Supporting Information

Hypoxia-Triggered Singlet Oxygen and Nitrogen Mustard Release Towards a Synergistic Therapeutic Action

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1. MATERIALS AND METHODS

Materials: Methylene blue hydrate and cholesterol were purchased from TCI Shanghai, DSPE-PEG2000 was purchased from Bide Pharmatech Ltd., 2,2'-(phenylazanediyl)bis(ethan-1-ol), phosphorus oxychloride ($POCl_3$), (4-aminophenyl)methanol, hydrochloric acid, 3-methylpyridin-2(1*H*)-one, ethyl 4-iodobenzoate, copper iodide (CuI), *N,N*-dimethylformamide (DMF), 4-Dimethylaminopyridine (DMAP), triethylamine (TEA) and soy lecithin were purchased from Energy Engineering Technologies co.,Ltd., Annexin V-FITC/PI Apoptosis Detection Kit and Calcein AM/PI Double Stain Kit were bought from Beyotime Biotechnology Co., Ltd. Reduced Glutathione(GSH) Content Assay Kit and 2′,7′-Dichlorofluorescein diacetate (DCFH-DA) were obtained from Solarbio.

1.2 Syntheses

*Synthesis of compound **1***: $POCl_3$ (5 mL, 53.8 mmol) was added into a round bottomed flask and cooled to 0 °C. And then 2,2'-(phenylazanediyl)bis(ethan-1-ol) (3.9 g, 21.5 mmol) was added slowly. After which, the mixture was heated to reflux at 110 °C for 1 h. Subsequently, the mixture was cooled to room temperature and concentrated by rotary evaporating. The residue was dissolved in ethyl acetate and washed thrice with water. The organic layer was dried over anhydrous magnesium sulfate. Finally, the residue was purified by silica gel chromatography (Hexane: EtOAc = 10: 1, v/v) to obtain compound **1** as a light yellow viscous liquid with 90% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.29 – 8.18 (m, 2H), 7.97 – 7.83 (m, 4H), 7.60 – 7.45 (m, 4H), 7.30 –

7.27 (m, 1H), 7.24 – 7.16 (m, 1H), 6.87 – 6.71 (m, 2H), 6.20 (t, *J* = 6.8 Hz, 1H), 5.45 (s, 2H), 3.85 (t, *J* = 7.0 Hz, 4H), 3.70 (t, *J* = 6.9 Hz, 4H), 2.19 (t, *J* = 0.9 Hz, 3H).

*Synthesis of compound **2***: A solution of $NaNO_2$ (555 mg, 8 mmol) in water (30 mL) was added into the solution of (4-aminophenyl)methanol (1 g, 8.1 mmol) in water (120 mL) containing concentrated HCl (1.675 mL). After stirring for 20 min at 0 °C, the obtained solution was added to the solution of compound **1** (1.5 g, 6.8 mmol) in EtOH. After 2 h, the solution was diluted with DCM (500 mL) and wash twice with water. The organic layer was collected and dried over Na_2SO_4. The concentrated residue was purified by silica gel chromatography (Hexane: EtOAc = 5: 1, v/v) to obtain compound **2** in 35% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.94 – 7.78 (m, 4H), 7.47 (d, *J* = 8.1 Hz, 2H), 6.81 – 6.71 (m, 2H), 4.75 (d, *J* = 4.6 Hz, 2H), 3.82 (q, *J* = 6.1, 5.1 Hz, 4H), 3.69 (t, *J* = 6.8 Hz, 4H).

*Synthesis of compound **3***: To a flame dried sealed tube containing pyridin-2(H)-one (1 g, 10 mmol) in dry DMF, was added corresponding aryl iodide (4.14 g, 15 mmol), To the resulting solution was added anhydrous K_2CO_3 (2.76 g, 20 mmol) and CuI (190.45 mg, 1 mmol). The reaction mixture was then heated to 150 °C. After 6 h, the reaction mixture was cooled to room temperature, quenched with chilled water, and extracted with EtOAc (EA) (3 times). The combined organic layers were washed with brine, dried with anhydrous Na_2SO_4, and concentrated in vacuo, The crude residue was purified by column chromatography to afford compound **3** with 66% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.16 (d, *J* = 8.6 Hz, 2H), 7.49 (d, *J* = 8.6 Hz, 2H), 7.31 – 7.20 (m, 2H), 6.20 (t, *J* = 6.8 Hz, 1H), 4.41 (q, *J* = 7.1 Hz, 2H), 2.20 (s, 3H), 1.42 (t, *J* = 7.1 Hz,

3H); ^{13}C NMR (100 MHz, Methanol-d_4) δ 165.64, 162.99, 144.95, 138.61, 135.44, 130.41, 130.15, 129.82, 126.76, 106.95, 61.09, 15.83, 13.18.

*Synthesis of compound **4***: A solution of compound **3** (900 mg, 3.5 mmol) in EtOH (135 mL) was added to the solution of NaOH (684 mg, 17.1 mmol) in water (45 mL). The solution was stirred for 5 h at 83 °C. The solvent was extracted with EA and concentrated under vacuo without further purification. The product was white solid form with 85% yield. ^{1}H NMR (400 MHz, CD_3OD) δ 8.24 – 8.13 (m, 2H), 7.63 – 7.49 (m, 4H), 6.53 (t, J = 6.8 Hz, 1H), 2.18 (s, 3H); ^{13}C NMR (100 MHz, Methanol-d_4) δ 167.30, 163.04, 144.84, 138.64, 135.49, 130.81, 130.45, 129.81, 126.66, 106.98, 15.84.

*Synthesis of **Pyd***: T To a dried 50 mL two-necked round-bottom flask, compound **4** (150 mg, 0.65 mmol) was added followed by DCM (3 mL) and then DMF (2 μL) sequentially. Thionyl chloride (57 μL, 0.785 mmol) was then added dropwise. The resulting mixture was stirred at room temperature for 2 h and concentrated under reduced pressure. The residue was dissolved in DCM (20 mL) again, and to the stirred solution, DMAP (0.611 mg, 0.005 mmol) and compound **2** (345 mg, 0.98 mmol) were added at room temperature. Then, NEt_3 (272 μL, 1.96 mmol) was added dropwise at 0 °C. The reaction mixture was stirred at room temperature. After 2 h, the solution was diluted with EtOAc (20 mL). After separation, the aqueous layer was extracted with EtOAc (20 mL). The combined organic layers were washed with brine (10 mL), dried over $MgSO_4$, and then evaporated under reduced pressure. The resulting crude product was purified by silica gel column chromatography (Hexane: EtOAc = 2: 1, v/v) to afford it as yellow solid with 60% yield.^{1}H NMR (400 MHz, $CDCl_3$) δ 8.29 –

8.18 (m, 2H), 7.97 – 7.83 (m, 4H), 7.60 – 7.45 (m, 4H), 7.30 – 7.27 (m, 1H), 7.24 – 7.16 (m, 1H), 6.87 – 6.71 (m, 2H), 6.20 (t, J = 6.8 Hz, 1H), 5.45 (s, 2H), 3.85 (t, J = 7.0 Hz, 4H), 3.70 (t, J = 6.9 Hz, 4H), 2.19 (t, J = 0.9 Hz, 3H); ^{13}C NMR (100 MHz, Chloroform-*d*) δ 165.63, 162.62, 153.01, 148.74, 145.37, 144.76, 137.24, 134.74, 131.27, 130.87, 129.90, 128.94, 126.89, 125.49, 122.75, 111.75, 106.18, 66.71, 40.38, 17.46.

Synthesis of ***Pyd-endo***: Compound **Pyd** (20 mg, 0.34 mmol) was dissolved in 3 mL $CDCl_3$. The reaction mixture was cooled to 0 °C in the ice bath. Methylene blue (10 mg, 0.0296 mmol) was added into the solution and mixture was stirred for 2 h under oxygen atmosphere. During the reaction, a 18 W, 630 nm red light was used. After removal of the solvent by rotary evaporator, the methylene blue was removed by activated carbon. The resulting compound, **Pyd-endo**, was obtained as a yellow solid with a yield of 97%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.13 (d, J = 8.8 Hz, 2H), 7.90 (dd, J = 14.5, 8.8 Hz, 4H), 7.55 (d, J = 8.5 Hz, 2H), 7.43 (d, J = 8.8 Hz, 2H), 6.97 (dd, J = 7.9, 5.4 Hz, 1H), 6.78 (d, J = 9.2 Hz, 2H), 6.60 (dd, J = 7.9, 1.8 Hz, 1H), 6.18 (dd, J = 5.4, 1.8 Hz, 1H), 5.42 (s, 2H), 3.85 (t, J = 7.0 Hz, 4H), 3.70 (t, J = 7.0 Hz, 4H), 1.70 (s, 3H); ^{13}C NMR (100 MHz, Chloroform-*d*) δ 168.08, 152.94, 148.73, 144.68, 137.56, 134.11, 133.95, 131.02, 128.87, 127.90, 125.45, 122.70, 122.67, 111.71, 85.37, 82.11, 66.54, 40.36, 14.74, 1.12.

Synthesis of ***endoperoxide 1***: Compound **3** (20 mg, 0.078 mmol) was dissolved in 3 mL $CDCl_3$. The reaction mixture was cooled to 0 °C in the ice bath. Methylene blue (10 mg, 0.0296 mmol) was added into the solution and mixture was stirred for 2 h

under oxygen atmosphere. During the reaction, 18 W, 630 nm red light was used. After removal of the solvent by rotary evaporator, the methylene blue was removed by activated carbon. The compound **endoperoxide 1** was white solid with 97% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.10 – 8.04 (m, 2H), 7.39 (dd, J = 8.8, 2.2 Hz, 2H), 6.95 (m, 1H), 6.58 (m, 1H), 6.17 (dd, J = 5.5, 1.8 Hz, 1H), 4.41 – 4.33 (m, 2H), 1.68 (d, J = 3.0 Hz, 3H), 1.38 (m, 3H).

2. Temporal Evolution of Pyd-endo ^{1}H NMR at 37 °C

The half-life of endoperoxides is tested by ^{1}H NMR spectroscopy. 3 mg of **Pyd-endo** is dissolved in 0.6 mL of deuterated solvents, and ^{1}H NMR is performed at 0 h, 19 h, 48 h, 72 h, 96 h, 120 h, and 168 h. The NMR tube needs to be maintained in a constant temperature water bath at 37 °C to observe the changes in the characteristic peak area of endoperoxides. The proportion is determined by the ratio value to determine whether the decay has ended.

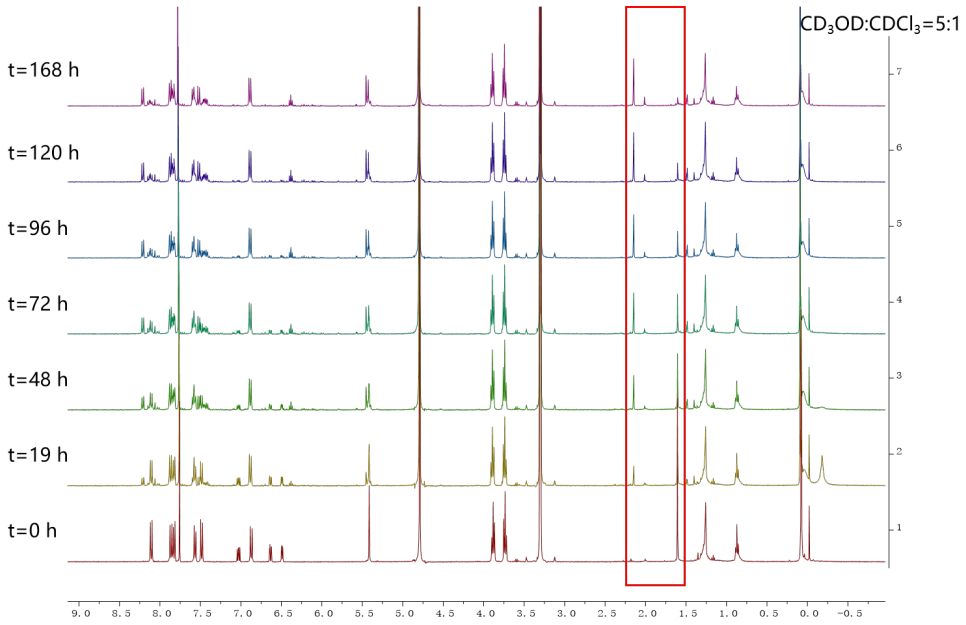

Figure S1. ^{1}H NMR spectrum of half-life of **Pyd-endo** in CD_3OD:$CDCl_3$=5:1 (37 °C)

The half-life $t_{1/2}$ of singlet oxygen releasing follows a first-order kinetic equation, and the red highlighted part indicated the signal of methyl group from **Pyd-endo** and **Pyd** (Figure S1). The equations are given below:

$$\ln[A] = -kt + \ln[A_0]\,, t_{0.5} = 0.693/k$$

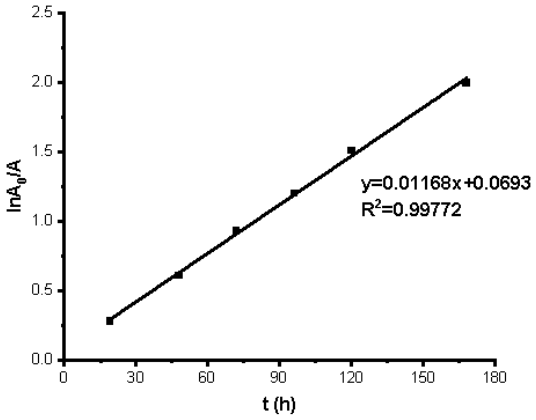

Figure S2. $\ln(A_0/A)/t$ for **Pyd-endo** at 37 °C ($t_{1/2}$ = 59.2 h)

3. Singlet Oxygen Detection of endoperoxide 1 with DPBF

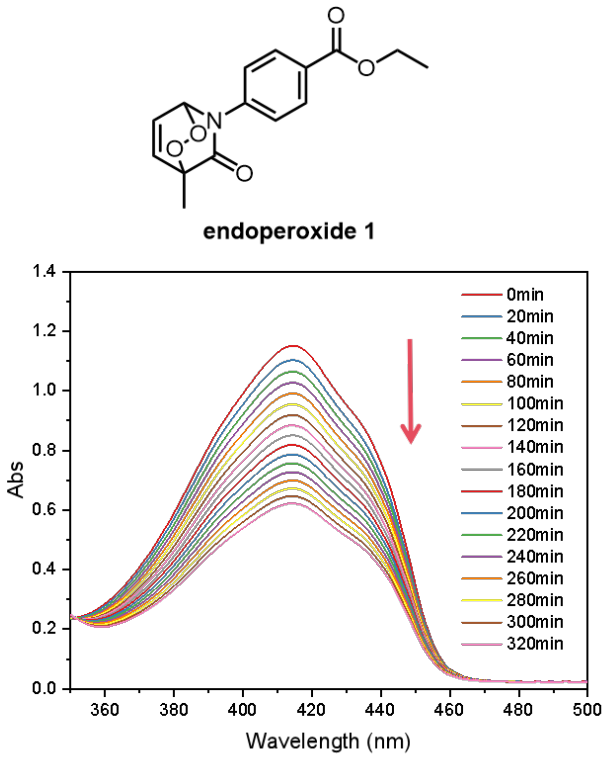

Figure S3. The decrease in the absorption peak of DPBF (0.05 mM) in the presence of **endoperoxide 1** (2.5 mM) in DMF in dark at 37 °C

4. Absorption spectra

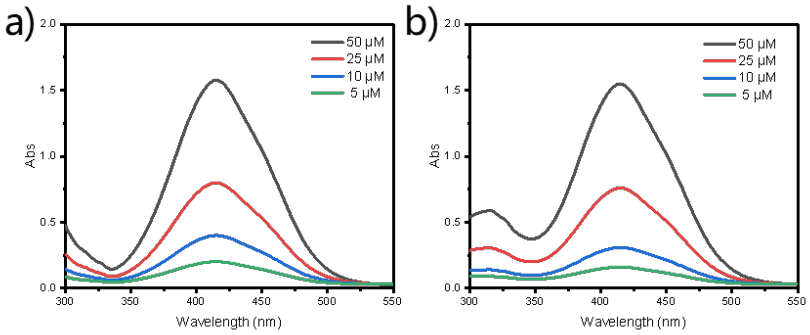

Figure S4. Absorption spectra of a) **Pyd-endo** and b) **Pyd** at different concentrations in DMSO.

5. MTT Test

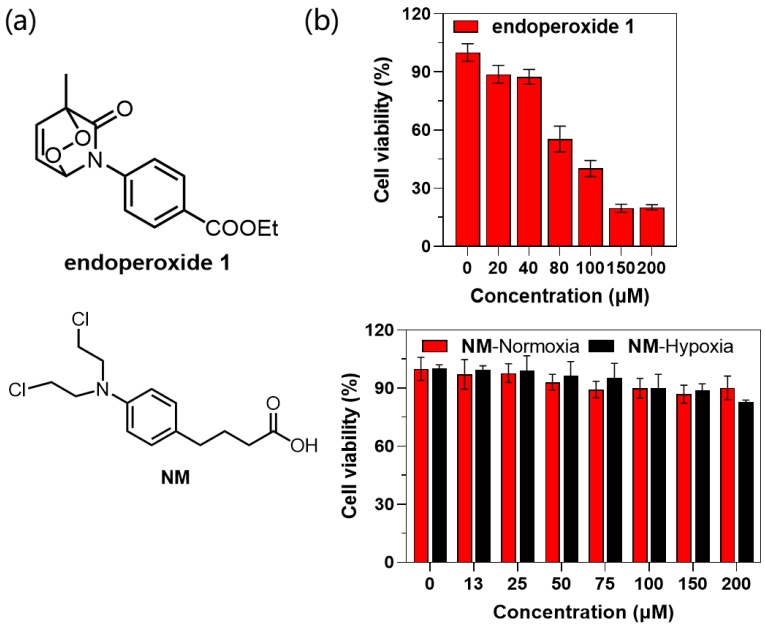

Figure S5. (a) Structure of **endoperoxide 1** and **NM** and (b) cell viability of tumor cells after incubation with **endoperoxide 1** and **NM** for 24 hours under normoxia or hypoxia. Data were assessed as mean ± SD (n = 4)

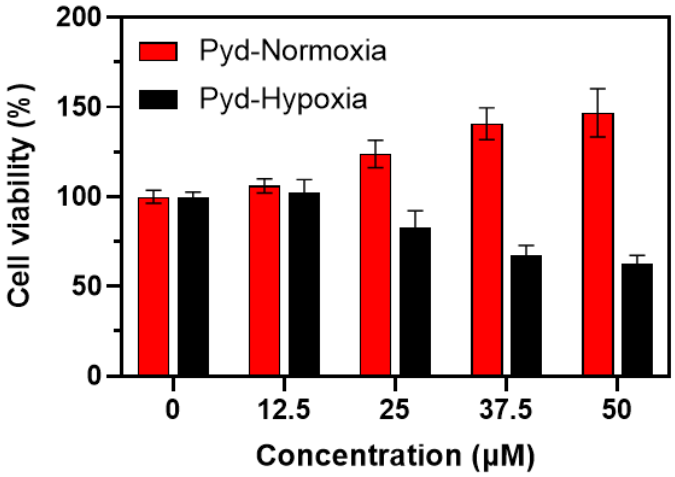

Figure S6. Hela cell viability of tumor cells after incubation with various concentrations of **Pyd** for 24 hours in normoxia or hypoxia. Data were assessed as

mean ± SD (n = 4)

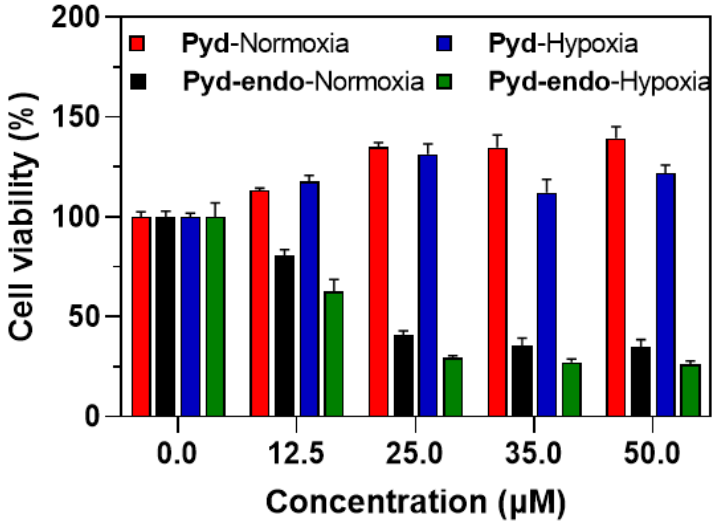

Figure S7. MCF-7 cell viability of tumor cells after incubation with various concentrations of **Pyd** and **Pyd-endo** for 24 hours in normoxia or hypoxia. Data were assessed as mean ± SD (n = 4)

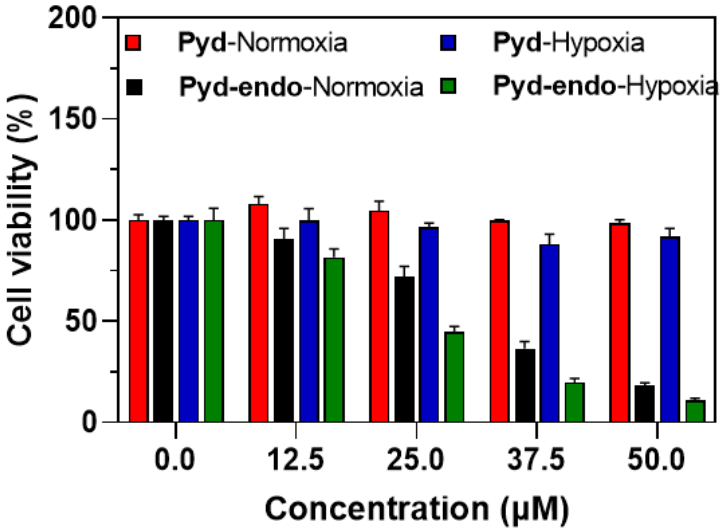

Figure S8. SK-OV-3 cell viability of tumor cells after incubation with various concentrations of **Pyd** and **Pyd-endo** for 24 hours in normoxia or hypoxia. Data were

assessed as mean ± SD (n = 4)

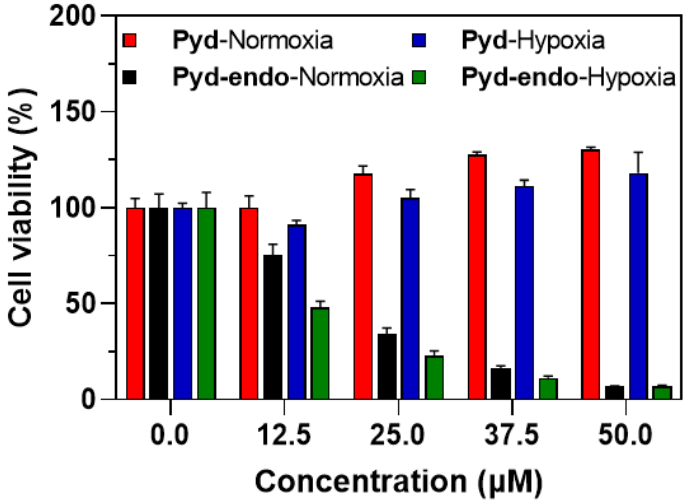

Figure S9. 4T1 cell viability of tumor cells after incubation with various concentrations of **Pyd** and **Pyd-endo** for 24 hours in normoxia or hypoxia. Data were assessed as mean ± SD (n = 4)

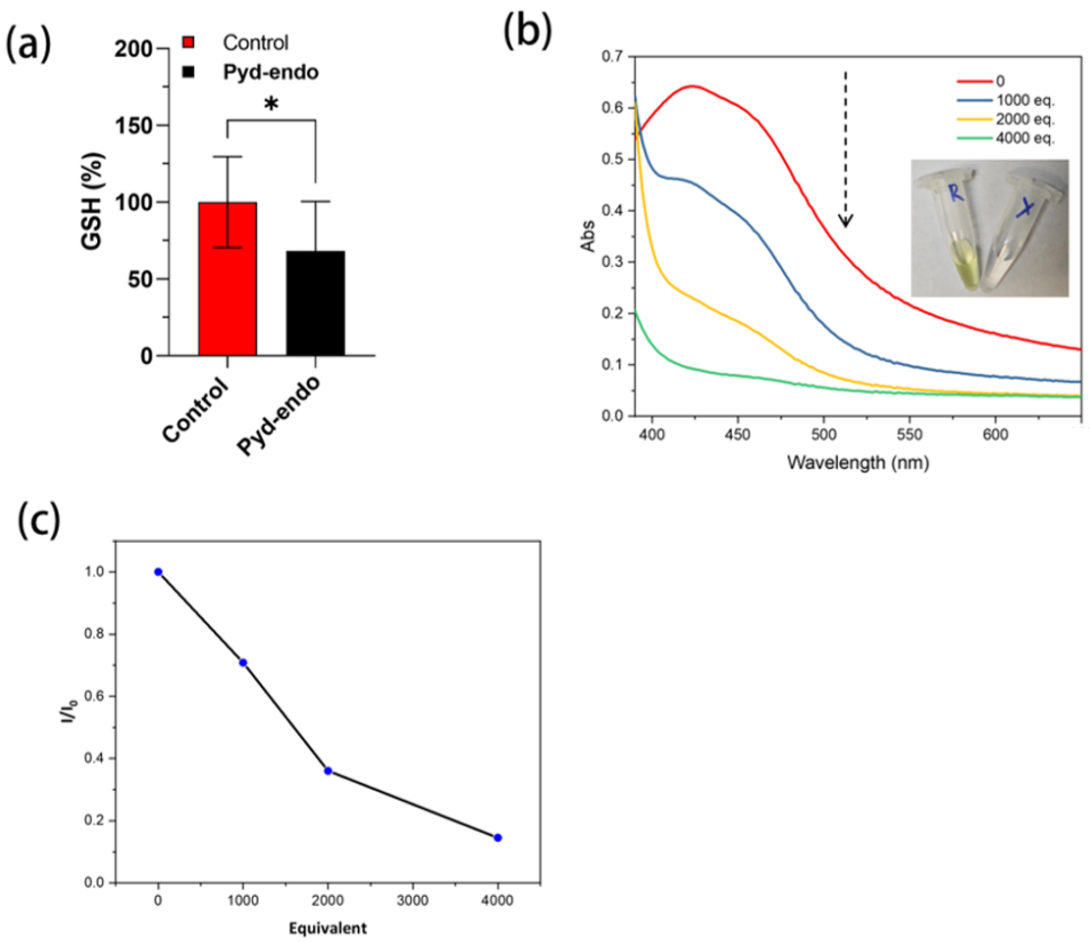

Figure S10. (a) Relative GSH of Hela cells after **Pyd-endo** treatment (**Pyd-endo**: 10 μM), (b) UV-vis absorption of **Pyd** (50 μM) after incubation with various concentrations of $Na_2S_2O_4$ and the digital photograph of color variations of **Pyd** before (R) and after (X) addition of $Na_2S_2O_4$, (c) Cleavage profile of **Pyd** (50 μM) triggered by various concentrations of $Na_2S_2O_4$ (calculated by 425 nm).

6. Intracellular Releasing of Singlet Oxygen

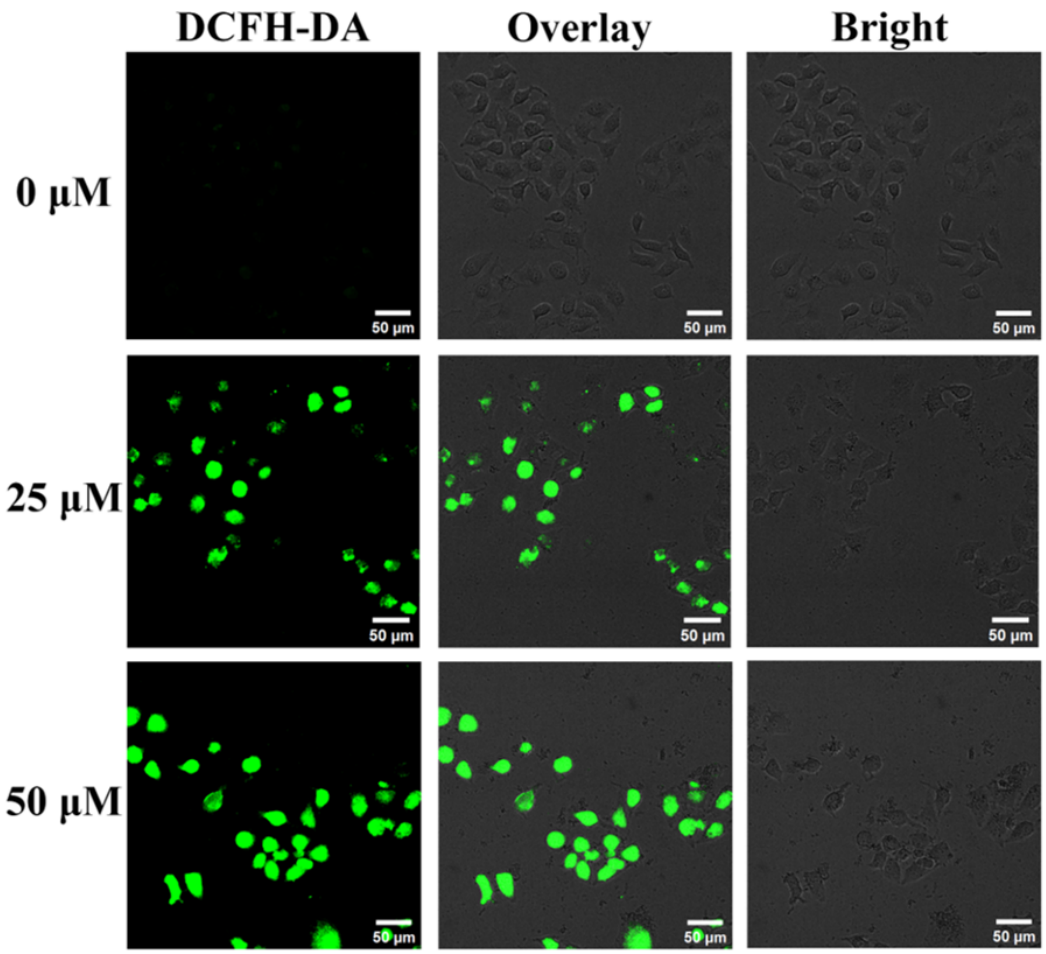

Figure S11. Pyd-endo-induced (25 μM or 50 μM) intracellular ROS detection by DCFH-DA probe. (Incubation time: 3 h)

7. Drug Encapsulation

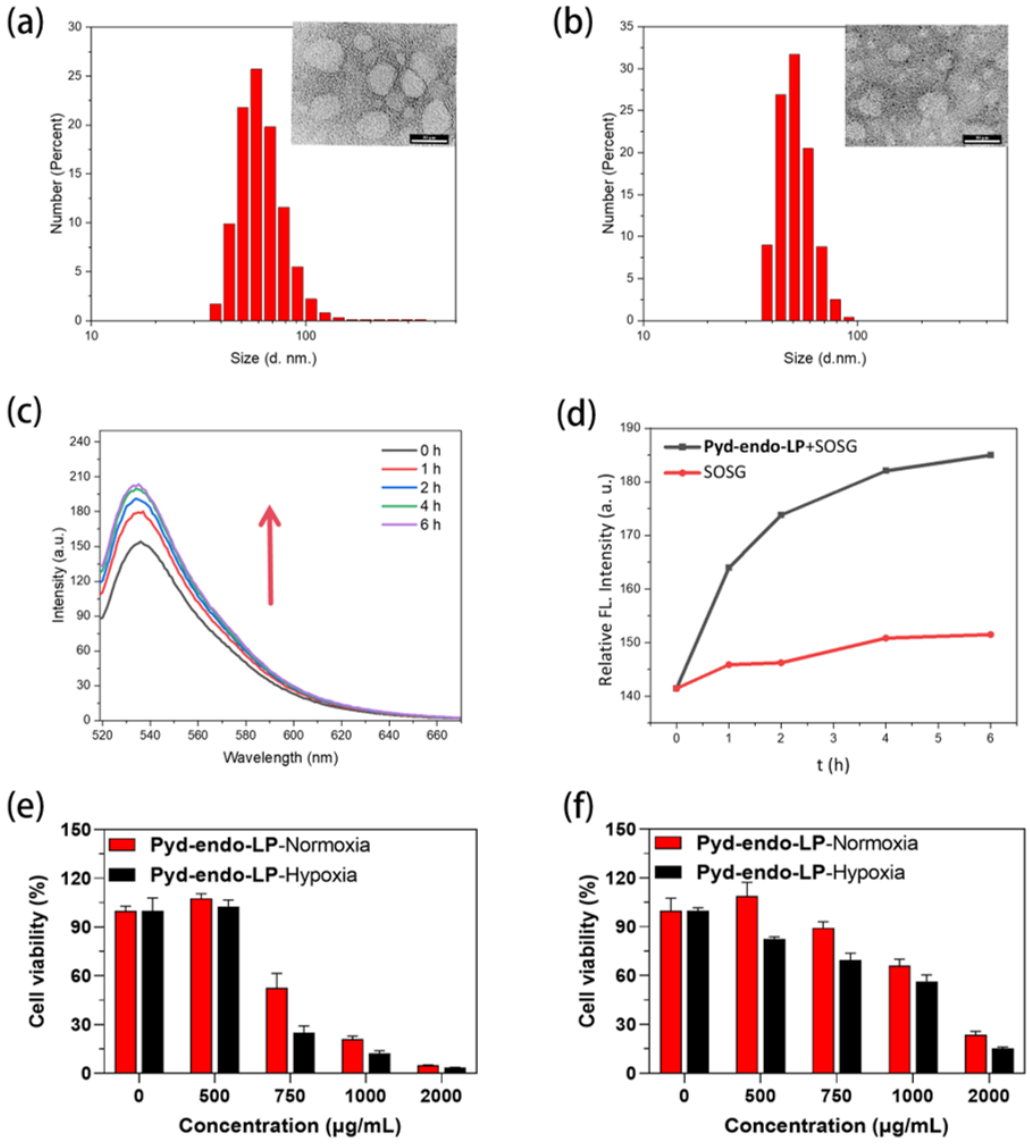

Figure S12. Transmission electron microscopy and dynamic light scattering data of (a) **Pyd-endo-LP** and (b) **Pyd-LP**, bar = 50 μm. (c) Fluorescence changes of SOSG (30 μM) treated with **Pyd-endo-LP** (20 mg/mL), (d) Normalized fluorescence intensity of SOSG/**Pyd-endo-LP** and SOSG at 530 nm, (e) Cytotoxicity of **Pyd-endo-LP** to Hela cells in normoxia or hypoxia, (f) Cytotoxicity of **Pyd-endo-LP** to 4T1 cells in normoxia or hypoxia. Data were assessed as mean ± SD (n = 4).

Establising a standard curve of absorbance /concentration of **Pyd-endo**. 27.83 mg of Liposome powder was dissolved using 2 mL of DMSO to break the emulsion. Test its absorbance at 415 nm and obtain the actual concentration corresponding to the standard curve. Based on the tested concentration, the mass of **Pyd-endo** loaded was obtained, and the drug loading was obtained by quotient with the total dosage of **Pyd-endo**. The calculation formula is: EE (%) = (**Pyd-endo** encapsulated in liposomes/total **Pyd-endo** added) × 100%.

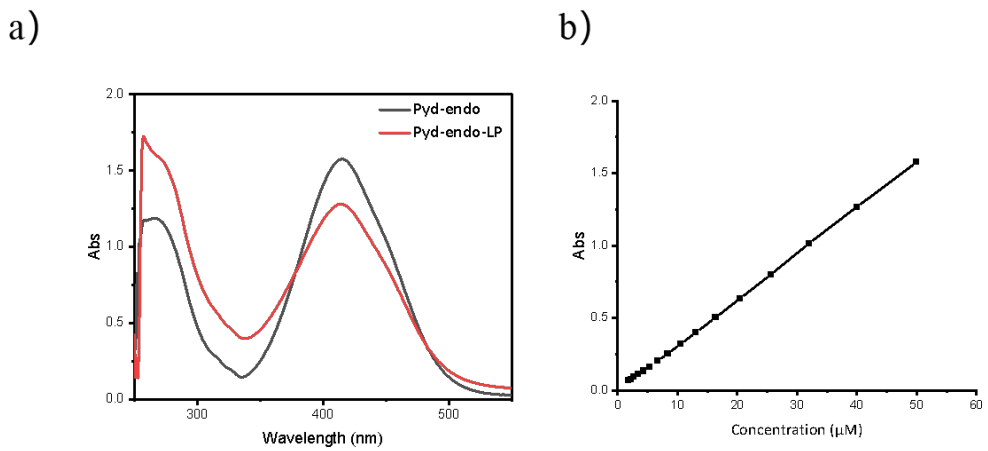

Figure S13. Calculation of liposome drug encapsulation capacity. a) UV-Vis spectra of **Pyd-endo**: 50 μM, **Pyd-endo-LP**: 2.3 mg/mL); b) Standard curve of concentration and absorbance of **Pyd-endo** at 415 nm.

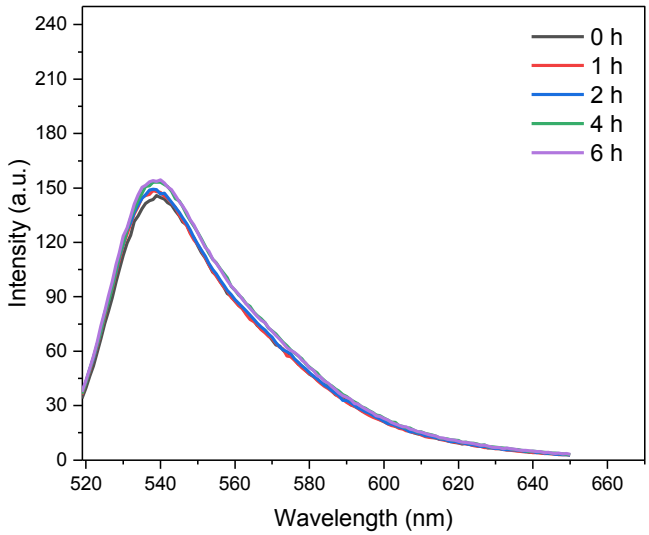

Figure S14. Fluorescence changes of SOSG (30 μM).

8. Antitumor Assay in the 4T1 Tumor-Bearing Mice

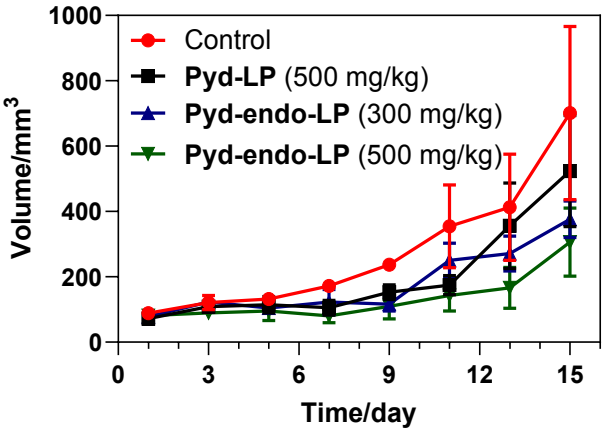

Figure S15. Tumor volume change in different groups.

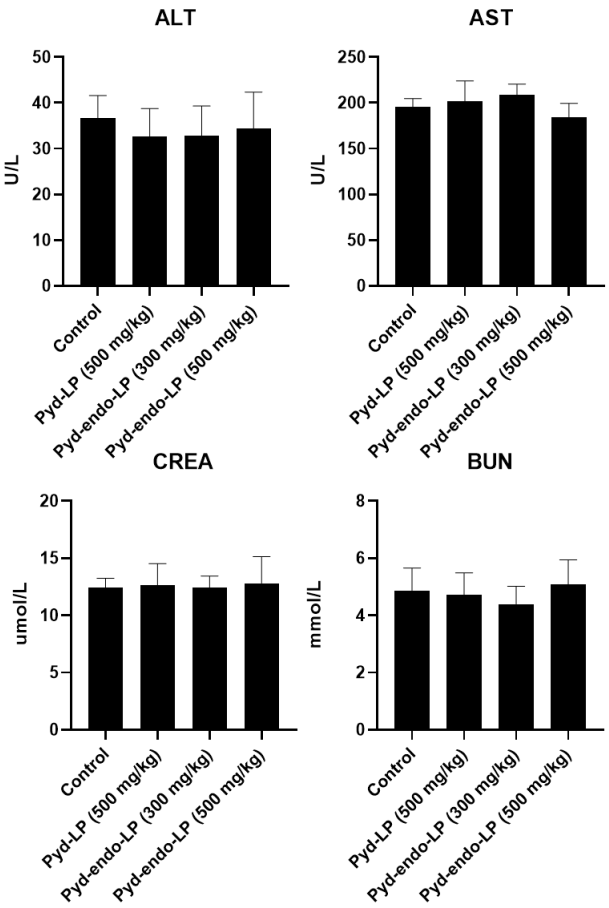

Figure S16. Blood biochemistry indexes of mice at day 15 after treatments including liver and renal function test: ALT, AST, CREA and BUN

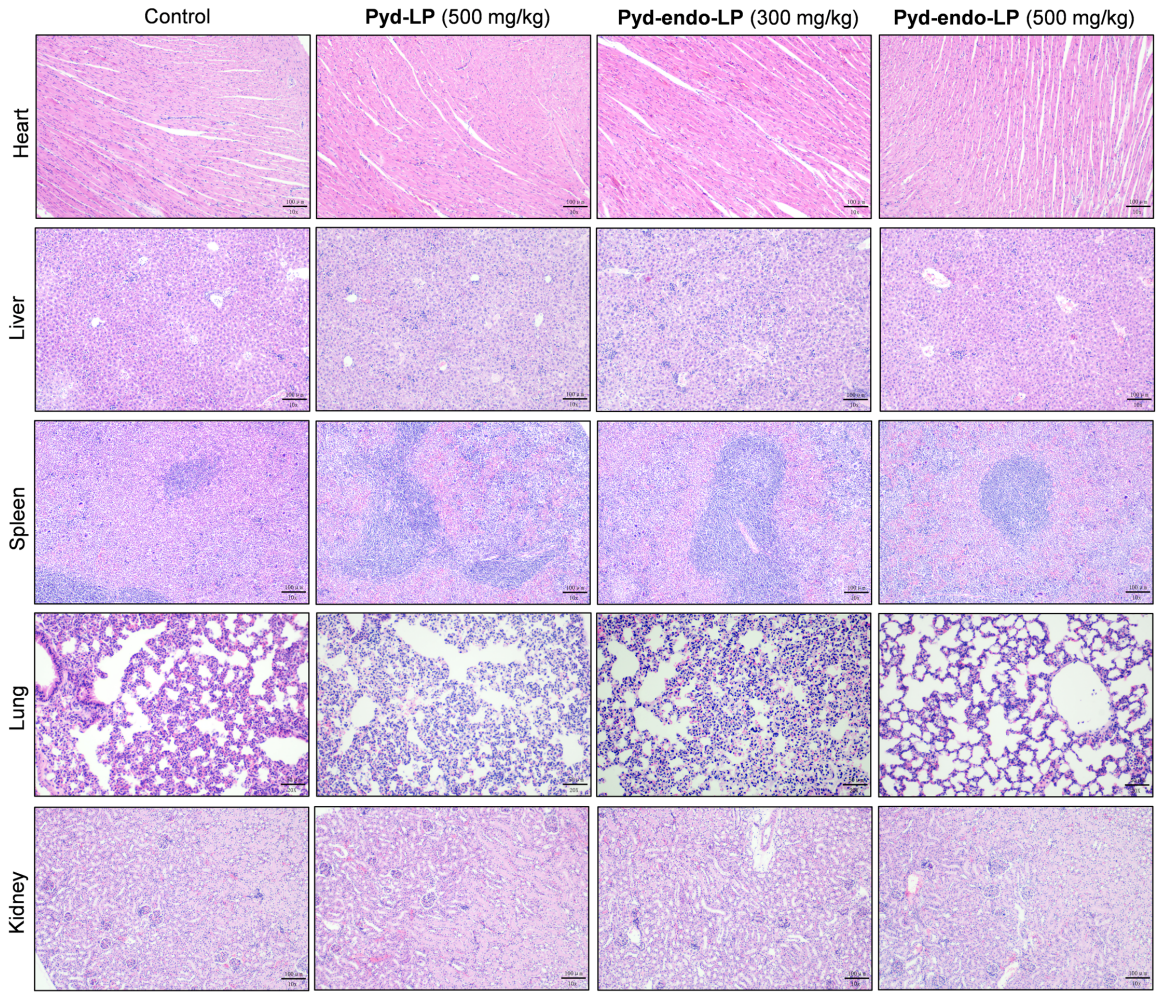

Figure S17. H&E staining histological analysis of normal tissues in various groups of tumor-bearing mice models. Scale bar: 100 μm.

9. NMR Spectra

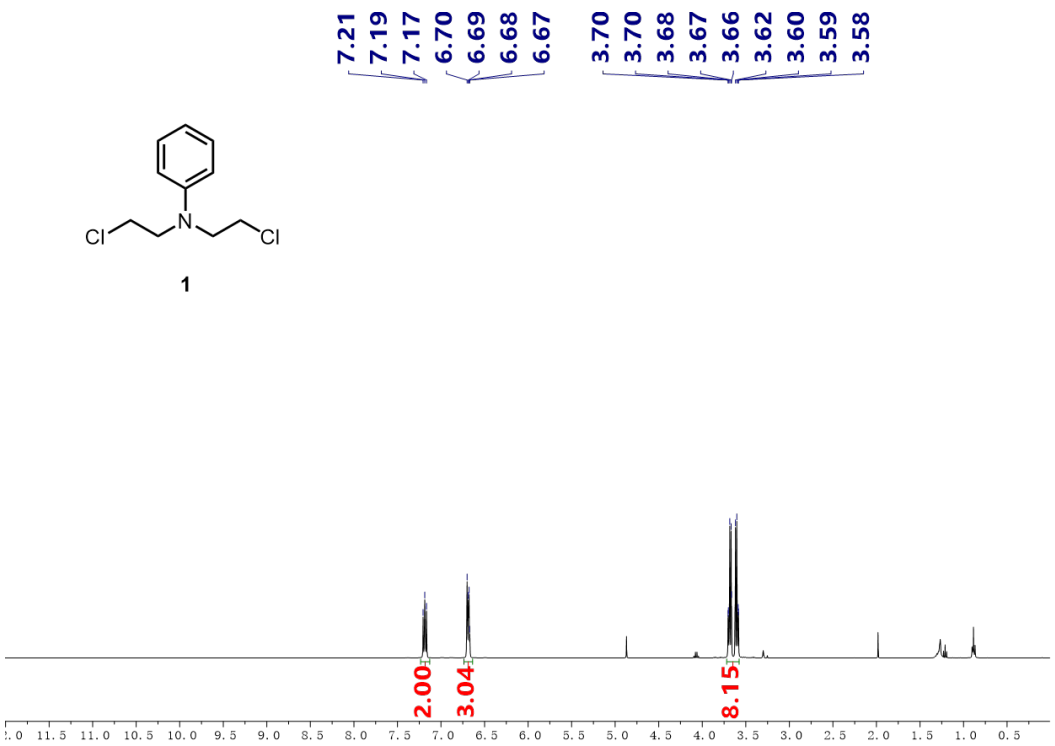

Figure S18. ^{1}H NMR Spectrum of compound **1**

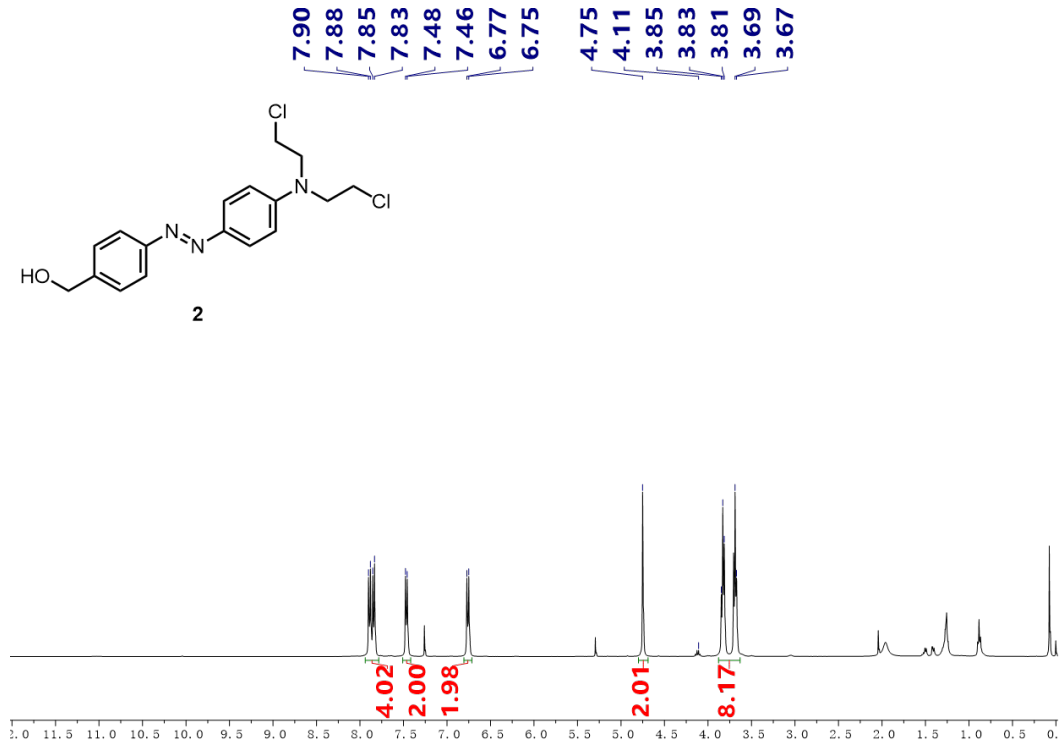

Figure S19. ^{1}H NMR Spectrum of compound **2**

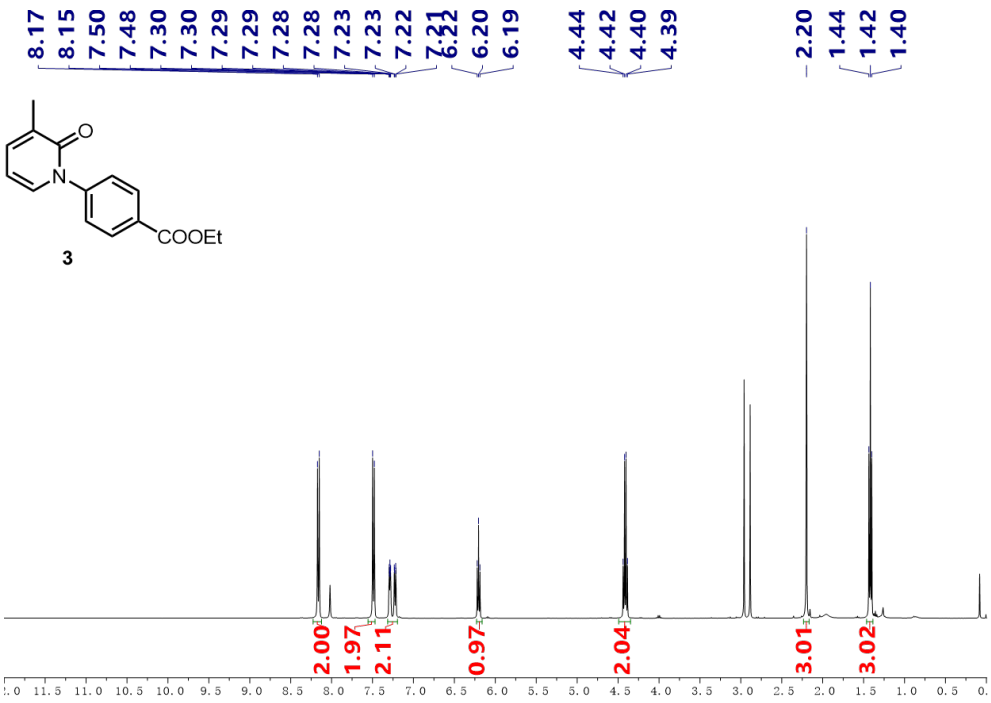

Figure S20. ^{1}H NMR Spectrum of compound **3**

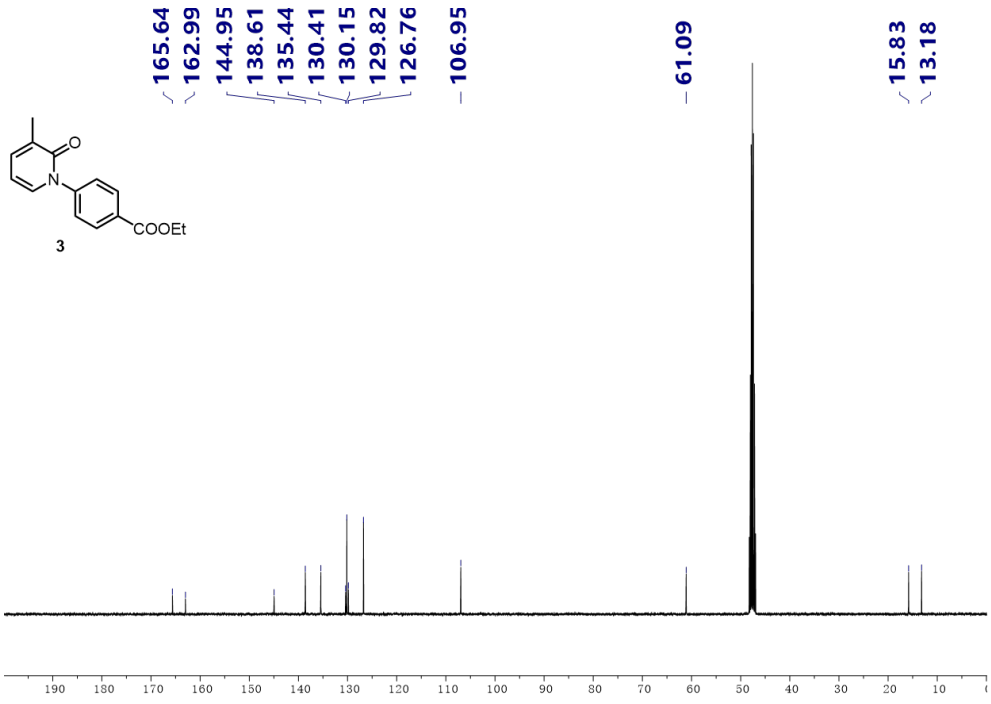

Figure S21. ^{13}C NMR Spectrum of compound **3**

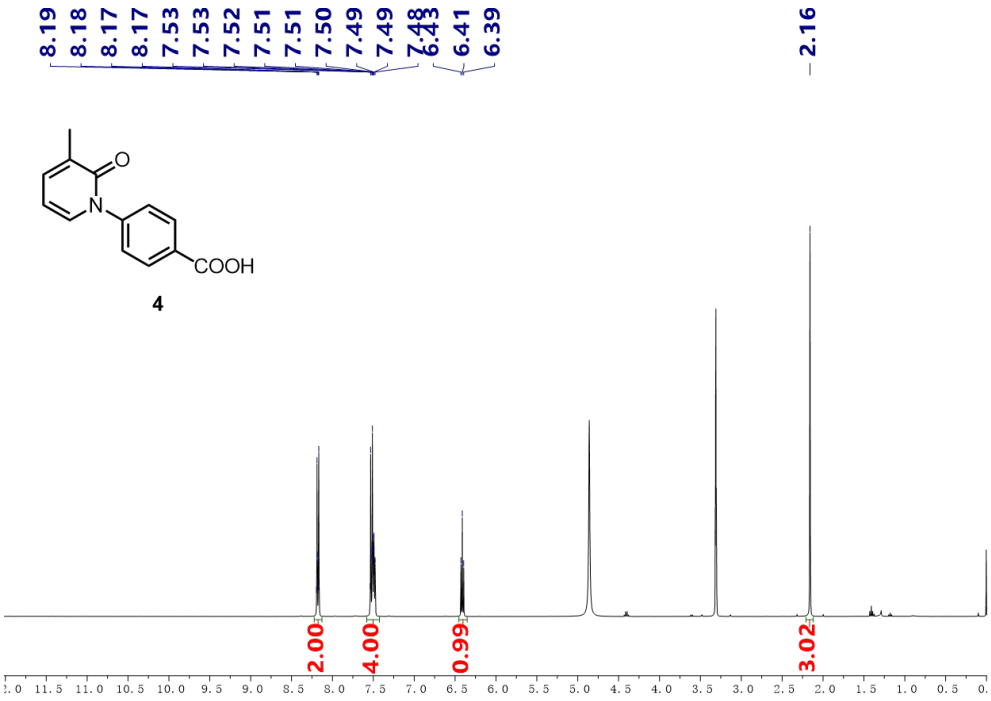

Figure S22. ^{1}H NMR Spectrum of **4**

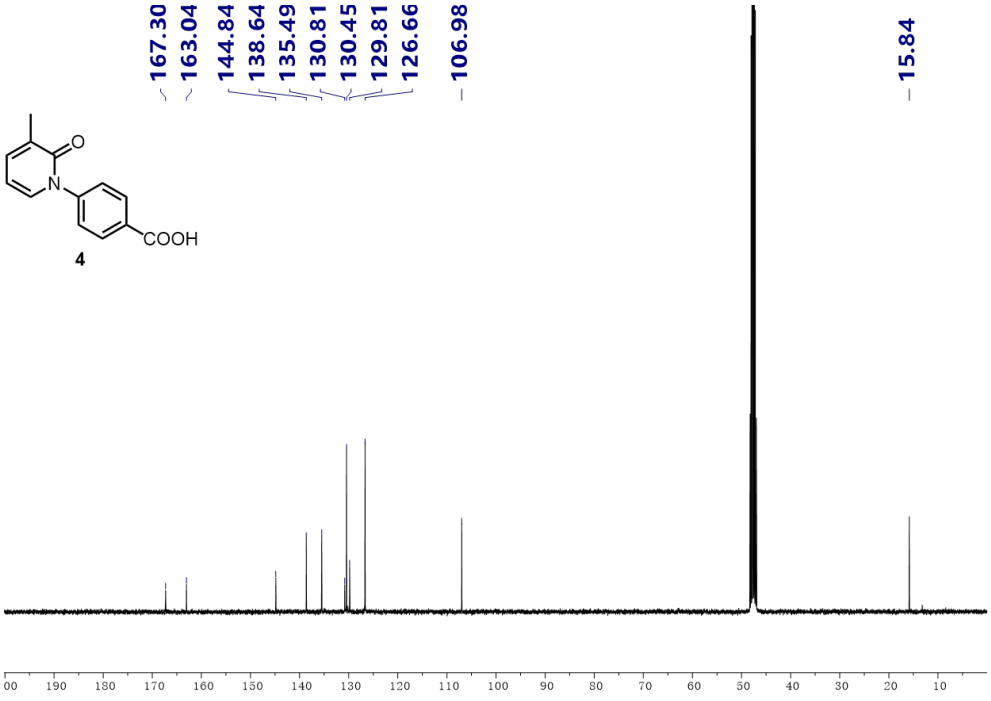

Figure S23. ^{13}C NMR Spectrum of **4**

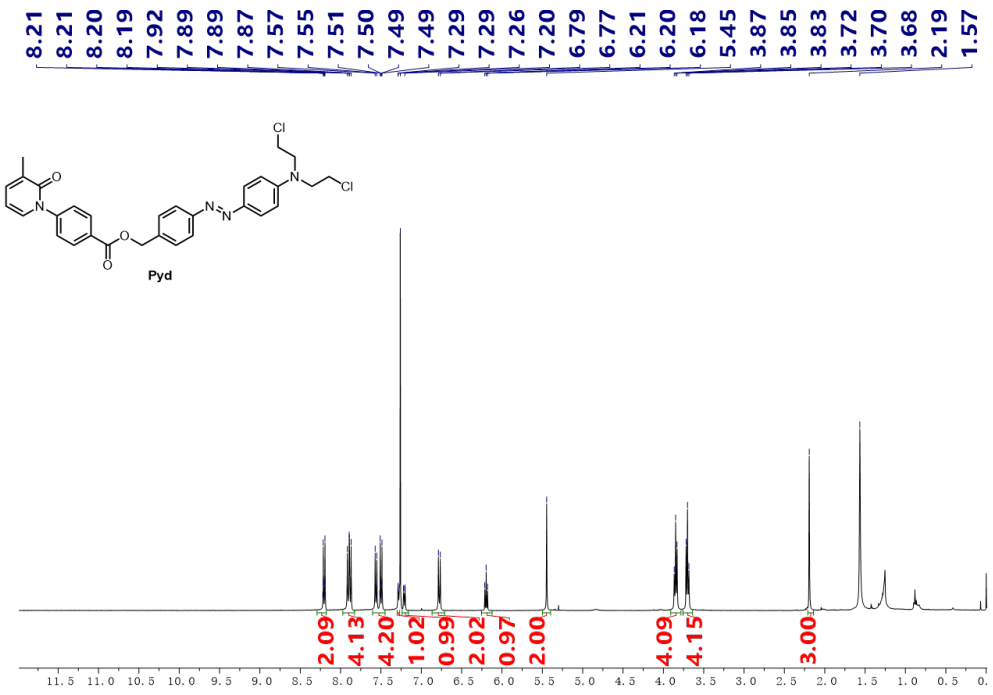

Figure S24. ^{1}H NMR Spectrum of compound **Pyd**

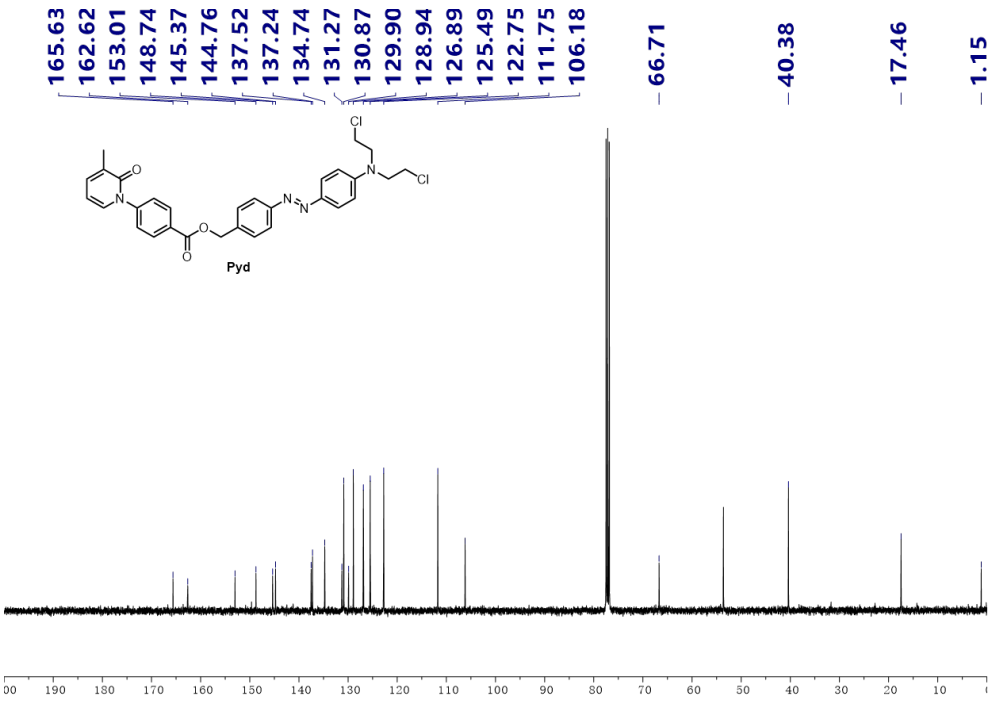

Figure S25. ^{13}C NMR Spectrum of compound **Pyd**

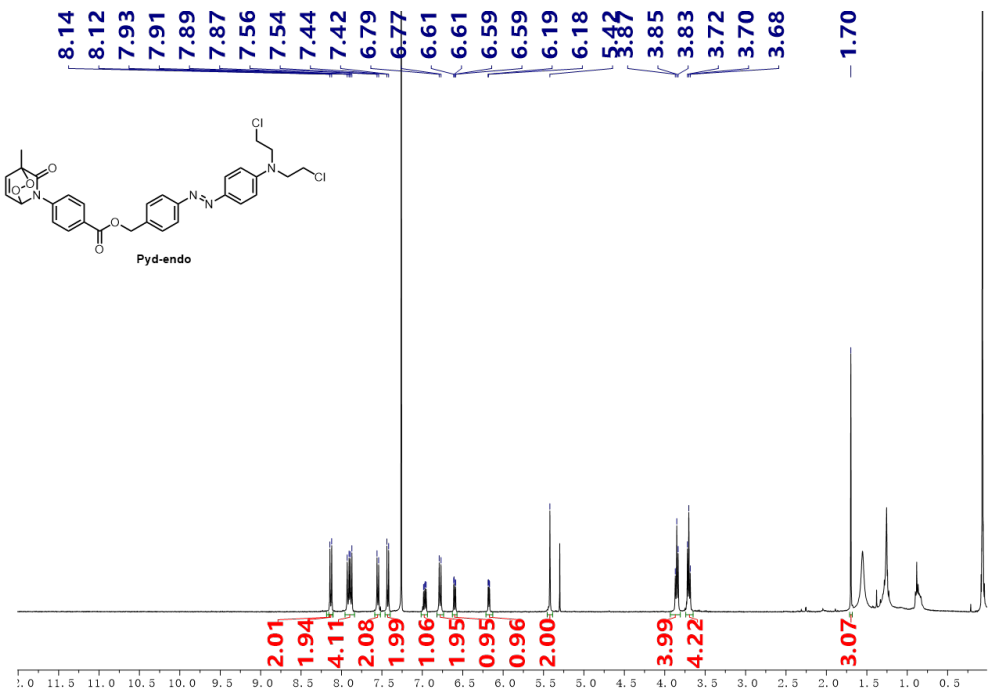

Figure S26. ^{1}H NMR Spectrum of compound **Pyd-endo**

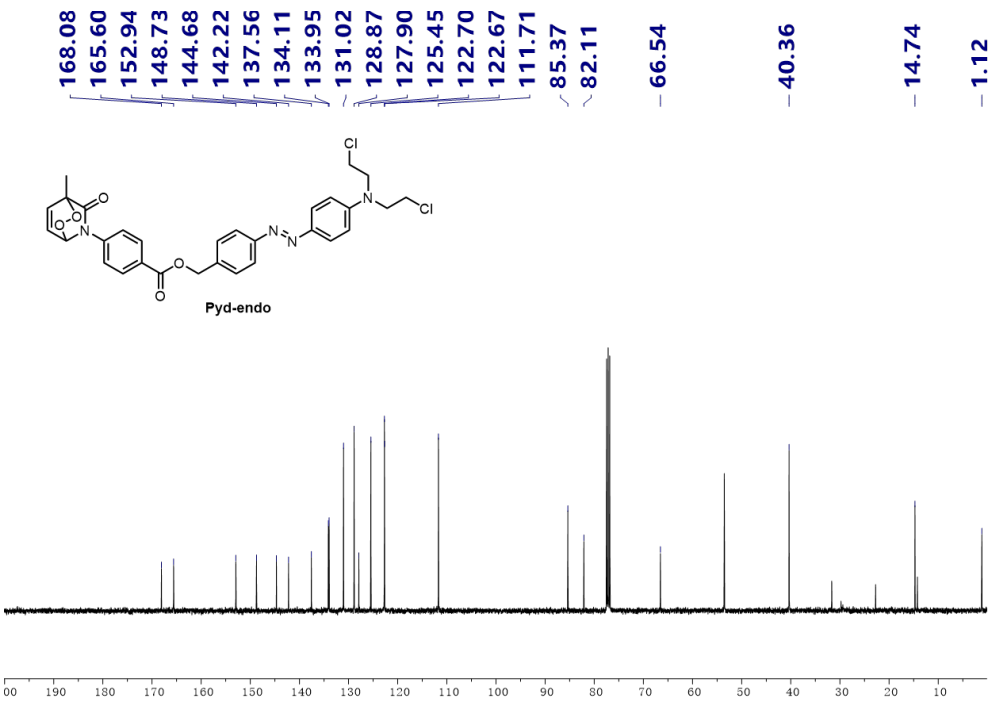

Figure S27. ^{13}C NMR Spectrum of compound **Pyd-endo**

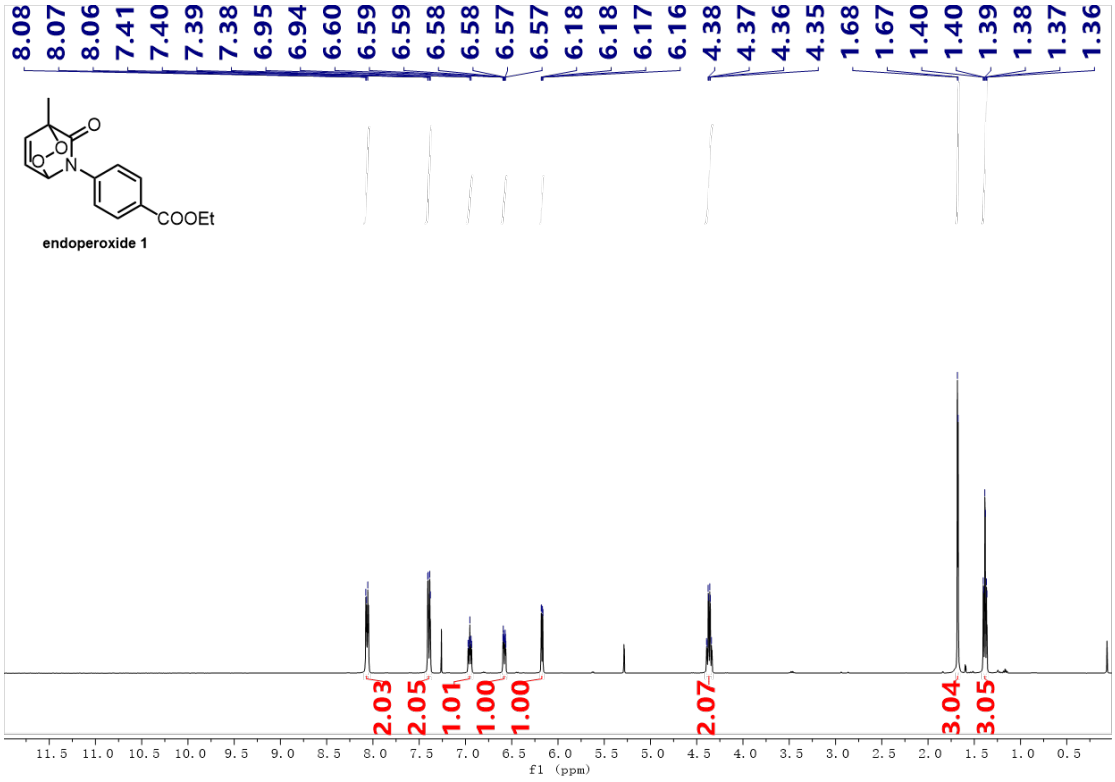

Figure S28. ^{1}H NMR Spectrum of compound endoperoxide **1**