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Supporting Information

Synthesis of Highly Selective Submicromolar Microcystin-Based Inhibitors of Protein Phosphatase (PP)2A over PP1

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1. Supporting Figures

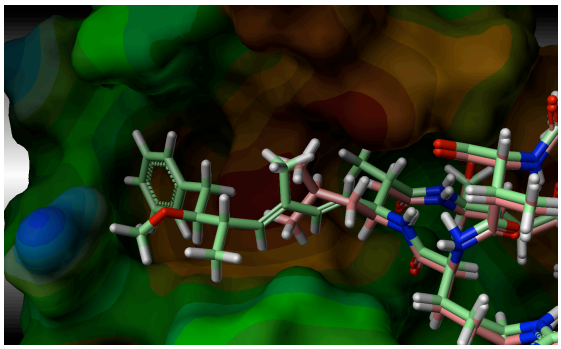


Figure S1. The isobutyl residue of compound **2** should be better accommodated in the Adda-binding pocket than the corresponding shorter residues in compounds **9** and **12**, which should enhance the binding to PP1 and PP2A. In order to check how well the isobutyl group at position 5 of compound **2** can fit into the binding pocket, we modeled the compound **2** (pink) by replacing the Adda-group in the crystal structure of MC (green) bound to PP1 (pdb code 2BDX) with the isobutyl group. After this modification this new part got minimized inside the protein pocket. The picture shows that this group is fitting into the pocket well and that no clashes to the protein occur. In spite of this finding, compounds **9** and **12** were more potent.

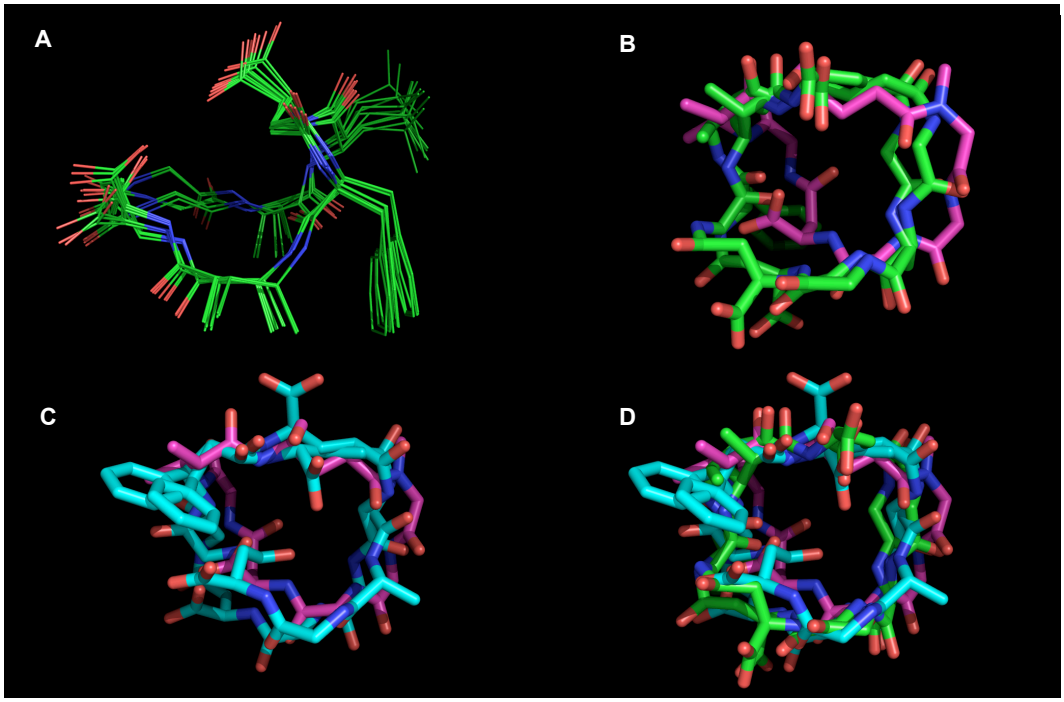


Figure S2. (A) Ensemble of calculated NMR structures for compound **10**. The calculation is based on 78 NOE derived distances (47 intraresidue, 29 sequential and 2 non-adjacent) and four ϕ dihedral angle restraints (residues Leu-Asp-Phe-Amba). The average rmsd of all heavy atoms to the mean structure is 0.61 +/- 0.11 Å. Note that these structures are generated under the assumption that the measured data result from a single conformation of the peptide in solution, an assumption that does most likely not hold for this compound given the loss of hydrogen bonding within the molecule and the reduced compaction compared to MC. (B-D) Superposition of possible conformers out of a pool of 500 randomly generated structures for compounds **3** (blue) and **10** (green) respectively to the solution structure of MC (1clm.pdb) (pink). (B) Superposition of the solution structure of MC-LF (pdb code 1LCM) (pink) with two solution structures of compound **10** (green) with the lowest rmsd deviation for the backbone Cα of Amba-(D)Glu-Gly selected from a pool of 500 geometrically correct structures. (C) Superposition of MC-LF (pink) with two solution structures of compound **3** (blue) with the lowest rmsd deviation for the backbone Cα of L-β-Phe-(D)Glu-Gly selected from a pool of 500 geometrically correct structures. (D) Superposition of MC-LF (pink), compound **10** and compound **3** (blue).

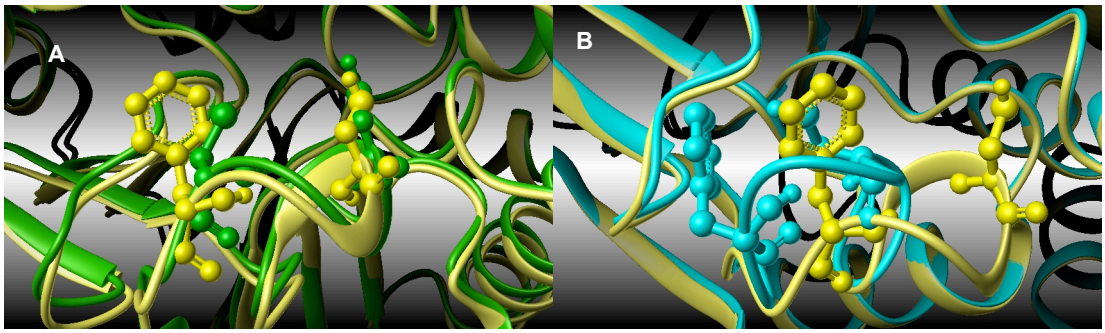


Figure S3. Alignment of PP2A and PP1 when bound to toxins. (A) PP2A covalently bound to MC (green, pdb code 2IE3) aligned with PP1 non-covalently bound to motuporin (yellow, pdb code 2BCD). The backbones align completely. Highlighted in the stick presentation: Phe276 (PP1) aligns with Cys269 (PP2A), Cys273 (PP1) aligns with Cys266 (PP2A); (B) PP1 from the structure covalently bound to MC (cyan, pdb code 1FJM) was aligned with PP1 from the structure in which it is non-covalently bound to motuporin (yellow, pdb code 2BCD). Phe276 und Cys273 are shown as sticks. The loop movement is clearly visible. In all other structures of PP1 non-covalently bound to toxins, PP1 adopts the conformation shown in yellow that matches the one of PP2A (A). Structures of PP2A do not differ in this region for covalently and non-covalently bound ligands (not shown). Of note, previous studies that were in general done before the PP2A crystal structure was available, erroneously assumed that PP2A would bind to MC with Cys266 that aligns in the canonical sequence with Cys273 in PP1. Inhibitor design was based on this assumption, which could be one explanation for the only modest selectivity achieved previously.[S1,S2]

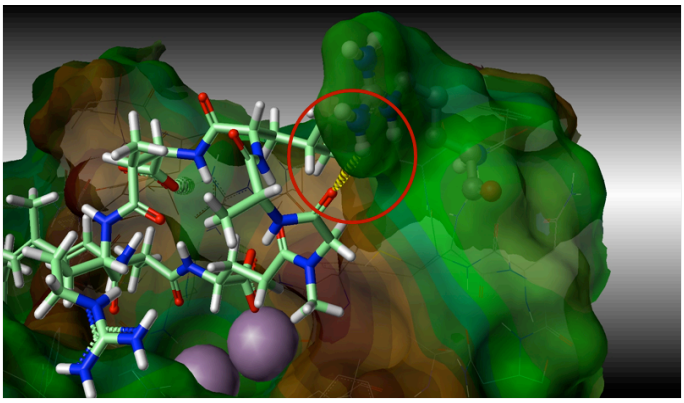


Figure S4. Two PP2A crystal structures with co-crystallized MCs are aligned (pdb codes 4I5L, 3FGA). The possible hydrogen bond is encircled.

2. General methods

Solvents were purchased and used as received from the following suppliers: dichloromethane (DCM) and cyclohexane from VWR; *N*,*N*-dimethylformamide (DMF) and dimethylsulfoxide (DMSO) from Merck; *N*,*N*-diisopropylethylamine (DIPEA) from abcr; *N*-methyl-2-pyrrolidone (NMP) and piperidine from ROTH; ethyl acetate, dichloromethane anhydrous ≥99.8%, methanol anhydrous 99.8%, acetonitrile (ACN) ≥99.9% HPLC grade, HPLC water LCMS chromasolv and methanol (MeOH) ≥99.9% HPLC grade from Sigma-Aldrich; anhydrous *N*,*N*-dimethylformamide (dry DMF) 99.8% from Alfa Aesar; dimethylsulfoxide-d6 (DMSO-d_6) 99.8%, deuterium oxide (D_2O) 99.95% and methyl alcohol-d_4 (CD_3OD) 99.8% from deutero GmbH.

Materials were used without further purification from the following comercial sources: Fmoc-Ala-OH, Fmoc-Gly-OH, Fmoc-Leu-OH, Fmoc-D-Ala-OH, Fmoc-*N*-hydroxysuccinimide ester, triethylamine (TEA), sodium hydrogen carbonate ($NaHCO_3$) and sodium chloride (NaCl) from Merck; Fmoc-Sar-OH, Fmoc-(D)Glu-OtBu, H-Phe-2-chlorotrityl resin 200-400 mesh 0.5-0.9 mmol/g substitution and 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) from Bachem; Fmoc-(D)Asp-OtBu, *N*-Fmoc-*L*-β-homophenylalanine (Fmoc-*L*-β-homo-Phe-OH), (*R*)-3-Fmoc-amino-3-phenylpropionic acid (Fmoc-*L*-β-Phe-OH) and triisopropylsilane (TIS) from Alfa Aesar; (1*S*, 2*S*)-2-aminocyclohexanecarboxylic acid, 2,2,2-trifluoroethanol (TFE), acetic acid (AcOH) ≥99.7%, imidazole ≥99.5%, sodium sulfate anhydrous ≥99%, dithiothreitol (DTT), manganese(II) chloride tetrahydrate ≥99% and Triton X-100 from Sigma-Aldrich; (*S*)-3-amino-5-methyl-hexanoic acid and (*R*)-3-amino-3-(naphthalen-2-yl)propanoic acid from Fluorochem; hydroxybenzotriazole (HOBt) from MOLEKULA; 1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate (HATU) and 1-hydroxy-7-azabenzotriazole (HOAt) from GL Biochem (Shanghai) Ltd; trifluoroacetic acid (TFA), silica gel 60 0.04-0.063mm (230-400mesh) and see sand from ROTH; 6,8-difluoro-4-Methylumbelliferylphosphate (DIFMUP) from Life Technologies and microcystin-LF from Enzo Life Science.

Analytical thin layer chromatography (TLC) was performed on Merck precoated aluminium silica gel 60 plates F254 and exposed to UV light for detection. Melting point was measured with Büchi melting point B-540 and optical rotation with Polatronic H532 polarimeter (Schmidt Haensch). $[\alpha]^{20}_D$ are reported in $degmlg^{-1}dm^{-1}$ and the concentration is in g in 100 ml. ^1H and ^{13}C NMR spectra were recorded with Bruker UltraShield Avance III HD 400 MHz or 500 MHz for compounds **2**-**15** using Topspin 3.2 as acquisition software and MestReNova 10.0 to analyze the date. Bruker Avance III 400 or 600 were used for compounds **16**-**19** using Topspin 3.2 as acquisition software and MestReNova. 8.1.4 to analyse the data. All chemical shifts are quoted in ppm and were reported using solvent as an internal standard (^1H NMR: 2.50 ppm (DMSO-d6), 7.26 ppm ($CDCl_3$), 3.31 ppm (CD_3OD); ^{13}C NMR: 39.51 ppm (DMSO-d6), 77.16 ppm ($CDCl_3$), 49.00 ppm (CD_3OD). When using D_2O as a solvent, ^1H NMR peaks were referenced using solvent as an internal standard (4.766 ppm) considering the temperature dependence and ^{13}C NMR peaks were referenced indirectly relative to the proton reference with the known gyromagnetic ratio.[S3] 2D COSY and HSQC were used to assist the NMR signal assignments. Synthesis of linear peptides was performed with a MultiSynTech Syro I Parallel Peptide Synthesis System. Analytical HPLC runs (1.5 mL/min) and purification (5 mL/min) of microcystin derivatives was performed on a Shimadzu HPLC-MS 2010EV Evolution system with a reversed phase column from

Macherey-Nagel (NUCLEODUR 100-5C18ec 250/4.0 or NUCLEODUR C18pyramid VP250/10 5µm particle size) with a UV/Vis detector operating at λ=215 and 254 nm. As mobile phases, ACN/0.05%TFA and H_2O/0.05%TFA were used. Additionally, Agilent 1290 UPLC with Acquity UPLC BEH C18 2.1X50 mm, 1.7 µM column and UV/Vis detector operating at 220 nm and 254 nm and ELSD detector was used. 3 µl were injected, using a flow rate of 1 mL/min for 3 minutes considering ACN/0.1%TFA and H_2O/0.1%TFA as mobile phases with a gradient from 5% to 100% ACN. Different colors of the peaks correspond to different target masses. High resolution mass spectrometry measurements were performed with either ESI, MALDI Bruker ICR ApexQe or Bruker Autoflex Speed MALDI-TOF in the positive or negative mode using 2,5-dihydroybenzoic acid (DHB) as a matrix for MALDI in the Mass Spectrometry Facility of Heidelberg University for compounds **2**-**15**. ESI HRMS Esquire 3000 Bruker Daltonics at Konstanz University was used for compounds **17**-**20**.

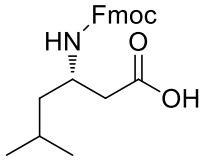
Fluorescence measurements were performed with Tecan Infinite M1000 PRO using a 358 nm excitation wavelength and 452 nm emission wavelength and opti-plate 96F black 96well microplates (Perkin Elmer). Fifty percent inhibitory concentration (IC_{50}) values were estimated by fitting the inhibition data GraphPad Prism considering the linear kinetic part of the curves.

3. Experimental procedures

3.1 Synthesis of Fmoc protected amino acids

Reactions were carried out under argon atmosphere. The amino acid (1.0 equiv.) was dissolved in a mixture of dry methanol/dichloromethane (1:1 v/v) and stirred at 0ºC for 30 min. Fmoc-*N*-hydroxysuccinimide ester (1.5 equiv.) was slowly added to the reaction mixture and then triethylamine (TEA) (1.5 equiv.) was added dropwise. The reaction mixture was stirred at room temperature overnight. After removal of the solvents, ethyl acetate was added and the mixture was washed with water. The organic phase was dried over sodium sulfate and the solvent was removed in vacuum. A column chromatographic separation was performed.

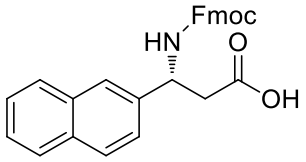
Synthesis of (S)-3-Fmoc-amino-5-methyl-hexanoic acid (Fmoc-*L*-β-homo-Leu-OH) (13)



The reaction was carried out according to the general procedure with (*S*)-3-amino-5-methyl-hexanoic acid (794.8 mg, 5.55 mmol), Fmoc-*N*-hydroxysuccinimide ester (2765.4 mg, 8.20 mmol) and TEA (1.142 mL, 8.20 mmol). The residue was purified by flash chromatography (DCM to DCM/MeOH 9:1) to give **13** as a yellow powder (1.4095 g, 3.84 mmol, 70%). Rf = 0.30 (DCM); HPLC analysis t_R = 8.2 min (50-100% ACN/0.05%TFA in 16 min); mp = 83-85.3 ºC; $[\alpha]^{20}_D$ = -4.6±0.4 (c. 0.8, DMF); ^{1}H NMR (400 MHz, DMSO-d6): δ 12.12 (s, 1H, COO**H**), 7.89 (d, *J* = 7.5 Hz, 2H, Ar), 7.68 (dd, *J* = 7.2, 4.3 Hz, 2H, Ar), 7.41 (t, *J* = 7.4 Hz, 2H, Ar), 7.32 (m, 2H, Ar), 7.17 (d, *J* = 8.9 Hz, 1H, N**H**-), 4.26 (m, 3H, Ar-C**H**-C$\mathbf{H}_2$), 3.86 (m, 1H, C_βH), 2.30 (dd, *J* = 9.3 Hz, 2H, $C_\alpha H_2$), 1.56 (m, 1H, CH), 1.34 (m, 1H, CH_2), 1.15 (m, 1H, CH_2),

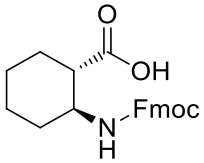
0.84 (d, *J* = 6.6 Hz, 6H, $2CH_3$) ppm; ^{13}C NMR (101 MHz, DMSO-d6): δ 173.1 (C=O), 142.7 (C=O(Fmoc)), 139.59 (2C(Ar)), 137.60 (2C(Ar)), 129.1 (2C(Ar)), 127.5 (2C(Ar)), 121.5 (2C(Ar)), 120.2 (2C(Ar)), 46.9 (Ar-CH-**C**H_2), 43.0 (Ar-**C**H-CH_2), 40.3 ($C_\beta H$), 40.1 ($C_\alpha H_2$), 39.9 (CH_2), 23.7 (CH), 22.52 (CH_3), 22.56 (CH_3) ppm; HRMS (ESI-pos): m/z calcd. for $C_{22}H_{25}NNaO_4^+$: 390.16758 $[M+Na]^+$; found: 390.16795.

Synthesis of (*R*)-3-Fmoc-amino-3-(naphthalen-2-yl)propanoic acid [Fmoc-(*R*)-2-naphthyl-beta-Ala-OH] (14)



The reaction was carried out according to the general procedure with (*R*)-3-amino-3-(naphthalen-2-yl)propanoic acid (250 mg, 1.16 mmol), Fmoc-*N*-hydroxysuccinimide ester (590.3 mg, 1.75 mmol) and TEA (0.243 mL, 1.74 mmol). The residue was purified by flash chromatography (DCM to DCM/MeOH 94:6) to give **14** as a white powder (443.9 mg, 1.01 mmol, 87%). Rf = 0.37 (DCM/MeOH 95:5); HPLC analysis t_R = 3.8 min (10-100% ACN/0.05%TFA in 16 min); mp>182.8 (dec.); $[\alpha]^{20}_D$ = -27.2±1.3 (c. 0.64, DMF); ^1H NMR (400 MHz, DMSO-d6): δ 12.34 (s, 1H, COO**H**), 8.19 (d, *J* = 8.4 Hz, 1H, N**H**-), 8.12 (d, *J* = 10.0 Hz, 1H, naphthyl), 7.96 (d, *J* = 7.7 Hz, 1H, naphthyl), 7.89 (d, *J* = 7.6 Hz, 2H, Fmoc), 7.84 (d, *J* = 10.1 Hz, 1H, naphthyl), 7.69 (t, *J* = 8.1 Hz, 2H, Fmoc), 7.56 (m, 4H, naphthyl), 7.41 (m, 2H, Fmoc), 7.29 (m, 2H, Fmoc), 5.79 (m, 1H, $C_\beta H$), 4.29 (m, 2H, CH_2), 4.21 (m, 1H, CH), 2.70 (m, 2H, $C_\alpha H_2$) ppm; ^{13}C NMR (101 MHz, DMSO-d6): δ 172.5 (C=O), 155.8 (C=O(Fmoc)), 144.4 (C(Ar)), 144.2 (C(Ar)), 143.0 (C(Ar)), 141.2 (C(Ar)), 139.9 (C(Ar)), 138.9 (C(Ar)), 137.9 (C(Ar)), 133.8 (C(Ar)), 129.4 (C(Ar)), 129.2 (C(Ar)), 128.1 (C(Ar)), 127.8 (C(Ar)), 127.5 (C(Ar)), 126.9 (C(Ar)), 126.2 (C(Ar)), 125.9 (C(Ar)), 125.7 (C(Ar)), 123.5 (C(Ar)), 123.3 (C(Ar)), 121.9 (C(Ar)), 120.6 (C(Ar)), 120.5 (C(Ar)), 65.9 (CH_2), 48.4 ($C_\beta H$), 47.2 (CH), 41.1 ($C_\alpha H_2$) ppm; HRMS (ESI-pos): m/z calcd. for $C_{28}H_{23}NaNO_4^+$: 460.15248 $[M+Na]^+$; found: 460.15280.

Synthesis of (1*S*,2*S*)-2-Fmoc-aminocyclohexanecarboxylic acid [Fmoc-(2*S*,3*S*)-Chx-β-Ala-OH] (15)

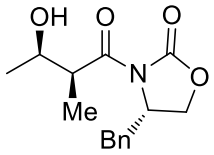


The reaction was carried out according to the general procedure with (1*S*,2*S*)-2-aminocyclohexanecarboxylic acid (400 mg, 2.76 mmol), Fmoc-*N*-hydroxysuccinimide ester (1422.7 mg, 4.22 mmol) and TEA (0.585 mL, 4.20 mmol). The residue was purified by flash chromatography (cyclohexane to cyclohexane/ethylacetate 4:1) to give **15** as a white powder (371.3 mg, 1.02 mmol, 37%). Rf = 0.37 (DCM/MeOH 95:5); HPLC analysis t_R = 7.2 min (50-100% ACN/0.05%TFA in 16 min); mp >195.4 (dec.); $[\alpha]^{20}_D$ = 10.1±2.2 (c. 0.4, DMF); ^1H NMR (400 MHz, DMSO-d6): δ 12.06 (s, 1H, COO**H**), 7.89 (d, *J* = 7.5 Hz, 2H, Ar), 7.68 (dd, *J* = 7.2 Hz, 2H, Ar), 7.41 (m, 2H, Ar), 7.32 (m, 3H, Ar, N**H**-), 4.20 (m, 3H, CH-CH_2), 3.49 (m, 1H,

CH), 2.26 (td, *J* = 11.7, 3.5 Hz, 1H, CH), 1.72 (m, 4H, $2CH_2$), 1.23 (m, 4H, $2CH_2$) ppm; ^{13}C NMR (101 MHz, DMSO-d6): δ 176.1 (C=O), 143.0 (C=O(Fmoc)), 139.90 (2C(Ar)), 137.91 (2C(Ar)), 129.4 (2C(Ar)), 127.8 (2C(Ar)), 121.9 (2C(Ar)), 120.5 (2C(Ar)), 61.2 (Ar-CH-**C**H_2), 52.5 (CH), 46.4 (Ar-**C**H-CH_2), 45.8 (CH), 32.0 (CH_2), 28.7 (CH_2), 25.9 (CH_2), 24.9 (CH_2) ppm; HRMS (ESI-pos): m/z calcd. for $C_{22}H_{23}NNaO_4^+$: 388.15193 $[M+Na]^+$; found: 388.15241.

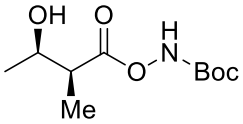
3.2. Synthesis of Fmoc-Amba (20)

(*S*)-4-Benzyl-3-((2*S*,3*R*)-3-hydroxy-2-methylbutanoyl)oxazolidin-2-one[S4] (17)



Under nitrogen (*S*)-4-benzyl-3-propionyloxazolidin-2-one (**16**) (3.5 g, 15 mmol) was dissolved in dry DCM (50 mL) and cooled to –5 °C (inside temperature). Di-*n*-butyl-boryl triflate (5.3 mL, 21 mmol) and dry triethylamine (3.1 mL, 23.5 mmol) were added dropwise at a rate to maintain a temperature < –2 °C. The resulting pale yellow solution was stirred at –5 °C for 15 min and then cooled to –78 °C. Distilled acetaldehyde (2 mL, 35 mmol) was added in one portion and the reaction mixture was stirred 1h at –78 °C. Then it was warmed up to –5 °C, stirred for an additional 1h and quenched with 1 M phosphate buffer pH 7 (25 mL) and methanol (75 mL). A mixture of 30% aqueous hydrogen peroxide and methanol (1:2, 75 mL) was added via a dropping funnel at a rate to maintain a temperature < 10 °C and the reaction was stirred for additional 30 min. After warming up to room temperature, water (100 mL) and DCM (100 mL) were added and the phases were separated. The aqueous phase was extracted additionally with DCM (4 x 100 mL). The combined organic phases were washed with sat. $NaHCO_3$ solution (150 mL), dried over Na_2SO_4, and concentrated in vacuo. The crude product was purified via column chromatography (200 g silica, 50% ethyl acetate in hexanes) to yield product **17** as a colorless oil (4.03 g, 14.53mmol, 97 %). R_f = 0.45 (EtOAc/petroleum ether, 1:1). ^1H and ^{13}C NMR and ESI-MS data were in agreement with the published ones.[S4]

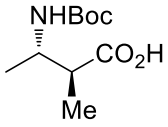
(2*S*,3*R*)-1-*O*-(*tert*-Butoxycarbamoyl)-3-hydroxy-butanoate (18)



Under nitrogen *N*-Boc-hydroxylamine was dissolved in dry THF (40 mL) and cooled to 0 °C. Sodium hydride (0.81 g, 20.2 mmol, 60%-dispersion in mineral oil) was added in small portions. After 30 min at 0 °C, the reaction mixture was cooled to –25 °C and compound **17** (2.0 g, 7.2 mmol), dissolved in dry THF (20 mL), was added dropwise at a rate to maintain the temperature below –20 °C. After 2 h at < –25 °C the reaction was quenched with 1 M phosphate buffer pH 4 (25 mL) and warmed to rt. The final pH of the mixture was adjusted to pH 7–8 with sat. $NaHCO_3$ solution. A small amount of water was added and the mixture was extracted with EtOAc (4 x 100 mL). The combined organic phases were washed with sat. $NaHCO_3$ solution (150 mL), dried over Na_2SO_4, and concentrated in vacuo. The crude

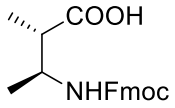
product was purified via flash chromatography (200 g silica, 5% methanol in DCM) to yield product **18** as a colorless oil (4.03 g, 17.28 mmol, 97 %). R_f = 0.41 (5% methanol in DCM); ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.95 (s, 1H, N**H**-), 4.19 (ddq, J = 6.5 Hz, 4.5 Hz, 3.9 Hz, 1H, HO-C**H**-), 2.87 (d, J = 4.5 Hz, 1H, -O**H**), 2.73 (dq, J = 7.1 Hz, 3.9 Hz, 1H, CO-C**H**-CH_3), 1.48 (s, 9H, tBu), 1.23 (d, J = 7.1 Hz, 3H, CO-CH-C**H**$_3$), 1.22 (d, J = 6.5 Hz, 3H, HO-CH-C**H**$_3$); ^{13}C NMR (101 MHz, $CDCl_3$): 174.9 (C=O), 156.2 (C=O), 83.9 (O-**C**$(CH_3)_3$), 68.4 (HO-**C**H-), 44.6 (CO-**C**H-CH_3), 28.2 (O-C(**C**H$_3$)$_3$), 19.6 (HO-CH-**C**H$_3$), 10.5 (CO-CH-**C**H$_3$); HRMS (ESI-pos) *m/z* calcd. for $C_{10}H_{19}NO_5$: 256.1155 $[M+Na]^+$; found 256.1151.

(2*S*,3*S*)-2-Methyl-3-[(*tert*-butoxycarbonyl)amino]-butanoic acid (Boc-Amba-OH) (19)



Under nitrogen freshly recrystallized naphthalene (3.27 g, 25.8 mmol) was dissolved in dry THF (40 mL) and small lumps of sodium (585 mg, 25.8 mmol) were added. The dark green solution was sonicated for 1 h. In a separate Schlenk-flask, **18** (0.30 g, 1.29 mmol) and triphenylphosphane (1.01 g, 3.86 mmol) were dissolved in dry THF (20 mL) and cooled to –78 °C. DEAD (0.67 mL, 3.86 mmol) was added dropwise during 15 min. The light-yellow reaction mixture was stirred for 3h at –78 °C and sodium naphthalenide solution was added dropwise until the green color remained for 5 min (completeness controlled by TLC). Then, water (20 mL) was added and the reaction mixture was warmed up to rt. The pH was adjusted to 2 with 1 M $NaHSO_4$ solution and the reaction mixture was extracted with EtOAc (4 x 50 mL). The combined organic phases were dried over Na_2SO_4 and concentrated in vacuo. The crude product was purified via column chromatography (50 g silica, 74% ethyl acetate, 24% petroleum ether, 1% acetic acid) to yield product **19** as a white solid (162 mg, 0.75 mmol, 58 %). R_f = 0.28 (74% ethyl acetate, 24% petroleum ether, 1% acetic acid). ^{1}H and ^{13}C NMR and ESI-MS data were in agreement with the published ones.[S5] $[\alpha]^{23}_D$ = = –7.20 (c = 0.795, $CHCl_3$), lit.: $[\alpha]^{rt}_D$ = –7.4 (c = 1, $CHCl_3$).

(2*S*,3*S*)-2-Methyl-3-[(((9H-fluoren-9-yl)methoxy)carbonyl)-amino]-butanoic acid (Fmoc-Amba-OH) (20)



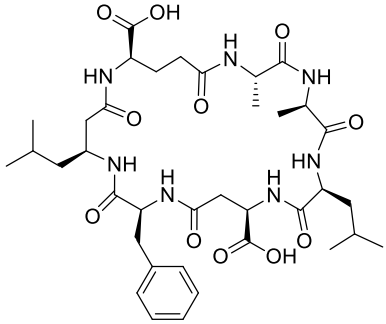
Boc-Amba-OH (**19**) (60 mg, 0.276 mmol) was dissolved in TFA (0.5 mL). After 25 min stirring at rt TFA was removed in vacuo and the residue was coevaporated with toluene (3 x 0.5 mL). Aqueous Na_2CO_3 solution (10% w/v, 0.5 mL) was added and the mixture was cooled to 0 °C. Fmoc-OSu (121 mg, 0.359 mmol), dissolved in dioxane (0.75 mL), was added and the reaction mixture was stirred for 10 min at 0 °C and overnight at rt. Then, the reaction mixture was diluted with H_2O and EtOAc (each 5 mL) and acidified to pH 2 with 1 M $NaHSO_4$ solution and the reaction mixture was extracted with EtOAc (3 x 5 mL). The combined organic phases were dried over Na_2SO_4 and concentrated in vacuo. The crude product was purified via flash chromatography (20 g silica, 74% ethyl acetate, 24% petroleum ether, 1% acetic acid) to yield product **20** as a white solid (72 mg, 0.21 mmol, 77

%). R_f = 0.23 (74% ethyl acetate, 24% petroleum ether, 1% acetic acid);); $[\alpha]^{20}_D$ = -4.2±0.3 (c. 0.63, DCM); ^{1}H NMR (600 MHz, CD_3OD): *δ* 7.80 (d, *J* = 7.6 Hz, 2H, Ar), 7.65 (dd, *J* = 7.4, 2.9 Hz, 2H, Ar), 7.39 ('t', *J* = 7.5 Hz, 2H, Ar), 7.31 ('t', *J* = 7.4 Hz, 2H, Ar), 4.39 (dd, *J* = 10.5, 7.0 Hz, 1H, CH_2), 4.30 (dd, *J* = 10.5, 7.0 Hz, 1H, CH_2), 4.21 (t, *J* = 6.9 Hz, 1H, -C**H**-CH_2), 3.94 (dq, *J* = 6.7 Hz, *J* = 6.7 Hz, 1H, $C_\alpha H$), 2.63 (dq, *J* = 7.0 Hz, *J* = 7.0 Hz, 1H, $C_\beta H$), 1.13 (d, *J* = 7.1 Hz, 3H, CH_3), 1.11 (d, *J* = 7.4 Hz, 3H, CH_3); ^{13}C NMR (151 MHz, CD_3OD): δ 178.4 (C=O), 158.0 (C=O(Fmoc)), 145.3 (2C(Ar)), 142.6 (2C(Ar)), 128.8 (2C(Ar)), 128.1 (2C(Ar)), 126.2 (2C(Ar)), 120.9 (2C(Ar), 67.6 (CH-**C**H_2), 49.9 ($C_\alpha H$), 49.6 (**C**H-CH_2), 45.3 ($C_\beta H$), 17.6 (CH_3), 13.2 (CH_3); HRMS (ESI-pos) *m/z* calcd. for $C_{20}H_{21}NO_4$: 340.1543 $[M+Na]^+$; found: 340.1539.

3.3 Synthesis of microcystin analogs (2-12)

All microcystin analogs were synthesised following this general procedure unless otherwise indicated. Protected linear peptides were synthesized manually (one coupling overnight) or on a peptide synthesizer (double couplings for 40 minutes) with Fmoc-amino acids (5 equiv.), HBTU (5 equiv.), HOBt (0.2 mol/L) and DIPEA (10 equiv.) in DMF using H-Phe-2-chlorotrityl resin. Fmoc deprotection was carried out using 40% piperidine for 3 minutes and then 20% piperidine for 14 minutes. Linear peptides were cleaved from the resin after manual washing with DMF and DCM by gentle shaking in a cleavage cocktail (AcOH/TFE/DCM 1:1:3) for 2.5 hours. Acetic acid was removed by coevaporation with cyclohexane and peptides were dried under vacuum. Macrocyclization was achieved by vigorously stirring a diluted solution of linear peptides in dry DMF (0.2 mM) with HATU (3 equiv.), HOAT (3 equiv.) and DIPEA (4.5 equiv.) under argon atmosphere overnight. After removal of the solvent, ethyl acetate was added to dissolve the crude product and the organic phase was washed with water, $NaHCO_3$ (5%) and brine. Organic solvent was removed and final deprotection was carried out by dissolving the crude peptide in TFA/TIPS/H_2O 95:2.5:2.5 and stirring for 2.5 hours. After removal of the solvent, peptides were dried under high vacuum, purified via HPLC and lyophilized. Overall yields of the synthesis of microcystin analogs were calculated considering a resin loading of 0.7 mmol/g as stated in the specifications of the resin.

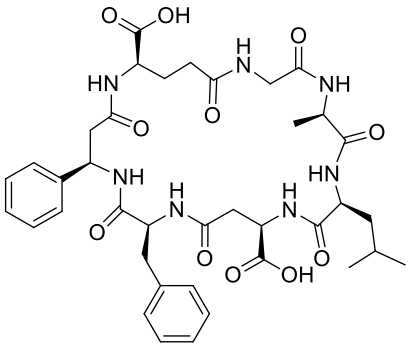
Synthesis of cyclo[(D)Ala-Leu-(D)Asp-Phe-(*L*-β-homo-Leu)-(D)Glu-Ala] (2)



Synthesis of the protected linear peptide was carried out with the MultiSynTech Syro I starting with 108 mg of resin. HPLC analysis t_R = 10.3 min (10-100% ACN/0.05%TFA in 16 min); MS (ESI-pos): m/z calcd. for $C_{45}H_{74}N_7O_{12}^+$: 904.54 $[M+H]^+$; found: 904.7. Synthesis and purification of **2** was carried out according to the general procedure to give a white

powder (14.5 mg, 18.76 µmol, 25%). HPLC analysis t_R = 9.6 min (10-80% ACN/0.05%TFA in 16 min); $[\alpha]^{20}_D$ = -21.9±0.3 (c. 0.89, MeOH); ^{1}H NMR (500 MHz, CD_3OD): δ 8.37 (m, 1H, N**H**-), 7.27 (m, 5H, Ar Phe), 4.81 (m, 1H, $C_\alpha H$), 4.42 (m, 2H, $C_\alpha H$, $C_\alpha H$ L-β-homo-Leu), 4.34 (m, 3H, $C_\alpha H$, $C_\alpha H$, $C_\alpha H$), 4.00 (q, *J* = 6.9 Hz, 1H, $C_\alpha H$ Ala), 3.27 (m, 1H, $C_Y H_2$), 2.81 (m, 2H, $C_X H_2$, $C_Y H_2$), 2.41 (m, 4H, CH-CH_2-C**H**$_2$ (D)Glu, CH_2, $C_X H_2$), 2.27 (ddd, *J* = 15.3, 10.4, 5.2 Hz, 1H, CH-CH_2-C**H**$_2$ (D)Glu), 2.10 (m, 1H, CH-C**H**$_2$-CH_2 (D)Glu),1.96 (m, 1H, CH-C**H**$_2$-CH_2 (D)Glu), 1.77 (m, 1H, CH_2 Leu), 1.69 (m, 1H, CH Leu), 1.62 (m, 1H, CH_2 Leu), 1.57 (m, 1H, CH L-β-homo-Leu), 1.48 (m, 1H, CH_2 L-β-homo-Leu), 1.37 (dd, *J* = 6.97 Hz, 3H, CH_3 Ala), 1.34 (m, 1H, CH_2 L-β-homo-Leu), 1.17 (d, *J* = 7.3 Hz, 3H, CH_3 (D)Ala), 0.89 (m, 12H, 2CH_3 Leu, 2CH_3 L-β-homo-Leu) ppm; ^{13}C NMR (125 MHz, CD_3OD): δ 175.3 (C=O), 175.2 (C=O), 175.0 (2C=O), 174.7 (C=O), 174.6 (C=O), 173.8 (C=O), 173.2 (C=O), 172.6 (C=O), 138.8 (C(Ar) Phe), 130.1 (2CH(Ar) Phe),129.6 (2CH(Ar) Phe), 127.9 (C4H(Ar) Phe), 57.1 ($C_\alpha H$), 54.3 ($C_\alpha H$), 53.4 ($C_\alpha H$), 51.5 ($C_\alpha H$ Ala), 50.3 ($C_\alpha H$), 50.1 ($C_\alpha H$), 46.9 ($C_\alpha H$ L-β-homo-Leu), 45.6 (CH_2 L-β-homo-Leu), 42.8 (CH_2), 41.2 (CH_2 Leu), 38.2 (CH_2), 36.9 (CH_2), 33.7 (CH-CH_2-**C**H_2 (D)Glu), 28.6 (CH-**C**H_2-CH_2 (D)Glu), 26.0 (CH L-β-homo-Leu), 25.7 (CH Leu), 23.5 (2CH_3 Leu or L-β-homo-Leu), 22.3 (CH_3 Leu or L-β-homo-Leu), 21.5 (CH_3 Leu or L-β-homo-Leu), 17.0 (CH_3 Ala), 16.9 (CH_3 (D)Ala) ppm; HRMS (MALDI-pos): m/z calcd. for $C_{37}H_{55}N_7NaO_{11}^+$: 796.38518 $[M+Na]^+$; found: 796.38855.

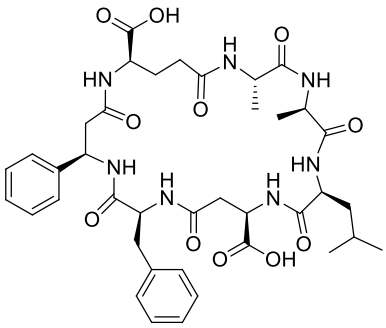
Synthesis of cyclo[(D)Ala-Leu-(D)Asp-Phe-(*L*-β-Phe)-(D)Glu-Gly] (3)



Synthesis of the protected linear peptide was carried out with the MultiSynTech Syro I starting with 104 mg of resin. HPLC analysis t_R = 8.6 min (40-100% ACN/0.05%TFA in 16 min), MS (ESI-pos): m/z calcd. for $C_{46}H_{68}N_7O_{12}^+$: 910.49 $[M+H]^+$; found: 910.65. Synthesis and purification of **3** was carried out according to the general procedure to give a white powder (15.9 mg, 20.35 µmol, 28%). HPLC analysis t_R = 9.3 min (10-100% ACN/0.05%TFA in 16 min); $[\alpha]^{20}_D$ = -19.4±2.1 (c. 0.20, MeOH); ^{1}H NMR (500 MHz, CD_3OD): δ 7.28 (m, 10H, 2Ar), 5.52 (d, *J* = 11.7 Hz, 1H, $C_\alpha H$ L-β-Phe), 4.94 (m, 1H, $C_{X\alpha}H$), 4.55 (d, *J* = 10.8 Hz, 1H, $C_{Y\alpha}H$), 4.48 (m, 1H, $C_\alpha H$ (D)Glu), 4.35 (d, *J* = 7.5 Hz, 1H, $C_\alpha H$ (D)Ala), 4.22 (d, *J* = 10.4 Hz, 1H, $C_\alpha H$ Leu), 3.87 (d, *J* = 17.0 Hz, 1H, CH_2 Gly), 3.73 (d, *J* = 16.4 Hz, 1H, CH_2 Gly), 3.40 (m, 1H, $C_Y H_2$), 2.96 (t, *J* = 13.2 Hz, 1H, CH_2 L-β-Phe), 2.76 (t, *J* = 11.7 Hz, 1H, $C_Y H_2$), 2.65 (dd, *J* = 14.9, 14.6 Hz, 2H, CH_2 L-β-Phe, $C_X H_2$), 2.43 (m, 1H, CH-CH_2-C**H**$_2$ (D)Glu), 2.31 (m, 2H, CH-CH_2-C**H**$_2$ (D)Glu, $C_X H_2$), 2.18 (m, 1H, CH-C**H**$_2$-CH_2 (D)Glu), 1.81 (m, 2H, CH_2 Leu, CH-C**H**$_2$-CH_2 (D)Glu), 1.74 (m, 1H, CH Leu), 1.66 (m, 1H, CH_2 Leu), 1.02 (d, *J* = 7.0 Hz, 3H, CH_3 (D)Ala), 0.95 (d, *J* = 6.1 Hz, 3H, CH_3 Leu), 0.89 (d, *J* = 6.1 Hz, 3H, CH_3 Leu) ppm; ^{13}C NMR (125 MHz, CD_3OD): δ 175.5 (2C=O), 174.4 (C=O), 174.0 (C=O), 173.5 (C=O), 174.3 (C=O), 172.1 (3C=O), 129.9 (2C(Ar)), 129.6 (4C(Ar)), 129.4 (4C(Ar)), 127.3 (2C(Ar)), 54.4 ($C_\alpha H$ Leu), 53.0 ($C_\alpha H$ (D)Glu), 52.1 ($C_{X\alpha}H$), 50.2 ($C_\alpha H$ (D)Ala), 50.0 ($C_{Y\alpha}H$), 46.8 ($C_{Z\alpha}H$), 44.3

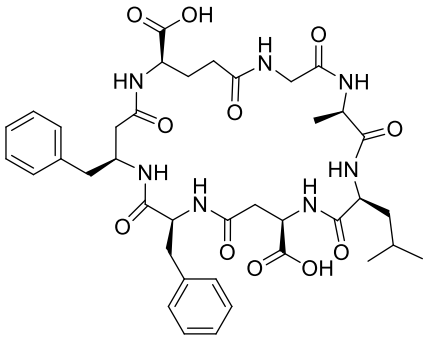
(CH_2 Gly), 43.8 (C_XH_2), 40.4 (CH_2 Leu), 37.1 (C_YH_2), 36.9 (C_ZH_2), 33.5 (CH-CH_2-**C**H_2 (D)Glu), 29.7 (CH Leu), 28.8 (CH-**C**H_2-CH_2 (D)Glu), 23.3 (CH_3 Leu), 21.2 (CH_3 Leu), 16.6 (CH_3 (D)Ala) ppm; HRMS (MALDI-pos): m/z calcd. for $C_{38}H_{50}N_7O_{11}^+$: 780.35628 $[M+H]^+$; found: 780.35706.

Synthesis of cyclo[(D)Ala-Leu-(D)Asp-Phe-(*L*-β-Phe)-(D)Glu-Ala] (4)



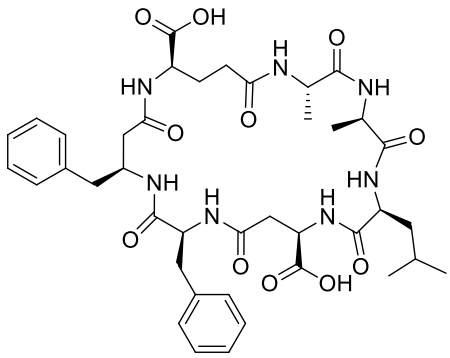
Synthesis of the protected linear peptide was carried out with the MultiSynTech Syro I starting with 72 mg of resin. HPLC analysis t_R = 10.0 min (10-100% ACN/0.05%TFA in 16 min), MS (ESI-pos): m/z calcd. for $C_{47}H_{70}N_7O_{12}^+$: 924.51 $[M+H]^+$; found: 924.65. Synthesis and purification of **4** was carried out according to the general procedure to give a white powder (10.6 mg, 13.38 µmol, 27%). HPLC analysis t_R = 10.9 min (10-100% ACN/0.05%TFA in 16 min); $[\alpha]^{20}_D$ = -97.7±15.3 (c. 0.08, MeOH); ^{1}H NMR (500 MHz, CD_3OD): δ 7.27 (m, 10H, 2Ar), 5.51 (dd, *J* = 11.2, 3.4 Hz, 1H, C_αH L-β-Phe), 4.90 (t, *J* = 4.6Hz, 1H, C_αH), 4.56 (dd, *J* = 10.5, 4.1 Hz, 1H, C_αH), 4.46 (dd, *J* = 9.0, 5.3 Hz, 1H, C_αH), 4.35 (m, 2H, C_αH (D)Ala, C_αH Ala), 4.00 (q, *J* = 7.0 Hz, 1H, C_αH Ala), 3.36 (m, *J* = 14.7, 4.2 Hz, 1H, C_ZH_2), 2.93 (dd, *J* = 14.7, 11.3 Hz, 1H, C_YH_2), 2.76 (m, 2H, C_XH_2, C_ZH_2), 2.66 (dd, *J* = 14.7, 3.5 Hz, 1H, C_YH_2), 2.41 (m, 1H, CH-CH_2-C**H**$_2$ (D)Glu), 2.34 (dd, *J* = 15.4, 4.7 Hz, 1H, C_XH_2), 2.25 (m, 1H, CH-CH_2-C**H**$_2$ (D)Glu), 2.14 (m, 1H, CH-C**H**$_2$-CH_2 (D)Glu), 1.89 (m, 1H, CH-C**H**$_2$-CH_2 (D)Glu), 1.80 (m, 1H, CH_2 Leu), 1.72 (m, 1H, CH Leu), 1.62 (m, 1H, CH_2 Leu), 1.40 (d, *J* = 7.0 Hz, 3H, CH_3 Ala), 1.13 (d, *J* = 7.3 Hz, 3H, CH_3 (D)Ala), 0.93 (d, *J* = 6.5 Hz, 3H, CH_3 Leu), 0.89 (d, *J* = 6.4 Hz, 3H, CH_3 Leu) ppm; ^{13}C NMR (125 MHz, CD_3OD): δ 176.8 (C=O), 175.6 (C=O), 175.4 (4C=O), 174.6 (C=O), 174.5 (2C=O), 130.1 (2C(Ar)), 129.7 (4C(Ar)), 129.5 (4C(Ar)), 127.5 (2C(Ar)), 57.7 (C_αH), 56.3 (C_αH), 52.0 (C_αH), 51.5 (C_αH), 51.1 (C_αH Ala), 50.1 (C_αH), 49.6 (C_αH), 43.4 (C_YH_2), 41.2 (CH_2 Leu), 37.4 (C_ZH_2), 37.0 (C_xH_2), 33.7 (CH-CH_2-**C**H_2 (D)Glu), 29.3 (CH-**C**H_2-CH_2 (D)Glu), 25.7 (CH Leu), 23.5 (CH_3 Leu), 21.5 (CH_3 Leu), 17.0 (CH_3 (D)Ala), 16.9 (CH_3 Ala) ppm; HRMS (MALDI-neg): m/z calcd. for $C_{39}H_{50}N_7O_{11}^-$: 792.35738 $[M-H]^-$; found: 792.35626.

Synthesis of cyclo[(D)Ala-Leu-(D)Asp-Phe-(*L*-β-homo-Phe)-(D)Glu-Gly] (5)



Synthesis of the protected linear peptide was carried out with the MultiSynTech Syro I starting with 104 mg of resin. HPLC analysis t_R = 9.0 min (40-100% ACN/0.05%TFA in 16 min), MS (ESI-pos): m/z calcd. For $C_{47}H_{70}N_7O_{12}^+$: 924.51 $[M+H]^+$; found: 924.7. Synthesis and purification of **5** was carried out according to the general procedure to give a white powder (27.8 mg, 35.08 µmol, 48%). HPLC analysis t_R = 9.7 min (10-100% ACN/0.05%TFA in 16 min); $[\alpha]^{20}_D$ = -39.4±0.4 (c.0.84, MeOH); ^{1}H NMR (400 MHz, CD_3OD): δ 8.21 (m, 1H, N**H**-), 7.23 (m, 10H, 2Ar), 4.74 (m, 1H, $C_{Y\alpha}H$), 4.59 (m, 1H, $C_\beta H$ β-homo-Phe), 4.38 (m, 2H, $C_\alpha H$ (D)Glu, $C_{X\alpha}H$), 4.32 (d, *J* = 7.2 Hz, 1H, $C_\alpha H$ (D)Ala), 4.23 (dd, *J* = 13.0, 6.4 Hz, 1H, $C_\alpha H$ Leu), 3.86 (d, *J* = 16.74 Hz, 1H, CH_2 Gly), 3.70 (d, *J* = 17.5 Hz, 1H, CH_2 Gly), 3.12 (m, 1H, C_xH_2), 2.87 (m, 2H, Ar-C**H**$_2$ β-homo-Phe), 2.70 (m, 2H, C_xH_2,C_YH_2,), 2.51 (m, 1H, $C_\alpha H_2$ β-homo-Phe), 2.38 (m, 2H, CH-CH_2-C**H**$_2$ (D)Glu, $C_\alpha H_2$ β-homo-Phe), 2.29 (m, 2H, CH-CH_2-C**H**$_2$ (D)Glu, C_YH_2), 2.14 (m, 1H, CH-C**H**$_2$-CH_2 (D)Glu), 1.82 (m, 2H, CH-C**H**$_2$-CH_2 (D)Glu, CH_2 Leu), 1.68 (m, 2H, CH-CH_2 Leu), 1.07 (d, *J* = 13.2 Hz, 3H, CH_3 (D)Ala), 0.95 (d, *J* = 7.3 Hz, 3H, CH_3 Leu), 0.89 (d, *J* = 6.6 Hz, 3H, CH_3 Leu) ppm; ^{13}C NMR (101 MHz, CD_3OD): δ 175.5 (C=O), 175.3 (C=O), 175.0 (C=O), 174.5 (C=O), 173.9 (C=O), 173.2 (C=O), 173.0 (C=O), 172.8 (C=O), 171.6 (C=O),139.2 (C(Ar)), 139.0 (C(Ar)), 130.6 (2C(Ar)), 130.0 (2C(Ar)), 129.6 (2C(Ar)), 129.5 (2C(Ar)), 127.8 (C(Ar)), 127.7 (C(Ar)), 56.9 ($C_{X\alpha}H$), 54.4 (C_αHLeu), 53.2 ($C_\alpha H$ (D)Glu), 50.1 ($C_\alpha H$ (D)Ala), 50.2 ($C_\beta H$ β-homo-Phe), 49.8 ($C_{Y\alpha}H$), 42.5 (CH_2 Gly), 41.2 (Ar-**C**H_2 β-homo-Phe), 40.7 (CH_2 Leu), 40.6 ($C_\alpha H_2$ β-homo-Phe), 37.8 (C_xH_2), 37.3 (C_YH_2), 33.5 (CH-CH_2-**C**H_2 (D)Glu), 28.7 (CH-**C**H_2-CH_2 (D)Glu), 26.0 (CH Leu), 23.5 (CH_3 Leu), 21.4 (CH_3 Leu), 16.8 (CH_3 (D)Ala) ppm; HRMS (MALDI-neg): m/z calcd. for $C_{39}H_{50}N_7O_{11}^-$: 792.35738 $[M-H]^-$; found: 792.35589.

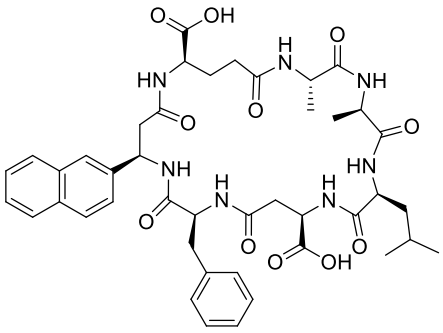
Synthesis of cyclo[(D)Ala-Leu-(D)Asp-Phe-(*L*-β-homo-Phe)-(D)Glu-Gly] (6)



Synthesis of the protected linear peptide was carried out with the MultiSynTech Syro I starting with 72 mg of resin. HPLC analysis t_R = 11.9 min (10-100% ACN/0.05%TFA in 16

min), MS (ESI-pos): m/z calcd. for $C_{48}H_{72}N_7O_{12}^+$: 938.52 $[M+H]^+$; found: 938.75. Synthesis and purification of **6** was carried out according to the general procedure to give a white powder (17.3 mg, 21.40 µmol, 42%). HPLC analysis t_R = 9.5 min (10-100% ACN/0.05%TFA in 16 min); $[\alpha]^{20}_D$ = -23.1±0.2 (c. 0.56, MeOH); ^{1}H NMR (500 MHz, CD_3OD): δ 7.21 (m, 10H, 2Ar), 4.80 (m, 1H, $C_\alpha H$), 4.52 (m, 1H, CH), 4.34 (m, 4H, $4C_\alpha H$), 3.99 (q, *J* = 6.9 Hz, 1H, $C_\alpha H$ Ala), 3.05 (dd, *J* = 14.2, 4.4 Hz, 1H, CH), 2.88 (d, *J* = 7.3 Hz, 2H, C_XH_2), 2.81 (dd, *J* = 15.5, 5.0 Hz, 1H, C_XH_2), 2.65 (dd, *J* = 14.2, 10.3 Hz, 1H, C_YH_2), 2.52 (dd, *J* = 14.5, 9.7 Hz, 1H, C_ZH_2), 2.40 (m, 3H, CH-CH_2-C**H**$_2$ (D)Glu, C_XH_2, C_ZH_2), 2.24 (ddd, *J* = 15.3, 10.2, 5.3 Hz, 1H, CH-CH_2-C**H**$_2$ (D)Glu), 2.11 (ddd, *J* = 19.1, 10.2, 5.0 Hz, 1H, CH-C**H**$_2$-CH_2 (D)Glu), 1.94 (m, 1H, CH-C**H**$_2$-CH_2 (D)Glu), 1.76 (m, 1H, CH_2 Leu), 1.68 (m, 1H, CH Leu), 1.62 (m, 1H, CH_2 Leu), 1.37 (d, *J* = 7.0 Hz, 3H, CH_3 Ala), 1.17 (d, *J* = 7.3, 3H, CH_3 (D)Ala), 0.92 (d, *J* = 6.4 Hz, 3H, CH_3 Leu), 0.88 (d, *J* = 6.3 Hz, 3H, CH_3 Leu) ppm; ^{13}C NMR (125 MHz, CD_3OD): δ 175.3 (C=O), 175.2 (C=O), 175.0 (C=O), 174.9 (C=O), 174.6 (2C=O), 173.8 (C=O), 173.1 (C=O), 172.8 (C=O), 139.4 (C(Ar)), 138.8 (C(Ar)), 130.5 (2C(Ar)), 129.9 (2C(Ar)), 129.6 (2C(Ar)), 129.5 (2C(Ar)), 127.8 (C(Ar)), 127.7 (C(Ar)), 57.1 ($C_\alpha H$), 54.3 ($C_\alpha H$), 53.4 ($C_\alpha H$), 50.3 ($C_\alpha H$), 51.5 ($C_\alpha H$ Ala), 50.1 ($C_\alpha H$), 49.6 ($C_\alpha H$), 42.1 (C_XH_2), 41.4 (C_ZH_2), 41.2 (CH_2 Leu), 37.9 (C_YH_2 Phe), 36.9 (C_XH_2), 33.6 (CH-CH_2-**C**H_2 (D)Glu), 28.7 (CH-CH_2-**C**H_2 (D)Glu), 25.7 (CH Leu), 23.5 (CH_3 Leu), 21.6 (CH_3 Leu), 17.03 (CH_3 Ala), 16.98 (CH_3 (D)Ala) ppm; HRMS (MALDI-pos): m/z calcd. for $C_{40}H_{53}N_7NaO_{11}^+$: 830.36953 $[M+Na]^+$; found: 830.36329.

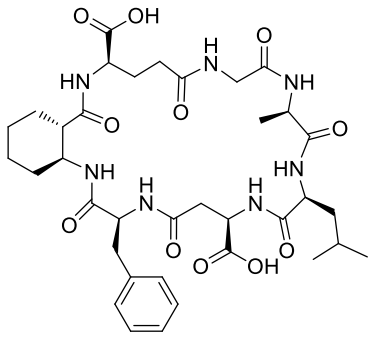
Synthesis of cyclo{(D)Ala-Leu-(D)Asp-Phe-[(*R*)-2-naphthyl-beta-Ala]-(D)Glu-Ala} (7)



Synthesis of the protected linear peptide was carried out with the MultiSynTech Syro I starting with 36 mg of resin. HPLC analysis t_R = 10.6 min (10-100% ACN/0.05%TFA in 16 min), MS (ESI-pos): m/z calcd. for $C_{51}H_{72}N_7O_{12}^+$: 974.52 $[M+H]^+$; found: 974.75. Synthesis and purification of **7** was carried out according to the general procedure to give a white powder (4.1 mg, 4.91 µmol, 19%). HPLC analysis t_R = 11.5 min (10-100% ACN/0.05%TFA in 16 min); $[\alpha]^{20}_D$ = -31.7±5.6 (c. 0.20, MeOH); ^{1}H NMR (500 MHz, CD_3OD): δ 8.32 (d, *J* = 8.4 Hz, 1H, naphthyl), 7.91 (d, *J* = 8.0 Hz, 1H, naphthyl), 7.83 (d, *J* = 8.2 Hz, 1H, naphthyl), 7.61 (m, 2H, naphtyl), 7.53 (t, *J* = 7.4 Hz, 1H, C6 or C7 naphthyl), 7.48 (t, *J* = 7.7 Hz, 1H, C6 or C7 naphthyl), 7.17 (m, 5H, Ar Phe), 6.36 (dd, *J* = 10.8, 2.9 Hz, 1H, $C_\alpha H$ (R)-2-naphthyl-beta-Ala), 4.92 (t, *J* = 4.6 Hz, 1H, $C_\alpha H$), 4.57 (dd, *J* = 10.5, 4.1 Hz, 1H, $C_\alpha H$), 4.51 (dd, *J* = 9.0, 5.3 Hz, 1H, $C_\alpha H$), 4.35 (m, 2H, $2C_\alpha H$), 4.01 (q, *J* = 6.9 Hz, 1H, $C_\alpha H$ Ala), 3.29 (m, 1H, $C_{Y2}H$), 2.98 (dd, *J* = 14.8, 11.0 Hz, 1H, $C_{Z2}H$), 2.83 (m, 2H, $C_{X2}H$, $C_{Z2}H$), 2.70 (dd, *J* = 14.1, 10.6 Hz, 1H, $C_{Y2}H$), 2.45 (m, 1H, CH-CH_2-C**H**$_2$ (D)Glu), 2.35 (dd, *J* = 11.0, 5.5 Hz, 1H, $C_{X2}H$), 2.30 (m, 1H, CH-CH_2-C**H**$_2$ (D)Glu), 2.16 (m, 1H, CH-C**H**$_2$-CH_2 (D)Glu), 1.94 (m, 1H, CH-C**H**$_2$-CH_2 (D)Glu), 1.81 (m, 1H, CH_2 Leu), 1.72(m, 1H, CH Leu), 1.62 (m, 1H, CH_2 Leu), 1.41 (d, *J* = 7.0 Hz, 3H, CH_3 Ala), 1.14 (d, *J* = 7.3 Hz, 3H, CH_3 (D)Ala), 0.92 (d, *J*=6.5 Hz, 3H, CH_3

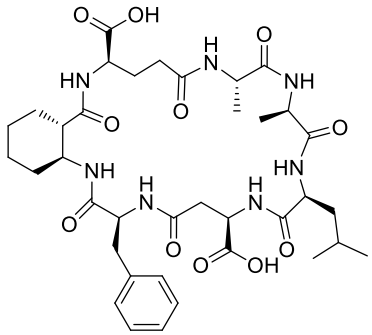
Leu) 0.89 (d, *J* = 6.5 Hz, 3H, CH_3 Leu) ppm; ^{13}C NMR (125 MHz, CD_3OD): δ 175.6 (C=O), 175.4 (2C=O), 174.8 (C=O), 174.7 (C=O), 174.5 (C=O), 174.2 (C=O), 172.34 (C=O), 172.29 (C=O), 138.9 (C(Ar)), 138.7 (C(Ar)), 135.5 (C(Ar)), 131.8 (C(Ar)), 130.1 (2CH(Ar)Phe), 130.0 (C(naphthyl)), 129.5 (2C(Ar)Phe), 129.3 (C(naphthyl)), 127.8 (C(naphthyl)), 127.6 (C(Ar)Phe), 126.95 (C6 or C7(naphthyl)), 126.5 (C6 or C7(naphthyl)), 124.2 (C(naphthyl)), 124.0 (C(naphthyl)), 56.6 ($C_\alpha H$), 54.5 ($C_\alpha H$), 53.2 ($C_\alpha H$), 51.5 ($C_\alpha H$ Ala), 50.3 ($C_\alpha H$), 50.1 ($C_\alpha H$), 48.3 ($C_\alpha H$ (R)-2-naphthyl-beta-Ala), 43.1 ($C_{Z2}H$), 41.2 (CH_2 Leu), 37.5 ($C_{Y2}H$), 36.9 ($C_{X2}H$), 33.8 (CH-CH_2-**C**H_2 (D)Glu), 29.3 (CH-**C**H_2-CH_2 (D)Glu), 25.7 (CH Leu), 23.5 (CH_3 Leu), 21.5 (CH_3 Leu), 16.99 (CH_3 Ala), 16.92 (CH_3 (D)Ala) ppm; HRMS (MALDI-neg): m/z calcd. for $C_{43}H_{52}N_7O_{11}^-$: 842.37303 $[M-H]^-$; found: 842.37303.

Synthesis of cyclo{(D)Ala-Leu-(D)Asp-Phe-[(2*S*,3*S*)-Chx-beta-Ala]-(D)Glu-Gly} (8)



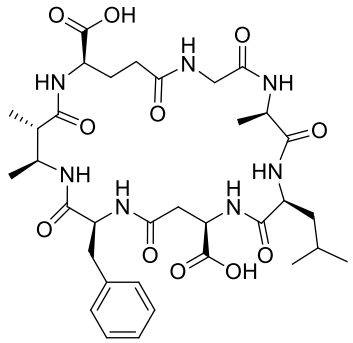
Synthesis of the protected linear peptide starting with 160 mg of resin was carried out with the MultiSynTech Syro I except coupling of **15** (4 equiv.), that was done manually. HPLC analysis t_R = 15.1 min (10-100% ACN/0.05%TFA in 16 min), MS (ESI-pos): m/z calcd. for $C_{59}H_{80}N_7O_{14}^+$: 1110.58 $[FmocM+H]^+$; found: 1110.7. Synthesis and purification of **8** was carried out according to the general procedure to give a white powder (17.9 mg, 23.62 µmol, 21%). HPLC analysis t_R = 11.5 min (10-80% ACN/0.05%TFA in 16 min); $[\alpha]^{20}_D$ = -10.5±1.0 (c. 0.24, H_2O); ^1H NMR (500 MHz, D_2O): δ 7.28 (m, 5H, Ar Phe), 4.65 (t, *J* = 5.4 Hz, 1H, $C_{X\alpha}H$), 4.54 (dd, *J* = 8.9, 4.8 Hz, 1H, $C_{Y\alpha}H$), 4.38 (q, *J* = 7.3 Hz, 1H, $C_\alpha H$ (D)Ala), 4.28 (m, 2H, $C_\alpha H$ Leu, $C_\alpha H$ (D)Glu), 3.94 (d, *J* = 17.3 Hz, 1H, CH_2 Gly), 3.89 (m, 1H, $C_\beta H$ (2*S*,3*S*)-Chx-beta-Ala), 3.72 (d, *J* = 17.3 Hz, 1H, CH_2 Gly), 3.26 (dd, *J* = 14.2, 4.7 Hz, 1H, C_YH_2), 2.84 (dd, *J* = 14.1, 9.1 Hz, 1H, C_YH_2), 2.77 (dd, *J* = 15.6, 5.3 Hz, 1H, C_XH_2), 2.51 (m, 2H, C_XH_2, $C_\alpha H$ (2*S*,3*S*)-Chx-beta-Ala), 2.36 (m, 2H, CH-CH_2-C**H**$_2$ (D)Glu), 2.14 (m, 1H, CH-C**H**$_2$-CH_2 (D)Glu), 1.78 (m, 6H, CH-C**H**$_2$-CH_2 (D)Glu, CH-CH_2 Leu, C**H**$_2$-$C_\alpha H$ (2*S*,3*S*)-Chx-beta-Ala, C**H**$_2$-$C_\beta H$ (2*S*,3*S*)-Chx-beta-Ala), 1.34 (m, 6H, C**H**$_2$-$C_\alpha H$ (2*S*,3S)-Chx-beta-Ala, C**H**$_2$-$C_\beta H$ (2*S*,3*S*)-Chx-beta-Ala, 2CH_2 (2*S*,3*S*)-Chx-beta-Ala), 1.19 (d, *J* = 7.2 Hz, 3H, CH_3 (D)Ala), 0.93 (d, *J* = 5.5 Hz, 3H, CH_3 Leu), 0.87 (d, *J* = 5.5 Hz, 3H, CH_3 Leu) ppm; ^{13}C NMR (101 MHz, CD_3OD): δ 137.3 (C(Ar)Phe), 130.1 (2CH(Ar)Phe), 129.6 (2CH(Ar)Phe), 127.8 (C4H(Ar)Phe), 56.1 ($C_\alpha H$), 54.7 ($C_\alpha H$), 52.9 ($C_\alpha H$), 51.2 ($C_\alpha H$), 50.9 ($C_\beta H$ (2*S*,3*S*)-Chx-beta-Ala), 50.1 ($C_\alpha H$), 50.0 ($C_\alpha H$), 44.5 (CH_2 Gly), 41.9 (CH_2), 38.3 (CH_2), 35.2 (CH_2), 33.7 (CH_2), 31.8 (CH_2), 30.8 (CH_2), 30.4 (CH_2), 28.3 (CH_2), 26.0 (CH Leu), 25.7 (CH_2), 21.3 (2CH_3 Leu), 15.4 (CH_3 (D)Ala) ppm; HRMS (MALDI-pos): m/z calcd. for $C_{36}H_{51}N_7NaO_{11}^+$: 780.35443 $[M+Na]^+$; found: 780.35758.

Synthesis of cyclo{(D)Ala-Leu-(D)Asp-Phe-[(2*S*,3*S*)-Chx-beta-Ala]-(D)Glu-Ala} (9)



Synthesis of the protected linear peptide starting with 90 mg of resin was carried out with the MultiSynTech Syro I except coupling of **15**, that was done manually. HPLC analysis t_R = 10.2 min (10-100% ACN/0.05%TFA in 16 min), MS (ESI-pos): m/z calcd. for $C_{45}H_{72}N_7O_{12}^+$: 902.52 $[M+H]^+$; found: 902.6. Synthesis and purification of **9** was carried out according to the general procedure to give a white powder (2.9 mg, 3.75 µmol, 6%). HPLC analysis t_R = 8.7 min (10-100% ACN/0.05%TFA in 16 min); $[\alpha]^{20}_D$ = -8.1±1.4 (c. 0.29, MeOH); ^{1}H NMR (500 MHz, CD_3OD): δ 7.22 (m, 5H, Ar Phe), 4.86 (m, 1H, $C_\alpha H$), 4.54 (dd, *J* = 10.6, 3.9 Hz, 1H, $C_\alpha H$), 4.38 (dd, *J* = 8.8, 5.7 Hz, 1H, $C_\alpha H$), 4.31 (m, 2H, $2C_\alpha H$), 4.03 (td, *J* = 11.4, 4.1 Hz, 1H, $C_\alpha H$), 3.96 (q, *J* = 6.9 Hz, 1H, $C_\alpha H$ Ala), 3.42 (dd, 1H, *J* = 14.1, 3.8 Hz, C_ZH_2), 2.73 (m, 2H, C_YH_2, C_ZH_2), 2.45 (m, 1H, $C_\alpha H$ (2*S*,3*S*)-Chx-beta-Ala), 2.36 (m, 1H, CH_2), 2.26 (m, 1H, C_YH_2), 2.20 (m, 1H, CH_2), 2.08 (m, 1H, C_XH_2), 1.92 (m, 1H, CH_2), 1.78 (m, 2H, C_XH_2, CH_2), 1.70 (m, 1H, CH Leu), 1.57 (m, 2H, CH_2), 1.39 (d, *J* = 7.0 Hz, 3H, CH_3 Ala), 1.31 (m, 5H, CH_2), 1.08 (d, *J* = 7.3 Hz, 3H, CH_3 (D)Ala), 0.92 (d, *J* = 6.5 Hz, 3H, CH_3 Leu), 0.88 (d, *J* = 6.5 Hz, 3H, CH_3 Leu) ppm; ^{13}C NMR (125 MHz, CD_3OD): δ 176.1 (C=O), 175.42 (C=O), 175.40 (C=O), 175.38 (C=O), 174.8 (C=O), 174.7 (C=O), 174.3 (C=O), 173.7 (C=O), 172.0 (C=O), 139.2 (C(Ar)Phe), 129.5 (2CH(Ar)Phe), 130.1 (2CH(Ar)Phe), 127.8 (C4H(Ar)Phe), 55.9 ($C_\alpha H$), 54.5 ($C_\alpha H$), 52.9 ($C_\alpha H$), 51.4 ($C_\alpha H$ Ala), 51.1 ($C_\alpha H$), 50.9 ($C_\alpha H$ (2*S*,3*S*)-Chx-beta-Ala), 50.3 ($C_\alpha H$), 50.1 ($C_\alpha H$), 41.1 (CH_2), 37.9 (C_ZH_2), 36.9 (C_YH_2), 33.8 ($2CH_2$), 30.8 ($2CH_2$), 29.2 (C_XH_2), 26.1 (CH Leu), 25.7 (CH_2), 23.5 (CH_3 Leu), 21.4 (CH_3 Leu), 17.0 (CH_3 (D)Ala), 16.9 (CH_3 Ala) ppm; HRMS (MALDI-pos): m/z calcd. for $C_{37}H_{53}N_7NaO_{11}^+$: 794.37007 $[M+Na]^+$; found: 794.37099.

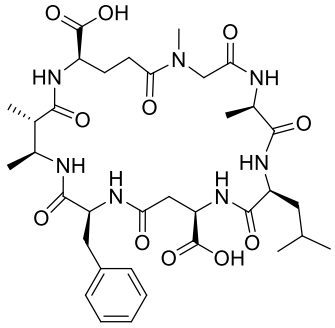
Synthesis of cyclo[(D)Ala-Leu-(D)Asp-Phe-Amba-(D)Glu-Gly] (10):



Synthesis of the protected linear peptide starting with 35 mg of resin was carried out with the MultiSynTech Syro I except coupling of **19** (3 equiv.) that was done manually. HPLC analysis t_R = 14.6 min (10-100% ACN/0.05%TFA in 16 min), MS (ESI-pos): m/z calcd. for $C_{57}H_{78}N_7O_{14}^+$: 1084.56 $[Fmoc-M+H]^+$; found: 1084.7. Synthesis and purification of **10** was

carried out according to the general procedure to give a white powder (5.2 mg, 7.11 µmol, 29%). HPLC analysis t_R = 9.1 min (10-80% ACN/0.05%TFA in 16 min); $[\alpha]^{20}_D$ = -345.3±88.1 (c. 0.12, H_2O); ^{1}H NMR (500 MHz, D_2O): δ 7.29 (m, 5H Ar Phe), 4.66 (t, *J* = 5.5 Hz, 1H, C_αH (D)Asp), 4.51 (dd, *J* = 8.3, 5.8 Hz, 1H, C_αH Phe), 4.36 (q, *J* = 7.0 Hz, 1H, C_αH (D)Ala), 4.28 (dd, *J* = 9.7, 6.6 Hz, 2H, C_αH Leu, C_αH (D)Glu), 3.97 (m, 2H, C_βH Amba, CH_2 Gly), 3.75 (d, *J* = 17.2 Hz, 1H, CH_2 Gly), 3.22 (dd, *J* = 13.9, 5.3 Hz, 1H, CH_2 Phe), 2.88 (dd, *J* = 14.0, 8.6 Hz, 1H, CH_2 Phe), 2.77 (dd, *J* = 15.7, 5.5 Hz, 1H, CH_2 (D)Asp), 2.59 (m, 2H, C_αH Amba, CH_2 (D)Asp), 2.37 (m, 2H, CH-CH_2-C**H**$_2$ (D)Glu), 2.14 (m, 1H, CH-C**H**$_2$-CH_2 (D)Glu), 1.91 (m, 1H, CH-C**H**$_2$-CH_2 (D)Glu), 1.68 (m, 3H, CH-CH_2 Leu), 1.24 (d, *J* = 7.2 Hz, 3H, (D)Ala), 1.13 (d, *J* = 6.7 Hz, 3H, C_βH-C**H**$_3$ Amba), 1.05 (d, *J* = 6.9 Hz, 3H, C_αH-C**H**$_3$ Amba), 0.93 (d, *J* = 5.4 Hz, 3H, CH_3 Leu), 0.87 (d, *J* = 5.5 Hz, 3H, CH_3 Leu) ppm; ^{13}C NMR (125 MHz, D_2O): δ 185.0 (C=O), 183.2 (C=O), 182.7 (C=O), 182.6 (C=O), 181.7 (C=O), 181.5 (C=O), 179.6 (C=O), 179.0 (C=O), 178.5 (C=O), 144.0 (C(Ar) Phe), 136.5 (2CH(Ar) Phe), 136.1 (2CH(Ar) Phe), 134.5 (C4H(Ar) Phe), 62.0 (C_αH Phe), 60.0 (C_αH Leu), 59.8 (C_αH (D)Glu), 57.1 (C_αH (D)Ala), 56.9 (C_αH (D)Asp), 55.5 (C_βH Amba), 52.7 (C_αH Amba), 50.1 (CH_2 Gly), 46.6 (CH_2 Leu), 44.0 (CH_2 Phe), 43.7 (CH_2 (D)Asp), 39.3 (CH-CH_2-**C**H_2 (D)Glu), 33.9 (CH-**C**H_2-CH_2 (D)Glu), 31.8 (CH Leu), 29.7 (CH_3 Leu), 27.5 (CH_3 Leu), 24.9 (C_βH-**C**H_3 Amba), 23.5 (CH_3 (D)Ala), 21.6 (C_αH-**C**H_3 Amba) ppm; HRMS (MALDI-pos): m/z calcd. for $C_{34}H_{49}N_7NaO_{11}^+$: 754.33877 $[M+Na]^+$; found: 754.33783.

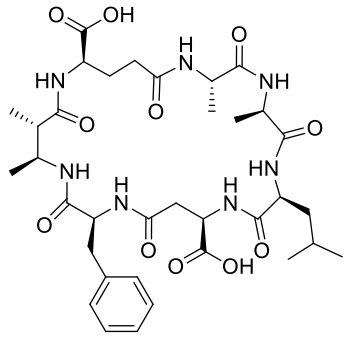
Synthesis of cyclo[(D)Ala-Leu-(D)Asp-Phe-Amba-(D)Glu-Sar] (11)



Synthesis of the protected linear peptide starting with 45 mg of resin was carried out with the MultiSynTech Syro I but coupling of Fmoc-D-Glu(OtBu)-OH lasted for 2 hours. Coupling of **19** was done manually. HPLC analysis t_R = 11.1 min (10-100% ACN/0.05%TFA in 16 min), MS (ESI-pos): m/z calcd. for $C_{43}H_{70}N_7O_{12}^+$: 876.51 $[M+H]^+$; found: 876.5. Synthesis and purification of **11** was carried out according to the general procedure to give a white powder (3.9 mg, 5.23 µmol, 17%). HPLC analysis t_R = 9.2 min (10-100% ACN/0.05%TFA in 16 min); $[\alpha]^{20}_D$ = = 17.8±2.2 (c. 0.13, H_2O); ^{1}H NMR (500 MHz, D_2O): δ 7.30 (m, 5H, Ar Phe), 4.62 (m, 1H, $C_{X\alpha}$H), 4.50 (m, 1H, $C_{Y\alpha}$H), 4.36 (q, *J* = 7.3 Hz, 1H, CH (D)Ala), 4.30 (m, 2H, C_αH Leu, C_αH (D)Glu), 4.18 (d, *J* = 16.8 Hz, 1H, CH_2 Sar), 3.99 (m, 1H, C_βH Amba), 3.90 (dd, *J* = 17.1, 11.3 Hz, 1H, CH_2 Sar), 3.23 (dd, *J* = 14.0, 5.6 Hz, 1H, C_YH_2), 3.12 (s, 1H, CH_3 Sar), 2.86 (dd, *J* = 14.0, 9.1 Hz, 1H, C_YH_2), 2.73 (m, 1H, C_XH_2), 2.55 (m, 4H, C_αH Amba, CH-CH_2-C**H**$_2$ (D)Glu, C_XH_2), 2.10 (td, *J* = 17.2, 16.4, 9.4 Hz, 1H, CH-C**H**$_2$-CH_2 (D)Glu), 1.91 (m, 1H, CH-C**H**$_2$-CH_2 (D)Glu), 1.66 (m, 3H, CH_2-CH Leu), 1.39 (d, *J* = 7.3, 3H, CH_3 (D)Ala), 1.14 (d, *J* = 6.9 Hz, 3H, C_βH-C**H**$_3$ Amba), 1.04 (dd, *J* = 8.7, 6.8 Hz, 3H, C_αH-C**H**$_3$ Amba), 0.92 (d, *J* = 5.8 Hz, 3H, CH_3 Leu), 0.86 (d, *J* = 5.7 Hz, 3H, CH_3 Leu) ppm; ^{13}C NMR (125 MHz, D_2O): δ 184.8 (C=O), 183.4 (C=O), 182.7 (C=O), 182.5 (C=O), 181.7 (C=O), 181.1 (C=O), 179.7 (C=O), 179.1 (C=O), 178.3 (C=O), 144.1 (C(Ar)Phe), 136.5 (2CH(Ar)Phe), 136.2

(2CH(Ar)Phe), 134.5 (C4H(Ar)Phe), 62.1 ($C_{X\alpha}H$), 60.2 (CH_2 Sar), 59.7 ($C_\alpha H$ Leu), 59.5 ($C_\alpha H$(D)Glu), 57.0 ($C_\alpha H$ (D)Ala), 56.7 ($C_{X\alpha}H$), 55.6 ($C_\beta H$ Amba), 52.7 ($C_\alpha H$ Amba), 46.7 (CH_2 Leu), 44.7 (C_YH_2), 44.1 (C_XH_2), 37.3 (CH_3 Sar), 33.8 (CH-CH_2-**C**H_2 (D)Glu), 31.7 (CH-**C**H_2-CH_2 (D)Glu), 29.6 (CH Leu), 27.6 (CH_3 Leu), 24.9 (CH_3 Leu), 24.7 ($C_\beta H$-**C**H_3 Amba), 23.5 (CH_3 (D)Ala), 21.5 ($C_\alpha H$-**C**H_3 Amba) ppm; HRMS (MALDI-pos): m/z calcd. for $C_{35}H_{51}N_7NaO_{11}^+$: 768.35443 $[M+Na]^+$; found: 768.35579.

Synthesis of cyclo[(D)Ala-Leu-(D)Asp-Phe-Amba-(D)Glu-Ala] (12)



Synthesis of the protected linear peptide starting with 38 mg of resin was carried out with the MultiSynTech Syro I except coupling of **19** (3 equiv.) that was done manually. HPLC analysis t_R = 9.9 min (10-100% ACN/0.05%TFA in 16 min), MS (ESI-pos): m/z calcd. for $C_{43}H_{70}N_7O_{12}^+$: 876.51 $[M+H]^+$; found: 876.7. Synthesis and purification of **12** was carried out according to the general procedure to give a white powder (4.2 mg, 5.63 µmol, 21%). HPLC analysis t_R = 9.2 min (10-80% ACN/0.05%TFA in 16 min); $[\alpha]^{20}_D$ = -1.5±0.8 (c. 0.29, H_2O); ^{1}H NMR (500 MHz, D_2O): δ 7.28 (m, 5H, Ar Phe), 4.67 (t, *J* = 5.6 Hz, 1H, $C_{X\alpha}H$), 4.49 (t, *J* = 7.0 Hz, 1H, $C_{Y\alpha}H$), 4.35 (m, 2H, $C_\alpha H$ (D) Ala, $C_\alpha H$ Leu), 4.31 (dd, *J* = 8.8, 5.3 Hz, 1H, $C_\alpha H$ (D)Glu), 4.14 (q, *J* = 6.9 Hz, 1H, $C_\alpha H$ Ala), 3.89 (m, 1H, $C_\beta H$ Amba), 3.13 (dd, *J* = 14.0, 6.6 Hz, 1H, C_YH_2), 2.91 (dd, *J* = 14.1, 7.6 Hz, 1H, C_YH_2), 2.72 (m, 2H, C_XH_2), 2.56 (m, 1H, $C_\alpha H$ Amba), 2.35 (m, 1H, CH-CH_2-C**H**$_2$ (D)Glu), 2.24 (m, 1H, CH-CH_2-C**H**$_2$ (D)Glu), 2.12 (m, 1H, CH-C**H**$_2$-CH_2 (D)Glu), 1.90 (m, 1H, CH-C**H**$_2$-CH_2 (D)Glu), 1.68 (m, 1H, CH_2 Leu), 1.61 (m, 2H, CH Leu, CH_2 Leu), 1.32 (t, *J* = 6.4 Hz, 6H, CH_3 (D)Ala, CH_3 Ala), 1.11 (d, *J* = 6.7 Hz, 3H, $C_\beta H$-C**H**$_3$ Amba), 0.98 (d, *J* = 6.9 Hz, 3H, $C_\alpha H$-C**H**$_3$ Amba), 0.91 (d, *J* = 6.0 Hz, 3H, CH_3 Leu), 0.86 (d, *J* = 5.8 Hz, 3H, CH_3 Leu) ppm; ^{13}C NMR (125 MHz, D_2O): δ 184.8 (C=O), 182.5 (C=O), 182.4 (C=O), 181.9 (C=O), 181.7 (C=O), 181.6 (C=O), 181.5 (C=O), 179.2 (C=O), 179.0 (C=O), 143.7 (C(Ar) Phe), 136.5 (2CH(Ar) Phe), 136.1 (2CH(Ar) Phe), 134.5 (C4H(Ar) Phe), 62.2 ($C_{Y\alpha}H$), 59.8 ($C_\alpha H$ Leu), 59.5 ($C_\alpha H$ (D)Glu), 57.1 ($C_\alpha H$ (D)Ala), 57.05 ($C_\alpha H$ Ala), 56.99 ($C_{X\alpha}H$), 55.7 ($C_\beta H$ Amba), 52.3 ($C_\alpha H$ Amba), 46.9 (CH_2 Leu), 44.1 (C_YH_2), 43.7 (C_XH_2), 38.9 (CH-CH_2-C**H**$_2$ (D)Glu), 33.9 (CH-C**H**$_2$-CH_2 (D)Glu), 31.7 (CH Leu), 29.8 (CH_3 Leu), 27.5 (CH_3 Leu), 24.7 ($C_\beta H$-C**H**$_3$ Amba), 24.0 (CH_3 (D)Ala or Ala), 23.9 (CH_3 (D)Ala or Ala), 21.2 ($C_\alpha H$-C**H**$_3$ Amba) ppm; HRMS (MALDI-pos): m/z calcd. for $C_{35}H_{51}NaN_7O_{11}^+$: 768.35442 $[M+Na]^+$; found: 768.35361.

4. Conversion during macrocyclization

Linear peptides were obtained with purities around 90% directly after mild cleavage from the resin without further purification. Protected cycles after macrocyclization were analyzed without purification using Agilent 1290 UPLC. Consumption of the linear peptide to a single product peak visible in the UPLC gradient with different retention time and a mass peak according to the cyclized product was detected in all cases. The injection peak contains DMF, which is the solvent selected for the macrocyclization reaction, and possibly salts from the reaction, and it cannot be excluded that side products or polymeric peptides that are not visible in the mass are included as well.

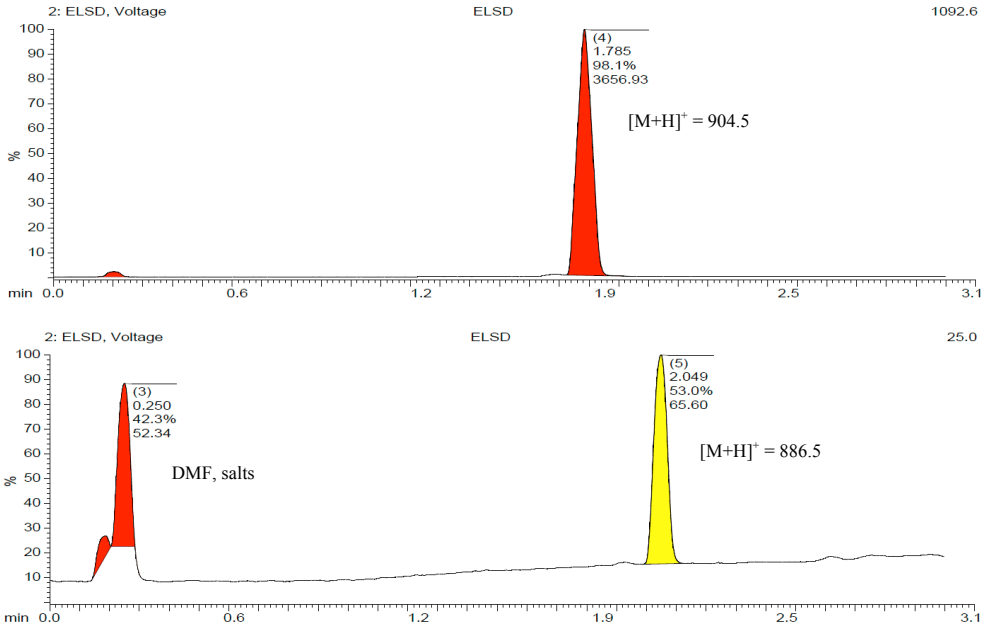


Figure S5. Total conversion of linear peptide (up) into the cycle (down) detected by UPLC/MS ELSD detector (synthesis of **2**).

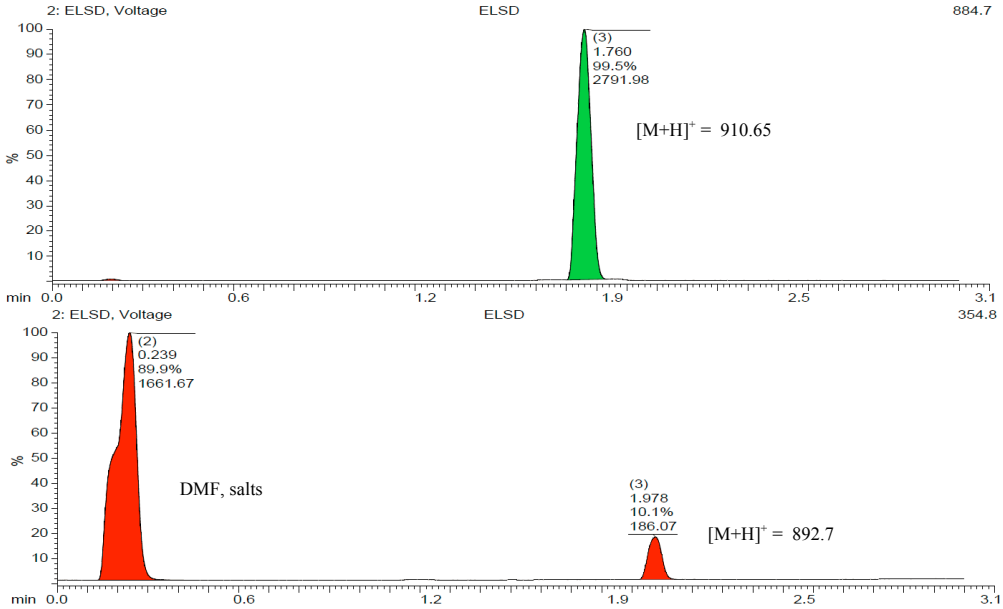


Figure S6. Total conversion of linear peptide (up) into the cycle (down) detected by UPLC/MS ELSD detector (synthesis of **3**).

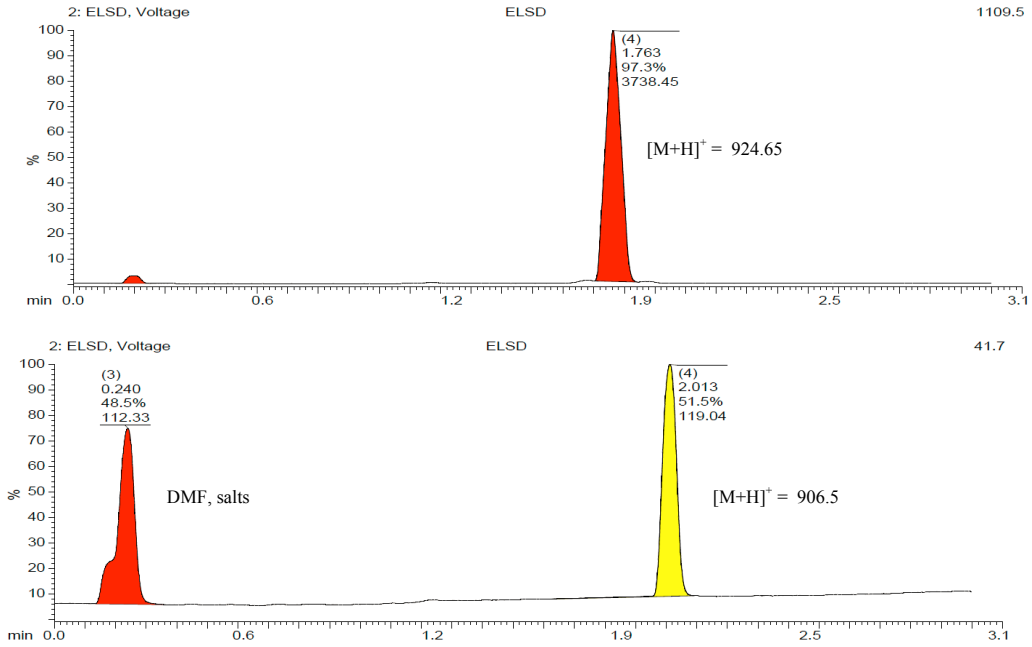


Figure S7. Total conversion of linear peptide (up) into the cycle (down) detected by UPLC/MS ELSD detector (synthesis of **4**).

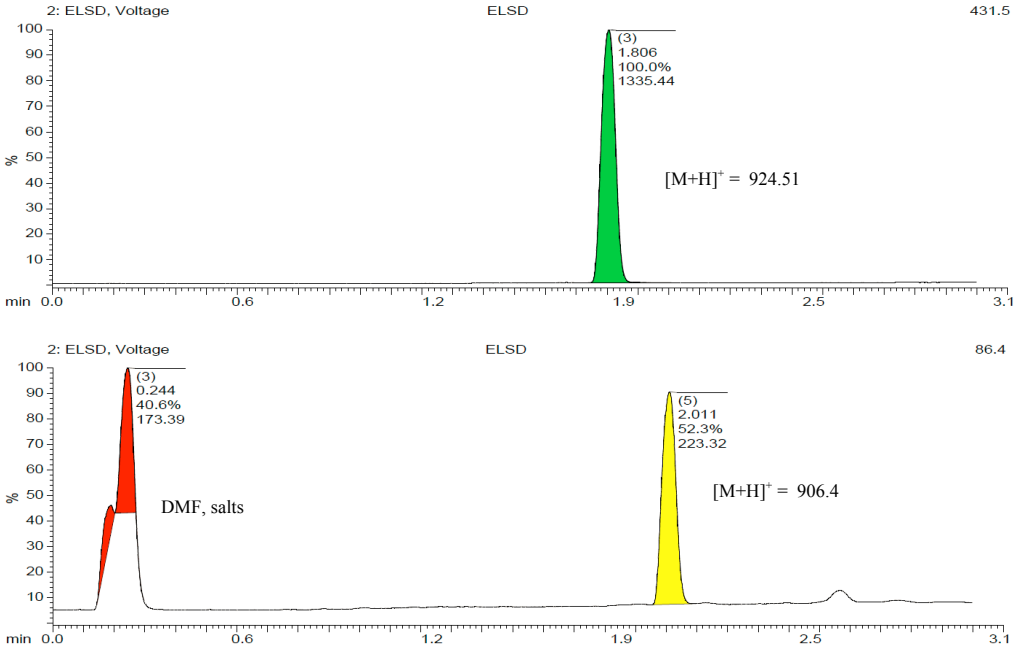


Figure S8. Total conversion of linear peptide (up) into the cycle (down) detected by UPLC/MS ELSD detector (synthesis of **5**).

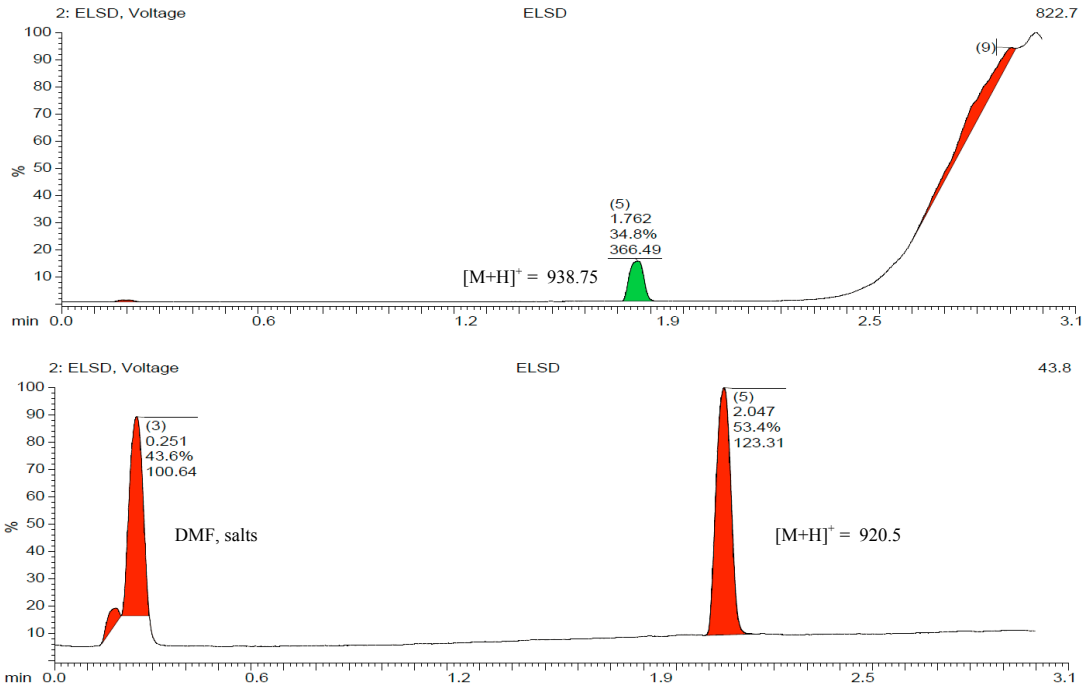


Figure S9. Total conversion of linear peptide (up) into the cycle (down) detected by UPLC/MS ELSD detector (synthesis of **6**).

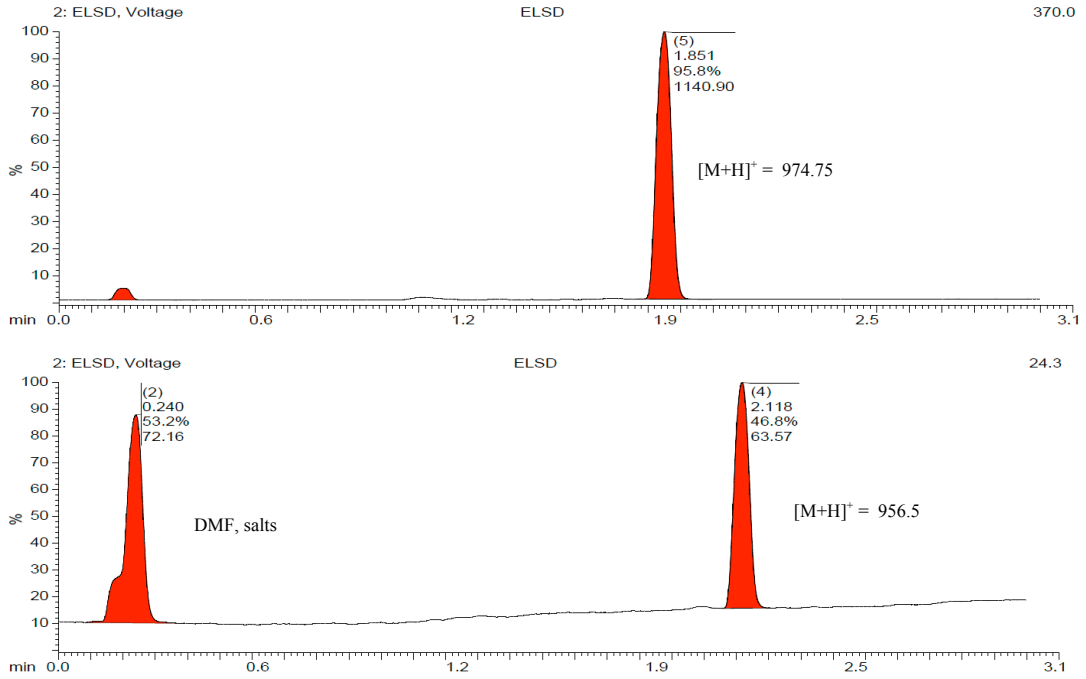


Figure S10. Total conversion of linear peptide (up) into the cycle (down) detected by UPLC/MS ELSD detector (synthesis of **7**).

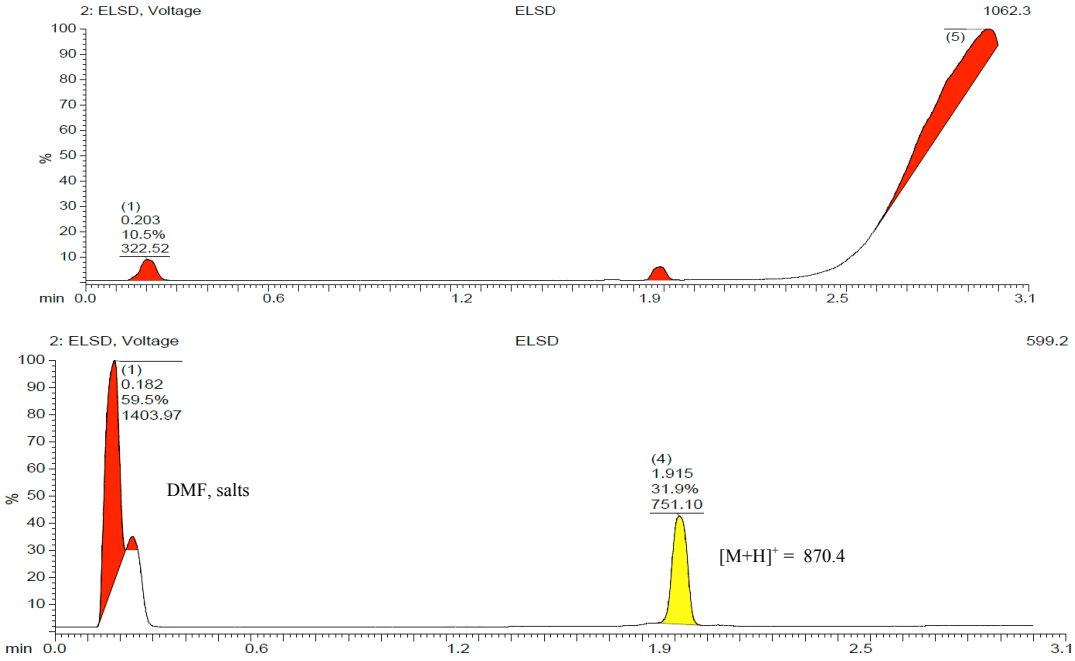


Figure S11. Total conversion of linear peptide (up, low concentration of peptide injected) into the cycle (down) detected by UPLC/MS ELSD detector (synthesis of **8**).

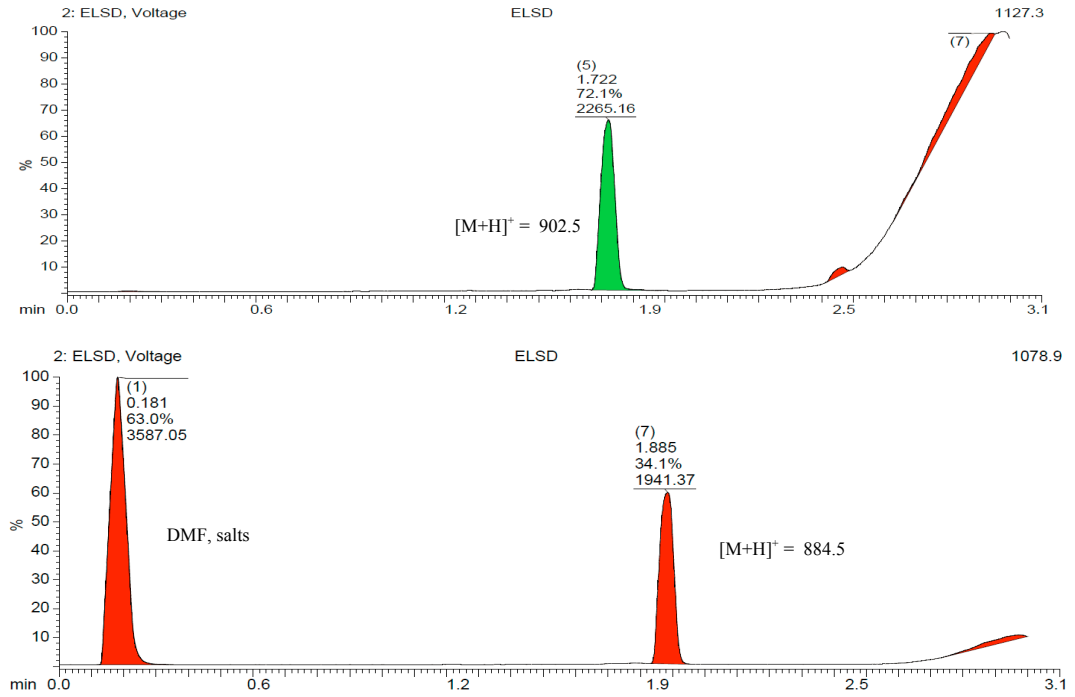


Figure S12. Total conversion of linear peptide (up) into the cycle (down) detected by UPLC/MS ELSD detector (synthesis of **9**).

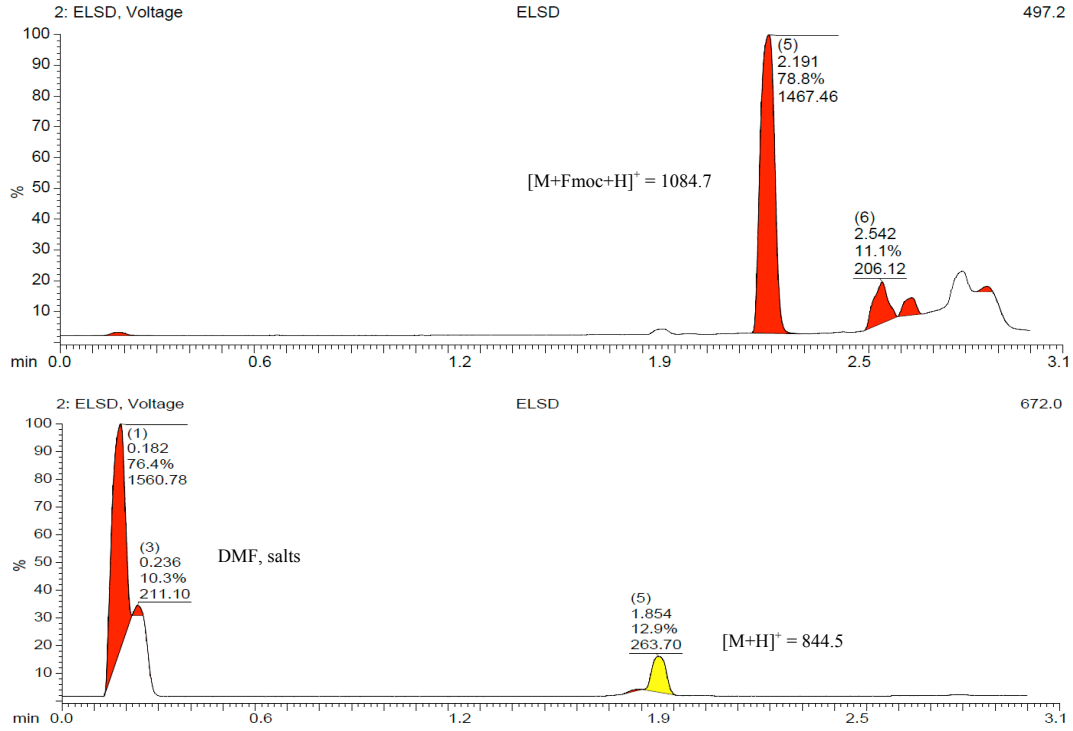


Figure S13. Total conversion of linear peptide (up) into the cycle (down) detected by UPLC/MS ELSD detector (synthesis of **10**).

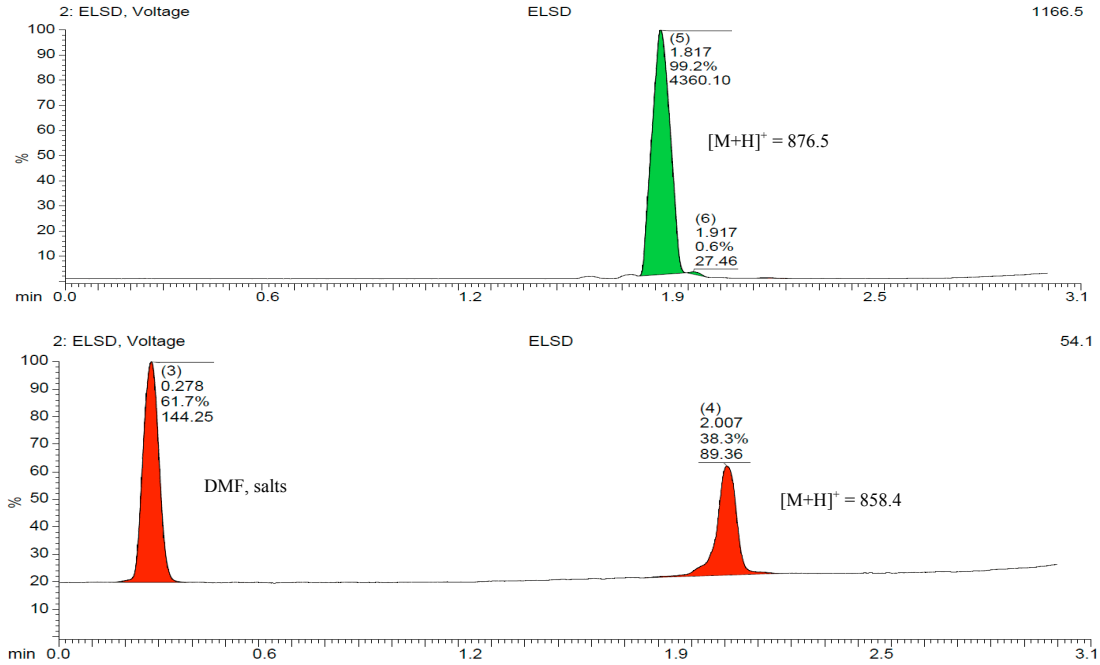


Figure S14. Total conversion of linear peptide (up) into the cycle (down) detected by UPLC/MS ELSD detector (synthesis of **11**).

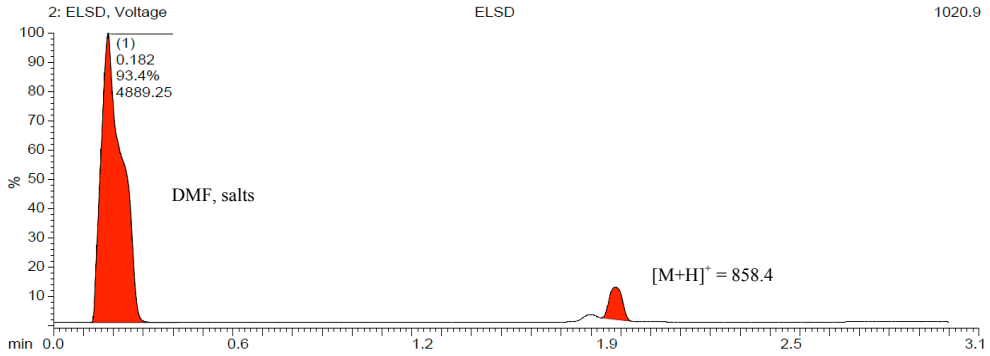


Figure S15. Total conversion of linear peptide detected by HPLC/MS (not shown) into the cycle detected by UPLC/MS ELSD detector (down) (synthesis of **12**).

5. No evidence of epimerization

Only one sharp peak with the corresponding right mass was detected in the HPLC and UPLC in all cases. In order to further evaluate the macrocyclization reaction, this reaction was further studied in the case of one microcystin analog (**6**). The macrocyclization was studied by checking ^{1}H, ^{13}C NMR and HRMS but also using Agilent 1290 UPLC with Acquity UPLC BEH C18 2.1X50 mm, 1.7 µM column and UV/Vis detector operating at 220 nm and 254 nm and ELSD detector. Only one peak with the expected mass could be detected (Figure S16).

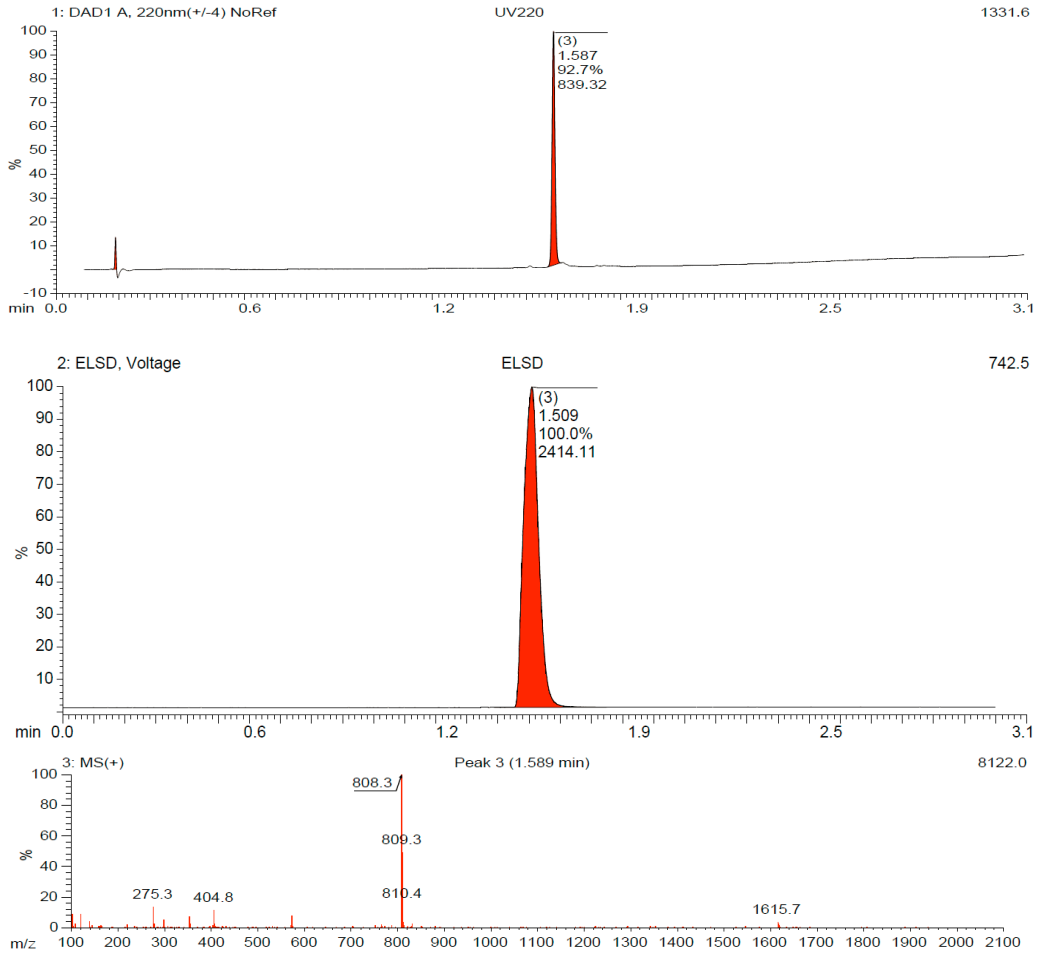


Figure S16. Purity of compound 6 checked with UPLC using UV and ELSD detector.

Additionally, the enantiomeric purity of compound **6** was checked using a chiral column (ChiraDex (5µm) LiChroCART 250-4 Merck) and only one single peak was detected (Figure S17).

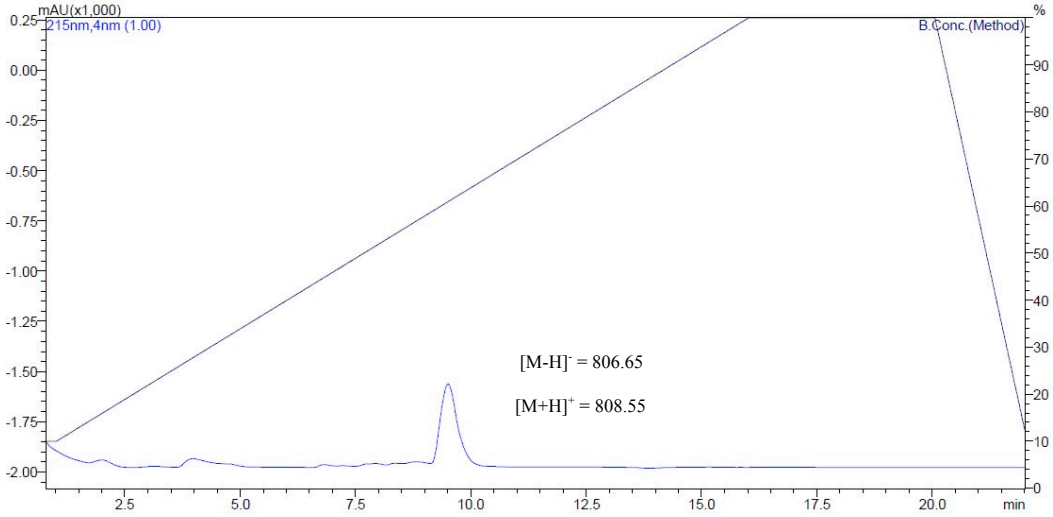


Figure S17. Purity of compound 6 checked with HPLC using a chiral column and detected by UV.

However, in order to get a conclusive result about epimerization during the cyclization step, compound **6** was hydrolyzed and the presence of D and L amino acids, which can be detected with higher sensitivity than with techniques such as HPLC or UPLC, was evaluated. Compound **6** was hydrolyzed with 6N DCl in D_2O and injected on a gas chromatograph (GC-MS) with a detection limit lower than 0.1% following the standard procedure in C.A.T. GmbH&Co Chromatographie und Analysentechnik KG. The results show 99.529% enantiomeric purity (Figure S18). Consequently, no evidence of isomerization during the macrocyclization reaction is detected.

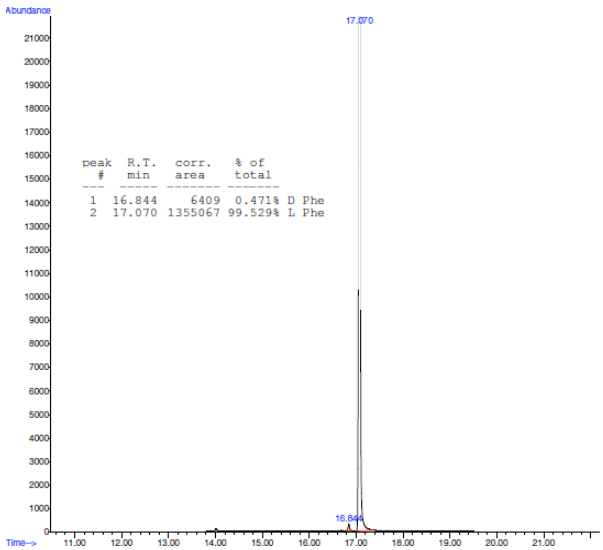


Figure S18. Enantiomeric purity of microcystin analogue **6** showing 99.529% enantiomeric purity.

6. UPLC/ELSD traces of final pure compounds (2-12).

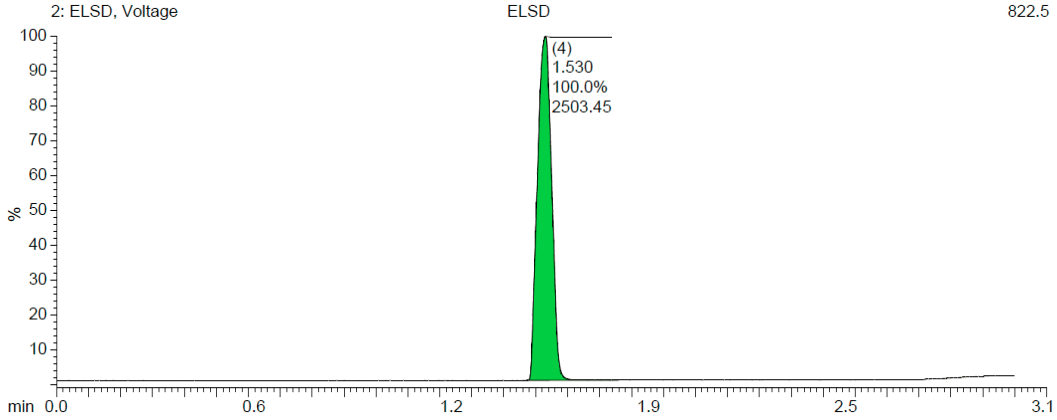


Figure S19. UPLC/ELSD of compound **2**.

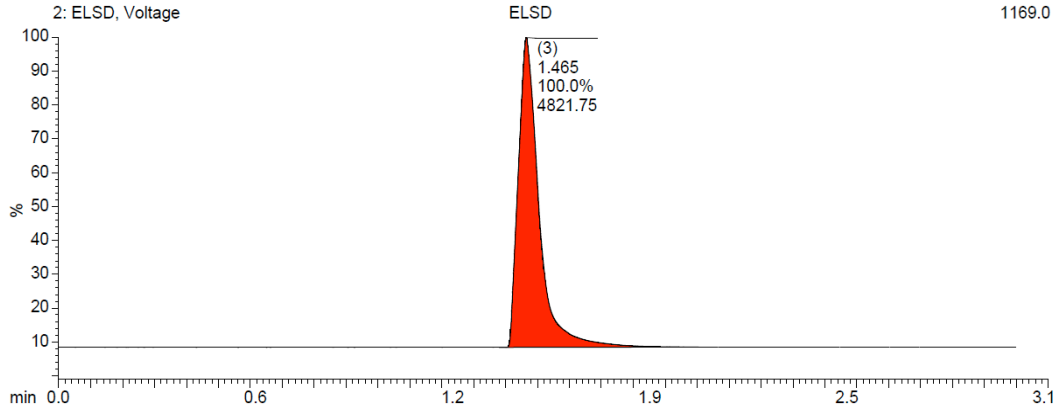


Figure S20. UPLC/ELSD of compound **3**.

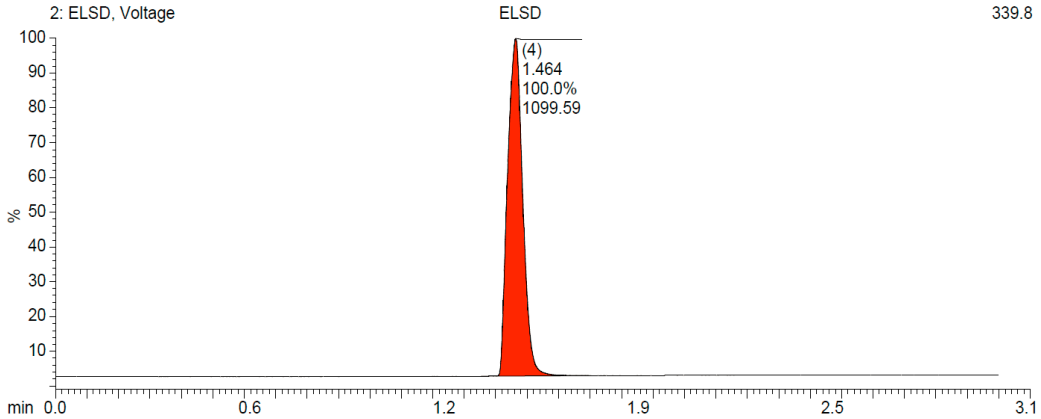


Figure S21. UPLC/ELSD of compound **4**.

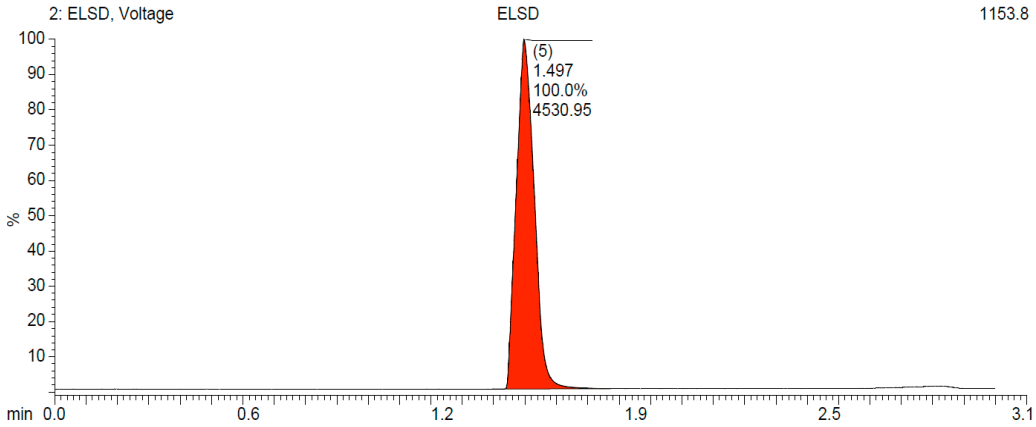


Figure S22. UPLC/ELSD of compound **5**.

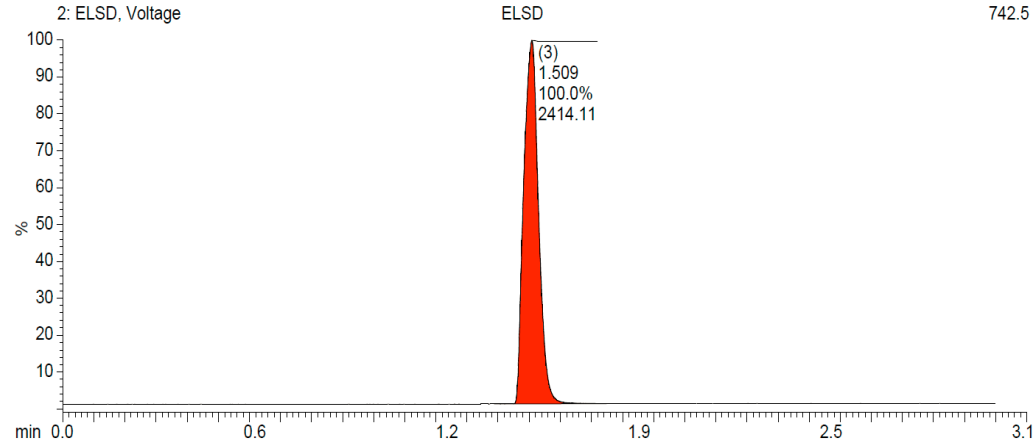


Figure S23. UPLC/ELSD of compound **6**.

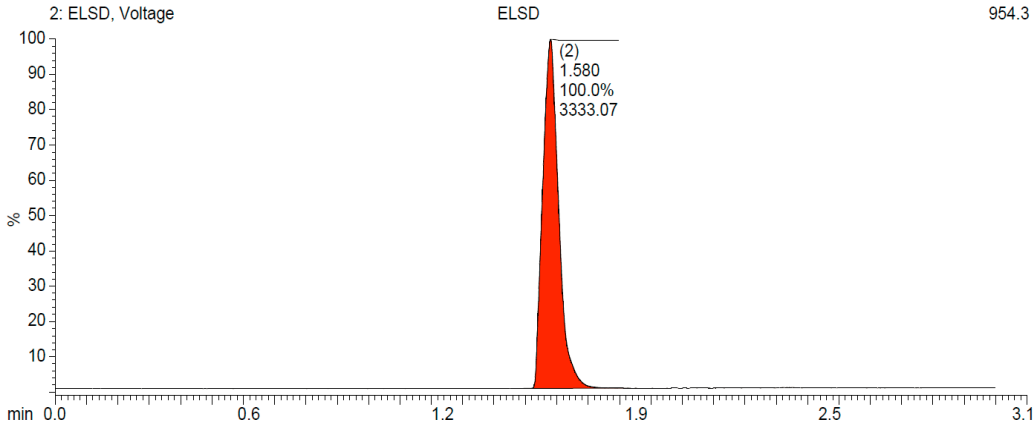


Figure S24. UPLC/ELSD of compound **7**.

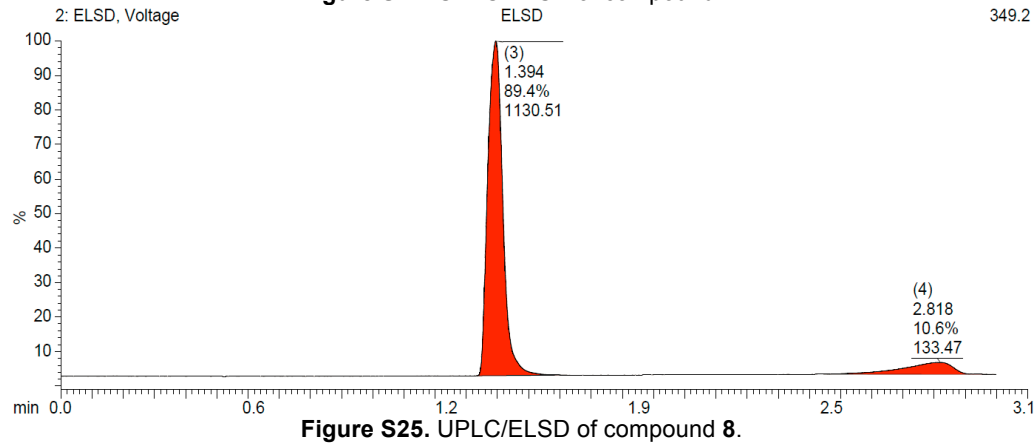


Figure S25. UPLC/ELSD of compound **8**.

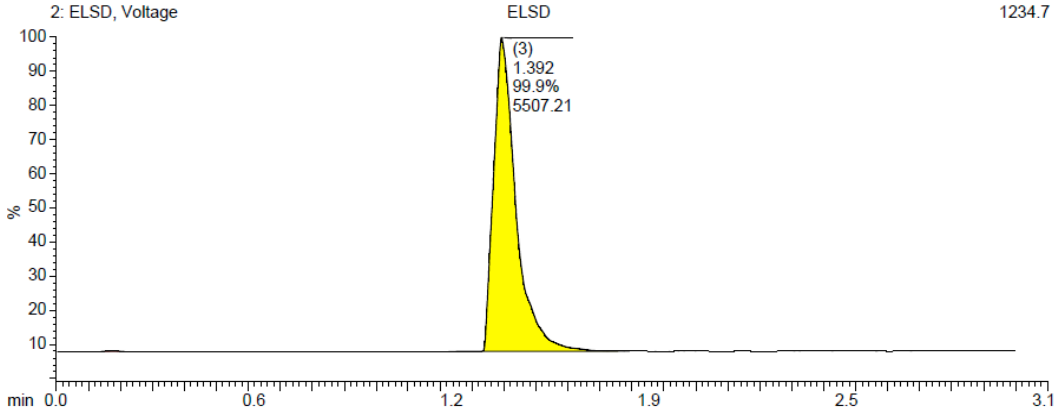


Figure S26. UPLC/ELSD of compound **9**.

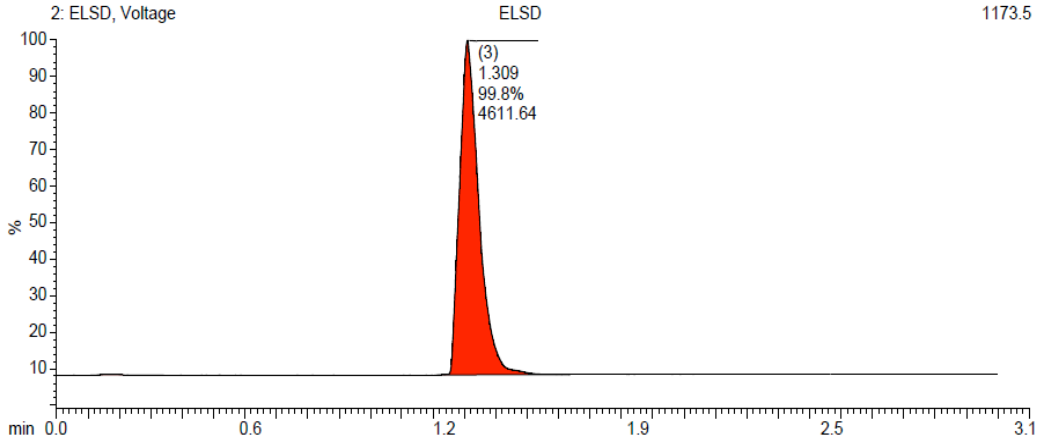


Figure S27. UPLC/ELSD of compound **10**.

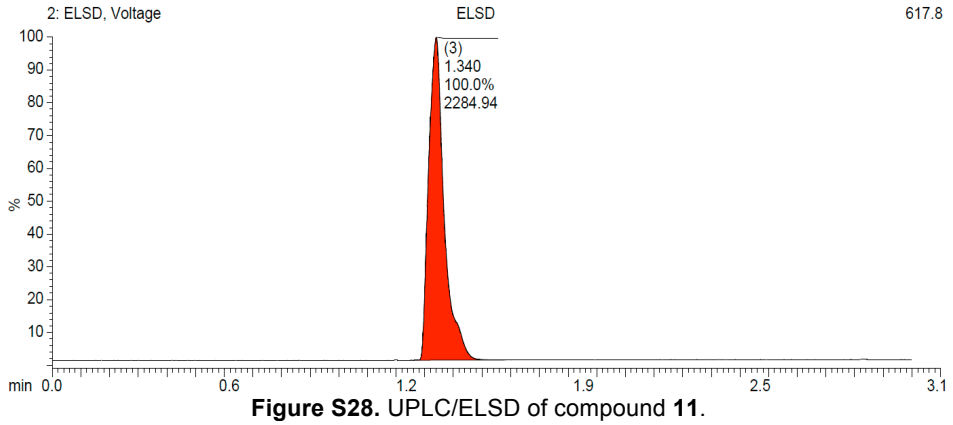


Figure S28. UPLC/ELSD of compound **11**.

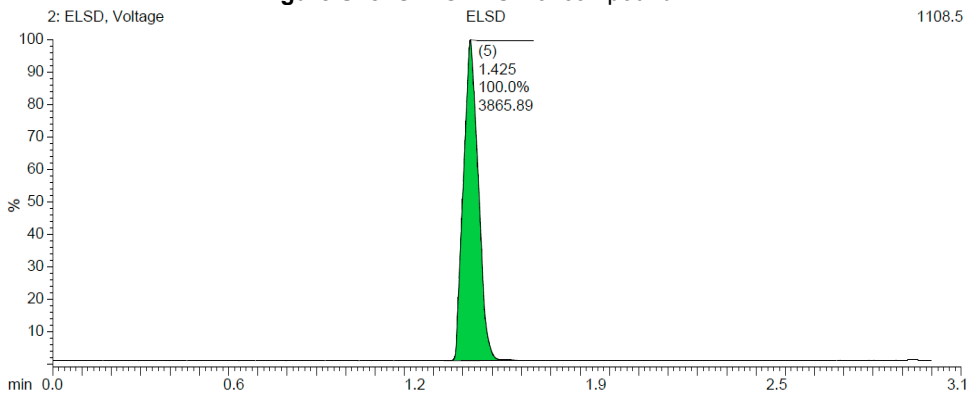
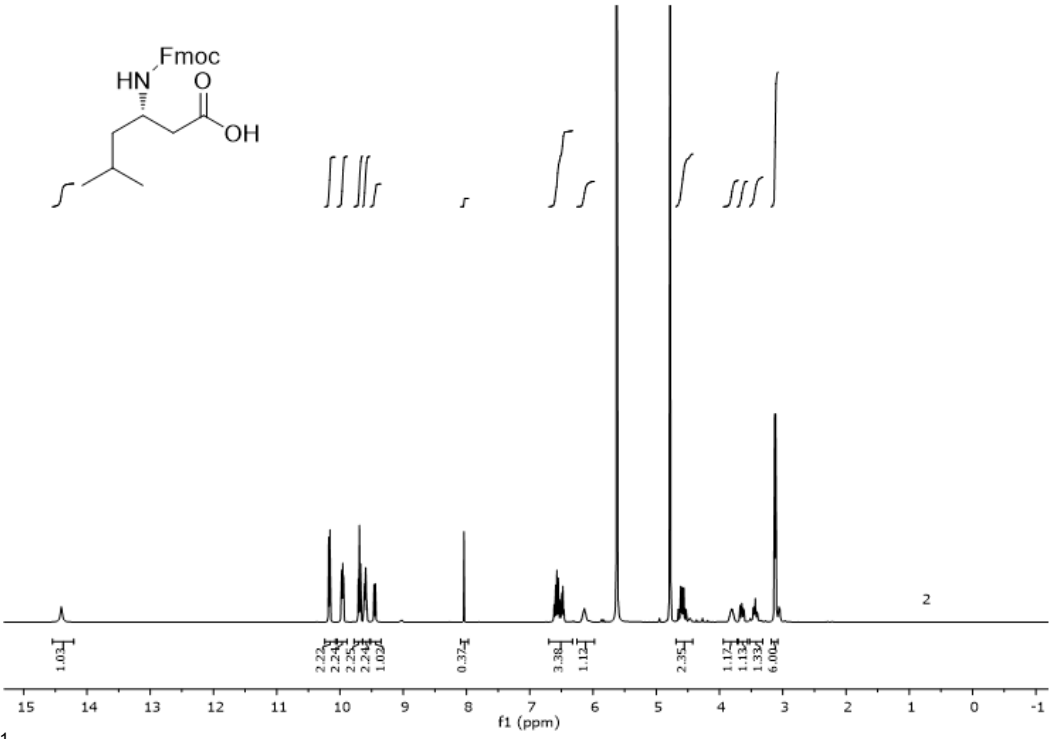
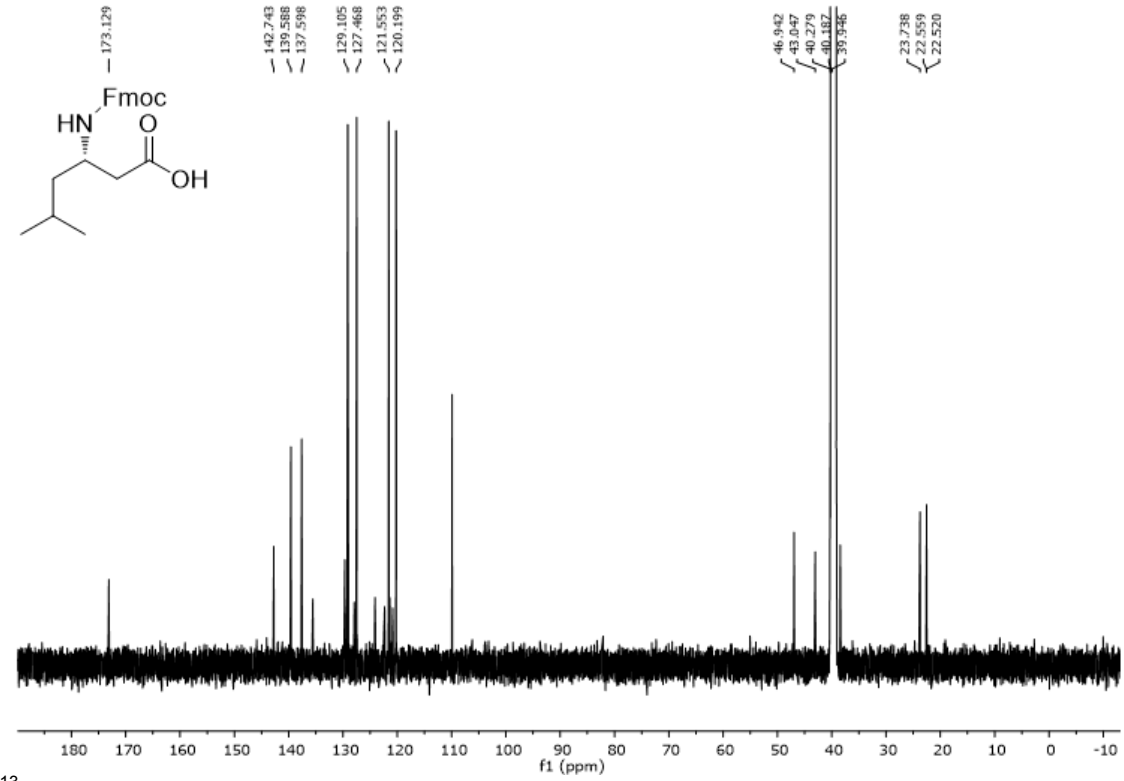


Figure S29. UPLC/ELSD of compound **12**.

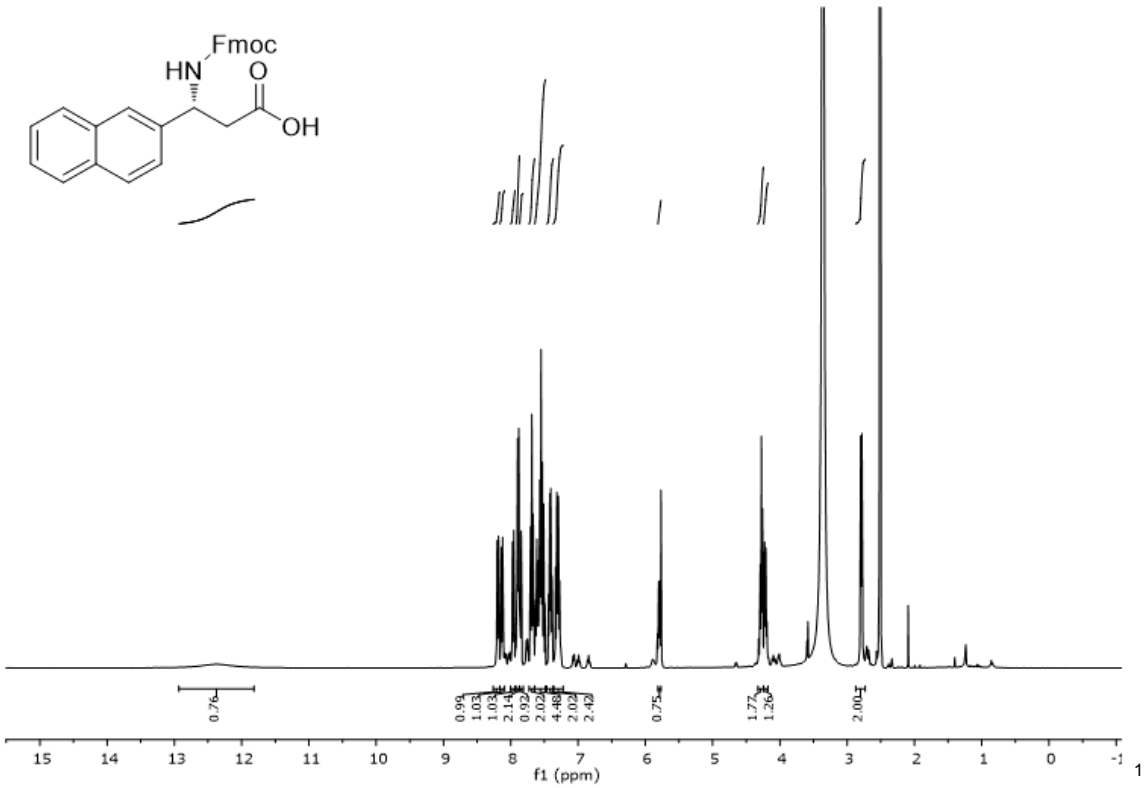
7. ^{1}H, ^{13}C NMR spectra



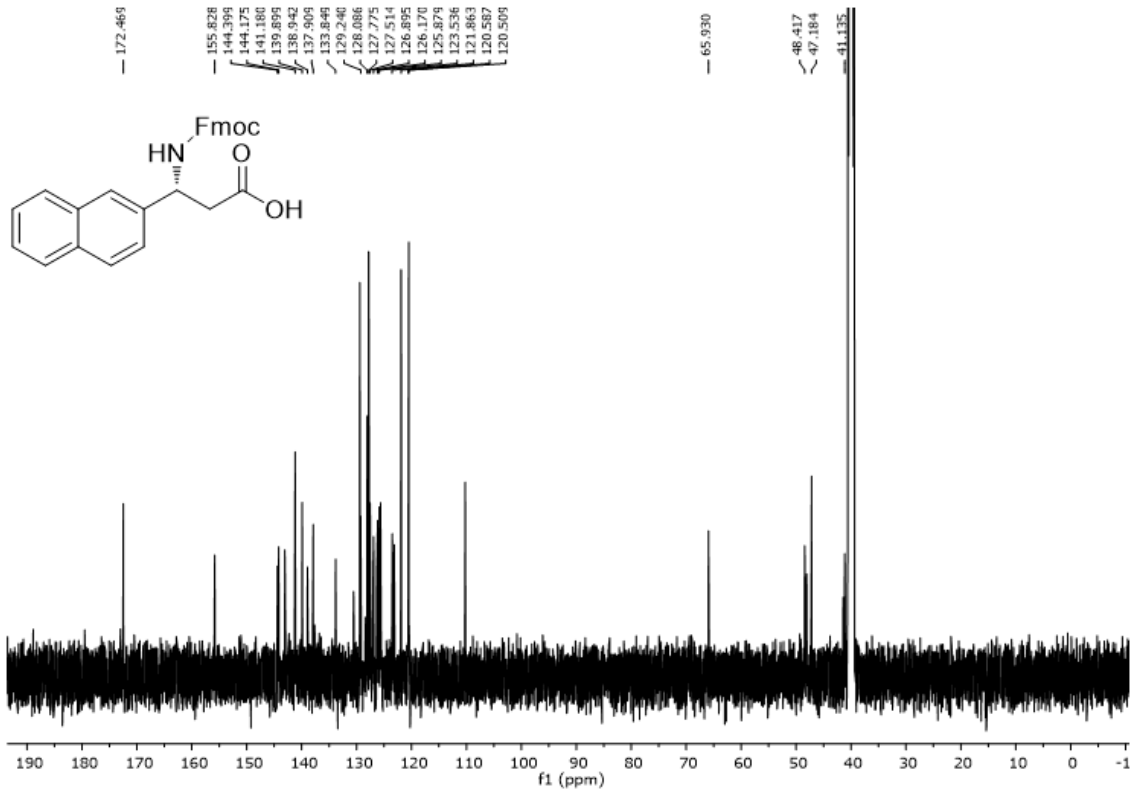
^{1}H NMR spectrum (400 MHz, 300 K, DMSO-d6) of **13**.



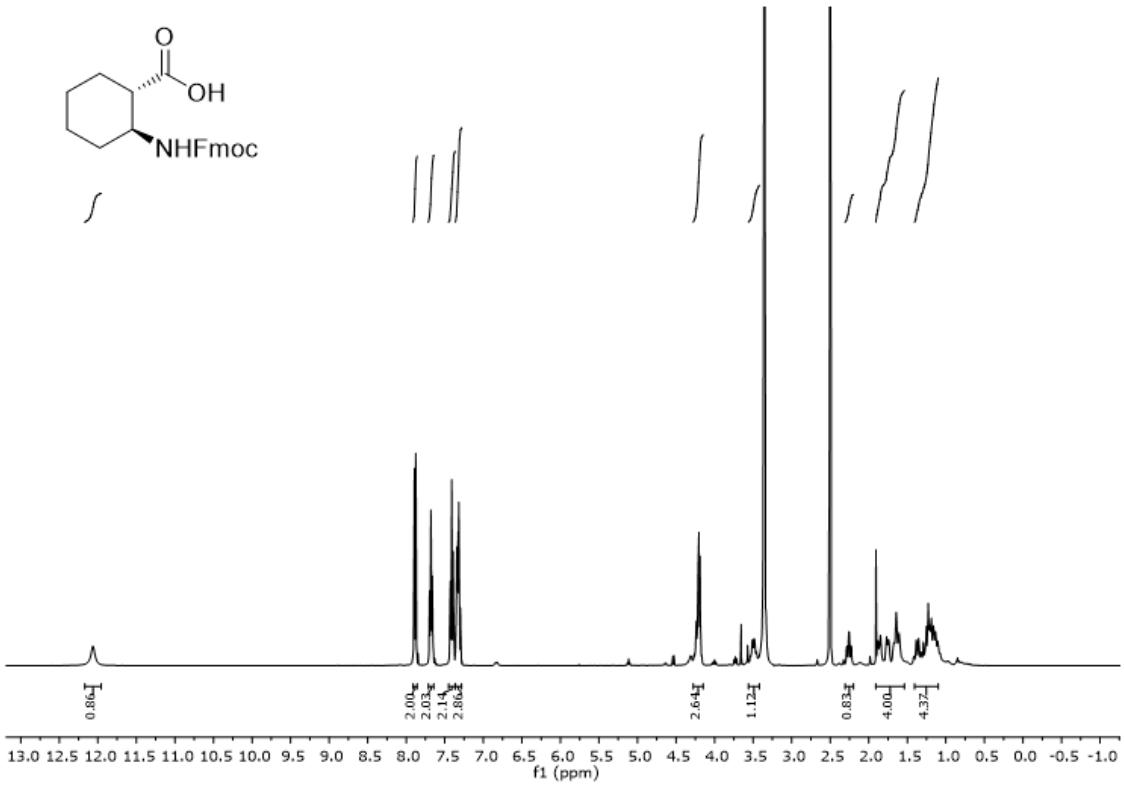
^{13}C NMR spectrum (101 MHz, 300 K, DMSO-d6) of **13**.



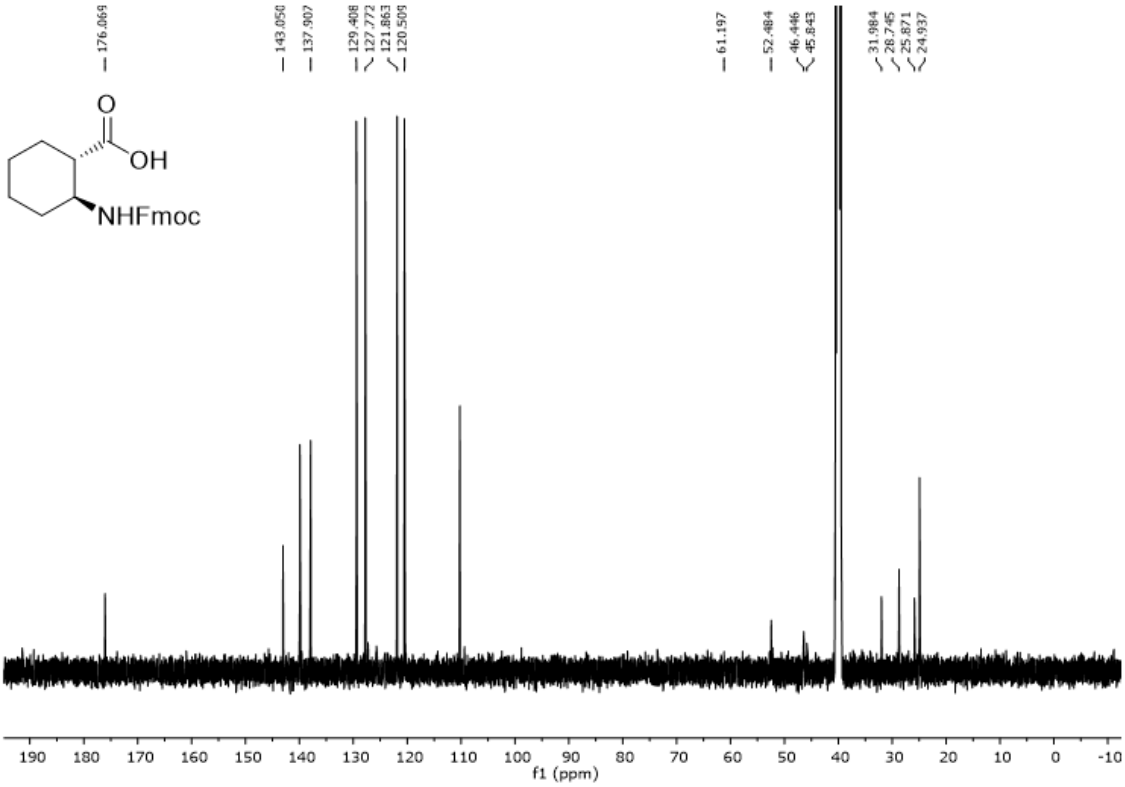
H NMR spectrum (400 MHz, 300 K, DMSO-d6) of **14**.



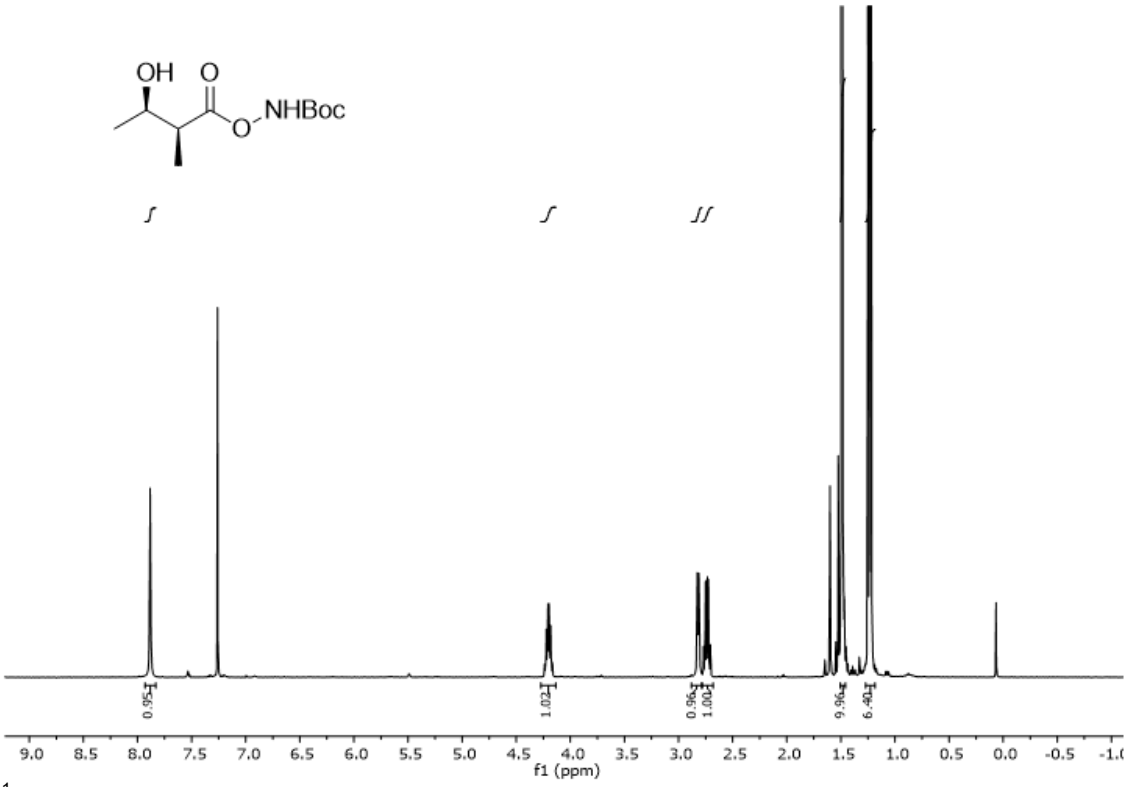
^{13}C NMR spectrum (101 MHz, 300 K, DMSO-d6) of **14**.



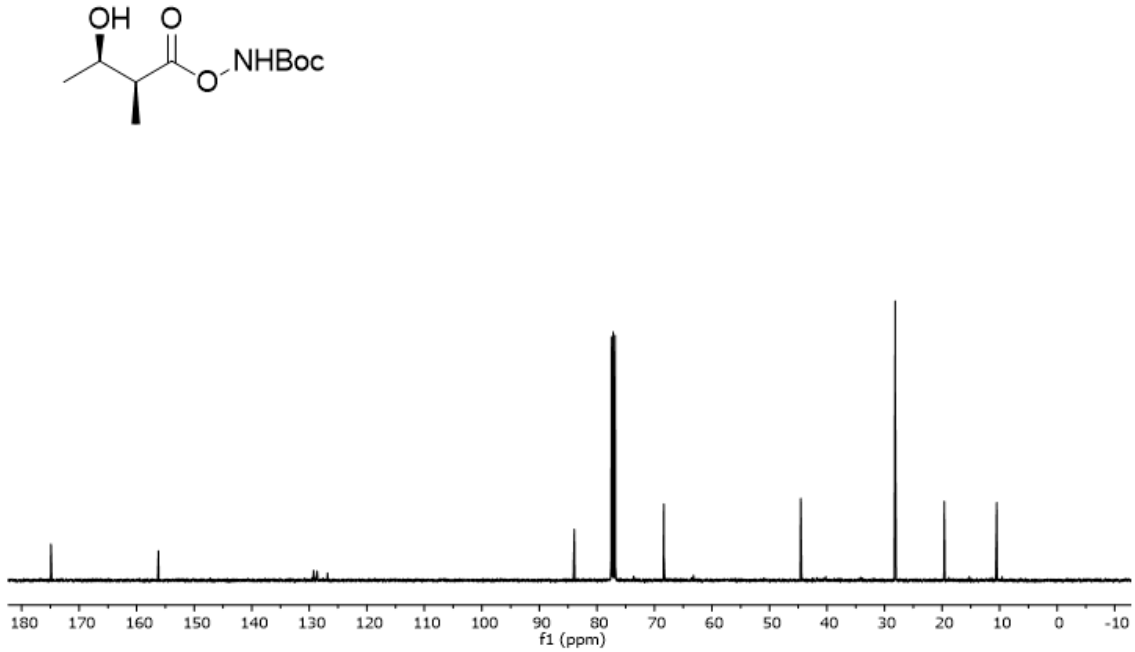
^{1}H NMR spectrum (400 MHz, 300 K, DMSO-d6) of **15**.



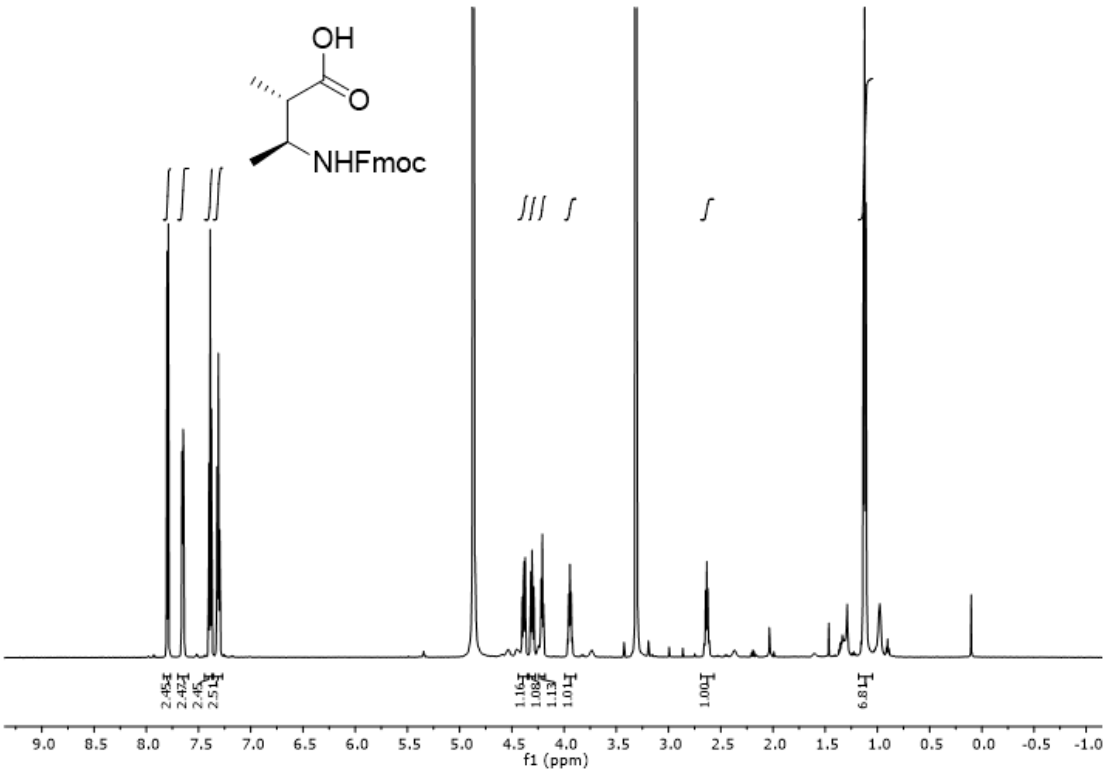
^{13}C NMR spectrum (101 MHz, 300 K, DMSO-d6) of **15**.



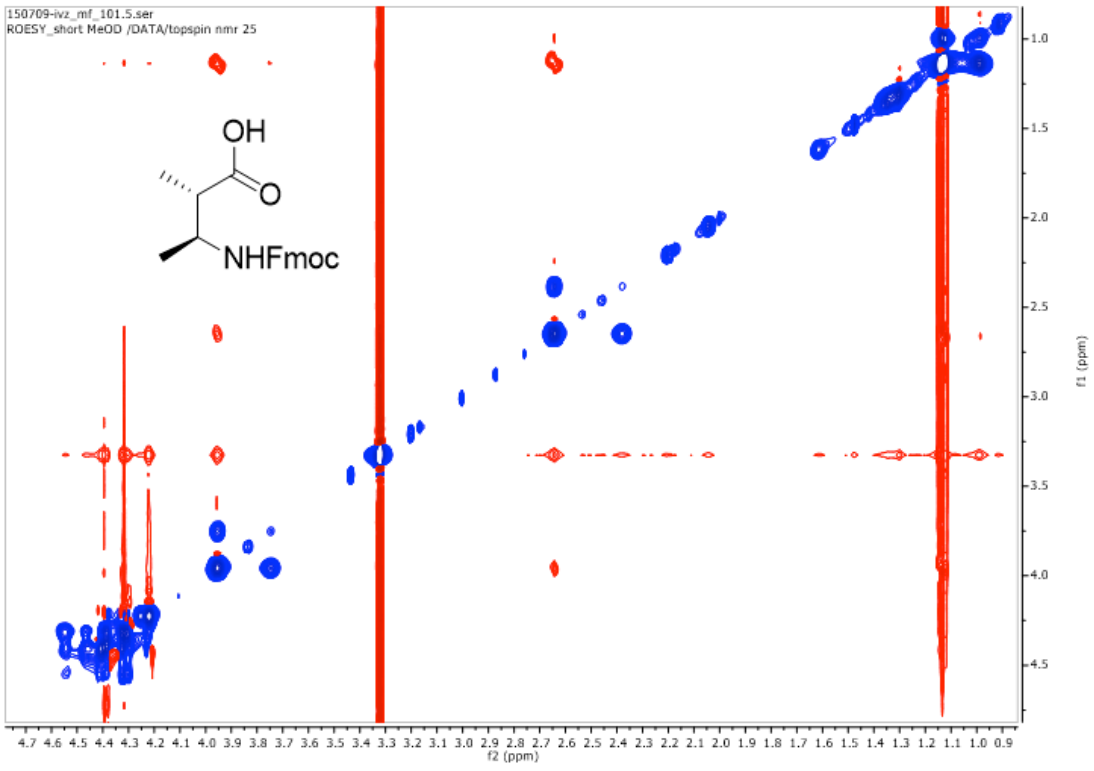
^{1}H NMR spectrum (400 MHz, 300 K, $CDCl_3$) of **8.**



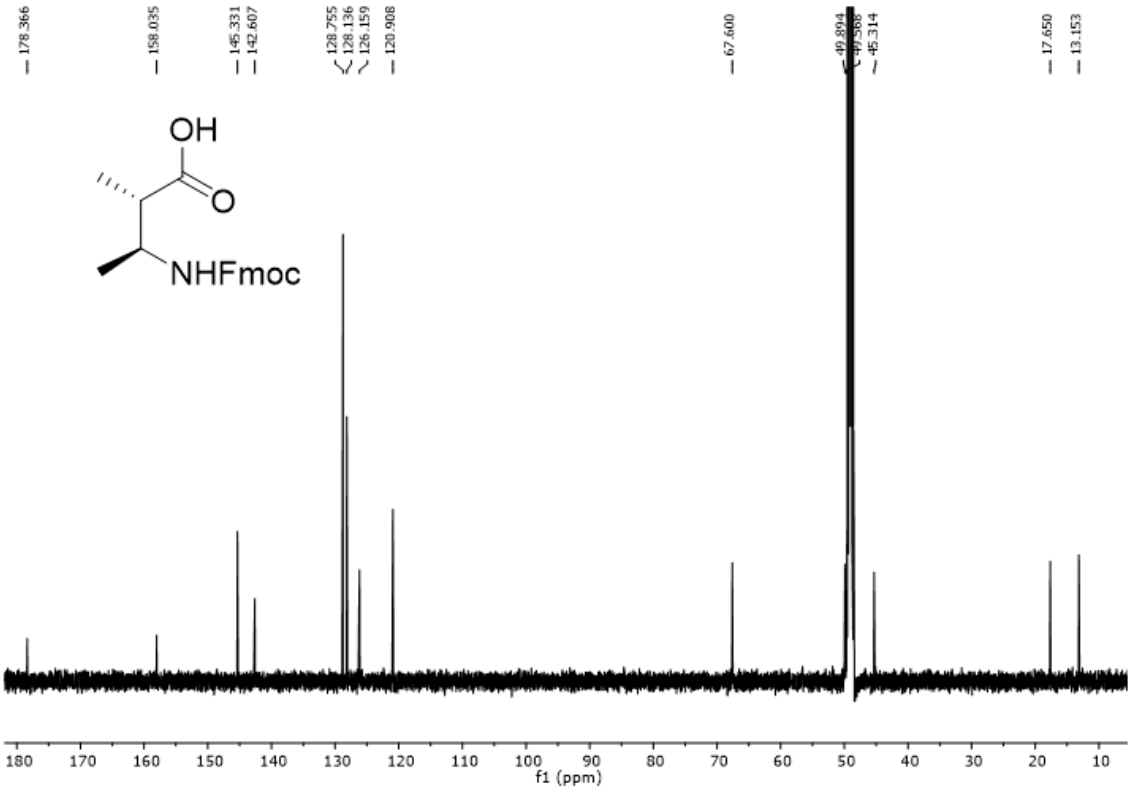
^{13}C NMR spectrum (101 MHz, 300 K, $CDCl_3$) of **18**.



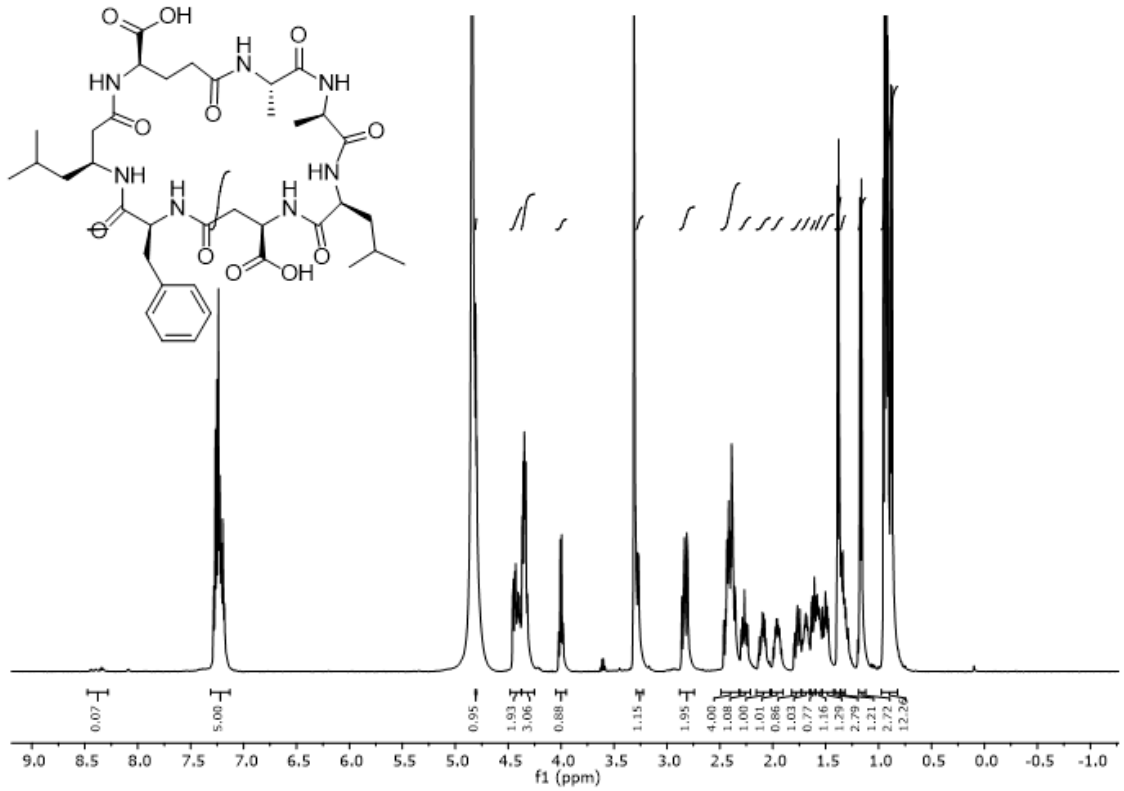
^{1}H NMR spectrum (600 MHz, 300 K, CD_3OD) of Fmoc-Amba-OH (**20**). The set of signals with lower intensity originates from a minor isomer due to *cis-trans* isomerization of the carbamate moiety, as was shown by observation of corresponding exchange signals in ROESY spectra.



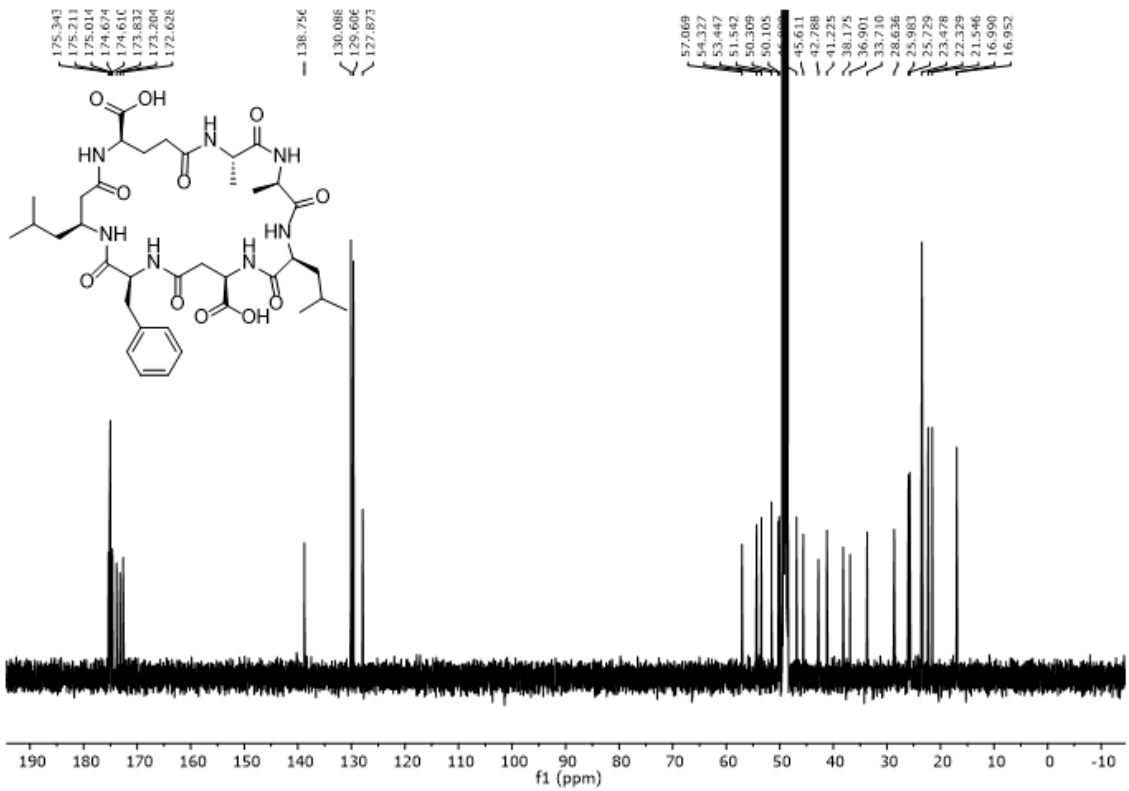
ROESY spectrum of Fmoc-Amba-OH (**20**) (600 MHz, 300 K, CD_3OD) Blue cross peaks (with the same sign as the diagonal) indicate chemical exchange whereas red cross peaks originate from dipolar cross relaxation (spatial vicinity).



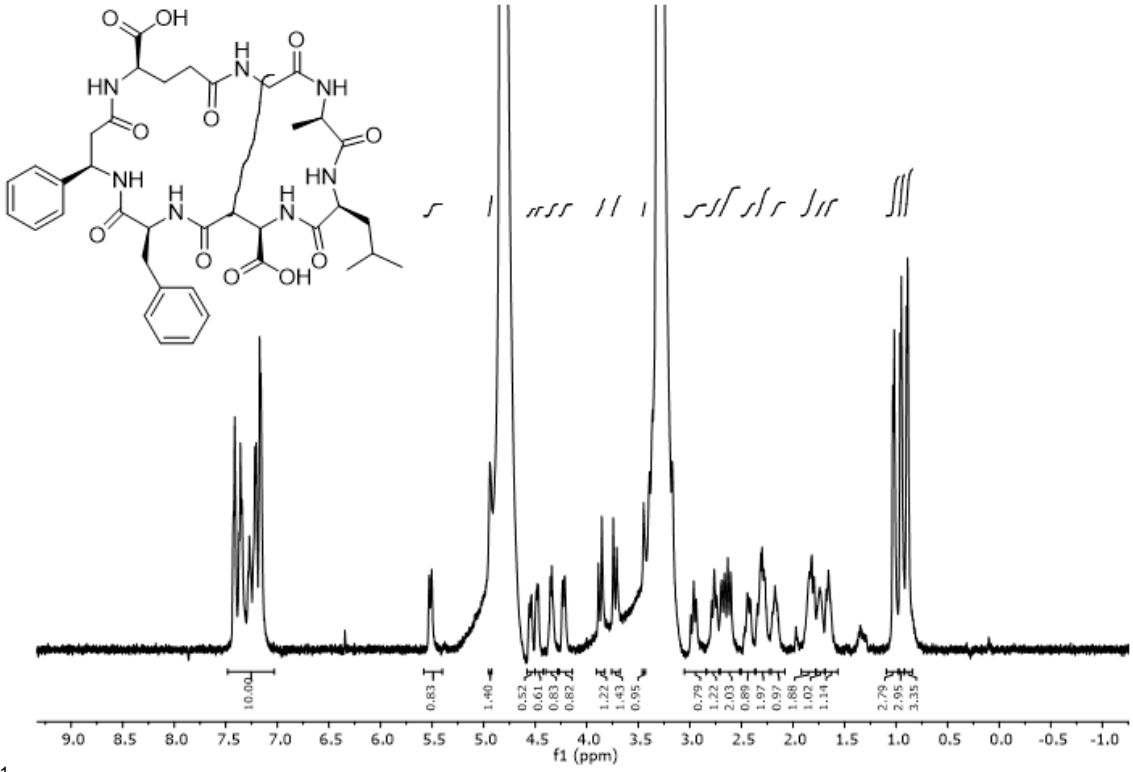
^{13}C NMR spectrum (151 MHz, 300 K, CD_3OD) of Fmoc-Amba-OH (**20**).



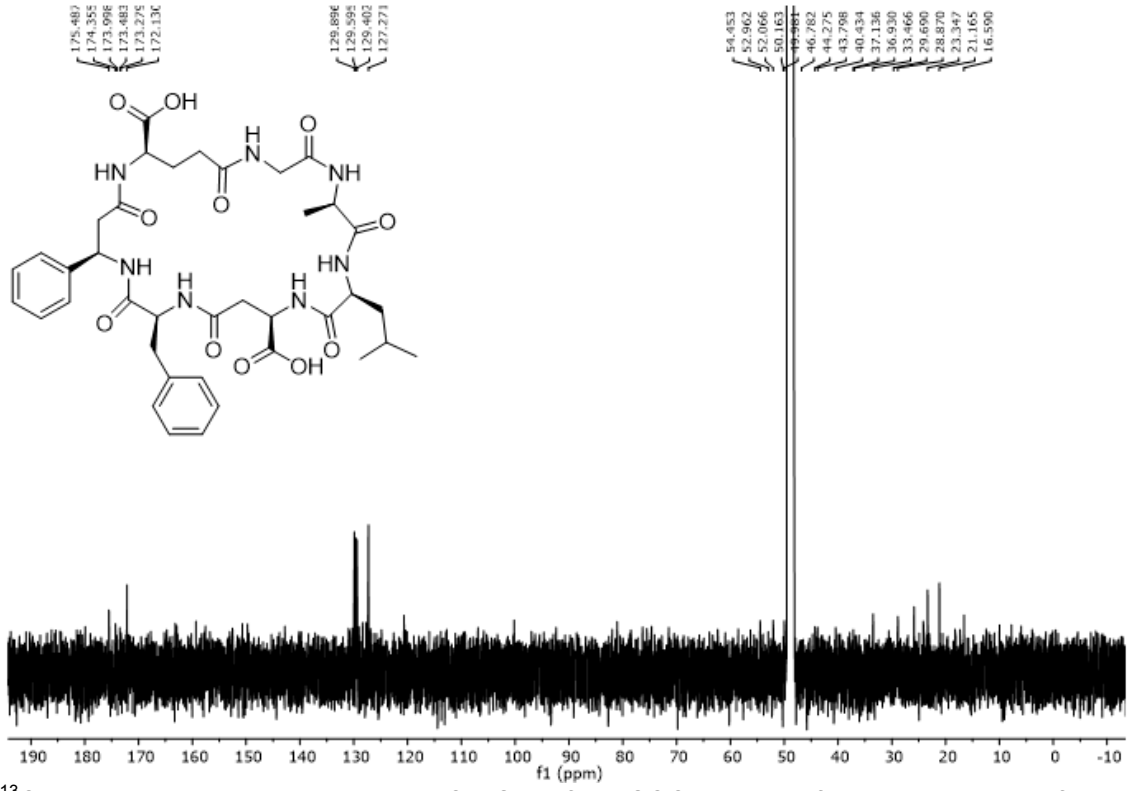
^{1}H NMR spectrum (500 MHz, 300 K, CD_3OD) of **2**.



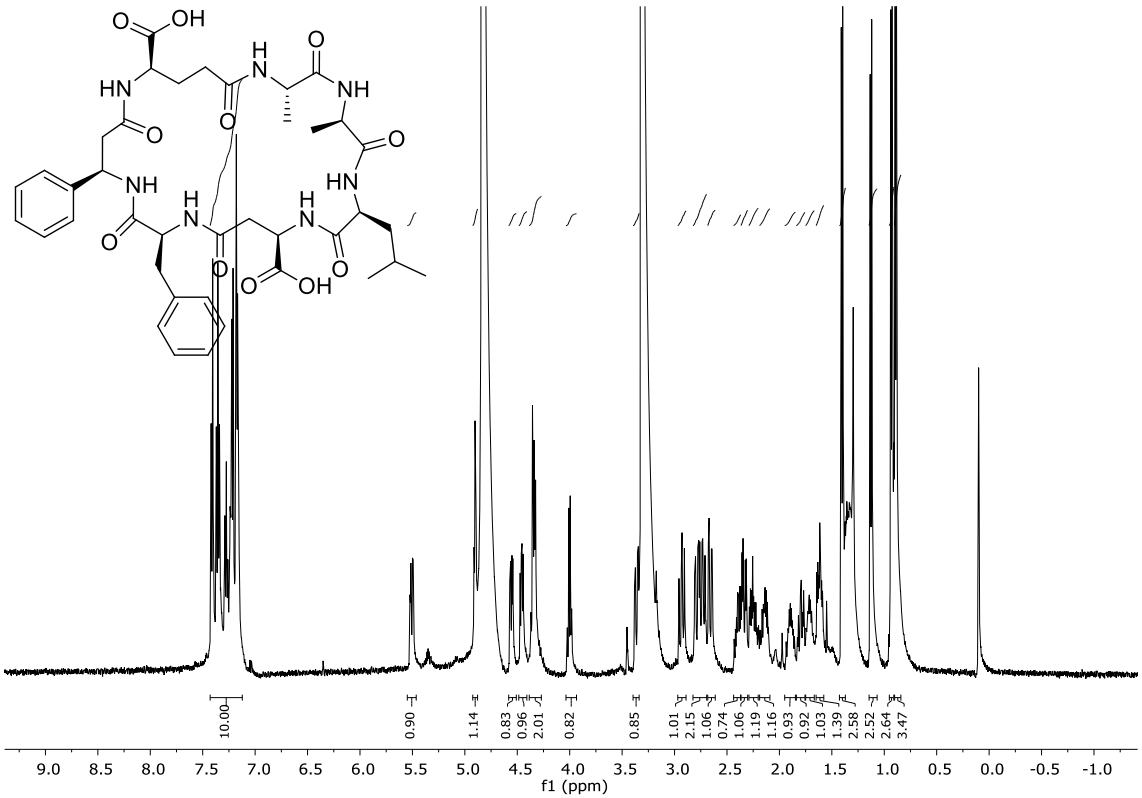
^{13}C NMR spectrum (125 MHz, 300 K, CD_3OD) of **2**.



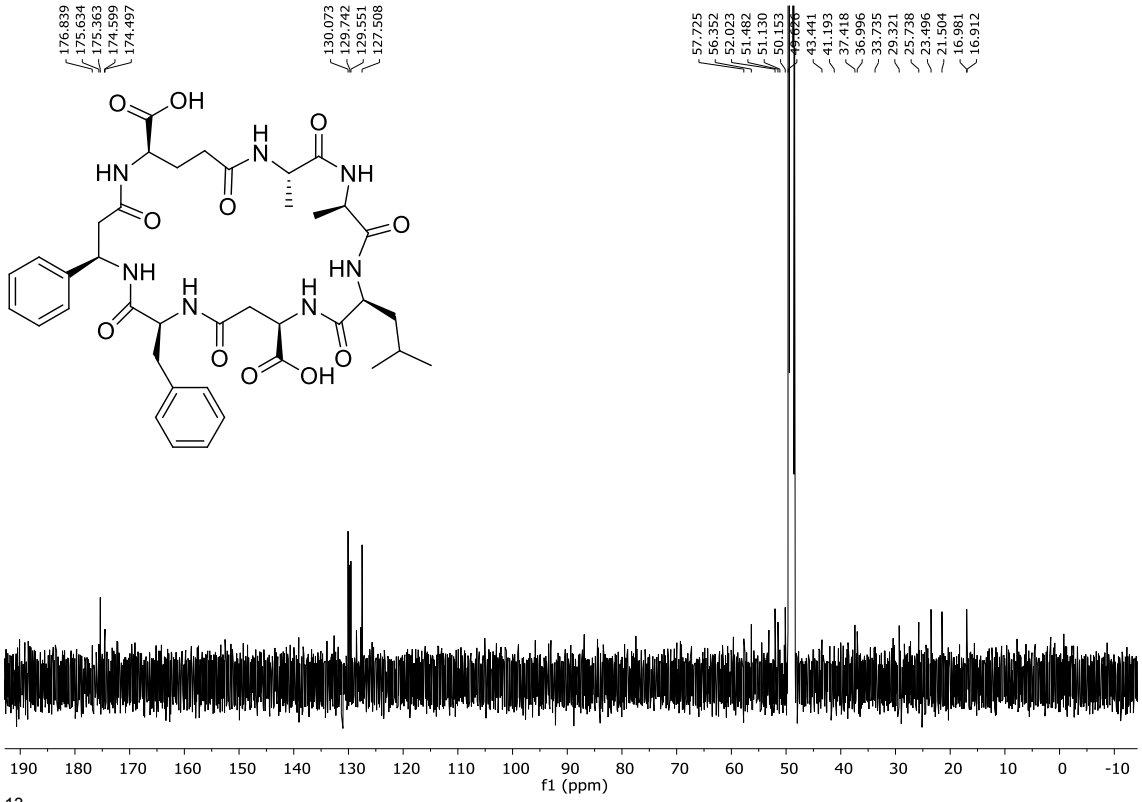
^{1}H NMR spectrum (500 MHz, 300 K, CD_3OD) of **3**.



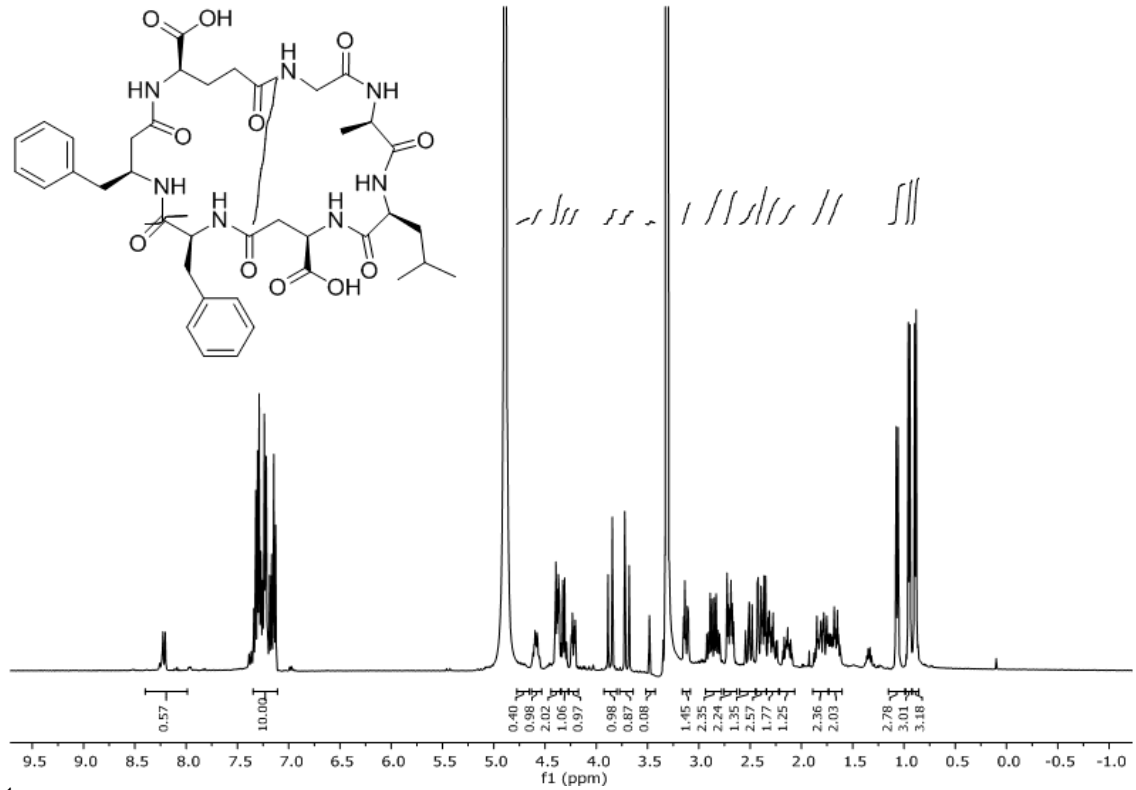
^{13}C NMR spectrum (125 MHz, 300 K, CD_3OD) of **3**. HSQC was used for the assignment of the signals.



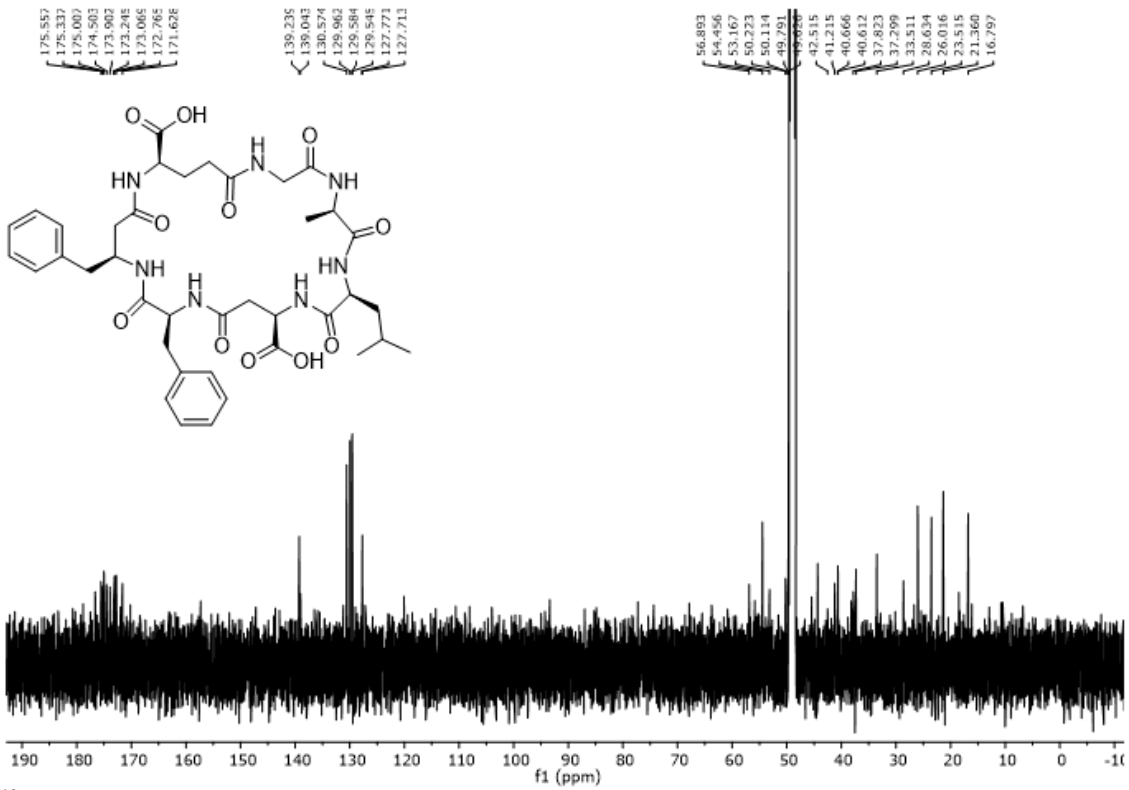
^{1}H NMR spectrum (500 MHz, 300 K, CD_3OD) of **4**.



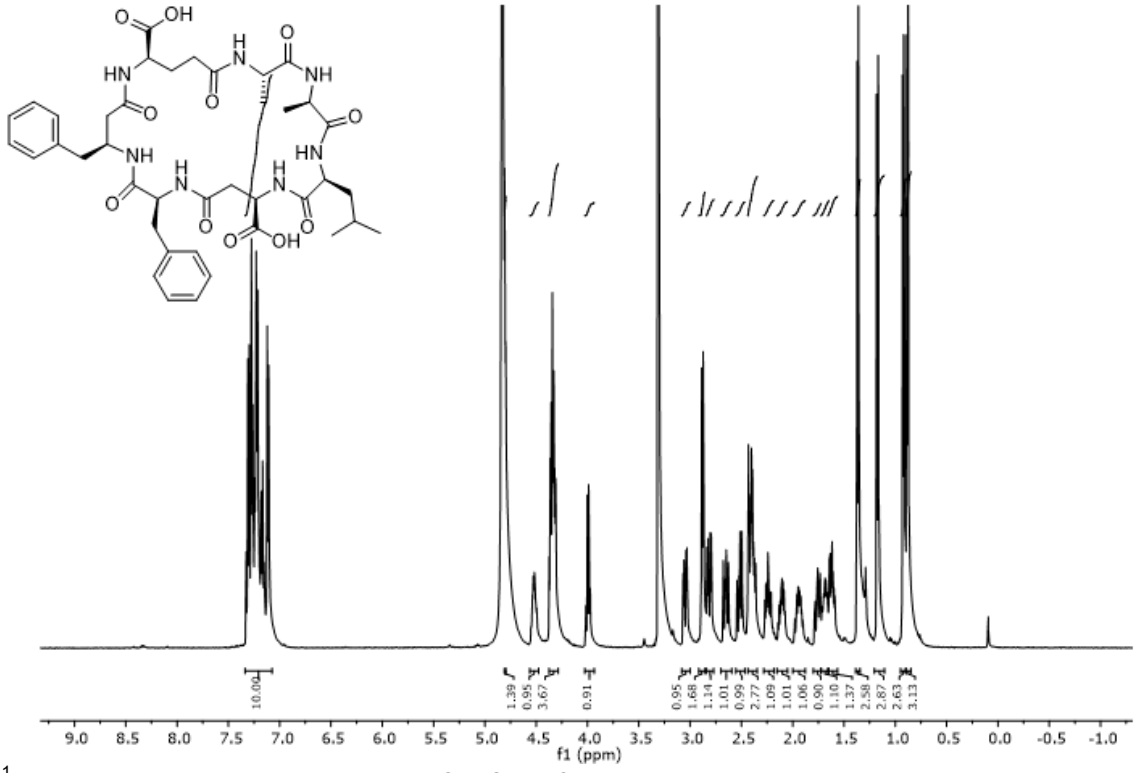
^{13}C NMR spectrum (125 MHz, 300 K, CD_3OD) of **4**. HSQC was used for the assignment of the signals.



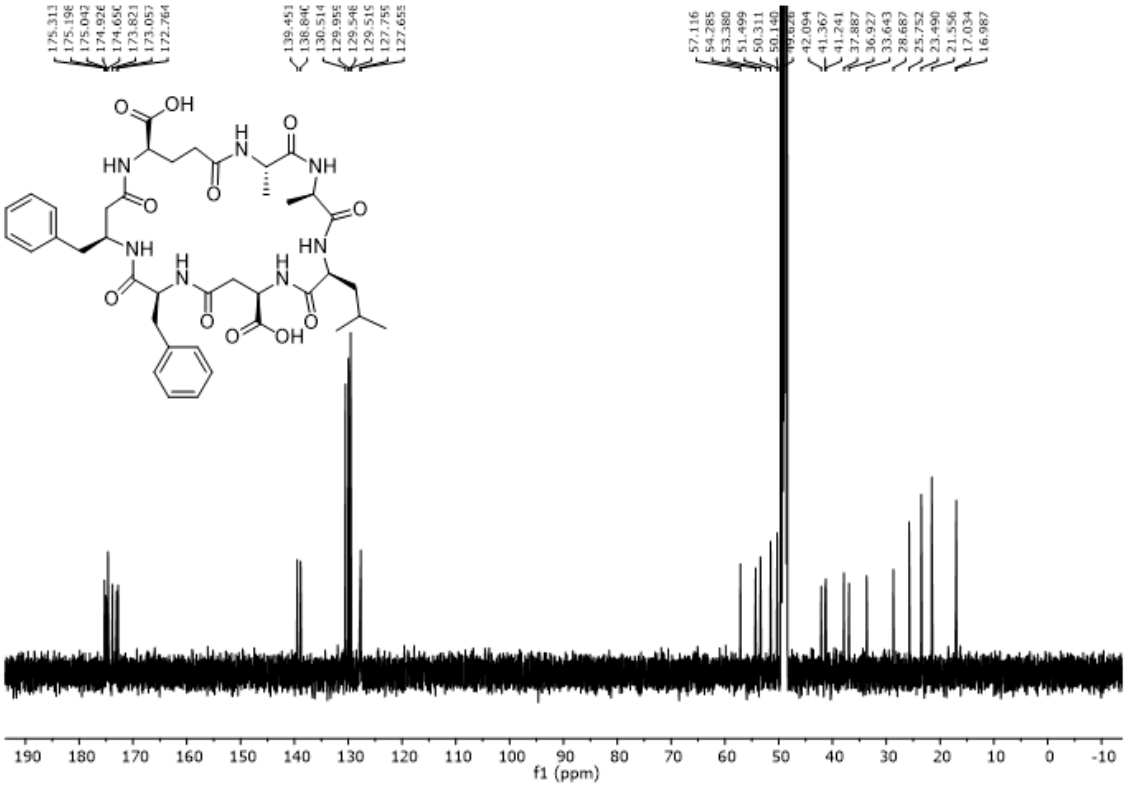
^{1}H NMR spectrum (400 MHz, 300 K, CD_3OD) of **5**.



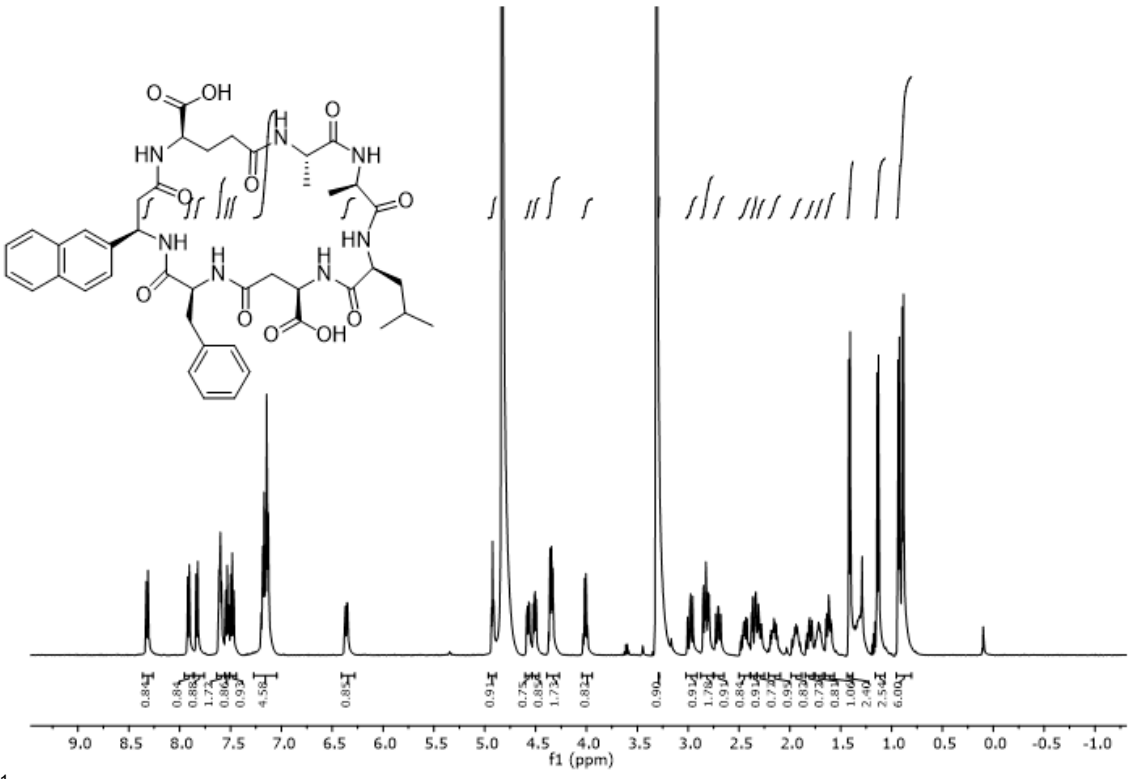
^{13}C NMR spectrum (101 MHz, 300 K, CD_3OD) of **5**.



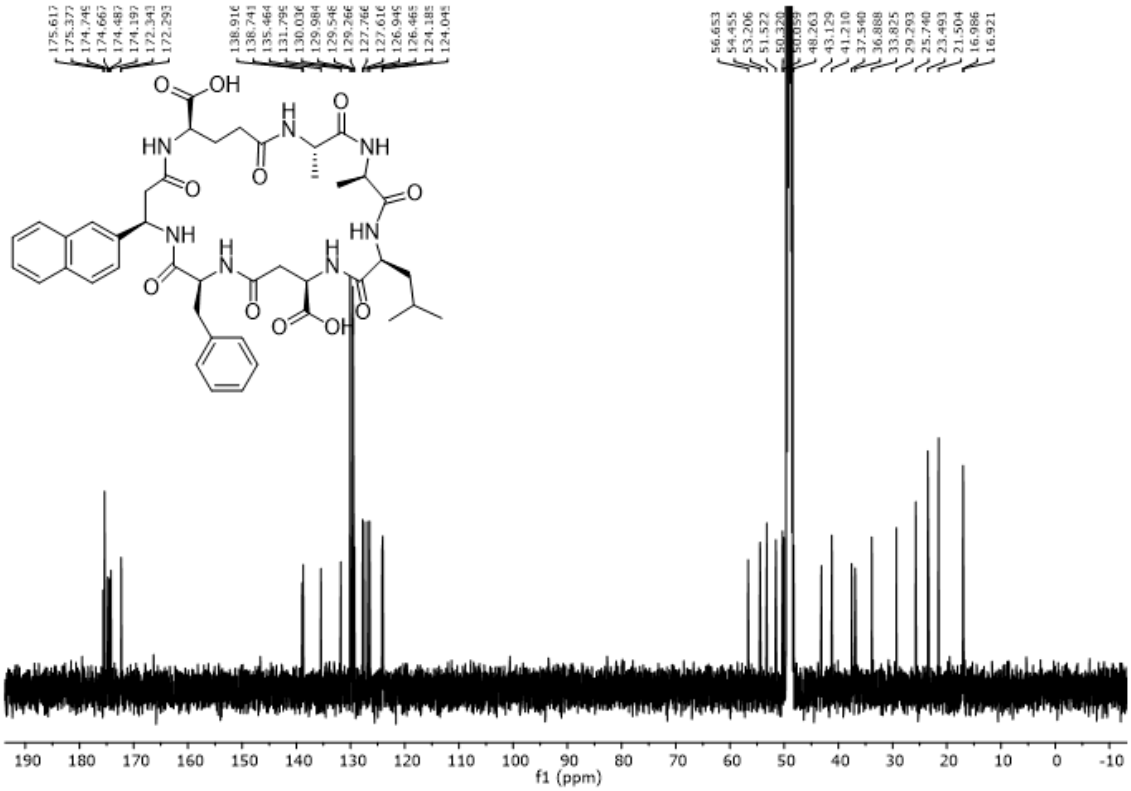
^{1}H NMR spectrum (500 MHz, 300 K, CD_3OD) of **6**.



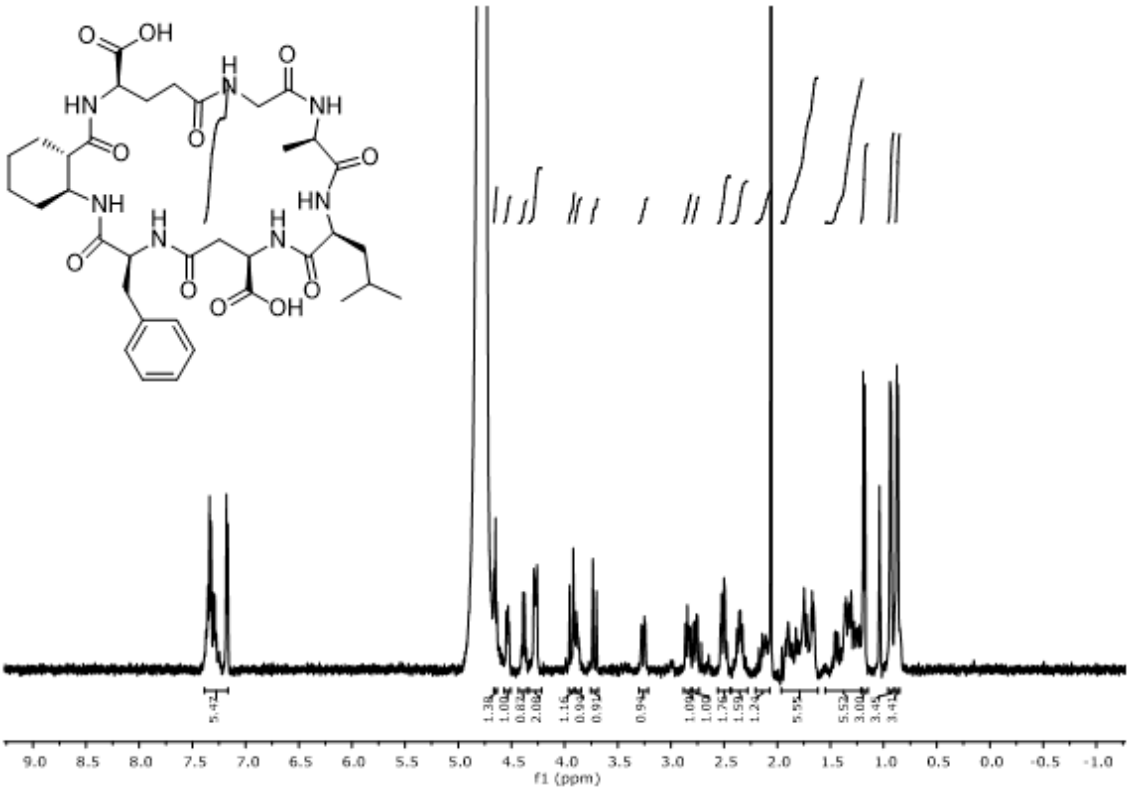
^{13}C NMR spectrum (125 MHz, 300 K, CD_3OD) of **6**.



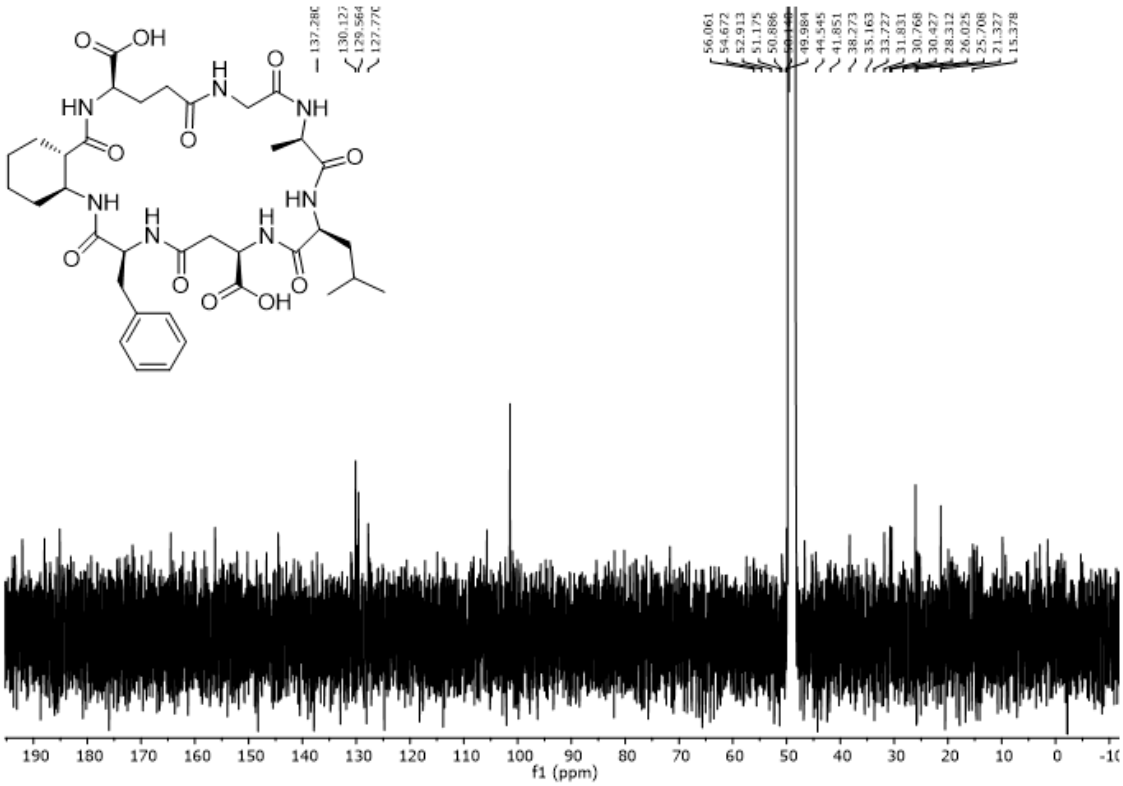
^{1}H NMR spectrum (500 MHz, 300 K, CD_3OD) of **7**.



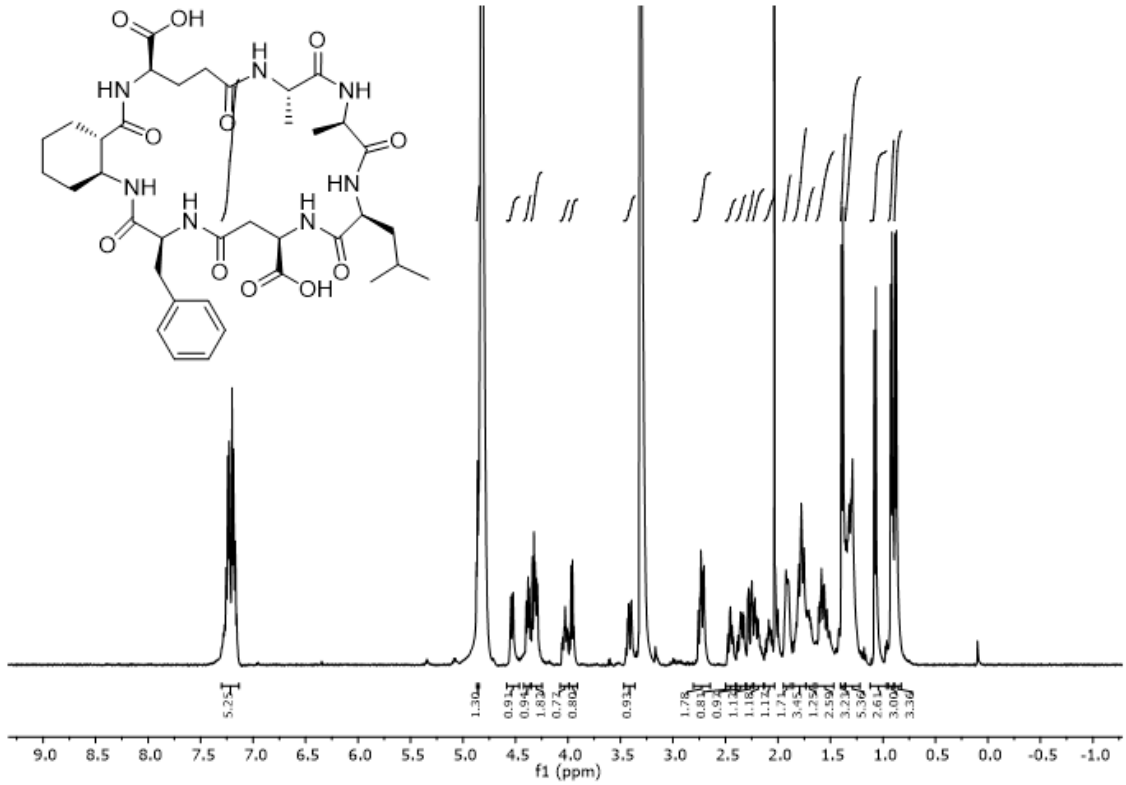
^{13}C NMR spectrum (125 MHz, 300 K, CD_3OD) of **7**.



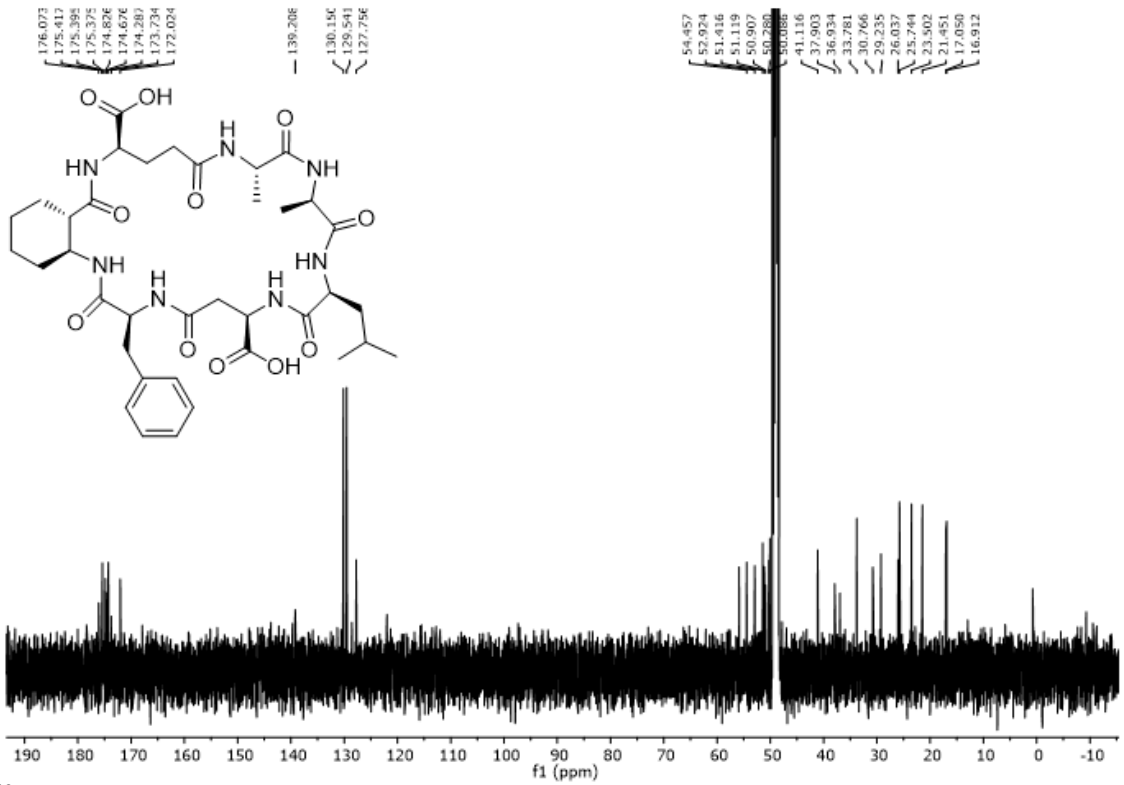
^{1}H NMR spectrum (500 MHz, 300 K, D_2O) of **8**.



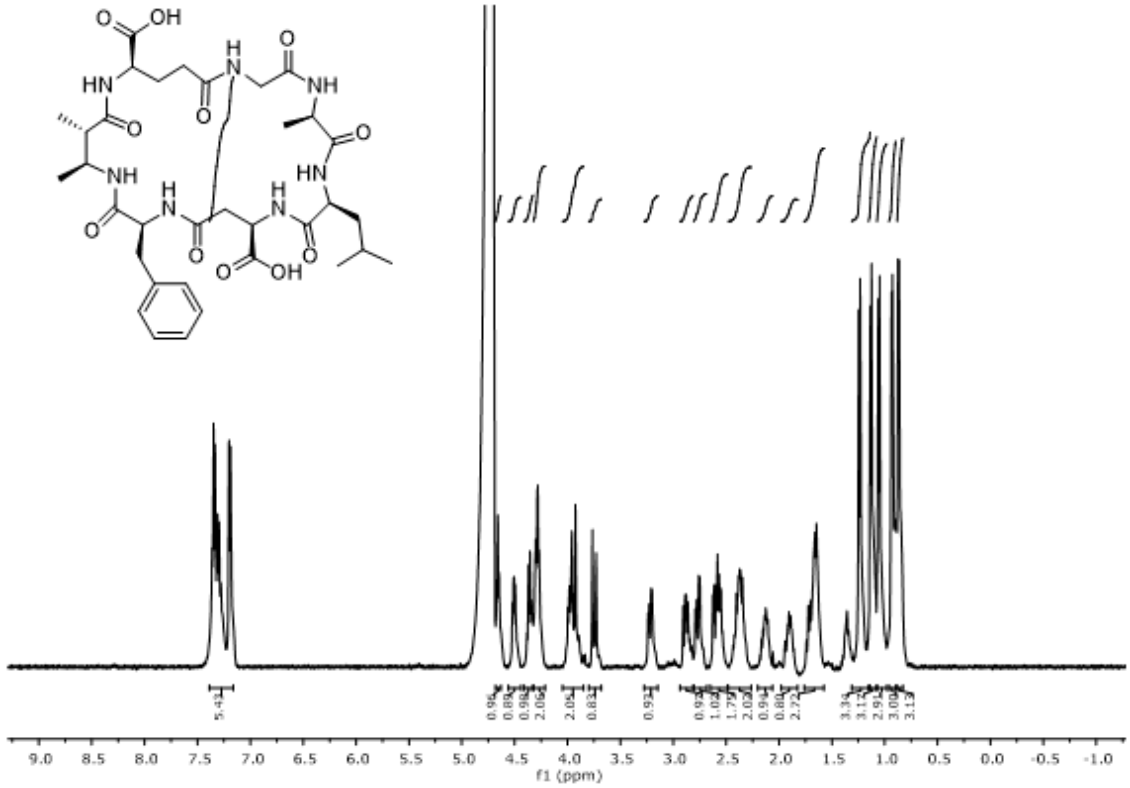
^{13}C NMR spectrum (101 MHz, 300 K, CD_3OD) of **8**. HSQC was used for the assignment of the signals.



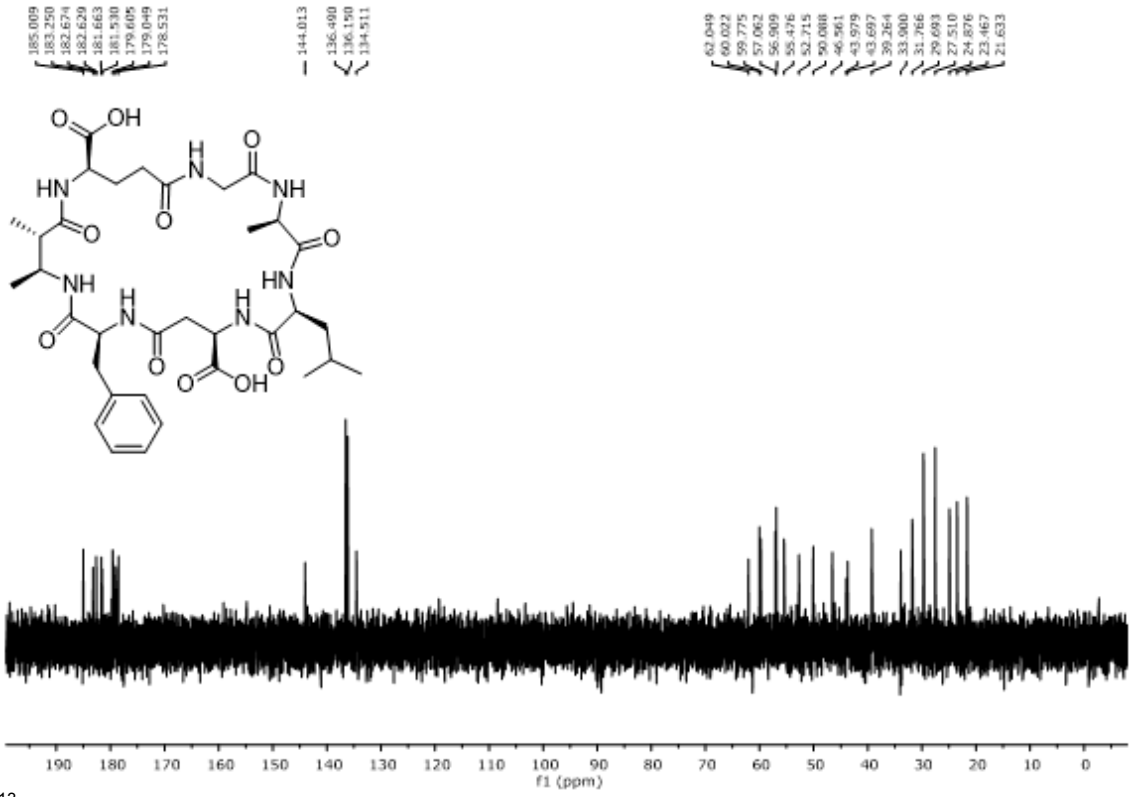
^{1}H NMR spectrum (500 MHz, 300 K, CD_3OD) of **9**.



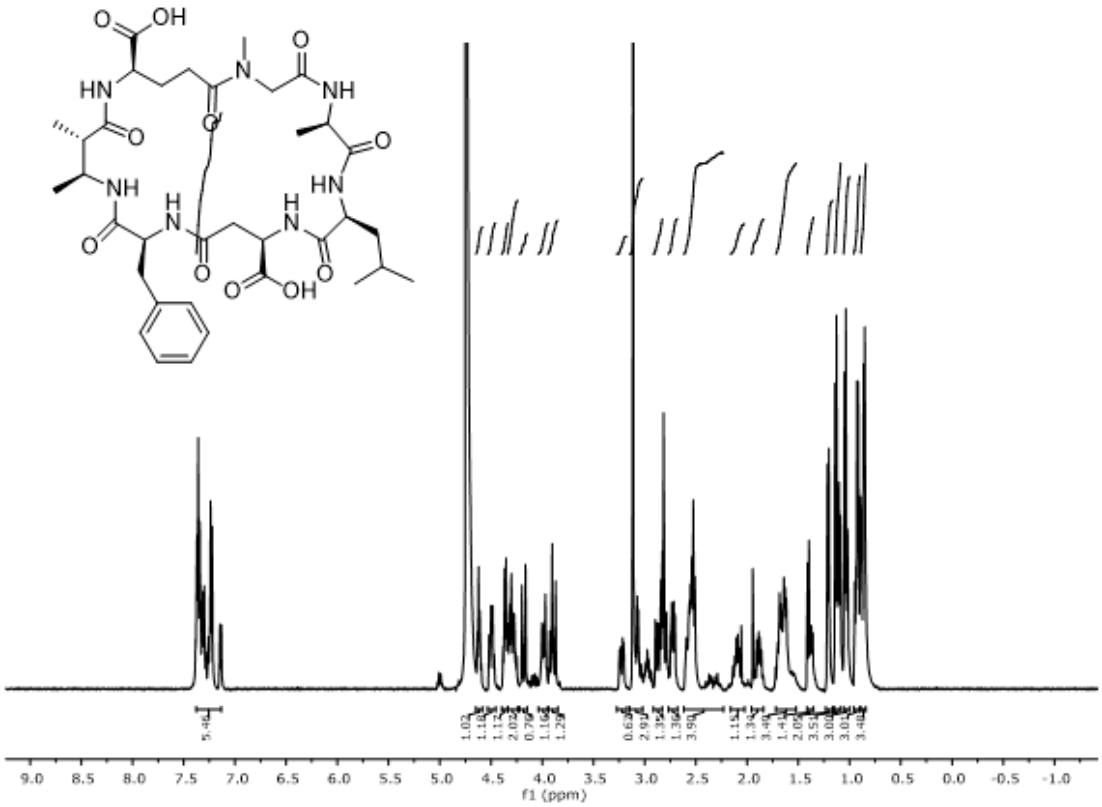
^{13}C NMR spectrum (125 MHz, 300 K, CD_3OD) of **9**.



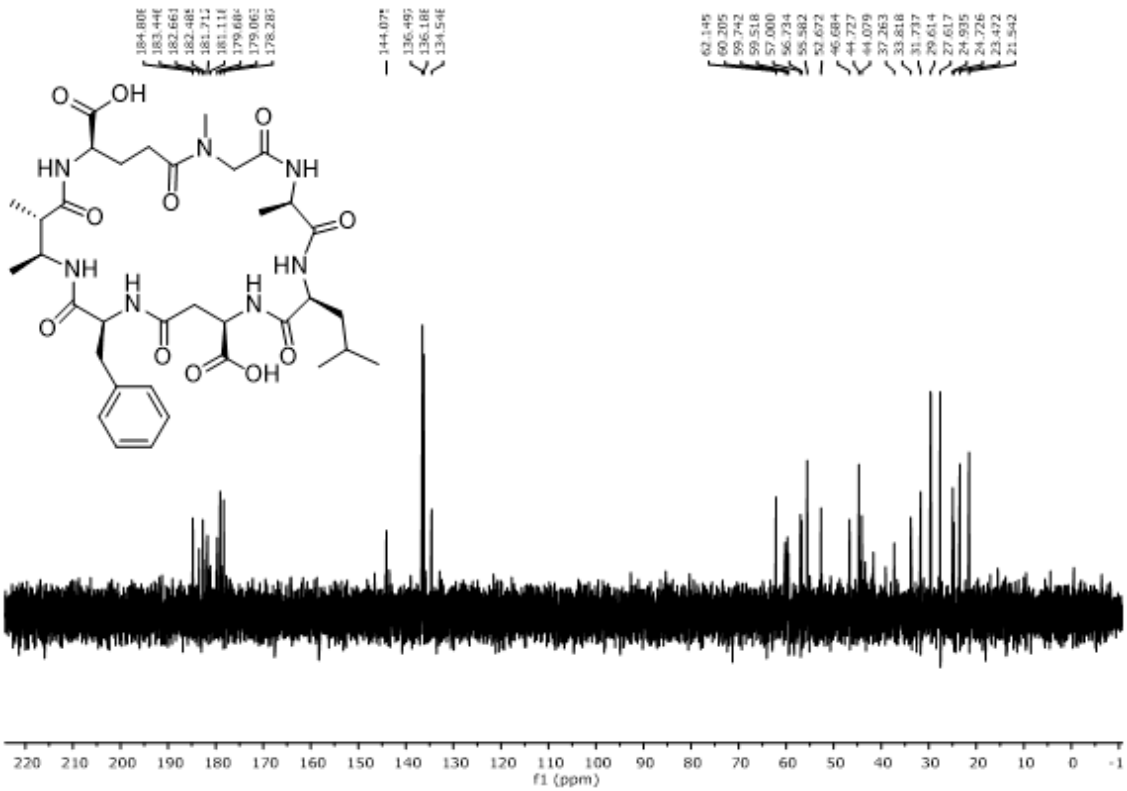
^{1}H NMR spectrum (500 MHz, 300 K, D_2O) of **10**.



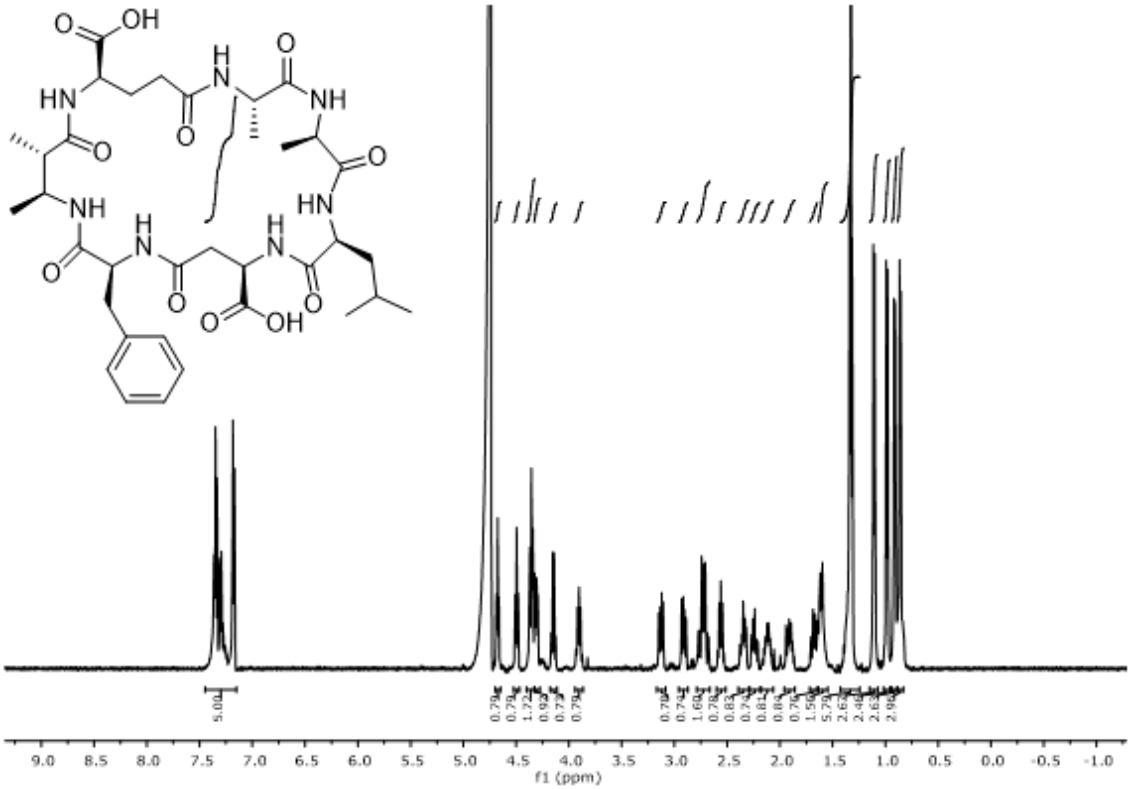
^{13}C NMR spectrum (125 MHz, 300 K, D_2O) of **10**.



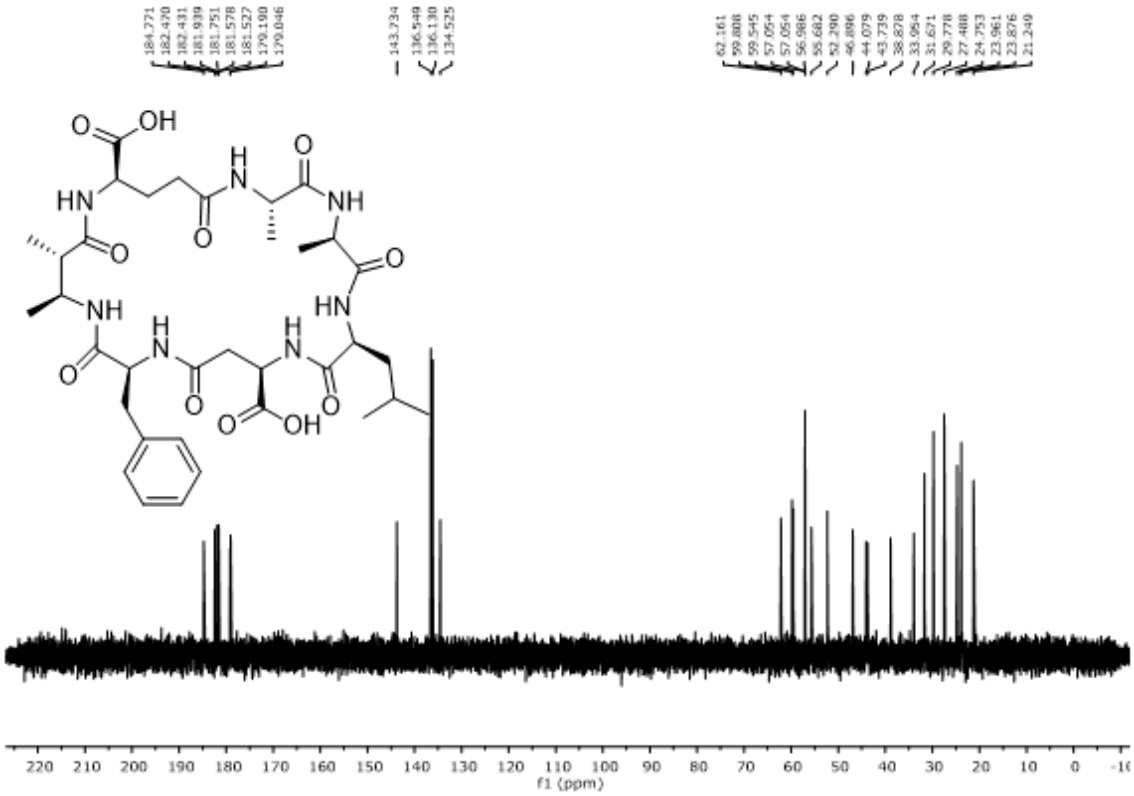
^{1}H NMR spectrum (500 MHz, 300 K, D_2O) of **11**.



^{13}C NMR spectrum (125 MHz, 300 K, D_2O) of **11**.



^{1}H NMR spectrum (500 MHz, 300 K, D_2O) of **12**.



^{13}C NMR spectrum (125 MHz, 300 K, D_2O) of **12**.

8. Solution structure determination

Compounds **10** and **3** were lyophilized and dissolved in H_2O with 5% D_2O with a final pH of 3.3 and 4 respectively and a concentration of 1.5 mM and 0.15 mM (due to the limited solubility of the compound in H_2O), respectively. NMR spectra were recorded on Bruker Avance III spectrometers operating at 700 and 800 MHz. For the assignment ^{1}H-^{13}C HSQC, ^{1}H-^{1}H TOCSY (mixing time 70ms) and NOESY (mixing time 150ms) where recorded at a temperature of 274K. ^3J $H_NH\alpha$ coupling constants (Table S1) where extracted from J-resolved spectra recorded between 274K and 308K, fitting the peak positions using the nLinLS routine of NMRPipe.[S6] For structure generation, the couplings where converted into dihedral angle ranges using the Karplus relations ^3J $H_NH\alpha$ = 6.41*cos^2(ϕ-60º) -1.38*cos(ϕ-60º)+1.72.[S7]. Couplings were converted to ϕ angle ranges adding an error of +/- 0.5 Hz and used only if the resulting angles were in the negative ϕ range (e.g. for couplings > 7.9Hz). Dihedral angles where used with assigned NOESY peak intensities to calculate structures with Aria1.2/CNS1.2[S8] employing a log-harmonic shape for distance restraint potential.[S9]

The structure calculation protocol was modified to include active randomization of all dihedral angles in the peptide ring. A Cartesian dynamics simulated annealing protocol was used, with a high-temperature and two cooling protocols with 120000/100000 and 80000 MD steps of 0.003 fs respectively calculating 20 structures in iterations 0-7 and 100 structures in iteration 8. The pool of unrestrained structures was generated with the same protocol, removing all experimental restraints and stacking dihedral energy terms and generating 500 structures in iteration 8.

NMR structures (Fig. S2 A) of compound **10** are based on 78 NOEs (47 intraresidue, 29 sequential and 2 non-adjecent) and four dihedral angle restraints (residues Leu-Asp-Phe-Amba). NOE distances were automatically calibrated within Aria1.2 from the peak intensities

of the NOESY spectrum. ϕ dihedral angle restraints were defined at the center of the possible ϕ range derived from the Karplus curve for the measured value +/- 0.5 Hz. Due to the lower concentration of compound **3** we could observe 27 strong NOEs only, which together with the coupling constants were insufficient to determine a converging ensemble of structures.

Table S1. Measured coupling constants and temperature dependence of the chemical shift values of the amide protons for compound 10 and 3 in comparison to the published values of MC-LF. [S10, S11]

* Values are the observed couplings without adjustment for electronegativity, e.g. the values of Table 7[S11] divided by 1.09.

	Compound **10**		Compound **3**		MC-LR		
	$^3J_{HNH_\alpha}$	$-\Delta\delta/\Delta T$	$^3J_{HNH_\alpha}$	$-\Delta\delta/\Delta T$	$^3J_{HNH_\alpha}$[S10]	$^3J_{HNH_\alpha}$[S11]*	$-\Delta\delta/\Delta T$[S11]
Ala	6.0	8.6	6.2	9.3	-	6.5	6.4
Leu	7.8	8.0	7.4	7.6	-	6.5	4.9
(D)Asp	8.4	1.4	8.7	3.3	9.8	9.8	-0.1
Phe	8.3	5.4	-	6.1	9.5	9.7	8.8
Position 5	9.3	4.8	8.7	7.6	9.6	9.4	1.7
(D)Glu	7.2	6.8	7.7	9.1	5.8	3.7	3.7
Gly	-	7.0	-	6.1	-	-	-

9. In vitro PP1 and PP2A inhibition assays

Recombinant PP1α catalytic subunit was purified by the Protein Expression and Purification Core Facility at EMBL, Heidelberg, according to a published protocol.[S12] The PP1α single and double mutant (E275R and E275R F276C, respectively) were prepared by site-directed mutagenesis using as template the gene coding for wild-type PP1α full length (1-330) with an N-terminal cleavable histag. After amplification of the custom design mutagenic primers by polymerase chain reaction, the template DNA was removed by treatment with *Dpn*I. The mutated gene was sequenced by GATC Biotech. The variant enzyme was expressed in *E. coli* and purified with a modified version of the published protocol[S12] by adding a C-terminal cleavable intein tag to increase expression levels (expression vector pTXB1) and substituting the TALON beads with a HisTrap™ HP nickel column. During the overnight incubation at 4 ℃ with TEV protease betamercaptoethanol was added in order to cleave the C-terminal intein tag. The cleaved intein tag was removed by incubation with chitin resin from NEB. The cleavage of the intein tag does not leave any extra amino acid at the C terminus. The purified variant enzymes were more than 90% pure from SDS-PAGE.
Human recombinant PP2A C subunit with L309 deletion was purchased from Biomol GmbH. Assays were carried out using 25 pM of protein in a buffer at pH 7.4 containing 50 mM imidazole, 10 mM NaCl, 2 mM DTT, 1 mM $MnCl_2$ and 0.05 % Triton. Assays were performed in 96 well plates and all inhibitors were dissolved in water or mixtures of water and DMSO and then diluted in assay buffers. Inhibitors were preincubated with the protein for 10 minutes at room temperature and assays were initiated by adding a fluorescence substrate 6,8-difluoro-4-methylumbelliferyl phosphate (DIFMUP) with a concentration in the Km of the protein (50 µM for PP1, 26 µM for the single variant PP1α E275R, 31 µM for the double variant PP1α E275R F276C and 35 µM for PP2A). Fluorescence of the phosphatase mediated hydrolysis product, 6,8-difluoro-4-methylumbelliferone (DIFMU) was measured at 25 °C every 15 seconds during 30 minutes measuring in the initial linear part of the slopes.

Experiments were done in triplicate and two or three independent experiments were conducted. Initially, the activity of both PP1 and PP2A was evaluated in presence of compounds **2**-**7** at a concentration of 250 μM and natural MC-LF at 1 nM (Table S2). Half maximal inhibitory concentration (IC_{50}) was measured for MC-LF and the most active compounds for both PP1 and PP2A (Table 1). Additionally, the activity of PP1, PP1 E275R F276C and PP2A was evaluated in presence of compounds **8**-**12** at a concentration of 250 μM (Table S3). Half maximal inhibitory concentration (IC_{50}) was measured for MC-LF and the most active compounds for both PP1 and PP2A (Table 1). The IC_{50} for MC-LF and compound **11** was also evaluated for both PP1 E275R and PP1 E275R F276C (Figure S30 and S31 respectively).

Table S2. Percentage of activity of PP1 and PP2A using 250 μM of compounds 1-7.

	PP1	PP2A
Compound	% activity	% activity
1	0.2 ± 0.1	0.7 ± 0.3
2	96.3 ± 4.3	37.5 ± 2.7
3	96.3 ± 2.9	11.9 ± 1.5
4	87.4 ± 2.4	16.7 ± 2.2
5	107.9 ± 2.8	24.3 ± 1.4
6	98.9 ± 2.1	49.6 ± 3.6
7	103.9 ± 1.7	77.3 ± 2.2

Table S3. Percentage of activity of PP1, PP1 (E275R F276C) and PP2A using 250 μM of compounds 8-12.

	PP1	PP1 (E275RF276C)	PP2A
Compound	% activity	% activity	% activity
8	47.3 ± 4.9	29.8±1.0	2.5±1.8
9	70.9 ± 2.3	59.1±0.4	3.6±0.5
10	35.6 ± 0.4	8.1±0.8	2.6±0.4
11	57.5 ± 7.1	6.1±1.2	1.8±0.1
12	42.4 ± 1.2	12.0±1.2	1.4±1.9

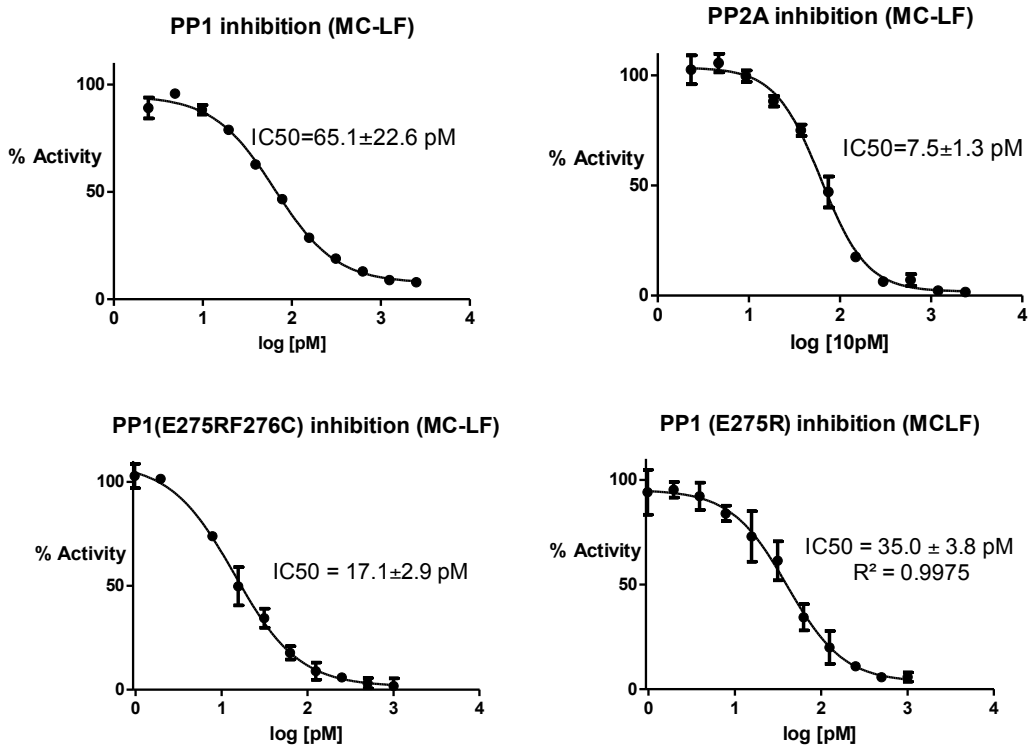


Figure S30. Percentage of phosphatase activity (PP1 top left, PP2A top right, PP1(E275R F276C) bottom left, PP1(E275R) bottom right vs. logarithm of the concentration of MC-LF.

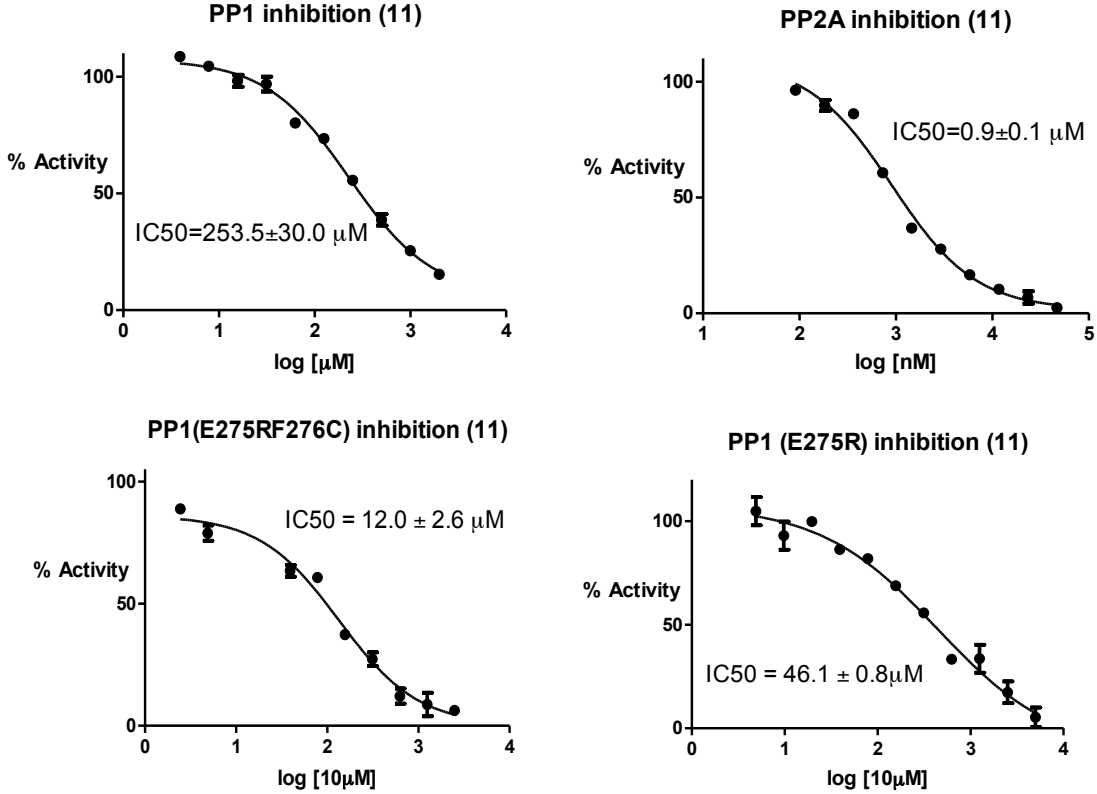


Figure S31. Percentage of phosphatase activity (PP1 top left, PP2A top right, PP1(E275R F276C) bottom left, PP1(E275R) bottom right vs logarithm of the concentration of **11**.

10. Computational methods

Pdb codes used for PP1 visualization are 3HVQ, 2BDX, 2BCD and 1FJM, and for PP2A 4I5L, 2IE3 and 3FGA.

The potential of additional interactions between a MC-analog and PP1 or PP2A proteins was analyzed by merging MC-ligands into the active site of diverse crystal structures of these phosphatases. MC-LR from 3FGA merged into the active site of 4I5L demonstrates that a hydrogen bond can be formed between Arg268 of PP1 and the carbonyl-group of position 7 of MC-LR.

Ligand preparations and alignments were done with the software package SYBYL-X 1.3 (SYBYL-X 1.3, Tripos, 1699 South Hanley Rd., St. Louis, Missouri, 63144, USA).

Images were created using the software Benchware® 3D Explorer 2.7 (Benchware® 3D Explorer, Tripos, 1699 South Hanley Rd., St. Louis, Missouri, 63144, USA).

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