

## **Supplementary Material**

# **Studies Toward the Total Synthesis and Stereochemical Assignment of Microspinosamide**

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## General Methods

Optical rotations ( $[\alpha]_D^{22.5}$ ) were obtained using a Perkin Elmer model 341 polarimeter at 589 nm (sodium D line) with a cell path length of 2 cm, using the indicated spectroscopic grade solvent and concentration (g/100 mL).

Nuclear magnetic resonance (NMR) spectroscopy was conducted at 300 K (unless stated otherwise) using a Bruker Avance DPX 500 spectrometer, a Bruker Avance DPX 400 spectrometer, a Bruker Avance DPX 300 spectrometer or a Bruker Avance DPX 200 spectrometer. Proton NMR ( $^1\text{H NMR}$ ) spectroscopy was conducted at the indicated frequency, in the deuterated NMR solvent as specified. Chemical shifts ( $\delta_{\text{H}}$ ) are reported as parts per million (ppm) downfield from trimethylsilane (TMS), using residual NMR solvent peaks as internal references in the absence of internal TMS. Multiplicity (br = broad, s = singlet, d = doublet, q = quartet, m = multiplet), coupling constants ( $J_{\text{HH}}$ ), relative integral (nH) and structural assignments are reported where possible. Carbon-13 NMR ( $^{13}\text{C NMR}$ ) was conducted at the indicated frequency in deuterated NMR solvent as specified. Chemical shifts ( $\delta_{\text{C}}$ ) are reported as parts per million (ppm) using residual NMR solvent peaks as internal references. Infrared (**IR**) absorption spectra were recorded on a Bruker Alpha-E FT-IR spectrometer using attenuated total reflection (ATR) of a thin film. Results were interpreted using OPUS 6.5 software and notable vibrational wavenumbers are reported ( $\nu_{\text{max}} / \text{cm}^{-1}$ ).

Low resolution mass spectrometry (**LRMS**) was carried out on a Shimadzu 2020 mass spectrometer using electrospray ionization (ESI in positive mode unless otherwise stated). High resolution mass spectrometry (**HRMS**) using ESI was obtained using a Bruker BioApex Fourier Transform Cyclotron Resonance mass spectrometer (FTICR, 7T). High-resolution MALDI MS experiments were conducted by MALDI-FTICR on a Bruker Apex Qe 7T Fourier Transform Ion Cyclotron Resonance Mass spectrometer. The instrument was externally mass calibrated and when required internally calibrated using PEG1500 via an ESI/MALDI APIII Dual source. Laser power was set to the minimum level possible for each sample (20-40%). The time of flight delay time was optimized for the expected mass and Q1 isolation set to allow ions  $> 500 m/z$  to the detector. All samples ( $\sim 1 \text{ mg/mL}$  in 7:3 (v/v)  $\text{CH}_3\text{CN}/\text{H}_2\text{O}$  both with 0.1% TFA) were prepared with  $\alpha$ -cyano-4-hydroxycinnamic acid matrix (10 mg/mL in 7:3 (v/v)  $\text{CH}_3\text{CN}/\text{H}_2\text{O}$  both with 0.1% TFA) in a ratio of 20:2 matrix:sample. A 1  $\mu\text{L}$  aliquot of this mixture was pipetted onto a 384 AnchorChip MTP plate and allowed to air dry prior to analysis.

**Analytical reverse-phase high performance liquid chromatography (HPLC)** was performed on a Waters System 2695 photodiode array detector and an Alliance series column heater set at 30  $^{\circ}\text{C}$ . A

Waters Sunfire 5  $\mu\text{m}$ , 2.1  $\times$  150 mm column (C18) was used unless otherwise indicated at a flow rate of 0.2 mL min<sup>-1</sup> using a mobile phase of 0.1% TFA in water (solvent A) and 0.1% TFA in acetonitrile (solvent B) in a linear gradient as indicated. Results were analysed with Waters Empower software and retention times ( $R_t$  / min) of pure compounds are reported.

Preparative and semi-preparative reverse-phase HPLC was performed using a Waters 600E Multisolvant Delivery System with a Rheodyne 7725i Injection Valve (4 mL loading loop) and Waters 500 pump with a Waters 490E programmable wavelength detector operating at 214, 230 or 254 nm. Preparative reverse-phase HPLC was performed using a Waters Sunfire C18 column (5  $\mu\text{m}$ , 19  $\times$  150 mm) at a flow rate of 7 mL min<sup>-1</sup>. Semi-preparative reverse-phase HPLC was performed using a Waters Sunfire C18 column (5  $\mu\text{m}$ , 10  $\times$  250 mm) at a flow rate of 4 mL min<sup>-1</sup>.

High performance liquid chromatography-mass spectrometry (HPLC-MS) was conducted on a Shimadzu LC-MS 2020 instrument consisting of a pump and a SPD-20A UV/Vis detector coupled to a Shimadzu 2020 mass spectrometer. Separation was performed on a Waters Sunfire C18 column (5  $\mu\text{m}$ , 2.1  $\times$  150 mm) operating at a flow rate of 0.2 mL min<sup>-1</sup> using a mobile phase of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B) in a linear gradient as indicated.

Analytical thin layer chromatography (TLC) was performed on commercially prepared silica plates (Merck Kieselgel 60 0.25 mm F254). Flash column chromatography was performed using 230-400 mesh Kieselgel 60 silica eluting with distilled solvents as described. Commercial materials were used as received unless otherwise noted. Amino acids, coupling reagents and resins were obtained from Novabiochem or GL Biochem. CH<sub>2</sub>Cl<sub>2</sub> and MeOH were distilled from calcium hydride. THF and Et<sub>2</sub>O were obtained anhydrous from a Pure Solv<sup>TM</sup> solvent purification system using alumina packed columns. DMF was obtained as peptide synthesis grade from Merck or Labscan. Petroleum ether (40 – 60 °C) obtained from Merck Biosciences was used without further purification. Solid-phase peptide synthesis (SPPS) was performed in polypropylene syringes equipped with Teflon filters, purchased from Torviq.



## Solid Phase Peptide Synthesis (SPPS) (A)

### Preloading 2-chlorotrityl chloride Resin

2-Chlorotrityl chloride resin (1.22 mmol/g) (1 eq) was initially washed with CH<sub>2</sub>Cl<sub>2</sub> for 30 min, after which a solution of Fmoc-Xaa-OH (2 eq), <sup>i</sup>Pr<sub>2</sub>NEt (4 eq), in 1:1 (v/v) DMF:CH<sub>2</sub>Cl<sub>2</sub> (final conc. 0.1 M) was shaken with the prewashed resin for 16 h. The resin was then washed with DMF (5 × 3 mL), CH<sub>2</sub>Cl<sub>2</sub> (5 × 3 mL) and DMF (5 × 3 mL), and capped with 17:2:1 (v/v/v) CH<sub>2</sub>Cl<sub>2</sub>:MeOH:<sup>i</sup>Pr<sub>2</sub>NEt for 30 min.

### Iterative Peptide Assembly For 2-CTC and Wang resin (Fmoc-SPPS)

*Deprotection:* (× 2): The resin was treated with 20 vol.% piperidine in DMF (2 × 3 min), after which the resin was washed with DMF (5 × 3 mL), CH<sub>2</sub>Cl<sub>2</sub> (5 × 3 mL), and DMF (5 × 3 mL).

*Proteinogenic amino acid coupling:* A preactivated solution of protected amino acid (4 eq), PyBOP (4 eq) and NMM (8 eq) in DMF (final concentration 0.1 M) was added to the resin. After 1 h the resin was washed with DMF (5 × 3 mL), CH<sub>2</sub>Cl<sub>2</sub> (5 × 3 mL) and DMF (5 × 3 mL).

*Non-proteinogenic amino acid coupling:* A preactivated solution of protected amino acid (1.2 eq), HATU (1.2 eq), and <sup>i</sup>Pr<sub>2</sub>NEt (2.4 eq) in DMF (final concentration 0.1 M) was added to the resin. After 16 h the resin was washed with DMF (5 × 3 mL), CH<sub>2</sub>Cl<sub>2</sub> (5 × 3 mL) and DMF (5 × 3 mL).

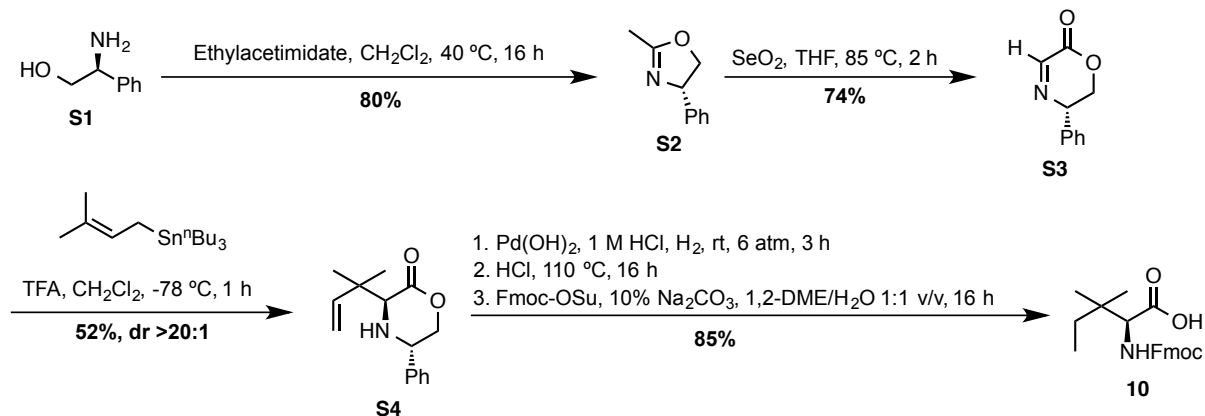
*On-resin coupling of Fmoc-Thr-OH to N-Me asparagine:* A preactivated solution of Fmoc-L-Thr-OH (2 eq), HATU (2 eq), <sup>i</sup>Pr<sub>2</sub>NEt (4 eq) in DMF (final concentration 0.1 M) was added to the resin. After 2 h the resin was washed with DMF (5 × 3 mL), CH<sub>2</sub>Cl<sub>2</sub> (5 × 3 mL) and DMF (5 × 3 mL).

*Capping:* Acetic anhydride/pyridine (1:9 v/v, 5 mL) was added to the resin. After 3 min the resin was washed with DMF (5 × 3 mL), CH<sub>2</sub>Cl<sub>2</sub> (5 × 3 mL), and DMF (5 × 3 mL).

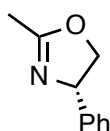
*Cleavage (CTC):* The resin was subjected to a 30 vol.% mixture of HFIP in CH<sub>2</sub>Cl<sub>2</sub>. After 2 h, the solution was transferred into a vial, and the resin was washed with CH<sub>2</sub>Cl<sub>2</sub> (3 × 2 mL). The combined solutions were concentrated to yield the crude peptide.

## Procedures and Analytical Data

### Synthesis of Fmoc- $\beta$ -Me-I-Ile 10

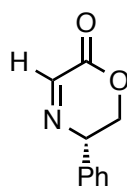


### Synthesis of (S)-2-methyl-4-phenyl-4,5-dihydrooxazole S2



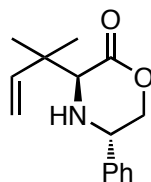
(S)-Phenylglycinol was dissolved in  $\text{CH}_2\text{Cl}_2$  (10 mL) and added dropwise to a cooled solution (0  $^\circ\text{C}$ ) of ethylacetimidate hydrochloride (3 g, 19 mmol), in  $\text{CH}_2\text{Cl}_2$  (50 mL). After complete addition, the reaction mixture was left to stir for 16 h under reflux. After cooling the reaction it was poured into  $\text{H}_2\text{O}$  (200 mL) and extracted with  $\text{CH}_2\text{Cl}_2$  (200 mL). The organic layers were collected, dried and concentrated *in vacuo* to yield a yellow liquid. The crude product was used without further purification (2.6 g, 80%).  $[\alpha]_{\text{D}}^{22.5}$ : +25.4 (*c* 1.0  $\text{CHCl}_3$ )  $^1\text{H NMR}$  (500 MHz,  $\text{CDCl}_3$ )  $\delta$  7.35 – 7.31 (m, 2H), 7.28 – 7.23 (m, 3H), 5.16 (brt,  $J = 10.0$  Hz 1H), 4.56 (dd,  $J = 10.0$  Hz,  $J = 8.0$  Hz, 1H) 4.08 (app,  $J = 8.0$  Hz, 1H), 2.09 (d,  $J = 1.2$  Hz, 3H). These data were in agreement with those previously reported Schafer *et al.*<sup>1</sup>

### Synthesis of (S)-5-phenyl-5,6-dihydro-2H-1,4-oxazin-2-one S3



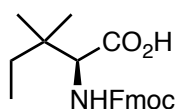
To a suspension of  $\text{SeO}_2$  (1.02 g, 9.32 mmol) in anhydrous THF (18.7 mL) under argon, was added a solution of **S2** (1 g, 6.21 mmol) in dry THF (18.8 mL). The mixture was heated at reflux for 2 h, after which time the reaction had reached completion, as shown by TLC analysis. The reaction mixture was filtered through a pad of silica, and eluted with  $\text{Et}_2\text{O}$ . The eluent was collected and concentrated *in vacuo*, to yield red oil. The crude product was used without further purification (715 mg, 71%).  $R_f$  0.29 (20% EtOAc in hexane),  $[\alpha]_{\text{D}}^{22.5}$ : +207.4 (*c* 1.0  $\text{CHCl}_3$ )  $^1\text{H NMR}$  (500 MHz,  $\text{CDCl}_3$ )  $\delta$  8.05 (d,  $J$  = 3.0 Hz, 1H), 7.44 – 7.34 (m, 5H, Ph), 4.91 (ddd,  $J$  = 11.0 Hz,  $J$  = 5.0 Hz,  $J$  = 3.0 Hz, 1H) 4.60 (dd,  $J$  = 12.0 Hz,  $J_{\text{HH}}$  = 5.0 Hz, 1H), 4.28 (dd,  $J$  = 12.0 Hz,  $J_{\text{HH}}$  = 11.0 Hz, 1H). These data were in agreement with those previously reported by Pigza *et al.*<sup>2</sup>

### Synthesis of (3S,5S)-3-(2-methylbut-3-en-2-yl)-5-phenylmorpholin-2-one S4



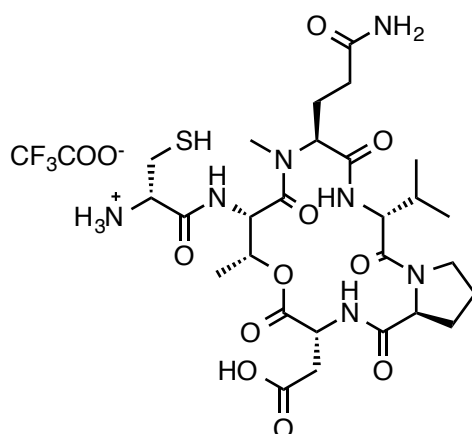
To a solution of the oxazinone **S3** (120 mg, 0.69 mmol) and dimethylallyltributylstannane (253 ml, 0.75 mmol) in  $\text{CH}_2\text{Cl}_2$  (6.5 mL) cooled to  $-78\text{ }^\circ\text{C}$  was added TFA (131 ml, 1.75 mmol) dropwise. After 1 h the reaction was quenched with sat. aqueous  $\text{NaHCO}_3$  and allowed to warm to rt. The reaction was extracted with  $\text{CH}_2\text{Cl}_2$ , and the combined layers were collected, dried and concentrated *in vacuo*. The crude material was purified via column chromatography (10 weight% KF in  $\text{SiO}_2$ ,  $\text{CH}_2\text{Cl}_2$ ) to provide a mixture of diastereomers (14:1). This mixture was recrystallized from pentane with a few drops of  $\text{Et}_2\text{O}$ , to yield white crystals of diastereomerically enriched product - dr > 20:1. (95 mg, 52%).  $R_f$  0.35 (20 % EtOAc in hexane),  $[\alpha]_{\text{D}}^{22.5}$ : -42.5 (*c* 1.0  $\text{CHCl}_3$ )  $^1\text{H NMR}$  (500 MHz,  $\text{CDCl}_3$ )  $\delta$  7.40 - 7.28 (m, 5H, Ph), 6.11 (dd,  $J$  = 17.5 Hz,  $J$  = 10.9 Hz, 1H), 5.15 – 5.13 (m, 2H), 4.43 (m, 1H), 4.25 (dd,  $J$  = 10.6 Hz,  $J$  = 9.0 Hz, 1H), 3.50 (s, 1H), 1.95 (brs, 1H), 1.33 (s, 3H), 1.32 (s, 3H). These data were in agreement with those previously reported Pigza *et al.*<sup>2</sup>

## Synthesis of (S)-2-(((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3,3-dimethylpentanoic acid 10

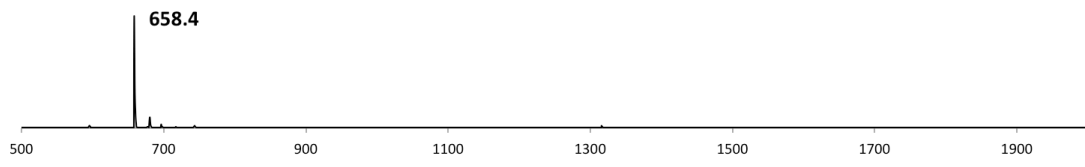
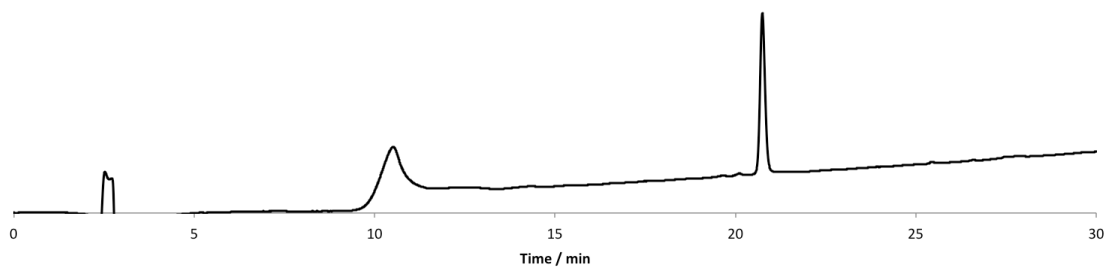


Morpholinone **S4** (85 mg, 0.35 mmol), Pd(OH)<sub>2</sub> (56 mg, 0.09 mmol, 20% Pd content), 1 M HCl (1.6 mL), and MeOH (9.5 mL) were added to a thick-walled flask and placed in a Parr hydrogenation apparatus, pressurized to 90 psi with H<sub>2</sub> (~6 atm). The reaction was allowed to stir for 3 h, then filtered through celite. The filtrate was concentrated to a yellow film. HCl (3 mL, conc.) was added and the reaction was heated to 100 °C for 16 h. The reaction was concentrated *in vacuo*. The crude mixture was extracted with H<sub>2</sub>O (5 mL) and Et<sub>2</sub>O (5 mL). The aqueous layer was collected and lyophilized. The crude material was dissolved in 10% aqueous Na<sub>2</sub>CO<sub>3</sub> solution (1.34 mL) and 1,2-dimethoxyethane (1,2-DME) (0.8 mL). To this solution was added Fmoc-OSu (137.4 mg, 0.39 mmol) in 1,2-DME (0.8 mL). The reaction mixture was stirred for 16 h, after which the solid that had precipitated from solution was filtered off and the filtrate was acidified to pH 3 using 1 M aqueous HCl. The filtrate was extracted using EtOAc (5 mL), and the organic layer was collected, dried and concentrated *in vacuo*. Purification by column chromatography (20% EtOAc in hexane, 1% AcOH) yielded a white solid after concentration (109 mg, 85%). *R*<sub>f</sub> 0.20 (20% EtOAc in hexane, 1% AcOH), [α]<sub>D</sub><sup>22.5</sup>: +10.2 (*c* 1.0 CHCl<sub>3</sub>) <sup>1</sup>H NMR (300 MHz, 10 vol.% MeOD in CDCl<sub>3</sub>) δ 7.74 (d, *J* = 6.0 Hz, 2H), 7.58 (d, *J* = 6.0 Hz, 2H), 7.37 (t, *J* = 6.0 Hz, 2H), 7.28 (t, *J* = 6.0 Hz, 2H), 4.36 (m, 2H), 4.17 (d, *J* = 6.0 Hz, 1H), 1.34 (m, 2H), 0.93 (m, 6H), 0.86 (m, 3H). These data were in agreement with those previously reported by Inoue *et al.*<sup>3</sup>

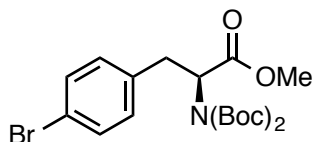
## Synthesis of 4



2-Chlorotrityl chloride resin (0.2 mmol) was loaded with Fmoc-L-Pro-OH (following general procedure A), followed by elongation of the linear peptide *via* general procedure A (Fmoc-SPPS). To conduct the on-resin esterification, Fmoc-D-Asp(OtBu)-OH (657 mg, 1.6 mmol) was dissolved in CH<sub>2</sub>Cl<sub>2</sub> (9 mL) at 0 °C. To this solution was added *N,N'*-diisopropylcarbodiimide (DIC) (125 ml, 0.8 mmol), after which the reaction mixture was warmed to rt, and stirred for 30 min. The reaction mixture was concentrated *in vacuo*, dissolved in DMF (1.5 mL) and this solution was shaken with the resin. In a separate vial DMAP (cat.) was dissolved in DMF (0.5 mL) and this solution was added to the resin and activated acid solution. The resin was left to shake for 16 h, after which time it was washed with DMF (5 × 3 mL), CH<sub>2</sub>Cl<sub>2</sub> (5 × 3 mL) and DMF (5 × 3 mL), and the *N*-terminus was deprotected following general procedure A. The peptide was cleaved from the resin with HFIP (see general procedure A, cleavage CTC). The crude material was dissolved in CH<sub>2</sub>Cl<sub>2</sub> (80 mL), which was added dropwise to a solution HATU (83.2 mg, 0.16 mmol) and *i*Pr<sub>2</sub>NEt (56 ml, 0.32 mmol) in 120 mL of CH<sub>2</sub>Cl<sub>2</sub>. The solution was stirred for 16 h. Following completion of the reaction (as judged by HPLC-MS), the reaction mixture was concentrated *in vacuo*. The crude material was stirred in a cocktail of TFA:*i*Pr<sub>3</sub>SiH:H<sub>2</sub>O (90:5:5 v/v/v) for 2 h, after which the reaction mixture was concentrated *in vacuo*. The crude material was subjected to reverse phase HPLC purification, which yielded a white fluffy solid after lyophilisation, (12 mg, 12%). *R*<sub>t</sub> = 38 min (0 – 25 % MeCN in H<sub>2</sub>O, 1 % TFA over 40 min), [α]<sub>D</sub><sup>22.5</sup>: -56.7 (c 0.7 in H<sub>2</sub>O), <sup>1</sup>H NMR (500 MHz, D<sub>2</sub>O) δ: 5.40 (dd, *J* = 6.7 Hz, *J* = 2.7 Hz, 1H), 5.09 (m, 1H), 5.02 (m, 1H), 4.61 (t, *J* = 7.6, 1H), 4.32 – 4.29 (m, 1H), 4.26 (t, *J* = 7.0, 1H), 3.89 (m, 1H), 3.64 (m, 1H), 3.07 (m, 5H), 2.87 (m, 1H), 2.77 (m, 1H), 2.26 – 2.14 (m, 2H), 2.12 – 2.05 (m, 3H), 2.04 – 1.19 (m, 5H), 1.24 (d, *J* = 7.2 Hz, 3H), 0.93 (d, *J* = 6.7 Hz, 3H), 0.88 (d, 7.2 Hz, 3H) <sup>13</sup>C NMR (125 MHz, D<sub>2</sub>O) δ: 177.7, 174.6 173.9, 172.7, 171.7,169.7, 169.6, 168.0, 70.6, 60.4, 57.4, 55.9, 54.4, 53.2, 49.8, 47.8, 34.7, 31.2, 30.8, 29.8, 27.9, 24.9, 23.9, 23.7, 18.1, 17.7, 15.3. **LRMS** (ESI<sup>+</sup>) *m/z* found 658.4 [M+H]<sup>+</sup>. **Analytical HPLC** *R*<sub>t</sub>20.7 min (0 – 25 min over 30 (0.1% Formic acid) λ = 220 nm).

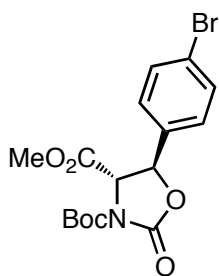


## Synthesis of 7



To a suspension of L-4-bromophenylalanine (5.0 g, 20.5 mmol) in MeOH (60.0 mL) was added  $\text{SOCl}_2$  (2.3 mL, 30.7 mmol) dropwise. The reaction mixture was stirred for 16 h before concentrating *in vacuo*. The crude solid was dissolved in  $\text{CH}_2\text{Cl}_2$  (52.0 mL) and  $(\text{Boc})_2\text{O}$  (5.2 mL, 22.5 mmol) and  $i\text{Pr}_2\text{NEt}$  (7.1 mL, 41 mmol) were added. The reaction was stirred for 16 h, after which the reaction was washed with sat. aqueous  $\text{NH}_4\text{Cl}$  solution (30 mL), sat. aqueous  $\text{NaHCO}_3$  solution (30 mL) and brine (30 mL). The organic layer was collected, dried and concentrated *in vacuo*. The crude oil was dissolved in MeCN (68 mL), before addition of DMAP (2.9 g, 23.4 mmol) and  $(\text{Boc})_2\text{O}$  (6.1 mL, 28.6). The reaction was stirred for 16 h, before concentration *in vacuo*. The crude product was purified via flash chromatography (10% EtOAc in hexane) to afford the title compound as a white solid (7.3 g, 78%),  $R_f$  0.20 (10 % EtOAc in hexane),  $[\alpha]_{\text{D}}^{22.5} = -119.2$  ( $c$  1.0 in  $\text{CH}_2\text{Cl}_2$ ),  $^1\text{H NMR}$  (500 MHz,  $\text{CDCl}_3$ )  $\delta$ : 7.38 (d,  $J = 9.0$  Hz, 2H), 7.06 (d,  $J = 9.0$  Hz, 2H), 5.10 (dd,  $J = 10.0$  Hz,  $J = 6.0$  Hz, 1H), 3.75 (s, 3H), 3.38 (dd,  $J = 14.0$  Hz,  $J = 6.0$  Hz, 1H), 3.17 (dd,  $J = 14.0$  Hz,  $J = 10.0$  Hz, 1H), 1.40 (s, 18H, Boc),  $^{13}\text{C NMR}$  (125 MHz,  $\text{CDCl}_3$ )  $\delta$ : 170.7, 151.8, 136.7, 131.4, 131.3, 120.5, 83.2, 59.1, 52.3, 35.7, 27.9, **IR** (ATR)  $\nu_{\text{max}}$  ( $\text{cm}^{-1}$ ), 1730, 1693, 1364, 1259, 1137, 1116, 1012, **HRMS** ( $\text{ESI}^+$ )  $\text{C}_{20}\text{H}_{28}^{79}\text{BrNO}_6\text{Na}$  calculated  $m/z$  480.0992 found  $m/z$  480.0995.

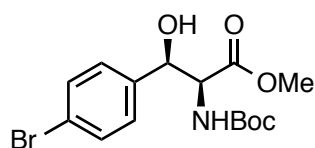
**Synthesis of 3-(*tert*-butyl) 4-methyl (4*S*,5*R*)-5-(4-bromophenyl)-2-oxooxazolidine-3,4-dicarboxylate 8**



To a solution of **7** (3.0 g, 5.58 mmol) in  $\text{CCl}_4$  (68 mL) was added NBS (2.2 g, 19.5 mmol). The reaction mixture was heated to reflux and irradiated with a 250 W Kr lamp for 45 min. After the reaction had reached completion, it was cooled to rt and filtered. The filtrate was concentrated *in vacuo* and the crude bromide was immediately dissolved in acetone (76 mL). To this solution was added  $\text{AgNO}_3$  (1.6 g, 9.45 mmol) and the suspension was stirred in the dark for 2 h. Once the reaction had reached completion the reaction mixture was filtered and concentrated *in vacuo*. The residue was taken up in EtOAc (30 mL) and washed with sat. aqueous  $\text{NH}_4\text{Cl}$  solution (30 mL), brine (30 mL) and  $\text{H}_2\text{O}$  (30 mL). The organic layer was collected, dried and purified via flash column chromatography (20% EtOAc in hexane) to afford the title compounds as a colourless oil (1.35 g, 52 %).  $R_f = 0.25$  (20% EtOAc in hexane),  $[\alpha]_{\text{D}}^{22.5} = -48.6$  ( $c$  1.0 in  $\text{CH}_2\text{Cl}_2$ ),  $^1\text{H NMR}$  (500 MHz,  $\text{CDCl}_3$ )  $\delta$ : 7.53 (d,  $J = 9.0$  Hz, 1H), 7.23 (d,  $J = 9.0$  Hz, 1H), 5.32 (d,  $J = 5.0$  Hz, 1H), 4.55 (d,  $J = 5.0$  Hz, 1H), 3.86 (s, 3H), 1.45 (s, 9H),  $^{13}\text{C NMR}$  (125 MHz,  $\text{CDCl}_3$ )  $\delta$  168.7, 150.4, 148.2, 136.1, 132.3, 126.7, 123.6, 85.0, 75.2, 63.5, 53.3, 27.8. **IR** (ATR)  $\nu_{\text{max}}$  ( $\text{cm}^{-1}$ ) 825, 912, 1006, 1057, 1151, 1211, 1253, 1318, 1368, 1438, 1490, 1595, 1727, 1752, 1820, 2980, **HRMS** (ESI $^+$ )  $\text{C}_{16}\text{H}_{18}^{79}\text{BrNO}_6\text{Na}$  calculated  $m/z$  420.0210 found  $m/z$  420.0215.

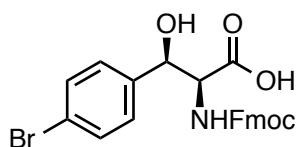


### Synthesis of methyl (2*S*,3*R*)-3-(4-bromophenyl)-2-((*tert*-butoxycarbonyl)amino)-3-hydroxypropanoate **9**



To a cooled (0 °C) solution of **8** (500 mg, 1.25 mmol) in anhydrous MeOH (15 mL) was added CsCO<sub>3</sub> (45 mg, 0.25 mmol). The reaction mixture was stirred at 0 °C for 1 h, after which it was concentrated and taken up into EtOAc (5 mL), washed with sat. aqueous NH<sub>4</sub>Cl solution (5 mL), H<sub>2</sub>O (5 mL) and brine (5 mL). The organic layers were collected, dried and concentrated *in vacuo*. The crude material was purified via flash chromatography (30% EtOAc in hexane) to yield the title compounds as a white solid (368 mg, 64%).  $R_f = 0.30$  (20% EtOAc in hexane),  $[\alpha]_D^{22.5} = -32.8$  ( $c$  0.5 in CH<sub>2</sub>Cl<sub>2</sub>), <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>)  $\delta$ : 7.48 (d,  $J = 9.0$  Hz, 1H), 7.25 (d,  $J = 9.0$  Hz, 1H), 5.27 (m, 1H), 5.20 (s, 1H), 4.51 (m, 1H), 3.77 (s, 3H) 1.33 (s, 9H) <sup>13</sup>C NMR (125 MHz, CDCl<sub>3</sub>)  $\delta$ : 171.1, 155.6, 138.3, 131.5, 127.8, 122.0, 80.4, 73.4, 59.2, 52.7, 28.1. IR (ATR)  $\nu_{\max}$  (cm<sup>-1</sup>) 830.9, 916.7, 1010.1, 1057.4, 1070.2, 1113.4, 1159.0, 1211.4, 1248.4, 1281.9, 1348.4, 1366.3, 1392.7, 1436.2, 1454.4, 1488.2, 1504.6, 1693.9, 1713.5, 2978.2, 3410.6 (br), HRMS (ESI<sup>+</sup>) C<sub>15</sub>H<sub>20</sub><sup>79</sup>BrO<sub>5</sub>NNa calculated  $m/z$  396.0417 found  $m/z$  396.0420.

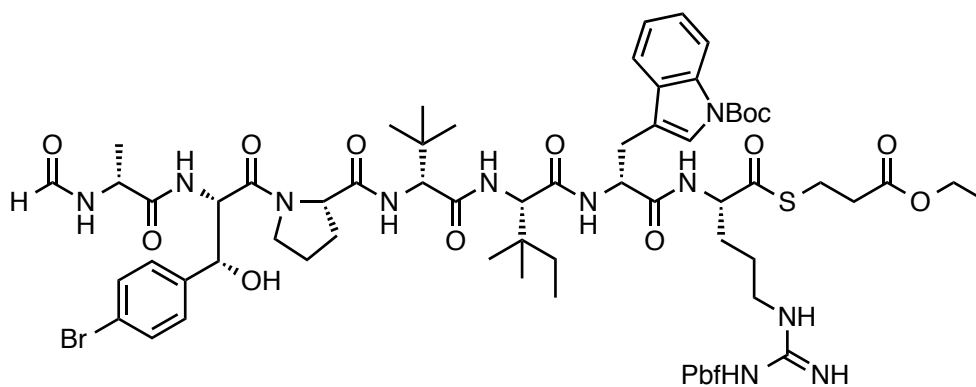
### Synthesis of (2*S*,3*R*)-2-(((9*H*-fluoren-9-yl)methoxy)carbonyl)amino)-3-(4-bromophenyl)-3-hydroxypropanoic acid **5**



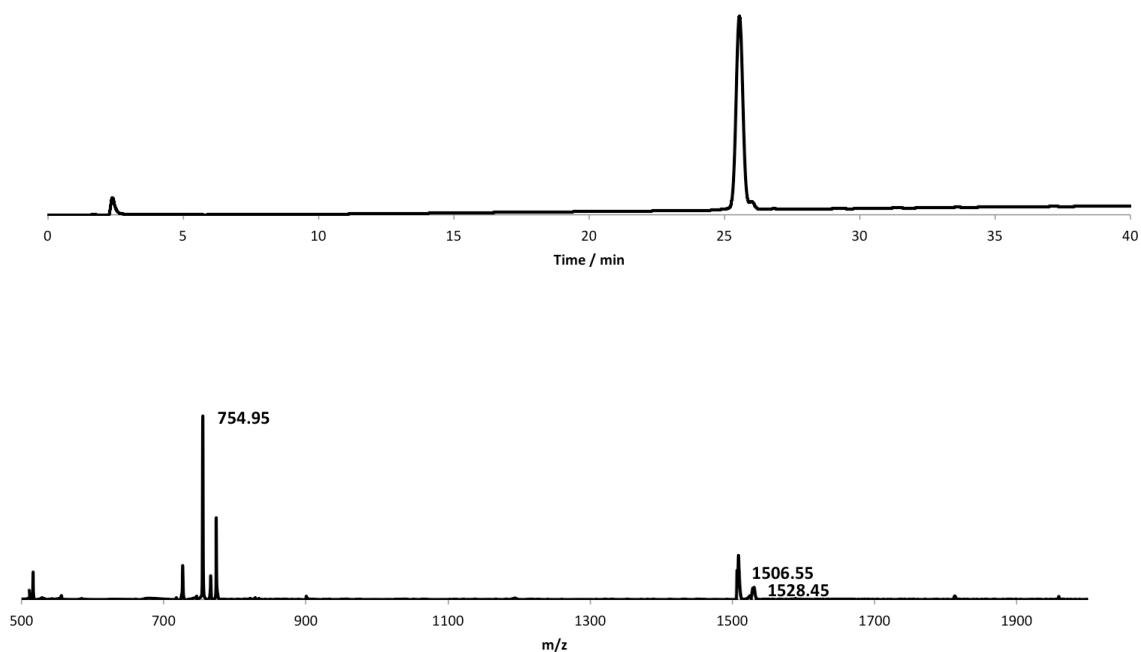
To a solution of **9** (365 mg, 0.98 mmol) in THF (4.9 mL) was added 1 M aqueous KOH (4.9 mL, 4.9 mmol). The reaction was stirred for 1 h, after which the reaction mixture was concentrated *in vacuo*, and acidified with 1 M aqueous HCl (pH = 4). The aqueous phase was extracted with EtOAc (2 x 20 mL), and the organic layers were collected, dried and concentrated *in vacuo*. The crude product was dissolved in CH<sub>2</sub>Cl<sub>2</sub> (6 mL) to which was added 4 M HCl in dioxane (6 mL, 9.8 mmol). The reaction was stirred for 1 h, after which time a white precipitate formed. The solid was collected and dried under reduced pressure. The amine salt was dissolved up in DME (3.5 mL) and 10% aqueous Na<sub>2</sub>CO<sub>3</sub> solution (7 mL), to this was added Fmoc-OSu (363 mg, 1.08 mmol) in DME (3.5 mL). The reaction was allowed to stir for 16 h, after which it was filtered, the filtrate was collected and washed Et<sub>2</sub>O (10

mL). The aqueous layer was collected and acidified to pH 3, the acidified solution was washed with EtOAc (10 mL), and the organic layer was collected, dried and concentrated *in vacuo*. The crude material was purified *via* flash column chromatography (20% EtOAc in hexane with 1% AcOH) to afford the title compound as a white solid (368 mg, 58%).  $R_f = 0.25$  (20% EtOAc in hexane with 1% AcOH),  $[\alpha]_D^{22.5} = -17.3$  (c 0.3 in  $\text{CH}_2\text{Cl}_2$ ),  $^1\text{H NMR}$  (500 MHz, 10% MeOD in  $\text{CDCl}_3$ )  $\delta$ : 7.68 (t,  $J = 6.4$  Hz, 2H), 7.45 (d,  $J = 8.8$  Hz, 2H), 7.36 (d,  $J = 8.0$  Hz, 2H), 7.33 – 7.29 (m, 2H), 7.21 (m, 4H), 5.22 (m, 1H), 4.46 (m, 1H), 4.23 – 4.20 (m, 1H, Fmoc), 4.16 – 4.13 (m, 1H), 4.06 (t,  $J = 7.3$ , 1H), 3.28 (m, 1H),  $^{13}\text{C NMR}$  (125 MHz,  $\text{CDCl}_3$ )  $\delta$ : 172.5, 156.6, 143.2, 143.1, 141.2, 141.2, 131.3, 127.0, 124.9, 121.4, 119.9, 119.8, 72.2, 67.0, 59.5, 46.9 **IR** (ATR)  $\nu_{\text{max}}$  ( $\text{cm}^{-1}$ ) 830.0, 1009.4, 1052.1, 1070.2, 1105.0, 1154.6, 1220.4, 1334.9, 1359.1, 1402.6, 1449.1, 1488.1, 1517.9, 1593.1, 1697.4, 2952.6 (br), 3402.9 (br). **HRMS** (ESI<sup>+</sup>)  $\text{C}_{24}\text{H}_{18}^{79}\text{BrNO}_5\text{Na}$  calculated  $m/z$  504.0417 found  $m/z$  504.0418.

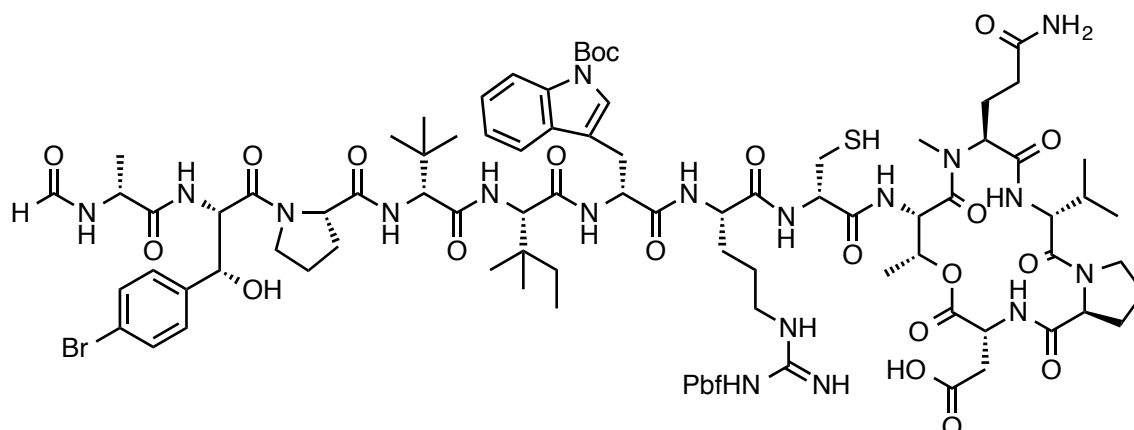
## Synthesis of **3**



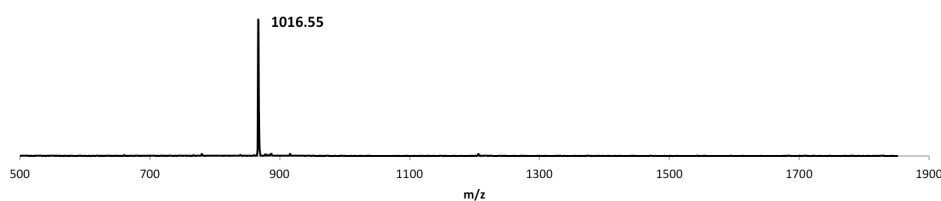
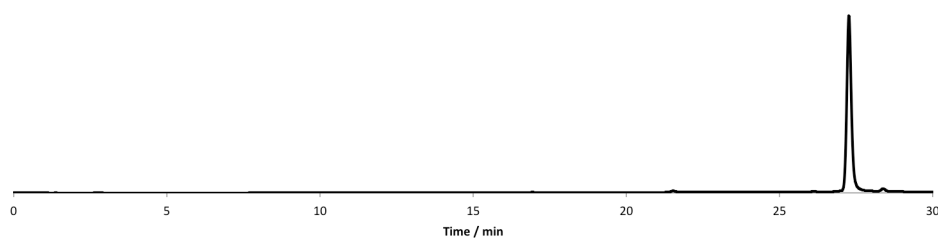
A solution of protected peptide **12** (25  $\mu\text{mol}$  scale) in dry DMF (final concentration 0.1 M) was prepared under argon and cooled to  $-30\text{ }^\circ\text{C}$ . Ethyl 3-mercaptopropionate (30 eq),  $\text{Pr}_2\text{NEt}$  (5 eq) and PyBOP (5 eq) were added sequentially and the solution was allowed to stir at  $-30\text{ }^\circ\text{C}$  for 1 h. The reaction was quenched by the addition of a solution of 0.1% TFA in water, filtered through a  $0.2\text{ }\mu\text{m}$  membrane filter and immediately purified by reverse-phase, preparative HPLC (50 – 100% MeCN in  $\text{H}_2\text{O}$  with 0.1% TFA over 40 min). Peptide thioester **3** was obtained as a white fluffy solid following RP-HPLC purification and lyophilisation (7.01 mg, 15%). **Analytical HPLC**  $R_t$  25.3 min (50 - 100% B over 40 min, ( $\lambda = 220\text{ nm}$ )), **LRMS** ( $\text{ESI}^+$ ) observed  $m/z$  754.0  $[\text{M}+2\text{H}]^{2+}$ , 1506.6  $[\text{M}+\text{H}]^+$ , 1528.5  $[\text{M}+\text{Na}]^+$ .



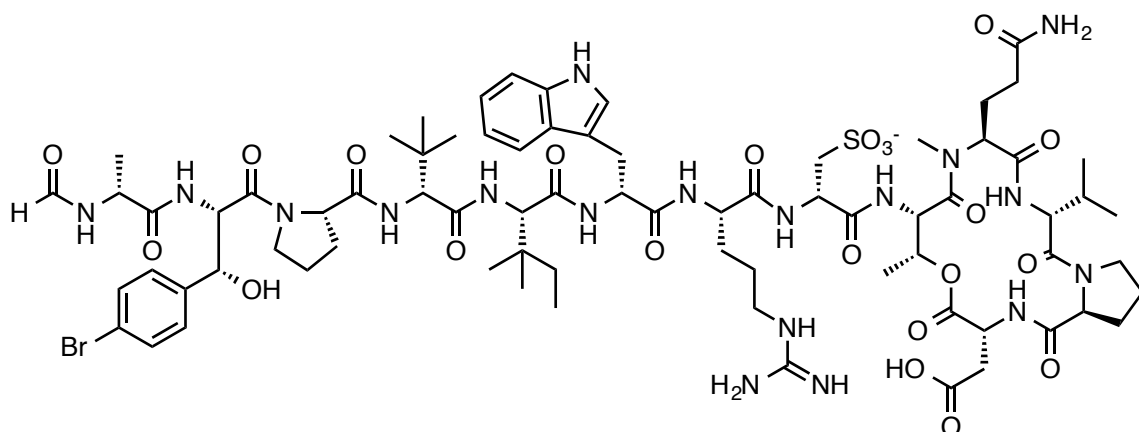
## Synthesis of 13



Peptide thioester **3** (2.45 mg, 1.63  $\mu\text{mol}$ ) was dissolved (final concentration 5 mM) in a degassed 1:1 (v/v) mixture of *N*-methylpyrrolidinone:buffer solution (6 M guanidine hydrochloride, 1 M HEPES, 200 mM PhSH, adjusted to pH 7.5). The solution was then added to the thiol-containing cyclic peptide **4** (1.18 mg, 1.79  $\mu\text{mol}$ ). The final pH of the solution was measured and adjusted to 7.0-7.3 by careful addition of 2 M NaOH. The solution was flushed with argon and placed in a water bath for 16 h at 37  $^{\circ}\text{C}$ . After this time the reaction mixture was concentrated under a stream of nitrogen, then dissolved in 10% MeOH in  $\text{CH}_2\text{Cl}_2$  (1% 1.0 M  $\text{P}(\text{CH}_3)_3$  in THF as an additive) and was loaded on a silica column. The final ligation product was eluted using a gradient of 0 – 5 vol% MeOH in  $\text{CH}_2\text{Cl}_2$  (1% AcOH). The eluent containing the product was concentrated *in vacuo*, the resulting residue was taken up in 1:1 v/v MeCN:H<sub>2</sub>O and lyophilized. The title compound was obtained as a white fluffly (2.13 mg, 64 %). **Analytical HPLC** R<sub>t</sub> 27.3 min (0 - 100% B with 0.1% Formic acid over 30 min,  $\lambda$  = 254 nm). **LRMS** (ESI<sup>+</sup>) *m/z* found 1016.55 [ $\text{M}+2\text{H}$ ]<sup>2+</sup>.



## Synthesis of 2



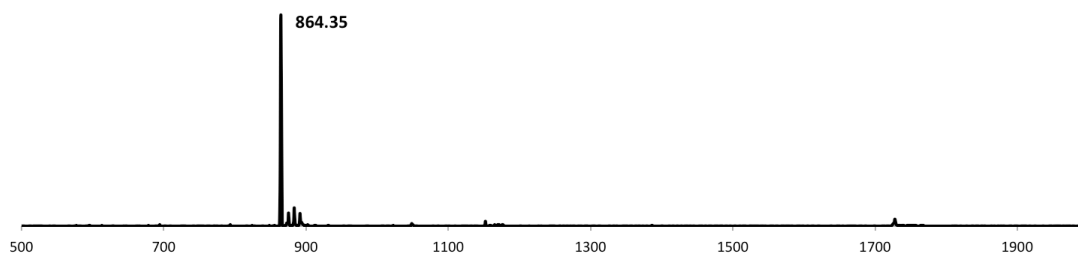
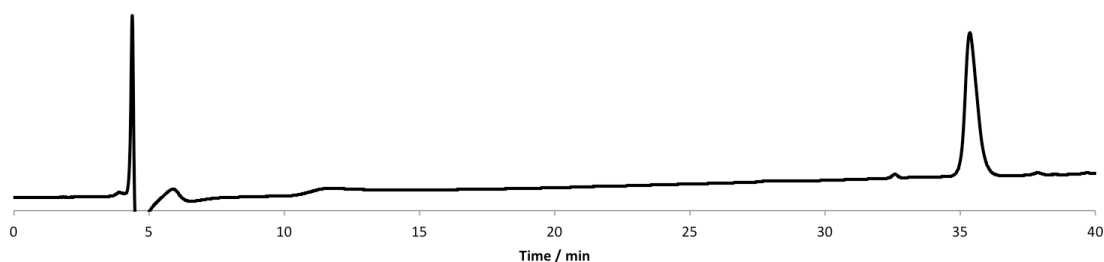
A cooled solution (0 °C) of freshly prepared 1 vol% performic acid (1 ml, 10 eq), (prepared through the addition of 30% H<sub>2</sub>O<sub>2</sub> to formic acid followed by incubation for 1 h at rt) was added to the thiol containing peptide **13** (2.13 mg, 1.05 μmol) at 0 °C. The reaction mixture was left to stir at 0 °C for 40 min, after which the reaction was quenched with 20 μl of DMS in 15 mL milliQ H<sub>2</sub>O (0.1% TFA) and the reaction mixture was lyophilized. The lyophilized peptide was then stirred in a mixture of TFA:<sup>i</sup>Pr<sub>3</sub>SiH:H<sub>2</sub>O (90:5:5 v/v/v) for 1 h, after which the reaction mixture was concentrated *in vacuo* and the crude peptide was subjected to reverse phase HPLC purification (0 – 70% MeCN in H<sub>2</sub>O in 0.1% TFA over 40 min). A white fluffy solid was obtained after lyophilisation, (1.00 mg, 55%).

<sup>1</sup>H NMR (600 MHz, DMSO-*d*<sub>6</sub>) δ: 10.71 (s, 1H, NH-indole), 8.26 (m, 1H, NH-BrPhe), 8.21 (m, 1H, NH-Cys), 8.15 (m, 1H, NH-Trp), 8.10 (m, 1H, NH-Ala), 7.98 (m, 1H, NH-Arg), 7.93 (s, 1H, HCO-Ala), 7.91 (m, 1H, NH-*t*Leu), 7.89 (m, 1H, NH-Thr), 7.60 (m, 1H, NH-β-Melle), 7.70 (m, 1H, NH-Val), 7.56 (d, *J* = 8.0 Hz, 1H), 7.47 (d, *J* = 8.5 Hz, 2H, C3/C5-BrPhe), 7.33 (d, *J* = 8.5 Hz, 2H, C2/C6-BrPhe), 7.33 (m, 1H, C7-Trp), 7.25 (m, 1H, NH-Asp), 7.17 (m, 1H, Trp)<sup>i</sup>, 7.06 (m, 1H, Trp)<sup>ii</sup>, 6.97 (t, 1H, *J* = 8.0 Hz), 5.50 (m, 1H, β-H Thr), 5.17 (m, 1H, α-H, *N*Me-Gln), 5.13 (m, 1H, α-H Thr), 4.93 (m, 1H, α-H BrPhe), 4.80 (m, 1H, β-H BrPhe), 4.86(m, 1H, α-H Cys), 4.66 (m, 1H, α-Trp), 4.63 (m, 1H, α-H Pro), 4.61 (m, 1H, α-H Asp), 4.43 (m, 1H, α-H *t*Leu), 4.40 (m, 1H, α-H Pro), 4.40 (m, 1H, α-H Val), 4.35 (m, 1H, α-H Arg), 4.16 (m, 1H, α-H, β-Melle), 3.91 (m, 1H, δ-H Pro (i)), 3.82 (m, 1H, δ-H Pro) 3.62 (m, 2H, δ-H Pro), 3.17 (m, β-H Trp), 3.01 (m, δ-H Arg), 2.99 (s, Me, *N*-MeGln), 2.93 (m, β-H Asp), 1.21 (m, β-H Cys), 2.06 (m, 4H, β + γ-H, Pro), 1.90 (2H, β-H Pro(ii)), 1.87 (m, 2H, γ-H Pro(ii)), 1.59 (m, 1H, β-H Arg), 1.50 (m, 1H, β-H Arg), 1.30 (m, 2H, γ-H Arg), 1.15 (d, *J* = 7.0 Hz, 3H, γ-H, Thr), 0.97 (d, 6H, *J* = 7.0 Hz, δ-H, Val), 0.87 (s, 9H, γ-H, *t*Leu), 0.85 (m, 1H, γ-H, Val), 0.73 (s, 3H, χ''-H β-Melle), 0.61 (m, 5H χ' + γ-H, β-Melle) <sup>13</sup>C NMR (125 MHz, DMSO-

<sup>i</sup> Observed via <sup>1</sup>H-<sup>13</sup>C HMBC cross peak

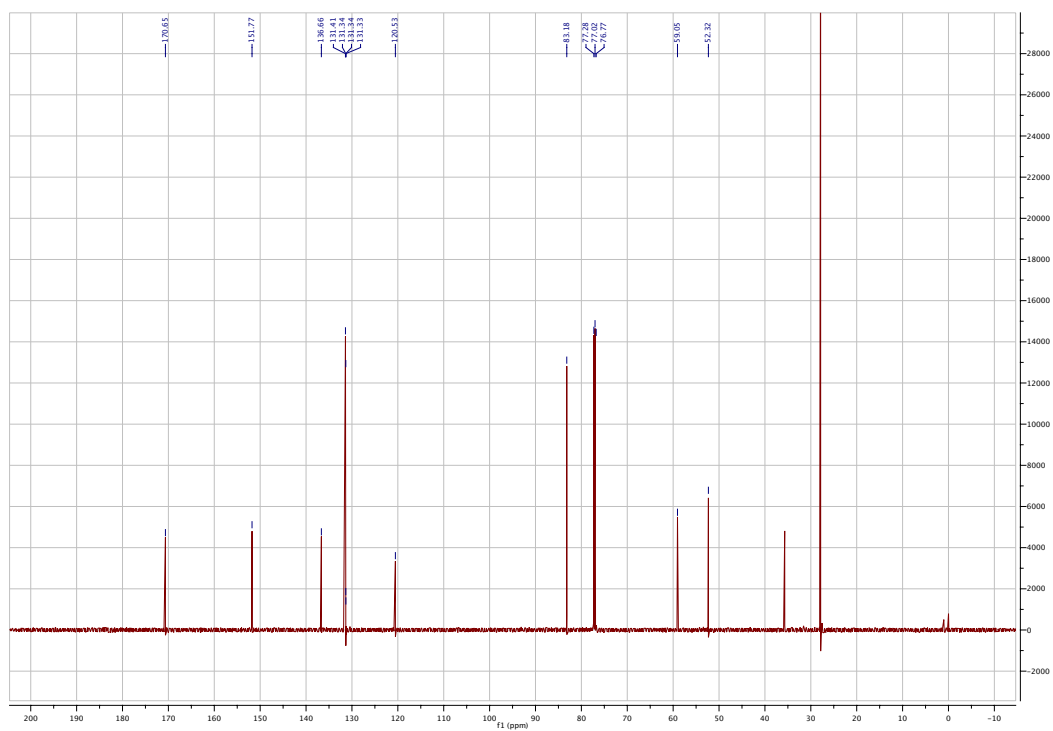
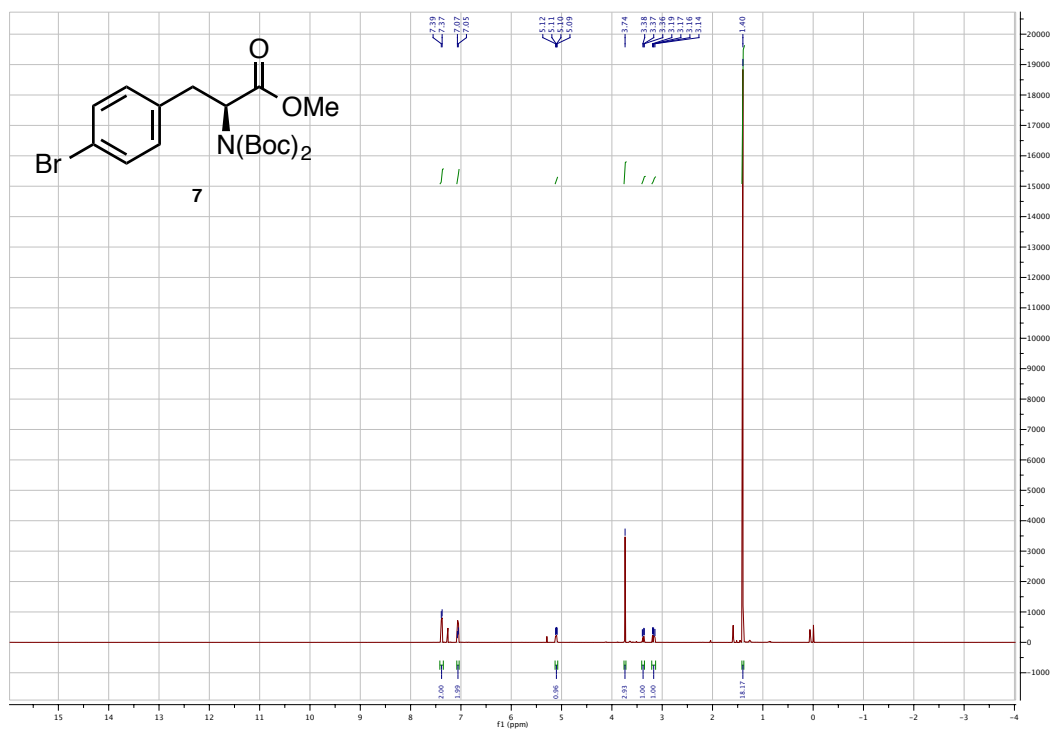
<sup>ii</sup> Observed via <sup>1</sup>H-<sup>13</sup>C HMBC cross peak

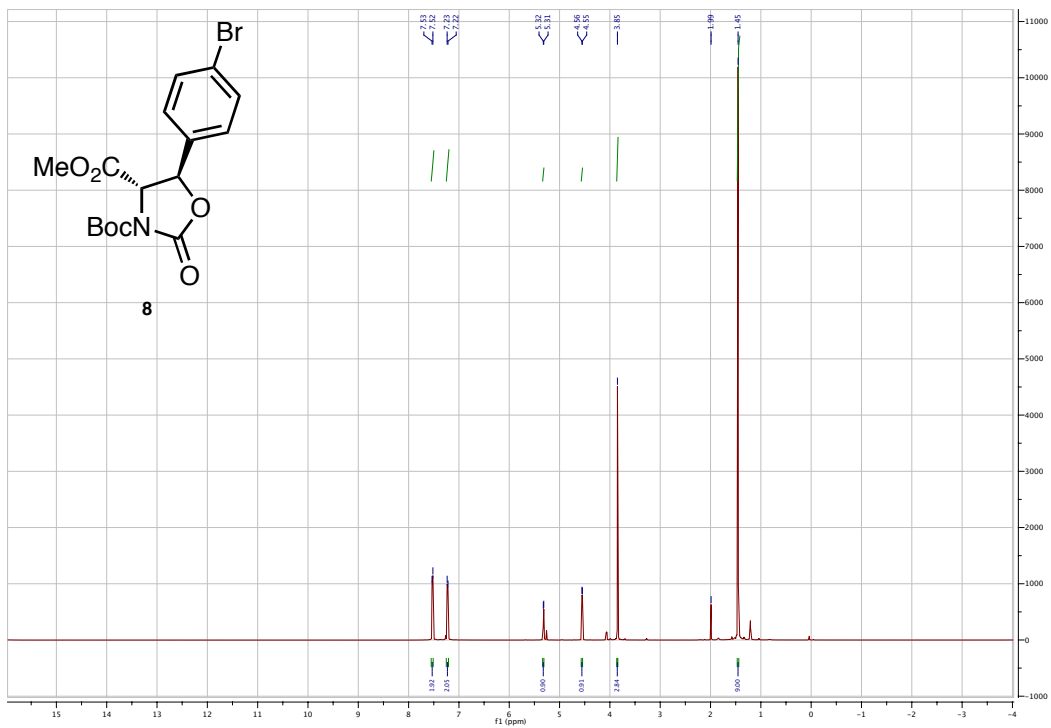
$d_6$ )  $\delta$ :179.8, 179.4,177.3, 176.9, 176.5, 174.8, 174.0, 173.7, 173.4, 172.3, 171.5, 171.5, 171.1, 168.7, 161.4, 141.5, 136.5, 131.0, 129.0, 127.7, 127.4, 121.4, 120.4, 118.1, 118.5, 111.6, 110.0, 72.2, 71.5, 70.7, 69.4, 65.7, 60.4, 60.3, 60.2, 59.8, 56.5, 56.0, 54.0, 52.3, 46.9, 46.8, 46.0, 36.3, 36.0, 35.6, 35.5, 34.7, 32.3, 32.2, 31.8, 31.8, 31.3, 30.5, 30.1, 29.3, 28.9, 27.5, 27.0, 23.3, 22.8, 22.7, 20.5, 20.4, 18.3, 17.1<sup>iii</sup> **Analytical HPLC**  $R_t$  25.3 min (0 - 70% B with 0.1% TFA over 40 min,  $\lambda = 220$  nm) **LRMS** (ESI<sup>+</sup>):  $m/z$  found 864.4 [M+2H]<sup>2+</sup> **HRMS:** (MALDI ESI<sup>+</sup>):  $m/z$  calculated for [C<sub>75</sub>H<sub>109</sub><sup>79</sup>BrN<sub>18</sub>O<sub>22</sub>S+H]<sup>+</sup>, 1725.6941,  $m/z$  found for [C<sub>75</sub>H<sub>109</sub><sup>79</sup>BrN<sub>18</sub>O<sub>22</sub>S+H]<sup>+</sup>, 1725.6941.



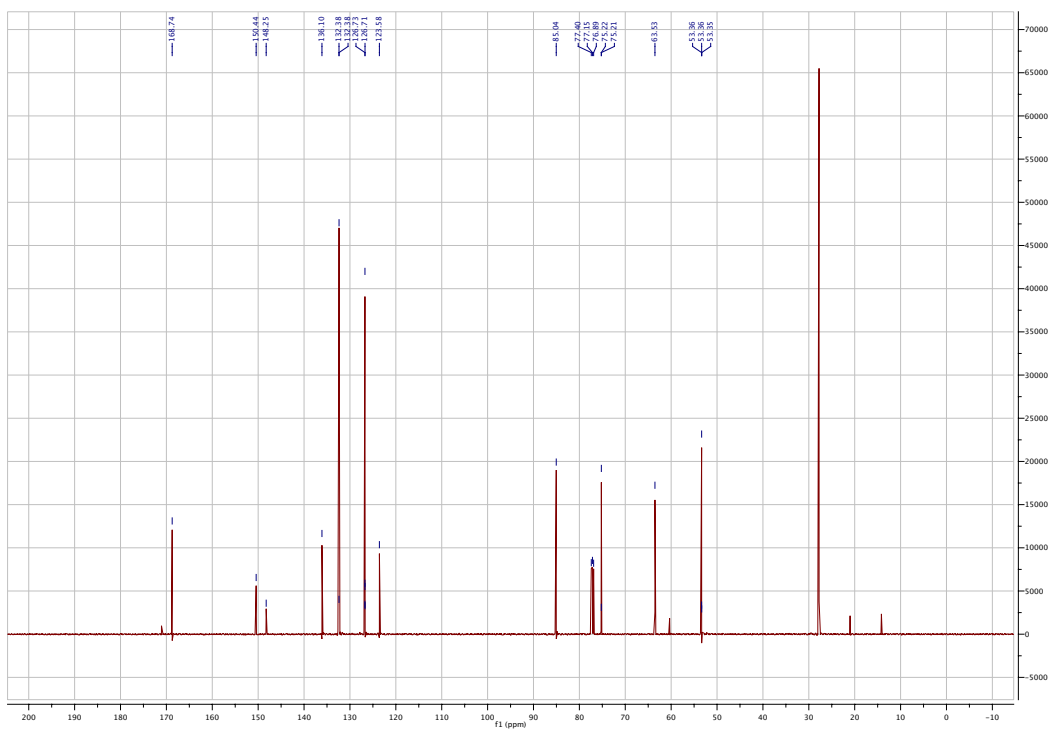
<sup>iii</sup> Some <sup>13</sup>C-<sup>1</sup>H cross peaks were not observed in the HMBC spectra, and therefore have not been reported

# Spectra of Novel Compounds



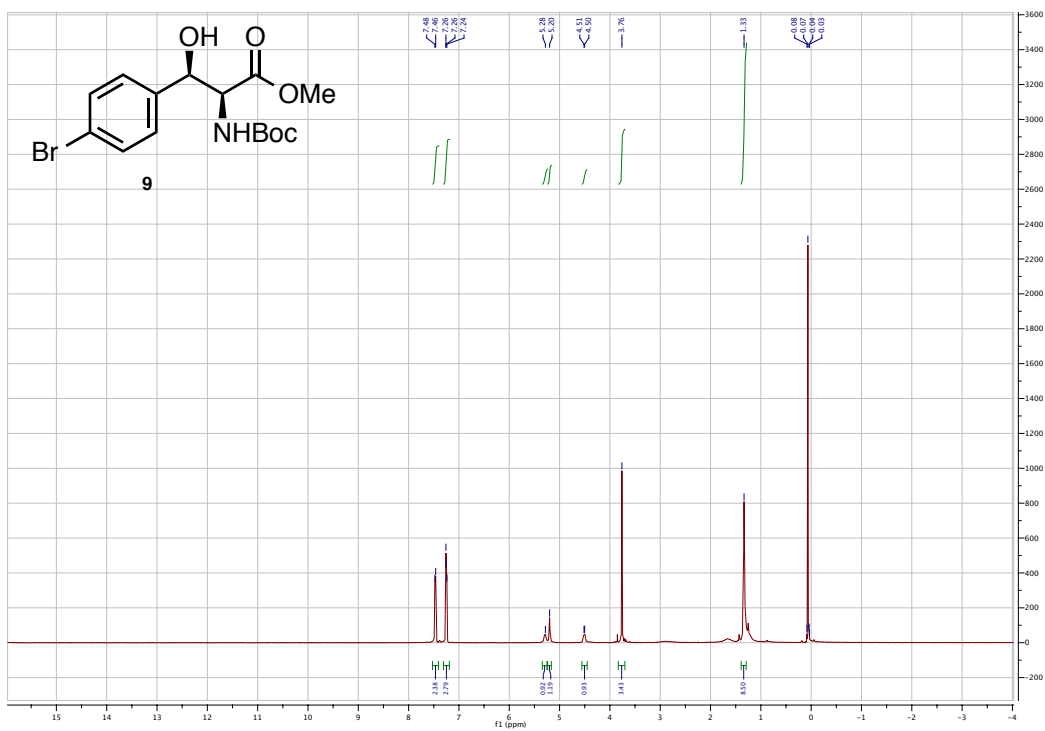


**Figure 3** <sup>1</sup>H NMR of **8** (500 MHz, CDCl<sub>3</sub>).

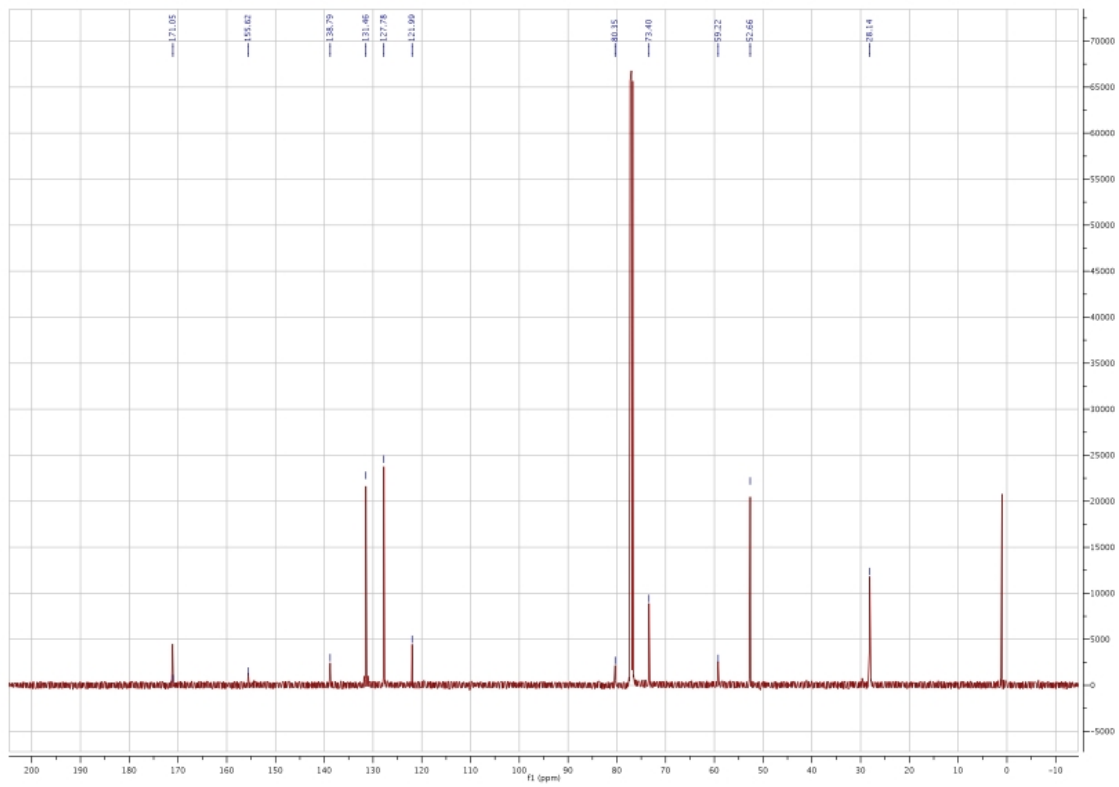


**Figure 4** <sup>13</sup>C NMR of **8** (125 MHz, CDCl<sub>3</sub>).

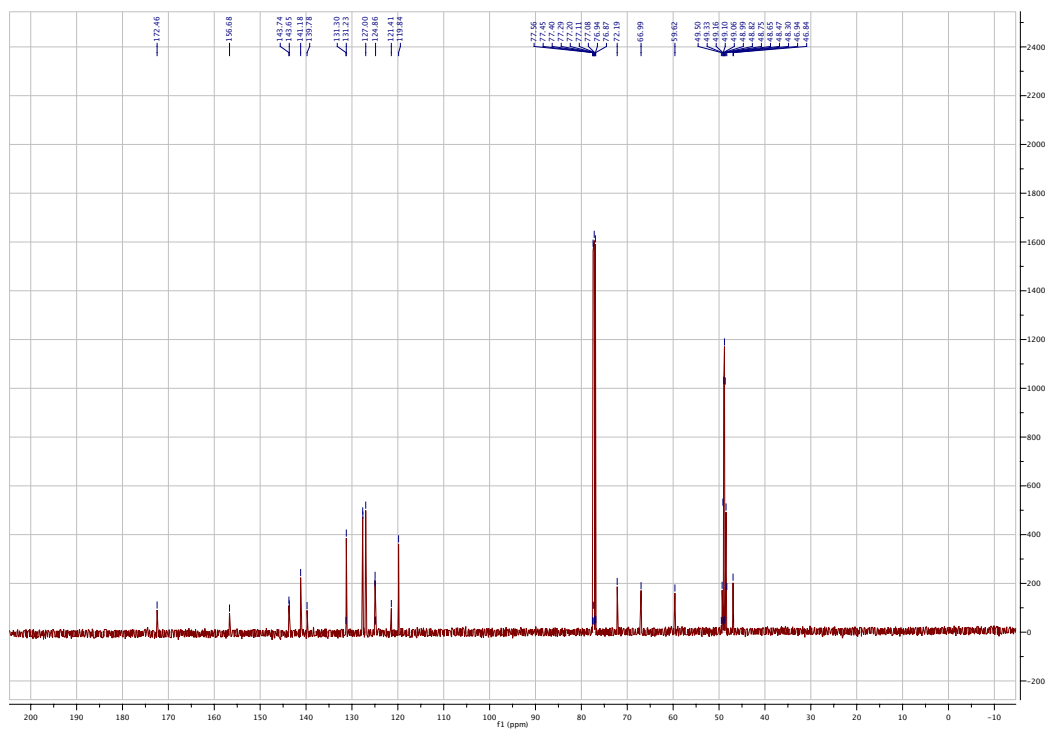
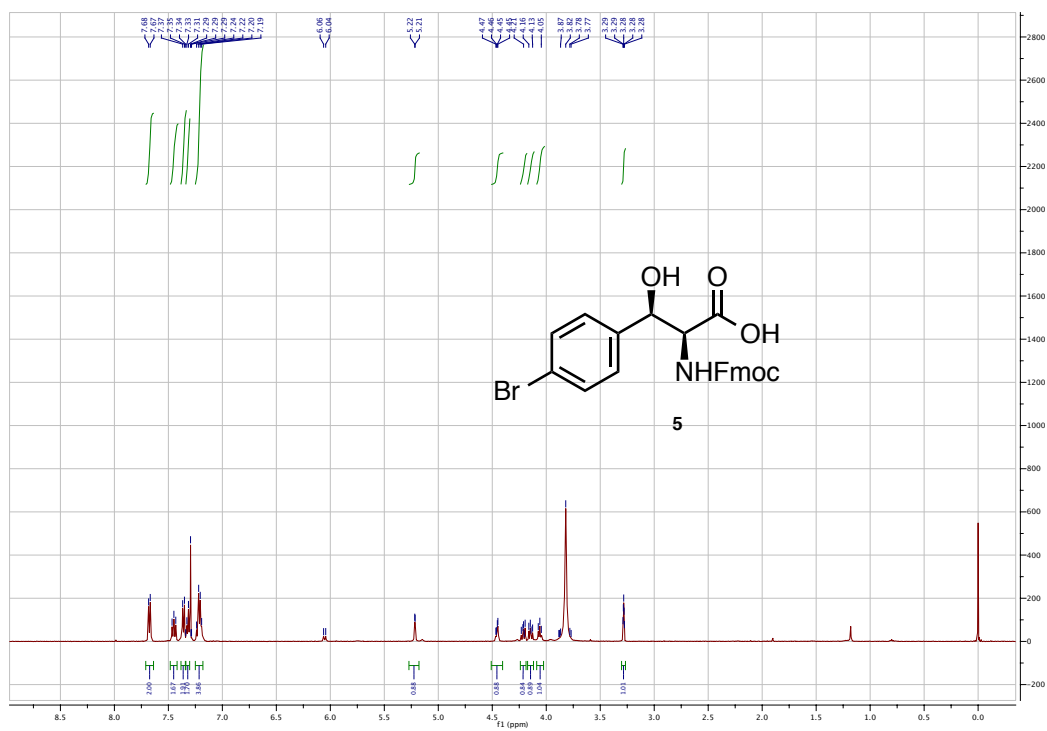




**Figure 5** <sup>1</sup>H NMR of **9** (500 MHz, CDCl<sub>3</sub>).



**Figure 6** <sup>13</sup>C NMR of **9** (125 MHz, CDCl<sub>3</sub>).



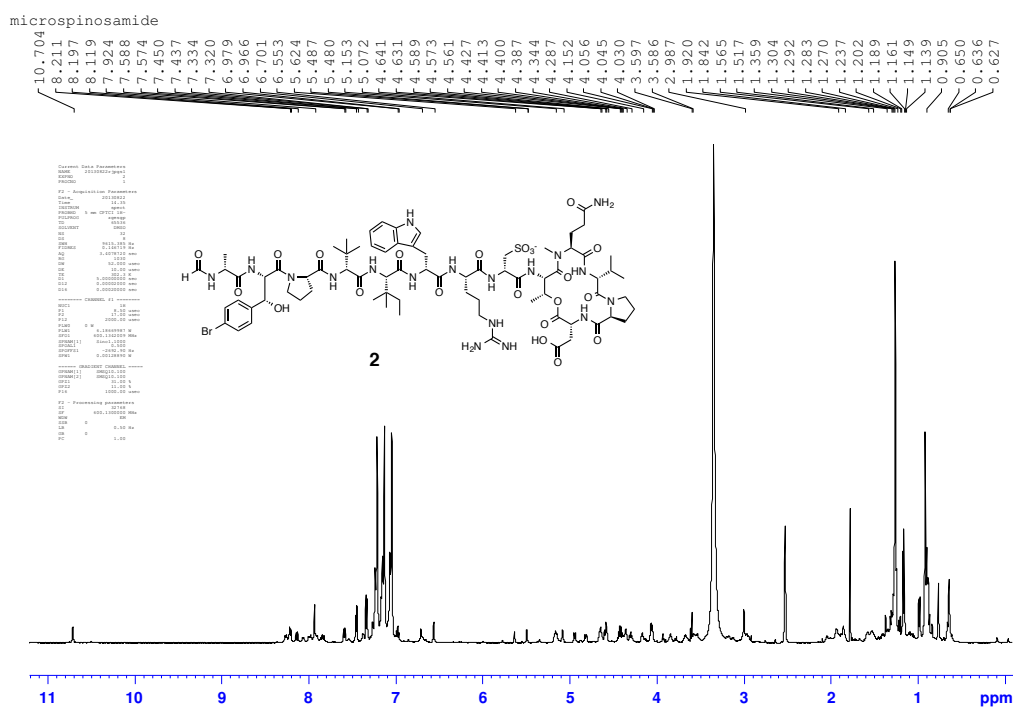


Figure 9  $^1\text{H}$  NMR of **2** (600 MHz,  $\text{DMSO}-d_6$ ).

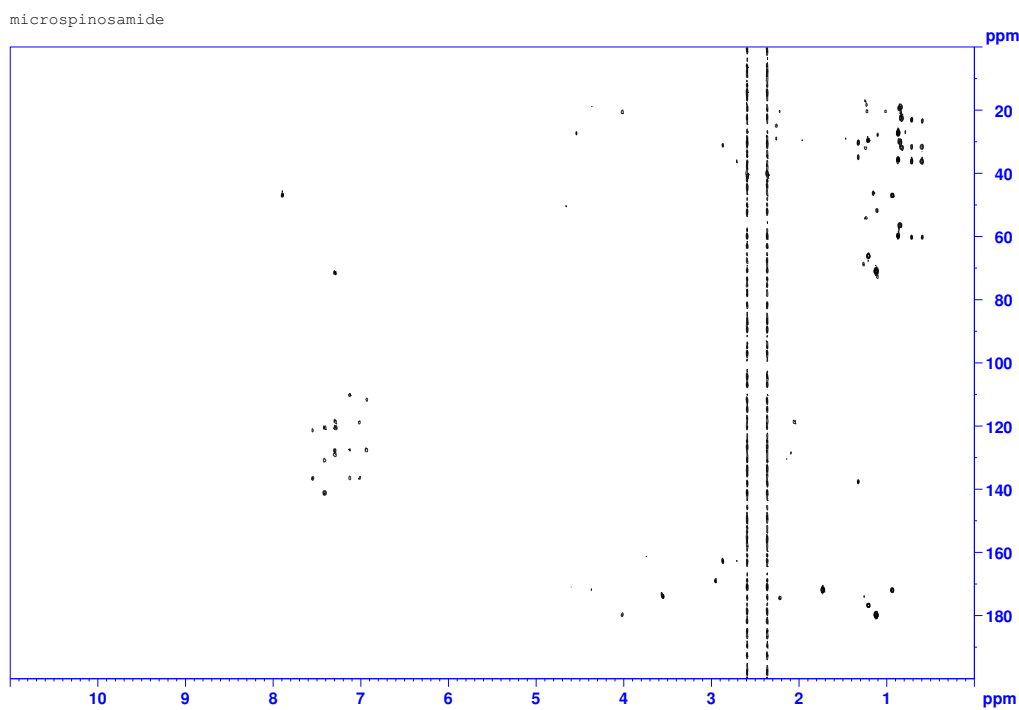


Figure 10  $^{13}\text{C} - ^1\text{H}$  HMBC of **2** (150 MHz,  $\text{DMSO}-d_6$ ).

# Generic Display Report

## Analysis Info

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Method 1MW Positive ESI  
Sample Name Microspinosamide  
Comment premixed. CHCA(10mg/mL, CH3CN:H2O (7:3))/sample(1 mg/mL, CH3CN:H2O (7:3) with 0.1% TFA) (10:1)

Acquisition Date 11/06/2013 5:28:49 PM

Operator

Instrument apex-Ultra

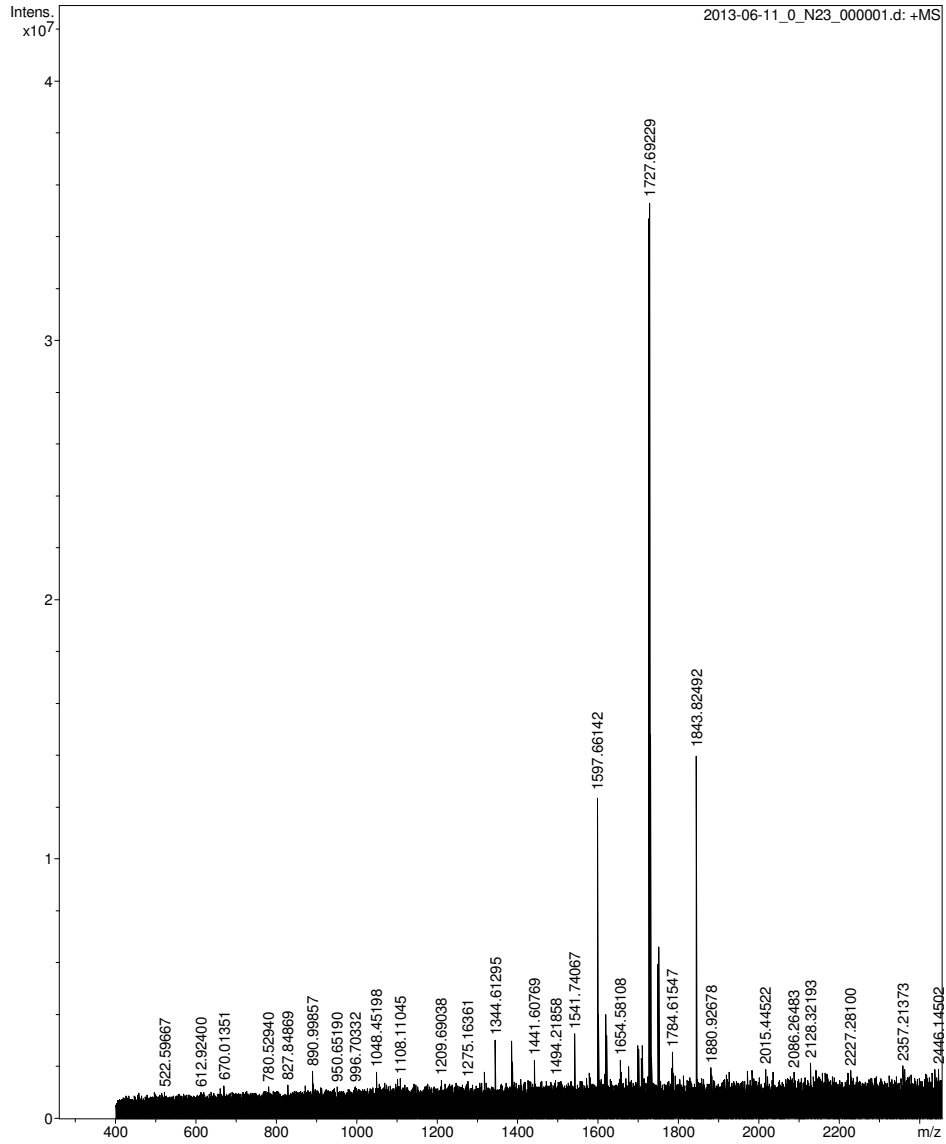


Figure 11 MALDI-FTICR of 2

## Generic Display Report

### Analysis Info

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Sample Name Microspinosamide Instrument apex-Ultra  
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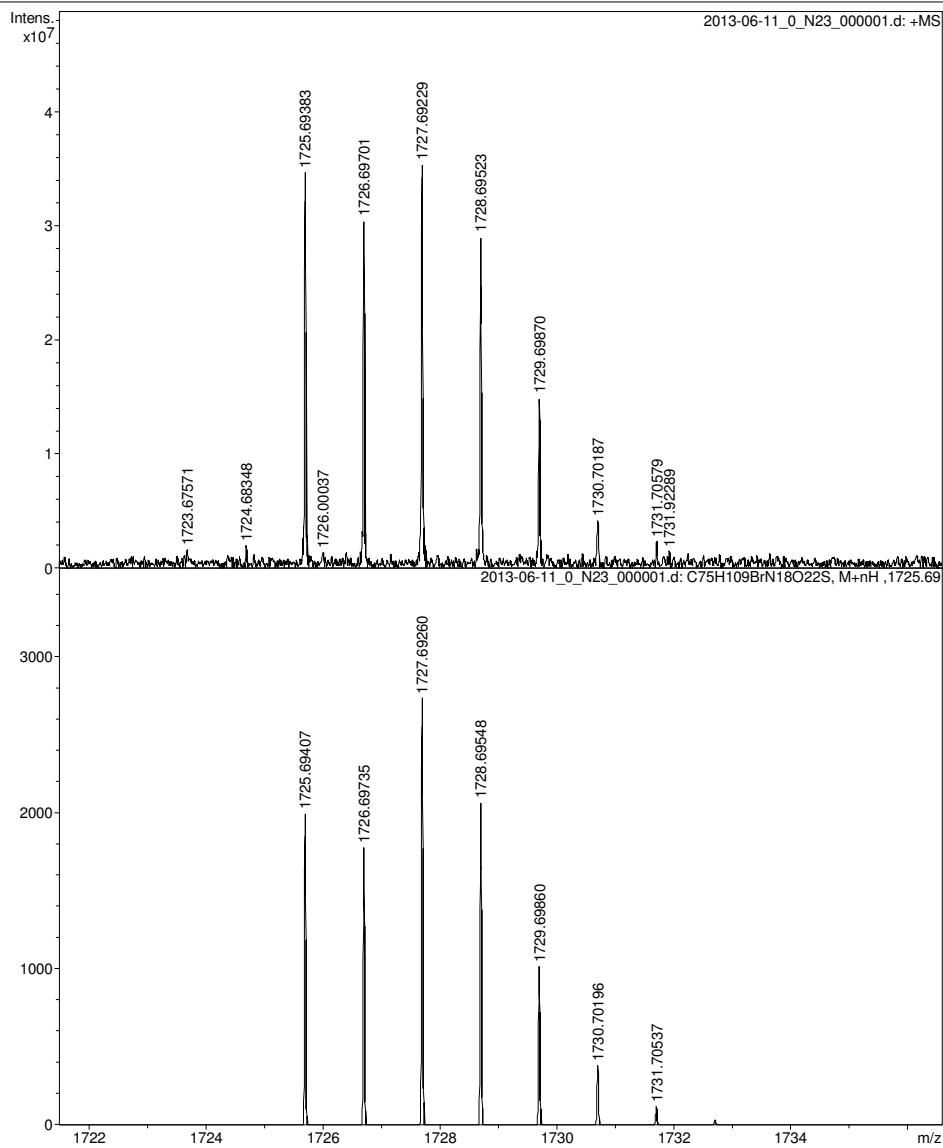
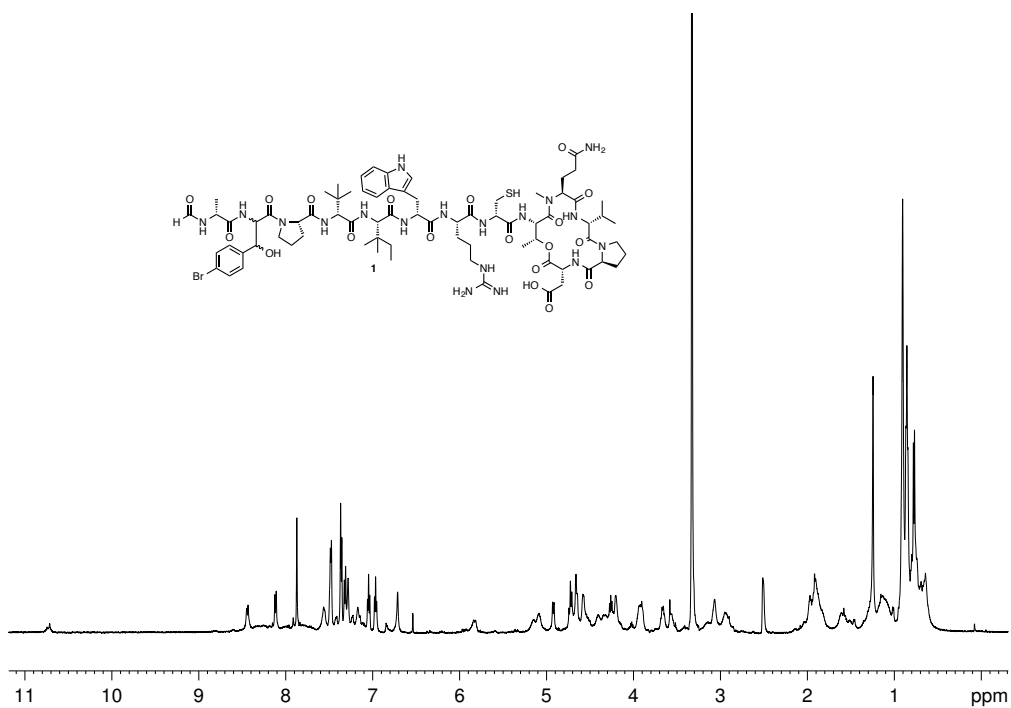
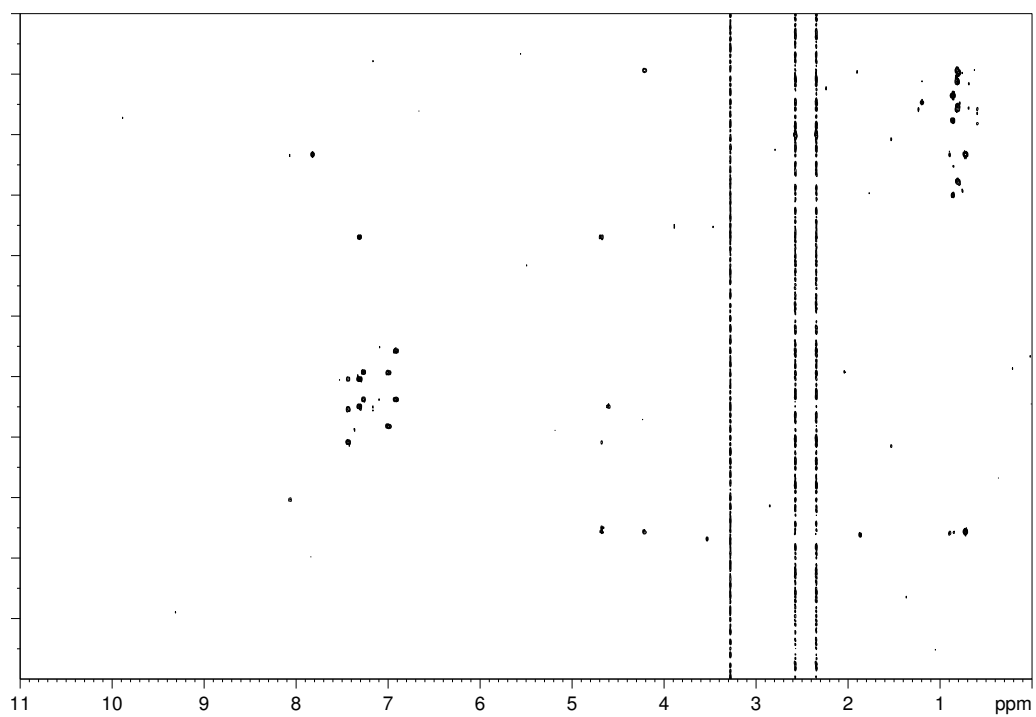


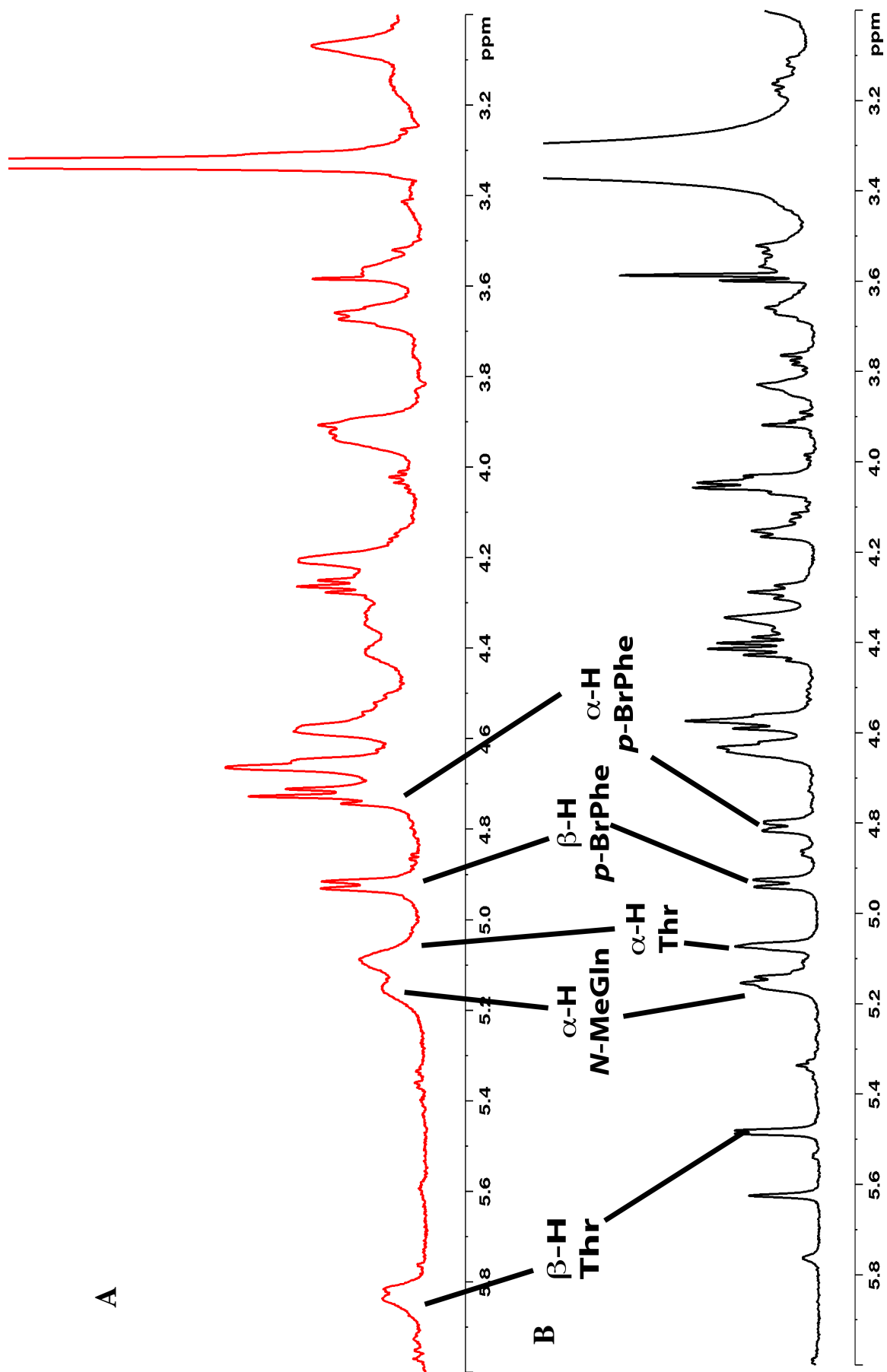
Figure 12 MALDI-FTICR of 2.



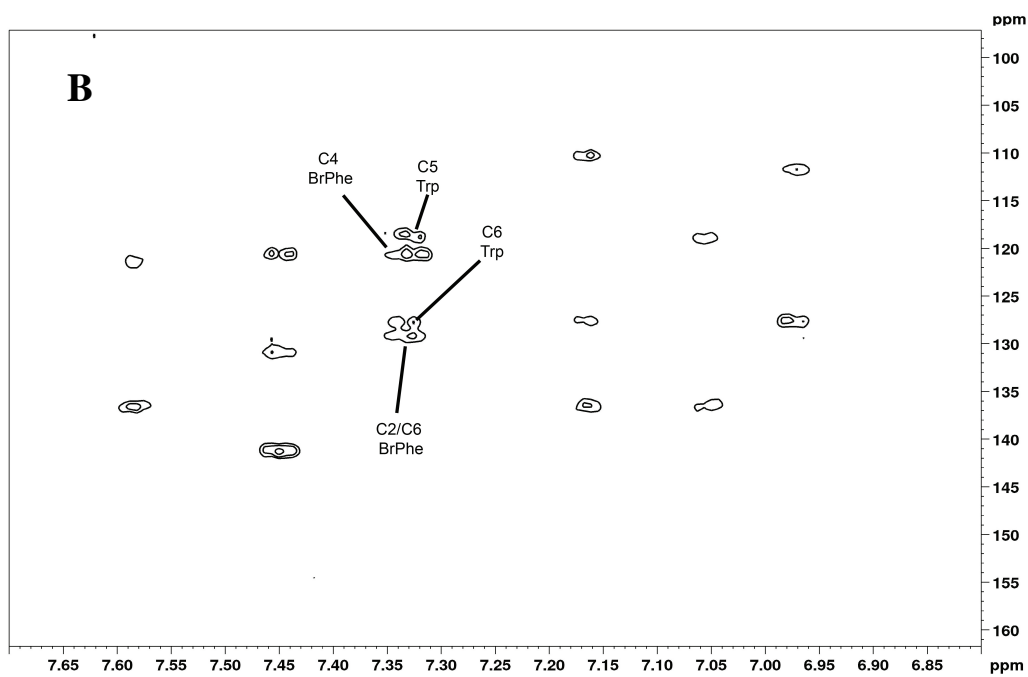
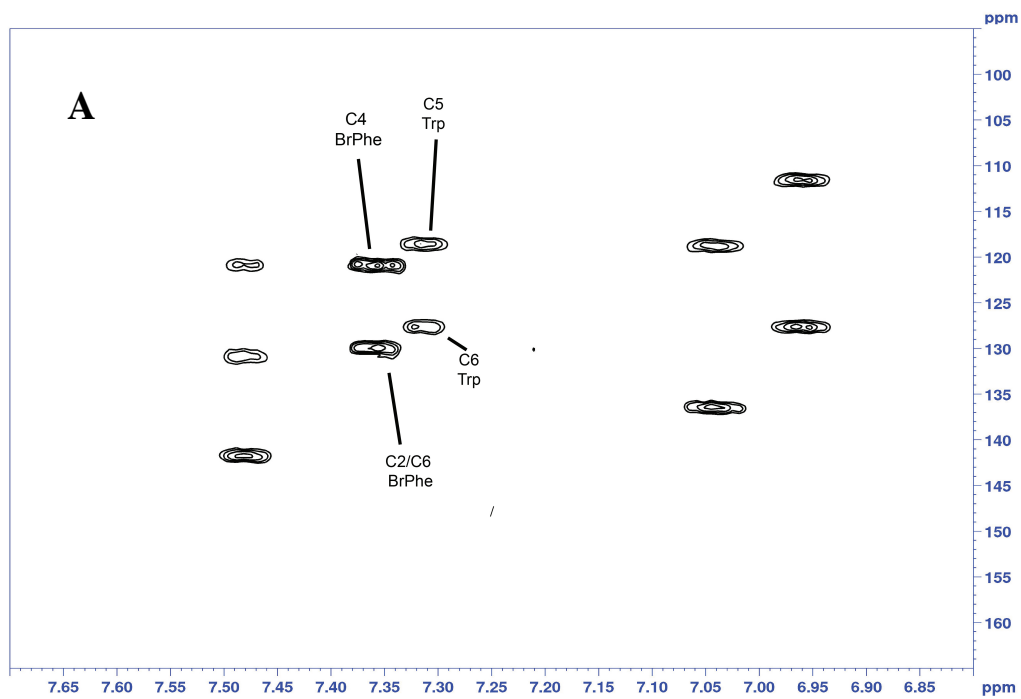
**Figure 13**  $^1\text{H}$  NMR of the isolated microspinosamide **1**



**Figure 14**  $^1\text{H} - ^{13}\text{C}$  HMBC of isolated microspinosamide **1**



**Figure 15** Comparison of the <sup>1</sup>H NMR Spectra (6.0 - 2.5 ppm) of the Synthetic and Isolated Material, <sup>1</sup>H NMR (600 MHz, DMSO-*d*<sub>6</sub>) of A) isolated natural product **1**, B) synthetic **2**



**Figure 16** Comparison of the  $^1\text{H}$ - $^{13}\text{C}$  HMBC (Aromatic Region) of the Synthetic and Isolated Material.  $^1\text{H}$ - $^{13}\text{C}$  HMBC (600 MHz,  $\text{DMSO}-d_6$ ) of A) isolated natural product **1**, B) synthetic **2**.



## References

- (1) Shafer, C. M.; Molinski, T. F. *J. Org. Chem.* **1996**, *61*, 2044-2050.
- (2) Pigza, J. A.; Molinski, T. F. *Org. Lett.* **2010**, *12*, 1256-1259.
- (3) Inoue, M.; Shinohara, N.; Tanabe, S.; Takahashi, T.; Okura, K.; Itoh, H.; Mizoguchi, Y.; Iida, M.; Lee, N.; Matsuoka, S. *Nat. Chem.* **2010**, *2*, 280-285.