Development of a novel H_2S and GSH detection cocktail for fluorescence imaging

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SUPPORTING INFORMATION

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General experimental for synthesis

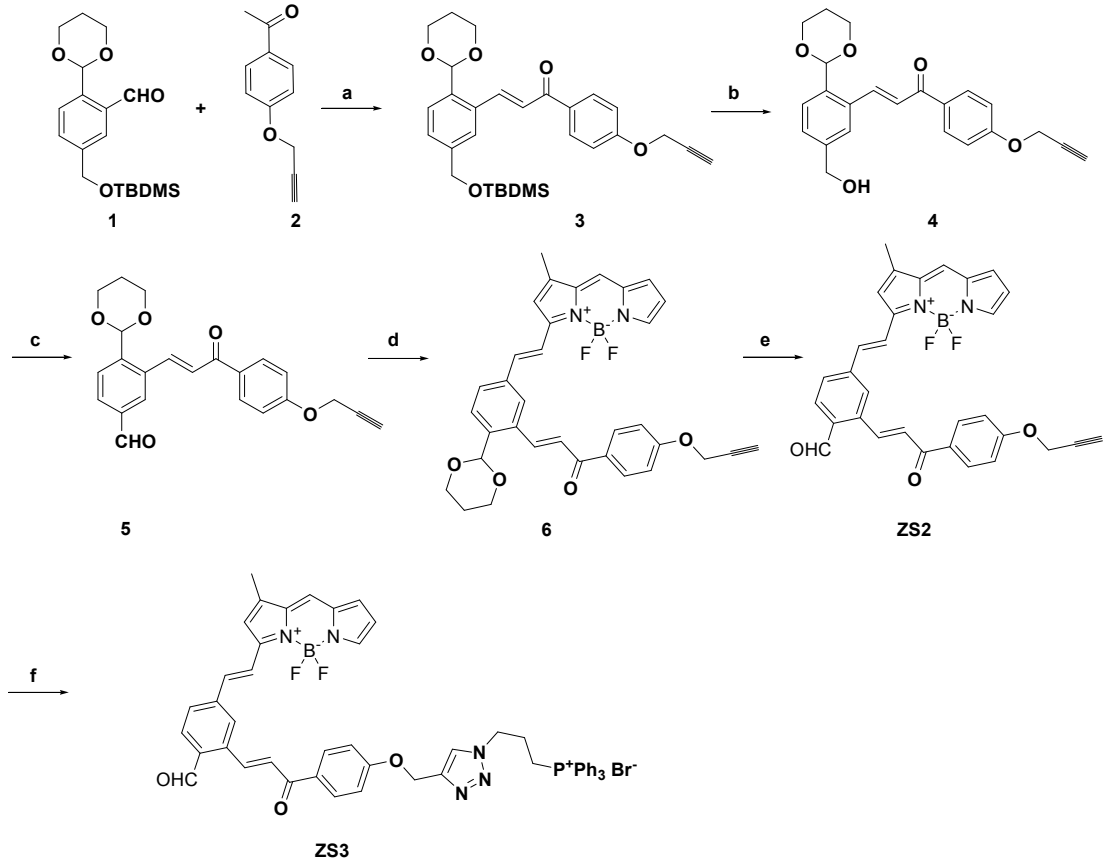
All reactions were carried out under an inert nitrogen atmosphere unless otherwise stated. All reagents were purchased from commercial suppliers and used without further purification unless otherwise stated. Acetonitrile (MeCN) and triethylamine (Et_3N) were purified by distillation over CaH_2. 3-Bromo-4-((tert-butyldimethylsilyloxy)methyl)benzaldehyde (**1**) and 4, 4-difluoro-1,3-dimethyl-4-bora-3a,4a-diaza-s-indacene were prepared referring to literature procedures with some modifications.[1]

Isolated yields refer to chromatographically and spectroscopically (^{1}H NMR) homogeneous materials. Reactions were monitored by thin-layer chromatography (TLC) carried out on Silica gel 60 F254 plates supplied by Qingdao Puke Separation Material Corporation using UV light as the visualizing agent and iodine as the developing agent. Flash column chromatography was performed using 200-300 mesh silica gel supplied by Qingdao Marine Chemical Factory, Qingdao, P. R. ^{1}H NMR spectra were recorded on a Bruker Fourier transform spectrometer (500 MHz or 400 MHz) at 25 °C. ^{13}C NMR spectra were recorded on a Bruker Fourier transform spectrometer (125 MHz or 100 Hz) spectrometer and were calibrated using residual undeuterated solvent as an internal reference (for $CDCl_3$: ^{1}H NMR = 7.26, ^{13}C NMR = 77.16; for DMSO: ^{1}H NMR = 2.50, ^{13}C NMR = 39.52). All chemical shifts were given in ppm and coupling constants (*J*) in Hz. The following abbreviations or combinations thereof were used to explain the multiplicities: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, b = broad. IR spectra were recorded on a Bruker Vector 22 spectrophotometer as KBr pellets. High resolution mass spectra (HRMS) were recorded on an Agilent 6224 TOF LC/MS spectrometer using ESI-TOF (electrospray ionization-time of flight).

[1] Y. Qian, J. Karpus, O. Kabil, S. Zhang, H. Zhu, R. Banerjee, J. Zhao and C. He, *Nat. Commun.*, 2011, **2**, 495; J. S. Lee, N. Y. Kang, Y. K. Kim, A. Samanta, S. Feng, H. K. Kim, M. Vendrell, J. H. Park and Y. T. Chang, *J. Am. Chem. Soc.*, 2009, **131**, 10077-82.

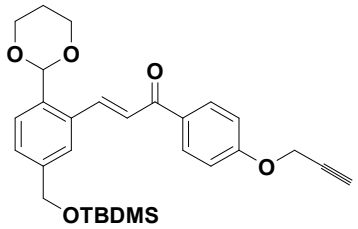
Synthesis and structure characterization data

Scheme S1. Synthesis of ZS series probes



Reagents and conditions: (a) MeOH, 5 N NaOH, ambient temperature, 10 h, 44%; (b) THF, *n*-$Bu_4N^+F^-$, ambient temperature, 2 h, 81%; (c) CH_2Cl_2, PCC, Na_2SO_4, ambient temperature, 30 min, 80%; (d) 4, 4-difluoro-1,3-dimethyl-4-bora-3a,4a-diaza-s-indacene, piperidine, CH_3COOH, toluene, reflux, Dean-stark trapper, 4 hours, 17%; (e) acetone, HCl (Conc.), ambient temperature, 10 min, 72%; (f) (3-azidopropyl)triphenylphosphonium bromide, ascorbic acid, Cu_2SO_4, THF/H_2O (5/2, v/v), ambient temperature, 12 h, 29%.

Synthesis of intermediate 3



3

To a solution of the aldehyde **1** (2.00 g, 5.95 mmol) and the ketone **2** (1.04 g, 5.85 mmol) in MeOH (20.0 mL) was added an aqueous solution of NaOH (5 N, 1.00 mL). After being stirred at ambient temperature for 10 hours, the reaction was quenched by the addition of H_2O (20 mL). The mixture was extracted with EtOAc (40 mL × 2). The combined organic phases were washed with H_2O (20 mL × 1) and brine (20 mL × 1) subsequently, dried over anhydrous Na_2SO_4 and concentrated by rotary evaporation. The remaining residue was purified by flash column chromatography (SiO_2, petroleum ether/EtOAc, 7:1) to give the product as a white solid (1.26 g, 44% yield).

Rf = 0.45 (5:1, petroleum ether:EtOAc)

m. p.: 99.7-100.8 °C

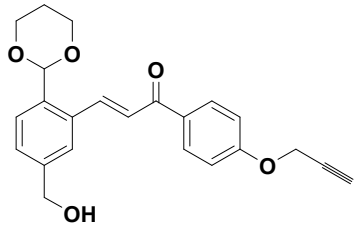
^{1}H NMR (400 MHz, $CDCl_3$) δ 8.31 (d, *J* = 15.6 Hz, 1H), 8.05 (d, *J* = 8.7 Hz, 2H), 7.72 (s, 1H), 7.59 (d, *J* = 8.0 Hz, 1H), 7.43 (d, *J* = 15.6 Hz, 1H), 7.35 (d, *J* = 8.0 Hz, 1H), 7.06 (d, *J* = 8.7 Hz, 2H), 5.75 (s, 1H), 4.78 (s, 4H), 4.27 (dd, *J* = 11.3, 4.7 Hz, 2H), 4.01 (t, *J* = 11.5 Hz, 2H), 2.57 (s, 1H), 2.34 – 2.17 (m, 1H), 1.45 (d, *J* = 13.5 Hz, 1H), 0.96 (s, 9H), -0.11 (s, 6H).

^{13}C NMR (101 MHz, $CDCl_3$) δ 189.16, 161.26, 142.53, 141.97, 136.43, 133.36, 131.98, 130.92, 127.56, 127.18, 124.17, 123.76, 114.78, 100.30, 77.92, 76.26, 67.65, 64.50, 55.97, 26.03, 25.78, 18.50, -5.11.

IR (cm^{-1}): 3230, 2933, 2857, 1650, 1601, 1246, 1109, 1011, 841

ESI-HRMS (m/z): $[M+H]^+$ calc'd. for $C_{29}H_{37}O_5Si$: 493.2410; found 493.2413.

Synthesis of intermediate 4



4

To the stirred solution of **3** (1.00 g, 2.02 mmol) in THF (50 mL) was added *n*-$Bu_4N^+F^-$ trihydrate (1.03 g, 3.30 mmol). The reaction was stirred at ambient temperature till the disappearance of the starting material as shown by TLC analysis, which required about 2 hours. After that, H_2O (30 mL) was added to quench the reaction and the crude product was extracted with EtOAc (50 mL × 2). The combined organic phases were washed with H_2O (30 mL × 1) and brine (30 mL × 1), dried over anhydrous Na_2SO_4 and concentrated under reduced pressure to give a white solid which was used without further purification (620 mg, 81% yield).

Rf = 0.38 (1:1, petroleum ether:EtOAc)

m. p.: 135.3-136.8 °C

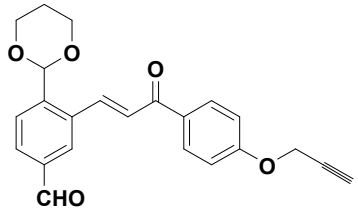
^{1}H NMR (500 MHz, $CDCl_3$) δ 8.26 (d, *J* = 15.6 Hz, 1H), 8.03 (d, *J* = 8.9 Hz, 2H), 7.70 (s, 1H), 7.59 (d, *J* = 7.9 Hz, 1H), 7.41 (d, *J* = 15.6 Hz, 1H), 7.35 (d, *J* = 7.9 Hz, 1H), 7.04 (d, *J* = 8.9 Hz, 2H), 5.73 (s, 1H), 4.77 (d, *J* = 2.4 Hz, 2H), 4.71 (s, 2H), 4.27 (dt, *J* = 10.9, 5.0 Hz, 2H), 4.01 (td, *J* = 12.4, 2.3 Hz, 2H), 2.57 (t, *J* = 2.4 Hz, 1H), 2.35-2.20 (m, 1H), 1.45 (d, *J* = 11.6 Hz, 1H).

^{13}C NMR (126 MHz, $CDCl_3$) δ 189.15, 161.32, 142.06, 141.60, 136.91, 133.58, 131.75, 131.02, 128.34, 127.39, 124.97, 123.77, 114.78, 100.13, 77.89, 76.32, 67.69, 64.73, 55.97, 25.75.

IR (cm^{-1}): 3440, 3185, 2861, 1659, 1606, 1502, 1390, 1327, 1244, 1175, 1089, 1019, 838

ESI-HRMS (m/z): $[M+H]^+$ calc'd. for $C_{23}H_{23}O_5$: 379.1545; found 379.1548.

Synthesis of intermediate 5



5

The intermediate **4** (800 mg, 2.11 mmol) was dissolved in dry CH_2Cl_2 (20 mL). The solution was cooled to 0°C with

an ice bath, to which was added rapidly anhydrous Na_2SO_4 (652 mg, 4.59 mmol) and PCC (682 mg, 3.16 mmol). After being stirred at ambient temperature for 30 min, TLC analysis was carried out which showed the total transformation of the starting material. The mixture was then filtered over celite. The filtrate was concentrated by rotary evaporation and purified by flash column chromatography (SiO_2, petroleum ether/EtOAc, 5:1) to give the product as a white solid (636 mg, 80% yield).

Rf = 0.45 (3:1, petroleum ether:EtOAc)

m. p.: 139.3-140.5 °C

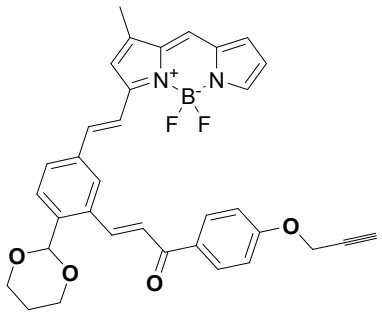
^{1}H NMR (500 MHz, $CDCl_3$) δ 10.05 (s, 1H), 8.23 (d, *J* = 15.6 Hz, 1H H), 8.20 (s, 1H), 8.06 (d, *J* = 8.8 Hz, 2H), 7.88 (dd, *J* = 8.0, 1.2 Hz, 1H), 7.84 (d, *J* = 7.9 Hz, 1H), 7.53 (d, *J* = 15.6 Hz, 1H), 7.06 (d, *J* = 8.8 Hz, 2H), 5.78 (s, 1H), 4.77 (d, *J* = 2.3 Hz, 2H), 4.27 (dt, *J* = 13.2, 6.6 Hz, 2H), 4.04 (td, *J* = 12.4, 2.3 Hz, 2H), 2.58 (t, *J* = 2.4 Hz, 1H), 2.30 – 2.19 (m, 1H), 1.49 (d, *J* = 11.6 Hz, 1H).

^{13}C NMR (126 MHz, $CDCl_3$) δ 191.80, 188.40, 161.46, 143.03, 140.06, 136.64, 134.61, 131.44, 131.01, 127.95, 127.55, 124.99, 114.84, 99.29, 77.78, 76.37, 67.67, 55.94, 25.63.

IR (cm^{-1}): 3256, 1697, 1660, 1601, 1224, 1089, 1013, 830

ESI-HRMS (m/z): $[M+H]^+$ calc'd. for $C_{23}H_{21}O_5$: 377.1389; found 377.1391.

Synthesis of intermediate 6



6

The intermediate **5** (92 mg, 0.24 mmol) and 4,4-difluoro-1,3-dimethyl-4-bora-3a,4a-diaza-s-indacene (57 mg, 0.26 mmol) was dissolved in toluene (10 mL), to which was added a solution of AcOH (0.10 mL) and piperidine (0.10 mL) in toluene (10 mL). The mixture was stirred under reflux for 4 hours with continuous separation of H_2O by a Dean-stark apparatus. After being cooled to ambient temperature, the mixture was diluted with EtOAc (20 mL), transferred to a separation funnel, washed with H_2O (10 mL × 1) and brine (10 mL × 1), dried over anhydrous Na_2SO_4, and concentrated by rotarory evaporation. The crude product was then purified by flash column chromatography (SiO_2, petroleum ether/EtOAc, 4:1) to give the product as a red solid (24 mg, 17% yield).

Rf = 0.55 (2:1, petroleum ether:EtOAc)

m. p.: >250 °C

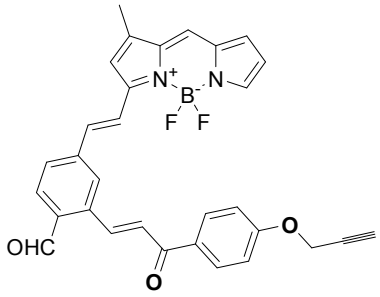
^{1}H NMR (500 MHz, DMSO) δ 8.21 (d, *J* = 14.2 Hz, 1H), 8.19 (s, 1H), 8.16 (d, *J* = 8.8 Hz, 2H), 7.87 (s, 1H), 7.83 (d, *J* = 16.4 Hz, 1H), 7.81-7.74 (m, 3H), 7.67 (d, *J* = 8.1 Hz, 1H), 7.51 (d, *J* = 16.4 Hz, 1H), 7.21 – 7.16 (m, 3H), 7.11 (s, 1H), 6.55 (dd, *J* = 3.5, 1.9 Hz, 1H), 5.81 (s, 1H), 4.96 (d, *J* = 2.0 Hz, 2H), 4.19 (dd, *J* = 11.1, 4.5 Hz, 2H), 4.00 (t, *J* = 11.3 Hz, 2H), 3.68 (s, 1H), 2.36 (s, 3H), 2.10 – 1.97 (m, 1H), 1.50 (d, *J* = 13.2 Hz, 1H).

^{13}C NMR (126 MHz, DMSO) δ 188.00, 161.14, 157.45, 145.52, 140.36, 139.65, 139.40, 138.90, 137.28, 136.19, 133.86, 133.22, 130.94, 130.92, 128.05, 127.75, 127.39, 127.03, 125.66, 124.13, 118.52, 117.61, 117.05, 114.94, 99.48, 78.87, 78.74, 66.88, 55.79, 25.36, 11.30.

IR (cm^{-1}): 2925, 2856, 1597, 1395, 1260, 1068, 1027, 807

ESI-HRMS (m/z): $[2M+Na]^+$ calc'd. for $C_{68}H_{58}B_2F_4N_4NaO_8$: 1179.4275; found 1179.4282.

Synthesis of ZS2



ZS2

The intermediate **6** (25 mg, 0.043 mmol) was dissolved in acetone (2 mL) and the solution was cooled to 0°C with an ice bath. HCl (10 N, 0.05 mL) was added dropwise to the vigorously stirred solution and the ice bath was removed after the addition. After 10 min, H_2O (2 mL) was added to quench the reaction. The mixture was diluted with EtOAc (15 mL) and then transferred to a separating funnel. The organic phase was washed with H_2O (5 mL × 1) and brine (5 mL × 2), dried over anhydrous Na_2SO_4, and concentrated by rotary evaporation. The crude product was then purified by flash column chromatography (SiO_2, petroleum ether/EtOAc, 3:1) to give the product as a red solid (16 mg, 72% yield).

Rf = 0.40 (2:1, petroleum ether:EtOAc)

m. p.: >250 °C

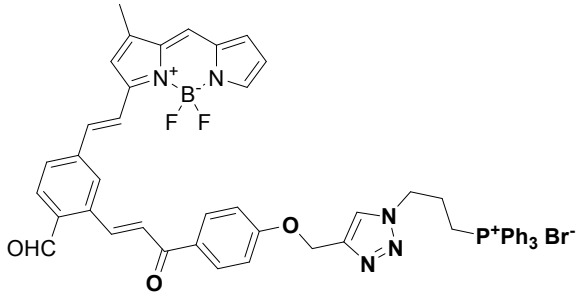
^{1}H NMR (500 MHz, DMSO) δ 10.25 (s, 1H), 8.54 (d, *J* = 15.5 Hz, 1H), 8.23 – 8.16 (m, 3H), 8.06 (d, *J* = 8.6 Hz, 1H), 7.95 – 7.83 (m, 5H), 7.61 (d, *J* = 16.4 Hz, 1H), 7.21 (d, *J* = 3.7 Hz, 1H), 7.17 (d, *J* = 8.9 Hz, 2H), 7.12 (s, 1H), 6.58 (dd, *J* = 3.9, 2.0 Hz, 1H), 4.96 (d, *J* = 2.3 Hz, 2H), 3.67 (t, *J* = 2.3 Hz, 1H), 2.37 (s, 3H).

^{13}C NMR (126 MHz, DMSO) δ 192.69, 187.44, 161.29, 156.35, 145.28, 140.74, 140.56, 139.67, 137.91, 137.23, 137.03, 134.01, 133.66, 133.60, 131.00, 130.76, 128.62, 128.28, 127.23, 126.78, 126.36, 120.77, 117.69, 117.54, 114.97, 78.86, 78.70, 55.80, 11.28.

IR (cm^{-1}): 2923, 2854, 1682, 1586, 1399, 1285, 1140, 1066, 830

ESI-HRMS (m/z): $[M+Na]^+$ calc'd. for $C_{31}H_{23}BF_2N_2NaO_3$: 543.1667; found 543.1667.

Synthesis of ZS3



ZS3

(3-Azidopropyl)triphenylphosphonium bromide (29 mg, 0.068 mmol) was dissolved in a mixture of THF (5 mL) and H_2O (2 mL), followed by the addition of probe **ZS2** (40 mg, 0.077 mmol) and an aqueous solution of $CuSO_4$ (4 μmol). The atmosphere of the system was thoroughly displaced with N_2. Ascorbic acid (2 mg, 0.01 mmol) was then added quickly. After being stirred at ambient temperature overnight (ca. 12 h), the mixture was diluted with CH_2Cl_2 (20 mL). The organic phase was washed with H_2O (5 mL × 1) and brine (5 mL × 1), dried over anhydrous

Na_2SO_4, and concentrated by rotary evaporation. The crude product was then purified by flash column chromatography (SiO_2, CH_2Cl_2/MeOH, 30:1) to give the product as a red solid (19 mg, 29% yield).

Rf = 0.38 (8:1, CH_2Cl_2:MeOH)

m. p.: >250 ^oC

^{1}H NMR (500 MHz, $CDCl_3$) δ 10.33 (d, *J* = 4.4 Hz, 1H), 8.56 (s, 1H), 8.50 (d, *J* = 15.5 Hz, 1H), 8.02 (d, *J* = 8.5 Hz, 2H), 7.93 (d, *J* = 8.0 Hz, 1H), 7.86-7.64 (m, 20H), 7.46-7.38 (m, 2H), 7.10 (d, *J* = 8.6 Hz, 2H), 7.01 (d, *J* = 7.6 Hz, 1H), 6.81 (s, 1H), 6.50 (dd, *J* = 3.9, 1.9 Hz, 1H), 5.29 (s, 2H), 5.02 (t, *J* = 7.5 Hz, 2H), 3.83 (t, *J* = 7.5 Hz, 2H), 2.37 – 2.31 (m, 5H).

IR (cm^{-1}): 2955, 2923, 2853, 1685, 1586, 1438, 1397, 1285, 1261, 1141, 1064, 815

ESI-HRMS (m/z): $[M\text{-}Br]^+$ calc'd. for $C_{52}H_{44}BF_2N_5O_3P$: 866.3243; found 866.3254.

Fluorometric analysis method

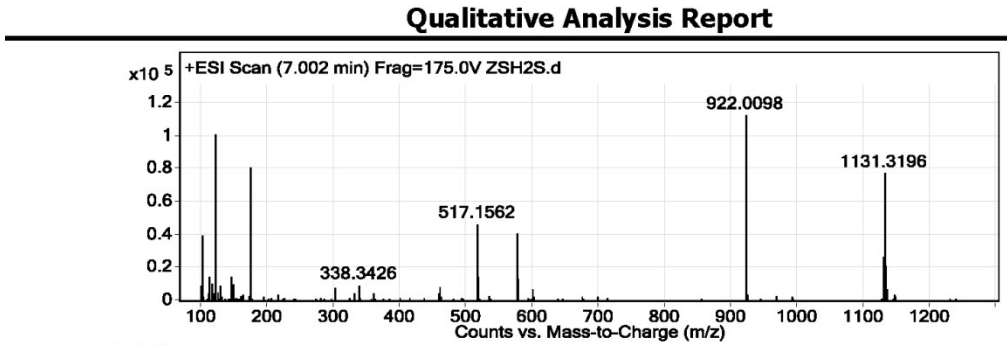
Phosphate buffer saline (PBS, 10 mM, pH 7.4) was prepared with deionized water and purged with nitrogen for 5 minutes before use. **ZS1** or **ZS2** was dissolved in DMSO to make a 5.0 mM stock solution, which was diluted to 5 μM for measurements. Sodium bisulfide (NaHS) was used as an aqueous sulfide source. Both NaHS and other analytes were dissolved in the above mentioned deoxygenated PBS to make stock solutions of 100 mM which were diluted to desired concentrations for use. All fluorescence measurements were carried out at ambient temperature in PBS with 20% CH_3CN and 0.1% DMSO as cosolvents. Excitation wavelength was kept at 530 nm and fluorescent spectra between 540 nm and 650 nm were collected. Fluorescence measurements were carried out on a JASCO FP 6500 spectrofluorimeter. Slit widths for excitation and emission were kept at 3 nm and 5 nm respectively and the sensitivity of the instrument was kept low. All fluorometric experiments were performed in triplicate.

Quantum yield

The fluorescence quantum yields of **ZS2** in PBS (10 mM, pH 7.4, 20% acetonitrile) before and after incubation with GSH/H_2S were were obtained by comparison with fluorescein (Φ 0.95, in 0.1 M NaOH) with the following equation where Σ[F] is the integrated fluorescence intensity, Abs is absorbance at λ_{ex} 496 nm, and *n* represents the refractive index. For PBS and 0.1 M NaOH, we used refractive indices of 1.334 and 1.335 respectively.

$$\phi_{sample} = \phi_{standard} \bullet \frac{Abs_{standard} \bullet \sum F_{sample}}{Abs_{sample} \bullet \sum F_{standard}} \bullet \frac{n_{sample}^2}{n_{standard}^2}$$

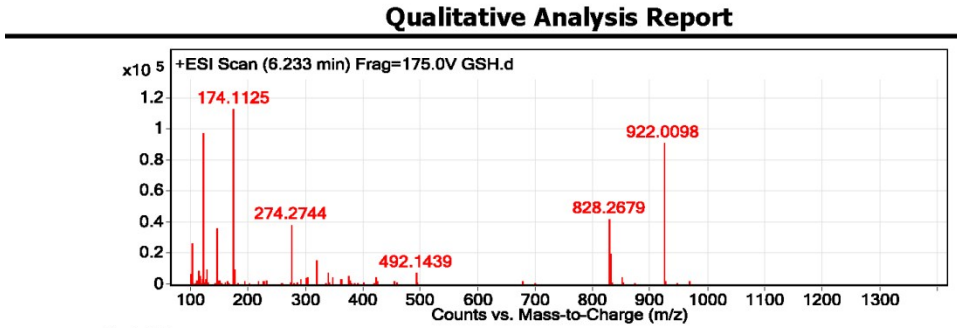
	λ_{em} (max)	ε (cm^2/mol)	Φ
ZS2	-	0.1228 (604 nm)	0.007
ZS2 (5 μM) + NaHS (400 μM)	562 nm	0.0955 (552 nm)	0.175
ZS2 (5 μM) + GSH (1 mM)	562 nm	0.1076 (554 nm)	0.162



Peak List

m/z	z	Abund
102.1281		39540
121.0509	1	101329.4
174.1126	1	81288.4
517.1562	1	46647.1
577.155	1	41107.3
922.0098	1	112811.6
1130.3243		26752.2
1131.3196	1	77505.3
1132.3219	1	48068.7
1133.322	1	21520.3

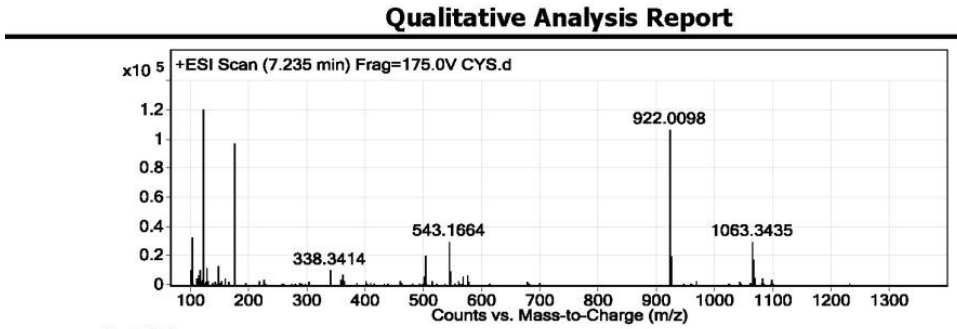
Fig. S1 HRMS analysis of the **ZS2**-NaHS reaction mixture. Both the *m/z* 577.1550 and *m/z* 1131.3196 signals are in agreement with the **ZS2**-H_2S (1:1) adduct ($[M + Na]^+$ Cal. 577.1545, and $[2M+Na]^+$ Cal. 1131.3192 for the latter).



Peak List

m/z	z	Abund
102.1279		26941.9
121.0509	1	97630.4
146.1175		36776.1
174.1125	1	113135.9
274.2744	1	38808.8
318.3004		16486
828.2679	1	42496.2
829.27	1	20068.2
922.0098	1	91293.8
923.0114	1	17281.2

Fig. S2 HRMS analysis of the **ZS2**-GSH reaction mixture. The *m/z* 828.2679 signal is in agreement with the **ZS2**-GSH (1:1) adduct ($[M+H]^+$ Cal. 828.2686).



Peak List

m/z	z	Abund
102.128		33018.2
121.0509	1	120864.5
146.1171		13763
174.1127	1	97940.3
501.1783	1	21312.2
543.1664	1	30289.1
922.0098	1	107054.2
923.0119	1	20588.9
1063.3435	1	30152.3
1064.3461	1	17764.3

Fig. S3 HRMS analysis of the **ZS2**-Cys reaction mixture. No signals other than that of **ZS2** ([M + Na]$^+$ Cal. 543.1667) was found.

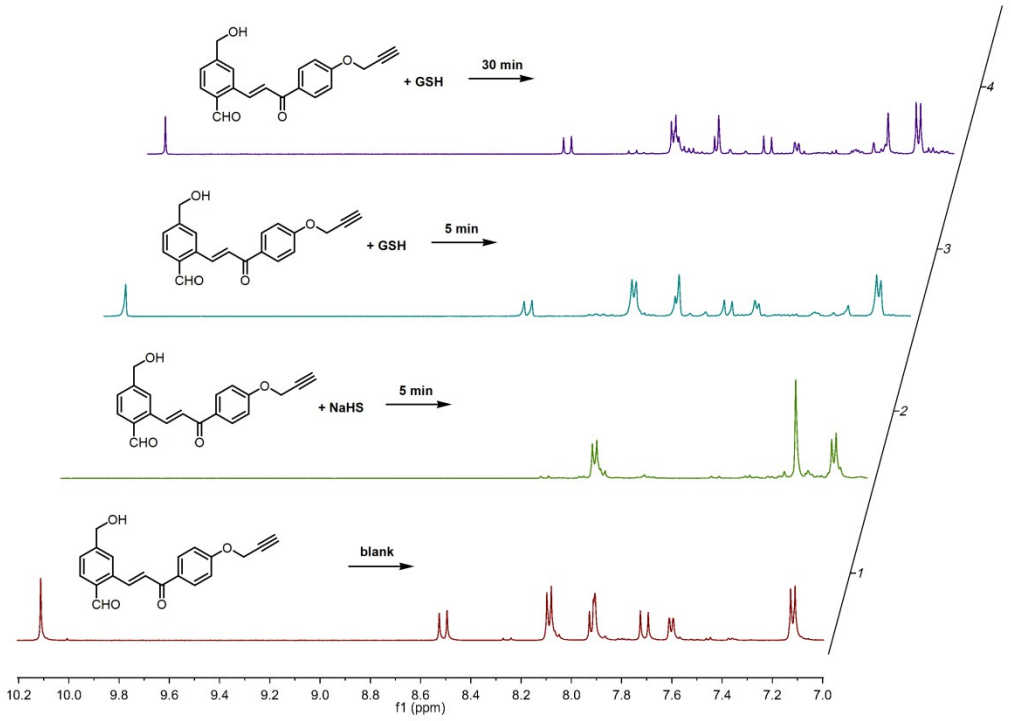


Fig. S4 ^{1}H NMR traces of the thiol-reaction trigger, compound **7**, in the presence of H_2S or GSH.

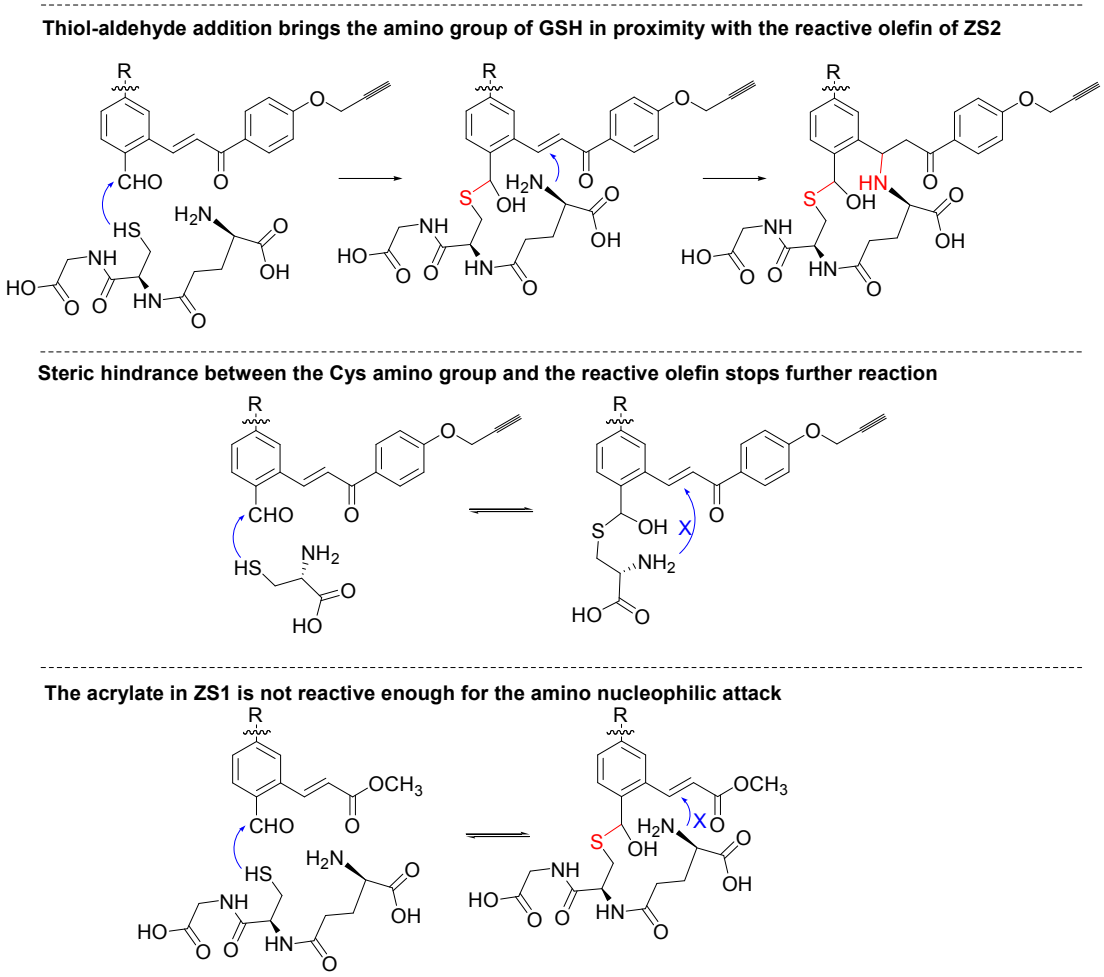


Fig. S5 Postulated detection mechanism by which **ZS2** responds to both GSH and H_2S, and **ZS1** only responds to H_2S. It is hypothesized that both favorable spatial distance and olefin reactivity are required for the amino-olefin nucleophilic addition to take place.

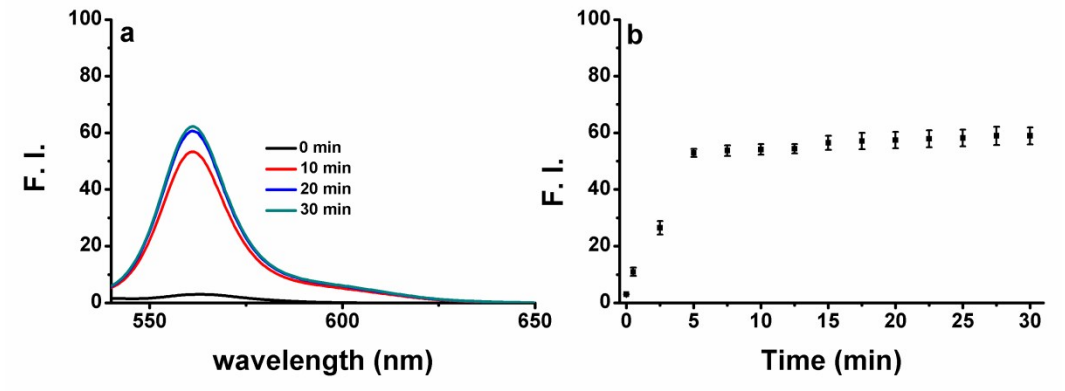


Fig. S6 Fluorescence spectra of **ZS2** (5 μM) in PBS (10 mM, pH 7.4, 20% CH_3CN) after incubating with NaHS (50 μM) for various time with excitation at 530 nm.

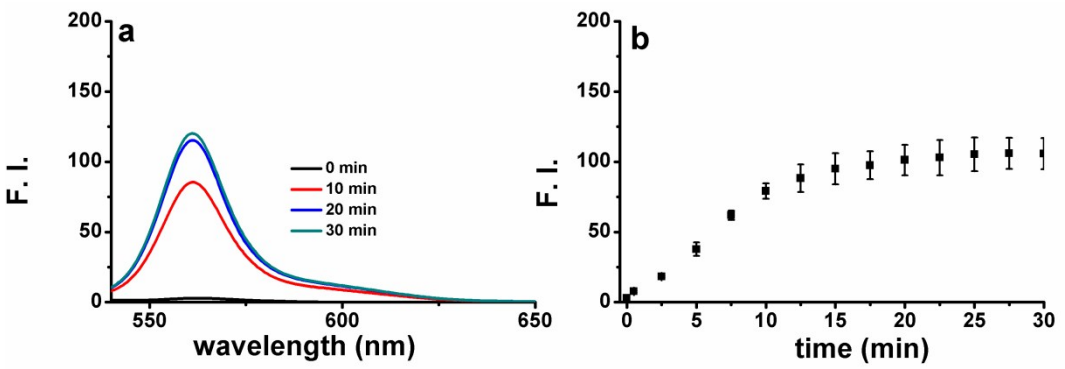


Fig. S7 Fluorescence spectra of **ZS2** (5 μM) in PBS (10 mM, pH 7.4, 20% CH_3CN) after incubating with NaHS (100 μM) for various time with excitation at 530 nm.

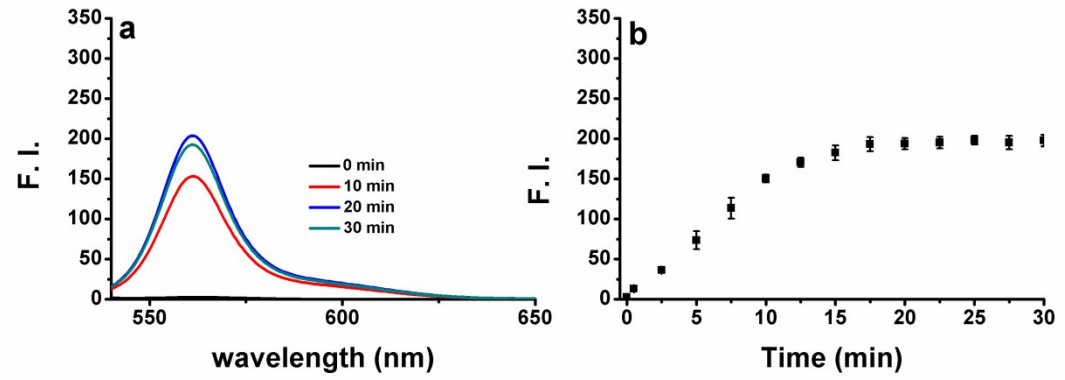


Fig. S8 Fluorescence spectra of **ZS2** (5 μM) in PBS (10 mM, pH 7.4, 20% CH_3CN) after incubating with NaHS (200 μM) for various time with excitation at 530 nm.

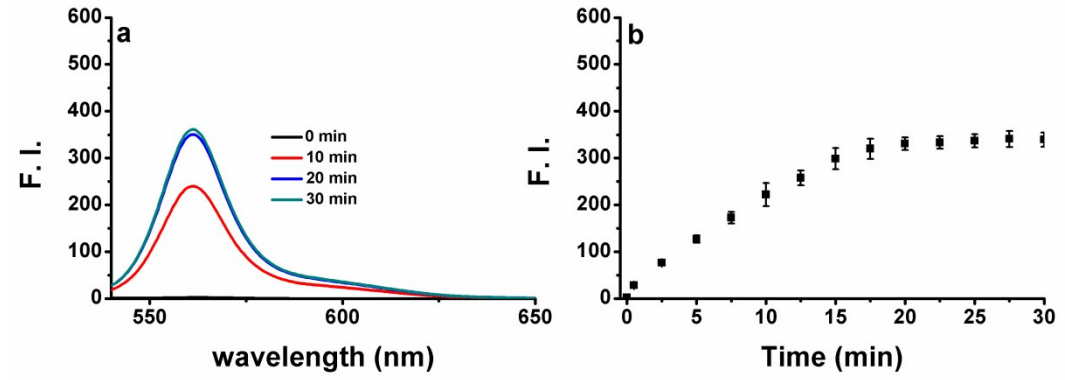


Fig. S9 Fluorescence spectra of **ZS2** (5 μM) in PBS (10 mM, pH 7.4, 20% acetonitrile) after incubating with NaHS (400 μM) for various time with excitation at 530 nm.

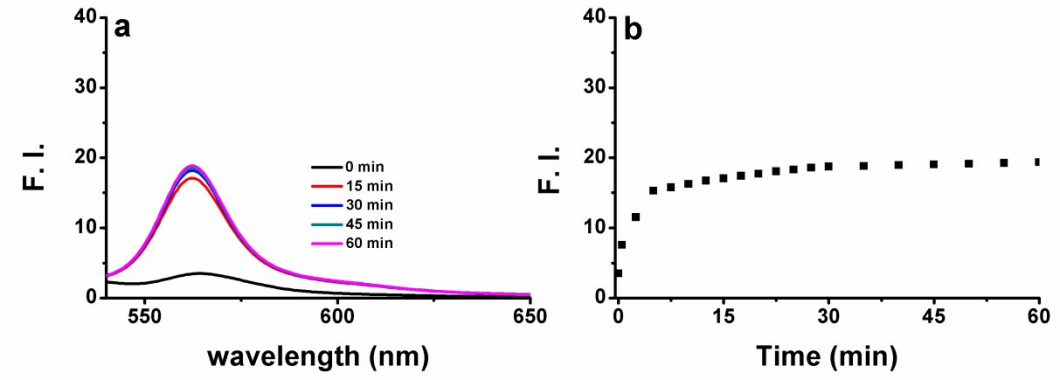


Fig. S10 Fluorescence spectra of **ZS2** (5 μM) in PBS (10 mM, pH 7.4, 20% acetonitrile) after incubating with GSH (50 μM) for various time with excitation at 530 nm.

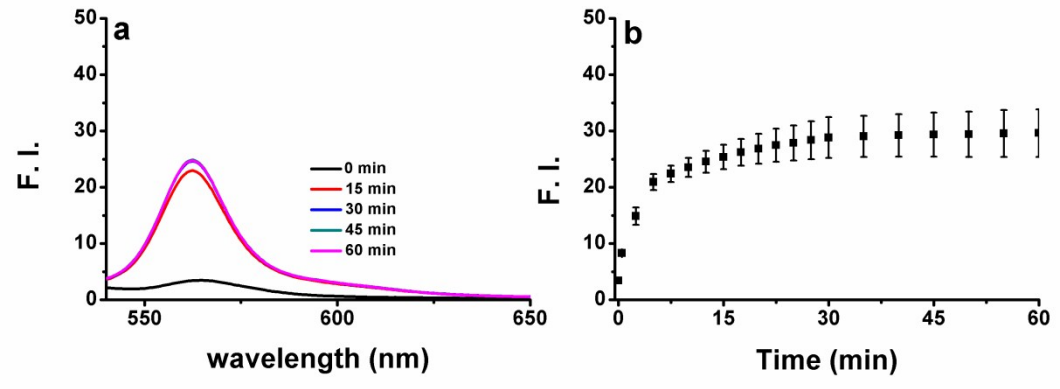


Fig. S11 Fluorescence spectra of **ZS2** (5 μM) in PBS (10 mM, pH 7.4, 20% acetonitrile) after incubating with GSH (100 μM) for various time with excitation at 530 nm.

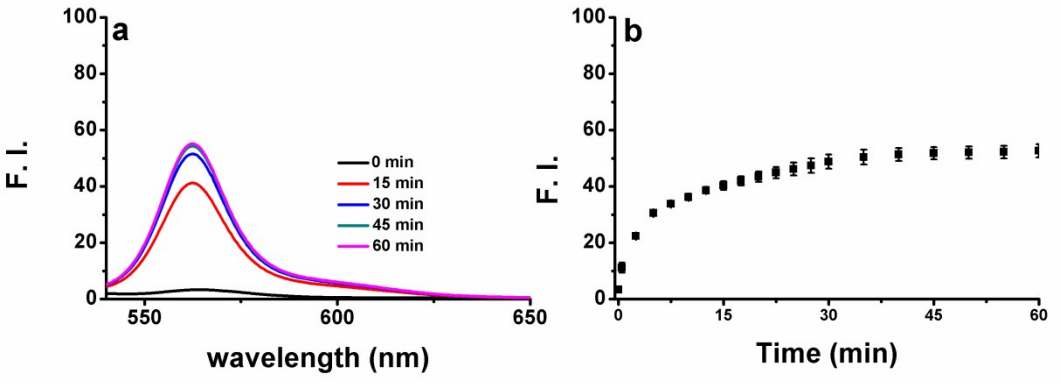


Fig. S12 Fluorescence spectra of **ZS2** (5 μM) in PBS (10 mM, pH 7.4, 20% acetonitrile) after incubating with GSH (200 μM) for various time with excitation at 530 nm.

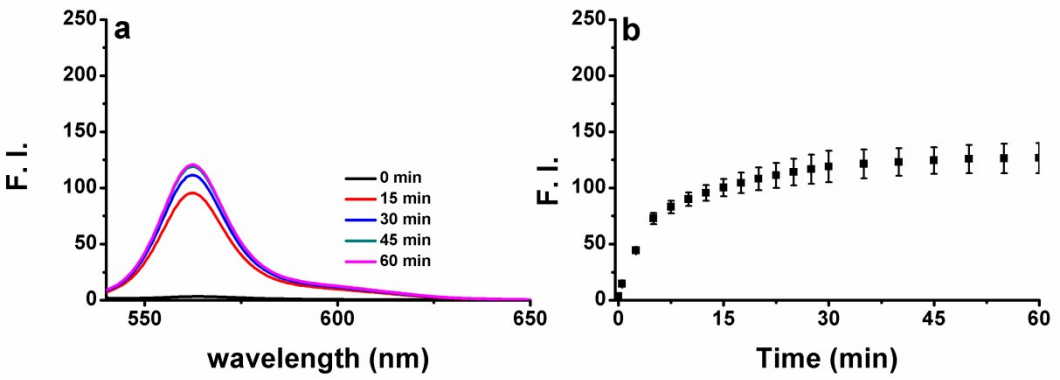


Fig. S13 Fluorescence spectra of **ZS2** (5 μM) in PBS (10 mM, pH 7.4, 20% acetonitrile) after incubating with GSH (400 μM) for various time with excitation at 530 nm.

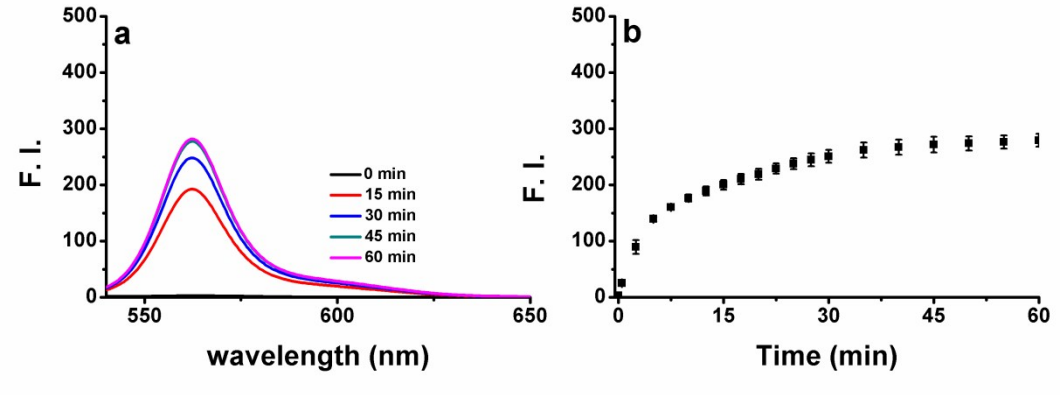


Fig. S14 Fluorescence spectra of **ZS2** (5 μM) in PBS (10 mM, pH 7.4, 20% acetonitrile) after incubating with GSH (800 μM) for various time with excitation at 530 nm.

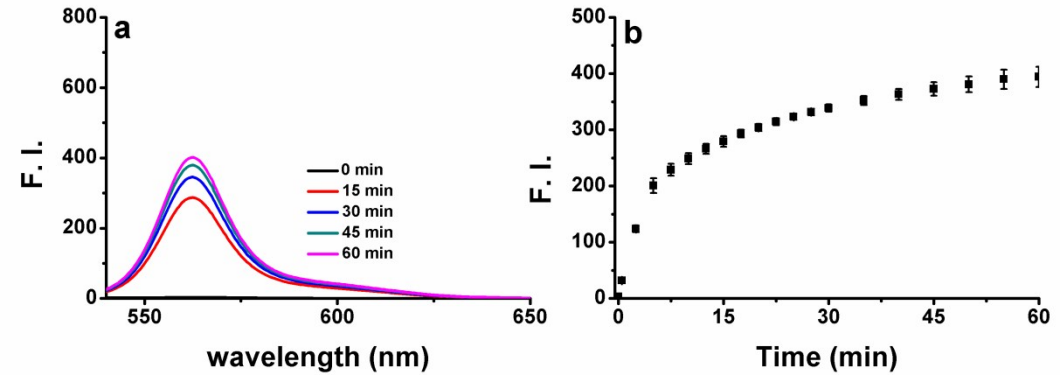


Fig. S15 Fluorescence spectra of **ZS2** (5 μM) in PBS (10 mM, pH 7.4, 20% acetonitrile) after incubating with GSH (1000 μM) for various time with excitation at 530 nm.

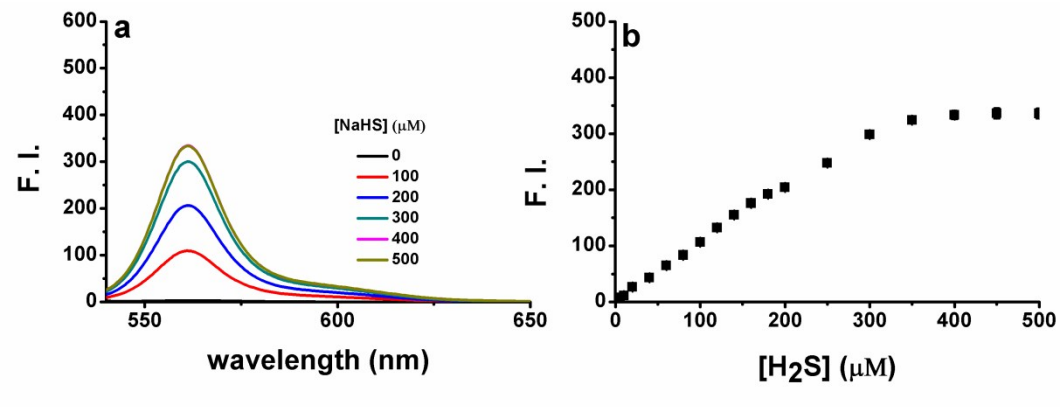


Fig. S16 Fluorescence spectra of **ZS2** (5 μM) in PBS (10 mM, pH 7.4, 20% acetonitrile) after incubating with various concentrations of NaHS for 1 hour (λ_{ex} 530 nm).

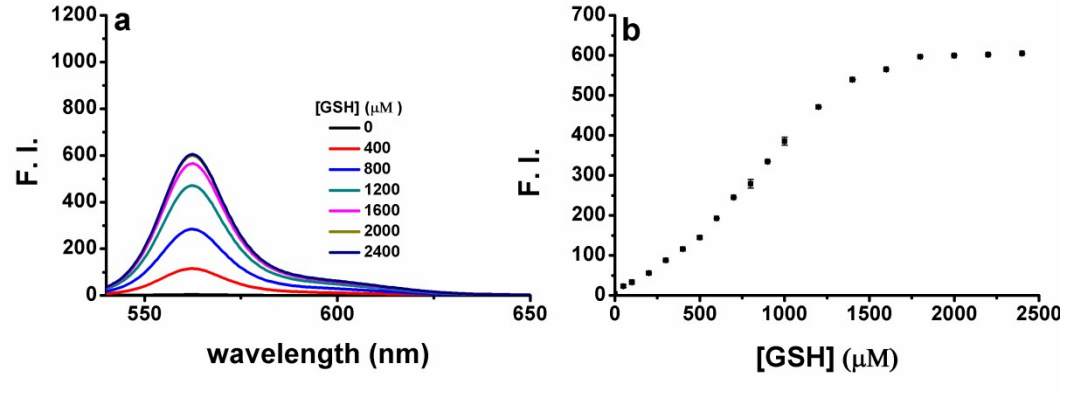


Fig. S17 Fluorescence spectra of **ZS2** (5 μM) in PBS (10 mM, pH 7.4, 20% acetonitrile) after incubating with various concentrations of GSH for 1 hour (λ_{ex} 530 nm).

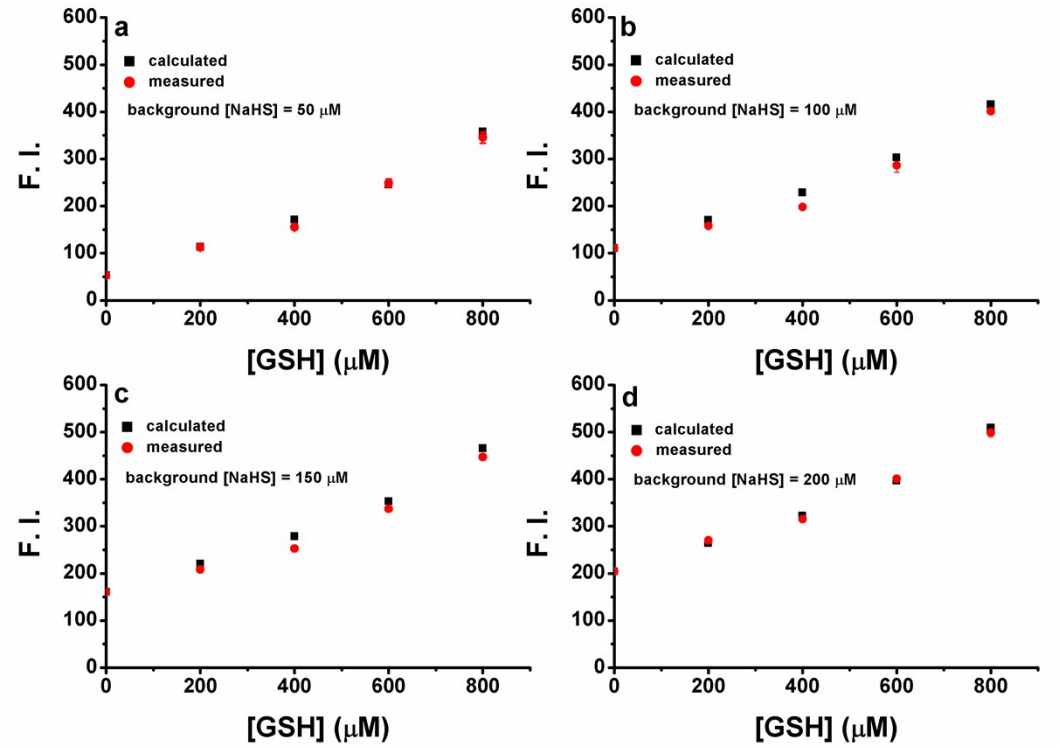


Fig. S18 Fluorescence responses of probe **ZS2** to the co-existing GSH and H_2S. Results showed that the measured fluorescence intensity of **ZS2** (5 μM) at 562 nm in the presence of various concentrations of H_2S and GSH is almost equal to the sum (calculated) of the intensities when **ZS2** was treated respectively with H_2S and GSH of corresponding concentrations. Data shown were the fluorescence intensity at 562 nm of **ZS2**/GSH/H_2S mixture after 1 hour of incubation in PBS (10 mM, pH 7.4, 20% acetonitrile) with excitation at 530 nm.

Biology Methods

Methods for the quantification of endogenous GSH and H_2S in fresh rat plasma employing ZS1/ZS2 detection cocktail

Sprague-Dawley (SD) rats (male, 2-3 months old, 200–250 g) were group housed on a 12-h light/dark cycle at a constant temperature of 22 ± 1 °C with 40–60% humidity. All animal studies were approved by the Committees for Animal Experiments of Zhejiang University in China. All the sample preparation procedures below were carried out at 4°C.

Endogenous H_2S measurement

Freshly collected rat blood (1.2 mL) was divided into two parts. One aliquot (0.60 mL) was treated with acetonitrile (0.40 mL) to precipitate the protein. The mixture was centrifuged in an eppendorf tube at 10 000 rpm for 5 min. The supernatant liquid was added into equal volume of PBS buffer (1.0 mL) to get the plasma sample (plasma 30%, v/v) for endogenous H_2S detection. The sample was pipetted to different eppendorf tubes with each tube containing 98 μL liquid. 1 μL $Zn(Ac)_2$ (100 mM, final concentration 1 mM, as 0 point), deionized H_2O (as X point), NaHS (1.0 mM, final concentration 10 μM, as X+10 μM point), NaHS (2.0 mM, final concentration 20 μM, as X+20 μM point), NaHS (3.0 mM, final concentration 30 μM, as X+30 μM point), NaHS (4.0 mM, final concentration 40 μM, as X+40 μM point), were spiked into the tubes respectively, then 1 μL **ZS1** (0.5 mM, final concentration 5 μM) was added. The fluorescence spectra were collected after the mixtures were incubated at 37 °C for 60 min and the intensity at 562 nm was plotted against H_2S concentration to calculate endogenous H_2S concentration (λ_{ex} = 530 nm). Rat 1, rat 2 and rat 3 represent the results of three different rats.

Endogenous GSH

The other aliquot of fresh plasma (0.60 mL) was treated with both acetonitrile (0.40 mL) to precipitate the protein and $ZnCl_2$ (final 1 mM) to precipitate endogenous H_2S. The mixture was centrifuged in an eppendorf tube at 10 000 rpm for 5 min. The supernatant liquid was added into equal volume of PBS buffer (1.0 mL) to get the plasma sample (plasma 30%, v/v) for endogenous GSH detection. The sample was pipetted to different eppendorf tubes with each tube containing 98 μL liquid. 1 μL *N*-methyl maleimide (1.0 M, final concentration 10 mM, as 0 point), deionized H_2O (as X point), GSH (10.0 mM, final concentration 100 μM, as X+100 μM point), GSH (20.0 mM, final concentration 200 μM, as X+200 μM point), GSH (30.0 mM, final concentration 300 μM, as X+300 μM point), GSH (40.0 mM, final concentration 400 μM, as X+400 μM point), were spiked into the tubes respectively, then 1 μL **ZS2** (0.5 mM, final concentration 5 μM) was added. The fluorescence spectra were collected after the mixtures were incubated at 37 ^oC for 60 min and the intensity at 562 nm was plotted against GSH concentration to calculate endogenous GSH concentration (λ_{ex} = 530 nm).

Cell imaging methods

Cell culture.

MEF, EA.hy926 and Hela cells were maintained in Dulbecco's Modified Eagle's Medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% Fetal bovine serum (Gibco, GlandIsland, NY, USA). All the cell lines were passaged every 2-3 days and cultured in a 37 ^oC humidified incubator under an atmosphere of 5% CO_2 in air.

Imaging assay in MEF and EA. hy926 cells.

For the experiments, cells (12000 per well) were seeded on a black 96-well microplate with optically clear bottom (Greiner bio-one, Germany) and were allowed to adhere overnight. Before treatment, MEF and EA.hy926 cells were cultured in DMEM without FBS for 3h. Then Cells were incubated with **ZS2** (5 μM, in DMEM) for 20 min to load the probe. After three times of quick wash with PBS (pH = 7.4), the cells were then incubated with GSH (0, 0.25, 0.5, 1mM) and NaHS (0, 0.1, 0.25, 0.5 mM), or both of them for 30 min at 37 ^oC. Before observation, the cells were washed three times with PBS buffer solution (pH = 7.4) .

For the MEF cells, the fluorescent images were recorded using Operetta high content imaging system (Perkinelmer, USA). The excitation channel of 520 to 550 nm and the emission channel of 560 to 630 nm were used. Data were mean of three independent experiments and at least 500 cells for each condition were analyzed and plotted by Columbus analysis system (Perkinelmer, USA).

For the EA. hy926 cells, the fluorescent images were recorded on a ZEISS LSM780 confocal microscopy with excitation at 561 nm and emission at 565-617 nm. Data were mean of three independent experiments.

Colocalization assay with ZS3 and MitoTracker Green in Hela cells

HeLa Cells were incubated with **ZS3** (20 μM) and Mito-Tracker Green (Beyotime) in DMEM for 30 min. After being washed with PBS three times, the cells were fixed with 4% paraformaldehyde. The fluorescent images were recorded using confocal microscope (Olymbus, Japan). The green fluorescence of Mito-Tracker Green was excited with 490 nm, and the emission wavelengths was 516nm. The red fluorescence of **ZS3** was excited with 559 nm, and the emission wavelengths was 603nm.

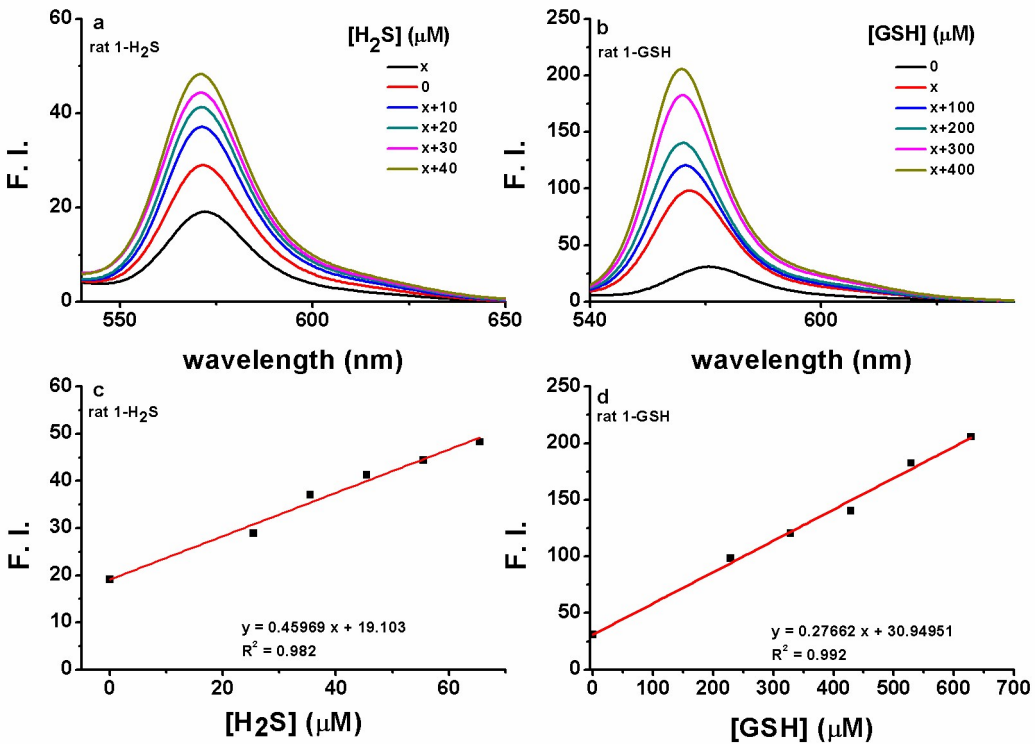


Fig. S19 Determination of endogenous H_2S (a, c) and GSH (b, d) in rat 1. Concentrations of endogeous H_2S and GSH in the 30% plasma solution of rat 1 were 25.5 μM, 228.5 μM, respectively.

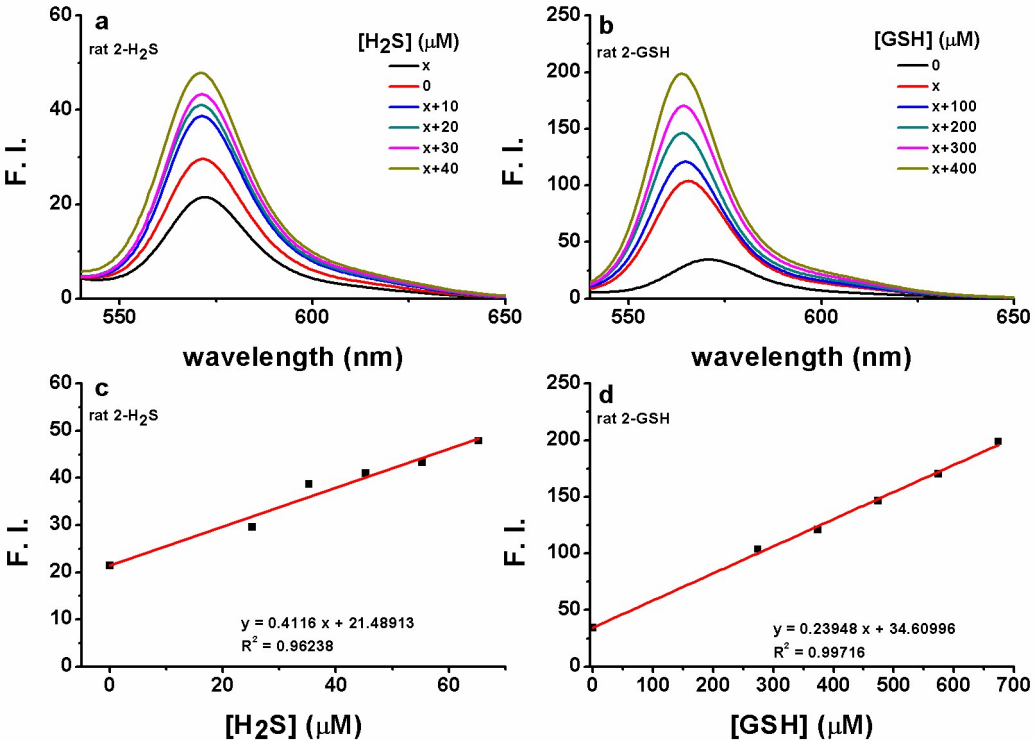


Fig. S20 Determination of endogenous H_2S (a, c) and GSH (b, d) in rat 2. Concentrations of endogeous H_2S and GSH in the 30% plasma solution of rat 1 were 25.2 μM, 273.9 μM, respectively.

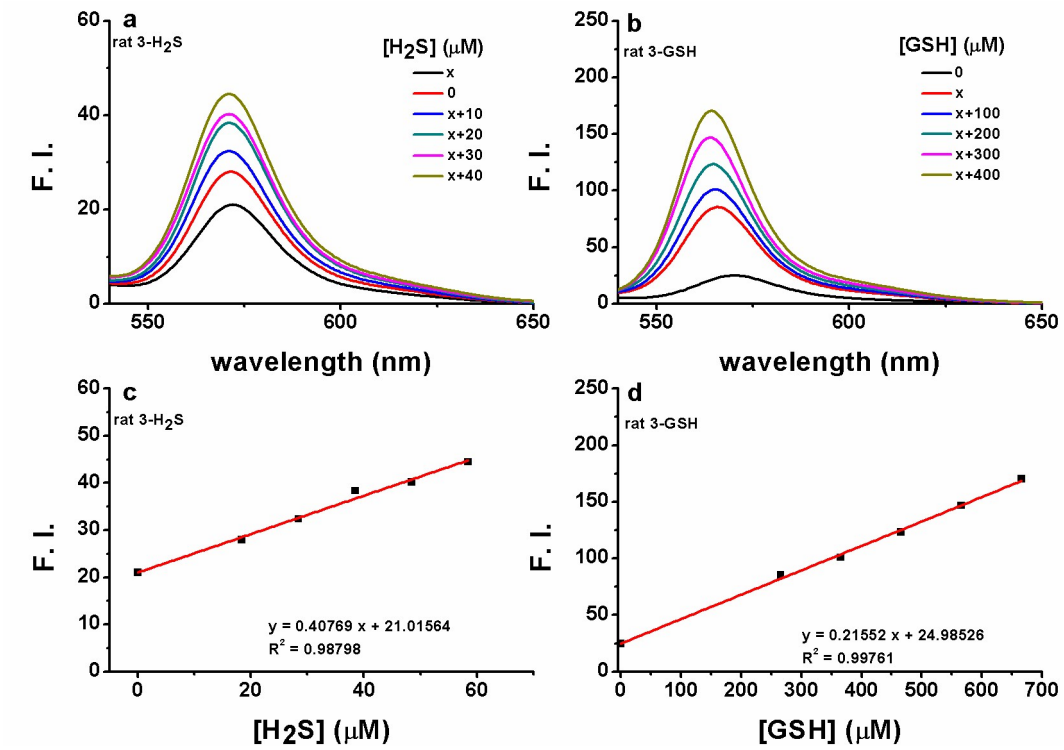


Fig. S21 Determination of endogenous H_2S (a, c) and GSH (b, d) in rat 3. Concentrations of endogeous H_2S and GSH in the 30% plasma solution of rat 1 were 18.4 μM, 265.6 μM, respectively.

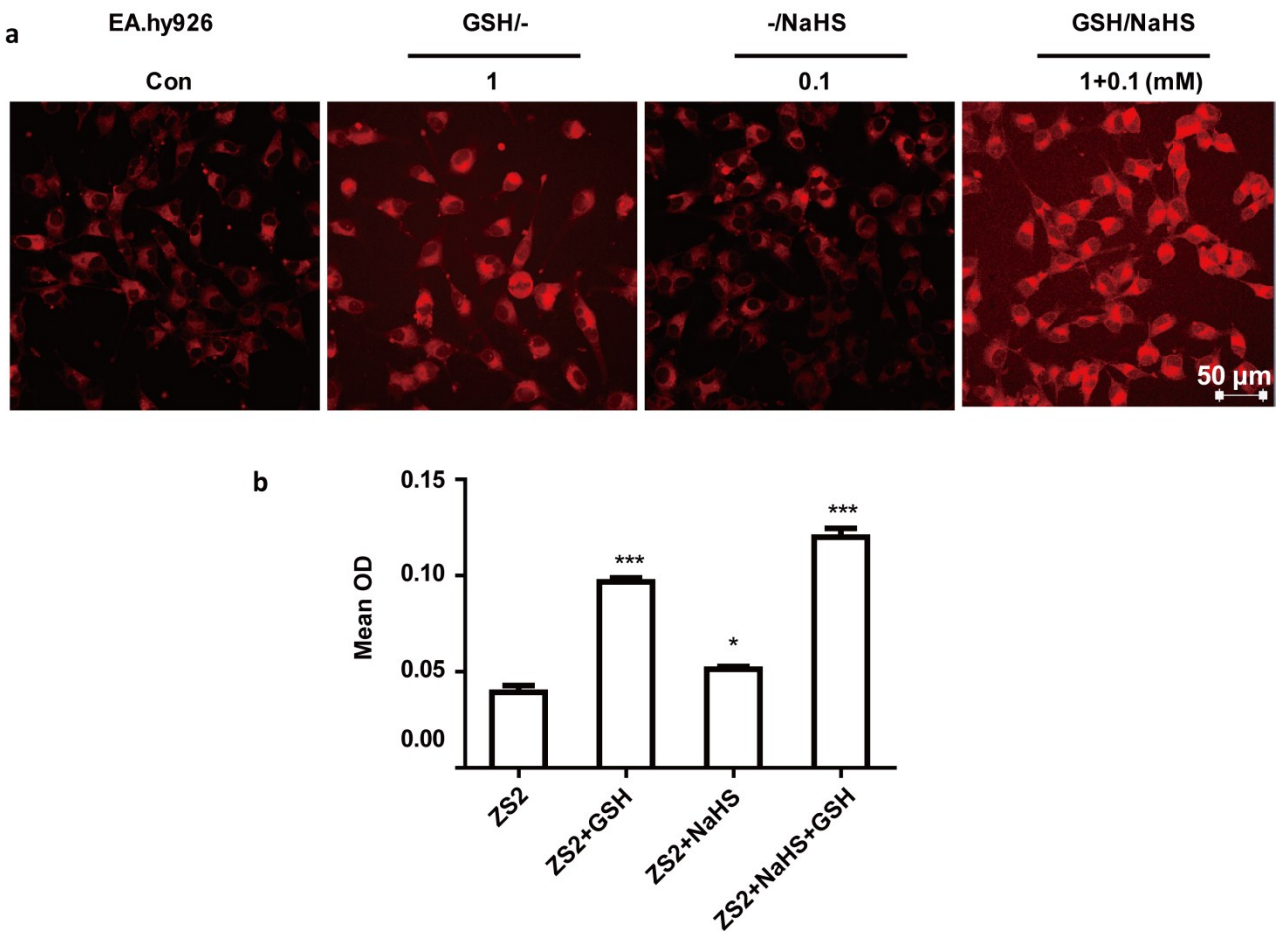


Fig. S22 Fluorescence confocal microscopic images of GSH and H_2S in EA. hy926 cells. Cells were preloaded with **ZS2** (5 μM) and then incubated without or with GSH (1 mM), NaHS (0.1 mM), both GSH (1 mM) and NaHS (0.1 mM). Emission was collected at 565-617 nm with excitation at 561 nm on a ZEISS LSM780.

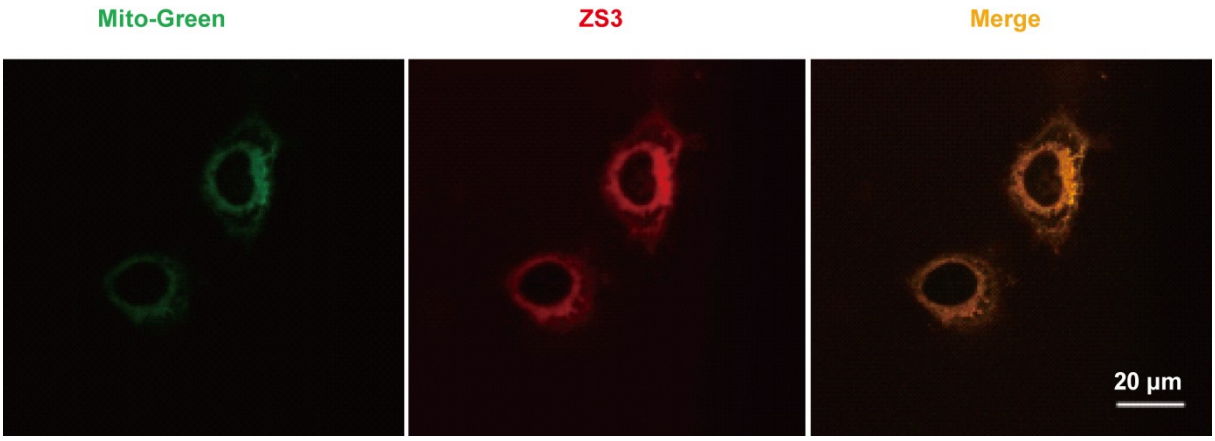
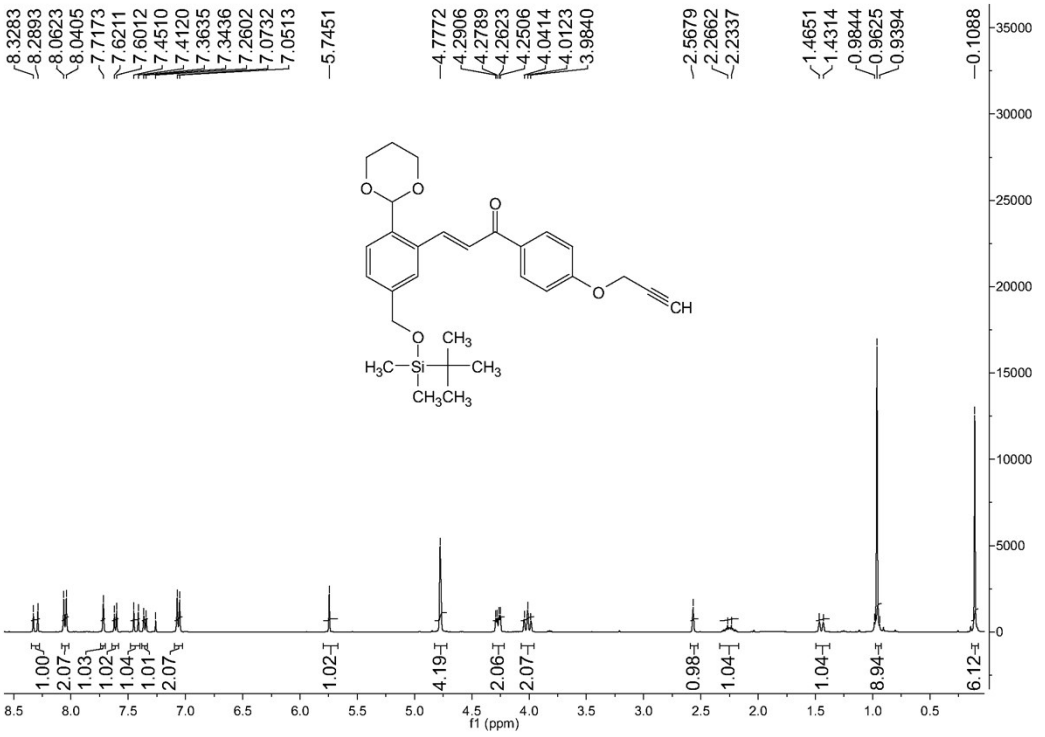
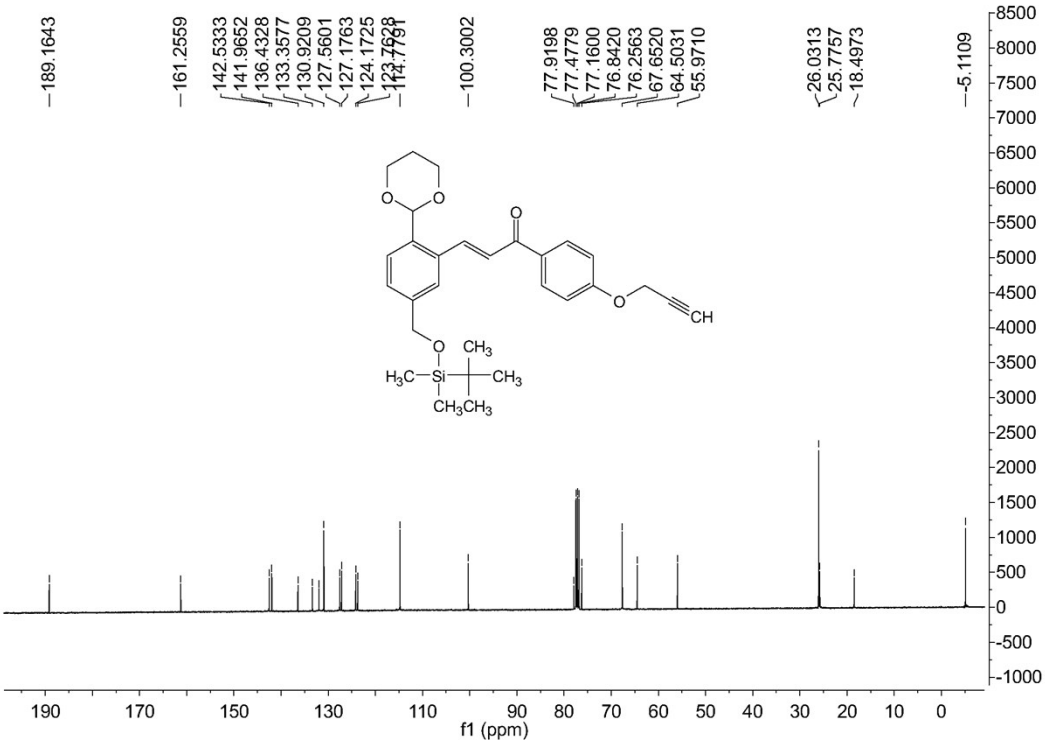
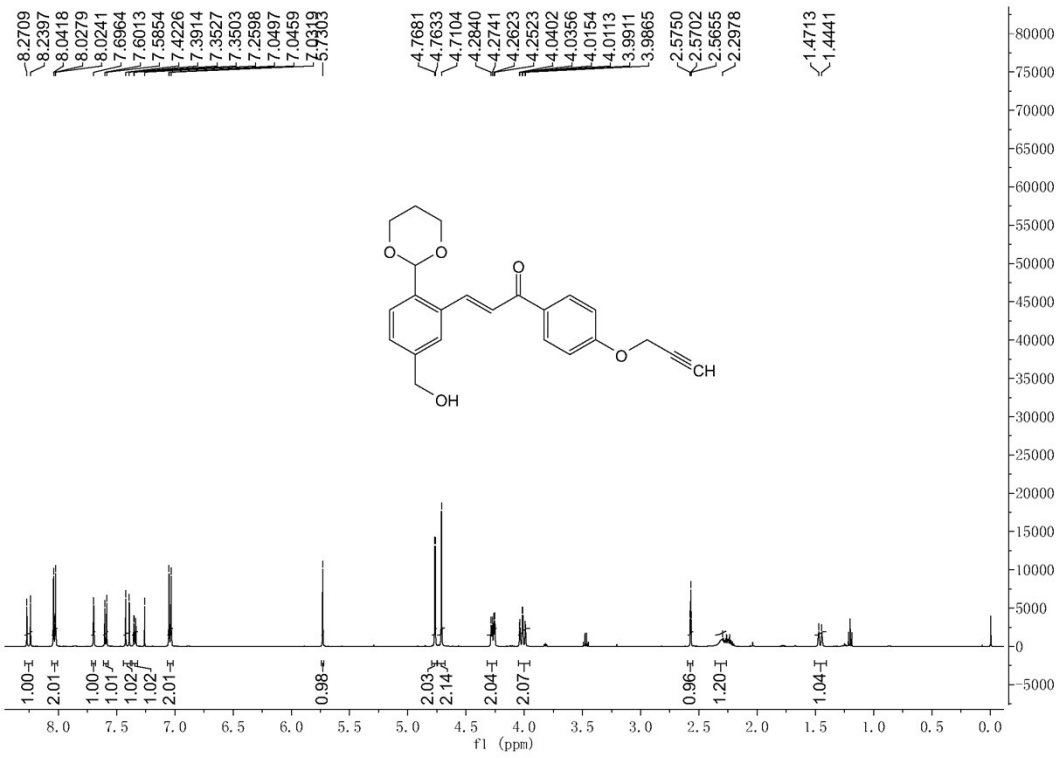


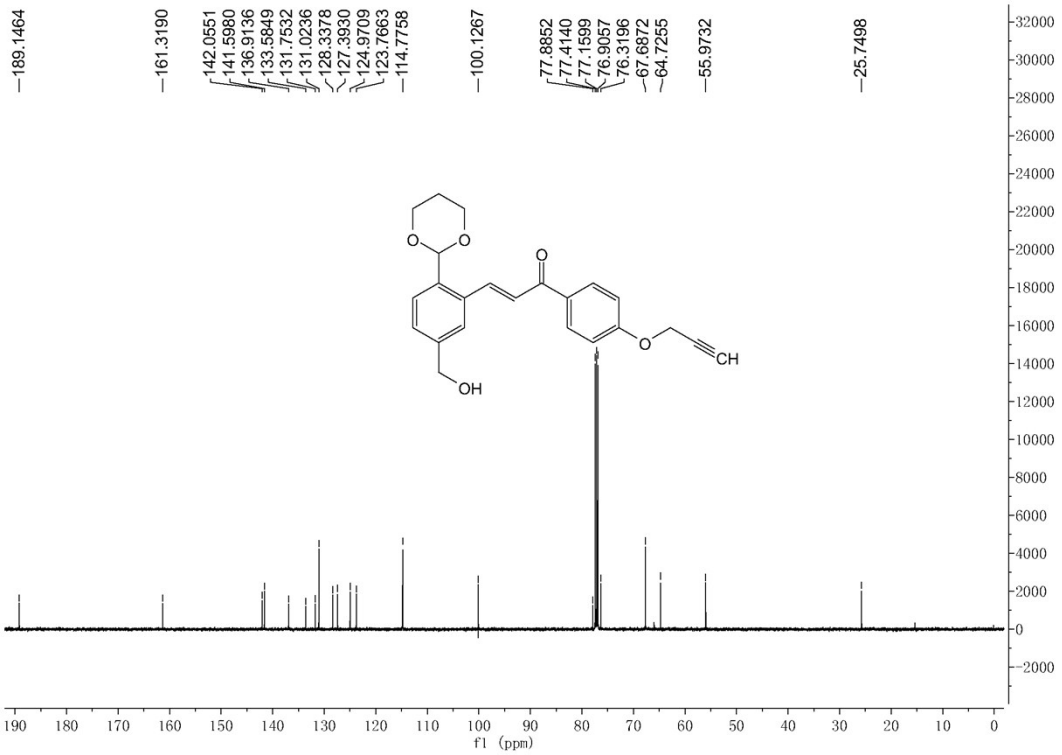
Fig. S23 Fluorescence confocal microscopic images of GSH and H_2S in the mitochondria of Hela cells. Distribution of **ZS3** fluorescence in the mitochondrial component of Hela cells was confirmed by counterstaining with MitoTracker Green. The fluorescent images were recorded using confocal microscope (Olymbus, Japan). The green fluorescence of Mito-Tracker Green was excited with 490 nm, and the emission wavelengths was 516nm. The red fluorescence of **ZS3** was excited with 559 nm, and the emission wavelengths was 603nm.



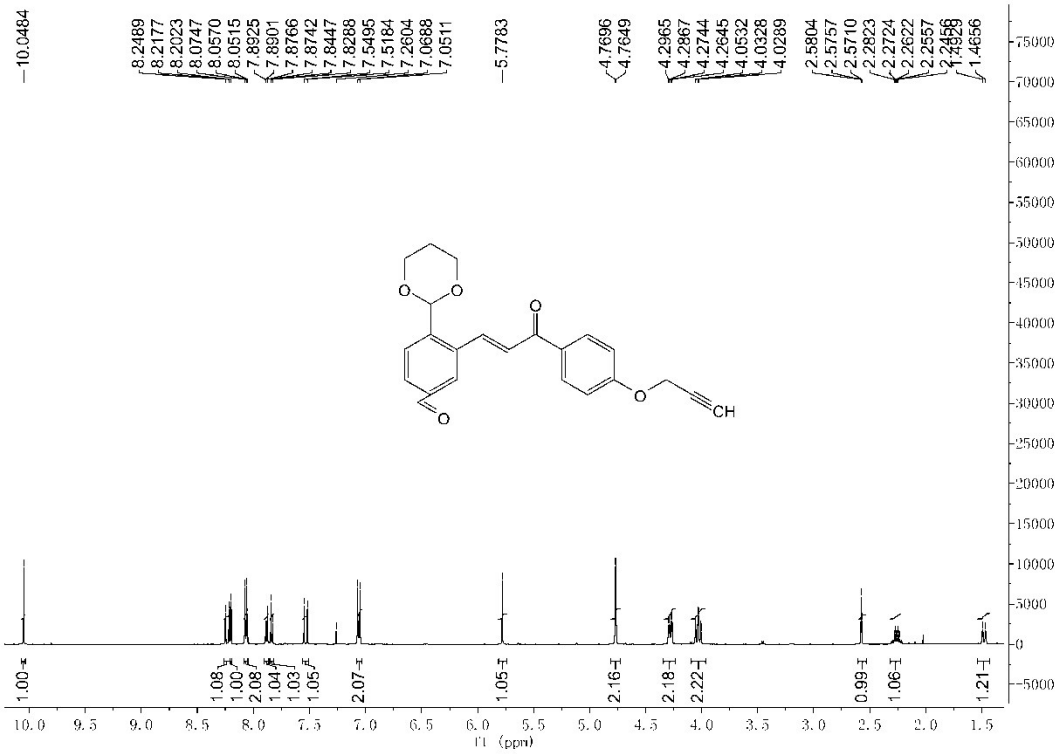


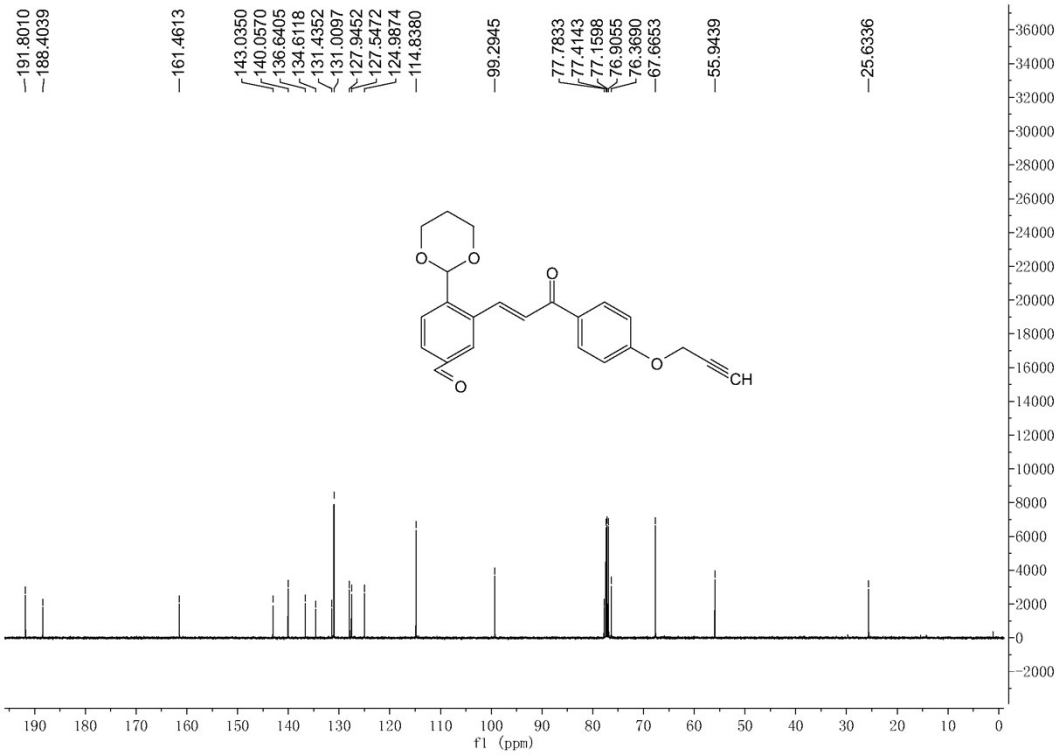
^{1}H NMR and ^{13} C NMR spectra of **3**



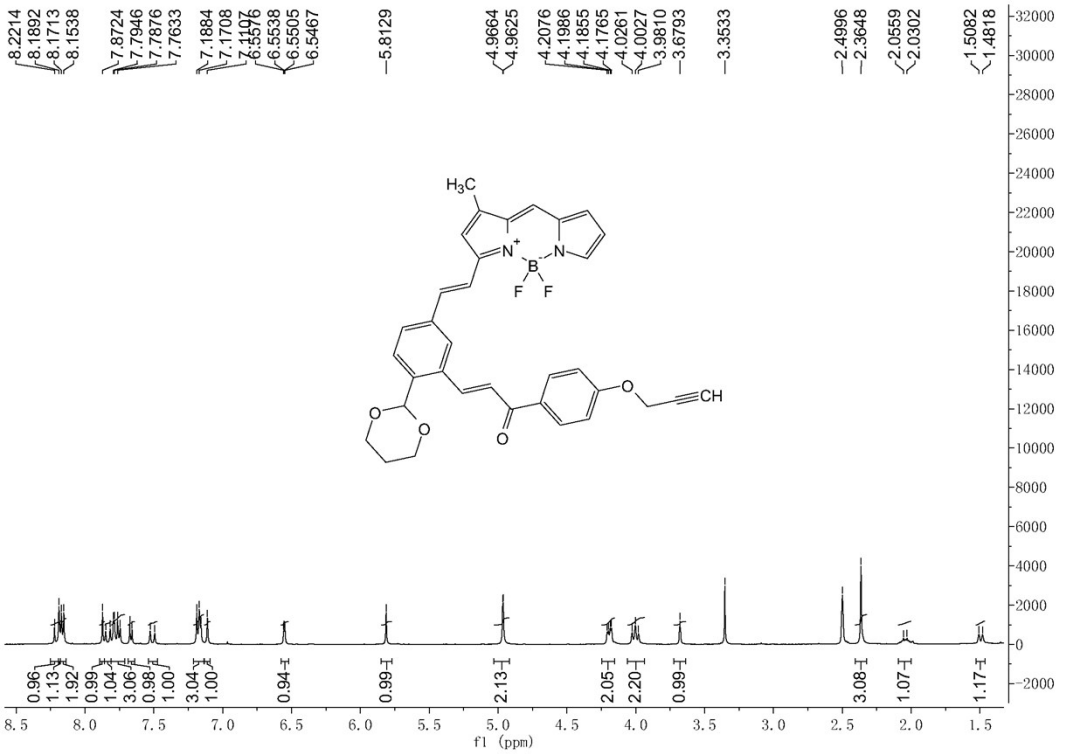


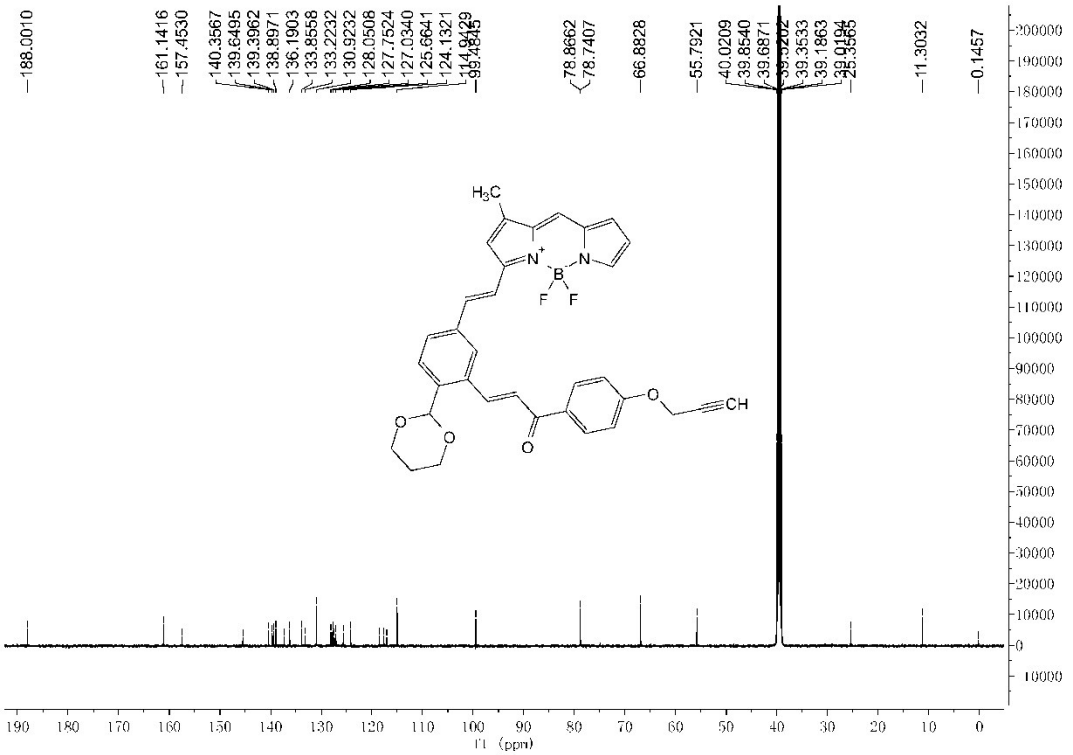
^{1}H NMR and ^{13}C NMR spectra of **4**



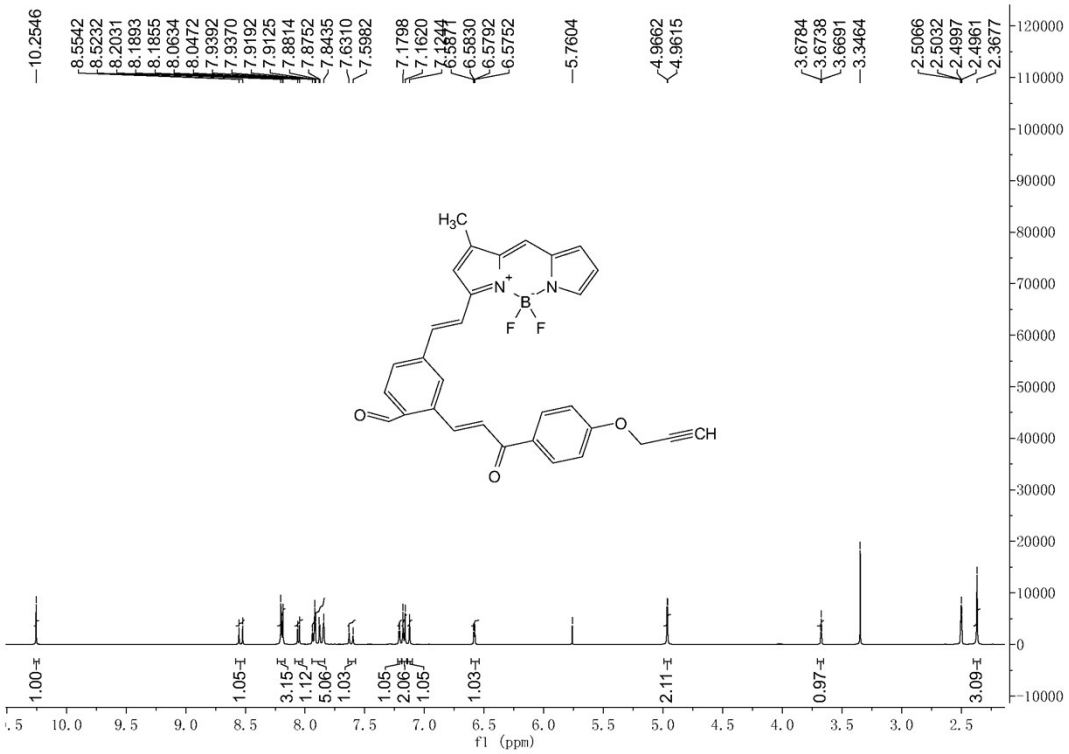


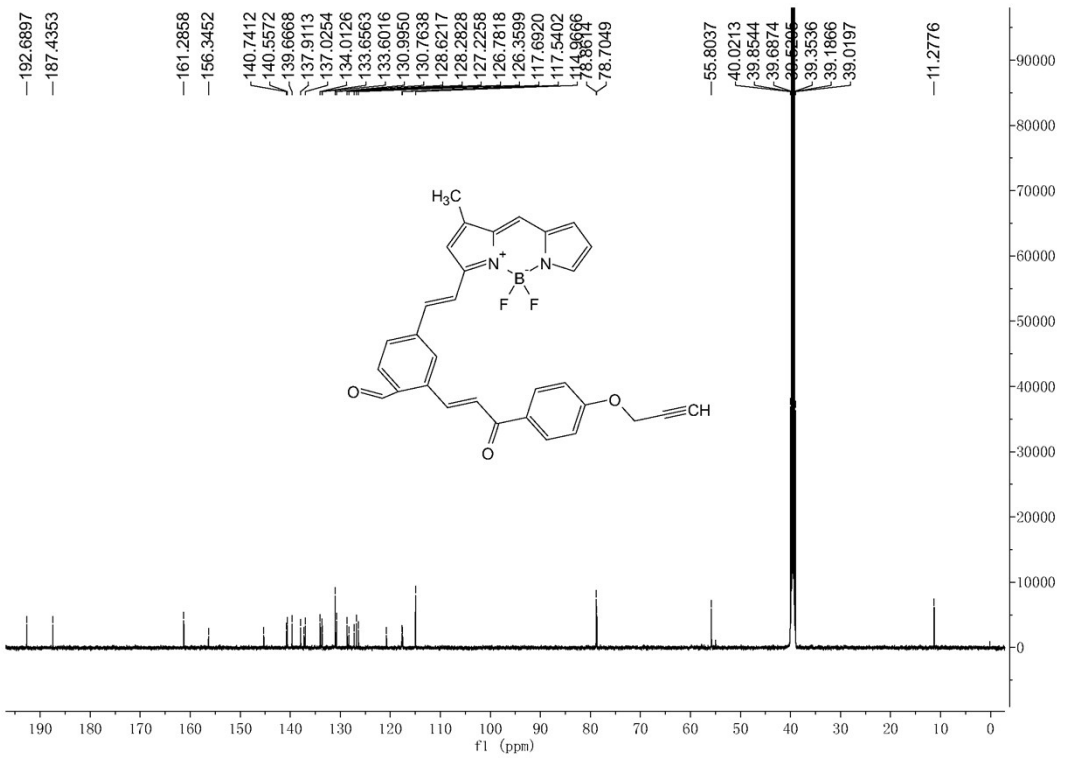
^{1}H NMR and ^{13}C NMR spectra of **5**



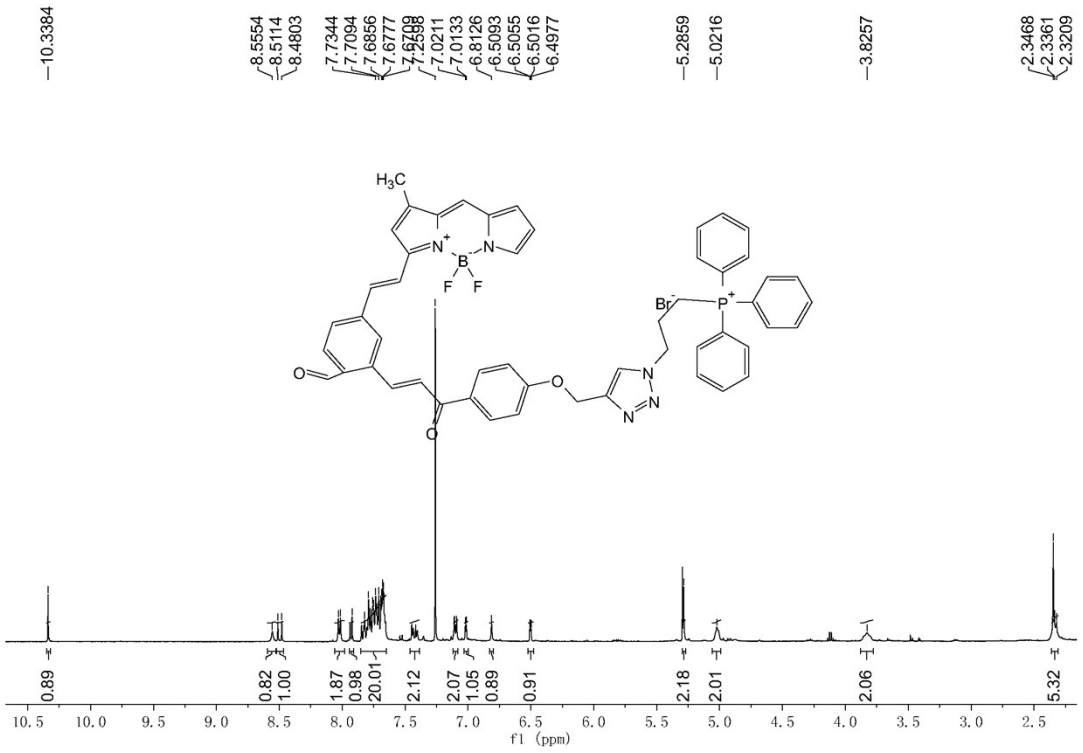


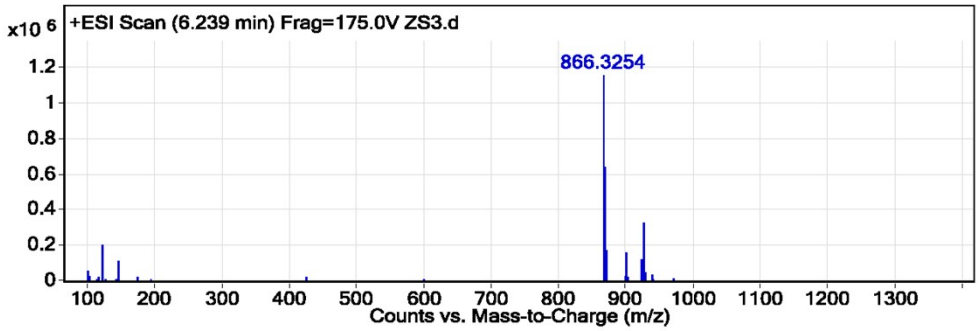
^{1}H NMR and ^{13} C NMR spectra of **6**





^{1}H NMR and ^{13} C NMR spectra of **ZS2**





Peak List

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925.3695	1	181141

^{1}H NMR and HRMS spectra of ZS**3**