Diasteltoxins A-C, asteltoxin-based dimers from a mutant of the sponge-associated *Emericella variecolor* fungus

Hailin Long,[†] Zhongbin Cheng,[†] Wei Huang,[†] Qi Wu,[†] Xiaodan Li,[†] Jingrong Cui,[†] Peter Proksch,[‡] Wenhan Lin*[†]

†State Key Laboratory of Natural and Biomimetic Drugs, Peking University, Beijing 100191, P.R. China, and
‡Institute of Pharmaceutical Biology and Biotechnology, Heinrich-Heine University, 40225 Duesseldorf, Germany

Supporting Information

Table of Contents

Experimental Section

General Experimental Procedures. IR spectra were recorded on a Nicolet iS50 FT-IR Microscope spectrometer (FT-IR Microscope Transmission). Optical rotations were measured using an Autopol III automatic polarimeter (Rudolph Research Co.). UV spectra were recorded by a 3300-ELSD UV detector (Alltech Co.). 1D and 2D NMR spectra were taken on either a Varian Inova 600 MHz or a Bruker Avance-III 400 MHz NMR spectrometer using DMSO-d_6 as a solvent. CD spectra were recorded at room temperature on a Jasco J-815 spectrophotometer in 1 cm cuvettes. HPLC analysis were carried out using a Shimadzu LC-20AD instrument with a SPD-M20A detector and a reversed-phase C_{18} column (Thermo Scientific BDS Hypersil, 4.6 × 150 mm, 5 μm). Preparative HPLC procedure was performed on a All-tech LabAlliance Series III instrument with a UV detector and a reversed-phase C_{18} column (Waters Sunfire^{TM}, 250×10 mm, particle size 5 μm). Column chromatography (CC) was performed using a silica gel (200-300 mesh; Qingdao Marine Chemical Inc., Qingdao, China), Size exclusion chromatography (Sephadex LH-20, 20-80 μm, Pharmacia Fine Chemicals, Uppsala, Sweden), precoated GF254 silica gel plates (Qingdao Marine Chemical Inc., Qingdao, China) was used for TLC analyses. The spots were visualized by spraying with 2.5% anisaldehyde in aqueous 95% EtOH and followed by heating under hair dryer. Both ESIMS and HRESIMS experiments were conducted on an Agilent 1100 series LC/MSD Trap SL mass spectrometer.

Fungal Material. Fungal strain *Emericella variecolor* XSA-07-2 was isolated from a marine *Cinachyrella* sp. sponge, which was collected from Yongxin Island in the South China Sea, in April 2013. The fungal clone germinated from the cut of sponge tissue was repurified under sterile conditions using standard methods. Morphological scrutiny of hyphae and spores combined with the 18S rDNA ITS sequence (GenBank number KP202154) led to the identification of its species. This strain (XSA-07-2) was deposited at the State Key Laboratory of Natural and Biomimetic Drugs, Peking University, China.

Mutation. The initial fungal strain, *Emericella variecolor* XSA-07-2 was used as control strain in the present study. The mutants were obtained by the DES mutagenesis. Fresh XSA-07-2 spores in 50% (v/v) DMSO were treated with 1% (v/v) DES at 4 ℃ for 1 day, and single colony isolates on the treated spores were collected. Eight mutants were isolated and each was inoculated onto potato dextrose agar (PDA) plates from a PDA slant stock stored at 4 ℃ and incubated at

28 ℃ for 4 days. Fresh spores formed on the PDA plates were harvested and suspended in 80 mL of sterilized, distilled water with several glass beads in a 100 mL cone-shaped flask and scattered well by shaking enough to prepare a crude spore suspension. A 100 μL portion of this crude spore suspension was added into a well of 96-well plates, diluted with water with its OD at 600 nm measured using a VERSAmax-BN03152 plate reader, and the dilution ratio was recorded when the OD value reached 0.35. Subsequently, the remaining whole crude spore suspension was diluted with sterilized, distilled water in the same proportion to obtain the mutant spore suspension. Each mutant spore suspension was used for the producing fermentation in the following experiments.

Fermentation of the mutants. Fermentation of mutated strains was initiated in 50×500 mL sized Erlenmeyer flasks, each preloaded with 100 g of rice and 100 mL of sterilized artificial seawater (NaCl 26.726 g, $MgCl_2$ 2.26 g, $MgSO_4$ 3.248 g, $CaCl_2$ 1.153 g, $NaHCO_3$ 0.198 g, KCl 0.721 g, NaBr 0.058 g, H_3BO_3 0.058 g, Na_2SiO_3 0.0024 g, $Na_2Si_4O_9$ 0.0015 g, H_3PO_4 0.002 g, Al_2Cl_6 0.013 g, NH_3 0.002 g, $LiNO_3$ 0.0013g, H_2O 1 L). The seed was prepared by inoculating activated fungal cakes from an agar Petri dish into 200 mL of potato dextrose broth medium. Approximately 20 mL aliquots of the inoculum were then transferred to fermentation medium and further incubated for 30 days at 28 ℃ statically.

After analyses of HPLC, ^{1}H NMR and ESIMS data of the EtOAc extract of each mutated strain, the mutant of *E. variecolor* XSA-07-2-M3 was selected for large scale solid fermentation.

Extraction and Isolation. The rice cultures of XSA-07-2-M3 were extracted with EtOAc (3 times), while the EtOAc solution was concentrated in vacuo to yield a residue (2 g), The EtOAc extract was partitioned between 80% EtOH and petroleum ether to remove lipid layer. After concentration in vacuo, The EtOH fraction (1.5 g) was subjected to an ODS column and eluted with MeOH-H_2O (3:7) to yield asteltoxin (403 mg) and a subfraction M3-2 (50 mg). M3-2 was separated by the preparative RP-HPLC chromatography eluting with CH_3CN/H_2O (40%, v/v) as the mobile phase to yield **1** (4.1 mg), **2** (3.8 mg) and **3** (2.3 mg).

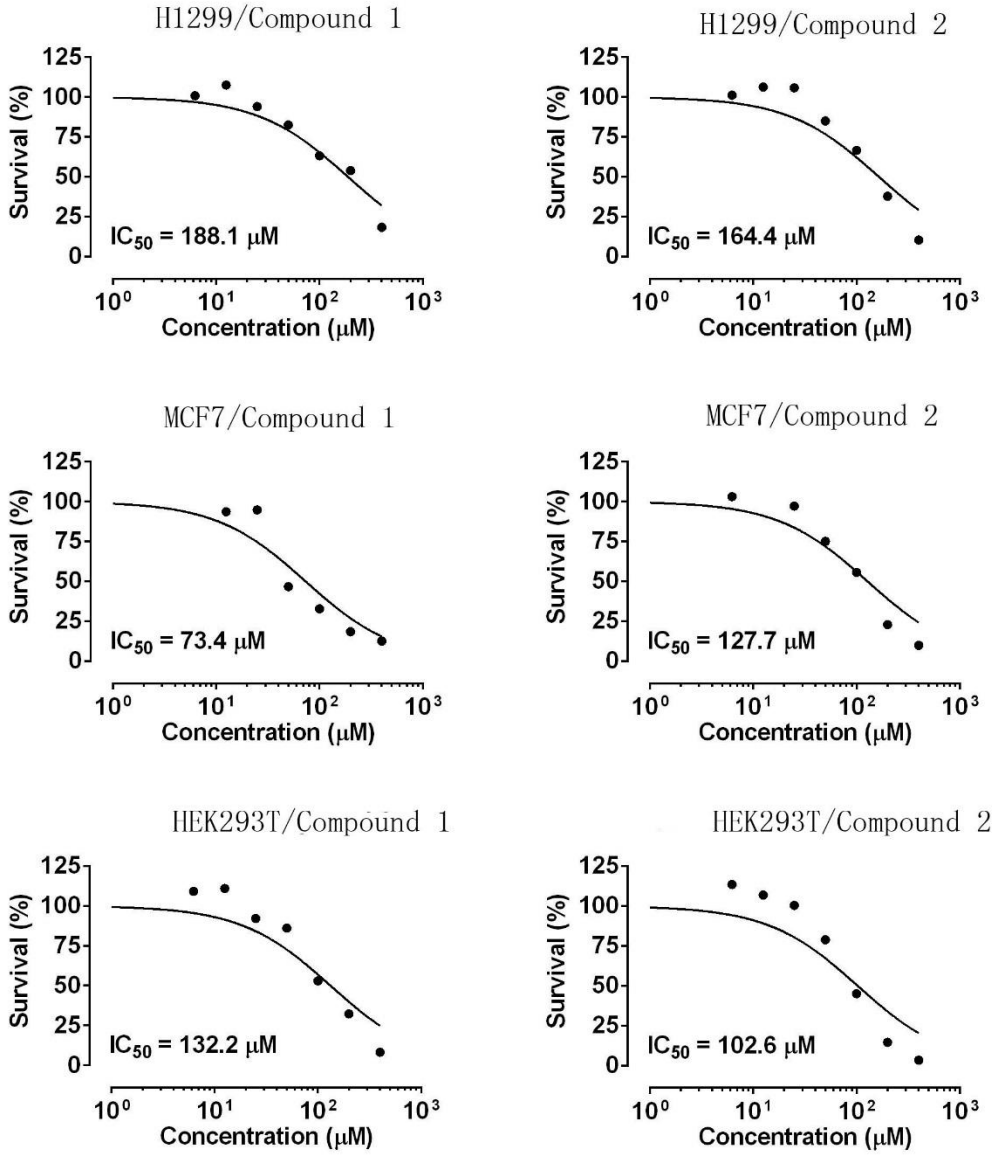
*Diasteltoxin A (**1**)*. White amorphous powder; ($[\alpha]_D^{20}$ +3.3 (c 0.3, MeOH); UV: λ_{max}^{MeOH}: 266, 276 nm; IR (KBr) ν_{max}: 3440, 2968, 2922, 2247, 1720, 1653, 1245, 1382 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table S1; HRESIMS *m/z* 837.4058 $[M + H]^+$ (calcd for $C_{46}H_{61}O_{14}$, 837.4061).

*Diasteltoxin B (**2**)*. White amorphous powder; ($[\alpha]_D^{20}$ +12.0 (c 0.3, MeOH); UV: λ_{max} MeOH: 266,

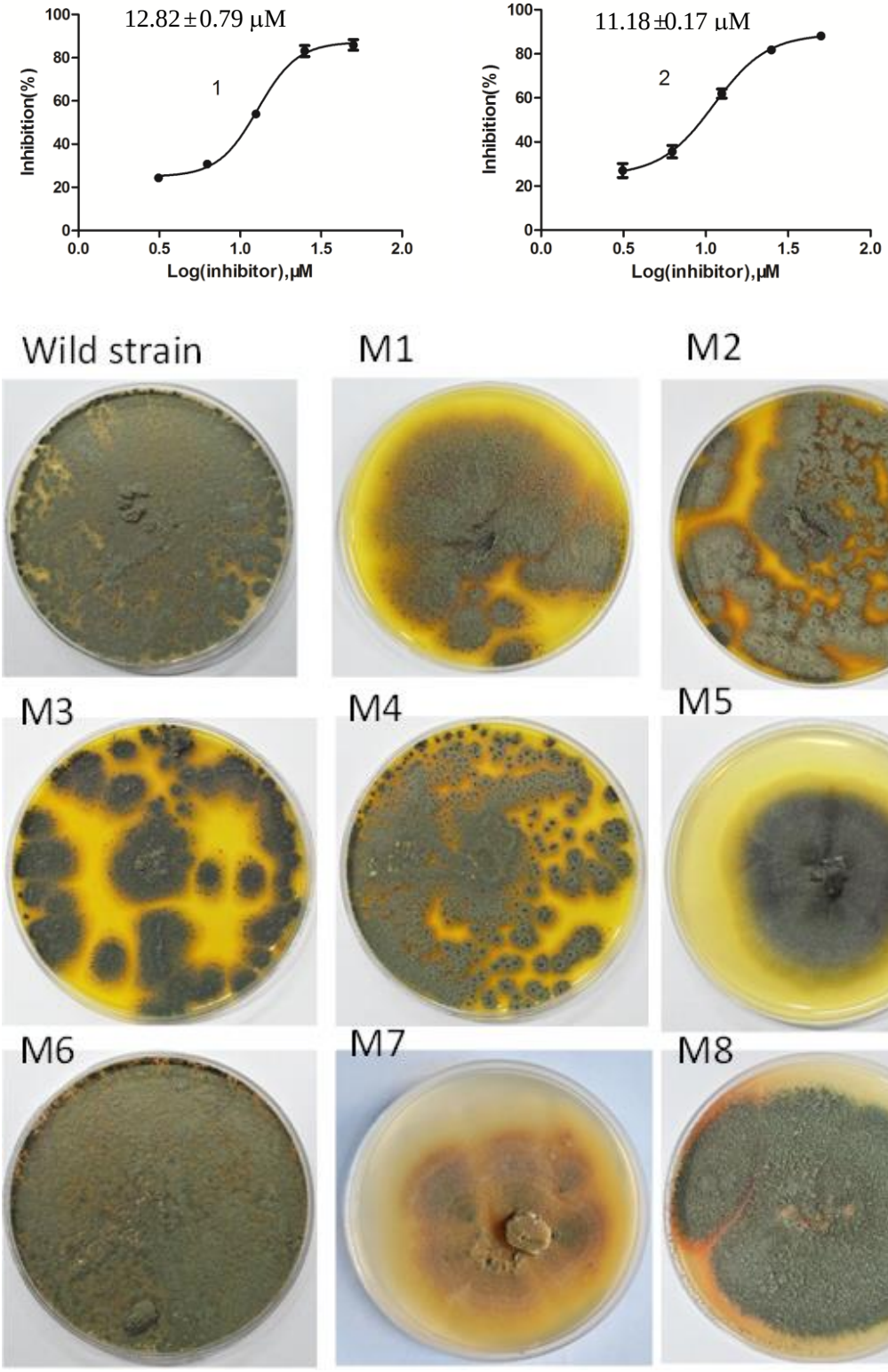
276 nm; IR (KBr) νmax: 3434, 2958, 2922, 1715, 1622 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table S2; HRESIMS *m/z* 837.4059 $[M + H]^+$ (calcd for $C_{46}H_{61}O_{14}$, 837.4061).

*Diasteltoxin C (**3**).* White amorphous powder; ($[\alpha]_D^{20}$ +4.5 (*c* 0.2, MeOH); UV: λ_{max} MeOH: 243, 295 nm; IR (KBr) νmax: 3417, 2958, 2923, 1715 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table S3; HRESIMS *m/z* 837.4058 $[M + H]^+$ (calcd for $C_{46}H_{61}O_{14}$, 837.4061).

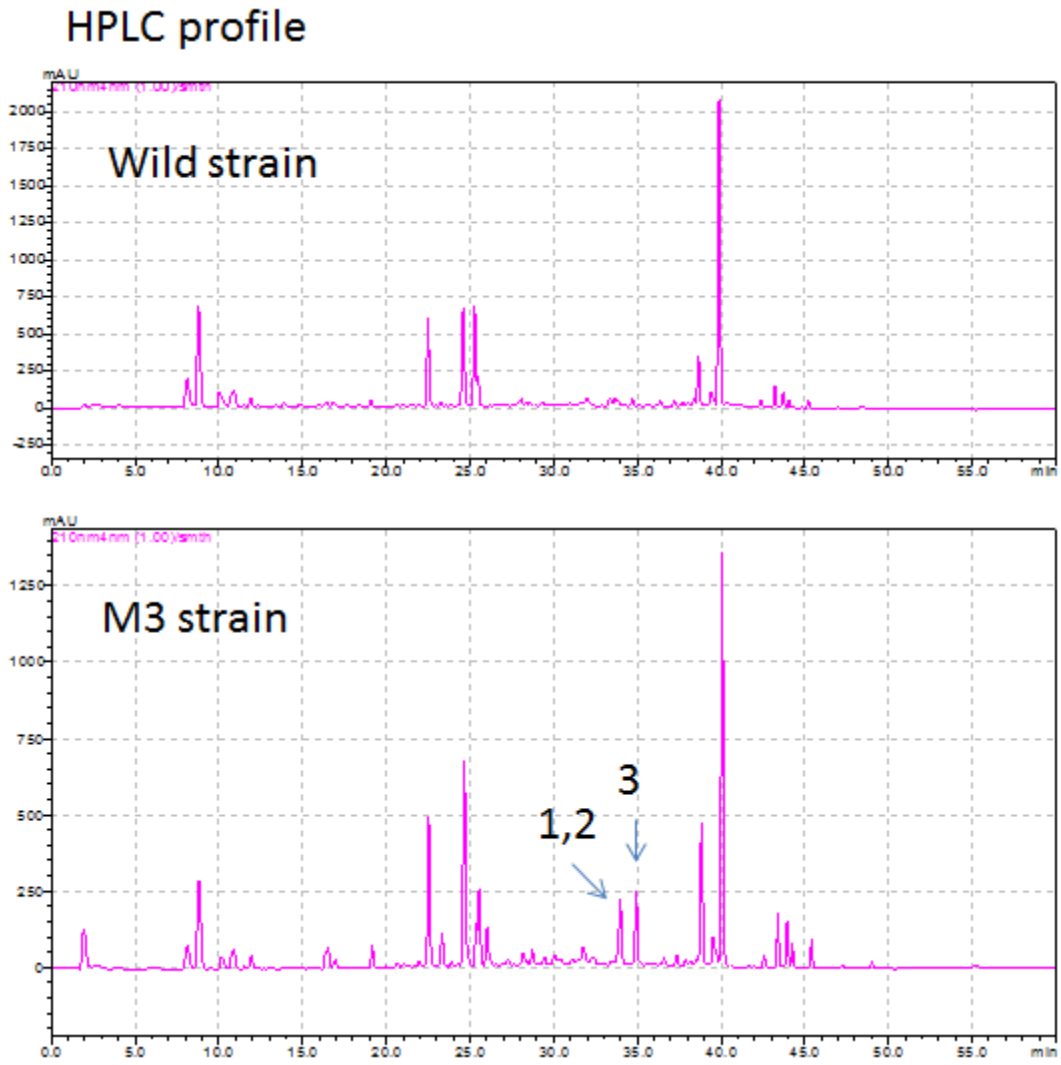
Cytotoxicity Assays. The *in-vitro* effect of the compounds on cell survival was determined using sulforhodamine B (SRB) assay against H1299 (human lung cancer) and MCF7 (human breast cancer) cells as well as HEK293T (human embryonic kidney) cells. H1299 and MCF7 cells were maintained in RPMI 1640 medium (Macgene, Beijing, China) containing 10% fetal bovine serum (FBS; PAN-Biotech GmbH, Aidenbach, Germany) and 1% penicillin/streptomycin (Macgene) in a 5% CO_2 incubator at 37℃. HEK293T cells were cultured in DMEM, supplemented with 10% FBS and 1% penicillin/streptomycin (Macgene) in a 37℃ humidified atmosphere containing 5% CO_2. To determine the cytotoxicity of the compound, H1299, MCF7 and HEK293T were seeded on a 96-well plate each at a density of $3\sim5\times10^3$ cells /well. Following 24 hours, cells were exposed to increasing concentrations of indicated compounds for 72 hours followed by SRB assay. The cell survival curve was plotted by the survival fractions on y-axis against the logarithmic concentrations on x-axis. IC_{50} of indicated compounds for each cell line was finally computed using the GraphPad Prism software (GraphPad Software, La Jolla, CA).



TrxR Inhibitory Activities. For determining the TrxR inhibitory activity of the compounds, the DTNB reduction assay was employed. All assays were conducted at 25 ℃ in a total volume of 40 μL. In each measurement, 0.3 μL of TrxR (0.04 μM) was added to an assay buffer containing 1 M potassium phosphate (pH 7.0), 500 mM EDTA (pH 7.4), NADPH (0.48 mM) and 1μL of inhibitor at various concentrations. After 5 min pre-incubation, the reaction was initiated with the addition of 3.2 μL of DTNB (final concentration of 5.0 mM). The control was incubated with the same amount of DMSO (2.5%, v/v). The increase in absorbance at 412 nm ($\Delta\varepsilon$ 412 = 13.6 $mM^{-1}cm^{-1}$) was monitored in the initial 120 s. The IC_{50} values were calculated to represent the TrxR inhibitory effect of compounds.



Wild strain of *Emericella variecolor* XSA-07-2 and its mutants (M1-M8)



HPLC profiles of Wild strain and mutant M3

Table 1. ^{1}H and ^{13}C NMR Data **1** and **2** in DMSO-d_6

	1		**2**	
no.	δ_C mult.	δ_H mult. (*J* in Hz)	δ_C mult.	δ_H mult. (*J* in Hz)
1	11.9, CH_3	0.89, t (7.2)	11.8, CH_3	0.88, t (7.2)
2	21.9, CH_2	1.35, m; 1.42, m	21.8, CH_2	1.38, m; 1.44, m
3	89.3, CH	4.15, t (6.0)	89.2, CH	4.15, dd (3.6, 8.4)
4	79.8, C		79.7, C	
5	61.6, C		61.5, C	
6	111.9, CH	5.00, s	111.8, CH	5.00, s
7	78.9, CH	3.54, dd (3.6, 6.0)	78.7, CH	3.54, brd (3.0)
8	84.5, CH	4.46, dd (3.6, 7.8)	84.4, CH	4.43, dd (3.0, 7.8)
9	132.6, CH	5.86, dd (7.8, 15.6)	131.1, CH	5.78, dd (7.8, 15.6)
10	131.8, CH	6.23, dd (10.0, 15.6)	132.0, CH	6.25, dd (10.0, 15.6)
11	134.3, CH	6.36, dd (10.0, 15.6)	134.2, CH	6.37, dd (10.0, 15.0)
12	132.5, CH	6.27, dd (10.0, 15.6)	132.5, CH	6.25, dd (10.0, 15.0)
13	131.5, CH	6.46, dd (10.0, 15.6)	131.5, CH	6.11, dd (10.0, 15.0)
14	127.1, CH	6.00, d (15.6)	126.9, CH	5.98, d (15.0)
15	83.6, C		83.5, C	
16	49.4, C		49.3, C	
17	174.2, C		174.0, C	
18	91.3, CH	5.47, s	91.3, CH	5.46, s
19	163.5, C		163.5, C	
20	18.2, CH_3	1.18, s	18.1, CH_3	1.18, s
21	16.5, CH_3	1.01, s	16.4, CH_3	1.01, s
22	19.9, CH_3	1.25, s	19.8, CH_3	1.24, s
23	56.9, CH_3	3.75, s	56.8, CH_3	3.73, s
4-OH		4.25, s		4.25, br.s
7-OH		5.06, d (6.0)		5.05, br.s
1´	11.9, CH_3	0.89, t (7.2)	11.8, CH_3	0.90, t (7.2)
2´	21.9, CH_2	1.35,m; 1.42, m	21.8, CH_2	1.38, m; 1.44, m
3´	89.3, CH	4.13, t (6.0)	89.2, CH	4.14, dd (3.6, 8.4)
4´	79.8, C		79.7, C	
5´	61.7, C		61.6, C	
6´	111.9, CH	5.01, s	111.8, CH	5.01, s
7´	78.8, CH	3.52, dd (3.6, 6.0)	78.8, CH	3.55, brd (3.0)
8´	84.4, CH	4.43, dd (3.6, 7.8)	84.4, CH	4.45, dd (3.0, 7.2)
9´	131.2, CH	5.81, dd (7.8, 15.6)	132.5, CH	5.86, dd (7.2, 15.6)
10´	132.2, CH	6.24, dd (10.0, 15.6)	131.7, CH	6.25, dd (10.0, 15.0)
11´	131.8, CH	6.13, dd (10.0, 15.6)	131.4, CH	6.45, dd (10.0, 15.0)
12´	130.9, CH	5.60, dd (8.4, 15.6)	130.6, CH	5.65, dd (7.8,15.0)
13´	43.5, CH	3.32, dd (8.4, 10.8)	43.2, CH	3.32, dd (7.8, 10.8)
14´	50.3, CH	4.03, d (10.8)	49.9, CH	4.07, d (10.8)
15´	155.2, C		155.0, C	
16´	109.0, C		108.9, C	
17´	170.6, C		170.5, C	
18´	88.4, CH	5.57, s	88.3, CH	5.56, s
19´	163.1, C		163.0, C	
20´	18.2, CH_3	1.18, s	18.1, CH_3	1.17, s
21´	16.5, CH_3	1.01, s	16.4, CH_3	1.01, s
22´	9.3, CH_3	1.75, s	9.2, CH_3	1.77, s
23´	57.2, CH_3	3.79, s	57.1, CH_3	3.80, s
4´-OH		4.25, s		4.25, brs
7´-OH		5.07, d (6.0)		5.07, brs

Table 2. ^{1}H and ^{13}C NMR Data of **3** in $CDCl_3$

no.	δ_C mult.	δ_H mult. (J in Hz)	no.	δ_C mult.	δ_H mult. (J in Hz)
1	11.2, CH_3	1.05, t (7.8)	1´	11.2, CH_3	1.05, t (7.8)
2	21.6, CH_2	1.53, m; 1.57, m	2´	21.8, CH_2	1.53, m; 1.57, m
3	89.5, CH	4.29, t (6.0)	3´	89.5, CH	4.27, m
4	81.0, C		4´	81.0, C	
5	62.0, C		5´	62.0, C	
6	111.6, CH	5.24, s	6´	111.7, CH	5.24, br.s
7	78.4, CH	3.67, m	7´	78.9, CH	3.65, m
8	82.7, CH	4. 64, m	8´	84.2, CH	4.60, dd (3.0, 7.2)
9	126.2, CH	5.60, dd (6.0, 15.6)	9´	126.6, CH	5.66, dd (6.0, 15.6)
10	133.9, CH	6.41, dd (10.0, 15.6)	10´	133.9, CH	6.34, dd (10.5, 15.6)
11	131.7, CH	6.11, dd (10.0, 15.6)	11´	131.7, CH	6.09, dd (10.5, 15.6)
12	132.9, CH	5.78, dd (8.8, 15.6)	12´	132.7, CH	5.87, dd (9.2, 15.0)
13	41.2, CH	3.99, dd (8.8, 9.8)	13´	41.4, CH	3.97, dd (6.8, 9.2)
14	42.8, CH	3.86, dd (6.8, 9.8)	14´	43.2, CH	3.84, dd (6.8, 9.8)
15	157.7, C		15´	157.7, C	
16	108.6, C		16´	108.7, C	
17	170.7, C		17´	170.8, C	
18	88.1, CH	5.47, s	18´	88.1, CH	5.47, s
19	164.1, C		19´	164.2, C	
20	17.9, CH_3	1.37, s	20´	17.9, CH_3	1.37, s
21	16.0, CH_3	1.16, s	21´	16.0, CH_3	1.16, s
22	9.2, CH_3	1.83, s	22´	9.2, CH_3	1.82, s
23	56.1, CH_3	3.83, s	23´	56.1, CH_3	3.83, s

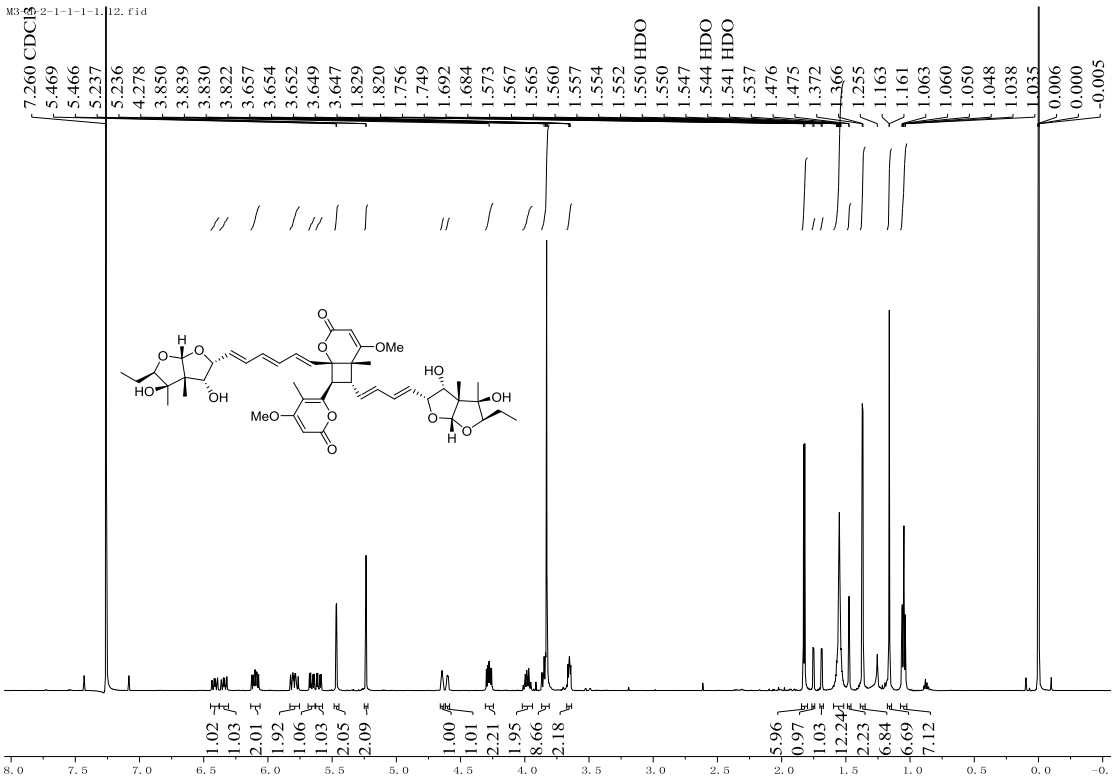


Figure S1. ^{1}H NMR spectrum of **1** (600 MHz, $CDCl_3$)

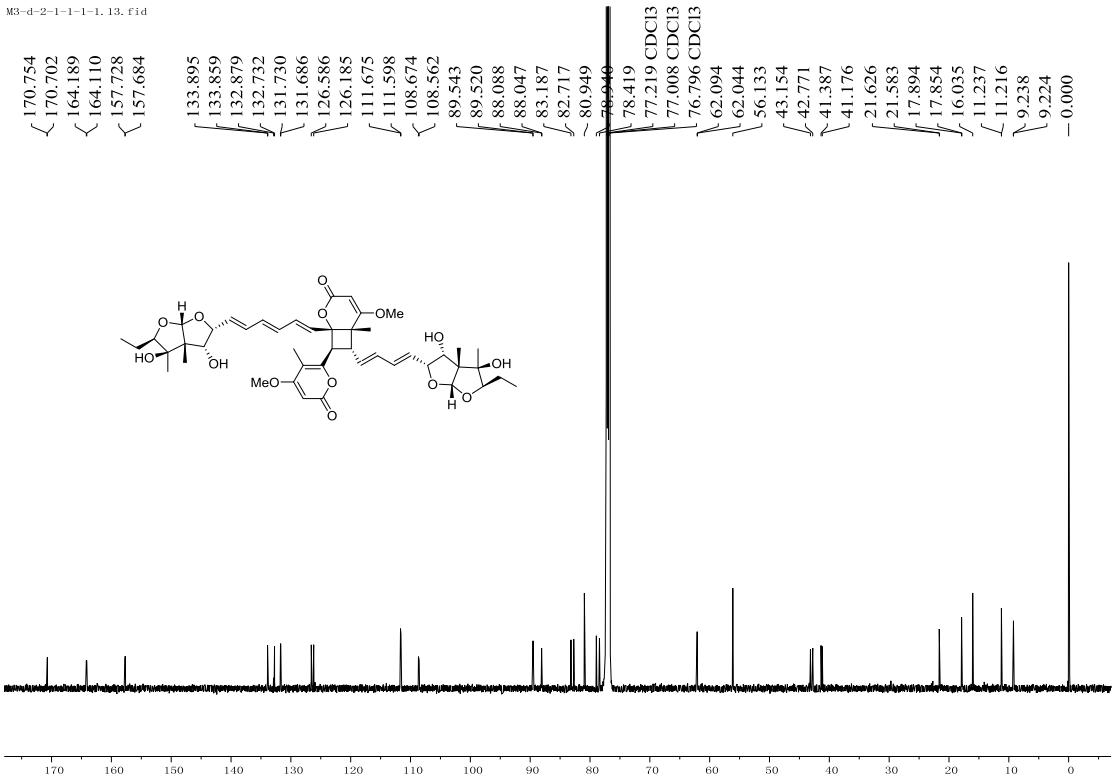


Figure S2. ^{13}C NMR spectrum of **1** (1**50** MHz, $CDCl_3$)

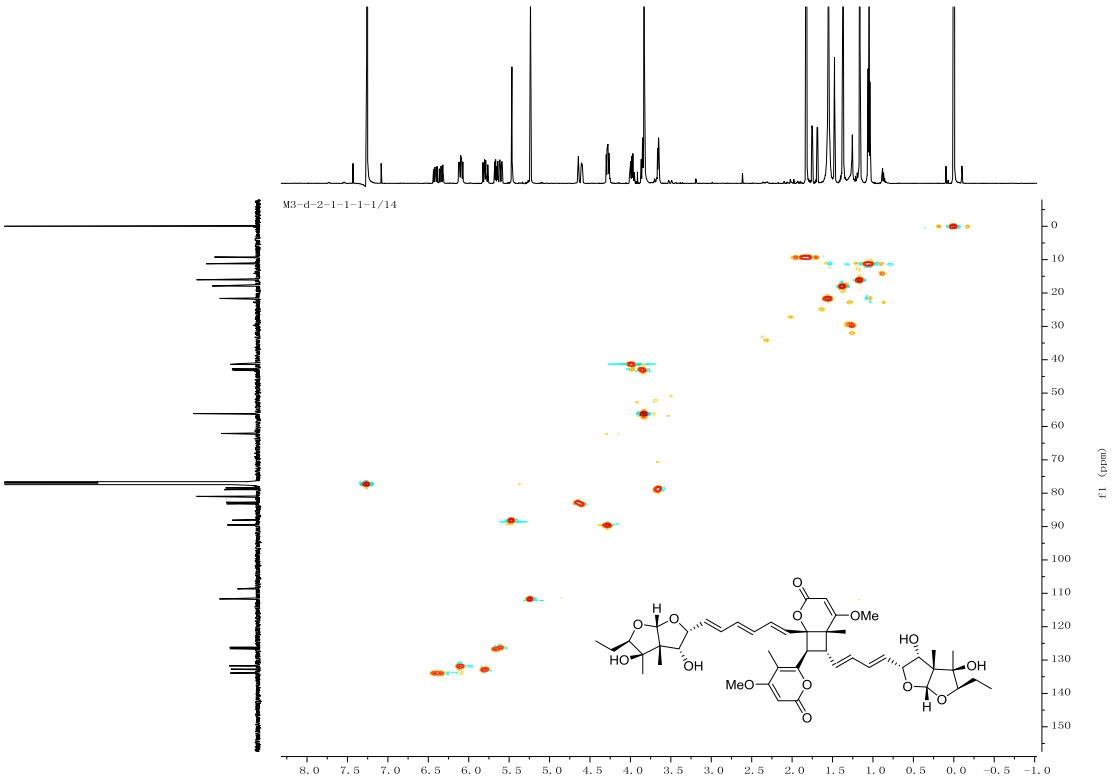


Figure S3. HSQC spectrum of **1** (600 MHz, $CDCl_3$)

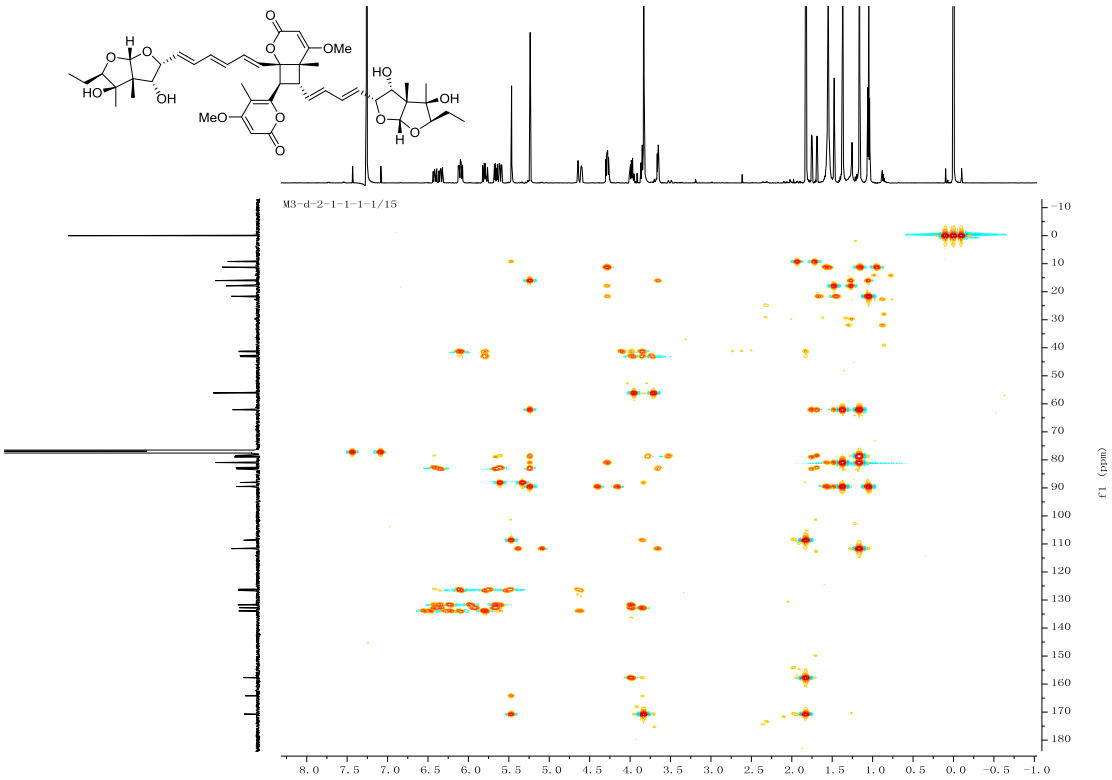


Figure S4. HMBC spectrum of **1** (600 MHz, $CDCl_3$)

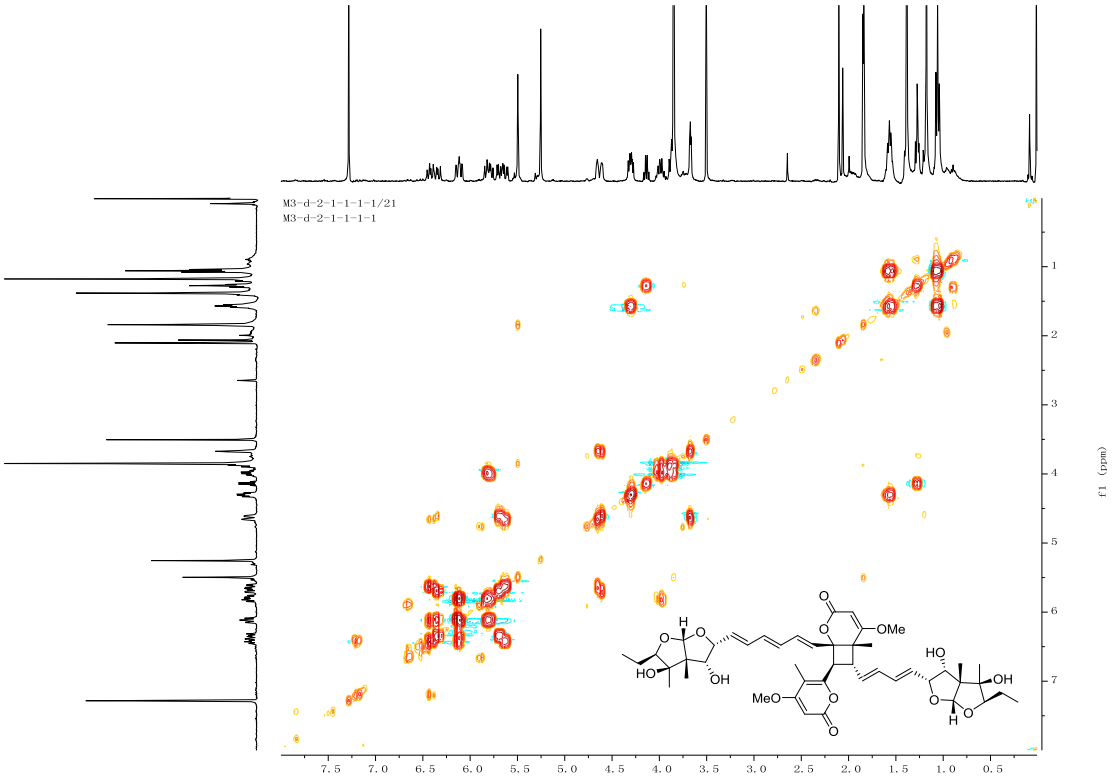


Figure S5. COSY spectrum of **1** (400 MHz, $CDCl_3$)

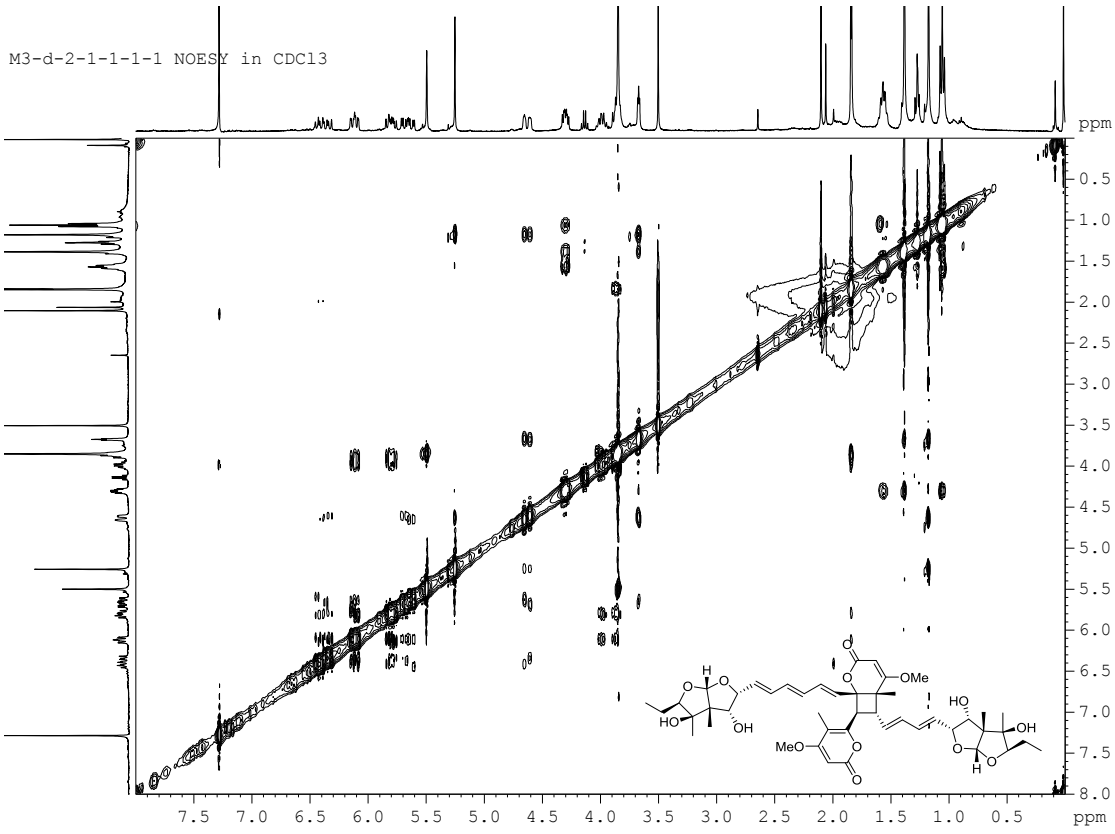


Figure S6. NOESY spectrum of **1** (400 MHz, $CDCl_3$)

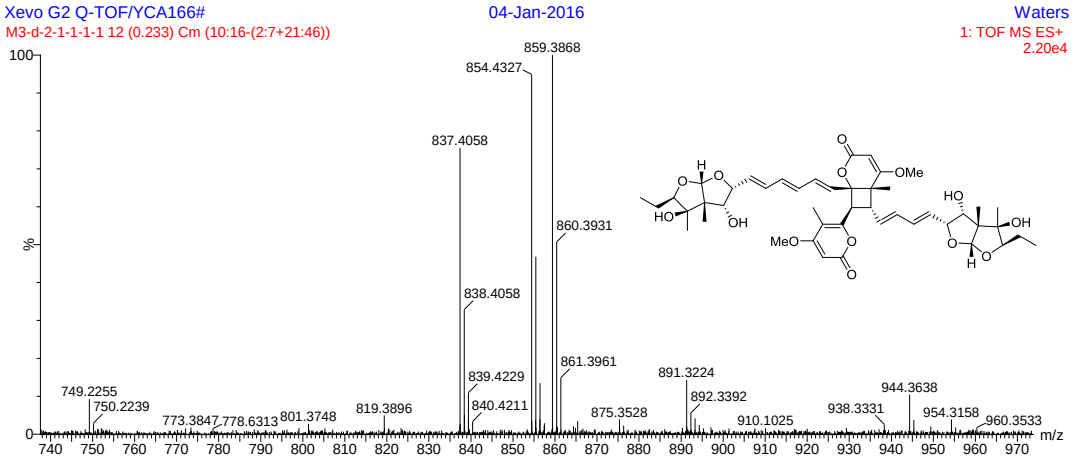


Figure S7. HRESIMS spectrum of **1**

UV (MeOH) spectrum for 1

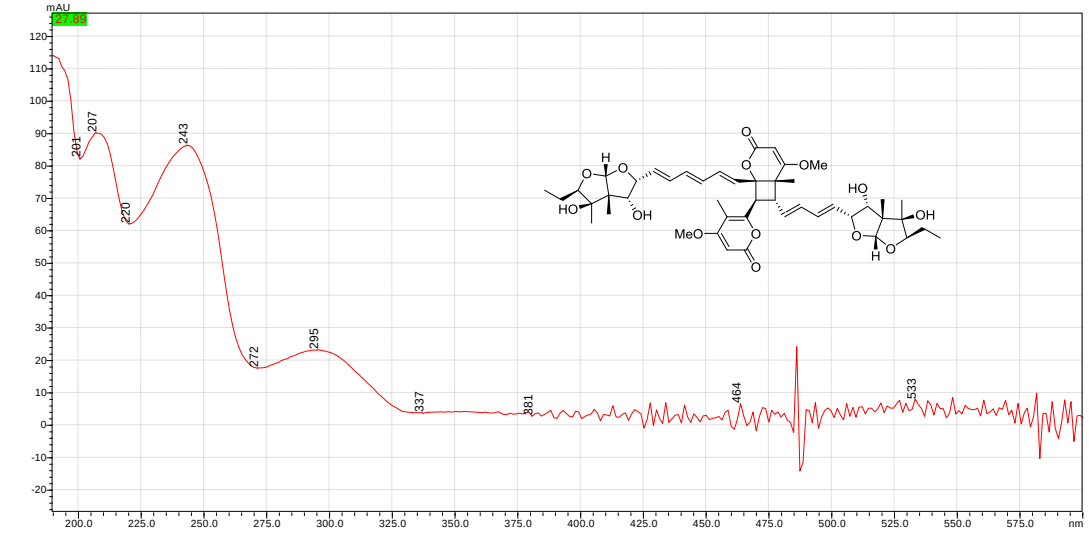


Figure S8. UV spectrum of **1**

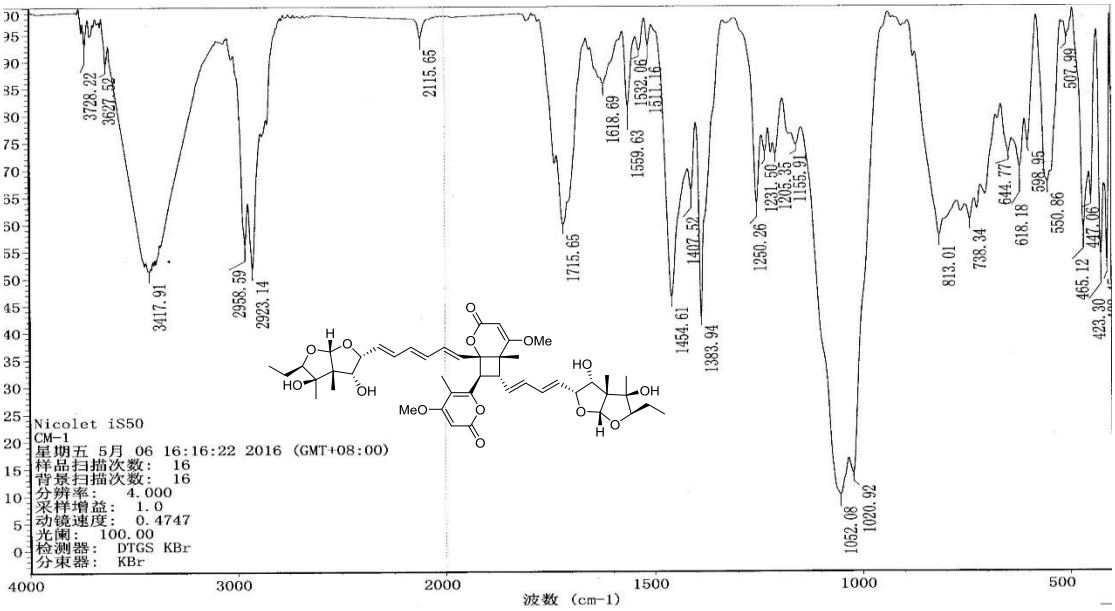


Figure S9. IR spectrum of **1**

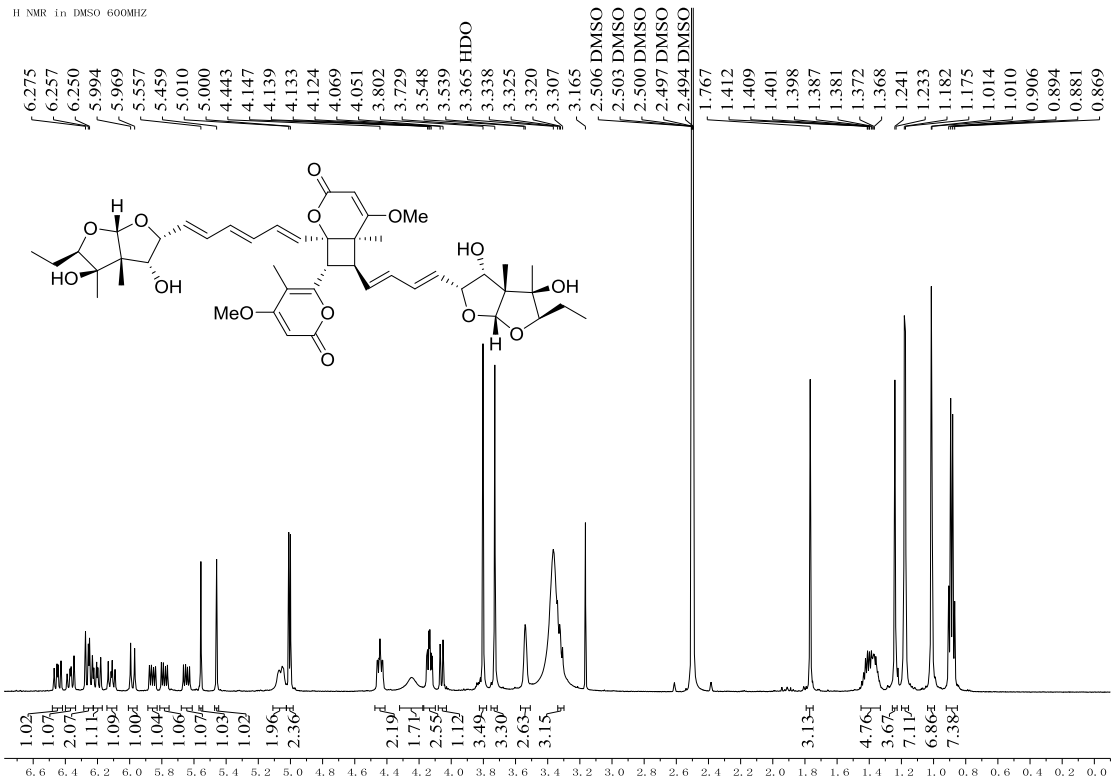
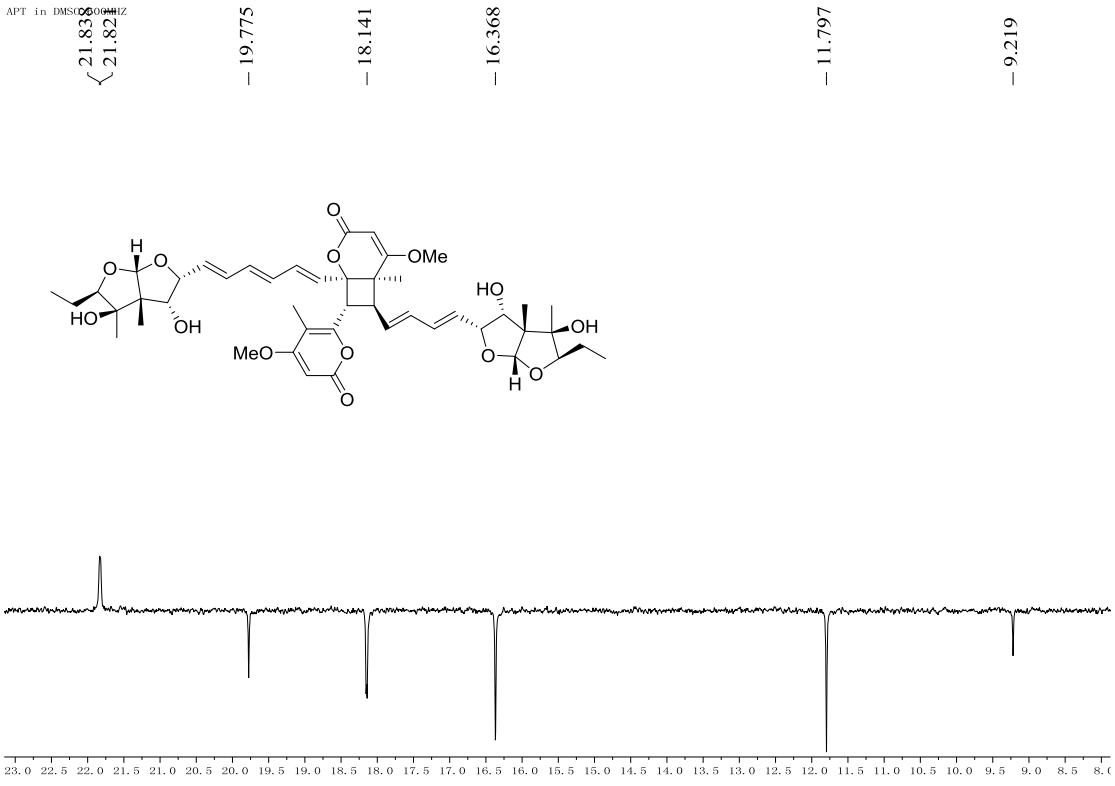
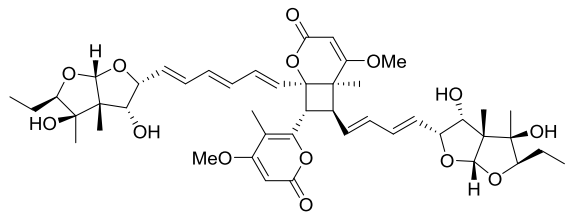


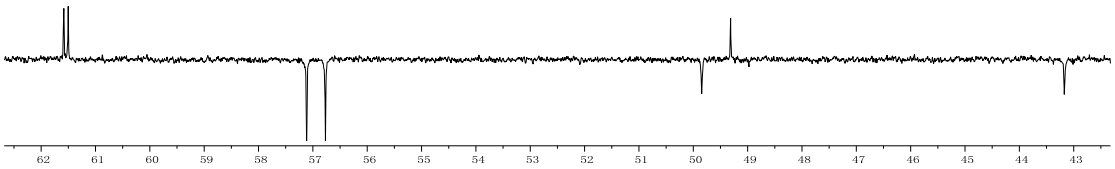
Figure S10. ^{1}H NMR spectrum of 2 (600 MHz, DMSO-d_6)



APT in DMSO 600MHZ

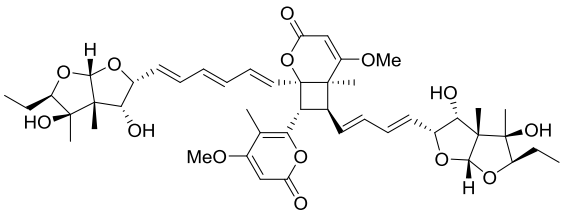
61.583
61.502
57.114
56.769
49.845
49.314
43.172

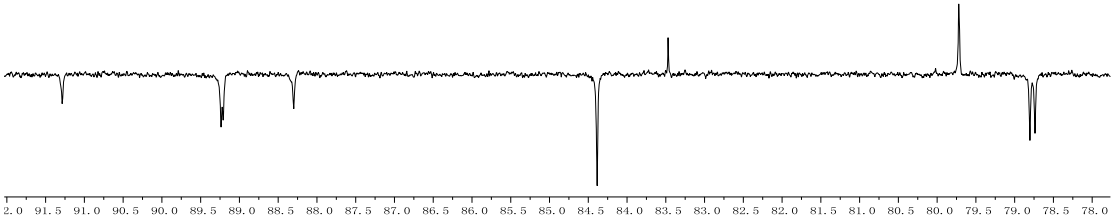




APT in DMSO 600MHZ

91.286
89.237
89.210
88.300
84.386
83.470
79.719
78.803
78.738





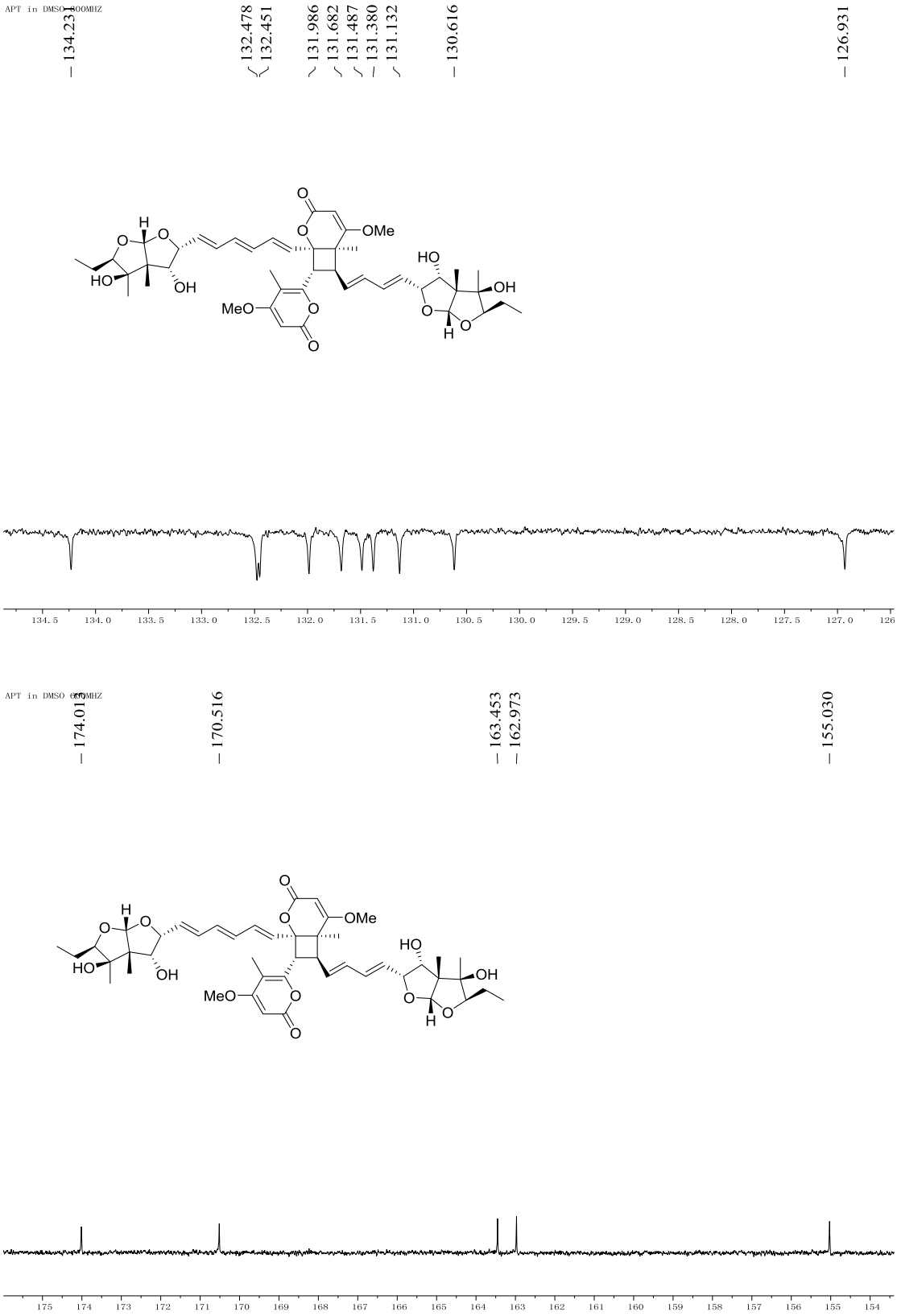


Figure S11. ^{13}C NMR spectrum of **2** (150 MHz, DMSO-d_6)

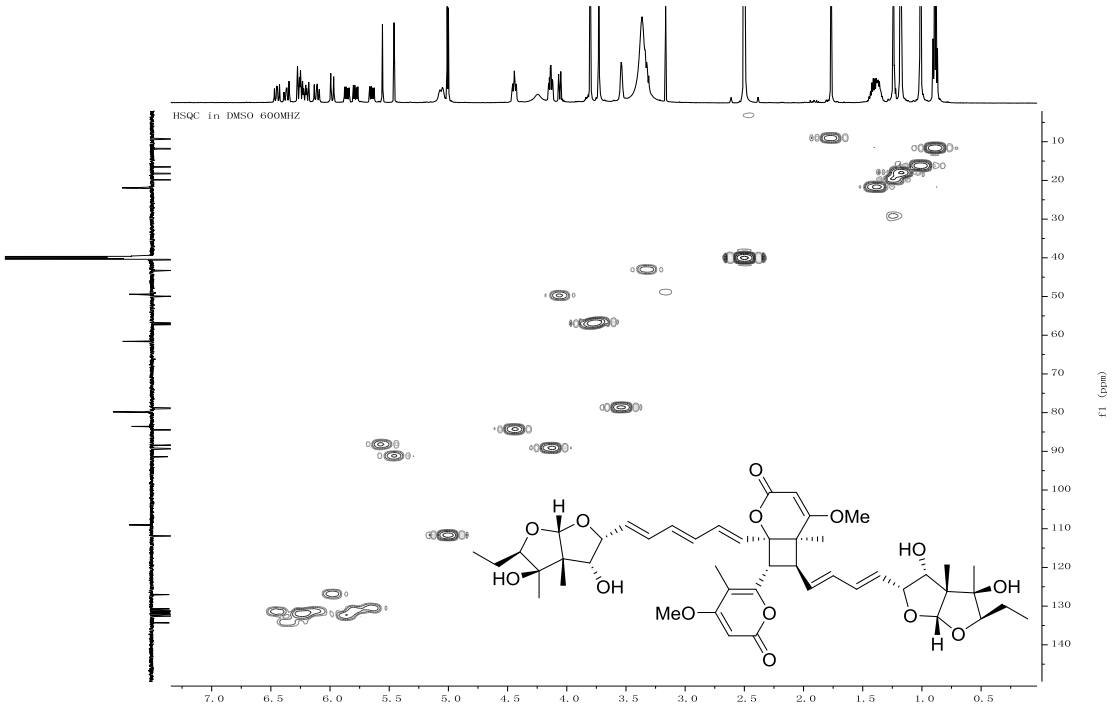


Figure S12. HSQC spectrum of **2** (600 MHz, DMSO-d_6)

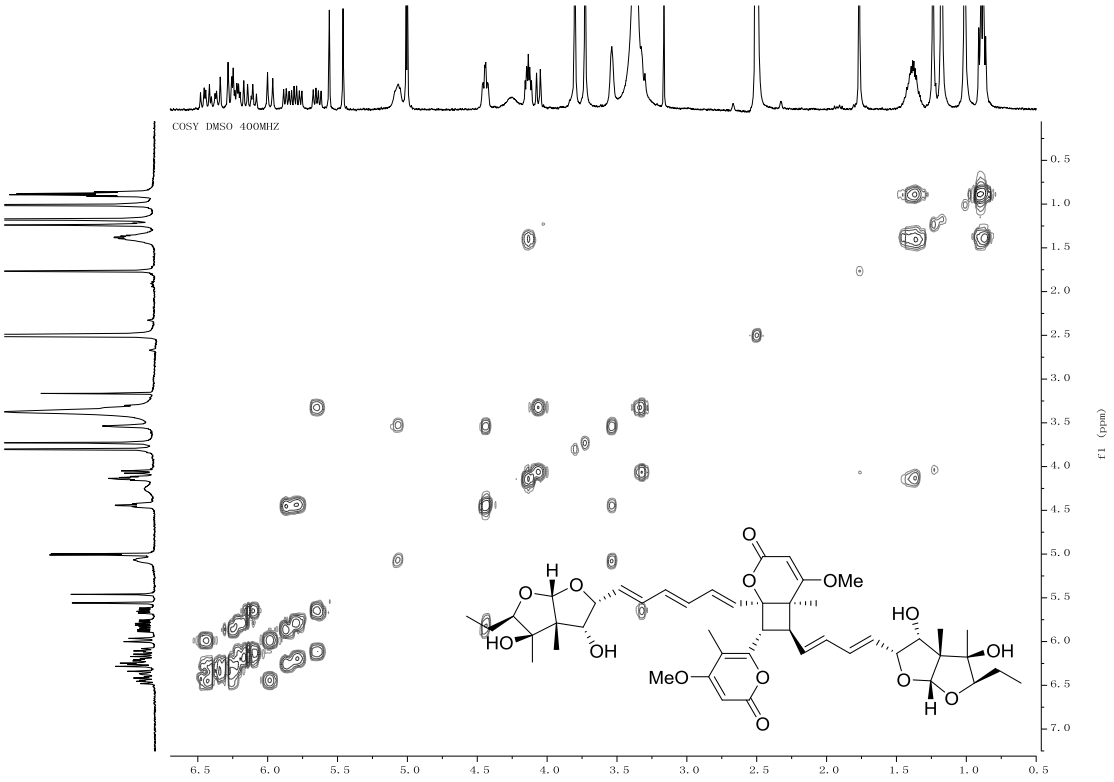
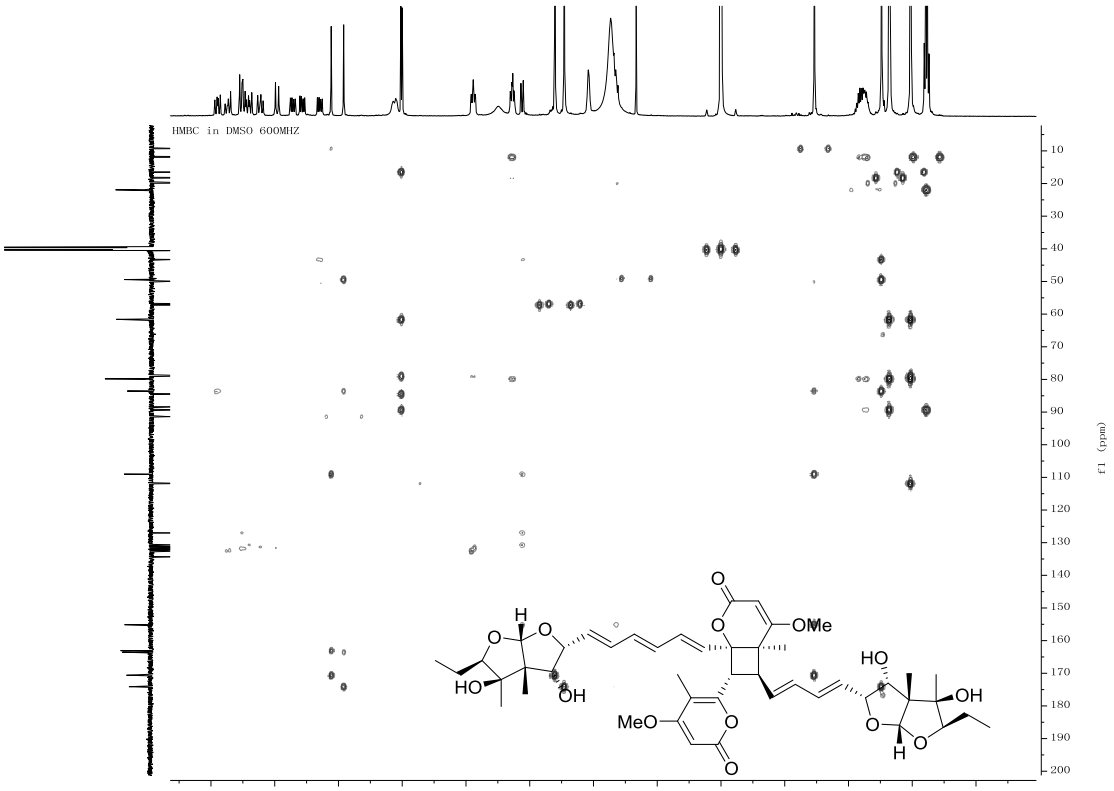
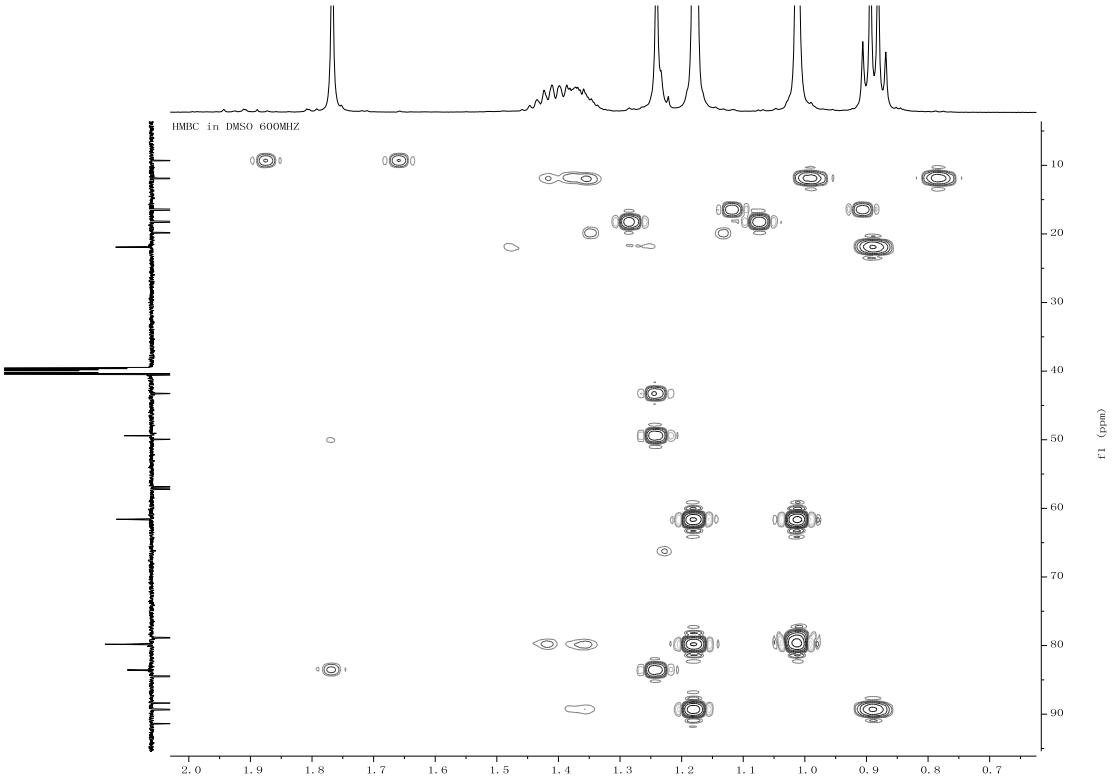
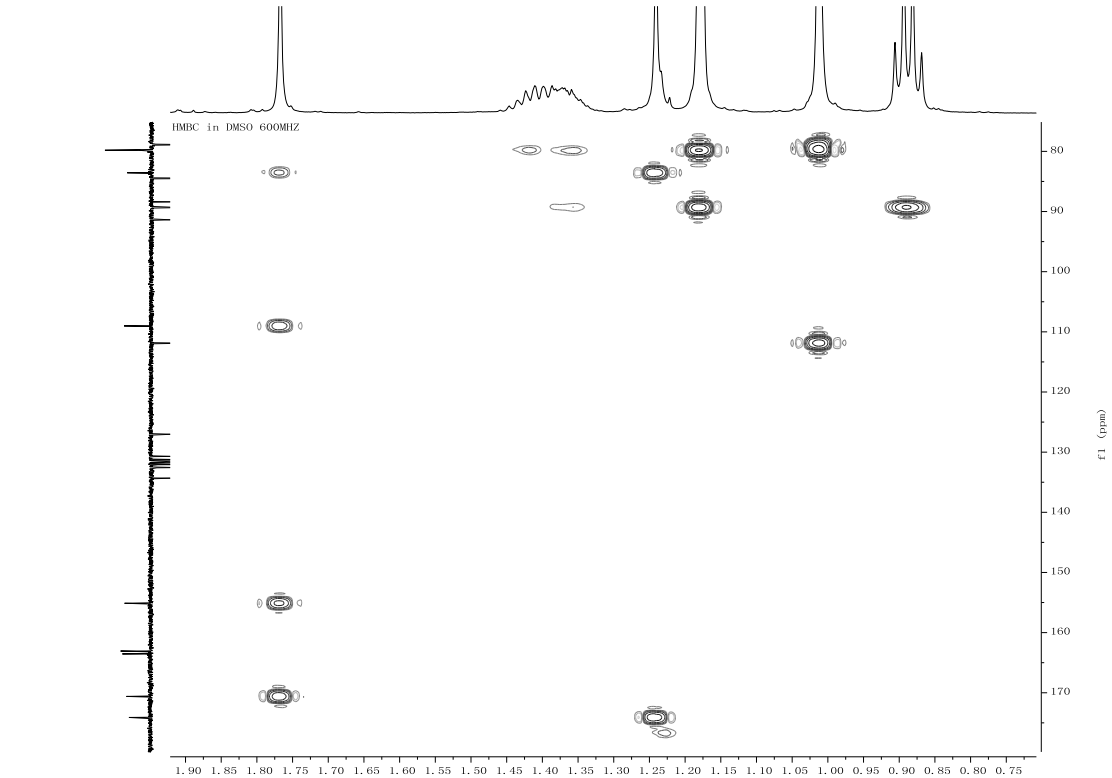
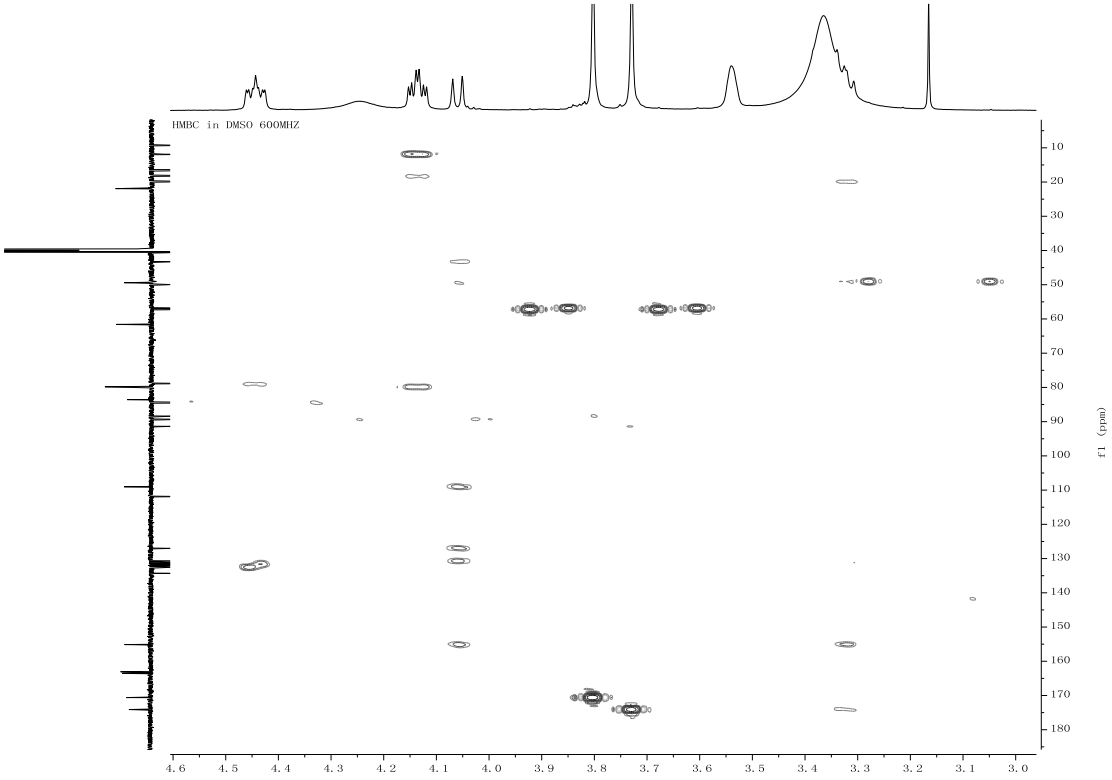


Figure S13. COSY spectrum of **2** (400 MHz, DMSO-d_6)


HMBC in DMSO 600MHZ
f1 (ppm)
OMe
HO
OH
MeO
H


HMBC in DMSO 600MHZ
f1 (ppm)


HMBC in DMSO 600MHZ
80
90
100
110
120
130
140
150
160
170
f1 (ppm)
1.90 1.85 1.80 1.75 1.70 1.65 1.60 1.55 1.50 1.45 1.40 1.35 1.30 1.25 1.20 1.15 1.10 1.05 1.00 0.95 0.90 0.85 0.80 0.75


HMBC in DMSO 600MHZ
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160
170
180
f1 (ppm)
4.6 4.5 4.4 4.3 4.2 4.1 4.0 3.9 3.8 3.7 3.6 3.5 3.4 3.3 3.2 3.1 3.0

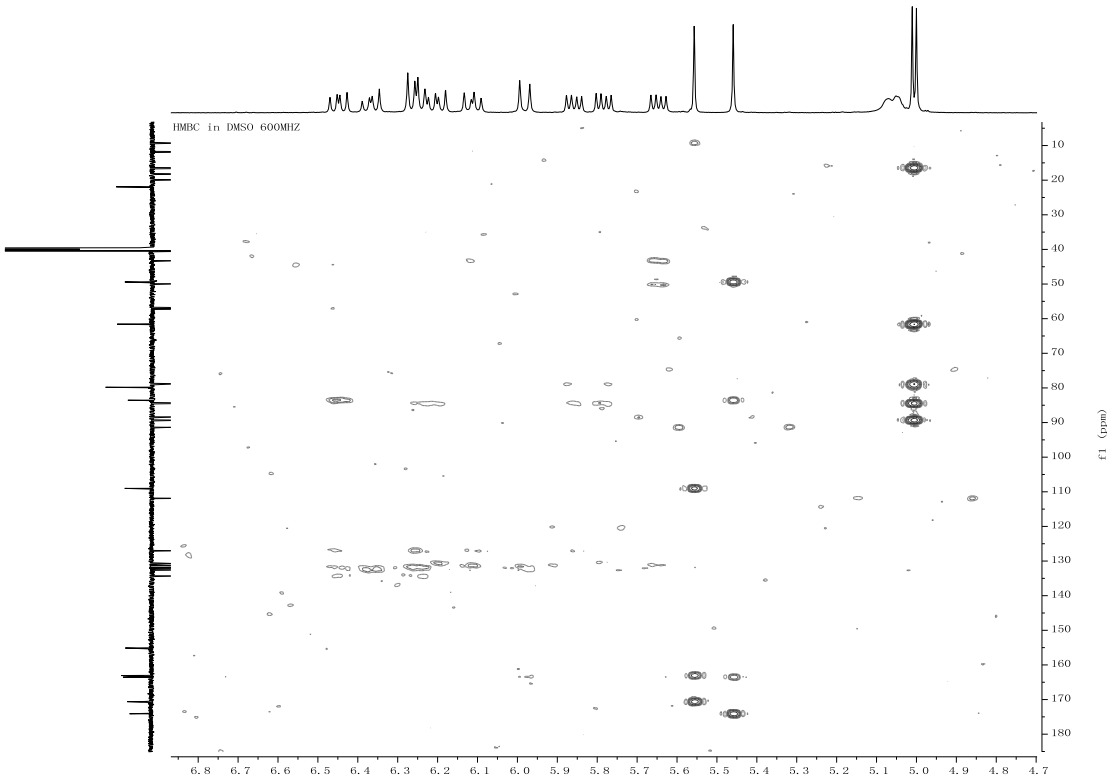
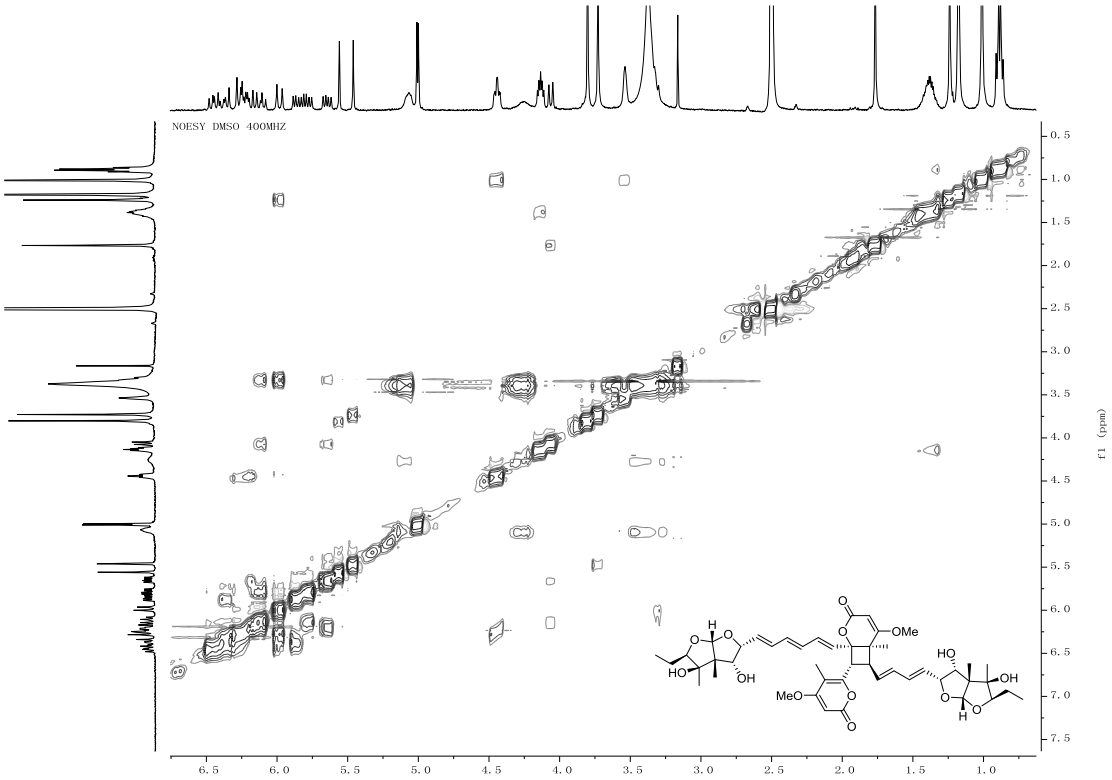


Figure S14. HMBC spectrum of **2** (600 MHz, DMSO-d_6)



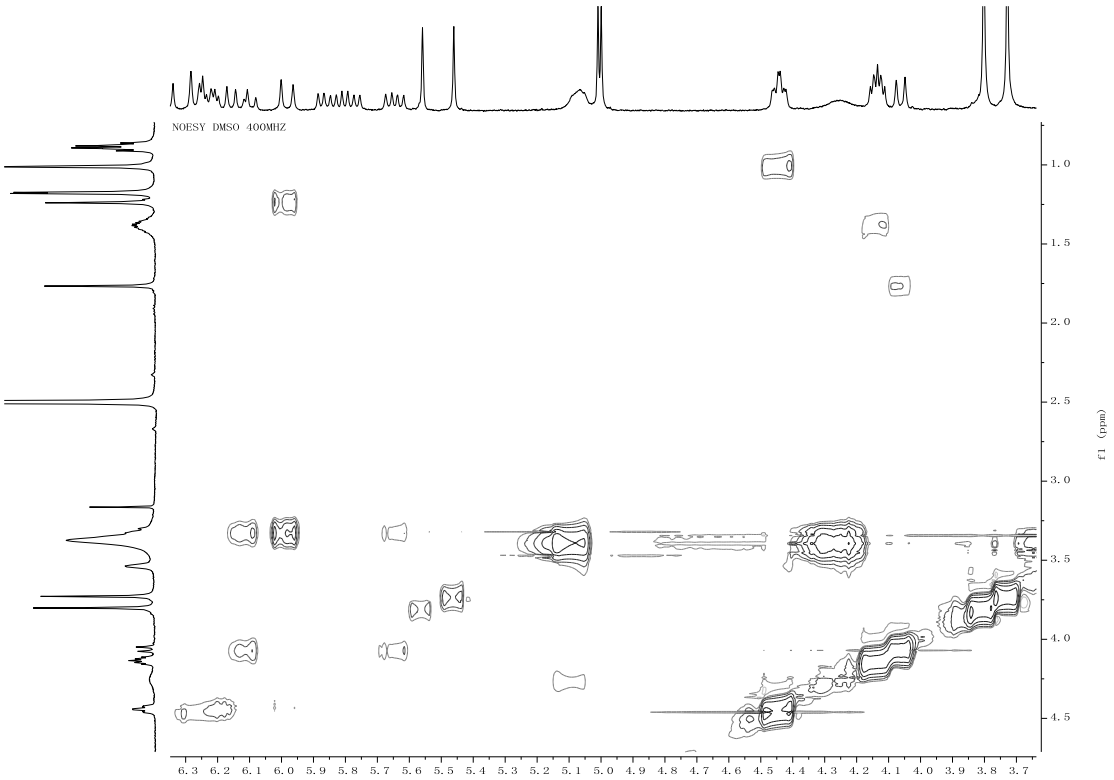


Figure S15. NOESY spectrum of **2** (400 MHz, DMSO-d_6)

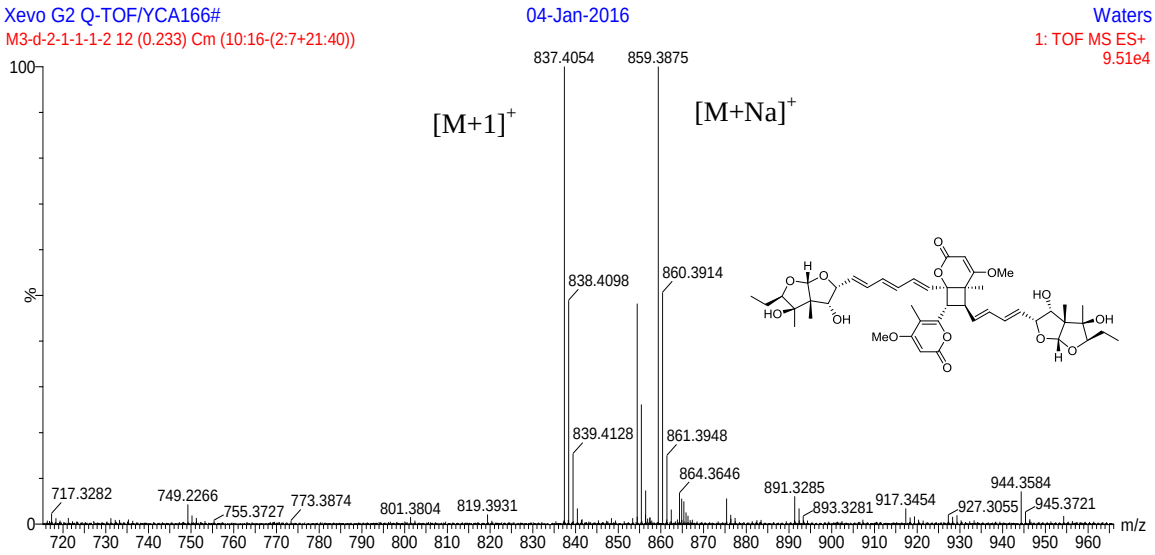


Figure S16. HRESIMS spectrum of **2**

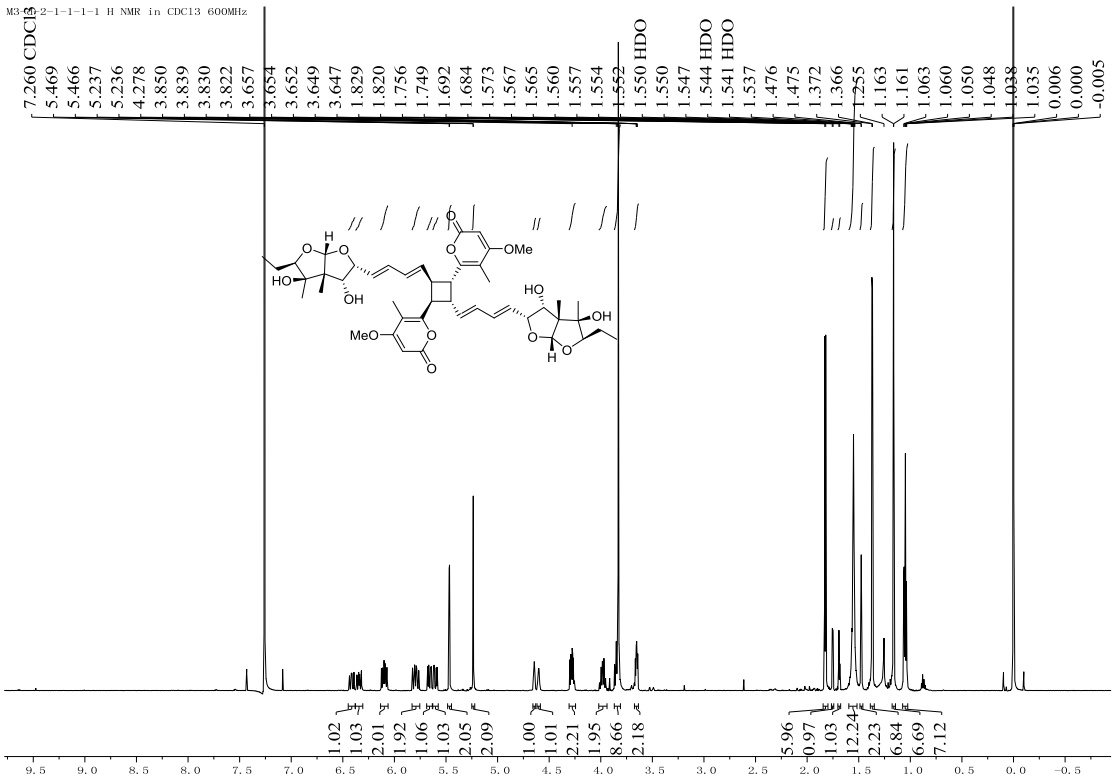


Figure S17. ^{1}H NMR spectrum of **3** (600 MHz, $CDCl_3$)

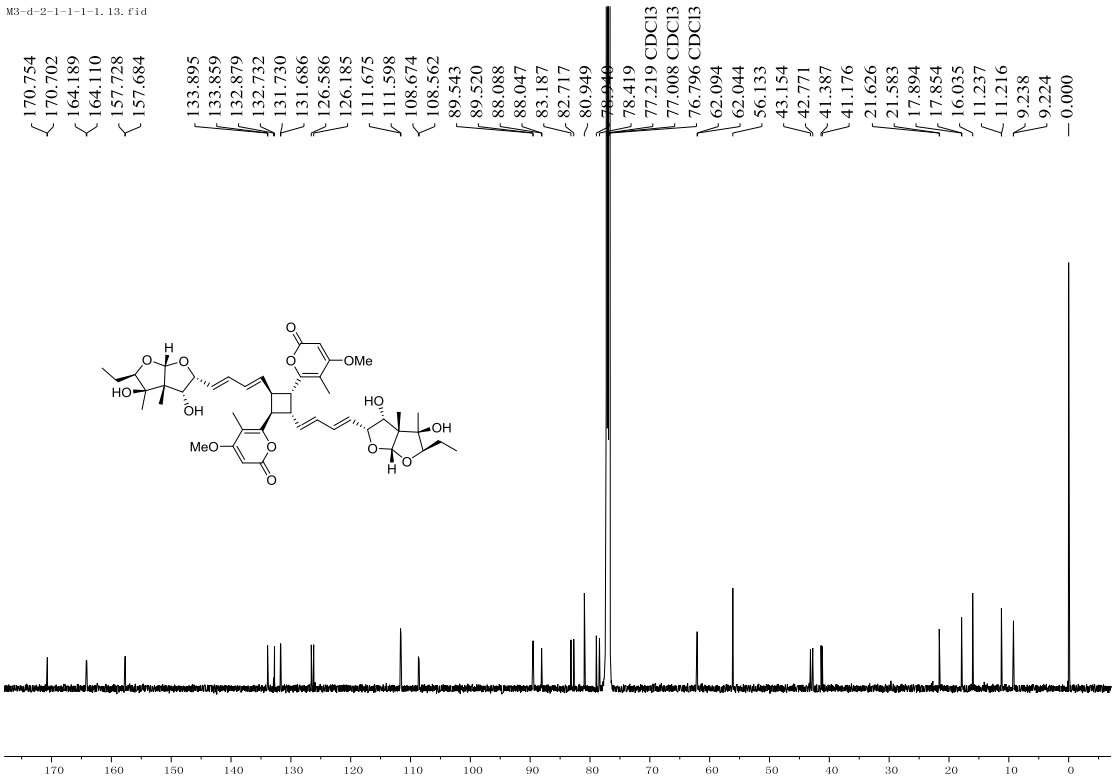


Figure S18. ^{13}C NMR spectrum of **3** (15**0** MHz, $CDCl_3$)

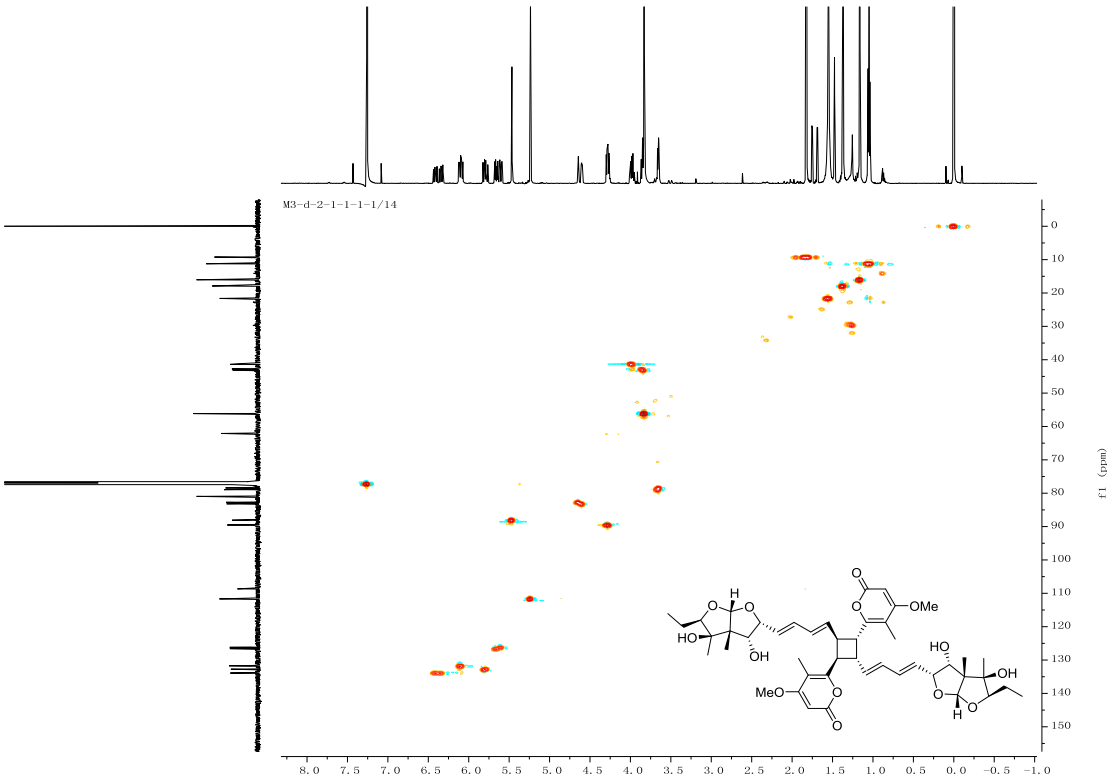


Figure S19. HSQC spectrum of **3** (600 MHz, $CDCl_3$)

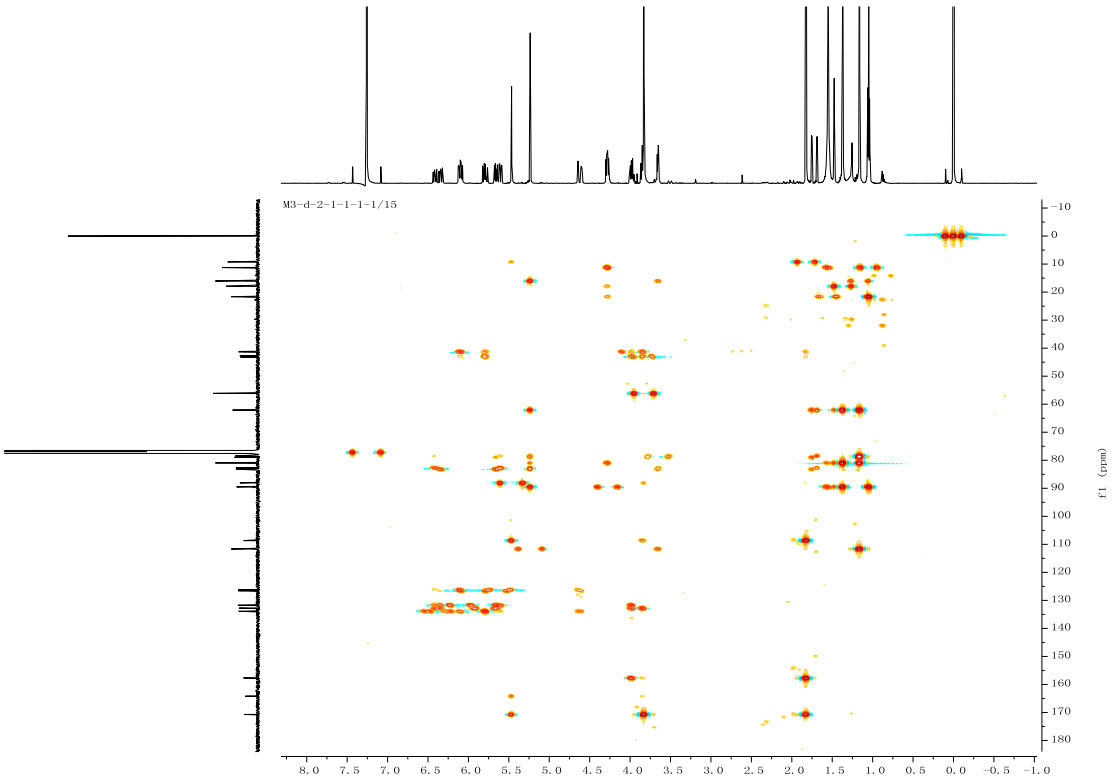


Figure S20. HMBC spectrum of **3** (600 MHz, $CDCl_3$)

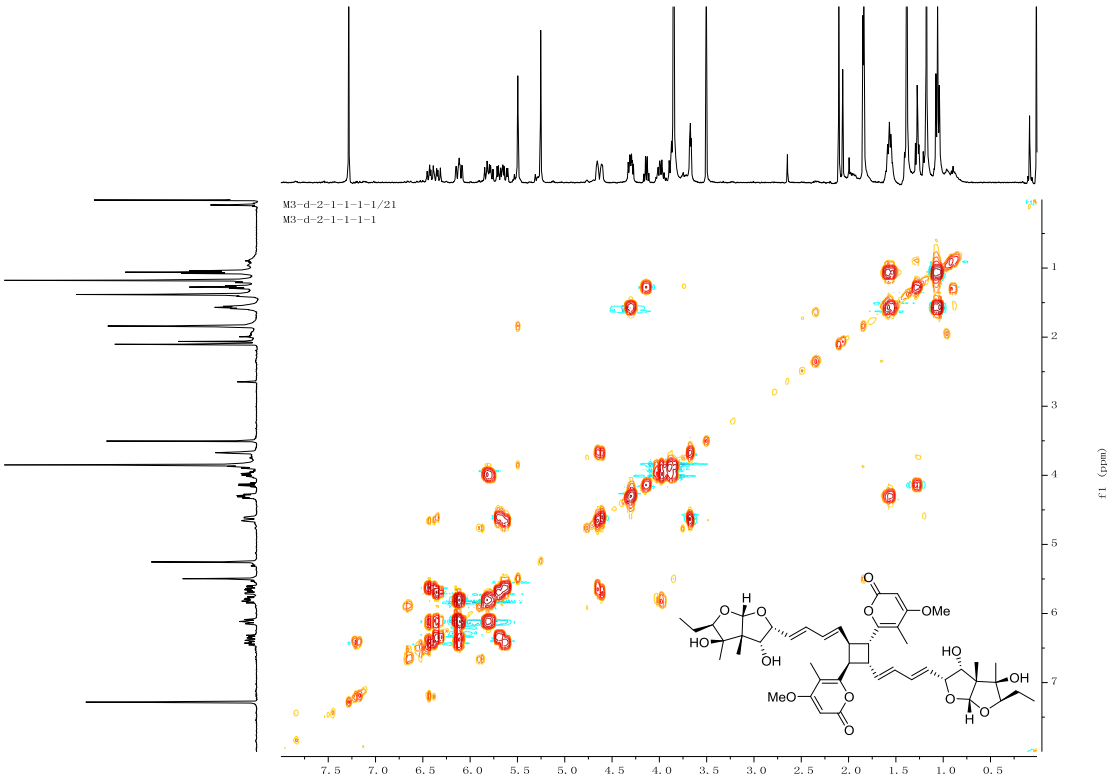


Figure S21. COSY spectrum of **3** (400 MHz, $CDCl_3$)

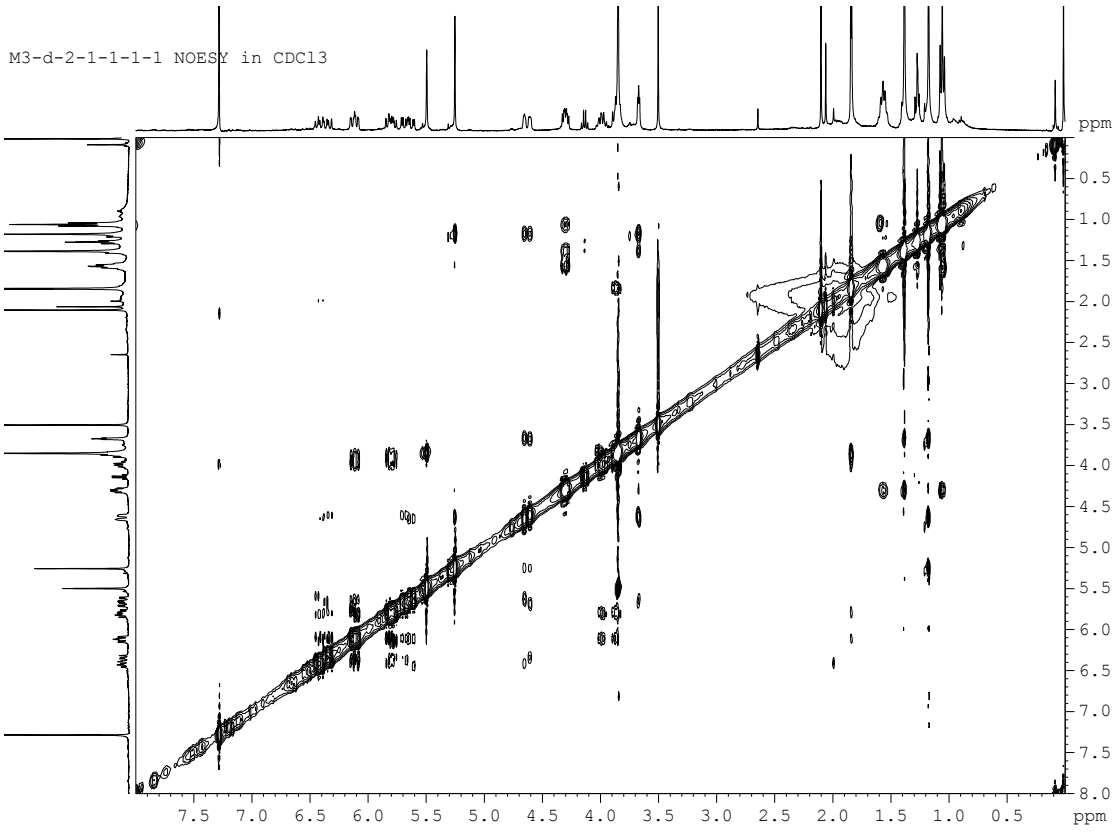
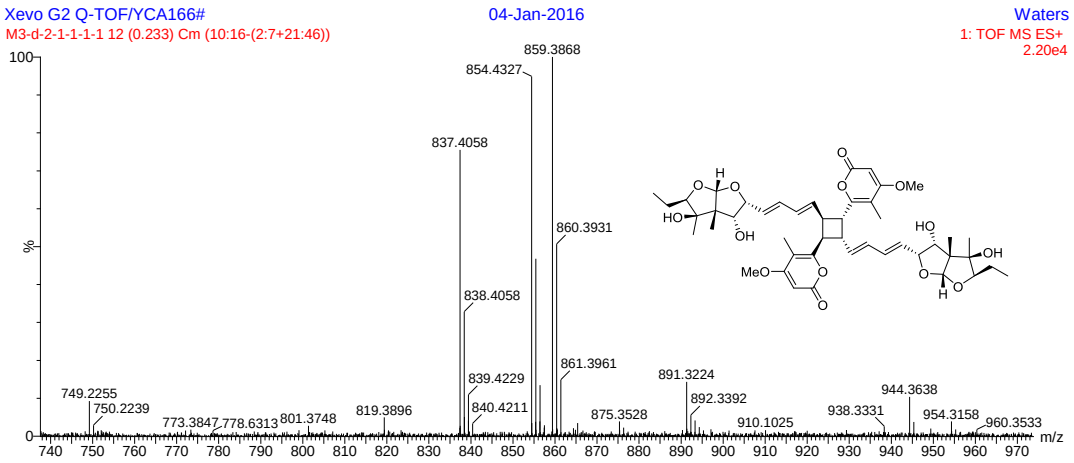


Figure S22. NOESY spectrum of **3** (400 MHz, $CDCl_3$)



Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	Norm	Conf (%)	Formula
837.4058	837.4061	-0.3	-0.4	16.5	244.3	0.009	99.07	$C_{46}H_{61}O_{14}$
	837.4096	-3.8	-4.5	38.5	248.9	4.675	0.93	$C_{64}H_{53}O$

Figure S23. HRESIMS spectrum of **3**

UV (MeOH) spectrum for 1

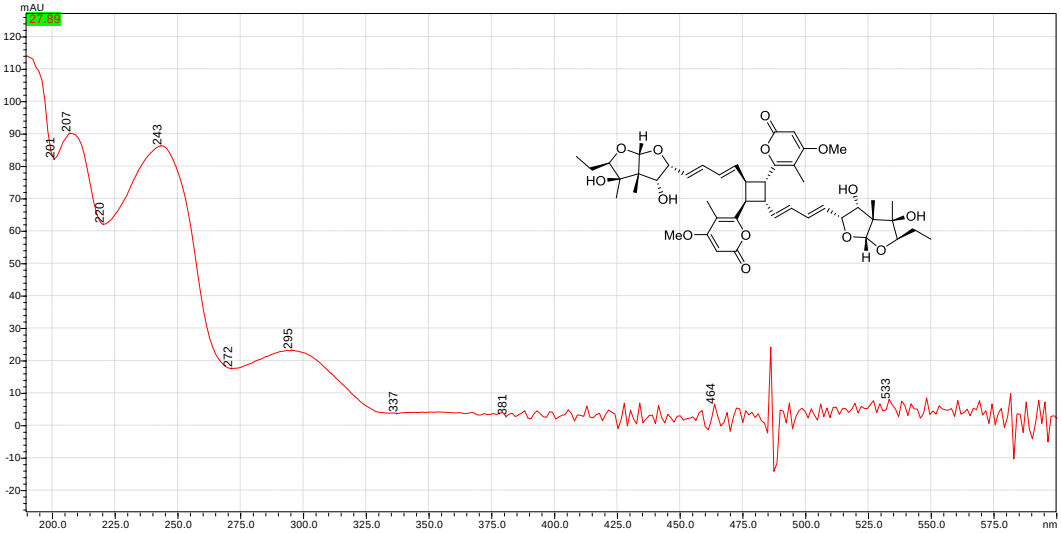


Figure S24. UV spectrum of **3**

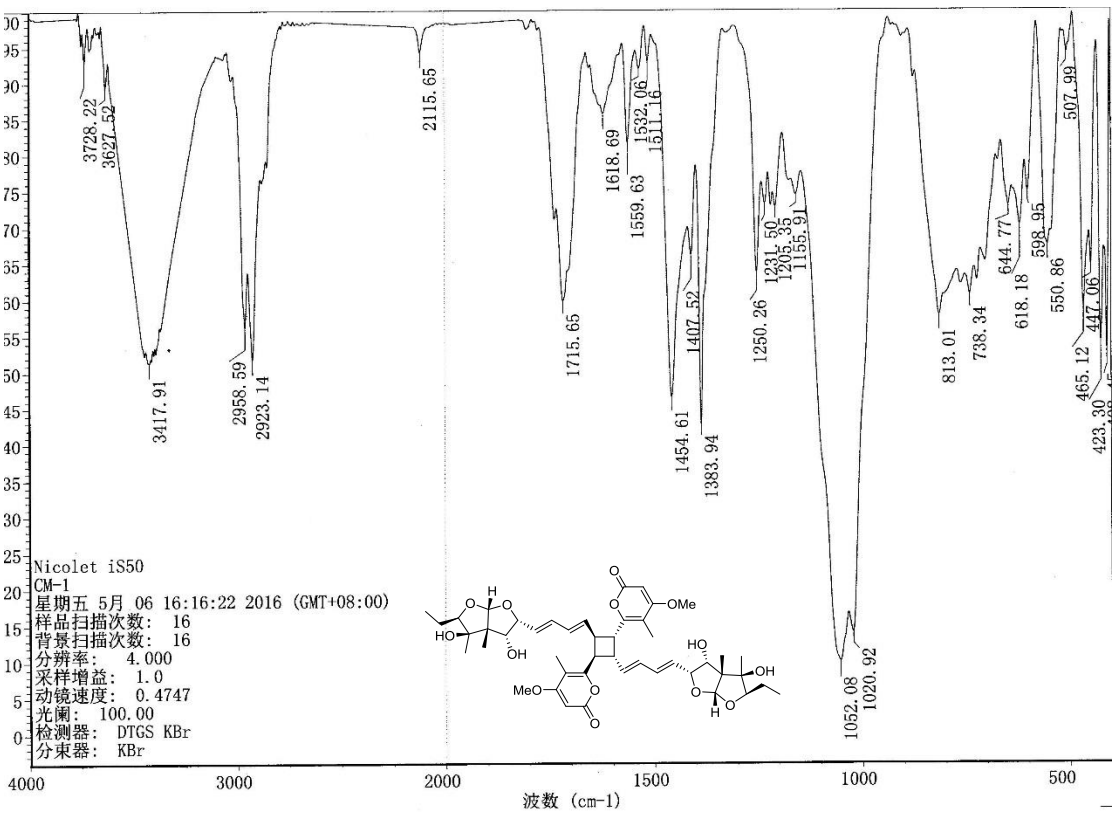


Figure S25. IR spectrum of **3**