Electronic Supplementary Material (ESI) for Chemical Communications.

Electronic Supplementary Information

A bodipy based dual functional probe for the detection of hydrogen sulfide and H_2S induced apoptosis in cellular system

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Instruments and experimental procedures

General information

All reagents were purchased from Aldrich and were used without further purification. HPLC grade solvent (CH_3CN, DMSO, DMF, Dioxane, EtOH, MeOH, THF, DCM) was used in UV-vis and fluorescence studies. UV-vis spectra were recorded on a SHIMADZU UV-2450 spectrophotometer, with a quartz cuvette (path length 1 cm). The cell holder was thermostatted at 25 °C. The fluorescence spectra were recorded with a SHIMADZU 5301 PC spectrofluorimeter. ^{1}H spectra were recorded on a Bruker Avance 400 and 500 MHz spectrophotometer using $CDCl_3$ as solvent and tetramethylsilane as the internal standard. Data are reported as follows: chemical shift in ppm (δ), multiplicity (s = singlet, d = doublet, t = triplet, m = multiplet, br = broad singlet), coupling constants *J* (Hz). Fluorescence quantum yields[1] were determined by using optically matching solution of fluorescein (Φ_{fr} = 0.95 in 0.1M NaOH solution) as standard at an excitation wavelength of 470 nm and quantum yield is calculated using the equation:

$$\Phi_{fs} = \Phi_{fr} \times \frac{1\text{-}10^{-ArLr}}{1\text{-}10^{-AsLs}} \times \frac{N_s^2}{N_r^2} \times \frac{D_s}{D_r}$$

Φ_{fs} and Φ_{fr} are the radiative quantum yields of sample and the reference respectively, A_s and A_r are the absorbance of the sample and the reference respectively, D_s and D_r the respective areas of emission for sample and reference. L_s and L_r are the lengths of the

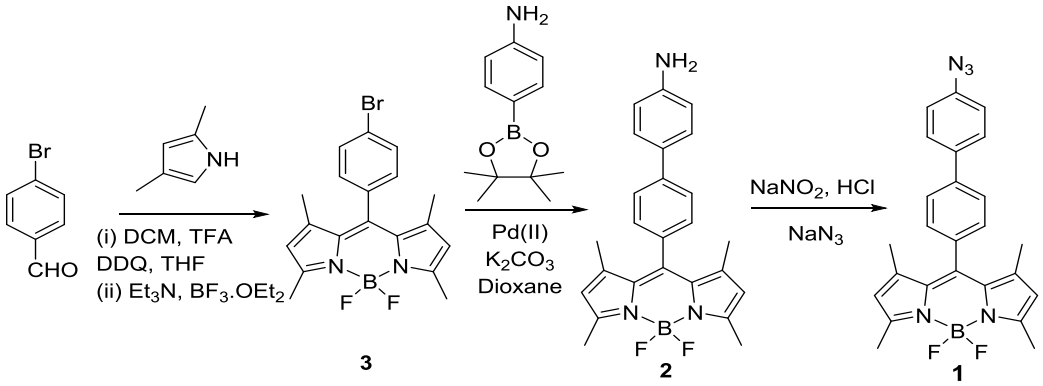
[1] Deams, J. N.; Grosby, G. A. *J. Phys. Chem.* **1971**, *75*, 991.

absorption cells of sample and reference respectively. N_s and N_r are the refractive indices of the sample and reference solutions.

Procedure for sensing

UV-vis and fluorescence titrations were performed on 5.0 μM solution of ligand in H_2O/DMSO (8:2, v/v; buffered with HEPES, pH = 7.02; at 25 °C) mixture. Typically, aliquots of freshly prepared anion (SO_4^{2-} as tetrabutylammonium salt) standard solutions (10^{-1} M to 10^{-3} M) were added to record the UV-vis and fluorescence spectra. Hydrogen peroxide (H_2O_2), and hypochlorite (OCl^-) were delivered from 30%, and 5% aqueous solutions, respectively. Hydroxyl radical (•OH) and tert-butoxy radical (•OtBu) were generated by reaction of 1 mM Fe^{2+} with 100 μM H_2O_2 or 100 μM TBHP, respectively. The reactive sulphur species (GSH, Cys, $S_2O_3^{2-}$, SO_4^{2-}, SO_3^{2-}) the standard solution (10^{-1} to 10^{-3} M) were added to record the spectra. In titration experiments, each time a 3 ml solution of ligand was filled in a quartz cuvette (path length, 1 cm) and spectra were recorded after the addition of appropriate analyte.

Synthetic routes and characteristic data



Scheme S1: Synthesis of probe **1**

Synthesis of probe 3:

4-bromobenzaldehyde (2.2 g, 12 mmol) and 2, 4-dimethylpyrrole (2.38 g, 25 mmol) were dissolved in dry and deaerated CH_2Cl_2 (150 mL) under nitrogen atmosphere. 60 µL trifluoroacetic acid (TFA) was added to the solution and reaction mixture was stirred at RT overnight. After disappearance of the benzaldehyde, a solution of DDQ (12 mmol, 2.9 g) in absolute THF was added and continued to stir for 4 h. Absolute triethylamine (12 mL) was then added to the mixture. After stirring for 15 min, 12 ml $BF_3.OEt_2$ was added drop wise in ice bath. After stirring for overnight, the reaction mixture was washed with water several times and extracted with chloroform (100 mL). The organic phase was dried over Na_2SO_4. The solvent was evaporated and the residue was purified by silica gel (60-120 mesh) column chromatography (hexane/chloroform; 9:1) to obtain red solid 0.9 g. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.63 (d, 2 H, *J* = 8 Hz), 7.20 (d, 2 H, *J* = 8 Hz), 6.0 (s, 2 H), 2.57 (s, 6 H), 1.42 (s, 6 H); ESI- MS ($C_{19}H_{18}BF_2N_2Br$) calc. m/z = 402.07, found m/z = 402.07.

Synthesis of probe 2:

To a solution of **3** (0.30 g, 0.744 mmol) and boronic ester of aniline (0.19 g, 0.893 mmol) in dioxane (20 ml) were added K_2CO_3 (0.41 g, 2.977 mmol), distilled water (1 mL), and $[Pd(Cl)_2(PPh_3)_2]$ (0.31 g, 0.44 mmol) under nitrogen, and the reaction mixture was refluxed overnight. The dioxane was then removed under vacuum, and the residue so obtained was treated with water, extracted with dichloromethane, and dried over

anhydrous Na_2SO_4. The organic layer was evaporated, and the compound was purified by column chromatography using hexane/chloroform (6:4) as an eluent to give 0.176 g (57 %) of compound **2** as orange coloured solid. ^{1}H NMR ($CDCl_3$, 400 MHz, ppm) 1.40 (s, 6 H), 2.56 (s, 6 H), 3.70 (s, 2H), 5.99 (s, 2 H), 6.80 (d, *J* = 8 Hz, 2 H), 7.29 (d, *J* = 8, 2 H), 7.51 (d, *J* = 8, 2 H), 7.68 (d, *J* = 8, 2 H). ^{13}C NMR ($CDCl_3$, 100 MHz, δ = ppm) = 29.32, 66.84, 115.16, 117.17, 121.14, 126.39, 127.99, 128.37, 131.29, 134.93, 135.24, 143.16, 145.46, 155.30; IR (KBr) v_{max} = 3496 cm^{-1} (primary aromatic amine) and 3399 cm^{-1} (NH-stretch). TOF MS ES^+, m/z: 416.2136 ($M+H^+$). HRMS data for $C_{25}H_{24}BF_2N_3$: Found: 416.2105 ($M+H^+$); calculated: 416.2109 ($M+H^+$).

Synthesis of probe 1:

To a solution of compound **2** (0.1 g, 0.2410 mmol) and HCl (8 ml) were dissolved in 25 ml ethanol and allowed to stir for 5 min. Then the reaction mixture was cooled to 0 ºC and $NaNO_2$ (33.26 mg, 0.4820 mmol) was added and then the reaction mixture was stirred for 1 hr. After that NaN_3 was added slowly and the reaction mixture was stirred for another 2 hrs at room temperature. The suspension was extracted with DCM and the organic layer was concentrated and then purified by column chromatography using hexane/chloroform (7:3) as eluent to give 0.04g (38% yield) of compound **1** as shiny orange coloured solid. ^{1}H NMR ($CDCl_3$, 500 MHz, ppm) 1.47 (s, 6 H), 2.50 (s, 6 H), 6.02 (s, 2 H), 7.16 (d, *J* = 10 Hz, 2 H), 7.38 (d, *J* = 5 Hz, 2 H), 7.70 (d, *J* = 5 Hz, 2 H), 7.74 (d, *J* = 5 Hz, 2 H). ^{13}C NMR ($CDCl_3$, 125 MHz, δ = ppm) = 14.60, 29.61, 119.25, 121.10, 127.45, 128.22, 128.76, 131.15, 133.78, 136.64, 139.75, 140.66, 141.14, 143.28, 155.57; IR (KBr) v_{max} = 2121 cm^{-1} (N=N=N) azide stretching frequency. TOF MS ES^+, data for $C_{25}H_{22}BF_2N_5$: Found: 441.2928 (M^+); calculated: 441.2886 (M^+).

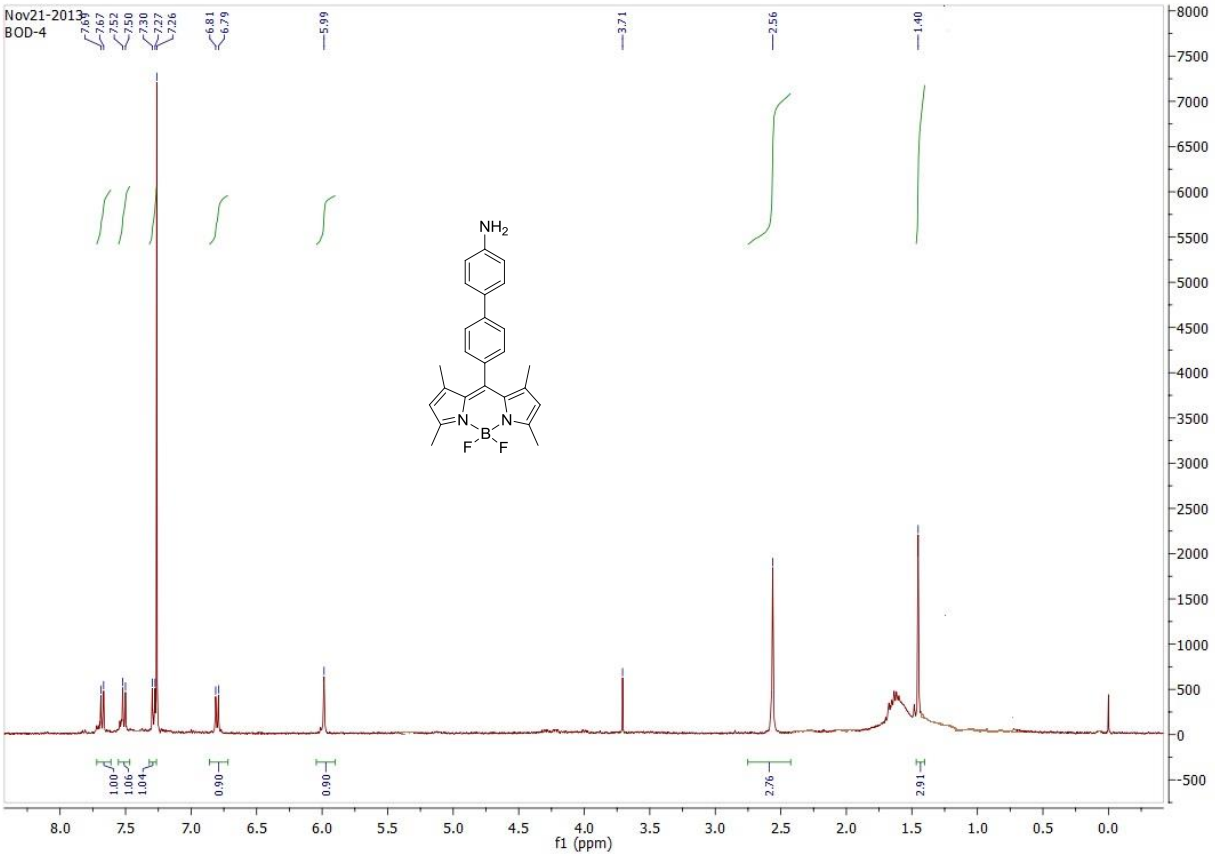


Figure S1: ^{1}H NMR spectrum of probe **2** in $CDCl_3$ (400 MHz)

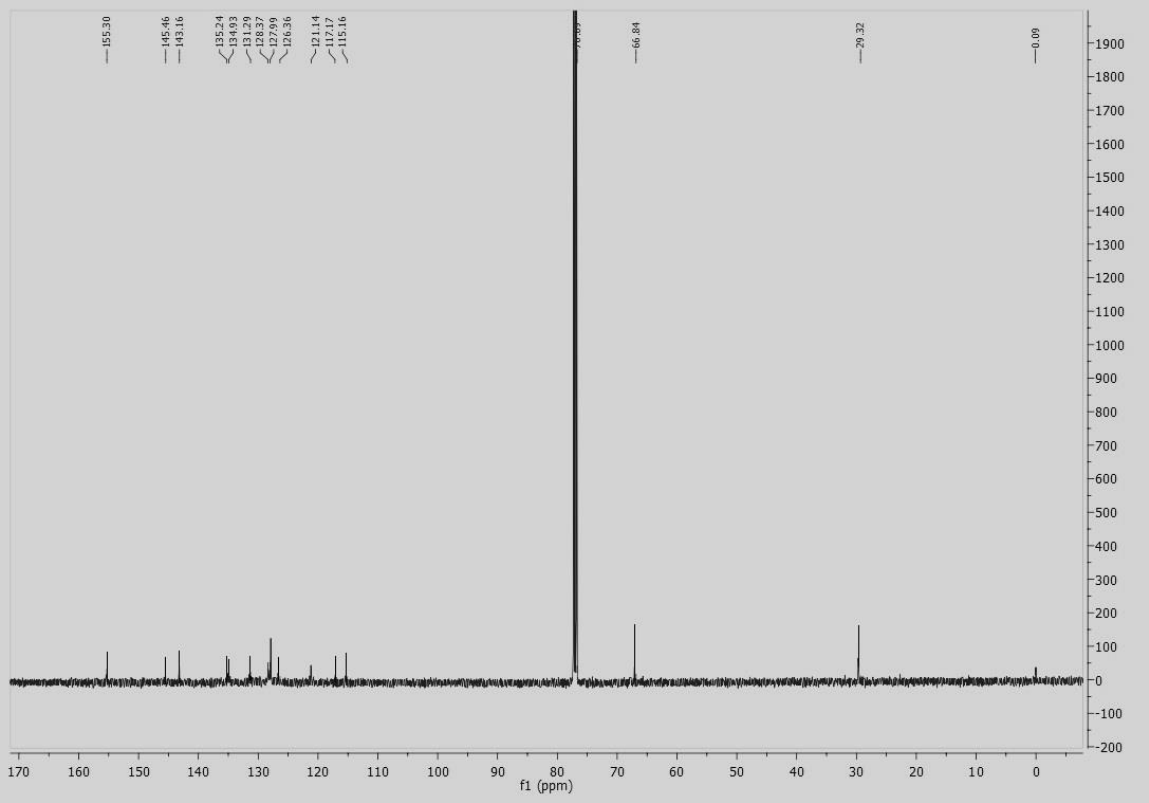


Figure S2: ^{13}C NMR spectrum of probe **2** in $CDCl_3$ (100 MHz)

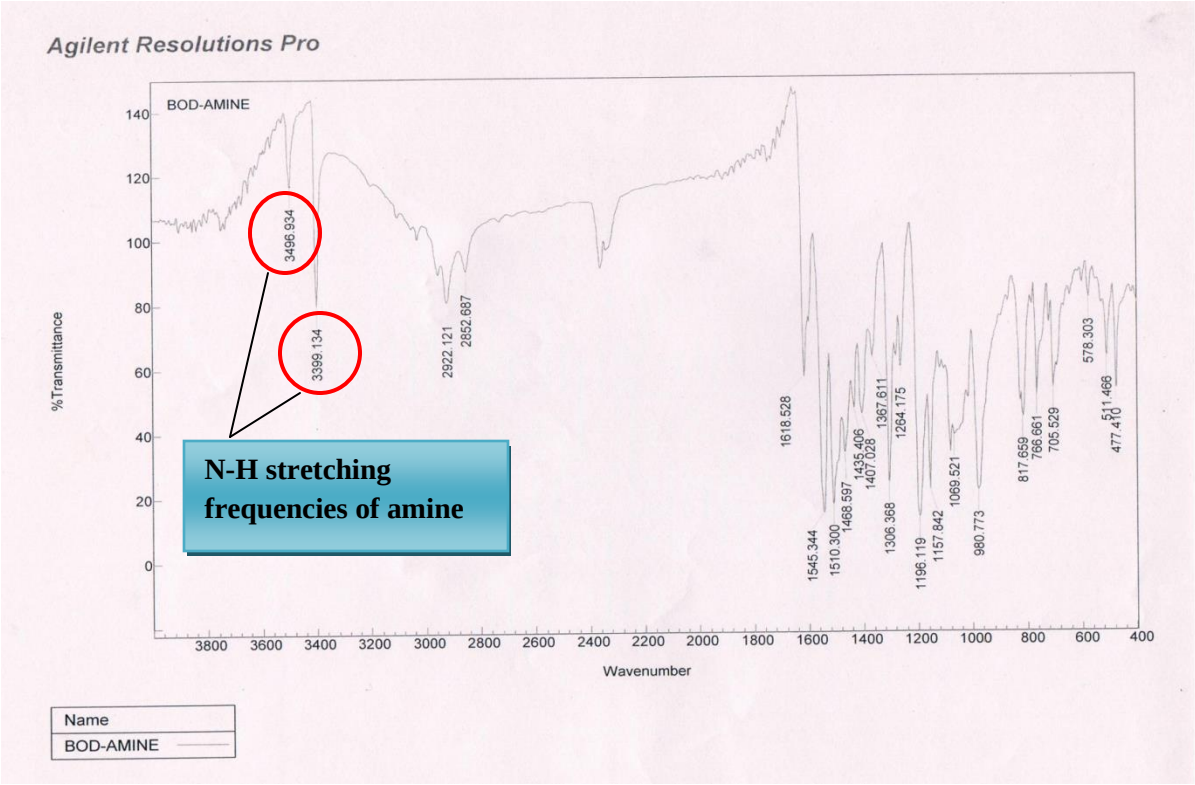


Figure S3: IR spectra of probe **2** (using KBr pallet)

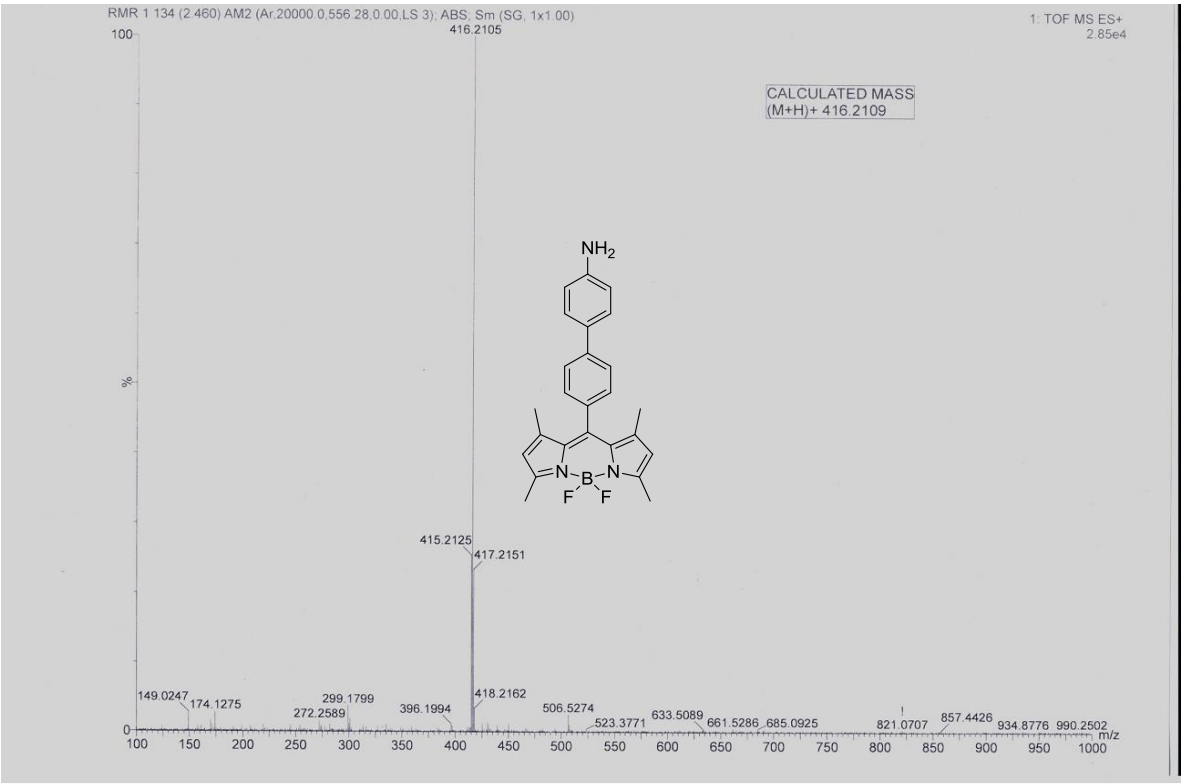


Figure S4: Mass spectrum of probe **2**

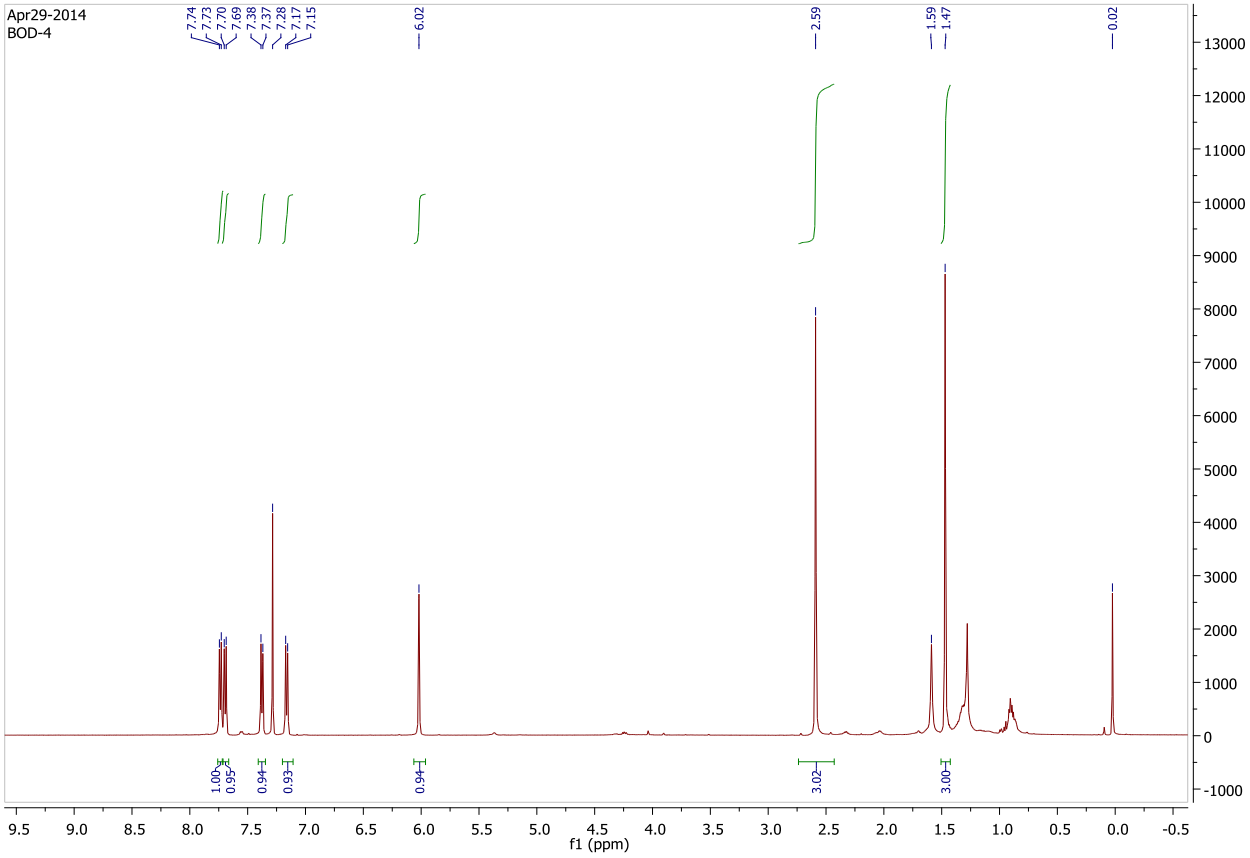


Figure S5: ^{1}H NMR spectrum of probe **1** $CDCl_3$ (500 MHz)

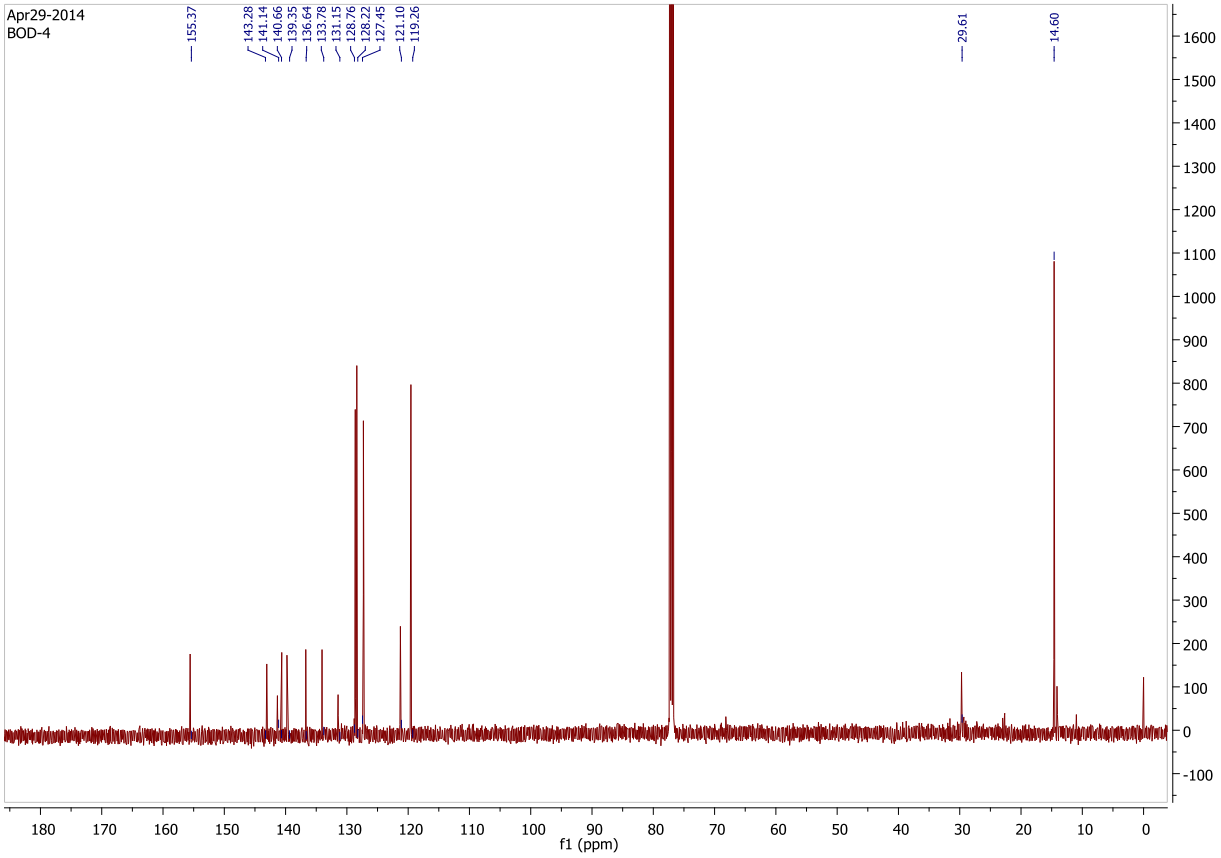


Figure S6: ^{13}C NMR spectrum of probe **1** $CDCl_3$ (125 MHz)

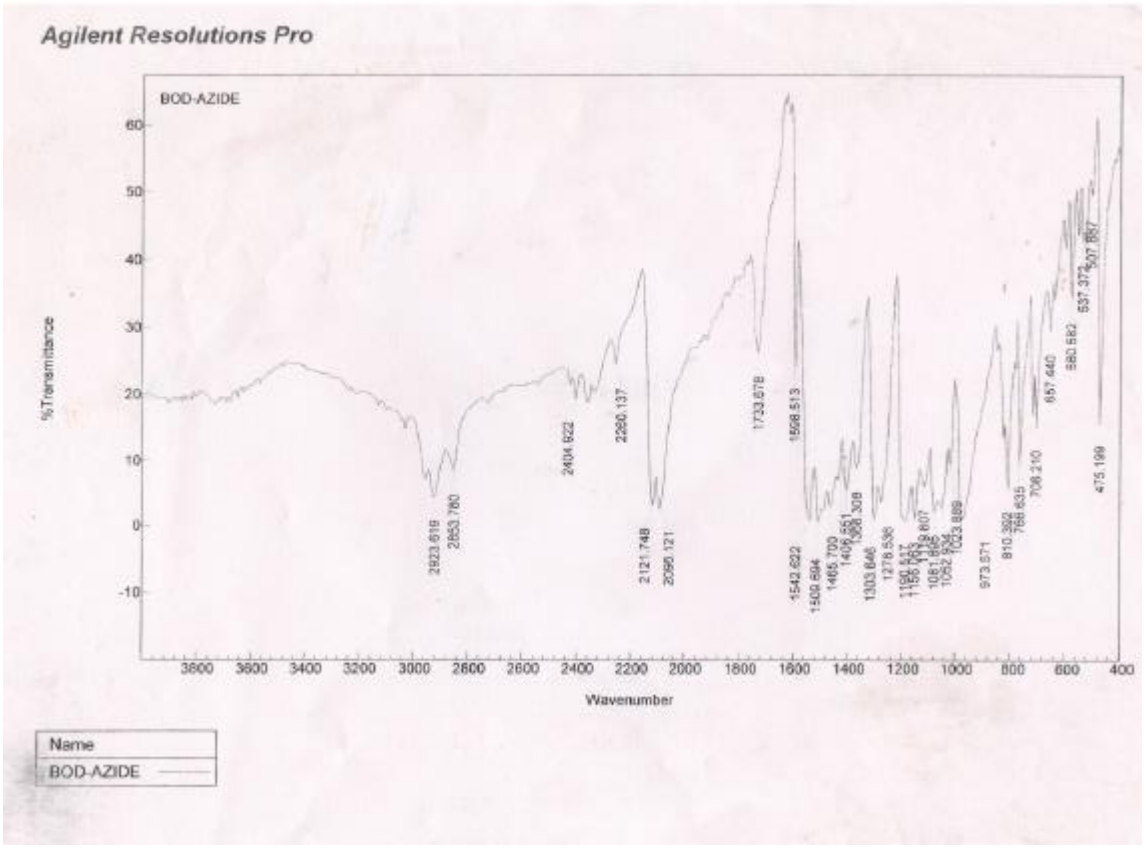


Figure S7: IR spectrum of probe **1** (using KBr pallet)

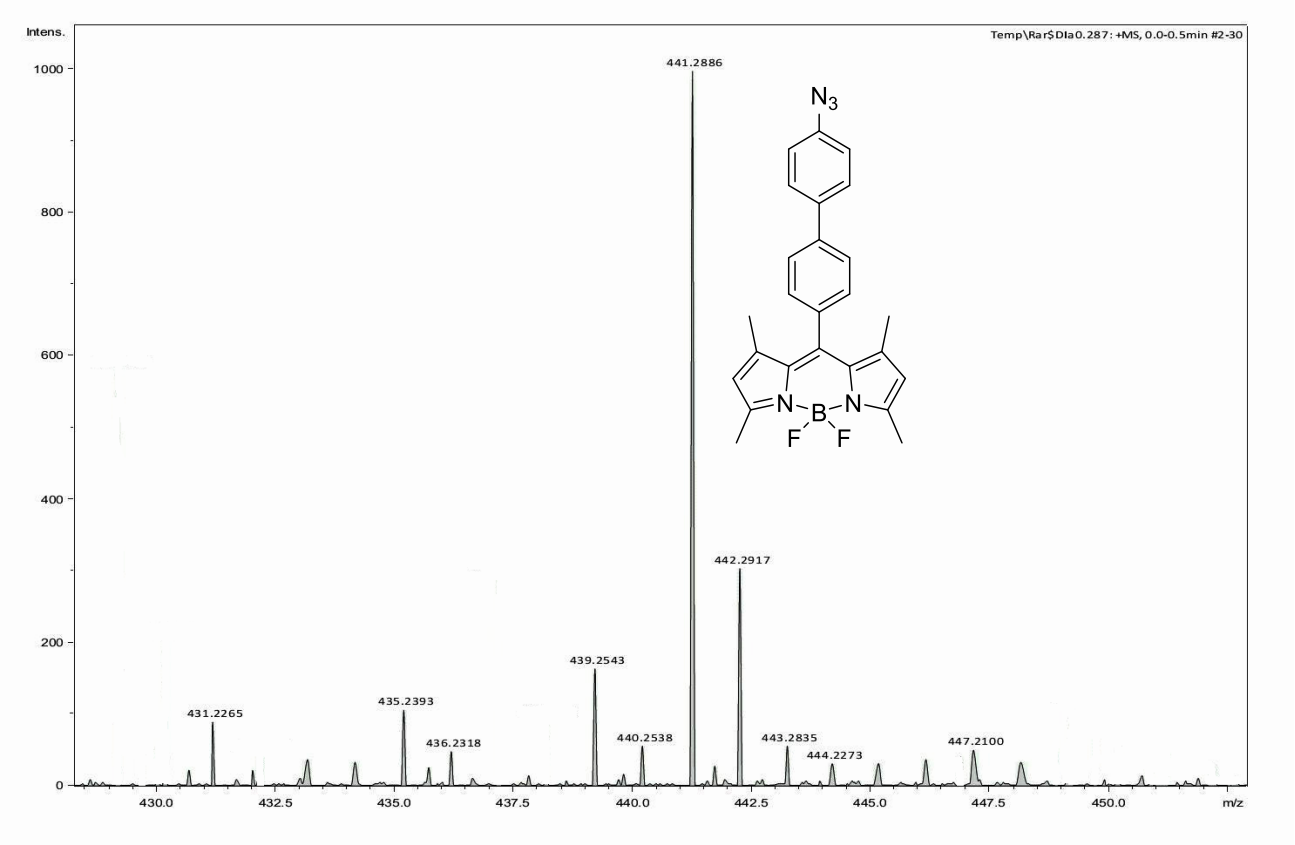


Figure S8: Mass spectrum of probe **1**

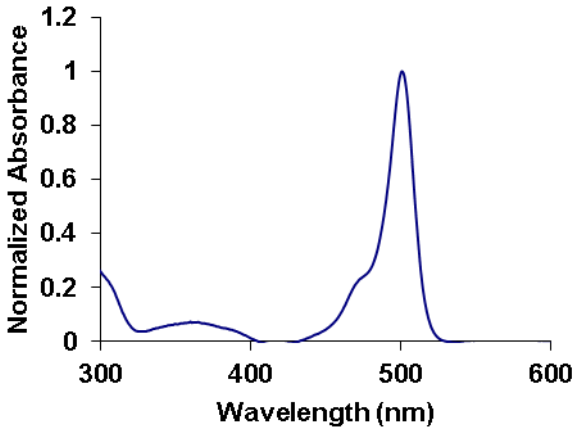


Figure S9: Normalized UV-vis spectra of probe **1** (5.0 µM) in H_2O/DMSO (8:2, v/v) buffered with HEPES, pH = 7.02. The spectra showing the transitions of S_0-S_1 and S_0-S_2

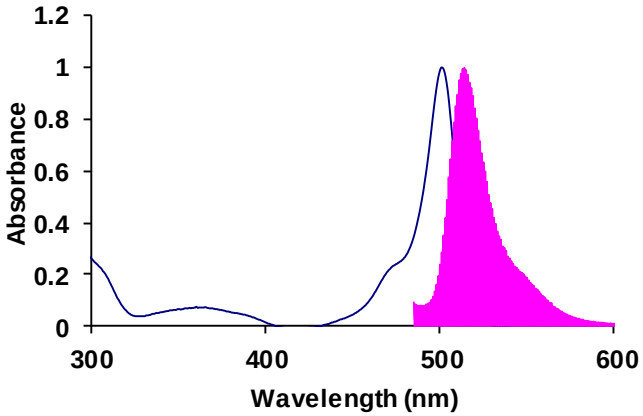


Figure S10: Normalized UV-vis and fluorescence spectra of probe **1** (5.0 µM) in H_2O/DMSO (8:2, v/v) buffered with HEPES, pH = 7.02. The blue line indicates the normalized UV-vis spectra and pink line indicate the normalized fluorescence spectra.

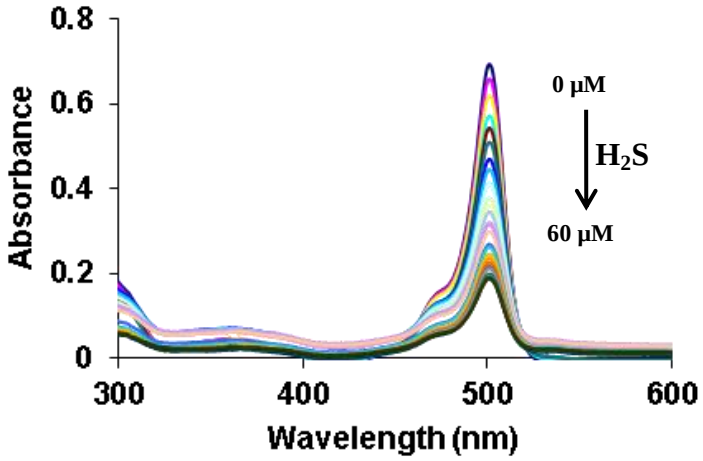


Figure S11: UV-vis spectra of probe **1** (5.0 µM) on addition of H_2S (0-60 µM) in H_2O/DMSO (8:2, v/v) buffered with HEPES, pH= 7.02. The spectra showing decrease in absorption with increase in H_2S concentration.

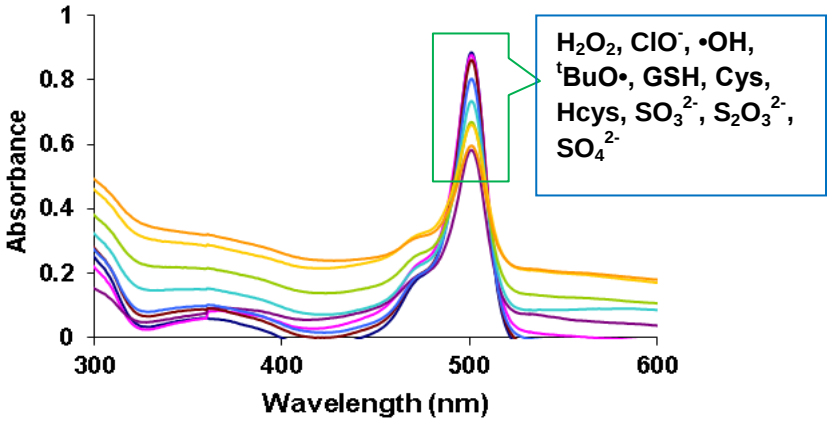


Figure S12: UV-vis spectra of probe **1** (5.0 µM) on addition of different analytes in H_2O/DMSO (8:2, v/v) buffered with HEPES, pH= 7.02.

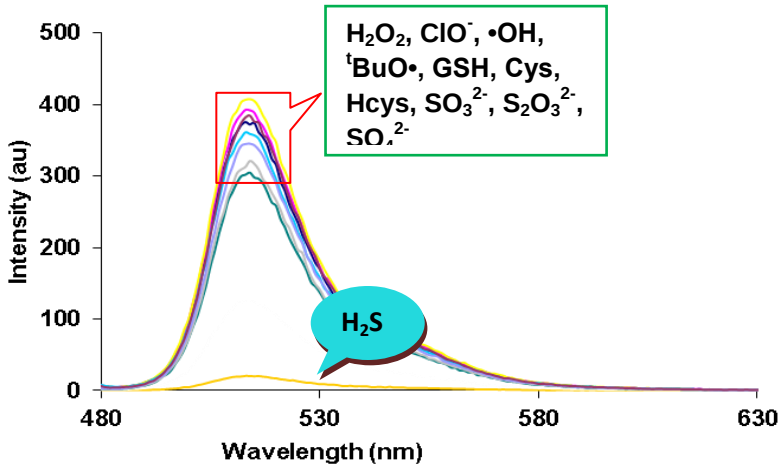


Figure S13: Fluorescence response of probe **1** (5.0 μM) in H_2O: DMSO (8:2, v/v) with HEPES buffer at pH = 7.02 (λ_{ex}= 470 nm and λ_{em} = 515 nm) with various analyte (60 μM each; but in case of cysteine and glutathione 10 mM conc. were used).

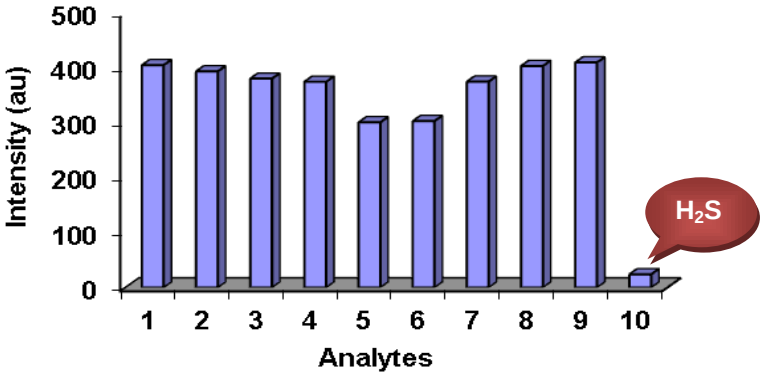


Figure S14: Fluorescence response of probe **1** (5.0 μM) to various reactive sulphur species (RSS) and anions (60 μM each) in H_2O: DMSO (8:2, v/v) buffered with HEPES, pH=7.02; λ_{ex} = 470 nm. Bars represent selectivity of probe **1** upon addition of different analyte (1= SO_4^{2-}, 2= SO_3^{2-}, 3= $S_2O_3^{2-}$, 4= •OH, 5= Cys, 6= GSH, 7= Hcys, 8= ^tBuO•, 9= H_2O_2, 10= H_2S. Fluorescence intensity measured at 515 nm.

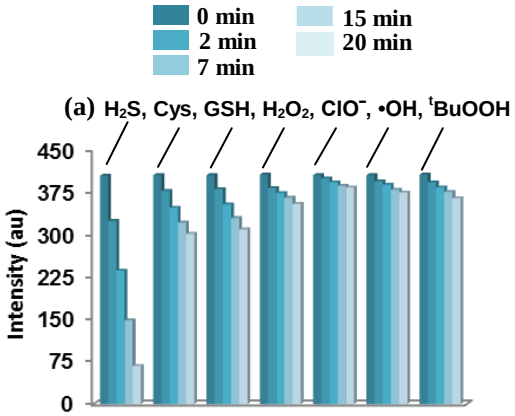


Figure S15: Fluorescence response of probe **1** (5.0 μM) in H_2O: DMSO (8:2, v/v) with HEPES buffer at pH = 7.02 (λ_{ex}= 470 nm) with various analyte (60 μM each; but in case of cysteine and glutathione 10 mM conc. were used). Bars represent selectivity (fluorescence intensity at 515 nm after the addition of analyte) of probe **1** upon addition of different analyte. Data were collected after incubation with the appropriate analyte after 0, 2, 7, 15, 20 min.

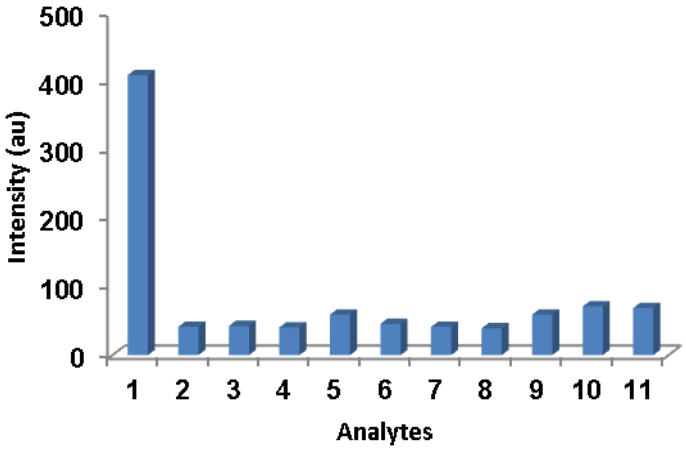


Figure S16: Fluorescence response of probe **1** (5.0 μM) with H_2S (60 μM) in the presence of various reactive sulphur species (RSS) and reactive oxygen species (ROS) (60 μM each) in H_2O: DMSO (8:2, v/v) buffered with HEPES, pH=7.02; λ_{ex} = 470 nm. Bars represent competitive of H_2S to probe **1** in the presence of different analyte (1= Only probe **1**, 2= SO_4^{2-}, 3= SO_3^{2-}, 4= $S_2O_3^{2-}$, 5= ClO^-, 6= Cys, 7= GSH, 8= Hcys, 9= $^tBuO•$, 10= •OH, 11= H_2O_2. Fluorescence intensity measured at 515 nm.

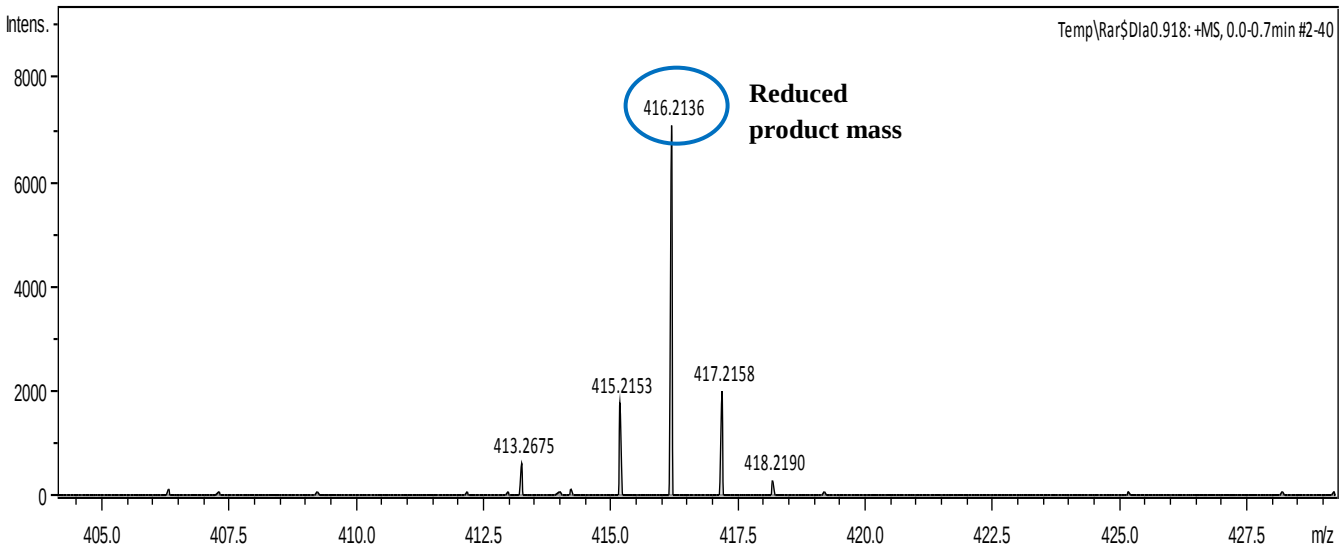


Figure S17: Mass spectrum of reduced product **2** (The reaction was carried out with probe **1** on addition of H_2S)

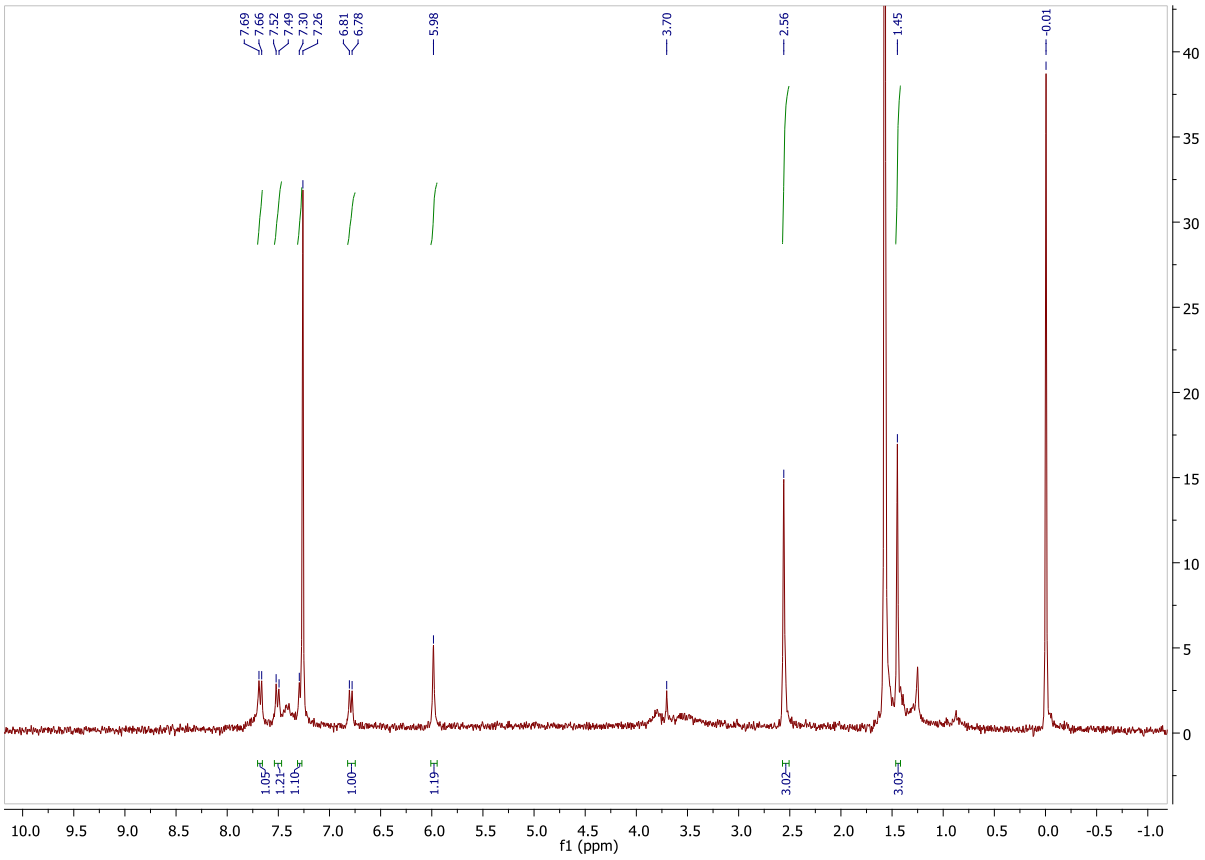


Figure S18: ^{1}H NMR spectrum of reduced product **2** (500 MHz in $CDCl_3$ as solvent) (The reaction was carried out with probe **1** on addition of H_2S)

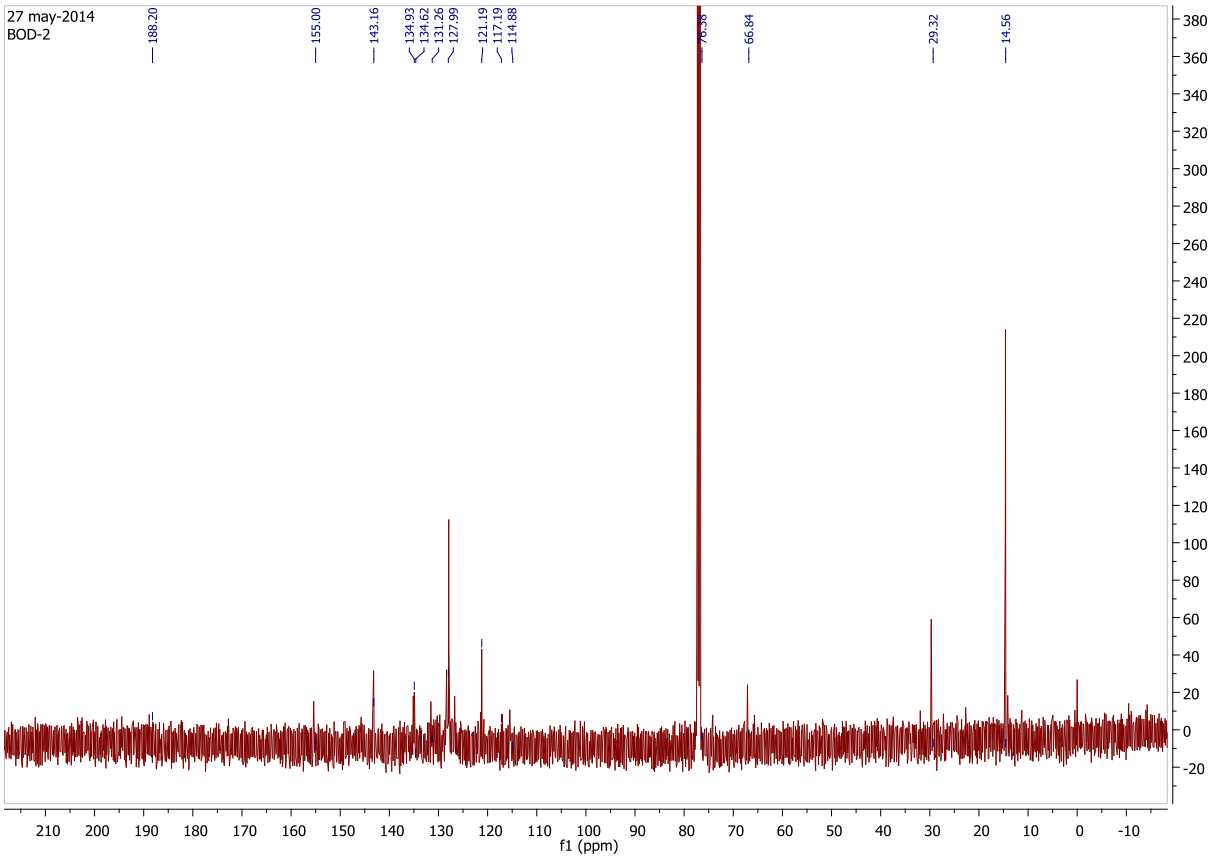


Figure S19: ^{13}C NMR spectrum of reduced product **2** (125 MHz in $CDCl_3$ as solvent) (The reaction was carried out with probe **1** on addition of H_2S)

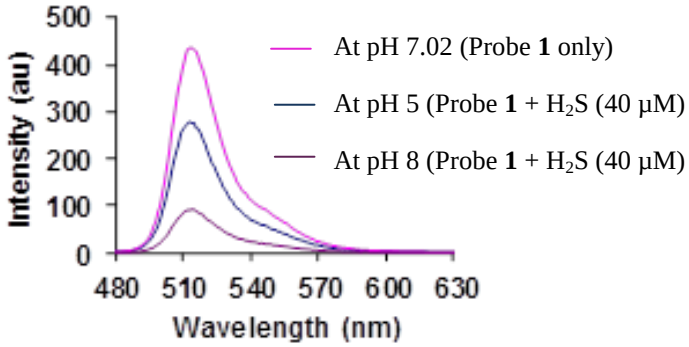
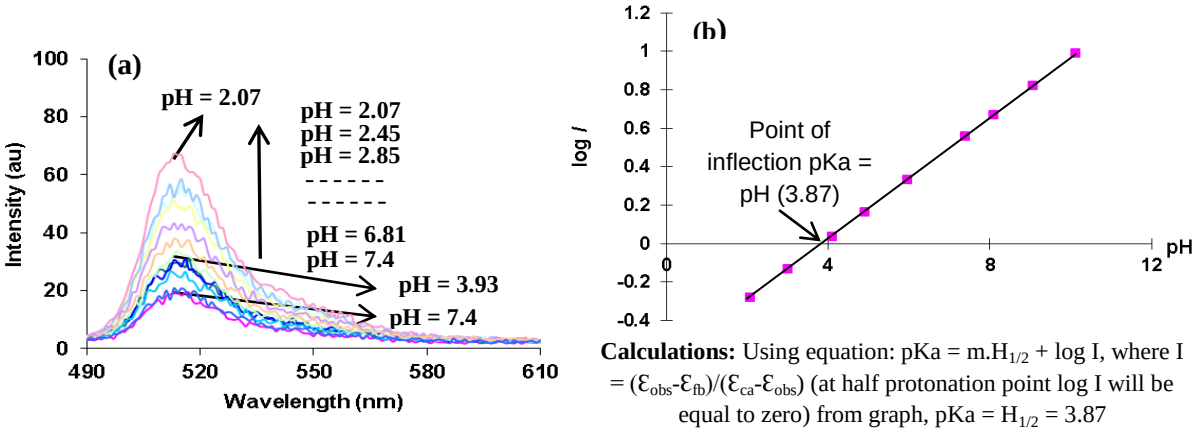


Figure S20: Fluorescence response of probe **1** (5.0 µM) in H_2O: DMSO (8:2, v/v) on addition of H_2S (40 µM) in different pH value pH 5 and pH 8 (λ_{ex}= 470 nm) and spectra recorded after 20 min.



Calculations: Using equation: $pKa = m.H_{1/2} + \log I$, where $I = (\varepsilon_{obs}-\varepsilon_{fb})/(\varepsilon_{ca}-\varepsilon_{obs})$ (at half protonation point log I will be equal to zero) from graph, $pKa = H_{1/2} = 3.87$

Figure S21: (a) Change in fluorescence spectra of probe **2** (5 µM) in aqueous solution containing 20% DMSO as cosolvent in different pH values (λ_{ex} = 470 nm). **(b)** The log I−pH plot for the probe **2** (amine).

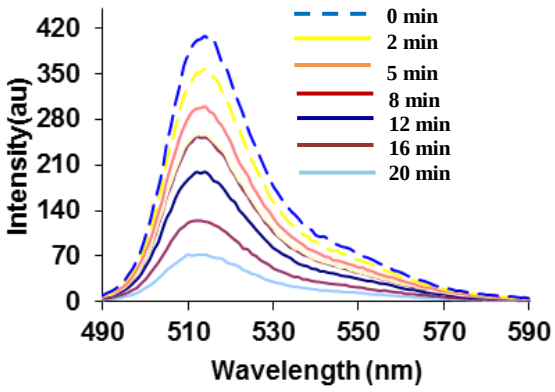
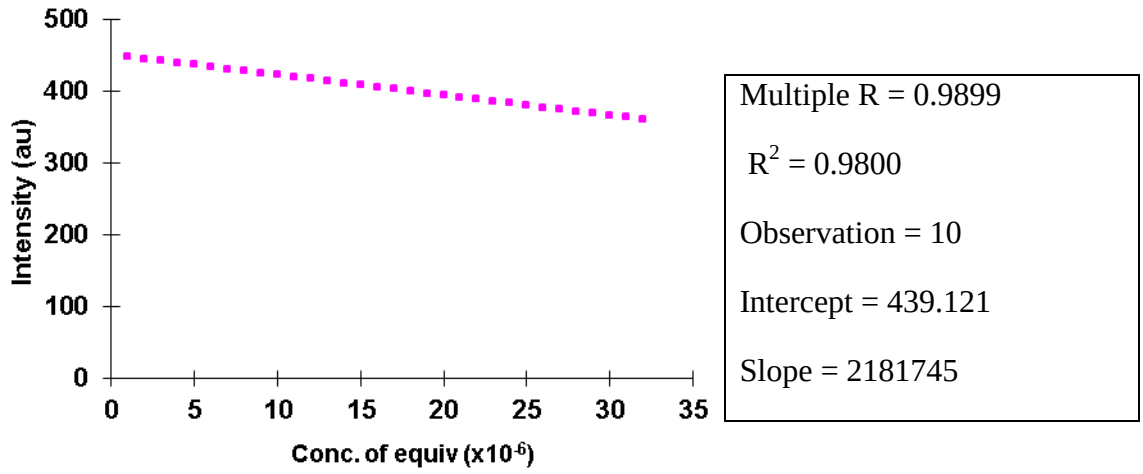


Figure S22: Time dependent fluorescence emission response of probe **1** in H_2O: DMSO (8:2, v/v) buffered with HEPES, pH=7.02; on addition of H_2S (60 μM); λ_{ex}= 470 nm.

Figure S23: Detection limit and its calculation



The detection limit was calculated based on the fluorescence titration. To determine the S/N ratio, the emission intensity of receptor **1** without H_2S was measured by 10 times and the standard deviation of blank measurements was determined. The detection limit is then calculated with the following equation:

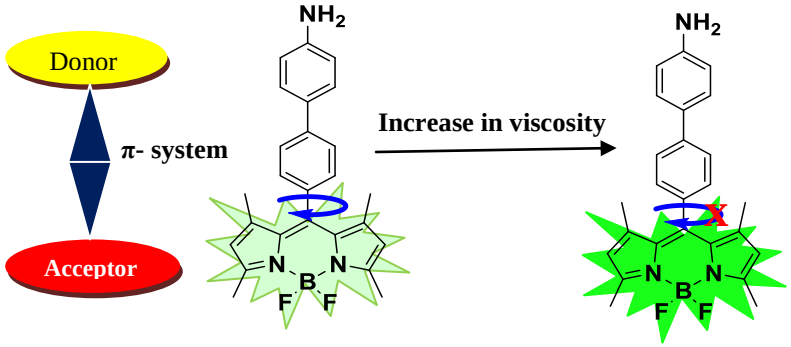
DL = 3 × SD/S

Where SD is the standard deviation of the blank solution measured by 10 times; S is the slope of the calibration curve.

From the graph we get slope (S) = 2181745, and SD value is 0.026

Thus using the formula we get the Detection Limit (DL) = 3.575119×10^{-8} M *i.e*. probe **1** can detect H_2S in this minimum concentration through fluorescence method.

Studies to prove that reduced product (amine) act as fluorescent molecular rotor.



Reduced product (amine) work as fluorescent molecular rotor

(A) Fluorescence studies:

Fluorescence studies were carried out in 5 μM of amine (probe **2**) in different methanol: glycerol fractions at excitation wavelength of 470 nm. We observed that with increasing glycerol fraction the fluorescence intensity increases. The increase in fluorescence intensity with increase in viscosity is attributed to the fact that high viscosity restricts the rotation of meso-substituted phenyl group due to which the fluorescence emission by non-radiative pathway gets decreased.

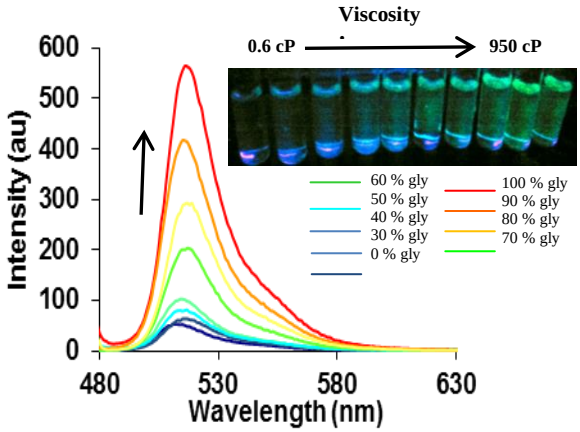


Figure S24: Fluorescence emission spectra of probe **2** (5.0 μM) at λ_{em}= 517 nm with the variation of solution viscosity (methanol/glycerol system); λ_{ex} = 470 nm. Inset figure showing increase in fluorescence intensity with increase in viscosity. The image was taken under 365 nm illumination.

(B) Fluorescence lifetime studies: Lifetime studies were carried out in 5.0 μM of probe **2** in different methanol: glycerol fractions at excitation wavelength 488 nm. With the gradual increase in viscosity from 0.6 to 950 cP, the fluorescence lifetime also increases from 2.3 ns to 4.4 ns. The increase in lifetime with increase in viscosity depicts the rotation restriction in high viscous medium which justifies the molecular rotation property of amine.

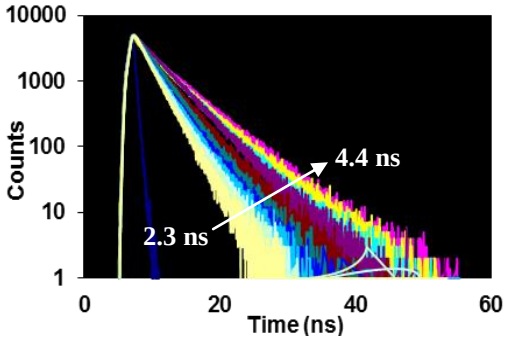


Figure S25: Fluorescence lifetime spectra of probe **2** (5.0 μM) at 517 nm in methanol-glycerol system with varying proportions to adjust the viscosity at 25 °C; λ_{ex} = 488 nm

(C) Fluorescence studies of probe 1 on addition of H_2S in different glycerol fractions: We prepared stock solution using 1mM of probe **1** by addition of 12 mM of H_2S for 30 min. Further, we used this solution for carrying out fluorescence studies in different viscosity environment. Probe **1** on reaction with H_2S forms amine and amine shows increase in fluorescence intensity with increasing glycerol fractions. This enhancement is due to the reason that in high viscosity the rotation of amine gets restricted and fluorescence emission by non-radiative pathway gets decreased. These studies proved that amine act as fluorescent molecular rotor.

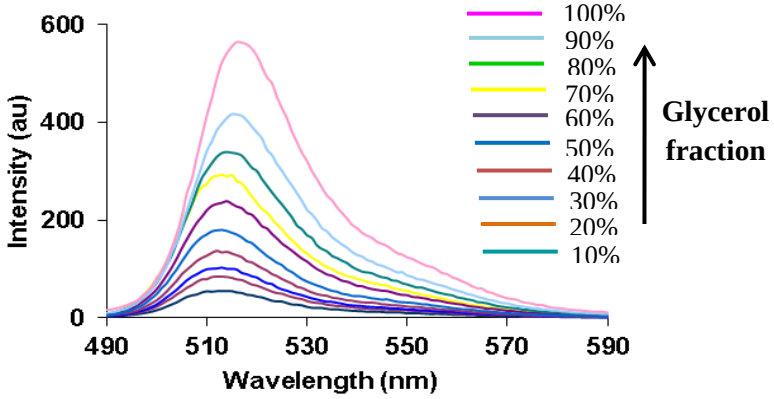


Figure S26: Fluorescence spectra of probe **1** (5.0 μM, from stock solution) with increasing glycerol fractions at 25 °C; λ_{ex} = 470 nm

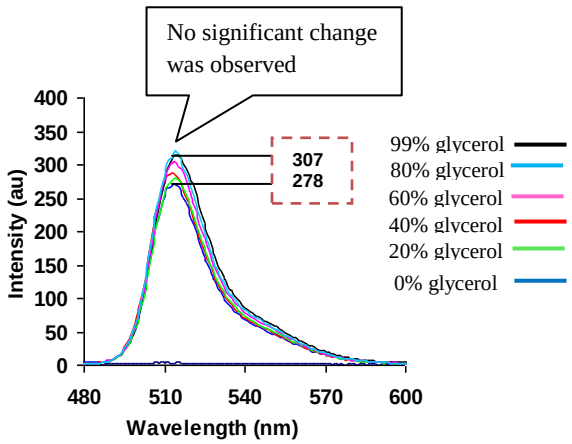


Figure S27: Fluorescence spectra of probe **1** (5.0 μM) at 515 nm in methanol: glycerol mixture with different viscosities (0.6 cP-950 cP) at 25 °C; λ_{ex} = 470 nm.

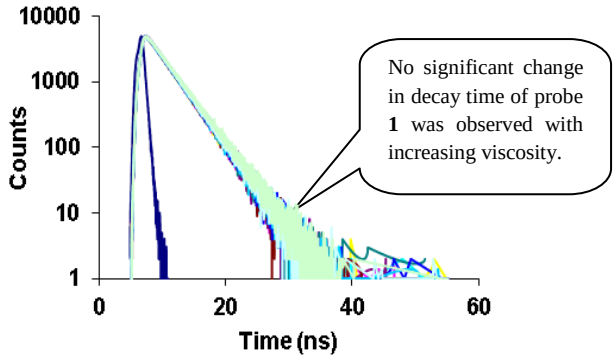


Figure S28: Fluorescence lifetime spectra of probe **1** (5.0 μM) at 515 nm in methanol: glycerol mixture with different viscosities (0.6 cP-950 cP) at 25 °C; λ_{ex} = 488 nm.

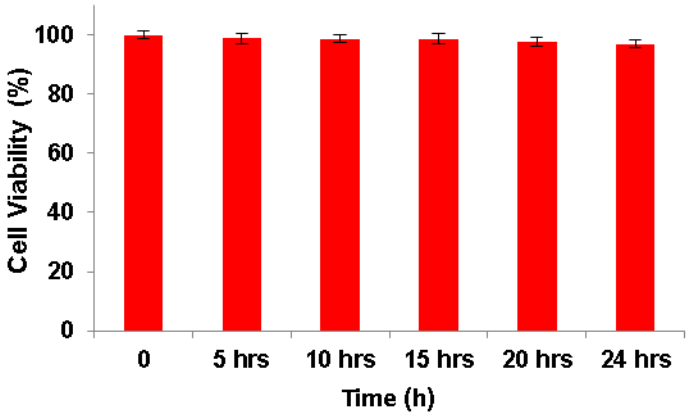


Figure S29: Cell viability of probe **1** (1.0 μM) at different time interval

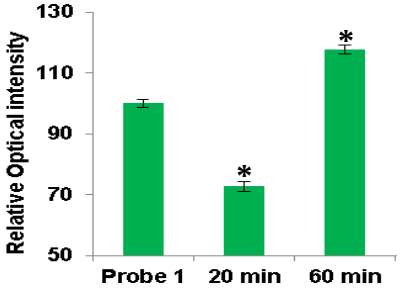


Figure S30: Intensity analysis of probe **1** (1.0 μM) with addition of exogenous H_2S (20 μM) on 20 min and 60 min incubation period.

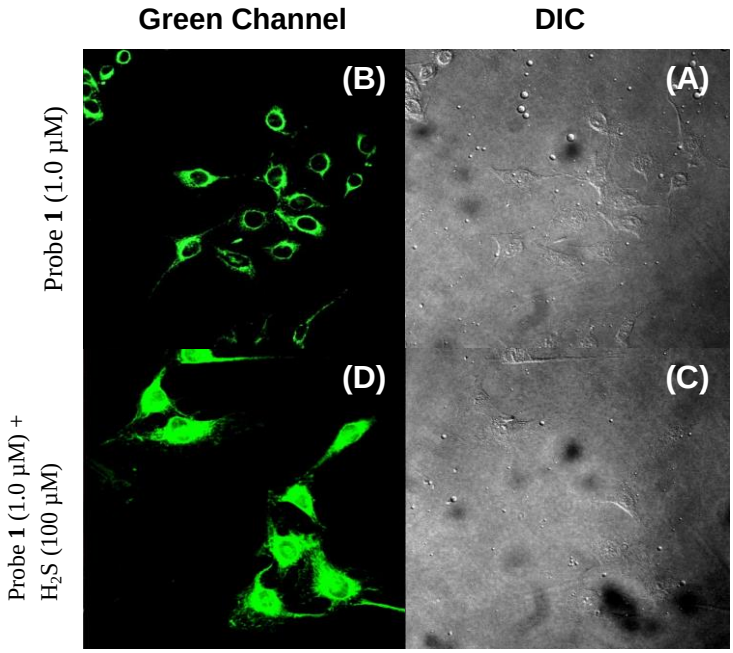


Figure S31: Cell images of C6 cell lines. (A) DIC image of cells were treated with probe **1** (1.0 μM) for 20 min at 37 °C. (B) Fluorescence image of cells were treated with probe **1** (1.0 μM) for 20 min at 37 °C. (C) DIC image of cells were treated with probe **1** (1.0 μM) for 20 min and then and then incubated with H_2S (100 μM) for 20 min at 37 °C. (D) DIC image of cells were treated with probe **1** (1.0 μM) for 20 min and then and then incubated with H_2S (100 μM) for 20 min at 37 °C. Images were taken at λ_{ex} = 488 nm and λ_{em} range 500-550 nm.

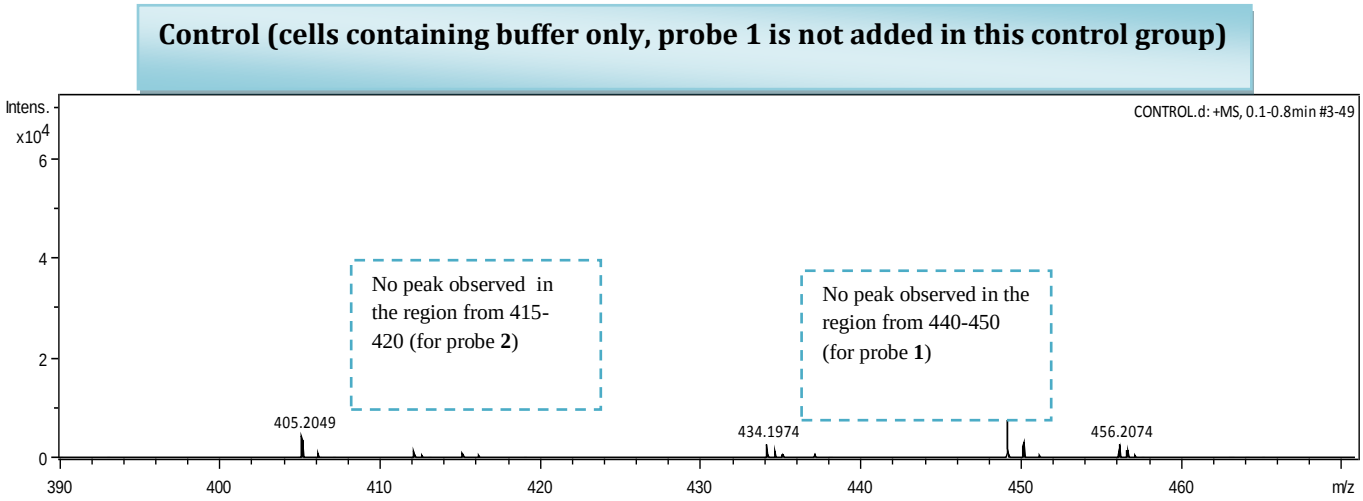


Figure S32: Mass spectral analysis of cell fluid of control group in which probe **1** is not added.

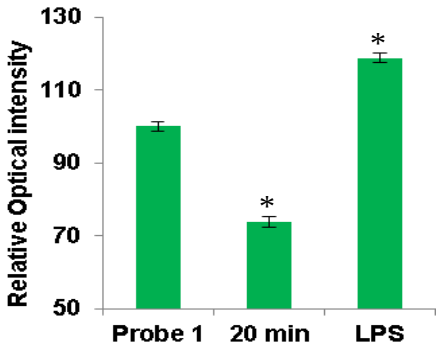


Figure S33: Intensity analysis of probe **1** (1.0 µM) with addition of exogenous H_2S (20 µM) on 20 min incubation period and lipopolysaccharide stimulated endogenously generated H_2S (2 µg/ml LPS for 24 hrs) then exposed to the probe **1** for 20 min.

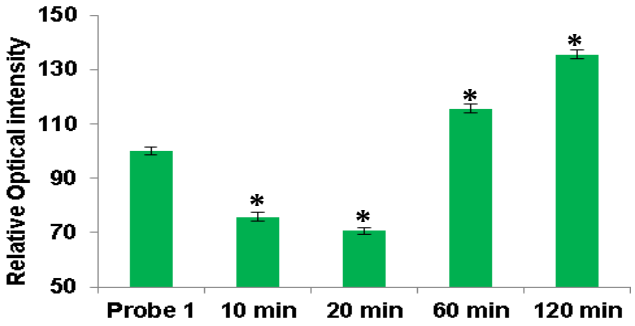
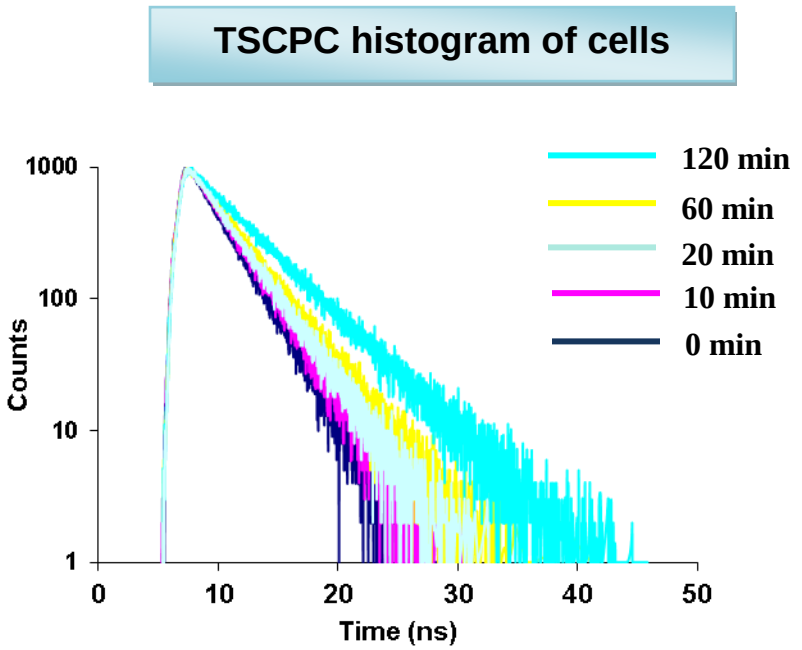


Figure S34: Intensity analysis of probe **1** (1.0 µM) with addition of exogenous H_2S (20 µM) with different time interval 10, 20, 60 and 120 min incubation period.



Time (min)	Life time (ns)
0	2.79
10	2.81
20	2.83
60	3.34
120	3.68

Figure S35: FLIM histogram of cells treated with probe **1** (1.0 µM) then incubated with H_2S (20 µM)

Procedure of cell imaging

Cell culture and treatments

C6 glioma cell was obtained from National Centre for Cell Sciences, Pune, India. Cells were maintained in DMEM supplemented with 1X PSN (GIBCO), 10% FBS (Biological Industries) at 37 °C and humid environment containing 5% CO_2. For fluorescence detection, cells were seeded on 18 mm coverslips in 12 well plates. For the purpose of the study, three groups were chosen:

I, Group in which C6 glioma cells treated with 1 μM probe **1** only, II, Exogenous H_2S detection: Cells were incubated with 1 μM probe for 20 min then exposed to 20 μM exogenous H_2S source for different time intervals (10 min, 20 min, 60 min, 120 min), III, Endogenous H_2S detection: cells were treated with 2 μg/ml LPS for 24 hrs then exposed to the probe **1** for 20 min.

For Fluorescence Detection, Firstly, cells were washed with 1X PBS thrice for 5 minutes each and fixed with acetone: methanol in 1:1 ratio for 10 min. After fixing cells were washed with 1X PBS for 5 min followed by mounting on the slides using anti-fading medium. Images were taken with A1R Nikon Laser Scanning Confocal microscope at 488 nm channel.

DAPI Staining: Firstly, cells were washed with 1X PBS thrice for 5 minutes each and fixed with acetone: methanol in 1:1 ratio for 10 min. After fixing cells were washed with 1X PBS for 5 min, then stained with DAPI for 5 min followed by mounting on the slides using anti-fading medium. Images were taken with A1R Nikon Laser Scanning Confocal microscope at 488 nm channel.

Statistical analysis

Values are expressed as mean ± SEM. The SigmaStat for Windows (version 3.5) was adopted to analyse the results by One Way ANOVA test in order to determine the significance of the means. Values of $p< 0.05$ were considered as statistically significant.

For MS Analysis:

C6 glioma cells were seeded in 90 mm petri plates with seeding density 25,000 cells/ml. For this study, four groups were chosen:

(a) I, Control group with untreated cells

(b) II, Group in which C6 glioma cells treated with 1.0 μM probe **1** only

(c) III, Exogenous H_2S detection: Cells were incubated with to1.0 μM probe **1** for 20 min and then exposed to 20 μM exogenous H_2S source for 20 min.

(d) IV, Endogenous detection: cells were treated with 2 μg/ml LPS for 24 hrs then exposed to the probe **1** for 20 min.

Cells were washed with 1X PBS thrice for 5 min each, then harvested using PBS-EDTA and pelleted down at 1500 rpm for 5 min at 4 °C. Amount of cell pellet was weighed and homogenized in 2 volumes of chilling homogenizing RIPA buffer and centrifuged for 10 min at 10,000 rpm. Thus the cell supernatant obtained was used for MS analysis for all the treatment groups and RIPA buffer was used as the blank.

Procedure of fluorescence lifetime imaging (FLIM)

The fluorescence lifetime imaging (FLIM) was measured with an inverted-type laser scanning confocal microscope with a 60Xobjective. The emission was collected through a 500 ± 30 nm band pass filter. For imaging studies, C6 cells were supplemented with 10% fetal bovine serum. The cells were seeded in 24-well flat-bottomed plates and then incubated for 24 hrs at 37 °C under 5% CO_2. Probe **1** (1.0 μM) was then added to the cells and then the cells are treated with H_2S (20 μM) incubated. The cells were washed three times with 2 mL PBS at room temperature, and then observed under a confocal microscopy with time.