

Electronic Supplementary Information

Multi-Stimuli Responsive Polymeric Prodrug Micelles for Combined Chemotherapy and Photodynamic Therapy

Cheng Hu,[†] Weihua Zhuang,[†] Tao Yu,[†] Liang Chen,[†] Zhen Liang,[†] Gaocan
Li,^{*,†} Yunbing Wang^{*,†}

[†] National Engineering Research Center for Biomaterials, Sichuan
University, Chengdu, Sichuan 610064, People's Republic of China

1. Supplemented Experimental Section

1.1 Materials

2-Mercaptoethanol, cis-1,2-dichloroethylene, 4-nitrophenyl chloroformate, N6-Cbz-L-Lysine, triphosgene, 2,3-dimethylmaleic anhydride and gemcitabine were purchased from Tansoole (China). Cuprous bromide (CuBr) and 2,2-bipyridine (bpy) were obtained from Chengdu Best Reagent Co., LTD (Chengdu, China). 2-Methacryloyloxyethyl phosphorylcholine (MPC) was obtained from Nanjing Natural Science and Technology Institute. Cell counting kit-8 (CCK-8), propidium iodide (PI) and Annexin V FITC/PI Apoptosis Detection Kit were purchased from Dojindo Laboratories (Japan). DCFH-DA Reactive Oxygen Species Assay Kit, Lyso-Tracker Green and Hoechst 33342 were purchased from Beyotime, Shanghai, China. Papain was purchased from Dalian Meilun Biotechnology Co., LTD (Dalian, China). Singlet oxygen sensor green (SOSG) was purchased from ThermoFisher Scientific Inc., Waltham, MA, USA. Fluorescein diacetate (FDA) was obtained from Fanbo biochemicals.

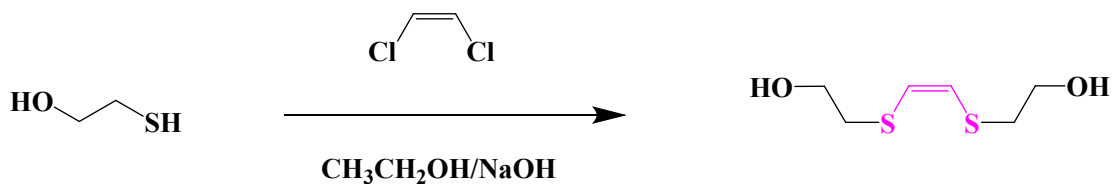
1.2 Cell culture

4T1 (mouse breast cancer cell) was purchased from Chinese Academy of Science Cell Bank for Type Culture Collection (Shanghai, China). Cell culture media RPMI-1640, penicillin, streptomycin, and fetal bovine serum (FBS) were obtained from Gibco (USA). BALB/c female mice (20 ± 3 g, 5-6 weeks old) were obtained from Dashuo Experimental Animal Company (Sichuan, China).

1.3 Characterizations

^1H -NMR spectra were obtained by a Bruker AMX-400 NMR spectrometer. The molecular weight distribution of polymer was detected using THF as the eluent by an Agilent 1260 gel permeation chromatography (GPC). The ability of intracellular singlet oxygen generation for micelles was detected by a fluorescence spectrophotometer (Hitachi F-7000, Japan).

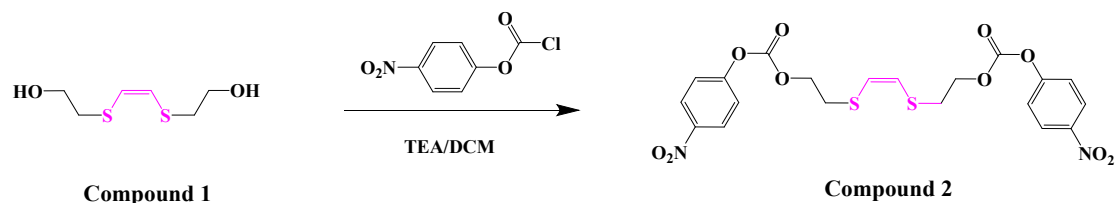
1.4 Synthesis of compound 1



Compound 1

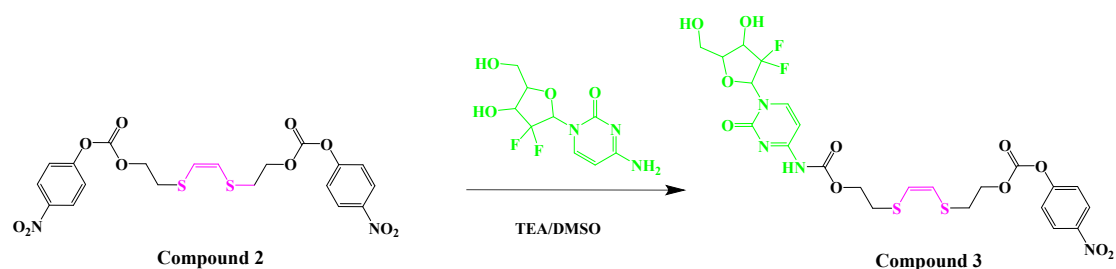
A mixture of 2-mercaptoethanol (6.00 g, 76.8 mmol) and NaOH (3.07 g, 78.8 mmol) in 30 mL of ethanol was stirred at 0 °C for 30 min. After the dropwise addition of cis-1,2-dichloroethylene (3.72 g, 38.4 mmol) in 4 mL of ethanol, the resulting solution was heated at 80 °C for 18 h. Then, the solution was cooled to ambient temperature, diluted with 20 mL water and washed with diethyl ether for three times. The combined organic layers were washed with water for two times, dried with anhydrous MgSO_4 for 12 h and concentrated to give the crude product. The product was purified by column chromatography on silica gel using the mixture of ethylacetate/hexane (7:3, v/v) to obtain the desired pure (Z)-2,2'-(ethene-1,2-diylbis(sulfanediyl))diethanol (yield: 70%).

1.5 Synthesis of compound 2



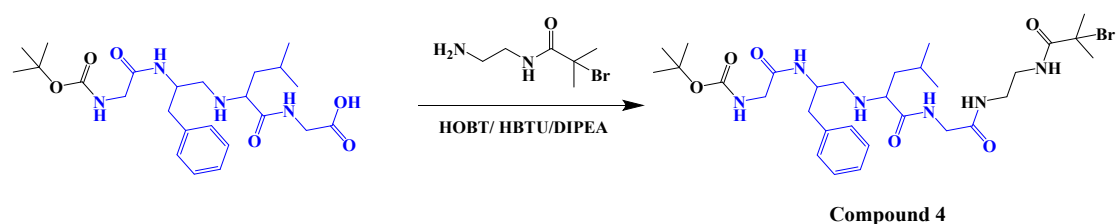
After (Z)-2,2'-(ethene-1,2-diylbis(sulfanediyl))diethanol (1.43 g, 7.9 mmol) was dissolved in CH_2Cl_2 , triethylamine (3.64 mL, 26.2 mmol, 3.3 equiv) was added and the mixture was kept stirring at ice-bath. After the dropwise addition of 4-Nitrophenyl chloroformate (3.53 g, 17.5 mmol) in 4 mL of CH_2Cl_2 , the resulting solution was kept stirring at ambient temperature for 24 h. The mixture was washed with saturated NaHCO_3 and water three times. The solution was dried with anhydrous MgSO_4 for 12 h and concentrated to give the crude product. The crude product was purified by column chromatography (EA: petroleum ether = 1:4, v/v) to afford desired pure products (yield: 47 %).

1.6 Synthesis of compound 3



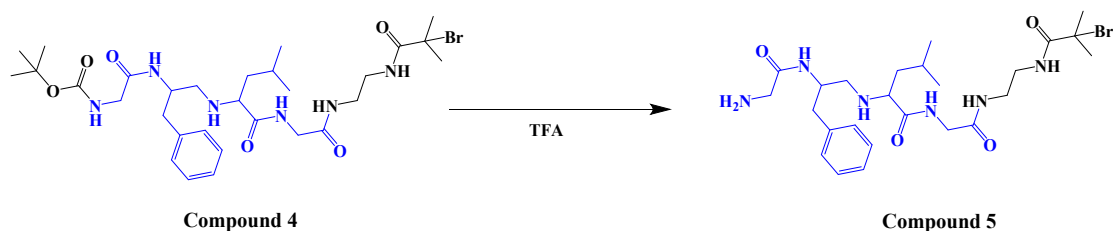
Compound 2 (1.20 g, 2.2 mmol) and gemcitabine (0.59 g, 2.2 mmol) were dissolved in DMSO, and triethylamine (0.31 mL, 2.23 mmol, 1 equiv) was added. The resulted mixture was kept stirring at ambient temperature for 48 h. Then, the mixture was added 40 mL EA and washed with water for three times. The solution was dried with anhydrous MgSO_4 for 12 h and concentrated to give the crude product. The crude product was purified by column chromatography with EA to afford desired pure product **compound 3** (yield: 42%).

1.7 Synthesis of compound 4



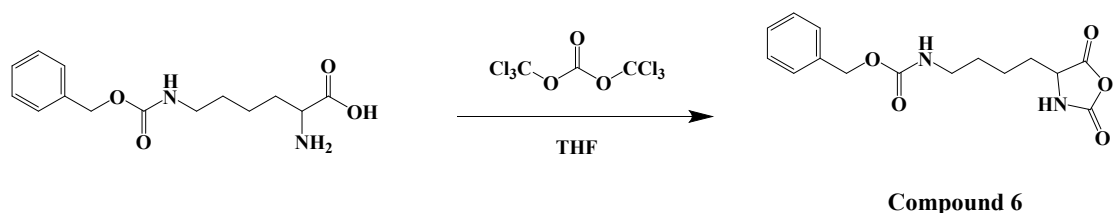
The initiator N-(2-aminoethyl)-2-bromo-2-methylpropanamide and Boc-GFLG-OH were synthesized as described in previous literature, respectively. Boc-GFLG-OH (1.30 g, 2.7 mmol), N-(2-aminoethyl)-2-bromo-2-methylpropanamide (1.60 g, 5.2 mmol), HBTU (1.60 g, 4.2 mmol) and HOBT (0.60 g, 4.4 mmol) were dissolved in DMF in argon atmosphere. Subsequently, DIPEA (2.30 mL, 13.7 mmol) was added to the mixture solution. The mixture was kept stirring for 2 days. DMF was removed under vacuum and the residue was dissolved in chloroform. The mixture was washed with saturated NaCl, NaHCO_3 and HCl (1 mol/L) several times. The solution was dried with anhydrous MgSO_4 for 12 h and concentrated to give the crude product. The crude product was purified by column chromatography (CH_3OH : EA: DCM = 1:5:10, v/v/v) to afford **compound 4** (yield: 38 %).

1.8 Synthesis of compound 5



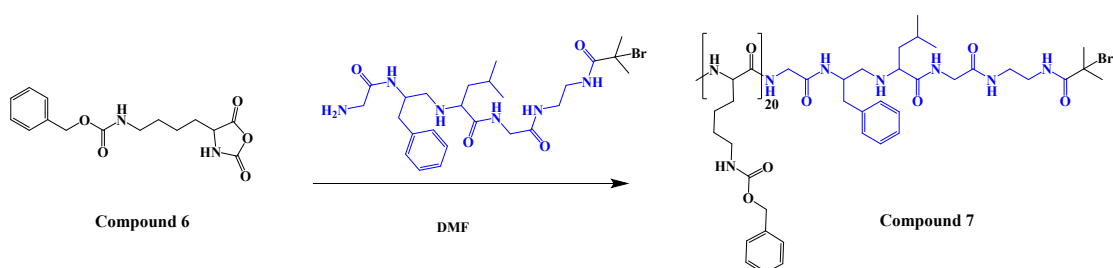
Compound 4 (0.50 g, 0.6 mmol) was dissolved in CH_2Cl_2 in argon atmosphere, and TFA (0.50 mL, 212.2 mmol) was added. The mixture was kept stirring at ice-bath for 12 h to remove t-Butyloxy carbonyl groups and **compound 5** was obtained (yield: 86 %).

1.9 Synthesis of compound 6



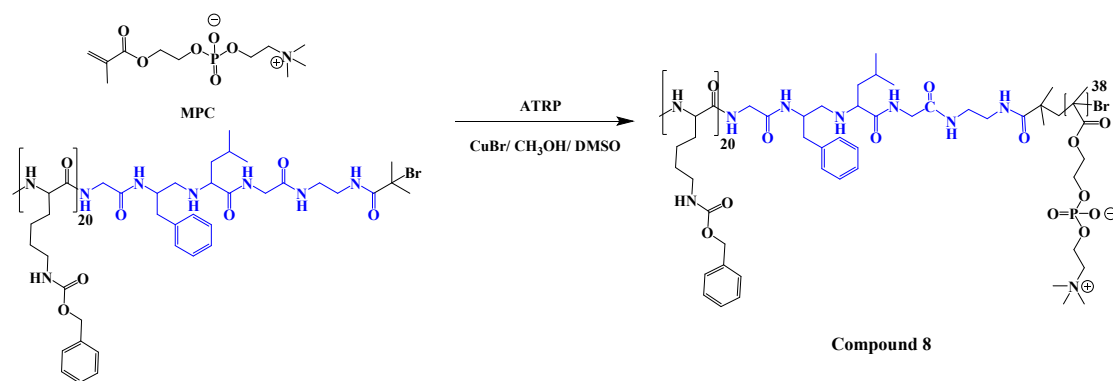
N6-Cbz-L-Lysine (5.00 g, 17.8 mmol) was dissolved in THF in argon atmosphere. Subsequently, triphosgene (2.70 g, 8.9 mmol) in 4 mL of THF was rapidly added to this solution and the resulting solution was heated at 50 °C until the solution clarification. Then, n-hexane was added and the mixture was filtrated to obtain a white solid product **compound 6** (yield: 75 %).

1.10 Synthesis of compound 7



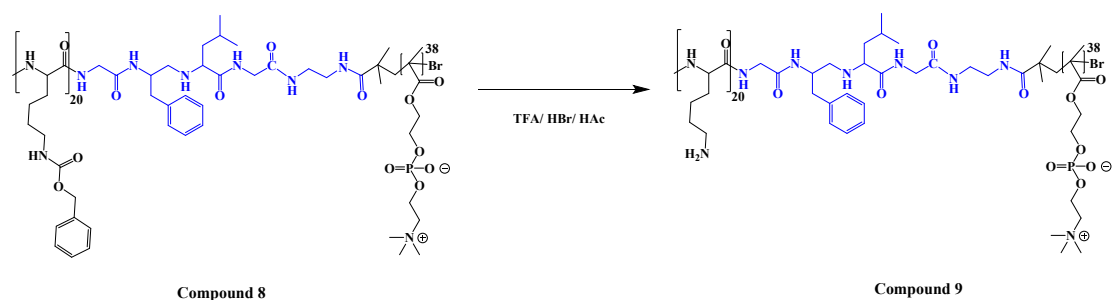
Compound 6 (1.20 g, 3.9 mmol) was dissolved in DMF and **compound 5** (0.40 g, 0.6 mmol) in 1 mL of DMF was added slowly to this solution. After 72 h, DMF was removed under vacuum and water was added drop by drop. A white solid product **compound 7** was obtained by centrifuging (yield: 68 %).

1.11 Synthesis of compound 8



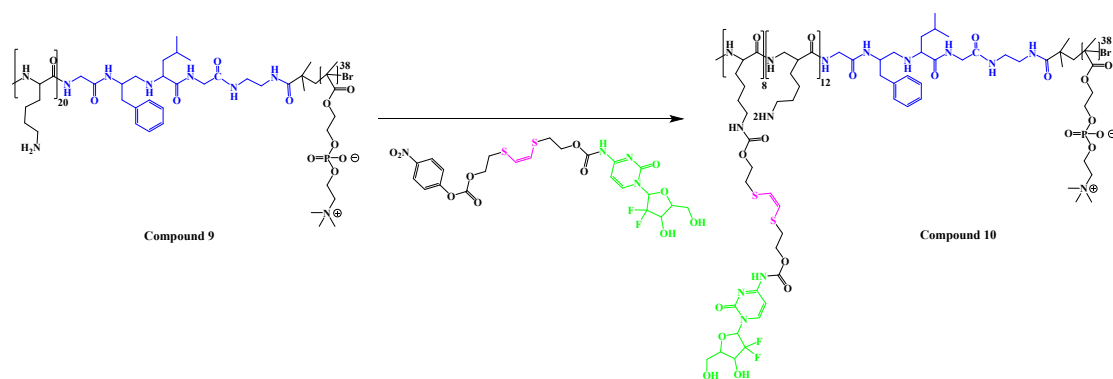
Compound 8 was synthesized by atom transfer radical polymerization (ATRP). Macroinitiator **compound 7** (1.00 g) and MPC (1.50 g, 5.0 mmol) were dissolved in 40 mL organic solvent (DMSO/MeOH, 1/1, v/v) in a schlenk flask. CuBr (28.80 mg, 0.2 mmol) and bpy (62.40 mg, 0.4 mmol) were added quickly into the schlenk flask under the protection of argon. After three cycles of freeze-pump-thaw procedure, the mixture was kept stirring at 40 °C for 48 h. A neutral aluminum oxide column was used to remove the catalyst with the solvent of DMF/MeOH (1:2, v/v). Then, the solution was concentrated and excess cold ethyl ether was added. **Compound 8** was obtained by drying in vacuum for 24 h (yield: 63 %).

1.12 Synthesis of compound 9



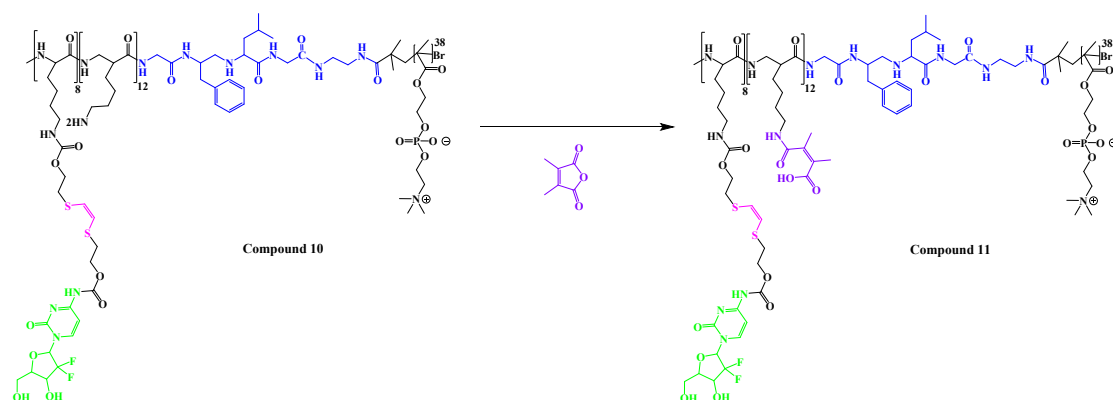
Compound 8 (1.00 g) was treated with TFA (5.94 mL, 72.9 mmol, 20 equiv), HBr (2.38 mL, 72.9 mmol, 20 equiv) and acetic acid (4.57 mL, 72.9 mmol, 20 equiv) for 24 h at room temperature to remove carbobenzyloxy groups. The solution was concentrated and transferred to the MWCO 3000 Da dialysis bags for dialyzing against deionized water for 48 h. Finally, the solution was freeze-dried to obtain a white solid product **compound 9** (yield: 88 %).

1.13 Synthesis of compound 10



Compound 9 (0.52 g) was dissolved in the mixed solvent of DMSO/MeOH (1:1, v/v). Subsequently, triethylamine (0.22 mL, 1.6 mmol) was added to this solution. Then, **compound 3** (0.50 g, 0.8 mmol) in 1 mL of DMSO/MeOH (1:1, v/v) was slowly added to mixture solution and kept stirring at ambient temperature for 48 h. The solution was concentrated and transferred to the MWCO 3000 Da dialysis bags for dialyzing against deionized water for 48 h. A white solid product **compound 10** was obtained by freeze-drying (yield: 73 %).

1.14 Synthesis of compound 11



Compound 10 (0.46 g) was dissolved in the mixed solvent of DMSO/MeOH (1:1, v/v). Then, 2,3-Dimethylmaleic anhydride (0.07 g, 0.52 mmol) in 1 mL of DMSO/MeOH (1:1, v/v) was slowly added to this solution and kept stirring at room temperature for 24 h. The solution was concentrated and transferred to the MWCO 3000 Da dialysis bags for dialyzing against deionized water for 48 h. A white solid product **compound 11** was obtained by freeze-drying (yield: 78 %).

1.15 Stability analysis of micelle

Micelles were incubated in PBS (pH 7.4) or 10% FBS to study the stability of micelle for different times at 25 °C. The average size was determined by DLS.

1.16 Critical micelle concentration of micelle

The pyrene was dissolved in aqueous medium and the concentration was 6.0×10^{-7} mol/L. The micelles were dissolved in the prepared solution of pyrene with the concentration from 1.0×10^{-4} to 200 $\mu\text{g/mL}$. Excitation spectra of pyrene were recorded using fluorescence spectrophotometer from 300 nm to 380 nm with an emission wavelength at 395 nm. The fluorescence values of I_{338} and I_{334} were tested and used to calculate the values of I_{338}/I_{334} .

1.17 Cellular uptake

4T1 cells with a density of 8×10^3 cell/well were inoculated into a 96-well black microtiter plate. 10 $\mu\text{g mL}^{-1}$ micelle was added and incubated for 1 h, 2 h, 4 h, 8 h and 24 h with cell culture media (pH 7.4 and pH 6.5). Then, cells were quantified by using a microplate reader excitation at 660 nm and emission at 692 nm.

1.18 Hemolysis ratio of micelles

Hemolysis ratio of micelles was evaluated by mouse RBCs. Fresh mouse blood was obtained from BALB/c mouse. The blood was centrifuged at 1500 rpm for 10 min to acquire RBCs. Then, RBCs were diluted with 9 volumes pH 7.4 PBS solution. 200 μL diluted RBCs were mixed with 800 μL micelles for 2 h. H_2O was used as a positive control and PBS was used as a negative control. Next, all samples were centrifuged for 6 min at 1200 rpm. The supernatants were tested by a microplate reader (541 nm). The hemolysis ratio was counted using the following equation: hemolysis percent = $(A_{\text{sample}} - A_{\text{negative}})/(A_{\text{positive}} - A_{\text{negative}}) \times 100\%$. All experiments were repeated in 6 times.

1.19 Lysosome colocalization

4T1 cells were inoculated to glass-bottomed dishes. After 24 h, 4T1 cells were incubated with 10 $\mu\text{g mL}^{-1}$ micelle for 1 h, 2 h, 4 h, 8 h and 24 h. Then the cells were stained with 1 μM Lyso-Tracker Green for 30 min, and Hoechst 33342 for 15 min in the dark. Finally, the cells were viewed by confocal laser scanning microscope (CLSM, Leica TCS SP5, Germany).

1.20 FDA/PI staining of tumor spheroids

Tumor spheroids were treated with Ce6 ($1.75 \mu\text{g mL}^{-1}$) and micelle ($16 \mu\text{g mL}^{-1}$), respectively. After 4 h, tumor spheroids were irradiation with a 660 nm laser (5 min, 0.1 W/cm^2). After 24 h, tumor spheroids were washed and stained with FDA/PI solution for 15 min. Then, tumor spheroids were imaged by CLSM with $5 \mu\text{m}$ per section from top to bottom.

1.21 ROS detection of tumor spheroids

Tumor spheroids were treated with Ce6 ($1.75 \mu\text{g mL}^{-1}$) and micelle ($16 \mu\text{g mL}^{-1}$), respectively. After 4 h, tumor spheroids were irradiation with a 660 nm laser (5 min, 0.1 W/cm^2). Tumor spheroids were washed and stained with Reactive Oxygen Species Assay Kit in the dark. Then, tumor spheroids were imaged by CLSM.

1.22 *In vivo* singlet oxygen detection

For detecting of singlet oxygen *in vivo*, tumor bearing mice were intravenous injected with free Ce6 (5 mg kg^{-1} Ce6), and micelle (5 mg kg^{-1} of Ce6 equivalents), respectively. Meanwhile, the same volume of saline was injected as control. The mice were intraperitoneal injected with SOSG ($6.25 \mu\text{g/mice}$) after 12 h, then the mice were irradiated with a 660 nm laser (10 min, 0.15 W/cm^2). Finally, tumors were dissected from the mice.

2. Characterizations

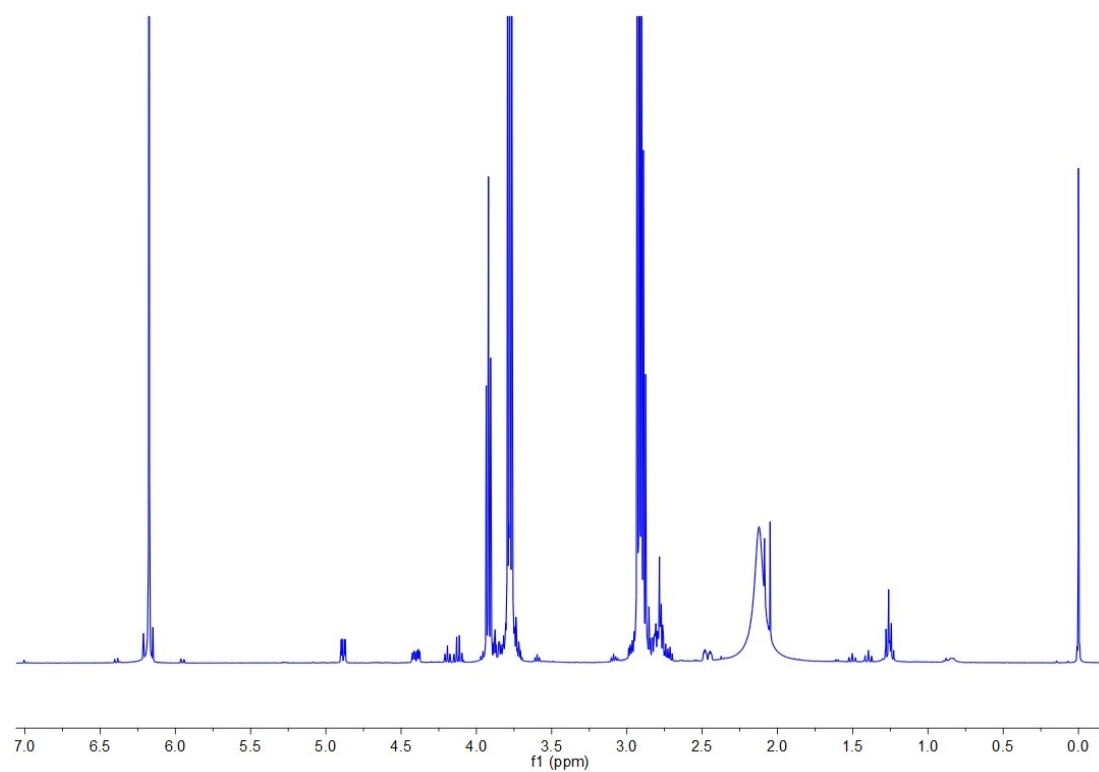


Figure S1. ^1H -NMR spectrum of **compound 1** in CDCl_3 .

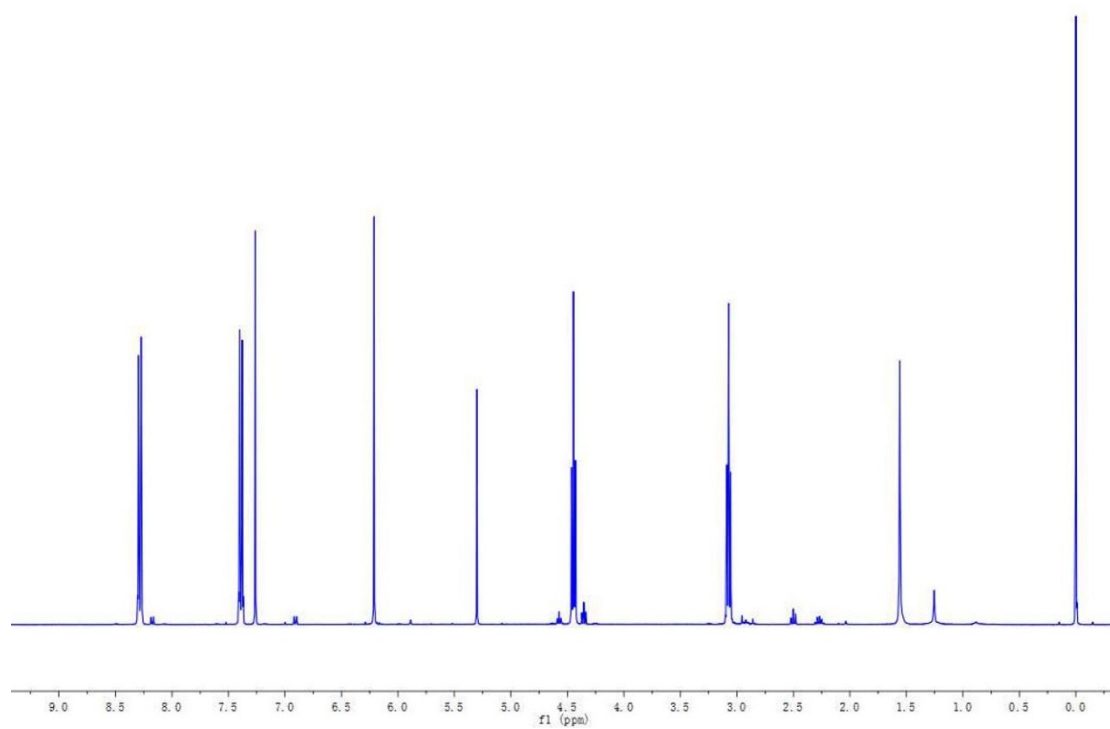


Figure S2. ^1H -NMR spectrum of **compound 2** in $\text{DMSO}-d_6$.

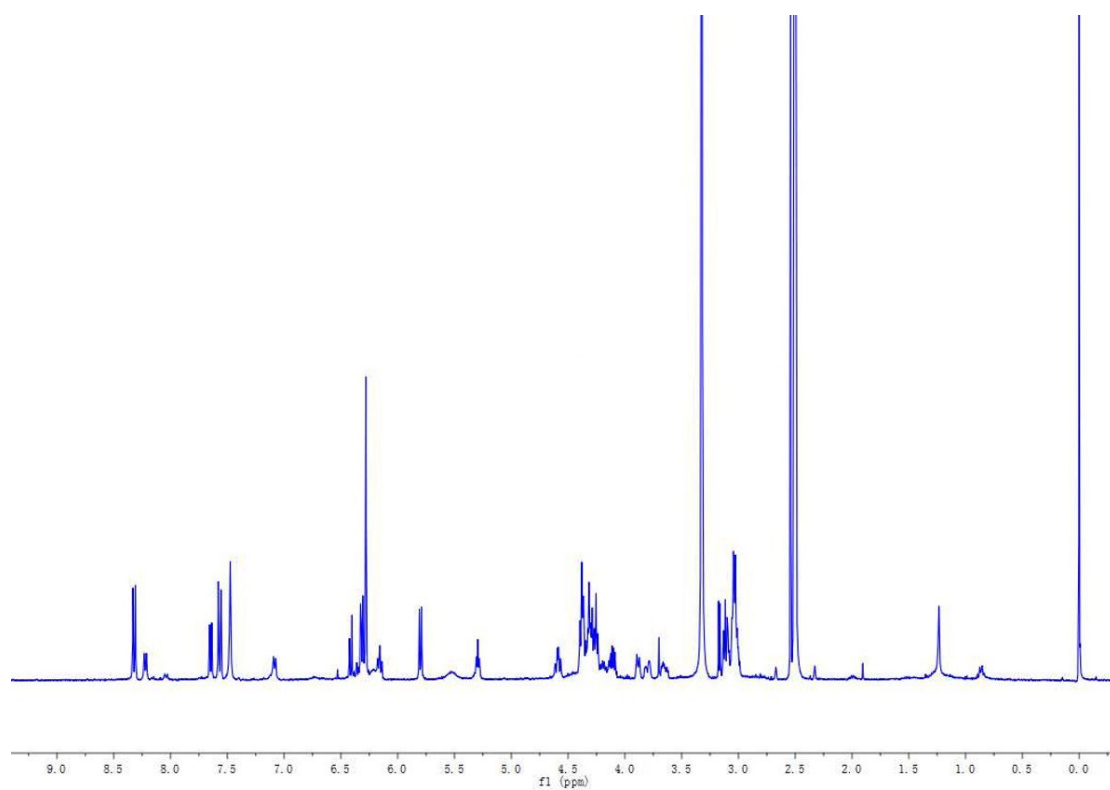


Figure S3. ^1H -NMR spectrum of **compound 3** in DMSO- d_6 .

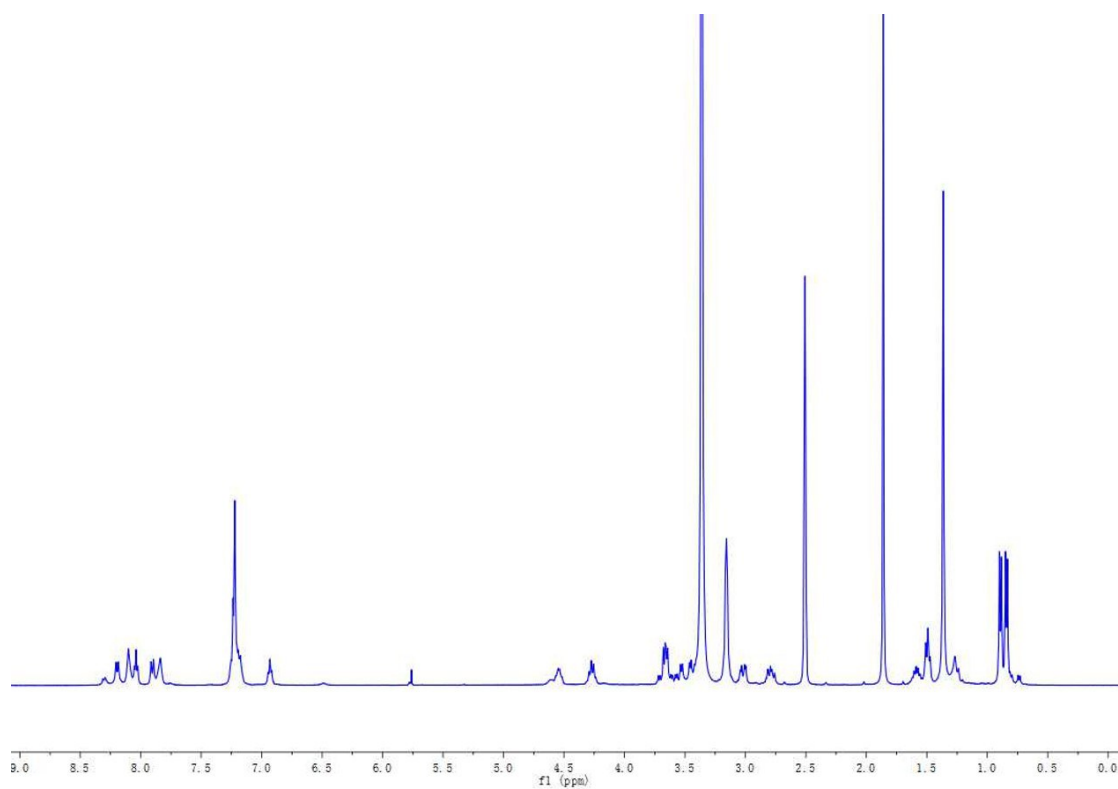


Figure S4. ^1H -NMR spectrum of **compound 5** in DMSO- d_6 .

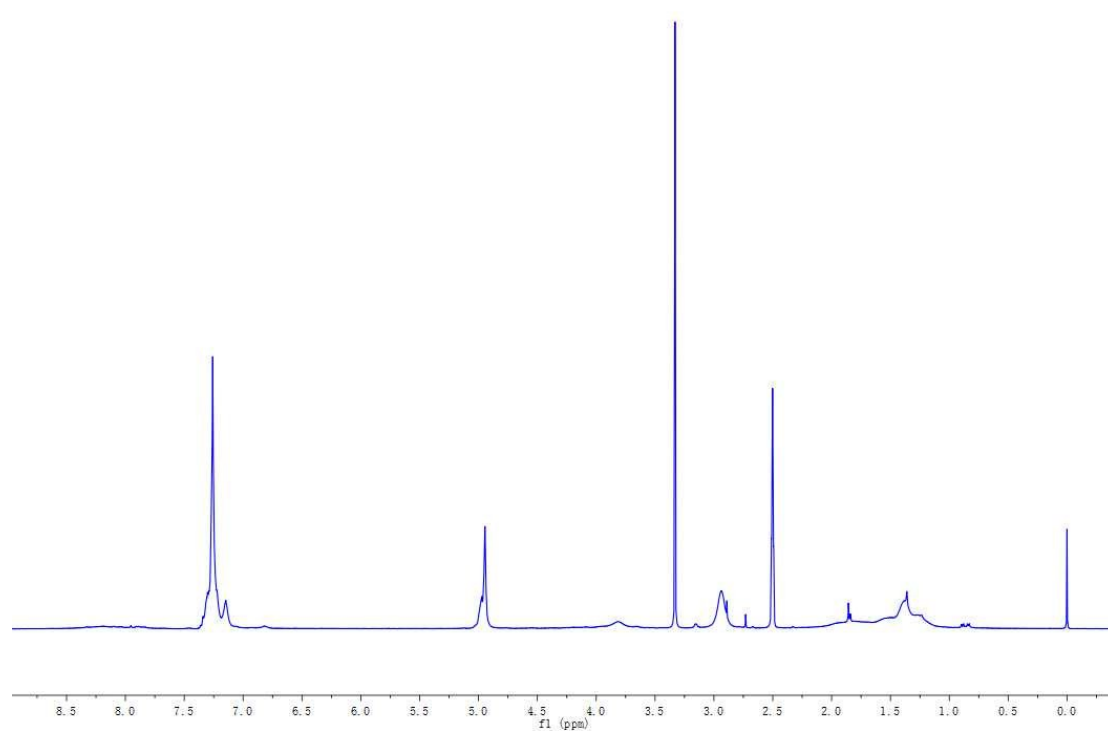


Figure S5. ^1H -NMR spectrum of **compound 7** in DMSO- d_6 .

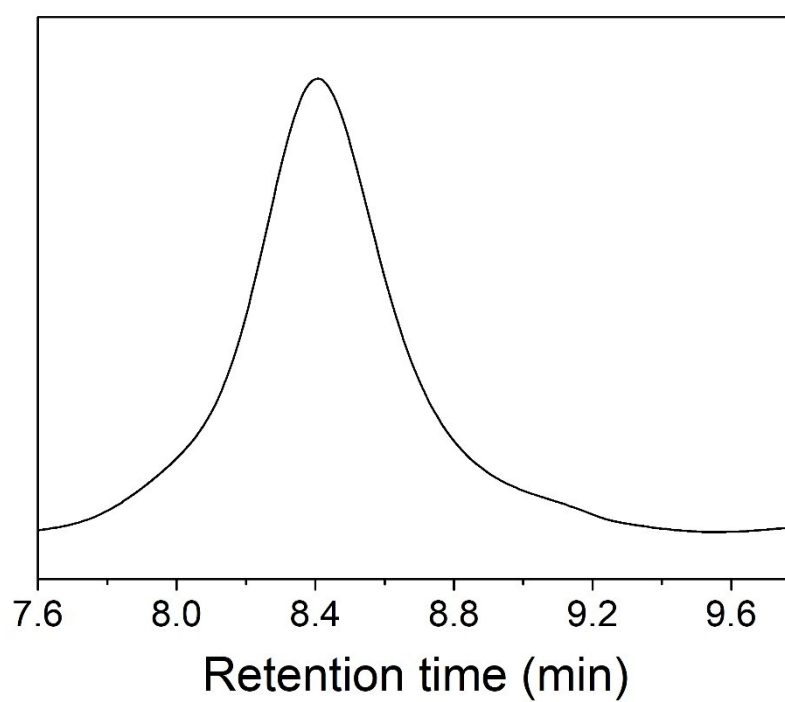


Figure S6. GPC traces of **compound 7**.

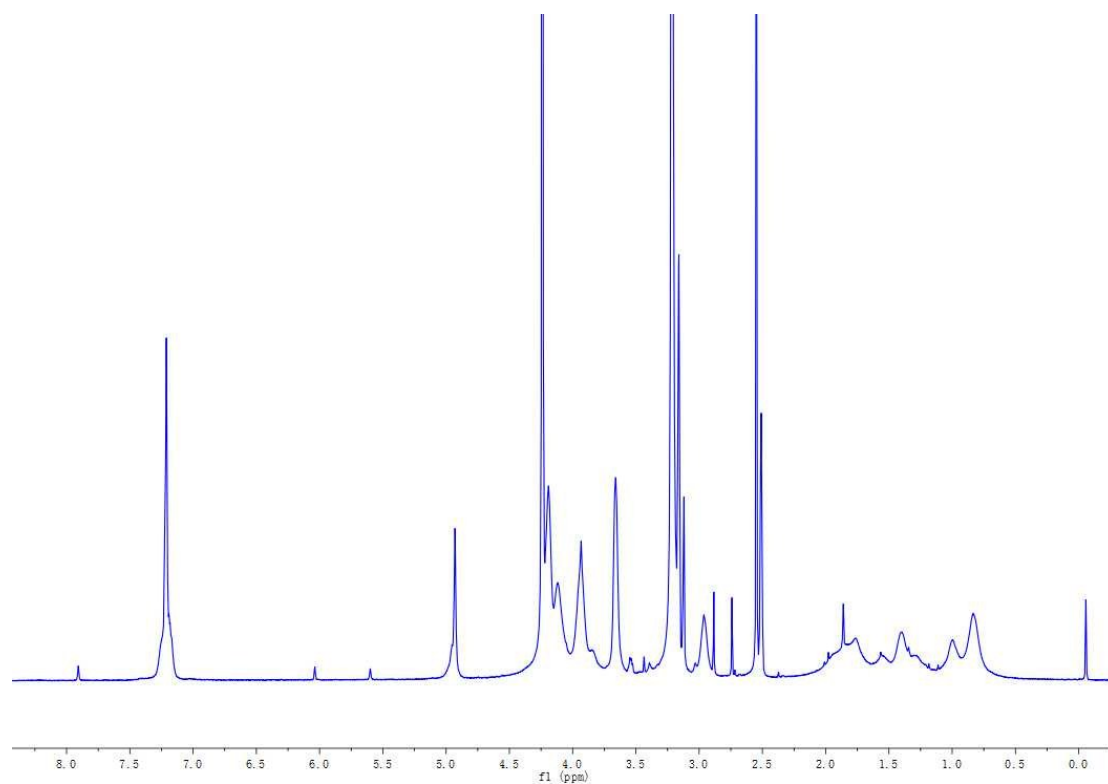


Figure S7. ^1H -NMR spectrum of **compound 8** in DMSO-d_6 .

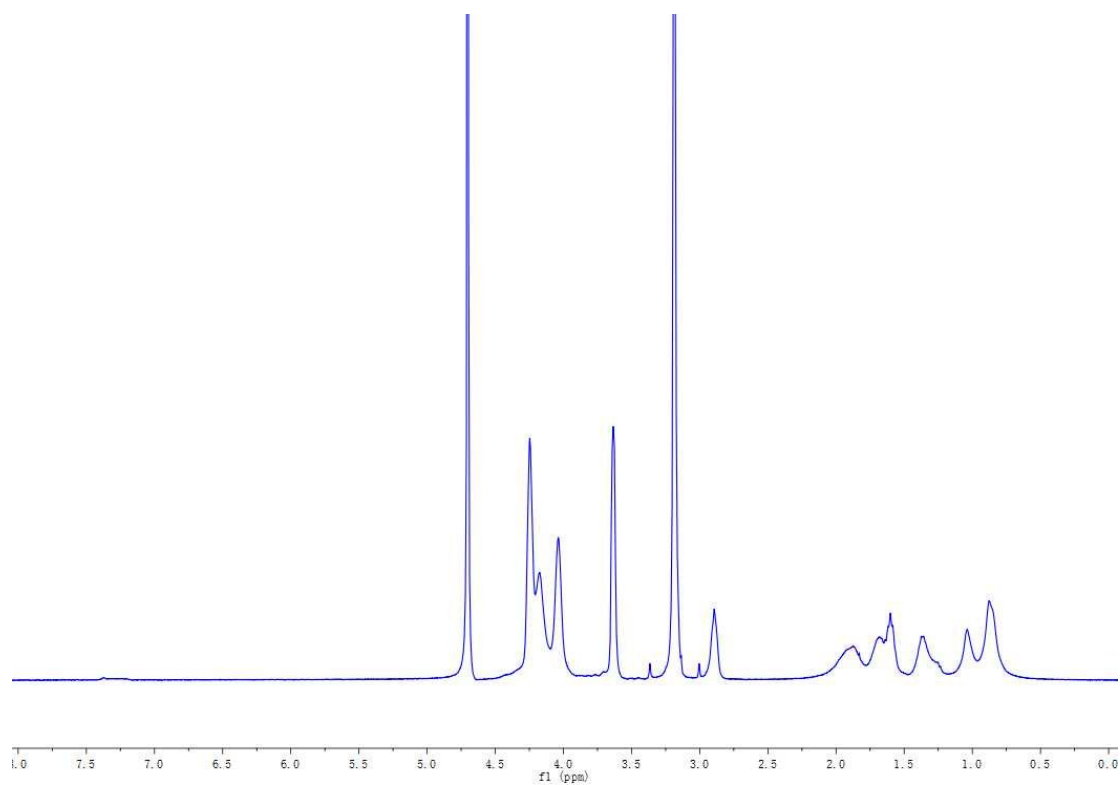


Figure S8. ^1H -NMR spectrum of **compound 9** in DMSO-d_6 .

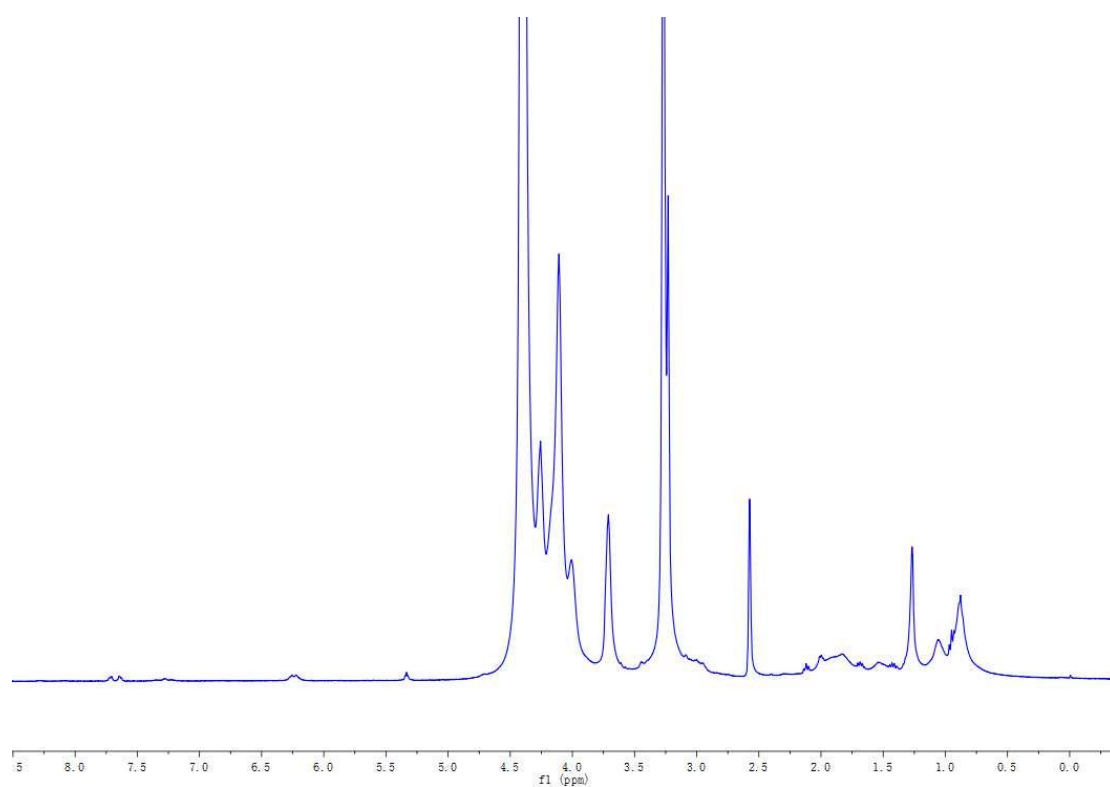


Figure S9. ^1H -NMR spectrum of **compound 10** in DMSO-d_6 .

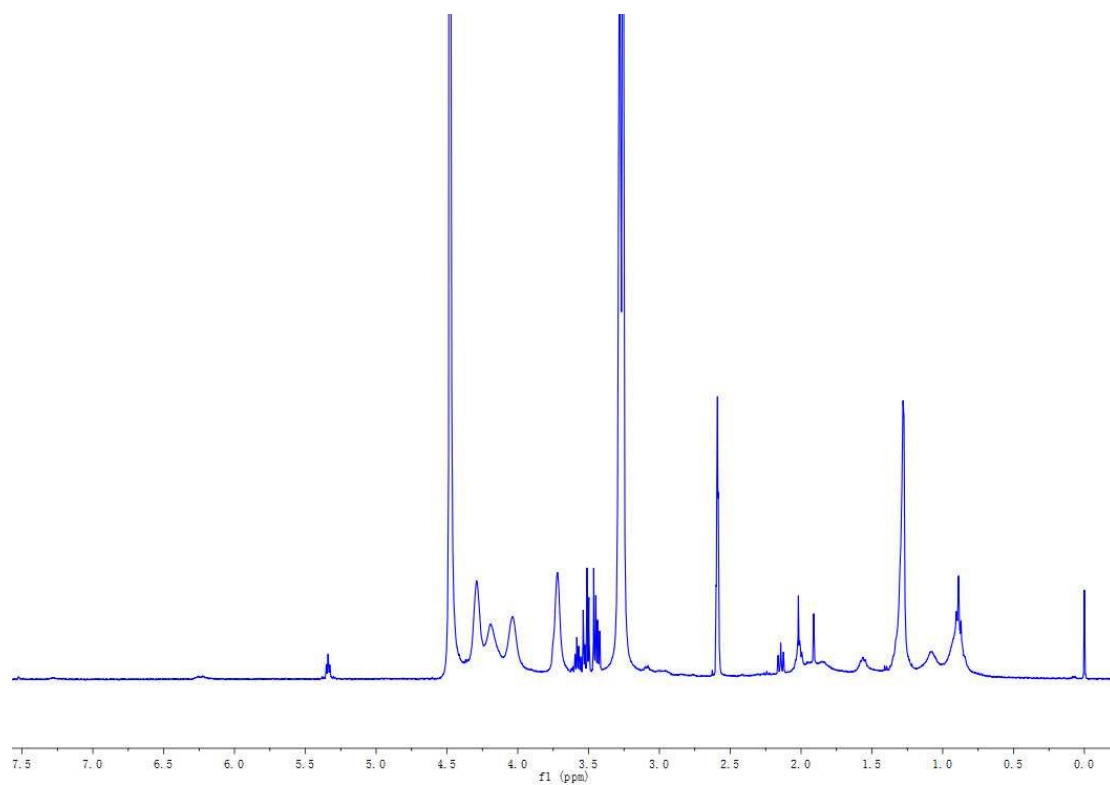


Figure S10. ^1H -NMR spectrum of **compound 11** in DMSO-d_6 .

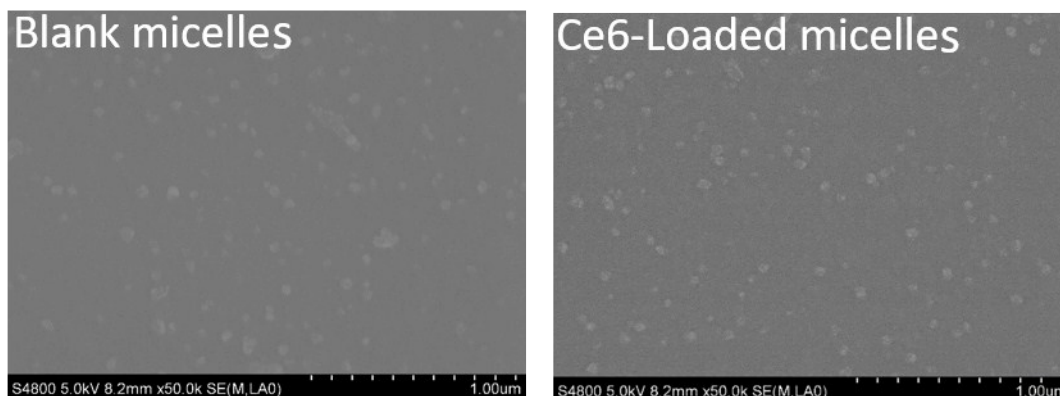


Figure S11. SEM images of blank micelles and Ce6-loaded micelles with a concentration of 100 $\mu\text{g/mL}$ in aqueous medium (pH 7.4).

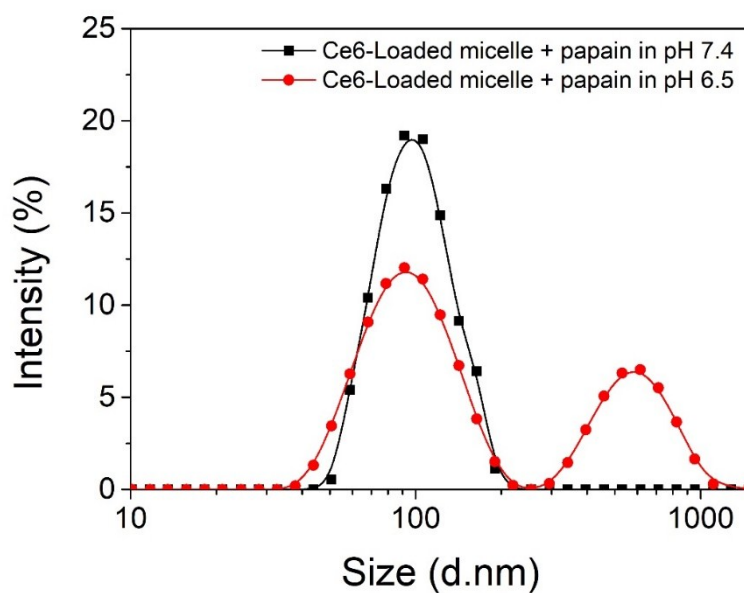


Figure S12. Size distributions of micelles in different conditions of pH 7.4, pH 6.5 with papain for 4 h measured by DLS.

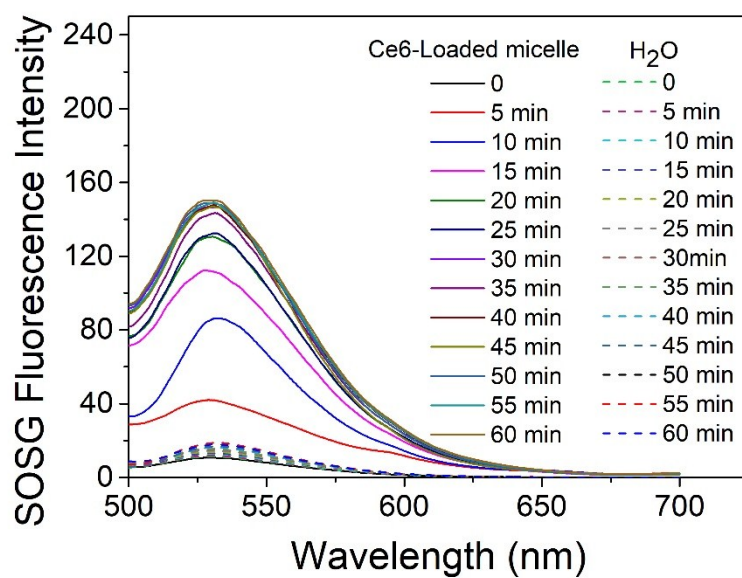


Figure S13. Fluorescence curves of SOSG changes under light irradiation at different times.

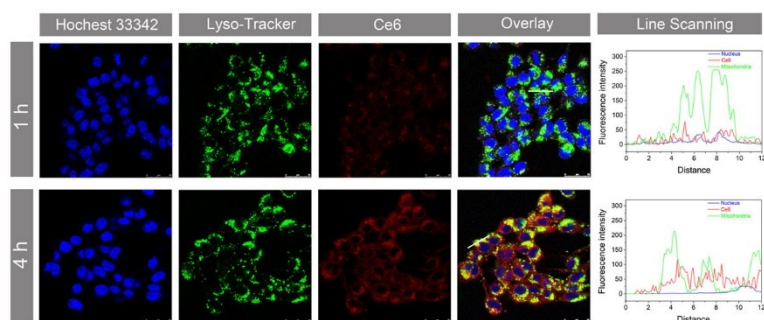


Figure S14. CLSM images of 4T1 cells treated with micelles at different times to show the colocalization of micelles and lysosomes.

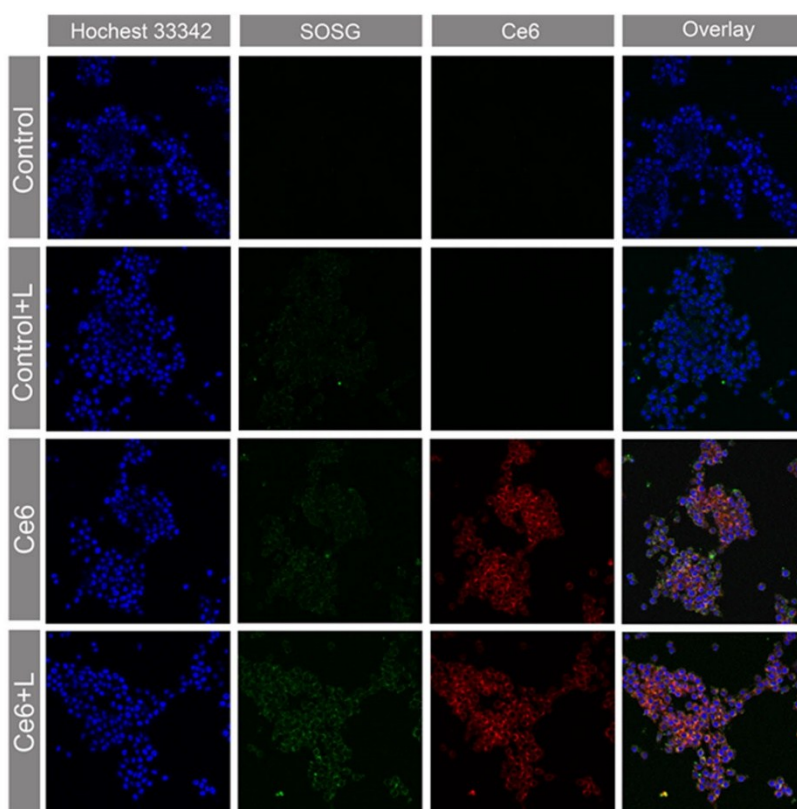


Figure S15. CLSM images for intracellular $^1\text{O}_2$. 4T1 cells were incubated with SOSG (5 μM) probe after being treated with or without **Ce6** and irradiated by 660 nm laser.

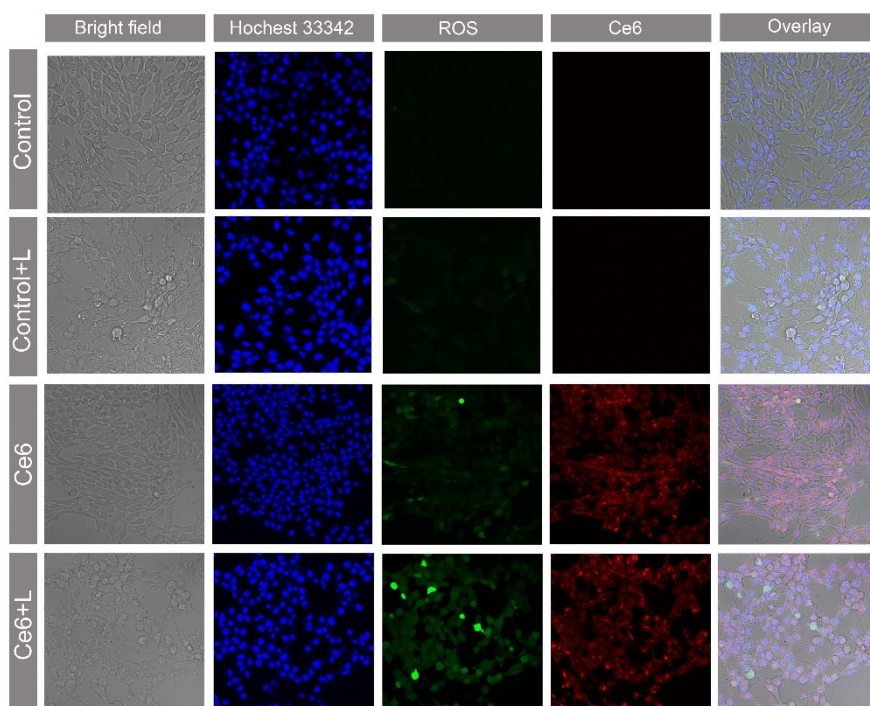


Figure S16. Intracellular ROS level of 4T1 cells after treatment the **Ce6** with or without laser irradiation for 24 h.

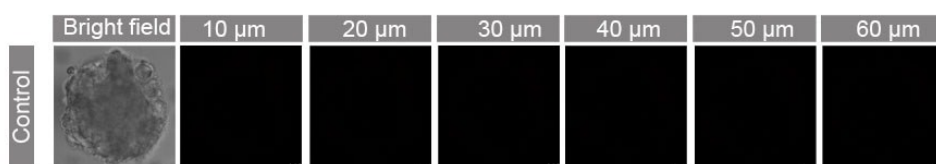


Figure S17. CLSM images of 4T1 cell spheres (Control group).

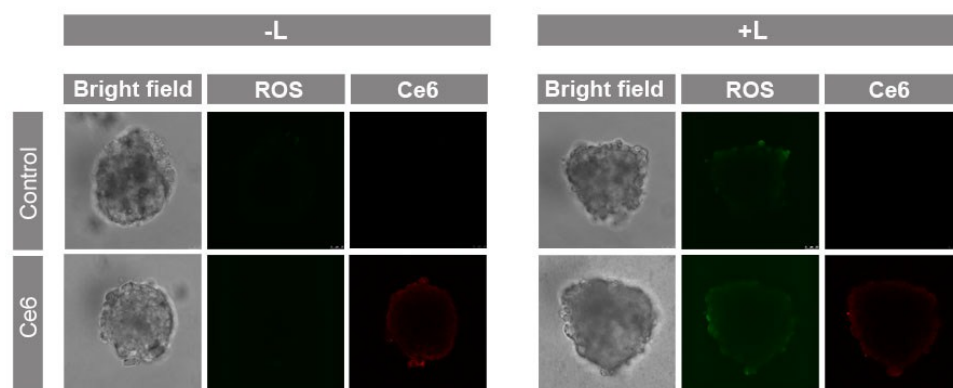


Figure S18. ROS level of 4T1 cell spheres after treatment the Ce6 with or without laser irradiation for 24 h.

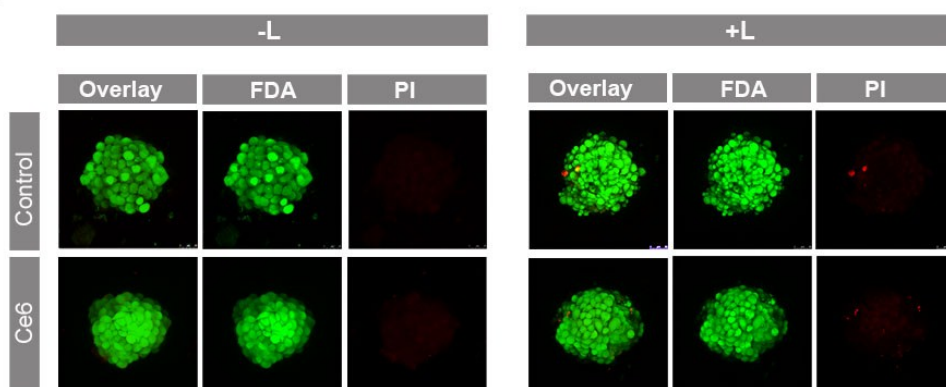


Figure S19. CLSM images for FDA/PI stained 4T1 cell spheres treated with the Ce6 with or without laser irradiation.

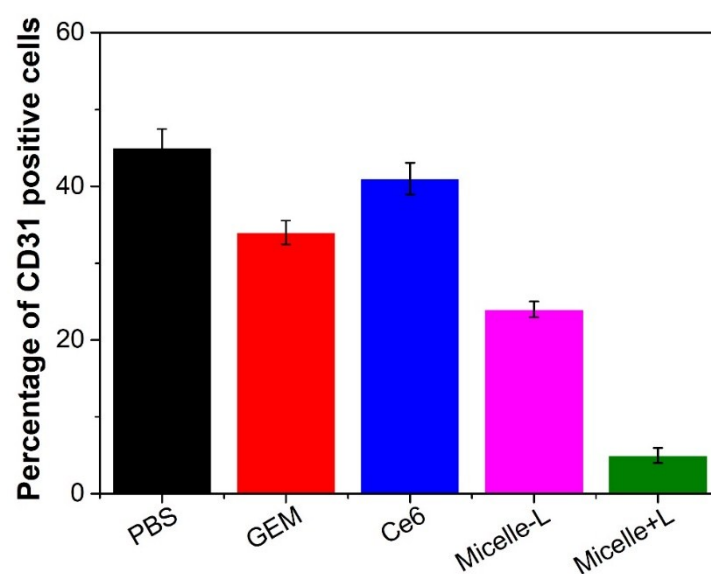


Figure S20. The percentage of CD31-positive cells of tumor tissues in the groups treated with various formulations (n=3).

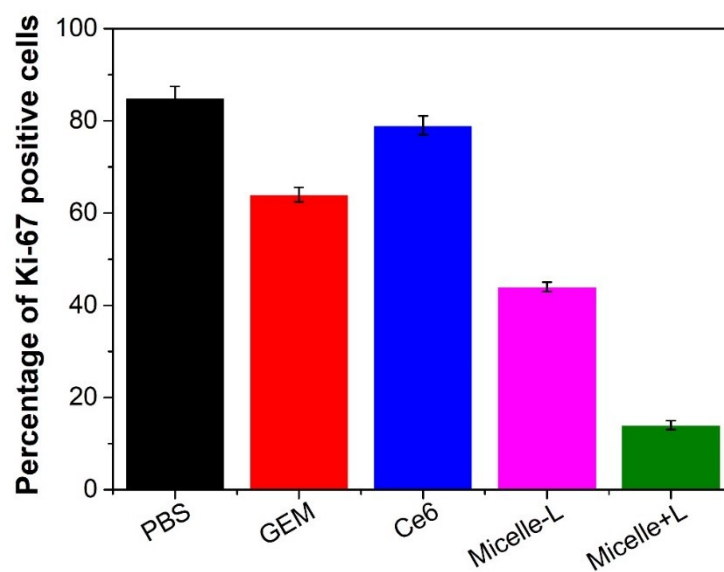


Figure S21. The percentage of Ki-67 positive cells of tumor tissues in the groups treated with various formulations (n=3).

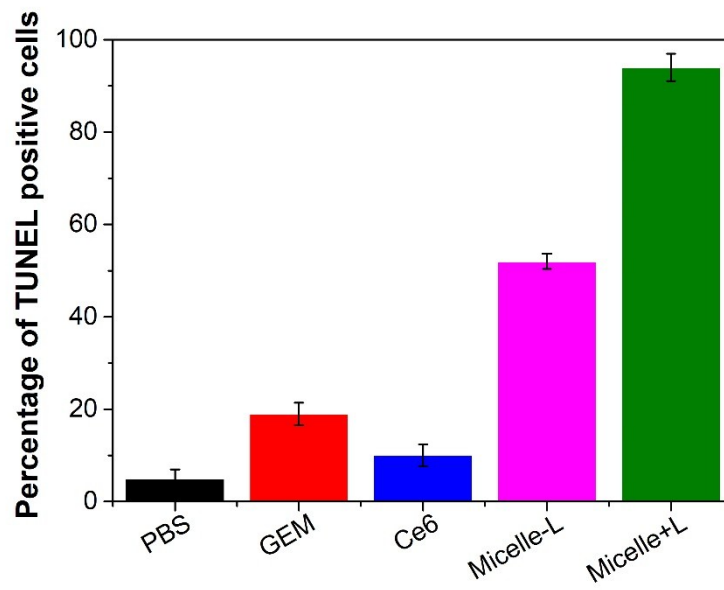


Figure S22. The percentage of TUNEL-positive cells of tumor tissues in the groups treated with various formulations (n=3).

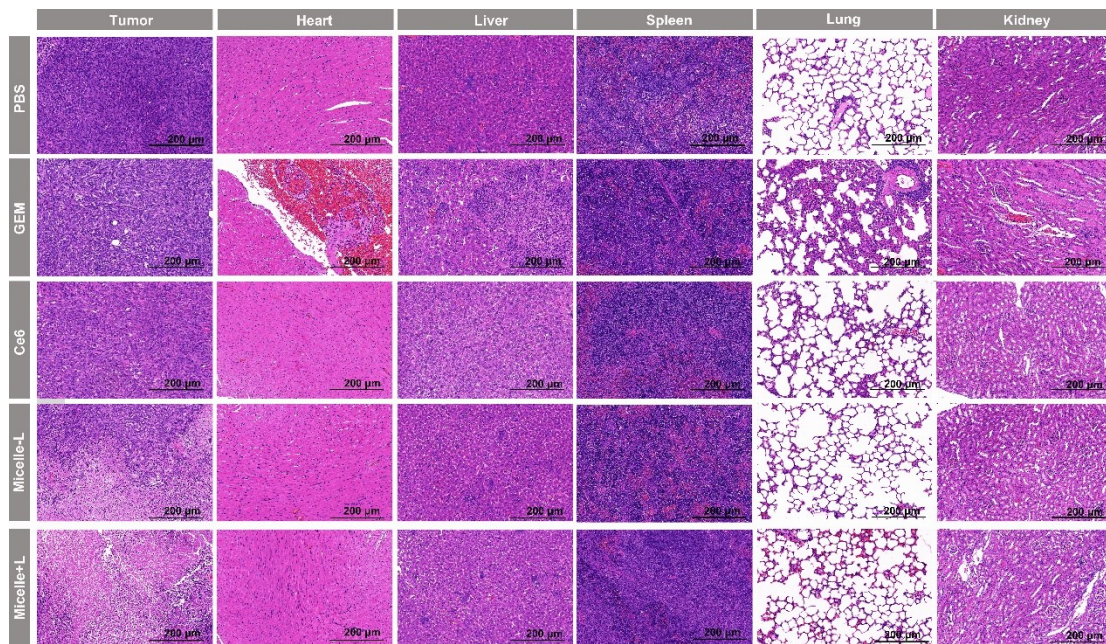


Figure S23. H&E stained results of major organs (heart, liver, spleen, lung, and kidney) and tumors for all tested groups. All scale bars: 200 µm.