Supporting Information

Engineering DNA templated nonribosomal peptide synthesis

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Experimental Procedures

Plasmid construction

Plasmids are listed in Table S1 and representative sequences are provided in GenBank file format (Table S2). DNA polymerases, T5 exonuclease, and restriction enzymes were obtained from New England Biolabs. Synthetic oligos, gene synthesis, and Sanger sequencing services were provided by Eurofins Genomics (Ebersberg, Germany).

Nonribosomal peptide synthetase (NRPS) modules were cloned from plasmids pTrc99a-*tycB1*,[1] pSU18-*grsA*[2] and pTrc99a_*grsB*_M3574L.[3] Linkers between NRPS module and zinc finger domain are listed in Table S3. Zinc finger domains *zif268* (Z), *pbsII* (P), *nre* (N)[4], and *zfb* (B) were synthesized or cloned from synthetic fragments followed by a C-terminal His_6 tag unless otherwise specified. The gene for the N-terminal docking domain (Dn) was cloned from *inxB* of *Xenorhabdus innexi* DSM 16336. The cognate C-terminal docking domain (Dc) was synthesized according to the peptide sequence of *inxA* (GenBank: KR871226)[5] with a conservative mutation from Ile to Leu in order to embed an *Afl*II restriction site.

Recombinant genes were assembled into pSU18 vector (Chl^R) using the Hot Fusion cloning method.[6] Chemically competent cells were prepared from *E. coli* HM0079,[1] and transformed following the TSS protocol.[7]

Protein expression and purification

Proteins were expressed using *E. coli* HM0079 as host which genomically encodes a phosphopantetheinyl transferase (Sfp) and generates the holo-form of NRPS modules. Media were supplemented with 25 µg/mL chloramphenicol. Bacterial cultures were incubated at 37 °C shaking at 230 rpm with an orbit diameter of 5.1 cm unless otherwise specified.

For protein expression, a preculture in LB broth was inoculated from a single colony of HM0079 freshly transformed with the relevant plasmid and incubated overnight (14-16 h). As growth medium, we used 2YT medium containing 16 g tryptone, 10 g yeast extract, 5 g NaCl in 1 L water adjusted to pH 7.0. A 500 mL Erlenmeyer flask containing 100 mL 2YT media was inoculated with 100 µL preculture and supplemented with sterile 10 µM $ZnCl_2$. The resulting culture was grown to mid-log phase (ca. 4 h, OD_{600} ~ 0.5) and protein expression induced with 0.25 mM IPTG. Incubation was continued at 20 °C for 15-16 h. Bacteria were pelleted by centrifugation at 10,000 x g and 7 °C for 3 min and stored at -20 °C.

All reagents for protein purification were precooled to 4 °C. Procedures were performed below 10 °C except for nickel affinity chromatography. Bacterial pellets were thawed and thoroughly resuspended in one tenth of the original culture volume with buffer A1 (500 mM NaCl, 20 mM imidazole, 2 mM tris(2-carboxyethyl)phosphine [TCEP], 50 mM Tris-HCl, pH 7.4). For cell lysis, up to 25 mL suspension were contained in a 50 mL conical tube cooled on ice. Immediately before cell lysis, 0.02% of the original culture volume of a protease inhibitor cocktail (Cat.No. P8849, Sigma) were added. Ultrasonic lysis was performed for a total of 10 min using an ultrasonic homogenizer UP200St (Hielscher) with sonotrode S26d7 (7 mm diameter) at 40% amplitude and periodic on/off cycles of 2 and 3 s. Cellular debris was removed by centrifugation at 19,000 x g for 30 min. To capture His-tagged protein, the supernatant was applied to 1 mL Rotigarose-His/Ni IDA-agarose beads suspension (Cat.No. 1308.1, Carl Roth) pre-equilibrated with buffer A1 in an Econo-Column (1.5 x 15 cm; Bio-Rad). The suspension was incubated for 12.5 min for resin to settle before draining the lysate. The beads were then washed three times with 6 mL buffer A1 followed by elution with 200 µL buffer B1 (500 mM NaCl, 300 mM imidazole, 2 mM TCEP, 50 mM Tris-HCl, pH 7.4) which were discarded and two times 0.5 mL buffer B1 which contained the protein of interest.

For further purification and to remove contaminating genomic DNA, the protein was subjected to anion exchange chromatography unless specified otherwise. The protein was buffer exchanged using Vivaspin 500 centrifugal filters (30 kDa cut-off, Sartorius) into low salt buffer (20 mM NaCl, 20 mM Tris-HCl, pH 8.0, filtered) and then injected onto a MonoQ 5x50 GL anion exchange column (GE Healthcare) connected to an NGC Quest 10 Plus Chromatography System (Bio-Rad). Elution was performed at a flow rate of 1 mL/min at 10 °C with a linear gradient of 20 to 650 mM NaCl in 20 mM Tris buffer (pH 8.0) in a total volume of 7.85 mL. Fractions containing target protein were pooled, concentrated, and exchanged into DKP-M buffer (100 mM NaCl, 1 mM TCEP, 50 mM HEPES, pH 8.0) using Vivaspin 500 centrifugal filters.

Protein concentrations were determined spectrophotometrically using calculated extinction coefficients. Of each protein, two 2.5 µL aliquots were loaded onto a Take3 Plate (Biotek) and absorbance at 280 nm determined on an Epoch2 microplate reader (Biotek) equipped with Gen5 software (Biotek). The purified protein was adjusted to a concentration of 50 µM in DKP-M buffer, supplemented with 10% glycerol, frozen in liquid nitrogen, and stored at -20 °C until further assays were performed. Protein purity was verified by SDS-PAGE (Figure S6).

Preparation of template DNA for peptide synthesis

DNA templates for peptide production assays were prepared by annealing synthetic oligos (Eurofins Genomics; Table S4). The oligos were dissolved in H_2O and annealing reactions were performed in buffer AN (100 mM NaCl, 10 mM Tris-HCl, pH 7.4) containing 10 µM forward and reverse oligos. The solution was heated to 95 °C in a water bath for 3 min to denature secondary structure; the water bath was then switched off to allow gradual cooling over the course of 3 hours. The annealed dsDNA templates were stored at -20 °C.

Peptide formation assay

The assay buffer (100 mM NaCl, 50 mM HEPES, 1 mM of each amino acid substrate, 0.1 mM $MgCl_2$, 10 µM $ZnCl_2$, 2% glycerol, 0.5 mM TCEP, 5 mM adenosine-5'-triphosphate [ATP]) was formulated to enable zinc-finger binding while minimizing zinc toxicity on NRPS enzyme activity (Figure S1). NaCl and HEPES were supplied from 20x DKP-M buffer (2 M NaCl, 1 M HEPES, pH 8.0). ATP disodium salt (Cat. No. 34369-07-8, Sigma) was freshly dissolved in H_2O before use. The components were mixed thoroughly before adding enzymes and DNA templates. In the final reaction mixture, a pH of 7.5 ± 0.5 was determined with pH-Fix 2.0 - 9.0 test strips (Macherey-Nagel). Unless indicated otherwise, reactions were run at 37°C for 1 hour in a water bath.

Chemical synthesis

To verify the identities of the nonribosomal peptide products, cyclo-(FP) was purchased from Bachem, fPVOL from Cambridge Research Biochemicals and further synthetic standards were synthesized in-house. Cyclic ornithine (*S*-3-aminopiperidin-2-one) is abbreviated as cOrn or O*.

Analytics

NMR spectra were recorded in deuterated solvents (Carl Roth, Germany) on a Bruker AVANCE II 300 or Bruker AVANCE III 600 MHz spectrometer, equipped with a Bruker Cryoplatform. The chemical shifts (δ) are reported in parts per million (ppm) relative to the solvent residual peak of DMSO-d_6 (^{1}H: 2.50 ppm, quintet; ^{13}C: 39.5 ppm, heptet) or $CDCl_3$ (^{1}H: 7.24, singlet; ^{13}C: 77.2, triplet). Some compounds show two sets of peaks due to conformational isomerism in which case only the major isomer was interpreted. **High resolution MS measurements** were performed on a Vanquish Horizon UHPLC system coupled with a Thermo QExactive HF-X Quadrupole-Orbitrap mass spectrometer (Thermo Scientific) equipped with a Acquity BEH C8 column (1.7 µm, 2.1 × 100 mm). Masses were detected by ESI as $M+H^+$ adducts in positive mode.
All reagents used were reagent grade and used as supplied (purchased from Sigma-Aldrich, Bachem, Fluorochem or Carl Roth). Reactions were performed at ambient temperature under argon atmosphere in anhydrous solvents (Acros Organics) unless otherwise stated. **Analytical thin-layer chromatography** was performed on silica 60 F_{254} plates (0.25 mm, Merck). Compounds were visualized by dipping the plates in a ninhydrin/acetic acid solution followed by heating.

HPLC purification of peptides

Semi-preparative HPLC of peptide products was performed using a Phenomenex Luna C18(2) (5 µm, 250 x 10 mm) column connected to a Shimadzu Nexera LC-20AR system. Hydrochloride salts of peptides were dissolved in 5% MeCN and purified by semi-preparative HPLC using the following gradient: 0 - 5 min, 5% B; 5 - 25 min, 5% - 35% B; 25-30 min, 80% B; 30-38 min, 5% B (A: H_2O with 0.1% (*v*/*v*) TFA, B: MeCN with 0.1% (*v*/*v*) TFA) with a flow rate of 8 mL/min. Fractions were collected from 5 min to 30 min. After purification, fractions containing the desired compounds (H_2N-D-Phe-L-Pro-L-cOrn: R_t = 14.1 min, H_2N-D-Phe-L-Pro-L-Val-L-cOrn: R_t = 17.4 min) were freeze dried, samples submitted to NMR measurements and pure compounds stored at -20 °C as TFA salts.

Method A: Solution phase peptide coupling

One equivalent of the carboxylic acid was dissolved in dry dichloromethane (DCM, 0.2 M solution) and cooled to 0 °C. *N*,*N*-Diisopropylethylamine (3 equivalents) was added drop-wise and the reaction was allowed to warm to room temperature. The acid was pre-activated through addition of hexafluorophosphate azabenzotriazole tetramethyl uronium (HATU, 1.2 equivalents) for 5 min at room temperature. Afterwards the amine (1.1 equivalents) was added and the reaction stirred at room temperature until completion as indicated by TLC (1 to 3 h). The reaction was quenched by pouring it onto 10% citric acid. Phases were separated and the aqueous phase extracted with DCM three times. The combined organic phases were washed with saturated $NaHCO_3$ solution and brine (one time each) and dried over Na_2SO_4. Volatiles were removed under reduced pressure. Purification by column chromatography (silica 60, DCM/MeOH 95:5) yielded the peptide product.

Method B: Removal of Boc-protecting groups

To the Boc-protected amine was added 4 M HCl in 1,4-dioxane (0.1 M final solution of the amine) at 0 °C (ice). After stirring for 5 min on ice the solution was allowed to warm to room temperature and stirred for further 3 h. Afterwards the volatile components were removed under vacuum, the residue dissolved in H_2O and freeze dried to yield the respective amine hydrochloride salt.

*Synthesis of cyclo-L-ornithine (**2**)*

L-Orn (**1**, 3.0 g, 17.8 mmol) was dissolved in dry MeOH (40 mL) under argon atmosphere. Trimethylsilyl chloride (10 mL, 79.3 mmol) was added portion wise and the reaction stirred for 17 h at room temperature. Afterwards, the reaction was cooled to 0 °C (ice) and a 5 M solution of NaOMe in MeOH (27 mL, 135 mmol) added. After stirring at 0 °C for 10 min, the reaction was allowed to warm to room temperature and stirred for 2.5 h. Then, the reaction was acidified to pH 6 using concentrated HCl. The solvent was removed under reduced pressure and the residue re-dissolved in iPrOH (100 mL) and filtered. The filtrate was concentrated again under reduced pressure. Purification by column chromatography (silica 60, DCM/MeOH 7:3) yielded cyclo-L-ornithine **2** (1.74 g, 15.3 mmol, 86% yield) as a white-red foam.

^{1}H NMR (600 MHz, DMSO): δ = 8.43 (s, 3H), 8.04 (s, 1H), 3.69 (dd, J = 11.0, 6.1 Hz, 1H), 3.19 – 3.04 (m, 2H), 2.27 – 2.11 (m, 1H), 1.89 – 1.80 (m, 1H), 1.78 – 1.69 (m, 2H) ppm.
^{13}C NMR (151 MHz, DMSO): δ = 167.3, 48.9, 40.8, 25.0, 20.2 ppm.
UPLC-MS: *m*/*z* = 114.98 $[M+H]^+$

*Synthesis of Boc-D-Phe-L-Pro-OMe (**5**)*

Method A with Boc-D-Phe-OH (**3**, 2.0 g, 7.7 mmol) as free acid and L-Pro-OMe (**4**, 1.1 g, 8.5 mmol) as amine yielded Boc-D-Phe-L-Pro-OMe **5** (2.2 g, 5.9 mmol, 77% yield) as an orange-white foam.

^{1}H NMR (300 MHz, $CDCl_3$) δ = 7.31 – 7.13 (m, 5H), 7.02 (d, *J* = 8.7 Hz, 1H), 4.43 (dd, *J* = 15.1, 8.5 Hz, 1H), 4.18 (dd, *J* = 8.4, 3.9 Hz, 1H), 3.58 (s, 3H), 3.28 – 3.12 (m, 1H), 2.92 – 2.67 (m, 3H), 2.06 – 1.93 (m, 1H), 1.90 – 1.59 (m, 3H) 1.32 (s, 9H).
^{13}C NMR (75 MHz, DMSO): δ = 172.2, 170.1, 154.9, 137.5, 129.3, 128.0, 126.3, 78.0, 58.6, 53.3, 51.6, 46.3, 37.3, 28.5, 28.1, 24.3 ppm.
UPLC-MS: *m*/*z* = 377.13 $[M+H]^+$

*Synthesis of Boc-D-Phe-L-Pro-OH (**6**)*

Compound **5** (530 mg, 1.4 mmol) was dissolved in THF (25 mL) and kept on ice (0 °C). 1 M LiOH (25 mL) was added, and the reaction stirred for 5 min at 0 °C. The reaction was allowed to warm to room temperature and stirred for further 2 h. THF was removed under vacuum, and the remaining aqueous solution acidified to pH 4 using 1 M HCl. The reaction mixture was extracted with DCM (20 mL) five times, the combined organic phases were washed with brine (100 mL) once, dried over Na_2SO_4 and concentrated under vacuum. Purification by column chromatography (silica 60, DCM/MeOH 95:5) yielded Boc-D-Phe-L-Pro-OH **6** (467 mg, 1.3 mmol, 93% yield) as a yellow-white solid.

^{1}H NMR (300 MHz, DMSO) δ = 12.54 (s, 1H), 7.35 – 7.10 (m, 5H), 6.95 (d, *J* = 8.8 Hz, 1H), 4.44 (dd, *J* = 15.0, 8.2, 1H), 4.11 (dd, *J* = 8.3, 3.5 Hz, 1H), 3.27 – 3.13 (m, 1H), 2.92 – 2.71 (m, 3H), 2.06 – 1.88 (m, 1H), 1.88 – 1.64 (m, 3H), 1.31 (s, 9H) ppm.
^{13}C NMR (75 MHz, DMSO): δ = 173.1, 169.8, 154.8, 137.5, 129.3, 128.0, 126.3, 77.9, 58.6, 53.3, 46.2, 37.5, 28.5, 28.1, 24.2 ppm.
UPLC-MS: *m*/*z* = 385.03 $[M+Na]^+$, *m*/*z* = 361.09 $[M-H]^-$

*Synthesis of H_2N-D-Phe-L-Pro-L-cOrn HCl (**8; fPO***)*

Method A with **6** (1.1 g, 2.9 mmol) as free acid and **2** (500 mg, 3.3 mmol) as amine yielded Boc-D-Phe-L-Pro-L-cOrn **7** (1.2 g, 2.6 mmol, 90% yield) as a yellow-white foam. Method B with **7** (1.2 g, 2.6 mmol) yielded H_2N-D-Phe-L-Pro-L-cOrn hydrochloride **8** (0.9 g, 2.7 mmol, quantitative yield) as a white solid.

^{1}H NMR (600 MHZ, DMSO): δ = 8.28 (s, 2H), 8.05 (d, *J* = 8.3 Hz, 1H), 7.57 (s, 1H), 7.37 – 7.20 (m, 5H), 4.36 (s, 1H), 4.21 (dd, *J* = 7.8, 2.8 Hz, 1H), 4.08 (ddd, *J* = 10.4, 8,1, 6.4 Hz, 1H), 3.58 – 3.46 (m, 1H), 3.14 – 3.09 (m, 2H), 3.07 (dd, *J* = 13.4, 6.1 Hz, 1H), 2.96 (dd, *J* = 13.3, 8.4 Hz, 1H), 2.68 (dd, *J* = 16.8, 7.1 Hz, 1H), 1.94 – 1.86 (m, 2H), 1.84 – 1.68 (m, 4H), 1.67 – 1.56 (m, 1H), 1.49 – 1.41 (m, 1H) ppm.
^{13}C NMR (151 MHz, DMSO): δ = 170.6, 169.6, 166.4, 134.5, 129.5, 128.5, 127.4, 59.7, 51.9, 48.8, 46.6, 41.0, 36.7, 29.2, 27.6, 23.7, 21.0 ppm.
UPLC-MS: *m*/*z* = 359.27 $[M+H]^+$

HRMS: calculated for $C_{19}H_{27}N_4O_3$ *m*/*z* = 359.2078 $[M+H]^+$; measured *m*/*z* = 359.2076 $[M+H]^+$
HPLC: R_t = 14.1 min

*Synthesis of Boc-L-Val-L-cOrn (**10**)*

Method A with Boc-L-Val-OH (**9**, 1.7 g, 7.7 mmol) as free acid and **2** (0.8 g, 7 mmol) as amine yielded Boc-L-Val-L-cOrn **10** (1.4 g, 4.6 mmol, 66% yield) as a white solid.

^{1}H NMR (300 MHz, CDCl3): δ = 7.28 (d, *J* = 4.3 Hz, 1H), 6.67 (s, 1H), 5.33 (d, *J* = 8.6 Hz, 1H), 4.34 – 4.18 (m, 1H), 4.10 – 3.96 (m, 1H), 3.40 – 3.23 (m, 2H), 2.58 – 2.39 (m, 1H), 2.22 – 2.01 (m, 1H), 1.96 – 1.82 (m, 2H), 1.71 – 1.50 (m, 1H), 1.42 (s, 9H), 0.95 (d, *J* = 6.8 Hz, 3H), 0.90 (d, *J* = 6.8 Hz, 3H) ppm.
^{13}C NMR (75 MHz, CDCl3): δ = 171.9, 171.6, 155.8, 79.5, 59.6, 50.3, 41.6, 31.3, 28.3, 27.1, 20.9, 19.2, 17.6 ppm.
UPLC-MS: *m*/*z* = 314.14 $[M+H]^+$

*Synthesis of H_2N-L-Val-L-cOrn HCl (**11**)*

Method B with **10** (500 mg, 1.6 mmol) yielded H_2N-L-Val-L-cOrn hydrochloride **11** (400 mg, 1.6 mmol, quantitative yield).

^{1}H NMR (300 MHz, DMSO): δ = 8.63 (d, *J* = 8.4 Hz, 1H), 8.27 (d, *J* = 3.5 Hz, 3H), 7.63 (s, 1H), 4.35 – 4.10 (m, 1H), 3.64 – 3.57 (m, 1H), 3.22 – 3.00 (m, 2H), 2.17 – 2.03 (m, 1H), 2.02 – 1.91 (m, 1H), 1.87 – 1.56 (m, 3H), 0.99 (d, *J* = 3.6 Hz, 3H), 0.96 (d, *J* = 3.6 Hz, 3H) ppm.
^{13}C NMR (75 MHz, DMSO): δ = 169.1, 167.4, 66.4, 57.3, 48.9, 41.0, 29.8, 27.5, 20.9, 18.2, 18.0 ppm.
UPLC-MS: *m*/*z* = 214.03 $[M+H]^+$

*Synthesis of H_2N-D-Phe-L-Pro-L-Val-L-cOrn HCl (**13; fPVO***)*

Method A with **6** as free acid and **11** (0.8 g, 7 mmol) as amine yielded Boc-D-Phe-L-Pro-L-Val-cOrn **12** (*m*/*z* = 558.38 $[M+H]^+$). Method B with **12** (176 mg, 0.3 mmol) yielded H_2N-D-Phe-L-Pro-L-Val-L-cOrn hydrochloride **13** (67.4 mg, 0.15 mmol, 50% yield) as a white solid.

^{1}H NMR (600 MHz, DMSO): δ = 8.26 (s, 3H), 7.93 (d, *J* = 9.0 Hz, 1H), 7.87 (d, *J* = 7.6 Hz, 1H), 7.58 (s, 1H), 7.37 – 7.28 (m, 3H), 7.25 – 7.18 (m, 2H), 4.44 – 4.29 (m, 2H), 4.17 – 4.04 (m, 2H), 3.58 – 3.47 (m, 1H), 3.15 – 3.09 (m, 2H), 3.06 (dd, *J* = 13.3, 6.0 Hz, 1H), 2.95 (dd, *J* = 13.3, 8.3 Hz, 1H), 2.76 – 2.64 (m, 1H), 2.05 – 1.94 (m, 2H), 1.81 – 1.69 (m, 4H), 1.60 – 1.48 (m, 1H), 1.47 – 1.39 (m, 1H), 0.89 (d, *J* = 6.8 Hz, 3H), 0.87 (d, *J* = 6.8 Hz, 3H) ppm.
^{13}C NMR (151 MHz, DMSO): δ = 170.9, 170.4, 169.6, 166.3, 134.5, 129.5, 128.5, 127.4, 59.4, 57.8, 51.9, 49.0, 46.7, 40.8, 36.8, 30.6, 29.3, 27.4, 23.8, 20.9, 19.3, 18.1 ppm.
UPLC-MS: *m*/*z* = 458.43 $[M+H]^+$
HRMS: calculated for $C_{24}H_{36}N_5O_4$ $[M+H]^+$ *m*/*z* = 458.2762 $[M+H]^+$; measured *m*/*z* = 458.2762 $[M+H]^+$
HPLC: R_t = 17.4 min

Peptide quantification by UPLC-MS/MS

For quantification of peptides, a Xevo TQ-S micro tandem mass spectrometer (Waters) was used. Samples were diluted 20-fold with 50% ethanol containing 0.1% formic acid (FA) when analyzing cyclo-(fP), fPO*, and fPVO*. A 2 µL aliquot was injected onto a CORTECS UPLC C18 column (1.6 µm, 2.1 x 50 mm; Waters) equipped with a VanGuard pre-column (2.1 x 5 mm; Waters) connected to an H-class UPLC system (Waters). Elution was performed at 40 °C with acetonitrile (MeCN) in H_2O containing 0.1% FA at a flow rate of 0.5 mL/min (2% MeCN at 0.00 - 0.30 min, linear gradient from 2 - 50% MeCN at 0.30 - 1.50 min, 95% MeCN at 1.51 - 1.80 min, and 2% MeCN at 1.81 - 2.80 min). Ionization was performed in an ESI Z-Spray probe operated in positive mode at a capillary voltage of 0.5 kV and a desolvation temperature of 600 °C. The cone voltage was set to 20 V and multiple reaction monitoring (MRM) was performed using argon as collision gas (Table S5).

Fluorescence polarization assay

Affinity between zinc finger domains and DNA was measured in a fluorescence polarization assay. The DNA probes (Table S4) were designed according to a previous report.[8] In short, a fluorescein-dT base was incorporated two bases upstream of the zinc finger binding site on the reverse oligo. Synthetic oligos (Eurofins) were dissolved in buffer NE (5 mM Tris-HCl, pH 8.5) at 100 µM concentration for long term storage. Double stranded DNA probes were generated by annealing of 10 µM forward and reverse oligos in buffer AN (100 mM NaCl, 10 mM Tris-HCl, pH 7.4). The DNA solutions were heated to 80 °C for 4 min to resolve secondary structure followed by gradual cooling to room temperature for 30 min. The resulting 10 µM probes were stored in the dark at -20 °C.

The fluorescence polarization assay buffer (100 mM NaCl, 50 mM HEPES, 0.1 mM $MgCl_2$, 9 µM $ZnCl_2$, 5% glycerol, 0.5 mM TCEP, 100 µg/mL bovine serum albumin [BSA; Roth Cat.No. 90604-29-8]) resembled the buffer for peptide formation as closely as possible. The buffer was freshly prepared, filtered through a cellulose acetate (CA) filter with a pore size of 0.22 µm (TH Geyer), and kept at room temperature for all following dilution procedures. DNA probes were diluted to 100 nM. Proteins were first diluted to 2 µM, centrifuged at 16,000 x g and 16 °C for 8 min to remove potential precipitates, and then subjected to 2-fold serial dilutions. In black 96-well plates (BRANDplates, Cat.No. 781668, BrandTech), 90 µL protein dilution were added to 10 µL of 100 nM DNA probe. After thoroughly mixing by pipetting, the sample was kept at room temperature for 10 min and measured on a Synergy H1 fluorescence microplate reader (Biotek) using a polarization filter set (485/528 nm) at 24.5 ± 1.0 °C. The polarization output (mP; Equation 1) was plotted against protein concentration (nM) and fitted to a bimolecular binding model (Equation 2) with R version 3.4.3 (Table S6).

Equation 1:

$$\text{polarization [mp]} = \frac{\text{Intensity}_{\parallel} - \text{Intensity}_{\perp}}{\text{Intensity}_{\parallel} + \text{Intensity}_{\perp}} \times 1000$$

where the incident light is subjected to a linear polarizer and
$\text{Intensity}_{\parallel}$: the intensity of emitted light measured using a parallel polarizing filter
$\text{Intensity}_{\perp}$: the intensity of emitted light measured using a perpendicular polarizing filter

Equation 2:

$$\mathrm{F} = \mathrm{F}_0 + \frac{\mathrm{F}_{\max} - \mathrm{F}_0}{\mathrm{A}_0} \times \frac{(\mathrm{A}_0 + \mathrm{P}_0 + K_\mathrm{d}) - \sqrt{(\mathrm{A}_0 + \mathrm{P}_0 + K_\mathrm{d})^2 - 4\mathrm{A}_0\mathrm{P}_0}}{2}$$

where these parameters are measured or controlled:
F: polarization
F_0: polarization in the absence of protein
A_0: DNA probe concentration
P_0: protein concentration
and the following parameters are predicted by the model:
$\mathrm{F}_{\max}$: polarization at saturating protein concentration
K_d: dissociation constant

Isothermal titration calorimetry (ITC)

ITC measurements (Figure S4) were performed at 25 °C in 50 mM HEPES buffer pH 8, 150 mM NaCl and 5% (v/v) glycerol using a MicroCal PEAQ-ITC calorimeter (Malvern Instruments). A solution containing 50 µM DnGrsB3 was titrated with 1 mM of the respective Dc peptides. Peptides InxA-Dc8 (QALLKGDI), InxA-Dc8-GS (QALLKGDIGS) and InxA-Dc6-GS (QALLKGGS) were custom synthesized on a 20-24 mg scale at >95% HPLC purity by GenScript Biotech (Netherlands) as HCl salts. ITC measurements started with an initial delay of 60 s. The first injection of 0.4 µl was followed by 24 injections with 3 µl each for titration with peptides InxA-Dc8 and InxA-Dc8-GS or 12 injections with 3 µl each for titration with peptide InxA-Dc6-GS. Injections were performed in intervals of 150 s. The reference power was set to 10 µcal/s and the stir speed to 750 rpm. For each measurement, the “high feedback” mode was selected. The thermograms were analyzed using the MicroCal PEAQ-ITC Analysis Software v1.22 assuming a one site binding model. Signals in thermograms resulting from titration of the respective peptides into buffer were subtracted point-by-point as background correction. *c*-Values were calculated using equation 3:

Equation 3:

$$c = nK_a[M]_T$$

where K_a is the binding constant, $[M]_T$ the total macromolecule concentration in the cell and *n* the binding stoichiometry. With InxA-Dc8 and InxA-Dc8-GS, binding was detected but the c-value <1 indicated unreliable quantification of the binding constants. In both cases the apparent interaction was only qualitatively noted as weak binding. With InxA-Dc6-GS, no binding was observed.

Analytical size exclusion chromatography

To physically confirm module assembly on DNA template, 2.5 µM each of TycB1-N, GrsB2-P, and GrsB3-Z were added to 2 µM of SEC-DNA in SEC sample buffer (100 mM NaCl, 50 mM HEPES pH 8.0, 35 µM $ZnCl_2$, 2% glycerol, and 0.5 mM TCEP). The solution was centrifuged at 16,000 x g and 16 °C for 6 min and equilibrated at 10 °C for 20 min. A 20 µL aliquot was injected onto a Superdex 200 Increase 3.2/300 analytical size exclusion column (GE Healthcare) connected to an NGC Quest 10 Plus Chromatography System (Bio-Rad) while bypassing the column selection valve. Elution was performed at 10 °C in 2.4 mL buffer MT (100 mM NaCl, 50 mM HEPES pH 8.0) at a flow rate of 0.075 mL/min. Absorbance at 260 and 280 nm was recorded in a 5 mm flow cell.

Molecular weight standards were prepared by dissolving components of Gel Filtration Calibration Kit (GE Healthcare) separately in buffer T (150 mM NaCl, 50 mM Tris pH 7.5) at the following concentrations: 20 mg/mL thyroglobin (T), 2 mg/mL ferritin (F), 20 mg/mL aldolase (A), and 20 mg/mL conalbumin (C). A mixture containing equal volumes of each protein was stored at -80 °C. Prior to analysis, the mixture was thawed, diluted 2.5-fold with buffer MT, and centrifuged at 16,000 x g and 16 °C for 6 min. A 20 µL aliquot was analyzed via size exclusion as described above.

Homology modelling

A homology model of a two-modular DT-NRPS was built based on the sequence of GrsB3-Z and a DNA template with two corresponding binding sites and a 20 bp spacer. The DNA was modelled as ideal B-form DNA with the script “build_DNA.mcr” in YASARA (version 19.12.14).[9] Two models of Zif268 prepared on the Swiss-Model[10] server were aligned with the DNA in PyMOL.[11] After energy minimization in Yasara with default settings, a homology model of the Dn/Dc domain (Swiss-Model) was changed to the sequence from GrsB3-Z at the termini and appended to one of the ZF. Next, the homology model of GrsB3-CAT (Swiss-Model) was added. The linkers between the homology models were modelled in an arbitrary low energy conformation not based on experimental data that was not further optimized. The Dn-CAT-Dc-ZF2 was duplicated and overlayed on the ZF bound to the other binding site. Since there was a slight clash between both CAT modules, a dihedral in the Dn-CAT linker was adjusted in both modules until the clash disappeared.

Sinusoidal fit of spacer length dependence

The spacer length dependence observed with the trimodular DT-NRPS (Figure 4) was nonlinearly fit to a cosine curve in R version 3.6.3 using the following code:

```
sta <- list(a=40, b=10, c=100, d=-6)
m <- nls(y~a*cos(x*2*pi/b+d) + c, start = sta)
summary(m)
```

The optimized parameters of fit are a = 43 ± 6 nM (amplitude), b = 10.1 ± 0.3 bp (wavelength), c = 90 ± 4 nM (y-axis offset), and d = -6.6 ± 0.4 bp (x-axis offset) with a residual standard error of 35.5 nM for 70 degrees of freedom.

Supplementary Figures and Tables

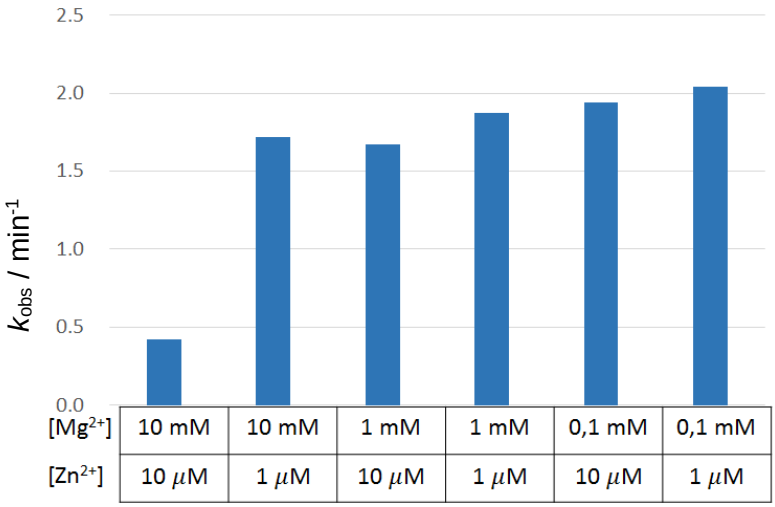

Figure S1.
Optimization of Zn^{2+} and Mg^{2+} concentration. Reactions were performed with 0.5 µM GrsA and TycB1 in buffer containing 100 mM NaCl, 50 mM HEPES pH 8.0, 2 % glycerol, and the indicated metal ions. Cyclo-(fP) was quantified by LC-MS/MS after 1 h at 37°C and divided by the enzyme concentration to obtain k_{obs}.

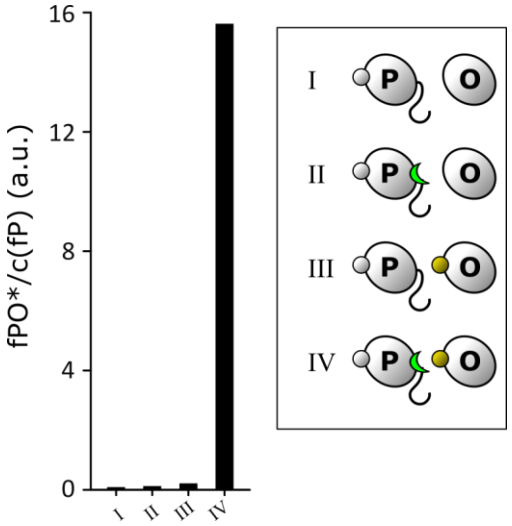

Figure S2. Portability of the docking domain.
GrsA was added to all reactions. In addition, proteins TycB1-linker o-P (I & III), TycB1-linker Dc-P (II & IV), GrsB3 (I & II), and Dn-GrsB3 (III & IV) were supplied (Table S1 and S3). The data were collected from a single batch of protein purified through nickel affinity chromatography. Reaction conditions: 1 µM of each protein, no DNA template or $ZnCl_2$; incubation at 37°C for 3 h. The ratio of fPO* to cyclo-(fP) determined by tandem UPLC-MS is a robust parameter to assess docking domain efficiency since the ratio is less sensitive to variations in enzyme purity than the overall yield.

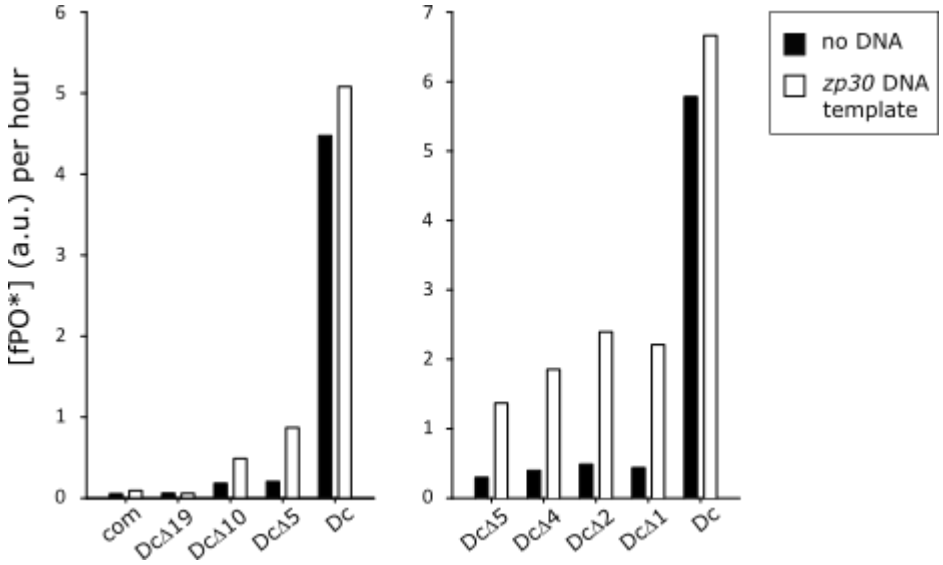

Figure S3. Docking domain truncation tests.
Reactions contained GrsA, TycB1-"X"-P, and Dn-GrsB3-linker com-Z. The linker "X" is specified on the x axes. Module assembly was tested on a DNA template with 30-bp spacer. Proteins were purified by nickel affinity chromatography only and the data was collected using a single batch of protein. Reaction conditions: 1 μM of each protein, and 0.5 μM DNA template zp30; incubation at 37°C for 2 h (left panel) or 1 h (right panel).

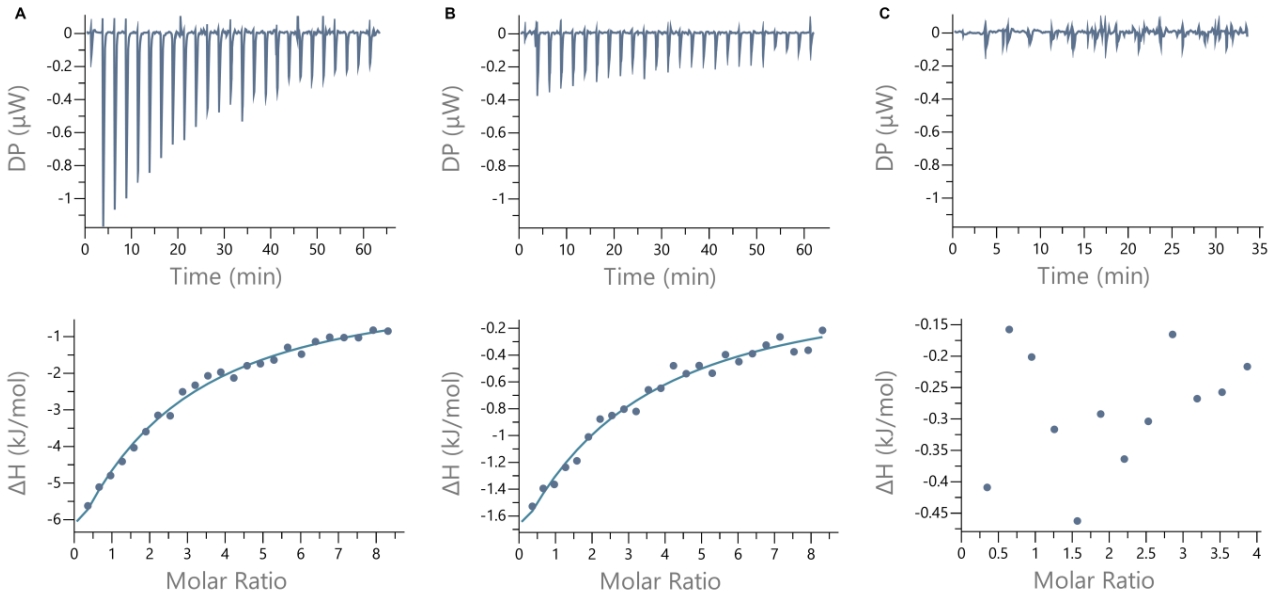

Figure S4. Docking domain binding affinity.
ITC thermograms (top) and derived binding curves (bottom) for titration of module Dn-GrsB3 with different variants of the InxA-Dc fragment responsible for interaction.[12] DP: power differential between reference and sample cell. (A) Titration with full length InxA-Dc8 (K_D: 280 ± 40 µM, ΔH: -41 ± 6 kJ/mol, c: 0.177). (B) Titration with peptide InxA-Dc8-GS containing additional C-terminal amino acids GS to mimic the linker region in ZF containing constructs (K_D: 350 ± 80 µM, ΔH: -13 ± 3 kJ/mol, c: 0.141). (C) Titration with peptide InxA-Dc6-GS truncated by two amino acids.

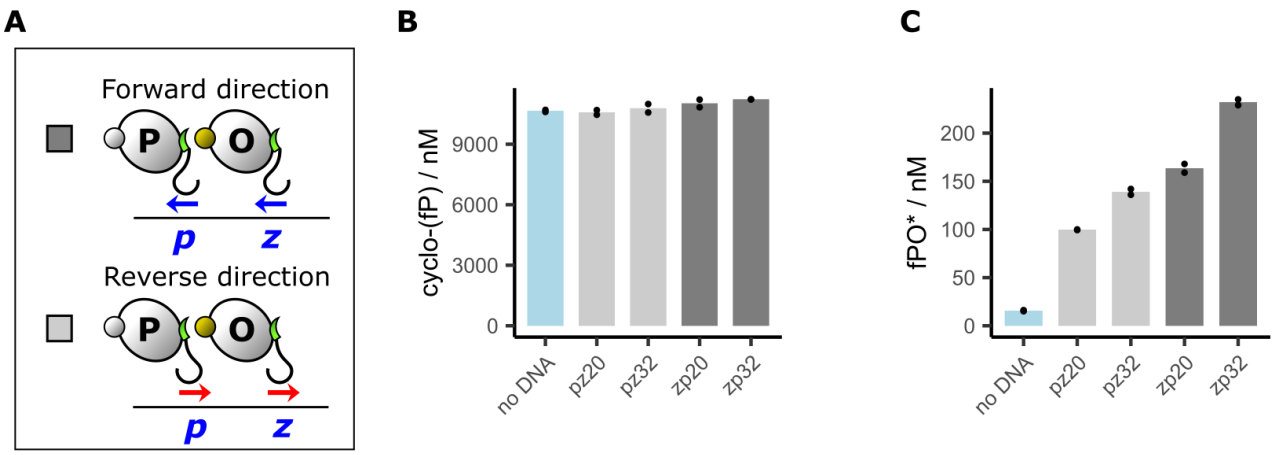

Figure S5. Influence of binding site orientation on peptide formation efficiency.
(A) Scheme of the ZF orientation. (B) DNA-independent formation of the control cyclo-(fP). (C) DNA-dependent formation of fPO* on templates with different ZF binding site orientations and linker lengths. Reaction conditions: 0.4 µM GrsA, 0.1 µM TycB1-P, 0.5 µM GrsB3-Z, and 0.4 µM DNA template. Data points are shown for a biological duplicate.

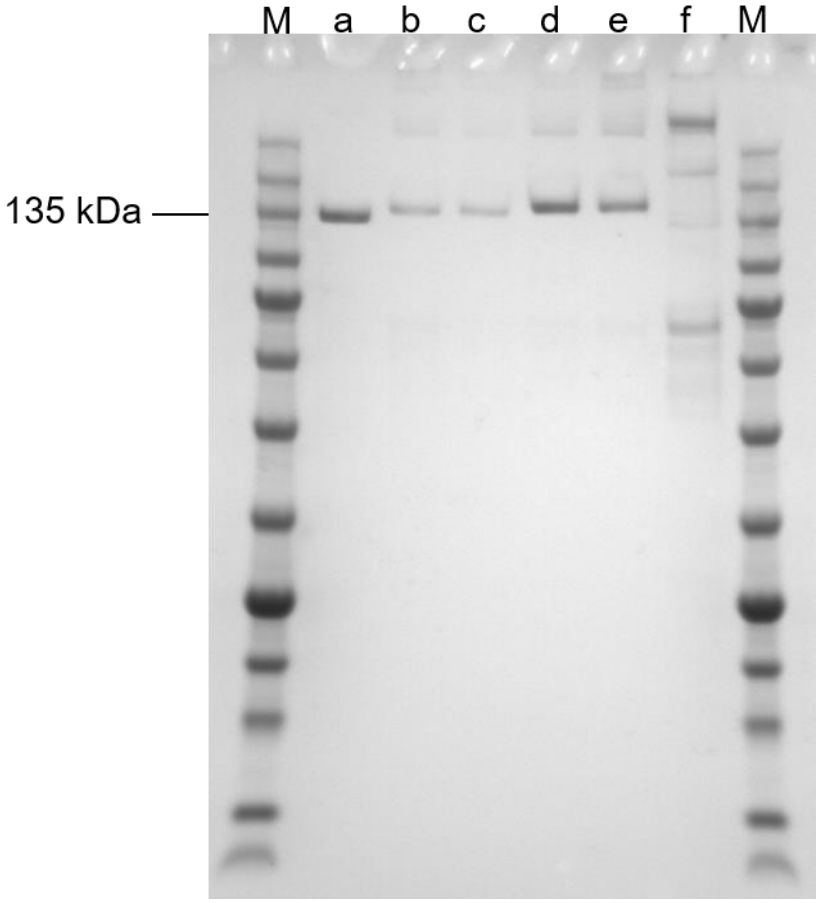

Figure S6. SDS PAGE of purified proteins.
Electrophoretic separation was performed on Bolt 4-12% Bis-Tris Plus Gels (Thermo Fisher). Per well, 500 ng protein were applied. M: Marker (SERVA Triple Color Protein Standard III, 5 µl used), a: GrsA (size 128 kDa), b: TycB1-P (size 131 kDa), c: TycB1-N (size 131 kDa), d: GrsB2-P (size 139 kDa), e: GrsB3-Z (size 141 kDa), f: GrsB123 (size 358 kDa). The gel was run at 200 V for 22 min in MES SDS buffer and stained with Quick Coomassie stain (Serva).

Table S1. Plasmids and proteins used in this study.

Plasmid name	Genetic layout*	Encoded protein	Usage	Source
pSU18-grsA	pSU18 vector; Plac-grsA; Chl^R	GrsA	Source of NRPS modules; Source of pSU18 vector; GrsA expression	[3]
pTrc99a-tycB1	Ptrc-tycB1; Amp^R	not used	Source of NRPS genes	[1]
pTrc99a_grsB_M3574L	Ptrc-grsB; Amp^R	not used	Source of NRPS genes	[3]
pSU18F-tycB1-P (o)	Plac-tycB1-linker o-pbsII; N' His6 tag	TycB1-linker o-P	Verify Dn & Dc portability	This study
pSU18F-tycB1-P (Dc)	Plac-tycB1-linker Dc-pbsII; N' His6 tag	TycB1-linker Dc-P	Verify Dn & Dc portability	This study
pSU18-grsB3	Plac-grsB3	GrsB3	Verify Dn & Dc portability	This study
pSU18-Dn-grsB3	Plac-Dn-grsB3	Dn-GrsB3	Verify Dn & Dc portability	This study
pSU18-tycB1-P (com)	Plac-tycB1-linker com-pbsII	TycB1-linker com-P	Dc truncation test	This study
pSU18-tycB1-P (Dc)	Plac-tycB1-linker Dc-pbsII	TycB1-linker Dc-P	Dc truncation test	This study
pSU18-tycB1-P (DcΔ1)	Plac-tycB1-linker DcΔ1-pbsII	TycB1-linker DcΔ1-P	Dc truncation test	This study
pSU18-tycB1-P (DcΔ2)	Plac-tycB1-linker DcΔ2-pbsII	TycB1-linker DcΔ2-P (TycB1-P)	Dc truncation test; DNA spacer length test	This study
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pSU18-Dn-grsB3-Z (com)	Plac-Dn-grsB3-linker com-zif268	Dn-GrsB3-linker com-Z	Dc truncation test	This study
pSU18-grsB123	Plac-grsB123	GrsB123	Positive control (fused multimodule)	This study
pSU18-tycB1-N (DcΔ2)	Plac-tycB1-linker DcΔ2-nre	TycB1-N	Multimodule assembly	This study
pSU18-Dn-grsB2-P (DcΔ2)	Plac-Dn-grsB2-linker DcΔ2-pbsII	GrsB2-P	Multimodule assembly	This study
pSU18-Dn-grsB3-Z (DcΔ2)	Plac-Dn-grsB3-linker DcΔ2-zif268	GrsB3-Z	DNA spacer length test; Multimodule assembly	This study
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pSU18-B-Dn-grsB4	Plac-zfb-Dn-grsB4	B-GrsB4	Multimodule assembly	This study

*For protein sequences encoded by the linkers, see Table S3. Genes of NRPS modules are highlighted in yellow, genes of linkers in green and genes of zinc fingers in cyan.

Table S2. Coding sequences of recombinant NRPS proteins used in multimodule assembly.
Sequences along with annotations are shown in GenBank file format, each starting with "LOCUS" and ending with "//". The text can be saved with ".gb" file extension using text editors and visualized by molecular biology software.

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     1321 TGGATTGCAA AATTTGATAT TTCATTCGAT ATTTTAGAAA AGCAAGATGA CATTCAATTT
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       61 AAACTGGTTC GGCACCAACG CACCCATACC GGTGAAAAGC CGTATAAATG CCCGGAATGT
      121 GGCAAATCCT TTTCGACCTC TGGCGAATTA GTCCGCCATC AGCGCACTCA CACGGGTGAG
      181 AAACCGTACA AATGCCCAGA ATGCGGCAAA TCGTTCAGTC GCTCAGATGA TCTGGTGCGT
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4441 AGATCTCATC ACCATCACCA TCACTAA
//
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Table S3. Linkers connecting NRPS module and zinc finger domain.

Linker	Peptide sequence	Description (underlined)
linker o	VEGGGESAYLAIPQAEGSAAA	TycB-derived, original linker following TycB1
linker com	IKDRSELTPSDFSFGSRSSSTSAAA	GrsA-derived, designed in reference to pTrcHis-tycA::COM$^{D}\Delta$E [13]
Dn	MKDAAQIVNEALDQGITLFVAEDRLQYETRLSNIPADLISE WKQHKQELIDFLNQLDSSEEQVSTHQLQGIPRYERA	N-terminus of InxB
linker Dc	VTVHLLKEKRKHFQAGQETAQALLKGDIGSAAA	C-terminus of InxA
linker Dc_1	VTVHLLKEKRKHFQAGQETAQALLKGDGSAAA	Truncated C-terminus of InxA
linker Dc_2	VTVHLLKEKRKHFQAGQETAQALLKGGSAAA	Truncated C-terminus of InxA
linker Dc_4	VTVHLLKEKRKHFQAGQETAQALLGSAAA	Truncated C-terminus of InxA
linker Dc_5	VTVHLLKEKRKHFQAGQETAQALGSAAA	Truncated C-terminus of InxA
linker Dc_10	VTVHLLKEKRKHFQAGQEGSAAA	Truncated C-terminus of InxA
linker Dc_19	VTVHLLKEKGSAAA	Truncated C-terminus of InxA

Table S4. DNA templates and probes.[#]

Oligos	Sequence (5' to 3')
	Fluorescence polarization assay
z_F	tacaGCGTGGGCGtaat
z_R (FdT)	at[FdT]aCGCCCACGCtgta
p_F	ggacGTGTGGAAAaacg
p_R (FdT)	cg[FdT]tTTTCCACACgtcc
n_F	ggacAAGGGTTCAgatg
n_R (FdT)	ca[FdT]cTGAACCCTTgtcc
b_F	tacaGCGGCTGGGtaat
b_R (FdT)	at[FdT]aCCCAGCCGCtgta
	DNA spacer test
zp9_F	ggtaccGCGTGGGCGGTGTGGAAAtattc
zp9_R	gaataTTTCCACACCGCCCACGCggtacc
zp10_F	ggtaccGCGTGGGCGtGTGTGGAAAtattc
zp10_R	gaataTTTCCACACaCGCCCACGCggtacc
zp12_F	ggtaccGCGTGGGCGtgaGTGTGGAAAtattc
zp12_R	gaataTTTCCACACtcaCGCCCACGCggtacc
zp14_F	ggtaccGCGTGGGCGtgaaaGTGTGGAAAtattc
zp14_R	gaataTTTCCACACtttcaCGCCCACGCggtacc
zp16_F	ggtaccGCGTGGGCGtgattaaGTGTGGAAAtattc
zp16_R	gaataTTTCCACACttaatcaCGCCCACGCggtacc
zp18_F	ggtaccGCGTGGGCGtgatcataaGTGTGGAAAtattc
zp18_R	gaataTTTCCACACttatgatcaCGCCCACGCggtacc
zp19_F	ggtaccGCGTGGGCGtgatccataaGTGTGGAAAtattc
zp19_R	gaataTTTCCACACttatggatcaCGCCCACGCggtacc
zp20_F	ggtaccGCGTGGGCGtgatccaataaGTGTGGAAAtattc
zp20_R	gaataTTTCCACACttattggatcaCGCCCACGCggtacc
zp21_F	ggtaccGCGTGGGCGtgatcctaataaGTGTGGAAAtattc
zp21_R	gaataTTTCCACACttattaggatcaCGCCCACGCggtacc
zp22_F	ggtaccGCGTGGGCGtgatccttaataaGTGTGGAAAtattc
zp22_R	gaataTTTCCACACttattaaggatcaCGCCCACGCggtacc
zp24_F	ggtaccGCGTGGGCGtgatcctgataataaGTGTGGAAAtattc
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zp26_F	ggtaccGCGTGGGCGtgatcctgccataataaGTGTGGAAAtattc
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zp28_F	ggtaccGCGTGGGCGtgatcctgccccataataaGTGTGGAAAtattc
zp28_R	gaataTTTCCACACttattatggggcaggatcaCGCCCACGCggtacc
zp30_F	ggtaccGCGTGGGCGtgatcctgcccgccataataaGTGTGGAAAtattc
zp30_R	gaataTTTCCACACttattatggcgggcaggatcaCGCCCACGCggtacc
zp31_F	ggtaccGCGTGGGCGtgatcctgcccagccataataaGTGTGGAAAtattc

zp31_R	gaataTTTCCACACttattatggctgggcaggatcaCGCCCACGCggtacc
zp32_F	ggtaccGCGTGGGCGtgatcctgcccacgccataataaGTGTGGAAAtattc
zp32_R	gaataTTTCCACACttattatggcgtgggcaggatcaCGCCCACGCggtacc
zp33_F	ggtaccGCGTGGGCGtgatcctgcccatcgccataataaGTGTGGAAAtattc
zp33_R	gaataTTTCCACACttattatggcgatgggcaggatcaCGCCCACGCggtacc
zp34_F	ggtaccGCGTGGGCGtgatcctgcccatccgccataataaGTGTGGAAAtattc
zp34_R	gaataTTTCCACACttattatggcggatgggcaggatcaCGCCCACGCggtacc
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zp36_R	gaataTTTCCACACttattatggcggacatgggcaggatcaCGCCCACGCggtacc
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zp40_R	gaataTTTCCACACttattatggcggagacgcatgggcaggatcaCGCCCACGCggtacc
zp44_F	ggtaccGCGTGGGCGtgatcctgcccatgcgcacgtctccgccataataaGTGTGGAAAtattc
zp44_R	gaataTTTCCACACttattatggcggagacgtgcgcatgggcaggatcaCGCCCACGCggtacc
	Multimodular templating
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zpn9_R	ccagcTGAACCCTTTTTCCACACCGCCCACGCtgtac
zpn20_F	gtacaGCGTGGGCGtgatcctgcccGTGTGGAAAactcccaggacAAGGGTTCAgctgg
zpn20_R	ccagcTGAACCCTTgtcctgggagtTTTCCACACgggcaggatcaCGCCCACGCtgtac
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zpn32_R	ccagcTGAACCCTTgtcctgggagacgtgcgcatgttTTTCCACACggctattatggcgggcaggatcaCGCCCACGCtgtac
b'zpn_F	gtacaCCCAGCCGCtGCGTGGGCGtgatcctgcccGTGTGGAAAactcccaggacAAGGGTTCAgctgg
b'zpn_R	ccagcTGAACCCTTgtcctgggagtTTTCCACACgggcaggatcaCGCCCACGCaGCGGCTGGGtgtac
	DNA spacer test and direction test
p'z9_F	gaataCGCCCACGCGTGTGGAAAagtacc
p'z9_R	ggtactTTTCCACACGCGTGGGCGtattc
p'z10_F	gaataCGCCCACGCtGTGTGGAAAagtacc
p'z10_R	ggtactTTTCCACACaGCGTGGGCGtattc
p'z11_F	ggtactTTTCCACACgaGCGTGGGCGtattc
p'z11_R	gaataCGCCCACGCtcGTGTGGAAAagtacc
p'z13_F	gaataCGCCCACGCtaccGTGTGGAAAagtacc
p'z13_R	ggtactTTTCCACACggtaGCGTGGGCGtattc
pz20_F	ggtaccGTGTGGAAAagatccaataaGCGTGGGCGtattc
pz20_R	gaataCGCCCACGCttattggatctTTTCCACACggtacc
pz32_F	ggtaccGTGTGGAAAagatcctgcccacgccataataaGCGTGGGCGtattc
pz32_R	gaataCGCCCACGCttattatggcgtgggcaggatctTTTCCACACggtacc
	SEC*
SEC-DNA_F	gtacaCCCAGCCGCtGCGTGGGCGtgatcctgcccGTGTGGAAAactcccaggacAAGGGTTCAgctgg
SEC-DNA_R	ccagcTGAACCCTTgtcctgggagtTTTCCACACgggcaggatcaCGCCCACGCaGCGGCTGGGtgtac

[#]ZF domain binding sites are shown in capital letters. The linkers in between are random sequences (lower case). [FdT]: fluoresceindT; _F: forward oligo; _R: reverse oligo. A prime after the ZF abbreviation indicates an inverted direction of the binding site. ***The SEC-DNA contains a fourth zinc finger binding site not used in this work.

Table S5. Tuning parameters for peptide quantification by tandem mass spectrometry.

Compound	Retention time (min)	Parent ion (m/z)	Daughter ion (m/z)	Collision energy (V)	Concentrations tested[#]
cyclo-(fP)	1.78	245.1	70.1	18	10 nM – 1µM
fPO*	1.59	359.2	212.1	18	20 pM – 200 nM
fPVO*	1.69	458.3	245.1	21	200 pM – 200 nM
fPVOL	1.73	295.2	120.1	18	172 pM – 172 nM

[#]In this concentration range, the signal was linear but the full dynamic range may be larger. Only relevant concentrations were tested.

Table S6. R script for modelling bimolecular binding.

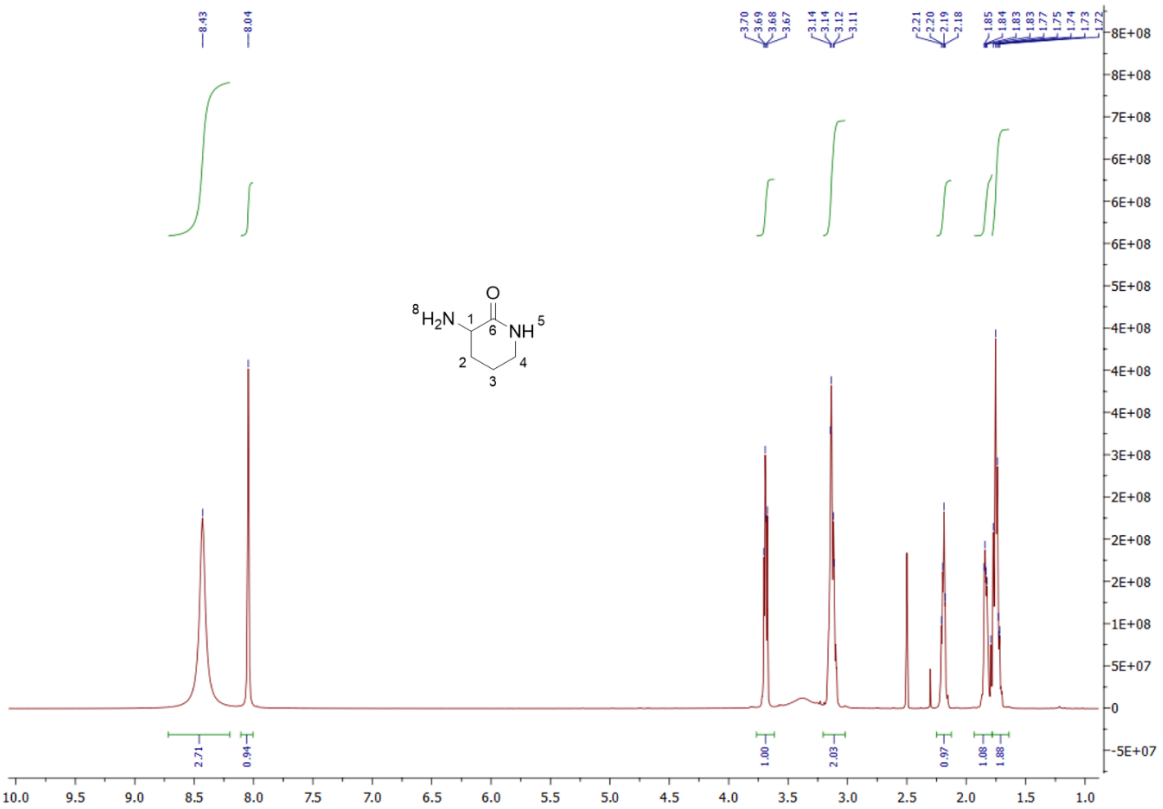
```
# Input data are organized in three columns in a csv (comma delimited) file
# 1st column starts with title "A0", the following rows specify DNA concentrations
# 2nd column starts with title "P0", the following rows specify protein concentration
# a data point without protein is required to calculate F0
# 3rd column starts with title "F", the following rows specify fluorescence polarization

library(dplyr)
library(stringr)

# Load input data
dat1 <- read.csv(file=file.choose())
F0 = dat1[dat1$P0==0,3]
print(dat1)

# Single-ligand model, requires preassigned variable F0
lig1 <- function(P0,A0,Ka,Fmax) {
  return((P0+A0+Ka-((P0+A0+Ka)^2-4*P0*A0)^0.5)/(2*A0)*(Fmax-F0)+F0)
}
# fit to model 1
m1<-nls(F~lig1(P0, A0, Ka, Fmax),data=dat1, start = list(Ka=20,Fmax = max(dat1$F)))
summary(m1)

# plot original data in log scale
plot.new()
x_label<- "P0 (nM)"; y_label<- "F (mP)"
plot(dat1[dat1$P0!=0,]$P0, dat1[dat1$P0!=0,]$F, log="x", xlab=x_label, ylab=y_label)

# draw regression line
Pseq=seq(log10(min(dat1[dat1$P0!=0,]$P0)),log10(max(dat1[dat1$P0!=0,]$P0)),len = 100)
Pseq=10^Pseq
lines(Pseq,predict(m1,list(P0= Pseq, A0=dat1$A0[1])),col="blue",lwd=2)
abline(h=F0, lty=3)
```

Table S7. Quantification of peptides in tetramodular spacer adjustment (related to Figure 5).[#]

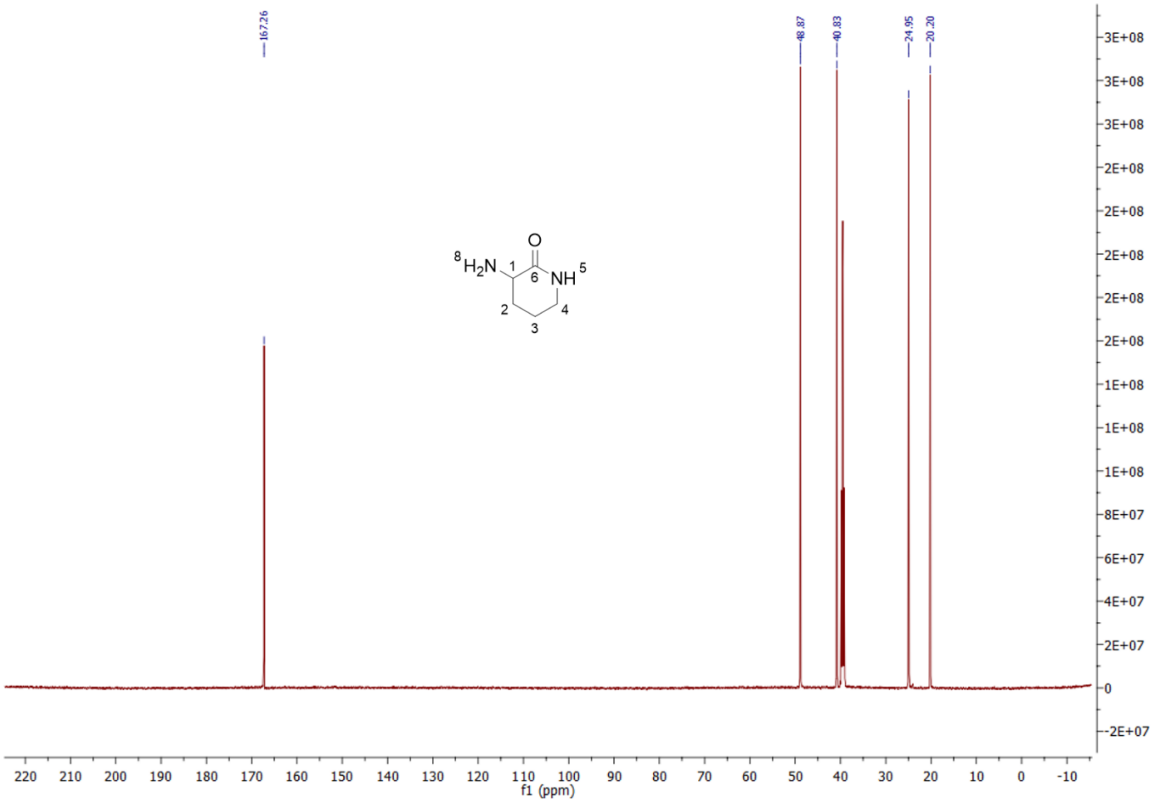
Experiment	Cyclo-(fP) (nM)	Std. dev.	fPO* (nM)	Std. dev.	fPVO* (nM)	Std. dev.	fPO*/ cyclo-[fP] (%)	Std. dev.	fPVO*/ cyclo-[fP] (%)	Std. dev.
No DNA	8860	397	25	3	20	3	0.28	0.02	0.22	0.02
9-bp spacer	8386	629	106	19	1256	228	1.25	0.13	14.92	1.60
20-bp spacer	8635	651	34	5	868	161	0.40	0.03	10.00	1.11
32-bp spacer	8887	577	23	4	512	70	0.25	0.02	5.75	0.41
GrsB123	4564	348	8	3	1336	377	0.18	0.05	29.04	6.05

[#]Concentrations are the average of two independent measurements.

NMR spectra

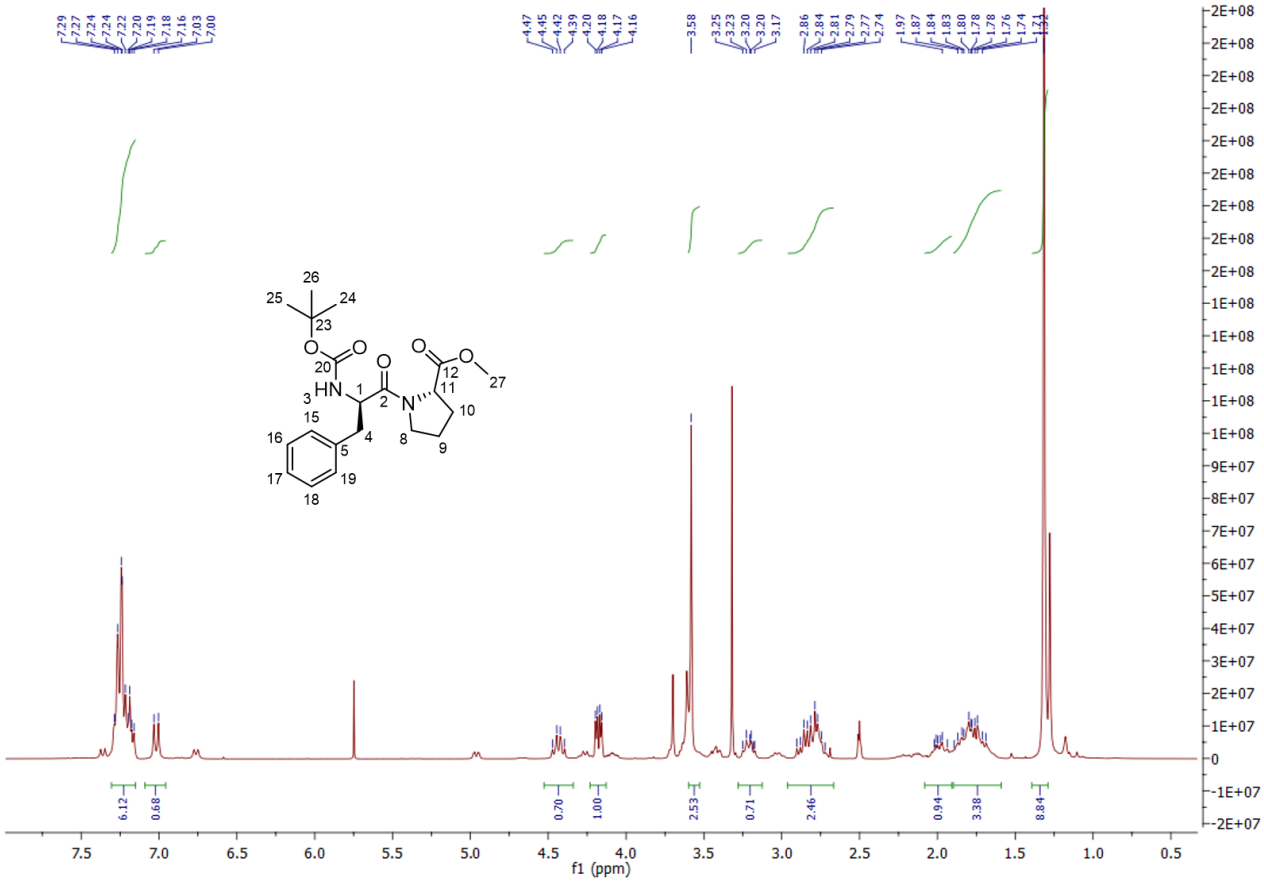


^{1}H NMR of compound **2** measured in DMSO-d_6

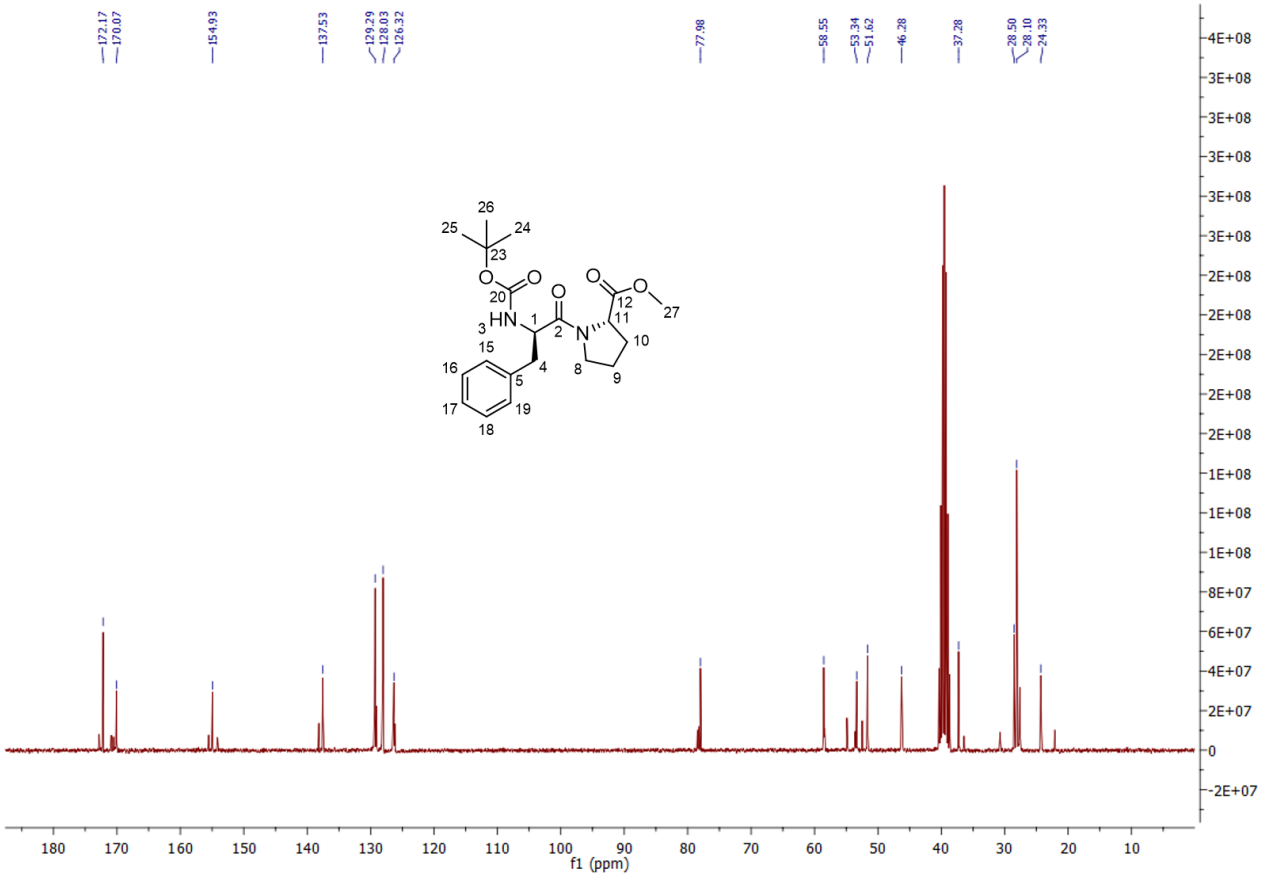


^{13}C NMR of compound **2** measured in DMSO-d_6

Position	**δ_C**	**δ_H, mult. (*J* in Hz)**
1	48.9, CH	3.69, dd (11.0, 6.1)
2a	25.0, CH_2	2.27 – 2.11, m
2b		1.89 – 1.80, m
3	20.2, CH_2	1.78 – 1.69, m
4	40.8, CH_2	3.19 – 3.04, m
5		8.04, s
6	167.3, qC	
8		8.43, broad s

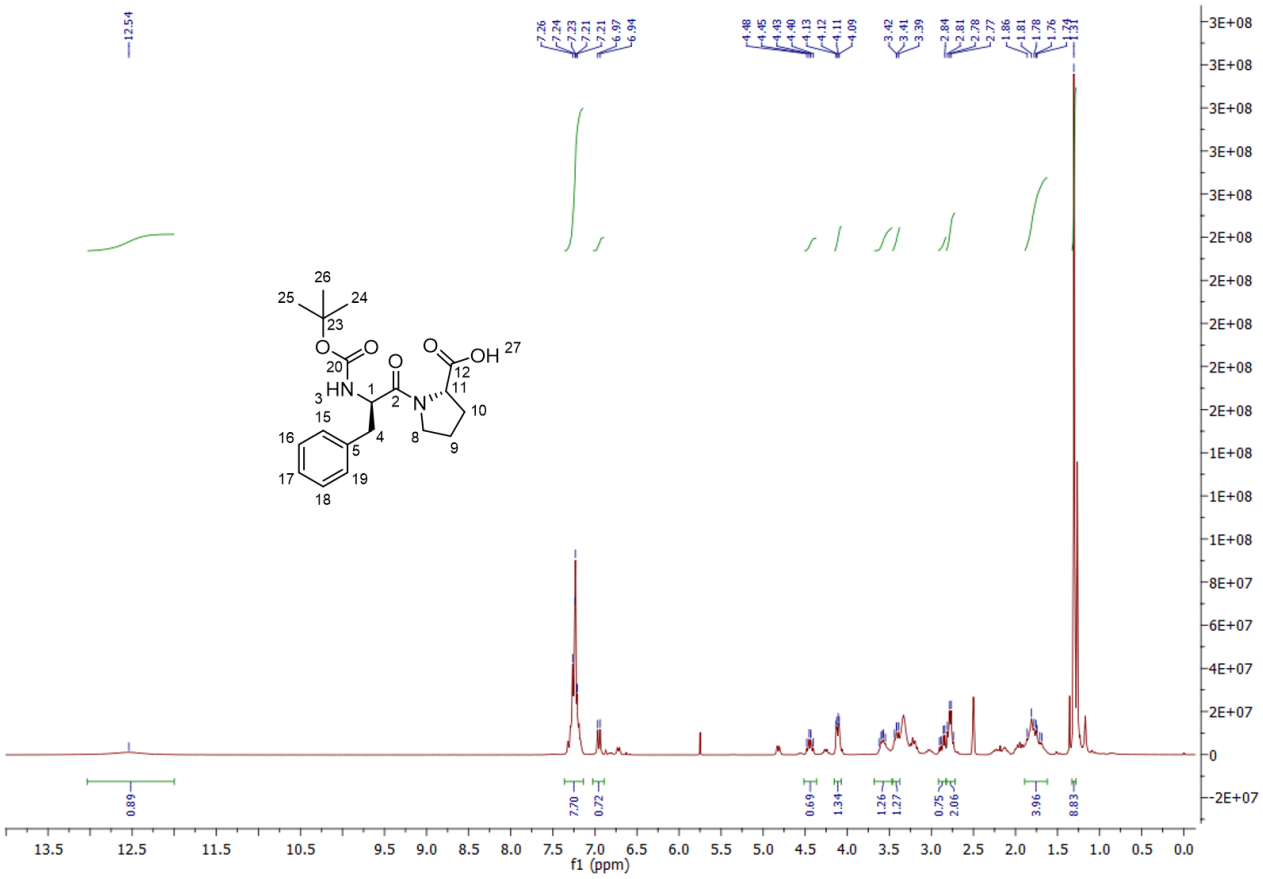


^{1}H NMR of compound **5** measured in DMSO-d_6

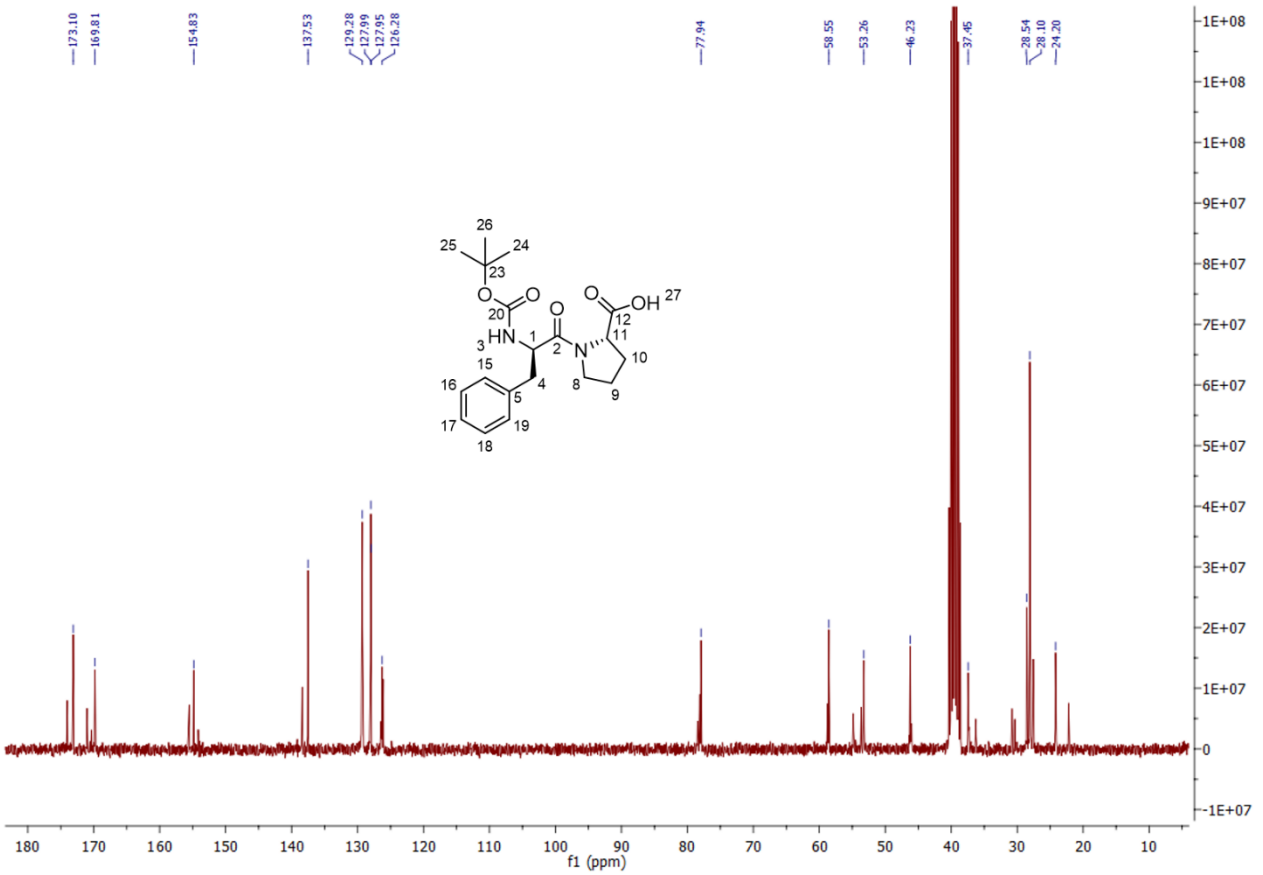


^{13}C NMR of compound **5** measured in DMSO-d_6

Position	**δ_C,**	**δ_H, mult. (*J* in Hz)**
1	53.3, CH	4.43, dd (15.1, 8.5)
2	170.1, qC	
3		7.02, d (8.7)
4	37.3, CH_2	2.92 – 2.67, m
5	137.5, qC	
8a 8b	46.3, CH_2	3.28 – 3.12, m 2.92 – 2.67, m
9	24.3, CH_2	1.90 – 1.59, m
10a 10b	28.5, CH_2	2.06 – 1.93, m 1.90 – 1.59, m
11	58.6, CH	4.18, dd (7.7, 4.3)
12	172.2, qC	
15	128.0, CH	7.31 – 7.13, m
16	129.3, CH	7.31 – 7.13, m
17	126.3, CH	7.31 – 7.13, m
18	129.3, CH	7.31 – 7.13, m
19	128.0, CH	7.31 – 7.13, m
20	154.9, qC	
23	78.0, qC	
24	28.1, CH_3	1.32, s
25	28.1, CH_3	1.32, s
26	28.1, CH_3	1.32, s
27	51.6, CH_3	3.58, s

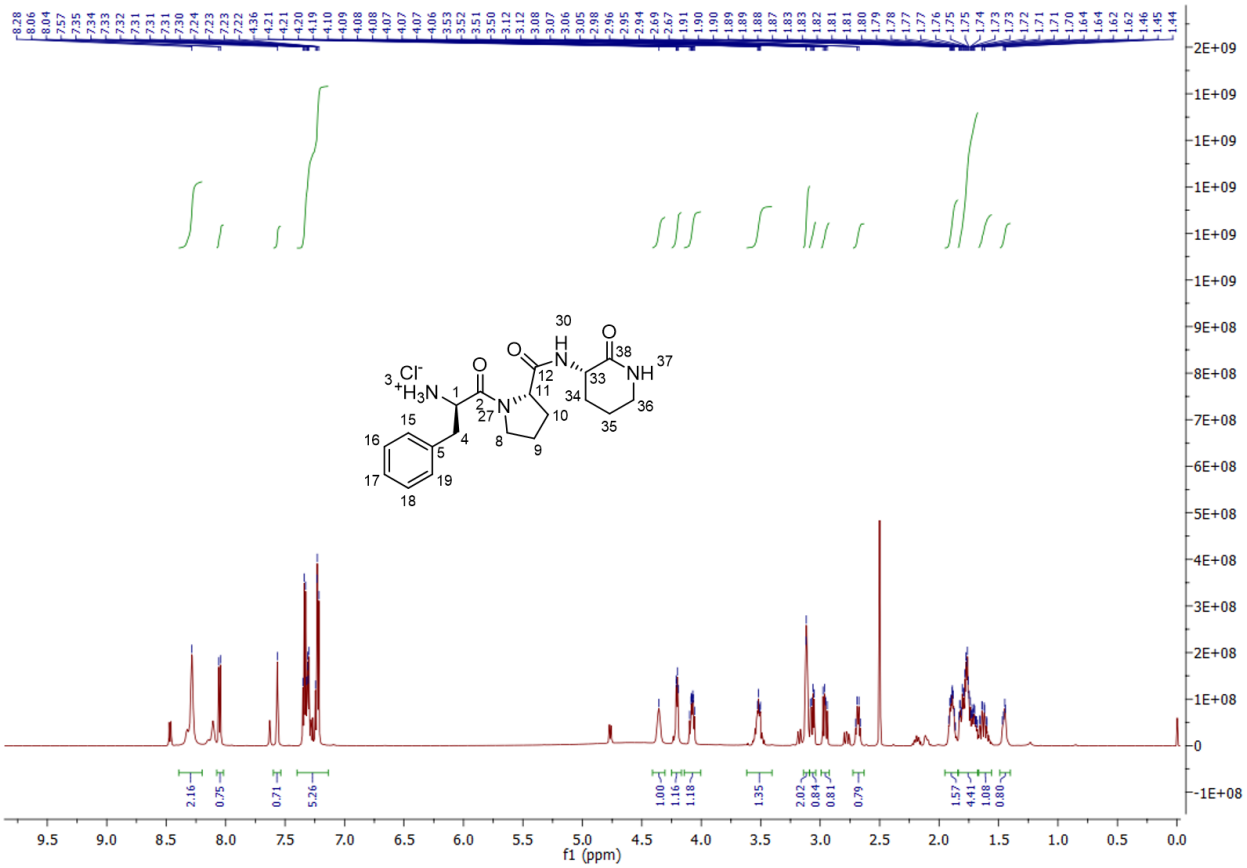


^{1}H NMR of compound **6** measured in DMSO-d$_6$

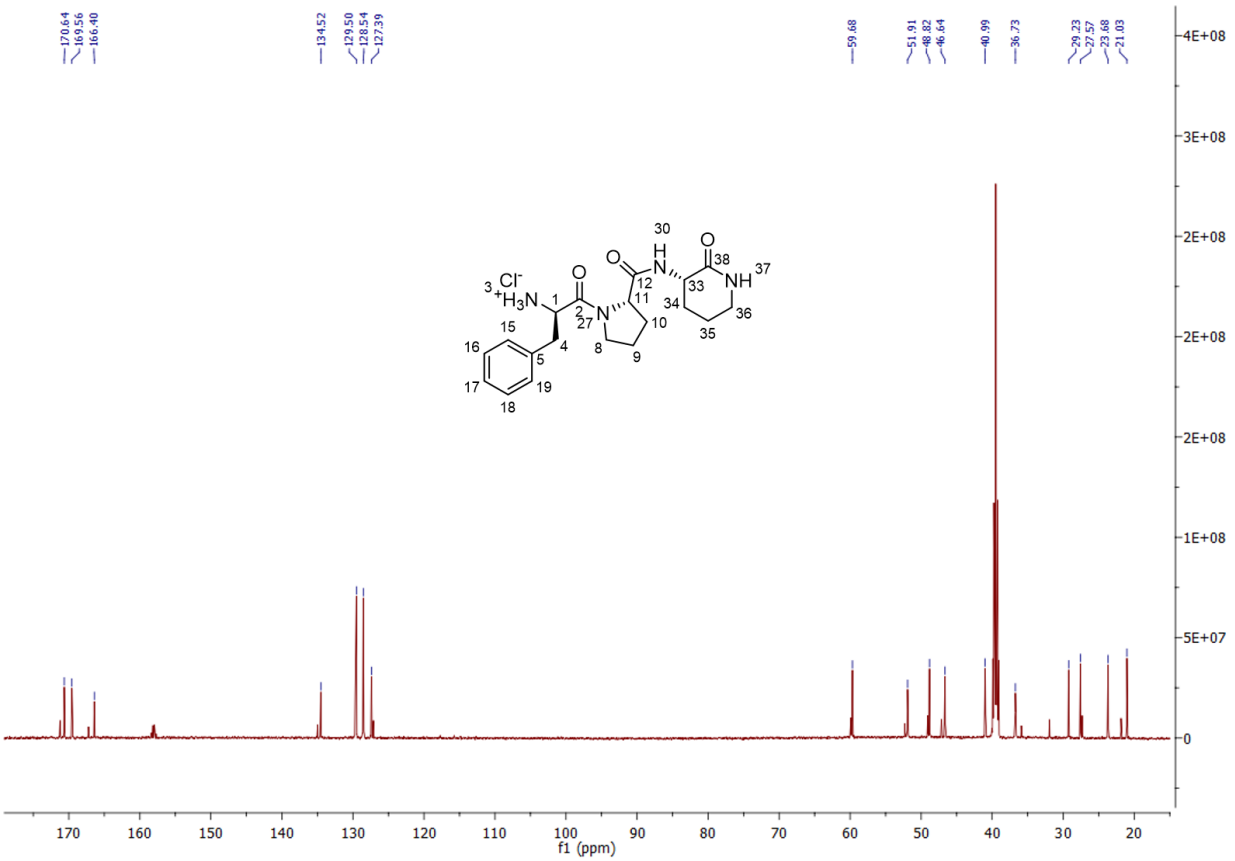


^{13}C NMR of compound **6** measured in DMSO-d$_6$

Position	**δ_C**	**δ_H, mult. (*J* in Hz)**
1	53.3, CH	4.44, dd (15.0, 8.3)
2	169.8, qC	
3		6.95, d (8.8)
4	37.5, CH_2	2.92 – 2.71, m
5	137.5, qC	
8a 8b	46.2, CH_2	3.27 – 3.13, m 2.92 – 2.71, m
9	24.2, CH_2	1.88 – 1.64, m
10a 10b	28.5, CH_2	2.06 – 1.88, m 1.88 – 1.64, m
11	58.6, CH	4.11, dd (8.3, 3.5)
12	173.1, qC	
15	128.0, CH	7.35 – 7.10, m
16	129.3, CH	7.35 – 7.10, m
17	126.3, CH	7.35 – 7.10, m
18	129.3, CH	7.35 – 7.10, m
19	128.0, CH	7.35 – 7.10, m
20	154.8, qC	
23	77.9, qC	
24	28.1, CH_3	1.31, s
25	28.1, CH_3	1.31, s
26	28.1, CH_3	1.31, s
27		12.54, broad s

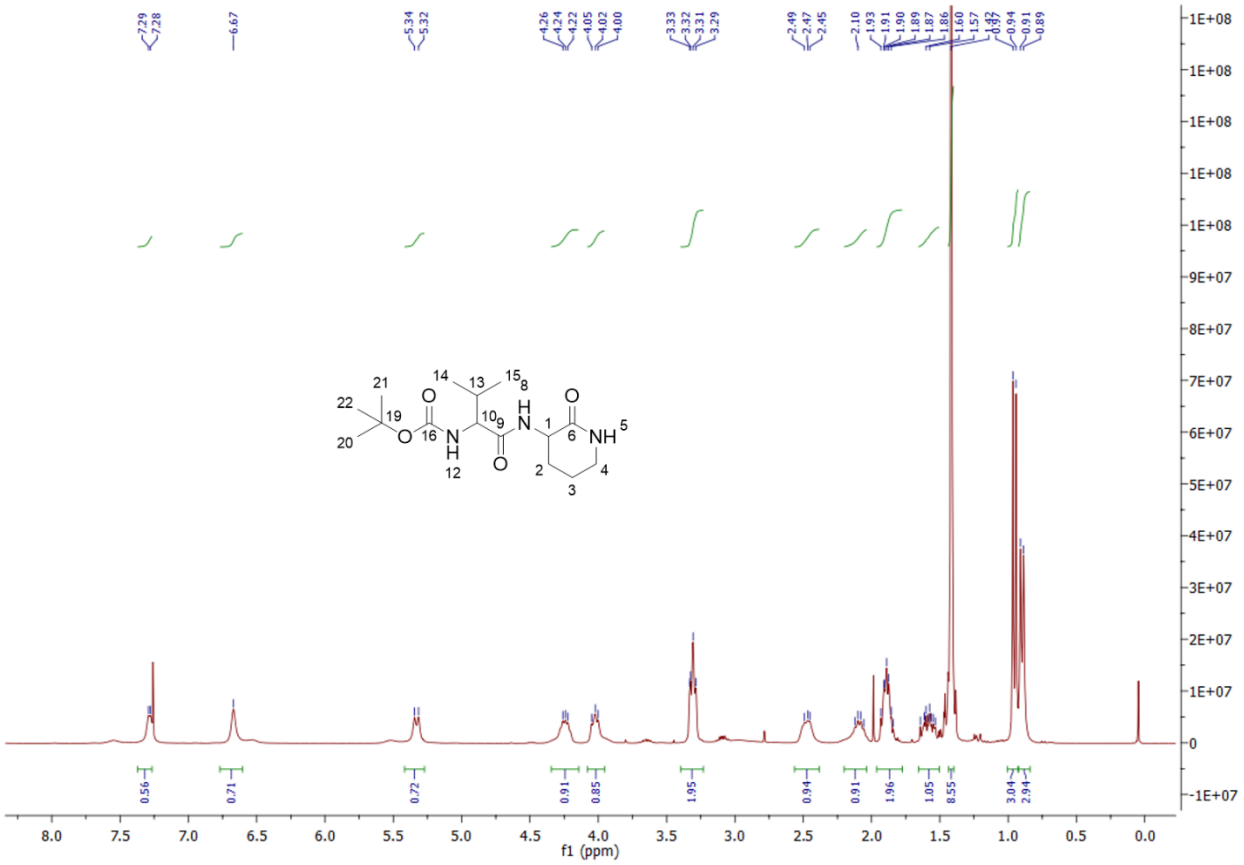


^{1}H NMR of compound **8** measured in DMSO-d_6

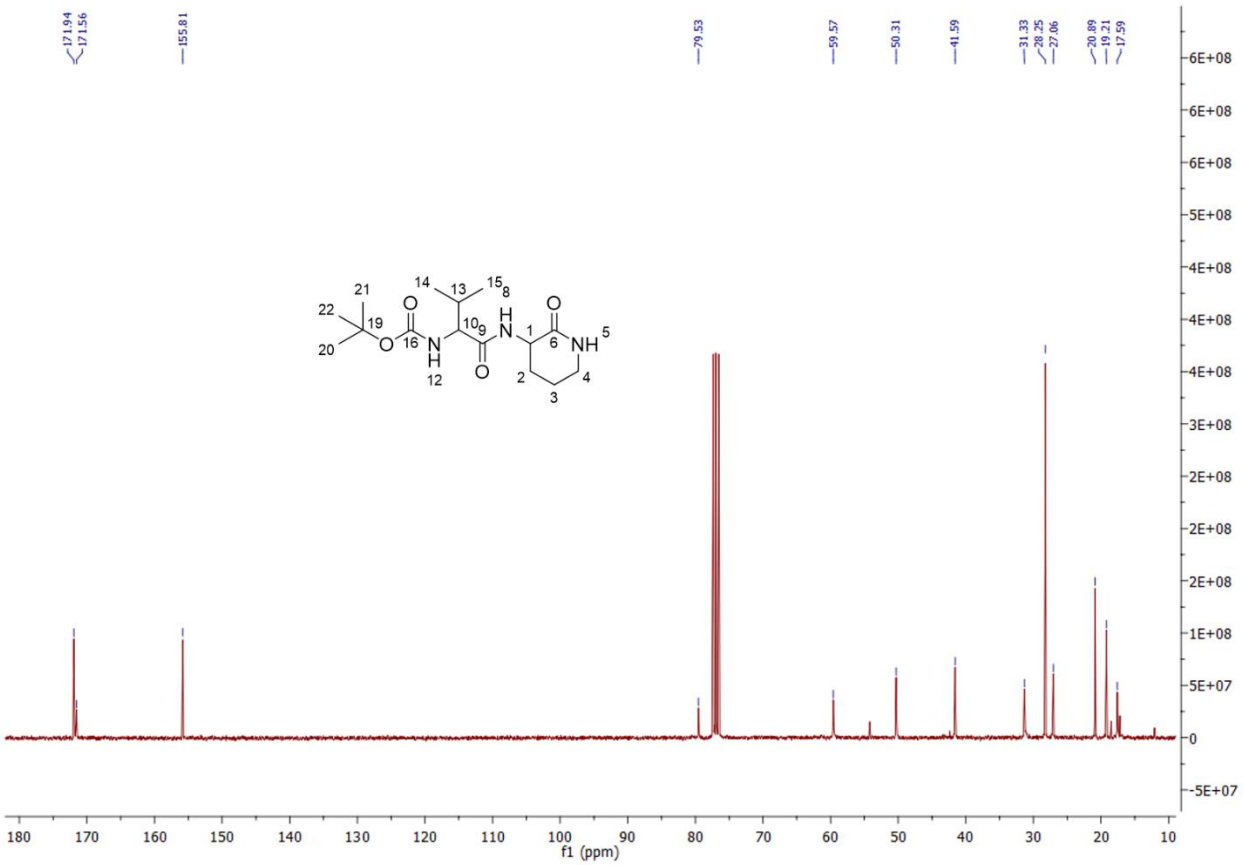


^{13}C NMR of compound **8** measured in DMSO-d_6

Position	**δ_C**	**δ_H, mult. (*J* in Hz)**
1	51.9, CH	4.36, broad s
2	170.6, qC	
3		8.28, broad s
4a 4b	36.7, CH_2	3.07, dd (13.4, 6.1) 2.96, dd (13.3, 8.4)
5	134.5, qC	
8a 8b	46.6, CH_2	3.58 – 3.46, m 2.68, dd (16.8, 7.1)
9a 9b	23.7, CH_2	1.84 – 1.68, m 1.49 – 1.41, m
10	29.2, CH_2	1.84 – 1.68, m
11	59.7, CH	4.21, dd (7.8, 2.8)
12	166.4, qC	
15	128.5, CH	7.37 – 7.20, m
16	129.5, CH	7.37 – 7.20, m
17	127.4, CH	7.37 – 7.20, m
18	129.5, CH	7.37 – 7.20, m
19	128.5, CH	7.37 – 7.20, m
30		8.05, d (8.3)
33	48.8, CH	4.08, ddd (10.4, 8.1, 6.4)
34a 34b	27.6, CH_2	1.94 – 1.86, m 1.67 – 1.56, m
35	21.0, CH_2	1.84 – 1.68, m
36	41.0, CH_2	3.14 – 3.09, m
37		7.57, s
38	169.6, qC	

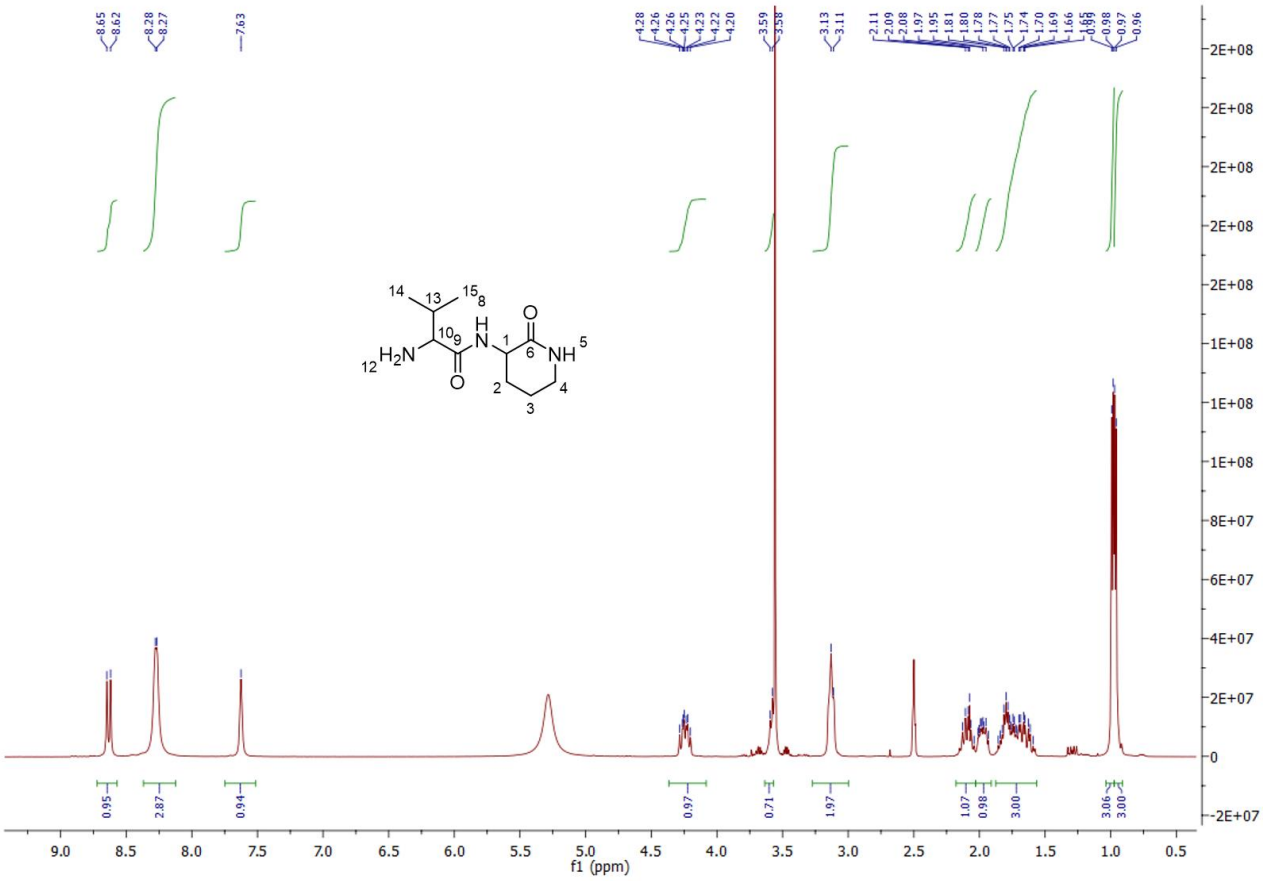


^{1}H NMR of compound 10 measured in $CDCl_3$

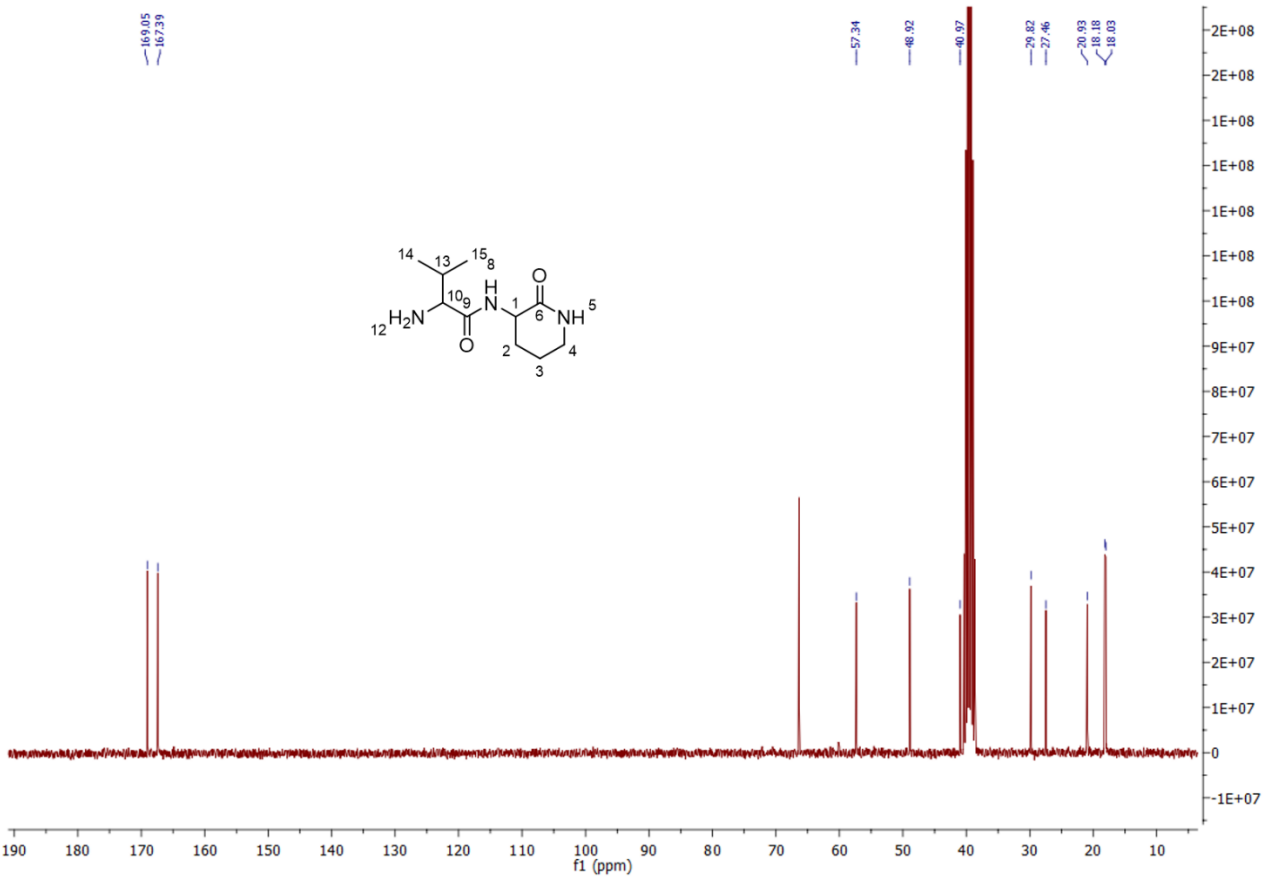


^{13}C NMR of compound 10 measured in $CDCl_3$

Position	**δ_C**	**δ_H, mult. (*J* in Hz)**
1	50.3, CH	4.10 – 3.96, m
2a 2b	27.1, CH_2	2.22 – 2.01, m 1.71 – 1.50, m
3	20.9, CH_2	1.96 – 1.82, m
4	41.6, CH	3.40 – 3.23, m
5		6.67, s
6	171.6, qC	
8		7.28, d (4.3 Hz)
9	171.9, qC	
10	59.6, CH	4.34 – 4.18, m
12		5.33, d (8.6)
13	31.3, CH	2.58 – 2.39, m
14	17.6, CH_3	0.90, d (6.8)
15	19.2, CH_3	0.95, d (6.8)
16	155.8, qC	
19	79.5, qC	
20	28.3, CH_3	1.42, s
21	28.3, CH_3	1.42, s
22	28.3, CH_3	1.42, s

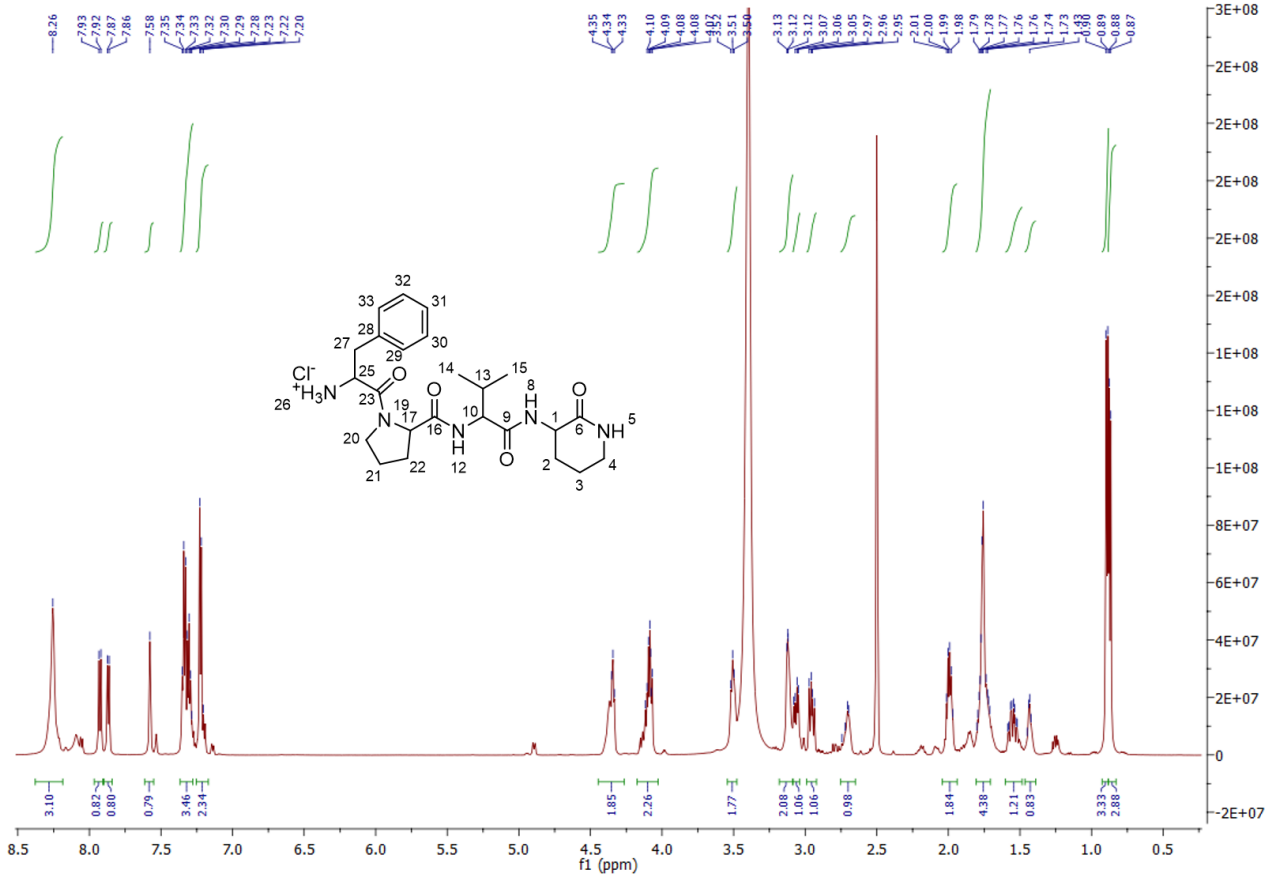


^{1}H NMR of compound **11** measured in DMSO-d_6

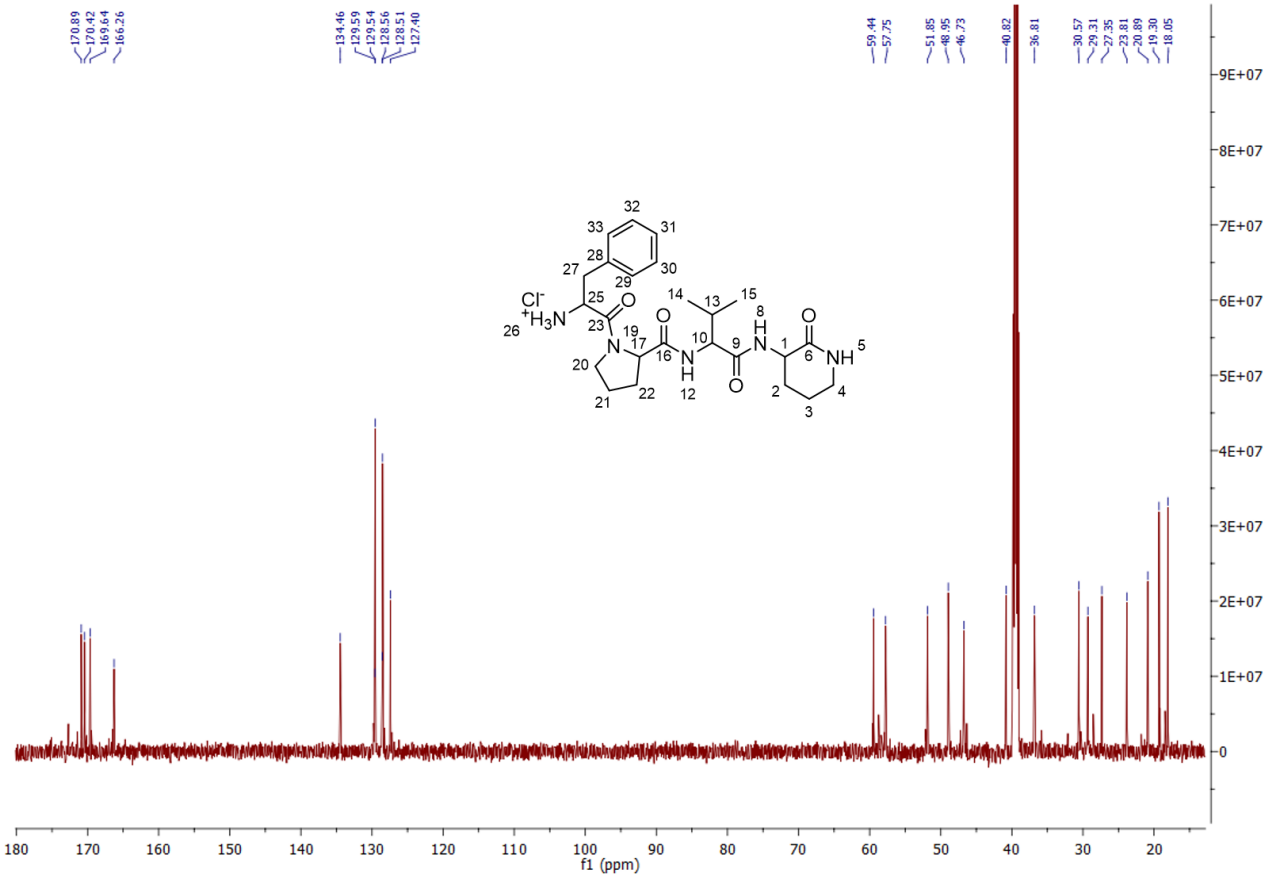


^{13}C NMR of compound **11** measured in DMSO-d_6

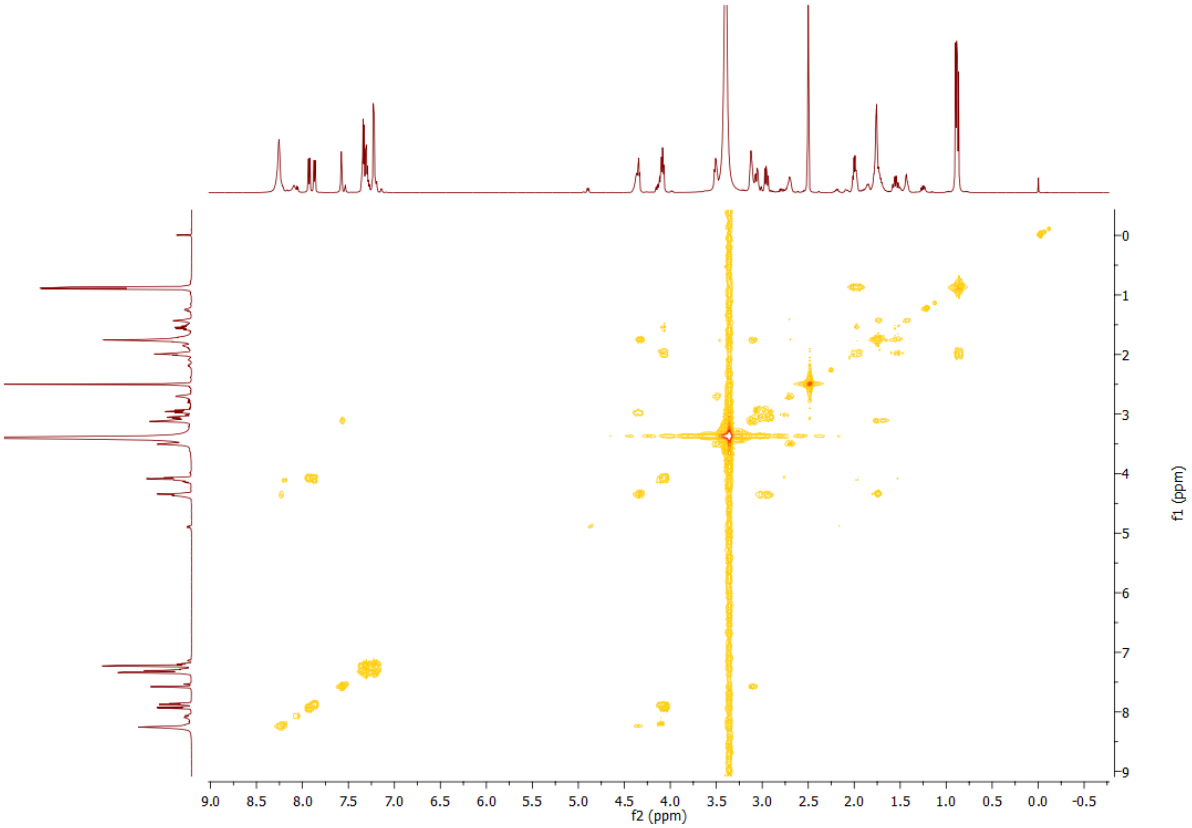
Position	**δ_C**	**δ_H, mult. (*J* in Hz)**
1	48.9, CH	3.64 – 3.57, m
2a 2b	27.5, CH_2	2.02 – 1.91, m 1.87 – 1.56, m
3	20.9, CH_2	1.87 – 1.56, m
4	41.0, CH_2	3.22 – 3.00, m
5		7.63, s
6	169.1, qC	
8		8.63, d (8.4)
9	167.4, qC	
10	57.3, CH	4.35 – 4.10, m
12		8.27, d (3.5)
13	29.8, CH	2.17 – 2.03, m
14	18.2, CH_3	0.99, d (3.6)
15	18.0, CH_3	0.96, d (3.6)



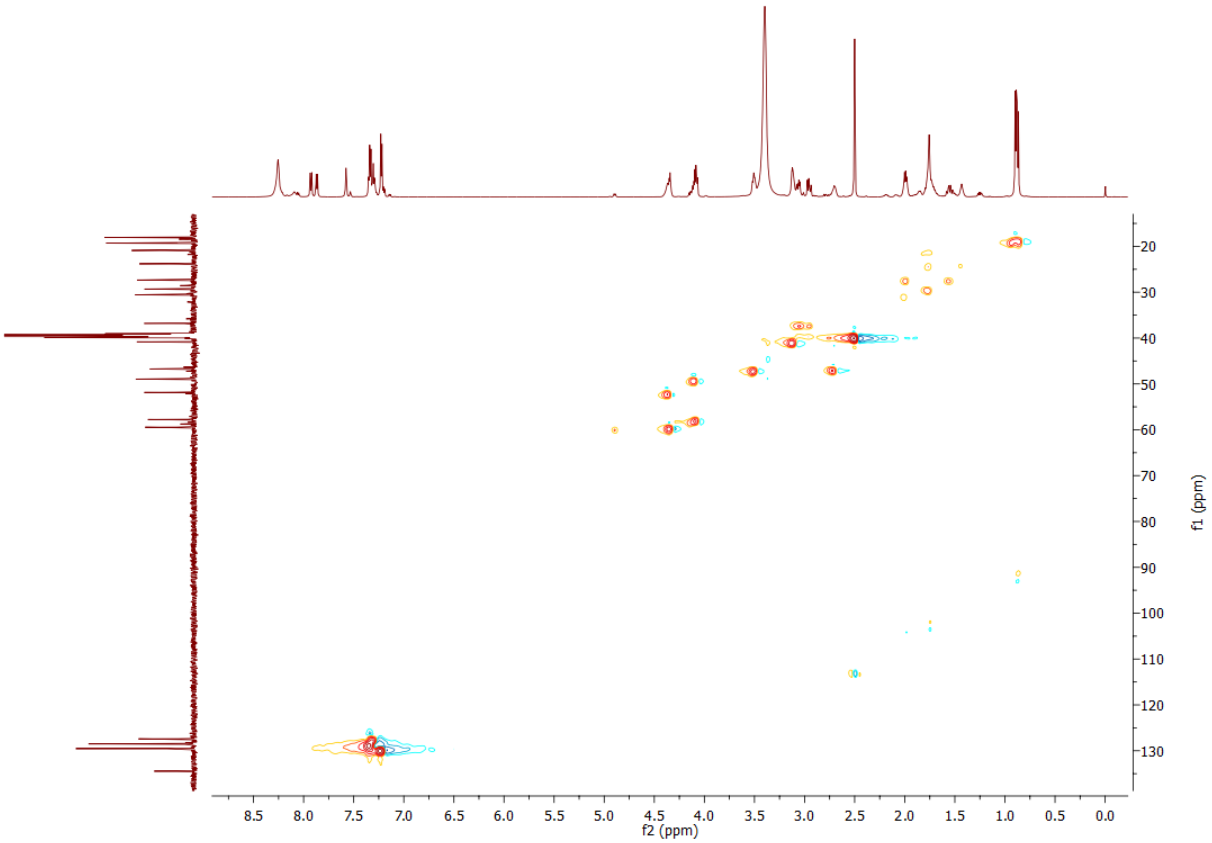
^{1}H NMR of compound **13** measured in DMSO-d_6



^{13}C NMR of compound **13** measured in DMSO-d_6



COSY spectra of compound **13** measured in DMSO-d_6



HSQC spectra of compound **13** measured in DMSO-d_6

Position	**δ_C**	**δ_H, mult. (*J* in Hz)**
1	49.0, CH	4.17 – 4.04, m
2a 2b	27.4, CH_2	2.05 – 1.94, m 1.60 – 1.48, m
3	20.9, CH_2	1.81 – 1.69, m
4	40.8, CH_2	3.15 – 3.09, m
5		7.58, s
6	169.6, qC	
8		7.87, d (7.6)
9	170.4, qC	
10	57.8, CH	4.17 – 4.04, m
12		7.93, d (9.0)
13	30.6, CH	2.05 – 1.94, m
14	19.3, CH_3	0.89, d (6.8)
15	18.1, CH_3	0.87, d (6.8)
16	170.9, qC	
17	59.4, CH	4.44 – 4.29, m
20a 20b	46.7, CH_2	3.58 – 3.47, m 2.76 – 2.64, m
21a 21b	23.8, CH_2	1.81 – 1.69, m 1.47 – 1.39, m
22	29.3, CH_2	1.81 – 1.69, m
23	166.3, qC	
25	51.9, CH	4.44 – 4.29, m
26		8.26, s
27a 27b	36.8, CH_2	3.06, dd (13.3, 6.0) 2.95, dd (13.3, 8.3)
28	134.5, qC	
29	128.5, CH	7.25 – 7.18, m
30	129.5, CH	7.37 – 7.28, m
31	127.4, CH	7.37 – 7.28, m
32	129.5, CH	7.37 – 7.28, m
33	128.5, CH	7.25 – 7.18, m

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