Supporting Information

A multi-signal fluorescent probe for simultaneously distinguishing and sequentially sensing cysteine/homocysteine, glutathione and hydrogen sulfide in living cells

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Materials and instruments: Unless otherwise stated, all reagents were purchased from commercial suppliers and used without further purification. Solvents used were purified by standard methods prior to use. Twice-distilled water was used throughout all experiments; Mass spectrometric analyses were measured on a Finnigan MAT 95 XP spectrometer; High resolution mass spectrometric (HRMS) analyses were measured on an Agilent 1100 HPLC/MSD spectrometer; NMR spectra were recorded on an AVANCE III 400 MHz Digital NMR Spectrometer, using TMS as an internal standard; Electronic absorption spectra were obtained on a Shimadzu UV-2700 power spectrometer; Photoluminescent spectra were recorded with a HITACHI F4600 fluorescence spectrophotometer with a 1 cm standard quartz cell; The fluorescent images of solution and filter paper strip were excited by a 365 nm lighting of ZF-1 UV analyzer; The visual pictures were captured by LG (G4) mobile phone built-in camera; The fluorescence imaging of cells was performed with a Nikon A1MP confocal microscope; Ultrasonic extraction were carry out on KUDOS ultrasonic cleaner (SK2210HP); Centrifugalization was carried out on a ZONKIA high speed centrifuge (HC-2518); The pH measurements were carried out on a Mettler-Toledo Delta 320 pH meter; TLC analysis was performed on silica gel plates and column chromatography was conducted over silica gel (mesh 200–300), both of which were obtained from the Qingdao Ocean Chemicals.

Cells culture. HeLa cells were cultured in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% FBS (fetal bovine serum) in an atmosphere of 5% CO_2 and 95% air at 37 °C.

All experiments for this study were performed in compliance with the requirements of the National Act on the use of experimental animals (China) and approved by the Animal Ethical Experimentation Committee of Shandong University (Jinan, China). The HeLa cells were a kind gift by the College of Life Science, Nankai University (Tianjin, China). Bovine serum was purchased from GE Healthcare Life Sciences,

HyClone Laboratories (USA).

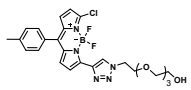
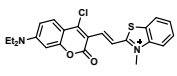
Discriminative fluorescence imaging of biothiols in living cells. The cell experiment was divided into five groups. The first group is that HeLa cells were incubated with **HMN** (5 μM) for 30 min, then washed by PBS buffer before imaging. The second group is that HeLa cells were pretreated with NEM (0.5 mM) for 30 min, subsequently incubated with **HMN** (5 μM) for 30 min, then imaged after washing by PBS buffer. In the third and fourth group, HeLa cells were pretreated with NEM (0.5 mM) for 30 min, subsequently incubated with Cys or Hcy (250 μM) for 15 min, washed by PBS buffer and continued to be incubated with **HMN** (5 μM) for 30 min, then imaged after washing by PBS buffer. In the fifth group, HeLa cells were pretreated with NEM (0.5 mM) for 30 min, subsequently incubated with Na_2S (2 mM) for 15 min, washed by PBS buffer and continued to be incubated with **HMN** (5 μM) for 30 min, then imaged after washing by PBS buffer.The confocal microscopic imaging uses Nikon A1MP confocal microscope withexcitation filter of 405, 488, and 561 nm and the collectionwavelength range is from 465-500 nm (blue channel), 525-555 nm (green channel), and 595-630 nm (red channel).

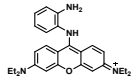
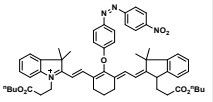
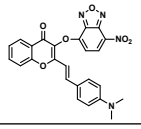
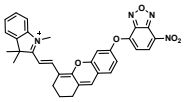
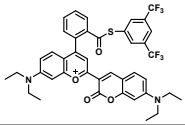
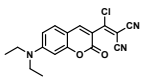
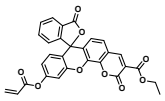
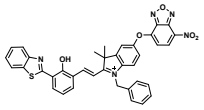
Imaging of exogenous and endogenous H_2S in living cells. HeLa cells were pretreated with NEM (0.5 mM) for 30 min, subsequently incubated with **HMN** (5 μM) for 30 min, then imaged after washing by PBS buffer, as the control group. HeLa cells were pretreated with NEM (0.5 mM) for 30 min, subsequently incubated with Na_2S (200 μM) for 15 min, washed by PBS buffer and continued to be incubated with **HMN** (5 μM) for 30 min, then imaged after washing by PBS buffer, as the experimental group of imaging exogenous H_2S. HeLa cells were pretreated with Cys (200 μM) for 60 min, subsequently washed by PBS buffer and incubated with **HMN** (5 μM) for 30 min, then imaged after washing by PBS buffer, as the experimental group of imaging endogenous H_2S. The confocal microscopic imaging uses Nikon A1MP confocal microscope with

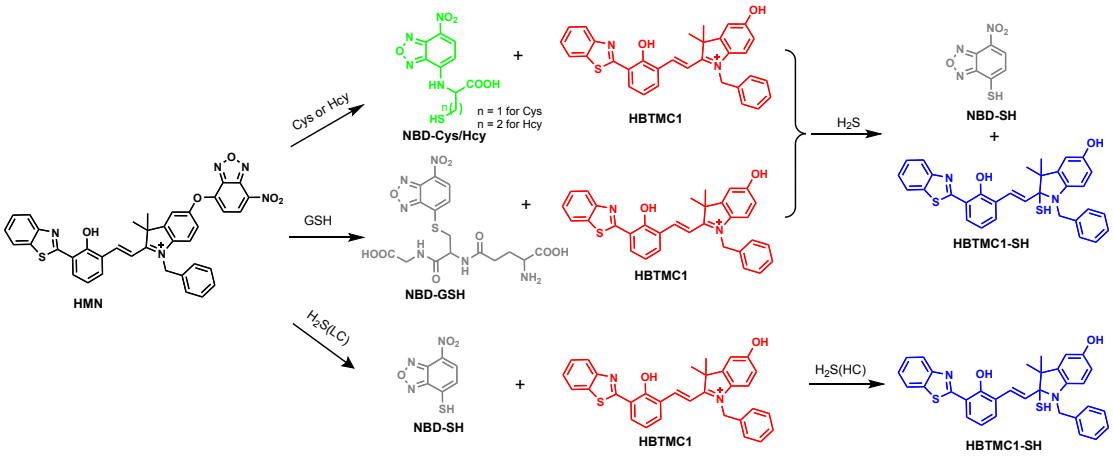
excitation filter of 405, 488, and 561 nm and the collection wavelength range is from 465-500 nm (blue channel), 525-555 nm (green channel), and 595-630 nm (red channel).

Sequential fluorescence imaging of biothiols in living cells. The cell experiment was divided into five groups. The first group is that HeLa cells were pretreated with NEM (0.5 mM) for 30 min, subsequently incubated with **HMN** (5 μM) for 30 min, then imaged after washing by PBS buffer, as the control group. In the second and third group, HeLa cells were pretreated with NEM (0.5 mM) for 30 min, subsequently incubated with Cys (250 μM) for 15 min, washed and continued to be incubated with **HMN** (5 μM) for 30 min, and then added 1 or 2 mM Na_2S to the culture dish, after 25 min, imaged after washing by PBS buffer. The fourth group is that HeLa cells were incubated with **HMN** (5 μM) for 30 min, then imaged after washing by PBS buffer.In the fifth group, HeLa cells were pretreated with **HMN** (5 μM) for 30 min, and then added 1 or 2 mM Na_2S to the culture dish, after 25 min, imaged after washing by PBS buffer. The confocal microscopic imaging uses Nikon A1MP confocal microscope withexcitation filter of 405, 488, and 561 nm and the collection wavelength range is from 465-500 nm (blue channel), 525-555 nm (green channel), and 595-630 nm (red channel).

Table S1. Summary of the optical properties of representative fluorescent probes for distinguishing biothiols with multiple sets of fluorescence signals.

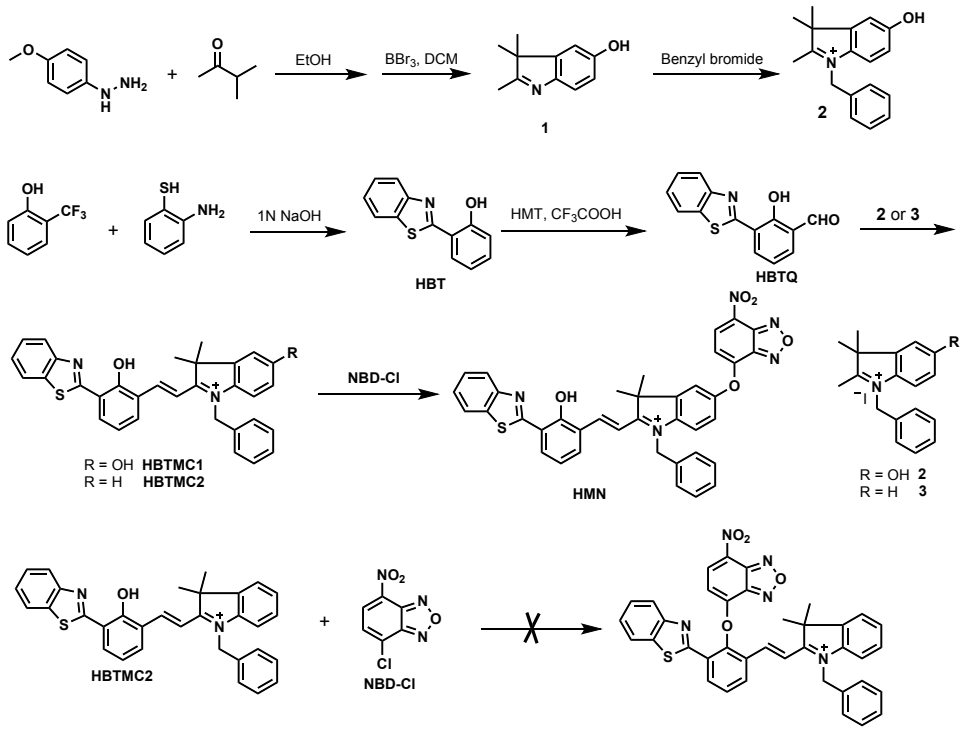
Probes	Chemical structures	Distinguishing targets for detection	Number of emission bands	Emission wavelength /nm	Emissiond istance /nm	References
1a		GSH and Cys/Hcy	2	556/588	32	1
1		Cys and GSH	2	420/512	92	2

1		Cys and GSH	2	536/618	82	3
MitoGP		GSH and Cys/Hcy	2	764/810	46	4
4F-2CN		Cys, Hcy, and GSH	2	450/500	50	5
NBD-OF		GSH and Cys/Hcy	2	545/621	76	6
NR-NBD		GSH and Cys/Hcy	2	550/716	166	7
1		Cys/Hcy	2	474/694	220	8
1		GSH and H_2S	2	517/564	47	9
2		Cys and Hcy/GSH	2	472/540	68	10
HMN		Cys/Hcy, GSH, and H_2S	3	485/546/609	61/63	This work



Scheme S1. The proposed responding mechanism of probe **HMN** for simultaneously distinguishing biothiols and sequentially sensing Cys/Hcy/GSH and H_2S with three emission bands. H_2S(LC)

andH_2S (HC) denote H_2S at low and high concentration, respectively.



Scheme S2. The synthetic route of probe **HMN**.

Synthesis of 2. Compound **1** was prepared via previous method.[11] The mixture of compound **1** (175 mg, 1.0 mmol), Benzyl bromide (342 mg, 2.0 mmol) and potassium iodide (232 mg, 2.0 mmol) was dissolved absolutely in acetonitrile. Under the protection of nitrogen, the mixture was heated and reflux for 6 hours. After complete reaction, the solvent was removed under reduced pressure affording the crude product, which was purified by flash chromatography column using ethanol/dichloromethane (v/v 1:50) to afford brown solid as compound **2** (252 mg, yield 64 %). ^{1}H NMR (400 MHz, CD_3OD), δ (ppm): 1.64 (s, 6H), 5.77 (s, 2H), 6.91-6.94 (dd, J = 8.8, 2.4 Hz, 1H), 7.139 (d, J = 2.4 Hz, 1H), 7.37-7.38 (2H), 7.42-7.48 (3H), 7.56-7.58 (d, J = 8.8 Hz, 1H); ^{13}C NMR (100 MHz, CD_3OD): δ (ppm): 14.61, 22.80, 51.04, 54.46, 111.01, 115.79, 117.43, 127.81, 129.15, 129.74, 132.73, 133.52, 144.61, 159.66, 197.29.HRMS (ESI) *m/z*calcd for $C_{18}H_{20}NO$ (M^+): 266.1539. Found 266.1537.

Synthesis of HBTMC1/2. Compounds **3**, **HBT** and **HBTQ** were prepared via previous method.[12,13] The mixture of compound **HBTQ** (51 mg, 0.2mmol) and comound **2** or **3** (2.0 mmol) was dissolved absolutely in acetonitrile.Under the protection of nitrogen, the mixture was heated and reflux for 4 hours. After complete reaction, the solvent was removed under reduced pressure affording the crude product, which was purified by flash chromatography column using ethanol/dichloromethane (v/v 1:20) to afford deep red solid as compound **HBTMC1** or **HBTMC2**.

HBTMC1(86 mg, yield 68 %): ^{1}H NMR (400 MHz, DMSO-d6), δ (ppm): 1.86 (s, 6H), 5.99 (s, 2H), 6.91 (dd, *J* = 8.8, 2.4 Hz, 1H), 7.27-7.26 (2H), 7.36-7.44 (m,5H), 7.48-7.52 (t, *J* = 7.2 Hz, 1H), 7.60-7.62 (2H), 7.70-7.74 (d, *J* = 16.8, 1H), 8.11-8.13 (d, *J* = 8.0 Hz, 1H), 8.18-8.22 (1H), 8.24-8.37 (dd, *J* = 8.8, 2.0 Hz, 1H), 8.60-8.64 (d, *J* = 16.0 Hz, 1H), 8.99-9.00 (d, *J* = 2.0 Hz, 1H); ^{13}C NMR (100 MHz, DMSO-d6): δ (ppm): 14.49, 26.44, 52.47, 63.44, 110.57, 111.07, 116.24, 117.08, 118.55, 120.09, 122.65, 125.80, 127.32, 128.91, 129.78, 132.85, 133.38, 134.42, 135.29, 146.64, 151.79, 152.69, 159.80, 161.07, 163.74, 180.02. HRMS (ESI) *m/z*calcd for $C_{32}H_{27}N_2O_2S$ (M^+): 503.1788. Found 503.1781.

HBTMC2(93 mg, yield 76 %):^{1}H NMR (400 MHz, DMSO-d6), δ (ppm): 1.83 (s, 6H), 5.69 (s, 2H), 6.62-6.64 (d, *J* = 8.4Hz, 1H), 7.06-7.10 (d, *J* = 14.8 Hz, 1H), 7.35-7.43 (m,9H), 7.48-7.52 (t, *J* = 8.2 Hz, 1H), 7.71-7.73 (d, *J* = 7.6, 1H), 7.97-7.99 (d, *J* = 8.4 Hz, 1H), 8.07-8.09 (d, *J* = 7.6 Hz, 2H), 8.44-8.47 (d, *J* = 14.8 Hz, 1H), 8.89(s,1H); ^{13}C NMR (100 MHz, DMSO-d6): δ (ppm): 15.64, 26.58, 52.34, 65.38, 107.98, 108.53, 115.25, 119.22, 121.14, 121.40, 122.59, 123.55, 125.37, 125.59, 127.33, 127.72, 129.47, 129.75, 130.13, 134.53, 137.22, 139.19, 141.48, 147.91, 151.82, 155.15, 162.02, 163.62, 172.48.HRMS (ESI) *m/z*calcd for $C_{18}H_{20}NO$ (M^+): 487.1839. Found 487.1834.

Synthesis of HMN. The mixture of compound **HBTMC1** (63 mg, 0.1mmol)and **NBD-Cl** (24 mg, 0.12mmol) was dissolved absolutely in dry dichloromethane. Under the protection of nitrogen, the mixture was stirred for 2 hours at room temperature. After complete reaction, the solvent was removed under reduced pressure

affording the crude product, which was purified by flash chromatography column using ethanol/dichloromethane (v/v 1:25) to afford blue solid as probe**HMN**(35 mg, yield 44 %).^{1}H NMR (400 MHz, $CDCl_3$), δ (ppm): 1.85 (s, 6H), 5.65 (s, 2H), 6.52-6.55 (d, *J* = 11.2 Hz, 1H), 6.81-6.83 (d, J = 8.4 Hz, 1H), 6.96-6.99 (d, J = 14.4 Hz, 1H), 7.34-7.37 (2H), 7.41-7.50 (7H), 7.86 (s, 1H), 7.93-7.95 (d, *J* = 8.4 Hz, 1H), 8.05-8.07 (d, *J* = 7.6 Hz, 2H), 8.44-8.47 (d, *J* = 11.2 Hz, 1H), 8.67-8.69 (d, *J* = 8.4 Hz, 2H), 8.86 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm): 14.47, 27.59, 50.13, 110.59, 113.52, 116.34, 121.80, 122.24, 124.30, 126.25, 127.28, 128.42, 129.60, 130.92, 135.68, 135.99, 136.22, 140.80, 144.33, 144.86, 145.83, 151.07, 151.98, 153.19, 153.58, 163.47, 175.55.HRMS (ESI) *m/z*calcd for $C_{18}H_{20}NO$ (M^+): 666.1806. Found 666.1802.

As control experiment, compounds **HBTMC2** and **NBD-Cl** were mixed with identical equivalent proportion under the same conditions. No desired product was found by TLC monitor method.

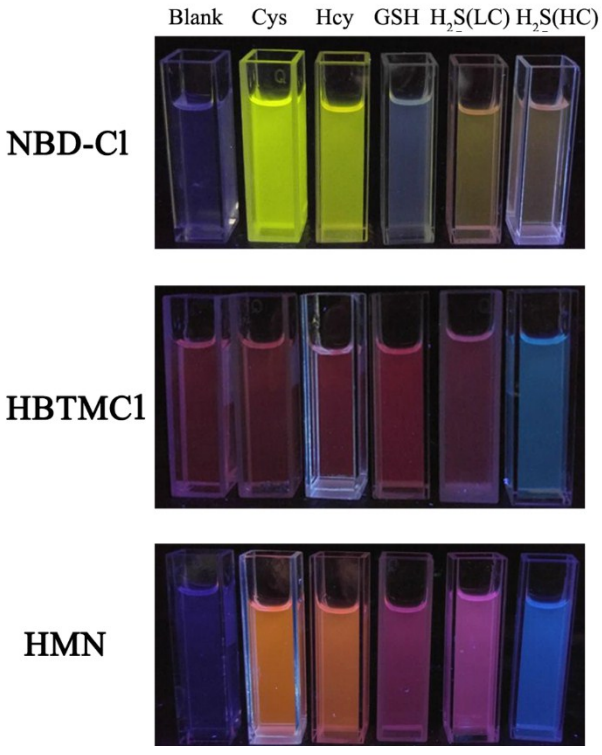


Figure S1. The fluorescent colour changes of 10 μM **NBD-Cl**, **HBTMC1**, and **HMN** by addition of Cys (550 μM), Hcy (550 μM), GSH (650 μM), H_2S(LC) (500 μM), or H_2S(HC) (5 mM) in aqueous solution, respectively. The pictures were captured under the irradiation of by a UV lamp (365 nm). H_2S(LC) and H_2S (HC) denote H_2S at low and high concentration, respectively.

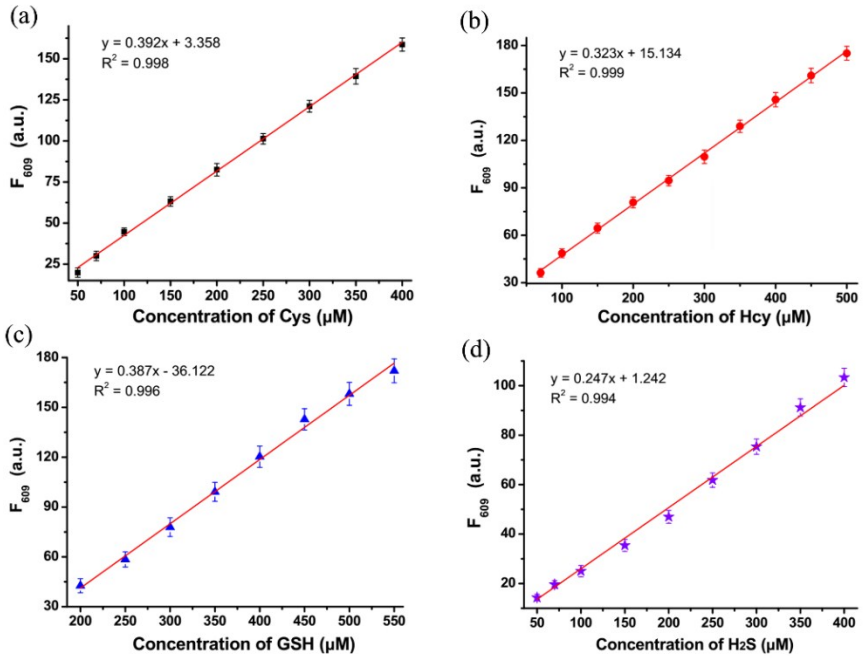


Figure S2. Linear correlation between the fluorescence emission intensity of **HMN** (10 μM) and (a) Cys (50-400 μM), (b) Hcy (70-500 μM), (c) GSH (200-550 μM), and (d) H_2S (50-400 μM) in PBS buffer (25 mM, pH 7.4, containing 20% acetonitrile).

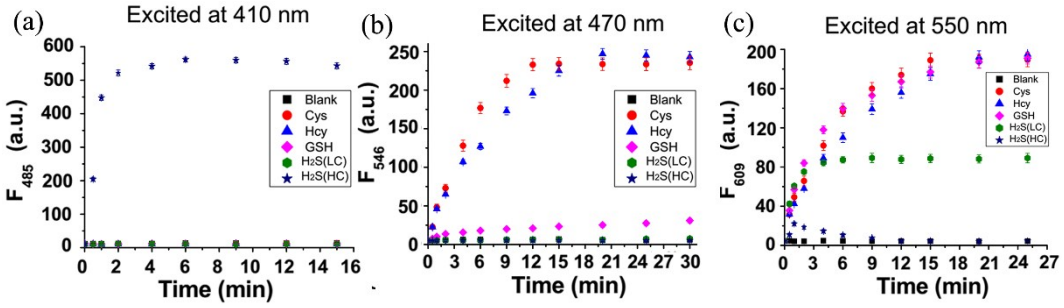


Figure S3. Reaction-time profiles of the probe **HMN** in the presence or absence of Cys (550 μM), Hcy (550 μM), GSH (650 μM), H_2S(LC) (500 μM), or H_2S(HC) (5 mM). (a) Fluorescence intensity at 485 nm, excitation at 410 nm; (b) Fluorescence intensity at 546 nm, excitation at 470 nm; (c) Fluorescence intensity at 609 nm, excitation at 550 nm. H_2S(LC) and H_2S(HC) denote H_2S at low and high concentration, respectively.

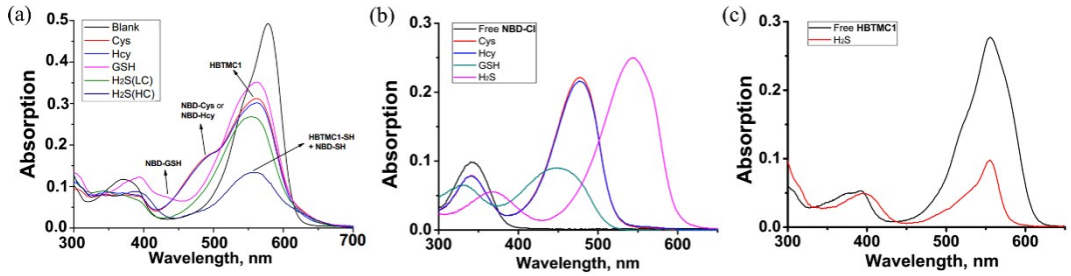


Figure S4. Absorption spectra of (a) **HMN**(10 μM), (b) **NBD-Cl** (10 μM), and (c) **HBTMC1** (10μM) in the presence of respective biothiolsin PBS buffer (25 mM, pH 7.4, containing 20% acetonitrile). 550μM for Cys/Hcy, 650 μM for GSH and 500 μM for H_2S in a and b; 5 mM for H_2S in a and c.

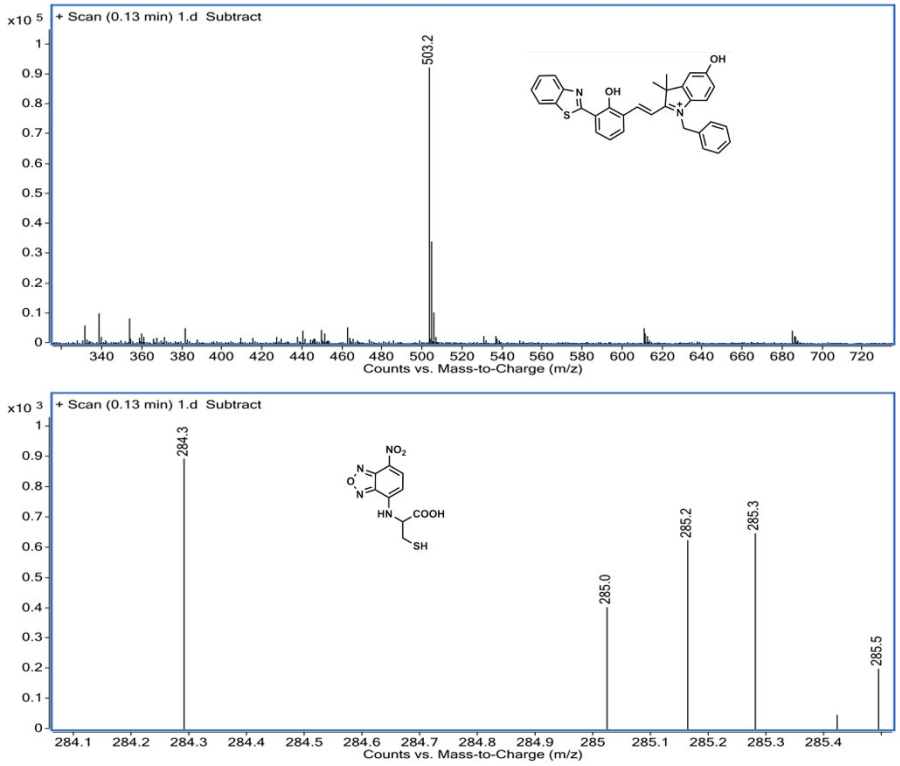


Figure S5. Mass spectra of **HMN** in the presence of Cys in aqueous solution. *m/z* 503.2 for **HBTMC1** and *m/z* 284.3 for **NBD-Cys**.

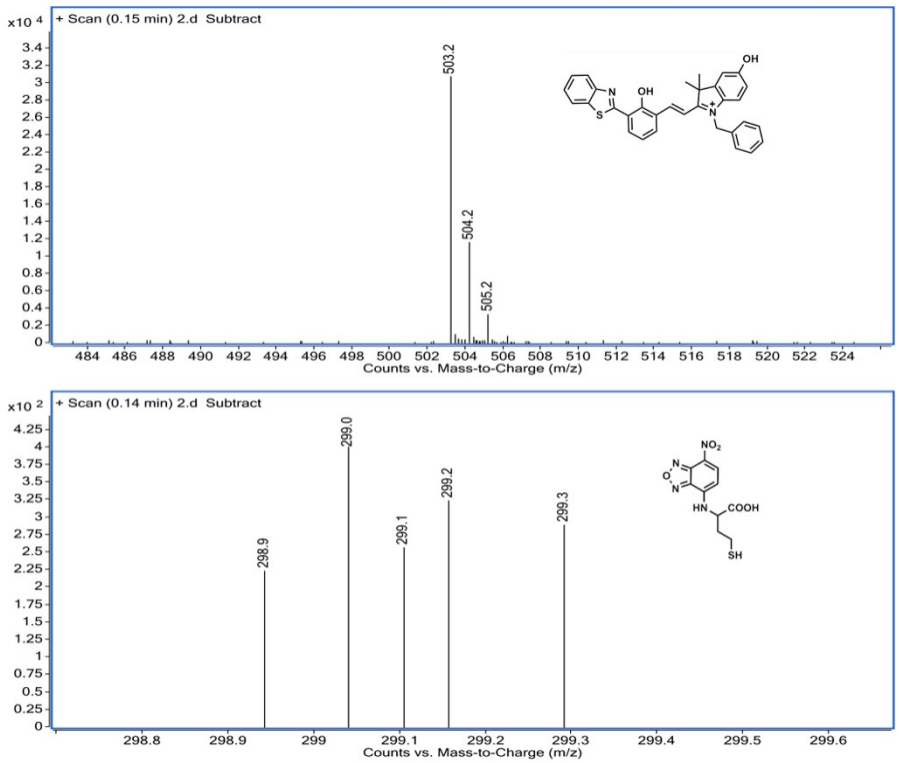


Figure S6. Mass spectra of **HMN** in the presence of Hcy in aqueous solution. *m/z* 503.2 for **HBTMC1** and *m/z* 299.2 for **NBD-Hcy**.

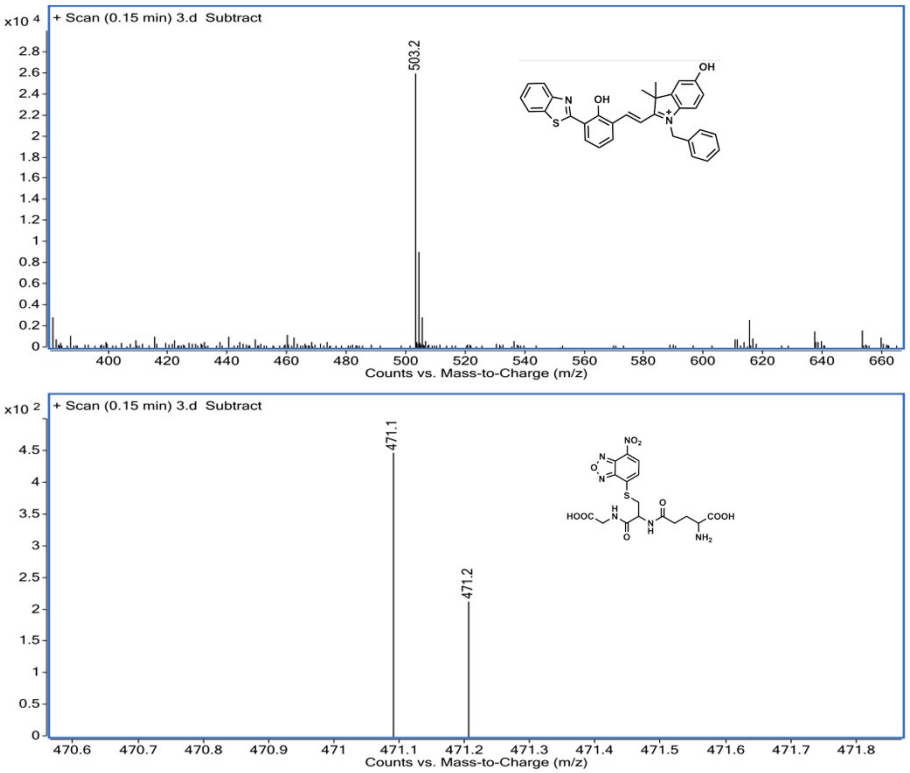


Figure S7. Mass spectra of **HMN** in the presence of GSH in aqueous solution. *m/z* 503.2 for **HBTMC1** and *m/z* 471.1 for **NBD-GSH**.

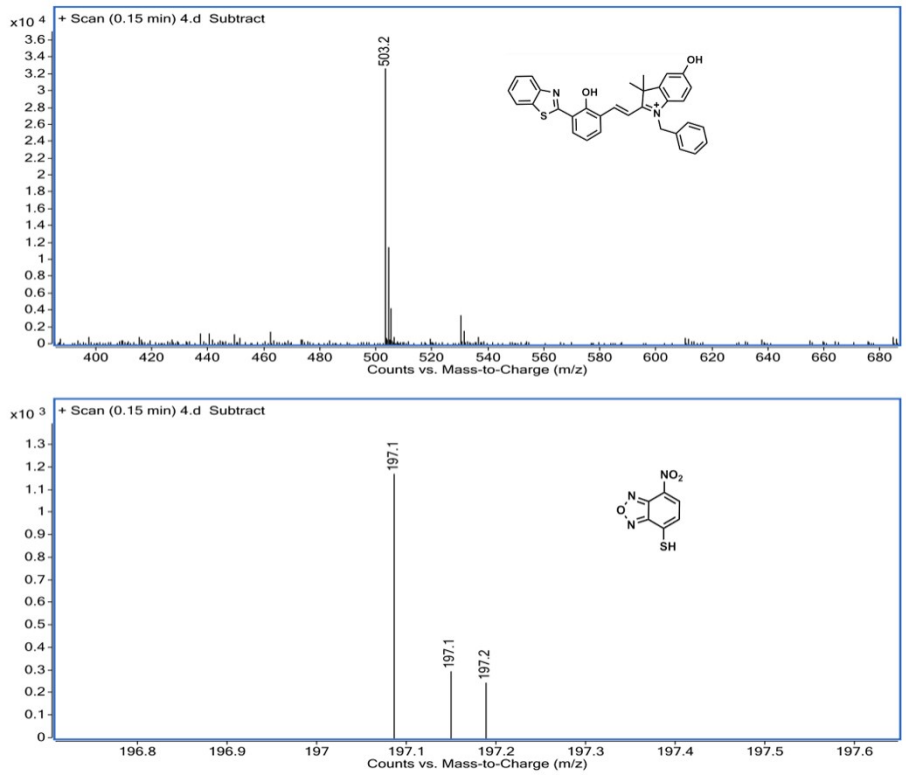


Figure S8. Mass spectra of **HMN** in the presence of low level of H_2S in aqueous solution. *m/z* 503.2 for **HBTMC1** and *m/z* 197.1 for **NBD-SH**.

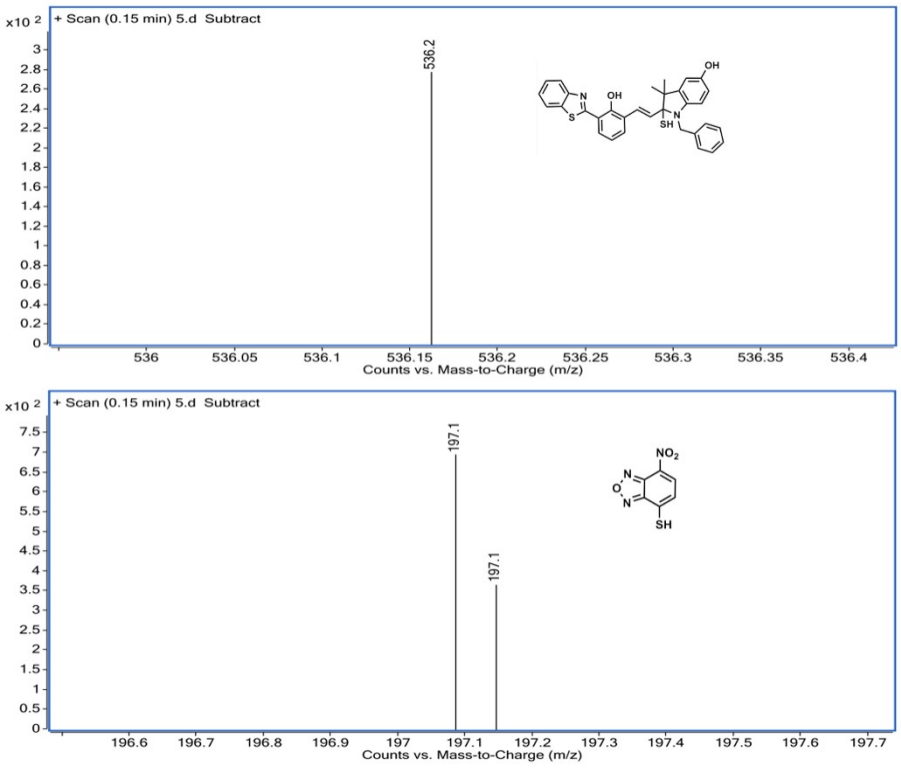


Figure S9. Mass spectra of **HMN** in the presence of high level of H_2S in aqueous solution. *m/z* 536.2 for **HBTMC1-SH** and *m/z* 197.1 for **NBD-SH**.

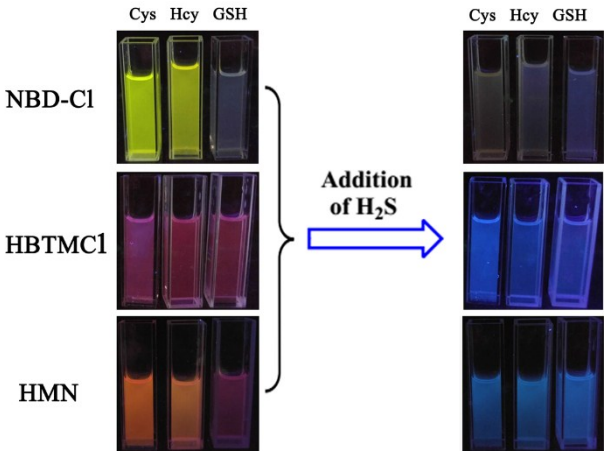


Figure S10. The fluorescent colour changes of 10 μM **NBD-Cl**, **HBTMC1**, and **HMN** by sequential addition of Cys (550 μM)/Hcy (550 μM)/GSH (650 μM) and H_2S (5 mM) in aqueous solution, respectively. The pictures were captured under the irradiation of by a UV lamp (365 nm).

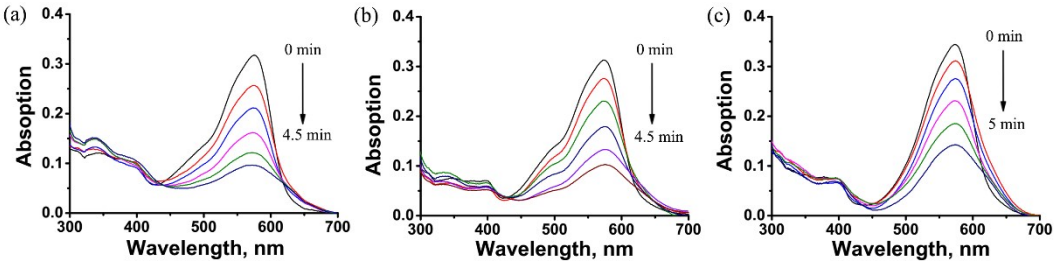


Figure S11. The absorption spectra of **HMN** (10 μM) pretreated with (a) Cys (100 equiv.), (b) Hcy (100 equiv.), and (c) GSH (100 equiv.) upon continued addition of H_2S (500 equiv.) within 4.5 (or 5.0) min in PBS buffer (25 mM, pH 7.4, containing 20% acetonitrile), respectively.

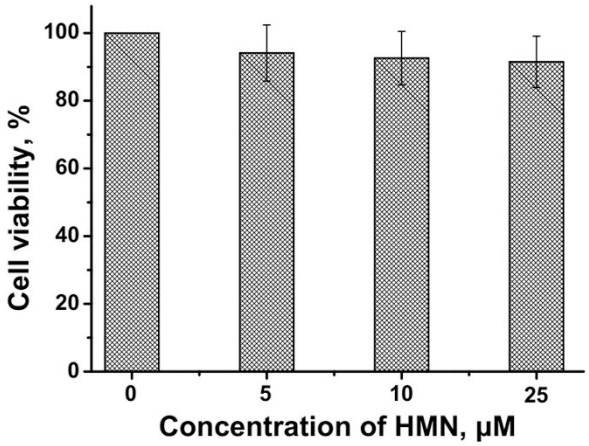


Figure S12. The cell viability of living HeLa cells treated with 5, 10, or 25 μM **HMN** for 24 hours measured by standard MTT assay.

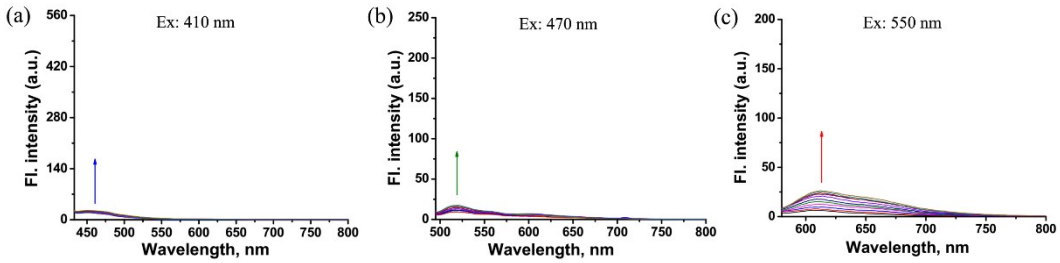


Fig. 13 The fluorescence spectra of **HMN** (10 μM) upon addition of BSA (0-1000 μg/mL) in PBS buffer (25 mM, pH 7.4, containing 20% acetonitrile). Excitation at 410 nm for (a), 470 nm for (b), and 550 nm for (c).

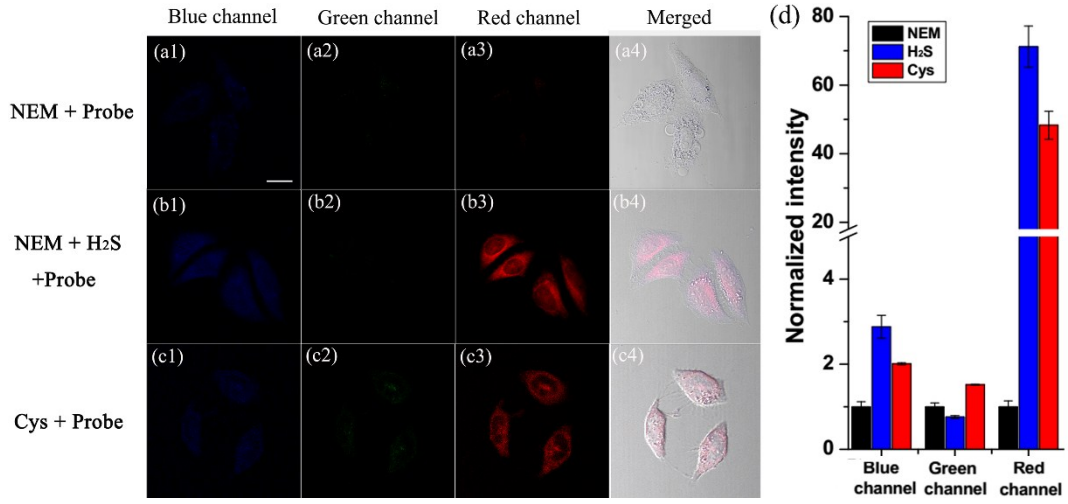


Figure S14. Fluorescence images of probe **HMN** responding to exogenous and endogenous H_2S in living HeLa cells by confocal fluorescence imaging. (a) Cells were pretreated with NEM (0.5 mM, 30 min), subsequently incubated with probe **HMN** (5 μM, 30 min), then imaged;(b) Cells were pretreated with NEM (0.5 mM, 30 min), subsequently incubated with Na_2S (200 μM, 15 min) and probe **HMN** (5 μM, 30 min), then imaged; (c) Cells were pretreated with Cys (200 μM, 60 min), subsequently incubated with probe **HMN**(5 μM, 30 min), then imaged.The fluorescence images were captured from the blue channel of 465-500 nm (firstcolumn), green channel of 525-555 nm(second column), and red channel of 595-630 nm (thirdcolumn) with excitation at 405, 488, and 561 nm, respectively. Fourth column: merged bright field images with blue, green, and red channel images. Scale bar: 20 μm. (f) Normalized average fluorescence intensity of blue, green, and red channel in Figure a-c. The normalized intensity of group (a) in respective channels is taken as the reference standard. Data are expressed as mean ± SD of three parallel experiments.

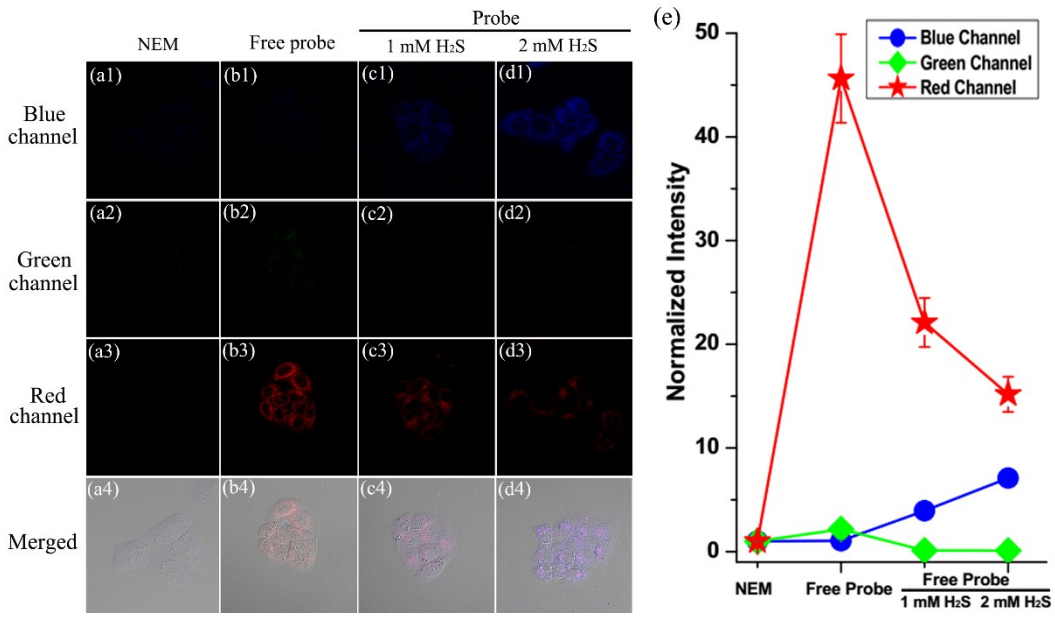


Figure S15. Fluorescence images of probe **HMN** sequentially sensing GSH and H_2S in living HeLa cells by confocal fluorescence imaging. (a) Cells were pretreated with NEM (0.5 mM, 30 min), subsequently incubated with probe **HMN**(5 μM, 30 min), then imaged; (b) Cells were incubated with probe **HMN** (5 μM, 30 min), then imaged; (c, d) Cells were pre-incubated with probe **HMN**(5 μM, 30 min), subsequently treated with 1 or 2 mM Na_2S for 25 min, then imaged. The fluorescence images were captured from the blue channel of 465-500 nm (first row), green channel of 525-555 nm (second row), and red channel of 595-630 nm (third row) with excitation at 405, 488, and 561 nm, respectively. Fourth row: merged bright field images with blue, green, and red channel images. Scale bar: 20 μm. (e) Normalized average fluorescence intensity of blue, green, and red channel in Figure a-d.The normalized intensity of group (a) in respective channels is taken as the reference standard. Data are expressed as mean ± SD of three parallel experiments.

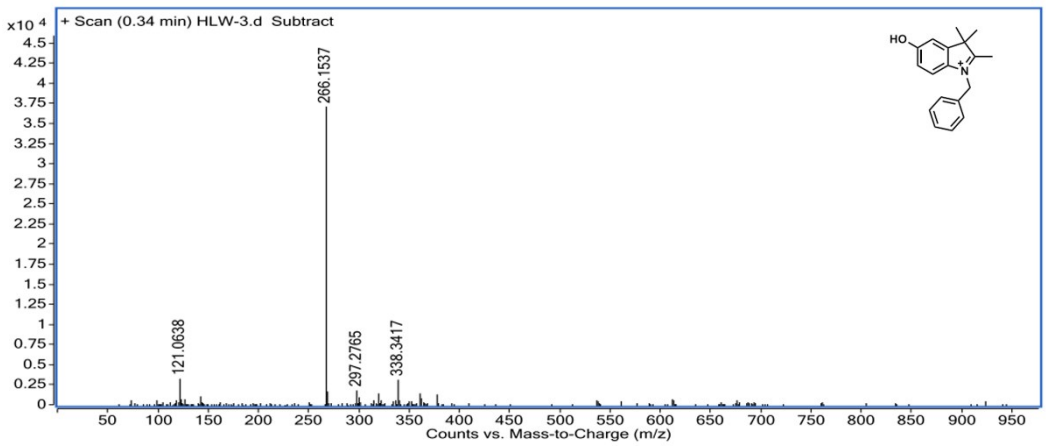


Figure S16. HRMS (ESI) of compound **2**. *m/z* calcd for $C_{18}H_{20}NO$ (M^+): 266.1539. Found 266.1537.

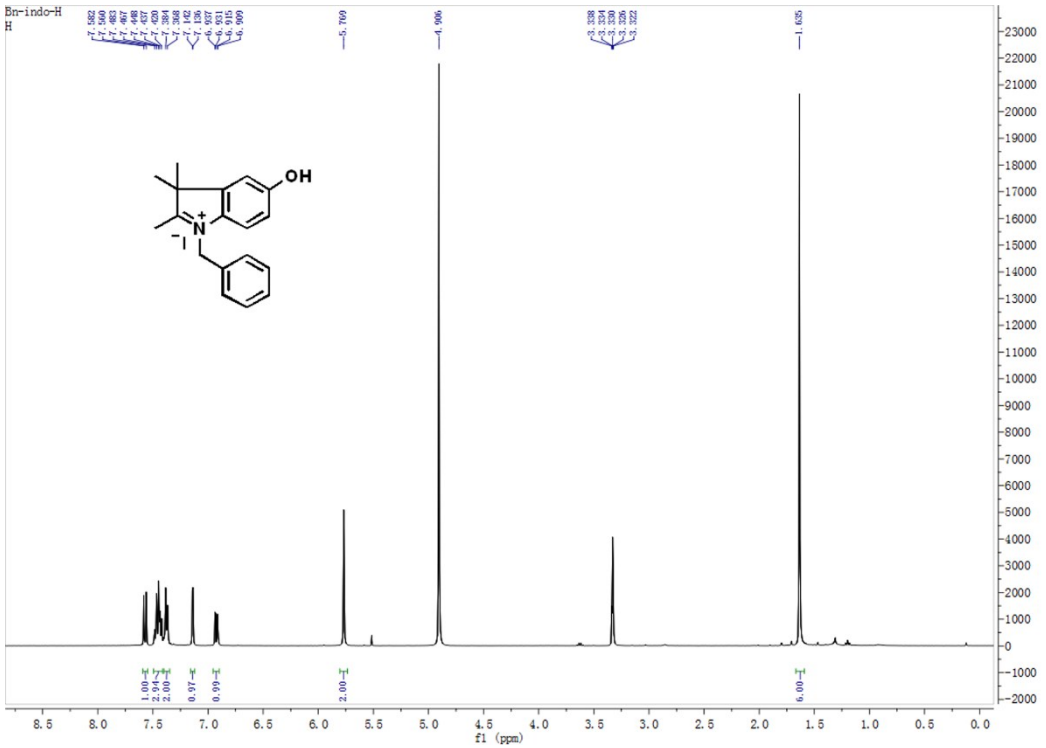


Figure S17. ^1H NMR of compound **2** in CD_3OD.

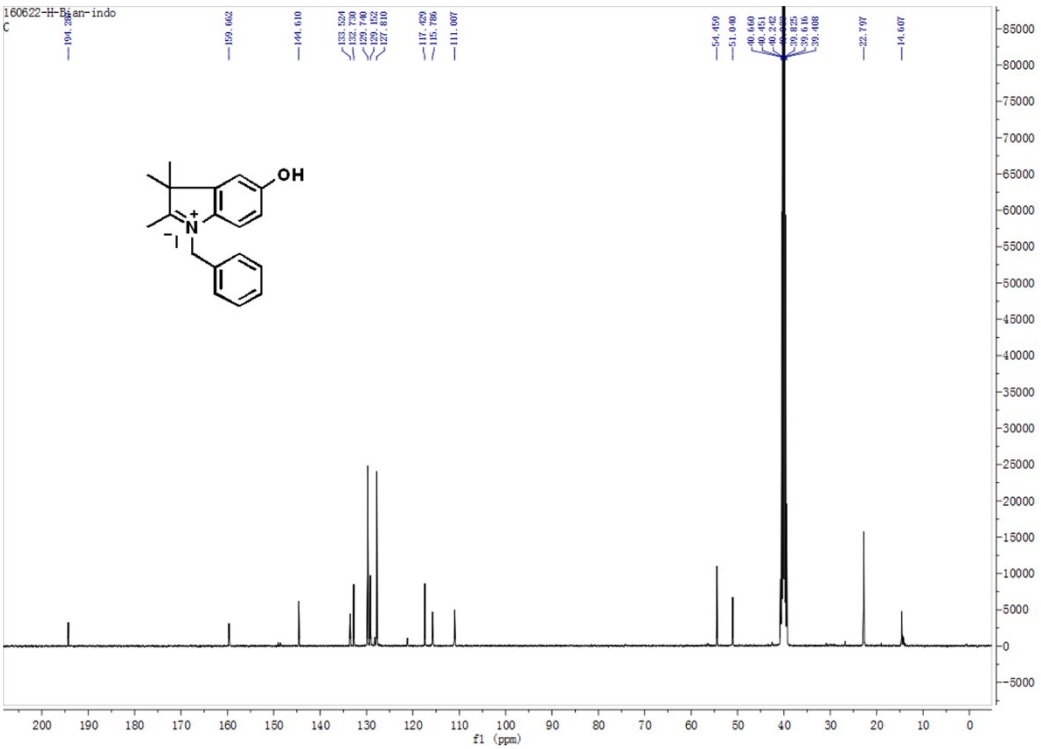


Figure S18. ^{13}C NMR of compound **2** in CD_3OD.

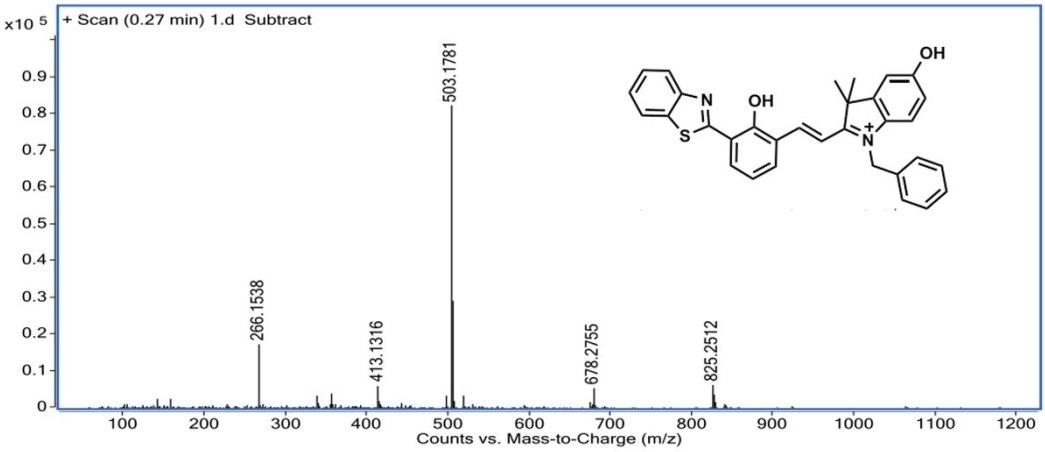


Figure S19. HRMS (ESI) of compound **HBTMC1**. *m/z* calcd for $C_{32}H_{27}N_2O_2S$ (M^+): 503.1788. Found 503.1781.

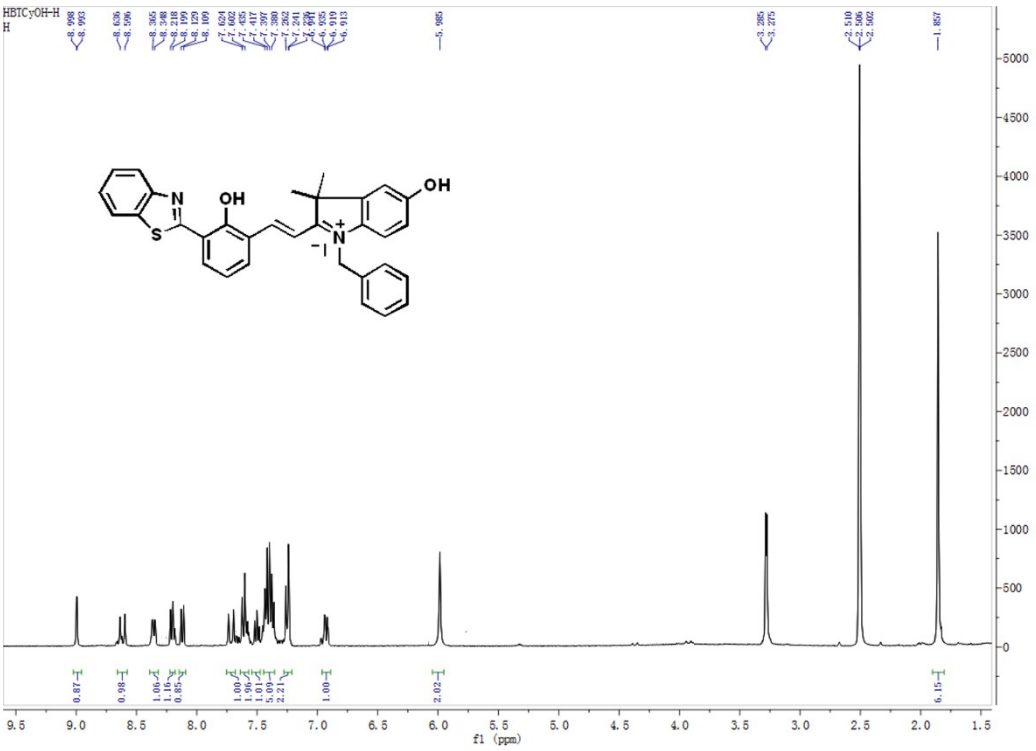


Figure S20. [1]H NMR of compound **HBTMC1** in DMSO-d6.

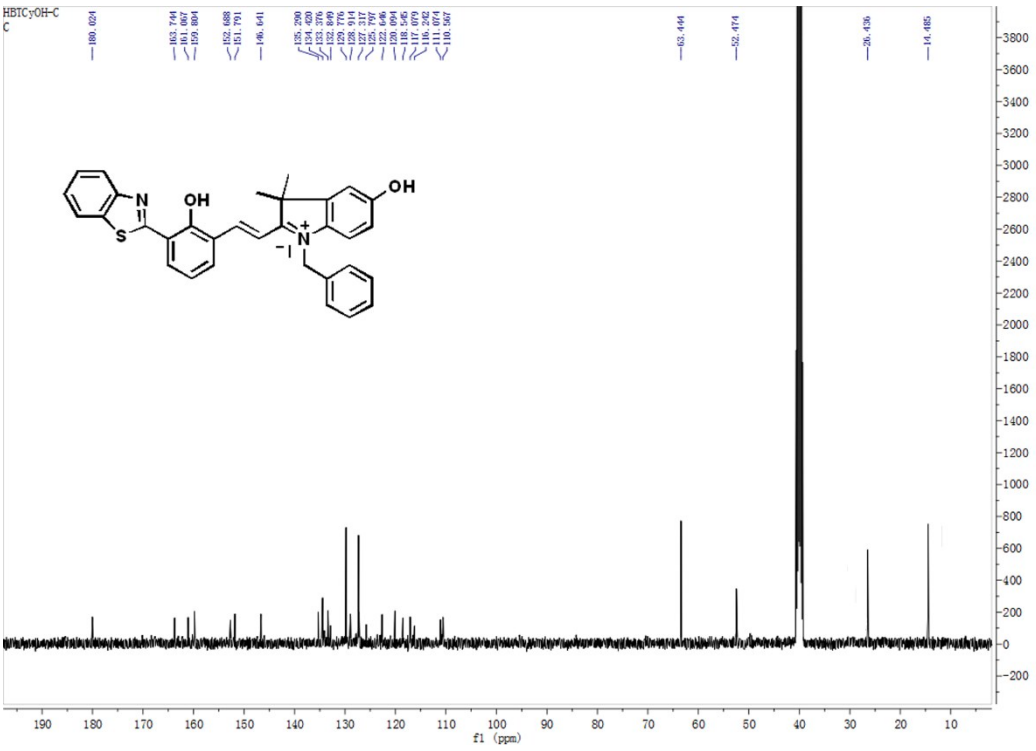


Figure S21. [13]C NMR of compound **HBTMC1** in DMSO-d6.

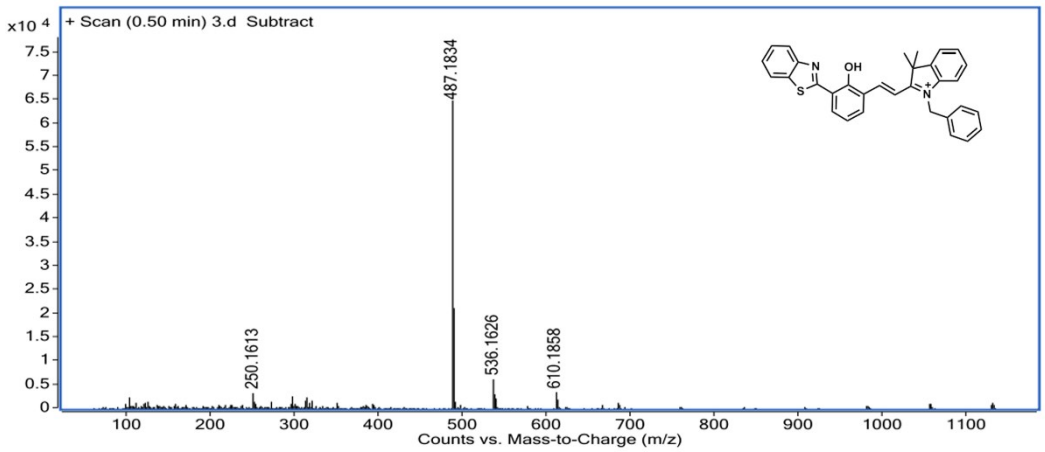


Figure S22. HRMS (ESI) of compound **HBTMC2**. *m/z* calcd for $C_{18}H_{20}NO$ (M^+): 487.1839. Found 487.1834.

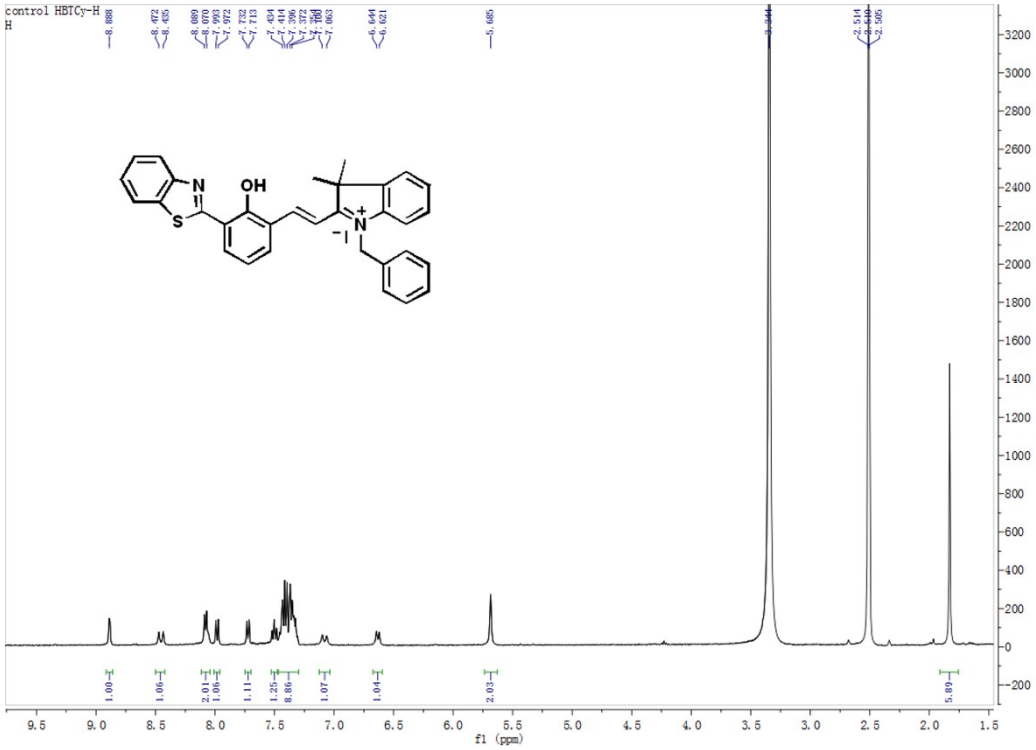


Figure S23. ^{1}H NMR of compound **HBTMC2** in DMSO-d6.

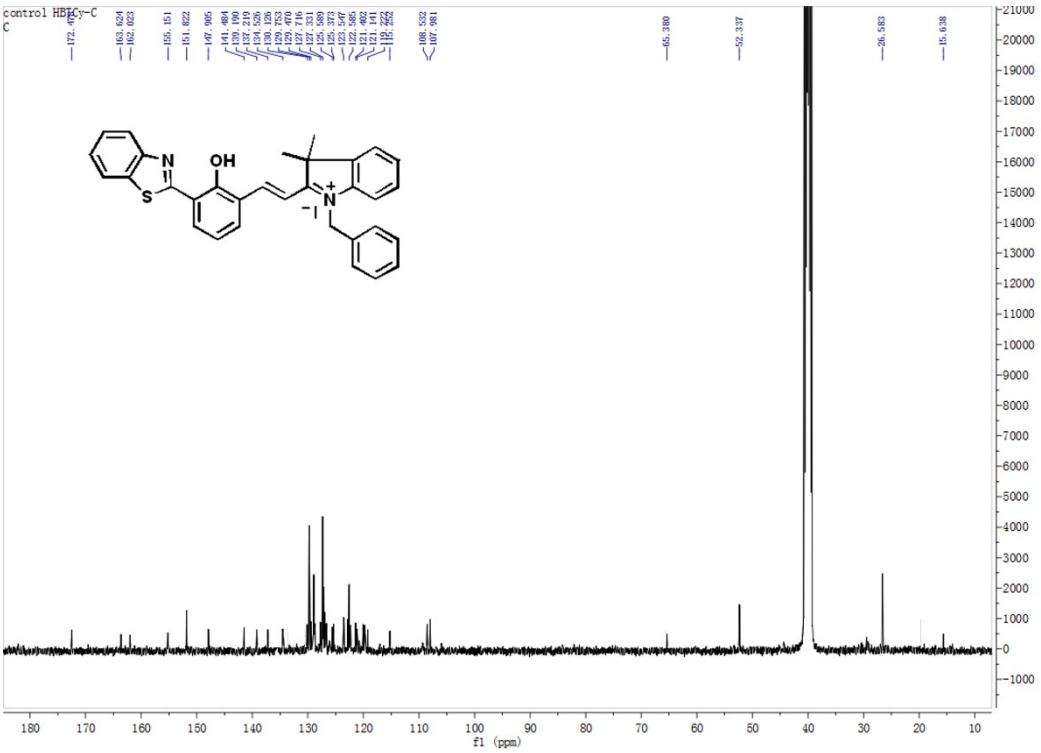


Figure S24. ^{13}C NMR of compound **HBTMC2** in DMSO-d6.

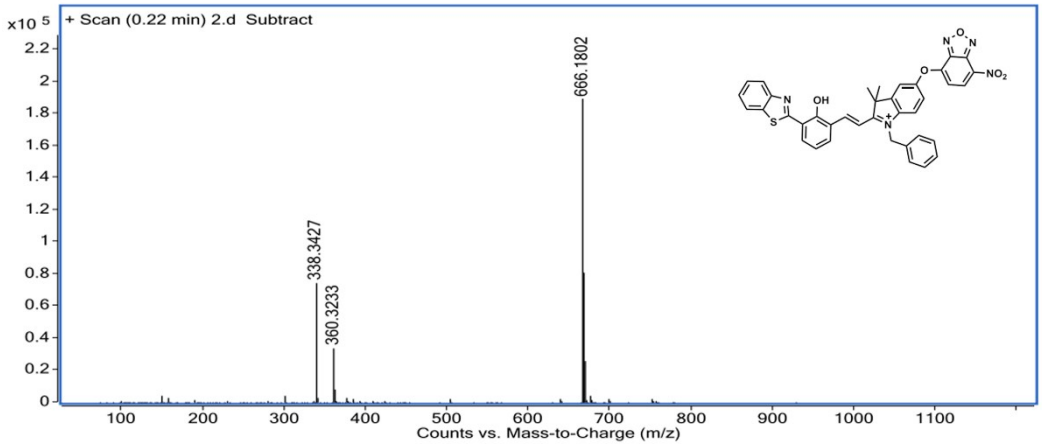


Figure S25. HRMS (ESI) of probe **HMN**. *m/z* calcd for $C_{18}H_{20}NO$ (M^+): 666.1806. Found 666.1802.

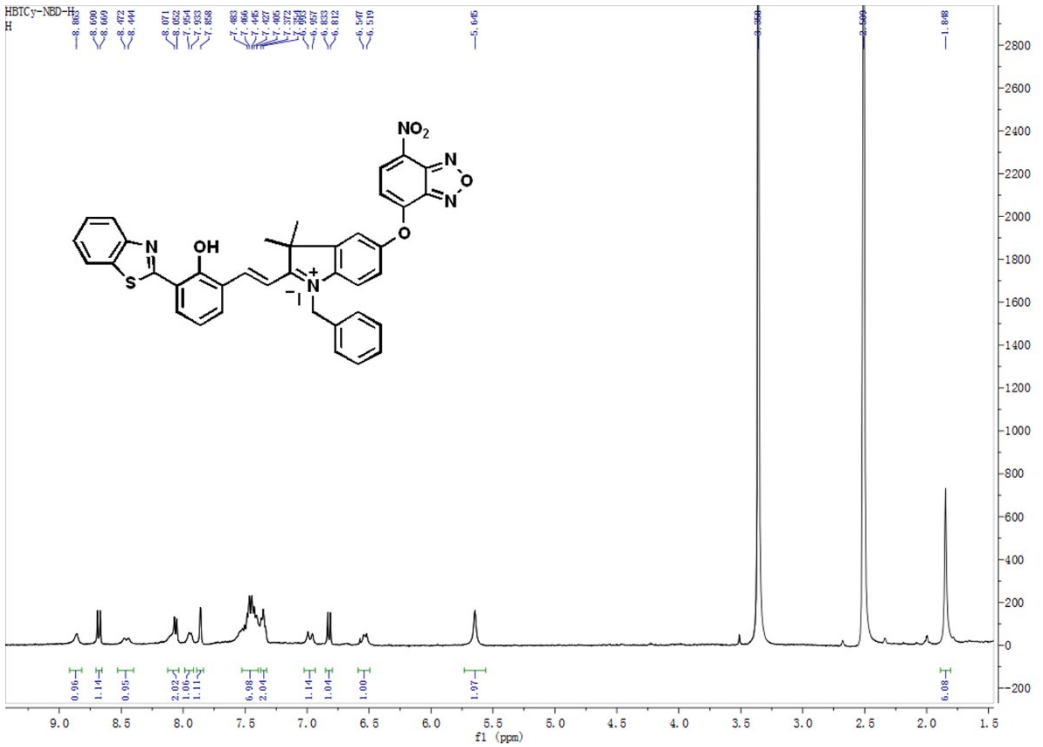


Figure S26. [1]H NMR of probe **HMN** in DMSO-d6.

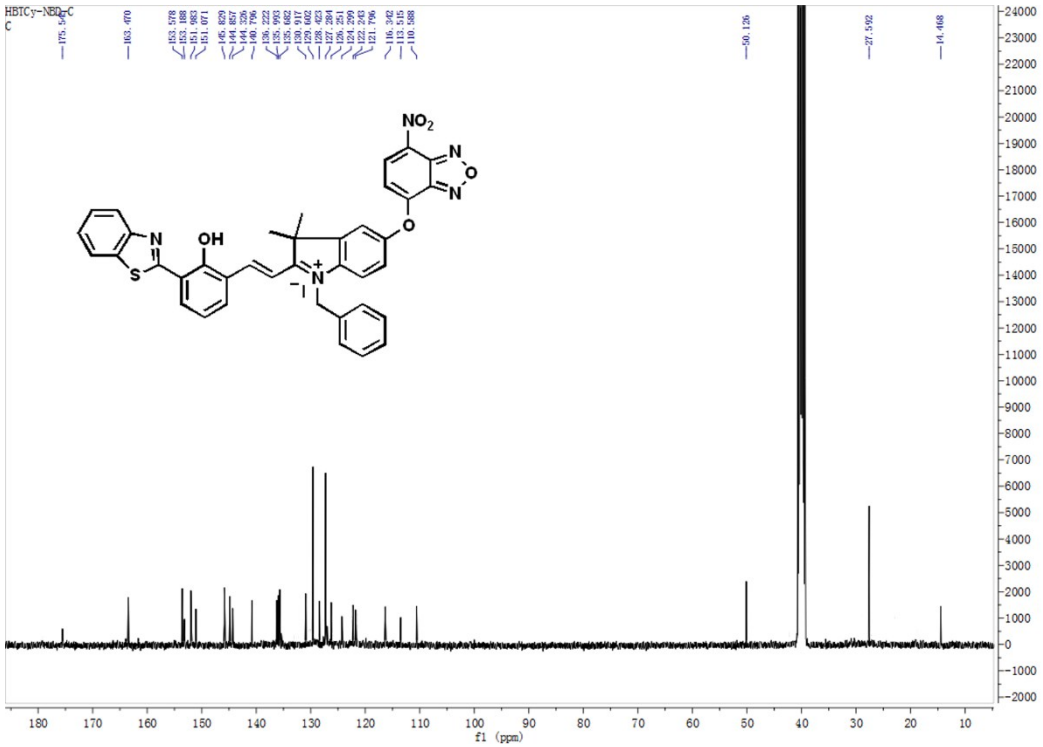


Figure S27. [13]C NMR of probe **HMN** in DMSO-d6.

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