*Supporting Information*

# Characterization of FK506 biosynthetic intermediates involved in post-PKS elaboration

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## Table of Contents

**Materials and Bacterial Strains.** FK506, nicotinamide adenine dinucleotide phosphate (NADPH), ferredoxin from *Spinacia oleracea* (spinach), ferredoxin-$NADP^+$ reductase from *S. oleracea* (spinach), *S*-(5′-Adenosyl)-L-methionine chloride dihydrochloride (SAM), ampicillin, apramycin, kanamycin, and nalidixic acid were purchased from Sigma. Ammonium acetate was purchased from Fluka, and HPLC-grade ethyl acetate, acetonitrile, methanol, glacial acetic acid, and water were supplied by J.T. Baker. His-Bind nickel chelate chromatography resin was obtained from Novagen. Restriction endonucleases and T4 DNA ligase were purchased from New England Biolabs. Polymerase chain reactions were carried out using *PfuTurbo* DNA Polymerase from Agilent Technologies. All other chemicals were of the highest purity available.

Bacterial strains used in this study are listed in Table S1. The FK506-producing strain *Streptomyces* sp. KCTC 11604BP was obtained from GenoTech (Daejeon, Republic of Korea). *Escherichia coli* DH5α[1] was used for routine subcloning, while *E. coli* BL21(DE3) and *E. coli* BL21(DE3)pLysS (Novagen) were used as heterologous hosts for expression of recombinant enzymes. *E. coli* ET12567/pUZ8002 was the nonmethylating plasmid donor strain[2] for intergeneric conjugation with *Streptomyces* sp. KCTC 11604BP.

**Gene inactivation of *fkbD* by in-frame deletion.** For in-frame deletion of the *fkbD* gene, the deletion plasmid was constructed by PCR amplification of the left- and right-flanking fragments from fosmid (fos1006D05; GenBank accession no. HM116536) DNA derived from *Streptomyces* sp. KCTC 11604BP. The primer pairs $FkbD_{in\text{-}frame}$-LF and $FkbD_{in\text{-}frame}$-LR were designed for the amplification of left-flanking fragment of *fkbD* gene, whereas $FkbD_{in\text{-}frame}$-RF and $FkbD_{in\text{-}frame}$-RR were for right-flanking fragment (Table S2). A total of 2 PCR fragments were separately cloned in pGEM-T Easy vector (Promega) and sequenced. After digestion with appropriate restriction enzymes, the fragments were cloned into pKC1139[3] digested with *Hind*III-*Xba*I or *Xba*I-*Eco*RV, to construct in-frame deletion plasmid. The *fkbD* in-frame deletion plasmid was then transferred by conjugation from *E. coli* ET12567/pUZ8002[2] to *Streptomyces* sp. KCTC 11604BP, as described elsewhere.[4] The ΔfkbD double cross-over mutant was selected by their apramycin-sensitive phenotype, verified by southern analysis (Figure S1).

**Gene inactivation of *fkbD* and *fkbM* by an apramycin resistance gene replacement.** The *fkbD* and *fkbM* were inactivated by gene replacement using the λ-RED PCR targeting method.

An apramycin resistance gene/*oriT* cassettes (*aac(3)IV-oriT*) for replacement were amplified. The primer pairs $FkbD_{apr}$-F and $FkbD_{apr}$-R were designed for deletion of *fkbD*, whereas $FkbM_{apr}$-F and $FkbM_{apr}$-R were for deletion of *fkbM* (Table S2). The amplified cassettes separately were then transferred by conjugation from *E. coli* ET12567/pUZ8002 to *Streptomyces* sp. KCTC 11604BP, as described elsewhere.[5] The double cross-over mutants were selected by their apramycin-resistant phenotype, verified by PCR analysis (Figure S2 and S3).

**Extraction and Isolation of the FK506 biosynthetic intermediates.** The 9 L culture broth of $\Delta fkbM_{apr}$ strain was centrifuged and the supernatant was subjected to solvent-solvent partition with ethyl acetate. The obtained extract was evaporated and the resultant brown residue was separated by preparative reversed-phase HPLC employing 90% aqueous methanol as mobile phase with a flow rate of 6 mL/min to afford five fractions. Obtained five fractions were subjected to HPLC-ESI-MS analysis and the fraction, major one, containing compound **2** was selected for further separation. This fraction was again injected to semipreparative reversed-phase HPLC separation using 53% aqueous acetonitrile as mobile phase with a flow rate of 2 mL/min to obtain four subfractions. Third subfraction was selected for purification after confirming the presence of the compound **2** by HPLC-ESI-MS and was purified on the same column using above conditions to yield pure compound as colorless solid (20 mg, $t_R$ 68 min).

Similarly the 8 L culture broth of $\Delta fkbD_{apr}$ strain was processed to obtain reddish brown extract which was fractionated by preparative reversed-phase HPLC using 80% aqueous acetonitrile as mobile phase with a flow rate of 6 mL/min. Resulting seven fractions were monitored for the presence of compound **3** using LC-ESI-MS analysis. The major fraction, fraction2, was further separated by semipreparative reversed-phase HPLC employing 50% aqueous acetonitrile as mobile phase with a flow rate of 2 mL/min to obtain four subfractions. Subfraction 4 containing compound **3** was purified by same column using 49% aqueous acetonitrile as mobile phase with a flow rate of 2 mL/min to afford pure compound **3** as an amorphous white solid (7 mg, $t_R$ 100 min).

The culture broth (8 L) of $\Delta fkbD_{in\text{-}frame}$ strain was partitioned with ethyl acetate leading a reddish brown extract followed by its fractionation by preparative reversed-phase HPLC using 80% aqueous methanol as mobile phase with a flow rate of 2 mL/min. Resulting five fractions were

monitored for the presence of compound **4** using LC-ESI-MS analysis. The major fraction, fraction5, was further separated by semipreparative reversed-phase HPLC employing 55% aqueous acetonitrile as mobile phase with a flow rate of 2 mL/min to obtain three subfractions. Subfraction 3 containing compound **4** was purified by same column using same HPLC conditions to afford pure an amorphous white solid (9 mg, $t_R$ 116 min).

The obtained crude extract from the culture broth 9 L of the strain *Streptomyces* sp. KCTC 11604BP was subjected to solvent-solvent partition between 90% methanol and *n*-hexane and the combined methanol layer was evaporated using rotary vacuum evaporator to yield brown residue. Obtained residue was chromatographed on a reversed-phase silica gel (YMC Gel ODS-A, 60 Å, 220 mesh) eluting with a step-gradient solvent system of 50 to 100% methanol/water to afford 20 fractions. Fraction 5 was subjected to reversed-phase HPLC on preparative column using 77% aqueous methanol with flow rate of 6 mL/min to obtain 3 subfractions. Subfraction 2 was found to contain trace amount of **5** on the basis of HPLC-ESI-MS/MS.

**Construction of protein expression plasmids.** pET28a (Novagen) containing an N, C-terminal $His_6$-tag was used for the expression of recombinant FkbD, whereas N-terminal $His_6$-tagged pET15b (Novagen) was used for FkbM. Amplification of FkbD was accomplished with the primers FkbD-ExF and FkbD-ExR. The PCR product was cloned into pET28a to generate pFKBD with an N, C-terminal $His_6$-tag (Tables S1, S2). Amplification of the DNA fragments containing FkbM was accomplished with the primers FkbM-ExF and FkbM-ExR. The PCR product was cloned into pET15b to produce pFKBM with an N-terminal $His_6$-tag (Tables S1, S2).

**Preparation and purification of recombinant FkbD and FkbM.** For the expression and purification of FkbD, the expression plasmid pFKBD was introduced into *E. coli* BL21(DE3) and the BL21(DE3)/pFKBD strain was grown in LB medium supplemented with 50 $\mu$g/mL kanamycin. Each liter of culture was inoculated with 10 mL of overnight starter culture. The culture was grown at 37 °C to an optical density ($OD_{600}$) of 0.6, and then expression was induced with 0.1 mM isopropyl-$\beta$-D-thiogalactopyranoside (IPTG). At the same time, the incubation temperature was shifted from 37 to 28 °C and the culture was grown for another 15 to 16 h. Cells were harvested by centrifugation (10 min at 6,000 × *g*), re-suspended in lysis buffer (50 mM sodium phosphate buffer, pH 8.0, containing 300 mM NaCl and 10 mM imidazole), and then

lysed by sonication. The lysate was clarified by centrifugation (30 min at 15,000 × *g*). Recombinant FkbD was purified by Ni-affinity chromatography according to the manufacturer's recommendations (Qiagen). The purified protein was subjected to 13% SDS-PAGE and visualized with Coomassie blue staining. The resultant protein was dialyzed against 50 mM phosphate buffer (pH 7.4) containing 1 mM EDTA, 1 mM dithiothreitol (DTT) and 10% glycerol, and then stored at -80 °C before use in the *in vitro* reaction.

For the expression and purification of FkbM, the expression plasmid pFKBM was introduced into *E.coli* BL21(DE3)pLysS, and the BL21(DE3)pLysS/pFKBM strain was grown in LB medium supplemented with 50 $\mu$g/mL ampicillin, and 25 $\mu$g/mL chloramphenicol. Each liter of culture was inoculated with 10 mL of overnight starter culture. The culture was grown at 37 °C to an $OD_{600}$ of 0.6, and then expression was induced with 0.1 mM IPTG. At the same time, the incubation temperature was shifted from 37 to 28 °C and the culture was grown for another 15 to 16 h. Recombinant FkbM was purified as described above. Protein concentrations were determined with a commercial Bio-Rad protein assay kit, and then corrected for the *in vitro* reactions.

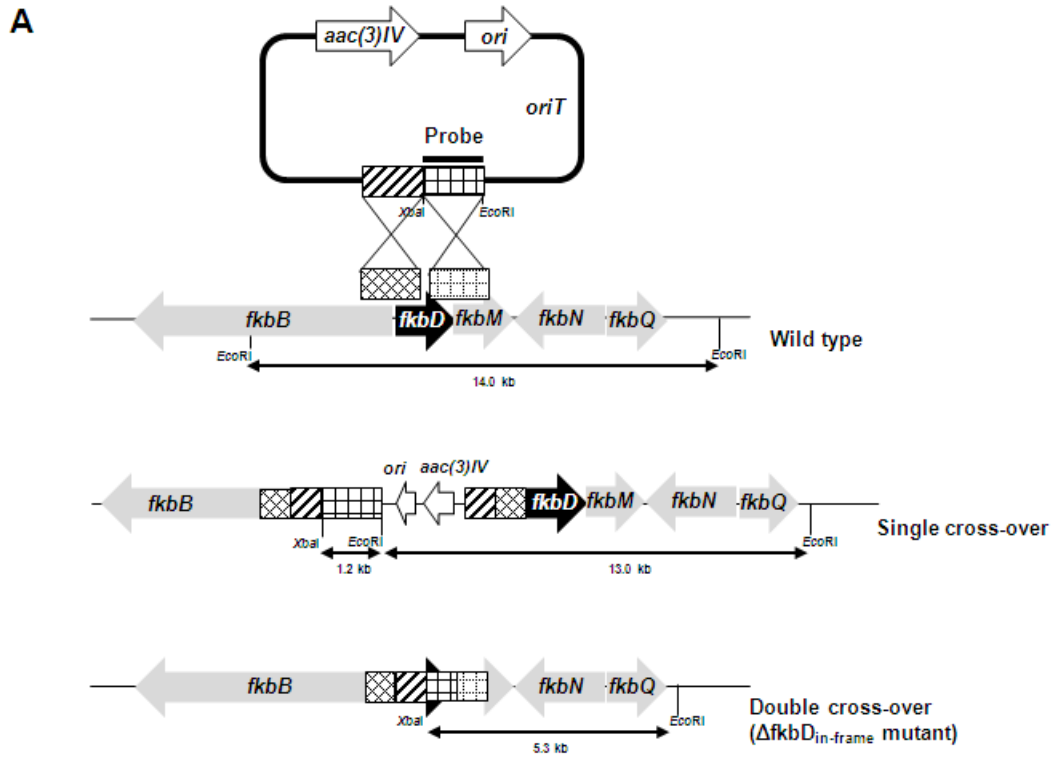
## Table S1. Bacterial strains and plasmids used in this study

| Strain/vector | Relevant characteristics | Reference |
|---|---|---|
| **Bacterial strains** | | |
| *Escherichia coli* | | |
| DH5α | Host for general cloning | New England Biolabs |
| BL21(DE3) | Host for protein expression | New England Biolabs |
| BL21(DE3)pLysS | Host for protein expression | New England Biolabs |
| ET12567/pUZ8002 | Donor strain for intergeneric conjugation between *E. coli* and *Streptomyces* | MacNeil, D.J. *et al*. |
| BL21(DE3)/pFKBD | Strain for FkbD protein expression | This study |
| BL21(DE3)pLysS/pFKBM | Strain for FkbM protein expression | This study |
| *Streptomyces* | | |
| KCTC 11604BP | Wild-type FK506-producing strain | This study |
| ΔFkbD$_{in\text{-}frame}$ | Mutant of KCTC 11604BP with an in-frame deletion of *fkbD*, produces **4** | This study |
| ΔFkbD$_{apr}$ | Mutant of KCTC 11604BP with an apramycin resistance gene replacement of *fkbD*, produces **3** | This study |
| ΔFkbM$_{apr}$ | Mutant of KCTC 11604BP with an apramycin resistance gene replacement of *fkbM*, produces **2** | This study |
| **Plasmids** | | |
| pCCFOS1 (fosmid) | Vector for genomic library construction | Epicentre Biotechnol. |
| Litmus 28 | Multi-purpose *E. coli* cloning vector | New England Biolabs |
| pGEM-Teasy | PCR fragment cloning vector | Promega |
| pKC1139 | High-copy-number temperature-sensitive *E. coli-Streptomyces* shuttle vector | Bierman, M. *et al*. |
| pET15b, pET28a | *E. coli* protein expression vector | Novagen |
| pΔFKBD$_{in\text{-}frame}$ | Deletion plasmid with in-frame deletion of 48-bp internal *fkbD* fragment | This study |
| pΔFKBD$_{apr}$ | Deletion plasmid with an apramycin resistance gene replacement of *fkbD* | This study |
| pΔFKBM$_{apr}$ | Deletion plasmid with an apramycin resistance gene replacement of *fkbM* | This study |
| pFKBD | N, C-terminal His$_6$-tagged FkbD expression plasmid based on pET28a(+) | This study |
| pFKBM | N-terminal His$_6$-tagged FkbM expression plasmid based on pET15b(+) | This study |

**Table S2. Primers used in this study**

| Primer | Sequence 5' to 3' (restriction site underlined) | Restriction enzyme |
|---|---|---|
| $FkbD_{in\text{-}frame}$-LF | TATAAAGCTTCGGAGCCCCGGTGGACCT | *Hind*III |
| $FkbD_{in\text{-}frame}$-LR | TTAATCTAGACGTCGCCTCGTCGTCGCT | *Xba*I |
| $FkbD_{in\text{-}frame}$-RF | GTAATCTAGAGTCGGCTACTGCCTCTAC | *Xba*I |
| $FkbD_{in\text{-}frame}$-RR | GAATGAATTCCGACGAACAGCGGTTCCT | *Eco*RI |
| $FkbD_{apr}$-F | ACGACGCCCGCCGGACGCTGCCCGTTCGCGATCCAGGACATTCCGGGGATCCGTCGACC | |
| $FkbD_{apr}$-R | GGCGAGGCGGACGTCCGGGAAGCGTTCGAACAACCGCAGTGTAGGCTGGAGCTGCTTC | |
| $FkbM_{apr}$-F | CAGCCCGGCCGAACTGCGGATCACCTGGGGTACGGCGTGATTCCGGGGATCCGTCGACC | |
| $FkbM_{apr}$-R | CCGGGGCGACCGGTGCGGGTAGCCGCCCCCGGCGGCTCATGTAGGCTGGAGCTGCTTC | |
| $FkbD_{apr}$-CheckF | CACCGTGCTCGAACTCCTGAC | |
| $FkbD_{apr}$-CheckR | CACGTCACCCGGGAGCAGTTC | |
| $FkbM_{apr}$-CheckF | CCGGCTGCTCATCAAGGTCGC | |
| $FkbM_{apr}$-CheckR | TGGGGCGTACGAACAGGGAGA | |
| FkbD-ExF | TATTCCCATATGAGCACCGACACACCCG | *Nde*I |
| FkbD-ExR | TAATCTCGAGCGCCGTACCCCAGGTGAT | *Xho*I |
| FkbM-ExF | TTAATTCATATGACCCCGGTGCCGGAGA | *Nde*I |
| FkbM-ExR | AATTAACTCGAGTCAGCCGGCCGGCCGCCGT | *Xho*I |

**Figure S1.** Construction and verification of *fkbD* in-frame deletion in *Streptomyces* sp. KCTC 11604BP. (A) Schematic representation of *fkbD* in-frame deletion by homologous recombination. (B) Southern blot analysis. 1, DNA fragments (14.0 kb) obtained from wild type strain;2, DNA fragments (1.2 kb and 13.0 kb) obtained from crossover mutant;3 and 4, DNA fragments (14.0 kb) obtained from wild-type revertant; 5 and 6, DNA fragments (5.3 kb) obtained from $\Delta fkbD_{in\text{-}frame}$ mutant; L, DNA ladder. The indicated *Xba*I-*Eco*RI fragment of 1.2 kb was used a probe.



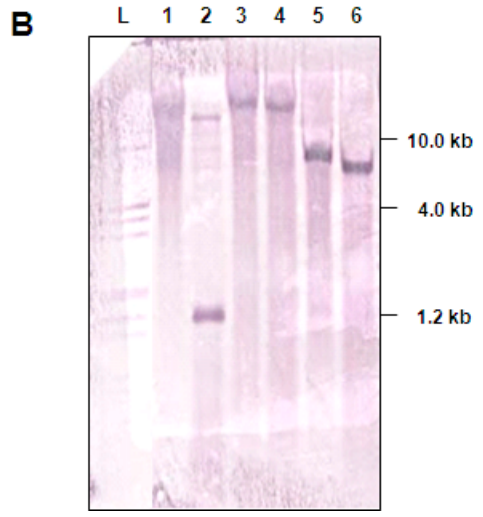
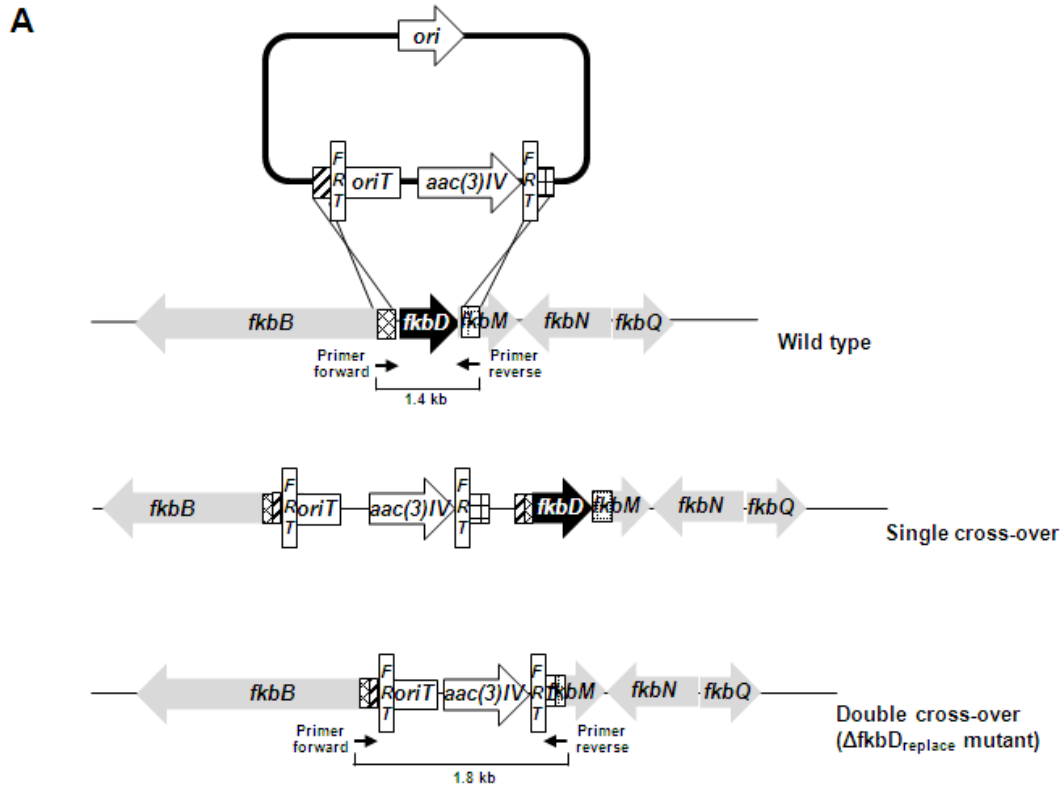



**Figure S2.** Construction and verification of *fkbD* inactivation with an apramycin resistance gene replacement in *Streptomyces* sp. KCTC 11604BP. (A) Schematic representation of *fkbD* deletion with gene replacement by homologous recombination. (B) PCR analysis. 1, PCR products (1.4 kb) obtained from wild type strain; 2, PCR products (1.8 kb) obtained from $\Delta$fkbD$_{apr}$ mutant; L, DNA ladder.



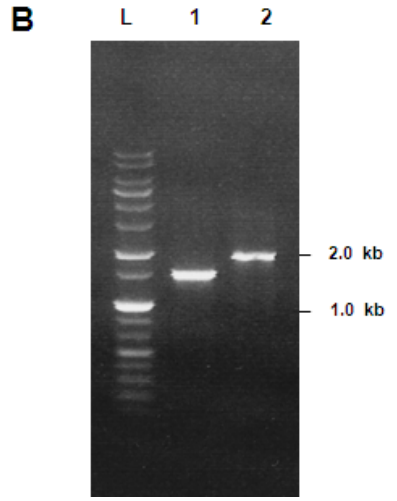



**Figure S3.** Construction and verification of *fkbM* inactivation with an apramycin resistance gene replacement in *Streptomyces* sp. KCTC 11604BP. (A) Schematic representation of *fkbM* deletion with gene replacement by homologous recombination. (B) PCR analysis. 1, PCR products (1.1 kb) obtained from wild type strain; 2, PCR products (1.7 kb) obtained from $\Delta$fkbM$_{apr}$ mutant strain; L, DNA ladder.

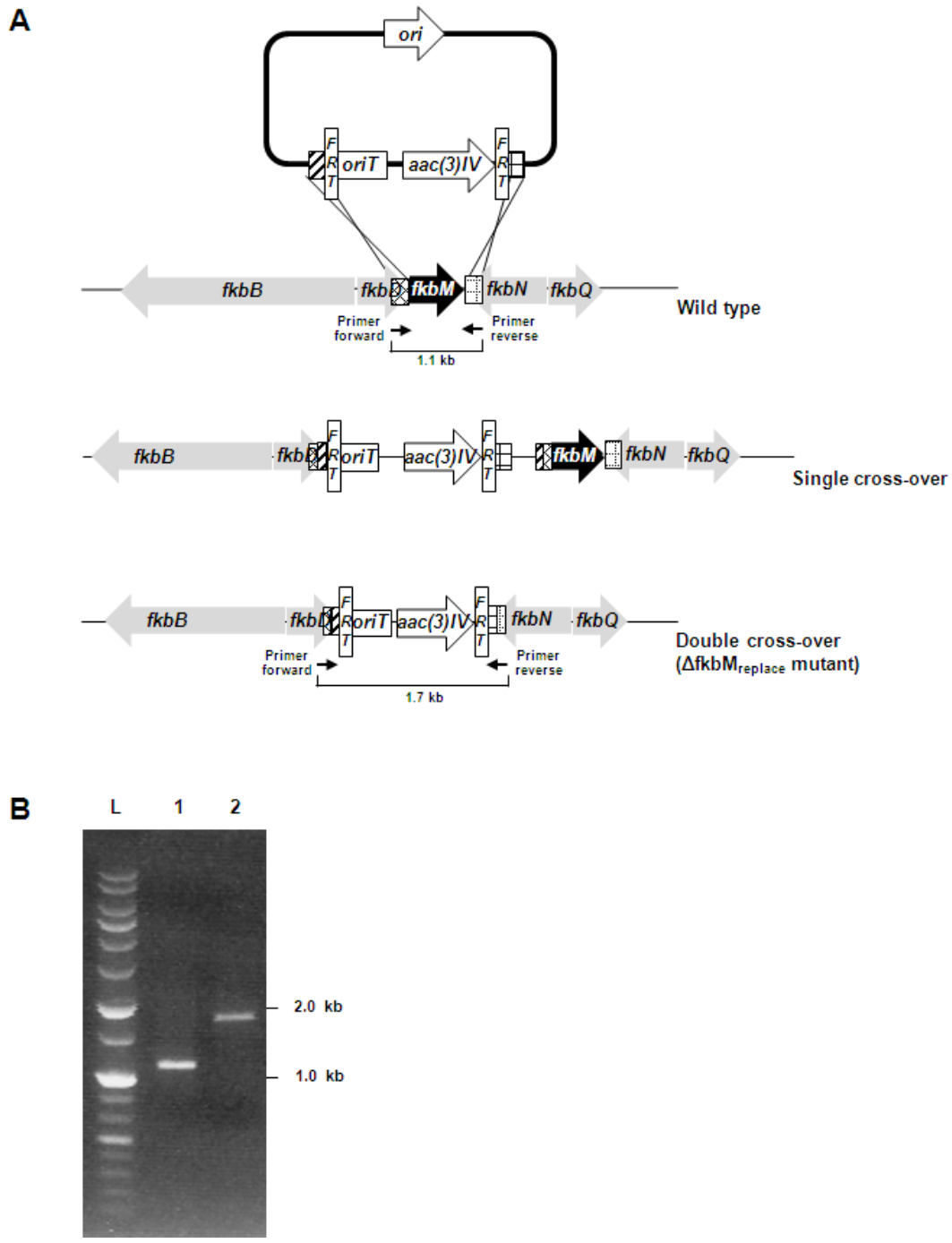
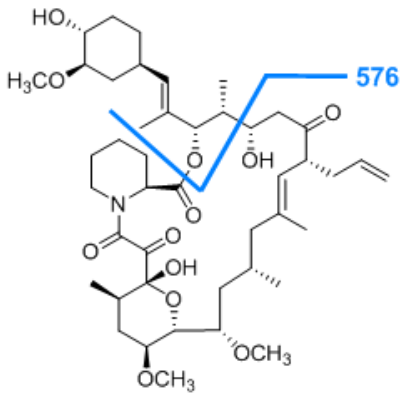


**Figure S4.** ESI-MS/MS analysis of FK506 (**1**). (A) ESI-MS/MS fragmentation pattern. (B) MS/MS spectra

**A**




**FK506**
**(1)**

**B**

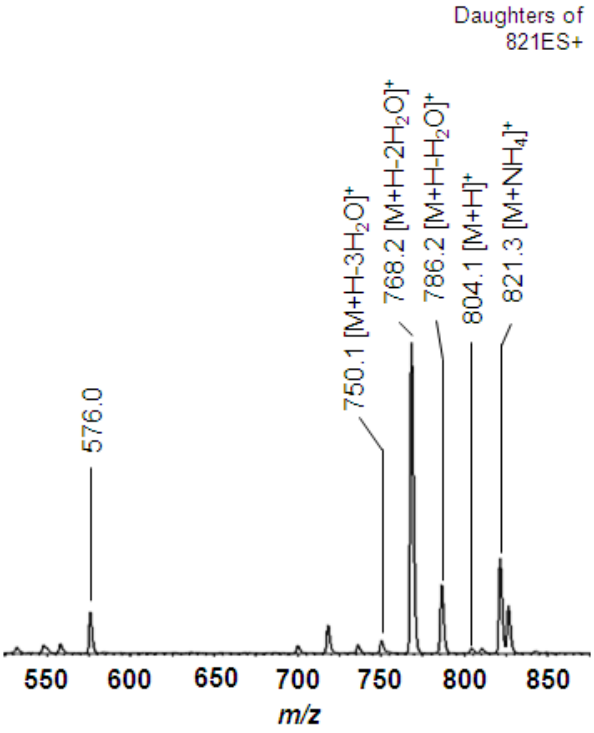
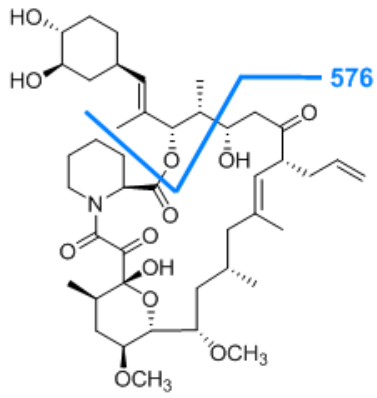


**Figure S5.** ESI-MS/MS analysis of 31-*O*-demethylFK506 (**2**) obtained from ΔfkbM$_{apr}$ strain. (A) ESI-MS/MS fragmentation pattern. (B) MS/MS spectra.

**A**




**31-*O*-demethylFK506**
**(2)**

**B**

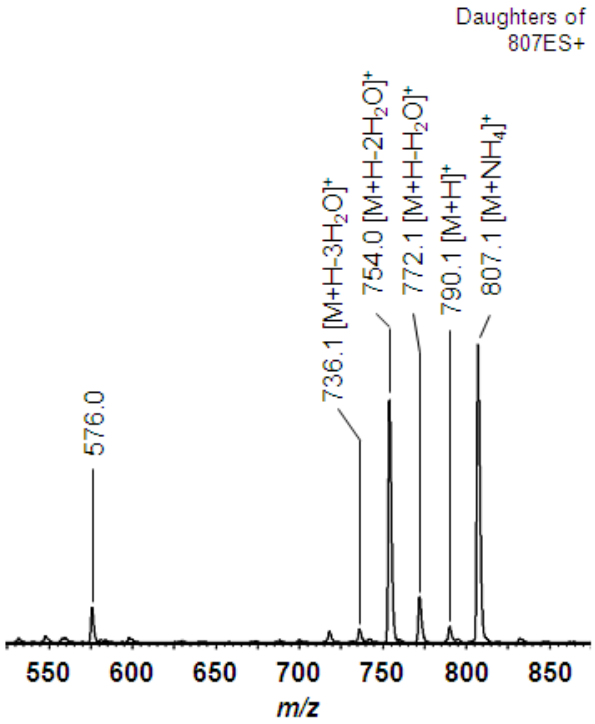
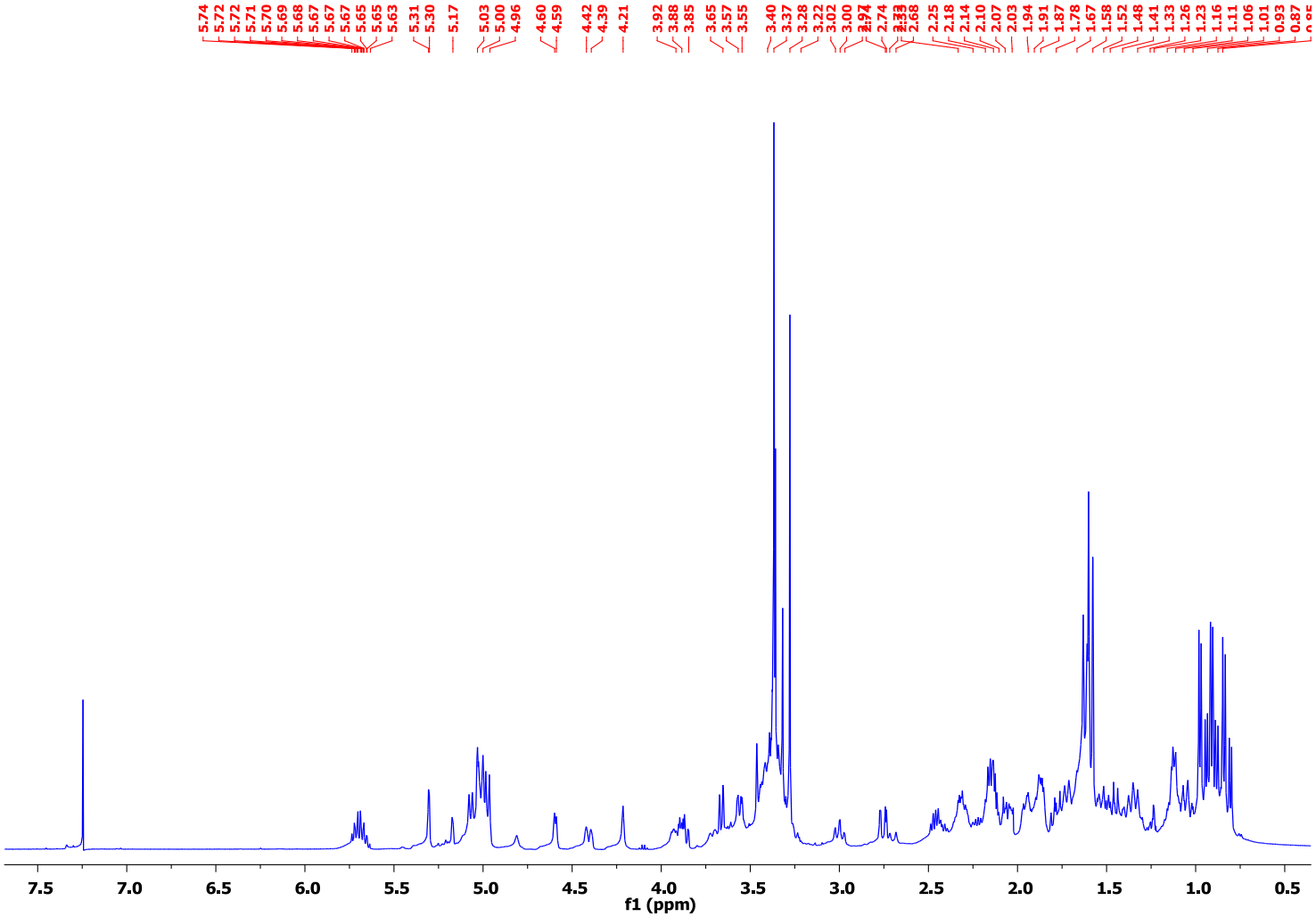
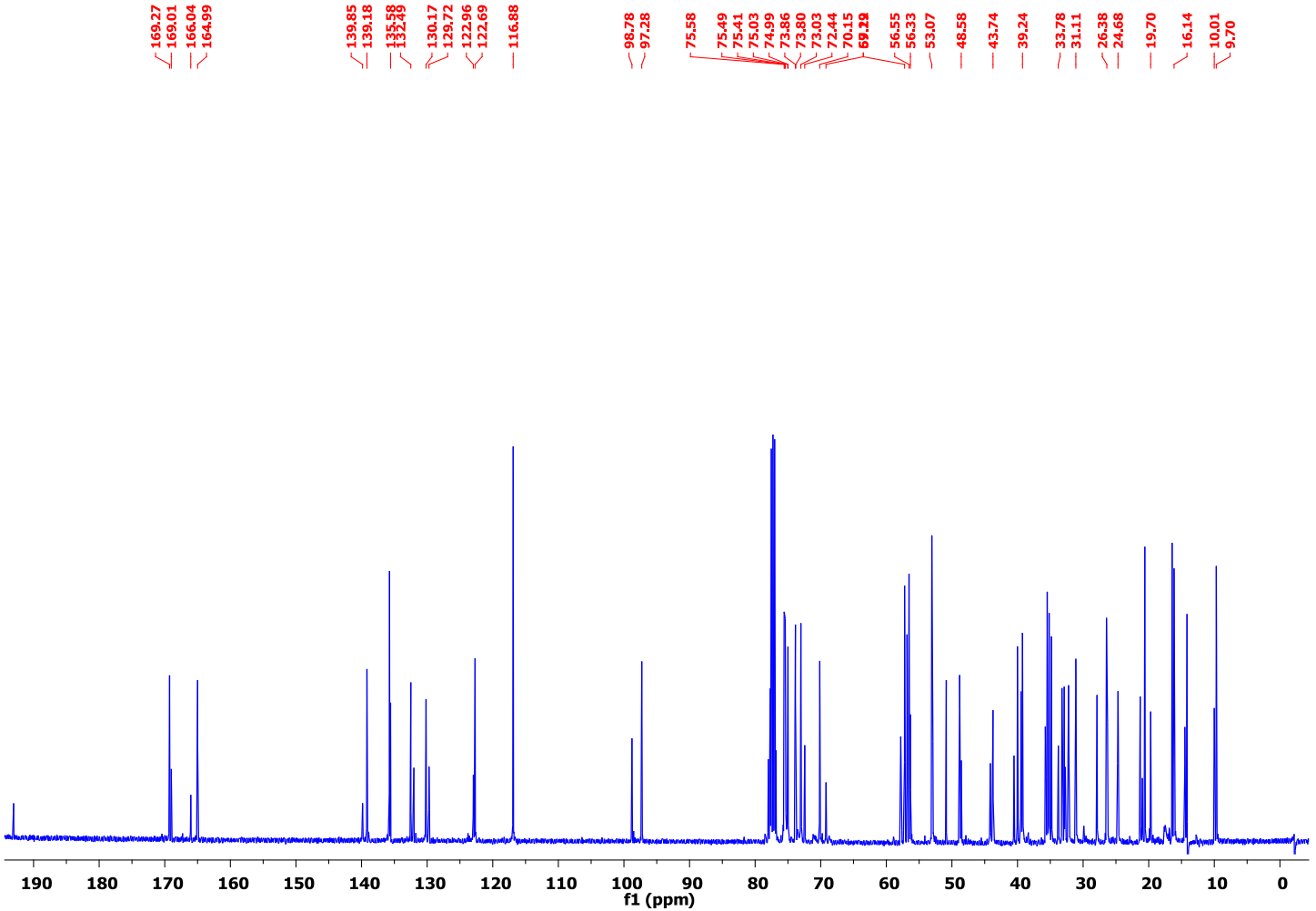


**Figure S6.** $^{1}$H NMR spectrum of 31-*O*-demethylFK506 (**2**) in $CDCl_3$.[a]




[a]Signals from minor rotamer were also observed in $^{1}$H NMR spectrum.

**Figure S7.** $^{13}$C NMR spectrum of 31-*O*-demethylFK506 (**2**) in $CDCl_3$.[a]




[a]Signals from minor rotamer were also observed in $^{13}$C NMR spectrum.

**Figure S8.** COSY spectrum of 31-*O*-demethylFK506 (**2**) in $CDCl_3$.

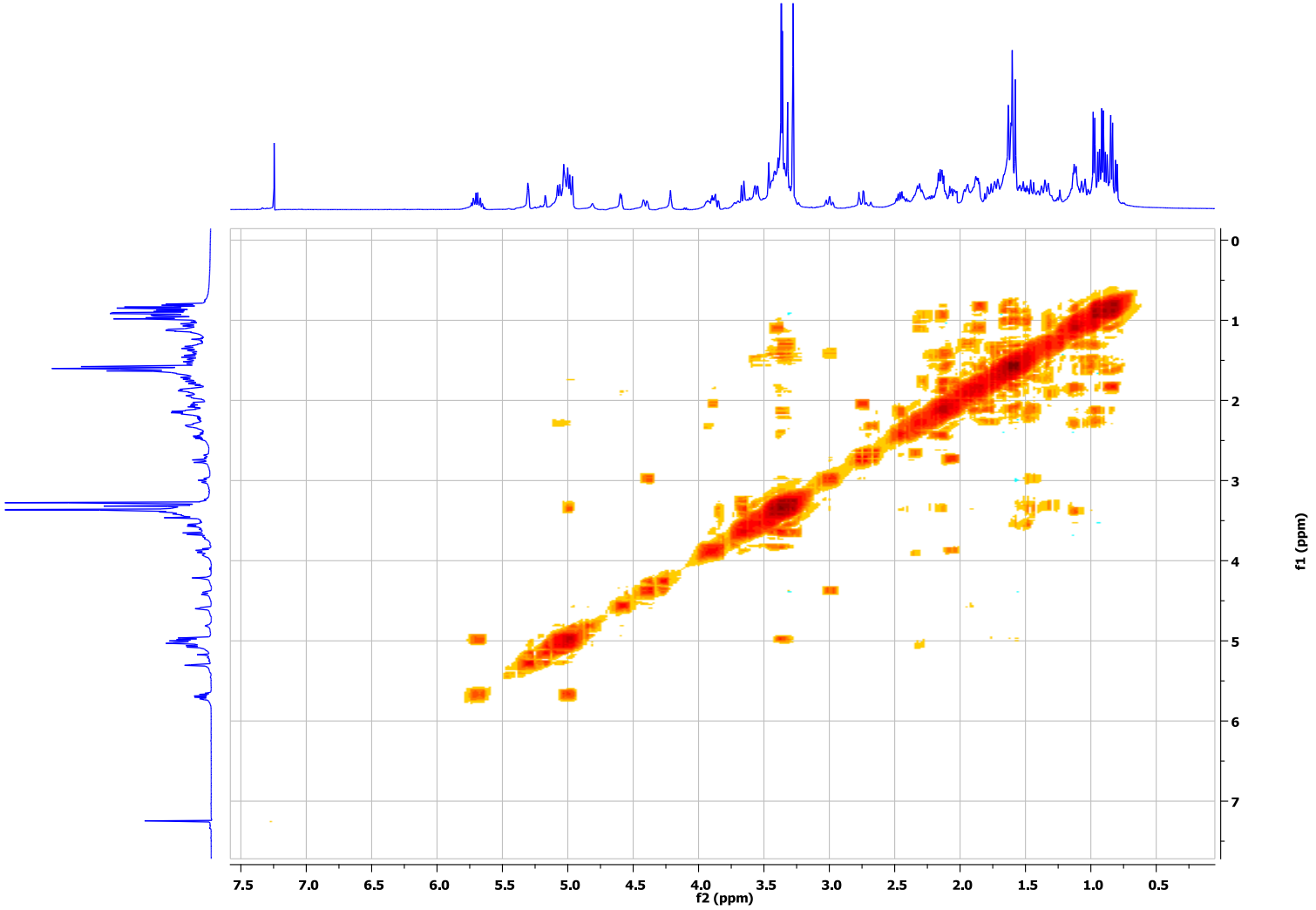


**Figure S9.** HSQC spectrum of 31-*O*-demethylFK506 (**2**) in $CDCl_3$.

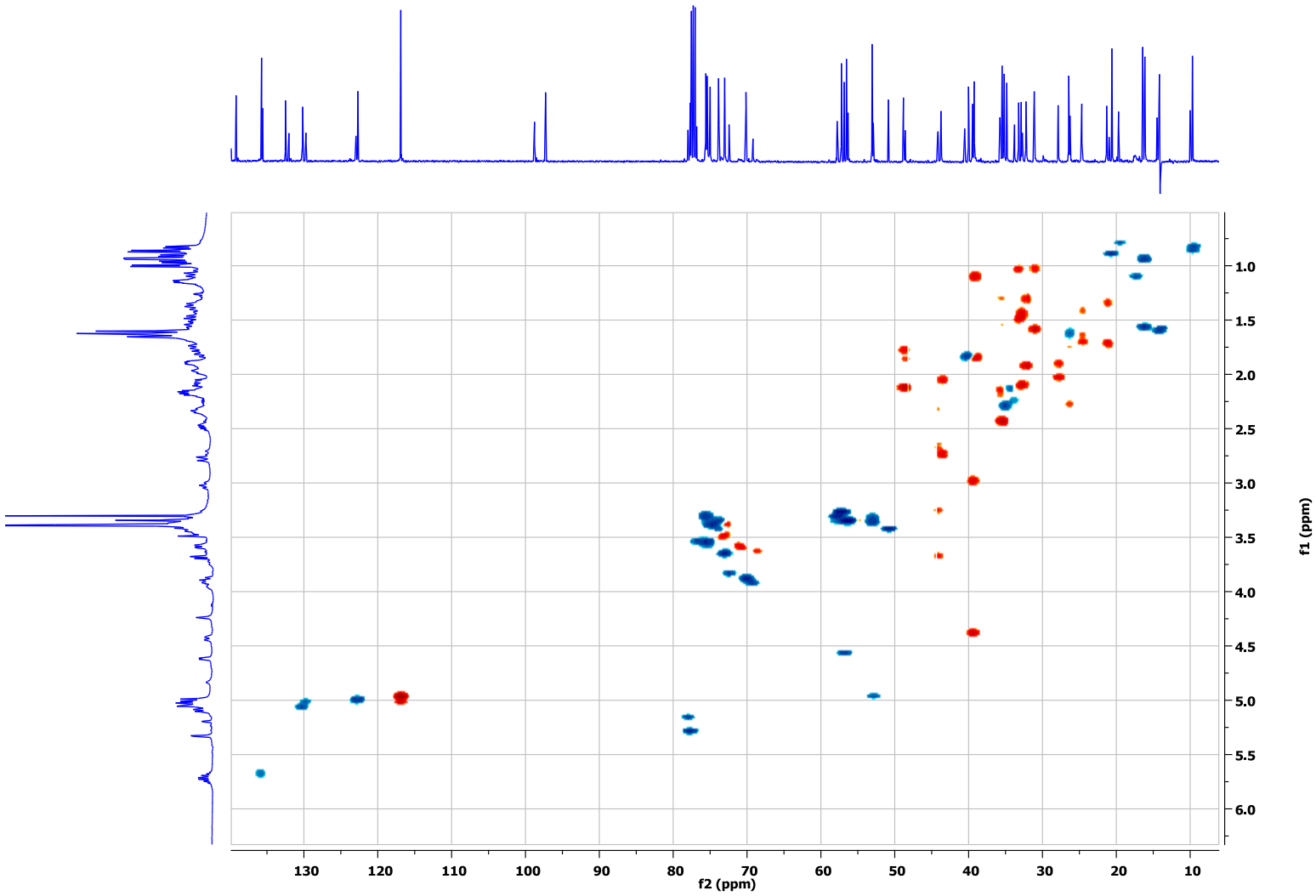


**Figure S10.** HMBC spectrum of 31-*O*-demethylFK506 (**2**) in $CDCl_3$.

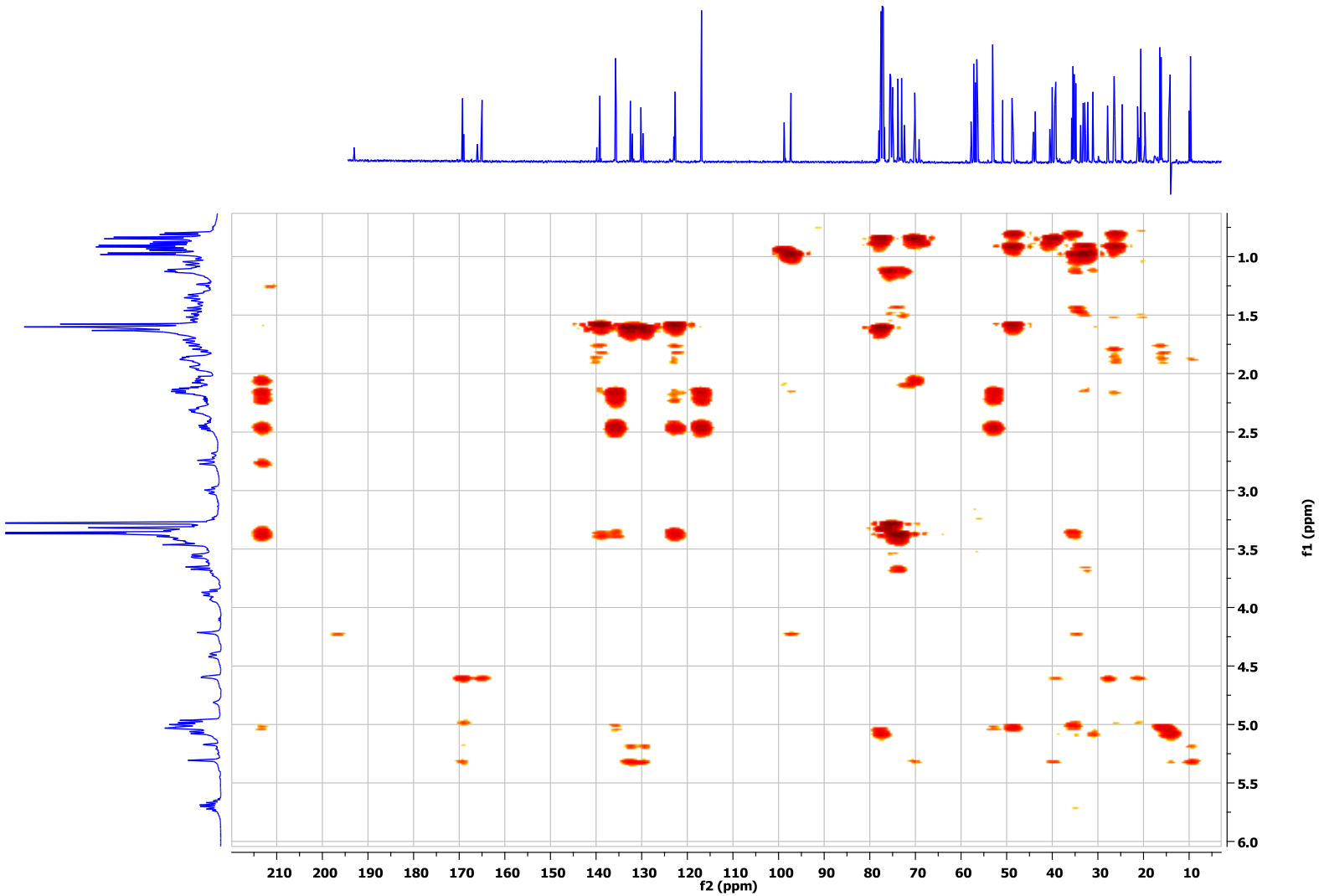


**Figure S11.** Selected COSY and HMBC correlations for 31-*O*-demethylFK506 (**2**).

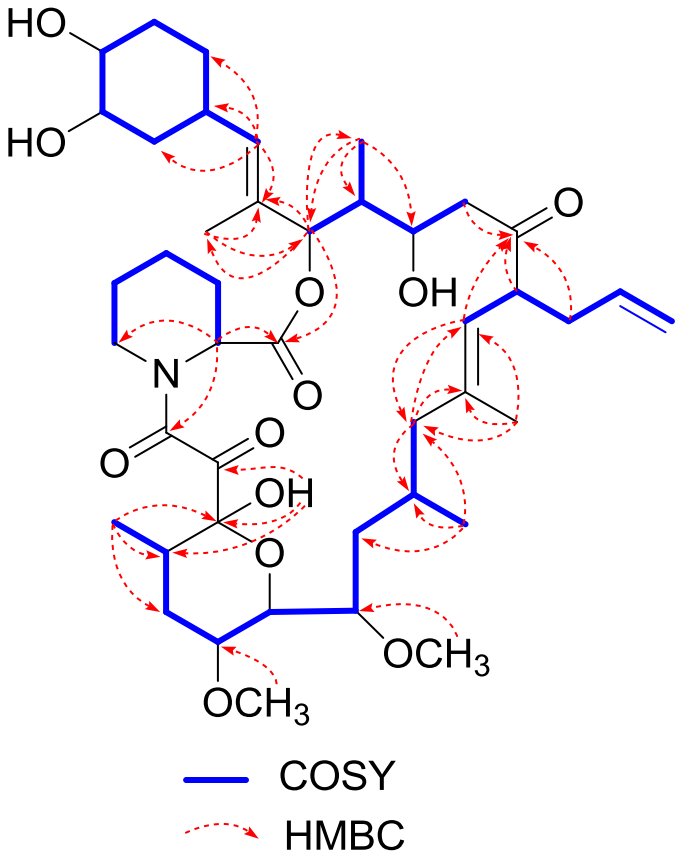
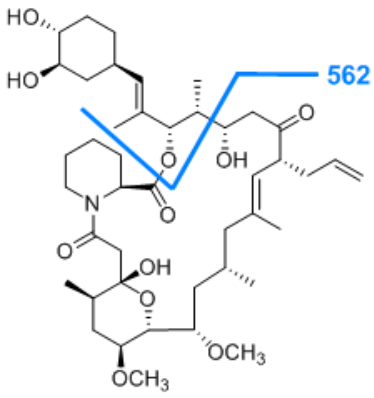


**Figure S12.** ESI-MS/MS analysis of 9-deoxo-31-*O*-demethylFK506 (**3**) obtained from $\Delta fkbD_{apr}$ strain. (A) ESI-MS/MS fragmentation pattern. (B) MS/MS spectra.

**A**




**9-deoxo-31-*O*-demethylFK506**
**(3)**

**B**

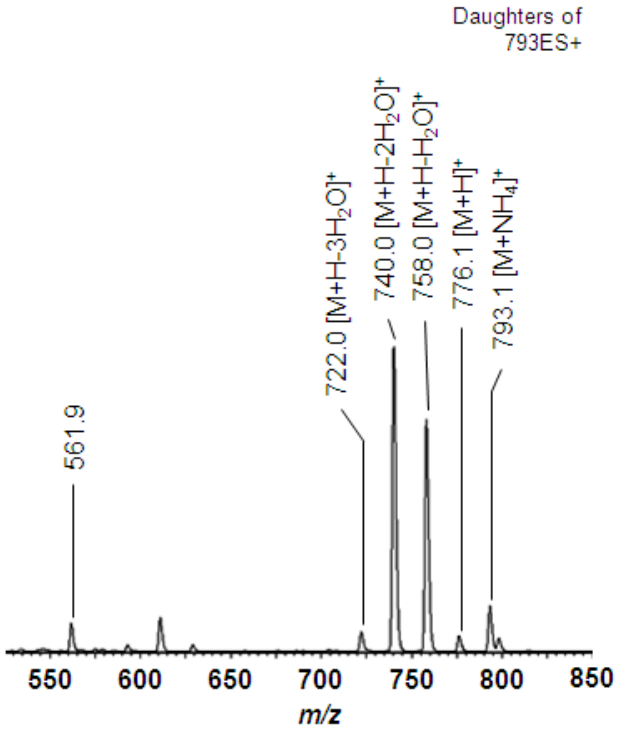


**Figure S13.** $^{1}$H NMR spectrum of 9-deoxo-31-*O*-demethylFK506 (**3**) in $CDCl_3$.

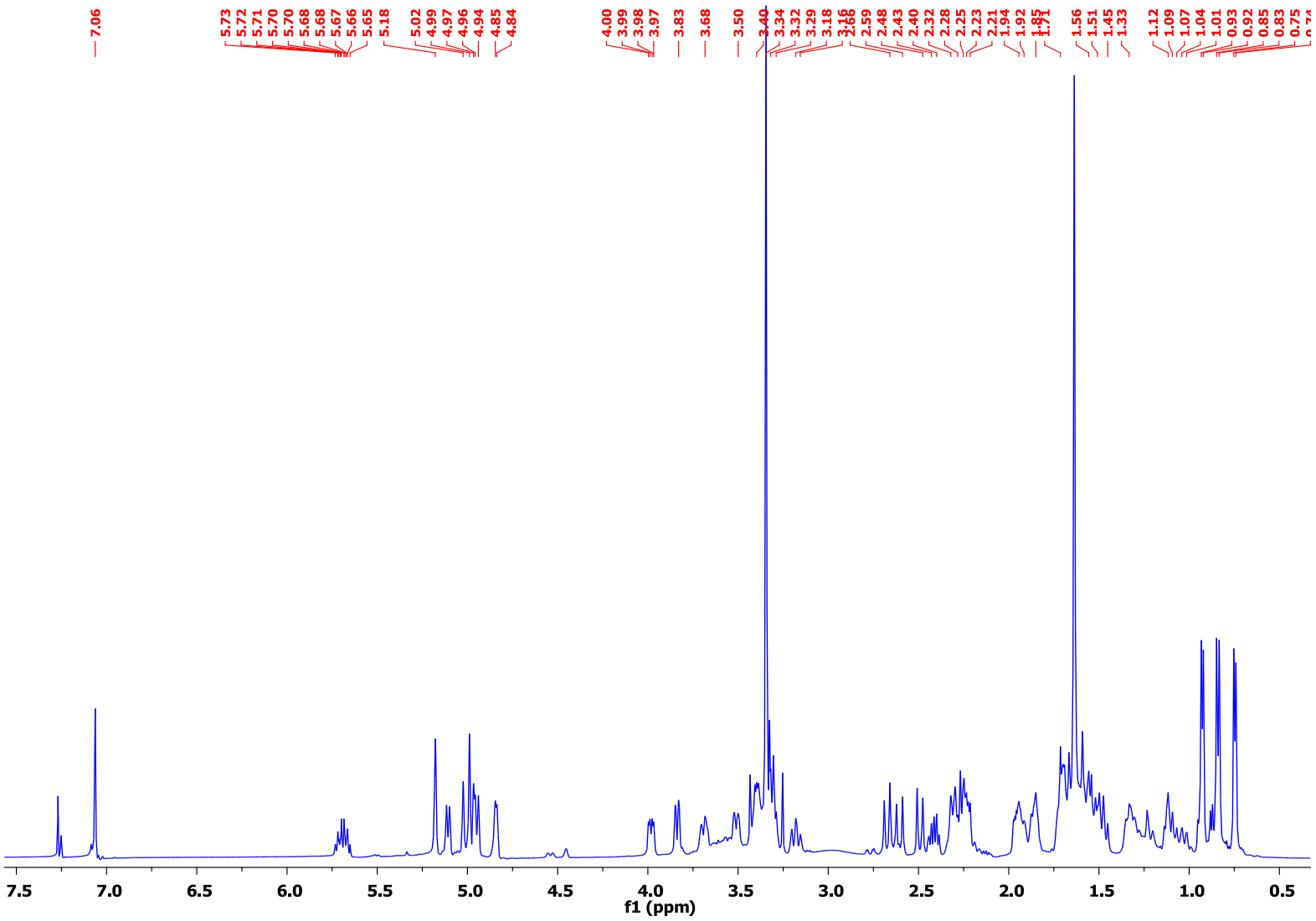
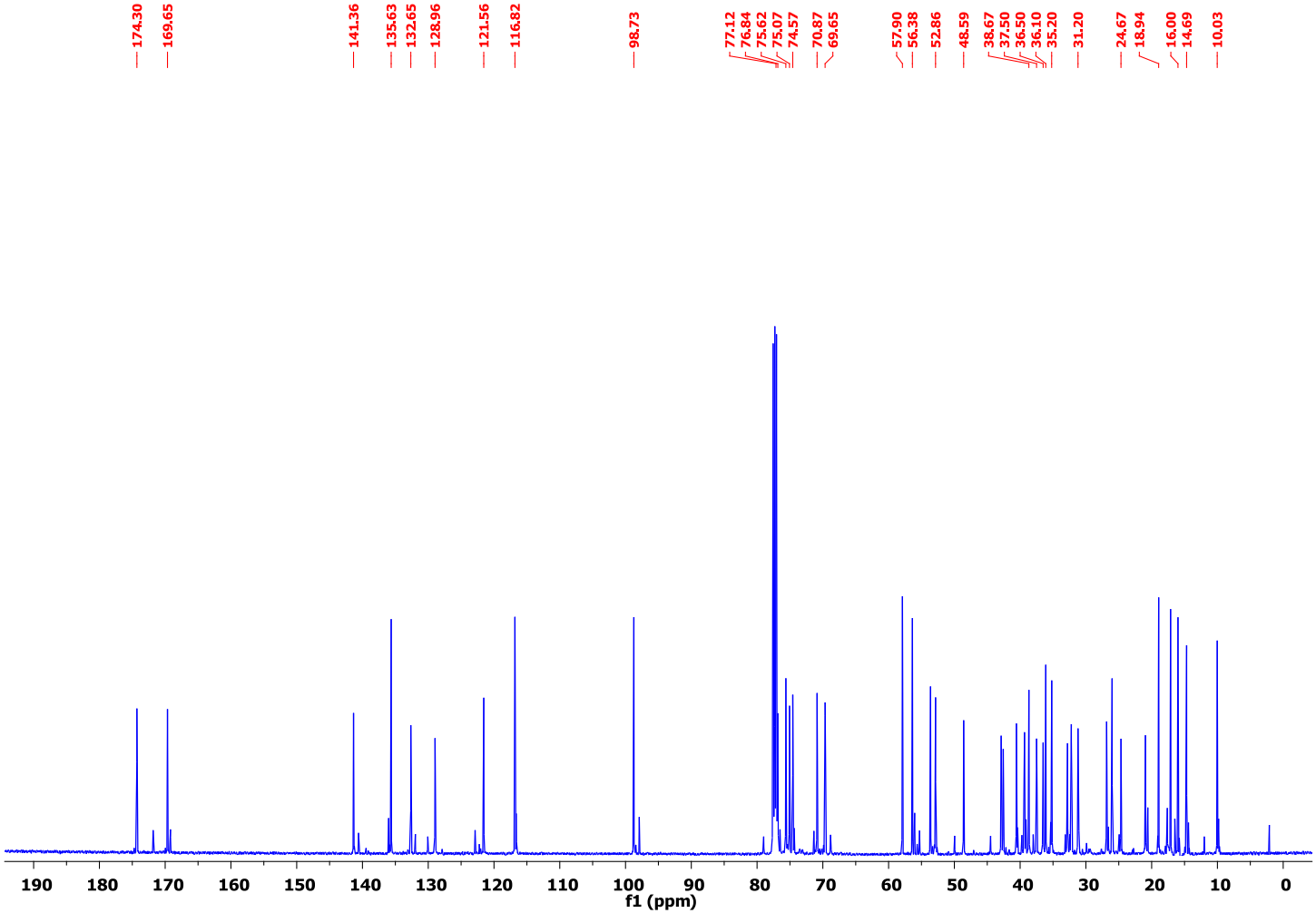


**Figure S14.** $^{13}$C NMR spectrum of 9-deoxo-31-*O*-demethylFK506 (**3**) in $CDCl_3$.[a]




[a]Signals from minor rotamer were also observed in $^{13}$C NMR spectrum.

**Figure S15.** COSY spectrum of 9-deoxo-31-*O*-demethylFK506 (**3**) in $CDCl_3$.

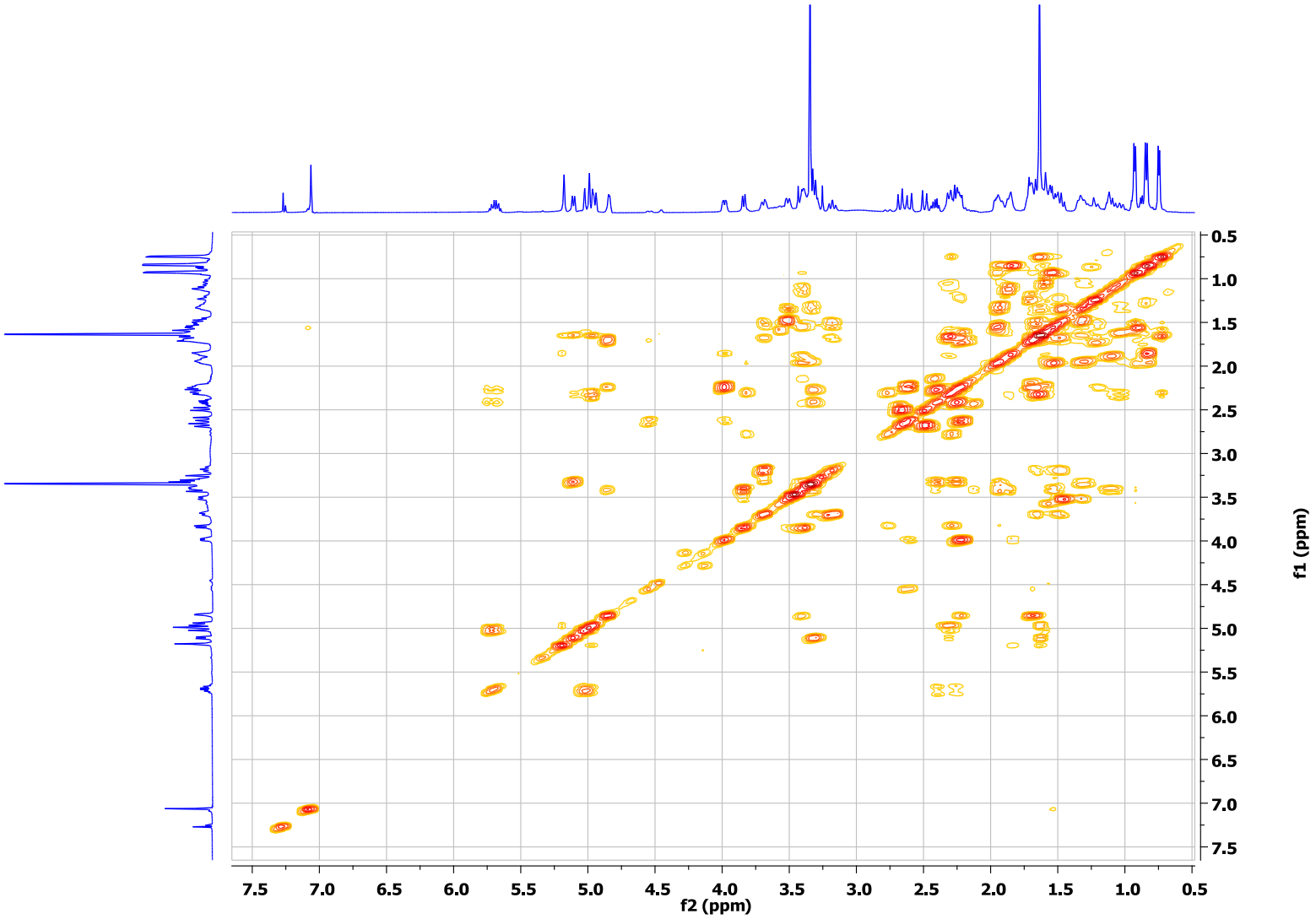


**Figure S16.** HSQC spectrum of 9-deoxo-31-*O*-demethylFK506 (**3**) in $CDCl_3$.

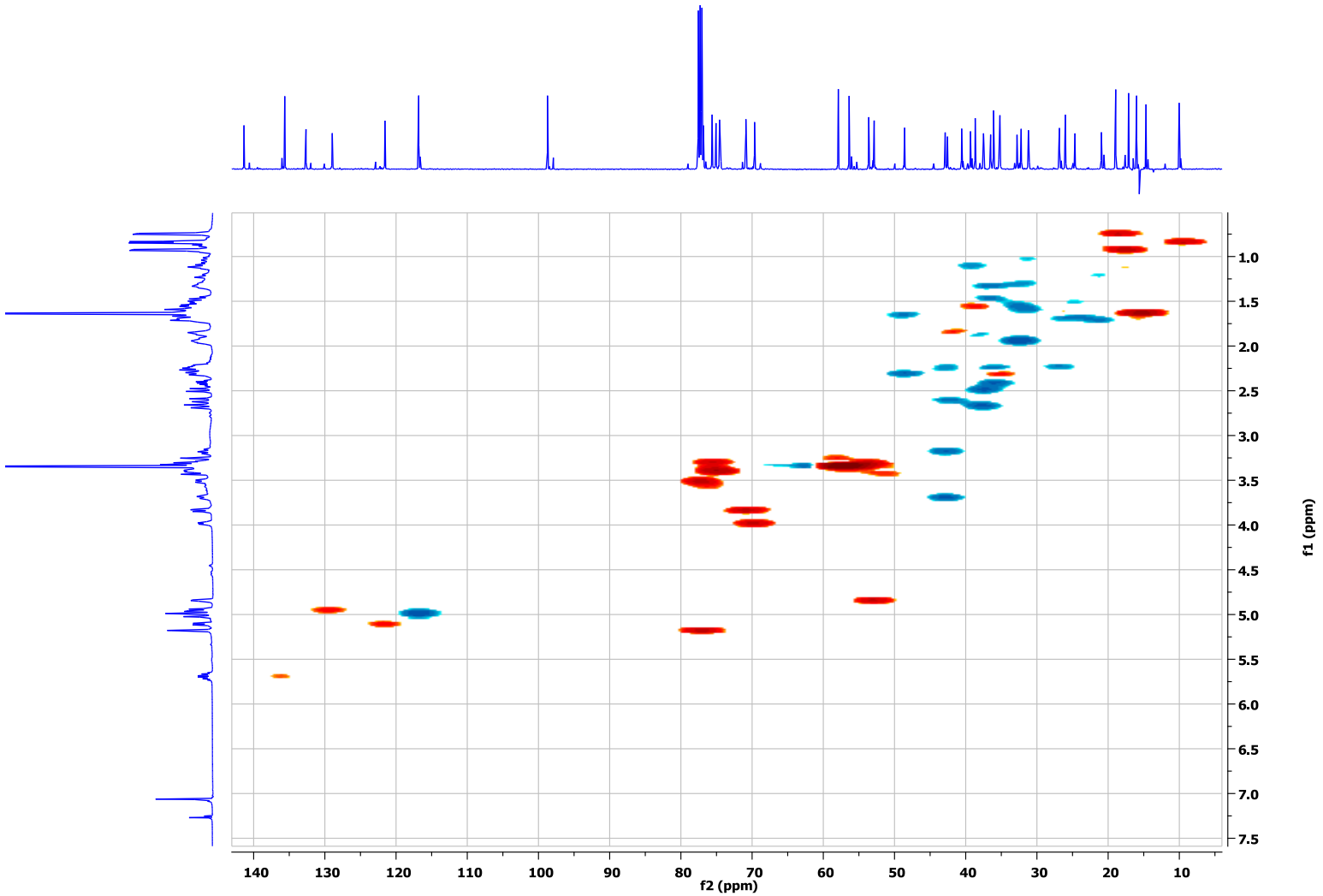


**Figure S17.** HMBC spectrum of 9-deoxo-31-*O*-demethylFK506 (**3**) in $CDCl_3$.

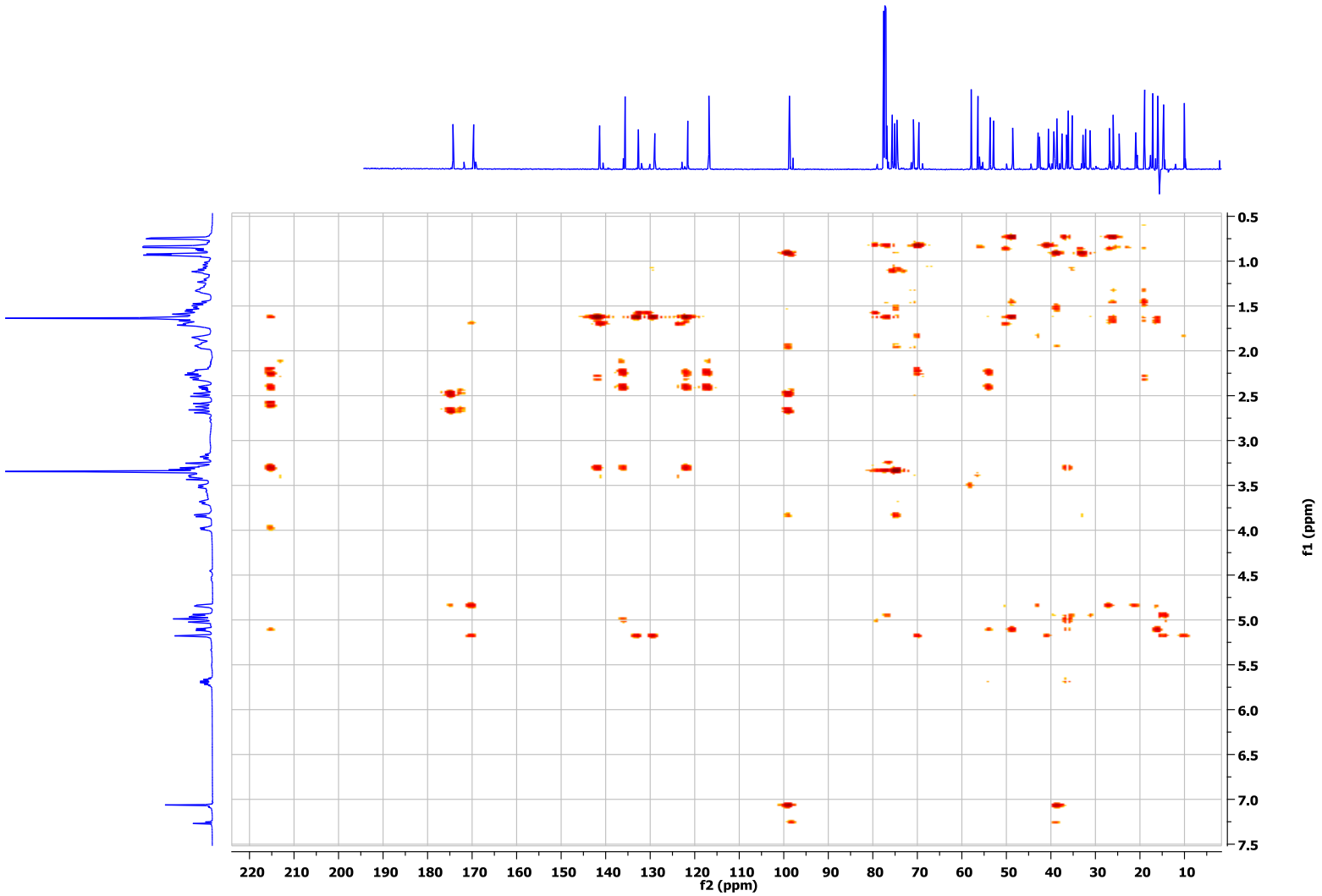


**Figure S18.** Selected COSY and HMBC correlations for 9-deoxo-31-*O*-demethylFK506 (**3**).

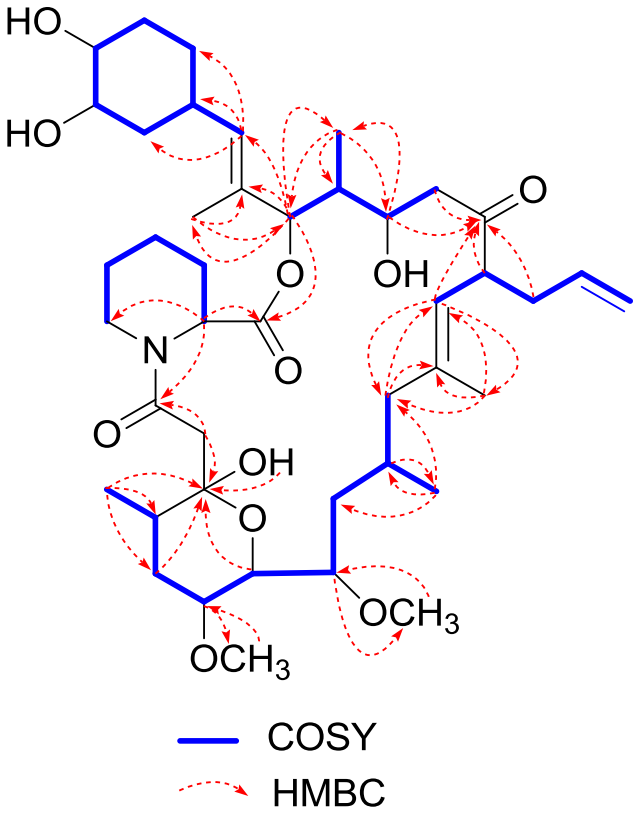
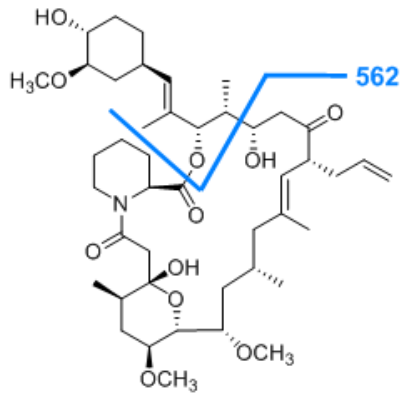


**Figure S19.** ESI-MS/MS analysis of 9-deoxoFK506 (**4**) obtained from ΔfkbD$_{in\text{-}frame}$ strain. (A) ESI-MS/MS fragmentation pattern. (B) MS/MS spectra.

**A**




**9-deoxoFK506**
**(4)**

**B**

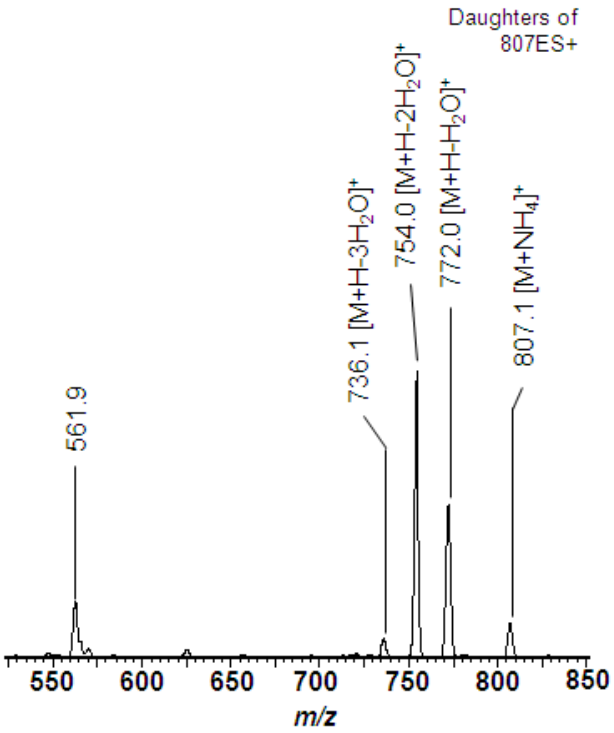


**Figure S20.** $^{1}$H NMR spectrum of 9-deoxoFK506 (**4**) in $CDCl_3$.

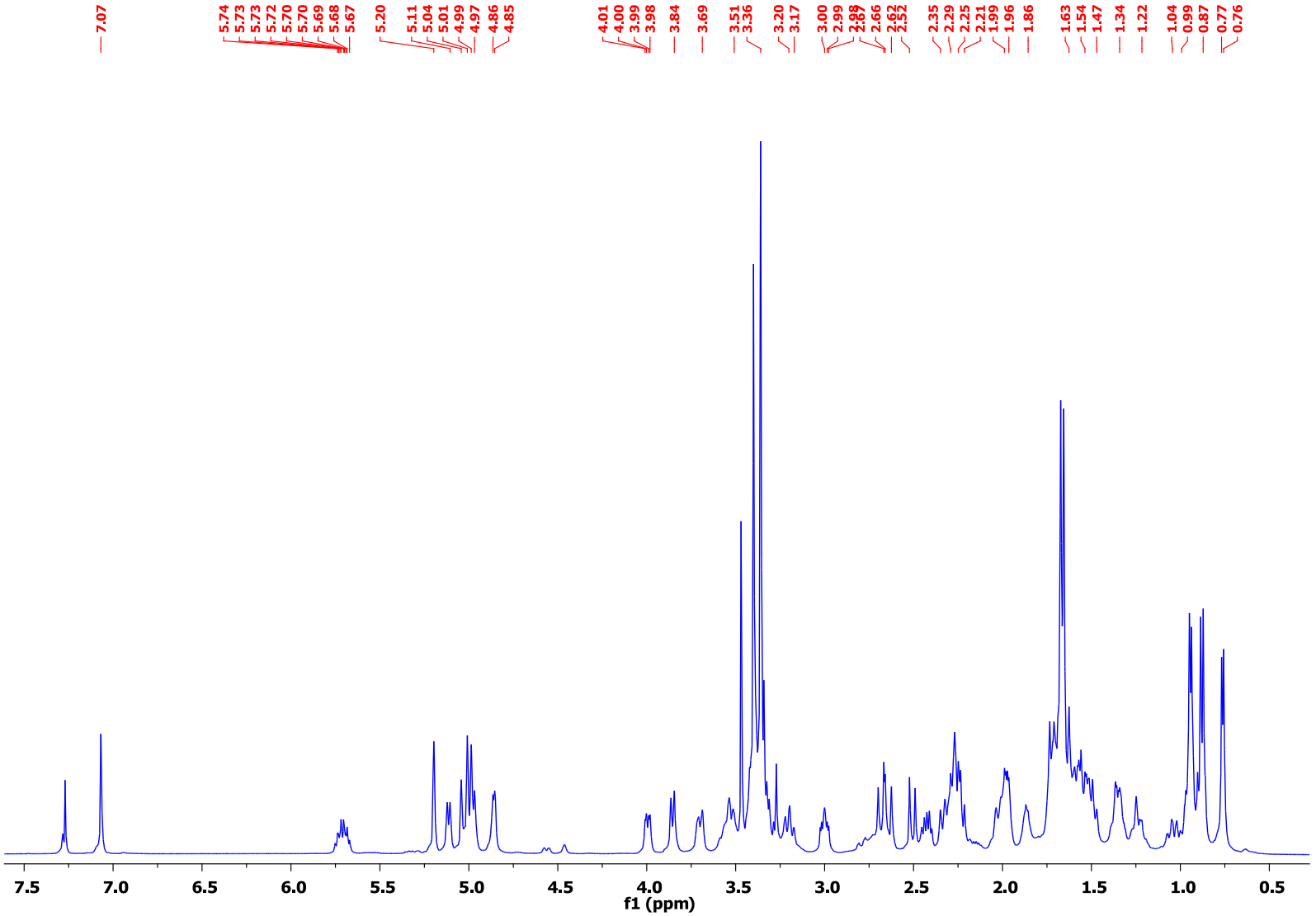
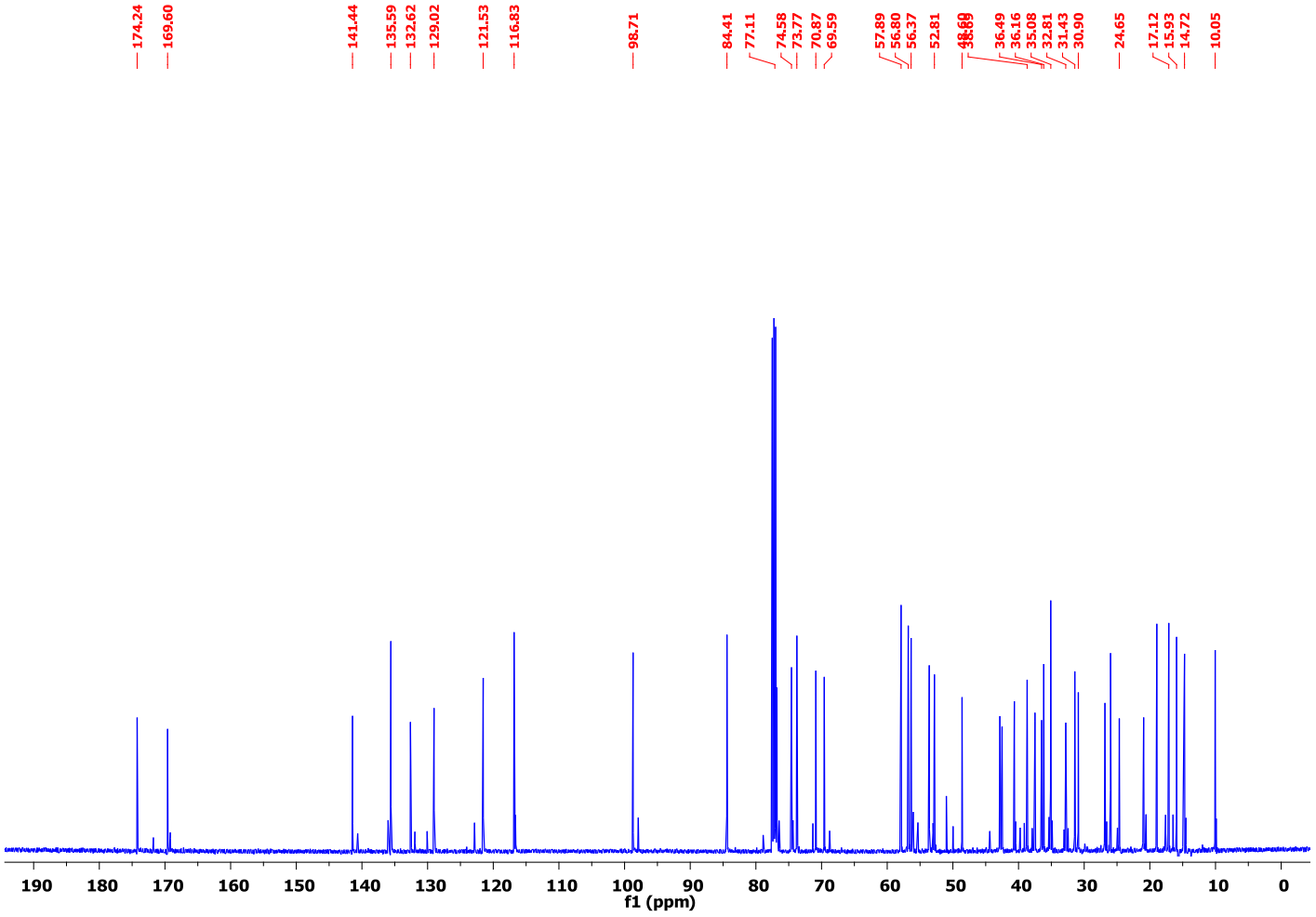


**Figure S21.** $^{13}C$ NMR spectrum of 9-deoxoFK506 (**4**) in $CDCl_3$.[a]




[a]Signals from minor rotamer were also observed in $^{13}C$ NMR spectrum.

**Figure S22.** COSY spectrum of 9-deoxoFK506 (**4**) in $CDCl_3$.

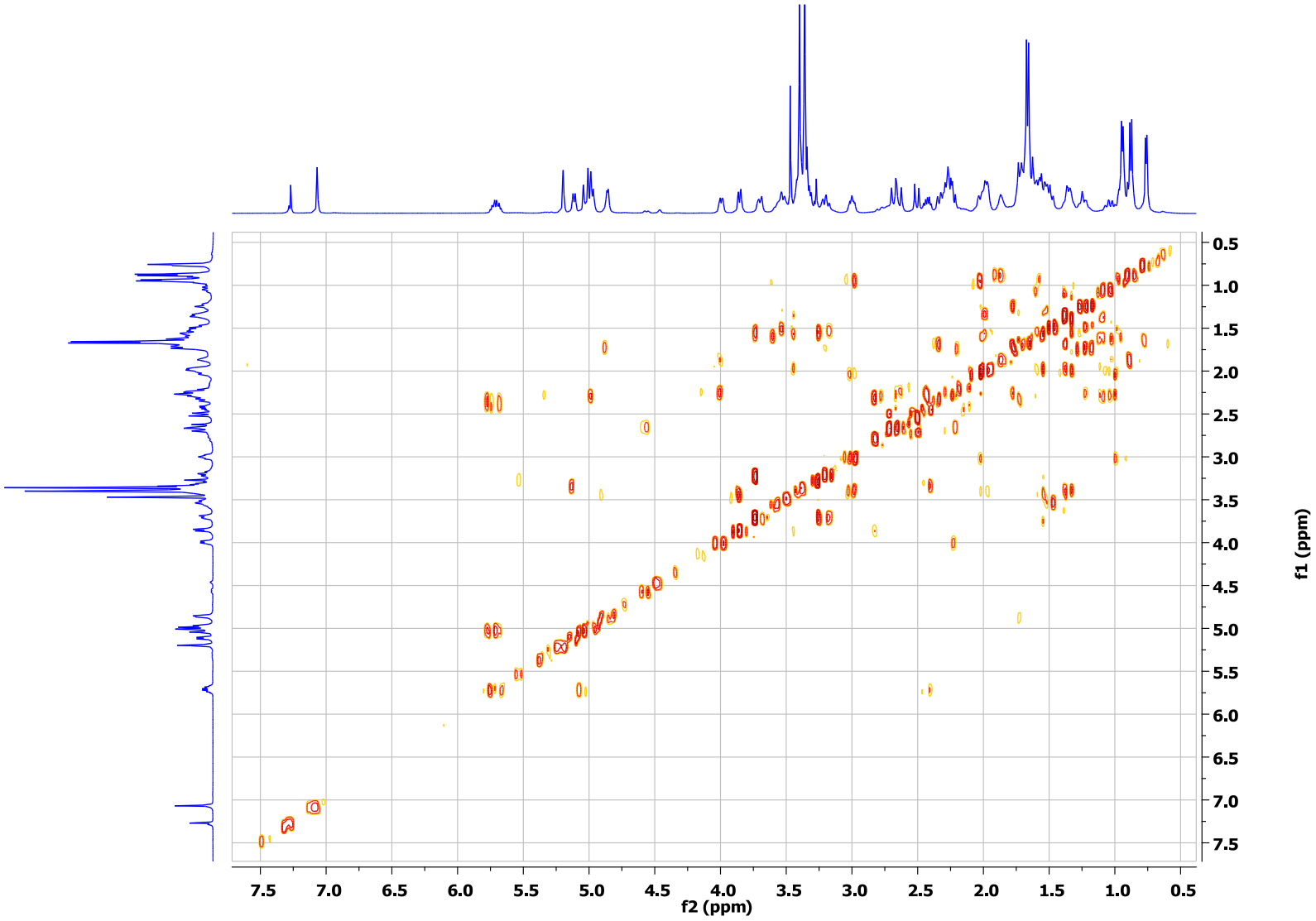


**Figure S23.** HSQC spectrum of 9-deoxoFK506 (**4**) in $CDCl_3$.

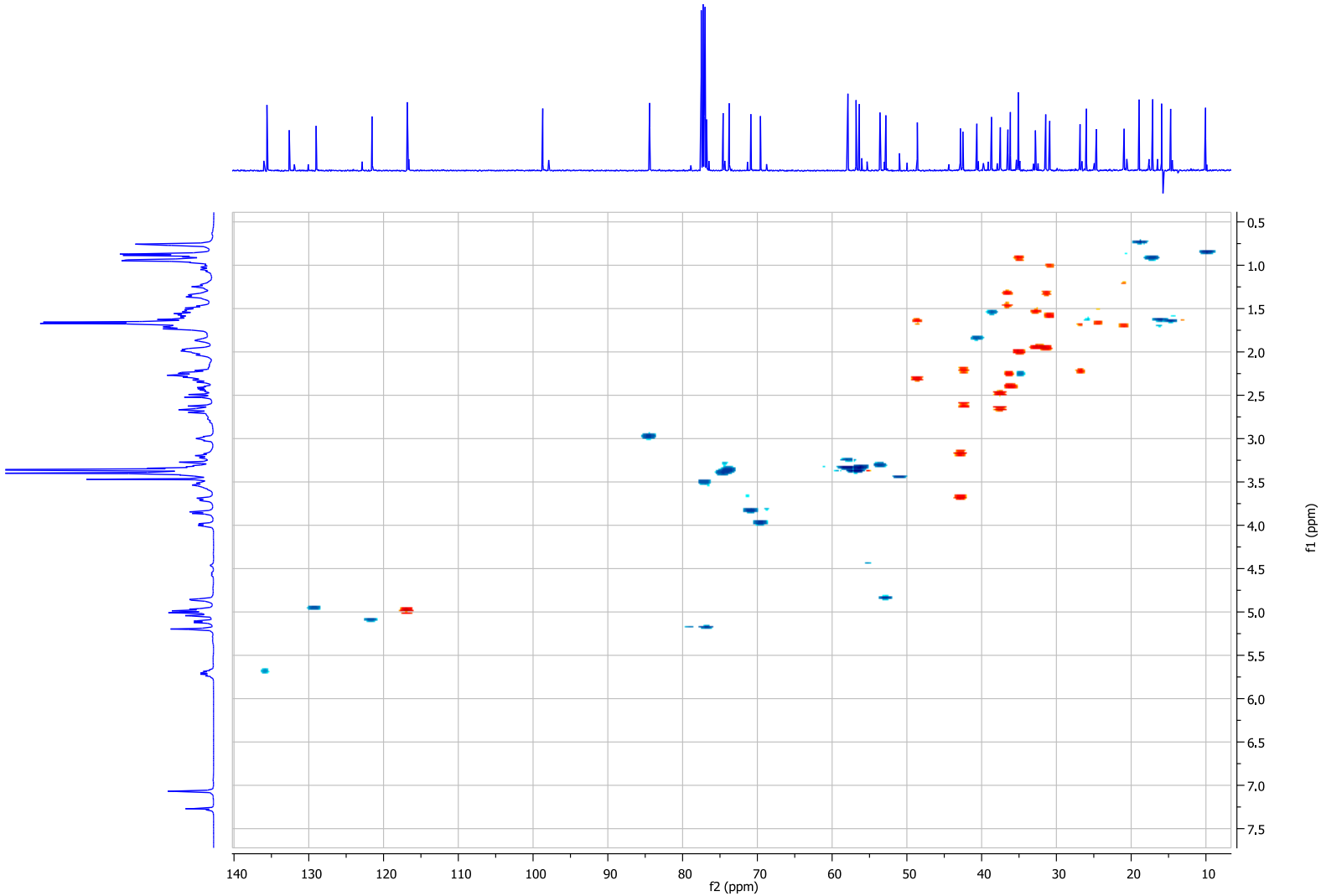


**Figure S24.** HMBC spectrum of 9-deoxoFK506 (**4**) in $CDCl_3$.

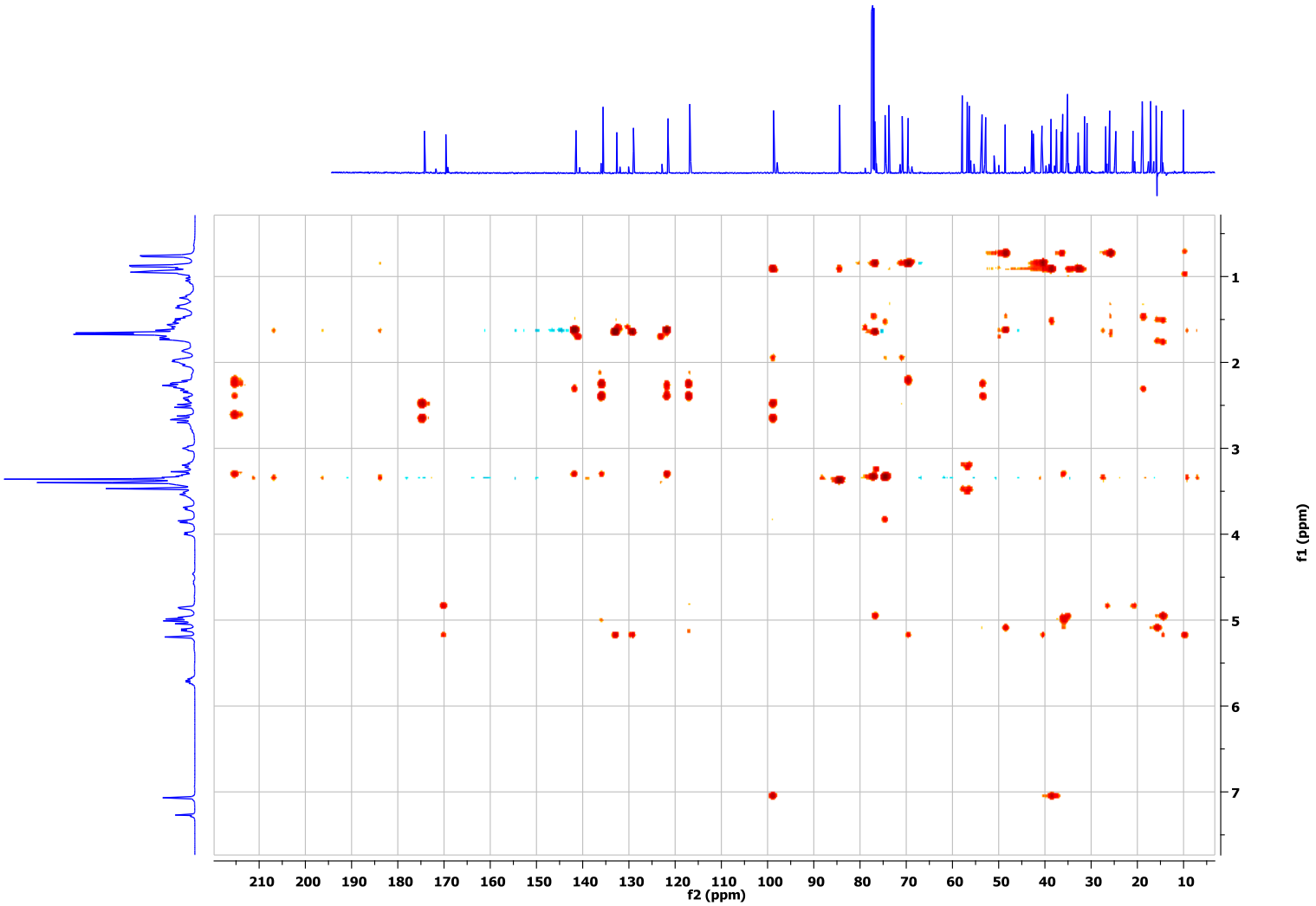


**Figure S25.** Selected COSY and HMBC correlations for 9-deoxoFK506 (**4**).

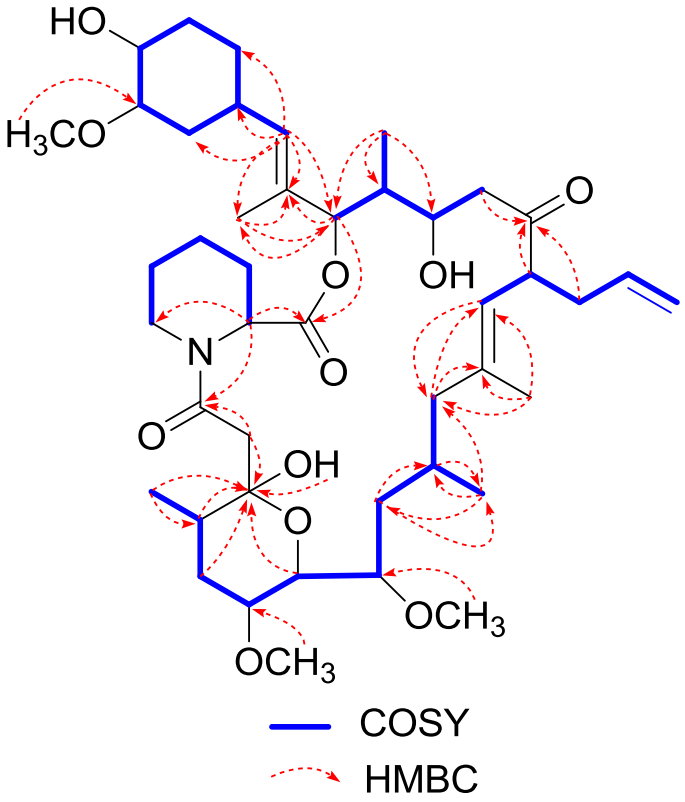
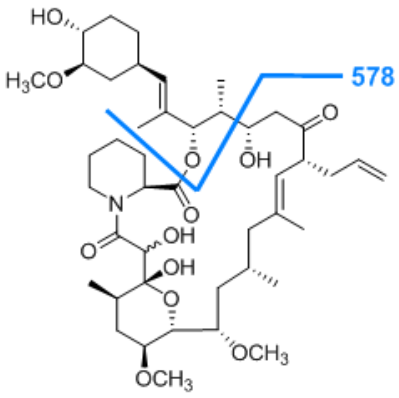


**Figure S26.** ESI-MS/MS analysis of 9-hydroxyFK506 (**5**) obtained from wild-type *Streptomyces* sp. KCTC11604BP strain. (A) ESI-MS/MS fragmentation pattern. (B) MS/MS spectra.

**A**




**9-hydroxyFK506**
**(5)**

**B**

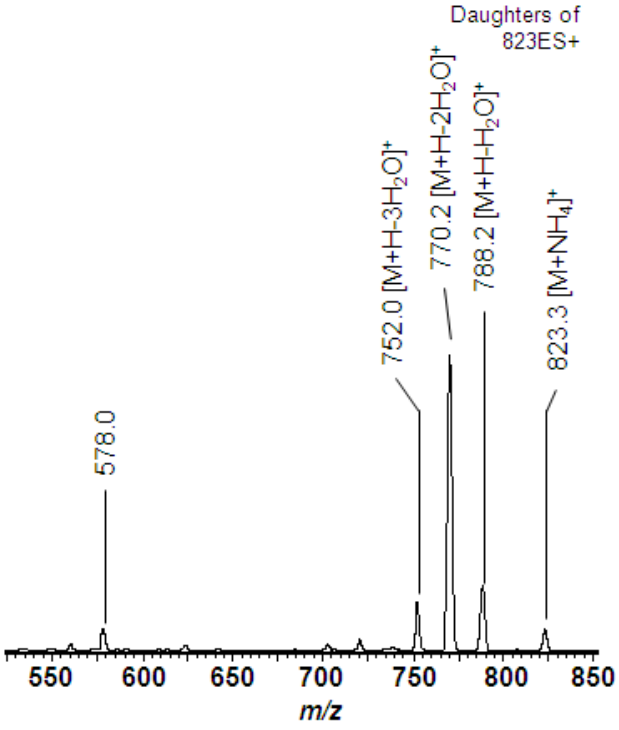
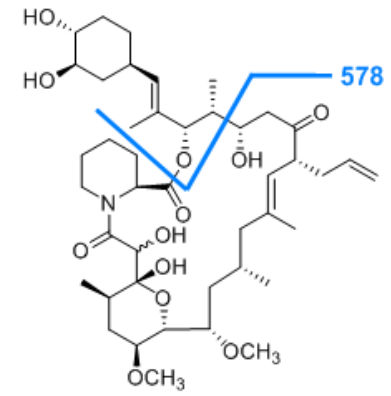


**Figure S27.** ESI-MS/MS analysis of 9-hydroxy-31-*O*-demethylFK506 (**6**) identified from *in vitro* reaction. (A) ESI-MS/MS fragmentation pattern. (B) MS/MS spectra.

**A**




**9-hydroxy31-*O*-demethylFK506**
**(6)**

**B**

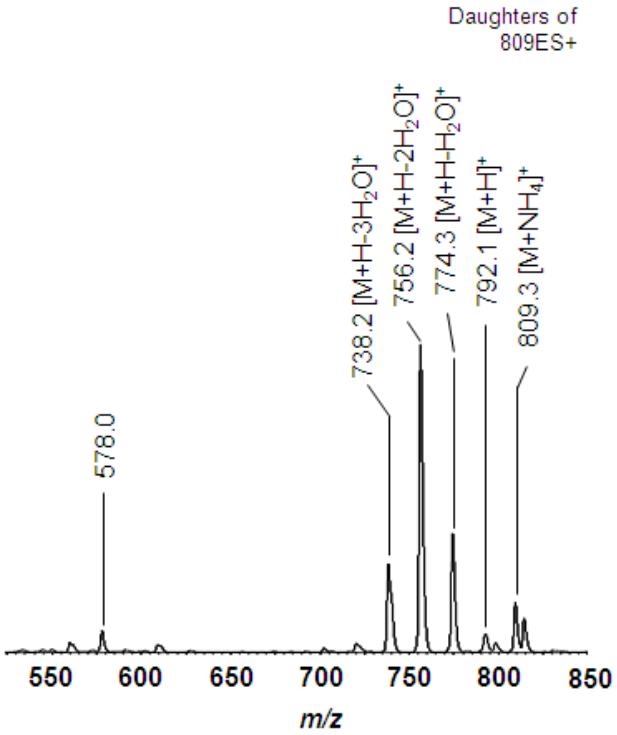
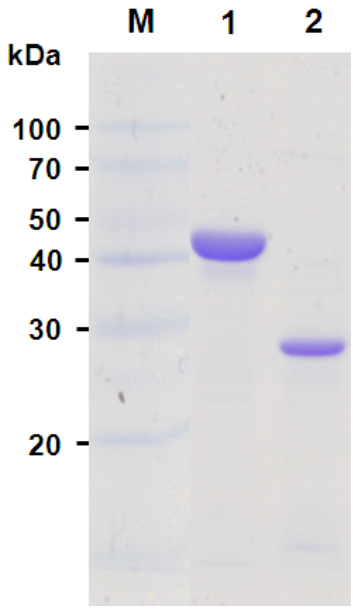


**Figure S28.** SDS-PAGE analysis of the purified FkbD and FkbM. 1, Purified $FkbD_{His6}$ (a molecular mass 44.6 kDa); 2, Purified $FkbM_{His6}$ (a molecular mass 28.5 kDa); M, molecular weight marker.



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