Supporting Information

Two isophorone fluorophores-based design of ratiometric fluorescent probe and its application for sensing of biothiols

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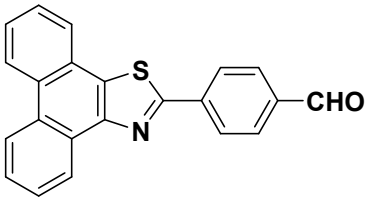
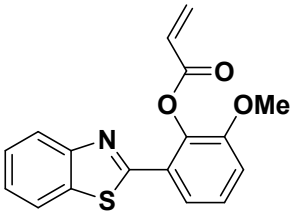
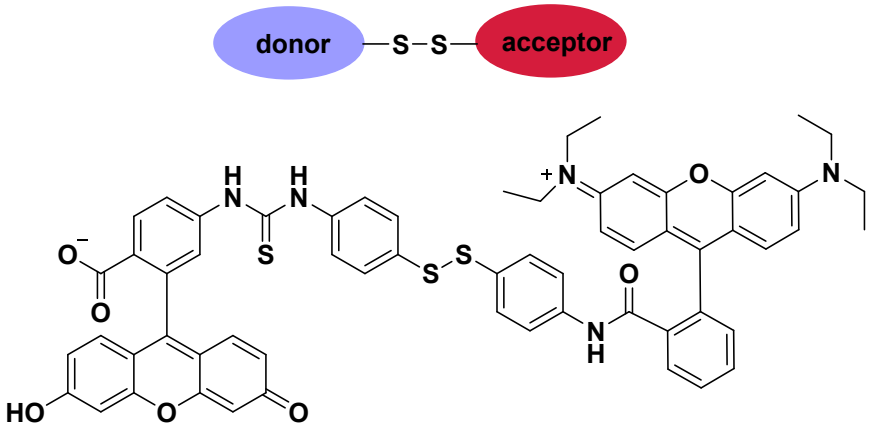


ICT based ratiometric probe
Org. Lett., 2008, 10, 5577-5580.



ESIPT based ratiometric probe
Angew. Chem. Int. Ed. 2011, 50, 10690-10693.



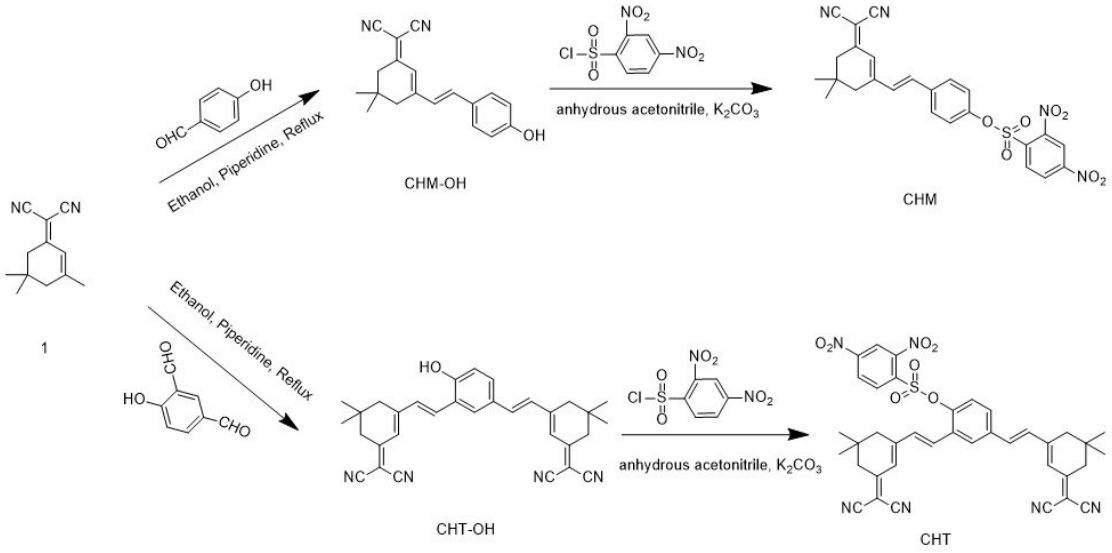
FRET based ratiometric probe
Ana. Biochem. 2006, 352, 265-273.

Scheme S1. Previously reported design of ratiometric fluorescent probes for biothiols.

1. Synthetic experiments

Materials and methods. All chemicals and reagents in the experiment were purchased from commercial sources and used without further purification unless specific notification. Anhydrous acetonitrile (CH_3CN), Absolute ethanol (EtOH), Piperidine, Potassium carbonate (K_2CO_3), 2,4-Dinitrobenzenesulfonyl chloride, 4-hydroxyisophthalaldehyde, N-Ethylmaleimide (NEM), Malononitrile, Isophorone ware purchased from Energy Chemical and Macklin Chemical. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and various of amino acids were purchased from Sigma-Aldrich. Thin layer chromatography (TLC) was performed on silica gel plates and visualized by UV. Column chromatography was performed using silica gel (Huanghai, Qingdao) 300-400 mesh. All pH measurements were carried out using a pH meter (Leici PHS-3E) which was calibrated with pH 4.00 and pH 6.86 buffers before use. ^1H and ^{13}C NMR spectra were recorded by using a Bruker AV-400 spectrometer with chemical shifts expressed in parts per million (Me_4Si as internal standard). ESI-High Resolution Time-of-Flight Mass Spectrometer.

Fluorescence measurements were determined on a Hitachi Fluorescence spectrometer F-7000. Excitation and emission slit widths were modified to adjust the fluorescence intensity to a suitable range. Absorption spectra were measured on a Hitachi U-3900 UV/Vis spectrometer.



Scheme S2. Synthesis of target probe **CHT** and control probe **CHM**.

Synthesis of compound 1. A solution of isophorone (2.05 mL, 13.7 mmol), malononitrile (1.36 g, 20.6 mmol), piperidine (0.45 mL, 4.5 mmol) were mixed in the absolute ethanol (25 mL). And the reaction mixture was refluxed at 65 ℃ for 18 h under a nitrogen atmosphere. After being cooled to room temperature, the reaction mixture was poured into ice water. The crude solid was collected and washed with ice water, afford the final product (yield, 65%). ^{1}H NMR (400 MHz, $CDCl_3$), δ 6.62 (s, 1H), 2.51 (s, 2H), 2.18 (s, 2H), 2.03 (s, 3H), 1.01 (s, 6H).

Synthesis of CHT-OH. 4-hydroxyisophthalaldehyde (0.300 g, 2 mmol), **compound 1** (0.744 g, 4 mmol), Piperidine (2 drop) were mixed in the absolute ethanol (20 mL), And the reaction mixture was heated at 60 ℃ under nitrogen protection about 1.5 h. The solvent was removed under reduced pressure and the product was purified by flash column chromatography (yield, 45%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.72 (s, 1H), 8.13(d, *J*=1.2 Hz, 1H), 7.53 (d, *J*=6.8 Hz, 1H), 7.45 (d, *J*=3.2 Hz, 2H), 7.29 (d, *J*=16.4 Hz, 2H), 6.95 (d, *J*=8.4 Hz, 1H), 6.86 (d, *J*=10.8 Hz, 2H), 2.61 (s, 4H), 2.53 (s, 4H), 1.02 (d, *J*=3.6 Hz, 12H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 191.47, 170.77, 161.83, 158.27, 156.89, 156.52, 138.28, 131.89, 129.70, 128.17, 127.55, 126.90, 123.63, 123.04, 122.18, 117.11, 114.50, 114.34, 113.80,

113.64, 76.58, 42.81, 40.63, 40.42, 40.21, 40.00, 39.79, 39.58, 39.37, 38.71, 38.60, 32.17, 32.15, 27.92, 27.87.

Synthesis of CHT. 2,4-Dinitrobenzenesulfonyl chloride (0.770 g, 0.29 mmol), **CHT-OH** (0.100 g, 0.21 mmol), K_2CO_3 (0.042 g, 0.30 mmol) were mixed in the anhydrous acetonitrile (8 mL), And the reaction mixture was heated at 50 ℃ under nitrogen protection about 0.5 h. The solvent was removed under reduced pressure and the product was purified by flash column chromatography (yield, 40%). ^{1}H NMR (400 MHz, DMSO-d_6), δ 9.18 (d, *J*=2.4 Hz, 1H), 8.61 (d, *J*=2 Hz, 1H), 8.24 (d, *J*=1.6 Hz, 2H), 7.76 (d, *J*=2 Hz, 1H), 7.53 (d, *J*=16 Hz, 1H), 7.38 (d, *J*=8.8 Hz, 1H), 7.33 (d, *J*=3.6 Hz, 1H), 7.29 (d, *J*=4 Hz, 1H), 7.02 (d, *J*=16.4 Hz, 1H), 6.94 (s, 1H), 6.87 (s, 1H), 2.50 (d, *J*=3.2 Hz, 8H), 1.01 (d, *J*=14.0 Hz, 12H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 170.70, 170.47, 155.52, 154.40, 152.10, 148.59, 146.90, 136.88, 135.73, 134.09, 133.60, 131.98, 131.19, 130.80, 130.47, 128.21, 128.00, 126.75, 124.84, 124.52, 124.13, 121.25, 114.09, 113.92, 113.40, 113.13, 78.69, 77.88, 42.75, 42.57, 38.70, 38.46, 32.16, 32.06, 27.89, 27.75. HRMS (ES+): calcd for $C_{38}H_{32}N_6O_7S$ $[M+Na]^+$ 739.2053, found 739.1953.

Synthesis of CHM-OH. 4-hydroxy-benzaldehyde (0.122 g, 1.00 mmol), **compound 1** (0.300 g, 1.61 mmol) and Piperidine (2 drop) were mixed in the absolute ethanol (20 mL). And the reaction mixture was heated at 70 ℃ under nitrogen protection about 2 h. Then, the solvent was evaporated under reduced pressure and the product was purified by recrystallization from ethanol, or by column chromatography (silica/dichloromethane), (yield, 89%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.43 (d, 2H), 7.26 (d, 2H), 6.80 (t, 3H), 2.59 (d, 2H), 2.46 (d, 2H), 1.08 (s, 6H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 170.75, 159.81, 138.76, 130.33, 129.46, 127.61, 126.74, 121.84, 116.36, 115.79, 114.59, 113.77, 75.28, 42.83, 40.66, 40.45, 40.25, 40.04, 39.83, 39.62, 39.41, 38.72, 32.13, 27.91.

Synthesis of CHM. 2,4-dinitrobenzenesulfonyl chloride (0.080 g, 0.28 mmol), **CHM-OH** (0.110 g, 0.41 mmol) and two drops of $(CH_3CH_2)_3N$ were mixed in the anhydrous acetonitrile (10 mL). And the reaction mixture was heated at 50 ℃ under nitrogen protection about 1 h. Then, the mixture was evaporated under reduced pressure and purified by flash column chromatography (hexane/EtOAc = 4 : 1) to give compound as a yellow solid, (yield, 76%); ^{1}H NMR (400 MHz, DMSO-d_6) d 8.69 (s, 1H), 8.52 (s, 1H), 8.26 (s, 1H), 7.54 (d, 2H), 7.28 (d, 2H), 6.97 (d, 2H), 6.88 (s, 1H), 2.63 (d, 2H), 2.47 (d, 2H), 1.10 (s, 6H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 170.71,

155.66, 151.99, 149.18, 148.59, 136.61, 135.87, 134.06, 131.57, 130.10, 127.94, 123.99, 122.93, 121.57, 114.14, 113.35, 77.66, 42.76, 40.66, 40.45, 40.24, 40.03, 39.82, 39.62, 39.41, 38.64, 32.14, 27.88. HRMS (ES+): calcd for $C_{25}H_{20}N_4O_7S$ $[M]^+$ 520.1053, found 520.1003.

2. Spectral experiment

Preparation of the test solution.

A stock solution of probe **CHT** (10 μM) and **CHM** (10 μM) ware prepared in THF. Milli-Q Water was used to prepare all aqueous solutions. The test solution is potassium phosphate buffer (0.01 M, pH 7.4) containing 50% THF (PBS-THF buffer). The solutions of various testing species were prepared from cysteine (Cys), homocysteine (Hcy), glutathione (GSH), glycine (Gly), L-Proline (Pro), aspartic acid (Asp), L-tyrosine (Tyr), L-arginine (Arg), L-glutamic acid (Glu), L-alanine (Ala), L-Threonine (Thr), phenylalanine (Phe), DL-serine (Ser), L-methionine (Met), and glutamine (Gln) respectively. The resulting solution was shaken well and incubated for 30 min at room temperature before recording the spectra.

Generation of various ROS and RNS

$ONOO^-$

Simultaneously, 0.6 M KNO_2, 0.6 M in HC1, 0.7 M in H_2O_2 were added at to a 3 M NaOH solution at 0 ºC. The concentration of peroxynitrite was estimated by using extinction co-efficient of 1670 $cm^{-1}M^{-1}$ at 302 nm in 0.1 M sodium hydroxide aqueous solutions.

^-OCl

The concentration of ^-OCl was determined from the absorption at 292 nm (Ɛ = 350 $M^{-1}cm^{-1}$).

H_2O_2

The concentration of H_2O_2 was determined from the absorption at 240 nm (Ɛ = 43.6 $M^{-1}cm^{-1}$).

HNO

Angeli's salt as the precursor of HNO. Angeli's salt ($Na_2N_2O_3$, AS) was purchased from Cayman Chemical.

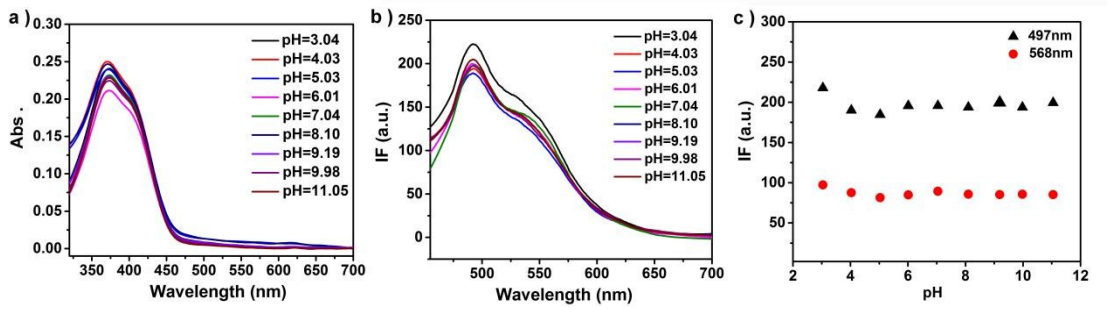


Figure S1. a) Absorption spectra of target probe **CHT** (10 μM) at different pH values in water. b) Emission spectra of **CHT** (10 μM) at different pH in water (λ_{ex}=425 nm). c) pH effect on the fluorescence intensity of **CHT** at 497 nm (in black) and 568 nm (in red) in water (λ_{ex} = 425 nm). The resulting solution was shaken well and incubated for 30 min at room temperature before recording the spectra.

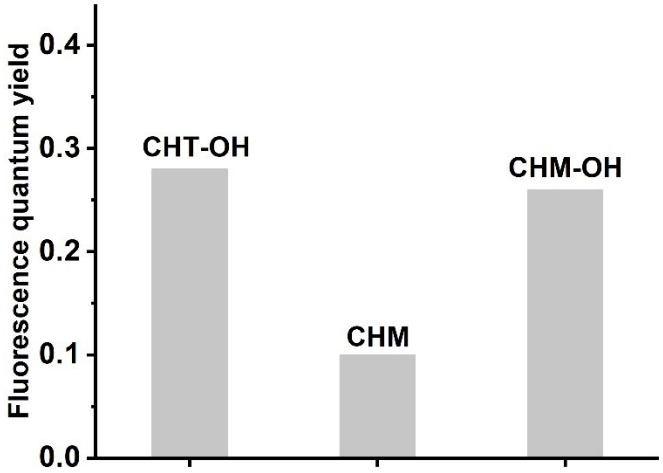


Figure S2. Fluorescence quantum yield (Φ_F) of **CHT-OH**, **CHM** and **CHM-OH** in PBS/DMSO (0.01 M, pH 7.4, v/v, 1/1). Fluorescein in 0.1M NaOH (Φ_F =0.95) as the standard for the assay.

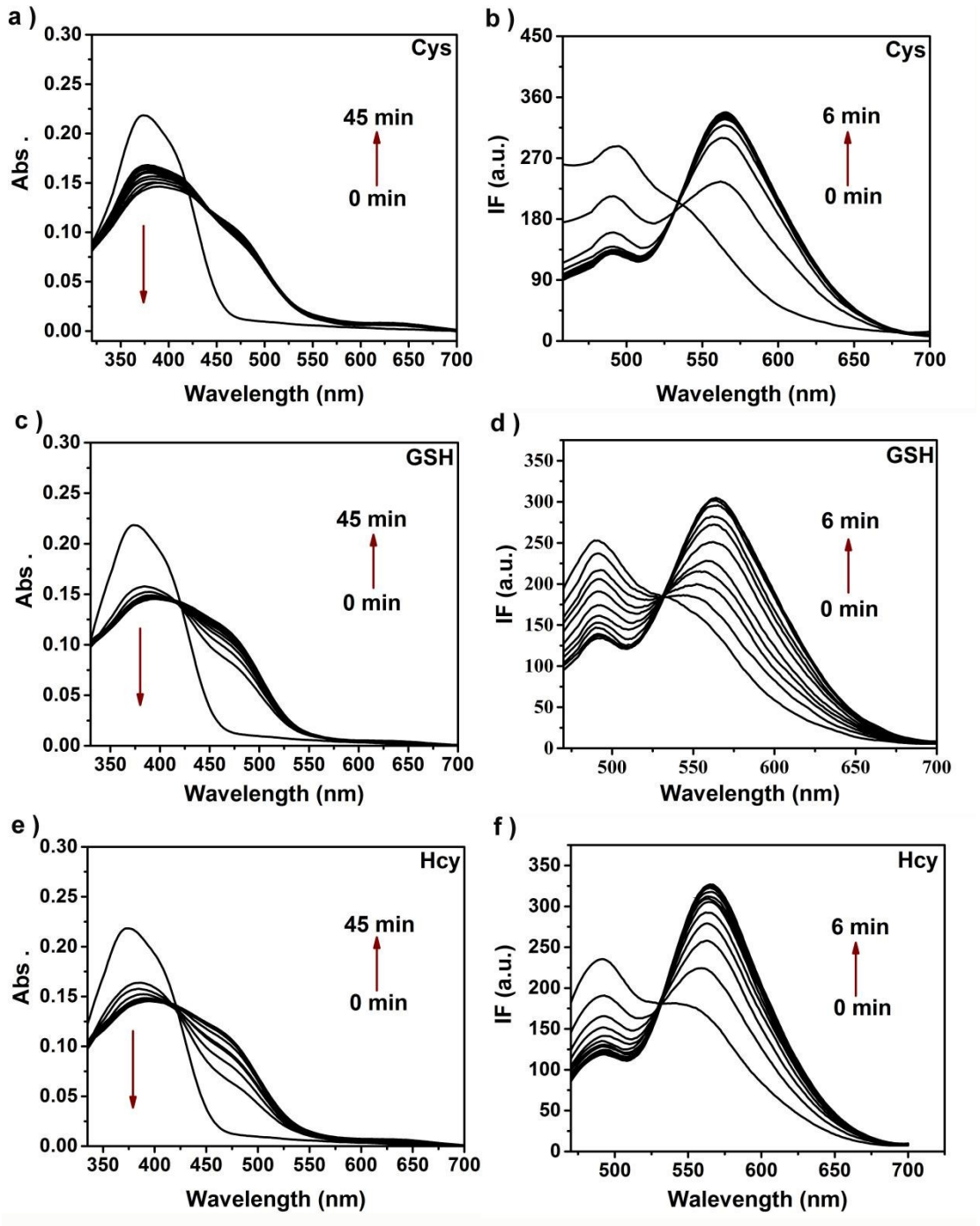


Figure S3. Time-dependent absorption and fluorescence changes of target probe **CHT** (10 μM) towards (a) (b) Cys (500 μM), (c) (d) Hcy (500 μM) and (e) (f) GSH (500 μM) in PBS/THF solution (0.01 M, pH 7.4, v/v, 1/1) at 25 ℃. (λ_{ex} = 425 nm).

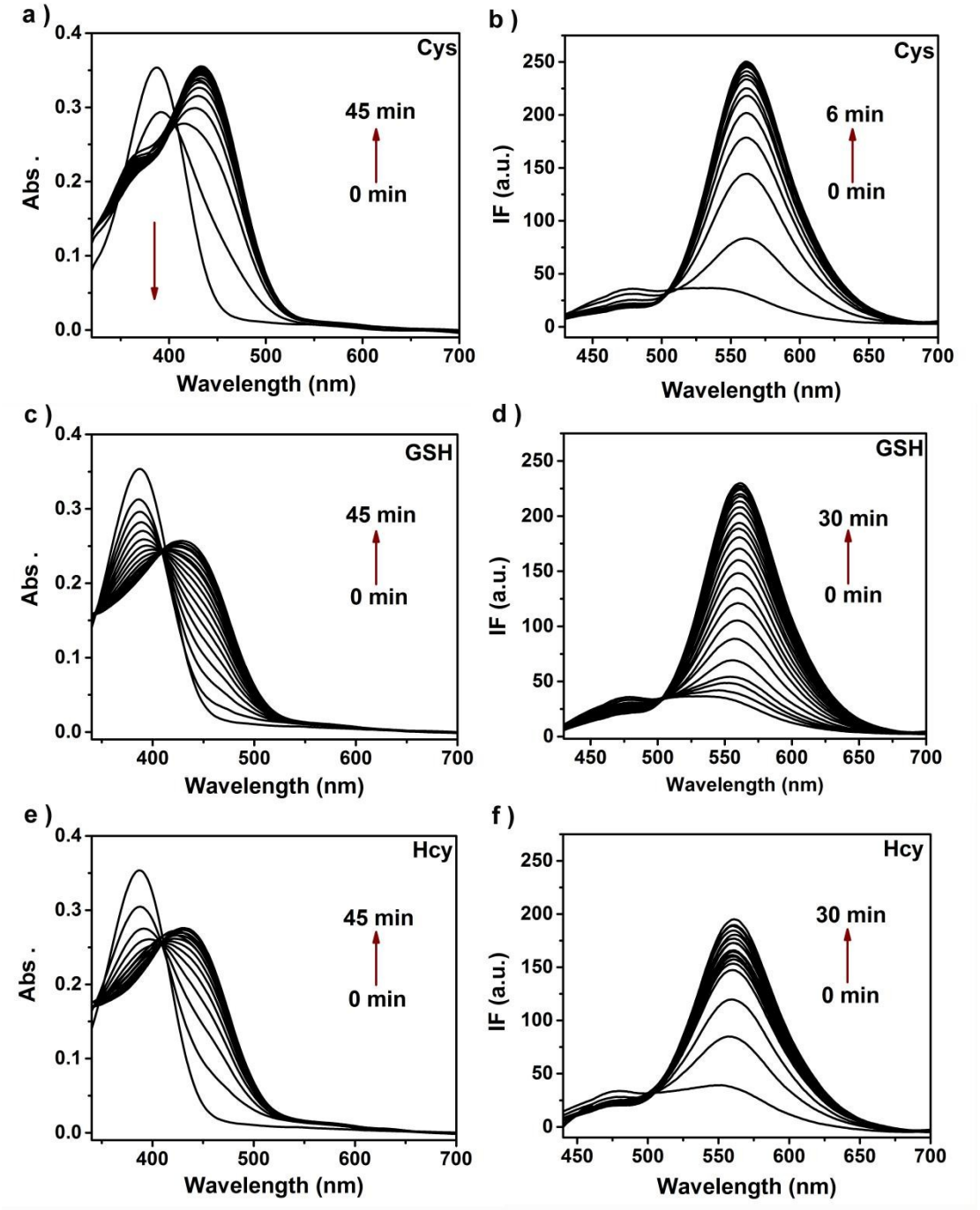


Figure S4. Time-dependent absorption and fluorescence changes of the control probe **CHM** (10 μM) towards (a) (b) Cys (500 μM), (c) (d) GSH (500 μM) and (e) (f) Hcy (500 μM) in PBS/THF solution (0.01 M, pH 7.4, v/v, 1/1) at 25 ℃. (λ_{ex} = 410 nm).

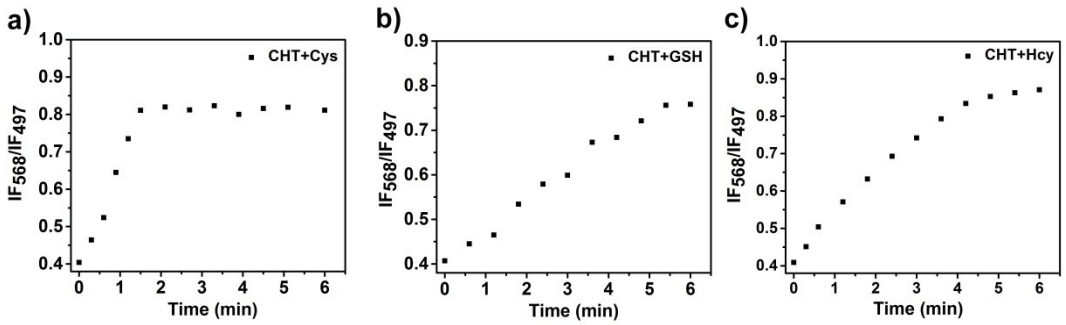
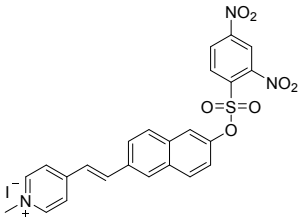
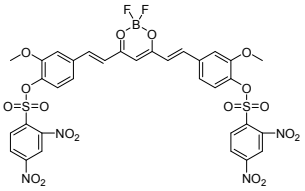
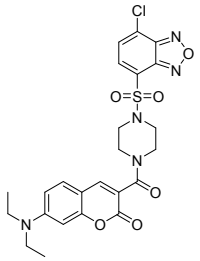
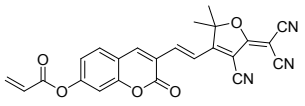
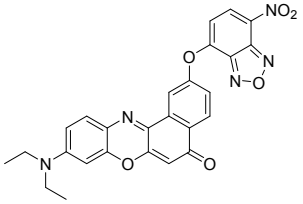
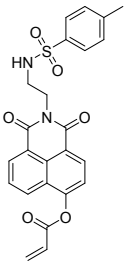
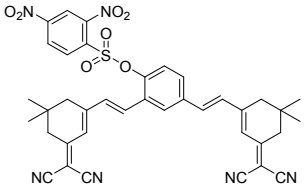


Figure S5. A plot of the fluorescence intensity changes of **CHT** (10 μM) (based on the peak heights of the maxima emission at 568 nm and 497 nm, respectively) depending on time, in the presence of (a) 50 μM Cys, (b) 50 μM GSH, (c) 50 μM Hcy, λ_{ex} = 425 nm, the tests were conducted in PBS/THF solution (0.01 M, pH 7.4) at 25 ℃.

Table S1. Comparison of the response time between CHT and other reported probes for cysteine

Probe	Time dependence response of target probe for cysteine	Literature
	2 min	*Analyst*, 2019, 144, 439.
	120 min	*J. Mater. Chem. B*, 2018, 6, 5248.
	70 min	*Anal. Chem*. 2017, 89, 9567−9573.
	10 min	*Dyes and Pigments*. 146 (2017) 103-111.

	20 min	*Analyst*, 2019, 144, 3676.
	20 min	*Anal. Chem*. 2019, 91, 5513−5516.
	90 s	This work

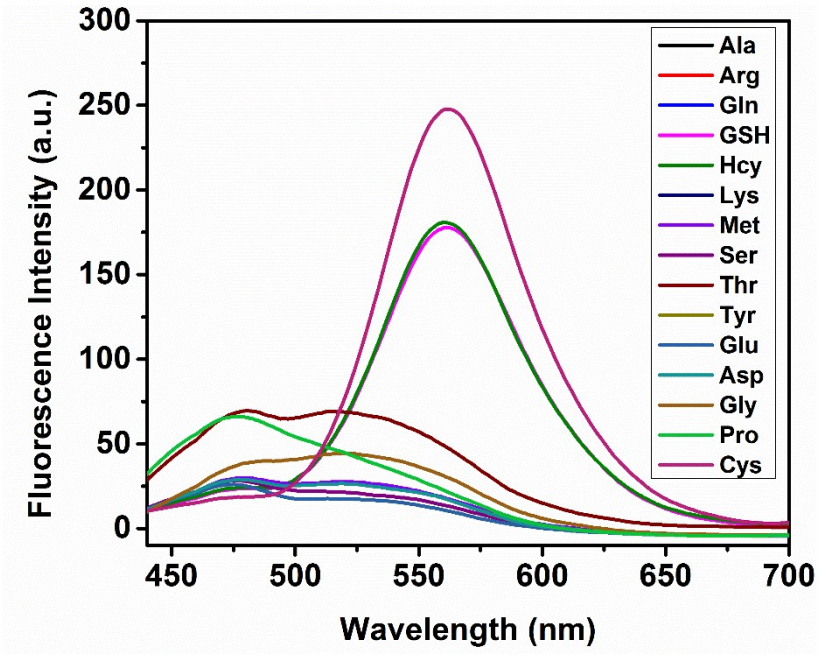


Figure S6. Changes in fluorescence emission of **CHM** (10 μM) with addition of Cys, Hcy, GSH (500 μM) and other amino acids after 30 min in PBS/THF solution (0.01 M, pH 7.4, v/v, 1/1) at 25 ℃, (λ_{ex} = 410 nm). The resulting solution was shaken well and incubated for 30 min at room temperature before recording the spectra.

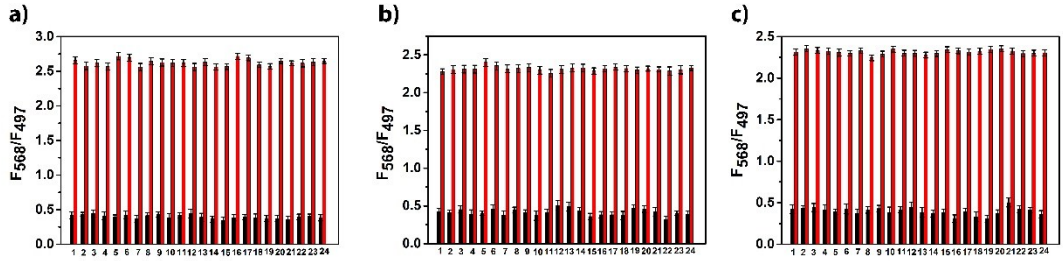


Figure S7. Fluorescence responses of **CHT** (10 μM) toward various relevant species (50 μM for RONS, 500 μM for others) without (black bar) and with (red bar) the presence of 500 μM Cys (a), 500 μM GSH (b) and 500 μM Hcy (c) in PBS/THF solution (0.01 M, pH 7.4, v/v,1/1) at 25 ^{o}C. The magnitudes of the error bars were calculated from the uncertainty given by three independent measurements. 1. Blank, 2. Ala, 3. Arg, 4. Asp, 5. Gln, 6. Glu, 7. Gly, 8. Met, 9. Phe, 10. Pro, 11. Ser, 12. Thr, 13. Tyr, 14. Na^{+} , 15. Ca^{2+}, 16. Zn^{2+}, 17. Fe^{3+}, 18. Ba^{2+}, 19. Cu^{2+}, 20. K^{+}, 21. H_2O_2, 22. HClO, 23. HNO, 24. $ONOO^{-}$. The resulting solution was shaken well and incubated for 30 min at room temperature before recording the spectra.

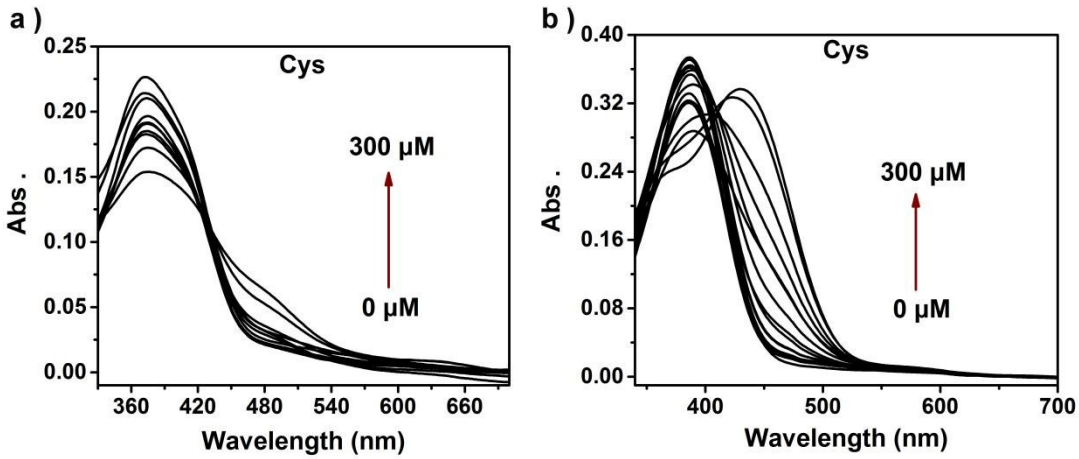


Figure S8. (a) (b) are the absorption spectral changes of **CHT** (10 μM) and **CHM** (10 μM) with addition of Cys (0-300 μM) in PBS/THF solution (0.01 M, pH 7.4, v/v, 1/1) at 25 ℃. The resulting solution was shaken well and incubated for 30 min at room temperature before recording the spectra.

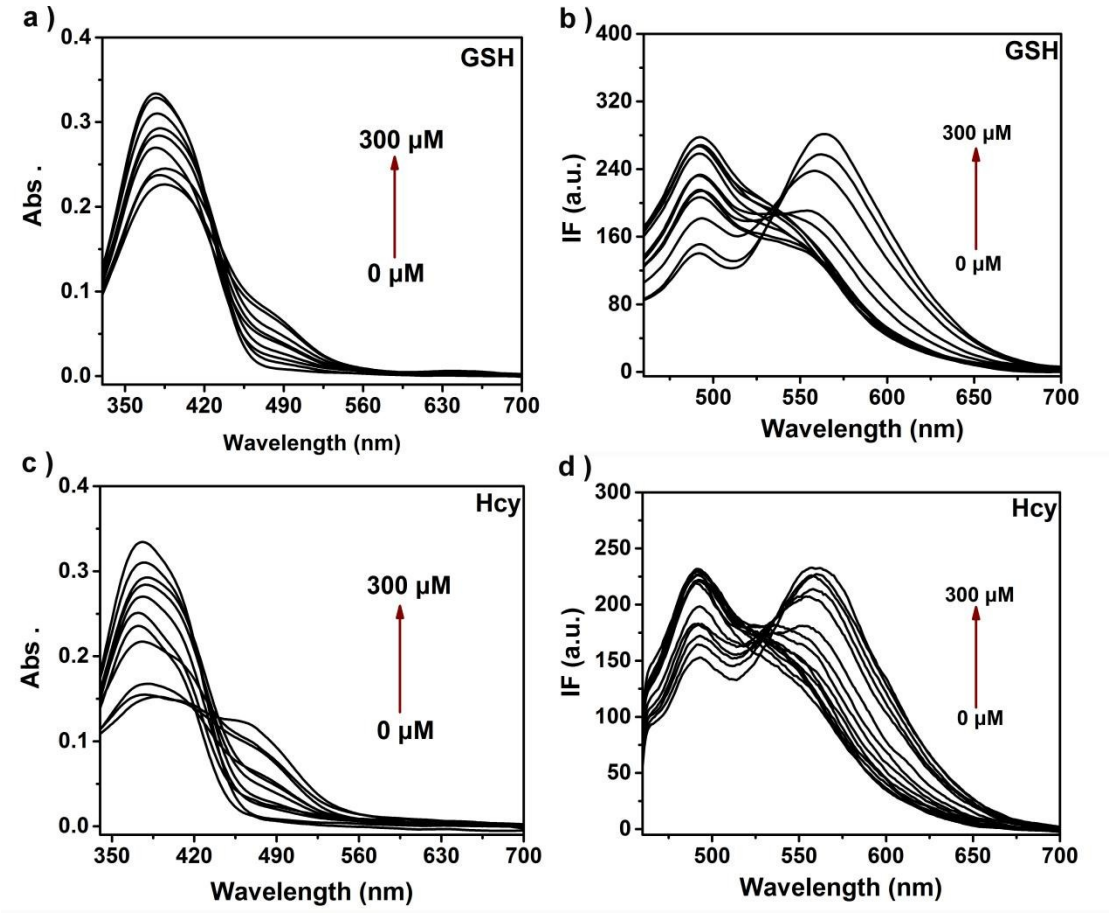


Figure S9. (a) (b) (c) (d) are the absorption and fluorescence spectrum of **CHT** (10 μM) with addition of GSH (0-300 μM) and Hcy (0-300 μM), respectively. All the tests were in PBS/THF solution (0.01 M, pH 7.4, v/v, 1/1) at 25 ℃, (λ_{ex} = 425 nm). The resulting solution was shaken well and incubated for 30 min at room temperature before recording the spectra.

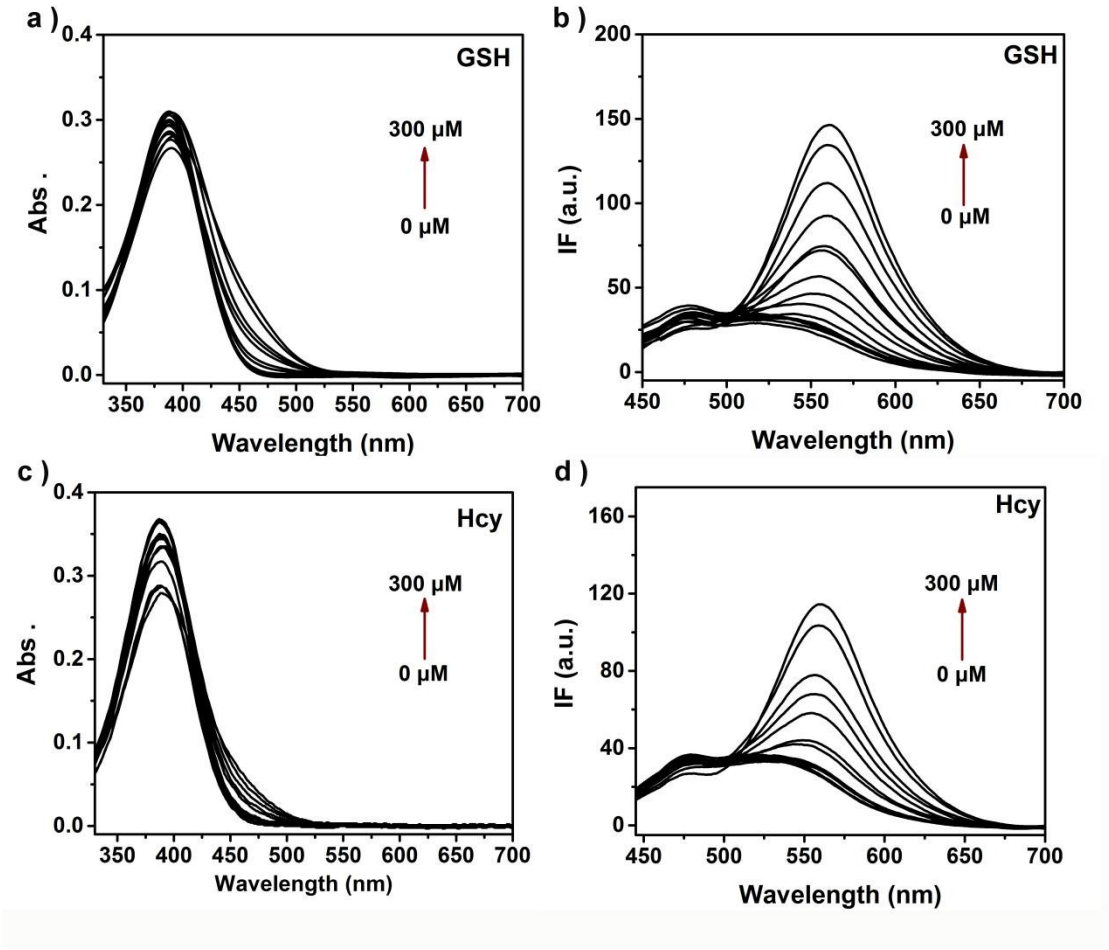


Figure S10. (a) (b) (c) (d) are the absorption and fluorescence spectrum of **CHM** (10 μM) with addition of GSH (0-300 μM) and Hcy (0-300 μM), respectively. All the tests were in PBS/THF solution (0.01 M, pH 7.4, v/v, 1/1) at 25 ℃, (λ_{ex} = 410 nm). The resulting solution was shaken well and incubated for 30 min at room temperature before recording the spectra.

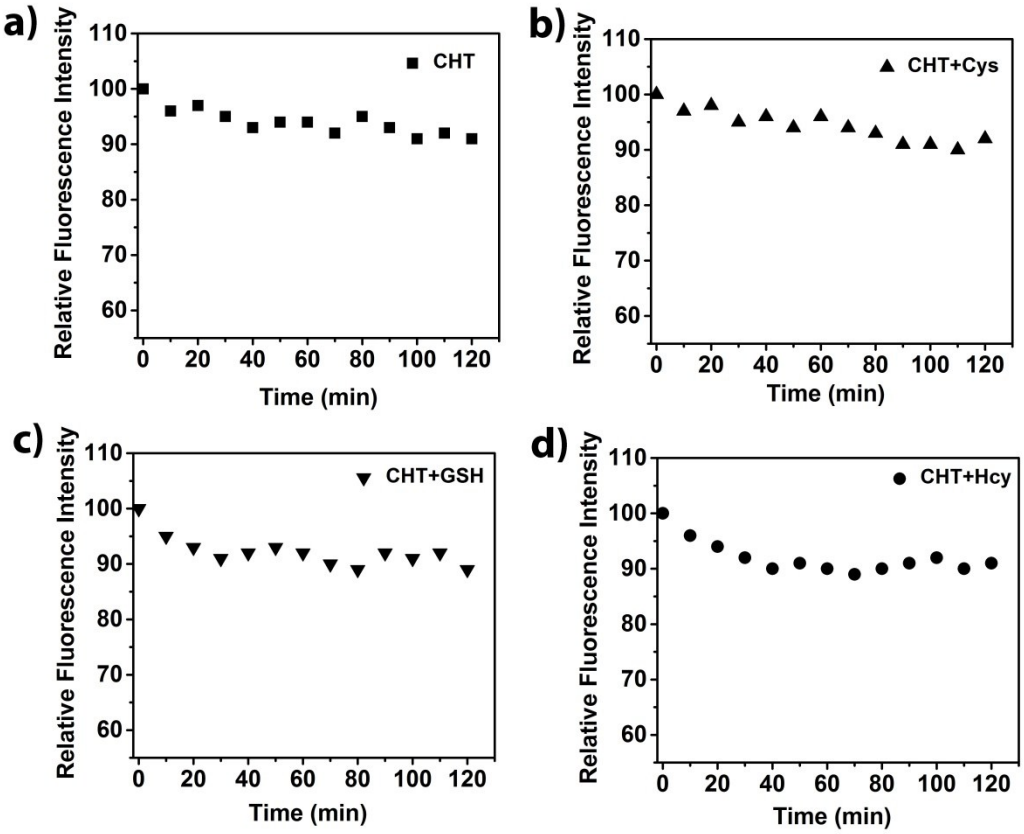


Figure S11. Photostability of **CHT** (a), **CHT**+Cys (b), **CHT**+GSH (c), **CHT**+Hcy (d). Relative fluorescence intensity of 10 μM compounds in THF/PBS buffer plotted as exposure time. The fluorescent intensity was obtained at different time during the continuous irradiation with 100 W soft white bulb, respectively. The fluorescent intensity was obtained with Hitachi Fluorescence Spectrophotometer F-7000 under excitation at 425 nm.

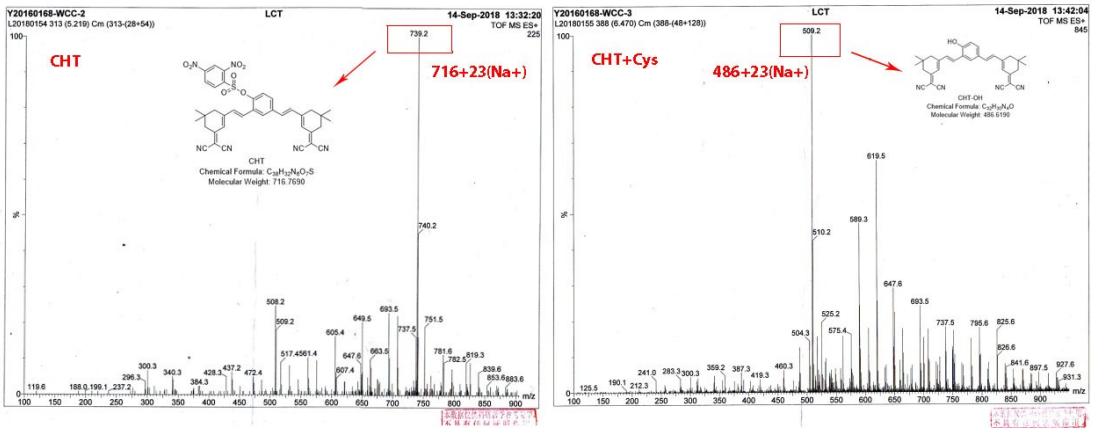


Figure S12. Mass spectra of **CHT** and **CHT** reacted with Cys.

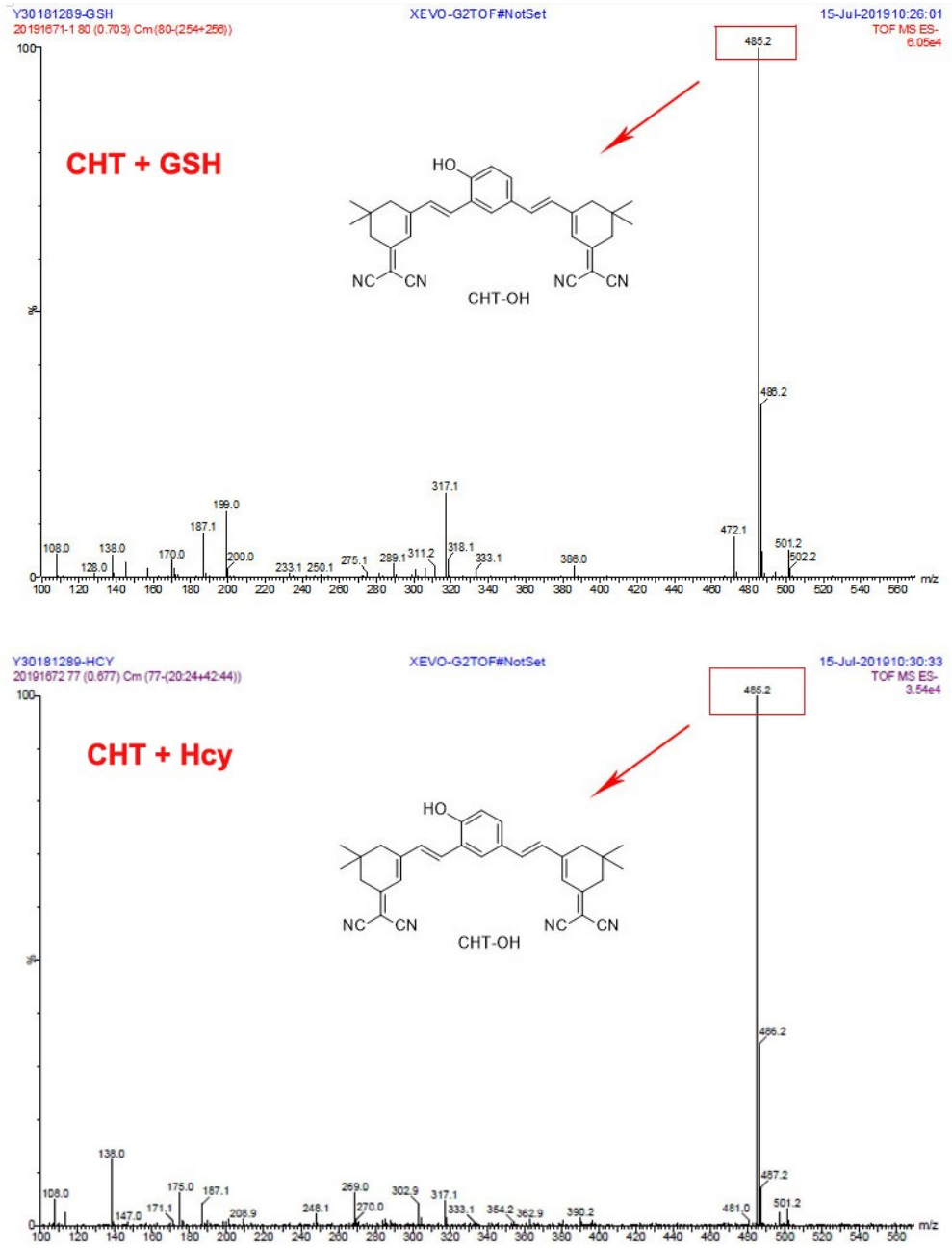


Figure S13. Mass spectra of **CHT** reacted with GSH and Hcy, respectively.

3. Calculation of limit of detection

The limit of detection (LOD) was calculated according to IUPAC and ACS method.

$$LOD = \frac{3\sigma}{s} \qquad \text{formula 1}$$

In this formula, 3 represents confidence level, σ is the standard deviation of blank

samples, and s is defined as the sensitivity of analysis (the slope of linear equation). σ are afforded through calculating the standard deviations of 11 times of independent fluorescence intensity ratios (F_{568}/F_{497}) of target probe **CHT** (10 μM) without adding biothiols in PBS/THF solution (0.01 M, pH 7.4, v/v, 1/1) at 25 ℃, the value is 0.004609. As shown in the Figure. 4b, the relationships between fluorescence intensity ratios (F_{568}/F_{497}) of target probe **CHT** (10 μM) and concentrations of Cys (0-200 μM) meets the liner equation (y = 0.41678 + 0.00837 x), so the slope of this liner equation is 0.00837. Then put the generated values into above formula, and the LOD was calculated to be about 1.65 μM. For the control probe **CHM,** σ are afforded through calculating the standard deviations of 11 times of independent fluorescence intensity (F_{562}) of **CHM** (10 μM) without adding biothiols in PBS/THF solution (0.01 M, pH 7.4, v/v, 1/1) at 25 ℃, the value is 0.763278, as shown in the Figure. 4d, the relationships between fluorescence intensity (F_{562}) of control probe **CHM** (10 μM) and concentrations of Cys (0-140 μM) meets the liner equation (y = 19.30610 + 0.99343 x), so s is the slope of this liner equation, that is 0.99343, according to the above formula, the LOD was calculated to be about 2.30 μM.

4. Cytotoxicity assay

Cell culture

CHO-K1 were obtained from American Type Culture collection, and grown in F-12 (High glucose) supplemented with 10% FBS. Cells were incubated in a 5% CO_2 humidified incubator at 37 ^{o}C and typically passaged with sub-cultivation ratio of 1:4 every two days.

Preparation of the stock solution of CHT for cytotoxicity and live cell imaging

The stock solutions (1 mM) of probe **CHT** and control probe **CHM** for assay in live cell assay were prepared by dissolving the solid compound **CHT** (**CHM**) in DMSO and stored under argon at -20 ^{o}C. Both of tubes of the stock solution were covered with aluminum foil to protect from light. Then the stock solution was diluted to the final working concentration during the assay with fresh FBS-free medium.

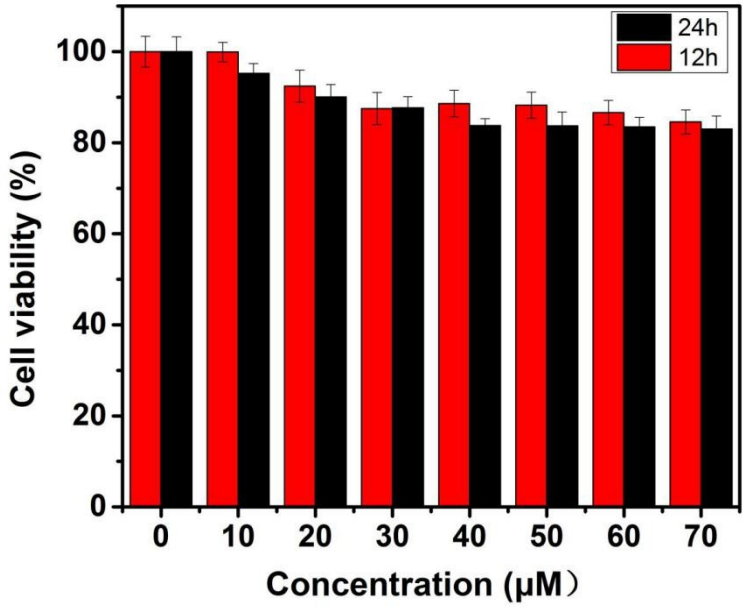


Figure S14. Cell viability (%) estimated by MTT assay versus incubation concentrations of **CHT**. CHO-K1 cells were incubated with **CHT** (0-70 μM) at 37 °C for 12 h and 24 h. The values are the mean ± s.d. for n = 3.

5. Fluorescence imaging in live cells.

We performed a fluorescence imaging experiments of probe **CHT** on ratio-type living cells (CHO-K1 cells) thiol-containing amino acid GSH and Hcy. CHO-K1 cells were incubated with probe in a confocal dish. After 10 minutes, GSH (200 μM) and Hcy (200 μM) were separately added and incubated for 20 minutes, then the cells were washed three times with PBS (0.01 M), and 2 mL of PBS (0.01 M) was loaded for imaging. The green channel (channel 1:450- 510 nm) was found to be dark. The red channel (channel 2: 550-700 nm) lights up.

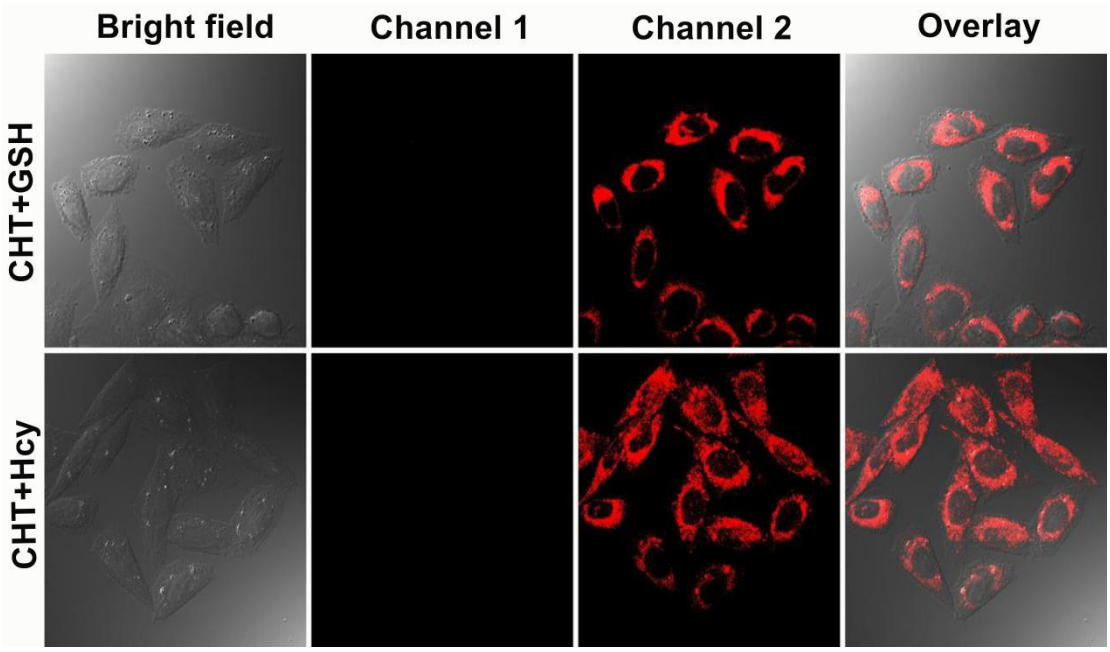


Figure S15. (**CHT**+GSH): Confocal image of CHO-K1 pretreated with **CHT** (50 μM) for 10 min, and then incubated with GSH (200 μM) for 20 min. (CHT+Hcy): Confocal image of CHO-K1 pretreated with **CHT** (50 μM) for 10 min, and then incubated with Hcy (200 μM) for 20 min.

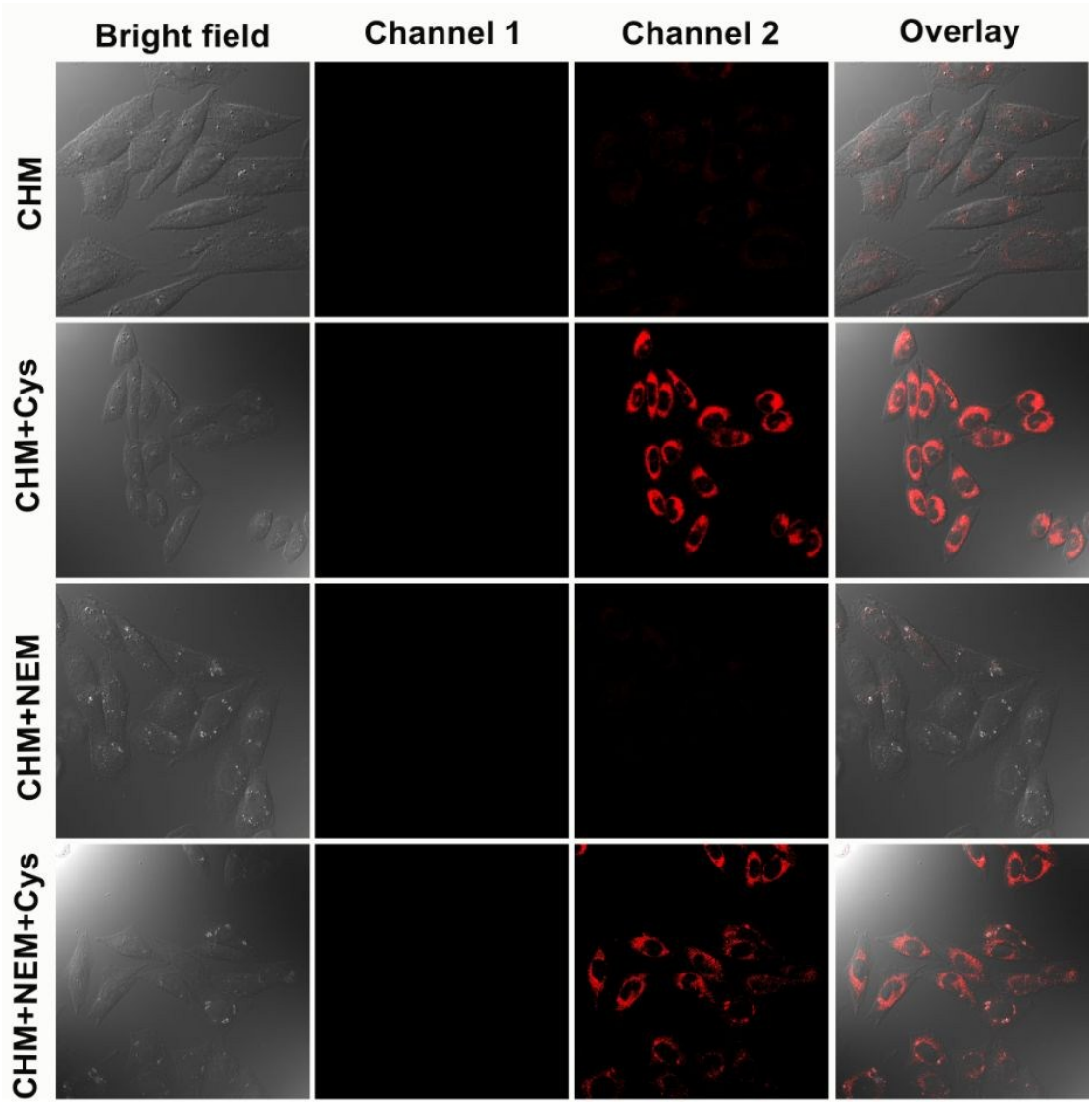


Figure S16. Fluorescence imaging of CHO-K1 cells with control probe **CHM** (50 μM). Excitation wavelength for **CHM**: 405 nm; emission collection, channel 1 (green): 415-510 nm; Channel 2 (red): 550-700 nm. (**CHM**): Confocal image of CHO-K1 cells pre-treated with **CHM** (50 μM) for 30 min; (**CHM**+Cys): Confocal image of CHO-K1 cells pre-treated with **CHT** (50 μM) for 10 min, and then incubated with Cys (200 μM) for 20 min. (**CHM**+NEM): Confocal image of CHO-K1cells pre-treated with NEM (200 μM) for 30 min, and then incubated with **CHM** (50 μM) for 30 min; (**CHM**+NEM+Cys): Confocal image of CHO-K1 cells pre-treated with NEM (200 μM) for 30 min, and then incubated with **CHM** (50 μM) for 10 min, and then incubated with Cys (200 μM) for 20 min.

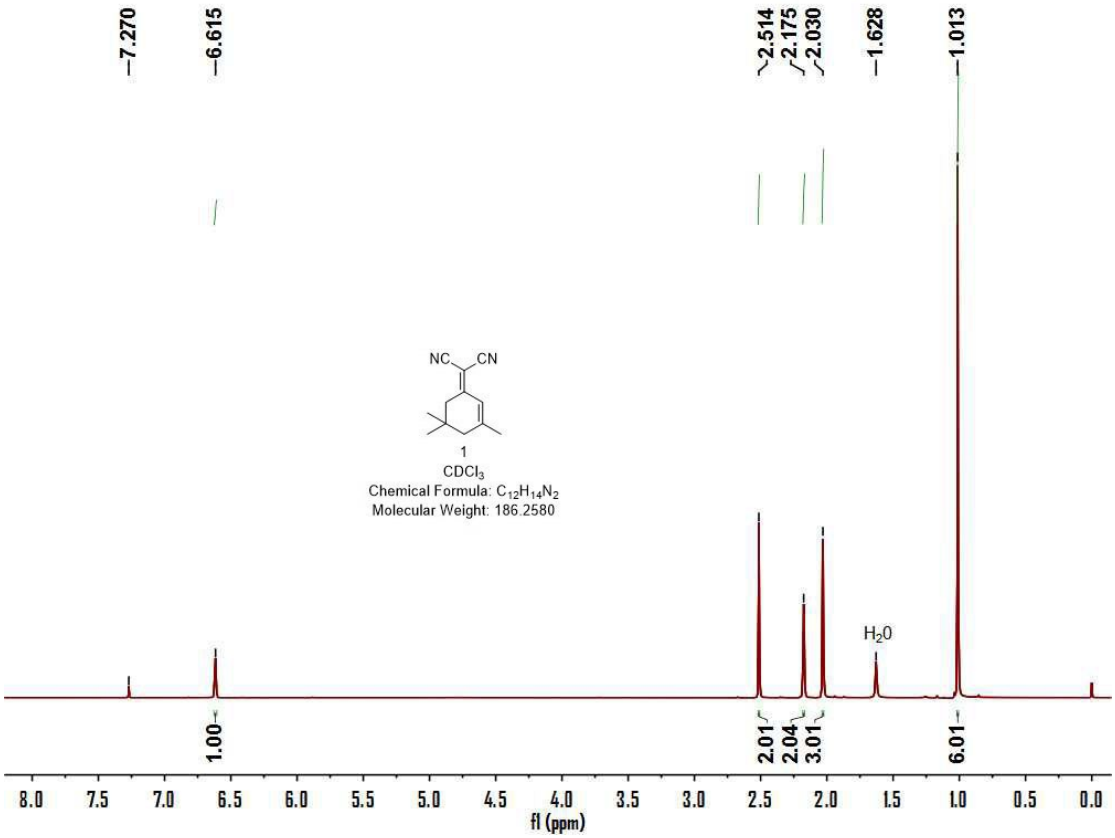


Figure S17. [1]H NMR spectra of **compound 1** in $CDCl_3$.

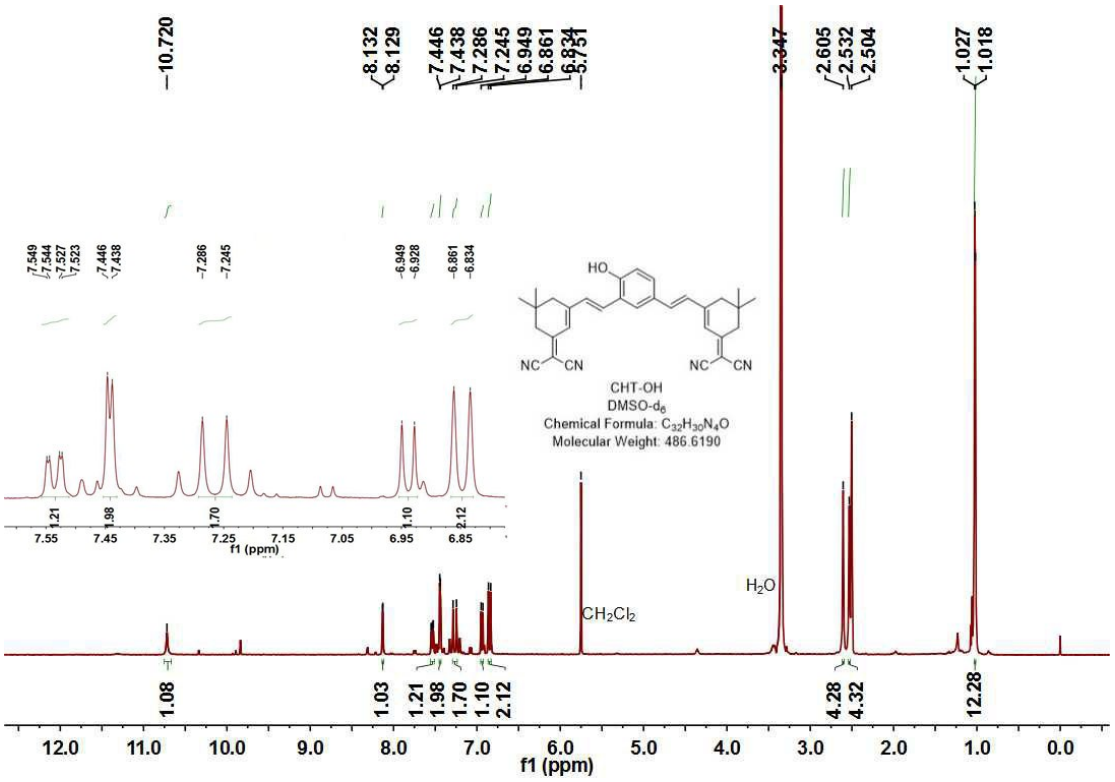


Figure S18. [1]H NMR spectra of **CHT-OH** in DMSO-d_6.

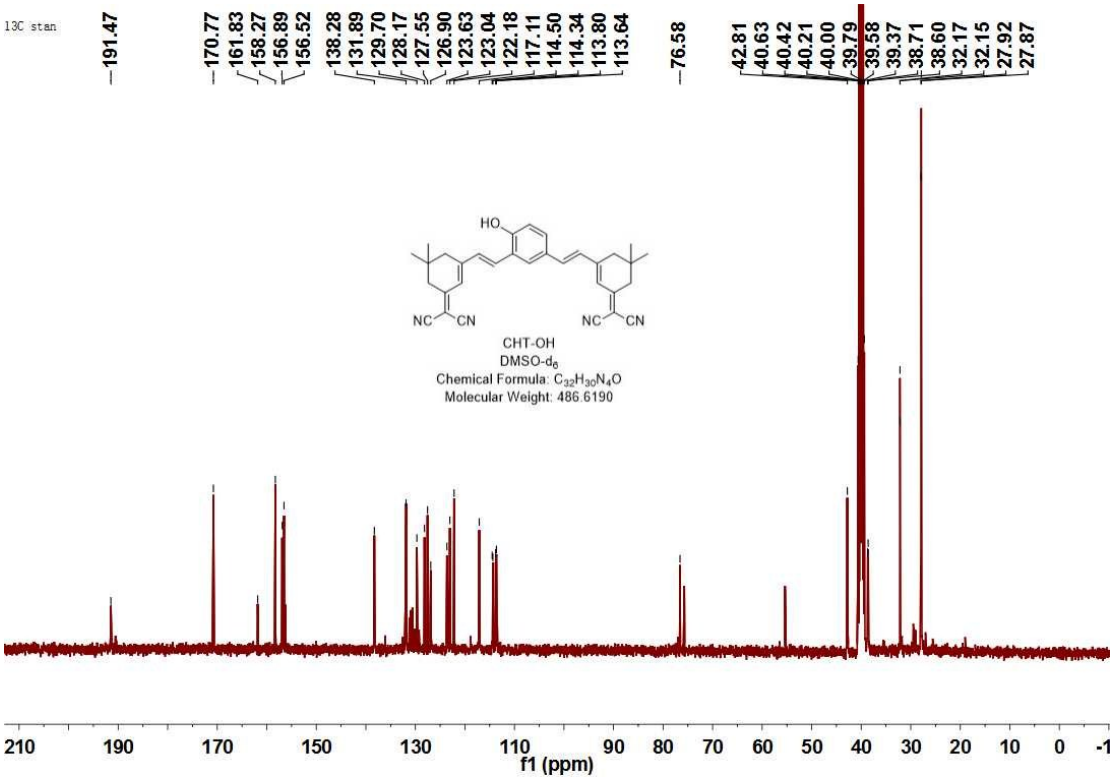


Figure S19. ^{13}C NMR spectra of **CHT-OH** in DMSO-d_6.

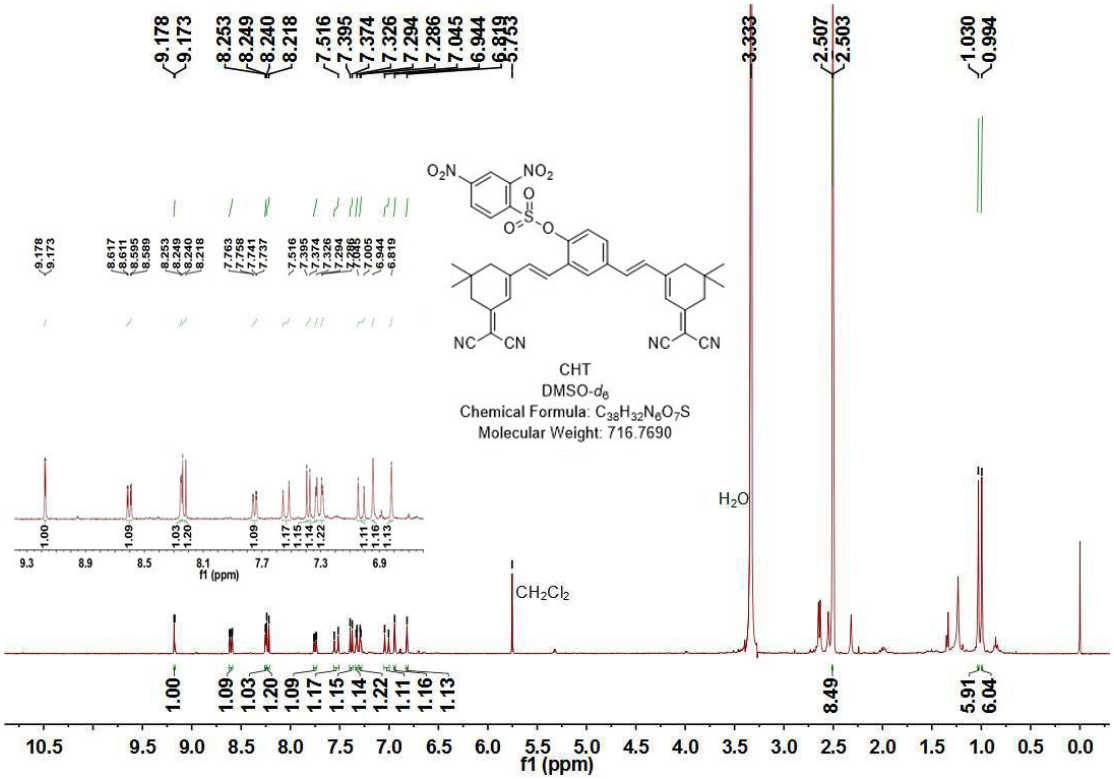


Figure S20. ^{1}H NMR spectra of **CHT** in DMSO-d_6.

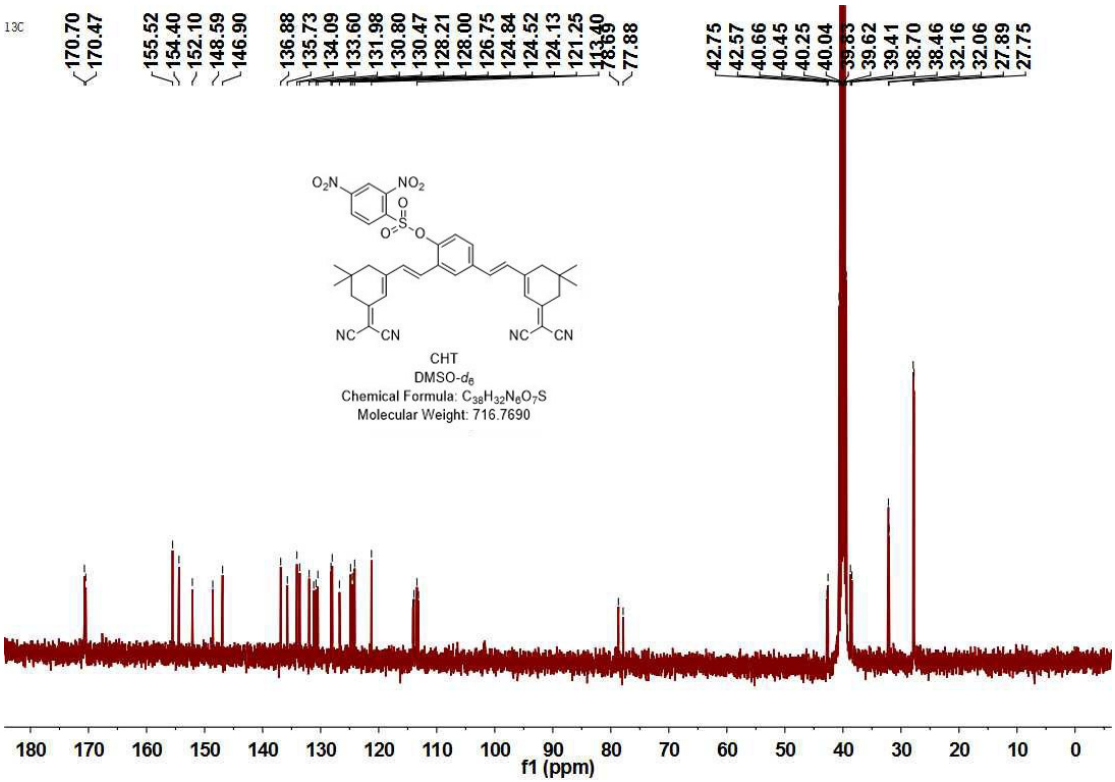


Figure S21. ^{13}C NMR spectra of **CHT** in DMSO-d_6.

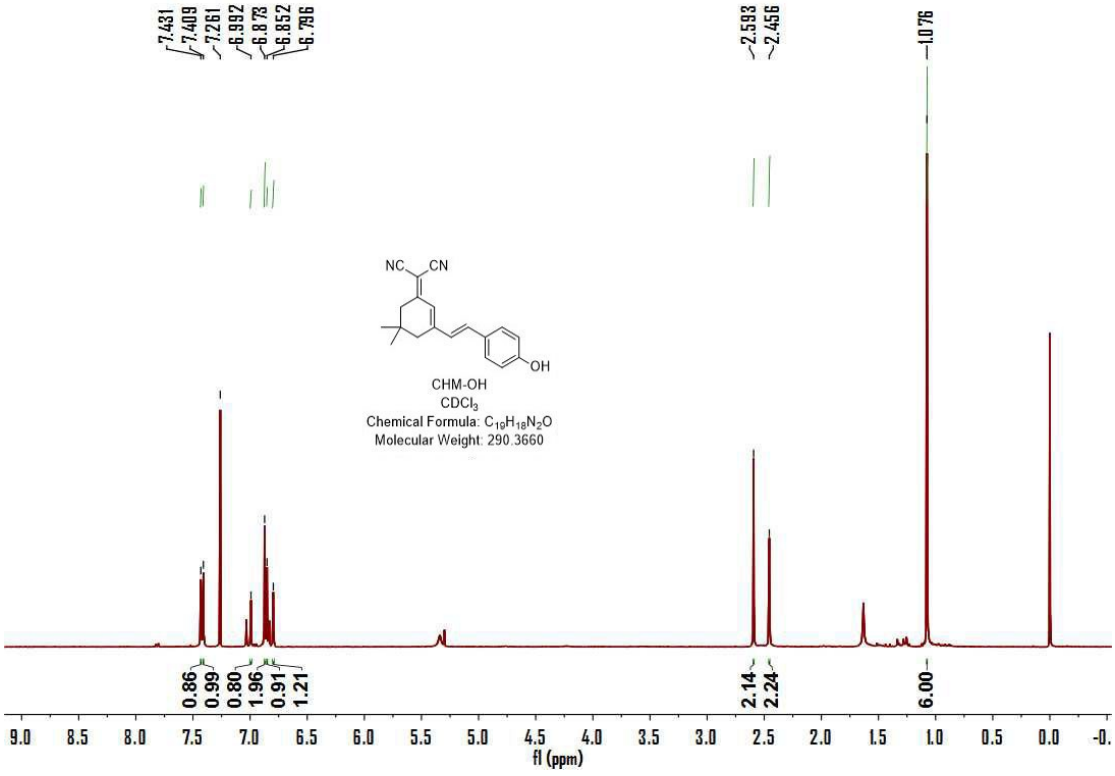


Figure S22. ^{1}H NMR spectra of **CHM-OH** in $CDCl_3$.

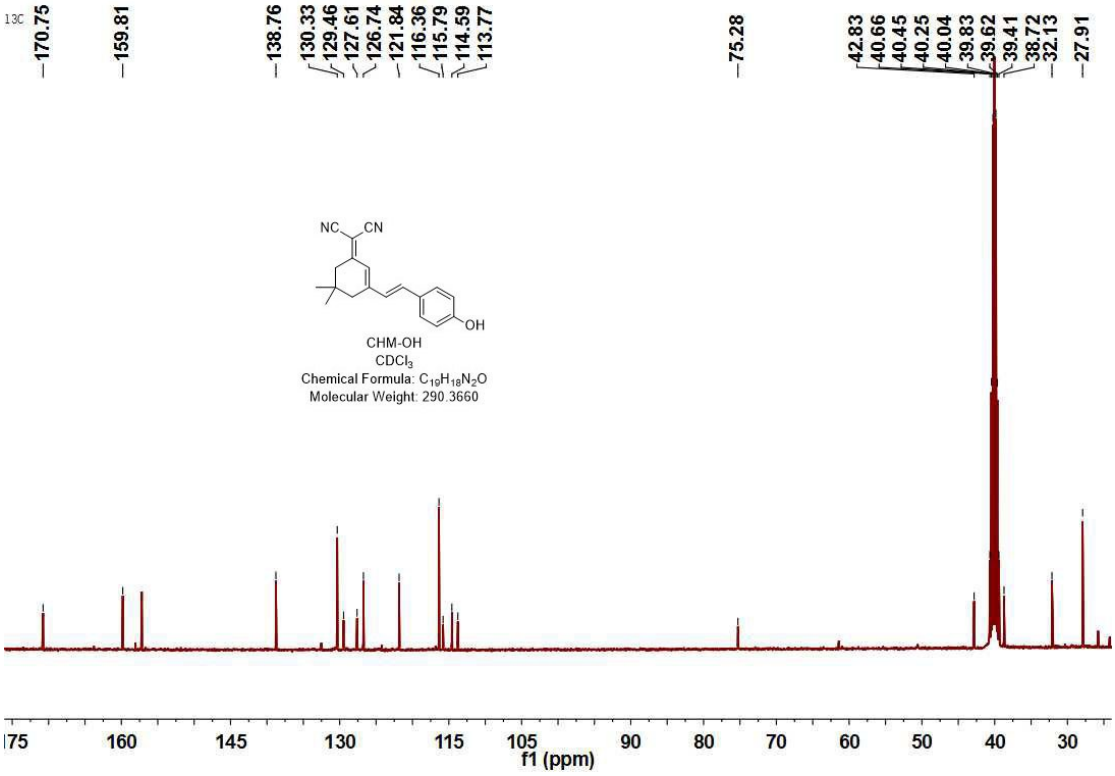


Figure S23. ^{13}C NMR spectra of **CHM-OH** in DMSO-d_6.

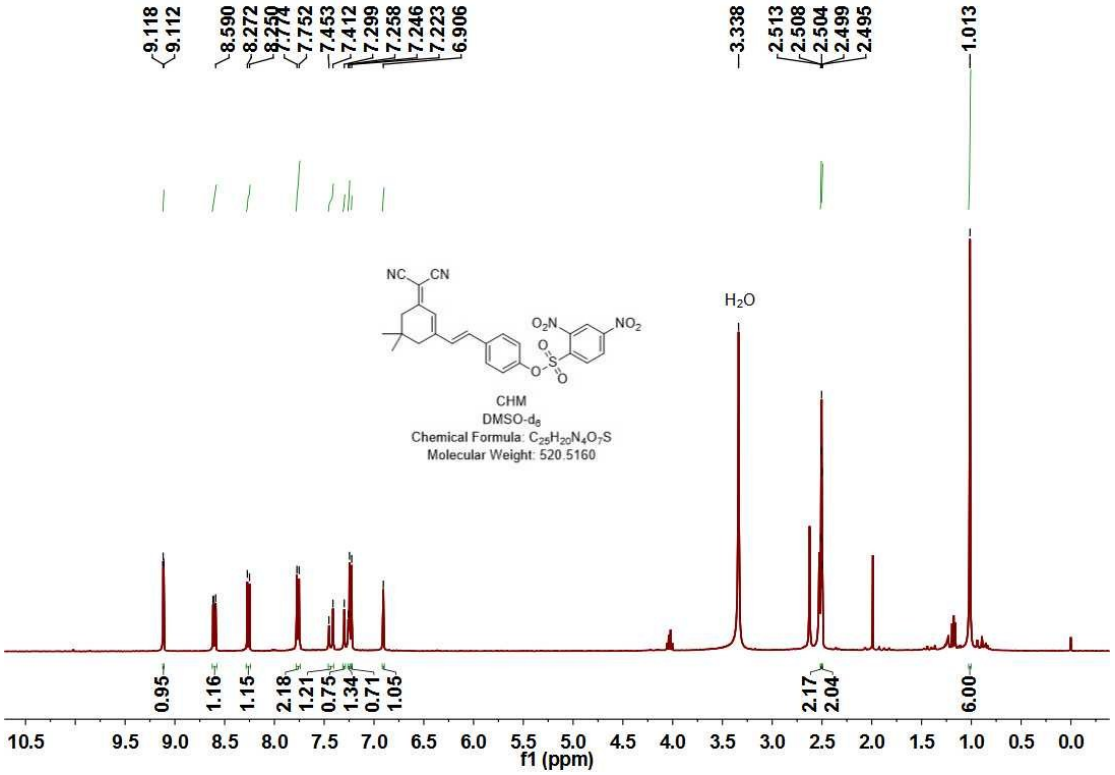


Figure S24. ^{1}H NMR spectra of **CHM** in DMSO-d_6.

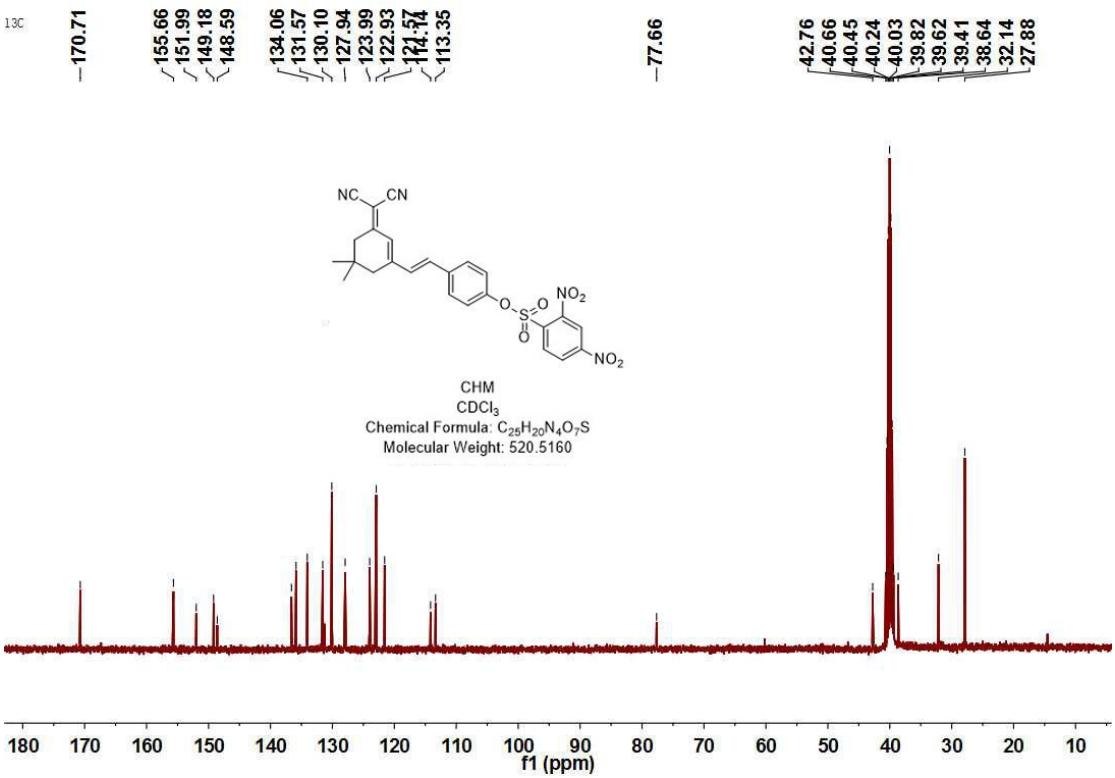
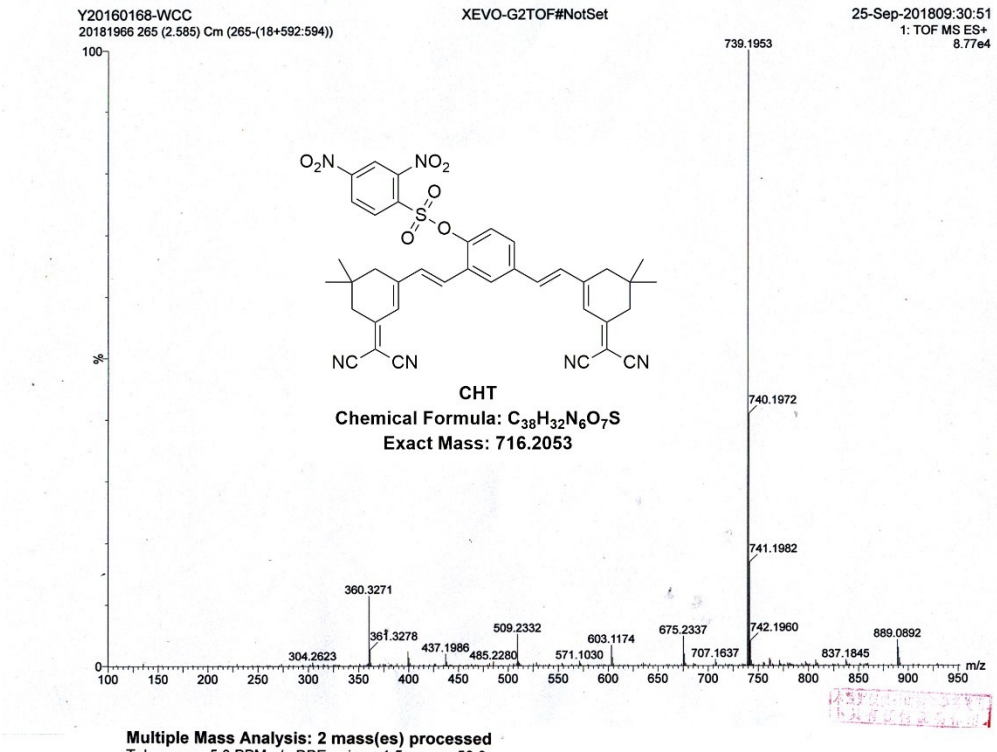
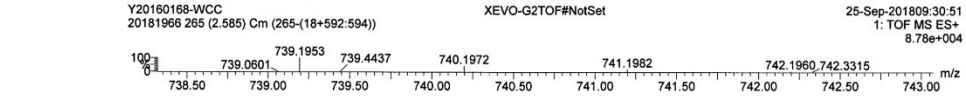


Figure S25. ^{13}C NMR spectra of **CHM** in DMSO-d_6.



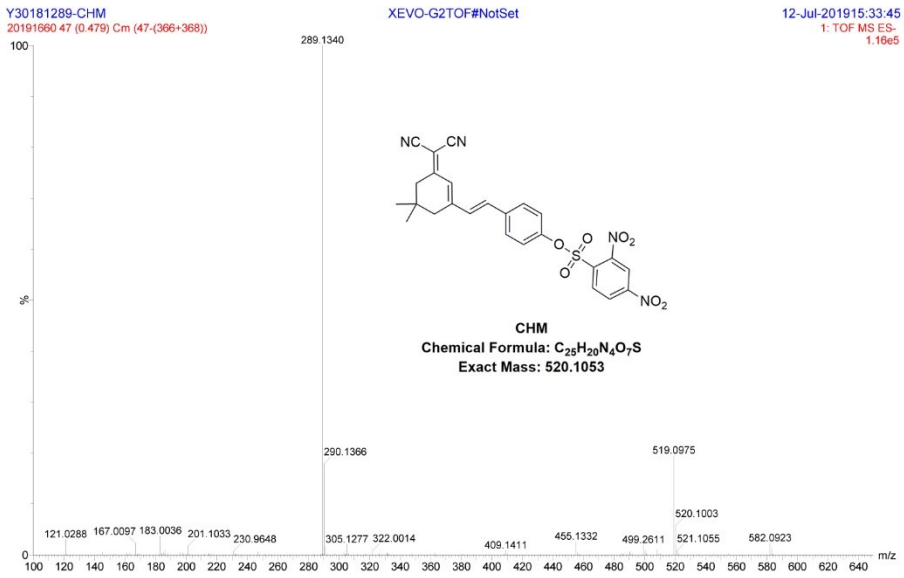
Multiple Mass Analysis: 2 mass(es) processed
Tolerance = 5.0 PPM / DBE: min = -1.5, max = 50.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions
30429 formula(e) evaluated with 30 results within limits (all results (up to 1000) for each mass)
Elements Used:
12C: 0-50 13C: 0-1 H: 0-50 N: 2-10 O: 4-14 Na: 0-1 S: 1-1



Mass	RA	Calc. Mass	mDa	PPM	DBE	i-FIT	Norm	Conf(%)	Formula
Minimum:	20.00				-1.5				
Maximum:	100.00		5.0	5.0	50.0				
739.1953	100.00	739.1951	0.2	0.3	25.5	64.1	3.147	4.30	12C38 H32 N6 O7 Na S
		739.1951	0.2	0.3	17.5	63.4	2.433	8.78	12C28 13C H33 N9 O11 Na S
		739.1957	-0.4	-0.5	33.5	64.6	3.616	2.69	12C42 13C H28 N7 O4 S
		739.1944	0.9	1.2	28.5	64.1	3.146	4.30	12C41 13C H32 N3 O8 S
		739.1962	-0.9	-1.2	23.5	63.3	2.366	9.38	12C39 H35 N2 O11 S
		739.1938	1.5	2.0	20.5	63.8	2.861	5.72	12C37 H36 N2 O11 Na S
		739.1969	-1.6	-2.2	12.5	62.6	1.684	18.57	12C26 H36 N8 O14 Na S
		739.1935	1.8	2.4	24.5	64.2	3.219	4.00	12C35 H31 N8 O9 S
		739.1933	2.0	2.7	30.5	65.0	4.061	1.72	12C40 13C H29 N7 O4 Na S
		739.1975	-2.2	-3.0	28.5	63.8	2.865	5.70	12C40 H31 N6 O7 S
		739.1976	-2.3	-3.1	20.5	63.1	2.174	11.37	12C30 13C H32 N9 O11 S
		739.1978	-2.5	-3.4	16.5	62.9	1.906	14.86	12C32 13C H37 N3 O13 Na S
		739.1921	3.2	4.3	19.5	64.0	3.054	4.72	12C34 H35 N4 O13 S
		739.1920	3.3	4.5	25.5	64.7	3.785	2.27	12C39 13C H33 N3 O8 Na S
		739.1917	3.6	4.9	29.5	65.1	4.123	1.62	12C37 13C H28 N9 O6 S
740.1972	40.88	740.1971	0.1	0.1	20.5	34.8	0.718	48.78	12C36 13C H36 N2 O11 Na S
		740.1968	0.4	0.5	24.5	37.6	3.510	2.99	12C34 13C H31 N8 O9 S
		740.1968	0.4	0.5	32.5	37.3	3.239	3.92	12C44 H30 N5 O5 S
		740.1962	1.0	1.4	16.5	37.4	3.284	3.75	12C30 H35 N7 O12 Na S
		740.1984	-1.2	-1.6	25.5	36.8	2.715	6.62	12C37 13C H32 N6 O7 Na S
		740.1986	-1.4	-1.9	19.5	37.0	2.912	5.43	12C32 H34 N7 O12 S
		740.1955	1.7	2.3	19.5	36.8	2.664	6.97	12C33 13C H35 N4 O13 S
		740.1995	-2.3	-3.1	23.5	36.3	2.193	11.16	12C38 13C H35 N2 O11 S
		740.1946	2.6	3.5	15.5	38.7	4.631	0.97	12C27 H34 N9 O14 S
		740.1944	2.8	3.8	29.5	38.9	4.847	0.79	12C42 H31 N5 O5 Na S
		740.1944	2.8	3.8	21.5	38.9	4.821	0.81	12C32 13C H32 N8 O9 Na S
		740.2002	-3.0	-4.1	20.5	37.4	3.348	3.52	12C35 H35 N5 O10 Na S
		740.2003	-3.1	-4.2	12.5	38.2	4.124	1.62	12C25 13C H36 N8 O14 Na S
		740.2008	-3.6	-4.9	28.5	38.1	4.029	1.78	12C39 13C H31 N6 O7 S
		740.1936	3.6	4.9	32.5	38.8	4.710	0.90	12C45 13C H31 N2 O6 S

Figure S26. HRMS of the target probe **CHT.**



Elemental Composition Report

Multiple Mass Analysis: 2 mass(es) processed

Tolerance = 5.0 PPM / DBE: min = -1.5, max = 50.0

Element prediction: Off

Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions

16656 formula(e) evaluated with 20 results within limits (all results (up to 1000) for each mass)

Elements Used:

12C: 0-60 13C: 0-1 H: 0-60 N: 0-10 O: 0-14 S: 1-1

Minimum:		10.00						-1.5
Maximum:		100.00			20.0		5.0	50.0
Mass		RA	Calc. Mass		mDa		PPM	DBE
	i-FIT	Norm	Conf(%)		Formula			
519.0975		100.00	519.0975		0.0	0.0	10.5	79.7
	2.574	7.62	12C15 13C H20 N7 O11 S					
			519.0974		0.1	0.2	18.5	79.7
	2.475	8.42	12C25 H19 N4 O7 S					

Figure S27. HRMS of the control probe **CHM.**