Supporting Information

Near-infrared light-triggered covalent nanodrug for combined singlet oxygen therapy and photothermal therapy

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1. General Information

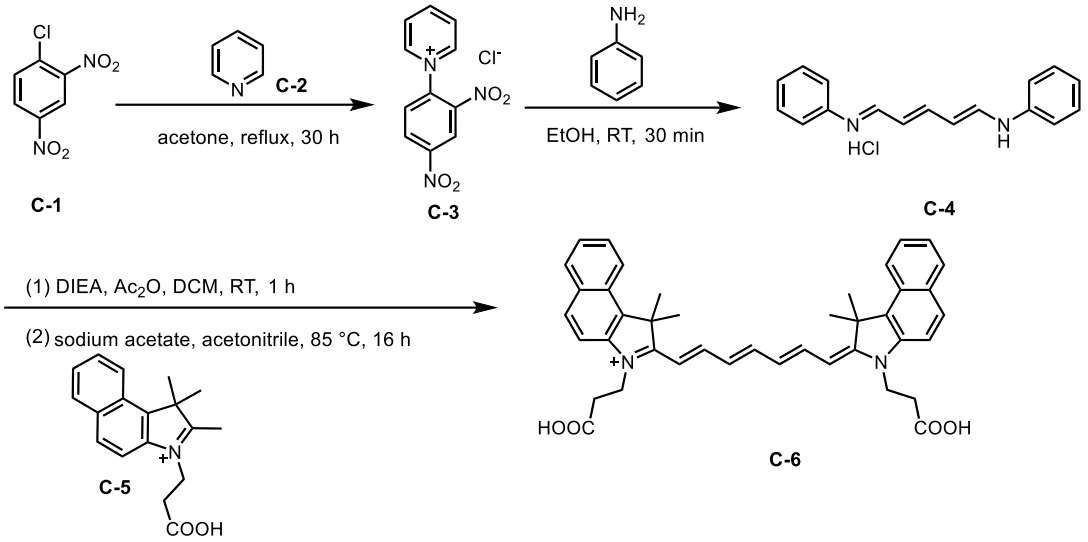
Materials

Unless otherwise specified, all reagents and solvents were purchased from commercial suppliers and used without further purification. Reactions were monitored by thin layer chromatography using Huang-hai TLC Silica gel 60 F-254. Column chromatography was performed by using Mei-gao Silica Gel 60 (particle size: 200-300 mesh). Reactive Oxygen Species Assay Kit (DCFH-DA) was bought from Beijing Solarbio Science & Technology Co., Ltd (Beijing, China). Calcein AM/propidium iodide (PI) Detection Kit was purchased from Beyotime Biotechnology Co. (China).

Characterization

The ^{1}H and ^{13}C NMR spectra were recorded using Bruker Vaian DLG400. Chemical shifts were reported in parts per million (ppm) and coupling constants (J values) are given in Hz. Splitting patterns are indicated as follows: singlet, d: doublet, t: triplet, m: multiplet. The UV-Vis absorption spectra were performed by using an Agilent Cary-3500 UV-vis spectrophotometer. The fluorescence spectrum was performed by using an Agilent CARY Eclipse fluorescence spectrometer. Transmission electron microscope (TEM) tests were performed using a JEM-2100F transmission electron microscope with an accelerating voltage of 200 kV. The MTT assay was performed on a TECAN-Spark R microplate reader (Tecan Trading AG, Switzerland). Animals' fluorescence imaging was carried out by IVIS LUMINA LT SYSTEM (Excitation wavelength: 660 nm, Emission wavelength: 700 nm). 980 nm laser diode and 808 nm laser diode were purchased from Changchun New Industries Optoelectronics Tech. Co., Ltd.

Syntheses:



Scheme S1 Synthetic route of **C-6**

Synthesis of Compound C-3.

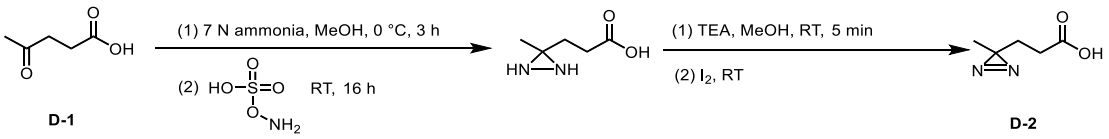
A mixture of compound **C-1** (993 mg, 4.9 mmol) and **C-2** (400 μL, 4.9 mmol) was refluxed in acetone for 30 h, then the mixture was cooled and concentrated under reduced pressure. The solid was washed several times with hexane and filtered to obtain the white powder compound **C-3** (Yield: 70%). ^{1}H NMR (400 MHz, CD_3OD) δ 9.96 (d, J = 5.8 Hz, 2H), 9.67 (d, J = 2.4 Hz, 1H), 9.53 (t, J = 7.9 Hz, 1H), 9.40 (d, J = 8.6 Hz, 1H), 8.98 – 8.90 (m, 3H); ^{13}C NMR (100 MHz, CD_3OD) δ 150.9, 150.0, 147.1, 144.3, 139.9, 132.5, 131.0, 129.3, 123.0.

Synthesis of Compound C-4.

Aniline (320 μL, 3.5 mmol) and compound **C-3** (488 mg, 1.7 mmol) were dissolved in ethanol which was stirring at r.t. for 30 minutes. The solvent was concentrated under reduced pressure, washed thrice with ethanol, and filtered to obtain compound **C-4** (Yield: 56%). ^{1}H NMR (400 MHz, CD_3OD) δ 8.97 (d, J = 2.7 Hz, 1H), 8.44 – 8.41 (m, 2H), 8.16 (dd, J = 9.4, 2.7 Hz, 1H), 7.91 (t, J = 12.9 Hz, 1H), 7.47 – 7.42 (m, 4H), 7.34 (d, J = 8.9 Hz, 3H), 7.25 (t, J = 7.4 Hz, 1H), 7.07 (d, J = 9.4 Hz, 1H), 6.32 (t, J = 12.1 Hz, 2H).

Synthesis of Compound C-6.

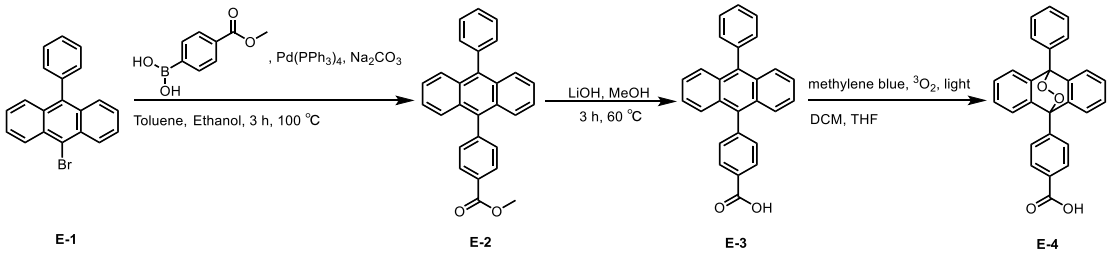
Compound **C-4** (142 mg, 0.5 mmol) and DIEA (130 mg, 1.0 mmol) were dissolved in DCM (1 mL). A solution of Ac_2O (60 mg, 0.6 mmol) in DCM (250 ul) was added dropwise into the reaction mixture at 0 °C for 5 min. The resulting solution was stirred for 1 h and added dropwise to a refluxing solution of compound **C-5** (410 mg, 1.1 mmol) and sodium acetate (160 mg, 2.0 mmol) in 5 mL of acetonitrile and water (v/v, 19:1). The mixture was refluxed for 16 h at 85 °C. Then, the reaction mixture was cooled to room temperature, the solution was filtered and washed with acetonitrile, 5% hydrochloric acid and ether to give **C-6** (Yield: 56%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.24 (d, J = 8.6 Hz, 2H), 8.11 – 7.94 (m, 6H), 7.81 (m, 1H), 7.72 (d, J = 9.0 Hz, 2H), 7.64 (t, J = 8.2 Hz, 2H), 7.50 (m, 2H), 6.60 (t, J = 12.6 Hz, 2H), 6.46 (d, J = 13.7 Hz, 2H), 4.43 (t, J = 7.1 Hz, 4H), 2.77 (t, J = 7.2 Hz, 4H), 1.91 (s, 12H).



Scheme S2 Synthetic route of **D-2**.

Synthesis of Compound D-2.

Under nitrogen protection, compound **D-1** (1.2 g, 10.1 mmol) and 10 ml of 7 N ammonia in MeOH were stirred at 0 °C for 3 h until hydroxylamine-O-sulfonic acid (1.3 g, 11.6 mmol) in 10 mL MeOH was added dropwise. The reaction mixture was further stirred at room temperature overnight and excess ammonia was removed by blowing air through the suspension in a fume hood. After filtration, the filtrate was concentrated to afford diaziridine acid. Diaziridine acid was dissolved in MeOH and the mixture was cooled to 0 °C. Triethylamine was added to the solution of which was stirred for 5 minutes. Iodine was added to the solution until a red-brown color remained. The solution was diluted with EtOAc and washed with 1 N HCl, 10% sodium thiosulfate and saturated aqueous NaCl, respectively. The organic layer was concentrated to afford diazirine **D-2** in 18% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 11.56 (s, 1H), 2.13 (t, J = 7.5 Hz, 2H), 1.58 (t, J = 7.9 Hz, 2H), 0.90 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 179.3, 29.7, 28.9, 25.4, 20.0.



Scheme S3 Synthetic route of **E-4**.

Synthesis of Compound E-2.

E-1 (452 mg, 1.4 mmol), phenylboronic acid (244 mg, 1.4 mmol), Pd $(PPh_3)_4$ (156 mg, 0.1 mmol), and Na_2CO_3 (7.0 g, 65.7 mmol) were mixted in toluene (18 mL), ethanol (9 mL) and water (6 mL). The reaction was heated for 3 h at 100 °C. The mixture was extracted with ethyl acetate and the organic phase was washed with brine, dried over anhydrous Na_2SO_4, and evaporated to dryness. The crude product was purified by silica gel column chromatography (DCM: hexane=3:1, v/v) (Yield: 34%).^{1}H NMR (400 MHz, DMSO-d_6) δ 8.24 (d, J = 8.0 Hz, 2H), 7.70 – 7.58 (m, 7H), 7.55 – 7.51 (m, 2H), 7.48 – 7.42 (m, 6H), 3.96 (s, 3H).

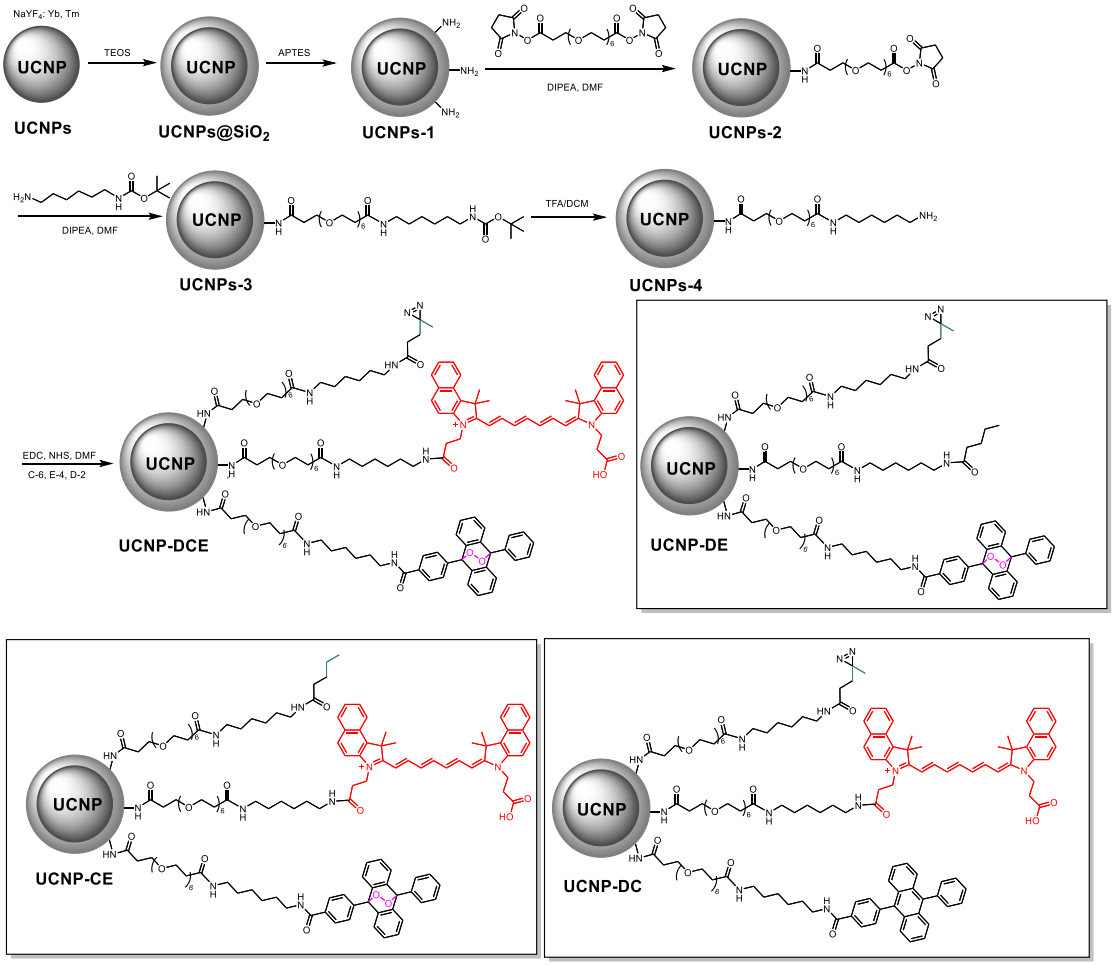
Synthesis of Compound E-3.

E-2 (80 mg, 0.2 mmol) was dissolved in 5 mL MeOH. 2M LiOH in 1 mL water was added to this solution. The mixture was stirred for 3 h at 60 °C then the solution was acidified to pH 2.0 followed by precipitation with water. Filtrate was collected and dried to give compound **E-3** (Yield: 71%)**.** ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.23 (d, J = 8.0 Hz, 2H), 7.70 – 7.58 (m, 7H), 7.57 – 7.52 (m, 2H), 7.49 – 7.42 (m, 6H).

Synthesis of Compound E-4.

To a solution of **E-3** (50 mg, 0.1 mmol) in DCM-THF (10 mL-5 mL), catalytic amount of methylene blue was added. The reaction mixture was stirred and irradiated with 630 nm light at 0 °C for 3 h. After the reaction is completed, methylene blue is removed by activated carbon, and the product was obtained under reduced pressure (Yield: 90%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.26 (d, J = 8.0 Hz, 2H), 7.78 (d, J = 8.0 Hz, 2H),

7.74 – 7.70 (m, 2H), 7.65 – 7.61 (m, 3H), 7.35 – 7.30 (m, 4H), 7.11 – 7.05 (m, 4H). MS-ESI m/z $[M-H]^-$ calcd for $C_{27}H_{18}O_4$, 405.1; found, 405.1.



Scheme S4 Synthetic route of **UCNP-DCE**.

Synthesis of $NaYF_4$: Yb, Tm Nanoparticles (UCNPs).

Briefly, YCl_3 (0.75 mmol, 146 mg), $YbCl_3$ (0.247 mmol, 69 mg), and $TmCl_3$ (0.003 mmol, 0.83 mg) were dissolved in 6 mL of oleic acid and 15 mL of 1-octadecene. The solution was heated to 130 °C for 22 min, degassed at 130 °C for 1 h, and then cooled to 50 °C. A solution of NH_4F (148 mg, 4 mmol) and NaOH (100 mg, 2.5 mmol) in 5 mL of methanol was added to the reaction mixture, and the solution was kept at 50 °C for 70 min. Then, the solution was heated to 70 °C and stirred for 1 h to remove methanol under vacuum. After heating to 300 °C for 23 min (10 °C/min), the solution was stirred at 300 °C for 1 h under argon. Finally, the solution was cooled to room

temperature. The **UCNPs** were precipitated by adding 30 mL of acetone to the reaction and further washed with hexane/ethanol three times.

Synthesis of Silica-Coated UCNPs@SiO_2.

To a solution of **UCNPs** (160 mg) in hexane, IGEPAL CO-520 (1.6 mL) was added. After sonication for 5 min and stirring for 15 min, 25% ammonia aqueous solution (330 μL) was added to the solution. After stirring for another 30 min, TEOS (330 μL) was added and the solution was further stirred overnight. **UCNPs@SiO_2** was precipitated by adding acetone and was then washed with ethanol.

Synthesis of UCNPs-1.

180 mg of **UCNPs@SiO_2** was suspended in ethanol and APTES (6.0 mL) was added. The solution was stirred at room temperature for 18 h, and the nanoparticles were obtained through centrifugation and washed with ethanol.

Synthesis of UCNPs-2.

30 mg of **UCNPs-1** was suspended in 0.5 mL of anhydrous dimethyl formamide (DMF), and Bis-PEG_6-NHS (10 mg) and DIPEA (6 μL) were added. After stirring overnight, the nanoparticles were obtained through centrifugation and washed with DMF.

Synthesis of UCNPs-3.

160 mg of **UCNPs-2** was suspended in 0.5 mL of DMF, and N-Boc-ethylenediamine (236 mg, 0.4 mmol) and DIPEA (0.2 mL, 1.2 mmol) were added. After stirring overnight, the nanoparticles were obtained through centrifugation and washed with DMF.

Synthesis of UCNPs-4.

160 mg of **UCNPs-3** was suspended in 1 mL of DCM at 0 °C, and TFA (100 μL) was added. The reaction mixture was stirred at room temperature for 5 h. The nanoparticles were obtained through centrifugation and washed with DCM.

Synthesis of UCNP-DCE.

Compound **C-6** (66 mg, 0.1 mmol), compound **D-2** (1.3 mg, 0.01 mmol), compound **E-4** (41 mg, 0.1 mmol), EDC (97 mg, 0.6 mmol) and NHS (72 mg, 0.6 mmol) were stirred in DMF (2 mL) at room temperature for 2 h, then **UCNPs-4** (160 mg) suspended in DMF was added dropwise, stirred for 12 h, and after the reaction, the centrifuge

speed was adjusted to 12000 rpm, 10 minutes to obtain solid precipitation. The precipitation was washed three times with DMF, washed once with DCM to obtain the **UCNP-DCE**, and finally, the **UCNP-DCE** was stored in DCM for subsequent characterization, in vitro and in vivo experiments.

***In Vitro* release of singlet oxygen**

A solution of **UCNP-DCE** (1 mg/mL) and DPBF (50 μM) were mixed and treated with different condition. The UV absorption of the solution was tested every 10 s to evaluate the release of singlet oxygen.

Photothermal Studies In Vitro

Different concentrations of **UCNP-DCE** (0, 0.5, 1, 2, 5 mg/mL) were suspended in DMF, and the temperature changes every 30 s under 808 nm laser irradiation for 3 min were measured.

Extracellular release of singlet oxygen and triplet oxygen

To calculate the ratio of singlet oxygen and triplet oxygen released from anthracene endoperoxide, tetramethylethylene was used as a probe which was incubated with anthracene endoperoxide in a 1-1 molar ratio. After anthracene endoperoxide was consumed, the generated anthracene and trapping product hydroperoxide were analyzed by ^{1}H NMR.

MTT Assays

Cells were incubated on the cell culture plate in RPMI-1640 medium (Gibco) supplemented with 10% FBS (PANTM SERATECH), and 1% penicillin-streptomycin (HyClone) at 37 ℃ in a humidified, 5% CO_2 atmosphere. MTT assays were carried out to evaluate the toxicity of nanoparticles towards cells. Briefly, cells with various treatments were further cultured for 24 h. Then, 10 μL of MTT (0.5 mg/mL) was added to each well of the plates and further incubated for 4 h. Thereafter, the culture medium was removed, and 150 μL dimethyl sulfoxide was added to dissolve the generated formazan. The absorption of the DMSO solution was measured using a microplate reader.

BSA Labeling

BSA (0.6 mg/mL) was mixed with **UCNP-DCE** (1 mg/mL) in a total volume of 100 μL PBS buffer and the mixture was vortexed for 1 hour to mix thoroughly at 4 ℃. The mixture was irradiated with or without 980 nm laser light (3 W/cm^2) for 10 min. After centrifugation three times, the supernatant was boiled for 10 min at 100 ℃ and then subjected to separation by using 8 % SDS-PAGE gel.

Singlet Oxygen Detection

For the intracellular singlet oxygen ($^1O_2$) detection assay, probe DCFH-DA was used according to the manufacturer's instructions. Briefly, 6 × 10^3 per well of 4T1 cells were seeded in 96-well plates and cultured for 24 h. Then, cells were incubated with **UCNP-DCE** (100 μg/mL) for 24 h. After washing, the cells were treated with indicated light irradiation. Then, serum-free DCFH-DA solution (1:1000) was incubated with the cancer cells for 20 min, followed with imaging study.

Live/Dead Assay

4T1 cells 6 × 10^3 per well were seeded in 96-well plates and cultured for 24 h. Then, cells were incubated with **UCNP-DCE** (100 μg/mL) for 24 h. After washing, the cells were treated with indicated light irradiation. Then, the cells were stained with calcein acetoxymethyl ester (Calcein-AM) and propidium iodide (PI) for 30 min, followed with imaging study.

Western blot

4T1 cells were seeded in 6 cm dish at a density of 5 × 10^5 cells per dish, and cultured for 24 h. Then, the medium was replaced by 5 mL new culture medium containing **UCNP-DCE**, or **UCNP-DC** at the concentration of 100 μg/mL, and further incubated in hypoxic incubator under 2% O_2 condition for 24 h. Free cells incubated under hypoxia were used as control. Then, the 4T1 cells were washed with PBS and the cell medium was replaced with 5 mL fresh medium, and the cells were irradiated upon 808 nm light (3 W/cm^2) for 5 min. After the 12 h, the 808 nm light irradiation was repeated, and the 808 nm laser was irradiated three times in total. After light treatment, cells were allowed to continue growing for 2 h under 2% O_2 condition. Then, the cells were lysed for Western blot.

***In Vivo* experiments**

All animal studies were approved by the ethics committee of the Dalian University of Technology (DUTSCE240801-01). Female BALB/c mice (5-weeks-old) were purchased from Changsheng Biology Laboratories. 4T1-tumor-bearing mice models were constructed by subcutaneous injection of 4T1 cells (5×10^5 cells per mouse, in 100 μL PBS) into the left and right back of BALB/c mice (for NIR imaging) or the right back of BALB/c mice (for anticancer study). To explore the long-term retention effect of **UCNP-DCE**, the mice bearing two 4T1 tumors were intratumorally injected with **UCNP-DCE** (1.5 mg/mL, 100 μL). At post injection, the right tumor was exposed to 980 nm laser light (3 W/cm^2) for 5 min. The mice were then anesthetized with 1.5% tribromoethyl alcohol and imaged using an IVIS spectrum at different time points.

When the tumor volume of 4T1 tumor reached to ~100 mm^3, the mice were divided into 3 groups: intratumorally injection of saline (100 μL) (Control); (2) intratumorally injection of **UCNP-DCE** (0.5 mg/mL, 100 μL) with 808 nm laser irradiation (3 W/cm^2, 5min) (**L$_{808}$**); (3) intratumorally injection of **UCNP-DCE** (0.5 mg/mL, 100 μL) with 980 nm (3 W/cm^2, 5 min) and 808 nm laser irradiation (3 W/cm^2, 5 min) (**L$_{980}$**+**L$_{808}$**). 980 nm light irradiation (3 W/cm^2, 5 min) was performed on day 0. 808 nm light irradiation (3 W/cm^2, 5 min) was performed on days 1, 3 and 5, respectively.

The size of the tumor, the body weight of the mice were recorded every 2 days. The tumor volume(V) was calculated according to the following formula: $V = L \times W^2/2$ by measuring the tumor length (L) and width (W) of each mouse. At the end of treatments, the mice were sacrificed. Tumor and major organs (heart, liver, spleen, lung, and kidney) were fixed with paraformaldehyde (10% vol/vol), sectioned into slices and analyzed via hematoxylin-eosin (H&E) and immunofluorescence (Ki-67 and HIF-1α) staining. The tumors were weighed and photographed and the blood of mice was collected to determine the side effect.

Statistical Analysis: GraphPad Prism 9 software was used to conduct the statistical analysis. The student's *t*-test was used to compare the two groups. Multiple group comparisons were conducted using one-way analysis of variance (ANOVA). Asterisks

represent different levels of significance: *p <0.05, **p <0.01, ***p <0.001, and ****p <0.0001; ns, not significant.

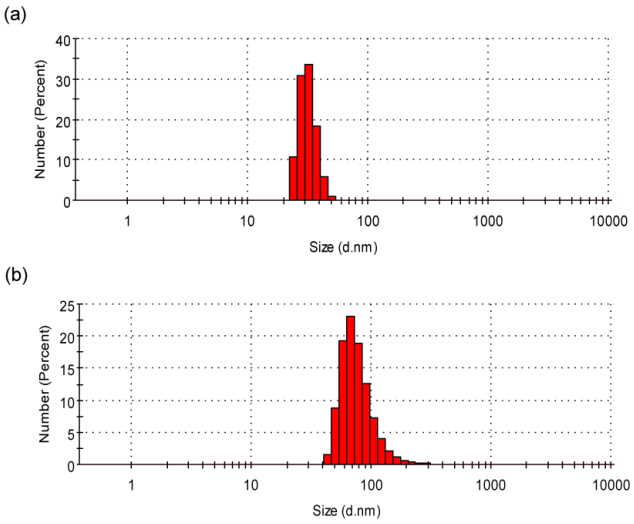


Fig. S1 (a) Diameter of UCNPs measured by DLS. (b) Diameter of UCNP@SiO_2 measured by DLS.

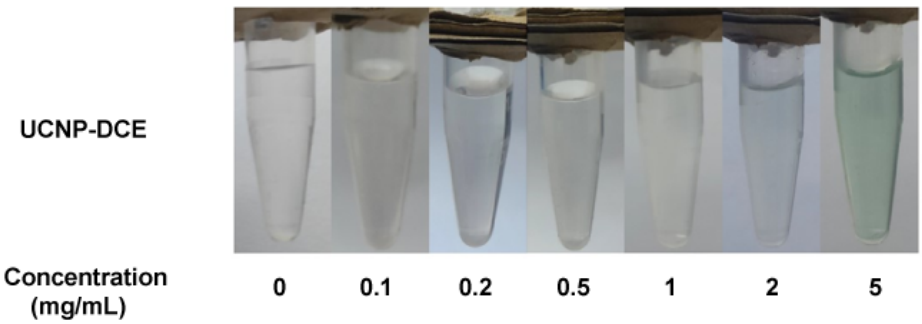


Fig. S2 UCNP-DCE at different concentrations in DMF.

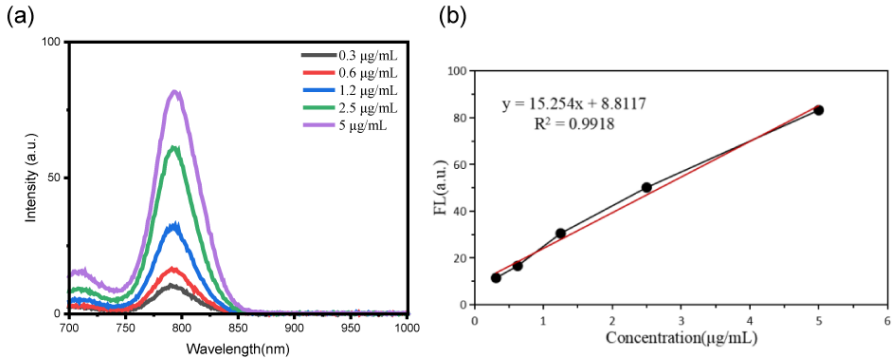


Fig. S3 The fluorescence of compound **C-6** at different concentrations and the standard curves.

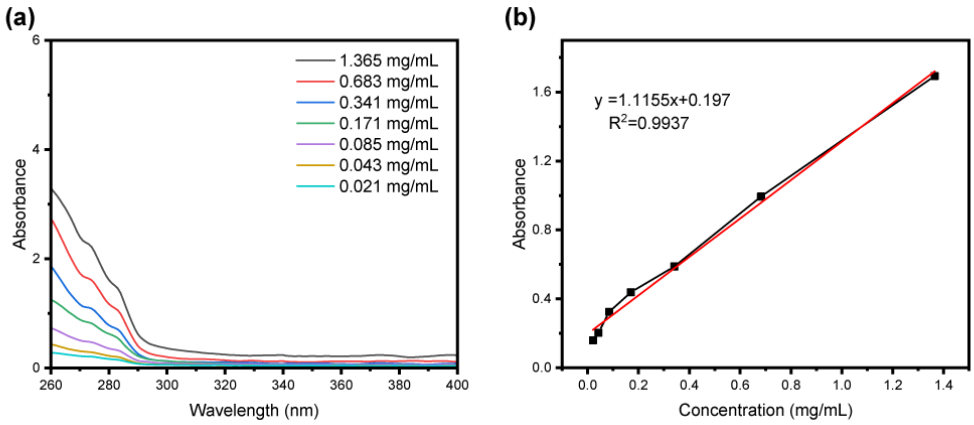


Fig. S4 The absorbance of compound **E-4** at different concentrations and the standard curves.

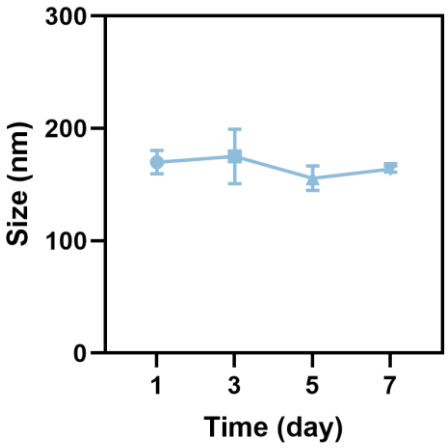


Fig. S5 The stability analysis of **UCNP-DCE** in RPMI 1640 medium containing 10 % fetal bovine serum (FBS) during 7 days.

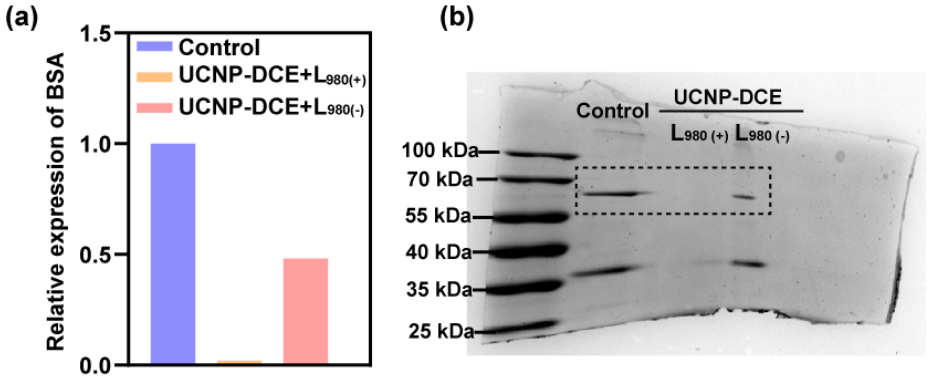


Fig. S6 (a) The relative BSA protein expression in supernatant after different treatments. (b) Image of the SDS-PAGE.

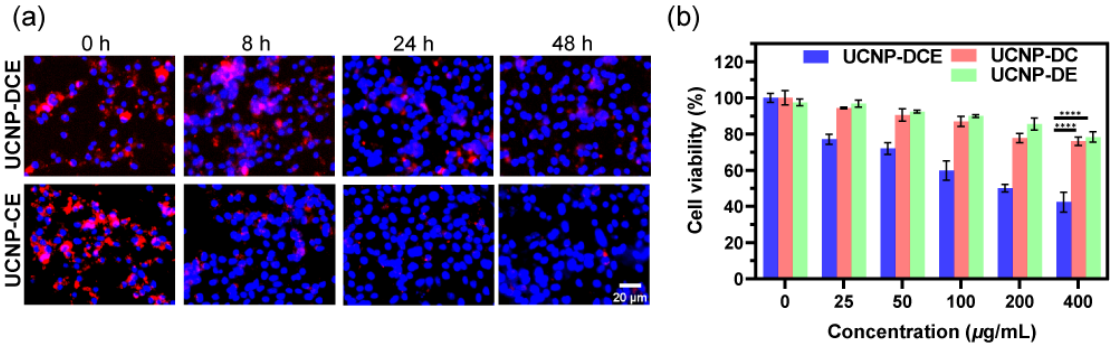


Fig. S7 (a) Fluorescence of cells treated with **UCNP-DCE** and **UCNP-CE** (100 μg/mL) under 980 nm light irradiation. Scale bars: 20 μm. (b) MTT assay of the **UCNP-DCE**, **UCNP-DC** and **UCNP-DE**.

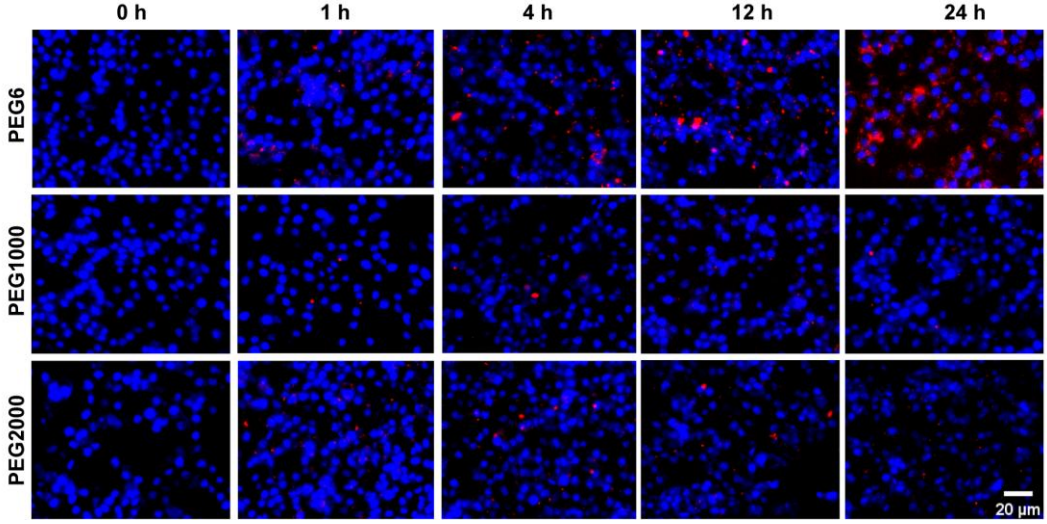


Fig. S8 Fluorescence of 4T1 cells treated with nanoparticles of various PEG chain.

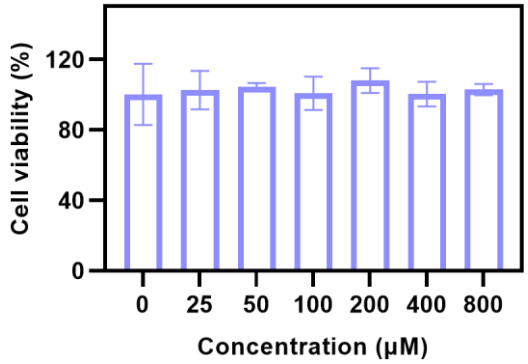


Fig. S9 Cell viability of 4T1 cells incubated with **UCNP-DCE** at various concentrations with 980 nm laser irradiation (3 W/cm^2).

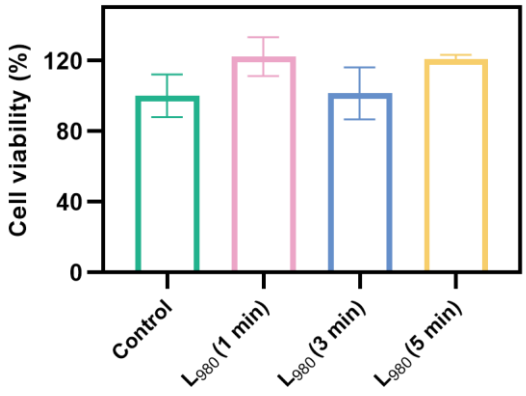


Fig. S10 Cell viability of 4T1 cells with 980 nm laser irradiation for different time (3 W/cm^2).

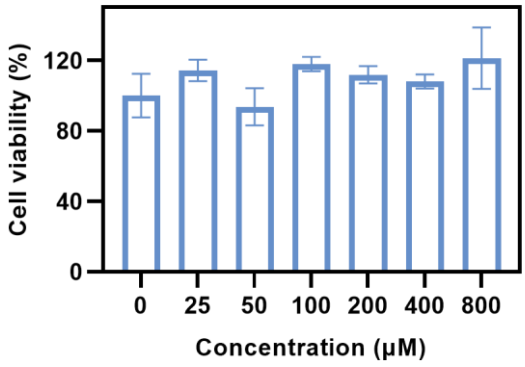


Fig. S11 Cell viability of 4T1 cells incubated with **UCNP-DCE** at various concentrations.

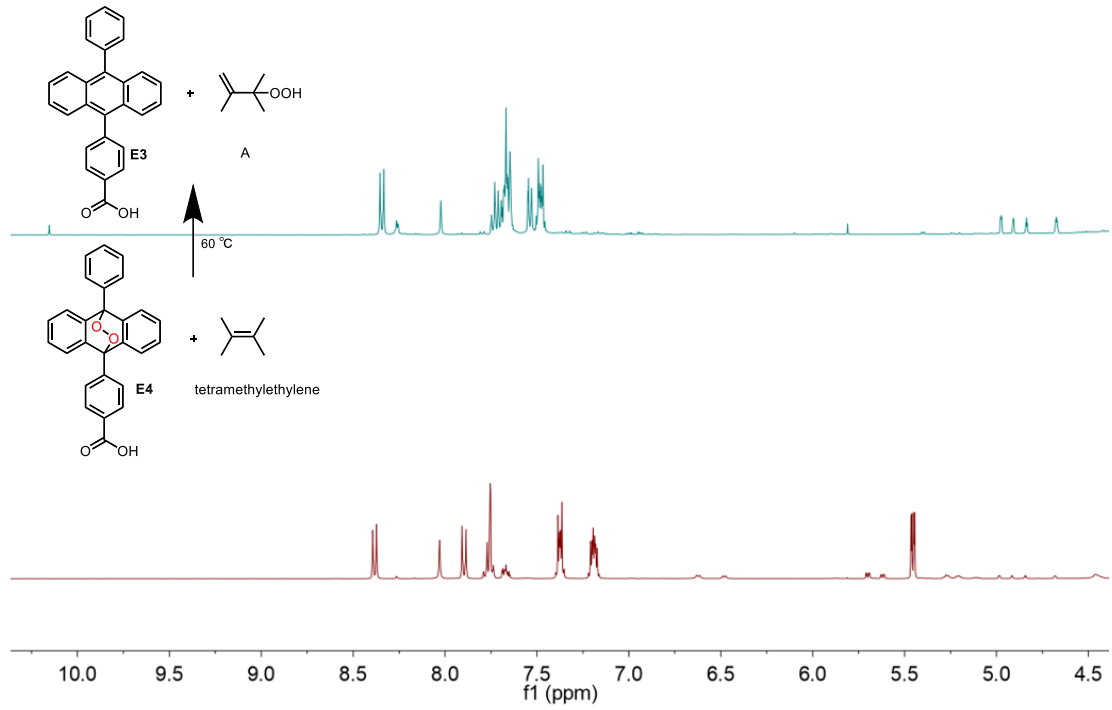


Fig. S12 Singlet oxygen trapping using tetramethylethylene (1-1 ratio to anthracene endoperoxide).

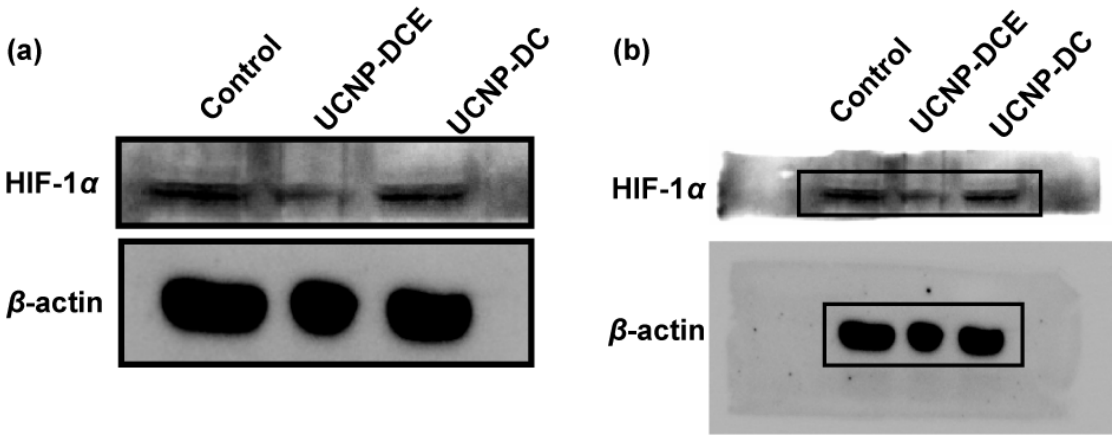


Fig. S13 Western blotting of HIF-1α of cells under different treatments. **UCNP-DCE**, **UCNP-DC** under 808 nm light irradiation.

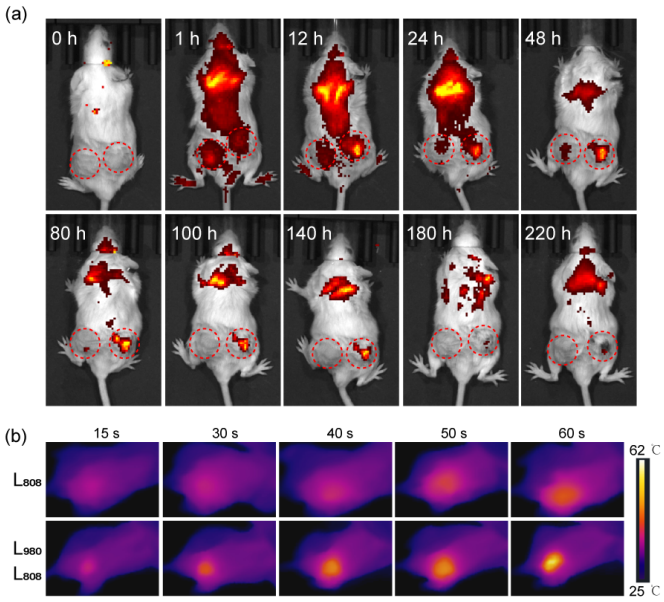


Fig. S14 (a) In vivo fluorescence images of dual tumor-bearing mice treated with **UCNP-DCE** (1.5 mg/mL, 100 μL, intratumoral injection). The right tumor site was irradiated with 980 nm light (3 W/cm^2, 5 min). The circles point the tumor site in mice. (b) Thermal images of the tumor under laser irradiation (980 nm, 3 W/cm^2, 5 min) and (808 nm, 3 W/cm^2, 5 min) with **UCNP-DCE** (0.5 mg/mL, 100 μL, intratumoral injection) or (808 nm, 3 W/cm^2, 5 min) as a control.

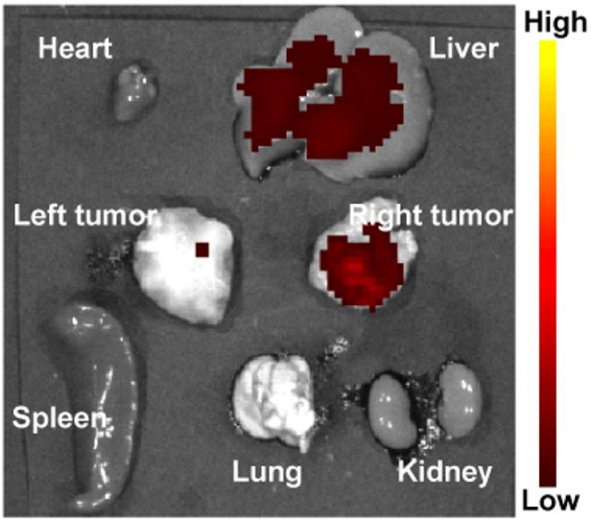


Fig. S15 Fluorescence imaging of the tumors and major organs at 240 h after **UCNP-DCE** (1.5 mg/mL, 100 μL) treatment and 980 nm light irradiation on the right tumor.

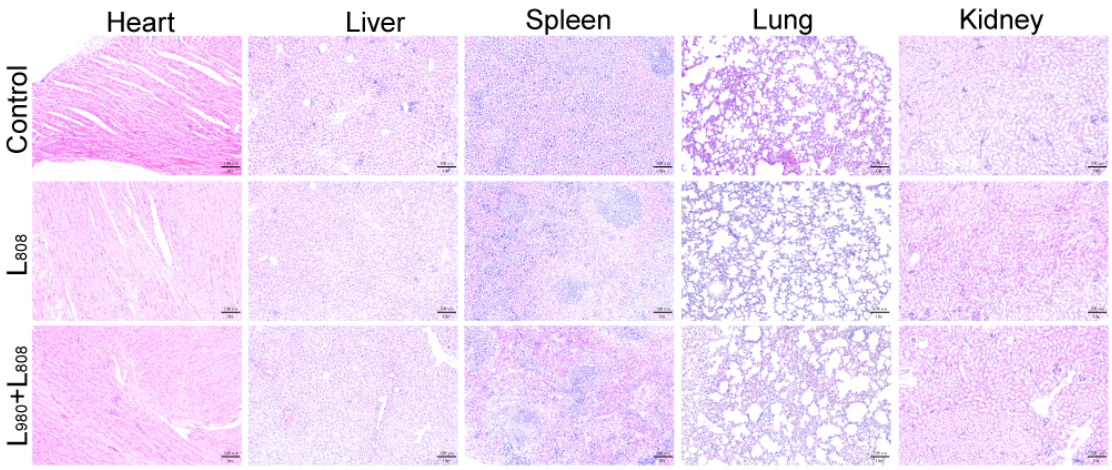


Fig. S16 H&E analysis of the major organs (heart, liver, spleen, lung and kidney) from mice in the different groups. Scale bars: 100 μm.

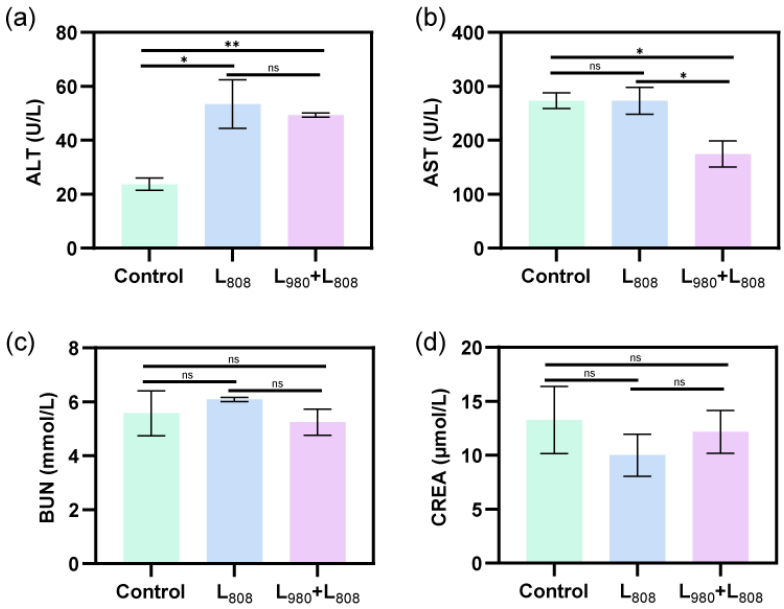


Fig. S17 Serum biochemistry study: ALT, AST, BUN and CREA. Data are presented as mean ± SD (n = 3).

NMR Spectra

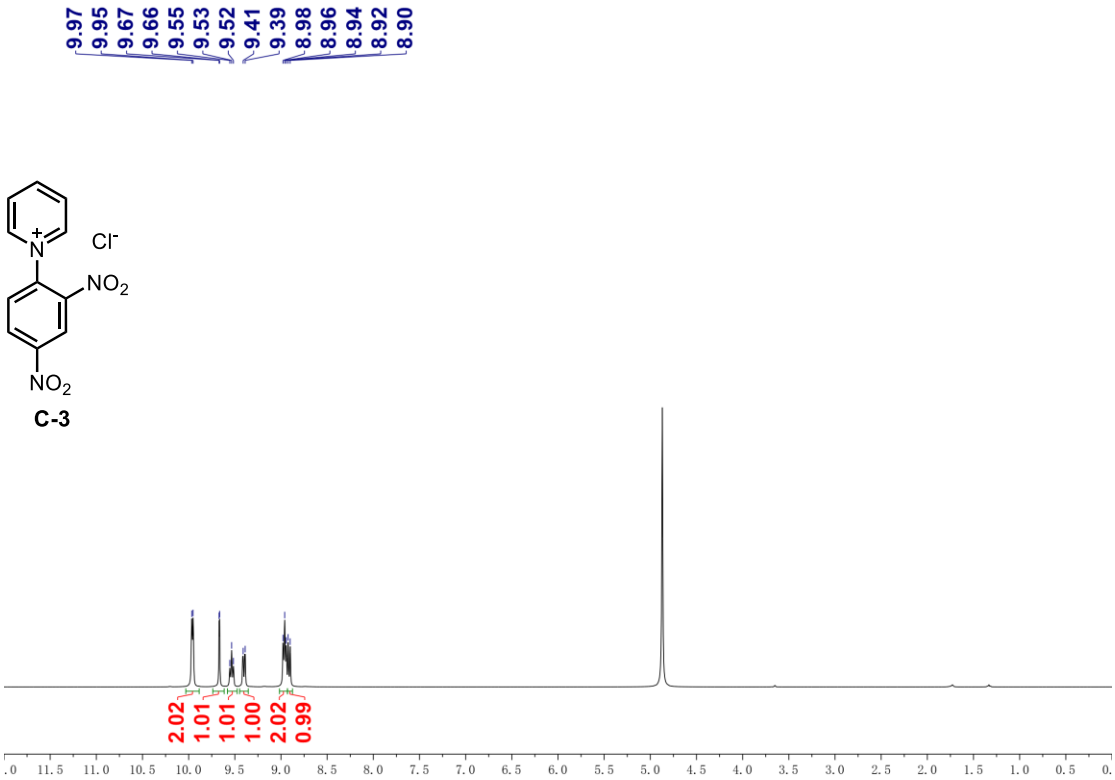


Fig. S18 ^{1}H NMR spectrum of compound **C-3**.

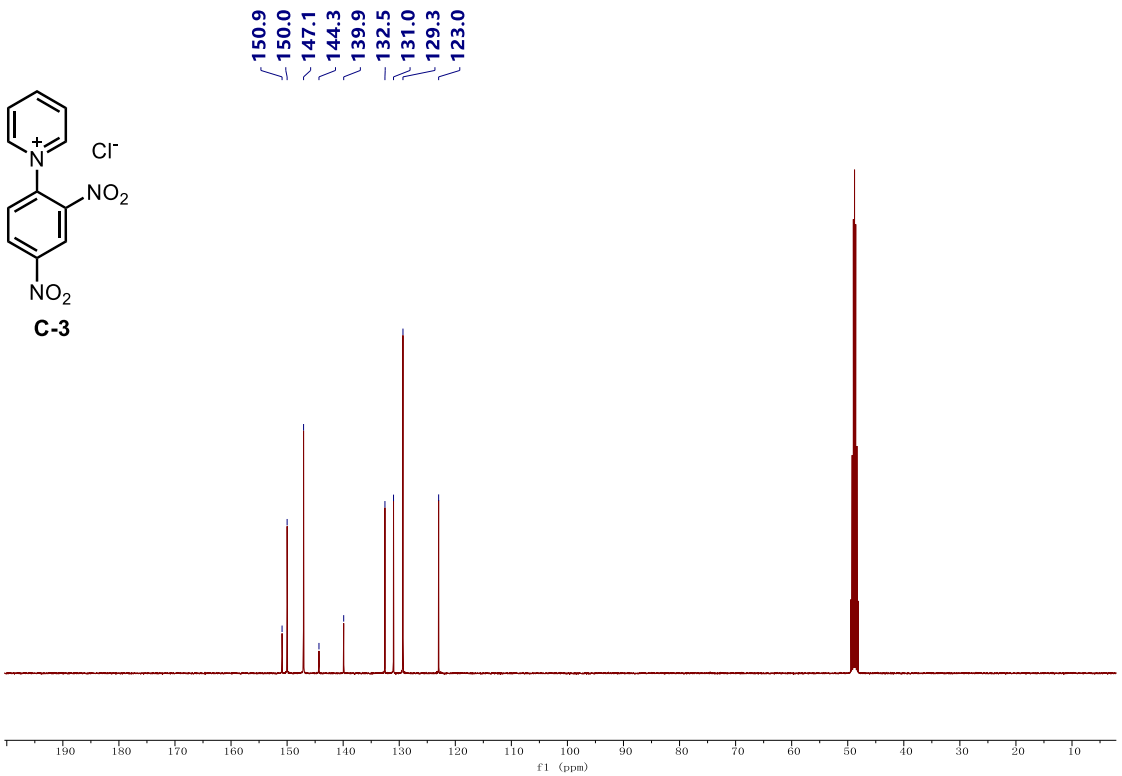


Fig. S19 ^{13}C NMR spectrum of compound **C-3**.

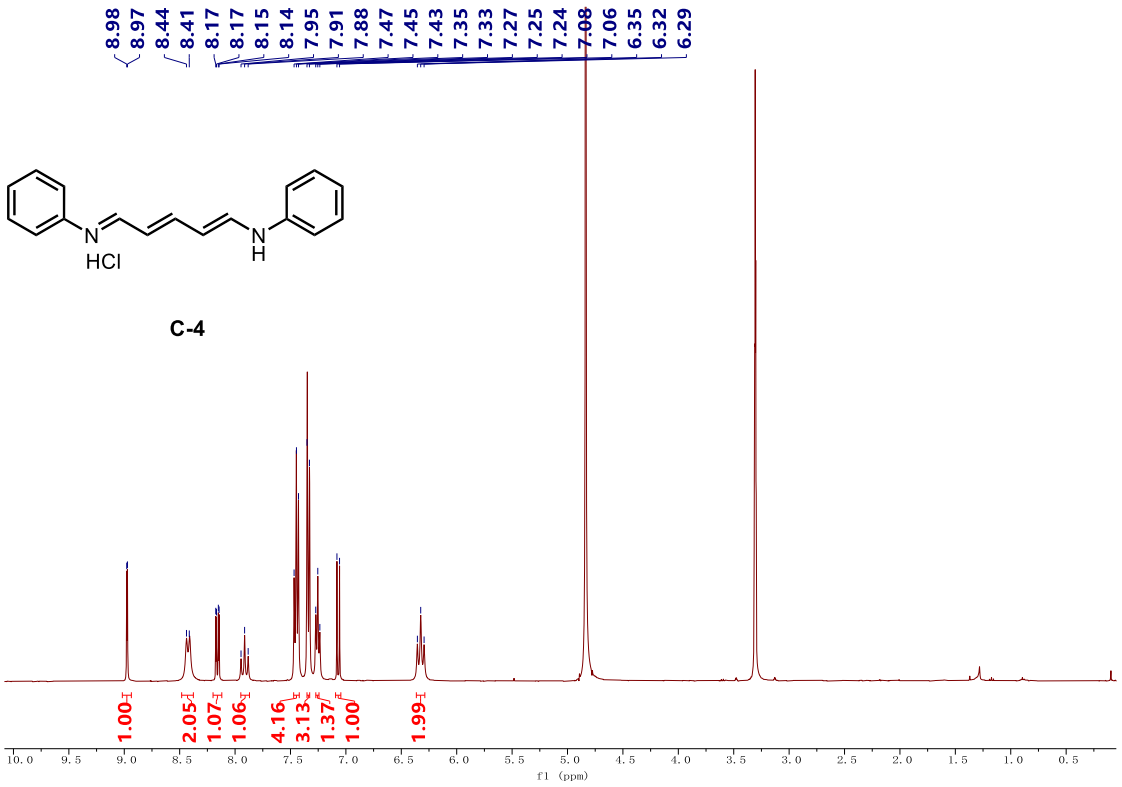


Fig. S20 ^{1}H NMR spectrum of compound **C-4**.

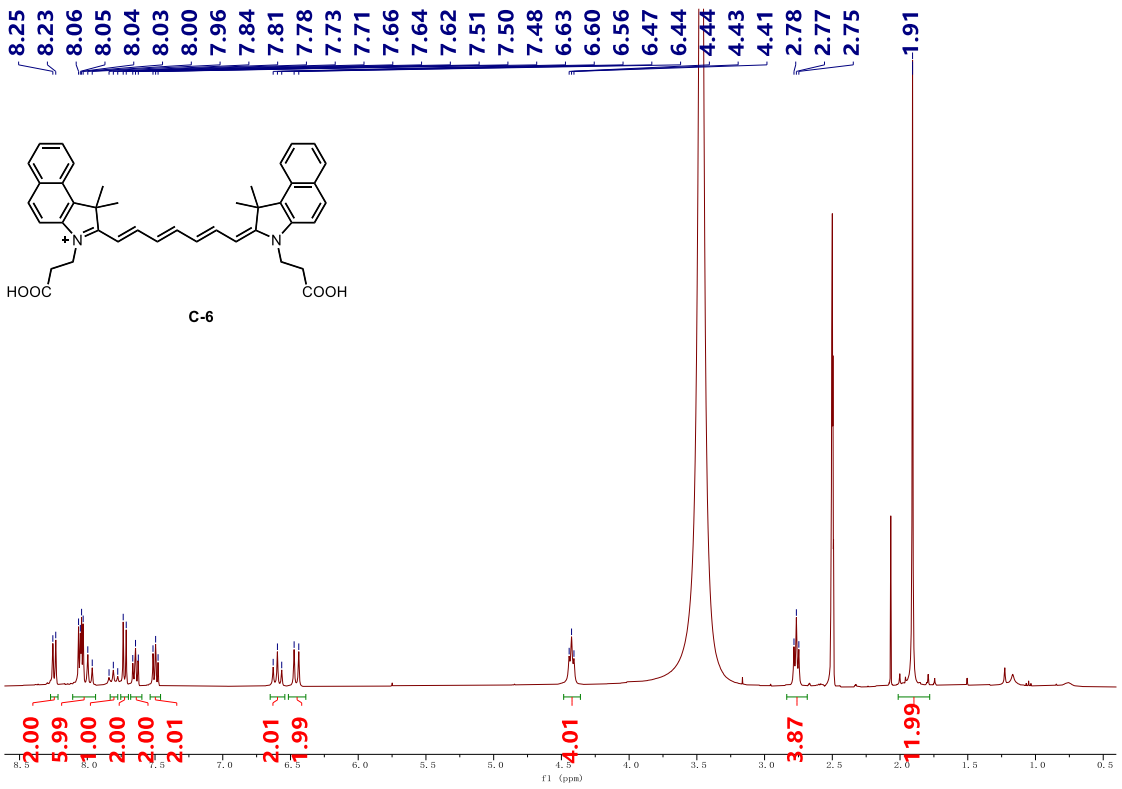


Fig. S21 ^{1}H NMR spectrum of compound **C-6**.

Fig. S22 ^{1}H NMR spectrum of compound **D-2**.

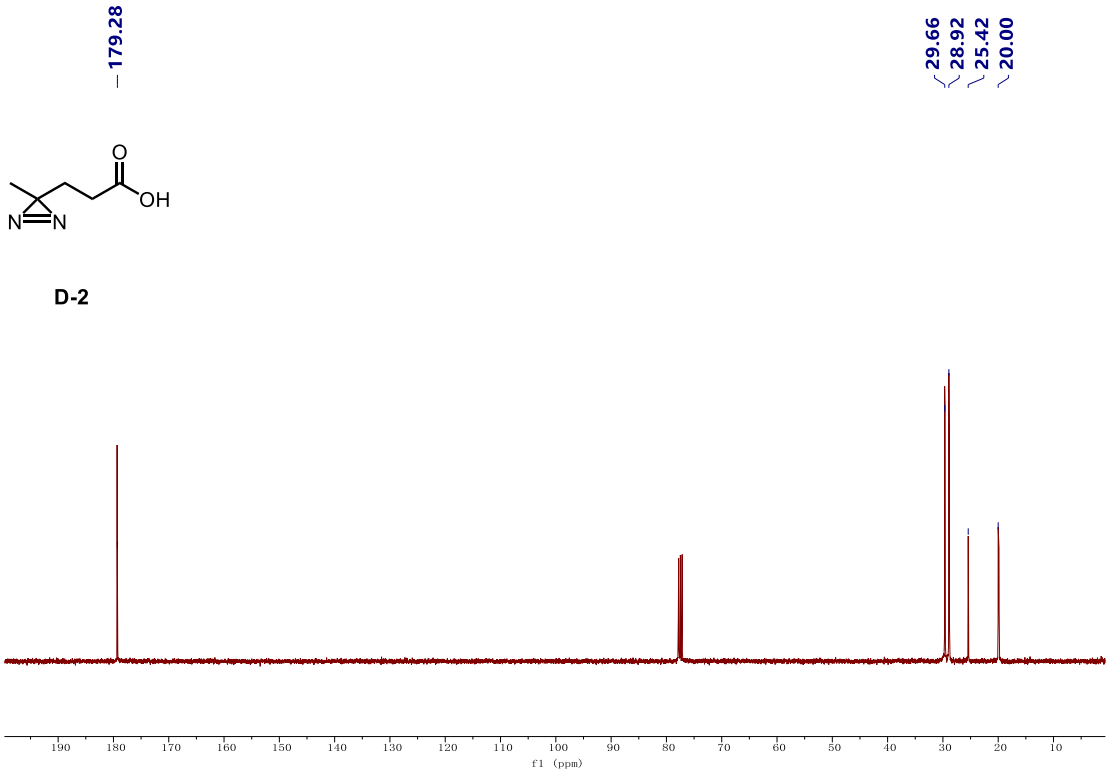


Fig. S23 ^{13}C NMR spectrum of compound **D-2**.

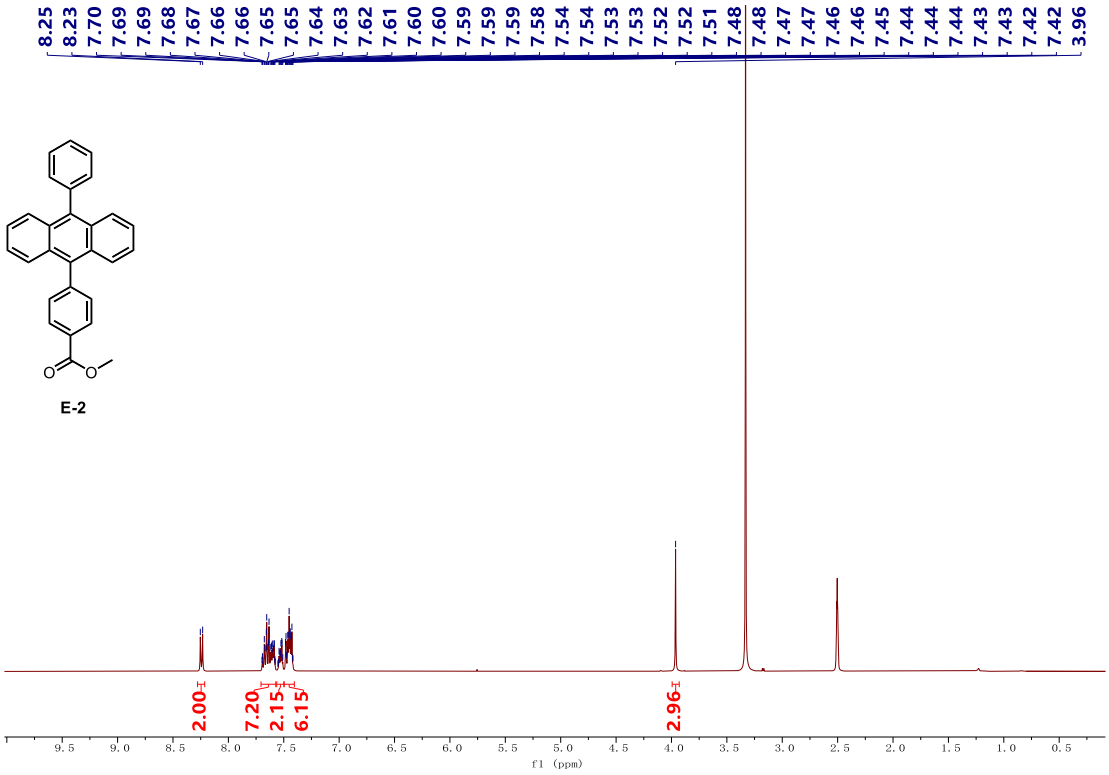


Fig. S24 ^{1}H NMR spectrum of compound **E-2**.

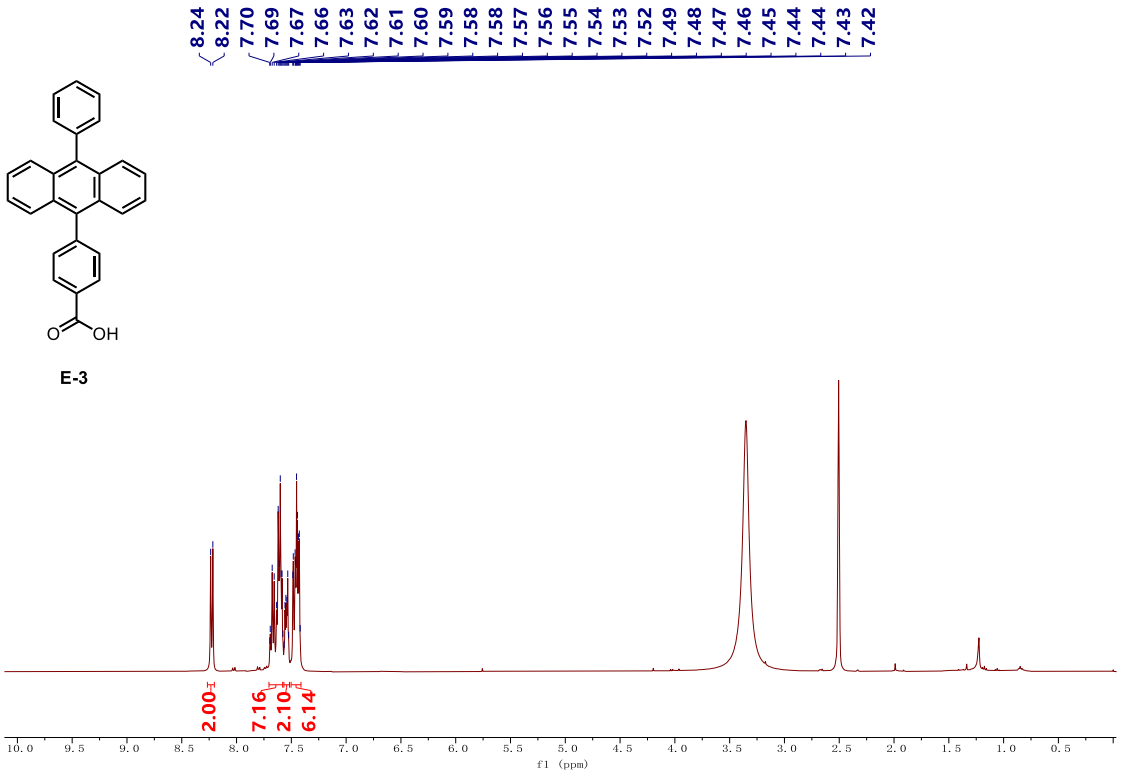


Fig. S25 ^{1}H NMR spectrum of compound **E-3**.

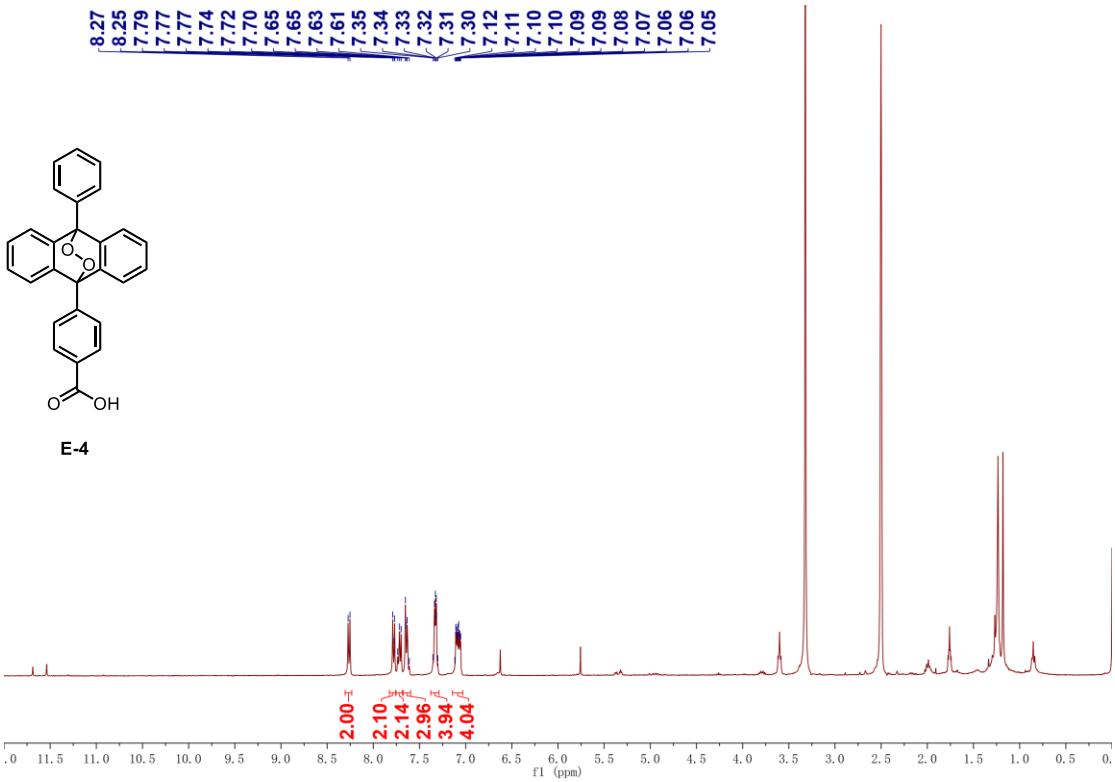


Fig. S26 ^{1}H NMR spectrum of compound **E-4**.