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Supporting Information for

Evaluation of the antiepileptic activity of hesperidin by fluorescence imaging

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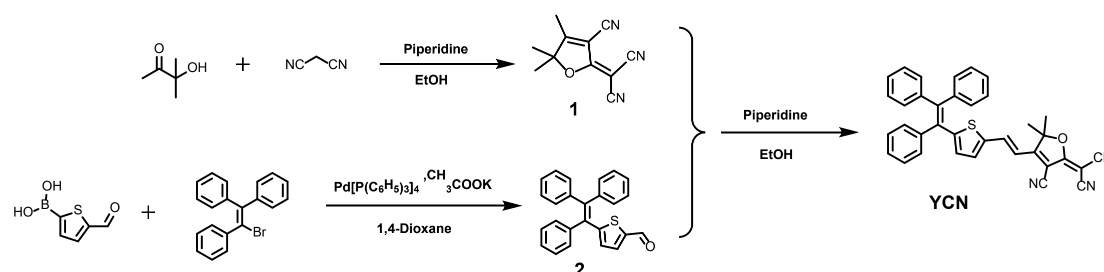
Materials and instruments

The reagents used in this experiment were commercially available and had not been subjected to any further treatment. TLC analysis was performed on silica gel plates and column chromatography was conducted over silica gel (mesh 200-300), both of which were obtained from the Qingdao Ocean Chemicals. The UV-Vis spectra were obtained by a UV-2700 spectrophotometer (Shimadzu, Japan). In addition, all fluorescence spectrum was tested at the room temperature using an F-4600 fluorescence spectrophotometer (Japan Hitachi). High resolution mass spectra were acquired on Aglient 7250& JEOL-JMS-T100LP AccuTOF (Bruker Daltonics, Billerica, MA, USA). And liquid chromatography-mass spectrometry (LC-MS) data were measured with an Agilent 6510 Q-TOF LC / MS (Agilent, USA). ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AVANCE III 400 MHz digital nuclear magnetic resonance spectrometer (Bruker Biospin, Billerica, MA, USA) with an internal standard of tetramethylsilane (TMS). The fluorescence imaging of cells was performed with a Leica TCS SP8 CARS confocal microscope.

The synthesis route of YCN



Scheme S1 The synthesis route of **YCN**.

Compound 1 was synthesized from existing literature[1]. 3-hydroxy-3-methyl-2-butanone (1.75 g, 15.98 mmol), propylene (2.58 g, 39.17 mmol) and sodium ethanol (0.2 g, 2.94 mmol) were dissolved in 20 mL of ethanol and stirred at 80 °C for 2 hours. Then, the solvent was depressurized and evaporated and added to cold ethanol to precipitate white solids under ice bath conditions.

5-aldehydo-2 thiopheneboronic acid (600 mg, 4 mmol), bromostilbene (1.34 g, 4 mmol), tetratriphenylphosphine) palladium (92 mg, 0.08 mmol) and potassium acetate (1.56 g, 16 mmol) were dissolved in 1,4-dioxane and stirred at 90°C for 12 hours under nitrogen conditions. Next, the solvent was spun dry under reduced pressure and the coarse product was purified by silica gel column chromatography (the volume ratio of petroleum ether and ethyl acetate was 10:1) to obtain **compound 2** (Yield：50%). ^{1}H NMR (600 MHz, Chloroform-*d*) δ 9.73 (s, 1H), 7.41 (d, *J* = 3.9 Hz, 1H), 7.26 (dd, *J* = 4.9, 1.9 Hz, 3H), 7.20 (dd, *J* = 6.5, 3.0 Hz, 2H), 7.18 – 7.15 (m, 3H), 7.15 – 7.12 (m, 2H), 7.08 – 7.06 (m, 3H), 6.98 (dt, *J* = 7.3, 3.6 Hz, 2H), 6.64 (d, *J* =

4.0 Hz, 1H). ^{13}C NMR (151 MHz, Chloroform-*d*) δ 182.83, 156.98, 144.74, 142.88, 142.64, 142.35, 142.22, 135.59, 133.14, 131.74, 131.33, 131.16, 130.88, 130.67, 130.58, 130.48, 130.03, 129.23, 128.55, 128.09, 128.06, 127.92, 127.86, 127.70, 127.47, 127.08.

Preparation of Solutions of probe YCN and Analytes

The solvents with different viscosity were obtained by mixing methanol-glycerol systems with different proportions. Viscosity value was measured by the NDJ-8S rotary viscometer. The test solutions of **YCN** (10 μM) with different viscosity were prepared by adding the stock solution (10 mM) 2 μL to 2 mL of solvent mixture (methanol-glycerol solvent systems). After standing for 1 hour at a constant temperature, the solutions were measured to obtain the UV-vis and fluorescence spectra.

Calculation of fluorescence quantum yield of YCN

The fluorescence quantum yields of **YCN** were evaluated by using Rhodamine B as a reference standard. The fluorescence quantum yield Φ_s is calculated by the formula as follow:

$$\Phi_u = \Phi_s \frac{F_u}{F_s} \frac{A_s}{A_u} \left(\frac{n_u}{n_s}\right)$$

Where Φ_s is the quantum yield of the sample, F is the area integral value of the corrected fluorescence spectrum, and A and n represent the absorbance and the refractive index of the solvent, respectively. The subscript "u" stands for the unknown to be tested and "s" is the standard.

The Förster-Hoffmann equation

The relationship between the fluorescence emission intensity of the probe **YCN** and the solvent viscosity could be formulated by the Förster-Hoffmann equation:

$$\log I = \mathrm{C} + x \log \eta$$

Where η is the viscosity, I is the emission intensity, C is a constant, and x is the sensitivity of the probe to viscosity.

Cytotoxicity assay

In vitro cytotoxicity was measured using the colorimetric methyl thiazolyl tetrazolium (MTT) assay on HeLa and SH-SY5Y cells. About 2×10^4 cells/mL HeLa or SH-SY5Y cells were incubated with various concentrations of **YCN** (0-50 μM) in 96-well plates for 24 h. Subsequent work was that 10 μL MTT (5 mg/mL) was added to each well and continued to incubate for another 4 h. In the end, the media was

discharged, and 100 μL of DMSO was loaded to dissolve the formazan crystals. The plate was shaken for about 10 min, and each well was analyzed by the microplate reader and detected at the absorbance of 490 nm.

$$\text{Cell viability} = \frac{OD_{sample} - OD_{blank}}{OD_{control} - OD_{blank}} \times 100\%$$

Cells culture

HeLa, SH-SY5Y and BV-2 cells were cultured in 90% Dulbecco's Modified Eagle Medium (DMEM, Gibico) supplemented with 10% FBS (Hyclone) and 1% antibiotics in an atmosphere of 37 °C and 5% CO_2. The cultured HeLa, SH-SY5Y and BV-2 cells were seeded into glass culture dishes and cultured to 4×10^5 cells/mL to fluorescence imaging.

HT-22 cells were cultured in RPIM 1640 containing 10% FBS (fetal calf serum) in the air with 5% CO_2 at 37 ℃. The cultured HT-22 cells were seeded into glass culture dishes and cultured to 4×10^5 cells/mL to fluorescence imaging.

Co-localization Experiment

The HeLa cells were seeded into glass bottom dishes with appropriate density. After 24 h, the cells were treated with 10 μM **YCN** for 30 min, and then treated with 10 μM **BODIPY 493/503** for another 10 min. Fluorescence images were acquired with a Leica TCS SP8 CARS confocal microscope.

Fluorescence imaging in live HeLa cells

(1) HeLa cells were cultured as above. The cultured DMEM cells were seeded into glass culture dishes and cultured to 4×10^5 cells/mL to fluorescence imaging. After 24 hours, the original medium in the dish were washed, then the first group HeLa cells was incubation with **YCN** only. The second and third group was treated with 200 μM Honokiol for 30 min or 100 μM Plumbagin for 30 min and then 10 μM **YCN** was added for extra 30 min, respectively. Besides, the fourth group were cultivated with 200 μM hesperidin for 30 min, and then 10 μM **YCN** was added for supererogatory 30 min. Last, the last group was treated with 200 μM Honokiol and 200 μM rapamycin for 30 min and then 10 μM **YCN** was added for additional 30 min. After that, confocal imaging was carried out on Leica TCS SP8 CARS confocal microscope.

(2) HeLa cells were cultured as above. The cultured DMEM cells were seeded into glass culture dishes and cultured to 4×10^5 cells/mL to fluorescence imaging. After 24 hours, the original medium in the dish were washed, and then the cell was

cultured with 10 μM **YCN** for 30 min. Next, 200 μM Honokiol was added to the cells to image for 120 min.

(3) SH-SY5Y cells were cultured as above. The cultured DMEM cells were seeded into glass culture dishes and cultured to 4×10^5 cells/mL to fluorescence imaging. After 24 hours, the original medium in the dish were washed, then the first group SH-SY5Y cells was incubation with **YCN** only. The second group was cultivated with 25 μM Kainic acid (KA) for 30 min, and next 10 μM **YCN** was supplied in the cell for another 30 min. The third and last group was trained with 50 μM or 100μM KA for 30 min, and next 10 μM **YCN** was added to the cell for another 30 min. After that, confocal imaging was carried out on Leica TCS SP8 CARS confocal microscope.

(4) SH-SY5Y, HT-22 and BV-2 cells culture as above. Then the cell was washed three times with sterile PBS buffer (pH 7.4) and incubated with 10 μM **YCN** for 30 min. After treatment, the cells were imaged *in situ* every 20 seconds (SH-SY5Y and BV-2) for 20 minutes or 30 seconds (HT-22) for 30 minutes.

(5) SH-SY5Y cells were cultured as described above. The cultured DMEM cells were seeded into glass culture dishes and cultured to 4×10^5 cells/mL to fluorescence imaging. After 24 hours, the original medium in the dish were washed, then the first group SH-SY5Y cells was incubation with **YCN** only. The second group was cultivated with 10 μM **YCN** for 30 min, and next 25 μM Kainic acid (KA) was supplied in the cell for another 30 min. The third group was treated with 10 μM **YCN** for 30 min, and next 25 μM KA and 20 μM hesperidin were supplied in the cell for another 30 min. The fourth group was disposed in 10 μM **YCN** for 30 min, and then 25 μM KA and 40 μM hesperidin were treated in the cell for another 30 min. The fifth group was incubated with 10 μM **YCN** for 30 min, and next 25 μM KA and 60 μM hesperidin were applied in the cell for 30 min. After that, confocal imaging was carried out on Leica TCS SP8 CARS confocal microscope.

Table S1 Fluorescence quantum yield (Φ) of YCN in the methanol/glycerol mixtures with different viscosities (η/cp).

Methanol/glycerol(v/v)	η (cp)	Fluorescence quantum yield(Φ)/%
0:10	937.48	14.39
1:9	312.84	6.56
2:8	111.36	2.99
3:7	54.74	2.96
4:6	34.84	0.94
5:5	25.23	0.83
6:4	14.57	0.53
7:3	7.89	0.33
8:2	3.3	0.25
9:1	2.63	0.19
10:0	1.29	0.16

Table S2 The fluorescence intensity of 10 μM YCN treated with various species and nature products in PBS (pH 7.4, 10 mM, 5% DMSO) and in glycerol at 715 nm. λ_{ex} = 561 nm.

Selective fluorescence spectrum			
Analytes	IF. Intensity (a.u.)	Analytes	IF. Intensity (a.u.)
Blank	29.85	$FeSO_4$	27.07
Cys	29.54	$CaCl_2$	30.05
Glucose	29.03	$CoCl_2$	28.55
GSH	28.93	$MgCl_2$	29.87
L-Leucine	25.73	$FeCl_3$	28.97
L-Tryptophan	29.78	$ZnCl_2$	30.52
L-Aspartic acid	27.58	OH •	37.91
Isoleucine	27.64	H_2O_2	27.09
TBHP	27.33	Na_2CO_3	28.28
NaClO	27.62	$HgNO_3$	29.13
NaF	29.3	NaBr	28.45
Glycine	27.76	Na_2SO_3	26.92
Na_2S	25.42	$NaNO_2$	28.49
$CuSO_4$	27.99	Honokiol	17.27
Hesperidin	8.413	Plumbagin	11.24
Glycerol	440.4	—	—

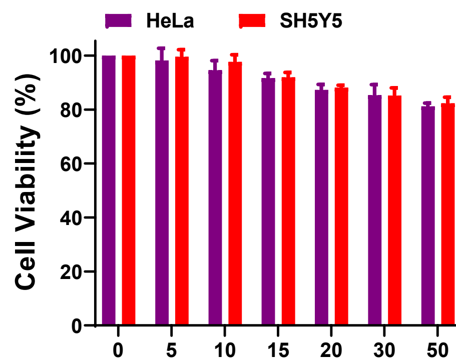


Figure S1 MTT assays of **YCN** at different concentrations for HeLa and SH-SY5Y.

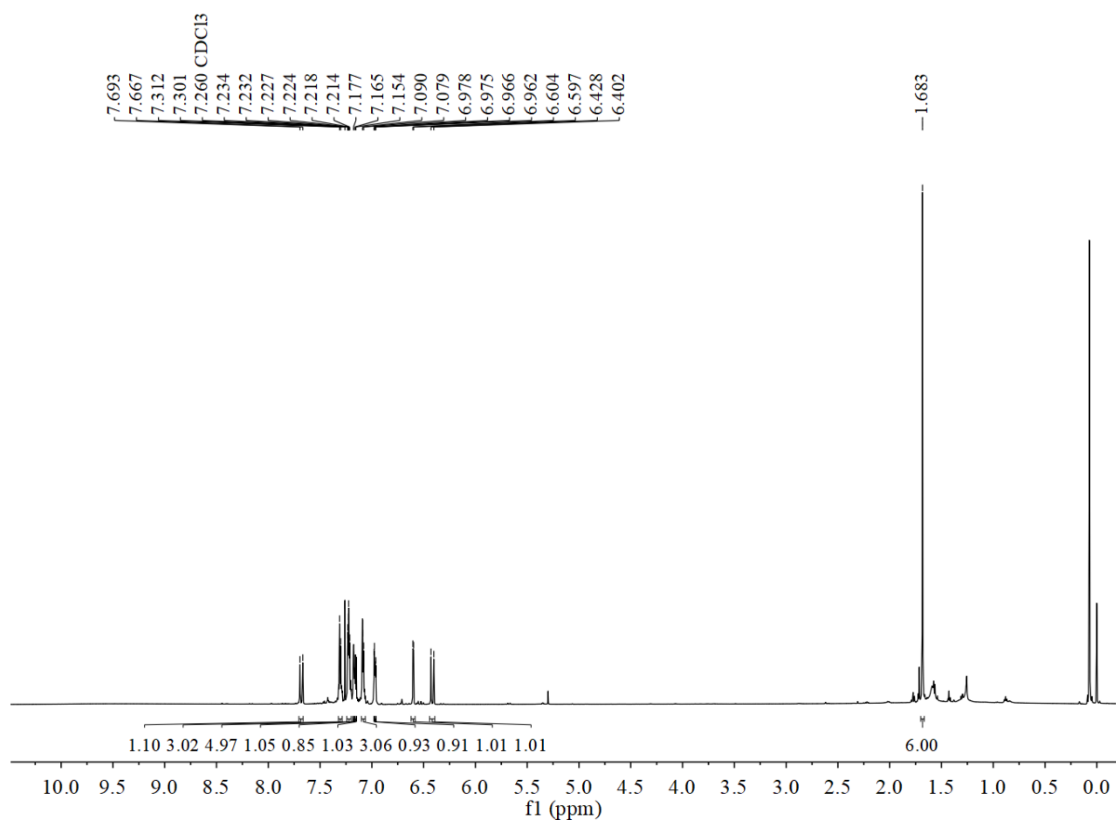


Figure S2 ^{1}H NMR data of **YCN** (Chloroform-*d*).

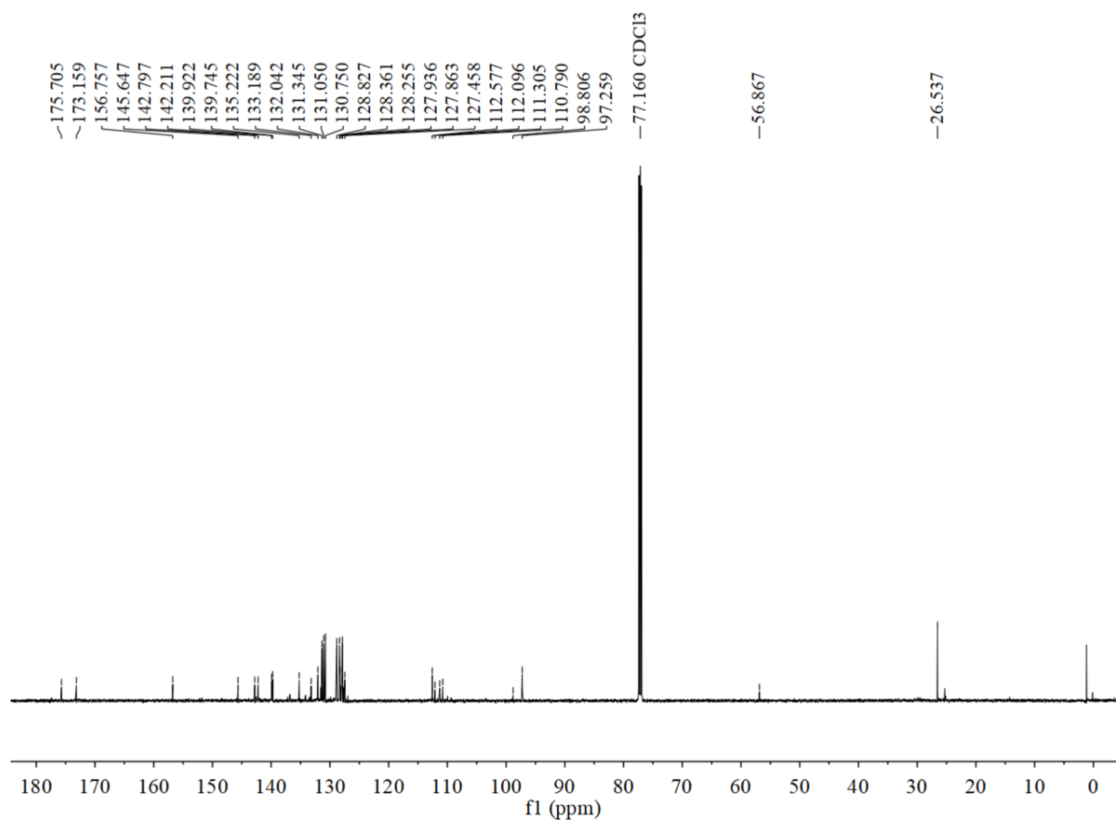


Figure S3 ^{13}C NMR data of **YCN** (Chloroform-*d*).

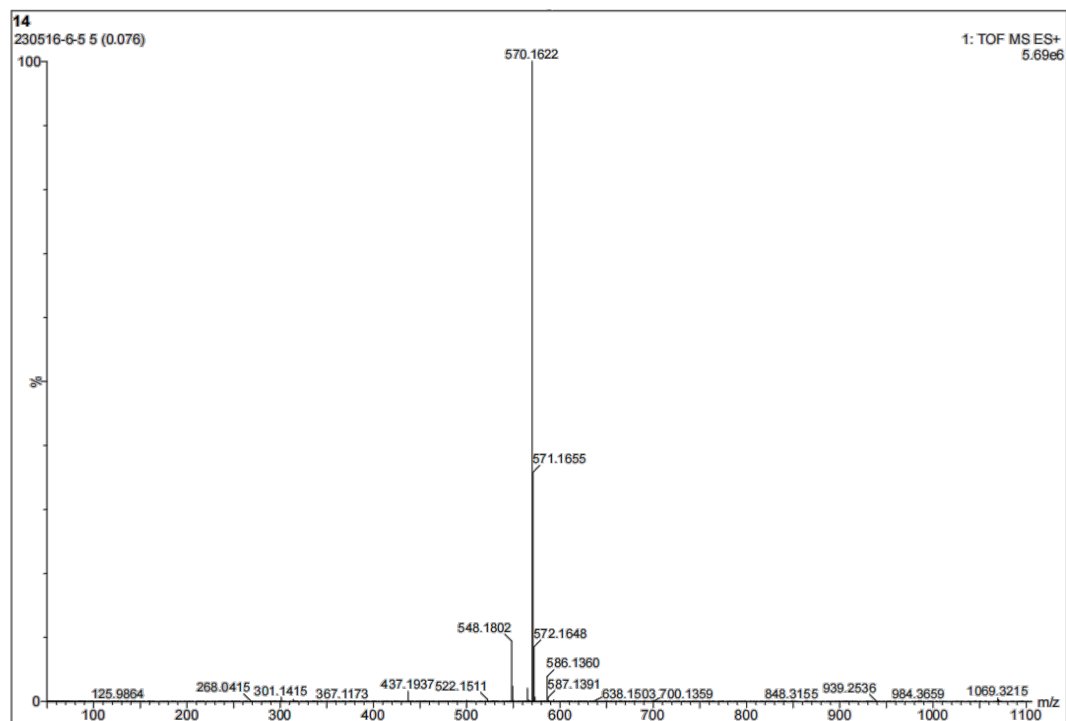


Figure S4 HRMS spectrum of probe **YCN.**

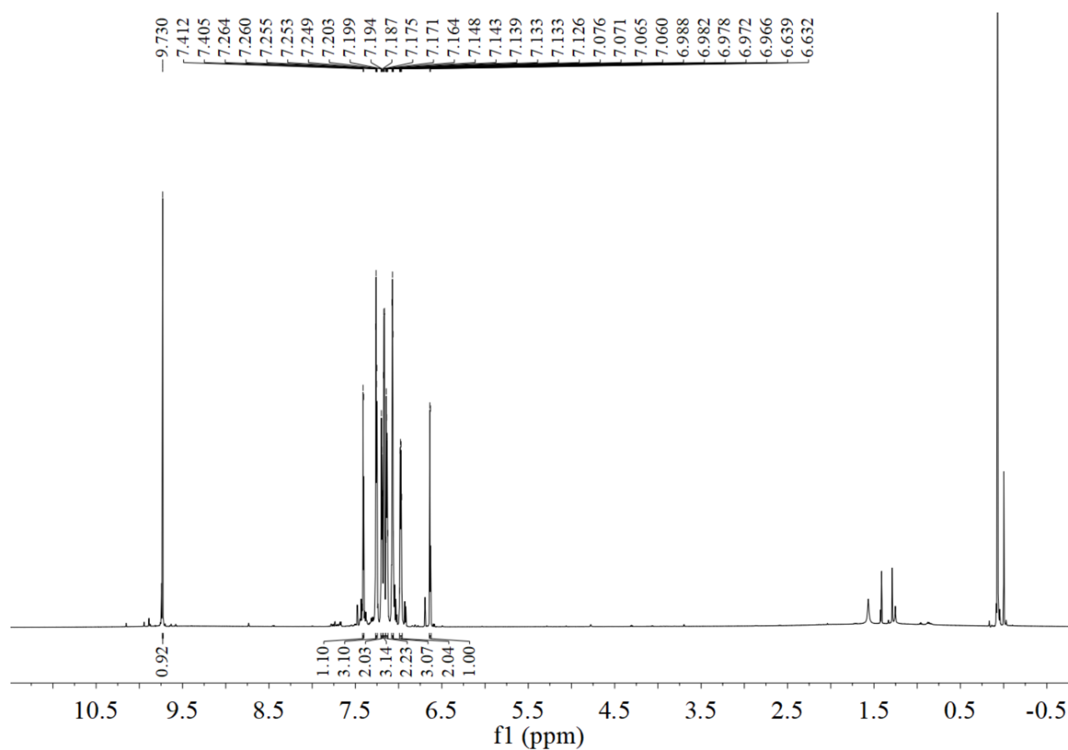


Figure S5 ^{1}H NMR data of **compound 2** (Chloroform-*d*).

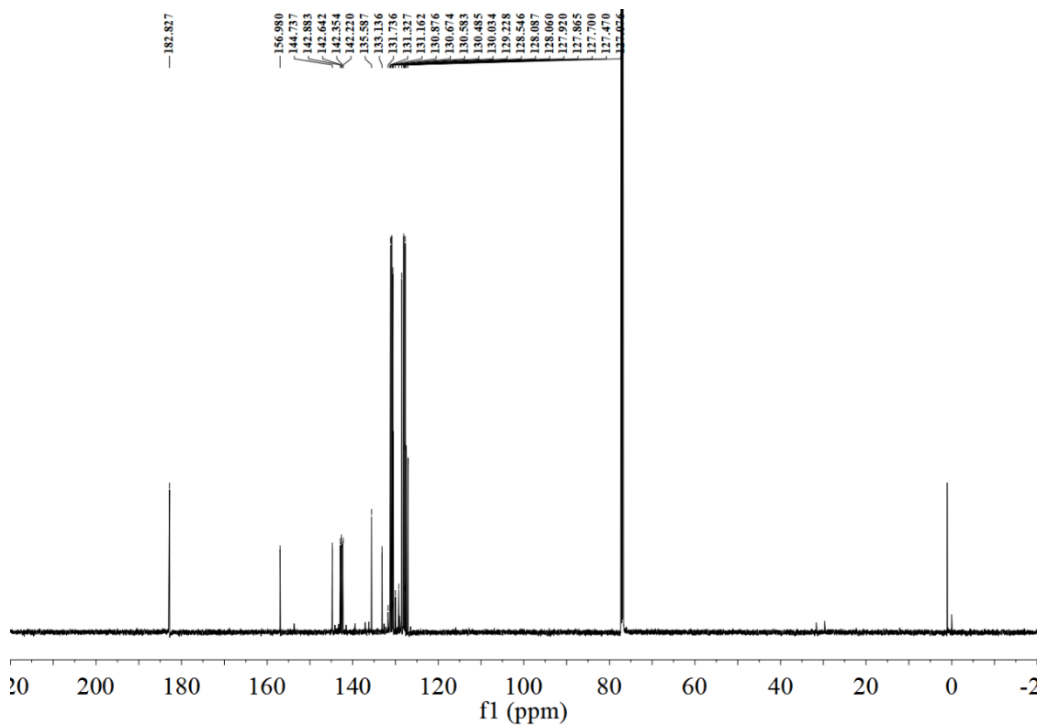


Figure S6 ^{13}C NMR data of **compound 2** (Chloroform-*d*).

References

1. T. T. Niu, T. Yu, G. X. Yin, H. M. Chen, P. Yin and H. T. Li, *Analyst*, 2019, **144**, 1546-1554.