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Supporting Information

Dual Mode 'Turn-on' Fluorescence-Raman (SERS) Response probe based on 1*H*-pyrrol-3(2*H*)-one scaffold for Monitoring H_2S Levels in Biological Samples

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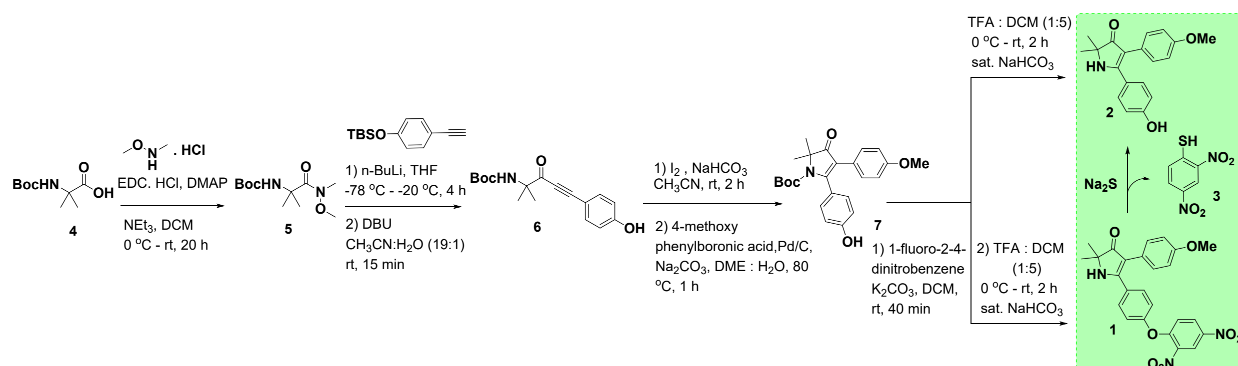
1. Experimental procedures, ^{1}H NMR and ^{13}C NMR data



Scheme S1. Synthesis of the probe **1**

Experimental procedure

1. *Synthesis of compound* **5**: Boc-protected 2-Aminoisobutyric acid (24 mmol, 5.0 g), N,O-dimethyl hydroxylamine.HCl (32 mmol, 3.1 g) and DMAP (2 mmol, 0.30 g) were dissolved in dry DCM (50 mL) and cooled to 0 °C in a 250 mL round bottom flask. Triethyl amine (39 mmol, 5.5 mL) was added slowly to the reaction mixture over 5 minutes, followed by 1-Ethyl-3-(3′ dimethylaminopropyl) carbodiimide hydrochloride salt (29 mmol, 5.6 g). The solution was allowed to stir for 1 h at 0 °C and then for 19 h at room temperature. It was then diluted with DCM (10 mL) and washed with 1N HCl (15 mL), sat. $NaHCO_3$ (15 mL) followed by brine (15 mL). The organic layer was concentrated under vacuum and the residue was purified by column chromatography (100-200 mesh silica) using ethyl acetate/ hexane solvent system to obtain the compound **5** in pure form. White solid; Yield = 99% (6.0 g); R_f = 0.36 (30% EtOAc/hexane). ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.21 (bs, 1H, N**H**), 3.68 (s, 3H, OC$\mathbf{H}_3$), 3.21 (s, 3H, NC$\mathbf{H}_3$), 1.55 (s, 6H, C$\mathbf{H}_3$), 1.43 (s, 9H, C$\mathbf{H}_3$) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ 174.8, 154.5, 79.6, 60.8, 56.8, 34.0, 28.5 (3C), 24.8 (2C) ppm. HRMS (ESI) m/z: $[M+Na]^+$ Calcd for $C_{11}H_{22}N_2O_4Na$ 269.1477; found 269.1457.

2. *Synthesis of compound* **6**: To a stirred solution of OTBS-protected 4-hydroxy phenyl acetylene (24 mmol, 5.57 g) in dry THF (50 mL) at -78 °C was added a solution of 1.6 M *n*-BuLi in hexane (24 mmol, 15.2 mL) dropwise, and the mixture was stirred for 45 minutes at the same temperature. To this was added **5** (8 mmol, 2.0 g) in THF (15 mL) dropwise at -78 °C, stirred at the same temperature for 1 h, and then at -20 °C for 2 h till completion of the reaction. The reaction was then quenched by adding saturated NH_4Cl solution and concentrated under vacuum. The mixture was diluted with ethyl acetate (20 mL), washed with water, the organic layer separated, dried with Na_2SO_4 and evaporated under vacuum. The crude residue

obtained was then purified on silica gel column using ethyl acetate/ hexane solvent system to get the OTBS-protected derivative of **6**. This intermediate (4 mmol, 1.9 g) was dissolved in Acetonitrile-H_2O mixture (19:1) and 1,8-Diazabicyclo[5.4.0]undec-7-ene (4 mmol, 0.68 ml) was added dropwise. The reaction mixture was stirred at room temperature for 15 min., solvent was evaporated and the residue was purified by column chromatography using ethyl acetate/ hexane solvent system to obtain the product **6** in pure form. White solid; Yield = 56% (1.3 g); $R_{f\,=}$ 0.50 (30% EtOAc/hexane). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.41 (d, *J* = 6.8 Hz, 2H, Ar**H**), 6.84 (d, *J* = 7.0 Hz, 2H, Ar**H**), 1.54 (s, 6H, C**H$_3$**), 1.43 (s, 9H, C**H**$_3$) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ 190.0, 159.6, 135.6 (2C), 129.2, 116.3 (2C), 115.4, 110.7, 85.3, 61.3, 28.4 (3C), 27.9, 24.6 (2C) ppm. HRMS (ESI) m/z: $[M+Na]^+$ Calcd for $C_{17}H_{21}NO_4Na$ 326.1368; found 326.1356.

2. *Synthesis of the compound* **7**: (a). To a solution of the compound **6** (4 mmol, 1.3 g) in acetonitrile (10 mL) taken in a 100 mL round bottom flask was added $NaHCO_3$ (12 mmol, 1.1 g), followed by Iodine (12 mmol, 1.6 g). The reaction mixture was stirred for 2 h at room temperature and quenched using sodium thiosulfate solution. It was then diluted with ethyl acetate (15 mL), washed with water, the organic layer was separated, dried using anhydrous Na_2SO_4 and concentrated under vacuum. The crude product obtained (**6a**) was purified by column chromatography on silica gel using ethyl acetate/ hexane solvent system. White solid; Yield = 63 % (1.1 g); R_f = 0.60 (40 % EtOAc/hexane). ^{1}H NMR (400 MHz, DMSO-d_6): δ 9.96 (bs, 1H, O**H**), 7.23 (d, *J* = 8.1 Hz, 2H, Ar**H**), 6.87 (d, *J* = 7.9 Hz, 2H, Ar**H**), 1.44 (s, 6H, 2C**H$_3$**), 1.17 (s, 9H, 3C**H$_3$**) ppm. ^{13}C NMR (100 MHz, DMSO-d_6): δ 199.3, 170.0, 158.9, 148.1, 129.5 (2C), 123.8, 114.7 (2C), 82.3, 75.8, 67.2, 27.1 (3C), 23.0 (2C) ppm. HRMS (ESI) m/z: $[M+Na]^+$ Calcd for $C_{17}H_{20}INO_4Na$ 452.0335; found 452.0322.

(b). A mixture of the intermediate **6a** (2 mmol, 1.1 g) obtained in the previous step, 4-methoxy phenylboronic acid (3 mmol, 0.47 g), 10% Palladium on carbon (0.2 mmol, 0.21 g) and sodium carbonate (6 mmol, 0.63 g) was taken in a round bottom flask and dissolved in DME/H_2O (1:1; 10 mL) mixture. The reaction mixture was heated at 80 ^{o}C for 1 h, filtered over celite, washed with DCM (4 x 5 mL) and the filtrate was concentrated under vacuum. The product was purified by column chromatography on silica gel using ethyl acetate/ hexane solvent system. Yellow solid; Yield = 99% (1.0 g); R_f = 0.56 (40% EtOAc/hexane). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.10 (d, *J* = 8.4 Hz, 2H, Ar**H**), 6.97 (d, *J* = 8.4 Hz, 2H, Ar**H**), 6.78 (d, *J* = 8.4 Hz, 2H, Ar**H**), 6.72 (d, *J* = 8.6 Hz, 2H, Ar**H**), 5.39 (s, 1H, O**H**), 3.74 (s, 3H, OC**H$_3$**), 1.61 (s, 6H, C**H$_3$**), 1.30 (s, 9H, C**H$_3$**) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ 201.1, 165.1, 158.3, 157.9,

148.8, 130.5 (2C), 129.4 (2C), 123.0, 122.4, 117.4, 114.9 (2C), 113.2 (2C), 81.7, 66.6, 54.9, 27.2 (3C), 22.9 (2C) ppm. HRMS (ESI) m/z: $[M+Na]^+$ Calcd for $C_{24}H_{27}NO_5Na$ 432.1787; found 432.1775.

4. *Synthesis of the compound* **3**: The compound **3** was prepared according to the literature procedure.[1]

2. ^{1}H NMR and ^{13}C NMR spectra

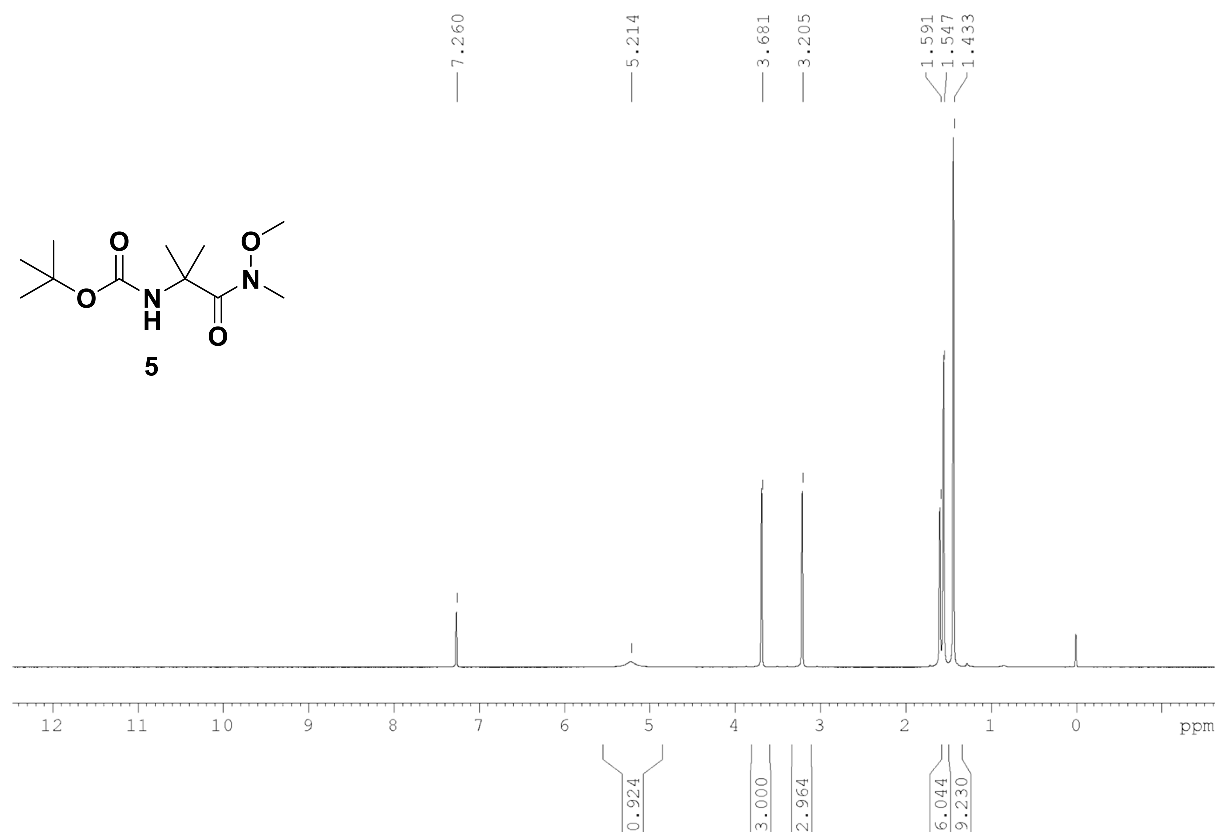


Figure S1. ^{1}H NMR spectrum of the compound **5** ($CDCl_3$; 400 MHz)

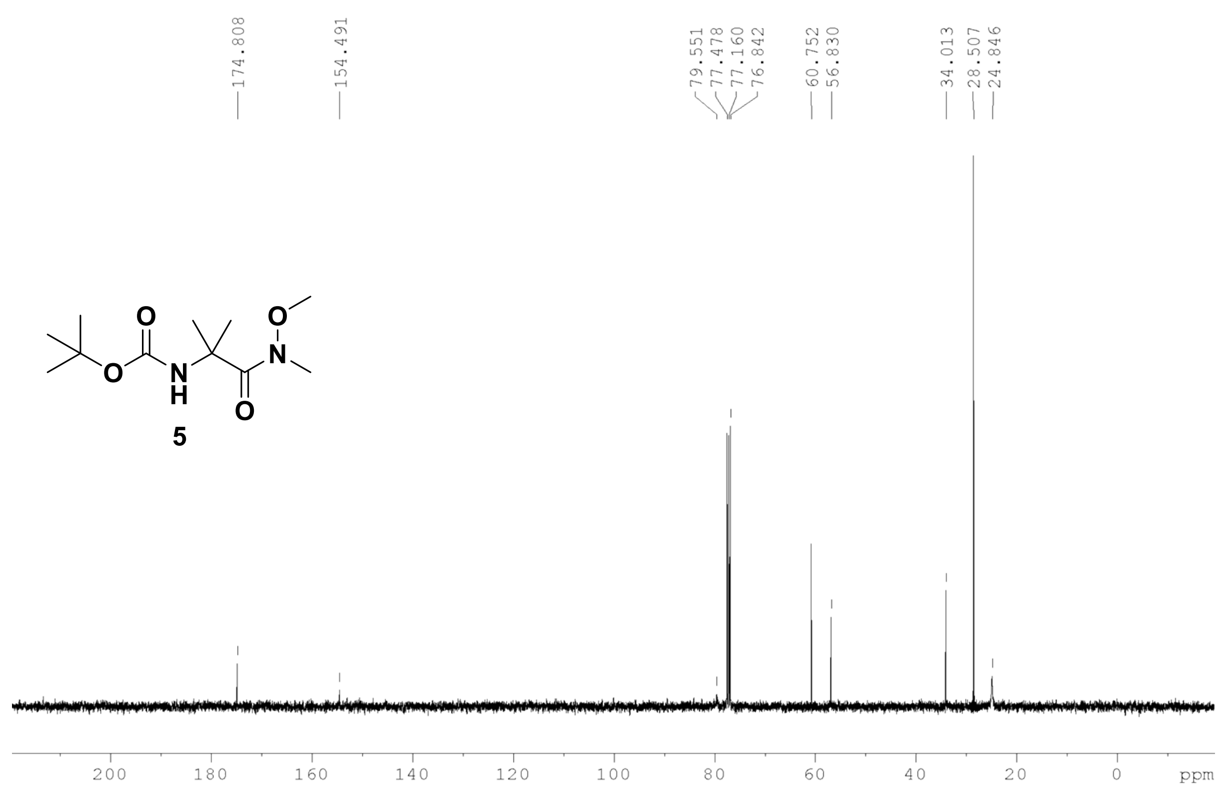


Figure S2. ^{13}C NMR spectrum of the compound **5** ($CDCl_3$; 100 MHz)

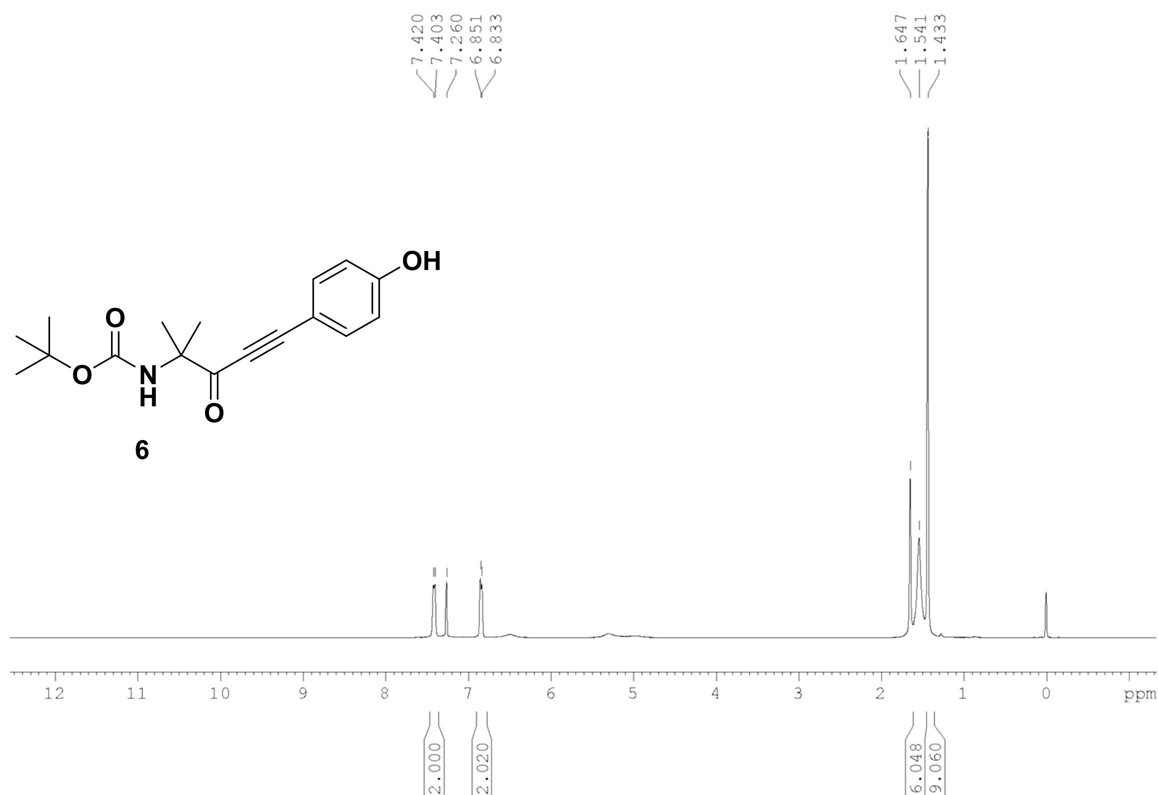


Figure S3. ^{1}H NMR spectrum of the compound **6** ($CDCl_3$; 400 MHz)

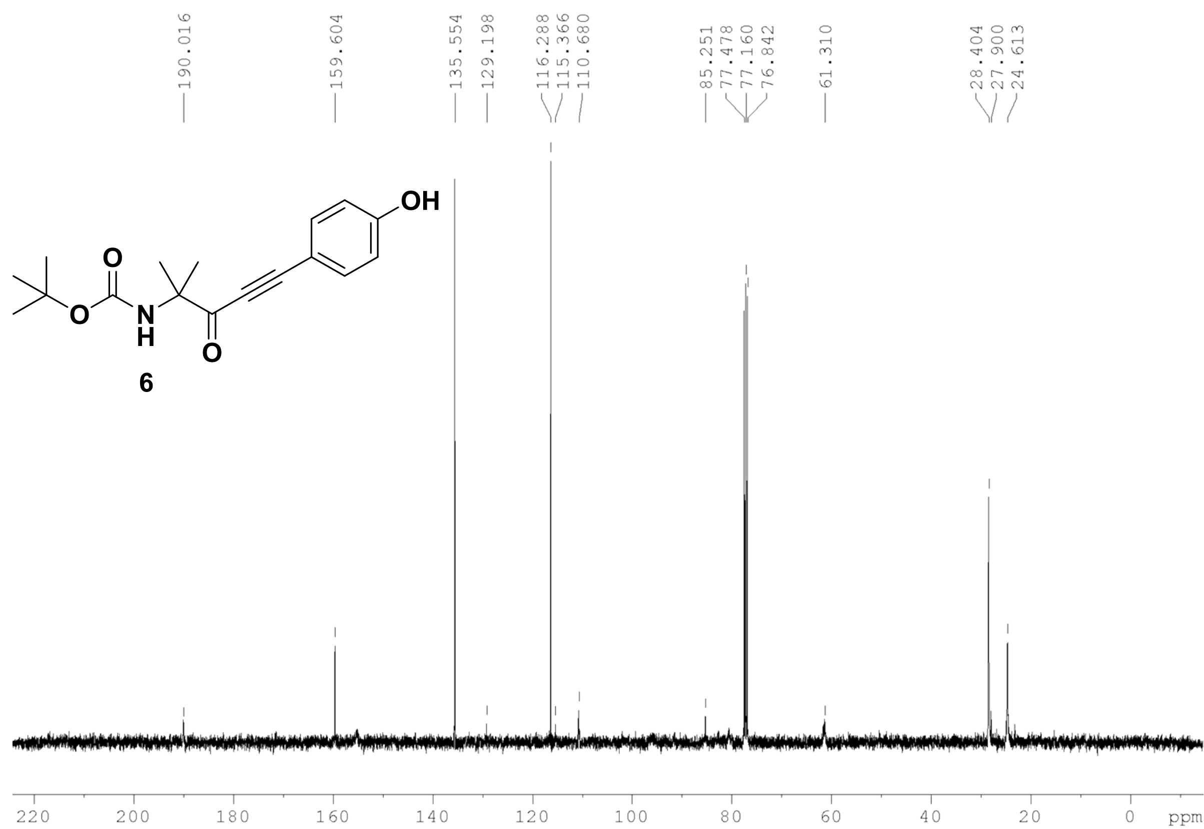


Figure S4. ^{13}C NMR spectrum of the compound **6** ($CDCl_3$; 100 MHz)

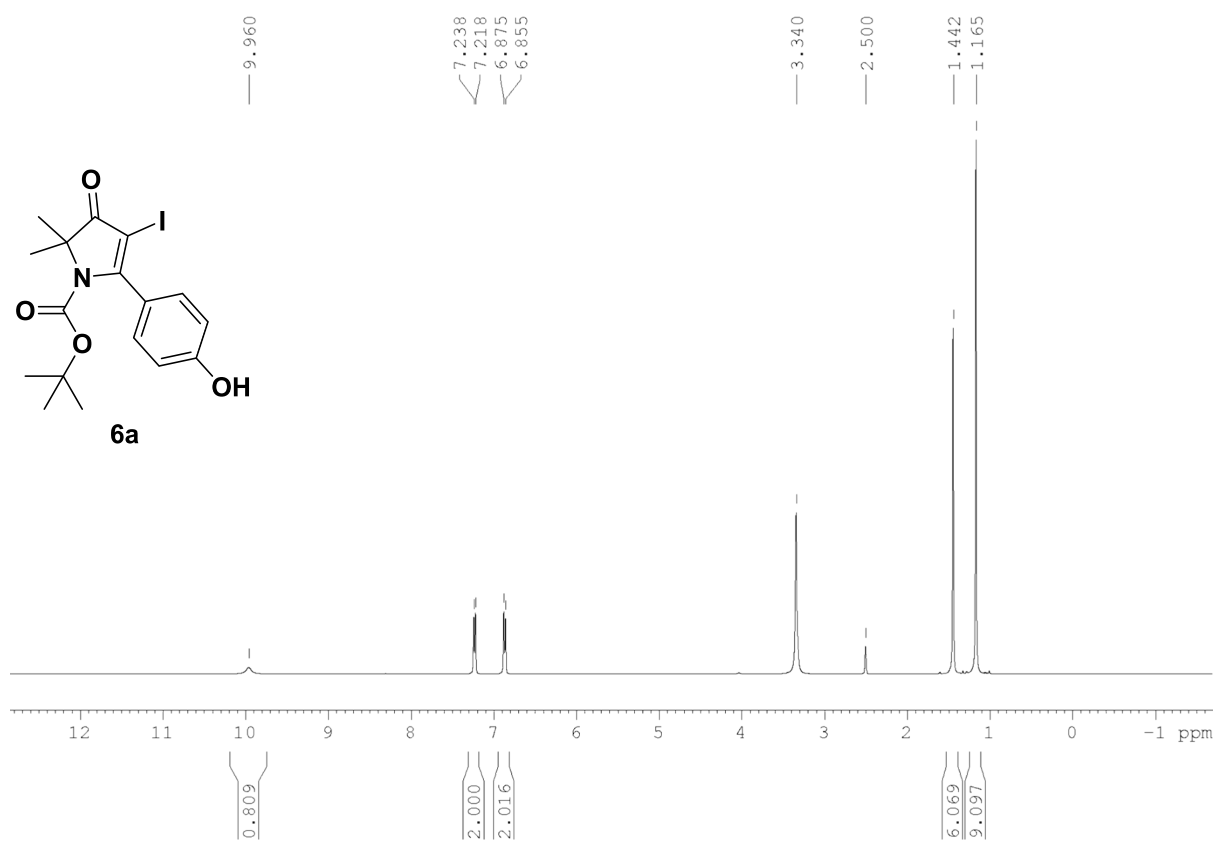


Figure S5. ^{1}H NMR spectrum of the iodo-cyclized intermediate **6a** (DMSO-d_6; 400 MHz)

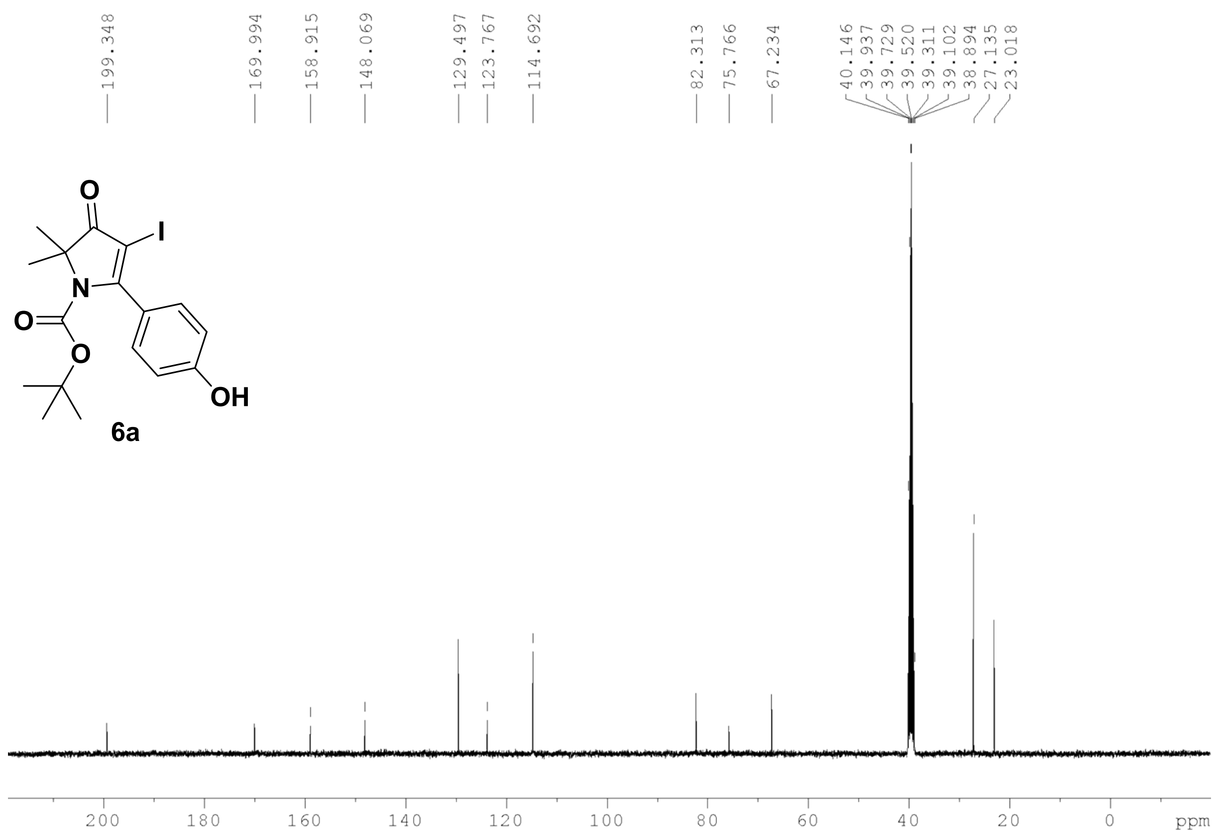
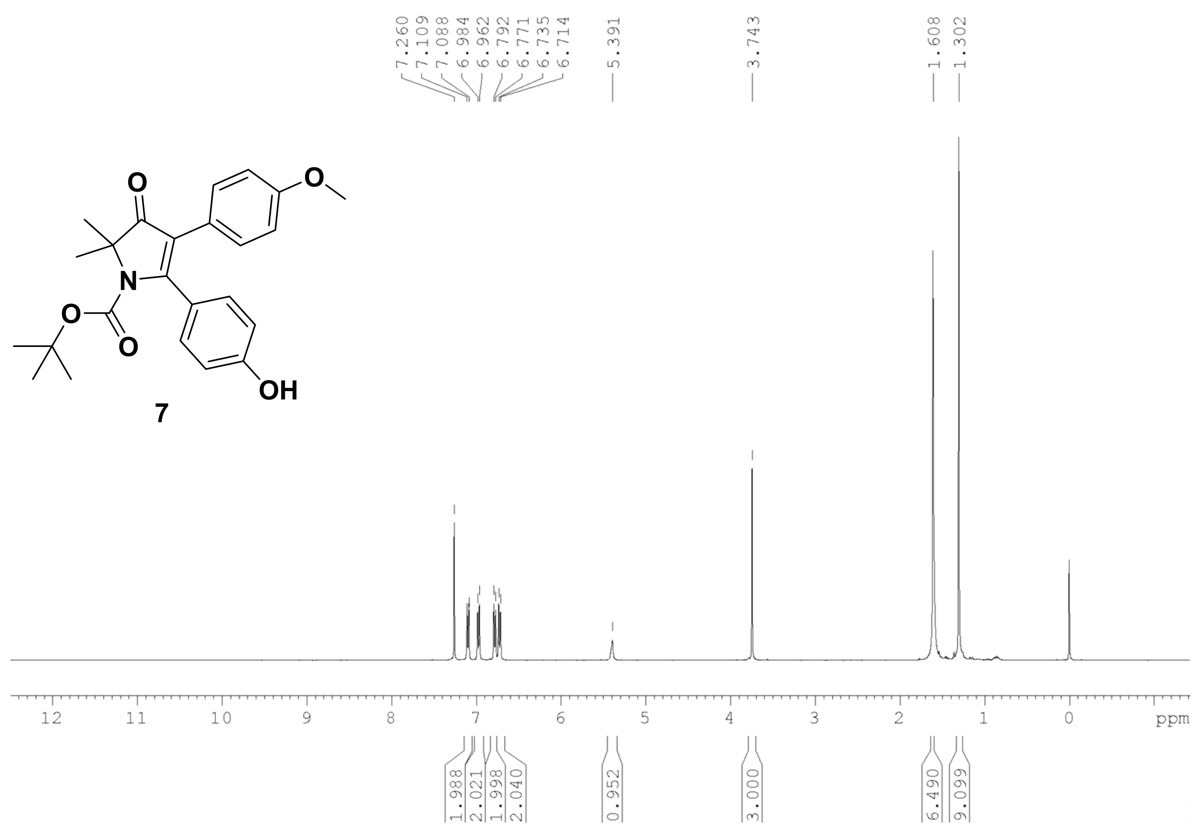


Figure S6. ^{13}C NMR spectrum of the iodo-cyclized intermediate **6a** (DMSO-d_6; 100 MHz)



F

igure S7. ^{1}H NMR spectrum of the compound **7** ($CDCl_3$; 400 MHz)

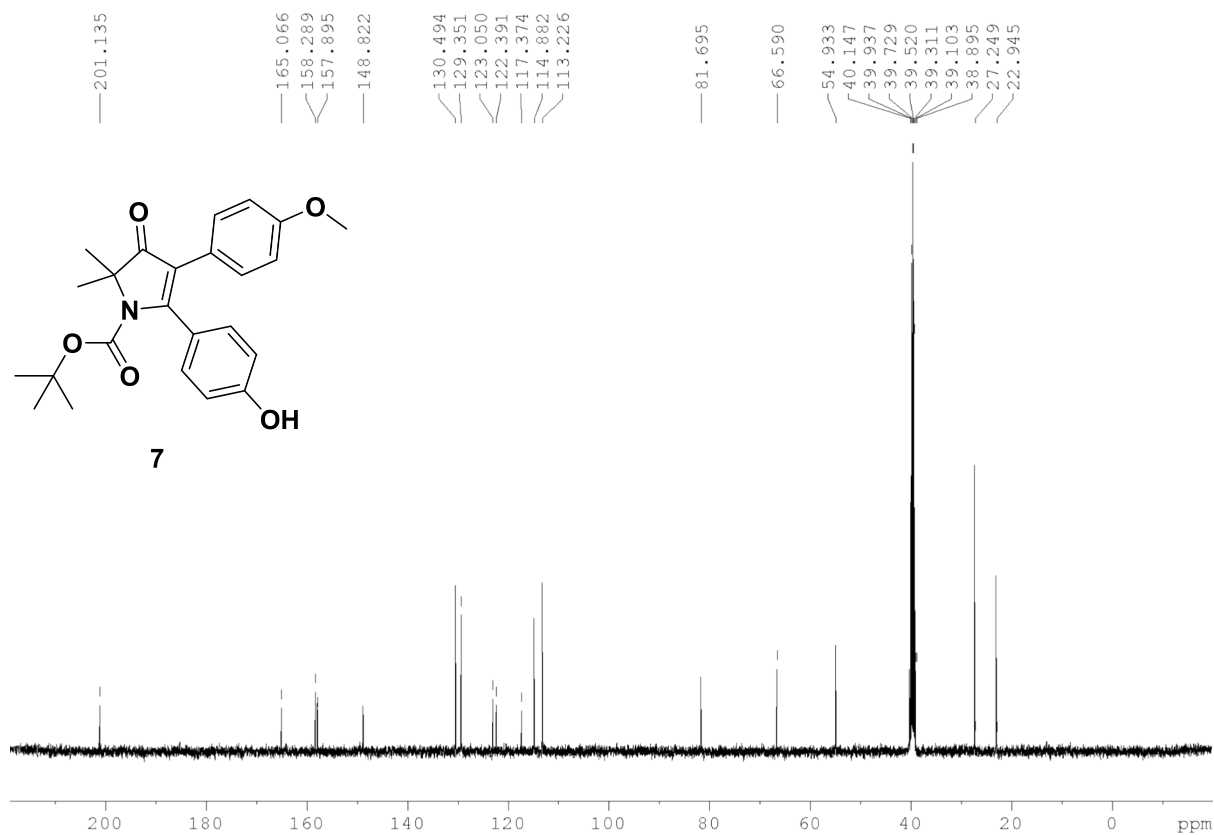


Figure S8. ^{13}C NMR spectrum of the compound **7** ($CDCl_3$; 100 MHz)

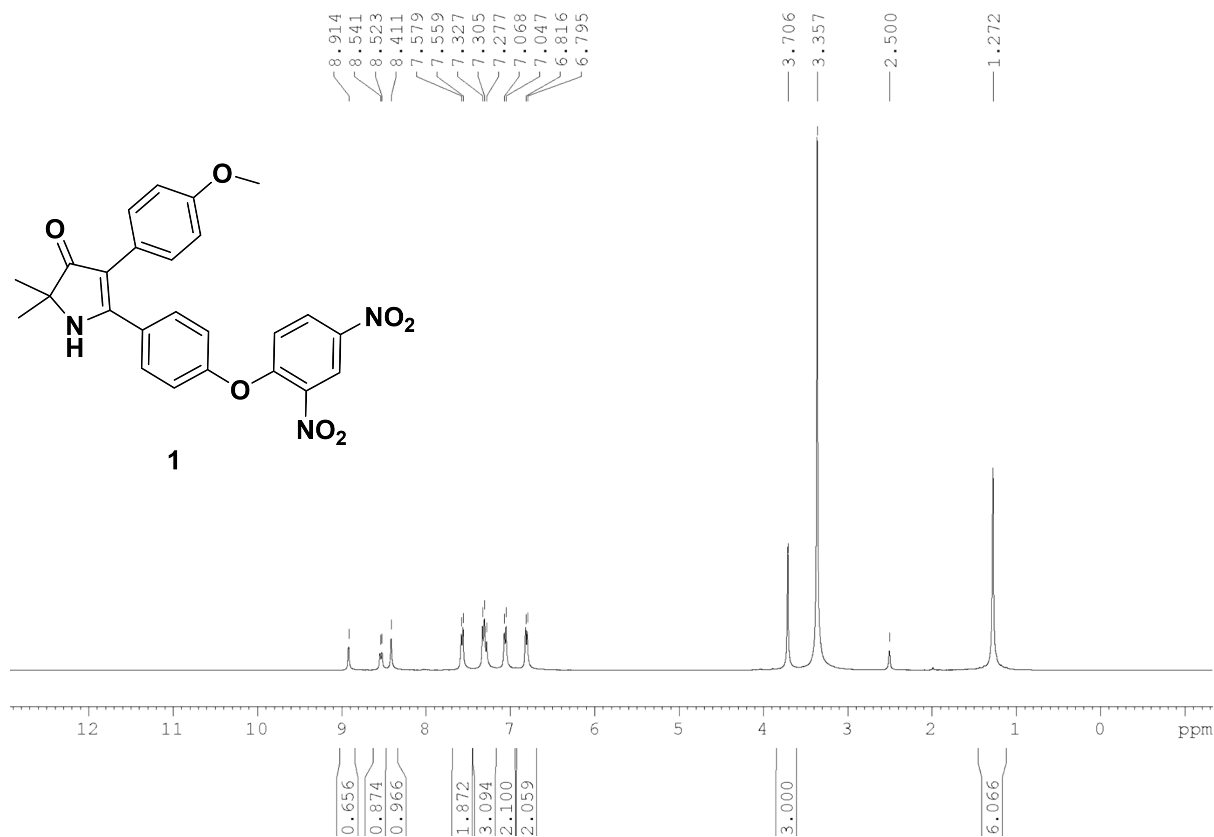


Figure S9. ^{1}H NMR spectrum of the compound **1** (DMSO-d_6; 400 MHz)

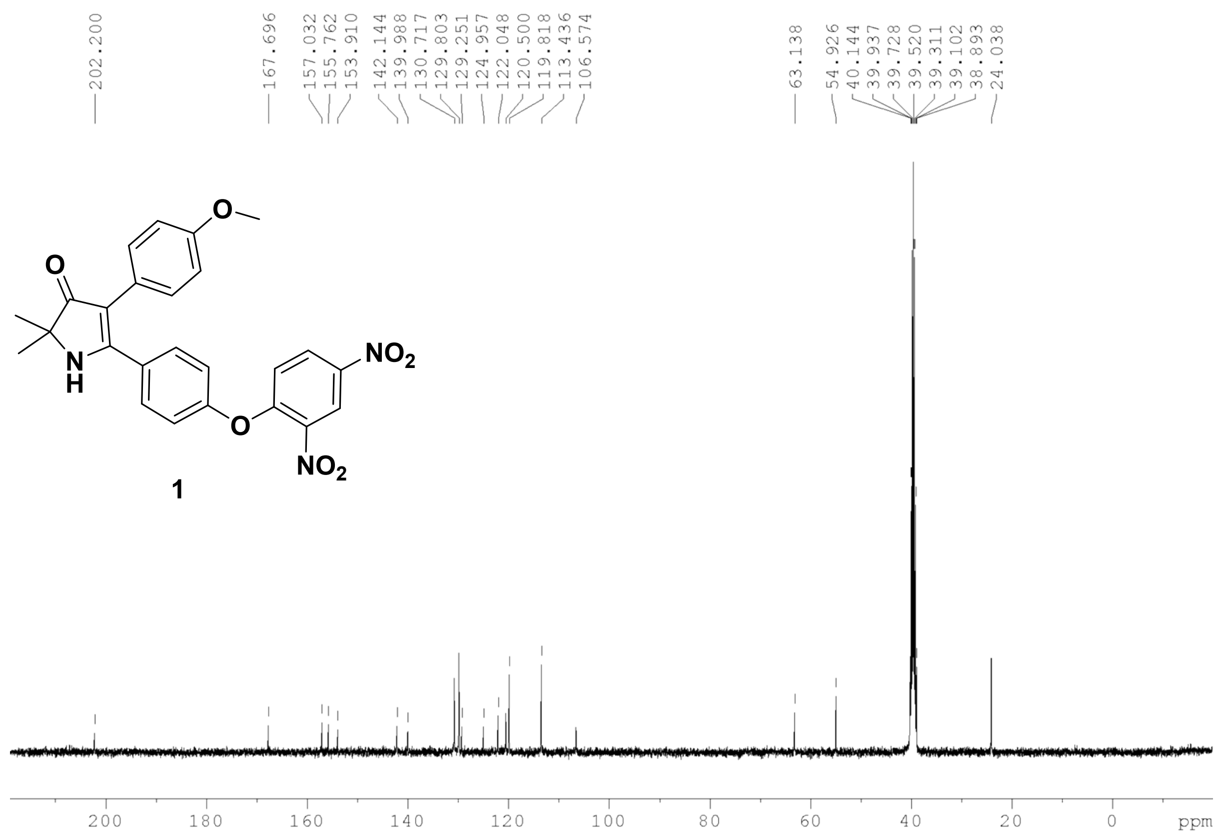


Figure S10. ^{13}C NMR spectrum of the compound **1** (DMSO-d_6; 100 MHz)

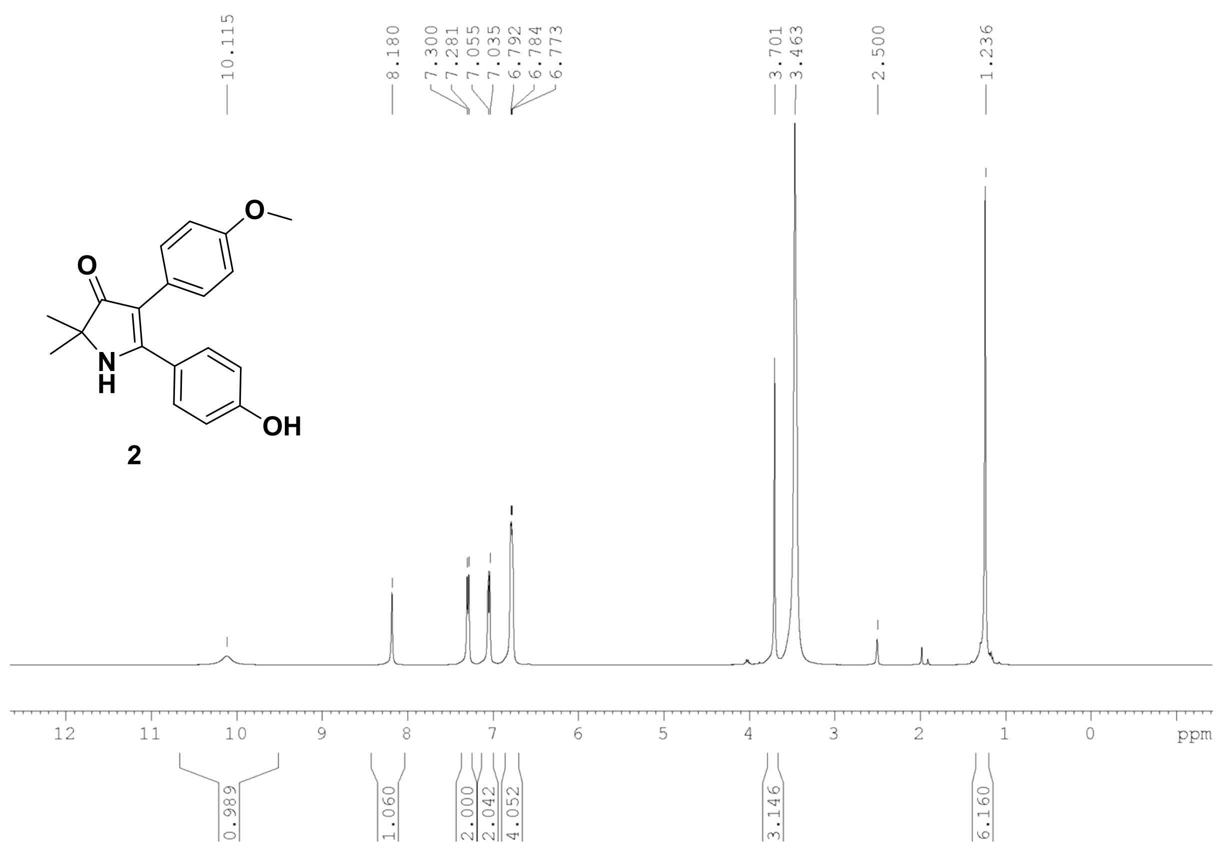


Figure S11. ^{1}H NMR spectrum of the compound **2** (DMSO-d_6; 400 MHz)

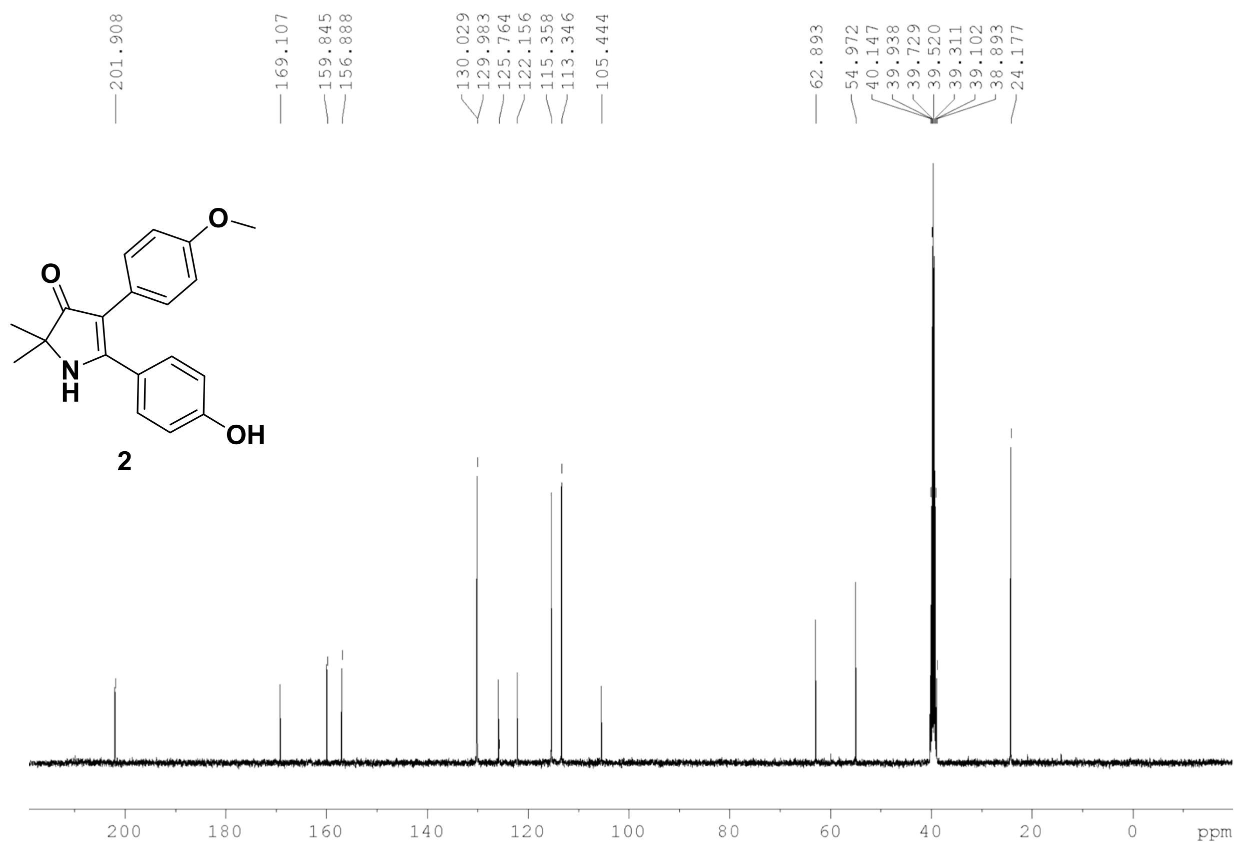


Figure S12. ^{13}C NMR spectrum of the compound **2** (DMSO-d_6; 100 MHz)

3. HRMS spectrum

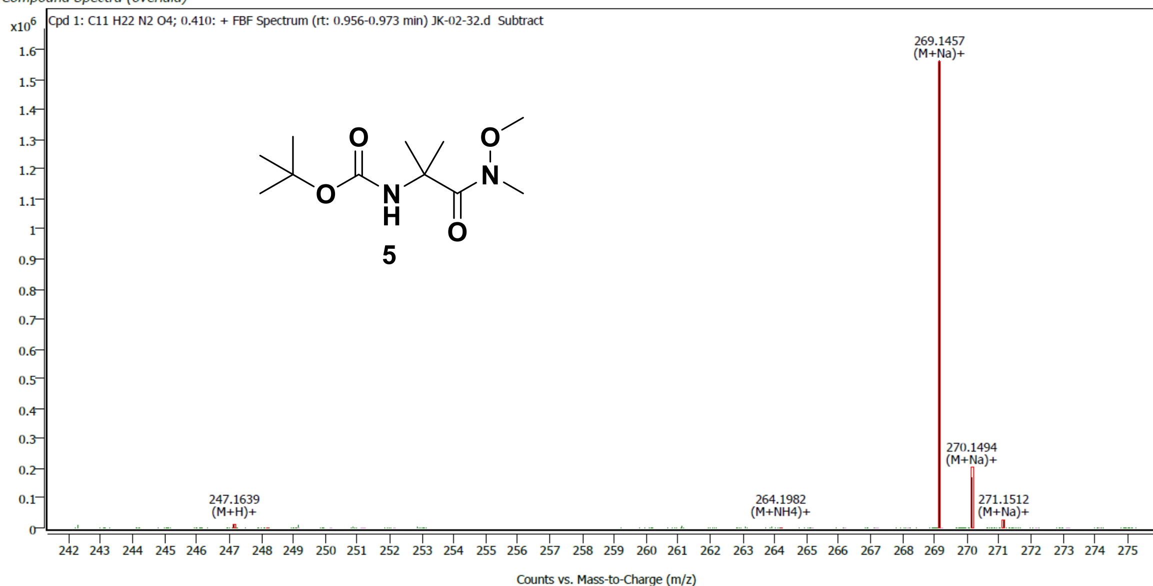


Figure S13. HRMS spectrum of the compound **5**

Compound Details

Cpd. 1: C17 H21 N O4

Compound Spectra (overlaid)

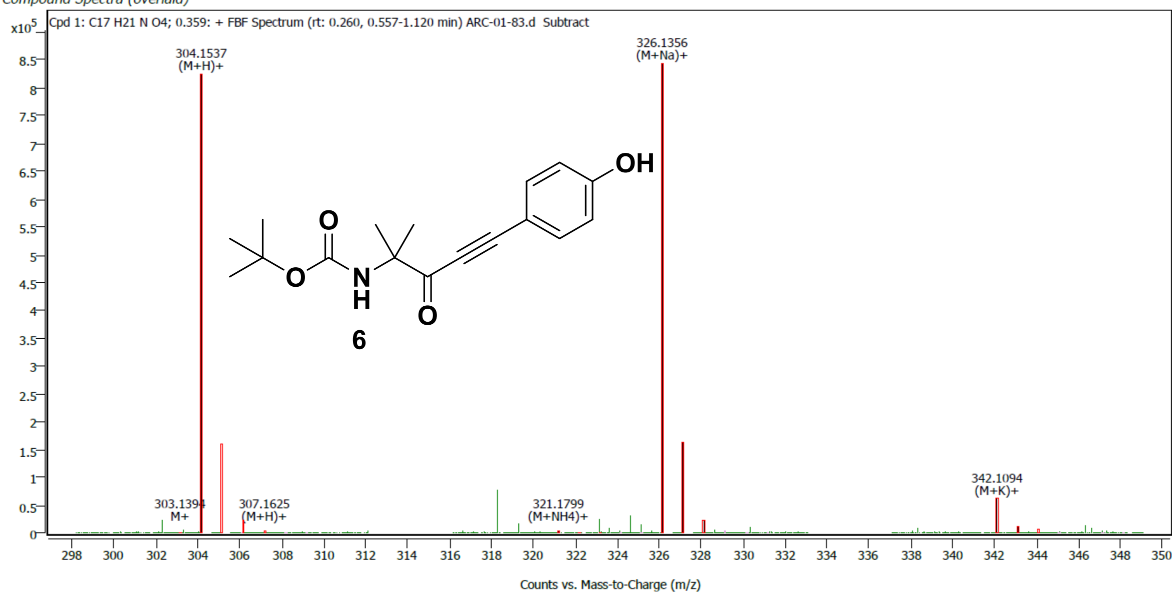


Figure S14. HRMS spectrum of the compound **6**

Compound Details

Cpd. 1: C17 H20 I N O4

Compound Spectra (overlaid)

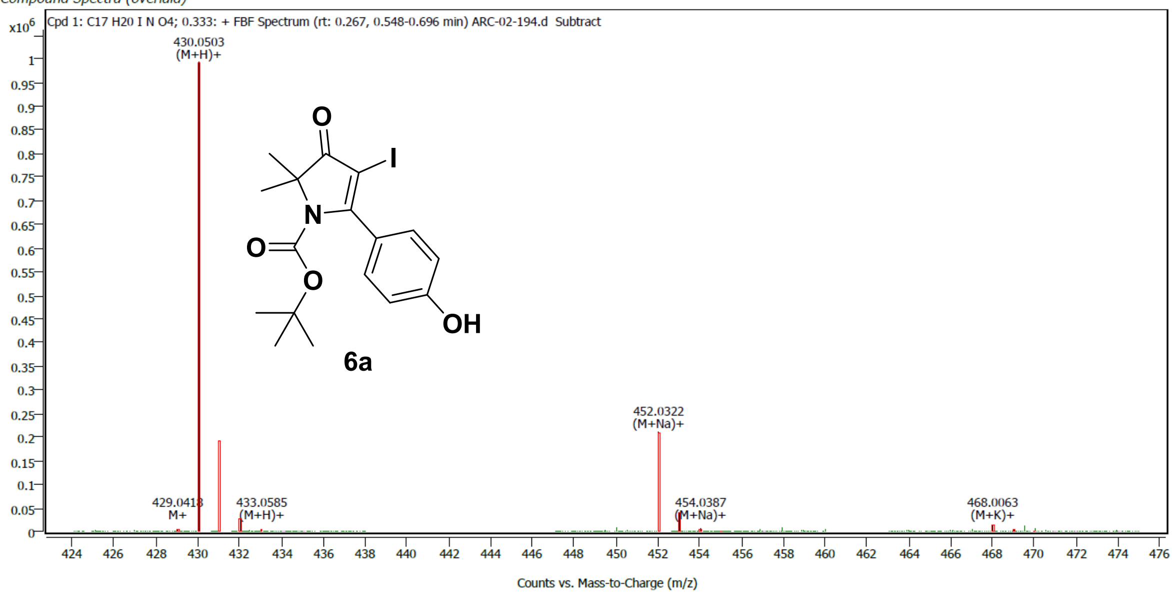


Figure S15. HRMS spectrum of the iodo-cyclized intermediate **6a**

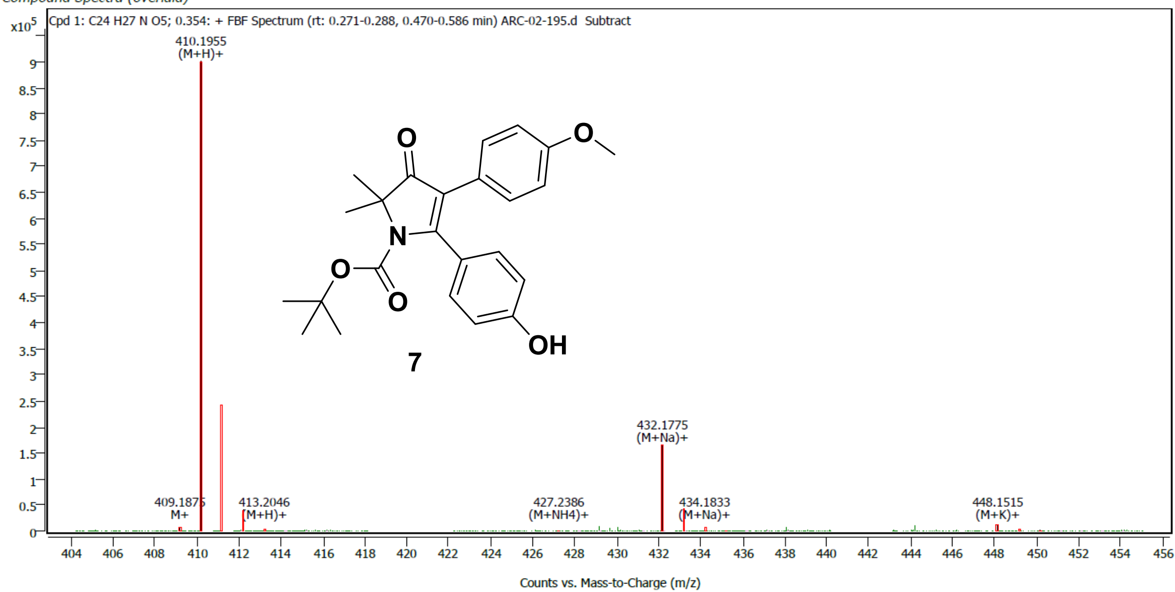


Figure S16. HRMS spectrum of the compound **7**

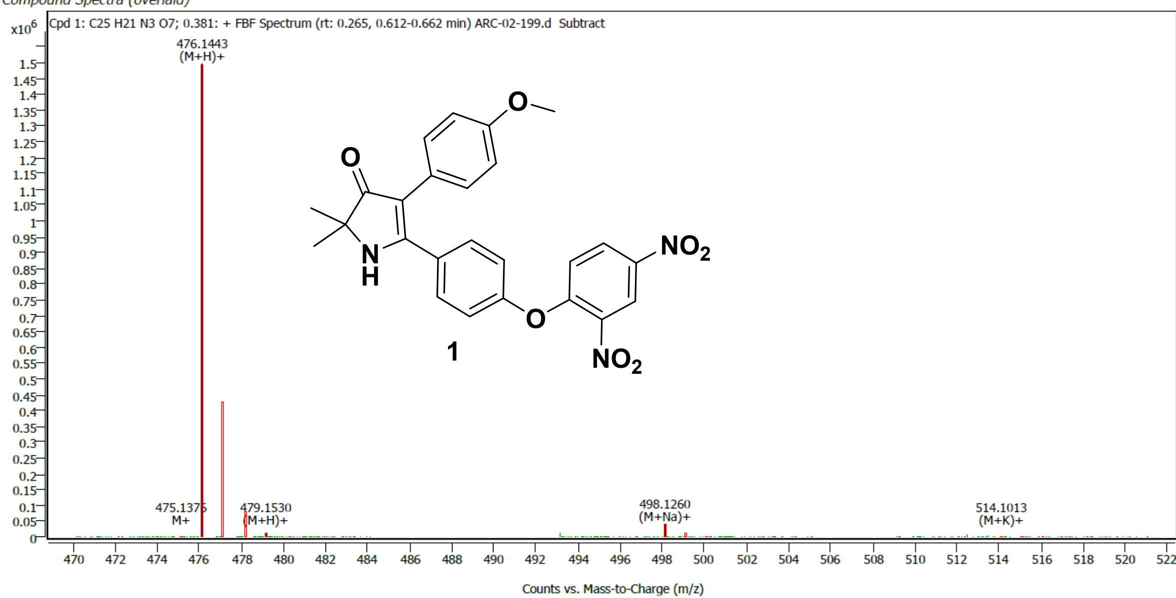


Figure S17. HRMS spectrum of the compound **1**

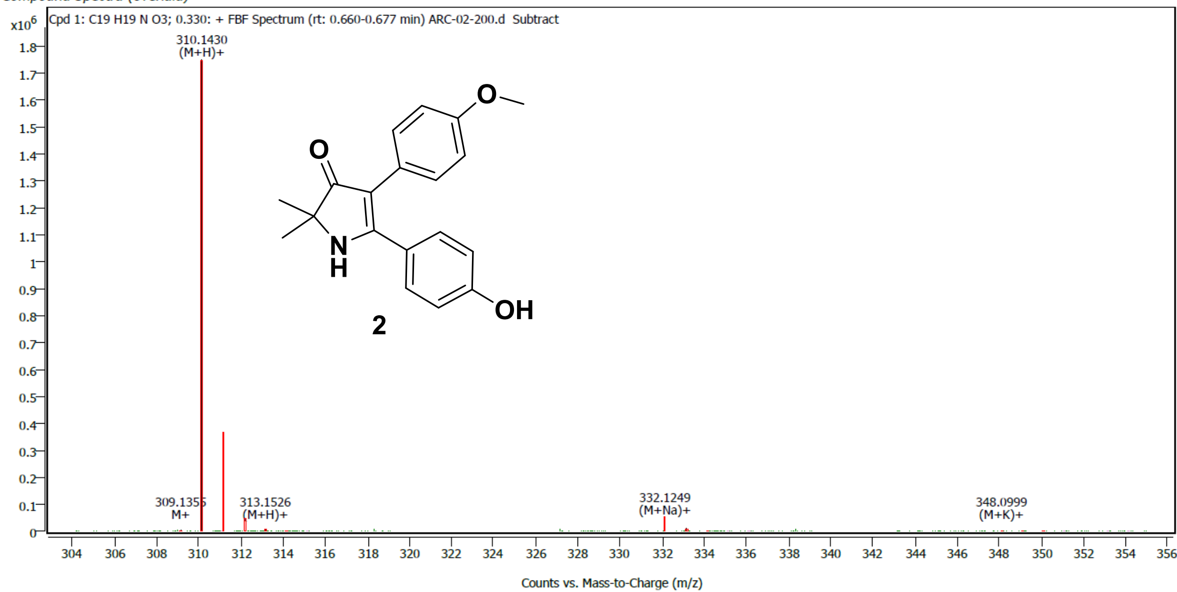


Figure S18. HRMS spectrum of the compound **2**

4. Photophysical response of the probe 1 towards H_2S

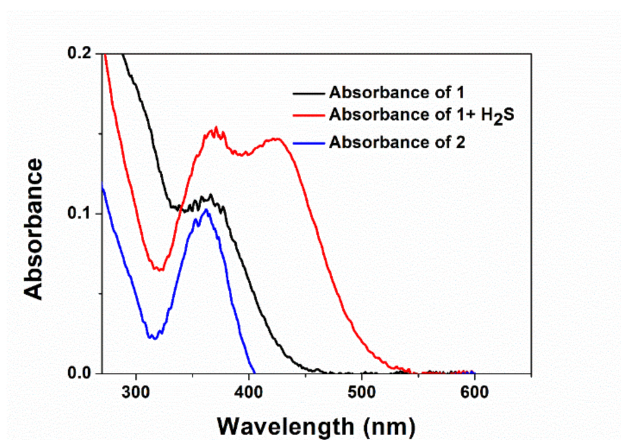


Figure S19. Absorption spectra of compounds **1**, **2** and that of the reaction mixture of probe **1** with H_2S in PBS buffer of pH = 7.4.

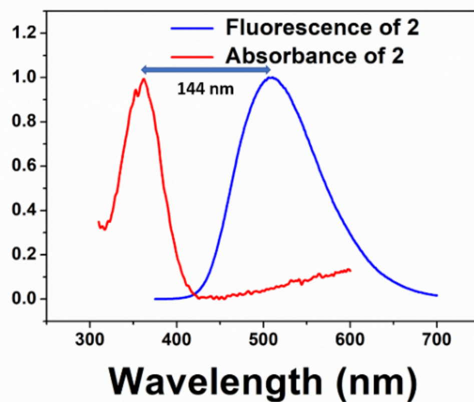


Figure S20. Normalized absorption and emission spectra of the compound **2** in PBS buffer of pH = 7.4.

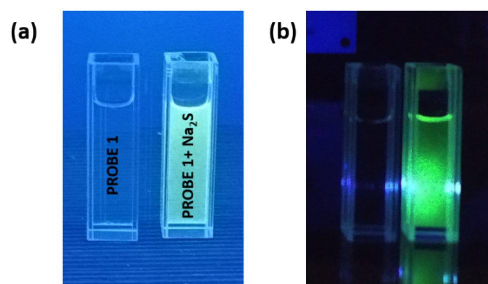


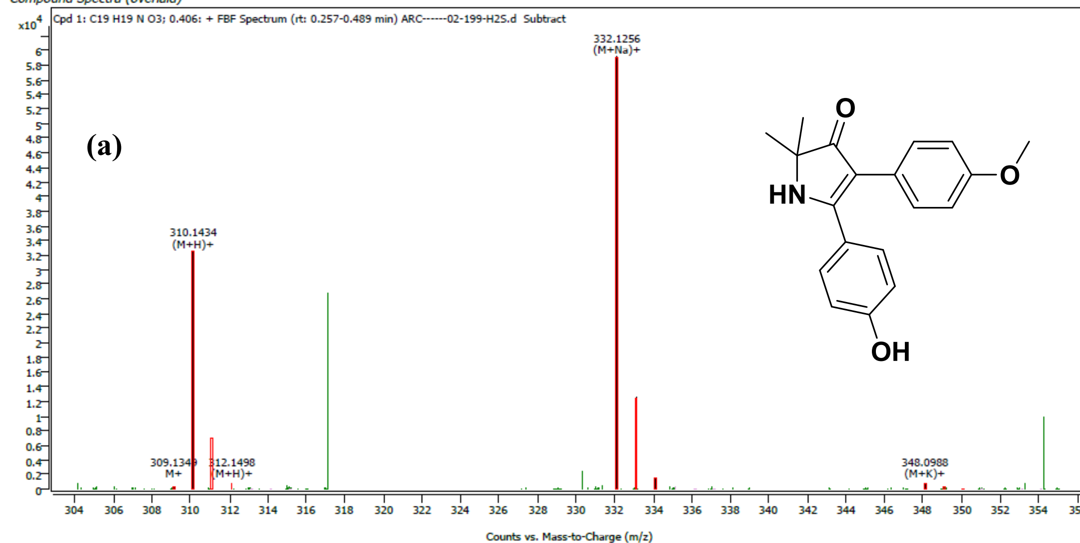
Figure S21. Visible fluorescence changes of the probe **1** (100 µM) before and after the addition of Na_2S (100 µM) when viewed under (a) UV light (365 nm), and (b) under 405 nm blue beam laser.

5. HRMS analysis of the reaction of probe 1 with H_2S

Compound Details

Cpd. 1: C19 H19 N O3

Compound Spectra (overlaid)



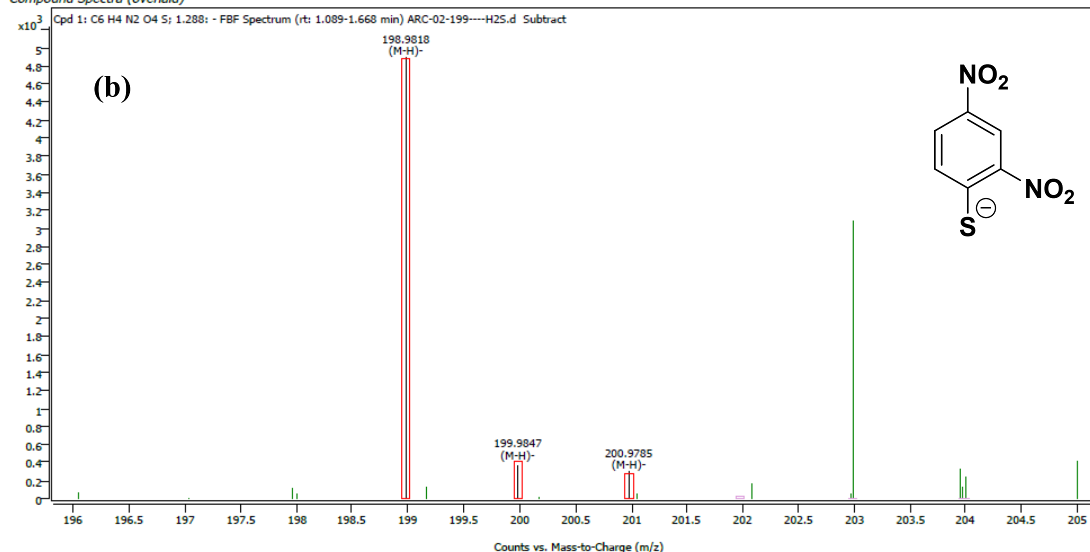
Compound ID Table

Cpd	Formula	Mass (Tgt)	Calc. Mass	Mass	Species	Diff(Tgt.ppm)	mDa
1	C19 H19 N O3	309.1365	309.1360	309.1349 310.1434 332.1256 348.0988	M+ (M+H)+ (M+Na)+ (M+K)+	-1.50	-0.46

Compound Details

Cpd. 1: C6 H4 N2 O4 S

Compound Spectra (overlaid)



Compound ID Table

Cpd	Formula	Mass (Tgt)	Calc. Mass	Mass	Species	Diff(Tgt.ppm)	mDa
1	C6 H4 N2 O4 S	199.9892	199.9891	198.9818	(M-H)-	-0.41	-0.08

Figure S22. HRMS spectra showing the formation of **2** (a) and **3** (b) during the reaction of the probe **1** (10 μM) with Na_2S (100 μM).

6. Effect of pH on the fluorescence response of the probe 1

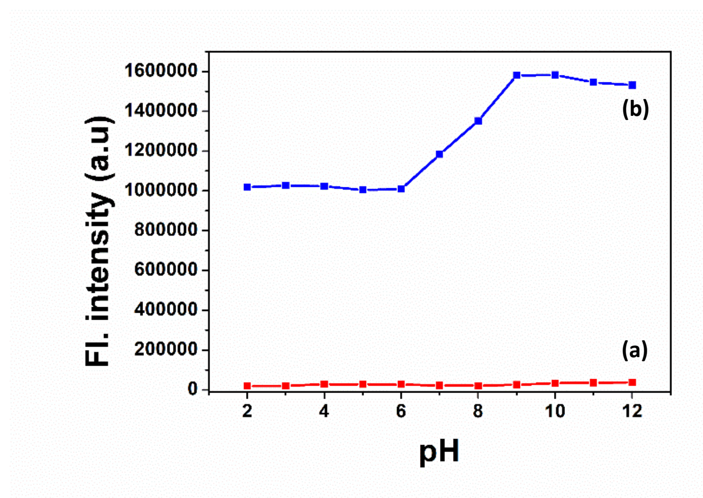


Figure S23. a) Effect of pH (2-11) on the emission intensity of the free probe **1** (100 µM) at 510 nm. b) Fluorescence intensity change as a function of pH (2-11) after mixing the probe **1** with H_2S (100 µM each).

7. Fluorescence response of probe 1 as a function of increasing H_2S concentration

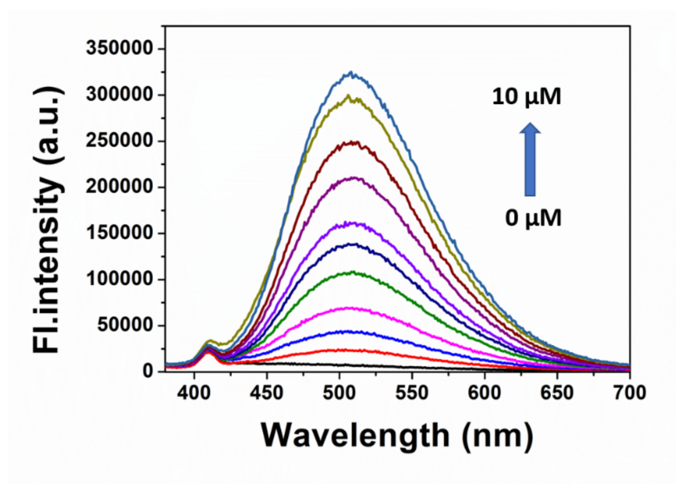


Figure S24. Fluorescence spectral responses of probe **1** (10 µM) to different concentrations of Na_2S (0-10 µM) in PBS buffer of pH= 7.4. λ_{ex} = 360 nm, λ_{em} = 510 nm; slit width: ex= 5 nm & em= 5 nm.

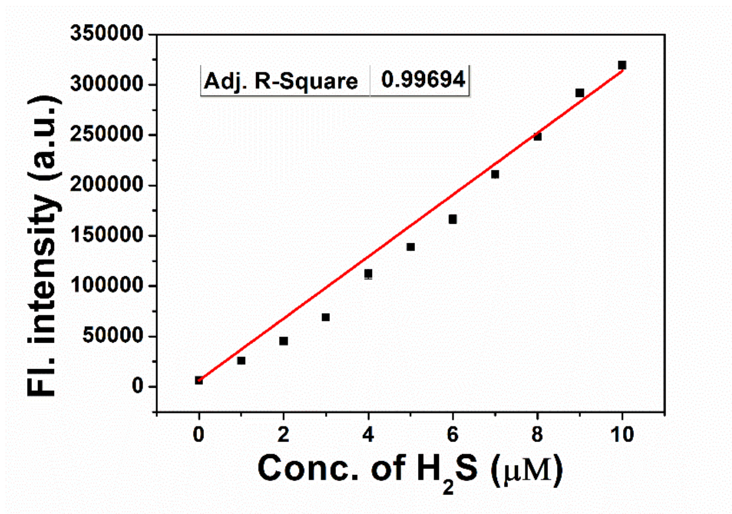


Figure S25. Linear correlation between the intensity of fluorescence at 510 nm with Na_2S concentration (0−10 μM) in PBS buffer (pH= 7.4, 10 mM); concentration of the probe **1** = 10 μM); λ_{ex} = 360 nm, λ_{em} = 510 nm; slit width: ex= 5 nm & em= 5 nm.

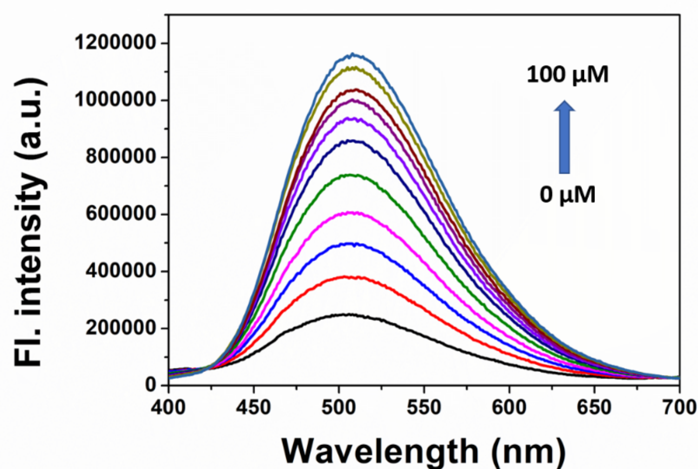


Figure S26. Fluorescence spectral responses of the probe **1** (100 μM) to different concentrations of externally added Na_2S (0-100 μM) in human blood plasma. λ_{ex} = 360 nm, λ_{em} = 510 nm; slit width: ex= 5 nm & em= 5 nm.

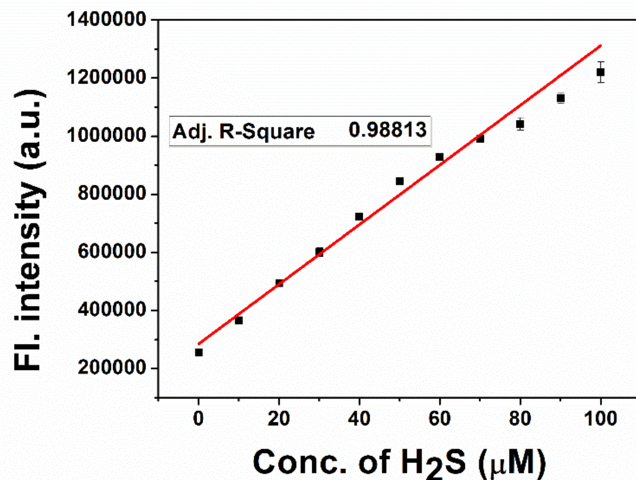


Figure S27. Linear correlation between the intensity of fluorescence at 510 nm and externally added Na_2S concentration (0–100 μM) in human blood plasma. λ_{ex} = 360 nm, λ_{em} = 510 nm; slit width: ex= 5 nm & em= 5 nm.

8. SERS response of the compounds 1, 2 and 3

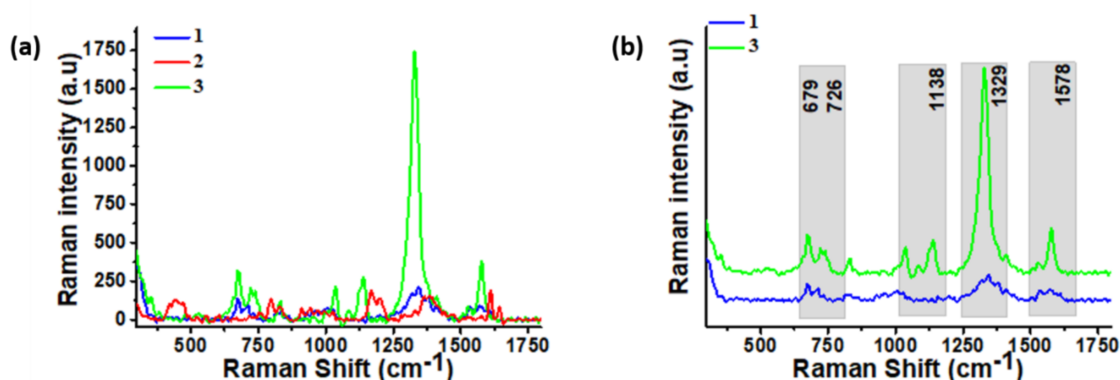


Figure S28. (a) SERS spectra of the compounds **1**, **2** and **3**, (b) distinct peaks appeared in SERS spectra for **3** when compared to **1**.

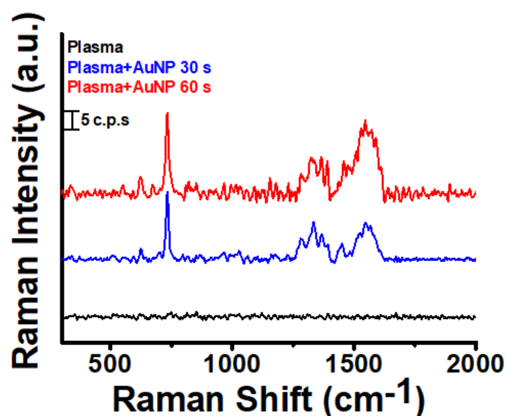


Figure S29. Bare Raman and SERS of plasma sample.

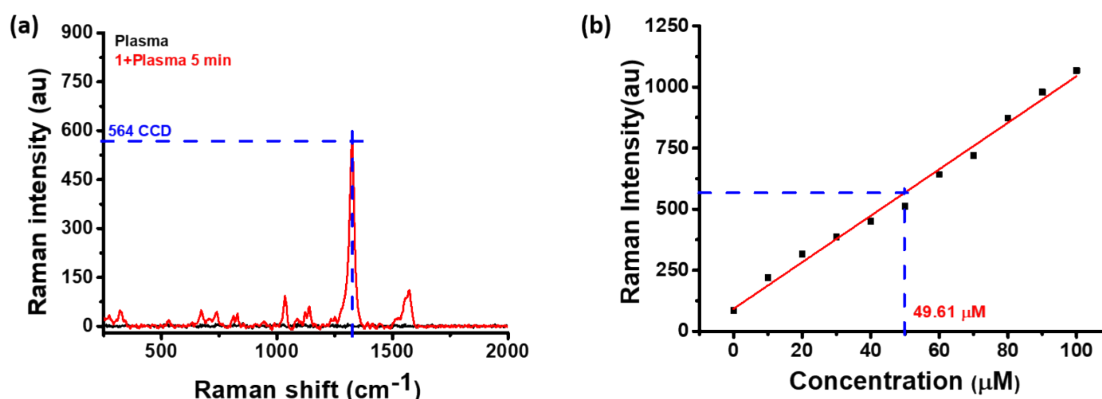


Figure S30. (a) SERS response after incubating the probe **1** (10 μM) with human blood plasma (10 μL) for 5 min; (b) H_2S concentration estimated based on the calibration curve.

9. MTT assay

MTT assay is based on the cleavage of an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) by mitochondrial dehydrogenases in viable cells. Quantities of 100 μL of the cell suspension of 1 x 10^4 cells/well were seeded in a 96-well plate and incubated at 37°C for 24 h in a CO_2 incubator. After the incubation cells were washed with 100 μL of PBS buffer (pH 7.4) twice. Then 100 μL of the compound under investigation at various concentrations along with positive control (1 μM doxorubicin) was similarly added to the appropriate wells. The plates were then incubated for 6 and 12 h in a CO_2 incubator at 37 °C. After incubation, cells were washed with 100 μL of PBS buffer (pH 7.4) twice followed by 100 μL MTT (0.5 mg/mL) was added to each well, and incubation was continued for an additional 2 to 4 h. The insoluble formazan crystals formed were solubilized by the addition of 100 μL DMSO followed by an incubation of 30 min and the absorbance was measured at 570 nm using a microplate spectrophotometer (BioTek, Power Wave XS).

The proliferation rate was calculated as: % Proliferation = (A_{sample} / $A_{control}$) x 100

The Inhibition rate was calculated as: % Inhibition = 100 - % Proliferation

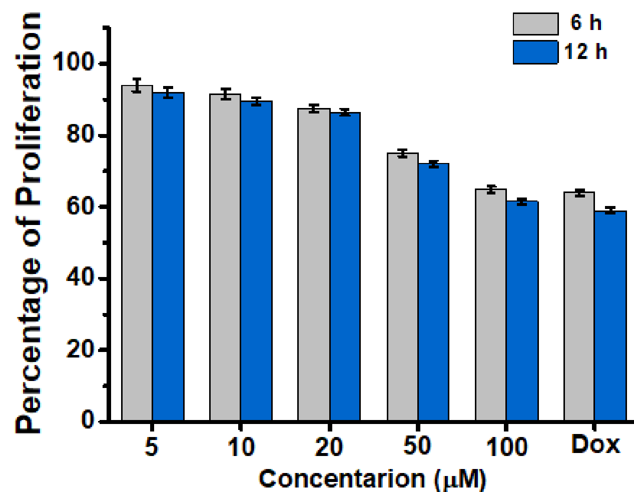


Figure S31. *In-vitro* cytotoxicity assay of Probe 1 at various concentrations (5,10,20,50 and 100 μM) in HepG2 cells at 6 and 12 h.

10. Cell line and cell culture information

The human hepatocarcinoma cancer cell line HepG2 was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were maintained in Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum and 5% CO_2 at 37 °C. Cells were cultured in glass-bottom, T-25 flasks, and 8-well chamber slides for various experiments 2 days prior to the conduction of experiments. All the experiments were performed in triplicate for accurate results.

11. Precision and accuracy of the measurements

Na_2S concentrations of 30 µM in buffer and 20 µM in plasma were analyzed in 3 independent runs on the same day (intra-day precision) and 3 successive days (inter-day precision) from three measurements of each sample. The precision of the analysis was determined by calculating the coefficient of variance or % relative standard deviation (% RSD). The % RSD values of intra-day and inter-day studies were 1.35 and 0.53 in buffer and 1.32 and 1.74 in plasma respectively. These values (Table S1) suggest that precision of the method is satisfactory.

Table S1. Intra-day precision and inter-day precision of the measurements in buffer and plasma.

In buffer						
Intra-day precision				**Inter-day precision**		
Concentration (µM)	Fluorescence measured (Mean±SD)	% RSD	±SE	Fluorescence measured (Mean±SD)	% RSD	± SE
30	614313± 8298	1.35	±4790.85	603415±3245	0.53	1873.50
In plasma						
Concentration (µM)	Fluorescence measured (Mean±SD)	% RSD	±SE	Fluorescence measured (Mean±SD)	% RSD	± SE
20	494300 ±6538	1.32	±3774.71	493946.7± 8606	1.74	4968.67

* Standard deviation (SD) = square root of $\Sigma (m-i)^2/n-1$ (m is mean and i is the measured value)

* Percentage relative standard deviation, %RSD = 100*(SD /m)

* Standard error (SE) = Standard deviation/√n

Accuracy is expressed in terms of percentage average recovery and percentage relative error. The average recoveries were found to be 96.8%, and 102.6% in the buffer for Na_2S concentrations 10, and 40 μM respectively. In plasma, it was 101.9%, and 102.4% for the concentration levels of 20, and 30 μM respectively (Table S2). Also, the percentage relative error was 3.16 and 2.62 for 10 and 40 μM Na_2S in buffer, and 1.92 and 2.42 for 20 and 30 μM of Na_2S in plasma respectively. The relatively low value of percentage relative error suggests that the method is having reasonable accuracy.

Table S2. Accuracy of the measurement in buffer and plasma.

Buffer			
Amount added [C] (μM)	Amount Found ($[C]^{\#}$ ±SD) (μM)	% Average recovery (r)	% Relative error (δ)
10	9.683623 ±1.331188	96.8	3.16
40	41.04933 ±0.770629	102.6	2.62
Plasma			
20	20.38566 ±0.636355	101.9	1.92
30	30.72694 ±1.465029	102.4	2.42

% Average recovery (r) = $100*[C]^{\#} / [C]$

% Relative error (δ)=$100*([C]^{\#}- [C])/ [C]$

12. References

1. Chaudhuri, A.; Venkatesh, Y.; Das, J.; Gangopadhyay, M.; Maiti, T.K.; Singh, N.D.P. One- And Two-Photon-Activated Cysteine Persulfide Donors for Biological Targeting. *J. Org. Chem.* **2019**, *84*, 11441–11449.