Supporting information

Design of a thiol-responsive, traceless prodrug with rapid self-immolation for cancer chemotherapy

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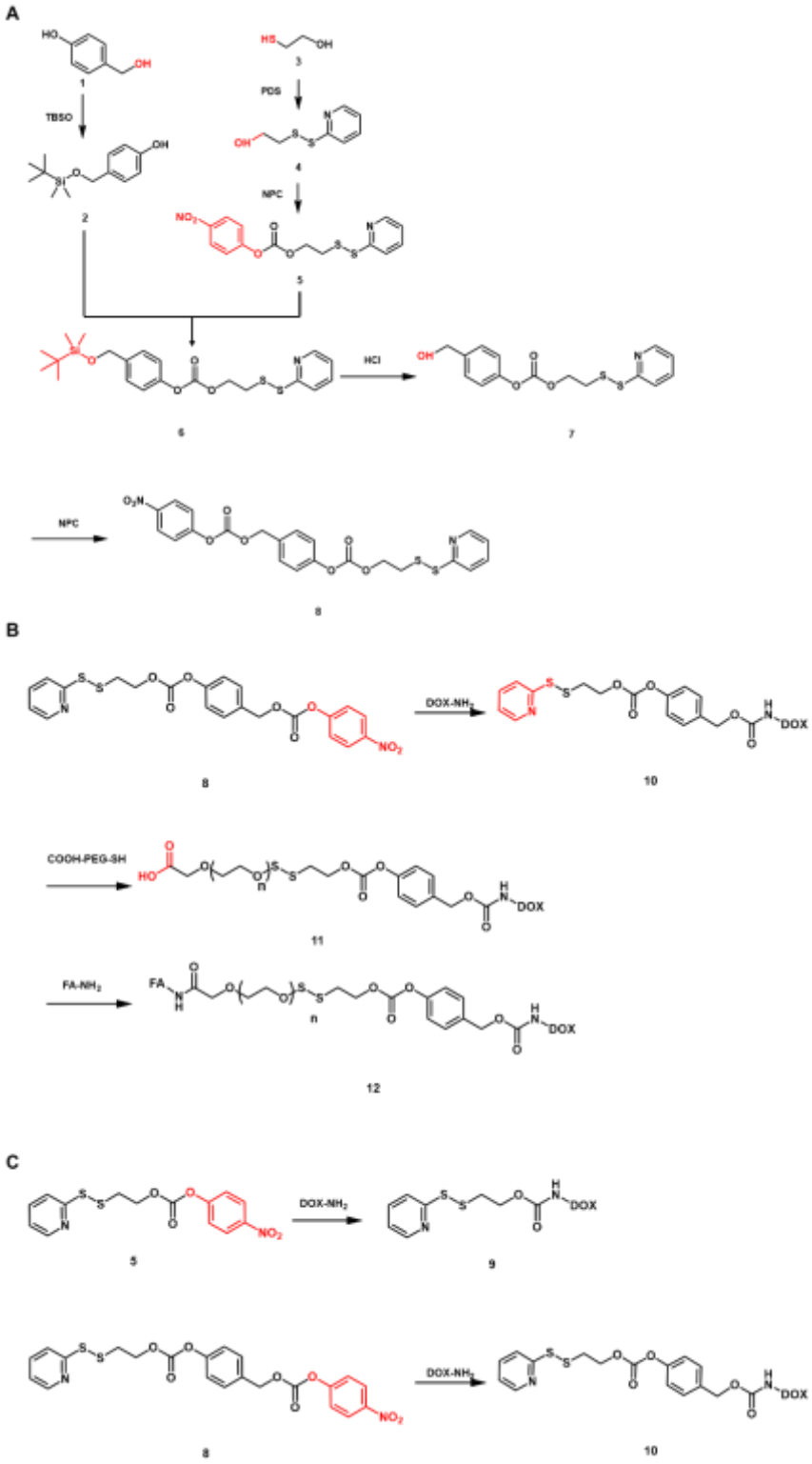
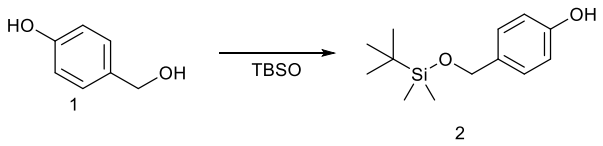


Figure S1. Synthetic route of crosslinkers and SIP-DOX. A) The synthesis of Self immolative crosslinker. The products obtained from the step 1 to step 6 are purified by column chromatography. B) The synthesis of KSIP-DOX (similar with that to CSIP-DOX). The products are purified by dialysis bag. The KSIP-DOX lyophilized to give

an orange-red powder, which is easily soluble in water. C) The synthesis of conjugates, compound 9 and 10 shown in Figure 2.

Synthesis of thiol-responsive self-immolative crosslinkers and drug conjugates.

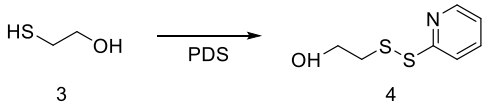
The key crosslinker was accomplished by five steps and its synthetic route was shown in Figure S1. The control crosslinker is just the intermediate, compound 5. The starting material for the synthesis was mercaptoethanol (1), which was activated with paranitro-chloroformate (generating 5, control crosslinker) and reacted with the silyl-protected phenol (compound 2), to generate the intermediate 6. Compound 6 was de-protected with tetrabutyl ammonium fluoride and then activated again with paranitro-chloroformate to yield the key SIL (8). The synthesis involves the protection and deprotection of 4- (hydroxyl methyl) phenol, which could probably be skipped and the final product could be purified using column chromatography. Optimization of the synthesis route will be investigaed in the future.



1 2

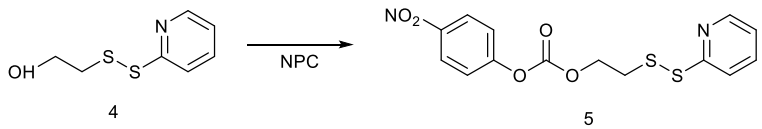
Synthesis of compound 2: Tert-butyldimethylsilyl chloride (3.6 g, 0.012 mmol) and imidazole (3.4 g, 0.025 mmol) were added to a solution of 4-hydroxybenzyl alcohol (2.48 g, 0.01 mmol) in DMF (15 mL), and then the mixture was stirred at room temperature under argon. After the reaction mixture was stirred for overnight, H_2O (30 mL) was added to the mixture. The resulting mixture was extracted with ethyl acetate (2 x 30 mL). The combined organic layers were dried over anhydrous Na_2SO_4, filtered,

and concentrated under reduced pressure. The residue was purified by column chromatography to provide compound 2 as a colorless oil (4.38 g, 92 %).[1]H NMR (400 MHz, $CDCl_3$, ppm) δ 7.17 (s, 1H), 7.09 (s, 2H), 6.71 (s, 2H), 4.58 (s, 2H), 0.84 (s, 9H), 0.00 (s, 6H). [13]C NMR (400 MHz, DMSO-d6, ppm) δ 158.92, 27.66, 23.25, 11.59. HRMS (EI) m/z 339.37.



3 4

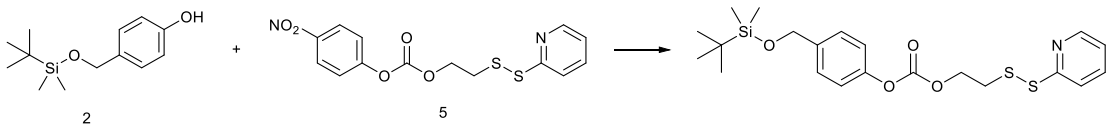
Synthesis of compound 4: 2-aldrithiol (5 g, 64 mmol) was added in MeOH (30 mL) with 3 mL acetic acid under argon. 2-mercaptoethanol (800 μL, 11.32 mmol) was added slowly and stirred for 24 h. Then, the solvent was evaporated in vacuum and the residue purified by column chromatography on silica gel (Hexane: ethyl acetate 3:2) to obtain compound 4 as a colorless oil (3.64 g, 86 %). [1]H NMR (400 MHz, $CDCl_3$, ppm) δ 8.47 (ddd, J = 5.0, 1.9, 0.9 Hz, 1H), 7.59 – 7.49 (m, 1H), 7.35 (dt, J = 8.1, 1.0 Hz, 1H), 7.11 (ddd, J = 7.4, 5.0, 1.1 Hz, 1H), 3.78 – 3.70 (m, 2H), 2.95 – 2.86 (m, 2H). [13]C NMR (400 MHz, DMSO-d6, ppm) δ 158.01, 150.08 (d, J = 34.0 Hz), 138.58, 122.25, 121.58, 59.64, 41.79. HRMS (EI) m/z 191.28.



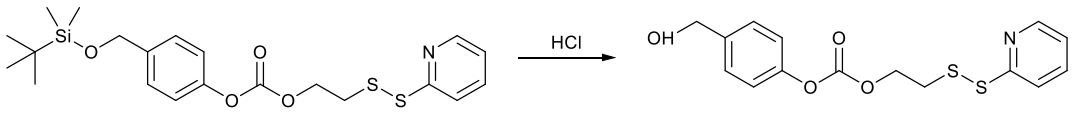
4 5

Synthesis of compound 5: A solution of compound 4 (3 g, 22.02 mmol 1 eq), Et_3N (2.4 mL, 1.1 eq) in ACN (30 mL) was cooled to 0 °C. The solution turned yellow as 4-nitrophenylchloroformate (3.72 g, 1.1 eq) was added, and was stirred for 24 h under Ar.

After concentration in vacuo, the residue was purified by column chromatography on silica gel (ethyl acetate/hexane 10 %-30 %) to get compound 5 as a pale-yellow oil (4.8 g, 85 %).^{1}H NMR (400 MHz, $CDCl_3$, ppm) δ 8.50 (d, J = 4.9 Hz, 1H), 8.29 (d, J = 9.1 Hz, 2H), 7.66 (q, J = 8.1, 7.2 Hz, 2H), 7.38 (d, J = 9.1 Hz, 2H), 7.13 (t, J = 5.6 Hz, 1H), 4.57 (t, J = 6.4 Hz, 2H), 3.16 (t, J = 6.4 Hz, 2H). ^{13}C NMR (400 MHz, DMSO-d6) δ 155.70, 150.19, 138.32, 125.98 (d, J = 17.4 Hz), 123.06, 121.91, 120.01, 67.06, 36.93. HRMS (EI) m/z 353.21.

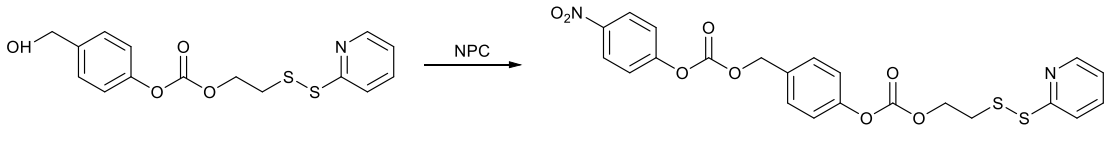


Synthesis of compound 6: Compound 3 (1 g, 2.84 mmol, 1.0 eq), Compound1 (811 mg, 3.41 mmol, 1.2 eq), DIPEA (44 μL, 4.26 mmol, 1.5 eq), and DMAP (35.5 mg, 0.284 mmol, 0.1 eq) were dissolved and stirred at room temperature in 30 mL of anhydrous CH_2Cl_2 for 15 h. The reaction was then washed with 1 M HCl followed by brine. The organic layer was dried over Na_2SO_4 and filtered, and the solvent was removed in vacuo. Compound 4 was then used in the next step without further purification (0.94 g, 73%). ^{1}H NMR (400 MHz, $CDCl_3$, ppm) δ 8.39 (d, J = 4.8 Hz, 1H), 7.66 – 7.52 (m, 2H), 7.23 (d, J = 8.5 Hz, 2H), 7.06 – 6.99 (m, 3H), 4.63 (s, 2H), 4.40 (t, J = 6.5 Hz, 2H), 3.05 (t, J = 6.5 Hz, 2H), 0.84 (s, 9H), -0.00 (s, 6H). ^{13}C NMR (400 MHz, DMSO-d6, ppm) δ 159.27, 156.89, 150.21 (d, J = 8.7 Hz), 138.34, 128.19 (d, J = 17.3 Hz), 121.47, 120.00 (d, J = 13.7 Hz), 115.40, 64.78, 64.37 (d, J = 52.8 Hz), 26.22 (d, J = 38.4 Hz), 18.52, 14.60, -4.72 (d, J = 16.2 Hz). HRMS (EI) m/z 452.38.



6 7

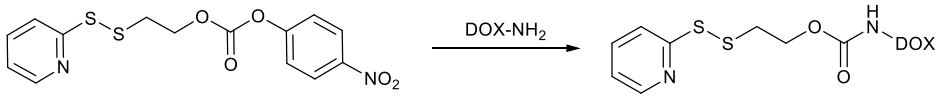
Synthesis of compound 7: Compound 6 (0.9 g, 2 mmol, 1.0 eq) was stirred at room temperature in 20 mL in ethanol (1 % HCl) for 3 h. The reaction was neutralized with minimal 1 M $NaHCO_3$ as determined with pH paper. The reaction was then diluted with 30 mL of CH_2Cl_2 and washed with water followed by brine. The organic layer was dried over Na_2SO_4 and filtered, and the solvent was removed in vacuo. The products were purified by column chromatography on silica gel (Hexane: ethyl acetate 9:1-7:3) to give compound 7 (0.54 g, 80%) as a clear oil. ^{1}H NMR (400 MHz, DMSO-d6, ppm) δ 8.48 (s, 1H), 7.91 – 7.73 (m, 2H), 7.36 (d, J = 8.7 Hz, 2H), 7.32 – 7.22 (m, 1H), 7.17 (d, J = 8.6 Hz, 2H), 5.25 (s, 1H), 4.56 – 4.31 (m, 4H), 3.22 (t, J = 6.0 Hz, 2H). ^{13}C NMR (400 MHz, DMSO-d6, ppm) δ 153.45, 152.32 , 150.06 (d, J = 43.7 Hz), 141.08, 138.35, 128.06, 125.95, 123.08, 121.94, 121.32, 67.06, 66.44, 62.79. HRMS (EI) m/z 376.36.



7 8

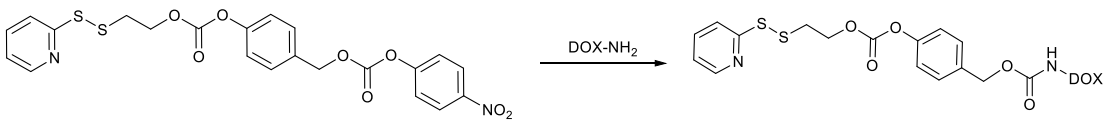
Synthesis of compound 8: A solution of compound 7 (0.54 g, 1.6 mmol 1 eq), Et_3N (430 µL, 1.1 eq) in anhydrous CH_2Cl_2 (20 mL) was cooled to 0 °C. The solution turned yellow as 4-nitrophenylchloroformate (353 mg, 1.1 eq) was added, and was stirred for 24 h under argon. After concentration in vacuo, the residue was purified by column chromatography on silica gel (Hexane: ethyl acetate 10:1-7:3) to give the compound 8

as a white solid (683 mg, 85%). ^{1}H NMR (400 MHz, DMSO-d6, ppm) δ 8.48 (d, J = 3.9 Hz, 1H), 8.33 (d, J = 9.2 Hz, 2H), 7.89 – 7.77 (m, 2H), 7.57 (dd, J = 17.2, 9.0 Hz, 4H), 7.29 (d, J = 8.7 Hz, 3H), 5.32 (s, 2H), 4.44 (s, 2H), 4.03 (d, J = 7.1 Hz, 2H). 13C NMR (151 MHz, DMSO-d6, ppm) δ 153.20, 152.46, 151.33, 150.19, 145.72, 138.36, 133.24, 130.50, 126.73, 125.95, 123.15, 121.94 (d, J = 9.4 Hz), 119.98, 70.18, 66.60. HRMS (EI) m/z 503.34.



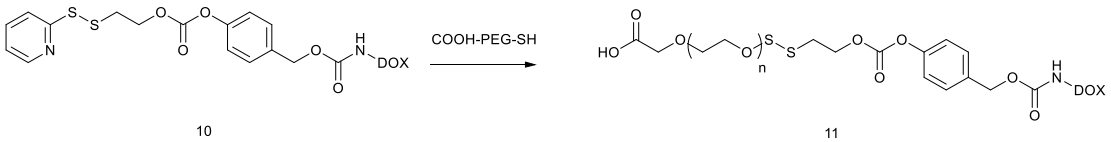
5 9

Synthesis of compound 9: Doxorubicin (50 mg, 0.085 mmol, 1.2 eq) was dissolved in 1.2 mL MeOH with Et_3N (47 μL, 0.355 mmol, 5 eq). 600 μL MeOH solution of compound 5 (25.1 mg, 0.071 mmol, 1eq) was dropwise added into the solution above. After the solution was stirred at room temperature overnight, 10 mL DCM was added. After the mixture was washed with brine, dried over Na_2SO_4, concentrated, the residue was purified by column chromatography on silica gel. (DCM: MeOH 20:1 to 10:1) The compound 9 was obtained as a red powder (42.26 mg, 85 %). ^{1}H NMR (600 MHz, DMSO-d6, ppm) δ 8.39 (d, J = 26.8 Hz, 1H), 7.91 – 7.50 (m, 6H), 7.26 – 7.14 (m, 1H), 4.93 – 4.46 (m, 4H), 4.15 – 3.88 (m, 6H), 3.64 (s, 2H), 3.42 (d, J = 46.5 Hz, 1H), 3.08 (t, J = 6.0 Hz, 1H), 3.00 – 2.83 (m, 4H), 2.21 – 2.00 (m, 2H), 1.84 – 1.76 (m, 1H), 1.41 (d, J = 14.7 Hz, 1H), 1.07 (d, J = 6.5 Hz, 3H). ^{13}C NMR (400 MHz, DMSO-d6, ppm) δ 138.12 (d, J = 67.9 Hz), 133.45, 126.65, 116.26, 113.19, 63.72, 60.02, 27.10. HRMS (EI) m/z 755.40.



8 10

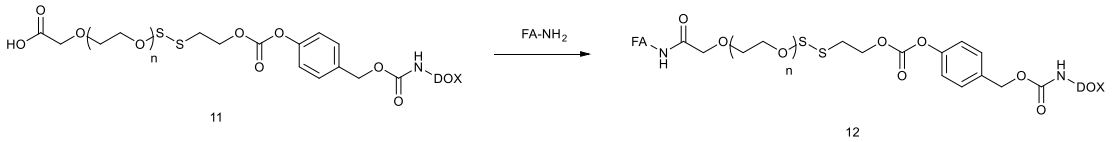
Synthesis of compound 10: Doxorubicin (50 mg, 0.085 mmol, 1.2 eq) was dissolved in 1.2 mL MeOH with Et3N (47 μL, 0.355 mmol, 5 eq). 600 μL MeOH solution of compound 8 (35.5 mg, 0.071 mmol, 1 eq) was dropwise added into the solution above. After the solution was stirred at room temperature overnight, 10 mL DCM was added. After the mixture was washed with brine, dried over Na_2SO_4, concentrated, the residue was purified by column chromatography on silica gel. (DCM: MeOH 20:1 to 10:1) The compound 9 was obtained as a red powder (54.6 mg, 85 %). ^{1}H NMR (400 MHz, DMSO-d6, ppm) δ 14.06 (s, 1H), 13.30 (s, 1H), 8.45 (s, 1H), 8.12 (d, J = 9.2 Hz, 1H), 7.98 – 7.64 (m, 4H), 7.42 – 7.11 (m, 4H), 6.93 (d, J = 9.2 Hz, 2H), 5.48 (s, 1H), 5.28 – 4.47 (m, 8H), 4.40 (s, 2H), 4.16 (d, J = 6.8 Hz, 1H), 3.99 (s, 2H), 3.44 (s, 1H), 3.20 (s, 2H), 2.93 (d, J = 34.8 Hz, 3H), 2.73 (s, 1H), 1.37 – 0.71 (m, 7H). ^{13}C NMR (400 MHz, DMSO-d6, ppm) δ 164.53 (d, J = 2.1 Hz), 160.04 – 148.59(m), 122.78 – 119.13 (m),68.74 ,57.06 ,40.95 ,17.54. HRMS (EI) m/z 905.86.



10 11

Synthesis of compound 11: To a solution of Compound 10 (50 mg, 0.055 mmol,1 eq) in DMSO (500 μL), COOH-PEG2000-SH (121 mg, 0.0605 mmol,1.1 eq) was added slowly and stirred for 24 h. Then, the solvent was removed by dialysis and then subjected to lyophilization to obtain compound 11 as the red power (143 mg, 90%). ^{1}H

NMR (600 MHz, DMSO-d6, ppm) δ 8.48 – 6.50 (m, 6H), 4.51 (d, J = 23.1 Hz, 1H), 4.31 – 4.21 (m, 0H), 4.10 – 4.02 (m, 2H), 3.96 (d, J = 11.7 Hz, 3H), 3.46 (s, 195H), 3.09 – 2.69 (m, 4H), 2.22 – 1.88 (m, 11H), 1.24 – 0.97 (m, 3H).



11 12

Synthesis of compound 12(KSIP-DOX): The 1.0 mL of compound 11(143 mg,0.049 mmol,1eq) aqueous solution was activated by 100 μL of EDC (46.7 mg,0.245 mmol, 5eq) solution in water for 5-10 minutes and 80 μL of folic acid (25.93 mg, 0.0588 mmol,1.2 eq) solution in DMSO was added and stirred for 12 h. Then, the solvent was purified by dialysis and subjected lyophilization to obtain compound 12 as the red power (153 mg, 95%). ^{1}H NMR (600 MHz, DMSO-d6, ppm) δ 8.12 – 6.54 (m, 11H), 4.59 – 4.32 (m, 1H), 4.10 – 4.04 (m, 3H), 3.95 (s, 2H), 3.62 – 3.52 (m, 5H), 3.46 (s, 315H), 3.01 – 2.70 (m, 9H), 2.22 – 1.98 (m, 26H), 1.16 (d, J = 30.4 Hz, 2H), 1.02 (t, J = 7.2 Hz, 1H), 0.93 (t, J = 7.1 Hz, 1H).

Synthesis of CSIP-DOX: The protocol of synthesis of CSIP-DOX is similar to that of KSIP-DOX, just replace Compound 10 with Compound 9. To a solution of Compound 9 (42 mg, 0.055 mmol,1 eq) in DMSO (500 μL), COOH-PEG2000-SH (121 mg, 0.0605 mmol,1.1 eq) was added slowly and stirred for 24 h. Then, the solvent was removed by dialysis and then subjected to lyophilization to obtain compound 11 as the red power (136 mg, 90%). The 1.0 mL of compound obtained in the previous step (136 mg,0.049 mmol,1eq) aqueous solution was activated by 100 μL of EDC (46.7 mg,0.245

mmol, 5eq) solution in water for 5-10 minutes and 80 µL of folic acid (25.93 mg, 0.0588 mmol,1.2 eq) solution in DMSO was added and stirred for 12 h. Then, the solvent was purified by dialysis and subjected lyophilization to obtain CSIP-DOX as the red power (148 mg, 95%).

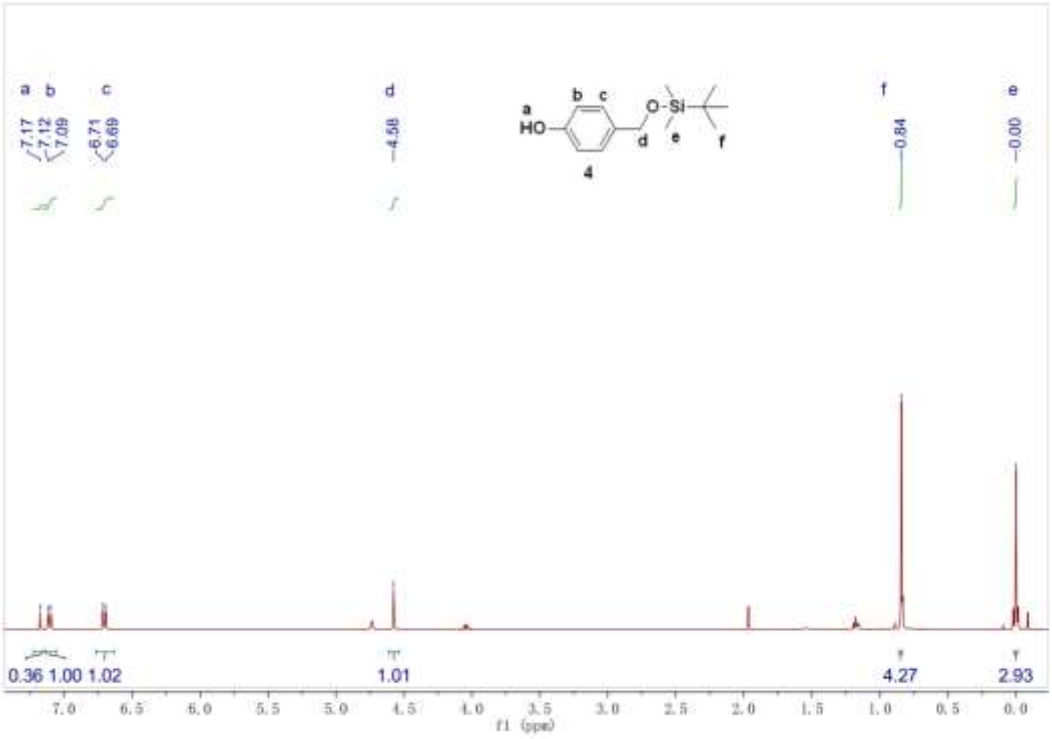


Figure S2: ^{1}H-NMR spectrum of compound 2 in $CDCl_3$

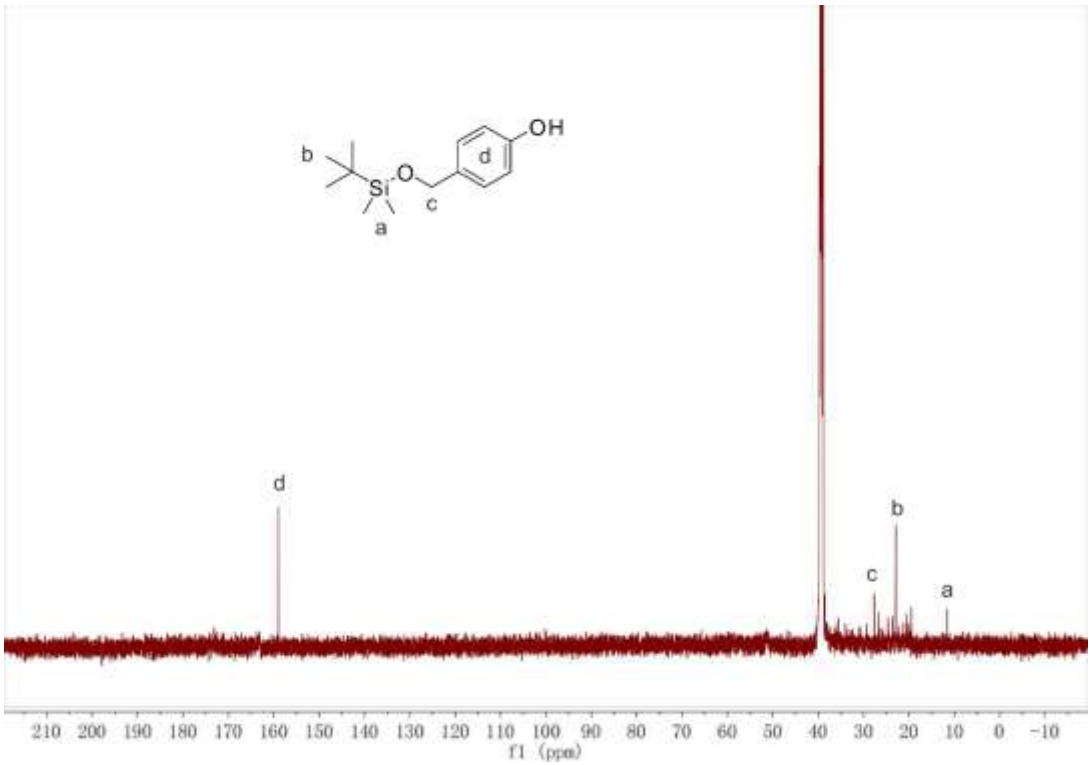


Figure S3: ^{13}C-NMR spectrum of compound 2 in DMSO-d6

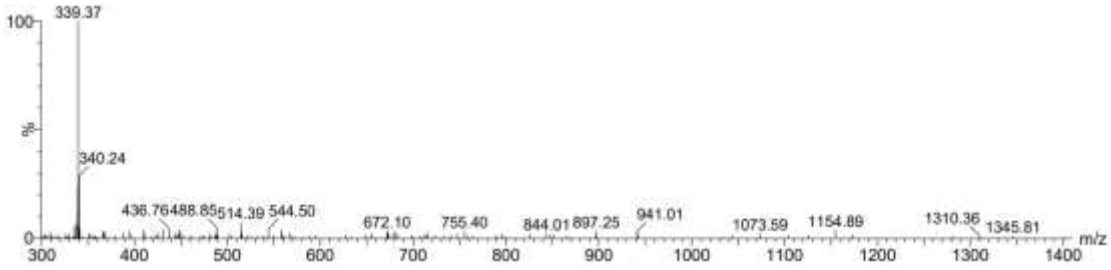


Figure S4: HR/MS spectrum of compound 2

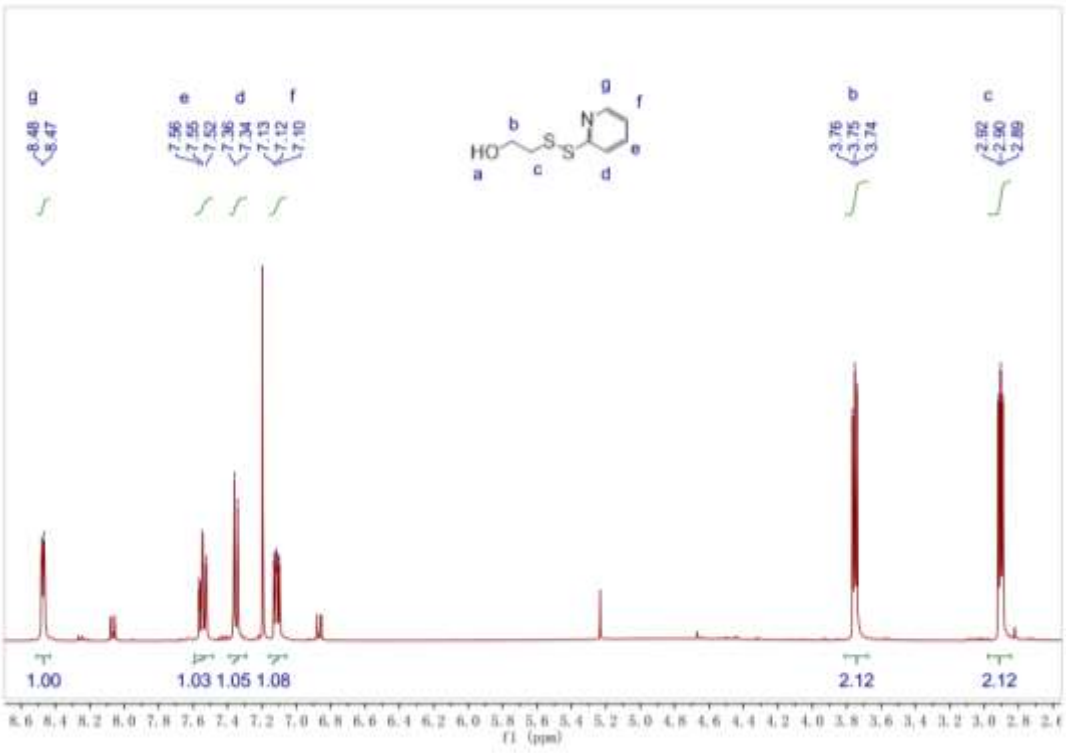


Figure S5: ^{1}H-NMR spectrum of compound 4 in $CDCl_3$

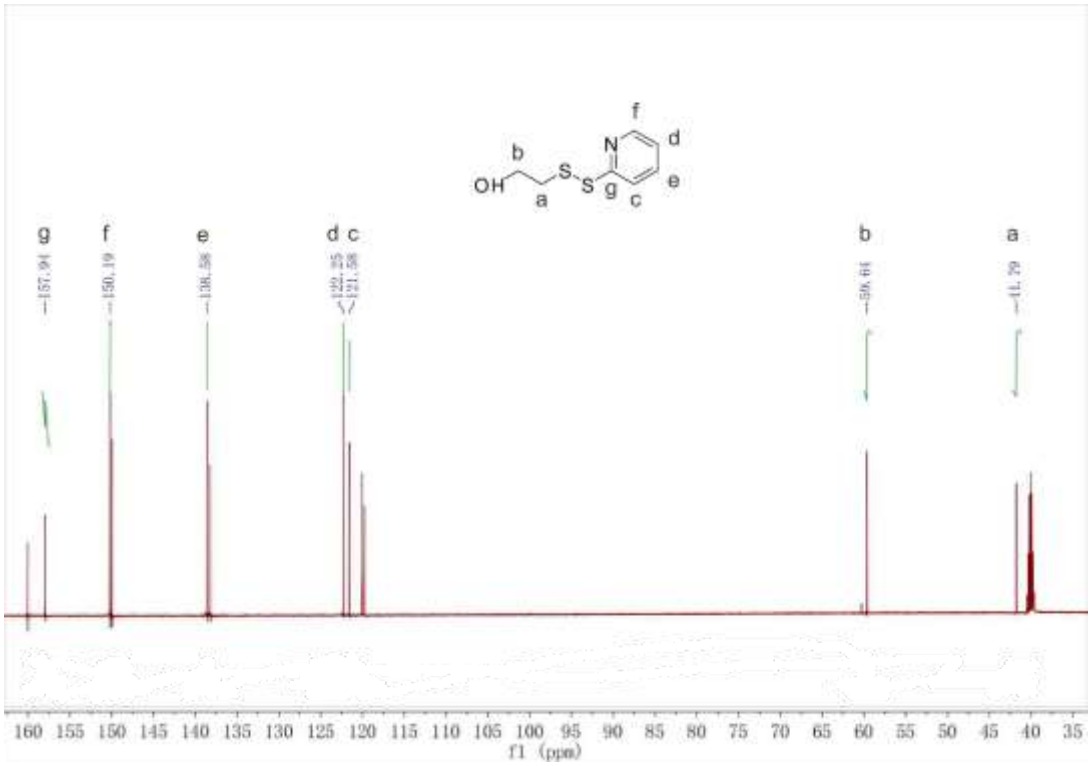


Figure S6: ^{13}C-NMR spectrum of compound 4 in DMSO-d6

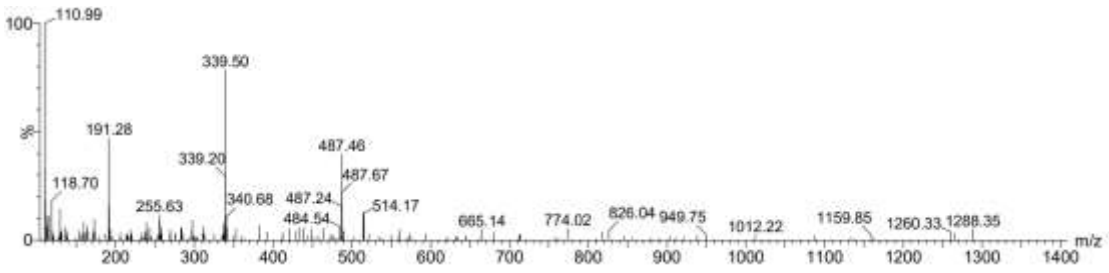


Figure S7: HR/MS spectrum of compound 4. M(EXT):191, M(THE):187.

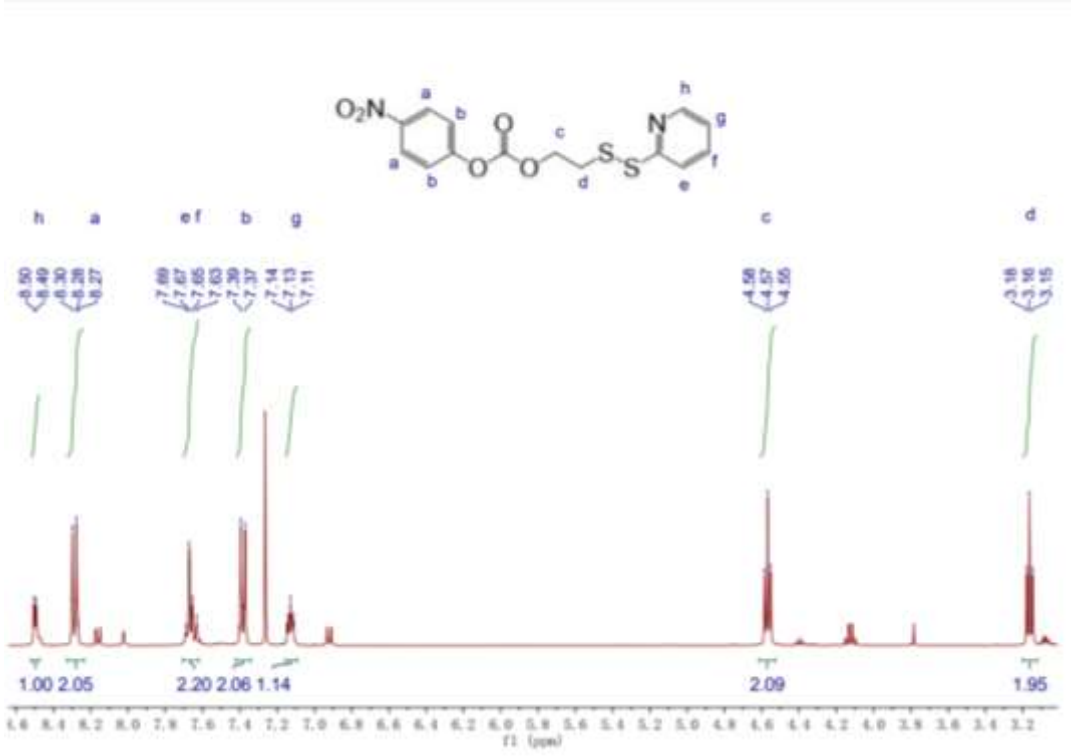


Figure S8: ^{1}H-NMR spectrum of compound 5 in $CDCl_3$

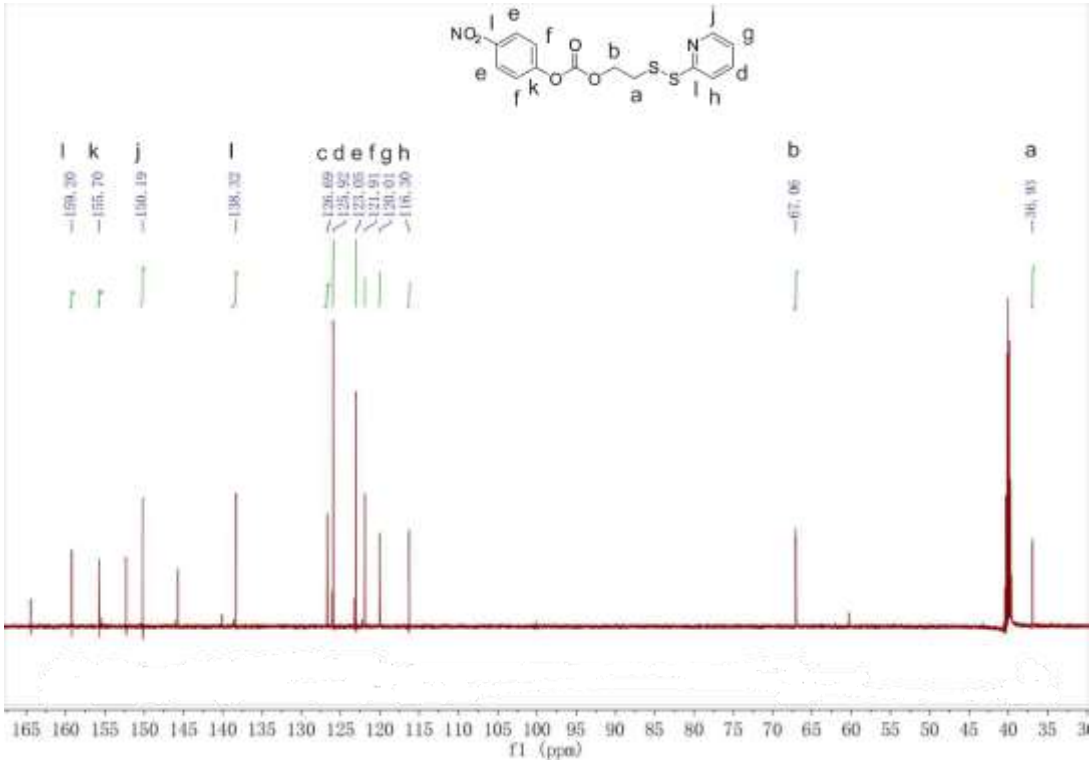


Figure S9 ^{13}C-NMR spectrum of compound 5 in DMSO-d6

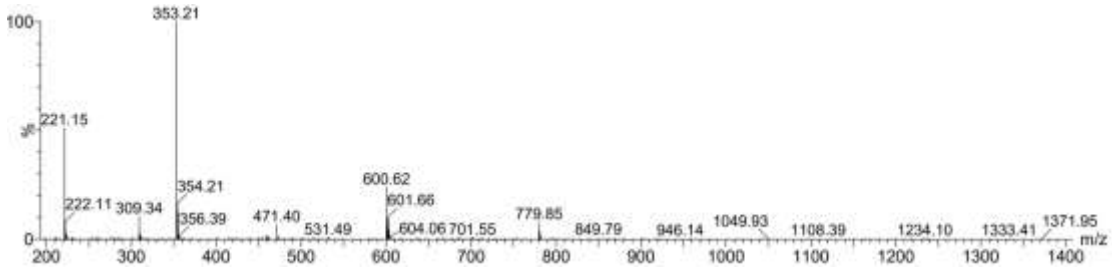


Figure S10: HR/MS spectrum of compound 5. M(EXT):353, M(THE):353.

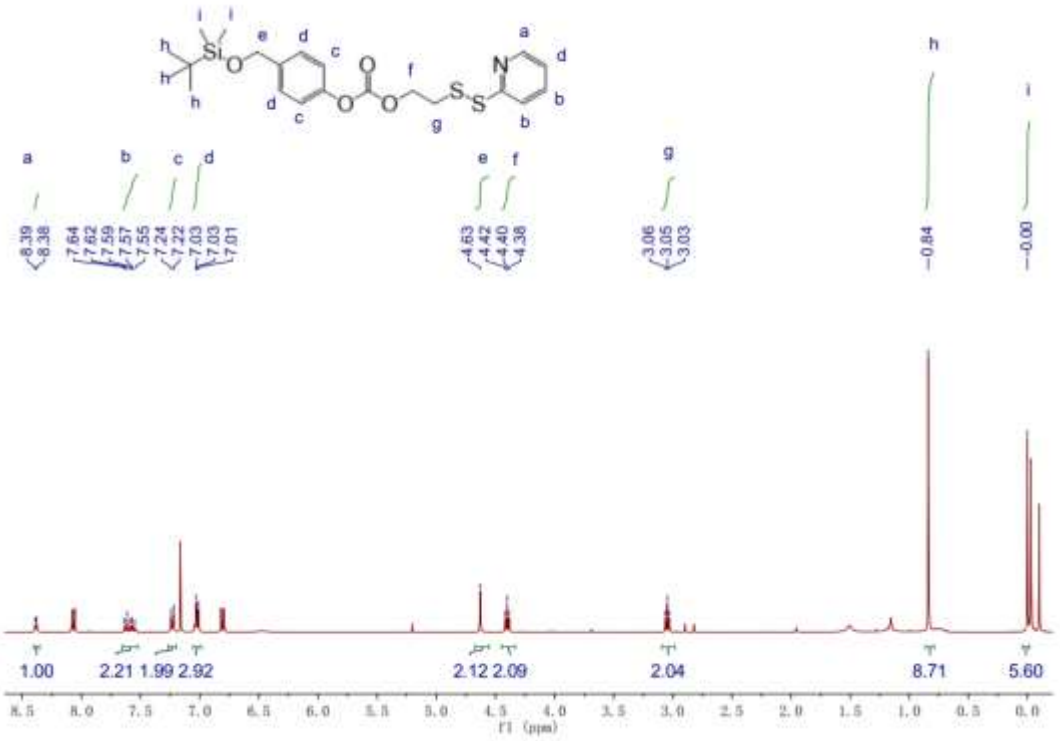


Figure S11: ^{1}H-NMR spectrum of compound 6 in $CDCl_3$

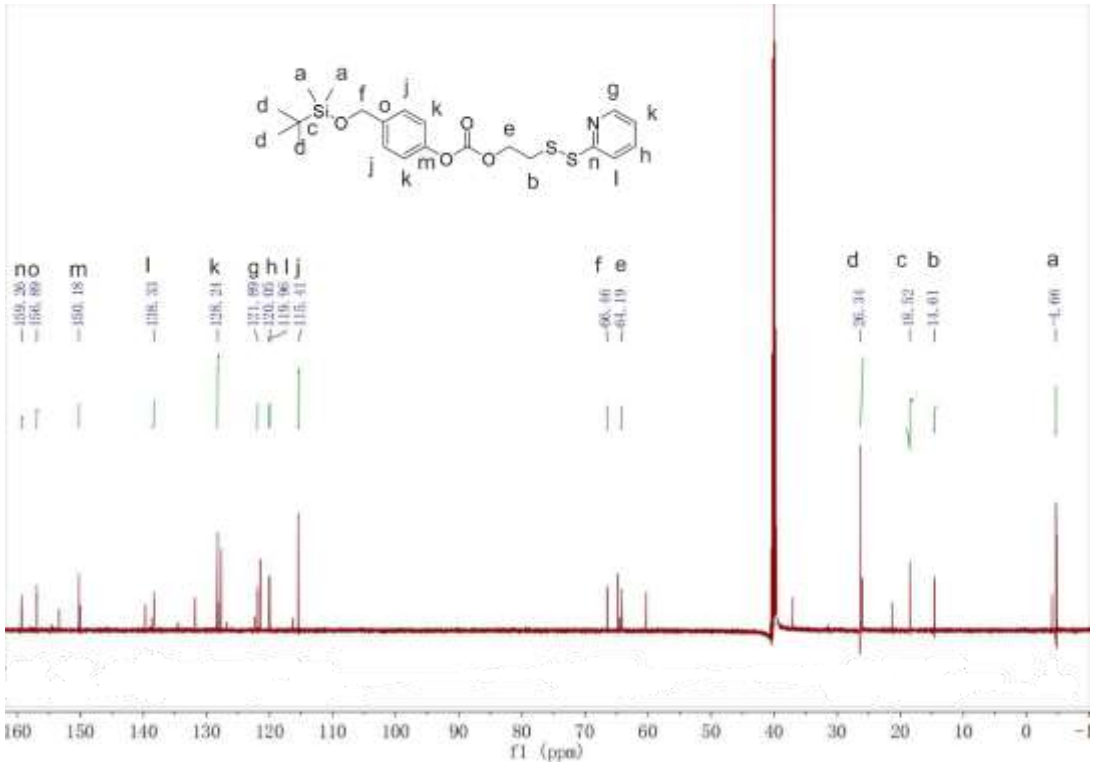


Figure S12: ^{13}C-NMR spectrum of compound 6 in DMSO-d6

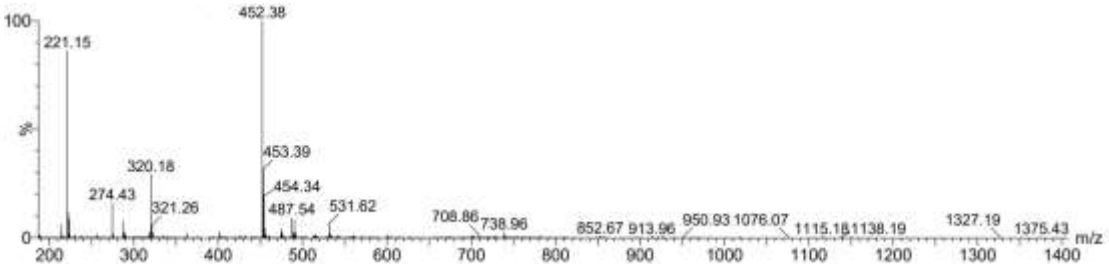


Figure S13: HR/MS spectrum of compound 6. M(EXT):452, M(THE):451.

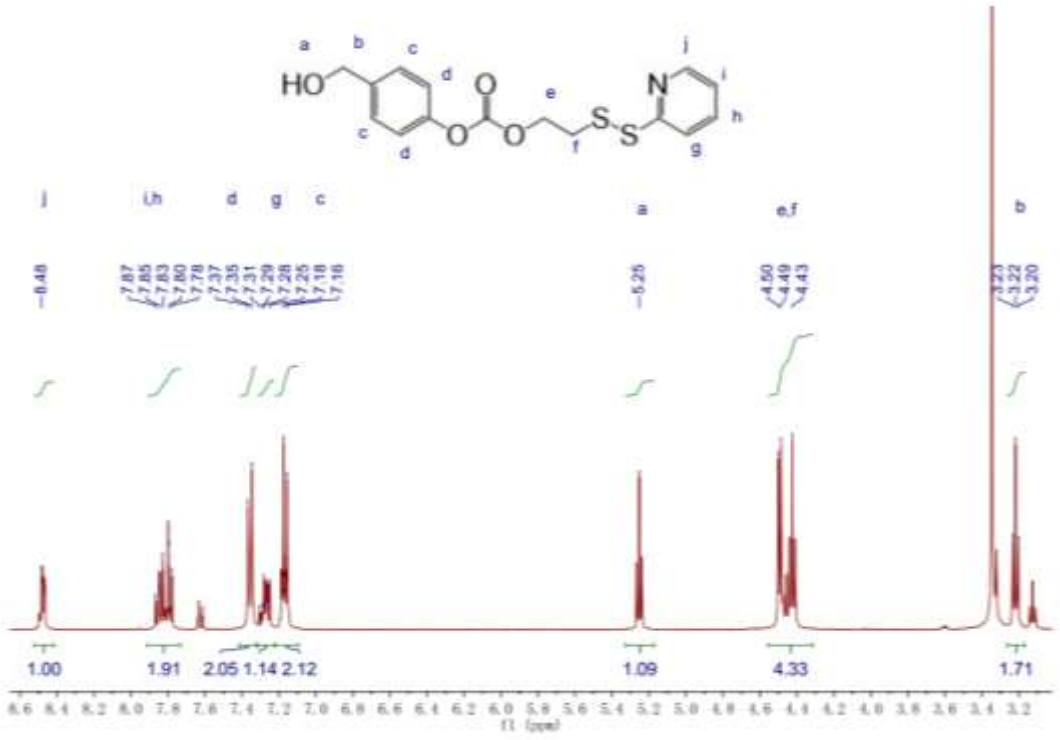


Figure S14: ^{1}H-NMR spectrum of compound 7 in DMSO-d6

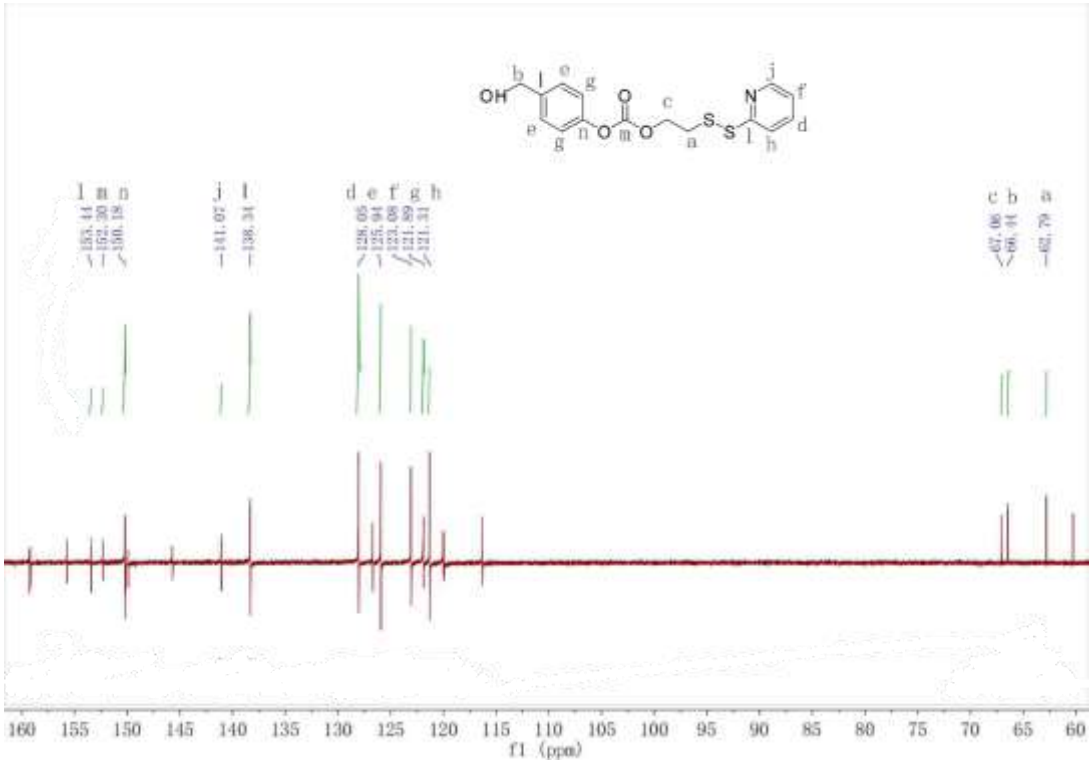


Figure S15: ^{13}C-NMR spectrum of compound 7 in DMSO-d6

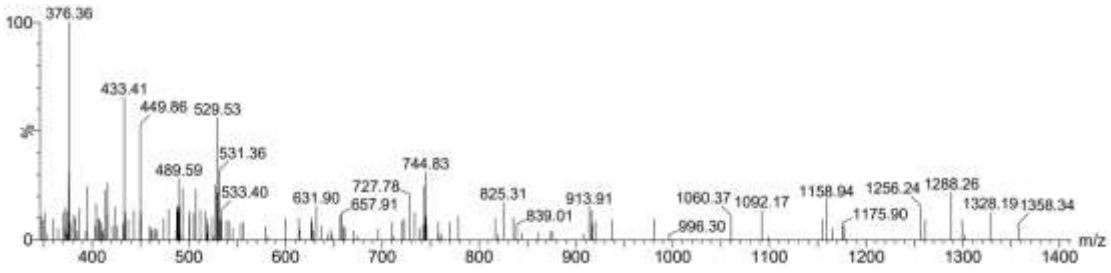


Figure S16: HR/MS spectrum of compound 7. M(EXT):376, M(THE):337.

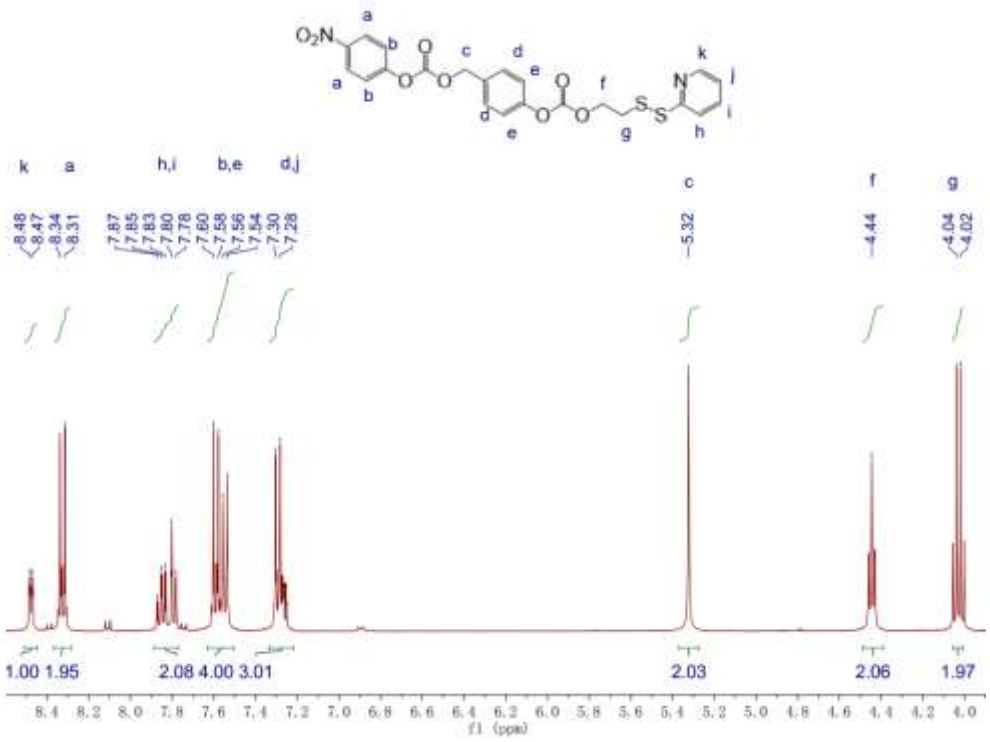


Figure S17: ^{1}H-NMR spectrum of compound 8 in DMSO-d6

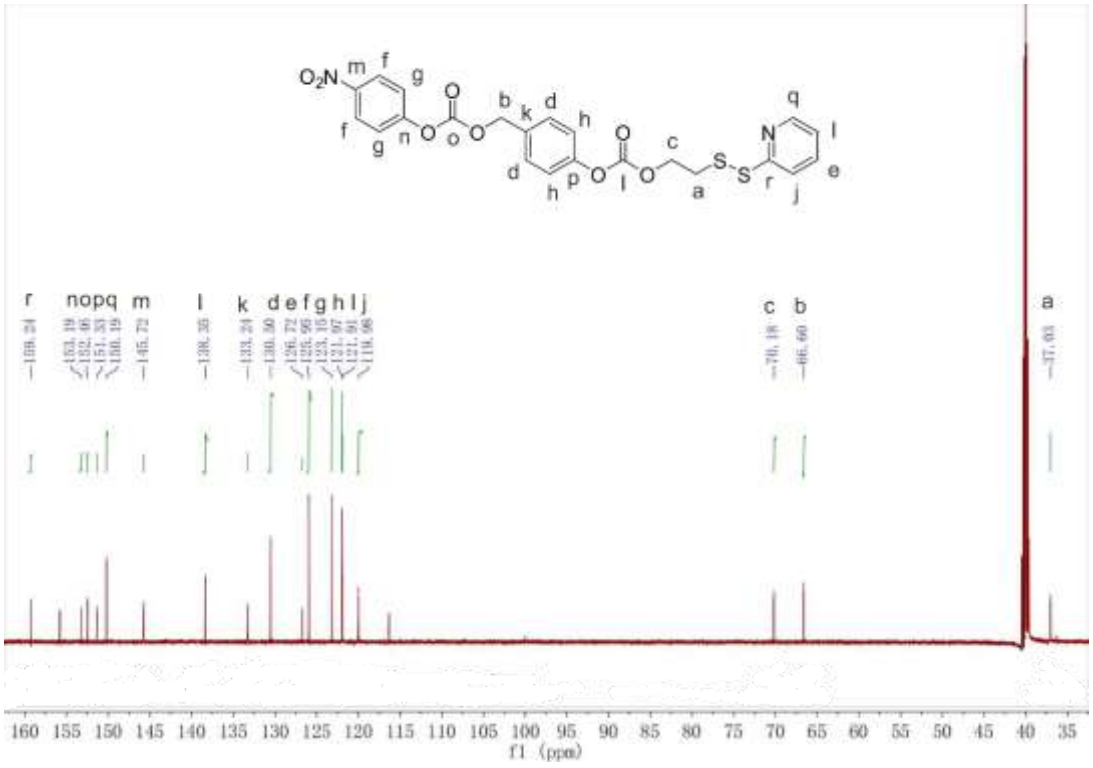


Figure S18: ^{13}C-NMR spectrum of compound 8 in DMSO-d6

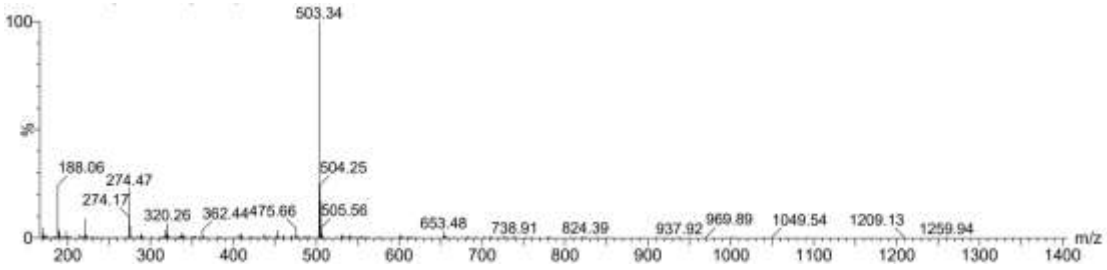


Figure S19: HR/MS spectrum of compound 8. M(EXT):503, M(THE):502.

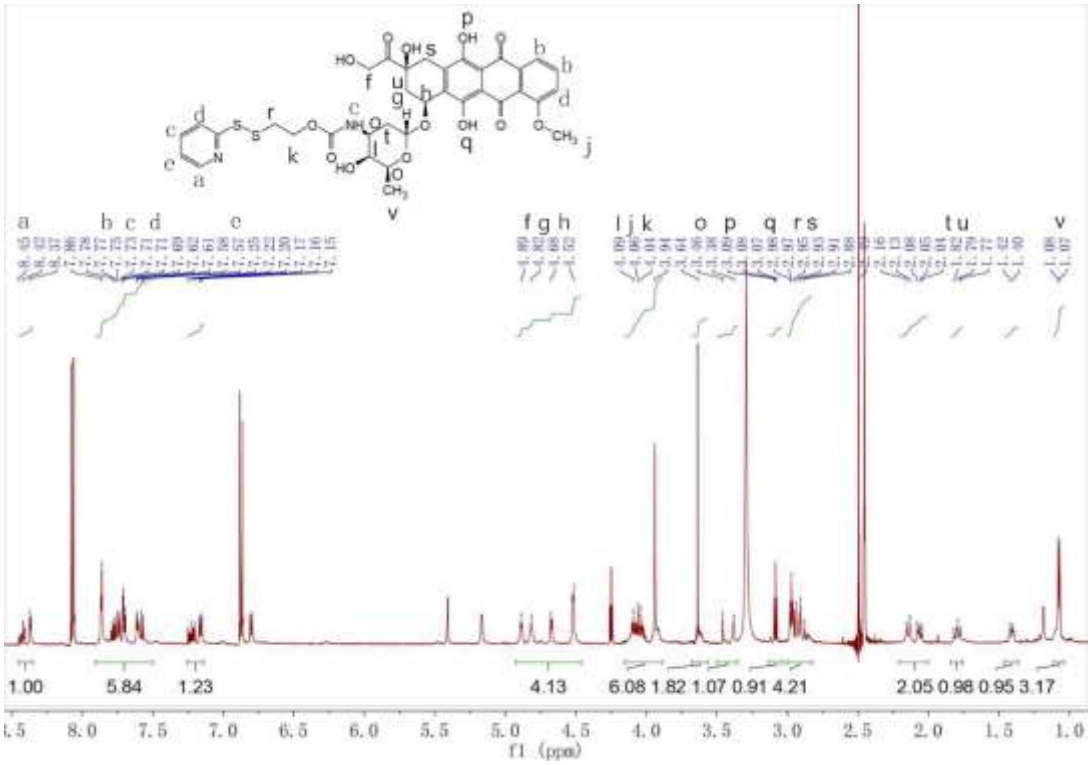


Figure S20: [1]H-NMR spectrum of compound 9 in DMSO-d6

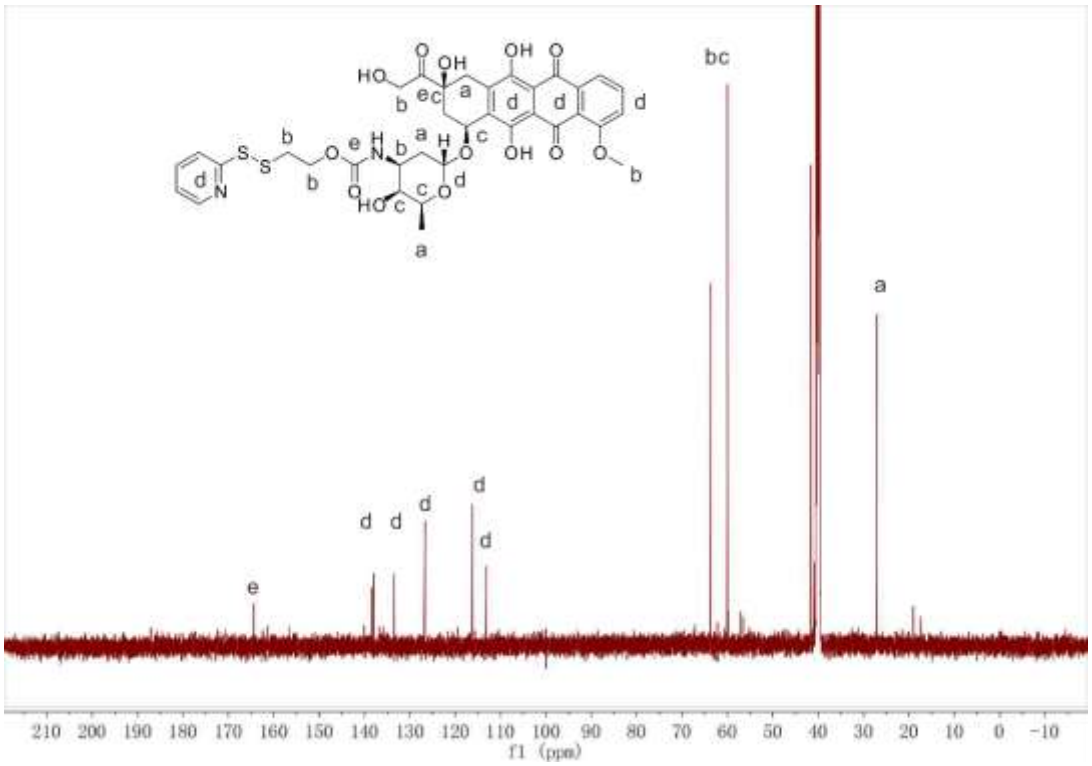


Figure S21: ^{13}C-NMR spectrum of compound 9 in DMSO-d6

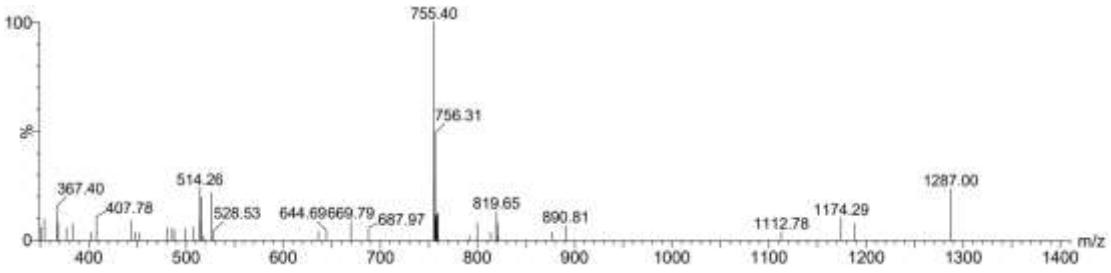


Figure S22: HR/MS spectrum of compound 9. M(EXT):755, M(THE):756.

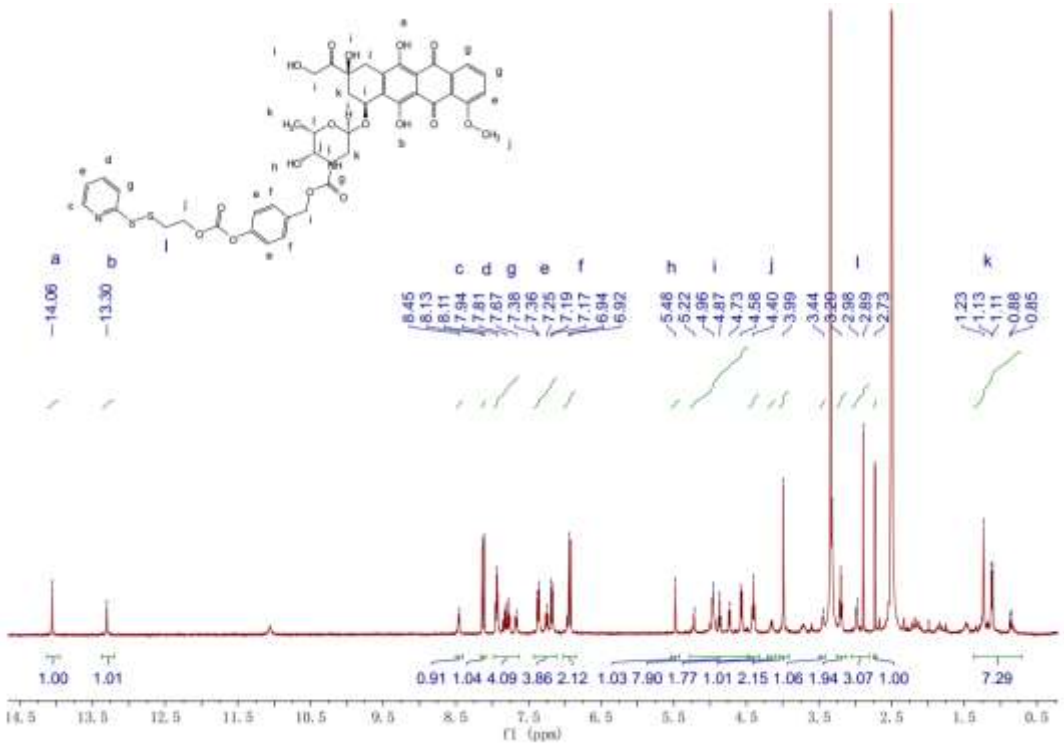


Figure S23: ^{1}H NMR spectrum of compound 10 in DMSO-d6

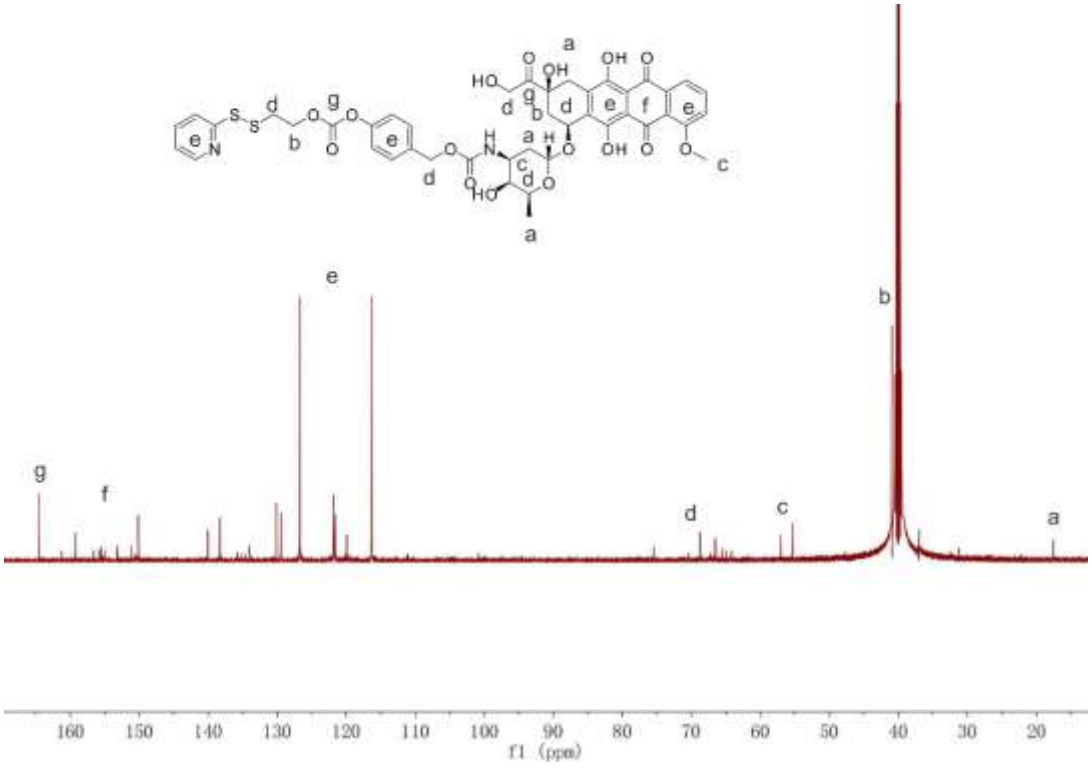


Figure S24: ^{13}C NMR spectrum of compound 10 in DMSO-d6

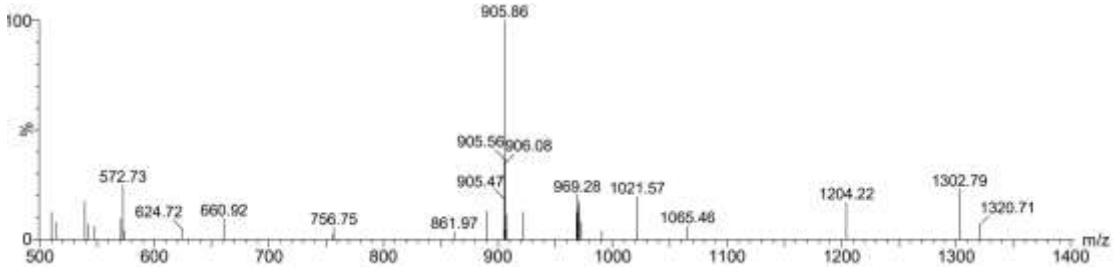


Figure S25: HR/MS spectrum of compound 10. M(EXT):905, M(THE):906.

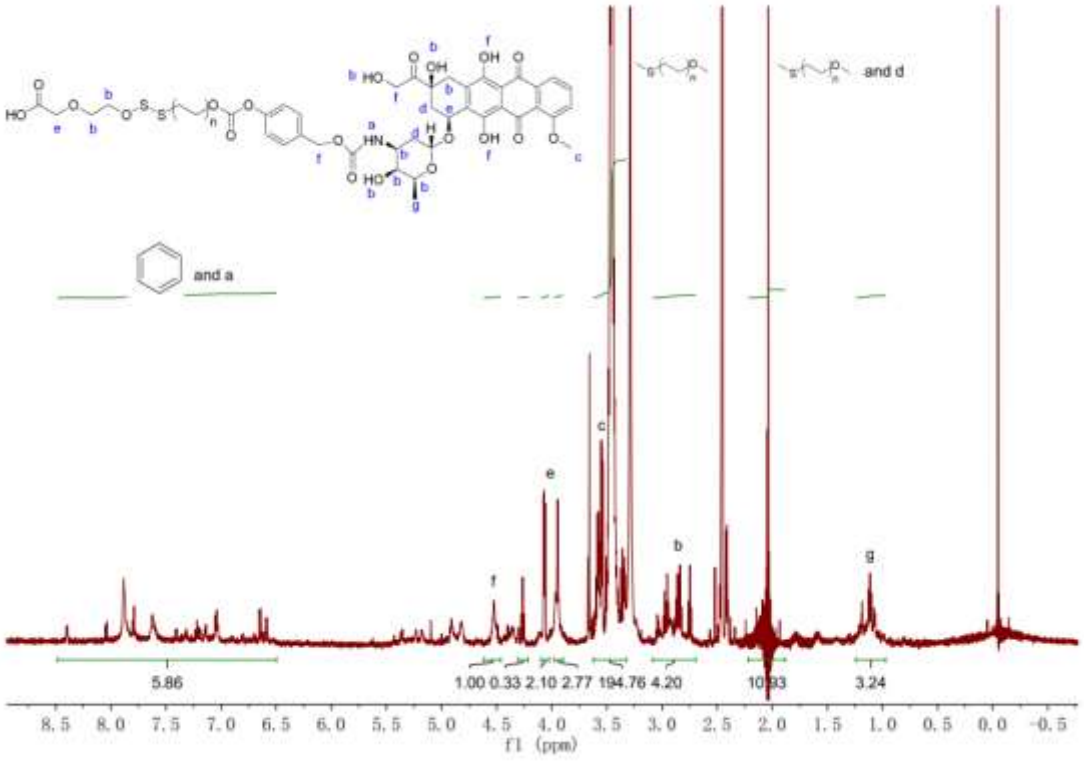


Figure S26: ^{1}H NMR spectrum of compound 11 in DMSO-d6

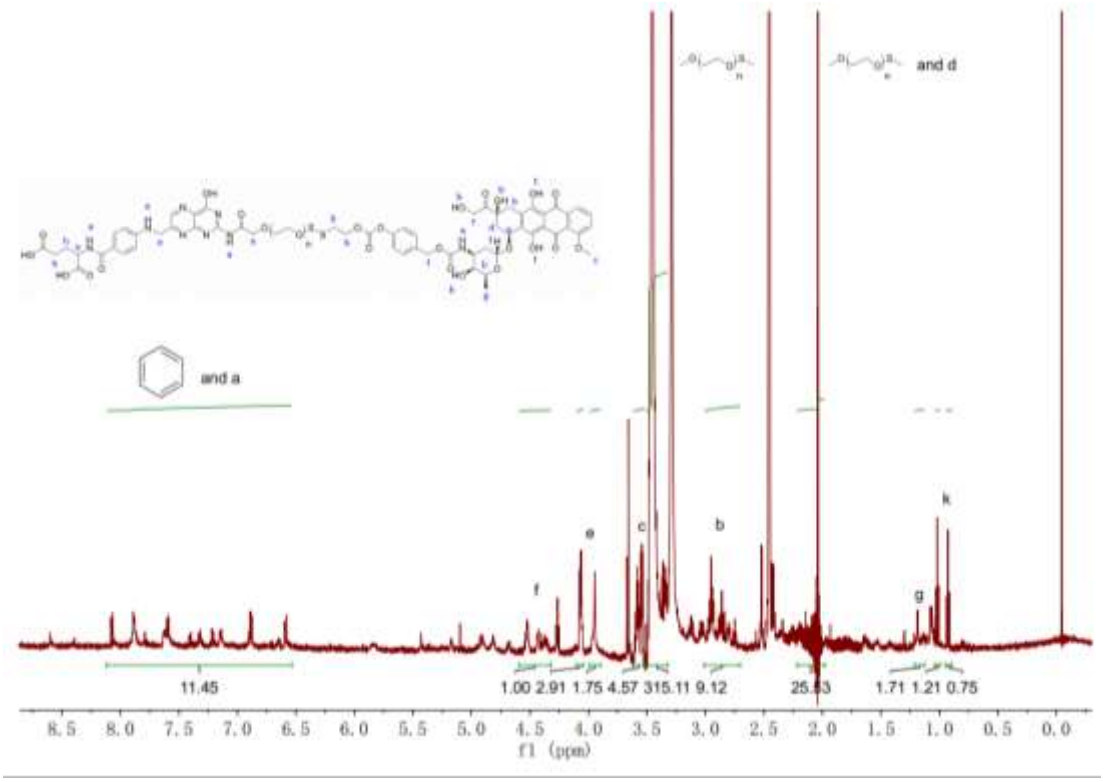


Figure S27: ^{1}H NMR spectrum of compound 12 in DMSO-d6

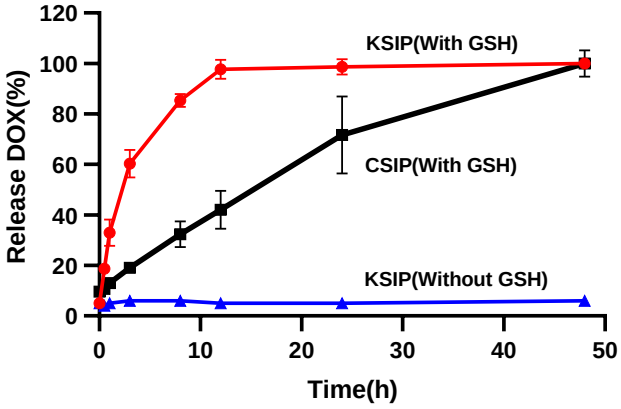


Figure S28. In vitro release profiles of KSIP-DOX and CSIP-DOX within 48 h in PBS buffer (pH=7.4, with 1% Tween 80) with/without 10 mM GSH.

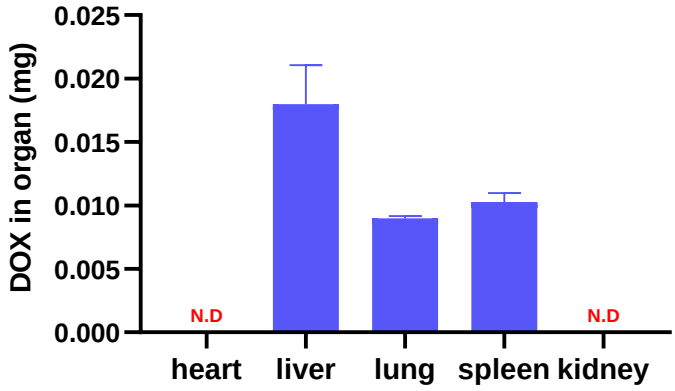


Figure S29. Biodistribution of DOX in various tissues after mice were intravenously administered 10 mg/kg KSIP-DOX at 24 h. Data are shown as mean ± SD, n = 3. (N.D means no detectable).

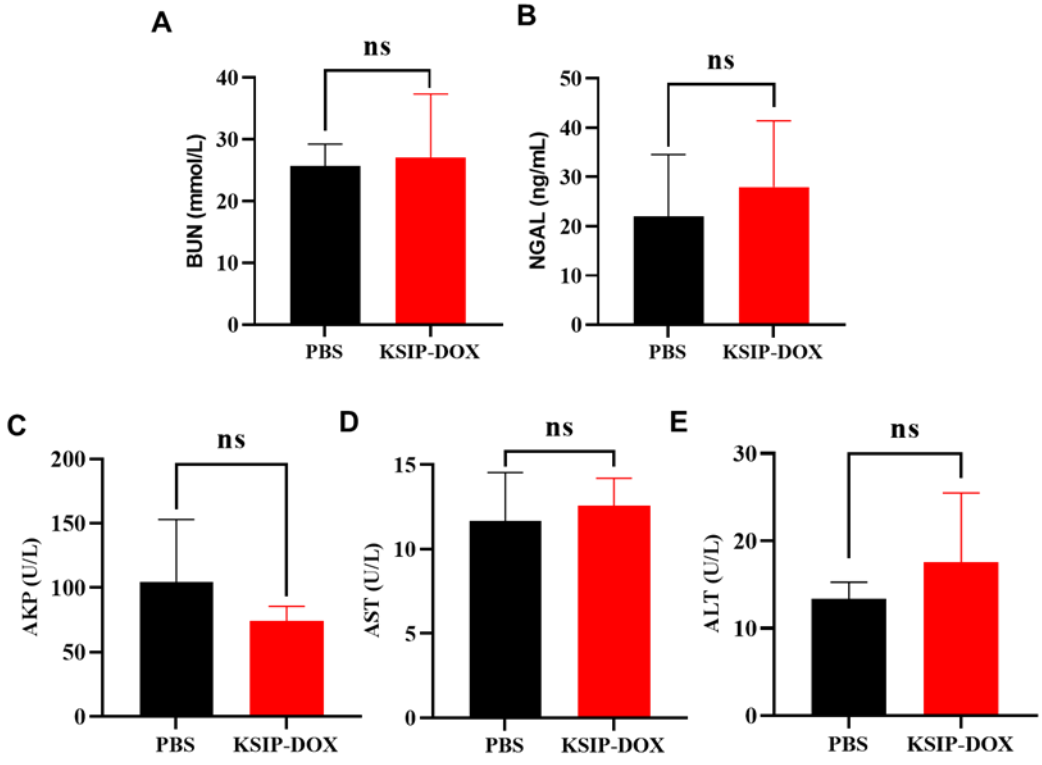


Figure S30. Toxicity evaluation of KSIP-DOX. The concentration of A) BUN, B) NGAL, C) AKP, D) AST, E) ALT in serum when mice were given 5 mg/kg (DOX)

KSIP-DOX on day 1, 3, 5 and blood were collected on day 8. n=3 independent mice for each group respectively. (One-way analysis of variance was used for significant difference analysis, ns $P>0.05$, * $P<0.05$, ** $P<0.01$).