Electronic Supplementary Information

A S-substituted Nile Blue-derived bifunctional near-infrared fluorescent probe for in vivo carboxylesterase imaging-guided photodynamic therapy of hepatocellular carcinoma

Beilei Wang[a], Yong Huang*[a], Dezhi Yang*[b], Jiayao Xu[a], Xiaohong Zhong[a], Shulin Zhao[a], Hong Liang*[a]

[a] *State Key Laboratory for the Chemistry and Molecular Engineering of Medicinal Resources, School of Chemistry and Pharmaceutical Science, Guangxi Normal University, Guilin 541004, P. R. China.*

E-mail: huangyong_2009@163.com; hliang@gxnu.edu.cn

[b] *School of Pharmacy, Zunyi Medical University, Zunyi 563000, P. R. China.*

E-mail: lpydz@163.com.

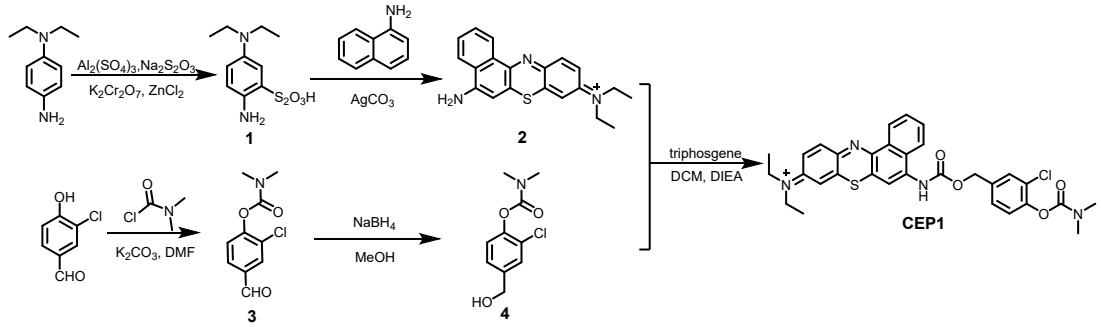
[*] *Corresponding authors.*

1. Apparatus and reagents

All solvents and reagents used were reagent grade, and were used without further purification. Mass spectrometric data were recorded in positive mode with a Thermo Scientific Exactive LC-MS instrument (US). ^{1}H NMR and ^{13}C NMR spectra were measured on Brucker DMX-400 spectrometer. Absorption spectra were measured on recorded on an Agilent Cary 60 UV-Visible Spectrophotometer (Agilent Technologies, USA). Fluorescence spectra were obtained with a LS-55 Fluorescence Spectrophotometer (Perkin-Elmer, USA). Fluorescence imaging was carried out on a LSM710 confocal laser scanning microscope (Zeiss, Germany). MTT assay was made on EL×800 Microplate Reader (BioTek, USA).

Carboxylesterase from porcine liver lyophilized, acetylcholinesterase (AChE), butyrylcholinesterase (BChE), and bovine serum albumin (BSA) were purchased from Sigma-Aldrich. Bis(4-nitrophenyl) phosphate) (BNPP) was obtained from Aladdin Co., Ltd. Lysosome-Tracker Green, Mitochondria- Tracker Green and Dihydroethidium (DHE) were purchased from Beyotime Biotechnology Co., Ltd. (Shanghai, China). Hydroxyphenyl fluorescein (HPF) was purchased from Macklin Co., Ltd. (Shanghai, China). Dihydrorhodamine 123 (DHR123) was obtained from Maokang Biotechnology Co., Ltd. (Shanghai, China). Dulbecco's Modified Eagle Medium (DMEM) was purchased from Thermo Fisher (Gibco, USA). Fetal bovine serum (FBS) was purchased from Gemini (USA). Tetramethylazazole salt (MTT), Trypsin with EDTA, penicillin/streptomycin were obtained from Solarbio (Beijing, China). Calcein-Am/PI Live/Dead Cell Double Staining Kit was obtained from Yeasen Biotechnology Co., Ltd. (Shanghai, China). A phosphate buffered saline solution was obtained from Solarbio (Beijing, China).

2. Synthesis



Scheme S1. Synthetic procedures of CEP1 and its intermediates.

2.1. Synthesis of Compound **1**[1]

To an aqueous solution (100 mL) of aluminum sulfate (9.2 g, 26.9 mmol), N, N-diethyl-p-phenylenediamine (5.0 g, 30.4 mmol) was added. Then, sodium thiosulfate (14.2 g, 57.3 mmol) and zinc chloride (3.6 g, 25.7 mmol) were added sequentially, and the reaction mixture was cooled in an ice bath. After that, the aqueous solution of potassium dichromate (2.0 g, 6.8 mmol) was slowly added. Thereafter, the reaction solution was stirred in an ice bath for 2 h with some precipitations was appearing. Then, the solution was filtered and washed with acetone, and the resulting residue was refluxed in MeOH (80 mL) for 1 h. Subsequently, the solution was cooled to room temperature and filtered to obtain a gray solid in 93% yield. This product was directly used for the next step.

2.2. Synthesis of Compound **2**

A mixture of 1-naphthylamine (2.0 g, 14 mmol) and compound **1** (7.8 g, 25 mmol) in 40 mL MeOH was refluxed at 80 ℃. Then, silver carbonate (7.7 g, 28 mmol) was slowly added to the reaction mixture to reflux at 70 ℃ for 30 min. After cooling, the resulting solution was filtered and evaporated to give a dark blue solid. Then, CH_2Cl_2 (30 mL) was added and washed with saturated sodium carbonate solution. The combined organic layer was dried over anhydrous $MgSO_4$, filtered and acidified with 0.4 mL of concentrated hydrochloric acid. The resulting product was purified by column chromatography (silica gel, gradient CH_2Cl_2/MeOH) to obtain a dark blue solid in 18% yield. ^{1}H-NMR (400 MHz, DMSO-d_6): δ ppm: 9.53 (br, 2H), 8.91 (s, 1H), 8.43

(s, 1H), 7.92 (br, 2H), 7.82 (s, 2H), 7.28 (br, 2H), 6.99 (s, 2H), 3.61(q, J = 8.0 Hz, 4H), 1.19 (t, J = 8.0 Hz, 6H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 156.53, 151.12, 138.95, 137.08, 134.05, 133.57, 132.11, 132.04, 130.99, 129.77, 124.98, 124. 22, 123.72, 117.41, 106.56, 105.64, 53.36, 45.61, 13.13(2C).

2.3. Synthesis of Compound **3**

A mixture of 3-chloro-4-hydroxybenzaldehyde (10 mmol), dimethylcarbamoyl chloride (11 mmol) and TEA (15 mmol) in dry CH_2Cl_2 (30 mL) was stirred at room temperature for 12 h. Then, the solution was cooled to room temperature, evaporated in vacuo. The resulting mixture was purified by column chromatography (silica gel, gradient PE/EA) to obtain a white solid in 81 % yield. ^{1}H-NMR (400 MHz, DMSO-d_6): δ ppm: 10.21 (s, 1H), 7.41 (s, 1H), 7.24 (br, 1H), 7.20 (s, 1H), 3.04 (s,3H), 2.88 (s.3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 189.90, 152.90, 152.41, 134.40, 131.11, 129.18, 128.35, 124.67, 36.93, 36.64.

2.4. Synthesis of Compound **4**

Compound **3** (2.3 g, 10.10 mmol) and sodium borohydride (0.76 g, 20.20 mmol) were added to a round bottom flask at 0 °C, and 15 mL of MeOH was added. The reaction was stirred at room temperature for 4 h, 20 mL of 10 % citric acid (w/w) was added, and then washed with 20% $NaHCO_3$ and water. The organic layer was dried with $MgSO_4$, filtered and concentrated to obtain white solid in 73 % yield. ^{1}H-NMR (400 MHz, $CDCl_3$): δ ppm: 7.40 (d, J = 4.0 Hz, 1H), 7.21 (dd, J = 4.0 Hz, J = 8.0 Hz, 1H), 7.16 (dd, J = 4.0 Hz, J = 8.0 Hz, 1H), 4.61(s, 2H), 3.14 (s,3H), 3.01(s,3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 154.06, 146.57, 140.00, 128.28, 126.96, 125.84, 123.9 2, 63.75, 36.86, 36.56.

2.5. Synthesis of Probe **CEP1**

A mixture of compound **2** (185 mg, 0.55 mmol), DIEA (0.8 mL) and triphosgene (330 mg, 1.1 mmol) in dry DCM was stirred in an ice bath for 2 h under Ar protection. Subsequently, the mixture solution was refluxed at 90°C for 12 h under N_2 protection, and then compound **4** (130 mg, 0.56 mmol) was added and stirred for 12 h. Thereafter, the reaction solution was cooled to room temperature and evaporated. The resulting

residue was purified by column chromatography (silica gel, gradient CH_2Cl_2/MeOH) to obtain a dark blue solid in 35 % yield. ^{1}H-NMR (400 MHz, DMSO-d_6): δ ppm: 8.69 (d, J = 8.0 Hz, 1H), 8.27 (d, J = 8.0 Hz, 1H), 7.76 (t, J = 4.0 Hz, 1H), 7.67 (m, 2H), 7.62 (d, J = 4.0 Hz, 1H), 7.43 (dd, J = 8.0 Hz, J = 4.0 Hz, J = 4.0 Hz, 1H), 7.30 (d, J = 8.0 Hz, 1H) 6.97 (s, 1H), 6.94 (d, J = 8.0 Hz, 1H), 6.89 (d, J = 4.0 Hz, 1H), 5.23 (s, 2H) 3.47 (q, J = 8.0 Hz, 4H), 3.05 (s, 3H), 2.84 (s, 3H), 1.13 (t, J = 8.0 Hz, 6H). 13C-NMR (600 MHz, DMSO-d6): δ ppm: 163.33, 156.15, 153.37, 149.42, 147.46, 137.30, 135.98, 135.77,135.28, 134.17, 131.34, 131.39, 130.09, 129.61, 128.59, 127.91, 126.93, 126.61, 126.39, 125.09, 124.69, 124.61, 113.55, 105.52, 66.44, 44.72(2C), 36.90, 36.65, 11.89(2C). ESI-HRMS Calc. for $C_{31}H_{30}ClN_4O_4S^+$, $[M+H]^+$, 589.17, found: 589.16.

3. Computational Methods

All of the DFT calculations in this study were performed using the GAUSSIAN 09 series of programs. Calculations are based by TDDFT at the PBEPBE/6-31G(d) level on ground state geometry. The solvent effects of water were taken into consideration using a SMD solvation model.

4. General Procedure for Carboxylesterase Analysis

The fluorescence of probe CEP1 reacting with carboxylesterase was determined in PBS buffer (pH 7.4). The stock solution (5.0 mM) of CEP1 was first prepared in DMSO. Stock solution of carboxylesterase was prepared in PBS. Briefly, the assay was performed in the 400 μL incubation mixture which including PBS (100 mM, pH 7.4), carboxylesterase and probe CEP1 (final concentration of 5 μM). After incubation for 3 h at 37℃, the reaction solution was transferred to a quartz cell to measure fluorescence with λex/em = 650/700 nm.

5. Enzyme Kinetic Analyses

Various concentrations of CEP1 (0-7 μM) solution were mixed with CEs (1 U/mL), respectively in mixture of PBS buffer (pH 7.4) at 37 ℃. The reaction was monitored by measuring the fluorescence intensity at 700 nm. The initial velocity was calculated and Michaelis-Menten curve was fitted plotting against the probe concentration. The

kinetic parameters were determined by the equation: V = Vmax*[probe] / (Km + [probe]), where V is reaction rate, [probe] is substrate concentration, and Km is the Michaelis constant.

6. Determination of the detection limit of CEP1 toward CEs [2]

Based on the linear fitting in Fig. 2A, the detection limit is estimated as follows:

Detection limit = 3σ/k

Where σ is the standard deviation of three individual fluorescence measurements, and k is the slope between the fluorescence intensity versus various CEs concentrations.

7. Cell Imaging and co-localization

HepG-2 cells and L02 cells were grown in DMEM medium supplemented with 10% of fetal bovine serum (FBS) in an atmosphere of 5% CO_2 at 37 °C, respectively. Cells were seeded in glass bottom dish and incubated overnight. After that, the cells were treated with probe CEP1 (10 μM) at different time, followed by washed with PBS for three times. Fluorescence imaging was captured on a LSM710 confocal laser scanning microscope (CLSM). λ_{ex} = 633 nm, λ_{em} = 670-780 nm.

HepG-2 cells were seeded in glass bottom dish and incubated overnight in a humidified 37℃, 5% CO_2 incubator. Cells were treated with CEP1 (10 μM) and washed with PBS for three times. Then, Lyso-Tracker Green (50 nm) was used to stain cell lysosome, λ_{ex} = 488 nm, λ_{em} = 511 nm. Mito-Tracker Green (50 nm) was used to stain cell mitochondrial, λ_{ex} = 488 nm, λ_{em} = 515 nm. Fluorescence imaging was captured on CLSM.

8. Cytotoxicity Assay

The cytotoxicity of CEP1 against HepG-2 and L02 cells were assessed using MTT assay. Two kinds of cells were plated into 96-well plates at a density of 5000 cells per well and then allowed to incubate for 24 h. Subsequently, the cells were incubated with CEP1 at 37℃ for 24 h. For phototoxicity assay, incubation with CEP1 at different concentrations in the dark for 3 h. Then, each well was irradiated (660 nm, 100 mW/cm^2) for 5 min and further cultured for 24 h. The MTT solution (20 μL) was added to each well and incubated for 4 h, then the optical density (OD) was recorded at 570

nm using EL×800 Microplate Reader (BioTek, USA). The cell viability was calculated with the equation: Cell viability (%) = (absorbance of sample × 100) / (absorbance of the control group).

9. Activity assay of CEs in cells

The CEs activity was measured using carboxyesterase activity assay kit following the instructions of the kit. Briefly, the HepG-2 cells were collected into a centrifuge tube, and the supernatant was discarded after centrifugation. Then, 400 μL reagent 1 from kit was added per 2 million cells, and the cells were sonicated (power 20%, sonication 3 seconds, 10 seconds interval, repeated 30 times). The samples were centrifuged at 12000 g at 4℃ for 30 min and the supernatant was taken to be tested.

10. Intracellular ROS Detection and Live/Dead Cell Staining

HepG-2 cells were incubated with **CEP1** (10 μM) for 3 h and then treated with DHE or HPF (10 μM) for 40 min. Subsequently, cells were irradiated (660 nm, 100 mW/cm^2) for 5 min and confocal imaging was performed with LSM710 confocal laser scanning microscope. For live/dead cell staining, HepG-2 cells were irradiated for 5 min after incubation with CEP1 for 3 h. Then, cells were further incubated for another 24 h and treated with Calcein AM and propidium iodide (PI) to visualize live and dead cells, respectively. For Calcein AM, DHE and HPF, excitation wavelength was 488 nm, emission wavelength was collected from 500-550 nm for green channel and from 570 to 630 nm for red channel. For PI, λ_{ex} = 543 nm and λ_{em} = 600-650 nm.

11. In vivo fluorescence imaging of HepG-2 bearing xenograft model

All animal procedures were carried out in accordance with the guidelines of the Experimental Animal Ethics Committee of Guangxi Normal University. Female BABL/c nude mice (5 weeks old, around 18-20 g) were purchased from Hunan SJA Laboratory Animal Co.,Ltd (China). A suspension of 3 × 10^6 HepG-2 cells was subcutaneously implanted into the right hind flank of each mouse. After 14 days inoculation, the mice was anesthetized during the experiments. CEP1 (20 μM,100 μL) was intratumorally injected into the mice bearing HepG-2 tumor. Fluorescence imaging were performed on FXPRO In Vivo imaging system (Bruker, Germany, λ_{ex} = 630 nm,

λ_{em} = 670-800 nm) at different time points post-injection. After that, mice were dissected and the tumor, heart, liver, spleen, lungs, and kidneys were collected for fluorescence imaging *ex vivo*.

12. In vivo evaluation of PDT effect

The HepG-2 tumor bearing mice were divided into four groups (3 mice per group) and were treated with the following different treatments: Group 1, saline; Group 2, and light irradiation; Group 3, CEP1; Group 4, CEP1 and light irradiation. After intratumor post-injection for 3 h, the tumor area was irradiated with a 660 nm laser irradiation at 100 mW/cm^2 for 10 min. The weight and tumor volume of all mice were recorded every two days from day 1 to day 14. Tumor volume was calculated as the following equation: volume = (tumor length) × (tumor width)^2/2. After 14 days of treatment, the mice were sacrificed, and the major organs (heart, liver, spleen, lung, and kidney) and tumor tissues were collected for H&E staining and histopathological analysis.

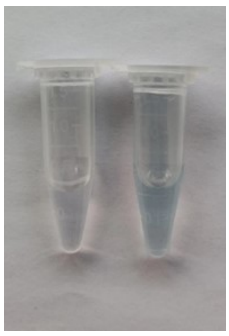


Fig. S1 The solvent color of CEP1 (5 μM) before (colorless) and after (blue) reaction with carboxylesterase (1 U/mL).

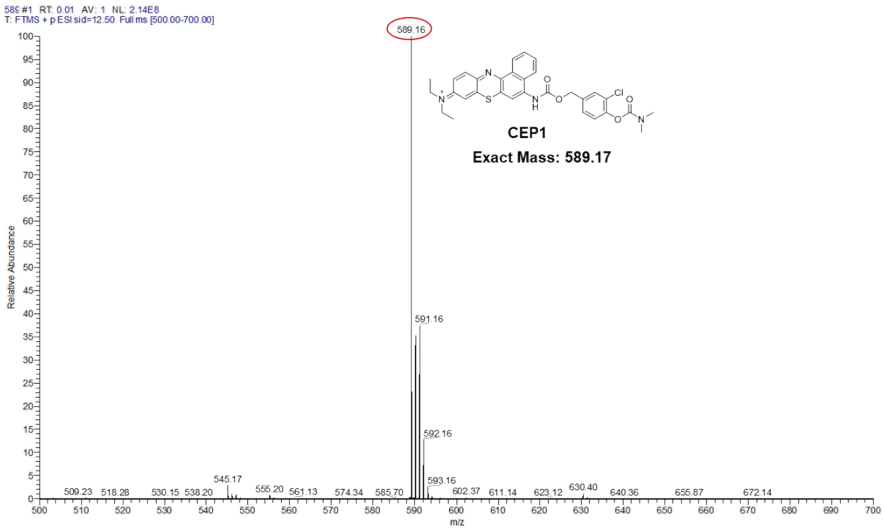


Fig. S2 HRMS spectrum of CEP1.

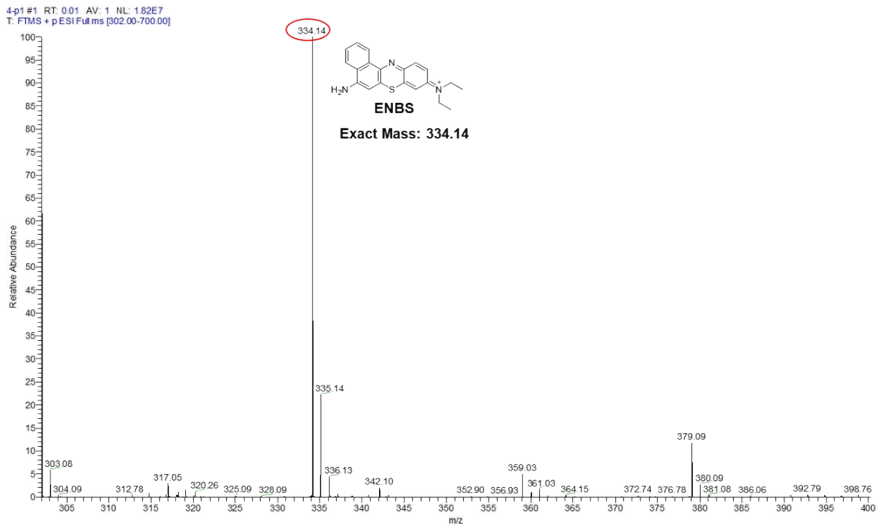


Fig. S3 HRMS analysis of the reaction solution of CEP1 with CEs.

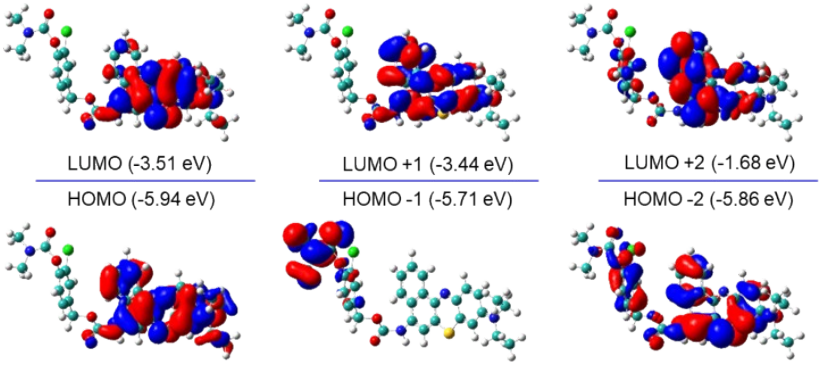


Fig. S4 The frontier molecular orbitals (MOs) of CEP1.

	TDDFT//PBE/6-31G(d)			
	Energy[a]	**f** [b]	**Composition**[c]	**CI**[d]
CEP1	1.7659 eV (702.08 nm)	0.0017	H-1→L	0.70578
	1.9117 eV (648.56 nm)	0.5179	H→L	0.66307
	1.9803 eV (626.09 nm)	0.0371	H→L	0.20107

Table S1 Selected parameters for the vertical excitation (UV-vis absorptions) of the compounds. Electronic excitation energies (eV) and oscillator strengths (f), configurations of the low-lying excited status of the CEP1. Calculated by TDDFT//PBE/6-31G(d), based on the optimized ground state geometries.

[a] The selected excited states were measured. The number in parentheses are the excitation energy in wavelength.

[b] Oscillator strength.

[c] H means the HOMO and L means the LUMO.

[d] Coefficient of the wavefunction for each excitation.

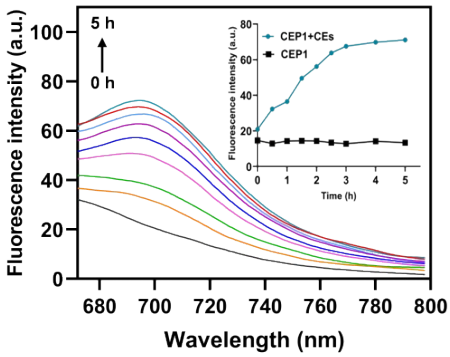


Fig. S5 (A) The fluorescence spectra change of CEP1 (5 μM) with CEs (1 U/mL) in 5 h at 37°C. (B) Time-dependent fluorescence enhancement at 700 nm upon incubation of CEP1(5 μM) with or without CEs (1 U/mL). λex/em = 650/700 nm.

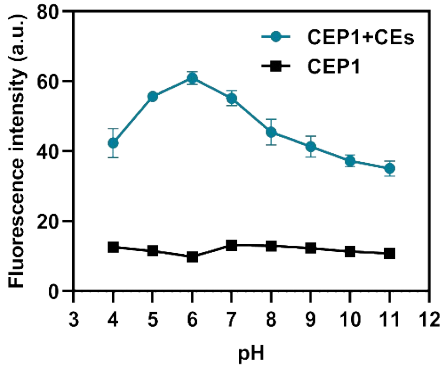


Fig. S6 Effects of pH on the fluorescence intensity of the reaction system of CEP1 (5 μM) and CEs (1U/mL). λex/em = 650/700 nm.

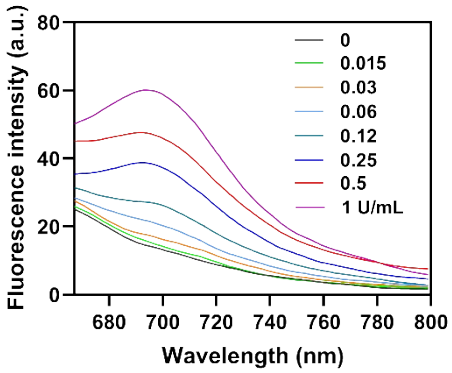


Fig. S7 The fluorescence response of **CEP1** with different concentrations of CEs (0-1.0 U/mL) for 3 h in PBS. λex/em = 650/700 nm.

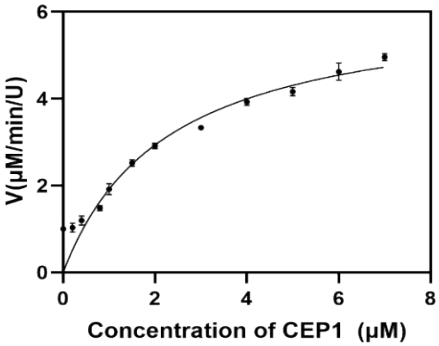


Fig. S8 Michaelis-Menten kinetics of CEP1 with CEs (1 U/mL). Relative reaction rate (V) of CEP1 in their corresponding concentration ranges. λex/em = 650/700 nm.

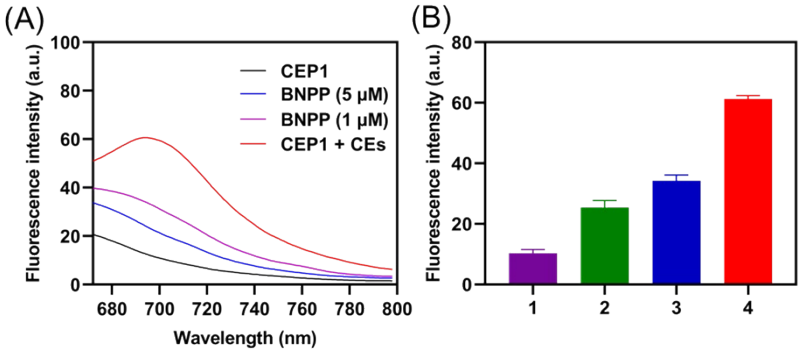


Fig. S9 (A, B) Inhibitory activity of CEP1 against BNPP under different conditions, including (1) CEP1 (5 μM), (2) CEP1 (5 μM) + BNPP (1 μM) + CEs (1 U/mL), (3) CEP1 (5 μM) + BNPP (5 μM) + CEs (1 U/mL), (4) CEP1 (5 μM) + CEs (1 U/mL).

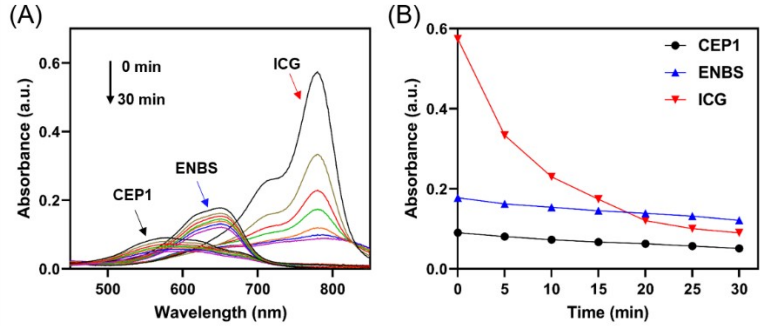


Fig. S10 Photostability of CEP1, ENBS and ICG in PBS detected via absorbance spectra. The samples (all at 5 μM) were continuously irradiated by a light source (100 mW/cm^2).

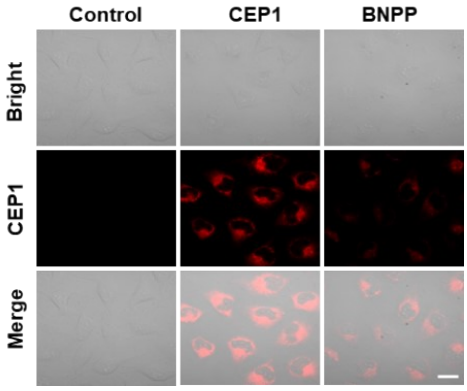


Fig. S11 Fluorescence imaging of HepG-2 cells treated with CEP1 (10 μM) for 3 h, and the BNPP group was pretreated with BNPP (100 μM) for 30 min before being incubated with CEP1 (10 μM) for 3 h. λex = 633 nm, λem = 670-780 nm. Scale bar: 20 μm.

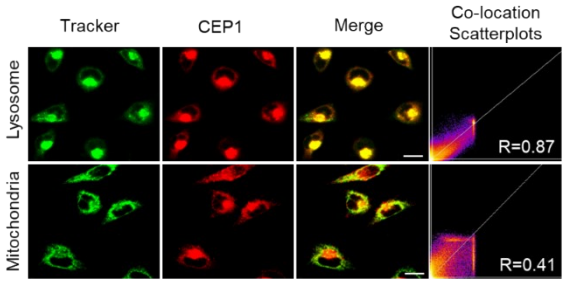


Fig. S12 Fluorescence co-localization images of CEP1 with Lysosome Tracker and Mitochondria Tracker in HepG-2 cells. Scale bar: 20 μm.

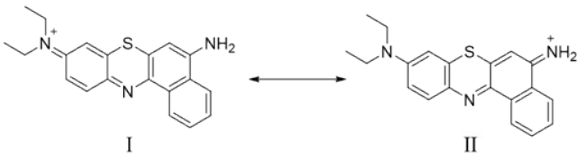


Fig. S13. Two electron delocalization forms of ENBS.

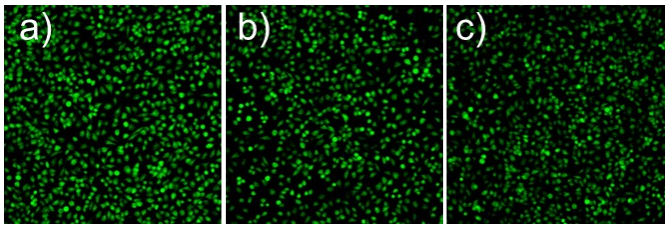


Fig. S14. The ROS generation dependent on CEs in the cells. (a) HepG-2 cells were incubated with probe CEP1 (10 μM) for 3 h, then ROS detection kit (DCFH-DA, 10 μM) was used and incubated for 30 min, then the cell fluorescence imaging was performed on CLMS after light irradiation. HepG-2 cells were pretreated with (b) BNPP (50 μM) or (c) BNPP (100 μM) for 30 min before being incubated with CEP1 for 3 h, and treated with DCFH-DA for another 30 min. λex = 488 nm, λem= 525 nm. The irradiation was set as 660 nm,100 mW/cm^2 for 5 min.

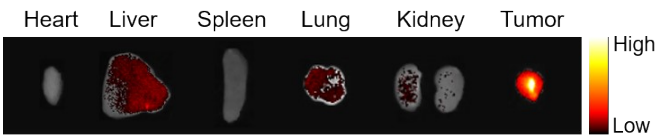


Fig. S15 Ex vivo fluorescence imaging of tumor and major organs following fluorescence imaging *in vivo*.

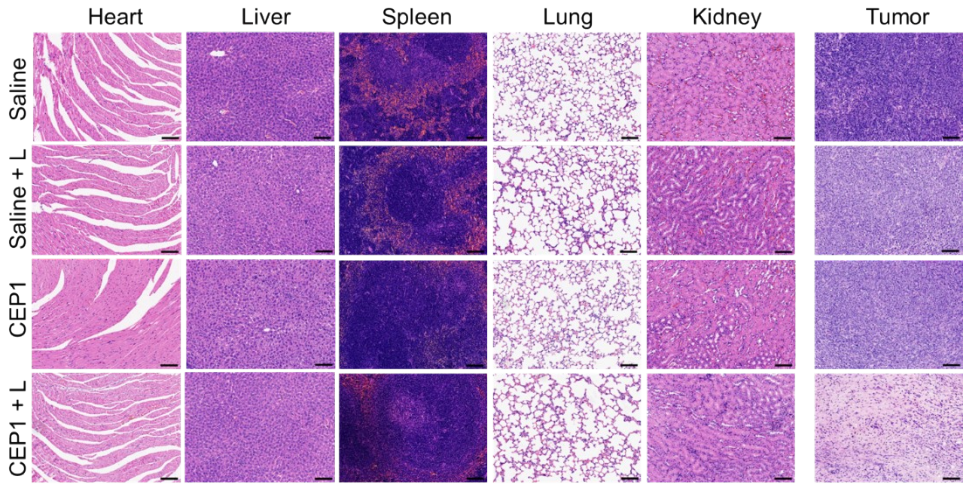


Fig. S16 H&E staining of organs and tumors in different groups after 14 days. The scale bars are 100 μm.

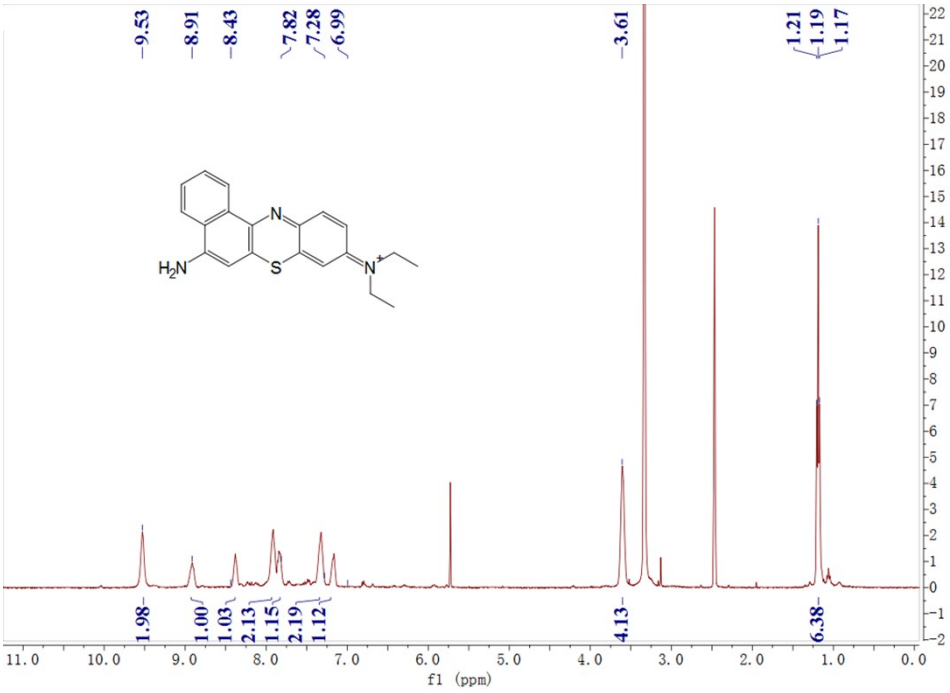


Fig. S17 ^{1}H NMR (400 MHz, DMSO-d_6) spectrum of compound **2**.

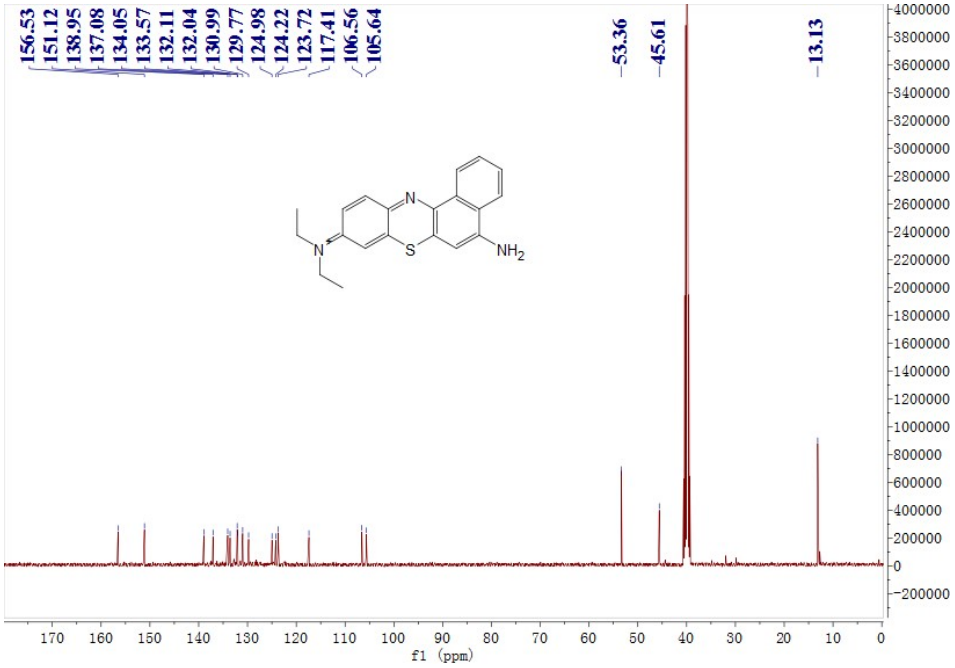


Fig. S18 ^{13}C NMR (101 MHz, DMSO-d_6) spectrum of compound **2**.

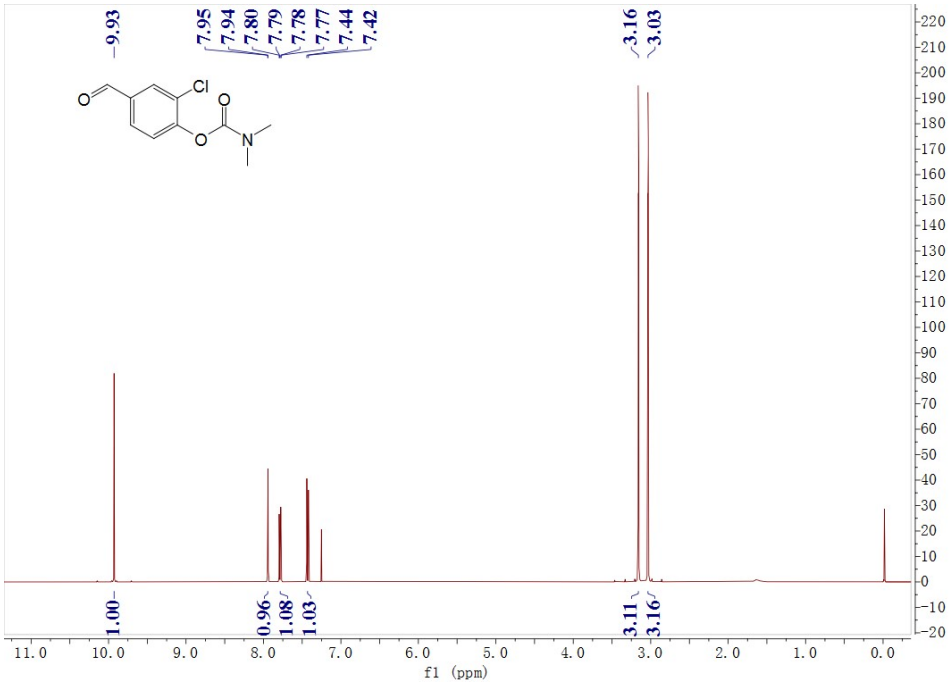


Fig. S19 ^{1}H NMR (400 MHz, DMSO-d_6) spectrum of compound **3**.

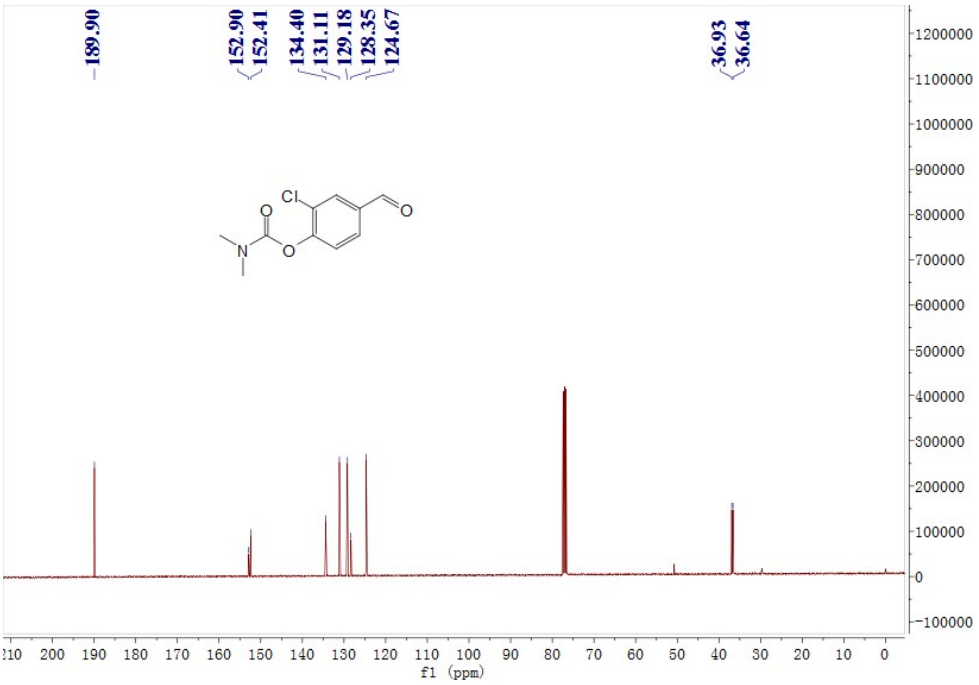


Fig. S20 ^{13}C NMR (101 MHz, $CDCl_3$) spectrum of compound **3**.

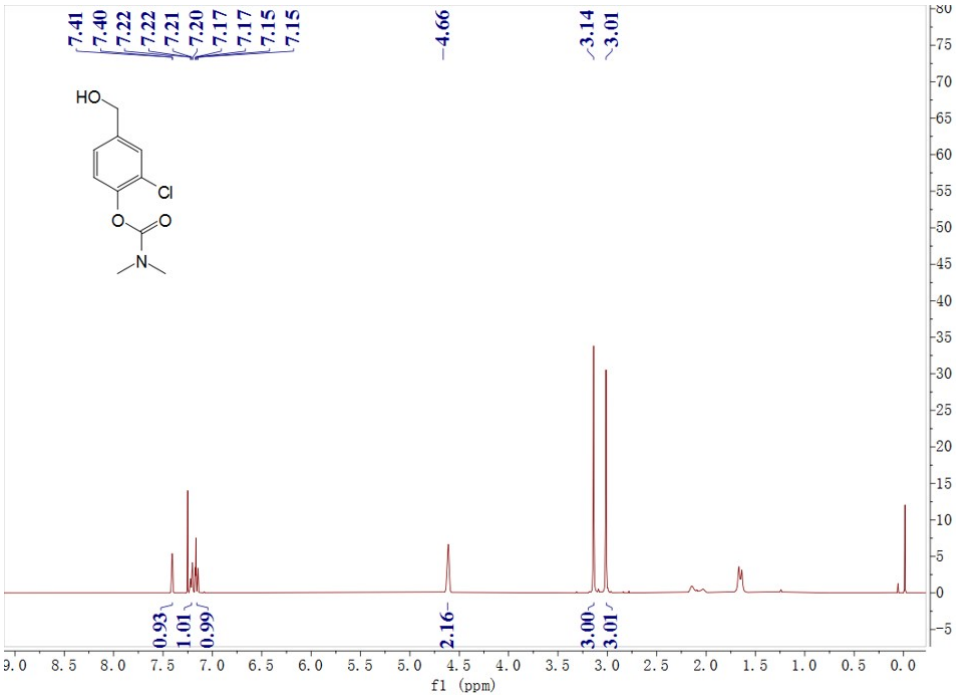


Fig. S21 ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of compound **4**.

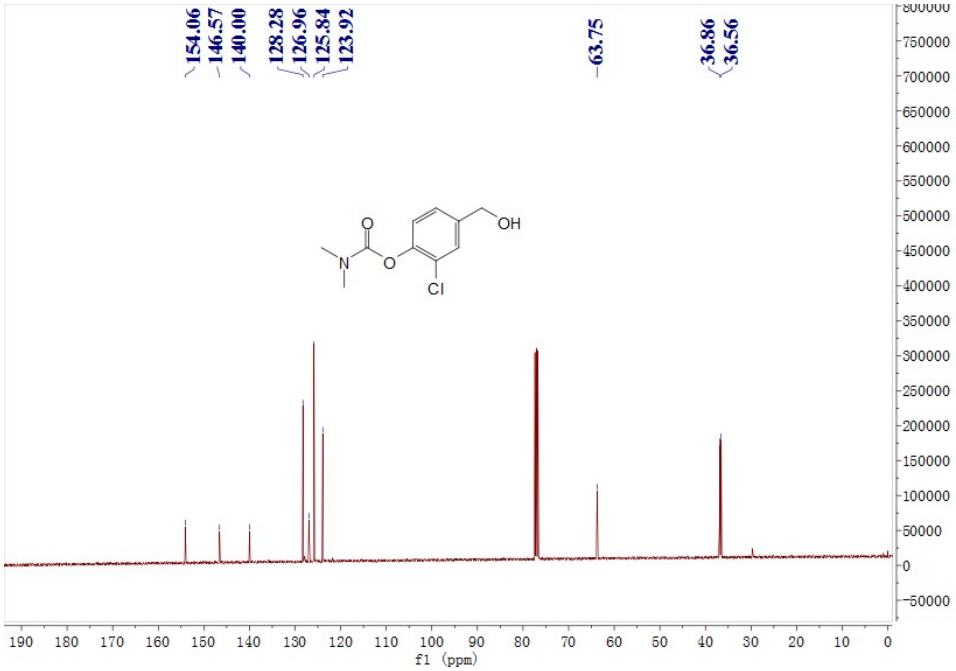


Fig. S22 ^{13}C NMR (101 MHz, $CDCl_3$) spectrum of compound **4**.

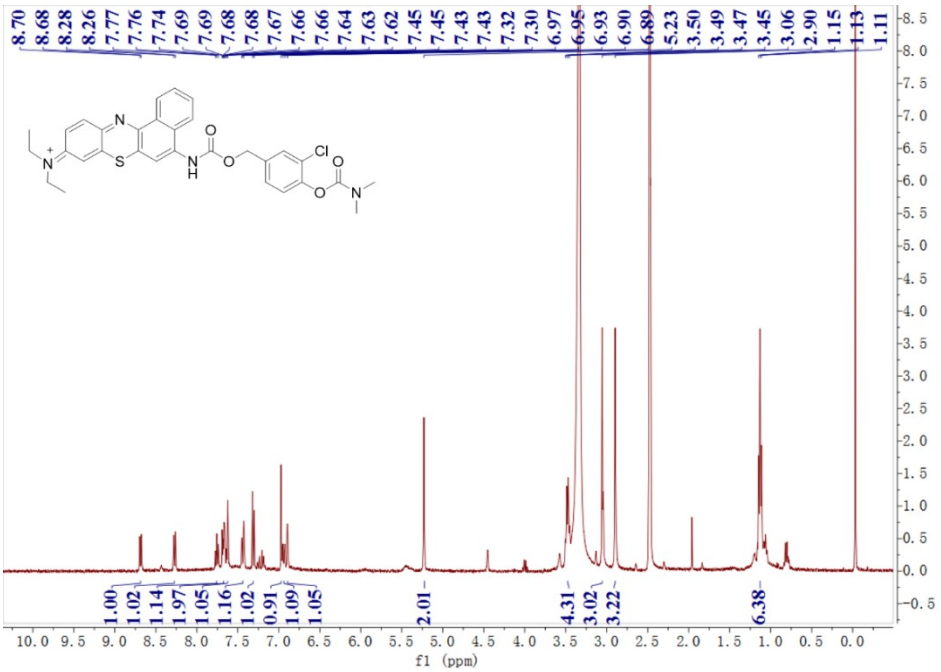


Fig. S23 ^{1}H NMR (400 MHz, DMSO-d_6) spectrum of **CEP1**.

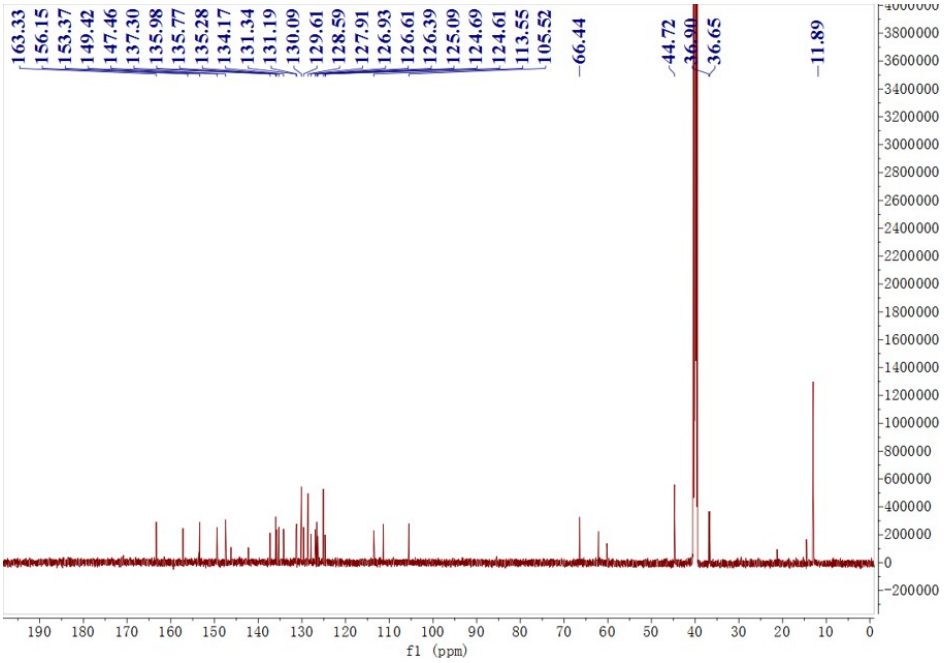


Fig. S24 ^{13}C NMR (400 MHz, DMSO-d_6) spectrum of **CEP1**.

References

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