Supporting Information

Performance comparison of two cascade reaction models in fluorescence *off-on* detection of hydrogen sulfide

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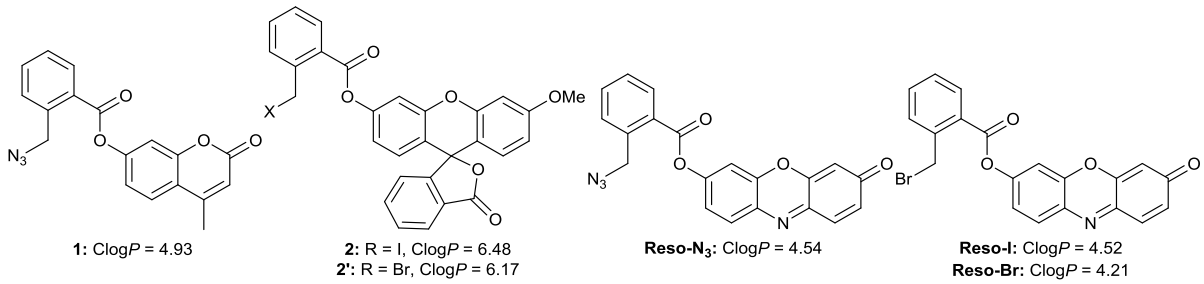
I. Estimation of Clog*P* Values



1: Clog*P* = 4.93 **2:** R = I, Clog*P* = 6.48 **2':** R = Br, Clog*P* = 6.17 **Reso-N_3:** Clog*P* = 4.54 **Reso-I:** Clog*P* = 4.52 **Reso-Br:** Clog*P* = 4.21

Fig. S1 Structures of probes and their Clog*P* values.

II. Photophysical Studies:

Procedures:

Preparation of the medium: All experiments were carried out either in Deionized water or in Phosphate buffer (10 mM, pH = 7.4) with 1% DMSO (maximum). Phosphate buffer (PB) was prepared by dissolving measured amount of solid Na_2HPO_4 and NaH_2PO_4 in deionized water.

Preparation of the solution of Reso-N_3, Reso-Br and Resorufin 7: A stock solution of **Reso-Br** and **Reso- N_3** (500 μM) were prepared in THF and Resorufin **7** (1000 μM) was prepared in DMSO. The final concentration of each of **Reso-Br, Reso-N_3** and Resorufin 7 during each assay was 10 μM with 2% THF or DMSO (maximum).

Preparation of the solution of analytes: Stock solutions of NaCl, NaBr, NaI, NaF, Na_2SO_3, Na_2SO_4, $Na_2S_2O_3$, NaSCN, Alanine (Ala), Serine (Ser), Cysteine (Cys), Glutathione (GSH), NaOH, $NaNO_2$, $NaNO_3$, were prepared in Deionized water (concentrations 20 mM). Calculated volumes of analytes were added from respective stock solutions to each fluorescence cuvette. All spectral data were recorded at 10 min after the addition of analyte(s) by exciting at 540 nm. The excitation and emission slit width were 2 nm and 3 nm, respectively.

Absorption spectra, emission spectra and determination of molar extinction coefficient of Reso-N_3, Reso-Br and Resorufin 7:

Absorption spectra of **Reso-N_3** (10 μM), **Reso-Br** (10 μM) and Resorufin **7** (10 μM) were recorded in the Water (Fig. S2). From absorption spectra, molar extinction coefficient of **Reso-Br**, **Reso-N_3** and resorufin **7** were determined using Beer-Lambert law.

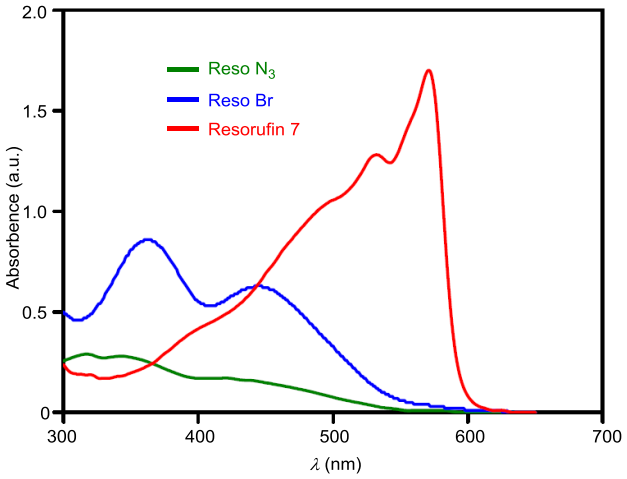


Fig. S2 UV-vis absorption spectra of **Reso-N_3** (10 μM), **Reso-Br** (10 μM) and Resorufin **7** (10 μM) recorded in water.

When fluorescence intensities of **Reso-N_3, Reso-Br** and Resorufin **7** were compared at λ_{ex} = 540 nm, Resorufin **7** was found stronger fluorescent than that of **Reso-Br** and **Reso-N_3** at identical condition (Fig. S3).

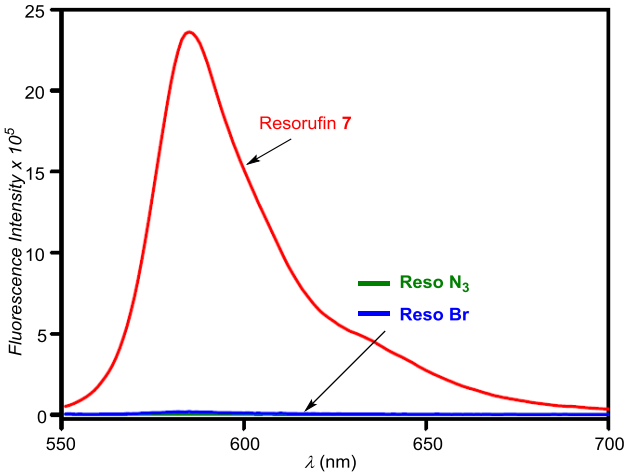


Fig. S3 Comparison of fluorescence intensities of **Reso-N_3, Reso-Br** and Resorufin **7** (10 μM each) recorded in water (λ_{ex} = 540 nm).

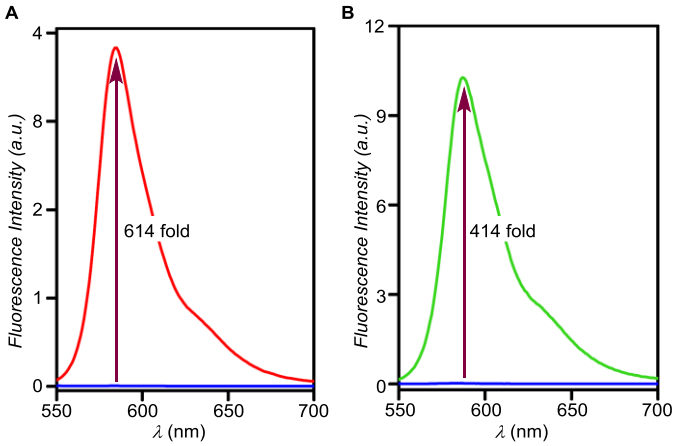


Fig. S4 Comparison of fluorescence intensities of **Reso-N_3** (**A**) and **Reso-Br** (**B**), before and after addition of Na_2S (5 mM) recorded in water (λ_{ex} = 540 nm).

III. H_2S Sensing

Reaction kinetics of probes upon H_2S sensing:

Fluorescence spectra of **Reso-N_3** and **Reso-Br** (10 μM) were recorded at definite time interval after addition of Na_2S (5 mM) in water. The fluorescence intensity at 585 nm of each spectrum were recorded (λ_{ex} = 540 nm) and plotted against time. Rate constant, k and reaction time for 50% completion ($t_{1/2}$) for each probe were determined according to the following equation.

$$Y = a \times [1 - e^{-(kt)}] \qquad \text{Eq. S1}$$

Where, Y = Fluorescence intensity, a = arbitrary constant, k = pseudo first order rate constant, t = time.

Half-life of the reaction ($t_{1/2}$) was calculated using Equation (S2)

$$t_{1/2} = 0.693/k \qquad \text{Eq. S2}$$

Where k = pseudo first order rate constant

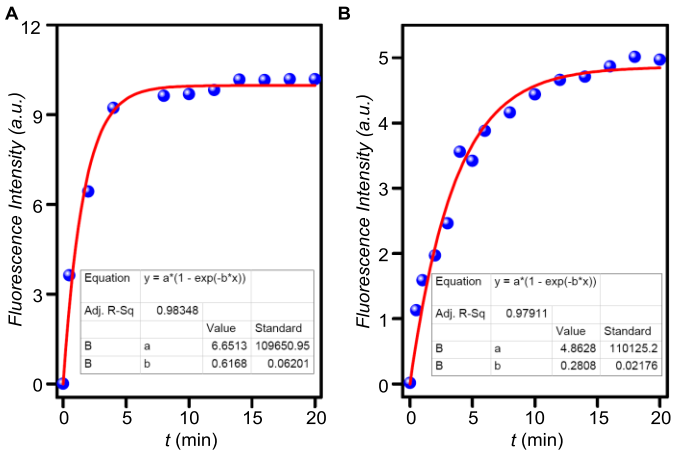


Fig. S5 Kinetics plot of **Reso-N_3** (**A**) and **Reso-Br** (**B**), 10 μM with Na_2S (5 mM) in water. Fluorescence intensity recorded at 585 nm (λ_{ex} = 540 nm) from each spectra.

Similarly the rate of the sensing process of **Reso-N_3** in phosphate buffer (pH = 7.4) was determined (Fig. 8 and S6).

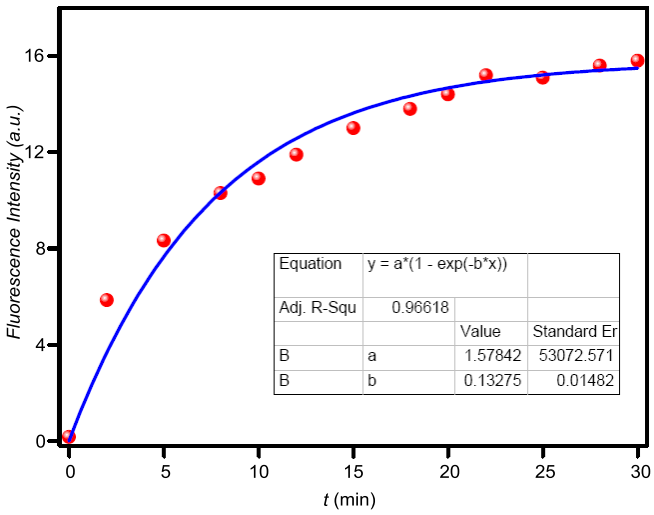


Fig. S6 Kinetics plot of **Reso-N_3** (10 μM) with Na_2S (5 mM) in phosphate buffer (pH = 7.4). Fluorescence intensity recorded at 585 nm (λ_{ex} = 540 nm) from each spectra.

Determination of detection limits of probes towards H_2S sensing in water:

The detection limits were determined based on the fluorescence titrations of **Reso-N_3** and **Reso-Br** (10 μM). To determine the *S/N* ratio, the emission intensity of **Reso-N_3** and

Reso-Br were measured without H_2S by 6-times and the standard deviations of blank measurements were calculated. Under these conditions, good linear relationships between the fluorescence intensities and the H_2S concentrations (Fig. S7) were obtained for **Reso-N_3** (R = 0.992) and **Reso-Br** (R = 0.993). Detection limits were then calculated with the equation: detection limit = $3\sigma/m$, where σ is the standard deviation of 6 blank measurements, m is the slope between intensity versus Na_2S concentration. The detection limits of **Reso-N_3** and **Reso-Br** towards H_2S were calculated to be 0.44×10^{-6} and 0.58×10^{-6} M respectively at S/N = 3 (signal-to-noise ratio of 3:1).

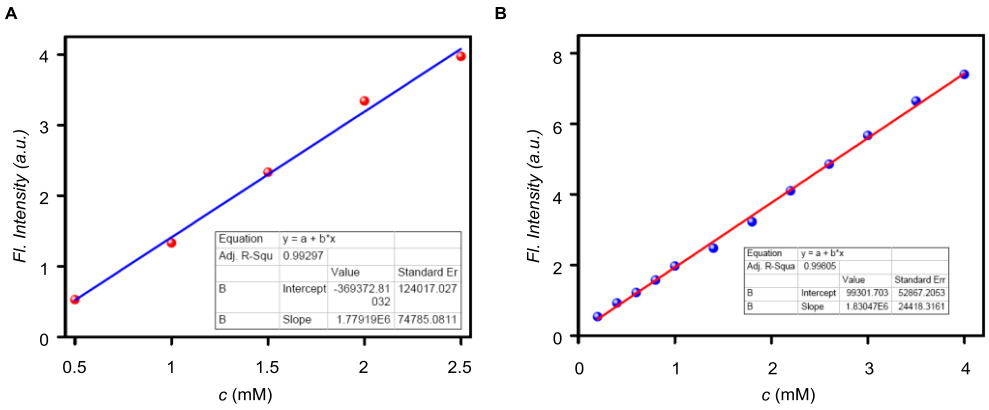


Fig. S7 Linear relationship between fluorescence intensity at 585 nm *versus* concentration of Na_2S added in Water for **Reso-N_3** (**A**) and **Reso-Br** (**B**).

Table S1. Calculation of detection limit for **Reso-N_3** in water.

	Reso N_3	
Reading 1	8287.36415	
Reading 2	8755.60198	
Reading 3	8826.09059	
Reading 4	8221.74833	
Reading 5	8518.18064	
Reading 6	8273.53117	
Standard deviation (σ)	262.1540489	
Slope (m)	17791900	
Detection Limit (3σ/m)	**0.000442081**	mM
	0.44	μM

Table S2. Calculation of detection limit for **Reso-Br** in water.

	Reso Br	
Reading 1	17692.6116	
Reading 2	17004.79289	
Reading 3	17500.99528	
Reading 4	17919.98993	
Reading 5	17867.55327	
Reading 6	18359.19323	
Standard deviation (σ)	354.9714082	
Slope (m)	1830470	
Detection Limit (3σ/m)	**0.000581771**	mM
	0.58	μM

Fluorescence spectra recorded for the selectivity studies of Probes:

Selectivity and inertness of **Reso-N_3** and **Reso-Br** towards other analytes were also confirmed by the UV-visible spectroscopy. Fluorescence spectra were recorded (λ_{ex} = 540 nm) after 10 minutes of mixing a probe (10 μM) with various analytes (5 mM). The emission profile was observed for each case was unchanged and identical with the emission spectra of probes (Fig. S8). Addition of Na_2S (200 μM) in the same solutions resulted increment in fluorescence intensities identical to the emission spectra of **7**. This data also confirms the selectivity of **Reso-N_3** and **Reso-Br** towards H_2S in the presence of other analytes.

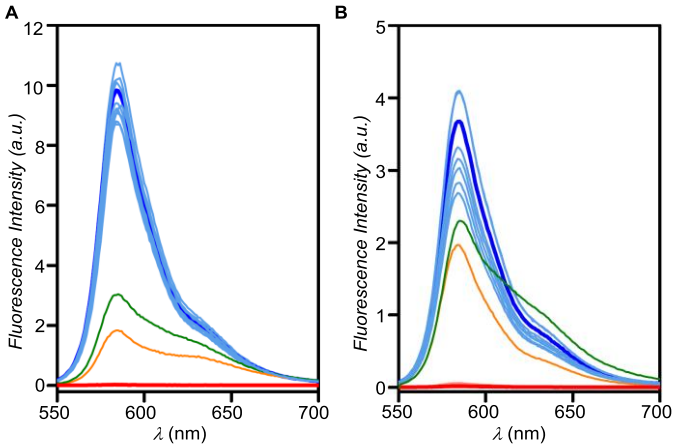


Fig. S8 Emission spectra of **Reso-N_3** and **Reso-Br** (10 μM) in presence of various analytes (5 mM, red colored) and in the same solution Na_2S (5 mM, blue colored) in water. Less

increments of intensity were observed in the case of Cys and GSH after addition of H_2S, represented by green and orange line in each spectrum.

The bar diagrams for selectivity studies (Fig. 7 and 10A) were normalized according to Eq. S3.

Relative Fluorescence Int. = $(I_M - I_0)/I_0$ Eq. S3

Where, I_M = Fluorescence intensity of probes at 585 nm after addition of different analyte.
I_0 = Fluorescence intensity of probes at 585 nm without the addition of analyte.

Stability of probes in buffered medium:

To evaluate the stability of probes in buffered system fluorescence spectra was recorded for both the probe (10 uM) in phosphate buffer (5 mM, pH = 7.4) with time. The background fluorescence of **Reso-N_3** is negligible and did not increase with time. But for **Reso-Br** the fluorescence intensity of the probe was considerable and found to be increased with increasing time.

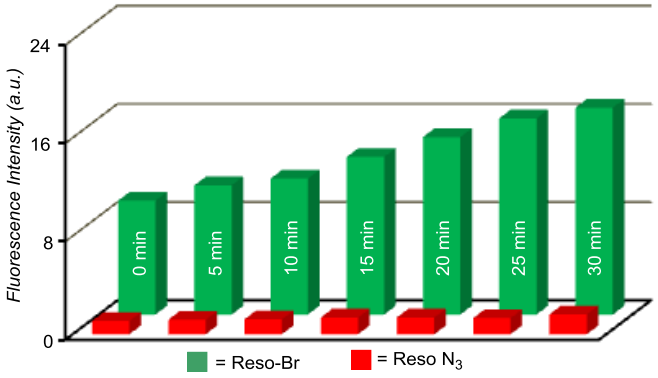


Fig. S9 Emission spectra of **Reso-N_3** and **Reso-Br** (10 μM) in phosphate buffer (5 mM, pH = 7.4), recorded at definite time intervals. Bar diagram plotted by taking the intensity at 585 nm in each case (λ_{ex} = 540 nm).

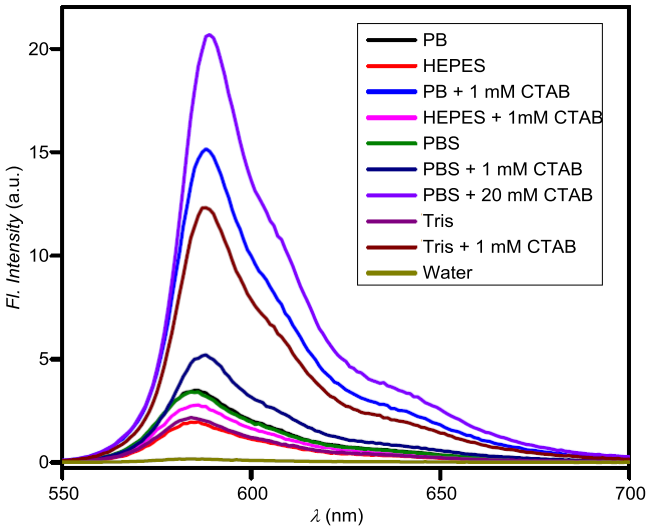
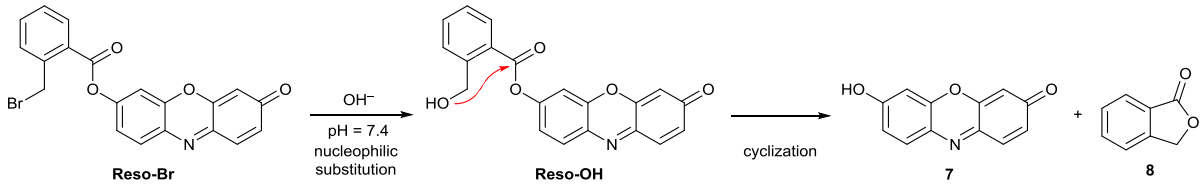


Fig. S10 Emission spectra of **Reso-Br** (10 μM) in different system in presence and in absence of CTAB (λ_{ex} = 540 nm). Each data was recorded after 10 minutes of addition.



Scheme S1. Mechanism of decomposition of **Reso-Br** in aqueous buffer conditions.

Determination of detection limit of Reso-N_3 towards H_2S sensing in Phosphate Buffer:

The detection limit of **Reso-N_3** towards H_2S sensing in phosphate buffer (5 mM, pH = 7.4) was determined based on fluorescence titrations as stated above. A linear relationships between the fluorescence intensities and the H_2S concentrations (Fig. S11) was obtained for **Reso-N_3** (R = 0.98095). Detection limit was then calculated with the equation: detection limit = $3\sigma/m$, where σ is the standard deviation of 6 blank measurements, m is the slope between intensity versus Na_2S concentration. The detection limits of **Reso-N_3** towards H_2S in phosphate buffer was calculated to be 4.15×10^{-6} M at S/N = 3 (signal-to-noise ratio of 3:1).

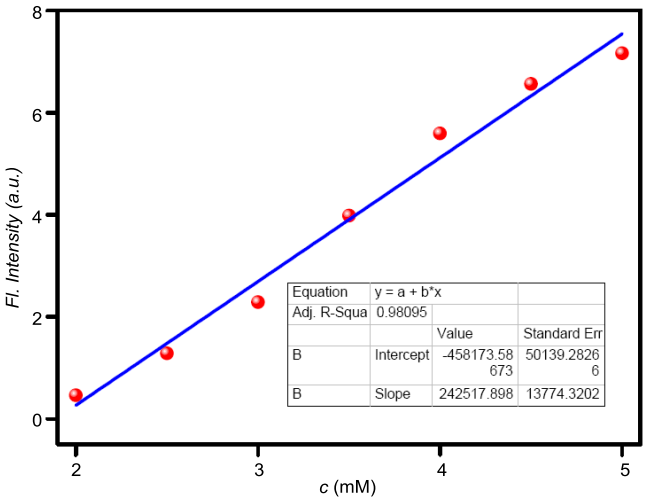


Fig. S11 Linear relationship between fluorescence intensity of **Reso-N_3** at 585 nm *versus* concentration of Na_2S added in phosphate buffer (5 mM, pH = 7.4).

Table S3. Calculation of detection limit for **Reso-N_3** in Phosphate Buffer (5 mM, pH = 7.4).

Reading 1	14422.29411	
Reading 2	15244.04831	
Reading 3	14622.43376	
Reading 4	14933.63767	
Reading 5	14354.08278	
Reading 6	14594.16937	
Standard deviation (σ)	335.9844159	
Slope (m)	242517	
Detection Limit (3σ/m)	0.004156217	mM
	4.156216875	**uM**

Interaction of Resorufin 7 with Glutathione:

Glutathione was added to the solution of Resorufin **7** in phosphate buffer (5 mM, pH = 7.4) and fluorescence spectra were recorded before and after addition of glutathione.

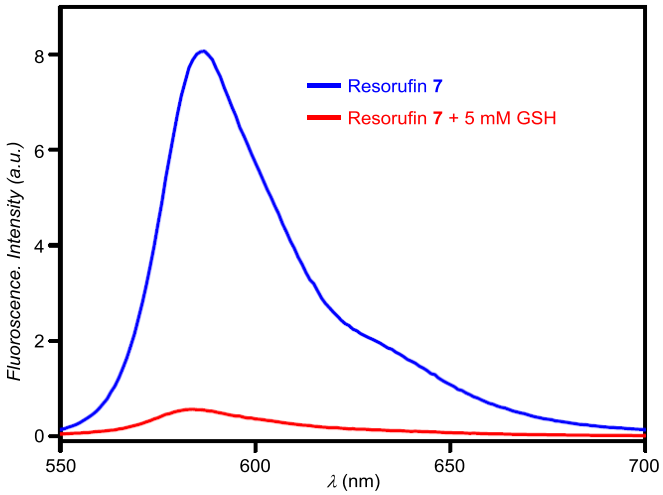


Fig. S12 Emission spectra of Resorufin **7** (10 μM) before and after addition of GSH in phosphate buffer (5 mM, pH = 7.4). Fluorescence spectra were acquired after 10 min of the addition of GSH.

Quantitative detection of H_2S in water sample:

Water sample was collected from the tap and definite amount of Na_2S was added to it. 10 μM of each probe were added to those water samples and fluorescence spectra were acquired (after 10 min). Intensity at 585 nm (λ_{ex} = 540 nm) were recorded from each spectrum and plotted to a bar diagram with increasing concentration of Na_2S added (Fig. 6).

IV. NMR Titration:

The H_2S mediated reduction of azide to amine followed by cyclization mechanism was studied by ^{1}H NMR titration (Fig. S13). **Reso-N_3** was titrated with increasing concentrations of Na_2S (0, 0.4, 1 and 2.0 equivalents) in deuterodimethylsulfoxide (DMSO-d_6) at room temperature and ^{1}H NMR spectra were recorded after 5 min of each addition. Signals corresponding to aromatic protons were monitored during the course of titration. With increasing equivalents of Na_2S, doublet at δ = 7.45 ppm (H_a protons) for **Reso-N_3** were disappeared with simultaneous increment of new doublet peak at δ = 7.3 ppm ($H_{a'}$ protons), corresponding to Resorufin **7** (Fig S13). Similarly, doublet of doublet peak at δ = 6.8 ppm (H_b) and doublet peak at δ = 6.3 ppm (H_c) for **Reso-N_3** were decreased and new peak at δ = 6.4 ppm ($H_{b'}$) and δ = 5.9 ppm ($H_{c'}$) corresponding to Resorufin **7** were appeared.

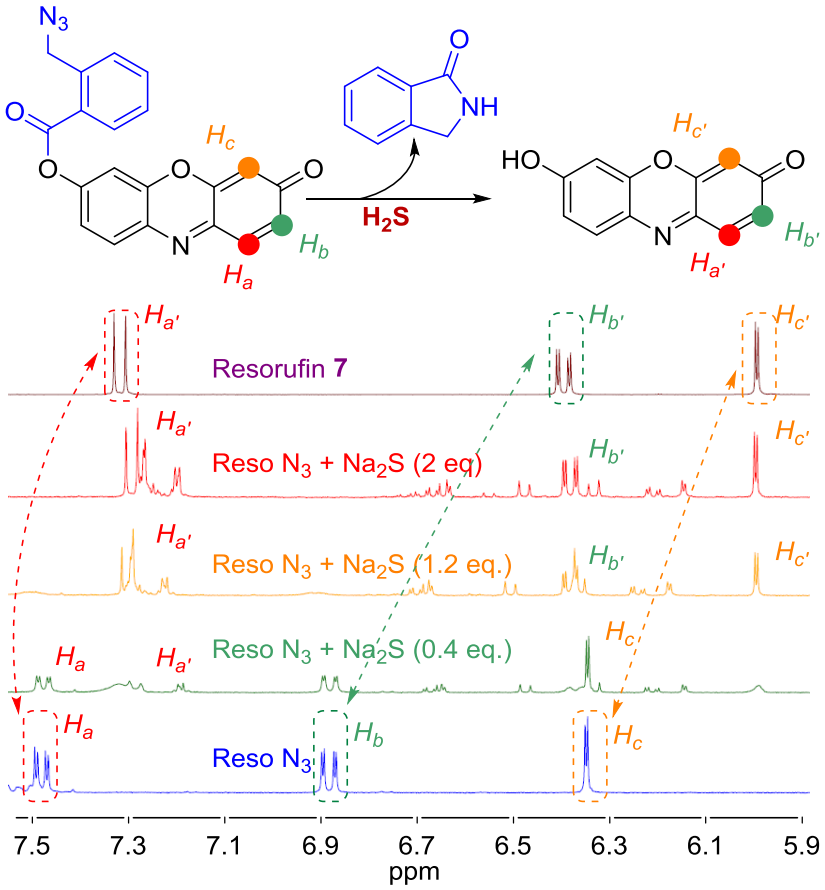


Fig. S13 ^{1}H NMR spectral change of **4** upon addition of Na_2S. (**i**) **4** only, (**ii**) **4** and 0.4 equiv. Na_2S, (**iii**) **4** and 0.8 equiv. Na_2S and (**iv**) **4** and 2.0 equiv. Na_2S. in CD_3CN at 25 ^{o}C.

The nucleophilic substitution-cyclization mechanism was studied by ^{1}H NMR titration (Fig. S14). **Reso-Br** was titrated with increasing concentrations of Na_2S (0, 0.4, 1 and 2.0 equivalents) in deuterodimethylsulfoxide (DMSO-d_6) at room temperature and ^{1}H NMR spectra were recorded after 5 min of each addition. Signals corresponding to aromatic protons were monitored during the course of titration. With increasing equivalents of Na_2S, doublet at δ = 7.45 ppm (H_a protons) for **Reso-Br** were disappeared with simultaneous increment of new doublet peak at δ = 7.3 ppm ($H_{a'}$ protons), corresponding to Resorufin **7** (Fig S14). Similarly, doublet of doublet peak at δ = 6.8 ppm (H_b) and doublet peak at δ = 6.3 ppm (H_c) for **Reso-Br** were decreased and new peak at δ = 6.4 ppm ($H_{b'}$) and δ = 5.9 ppm ($H_{c'}$) corresponding to Resorufin **7** were appeared.

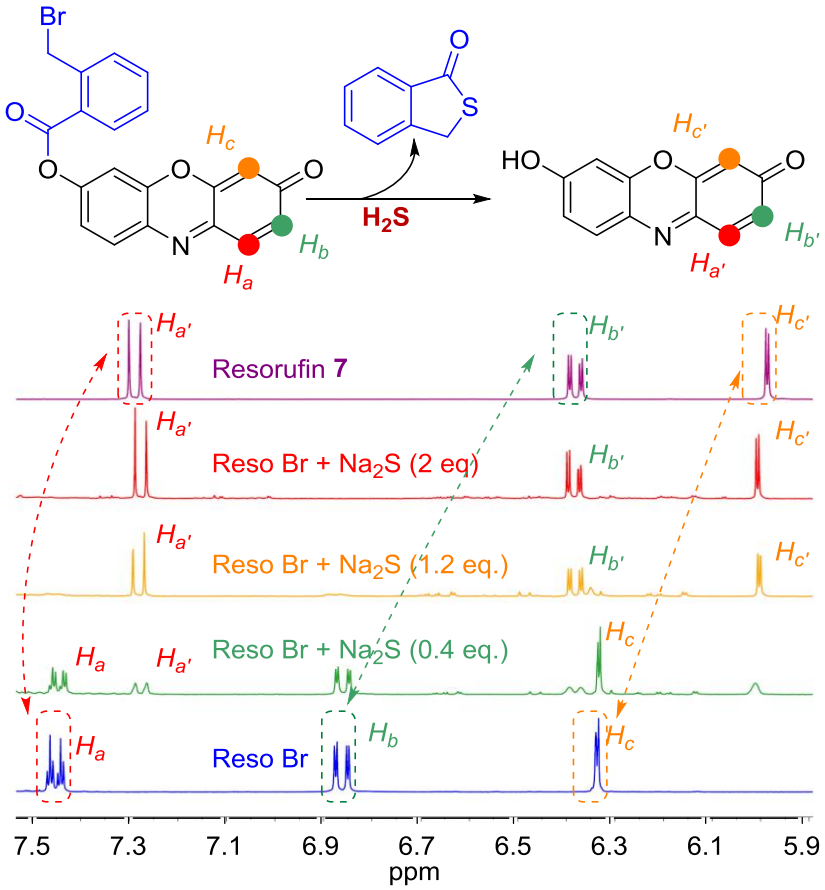


Fig. S14 ^{1}H NMR spectral change of 4 upon addition of Na_2S. (i) 4 only, (ii) 4 and 0.4 equiv. Na_2S, (iii) 4 and 0.8 equiv. Na_2S and (iv) 4 and 2.0 equiv. Na_2S. in CD_3CN at 25 ^oC.

V. Cell Imaging:

HeLa cells were purchased from National Centre for Cell Science, Pune (India). HeLa cells were grown in DMEM supplemented with 10% heat inactivated fetal bovine serum (FBS), 100 IU/ml penicillin, 100 mg/ml streptomycin and 2 mM L-glutamine. Cultures were maintained in a humidified atmosphere with 5% CO_2 at 37 ^oC. The cultured cells were subcultured twice in each week, seeding at a density of about 2 × 10^4 cells/mL. Typan blue dye exclusion method was used to determine Cell viability. The fluorescence images were taken using an Olympus Inverted IX81 equipped with a Hamamatsu Orca R2 microscope by exciting at λ_{ex} = 535-555 nm (by using RFP filter).

The HeLa cells were incubated with solution of the **Reso-N_3** (10 μM in 1:100 DMSO-PBS v/v, pH = 7.4) at 37 ^oC for 20 min. After rinsing with PBS the fluorescence images were taken. In this case less significant fluorescence was observed. Another set of HeLa cells were pre-incubated with **Reso-N_3** (10 μM in 1:100 DMSO-PBS v/v, pH = 7.4) at 37 ^oC for 20 min followed by washing with PBS and incubation with Na_2S (100 μM in 1:100 DMSO-PBS v/v,

pH = 7.4) at 37 ^{o}C for 20 min. After washing with PBS the fluorescence images showed strong red fluorescence (Fig. 11).

For measuring the intensity of cell fluorescence quantitatively, HeLa cells were first incubated with **Reso-N_3** (5 μM, at 37 ^{o}C for 30 min) the plate was thoroughly washed with PBS and placed under the microscope fitted with an incubator. The images were taken in 5 min time intervals after addition of Na_2S (100 μM). Five different region of interest (ROI) were selected and the intensity was obtained by using *image j* software. The average intensity of ROIs for each image was plotted in a bar diagram (Fig. 12F). The data presented in Fig. 12, Fig. S13 and Fig. S14 corresponds to 3 different set of experiment.

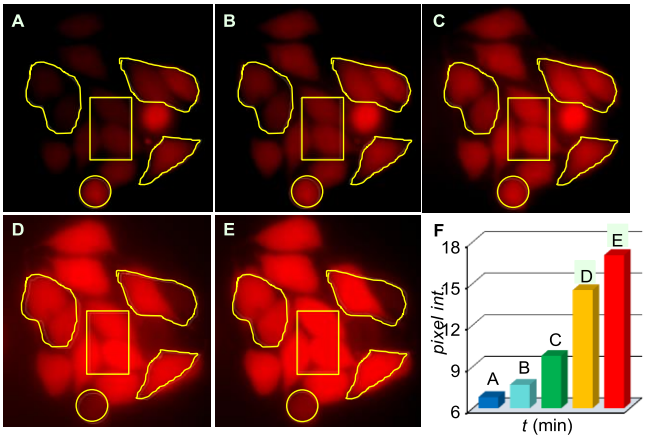


Fig. S15 Fluorescence images of HeLa cell acquired at different time intervals (0, 5, 10, 15 and 20 min) after incubating with Na_2S (**A**–**E**). Bar diagram was plotted by the average pixel intensity of selected ROI's *versus* time (**F**).

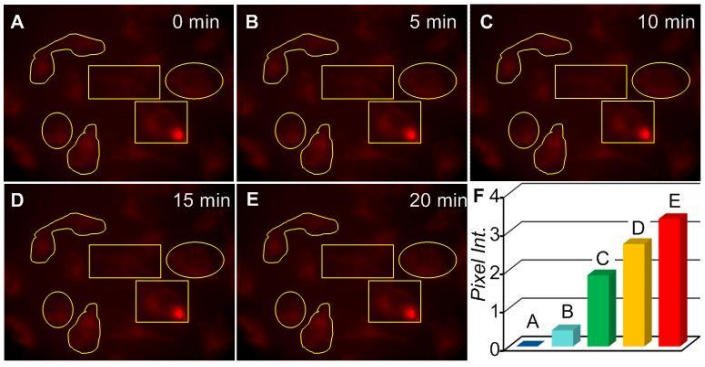


Fig. S16 Fluorescence images of HeLa cell acquired at different time intervals (0, 5, 10, 15 and 20 min) after incubating with Na_2S (**A**–**E**). Bar diagram was plotted by the average pixel intensity of selected ROI's *versus* time (**F**).

VI. NMR Spectra.

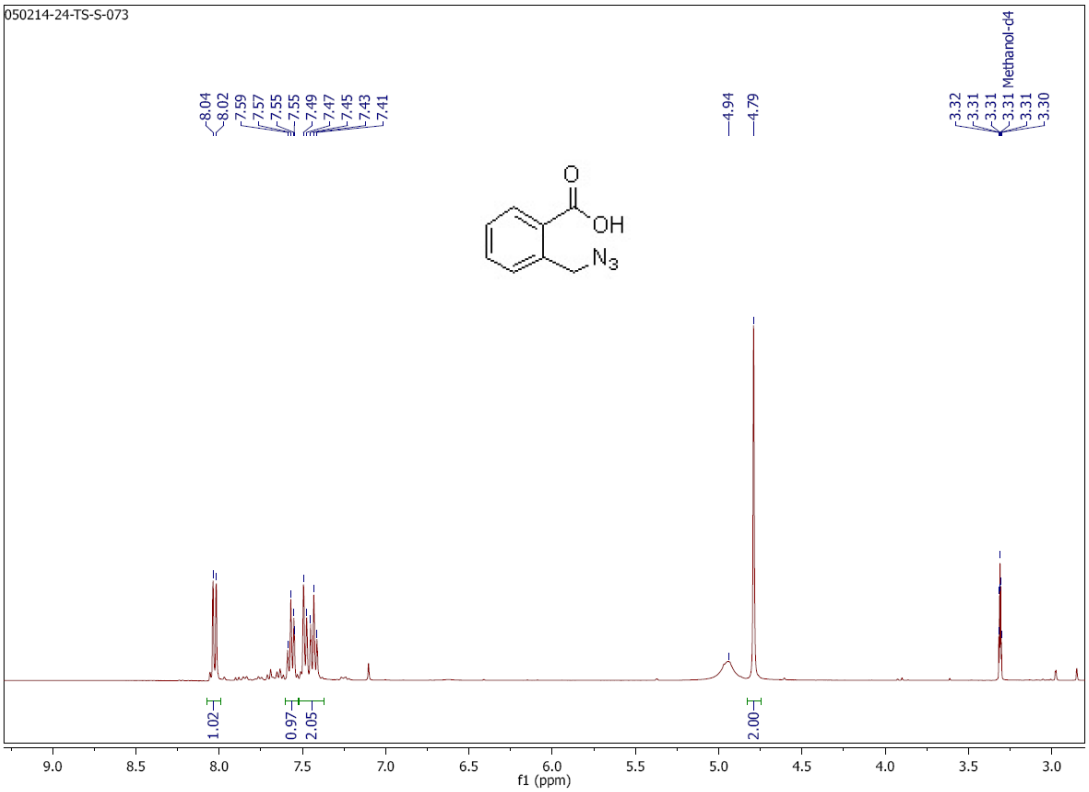


Fig. S17 ^{1}H NMR of **6** in Methanol-d_4.

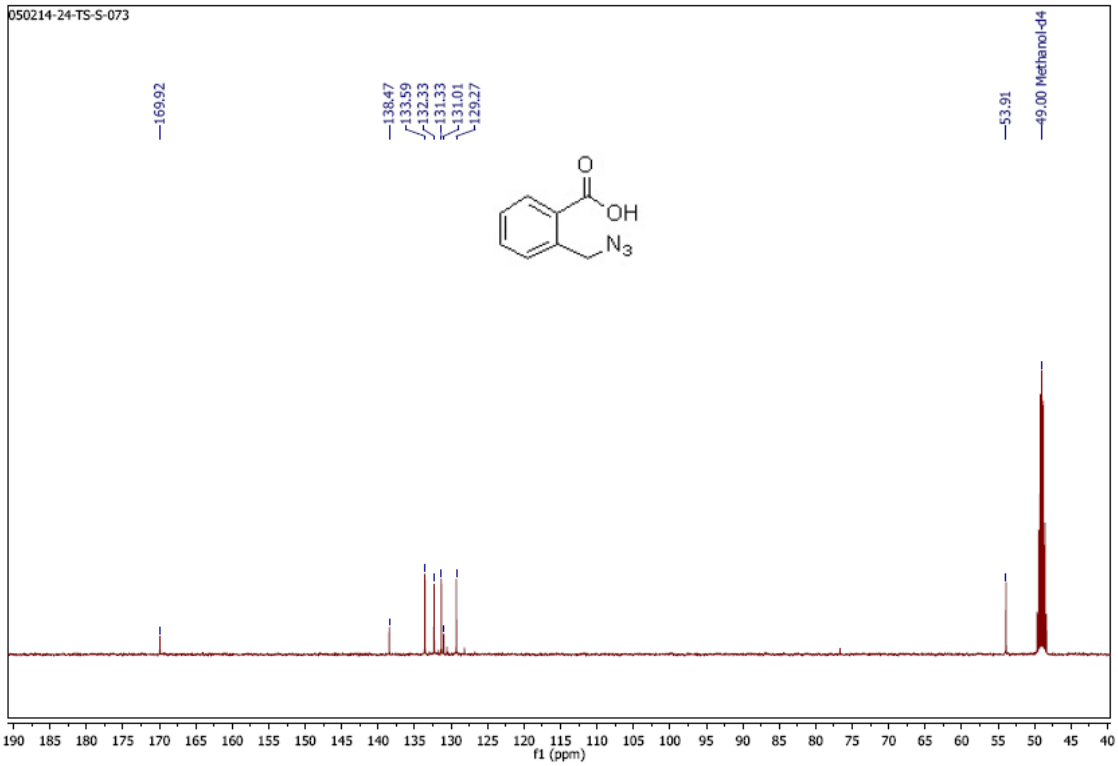


Fig. S18 ^{13}C NMR of **6** in Methanol-d_4.

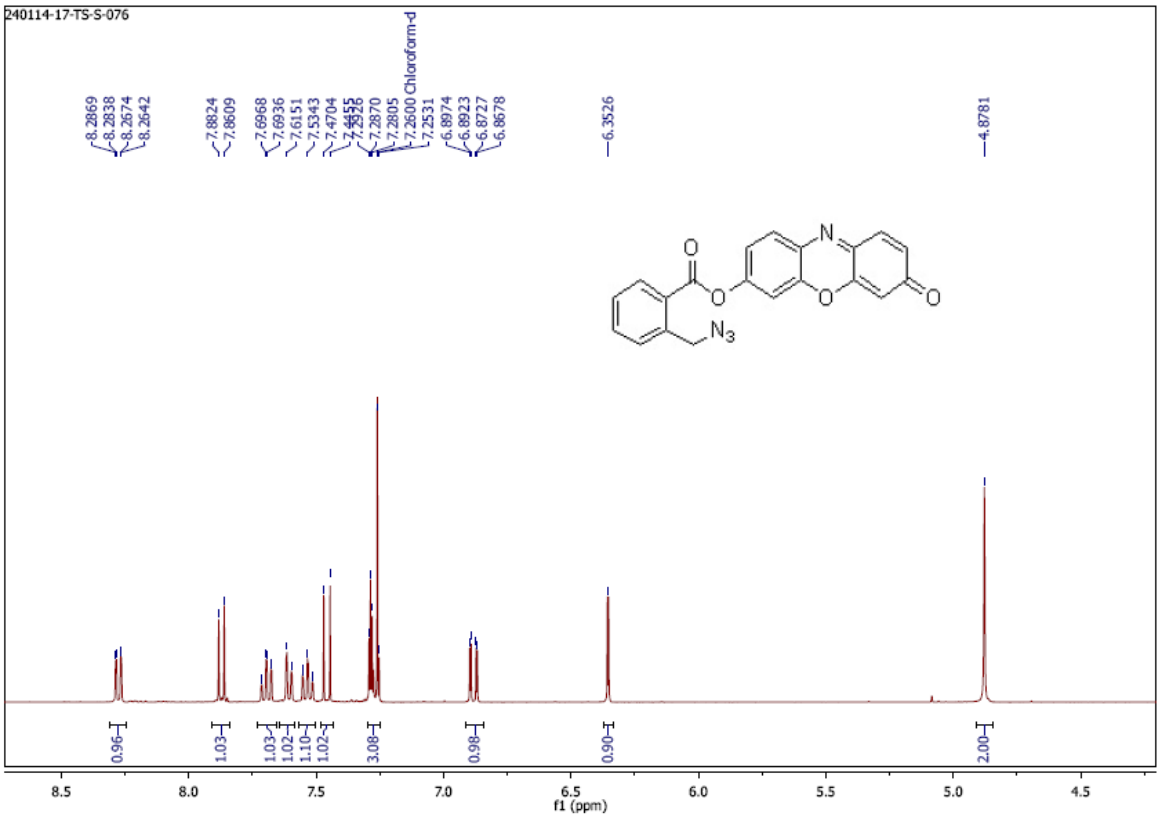


Fig. S19 ^{1}H NMR of **Reso-N_3** in $CDCl_3$.

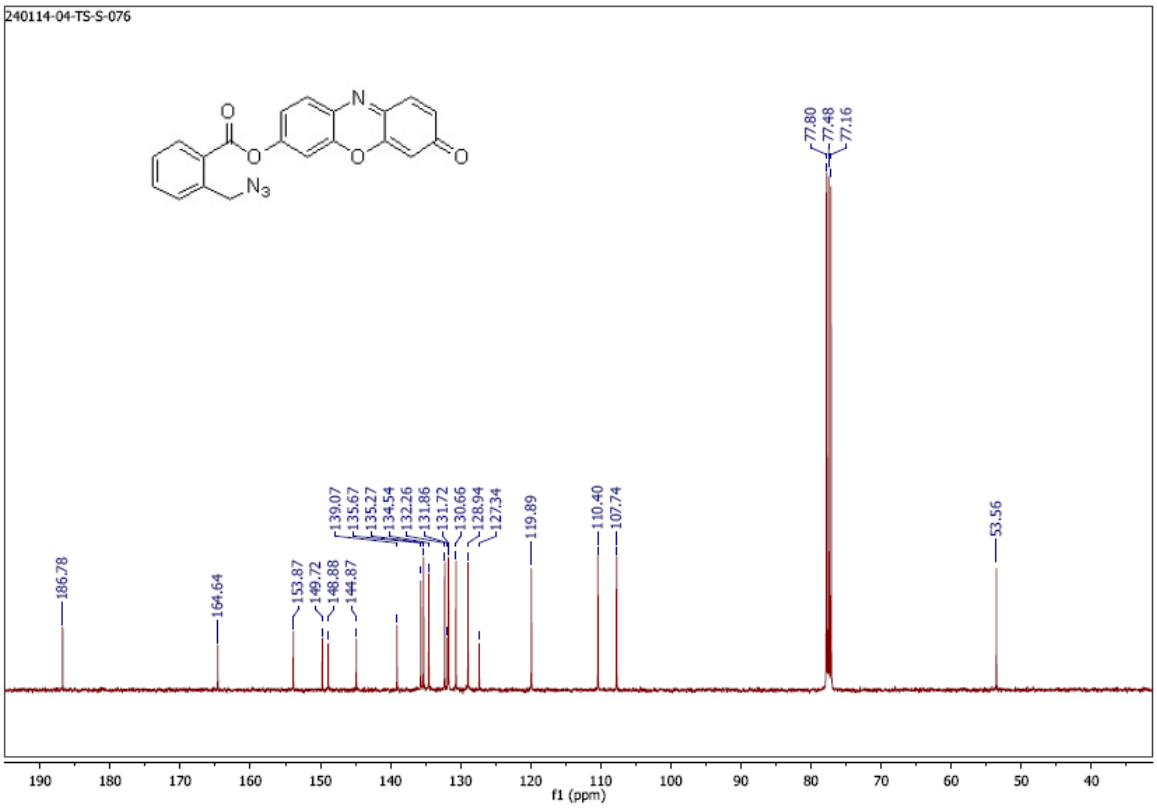


Fig. S20 ^{13}C NMR of **Reso-N_3** in $CDCl_3$.

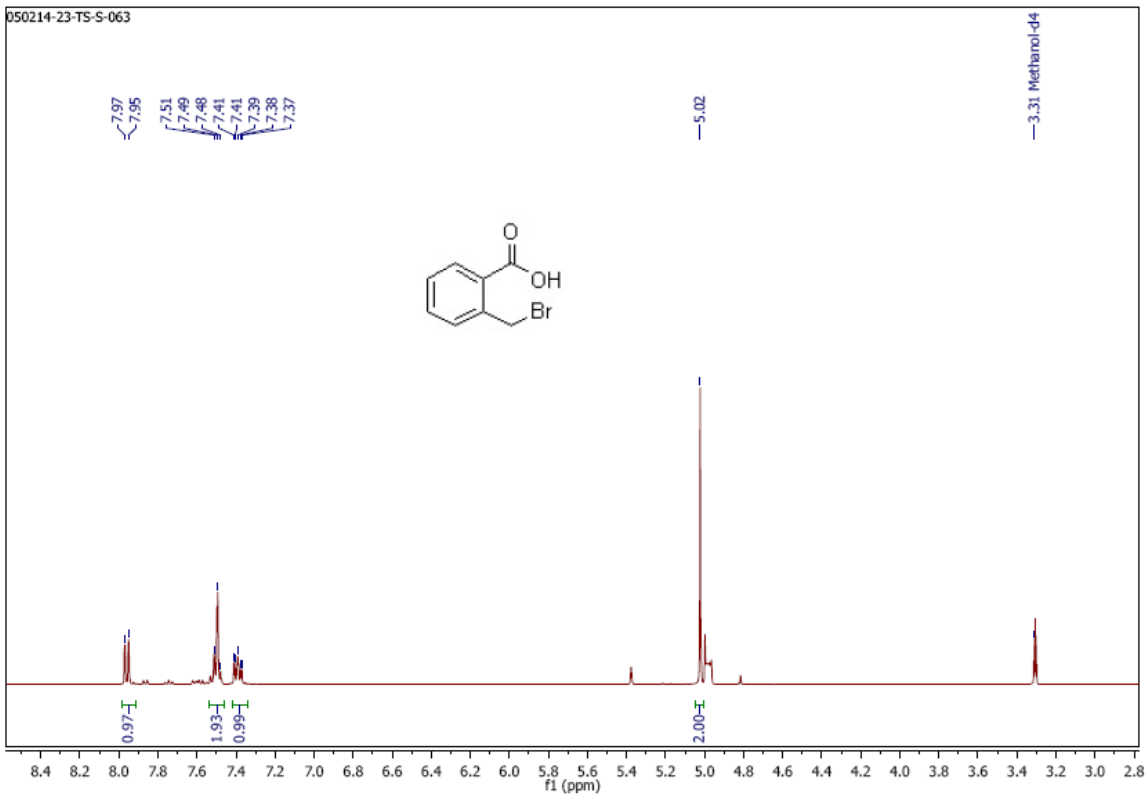


Fig. S21 ^{1}H NMR of **9** in Methanol-d_4.

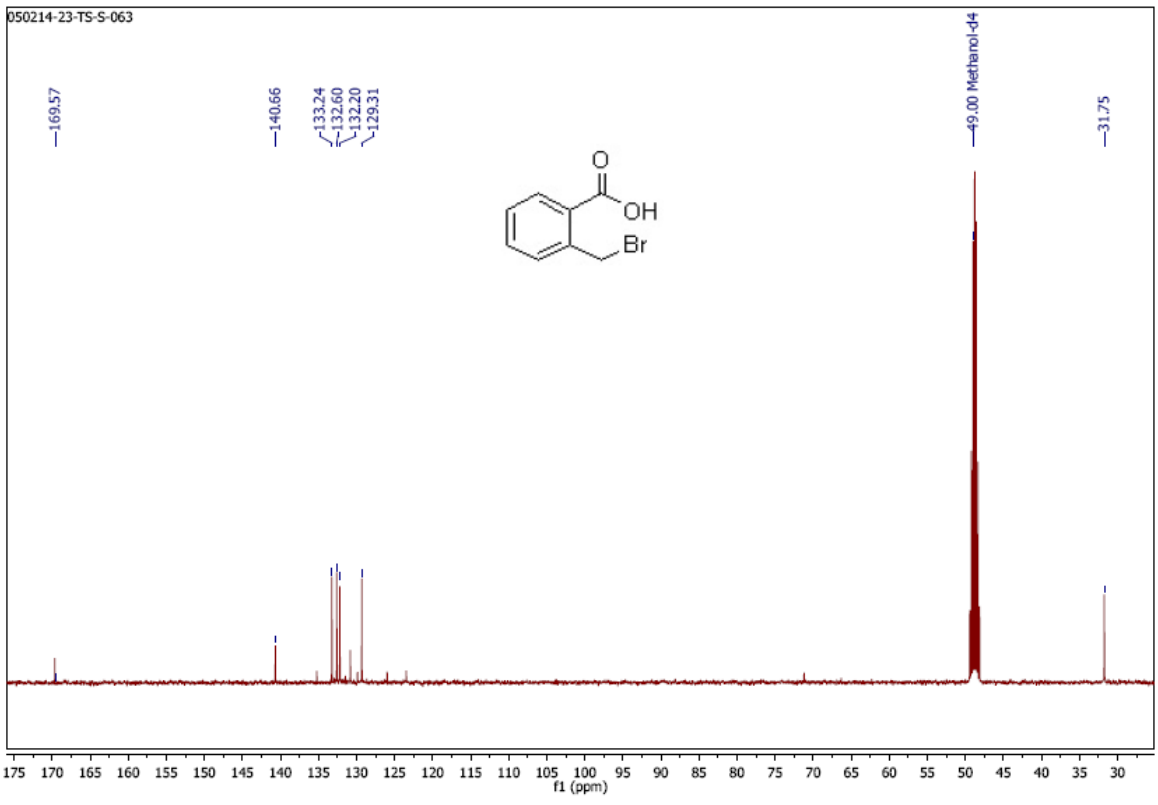


Fig. S22 ^{13}C NMR of **9** in Methanol-d_4.

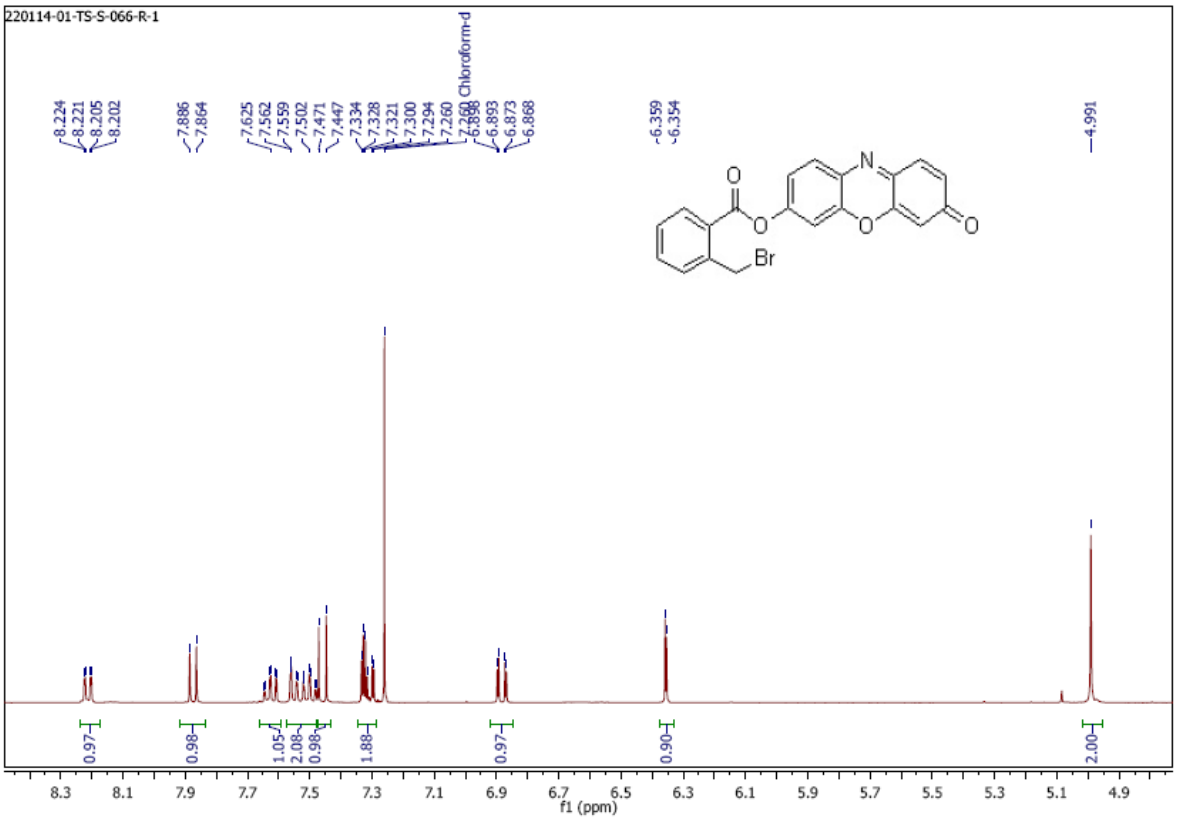


Fig. S23 ^{1}H NMR of **Reso-Br** in $CDCl_3$.

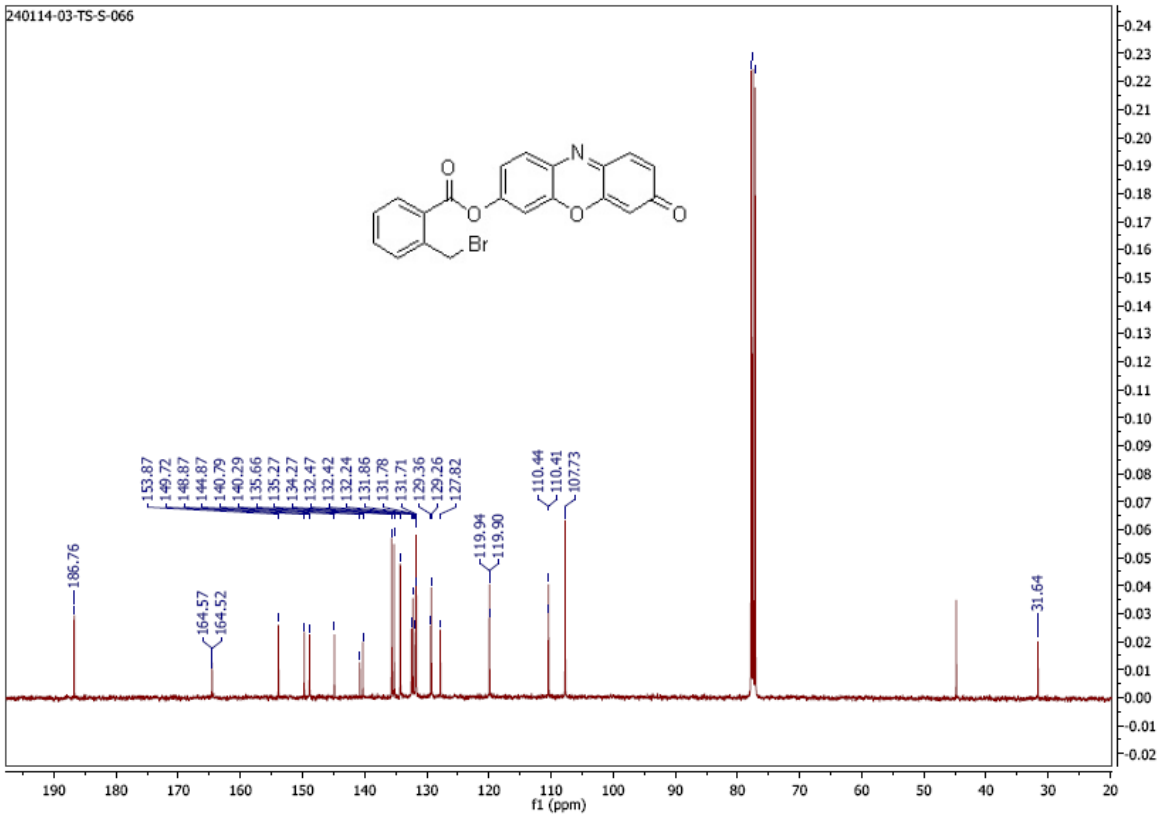


Fig. S24 ^{13}C NMR of **Reso-Br** in $CDCl_3$.

VII. HPLC Purity:

Column: Phenomenex (4.6 mm × 250 mm)

Flow: 1.0 mL/min

Method: Gradient

20 % Acetonitrile/water 0 min

100 % Acetonitrile 0 to10 min

100 % Acetonitrile 10 to 15 min

20 % Acetonitrile/water 15 to 20 min

20 % Acetonitrile/water 20 to 25 min

Wavelength: 250 nm.

Rentention time (t_R): **Reso-Br** = 11.38 min.

Reso- N_3 = 111.32 min

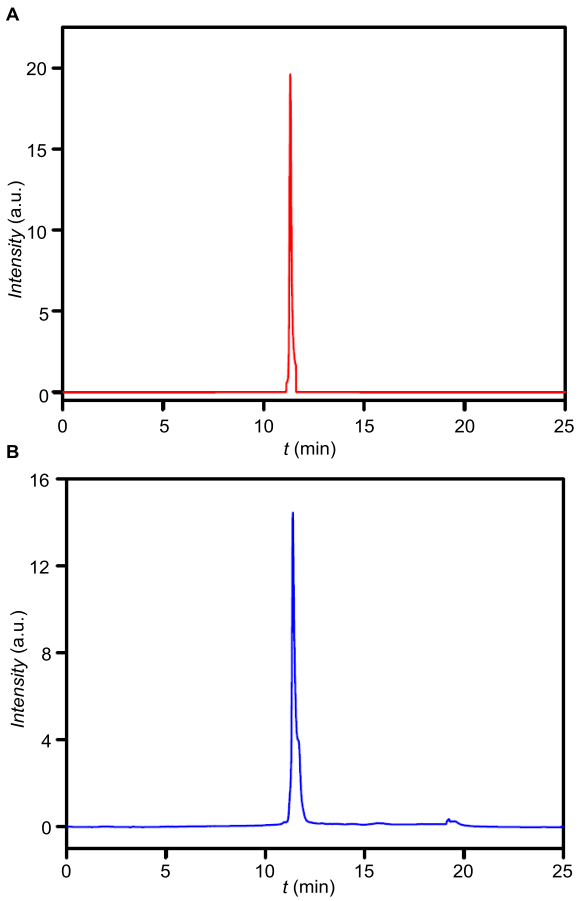


Fig. S25 HPLC Purity of **Reso-N_3** (**A**) and **Reso-Br** (**B**).