Electronic Supplementary Material (ESI) for Chemical Communications.

Supporting information

Increasing cytochrome P450 enzyme diversity by identification of two distinct cyclodipeptide dimerases

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Experimental Procedures

1. Computer-assisted sequence analysis

The gene and protein sequences used in this study were obtained from NCBI databases (http://www.ncbi.nlm.nih.gov). Protein sequences were compared with each other by using BLASTP program (http://blast.ncbi.nlm.nih.gov/). The phylogenetic tree of P450s showing in Fig. S2 was created by MEGA version 7.0 (http://www.megasoftware.net). Protein sequence alignments were performed with the program ClustalW and visualized with ESPript 3.0 (http://endscript.ibcp.fr/ESPript/cgi-bin/ESPript.cgi) to identify strictly conserved amino acid residues (Fig. S1 and S3).

2. Bacterial strains, plasmids, and growth conditions

Strains and plasmids used in this study are listed in Tables S2 and Table S3, respectively. Recombinant *E. coli* strains were cultivated in liquid or on solid Luria-Bertani (LB) medium with 100 μg/mL ampicillin, 50 μg/mL kanamycin, 50 μg/mL apramycin or 25 μg/mL chloramphenicol, when necessary.

Saccharopolyspora antimicrobica DSM 45119 was purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ). *Streptomyces albus* J1074[1] was kindly gifted by Prof. Luzhetskyy (Saarland University). *S. albus* J1074 and the generated exconjugants were maintained on MS plates (mannitol 20.0 g/L, soya flour 20.0 g/L, agar 20.0 g/L) at 28 °C for sporulation. For secondary metabolite production, *S. albus* J1074 transformants were cultivated in liquid modified R5 medium (sucrose 103.0 g/L, glucose 10.0 g/L, yeast extract 5.0 g/L, $MgCl_2 \cdot 6H_2O$ 10.12 g/L, K_2SO_4 0.25 g/L, Difco casaminoacids 0.1 g/L, MOPS 21.0 g/L, trace element solution 2 mL/L, pH 7.2) at 28 °C for 7 days.

3. Genetic manipulation, PCR amplification, and gene cloning

Genetic manipulation in *E. coli* was performed according to the protocol by Green and Sambrook.[2] Isolation of genomic DNA from actinomycetes was carried out as described in the literature.[3] The *cdps* and *p450* genes were amplified by PCR from genomic DNA of *S. antimicrobica* DSM 45119 by using primers listed in Table S3 and Phusion® High-Fidelity DNA Polymerase from New England Biolabs (NEB). The generated PCR fragments were cloned into pGEM-T Easy vector and the sequence integrity was confirmed by sequencing. Subsequently, the fragments were released with restriction endonucleases from pGEM-T Easy and ligated into pPWW50A[4] or pET28a (+) vector, which were digested with the same enzymes, previously. The

generated constructs (Table S3) were transformed into *S. albus* J1074 or *E. coli* BL21 (DE3) for gene expression.

4. Heterologous gene expression in *Streptomyces albus* J1074

The constructed plasmids harbouring different genes or gene clusters were firstly transformed into the non-methylating *E. coli* ET12567/pUZ8002, then conjugated with *S. albus* J1074. The positive conjugants were firstly selected by the phenotype showing apramycin resistance and further confirmed by PCR. The spores of the *S. albus* J1074 transformants were inoculated into 50 mL of modified R5 liquid media supplied with 50 μg/mL of apramycin in 250 mL baffled flasks and cultured at 28 °C and 200 rpm for 7 days. 1 mL of the cultures was extracted with the same volume of ethyl acetate for three times. The organic phases were combined, evaporated, and the dried residues were afterwards dissolved in 400 μL of methanol. 5 μL of such samples were subjected to LC-MS for analysis.

5. Overproduction and purification of P450s in *E. coli* and *Streptomyces*

For the purification of TtpB1, pJL80 was transformed into *E. coli* BL21 (DE3). The recombinant *E. coli* cells were cultivated for 16 h in 50 mL LB media supplied with 50 μg/mL kanamycin as preculture. 5 mL of the preculture were transferred into 500 mL LB media (with 50 μg/mL kanamycin) in 2 L-Erlenmeyer flasks and grew at 37 °C and 230 rpm to an absorption of 0.6 at 600 nm. The gene expression was induced with 0.1 mM IPTG at 16 °C for 20 h. The bacterial cultures were harvested by centrifugation (4,500 rpm, 20 min, 4 °C) and the cells were resuspended in lysis buffer (50 mM Tris-HCl, 10 mM imidazole, 300 mM NaCl, pH 8.0) with 2–5 mL/g wet weight. Lysozyme from the chicken egg white was added to the mixture at a final concentration of 1 mg/mL, which was incubated on ice for 30 min. The cells were then lysed by sonication on ice. Cell debris was removed by centrifugation at 13,000 rpm and 4 °C for 30 min. One-step purification of the recombinant His_6-tagged protein was performed by using Ni-NTA agarose (Macherey-Nagel, Düren, Germany) according to the manufacturer's instruction. The storage buffer was changed to 50 mM Tris-HCl (pH 7.5) containing 15 % (v/v) glycerol through a PD-10 column (GE Healthcare, Freiburg, Germany), which had been equilibrated with the same buffer. The obtained protein was stored frozen at -80 °C.

S. albus J1074 harbouring pJL84 (*ttpB2* in pPWW50A, Table S3) was cultivated in 50 mL tryptic soy broth (TSB) medium containing 50 μg/mL apramycin for 48 h as preculture. 5 mL of this pre-culture were transferred to 100 mL TSB with 50 μg/mL apramycin in 500 mL conical flasks. The cultures were further incubated at 28°C and 200 rpm for 3 days. Two litres of such cultures were harvested by centrifugation at 4 °C and 4,500 rpm for 20 min. The protein was purified as

described for *E. coli* cells.

The concentrations of TtpB1 and TtpB2 were determined on a Nanodrop C2000 (Thermo Scientific, Braunschweig, Germany) to be 0.65 mg/L and 0.05 mg/L culture, respectively. The purity of the recombinant P450s was proven by 12 % (w/v) SDS-PAGE (Fig. S29).

6. UV-Vis spectroscopic analysis of TtpB1

To measure the typical absorbance of P450 ferrous CO complex after reduction, carbon monoxide gas was bubbled into the TtpB1 solution (14 μM in 50 mM Tris-HCl containing 15% (v/v) glycerol, pH 7.5) for 2 min. After addition of 0.2 g/mL of sodium dithionite, a UV-Vis spectrum between 350 and 550 nm was taken on a Multiskan™ GO Microplate Spectrophotometer (Thermo Scientific, Dreieich, Germany). UV-Vis spectra of a protein sample without any treatment and another one only bubbled with CO were taken as controls. The spectra of TtpB1 is given in Fig. S30.

7. *In vitro* assays of P450s

cWW (**1,** 500 μM) was first assayed with 7 μM TtpB1, 5 mM NADPH, 2 μM spinach ferredoxin (Sigma-Aldrich), 0.1 unit/mL spinach ferredoxin-$NADP^+$ reductase (Sigma-Aldrich), 50 mM Tris-HCl buffer (pH 7.5) in a total volume of 50 μL at 30 °C for 30 min. Afterwards, 50 μM of **1** was incubated with 5 μM TtpB1 or 1.1 μM TtpB2 for 12 h. The reactions were quenched with 50 μL ice-cold MeOH. After centrifugation at 13,000 rpm for 5 min, 5 μL of the supernatants were subjected to LC-MS analysis. Incubations with heat-inactivated P450s, without ferredoxin, ferredoxin reductase, or NADPH were used as negative controls.

8. Determination of kinetic parameters for TtpB1

For determination of the kinetic parameters of TtpB1 towards cWW (**1**), the reaction mixtures (50 μL) contained 7 μM TtpB1, 5 mM NADPH, 2 μM spinach ferredoxin (Sigma-Aldrich), 0.1 unit/mL spinach ferredoxin-$NADP^+$ reductase (Sigma-Aldrich), 50 mM Tris-HCl buffer (pH 7.5) and **1** at final concentrations of 0.01, 0.02, 0.05, 0.1, 0.2, 0.3, and 0.4 mM. The reactions were carried out at 30 °C for 30 min and terminated by addition of 50 μL ice-cold MeOH. After removal of proteins by centrifugation, 50 μL of the supernatants were subjected to HPLC analysis.

The analysis was carried out on an Agilent HPLC 1200 series equipped with a photo diode array detector and an Eclipse XDB C18 column (5 μm, 4.6 x 150 mm). A linear gradient of 5 to 100% acetonitrile in water (0.1% formic acid) in 20 min was followed by 100% acetonitrile for 5 min and 5% acetonitrile in water for 5 min. The flow rate was set to 0.5 mL/min. Absorptions at 280 nm were illustrated in this study. The K_M and k_{cat} were proceeded with GraphPad Prism 8 (Fig. S32).

9. LC-MS analysis

LC-MS analysis was performed on an Agilent HPLC 1260 series system equipped with a photo diode array detector and a microTOF QIII mass spectrometer (Bruker, Bremen, Germany) by using a Multospher 120 RP-18 column (250 x 2 mm, 5 μm, CS-Chromatographie Service GmbH). For secondary metabolite analysis, a linear gradient of 5 – 100 % acetonitrile in water, both containing 0.1 % formic acid, in 40 min and a flow rate at 0.25 mL/min were used. The column was then washed with 100 % acetonitrile containing 0.1 % formic acid for 5 min and equilibrated with 5 % acetonitrile in water for 5 min. For enzyme assay analysis, a linear gradient of 5 – 100 % acetonitrile in water in 10 min was used, and the column was then washed and equilibrated as described as the former method. The parameters of the mass spectrometer were set as following: electrospray positive ion mode for ionization, capillary voltage with 4.5 kV, collision energy with 8.0 eV.

10. Isolation of generated metabolites from *S. albus* J1074 transformants

For structural elucidation of the accumulated compounds, the *S. albus* J1074 transformants harbouring *ttp(ABC)1* and *ttp(AB)2* were fermented in modified R5 medium on a large scale (4 L) at 28 °C for 7 days. The cultures were extracted with equal volume of ethyl acetate for three times. The organic phases were combined and evaporated to dryness. The extracts were applied to a silica gel column and eluted with a gradient CH_2Cl_2: MeOH in ratios of 100:2, 100:3, 100:5, 100:10. The target compounds **2** and **4** were mainly found in the fractions eluted with CH_2Cl_2: MeOH of 100:5 and **3** in 100:10, respectively. These fractions were further purified on an Agilent HPLC 1260 series equipped with a photo diode array detector by using a semi-preparative Agilent ZORBAX Eclipse XDB C18 HPLC column (9.4 x 250 mm, 5 μm) with 55 % ACN in water as solvent. The flow rate was set to 2.0 mL/min.

11. Precursor feeding experiments

Precursor feeding was carried out by using 20 mM cWW in DMSO. 150 µL of this solution were added to 30 mL of 2 day-old cultures of *Streptomyces* transformants in modified R5 media. After cultivation at 28°C for additional 7 days, the metabolites were extracted with EtOAc and analysed on LC-MS.

12. Determination of production yields of cWW dimers in *Streptomyces* transformants

An Agilent HPLC 1200 series equipped with a photo diode array detector and an Agilent Eclipse XDB C18 column (5 μm, 4.6 × 150 mm) were used for quantification. A linear gradient of 10 to 100 % acetonitrile in water in 40 min was followed by 100 % acetonitrile for 5 min and then 10 %

acetonitrile in water for 5 min. The flow rate was set to 0.5 mL/min. The absorption at 280 nm was used for quantification. To ensure complete extraction of cWW dimers from mycelia, precipitants and supernatants, 1 mL whole culture of *S. albus* J1074 transformants was extracted with 1 mL ethyl acetate for three times. The organic phases were combined and evaporated to dryness. The residues were dissolved in 200 μL of methanol and 100 μL were analysed on HPLC. The isolated products were used as authentic standards for quantification.

13. NMR analysis

The NMR spectra of the purified compounds **2** and **3** were recorded on a JOEL ECA-500 MHz spectrometer (JEOL, Tokyo, Japan) in DMSO-d_6. The NMR spectra of compound **4** in DMSO-d_6 were taken at 300 K on a Bruker AVIII spectrometer (500 MHz) equipped with a 5 mm cryo BBO probe Prodigy. To obtain a better NMR signal resolution, the ^{1}H NMR of **4** was also recorded in acetonitrile-d_3 at 273 K, 300 K, 310 K, and 320 K on a Bruker HD AVII spectrometer (500 MHz) equipped with a cryo BBO probe Prodigy. The ^{13}C and HSQC NMR spectra of **4** were then taken at the best found temperature 310 K on the same equipment.

All spectra were processed with MestReNova 5.2.2 (Metrelab Research, S5 Santiago de Compostella, Spain). The NMR data of the identified compounds are listed as physiochemical properties and the spectra are provided in Fig. S4 – S27.

14. The physiochemical properties of the identified compounds

Tetratryptomycin A (**2**): 30 mg, light yellow powder; slightly soluble in modified R5 media (approx. 20 mg/L); CD (MeOH) λ_{max} ($\Delta\varepsilon$) 306 (-18.4), 269 (-4.8), 249 (-29.4), 227 (+13) nm; HRMS (*m/z*): (ESI/[M+H]$^+$) calcd. for $C_{44}H_{38}N_8O_4$, 743.3089, found 743.3090. ^{1}H NMR (DMSO-d_6, 500 MHz) δ 10.68 (s, 2H, H-20 and H-20′), 7.66 (s, 2H, H-15 and H-15′), 7.55 (d, *J* = 7.8 Hz, 2H, H-25 and H-25′), 7.38 (d, *J* = 8.1 Hz, 2H, H-22 and H-22′), 7.19 (d, *J* = 7.4 Hz, 2H, H-4 and H-4′), 7.07 (s, 2H, H-19 and H-19′), 7.06 (t, *J* = 8.1 Hz, 2H, H-23 and H-23′), 7.04 (t, *J* = 7.8 Hz, 2H, H-6 and H-6′), 6.96 (t, *J* = 7.8 Hz, 2H, H-24 and H-24′), 6.67 (s, 2H, H-1 and H-1′), 6.66 (t, *J* = 7.4 Hz, 2H, H-5 and H-5′), 6.57 (d, *J* = 7.8 Hz, 2H, H-7 and H-7′), 5.10 (s, 2H, H-2 and H-2′), 4.34 (t, *J* = 5.0 Hz, 2H, H-14 and H-14′), 3.82 (dd, *J* = 12.1, 5.9 Hz, 2H, H-11 and H-11′), 3.25 (dd, *J* = 15.0, 5.7 Hz, 2H, H-17α and H-17′α), 3.04 (dd, *J* = 15.0, 5.7 Hz, 2H, H-17β and H-17′β), 2.35 (dd, *J* = 12.3, 5.9 Hz, 2H, H-10α and H-10′α), 2.25 (t, *J* = 11.9 Hz, 2H, H-10β and H-10′β). ^{13}C NMR (DMSO-d_6,125 MHz) δ 168.1 (C-16 and C-16′), 165.4 (C-13 and C-13′), 151.0 (C-8 and C-8′), 136.0 (C-21 and C-21′), 129.1 (C-6 and C-6′), 127.5 (C-26 and C-26′), 127.0 (C-9 and C-9′), 124.6 (C-4 and C-4′), 123.7 (C-19 and C-19′), 120.9 (C-23 and C-23′), 118.4 (C-25 and C-25′), 118.2 (C-24 and C-24′), 117.4 (C-5 and C-5′), 111.4 (C-22 and C-22′), 109.3 (C-18 and C-18′), 108.7 (C-7 and C-7′), 77.2

(C-2 and C-2′), 58.7 (C-3 and C-3′), 57.8 (C-11 and C-11′), 55.0 (C-14 and C-14′), 36.2 (C-10 and C-10′), 25.4 (C-17 and C-17′).

Tetratryptomycin B (**3**): 40 mg, light yellow powder; slightly soluble in modified R5 media (approx. 20 mg/L); CD (MeOH) λ_{max} ($\Delta\varepsilon$) 300 (+11.4), 268 (-5.34), 245 (+4.8), 240 (+7.0), 229 (+47.0), 214 (-66.5) nm; HRMS (*m/z*): (ESI/[M+H]$^+$) calcd. for $C_{44}H_{38}N_8O_4$, 743.3089, found 743.3119. ^{1}H NMR (DMSO-d_6, 500 MHz) δ_H 10.95 (s, 1H, H-20′), 10.86 (s, 1H, H-20), 8.10 (s, 1H, H-15′), 7.94 (s, 1H, H-15), 7.62 (d, *J* = 1.9 Hz, 1H, H-12′), 7.58 (d, *J* = 7.5 Hz, 1H, H-25), 7.54 (d, *J* = 7.7 Hz, 1H, H-25′), 7.38 (d, *J* = 8.0 Hz, 1H, H-22′), 7.34 (d, *J* = 8.0 Hz, 1H, H-22), 7.28 (s, 1H, H-19), 7.24 (d, *J* = 7.8 Hz, 1H, H-4′), 7.19 (d, *J* = 3.6 Hz, 1H, H-1), 7.15 - 7.06 (m, 4H, H-6, H-23, H-23′ and H-24′), 6.99 (t, *J* = 7.5 Hz, H-24), 6.95 (t, *J* = 7.8 Hz, 1H, H-5′), 6.88 (t, *J* = 7.8 Hz, 1H, H-6′), 6.88 (s, 1H, H-19′), 6.73 (d, *J* = 7.5 Hz, 1H, H-4), 6.70 (d, *J* = 7.8 Hz, 1H, H-7), 6.54 (d, *J* = 7.8 Hz, 1H, H-7′), 6.53 (t, *J* = 7.5 Hz, 1H, H-5), 6.47 (s, 1H, H-2′), 5.82 (d, *J* = 3.6 Hz, 1H, H-2), 4.67 (dd, *J* = 13.9, 5.9 Hz, 1H, H-11), 4.39 (t, *J* = 5.1 Hz, 1H, H-14), 4.04 (m, 1H, H-14′), 3.71 (m, 1H, H-11′), 3.48 (dd, *J* = 14.6, 5.9 Hz, 1H, H-10α), 3.39 (dd, *J* = 15.4, 5.1 Hz, 1H, H-17α), 3.08 – 2.98 (m, 2H, H-17β and H-17′β), 2.85 (dd, *J* = 14.1, 4.4 Hz, 1H, H-17′α), 2.64 (dd, *J* = 14.0, 3.3 Hz, 1H, H-10′α), 2.23 (t, *J* = 14.2, Hz, 1H, H-10β), 1.27 (dd, *J* = 14.0, 9.6 Hz, 1H, H-10′β). ^{13}C NMR (DMSO-d_6,125 MHz) δ 169.2 (C-16), 167.8 (C-13), 166.8 (C-16′), 166.6 (C-13′), 148.0 (C-8), 136.0 (C-21), 136.0 (C-21′), 135.0 (C-8′), 129.6 (C-6), 129.2 (C-9′), 128.6 (C-9), 127.7 (C-26′), 127.3 (C-26), 124.8 (C-19′), 124.7 (C-2′), 124.1 (C-19), 122.2 (C-4), 121.0 (C-6′), 120.9 (C-23′), 120.8 (C-23), 119.2 (C-5′), 119.0 (C-25′), 118.6 (C-4′), 118.5 (C-24′), 118.4 (C-25), 118.3 (C-24), 118.1 (C-5), 111.8 (C-7′), 111.5 (C-22′), 111.3 (C-22), 110.0 (C-7), 109.5 (C-18), 109.1 (C-3′), 108.6 (C-18′), 81.4 (C-2), 73.4 (C-3), 57.4 (C-11), 55.4 (C-14′), 55.2 (C-14), 54.5 (C-11′), 39.1 (C-10), 30.2 (C-10′), 29.8 (C-17′), 24.4 (C-17).

Tetratryptomycin C (**4**): 10 mg, light yellow powder; slightly soluble in modified R5 media (approx. 20 mg/L); CD (MeOH) λ_{max} ($\Delta\varepsilon$) 267 (-75.4), 216 (-2.7), 242 (+14.3), 224 (-1.4), 219 (+2.2) nm; HRMS (*m/z*): (ESI/[M+H]$^+$) calcd. for $C_{44}H_{38}N_8O_4$, 743.3089, found 743.3113. ^{1}H NMR (DMSO-d_6, 500 MHz) δ 10.81 (br s, 1H, H-20), 10.73 (br s, 1H, H-20′), 7.95 (br s, 1H, H-15′), 7.63 (d, *J* = 7.6 Hz 1H, H-25′), 7.60 (s, 1H, H-15), 7.58 (d, *J* = 7.8 Hz 1H, H-25), 7.33 (d, *J* = 7.9 Hz, 1H, H-22), 7.28 (d, *J* = 7.5 Hz, 1H, H-22′), 7.19 (s, 1H, H-19), 7.15 (s, 1H, H-19′), 7.08 (t, *J* = 7.9 Hz, 1H, H-23), 7.02 – 6.93 (m, 5H, H-6, H-6′, H-23′, H-24 and H-24′), 6.45 (m, 6H, H-4, H-4′, H-5, H-5′, H-7 and H-7′), 5.37 (br s, 1H, H-2), 5.13 (br s, 1H, H-2′), 4.35 (m, 1H, H-14′), 4.29 (m, 1H, H-14), 4.07 (m, 1H, H-11), 3.77 (m, 1H, H-11′), 3.36 (m, 1H, H-17α), 3.24 - 3.16 (m, 2H, H-17′α and H-17′β), 2.96 (dd, *J* = 14.4, 6.7 Hz, 1H, H-17β), 2.37 (m, 1H, H-10′α), 2.27 (dd, *J* = 13.0, 6.3 Hz, 1H,

H-10α), 1.76 (t, *J* = 11.8 Hz, 1H, H-10′β), 1.07 (m, 1H, H-10β). ^{13}C NMR (DMSO-d_6, 125 MHz) δ 169.6 (C-16), 168.6 (C-13), 167.0 (C-16′), 164.7 (C-13′), 151.2 (C-8), 148.9 (C-8′), 136.1 (C-21), 135.9 (C-21′), 130.0 (C-9), 128.9 (C-6), 128.5 (C-6′), 127.6 (C-26′), 127.5 (C-9′), 127.1 (C-26), 124.4 (C-19′), 124.2 (C-19), 123.8 (C-4), 123.7 (C-4′), 120.9 (C-23), 120.8 (C-23′), 118.9 (C-25′), 118.3 (C-25), 118.3 (C-24′), 118.3 (C-24), 117.5 (C-5), 117.4 (C-5′), 111.4 (C-22′), 111.4 (C-22), 109.5 (C-18), 108.9 (C-18′), 108.8 (C-7), 108.3 (C-7′), 78.1 (C-2′), 76.0 (C-2), 59.8 (C-3), 59.6 (C-3′), 57.3 (C-11′), 57.2 (C-11), 55.7 (C-14′), 55.4 (C-14), 38.6 (C-10′), 34.5 (C-10), 26.8 (C-17′), 24.3 (C-17). ^{1}H NMR (acetonitrile-d_3, 500 MHz, 310 K) δ 9.68 (br s, 1H, H-20), 9.19 (br s, 1H, H-20′), 7.65 (d, *J* = 8.0 Hz, 1H, H-25′), 7.63 (d, *J* = 8.0 Hz, 1H, H-25), 7.41 (d, *J* = 8.0 Hz, 1H, H-22′), 7.39 (d, *J* = 7.9 Hz, 1H, H-22), 7.18 – 7.14 (m, 3H, H-19, H-23′ and H-23), 7.11 – 7.04 (m, 5H, H-6, H-6′, H-19′, H-24′ and H-24), 6.58 – 6.51 (m, 6H, H-4, H-4′, H-5, H-5′, H-7 and H-7′), 6.45 (s, 1H, H-15′), 6.03 (s, 1H, H-15), 5.65 (br s, 1H, H-1), 5.47 (s, 1H, H-2), 5.23 (br s, 1H, H-1′), 5.12 (s, 1H, H-2′), 4.39 (t, *J* = 4.6 Hz,1H, H-14′), 4.36 (dd, *J* = 8.0, 4.9 Hz, 1H, H-14), 4.18 (t, *J* = 8.2 Hz, 1H, H-11), 3.78 (dd, *J* = 12.0, 5.4 Hz, 1H, H-11′), 3.47 (dd, *J* = 15.0, 4.5 Hz, 1H, H-17α), 3.36 (dd, *J* = 14.8, 5.0 Hz, 1H, H-17′α), 3.49 (dd, *J* = 15.0, 4.2 Hz, 1H, H-17′β), 3.11 (dd, *J* = 15.2, 7.6 Hz, 1H, H-17β), 2.42 – 2.38 (m, 2H, H-10′α and H-10α), 1.73 (t, *J* = 12.0 Hz, 1H, H-10′β), 1.31 (m, 1H, H-10β). ^{13}C NMR (acetonitrile-d_3, 125 MHz, 310 K) δ 169.3 (C-16), 168.8 (C-13), 166.6 (C-16′), 164.8 (C-13′), 151.1 (C-8), 148.8 (C-8′), 136.6 (C-21), 136.4 (C-21′), 130.3 (C-9), 129.2 (C-6), 128.8 (C-6′), 127.9 (C-9′), 127.8 (C-26′), 127.3 (C-26), 124.5 (C-4), 124.2 (C-4′), 124.0 (C-19), 123.9 (C-19′), 121.7 (C-23), 121.6 (C-23′), 119.1 (C-24), 119.1 (C-24′), 118.9 (C-25′), 118.7 (C-5), 118.5 (C-5′), 118.4 (C-25), 111.7 (C-22′), 111.5 (C-22), 109.3 (C-18), 109.3 (C-18′), 109.3 (C-7), 108.8 (C-7′), 78.9 (C-2′), 76.5 (C-2), 60.1 (C-3), 60.0 (C-3′), 57.4 (C-11′), 57.3 (C-11), 56.5 (C-14′), 55.4 (C-14), 39.0 (C-10′), 35.0 (C-10), 27.9 (C-17′), 25.2 (C-17).

Supplementary Tables

Table S1. Comparison of CDPSs and P450s from the two clusters in *Saccharopolyspora antimicrobica* DSM 45119

ttp cluster 1			*ttp* cluster 2			Sequence identity (%)
Protein	Accession No.	Length (aa)	Protein	Accession No.	Length (aa)	
TtpA1	WP_093145978.1	257	TtpA2	WP_093145813.1	254	80
TtpB1	WP_121505431.1	400	TtpB2	WP_170210414.1	400	71
TtpC1	WP_143121525.1	412				31 to TtpB1 39 to TtpB2

Table S2. Bacterial strains used in this study

Strain	Source	Cultivation media
E. coli DH5α	Invitrogen	LB
E. coli BL21(DE3)	Novagen	LB
E. coli ET12567/pUZ8002	[5]	LB
Streptomyces albus J1074	[1]	MS
Saccharopolyspora antimicrobica DSM 45119	DSMZ	modified R5

DSMZ: Deutsche Sammlung von Mikroorganismen und Zellkulturen

LB medium: tryptone 10.0 g/L, yeast extract 5.0 g/L, NaCl 10.0 g/L.

Modified R5 medium: sucrose 103.0 g/L, glucose 10.0 g/L, yeast extract 5.0 g/L, $MgCl_2.6H_2O$ 10.12 g/L, K_2SO_4 0.25 g/L, Difco casaminoacids 0.1 g/L, MOPS 21.0 g/L, trace element solution 2 mL/L, pH 7.2.

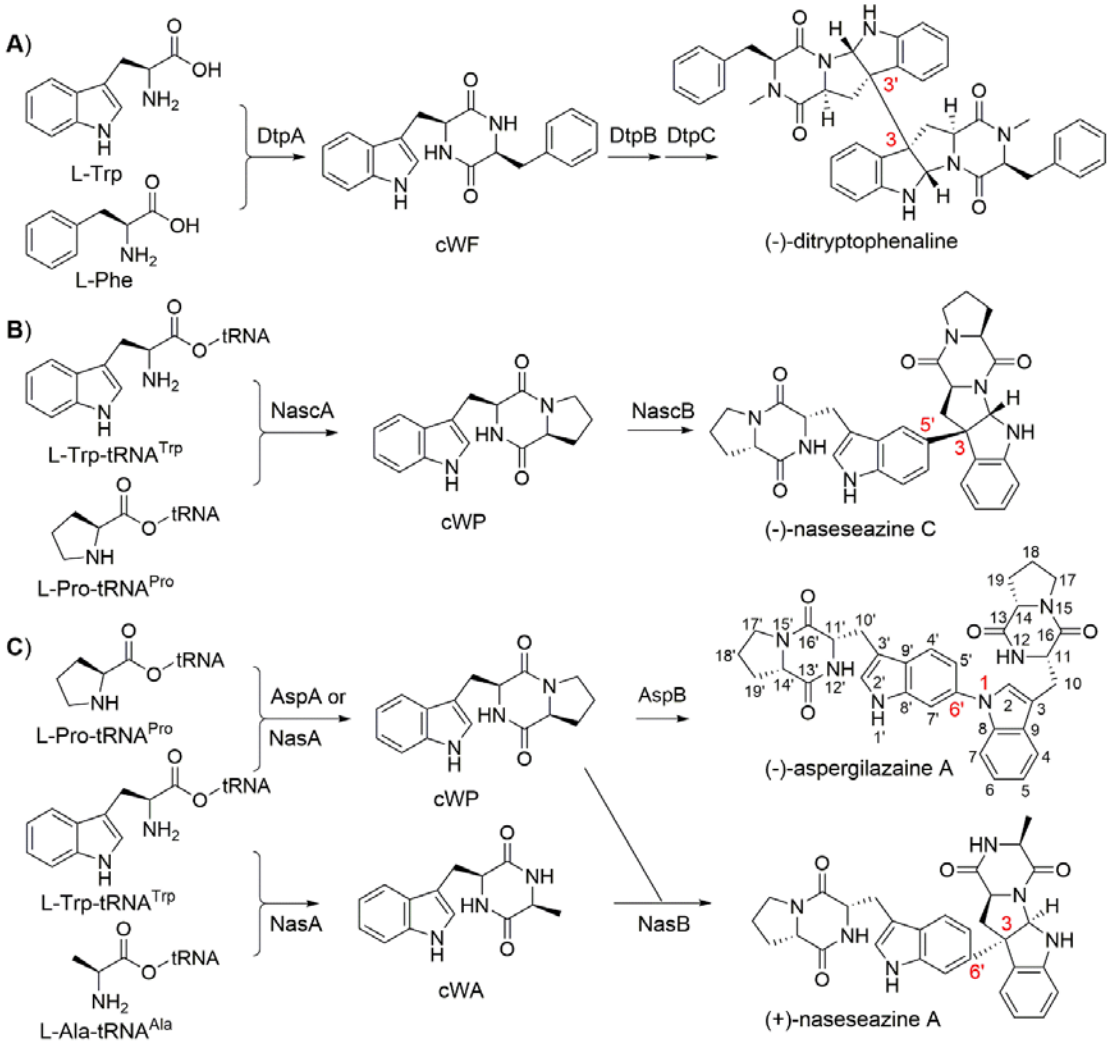
MS medium: mannitol 20.0 g/L, soya flour 20.0 g/L, agar 20.0 g/L.

Table S3. Cloning and expression constructs used in this study

Gene	Primer sequences (5'-3')	Cloning constructs	Expression vector	Cloning sites	Expression constructs
ttpA1	CATATGCCACCCACGCCTACCACTG GGATCCTCACGCGGTGCTGGCTCGTC	pJL66	pPWW50A	*Nde*I/*Bam*HI	pJL75
ttp(ABC)1	CATATGCCACCCACGCCTACCACTG GGATCC TCACGCGGTGCTGGCTCGTC	pJL67	pPWW50A	*Nde*I/*Bam*HI	pJL76
ttp(AB)1	CATATGCCACCCACGCCTACCACTG GGATCC CTACCAGCTCACGGGAAGGGC	pJL68	pPWW50A	*Nde*I/ *Bam*HI	pJL77
ttp(AC)1	CATATGCCACCCACGCCTACCACTG GGATCCTCACGCGGTGCTGGCTCGTC	pJL66	pPWW50A	*Nde*I/*Bam*HI	pJL78
	AGATCTGTGCCACCAGACAACGAGGCG ACTAGTTCACCAGCTGACGGGGAGCTG	pJL67		*Bgl*II/*Spe*I	
ttpB1	CATATGTTCGCCATCGACGACATCCCG GGATCC CTACCAGCTCACGGGAAGGGC	pJL70	pPWW50A	*Nde*I/*Bam*HI	pJL79
ttpB1	CATATGTTCGCCATCGACGACATCCCG GGATCC CTACCAGCTCACGGGAAGGGC	pJL70	pET28a (+)	*Nde*I/*Bam*HI	pJL80
ttpC1	CATATGCCACCAGACAACGAGGCG GGATCCTCACGTCAAGTCCCTTTCTCC	pJL71	pPWW50A	*Nde*I/*Bam*HI	pJL81
ttpA2	CATATGCATTCCACGTGTATCGACCGAG GGATCCTCACTGGACAGCATCGTTCCCCC	pJL72	pPWW50A	*Nde*I/*Bam*HI	pJL82
ttp(AB)2	CATATGCATTCCACGTGTATCGACCGAG GGATCC CTACCAGGTGACGGGCAGGG	pJL73	pPWW50A	*Nde*I/*Bam*HI	pJL83
ttpB2	CATATGCTGTCCAGTGATCAGATCCCGG GGATCC CTACCAGGTGACGGGCAGGG	pJL74	pPWW50A	*Nde*I/*Bam*HI	pJL84

Restriction sites for cloning are underlined in the primer sequences. Cloning constructs are based on pGEM T Easy vector

Supplementary Scheme



Scheme S1 Known biosynthetic pathways of CDP dimers in *Aspergillus flavus* (A) and *Streptomyces* species (B and C)

Supplementary Figures

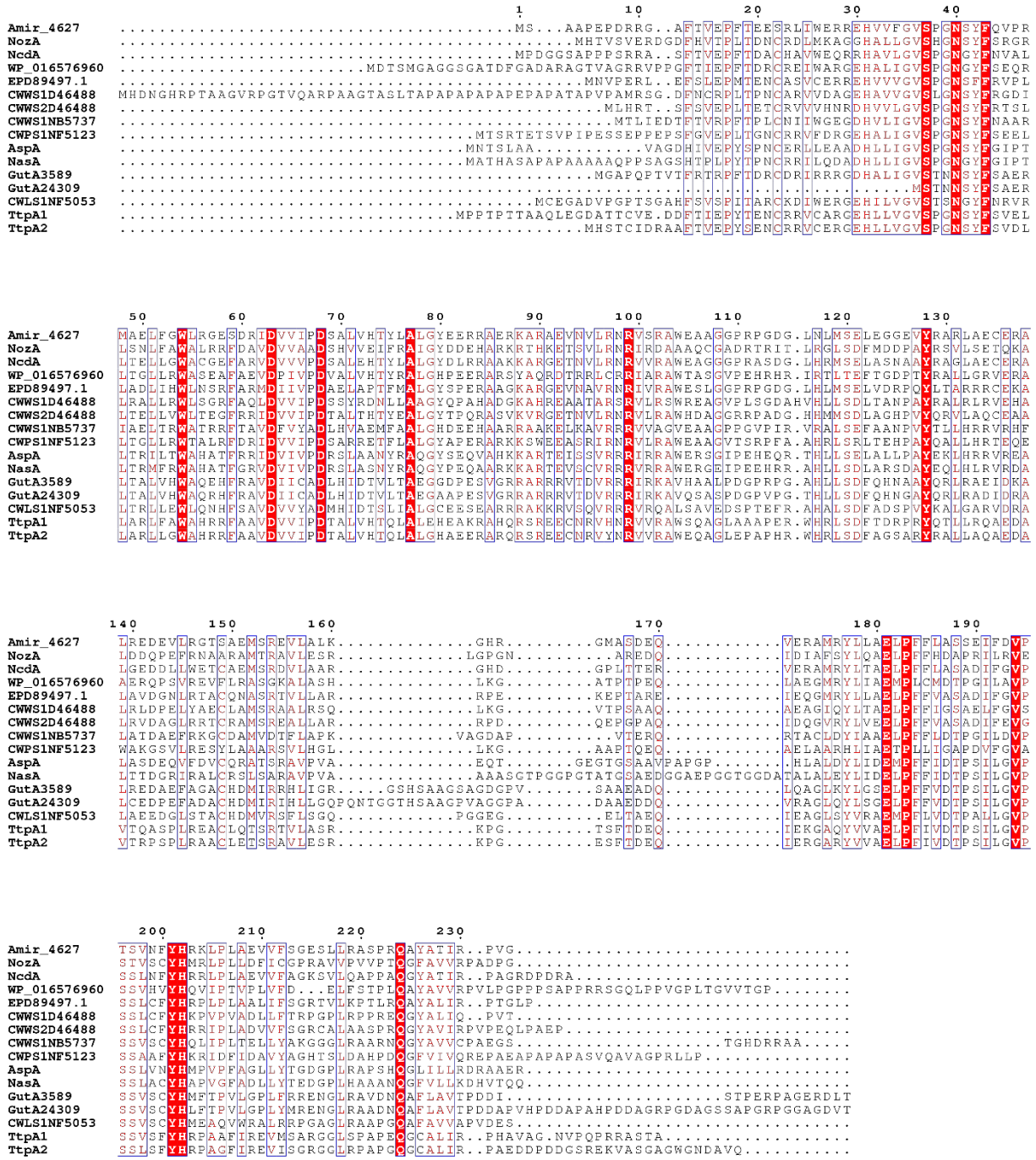


Fig. S1 Alignments of CDPSs from bacteria. Amir_4627,[6] NozA,[7] NcdA,[7] WP_016576960,[8] EPD89497.1,[8,9] $CWWS1_{D46488}$,[10] $CWWS2_{D46488}$,[10] $CWWS1_{NB5737}$,[10] $CWPS1_{NF5123}$,[10] AspA,[11] NasA,[11] $GutA_{3589}$,[12] $GutA_{24309}$[12] and $CWLS1_{NF5053}$[10] have been characterised as tryptophan-containing CDP synthases.

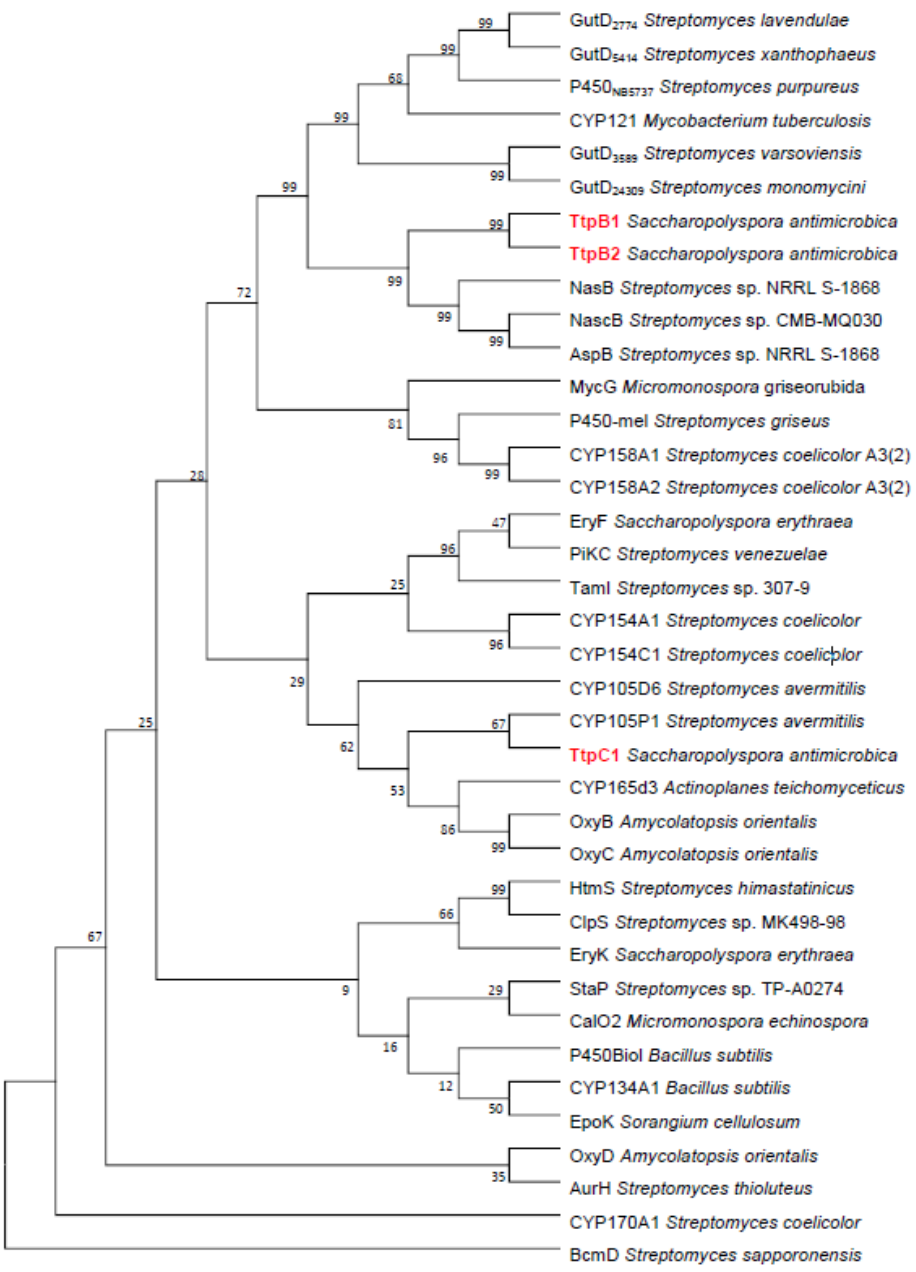


Fig. S2 Phylogenetic analysis of P450s investigated in this study (in bold red) and functionally characterised P450s from bacteria. $GutD_{2774}$,[13] $GutD_{5414}$,[13] $GutD_{24309}$,[12] $GutD_{3589}$,[12] $P450_{5737}$,[13] CYP121,[14] NasB,[11] NascB,[15] AspB,[11] CYP134A1[16] and BcmD[17] are members of the CDPS-related pathways. HtmS[18] and ClpS[19] are involved in the biosynthesis of himastatin and chloptosin, respectively. Other enzymes are structurally characterised natural product P450s mentioned in the review by Podust *et al*.[20] The protein sequences were downloaded from NCBI database.

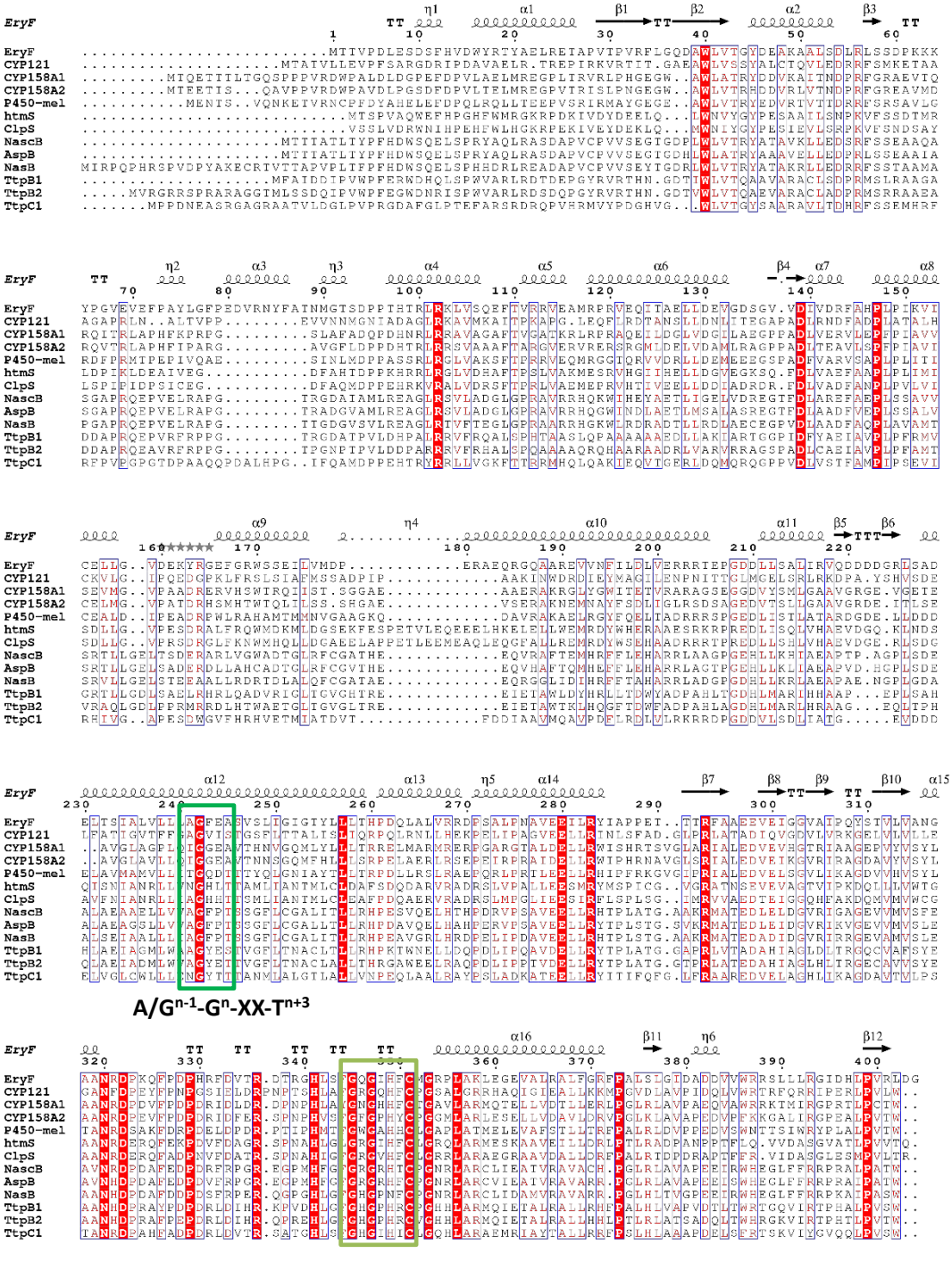


Fig. S3 Alignments of CDP dimerization P450s from bacteria. The origins of the enzymes were mentioned in the legend to Fig. S2.

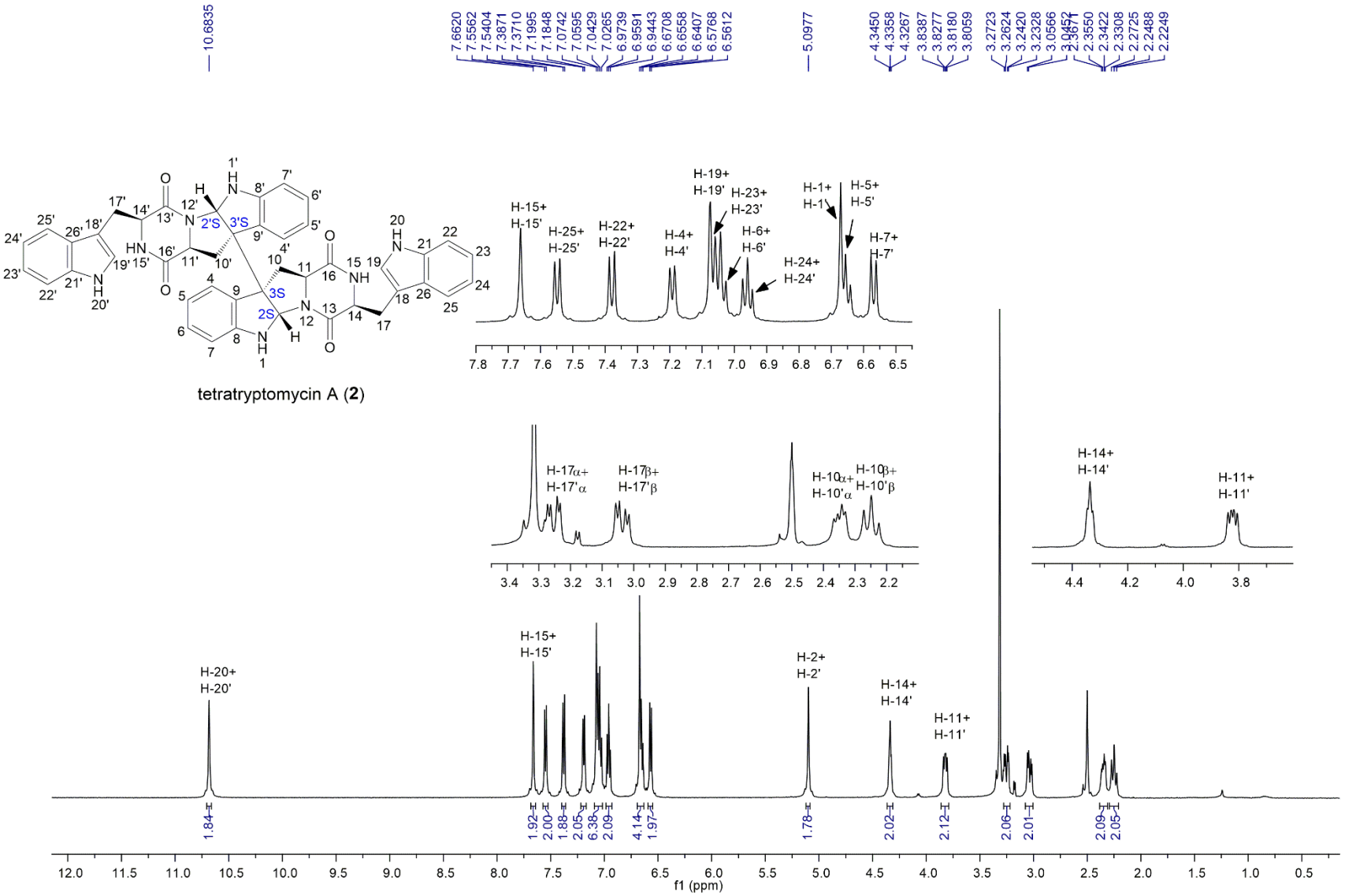


Fig. S4 ^{1}H NMR spectrum of tetratryptomycin A (**2**) in DMSO-d_6 at 300 K (500 MHz).

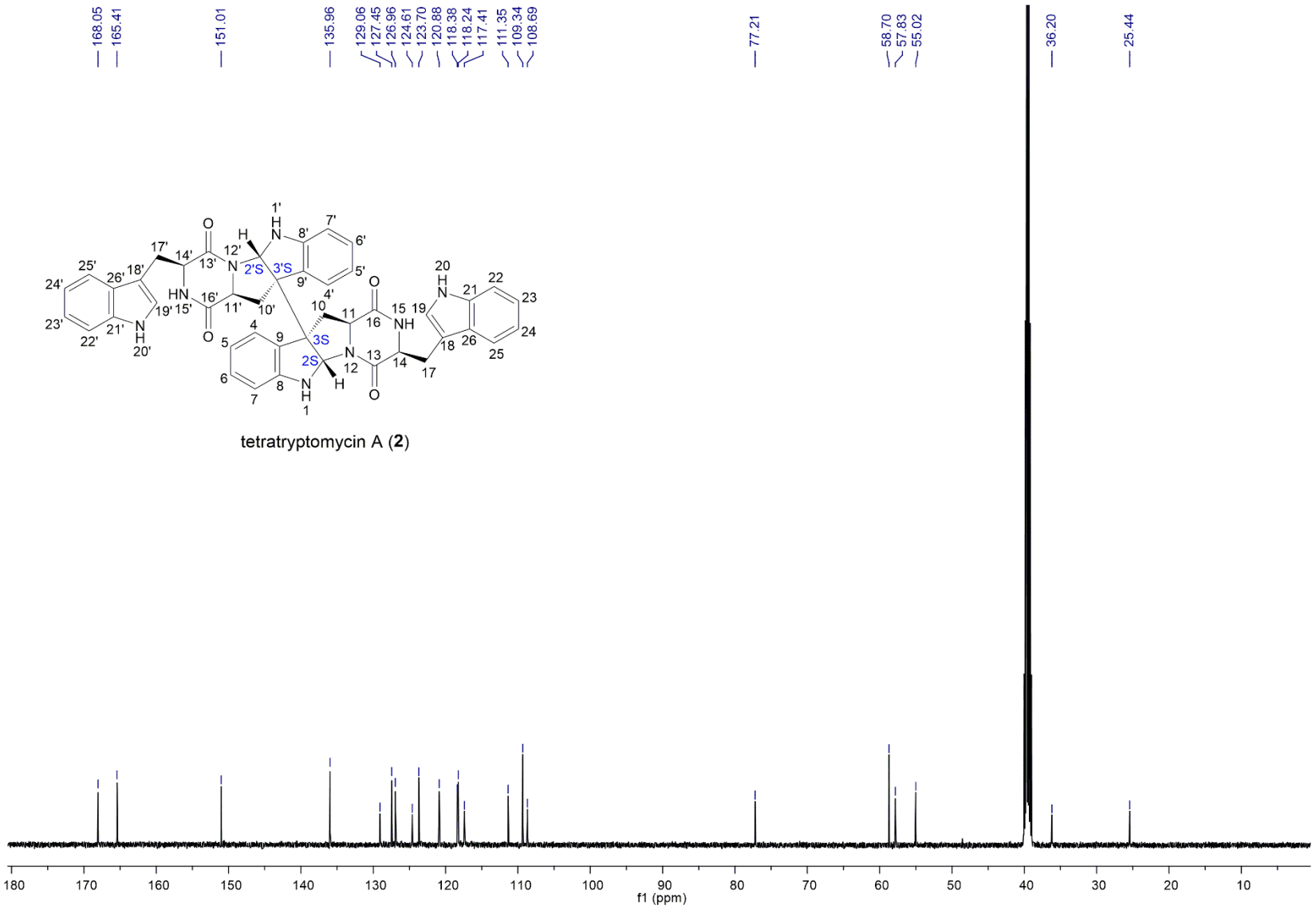


Fig. S5 ^{13}C NMR spectrum of tetratryptomycin A (**2**) in DMSO-d_6 at 300 K (125 MHz).

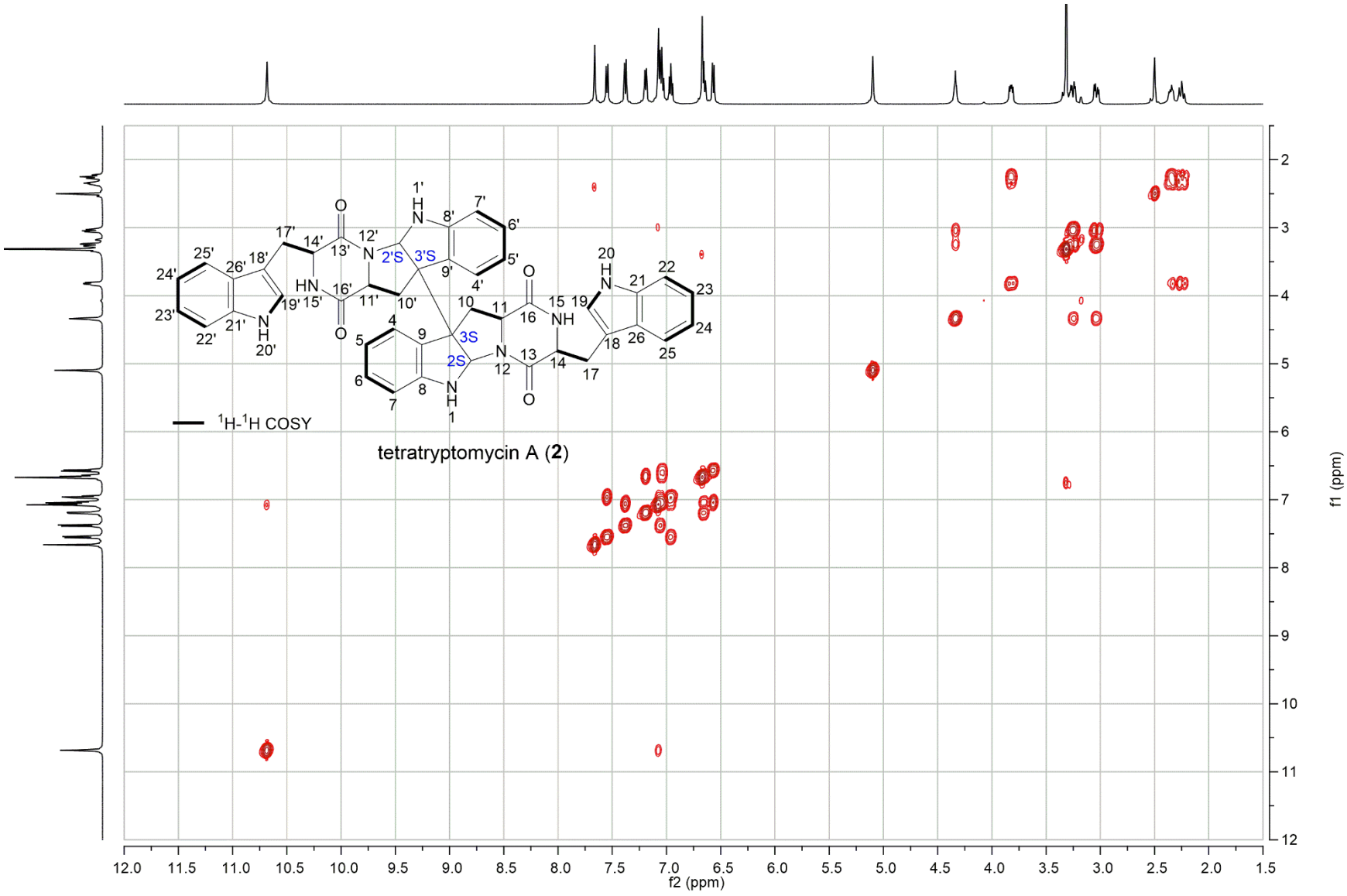


Fig. S6 ^{1}H-^{1}H COSY spectrum of tetratryptomycin A (**2**) in DMSO-d_6 at 300 K (500 MHz).

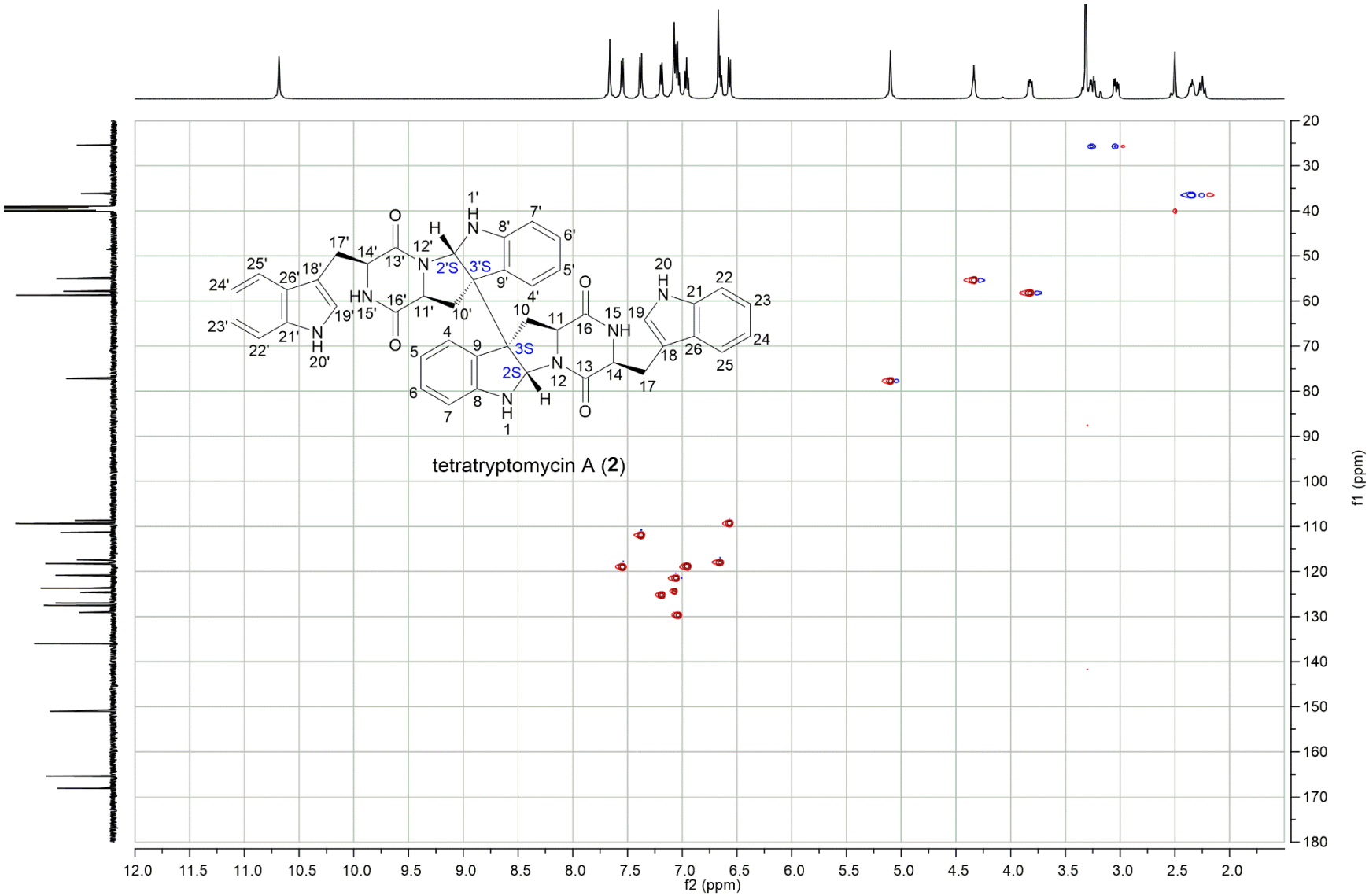


Fig. S7 HSQC spectrum of tetratryptomycin A (**2**) in DMSO-d_6 at 300 K (500 MHz, 125 MHz).

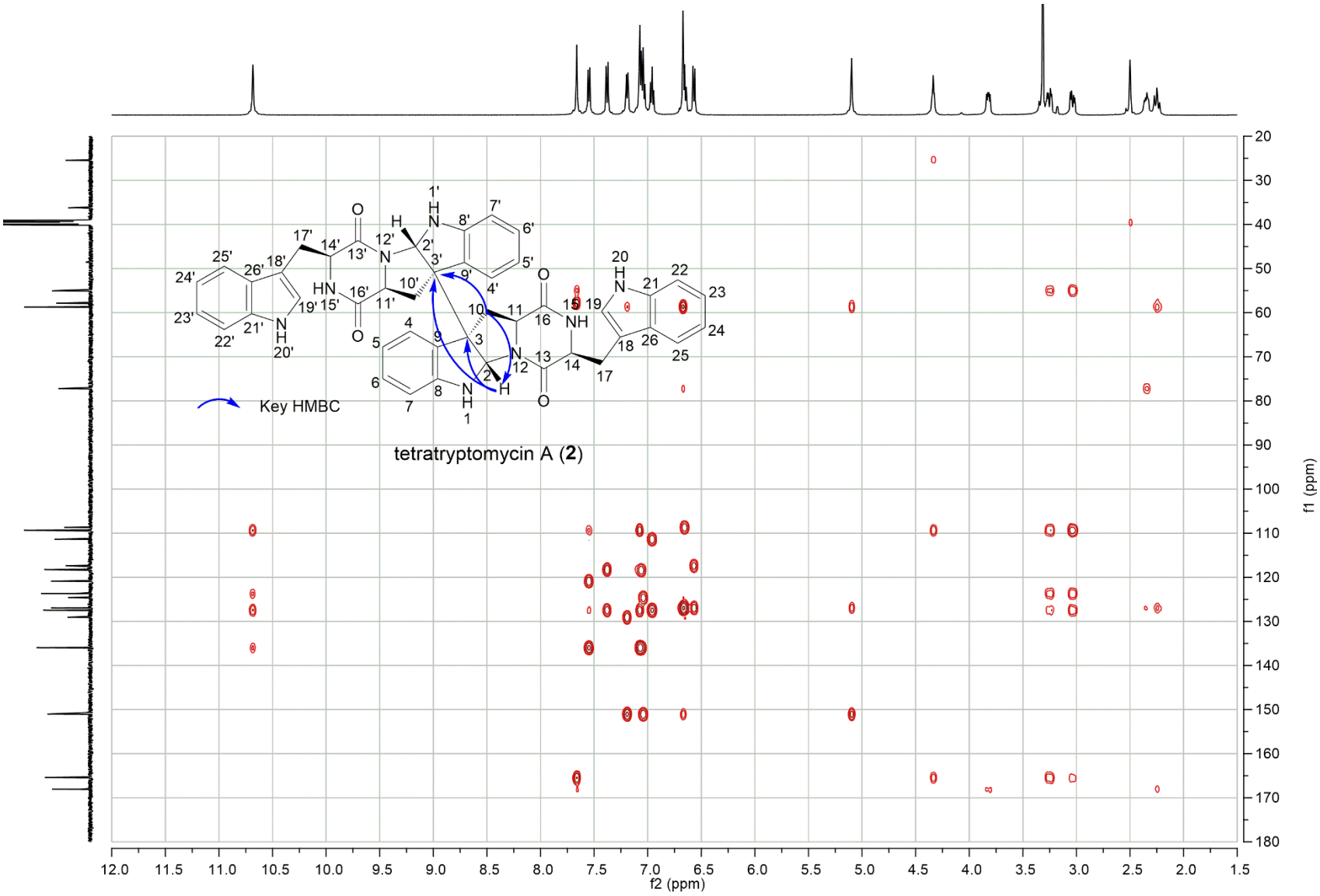


Fig. S8 HMBC spectrum of tetratryptomycin A (**2**) in DMSO-d_6 at 300 K (500 MHz, 125 MHz).

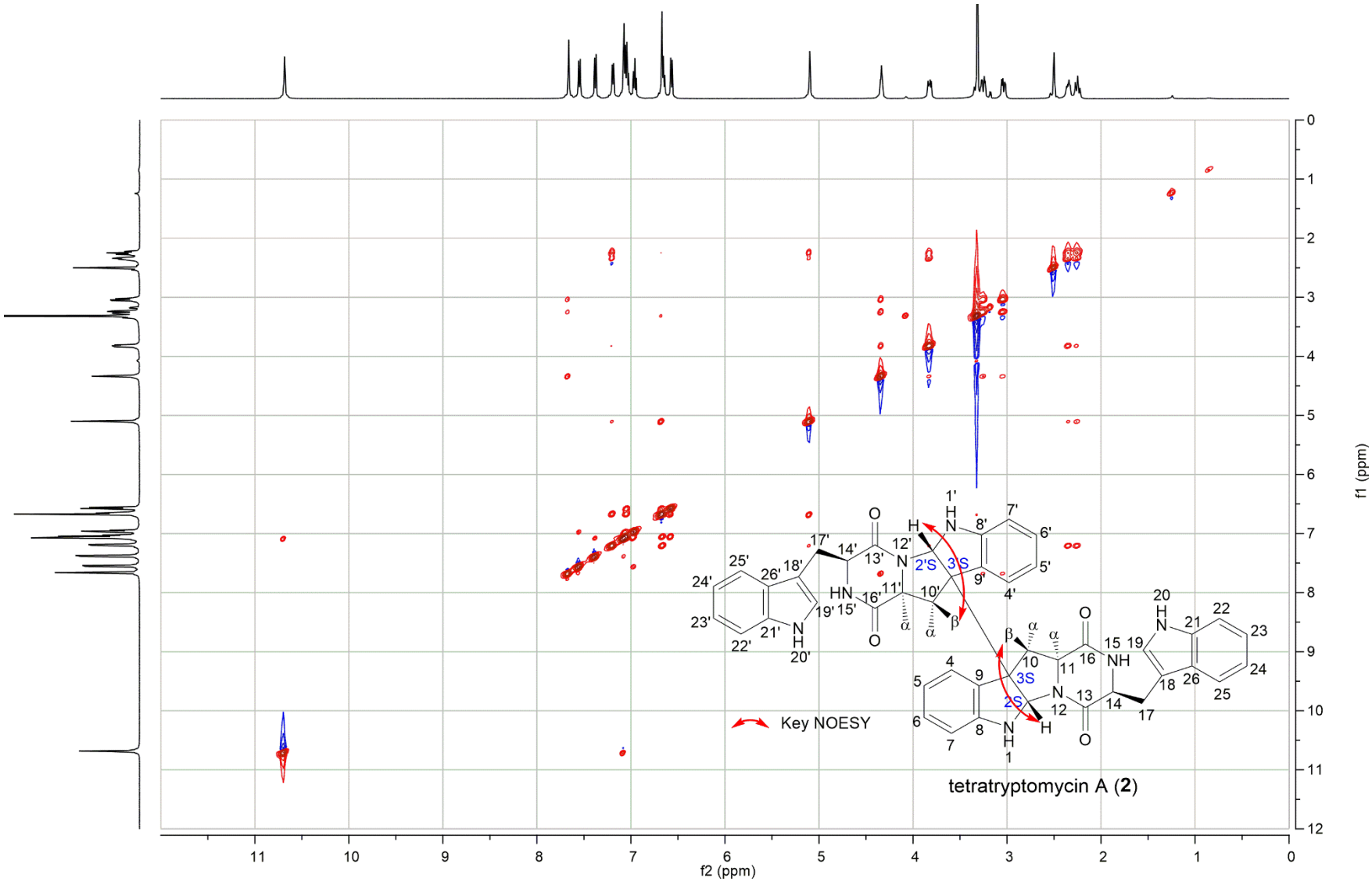


Fig. S9 NOESY spectrum of tetratryptomycin A (**2**) in DMSO-d_6 at 300 K (500 MHz).

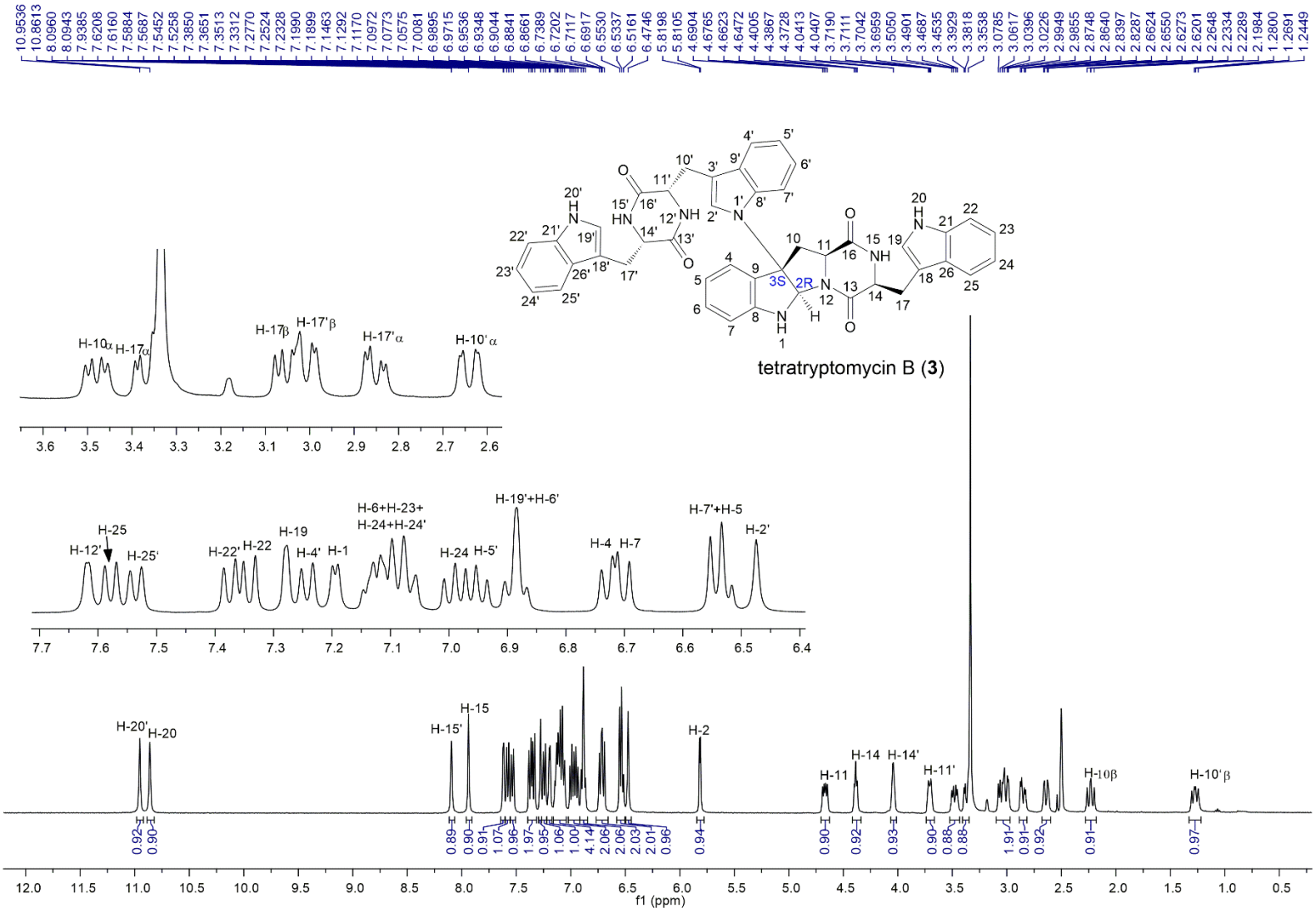


Fig. S10 ^{1}H NMR spectrum of tetratryptomycin B (**3**) in DMSO-d_6 at 300 K (500 MHz).

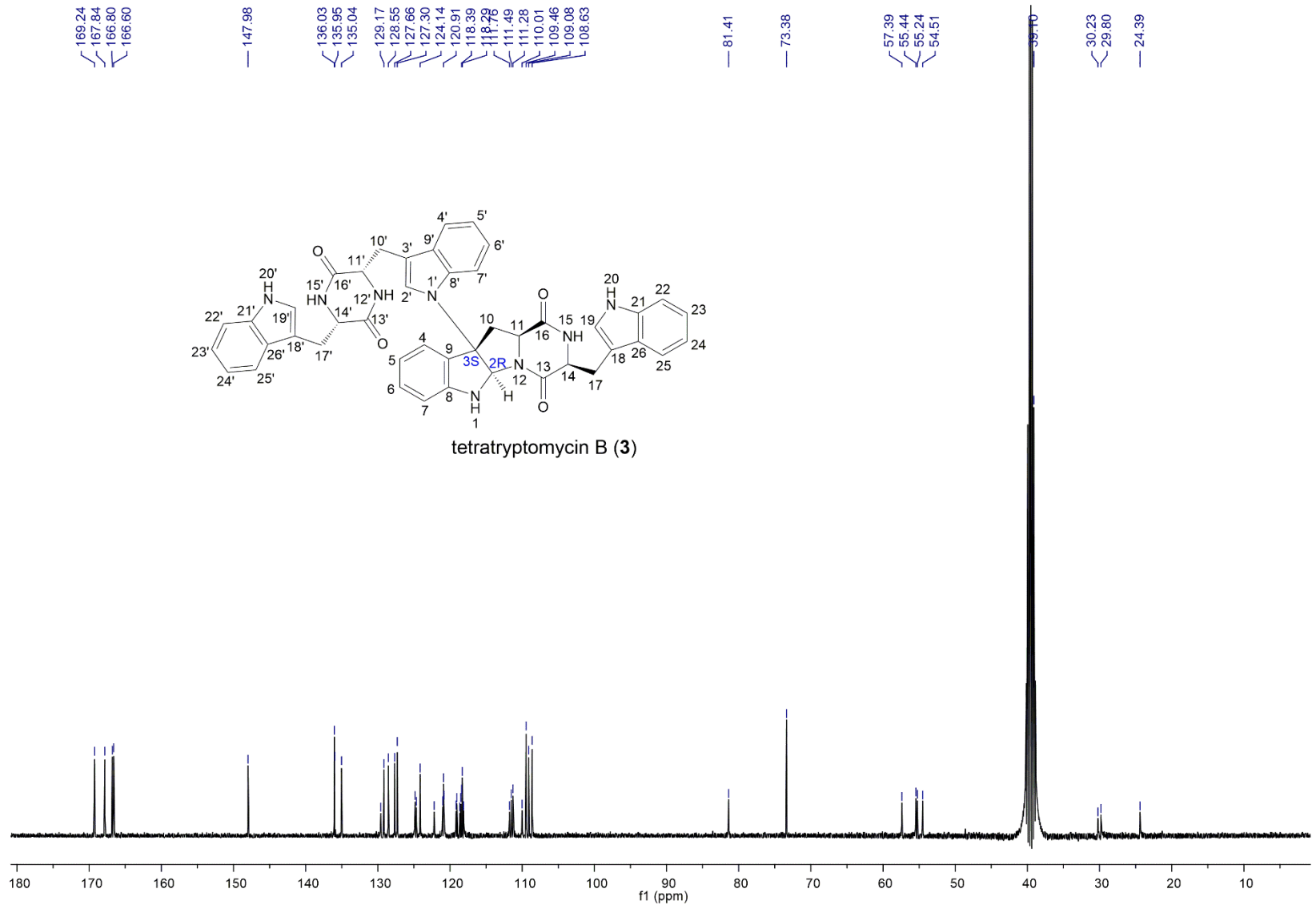


Fig. S11 ^{13}C NMR spectrum of tetratryptomycin B (**3**) in DMSO-d_6 at 300 K (125 MHz).

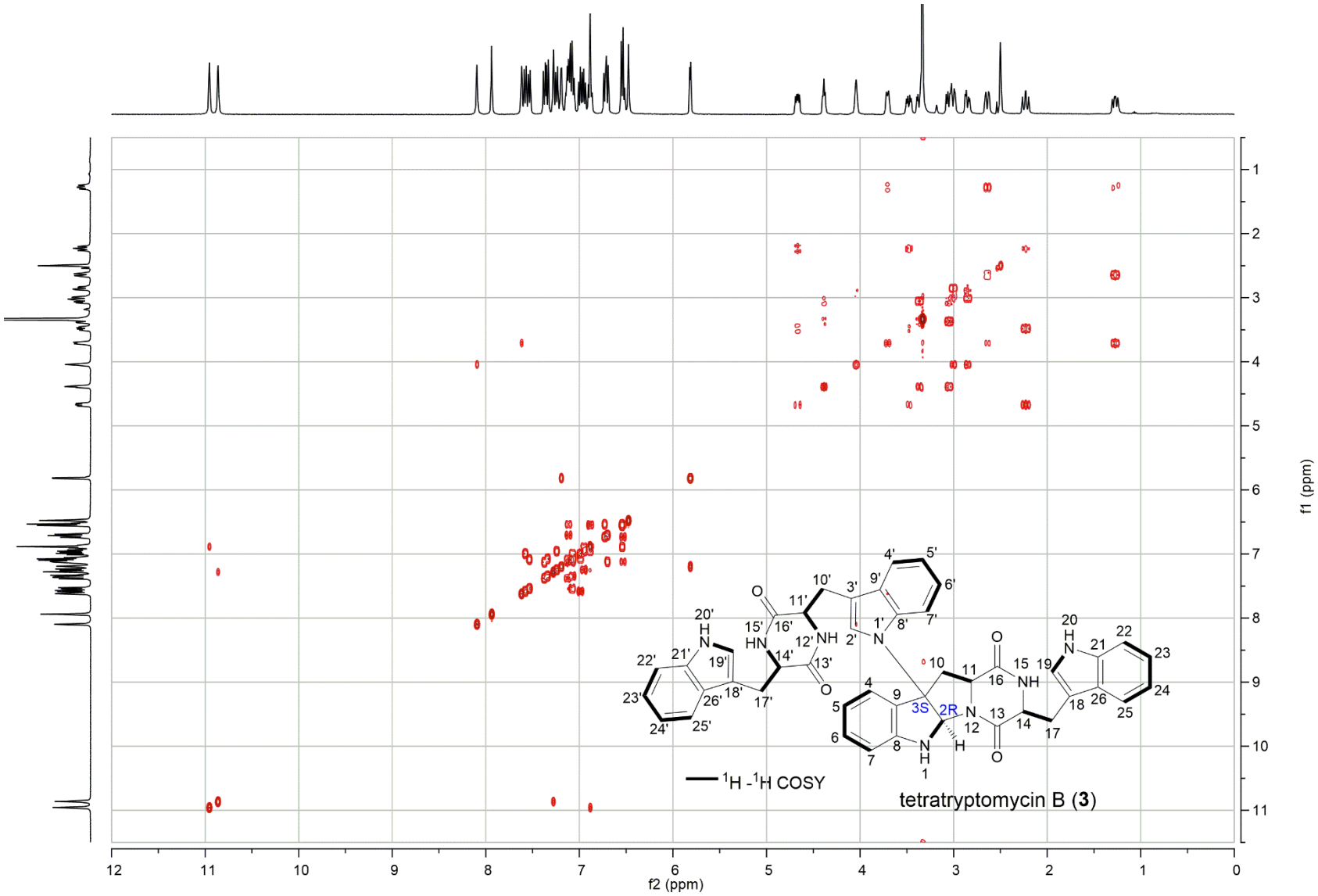


Fig. S12 ^{1}H-^{1}H COSY spectrum of tetratryptomycin B (**3**) in DMSO-d_6 at 300 K (500 MHz).

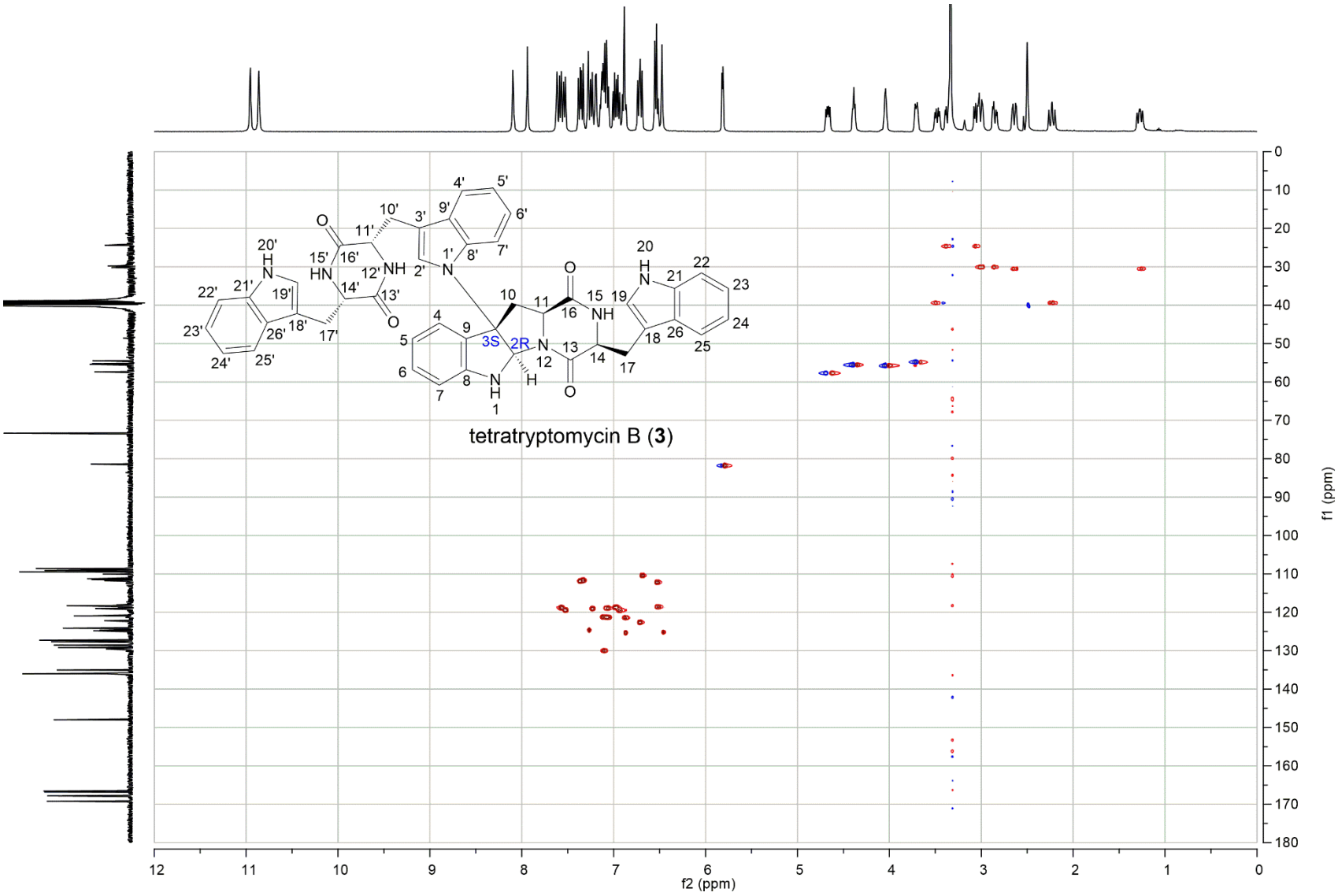


Fig. S13 HSQC spectrum of tetratryptomycin B (**3**) in DMSO-d_6 at 300 K (500 MHz, 125 MHz).

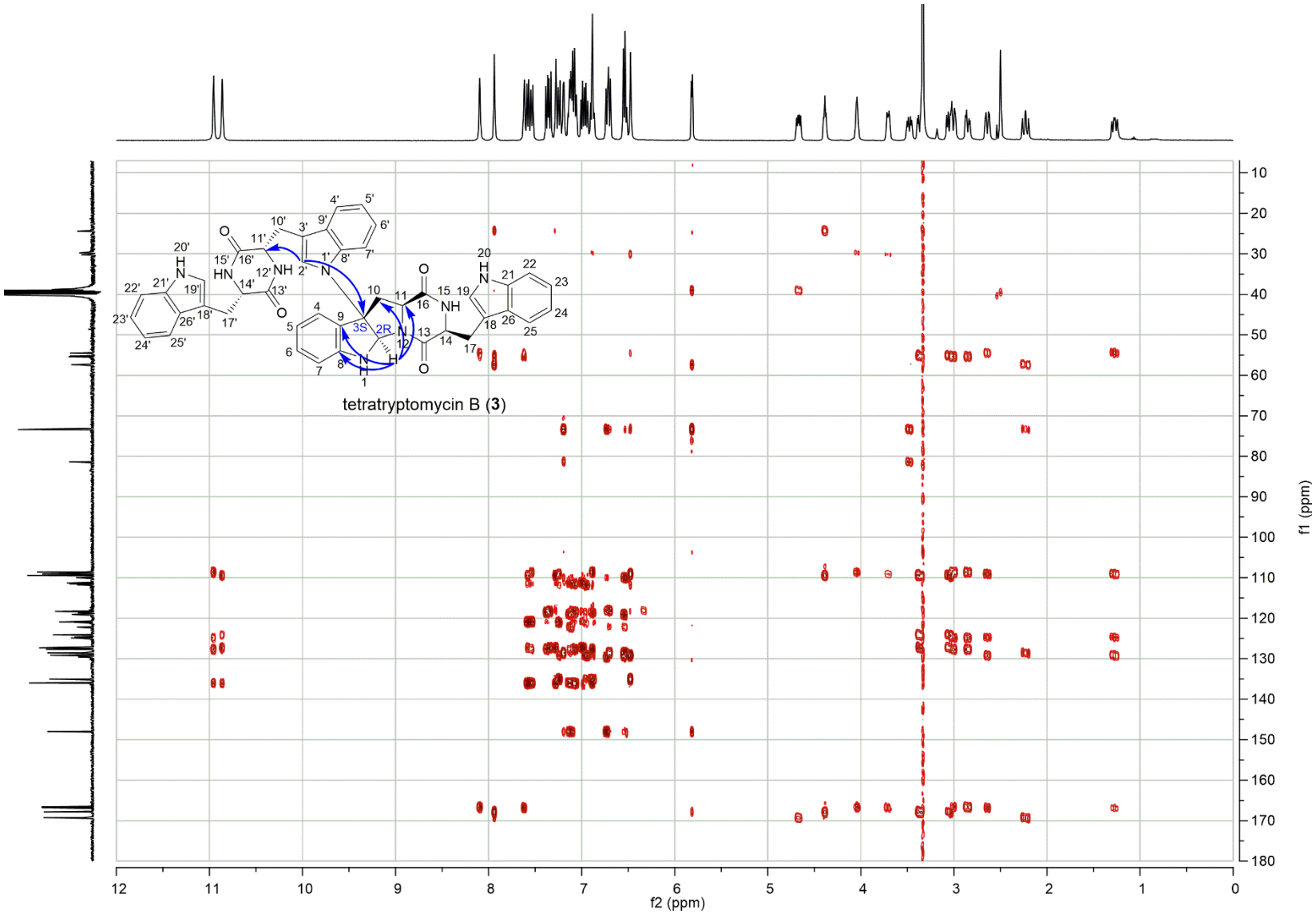


Fig. S14 HMBC spectrum of tetratryptomycin B (**3**) in DMSO-d_6 at 300 K (500 MHz, 125 MHz).

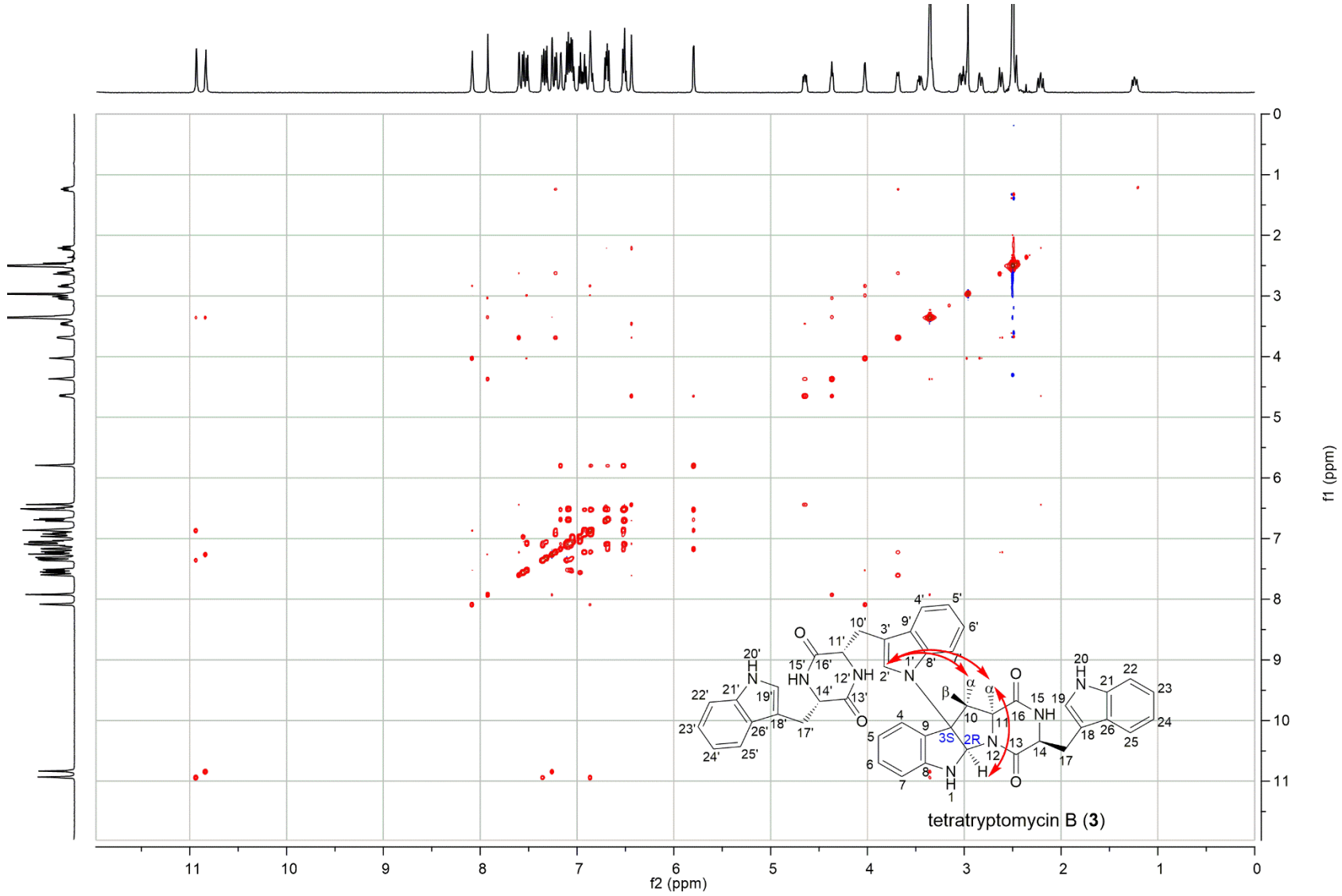


Fig. S15 NOESY spectrum of tetratryptomycin B (**3**) in DMSO-d_6 at 300 K (500 MHz).

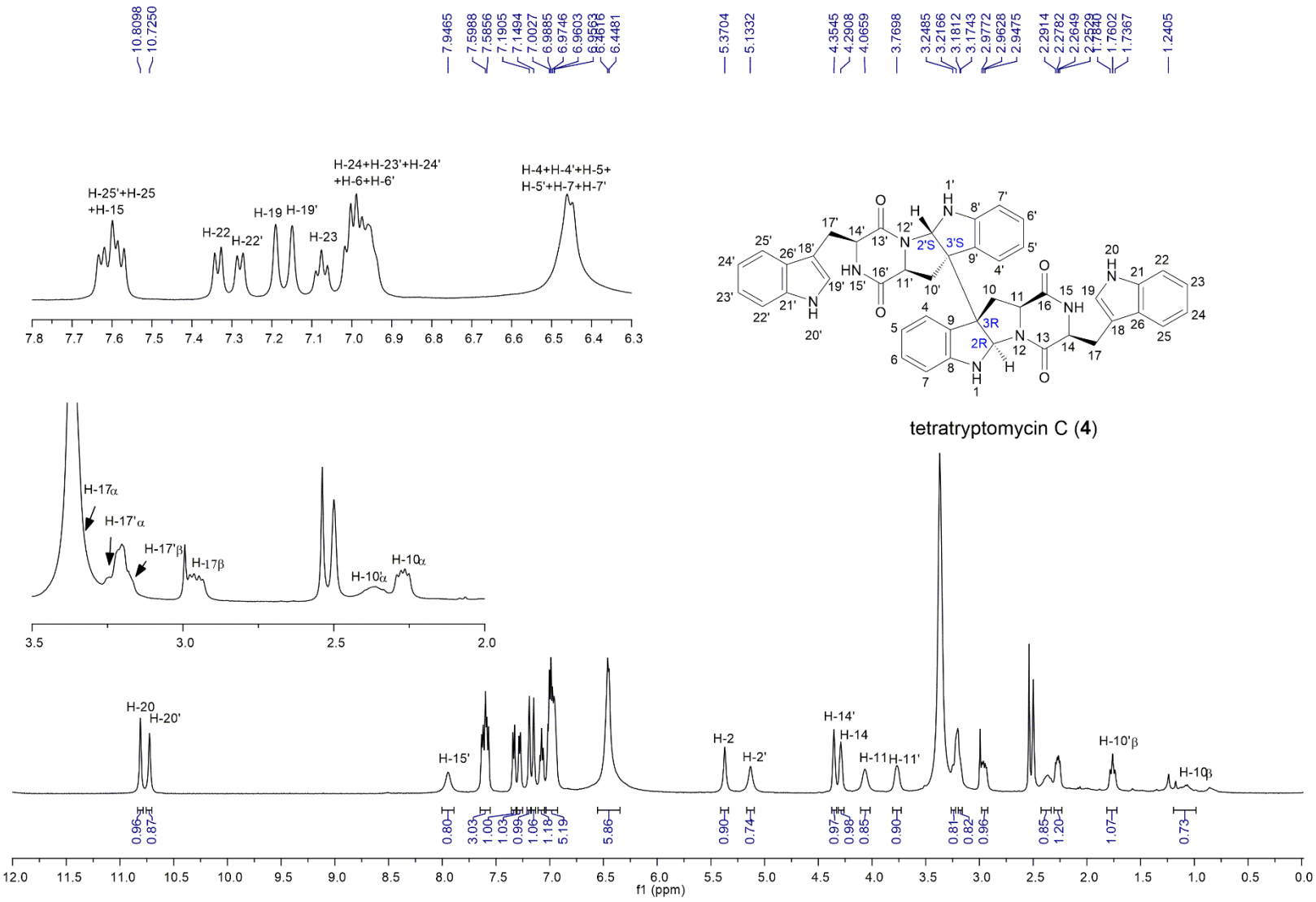


Fig. S16 ^{1}H NMR spectrum of tetratryptomycin C (**4**) in DMSO-d_6 at 300 K (500 MHz).

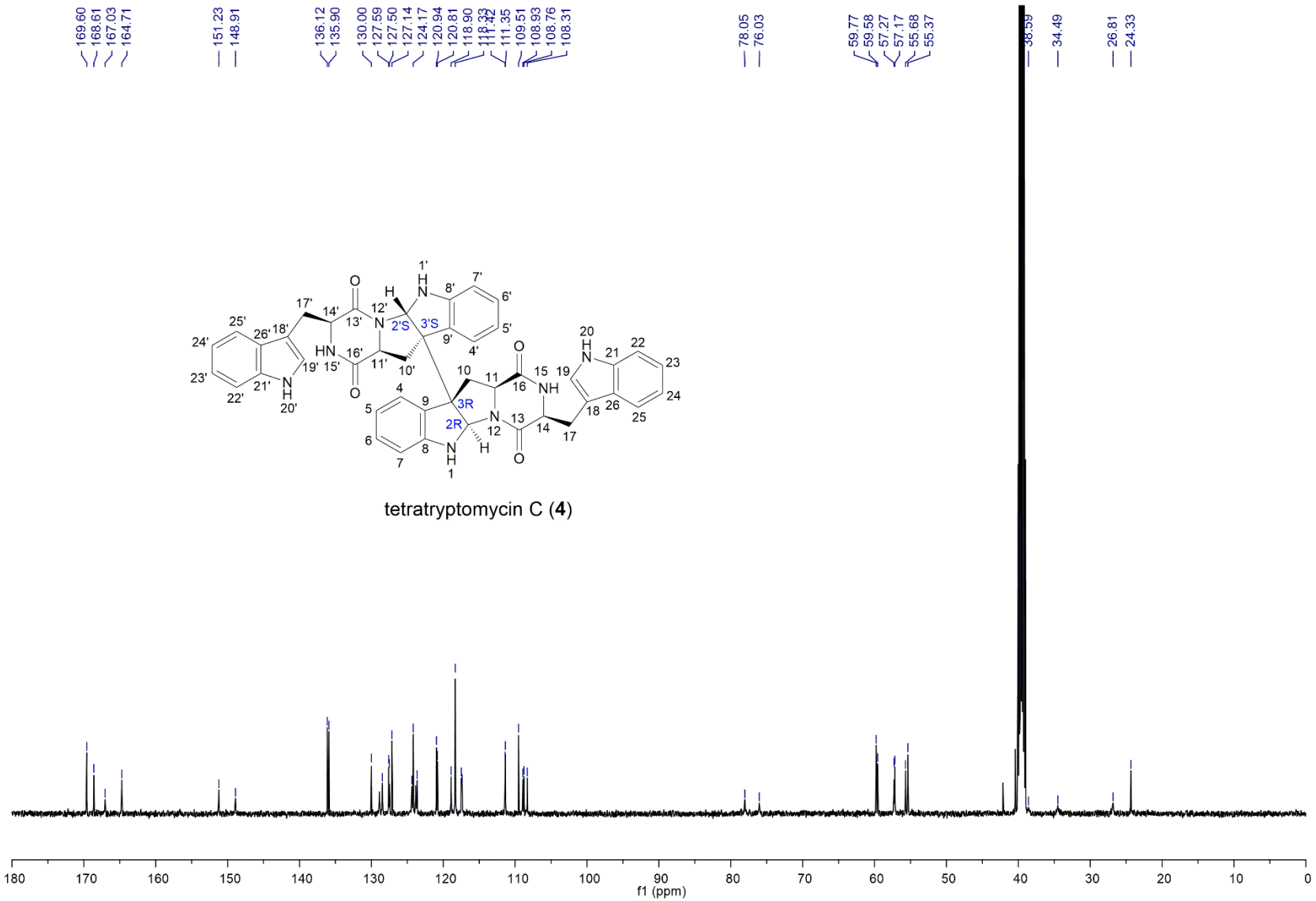


Fig. S17 ^{13}C NMR spectrum of tetratryptomycin C (**4**) in DMSO-d_6 at 300 K (125 MHz).

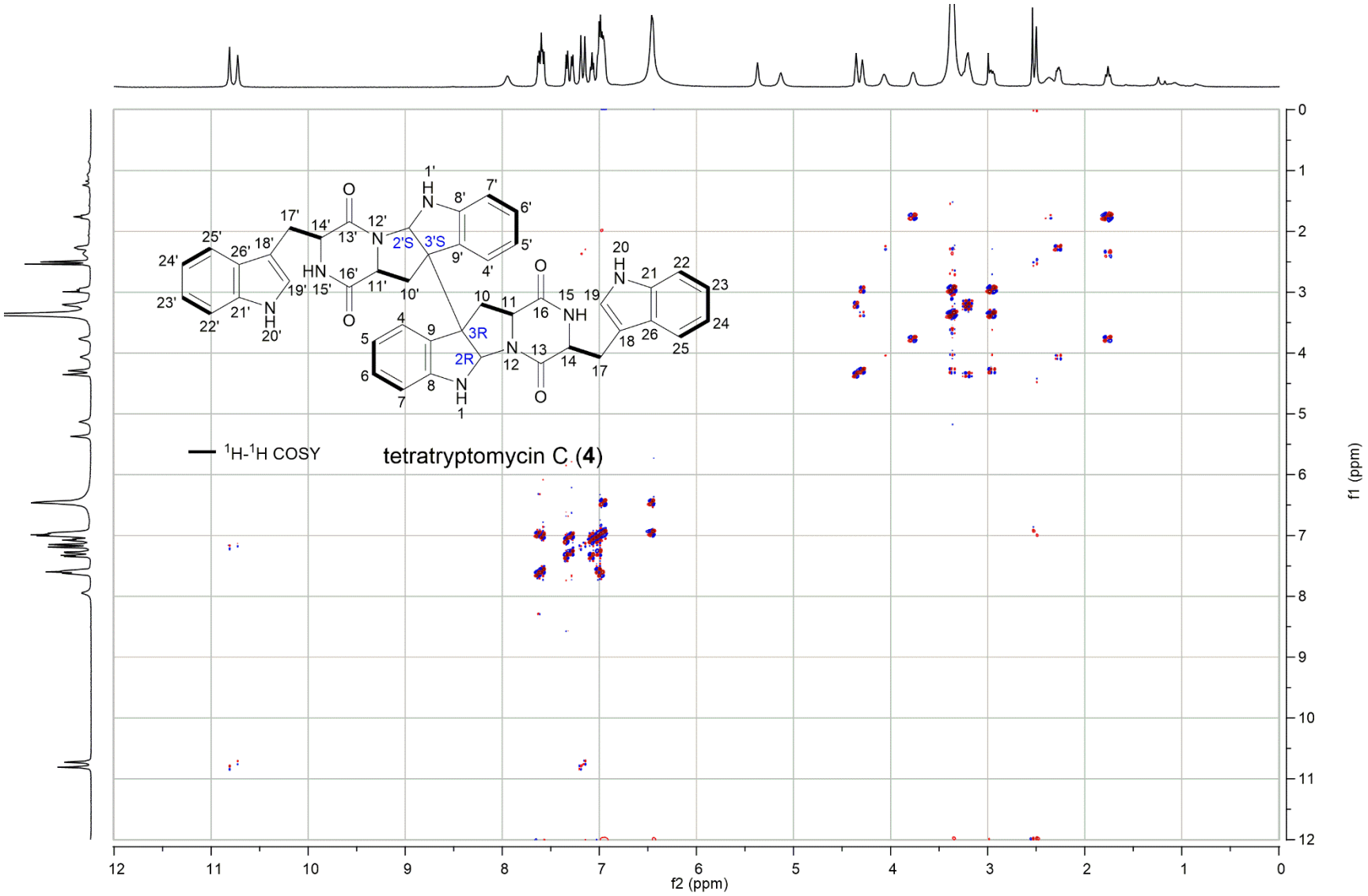


Fig. S18 ^{1}H-^{1}H COSY spectrum of tetratryptomycin C (**4**) in DMSO-d_6 at 300 K (500 MHz).

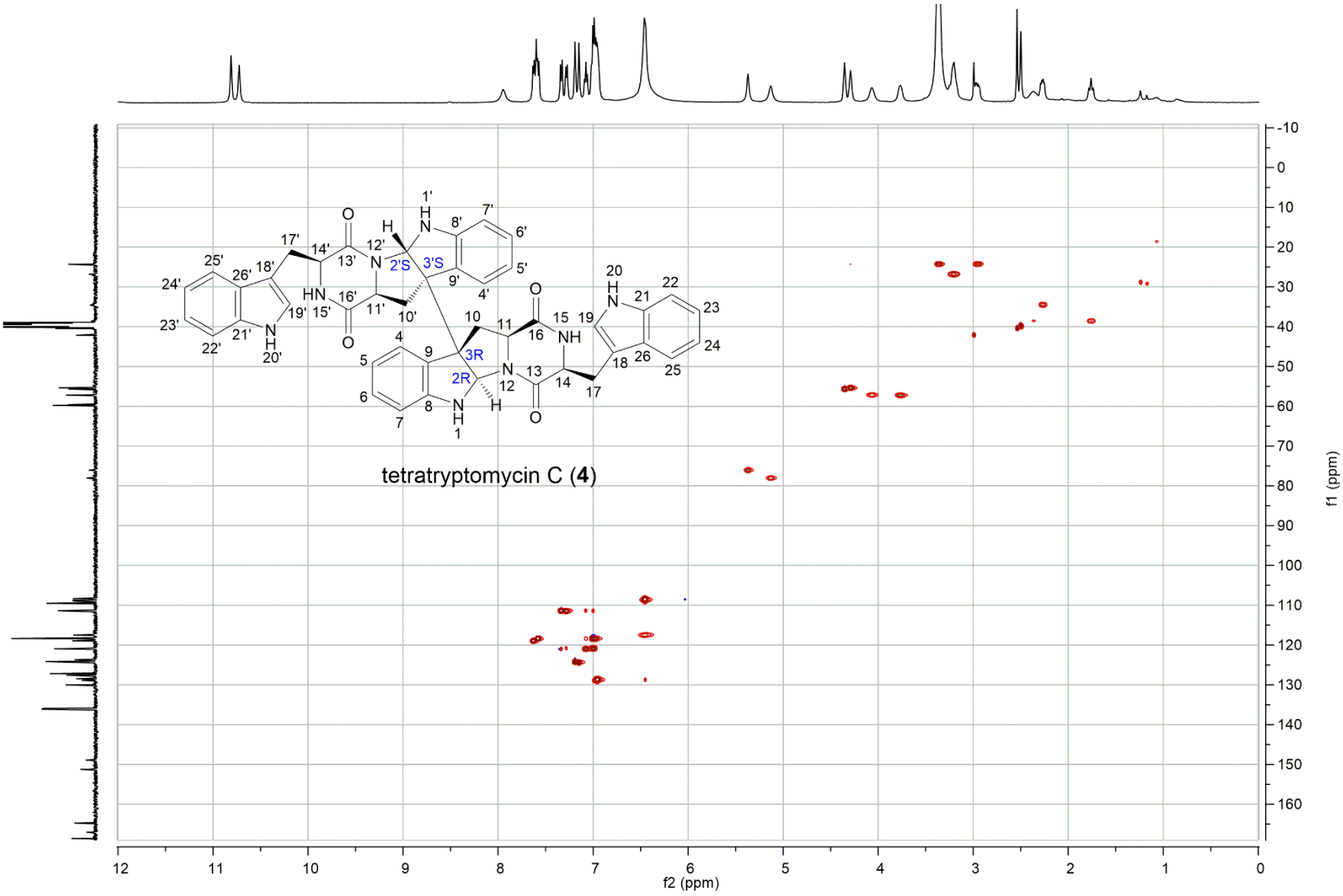


Fig. S19 HSQC spectrum of tetratryptomycin C (**4**) in DMSO-d_6 at 300 K (500 MHz, 125 MHz).

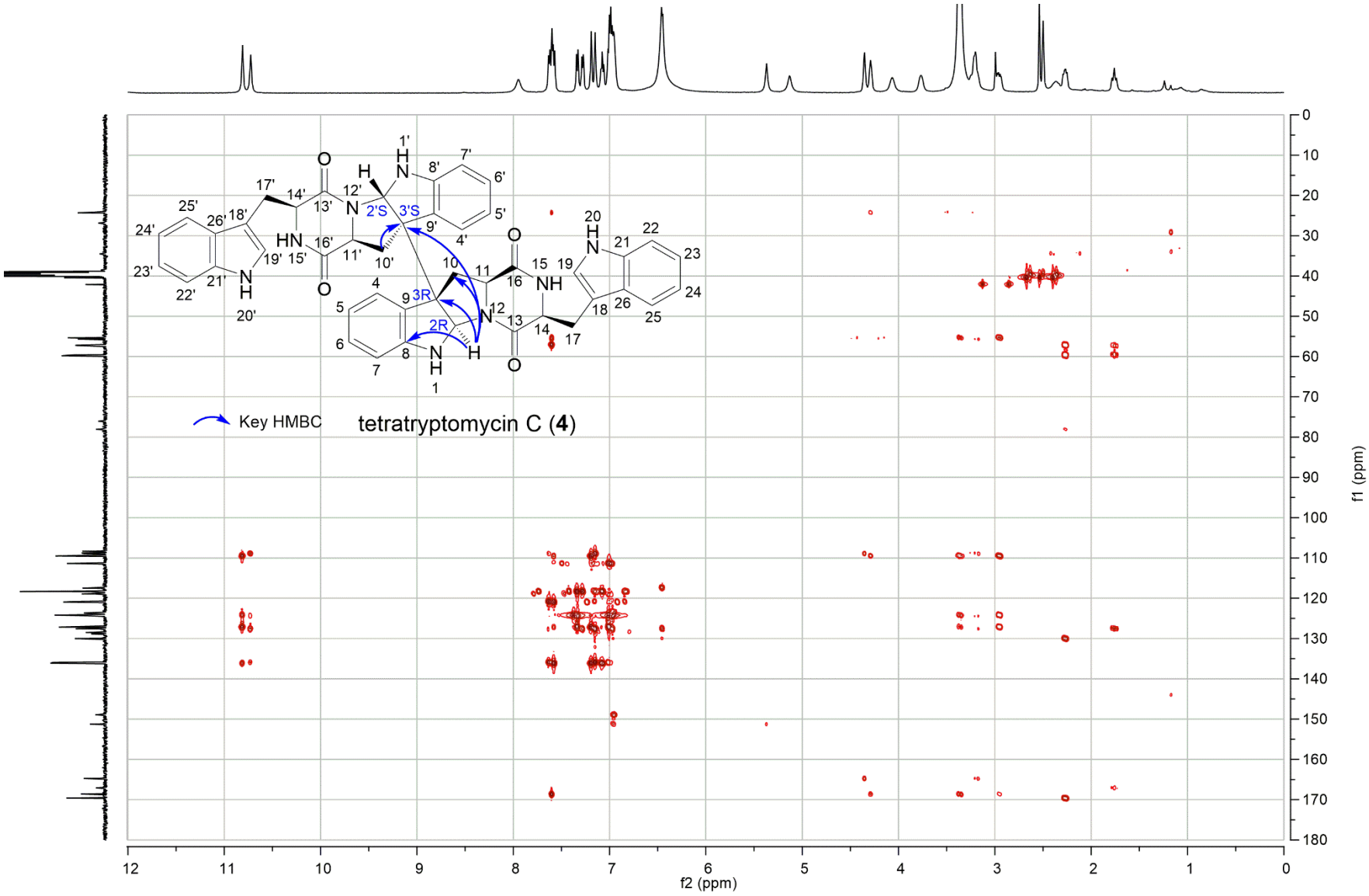


Fig. S20 HMBC spectrum of tetratryptomycin C (**4**) in DMSO-d_6 at 300 K (500 MHz, 125 MHz).

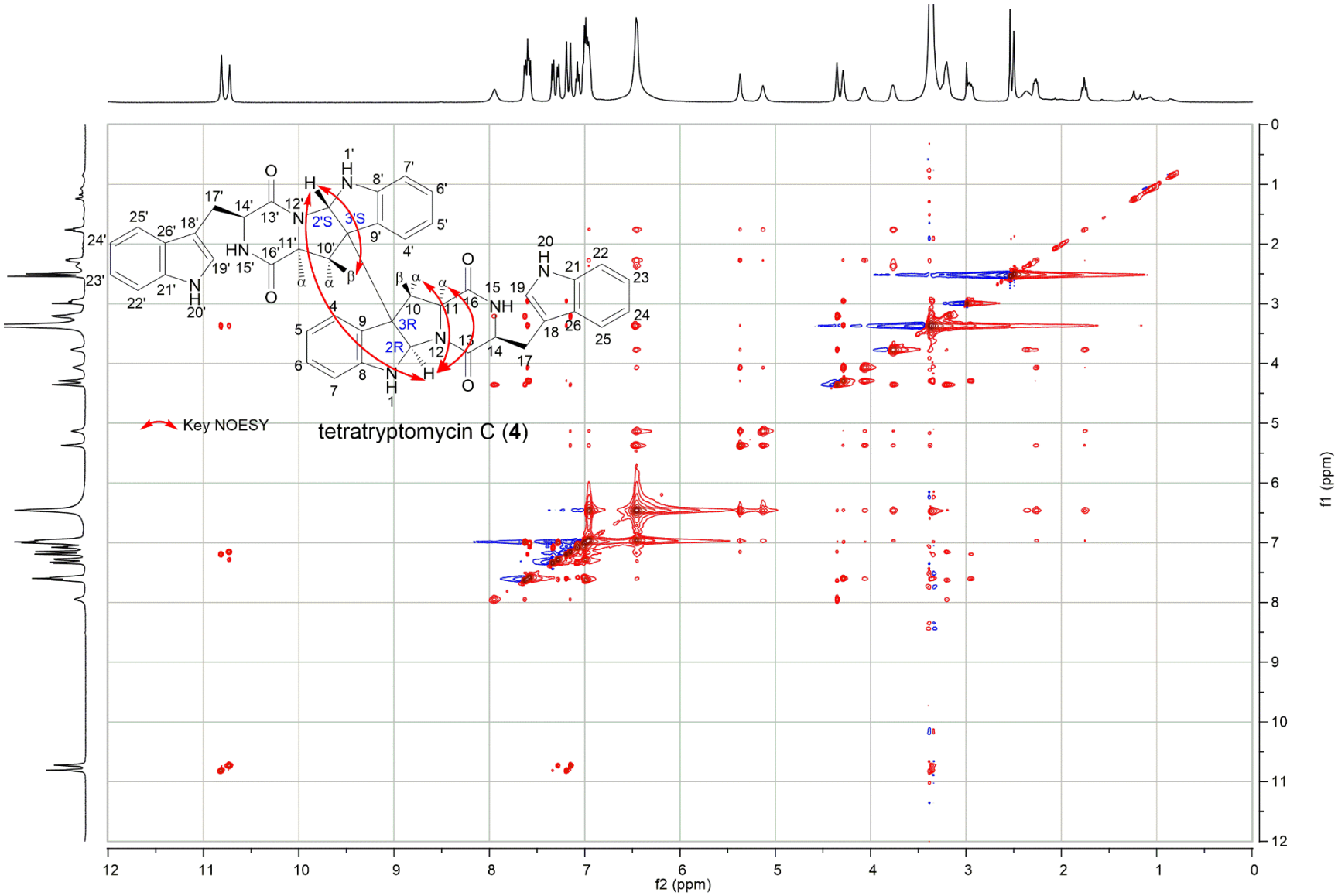


Fig. S21 NOSEY spectrum of tetratryptomycin C (**4**) in DMSO-d_6 at 300 K (500 MHz).

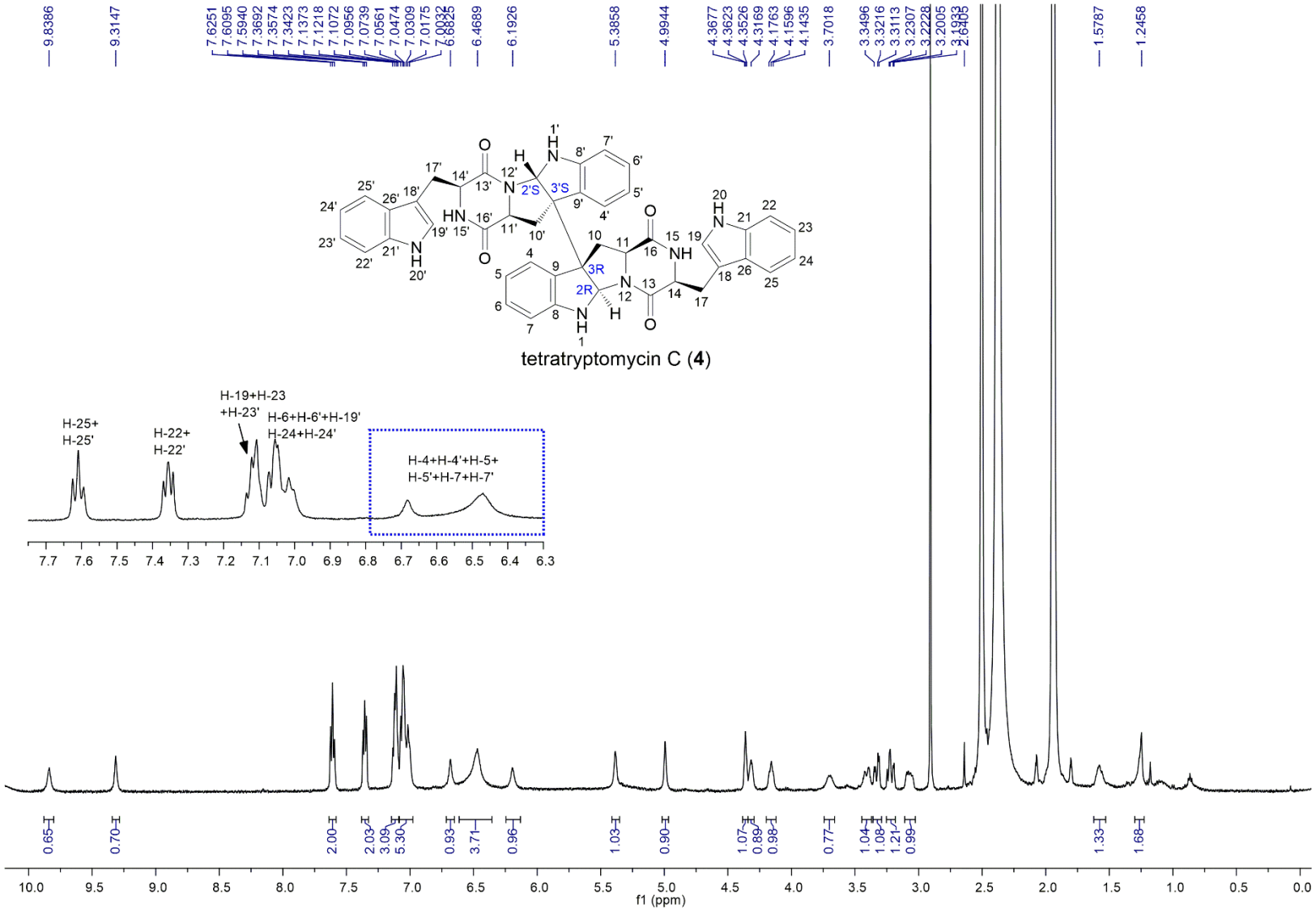


Fig. S22 ^{1}H NMR spectrum of tetratryptomycin C (**4**) in acetonitrile-d_3 at 273 K (500 MHz).

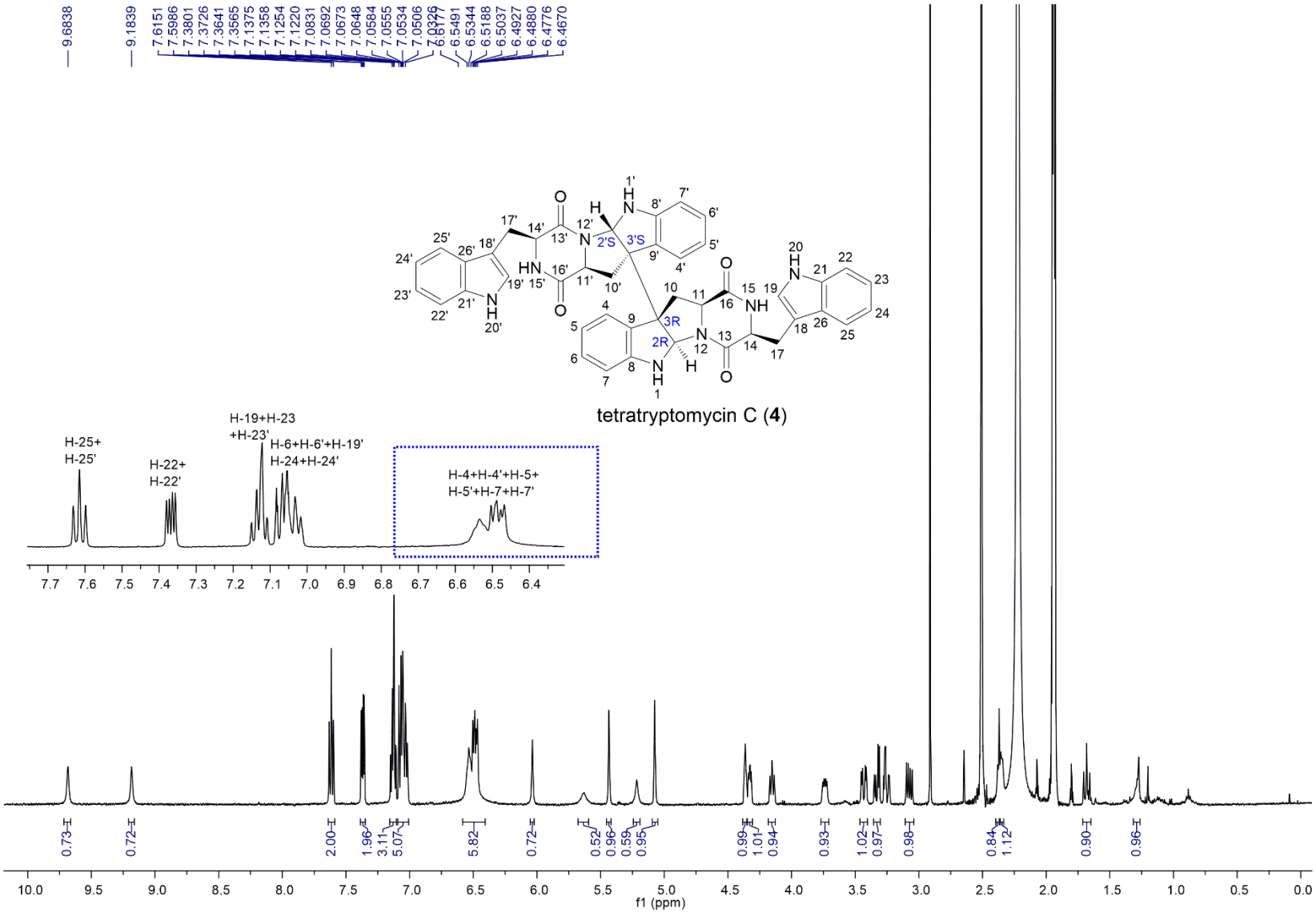


Fig. S23 ^{1}H NMR spectrum of tetratryptomycin C (**4**) in acetonitrile-d_3 at 300 K (500 MHz).

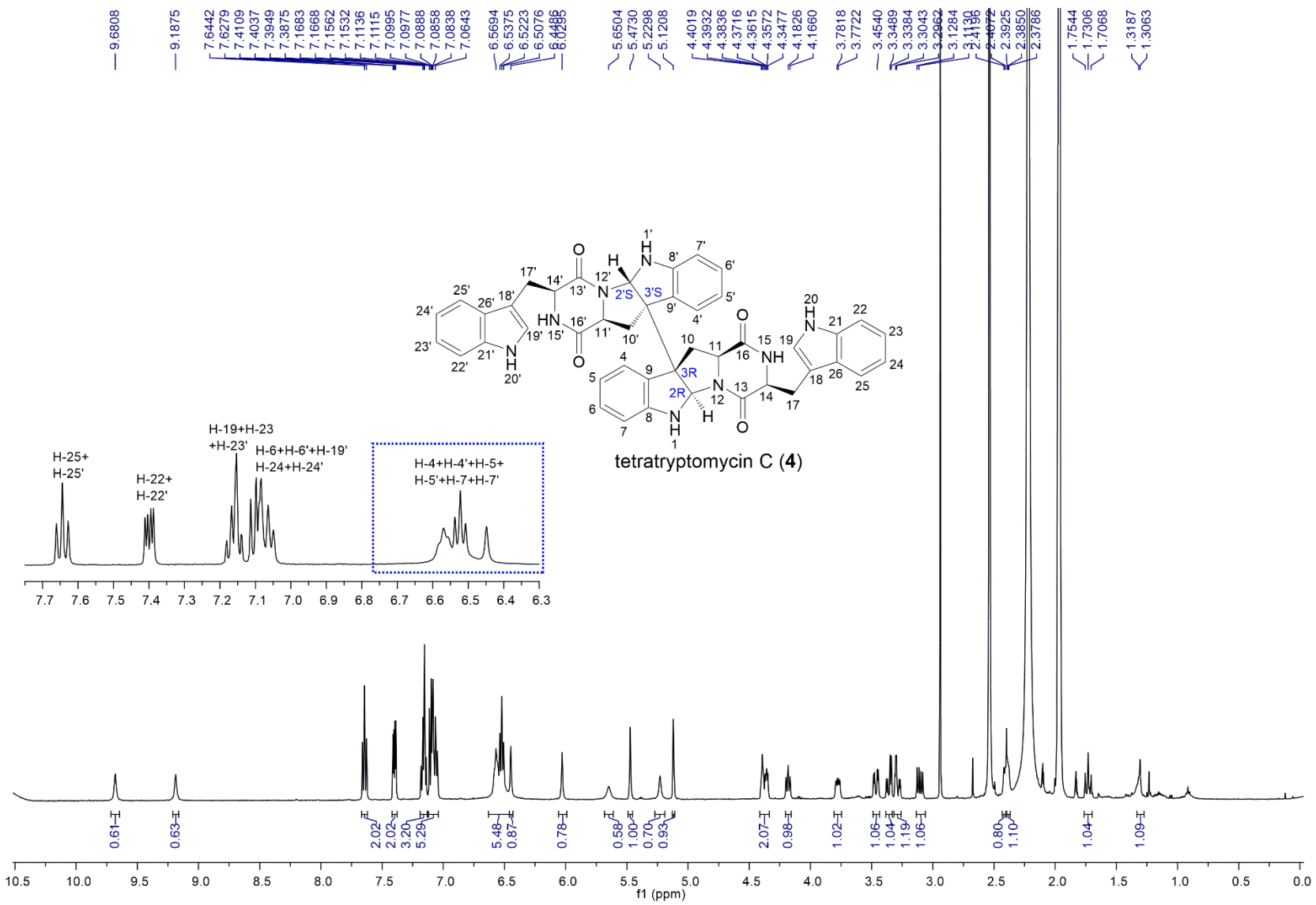


Fig. S24 ^{1}H NMR spectrum of tetratryptomycin C (**4**) in acetonitrile-d_3 at 310 K (500 MHz).

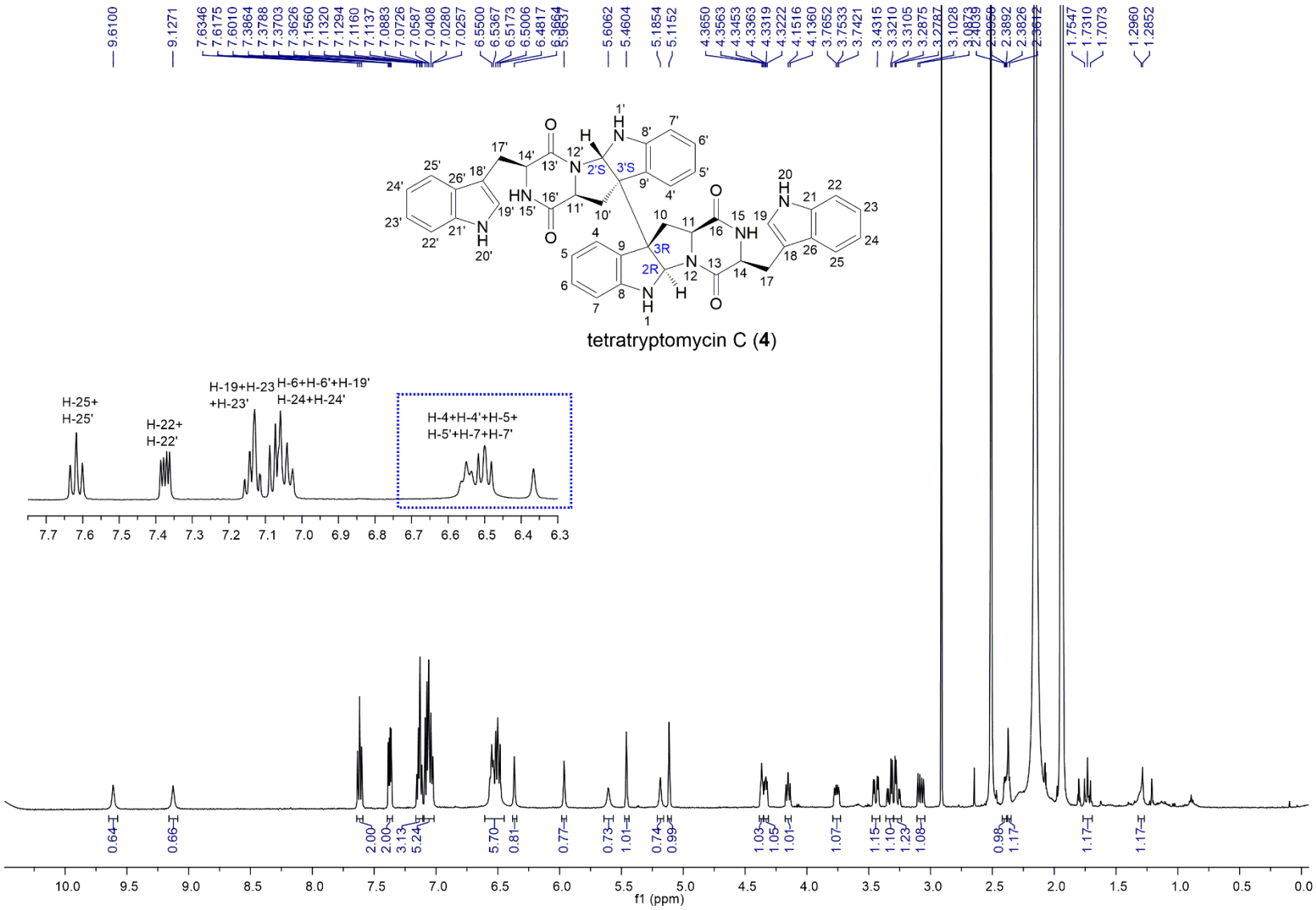


Fig. S25 ^{1}H NMR spectrum of tetratryptomycin C (**4**) in acetonitrile-d_3 at 320 K (500 MHz).

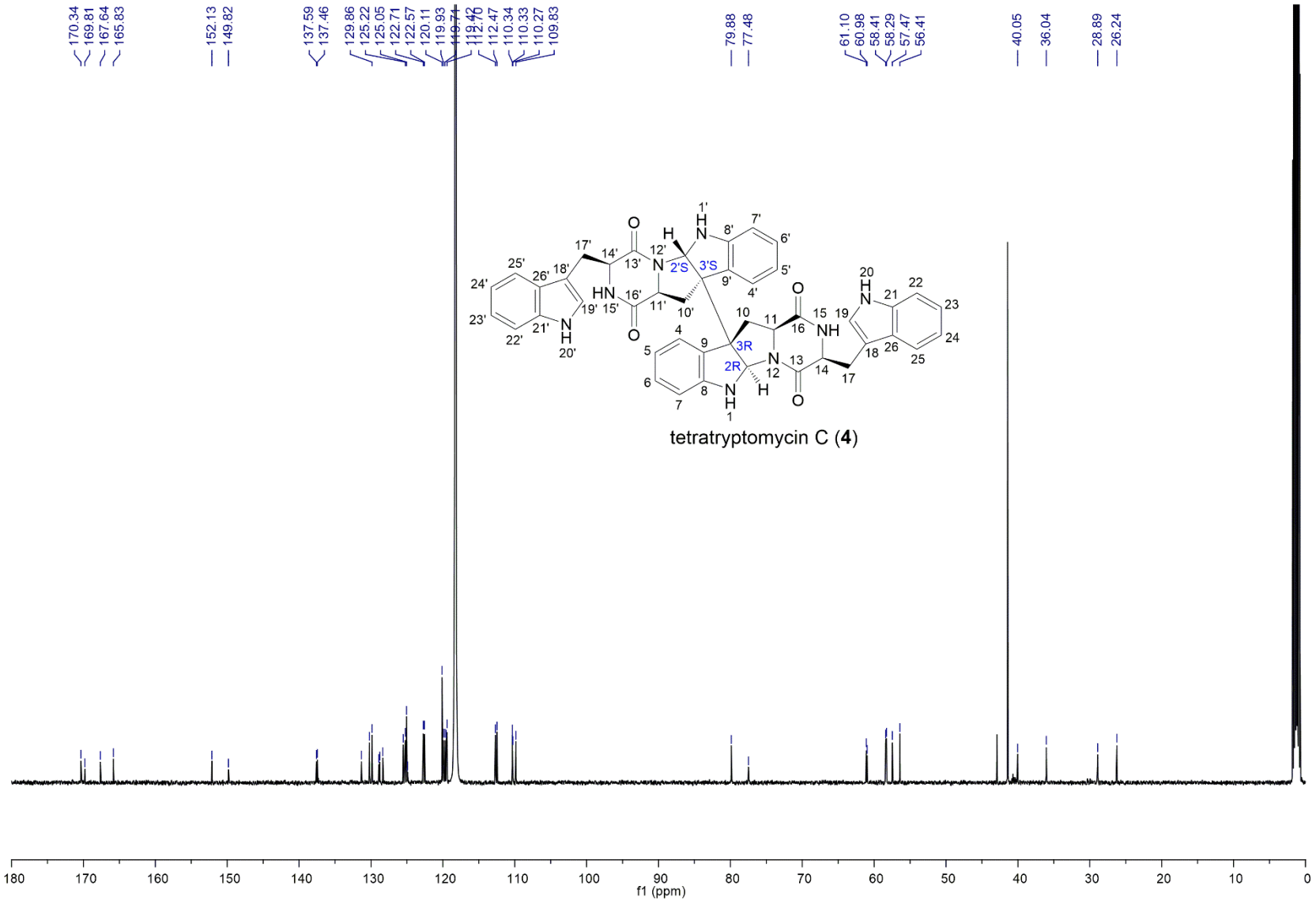


Fig. S26 ^{13}C NMR spectrum of tetratryptomycin C (**4**) in acetonitrile-d_3 at 310 K (125 MHz).

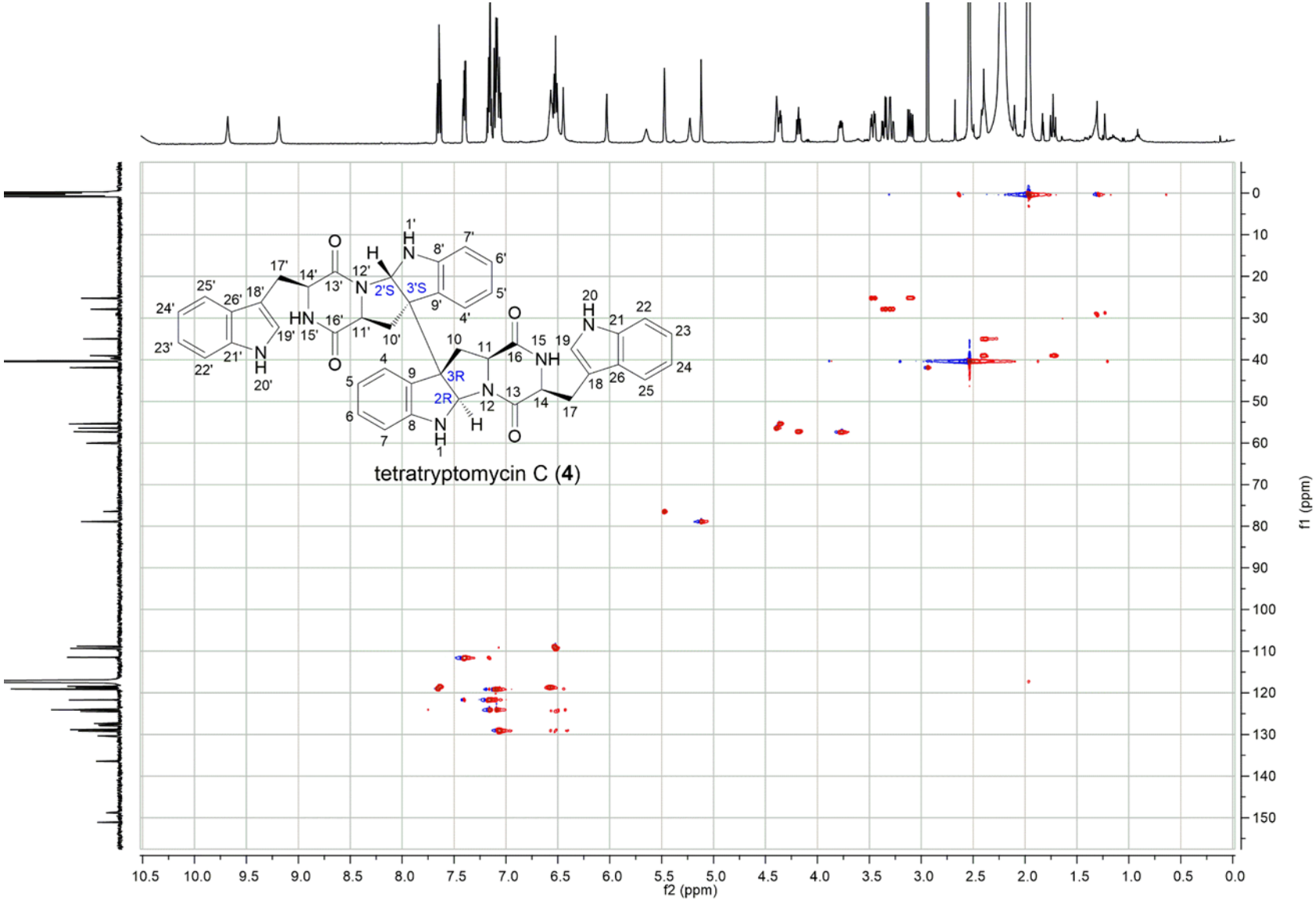


Fig. S27 HSQC spectrum of tetratryptomycin C (**4**) in acetonitrile-d_3 at 310 K (500 MHz, 125 MHz).

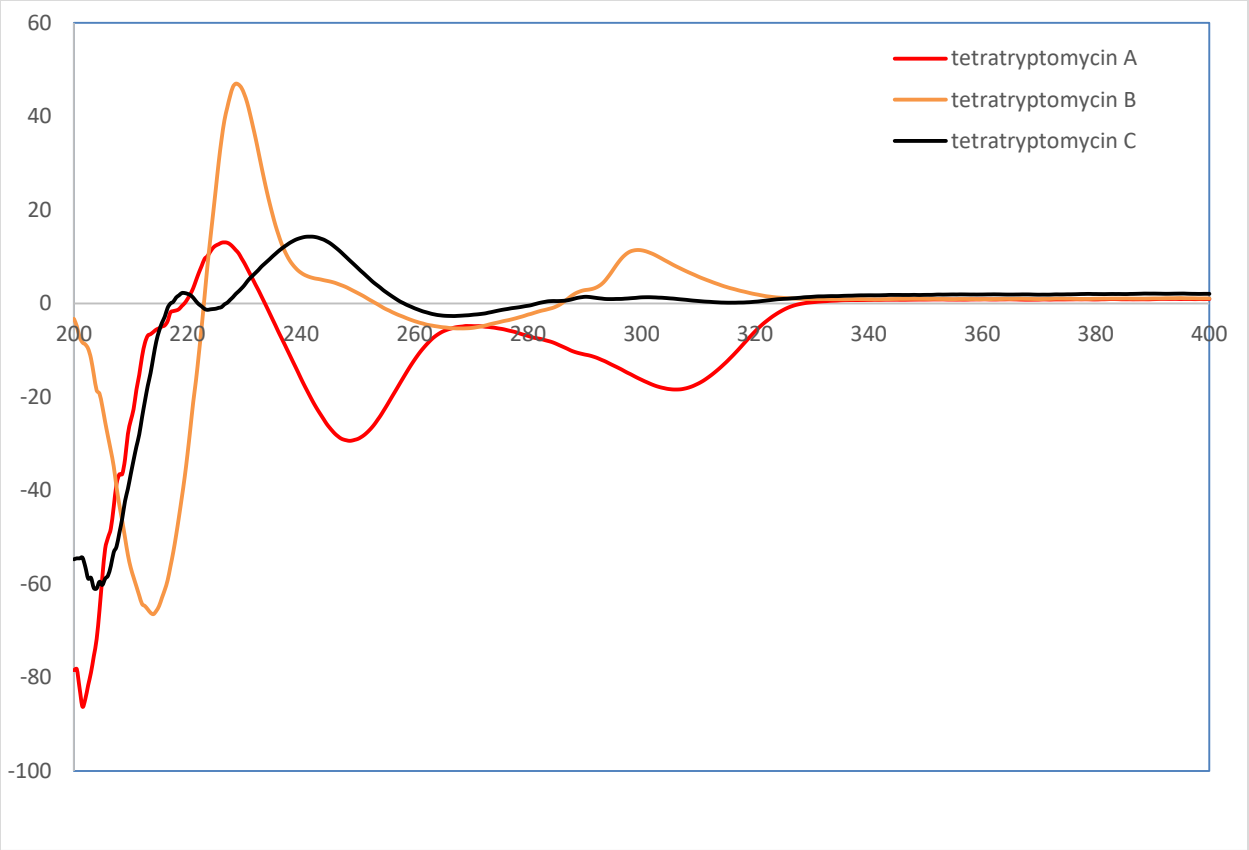


Fig. S28 CD spectra of tetratryptomycins.

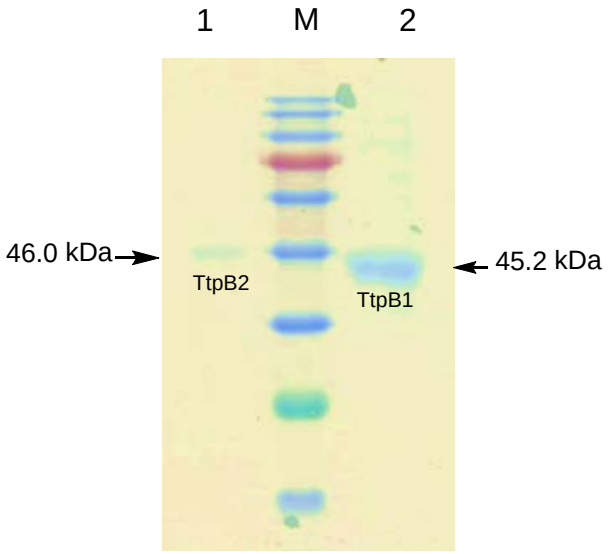


Fig. S29 SDS-PAGE analysis of the purified P450s.

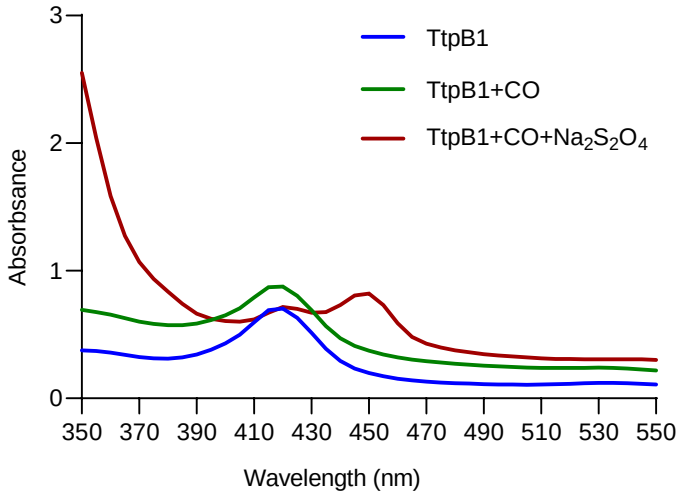


Fig. S30 UV-Vis spectroscopic analysis of TtpB1.

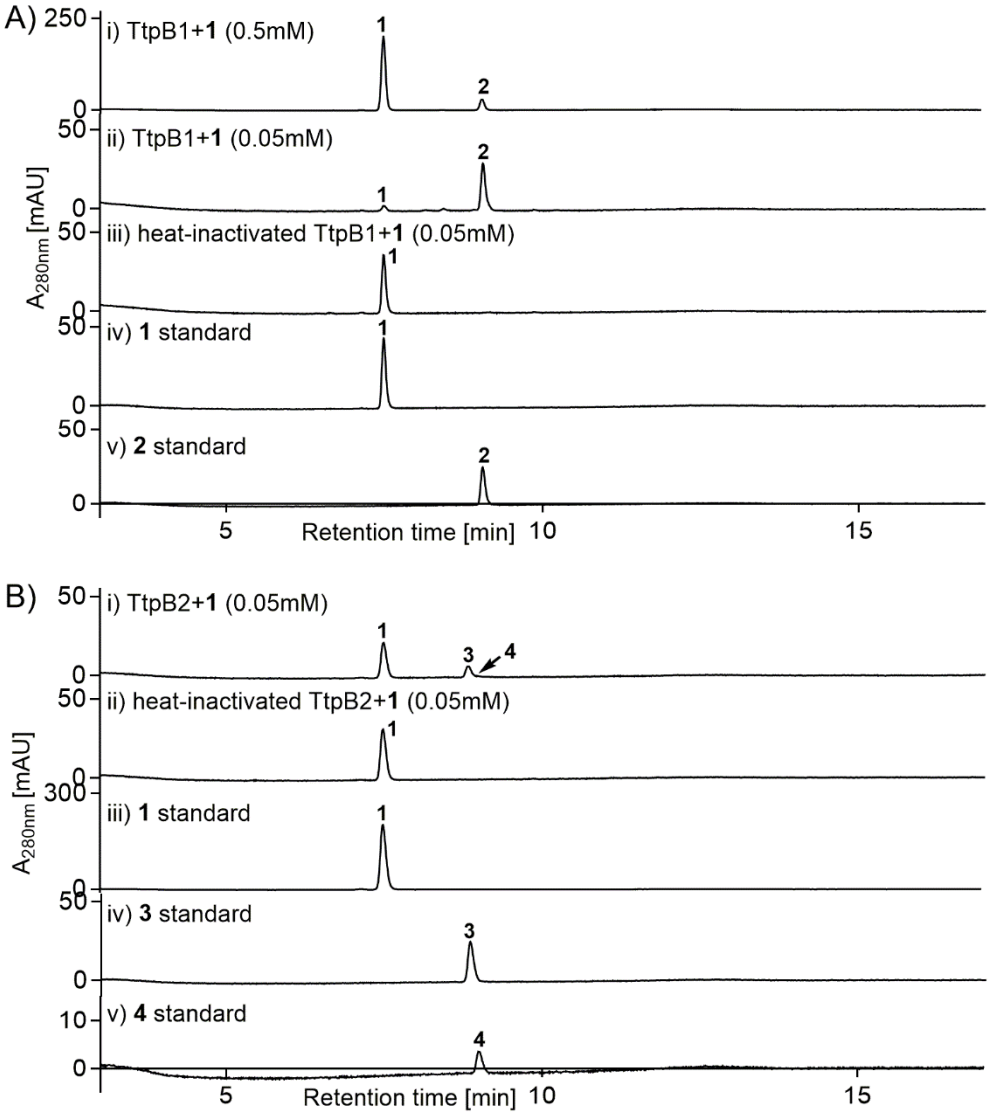


Fig. S31 HPLC analysis of the enzyme assays with TtpB1 (A) and TtpB2 (B).

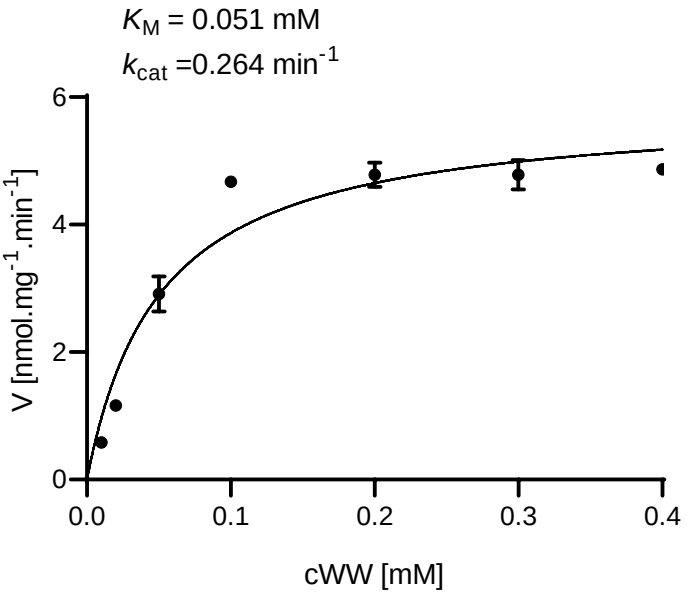


Fig. S32 Determination of kinetic parameters of TtpB1 for cWW (**1**).

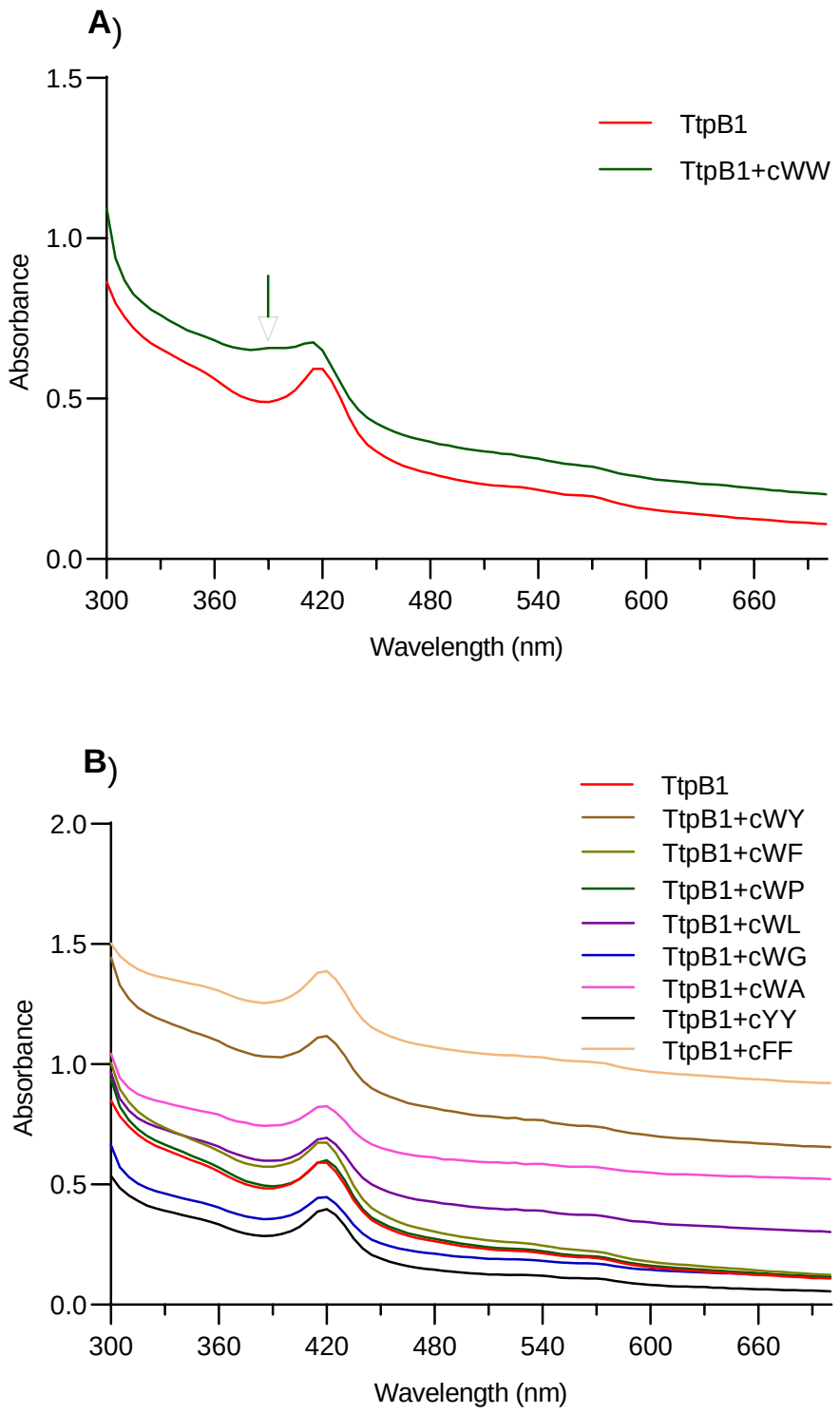


Fig. S33 UV-Vis spectroscopic analysis of TtpB1 with its substrate cWW (A) and other CDPs (B).

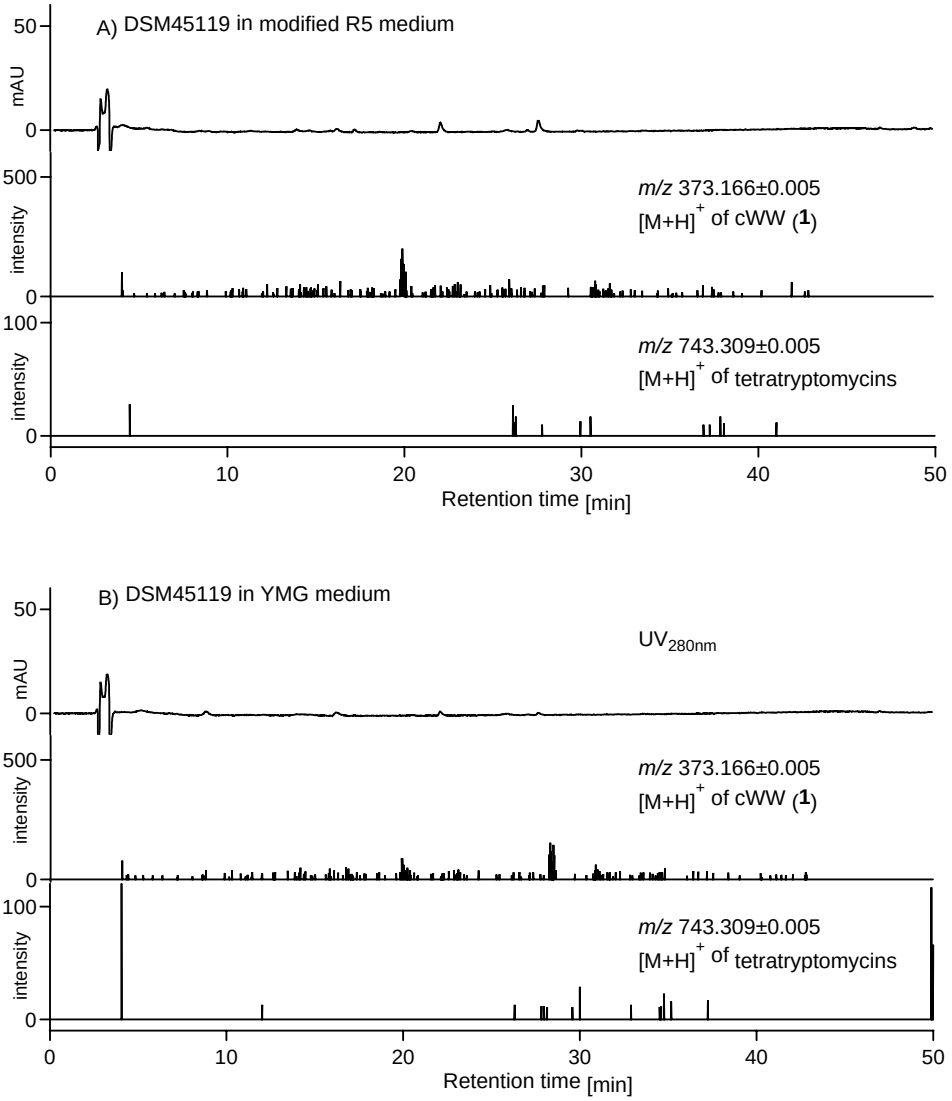


Fig. S34 LC-MS analysis for tetratryptomycin production in *S. antimicrobica* DSM 45119.

References

1 N. Zaburannyi, M. Rabyk, B. Ostash, V. Fedorenko, and A. Luzhetskyy, *Bmc Genomics*, 2014, **15**, 97.
2 M. R. Green and J. Sambrook, Molecular Cloning: a Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 2012.
3 T. Kieser, M. J. Bibb, M. J. Buttner, K. F. Chater, and D. A. Hopwood, Practical *Streptomyces* Genetics, John Innes Foundation, Norwich, UK, 2000.
4 Y. Zhu, P. Fu, Q. Lin, G. Zhang, H. Zhang, S. Li, J. Ju, W. Zhu, and C. Zhang, *Org Lett.*, 2012, **14**, 2666-2669.
5 B. Gust, G. L. Challis, K. Fowler, T. Kieser, and K. F. Chater, *Proc. Natl. Acad. Sci. U. S. A*, 2003, **100**, 1541-1546.
6 T. W. Giessen, A. M. von Tesmar, and M. A. Marahiel, *Biochemistry*, 2013, **52**, 4274-4283.
7 E. D. James, B. Knuckley, N. Alqahtani, S. Porwal, J. Ban, J. A. Karty, R. Viswanathan, and A. L. Lane, *ACS Synth. Biol.*, 2016, **5**, 547-553.
8 I. B. Jacques, M. Moutiez, J. Witwinowski, E. Darbon, C. Martel, J. Seguin, E. Favry, R. Thai, A. Lecoq, S. Dubois, J. L. Pernodet, M. Gondry, and P. Belin, *Nat. Chem Biol.*, 2015, **11**, 721-727.
9 H. Li, Y. Qiu, C. Guo, M. Han, Y. Zhou, Y. Feng, S. Luo, Y. Tong, G. Zheng, and S. Zhu, *Chem. Commun.*, 2019, **55**, 8390-8393.
10 J. Liu, H. Yu, and S.-M. Li, *Appl. Microbiol. Biotechnol*, 2018, **102**, 4435-4444.
11 H. Yu and S.-M. Li, *Org. Lett.*, 2019, **21**, 7094-7098.
12 J. Liu, X. Xie, and S.-M. Li, *Angew. Chem. Int. Ed. Engl.*, 2019, **58**, 11534-11540.
13 H. Yu, X. Xie, and S.-M. Li, *Org Lett.*, 2018, **20**, 4921-4925.
14 P. Belin, M. H. Le Du, A. Fielding, O. Lequin, M. Jacquet, J. B. Charbonnier, A. Lecoq, R. Thai, M. Courcon, C. Masson, C. Dugave, R. Genet, J. L. Pernodet, and M. Gondry, *Proc. Natl. Acad. Sci. U. S. A*, 2009, **106**, 7426-7431.
15 W. Tian, C. Sun, M. Zheng, J. R. Harmer, M. Yu, Y. Zhang, H. Peng, D. Zhu, Z. Deng, S. L. Chen, M. Mobli, X. Jia, and X. Qu, *Nat. Commun.*, 2018, **9**, 4428.
16 M. J. Cryle, S. G. Bell, and I. Schlichting, *Biochemistry*, 2010, **49**, 7282-7296.
17 S. Meng, W. Han, J. Zhao, X. H. Jian, H. X. Pan, and G. L. Tang, *Angew. Chem Int. Ed. Engl.*, 2018, **57**, 719-723.
18 J. Ma, Z. Wang, H. Huang, M. Luo, D. Zuo, B. Wang, A. Sun, Y. Q. Cheng, C. Zhang, and J. Ju, *Angew. Chem. Int. Ed. Engl.*, 2011, **50**, 7797-7802.
19 Y. Du, Y. Wang, T. Huang, M. Tao, Z. Deng, and S. Lin, *BMC Microbiol.*, 2014, **14**, 30.
20 L. M. Podust and D. H. Sherman, *Nat. Prod. Rep.*, 2012, **29**, 1251-1266.