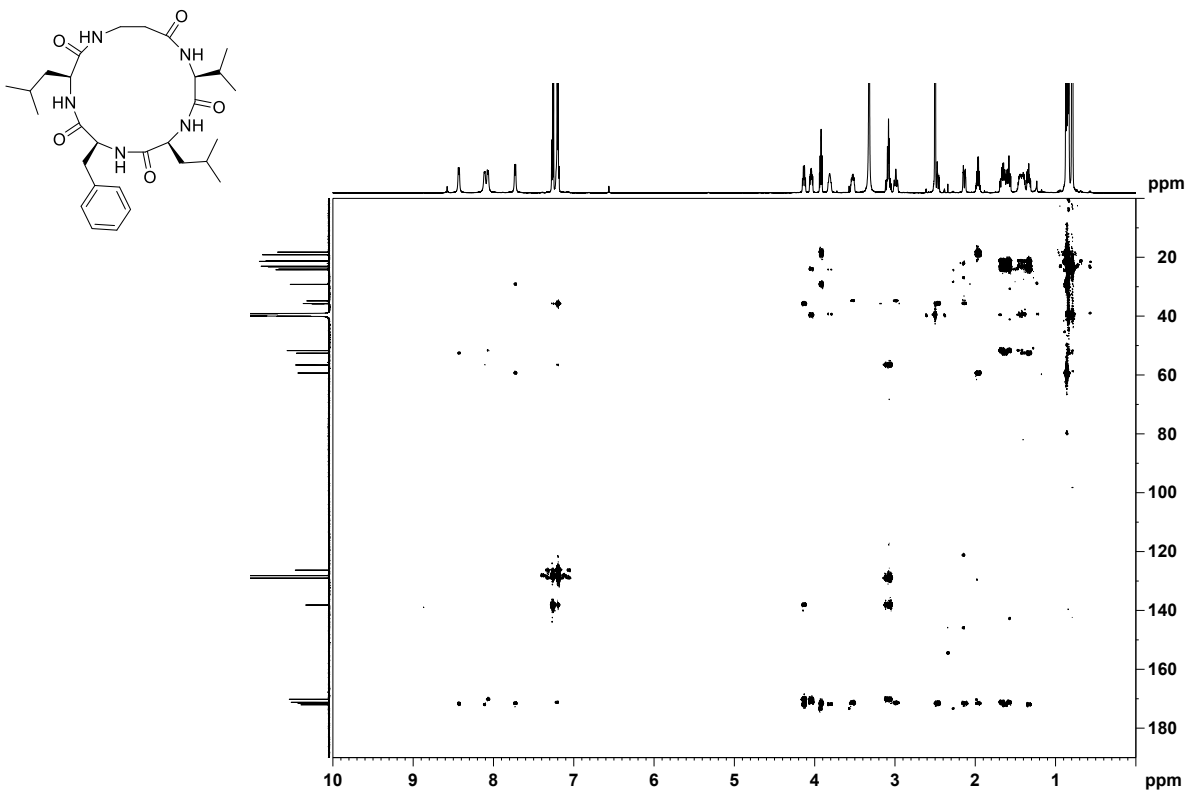
^{1}H-^{1}H COSY (600 MHz, DMSO-d_6) of **4**



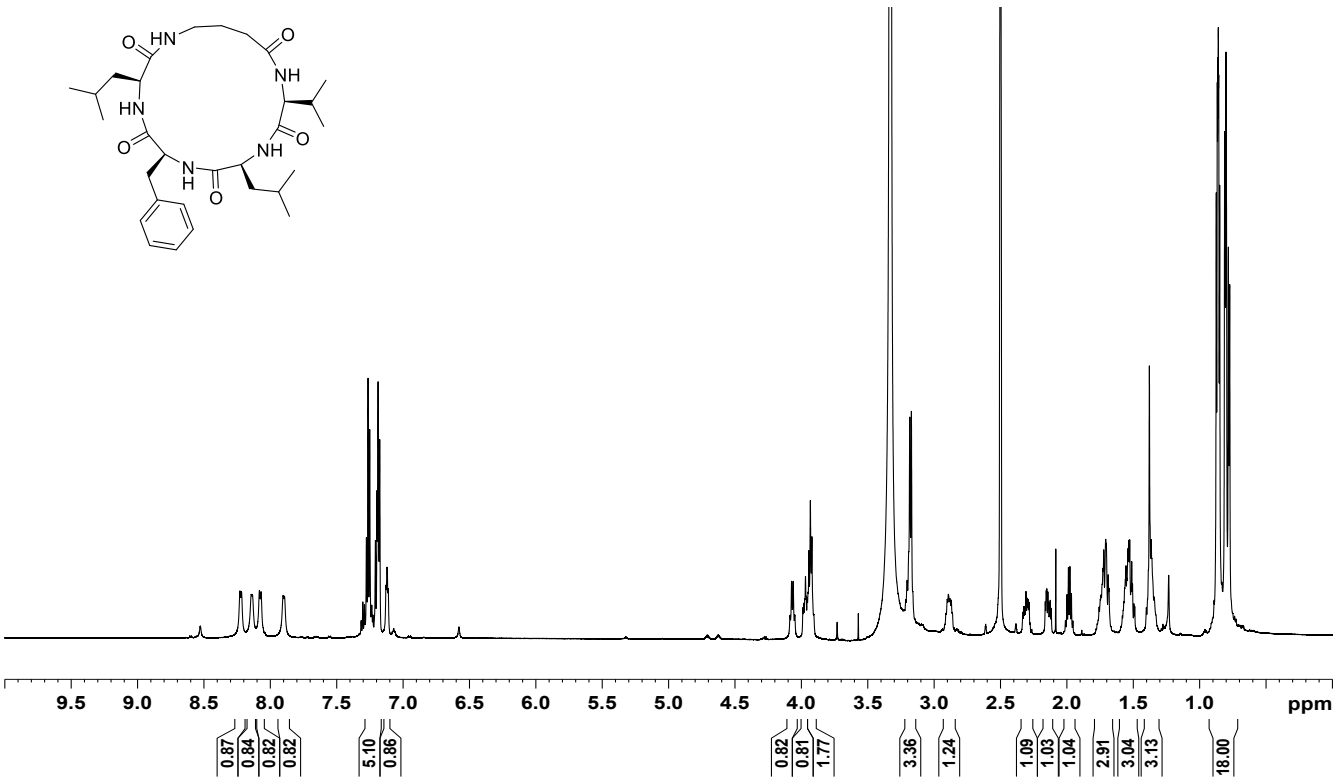
^{1}H-^{13}C HSQC (600 MHz, DMSO-d_6) of **4**



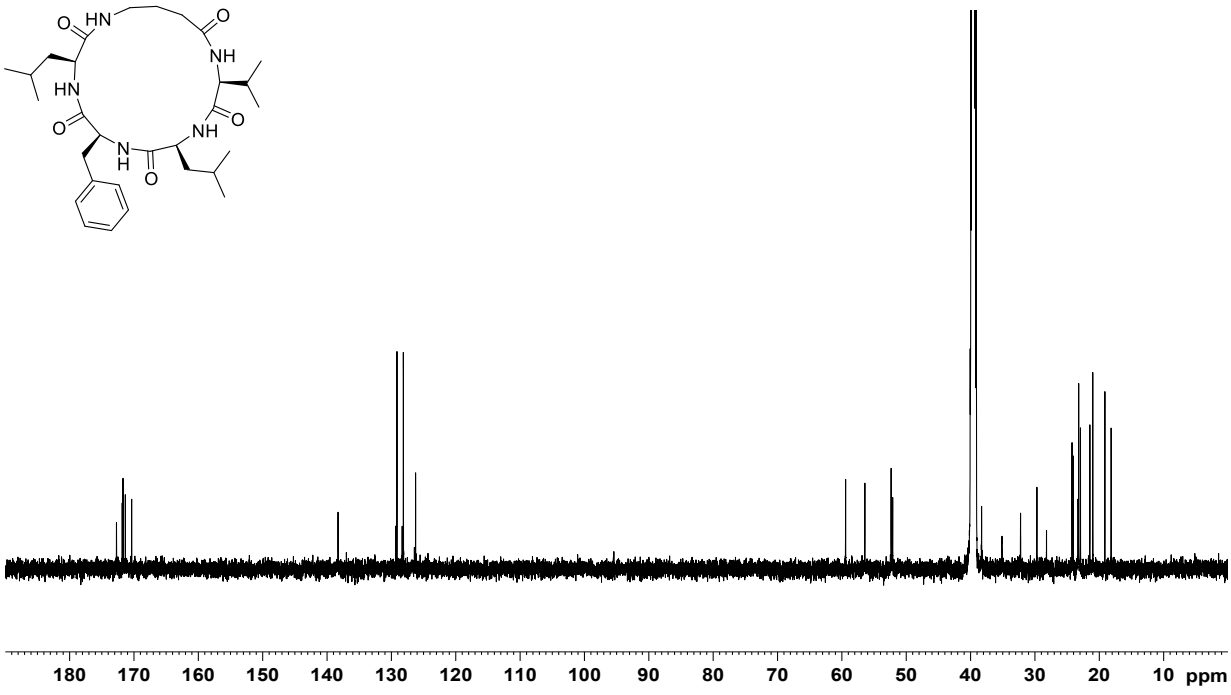
^{1}H-^{13}C HMBC (600 MHz, DMSO-d_6) of **4**



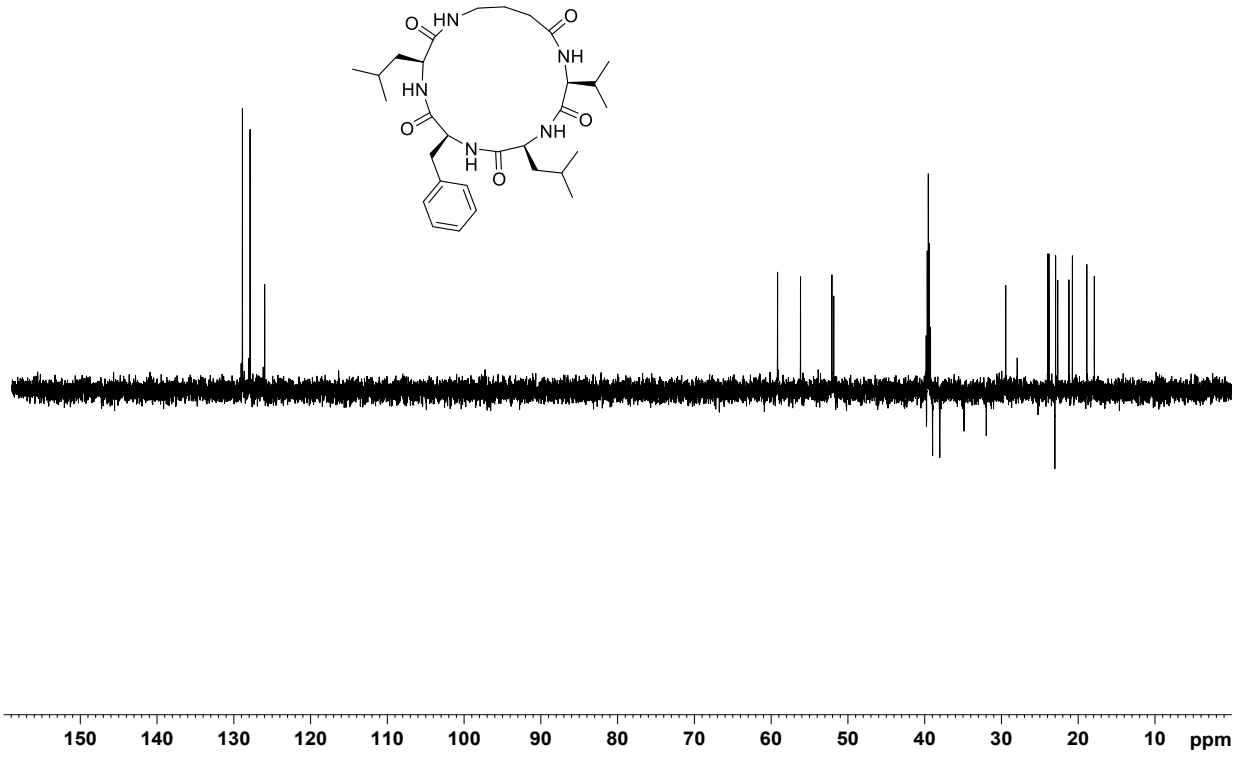
^{1}H NMR (600 MHz, DMSO-d_6) of **5**



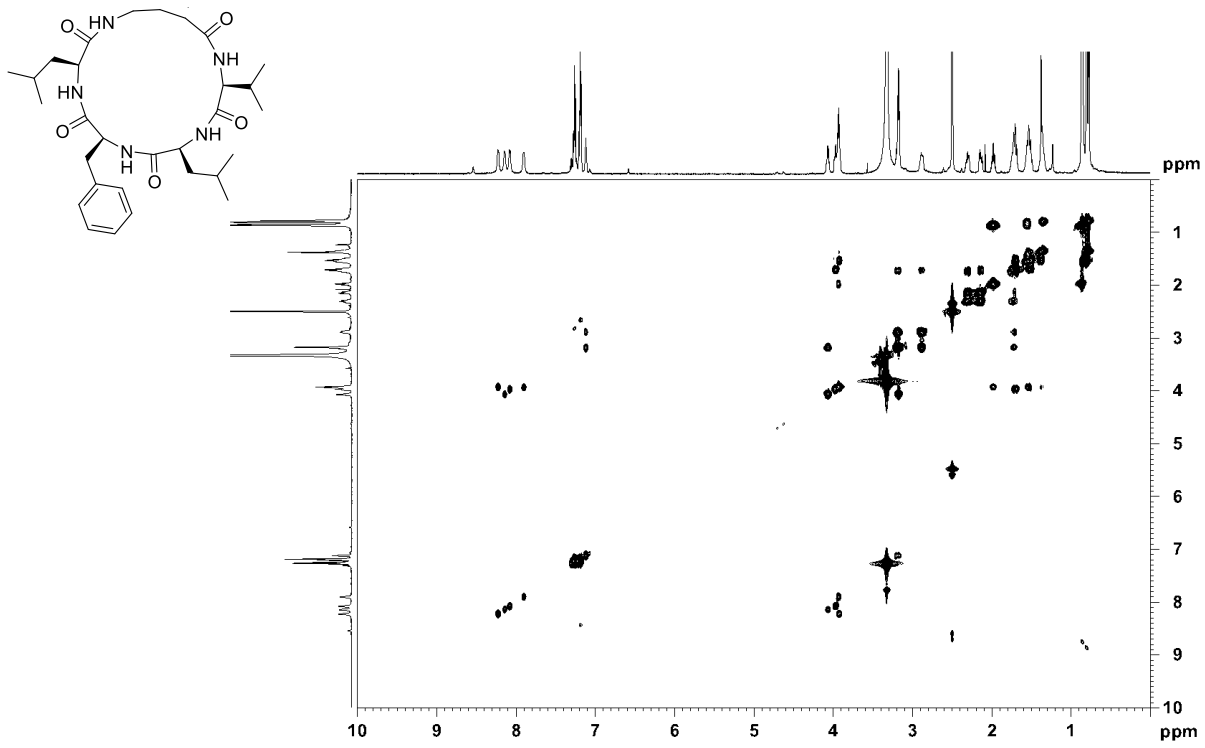
^{13}C NMR (150 MHz, DMSO-d_6) of **5**



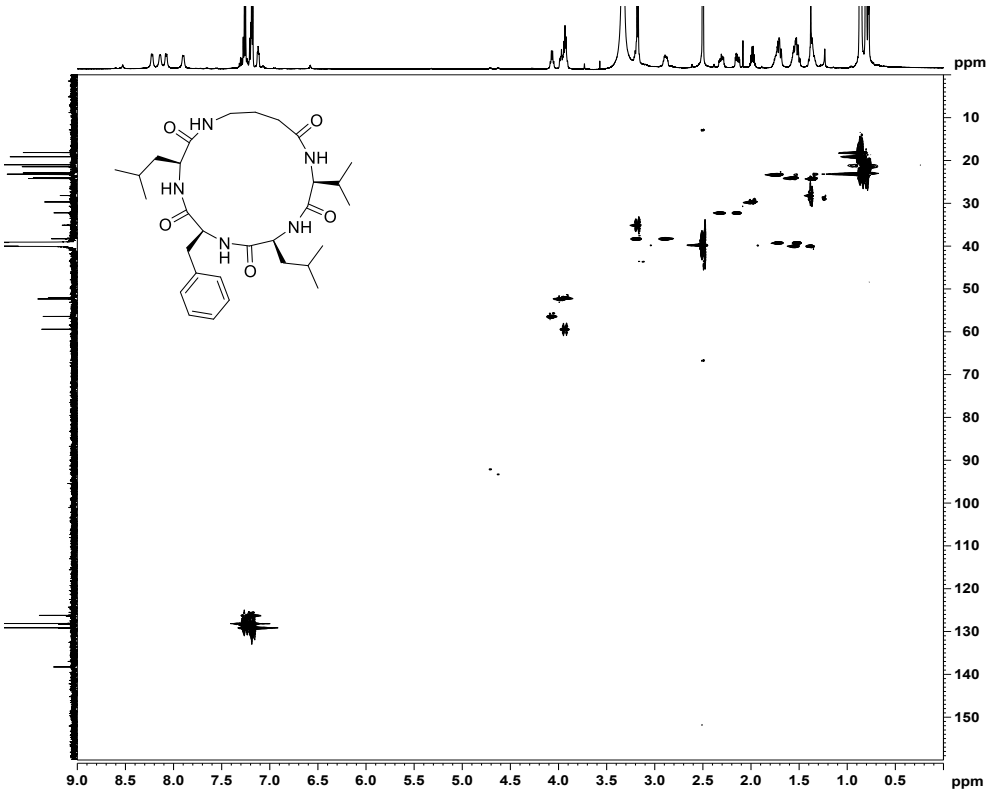
^{13}C DEPT (150 MHz, DMSO-d_6) of **5**



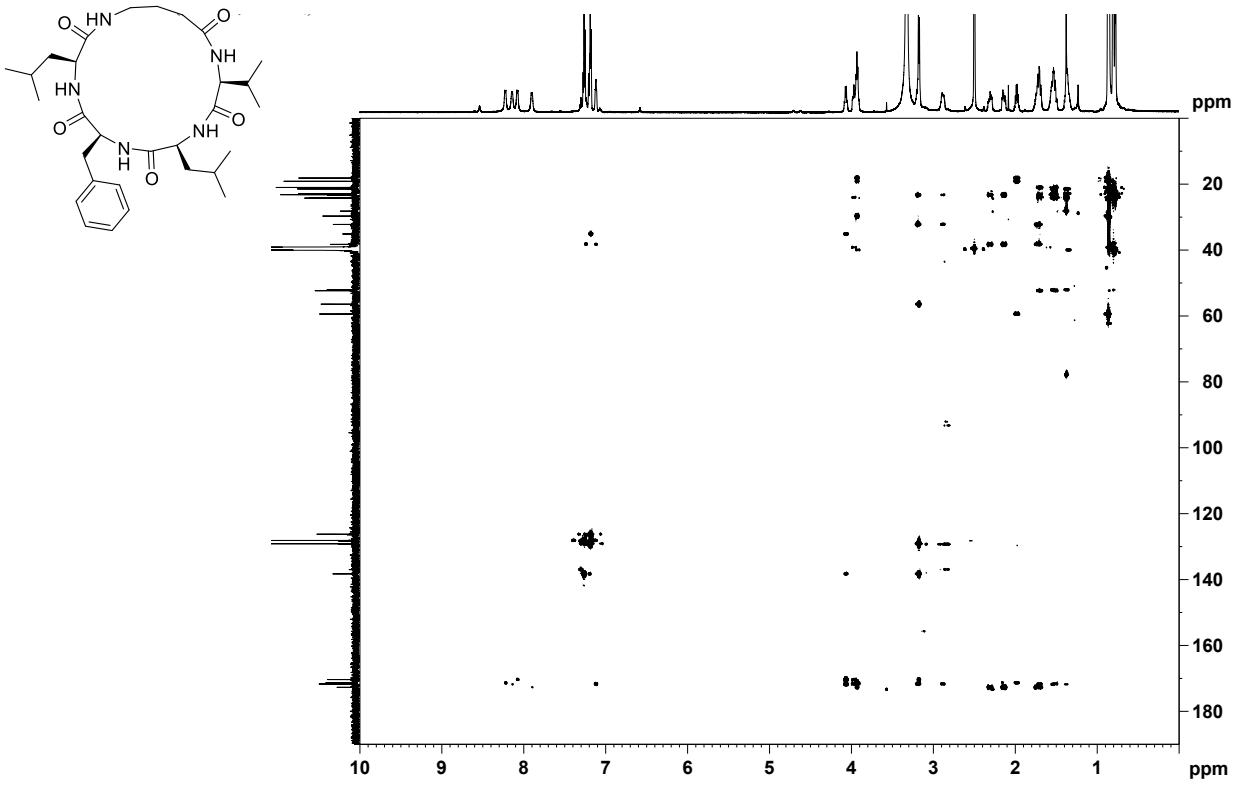
^{1}H-^{1}H COSY (600 MHz, DMSO-d_6) of **5**



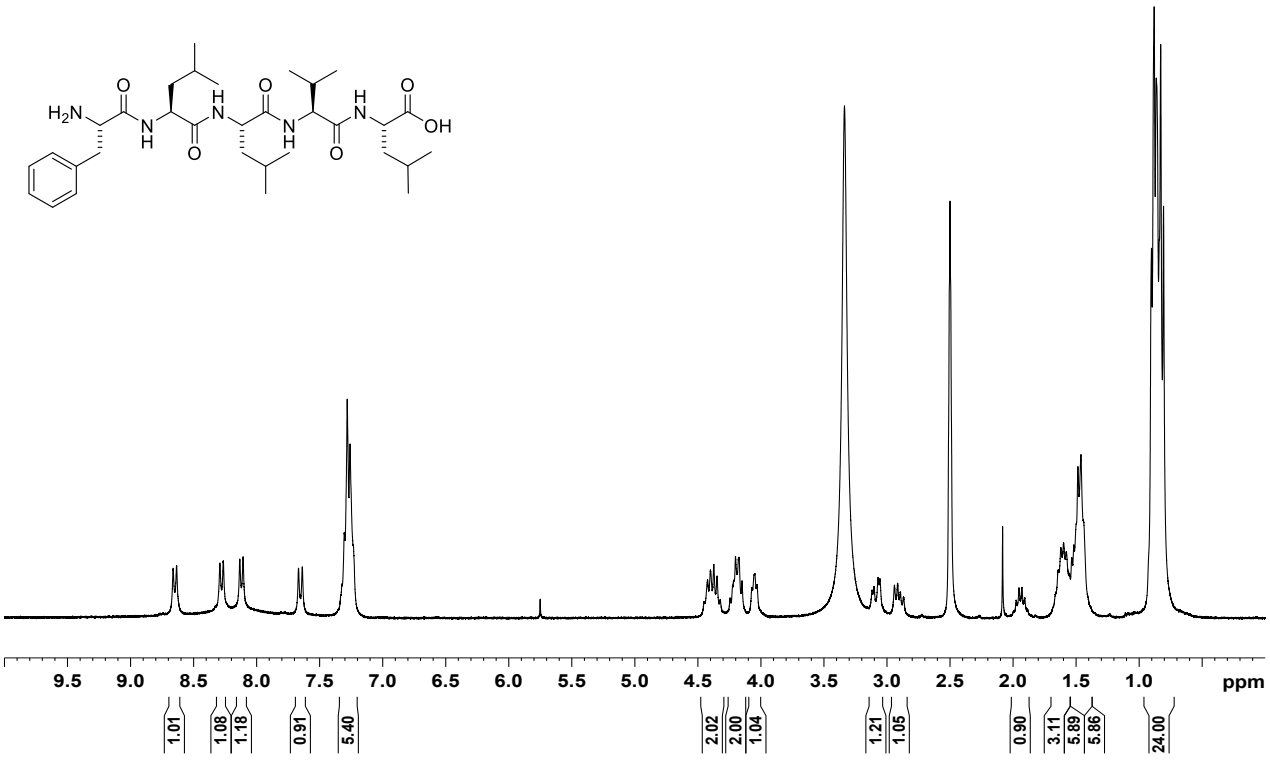
^{1}H-^{13}C HSQC (600 MHz, DMSO-d_6) of **5**



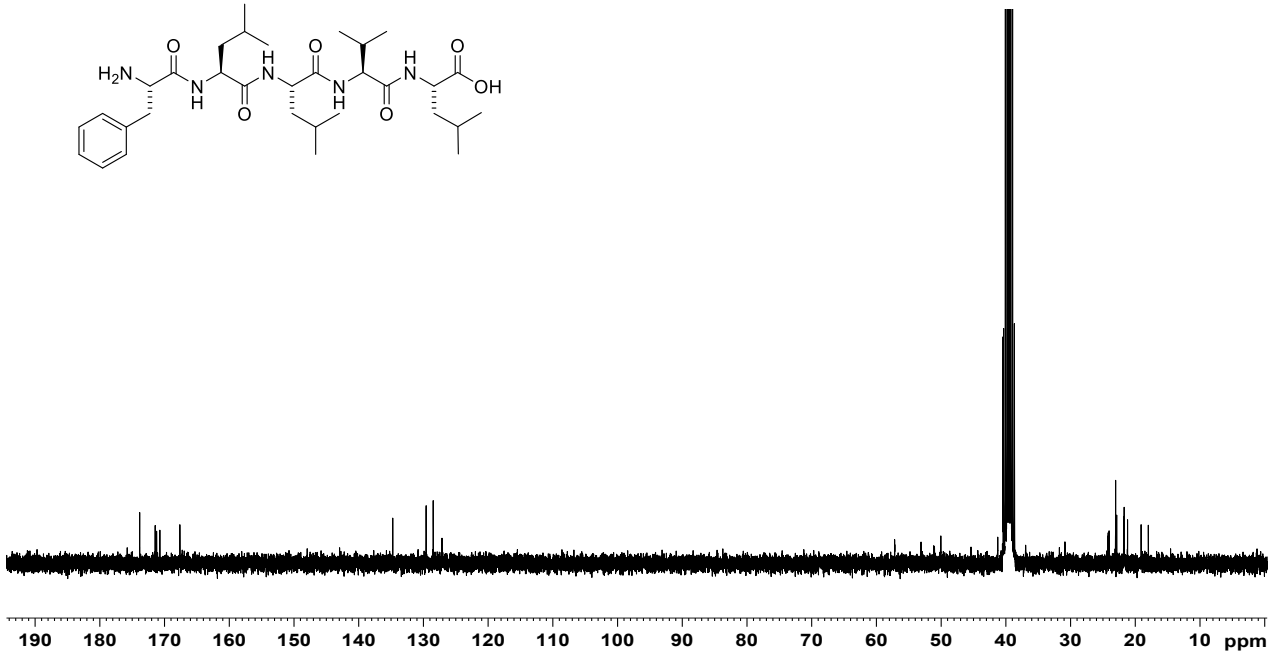
^{1}H-^{13}C HMBC (600 MHz, DMSO-d_6) of **5**



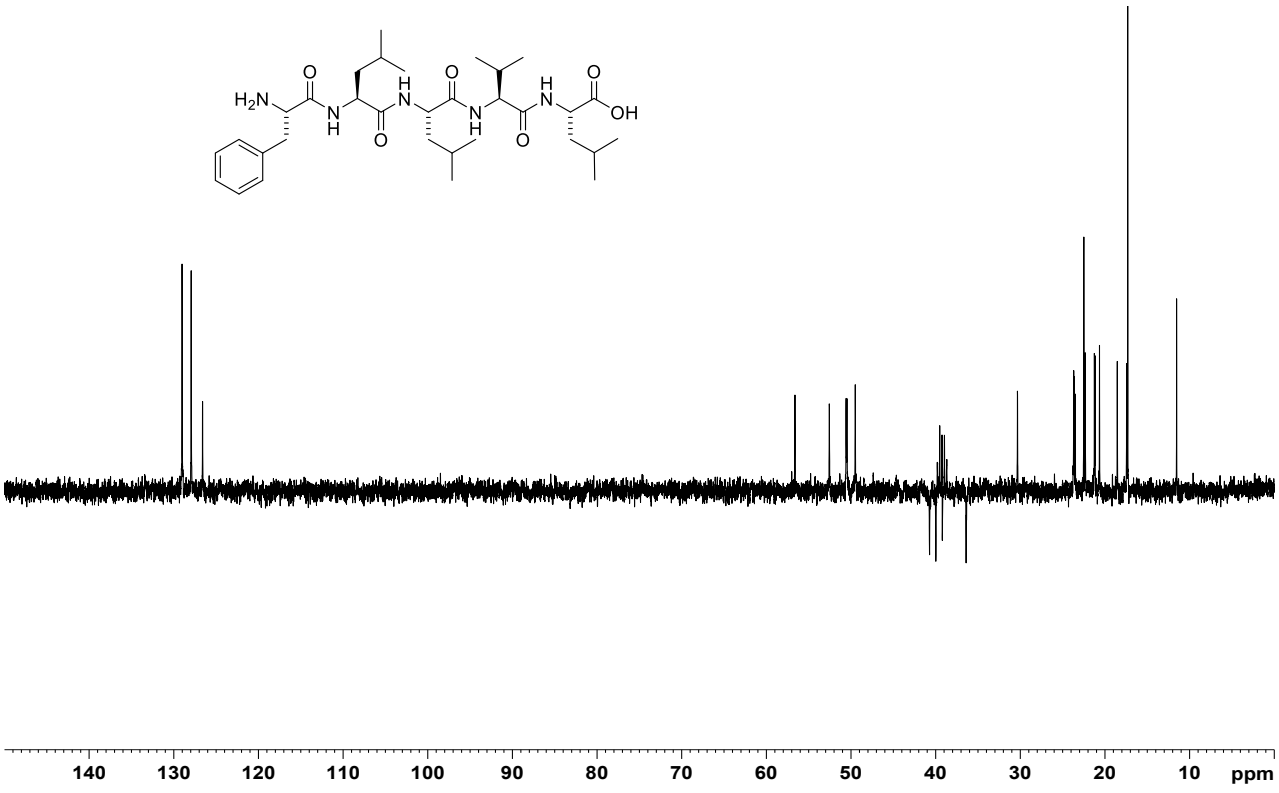
^{1}H NMR (300 MHz, DMSO-d_6) of **7**



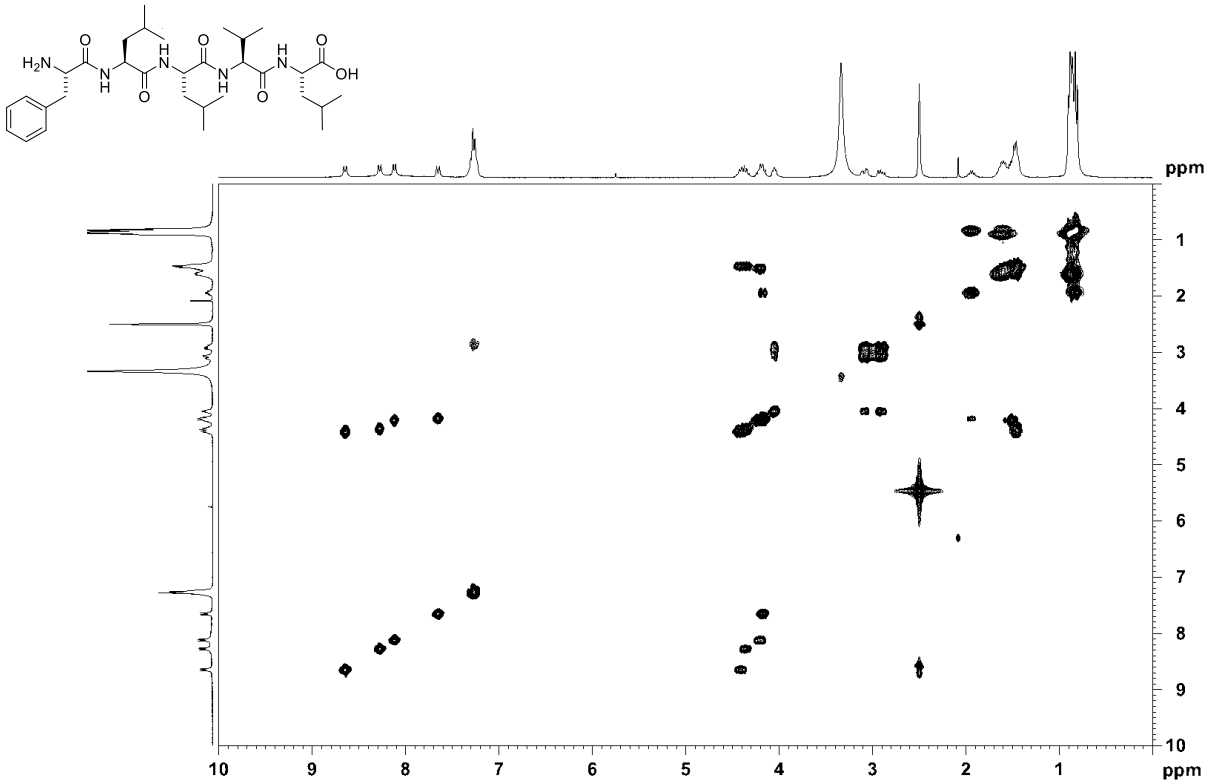
^{13}C NMR (75 MHz, DMSO-d_6) of **7**



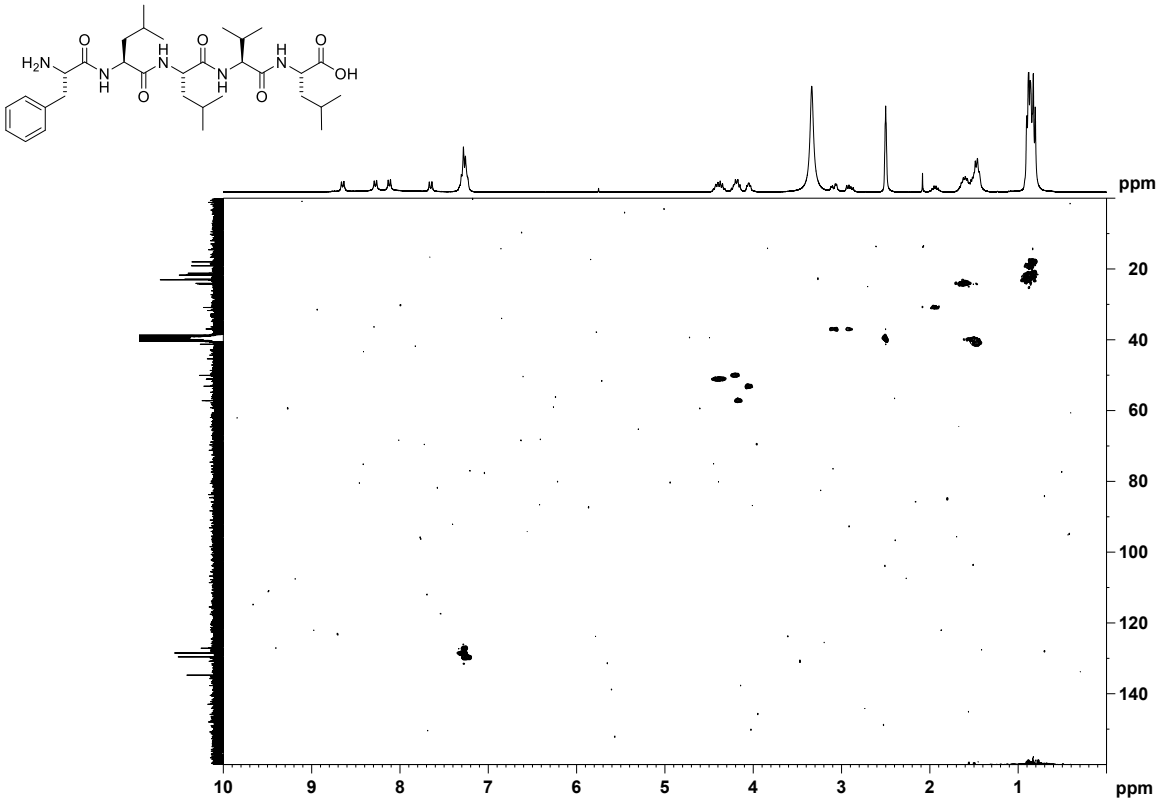
^{13}C DEPT (300 MHz, DMSO-d_6) of **7**



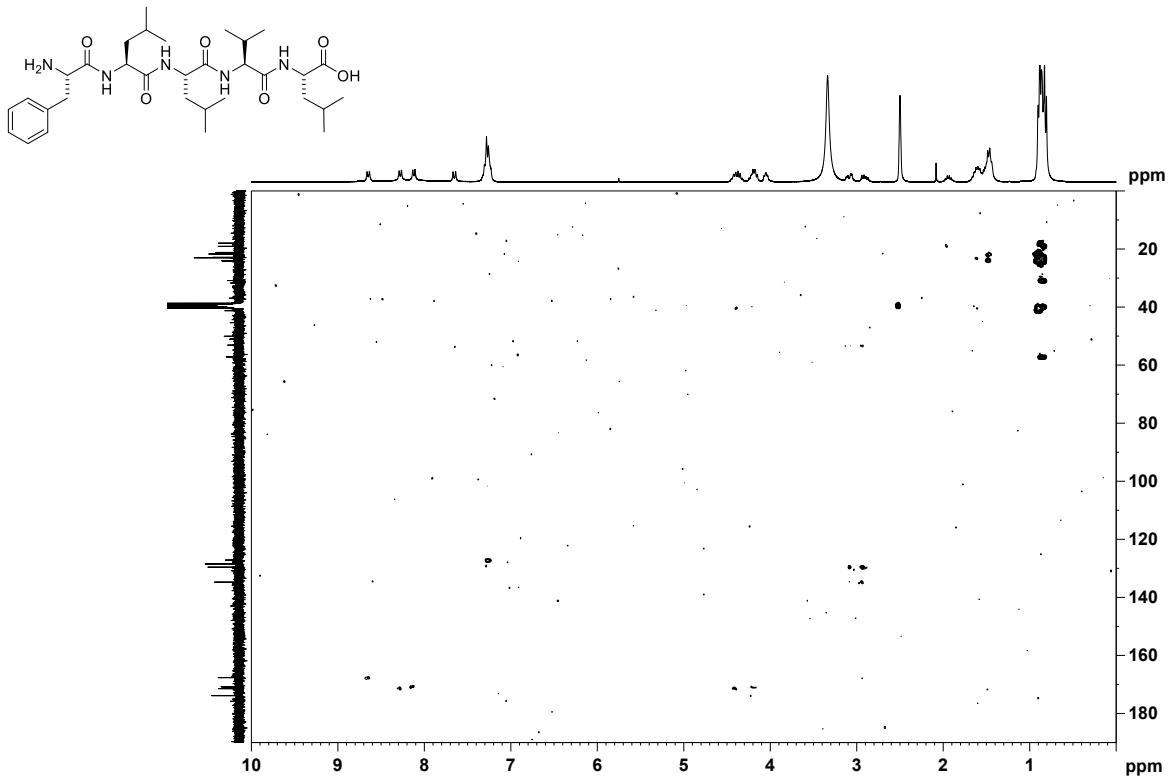
^{1}H-^{1}H COSY (300 MHz, DMSO-d_6) of **7**



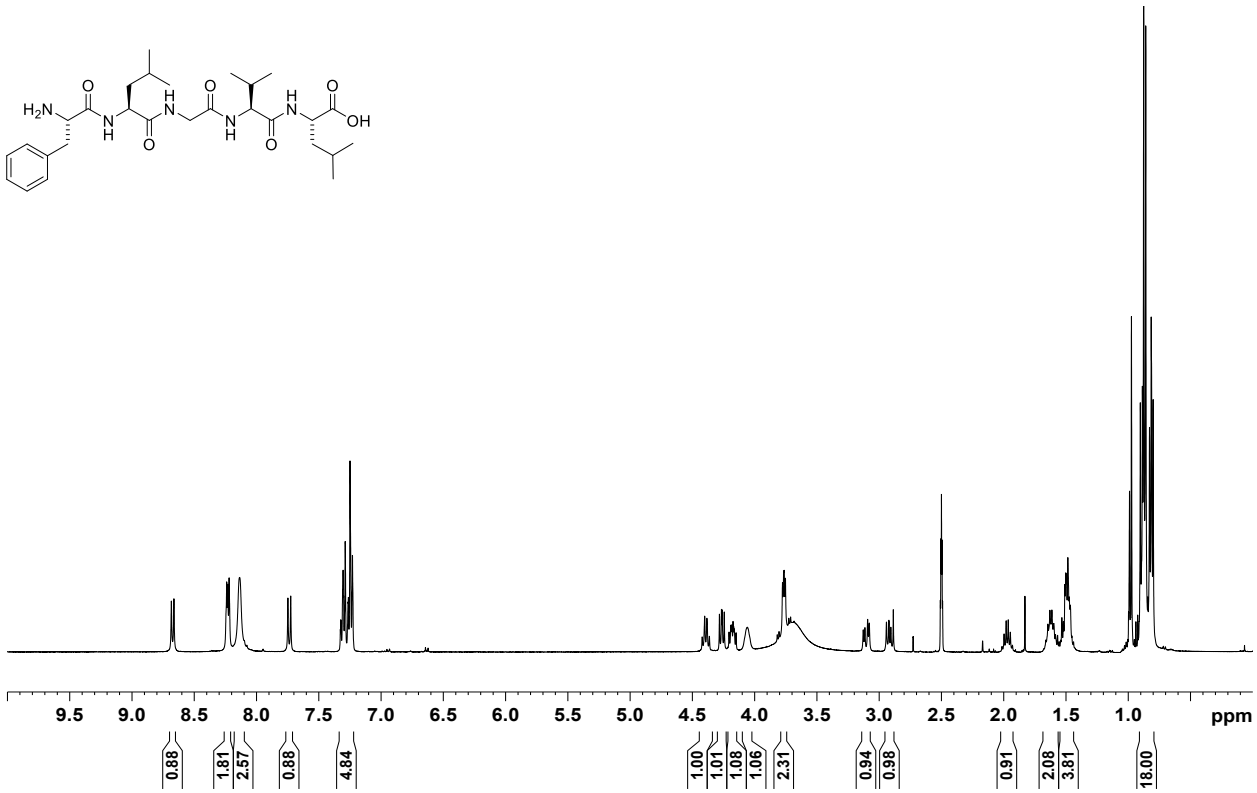
^{1}H-^{13}HSQC (300 MHz, DMSO-d_6) of **7**



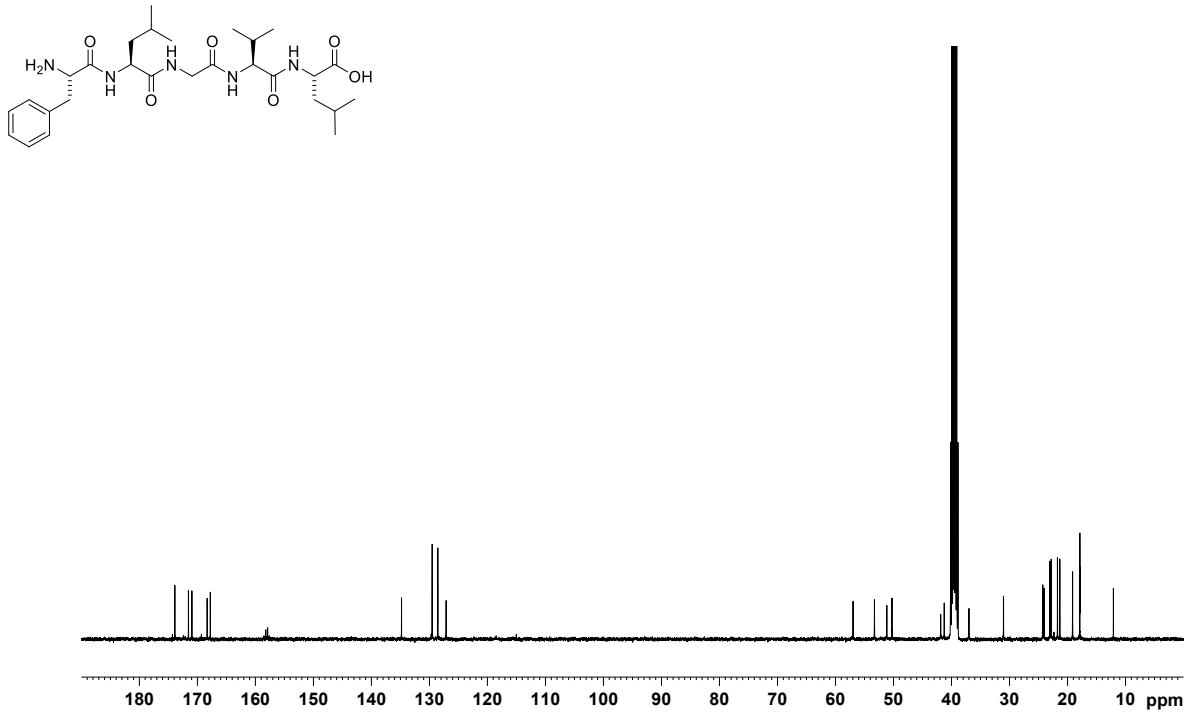
^{1}H-^{13}C HMBC (300 MHz, DMSO-d_6) of **7**



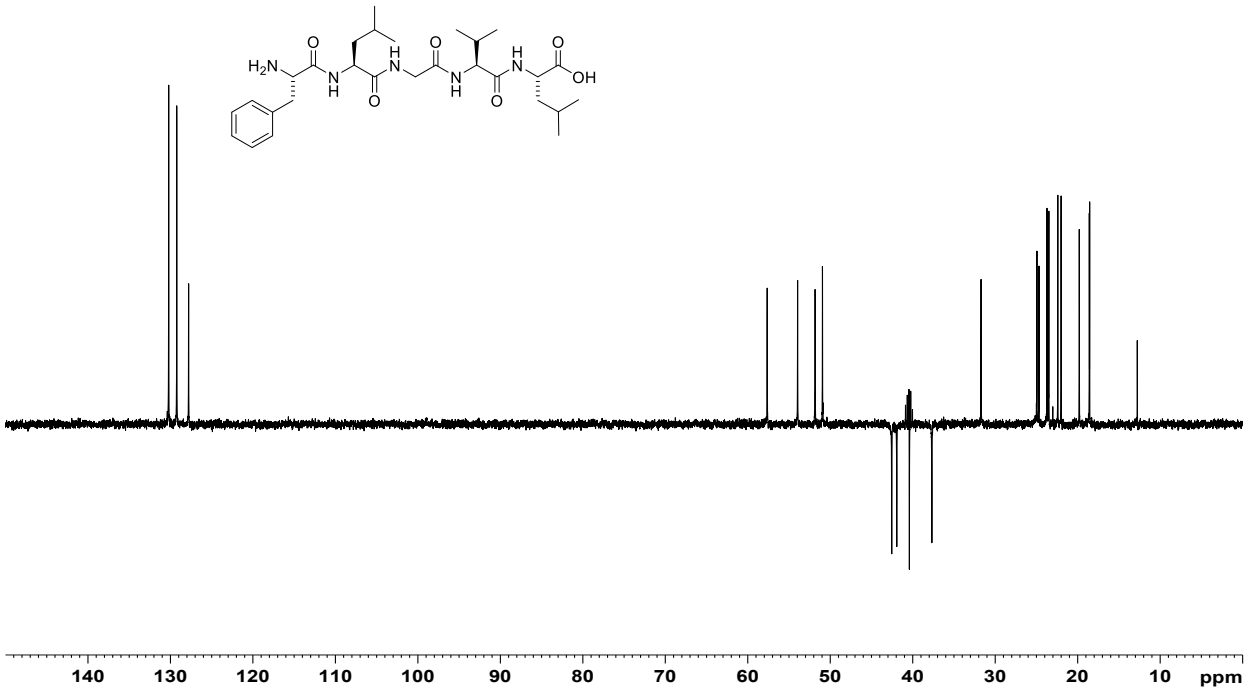
^{1}H NMR (400 MHz, DMSO-d_6) of **8**



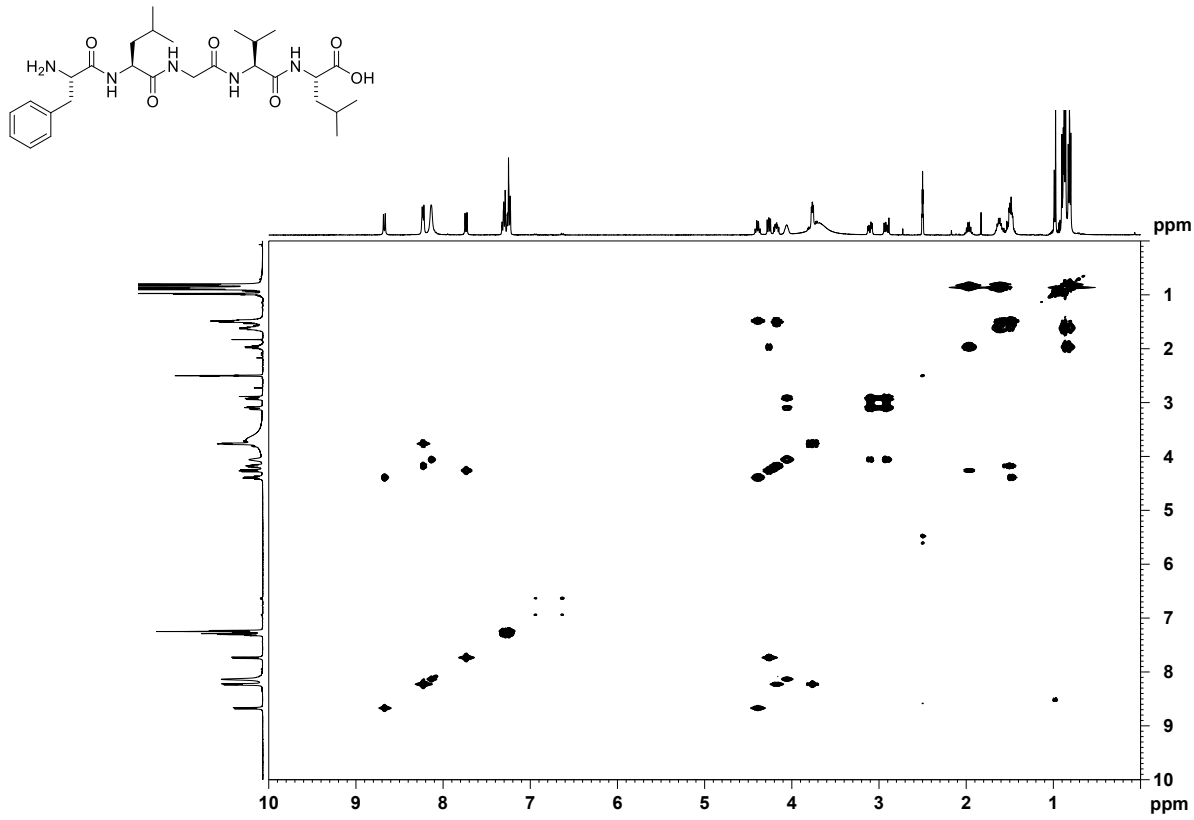
^{13}C NMR (100 MHz, DMSO-d_6) of **8**



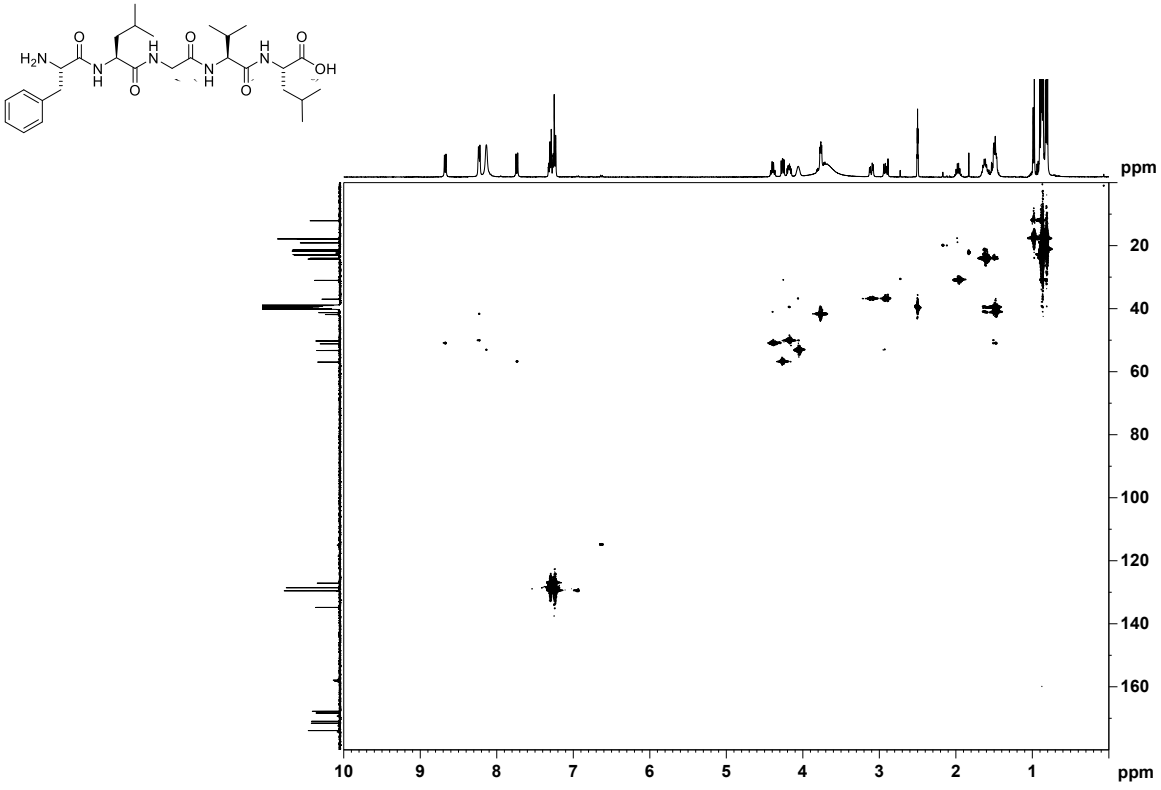
^{13}C DEPT (100 MHz, DMSO-d_6) of **8**



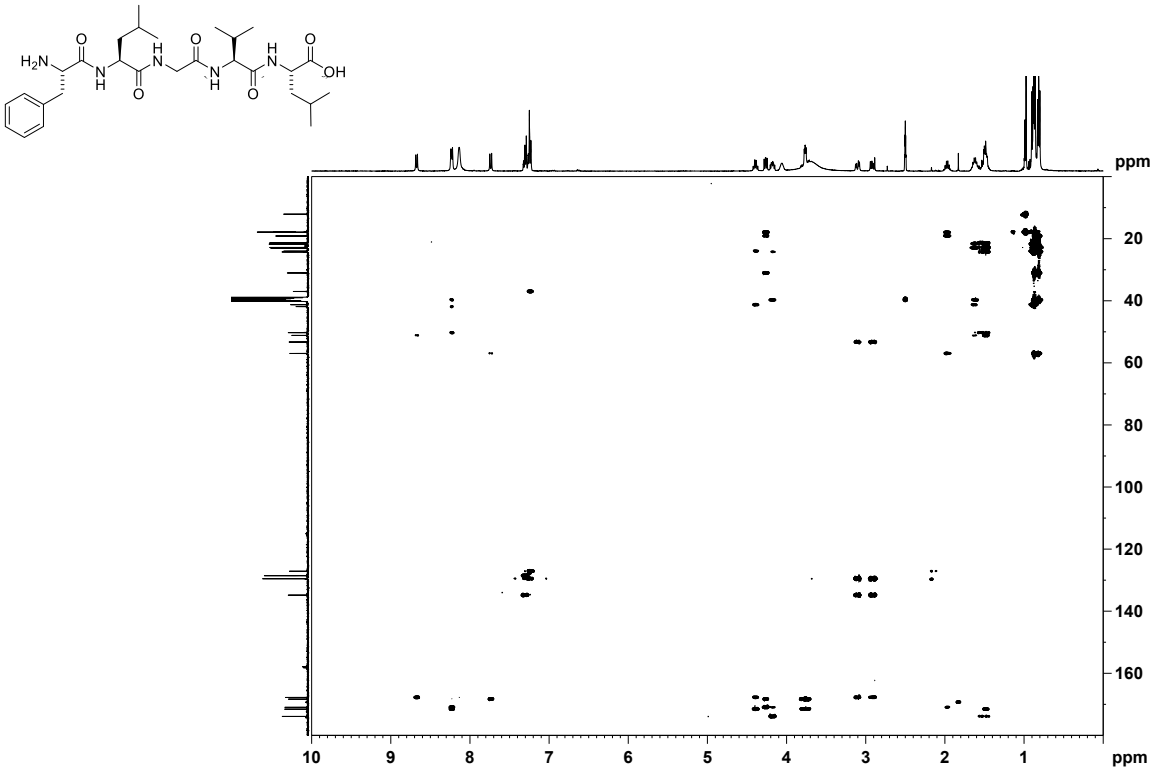
^{1}H-^{13}C HSQC (400 MHz, DMSO-d_6) of **8**



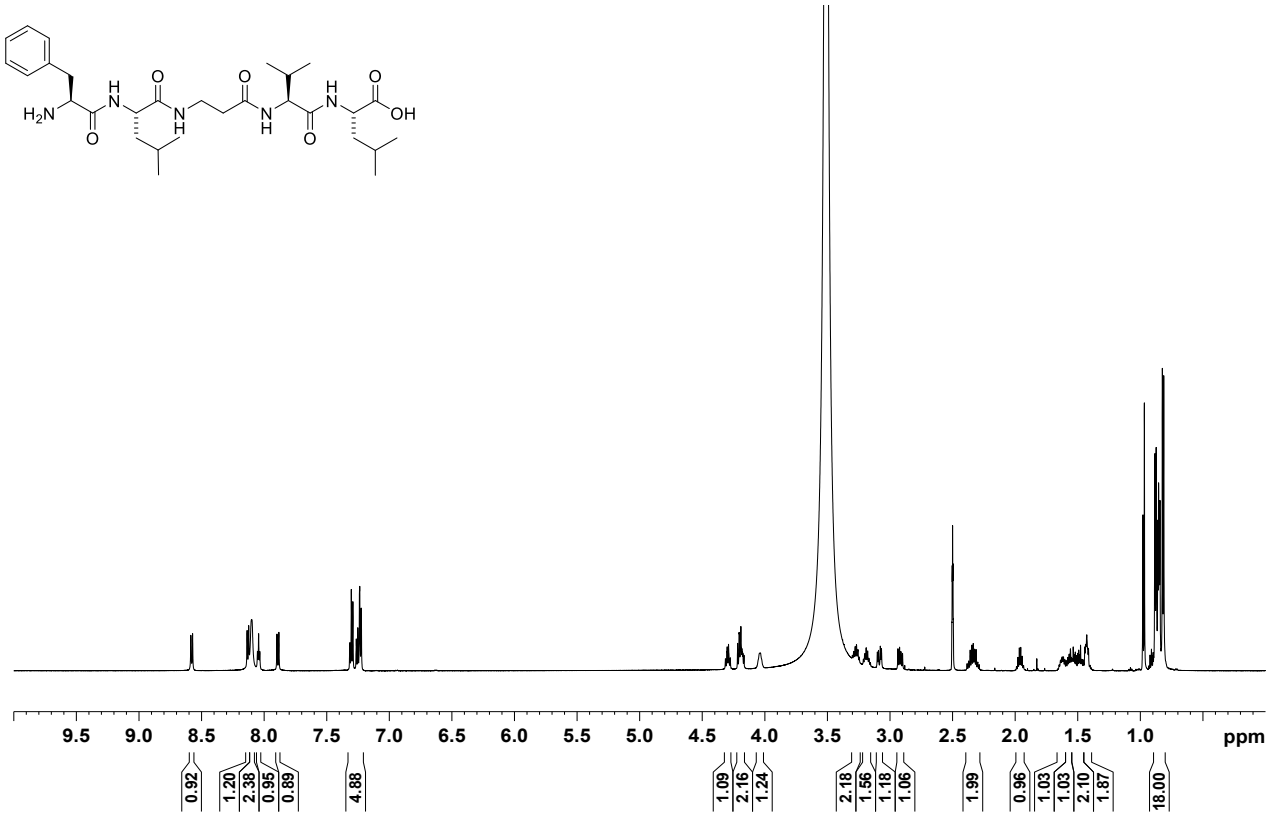
^{1}H-^{13}C HSQC (400 MHz, DMSO-d_6) of **8**



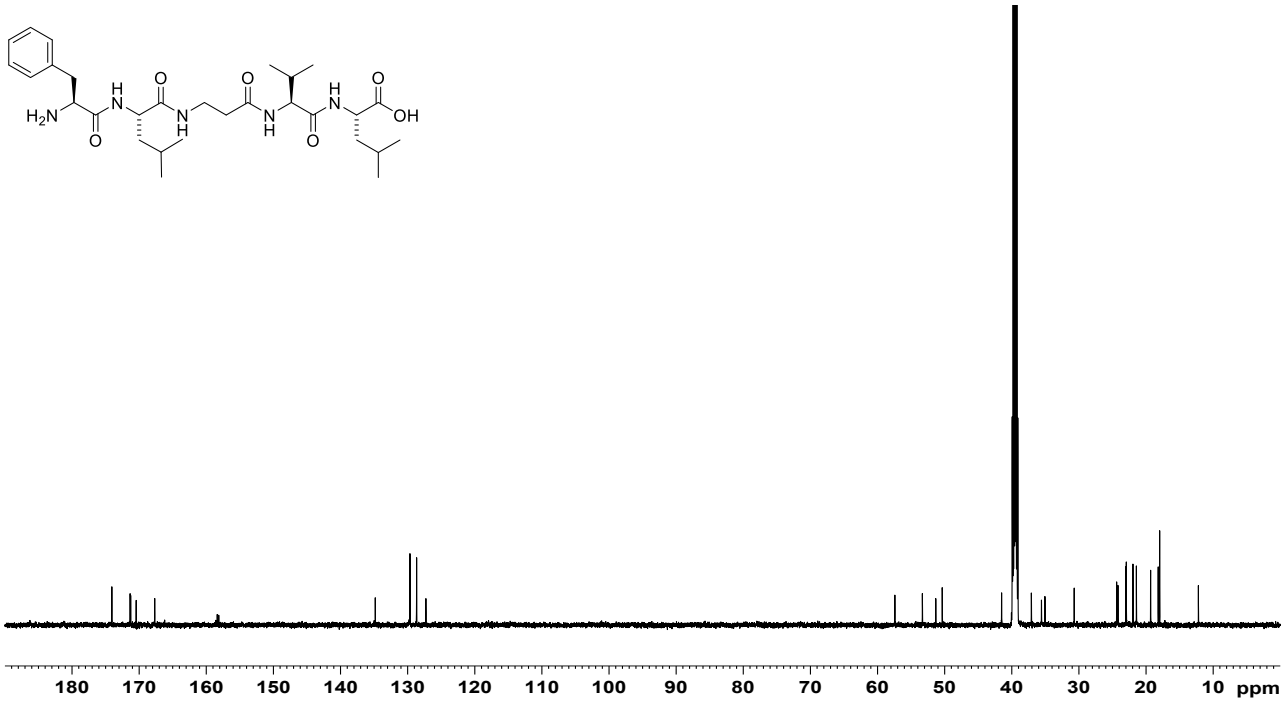
^{1}H-^{13}C HMBC (400 MHz, DMSO-d_6) of **8**



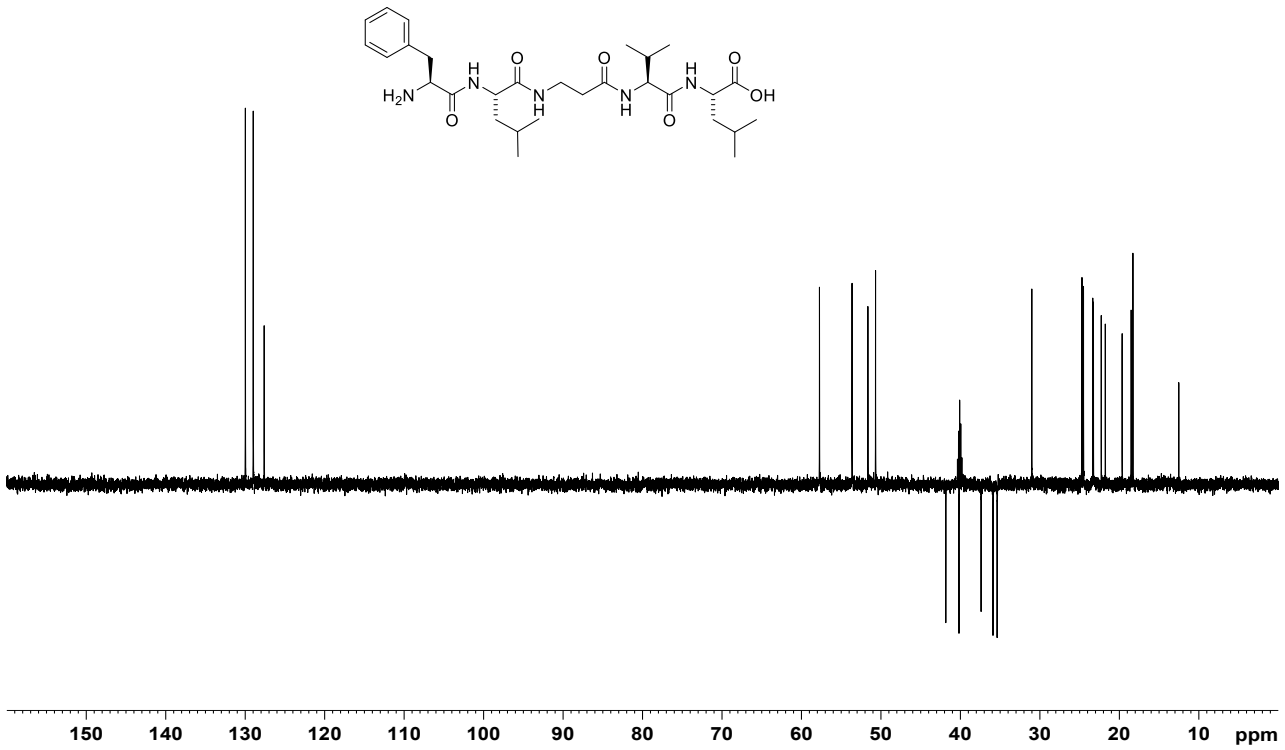
^{1}H NMR (600 MHz, DMSO-d_6) of **9**



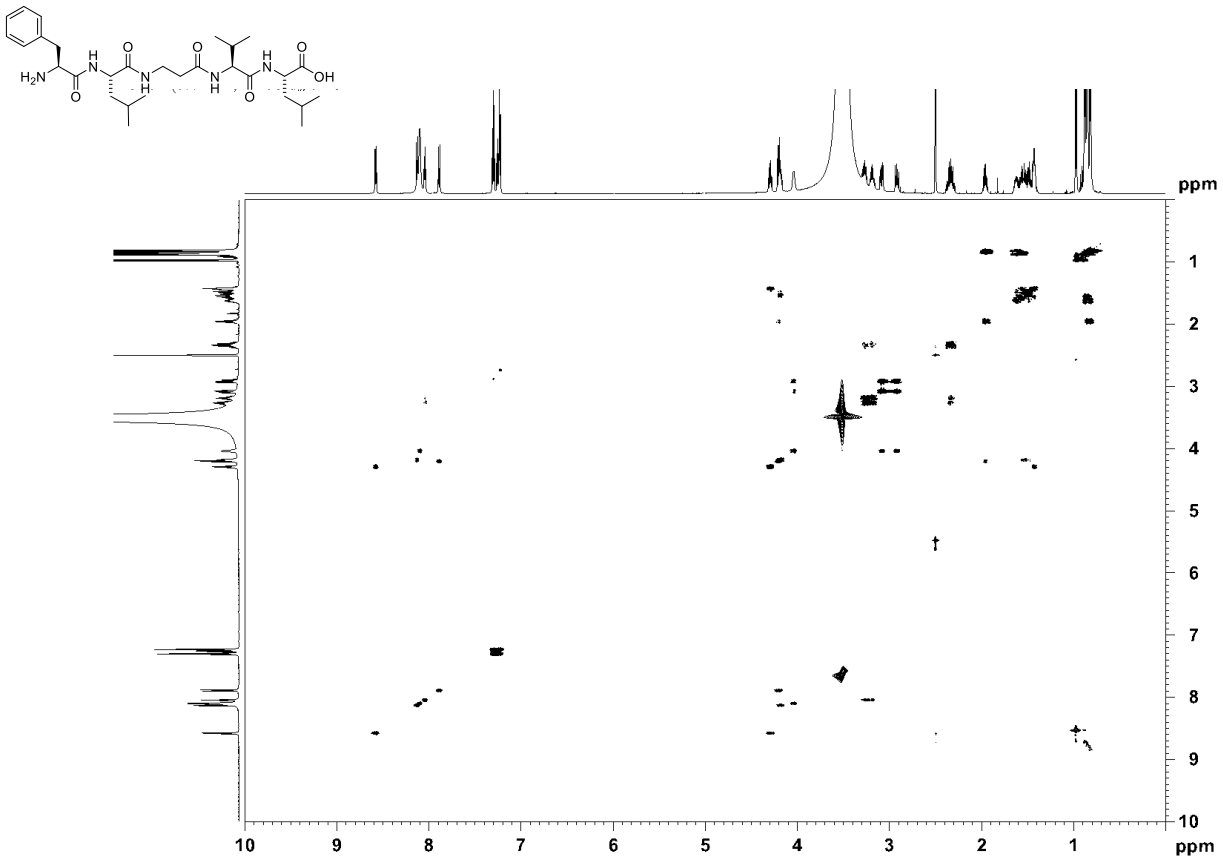
^{13}C NMR (150 MHz, DMSO-d_6) of **9**



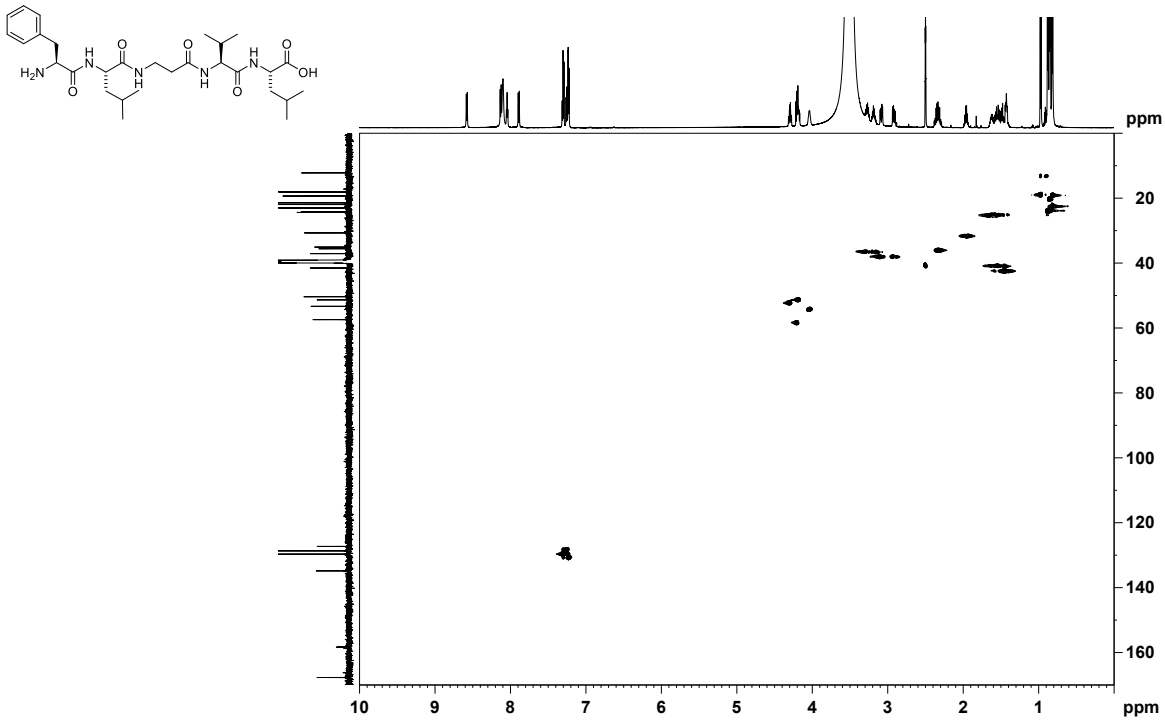
^{13}C DEPT (150 MHz, DMSO-d_6) of **9**



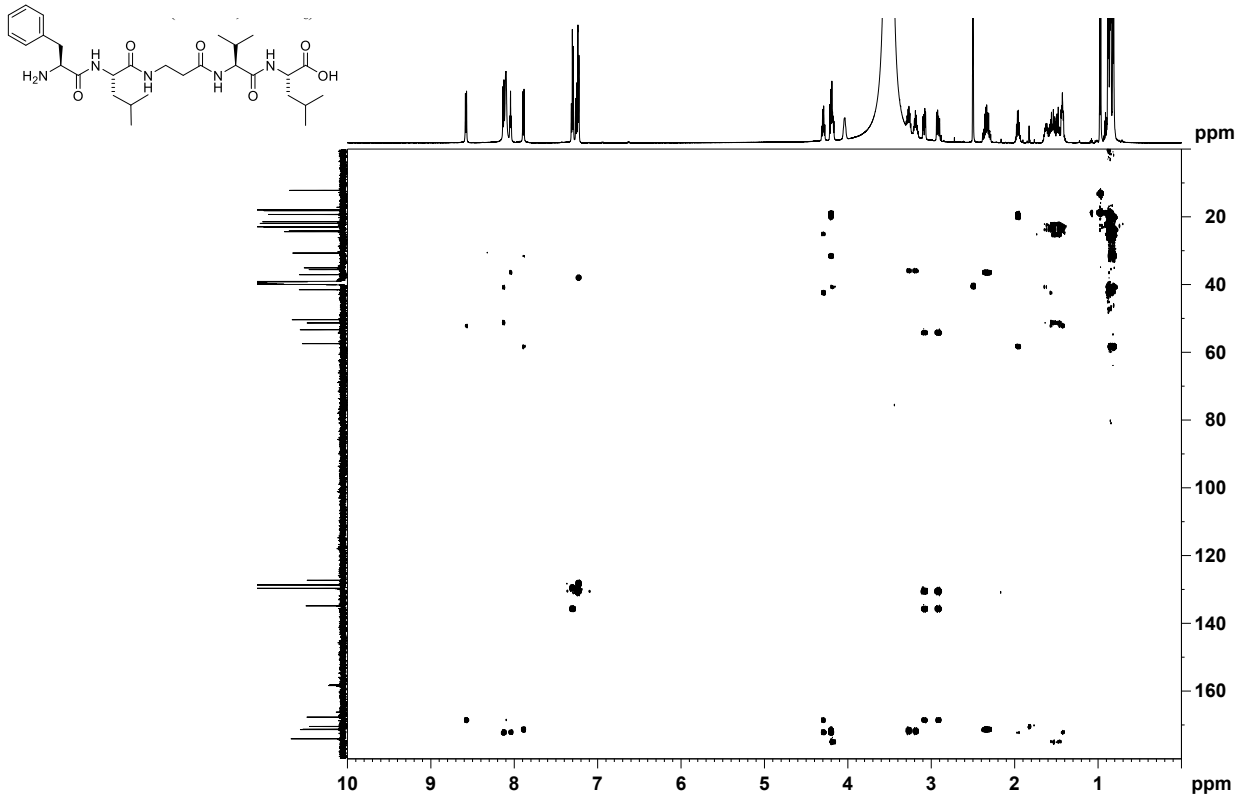
^{1}H-^{1}H COSY (600 MHz, DMSO-d_6) of **9**



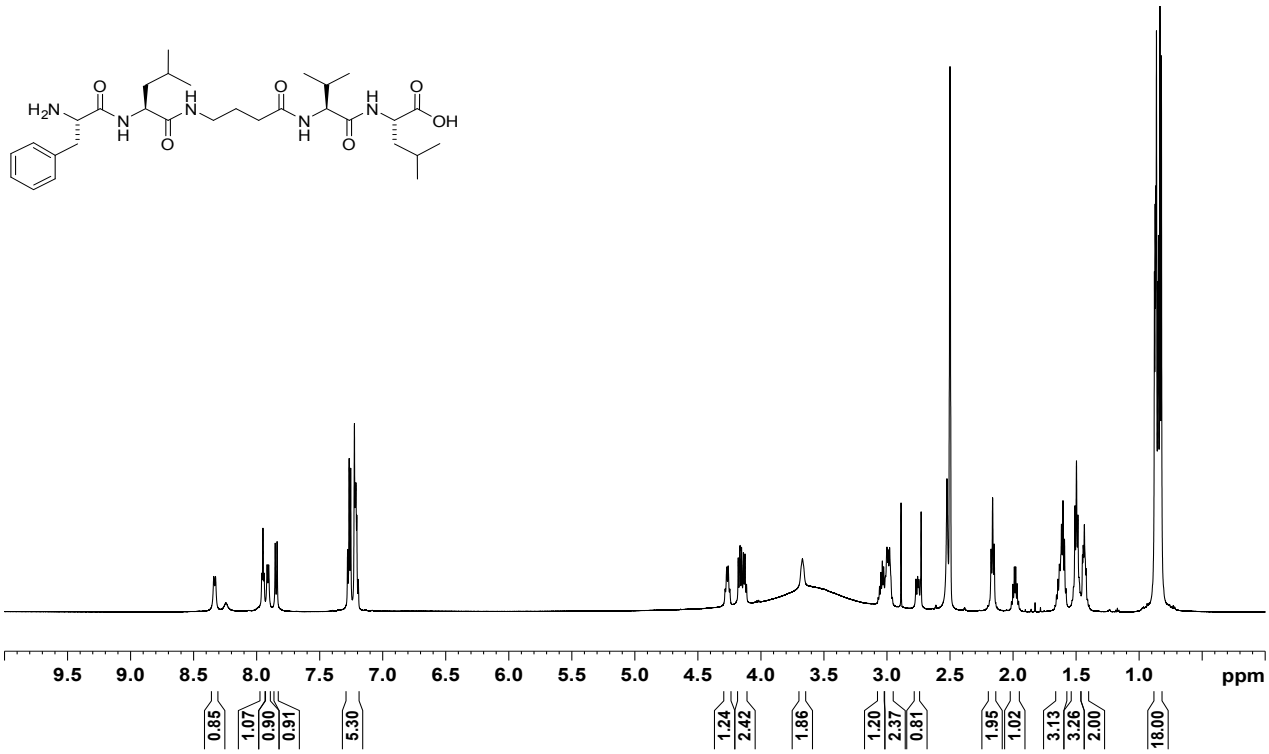
^{1}H-^{13}C HSQC (600 MHz, DMSO-d_6) of **9**



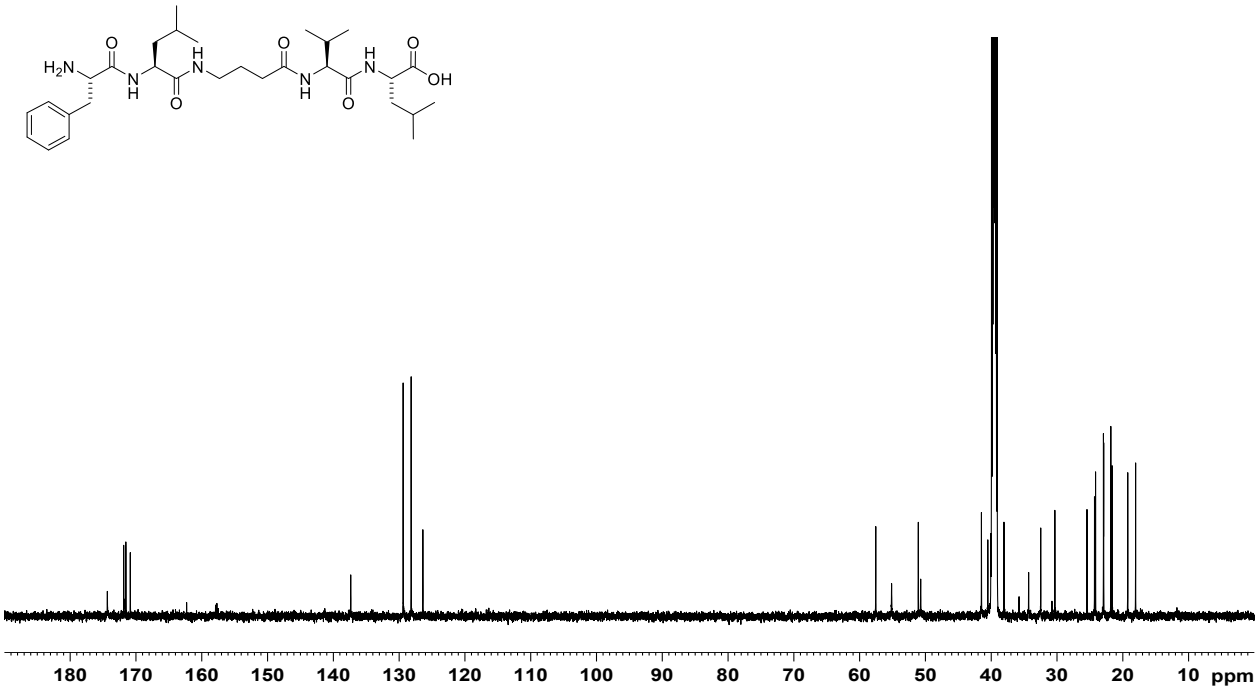
^{1}H-^{13}C HMBC (600 MHz, DMSO-d_6) of **9**



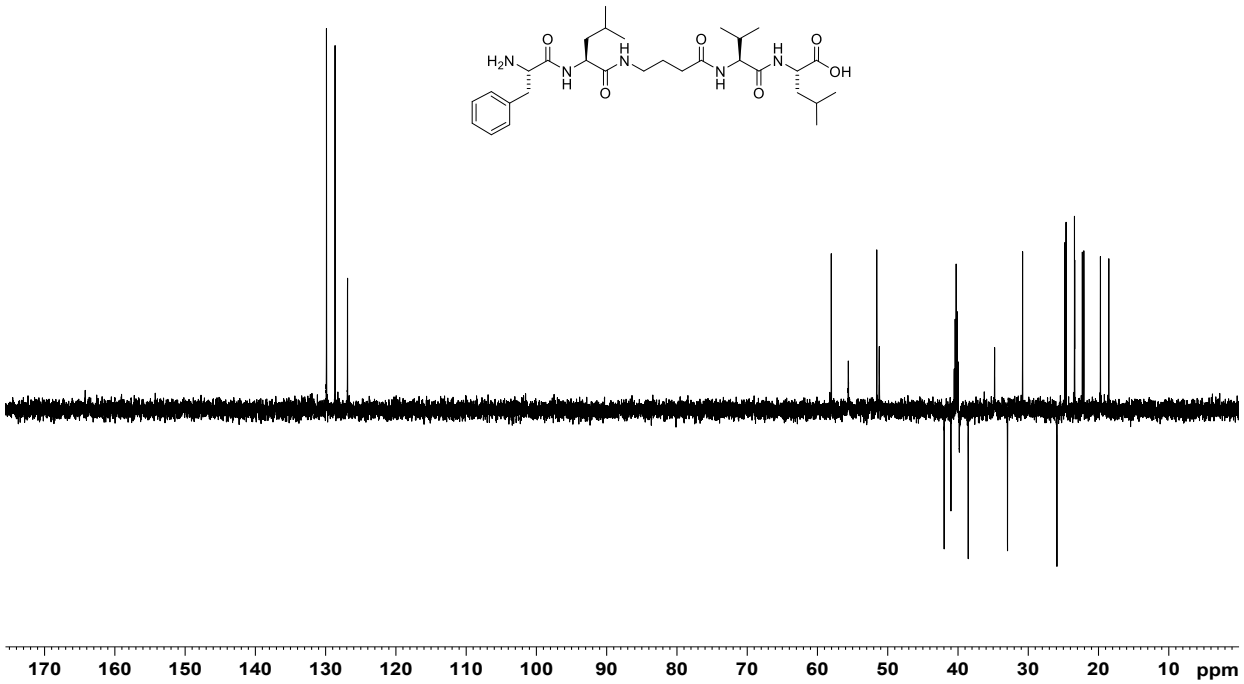
^{1}H NMR (600 MHz, DMSO-d_6) of **10**



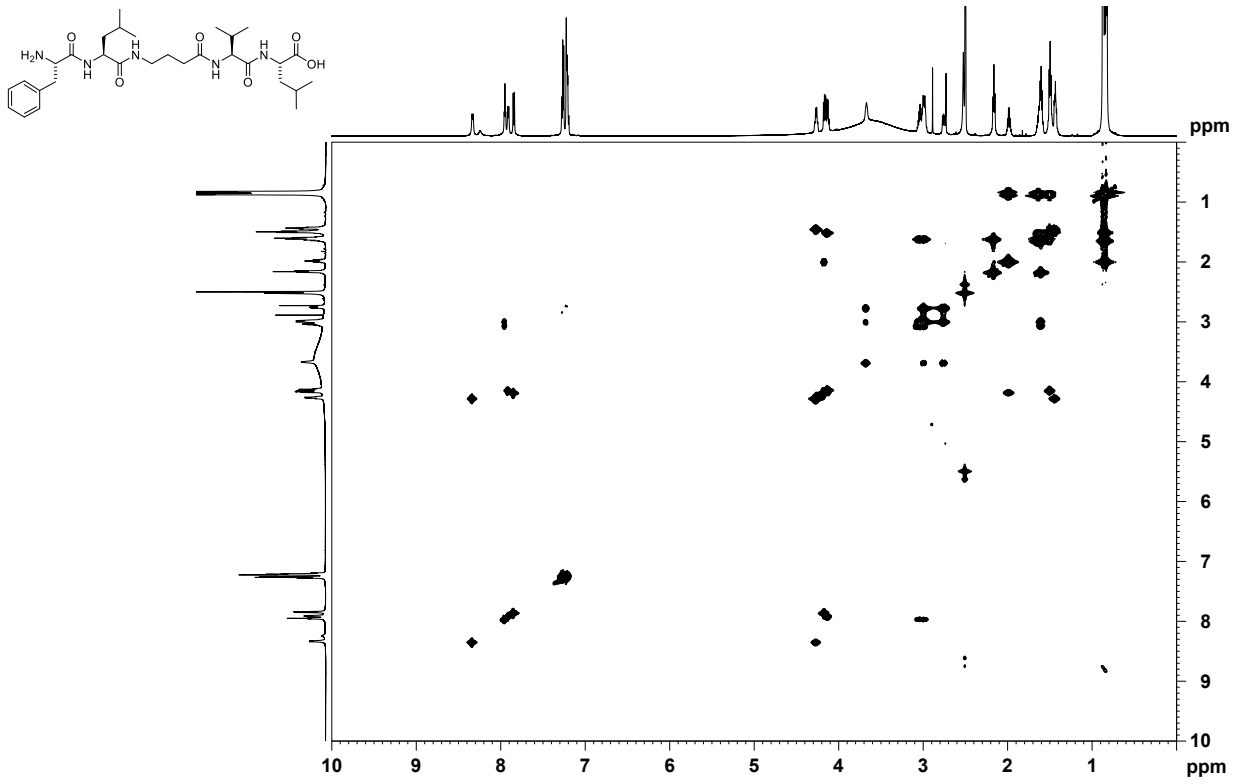
^{13}C NMR (150 MHz, DMSO-d_6) of **10**



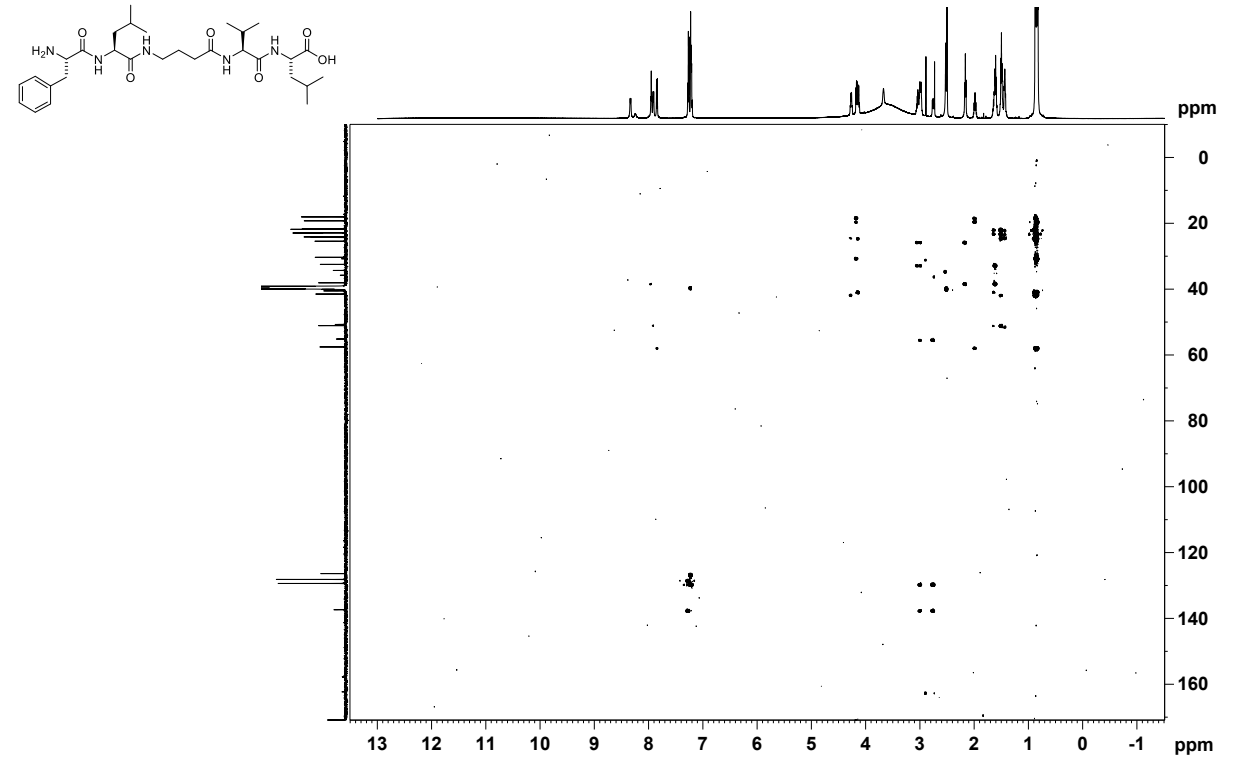
^{13}C DEPT (150 MHz, DMSO-d_6) of **10**



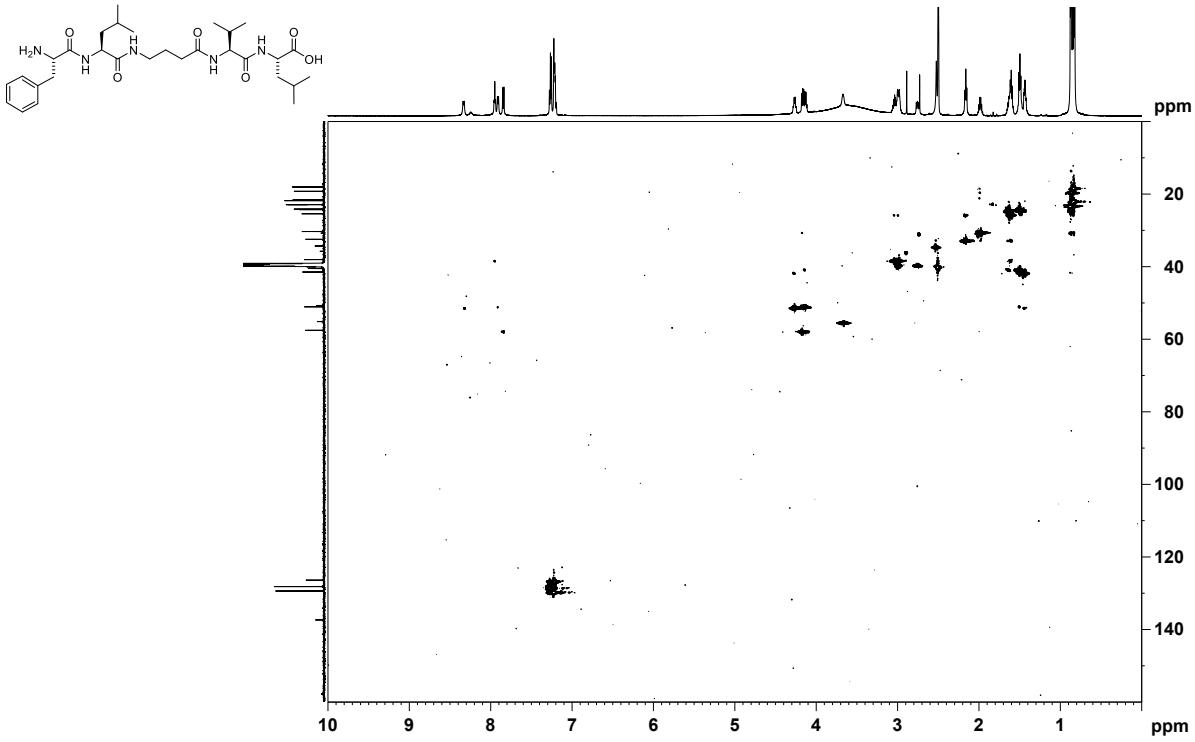
^{1}H-^{1}H COSY (600 MHz, DMSO-d_6) of **10**



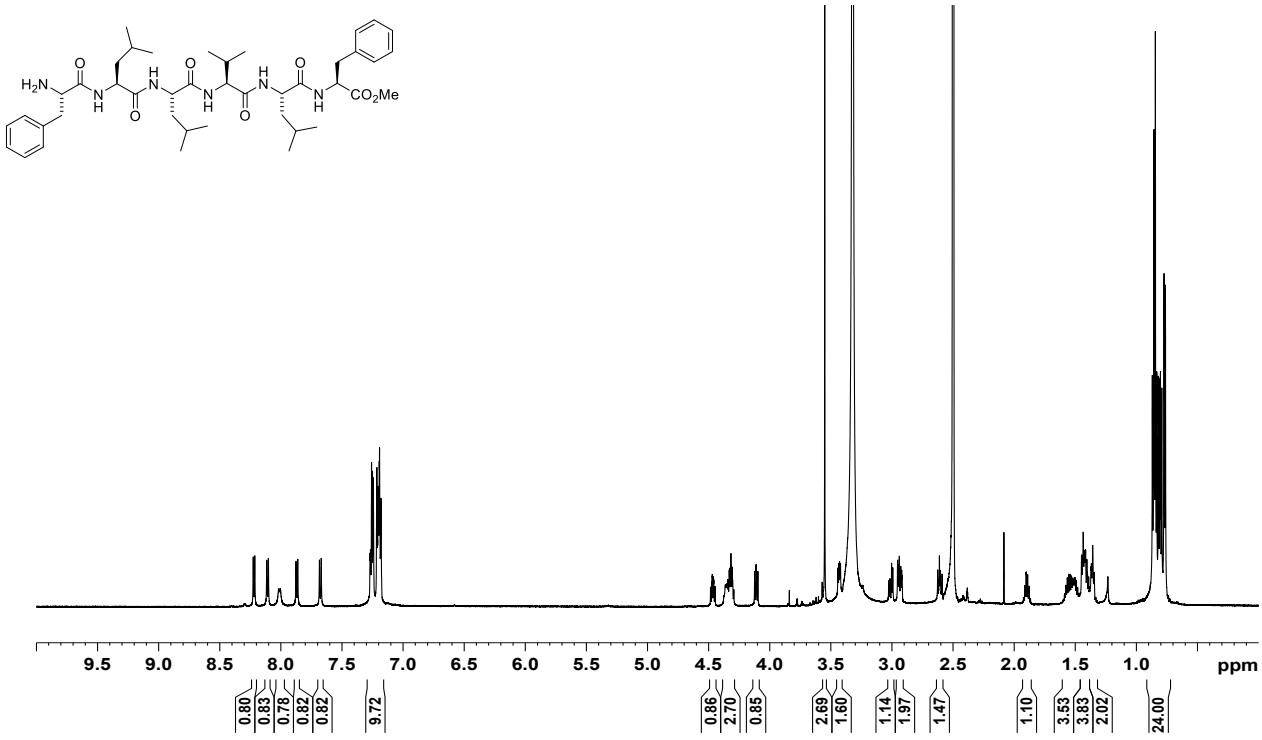
^{1}H-^{13}C HSQC (600 MHz, DMSO-d_6) of **10**



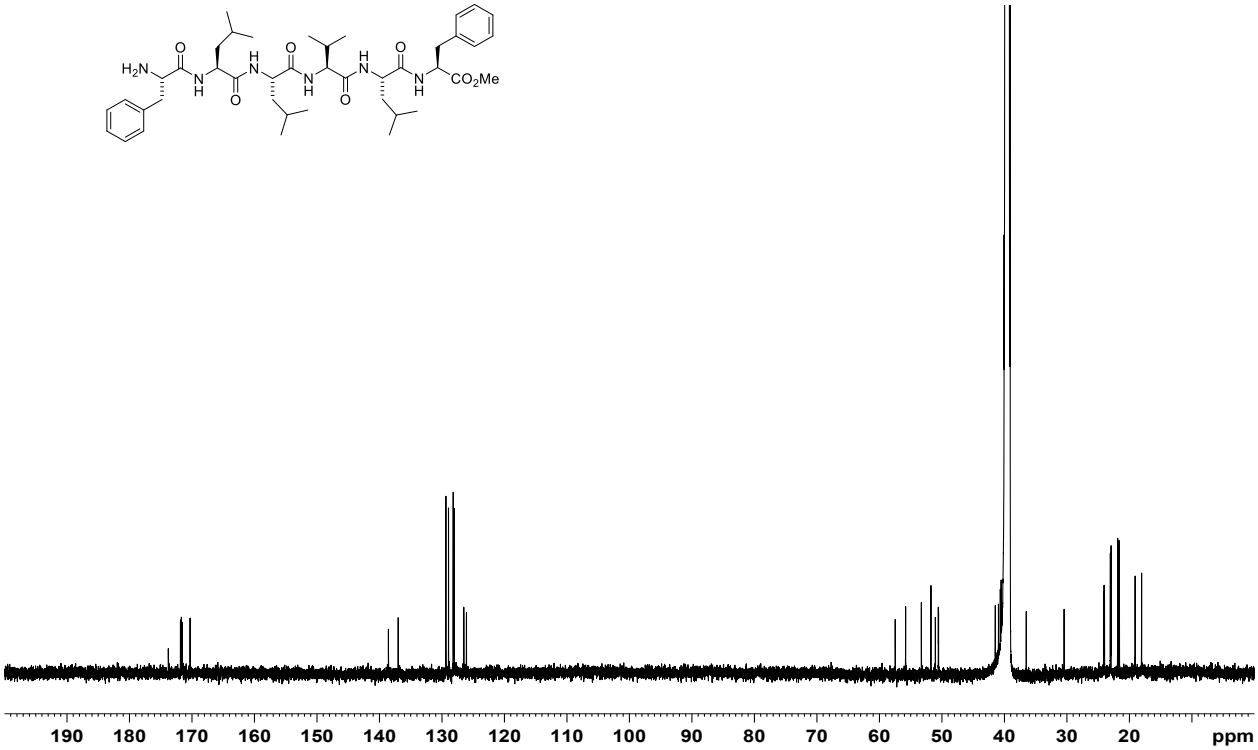
^{1}H-^{13}C HMBC (600 MHz, DMSO-d_6) of **10**



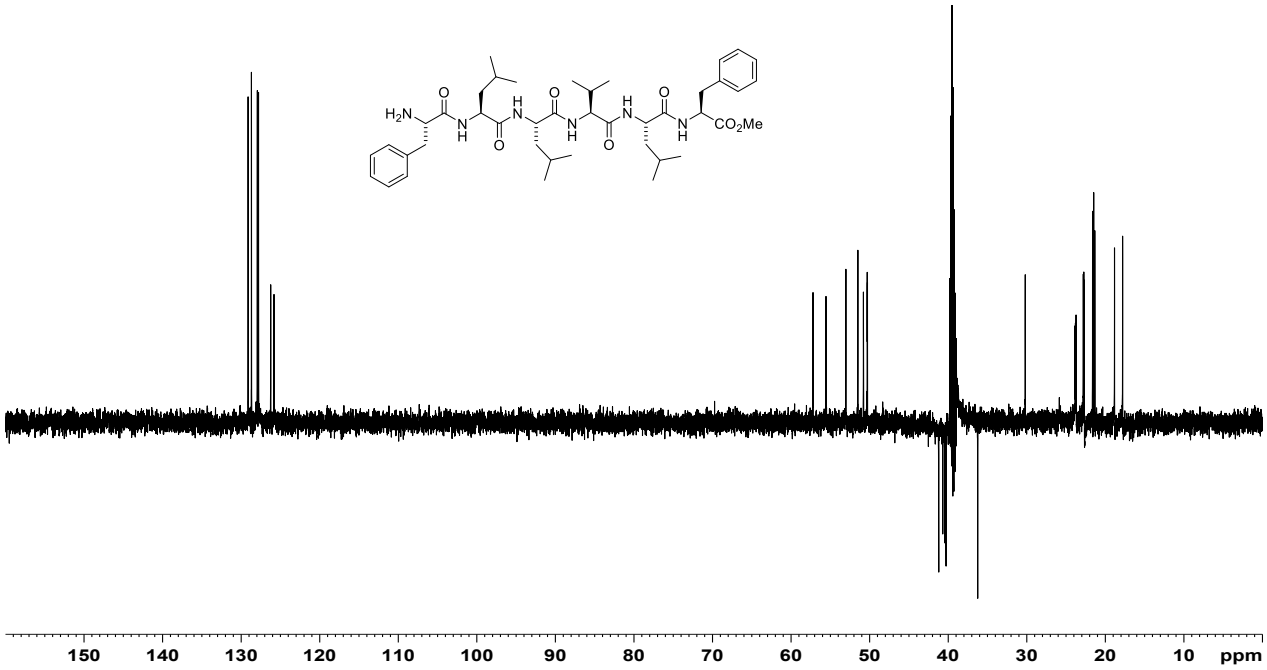
^{1}H NMR (600 MHz, DMSO-d_6) of **11**



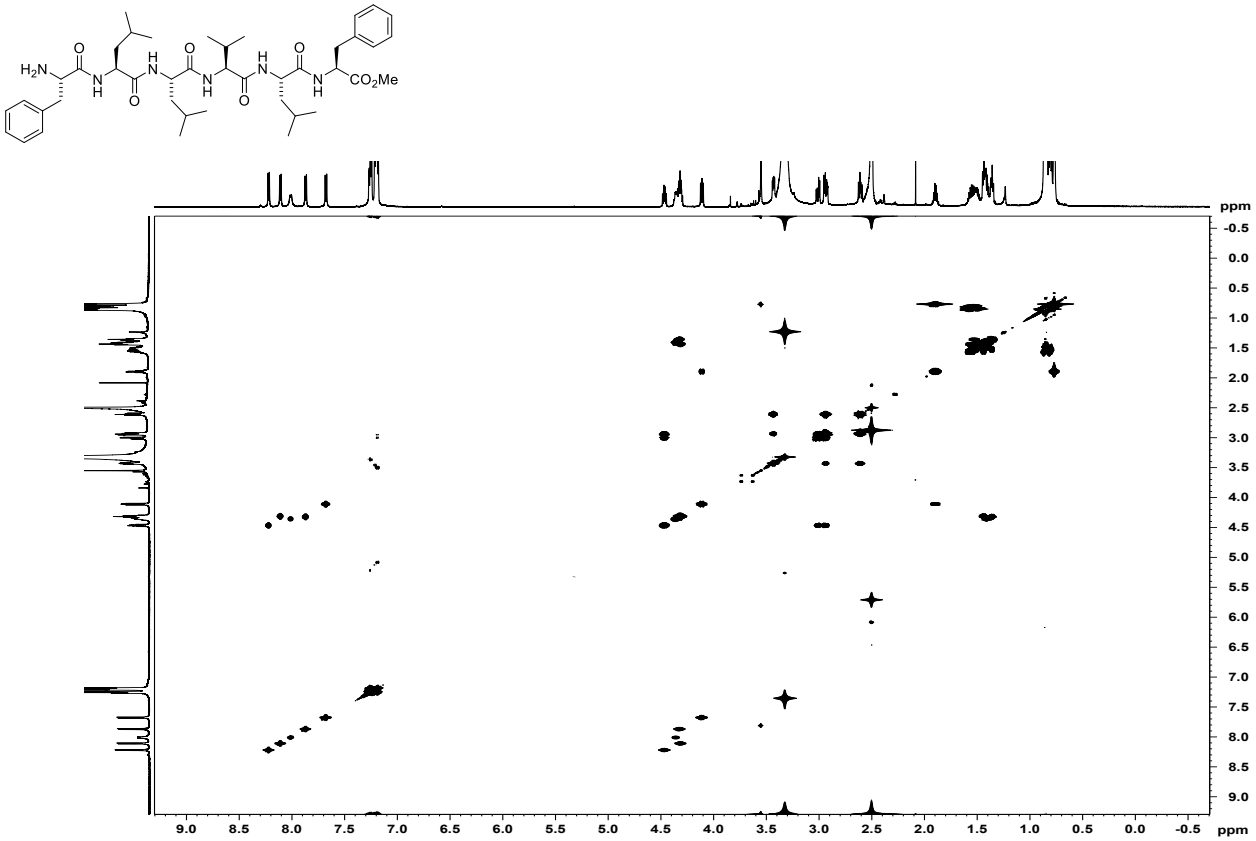
^{13}C NMR (600 MHz, DMSO-d_6) of **11**



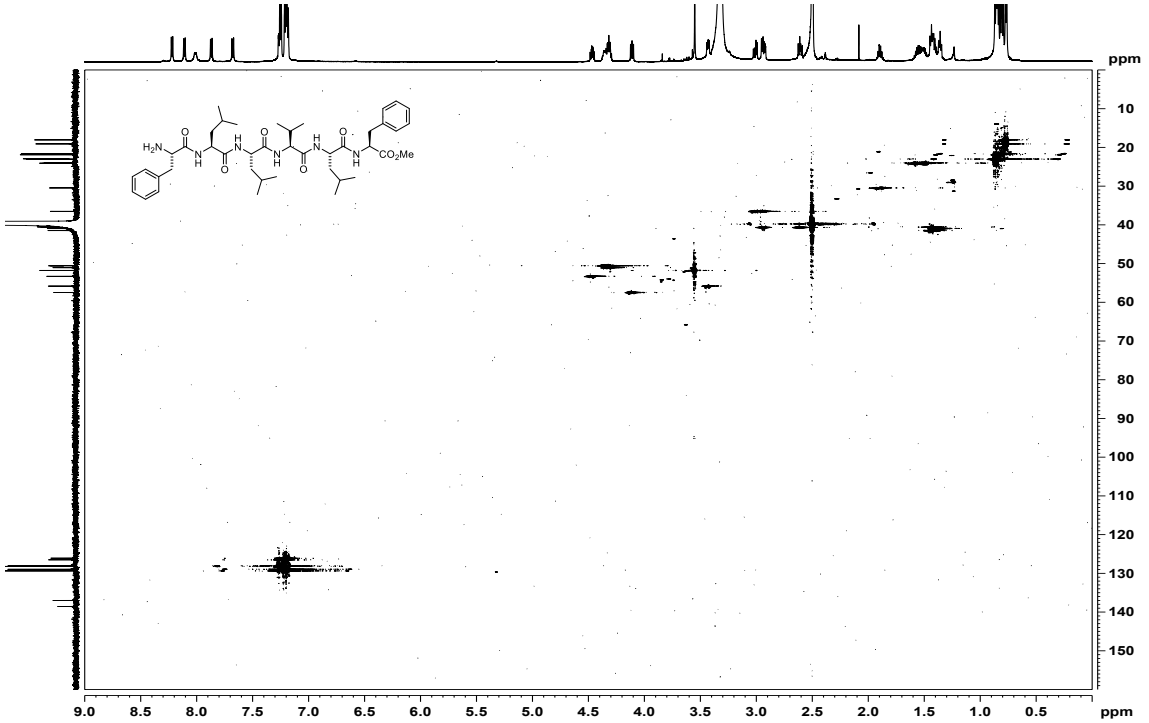
^{13}C DEPT (600 MHz, DMSO-d_6) of **11**



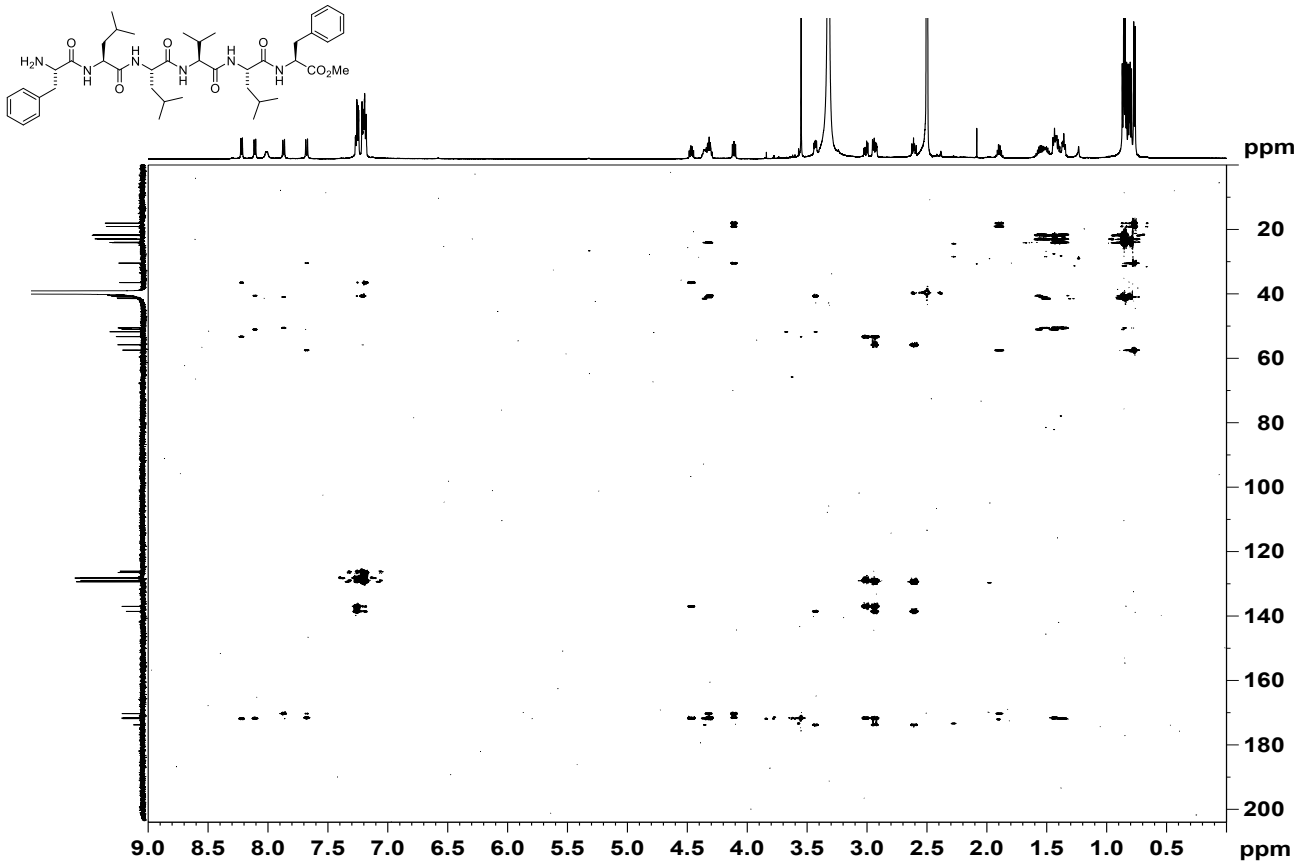
^{1}H-^{1}H COSY (600 MHz, DMSO-d_6) of **11**



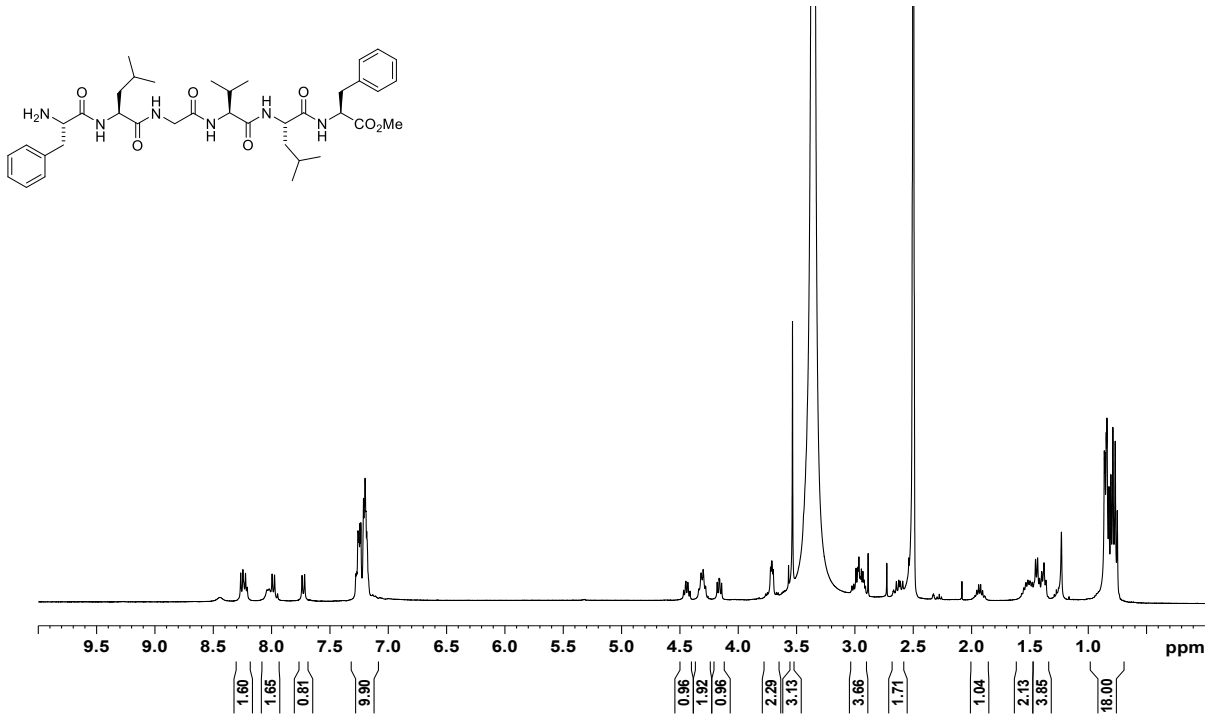
^{1}H-^{13}C HSQC (600 MHz, DMSO-d_6) of **11**



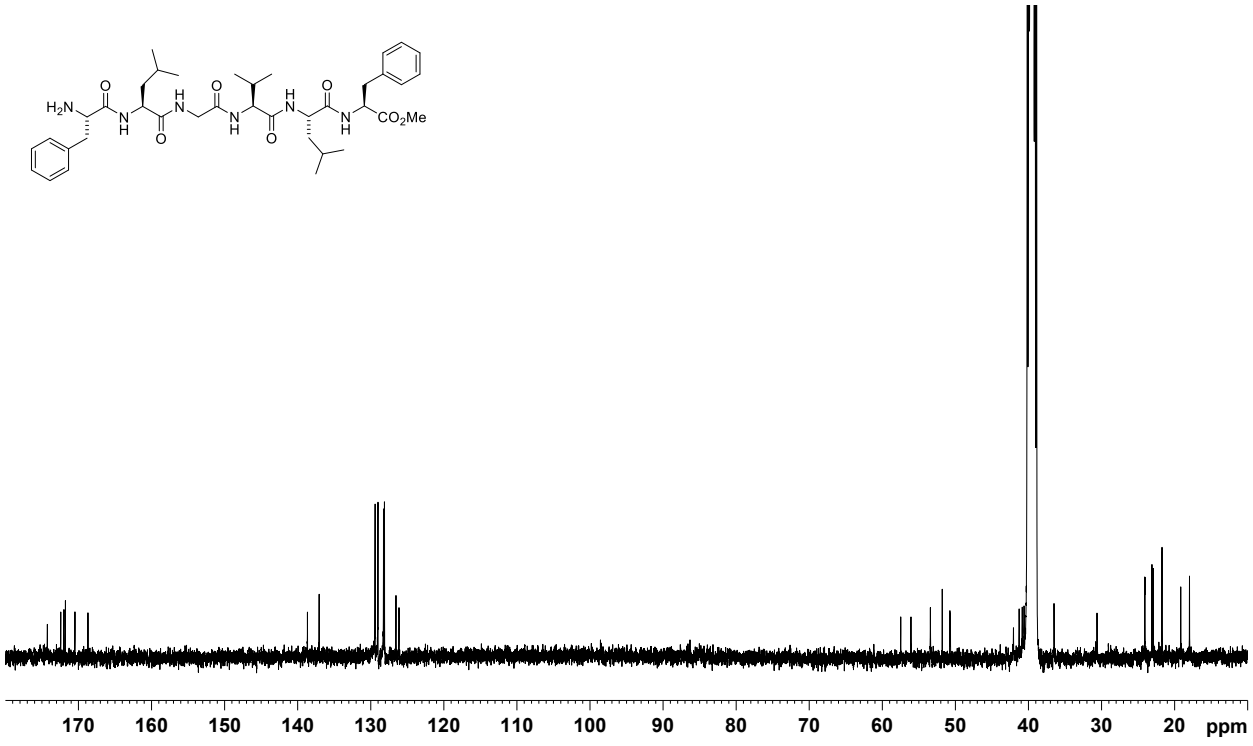
^{1}H-^{13}C HMBC (600 MHz, DMSO-d_6) of **11**



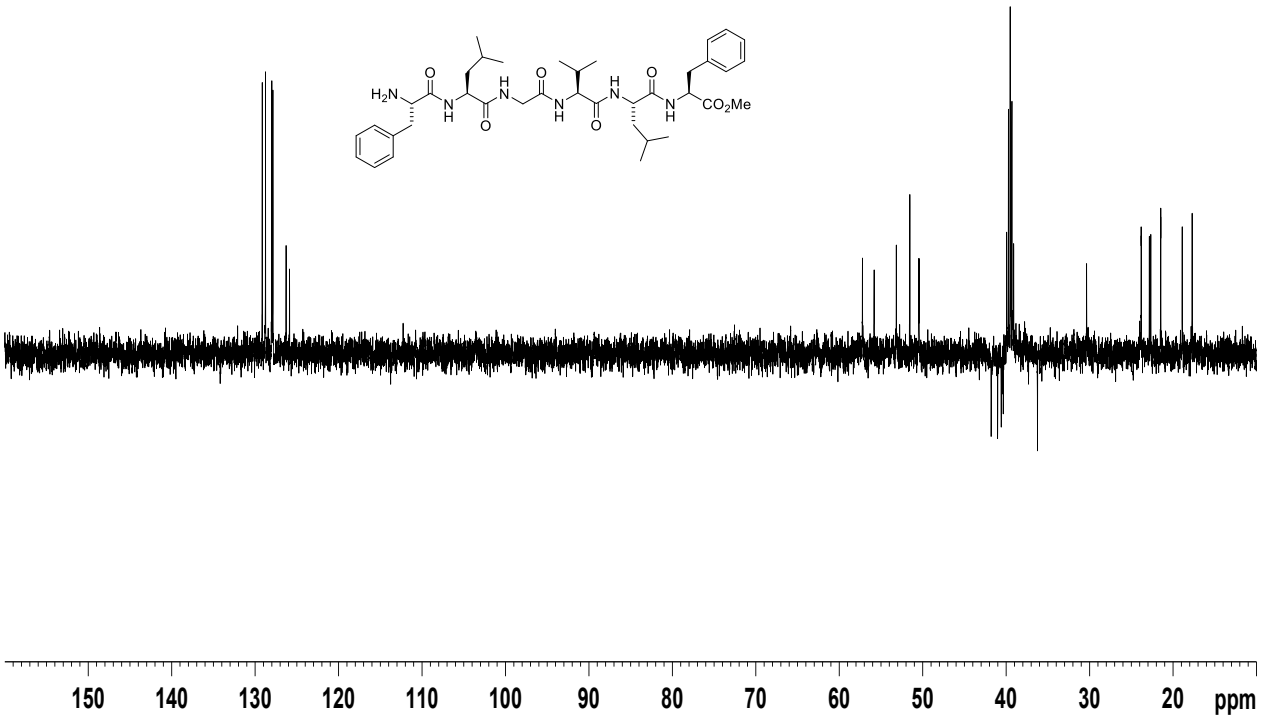
^{1}H NMR (600 MHz, DMSO-d_6) of **12**



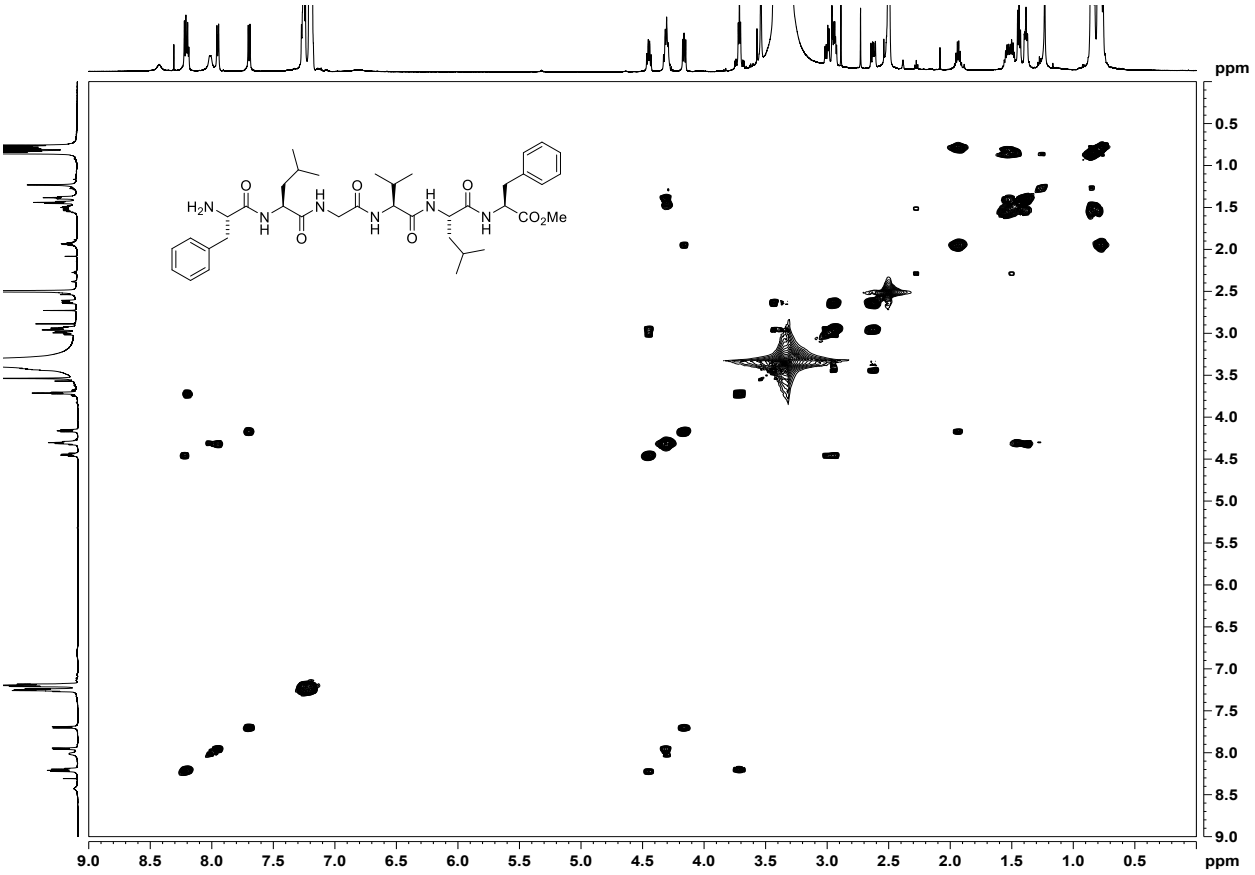
^{13}C NMR (400 MHz, DMSO-d_6) of **12**



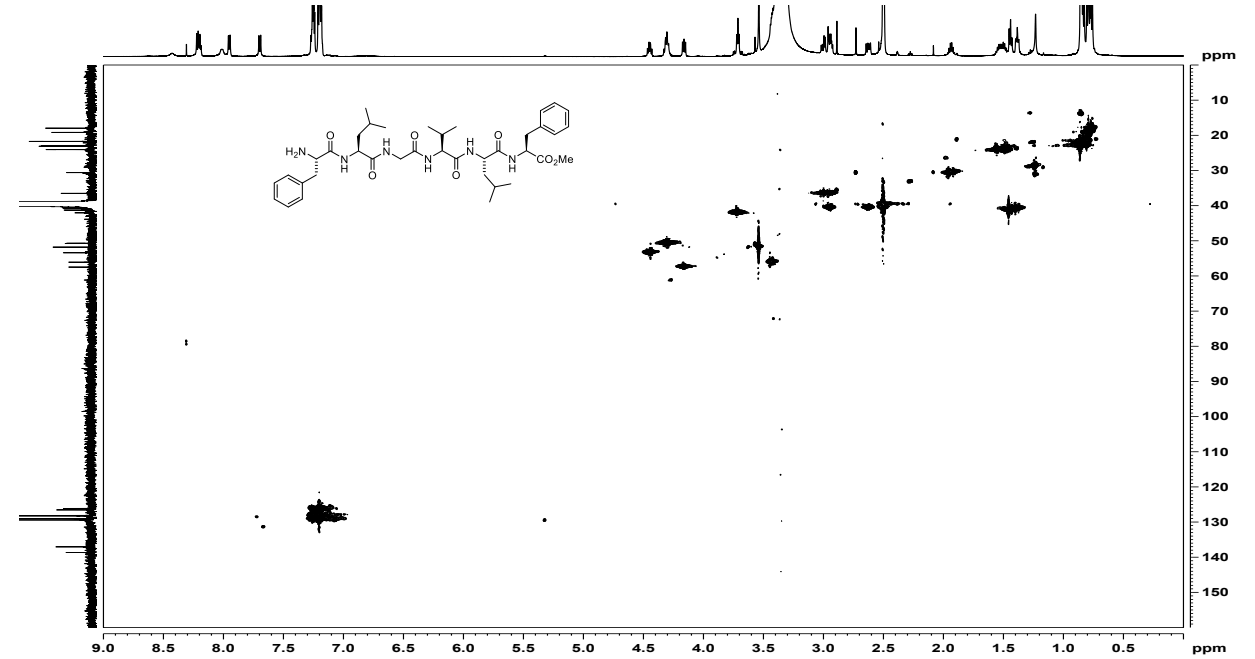
^{13}C DEPT (400 MHz, DMSO-d_6) of **12**



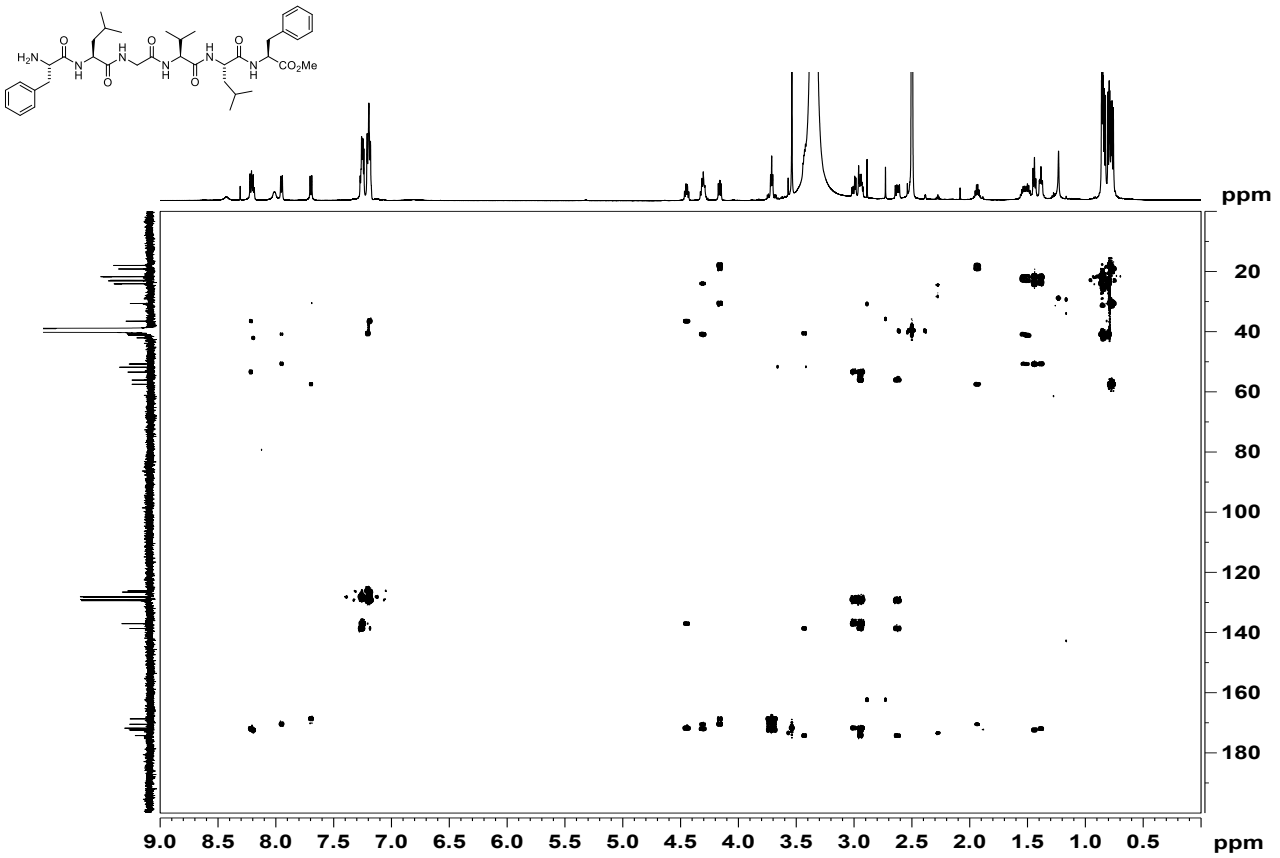
^{1}H-^{1}H COSY (600 MHz, DMSO-d_6) of **12**



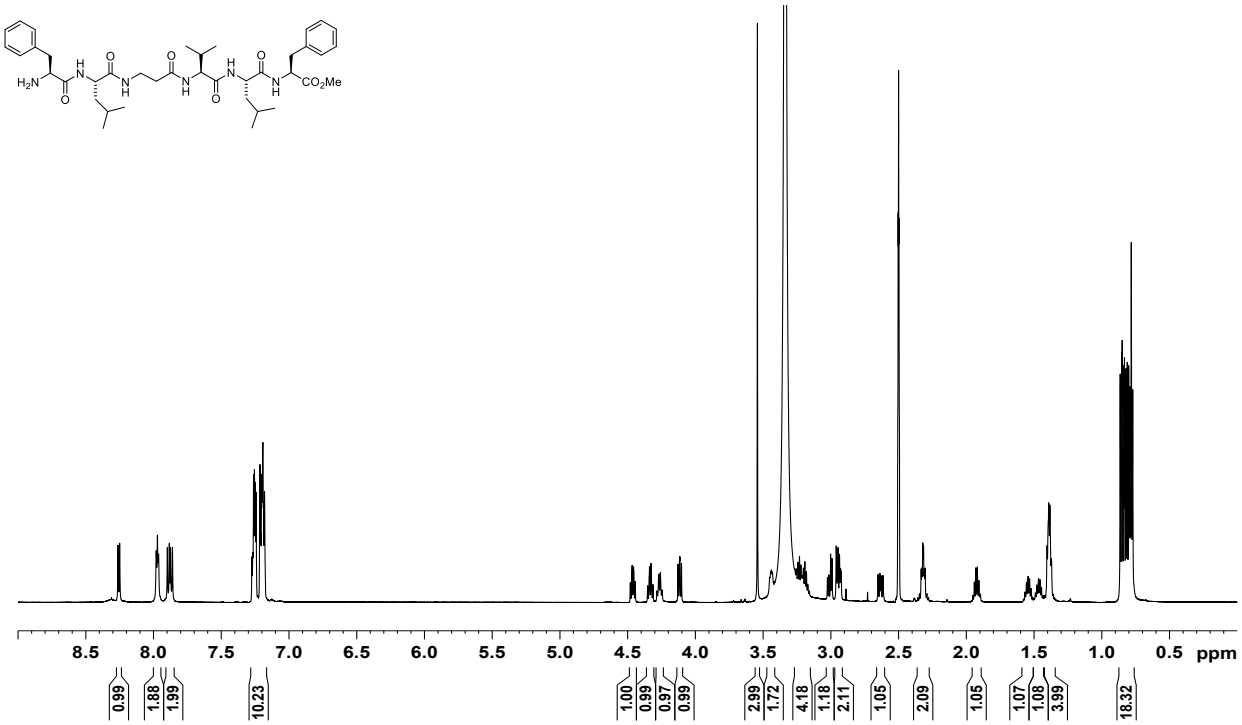
^{1}H-^{13}C HSQC (600 MHz, DMSO-d_6) of **12**



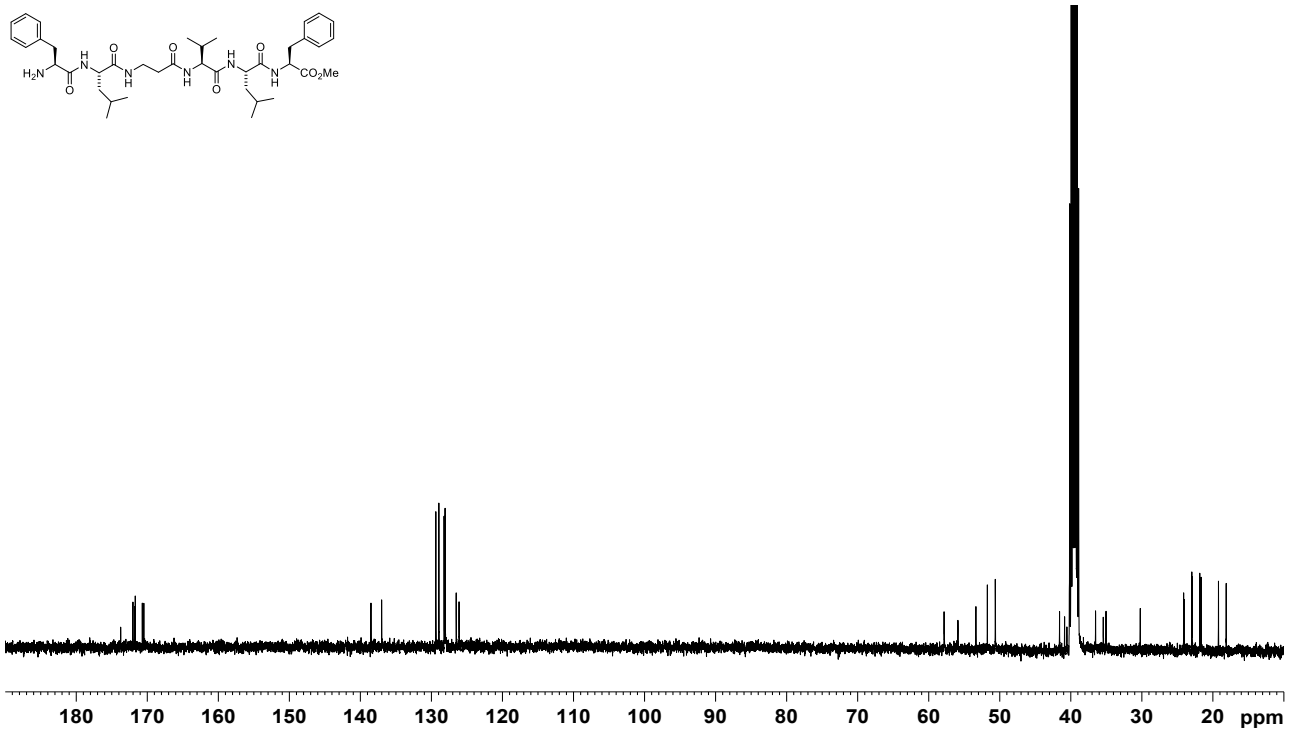
^{1}H-^{13}C HMBC (600 MHz, DMSO-d_6) of **12**



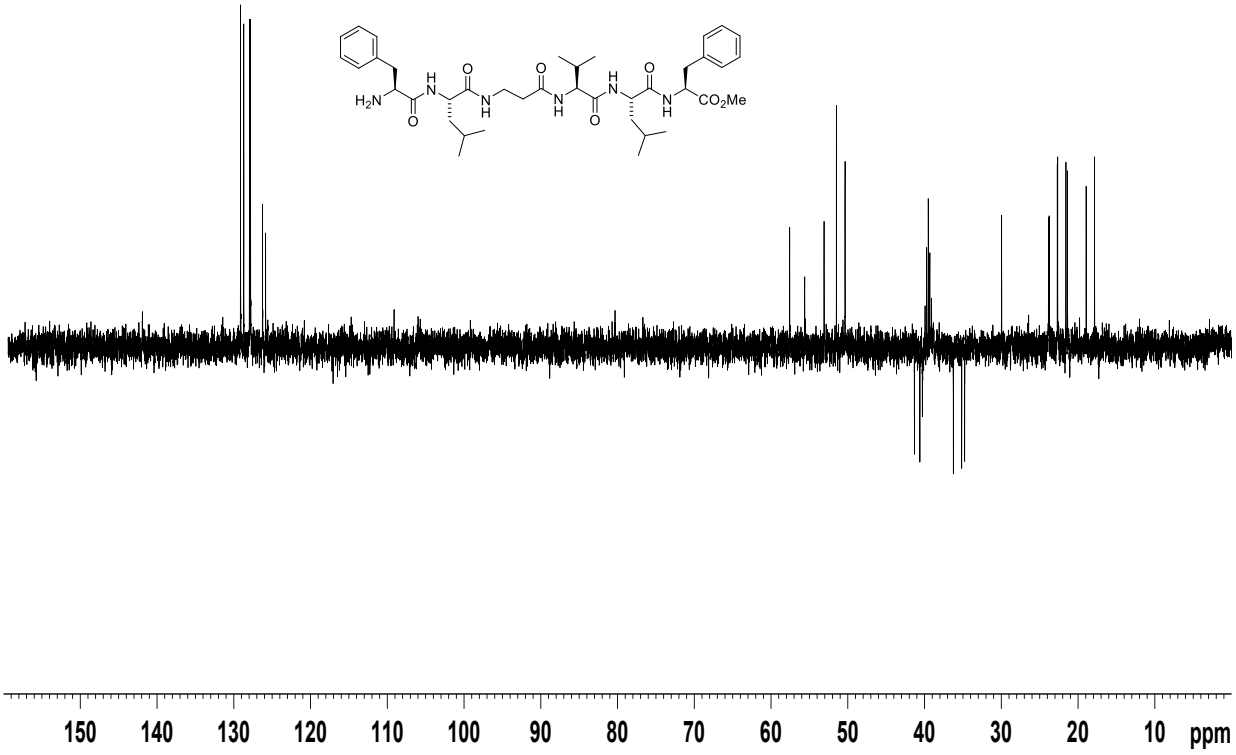
^{1}H NMR (600 MHz, DMSO-d_6) of **13**



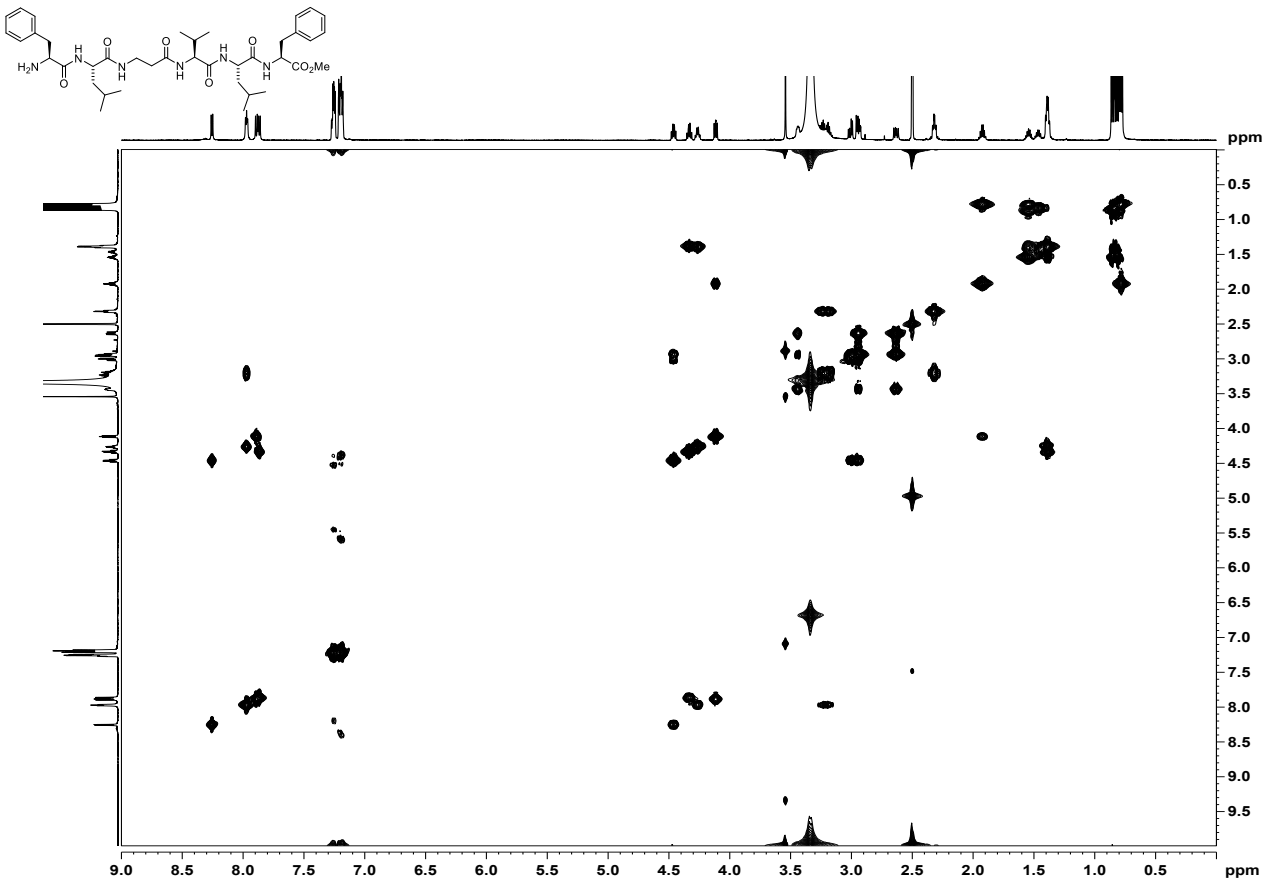
^{13}C NMR (400 MHz, DMSO-d_6) of **13**



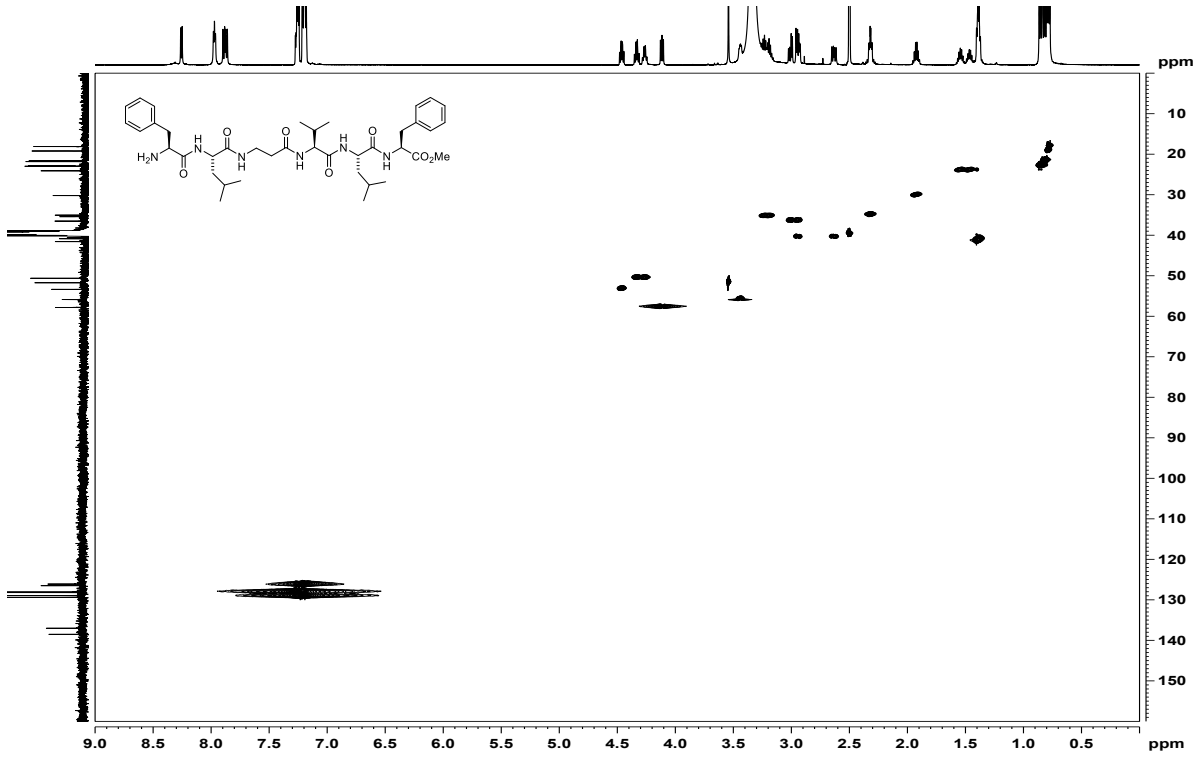
^{13}C DEPT (400 MHz, DMSO-d_6) of **13**



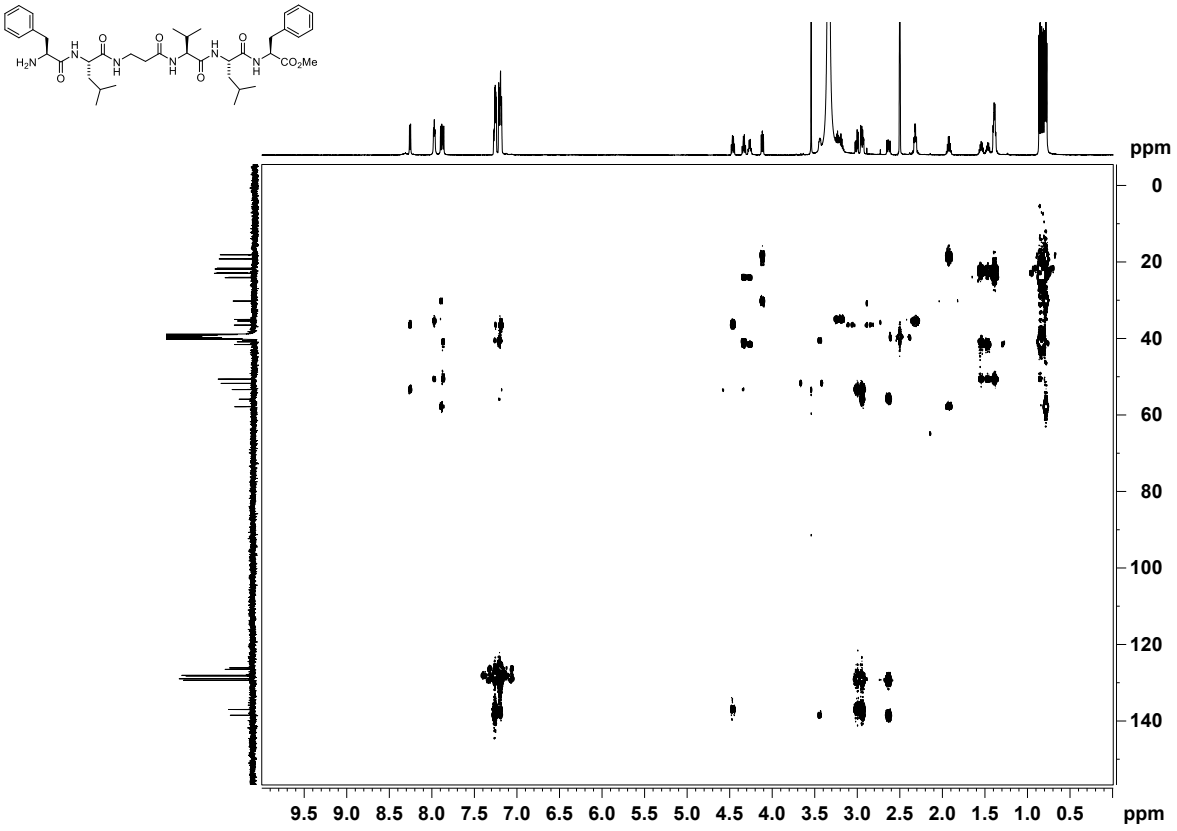
^{1}H-^{1}H COSY (600 MHz, DMSO-d_6) of **13**



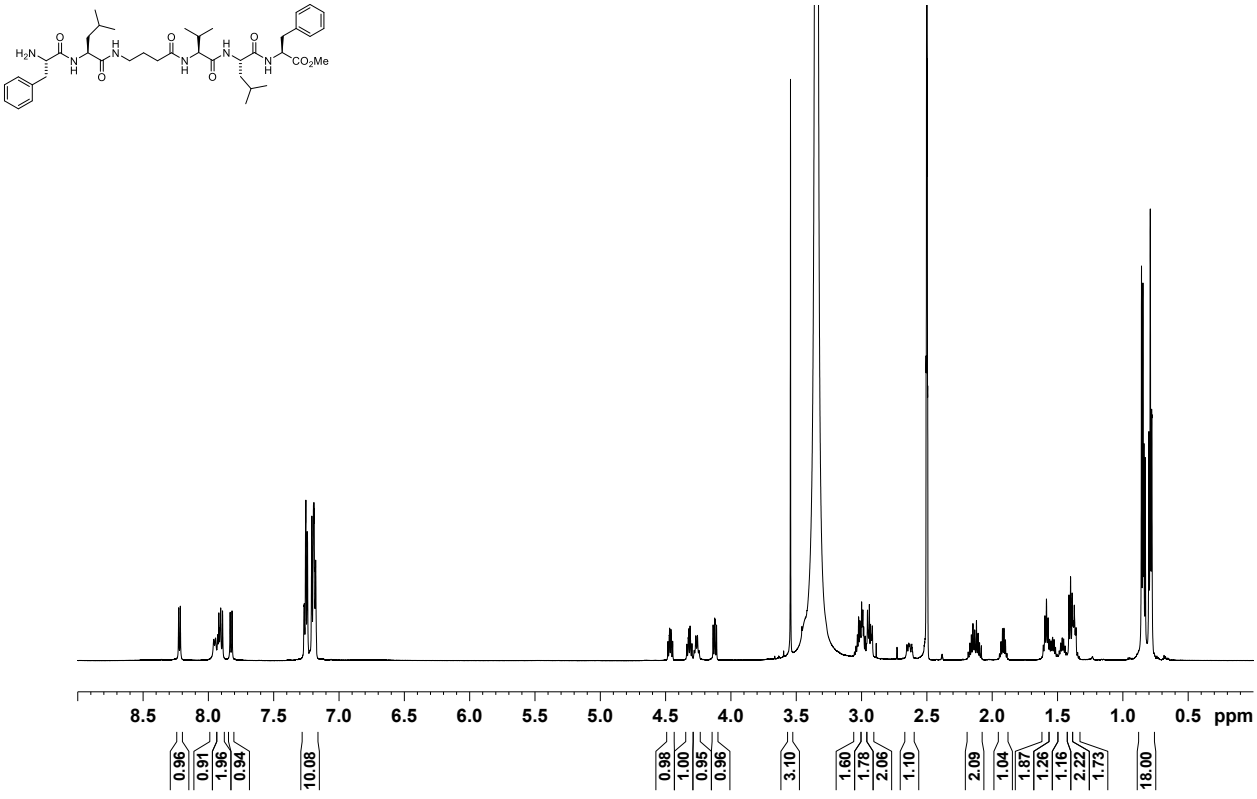
^{1}H-^{13}C HSQC (600 MHz, DMSO-d_6) of **13**



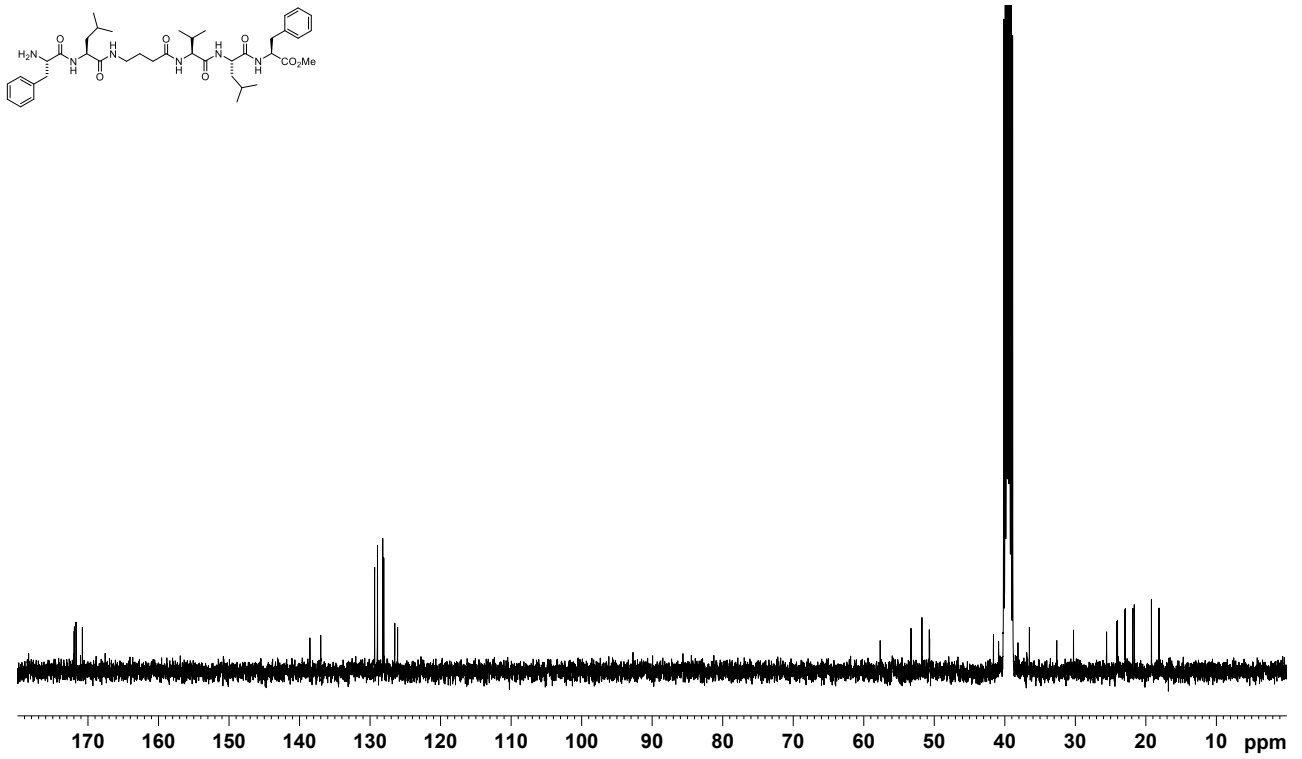
^{1}H-^{13}C HMBC (600 MHz, DMSO-d_6) of **13**



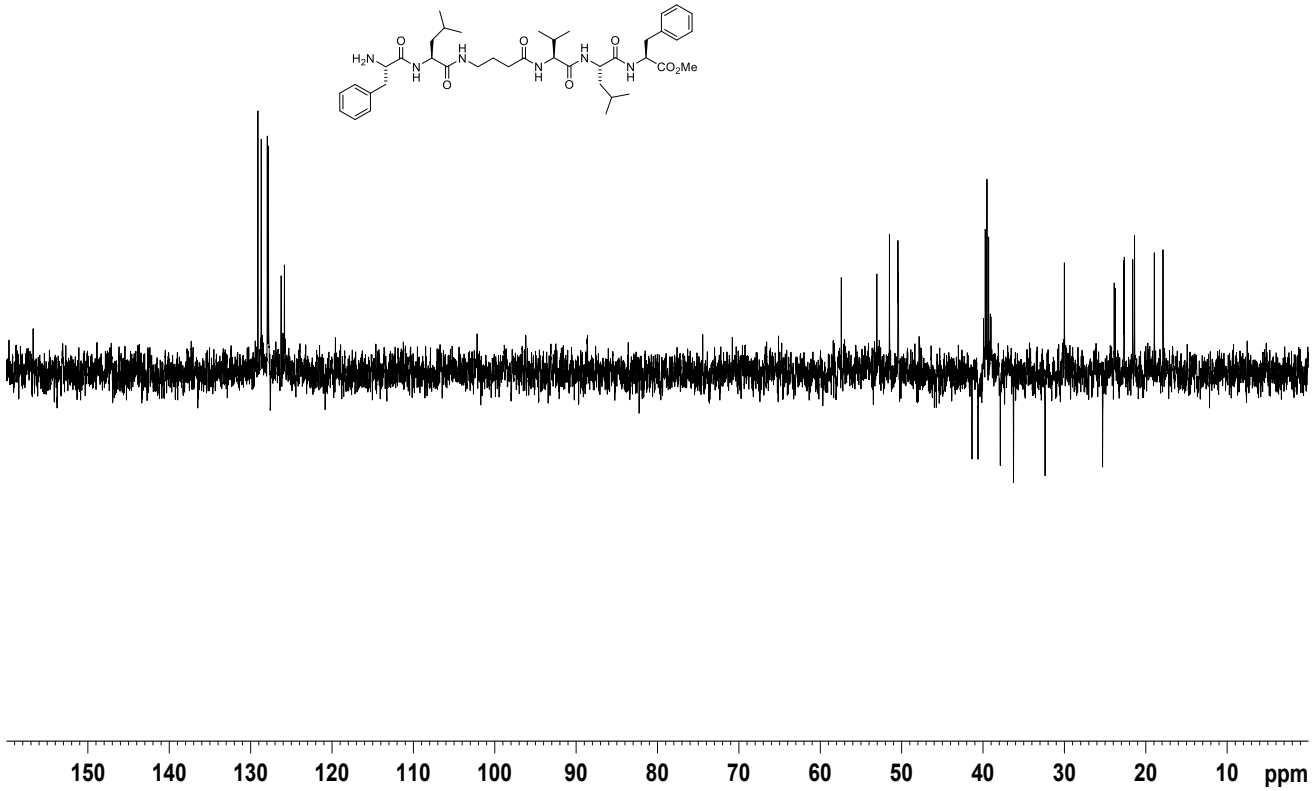
^{1}H NMR (600 MHz, DMSO-d_6) of **14**



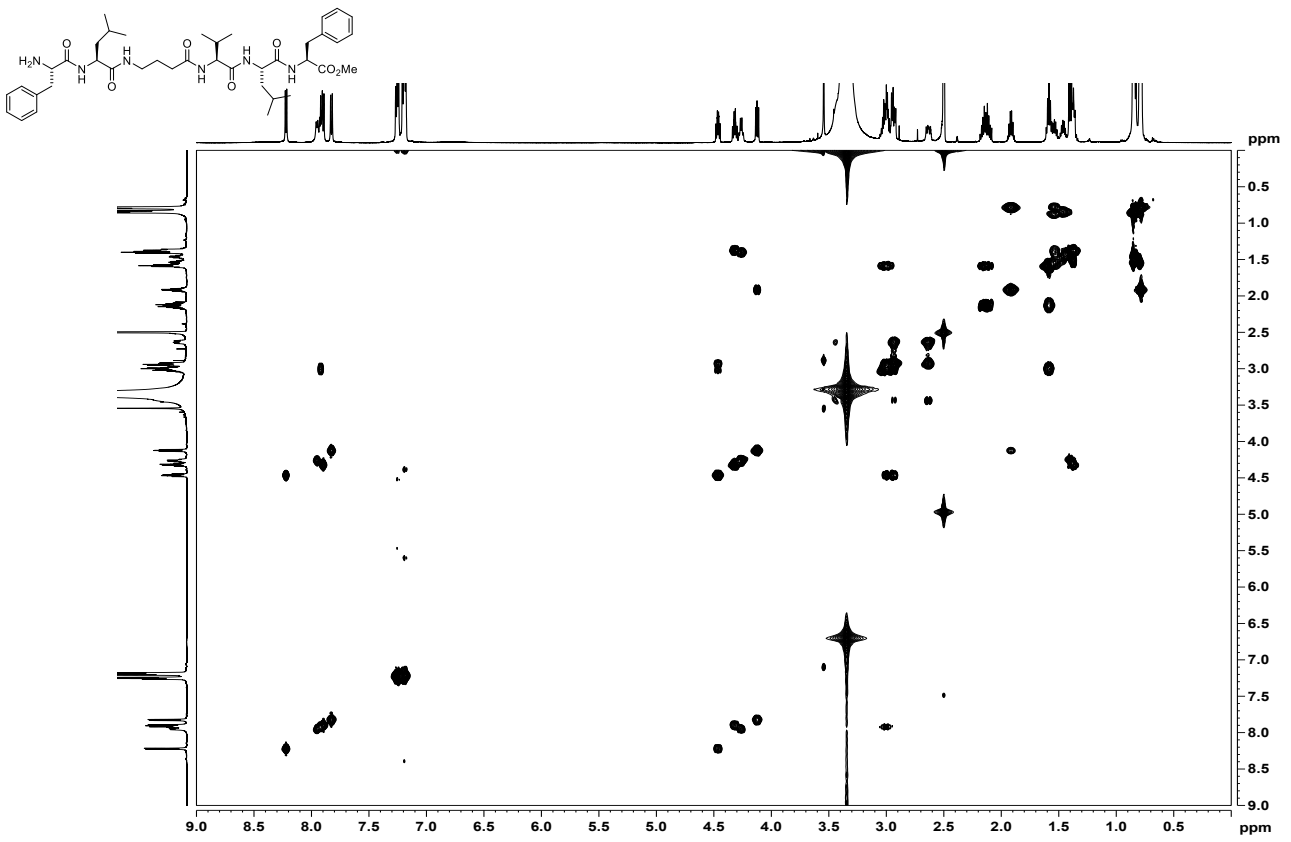
^{13}C NMR (400 MHz, DMSO-d_6) of **14**



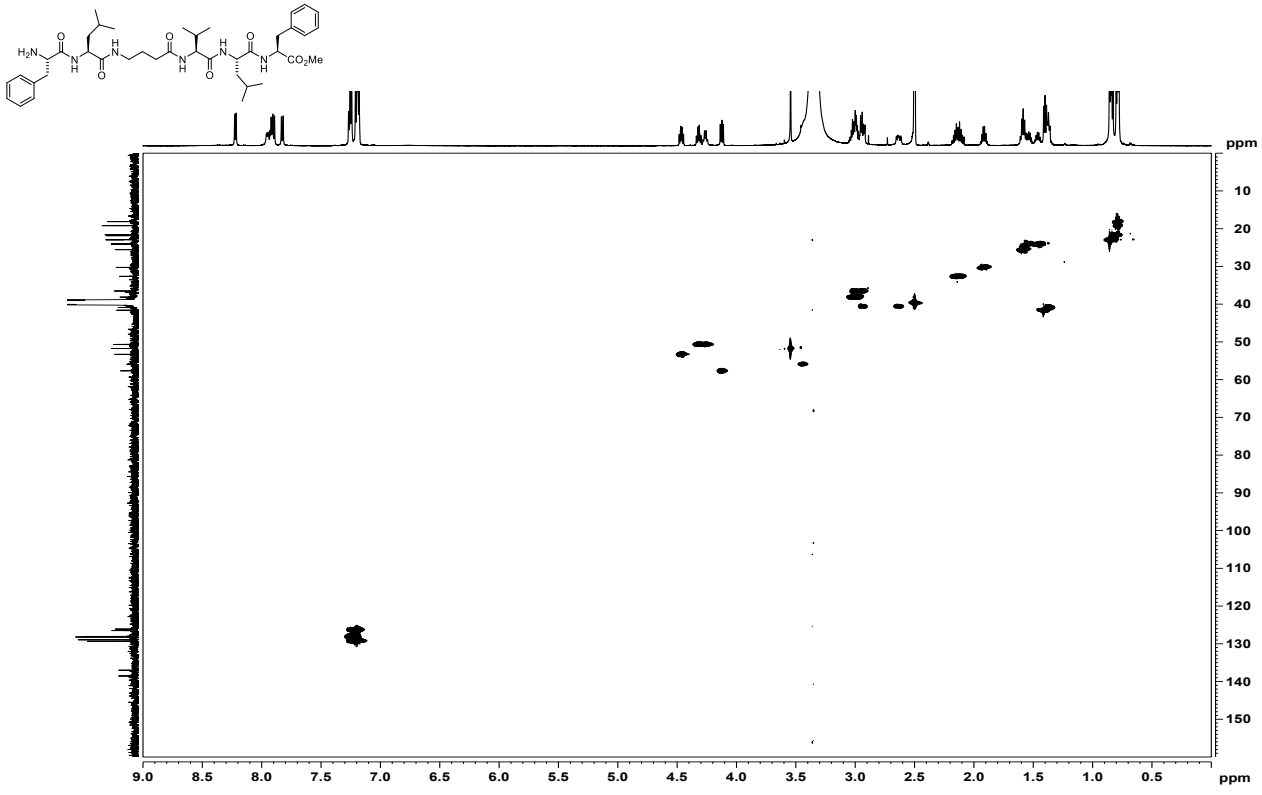
^{13}C DEPT (400 MHz, DMSO-d_6) of **14**



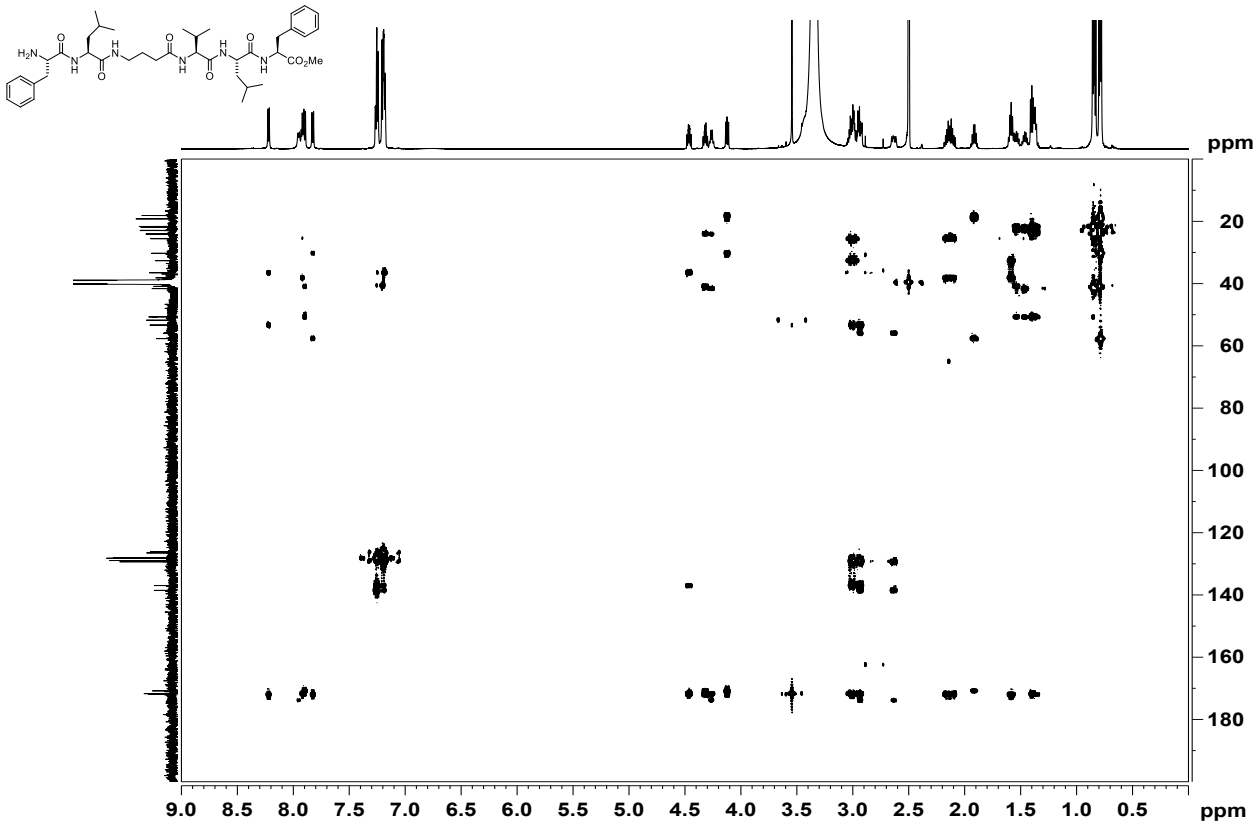
^{1}H-^{1}H COSY (600 MHz, DMSO-d_6) of **14**



^{1}H-^{13}C HSQC (600 MHz, DMSO-d_6) of **14**



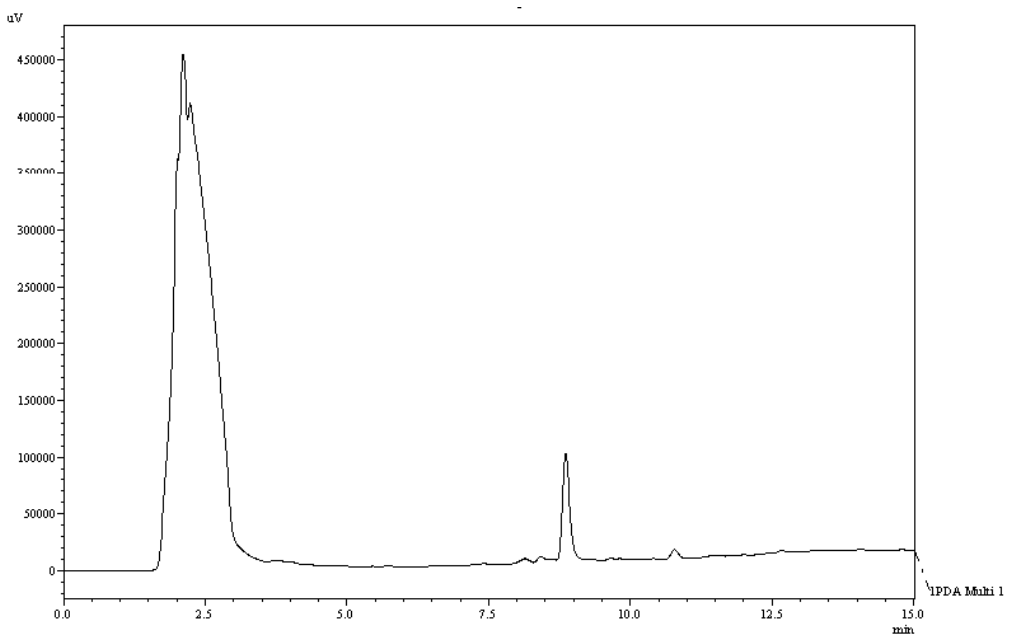
^{1}H-^{13}C HMBC (600 MHz, DMSO-d_6) of **14**



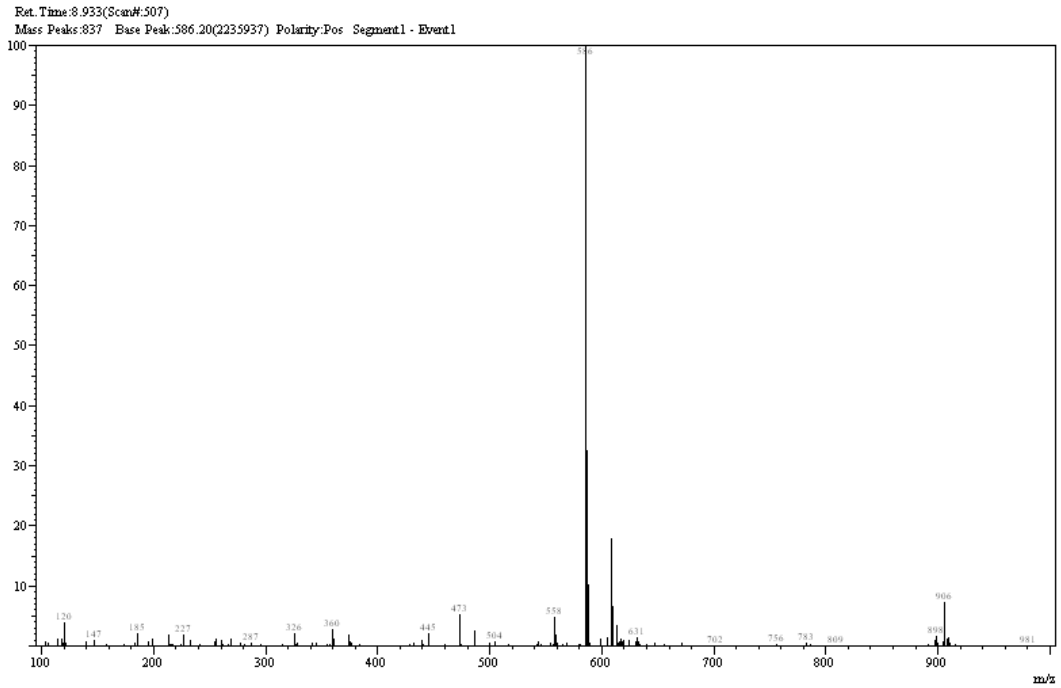
5. LC-MS traces of novel compounds

LC-MS of compound **2** (reverse-phase, 30 →100% (MeCN/H_2O) over 10 min)

Chromatogram (254 nm)

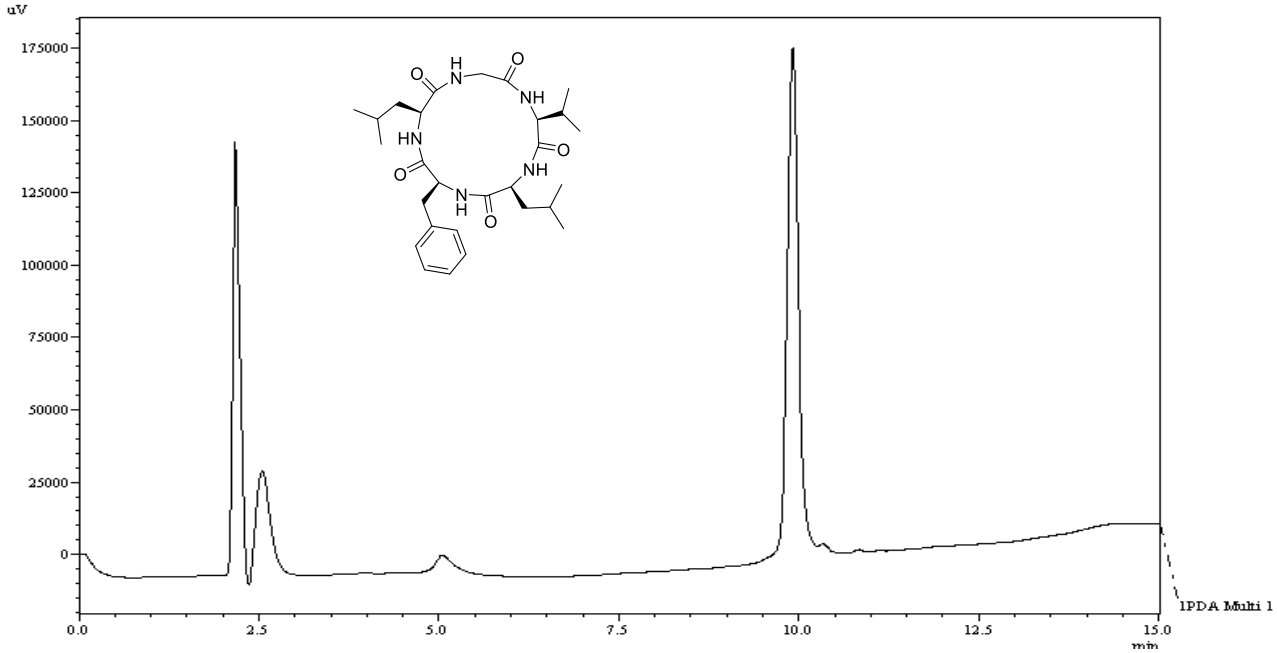


Mass Spectrum

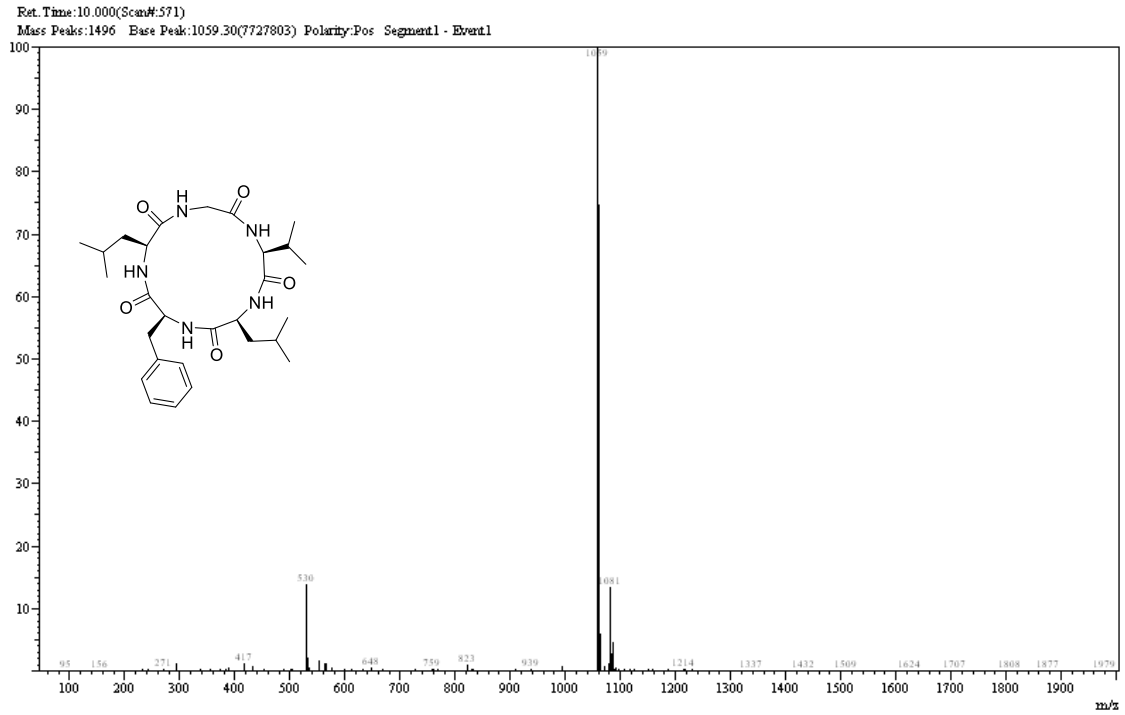


LC-MS of compound **3** (reverse-phase, 2→100% (MeCN/H_2O) over 10 min)

Chromatogram (254 nm)

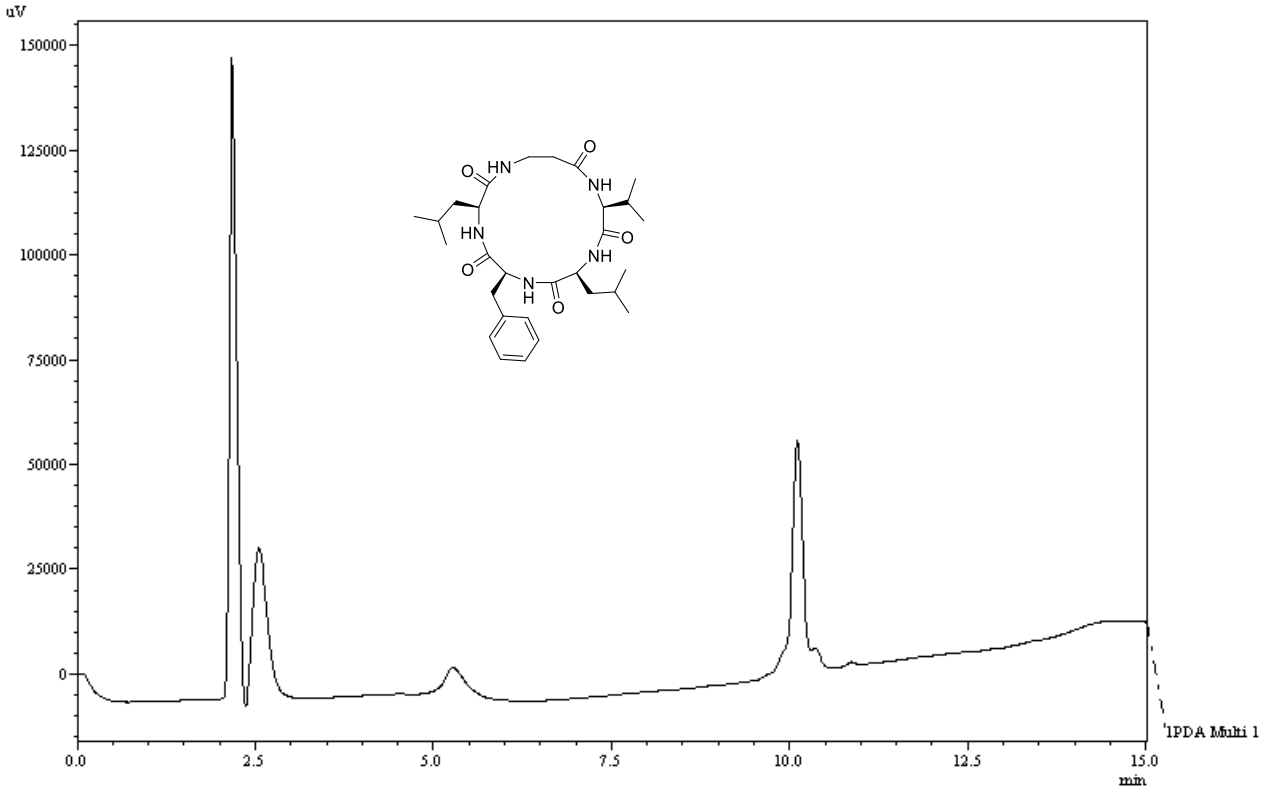


Mass Spectrum

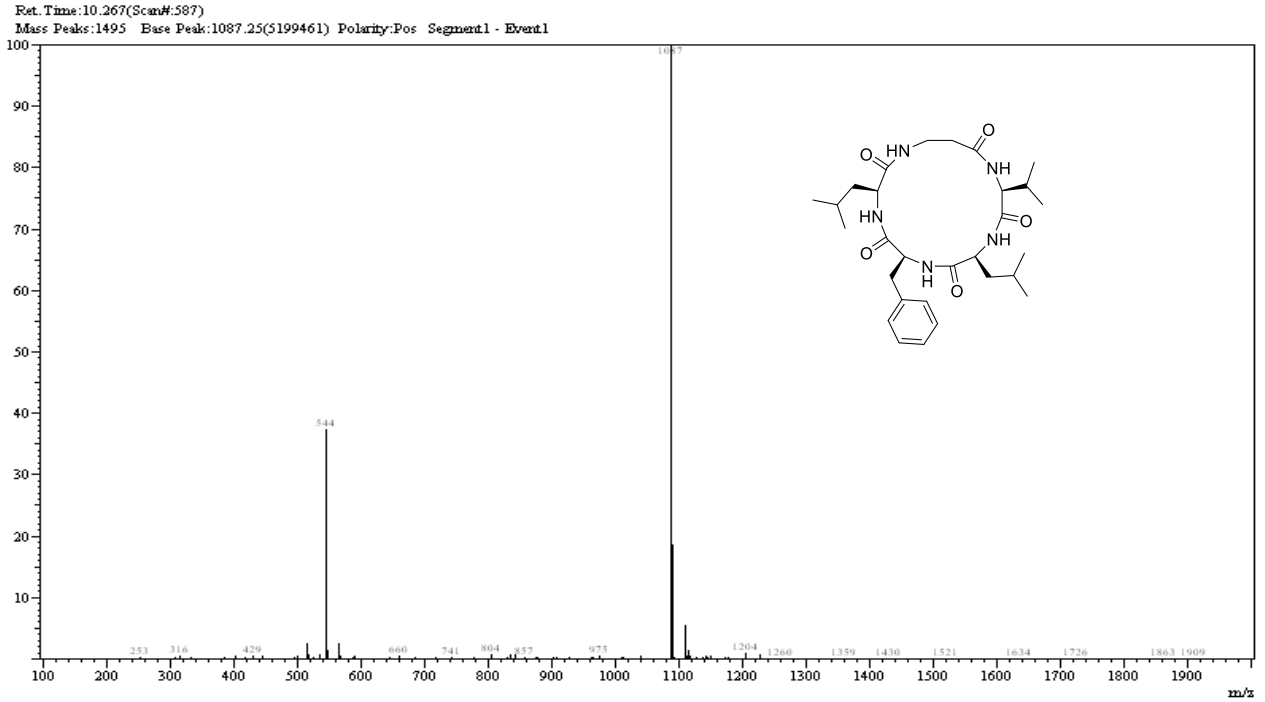


LC-MS of compound **4** (reverse-phase, 2→100% (MeCN/H_2O) over 10 min)

Chromatogram (254 nm)

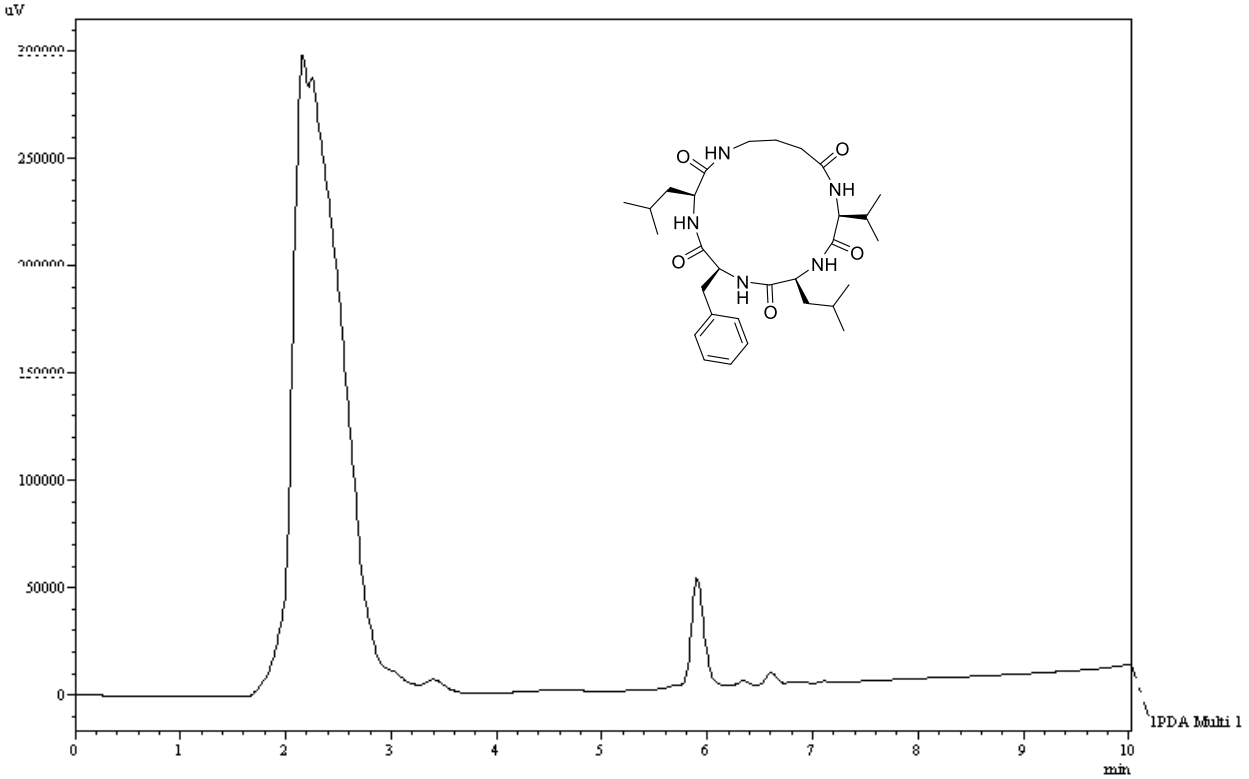


Mass Spectrum

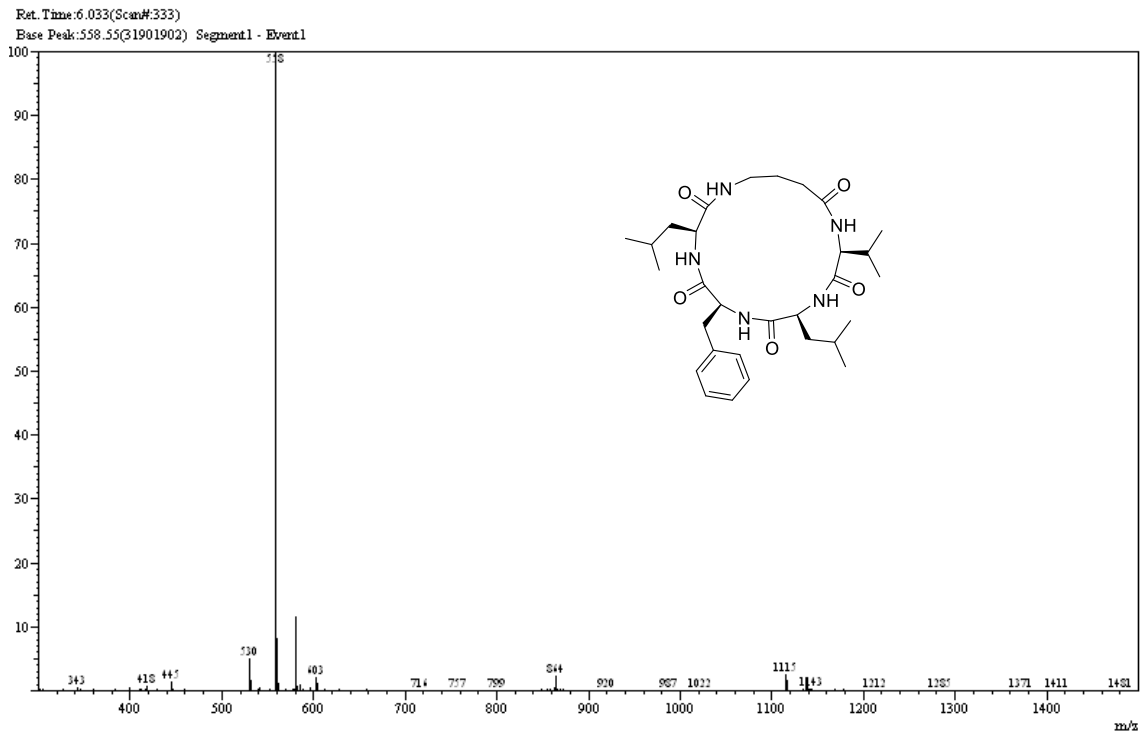


LC-MS of compound **5** (reverse-phase, 40→100% (MeCN/H_2O) over 6 min)

Chromatogram (254 nm)

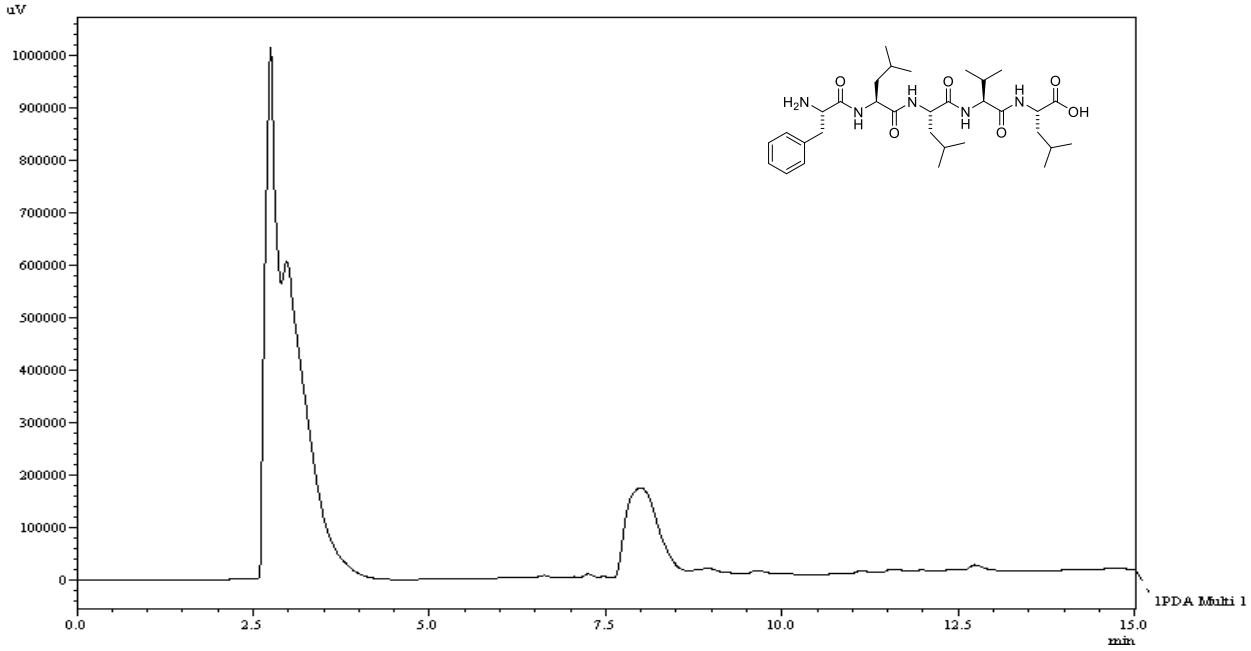


Mass Spectrum

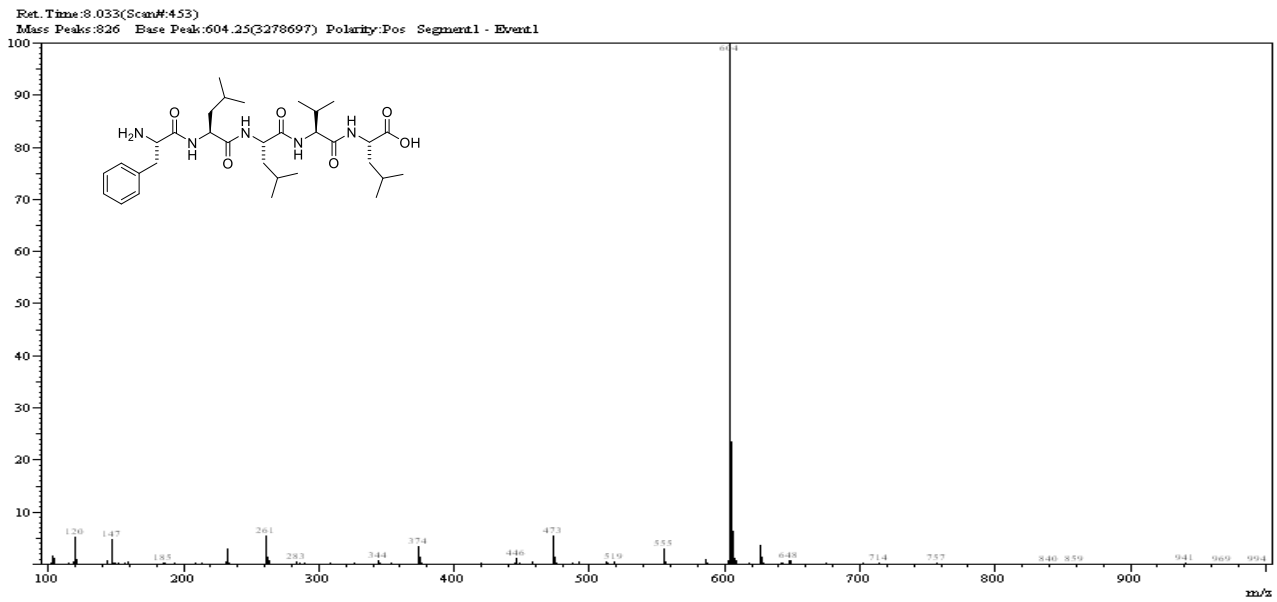


LC-MS of compound **7** (reverse-phase, 10→100% (MeCN/H_2O) over 10 min)

Chromatogram (254 nm)

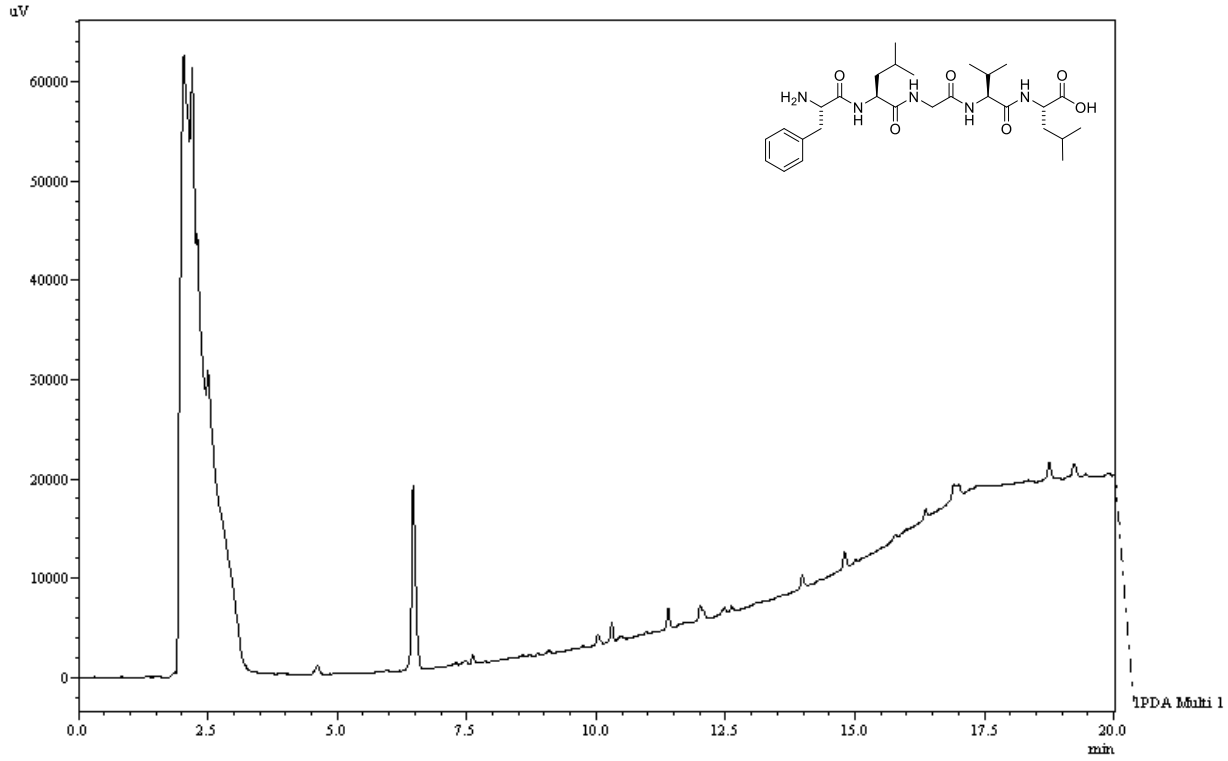


Mass Spectrum

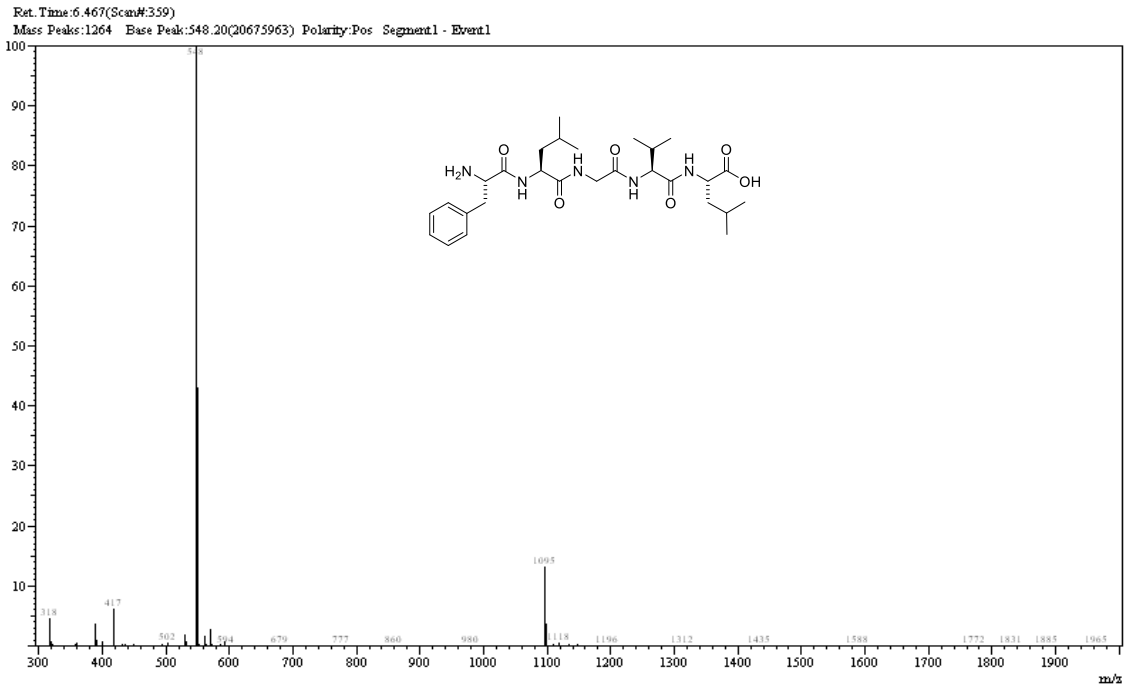


LC-MS of compound **8** (reverse-phase, 2→100% (MeCN/H_2O) over 15 min)

Chromatogram (254 nm)

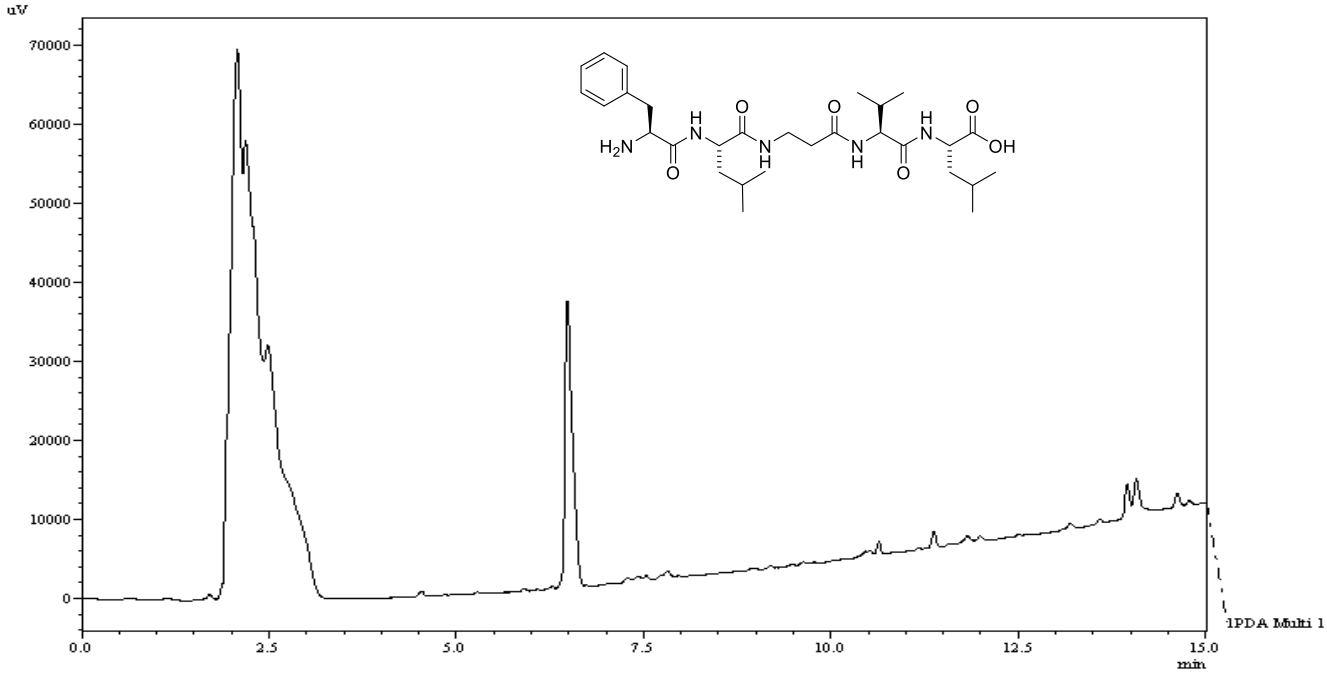


Mass Spectrum

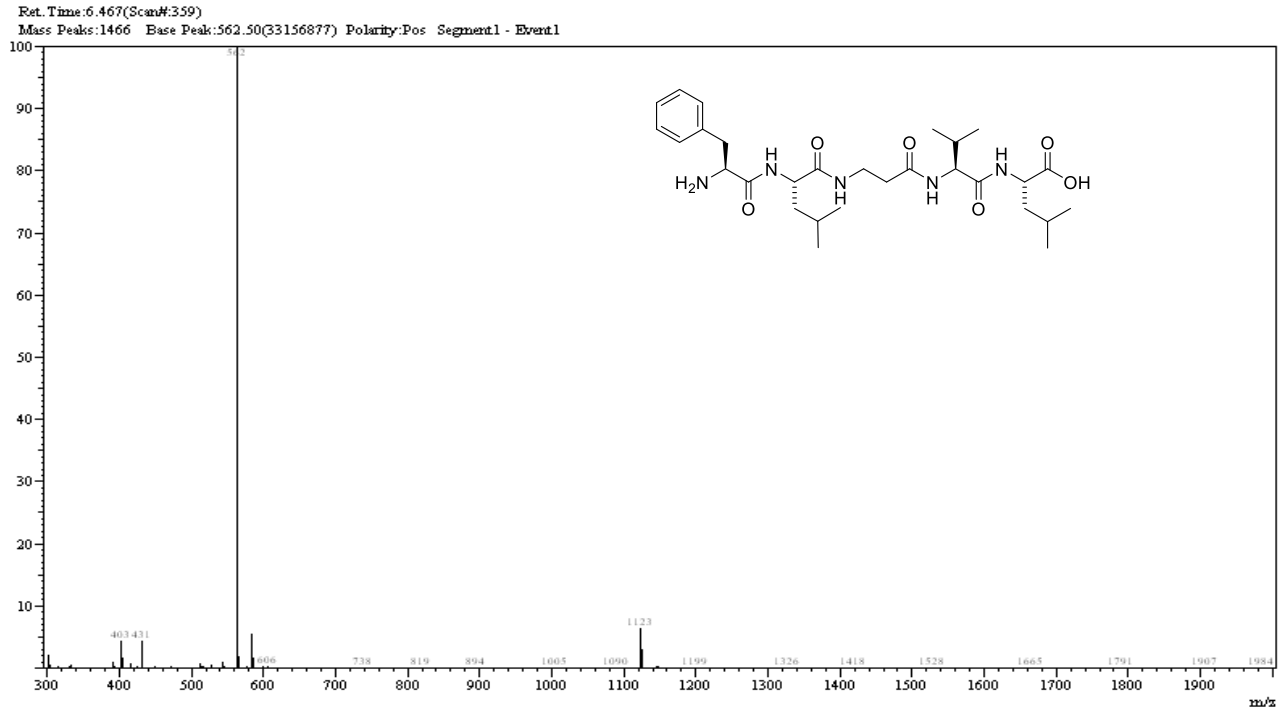


LC-MS of compound **9** (reverse-phase, 2→100% (MeCN/H_2O) over 15 min)

Chromatogram (254 nm)

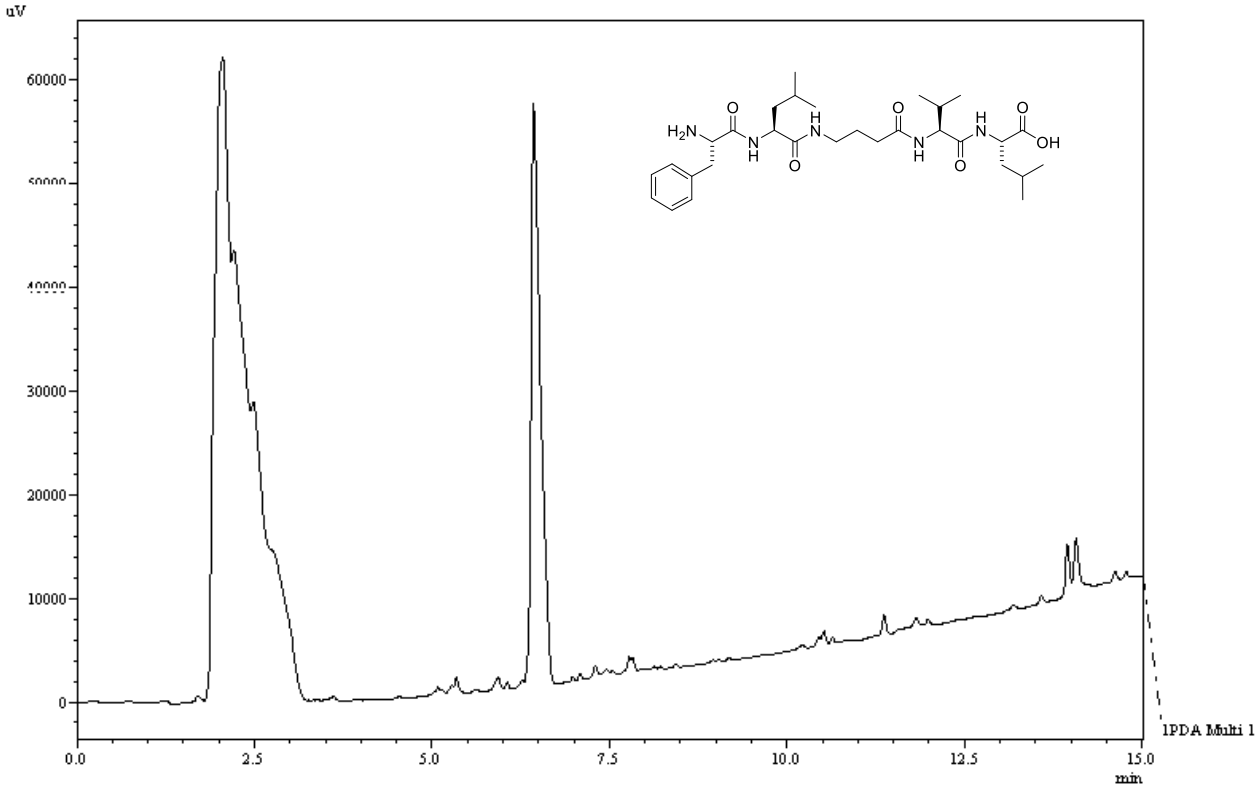


Mass Spectrum

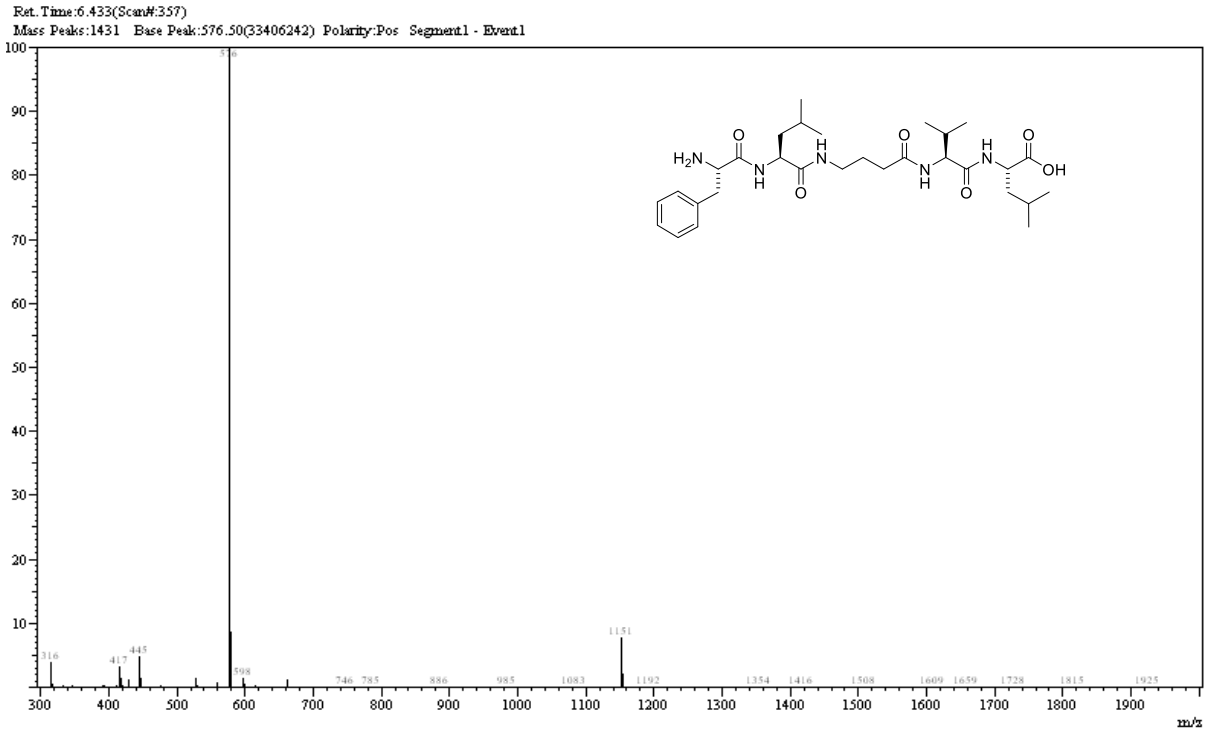


LC-MS of compound **10** (reverse-phase, 2→100% (MeCN/H_2O) over 15 min)

Chromatogram (254 nm)

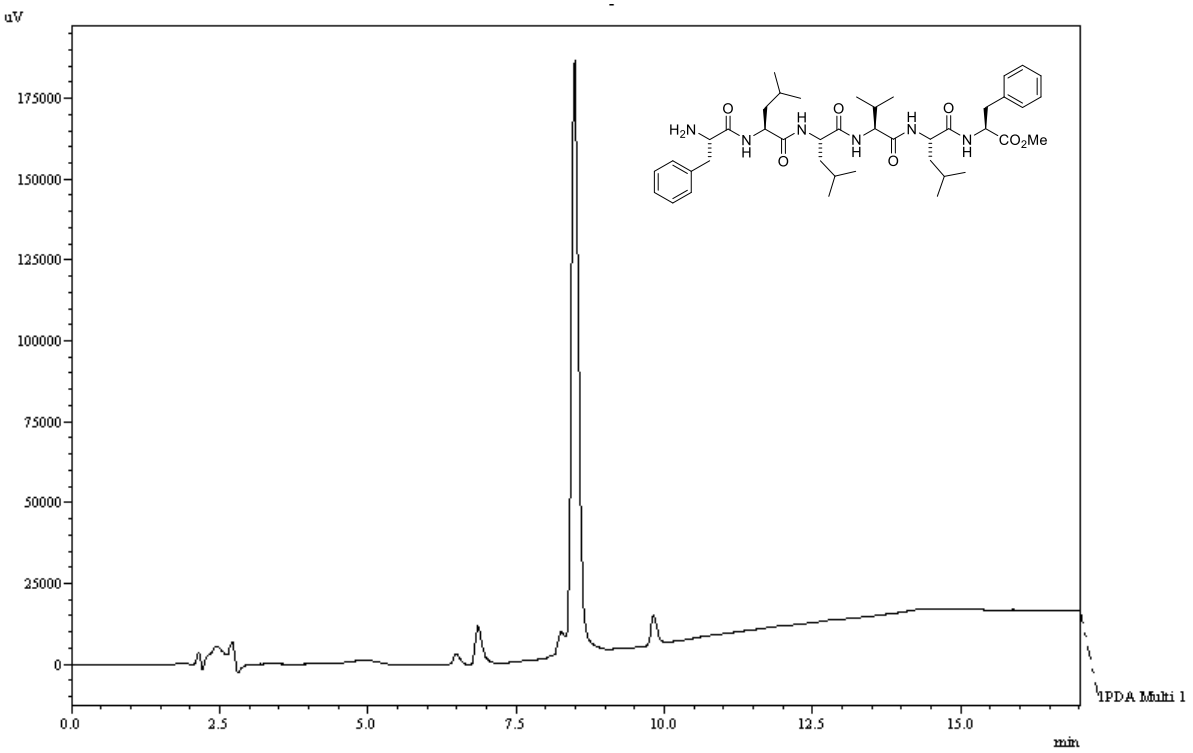


Mass Spectrum

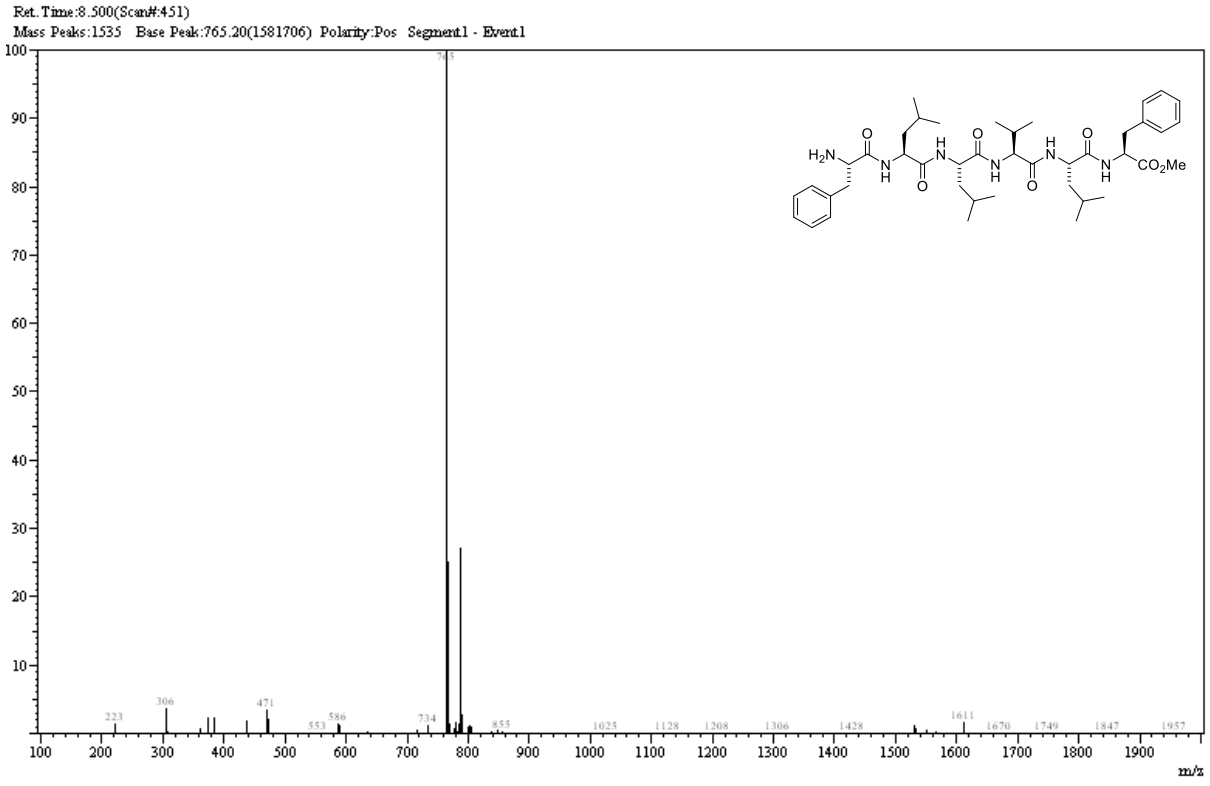


LC-MS of compound **11** (reverse-phase, 5→100% (MeCN/H_2O) over 10 min)

Chromatogram (254 nm)

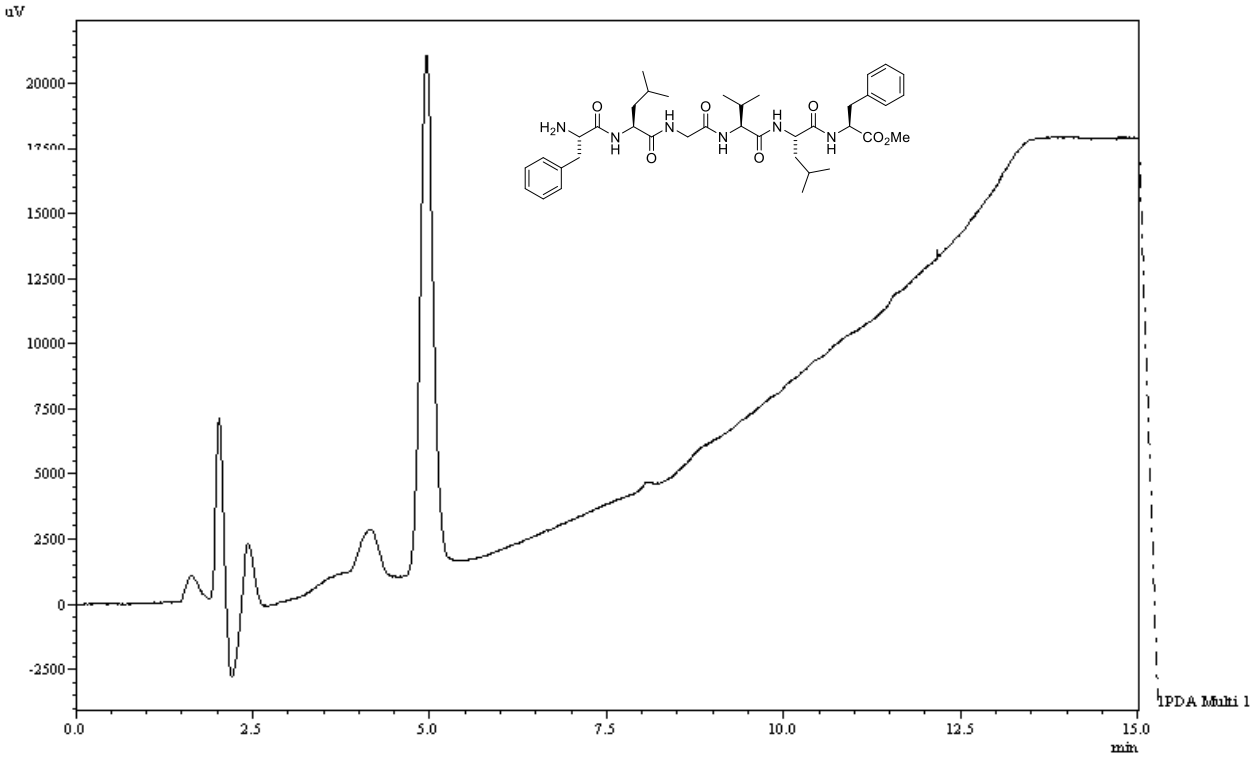


Mass Spectrum

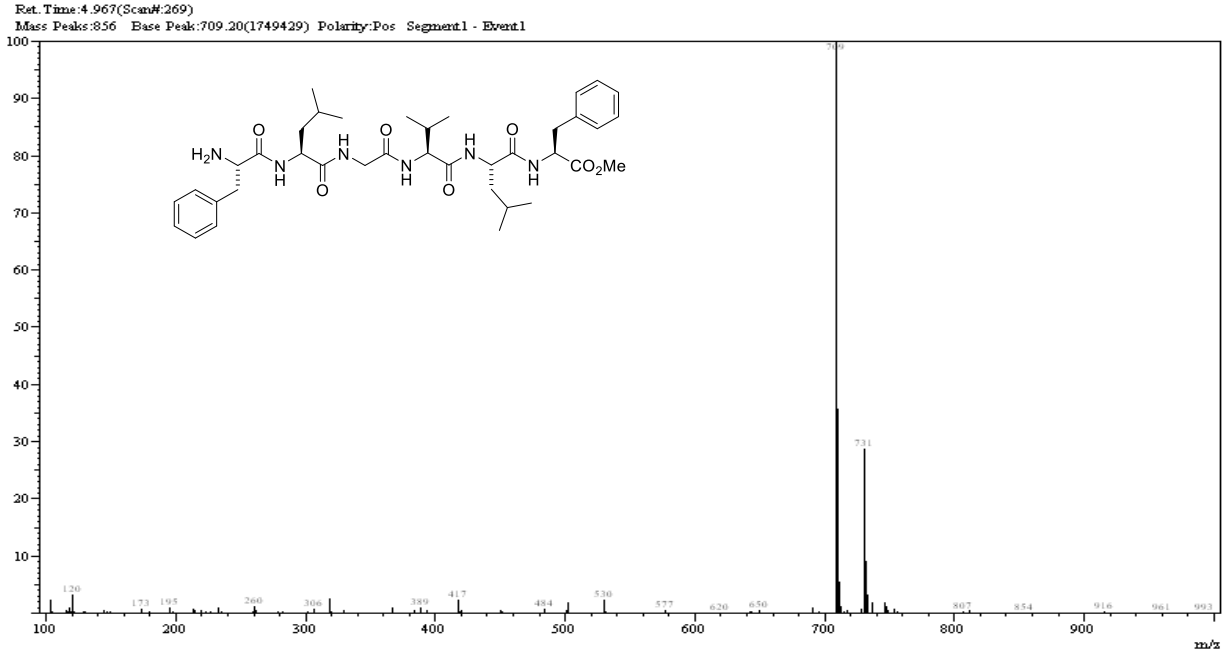


LC-MS of compound **12** (reverse-phase, 5→100% (MeCN/H_2O) over 10 min)

Chromatogram (254 nm)

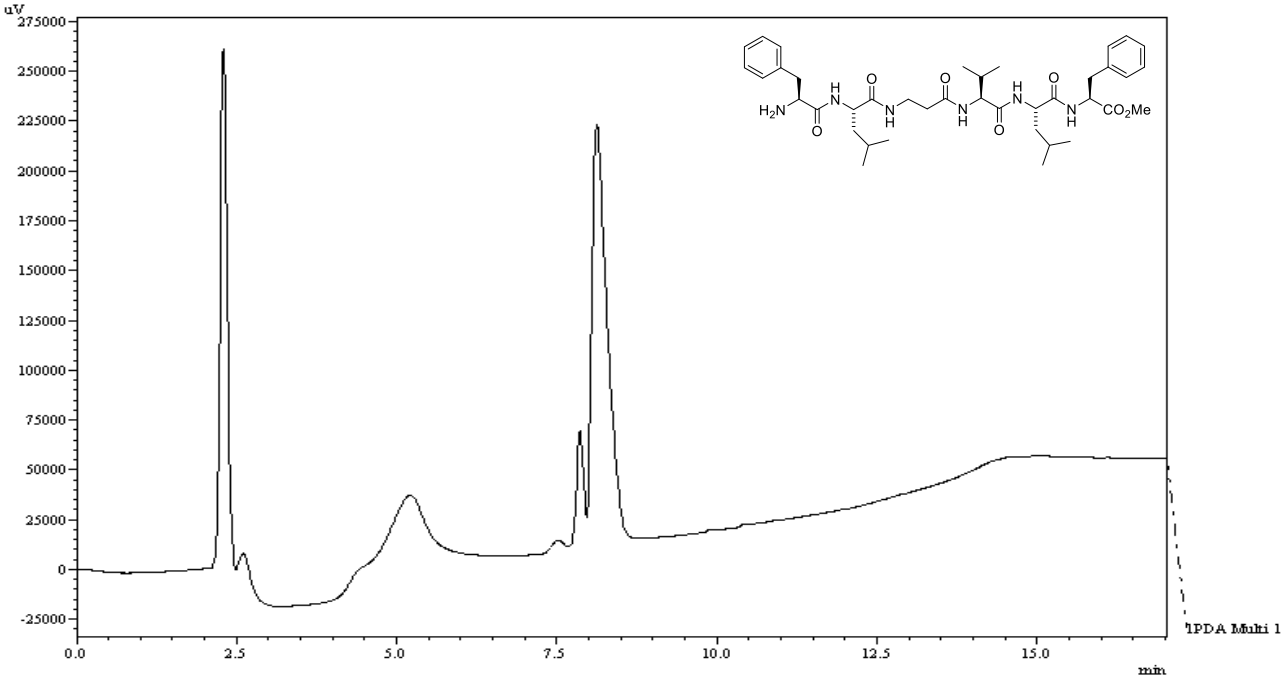


Mass Spectrum

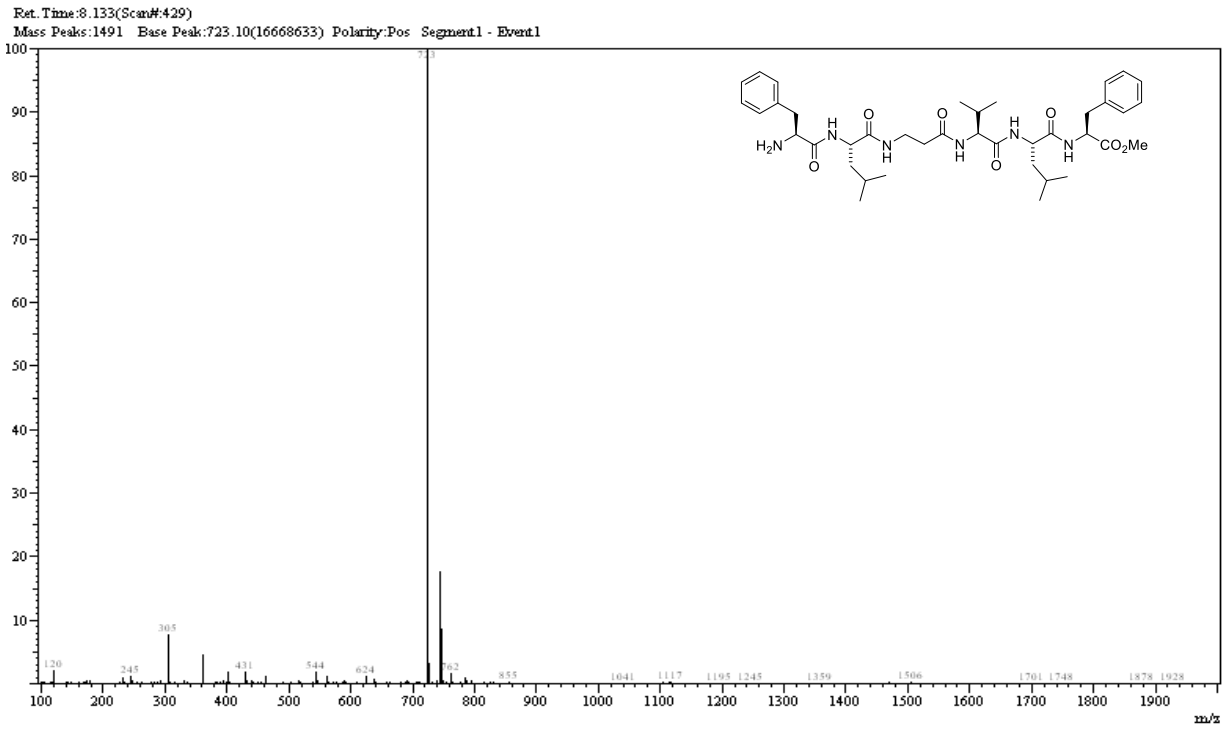


LC-MS of compound **13** (reverse-phase, 5→100% (MeCN/H_2O) over 10 min)

Chromatogram (254 nm)

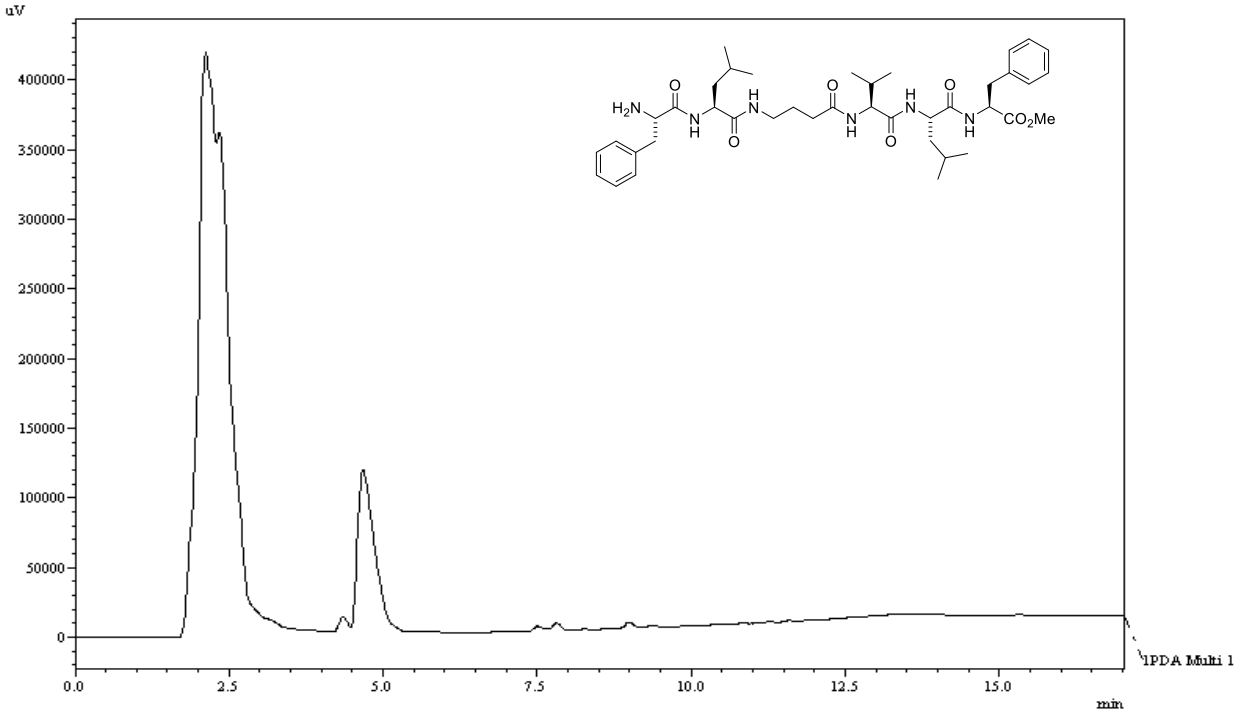


Mass Spectrum

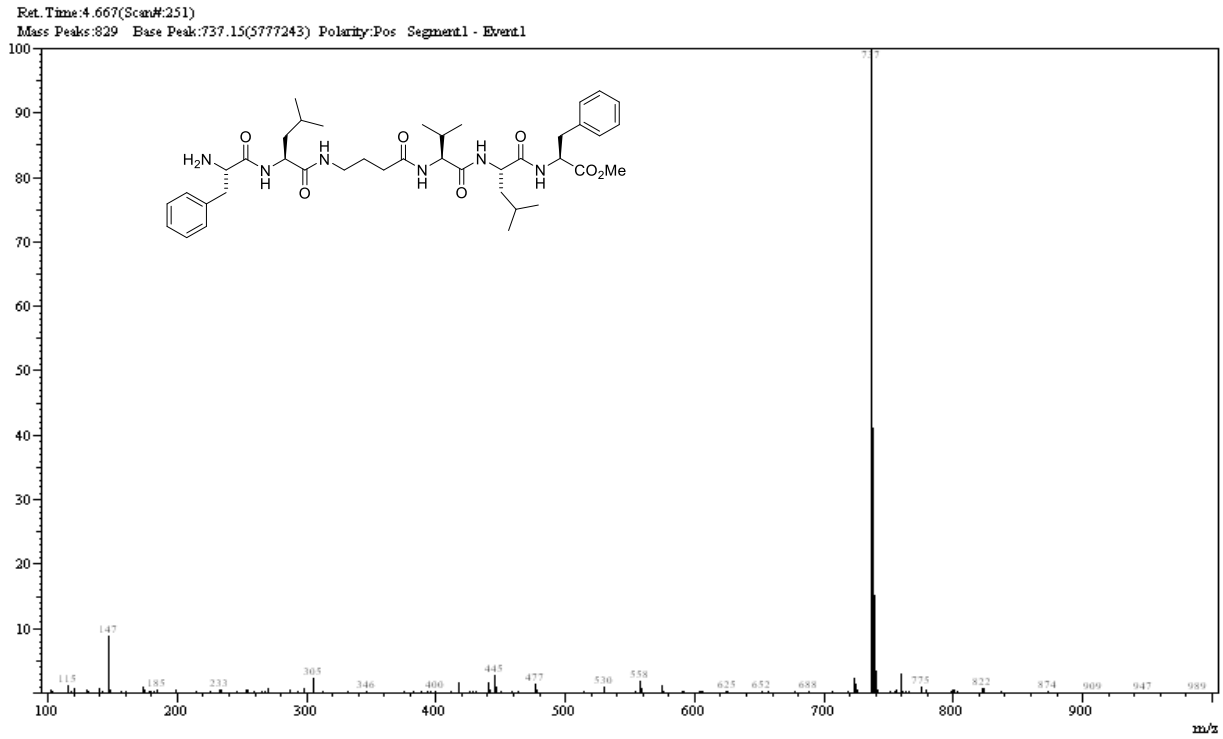


LC-MS of compound **14** (reverse-phase, 30 →100% (MeCN/H_2O) over 10 min

Chromatogram (254 nm)

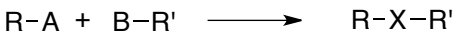


Mass Spectrum



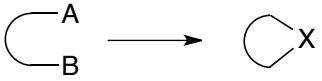
6. Determination of effective molarity (*EM*)

For an intermolecular reaction (between functionalities A and B to give X), the intermolecular rate constant is second order.



$$\text{Rate} = k_{2(\text{inter})}[\text{RA}][\text{R}'\text{B}]$$

For the corresponding intramolecular reaction, conceptually the rate is also dependent on the concentrations of both functional groups. However for a given concentration of one functional group (say A), the concentration of the other functional group is constant (assuming that the reaction is entirely intramolecular). This 'concentration' does not change as the reaction proceeds. This constant concentration term is incorporated into the *apparent* rate constant for the intramolecular process, which is first order. Overall then, the reaction can be thought of as being pseudo first order.



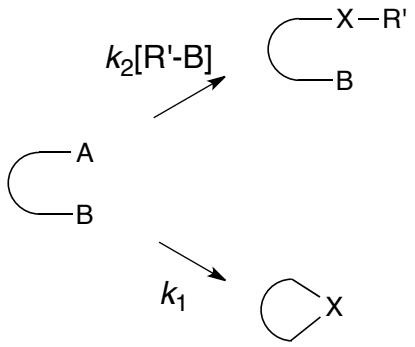
$$\text{Rate} = k_{2(\text{intra})}[\text{RA}][\text{R}'\text{B}]$$
$$= k_{1(\text{intra})}[\text{RA}]$$

The effective molarity (*EM*) of a given moiety, or more properly here the kinetic effective molarity, measures the importance of having the reactive sites within the same molecule. It is defined as the ratio of the rate constants for the intra- and inter-molecular processes. This can be thought of in terms of the competition reaction below. (Practically, this can be achieved by carrying out the reaction in the presence of an excess of RB, such that both processes are observed as first order, with a subsequent calculation allowing determination of $k_{2(\text{inter})}$.)

$$EM = \frac{k_{1(\text{intra})}}{k_{2(\text{inter})}}$$

The value for *EM* of a functional group in an intramolecular process can be considered in terms of the concentration of a species with the same functionality required to have the same observed rate constant for the intermolecular reaction as is observed for the intramolecular case. (Thus, a high value of *EM* is indicative of a very efficient intramolecular process.) *EM* can be thought of as the effective local concentration of the second functional group, with the assumption that the intrinsic second order rate constants for the intra and inter-molecular processes are the same.

Determining effective molarities does not necessarily require determination of the intra- and inter-molecular rate constants. Consider the competition experiment below.



The disappearance of A-B can be expressed in terms of a rate:

$$\text{rate} = -\frac{\text{d[A-B]}}{\text{d}t} = k_1[\text{A-B}] + k_2[\text{A-B}][\text{R'-B}] = (k_1 + k_2[\text{R'-B}])[\text{A-B}]$$

If an excess of R'-B is used, this simplifies to a first order process to give:

$$[\text{A-B}] = [\text{A-B}]_o e^{-(k_1 + k_2[\text{R'-B}])t}$$

Similarly, assuming integration from 0 to t and only A-B present initially, the formation of the cyclic material is given by:

$$\frac{\mathrm{d[cycle]}}{\mathrm{d}t} = k_1[\mathrm{A\text{-}B}] = k_1[\mathrm{A\text{-}B}]_o e^{-(k_1 + k_2[\mathrm{R'\text{-}B}])t}$$

$$[\mathrm{cycle}] = \frac{k_1[\mathrm{A\text{-}B}]_o}{(k_1 + k_2[\mathrm{R'\text{-}B}])}(1 - e^{-(k_1 + k_2[\mathrm{R'\text{-}B}])t})$$

And for the intermolecular reaction:

$$\frac{\mathrm{d[A\text{-}X\text{-}R']}}{\mathrm{d}t} = k_2[\mathrm{R'\text{-}B}][\mathrm{A\text{-}B}] = k_1[\mathrm{R'\text{-}B}][\mathrm{A\text{-}B}]_o e^{-(k_1 + k_2[\mathrm{R'\text{-}B}])t}$$

$$[\mathrm{A\text{-}X\text{-}R'}] = \frac{k_2[\mathrm{R'\text{-}B}][\mathrm{A\text{-}B}]_o}{(k_1 + k_2[\mathrm{R'\text{-}B}])}(1 - e^{-(k_1 + k_2[\mathrm{R'\text{-}B}])t})$$

Hence, the ratio of the products ***at any stage*** is given by:

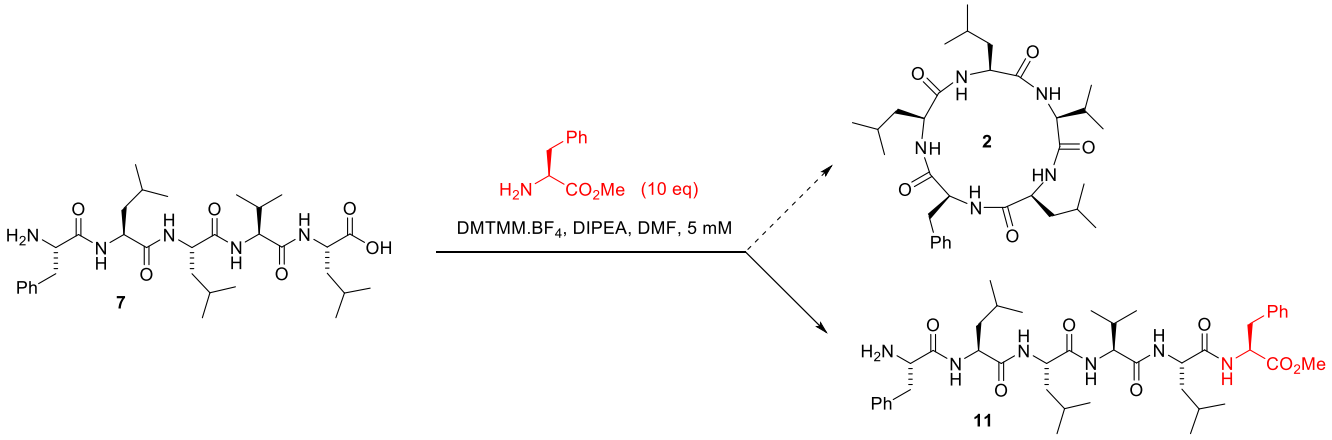
$$\frac{[\mathrm{cycle}]}{[\mathrm{A\text{-}X\text{-}R'}]} = \frac{\dfrac{k_1[\mathrm{A\text{-}B}]_o}{(k_1 + k_2[\mathrm{R'\text{-}B}])}(1 - e^{-(k_1 + k_2[\mathrm{R'\text{-}B}])t})}{\dfrac{k_2[\mathrm{R'\text{-}B}][\mathrm{A\text{-}B}]_o}{(k_1 + k_2[\mathrm{R'\text{-}B}])}(1 - e^{-(k_1 + k_2[\mathrm{R'\text{-}B}])t})}$$

$$= \frac{k_1}{k_2[\mathrm{R'\text{-}B}]}$$

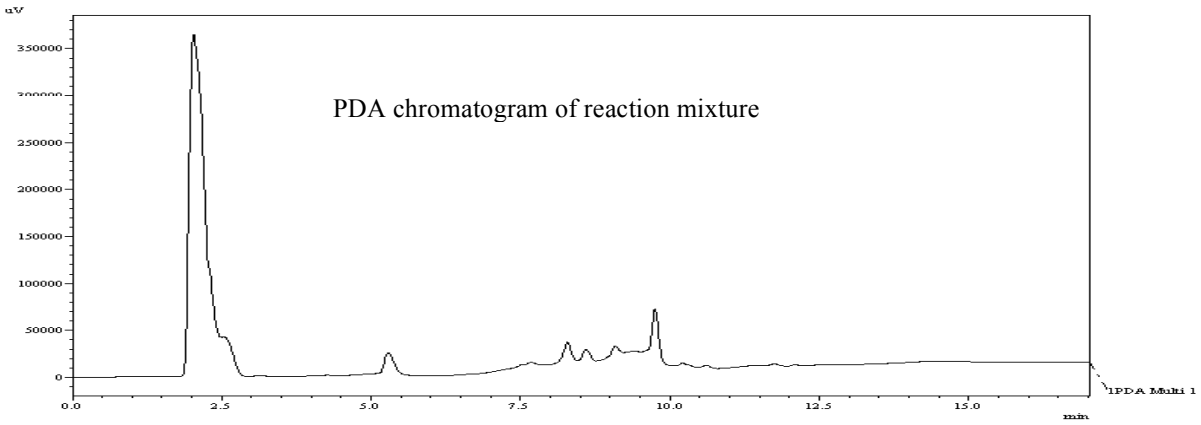
Conveniently, this can readily be converted to the effective molarity and was the method used in the following series of experiments.

Determining *EM* of linear peptide 7:

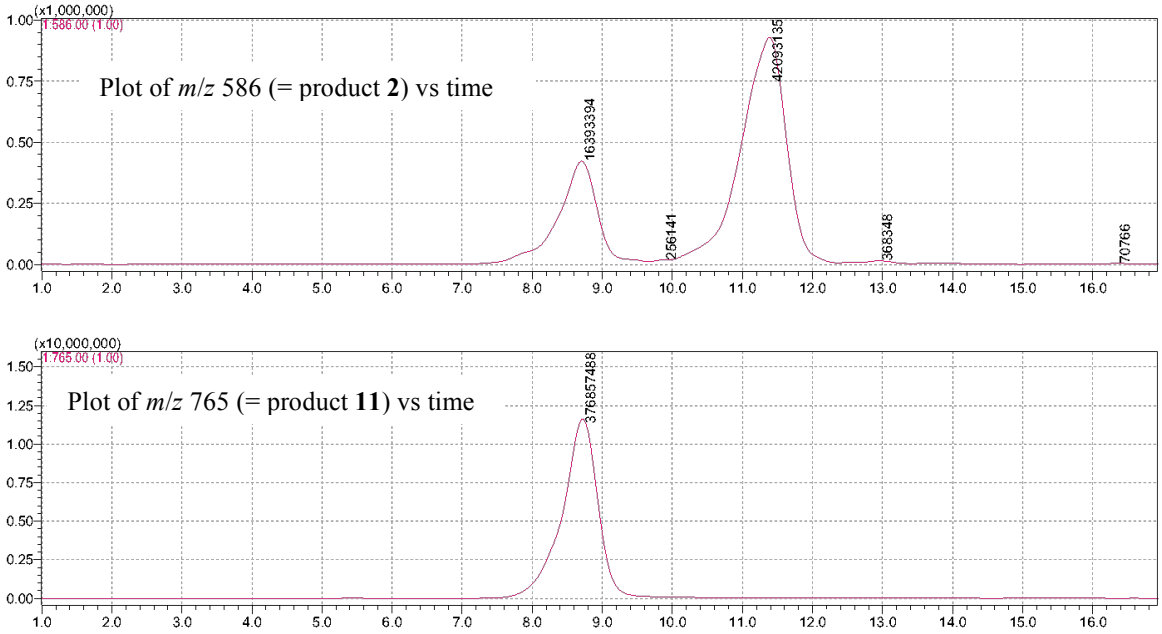
(i) Competition reaction



The competition reaction was performed in triplicate. The linear precursor peptide **7** (10.7 mg, 14.9 μmol) and PheOMe (32.4 mg, 150 μmol, 10 equiv. relative to peptide) were dissolved in minimal DMF. A solution of DMTMM.BF_4 (14.8 mg, 45.1 μmol, 3 equiv. relative to peptide) in DMF was added *via* cannula to give a final peptide concentration of 5 mM (3 mL DMF in total). Immediately, DIPEA (26.1 μL, 10 equiv. relative to peptide) was added. After 3 hours, an aliquot (4 μL) was withdrawn from the reaction mixture, diluted to 80 μL by addition of 0.1% TFA/MeCN, and directly analyzed by LCMS (see representative trace below). The eluent was a mixture of MilliQ H_2O and MeCN containing 0.1% formic acid, and the injection volume was 30 μL.

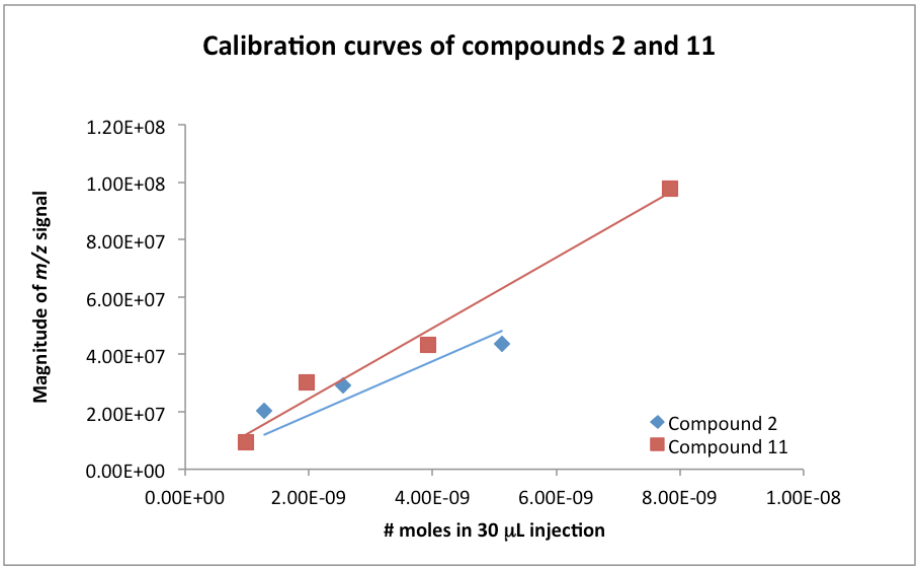


Due to the large number of overlapping peaks in the PDA chromatogram, the two products (cyclic pentapeptide **2** and linear hexapeptide **11**) were quantified by plotting the intensity of the appropriate mass signal over time, and measuring the resulting peak area (see below). Two peaks with mass 586 were observed; the minor peak was attributed to an epimerised cyclic product and was not included in the *EM* calculations.



*(ii) Quantifying the molar ratio of **2** : **11***

Authentic samples of **2** and **11**, of known concentration, were analysed by LCMS in an attempt to create calibration curves (see below). The data points are somewhat scattered, reflecting the uncertainty associated with quantifying samples by mass spectrometry. However the slopes of the two lines are quite close to one another. Therefore, for subsequent calculations it was assumed that compounds **2** and **11** ionise with equal efficiency. Hence, the ratio of peak areas in the traces above is assumed to be equal to the molar ratio of compounds **2** and **11**.



(iii) Calculation of EM

The molar ratio of **2** : **11** was calculated from the peak areas on the [*m/z* vs. time] plots above. This molar ratio was then converted to *EM* using the following formula:

$$EM \text{ of peptide } \mathbf{7} = \frac{[\text{cyclic peptide } \mathbf{2}] \,.\, [\text{Phe-OMe}]}{[\text{linear peptide } \mathbf{11}]}$$

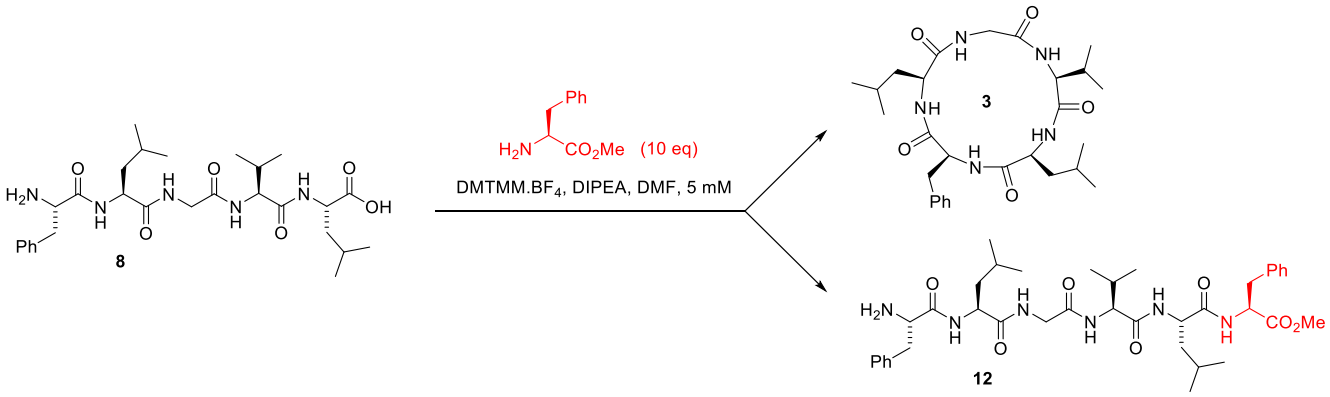
Experiment	Molar ratio of **2** : **11**	[Phe-OMe] (mM)	*EM* (mM)
1	0.112	50	5.60
2	0.171	50	8.55
3*	0.121	25	6.06

* Reaction performed at 2.5 mM peptide concentration. **Mean = 6.7±1.5 mM****

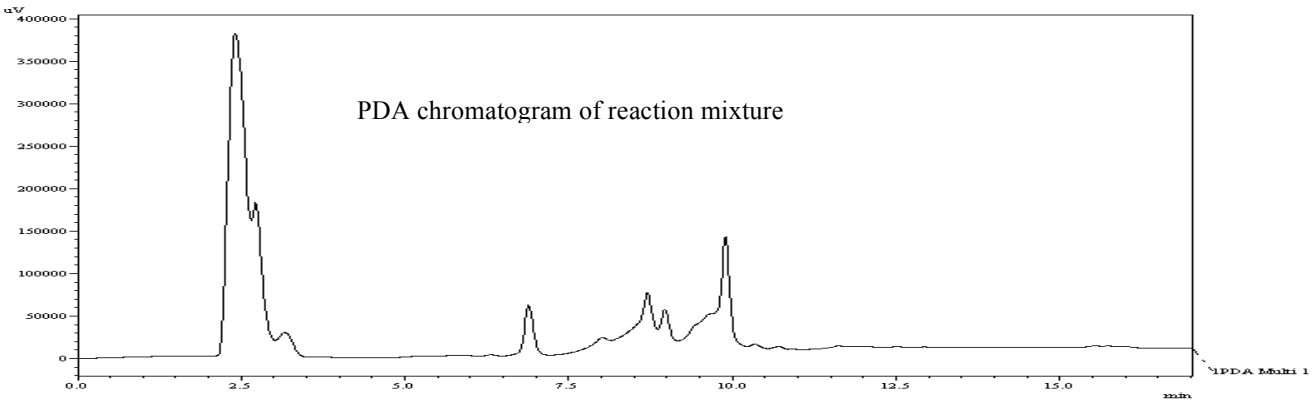
** Results are shown as mean ± half-the-range.

Determining *EM* of linear peptide 8:

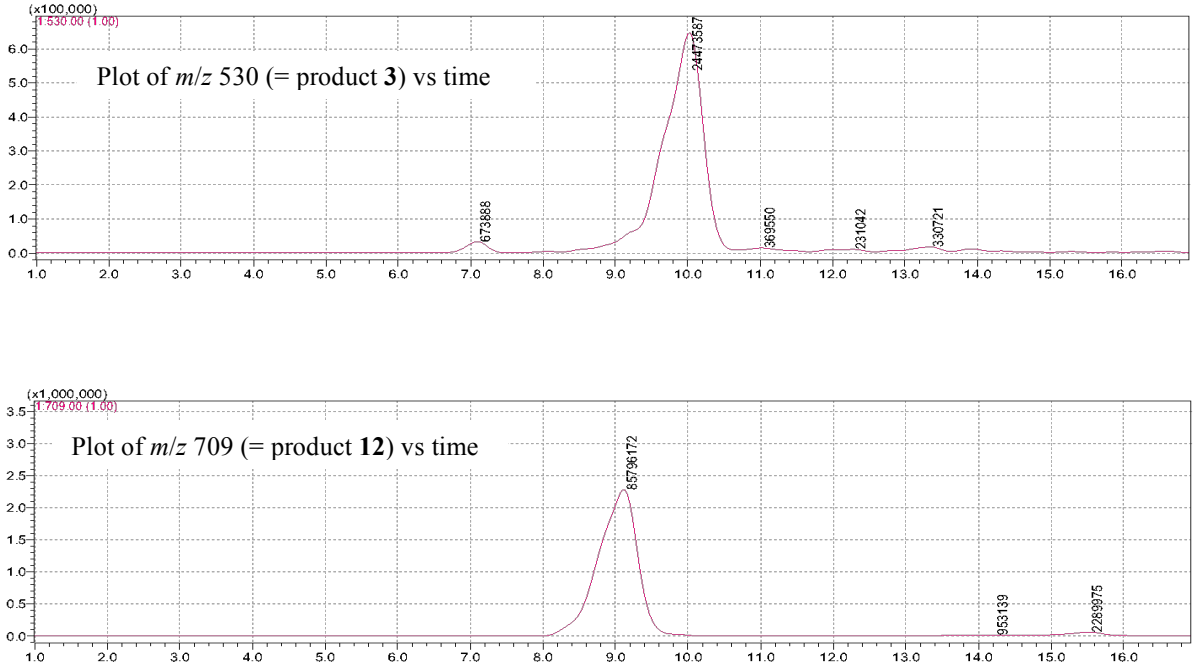
(i) Competition reaction



The competition reaction was performed in quadruplicate. The linear precursor peptide **8** (9.8 mg, 14.8 μmol) and PheOMe (32.4 mg, 150 μmol, 10 equiv. relative to peptide) were dissolved in minimal DMF. A solution of DMTMM.BF_4 (14.8 mg, 45.1 μmol 3 equiv. relative to peptide) in DMF was added *via* cannula to give a final peptide concentration of 5 mM (3 mL DMF in total). Immediately, DIPEA (26.1 μL, 10 equiv. relative to peptide) was added. After 3 hours, an aliquot (4 μL) was withdrawn from the reaction mixture, diluted to 80 μL by addition of 0.1% TFA/MeCN, and directly analyzed by LCMS (see representative trace below). The eluent was a mixture of MilliQ H_2O and MeCN containing 0.1% formic acid, and the injection volume was 30 μL.

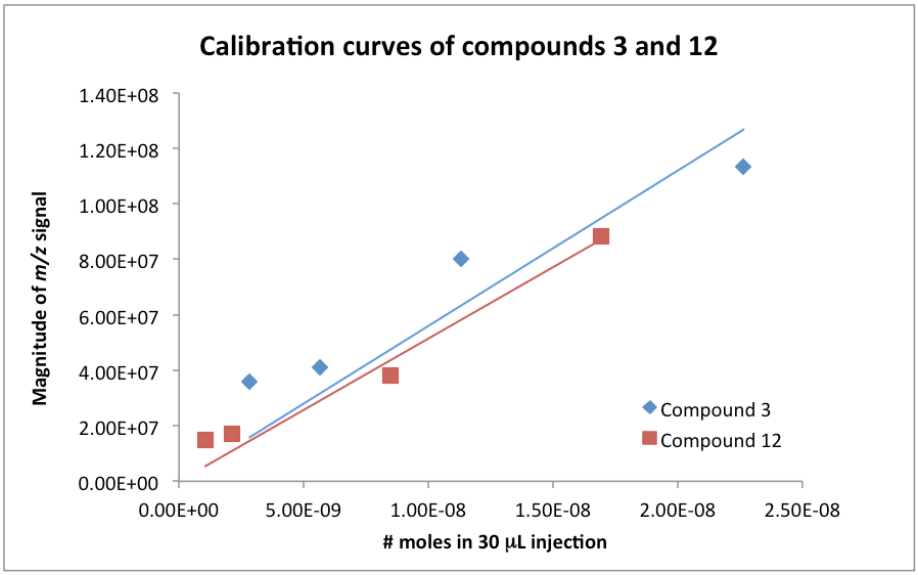


Due to the large number of overlapping peaks in the PDA chromatogram, the two products (cyclic pentapeptide **3** and linear hexapeptide **12**) were quantified by plotting the intensity of the appropriate mass signal over time, and measuring the resulting peak area (see below).



*(ii) Quantifying the molar ratio of **3** : **12***

Authentic samples of **3** and **12**, of known concentration, were analysed by LCMS in an attempt to create calibration curves (see below). The data points are somewhat scattered, reflecting the uncertainty associated with quantifying samples by mass spectrometry. However the slopes of the two lines are quite close to one another. Therefore, for subsequent calculations it was assumed that compounds **3** and **12** ionise with equal efficiency. Hence, the ratio of peak areas in the traces above is assumed to be equal to the molar ratio of compounds **3** and **12**.



(iii) Calculation of EM

The molar ratio of **3** : **12** was calculated from the peak areas on the [*m*/*z* vs. time] plots above. This molar ratio was then converted to *EM* using the following formula:

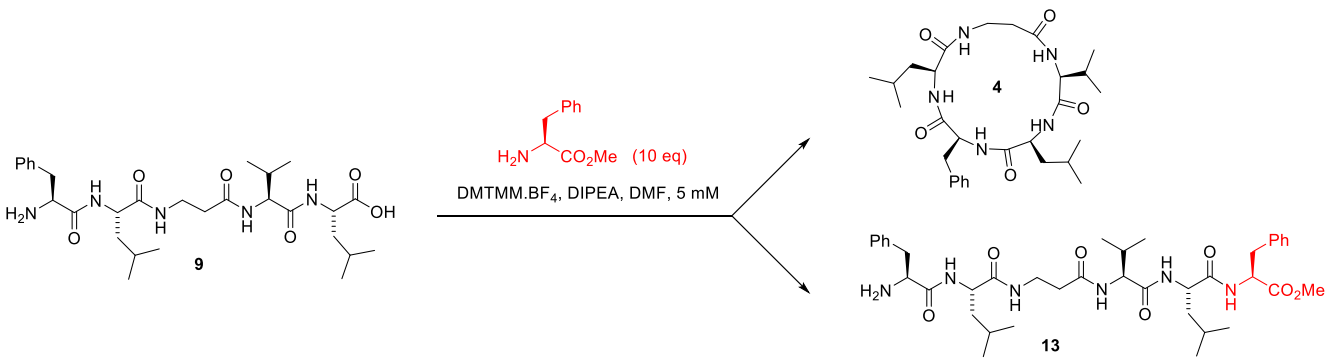
$$EM \text{ of peptide } \mathbf{8} = \frac{[\text{cyclic peptide } \mathbf{3}] \,.\, [\text{Phe-OMe}]}{[\text{linear peptide } \mathbf{12}]}$$

Experiment	Molar ratio of **3** : **12**	[Phe-OMe] (mM)	*EM* (mM)
1	0.207	50	10.4
2	0.257	50	12.9
3	0.286	50	14.3
4	0.317	50	15.8

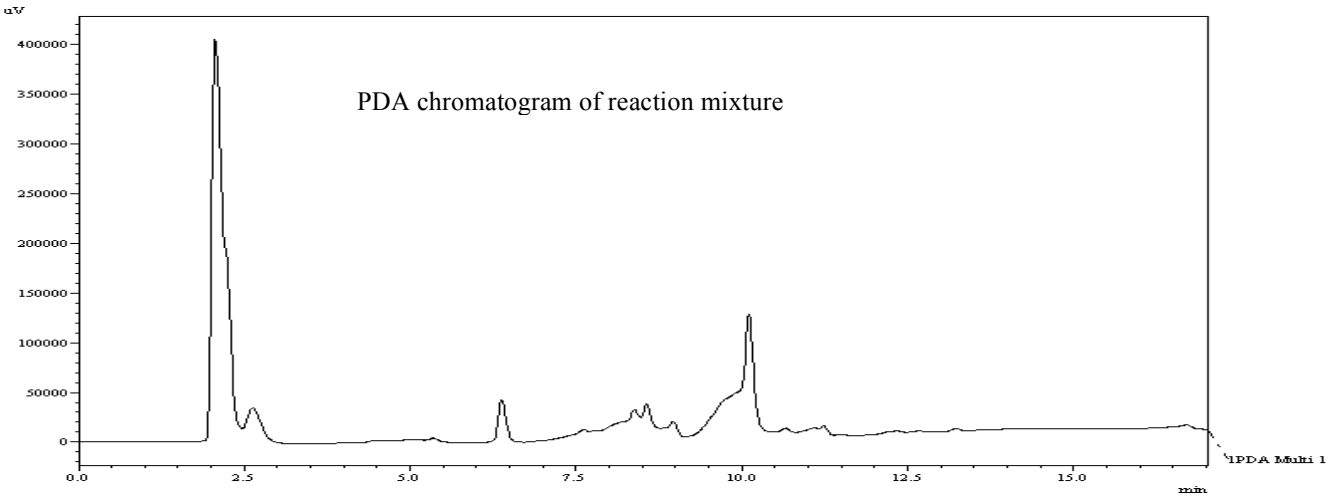
* Results are shown as mean ± half-the-range. **Mean = 13.4±2.7 mM***

Determining *EM* of linear peptide 9:

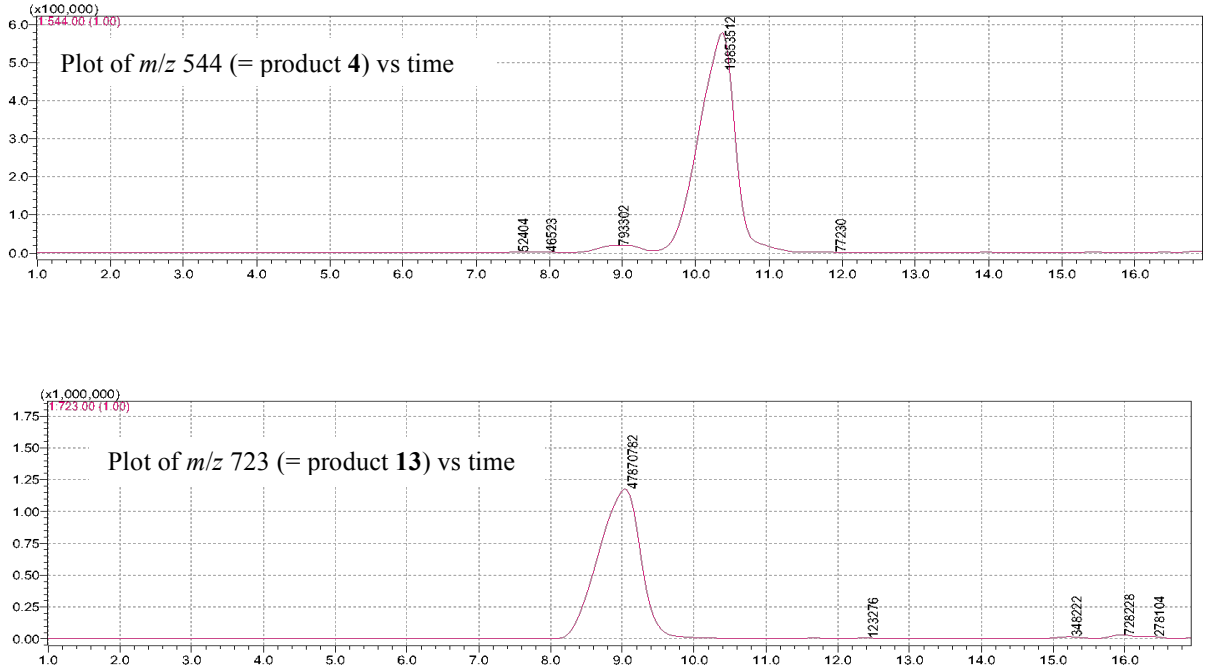
(i) Competition reaction



The competition reaction shown above was performed in quadruplicate. The linear precursor peptide **9** (10.8 mg, 15.9 μmol) and PheOMe (34.5 mg, 160 μmol, 10 equiv. relative to peptide) were dissolved in minimal DMF. A solution of DMTMM.BF_4 (15.8 mg, 48.2 μmol 3 equiv. relative to peptide) in DMF was added *via* cannula to give a final peptide concentration of 5 mM (3.2 ml DMF in total). Immediately, DIPEA (27.9 μl, 10 equiv. relative to peptide) was added. After 3 hours, an aliquot (4 μL) was withdrawn from the reaction mixture, diluted to 80 μL by addition of 0.1% TFA/MeCN, and directly analyzed by LCMS (see representative trace below). The eluent was a mixture of MilliQ H_2O and MeCN containing 0.1% formic acid, and the injection volume was 30 μL.

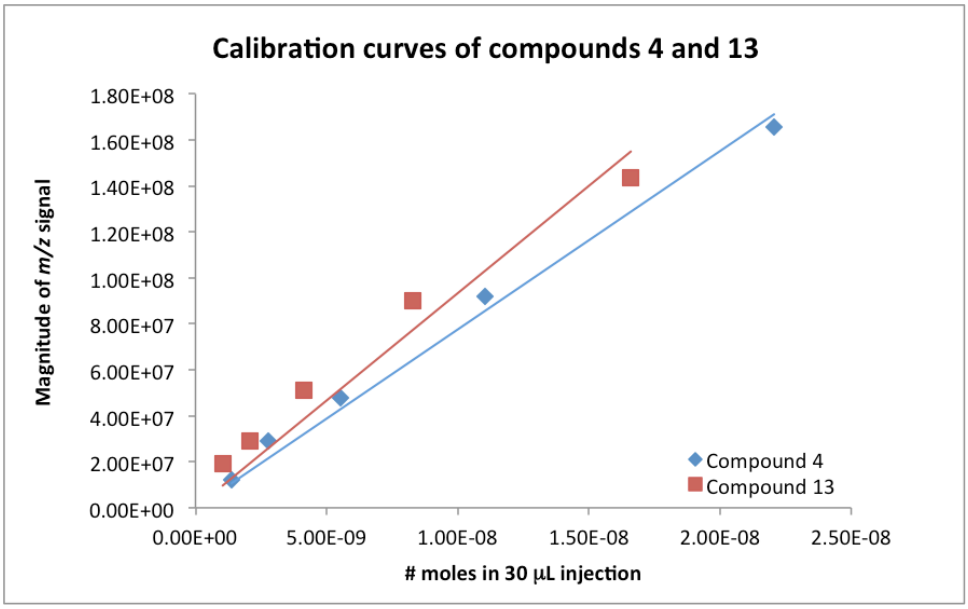


Due to the large number of overlapping peaks in the PDA chromatogram, the two products (cyclic pentapeptide **4** and linear hexapeptide **13**) were quantified by plotting the intensity of the appropriate mass signal over time, and measuring the resulting peak area (see below).



*(ii) Quantifying the molar ratio of **4** : **13***

Authentic samples of **4** and **13**, of known concentration, were analysed by LCMS in an attempt to create calibration curves (see below). The data points are somewhat scattered, reflecting the uncertainty associated with quantifying samples by mass spectrometry. However the slopes of the two lines are quite close to one another. Therefore, for subsequent calculations it was assumed that compounds **4** and **13** ionise with equal efficiency. Hence, the ratio of peak areas in the traces above is assumed to be equal to the molar ratio of compounds **4** and **13**.



(iii) Calculation of EM

The molar ratio of **4** : **13** was calculated from the peak areas on the [*m*/*z* vs. time] plots above. This molar ratio was then converted to *EM* using the following formula:

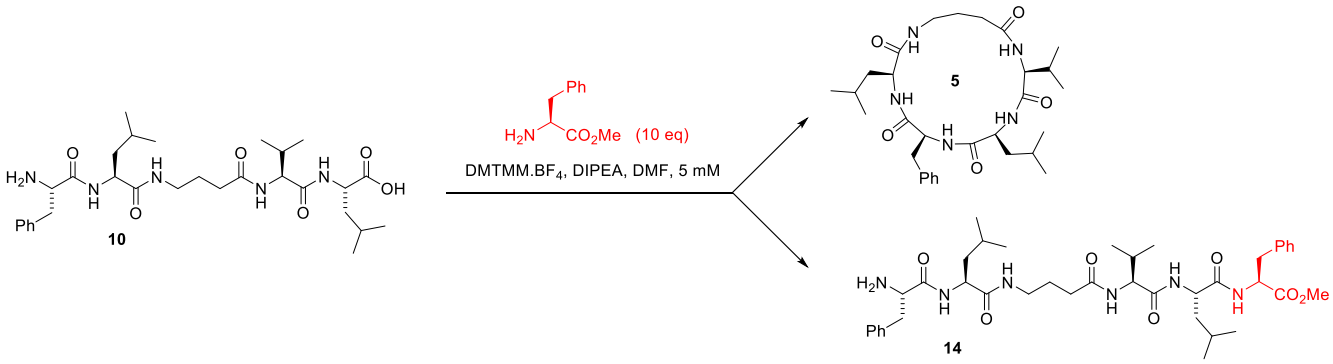
$$EM \text{ of peptide } \mathbf{9} = \frac{[\text{cyclic peptide } \mathbf{4}] \,.\, [\text{Phe-OMe}]}{[\text{linear peptide } \mathbf{13}]}$$

Experiment	Molar ratio of **4** : **13**	[Phe-OMe] (mM)	*EM* (mM)
1	0.323	50	16.2
2	0.415	50	20.8
3	0.412	50	20.6
4	0.315	50	15.8

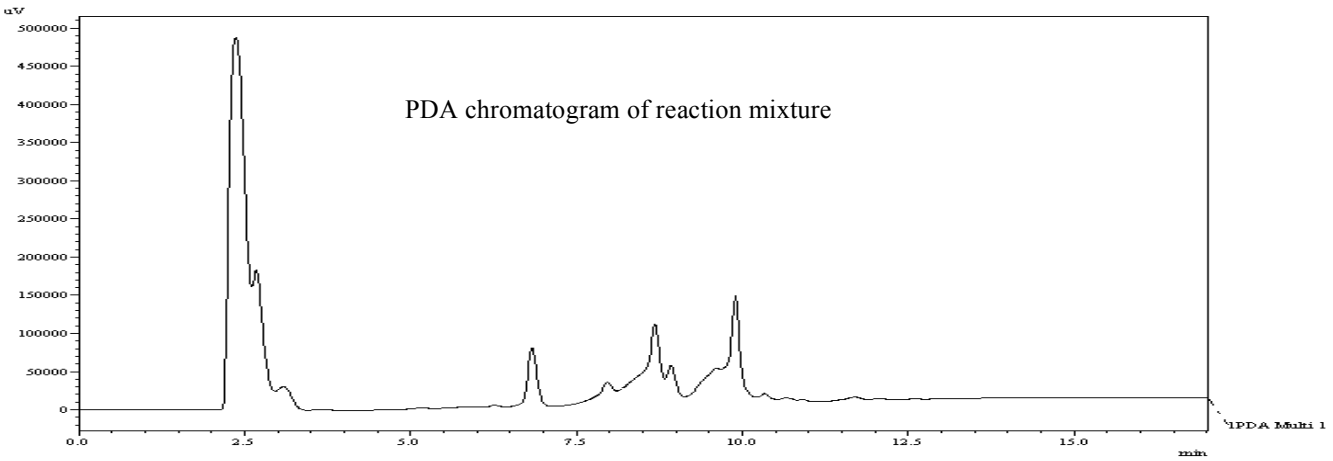
* Results are shown as mean ± half-the-range. **Mean = 18.4±2.5 mM***

Determining *EM* of linear peptide 10:

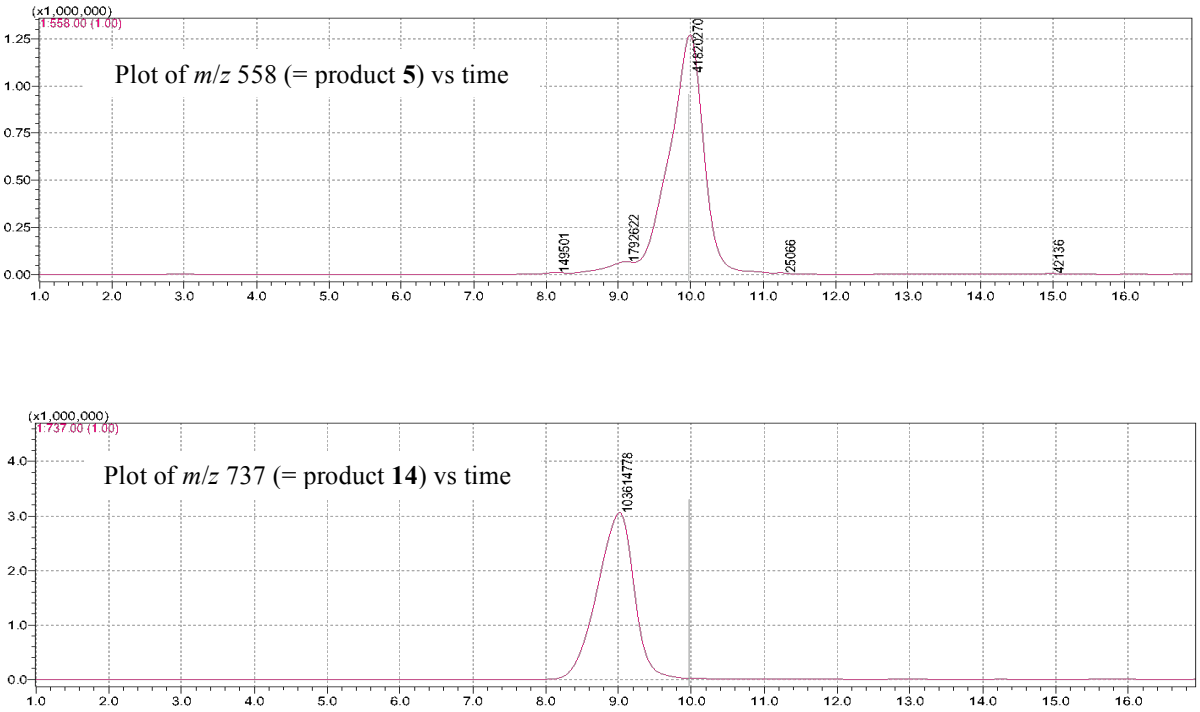
(i) Competition reaction



The competition reaction shown above was performed in quadruplicate. The linear precursor peptide **10** (9.8 mg, 14.2 μmol) and PheOMe (30.6 mg, 141.9 μmol, 10 equiv. relative to peptide) were dissolved in minimal DMF. A solution of DMTMM.BF_4 (13.8 mg, 42.1 μmol 3 equiv. relative to peptide) in DMF was added *via* cannula to give a final peptide concentration of 5 mM (2.8 mL DMF in total). Immediately, DIPEA (24.7 μl, 10 equiv. relative to peptide) was added. After 3 hours, an aliquot (4 μL) was withdrawn from the reaction mixture, diluted to 80 μL by addition of 0.1% TFA/MeCN, and directly analyzed by LCMS (see representative trace below). The eluent was a mixture of MilliQ H_2O and MeCN containing 0.1% formic acid, and the injection volume was 30 μL.

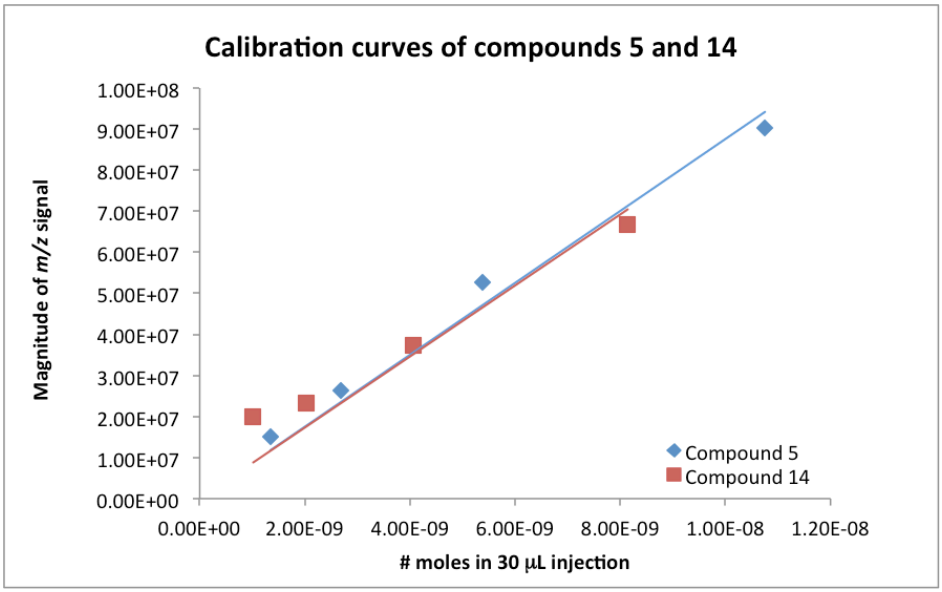


Due to the large number of overlapping peaks in the PDA chromatogram, the two products (cyclic pentapeptide **5** and linear hexapeptide **14**) were quantified by plotting the intensity of the appropriate mass signal over time, and measuring the resulting peak area (see below).



*(ii) Quantifying the molar ratio of **5** : **14***

Authentic samples of **5** and **14**, of known concentration, were analysed by LCMS in an attempt to create calibration curves (see below). The data points are somewhat scattered, reflecting the uncertainty associated with quantifying samples by mass spectrometry. However the slopes of the two lines are quite close to one another. Therefore, for subsequent calculations it was assumed that compounds **5** and **14** ionise with equal efficiency. Hence, the ratio of peak areas in the traces above is assumed to be equal to the molar ratio of compounds **5** and **14**.



(iii) Calculation of EM

The molar ratio of **5** : **14** was calculated from the peak areas on the [*m*/*z* vs. time] plots above. This molar ratio was then converted to *EM* using the following formula:

$$EM \text{ of peptide } \mathbf{10} = \frac{[\text{cyclic peptide } \mathbf{5}] \,.\, [\text{Phe-OMe}]}{[\text{linear peptide } \mathbf{14}]}$$

Experiment	Molar ratio of **5** : **14**	[Phe-OMe] (mM)	*EM* (mM)
1	0.305	50	15.2
2	0.406	50	20.3
3	0.402	50	20.1
4	0.304	50	15.2

* Results are shown as mean ± half-the-range. **Mean = 17.7±2.6 mM***

7. Cytotoxicity assay details

Media, antibiotics and cell cultures

The HCT-116 human colorectal carcinoma cell line was purchased from ATCC (Manassas, Virginia, USA). Cells were preserved in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Invitrogen/Life Technologies, Carlsbad, California, USA). Cells were proliferated in a humidifier at 37 °C with 5% CO_2. Stock solutions of compound were prepared by dissolving the solid compound in molecular biology grade dimethyl sulfoxide (DMSO, Sigma Aldrich).

Cytotoxicity assay protocol

Cells were seeded in a 96-well plate (2500 cells/well) and allowed to adhere to the dish for 24 hours. Seeded cells were treated in each well with compounds at a final concentration of 100 μM in a total volume of 100 μL. 17-AAG [17-(allylamino)-17-demethoxyfeldanamycin; 100 nM; Sigma Aldrich] was used as the positive control. HCT-116 cells were incubated with the treated compounds for 72 h at 37 °C with 5% CO_2.

Cell proliferation was measured using a Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Rockville, Maryland, USA) following the manufacturer's instructions. Reduction of formazen dye was measured using a Chromate 4300 microplate reader (450 nm; Awareness Technology Inc.). The absorbance of compound treated cells was compared to the media control and the average percent growth inhibition was determined for each compound tested. The assays were performed in triplicate.