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One- and Two-Photon Activated Cysteine Persulfide Donors for Biological Targeting

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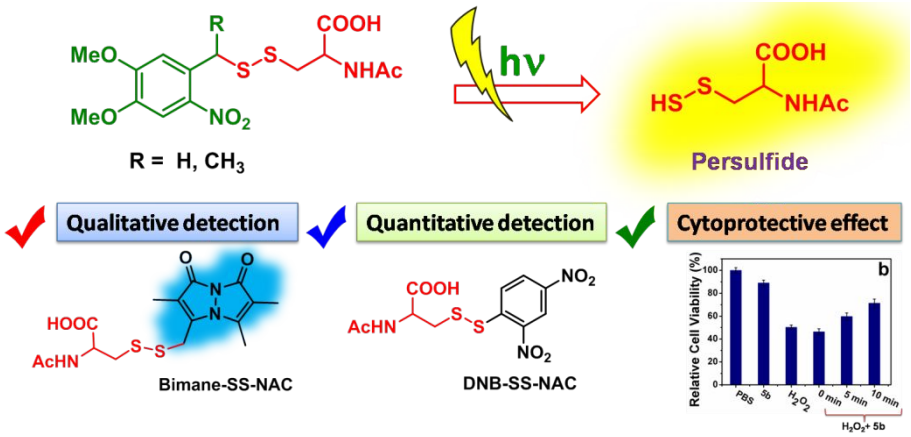


ABSTRACT: Persulfides (RSSH) have been considered as potential signaling compounds similar to the H_2S in "S-persulfidation", a sulfur-mediated redox cycle. The research of this sulfur-mediated species is hindered due to the lack of efficient persulfide donors. In this current study, we have developed a one- and two-photon activated persulfide donors based on *o*-nitrobenzyl (ONB) phototrigger, which releases the biologically active persulfide (*N*-acetyl *L*-cysteine persulfide, NAC–SSH) in a spatiotemporal manner. Next, we have demonstrated the detection of persulfide release both qualitatively and quantitatively using the well-known "turn on" fluorescence probe i.e., monobromobimane and the trapping agent i.e., 2,4-dinitrofluorobenzene, respectively. Furthermore, we examined the cytotoxicity of synthesized

persulfide donors on HeLa cells and the cytoprotective ability in the highly oxidizing cellular environment.

INTRODUCTION:

Hydrogen sulfide (H_2S) is an important small gaseous signaling molecule that has been recently explored as a novel therapeutic agent for the treatment of cancer, inflammation, metabolic syndrome, obesity, cardiovascular disease, and neurodegenerative disorders.[1] Looking into the therapeutic prospect and potential of H_2S, several research groups started understanding mechanisms of action that underlie the effects of H_2S.[2] Since H_2S is endogenously produced by various mammalian tissues at nanomolar concentrations, it is difficult to fully elucidate the sulfide-based signaling mechanism.[3] In order to advance the field of sulfur biology, it is important to devise chemical tools that facilitate precise exogenous production of various specific sulfur species to understand the role of these sulfur species at the molecular level.

To enable the exogenous production of various specific sulfur species, several research groups developed different types of H_2S-releasing compounds based on different triggering mechanisms (e.g., water, pH, thiols, enzymes, and light).[4-13] There are three main ways in which H_2S exerts its biological effects: (i) metal center interactions, (ii) reactive oxygen and nitrogen species, and (iii) persulfidation. Among these, persulfidation has been shown to be an important signaling pathway involving H_2S.[14] Protein persulfidation (sometimes referred to as S-sulfhydration) is an oxidative process, which involves the post-translational modification of cysteine to cysteine persulfide (thiol -SH is converted to a perthiol -SSH). Recent investigation has shown that much of the biological activity associated with H_2S can be attributed to persulfides and the closely related polysulfides.[15] It has even been suggested that H_2S could only be a degradation product

of persulfides.[16,17] Furthermore, persulfidation modulates the biological activity of proteins due to the decrease in pKa and increase in nucleophilicity of perthiols relative to thiols. In addition, other findings also support that reactive sulfur species (RSS) such as sulfane sulfurs or polysulfides are more effective in S-sulfhydration than H_2S.[18] Hence, there is a clear need for the investigation of persulfide chemistry in the sulfide-based signaling mechanisms. A major obstacle in the study of the biological roles of persulfides is a lack of chemical tools capable of generating well-defined persulfide species in response to specific biologically relevant triggers.

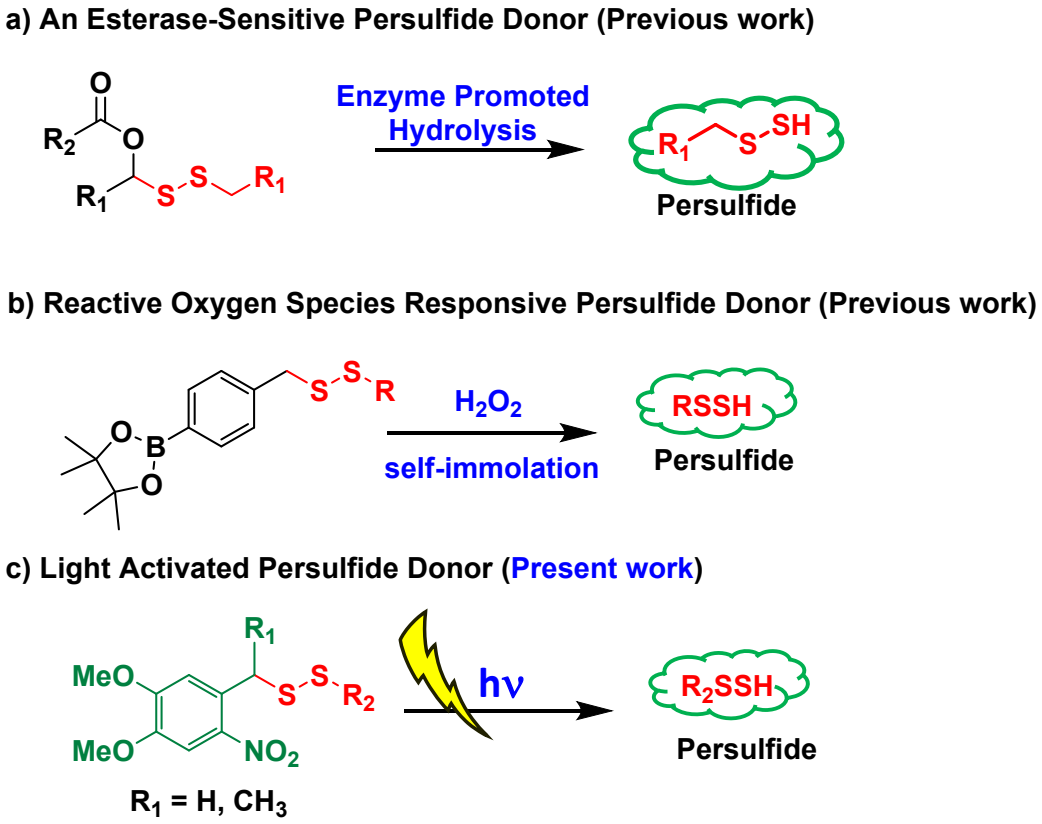


Figure 1. (**a**) An esterase-sensitive persulfide donor (**b**) H_2O_2 responsive persulfide donor and (**c**) Designed light activated persulfide donor based on *o*-nitrobenzyl phototrigger.

To date, very few donors have been developed for the release of persulfide.[19] Galardon and co-workers developed a pH-triggered persulfide analog of the nitrosothiol (SNAP).[20] Of late, Wang and co-workers developed esterase-triggered persulfide prodrugs capable of releasing either a persulfide or hydrogen persulfide (H_2S_2) (**Figure 1a**).[21-23] Recently, Xian group also reported a class of pH- and fluoride-dependent persulfide donors.[24] In addition, Matson and co-workers also developed ROS-responsive (ROS: reactive oxygen species), self-immolative persulfide donor (**Figure 1b**).[25] The main limitation of the abovementioned persulfide donors is their inability to provide temporal control over the persulfide release.

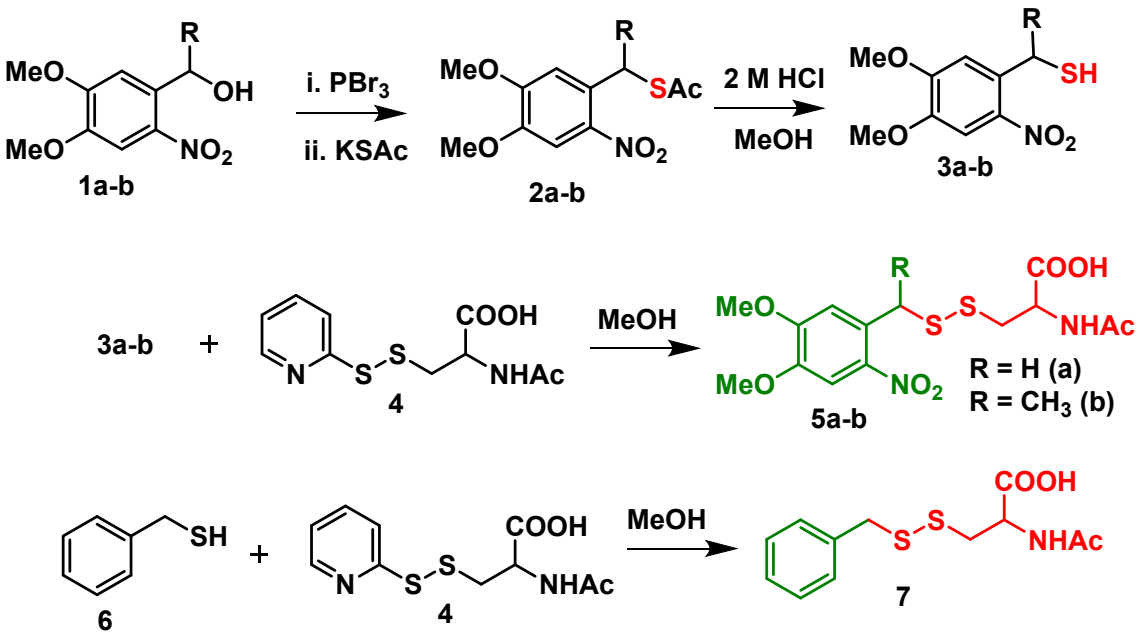
Light-triggered persulfide generation from a photochemically active chromophore will be a suitable activation strategy because it will provide precise spatial and temporal control over the persulfide release. However, to the best of our knowledge, there is no report in the literature for the release of persulfide using an external stimulus light. Herein, we designed for the first time one- and two-photon activated persulfide donors based on *o*-nitrobenzyl (ONB) phototrigger. To design photochemically active persulfide donor, *o*-nitrobenzyl (ONB) phototrigger was chosen for the following reasons (i) ONB is more widely reported as a phototrigger for caging several biologically active compounds for its fast and clean photorelease ability. (ii) It can be photolyzed upon both one- and two-photon excitation (1PE and 2PE) (**Figure 1c**).[26,27] Two-photon uncaging provides finer spatial resolution and less photo-toxicity with a deeper tissue penetration.[28]

RESULTS AND DISCUSSION:

Here, we have synthesized two persulfide donors based on *o*-nitrobenzyl phototrigger, namely (i) *N*-acetyl-*S*-((4,5-dimethoxy-2-nitrobenzyl)thio)cysteine (**5a)** and (ii) *N*-acetyl-*S*-((1-(4,5-dimethoxy-2-nitrophenyl)ethyl)thio)cysteine (**5b**). Next, we also synthesized *N*-acetyl-*L*-

(benzylthio)cysteine (**7**) lacking the ONB group (**Scheme 1**). The compound **5a** is well known for its two-photon uncaging efficiency and the significance of developing **5b** is that it undergoes rapid and clean photolysis with high quantum efficiency, which will be suitable for the biomedical applications, compared to **5a** under one photon excitation.[26] In the present study, we choose *N*-acetyl *L*-cysteine (NAC) due to its biocompatibility and antioxidant properties to protect cells in highly oxidative environments.[25]

Scheme 1. Synthesis of persulfide donors 5a-b and 7



In order to synthesize light activated persulfide donors **5a-b**, first, compounds **1a-b** were prepared according to literature procedures.[27,29] Then, compounds **1a-b** were treated with PBr_3, followed by reaction with potassium thioacetate afforded thioacetates **2a-b**. Deprotection of acetyl group was then carried out by refluxing compounds **2a-b** in the presence of 2N HCl to give thiols **3a-b**. Next, the treatment of **3a-b** with *N*-Acetylcysteine pyridine disulfide (**NAC-pyDS, 4**) gave our target persulfide donors **5a-b**. Finally, the compound **7** was obtained by the

treatment of benzyl mercaptan (**6**) with **NAC-pyDS** (**4**). The product obtained in each step of the synthesis was characterized by ^{1}H NMR, ^{13}C NMR, and mass spectrometry (**Fig. S1–S17** in the Supporting Information (**SI**)) and we also provided the purity information of final compounds (**5a**, **5b** and **7**) by RP-HPLC (**Fig. S18–S20**).

The photophysical properties of persulfide donors **5a-b** (10 μM) were investigated in ACN/PBS buffer (3:7 v/v) (**Figure 2**). The absorption spectrum of **5a-b** showed a broad absorption band in the wavelength range of 300-430 nm.

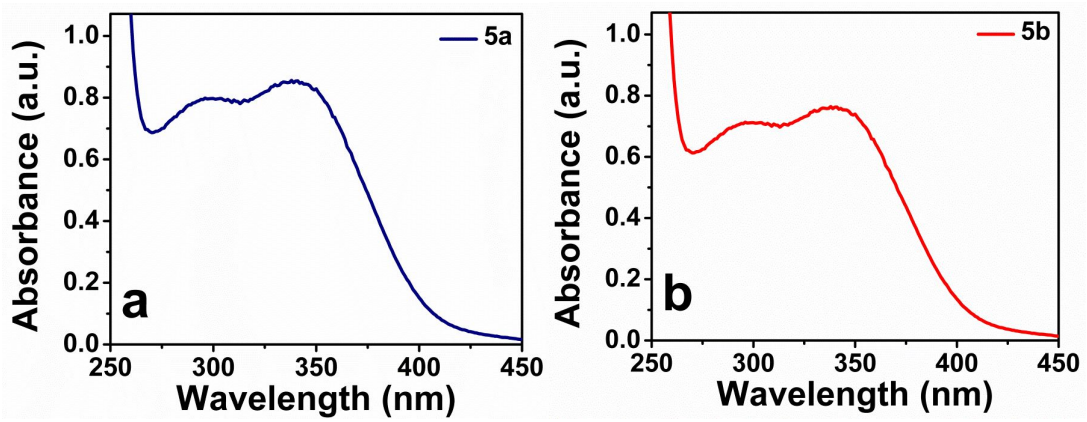


Figure 2. The absorption spectrum of (**a**) **5a** and (**b**) **5b** in ACN/PBS buffer (3:7 v/v) system.

Next, the hydrolytic stability of **5a-b** and **7** (100 μM) were tested in ACN/PBS (3:7 v/v, pH = 7.4) containing 10% fetal bovine serum and also in the presence of cellular thiols (1 mM) (e.g; NAC and GSH) separately for 10 days under dark conditions. The aliquots collected after 10 days were analyzed by HPLC and the results showed that the decomposition of the donors was less than 20% (average of three runs) (**Figure S21** in **SI**). Therefore, our designed persulfide donors are sufficiently stable and suitable for biological applications.

To analyze the photouncaging of persufide from **5a-b**, a solution of **5a** and **5b** (100 μM in ACN: PBS buffer (3:7 v/v); pH=7.4) was irradiated by one-photon excitation (1PE) at $\lambda \geq 365$

nm, individually. The progress of photodecomposition was monitored by reverse phase (RP)-HPLC (**Figure S22** in **SI**). As a proof of concept, we have represented the photolysis of **5b** by ^{1}H NMR study in CD_3OD/D_2O (1:1 v/v) solvent system (**Figure 3**). At 0 min, the ^{1}H NMR spectrum of **5b** showed two characteristic signals of aromatic protons at 7.55 ppm (doublet) and 7.19 ppm (doublet). With the gradual increase in an irradiation time, a decrease in the intensity of the signals at 7.55 and 7.19 ppm was observed, indicating the photodecomposition of **5b**. On the other hand, four new aromatic signals were appeared, corresponding to the photoproduct, 4,5-dimethoxy-2-nitrosoacetophenone (at 7.68 and 7.22 ppm) and might be an adduct, formed between photoproduct (reactive nitroso acetophenone, **13b)**[30] and released NAC-SSH (at 7.06 and 6.36 ppm). With the progress of irradiation time, a new signal at 2.12 ppm appeared with an increase in the intensity, represents the $-CH_3$ proton of free NAC-SSH or NAC (as persulfide species are not enough stable) (see full ^{1}H NMR spectra **Figure S23** in **SI**). In addition, the ^{1}H NMR spectra indicated the formation of few minor by-products, possibly due to the further decomposition of 4,5-dimethoxy-2-nitrosoacetophenone. The major photoproducts obtained (characterized by NMR study) from **5a** and **5b** were also characterized using LC-MS analysis by immediate injection of the photolysis mixture (**Figures S24-S27** in **SI**).

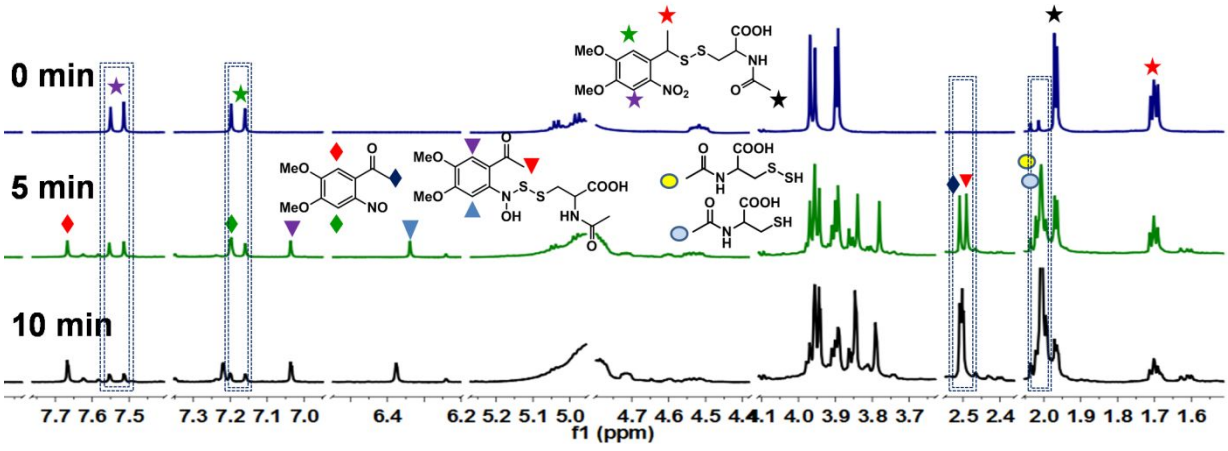


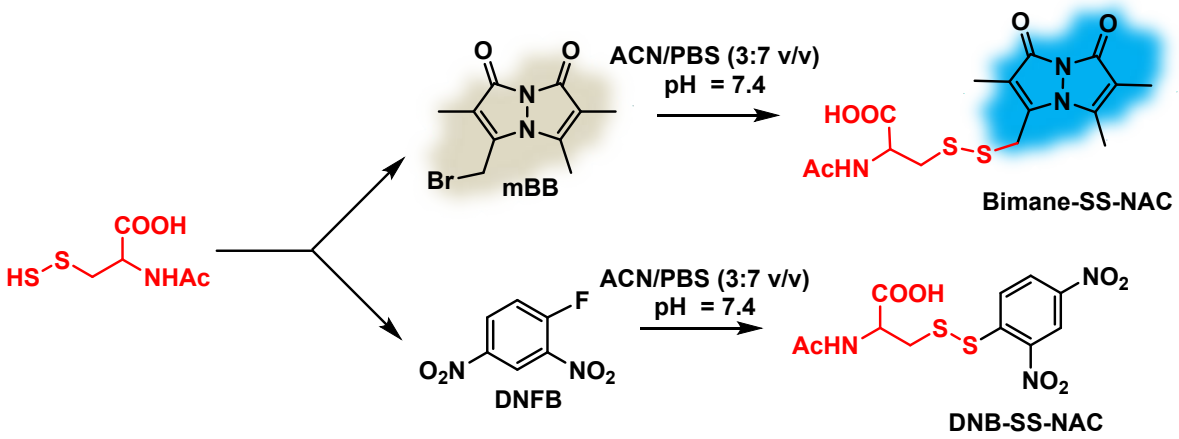
Figure 3. ^{1}H NMR study of **5b** (CD_3OD/D_2O 1:1 v/v) during photolysis (0-10 min). Irradiation wavelength of $\lambda \geq 365$ nm.

From the HPLC data, the photochemical quantum yields (Φ_p) for the decomposition of **5a** and **5b** (ACN: PBS buffer; 3:7 v/v) were calculated using potassium ferrioxalate as an actinometer[31] and they found to be 0.07 and 0.36, respectively.

To explore the two-photon uncaging ability of our persulfide donor **5a,** we have measured the two-photon uncaging cross-section (δ_u) of **5a** by comparing the photolysis rate of **5a** with that of **DMNB-OAc** (4,5-dimethoxy-2-nitrobenzyl acetate) as a reference (δ_u = 0.035 GM at 730 nm).[32] The individual solutions of **5a** and **DMNB-OAc** (100 μM in ACN/PBS buffer, 3:7 v/v, 100 μL), were irradiated by varying the time (0–30 min) with a 730 nm Ti: sapphire laser (pulse width 100 fs, 80 MHz) emitting at an average of 300 mW. The progress of photolysis was monitored by HPLC and the first-order decay constants were calculated. Then, the value of δ_u (where $\delta_u = \delta_a \times \Phi_u$) was calculated using the formula $\delta_a\Phi_u$(**5a**) = $\delta_a\Phi_u$(reference) × k_{obs}(**5a**)/k_{obs}(reference) and the two-photon uncaging cross-section for **5a** was found to be ~ 0.033 GM (See **Table S1** in **SI**).

Now, our main focus was to detect and quantify the release of NAC-SSH from **5a** and **5b**. For this purpose, we used monobromobimane (**mBB**) and 2, 4-dinitrofluorobenzene (**DNFB**) to detect the released persulfide by qualitative and quantitative fashion, respectively, as shown in **Scheme 2**.

Scheme 2. Quantitative and Qualitative Detection of released NAC-SSH



For the qualitative detection of persulfide, the solution of **5a** and **5b** (100 μM in ACN/PBS buffer (3:7 v/v); pH = 7.4) was irradiated, individually. The aliquots collected from the photolysis mixture at different time intervals were incubated with **mBB** (100 μM, ACN/PBS buffer (3:7 v/v); pH = 7.4) for 30 min.[16c,33] Then, the emission spectrum of the aliquots was recorded and we observed a gradual increase in fluorescence intensity at λ= 455 nm with an increase in irradiation time, indicating the formation of Bimane-SS-NAC (**Figure 4** and **Figure S28a** in **SI**), which was further confirmed by mass spectral (MS) analysis (**Figure S29**, **S30** in **SI**). Next, compound 7, which lacks the nitro group, was subjected to mBB experiment under light irradiation for 30 min and the results showed that the compound **7** was unable to produce Bimane-SS-NAC, indicating that the presence of nitro group is responsible for the generation of cysteine persulfide (**Figure S28b** in the **SI**). Furthermore, we also subjected the persulfide donors **5a** and **5b** to the mBB experiment against dark conditions (absence of light). However, the donors **5a** and **5b** showed no response towards mBB experiment, suggesting that the generation of cysteine persulfide is possible only under light irradiation (**Figure S28c** & **S28d** in the **SI**).

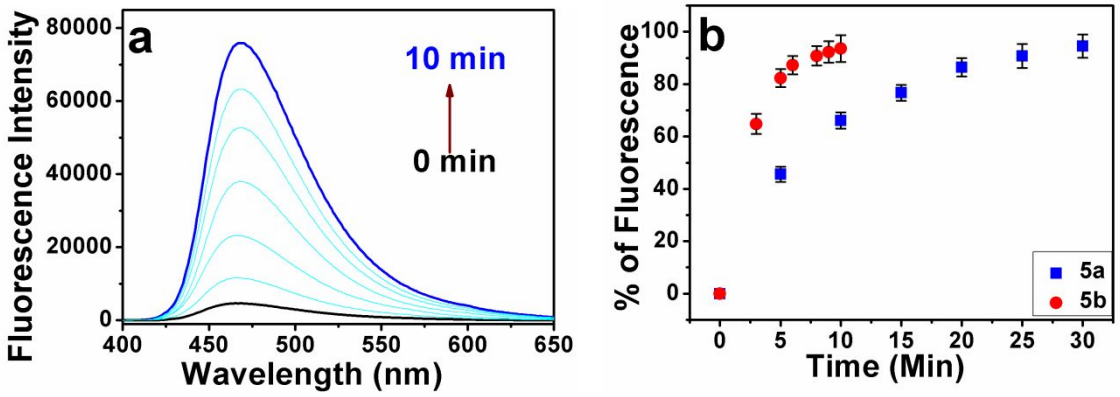
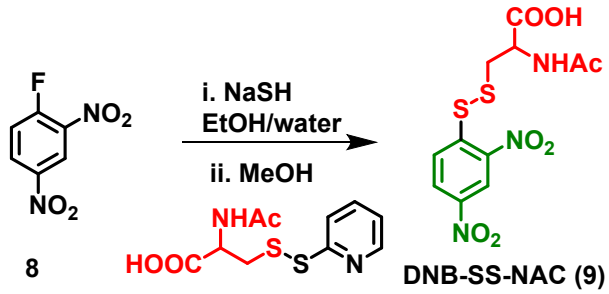


Figure 4. (**a**) Qualitative detection of persulfide release by **mBB**. Emission spectra recorded at a different interval of irradiation time (0-10 min) after incubation of photolysis mixture of **5b** (100 μM) with mBB and (**b**) % of fluorescence enhancement for **5a** (blue dots) and **5b** (red dots) by 1PE at different time intervals after incubation with mBB. Excitation wavelength: 380 nm. Values are presented as mean ± standard deviation (SD).

Next, to detect the persulfide release quantitatively, we carried out the photolysis of **5a** and **5b** (100 μM, ACN/PBS buffer (3:7 v/v) at pH= 7.4) in the presence of different concentration of **DNFB** [100 μM, 200 μM, 500 μM, 1 mM and 5 mM, ACN/PBS buffer (3:7 v/v, at pH= 7.4)] for 55 min and 20 min, respectively. The photolysis mixture was further incubated for 30 min at 37 °C and it was then analyzed by RP-HPLC to detect NAC-SSH as in the form of DNB-SS-NAC (**Figure S32** in **SI**). To quantify the amount of persulfide release, the modal compound **DNB-SS-NAC** (**9**) was synthesized by reacting DNFB with NaSH, followed by the treatment of **NAC-pyDS** (**4**) (**Scheme 3**, **Figure S31** in **SI**).

Scheme 3: Synthetic Scheme of modal compound DNB-SS-NAC.



From the HPLC data, the generation of DNB-SS-NAC was quantified as 57 ± 3 μM and 59 ± 4 μM from **5a** and **5b**, respectively at 5 mM DNFB (**Figure 5** and **Figure S33**). The formation of **DNB-SS-NAC** after photolysis was further confirmed by mass spectroscopy (**Figure S34** in **SI**). To confirm the persulfide release was due to photocleavage of the ONB protecting group, control experiments were performed using compound **7**. As shown in **Figure 5** & **S33c**, the irradiation (λ ≥ 365 nm) of compound **7** (100 μM) in ACN/PBS buffer (3:7 v/v, at pH= 7.4) solution in the presence of DNFB was incapable to generate DNB-SS-NAC. Henceforth, the compound **7**, which lacks the ONB group does not release persulfide upon photolysis, confirmed that the carbon-disulfide linkage is not photodegraded under light irradiation.

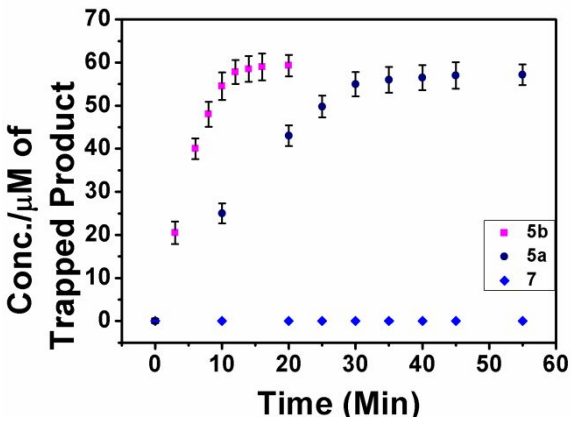


Figure 5. Time-dependent quantification of persulfide release from **5a** and **5b (**100 μM each**)** was determined in the form of DNB-SS-NAC. The DNFB concentration is 5 mM. Values are presented as mean ± SD.

Furthermore, to show the precise control over the persulfide release from **5a** and **5b** (100 μM) by external stimuli light, we monitored the formation of **DNB-SS-NAC** (in presence of 100 μM DNFB) under light and dark conditions periodically, respectively. The HPLC data clearly indicated that only external stimulus light induces the release of persulfide (**Figure 6**).

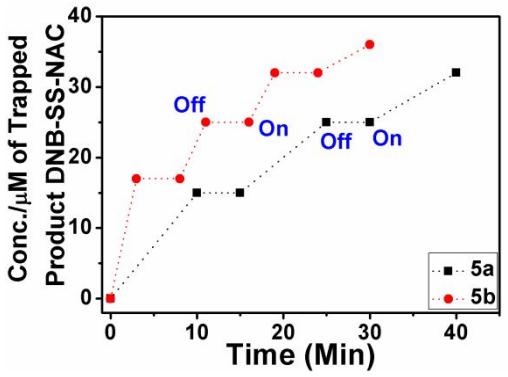


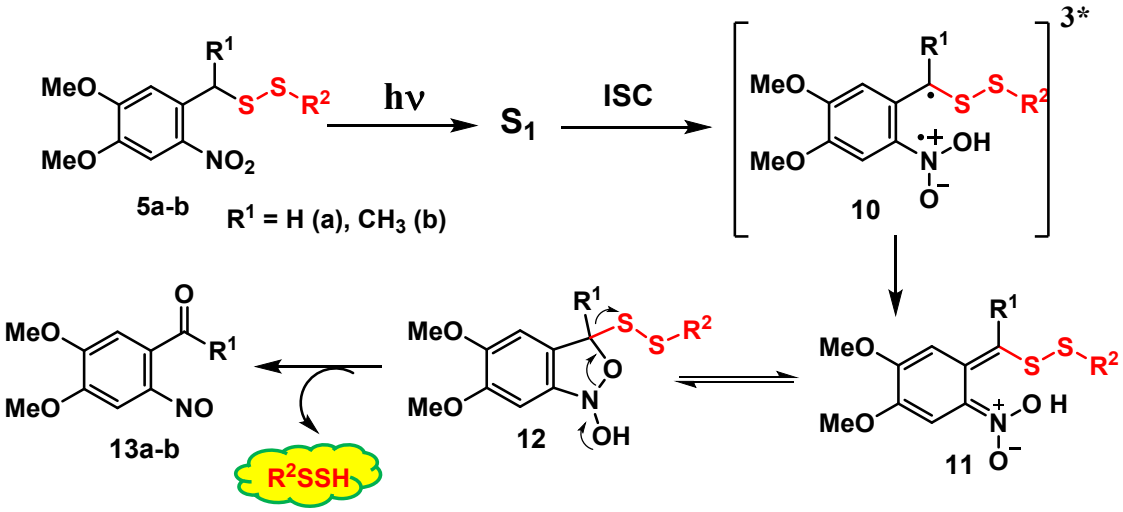
Figure 6: Progress of the formation of DNB-SS-NAC from persulfide donors **5a** and **5b** under light and dark conditions (ON indicates the start of light irradiation and OFF indicates the end of light irradiation).

Based on the literature,[26] we proposed a possible photorelease mechanism for NAC-SSH from **5a-b** (**Scheme 4**) and it is known to proceeds through the Norrish-type II reaction pathway. After absorption of a photon by *o*-nitro benzyl group, it gets excited to singlet state (S_1) and then undergoes rapid intersystem crossing (ISC) to its triplet excited state. In the triplet excited state, the oxygen atom of the nitro group abstracts a proton from the methylene carbon in the γ-H position, resulting in intramolecular electron redistribution and formation of the *aci*-nitro

tautomer **11**. The *aci*-nitro intermediate then cyclises to form benzisoxazoline intermediate **12**. Thereafter, subsequent decomposition of the resonance stabilized five-membered ring **12** rapidly generates NAC-SSH and photoproducts (**13a-b**).

It has been found from the literature studies that in the case of the ONB-type uncaging process, the released photoproduct **13** is known to undergo a "self-interaction" with the released thiol-type species and form a substantial amount of the adduct. This type of undesirable product formed between **13** and released NAC-SSH can reduce the yield of released persulfide, but not the rate of release [30c]

Scheme 4. Proposed photorelease mechanism for persulfide from ONB phototrigger



Further, to examine the rate and efficiency of persulfide release under biological environment, we carried out the photolysis experiment in PBS buffer solution containing 5% DMSO and no significant difference in persulfide release profile was observed compared to ACN/PBS buffer (3:7 v/v) solution (**Table S2**).

To investigate the cytotoxicity of persulfide donors **5a, 5b** and compound **7,** we carried out the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay using two set of experiments: i) persulfide donors incubated in HeLa cells without irradiation; ii) persulfide donors incubated in HeLa cells and subjected to the irradiation (**Figure 7**). From the MTT assay, we have found that there is no evidence for inhibition of cell proliferation by **5a**, **5b** and **7** up to a concentration of 200 μM before and after photolysis. Therefore, *in vitro* cytotoxicity studies showed that persulfide donors **5a-b** did not generate substantial toxicity at concentrations up to 200 uM under the studied experimental conditions.

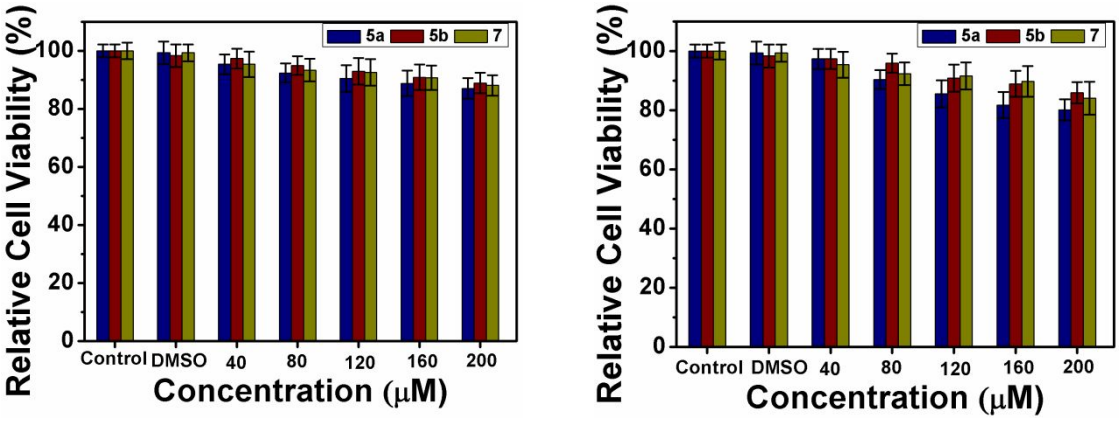


Figure 7: Cell viability assay of **5a**, **5b** and **7** on the HeLa cell line: (**a**) before and (**b**) after photolysis. Values are presented as mean ± SD from three independent experiments with triplicates per experiment.

The persulfide was found to be persuasive to exhibit the cytoprotective effects in a highly oxidative cellular environment. In this regard, we used persulfide donors **5a-b** to rescue the cells from the highly oxidizing cellular environment. First, we treated the HeLa cells with 200 μM of H_2O_2 and found that cell viability was reduced by ~ 50% compared to the cell line without H_2O_2 treatment. Next, the cells were incubated with persulfide donors **5a-b** (200 μM) in the presence

of 200 μM of H_2O_2 and then photolysis was carried out. From the MTT assay, we observed that the cell viability increased with increasing irradiation time and ~ 68% and ~ 71% cell viability was found for **5a** and **5b** after photolysis, respectively (**Figure 8**). Thus, we envisioned that the persulfide donors **5a** and **5b** are effective in rescuing cells under oxidative stress.[20] In addition, HeLa cells were also treated with different thiols for e.g; GSH, NAC, HCys, and Na_2S prior to the addition of H_2O_2 (**Figure S35**). The cell viability assay demonstrated that NAC-SSH was comparatively effective in defending cells from the highly oxidative environment than the studied thiols and H_2S. On the other hand, we also carried out the cell viability assay of compound **7** under the above experimental conditions, and the results showed that compound **7** was incapable to defend cells from the oxidising environment because compound **7** (lacks the nitro group**)** does not release NAC-SSH under light irradiation (see **Figure S36** in the **SI**).

Additionally, to validate our results, a DCFDA staining experiment was also performed using HeLa cells to realize intracellular ROS generation (oxidative stress) by H_2O_2 and reduction of ROS by NAC-SSH. Initially, DCFDA is nonfluorescent in nature. Addition of H_2O_2 to the HeLa cells in the presence of DCFDA produces an intense green color fluorescence, which can be attributed to the formation dichloro fluorescein (DCF) due to the ROS generation. On the other hand, irradiation of the persulfide donor **5b** in the presence of H_2O_2 for 10 min and subsequent addition of DCFDA resulted in relatively less intense green color fluorescence, clearly indicating that the cysteine persulfide, released from donor **5b** has the cytoprotective effects in the highly oxidative environment (**Figure S37** in the **SI**).

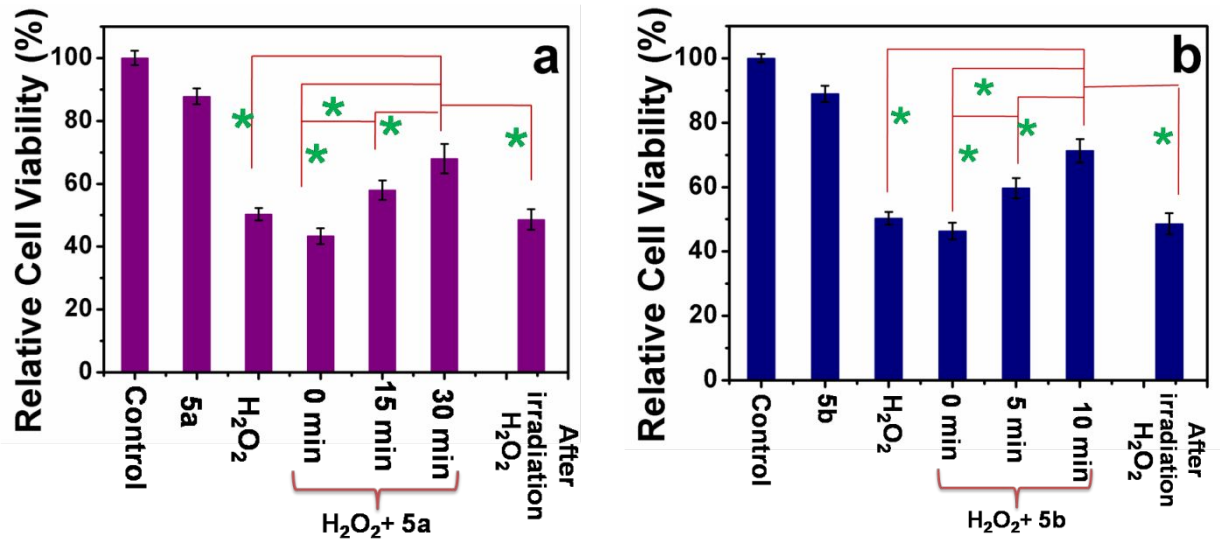


Figure 8. Cell viability assay of (**a**) **5a** and (**b**) **5b** (200 μM) before and after irradiation in presence of H_2O_2 (200 μM; to generate highly oxidative environment on HeLa cell line). Cell viability was evaluated by MTT assay. Data represent the mean (± standard deviation, SD) of three independent experiments; each performed in triplicates, and is presented relative to control. **Error bars** indicate SDs.* significant differences between groups at the level of $p<0.05$.

In summary, we have synthesized photoactivated persulfide donors **5a** and **5b**, which release persulfide NAC-SSH spatiotemporally under one- and two-photon excitation. Next, the photochemical properties of the persulfide donors under physiological conditions were followed by RP-HPLC, ^{1}H NMR, and MS analysis. Furthermore, we detected the persulfide release both qualitatively and quantitatively using the well-known "turn on" fluorescence probe i.e., monobromobimane and the trapping agent 2,4-dinitrofluorobenzene, respectively. Finally, we demonstrated the cytotoxic effect and the cytoprotective ability of persulfide donors in the highly oxidative cellular environment within the HeLa cells. We intend that the current light-activated

persulfide donors will help to understand the persulfide biology to a greater extent by providing insight into sulfur redox cycles and sulfur-mediated cell signaling.

EXPERIMENTAL SECTION:

1. General Information: All commercially available anhydrous solvents dimethylformamide (DMF), dichloromethane (DCM), petroleum ether (PE) and ethyl acetate (EtOAc) and other chemicals were used without further purification. Acetonitrile and dichloromethane were distilled from CaH_2 before use. NMR spectra were recorded on a 600 and 400 MHz instrument. ^1H NMR chemical shifts were referenced to the tetramethylsilane signal (0 ppm), ^{13}C NMR chemical shifts were referenced to the solvent resonance (77.23 ppm, Chloroform-d ($CDCl_3$) and 39.97 ppm, DMSO-d_6). Chemical shifts (δ) are reported in ppm, and spin-spin coupling constants (J) are given in Hz. The following abbreviations were used to explain multiplicities: s = singlet, d = doublet, t = triplet, q = quadruplet, m = multiplet. UV/vis absorption spectra were recorded on a Shimadzu UV-2450 UV/vis spectrophotometer and fluorescence spectra were recorded on a Hitachi F-7000 fluorescence spectrophotometer. High-resolution mass spectra (HRMS) were recorded on ESI-TOF (electrospray ionization-time-of-flight). Photolysis was carried out using a 125 W medium pressure mercury lamp. RP-HPLC was taken using mobile phase acetonitrile/water (1:1), at a flow rate of 1 mL /min (detection: UV 310 nm). Chromatographic purification was done with 60–120 mesh silica gel. For reaction monitoring, precoated silica gel 60 F254 TLC sheets were used. HRMS spectra were recorded by electron spray ionization (ESI) method on a Q-TOF Micro with lock spray source (High Resolution Q-Tof Mass).

EXPERIMENTAL PROCEDURE AND SPECTROSCOPIC DATA:

(4,5-dimethoxy-2-nitrophenyl)methanol (1a)[27]: Light yellow solid (0.28 g, 93%). ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.71 (s, 1H), 7.18 (s, 1H), 4.96 (d, *J* = 5.3 Hz, 2H), 4.01 (s, 3H), 3.96 (s, 3H), 2.63 (t, *J* = 6.0 Hz, 1H). ^{13}C {^{1}H} NMR (151 MHz, $CDCl_3$) δ 153.9, 147.9, 139.7, 132.3, 111.0, 108.1, 62.8, 56.5 (purity > 98% from HPLC).

1-(4,5-dimethoxy-2-nitrophenyl)ethan-1-ol (1b) [29]: Yellow solid (0.23 g, 94%). ^{1}H NMR (600 MHz, Chloroform-*d*) δ 7.58 (d, *J* = 2.1 Hz, 1H), 7.33 – 7.31 (m, 1H), 5.58 (qd, *J* = 6.2, 1.9 Hz, 1H), 4.02 (d, *J* = 1.6 Hz, 3H), 3.96 (d, *J* = 1.6 Hz, 3H), 1.57 (dd, *J* = 6.3, 2.0 Hz, 3H). ^{13}C {^{1}H} NMR (151 MHz, $CDCl_3$) δ 153.7, 147.7, 139.6, 136.8, 108.5, 107.6, 65.7, 56.4, 24.3 (purity > 98% from HPLC).

S-4,5-dimethoxy-2-nitrobenzyl ethanethioate (2a): PBr_3 (1.39 g, 0.48 mL 5.15 mmol) in DCM was added dropwise to the solution of compound **1a** (1 g, 4.7 mmol) in DCM (30 mL) under an N_2 atmosphere with external cooling. After stirring 1 h at room temperature the reaction mixture was poured into H_2O (50 mL) and was neutralized with a 2 N aqueous NaOH solution (100 mL). The organic phase was then separated, and the aqueous phase was rinsed twice with DCM (50 mL). The solution was then dried over Na_2SO_4 and the solvent was removed under vacuum. The residue was purified over silica gel column (10% EtOAc: hexane) (1.03 g, 80%) as a yellow solid. To the solution of brominated compound (1 g, 3.62 mmol) in ACN (40 mL), was added potassium thioacetate (0.496 g, 4.34 mmol) and the reaction mixture was stirred for 3 h. White solid formed during the reaction was filtered away and the filtrate was concentrated by removing the ACN under reduced pressure. The residue was purified over silica gel column (10 % EtOAc: hexane) to afford compound **2a** (0.90 g, 92%) as a yellow solid. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.69 (s, 1H), 7.09 (s, 1H), 4.44 (s,

2H), 4.01 (s, 3H), 3.96 (s, 3H), 2.35 (s, 2H). $^{13}C\{^{1}H\}$ NMR (150 MHz, $CDCl_3$) δ 195.9, 153.3, 148.2, 140.1, 128.9, 114.0, 108.24, 56.5, 56.4, 31.8, 30.2. HRMS (ESI^+) calcd for $C_{11}H_{13}NO_5S$ $[M + H]^+$, 272.0587; found: 272.0594.

S-(1-(4,5-dimethoxy-2-nitrophenyl)ethyl) ethanethioate (2b): PBr_3 (1.31 g, 0.45 mL, 5.15 mmol) in DCM was added dropwise to the solution of compound **1b** (1 g, 4.4 mmol) in DCM (30 mL) under a N_2 atmosphere with external cooling. After stirring 1 h at room temperature the reaction mixture was poured into H_2O (50 mL) and was neutralized with a 2 N aqueous NaOH solution (100 mL). The organic phase was then separated, and the aqueous phase was rinsed twice with DCM (50 mL). The solution was then dried over Na_2SO_4 and the solvent was removed under vacuum. The residue was purified over silica gel column (5% EtOAc: hexane) to afford compound (0.99 g, 78%) as a dirty yellow solid. To this solution of brominated compound (0.9 g, 3.44 mmol) in ACN (40 mL), was added potassium thioacetate (0.43 g, 4.12 mmol) and the reaction mixture was stirred for 3 h. White solid formed during the reaction was filtered away and the filtrate was concentrated by removing the ACN under reduced pressure. The residue was purified over silica gel column (10 % EtOAc: hexane) to afford compound **3b** (0.81 g, 92%) as a yellow solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.46 (s, 1H), 6.95 (s, 1H), 5.35 (q, J= 6.8 Hz, 1H), 3.96 (s, 3H), 3.91 (s, 3H), 2.25 (s, 2H), 1.68 (d, J= 8 Hz, 3H). $^{13}C\{^{1}H\}$ NMR (101 MHz, $CDCl_3$) δ 194.4, 152.9, 147.7, 141.3, 132.7, 110.8, 107.62, 56.4, 56.3, 38.8, 30.3, 21.7. HRMS (ESI^+) calcd for $C_{12}H_{19}N_2O_5S$ $[M + NH_4]^+$, 303.1009; found:303.1009.

(4,5-dimethoxy-2-nitrophenyl)methanethiol (3a): The methanolic solution of compound **3a** (0.9 g, 3.32 mmol) was refluxed in presence of 2 N HCl. After 3 h the

solvent was evaporated under reduced pressure and neutralized by $NaHCO_3$. Then the aqueous layer was extracted by EtOAc (3 × 15 mL). The combined organic layer was then dried over Na_2SO_4 and the solvent was removed under vacuum. The residue was purified over silica gel column (10% EtOAc: hexane) to afford compound **5a** as a yellow solid (0.67 g, 88%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.65 (s, 1H), 6.86 (s, 1H), 4.02 (d, J = 8.5 Hz, 2H), 3.99 (s, 3H), 3.94 (s, 3H), 2.21 (t, J = 8.4 Hz, 1H). ^{13}C{^{1}H} NMR (101 MHz, $CDCl_3$) δ 153.5, 148.1, 140.1, 131.9, 112.8, 108.6, 56.4, 26.9. HRMS (ESI$^+$) calcd for $C_9H_{15}N_2O_4S$ $[M + NH_4]^+$, 247.0747; found: 247.0741.

1-(4,5-dimethoxy-2-nitrophenyl)ethanethiol (3b): The methanolic solution of compound **3b** (0.8 g, 2.80 mmol) was refluxed in presence of 2 N HCl. After 3 h the solvent was evaporated under reduced pressure and neutralized by $NaHCO_3$. Then the aqueous layer was extracted by EtOAc (3 × 15 mL). The combined organic layer was then dried over Na_2SO_4 and the solvent was removed under vacuum. The residue was purified over silica gel column (10% EtOAc: hexane) to afford compound **5b** as a yellow solid (0.60 g, 88%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.43 (s, 1H), 7.18 (s, 1H), 5.02 (p, J = 6.4 Hz, 1H), 3.98 (s, 3H), 3.91 (s, 3H), 2.23 (d, J = 5.1 Hz, 1H), 1.66 (d, J = 6.9 Hz, 3H). ^{13}C{^{1}H} NMR (101 MHz, $CDCl_3$) δ 153.3, 147.6, 140.1, 135.7, 109.5, 107.5, 56.4, 32.9, 25.4. HRMS (ESI$^+$) calcd for $C_{10}H_{17}N_2O_4S$ $[M + NH_4]^+$, 261.0904; found: 261.0902.

2-acetamido-3-(pyridin-2-yldisulfanyl)propanoic acid (4): According to a previously reported procedure,[25] a round bottom flask was charged with *N*-acetyl-*L*-cysteine (2.0 g, 12.3 mmol), H_2O (17 mL), and a stirbar to give a clear solution. A solution of 2,2'-dipyridyl disulfide (5.40 g, 24.5 mmol) in MeOH (17 mL) was added in one portion resulting in a clear, yellow solution. The

reaction mixture was stirred at rt (16 h). Reaction progress was monitored by TLC (EtOAc), showing complete consumption of starting material. The resulting yellow solution was concentrated via rotary evaporation. The crude product, obtained as a yellow solid, was then purified by silica gel chromatography eluting with 5 % to 15 % MeOH in DCM, yielding a light yellow powder (2.30 g, 69% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.41 (dd, J = 22.5, 5.9 Hz, 2H), 7.83 – 7.72 (m, 2H), 7.26 – 7.21 (m, 1H), 4.46 (d, J = 8.7 Hz, 1H), 3.19 (dd, J = 13.7, 3.9 Hz, 1H), 3.06 (dd, J = 13.5, 9.1 Hz, 1H), 1.84 (s, 3H). ^{13}C{^{1}H} NMR (101 MHz, DMSO) δ 172.3, 169.9, 159.2, 150.1, 138.27, 121.7, 119.8, 51.8, 22.8. NMR spectral data are in accordance with those previously reported.[25]

3-(2-(4,5-dimethoxy-2-nitrobenzyl)disulfanyl)-2-acetamidopropanoic acid (5a): To a solution of compound **4a** (0.2 g, 0.87 mmol) in methanol **NAC-pyDS (4)** (0.213 g, 0.78 mmol) was added. The reaction mixture was stirred for 2 h at rt, monitoring reaction progress with TLC (50:50; hexane: EtOAc). Once complete, the solvent of the reaction mixture was evaporated in rotary evaporator to get a crude yellow solid which was further purified by silica gel chromatography (5% methanol in DCM), yielding a yellow solid (0.26 g, 78 % yield) (purity > 99% from HPLC chromatogram). ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.72 (s, 1H), 6.90 (s, 1H), 6.66 (s, 1H), 4.78 – 4.72 (m, 1H), 4.34 – 4.27 (m, 2H), 4.02 (s, 3H), 3.98 (s, 3H), 3.07 (d, J = 13.6 Hz, 1H), 2.95 (d, J = 13.4 Hz, 1H), 2.08 (d, J = 8.8 Hz, 3H). ^{13}C{^{1}H} NMR (151 MHz, $CDCl_3$) δ 172.9, 171.3, 153.2, 148.4, 140.1, 128.0, 113.9, 108.7, 56.7, 56.4, 52.3, 41.8, 39.8, 22.9. HRMS (ESI^{+}) calcd for $C_{14}H_{19}N_2O_7S_2$ $[M+H]^{+}$, 391.0628, found 391.0628.

2-acetamido-3-(2-(1-(4,5-dimethoxy-2-nitrophenyl)ethyl)disulfanyl)propanoic acid (5b): To a solution of compound **4a** (0.2 g, 0.82 mmol) in methanol **NAC-pyDS (4)** (0.201 g,

0.73 mmol) was added. The reaction mixture was stirred for 2 h at rt, monitoring reaction progress with TLC (50:50; hexane: EtOAc). Once complete, the solvent of the reaction mixture was evaporated in rotary evaporator to get a crude yellow solid which was further purified by silica gel chromatography (5% methanol in DCM), yielding regioselectively diastereomeric pair (1:1) (yellow solid, 0.25 g, 78 % yield) (purity > 99% as diastereomeric pair from HPLC chromatogram). ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.52 (d, J = 2.2 Hz, 1H), 7.06 (d, J = 6.6 Hz, 1H), 6.51 – 6.43 (m, 1H), 5.14 – 5.08 (m, 1H), 4.75 – 4.70 (m, 1H), 4.01 (d, J = 5.1 Hz, 3H), 3.97 (s, 3H), 3.04 – 2.96 (m, 2H), 2.84 (dd, J = 14.0, 6.3 Hz, 1H), 2.08 (dt, J = 6.1, 3.0 Hz, 3H), 1.71 (d, J = 6.9 Hz, 3H). ^{13}C{^{1}H} NMR (151 MHz, $CDCl_3$) δ 171.9, 171.2, 153.2, 153.0, 148.5, 148.3, 140.2, 140.1, 128.29, 127.8, 114.0, 113.9, 108.7, 108.5, 56.7, 56.6, 56.4, 56.3, 52.1, 42.1, 41.8, 39.5, 23.0. HRMS (ESI$^+$) calcd for $C_{15}H_{21}N_2O_7S_2$ $[M+H]^+$ 405.0785, found 405.0775.

***N*-acetyl-S-(benzylthio)cysteine (7):** To a solution of benzyl marcaptan (0.1 g, 0.80 mmol) in methanol **NAC-pyDS (4)** (0.196 g, 0.72 mmol) was added . The reaction mixture was stirred for 2 h at rt, monitoring reaction progress with TLC (50:50; hexane: EtOAc). Once complete, the solvent of the reaction mixture was evaporated in rotary evaporator to get a grey solid which was further purified by silica gel chromatography (2% methanol in DCM), yielding a white solid, 0.25 g, 78 % yield) (purity > 99% from HPLC chromatogram). ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.89 (s, 1H), 8.29 (d, J = 8.0 Hz, 1H), 7.30–7.32 (m, 4H), 7.26 (m, 1H), 4.46 (dt, J = 8.1, 4.5 Hz, 1H), 3.99 – 3.91 (m, 2H), 2.97 – 2.89 (m, 1H), 2.75 (dd, J = 13.6, 9.3 Hz, 1H), 1.84 (s, 3H). ^{13}C{^{1}H} NMR (101 MHz, $CDCl_3$) δ 172.5, 169.8, 137.8, 129.7, 128.9, 127.7, 51.6, 42.4, 40.1, 22.9. HRMS (ESI$^+$) calcd for $C_{12}H_{16}NO_3S_2$ $[M+H]^+$ 286.0572, found 286.0574.

***N*-acetyl-S-((2,4-dinitrophenyl)thio)cysteine (DNB-SS-NAC, 9):** NaSH (0.15g, 2.68 mmol) in water was added dropwise to an ethanolic solution of 2,4 dinitrofluorobenzene (0.5g, 2.68 mmol). The resulting solution was stirred 1 h at room temperature. After 1 h, the ethanol was evaporated in the rotary evaporator and acidified with 1 N HCl to make the pH of the solution to 6. The aqueous fraction was extracted with EtOAc (3 × 30 mL). The organic layers were dried over Na_2SO_4, evaporated and purified to get the pure compound (Yield: 0.318g, 60%). To the resulting yellow solid compound (0.1 g, 0.5 mmol) the methanolic solution of linker **4** (0.12 g, 0.44 mmol) was added and heated to 60°C for 1 h. After 1 h, the methanol was evaporated and purified by column chromatography to get the yellow solid (Yield: 0.16 g, 90%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.88 (s, 1H), 8.57 (d, J = 8.8 Hz, 1H), 8.49 (d, J = 8.9 Hz, 1H), 8.27 (d, J = 6.9 Hz, 1H), 4.41 – 4.35 (m, 1H), 3.22 (s, 1H), 3.14 (s, 1H), 1.84 (s, 3H). ^{13}C{^{1}H} NMR (101 MHz) δ 171.9, 169.9, 145.8, 145.2, 145.0, 129.4, 128.6, 121.9, 52.2, 40.1, 22.9. HRMS (ESI$^+$) calcd for $C_{11}H_{12}N_3O_7S_2$ $[M+H]^+$ 362.0117, found 362.0111.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: NMR and HRMS spectra of all synthesized compounds, stability studied of donors, All experimental details of photolysis, qualitative and quantitative analysis, Related emission, HPLC and LC-MS spectra, Details information of biological studies, Cell viability assay (PDF)

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