Supporting Information

Pyrone and isocoumarin derivatives from the endophytic fungus *Cytospora rhizophorae*

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1. Experimental Section

1.1 General experimental procedures

Melting point was measured by SGW X-4 micro melting point apparatus; Optical rotations were recorded on an Anton Paar MCP-500 spectropolarimeter (Anton Paar, Graz, Austria) at 25 ℃. UV spectra were taken on a Shimadzu UV-2600 spectrophotometer (Shimadzu, Kyoto, Japan). Circular dichroism (CD) measurements were performed on a Jasco 820 spectropolarimeter (Jasco Corporation, Kyoto, Japan). IR spectra were measured using a Shimadzu IR Affinity-1 spectrometer (Shimadzu, Kyoto, Japan). 1D and 2D NMR spectra were obtained on a Bruker Avance-500 spectrometer with TMS as an internal standard (Bruker, Fällanden, Switzerland). HRESIMS were acquired on a Thermo MAT95XP high resolution mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Silica gel (200−300 mesh, Qingdao Marine Chemical Inc., Qingdao, China), C_{18} reversed-phase silica gel (40−75 μm, Fuji Silysia Chemical Ltd., Kasugai, Japan) and Sephadex LH-20 (Amersham Biosciences, Uppsala, Sweden) were used for open column chromatography. TLC analysis was carried out on silica gel plates (Merck KGaA, Darmstadt, Germany). A Shimadzu LC-20 AT (Shimadzu, Kyoto, Japan) equipped with an SPD-M20A PDA detector (Shimadzu, Kyoto, Japan) was used for HPLC. Preparative HPLC was performed using a YMC-pack ODS-A column (250 × 20 mm, 5 μm, 12 nm, YMC CO., Ltd, Kyoto, Japan); while semi-preparative HPLC was performed utilizing a YMC-pack ODS-A/AQ column (250 × 10 mm, 5 μm, 12 nm, YMC CO., Ltd, Kyoto, Japan).

Fermentation, extraction and isolation

The strain 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 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 500 mL Erlenmeyer flasks, each containing 250 mL PDB medium. After five days of incubation at 28 °C

on a rotary shaker at 120 r/m, 25 mL seed cultures were transferred into the rice solid medium (each flask contained 300 g of rice, 380 mL of natural filtered water). The 17 flasks (3000 mL) were then kept for 30 days at 28 °C.

All fermentation products (5.0 Kg) were extensively extracted by MeOH (20 L) for three times, then, the MeOH phases were combined. The reduced pressure with temperature below 40 °C was used to remove the resulting solvent and offered a dark-brown gum (268 g).

The total of 268 g of dark-brown gum crude extract was obtained by MeOH extraction, which was further purified by silica gel (100-200 mesh) flash column chromatography (CC) and eluted with mixed solvent of *n*-hexane and EtOAc to obtain 12 fractions (Fr. A- Fr. L).

Fr. D (4.5 g) was purified by CC on RP C_{18} silica gel and eluted by MeOH/H_2O (60% → 100%) to generate 11 sub-fractions (Fr. D1-Fr. D11). Fr. D7 (96 mg) was further subjected by reversed phase semi-preparative HPLC with MeOH/H_2O (100:0, v/v, 3 mL/min) to obtain a mixture (7.8 mg, t_R = 6.7 min). Compound **1** (2.8 mg) was obtained from the mixture, which was further separated by silica gel (200-300 mesh) flash CC, eluting with $CHCl_3$ as the eluent solvent.

Fr. G (10.2 g) was then subjected to CC on RP C_{18} material with the eluent as MeOH/H_2O (50% → 100%) was eluted to further obtain 8 fractions (Fr. G1-G8). Fr. G3 was further isolated by silica gel (200-300 mesh) flash CC (*n*-hexane/EtOAc, 10:1 → 1:1, v/v) to obtain three fractions (Fr. G3-1-Fr. G3-3). Fr. G3-1 was further separated by reversed phase semi-preparative HPLC (MeOH/H_2O, 2:1, v/v, 3 mL/min) and provided compound **4** (3.0 mg, t_R = 22.6 min).

Fr. H (20.8 g) was subjected to RP C_{18} CC by eluting with MeOH/H_2O (50% → 100%) to further obtain six sub-fractions (Fr. H1-H6). Fr. H1 was then purified by silica gel (200-300 mesh) flash CC (CH_2Cl_2/MeOH, 100:1, 50:1, 20:1, 10:1, 5:1, 0:1, v/v) to obtain nine sub-fractions (Fr. H1-1-Fr. H1-9). Fr. H1-6 (1.4 g) was further isolated by silica gel (200-300 mesh) flash CC (*n*-hexane/EtOAc, 2:1, 1:1, 1:2, 1:5, 0:1, v/v) to give three sub-fractions (Fr. H1-6-1-Fr. H1-6-3). Fr. H1-6-3 (237 mg) was further separated by reversed phase semi-preparative HPLC (MeOH/H_2O, 45:55, v/v,

3 mL/min) to achieve compound **2** (5.0 mg, t_R = 20.0 min) and **3** (10.0 mg, t_R = 22.6 min).

Physical and chemical data

Cytospone A (**1**): yellowish crystal; m.p. 240-241 °C; $[\alpha]^{20}_D$ = +12.6 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 210 (5.28), 262 (4.94), 293 (4.81), 374 (5.28) nm; IR ν_{max} 2938, 1682, 1636, 1559, 1456, 1408, 1246, 1128, 1107, 1026, 997, 939, 812, 758 cm^{-1}. ^1H and ^{13}C NMR, see Table 1; HRESIMS: *m*/*z* 435.1409 $[M + Na]^+$ (calcd for $C_{23}H_{24}NaO_7$, 435.1414).

Cytospone B (**2**): colorless oil; UV (MeOH) λ_{max} (log ε) 210 (5.05), 286 (4.76) nm; IR ν_{max} 2938, 1682, 1636, 1559, 1456, 1408, 1246, 1128, 1107, 1026, 997, 939, 812, 758 cm^{-1}. ^1H and ^{13}C NMR, see Table 1; HRESIMS: *m*/*z* 241.1075 $[M + Na]^+$ (calcd for $C_{12}H_{17}O_5$, 241.1071).

Cytospone C (**3**): colorless oil; UV (MeOH) λ_{max} (log ε) 205 (4.24), 286 (4.81) nm; IR ν_{max} 3366, 1695, 1645, 1636, 1560, 1456, 1408, 1248, 1128, 1069, 999, 932, 812, 752, 669 cm^{-1}. ^1H and ^{13}C NMR, see Table 1; HRESIMS: *m*/*z* 227.1281 $[M + H]^+$ (calcd for $C_{12}H_{19}O_4$, 227.1278).

Cytospone D (**4**): yellow oil; $[\alpha]^{20}_D$ = +7.6 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 212 (5.18), 272 (4.82), 315 (4.63) nm; IR ν_{max} 3306, 2926, 2855, 1717, 1647, 1616, 1395, 1256, 1182, 1152, 1070, 1034, 847, 800, 725, 683, 669, 642 cm^{-1}. ^1H and ^{13}C NMR, see Table 1; HRESIMS: *m*/*z* 281.1019 $[M + H]^+$ (calcd for $C_{14}H_{17}O_6$, 281.1020).

Anti-inflammatory activity assay

The natural products **1**-**4** were evaluated for the anti-inflammatory activity through the inhibition of NO production in lipopolysaccharide induced RAW 246.7 mouse macrophages. Assays were performed by the Griess method and the detail procedure were reported by our group.[17]

In vitro Cytotoxicity assay

The anti-cancer activities of **1**-**4** were screened towards three human cancer cell lines (SF-268, MCF-7, and HepG-2) using adriamycin as positive control. All the

assays were performed in triplicate with use of SRB method.

Antibacterial assay

The antibacterial activity evaluation was performed in triplicate based on the standard microdilution method by using vancomycin as the positive control. The tested strains were *S. aureus* (CMCC 26003) and *E. coli* (ATCC 8739), which had been purchased from Guangdong Microbial Culture Collection Center (GDMCC) (Guangzhou, China).

1.2 X-ray crytallographic data of compound 1.

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 **1** reported in this paper have been deposited in the Cambridge Crystallographic Data Centre. (Deposition number: CCDC 2094230 for **1**). 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 compound 1.

Identification code	zhangyanjiang_ZYJ-A761-44_collect
Empirical formula	$C_{23}H_{24}O_7$
Formula weight	412.42
Temperature/K	106.6(8)
Crystal system	monoclinic
Space group	$P2_1/c$
a/Å	12.2709(5)
b/Å	15.7729(5)
c/Å	10.1394(3)
α/°	90
β/°	96.603(3)
γ/°	90
Volume/Å^3	1949.44(12)

Z	4
ρ_{calc}g/cm^3	1.405
μ/mm^{-1}	0.864
F(000)	872.0
Crystal size/mm^3	0.25 × 0.04 × 0.03
Radiation	CuKα (λ = 1.54184)
2Θ range for data collection/°	7.252 to 141.816
Index ranges	-14 ≤ h ≤ 14, -19 ≤ k ≤ 18, -6 ≤ l ≤ 11
Reflections collected	10730
Independent reflections	3628 [R_{int} = 0.0734, R_{sigma} = 0.0925]
Data/restraints/parameters	3628/0/277
Goodness-of-fit on F^2	0.983
Final R indexes [I>=2σ (I)]	R_1 = 0.0529, wR_2 = 0.1169
Final R indexes [all data]	R_1 = 0.1025, wR_2 = 0.1377
Largest diff. peak/hole / e Å^{-3}	0.27/-0.22

2. NMR, HRESIMS, CD, UV and IR spectra of compounds 1-4

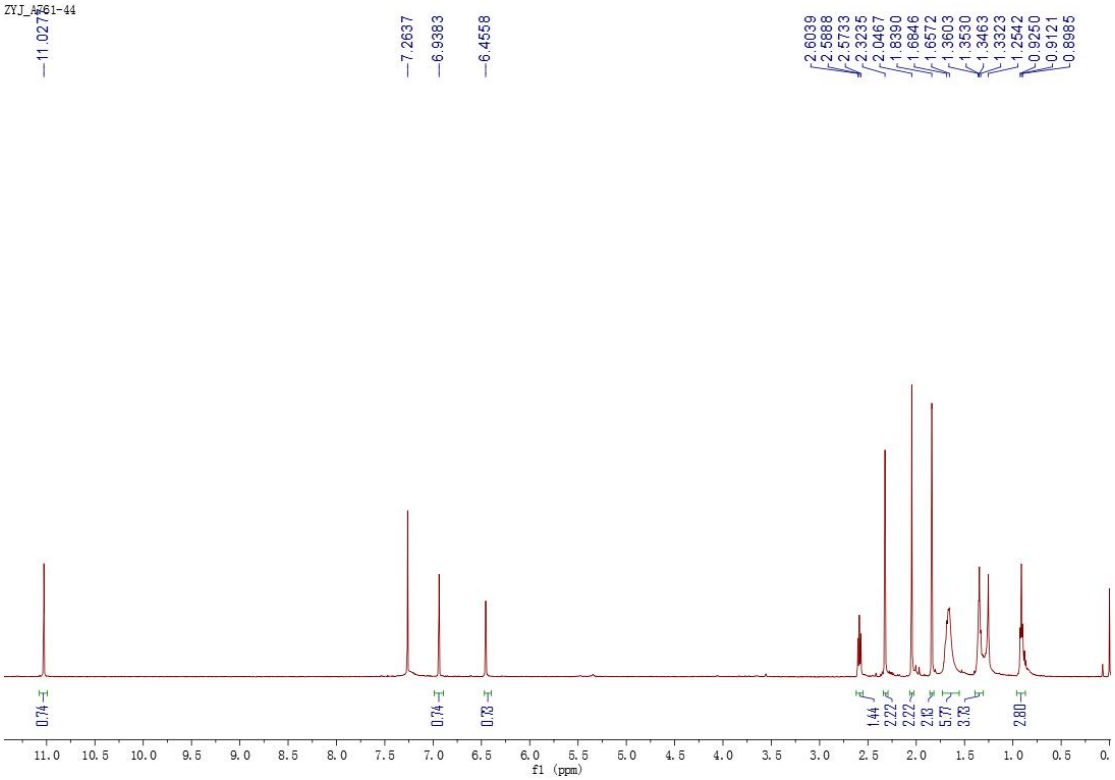


Figure S1. ^{1}H NMR spectrum (500 MHz, $CDCl_3$) of compound **1**

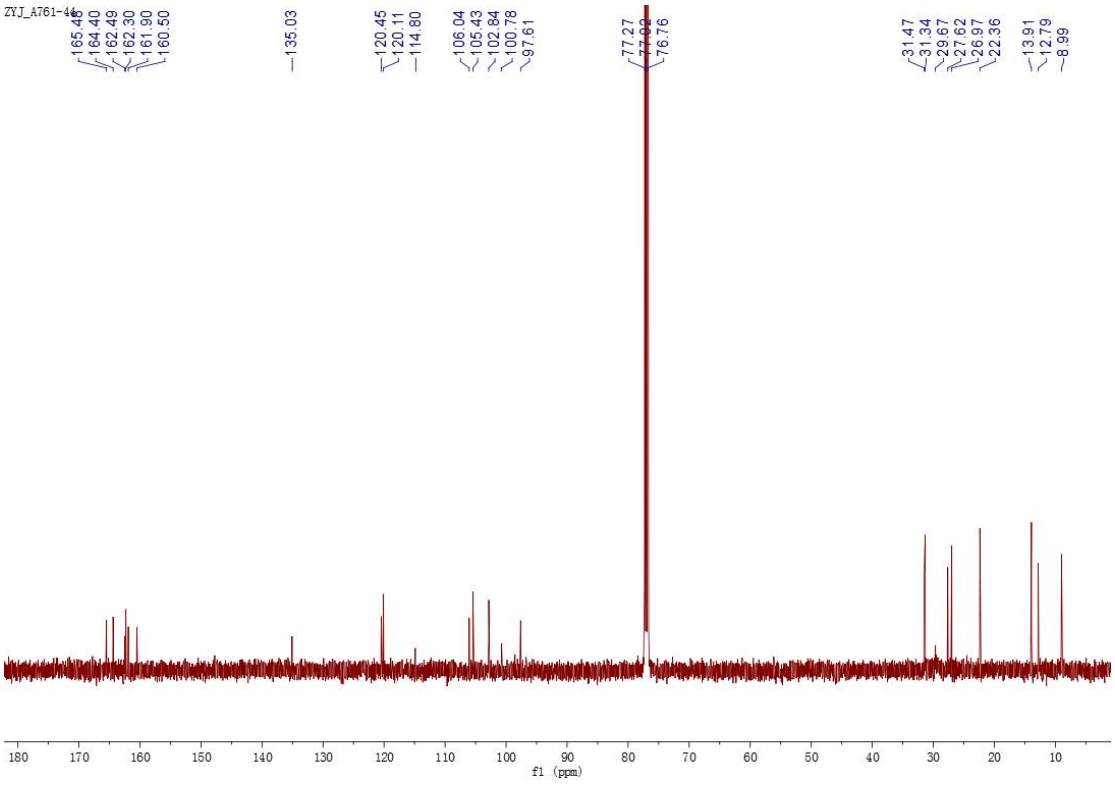


Figure S2. ^{13}C NMR spectrum (125 MHz, $CDCl_3$) of compound **1**.

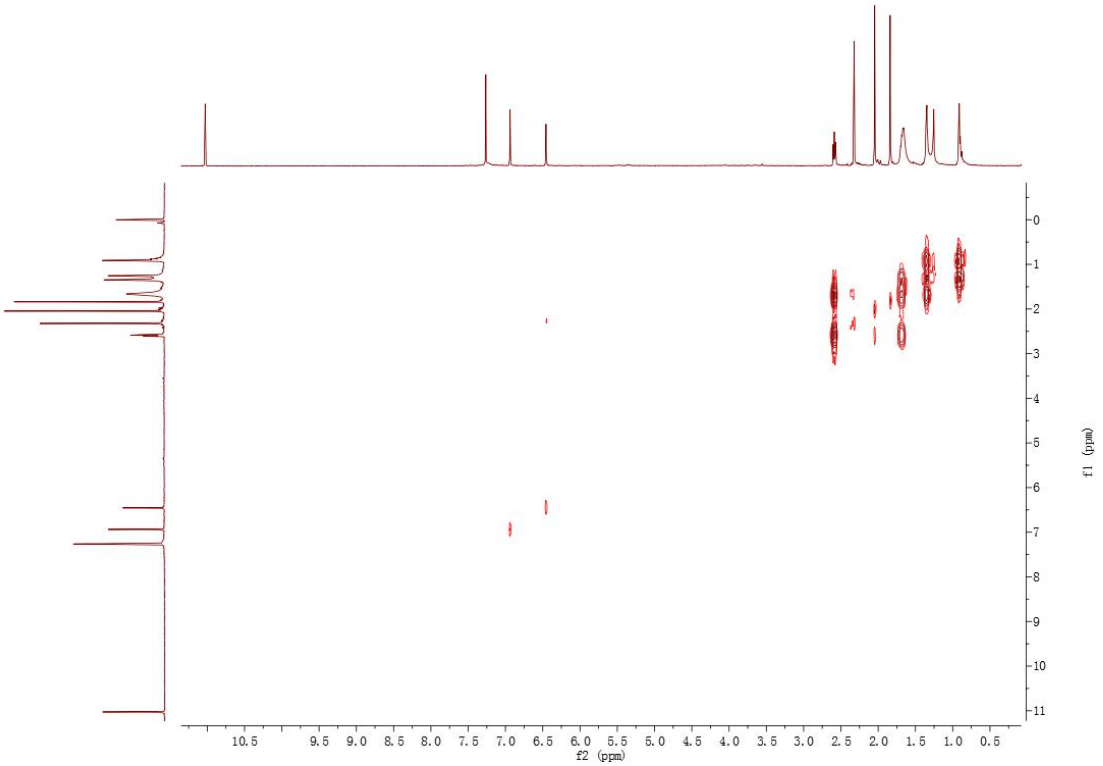


Figure S3. ^{1}H-^{1}H COSY spectrum (500 MHz, $CDCl_3$) of compound **1**.

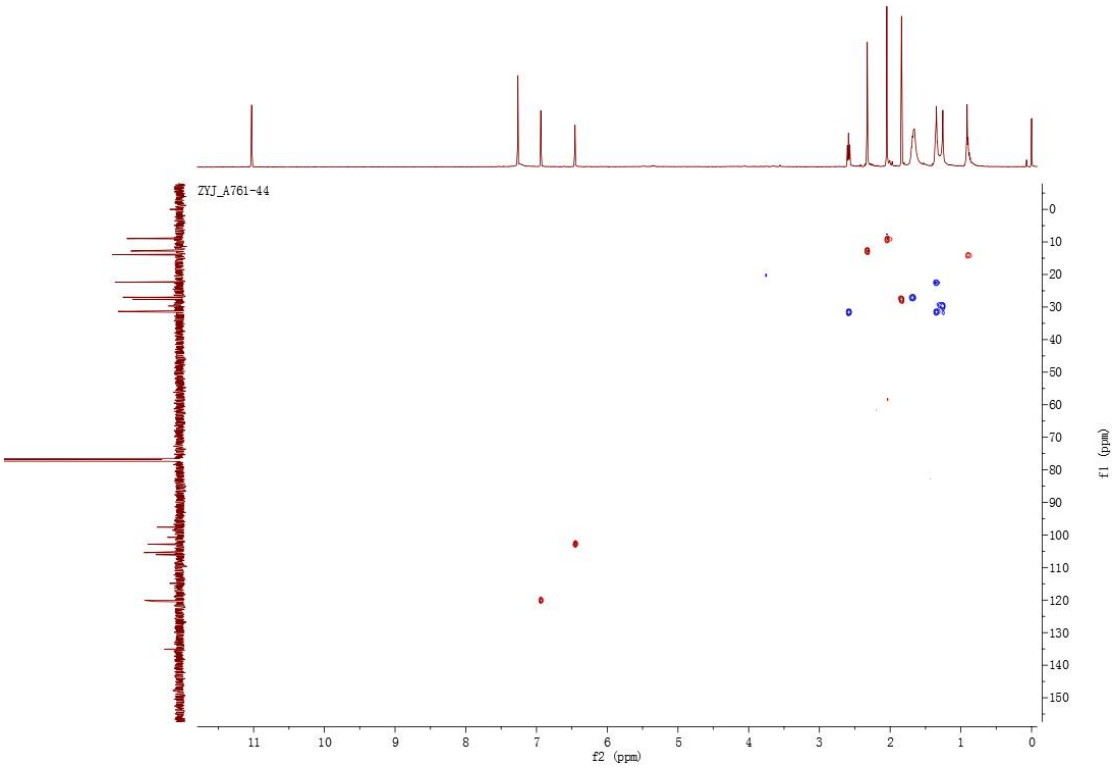


Figure S4. HSQC spectrum of compound **1**.

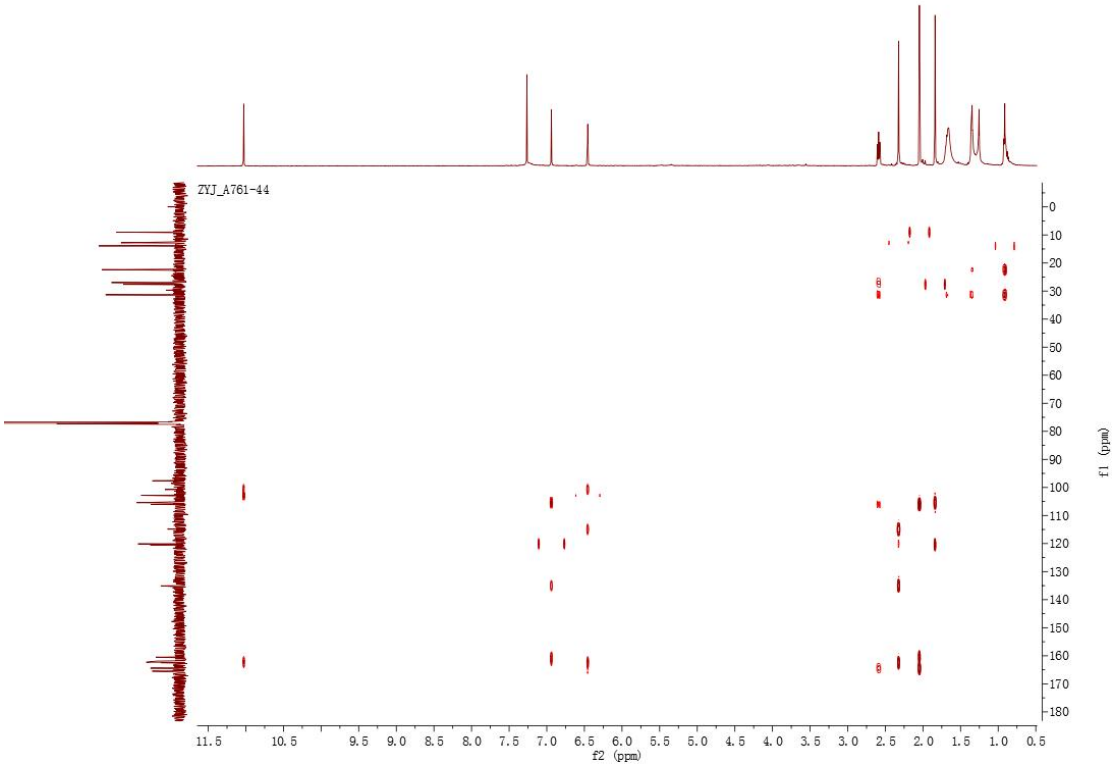


Figure S5. HMBC spectrum of compound **1**.

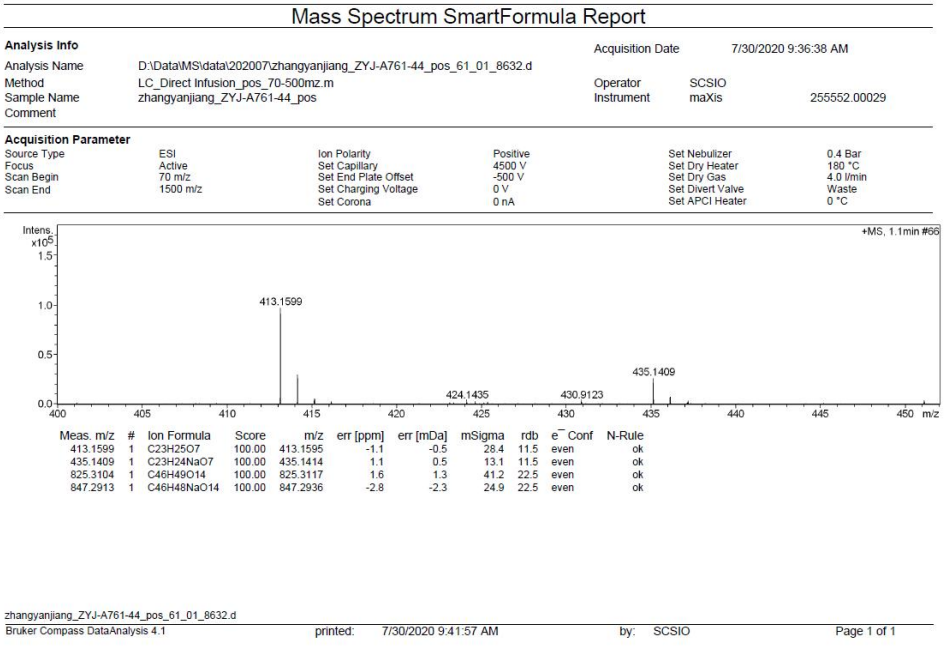


Figure S6. HRESIMS spectrum of compound **1**.

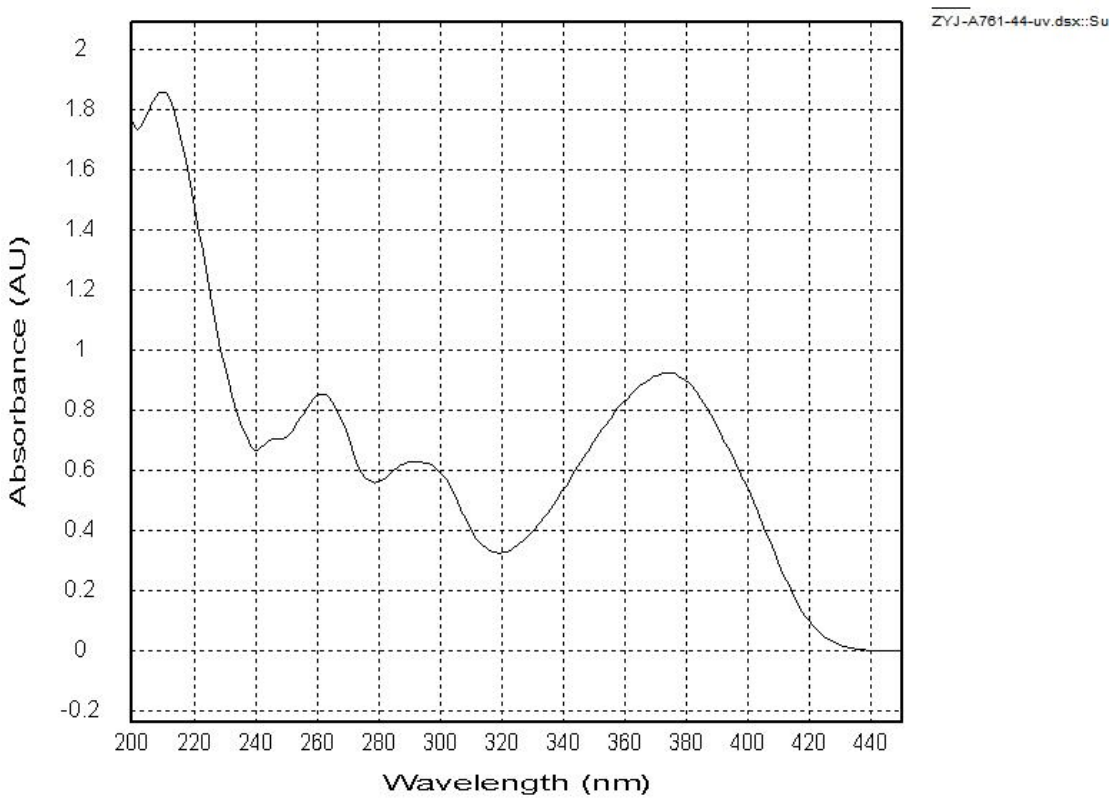


Figure S7. UV spectrum of compound **1**.

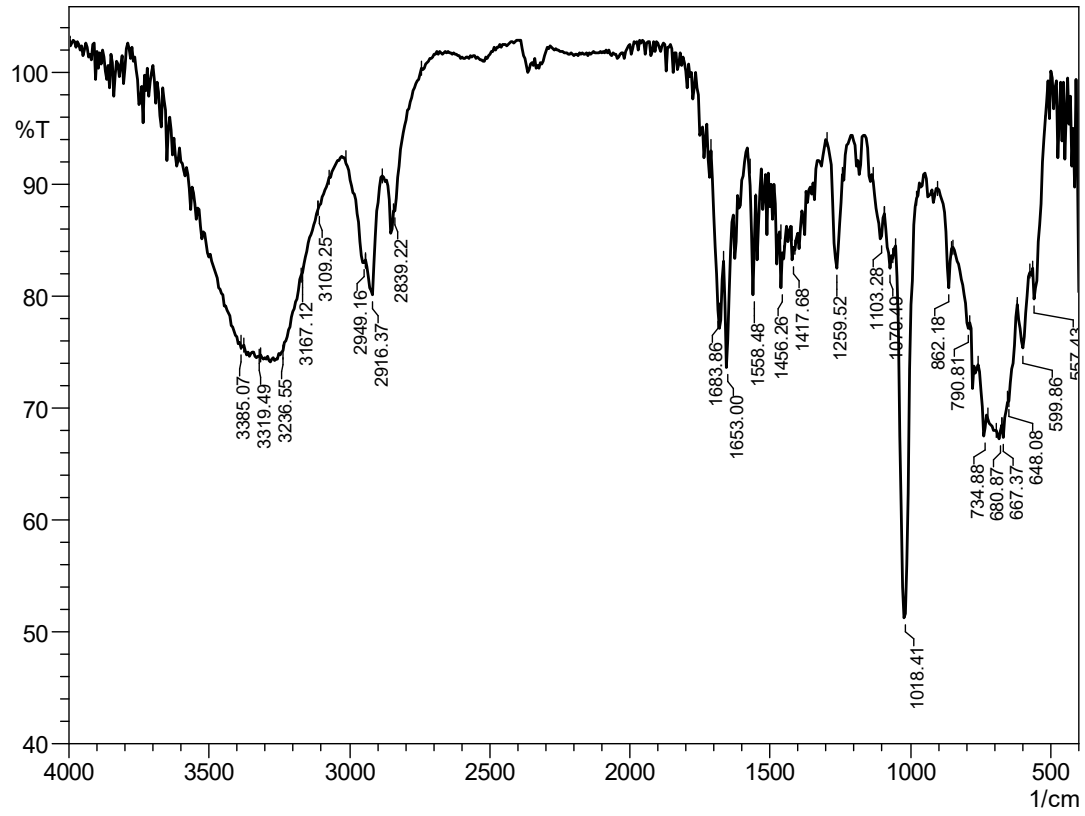


Figure S8. IR spectrum of compound **1**.

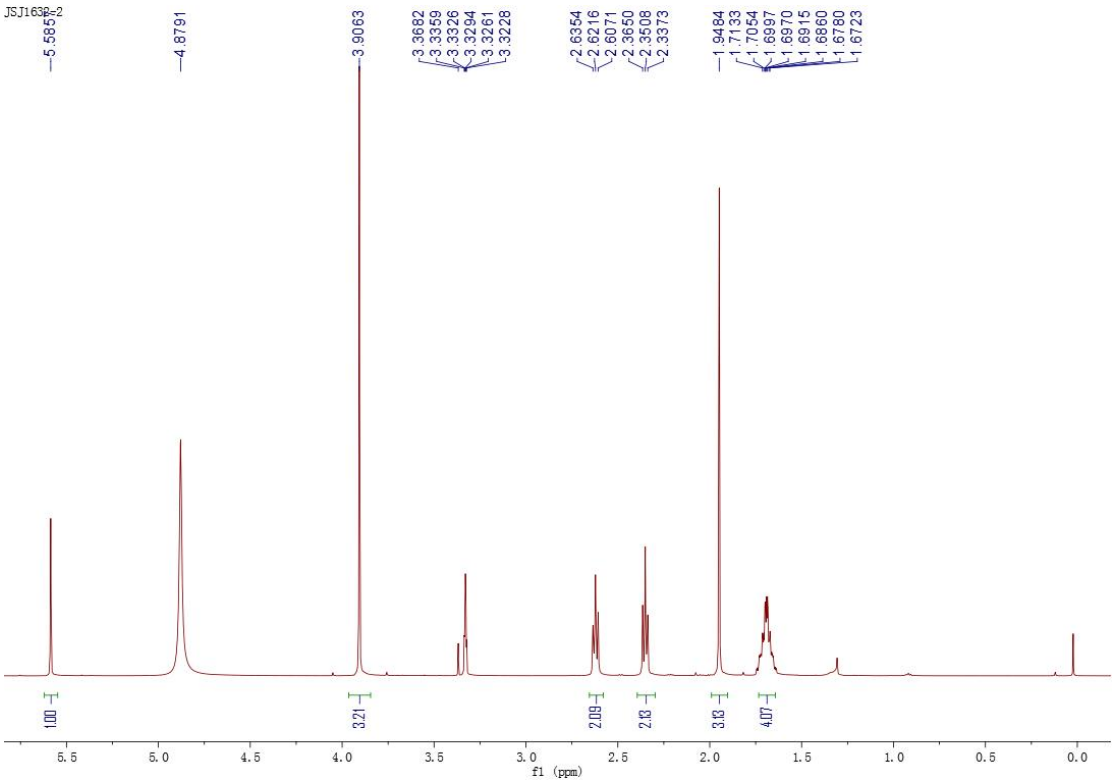


Figure S9. ^{1}H NMR spectrum (500 MHz, CD_3OD) of compound **2**.

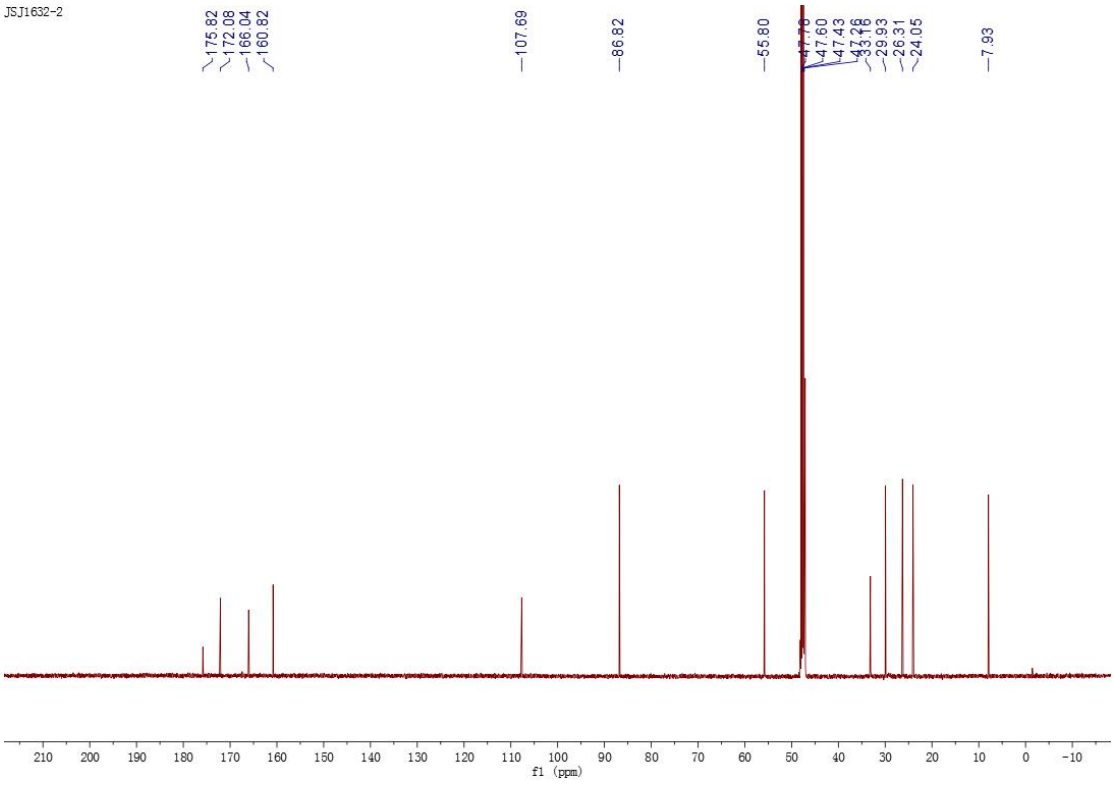


Figure S10. ^{13}C NMR spectrum (125 MHz, CD_3OD) of compound **2**.

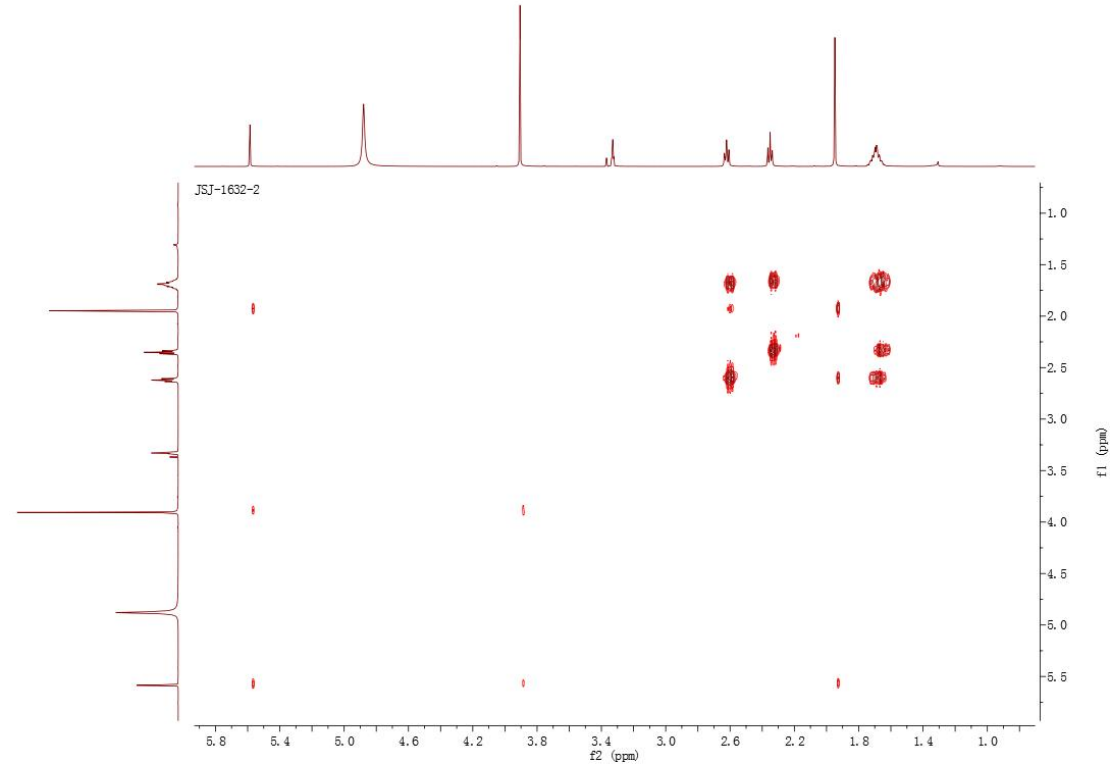


Figure S11. ^{1}H-^{1}H COSY spectrum (500 MHz, CD_3OD) of compound **2**.

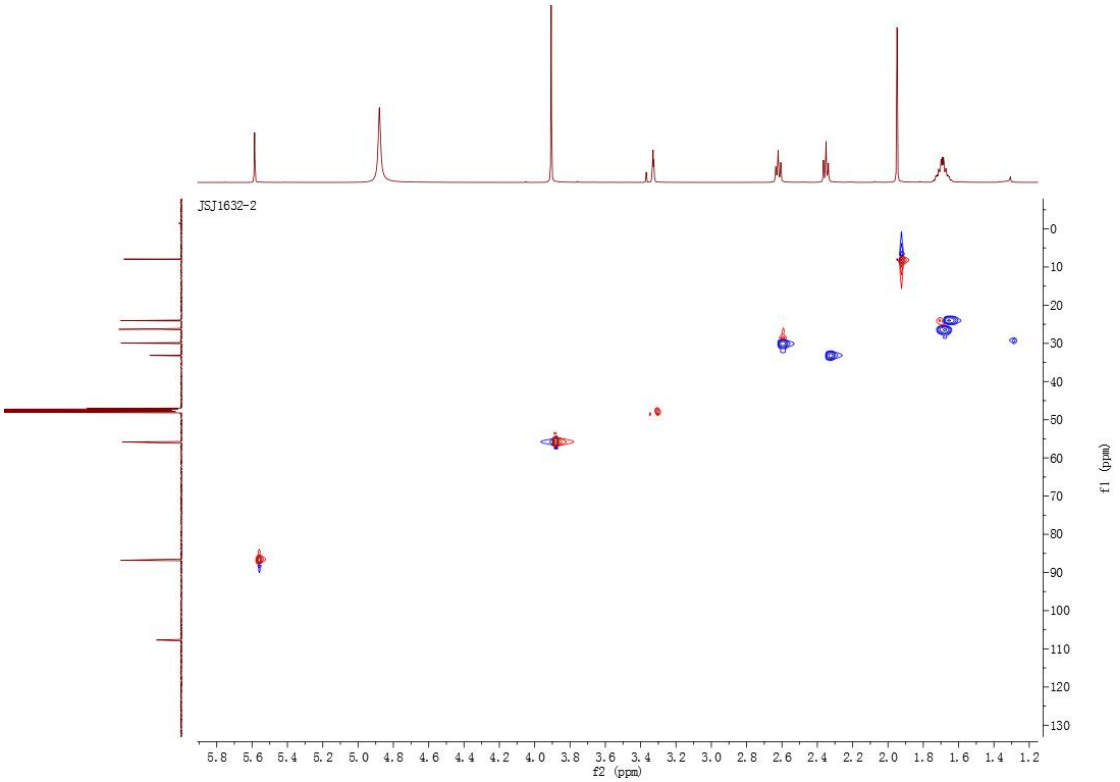


Figure S12. HSQC spectrum of compound **2**.

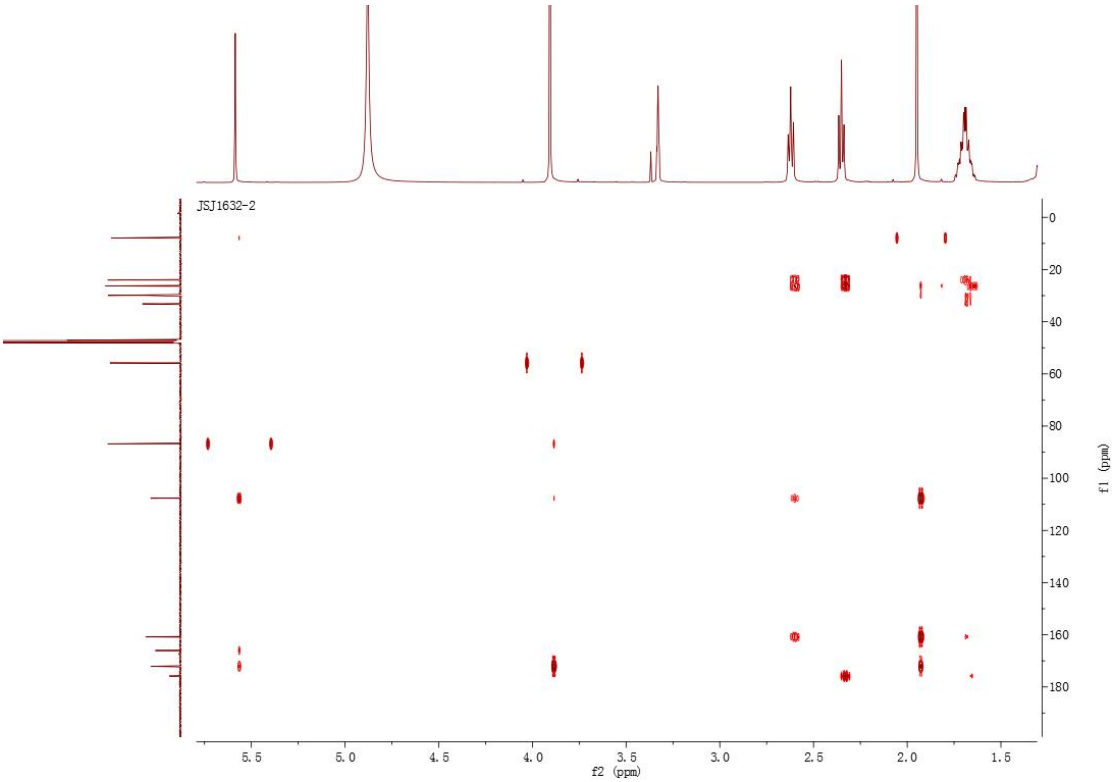


Figure S13. HMBC spectrum of compound **2**.

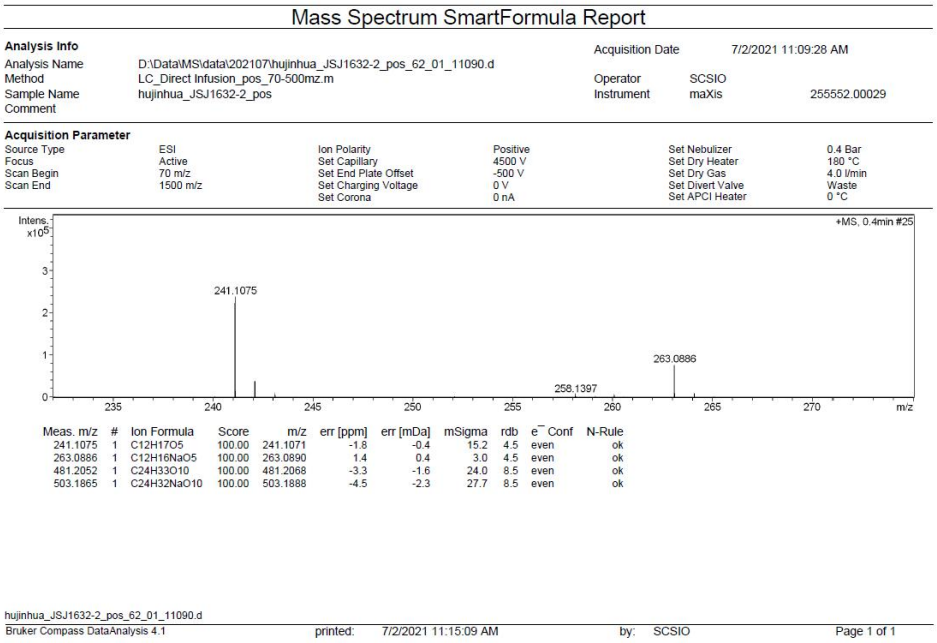


Figure S14. HRESIMS spectrum of compound **2**.

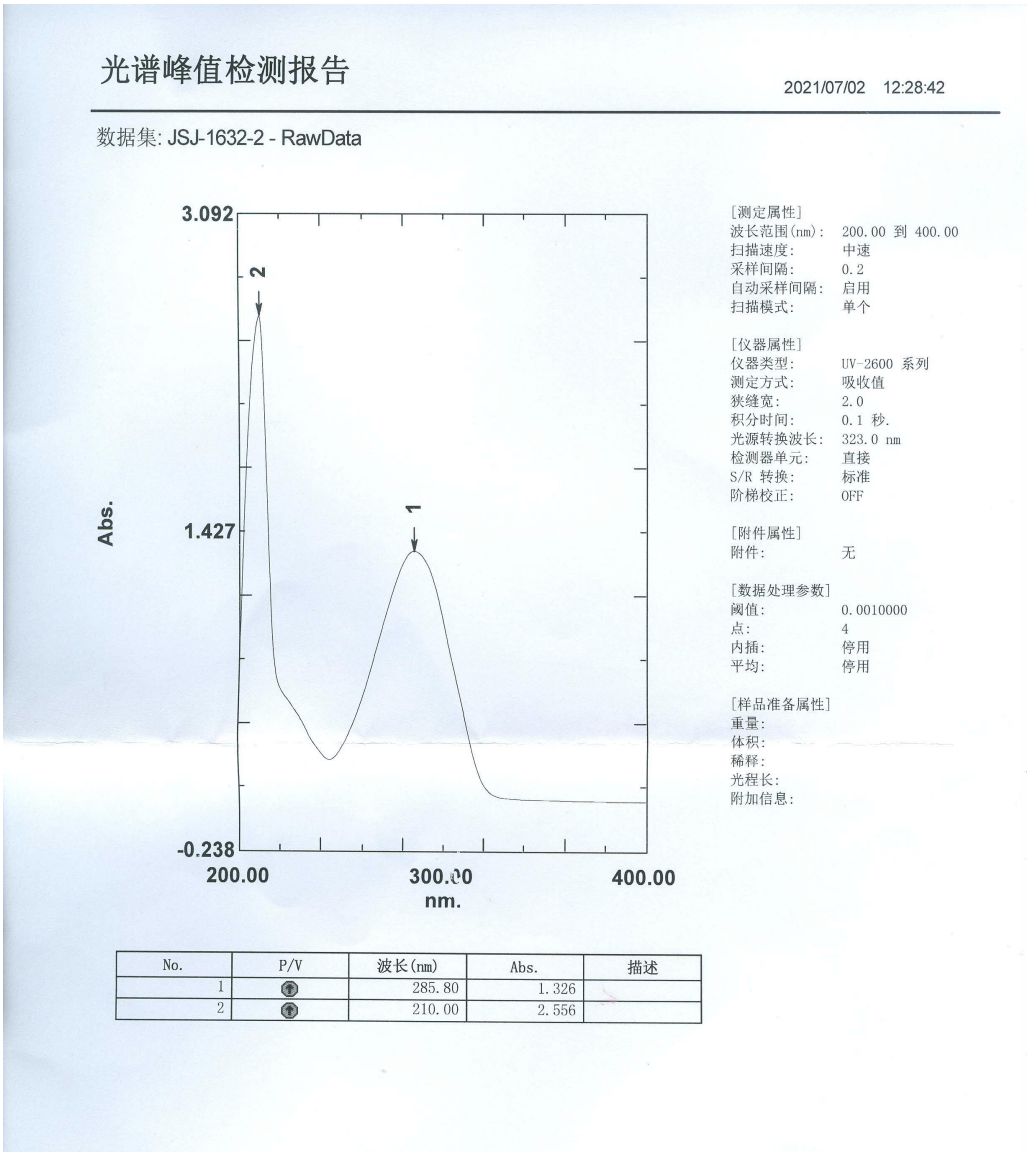


Figure S15. UV spectrum of compound **2**.

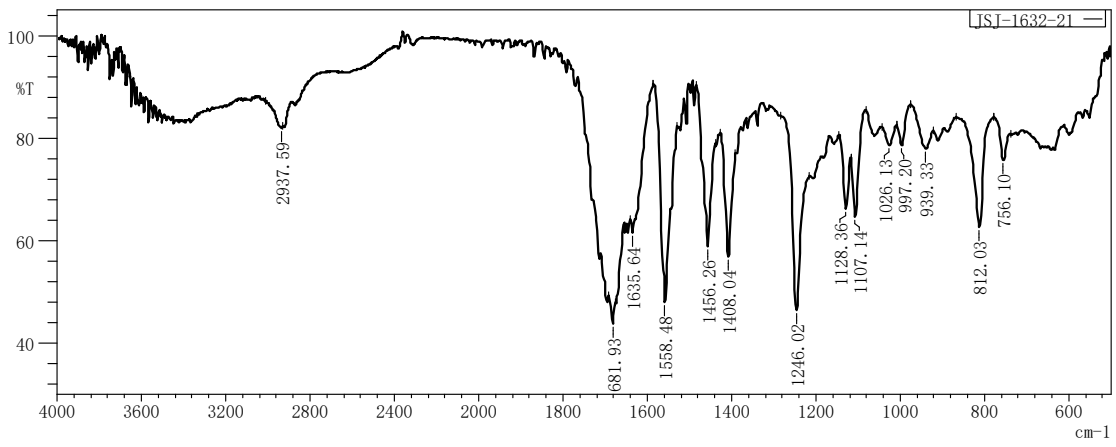


Figure S16. IR spectrum of compound **2**.

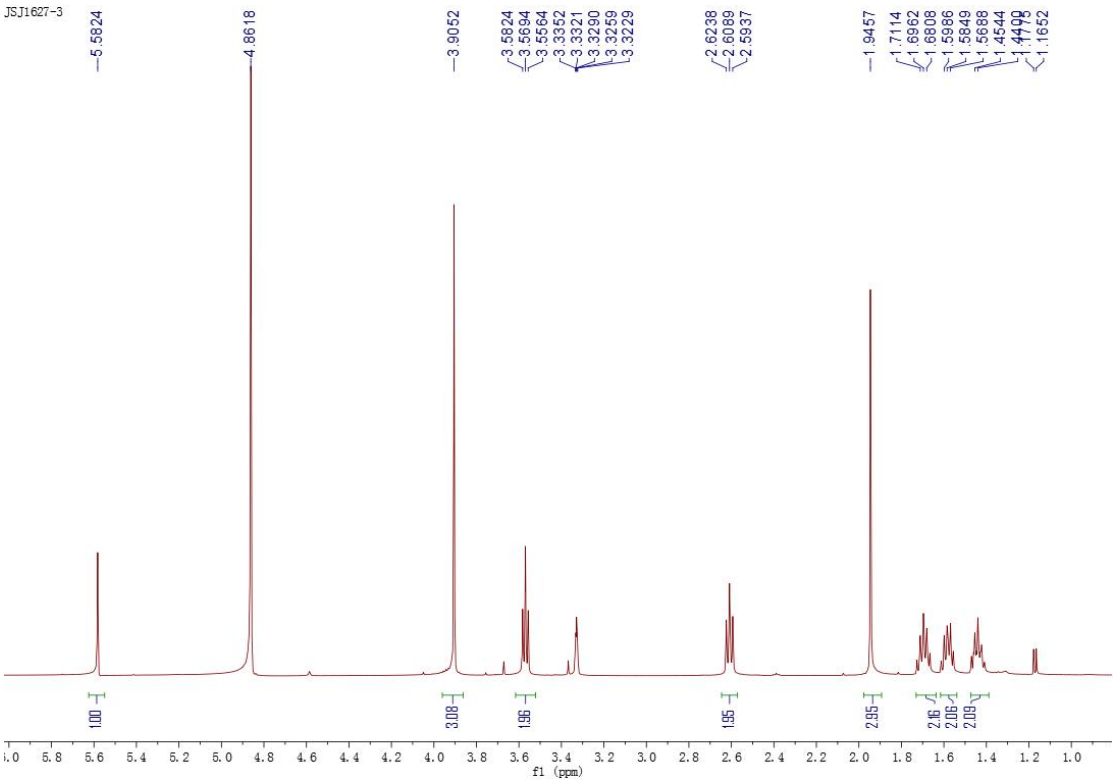


Figure S17. ^{1}H NMR spectrum (500 MHz, CD_3OD) of compound **3**.

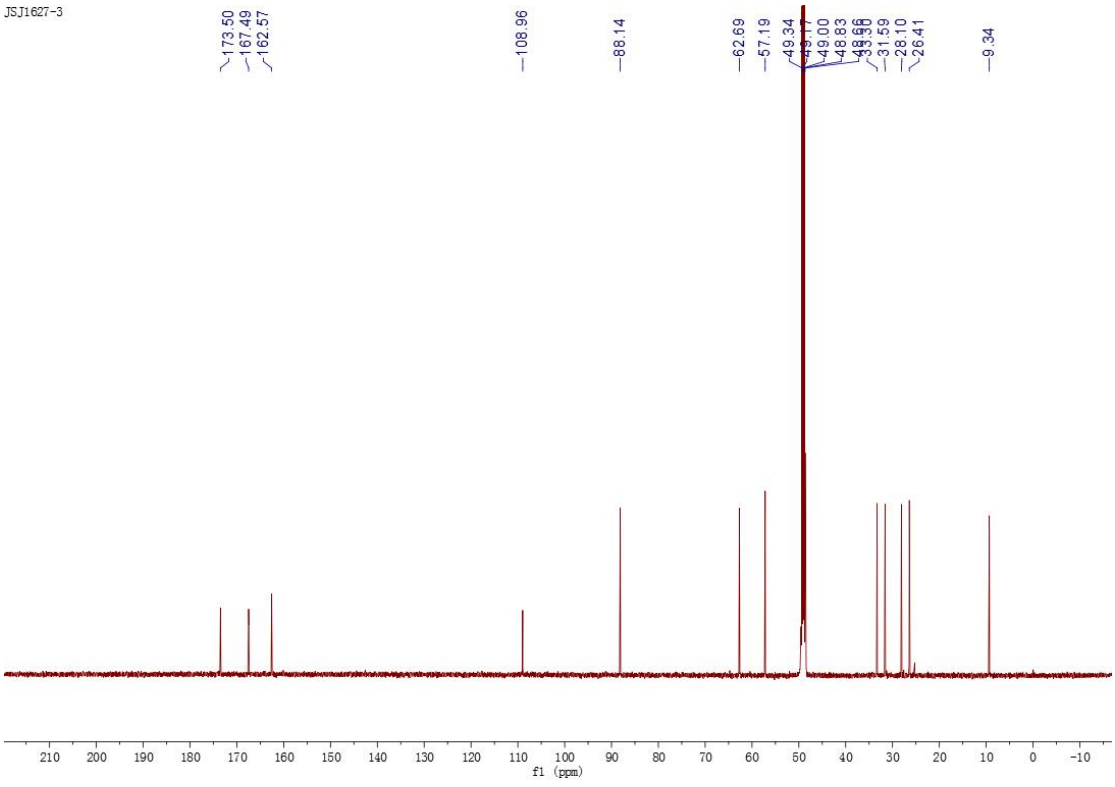


Figure S18. ^{13}C NMR spectrum (125 MHz, CD_3OD) of compound **3**.

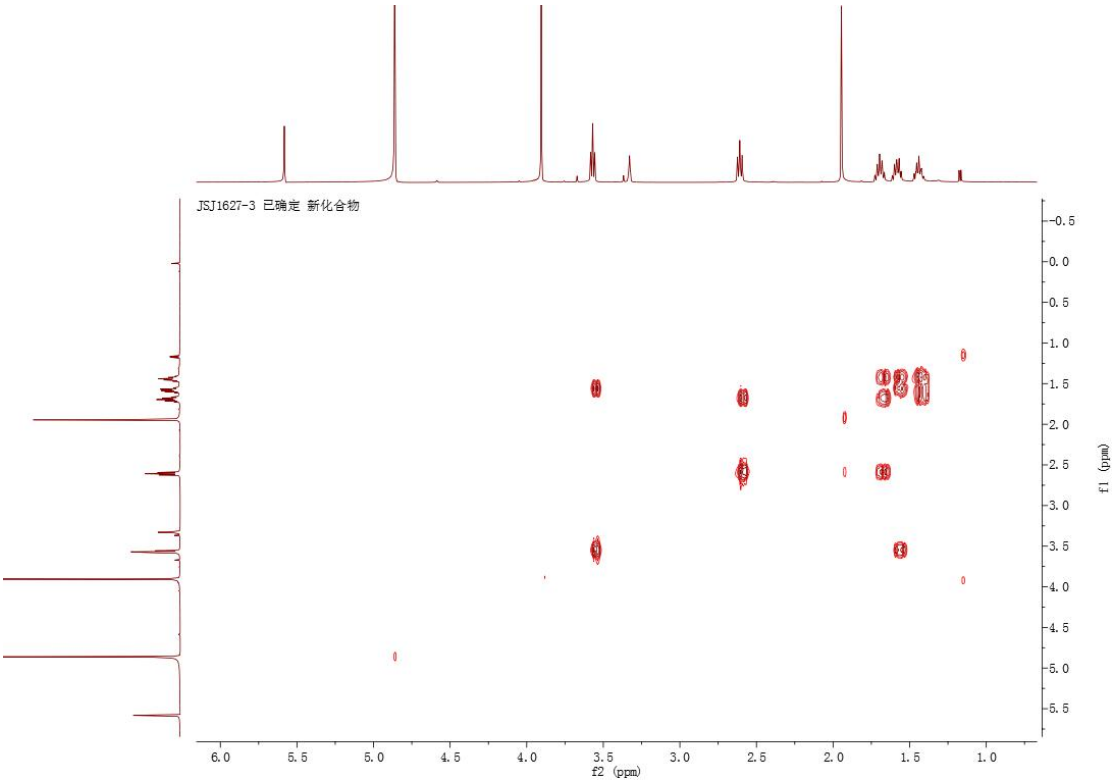


Figure S19. ^{1}H-^{1}H COSY spectrum (500 MHz, CD_3OD) of compound **3**.

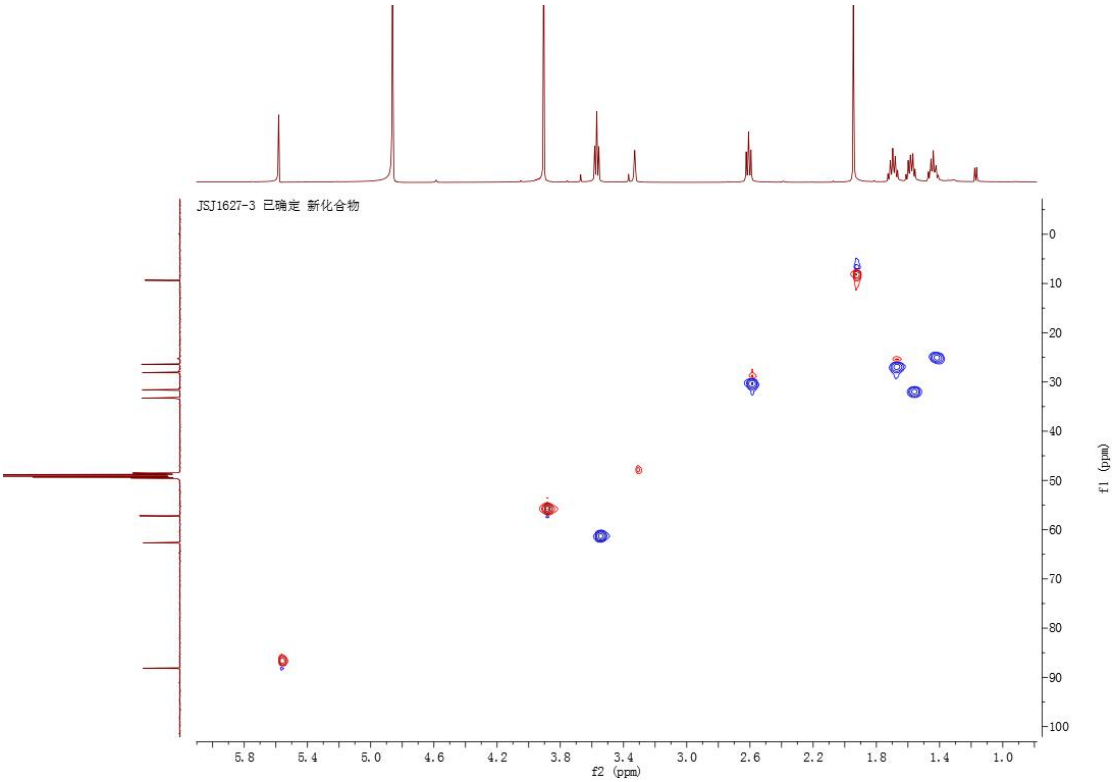


Figure S20. HSQC spectrum of compound **3**.

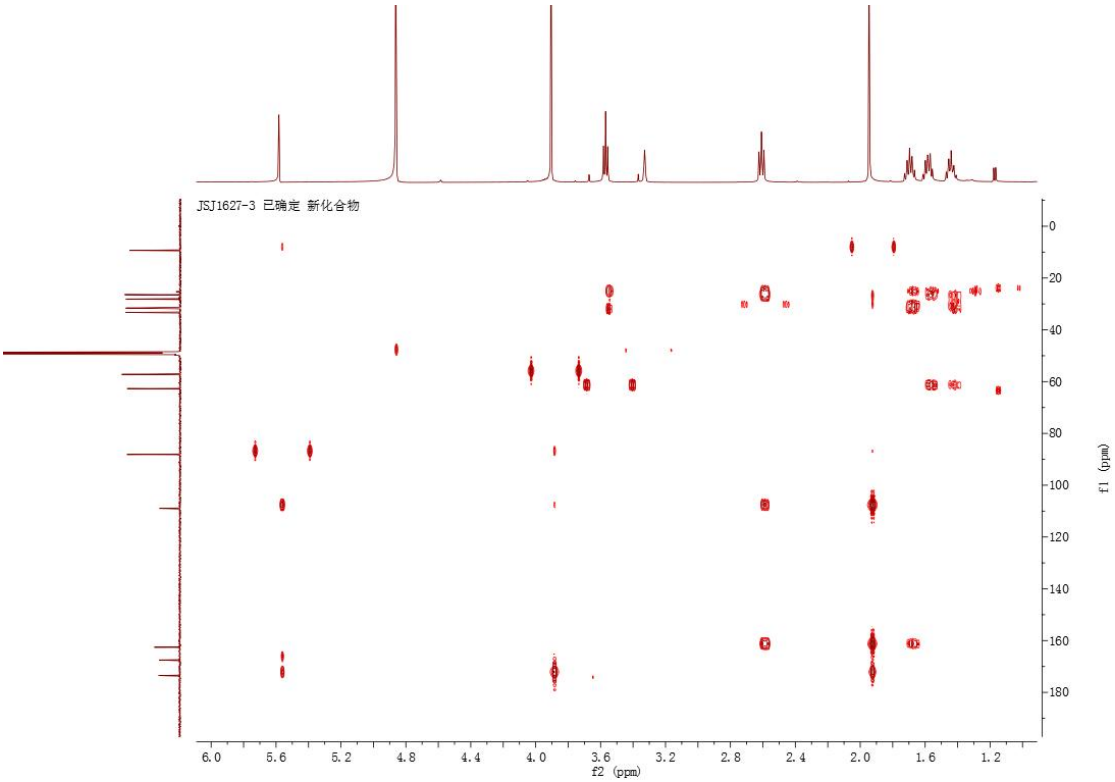


Figure S21. HMBC spectrum of compound **3**.

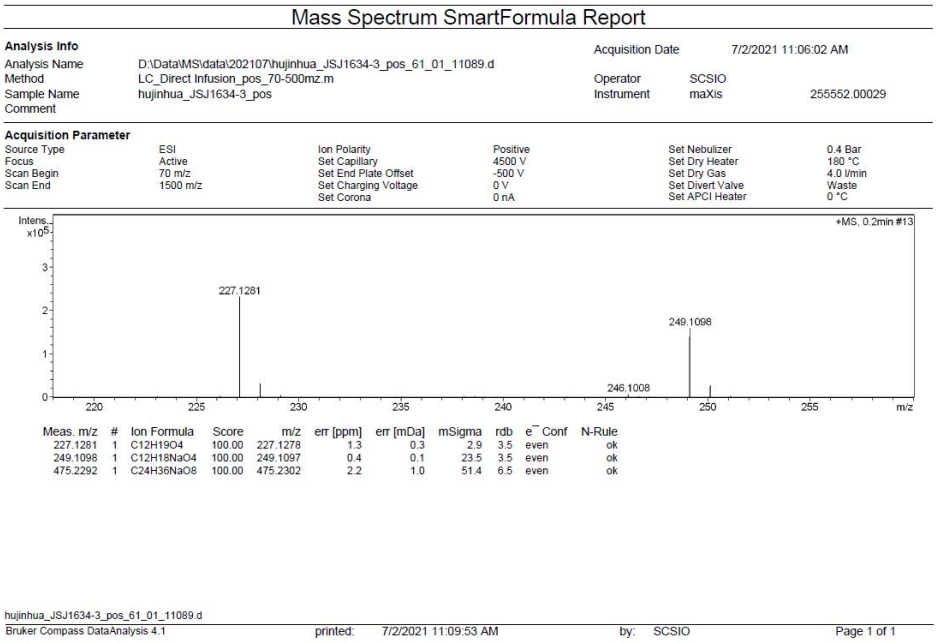
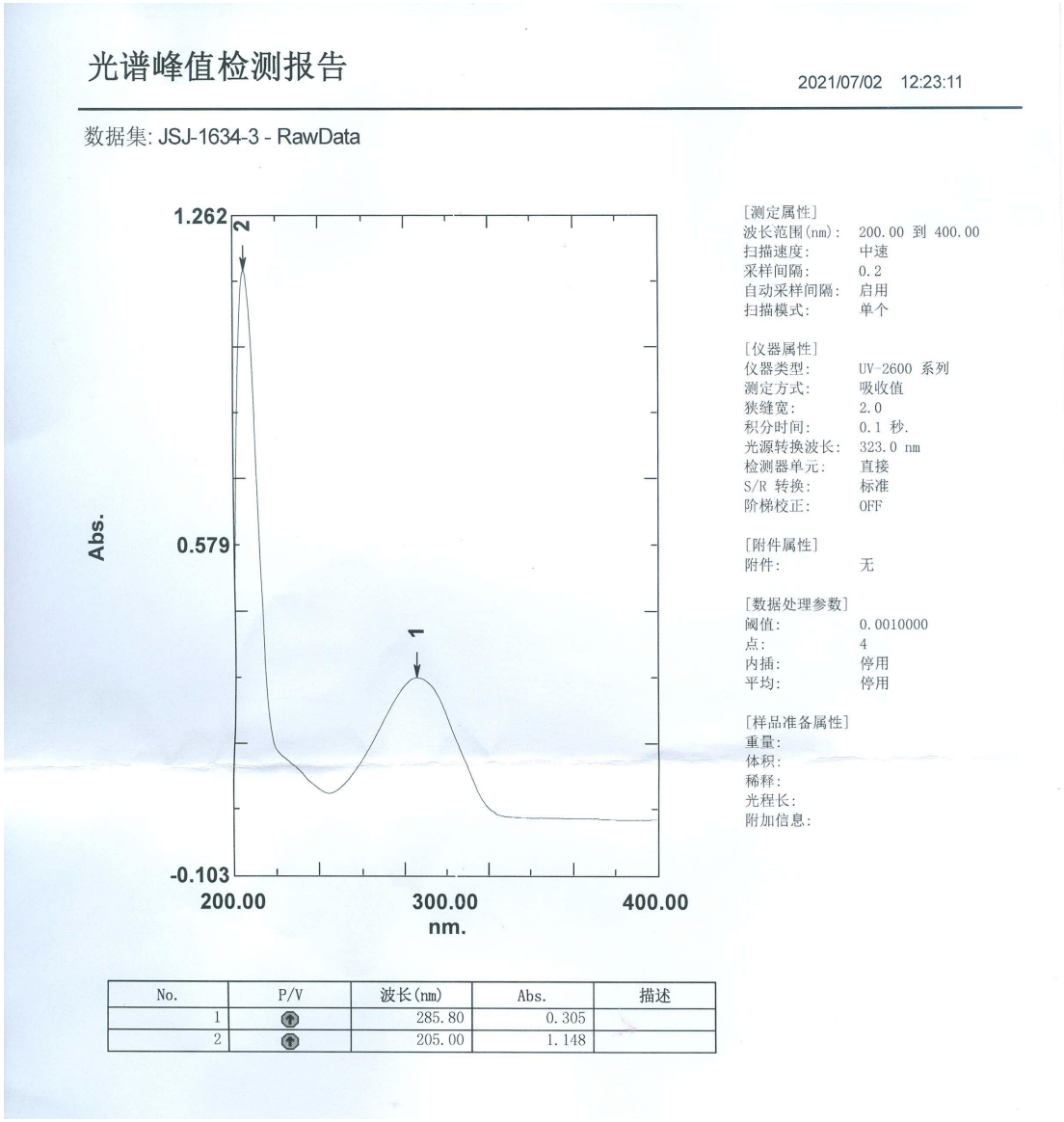


Figure S22. HRESIMS spectrum of compound **3**.



No.	P/V	波长(nm)	Abs.	描述
1	↑	285.80	0.305	
2	↑	205.00	1.148	

Figure S23. UV spectrum of compound **3**.

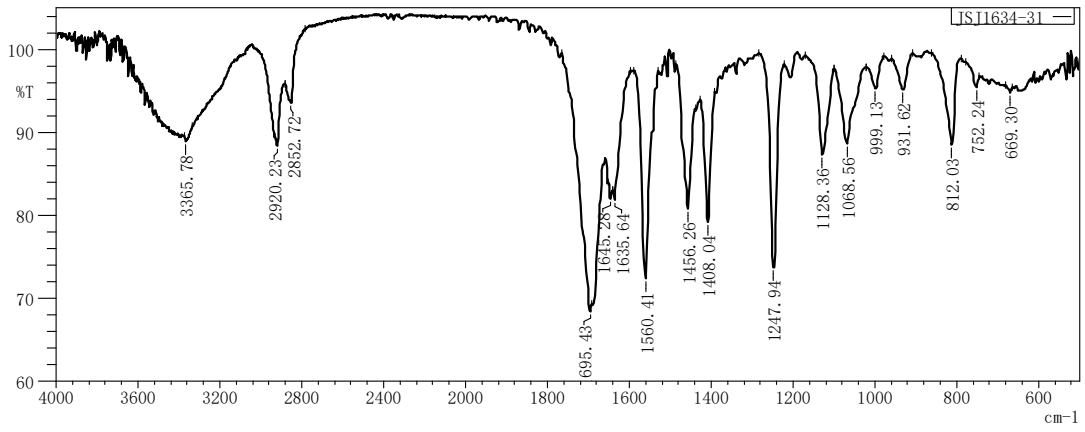


Figure S24. IR spectrum of compound **3**.

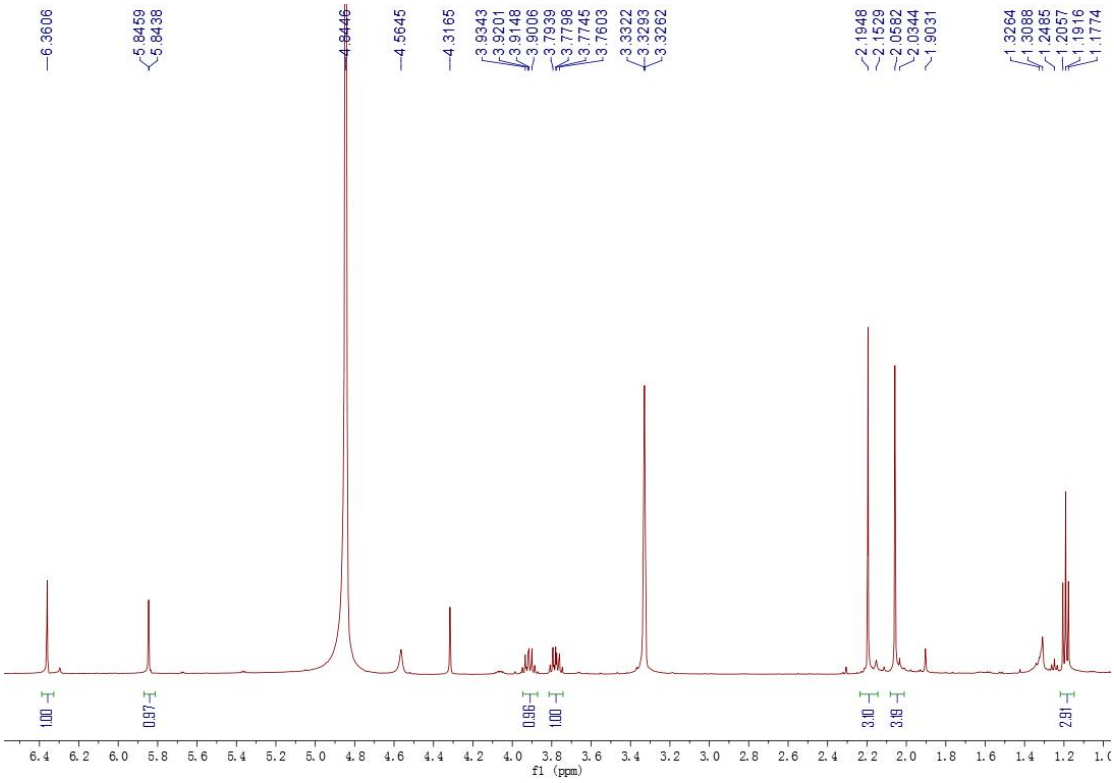


Figure S25. ^{1}H NMR spectrum (500 MHz, CD_3OD) of compound **4**.

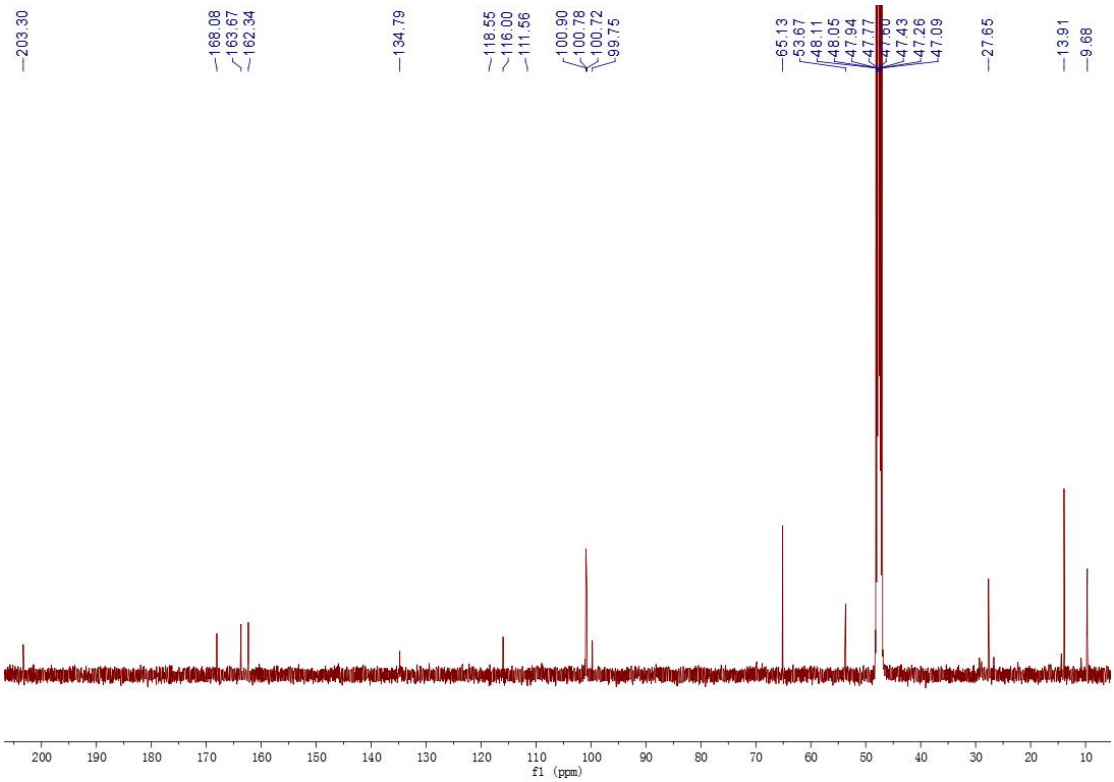


Figure S26. ^{13}C NMR spectrum (125 MHz, CD_3OD) of compound **4**.

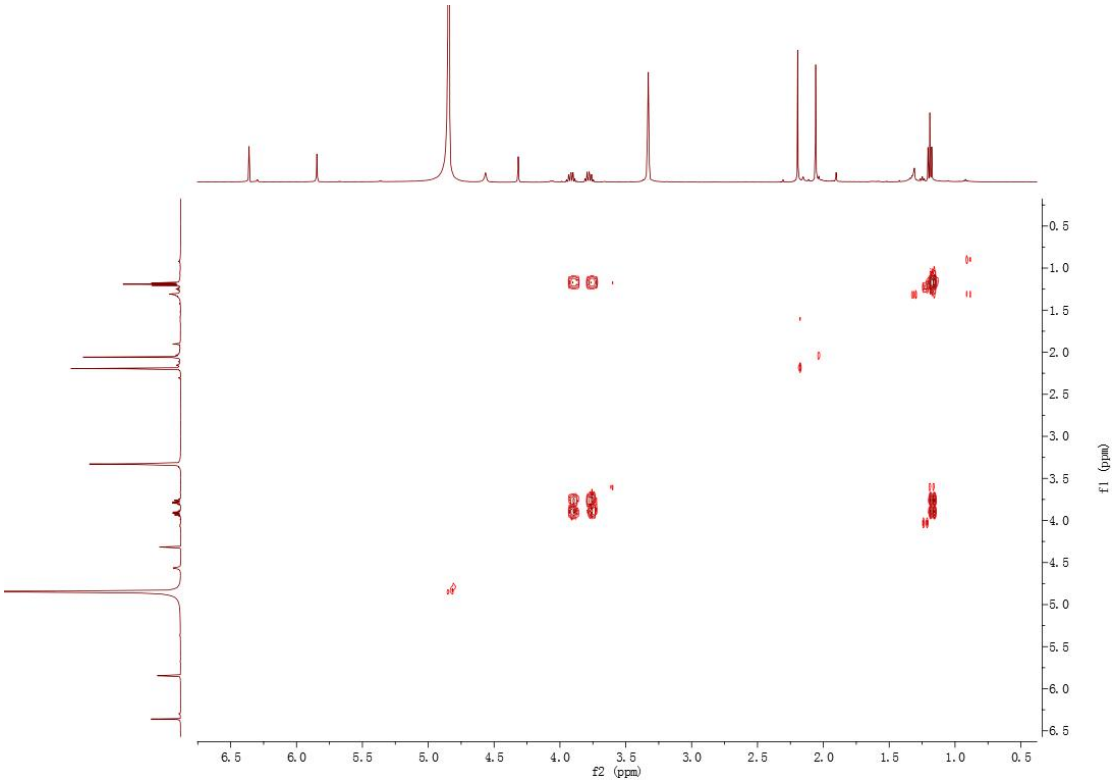


Figure S27. ^{1}H-^{1}H COSY spectrum (500 MHz, CD_3OD) of compound **4**.

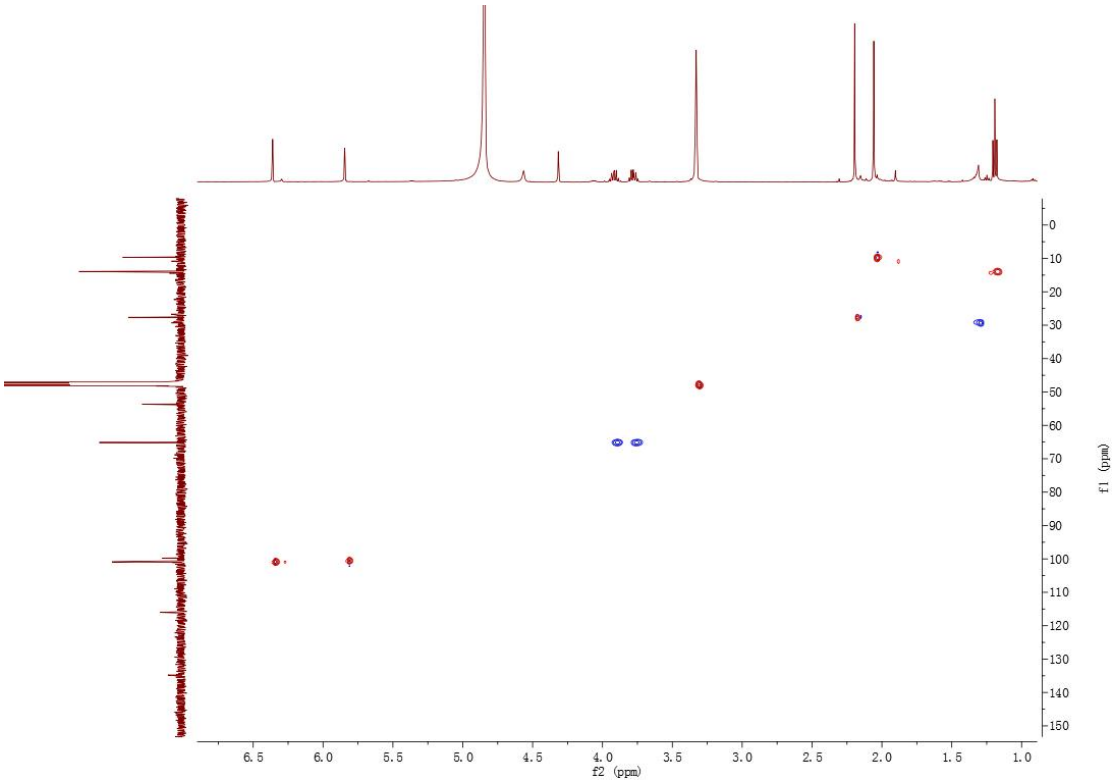


Figure S28. HSQC spectrum of compound **4**.

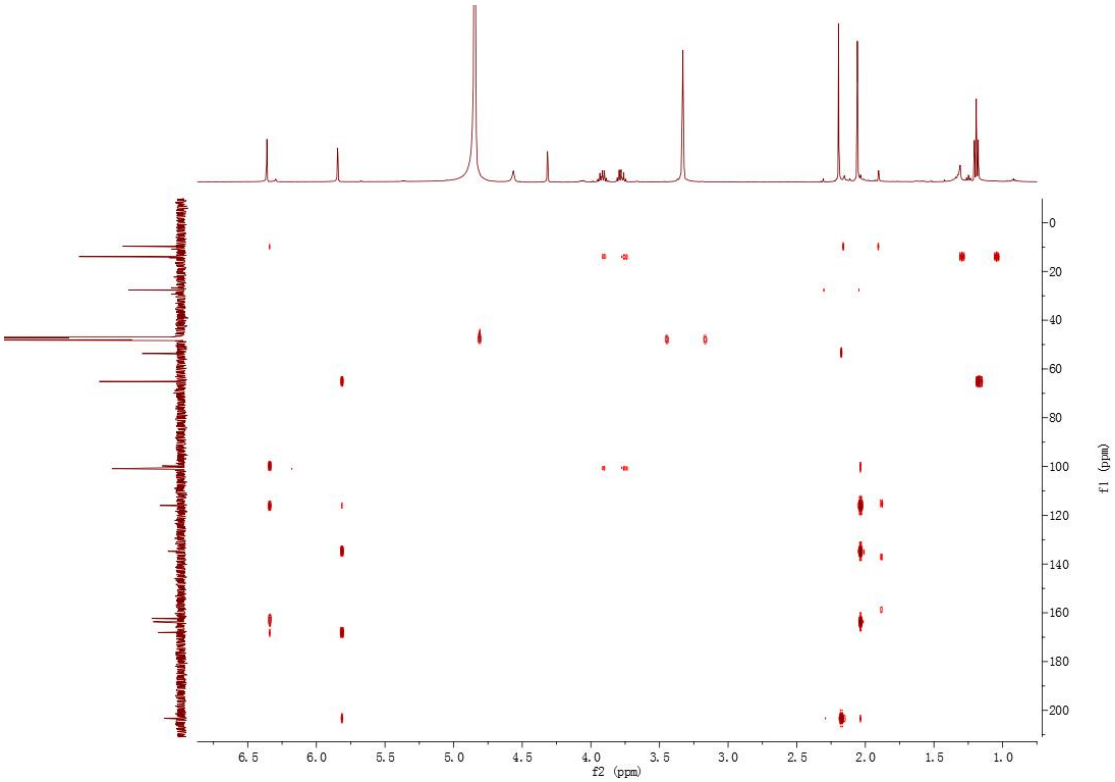


Figure S29. HMBC spectrum of compound **4**.

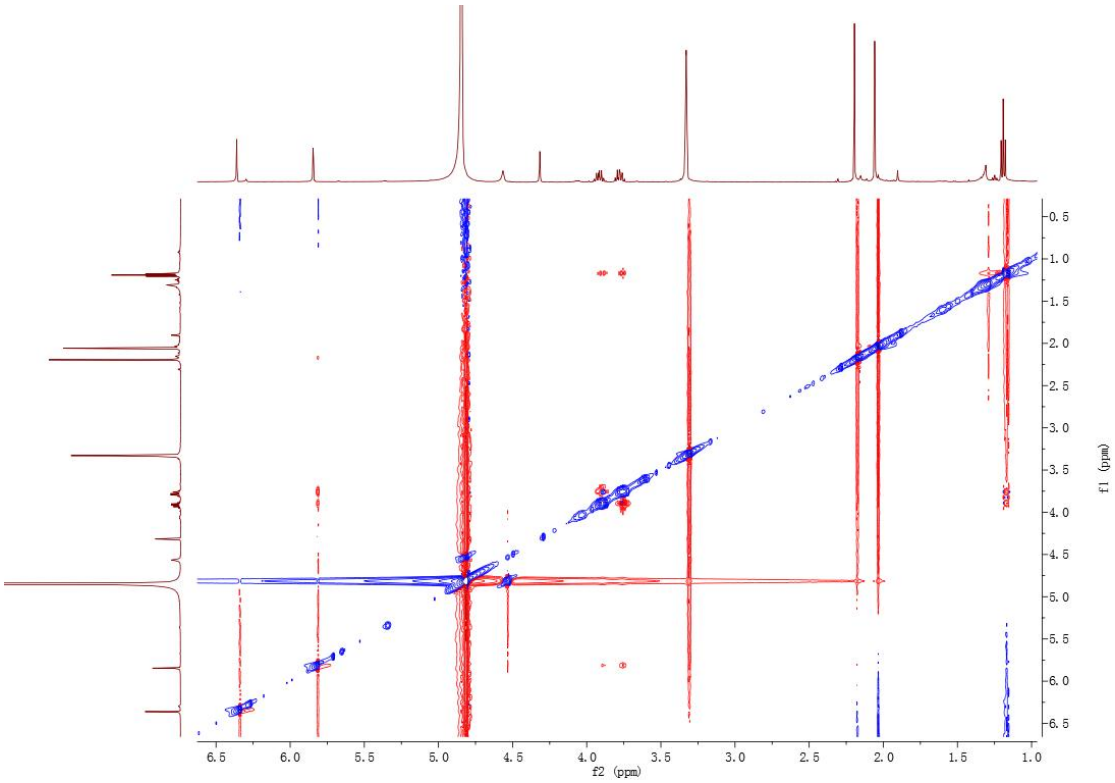


Figure S30. NOESY spectrum of compound **4**.

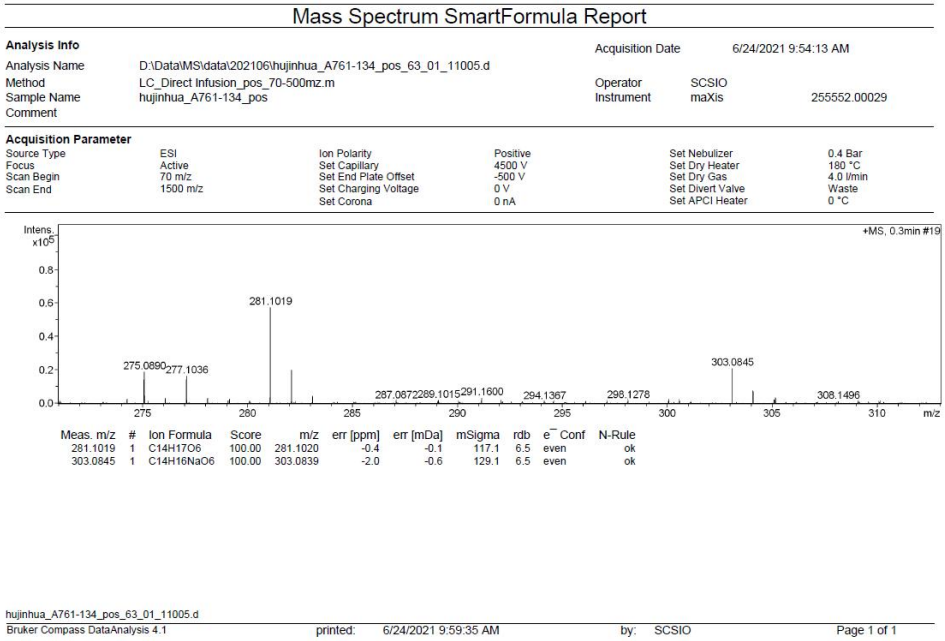


Figure S31. HRESIMS spectrum of compound **4**.

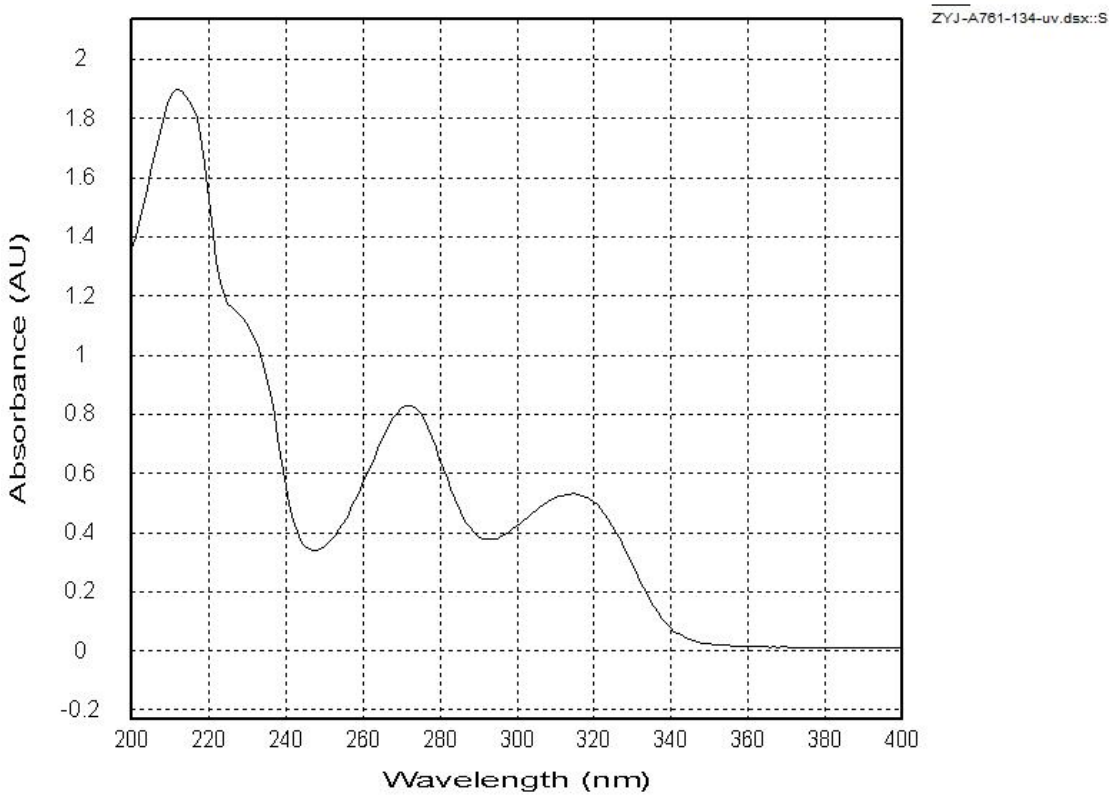


Figure S32. UV spectrum of compound **4**.

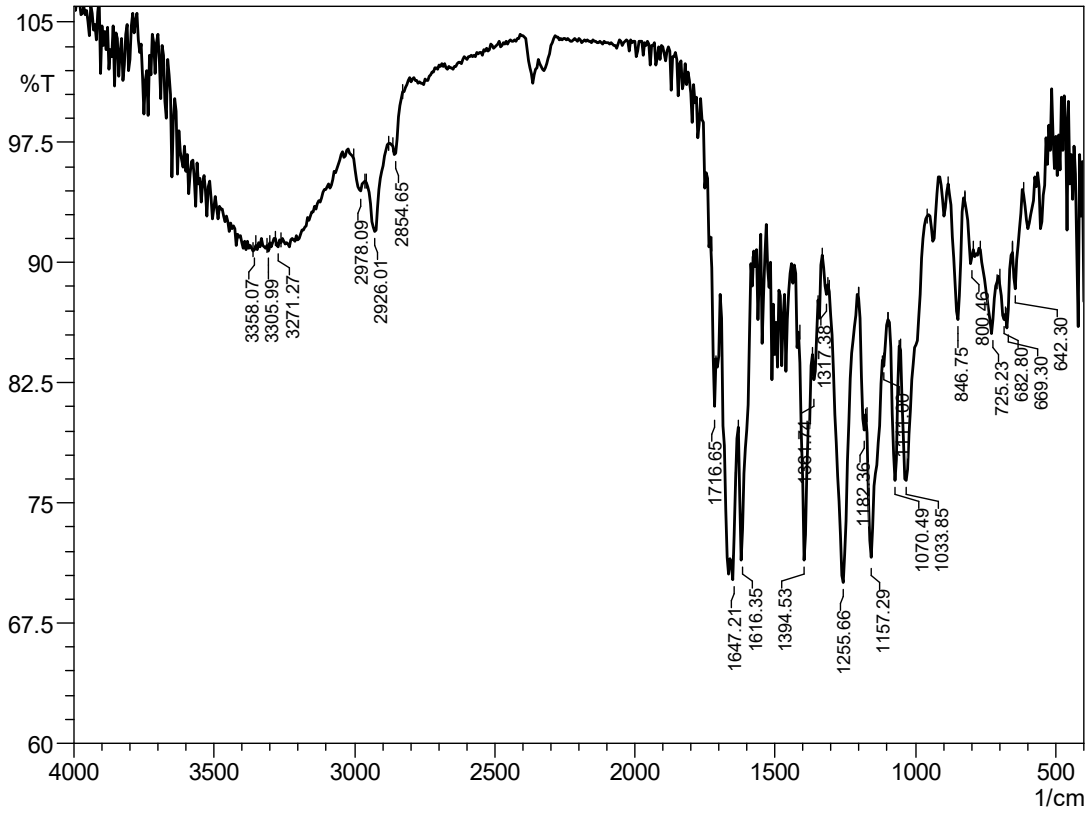


Figure S33. IR spectrum of compound **4**.