Rhizophols A and B, antioxidant and axially chiral benzophenones from endophytic fungus *Cytospora rhizophorae*

Zhaoming Liu,‡[a] Haibo Tan,‡[b] Kai Chen,[c,d] Yuchan Chen,[a] Wenge Zhang,[b] Shanchong Chen,[a] Hongxin Liu,*[a] Weimin Zhang*[a]

[a]State Key Laboratory of Applied Microbiology Southern China, Guangdong Provincial Key Laboratory of Microbial Culture Collection and Application, Guangdong Open Laboratory of Applied Microbiology, Guangdong Institute of Microbiology, Guangzhou 510070, China.

[b]Program for Natural Products Chemical Biology, Key Laboratory of Plant Resources Conservation and Sustainable Utilization, Guangdong Provincial Key Laboratory of Applied Botany, South China Botanical Garden, Chinese Academy of Sciences, Guangzhou 510650, China.

[c]State Key Laboratory of Chemical Oncogenomics, Peking University Shenzhen Graduate School, Shenzhen 518055, China

[d]College of Chemistry and Chemical Engineering, Central South University, Changsha 410083, China

S1. Experimental section

General experimental procedures

Optical rotation was measured on an Anton Paar MCP-500 spectropolarimeter (Anton Paar, Graz, Austria). UV spectra were measured on a SHIMADZU UV-2600 UV-visible spectrophotometer (Shimadzu, Kyoto, Japan). Circular dichroism (CD) spectra were obtained under N_2 gas on a Jasco 820 spectropolarimeter (Jasco Corporation, Kyoto, Japan). 1D and 2D NMR spectra were recorded on a Bruker Avance-600 spectrometers (Bruker, Fällanden, Switzerland) with TMS as internal standard, δ in ppm, J in Hz. ESIMS data were collected on an Agilent Technologies 1290-6430A Triple Quad LC/MS (Agilent Technologies, Palo Alto, CA, USA). HRESIMS were done with a Thermo MAT95XP high resolution mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). A Shimadzu LC-20AT (Shimadzu Corporation, Kyoto, Japan) equipped with an SPD-M20A PDA detector (Shimadzu Corporation, Kyoto, Japan) was used for HPLC separation. A YMC-pack ODS-A column (250 × 10 mm, 5 μm) was used for semipreparative HPLC separation, and a CHIRALPAK IC column (250 × 10 mm, 5 μm) column for chiral semipreparative HPLC separation. Silica gel (200-300 mesh) was used for column chromatography, and precoated silica gel GF_{254} plates (Qingdao Marine Chemical Inc., Qingdao, China) were used for TLC spotting. C_{18} reversed-phase silica gel (40-63 μm, Merck, German), and Sephadex LH-20 gel (Pharmacia Fine Chemical Co. Ltd., Sweden) were also used for column chromatography. TLC spots were visualized under UV light and by dipping into 10% H_2SO_4 in alcohol followed by heating.

Fungal material and identification

The strain A761 was isolated from the plant *Morinda officinalis*, which was collected from Gaoyao city of Guangdong province in January 2015. The strain was identified by sequence analysis of rDNA ITS (internal transcribed spacer) region. The sequence of ITS region of the fungus A761 has been submitted to GenBank (Accession No. KU529867). By using BLAST (nucleotide sequence comparison program) to search the GenBank database, A761 has 99.5% similarity to *Cytospora*

rhizophorae M225 (Accession No. KR056292). The strain is preserved at the Guangdong Provincial Key Laboratory of Microbial Culture Collection and Application, Guangdong Institute of Microbiology.

Fermentation, Extraction and Isolation

Cytospora rhizophorae A761 was cultured in potato dextrose broth (potato 20%, glucose 2%, K_2HPO_4 0.3%, $MgSO_4$•$7H_2O$ 0.15%, vitamin B 10 mg/L). The fungus *C. rhizophorae* A761 was maintained on potato dextrose agar (PDA) medium at 28 °C for 5 days, and then three pieces (0.5 × 0.5 cm^2) of mycelial agar plugs were inoculated into 20 × 500 mL Erlenmeyer flasks, each containing 250 mL potato dextrose broth. After 4 days of incubation at 28 °C on a rotary shaker at 120 rev/min, 25 mL seed cultures was aseptically transferred into each of a total of 150 flasks (1000 mL) containing 500 mL of potato dextrose broth. The liquid cultivation that followed was kept for 7 days at 28 °C and 120 rev/min on a rotary shaker.

The culture (75 L) was centrifuged to give the broth and mycelia. The fermented broth was subjected to macroporous resin column with ethanol as eluent. The EtOH fraction was evaporated under reduced pressure at a temperature not exceeding 40 °C to yield a dark brown gum (37 g). The crude extract was subjected to reversed-phase silica gel C_{18} (MeOH/H_2O, 60%→100%) column chromatography to afford 6 fractions (Fr. 1–Fr. 6).

Fr. 2 (8.92 g) was subjected to CC on silica gel flash column chromatography (*n*-hexane/EtOAc, 20:1, 10:1, 5:1, 2:1, 1:1, 0:1, v/v) to yield nine sub-fractions Fr. 2-1 to Fr.2-9. Fr. 2-7 (3.07 g) was further was subjected to CC on Sephadex LH-20 (CH_2Cl_2/MeOH, 1:1, v/v) to give ten sub-fractions Fr. 2-7-1 to Fr. 2-7-10. Fr. 2-7-5 was further purified by silica gel flash column chromatography (*n*-hexane/EtOAc, 8:1→1:1, v/v) to give five sub-fractions Fr. 2-7-5-1 to Fr. 2-7-5-5. Fr. 2-7-5-3 was further purified by semiprep-HPLC (MeOH/H_2O, 60:40, v/v, 3 mL/min) to obtain sub-fractions Fr. 2-7-5-3-1 to Fr. 2-7-5-3-7. Fr. 2-7-5-3-6 (20 mg, t_R = 11.0 min) was further purified by silica gel flash column chromatography (*n*-hexane/EtOAc, 4:1→1:1, v/v) to give **1** (5 mg) and **2** (10 mg).

Rhizophol A (**1**): yellow powder; $[\alpha]^{20}_{D}$ = + 11.5 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 280 (4.62) nm; IR ν_{max} 2980, 2941, 2833, 1682, 1626, 1616, 1589, 1454, 1348, 1302, 1279, 1227, 1117, 1020, 1015, 897, 882, 855, 810, 787 cm^{-1}. ^{1}H and ^{13}C NMR, see Table 1; HRESIMS: *m*/*z* 401.1594 $[M + H]^+$ (calcd for $C_{22}H_{25}O_7$, 401.1595).

Rhizophol B (**2**): yellow needle; $[\alpha]^{20}_{D}$ = + 38.5 (*c* 0.08, MeOH); UV (MeOH) λ_{max} (log ε) 371 (5.18), 289 (5.43) nm; IR ν_{max} 3288, 2927, 1616, 1472, 1015, 887, 794 cm^{-1}. ^{1}H and ^{13}C NMR, see Table 1; HRESIMS: *m*/*z* 383.1491 $[M + H]^+$ (calcd for $C_{22}H_{23}O_6$, 383.1489).

DPPH photometric assay

Sample stock solutions (10 mM) were diluted to final concentrations of 2, 4, 8, 16, 32, 64, and 128 μM, in ethanol. 100 μL 0.2 mmol/L DPPH ethanol solution was added to 100 μL sample solutions of different concentrations on 96-well plates, and allowed to react at room temperature. Ascorbic acid was used as a positive control possessing potent antioxidant acitivity. Afert 12 h, the absorbance values were measured at 517 nm and converted into the percentage antioxidant activity (AA) using the following formula: $AA(\%)= [1 - (A_{sample} - A_{blank})/A_{control}]\times 100\%$. All data were obtained in triplicate and are presented as means ±S.D. IC_{50} values were calculated with the SigmaPlot 10.0 software using a non-linear curve-fitting method.

Cytotoxicity assay

Cytotoxicities of compounds **1** and **2** were assayed against four human tumor cell lines, including MCF-7 (human breast adenocarcinoma cell line), NCI-H460 (human non-small cell lung cancer cell line), HepG-2 (human liver hepatocellular carcinoma), and SF-268 (human glioma cell line). Assays were performed by SRB (Sulforhodamine B) method.

S2. X-ray crytallographic data of compound 2.

The single-crystal X-ray diffraction data were collected at 100K for **1** on Agilent Xcalibur Nova single-crystal diffractometer using CuKα radiation. The crystal

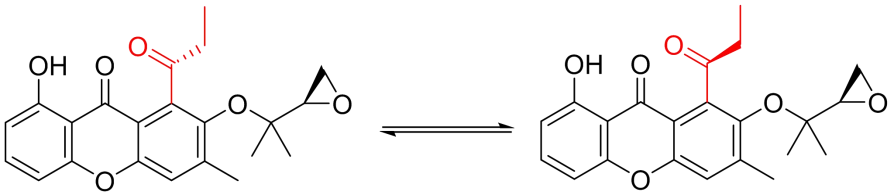
structure was refined by full-matrix least-squares calculation. Hydrogen atoms bonded to carbons were located by the geometrically ideal positions by the "ride on" method. Hydrogen atoms bonded to oxygen were placed on the difference Fourier method and were included in the calculation of structure factors with isotropic temperature factors. Crystallographic data for **2** reported in this paper has been deposited in the Cambridge Crystallographic Data Centre. (Deposition number: CCDC 1942463). Copies of these data can be obtained free of charge via www.ccdc.cam.au.ck/conts/retrieving.html.)

Table 1 Crystal data and structure refinement for liuhongxin_B3_collect.

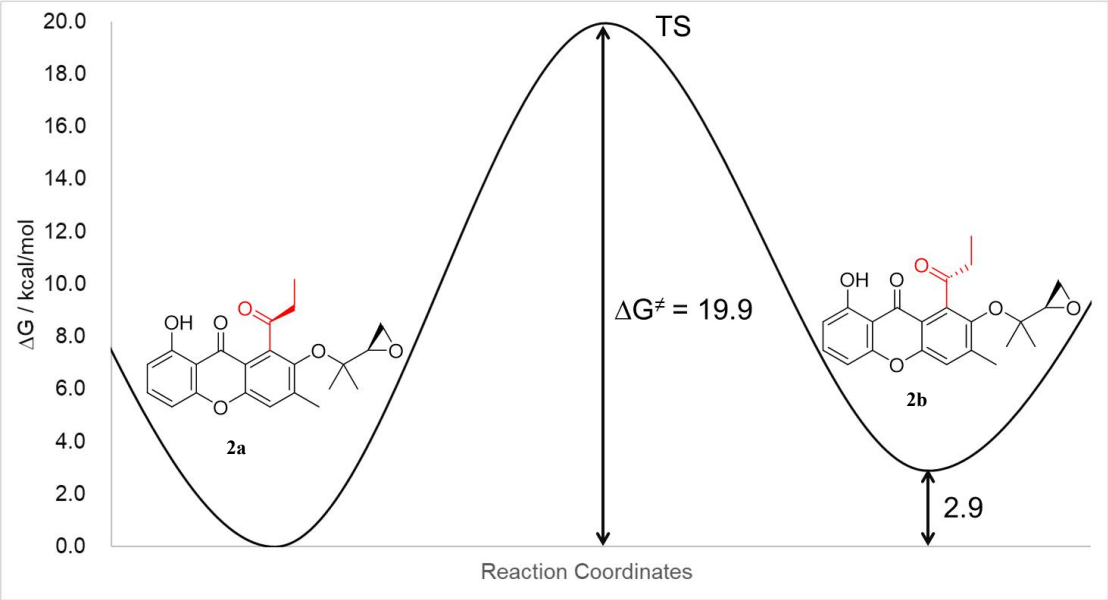
Identification code	liuhongxin_B3_collect
Empirical formula	$C_{88}H_{88}O_{24}$
Formula weight	1529.58
Temperature/K	99.98(12)
Crystal system	orthorhombic
Space group	$P2_12_12_1$
a/Å	6.8000(2)
b/Å	15.6030(4)
c/Å	17.7559(6)
α/°	90
β/°	90
γ/°	90
Volume/Å^3	1883.91(10)
Z	1
ρ_{calc}g/cm^3	1.348
μ/mm^{-1}	0.810
F(000)	808.0
Crystal size/mm^3	0.2 × 0.1 × 0.1
Radiation	CuKα (λ = 1.54184)
2Θ range for data collection/°	7.542 to 147.118
Index ranges	$-8 \le h \le 8$, $-18 \le k \le 16$, $-21 \le l \le 20$
Reflections collected	9486
Independent reflections	3675 [R_{int} = 0.0425, R_{sigma} = 0.0506]
Data/restraints/parameters	3675/0/258
Goodness-of-fit on F^2	1.069
Final R indexes [I>=2σ (I)]	R_1 = 0.0448, wR_2 = 0.1220
Final R indexes [all data]	R_1 = 0.0517, wR_2 = 0.1271
Largest diff. peak/hole / e Å^{-3}	0.16/-0.24
Flack parameter	-0.15(13)

S3. Computational methods

Two possible stable conformations:

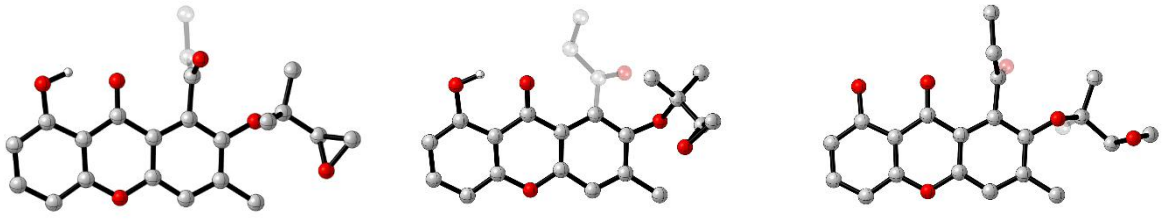


2a **2b**



Two stable conformations could be observed in the solution, and the conversion barrier is nearly 20 kcal/mol. According to the transition state theory (Evans, M.G.; Polanyi M. *Trans. Faraday Soc*. **1935**, *31*, 875), the conversion rate constant at 293 K is about 8.6 X 10^{-3} s^{-1}, and the corresponding $t^{1/2}$ should be 1.3 min.

The optimized structures of B3-A and B3-B.



2a **TS** **2b**

References

1. Evans, M.G.; Polanyi M. Some applications transitionstate method reactionvelocities, especially solution. *Trans. Faraday Soc*. **1935**, *31*, 875.

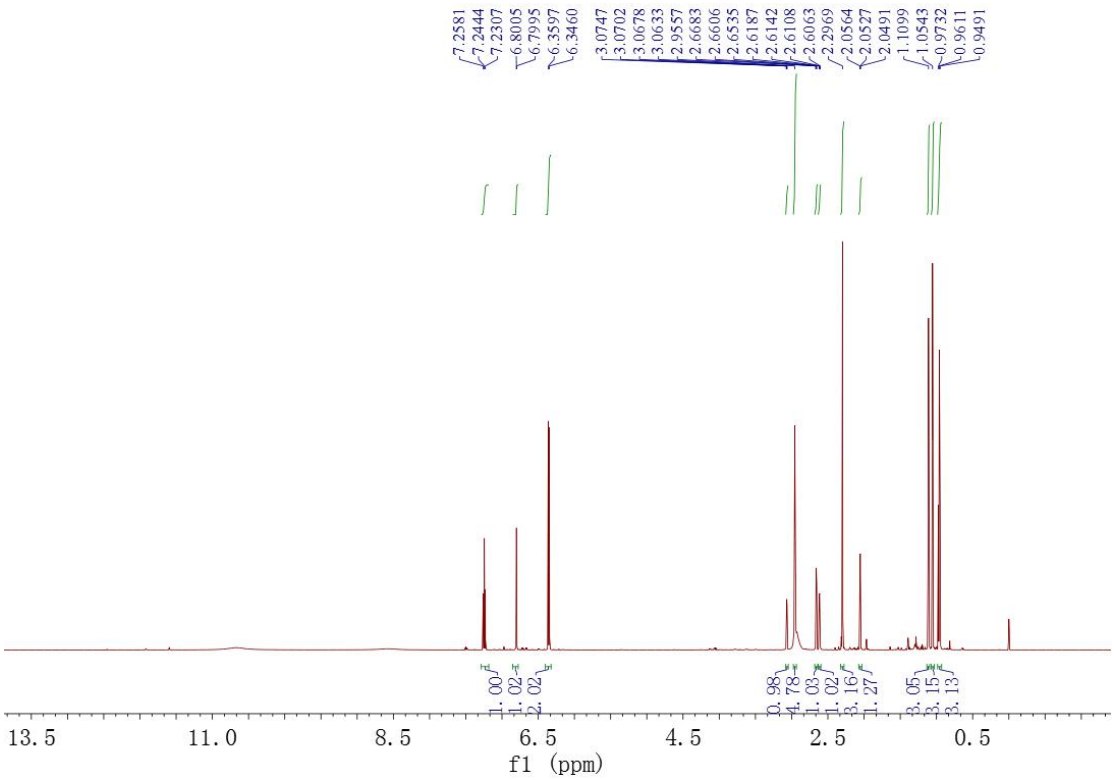


Figure S1. ^{1}H NMR spectrum (600 MHz, CD_3OCD_3) of **1**.

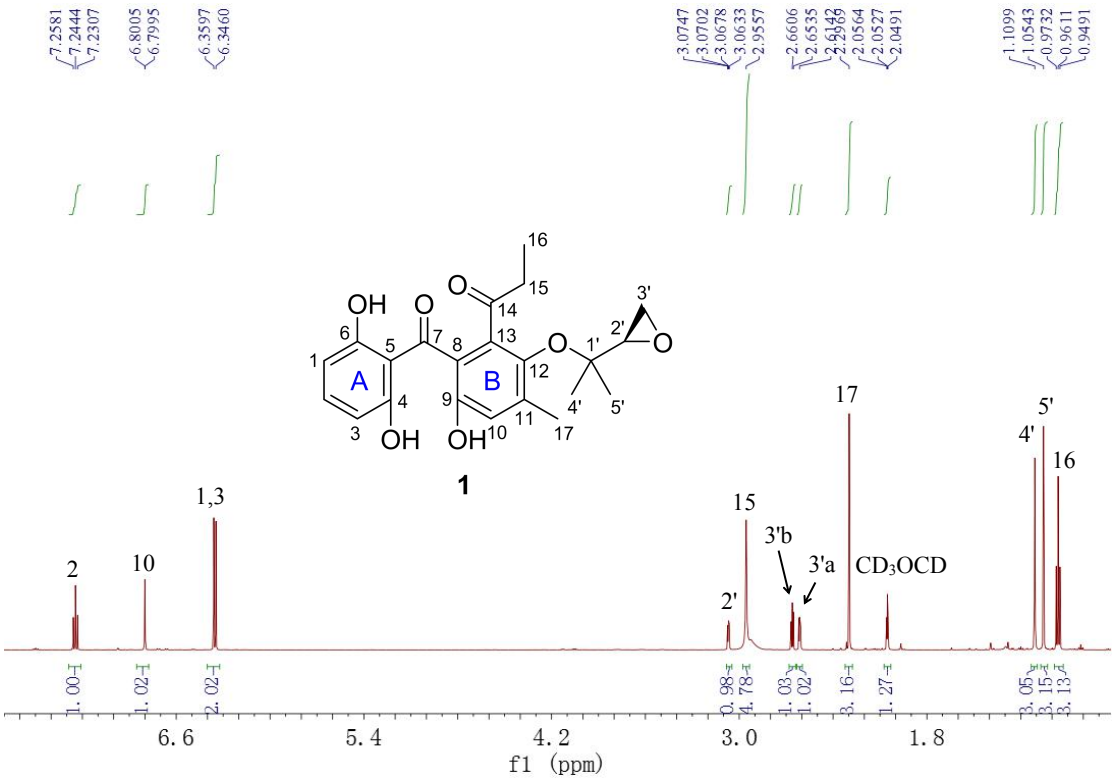


Figure S2. Enlarged ^{1}H NMR spectrum (600 MHz, CD_3OCD_3) of **1**.

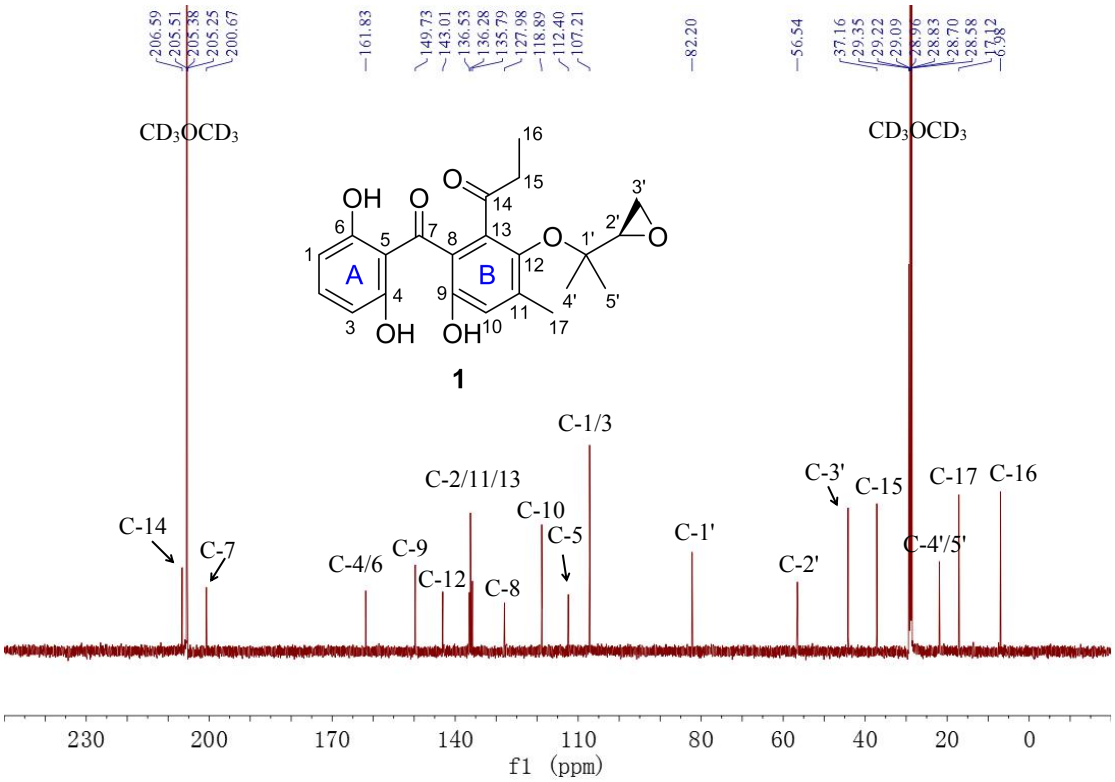


Figure S3. ^{13}C NMR spectrum (150 MHz, CD_3OCD_3) of **1**.

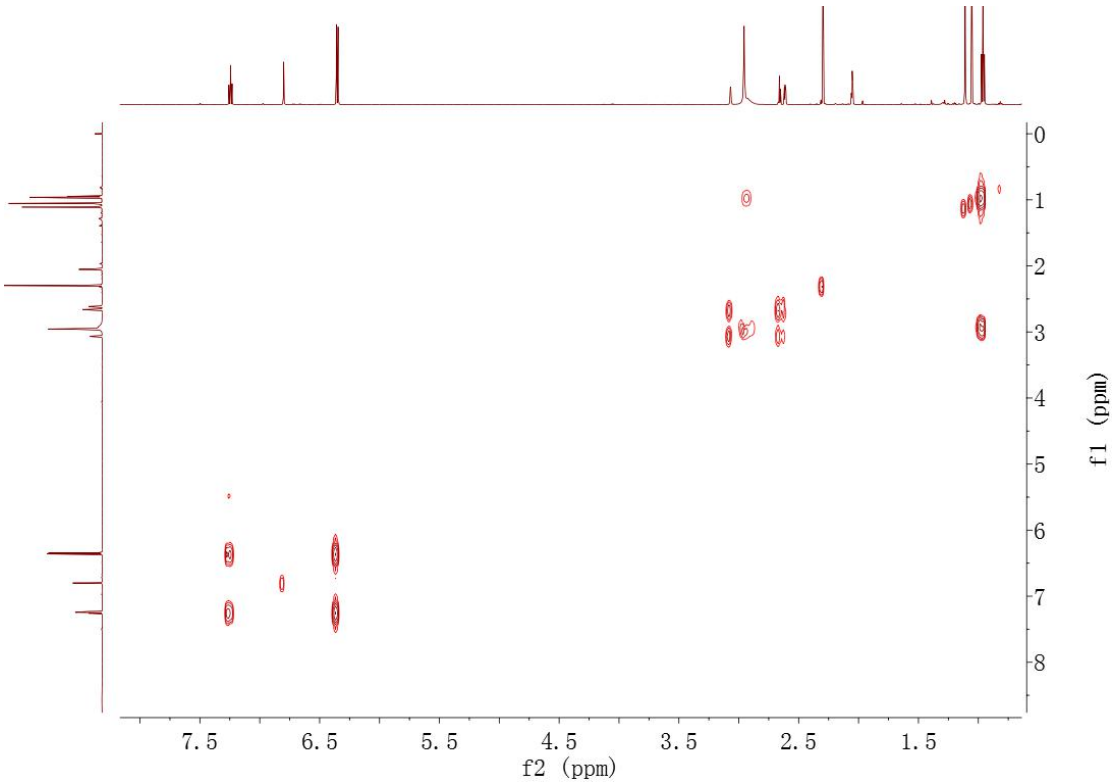


Figure S4. ^{1}H-^{1}H COSY spectrum (600 MHz, CD_3OCD_3) of **1**.

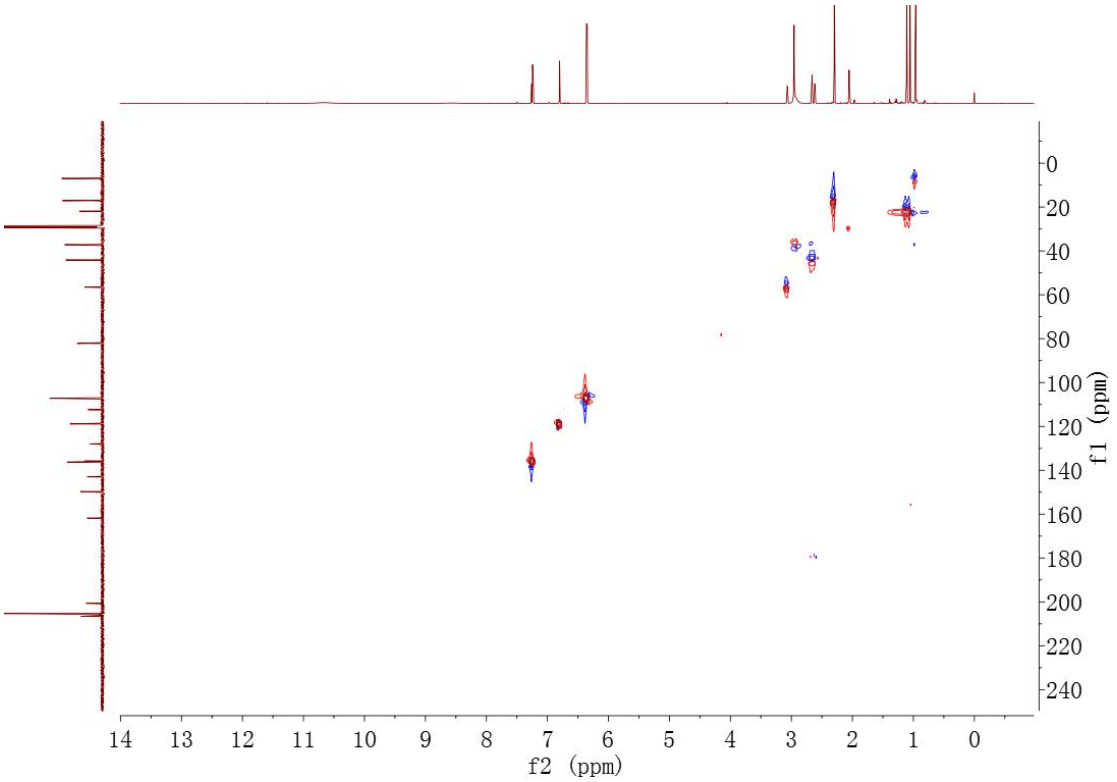


Figure S5. HSQC spectrum of **1**.

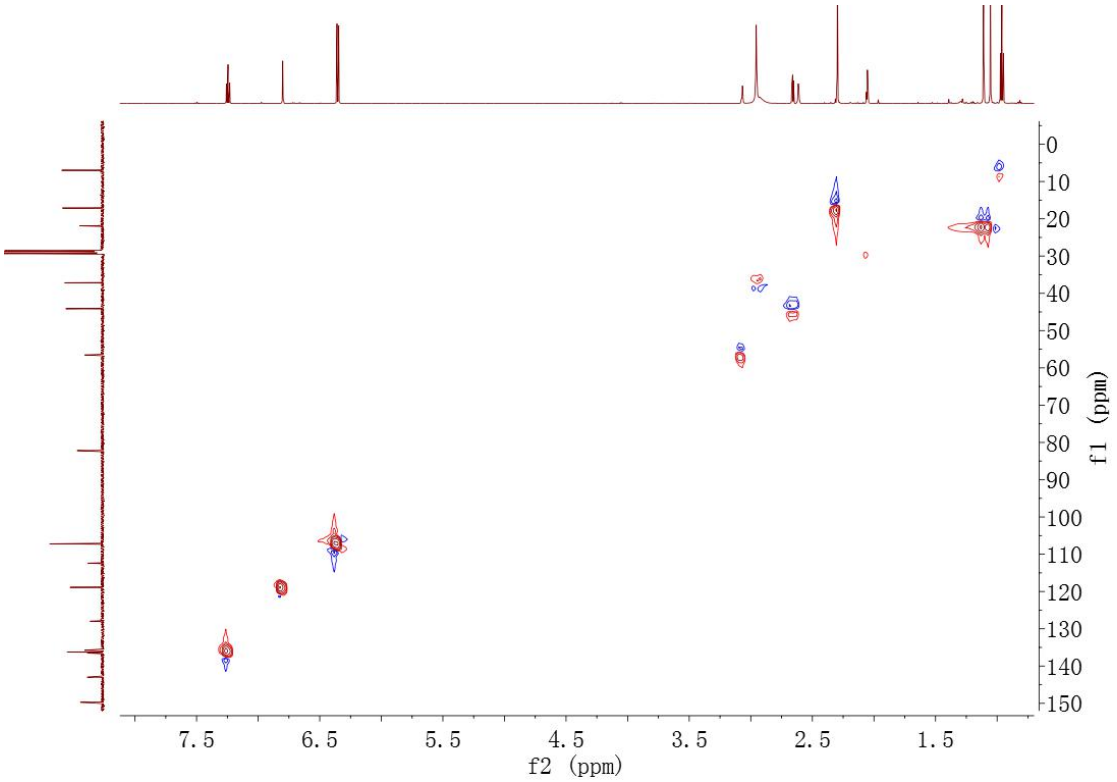


Figure S6. Partial enlarged HSQC spectrum of **1**.

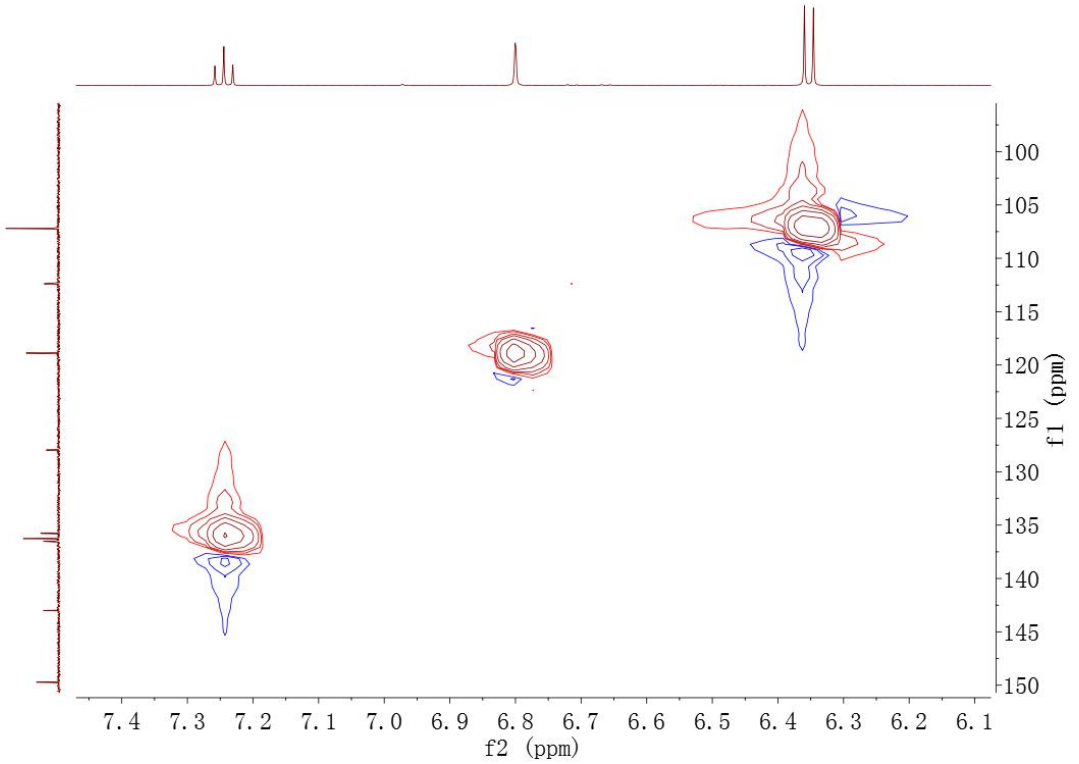


Figure S7. Partial enlarged HSQC spectrum of **1**.

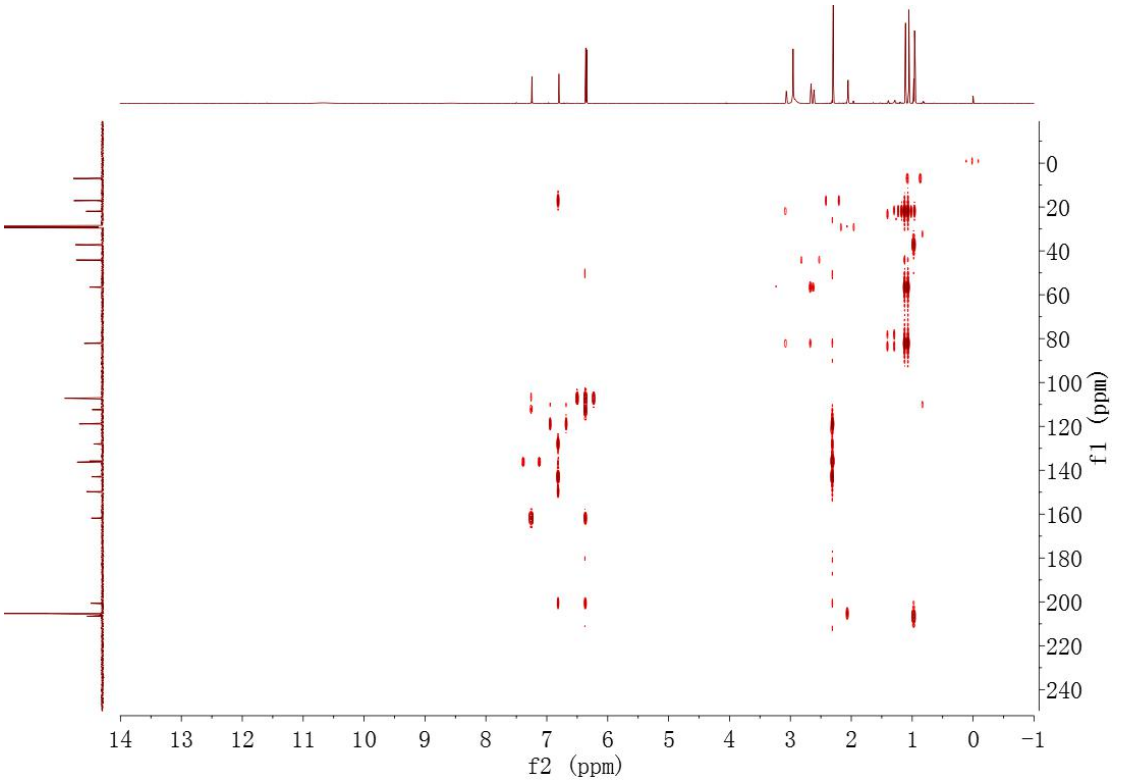


Figure S8. HMBC spectrum of **1**.

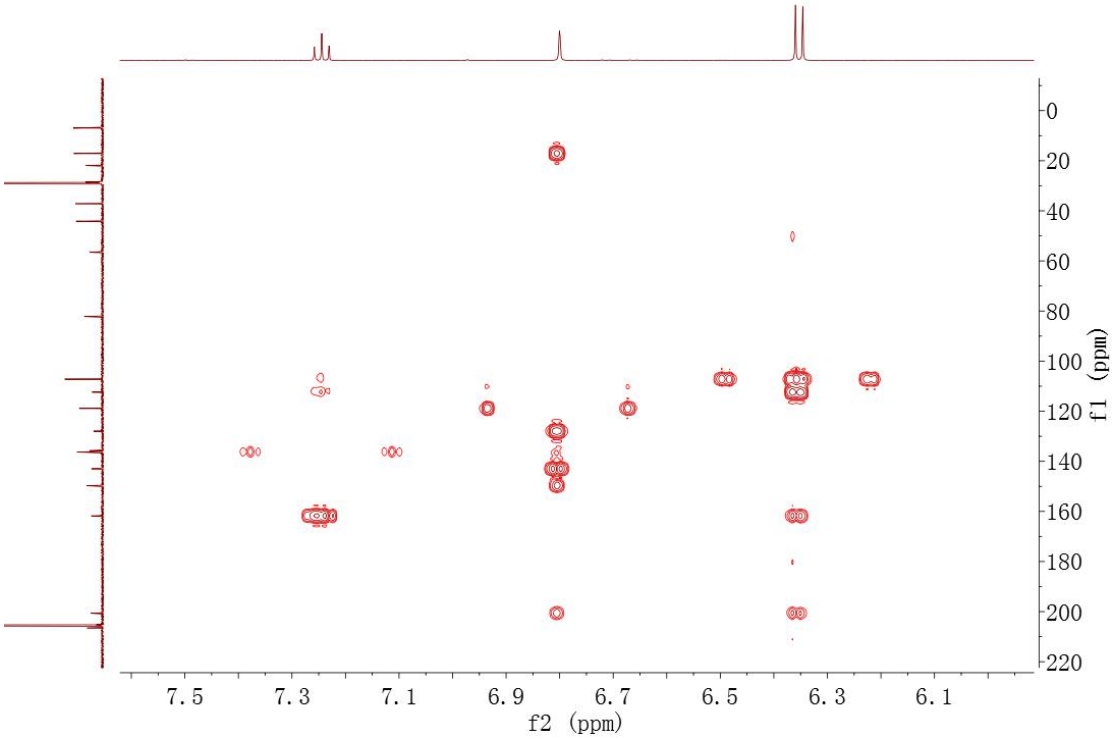


Figure S9. Partial enlarged HMBC spectrum of **1**

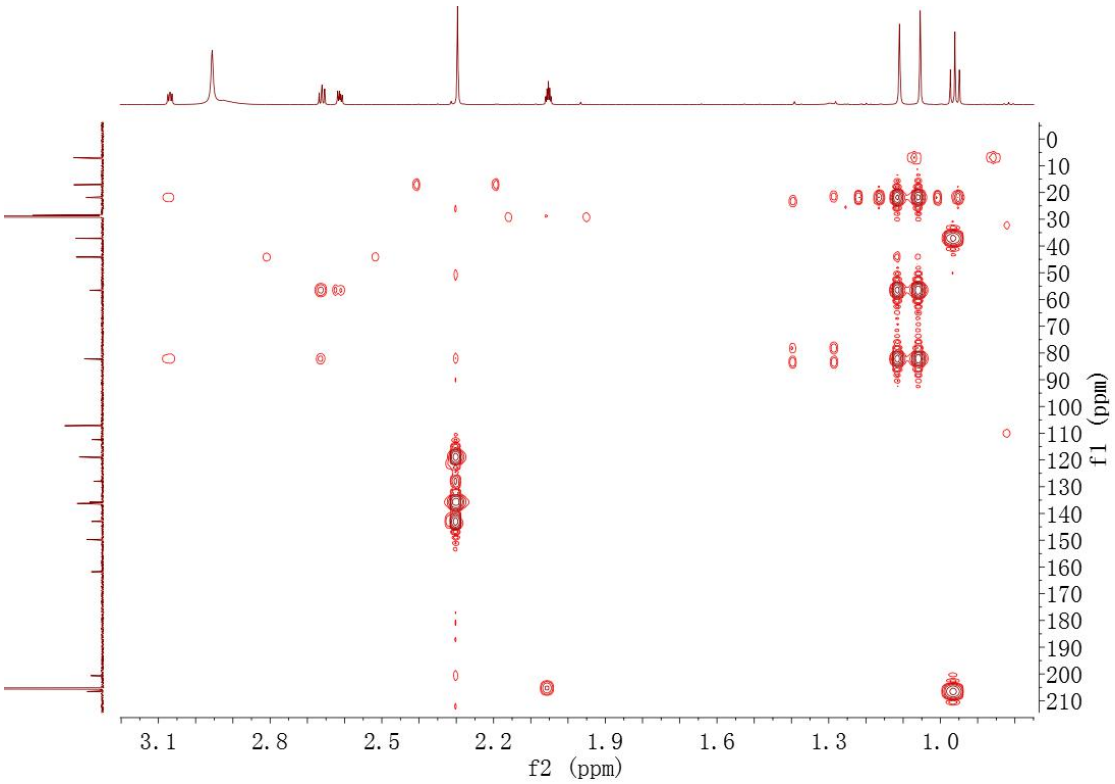


Figure S10. Partial enlarged HMBC spectrum of **1**.

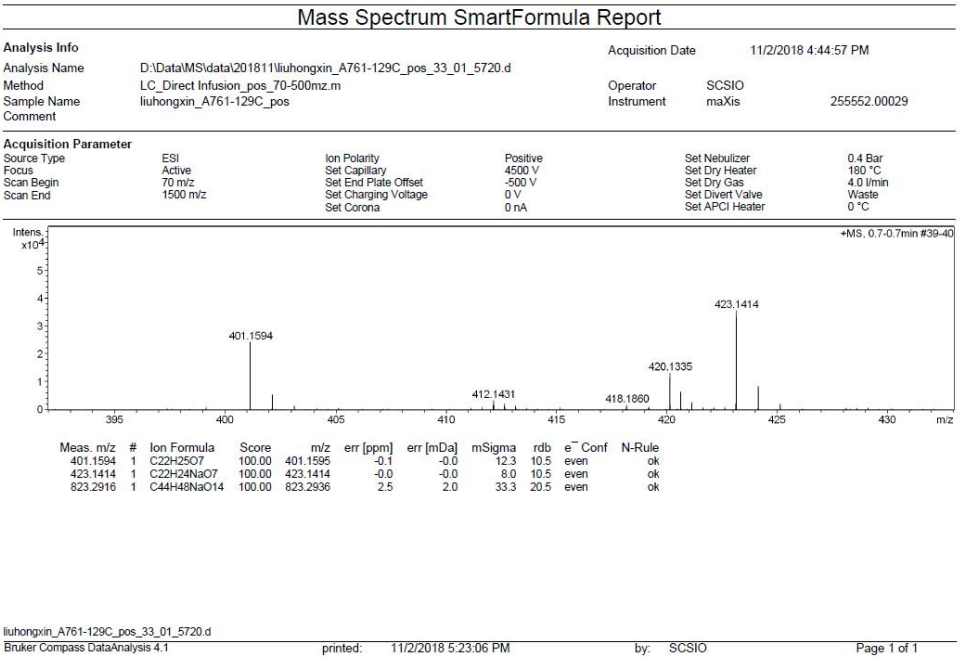


Figure S11. HRESIMS spectrum of **1**.

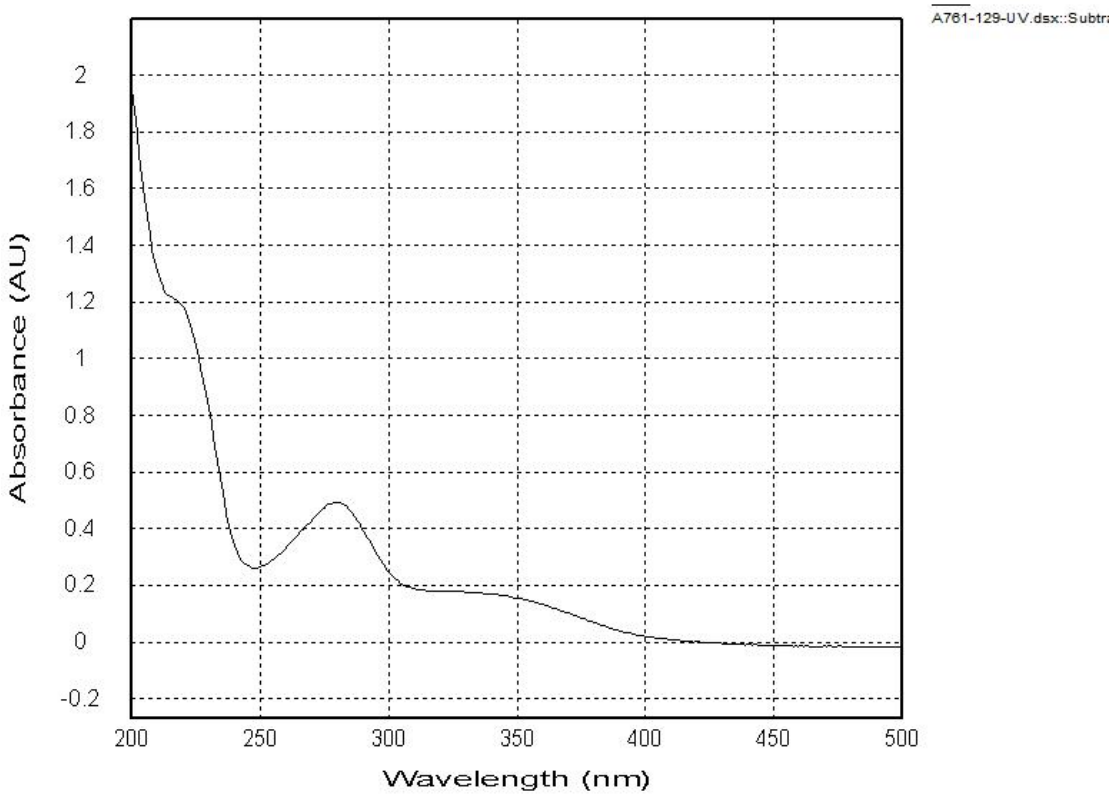


Figure S12. UV spectrum of **1**.

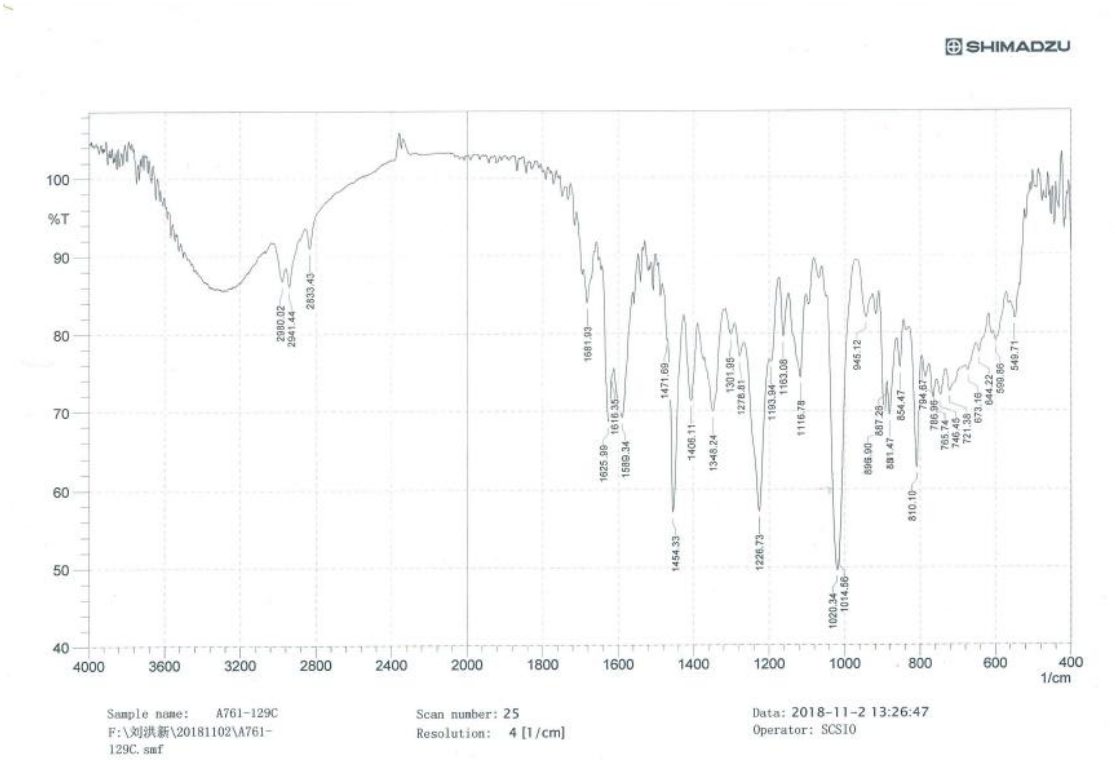


Figure S13. IR spectrum of **1**.

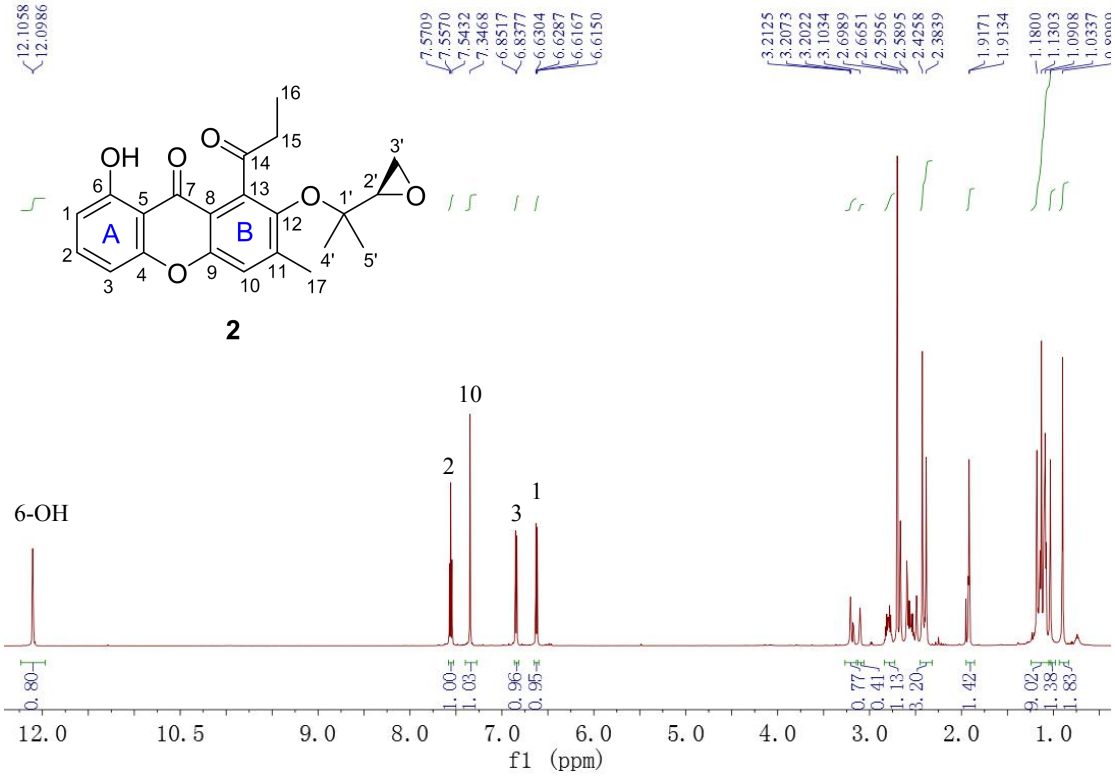


Figure S14. ^{1}H NMR spectrum (600 MHz, CD_3COCD_3) of **2**.

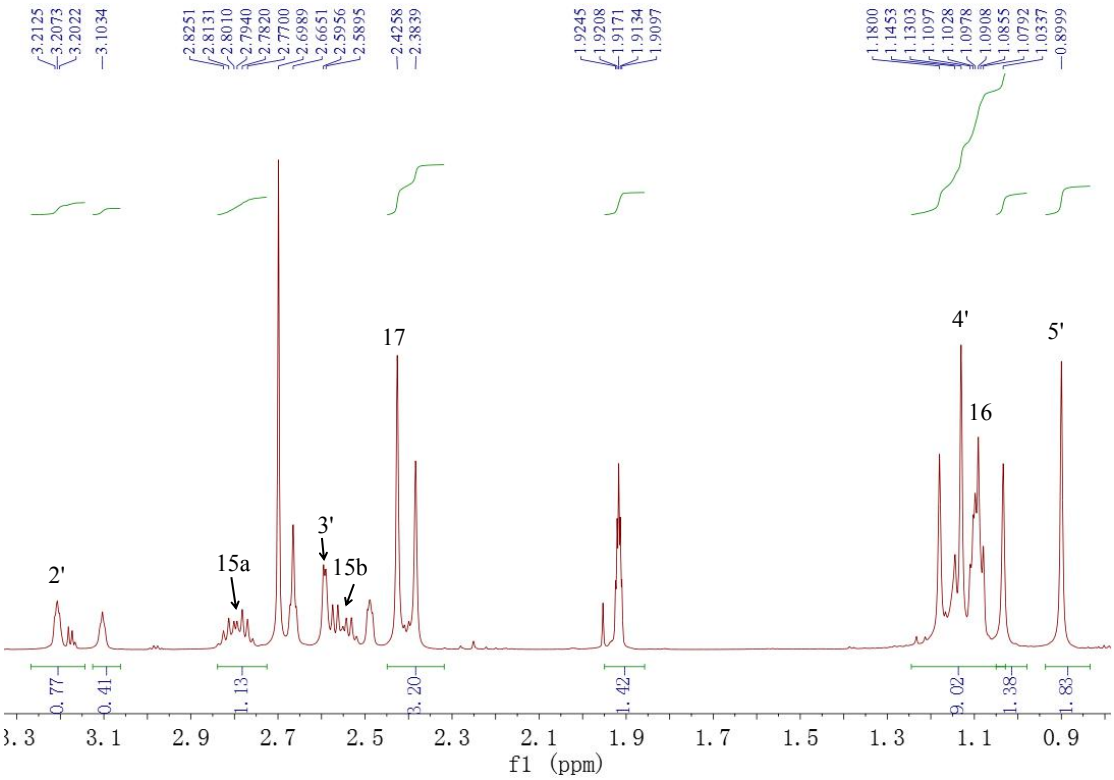


Figure S15. Partial enlarged ^{1}H NMR spectrum (600 MHz, CD_3COCD_3) of **2**..

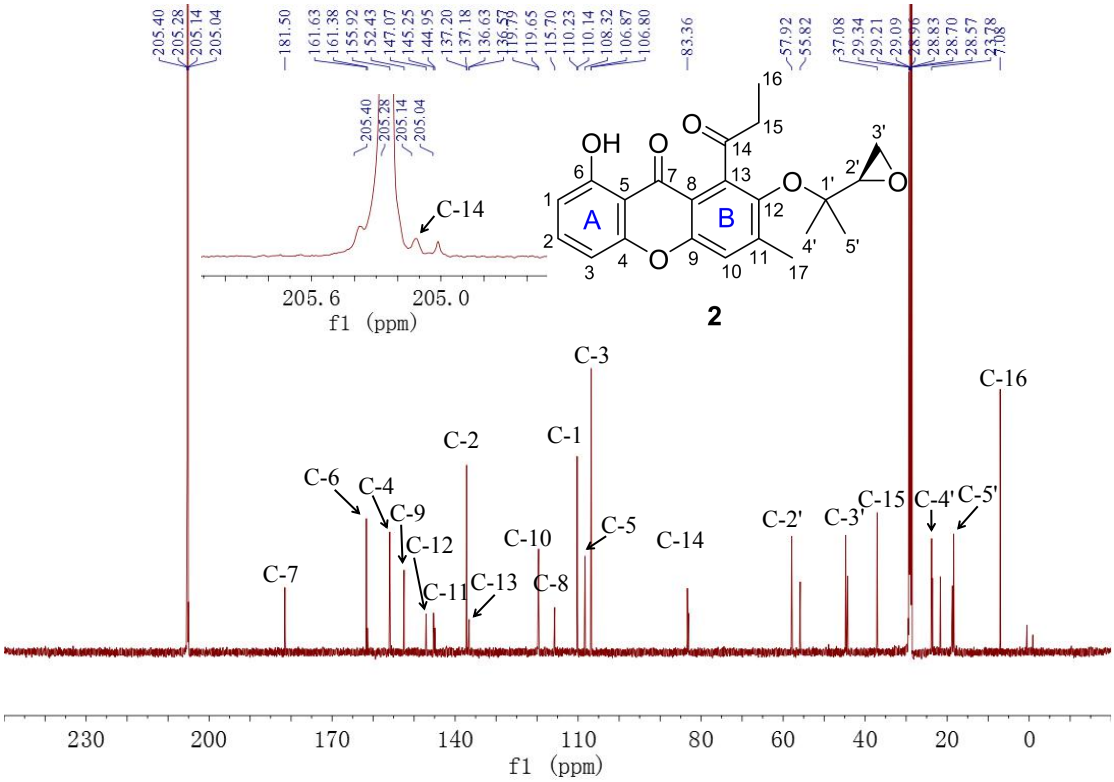


Figure S16. ^{13}C NMR spectrum (150 MHz, CD_3COCD_3) of **2**.

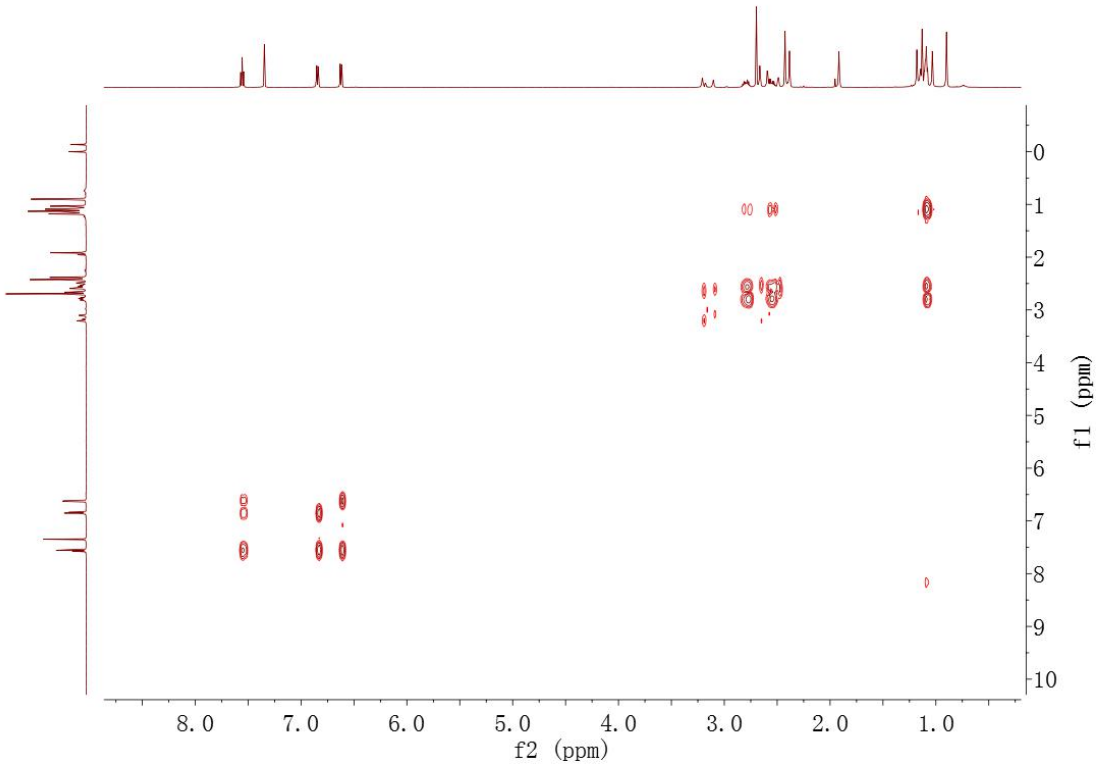


Figure S17. ^{1}H-^{1}H COSY spectrum (600 MHz, CD_3COCD_3) of **2**.

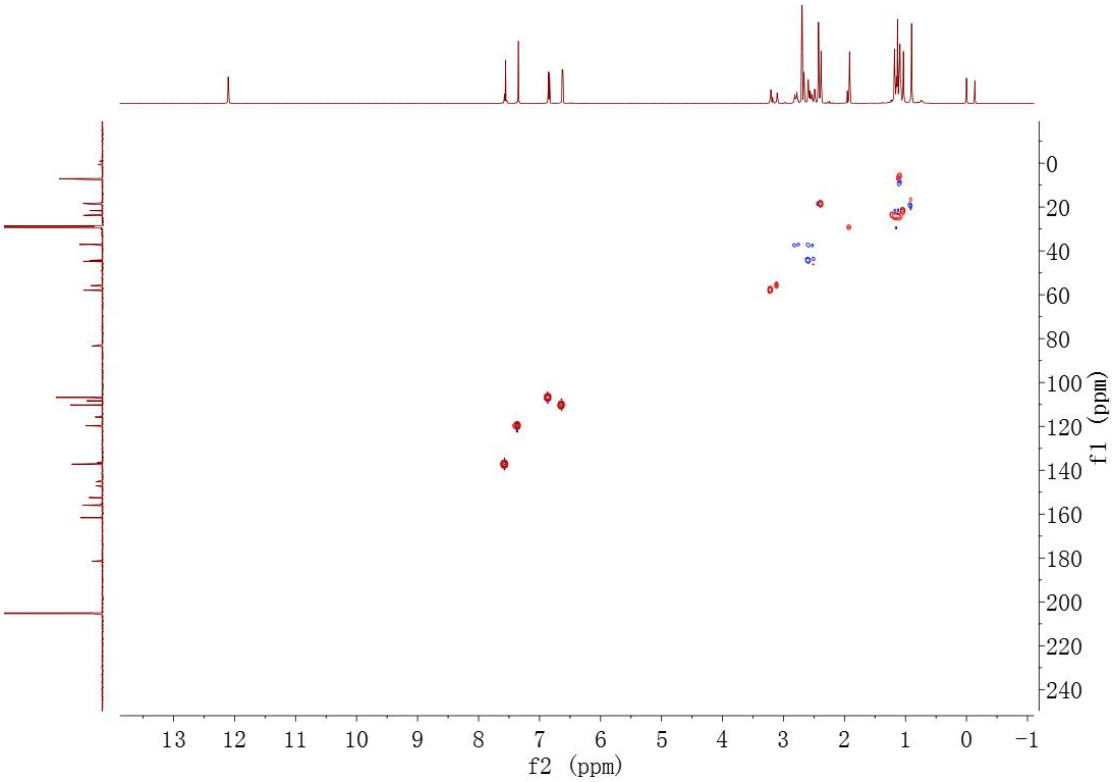


Figure S18. HSQC spectrum of **2**.

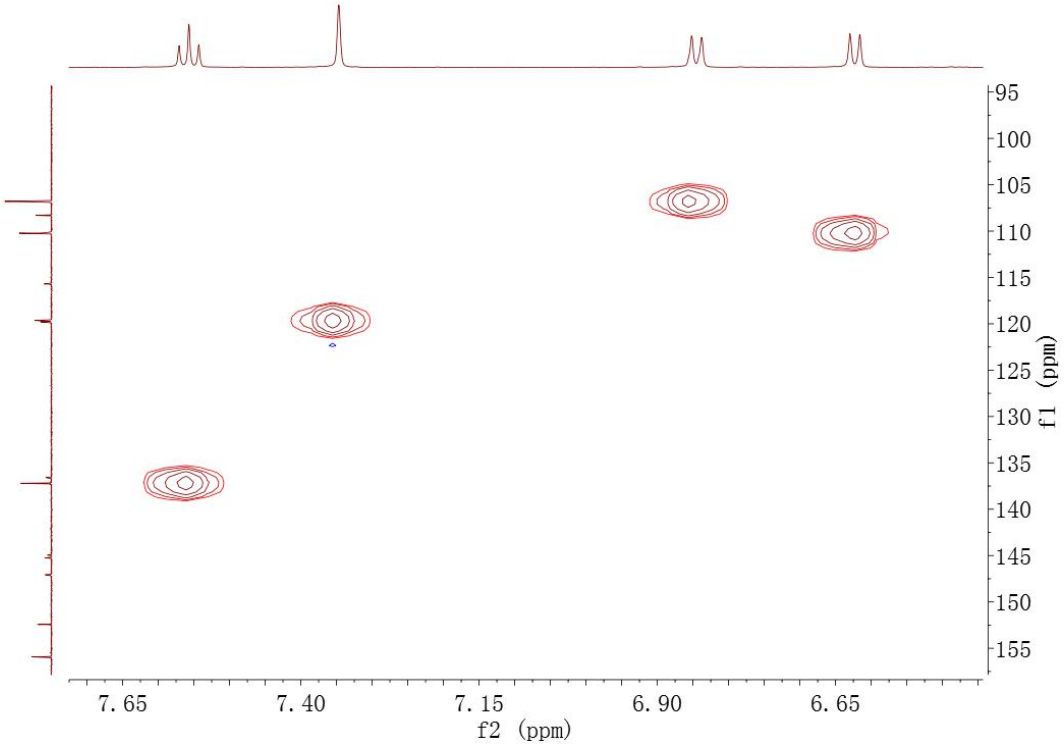


Figure S19. Partial enlarged HSQC spectrum of **2**.

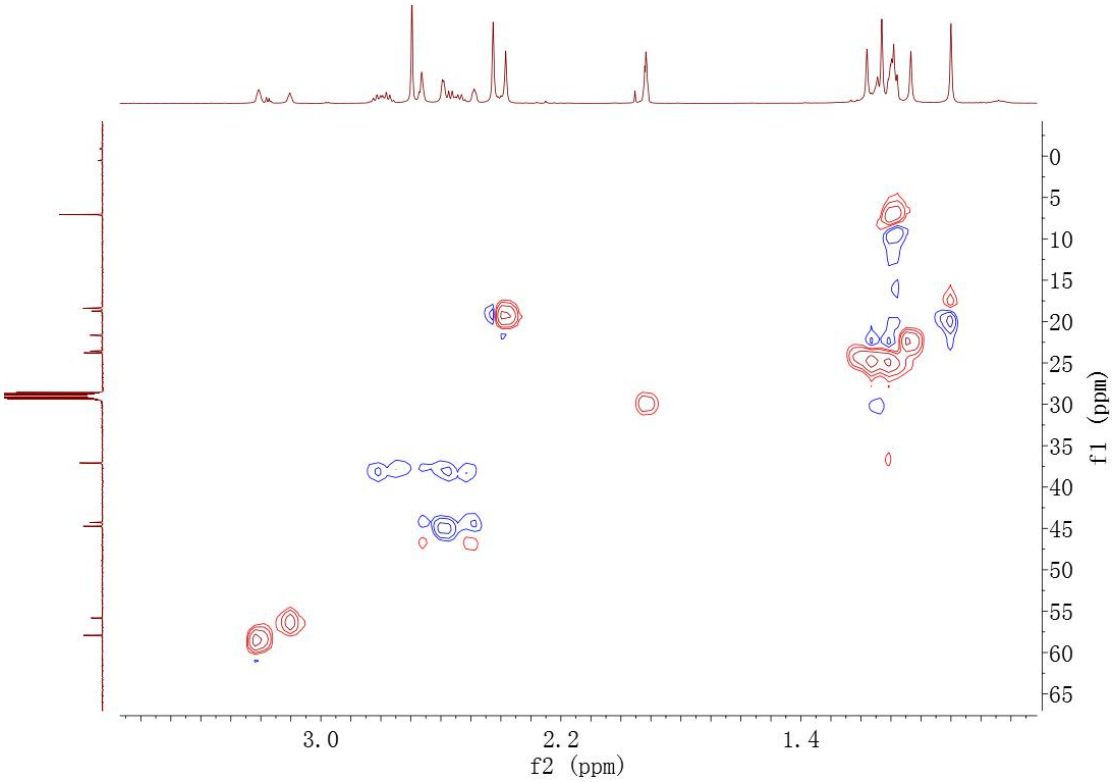


Figure S20. Partial enlarged HSQC spectrum of **2**.

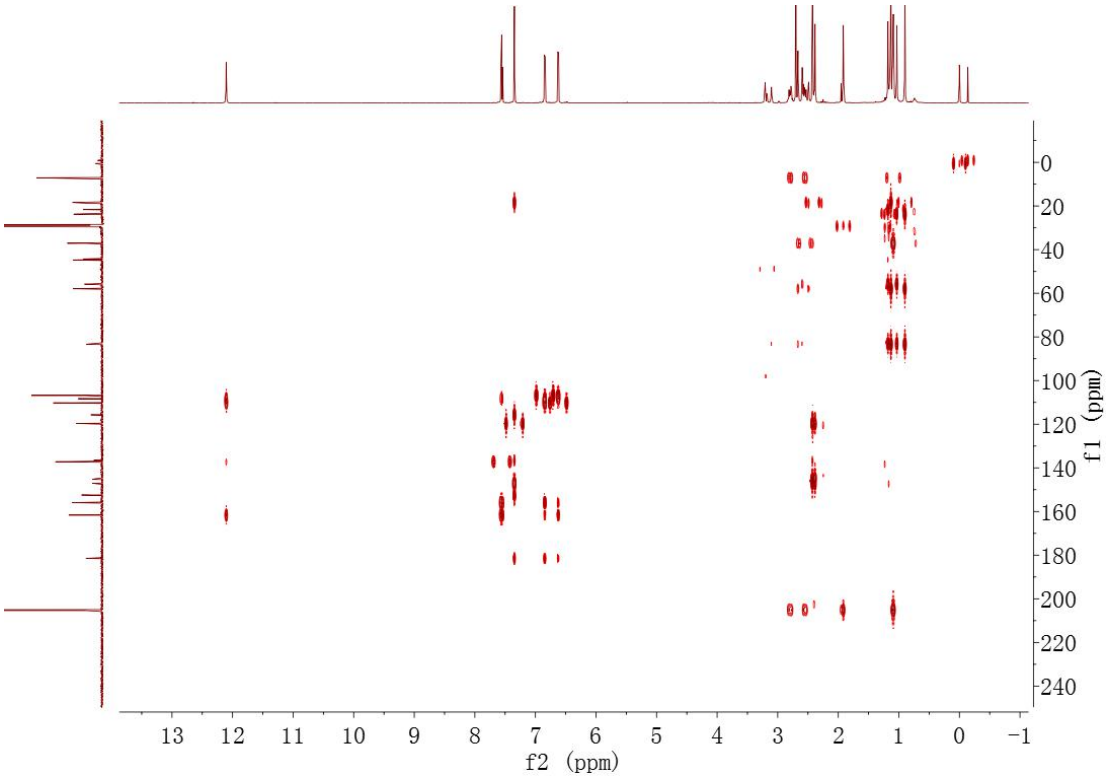


Figure S21. HMBC spectrum of **2**.

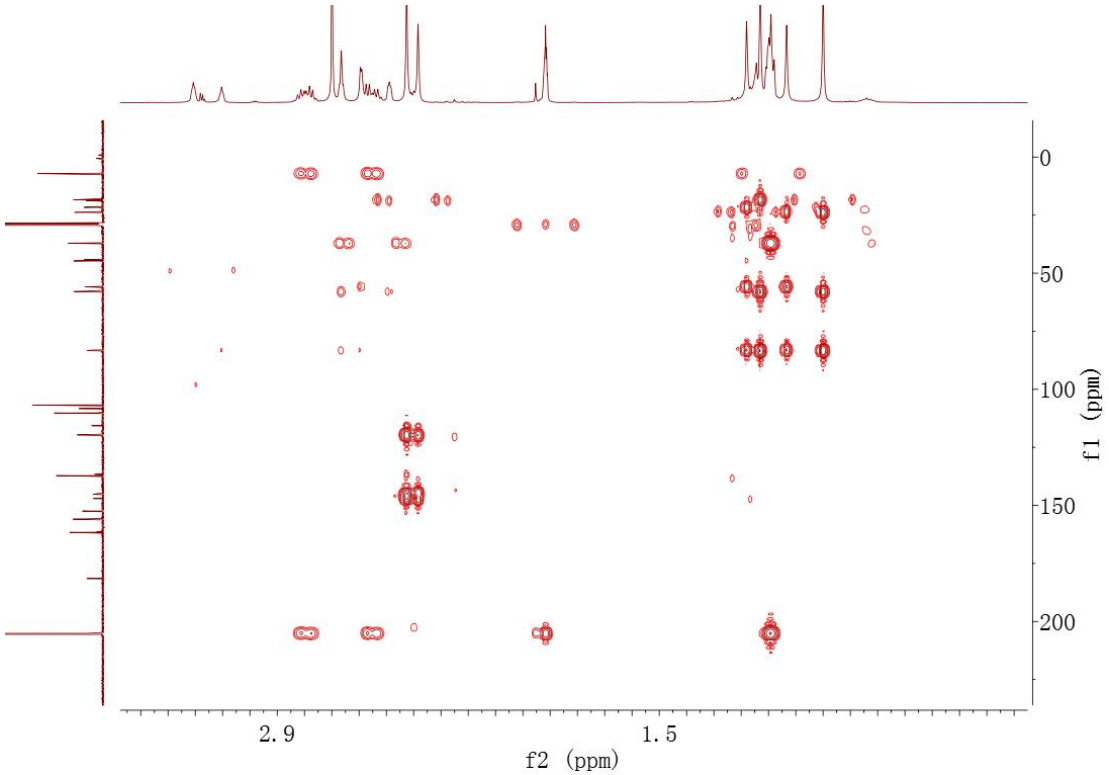


Figure S22. Partial enlarged HMBC spectrum of **2**.

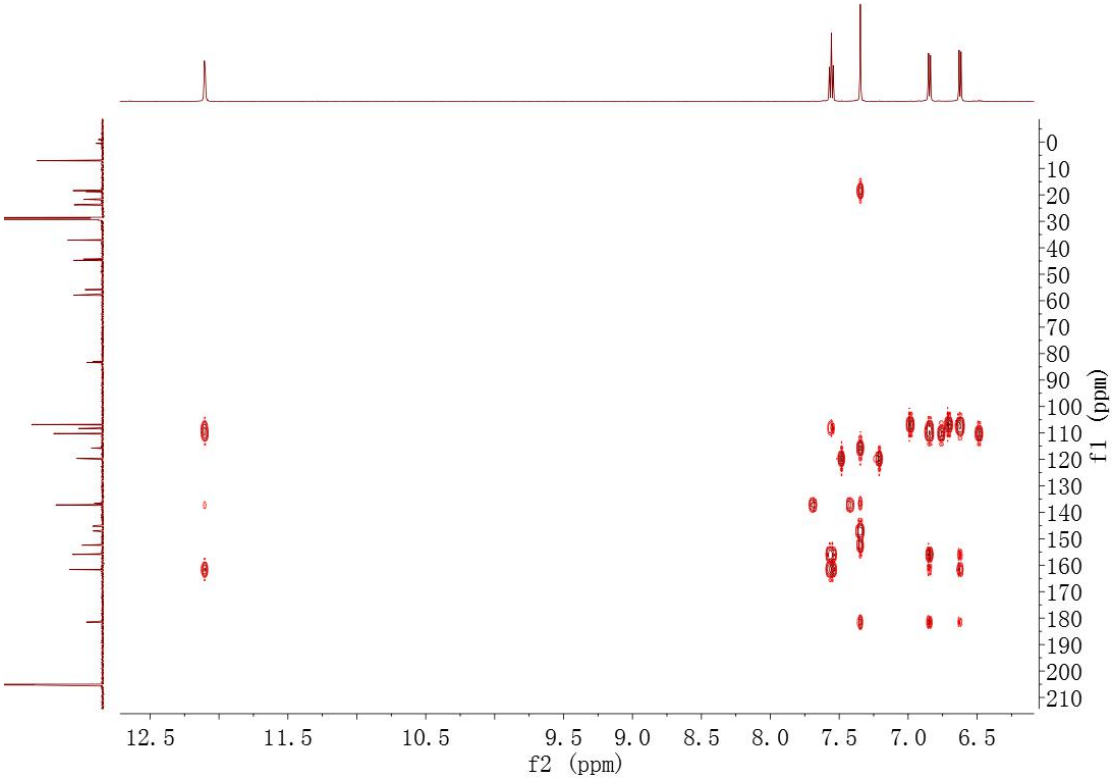


Figure S23. Partial enlarged HMBC spectrum of **2**.

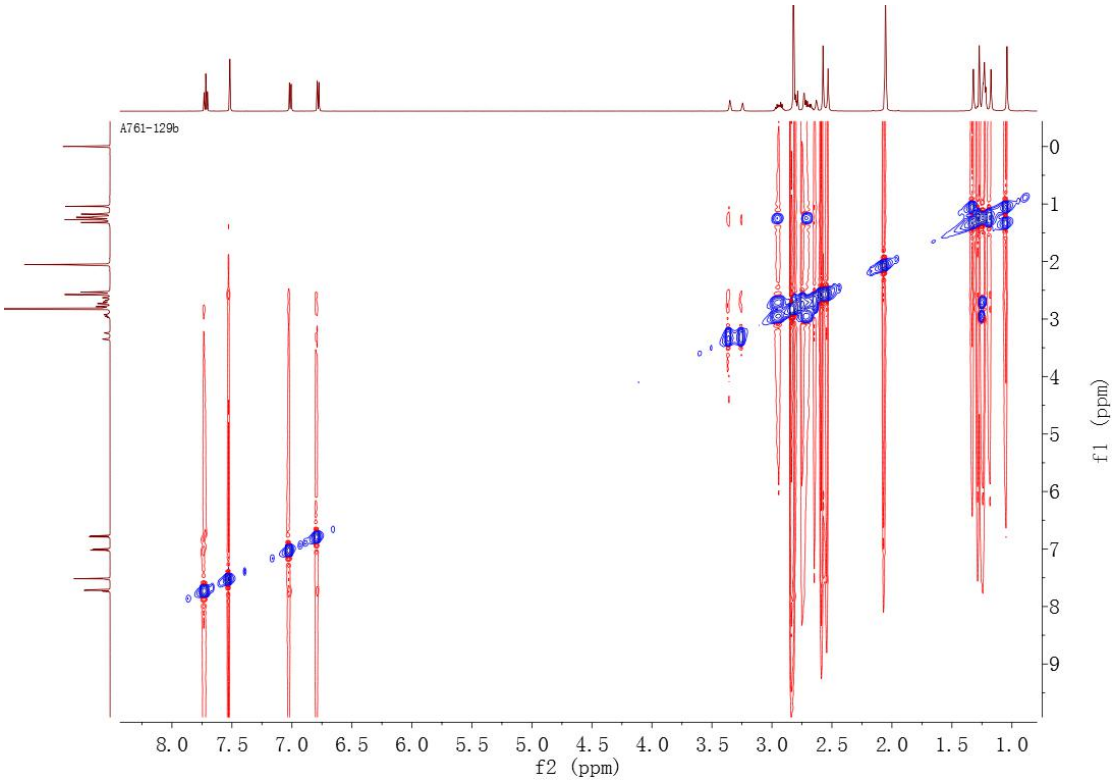


Figure S24. NOESY spectrum of **2**.

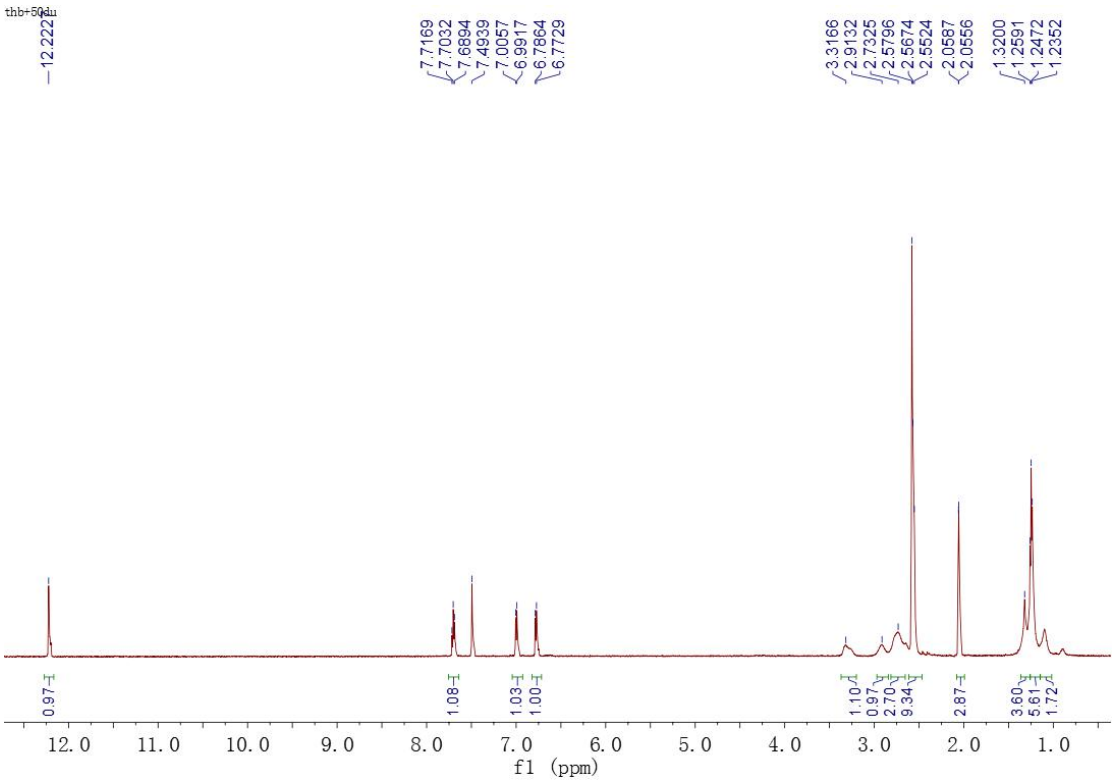


Figure S25. ^{1}H NMR spectrum (600 MHz, 323 K,CD_3COCD_3) of **2**.

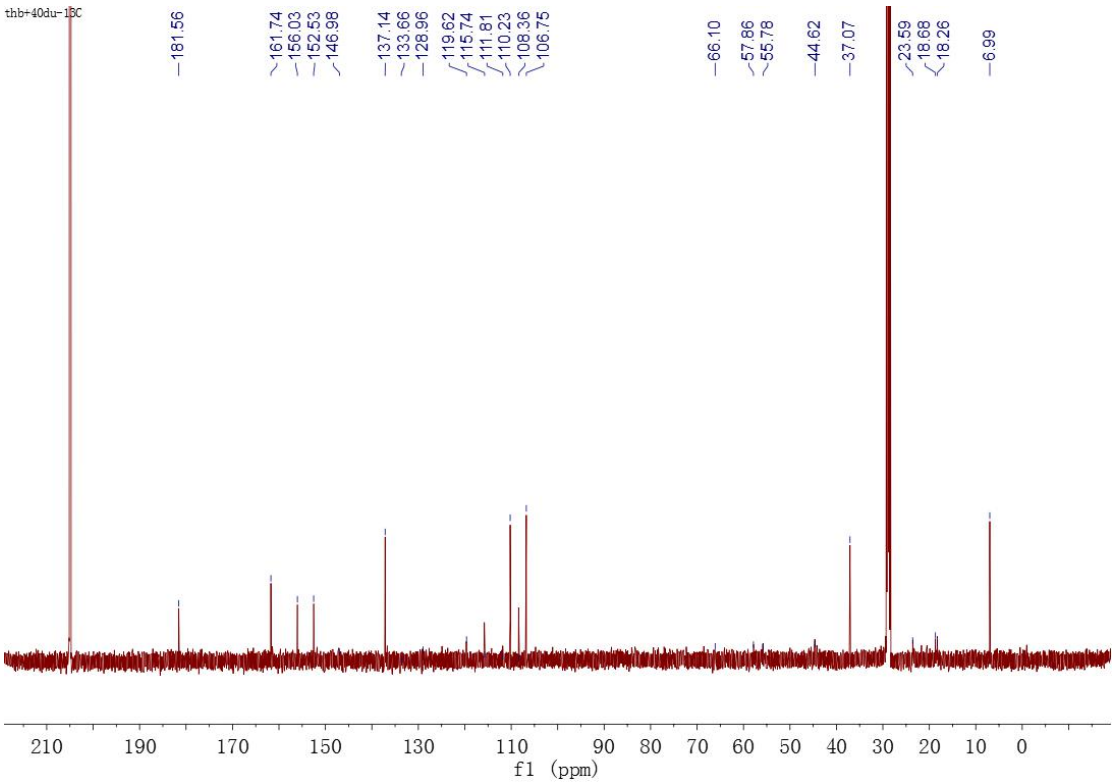


Figure S26. ^{13}C NMR spectrum (150 MHz, 313 K, CD_3COCD_3) of **2**.

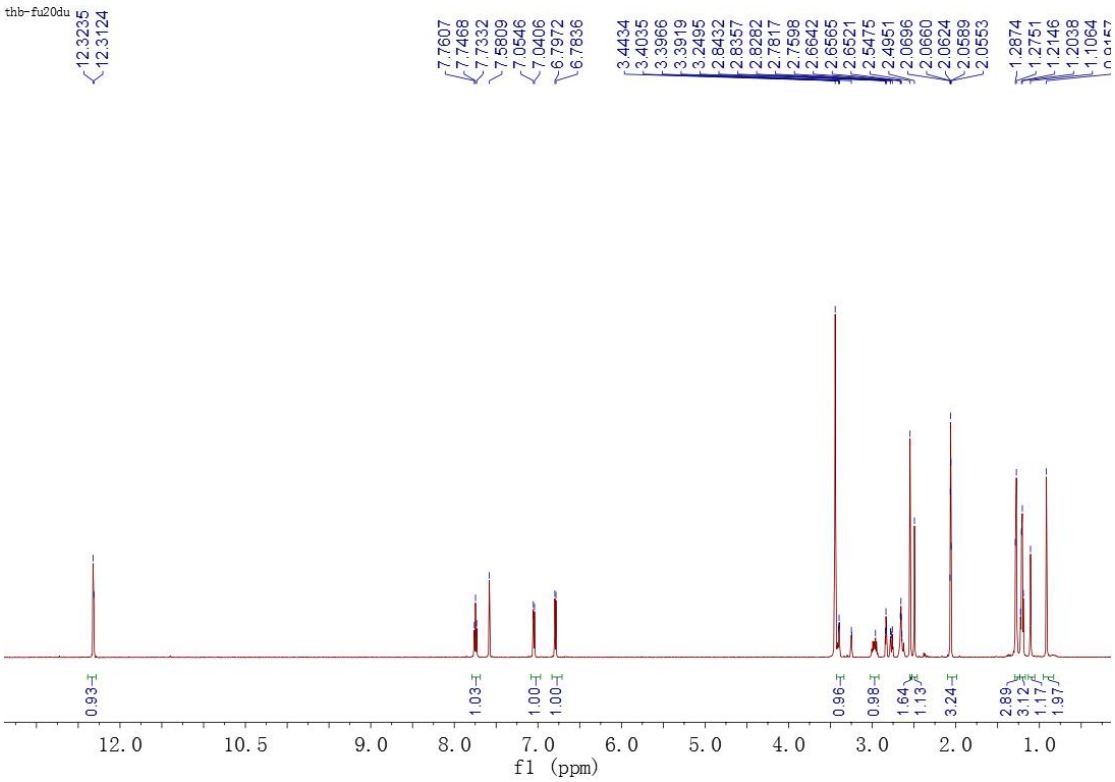


Figure S27. ^{1}H NMR spectrum (600 MHz, 253 K, CD_3COCD_3) of **2**.

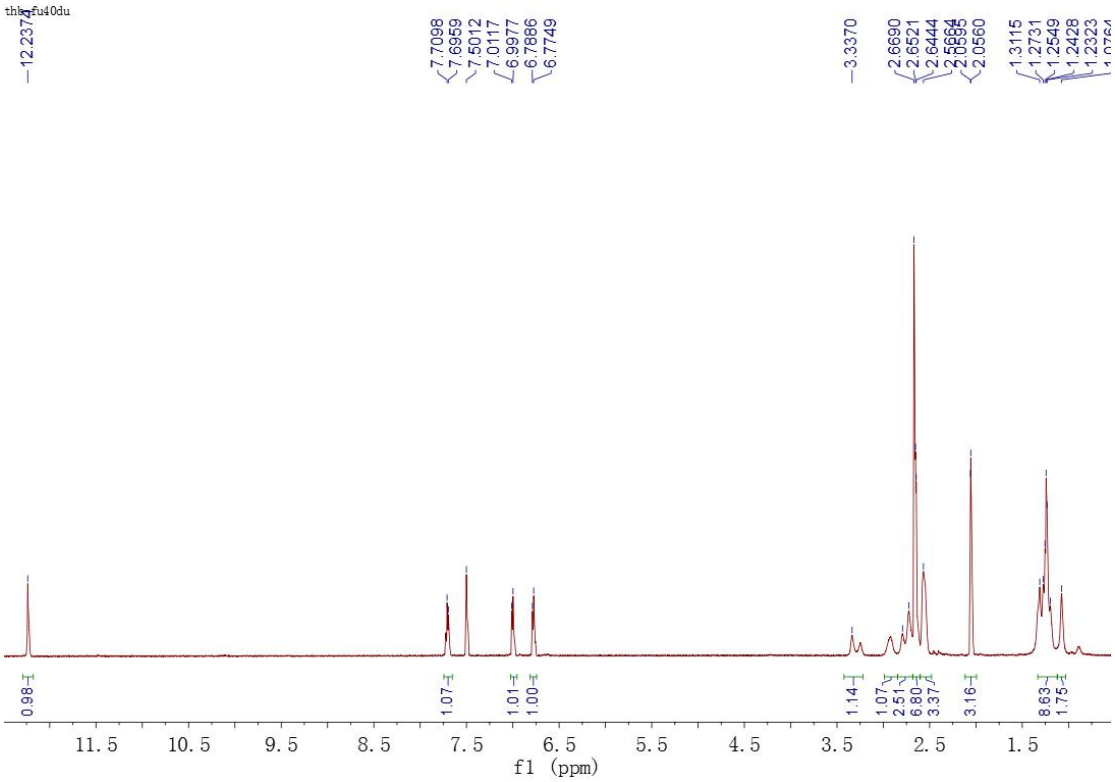


Figure S28. ^{1}H NMR spectrum (600 MHz, 233 K, CD_3COCD_3) of **2**.

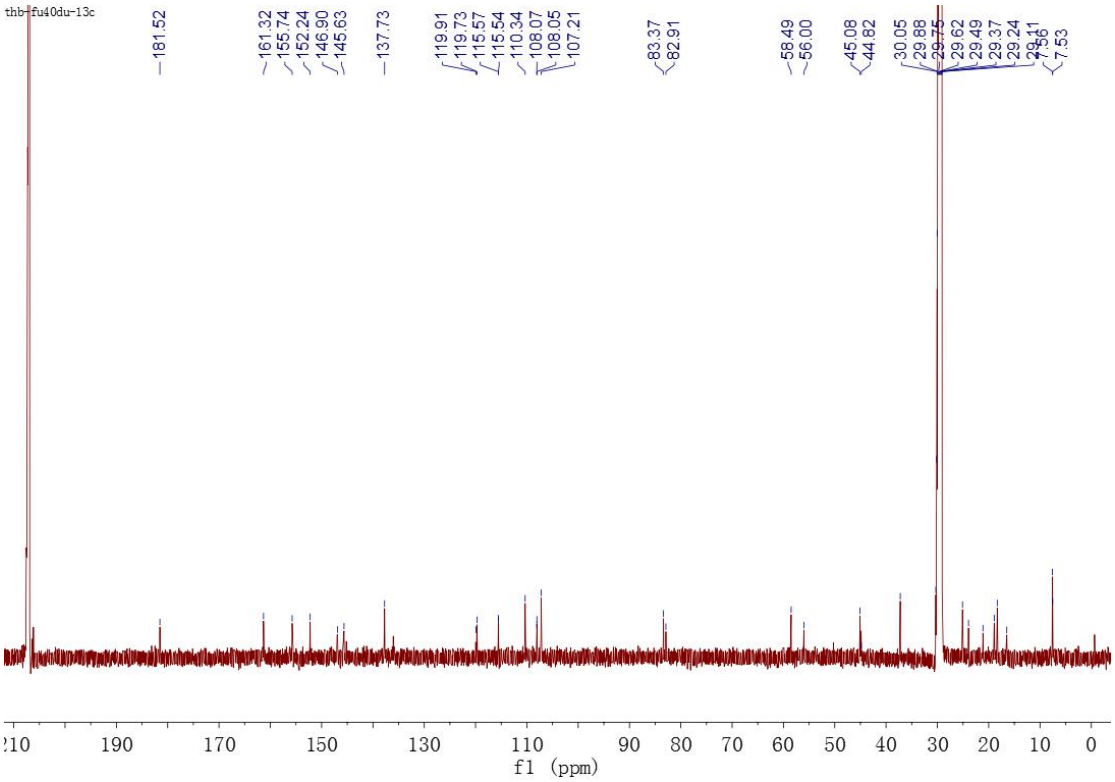


Figure S29. ^{13}C NMR spectrum (150 MHz, 273 K, CD_3COCD_3) of **2**.

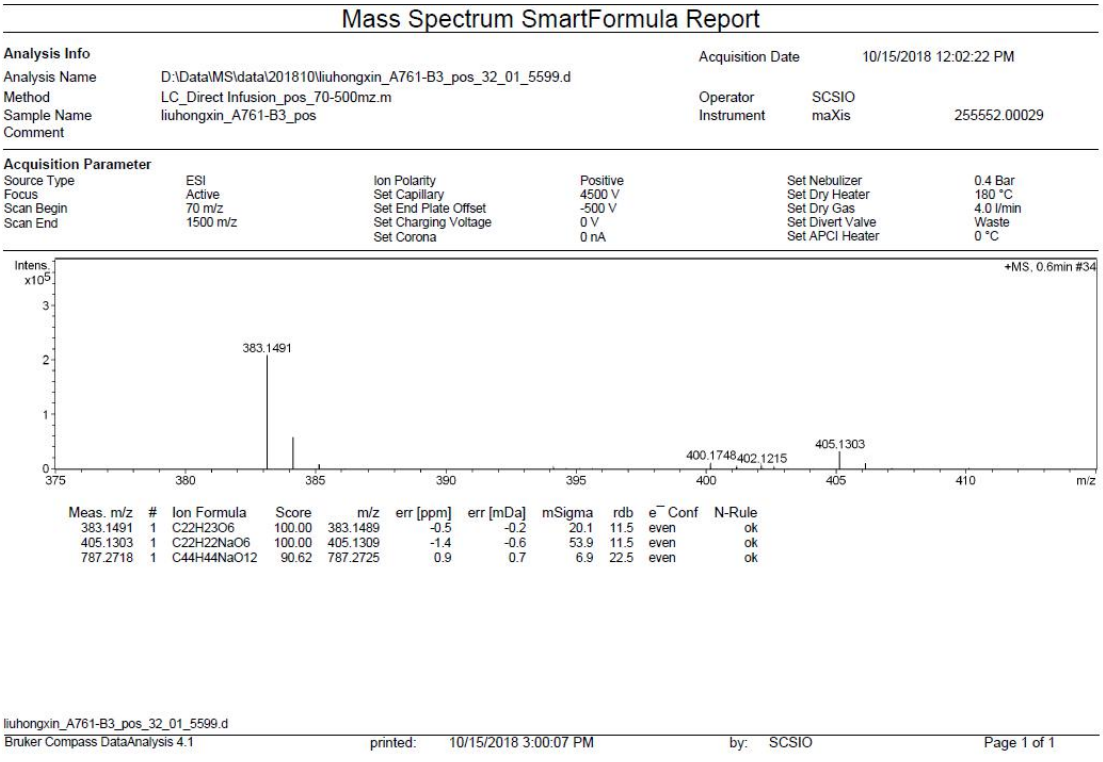
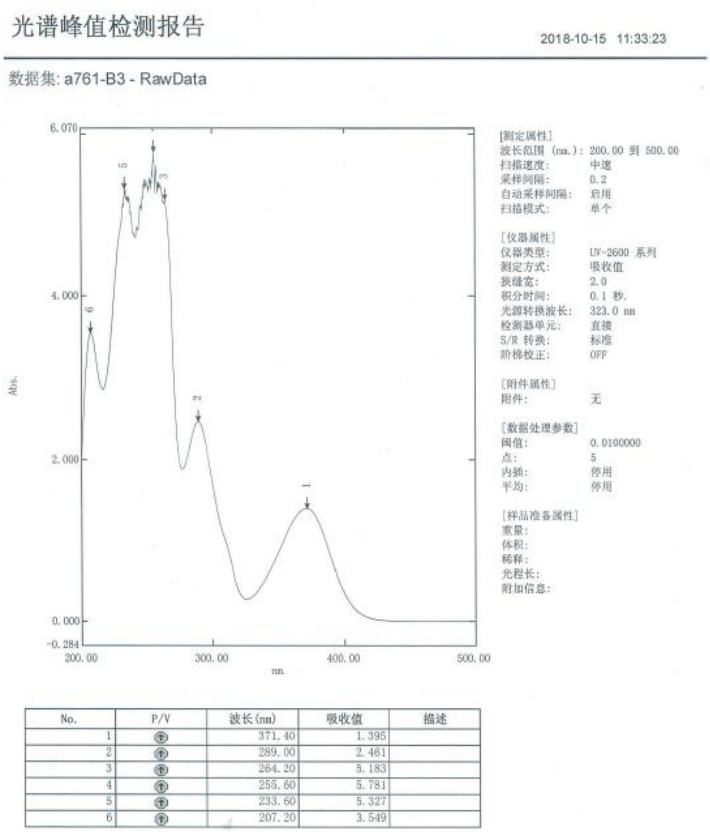


Figure S30. HRESIMS spectrum of **2**.



No.	P/V	波长(nm)	吸收值	描述
1	↑	371.40	1.395	
2	↑	289.00	2.461	
3	↑	264.20	5.183	
4	↑	255.60	5.781	
5	↑	233.60	5.327	
6	↑	207.20	3.549	

Figure S31. UV spectrum of **2**.

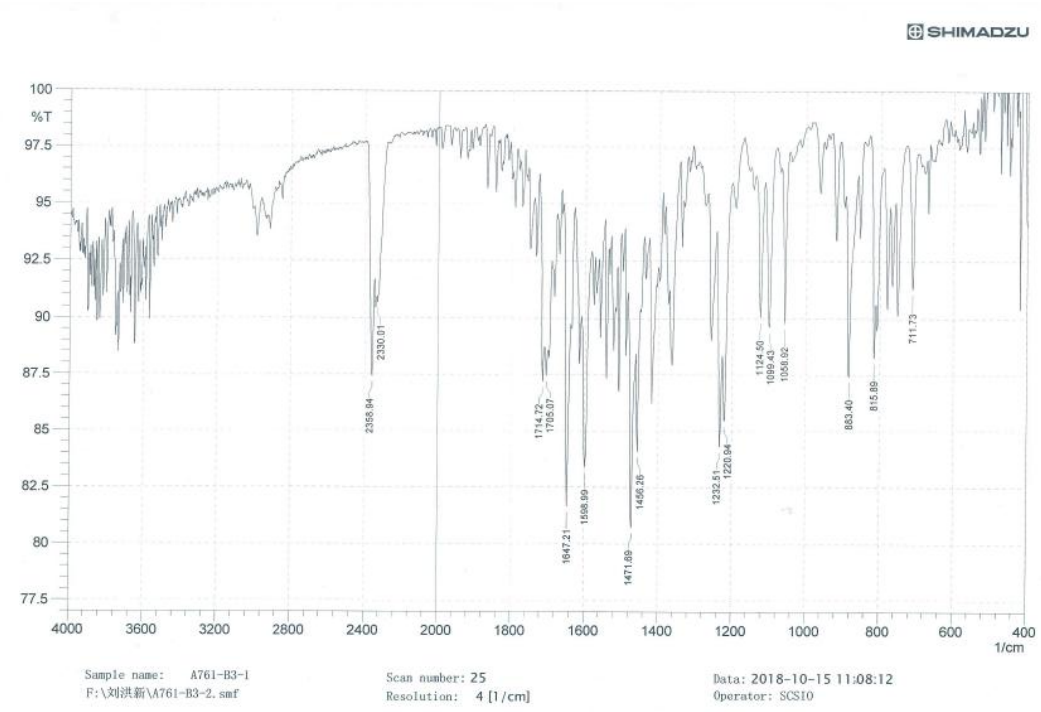


Figure S32. IR spectrum of **2**.