Support information for

Enabling Desaturation of Lactones by Reversible Catalytic Activity of 'Ene'-reductases

Fengxi Li [a], Yihang Dai [a], Shenhan Xie [a], Xinjia Yu [a], Xinyan Shi [a], Zhengqiang Li [a], Chuang Du *[b], Zhi Wang *[a], Lei Wang *[a]

[a] Key Laboratory of Molecular Enzymology and Engineering of Ministry of Education, School of Life Sciences, Jilin University, Changchun 130023 (P. R. China)

[b] Changchun Institute of Applied Chemistry, Chinese Academy of Sciences, No.5625 Renmin Rd., Changchun, 130022 (P. R. China)

E-mail: duch@ciac.ac.cn; wangzhi@jlu.edu.cn; w_lei@jlu.edu.cn.

Table of Contents

Materials and methods

Materials.

All the chemicals and reagents were purchased from commercial suppliers (Sigma- Aldrich, Bide Pharmatech Ltd., Aladdin, Energy Chemical, TCI, Shanghai Chemical Reagent Company) and used without any further purification, unless otherwise stated. Ni-NTA Superflow resin obtained from Beijing Solarbio Science & Technology Co., Ltd. *E. coli* BL21(DE3) Competent Cell, Spin Miniprep, and Gel Extraction Kits were all obtained from Tiangen. Silica gel chromatography purifications were carried out using AMD Silica Gel 60 230- 400 mesh. Thin Layer Chromatography (TLC) and preparative TLC were carried out using Merck Millipore TLC silica gel 60 F254 glass plates. Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded on a 400 MHz spectrometer in $CDCl_3$ or DMSO-d_6, and carbon nuclear magnetic resonance (^{13}C NMR) spectra were recorded on 100 MHz spectrometer in CDCl3. Chemical shifts for protons are reported in parts per million downfield from tetramethylsilane (TMS) and are referenced to residual protium in the NMR solvent ($CHCl_3$ = δ 7.26 ppm, DMSO-d_6 = δ 2.50 ppm). Chemical shifts for carbon are reported in parts per million downfield from tetramethylsilane (TMS) and are referenced to the carbon resonances of the solvent residual peak ($CDCl_3$ = δ 77.16 ppm). NMR data are presented as follows: chemical shift (δ ppm), multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad), coupling constant in Hertz (Hz), integration. The experiments were performed triplicate, and all data were obtained based on the average values. Mass spectra were recorded on the Bruker MicrOTOF Q II and an Orbitrap Fusion Tribrid mass spectrometer (Thermo Scientific, San Jose, CA, U.S.A.) coupled with HESI ion source.

Cloning and mutagenesis.

pET-22b (+) was used as a cloning and expression vector for all enzymes mutants in this study. The OYEs gene sequence from *Saccharomyces cerevisiae* were obtained from NCBI. Gene constructs were cloned directly between NdeI and XhoI restriction sites. Cloned plasmids were transformed into *E. coli. DH5-α* cell for storage, and *E. coli. BL21(DE3)* thermocompetent cells for expression. PCR was performed using *Transtart@ FastPfu* DNA polymerase (TransGen Biotech) and the resulting PCR products were digested with DpnI (NEB). Then gel was purified (Omega), repaired (Vazyme) and the product was directly transformed to *E. coli BL21*(DE3) by incubating at 42℃. Following transformation, cells were recovered for 45 min at 37℃ in Luria-Bertani (LB) medium, aliquots were plated on LB agar plates supplemented with 100 μg/mL ampicillin (LB-Amp plates), and plates were incubated at 37℃ overnight.

Protein expression and purification.

The recombinant plasmids were transformed into *E. coli BL21*(DE3) cells and selected on Luria-Bertani (LB) agar plates containing 100 μg/mL ampicillin. Single colonies were grown overnight at 37 ℃ in LB medium containing 100 μg/mL kanamycin or ampicillin. The overnight culture was then inoculated into Terrific-broth (TB) medium containing 100 μg/mL ampicillin. Isopropyl-beta-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM when OD600 of the culture reached 0.6-0.8, and the cultivation was continued at 20-28 ℃ for 18-22 h. Cells were harvested by centrifugation at 4000 rpm for 10 min at 4 ℃, washed with phosphate buffered saline (PBS). After sonication for 30 min on ice, the cell lysates were centrifuged at 12,000 rpm for 30 min. For purification, the lysate was transferred to a Ni-NTA column equilibrated with Ni-NTA lysis Buffer. The enzyme was eluted with buffer containing a gradient of imidazole from 10 to 250 mM at a flow rate of 1 mL/min, and five column volumes were used for the gradient elution. The fractions containing the target protein were collected and dialyzed against 10 mM PBS (pH 7.2-7.4), and then the appropriate fractions were concentrated to 20–40 mg/mL by ultrafiltration (Amicon Ultra-4 membrane, 10 000 NMWL). Purified enzymes were analyzed by SDS-PAGE and used for enzymatic assays. Determination of enzyme concentration based on methods from previous literature [1].

Small-scale reaction screening of mutant library

Small-scale biocatalytic desaturation were set up in 1.5 mL EP tubes. Typically, the reaction solution of **1** (10 mM) in 410 μL Tris-HCl and OYEs (0.2% mol, 40 μL), glycerol 50 μL were then added. The total volume of the reaction is 500 μL and the mixture was stirred for 24 hours at 37 °C. For sample work-up for HPLC analysis the reactions were quenched by adding extractive solvent (*n*-hexane: ethyl acetate=4:1, 400 μL/tube) supplemented. The sample solutions were incubated for 30 min, transferred to 1.5 mL tubes, and centrifuged at 13,000×g for 10 minutes. The cleared supernatant was transferred to clean 2 mL vials and analyzed by HPLC.

Preparative scale procedure for enzymatic desaturation (Procedure A, 0.1 mmol).

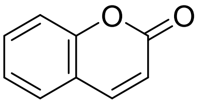
Preparative scale biocatalytic desaturation were set up in 5 mL EP tubes. Typically, the reaction solution of **1** (0.1 mmol) in 2.6 mL Tris-HCl and $\mathrm{OYE1_{LD}}$ (0.5% mol, 100 μL), glycerol 300 μL were then added. The total volume of the reaction is 3 mL and the mixture was stirred for 24 hours at 37 °C. The thus composed reaction mixture was stopped by extracting the content of the vial with EtOAc (3 × 5 mL). The organic layers were combined and dried over sodium sulfate, evaporated under reduced pressure, and the residue was purified by column chromatography (hexanes/ethyl ether, 50:1 to 20:1) to afford the product.

Molecular Docking Analysis.

The initial structure of VHb was taken from PDB code of 3tx9, structures of $\mathrm{OYE1_{LD}}$ was obtained by SWISS-MODEL[2] using homology modeling strategy. Benzofuran was docked into the active site of WT-OYE1 and $\mathrm{OYE1_{LD}}$ using AutoDock Vina tool in Chimera respectively [3], [4].

Supporting Experimental Tables and Figures

Product: Ethyl phenylvalinate **(2a)**



Column: Superchiral S-OD
Wavelength: 254 nm

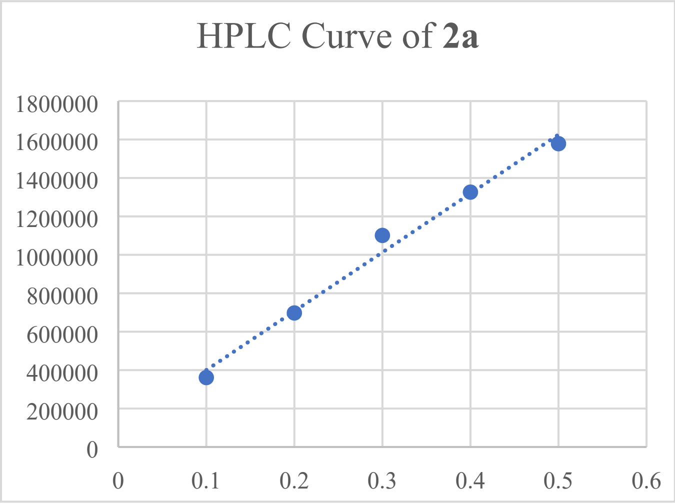


Figure S1. HPLC calibration curve for **2a**.

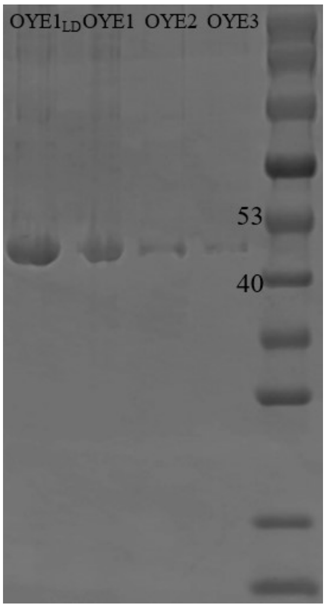


Figure S2. SDS-PAGE of expressed OYEs.

Table S1. Screening of OYEs.

Mutations	Yield of **2a** (TON)
OYE1	46% (230)
OYE2	17% (85)
OYE3	33% (165)
BSA	0 (0)
-	0 (0)

Table S2. Optimization of the desaturation reaction.

Entry	Modification from standard conditions	Yield (%) / TON
1	none	46%/230
2	DMSO instead of glycerol	27%/135

3	*i*-PrOH instead of glycerol	24%/120
4	20% glycerol	36%/180
5	5% glycerol	28%/140
6	No-cosolvents	19%/95
7	PBS buffer (pH = 7.4)	21%/105
8	Tris-HCl (pH = 7.0)	15%/75
9	Tris-HCl (pH = 9.0)	32%/160
10	Tris-HCl (pH = 10.0)	26%/130
11	25 ^{o}C	25%/125
12	OYE1$_{LD}$ (Recycling)	27%/135

Table S3. Green chemistry metrics for enzyme system and metal-catalysis.*

Parameter	OYE1$_{LD}$ (This work)	Metal Catalysis [Ref. 4a]
Yield (%)	87% (for **2a**)	67%
Reaction Conditions	37°C, aqueous buffer	50°C, organic solvent
Catalyst Loading	0.5 mol%	10 mol% Pd
Additive	none	Ag/Base
PMI	26.3	363.7
E-Factor	25.2	362.2
Solvent Toxicity	Low (water/glycerol)	High (toluene)

*Water and buffer components were excluded from PMI/E-Factor calculations due to their low environmental impact and role as reaction media.

PMI (Process Mass Intensity) =Mass of Product (kg)/Total Mass of Inputs (kg)
Calculation for OYE1$_{LD}$ (0.2 mmol scale):
Substrate (**1a**): 29.6 mg
Enzyme (OYE1$_{LD}$): 0.5 mol% of substrate ≈ 40 mg.
Glycerol: 600 mg.
Total Inputs: 29.6 mg + 40 mg + 600 mg = 669.6 mg.
Product (2a): 87% yield → 25.4 mg.
PMI = 669.6/25.4 = 26.3

Comparison to Metal Catalysis (0.2 mmol scale):
Substrate: 29.6 mg
Pd Catalyst: 10 mol% → 5 mg
Toluene Solvent: 6920 mg.
Additive: 30%AgTFA, 2 equiv. DAC, 1.3 equiv. Bu$_2$BOTf, 1.3 equiv. DIPEA → 174 mg.
Total Inputs: 29.6 + 5 + 6920 + 174 = 7128.6 mg.
Product: 67% yield → 19.6 mg.
PMI: 7128.6/19.6 = 363.7

E-Factor=Mass of Product (kg)/Mass of Waste (kg)
Waste: Enzyme + glycerol = 640 mg.

E-Factor: 640/25.4 = 25.2

Waste: Pd + toluene + additive = 7099 mg.

E-Factor: 7099/19.6 =362.2

Table S4. Optimum temperature of the OYE1$_{LD}$.

Temperature	Yield of **2a**
25 ^oC	62%
30 ^oC	79%
37 ^oC	**87%**
40 ^oC	86%
50 ^oC	81%
60 ^oC	59%

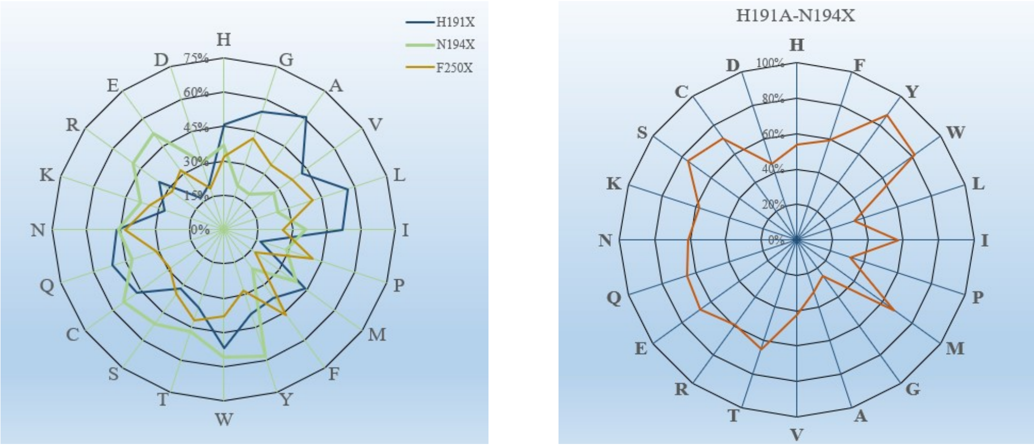


Figure S3. Results for directed evolution of OYE1: (a) yields for reactions using single mutants at positions H191X, N194X or F250X; (b) yields for reactions using double mutants at positions H191A-N194X.

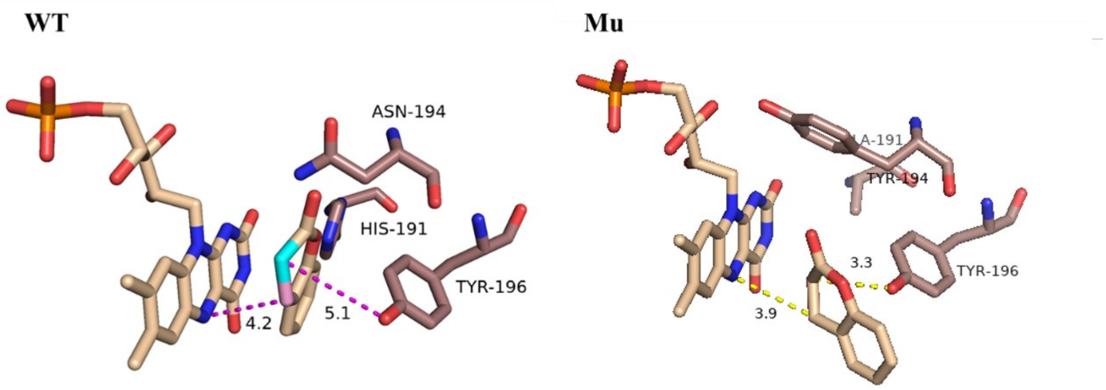


Figure S4. Simplified version of catalytic distance analysis.

Sequence of primers and variants:

Amino Acid Sequence of WT-OYE-1

MSFVKDFKPQALGDTNLFKPIKIGNNELLHRAVIPPLTRMRALHPGNIPNRDWAVEYYTQ
RAQRPGTMIITEGAFISPQAGGYDNAPGVWSEEQMVEWTKIFNAIHEKKSFVWVQLWVL
GWAAFPDNLARDGLRYDSASDNVFMDAEQEAKAKKANNPQHSLTKDEIKQYIKEYVQA
AKNSIAAGADGVEIHSANGYLLNQFLDPHSNTRTDEYGGSIENRARFTLEVVDALVEAIG
HEKVGLRLSPYGVFNSMSGGAETGIVAQYAYVAGELEKRAKAGKRLAFVHLVEPRVTNP
FLTEGEGEYEGGSNDFVYSIWKGPVIRAGNFALHPEVVREEVKDKRTLIGYGRFFISNPDL
VDR LEKGLPLNKYDRDTFYQMSAHGYIDYPTYEEALKLGWDKKHHHHHH*

Amino Acid Sequence of WT-OYE-2

MGSSHHHHHHSSGLVPRGSHNMPFVKDFKPQALGDTNLFKPIKIGNNELLHRAVIPPLTR
MRAQHPGNIPNRDWAVEYYAQRAQRPGTLIITEGTFPSPQSGGYDNAPGIWSEEQIKEWT
KIFKAIHENKSFAWVQLWVLGWAAFPDTLARDGLRYDSASDNVYMNAEQEEKAKKAN
NPQHSITKDEIKQYVKEYVQAAKNSIAAGADGVEIHSANGYLLNQFLDPHSNNRTDEYG
GSIENRARFTLEVVDAVVDAIGPEKVGLRLSPYGVFNSMSGGAETGIVAQYAYVLGELER
RAKAGKRLAFVHLVEPRVTNPFLTEGEGEYNGGSNKFAYSIWKGPIIRAGNFALHPEVVR
EEVKDPRTLIGYGRFFISNPDLVDRLEKGLPLNKYDRDTFYKMSAEGYIDYPTYEEALKL
GWDKN*

Amino Acid Sequence of WT-OYE-3

MPFVKGFEPISLRDTNLFEPIKIGNTQLAHRAVMPPLTRMRATHPGNIPNKEWAAVYYGQ
RAQRPGTMIITEGTFISPQAGGYDNAPGIWSDEQVAEWKNIFLAIHDCQSFAWVQLWSLG
WASFPDVLARDGLRYDCASDRVYMNATLQEKAKDANNLEHSLTKDDIKQYIKDYIHAA
KNSIAAGADGVEIHSANGYLLNQFLDPHSNKRTDEYGGTIENRARFTLEVVDALIETIGPE
RVGLRLSPYGTFNSMSGGAEPGIIAQYSYVLGELEKRAKAGKRLAFVHLVEPRVTDPSLV
EGEGEYSEGTNDFAYSIWKGPIIRAGNYALHPEVVREQVKDPRTLIGYGRFFISNPDLVYR
LEEGLPLNKYDRSTFYTMSAEGYTDYPTYEEAVDLGWNKNHHHHHH*

Amino Acid Sequence of OYE$_{LD}$

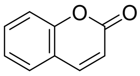
MSFVKDFKPQALGDTNLFKPIKIGNNELLHRAVIPPLTRMRALHPGNIPNRDWAVEYYTQ
RAQRPGTMIITEGAFISPQAGGYDNAPGVWSEEQMVEWTKIFNAIHEKKSFVWVQLWVL
GWAAFPDNLARDGLRYDSASDNVFMDAEQEAKAKKANNPQHSLTKDEIKQYIKEYVQA
AKNSIAAGADGVEIASAYGYLLNQFLDPHSNTRTDEYGGSIENRARFTLEVVDALVEAIG
HEKVGLRLSPYGVFNSMSGGAETGIVAQYAYVAGELEKRAKAGKRLAFVHLVEPRVTNP
FLTEGEGEYEGGSNDFVYSIWKGPVIRAGNFALHPEVVREEVKDKRTLIGYGRFFISNPDL
VDR LEKGLPLNKYDRDTFYQMSAHGYIDYPTYEEALKLGWDKKHHHHHH*

Table S5. Primers of PCR reactions

Primer	Sequence
H191-F	GAAATCNNKAGCGCTAACGGTTATCTGC
H191-R	AGCGCTMNNGATTTCAACGCCAT
N194-F	TAGCGCTNNKGGTTATCTGCTGAAC
N194-R	TAACCMNNAGCGCTATGGATTTCAACG

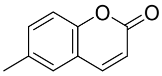
Y196A-F	ATTCACAGTGCTAACGGTGCGTTGTTAAAC
Y196A-R	CAAGAACTGGTTTAACAACGCACCGTTAGC
F250-F	CATACGGTGTTNNKAACAGTATGTCTGGTG
F250-R	ATACTGTTMNNAACACCGTATGGGGACAAT
H191A-N194-F	GTTGAAATTGCGAGTGCTNNKGGTTACTTG
H191A-N194-R	CAAGTAACCMNNAGCACTCGCAATTTCAAC

NMR data [5-7]



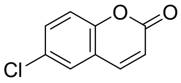
2a

Following the standard procedure A, white solid, ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.75 (d, J = 9.5 Hz, 1H), 7.63 – 7.48 (m, 2H), 7.39 – 7.30 (m, 2H), 6.45 (d, J = 9.5 Hz, 1H); ^{13}C NMR (101 MHz, Chloroform-*d*) δ 160.85, 154.10, 143.52, 131.90, 127.94, 124.50, 118.89, 116.95, 116.75. MS (ESI): m/z = (M + H) calcd for($C_9H_6O_2$)H147.0446, found 147.0441.



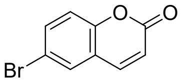
2b

Following the standard procedure A, white solid, ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.69 (d, J = 9.5 Hz, 1H), 7.37 (d, J = 8.5 Hz, 1H), 7.32 – 7.22 (m, 2H), 6.44 (d, J = 9.5 Hz, 1H), 2.44 (s, 3H); ^{13}C NMR (101 MHz, Chloroform-*d*) δ 161.14, 152.23, 143.48, 134.19, 132.89, 127.74, 118.64, 116.80, 116.66, 20.79. MS (ESI): m/z = (M + H) calcd for ($C_{10}H_8O_2$)H (M + H) 161.0603, found 161.0608.



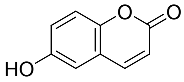
2c

Following the standard procedure A, white solid, ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.68 (d, J = 9.6 Hz, 1H), 7.61 – 7.53 (m, 1H), 7.36 – 7.24 (m, 2H), 6.52 (d, J = 9.6 Hz, 1H); ^{13}C NMR (101 MHz, Chloroform-*d*) δ 160.11, 152.51, 142.26, 131.85, 129.77, 127.19, 119.51, 118.42, 117.96. MS (ESI): m/z = (M + H) calcd for ($C_9H_5ClO_2$)H, 181.0051; found, 181.0060.



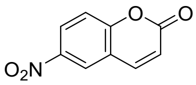
2d

Following the standard procedure A, white solid, ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.73 – 7.60 (m, 3H), 7.26 (d, J = 8.8 Hz, 1H), 6.50 (d, J = 9.5 Hz, 1H); ^{13}C NMR (101 MHz, Chloroform-*d*) δ 160.04, 152.97, 142.18, 134.66, 130.23, 120.38, 118.71, 117.93, 117.06. MS (ESI): m/z = (M + H) calcd for ($C_9H_5BrO_2$)H 224.9551, found 224.9555.



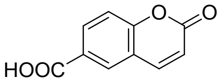
2e

Following the standard procedure A, pale yellow solid, ^{1}H NMR (400 MHz, DMSO-*d*$_6$) δ 9.78 (s, 1H), 7.99 (d, *J* = 9.5 Hz, 1H), 7.31 – 7.13 (m, 1H), 7.05 (dd, *J* = 7.3, 2.6 Hz, 2H), 6.45 (d, *J* = 9.6 Hz, 1H); ^{13}C NMR (101 MHz, DMSO) δ 160.81, 154.28, 147.28, 144.67, 120.35, 119.78, 117.68, 116.78, 112.99. MS (ESI): m/z = (M + H) calcd for ($C_9H_6O_3$)H 163.0395, found 163.0397.



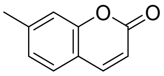
2f

Following the standard procedure A, yellow solid,^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.57 – 8.33 (m, 2H), 7.85 (d, *J* = 9.6 Hz, 1H), 7.51 (d, *J* = 9.1 Hz, 1H), 6.63 (d, *J* = 9.7 Hz, 1H); ^{13}C NMR (101 MHz, Chloroform-*d*) δ 158.90, 157.62, 144.11, 142.28, 126.70, 123.82, 118.92, 118.87, 118.17. MS (ESI): m/z = (M + H) calcd for ($C_9H_5NO_4$)H 192.0297, found 192.0296.



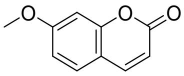
2g

Following the standard procedure A, white solid, ^{1}H NMR (400 MHz, DMSO-*d*$_6$) δ 13.26 (s, 1H), 8.37 (d, *J* = 2.2 Hz, 1H), 8.22 (d, *J* = 9.5 Hz, 1H), 8.14 (dd, *J* = 8.7, 2.1 Hz, 1H), 7.51 (d, *J* = 8.7 Hz, 1H), 6.60 (d, *J* = 9.6 Hz, 1H); ^{13}C NMR (101 MHz, DMSO-*d*$_6$) δ 166.72, 160.02, 156.74, 144.64, 133.03, 130.72, 127.49, 119.20, 117.42, 117.26. MS (ESI): m/z = (M + H) calcd for ($C_{10}H_6O_4$)H 192.0326, found 192.0291.



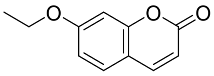
2h

Following the standard procedure A, white solid, ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.70 (dd, *J* = 9.5, 1.6 Hz, 1H), 7.39 (dd, *J* = 7.8, 1.6 Hz, 1H), 7.17 (s, 1H), 7.12 (d, *J* = 7.9 Hz, 1H), 6.38 (dd, *J* = 9.5, 1.6 Hz, 1H), 2.48 (s, 3H); ^{13}C NMR (101 MHz, Chloroform-*d*) δ 161.17, 154.23, 143.45, 143.17, 127.57, 125.66, 117.13, 116.52, 115.52, 21.84. MS (ESI): m/z = (M + H) calcd for ($C_{10}H_8O_2$)H (M + H) 161.0603, found 161.0605.



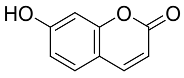
2i

Following the standard procedure A, white solid, ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.67 (d, *J* = 9.4 Hz, 1H), 7.40 (d, *J* = 8.4 Hz, 1H), 6.93 – 6.79 (m, 2H), 6.34 – 6.23 (m, 1H), 3.90 (d, *J* = 1.2 Hz, 3H); ^{13}C NMR (101 MHz, Chloroform-*d*) δ 162.88, 161.27, 155.95, 143.50, 128.83, 113.13, 112.64, 112.58, 100.88, 55.84. MS (ESI): m/z = (M + H) calcd for ($C_{10}H_8O_3$)H (M + H) 177.0552, found 177.0559.



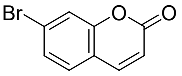
2j

Following the standard procedure A, white solid, ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.67 (d, *J* = 9.4 Hz, 1H), 7.40 (d, *J* = 8.5 Hz, 1H), 6.93 – 6.78 (m, 2H), 6.28 (d, *J* = 9.4 Hz, 1H), 4.12 (q, *J* = 7.0 Hz, 2H), 1.49 (t, *J* = 6.9 Hz, 3H). ^{13}C NMR (101 MHz, Chloroform-*d*) δ 162.27, 161.40, 155.95, 143.57, 128.79, 113.03, 112.99, 112.43, 101.31, 64.23, 14.63. MS (ESI): m/z = (M + H) calcd for ($C_{11}H_{10}O_3$)H, 191.0703, found, 191.0704.



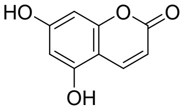
2k

Following the standard procedure A, orange solid,^{1}H NMR (400 MHz, DMSO-d_6) δ 10.60 (s, 1H), 7.95 (d, J = 9.5 Hz, 1H), 7.54 (d, J = 8.4 Hz, 1H), 6.80 (dd, J = 8.5, 2.2 Hz, 1H), 6.73 (d, J = 2.4 Hz, 1H), 6.22 (d, J = 9.4 Hz, 1H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 161.79, 160.95, 155.99, 145.06, 130.23, 113.62, 111.91, 111.78, 102.65. MS (ESI): m/z = (M + H) calcd for $(C_9H_6O_3)$H 163.0395, found 163.0398.



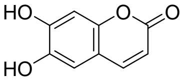
2l

Following the standard procedure A, white solid, ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.70 (d, J = 9.5 Hz, 1H), 7.54 (s, 1H), 7.45 (dd, J = 8.2, 1.5 Hz, 1H), 7.38 (d, J = 8.2 Hz, 1H), 6.47 (d, J = 9.5 Hz, 1H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 159.96, 154.32, 142.82, 128.86, 127.94, 125.86, 120.25, 117.80, 116.94. MS (ESI): m/z = (M + H) calcd for $(C_9H_5BrO_2)$H 224.9551, found 224.9554.



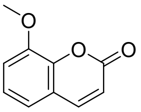
2m

Following the standard procedure A, pale yellow solid, ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.67 (s, 1H), 10.39 (s, 1H), 7.97 (d, J = 9.6 Hz, 1H), 6.27 (d, J = 2.8 Hz, 1H), 6.19 (d, J = 2.3 Hz, 1H), 6.04 (d, J = 9.6 Hz, 1H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 162.56, 161.24, 156.96, 156.43, 140.07, 109.15, 102.13, 98.68, 94.53. MS (ESI): m/z = (M + H) calcd for $(C_9H_6O_4)$Na: 201.0153, found 201.0154.



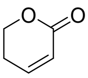
2n

Following the standard procedure A, pale yellow solid, ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.84 (s, 2H), 7.88 (d, J = 9.5 Hz, 1H), 6.99 (s, 1H), 6.75 (s, 1H), 6.18 (d, J = 9.4 Hz, 1H); ^{13}C NMR (101 MHz, DMSO) δ 161.30, 150.89, 148.98, 144.95, 143.37, 112.80, 112.00, 111.25, 103.13. MS (ESI): m/z = (M + H) calcd for calcd for $(C_9H_6O_4)$Na: 201.0153, found 201.0158.



2o

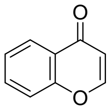
Following the standard procedure A, white solid, ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.72 (dd, J = 9.5, 1.3 Hz, 1H), 7.25 (t, J = 8.0 Hz, 1H), 7.15 – 7.05 (m, 2H), 6.47 (dd, J = 9.5, 1.3 Hz, 1H), 4.00 (d, J = 1.4 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 160.26, 147.32, 143.81, 143.66, 124.35, 119.52, 119.31, 117.02, 113.77, 56.31. MS (ESI): m/z = (M + H) calcd for$(C_{10}H_8O_3)$H (M + H) 177.0552, found 177.0556.



2p

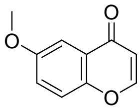
Following the standard procedure A, colorless liquid, ^{1}H NMR (400 MHz, Chloroform-*d*) δ 6.96 (dt, J = 9.4, 4.3 Hz, 1H), 6.04 (dd, J = 9.9, 2.0 Hz, 1H), 4.43 (t, J = 6.3 Hz, 2H), 2.47 (q, J = 5.7 Hz, 2H). ^{13}C

NMR (101 MHz, Chloroform-*d*) δ 163.88, 145.90, 121.63, 66.57, 24.06. MS (ESI): m/z = (M + H) calcd for($C_5H_6O_2$)H (M + H) 99.0421, found 99.0423.



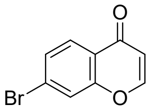
2q

Following the standard procedure A, white solid, ^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.22 (d, *J* = 8.0 Hz, 1H), 7.88 (d, *J* = 6.0 Hz, 1H), 7.68 (d, *J* = 7.2 Hz, 1H), 7.52 – 7.37 (m, 2H), 6.36 (d, *J* = 6.0 Hz, 1H). ^{13}C NMR (101 MHz, Chloroform-*d*) δ 177.70, 156.55, 155.41, 133.85, 125.84, 125.32, 124.92, 118.25, 113.04. MS (ESI): m/z = (M + H) calcd for ($C_9H_6O_2$)H 147.0440, found: 147.0441.



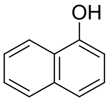
2r

Following the standard procedure A, white solid, ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.89 (dd, *J* = 6.1, 1.5 Hz, 1H), 7.65 – 7.57 (m, 1H), 7.44 (dd, *J* = 9.0, 1.5 Hz, 1H), 7.33 – 7.29 (m, 1H), 6.37 (dd, *J* = 6.0, 1.5 Hz, 1H), 3.94 (s, 3H). ^{13}C NMR (101 MHz, Chloroform-*d*) δ 177.64, 157.02, 155.13, 151.46, 125.53, 124.01, 119.69, 112.20, 104.83, 56.00. MS (ESI): m/z = (M + H) calcd for ($C_{10}H_8O_3$)H 177.0546, found: 177.0547.



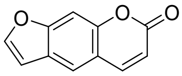
2s

Following the standard procedure A, white solid, ^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.10 (dd, *J* = 8.5, 1.6 Hz, 1H), 7.85 (dd, *J* = 6.1, 1.5 Hz, 1H), 7.69 (t, *J* = 1.6 Hz, 1H), 7.56 (dt, *J* = 8.5, 1.6 Hz, 1H), 6.38 (dd, *J* = 6.1, 1.6 Hz, 1H). ^{13}C NMR (101 MHz, Chloroform-*d*) δ 176.90, 156.58, 155.32, 129.01, 128.12, 127.34, 123.82, 121.37, 113.40. MS (ESI): m/z = (M + H) calcd for ($C_9H_5BrO_2$)H 224.9545, found: 224.9544.



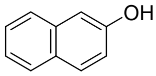
2t

Following the standard procedure A, brown solid, ^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.43 – 8.20 (m, 2H), 7.90 (d, *J* = 6.4 Hz, 2H), 7.65 – 7.48 (m, 6H), 7.38 (t, *J* = 7.8 Hz, 2H), 6.88 (s, 1H), 5.46 (d, *J* = 2.7 Hz, 2H). ^{13}C NMR (101 MHz, Chloroform-*d*) δ 151.38, 134.86, 127.82, 126.59, 125.96, 125.43, 124.43, 121.62, 120.87, 108.79. MS (ESI): m/z = (M + H) calcd for($C_{10}H_8O$)H (M + H) 145.0594, found 145.0599.



2u

Following the standard procedure A, white solid, ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.83 (d, *J* = 9.6 Hz, 1H), 7.74 – 7.68 (m, 2H), 7.49 (s, 1H), 6.87 (d, *J* = 2.3 Hz, 1H), 6.41 (d, *J* = 9.6 Hz, 1H); ^{13}C NMR (101 MHz, Chloroform-*d*) δ 161.10, 156.44, 152.06, 146.98, 144.17, 124.93, 119.92, 115.45, 114.69, 106.45, 99.91. MS (ESI): m/z = (M + H) calcd for ($C_{11}H_6O_3$)H 187.0395, found 187.0377.



2v

Following the standard procedure A, pale brown solid, ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.82 (t, *J* = 8.2 Hz, 2H), 7.73 (d, *J* = 8.3 Hz, 1H), 7.49 (t, *J* = 7.5 Hz, 1H), 7.39 (t, *J* = 7.5 Hz, 1H), 7.24 – 7.13 (m, 2H), 5.13 (s, 1H). ^{13}C NMR (101 MHz, Chloroform-*d*) δ 153.30, 134.63, 129.97, 129.03, 127.86, 126.65, 126.46, 123.75, 117.79, 109.60. MS (ESI): m/z = (M + H) calcd for($C_{10}H_8O$)H (M + H) 145.0594, found 145.0598.

Reference

[1]. M. Li, Y. Yuan, W. Harrison, Z. Zhang, H. Zhao, *Science* **2024**, *385*, 416-421

[2]. A. Waterhouse, M. Bertoni, S. Bienert, G. Studer, G. Tauriello, R. Gumienny, F. T. Heer, T. A P. de Beer, C. Rempfer, L. Bordoli, R. Lepore and T. Schwede, *Nucleic Acids Res.*, **2018**, *46*, W296-W303.

[3]. E. F. Pettersen, T. D. Goddard, C. C. Huang, G. S. Couch, D. M. Greenblatt, E. C. Meng and T. E. Ferrin, *J. Comput. Chem.*, **2004**, *25*, 1605-1612.

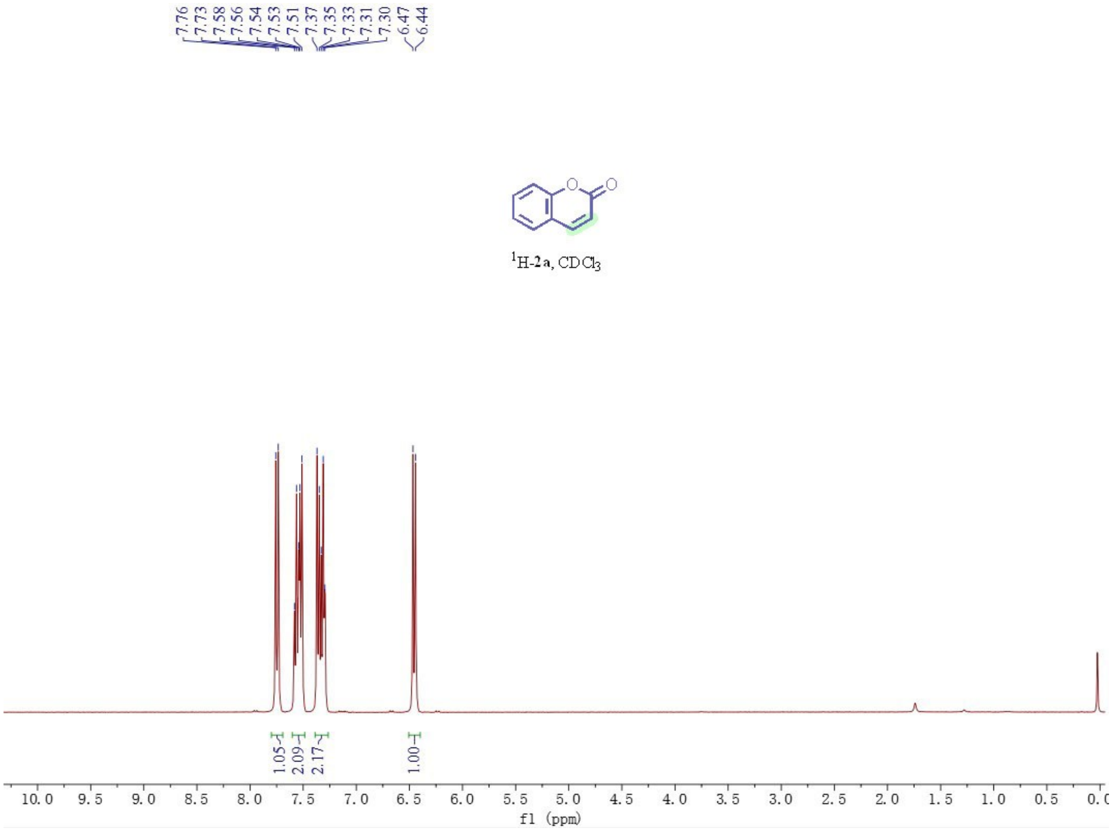
[4]. O. Trott and A. J. Olson, *J. Comput. Chem.*, **2010**, *31*, 455-461.

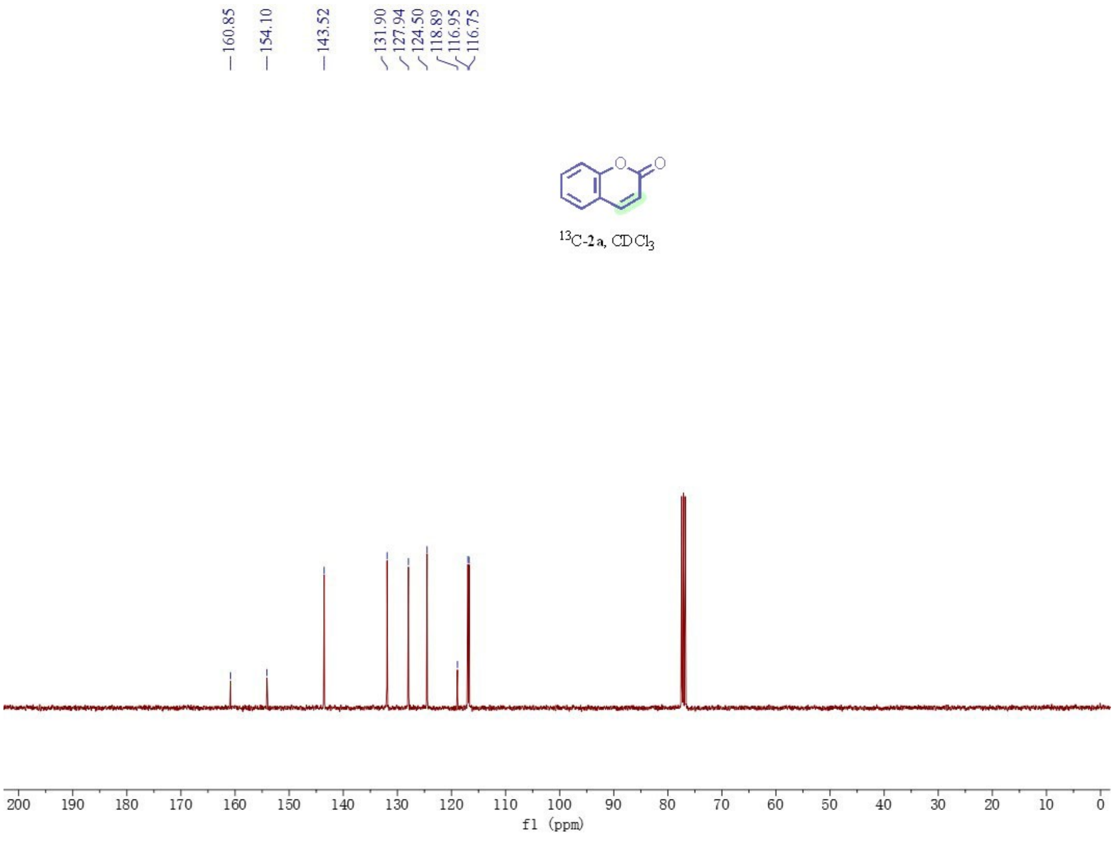
[5]. Z.-Y. Wang, T. Yang, K.-K. Wang, D.-F. Liu, X. Ma, N. Wang, H. Liu, A. Sun, H. Liu, *Green Chem.* **2023**, *25*, 3040-3045

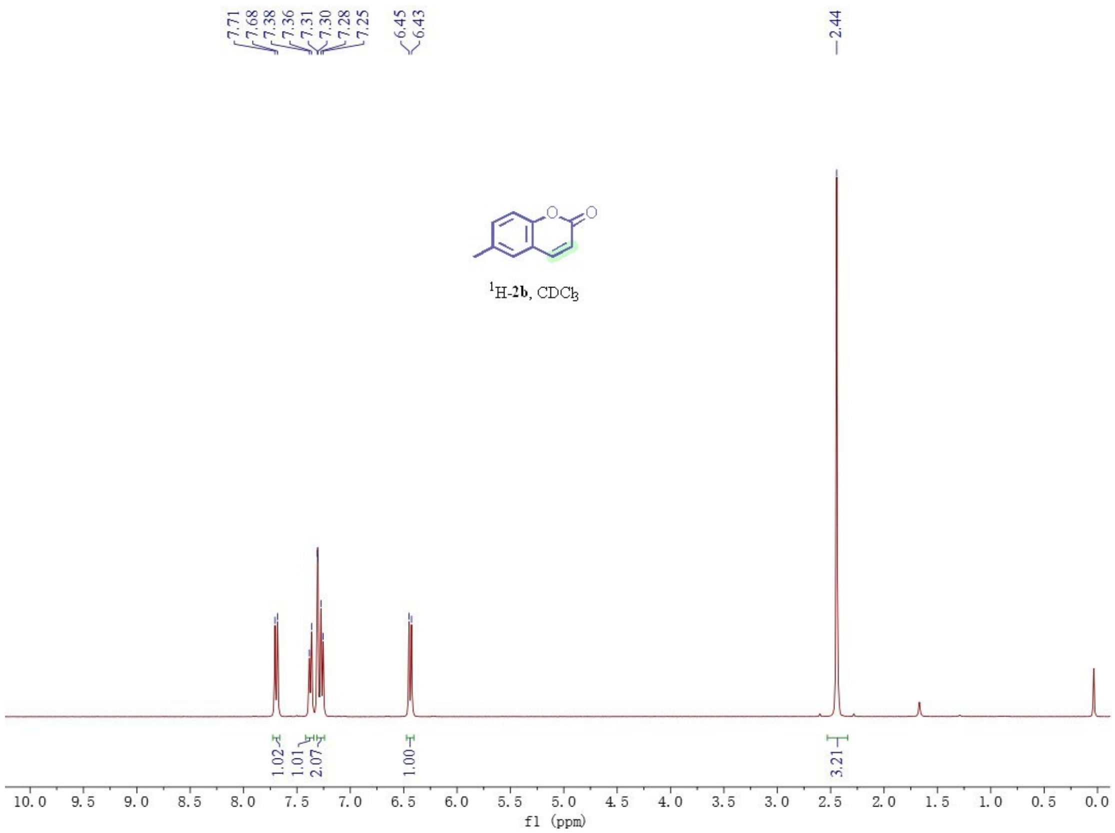
[6]. X.-S. Zhang, Z.-W. Li, Z.-J. Shi, *Org. Chem. Front.* **2014**, *1*, 44-49.

[7]. A. Cervi, Y. Vo, C. L. L. Chai, M. G. Banwell, P. Lan, A. C. Willis, *J. Org. Chem.* **2021**, *86*, 178-198.

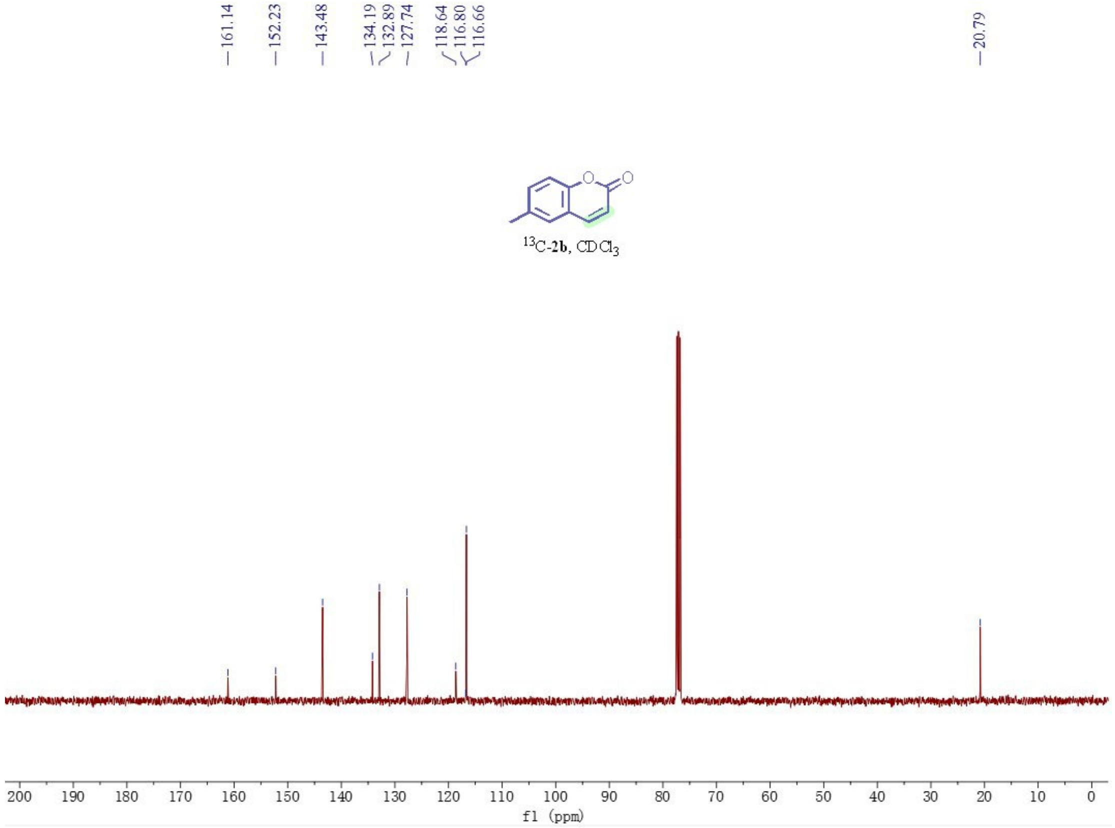
HPLC and NMR Spectra



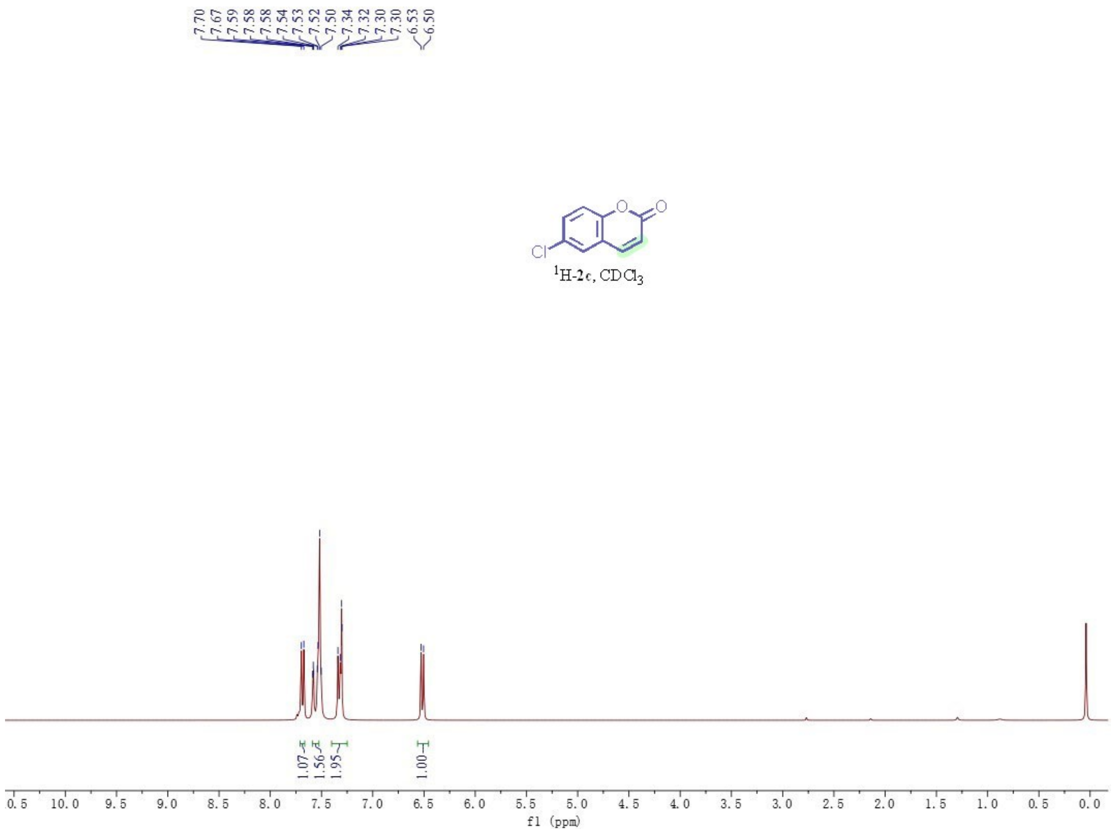


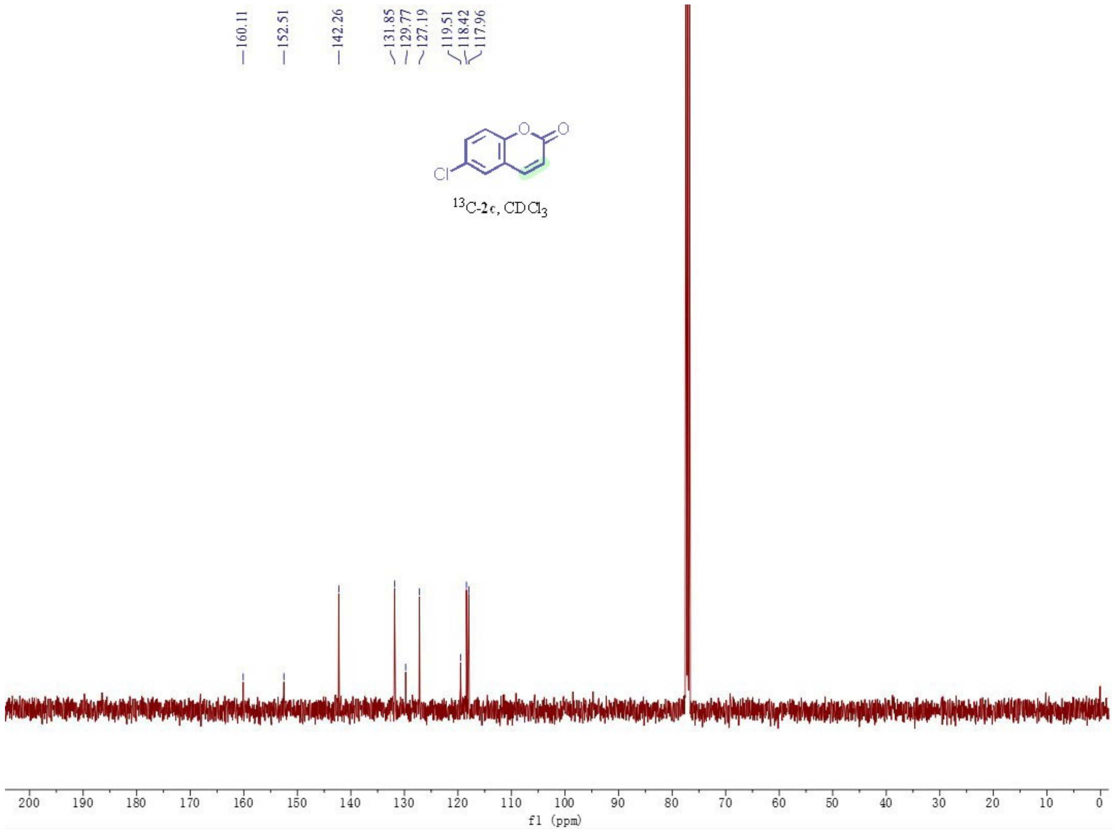


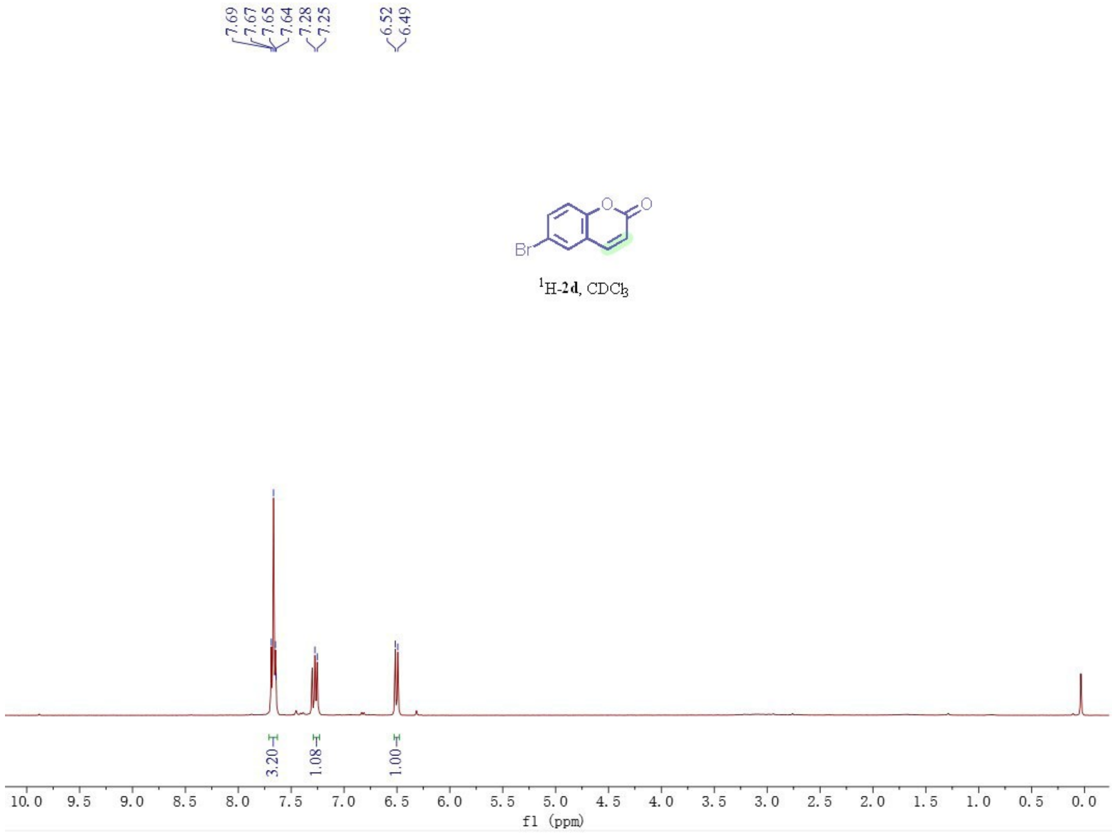
^{1}H-**2b**, $CDCl_3$

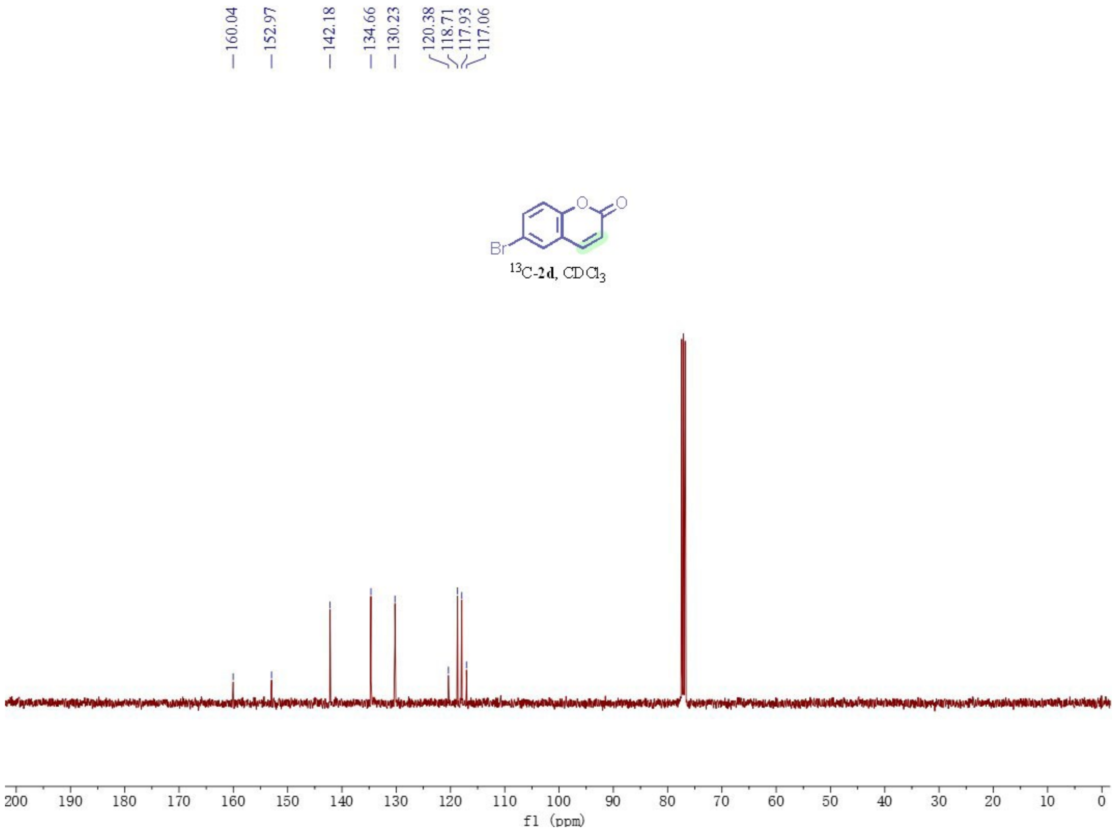


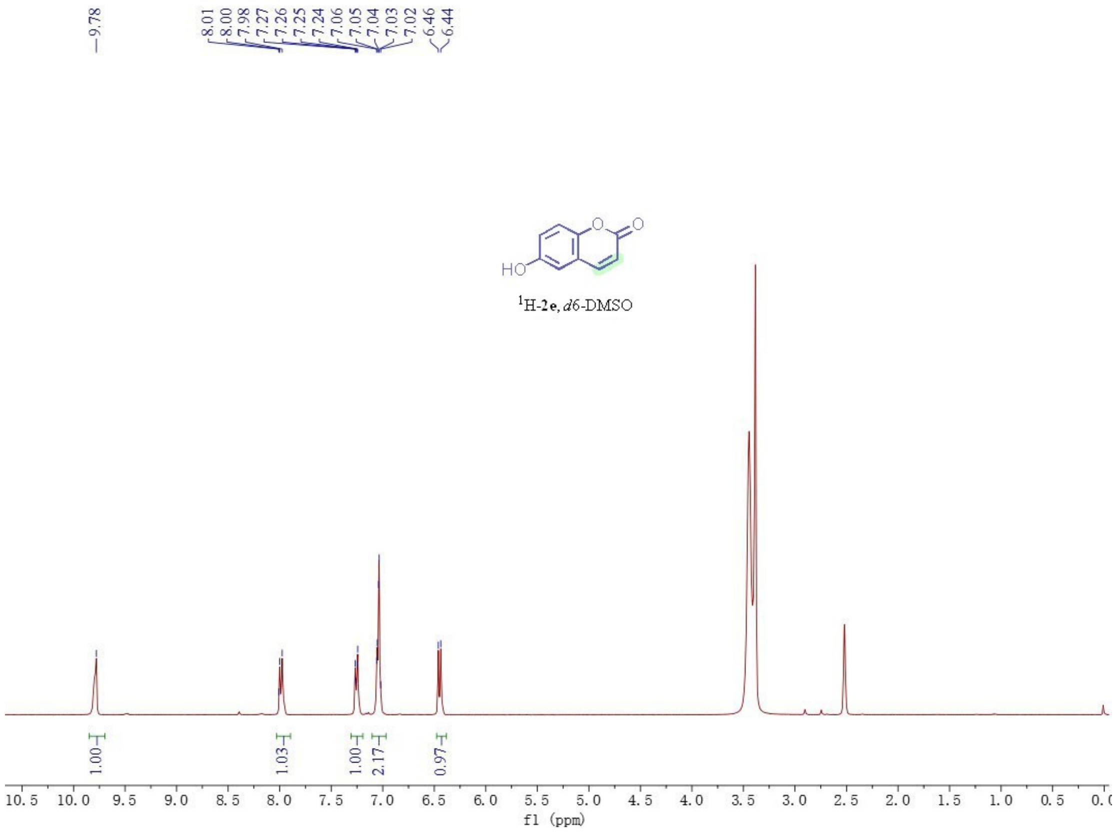
^{13}C-**2b**, $CDCl_3$


7.70
7.67
7.59
7.58
7.58
7.54
7.53
7.52
7.50
7.34
7.32
7.30
7.30
6.53
6.50
Cl
O
O
^{1}H-2c, CDCl$_3$
1.07
1.56
1.95
1.00
0.5 10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0
f1 (ppm)

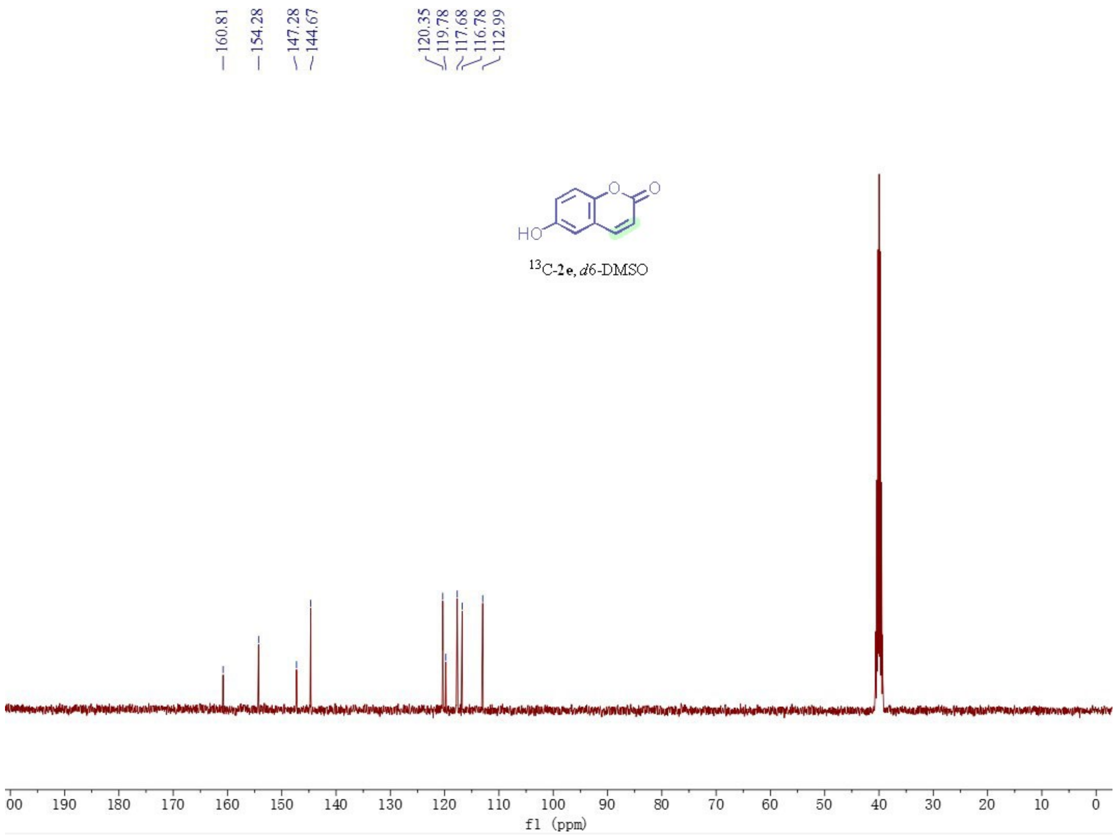

160.11
152.51
142.26
131.85
129.77
127.19
119.51
118.42
117.96
Cl
O
O
^{13}C-2c, CDCl$_3$
200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
f1 (ppm)


7.69
7.67
7.65
7.64
7.28
7.25
6.52
6.49
Br
O
O
^{1}H-**2d**, CDCl$_3$
3.20
1.08
1.00
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
f1 (ppm)

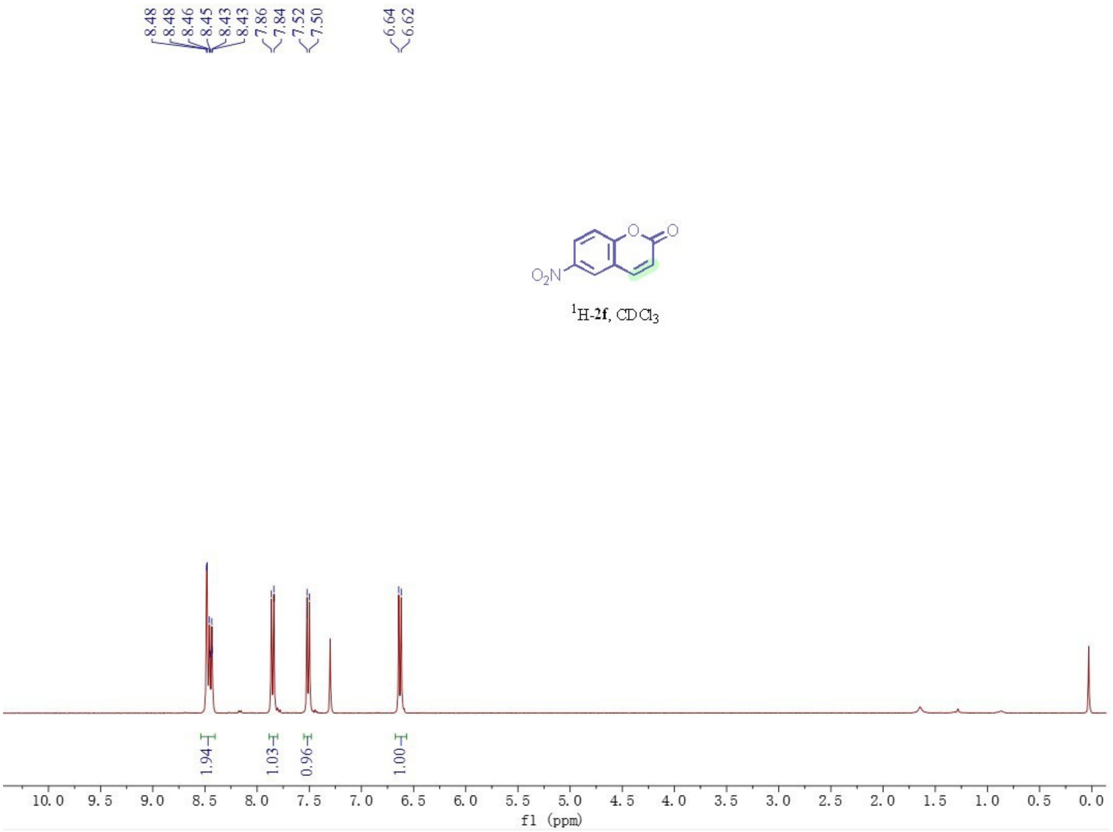

160.04
152.97
142.18
134.66
130.23
120.38
118.71
117.93
117.06
Br
O
O
^{13}C-**2d**, CDCl$_3$
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
f1 (ppm)



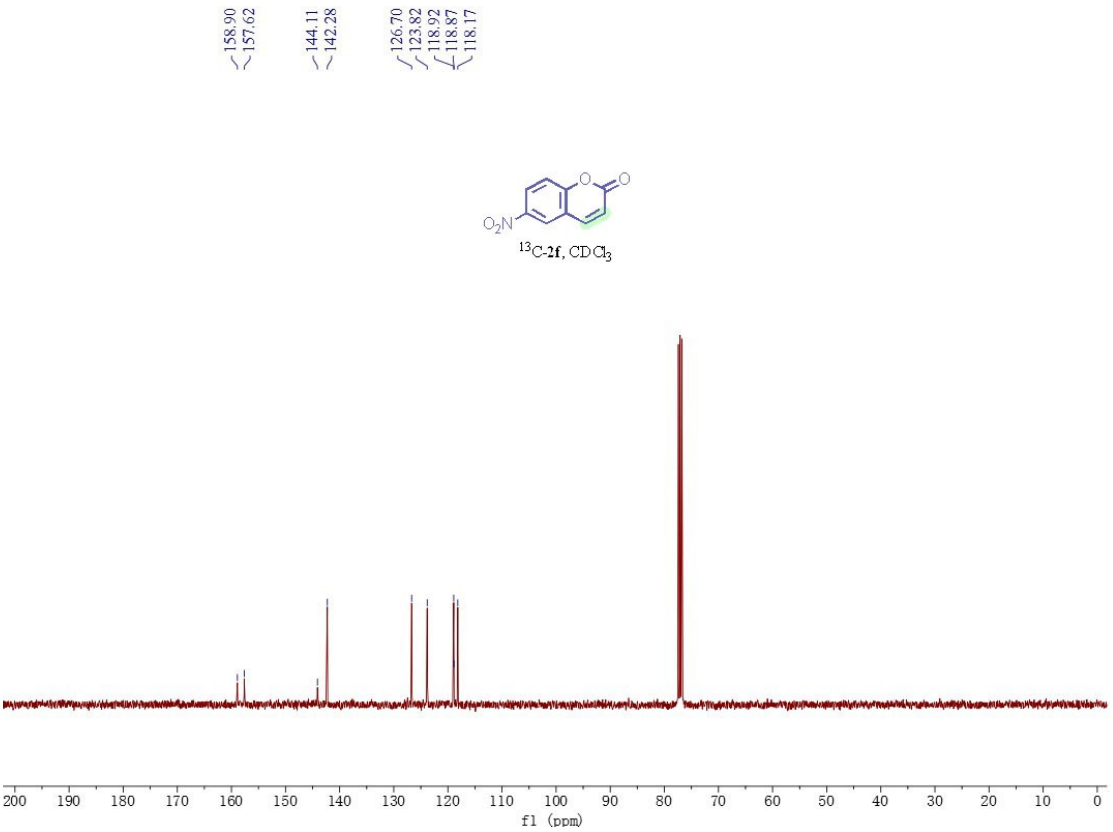
^{1}H-**2e**, *d6*-DMSO



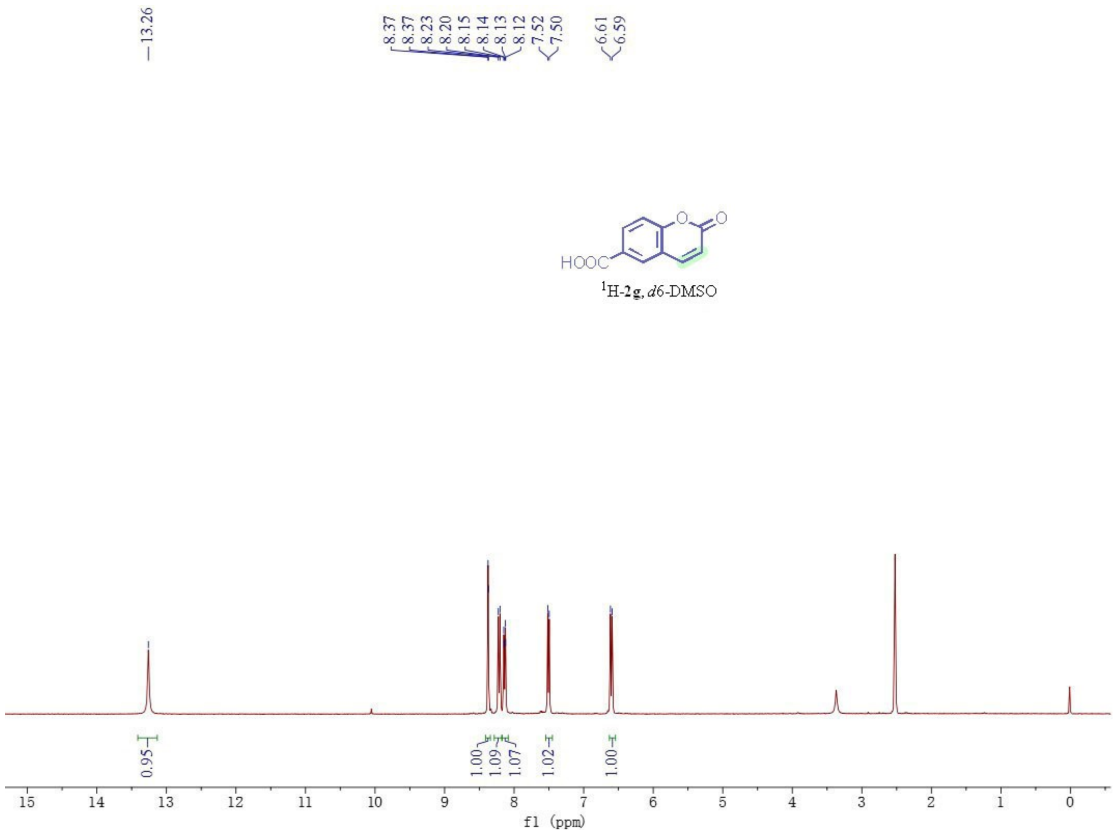
^{13}C-**2e**, *d6*-DMSO



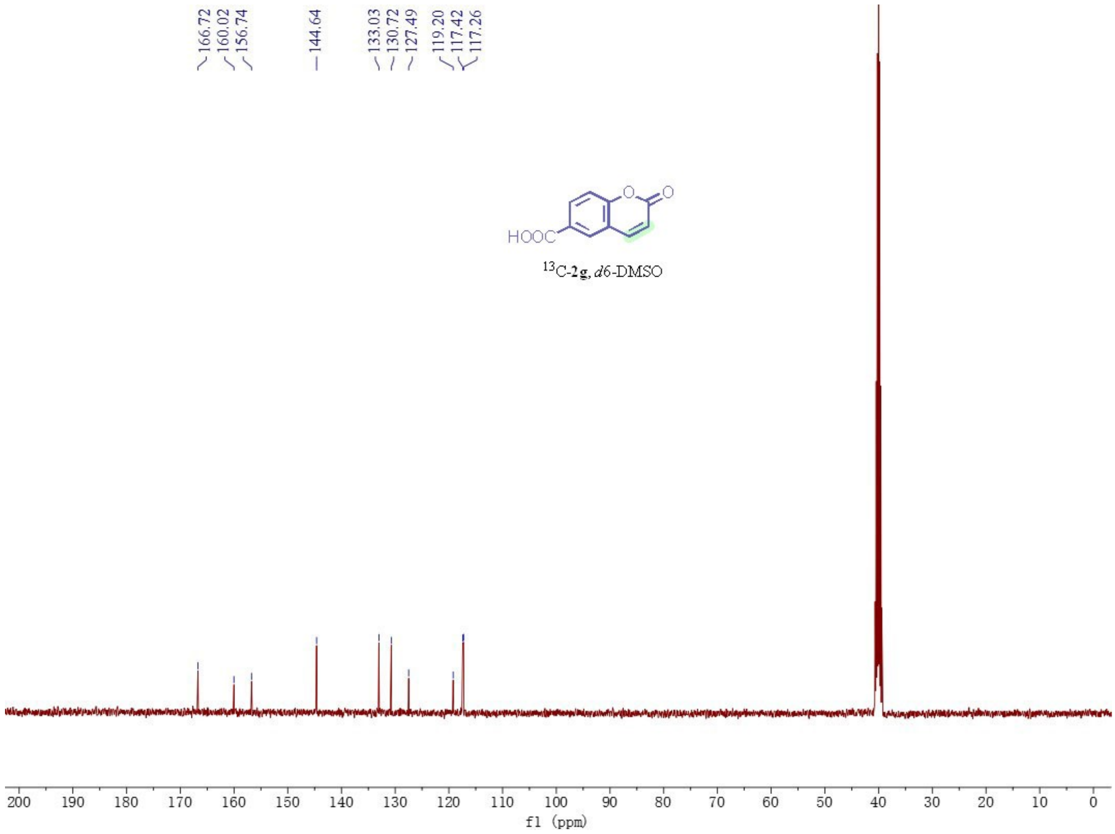
^{1}H-**2f**, CDCl$_3$



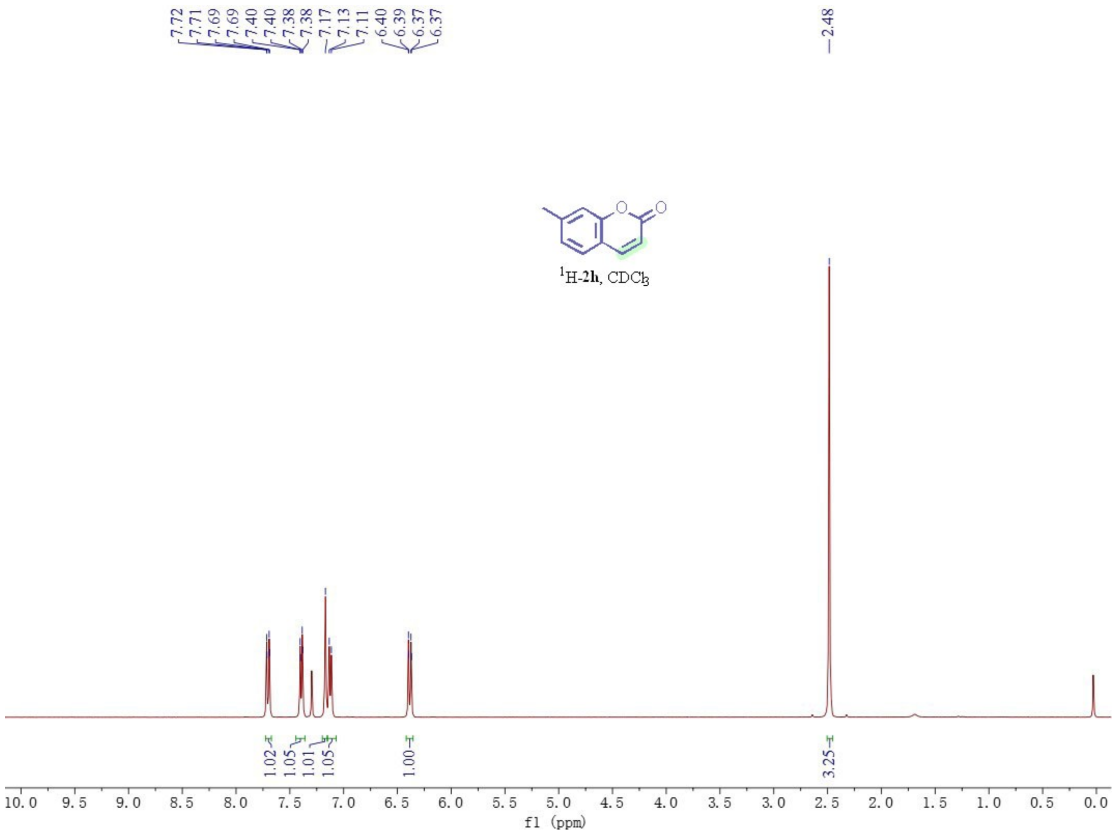
^{13}C-**2f**, CDCl$_3$

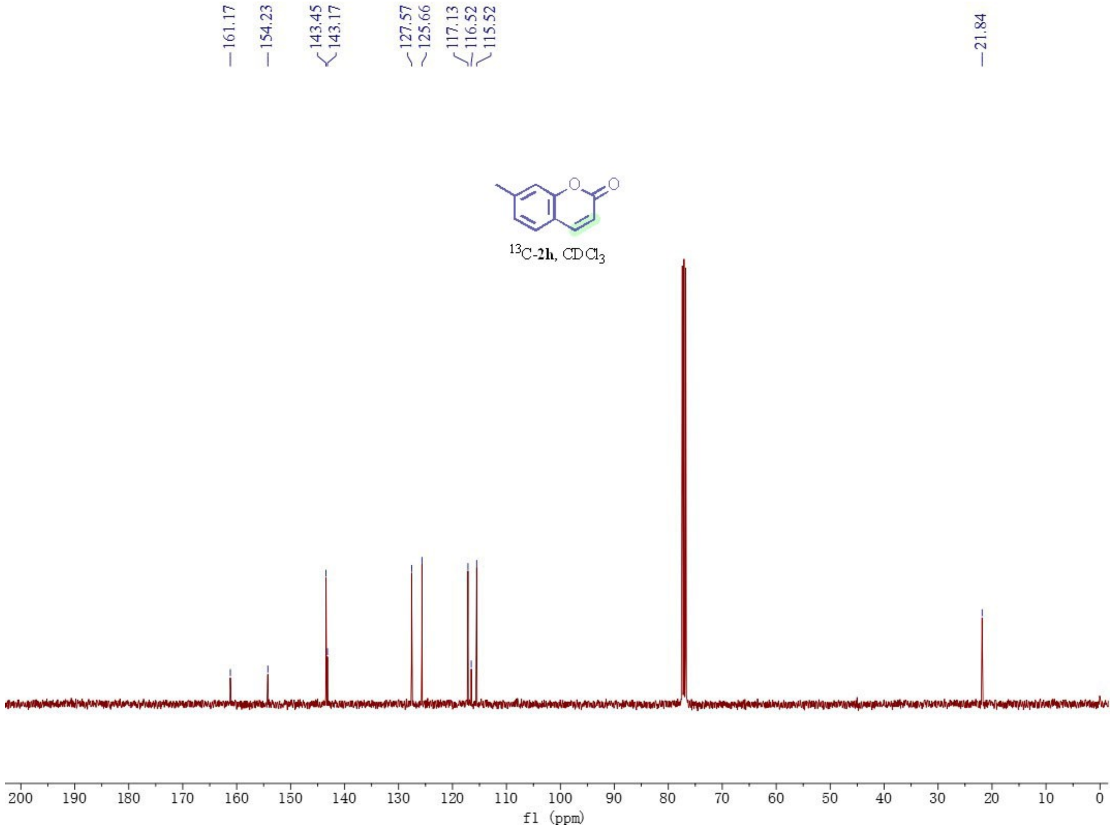


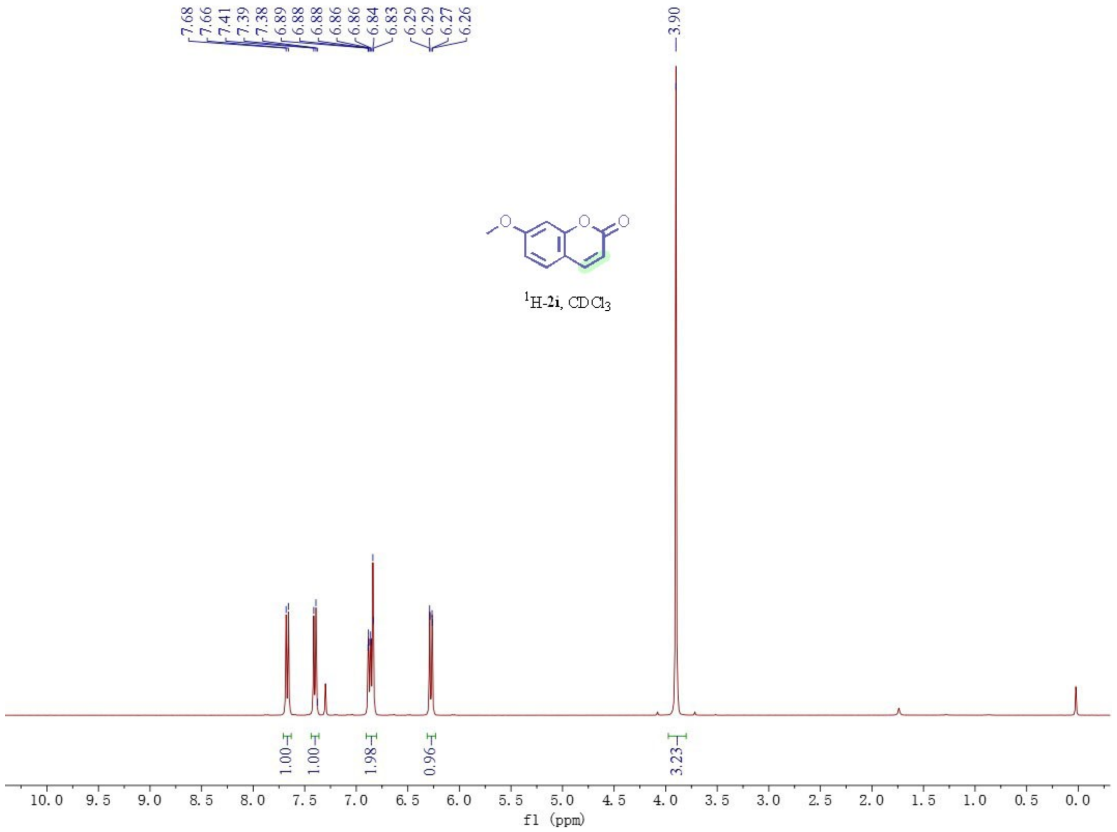
^{1}H-**2g**, *d6*-DMSO

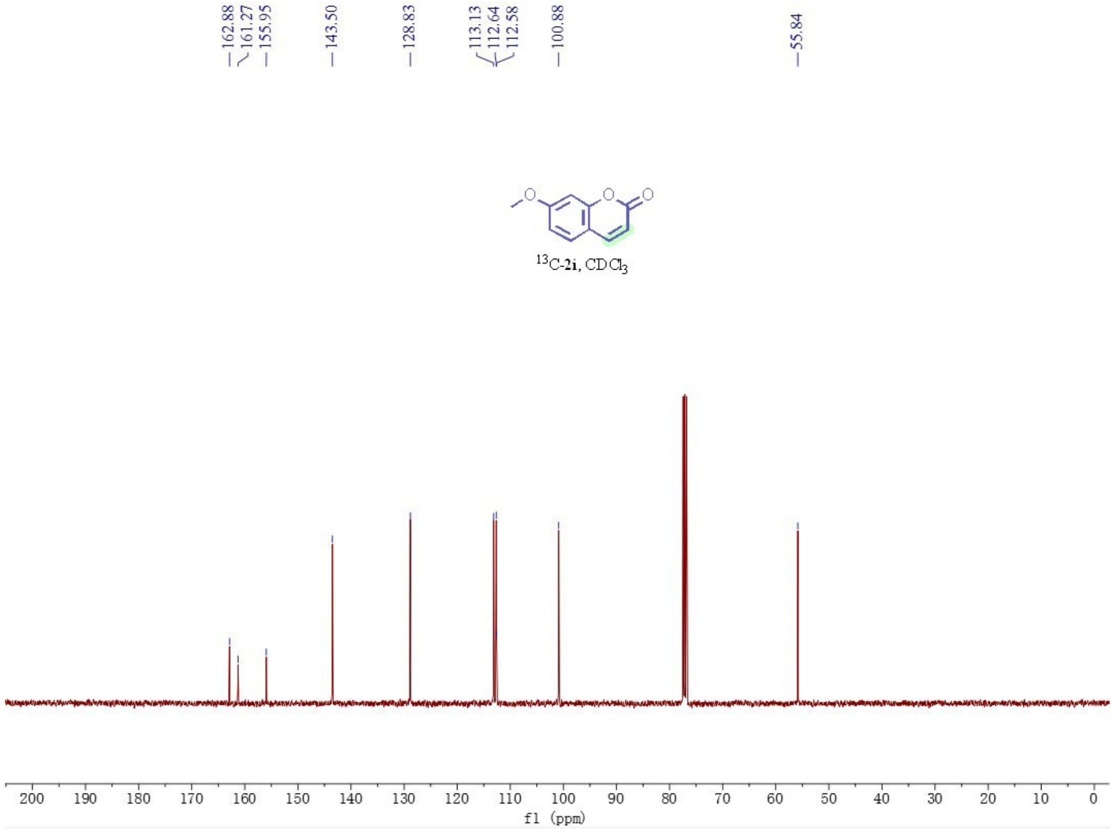


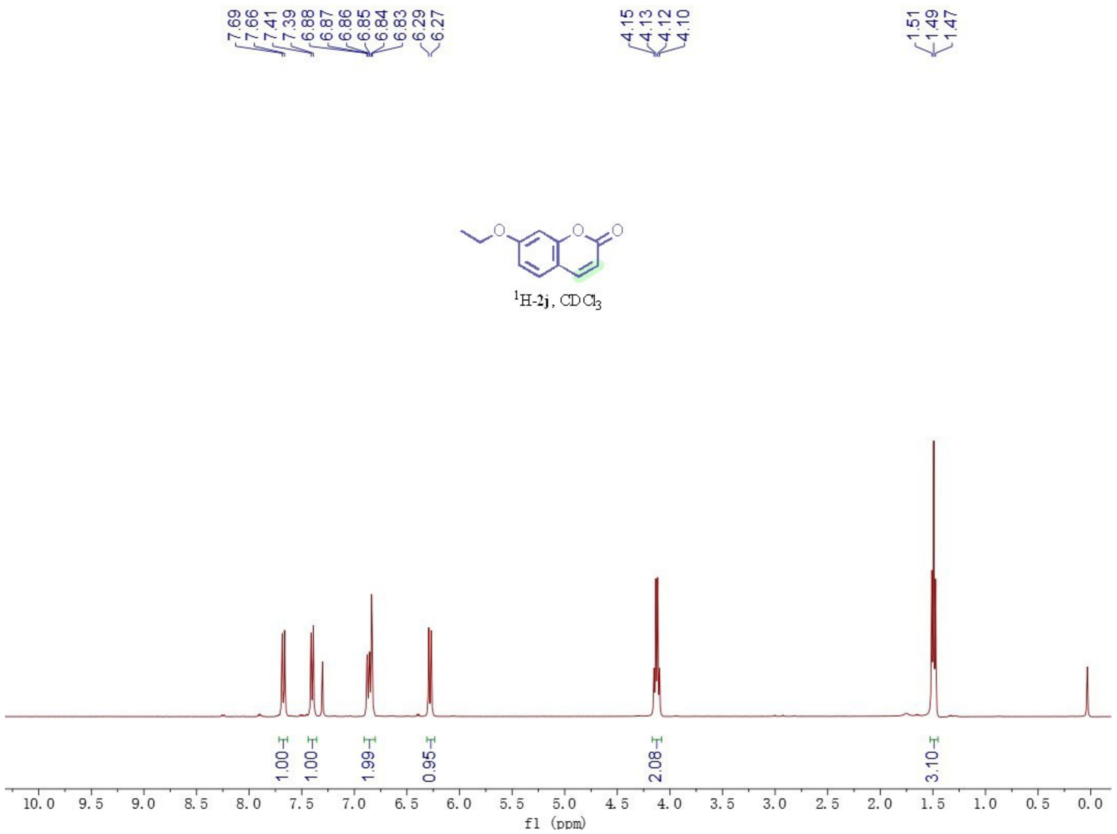
^{13}C-**2g**, *d6*-DMSO

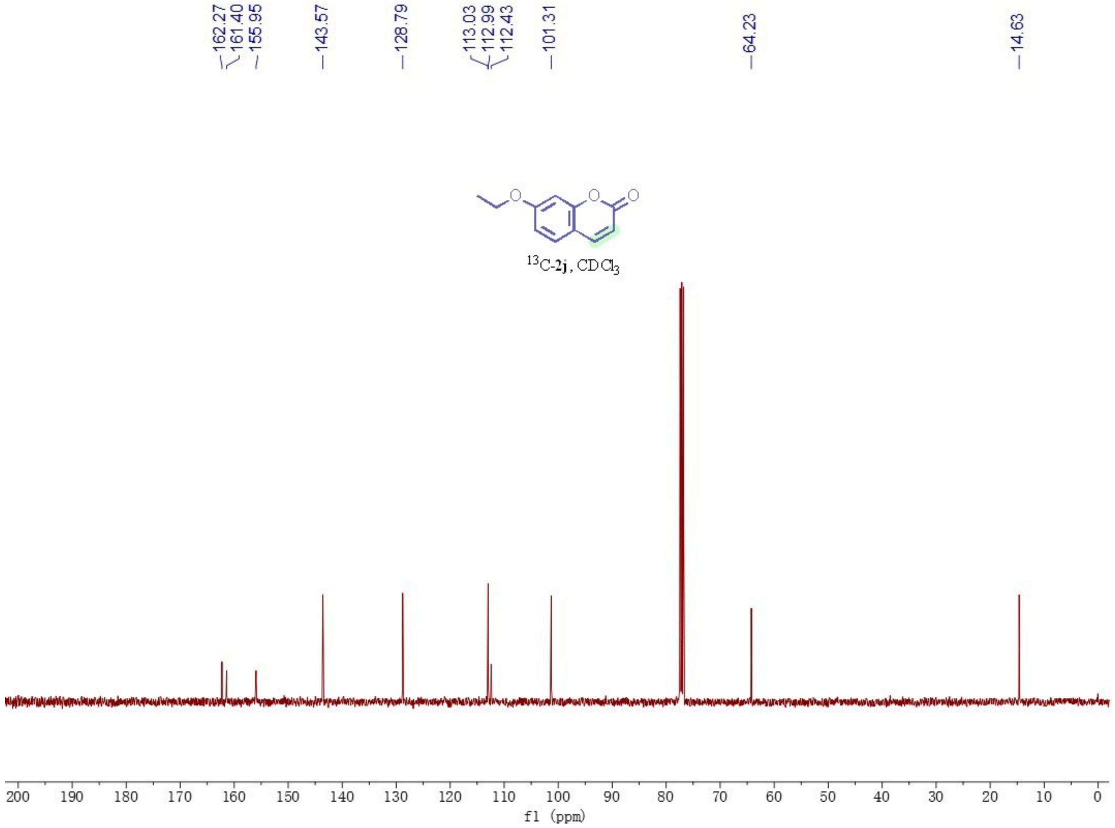

7.72
7.71
7.69
7.69
7.40
7.40
7.38
7.38
7.17
7.13
7.11
6.40
6.39
6.37
6.37
2.48
^{1}H-**2h**, CDCl$_3$
1.02
1.05
1.01
1.05
1.00
3.25
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
f1 (ppm)

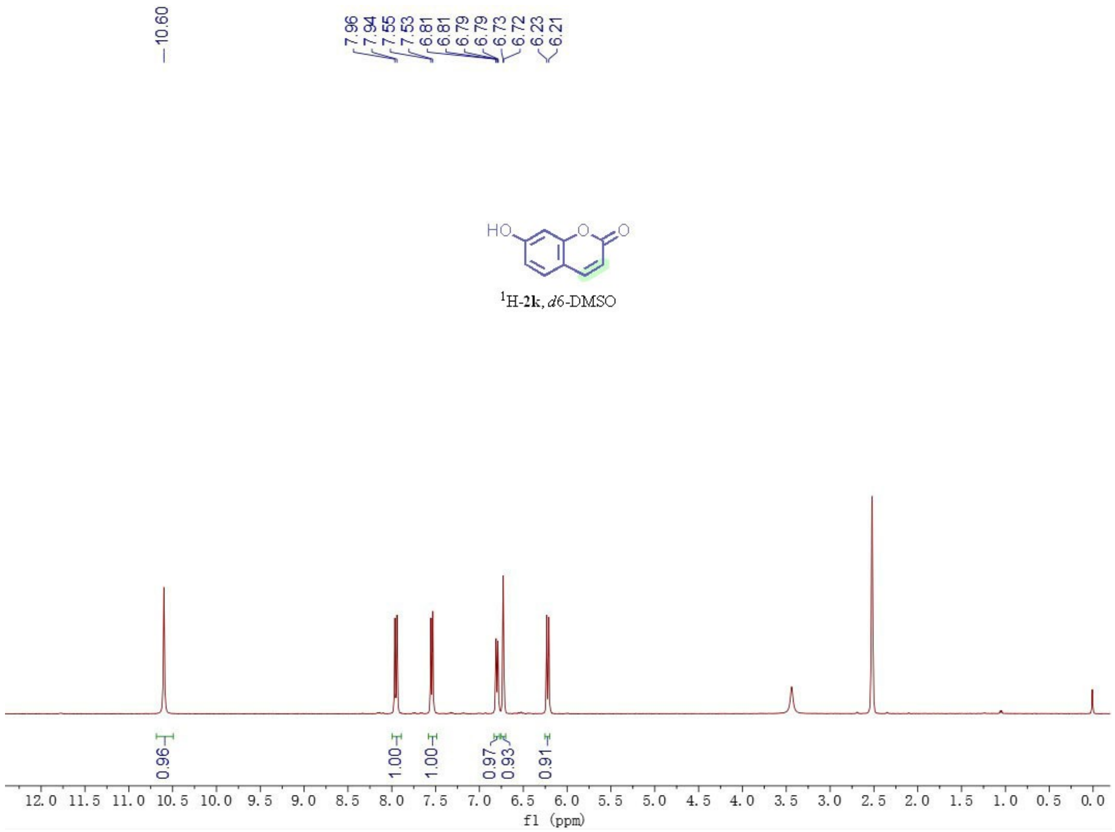

161.17
154.23
143.45
143.17
127.57
125.66
117.13
116.52
115.52
21.84
^{13}C-**2h**, CDCl$_3$
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
f1 (ppm)


7.68
7.66
7.41
7.39
7.38
6.89
6.88
6.88
6.86
6.86
6.84
6.83
6.29
6.29
6.27
6.26
3.90
^{1}H-**2i**, CDCl$_3$
1.00
1.00
1.98
0.96
3.23
10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0
f1 (ppm)

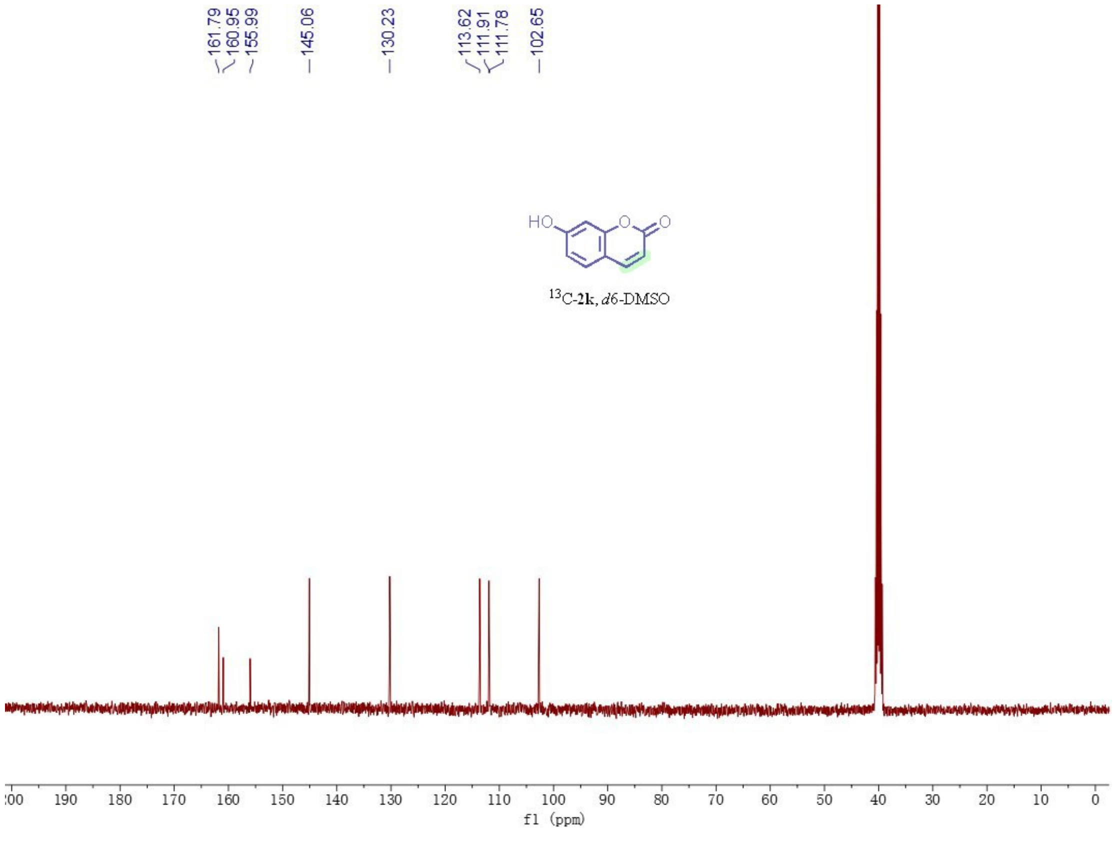

162.88
161.27
155.95
143.50
128.83
113.13
112.64
112.58
100.88
55.84
^{13}C-**2i**, CDCl$_3$
200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
f1 (ppm)


7.69
7.66
7.41
7.39
6.88
6.87
6.86
6.85
6.84
6.83
6.29
6.27
4.15
4.13
4.12
4.10
1.51
1.49
1.47
^{1}H-**2j**, CDCl$_3$
1.00
1.00
1.99
0.95
2.08
3.10
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
f1 (ppm)

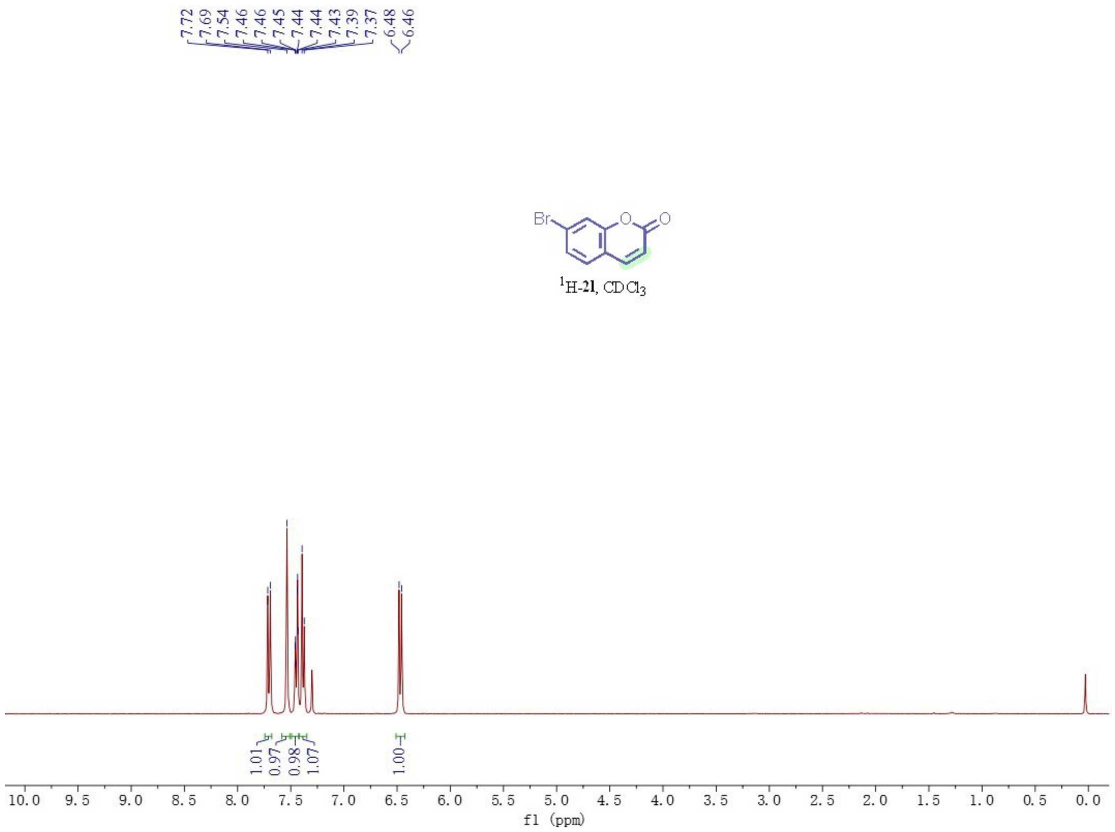

162.27
161.40
155.95
143.57
128.79
113.03
112.99
112.43
101.31
64.23
14.63
^{13}C-**2j**, CDCl$_3$
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
f1 (ppm)

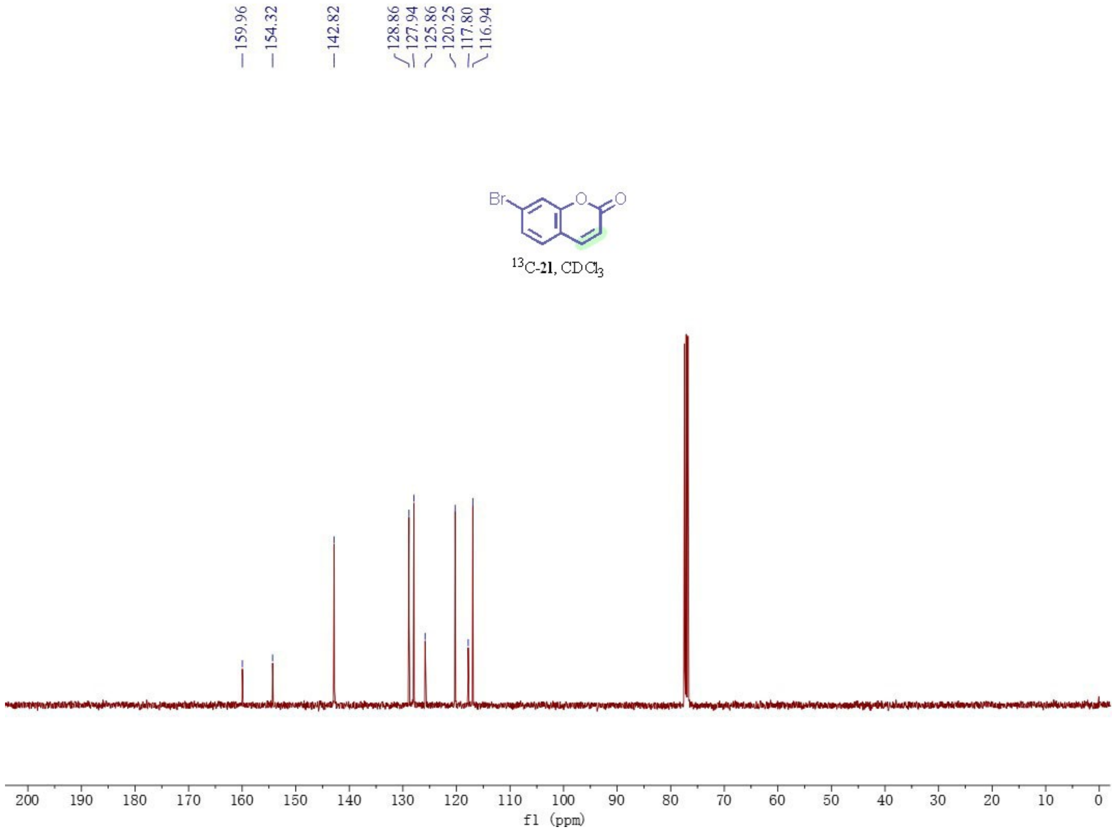


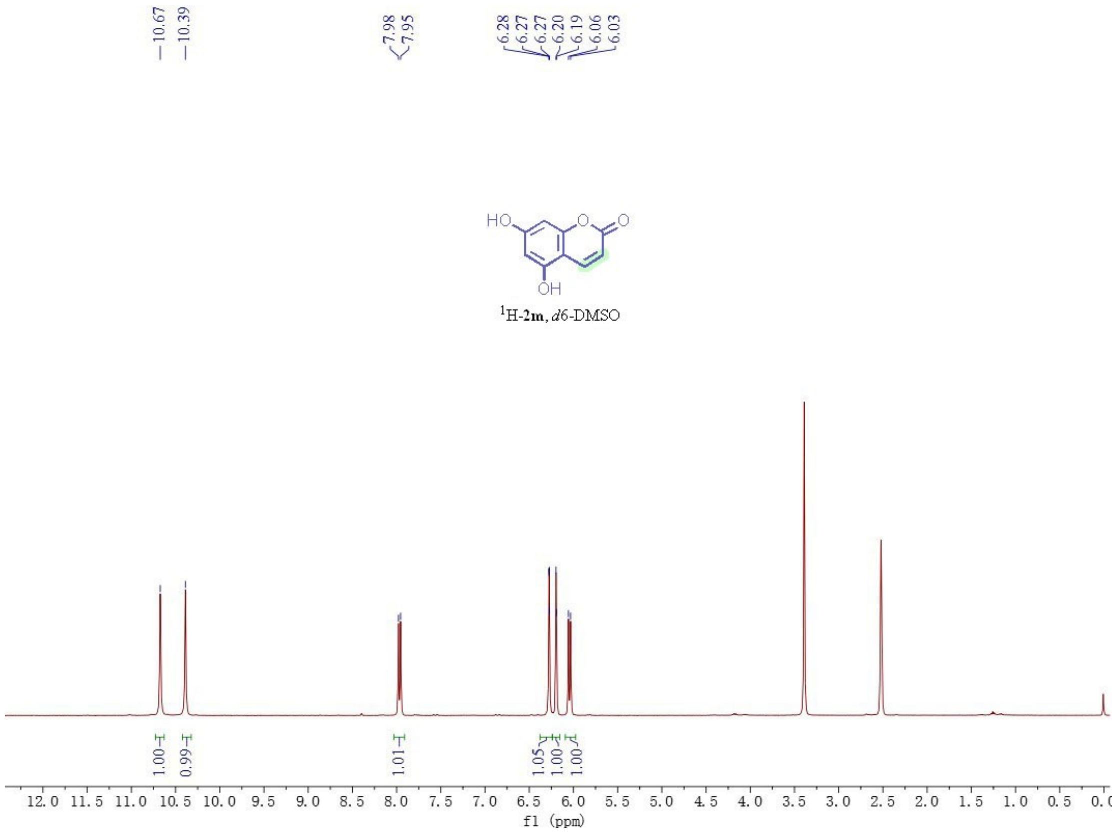
^{1}H-**2k**, *d6*-DMSO

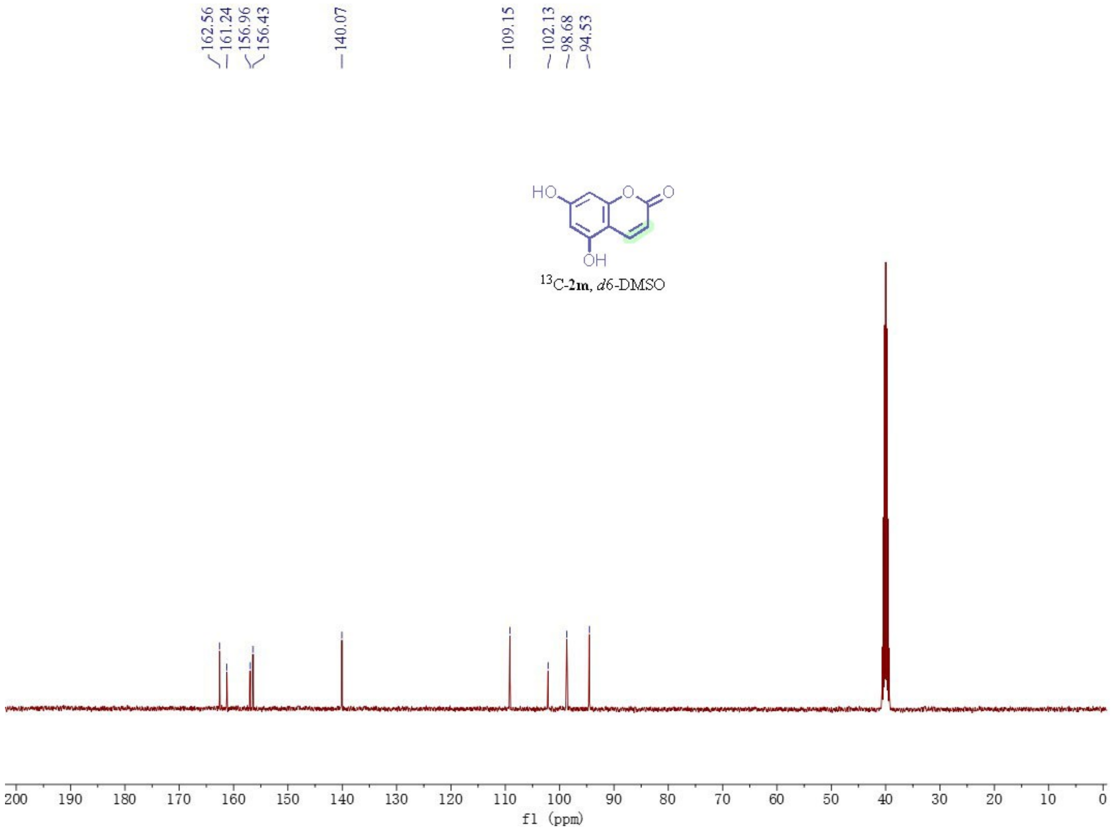


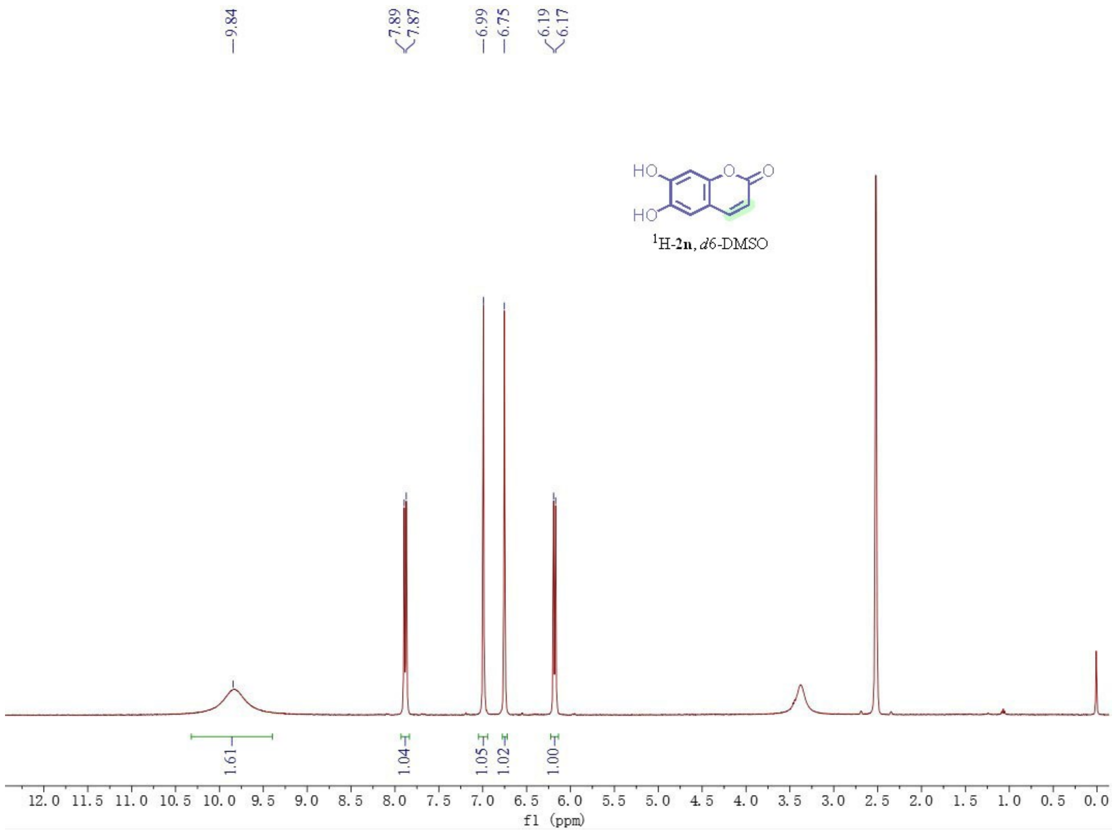
^{13}C-**2k**, *d6*-DMSO

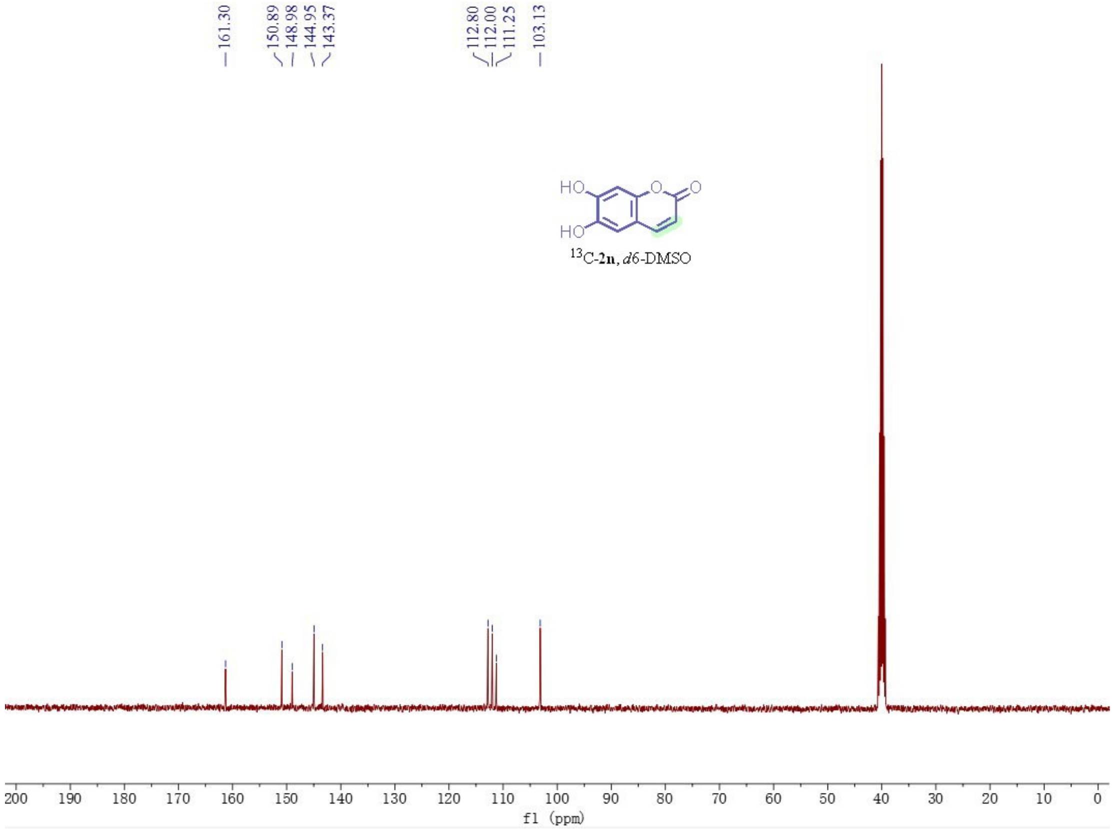

^{1}H-**21**, CDCl$_3$

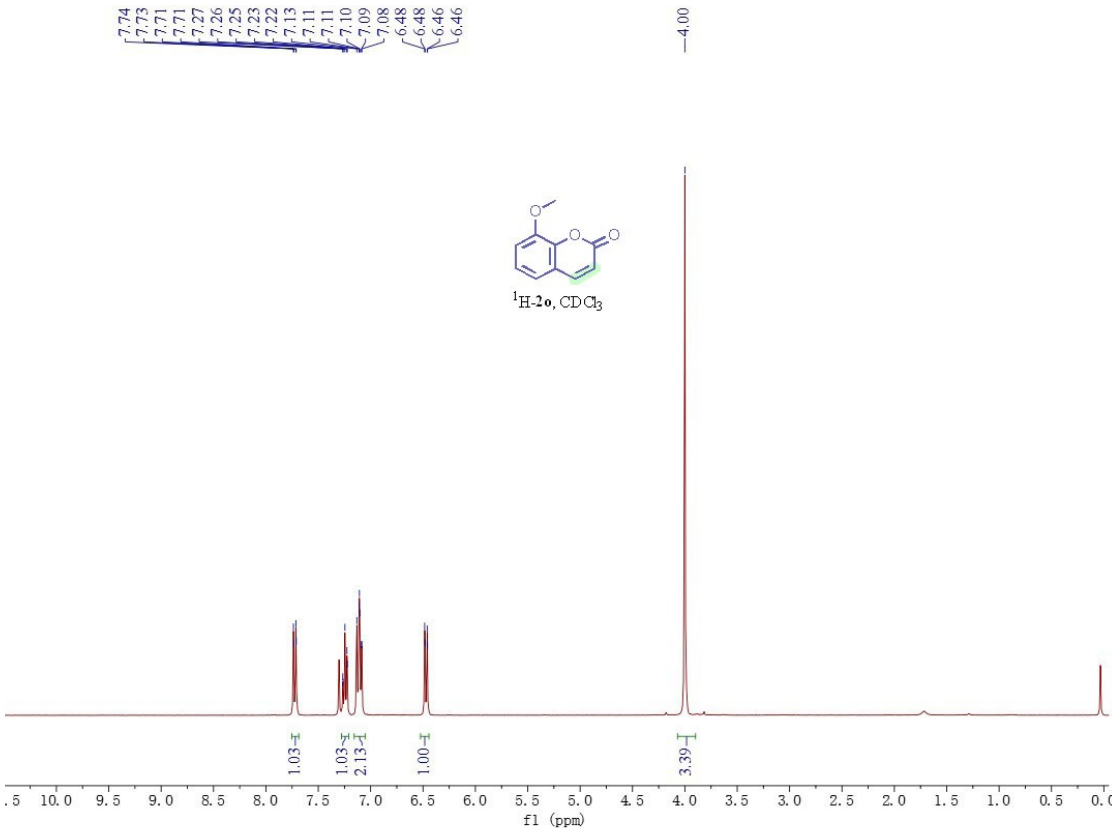

^{13}C-**21**, CDCl$_3$


10.67
10.39
7.98
7.95
6.28
6.27
6.27
6.20
6.19
6.06
6.03
HO
O
O
OH
^{1}H-**2m**, *d*6-DMSO
1.00
0.99
1.01
1.05
1.00
1.00
12.0 11.5 11.0 10.5 10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0
f1 (ppm)

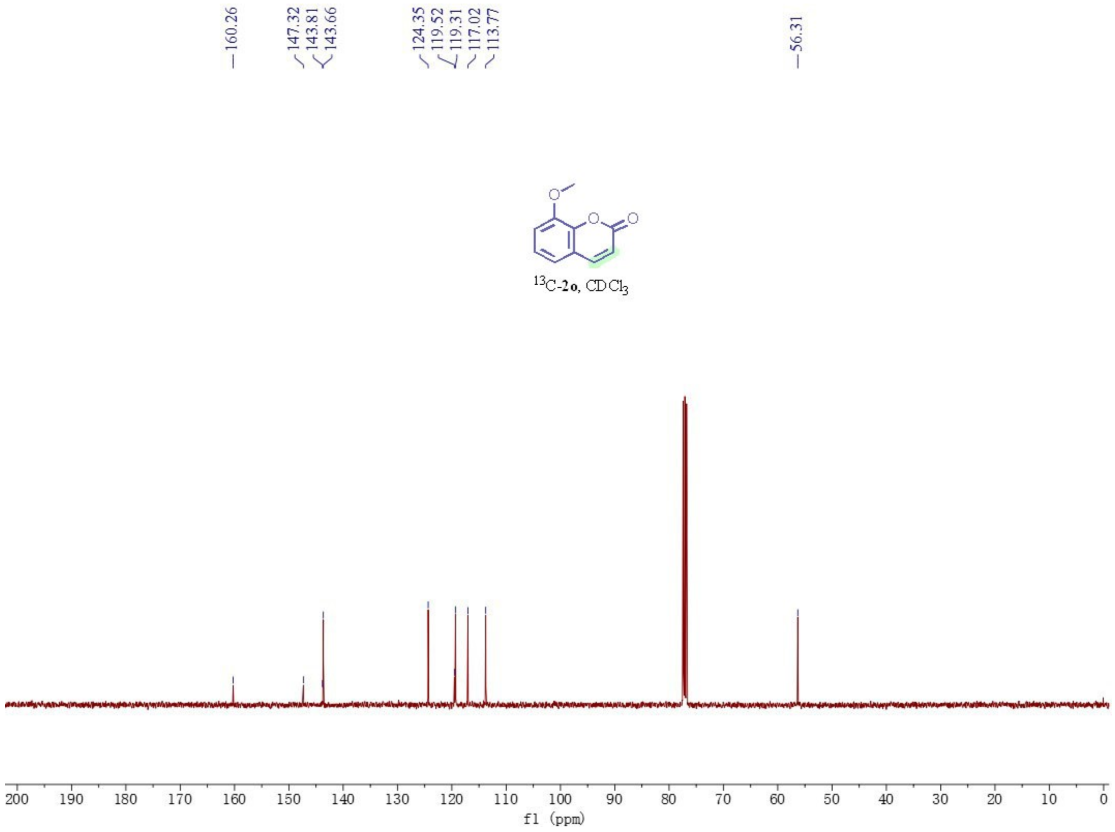

162.56
161.24
156.96
156.43
140.07
109.15
102.13
98.68
94.53
HO
O
O
OH
^{13}C-**2m**, *d*6-DMSO
200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
f1 (ppm)


9.84
7.89
7.87
6.99
6.75
6.19
6.17
HO
HO
O
O
^{1}H-**2n**, *d6*-DMSO
1.61
1.04
1.05
1.02
1.00
12.0 11.5 11.0 10.5 10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0
f1 (ppm)

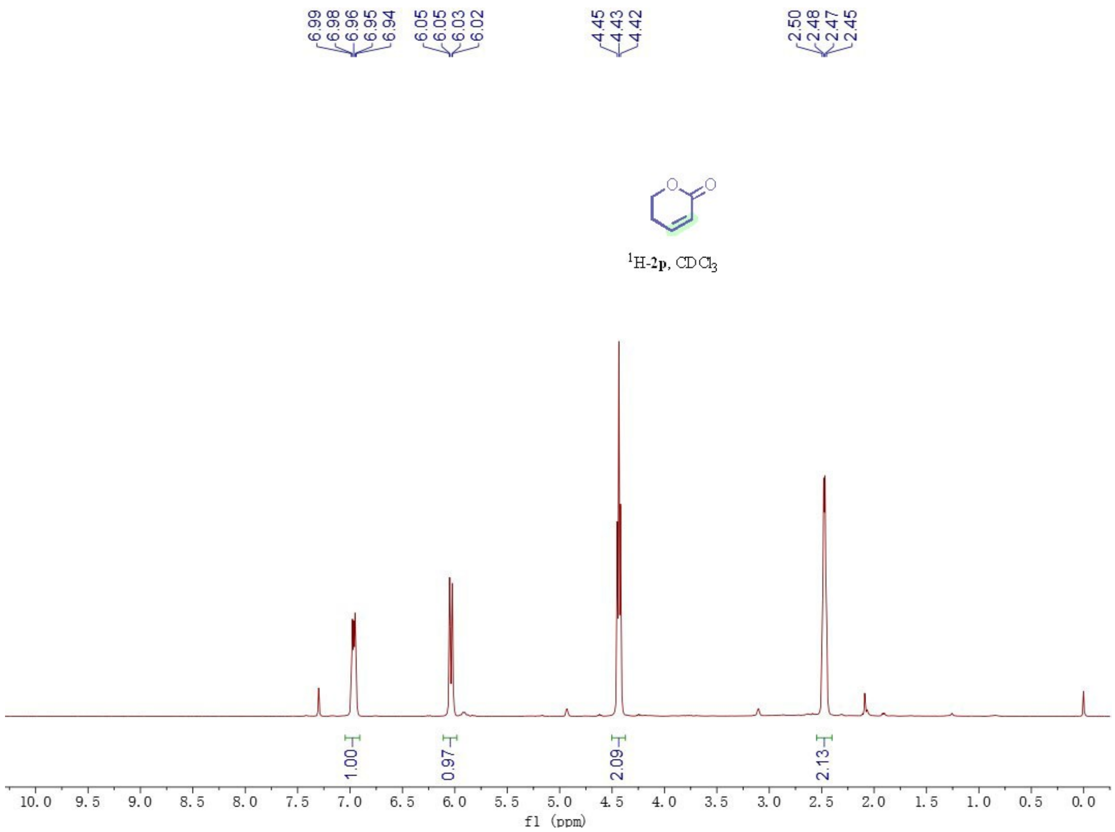

161.30
150.89
148.98
144.95
143.37
112.80
112.00
111.25
103.13
HO
HO
O
O
^{13}C-**2n**, *d6*-DMSO
200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
f1 (ppm)



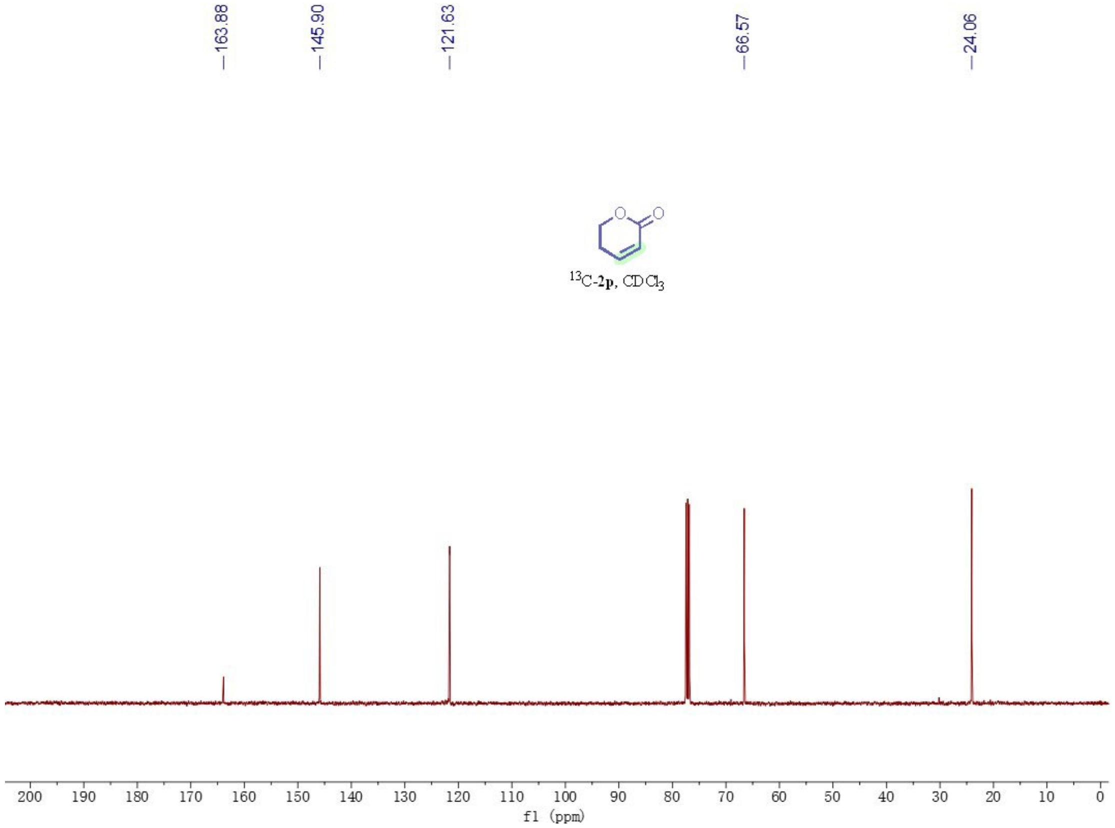
^{1}H-**2o**, $CDCl_3$



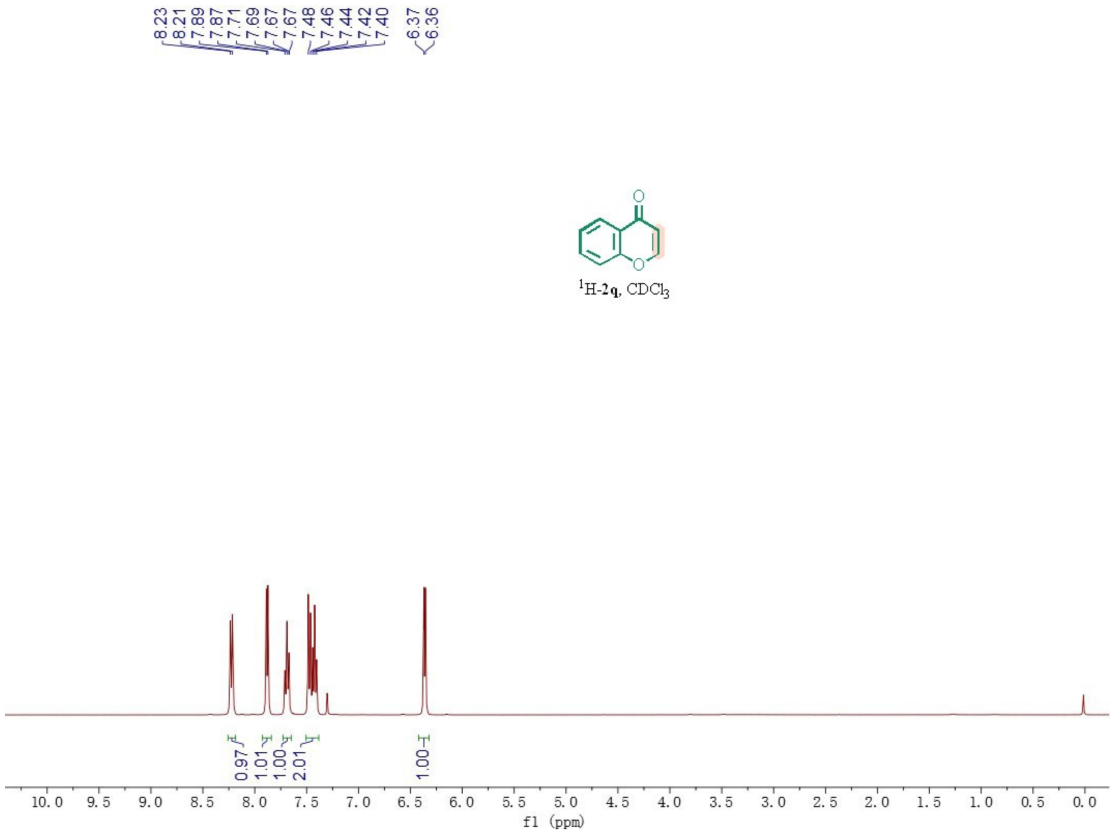
^{13}C-**2o**, $CDCl_3$

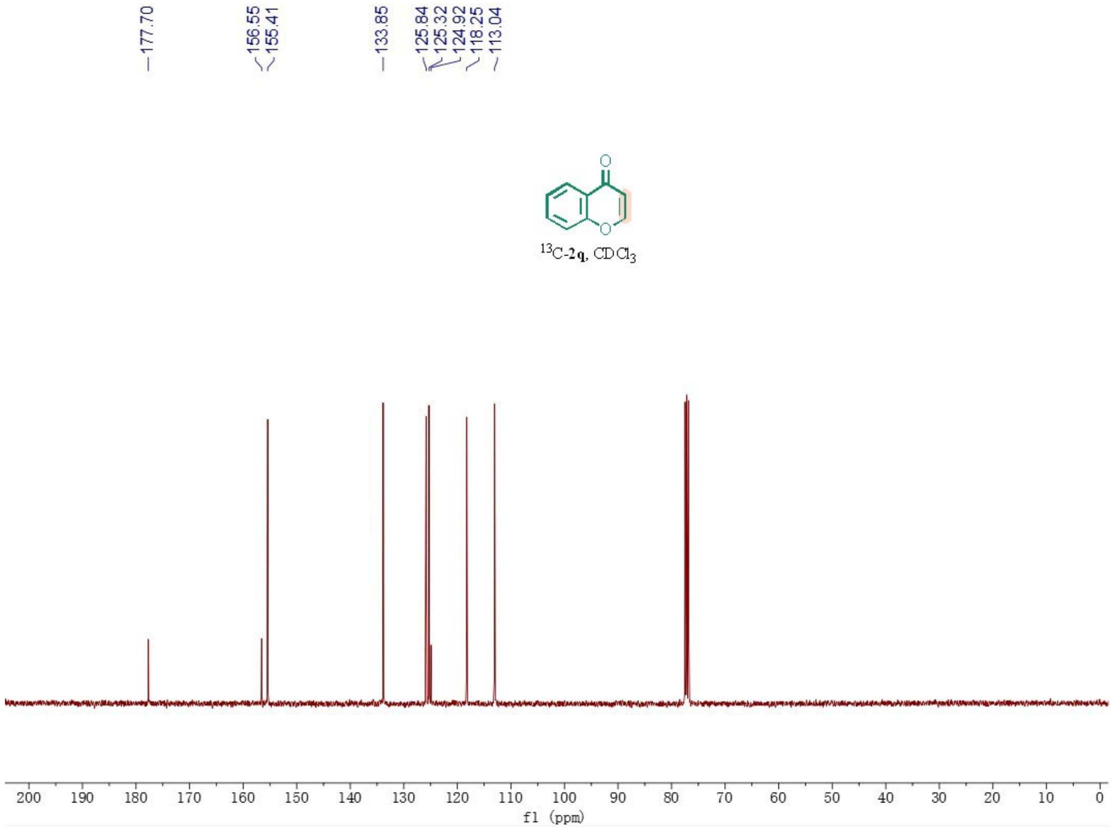


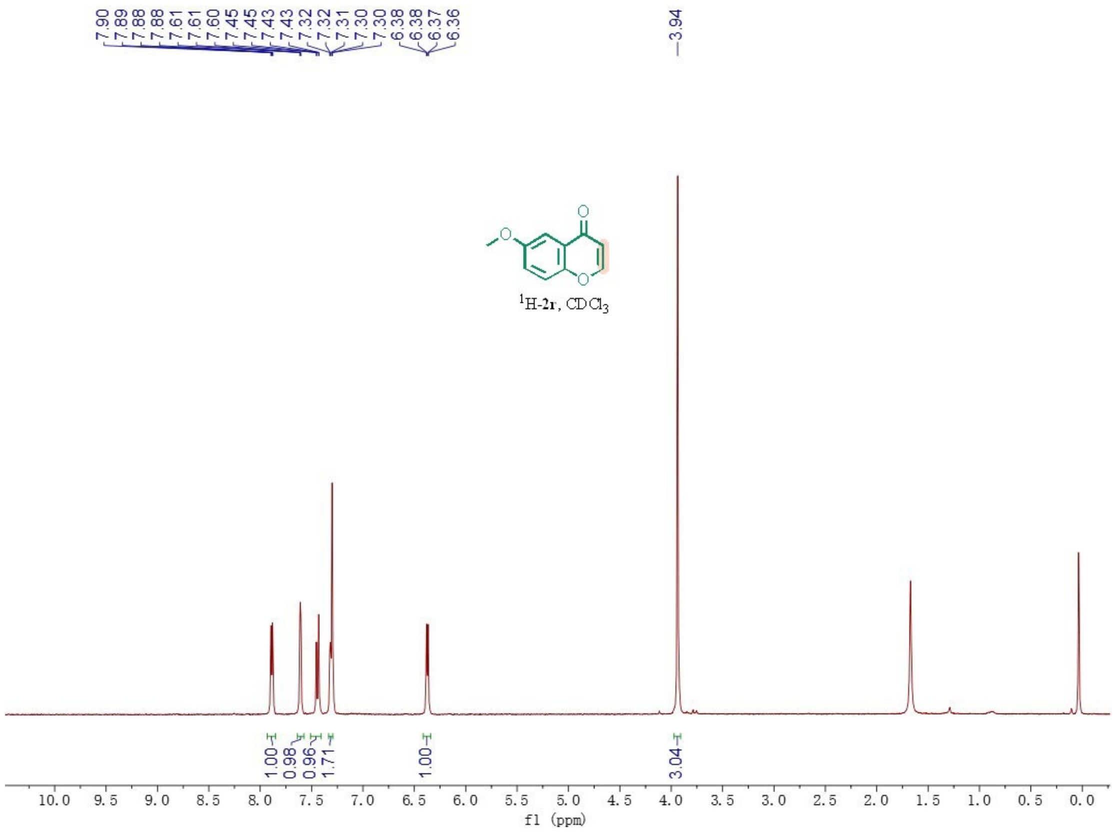
^{1}H-**2p**, CDCl$_3$

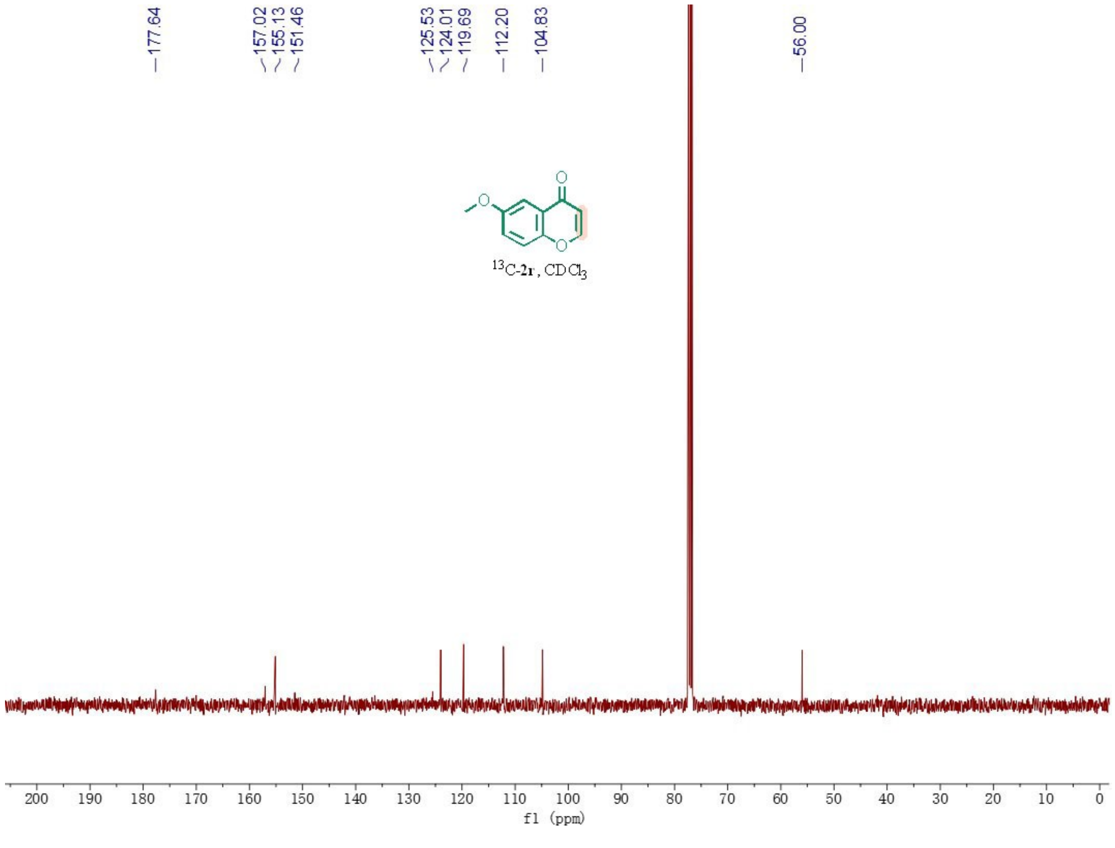


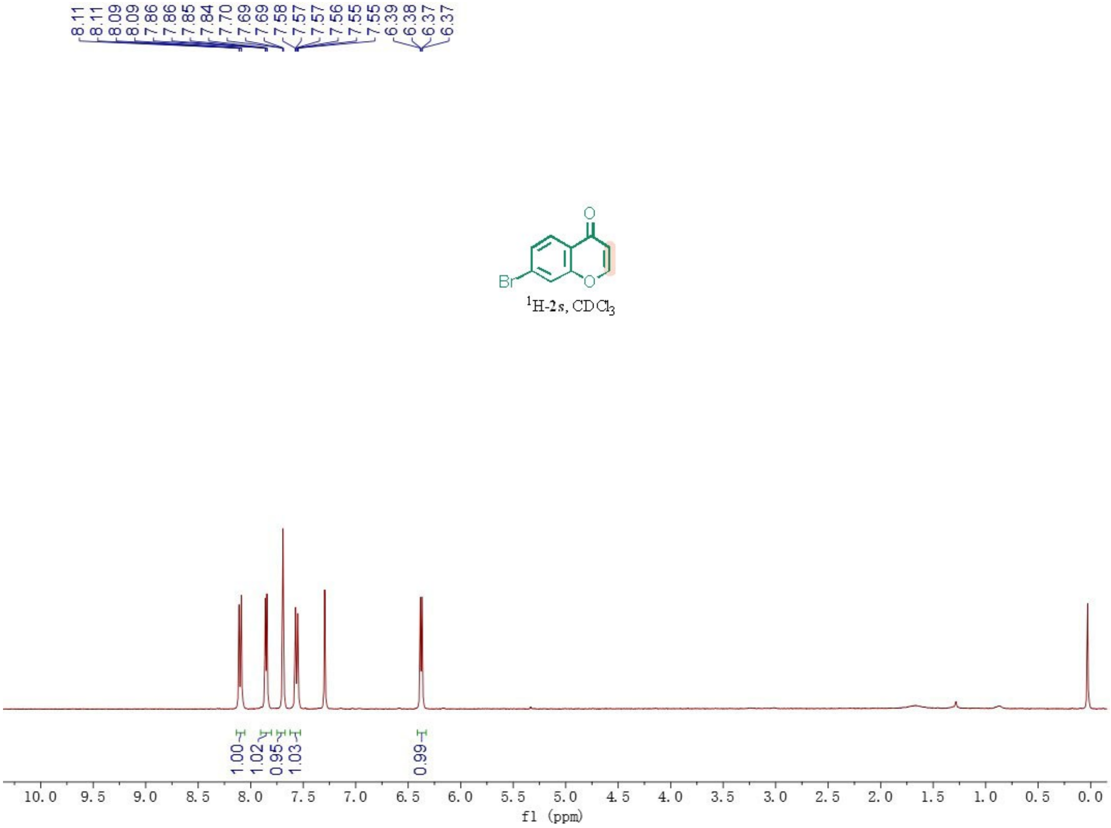
^{13}C-**2p**, CDCl$_3$

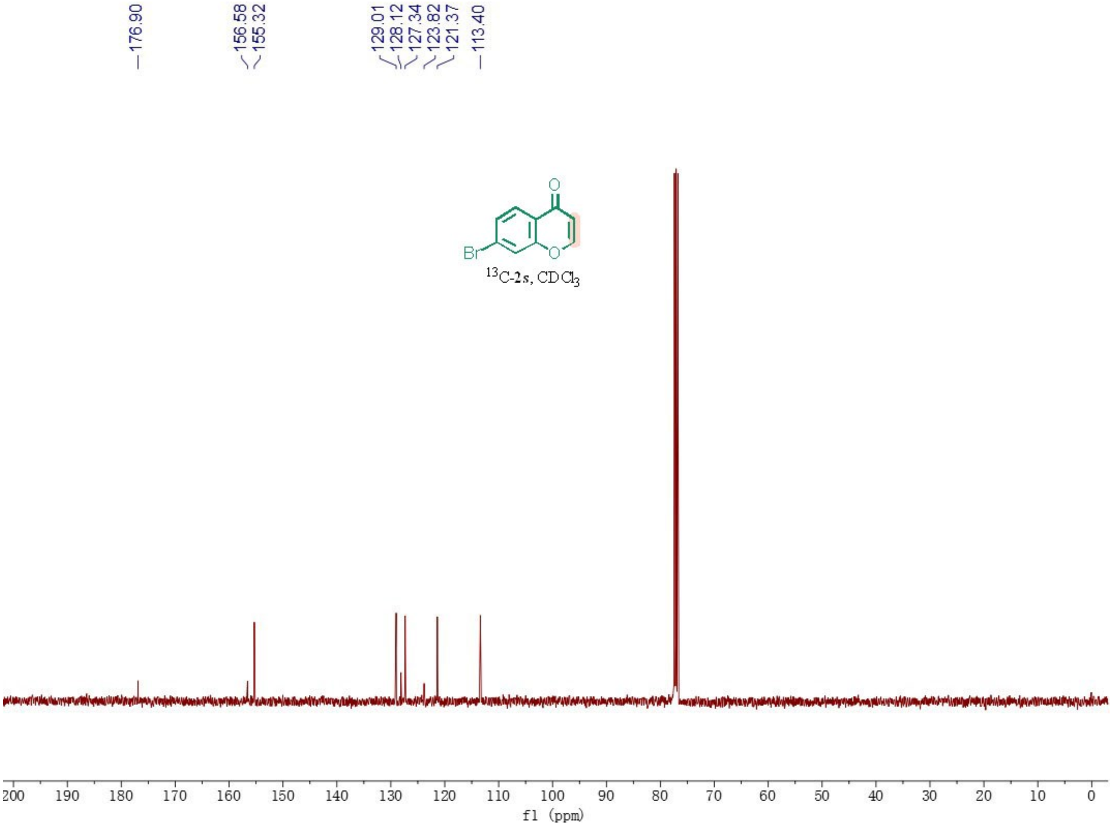

8.23
8.21
7.89
7.87
7.71
7.69
7.67
7.67
7.48
7.46
7.44
7.42
7.40
6.37
6.36
^{1}H-**2q**, CDCl$_3$
0.97
1.01
1.00
2.01
1.00
10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0
f1 (ppm)

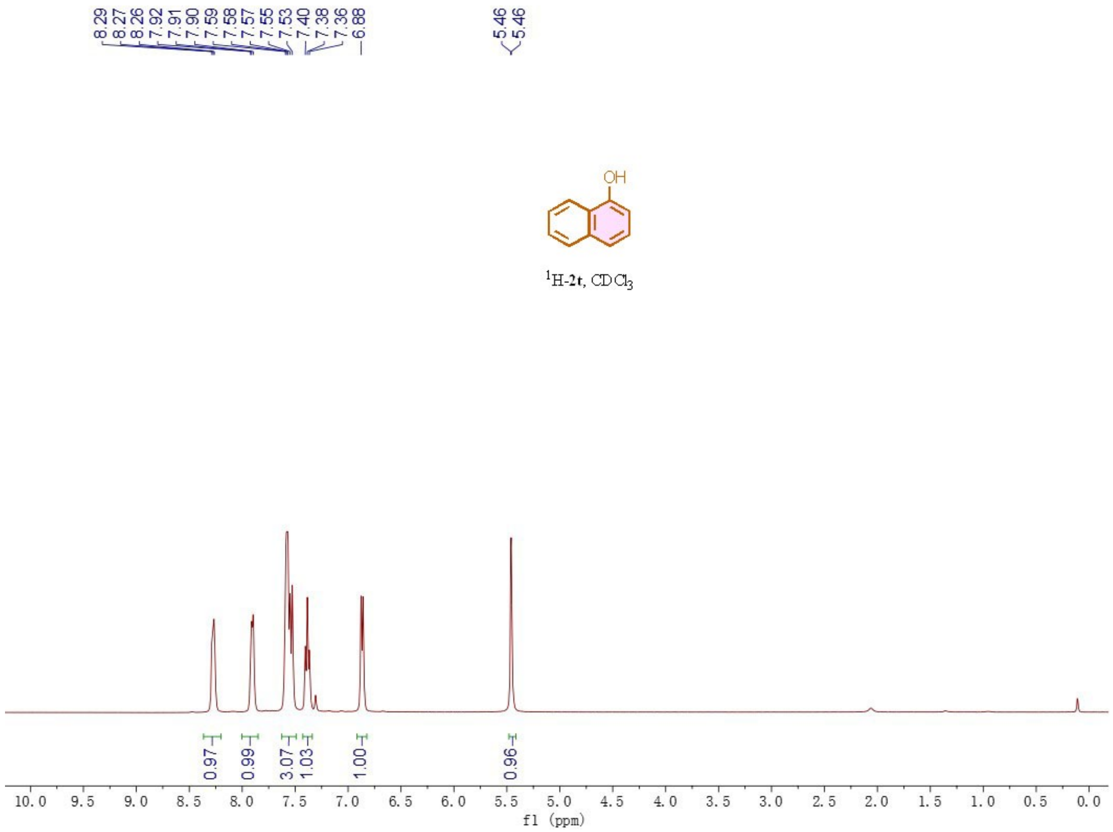

177.70
156.55
155.41
133.85
125.84
125.32
124.92
118.25
113.04
^{13}C-**2q**, CDCl$_3$
200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
f1 (ppm)


7.90
7.89
7.88
7.88
7.61
7.60
7.45
7.45
7.43
7.43
7.32
7.32
7.31
7.30
7.30
6.38
6.38
6.37
6.36
3.94
O
O
O
^{1}H-**2r**, CDCl$_3$
1.00
0.98
0.96
1.71
1.00
3.04
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
f1 (ppm)

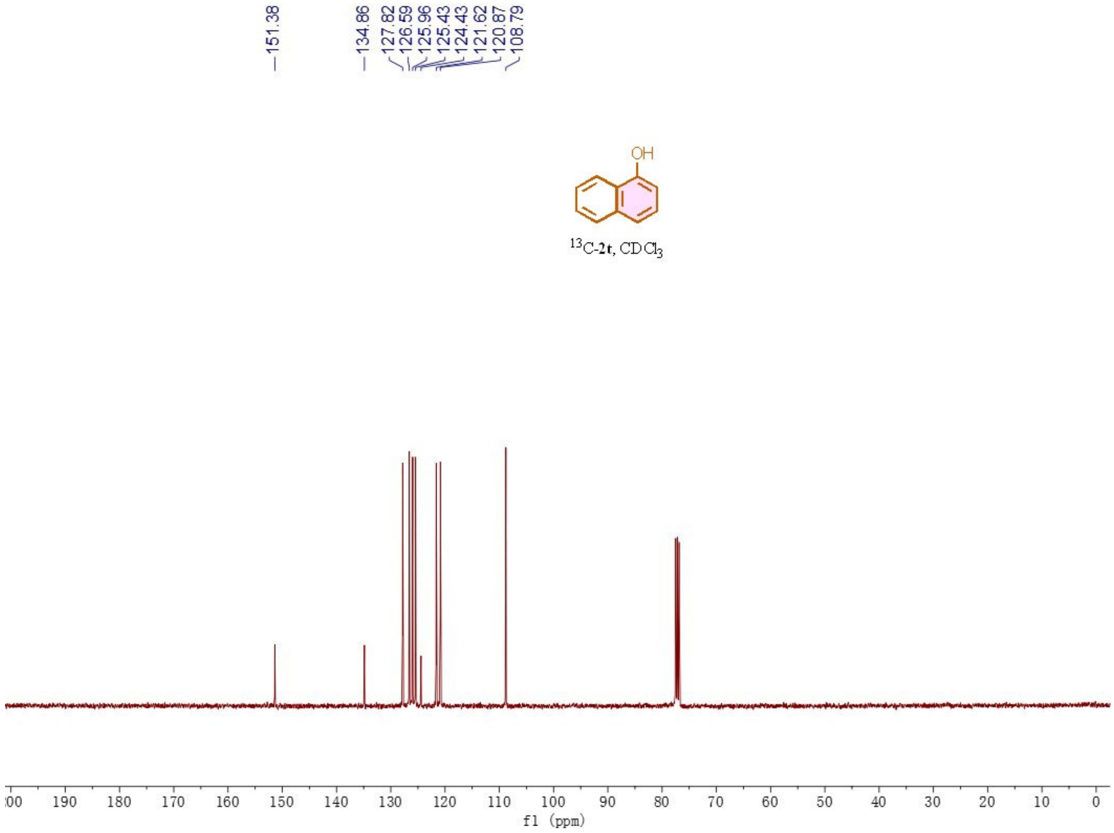

177.64
157.02
155.13
151.46
125.53
124.01
119.69
112.20
104.83
56.00
O
O
O
^{13}C-**2r**, CDCl$_3$
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
f1 (ppm)


8.11
8.11
8.09
8.09
7.86
7.85
7.84
7.70
7.69
7.69
7.58
7.57
7.57
7.56
7.55
6.39
6.38
6.37
6.37
O
Br
O
^{1}H-**2s**, CDCl$_3$
1.00
1.02
0.95
1.03
0.99
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
f1 (ppm)

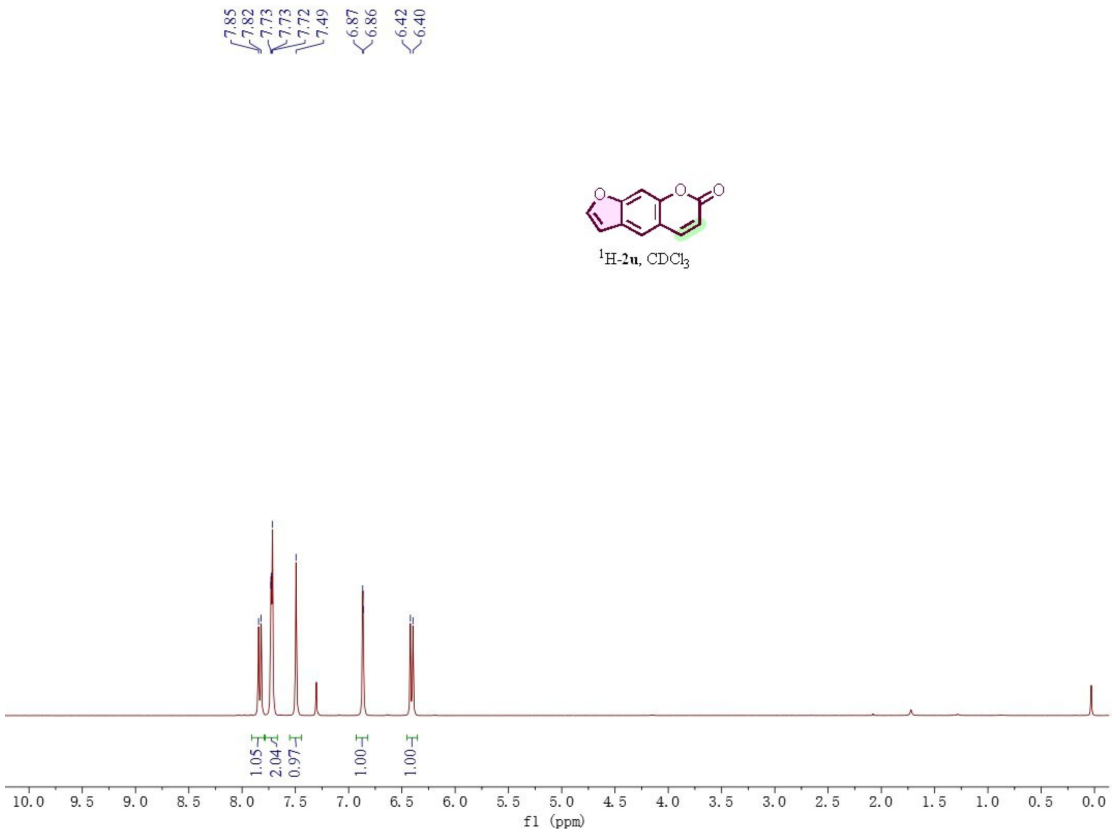

176.90
156.58
155.32
129.01
128.12
127.34
123.82
121.37
113.40
O
Br
O
^{13}C-**2s**, CDCl$_3$
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
f1 (ppm)

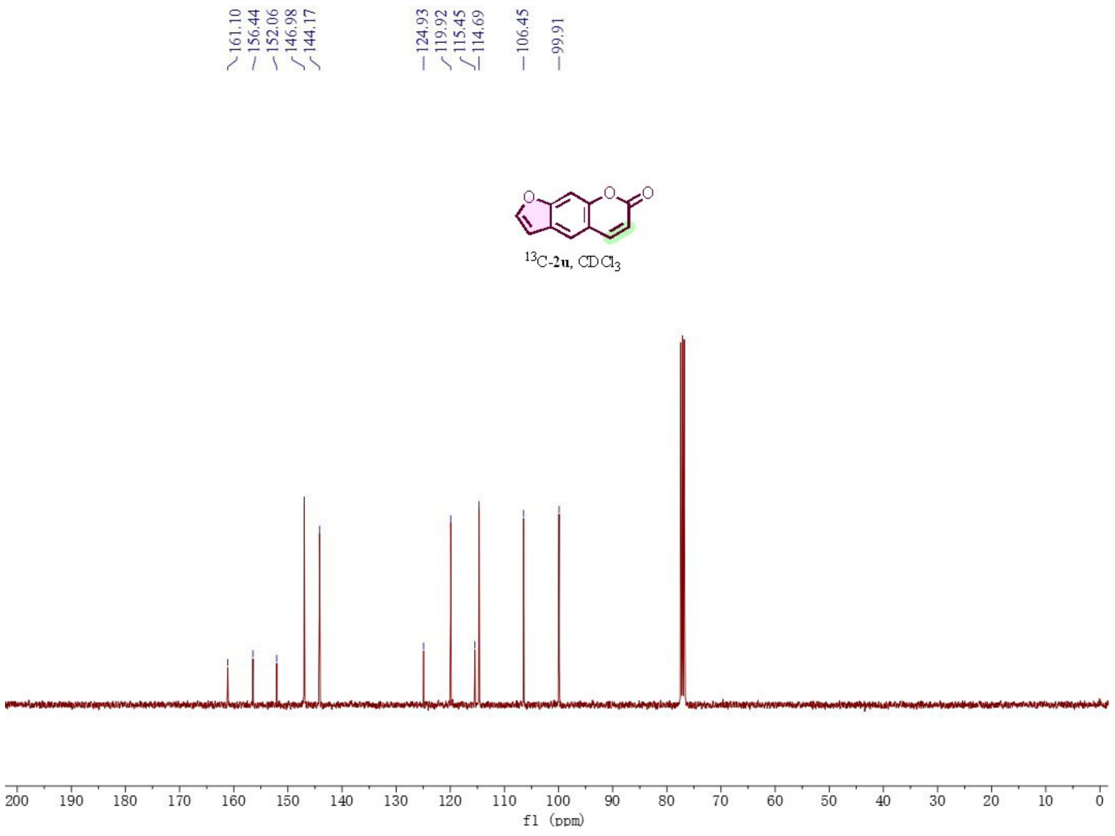


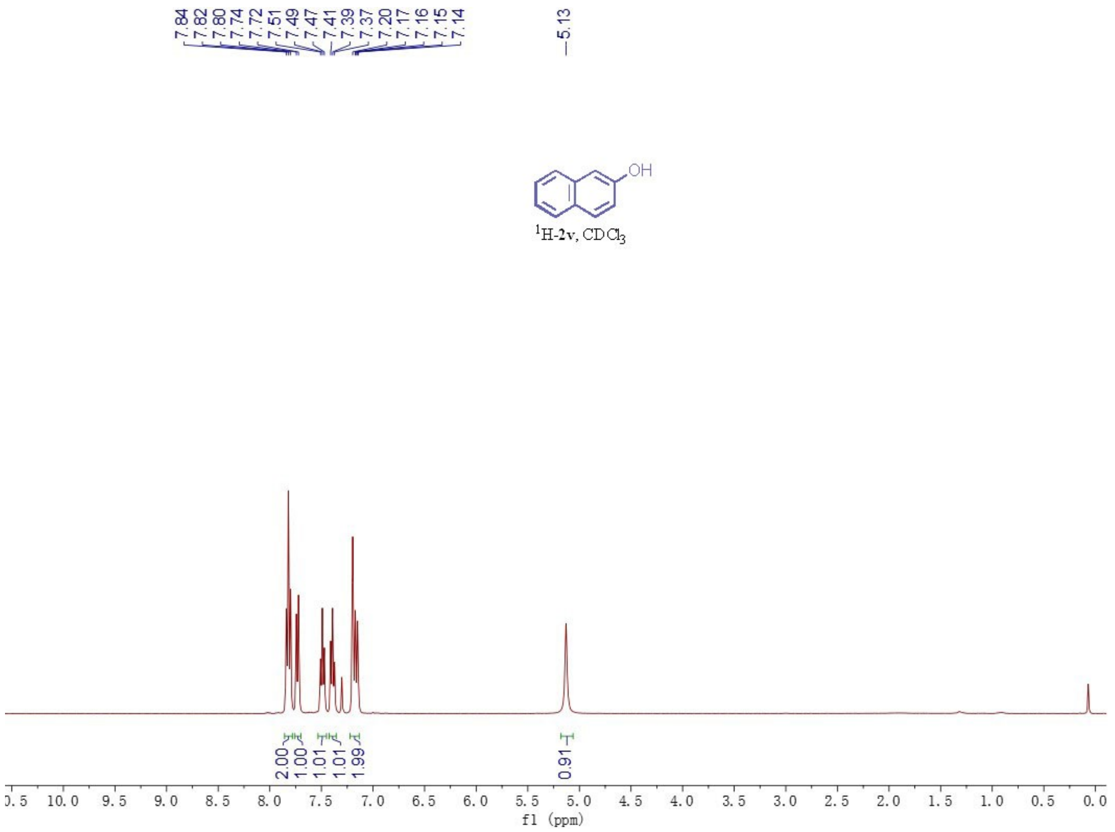
^{1}H-**2t**, CDCl$_3$

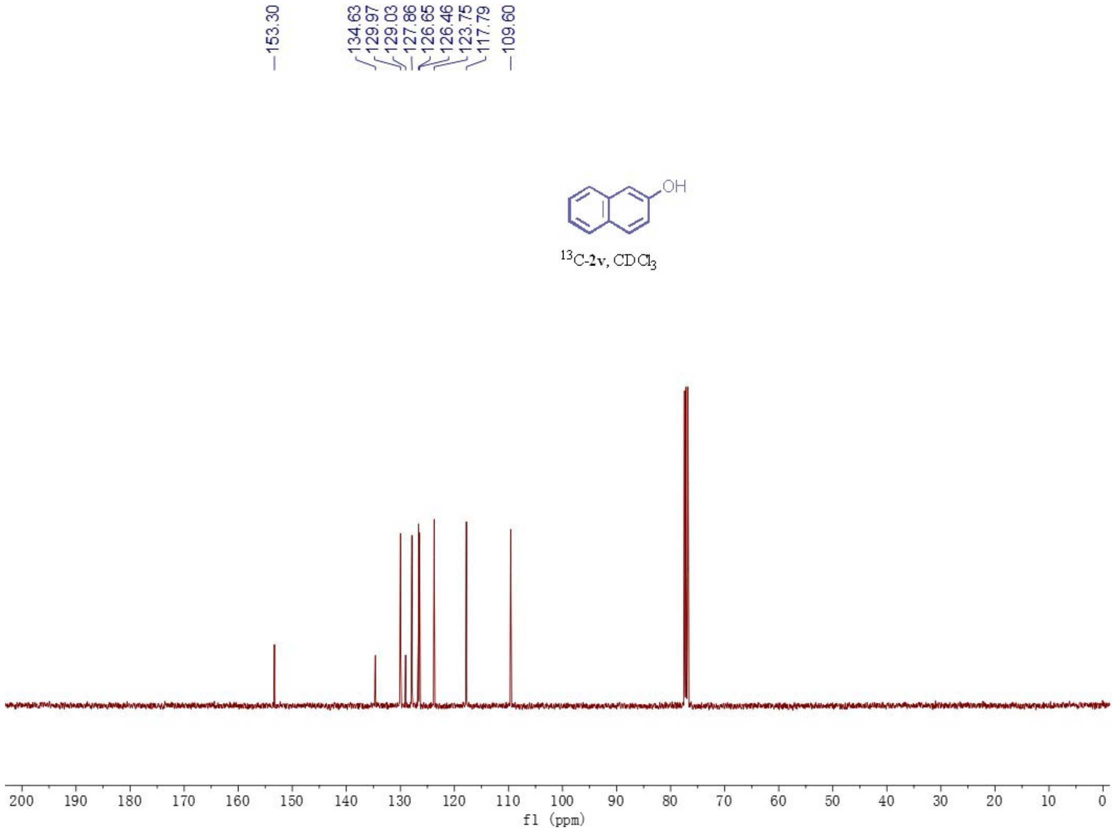


^{13}C-**2t**, CDCl$_3$


7.85
7.82
7.73
7.73
7.72
7.49
6.87
6.86
6.42
6.40
^{1}H-**2u**, CDCl$_3$
1.05
2.04
0.97
1.00
1.00
10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0
f1 (ppm)


161.10
156.44
152.06
146.98
144.17
124.93
119.92
115.45
114.69
106.45
99.91
^{13}C-**2u**, CDCl$_3$
200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
f1 (ppm)


7.84
7.82
7.80
7.74
7.72
7.51
7.49
7.47
7.41
7.39
7.37
7.20
7.17
7.16
7.15
7.14
5.13
OH
^{1}H-**2v**, CDCl$_3$
2.00
1.00
1.01
1.01
1.99
0.91
0.5
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
f1 (ppm)


153.30
134.63
129.97
129.03
127.86
126.65
126.46
123.75
117.79
109.60
OH
^{13}C-**2v**, CDCl$_3$
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
f1 (ppm)