Identification and characterization of ternary complexes consisting of FKBP12, MAPRE1 and macrocyclic molecular glues

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1. SUPPLEMENTAL INFORMATION

1.1 Materials and Methods for the characterization of the ternary complex

1.1.1 Native nano-ESI MS

HIS-FKBP1a and HIS-MAPRE1 were buffer-exchanged overnight at 4 °C into 200 mM ammonium acetate (pH 6.8) using a Slide-A-Lyzer™ MINI Dialysis Device (0.5 mL) with a molecular weight cutoff (MWCO) membrane of 10 kDa (ThermoFisher Scientific). The concentrations of the buffer-exchanged proteins were determined using A_{280nm} . A stock solution of 10 mM NVP-FKI774 was prepared in DMSO. Prior to native nano-ESI MS analysis, the proteins and ligand were diluted appropriately to provide final sample concentrations of 10 µM HIS-FKBP1a, 10 µM HIS-MAPRE1, and 25 µM NVP-FKI774. These two proteins without/with the ligand were incubated for at least 15 minutes at room temperature before MS acquisition.

Platinum-coated pulled glass capillaries (HUMANIX) of 2-3 µm inner diameter were used for nano-electrospray ionization using a Bruker static nanospray source. A Bruker 7T SolariX Fourier Transform Ion Cyclotron Resonance Mass Spectrometer (FT-ICR-MS) was used for MS acquisition. The ESI source was used with a capillary voltage of 1.7 kV, a drying gas temperature of 100 °C and a drying gas flow rate of 2.0 L/min. In the source optics, skimmer 1 voltage was set to 50 V. Data acquisition was performed with 1M data points between m/z 400 – 6000, with 32 scans averaged for each spectrum.

1.1.2 Protein NMR experiments

1.1.2.1 Production of ^{13}C,^{15}N-labeled MAPRE1 and unlabeled FKBP12

GST-HRV3C-MAPRE1 (G189-Y268) 1TXQ was transformed into BL21-DE3 Star cells. Overnight growth pelleted by centrifugation at 6000rpm for 15 minutes at 20C. 3L of M9 minimal media (Teknova M8008) with 0.5 g/L 15NH4Cl and 1 g/L $^{13}C6$-Glucose added was inoculated with the cell pellet resuspended in media, and incubated at 37°C at 230 RPM shaking. After ~ 3 hours, the O.D. reached 0.6 and the incubator was turned down to ^{19}C. Once the shaker reached temperature, the flasks were induced with 1 mM IPTG and allowed to grow for 20 hours. The cells were then harvested via centrifugation, and stored at –80C. Pellet was lysed protein purified from the supernatant using 3mls of Glutathione sepharose 4B (GE Life Sciences

17075601), washed with 3 x 10x the resin volume and eluted with 50mM Tris pH 8.0, 1mM DTT, 30mM reduced glutathione. Elution loaded onto a 1ml DEAE sepharose fast flow column (GE Life Sciences 17505501) equilibrated with 50mM Tris pH 8.0, 0.1mM EDTA, 1mM DTT. Protein was eluted off using a gradient to 50mM Tris pH 8.0, 400mM KCl, 0.1mM EDTA, 1mM DTT. Peak fractions cleaved with 2mg of GST-HRV3C protease at 4C dialyzing against 50mM potassium phosphate pH 7.0, 100mM KCl, 0.1mM EDTA, 1mM DTT (SEC buffer) overnight. 1ml GSTrap FF column (GE Healthcare 17513001) equilibrated and run with SEC buffer.

The flowthrough non containing the GST tag, or residual HRV3C protease was concentrated down to 2mls and loaded onto a Superdex S75 16/60 sizing column equilibrated in SEC buffer. Peak fractions concentrated using a 3k cutoff spin concentration device.

His-TEV-FKBP12 DNA was was transformed into BL21-DE3 Star cells. Protein was expressed at 18C overnight. Pellet lysed, and protein purified from the lysate using a 5ml Ni-NTA column. Eluted protein was desalted using a HiTrap desalt column into 50mM Tris, 150mM NaCl, 10% Glycerol, pH7.5.

1.1.2.2 Protein NMR experiments

NMR experiments were carried out at 303 K on a Bruker AV800 NMR spectrometer in a buffer containing 50mM K_2HPO_4 pH 7.0, 100mM KCl, 0.1mM EDTA, 1mM DTT. R-SLF-1 was first solubilized in 10mM dmso-d6, and then added in small aliquots to FKBP12 to form the binary complex, which was then added to MAPRE1.

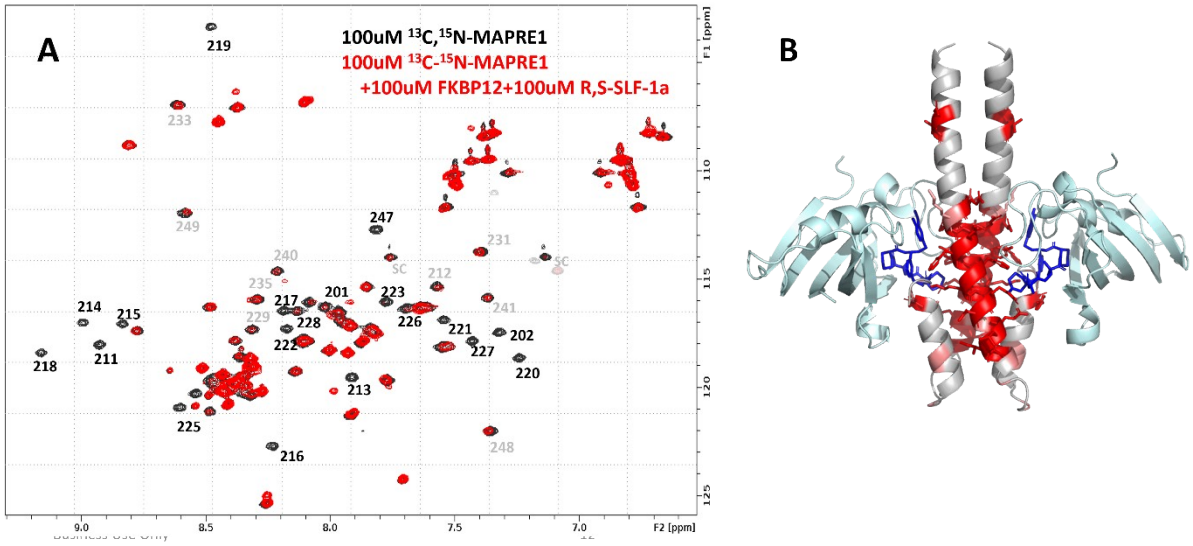


Figure S 1: **NMR characterization of the ternary complex FKBP12:R,S-SLF-1a:MAPRE1 (2:2:2)**

A) ^{15}N-HSQC NMR spectrum of ^{13}C,^{15}N-MAPRE1 (100uM, black) is superimposed on a ^{15}N-HSQC NMR spectrum of ^{13}C,^{15}N-MAPRE1 in presence of FKBP12:**R,S-SLF-1a**, generating the 2:2:2 ternary complex. Resonance assignments are given in the Figure.

B) MAPRE1 residues that are affected by the addition of FKBP1:R,S-SLF-1 are mapped onto the X-ray structure of the ternary complex. FKBP12 is shown in light cyan, **R,S-SLF-1a** is shown in blue, and MAPRE1 is shown in gray, except for the residues affected in the ternary complex, which are colored dark red or light red, according to the magnitude of the effect.

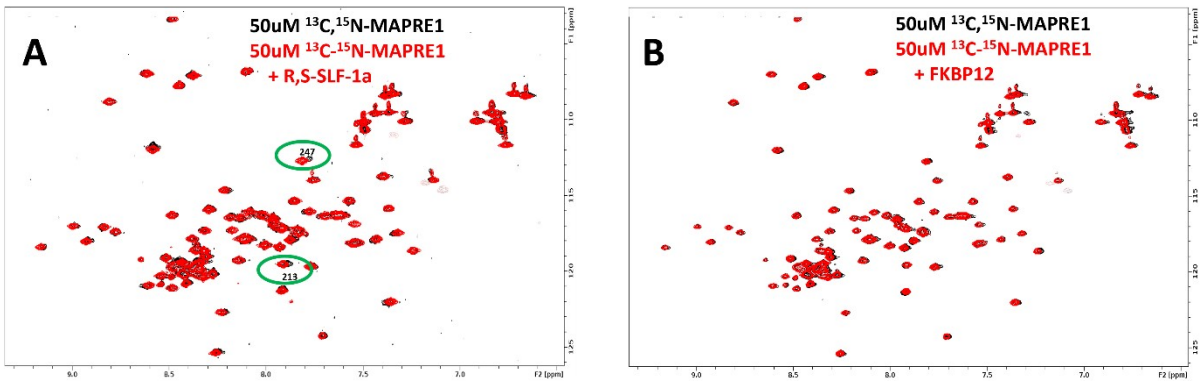


Figure S 2: **NMR investigation of weak intrinsic interactions in binary complexes**

A) The 15N-HSQC NMR spectrum of $^{13}C,^{15}N$-MAPRE1 (50uM, black) is superimposed on a 15N-HSQC NMR spectrum of $^{13}C,^{15}N$-MAPRE1 in presence of 100 uM **R,S-SLF-1a**, Note that the solubility of R-SLF-1 is only about 20 uM. Resonance assignments of the two residues that experience chemical shift changes are given in the Figure.

B) The ^{15}N-HSQC NMR spectrum of $^{13}C,^{15}N$-MAPRE1 (50uM, black) is superimposed on a ^{15}N-HSQC NMR spectrum of $^{13}C,^{15}N$-MAPRE1 in presence of 350 uM FKBP12, There are no chemical shift changes in the presence of FKBP12, indicating no intrinsic affinity between the two proteins in the absence of **R,S-SLF-1a**.

1.1.3 X-ray experiments

1.1.3.1 Binary complex FKBP12:R,S-SLF-1a

FKBP1a protein diluted to 125uM in 50mM HEPES pH 7.4, 100mM NaCl – 3ml final volume. Compound added to 250uM final concentration and incubated at room temperature for 1hr. Concentrated using a 3k cutoff spin concentration device down to 500uL. Screened against MCSG plates 1-4, 150nL complex, 150nL well solution in sitting drop. At 4C, crystal observed at day 1 in 0.1M CHES 9.5, 30% PEG3K, and picked at day 9 – no cryo buffer added – directly frozen in liquid nitrogen.

Data collected at APS. Structure was solved by molecular replacement, PDB 3MDY was used for FKBP12, and 5N74 for MAPRE1. The Phenix software suite was used for molecular replacement and refinement (COOT).

The PDB code for this deposition is 9DCW.

1.1.3.2 Ternary complex FKBP12:R,S-SLF-1a:MAPRE1

200uM FKBP12 protein and 200uM MAPRE1 (N183-Y268) protein mixed with 250uM FKI774 – 3mls final volume with 2% DMSO. Incubated at 4C with gentle rocking overnight, then concentrated down to ~500uL – 22.8mg/ml. Screened against MCSG plates 1-4, 150nL complex, 150nL well solution in sitting drop. Crystals observed in 1.5M LiSO4, 0.1M CH3COONa pH 4.6 and picked at day 17. Well solution supplemented with 20% glycerol added to drop, and crystals picked and flash frozen in liquid nitrogen.

Data collected at APS. Structure was solved by molecular replacement, PDB 3MDY was used for FKBP12, and 5N74 for MAPRE1. The Phenix software suite was used for molecular replacement and refinement (COOT).

The PDB code for this deposition is 9CO5.

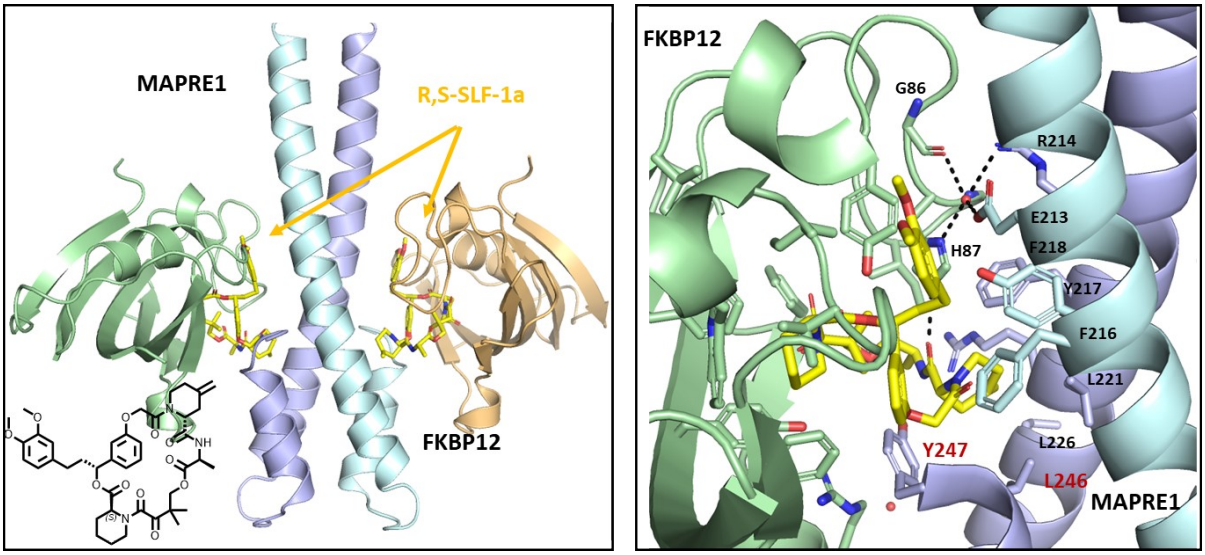


Figure S 3 **X-ray structure of the ternary complex FKBP12:R,S-SLF-1a:MAPRE1 (2:2:2)**

R,S-SLF-1a acts as a Molecular Glue with a predominant hydrophobic interface bridging MAPRE1 and FKBP12. Limited protein-protein interactions flank the R,S-SLF-1 binding pocket.

1.2 Experimentals on the protein array screening

1.2.1 Protein expression and array generation

To generate the protein array content, expression clones for each protein were generated by PIPE cloning[1]. The PDB was used as a guide for proteins and amino acid sequences representing domains of proteins deposited into the PDB. All cloning and protein expression steps were performed in 96-well format with several exceptions as noted. I-PIPE PCR primers (IDT) were designed to amplify PDB protein sequences from a human clone library (Thermo Fisher Ultimate ORF Collection). PCR reactions were mixed with a linearized PIPE-compatible expression vector containing an N-terminal 6His tag, created by V-PIPE. The mixture was then added to E. coli PB1 cells, heat shocked, and plated into 48-well divided agar plates. Single colonies were picked, grown and split for DNA purification for sequence confirmation and for protein induction. Protein expression was induced with 0.08% arabinose for 18 hrs at 18°C .Overnight inductions were pelleted, lysed and purified using Qiagen Ni-NTA agarose (Qiagen cat# 30210). Proteins were quantitated by capillary electrophoresis (Revvity LabChip GXII). Protein spotting onto nitrocellulose slides was outsourced (Arrayjet).

1.2.2 Protein array probing

For probing the protein arrays with bio-FKBP12 and compounds, a protocol closely based on the Invitrogen ProtoArray protocol (https://tools.thermofisher.com/content/sfs/manuals/protoarray_applicationsguide_man.pdf) was used. All steps were performed at 4° C unless otherwise noted. Arrays were first incubated in 5 mL of block buffer (50 mM HEPES pH 7.5, 200 mM NaCl, 0.08% Triton X-100, 25% glycerol, 20 mM reduced glutathione, 1 mM DTT, and 1X synthetic block [Invitrogen cat# PA017]) for one hour with gentle rocking, in a four-chamber microarray incubation tray (Sarstedt, Cat. no. 94.6077.307). Typically, three or four arrays were used in a single experiment. During the blocking step, bio-FKBP probe was prepared by diluting bio-FKBP12 to 500nM in 1X block buffer along with 500nM compound or DMSO. After blocking, block buffer was aspirated from each chamber containing an array, and 120 µL of each bio-FKBP12 probe was dispensed drop-wise directly onto the surface of the arrays and covered with a LifterSlip cover slip (Thermo cat# 25X60I-2-4789). The arrays were incubated with the probes for 90 minutes. The coverslips were removed using forceps, and the arrays were washed in 5 mL of wash buffer (1X PBS, 0.1% Tween-20, 1X synthetic block) + 500nM compound or DMSO for five minutes with gentle rocking. Streptavidin-Alexafluor 647 (Thermo cat# S-32357) was added to wash buffer at 1 µg/mL + 500nM compound or DMSO, and 5 mL was dispensed onto the arrays and incubated for 45 minutes with gentle rocking. Five additional washes + 500nM compound or DMSO were performed, 5 mL each for five minutes. The arrays were transferred to room temperature and dipped into room-temperature water in a 50 mL conical tube for one second to remove any residual components of wash buffer, then centrifuged at 200 x g for two minutes in the slide carrier provided with the Grace PATH arrays to remove any residual liquid. The dry slides were scanned on a GenePix 4000B slide scanner and the images were analyzed using GenePix software. The resulting data table was imported into Spotfire analysis software (Tibco) and Signal-background was calculated for each spot. Hits were identified by having significant signal-background over FKBP12 alone, and other compounds tested.

1.3 Biochemical assays

1.3.1 TR-FRET FKBP12 binding assay

FKBP12 binding was determined using an N-terminal biotinylated FKBP12 protein and a FITC-labeled SLF compound in TR-FRET. Compounds were tested in a 16 point 3 fold dilution series. Compounds were dispensed using an Echo acoustic dispenser (Beckman) into 1536-well white plates (Greiner). 4nM final biotion-FKBP12 protein in 50mM HEPES 50mM NaCl, 5mM MgCl2, 0.02% Brij-35 was dispensed – 2uL/well using a Multidrop Combi (Thermo

Scientific). Added streptavidin-TB (Life Technologies) at 2nM final to each well, 2uL/well and incubated 1hr at room temp. Read on Envision plate reader with excitation at 320nm, and emissions at 520 and 492. Ratio of 520 and 492 used for analysis.

Biotin-FKBP12 was generated by a HIS6-Avi-linker-FKBP12 vector. Protein was expressed in E. coli, purified using Ni-NTA chromatography. 70mgs of purified protein was incubated with 2mgs of GST-BirA protein (internal) + 5mM D-biotin + 1mM ATP + 1mM MgCl2 overnight at 4C. The next day, protein was purified over a Superdex S75 16/60 column equilibrated in 50mM Tris pH 7.5, 150mM NaCl, 10% glycerol.

1.3.2 TR-FRET FKBP12-MAPRE1 proximity assay

Biotin-FKBP12 and His-tagged MAPRE1 protein were diluted to 200nM in 50mM HEPES 50mM NaCl, 5mM MgCl2, 0.02% Brij-35. 2uL was dispensed to 1536-well white plates (Greiner) and dilution series of compounds were added using an Echo dispenser (Labcyte). 2uL of 30nM Streptavidin-TB (LifeTechnologies PV3965), 15nM anti-HIS FITC (GenScript A01620) added to each well. Incubated at room temp for 90 minutes and read out on an Envision plate reader. Luminescence values were normalized using the equation (100*[well value-NC]/NC), where NC is the neutral control (DMSO). Curves were fitted using Spotfire analysis software (Tibco).

1.4 NanoBiT assays

1.4.1 FKBP12-MAPRE1 recruitment experiments

For the FKBP12-MAPRE recruitment experiments, HEK293T cells were reverse transfected by plating 15 mL of cells at 0.625x10^6 cells/mL in complete medium (DMEM with 10% FBS) into a 75 cm2 flask (Corning). 90 µL of Fugene HD (BioRad #E2312) was combined with 1500 µL of Optimem I (Gibco # 31985-062) and added to 30 µg of DNA total (15 µg of HSVTK-driven FKBP12[M1-E108]-LgBiT plasmid and 15 µg of HSVTK-driven MAPRE[N183-Y268]-SmBiT plasmid). The transfection mixture was incubated for 15 minutes at room temperature before being added drop-wise to the plated cells. After 24 hours of incubation in a cell culture incubator (37° C, 5% CO_2), the transfected cells were trypsinized, normalized to 0.5x10^6 cells/mL in complete media and plated into 1536-well plates (Greiner cat#782073), 5 µL/well using a tip-based dispenser (GNF Systems One Tip Dispenser). The plates were incubated in a cell culture incubator (37°C, 5% CO_2) overnight. On the next day, 10 nL of compounds in 100% DMSO, or DMSO alone as a control, were dispensed into target wells using the Echo, and the plates were returned to the incubator for another 2 hours. The plates were allowed to cool to room temperature for 10 minutes, and 5 µL of NanoBiT NanoGlo reagent, consisting of NanoGlo substrate (BioRad # N2058) diluted 1:25 in a mixture of 50% NanoGlo LCS buffer

(BioRad # N206C) and 50% PBS, was dispensed into each well using the GNF dispenser. After a final incubation at room temperature for 10 minutes, luminescence was read on a Viewlux plate reader (Perkin Elmer). Luminescence values were normalized using the equation (100*[well value-NC]/NC), where NC is the neutral control (DMSO). Curves were fitted using Spotfire analysis software (Tibco).

1.4.2 MAPRE1 PPI competition experiments

For the MAPRE PPI competition experiments, in addition to the indicated LgBiT and SmBiT fusion constructs used to generate a NanoBiT signal, a third construct encoding the untagged competitor protein driven by the CMV promoter was included in the mix of plasmids transfected into cells. In these experiments, 10 µg of each construct was used to maintain the same 30 µg total DNA per transfection as in all other NanoBiT experiments.

1.4.3 FKBP ligand competition experiments

For the ligand competition experiments, the setup and execution was the same as for the recruitment experiments described in 1.4.1, with the exception that 40 nL of the competitive non-recruiting FKBP12 ligand (80 uM final well concentration) or DMSO was added to each well, in addition to the standard 10 nL of the recruiting FKBP12 ligand in dose response.

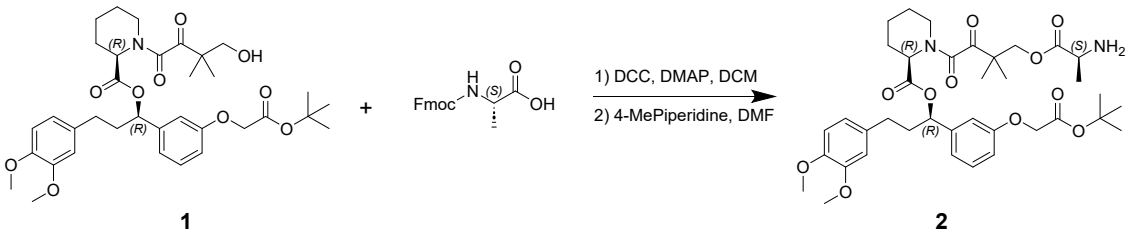
1.5 Synthesis of macrocyclic FKBP12 ligands

1.5.1 General Information

General methods: Reagents were purchased from different commercial sources and used without further purification. The removal of solvent under reduced pressure was carried out on a standard rotary evaporator.

Chromatography: For flash column chromatography silica gel 60 from Merck, with a particle size between 40 and 63 µm, was used. Crudes were often loaded onto columns using dry loading technique with Isolute HM-N (Biotage PN: 9800-5000).

1.5.2 Representative protocol for the first amino acid coupling to the FKBP12 binding scaffold

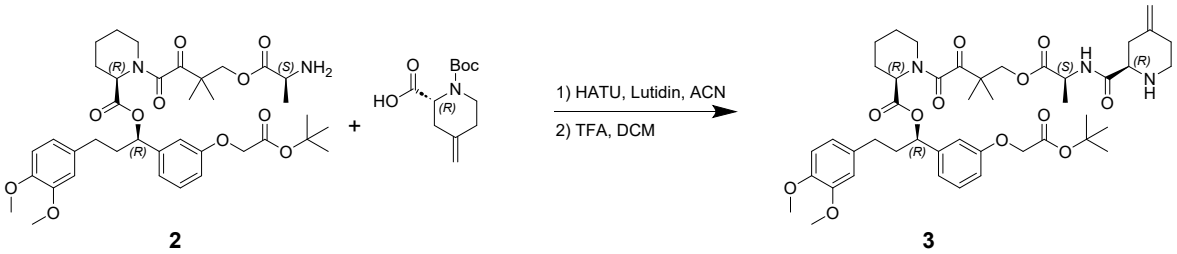


1 **2**

Scheme 1

1 was prepared according to a reported method [Ref **30** Holt et al.]. To a solution of **1** (800 mg, 1.247 mmol) in DCM (25 mL) was added Fmoc-Ala-OH (776 mg, 2.493 mmol). After that, DCC (643 mg, 3.12 mmol) and DMAP (76 mg, 0.623 mmol) were added. The reaction mixture showed after stirring for 4 h complete conversion to the desired product (m/z = 952.7 $(M+H_2O)^+$). The reaction mixture was filtered and concentrated. The crude residue was diluted with DCM and extracted with 0.2M HCl, dried and evaporated. The crude residue from the coupling step was dissolved in 20 mL of 4-MePiperidine/DMF (1:4) for Fmoc deprotection. The reaction was stirred for 30 min (complete conversion to the desired product (m/z = 713.4$(M+H)^+$)). After evaporation of DMF under vacuum, the crude product was purified by normal phase chromatography (0-50% Cyclohexane/AcOEt then 100% of AcOEt) to yield 656 mg, 0.920 mmol, 74% yield) of **2** as a clear oil.

1.5.3 Representative protocol for the second amino acid coupling and subsequent deprotection



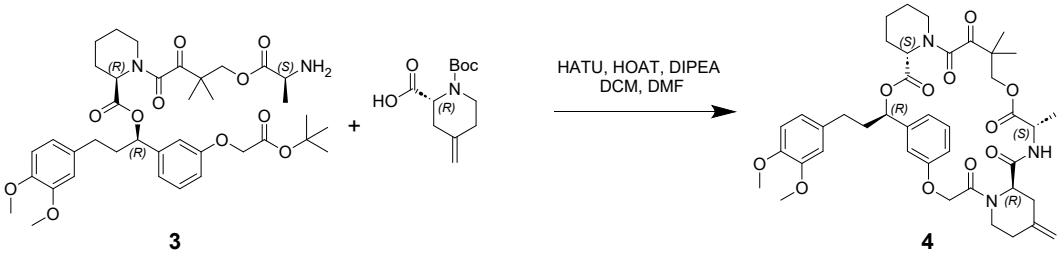
2 **3**

Scheme 2

(R)-4-Boc-piperidine-2-acid (55.9 mg, 0.231 mmol) was placed in a flask, after that solution of HATU (88 mg, 0.231 mmol) in ACN (2 mL) was added followed by lutidine (0.123 mL, 1.052 mmol). After stirring for 2 min, a solution of **2** (150 mg, 0.210 mmol) in ACN (2 mL) was added. The reaction was stirred for 16 h, showing complete conversion to coupled-product (m/z = 953.6 (M+H2O)).The reaction mixture was concentrated to 1 mL and supplemented with DCM (1 mL, 1:1 v/v) and TFA (0.811 mL, 10.52 mmol). Stirring 16 h showed complete conversion

to **3** (m/z = 780.3 $(M+H)^+$). The solvents were evaporated and the residue was dried over high-vacuum. The crude product was used without further purification for the cyclization step.

1.5.4 Representative protocol for the cyclisation



Scheme 3

To a flask containing DCM (270 mL), a solution of HATU (366 mg, 0.962 mmol) and HOAT (131 mg, 0.962 mmol) dissolved in DMF (1 mL) was added, followed by DIPEA (0.336 mL, 1.923 mmol). After that, a solution of crude **3** (250 mg) in DCM (30 mL) was added dropwise. LC-MS analysis after 2 hours showed 39% conversion to **4** (m/z = 762.4 $(M+H)^+$). LC-MS analysis after 6 hours showed completion of the reaction. The reaction mixture was concentrated. The crude product was purified by reverse phase prep-HPLC to yield **4** (**R,S-SLF-1a**) (56.6 mg, 0.068 mmol, 32 % yield) as a white amorphous powder. HR-LCMS see section 1.8.3

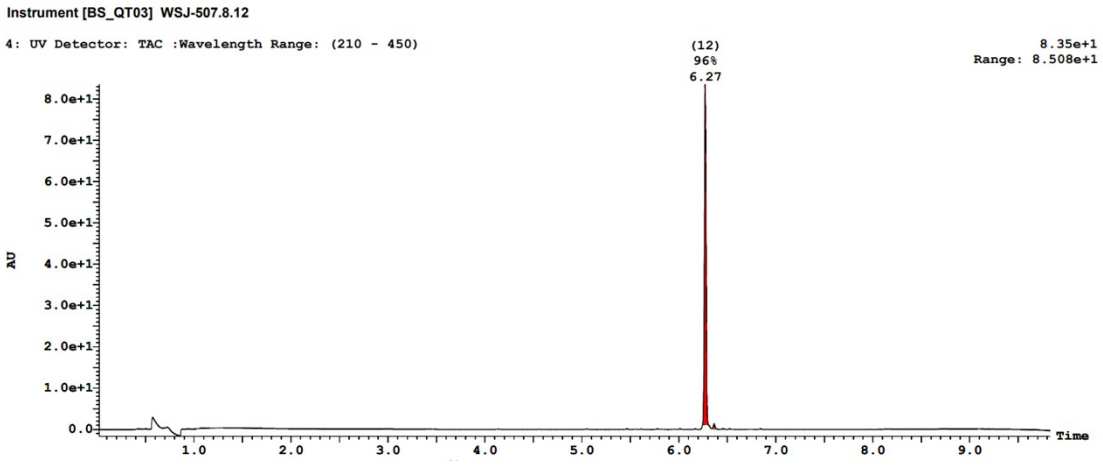
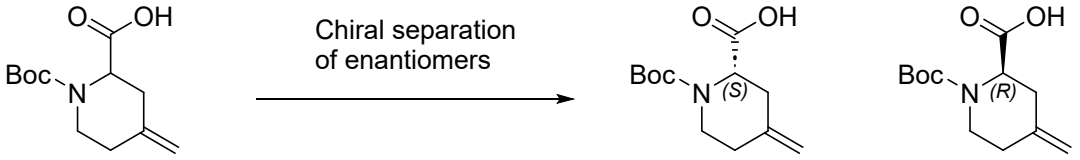


Figure S 4 Representative Analytical reversed phase LC-MS chromatogram for a final macrocyclic compound

1.6 Synthesis protocols of required building blocks to prepare the macrocyclic FKPB12 ligands

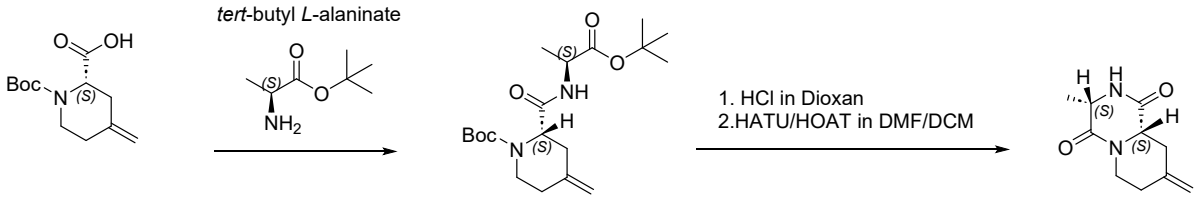
1.6.1 Chiral separation of (±)-N-Boc-4-methylenepiperidine-2-carboxylic acid incl. assessment of the assignment of the absolute stereochemistry



5 **6a** **6b**

(±)-N-Boc-4-methylenepiperidine-2-carboxylic acid (5) (500 mg, 2.07 mmol) was separated by chiral SFC (Column: Lux Cellulose-2 (250mm*30mm; 5µm); Mobile phase: CO2/(MeOH+0.05%NH_3H_2O) 90/10 isocratic elution mode; Flow rate: 80mL/min; BPR120 bars, Column temperature: 40°C) given P1 (>99%*ee*) as first eluting peak and P2 (>99%*ee*) as second eluting peak. To get the free acid, an acidic extraction was performed. The combined fractions of P1 and P2 were dissolved in 150 mL DCM and extracted twice with 100 mL of 0.1 N HCl. The two aqueous phases were back-extracted one time with 150 mL DCM. The combined organic phases were dried with Na_2SO_4 and evaporated yielding **(S)-N-Boc-4-methylenepiperidine-2-carboxylic acid** (**6a**; 231 mg, 0.957 mmol, >99%*ee*) and **(R)-N-Boc-4-methylenepiperidine-2-carboxylic acid** (**6b**; 210 mg, 0.87 mmol, >99%*ee*).

1.6.2 Conversion of (S)-N-Boc-4-methylenepiperidine-2-carboxylic acid to the diketopiperazine 1 Leading to (S,S) assignment



6a **7a** **8a**

tert-butyl L-alaninate (14.44 mg, 0.099 mmol) was placed in a flask, after that, a solution of HATU (37.8 mg, 0.099 mmol) in ACN (0.5 mL) was added followed by lutidine (0.048 mL, 0.414 mmol) and it was stirred for 2 min. Then a solution of **(S)-N-Boc-4-methylenepiperidine-2-carboxylic acid** (**6a**; 20 mg, 0.083 mmol) in ACN (0.5 mL) was added. Reaction was stirred for 16 h. After completion of the reaction, the reaction mixture

was concentrated. The crude product was purified using RP prep-HPLC to yield 12 mg (0.033 mmol, 39 % yield) of Intermediate dipeptide **7a**.

To cleave off the Boc protecting group, the product was dissolved in 4M HCl in dioxane (1 mL, 4.00 mmol). To increase the solubility, ACN (0.5 mL) was added. After completion of the deprotection, solvent was evaporated.

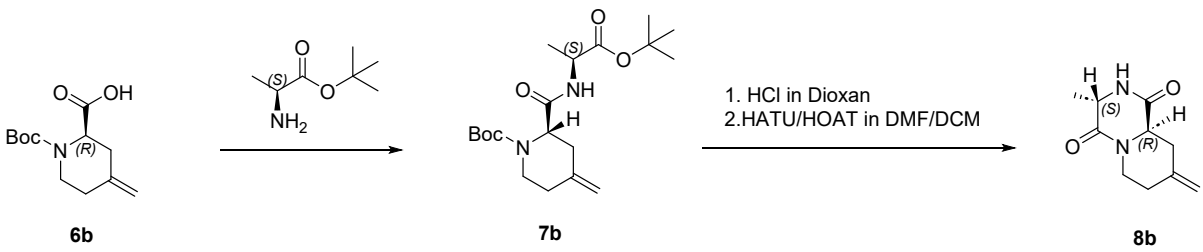
Cyclisation of dipeptide to diketopiperazine: To a flask containing DCM (20 mL) was added a solution of HATU (18.57 mg, 0.049 mmol) and HOAT (6.65 mg, 0.049 mmol) in DMF (1.0 mL), followed by DIPEA (0.028 ml, 0.163 mmol). After that, a solution of crude deprotected dipeptide in DCM (20 mL) was added dropwise. After completion of the cyclization, the reaction mixture was concentrated. The crude product was purified by reverse prep-HPLC to yield the desired diketopiperazine **8a** (2.2 mg, 0.011 mmol, 33 % yield) as a yellowish sticky oil.

^{1}H NMR (600 MHz, DMSO) δ 8.29 (s, 1H), 4.90 (q, *J* = 1.9 Hz, 1H), 4.86 (q, *J* = 1.9 Hz, 1H), 4.47 (ddd, *J* = 12.7, 5.5, 2.0 Hz, 1H), 3.93 (q, *J* = 6.9 Hz, 1H), 3.77 (ddd, *J* = 12.4, 3.3, 1.1 Hz, 1H), 2.71 (ddd, *J* = 13.4, 3.3, 1.7 Hz, 1H), 2.48 (d, *J* = 3.9 Hz, 1H), 2.28 (ddt, *J* = 13.6, 3.5, 1.9 Hz, 1H), 2.13 (t, *J* = 12.8 Hz, 1H), 2.01 (td, *J* = 13.3, 5.3 Hz, 1H), 1.33 (d, *J* = 7.0 Hz, 3H).

^{13}C NMR (151 MHz, DMSO) δ 165.53 (s, 1 C), 165.38 (s, 1 C), 142.93 (s, 1 C), 110.97 (s, 1 C), 58.13 (s, 1 C), 49.89 (s, 1 C), 41.97 (s, 1 C), 38.67 (s, 1 C), 32.86 (s, 1 C), 21.40 (s, 1 C).

This material was used for **2D NMR Experiments** (see chapter 1.7) to prove the *cis* geometry of the two α-hydrogens, leading to the **absolute stereochemistry of S,S** for the **diketopiperazine 8a**.

1.6.3 Conversion of (R)-N-Boc-4-methylenepiperidine-2-carboxylic acid to the diketopiperazine 2 Leading to (S,R) assignment



6b **7b** **8b**

tert-butyl L-alaninate (14.44 mg, 0.099 mmol) was placed in a flask, after that solution of HATU (37.8 mg, 0.099 mmol) in ACN (0.5 mL) was added followed by lutidine (0.048 mL, 0.414 mmol) and it was stirred for 2 min. Then a solution of **(R)-N-Boc-4-methylenepiperidine-2-carboxylic acid** (20 mg, 0.083 mmol) in 0.5 mL of ACN was added. Reaction was stirred for 16 h. After completion of the reaction mixture was concentrated. The

crude product was purified using RP prep-HPLC to yield 15 mg (0.041 mmol, 49 % yield) of Intermediate dipeptide **7b**.

To cleave off the Boc protecting group, the product was dissolved in 4M HCl in dioxane (1 mL, 4.00 mmol). To increase the solubility, ACN (0.5 mL) was added. After completion of the deprotection, solvent was evaporated.

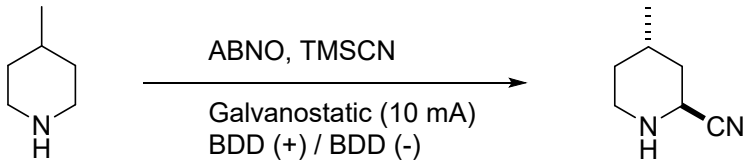
Cyclisation of dipeptide to diketopiperazine: To a flask containing DCM (20 mL) was added a solution of HATU (18.57 mg, 0.049 mmol) and HOAT (6.65 mg, 0.049 mmol) in DMF (1.0 mL), followed by DIPEA (0.028 mL, 0.163 mmol). After that, a solution of crude deprotected dipeptide in DCM (20 mL) was added dropwise. After completion of the cyclization, the reaction mixture was concentrated. The crude product was purified by reverse prep-HPLC to yield the desired diketopiperazine **8b** (3.8 mg, 0.020 mmol, 49% yield) as a yellowish sticky oil.

^{1}H NMR (600 MHz, DMSO) δ 8.27 (s, 1H), 4.89 (q, *J* = 2.0 Hz, 1H), 4.85 (q, *J* = 1.9 Hz, 1H), 4.48 (ddd, *J* = 12.7, 5.5, 2.0 Hz, 1H), 4.02 (qt, *J* = 6.9, 1.3 Hz, 1H), 3.80 (ddd, *J* = 12.4, 3.2, 1.1 Hz, 1H), 2.64 (ddd, *J* = 13.3, 3.3, 1.6 Hz, 1H), 2.49 – 2.45 (m, 1H), 2.26 (ddt, *J* = 13.6, 3.5, 1.9 Hz, 1H), 2.24 – 2.18 (m, 1H), 2.02 (tdd, *J* = 13.2, 5.5, 1.6 Hz, 1H), 1.31 (d, *J* = 7.0 Hz, 3H).

^{13}C NMR (151 MHz, DMSO) δ 165.6 (s, 1 C), 165.5 (s, 1 C), 143.1 (s, 1 C), 110.9 (s, 1 C), 58.3 (s, 1 C), 49.6 (s, 1 C), 42.1 (s, 1 C), 38.1 (s, 1 C), 32.8 (s, 1 C), 20.2 (s, 1 C).

This material was used for **2D NMR Experiments** (see chapter 1.7) to prove the *trans* geometry of the two α-hydrogens, leading to the **absolute stereochemistry of S,R** for the **diketopiperazine 8b**.

1.6.4 Synthesis of (±)-*trans*-4-methylpiperidine-2-carbonitrile



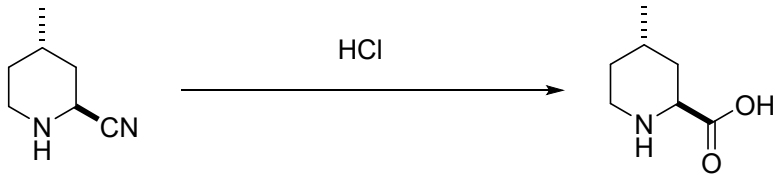
9 **10**

Electrochemical procedure in analogy as described by Stahl *et. al*.[2] On a IKA plate, multichannel potentiostat, BDD (Boron-Doped Diamond) electrodes. The BDD electrodes were conditioned with aqueous H_2SO_4 solution (2.5 mL of conc. H_2SO_4 in 1L of H_2O). The solution was stirred and current was then maintained at 0.49 A until 1000C were consumed (ca. 35min).

4-methylpiperidine (**9**; 2.98 mL, 25.2 mmol), ABNO (0.353 g, 2.52 mmol), TMSCN (4.73 mL, 37.8 mmol), Methanol (0.510 mL, 12.60 mmol) and tetra-n-butylammonium hexafluorophosphate (7.75 g, 20.01 mmol) were solubilized with ACN (200 mL). The resulting solution was transferred into the electrochemical reactor described above equipped with a magnetic stirrer. The solution was stirred and current was then maintained at 0.49 A until 6079 C were consumed (3.5 h, F=2.5 j= 7 mA/cm^2). Completion of the reaction was monitored by GC-MS analysis.

The reaction mixture was transferred to a round-bottom flask and concentrated under reduced pressure. TBME (ca. 5 x 4 mL) was used to extract the product from the solid mixture *via* trituration: a portion of solvent was added to the flask, stirred until the electrolyte (tetra-n-butylammonium hexafluorophosphate) crystallized out, and then filtered through a glass pipette filled with cotton wool into a pre-weighed vial and stir bar, and repeated with further portions of Et_2O. **(±)-*trans*-4-methylpiperidine-2-carbonitrile** (**10**; 3.6 g, 23.8 mmol, 94% yield) was obtained as a brown oil that was used crude in the next hydrolysis step.

1.6.5 Hydrolysis to the (±)-*trans*-4-methylpiperidine-2-carboxylic acid (HCl)

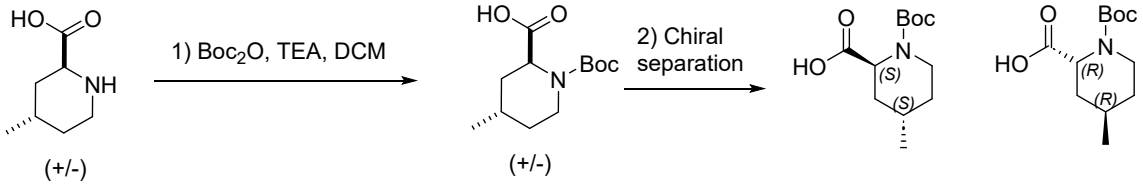


10 **11**

To a solution of **(±)-*trans*-4-methylpiperidine-2-carbonitrile** (**10**; 2.4 g, 19.33 mmol) in Dioxane (15 mL) was added HCl (aq., 36%, 15 mL, 183 mmol), the resulting solution was heated at 95°C for 2 days. Toluene (15 mL) was added to the reaction mixture and the resulting solution was concentrated to dryness under reduced pressure. Further three portions of toluene (5 mL) were added and then concentrated successively to eliminate the water via azeotropic distillation. The solid mixture was washed with DCM (5 mL) three times and then dried under high vacuum (0.2 mtorr) for 5 hours to give the desired **(±)-*trans*-4-methylpiperidine-2-carboxylic acid (HCl)** an amorphous white solid (**11**; 2.07 g, 11.5 mmol, 60% yield).

^{1}H NMR (400 MHz, Methanol-d4) δ 4.28 (t, J = 5.1 Hz, 1H), 3.32 – 3.26 (m, 2H), 2.27 – 2.16 (m, 1H), 1.94 – 1.72 (m, 3H), 1.48 (dtd, J = 13.9, 8.6, 5.2 Hz, 1H), 1.06 (d, J = 6.3 Hz, 3H).

1.6.6 Boc Protection of (±)-trans-4-methylpiperidine-2-carboxylic acid followed by chiral separation and assignment of absolute stereochemistry by X-ray



(+/-) (+/-)

11 **12** **12a** **12b**

Assignment of absolute stereochemistry according to X-Ray structures

To a solution of **(±)-4-methylpiperidine-2-carboxylic acid (HCl)** (**11**; 322 mg, 2.249 mmol) in DMF (22.5 mL) was added TEA (0.784 mL, 5.62 mmol), followed by Boc_2O (0.731 mL, 3.15 mmol). The reaction mixture was stirred for 1 h at room temperature. LC-MS after 1 h showed peak with expected mass. After completion of the reaction, the solvent was evaporated. The crude product was purified using reverse prep-HPLC to yield **(±)-N-Boc-4-methylpiperidine-2-carboxylic acid** (**12**; 132 mg, 0.540 mmol, 24% yield) as a white powder. The racemate

was then separated by SFC (Column: Lux i-Cellulose-5 (250mm*30mm; 5µm); Mobile phase: CO2/IPA 92/8 isocratic elution mode; Flow rate: 80ml/min; BPR:120 bars, Column temperature: 40°C) delivering **12a** (%ee>95%) as first eluting peak and **12b** (%ee>95%) as second eluting peak. Combined fractions yielded **12a** (45 mg, 0.184 mmol, 8% yield) and **12b** (47 mg, 0.192 mmol, 9% yield). After crystallization and X-ray crystallography, **12a** was assigned as **(S)-4-Methyl-Boc-piperidine-2-(S)-carboxylic acid** and **12b** as **(R)-4-Methyl-Boc-piperidine-(S)-2-carboxylic acid** (see next section for X-ray diffraction experiment).

1.6.6.1 Single crystal X-ray diffraction for (S)-4-Methyl-Boc-piperidine-(S)-2-carboxylic acid (12a) and (R)-4-Methyl-Boc-piperidine-(R)-2-carboxylic acid (12b).

Crystals suitable for diffraction experiments were obtained from a 1:1 mixture of dichloromethane and heptane by slow evaporation of the solvents at room temperature for compounds **12a** and **12b**.

Single-crystal X-ray diffraction data for **12a** and **12b** were collected at 100 K on a Bruker AXS three-circle diffractometer with monochromated Cu(Kα)-radiation (with multilayer mirrors), microfocus rotating anode generator, and a Smart 6000 CCD detector using the SMART software.[3],[4]

12a: 9 ω-scans at different ϕ-positions were performed to ensure appropriate data redundancy (6.47). P2: 10 ω-scans at different ϕ-positions were performed to ensure appropriate data redundancy (7.15).

Data processing and global cell refinement were performed with SAINT. A semiempirical absorption correction (SADABS) was applied based on the intensities of symmetry-related reflections measured at different angular settings. Crystal data, data collection parameters, and convergence results for **12a** and **12b** are listed in Table 1.

12a was solved by intrinsic phasing and subsequent difference Fourier syntheses, **12b** was solved by dual space-recycling methods and subsequent DF syntheses, both were refined based on full-matrix least-squares on F2 using ShelXle.[5]

Anisotropic displacement parameters were used for all non-hydrogen atoms. Hydrogen atoms were located in Difference Fourier maps and were refined in idealized positions using a riding model.

The absolute structure was determined based on the signal of the anomalous scatterers present (N, O). For **12a** (the C8S, C10S enantiomer) shown in **Figure S 5**, the Flack xq parameter based on 942 quotients refined to 0.06(9). For **12b** (the C8R, C10R

enantiomer) shown in Figure S 6, the Flack xq parameter based on 901 quotients refined to 0.04(9).

Identification code	MAF04a (**12a**)	MAF05a (**12b**)
Empirical formula	C12 H21 N O4	C12 H21 N O4
Formula weight	243.30	243.30
Temperature	100(2) K	100(2) K
Wavelength	1.54178 Å	1.54178 Å
Crystal system	Orthorhombic	Orthorhombic
Space group	$P2_12_12_1$	$P2_12_12_1$
Unit cell dimensions	a = 6.8875(2) Å b = 10.3983(3) Å c = 18.8853(5) Å α = 90 ° β = 90 ° γ = 90 °	a = 6.8873(3) Å b = 10.3941(5) Å c = 18.8746(8) Å α = 90 ° β = 90 ° γ = 90 °
Volume	1352.53(7) $Å^3$	1351.18(10) $Å^3$
Z	4	4
Density (calculated)	1.195 g/cm^3	1.196 g/cm^3
Absorption coefficient	0.734 mm^{-1}	0.735 mm^{-1}
F(000)	528	528
Crystal size	0.41 x 0.03 x 0.02 mm^3	0.25 x 0.07 x 0.02 mm^3
Theta range for data collection	4.683 to 68.367°	4.685 to 68.462°
Index ranges	-8<=h<=8 -12<=k<=12 -22<=l<=22	-8<=h<=8 -12<=k<=12 -22<=l<=22
Reflections collected	16097	17767
Independent reflections	2460 [R(int)=0.0504]	2451 [R(int)=0.0551]
Completeness to theta	Θ=68.367°, 99.7%	Θ=68.462°, 99.2%
Absorption correction	Semi-empirical from equivalents	Semi-empirical from equivalents
Max. and min. transmission	0.7531 and 0.6681	0.7531 and 0.6122
Refinement method	Full-matrix least-squares on F^2	Full-matrix least-squares on F^2
Data / restraints / parameters	2460 / 0 / 160	2451 / 0 / 159
Goodness-of-fit on F^2	1.085	1.131
Final R indices [I>2sigma(I)]	R1 = 0.0278 wR2 = 0.0694	R1 = 0.0335 wR2 = 0.0803
R indices (all data)	R1 = 0.0301 wR2 = 0.0704	R1 = 0.0378 wR2 = 0.0828
Absolute structure parameter	0.06(9)[942 quotients]	0.04(9)[901 quotients]
Largest diff. peak and hole	0.146 and -0.168 $e.Å^{-3}$	0.202 and -0.321 $e.Å^{-3}$

Table 1: Crystal data and structure refinement for 12a and 12b Summary of Data - CCDC deposition Number 2372678 (for 12a) and 2372681 (for 12b).

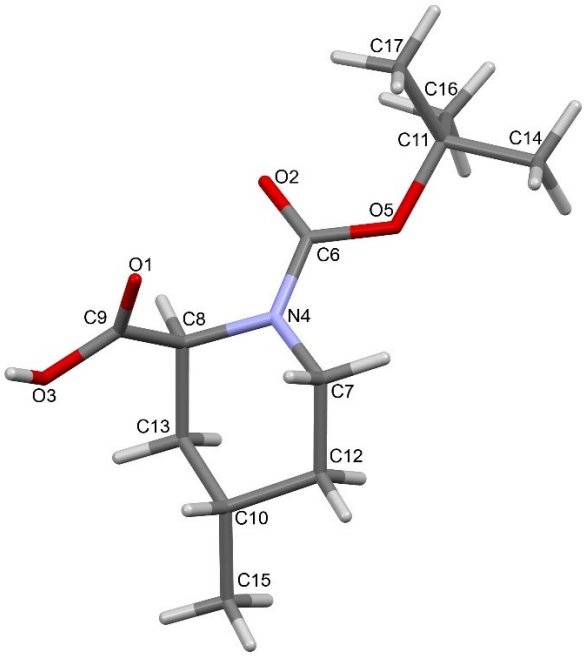


Figure S 5: Atomic numbering scheme for 12a = (S)-4-Methyl-Boc-piperidine-(S)-2-carboxylic acid. Figure created with MERCURY (Macrae CF (2020)).

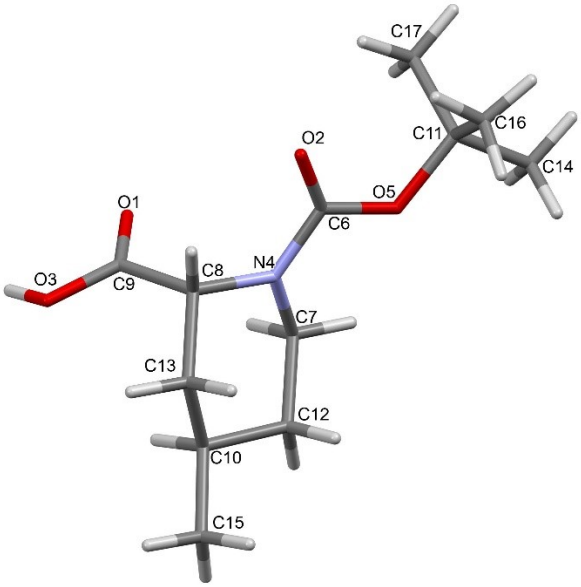
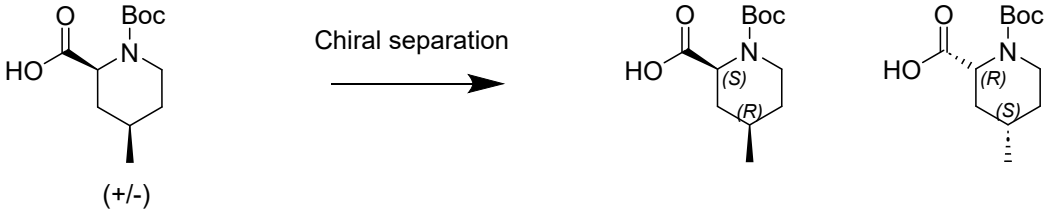


Figure S 6: Atomic numbering scheme for 12b = (R)-4-Methyl-Boc-piperidine-R-(2)-carboxylic acid (Figure created with MERCURY (Macrae CF (2020)).

1.6.7 Chiral separation of racemic *cis*-4-Methyl-Boc-piperidine-2-carboxylic acid.



13
Commerically available

13a **13b**

Assignment of absolute stereochemistry according to X-Ray experiments

Commercially available **(±)-*cis*-4-Methly-2-Boc-piperidine-2-carboxylic acid** (1 g) was purified was separated by SFC (Column: Chiralpak IC (250mm*30mm; 5µm); Mobile phase: CO2/IPA 90/10 isocratic elution mode; Flow rate: 80 mL/min; BPR:120 bars, Column temperature: 40°C) giving **P1** (>99%*ee*) as first eluting peak and **P2** (>99%*ee*) as second eluting peak. To get the free acid, the pooled fractions of the two separated enantiomers were dissolved in 150 mL DCM and extracted with 100 mL 0.1 N HCl. The two aqueous phases were back-extracted with 150 mL DCM. The combined organic phases were dried over Na_2SO_4 and evaporated. Fractions from first eluting peak gave **P1** (369,5 mg, 37% yield, >99%*ee*) and from second eluting peak **P2** (412 mg, 41% yield, >99%*ee*). Subsequent crystallization and X-ray experiments led to the following absolute stereochemistry assignment: **P1** = **(R)-Methyl-Boc-piperidine-(S)-2-carboxylic acid**; **P2 = (S)-Methyl-Boc-piperidine-(R)-2-carboxylic acid.**

1.6.7.1 Single crystal X-ray diffraction for (R)-4-Methyl-Boc-piperidine-(S)-2-carboxylic acid (13a) and (S)-4-Methyl-Boc-piperidine-(R)-2-carboxylic acid (13b)

Identification code	HJR24a (**13a**)	**HJR25a (13b)**
Empirical formula	C12 H21 N O4	C12 H21 N O4
Formula weight	243.30	243.30
Temperature	100(2) K	100(2) K
Wavelength	1.54178 Å	1.54178 Å
Crystal system	Orthorhombic	Orthorhombic
Space group	$P2_12_12_1$	$P2_12_12_1$
Unit cell dimensions	a = 9.2145(3) Å b = 11.6715(4) Å c = 12.0759(4) Å α = 90 ° β = 90 ° γ = 90 °	a = 9.20990(10) Å b = 11.6710(2) Å c = 12.0785(2) Å α = 90 ° β = 90 ° γ = 90 °
Volume	1298.73(7) $Å^3$	1298.30(3) $Å^3$
Z	4	4
Density (calculated)	1.244 g/cm^3	1.245 g/cm^3
Absorption coefficient	0.764 mm^{-1}	0.765 mm^{-1}
F(000)	528	528
Crystal size	0.52 x 0.05 x 0.02 mm^3	0.41 x 0.12 x 0.03 mm^3
Theta range for data collection	5.270 to 68.510°	5.270 to 68.310°
Index ranges	-10<=h<=11 -13<=k<=14 -14<=l<=14	-11<=h<=11 -13<=k<=13 -14<=l<=14
Reflections collected	19149	28203
Independent reflections	2371 [R(int)=0.0581]	2364 [R(int)=0.0470]
Completeness to theta	Θ=68.510°, 99.6 %	Θ=68.310°, 99.8 %
Absorption correction	Semi-empirical from equivalents	Semi-empirical from equivalents
Max. and min. transmission	0.7531 and 0.5803	0.7531 and 0.5727
Refinement method	Full-matrix least-squares on F^2	Full-matrix least-squares on F^2
Data / restraints / parameters	2371 / 0 / 160	2364 / 0 / 161
Goodness-of-fit on F^2	1.079	1.057
Final R indices [I>2sigma(I)]	R1 = 0.0279 wR2 = 0.0688	R1 = 0.0251 wR2 = 0.0664
R indices (all data)	R1 = 0.0297 wR2 = 0.0698	R1 = 0.0252 wR2 = 0.0664
Absolute structure parameter	0.10(10)[928 quotients]	0.04(3)[982 quotients]
Largest diff. peak and hole	0.151 and -0.154 $e.Å^{-3}$	0.208 and -0.220 $e.Å^{-3}$

Table 2: Crystal data and structure refinement for 13a and 13b CCDC deposition Number 2372679 for **13a** and 2372680 for **13b**.

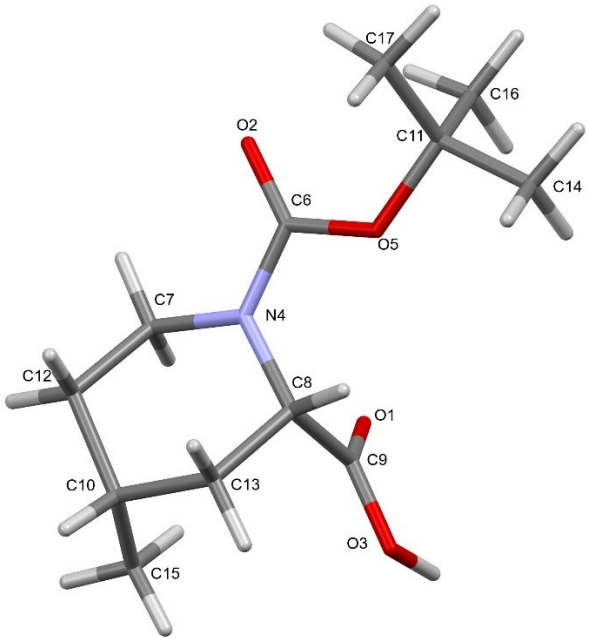


Figure S 7 Atomic numbering scheme for **13a** = (R)-4-Methyl-Boc-piperidine-S-(2)-carboxylic acid (Figure created with MERCURY (Macrae CF (2020)).

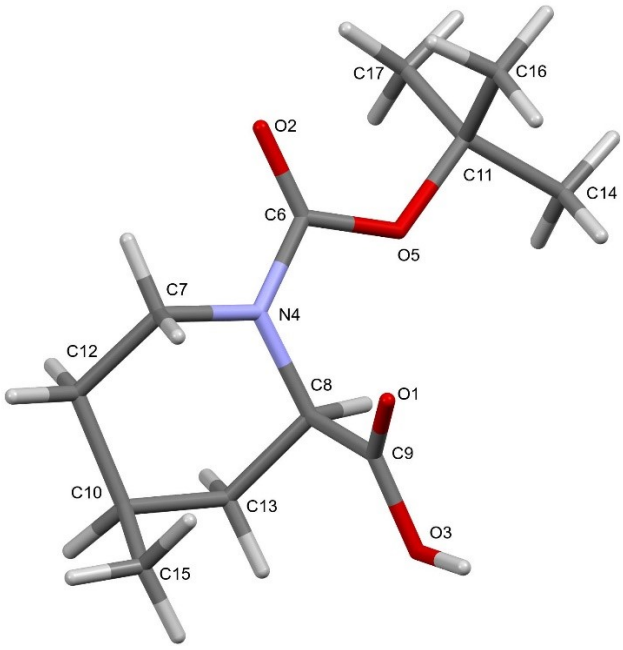


Figure S 8: Atomic numbering scheme for **13b** = (S)-4-Methyl-Boc-piperidine-R-(2)-carboxylic acid (Figure created with MERCURY (Macrae CF (2020)).

1.7 Assignment of absolute stereochemistry of the α-H proton of the 4-methylenepiperidine-2-carboxylic acid building block 6a and 6b through 8a and 8b

The conversion of enantiomerically pure but not assigned 4-methylenpiperidine-(R/S)-2-carboxylic acid (**6a** and **6b**) with L-Ala to the corresponding S,S and S,R diketopiperazine diastereomers **8a** and **8b** was used to determine the relative position of the α-H of 4-methylenpiperidine-2-carboxylic acid to the α-H of L-Ala. The presence or absence of NOEs between the two α protons allowed the absolute stereochemistry of the isolated enantiomers of the 4-methylenepiperidine-2-carboxylic acid building block to be unambiguously assigned. Peak 1 of the chiral separation corresponded to (S)-4-methylenepiperidine-2-carboxylic acid **6a** and peak 2 to (R)-4-methylenepiperidine-2-carboxylic acid **6b**.

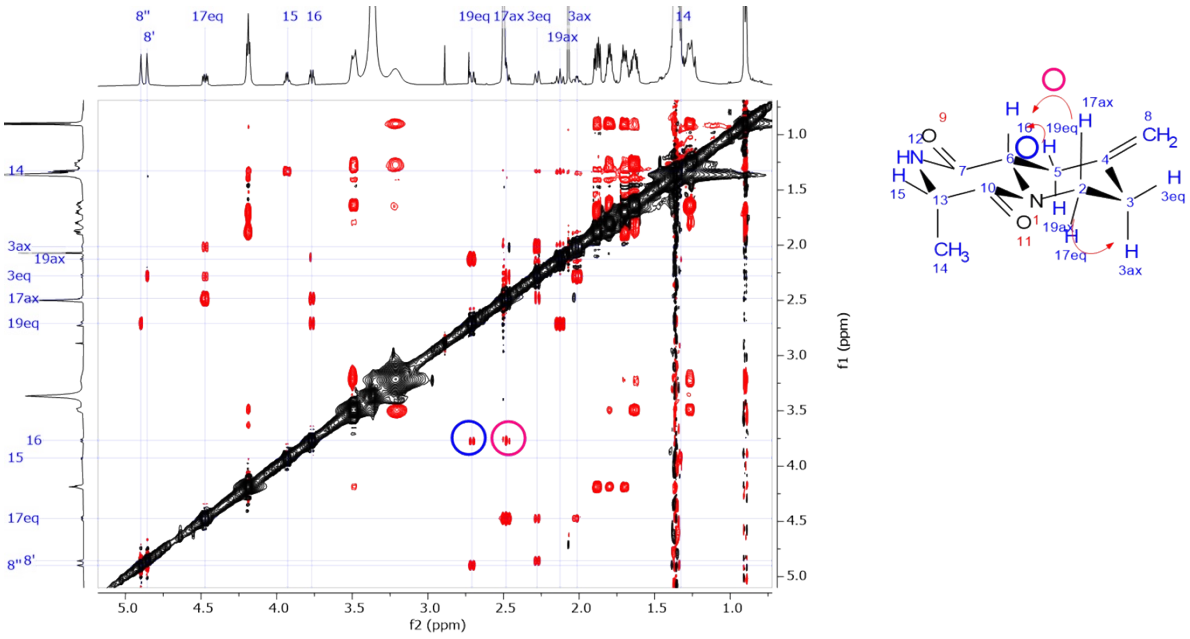


Figure S 9 ROESY of diketopiperazine **8a** originating from peak 1 of chiral building block separation (**6a**). The ROESY spectra of the diketopiperazine 1 derived from the 4-methylenepiperidine-2-carboxylic acid enantiomer with the earlier retention time ("peak 1") showed significant NOEs between the two α protons, indicating their cis position relative to each other. Together with the S-configuration of L-Ala, this information leads to the assignment of the S-configuration of 4-methylenepiperidine-2-carboxylic acid, resulting in the inactive macrocyclic FKBP12 ligand **S,S-SLF-1d**.

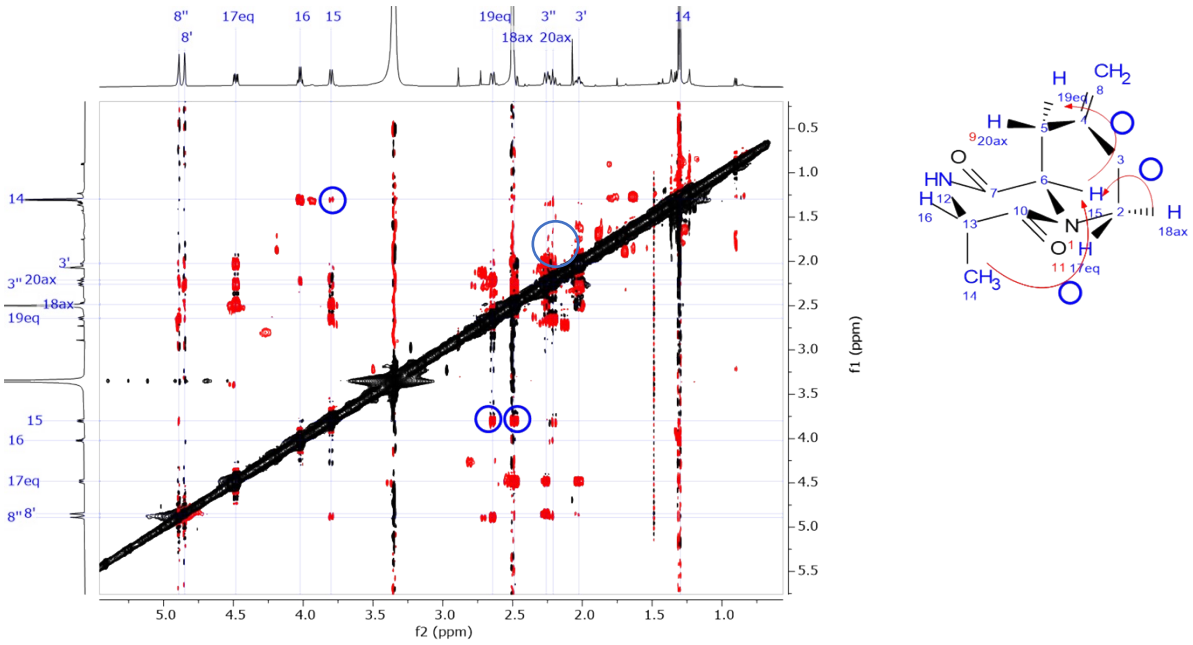


Figure S 10: The ROESY spectra of the diketopiperazine **8b** derived from the 4-methylenepiperidine-2-carboxylic acid enantiomer **6b** with the later retention time ("peak 2") showed no significant NOEs between the two α protons, indicating their trans position relative to each other. Together with the S-configuration of L-Ala, this information leads to the assignment of the R-configuration of 4-methylenepiperidine-2-carboxylic acid, resulting in the active macrocyclic FKBP12 ligand **R,S-SLF-1**.

1.7.1 NMR experiments

NMR spectra from the S,S (**8a**) and S,R (**8b**) diketopiperazines were prepared from approximately 2 mg in 500 µl DMSO-d_6, providing 20 mM solutions. All NMR experiments were recorded at 300K on a Bruker Avance III HD spectrometer equipped with a 5 mm BBO H&F CryoProbe™ with a z-gradient system, using a ^{1}H resonance frequency of 600.14 MHz and a ^{13}C resonance frequency of 150.92 MHz. Chemical shifts were measured relative to the DMSO-d_6 solvent signal at 2.50 ppm (^{1}H) and 39.5 ppm (^{13}C). A series of NMR spectra were measured: 1D-^{1}H, HSQC, COSY, ROESY, and TOCSY. The ROESY spectra were recorded using a standard pulse sequence from Bruker, 8 scans per increment, a data matrix of 256 x 2048 points, and a mixing time of 200 ms with a spin lock time of 60 µs.

The ROE correlations needed for the determination of the relative stereochemistry are illustrated in the correlation scheme in **Figure S 11** and **Figure S 12**, respectively for diketopiperazine **8a** and diketopiperazine **8b**. ^{1}H and ^{13}C spectra are consistent with the number and type of protons and carbons defined by the depicted structure of the diketopiperazines **8a** and **8b**.

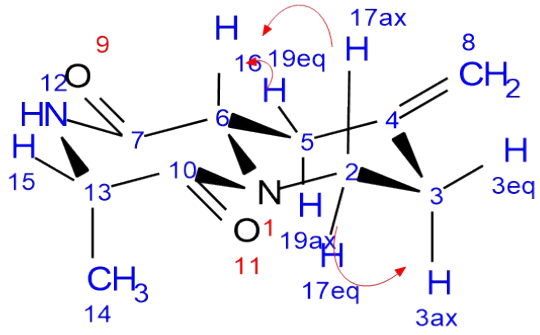


Figure S 11: NOE signals of the two α protons of the **diketopiperazine 8a**, originating from peak 1 of enantiomerically pure but unassigned 4-methylenpiperidine-2-carboxylic acid (plus L-Ala) demonstrated the relative cis orientation of the two α protons, leading to the assignment of (S)-4-methylenepiperidine-2-carboxylic acid (**6a**).

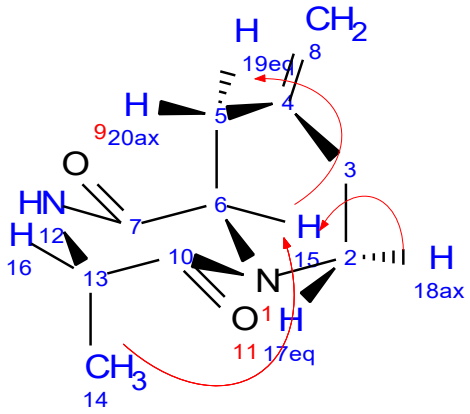


Figure S 12: NOE signals of the two α-H protons of the **diketopiperazine 8b**, originating from peak 2 of enantiomerically pure but unassigned 4-methylenpiperidine-2-carboxylic acid (plus L-Ala) demonstrated the relative cis orientation of the two α protons, leading to the assignment of (R)-4-methylenepiperidine-2-carboxylic acid (**6b**).

1.7.2 **Recorded NMR Experiments**

1H-NMR: 1D 1H experiment

1. 13C-NMR: 1D 13C experiment with 1H-broadband-decoupling
2. 2D: 1H,1H-COSY: gradient selected 2D homonuclear shift correlation (Hurd (1990))

2D: 1H,1H-ROESY: phase sensitive experiment with 180x/180-x spin-lock including a purge pulse (Bax and Davis (1985), Hwang and Shaka (1992))

2D: single bond 1H,13C correlation (13C-DEPT-HSQC): HSQC experiment with multiplicity editing, 1H detection and z-gradient (Kay, Keifer and Saarinen 1992)

2D: long range 1H,13C correlation (13C-HMBC): HMBC experiment with 1H detection and z-gradient (Bax and Summers 1986)

1.7.3 NMR spectroscopy – Spectra interpretation – Assignments of signals

The ^{1}H NMR spectra of the **diketopiperazines 8a** and **8b** show 12 signal groups besides the DMSO (2.50 ppm) and H_2O (3.36 ppm) and impurities which are not integrated in the NMR spectra. These 12 groups integrate all together to 14 protons. The 13C NMR spectra show 10 signal groups besides the seven DMSO (39.5 ppm) resonances and the impurities signals.

Structural elements and the molecular constitution were determined based on the chemical shift values and the identified spin systems. The connectivity of the individual elements was determined based on COSY, ROESY and HMBC data. The ^{1}H and ^{13}C chemical shifts are summarized in Table 3 and Table 4 for respectively **diketopiperazine 8a** and **diketopiperazine 8b**.

The observed connectivity's are illustrated in the correlation scheme in **Figure S 13**, **Figure S 14**, **Figure S 15**, **Figure S 16**. ^{1}H and ^{13}C spectra are consistent with the number and type of protons and carbons defined by the depicted structures. The unassigned ^{1}H and ^{13}C spectra of diketopiperazine **8a** and diketopiperazine **8b** are shown in **Figure S 17**, **Figure S 18**, **Figure S 24**, **Figure S 25**, respectively.

The technical details for the NMR experiments are described in **Table 4**, **Table 5**, **Table 6**, **Table 7** and **Table 8**.

Atom	Group	^{1}H			^{13}C
		δ [ppm]	Mult J in Hz	Number of Protons	δ [ppm]
1	N				
2	C				42.0
3	C				32.9
3ax	H	2.01	td (13.6, 5.3)	1	
3eq	H	2.28	ddt(13.6, 3.5, 1.9)	1	
4	C				142.9
5	C				38.7
6	C				58.1
7	C				165.5
8’	CH2	4.86	q (1.9)	1	111.0
8’’	CH2	4.90	q (1.9)	1	
9	O				
10	C				165.4
11	O				
12	NH	8.29	s	1	
13	C				49.9
14	CH3	1.33	d (7)	3	21.4
15	H	3.93	q (7)	1	
16	H	3.77	ddd (12.4, 3.3, 1.1)	1	
17ax	H	2.48	m	1	
17eq	H	4.47	ddd (12.7, 5.5, 2.0)	1	
19ax	H	2.13	t (12.8)	1	
19eq	H	2.71	ddd (13.4, 3.3, 1.7)	1	

Table 3 Diketopiperazine 8a

Atom	Group	^{1}H			^{13}C
		δ [ppm]	Mult J in Hz	Number of Protons	δ [ppm]
1	N				
2	C				42.1
3	C				32.8
3ax	H	2.02	tdd (13.2, 5.2, 1.6)	1	
3eq	H	2.26	ddt(13.6, 3.5, 1.9)	1	
4	C				143.1
5	C				38.1
6	C				58.3
7	C				165.6

8’	CH2	4.85	q (1.9)	1	110.9
8’’	CH2	4.89	q (1.9)	1	
9	O				
10	C				165.5
11	O				
12	NH	8.27	s	1	
13	C				49.6
14	CH3	1.30	d (7)	3	20.2
15	H	3.80	ddd (12.4, 3.2, 1.1)	1	
16	H	4.02	qt (6.9, 1.3)	1	
17eq	H	4.48	ddd (12.7, 5.5, 2.0)	1	
18ax	H	2.49	m	1	
19eq	H	2.64	ddd (13.3, 3.3. 1.6)	1	
20ax	H	2.21	m	1	

Table 4 Diketopiperazine 8b

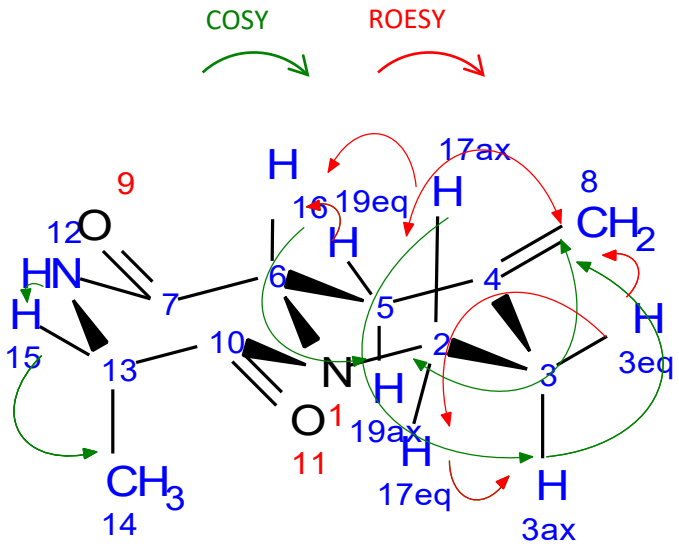


Figure S 13: COSY and ROESY Correlation scheme showing NMR connectivity’s for diketopiperazine **8a**

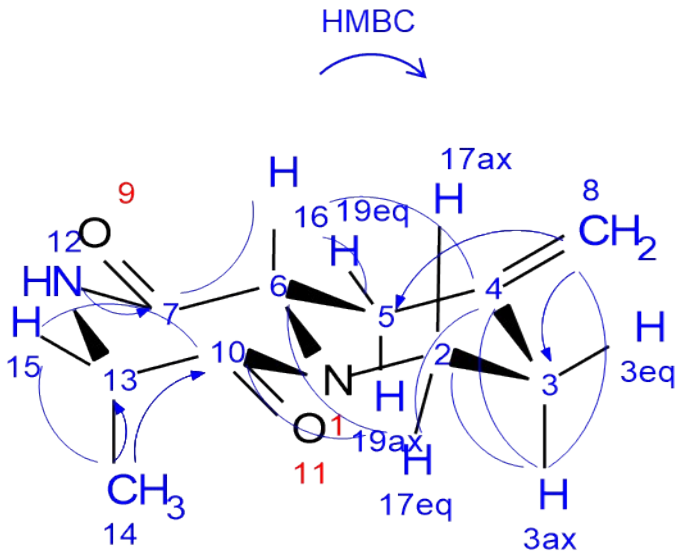


Figure S 14: HMBC correlation scheme showing NMR connectivity's for diketopiperazine **8a**

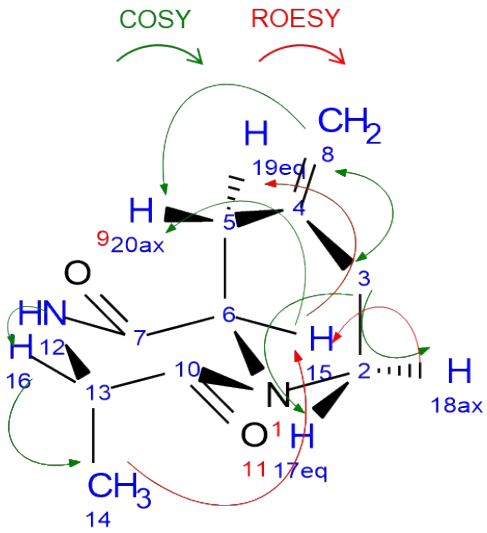


Figure S 15: COSY and ROESY Correlation scheme showing NMR connectivity's for diketopiperazine **8b**

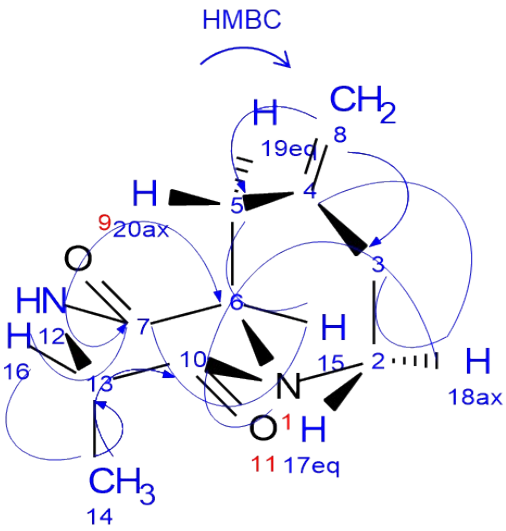


Figure S 16: HMBC correlation scheme showing NMR connectivity's for diketopiperazine **8b**

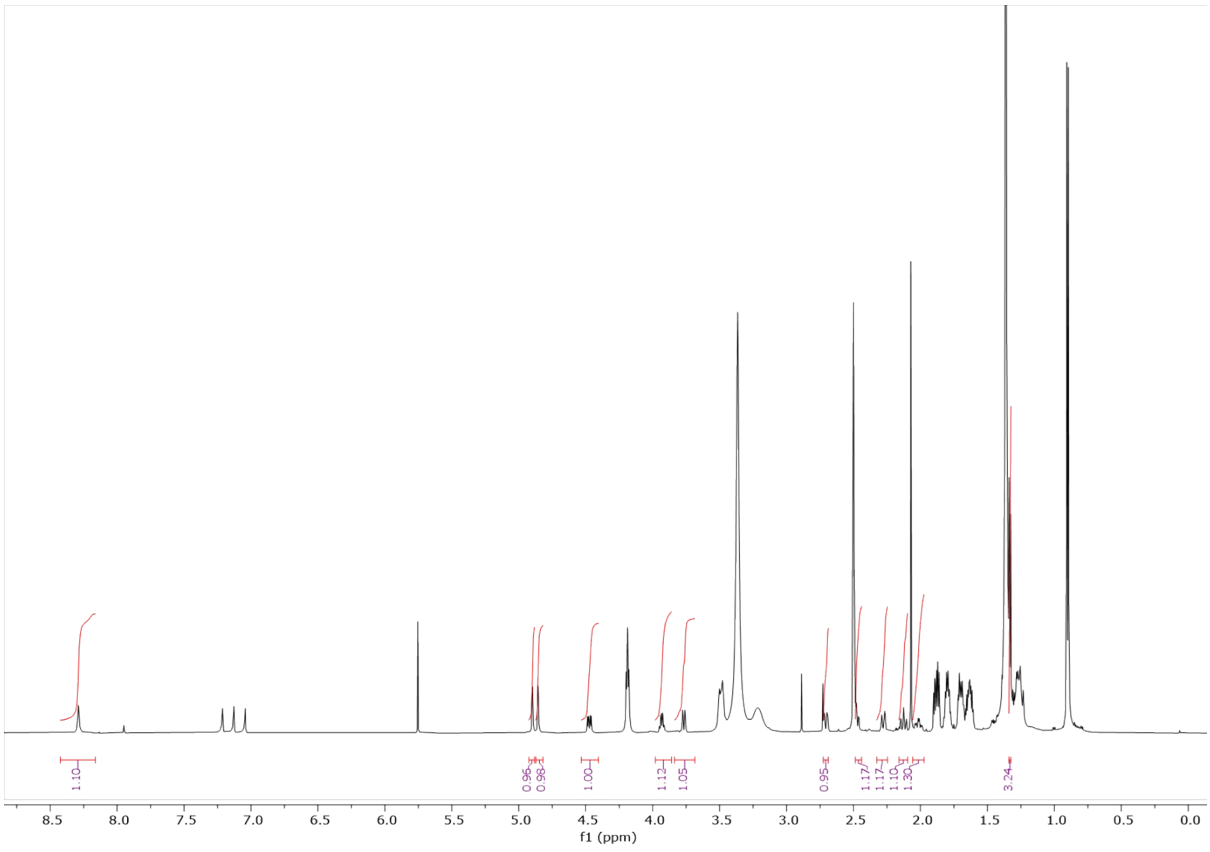


Figure S 17 1H-NMR spectra of Diketopiperazine 8a

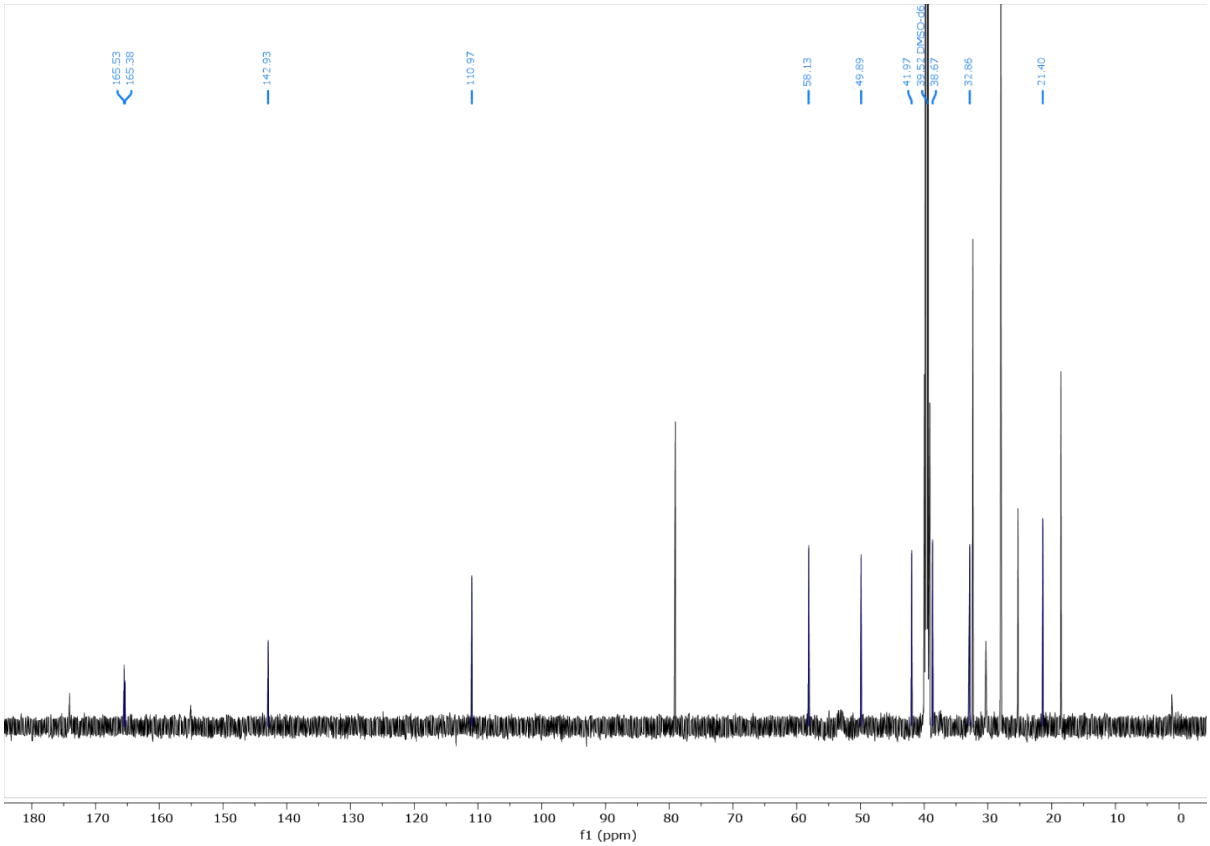


Figure S 18 13C-NMR spectra of Diketopiperazine 8a

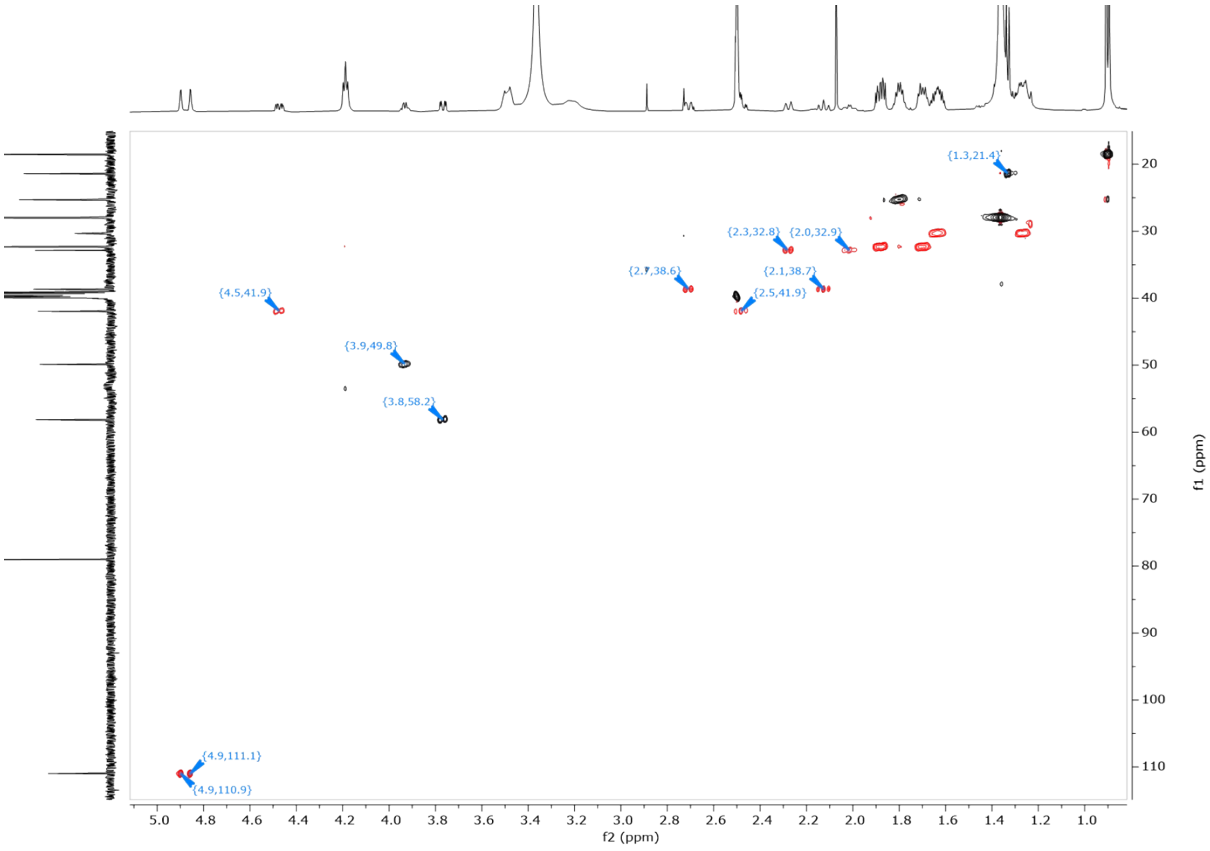


Figure S 19 HSQC spectra of Diketopiperazine 8a

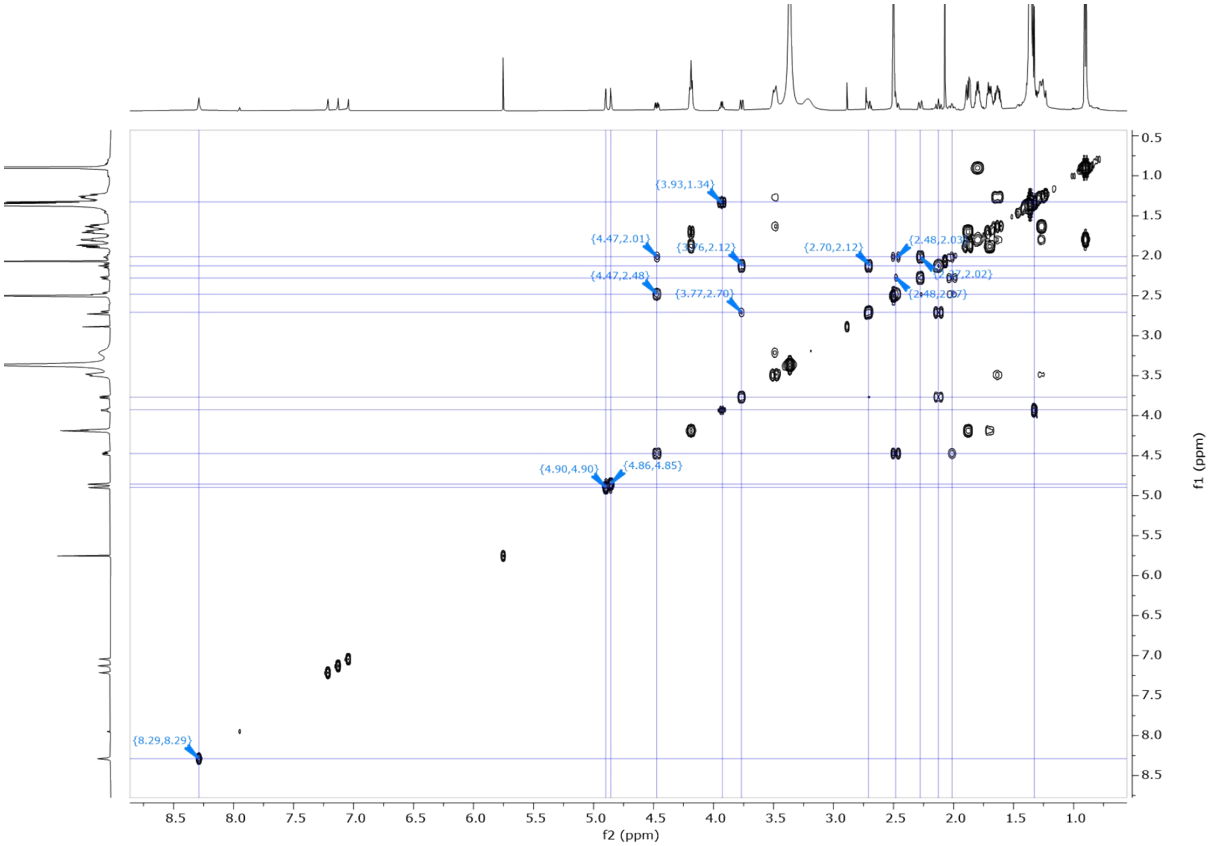


Figure S 20 COSY spectra of Diketopiperazine 8a

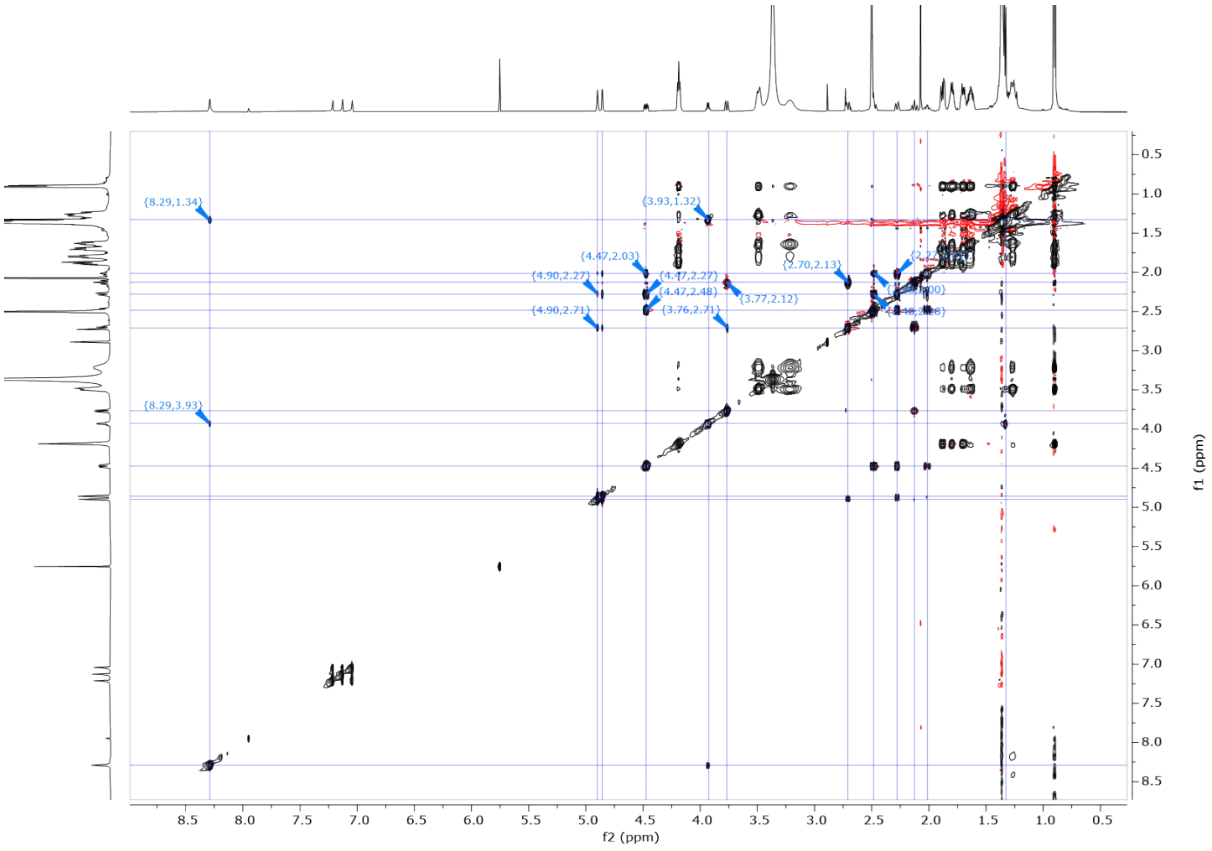


Figure S 21 TOCSY spectra of Diketopiperazine 8a

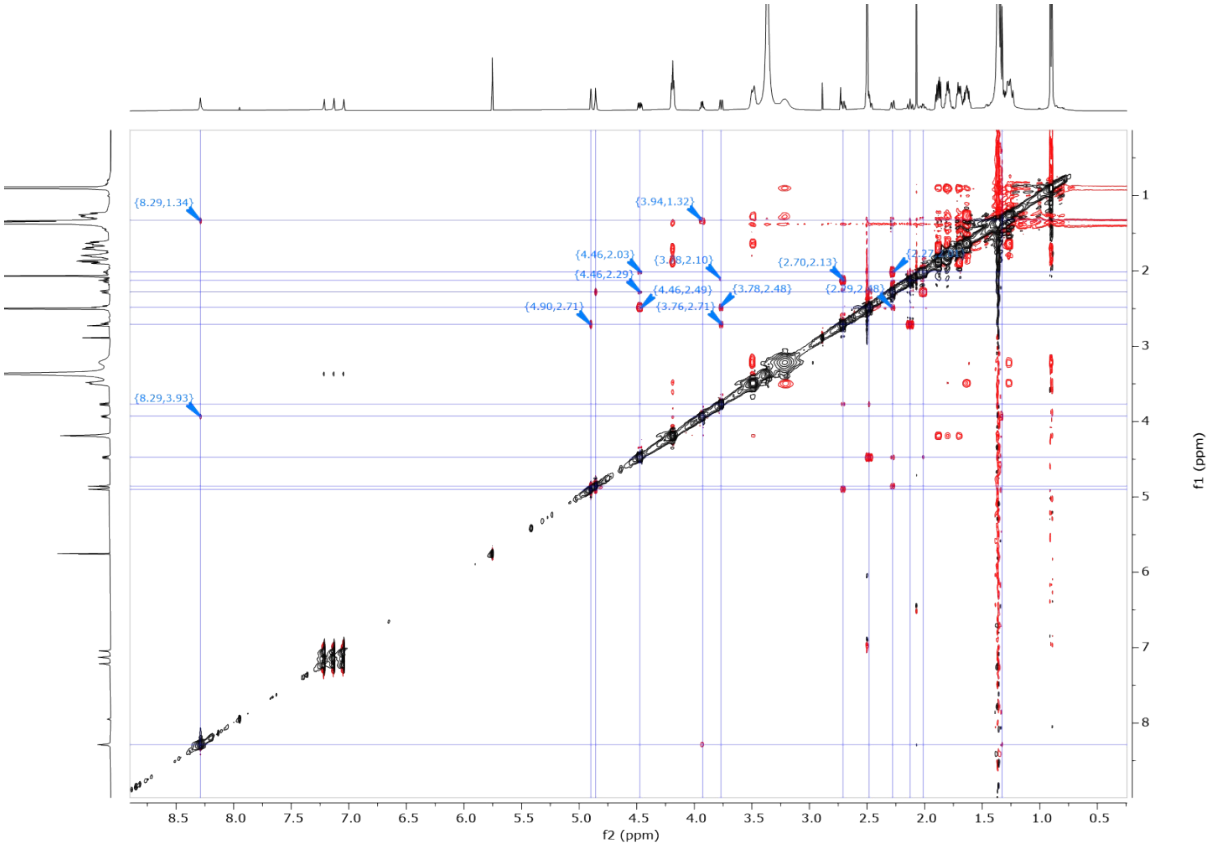


Figure S 22 ROESY spectra of Diketopiperazine 8a

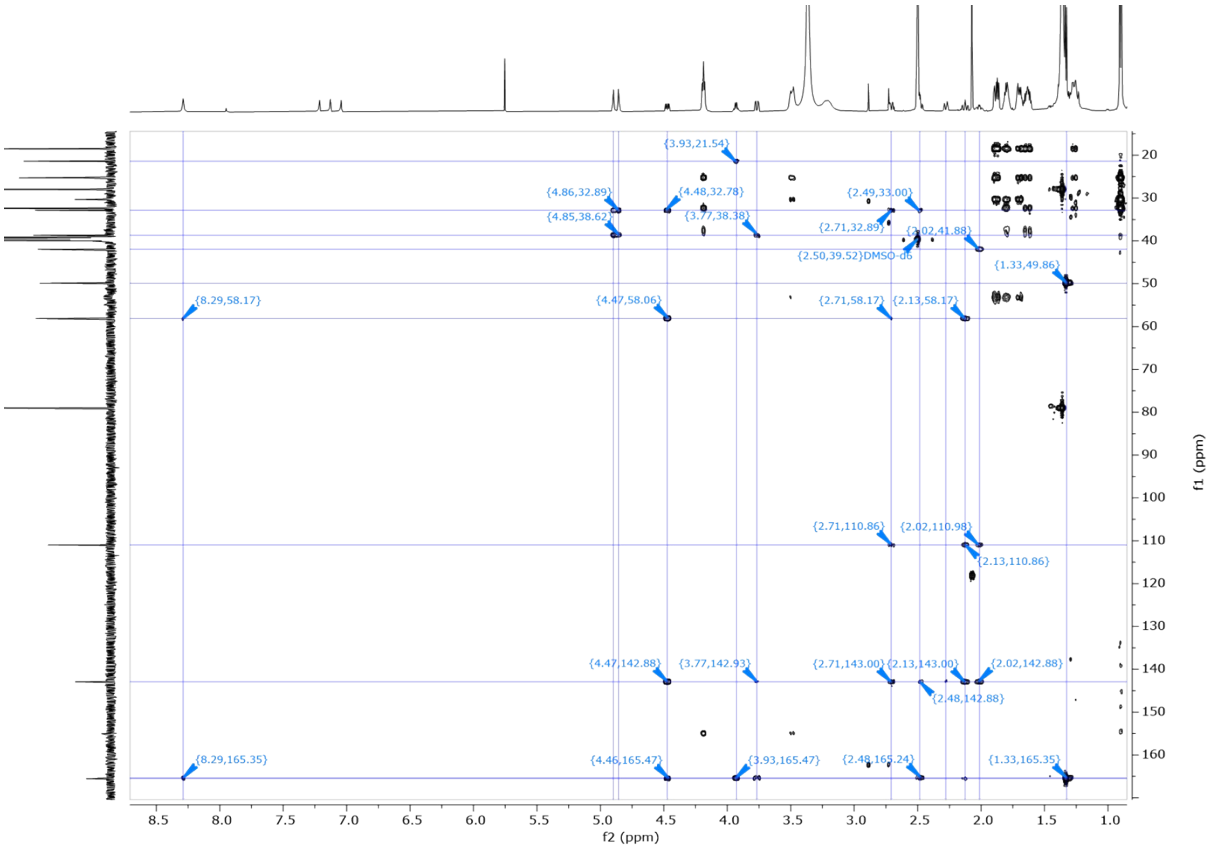


Figure S 23 HMBC spectra of Diketopiperazine 8a

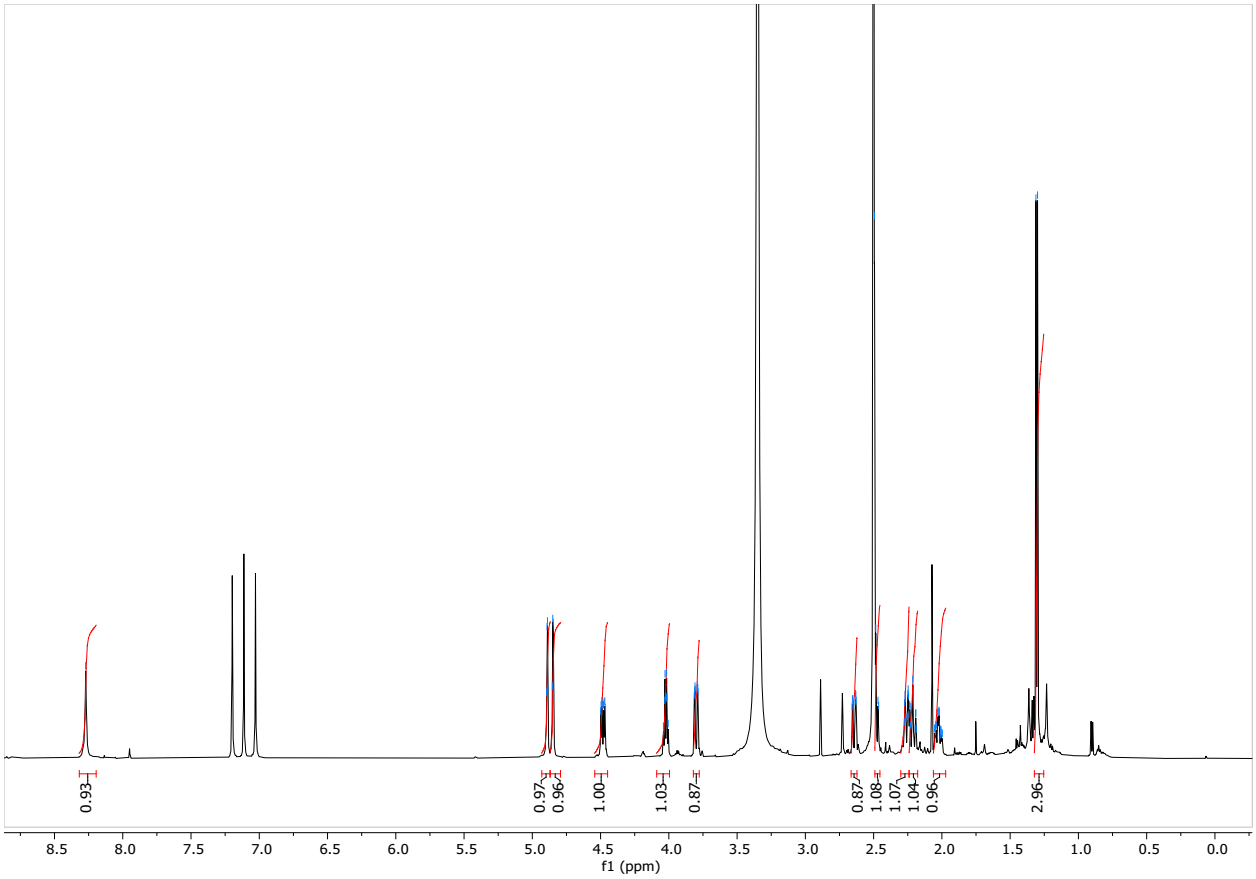


Figure S 24 1H-NMR spectra of Diketopiperazine 8b

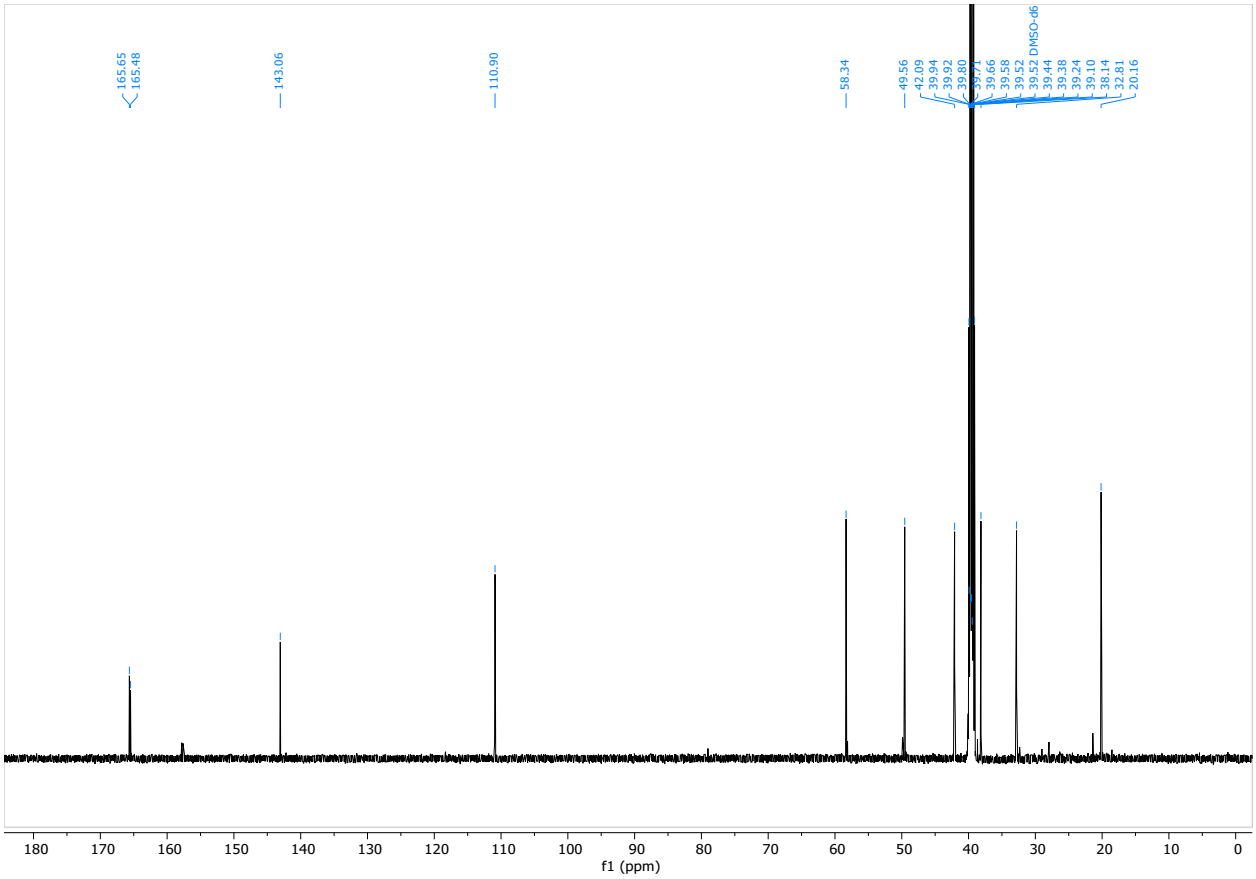


Figure S 25 13C-NMR spectra of Diketopiperazine 8b

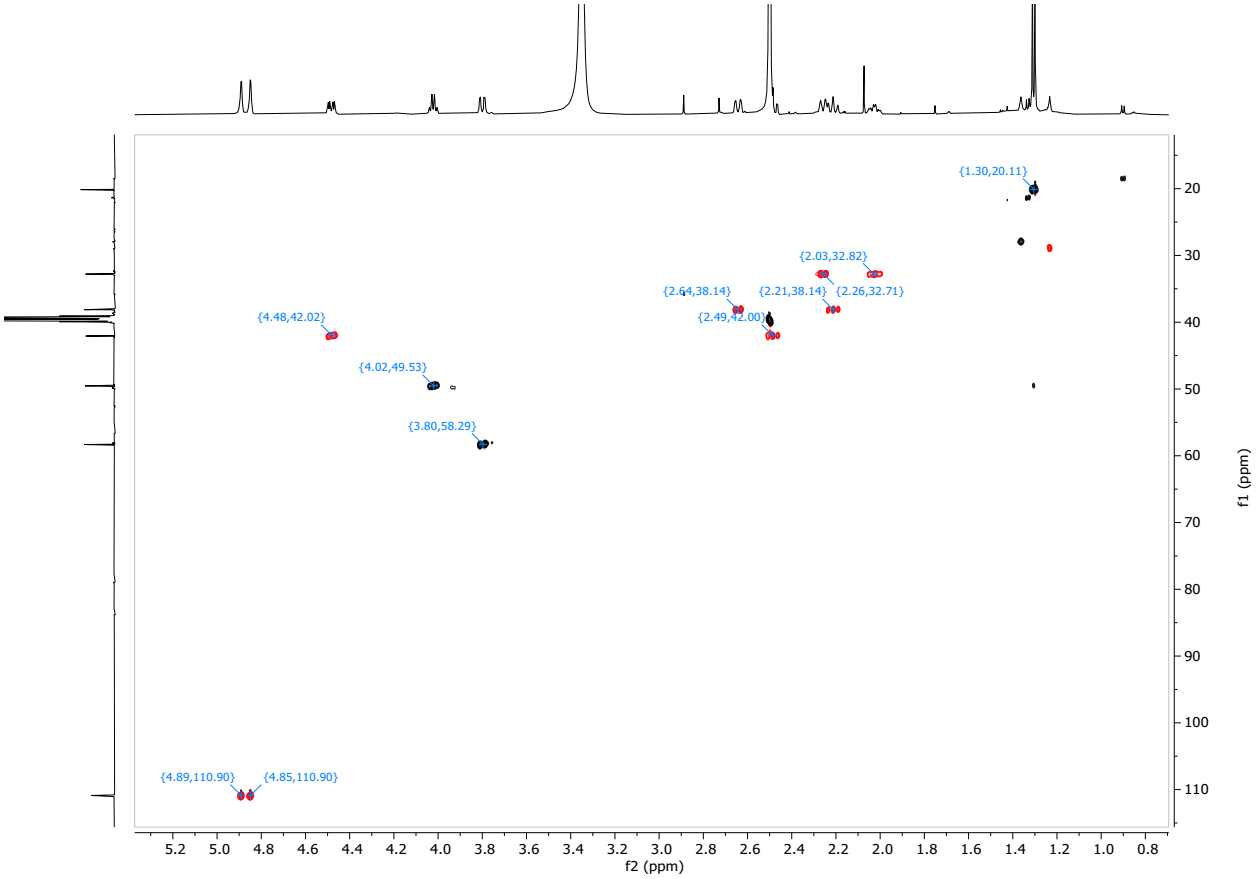


Figure S 26 HSQC spectra of Diketopiperazine 8b

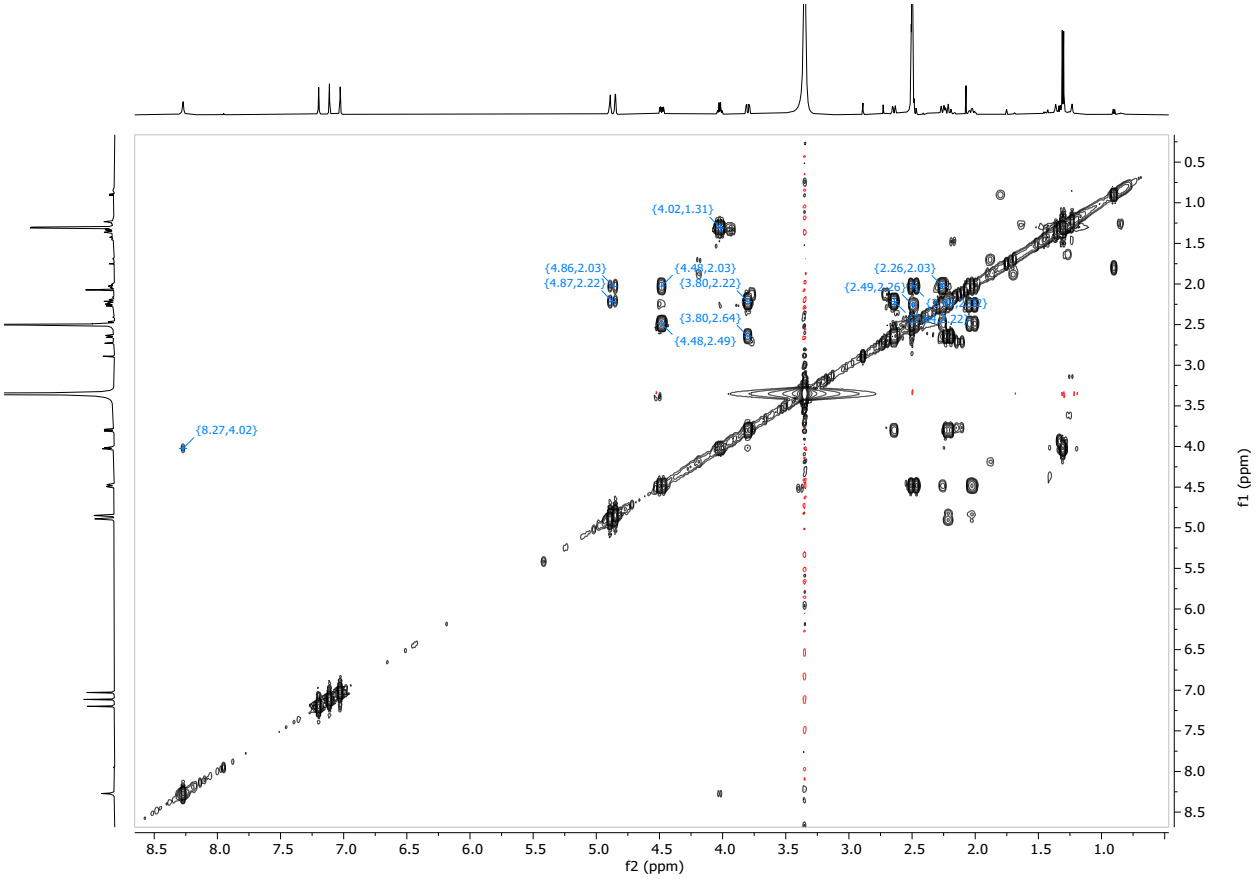


Figure S 27 COSY spectra of Diketopiperazine 8b

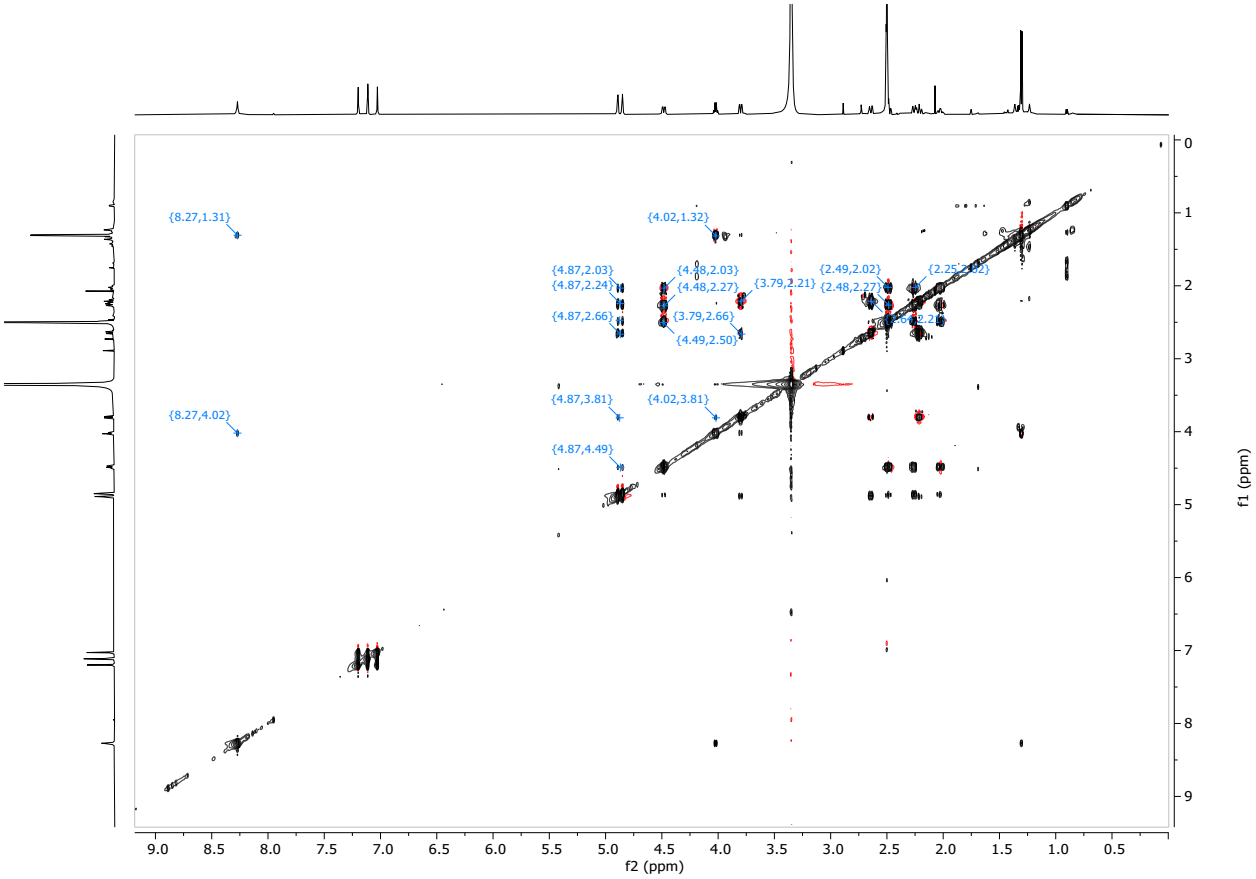


Figure S 28 TOCSY spectra of Diketopiperazine 8b

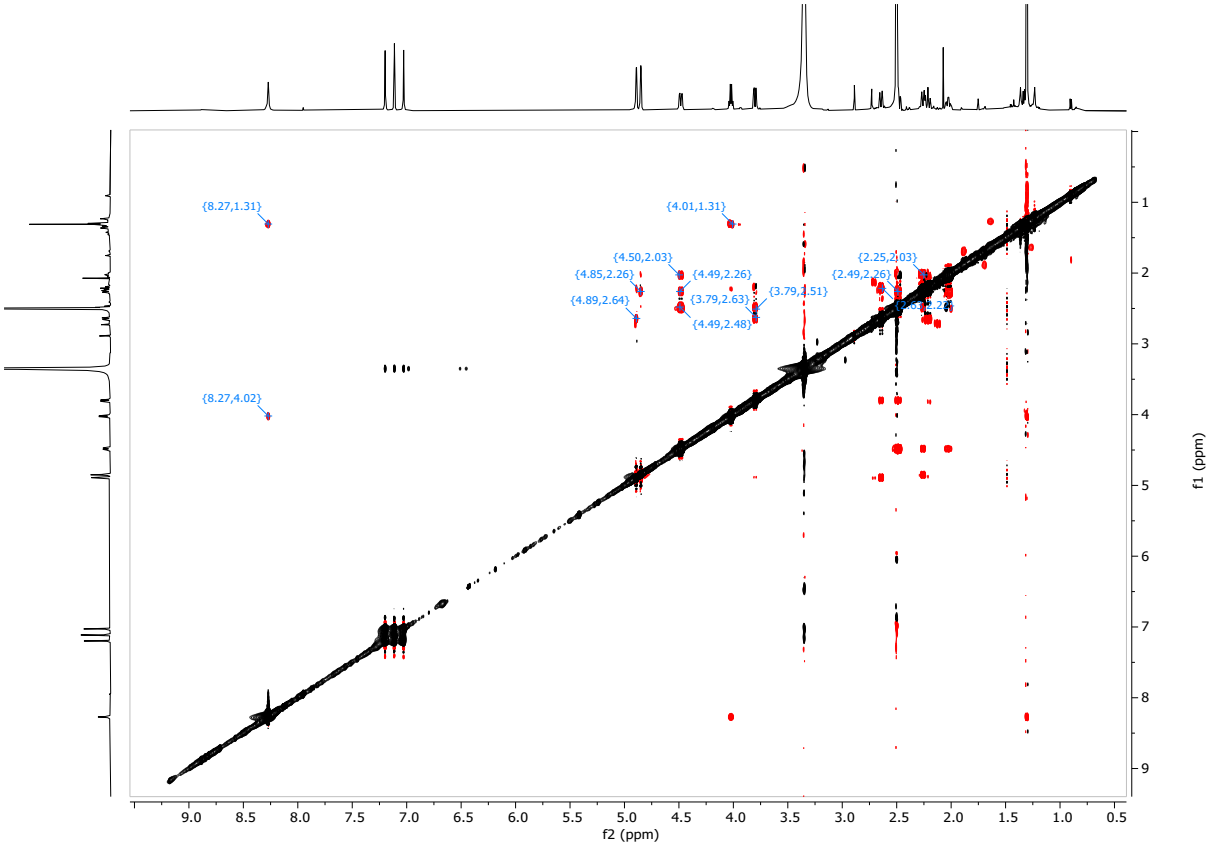


Figure S 29 ROESY spectra of Diketopiperazine 8b

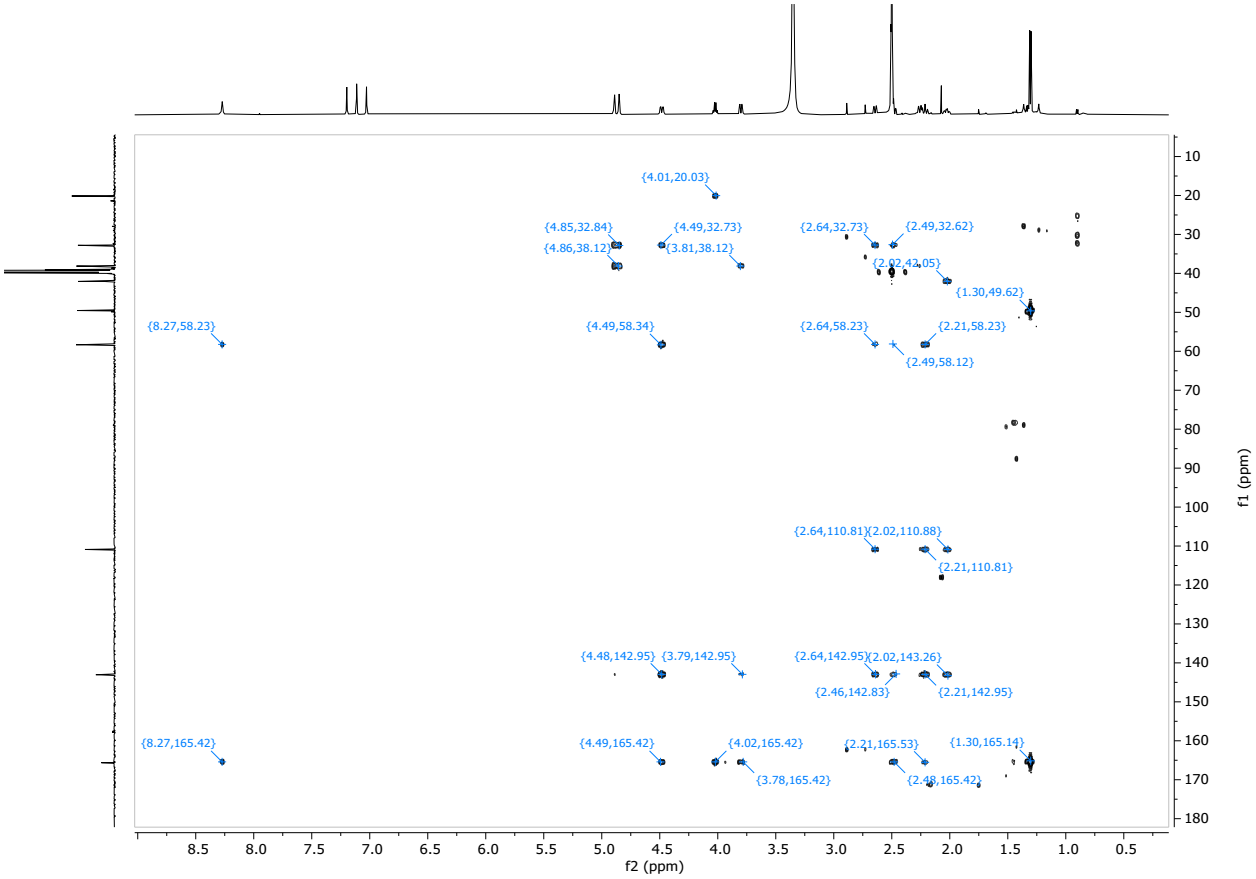


Figure S 30 HMBC spectra of Diketopiperazine 8b

1.7.4 NMR Experimental Parameters

1.7.4.1 Diketopiperazine 8a

1D ^{1}H-NMR

	acquisition
Pulse sequence	1D NMR sequence (zg)
Relaxation delay	D1 = 10sec
Scans	NS = 4
Time domain points	TD = 65536
Sweep width	SW = 14423.077 Hz
	processing
Frequency domain points	SI = 131072 (complex points)
Window Function	WDW = no
Line broadening	LB = 0.3 Hz

Table 5

1D ^{13}C-NMR

	acquisition
Pulse sequence	1D NMR sequence with 30 degree flip angle and composite pulse proton decoupling (zgpg)
Relaxation delay	D1 = 0.2sec
Scans	NS = 40
Time domain points	TD =130890
Sweep width	SW = 37500 Hz
	processing
Frequency domain points	SI = 524288 (complex points)
Window Function	WDW = exponential (EM)
Line broadening	LB = 0.5 Hz

Table 6

1.7.4.2 Diketopiperazine 8b

1D ^{1}H-NMR

	acquisition
Pulse sequence	1D NMR sequence (zg)
Relaxation delay	D1 = 10sec
Scans	NS = 4
Time domain points	TD = 65536
Sweep width	SW = 14423.077 Hz
	processing
Frequency domain points	SI = 131072 (complex points)
Window Function	WDW = no
Line broadening	LB = 0.3 Hz

Table 7

1D ^{13}C-NMR

	acquisition
Pulse sequence	1D NMR sequence with 30 degree flip angle and composite pulse proton decoupling (zgpg)
Relaxation delay	D1 = 0.2sec
Scans	NS = 680
Time domain points	TD =130890
Sweep width	SW = 37500 Hz
	processing
Frequency domain points	SI = 524288 (complex points)
Window Function	WDW = exponential (EM)
Line broadening	LB = 0.5 Hz

Table 8

1.8 High resolution LC-MS of all final compounds

1.8.1 LC-HRMS Analyses

The sample was dissolved with a concentration of 1mg/ml in ACN:H_2O (7:3). LC/ESI-MS data were recorded using an Orbitrap LumosTM (Thermo Fisher Scientific Inc.) mass spectrometer equipped with an electrospray ionization source and coupled to a Thermo Ultimate 3000 liquid chromatograph equipped with a diode array detector. The chromatography separation was achieved with an Acquity UPLC BEH C18 1.7µm, 1.0 x50 mm column. The accurate mass was obtained by averaging 6 scans at a mass resolution of ca 120000 (FWHM definition). The mass accuracy of the system has been found to be better than 2 ppm. The chromatography was performed at 150 µL/min flow rate (1 mm C_{18}-column) with a gradient from 5% to 100% B acetonitrile with 0.05% formic acide in 9 min. The mobile phase A was H_2O with 0.04% formic acid.

1.8.2 Data table

	Formula	Ion observed	Calculated	Measured	Deviation mmu	Deviation ppm
R,S-SLF-1a	C41H51N3O11	$[M+H]^+$	762.35964	762.36019	0.55	0.7
R,R-SLF-1b	C41H51N3O11	$[M+H]^+$	762.35964	762.35985	0.21	0.3
S,R-SLF-1c	C41H51N3O11	$[M+H]^+$	762.35964	762.36004	0.40	0.5
S,S-SLF-1d	C41H51N3O11	$[M+H]^+$	762.35964	762.36019	0.55	0.7
R,S-SLF-2a	C41H53N3O11	$[M+H]^+$	764.37529	764.37573	0.44	0.6
R,S-SLF-2b	C41H53N3O11	$[M+H]^+$	764.37529	764.37564	0.35	0.5
R,S-SLF-3	C42H53N3O11	$[M+H]^+$	776.37529	776.37586	0.57	0.7
R,S-SLF-4a	C43H55N3O11	$[M+H]^+$	790.39094	790.39155	0.61	0.8
S,S-SLF-4b	C43H55N3O11	$[M+H]^+$	790.39094	790.39135	0.41	0.5
R,S-SLF-4c	C43H53N3O11	$[M+H]^+$	788.37529	788.37585	0.56	0.7
R,S-SLF-5a	C43H55N3O11	$[M+H]^+$	790.39094	790.39154	0.60	0.8
S,S-SLF-5b	C43H55N3O11	$[M+H]^+$	790.39094	790.39113	0.19	0.2
R,S-SLF-6	C41H51N3O12	$[M+H]^+$	778.35455	778.35521	0.66	0.8
R,S-SLF-7	C41H52N4O11	$[M+H]^+$	777.37054	777.37071	0.17	0.2
R,S-SLF-8	C42H50F3N3O11	$[M+H]^+$	830.34702	830.34741	0.39	0.5
R,S-SLF-9	C43H55N3O12	$[M+H]^+$	806.38585	806.38635	0.50	0.6
R,--SLF-10	C42H53N3O11	$[M+H]^+$	776.37529	776.37547	0.18	0.2
R,--SLF-11	C42H51N3O11	$[M+H]^+$	774.35964	774.36001	0.37	0.5

Table 9 High resolution LC-MS data of all compounds

1.8.3 HR-LCMS Spectra

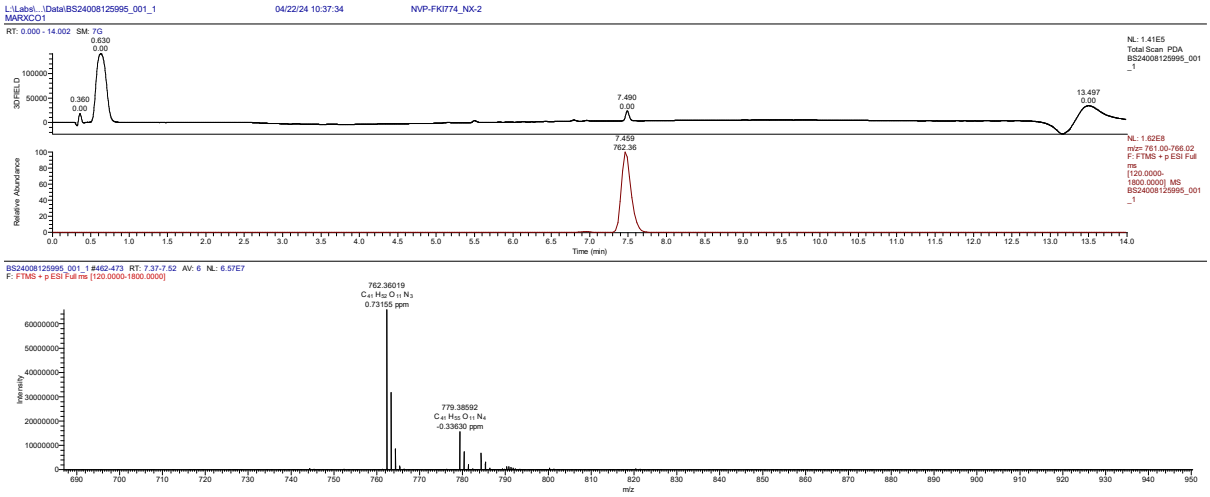


Figure S 31: R,S-SLF-1a

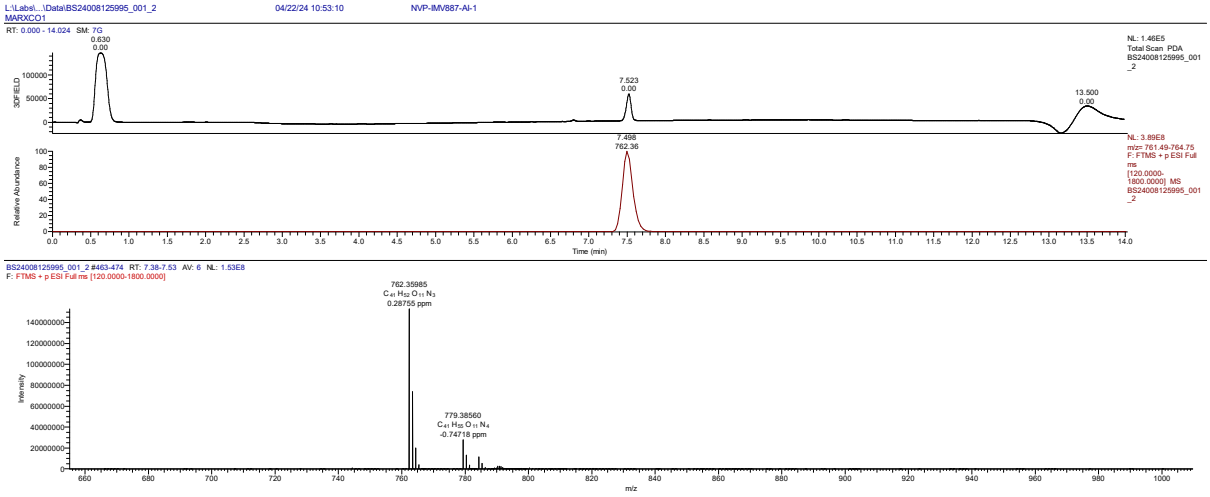


Figure S 32: R,R-SLF-1b

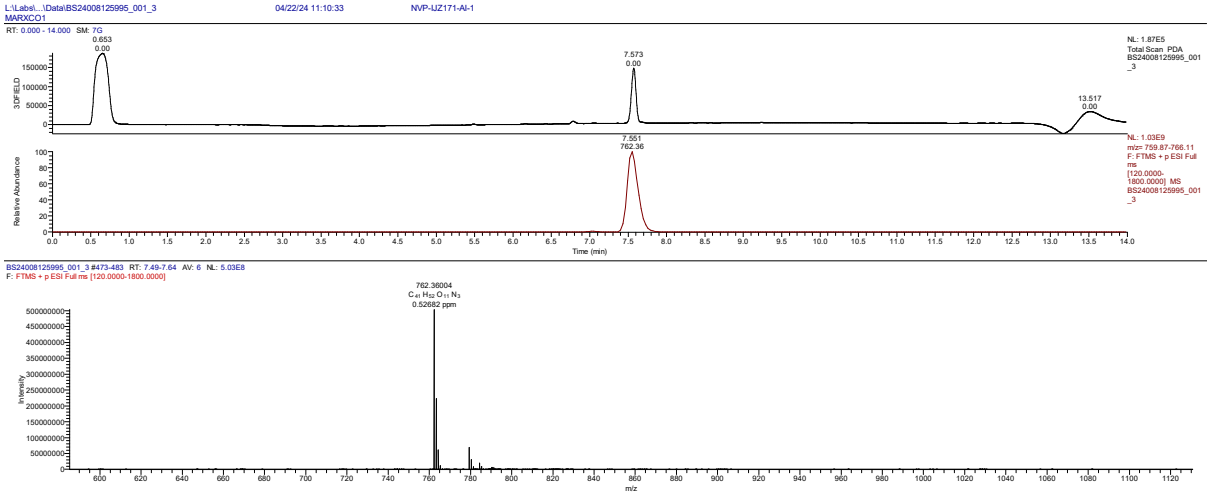


Figure S 33: S,R-SLF-1c

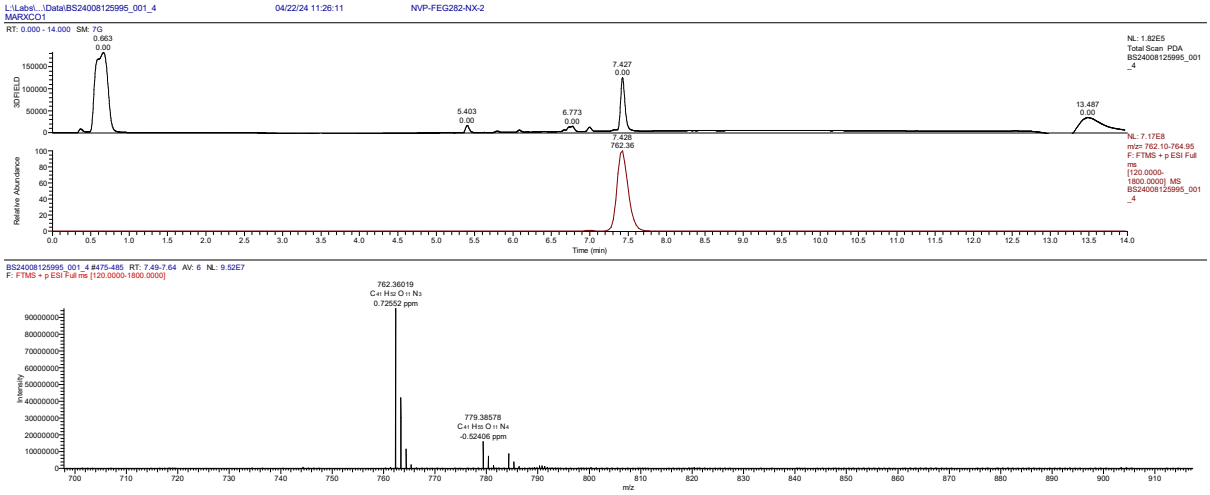


Figure S 34: S,S-SLF-1d

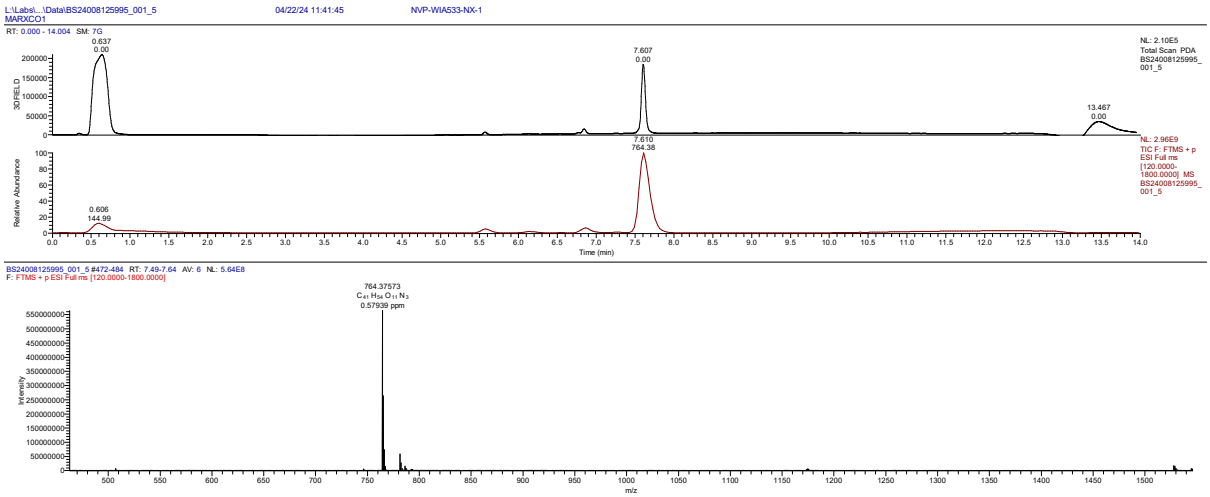


Figure S 35: R,S-SLF-2a

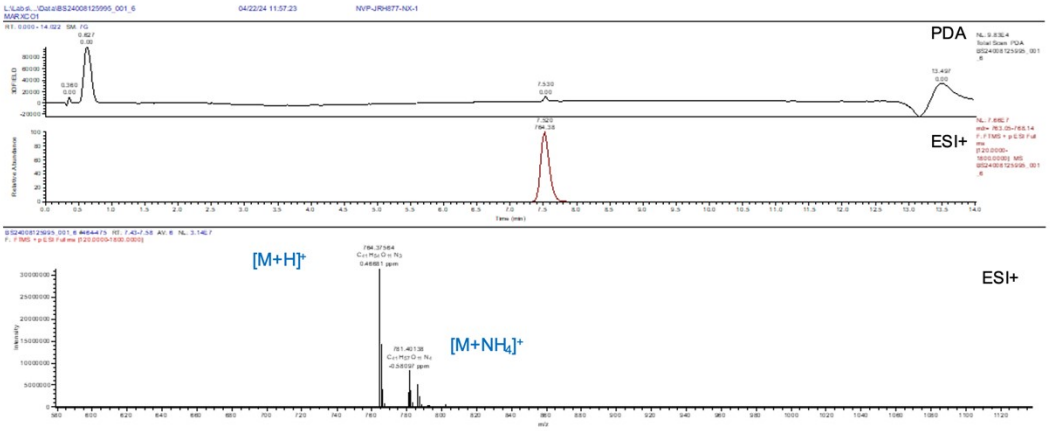


Figure S 36: R,S-SLF-2b

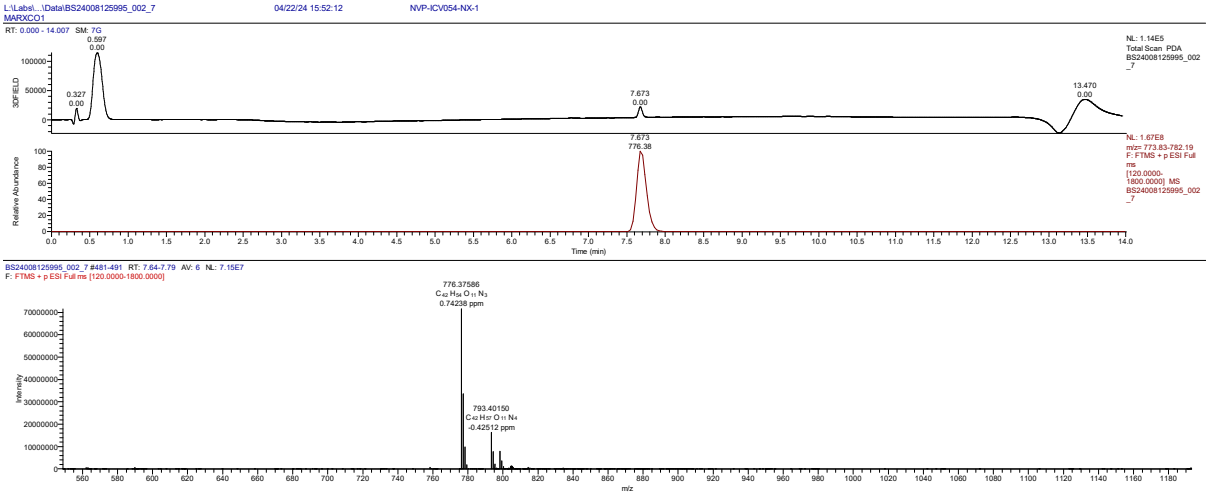


Figure S 37: R,S-SLF-3

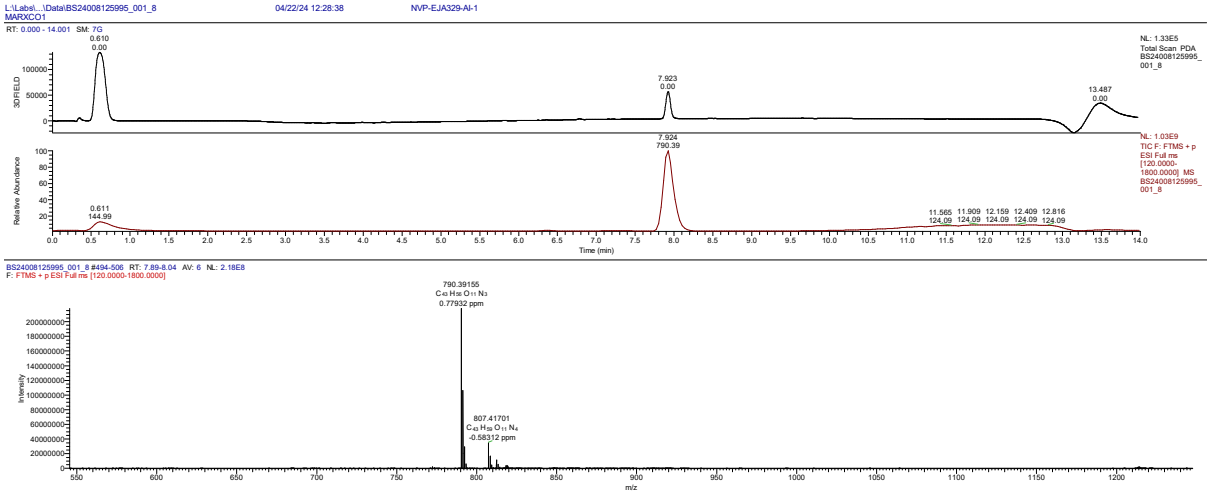


Figure S 38 R,S-SLF-4a

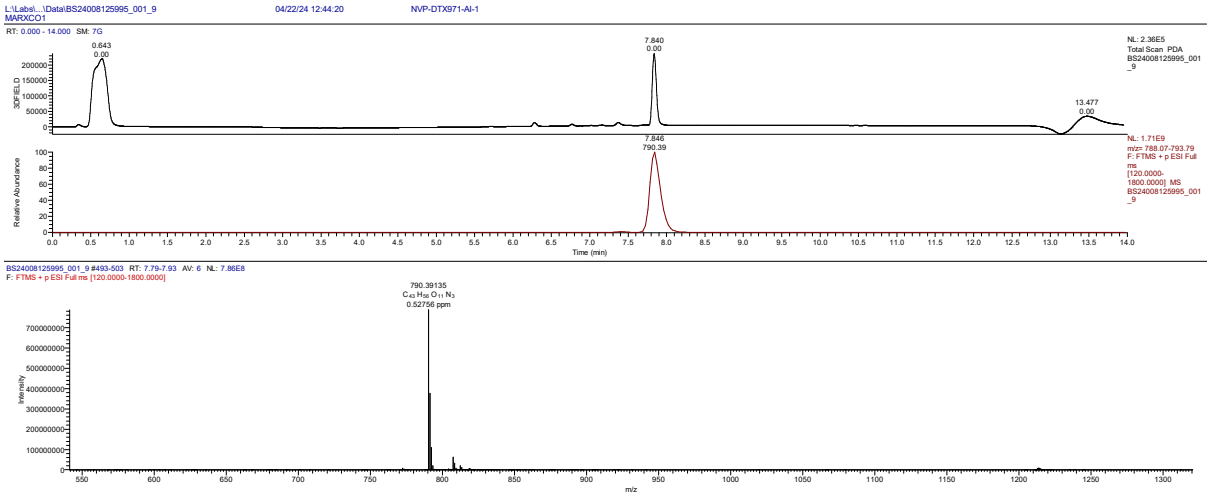


Figure S 39: S,S-SLF-4b

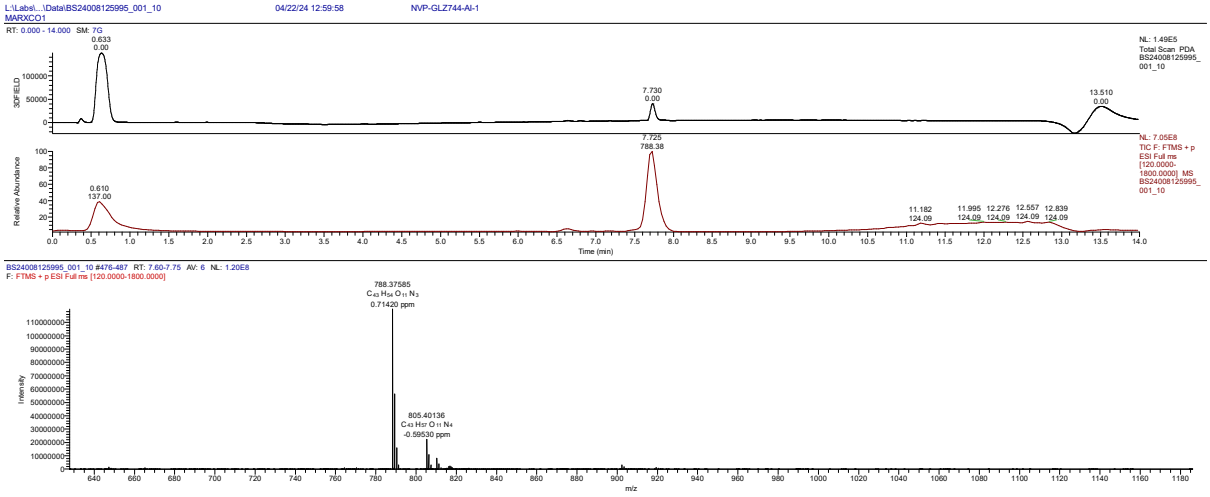


Figure S 40: R,S-SLF-4c

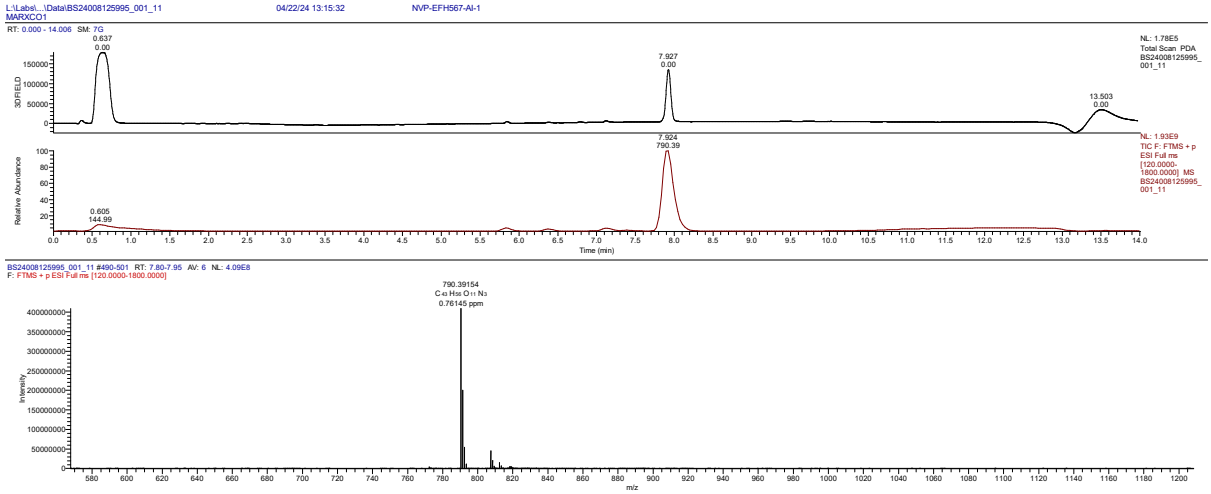


Figure S 41: R,S-SLF-5a

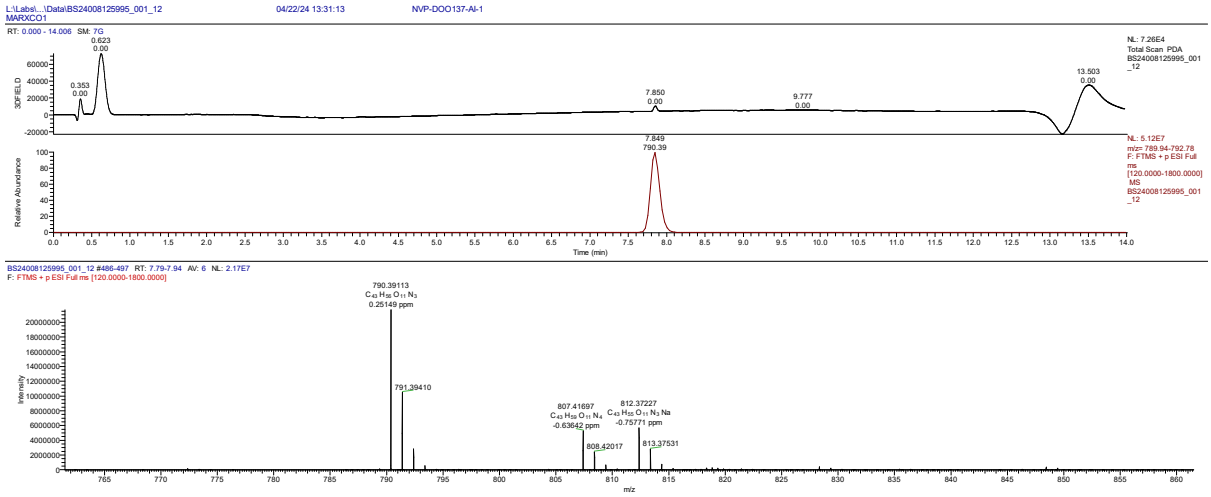


Figure S 42: S,S-SLF-5b

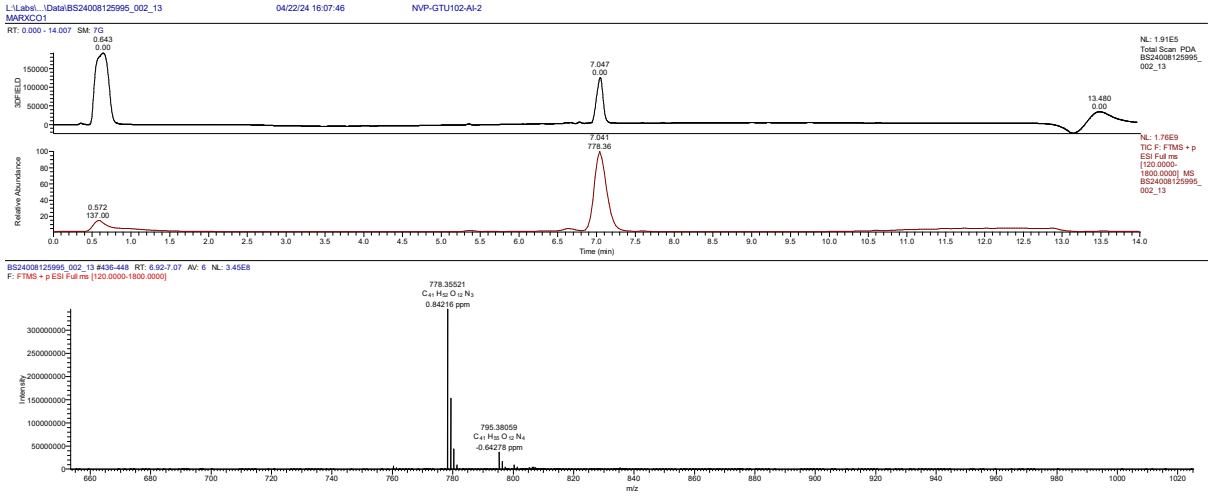


Figure S 43: R,S-SLF-6

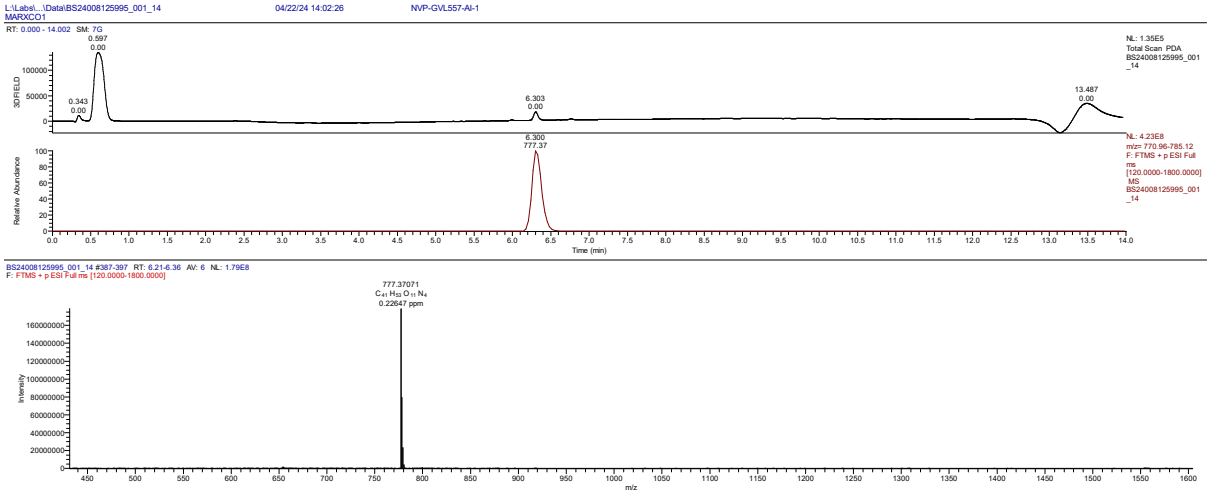


Figure S 44: R,S-SLF-7

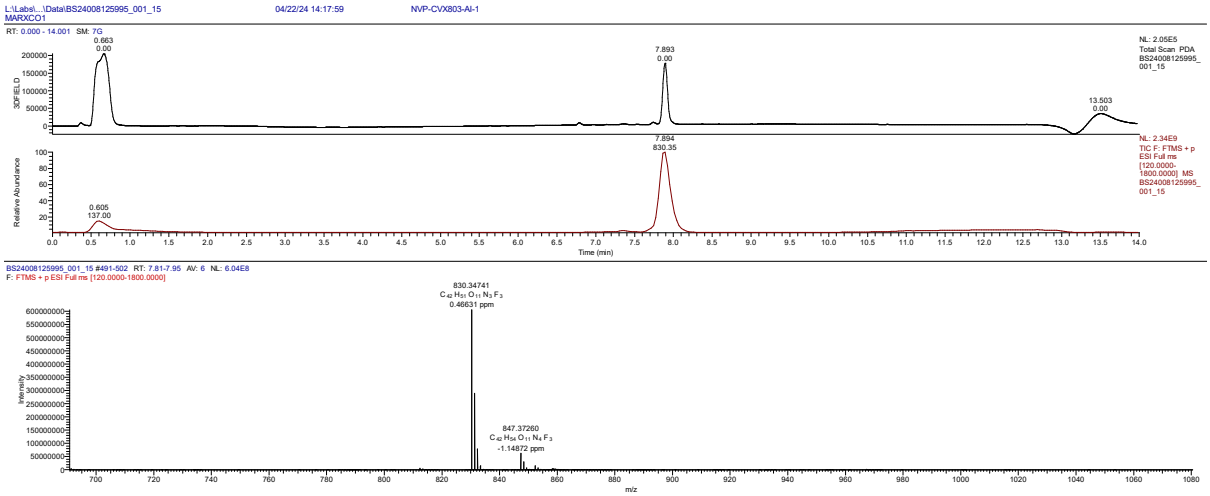


Figure S 45: R,S-SLF-8

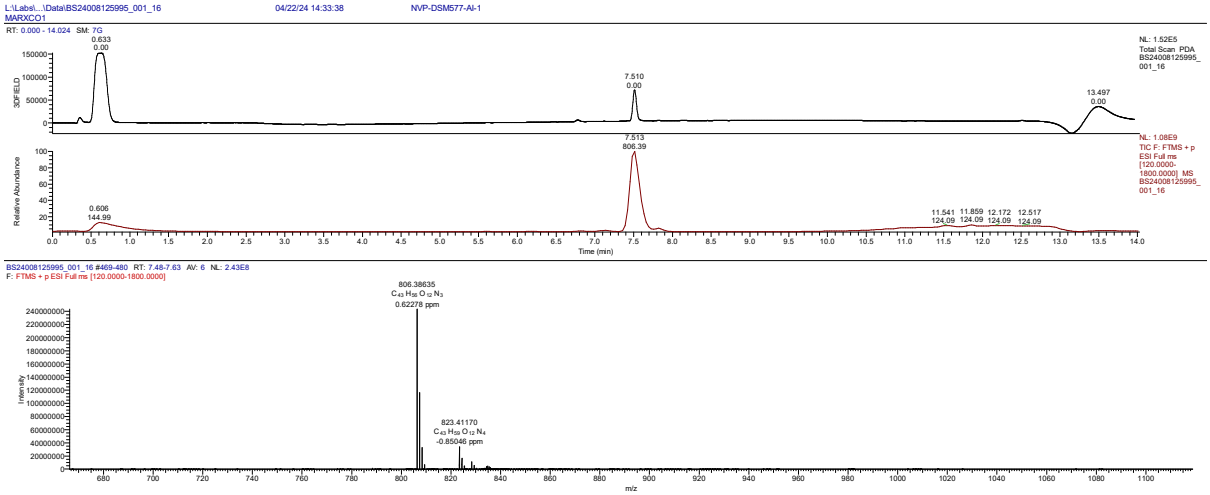


Figure S 46: R,S-SLF-9

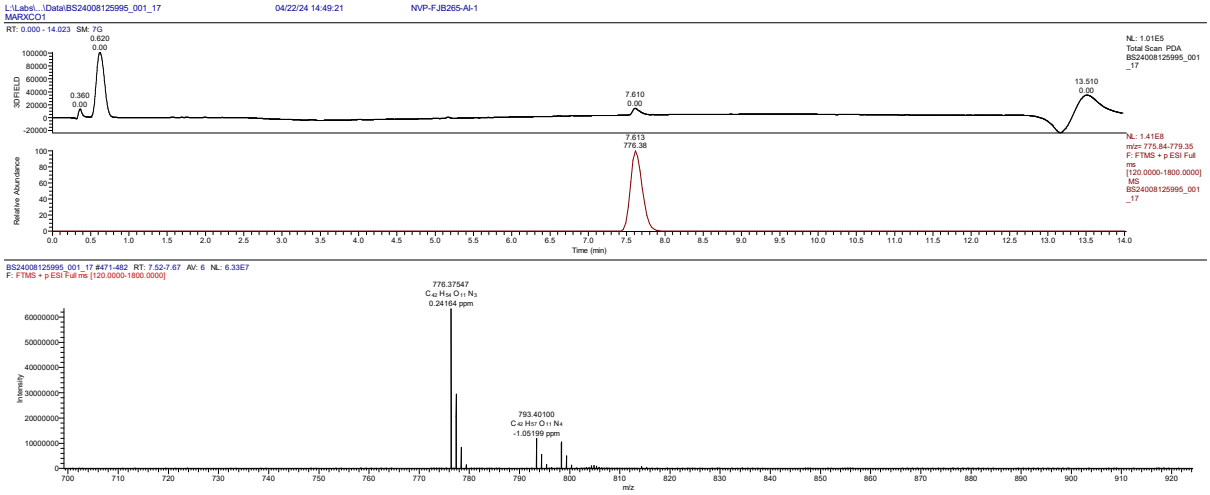


Figure S 47: R,--SLF-10

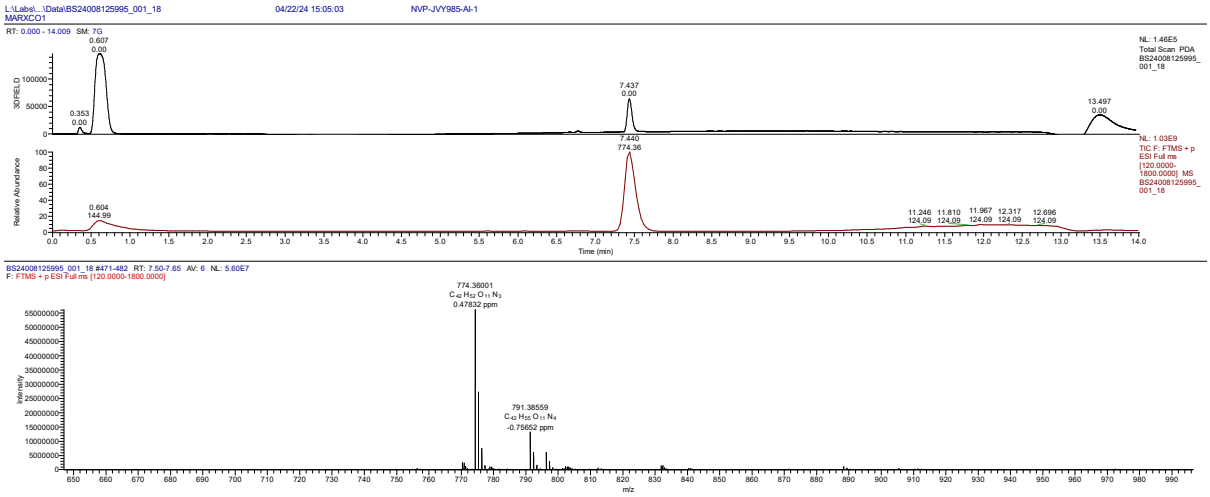


Figure S 48: R,--SLF-11

References

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