Supporting Information

Apratyramide, a Marine-Derived Peptidic Stimulator of VEGF-A and Other Growth Factors with Potential Application in Wound Healing

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Supplementary Methods

General Experimental Procedures

Optical rotations were measured on a Perkin-Elmer 341 polarimeter. Nuclear magnetic resonance (NMR) spectra were recorded on a Varian 400 MHz or Bruker Avance II 600 MHz spectrometer as indicated in the data list. Chemical shifts for proton nuclear magnetic resonance (^{1}H NMR) spectra are reported in parts per million relative to the signal residual $CDCl_3$ at 7.26 ppm and DMSO-d_6 at 2.50 ppm. Chemicals shifts for carbon nuclear magnetic resonance (^{13}C NMR) spectra are reported in parts per million relative to the center line of the $CDCl_3$ triplet at 77.0 ppm and DMSO-d_6 at 40.0 ppm. Data are described as following: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, br = broad, m = multiplet), coupling constants (Hz), integration, and assignment. HRESIMS data were obtained using an Agilent LC-TOF mass spectrometer equipped with an APCI/ESI multimode ion source detector.

Collection, Extraction and Isolation

Five different Guamanian collections of freeze dried apratoxin A-producing *M. bouillonii* were collected from Fingers Reef, Guam. Each were individually extracted with CH_2Cl_2 and MeOH (2:1) by vigorous stirring for 48 h. The extract obtained (~80 g) was then partitioned between hexanes, *n*-BuOH and 80% aqueous MeOH. The crude fractions (hexanes 13 g; *n*-BuOH ~7 g) from all the partitions were then subjected to flash chromatography over silica gel with increasing amountsof *i*-PrOH in CH_2Cl_2. The orange band eluting with 4-6% *i*-PrOH in CH_2Cl_2 was collected and utilized for the re-isolation of apratoxin A. A combined fraction (~10 g) obtained after re-isolating apratoxin A was then chromatographed over silica gel with CH_2Cl_2 and gradients of *i*-PrOH to afford 16 factions (F_1-F_{16}). Fraction F_{12} (20 mg, 10% *i*-PrOH in CH_2Cl_2), F_{13} (76 mg, 20% *i*-PrOH in CH_2Cl_2), and F_{14} (120 mg, 20% *i*-PrOH in CH_2Cl_2) were individually subjected to semi-preparative HPLC (Phenomenex Phenyhexyl, ODS 250 × 10 mm, 5μ, 2.0 mL/min; PDA detection) using a MeOH-H_2O linear gradient (90−100% MeOH for 30 min; and 100% MeOH for 10 min). Semipure apratyramide (**1**) (t_R 11.8− 12.2 min) co-eluted with lyngbyabellin E[1] was collected and purified over reversed-phase semi-preparative HPLC (Phenomenex Ultracarb ODS 250 × 10 mm, 5μ, 2.0 mL/min; PDA detection) using the same HPLC conditions mentioned above to afford lyngbyabellin E (t_R 8.9 min) and semipure apratyramide (**1**) (t_R 9.4 min). Final purification of **1** was achieved using reversed-phase semi-preparative HPLC (Phenomenex Synergy AQ, ODS 150 × 10 mm, 5μ, 2.0 mL/min; PDA detection) using HPLC conditions as described earlier to afford pure apratyramide (**1**) (t_R 14.3 min, 2.0 mg).

Apratyramide (**1**): colorless, amorphous powder; $[\alpha]_D^{20}$ −101.9 (*c* 0.59, MeOH); ^{1}H and ^{13}C NMR data, Table S1; UV (MeOH) λmax (log ε) 210 (4.36), 220 (4.37), 230 (4.24), 270 (3.63); HR-ESI/APCI-MS *m/z* $[M+H]^+$ 805.4388 (calcd for $C_{44}H_{60}N_4O_{10}$, 805.4343)

Absolute Configuration

A sample of compound **1** (100 μg) was hydrolyzed with 6 N HCl (0.35 mL) at 110°C for 20 h. The hydrolyzate was concentrated to dryness, reconstituted in 100 μL of H_2O, and then analyzed by chiral HPLC [Chirobiotic TAG (250 × 4.6 mm), Supelco; solvent: MeOH:10 mM NH_4OAc (40:60, pH 5.48); flow rate 0.5 mL/min; detection by ESIMS in positive ion mode (MRM scan)]. *N*-Me-L-Tyr, *N*,*N*-diMe-L-Tyr(*O*Me) and Val eluted at t_R 18.5, 190 and 8.2 min, respectively. The retention times (t_R, min; MRM ion pair, parent→ product) of the authentic amino acids were as follows: *N*-Me- L-Tyr (19.1;196.3 →77), *N*-Me- D-Tyr (37.6), *N*,*N*-diMe-L-Tyr(*O*Me) (190.0; 224.3→179.3), *N*,*N*-diMe-L-Tyr(*O*Me) (130.0), L-Val (8.2;118.3→72), D-Val (15.1). The compound-dependent MS parameters were as follows: *N*-Me- Tyr: DP 40, EP 5, CE 65, CXP 3, CEP 10; *N*,*N*-diMe-Tyr(*O*Me): DP 38, EP 8, CE 19, CXP 3, CEP 17; Val: DP 135, EP 9, CE 15, CXP 2, CEP 106. The source and gas-dependent MS parameters were as follows: CUR 30, CAD high, IS 4500, TEM 450, GS1 40, GS2 40.

Hmpa in the hydrolyzate of **1** was detected in negative ion mode [column, Chirobiotic TAG (4.6 × 250 mm), Supelco; solvent, MeOH-10 mM NH4OAc (40:60, pH 5.33); flow rate, 0.5 mL/min; detection by ESIMS in negative ion mode (MRM scan)]. The MS parameters used were as follows: DP -30, EP -5.0, CE -18, CXP -8, CEP -130, CUR 30, CAD high, IS -4500, TEM 450, GS1 40, and GS2 40. (2*R*,3*S*)-Hmpa from the hydrolyzate eluted at t_R 6.4 min. The retention times (t_R, min; MRM ion pair, parent → product) of the authentic standards were as follows: (2*S*, 3*R*)-Hmpa (6.73; 131→ 85), (2*S*, 3*S*)-Hmpa (6.9; 131→ 85), (2*R*,3*S*)-Hmpa (7.2; 131→ 85), and (2*R*,3*R*)-Hmpa (7.7; 131→ 85). The hydrolyzate was examined under different HPLC conditions in order to confirm this assignment [column, Chiralpak MA (+) (4.6 × 50 mm), Daicel Chemical Industries, Ltd.; solvent, 2 mM $CuSO_4$-CH_3CN (90:10); flow rate, 1.0 mL/min; detection by UV absorption at 254 nm]. (2*R*,3*S*)-Hmpa from the hydrolysate eluted at t_R 15.4 min. The retention times (t_R, min) of the authentic standards were as follows: (2*R*,3*S*)-Hmpa (11.0), (2*R*,3*R*)-Hmpa (14.5), (2*S*,3*R*)-Hmpa (18.0), and (2*S*,3*S*)-Hmpa (22.0).

Cell Culture

Human keratinocyte HaCaT cells and human colon cancer HCT116 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum at 37 ºC humidified air and 5% CO_2. Human normal colon (CCD-18Co) cells (ATCC) were cultured in Eagle's minimal essential medium (EMEM) supplemented with 10% fetal bovine serum at 37 ºC humidified air and 5% CO_2. Human Umbilical Vein Endothelial Cells (HUVEC, cat# CC-2519, Lonza) were cultured in EGM (Lonza cat# CC-3124) under the same condition.

Cell Viability Assay (MTT)

Cells were seeded in a 96-well clear bottom plate, and 24 h later cells were treated with various concentrations of the apratyramide or solvent control (DMSO). After 48 h of incubation, cell viability was detected using MTT according to the manufacturer's instructions (Promega,

Madison, WI). Nonlinear regression analysis was carried out using GraphPad Prism software for IC_{50} value calculations.

Measurement of Human VEGF-A Secretion

HaCaT cells were seeded in a 96-well clear bottom plate. Cells were treated with various concentrations of apratyramide or solvent control (DMSO). After 24 h incubation, culture supernatants were collected for detection of VEGF-A using AlphaLISA kits (PerkinElmer, Waltham, MA) following the manufacturer's instruction. Briefly, acceptor bead and anti-VEGF-A antibody were incubated with the supernatants for 60 min, donor beads were added later and incubated for another 30 min. Signal was detected using Envision (PerkinElmer). Levels of VEGF-A (pg/mL) were calculated using a standard curve and then normalized based on cell numbers.

Immunoblot Analysis

HaCaT cells were seeded in 6-well clear bottom plate the day before treatment. The next day, cells were treated with **1** or solvent control (DMSO). 24 h later, whole cell lysates were collected using PhosphoSafe buffer (EMD Chemicals, Inc, Gibbstown, NJ). Protein concentrations were measured with the BCA Protein Assay kit (Thermo Fisher Scientific, Rockford, IL). Lysates containing equal amounts of protein were separated by SDS polyacrylamide gel electrophoresis (4–12%), transferred to polyvinylidene difluoride membranes, probed with primary and secondary antibodies, and detected with the SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific). BIP, IRE1, β-actin and secondary anti-mouse and rabbit antibodies were from Cell Signaling Technology, Inc (Danvers, MA). ATF4 and ORP150 antibodies were obtained from Santa Cruz (CA).

In Vitro Angiogenesis Assay

HaCaT cells were seeded in 12-well plates in complete growth medium the day before treatment. The next day, the medium was replaced with fresh medium followed by the treatment of **1** or solvent control. After 24 h or incubation, the conditioned medium was collected for in vitro angiogenesis assay.

HUVECs (Lonza) were used at passage 4 for this assay. In vitro Angiogenesis Assay Kit (Chemicon) was used according to the manufacturer's recommendation. Briefly, an ice-cold mixture of ECMatrix was transferred into a precooled 96-well plate. After the matrix solution had solidified (> 1 h incubation at 37 °C, 10,000 cells were mixed with the CM or EGM with or without the presence of VEGF-A protein, 100 ng/mL and plated into each well. After incubation at 37 °C for 14 h, images were captured for each well using a Nikon inverted microscope equipped with NIS-Elements software. Branch point counting was used as quantification method. Three random microscope view-fields were counted and the number of branch points was averaged for each well. Branch point for each group was calculated by averaging eight replicates from two independent experiments.

RNA Isolation and Reverse Transcription

HaCaT cells were seeded in 6-well plates at a density of 2×10^5 per well and incubated further for 24 h in growth medium prior to treatment. RNA was isolated at 3, 12 or 16 h post treatment using the RNeasy mini kit (QIAGEN, Valencia, CA). Total RNA was quantified using NanoDrop 2000. From 2 μg of total RNA, cDNA synthesis was done using SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA) and oligo (dT) (Invitrogen).

Real-time Quantitative Polymerase Chain Reaction (qPCR) for Transcript Level Determination in HaCaT cells

qPCR after reverse transcription (RT-qPCR) was performed on a 25 μL reaction solution containing a 0.3 μL aliquot of cDNA, 12.5 μL of TaqMan gene expression assay mix, 1.25 μL of probes, and 11 μL RNase-free water. qPCR was carried out on an ABI 7300 sequence detection system using the thermocycler program: 2 min at 50 °C, 10 min at 95 °C, and 15 s at 95 °C (40 cycles) and 1 min at 60 °C. Each experiment was performed in triplicate. *VEGF-A* (Hs00900055_m1), *PDGFB* (Hs00266645_m1) and *bFGF* (Hs00966522_m1) were used as target genes, while *GAPDH* (Hs02758991_g1) was used as endogenous control. Graphs and data analysis were performed using the Prism software and analyzed using unpaired t test.

Transcriptome Profiling

RNA was analyzed using a NanoDrop spectrophotometer and Agilent 2100 Bioanalyzer to determine the RNA concentration and quality, respectively. RNA samples were processed using the GeneChip WT PLUS Reagent Kit (Affymetrix, Santa Clara, CA) according to the manufacturer's instruction. In brief, 250 ng of RNA were used for cDNA synthesis by reverse transcription, and the cDNA was utilized as a template for the biotin-labeled RNA prepared by in vitro transcription reaction. The labeled RNA was further purified, fragmented, and hybridized with rotation at 45 °C for 16 h to the GeneChip™ Human Transcriptome Array 2.0. The arrays were washed and stained using the GeneChip Hybridization Wash and Stain kit on an Affymetrix Fluidics Station 450. The chips were scanned using a GeneChip 7G Scanner. Analysis of the microarray data was done according to the reported method.[2] Statistical tests were performed with Bioconductor statistical software (http://www.bioconductor.org/) and R program. Raw data were normalized by the Robust Multichip Analysis approach. Then the probe set's detection call was estimated by using the Wilcoxon signed rank-based algorithm. Probe sets that are absent in all of the study samples were removed from further analyses. A linear modeling approach and the empirical Bayes statistics as implemented in the limma package in the R software were used for differential expression analysis. The P values obtained were controlled for multiple testing (false discovery rate) using the Benjamini-Hochberg method. Differentially expressed genes were then ranked by the P values, and genes with $P < 0.05$ (with FDR correction) and fold change >1.5 or <0.67 were considered as differentially expressed genes at a statistically significant level. Hierarchical clustering of the data was computed on log-transformed and normalized data by using complete linkage and Pearson correlation distances. The transcriptome data are deposited in NCBI's Gene Expression Omnibus with accession number GSE102100.

Ex Vivo Organ Culture of Rabbit Corneas

The central 6 mm diameter area of corneas of twelve fresh rabbit globes (Pelfreeze) was ablated to a total depth of 155 microns using a Nidek excimer laser in phototherapeutic keratectomy mode. Ablated corneas were then surgically dissected from the rabbit globes using sterile scalpel and forceps, grasping only the scleral rims and not the clear cornea. The corneas were cultured in DMEM/F-12 1:1 (Thermo Fisher) containing 40 mM HEPES, 10% FBS, 0.01 % dextran 40 (Tokyo Chemical Industry, Co., Ltd.) and 0.025% chondroitin sulfate (Chem-Impex International, Inc.) in 6-well plates. Twelve corneas were randomly distributed into four groups (three corneas in each group): DMSO, 25 μM, 50 μM or 100 μM apratyramide. Compounds or solvent (1% DMSO) were added to the media immediately to fully immerse the corneas in the 6-well plates. The corneas were incubated for 18 h at 37 °C in humidified atmosphere containing 5% CO_2 before total RNA was extracted.

Evaluation of mRNA Level of *VEGF-A* in Ablated Rabbit Corneas After Treatment with Apratyramide

The central scar-like tissue from each cornea was collected using an 8 mm punch biopsy. RNA was collected from the scar-like tissue using TRIzol Reagent (Life Technologies) according to manufacturer's procedure. To generate and validate the VEGF-A primer set (IDT DNA), cDNA was generated from whole rabbit eye RNA using the iScript™ Select cDNA Synthesis Kit (Bio-Rad) according to manufacturer's procedure. The amplicon obtained from the primer set was gel purified to ensure a single product and then sequenced to confirm the predicted sequence. The purified cDNA was then quantified using nanodrop and used to generate a standard curve. Serial 10- fold dilutions of the quantified cDNA amplicon was subjected to RT-qPCR using SYBR Select Master Mix (Applied Biosystems). A standard curve of Log [cDNA input]) vs observed CT values was made to demonstrate the proportional response to input cDNA for the mRNA target (Figure S22). GAPDH levels were found to vary more than that of the target gene. Among the biological replicates, the average standard deviation for GAPDH was 1.54 C_t & 1.98 C_t for control and 100 μM treatment, respectively; while it was only 0.09 C_t / 0.08 C_t for VEGF-A. This degree of variation in GAPDH per unit total RNA demonstrates that GAPDH is a poor internal control for our purposes. The high degree of consistency among biological replicates for mass-normalized VEGF-A supports the choice to normalize to input total RNA. The mass-normalized C_t values for the apratyramide treatment groups and DMSO control group (n = 3 biological replicates per group) were analyzed by a one-way ANOVA ($\alpha = 0.05$, Microsoft Excel 2010, Analysis Tool Pack) followed by Tukey's HSD *post hoc* test manually calculated in Excel 2010 (Figure S23).

General Experimental Procedures for Synthesis

Solvents were purified by standard methods. TLCs were carried out on Merck 60 F_{254} silica gel plates and visualized by UV irradiation or by staining with aqueous acidic ammonium molybdate solution as appropriate. Flash column chromatography was performed on silica gel (170–400 mesh, Fisher, USA).

Synthesis of compound 4. 7.87 g (60 mmol) of L-Ile was dissolved in ice-cold H_2SO_4 (40 mL, 2.5 mol/L). 6.21 g (90 mmol) of $NaNO_2$ was dissolved in 30 mL of H_2O, and was added to the above solution dropwise. The mixture was kept at 0 °C for 2 h, then warmed to rt and stirred for another 16 h. After extraction with EtOAc (200 mL × 1, 100 mL × 3), the combined organic phase was washed with brine (50 mL × 1), dried over Na_2SO_4, and concentrated in vacuo, giving the α-hydroxy acid (4.50 g, 57%) as colorless oil, which was used for next step without further purification.

To a solution of the α-hydroxy acid (2.64 g, 20 mmol) obtained above in a mixed solvent (MeOH/H_2O, 60 mL/15 mL), was added Cs_2CO_3 (3.91 g, 12 mmol) in 20 mL of H_2O. The mixture was concentrated to dryness after stirring for 5 min. The crude cesium salt was redissolved in 30 mL of DMF, followed by the addition of BnBr (2.85 mL, 24.0 mmol), and then stirring for 24 h.

After filtration and removal of the solvent, the residue was suspended in water (50 mL), and extracted with Et_2O (150 mL × 1, 50 mL × 3). The extracts were combined, dried over Na_2SO_4 and concentrated in vacuo. The residue was purified by chromatography on silica gel to give the desired benzyl ester (**4**) as colorless oil (3.45 g, 78%).

Compound **4**. $[\alpha]_D^{20}$ −13.4 (*c* 1.58, MeOH); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.30-7.28 (m, 5H, ArH), 5.23 (d, *J* = 12.2 Hz, 1H, PhC<u>Ha</u>Hb), 5.17 (d, *J* = 12.2 Hz, 1H, PhCHa<u>Hb</u>), 4.11 (dd, *J* = 3.2, 1.6 Hz, 1H, H-2), 2.79 (brs, 1H, OH), 1.88-1.75 (m, 1H, H-3), 1.35-1.30 (m, 1H, H-4a), 1.27-1.16 (m, 1H, H-4b), 0.96 (d, *J* = 6.9 Hz, 3H, CH_3), 0.84 (t, *J* = 8.2 Hz, 3H, CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 175.0, 135.4, 128.8, 128.7, 128.6, 75.0, 67.3, 39.3, 23.8, 15.6, 11.9; ESI-MS (*m/z*) 245.3 $[M+Na]^+$; HRESIMS $C_{13}H_{18}O_3Na$ calcd 245.1148 $[M+Na]^+$, found 245.1155.

Synthesis of 5. Boc-Tyr(OMe)-OH (708.7 mg, 2.4 mmol), alcohol **4** (444.6 mg, 2.0 mmol), and Ph_3P (786.9 mg, 3.0 mmol) were dissolved in 50 mL of dry THF. After cooling with an ice-water bath for 20 min, 1.40 mL (3.0 mmol) of DEAD (40% solution in toluene) was added dropwise to this mixture. After stirring at 0 °C for 1 h and then at rt for another 15 h, the solvent was removed in vacuo. The residue was purified by chromatography on silica gel, giving the desired compound (**5**) as a colorless oil (954.80 mg, 96%).

Compound **5**. $[\alpha]_D^{20}$ + 2.9 (*c* 0.51, MeOH); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.36-7.28 (m, 5H, ArH), 7.05 (d, *J* = 8.6 Hz, 2H, Ar =H), 6.80 (d, *J* = 8.5 Hz, 2H, Ar H), 5.18 (d, *J* = 12.2 Hz, 1H, C_6H_5C<u>Ha</u>HbO), 5.13 (d, *J* = 12.2 Hz, 1H, C_6H_5CHa<u>Hb</u>O), 5.01 (d, *J* = 3.4 Hz, 1H), 4.93 (d, *J* = 7.8 Hz, 1H), 4.61 (dd, *J* = 13.8, 6.6 Hz, 1H, Tyr αH), 3.75 (s, 3H, OCH_3), 3.08 (dd, *J* = 14.1, 6.1 Hz, 1H, *p*-$MeOC_6H_4C$<u>Ha</u>Hb), 2.96 (dd, *J* = 14.1, 6.6 Hz, 1H, *p*-$MeOC_6H_4$CHa<u>Hb</u>), 1.97-1.93 (m, 1H, Hmp H-3), 1.39 (s, 9H, Boc), 1.34-1.28 (m, 1H, Hmp H-4a), 1.24-1.14 (m, 1H, Hmp H-4b), 0.866 (d, *J* = 6.4 Hz, 3H, Hmp CH_3), 0.865 (t, *J* = 7.0 Hz, 3H, Hmp CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 171.9, 169.5, 158.8, 155.1, 135.5, 130.5, 128.8, 128.6, 128.5, 128.1, 114.1, 80.0, 75.8,

67.2, 55.4, 54.8, 37.6, 36.8, 28.5, 26.0, 14.4, 11.8; ESI-MS (*m/z*) 522.4 $[M+Na]^+$; HRESIMS $C_{28}H_{37}NO_7Na$ calcd 522.2469 $[M+Na]^+$, found 522.2483.

Synthesis of 2. 499.6 mg (1.0 mmol) of compound **5** was treated with 4M HCl-EtOAc (5 mL) for 1 h. After concentration to dryness, 5 mL of EtOAc was added and the solution concentrated again. The residue was dissolved in CH_3CN (5 mL), followed by the addition of DIEA (248 μL, 1.5 mmol) at 0 °C, and then 37% aqueous formaldehyde solution followed by AcOH (0.1 mL). After stirring for 1 h, $NaBH_3CN$ (188.5 mg, 3.0 mmol) was added carefully. AcOH was added periodically to maintain a pH of 5-7, and the mixture was stirred for another 24 h at rt. After concentration in vacuo, the residue was re-dissolved in EtOAc, washed with saturated Na_2CO_3 solution and brine, dried over Na_2SO_4, and then purified by column chromatography to give compound **2** as colorless oil (46 % Yield).

Compound **2.** $[\alpha]_D^{20}$ + 58.7 (*c* 1.46, MeOH); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.35-7.30 (m, 5H, ArH), 7.09 (d, *J* = 8.4 Hz, 2H, ArH), 6.77 (d, *J* = 8.5 Hz, 2H, ArH), 5.17 (d, *J* = 12.2 Hz, 1H, $C_6H_5CHaHbO$), 5.11 (d, *J* = 12.2 Hz, 1H, $C_6H_5CHaHbO$), 4.89 (d, *J* = 3.2 Hz, 1H, Hmp αH), 3.74 (s, 3H, OCH_3), 3.46 (dd, *J* = 9.7, 5.7 Hz, 1H, Tyr αH), 2.98 (dd, *J* = 13.2, 10.0 Hz, 1H, *p*-$MeOC_6H_4CHaHb$), 2.86 (dd, *J* = 13.4, 5.6 Hz, 1H, *p*-$MeOC_6H_4CHaHb$), 2.39 (s, 6H, NMe_2), 1.86-1.78 (m, 1H, Hmp H-3), 1.01-0.84 (m, 2H, Hmp H-4), 0.75 (d, *J* = 6.8 Hz, 3H, Hmp CH_3), 0.68 (t, *J* = 7.3 Hz, 3H, Hmp CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 171.7, 170.0, 158.4, 135.5, 130.3, 130.0, 128.7, 128.6, 114.0, 74.7, 69.5, 67.1, 55.4, 41.8, 36.6, 36.2, 25.8, 14.4, 11.7; ESI-MS (*m/z*) 428.5 $[M+H]^+$; HRESIMS $C_{25}H_{34}NO_5$ calcd 428.2431 $[M+H]^+$, found 428.2438.

Synthesis of dipeptide 6. H-MeTyr(OBn)-OMe hydrochloride (1007.5 mg, 3.0 mmol) and 1079.0 mg (2.8 mmol) of Boc-MeTyr(OBn)-OH were dissolved in 60 mL of iced-cold DCM. EDC (644.0 mg, 3.36 mmol), HOAt (457.0 mg, 3.36 mmol) and DIEA (926 μL, 5.6 mmol) were added, respectively. The mixture was stirred at 0 °C for 2 h, and then at rt for another 16 h before diluted with 200 mL of EtOAc. The organic phase was washed with 1 mol/L HCl (20 mL × 3), saturated $NaHCO_3$ (20 mL × 3), brine (20 mL × 3), dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by column chromatography to give the desired dipeptide **6** (1500.7 mg, 80%) as colorless oil.

Compound **6.** $[\alpha]_D^{20}$ −103.2 (*c* 0.50, MeOH); ^{1}H NMR ($CDCl_3$, 400 MHz, mixture of rotamers) δ 7.38-7.27 (m, 10H, ArH), 7.17-7.02 (m, 2H, ArH), 6.88-6.77 (m, 6H, ArH), 5.17-5.06 (m, 1H, Tyr αH), 5.03-4.96 (brd, overlapped, 4H, $C_6H_5CH_2O$ × 2), 4.72 (dd like overlapped, 1H, Tyr αH), 3.72, 3.702, 3.695, 3.688 (each s, total 3H, CO_2CH_3), 3.33-3.19, 3.02-2.96, 2.92-2.89, 2.73-2.72 (dd like overlapped, total 4H, *p*-$BnOC_6H_4CH_2$ × 2), 2.93, 2.85, 2.76, 2.68, 2.65, 2.60, 2.39, 2.28 (each s, total 6H, N-CH_3 × 2), 1.32, 1.27, 1.17, 1.14 (each s, total 9H, Boc); ^{13}C NMR (100 MHz, $CDCl_3$, mixture of rotamers) δ 171.1, 170.6, 157.7, 157.6, 155.3, 137.4, 137.2, 130.8, 130.7, 129.9, 128.8, 128.7, 128.13, 128.07, 127.6, 127.5, 115.3, 114.8, 80.5, 70.2, 58.6, 56.5, 52.5, 34.5, 33.7, 32.7,

29.3, 28.4; ESI-MS (*m/z*) 689.5 $[M+Na]^+$; HRESIMS $C_{40}H_{46}N_2O_7Na$ calcd 689.3197 $[M+Na]^+$, found 689.3197.

Synthesis of tripeptides 3. Dipeptide **6** (641.7 mg, 0.96 mmol) was treated with 4M HCl-EtOAc (5 mL) for 1 h. After concentration to dryness, 5 mL of EtOAc was added and the solution concentrated again. The resulting amine hydrochloride and Boc-Val-OH (209.0 mg, 0.96 mmol) were dissolved in 15 mL of dry DCM and cooled with ice-water bath. EDC (220.8 mg, 1.15 mmol), HOAt (156.8 mg, 1.15 mmol) and DIEA (317 μL, 1.9 mmol) were added, respectively. The mixture was stirred at this temperature for 2 h and at rt for another 16 h. Then the mixture was diluted with 150 mL of EtOAc, washed with 1 mol/L HCl (20 mL × 3), saturated $NaHCO_3$ (20 mL × 3), brine (20 mL × 3), dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by column chromatography to give the desired tripeptide **3** (220.9 mg, 30%) as white foam.

Compound **3.** $[\alpha]_D^{20}$ −165.2 (*c* 0.25, MeOH); ^{1}H NMR ($CDCl_3$, 400 MHz, mixture of rotamers) δ 7.38-7.27 (m, 10H, ArH), 7.15 (d, *J* = 8.4 Hz, 2H, ArH), 6.87 (d, *J* = 8.4 Hz, 2H, ArH), 6.80-6.75 (m, 4H, ArH), 5.49 (t like, *J* = 7.6, 6.6 Hz, 1H), 5.04-4.94 (m, 4H, $C_6H_5CH_2O$ × 2), 4.83-4.77 (m, 1H), 4.30 (dd, *J* = 9.1, 4.9 Hz, 1H), 3.67 (s, 3H, CO_2CH_3), 3.27-3.17 (m, 2H, $ArCH_2$), 2.96-2.81 (m, 2H, $ArCH_2$), 2.61 (s, 3H, N-CH_3), 2.59 (s, 3H, N-CH_3), 1.78-1.69 (m, 1H, Val βH), 1.41 (s, 9H, Boc), 0.89 (d, *J* = 6.6 Hz, 3H, Val CH_3), 0.77 (d, *J* = 6.3 Hz, 3H, Val CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz, mixture of rotamers) δ 171.9, 170.9, 169.9, 158.1, 157.8, 155.9, 137.3, 137.0, 130.8, 130.0, 129.4, 128.8, 128.7, 128.2, 128.0, 127.7, 127.62, 127.59, 115.1, 114.8, 79.7, 70.2, 70.1, 60.6, 55.2, 53.4, 52.5, 34.3, 33.6, 31.1, 30.7, 30.4, 28.5, 20.1, 16.8; ESI-MS (*m/z*) 788.5 $[M+Na]^+$; HRESIMS $C_{45}H_{55}N_3O_8Na$ calcd 788.3881 $[M+Na]^+$, found 788.3881.

Synthesis of 1. Hydrogenation of **2** (50.0 mg, 0.12 mmol) was carried out in MeOH (5 mL) in the presence of a catalytic amount of Pd-C (10%) under hydrogen at rt. Pd-C was removed by filtration through a celite and concentrated under reduced pressure to give the corresponding carboxylic acid, which was used directly in the next step.

The tripeptide **3** (89.6 mg, 0.12 mmol) was treated with 4M HCl-EtOAc (3 mL) for 1 h and the solution concentrated under reduced pressure. The residue was re-dissolved in 5 mL of EtOAc and concentrated again, giving the corresponding amine as its hydrochloride.

The carboxylic acid and the amine (as its hydrochloride) obtained above were dissolved in DMF (5 mL) and cooled with ice-water bath. HATU (54.0 mg, 0.14 mmol), HOAt (19.0 mg, 0.14 mmol), and DIEA (78 μL, 0.47 mmol) were added, respectively. The mixture was stirred at 0 °C for 1 h, and then at rt for another 16 h. The solvent was diluted with EtOAc (100 mL), washed with water (10 mL × 3), 1 mol/L HCl (10 mL × 3), saturated $NaHCO_3$ (10 mL × 3), and brine (10 mL × 3), dried over Na_2SO_4 and concentrated in vacuo. The residue was purified by preparative TLC to give the crude fully protected pentapeptide **7** {43.6 mg, 38%; ESI-MS (*m/z*) 985.6 $[M+H]^+$}, which was used for next step without further characterization.

Compound **7** (48.0 mg, 0.049 mmol) was hydrogenated for 2 h in the presence of catalytic palladium on charcoal (10 wt%), giving the crude pentapeptide **1**, which was finally purified on semi-prepared RP-HPLC (Phenomenex Luna 5 µm, 250 × 10 mm, 2.0 mL/min, gradient elution with 75% to 100% MeOH-H_2O over 30 min) to give the pure compound **1** (24.9 mg, 63%).

Compound **1.** $[\alpha]_D^{20}$ −101.8 (*c* 0.22, MeOH); ^{1}H NMR (DMSO-*d*$_6$, 400 MHz) 9.22 (brs, 2H, Tyr OH×2), 8.09 (d, *J* = 8.9 Hz, 1H, Val NH), 7.10 (d, *J* = 8.5 Hz, 2H, ArH), 6.99 (d, *J* = 8.4 Hz, 2H, ArH), 6.79 (d, *J* = 8.6 Hz, 2H, ArH), 6.68 (d, *J* = 8.4 Hz, 2H, ArH), 6.57 (d, *J* = 8.4 Hz, 2H, ArH), 6.51 (d, *J* = 8.4 Hz, 2H, ArH), 5.27 (dd, *J* = 9.8, 3.8 Hz, 1H, NMeTyr-1 αH), 4.81 (d, *J* = 3.7 Hz, 1H, Hmp αH), 4.54 (dd, *J* = 11.4, 4.2 Hz, 1H, NMeTyr-2 αH), 4.45 (t, *J* = 8.4 Hz, 1H, Val αH), 3.69 (s, 3H, *N*,*N* diMeTyr(OMe) *O*-Me), 3.59 (s, 3H, Hmp CO_2CH_3), 3.46 (dd, *J* = 8.8, 6.6 Hz, 1H, *N*,*N* diMeTyr(OMe) αH), 3.09 (dd, *J* = 12.4, 10.5 Hz, 1H, NMeTyr-1 H-3a), 2.98 (dd, *J* = 13.9, 4.1 Hz, 1H, NMeTyr-2 H-3a), 2.87-2.80 (dd, overlapped, 2H, $ArCH_2$), 2.76 (dd, *J* = 13.7, 6.5 Hz, 1H, ArCHaHb), 2.70 (s, 3H, NMeTyr-1 N-Me), 2.44 (s, 3H, NMeTyr-2 N-Me), 2.34 (dd, *J* = 13.2, 3.7 Hz, 1H, NMeTyr-1 H-3b), 2.29 (s, 6H, NMe_2), 1.94-1.85 (m, 1H, Val H-3), 1.76-1.68 (m, 1H, Hmp H-3), 1.08-0.98 (m, 1H, Hmp H-4a), 0.96-0.86 (m, 1H, Hmp H-4b), 0.78 (br d, *J* = 6.6 Hz, 6H, Val CH_3×2), 0.69 (t like, *J* = 7.2, 7.0 Hz, 6H, Hmp CH_3×2); ^{13}C NMR (DMSO-*d*$_6$, 100 MHz) 170.62, 170.58, 170.4, 168.7, 168.5, 157.8, 155.82, 155.78, 130.2, 130.0, 129.80, 129.76, 127.8, 127.2, 115.01, 114.97, 113.6, 75.1, 68.3, 61.2, 54.9, 53.7, 51.7, 41.1, 36.3, 34.4, 33.8, 32.5, 30.1, 29.6, 25.3, 19.3, 17.8, 13.8, 11.3; ESI-MS (*m/z*) 805.7 $[M+H]^+$; HRESIMS $C_{44}H_{61}N_4O_{10}$ calcd 805.4382 $[M+H]^+$, found 805.4371.

Supplementary References

(1) Han, B., McPhail, K. L., Gross, H., Goeger, D. E., Mooberry, S. L., and Gerwick, W. H. (2005) Isolation and structure of five lyngbyabellin derivatives from a Papua New Guinea collection of the marine cyanobacterium Lyngbya majuscula. *Tetrahedron 61*, 11723–11729.

(2) Salvador, L. A., Taori, K., Biggs, J. S., Jakoncic, J., Ostrov, D. A., Paul, V. J., and Luesch, H. (2013) Potent elastase inhibitors from cyanobacteria: Structural basis and mechanisms mediating cytoprotective and anti-inflammatory effects in bronchial epithelial cells. *J. Med. Chem. 56*, 1276–1290.

Supplementary Tables

Table S1. NMR spectroscopic data of apratyramide (**1**) in DMSO-d_6

Unit	C/H no	δ_H (*J* in Hz)	δc	COSY	HMBC	Key NOESY
N,*N*-di MeTyr(*O*Me)	1		170.62			
	2	3.46, dd (9.4, 6.2)	68.28	H-3a, H-3b	C-3, C-1, *N*Me	
	3a	2.82, dd (13.4, 9.4)	34.41	H-3b, H-2	C-9/C-5, C-4, C-2, C-1	
	3b	2.77, dd (13.4, 6.2)	34.41	H-3a, H-2	C-9/C-5, C-4, C-2, C-1	
	4		130.2			
	5, 9	7.10, d (8.7)	130	H-6, H-8	C-9/C-5, C-7, C-3	
	6, 8	6.79, d (8.7)	113.55	H-5, H-9	C-7, C-4	
	7		157.74			
	*N*Me	2.28, s	41.11		C-2, *N*Me	
	*O*Me	3.69	54.98		C-7	
Hmpa	1		168.71			
	2	4.81, d (3.8)	75.09	H-3	C-1, C-6, C-3, C-4, C-1 (*N*,*N*-diMeTyr(OMe))	NH (Val)
	3	1.72, m	36.28	H-2, H-6	C-6, C-4	NH (Val)
	4a	1.03, m	25.33	H-5, H-4b	C-5, C-6, C-3	
	4b	0.91, m	25.33	H-5, H-4a	C-5, C-6, C-3	
	5	0.69, t (7.5)	11.28	H-4a, H-4b	C-4, C-3	C-8 (*N*,*N*-diMeTyr(OMe)), C-9 (*N*,*N*-diMeTyr(OMe))
	6	0.68, d (7.0)	13.83	H-3	C-2	
Val	1		170.58			
	2	4.45, t (8.6)	53.65	H-3, NH	C-1, C-3, C-4, C-5, C-1 (Hmpa)	
	3	1.9, m	29.63	H-2, H-4, H-5	C-5, C-4, C-2	
	4	0.77, d (6.7)	17.83	H-3	C-5, C-3, C-2	
	5	0.78, d (6.7)	19.27	H-3	C-4, C-3, C-2	
	NH	8.08		H-2	C-2, C-1 (Hmpa)	H-2 (Hmpa), H-5 (Hmpa)

Supplementary Tables

Table S1. Continued

Unit	C/H no	δ_H (*J* in Hz)	δc	COSY	HMBC	Key NOESY
N-MeTyr	1		168.52			
	2	5.27, dd (10.0, 3.8)	54.93	H-3a, H-3b	C-4, C-3, C-1, C-1 (Val), *N*-Me	
	3a	3.09, dd (13.0, 10.0)	33.77	H-3b, H-2	C-9/C-5, C-4, C-2, C-1	
	3b	2.33, dd (13.0, 3.8)	33.77	H-3b, H-2	C-9/C-5, C-4, C-2, C-1	
	4		128			
	5, 9	6.98, d (7.9)	130.18	H-6/H-8	C-9/C-5, C-7, C-3	
	6, 8	6.68, d (7.9)	115	H-5/H-9	C-7, C-4	
	7		155.8			
	7-OH	9.27, brs				
	*N*Me	2.70, s	30.07		C-1 (Val)	
N-MeTyr (1-*O*Me)	1		170.42			
	2	4.53, dd (11.8, 4.5)	61.28	H-3a, H-3b	C-3, C-1, C-1 (*N*-MeTyr), *N*-Me	
	3a	2.98, dd (14.0, 4.5)	32.45	H-3b, H-2	C-9/C-5, C-4, C-2	
	3b	2.83, dd (14.0, 11.8)	32.45	H-3b, H-2	C-9/C-5, C-4, C-2, C-1	
	4		127.2			
	5, 9	6.57, d (8.5)	129.8	H-6/H-8	C-9/C-5, C-7, C-3	
	6, 8	6.51, d (8.5)	115.14	H-5/H-9	C-7, C-4	
	7		155.8			
	7-OH	9.28, brs				
	*N*Me	2.44, s	34.47		C-2, C-1 (*N*-MeTyr)	
	1-*O*Me	3.59, s	51.47		C-1	

Table S2. Full list of up and down regulated genes after 12 h treatment with 30 µM apratyramide

	Up-regulationed genes, FDR correction, 12 h	Down-regulationed genes, FDR correction, 12 h
Growth factor	*AREG, VEGFA, HBEGF, EREG*	
Cytokine	*IL1A, IL1B, MYDGF*	*SCGB1A1, CXCL1, CCL2, TNFSF10*
Transcription regulator	*DDIT3, CREB3L2, XBP1, ETV5, ATF4, JUND, ZNF165, ETV4, ATF3, MYC, NFE2L1, KLF6, SQSTM1, DAP, PREB, NFKB2, FOXE1, MEF2D, MAGED1, IRF2BP2, BHLHE40*	*GMNN, UHRF1, GTF2I, VGLL1, DLX5, E2F8, SMAD6, ID3, GRHL3*
Transporter	*SLC7A11, SEC24D, LCN2, SLC33A1, LDLR, SYVN1, COPG1, SLC1A5, SEC61A1, SLC6A9, SEC23B, SLC39A7, STX5, NPC1, SLC1A4, SLC43A1, TMCO3, SEC63, SLC50A1, SEC61B, TMED10, OSBP*	*ATP1B1, ATP1B3, TFRC, SCFD2, SLC12A2, AQP3, SLC39A10, SLC6A6, SLC1A3*
Kinase	*TRIB3, PCK2, ERN1, NDRG1*	*EPHA4*
G-protein coupled receptor	*GPR1*	*P2RY2*
Ion channel	*CLIC4, CLCN6, CACNB1*	*KCNJ15*
Transmembrane receptor	*F3*	*BCAM*
Phosphatase	*PLPP5, LPIN1, DUSP6, MTMR4*	*ALPPL2, DUSP10*
Peptidase	*ABHD4, PRSS8, LONP1*	*MMP13*
Enzyme	*CYP1A1, ASNS, GFPT1, CTH, WARS, SARS, MTHFD2, PSAT1, CYP1B1, AARS, UPP1, CYP51A1, FUT3, SCD, HSPA5, CBS/CBSL, MICAL2, DHCR7, CYP4F11, ALG2, PYCR1, FKBP11, EDEM2, MARS, PDIA4, GPT2, NCF2, EDEM1, CARS, PHGDH, OSTC, GARS, GMPPA, ANXA3, IARS, PYGB, SMOX, MSMO1, CHPF, SHMT2, UAP1, FDFT1, SND1, GTPBP2, YARS, MVD, LSS, SDR42E1, DNASE2, NEU1, PDIA6, RAB6A, P4HB, SQLE, MTHFD1L, TXNDC11, ODC1, DTD1, ACSL3*	*HSPA8, MSH2, DHFR, ESCO2, TYMS, GLUL, PCNA, RHOBTB3, MCM6, POP1, AKR1B10, HSPA1A/HSPA1B, CROT, HAS2, RNF152*
MicroRNA		*MIR3143, MIR-554, MIR-548*

Supplementary Figures

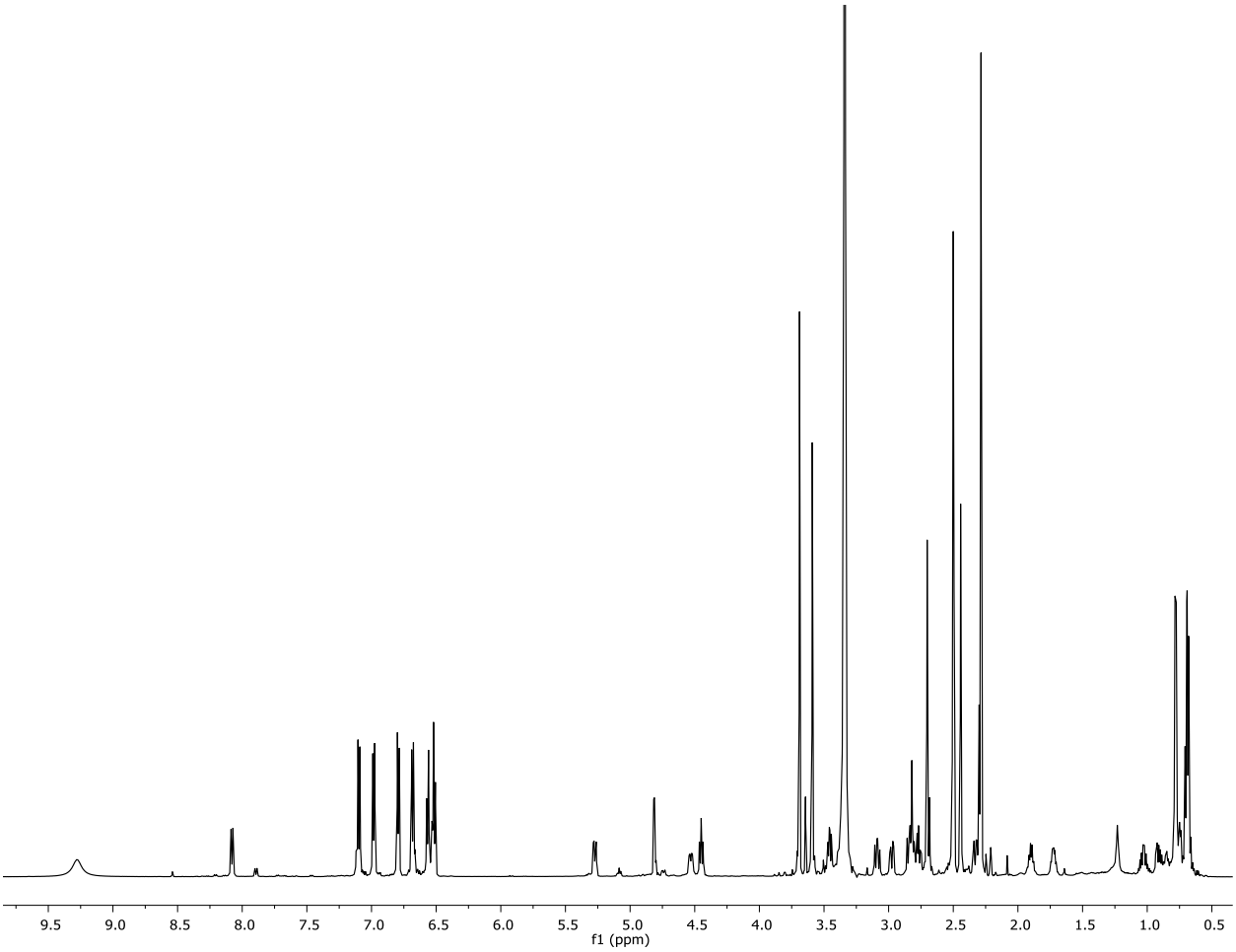

Figure S1. ^{1}H NMR spectrum of natural product apratyramide (**1**) in DMSO-d_6 (600 MHz).

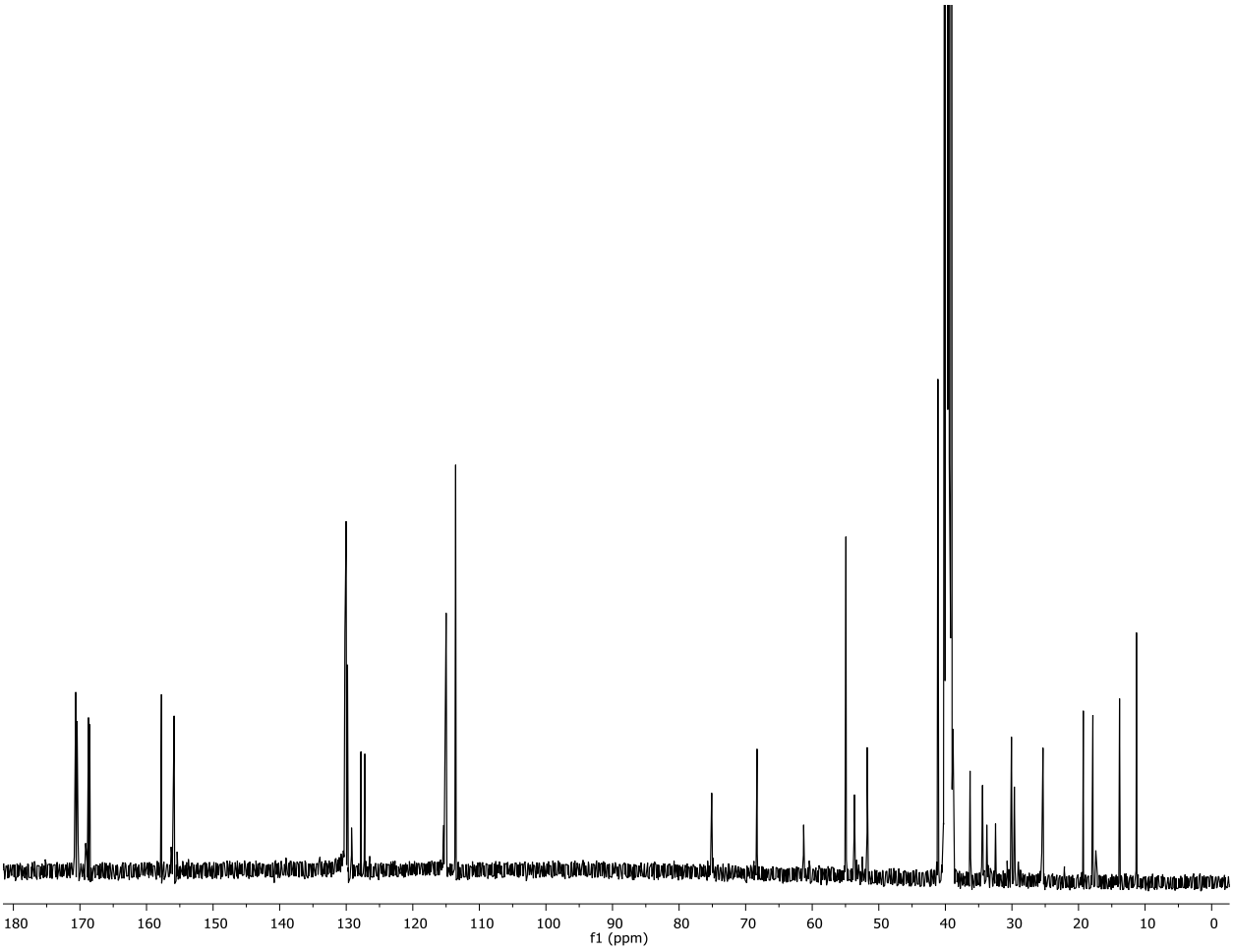

Figure S2. ^{13}C NMR spectrum of natural product apratyramide (**1**) in DMSO-d_6 (150 MHz).

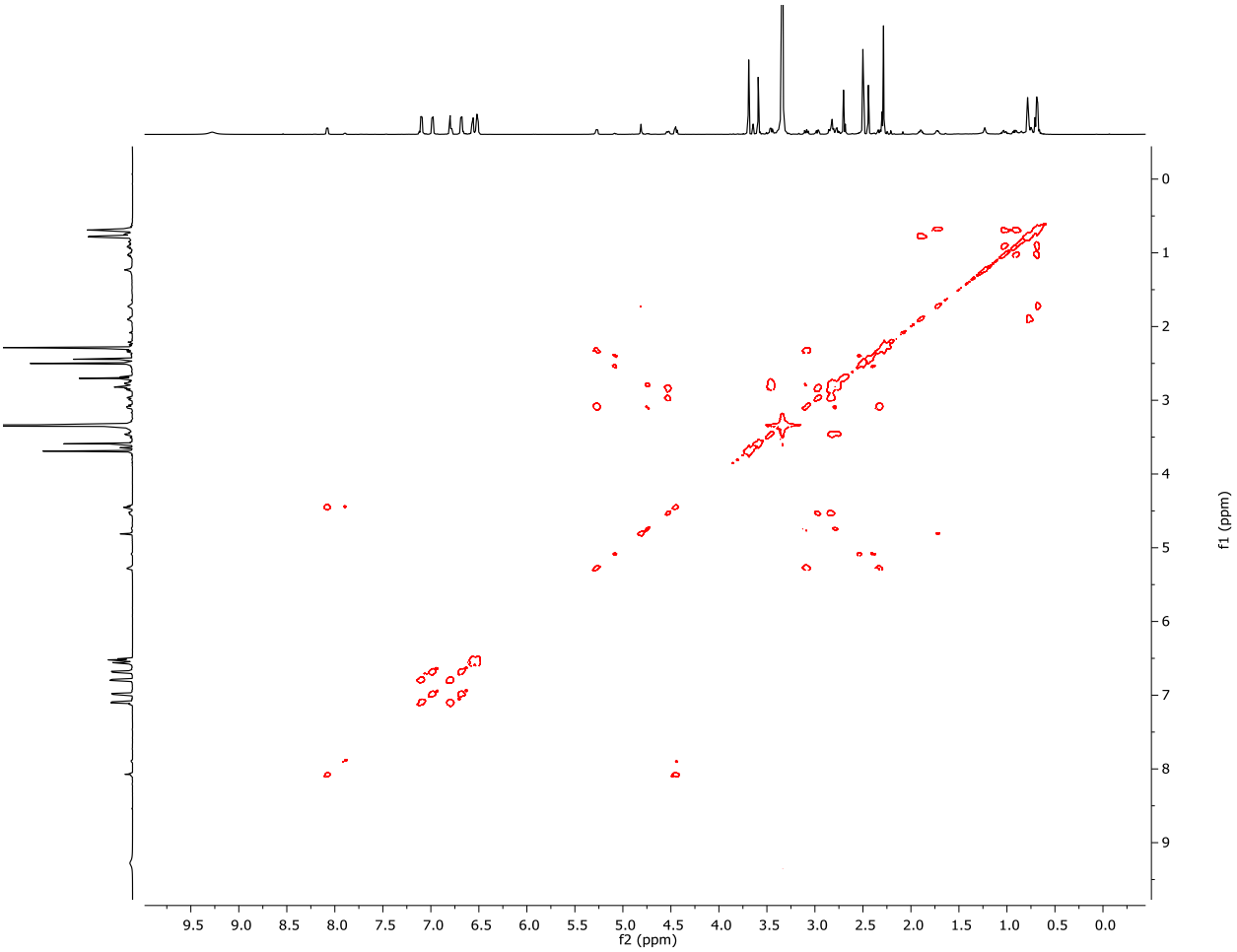

Figure S3. COSY spectrum of natural product apratyramide (**1**) in DMSO-d_6 (600 MHz).

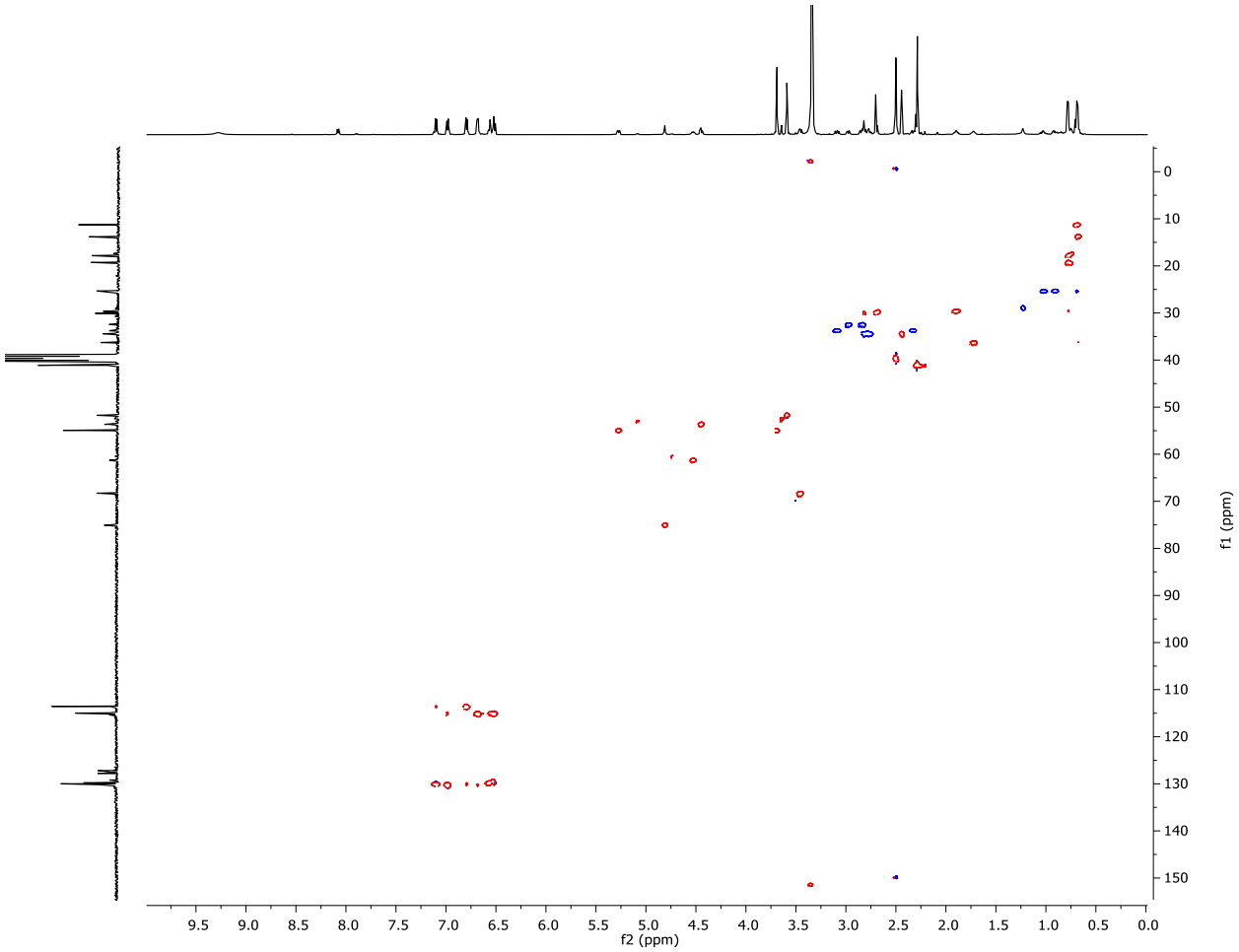

Figure S4. HSQC spectrum of natural product apratyramide (**1**) in DMSO-d_6 (600 MHz).

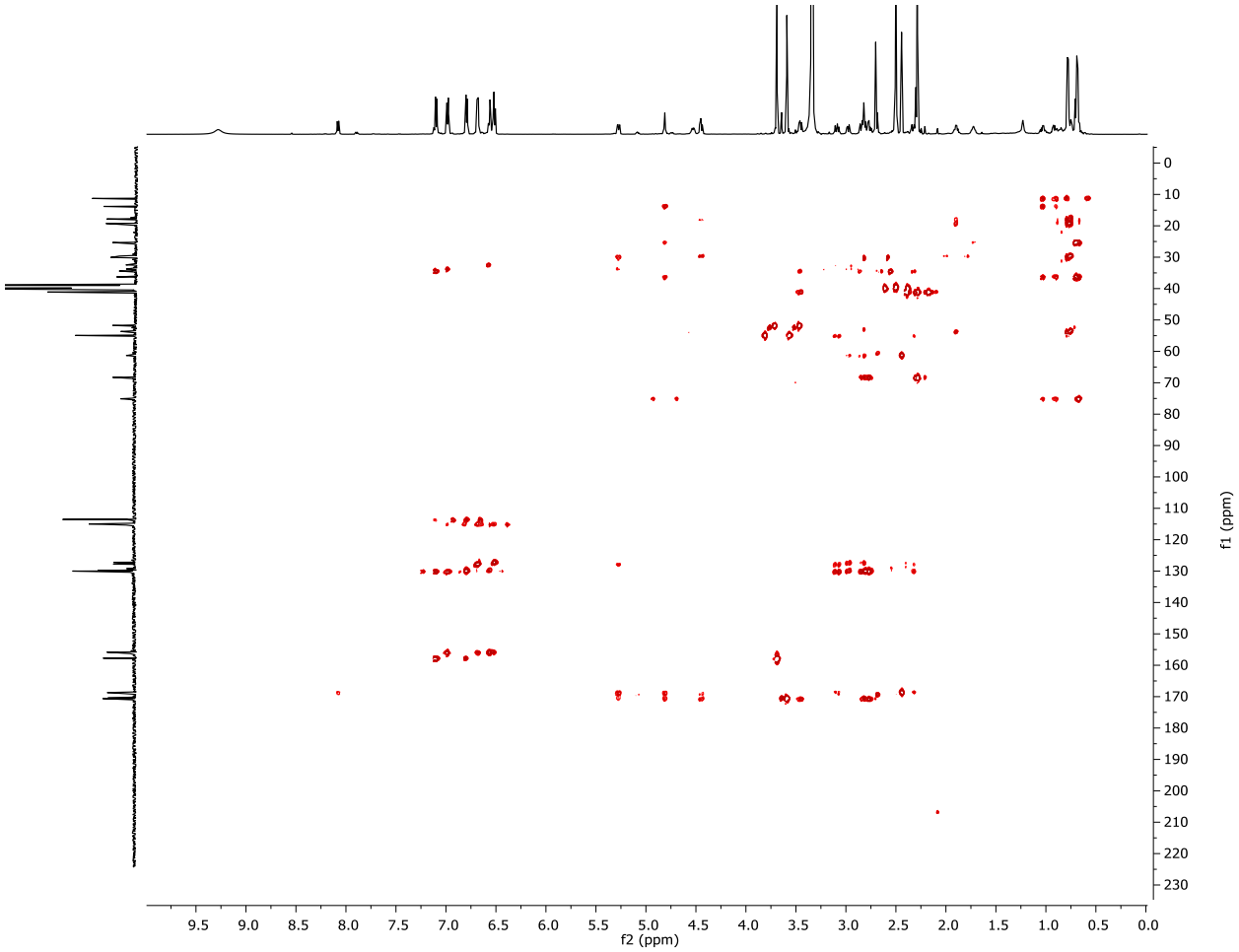

Figure S5. HMBC spectrum of natural product apratyramide (**1**) in DMSO-d_6 (600 MHz).

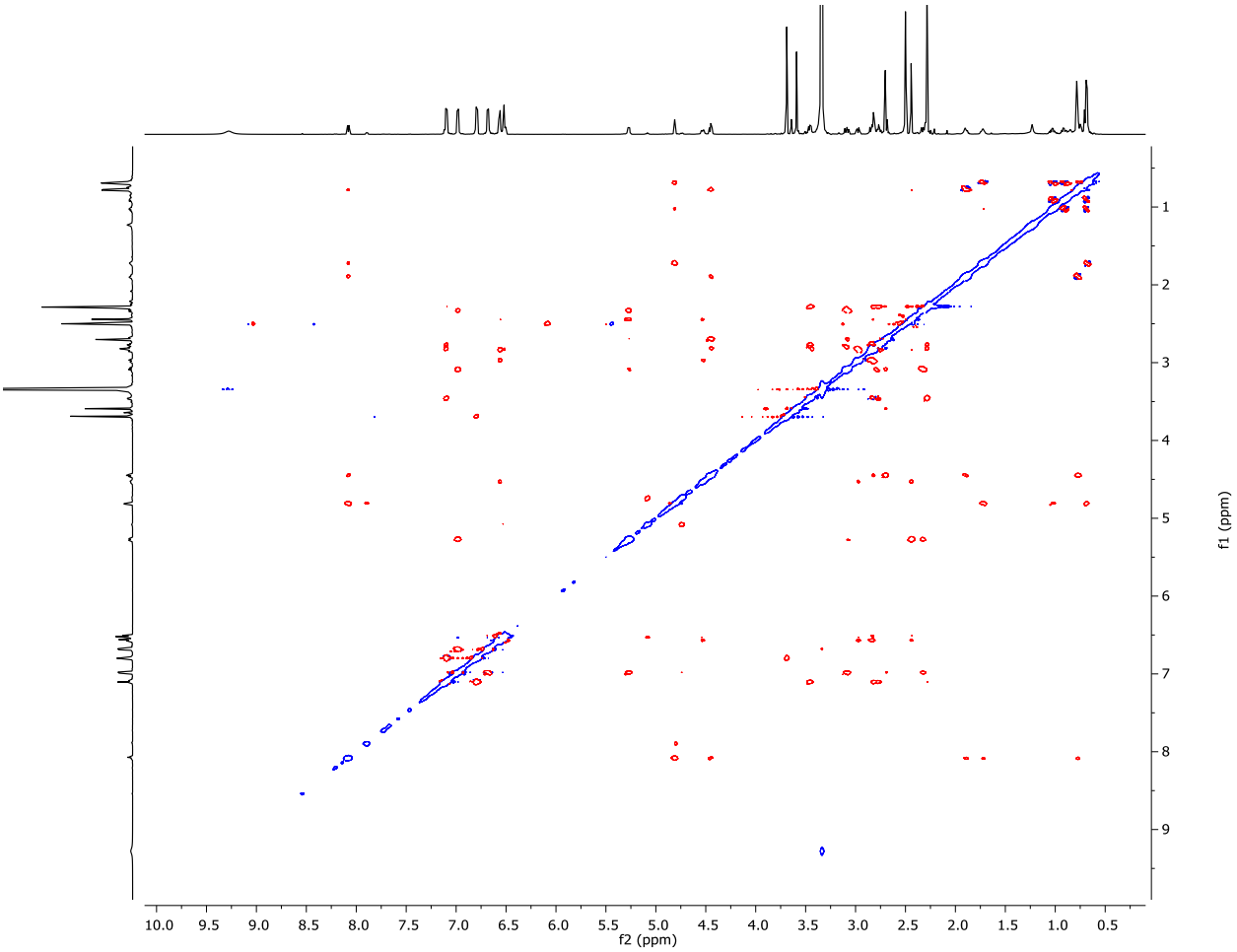

Figure S6. NOESY spectrum of natural product apratyramide (**1**) in DMSO-d_6 (600 MHz).

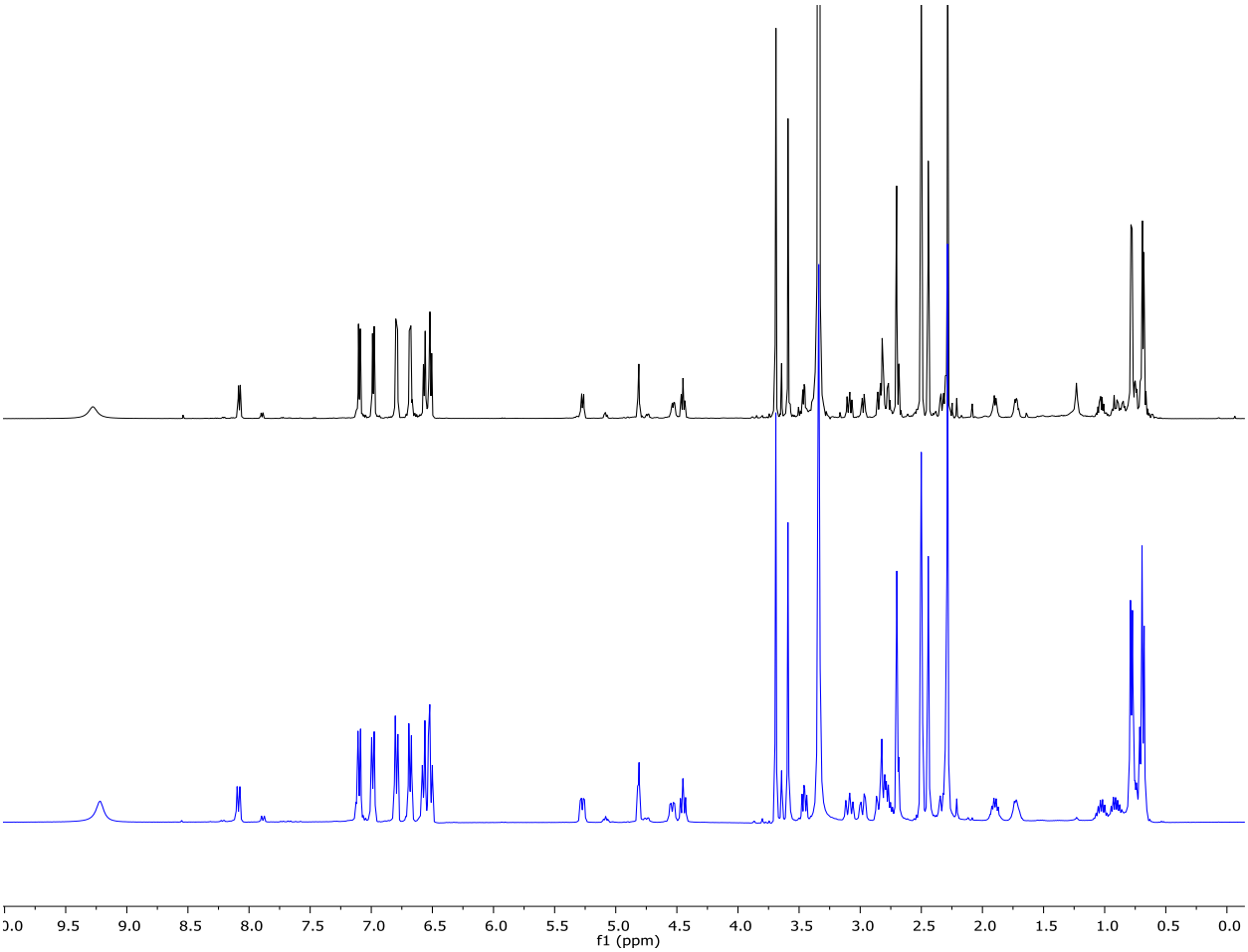

Figure S7. ^{1}H NMR spectrum of natural product (black, DMSO-d_6 (600 MHz)) and synthetic (blue, DMSO-d_6 (400 MHz)) apratyramide (**1**).

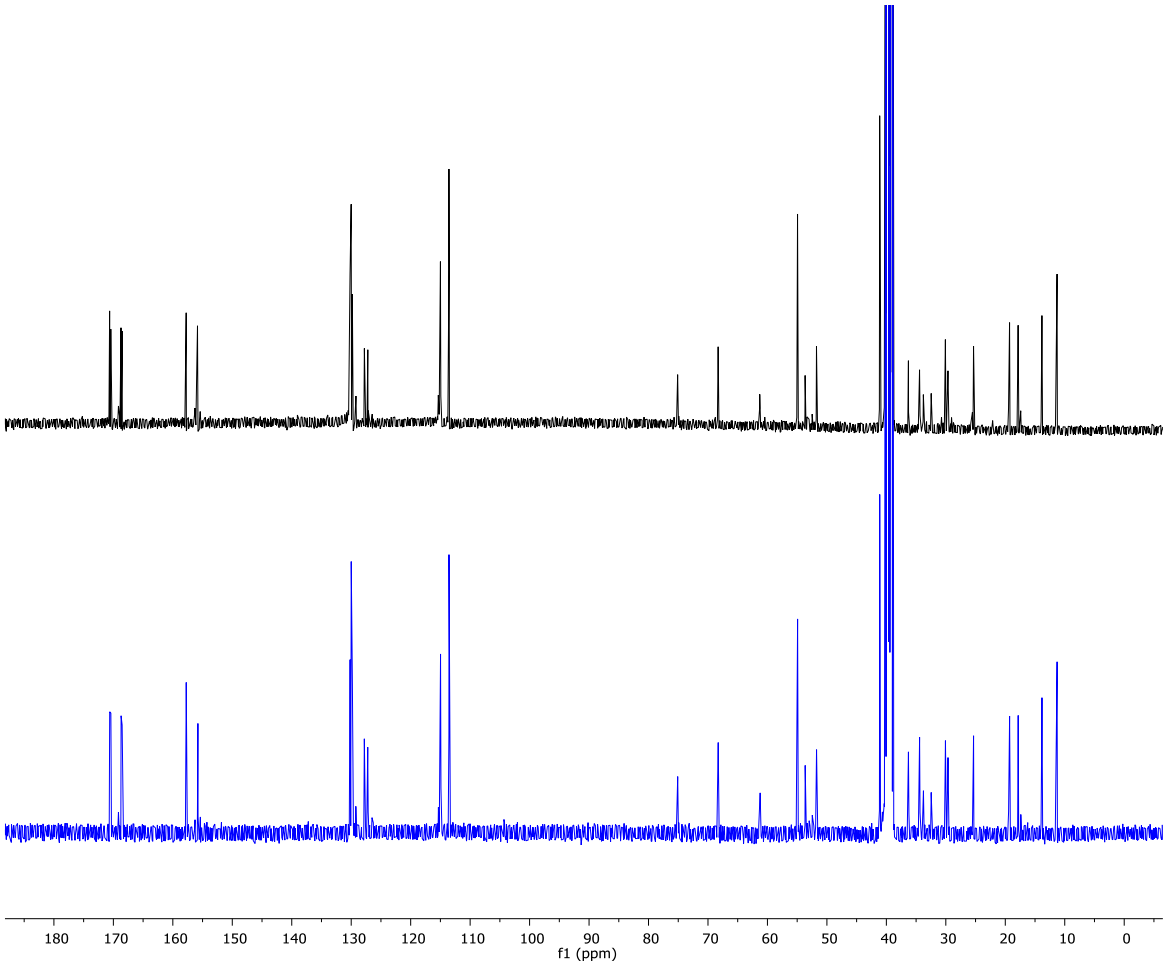

Figure S8. ^{13}C NMR spectrum of natural product (black, DMSO-d_6 (150 MHz)) and synthetic (blue, DMSO-d_6 (100 MHz)) apratyramide (**1**).

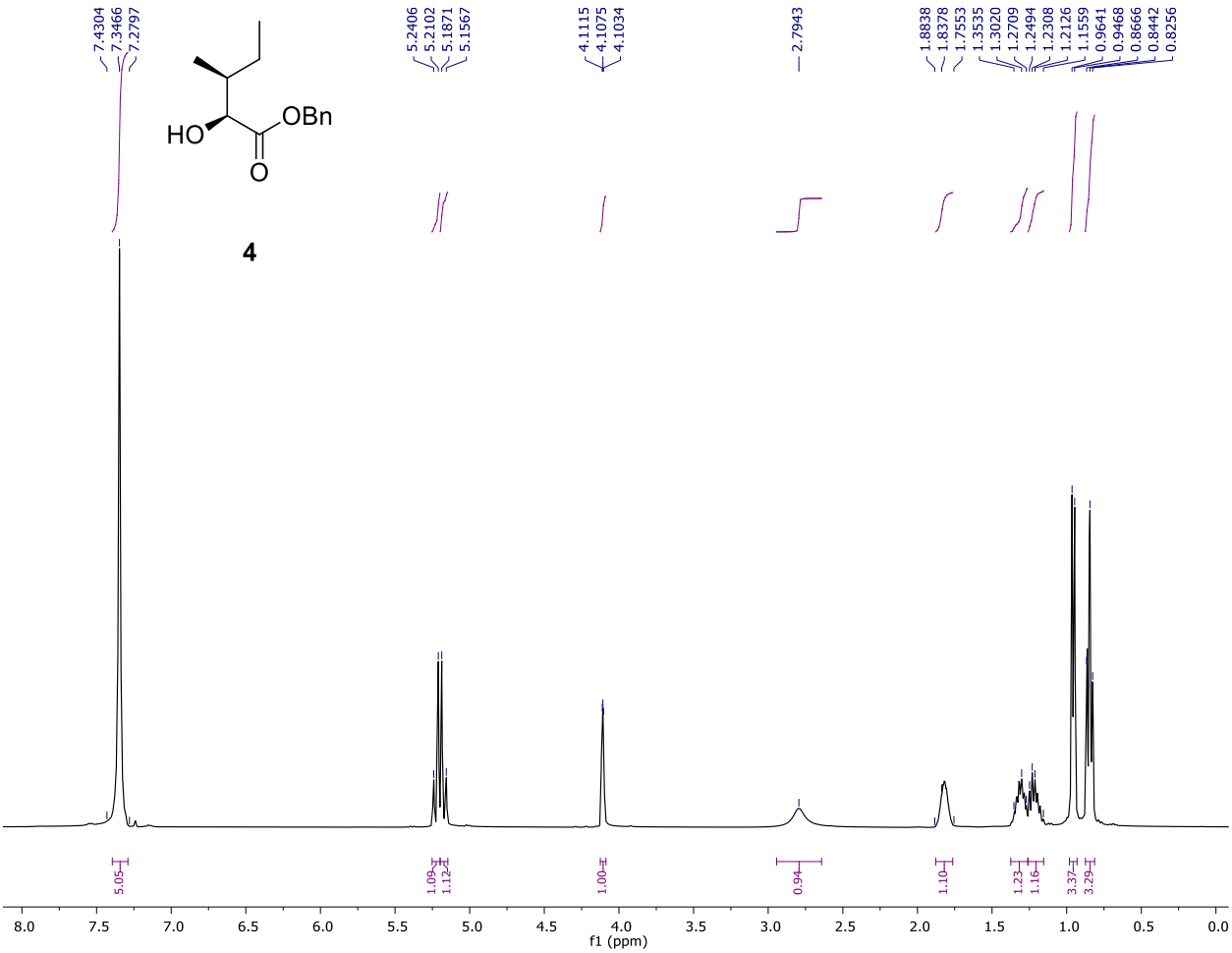

Figure S9. ^{1}H NMR spectrum of **4** in $CDCl_3$ (400 MHz).

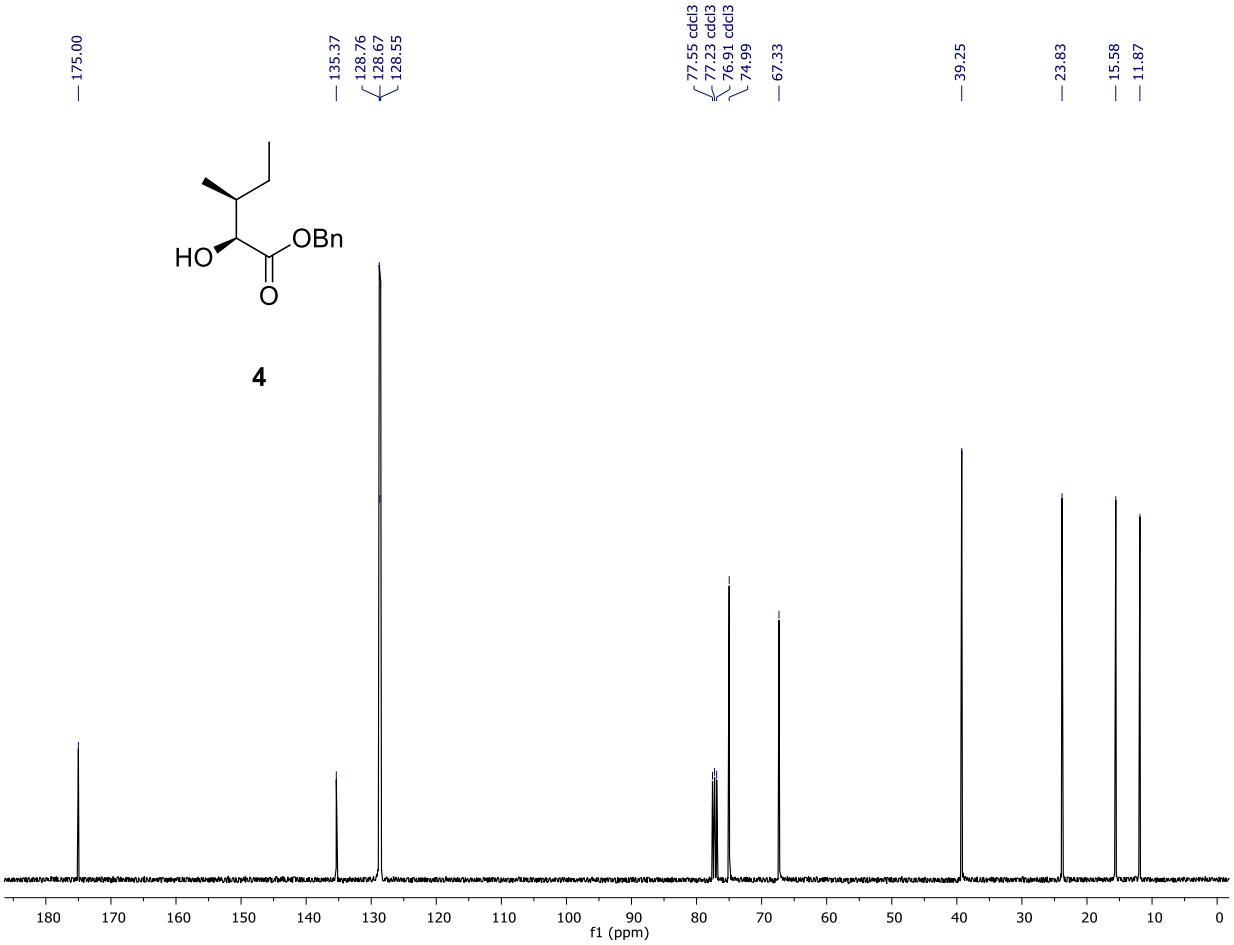

Figure S10. ^{13}C NMR spectrum of **4** in $CDCl_3$ (100 MHz).

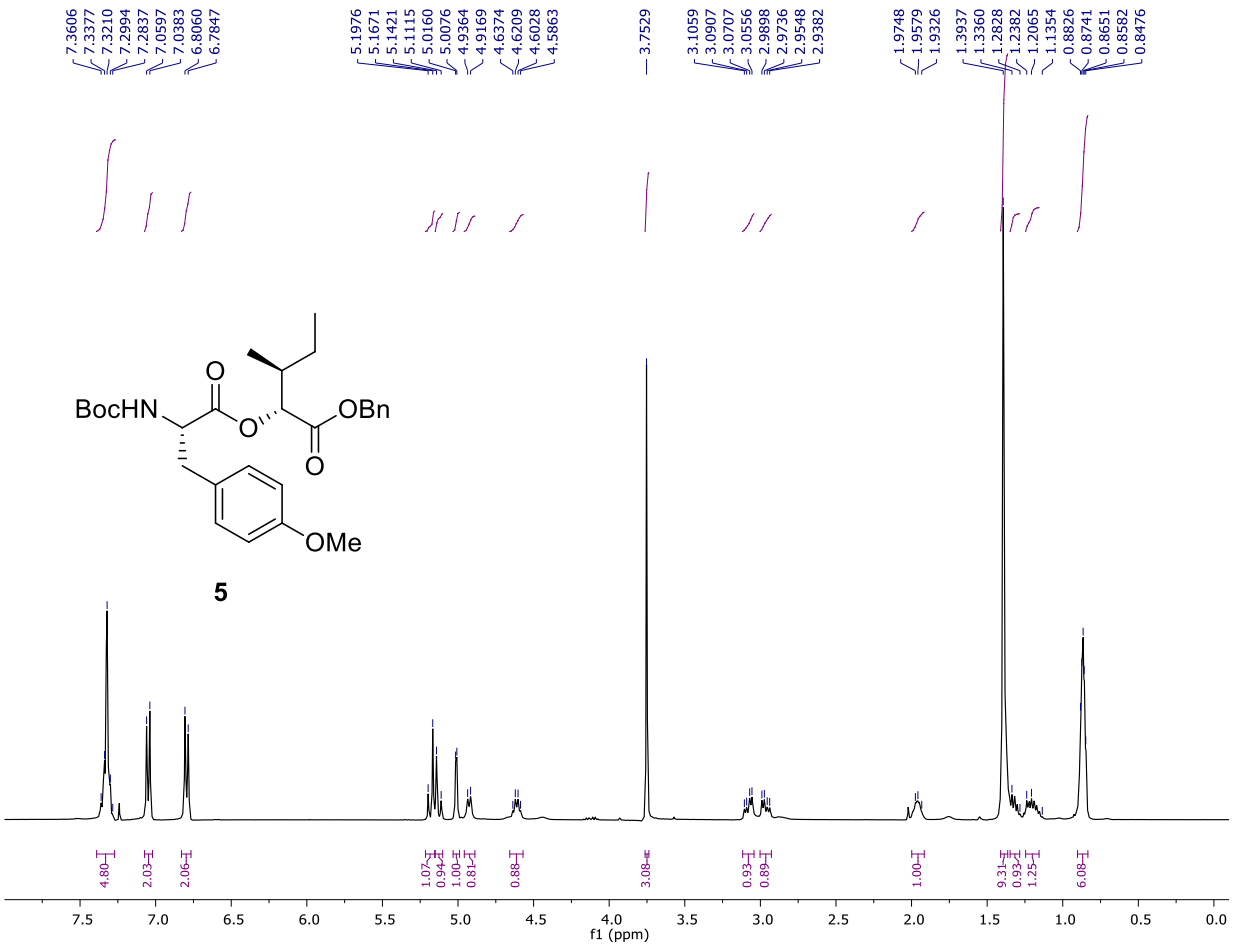

Figure S11. ^{1}H NMR spectrum of **5** in $CDCl_3$ (400 MHz).

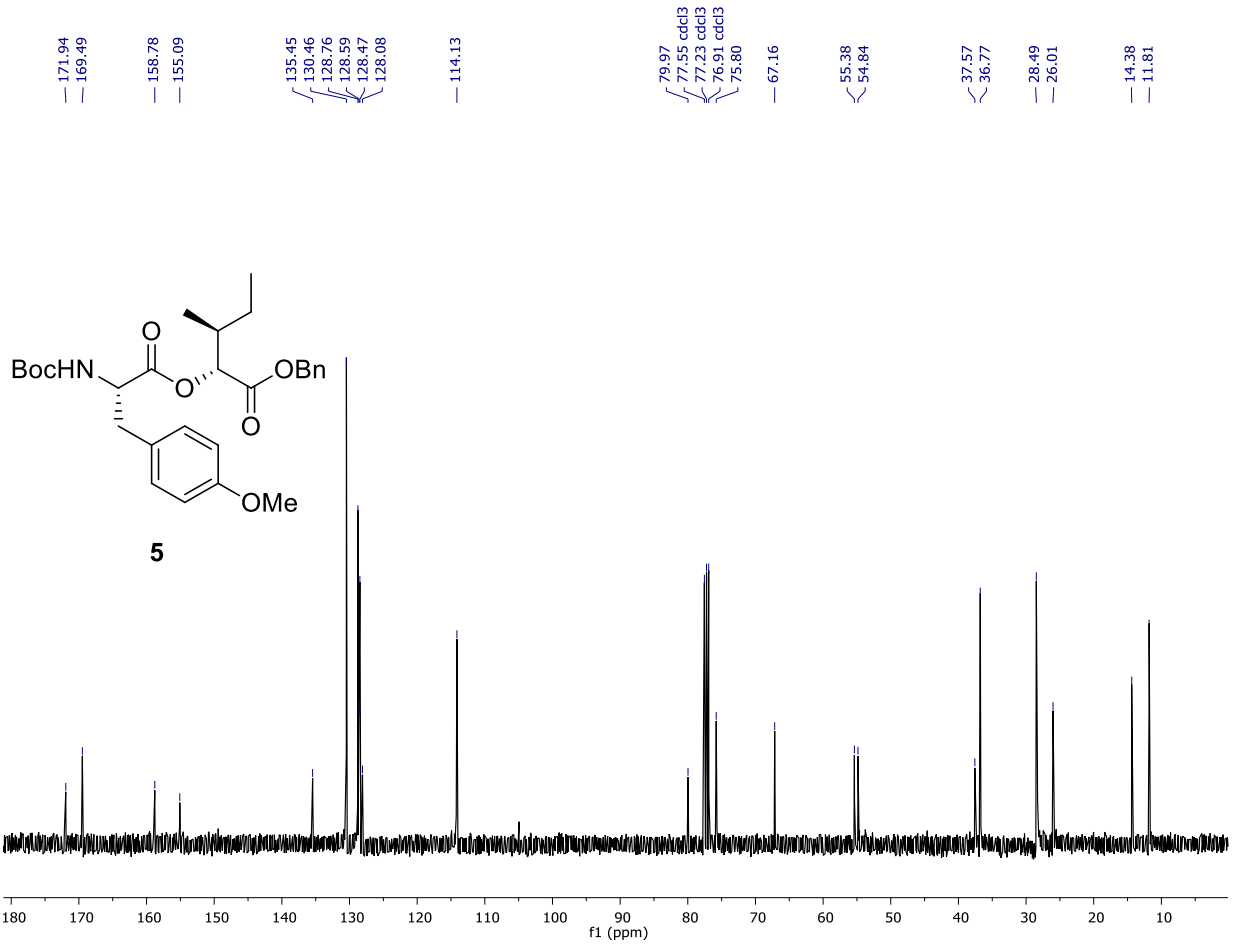

Figure S12. ^{13}C NMR spectrum of **5** in $CDCl_3$ (100 MHz).

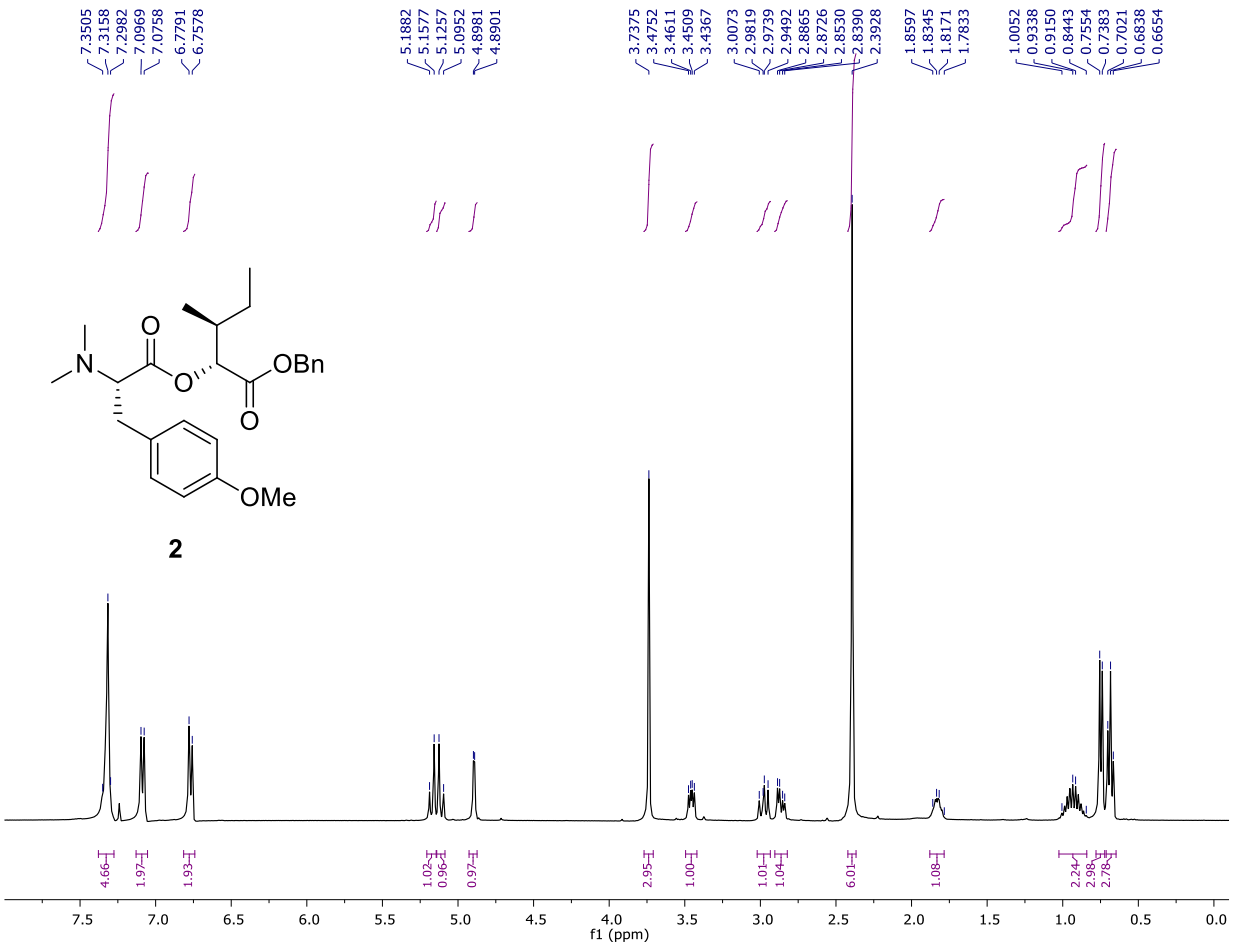

Figure S13. ^{1}H NMR spectrum of **2** in $CDCl_3$ (400 MHz).

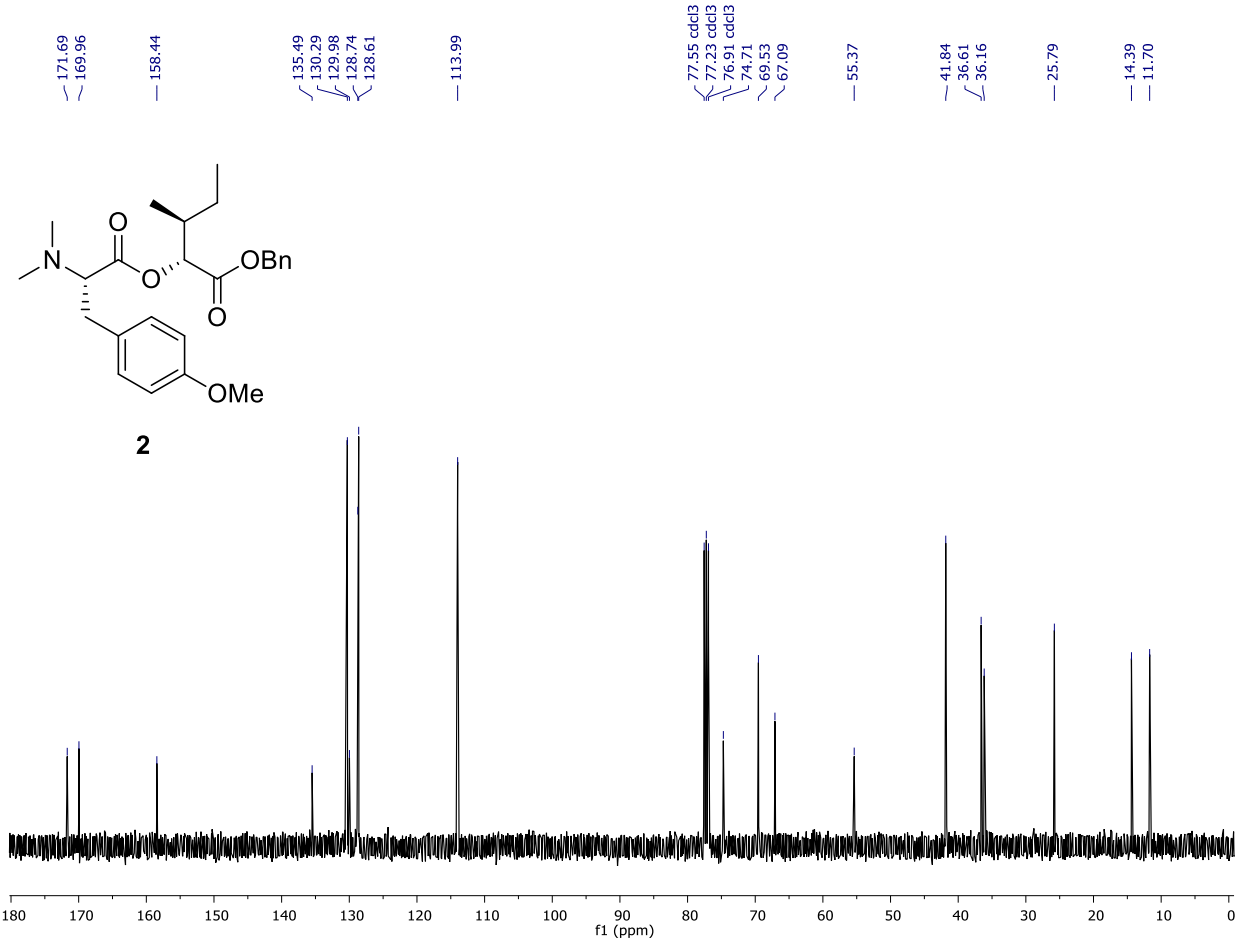

Figure S14. ^{13}C NMR spectrum of **2** in $CDCl_3$ (100 MHz).

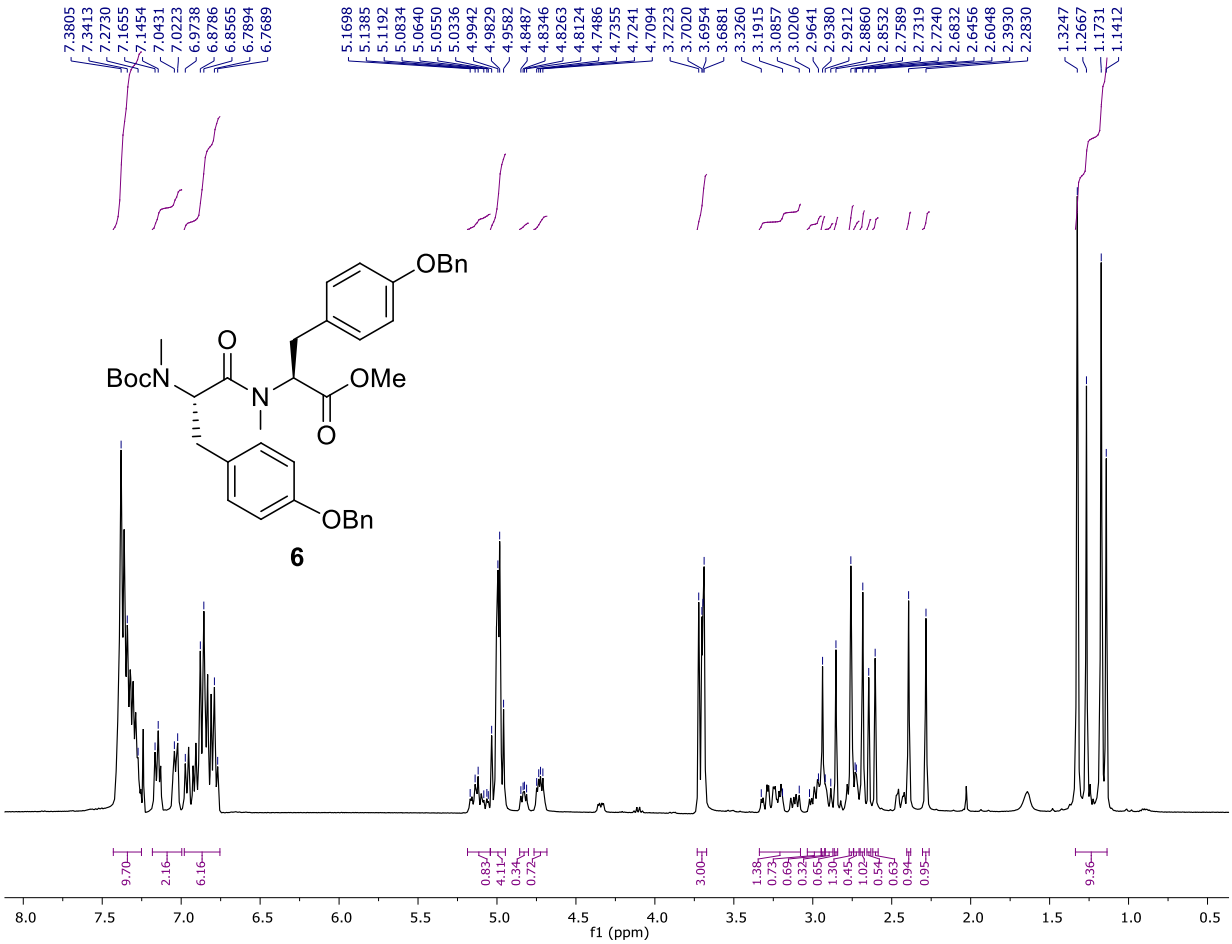

Figure S15. ^{1}H NMR spectrum of **6** in $CDCl_3$ (400 MHz).

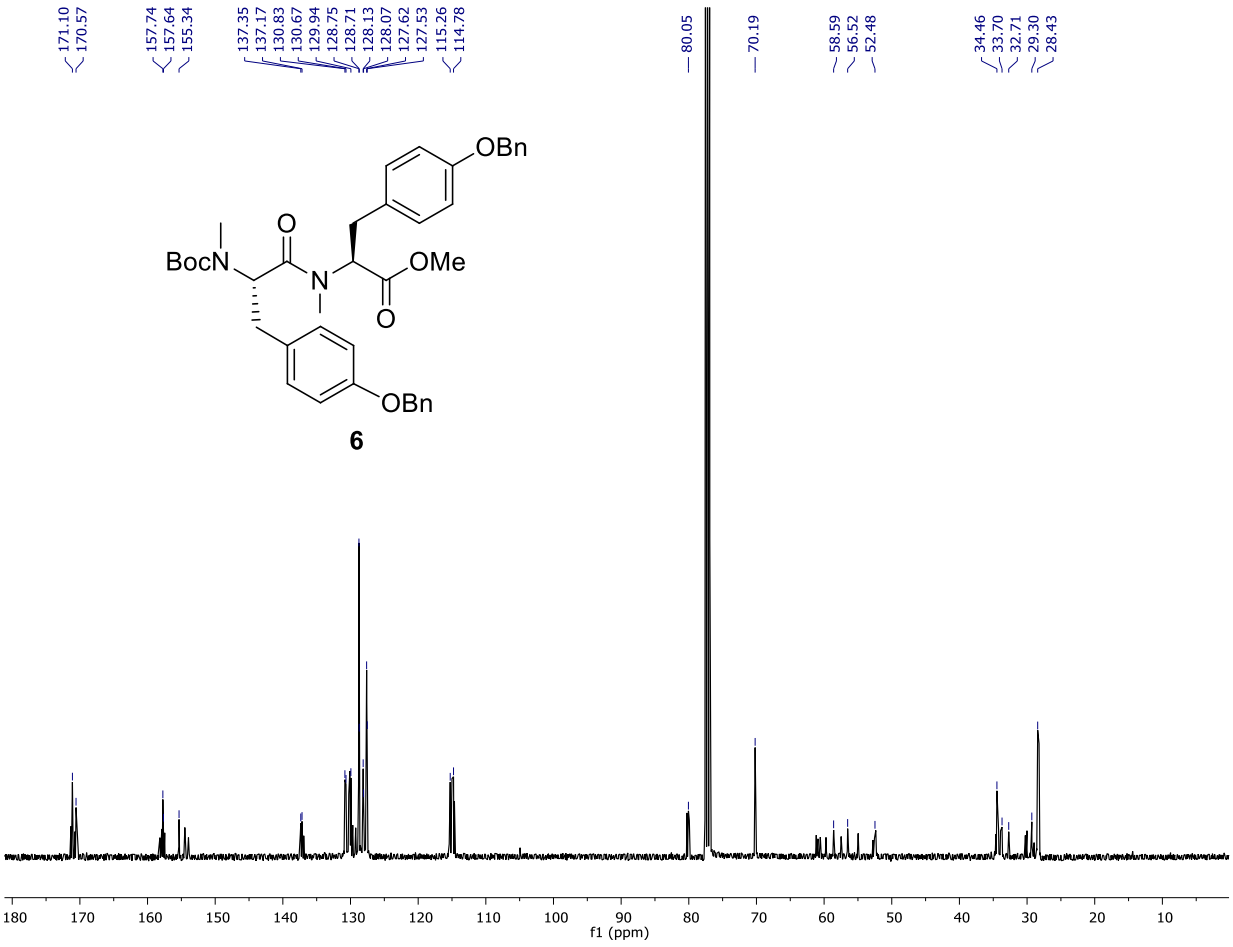

Figure S16. ^{13}C NMR spectrum of **6** in $CDCl_3$ (100 MHz).

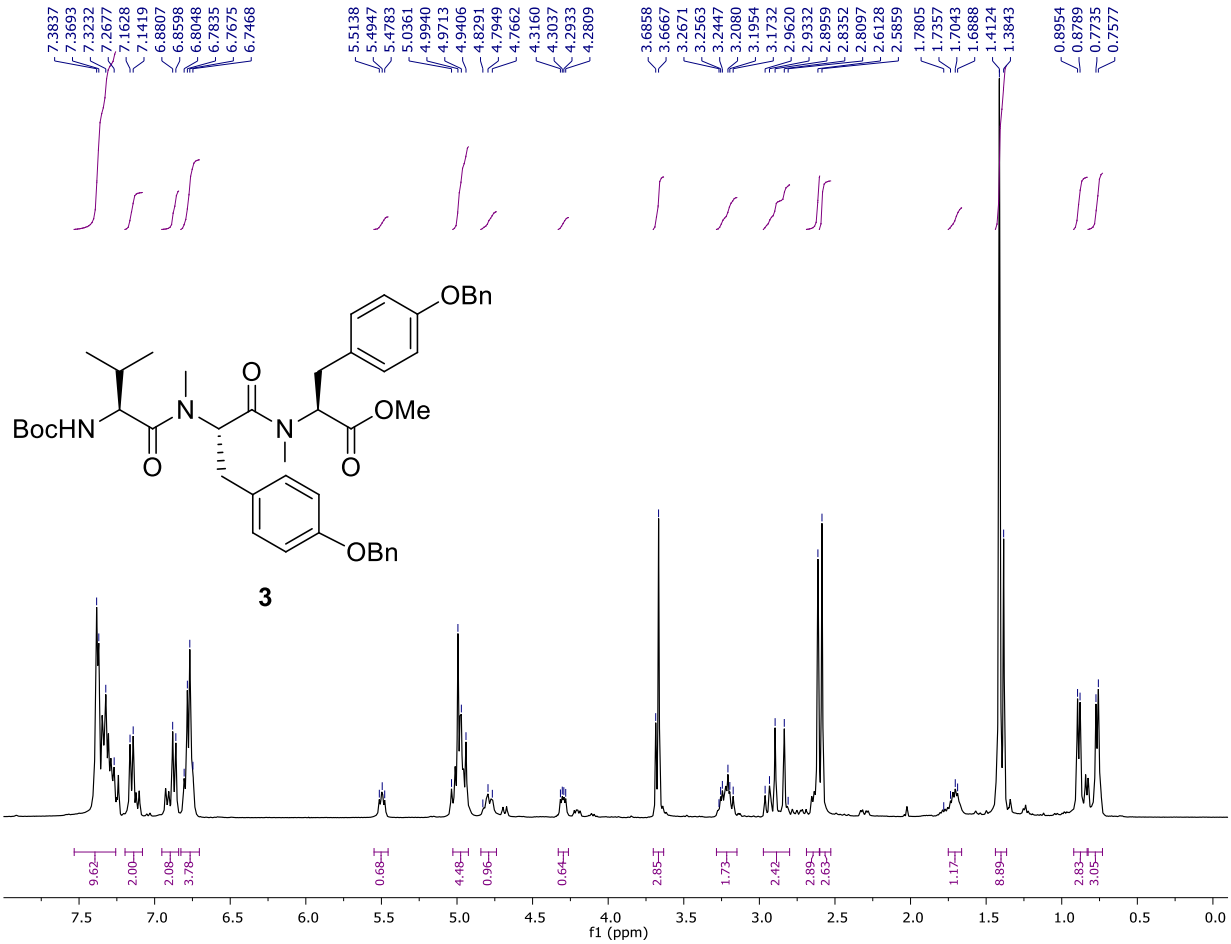

Figure S17. ^{1}H NMR spectrum of **3** in $CDCl_3$ (400 MHz).

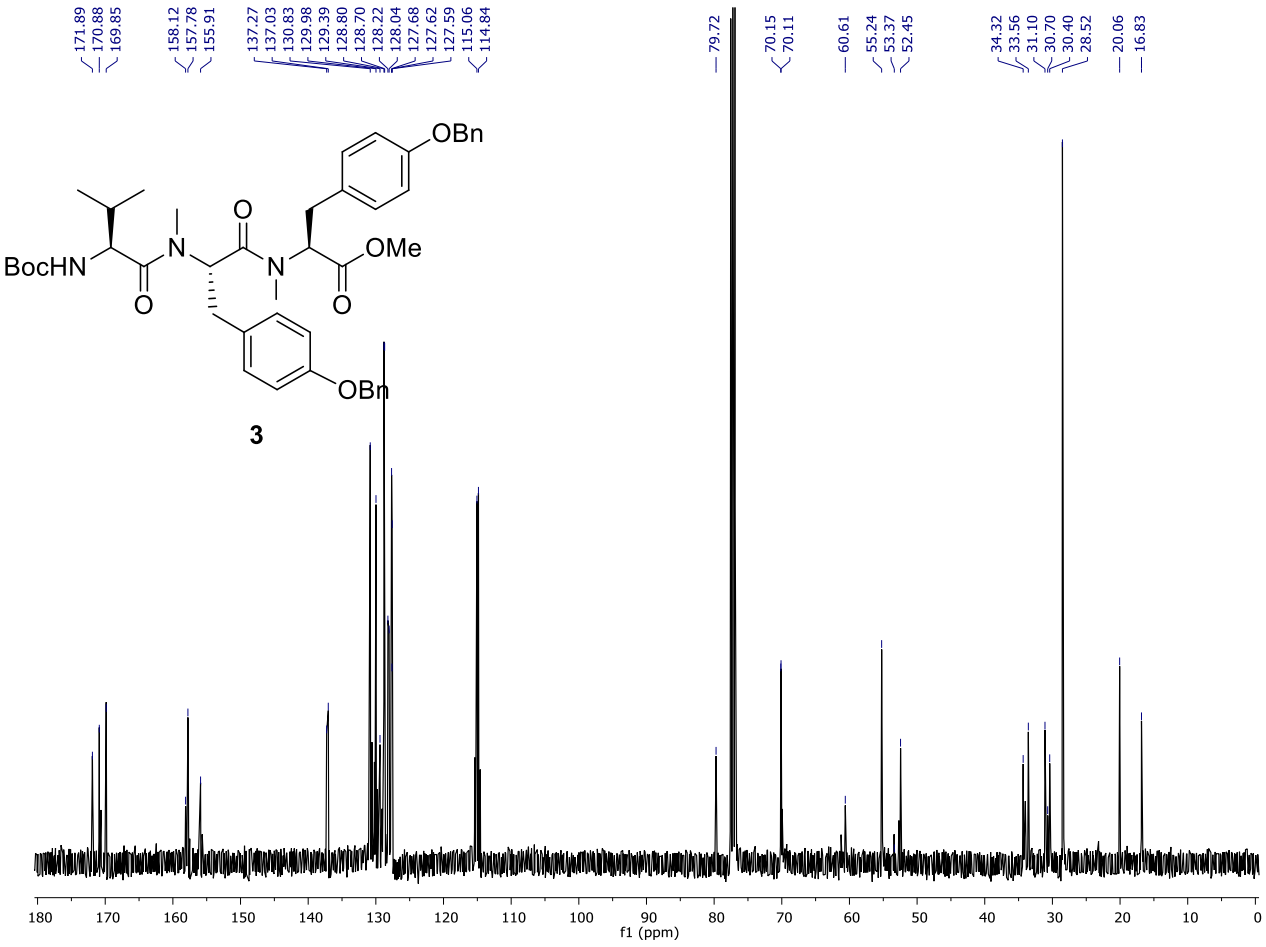

Figure S18. ^{13}C NMR spectrum of **3** in $CDCl_3$ (100 MHz).

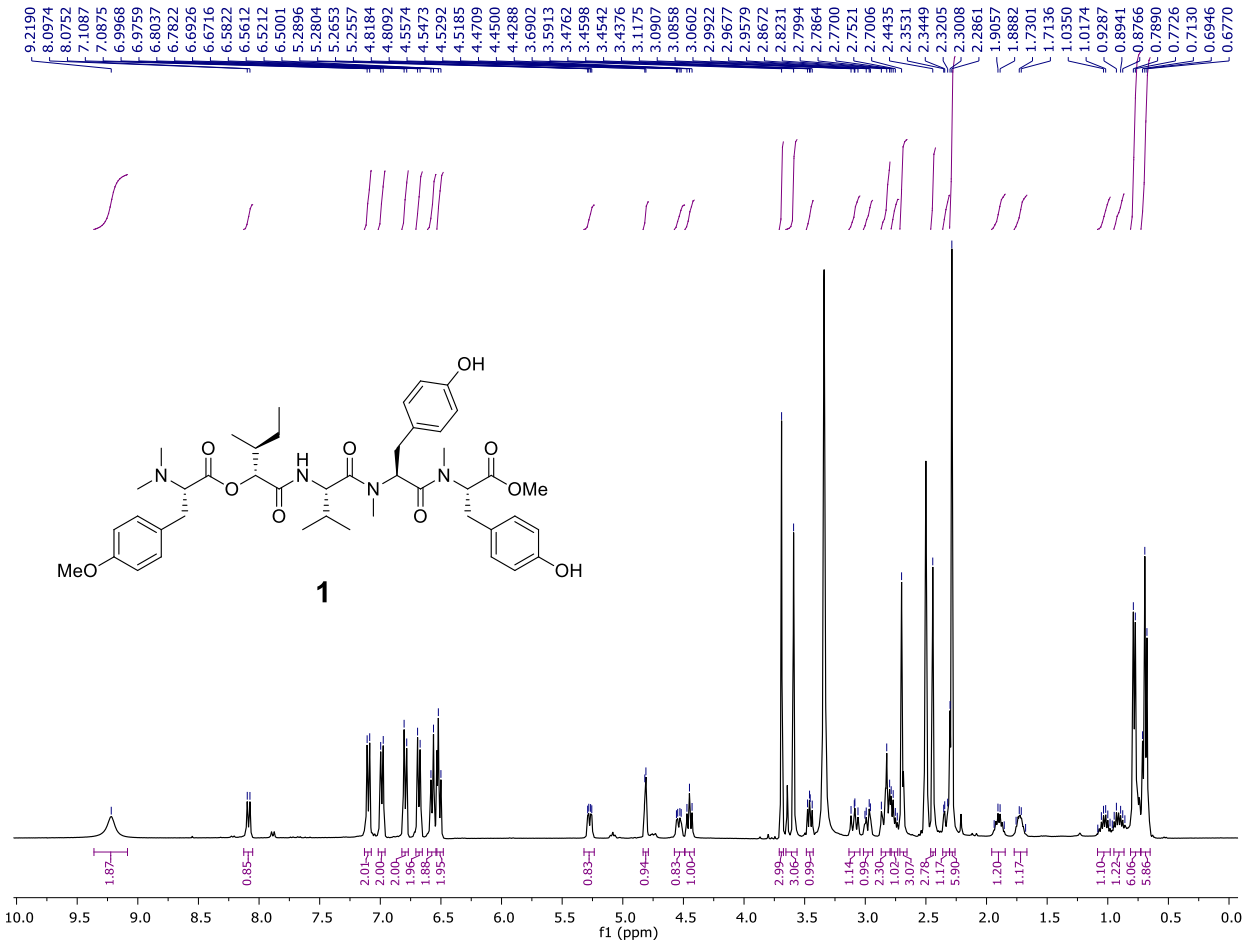

Figure S19. ^{1}H NMR spectrum of synthetic **1** in DMSO-d_6 (400 MHz).

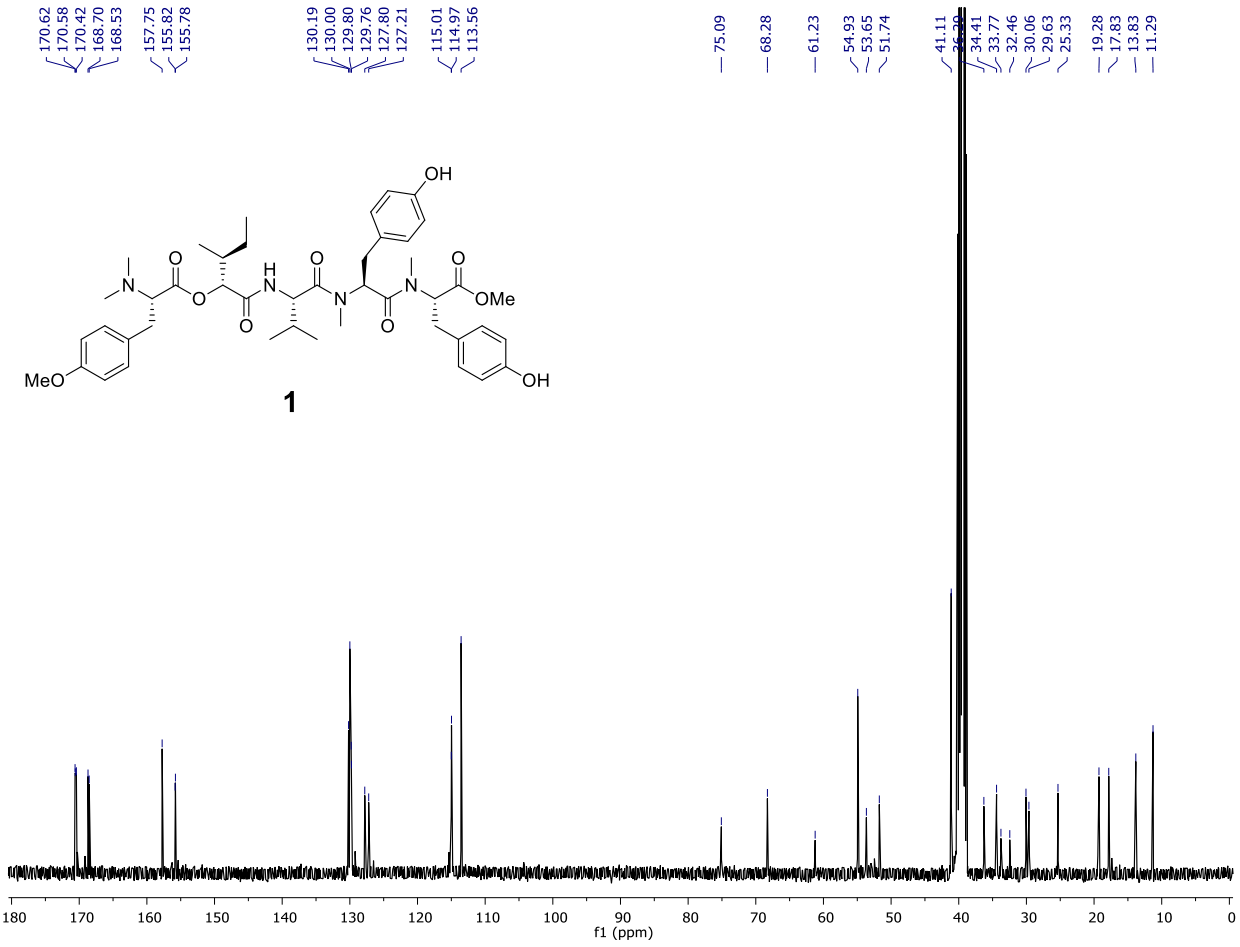

Figure S20. ^{13}C NMR spectrum of synthetic **1** in DMSO-d_6 (100 MHz).

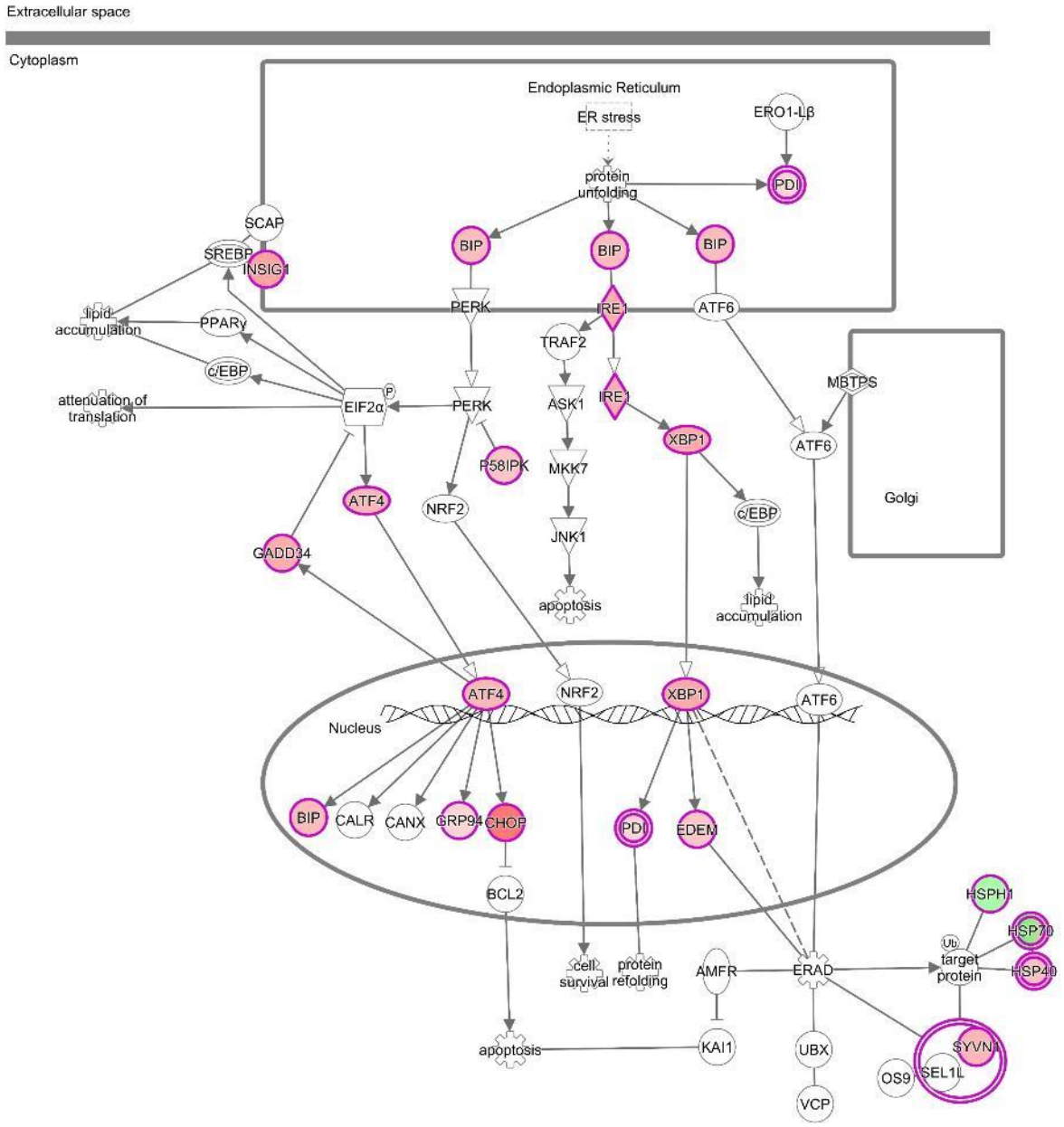

Figure S21. IPA analysis identified unfolded protein response (UPR) pathway as the top canonical pathway with a p-value of 1.45×10^{-16}. Red indicates up-regulated transcripts. Green indicates down-regulated transcripts.

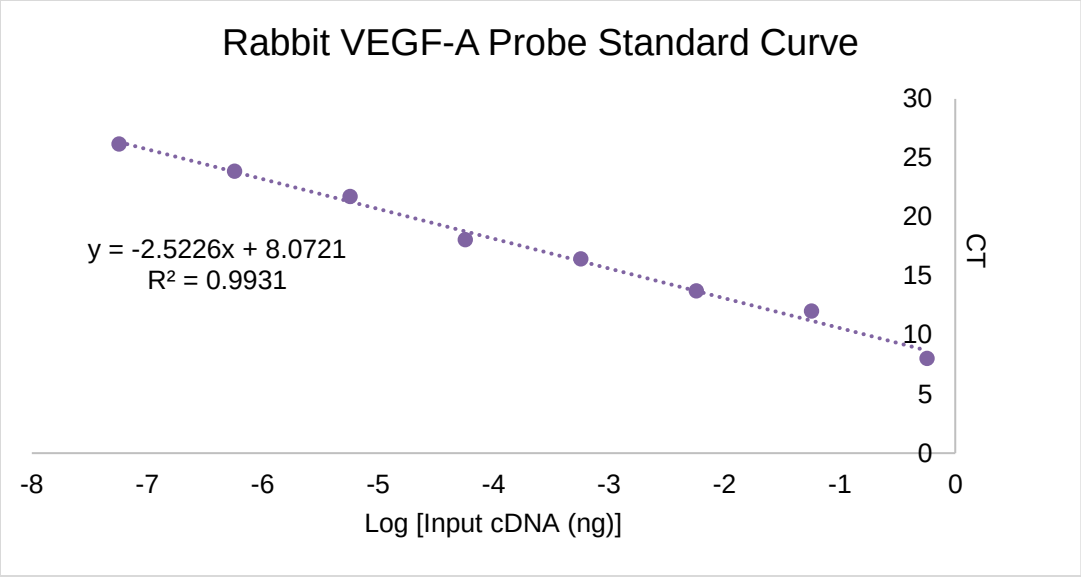

Figure S22. Standard Curve of Rabbit VEGF-A Probe for quantification.

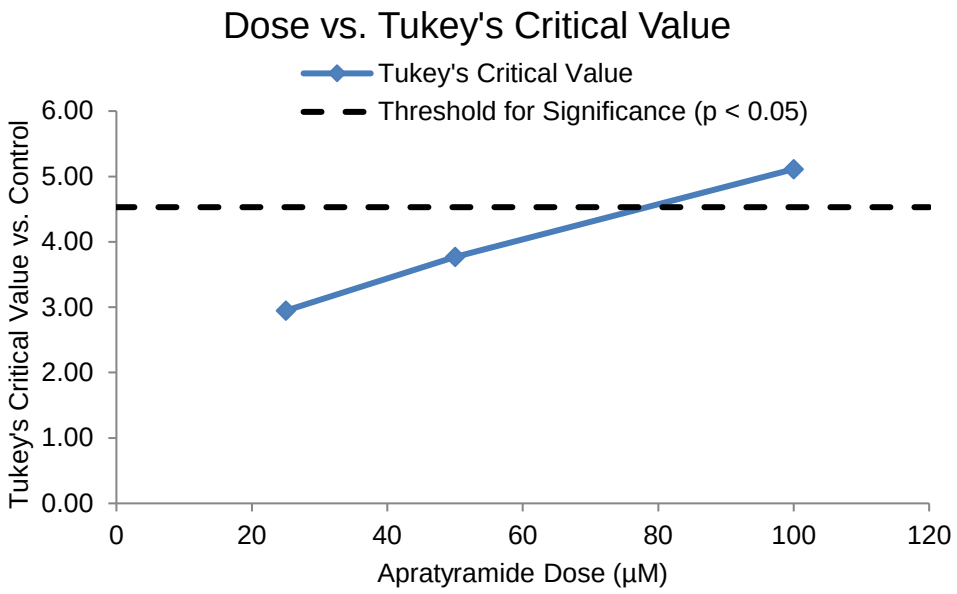

Figure S23. Figure representing increasing certainty of effect for ex vivo data analysis.