Electronic Supplementary Material (ESI) for Chemical Communications.
This journal is © The Royal Society of Chemistry 2023

Supporting Information

Two-Photon Responsive Hydroxyphenylquinazolinone (HPQ) Based Fluorescence Organic Nanoprodrug for H_2S Release Against Oxidative Stress

Amrita Chaudhuri,[a] Tara Singha,[b] Bikash Chandra Jena,[c] Maniklal Shee,[a] Prasanta Kumar Datta,[b] Mahitosh Mandal,[c] N. D. Pradeep Singh*[a]

[a]Department of Chemistry, [b]Department of Physics, [c]School of Medical Science and Technology, Indian Institute of Technology Kharagpur, 721302, West Bengal, India

Corresponding Author

* N. D. Pradeep Singh, E-mail: ndpradeep@chem.iitkgp.ac.in

1. General procedure and materials.

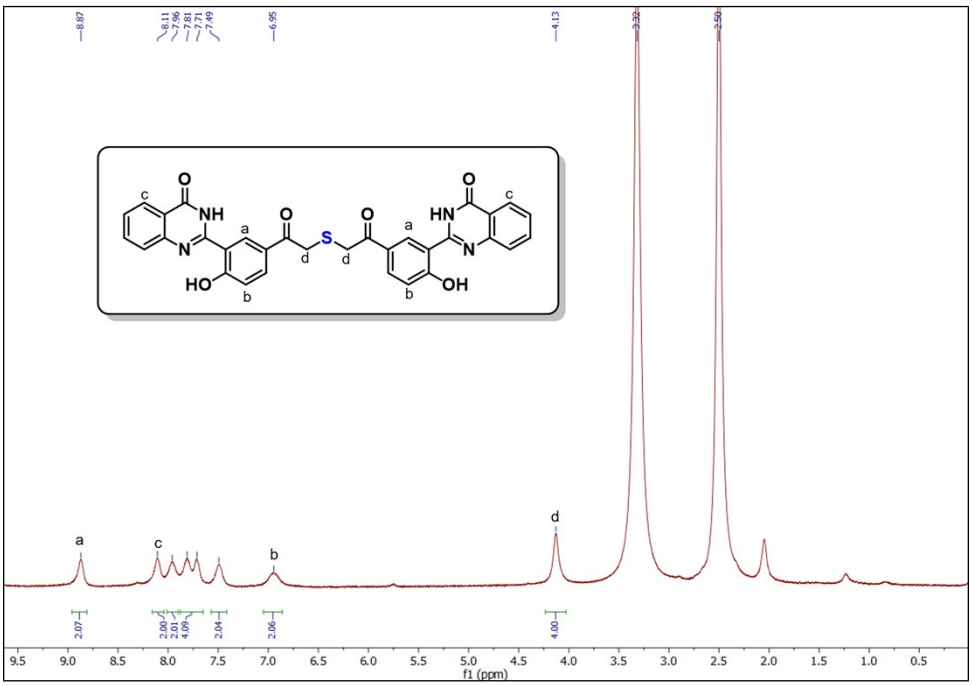
^{1}H NMR (500 MHz and 400 MHz) and ^{13}C NMR with complete proton decoupling (125 MHz and 100 MHz) spectra were recorded on a BRUKER-AC 500 MHz and a BRUKER-AC 400 MHz spectrometer, respectively. Chemical shifts are reported in parts per million from tetramethylsilane with the solvent resonance as the internal standard (deuterochloroform: 7.26 ppm, DMSO-d_6: 3.313 ppm and 2.484 ppm). Data are reported as follows: chemical shifts, multiplicity (s = singlet, d = doublet, t = triplet, m = multiplet), and coupling constant (Hz). Chemical shifts are reported in parts per million from tetramethylsilane with solvent resonance as the internal standard ($CDCl_3$: 77.23 ppm, DMSO-d_6: 39.96 ppm). UV/vis absorption spectra were recorded on a Shimadzu UV-2450 UV/vis spectrophotometer, fluorescence emission spectra were recorded on a Hitachi F-7000 fluorescence spectrophotometer, and HRMS spectra were recorded on a JEOL-Accu TOF JMS-T100L mass spectrometer. Photolysis of the H_2S nanoprodrug was carried out using a 125 W medium-pressure mercury lamp supplied by SAIC (India). RP-HPLC was recorded using a mobile phase, a gradient of ACN/water (water containing 0.1% trifluoroacetic acid) (phase 1/phase 2). Methods were performed with a flow rate of 1.0 mL/min. Compounds were detected at λ = 254 nm. Chromatographic purification was done with 60-120 mesh silica gel (Merck). Precoated silica gel 60 F254 TLC sheets (Merck) were used for reaction monitoring.

Anhydrous solvent dimethylformamide (DMF) was used without distillation. Dichloromethane was distilled from CaH_2 before use. Solvents for workup and column chromatography, such as petroleum ether (PE), ethyl acetate (EA), and other chemicals, were obtained from commercial vendors and used without further purification. All reagents were purchased from Sigma Aldrich and used without further purification. HPLC-grade acetonitrile and water were used for photolysis and HPLC.

2. Experimental Procedure and spectroscopic data:

2,2'-((2,2'-thiobis(acetyl))bis(6-hydroxy-3,1-phenylene))bis(quinazolin-4(3*H*)-one) (3):

5,5'-(2,2'-thiobis(acetyl))bis(2-hydroxybenzaldehyde) (**2**) (0.5 g, 1.39 mmol) is dissolved in 15 mL of ethanol. Then, I_2 (0.423 g, 1.67 mmol) and 2-aminobenzamide (0.23 g, 1.67 mmol) are added to the mixture and heated at 80 °C for 6 h. After 6 hours, the reaction mixture was evaporated, and added sodium thiosulfate solution to remove excess I_2. Then the solid part was filtered and washed with many solvents (water, ethyl acetate, DCM, hexane) to give the target compound **3** off-white colored solid (0.67 g, 81%). ^{1}H NMR (400 MHz, DMSO-*d*6) δ 8.87 (s, 2H), 8.11 (s, 2H), 7.96 (s, 2H), 7.76 (d, *J* = 40.3 Hz, 4H), 7.49 (s, 2H), 6.95 (s, 2H), 4.13 (s, 4H).^{13}C NMR (126 MHz, DMSO-*d6* with 1% Acetone-*d6*) δ 193.3, 165.4, 135.4, 133.9, 129.9, 127.6, 127.2, 126.5, 126.5, 121.3, 119.2, 113.1, 37.5 [Due to solubility issue all ^{13}C signal has not clearly visible]. HRMS (ESI) m/z: $[M+H]^+$ Calcd for $C_{32}H_{23}N_4O_6S$ 591.1333; found: 591.1352.



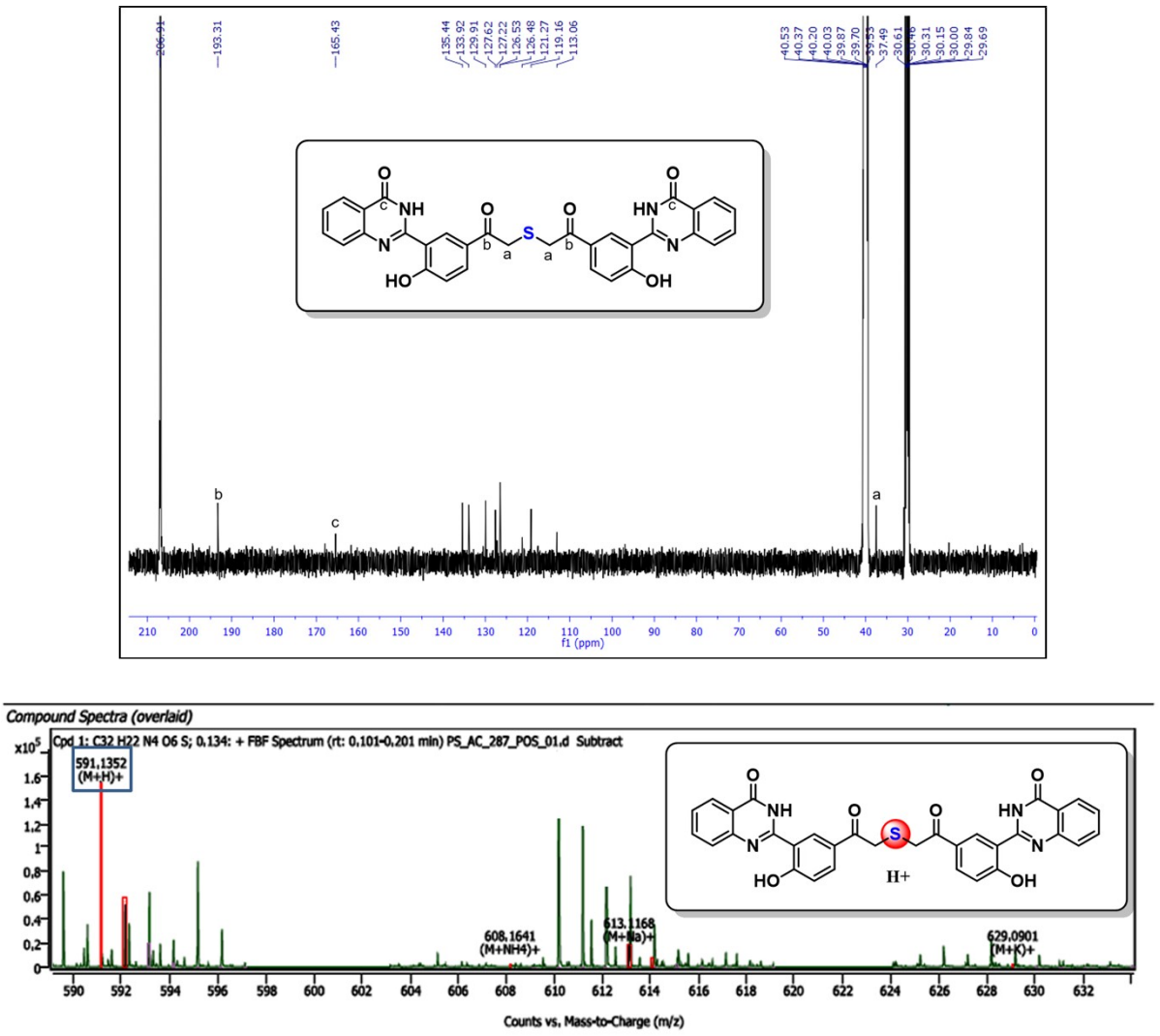


Fig. S1: ^{1}H &^{13}C NMR spectra and HRMS of compound **3**.

Characterization of photoproduct, 2-(4-hydroxy-3-(4-oxo-3,4-dihydroquinazolin-2-yl)phenyl)acetic acid (HPQ-COOH):

^{1}H NMR (500 MHz, $CDCl_3${1% DMSO-*d6*}) δ 13.94 (s, 1H), 12.03 (s, 1H), 8.20 (t, *J* = 6.6 Hz, 1H), 8.10 (d, *J* = 4.5 Hz, 1H), 7.70 (t, *J* = 6.9 Hz, 1H), 7.61 (t, *J* = 7.0 Hz, 1H), 7.44 – 7.39 (m, 1H), 7.39 – 7.35 (m, 1H), 7.30 – 7.26 (m, 1H), 6.93 (t, *J* = 7.6 Hz, 1H), 4.12 – 4.06 (m, 2H). ^{13}C NMR (126 MHz, $CDCl_3$ {1% DMSO-*d6*}) δ 171.50, 162.16, 160.01, 153.59, 146.41, 134.67, 134.52, 127.80, 126.64, 126.34, 125.92, 124.59, 120.97, 118.37, 112.99, 60.64. HRMS (ESI) m/z: $[M+H]^+$ Calcd for $C_{16}H_{13}N_2O_4$ 297.0870; found: 297.0830.

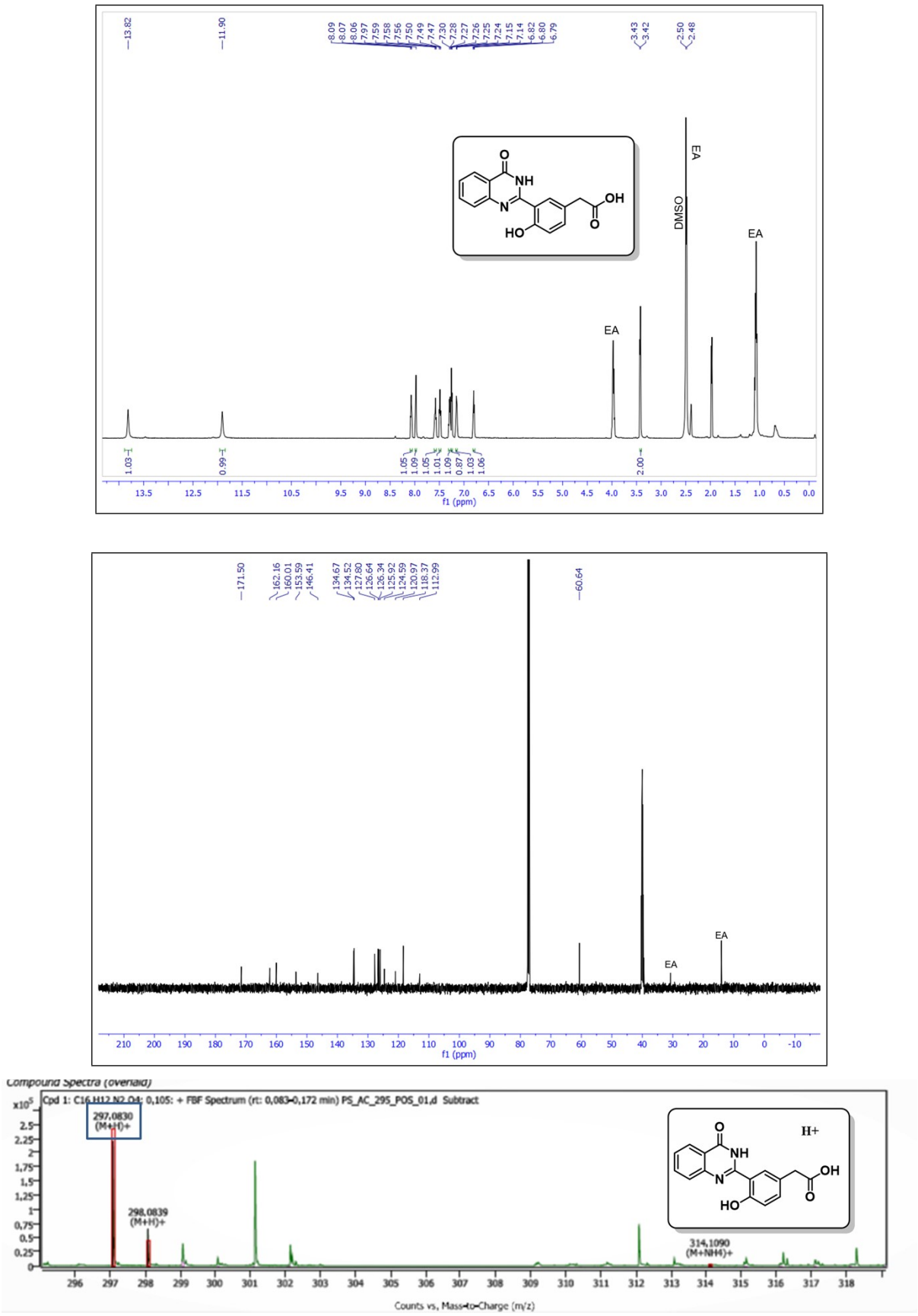


Fig. S2: ^{1}H and ^{13}C NMR and HRMS spectra of photoproduct (HPQ-COOH).

3. Photophysical studies of compound 3 in different solvent systems

The UV absorption and emission behaviour were determined in different solvent systems for compound **3** (HPQ-H_2S) to understand the ESIPT effect. In most solvent systems, we observed two absorption humps (between 310 nm to 360 nm) representing the keto-enol tautomeric form (**Fig. S3, a**). In **Fig. S3 (b**), we observed two emission maxima (between 410 nm to 460 nm), indicating enol emission and keto emission (except for polar aprotic solvents).

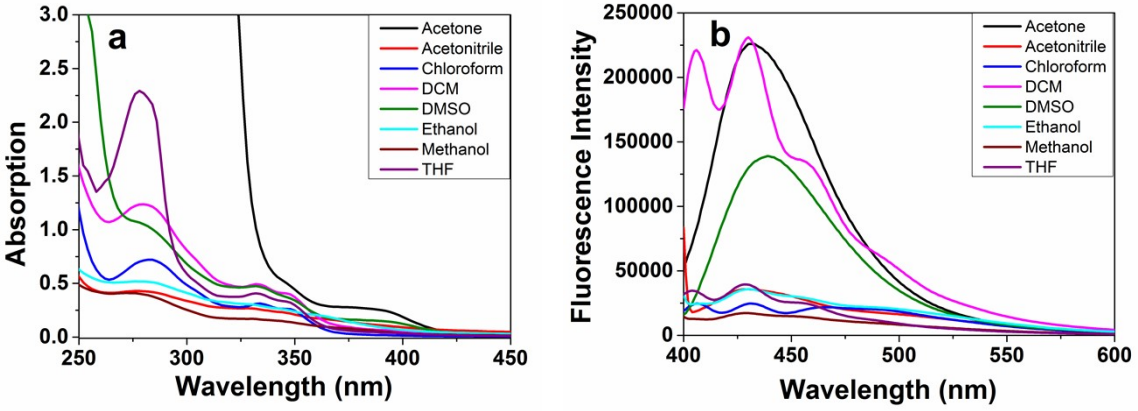


Fig. S3: (a) UV-vis and (b) fluorescence spectra of compound **3** in different solvent systems

4. Hydrolytic stability of 3:

To measure the hydrolytic stability, we prepared the solution of HPQ-H_2S conjugate (H_2S donor) **3** (1×10^{-4} M) in DMSO/PBS buffer (0.5:10 v/v) containing 10 % fetal bovine serum at pH = 7.4 and kept them in the dark conditions. The solution was incubated at R.T. for 7 days. Next, we also prepared the solution of thiol (GSH; 1 mM), amino acids (cysteine; 1 mM), and ROS (H_2O_2; 1 mM) added to the previously prepared solution of our H_2S donor **3** separately. The aliquots taken after 7 days were measured by HPLC. The results showed that the decomposition of the H_2S donor (**3**) was less than 12% (**Fig. S4**) in all solutions. Therefore, our designed H_2S donor in solution is sufficiently stable and suitable for biological applications.

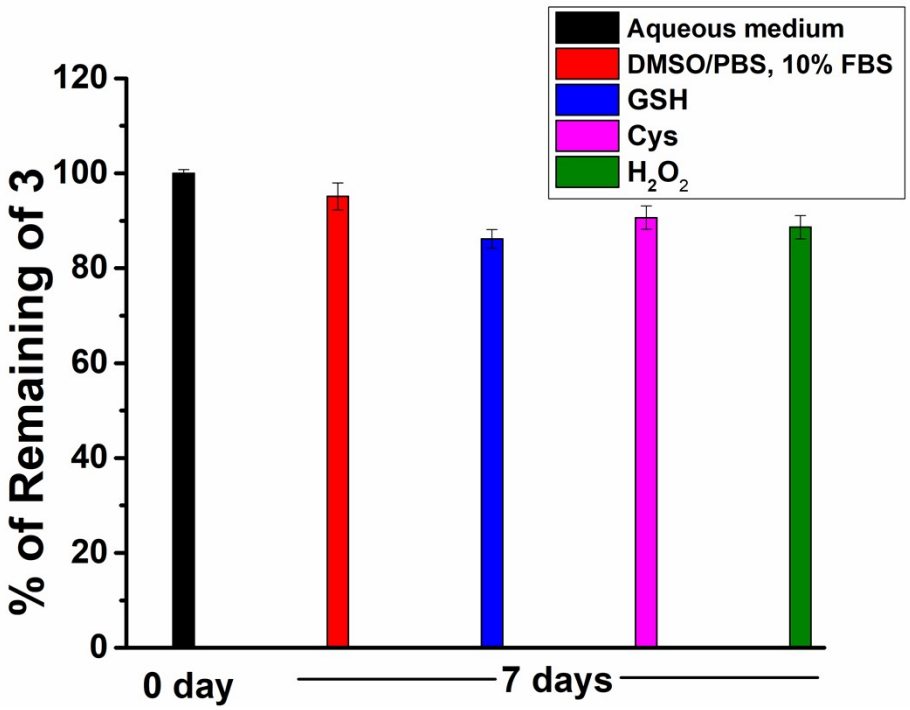


Fig. S4: % of remaining of **3** (1×10^{-4} M), calculated from HPLC peak area while testing the hydrolytic stability at 0 day and 7 days.

5. Preparation of H_2S nanoprodrug:

Photoresponsive H_2S nanoprodrug was prepared by a reprecipitation technique. Millipore water (25.0 mL) was taken into a 25 mL vial and placed in a sonicator. A solution of HPQ-H_2S (1 mM, 0.0006 g in 1 mL) in DMSO was prepared and kept in the dark. The 10 µL of the DMSO solution of HPQ-H_2S conjugate was slowly injected into the sonicated water kept in the vial, giving an effective concentration of H_2S nanoprodrug in the water of 1×10^{-5} M. The resulting solution was then sonicated for a period of 12 min, maintaining the temperature below 25 °C. Before starting the next sonication period, every 10 min interval, a short break was given to cool down the solution to 10 °C. The sonication was continued for up to one hour. Next, we recorded the UV absorption and fluorescence spectra of the H_2S nanoprodrug (**Fig. S5**).

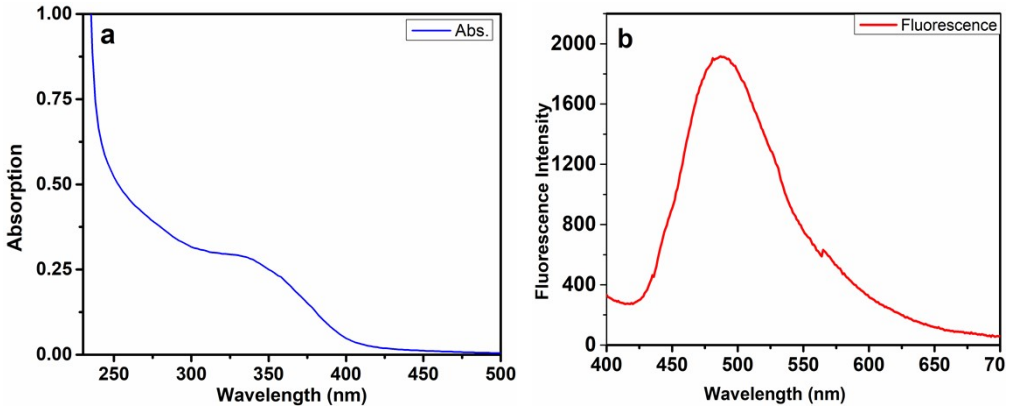


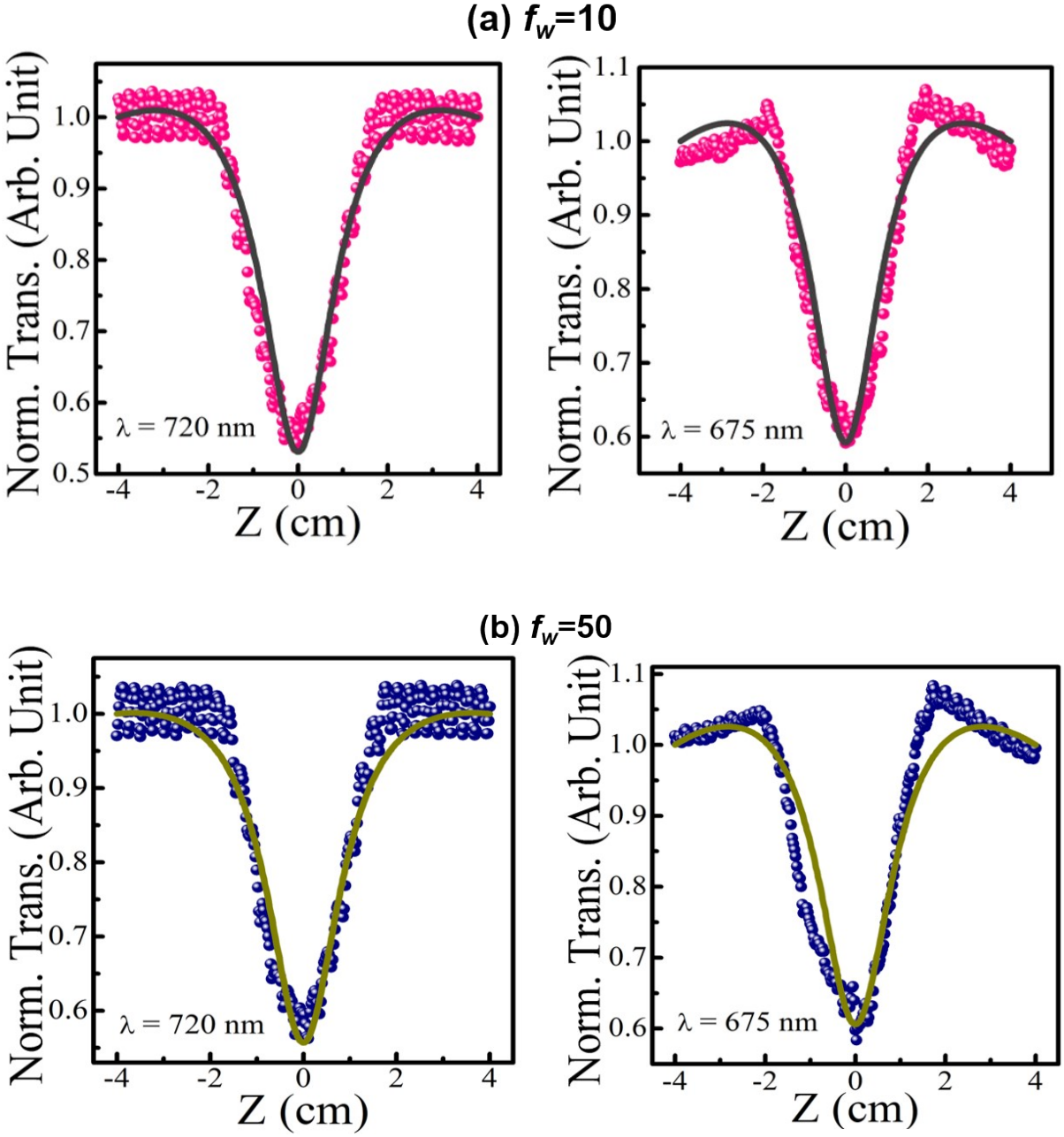
Fig. S5: (a) UV-vis and (b) fluorescence spectra of H_2S nanoprodrug.

6. Open aperture Z-scan measurement:

To study the two-photon absorption cross-section of H_2S nanoprodrug in a DMSO/water medium, open aperture (OA) Z-scan measurements were performed in the femtosecond regime. We used a femtosecond pulsed laser (100 fs, 1 kHz) beam of 720 and 675 nm wavelengths generated from a Ti-Sapphire laser (Coherent: Libra, 808 nm, 50 fs, 1 kHz) pumped Optical Parametric Amplifier (OPA, TOPAS-Prime) system. A 20 cm planoconvex lens is used to focus the beam at the beam waist of 40 µm and Rayleigh range of 0.71 cm. The samples are kept in the cuvette with an effective sample length of 1 mm and moved across the focused beam by a motorized translational stage (Newport, GTS-150). The transmitted beam is collected by a photodetector (Si-photodiode, Thorlabs PDA100A-EC) through a planoconvex lens of 10 cm. A lock-in amplifier (7225 DSP, Signal Recovery) is used for the data collection for a better signal-to-noise ratio. The lock-in-amplifier and the translational stage are incorporated in Labview 2012 software for data acquisitions. The experimental OA curves (**Fig. S6**) were fitted using the transmittance equation of the theoretical model associated with two-photon absorption (TPA) and saturable absorption.[1] The TPA coefficients extracted from the fitting parameters were used to calculate the TPA cross-section at different water fractions for HPQ-H_2S conjugate and H_2S nanoprodrug using a standard equation.[2]

TPA cross-section at different water fractions for HPQ-H_2S conjugate and H_2S nanoprodrug:

The two-photon absorption cross-section δa was measured for the HPQ-H_2S conjugate and H_2S nanoprodrug with a (10 mM) solution in DMSO / buffer (pH – 7.4) binary mixture with varying water fractions at 720 and 675 nm in all cases.



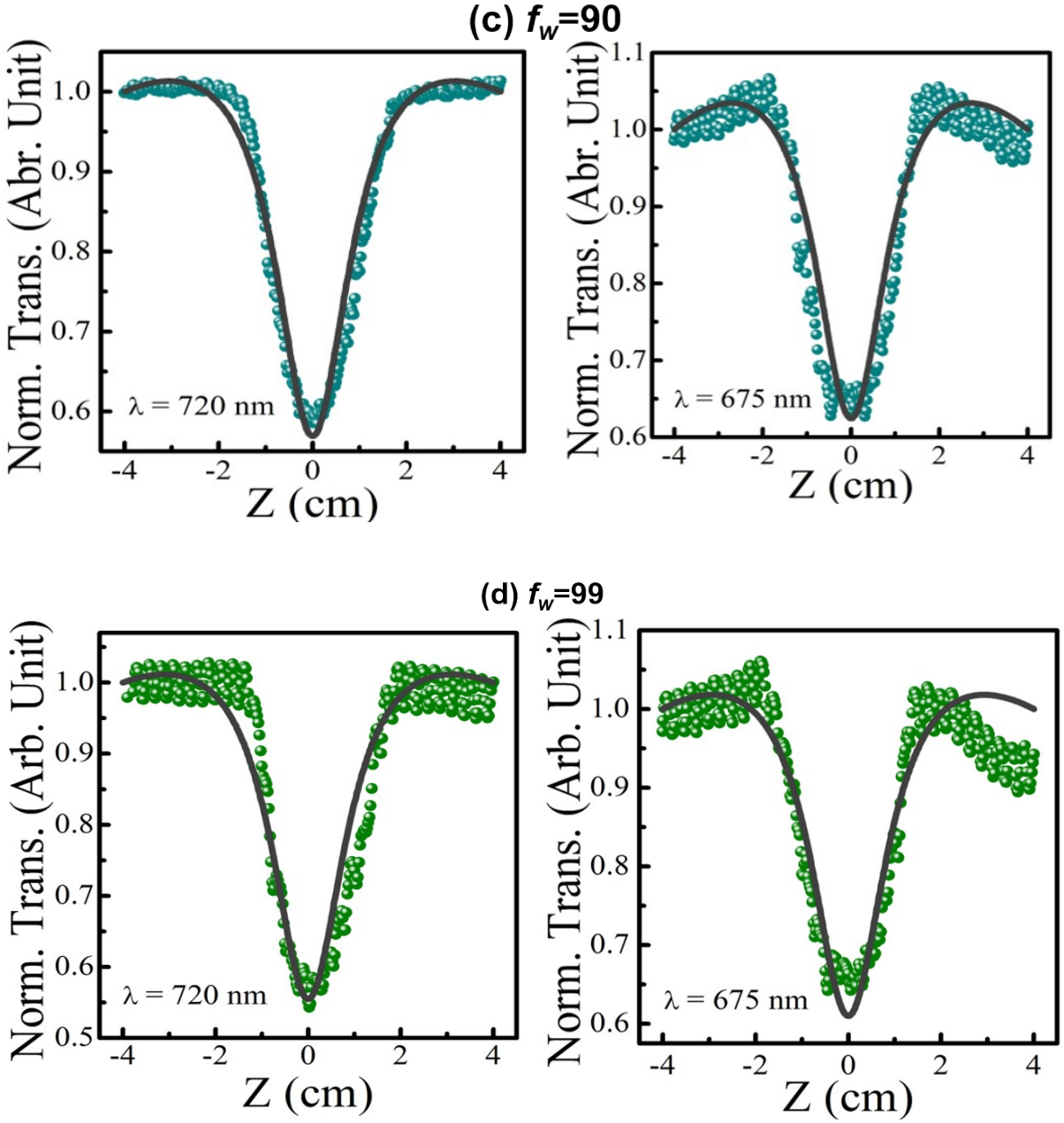


Fig. S6: Open aperture (OA) Z-scan traces of HPQ-H_2S conjugate and H_2S nanoprodrug at 720 and 675 nm in DMSO/ buffer (pH – 7.4) binary mixture with varying f_w. Normalized transmittance (Norm. Trans.): The dotted line represents experimental data, and the solid line represents theoretical fitting.

The TPA cross-section δ_a is calculated for both wavelengths and summarised in **Table S1**.

HPQ-H_2S	TPA cross-section at different water fractions (f_w) in GM			
	f_w=10	f_w=50	f_w=90	f_w=99
720 nm	310	260	269	283
675 nm	283	268	258	253

Table S1: The two-photon absorption cross section δa was measured for HPQ-H_2S conjugate and H_2S nanoprodrug with a (10 mM) solution in DMSO / buffer (pH – 7.4) binary mixture with varying water fraction at 720 and 675 nm in all cases (1 GM = 10^{-50} cm^4 s/photon).

7. Two-photon photolysis:

To observe the two-photon photorelease ability of our H_2S nanoprodrug, we irradiated the H_2S nanoprodrug (100 μM) with 720 nm light and monitored the photorelease by HPLC. After 1 h irradiation, we found 25% decomposition of H_2S nanoprodrug (**Fig. S7** in f_w = 99% PBS buffer in DMSO; pH-7.4).

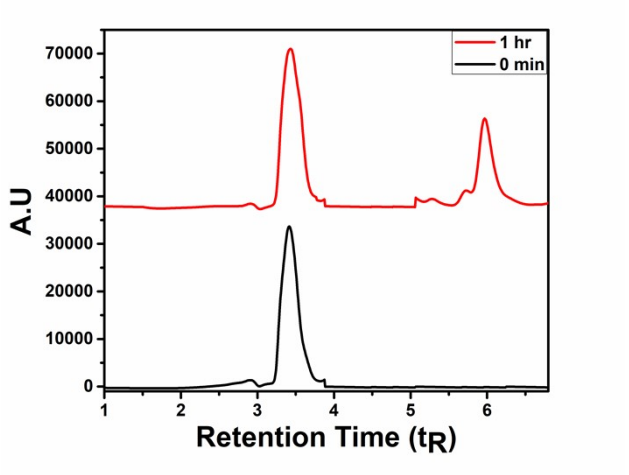


Fig. S7: HPLC chromatogram of two-photon photolysis of H_2S nanoprodrug.

We calculated the decomposition rate constant (H_2S nanoprodrug) under both one- and two-photon excitation, which in turn is comparable to the release rate of H_2S. From **fig. S8**, we have calculated k(1P)= 4.8×10^{-2} s^{-1}, and k(2P)= 7.1×10^{-3} s^{-1}.

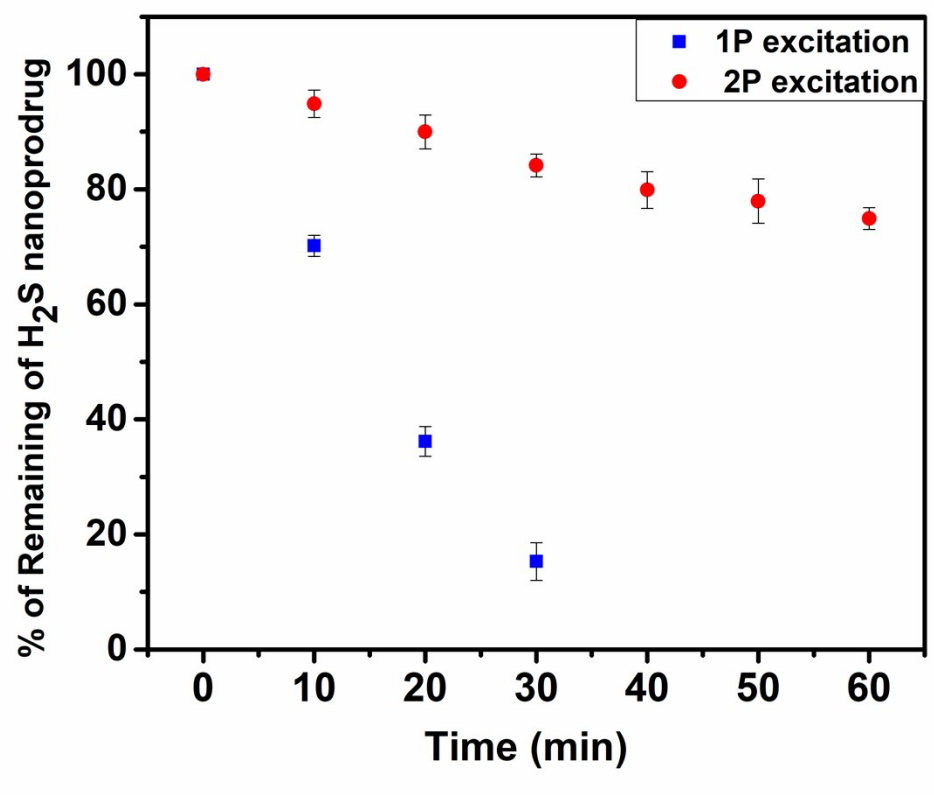


Fig. S8: The % remaining of H_2S nanoprodrug after 30 min irradiation under one photon (1P) excitation and after 1 h irradiation under two-photon (2P) excitation.

H_2S detection under two-photon excitation:

We have carried out the H_2S detection by the same methylene blue assay, from which we detected around 9.85 μM H_2S after 1 h irradiation.

8. Determination of incident photon flux (I_0) of the UV lamp by potassium ferrioxalate actinometry:

Potassium ferrioxalate actinometry was used to determine the incident photon flux (I_0) of the UV lamp used for irradiation. The solution of potassium ferrioxalate, 1, 10-phenanthroline, and the buffer solution was prepared following the literature procedure.[3,4]

Solution (0.006 M) of potassium ferrioxalate was irradiated using a 125 W medium pressure Hg lamp as a UV light source ($\lambda \geq 365$ nm) and 1 M $CuSO_4$ solution as a UV cut–off filter.

At a regular interval of time (3 min), 1 mL of the aliquots was taken out, 3 mL of 1,10 phenanthroline solution, and 2 mL of the buffer solution were added, and the whole solution was kept in the dark for 30 min. The absorbance of the red phenanthroline-ferrous complex formed was then measured spectrophotometrically at 510 nm. The amount of Fe^{2+} ions was determined from the calibration graph. The calibration graph was plotted by measuring the absorbance of the phenanthroline-ferrous complex at several known concentrations of Fe^{2+} ions in the dark. From the slope of the graph, the molar absorptivity of the phenanthroline-ferrous complex was calculated at 510 nm, which is found to be similar to the reported value. Using the known quantum yield for potassium ferrioxalate actinometer at 365 nm,[4] the number of Fe^{2+} ions formed during photolysis and the fraction of light absorbed by the actinometer, the incident photon flux (I_0) at 350 nm of the 125 W Hg lamp was determined as 1.55×10^{17} photons $s^{-1}cm^{-2}$.

9. Deprotection photolysis of H_2S nanoprodrug and measurement of photochemical quantum yields:

A solution of 1×10^{-4} M of the H_2S nanoprodrug was prepared in PBS buffer. Half of the solution was kept in the dark. The remaining half nitrogen was passed and irradiated using a 125 W medium-pressure Hg lamp as a UV light source ($\lambda \geq 365$ nm) and 1 M $CuSO_4$ solution as a UV cut–off filter. For H_2S nanoprodrug at different intervals, 20µl of the aliquots was taken and analyzed by RP-HPLC using mobile phase acetonitrile at a flow rate of 1ml / min (detection: UV 254 nm). Peak areas were determined by RP-HPLC, which indicated a gradual decrease of the H_2S nanoprodrug with time and the average of three runs. The reaction was followed until the consumption of the H_2S nanoprodrug was less than 10% of the initial area. Based on HPLC data, we plotted normalized [A] (HPLC peak area) versus irradiation time. We observed an exponential correlation for the disappearance of the H_2S nanoprodrug, which suggested a first-order reaction.

Further, the quantum yield for the photolysis of the H_2S nanoprodrug was calculated using Equation (**1**).

$$(\Phi_p)_{CG} = (\Phi_p)_{act} \frac{(k_p)_{CG}}{(k_p)_{act}} \frac{(F_{act})}{(F_{CG})} \qquad (1)$$

The subscript ‘CG’ and ‘act’ denotes H_2S nanoprodrug and actinometer, respectively. Potassium ferrioxalate was used as an actinometer. Φ_p is the photolysis quantum yield, k_p is

the photolysis rate constant, and F is the fraction of light absorbed.

In addition, we have recorded the ^{1}H NMR of the photolysis mixture after extraction with ethyl acetate (**Fig S9**) from the photolysis mixture (after 30 min irradiation). From the spectra, we can observe the photoproduct formation in the photolysis mixture.

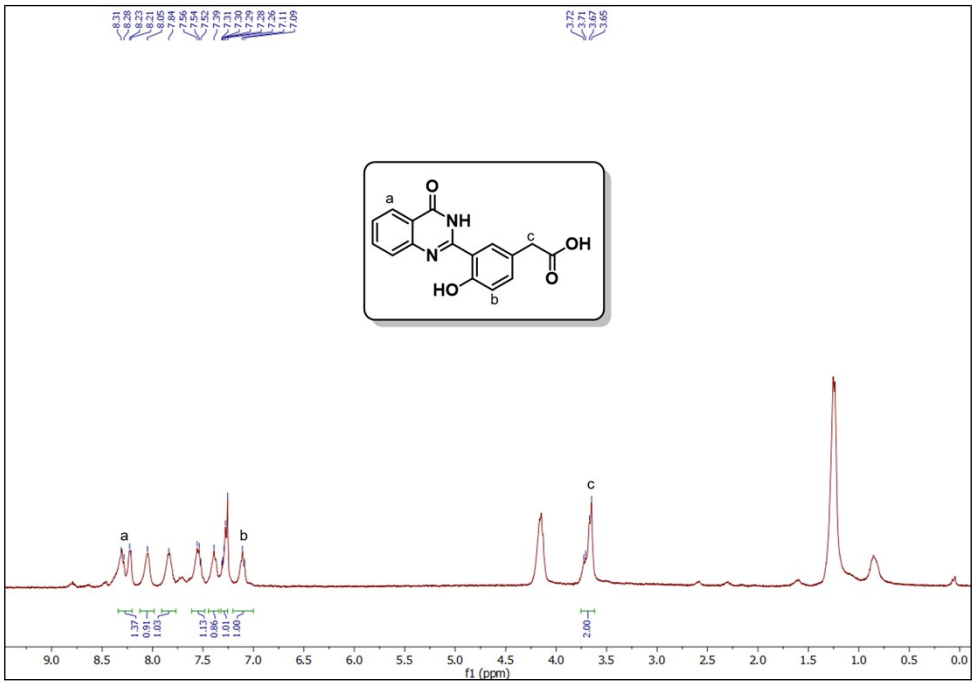
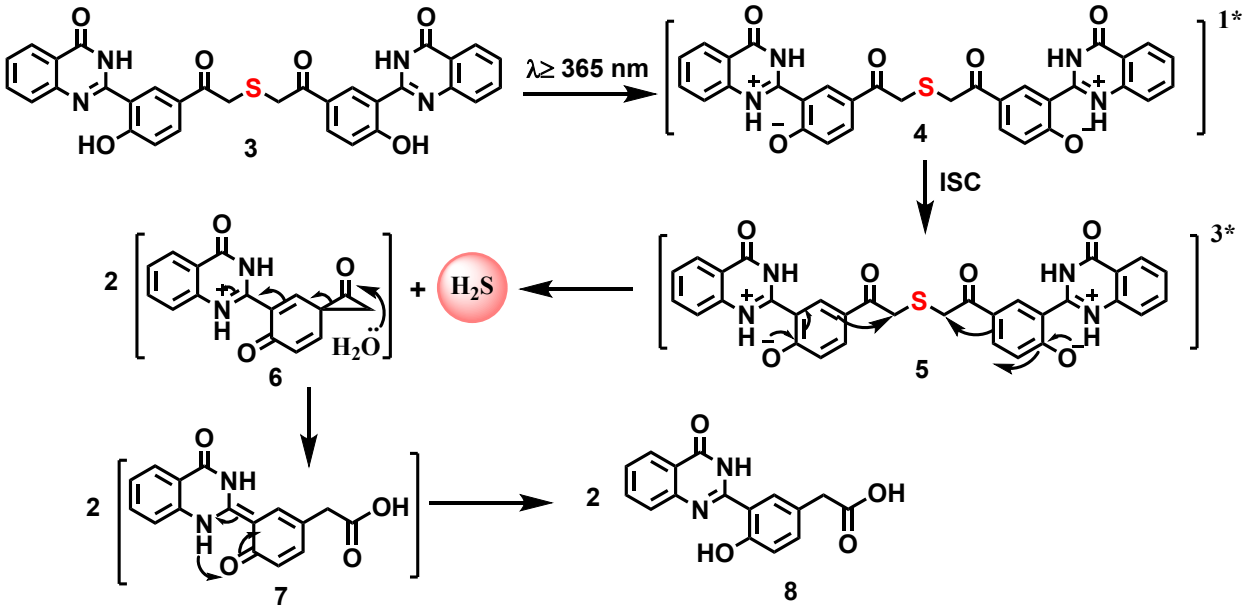


Fig. S9. ^{1}H NMR of photolysis mixture in $CDCl_3$ (with 1% DMSO-*d6*).

10. Possible photorelease mechanism of H_2S nanoprodrug;



Scheme S1: Proposed ESIPT-induced H_2S-Releasing Mechanism from H_2S nanoprodrug.

11. Methylene Blue assay:

A methylene blue assay was carried out as described previously with some modifications.[5]
A 5 mM solution of Na_2S in sodium phosphate buffer (20 mM, pH 7.4)/acetonitrile (HPLC grade) (7:3) was prepared ($Na_2S{\cdot}9H_2O$, 120.20 mg in 100 mL volumetric flask) and used as the stock solution. Aliquots of 100, 200, 300, 500, 700, and 1000 µL of the Na_2S stock solution were added into a 50 mL volumetric flask and dissolved in a mixture of sodium phosphate buffer/acetonitrile to obtain the standard solutions in 10, 20, 30, 50, 70, 100 µM, respectively.
1 ml aliquot of the respective solution was reacted with the methylene blue (MB^+) cocktail: 30 mM $FeCl_3$ (400 µL) in 1.2 M HCl, 20 mM of *N,N*-dimethyl-1,4- phenylenediamine sulfate (400 µL) in 7.2 mM HCl, 1% w/v of $Zn(OAc)_2$ (100 µL) in H_2O at room temperature for at least 15 min (each reaction was performed in triplicate). The absorbance of methylene blue was measured at λ_{max} = 663 nm. To obtain the molar absorptivity of (MB^+), a linear regression was plotted with the observed absorbance and concentration.

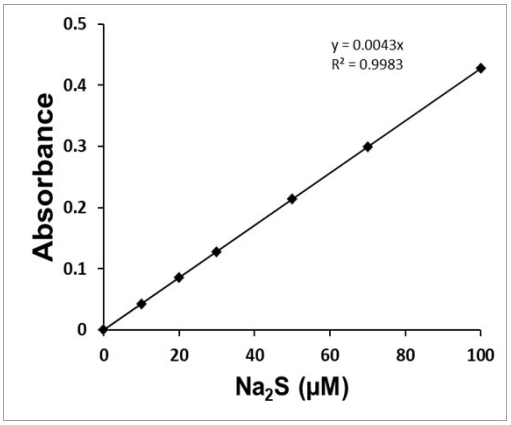


Fig. S10. A standard Calibration curve with different concentrations of Na_2S.

In this experiment, a 100 µM solution (total volume 20 mL) of the compound H_2S nanoprodrug **3** was prepared in a 7:3 solution of sodium phosphate buffer (20 mM, pH 7.4)/acetonitrile. This solution was placed in a 24 mL scintillation vial. The resulting reaction vessel was irradiated with a 125 W medium-pressure mercury lamp as the source of UV light ($\lambda \geq 365$ nm) using a suitable UV cut-off filter (1 M $CuSO_4$ solution) with continuous stirring. The aliquot (1 mL) was collected at different time intervals (5, 10, 15, and 20 min) and was mixed immediately with the methylene blue cocktail: 30 mM FeCl3 (200 µL) in 1.2 M HCl, 20 mM of *N,N*-dimethyl-1,4-phenylenediamine sulfate (200 µL) in 7.2 mM HCl, 1% w/v of Zn(OAc)2 (100 µL) in H_2O at room temperature for at least 20 min. The absorbance

of methylene blue was measured at λ_{max}= 663 nm against a blank: 30 mM $FeCl_3$ (400 µL) in 1.2 M HCl, 20 mM of *N,N*-dimethyl-1,4- phenylenediamine sulfate (400 µL) in 7.2 mM HCl, 1% w/v of $Zn(OAc)_2$ (100 µL) in H_2O, ACN (500 µL), 20 mM sodium phosphate buffer pH 7.4 (500 µL).

12. *In vitro* cellular imaging and cytotoxicity studies of H_2S nanoprodrug:

***In vitro* cellular uptake studies of the H_2S nanoprodrug:**

The MDA-MB-468 cells (1 × 10^4 cells / mL) were seeded on coverslips in MEM medium. After 24 h, one set of cells was treated with 10 µM of H_2S nanoprodrug and incubated for 4 h, and another set was used as a control (no treatment). Cells were fixed using 3.7% paraformaldehyde. Then, the slides were washed thrice with PBS and prepared with DPX mountant. Imaging was done using fluorescent microscopy (Zeiss Observer Z1, Carl Zeiss, Germany) before and during irradiation.

Cell viability of H_2S nanoprodrug on MDA-MB-468 cell line:

The cytotoxicity of the H_2S nanoprodrug in MDA-MB-468 was determined using the MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay on MDA-MB-468 cells before and after irradiation (**Fig. S11**).

Before photolysis:

The cells were seeded into a 96-well cell culture plate at 1 × 10^4 cells / mL. Afterward, different concentrations (40, 60, 80, and 100 µM) of H_2S nanoprodrug were added separately into the wells, and an equal volume of PBS buffer was added to the control wells. Then, fresh MTT solution (0.40 mg/mL) in PBS was added to the wells and incubated in the same cellular environment. After 4 h, a solution containing MTT was removed, and the formed Formazan crystals were dissolved in DMSO. The absorbance was recorded at 595 nm an iMark^{TM} microplate absorbance reader.

After photolysis:

MDA-MB-468 cultured in MEM (in a 96- well cell culture plate at a concentration of 1 × 10^4 cells / mL) were incubated independently with different concentrations (40, 60, 80, and 100 µM) H_2S nanoprodrug for 4 h at 37 °C in 5% CO_2. After that, the cells were irradiated under light (≥ 365 nm) for a certain time, keeping the cell culture plate minimum 5 cm apart from

the light source. After irradiation, the cell viability was measured using the MTT assay described above.

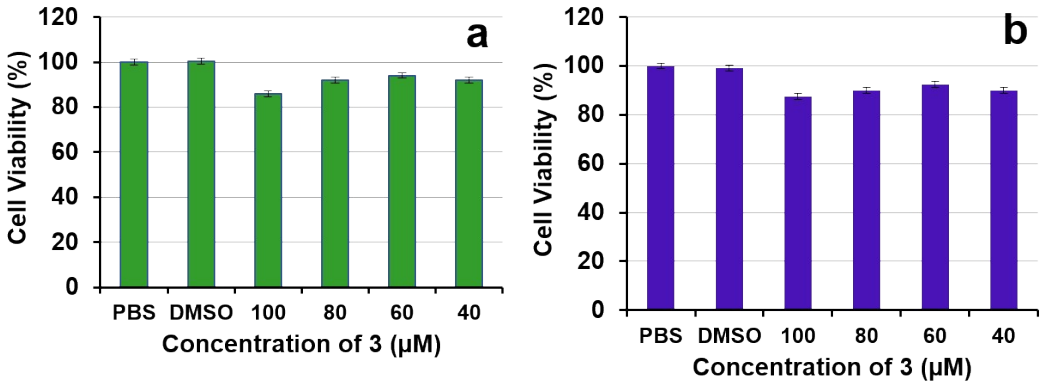


Fig. S11: Cell viability assay of H_2S nanoprodrug on the MDA-MB-468 cell line: (a) before and (b) after photolysis. Values are presented as mean ± SD from three independent experiments with triplicates per experiment.

Cell viability assay of H_2S nanoprodrug during irradiation on H_2O_2 treatment:

The cells were seeded into a 96-well cell culture plate at 1×10^4 cells per well. After that, 200 μM H_2O_2 was added to the wells without the control. Then, we measured the % of cell viability by MTT assay. Next, MDA-MB-468 cells were treated with our H_2S nanoprodrug (100 μM) separately for 3 h at 37 °C in the presence of 200 μM H_2O_2 and further incubated for 3 h. Then, the cells were irradiated under light (≥ 365 nm) for 30 minutes, keeping the cell culture plate a minimum of 5 cm apart from the light source. After irradiation, cell viability was measured using the MTT assay described above by three (n=3) independent experiments.

13. Reference:

1. Bhattachraya, S.; Maiti, R.; Das, A. C.; Saha, S.; Mondal, S.; Ray, S. K.; Bhaktha, S. N. B.; Datta, P. K. Efficient control of ultrafast optical nonlinearity of reduced graphene oxide by infrared reduction, *Journal of Applied Physics* **2016**, *120*, 013101.

2. Majumdar, S.; Singha, T.; Dhibar, S.; Mandal, A.; Datta, P. K.; Dey, B. *ACS Applied Electronic Materials* **2020** *2*, 3678-3685

3. Ryan, M. A.; Xiang, E. T.; Johnston, T.; Fox, K. P. Absorption and Fluorescence Studies of Acridine in Subcritical and Supercritical Water *J. Phys. Chem. A* **1997**, *101*, 1827–1835.

4. Jana, A.; Saha, B.; Ikbal, M.; Ghosh, S. K.; Singh, N. D. P. 1-(Hydroxyacetyl)pyrene a New Fluorescent Phototrigger for Cell Imaging and Caging of Alcohols, Phenol and Adenosine *Photochem. Photobiol. Sci.* **2012**, *11*, 1558–66.

5. Venkatesh, Y.; Das, J.; Chaudhuri, A.; Karmakar, A.; Maiti, T. K.; Singh, N. D. P. *Chem. Commun.* **2018**, *54*, 3106-3109.