Electronic Supplementary Material (ESI) for Molecular BioSystems

Electronic Supplementary Information

Mutational biosynthesis of FK506 analogue containing a non-natural starter unit

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Materials and methods

Materials, bacterial strains, and culture conditions. FK506 (**1**), FK520 (**2**) and 3-cyclohexene-1-carboxylic acid (**5**) were purchased from Sigma-Aldrich. Ammonium acetate was purchased from Fluka, and HPLC-grade ethyl acetate, acetonitrile, methanol, glacial acetic acid, and water were supplied by J.T. Baker. Restriction endonucleases and T4 DNA ligase were purchased from New England Biolabs. Polymerase chain reactions were carried out using Taq DNA polymerase from Stratagene. All other chemicals were of the highest purity available. The **1**-producing strains *Streptomyces* sp. KCTC 11604BP was obtained from GenoTech (Daejeon, Republic of Korea). Spores of *Streptomyces* sp. KCTC 11604BP and the *fkbO* deletion mutant were generated on ISP4 agar plates.[1]

Construction of deletion plasmid and in-frame deletion mutant strain. For in-frame deletion of the *fkbO* gene, the deletion plasmid was constructed by PCR amplification of the left- and right-flanking fragments from fosmid (fos1005D02; GenBank accession no. HM116536) DNA derived from *Streptomyces* sp. KCTC 11604BP. The left-flanking fragment was PCR-amplified by a forward primer, 5'-TTAAAAGCTTTCCGCCGTGAGCGAGGCG-3' and a reverse primer, 5'-TACCTCTAGAACCGGTGCCGTGCTCGAT-3'. The right-flanking fragment was PCR-amplified by a forward primer, 5'-TTAATCTAGATGCGCCGCCCGCTTGGCG-3' and a reverse primer, 5'-TATAGATATCTGGCCCGGCAGCGGCCGA-3'. 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[2] digested with *Hind*III-*Xba*I or *Xba*I-*Eco*RV, to construct in-frame deletion plasmid. The *fkbO* in-frame deletion plasmid was then transferred by conjugation from *E. coli* ET12567/pUZ8002[3] to *Streptomyces* sp. KCTC 11604BP, as described elsewhere.[4] The ΔfkbO double cross-over mutant was selected by their apramycin-sensitive phenotype, verified by PCR analysis (Figure S1).

Production and HPLC-ESI-MS/MS analysis of 32-dehydroxy-FK506 (6). ΔfkbO mutant of *Streptomyces* sp. KCTC 11604BP was cultivated at 28°C for 5 days on R2YE agar supplemented with 3-cyclohexene-1-carboxylic acid (**5**) at final concentration of 10mM at the time of inoculation. The grown cultures (3L) of the ΔfkbO mutant strain were blended with 1 volume of methanol and shaken at room temperature for 12 h. The cell debris was removed by centrifugation before rotary evaporation. The methanol extract residues dissolved in 1 volume of water, and then extracted with 1 volume of ethyl acetate. The organic extract was evaporated to dryness under reduced pressure, then analyzed by HPLC-ESI-MS/MS as described.[5] Sample was separated on an ACQUITY UPLC BEH C_{18} column (50X2.1 mm, 1.7 μm; Waters) interfaced with a Waters/Micromass Quattro micro/MS instrument tracing by MS/MS using a gradient of acetonitrile at a flow rate of 0.08 ml/min over 70 min starting with 80% (v/v) aqueous acetonitrile containing 10 mM ammonium acetate and 0.1% acetic acid. Tracing was done by MS/MS operated in multiple reactions monitoring mode choosing mass pairs specific for the selected analytes to detect the transition from parent ion as an ammonium adduct to product ion: 805 > 576 for 32-dehydroxy-FK506 (**6**) (Figure S2). Three separate cultivations and independent extractions were performed.

Isolation and identification of 32-dehydroxy-FK506 (6). The grown cultures (3 L) to which 38 mg of **5** had been added were extracted with methanol. Evaporation of the methanol under reduced pressure left of reddish foam. The methanol extract residues dissolved in water, and then subject to Diaion HP20 and ODS column chromatography with methanol system sequentially. HPLC was carried out with a Waters (Milford, MA, USA) Model 2690 separations module, using a 25-cm×4.6-mm BDS HYPERSIL C18 (5 μm) column (Thermo scientific, USA) with 60% (v/v) aqueous acetonitrile. The column was maintained at 50°C, the flow rate was 1 ml/min, and detection was at 205 nm. HPLC purification of this material gave **6** (0.6 mg).
For NMR characterization of FK506 and its analogue obtained from the culture media, samples

purified via reversed-phase HPLC were prepared by dissolving each compound in 250 µL of $CDCl_3$ (Sigma) and placing the solution in a 5-mm Shigemi advanced NMR microtube (Sigma) matched to the solvent. ^{1}H, ^{13}C, and 2D NMR spectra were acquired using a Bruker Avance II 900 spectrometer at 298K. Chemical shifts are given in ppm using tetramethylsilane (TMS) as an internal reference. All NMR data processing was done using the Mnova (Mestrelab Research S.L.) software.

***In vitro* T-cell activation assay.** The relative immunosuppressive properties of the mutasynthetic analogue **6** compared with authentic **1**, were determined using T lymphocytes as described elsewhere[4]. The secretion level of interleukin-2 was quantified from human T-cells (1 x 10^6 cells/well), which were activated with CD3/CD28 antibodies (BD Pharmingen; 0.5 µg/ml for each), then treated with the different concentrations of **1** and **6** for 16 to 20 hr (see Figure 3).

***In vitro* neurite outgrowth assay.** The relative nerve regeneration activities of the mutasynthetic analogue **6**, compared with authentic **1**, were determined using human neuroblastoma cells as described elsewhere[4]. The human neuroblastoma SH-SY5Y cells (1x10^3 cells/well) were treated with nerve growth factor (NGF; KOMA Biotech; 10 ng/mL) and the different concentrations of **1** and **6**. The neurite lengths were measured on photographic prints as previously described (see Figure 3).[6]

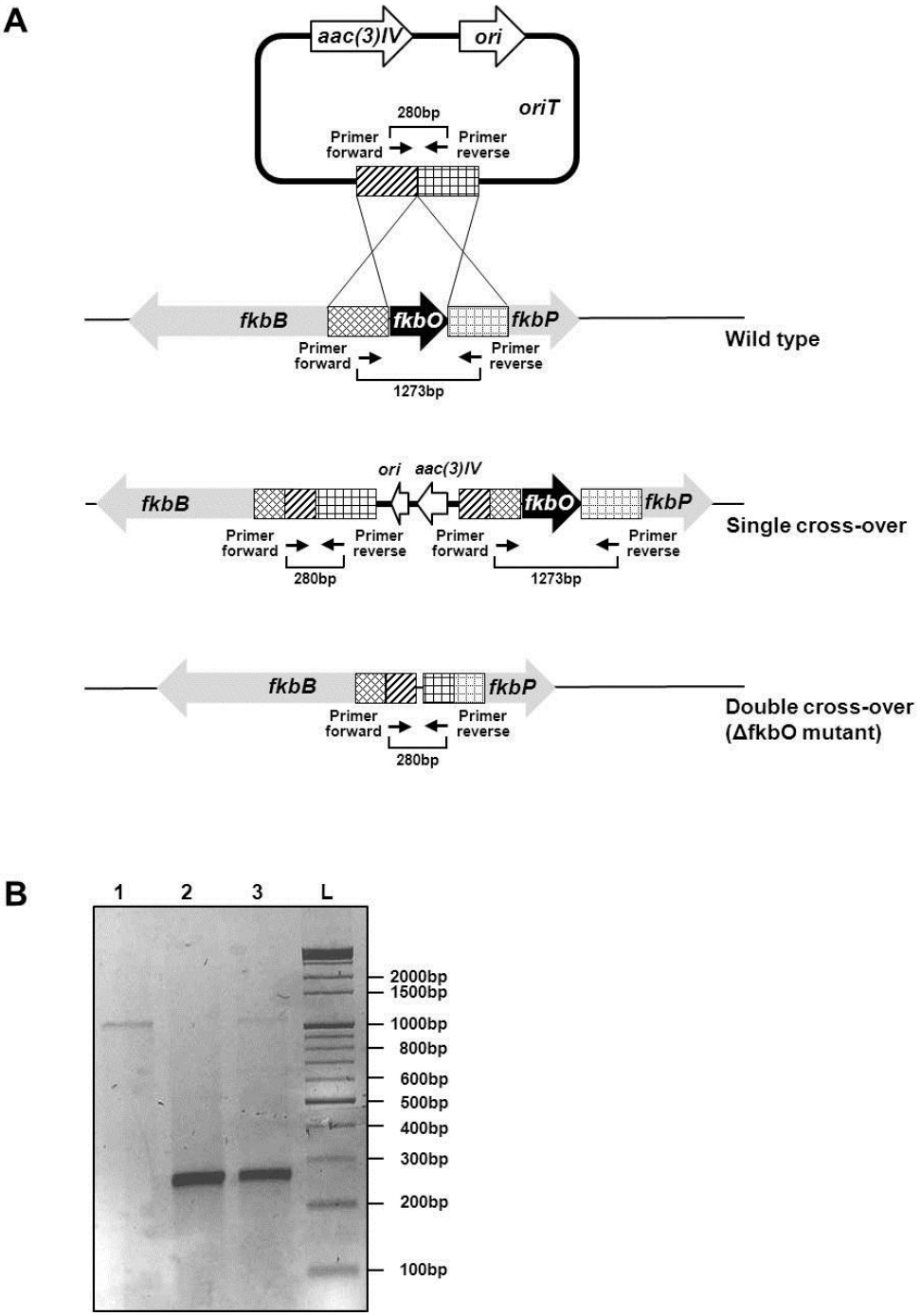
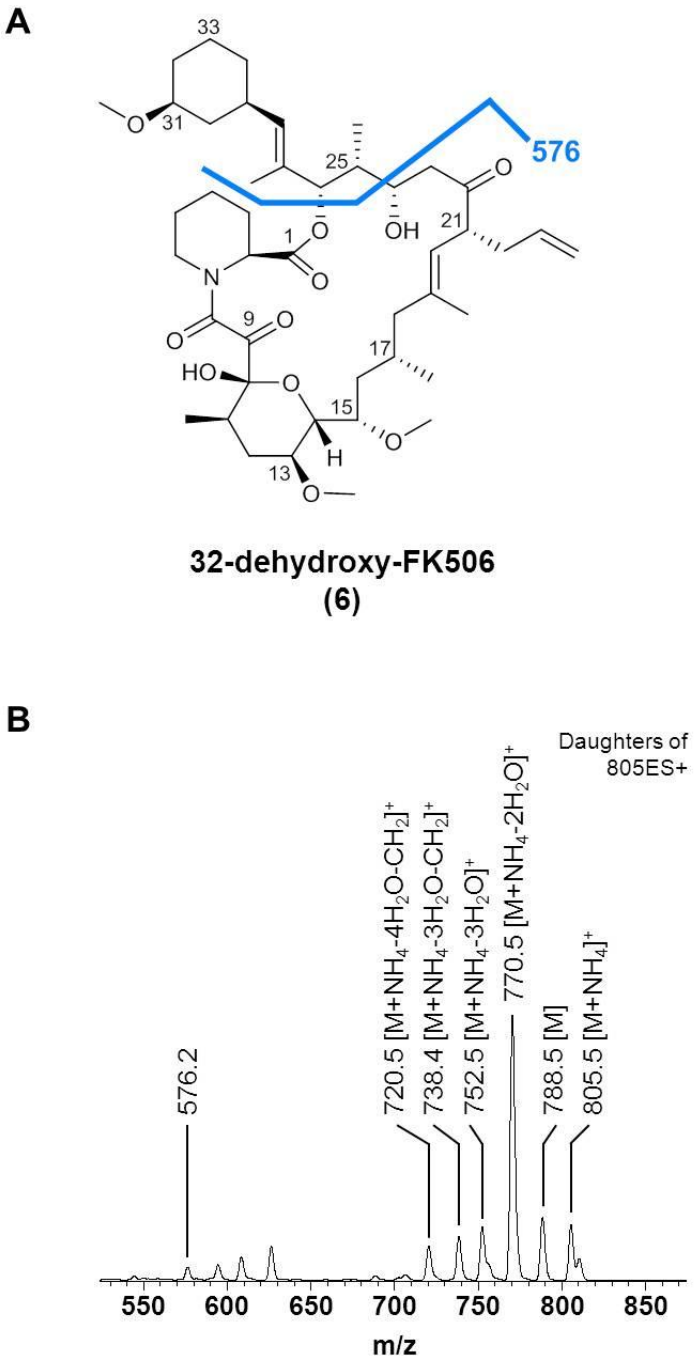


Figure S1. Construction and verification of *fkbO* in-frame deletion in *Streptomyces* sp. KCTC 11604BP. (A) Schematic representation of *fkbO* in-frame deletion by homologous recombination. (B) PCR analysis. 1, PCR products (1,273 bp) obtained from wild type strain; 2, PCR products (280 bp) obtained from ΔfkbO mutant strain; 3, PCR products (280 bp and 1,273 bp) obtained from single cross-over mutant strain; L, DNA ladder.



32-dehydroxy-FK506
(6)

***Figure S2*.** ESI-MS/MS analysis of a novel FK506 analogue, 32-dehydroxy-FK506 (**6**) obtained from the *fkbO* deletion mutant of *Streptomyces* sp. KCTC 11604BP (ΔfkbO strain) supplemented with 3-cyclohexene-1-carboxylic acid (**5**). (A) ESI-MS/MS fragmentation pattern of **6**. (B) MS/MS spectra of **6**. Structural estimation of FK506 congeners produced by the same strain was carried out as previously described.[5]

Figure S3. ^{1}H NMR (900 MHz, $CDCl_3$) spectrum of 32-dehydroxy-FK506 (**6**).

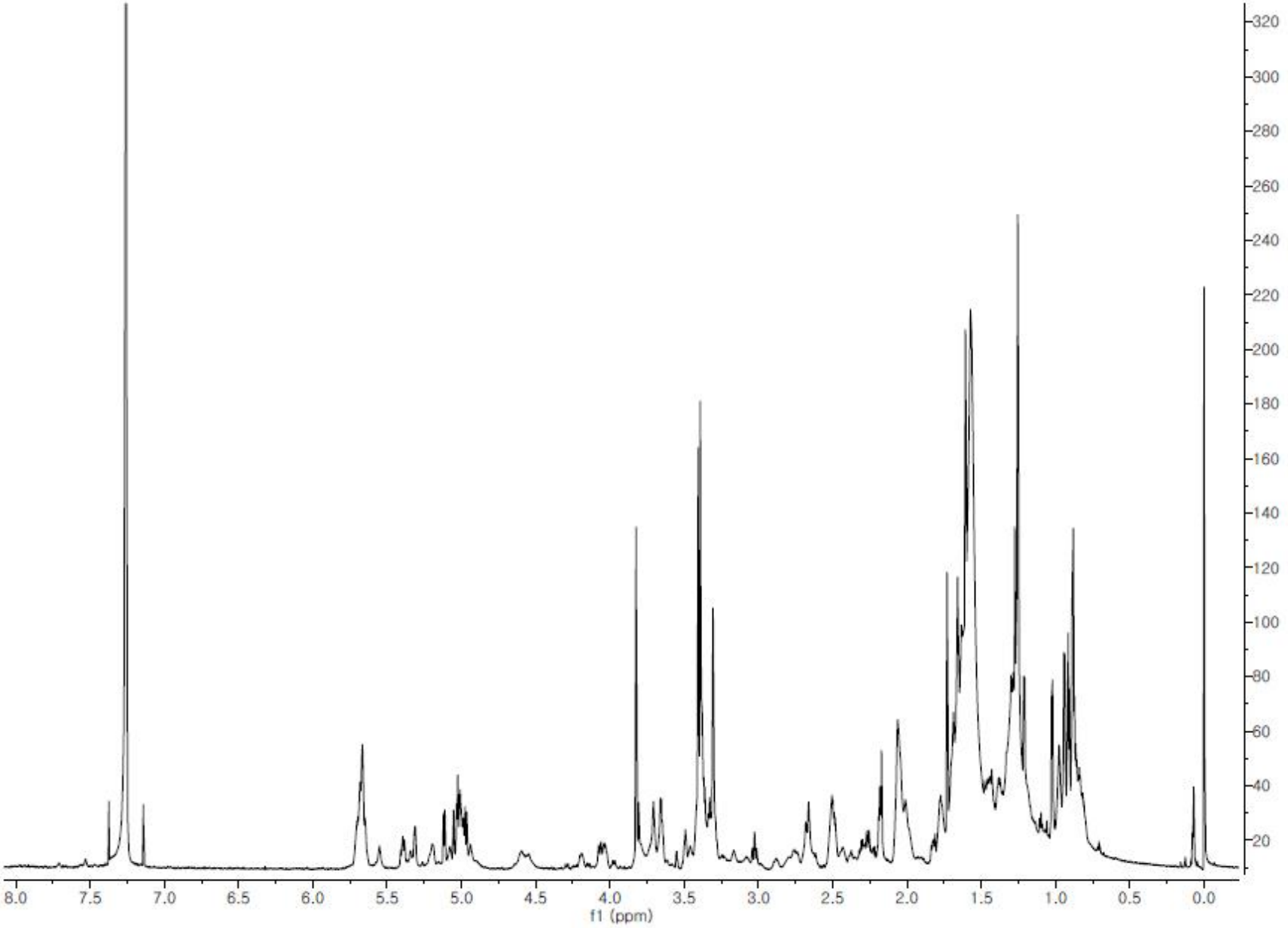


Figure S4. ^{13}C NMR (225 MHz, $CDCl_3$) spectrum of 32-dehydroxy-FK506 (**6**).

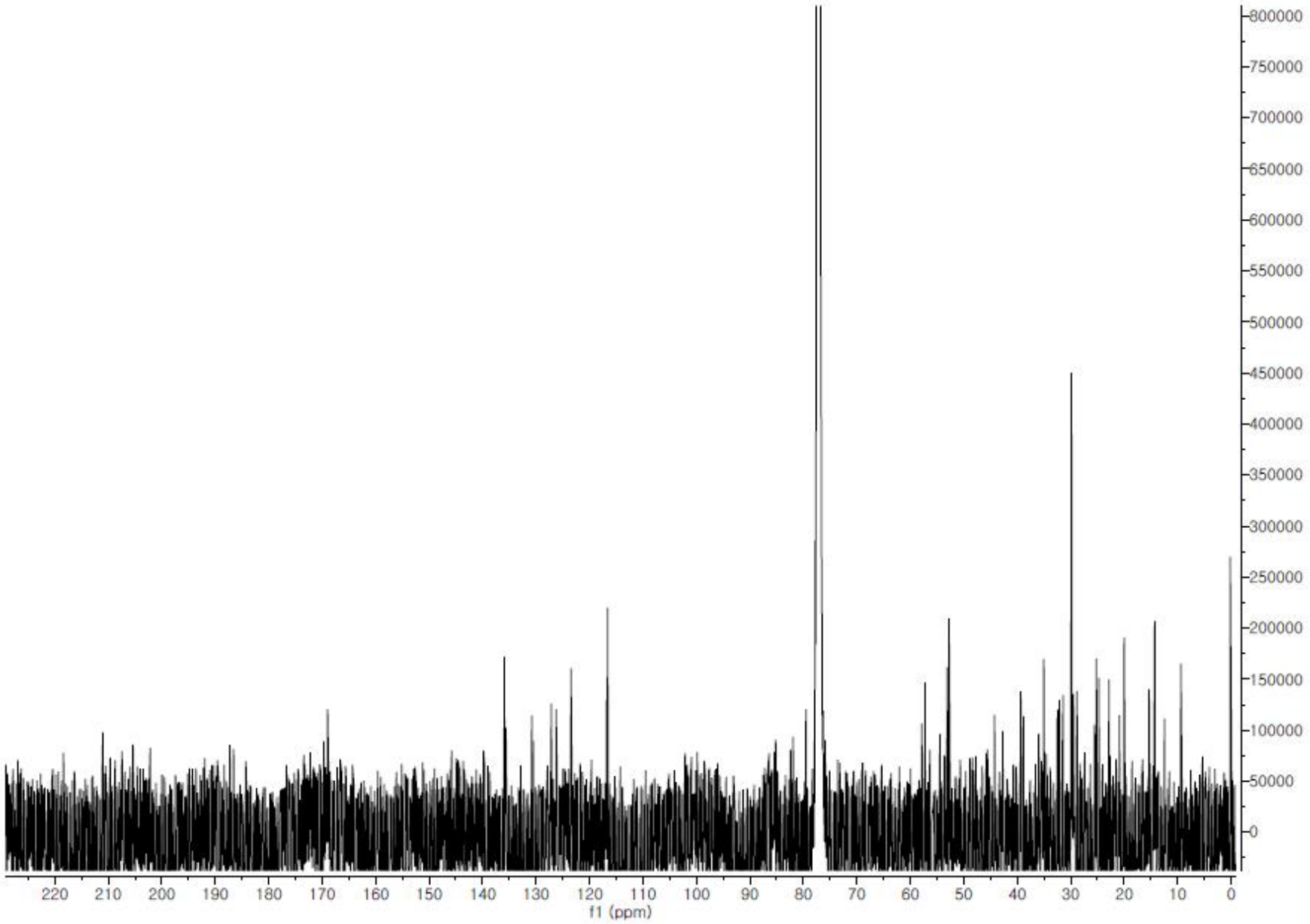


Figure S5. 2D ^{1}H-^{1}H COSY NMR spectrum of 32-dehydroxy-FK506 (**6**).

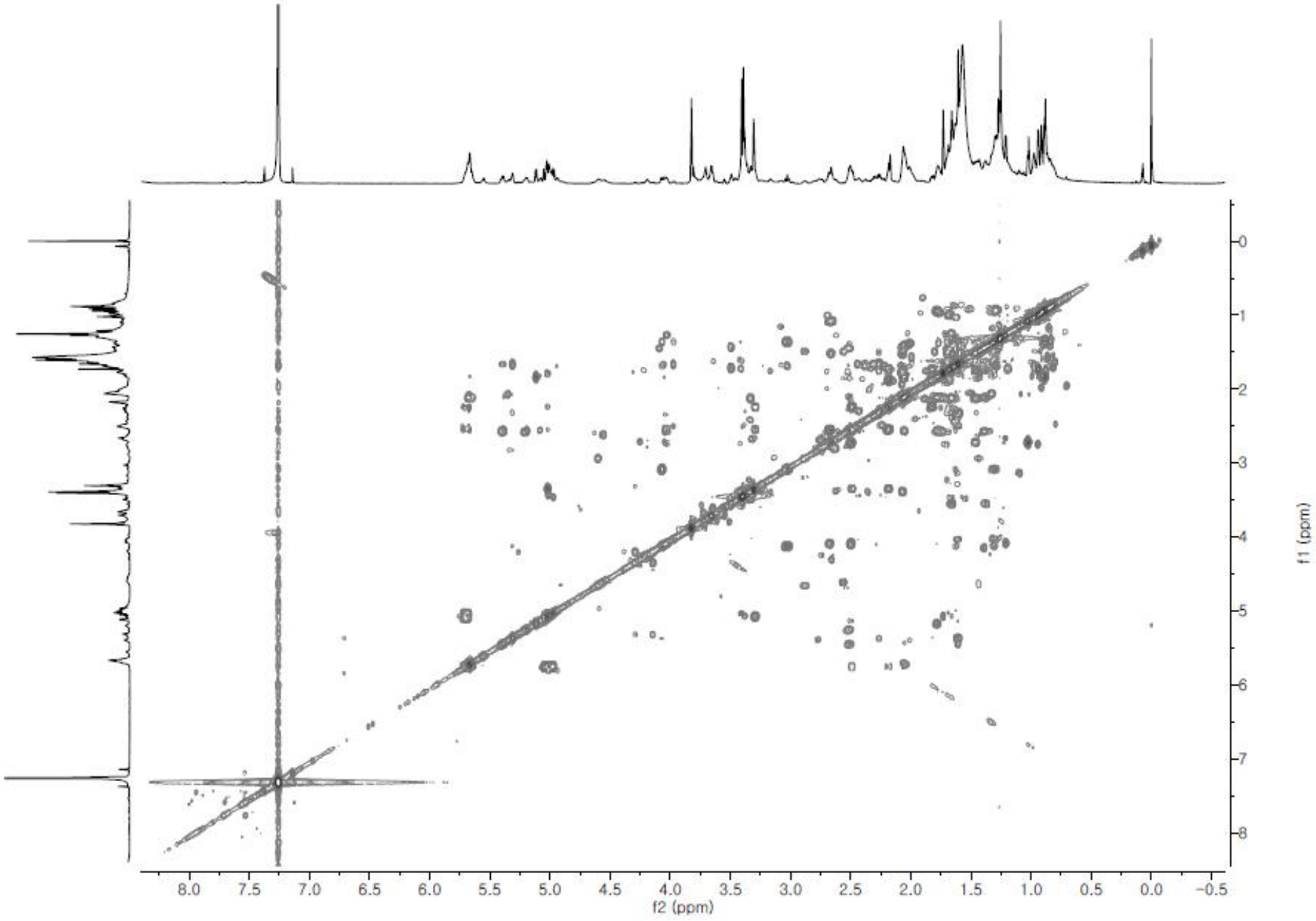


Figure S6. 2D HMQC NMR spectrum of 32-dehydroxy-FK506 (**6**).

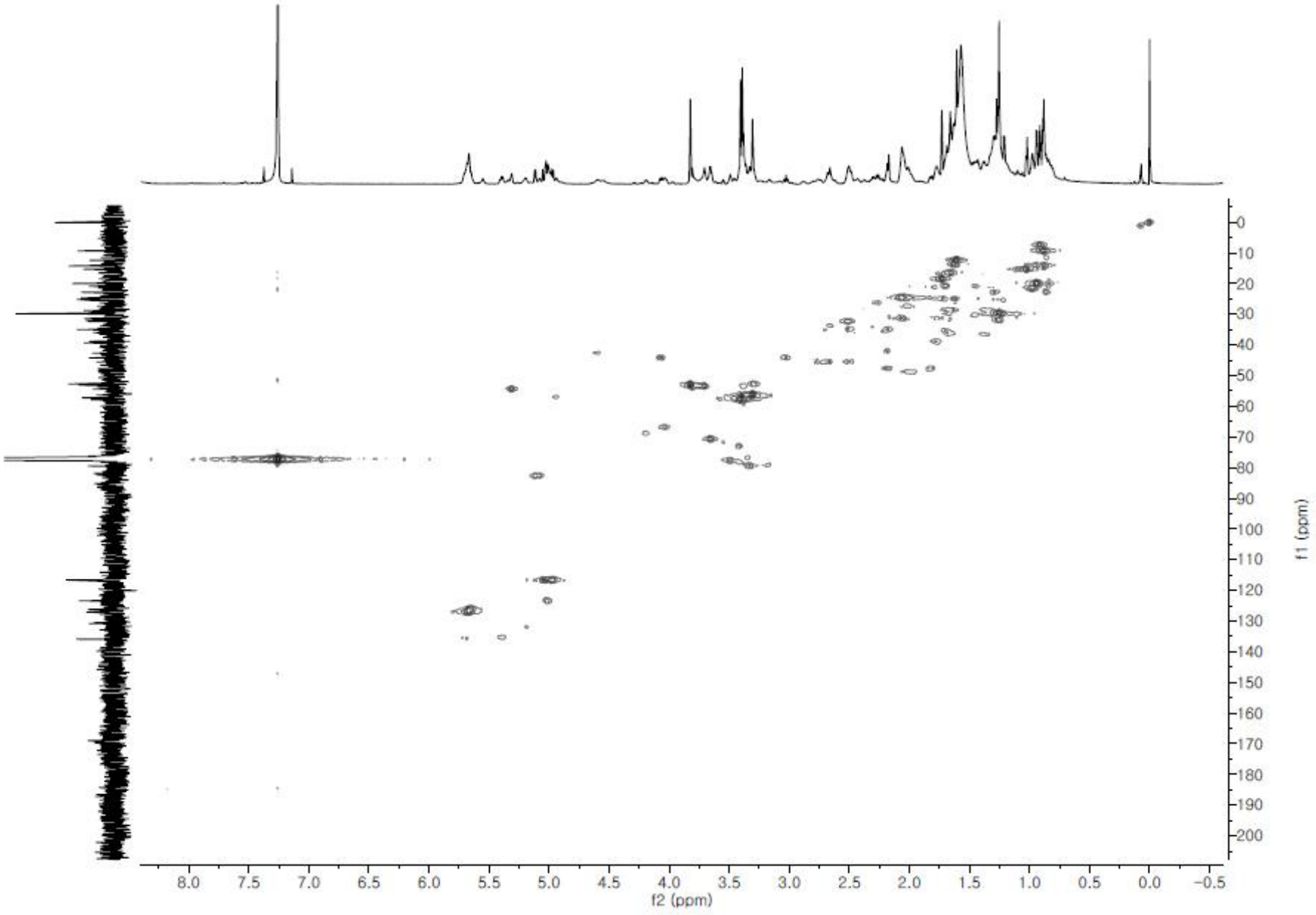


Figure S7. 2D HMBC NMR spectrum of 32-dehydroxy-FK506 (**6**).

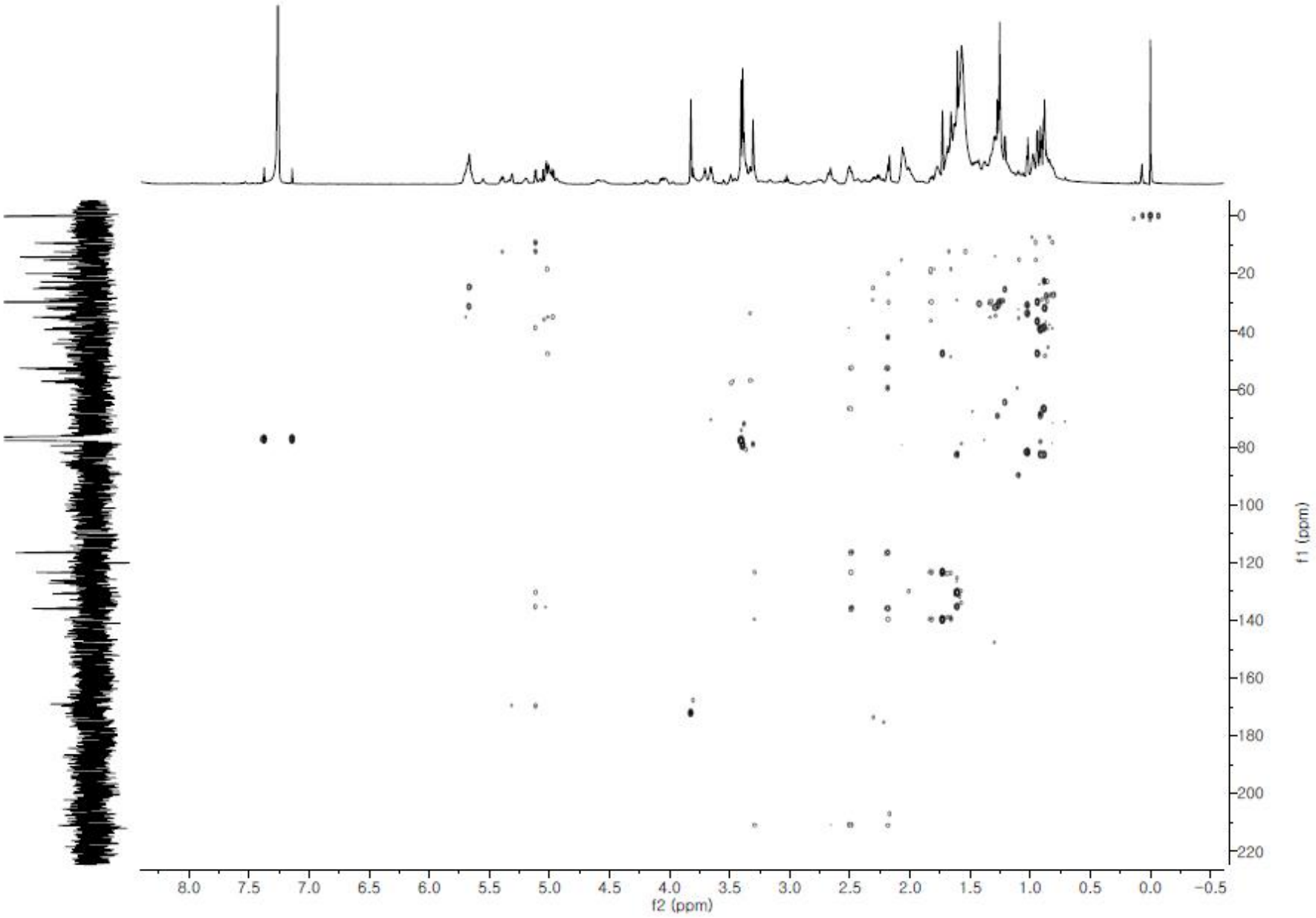


Table S1. NMR data for the novel FK506 analogue, 32-dehydroxy-FK506 (**6**)

Position	δ_C	δ_H	COSY correlations	HMBC correlations
1	168.98	-		
2	54.43	4.59	H-3b	C-1, 3, 4
3	27.36	2.06	H-3b	
		2.01	H-4b	
4	22.83	1.73	H-4b	C-3, 6
		1.39		
5	25.18	1.73	H-5b	C-3, 6
		1.48	H-4b	
6	38.81	4.43	H-5a/b, H-6b	C-2, 4, 5, 8
		3.03	H-5a/b	C-2, 8
7	-	-		
8	164.34	-		
9	197.40	-		
10	97.53	-		
11	33.83	2.19	H-12b, 38	
12	32.37	2.17		C-13
		1.46		C-11, 13
13	73.19	3.42	H-12a/b	
14	71.91	3.71	H-13, 15	C-10, 12, 13, 15
15	76.85	3.55	H-16a/b	
16	32.49	1.59	H-16b	
		1.06		
17	26.33	1.66	H-16a/b, 39	C-19
18	44.20	2.18	H-17, 18b	C-16, 17, 19, 20, 39, 40
		1.81		C-16, 17, 19, 20, 39, 40
19	135.92	-		
20	123.44	5.08	H-21	C-18, 21, 35, 40
21	52.75	3.55	H-35a/b	C-19, 20, 22, 35, 36

22	210.99	-		
23	42.70	2.76	H-23b	C-22, 24, 25
		2.17		C-21, 22
24	70.54	3.97	H-23a/b, 25	C-26
25	39.33	1.83	H-41	C-24, 41
26	77.65	5.31	H-25	C-24, 25, 27, 28, 41, 42
27	130.78	-		
28	127.13	5.12	H-29	C-26, 29, 30, 34, 42
29	34.56	2.31	H-30b, 34a	
30	34.40	2.04		C-28, 31
		0.98		C-28, 29, 31, 32, 34
31	82.42	3.03	H-30a/b	C-32, 45
32	31.39	1.78	H-31, 33b	C-31, 33
		1.21		
33	32.08	2.01	H-33b, 34a	
		1.37	H-34b	C-32
34	31.47	1.66		C-28, 29, 30
		1.10		
35	35.03	2.43		C-20, 21, 22, 36, 37
		2.17		C-20, 21, 22, 36, 37
36	135.92	5.64		
37	116.51	5.03		C-36
		4.97		
38	15.32	1.02		C-10, 12
39	20.00	0.94		C-16, 17, 18
40	12.44	1.61		C-18, 19, 20
41	14.25	0.88		C-24, 25, 26
42	18.48	1.73		C-26, 27, 28
43	57.77	3.40		C-13
44	57.33	3.39		C-15
45	56.37	3.31		C-31

Reference

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