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# Biotransformation-coupled mutasynthesis for the generation of novel pristinamycin derivatives by engineering the phenylglycine residue

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**Protocol S1**: Fermentation and purification of 6-chloropristinamycin I (**3**)

For batch cultivations, 100 mL of HT7T medium was inoculated with 1 mL of mycelium of *S. pristinaespiralis ΔpglAΔsnaE1* and cultivated for 72 h in 500 mL Erlenmeyer flasks (with baffles) on an orbital shaker (110 rpm) at 28°C as a preculture. 100 mL of preculture was used to inoculate 1 L of HT7T medium (supplemented with 100 µM 4-chloro-DL-Phg solubilized as mentioned above) in a 5-L Erlenmeyer flask (with baffles) on an orbital shaker (110 rpm) at 28°C for 48-72h. The whole culture was extracted with two times 1 L ethyl acetate and the organic phase was concentrated in a rotary evaporator. For initial purifications, the extract was first dissolved in 20 mL of methanol/water (ratio: 85/15) and mixed twice with 20 mL of heptane. After separation, the heptane phase was discarded. The mixture was adjusted to a 70/30 methanol/water ratio and mixed twice with 20 mL of dichloromethane. The dichloromethane phase was mixed with 20 mL of water. After discarding the water phase, the extract was evaporated in a rotary evaporator and weighed. In total, 12 L of culture were extracted. For isolation of **3**, 180 mg of the crude extract solved in MeOH were separated using a PLC 2250 preparative HPLC system (Gilson, Middleton, WI, USA) with a Gemini® 10u C18 110Å column (250 × 50 mm, 10 µm; Phenomenex, Torrance, CA, USA) as the stationary phase and in the following conditions: solvent A: $H_2O$ + 0.1% formic acid, solvent B: MeCN + 0.1% formic acid; flow: for 1 min 20 mL/min, afterwards 45 mL/min, fractionation: 15 mL, gradient: increase from 5% B to 100% B in 41 min followed by a final isocratic step of 100% B for 10 min. This yielded one pure fraction of **3** (F3: 0.6 mg, retention time (tR) = 34.02–35.15 min).

**Protocol S2**: Fermentation and purification of 6-fluoropristinamycin I (**4**)

For batch cultivations, 100 mL of HT7T medium was inoculated with 1 mL of mycelium of *S. pristinaespiralis ΔpglAΔsnaE1* and cultivated for 72h in 500-mL Erlenmeyer flasks (with baffles) on an orbital shaker (110 rpm) at 28°C as a preculture. 100 mL of preculture was used to inoculate 1 L of HT7T medium (supplemented with 100 µM 4-fluoro-L-Phg solubilized as mentioned above) in a 5-L Erlenmeyer flask (with baffles) on an orbital shaker (110 rpm) at 28°C for 48-72 h. The whole culture was extracted with two times 1 L ethyl acetate and the organic phase was concentrated in a rotary evaporator. For initial purifications, the extract was first dissolved in 20 mL of 85/15 methanol/water and mixed twice with 20 mL of heptane. After separation, the heptane phase was discarded. The extract was adjusted to 70/30 methanol/water and mixed twice with 20 mL of dichloromethane. The dichloromethane phase was mixed with 20 mL of water. After discarding the water phase, the extract was evaporated in a rotary evaporator and weighed. In total, 6 L of culture were extracted. 320 mg crude extract were subjected to flash chromatography (Grace Reveleris®, Columbia, MD, USA) (silica cartridge 12 g [FlashPure, Büchi Switzerland, USA], solvent A: DCM, solvent B: DCM/MeOH 95%/5%, solvent C: DCM:MeOH 9/1, solvent D: MeOH), gradient: 100% A for 10 min, increasing to 50% B in 2 min, followed by an isocratic step of 50% B for 2 min, increasing to 100% B in 2 min followed by an isocratic step at 100% B for 2 min, followed by a change from 100% B to 50 % A and 50% C with an increase to 80% C in 2 min, followed by a further increase to 100% C in 2 min, followed by a change to 100% D in 10 min and an isocratic step of 100% D for 10 min. One fraction containing **4** was collected with an amount of 73 mg at the retention time 19.6 min. The yielded fraction from the flash-chromatography was separated using a PLC 2250 preparative HPLC system (Gilson, Middleton, WI, USA) with a Gemini® 10u C18 110Å column (250 × 50 mm, 10 µm; Phenomenex, Torrance, CA, USA) as the stationary phase and

in the following conditions: solvent A: $H_2O$ + 0.1% formic acid, solvent B: MeCN + 0.1% formic acid; flow: 45 mL/min, fractionation: 10 mL, gradient: increase from 5% B to 40% B in 10 min followed by an increase to 42% B in 30 min and a isocratic step in 42% B for 3 min, followed by an increase to 100% B in 8 min and an isocratic step in 100% B for 10 min. This yielded to two pure fractions of **4** (F2: 1.54 mg, tR = 43.36–44.21 min; F2.1 (1.88 mg, tR = 42.23–43.36 min).

**Protocol S3:** Construction of plasmids pET28a-*hmo* and pACYC-*bcd-gdh* biotransformation approach for the synthesis of L-Phg derivatives

Cloning of pET28a-*hmo*

The synthetic codon optimized *hmo* gene (GeneArt, Regensburg, Germany) from *Streptomyces coelicolor* was cloned into pET28a(+). For this purpose, *hmo* as well as the pET28a plasmid were double-digested with *Nde*I and *Bam*HI. The digested *hmo* gene fragment was ligated into the pET28a plasmid with T4 DNA ligase (NEB). The ligation product was transferred to competent *E. coli* DH5α for plasmid multiplication. The isolated plasmid pET28a-*hmo* was verified by sequencing (Eurofins Genomics, Germany).

Cloning of pACYC-*bcd-gdh*

The gene *bcd* from *Bacillus thuringiensis* was amplified from chromosomal DNA using primers bcd-fw/-rv with KOD Hot Start Polymerase (Novagen). The PCR fragment and pET28a were digested with *Nco*I and *BamH*I and ligated with T4 DNA ligase (NEB). The ligation product was transferred to competent *E. coli* DH5α. The isolated plasmid pET28-*bcd* was verified by sequencing (Eurofins Genomics, Germany). The *Hin*dIII restriction sites in the plasmid were deleted by insertion of point mutations with the primer pairs pET28a-Hind-fw/-rv and bcd-Hind-fw/rv.

The glucose dehydrogenase gene (*gdh*) from *Bacillus megaterium* was amplified from chromosomal DNA using primers gdh-fw/-rv with KOD Hot Start Polymerase (Novagen). The PCR fragment and pET28a were digested with *Nde*I and *Bam*HI and ligated with T4 DNA ligase. The constructed plasmid pET28-*gdh* was verified by sequencing (Eurofins Genomics, Germany). The *Hin*dIII restriction site in the plasmid was deleted by insertion of a point mutation with the primer pET28-Hind-fw/rv. The *bcd* gene was amplified with OP-fw/-rv by using pET28-*bcd* as template with the KOD Hot Start Polymerase (Novagen). The *bcd* fragment was digested with *Kpn*I and *Spe*I. The *gdh* gene was amplified with OP-fw/-rv from the template pET28-*gdh* with the KOD Hot Start Polymerase (Novagen). The *gdh* fragment was digested with *Nhe*I and *Hin*dIII. The *Nhe*I restriction site in pACYC was deleted by insertion of a point mutation with the primer pACYC-Nhe-fw/rv. Afterwards, the pACYC plasmid was amplified with pACYC-fw/-rv with KOD Hot Start Polymerase (Novagen) and afterwards digested with *Kpn*I and *Hin*dIII. All three digested fragments were ligated with T4 DNA ligase to obtain pACYC-*bcd-gdh,* which was transferred to *E. coli* DH5α. The isolated plasmid was verified by sequencing (Eurofins Genomics, Germany).

**Table S1:** Bacterial strains, plasmids and oligonucleotides used in this study.

| Strains/ plasmids/ oligonucleotides | Genotype/phenotype/sequence | Source/ reference |
|---|---|---|
| ***S. pristinaespiralis*** | | |
| PR11 | Pristinamycin producing strain/wild-type, natural isolate of *S. pristinaespiralis* ATCC 25486 | Sanofi-Aventis |
| M*pglA* | gene interruption of *pglA*, $apra^R$, PI non-producing | Mast *et al*., 2011 |
| Δ*pglA*Δ*snaE1* | gene interruption of *pglA*, $apra^R$, PI non-producing gene interruption of *snaE1*, $tsr^R$, PII non-producing | This study |
| ***E. coli*** | | |
| Novablue | *endA1 hsdR17* ($r_{K12}^{-}$ $m_{K12}^{+}$) *supE44 thi-1 recA1 gyrA96 relA1 lac* F′[*proA*$^+$*B*$^+$ *lacI*$^q$*Z*Δ*M15*::Tn*10*] ($Tet^R$) | Novagen, SigmaAldrich |
| DH5α | *fhuA2 lac(del)U169 phoA glnV44 Φ80' lacZ(del)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17* | Novagen, Merck Millipore |
| **Bioassay test strains** | | |
| *Bacillus subtilis* ATCC 6633 | Bioassay test strain | Pelzer *et al*., 1999 |
| *Pseudomonas aeruginosa* DSM 1117 | Bioassay test strain | DSMZ strain collection |
| *Proteus vulgaris* DSM 2140 | Bioassay test strain | DSMZ strain collection |
| *Enterococcus faecium* DSM 20477 | Bioassay test strain | DSMZ strain collection |
| *Staphylococcus aureus* DSM 18827 | Bioassay test strain | DSMZ strain collection |
| *Staphylococcus aureus* CIP 111304 | Bioassay test strain; *vat(A), vgb(A)* | Collection de L´Institut Pasteur |
| *Staphylococcus aureus* CIP 108540 | Bioassay test strain; *vgb(A), vgb(B), vat(B), erm(A), erm(B)* | Collection de L´Institut Pasteur |
| *Candida albicans* DSM 1386 | Bioassay test strain | DSMZ strain collection |
| *Trichophyton rubrum* DSM 16111 | Bioassay test strain | DSMZ strain collection |
| **Plasmids** | | |
| pDRIVE | *lacZ'*-complementation system, $amp^R$, $kan^R$ | Qiagen |
| pDRIVE/thio | *lacZ'*-complementation system, $amp^R$, $kan^R$, $tsr^R$ | Moosmann et al., 2020 |
| pK18 | pUC derivative, *aph*II, *lac*Z'-complementation system | Pridmore, 1987 |
| pK18/snaE1tsr | pK18 derivative, *aph*II, $tsr^R$, *lac*Z'*α*, *snaE1ab* | This study |
| pET28a | pET28a, $kan^R$ | Novagen |
| pET28a-*hms* | pET28a derivate with *hms* from *A. mediterranei*, $kan^R$ | Youn *et al.,* 2019 |
| pET28a-*hmo* | pET28a derivate with *hmo* from *S. coelicolor*, $kan^R$ | This study |
| pET28a-*bcd* | pET28a derivate with *bcd* from *B. thuringiensis*, $kan^R$ | This study |
| pET28a-*gdh* | pET28a derivate with *gdh* from *B. megaterium*, $kan^R$ | This study |
| pACYC | pACYC, $cm^R$ | Chang & Cohen 1978 |
| pACYC-*leuDH-gdh* | pACYC derivate, $cm^R$ | This study |
| **Oligonucleotides** | **5'→3'** | |
| MsnaE1fw | AGCCGATGCTGTGCACGATG | This study |
| MsnaE1rv | CCACGTCGTCCTGGAAGAAG | This study |
| thio1 | CGTTGGTGATTGCCGGTCAG | This study |

| | | |
|---|---|---|
| thio2 | GGCGATGCCGAATGTCTTGG | This study |
| KsnaE1fw | TGTCCCACTCGGGTACCAGG | This study |
| KsnaE1rv | TTCGAGCGGCAGTCCTACTGG | This study |
| bcd-fw | ATACCATGGGCACATTAGAAATCTTCGAATACTTAGAAAAATATG | This study |
| bcd-rv | TTCGGATCCTTAGCGACGGCTAATAATATCGTG | This study |
| pET28-Hind-fw | GAGCTCCGTCGACATGCTTGCGGCCGC | This study |
| pET28-Hind-rv | GCGGCCGCAAGCATGTCGACGGAGCTC | This study |
| bcd-Hind-fw | TGAAGAGCGCATTGCTAGCTTGAAAAATTC | This study |
| bcd-Hind-rv | GAATTTTTCAAGCTAGCAATGCGCTCTTCA | This study |
| gdh-fw | TTTCATATGATGTATAAAGATTTAGAAGGAAAAGTAGTTGTCATAACAGG | This study |
| gdh-rv | TTTGGATCCTTATCCGCGT-CCTGCTTGGAATG | This study |
| OP-fw | GCAGGTACCGCGGCCGCGCTAGCGATATAGGCGCCAGCAACCGCAC | This study |
| OP-rv | AGCAAGCTTCTAGCCGGCCGCTACTAGTCAGCAAAAAACCCCTCAAGACCCGTTT | This study |
| pACYC-Nhe-fw | CAGTATACACTCCGCCAGCGCTGATGTCCGG | This study |
| pACYC-Nhe-rv | CCGGACATCAGCGCTGGCGGAGTGTATACTG | This study |
| pACYC-fw | GTCAAGCTTAAGCTGTCAAACATGAGAATTACAAC | This study |
| pACYC-rv | ATGGGTACCATTTCAGAATATTTGCCAGAACCGT | This study |

**Table S2**: MIC assay of pristinamycin IA (**1**) and its 6-fluoro derivative (**4**) against standard test organisms. Inhibited organisms are highlighted in bold; n.i. = not identified.

| | | **1** | **4** | |
|---|---|---|---|---|
| **test organism** | **DSM-Nr.** | **MIC µg/mL** | **MIC µg/mL** | **positive control** |
| *Schizosaccharomyces pombe* | 70572 | n.i. | n.i. | Nystatin |
| *Pichia anomala* | 6766 | n.i. | n.i. | Nystatin |
| *Mucor hiemalis* | 2656 | n.i. | n.i. | Nystatin |
| *Candida albicans* | 1665 | n.i. | n.i. | Nystatin |
| *Rhodotorula glutinis* | 10134 | n.i. | n.i. | Nystatin |
| *Acinetobacter baumannii* | 30008 | n.i. | n.i. | Ciprofloxacin |
| *Escherichia coli* | 1116 | n.i. | n.i. | Oxytetracyclin |
| ***Bacillus subtilis*** | **10** | **2,1** | **4,2** | Oxytetracyclin |
| *Mycobacterium smegmatis* | ATCC 700084 | n.i. | n.i. | Kanamycin |
| ***Staphylococcus aureus*** | **346** | **16,7** | **33,3** | Oxytetracyclin |
| *Pseudomonas aeruginosa* | PA14 | n.i. | n.i. | Gentamycin |

| *Chromobacter violaceum* | 30191 | n.i. | n.i. | Oxytetracyclin |
|---|---|---|---|---|

**Table S3:** Cytotoxicity assay of 6-fluoropristinamycin I (**4**) against human cell lines (* = no changed cells, no cytotoxic activity; ** = no changed cells, low inhibition in proliferation)

| **Compound** | **KB3.1 cell line** | **Tox-Nr.** | **L929 cell line** | **Tox-Nr.** |
|---|---|---|---|---|
| **4** | ** | **3548** | * | **3549** |

**Table S4:** Determination of enantiomeric excesses (ee) of L-Phg synthons from the whole cell biotransformation approach with *E. coli* BL21(DE3) pET28-*hmo/* pACYC-*bcd-gdh* with different mandelic acids. *The ee of 4-chloro-L-Phg produced by HMO-LeuDH-GDH could not be determined.

| **Product** | **Enantiomeric excess of L-phenylglycine derivatives [%]** |
|---|---|
| L-phenylglycine | >99 |
| 2-fluoro-L-phenylglycine | >95 |
| 3-fluoro-L-phenylglycine | >99 |
| 4-fluoro-L-phenylglycine | >99 |
| 2-chloro-L-phenylglycine | 50 |
| 3-chloro-L-phenylglycine | 67 |
| 4-chloro-L-phenylglycine | -* |

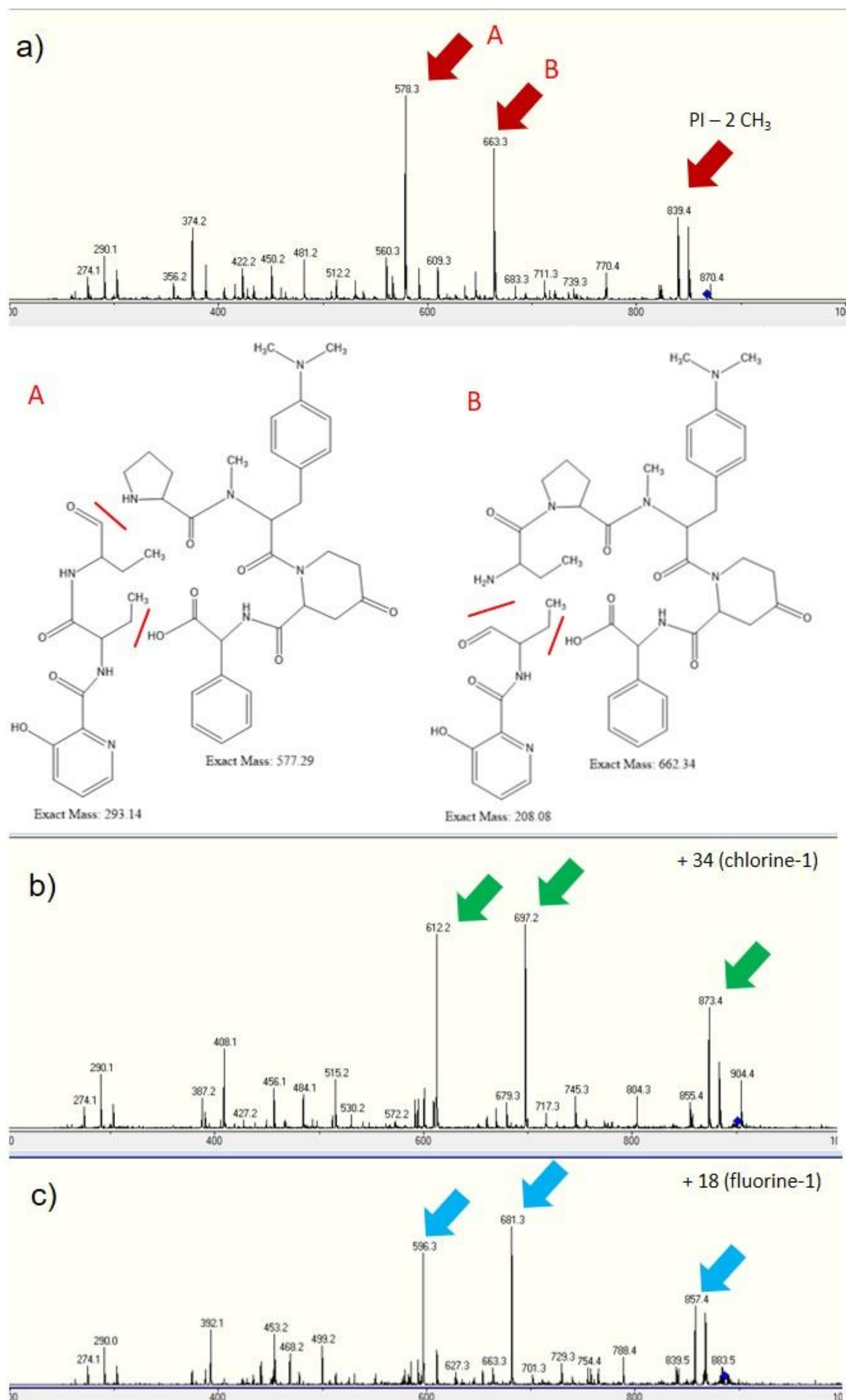



**Figure S1**: MS/MS fragmentation pattern of pristinamycin I (**1**) (a), 6-chloropristinamycin I (**3**) (b) and 6-fluoropristinamycin I (**4**) (c). Extracts of *S. pristinaespiralis* PR11 (1; m/z of precursor ion: 867.4) and *S. pristinaespiralis ΔpglAΔsnaE1* supplemented with 4-chloro-DL-Phg (2; *m/z* of precursor ion: 901.4) and 4-fluoro-L-Phg (3; *m/z* of precursor ion: 885.4). Distinct fragments of 1 are marked by arrows and likely fragmentation results are shown (A, B). Corresponding fragments that show the expected mass shift for the respective halogenation are marked by arrows in green (chlorination) or blue (fluorination). Measurements were conducted in positive mode.

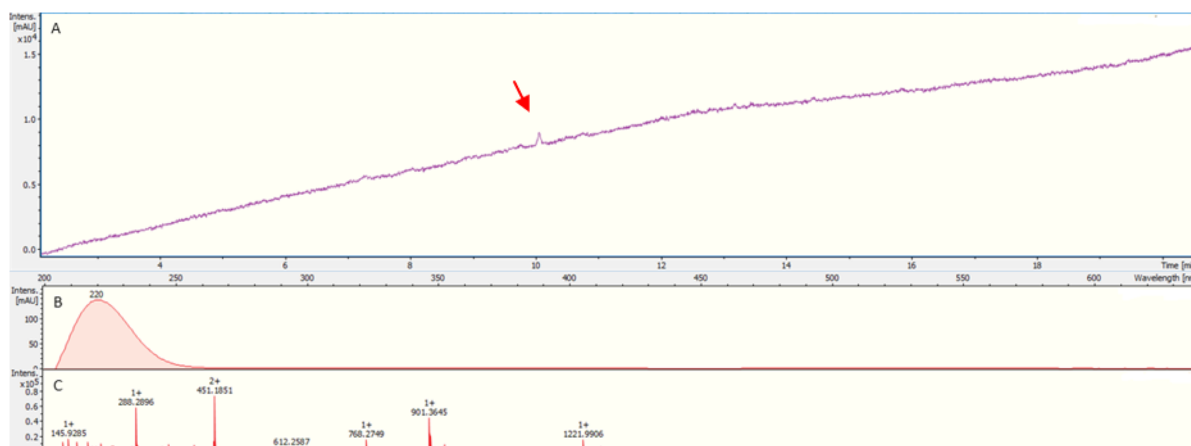



**Figure S2:** HRESIMS of 6-chloropristinamycin I (**3**). A is the chromatogram at 210 nm (peak indicated by red arrow). B and C are the UV spectrum and the mass spectrum of **3**.

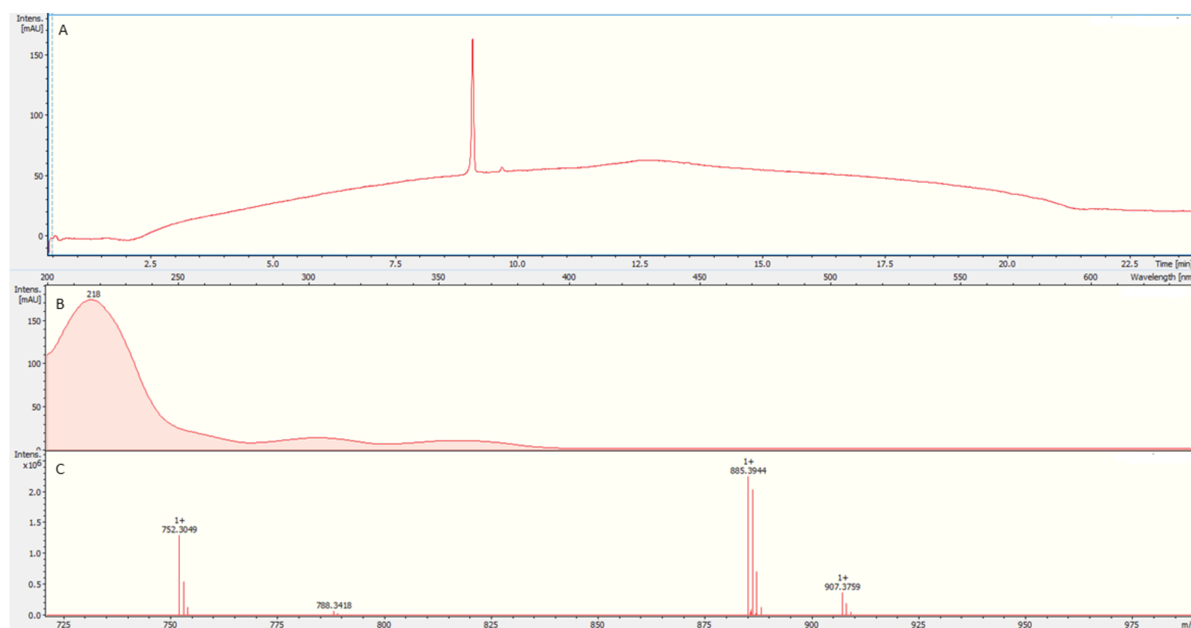



**Figure S3:** HRESIMS of 6-fluoropristinamycin I (**4**). A is the chromatogram at 210 nm. B and C are the UV spectrum and the mass spectrum of **4**.

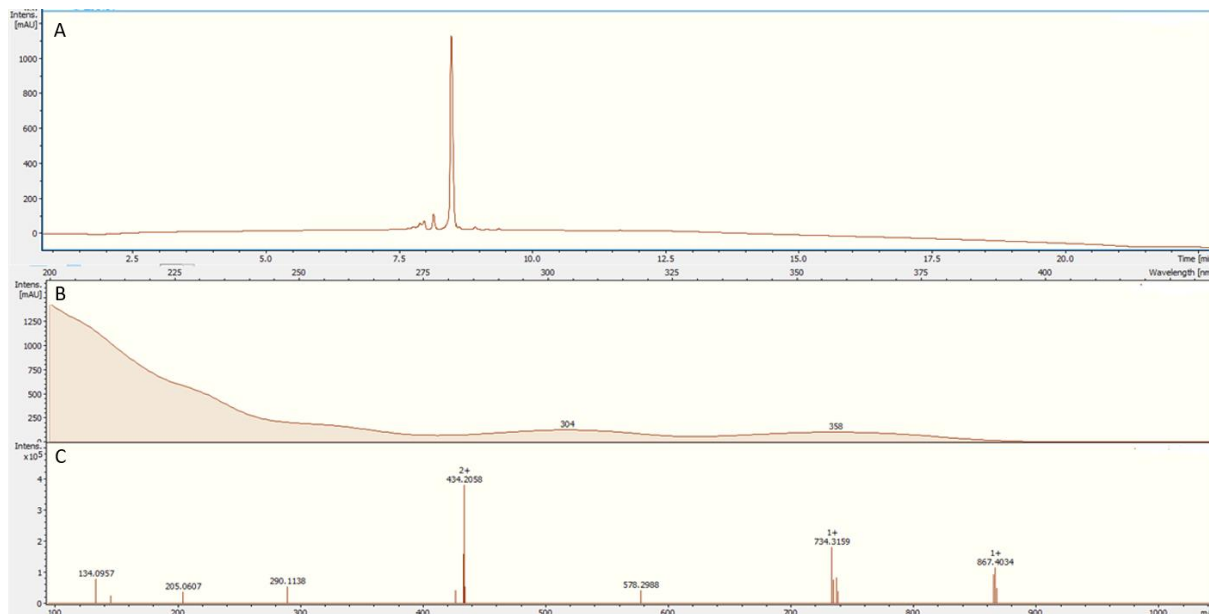



**Figure S4:** HRESIMS of pristinamycin I (**1**). A is the chromatogram 210 nm. B and C are the UV spectrum and the mass spectrum of **1**.

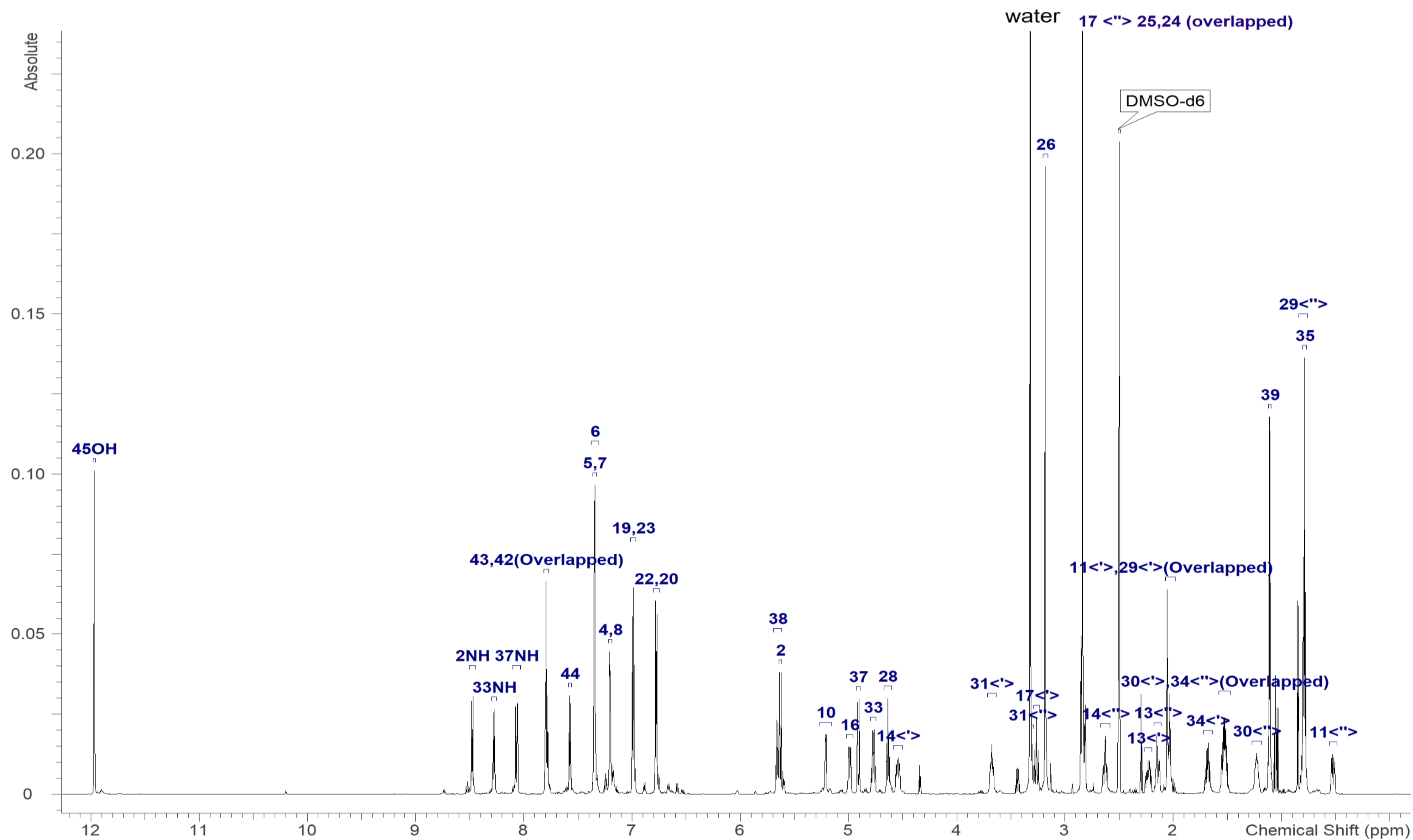



**Figure S5:** $^{1}$H NMR spectrum (700 MHz) pristinamycin I (**1**) in DMSO-$d_6$.

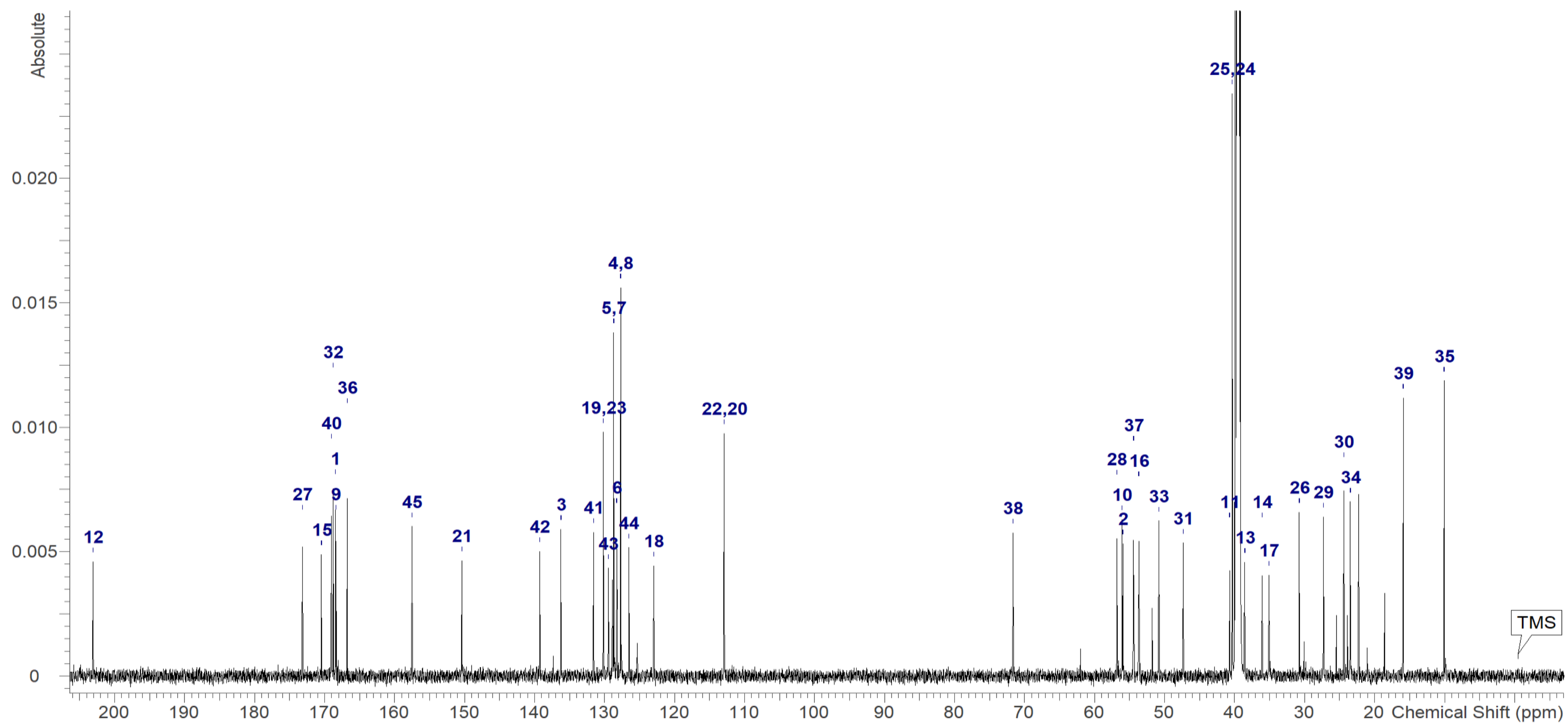



**Figure S6:** $^{13}C$ NMR spectrum (700 MHz) pristinamycin I (**1**) in DMSO-$d_6$.

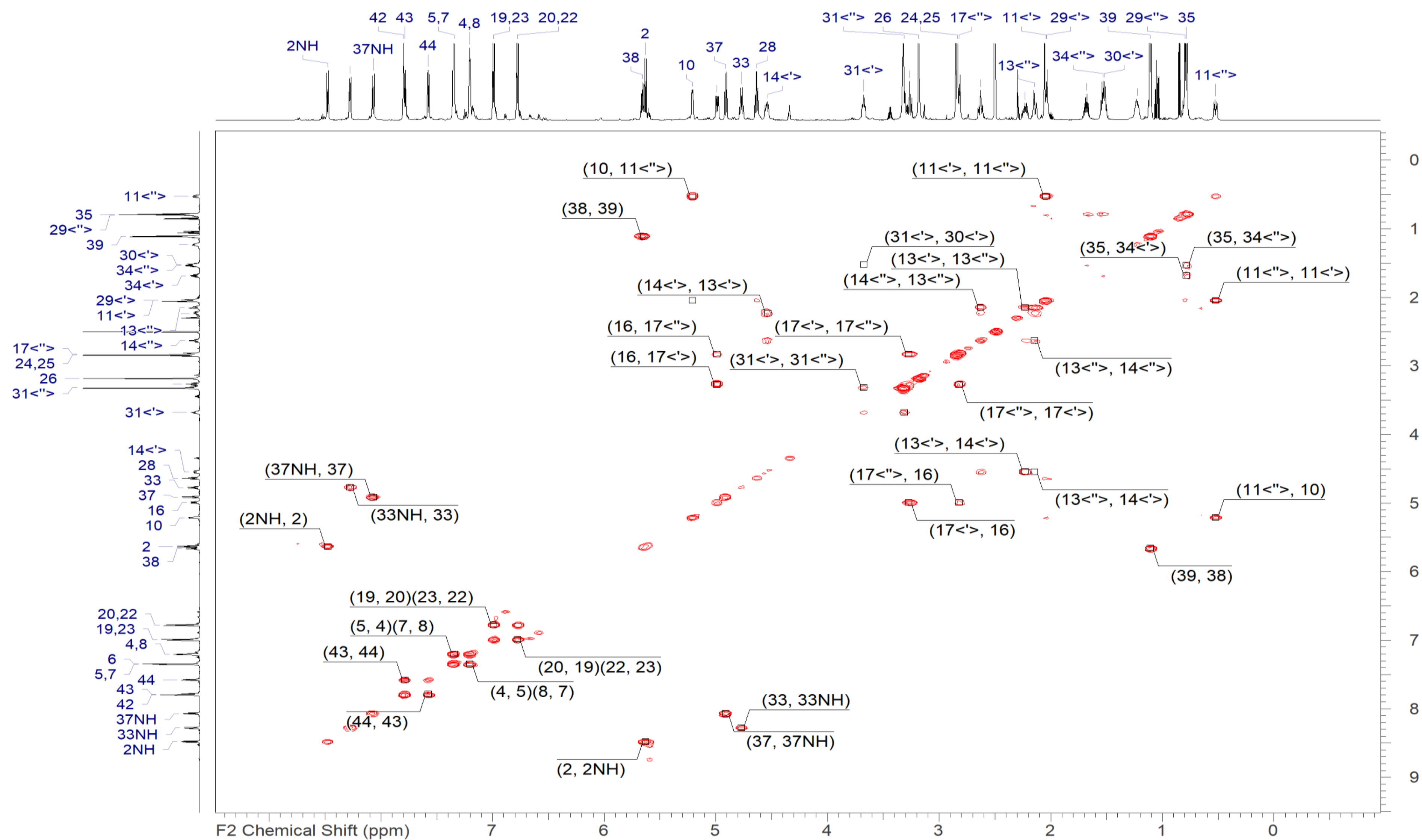



**Figure S7:** COSY NMR spectrum (700 MHz) pristinamycin I (**1**) in DMSO-$d_6$.

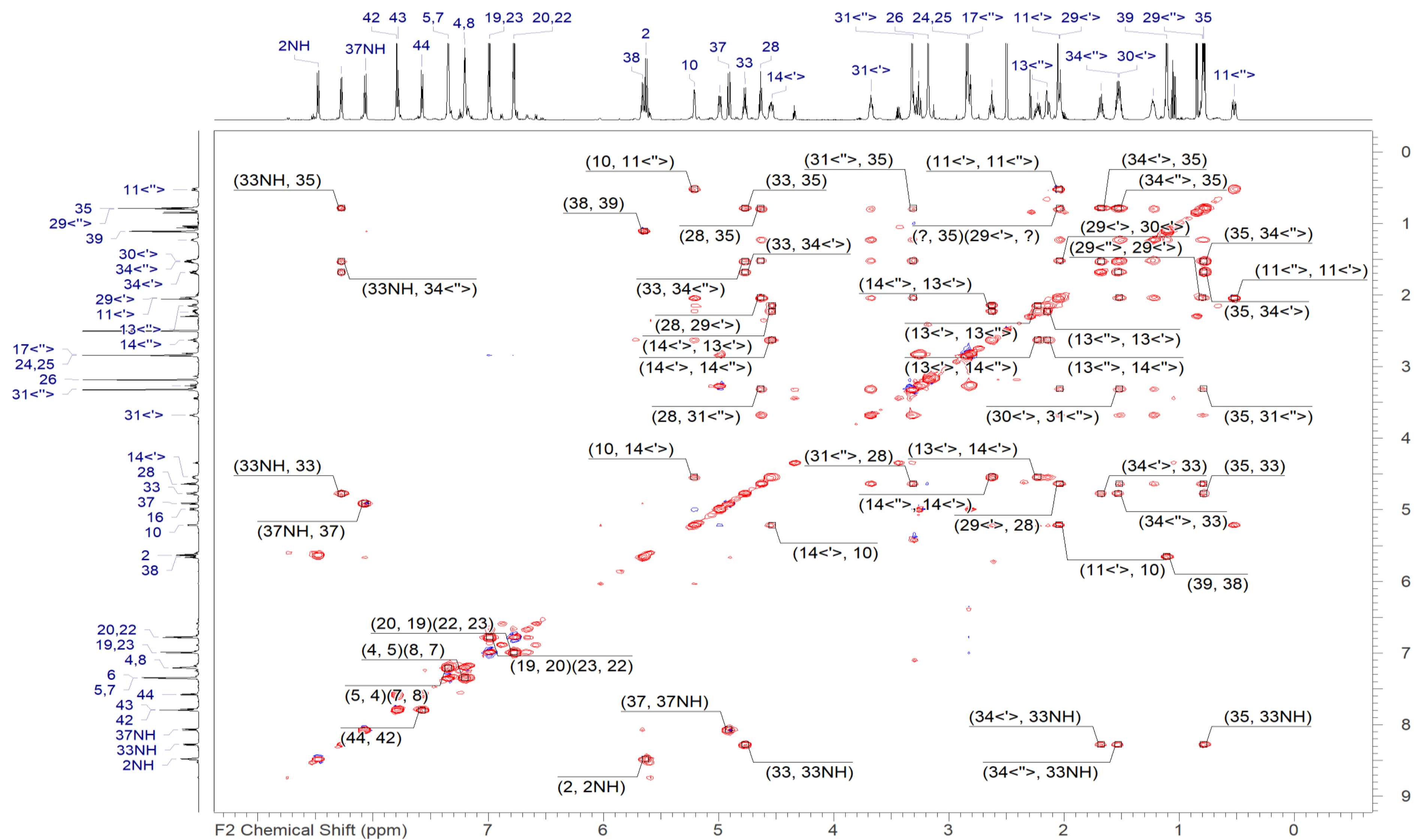



**Figure S8:** TOCSY NMR spectrum (700 MHz) pristinamycin I (**1**) in DMSO-$d_6$.

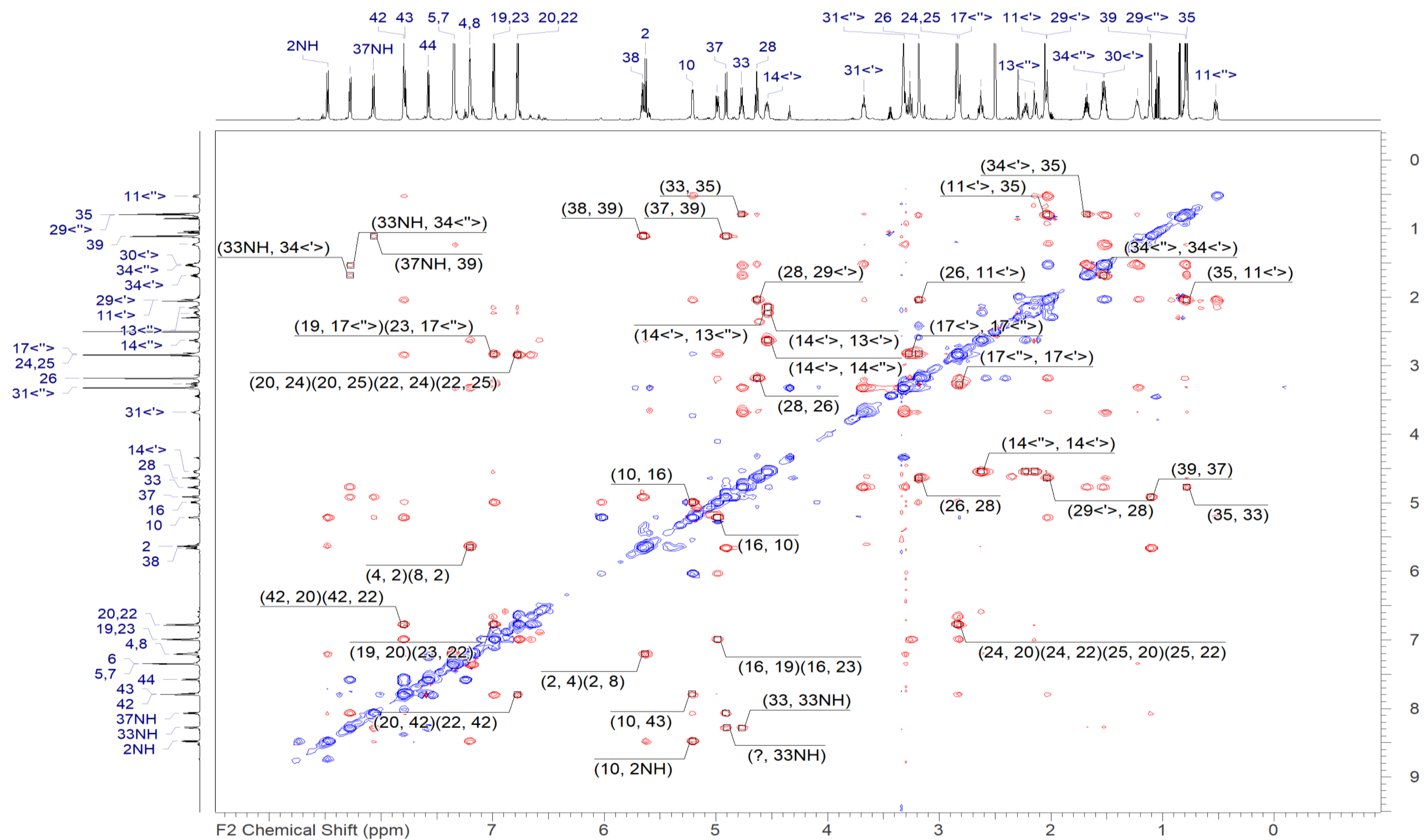



**Figure S9:** ROESY NMR spectrum (700 MHz) pristinamycin I (**1**) in DMSO-$d_6$.

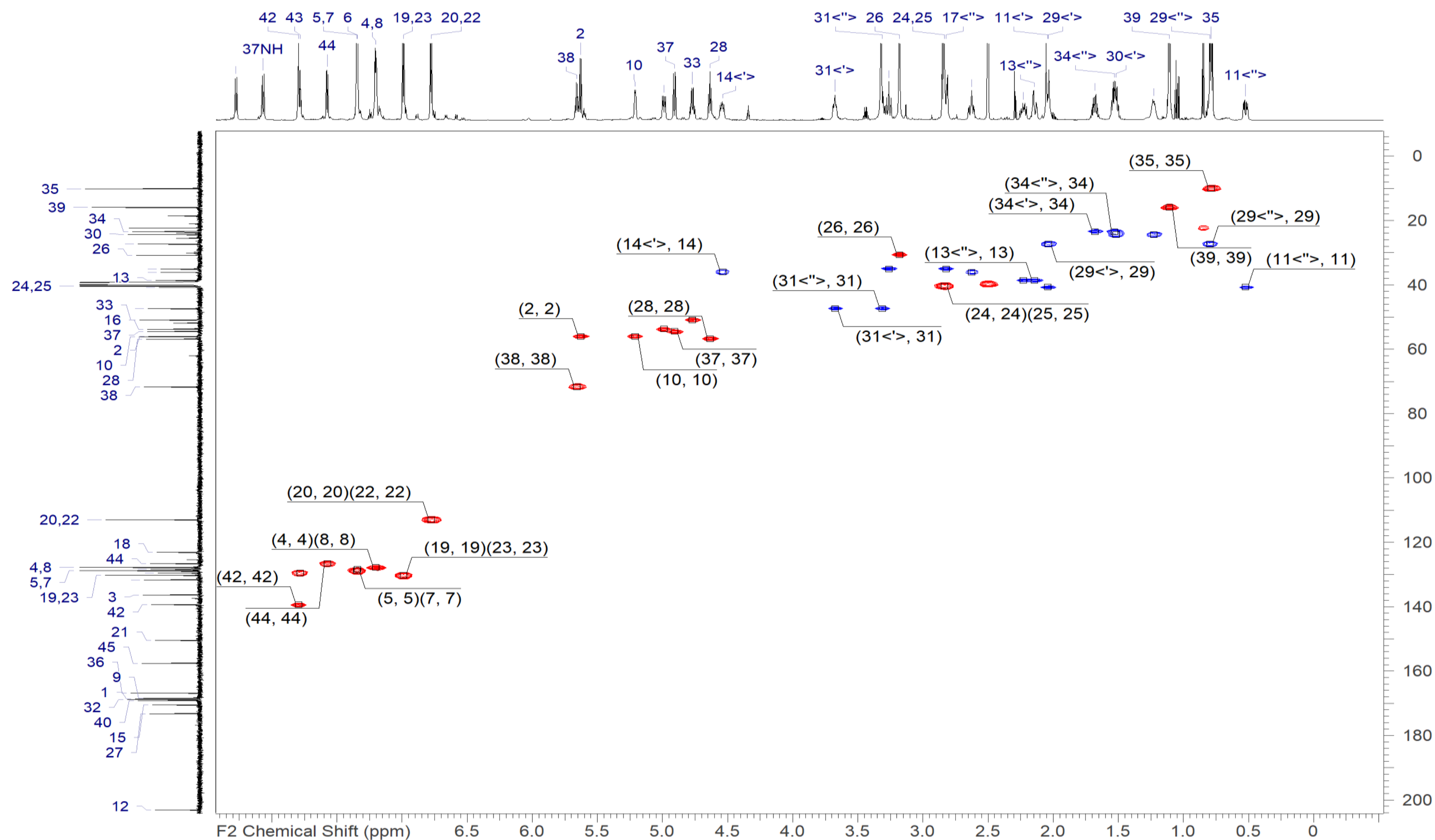



**Figure S10:** HSQC-DEPT NMR spectrum (700 MHz) pristinamycin I (**1**) in DMSO-$d_6$.

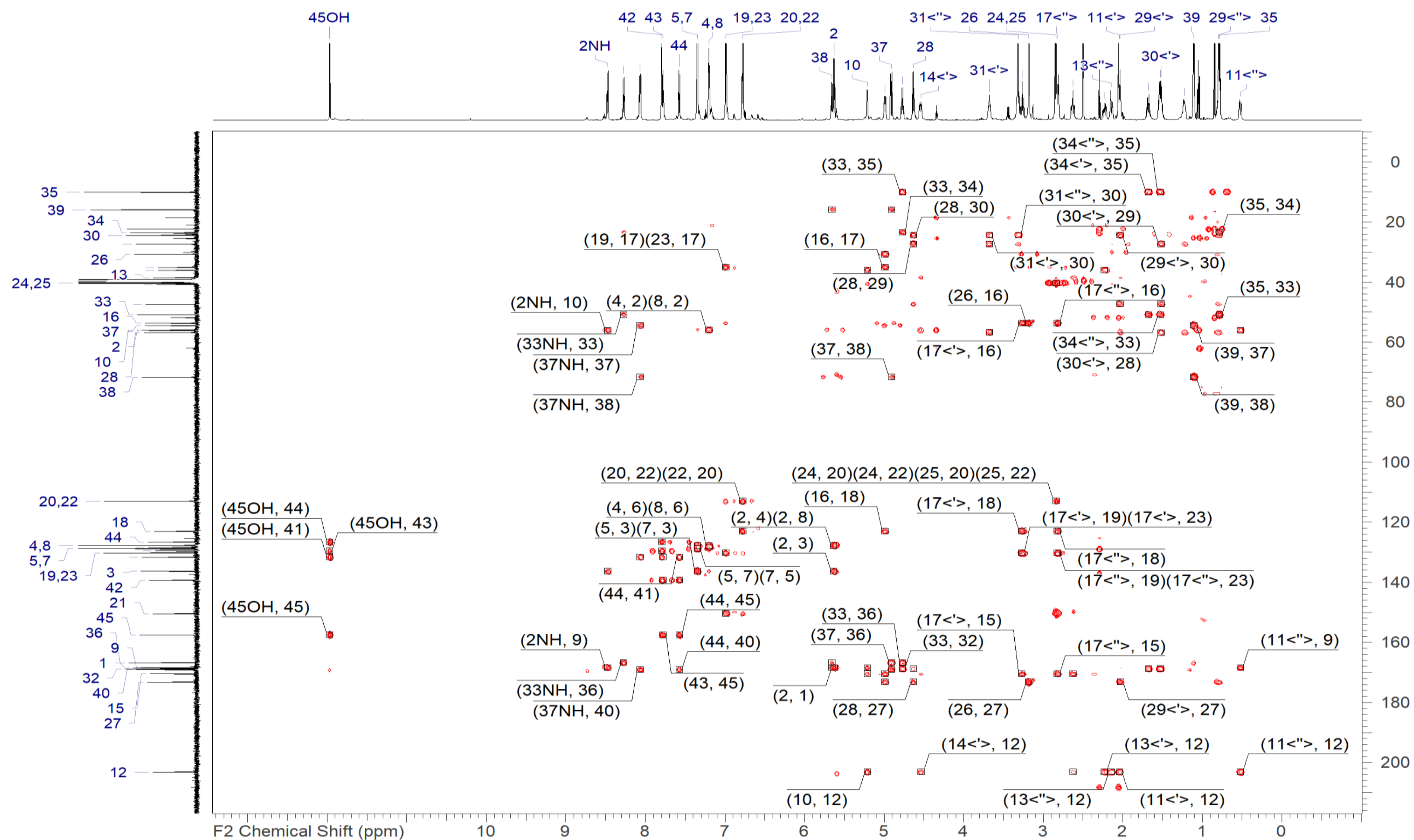



**Figure S11:** HMBC NMR spectrum (700 MHz) pristinamycin I (**1**) in DMSO-$d_6$.

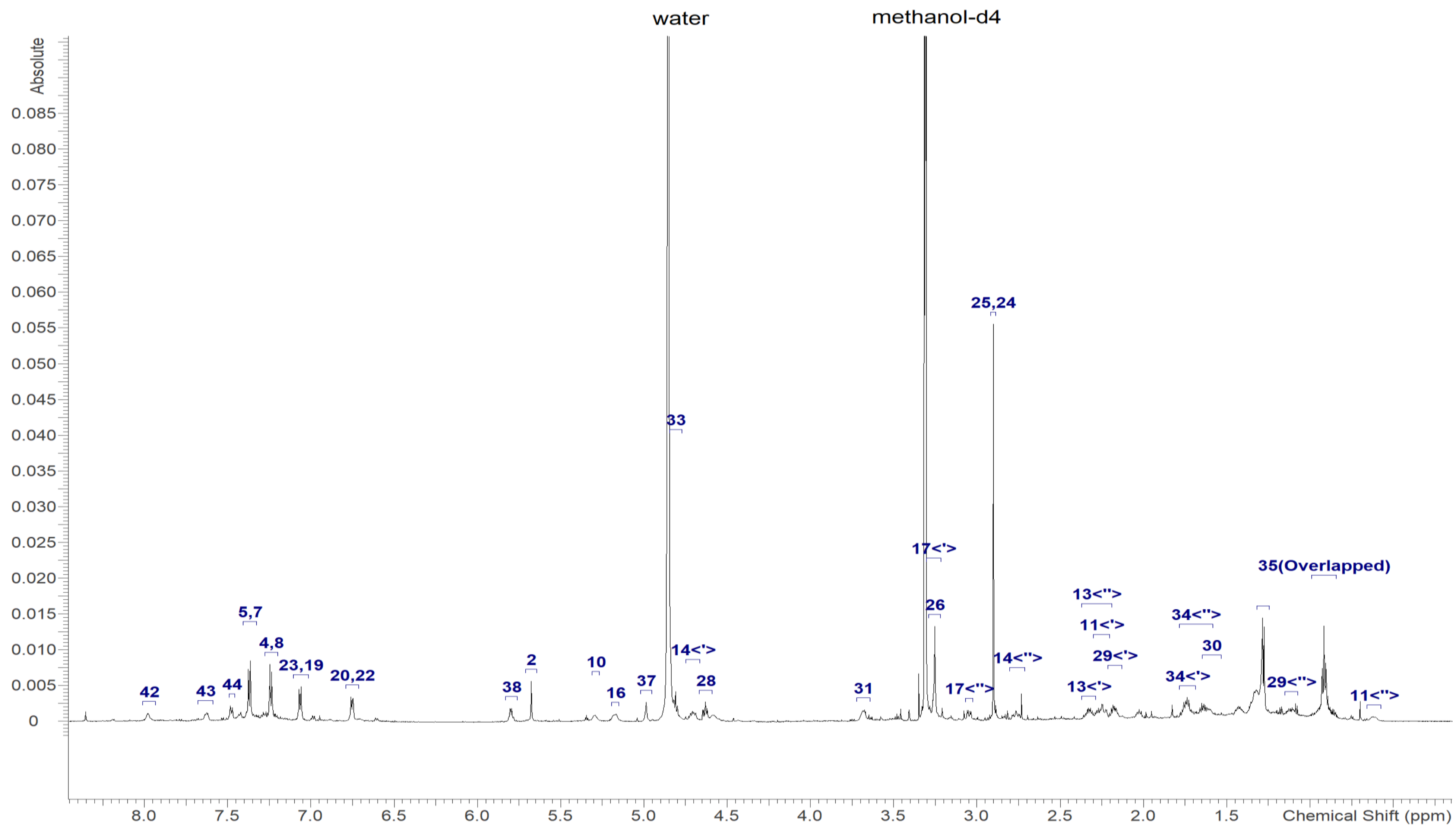



**Figure S12:** $^{1}H$ NMR spectrum (700 MHz) 6-chloropristinamycin I (**3**) in MeOD.

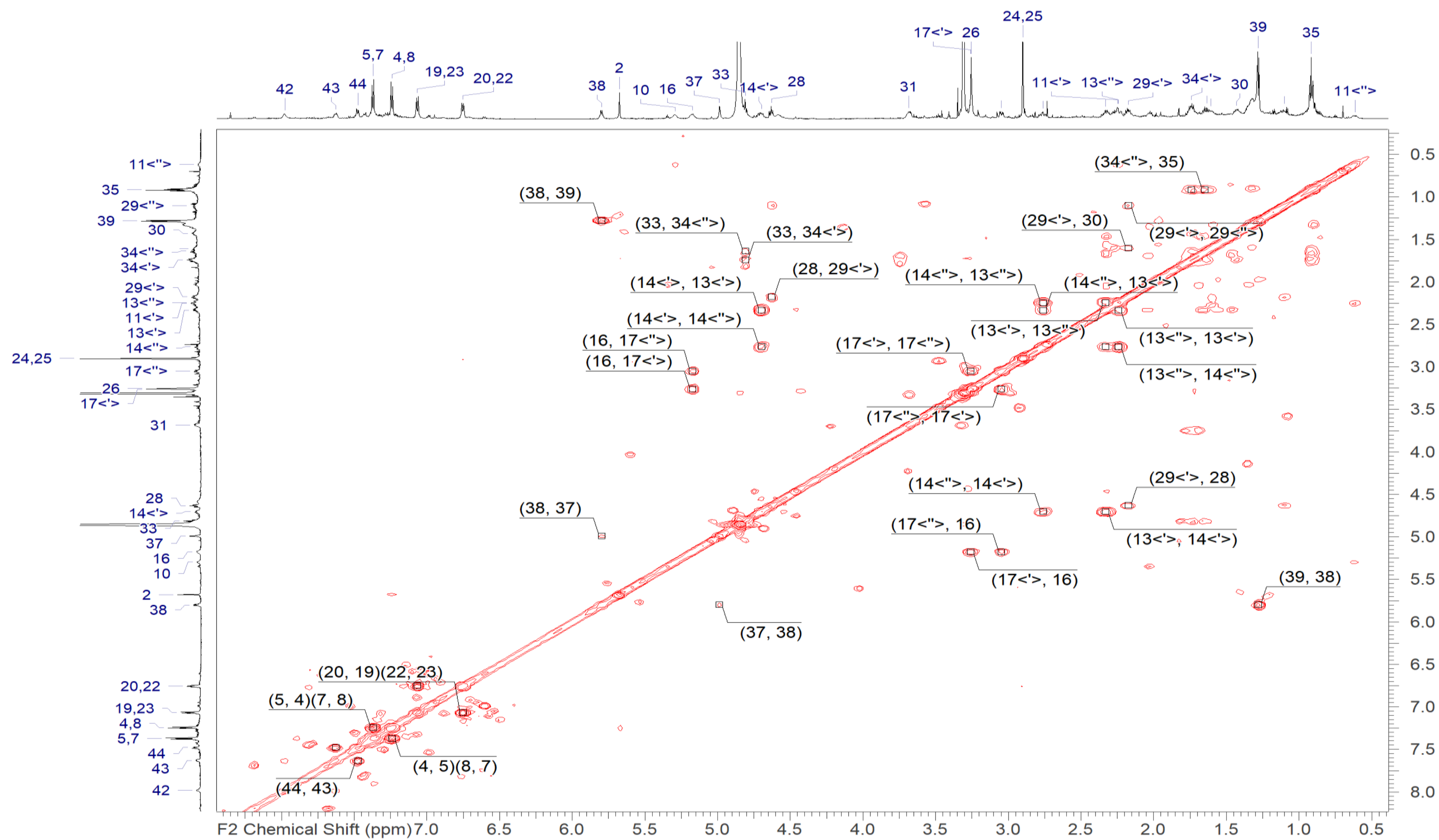


**Figure S13:** COSY NMR spectrum (700 MHz) 6-chloropristinamycin I (**3**) in MeOD.

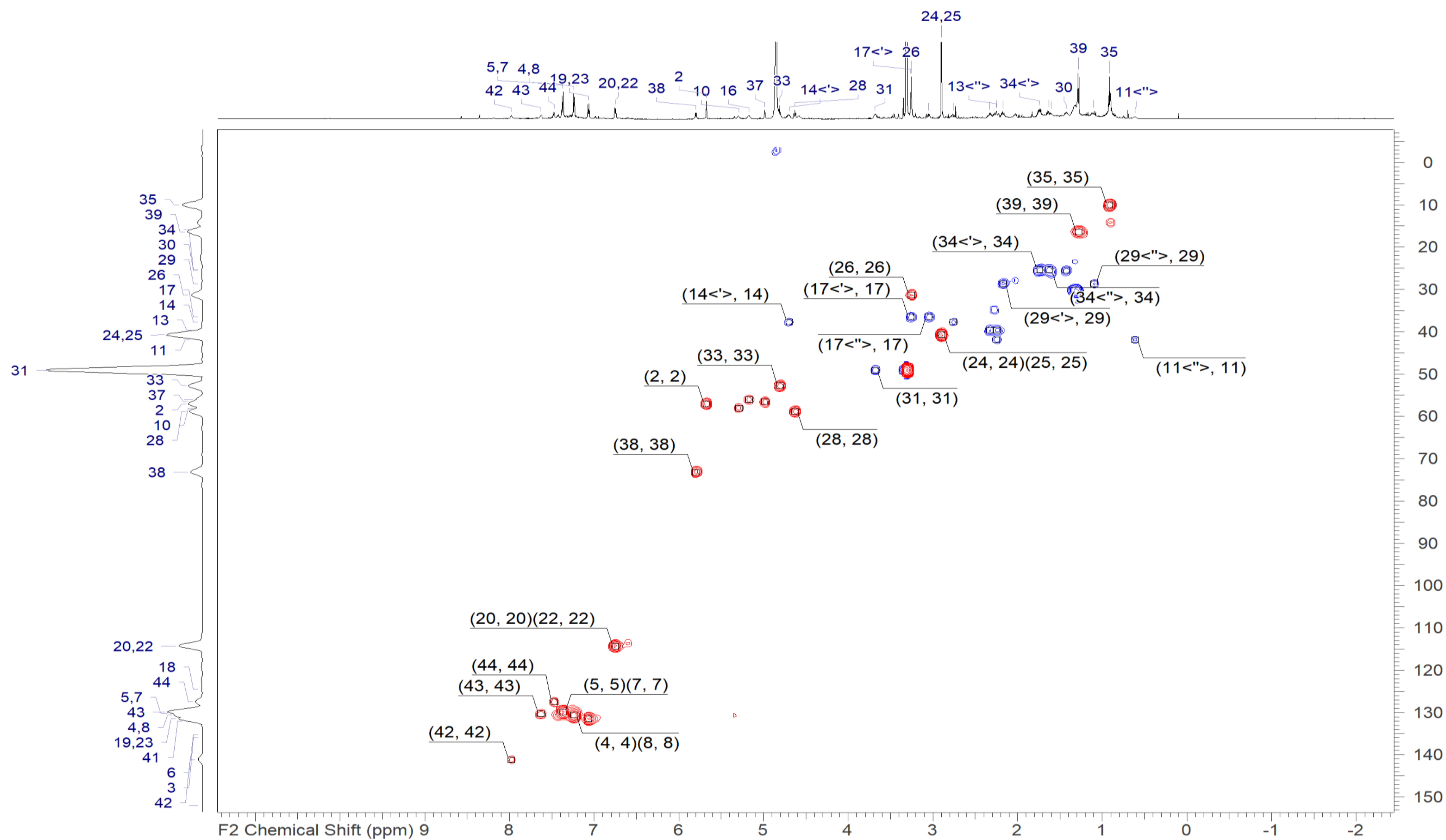



**Figure S14:** HSQC-DEPT NMR spectrum (700 MHz) 6-chloropristinamycin I (**3**) in MeOD.

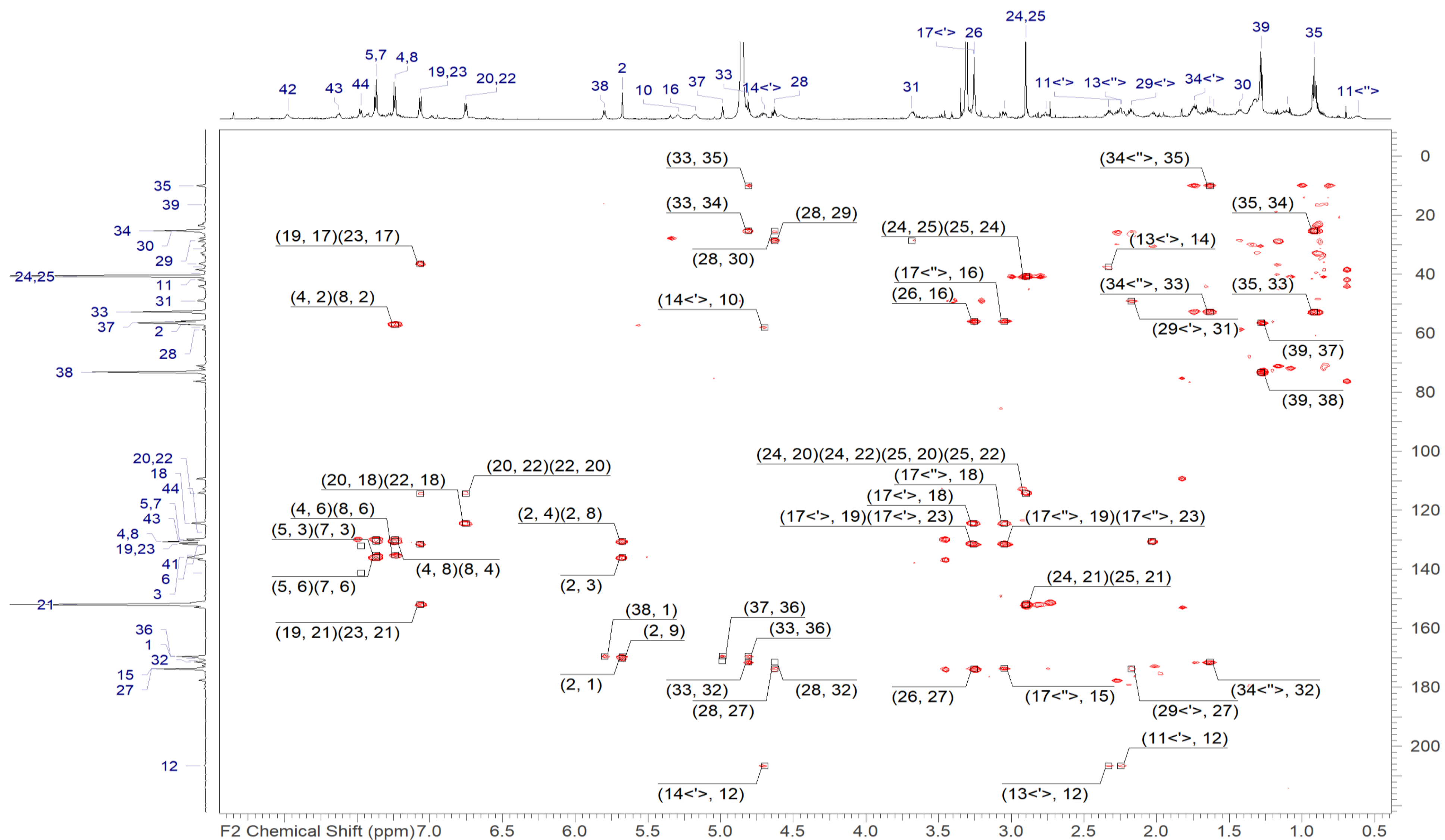



**Figure S15:** HMBC NMR spectrum (700 MHz) 6-chloropristinamycin I (**3**) in MeOD.

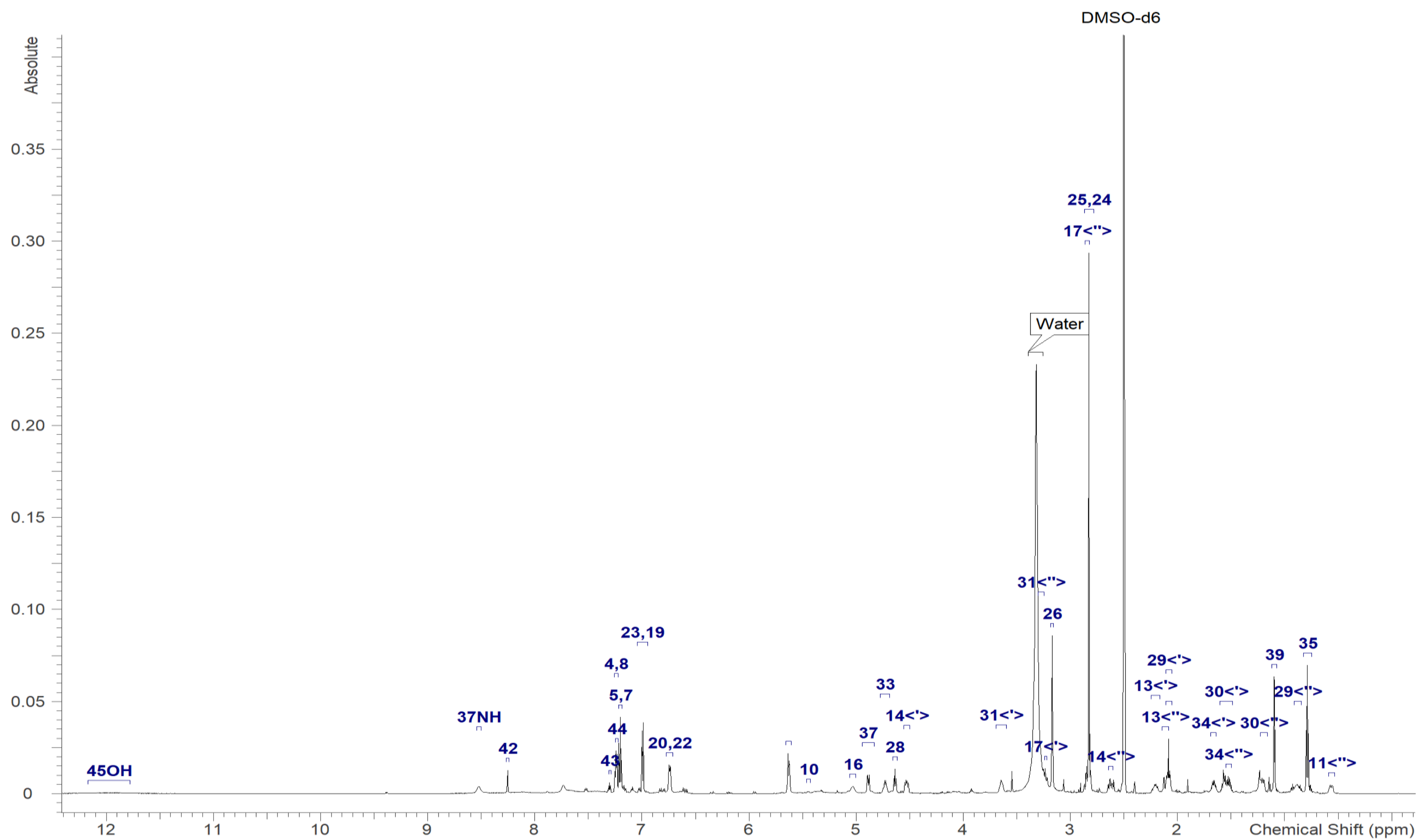



**Figure S16** $^{1}H$ NMR spectrum (700 MHz) 6-fluoropristinamycin I (**4**) in DMSO-$d_6$.

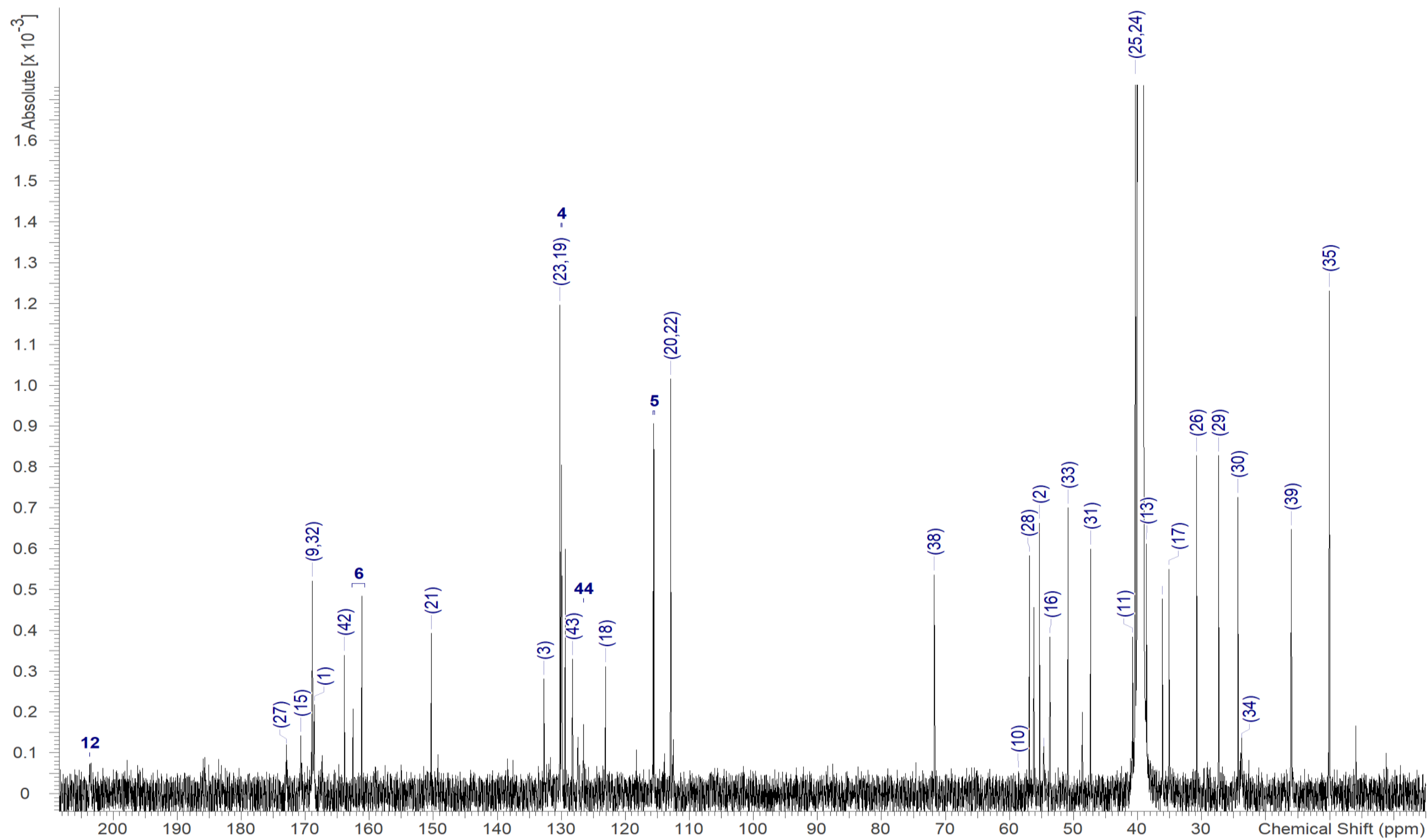



**Figure S17:** $^{13}$C NMR spectrum (700 MHz) 6-fluoropristinamycin I (**4**) in DMSO-$d_6$.

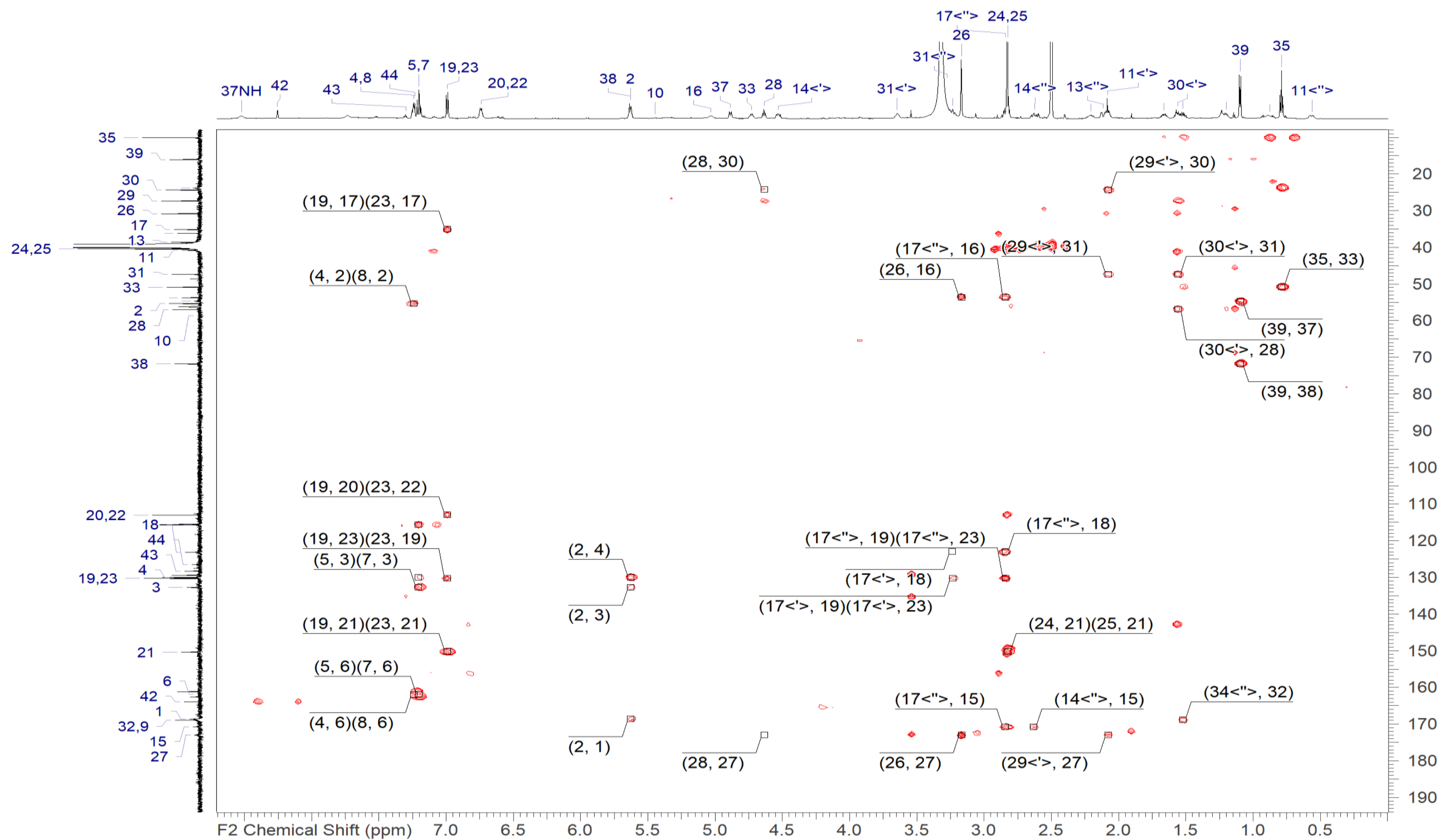



**Figure S18:** HMBC spectrum (700 MHz) 6-fluoropristinamycin I (**4**) in DMSO-$d_6$.

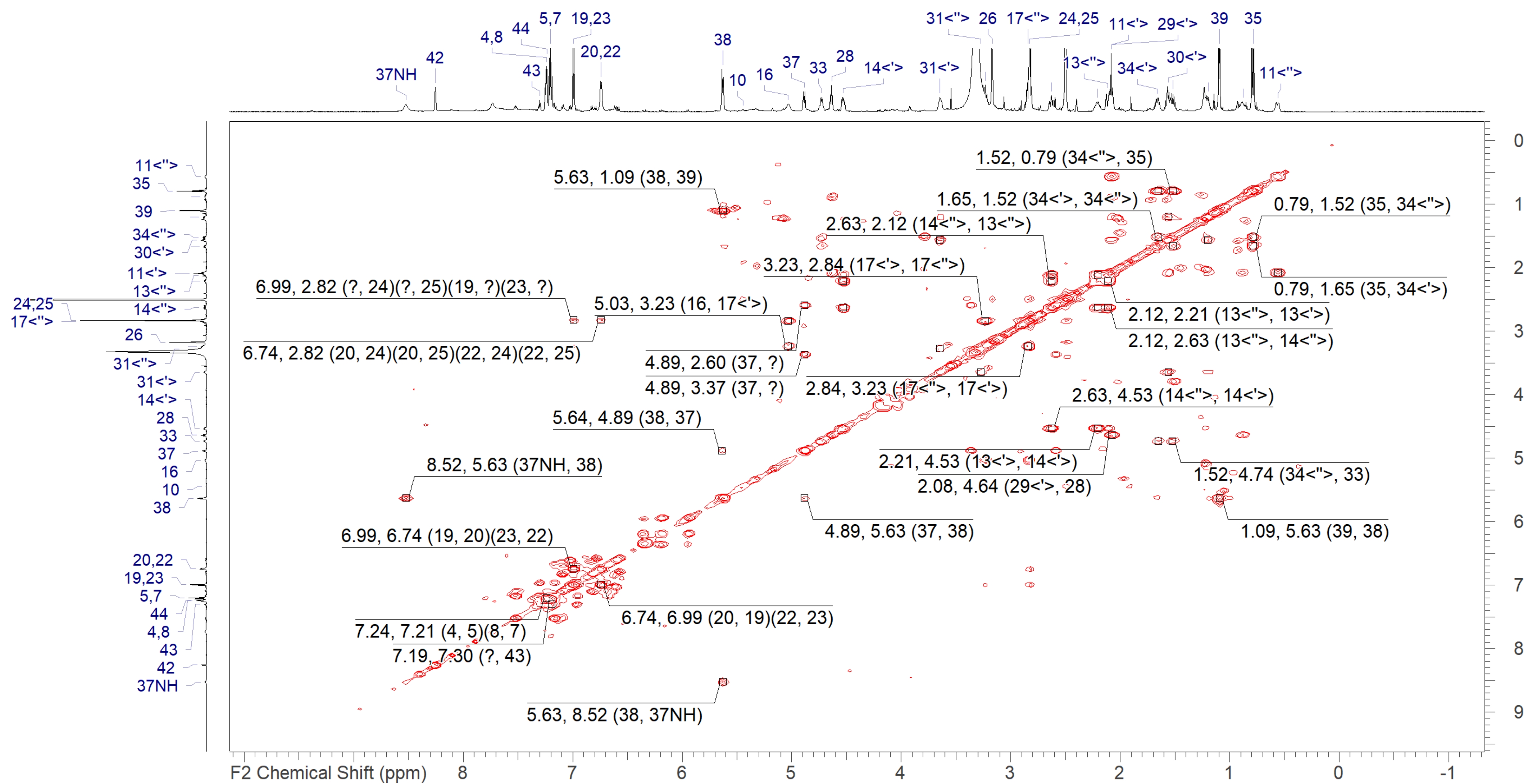



**Figure S19:** COSY spectrum (700 MHz) 6-fluoropristinamycin I (**4**) in DMSO-$d_6$.

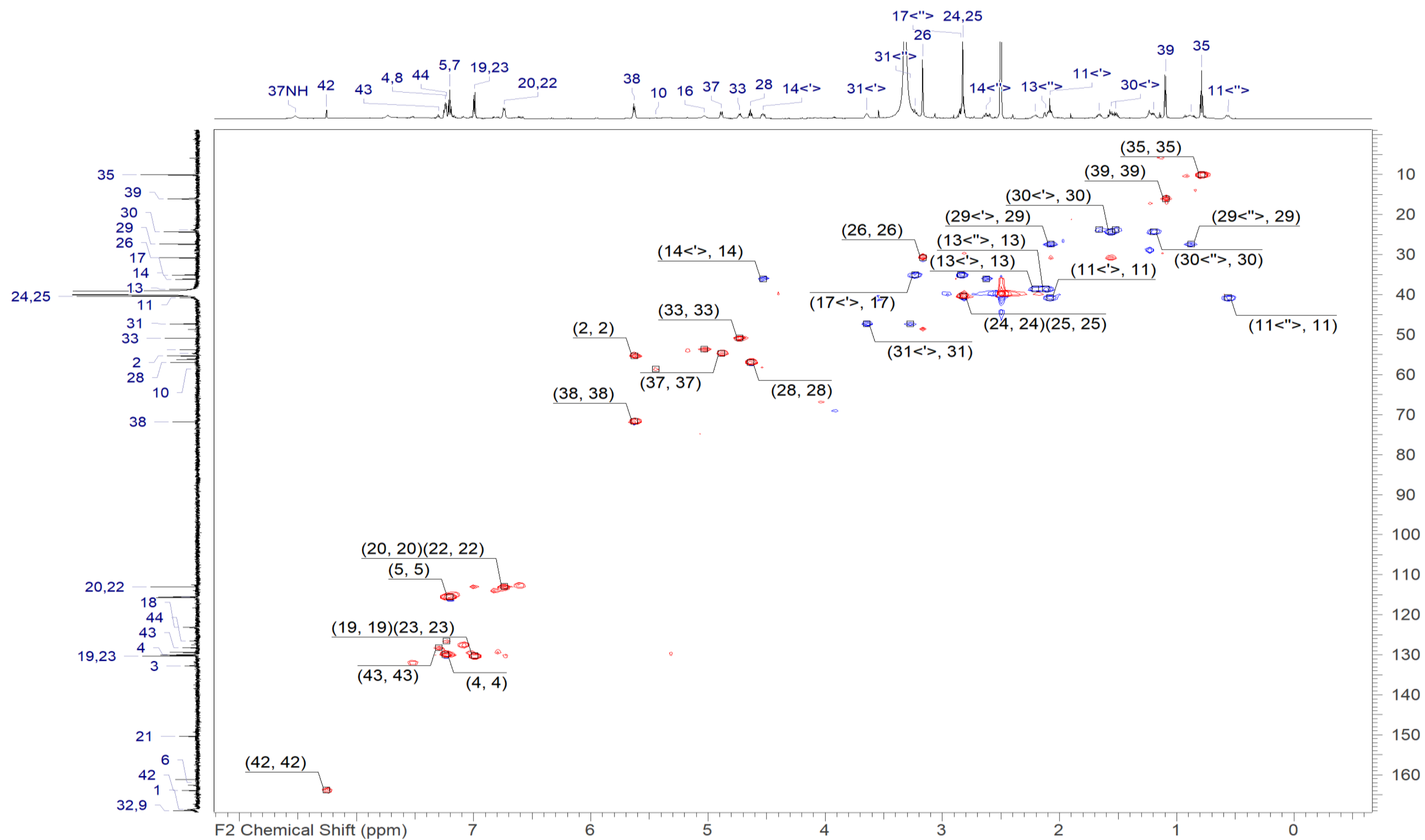



**Figure S20:** HSQC-DEPT NMR spectrum (700 MHz) 6-fluoropristinamycin I (**4**) in DMSO-$d_6$.

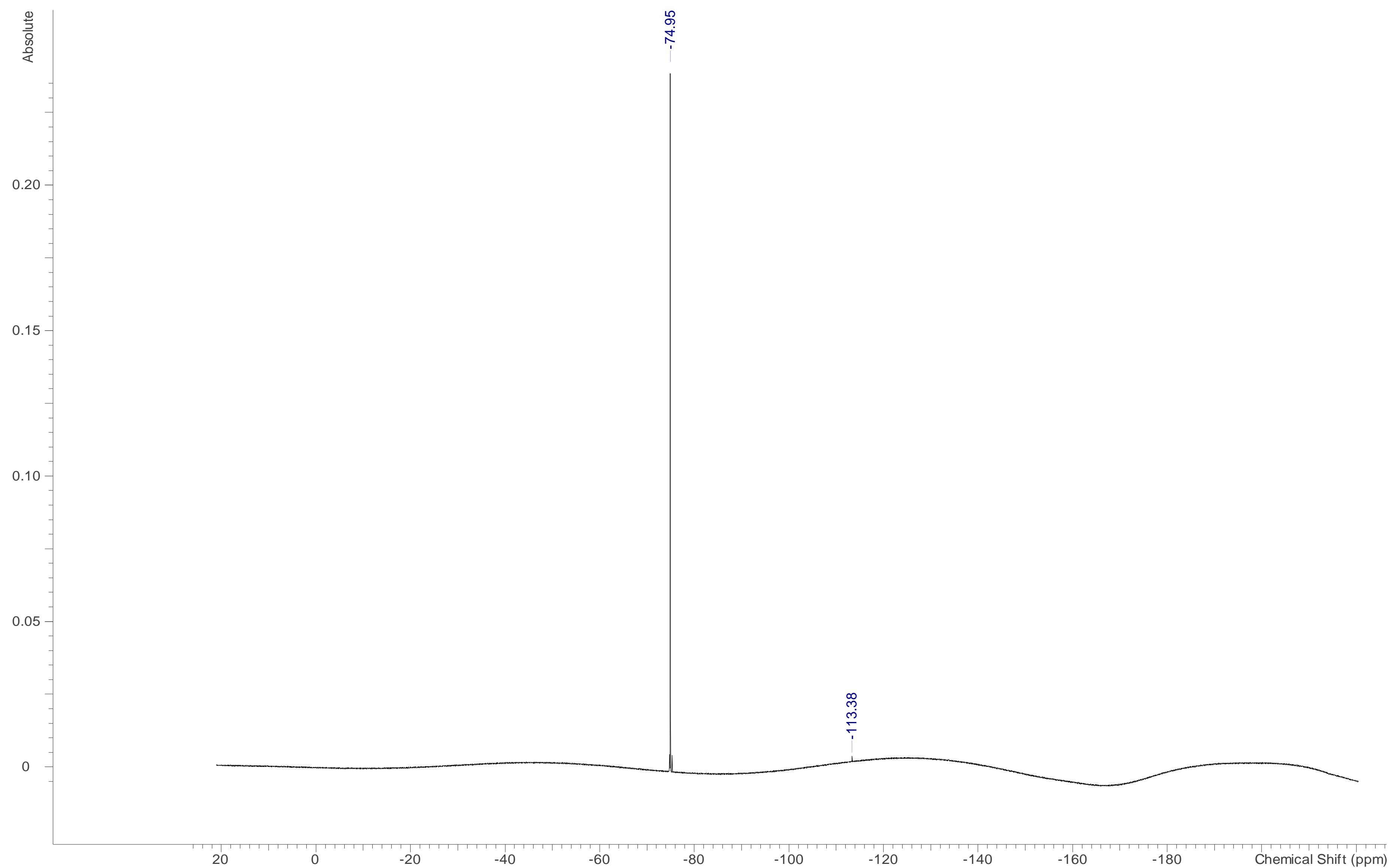
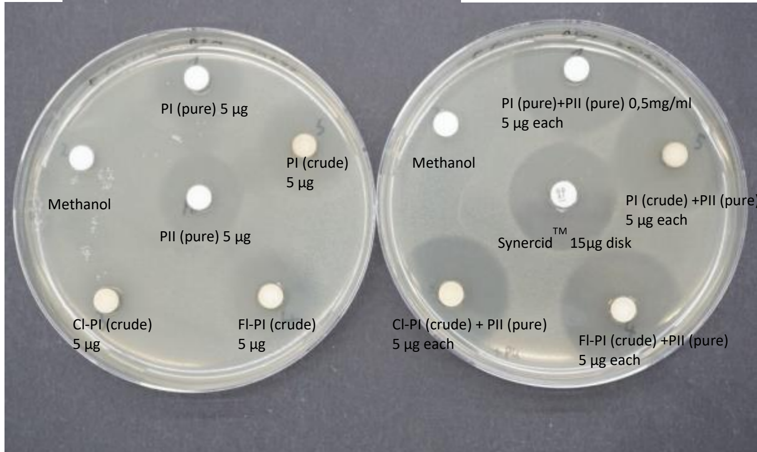
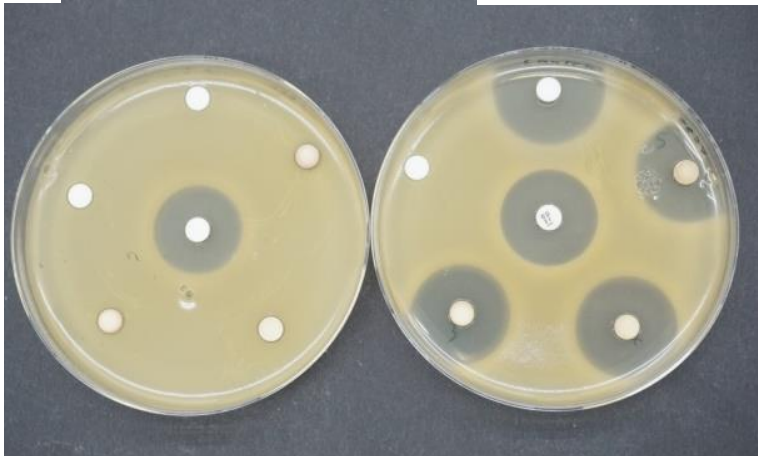


**Figure S21:** $^{19}$F NMR spectrum (470 MHz) 6-fluoropristinamycin I (**4**) in DMSO-$d_6$.

**A** *E. faecium* DSM 20477




**B** *S. aureus* DSM 18827



**C** *S. aureus* CIP 111304

**D** *S. aureus* CIP 108540

**Figure S22:** Results of the agar diffusion test of PI derivatives alone and in combination with PII against streptogramin susceptible clinical isolates. The pattern of sample application follows that of the *E. faecium* DSM 20477 example.

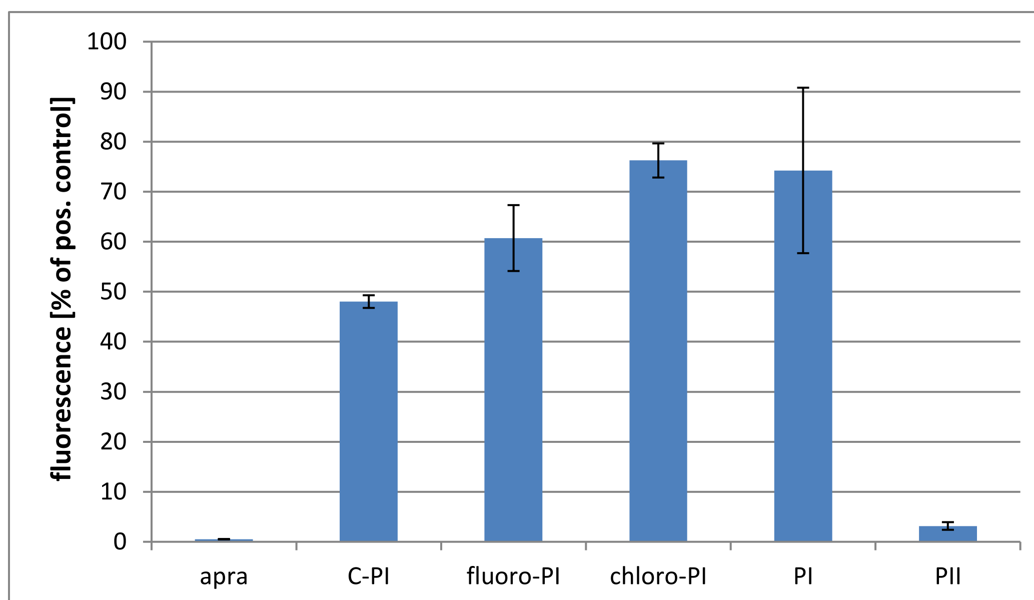



**Figure S23:** Results of the *in vitro* transcription translation assay used to investigate the effect of semi purified PI derivatives as well as pure pristinamycin I (PI) and II (PII) on bacterial protein biosynthesis. Apramycin (apra) served as a positive control (C-PI = PI isolated from *S. pristinaespiralis ΔpglAΔsnaE1* supplemented with L-Phg).

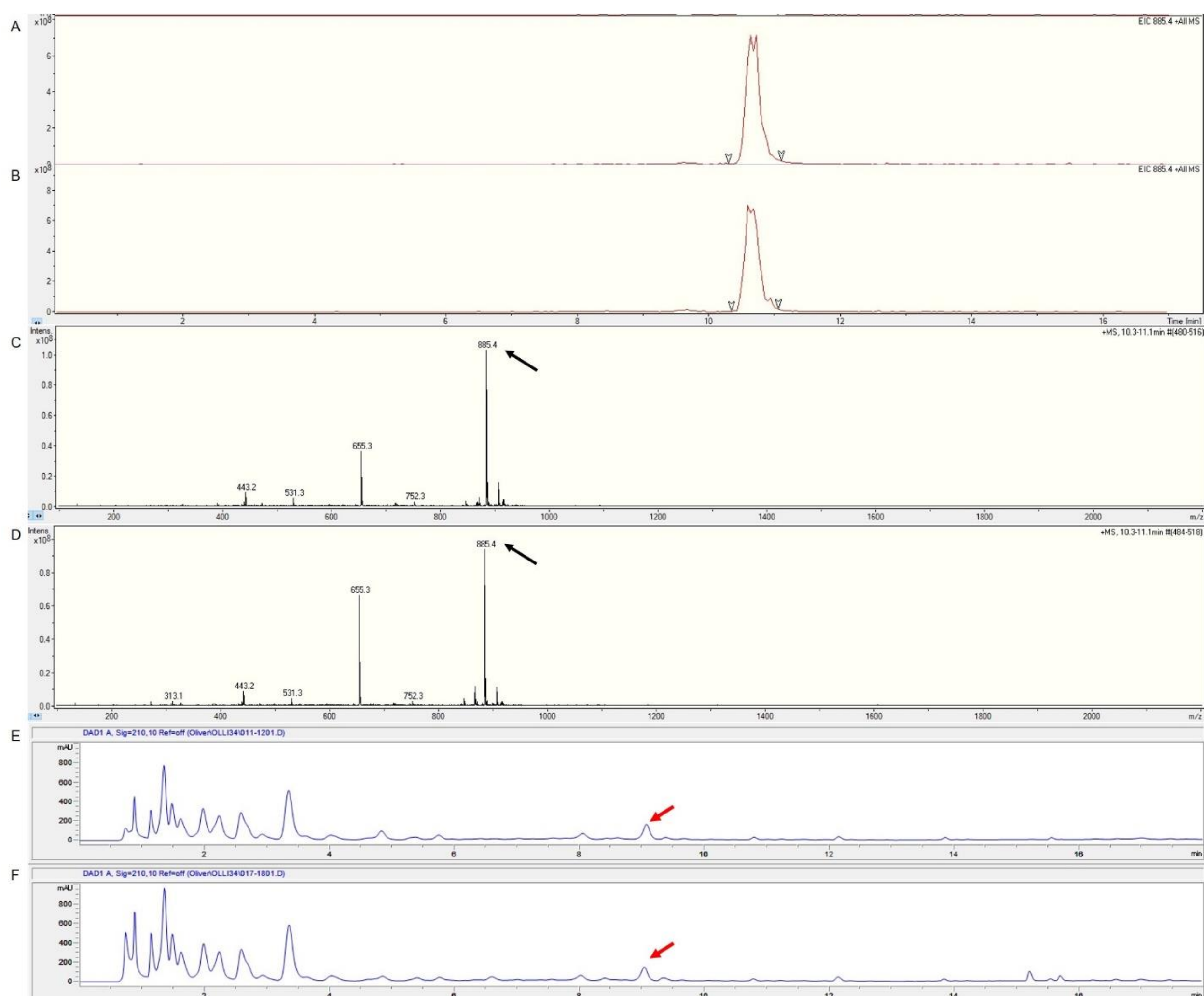



**Figure S24:** HPLC/MS analysis of *S. pristinaespiralis ΔpglAΔsnaE1* extracts from cultures supplemented with pure 4-fluoro-Phg (A, C, E) and 4-fluoro-Phg containing *E. coli* BL21(DE3) pET28-*hmo*/pACYC-*bcd-gdh* supernatant (B, D, F), respectively. A and B are the extracted ion chromatograms (EIC) for the mass corresponding to 6-fluoropristinamycin I (**4**) (*m/z* 885.4 $[M+H]^+$) in positive mode. C and D are the corresponding mass spectra. E and F are separate UV chromatograms at 210 nm (at the same scale). The mass signals for **4** are marked with black arrows and the respective UV signals are marked with red arrows.

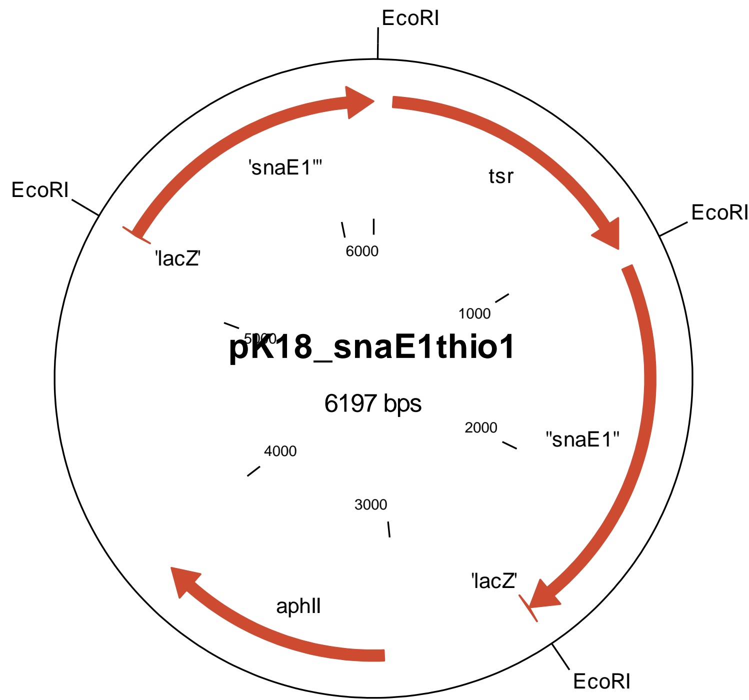



**Figure S25**: Cloning chart of mutagenesis plasmid pK18/snaE1tsr.

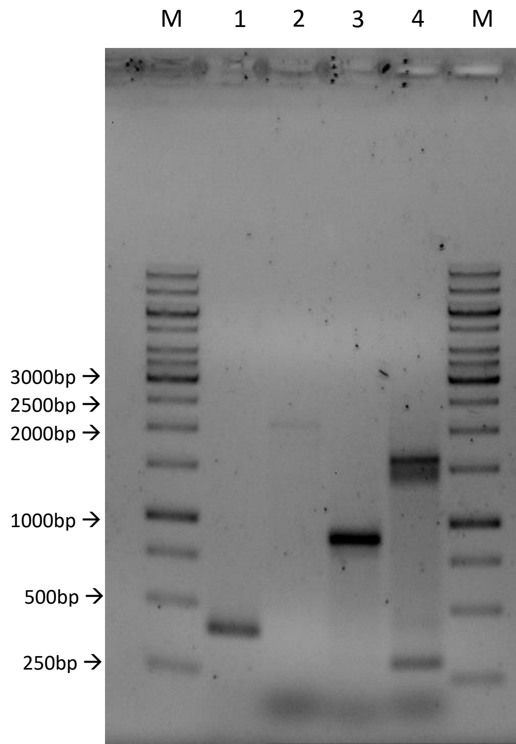



**Figure S26**: Verification of the *snaE1* mutation by PCR. Amplificates are shown for PCR with primer pairs thio1/2 (lane 1; ≈0.4 kb), KnsaE1fw/rv (lane 2; ≈2.1 kb), KnsaE1fw/thio2 (lane 3; ≈0.8 kb), and KnsaE1rv/thio1 (lane 4; ≈1.7 kb) separated in a 1% agarose gel. Marker (M): 1 kb length marker, Thermo Fisher Scientific.

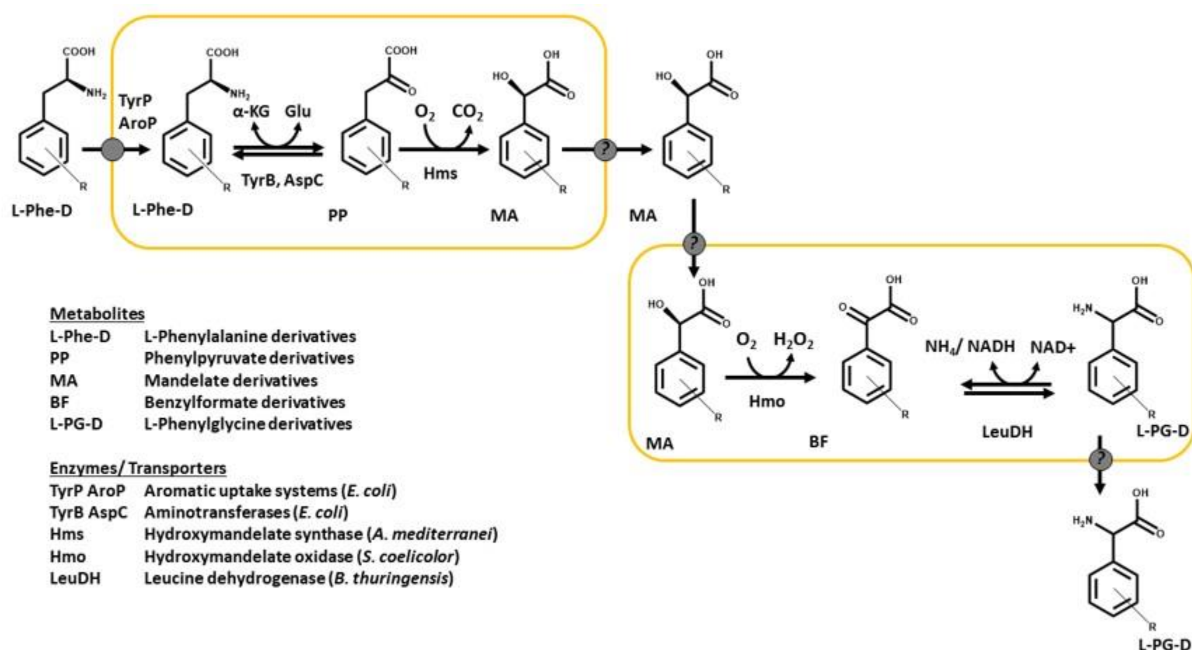



**Figure S27:** Schematic representation of the biotransformation approach for the synthesis of L-Phg derivatives including respective enzymes and metabolites involved in each step.

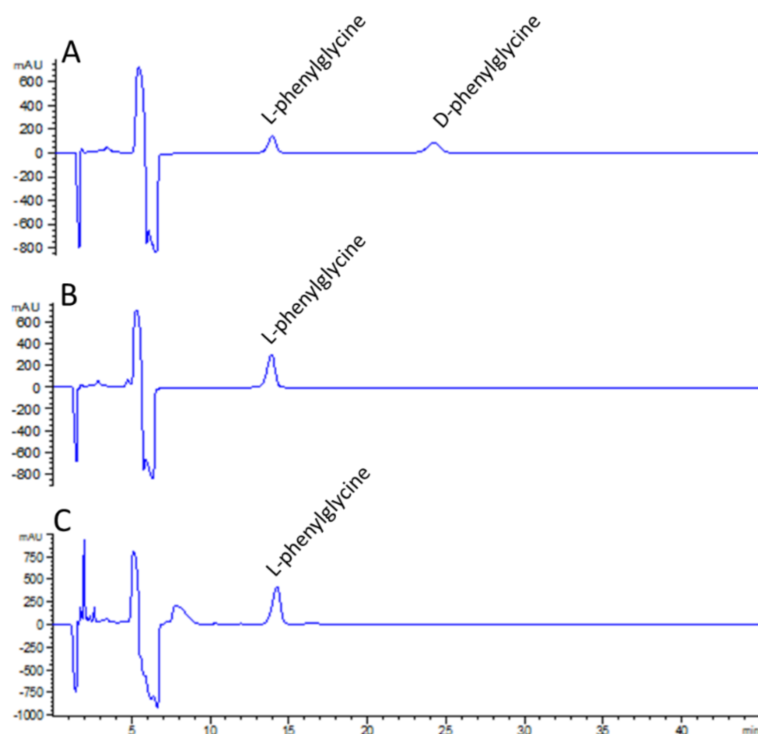



**Figure S28:** Representative chiral HPLC chromatogram of the separation of Phg enantiomers at 210 nm. Chromatogram A and B illustrate the separation of commercially available standards for DL-Phg and L-Phg. C is showing a typical chromatogram of the whole cell biotransformation approach with *E. coli* BL21(DE3) pET28-*hmo/* pACYC-*bcd-gdh* with L-mandelic acid.

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