Supporting Information

Efficient Strategy for the Synthesis and Modification of 2-Hydroxyethylluciferin for Highly Sensitive Bioluminescence Imaging of Endogenous Hydrogen Sulfide in Cancer Cells and Nude Mice

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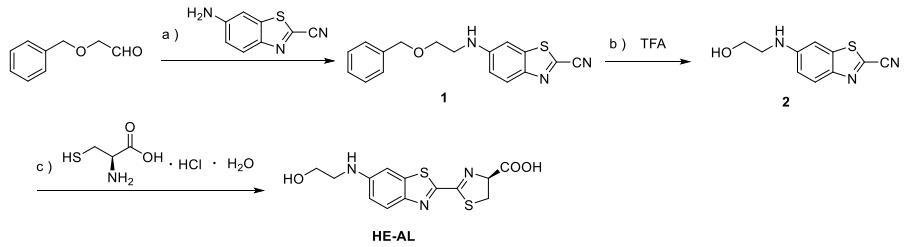
1. Synthesis of 2-hydroxyethylluciferin (HE-AL)



Scheme S1. Reaction pathway for the synthesis of **HE-AL**. (a) 5-amino-2-benzothiazolecarbonitrile (1.0 equiv.), Hantzsch ester (1.2 equiv.), iodine (0.05 equiv.), ambient temperature, overnight, 94%. (b) trifluoroacetic acid, 65 °C, 60 h, 71%. (c) H-D-Cys-OH·HCl·H_2O (1.0 equiv.), triethylamine (1.5 equiv), MeOH: DCM: H_2O = 4: 2: 1 (v/v/v), room temperature, overnight, 98%.

6-((2-(benzyloxy)ethyl)amino)benzo[d]thiazole-2-carbonitrile (1)

Under nitrogen atmosphere, Hantzsch ester (0.91 g, 3.6 mmol) was added to the mixture of 2-oxoethyl 2-((2,4-dinitrophenyl)thio)benzoate (0.42 mL, 3.0 mmol) and 5-amino-2-benzothiazolecarbonitrile (0.53 g, 3.0 mmol) in DCM, followed by the catalytic amount of iodine (0.038 g, 0.15 mmol). After the reaction was completed at room temperature overnight, saturated $Na_2S_2O_3$ solution was added to quench the reaction. The crude was extracted with DCM, concentrated, and purified by column chromatography to obtain the light yellow solid (0.87 g, yield 94%). ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.90 (d, J = 9.0 Hz, 1H), 7.39 – 7.29 (m, 5H), 6.94 (d, J = 2.2 Hz, 1H), 6.89 (dd, J = 9.0, 2.3 Hz, 1H), 4.58 (s, 2H), 3.75 (t, J = 5.2 Hz, 2H), 3.40 (t, J = 5.2 Hz, 2H).

6-((2-hydroxyethyl)amino)benzo[d]thiazole-2-carbonitrile (2)

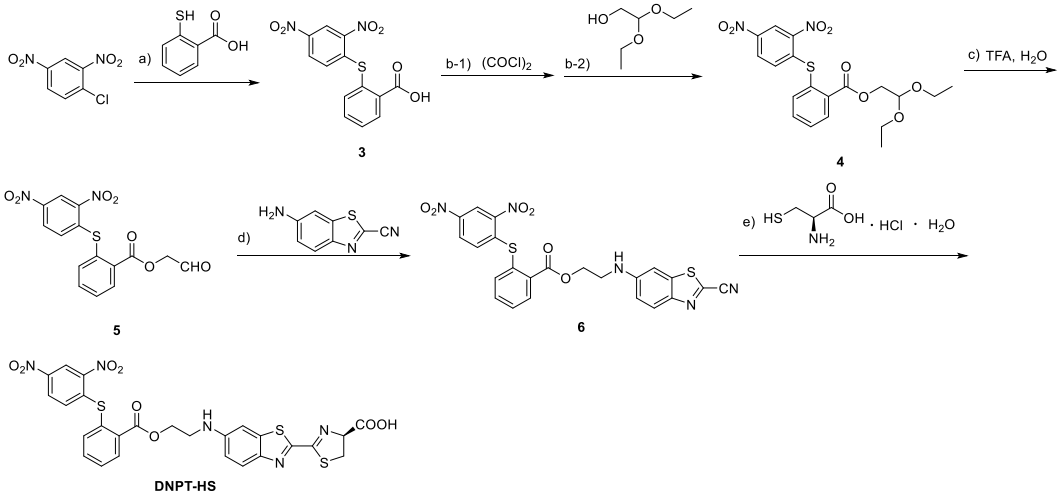
To the suspension of 6-((2-(benzyloxy)ethyl)amino)benzo[d]thiazole-2-carbonitrile **1** (0.097 g, 0.3 mmol) in DCM (2.0 mL), trifluoroacetic acid (45.0 mL) was added. Subsequently, the reaction was refluxed for 60 h under the protection of nitrogen atmosphere. After completion, the mixture was concentrated under vacumm, diluted with DCM, washed with saturated brine, dried over Na_2SO_4 and concentrated in vacuo to give the crude product. Then the raw product was purified with silica gel chromatograph (PE/EA = 8:1) to obtain the yellow solid (0.047 g, yield 71%). ^{1}H NMR (600 MHz, DMSO-d_6) δ 7.87 (d, J = 9.1 Hz, 1H), 7.19 (d, J = 2.2 Hz, 1H), 7.06 (dd, J = 9.1, 2.3 Hz, 1H), 6.68 (s, 1H), 3.60 (t, J = 5.7 Hz, 2H), 3.19 (q, J = 5.8 Hz, 2H).

2-hydroxyethylluciferin (HE-AL)

6-((2-hydroxyethyl)amino)benzo[d]thiazole-2-carbonitrile (0.033 g, 0.15 mmol) **2** and triethylamine

(0.033 g, 0.23 mmol) were placed in a flask containing MeOH:DCM:H_2O = 4:2:1 (1.5 mL) successively. Then D-Cysteine hydrochloride monohydrate (0.032 mL, 0.18 mmol) was added to the suspension and stirred for 2 h at ambient temperature. After completion, the mixture was acidified to 3, concentrated, purified with silica gel chromatography, and eluted with DCM/MeOH (50:1-30:1) to generate red solid (0.047 g, yield 98%). $C_{13}H_{13}N_3O_3S_2$ $[M + H]^+$ calcd. 324.05, found 324.0.

2. Synthesis of Probe DNPT-HS



Scheme S2. Reaction pathway for the synthesis of **DNPT-HS**. (a) 2-mercaptobenzoic acid (1.0 equiv.), Et_3N (2.0 equiv.), 80 ℃, 3 h, 97%. (b-1) oxalyl chloride (2.0 equiv.), room temperature, 2 h. (b-2) 2,2-diethoxyethanol (1.1 equiv.), Et_3N (3.0 equiv.), 0 ℃, 3 h, 73%. (c) trifluoracetic acid (5.0 equiv.), ambient temperature, overnight, 91%. (d) 5-amino-2-benzothiazolecarbonitrile (1.0 equiv.), Hantzsch ester (1.2 equiv.), iodine (0.05 equiv.), ambient temperature, overnight, 91%. (e) H-D-Cys-OH·HCl·H_2O (1.0 equiv.), MeOH: DCM: H_2O = 4 : 2 : 1 (v/v/v), room temperature, 98%.

2-((2,4-dinitrophenyl)thio)benzoic acid (**3**)

To the solution of 1-chloro-2,4-dinitrobenzene (0.41 g, 2.0 mmol) in isopropyl alcohol (6 mL), Et_3N (0.55 mL, 4.0 mmol) and 2-mercaptobenzoic acid (0.31 g, 2.0 mmol) were added. The mixture was refluxed for 2 h and monitored by TLC. After completion, the mixture was cooled to room temperature, and the pH value was adjusted to 2 using 1 M HCl and extracted with ethyl acetate three times. The organic phase was washed with saturated brine, dried over Na_2SO_4 and concentrated *in vacuo* to obtain the crude product that was purified by chromatography eluting with DCM/MeOH (100:1) to afford the desirable compound (0.62 g) as light yellow solid in 97% yield. ^{1}H NMR (600 MHz, DMSO-d_6) δ 13.36 (s, 1H), 8.89 (d, J = 2.5 Hz, 1H), 8.34 (dd, J = 9.0, 2.5 Hz, 1H), 8.00 – 7.95 (m, 1H), 7.70 (d, J = 3.1 Hz, 3H), 7.12 (d, J = 9.0 Hz, 1H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 167.1, 145.2, 144.6, 144.3, 136.6, 136.3,

132.8, 130.6, 130.7, 130.8, 129.0, 127.4, 120.8. $C_{13}H_8N_2O_6S$ [2M - H]$^-$ calcd. 639.01, found 638.8.

2,2-diethoxyethyl 2-((2,4-dinitrophenyl)thio)benzoate (**4**)

Under nitrogen atmosphere, 2-((2,4-dinitrophenyl)thio)benzoic acid **3** (1.60 g, 5.0 mmol) was dissolved in anhydrous THF (10.0 mL). After both oxalyl chloride (0.85 mL, 10.0 mmol) and two drops of anhydrous DMF were added, the suspension was stirred for 2 h at room temperature. Solvent was removed under reduced pressure to obtain the light yellow oil, then dry DCM (5.0 mL) was added. The intermediate in DCM was slowly added to the mixture of 2,2-diethoxyethanol (0.73 mL, 5.5 mmol) and trimethylamine (2.10 mL, 15.0 mmol) at 0 ℃ under nitrogen atmosphere. After 30 min, the reaction was stirred for 3 h at room temperature. Subsequently, the solution was diluted with DCM, washed with saturated brine, dried over Na_2SO_4 and concentrated under vacumm. The crude production was purified via silica gel chromatography (petrol/DCM =1:1) to afford the target compound (1.59 g, yield 73%). ^{1}H NMR (600 MHz, $CDCl_3$) δ 9.07 (d, J = 2.4 Hz, 1H), 8.11 (dd, J = 9.0, 2.5 Hz, 1H), 8.05 – 8.01 (m, 1H), 7.69 (dt, J = 6.8, 3.1 Hz, 1H), 7.67 – 7.63 (m, 2H), 6.96 (d, J = 9.0 Hz, 1H), 4.64 (t, J = 5.3 Hz, 1H), 4.21 (d, J = 5.3 Hz, 2H), 3.64 (m, 2H), 3.51 (m, 2H), 1.16 (t, J = 7.1 Hz, 6H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 164.8, 147.2, 143.7, 143.4, 137.4, 135.6, 132.7, 131.2, 130.6, 129.1, 128.8, 126.1, 120.6, 98.5, 64.1, 61.6 (2C), 14.6 (2C). $C_{19}H_{20}N_2O_8S$ [M+Na]$^+$ cald. 459.08, found 459.0.

2-oxoethyl 2-((2,4-dinitrophenyl)thio)benzoate (**5**)

2,2-diethoxyethyl 2-((2,4-dinitrophenyl)thio)benzoate **4** (0.26 g, 0.6 mmol) was dissolved to a mixture of trifluoracetic acid (0.22 mL, 3.0 mmol), DCM (2.0 mL), and water (10.0 μL). Suspension was vigorously stirred overnight at ambient temperature and separated between DCM/saturated brine. The DCM extracts were dried (Na_2SO_4) and concentrated to obtain raw product that was purified by chromatography eluted with DCM/PE (1:1-4:1) to give the light yellow solid 0.19 g with the yield 91%. ^{1}H NMR (600 MHz, DMSO-d_6) δ 9.50 (s, 1H), 8.89 (d, J = 2.5 Hz, 1H), 8.33 (dd, J = 9.0, 2.5 Hz, 1H), 8.12 – 8.09 (m, 1H), 7.80 (t, J = 2.4 Hz, 3H), 7.08 (d, J = 9.0 Hz, 1H), 4.97 (s, 2H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 196.3, 164.6, 145.2, 144.3 (2C), 137.4, 134.1, 133.8, 131.5, 131.1, 130.2, 129.3, 127.5, 120.9, 69.5. $C_{15}H_{10}N_2O_7S$ [M-H]$^-$ cald. 361.01, found 360.8.

2-((2-cyanobenzo[d]thiazol-6-yl)amino)ethyl-2-((2,4-dinitrophenyl)thio)benzoate (**6**)

Hantzsch ester (0.15 g, 0.6 mmol) was added to the mixture of 2-oxoethyl 2-((2,4-dinitrophenyl)thio)benzoate **5** (0.18 g, 0.5 mmol) and 5-amino-2-benzothiazolecarbonitrile (0.088 g, 0.5 mmol) in DCM, followed by the catalytic amount of iodine, under nitrogen atmosphere. After the

reaction was completed at r.t. overnight, saturated $Na_2S_2O_4$ solution was added to quench the reaction. The crude production was extracted with DCM, concentrated and purified by silica gel column chromatography to obtain the light yellow solid (0.24 g, yield 91%) ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.70 (d, J = 2.5 Hz, 1H), 8.23 (dd, J = 9.0, 2.5 Hz, 1H), 8.05 – 8.01 (m, 1H), 7.81 (d, J = 9.0 Hz, 1H), 7.76 – 7.72 (m, 3H), 7.09 (d, J = 2.2 Hz, 1H), 7.01 (d, J = 9.0 Hz, 1H), 6.92 (dd, J = 9.1, 2.3 Hz, 1H), 6.70 (t, J = 5.3 Hz, 1H), 4.38 (t, J = 5.2 Hz, 2H), 3.31 (t, J = 5.0 Hz, 2H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 165.6, 149.3, 145.4, 143.9, 143.7, 143.2, 138.5, 137.3, 135.5, 133.5, 131.3, 131.1, 130.1, 128.7, 127.9, 127.3, 124.6, 120.6, 116.9, 114.1, 99.2, 63.7, 41.4. $C_{23}H_{15}N_5O_6S_2$ [M-H]$^-$ cald. 520.04, found 519.8.

3. Identification of the products of the reaction between DNPT-HS and H_2S

For further investigation of the mechanism of the reaction between **DNPT-HS** and H_2S, the stock solution of **DNPT-HS** (10 mM) in DMSO and Na_2S (100 mM) in water was mixed together (V/V, 1:1) at room temperature for 30 min. After the reaction was completed, the suspension was diluted 100 times with acetonitrile, then measured by mass spectrometry. As depicted in Figure S3, ion peak m/z 324.0 was observed, which could be assigned to [**HE-AL**+H]$^+$ species. In addition, the peak at m/z 359.2 was attributed to 2-[(2,4-dinitrophenyl)thio]benzenecarbothioic S-acid (**DBS**), which was appointed to [**DBS**+Na]$^+$ species. The above results testified the hypothesis that the cleavage of **DBS** was triggered by the nucleophilicity of HS^-.

4. Kinetic assay of DNPT-HS

To measure the dynamics of **DNPT-HS**, the stock solution of **DNPT-HS** (1.0 mM) and various concentrations (0.1, 0.05, 1.0, 2.0, and 4.0 mM) of Na_2S in PBS (pH 7.2) buffer were freshly prepared. As a control, Na_2S was replaced by the same volume of PBS (pH 7.2) buffer. Then, 2 μL of ATP (100 mM) and 1 μL of luciferase (100 μg/mL) were added to each tube at specified times (15, 30, 45, 60, 75, 90, 105, and 120 min) and the total flux was immediately detected in photons per second. In the meantime, the control groups were measured at the same condition. The final concentrations were 100 μM for **DNPT-HS** and 0.01, 0.05, 0.1, 0.2 and 0.4 mM for Na_2S.

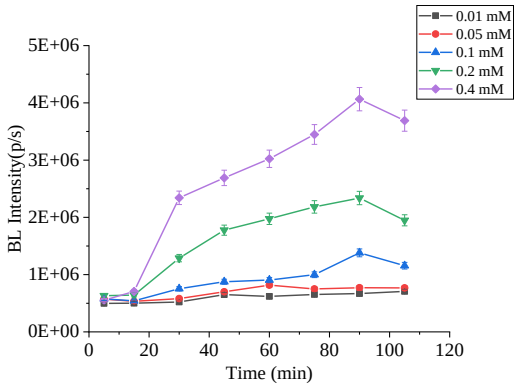
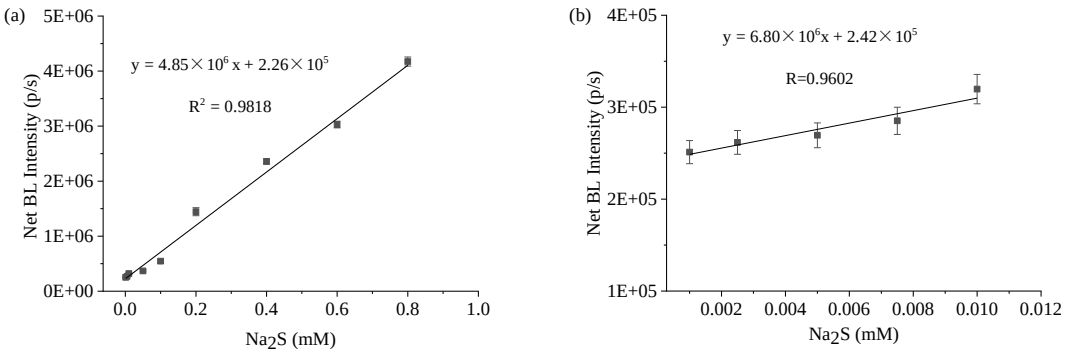


Figure S1. Time-dependent bioluminescence imaging changes of **DNPT-HS** with Na_2S (**DNPT-HS**, 100 μM; Na_2S, 0.01, 0.05, 0.1, 0.2 and 0.4 mM) in PBS (pH 7.2) at room temperature.

5. Establishing the calibration curves of DNPT-HS

Freshly prepared **DNPT-HS** was dissolved in DMSO to make 10 mM stock solution, then diluted to 1.0 mM with degassed sodium phosphate buffer (PBS, pH 7.2). Freshly prepared with PBS (pH 7.2), a set of Na_2S (0.01, 0.025, 0.05, 0.075, 0.1, 0.2, 0.5, 1.0, 1.50, 2.0, 4.0, 6.0, and 8.0 mM) stock solutions were added to **DNPT-HS**. The mixtures were incubated for 45 min in darkness at ambient temperature, followed by adding 2 μL of ATP (100 mM) and 1 μL of luciferase (100 μg/mL) for each tube, then the total flux was immediately measured in photons per second. The final concentrations were 0.1 mM for **DNPT-HS** and 0.001, 0.0025, 0.005, 0.0075, 0.01, 0.02, 0.05, 0.075, 0.1, 0.2, 0.4, 0.6, and 0.8 mM for Na_2S. The detection limits of different concentration regions were calculated to be 70 nM, 205 nM, and 91 nM, respectively.



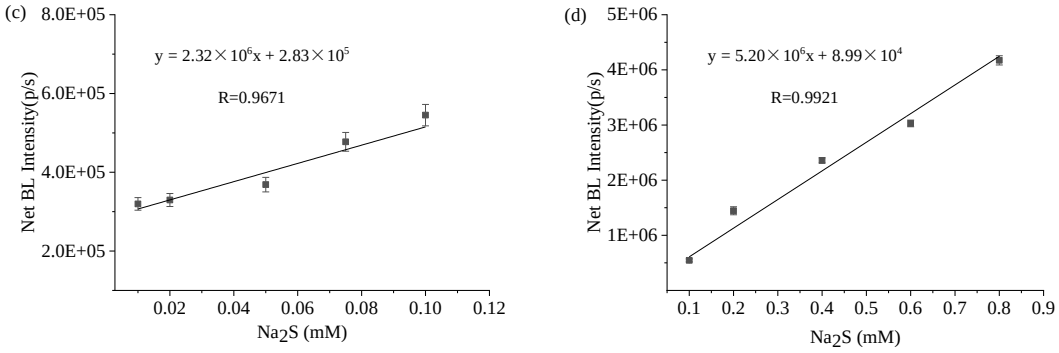


Figure S2. Linear relationships between net bioluminescence intensity and Na_2S. (a) The concentrations of Na_2S were 0.001, 0.005, 0.01, 0.05, 0.1, 0.2, 0.4, 0.6, and 0.8 mM. (b) The concentrations of Na_2S were 0.001, 0.0025, 0.005, 0.0075, and 0.01 mM. (c) The concentrations of Na_2S were 0.01, 0.02, 0.05, 0.075, and 0.1 mM. (d) The concentrations of Na_2S were 0.1, 0.2, 0.4, 0.6, and 0.8 mM. As a blank, Na_2S was replaced by the same volume of PBS buffer (pH 7.2). Samples and blank were incubated for 45 min in dark at ambient temperature prior to analysis.

6. Estimation of possible interferences

The stock solutions (10.0 mM) of possible interferences (Na_2NO_3, Na_2NO_2, KH_2PO_4, $NaHSO_3$, $NaSO_4$, Cys, GSH, $Na_2S_2O_3{\cdot}5H_2O$, Na_2SO_3, NaCl, $NaHCO_3$, Na_2CO_3, CH_3COONa, and NaF), 8.8 mM for NaClO, 0.67 mM for H_2O_2, and 7.8 mM for *t*-BuOOH were prepared in PBS (pH 7.2) buffer respectively. The same volume of PBS (pH 7.2) buffer was used as blank and Na_2S (10.0 mM) as control. The solutions were incubated with **DNPT-HS** (0.5 mM) for 30 min in dark at room temperature. The total flux was immediately evaluated in the presence of 2 μL of ATP (100 mM) and 1 μL of luciferase (100 μg/mL). The final concentrations were 0.05 mM for **DNPT-HS** and 1.0 mM for Na_2S.

7. Cell culture and cell viability assays

The cytotoxicity of **DNPT-HS** towards HEK 239T cells was evaluated by CCK8. Initially, HEK 239T cells were seeded in 96-well micro assay plate at a density of 5×10^3 cell/well in DMEM that was supplemented with 5% fetal bovine serum, 1% penicillin, 1% streptomycin sulfate, then incubated under humidified 5% CO_2/95% air at 37 ℃. After being cultured for 24 h, the adherent cells were washed by FBS-free DMEM one time, and the 100 μL of FBS-free DMEM containing **DNPT-HS** at the different concentrations (0.0, 10, 50, 100, 125, and 150 μM), then was incubated for 2 h. The culture media was removed and 100 μL of FBS-free DMEM containing 10% CCK-8 was added. After being incubated for 2 h, the absorbance was measured at 450 nm. The results from three individual experiments were

averaged. Following formula was used to calculate the viability of cell growth:

Percentage of cell viability (%) = (mean of treatment group – blank)/ (mean of control group – blank) × 100%

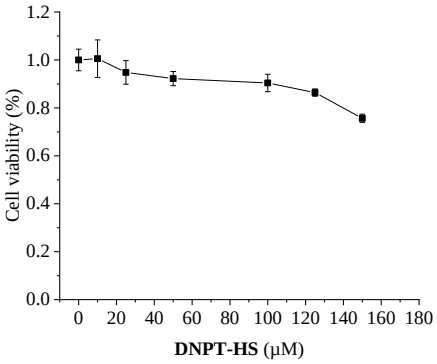


Figure S3. Cell viability under different concentrations of **DNPT-HS** (0, 10, 50, 100, 125, and 150 μM) in the HEK 293T cell line shown by the CCK8 assay.

8. Characterization of the reaction mechanism

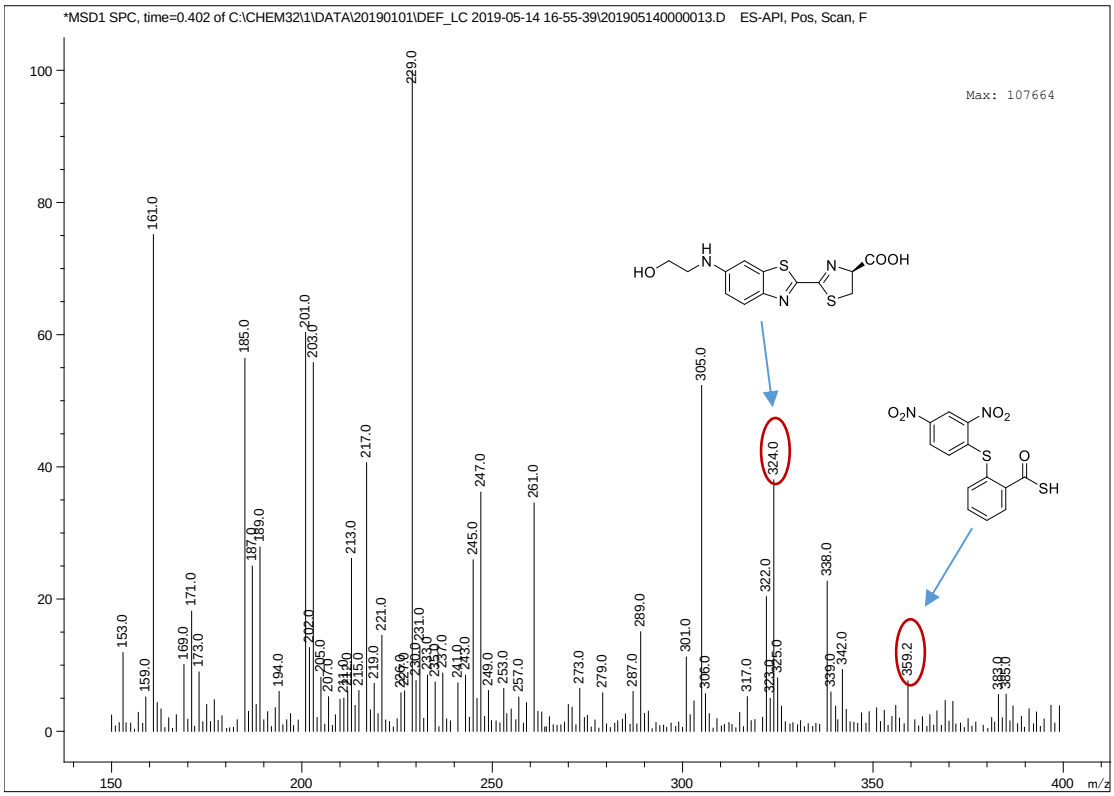


Figure S4. MS analysis of the products of **DNPT-HS** reacted with Na_2S

9. Characterizations of compound 3-6 and DNPT-HS

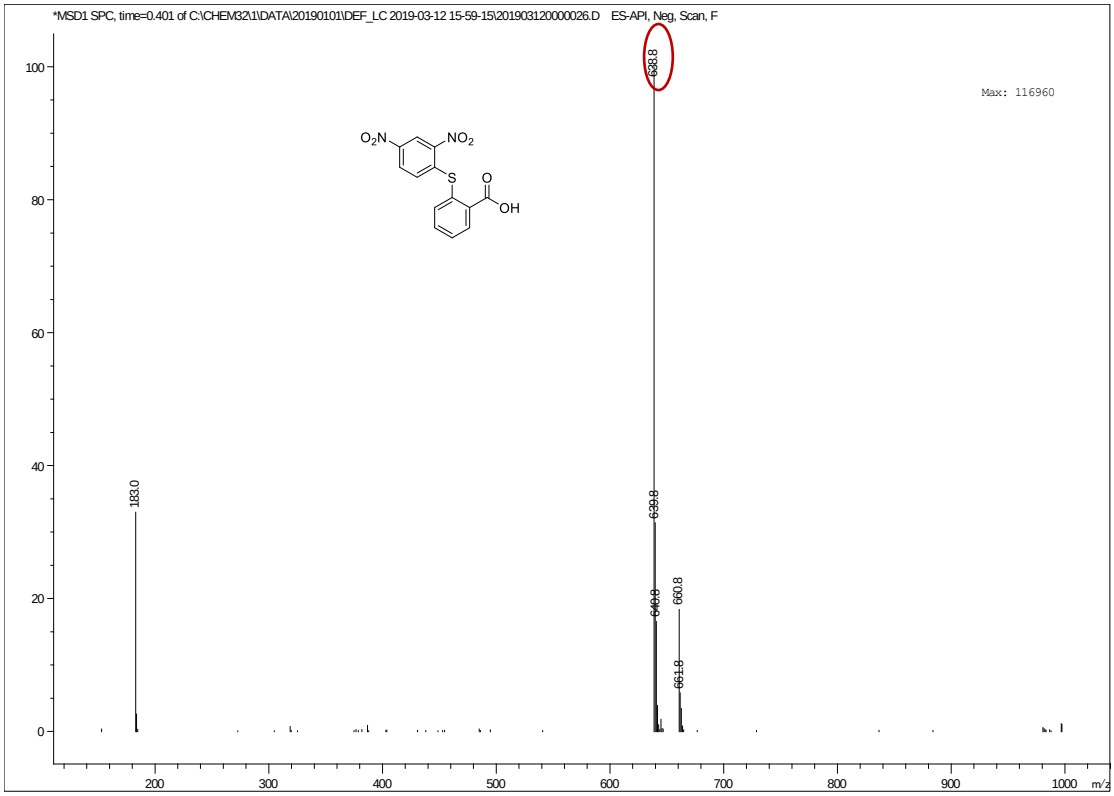


Figure S5. MS spectrum of compound **3**.

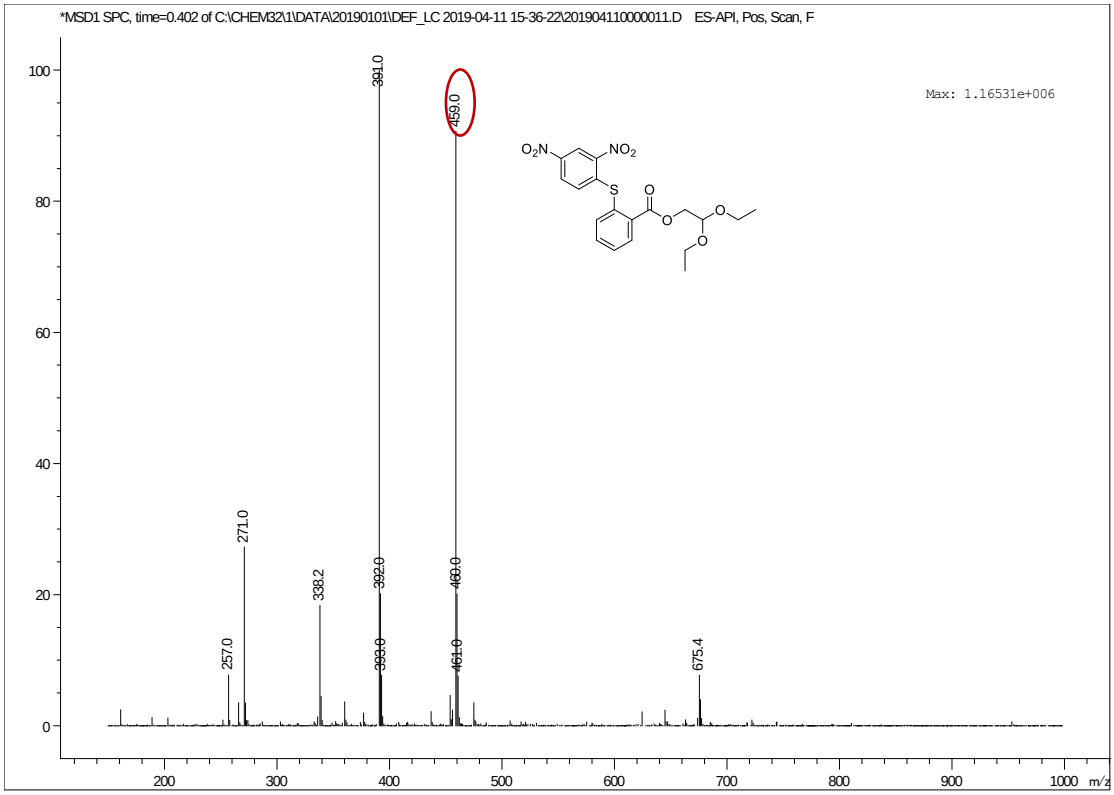


Figure S6. MS spectrum of compound **4**.

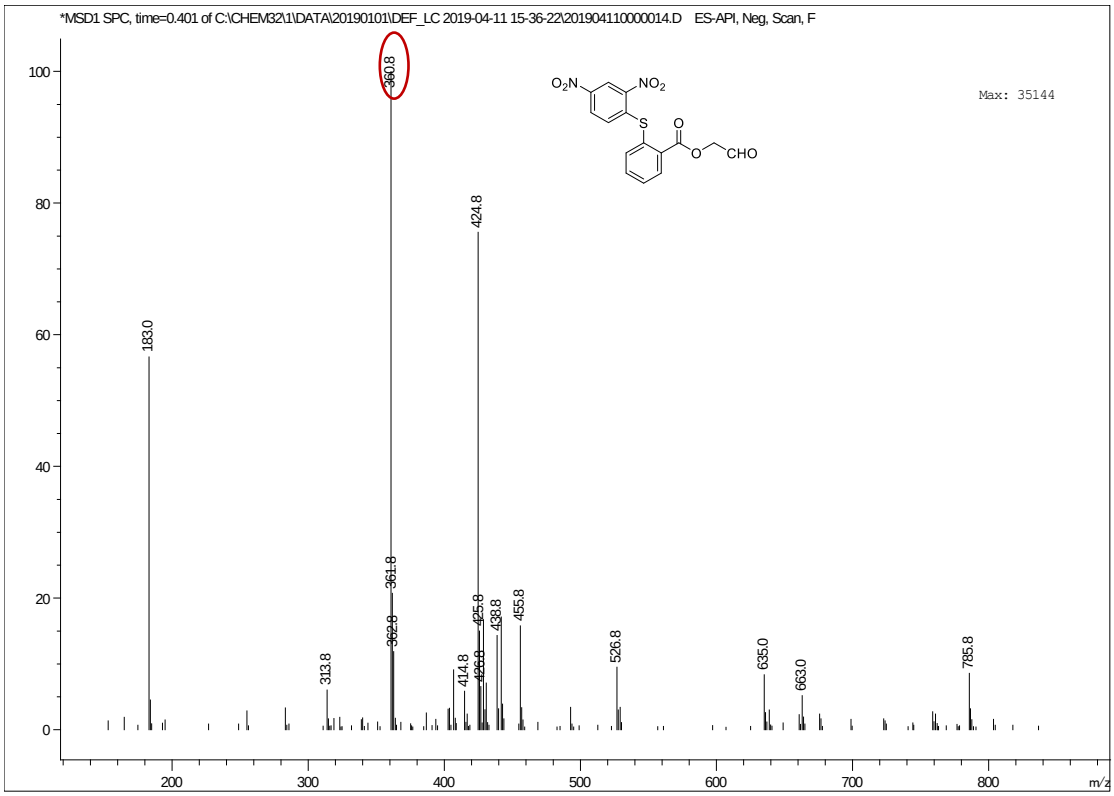


Figure S7. MS spectrum of compound **5**.

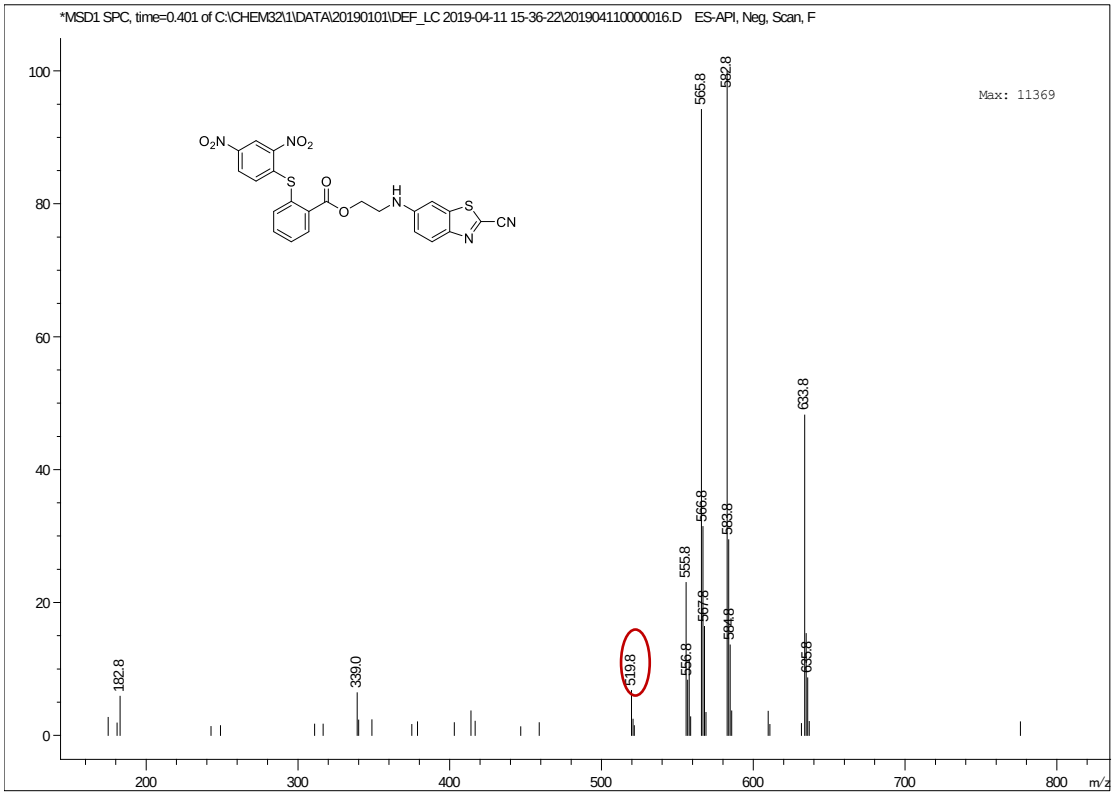


Figure S8. MS spectrum of compound **6**.

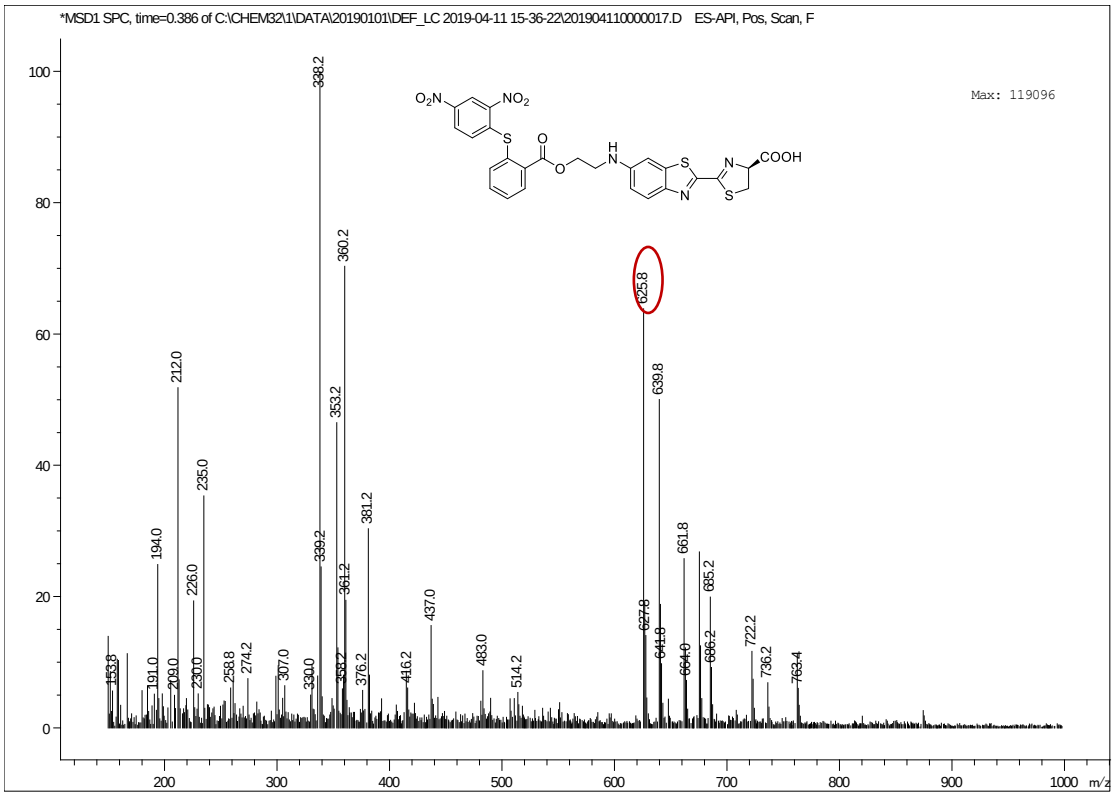


Figure S9. MS spectrum of **DNPT-HS**.

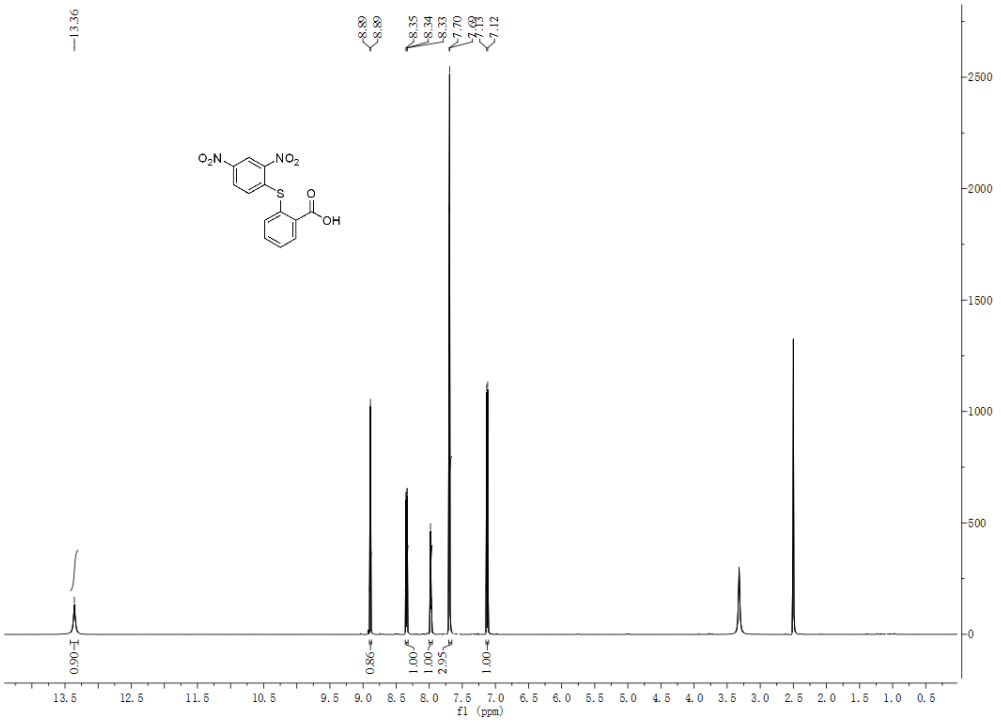


Figure S10. ^{1}H NMR spectrum of compound **3** (DMSO-d_6, 600 MHz).

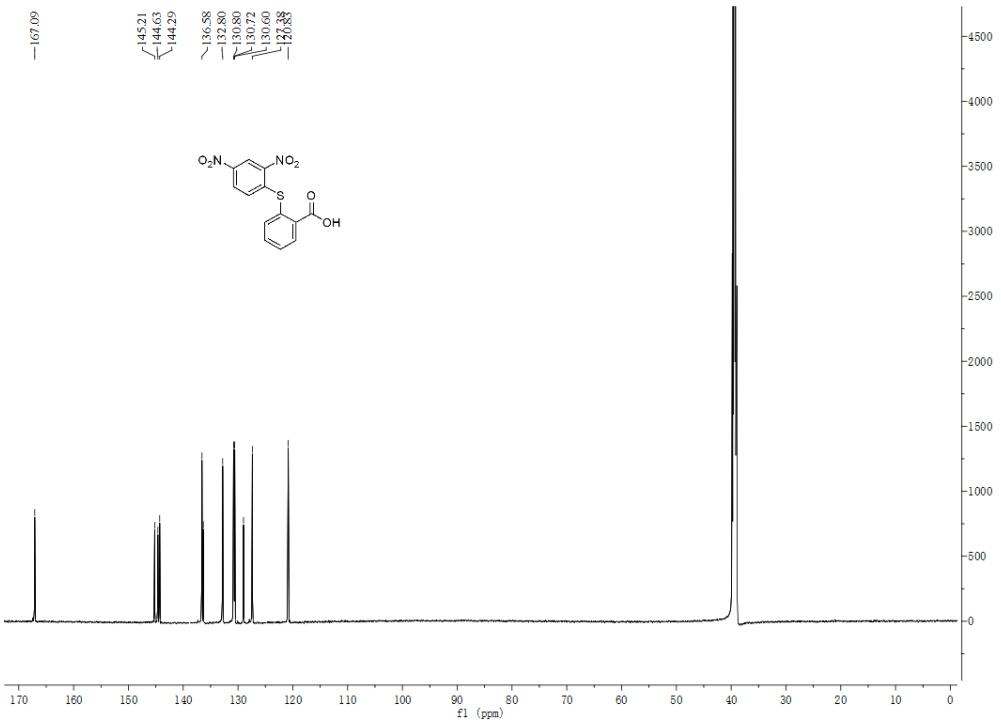


Figure S11. ^{13}C NMR spectrum of compound **3** (DMSO-d_6, 151 MHz).

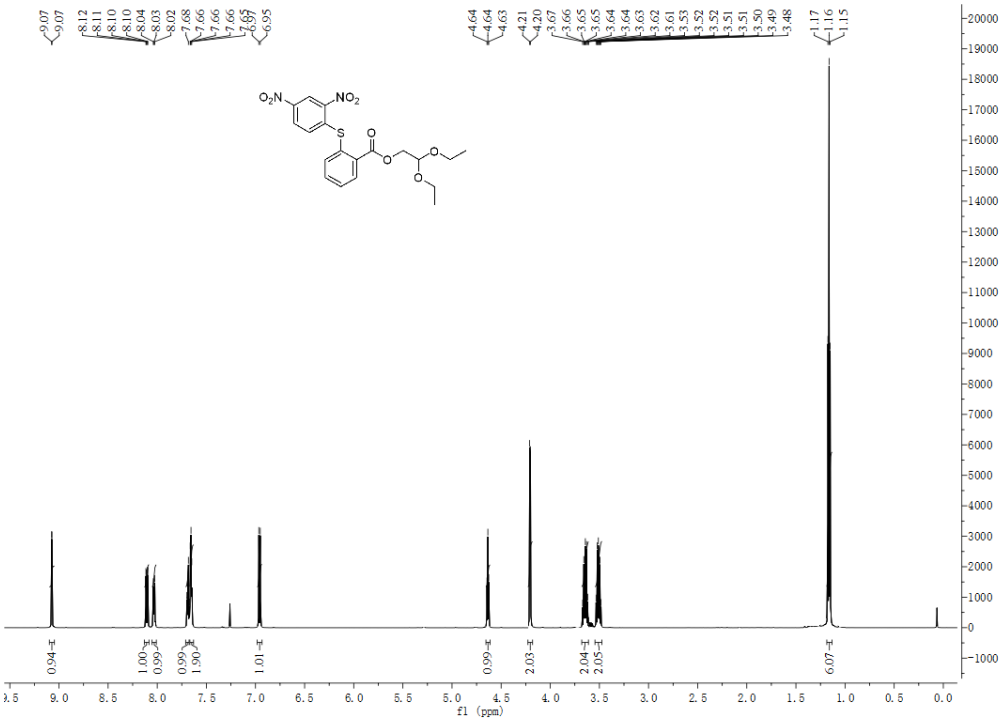


Figure S12. ^{1}H NMR spectrum of compound **4** ($CDCl_3$, 600 MHz).

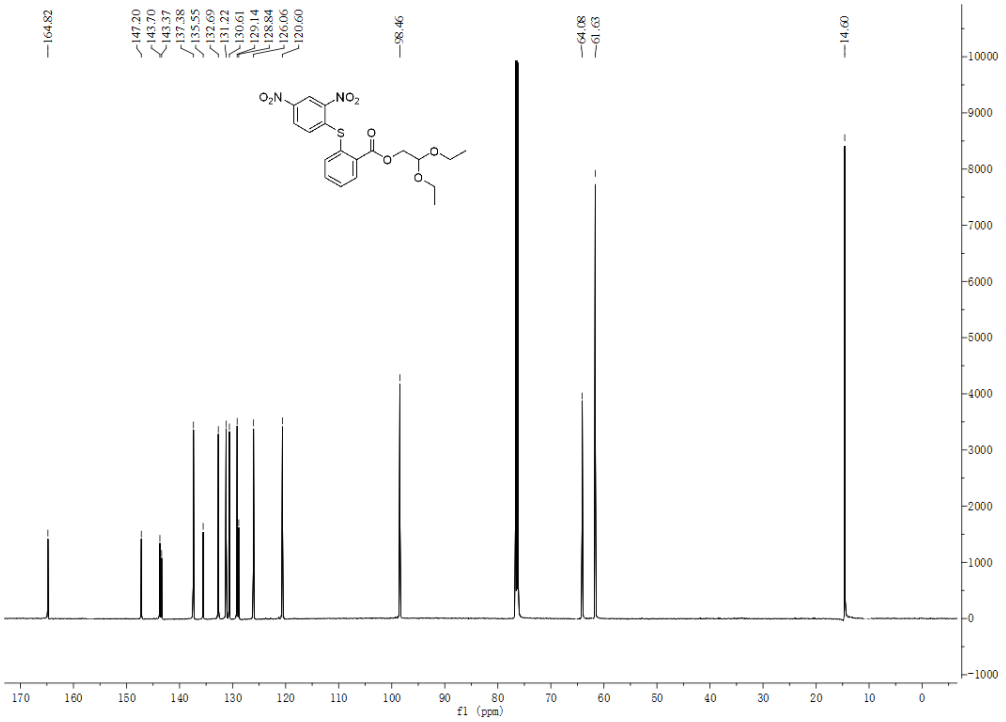


Figure S13. ^{13}C NMR spectrum of compound **4** ($CDCl_3$, 151 MHz).

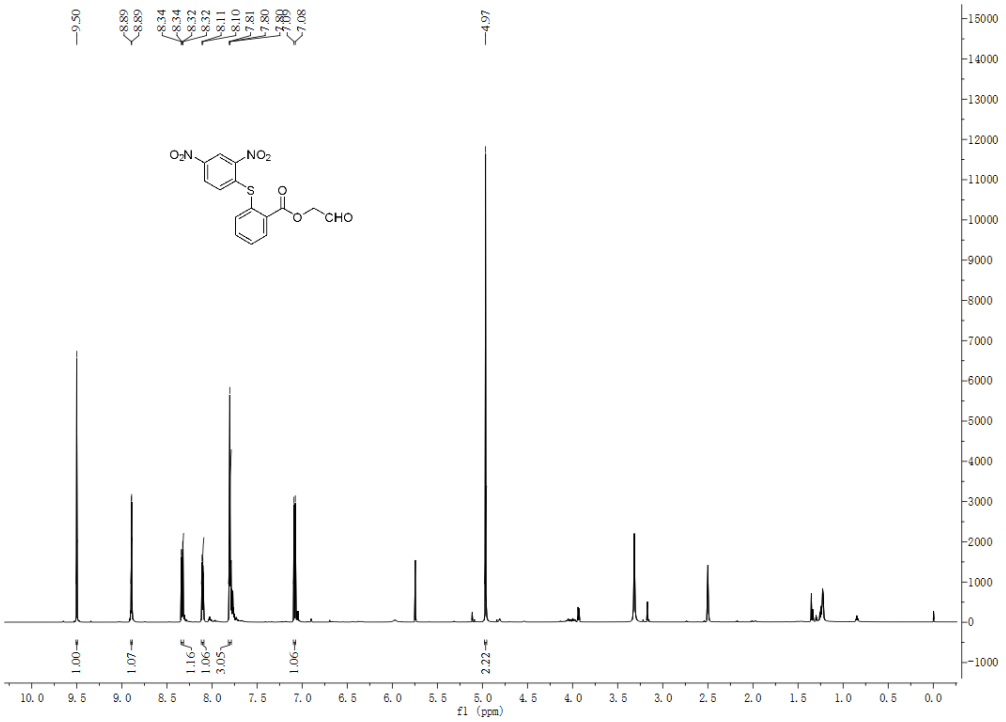


Figure S14. ^{1}H NMR spectrum of compound **5** (DMSO-d_6, 600 MHz).

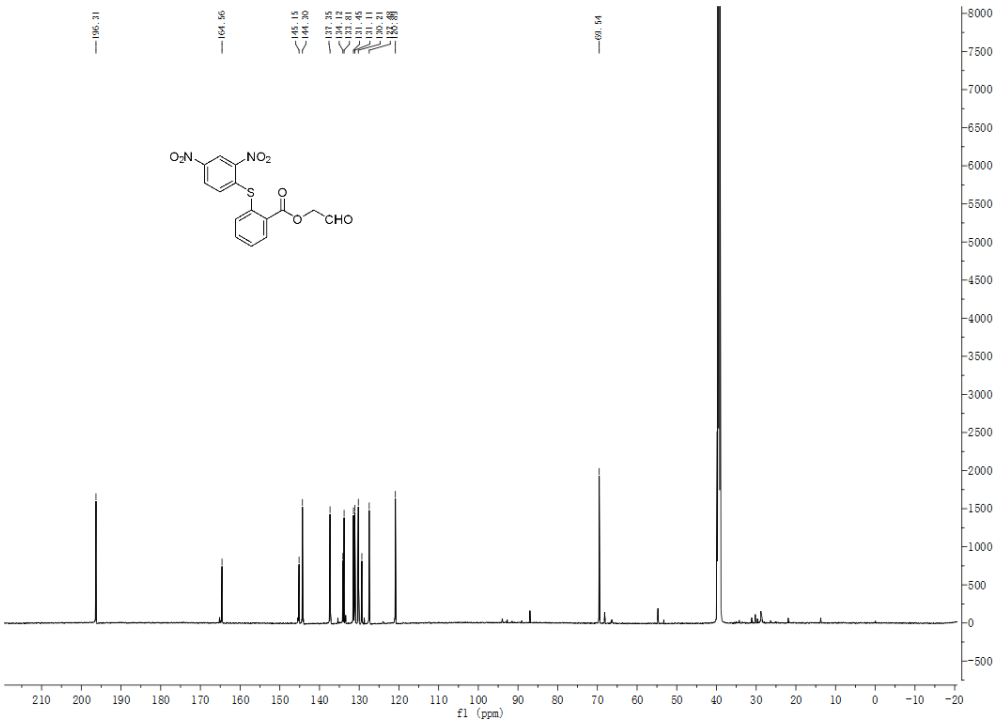


Figure S15. ^{13}C NMR spectrum of compound **5** (DMSO-d_6, 151 MHz).

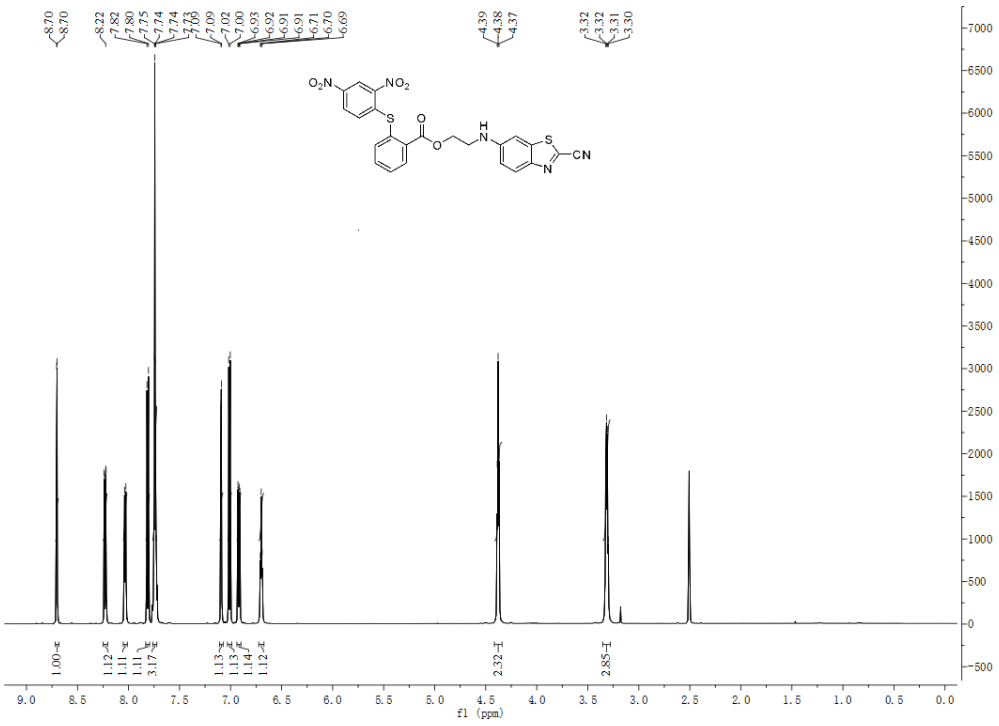


Figure S16. ^{1}H NMR spectrum of compound **6** (DMSO-d_6, 600 MHz).

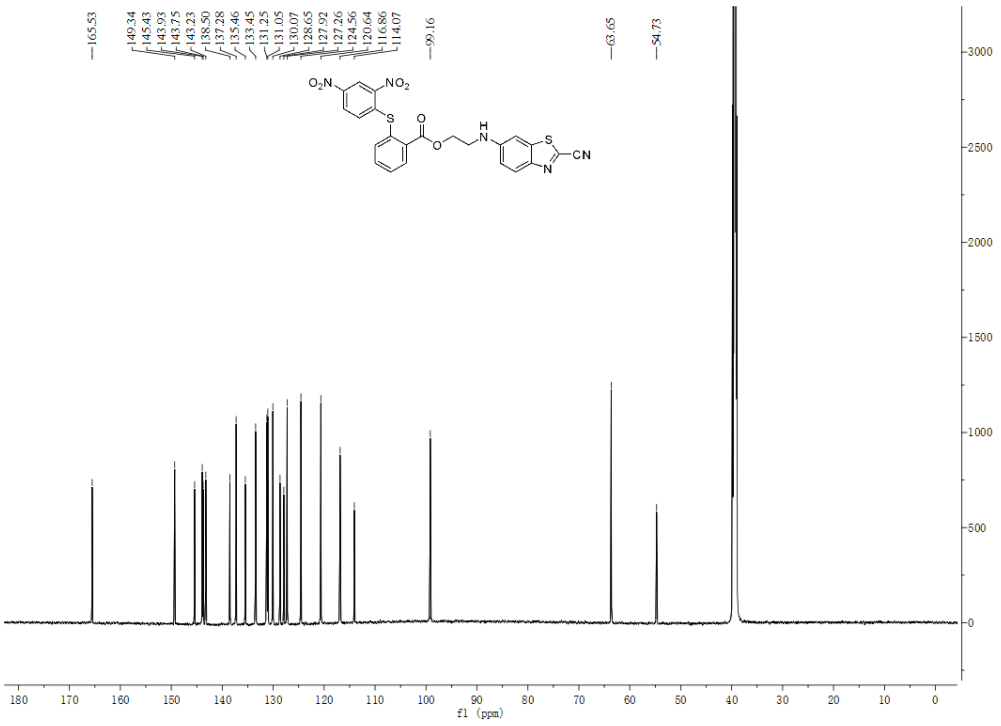


Figure S17. ^{13}C NMR spectrum of compound **6** (DMSO-d_6, 151 MHz).

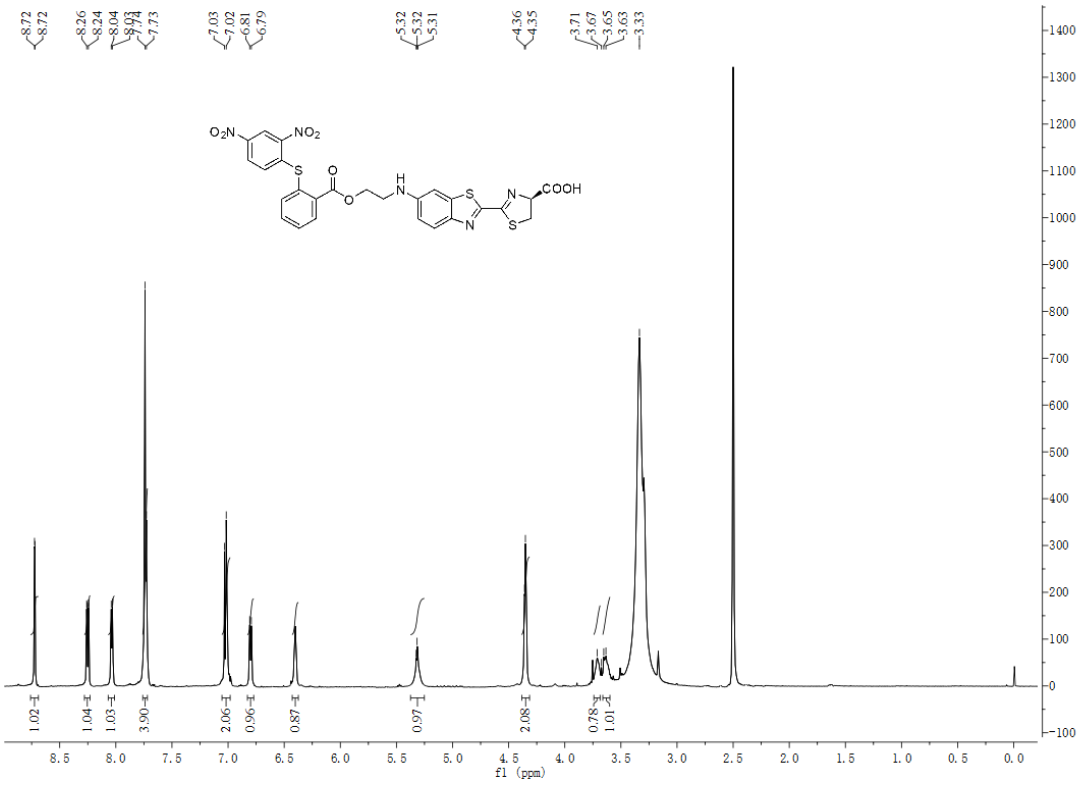


Figure S18. ^{1}H NMR spectrum of **DNPT-HS** (DMSO-d_6, 600 MHz).

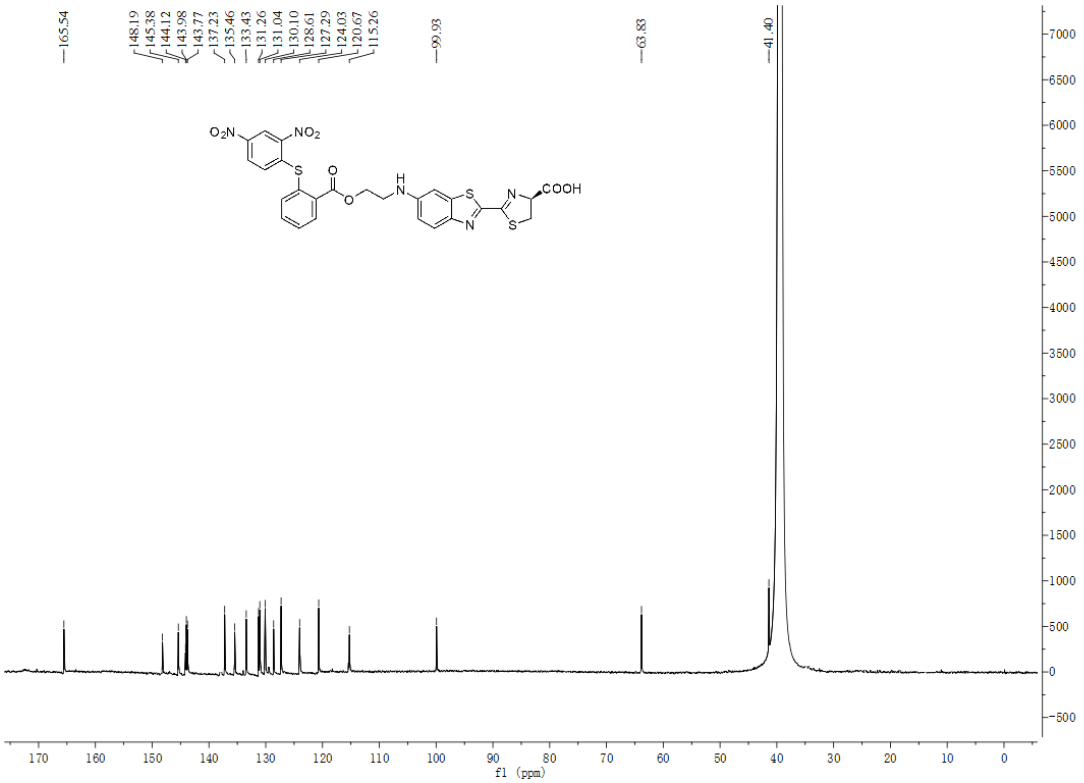


Figure S19. ^{13}C NMR spectrum of **DNPT-HS** (DMSO-d_6, 151 MHz).