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Supporting Information

A Linear Hydroxymethyl Tetramate Undergoes an Acetylation-Elimination Process for Exocyclic Methylene Formation in the Biosynthetic Pathway of Pyrroindomycins

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1. Supplementary Methods

General Experimental Procedures.

UV absorption spectra were recorded with a Varian Cary 100 UV/Vis spectrophotometer; wavelengths are reported in nm. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance III AV600 (Cryo) spectrometers (Bruker Co. Ltd., Germany), or on the Agilent ProPlus 500 MHz NMR spectrometer (Agilent Technologies Inc., USA), *J* values are expressed in Hz. The ^{1}H and ^{13}C NMR assignments were supported by ^{1}H-^{1}H COSY, HMQC, and HMBC experiments. The following abbreviations are used to describe spin multiplicity: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; brs, broad singlet; dd, doublet of doublets; ov, overlapped signals. The molecular mass was determined with high resolution ESIMS (HRESIMS) analysis with the 6230B Accurate Mass TOF LC/MS System (Agilent Technologies Inc., USA). An isopropyl alcohol solution of sodium iodide (2 mg/mL) was used as a reference compound. Reversed-phase HPLC was performed on an Agilent 1200 HPLC equipped with a DAD detector using either a semi-preparative (ZORBAX SB, 9.6 mm × 250 mm, 5 μm) C18 HPLC column or analytic (ZORBAX SB, 4.6 mm × 250 mm, 5 μm) C18 HPLC column. To analyze the species of the *in vitro* assay, reaction mixtures were analyzed by gradient elution of mobile phase A (H_2O supplemented with 0.1% formic acid) and mobile phase B (acetonitrile supplemented with 0.1% formic acid) with the flow rate of 1.0 mL/min: 0 to 5 min, 65% phase B; 5 to 15 min, 65% to 100% phase B; 15 to 20 min, 100% phase B; 20 to 25 min, 100% to 65% phase B; and 25 to 30 min, 65% phase B, λ at 220 nm). Acetyl-CoA was purchased from Sigma-Aldrich, USA. All the molecular subcloning experiments were conducted by using Phanta Max Super-Fidelity DNA polymerase (Vazyme Biotech Co. Ltd., China) and/or ClonExpress Entry One Step Cloning Kit (Vazyme Biotech Co. Ltd., China).

Strains and Proteins.

The double mutant *S. rugosporus* Δ*pyrD3/D4* was constructed as previously described.[1,2] The proteins PyrD3, PyrD4 and PyrE3 were expressed heterologously in *E. coli* BL21(DE3) as previously described.[1,3]

Fermentation and Isolation of Compound 2.

The fermentation was carried out according to the method described previously, with shorter fermentation time (96 hours). The metabolites were analyzed by gradient elution of mobile phase A (H_2O supplemented with 0.1% formic acid) and mobile phase B (acetonitrile supplemented with 0.1% formic acid) with the flow rate of 1 mL/min: 0 to 5 min, 10% to 30% phase B; 5 to 10 min, 30% to 65% phase B; 10 to 20 min, 65% to 100% phase B; 20 to 25 min, 100% phase B; 25 to 30 min, 100% to 10% phase B; and 30 to 35 min, 10% phase B (λ at 220 nm). A 50 L fermentation broth of *WL2004* was extracted three times with equal volume of EtOAc. The organic extract was dried and evaporated under reduced pressure to afford 50.0 g residue. The residue was subjected to column chromatography (CC) on ODS to give **2** containing fraction, eluted with MeOH/H_2O in gradient of 30% to 100%. Further chromatography on semi-preparative HPLC (75% CH_3CN/H_2O containing 0.1% HCOOH, 4.0 mL/min, 280 nm) yielded compound **2** (3.4 mg, 21.3 min).

Compound **2:** colorless, amorphous powder; UV (CH_3CN) λ_{max} 235, 245 nm; ^1H and ^{13}C NMR spectroscopic data, see Table 1. HRESIMS $[M + H]^+$ *m/z* 480.3144 (calcd for $C_{30}H_{46}NO_6$, 516.3320).

Biotransformation and Isolation of Compound 3.

Compound **3** was prepared by PyrD3-catalyzed biotransformation of compound **2**. The reaction proceeded at 30˚C in the 20 mL mixture containing 3.5 mg compound **2**, 5 mM acetyl-CoA and 50 μM PyrD3 in 50 mM Tris buffer (pH 7.0). After completion, the reaction mixture was extracted three times with EtOAc. The organic extracts were concentrated under reduced pressure, and the crude residue was subjected to semi-preparative HPLC (70% CH_3CN/H_2O supplemented with 0.1% formic acid, 4.0 mL/min, λ at 220 nm) to yield **3** (2.0 mg, 57% yield).

Compound **3:** colorless, amorphous powder; UV (CH_3CN) λ_{max} 235, 246 nm; ^1H and ^{13}C NMR spectroscopic data, see Table 1; HRESIMS $[M + H]^+$ *m/z* 558.3444 (calcd for $C_{32}H_{48}NO_7^+$, 558.3425); and $[M + Na]^+$ *m/z* 580.3237 (calcd for $C_{32}H_{47}NO_7Na^+$, 580.3250).

***In vitro* Assay of Compound 3 and PyrD4.**

Deacetylation of compound **3** was carried out in the assay containing 2 mM compound **3**, 50 μM PyrD4 in 50 mM Tris buffer (pH 7.0), incubated at room temperature for 30 mins. After completion, 20 μL reaction mixture was taken and

mixed with 20 μL methanol to terminate the reaction, then was subjected to HPLC analysis.

***In vitro* Assay of Compound 2 or 3 with PyrE3.**

To evaluate whether compound **2** or **3** were capable to proceed cycloaddition before acetylation or acetic acid elimination, *in vitro* assays were performed as previous described[3] in aqueous solution containing 1 mM **1** or **2**, 10 uM PyrE3, 10% methanol at room temperature for 30 mins. 20 μL aliquot of the solution was taken and mixed with 20 μL methanol to terminate the reaction, then was subjected to HPLC analysis.

2. Supplementary Tables

Table S1. ^{1}H and ^{13}C NMR data for compounds **2** and **3**.

			2 [a]			**3** [b]
no.	δ_C	type	δ_H (*J* in Hz)	δ_C	type	δ_H (*J* in Hz)
1	176.4	C		179.2	C	
2	109.5	C		101.5	C	
3	183.9	C		193.2	C	
4	129.3	C		136.3	C	
5	144.2	CH	7.03, m	140.2	CH	6.30, t (6.7)
6	28.3	CH_2	2.22, m	29.4	CH_2	2.24, m
7	24.1	CH_2	1.40, m	25.7	CH_2	1.51, m
8	36.9	CH_2	1.46, m	38.1	CH_2	1.59, m
9	70.3	CH	3.96, m	73.1	CH	4.06, m
10	135.6	CH	5.54, dd (14.8, 6.3)	135.5	CH	5.57, dd (15.0, 6.9)
11	129.0	CH	6.09, dd (14.9, 10.6)	131.6	CH	6.19, dd (15.0, 10.5)
12	131.4	CH	6.02, dd (14.8, 10.7)	133.1	CH	6.09, dd (15.0, 10.5)
13	131.2	CH	5.66, dd (14.9, 7.3)	132.4	CH	5.70, dt (15.0, 7.2)
14	37.3	CH_2	2.12, m	38.5	CH_2	2.24, m
15	70.7	CH	3.46, brs	73.2	CH	3.63, m
16	41.9	CH	1.68, m	43.5	CH	1.51, m
17a	41.0	CH_2	2.07, dd (13.9, 6.6)	42.2	CH_2	2.13, dd (13.2, 7.1)
17b			1.85, dd (13.6, 7.8)			1.96, dd (13.2, 7.1)
18	133.6	C		134.8	C	
19	130.3	CH	5.61, s	132.0	CH	5.66, s
20	133.3	C		134.8	C	
21	122.7	CH	5.25, q (4.5)	123.8	CH	5.23, q (6.7)
22a	61.0	CH_2	3.61, brs	65.5	CH_2	4.44, dd (11.4, 2.9)
22b						4.12, dd (11.4, 5.9)
23	63.5	CH	3.75, brs	60.5	CH	3.91, dd (5.9, 2.9)
24	182.7	C		193.3	C	
25	12.1	CH_3	1.80, s	13.0	CH_3	1.83, s
26a	20.9	CH_2	1.28, m	22.1	CH_2	1.43, m
26b			1.22, m			1.30, m
27	11.8	CH_3	0.83, t (7.5)	12.3	CH_3	0.91, t (7.4)

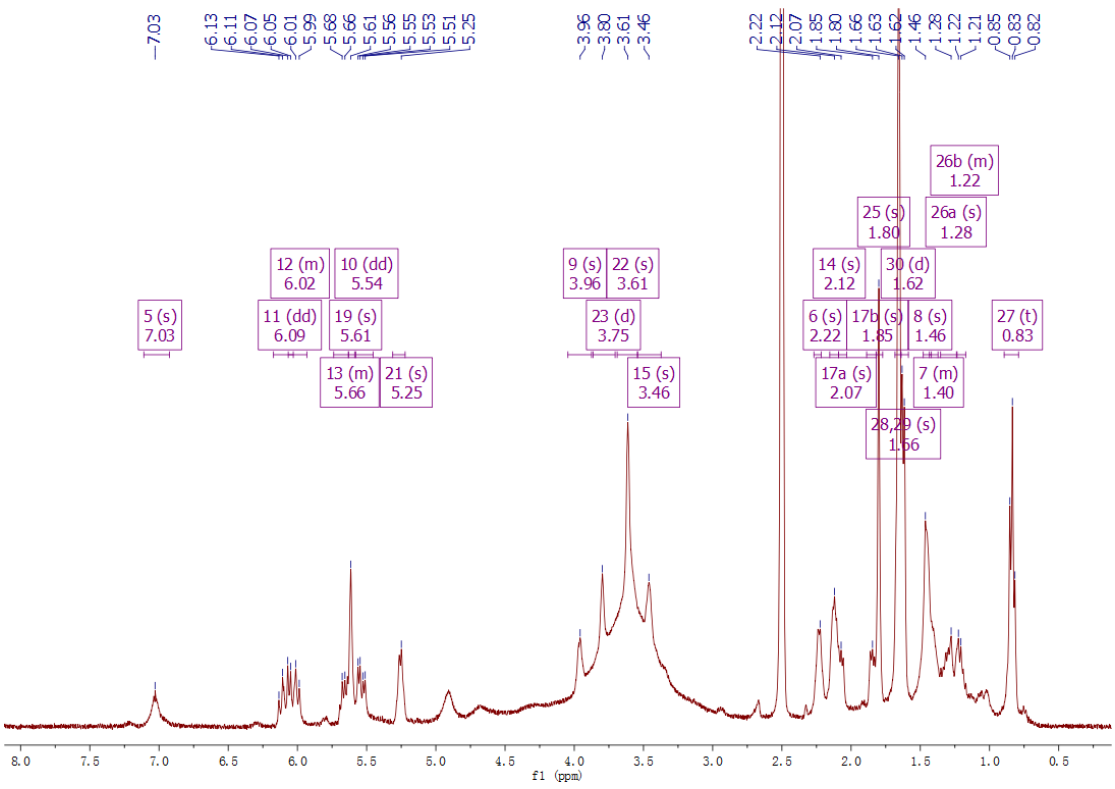
28	17.6	CH_3	1.66, s	17.9	CH_3	1.68, s
29	16.7	CH_3	1.66, s	16.9	CH_3	1.68, s
30	13.5	CH_3	1.62, d (6.8)	13.7	CH_3	1.66, d (6.7)
-OAc				172.7	C	
				20.7	CH_3	2.02, s

500 MHz for ^{1}H and 125 MHz for ^{13}C NMR. [a] in DMSO-d_6; [b] in CD_3OD.

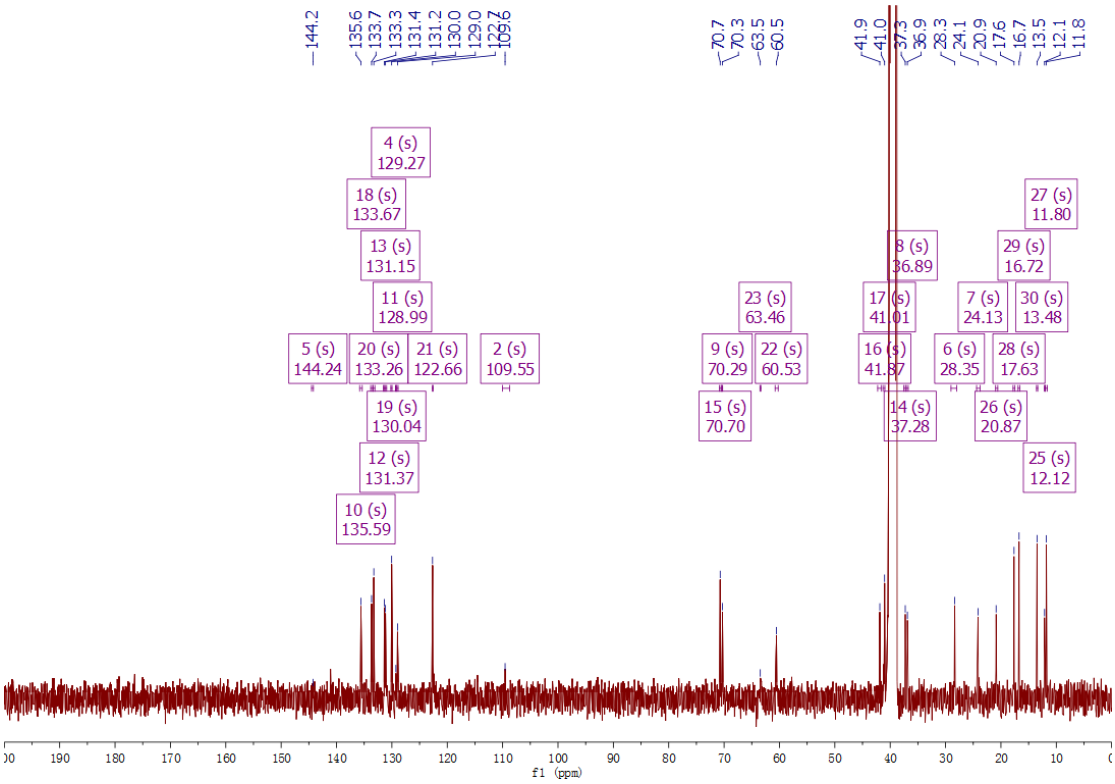
3. Supplementary Figures

Figure S1. NMR spectra of compound **2** (a-f)

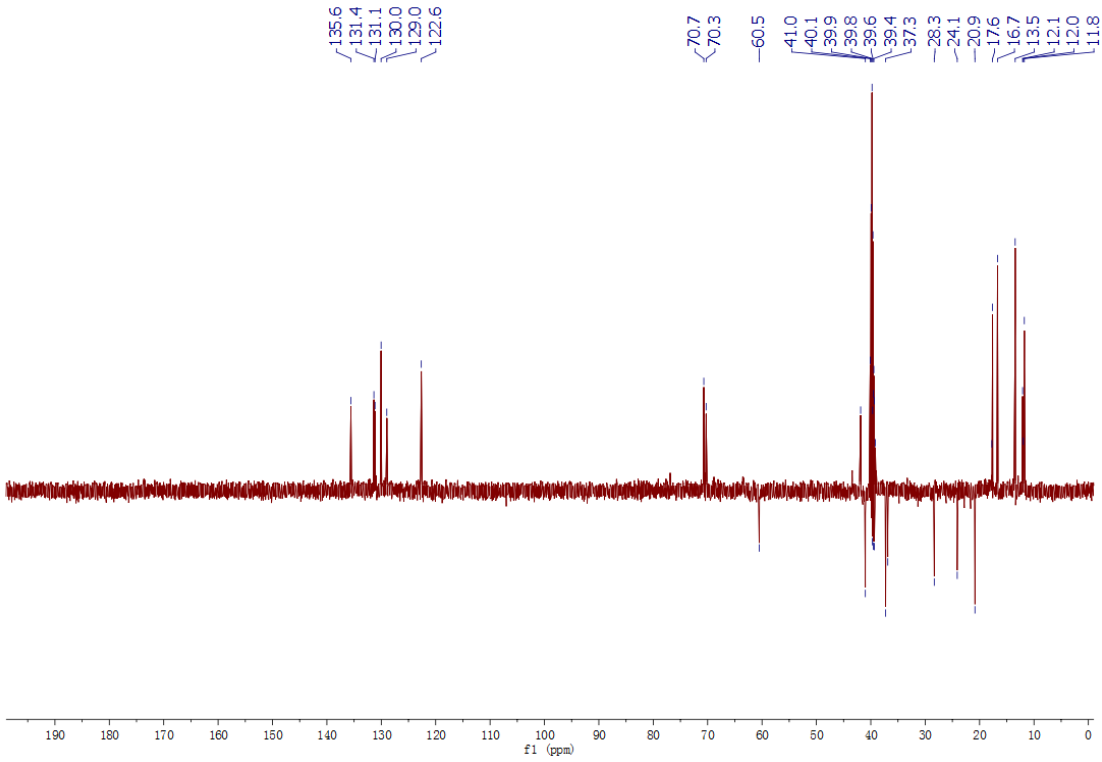
a) ^{1}H spectrum of compound **2**



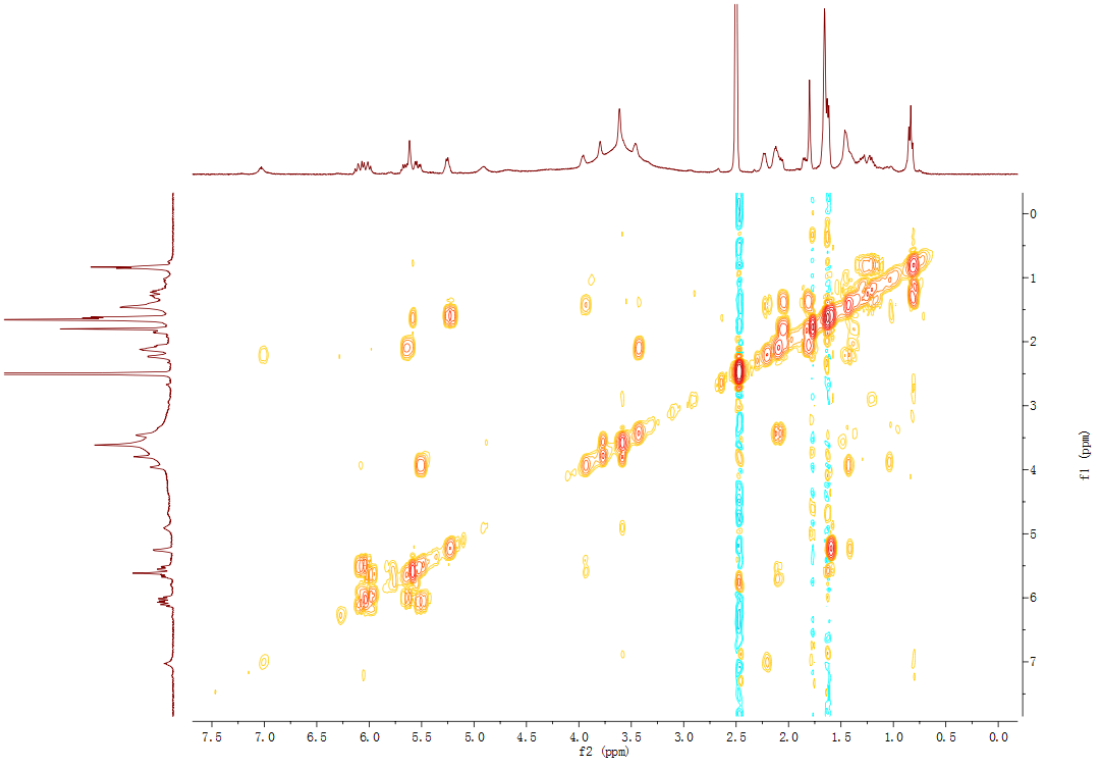
b) ^{13}C spectrum of compound **2**



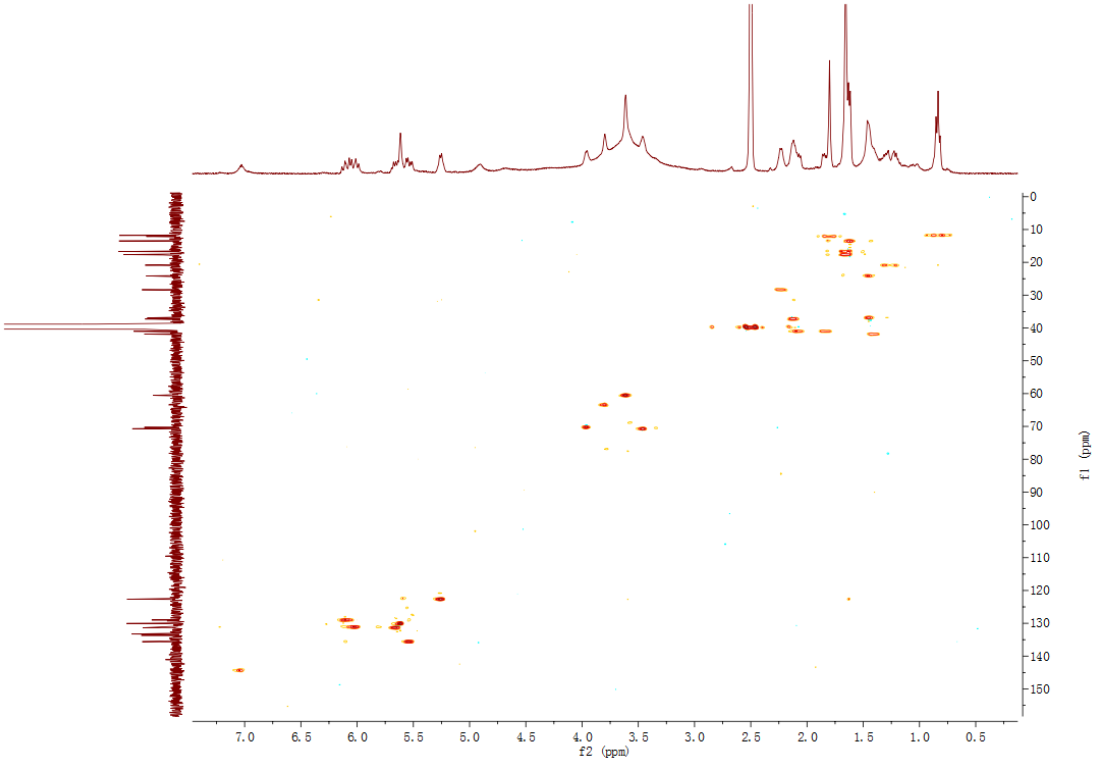
c) DEPT spectrum of compound **2**



d) COSY spectrum of compound **2**



e) HSQC spectrum of compound **2**



f) HMBC spectrum of compound **2**

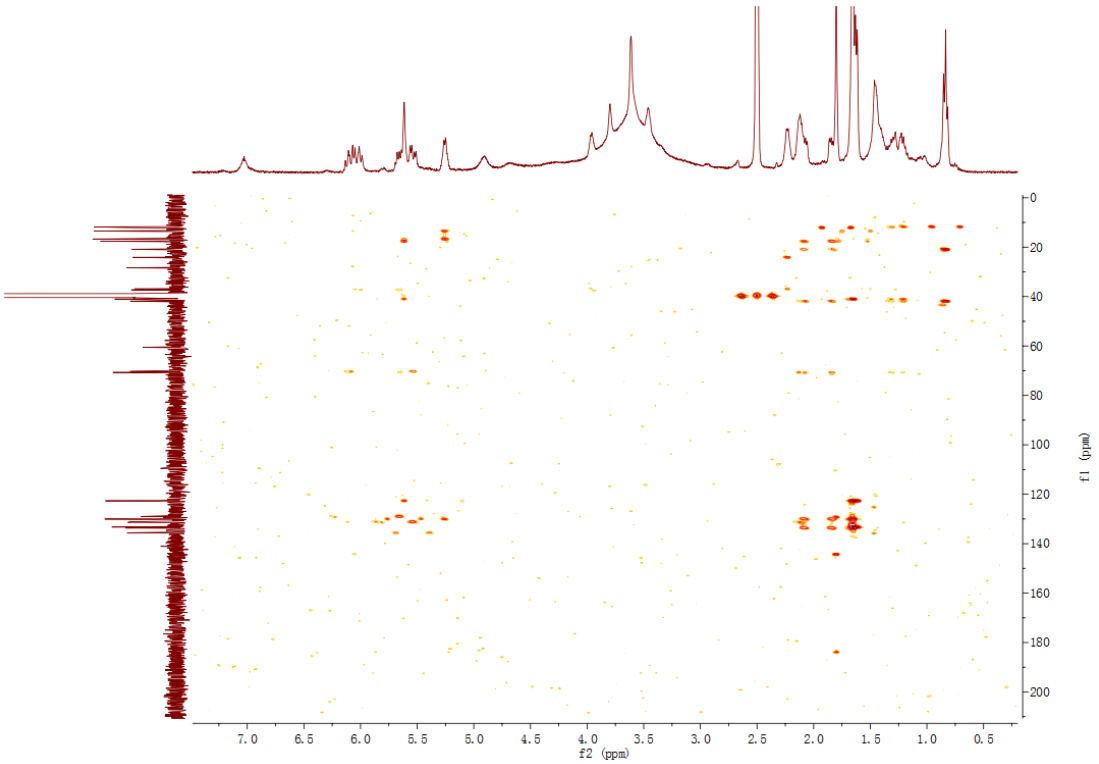
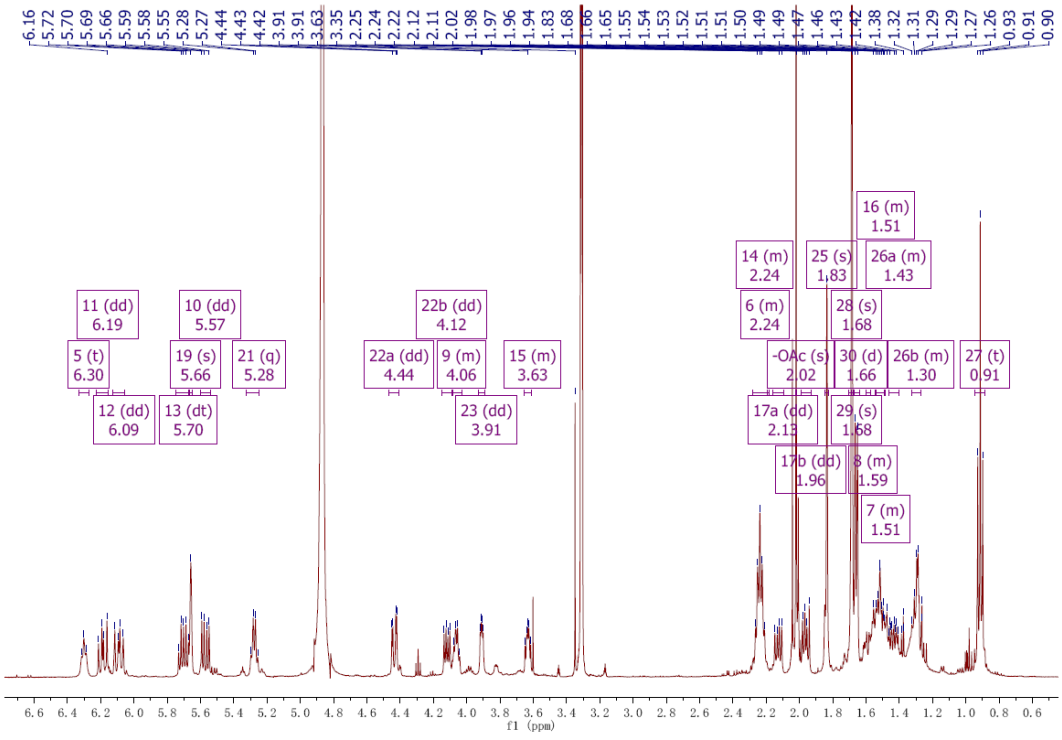
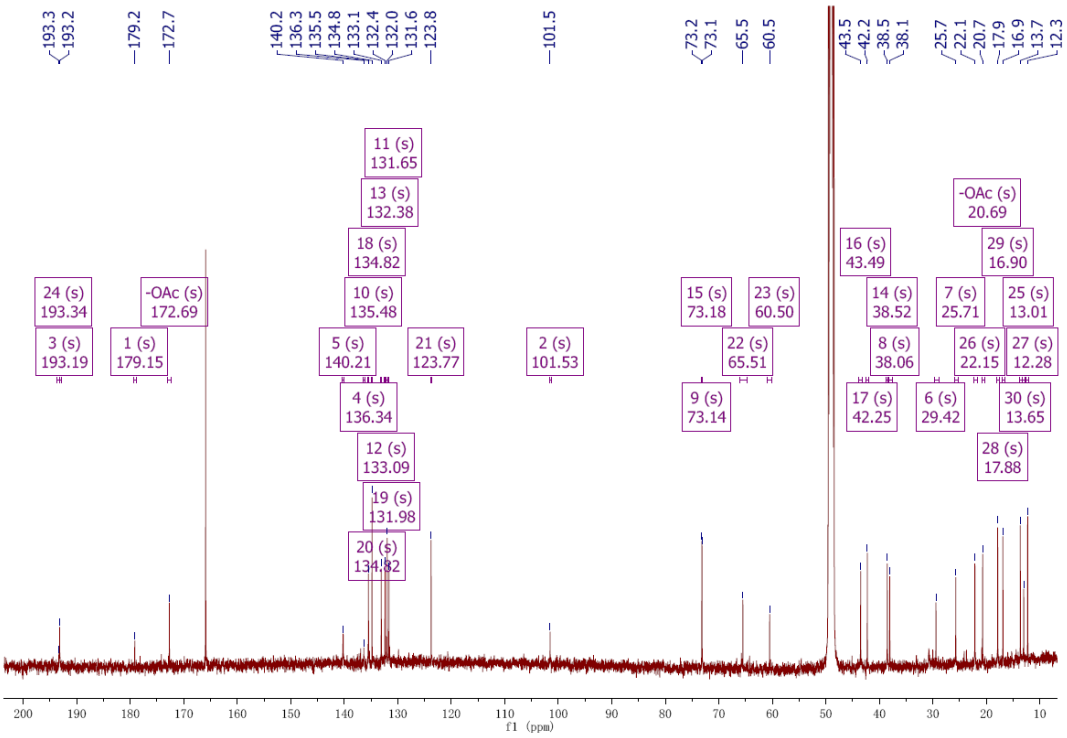


Figure S2. NMR spectra of compound **3** (a-f)

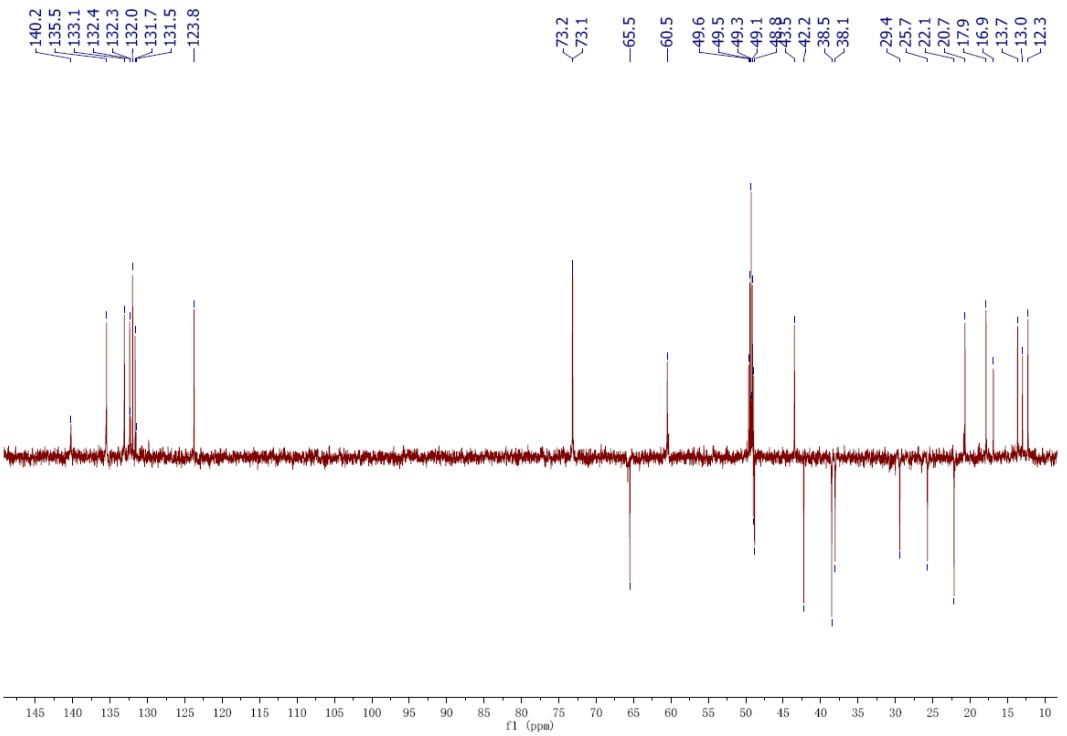
a) ^{1}H spectrum of compound **3**



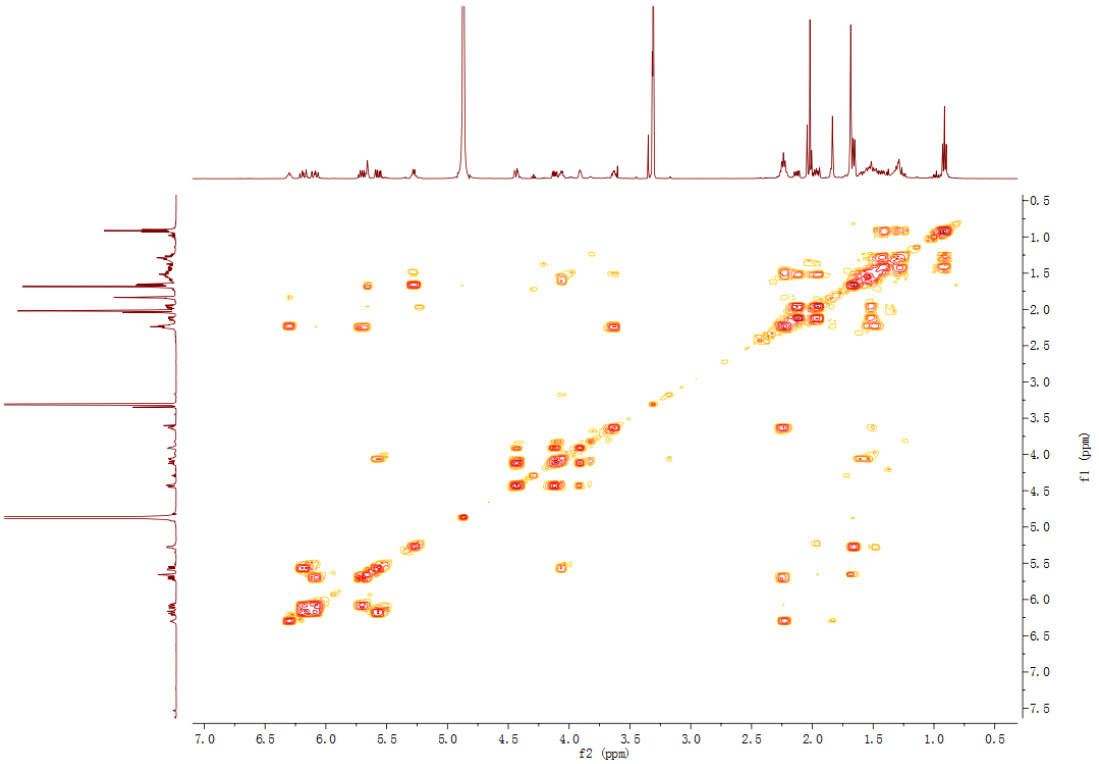
b) ^{13}C spectrum of compound **3**



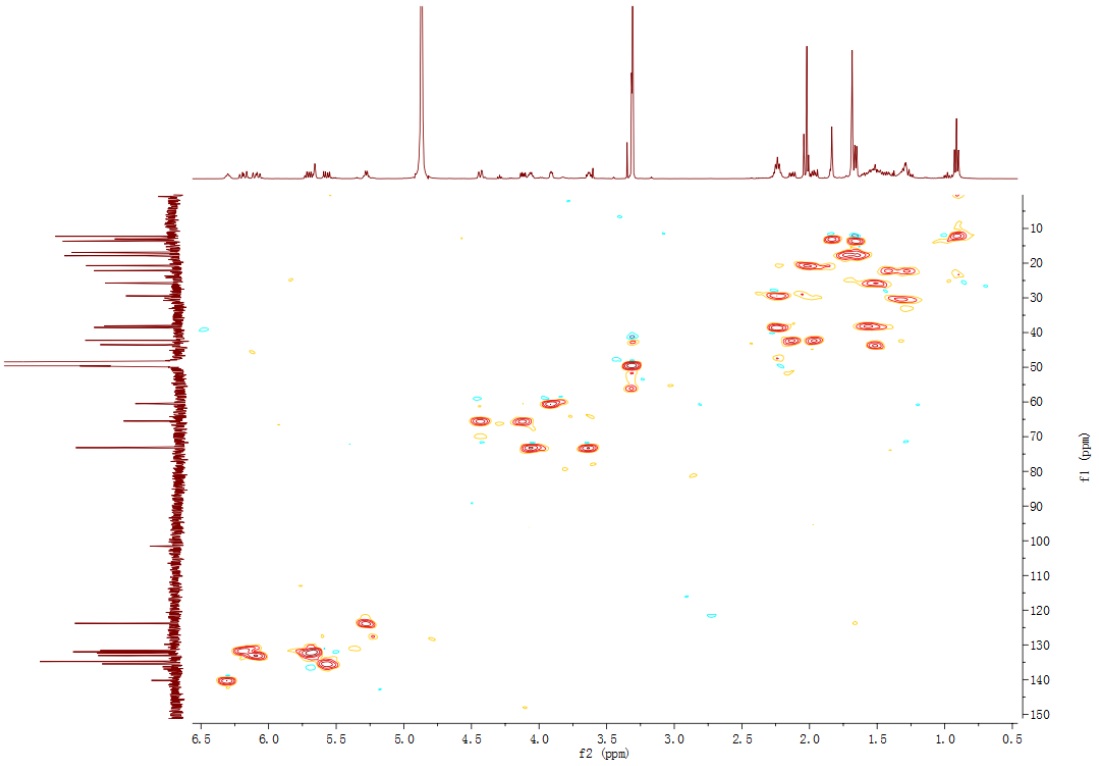
c) DEPT spectrum of compound **3**



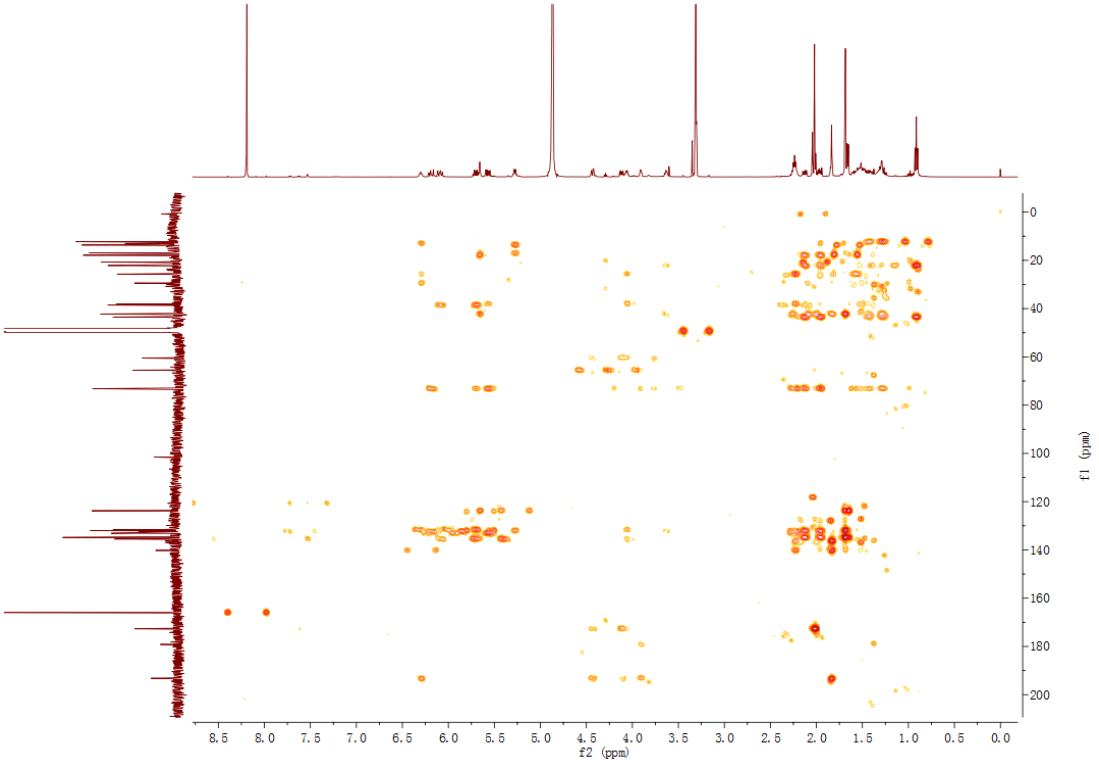
d) COSY spectrum of compound **3**



e) HSQC spectrum of compound **3**



f) HMBC spectrum of compound **3**



g) NOESY spectrum of compound **3**

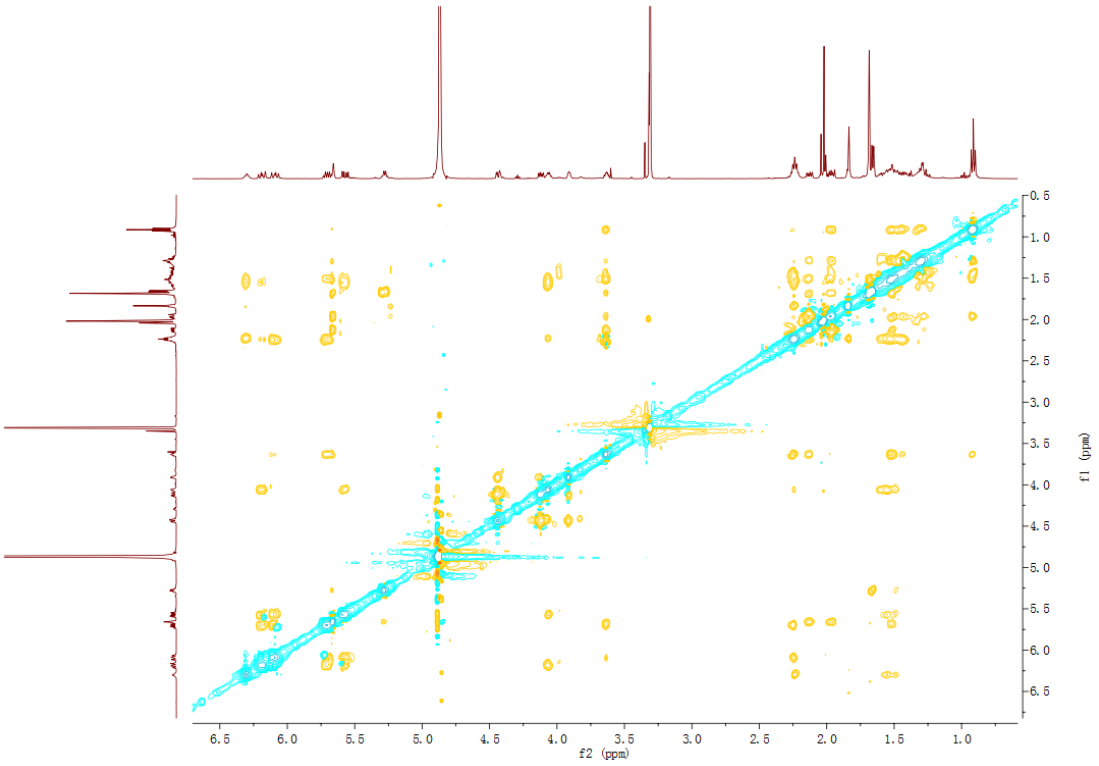
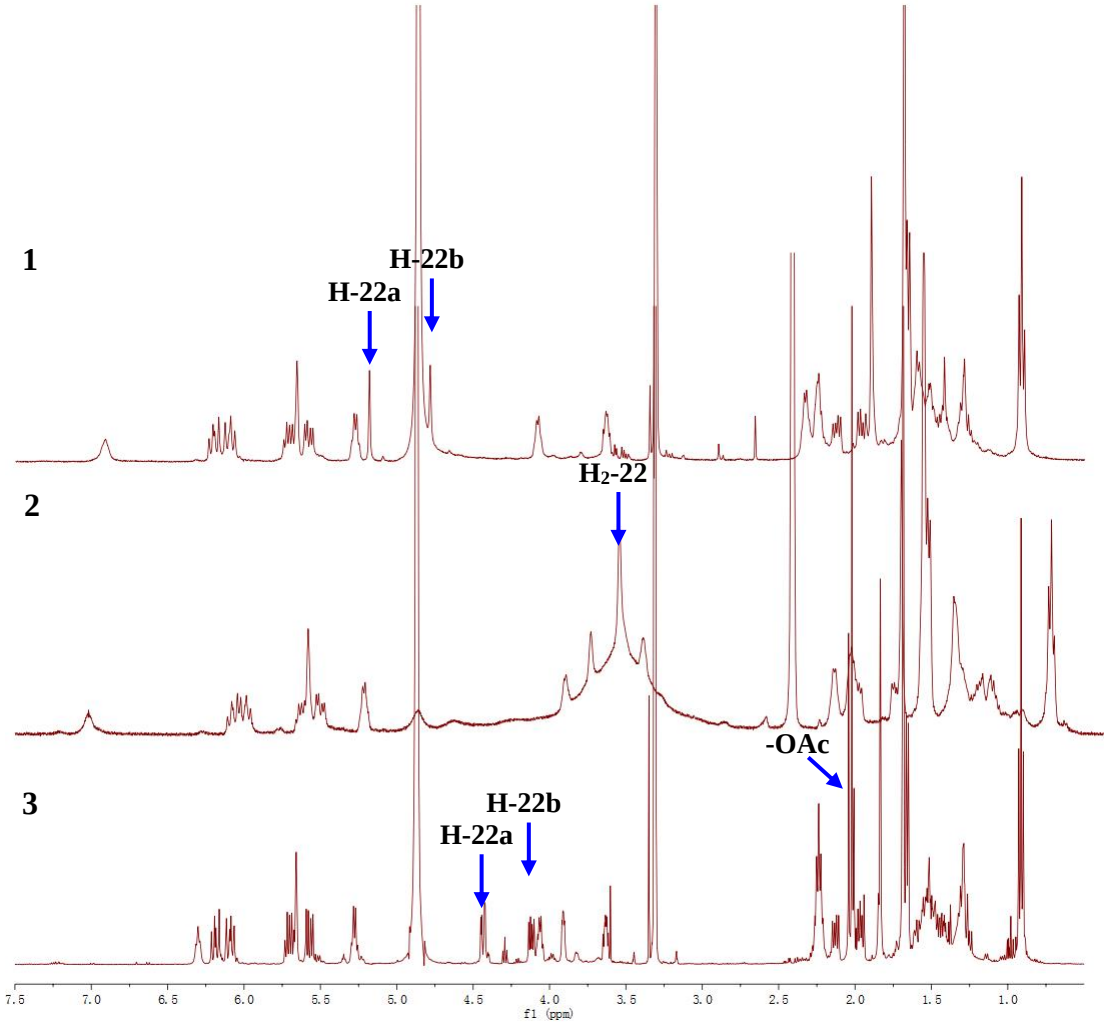


Figure S3. The comparison of ^{1}H NMR of compounds **1**, **2** and **3** (the key signals for H_2-22 and the additional acetyl group in **3** were marked).



4. Supplementary References

(1) Q. Wu, Z. Wu, X. Qu and W. Liu, *J. Am. Chem. Soc.*, 2012, **134**, 17342-17345.

(2) D. A. Hopwood, T. Kieser, M. Bibb, M. Buttner and K. Chater, *Practical Streptomyces Genetics*. John Innes Foundation: 2000; p 613.

(3) Z. Tian, P. Sun, Y. Yan, Z. Wu, Q. Zheng, S. Zhou, H. Zhang, F. Yu, X. Jia, D. Chen, A. Mándi, T. Kurtán and W. Liu, *Nat. Chem. Biol.*, 2015, **11**, 259-265.