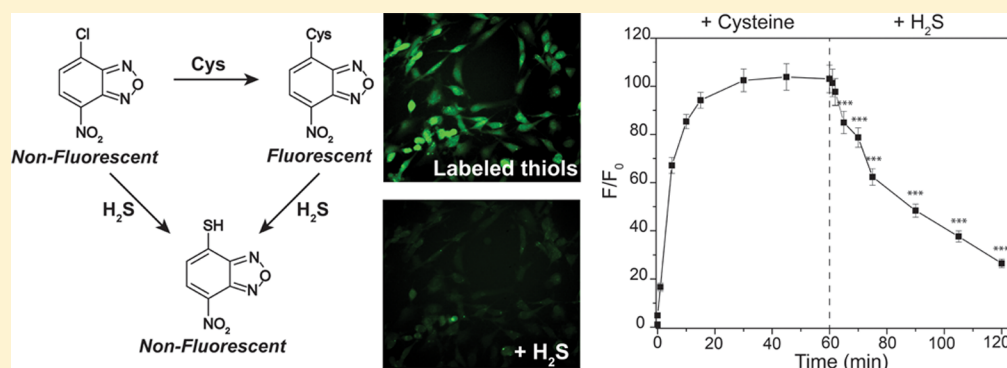


# Hydrogen Sulfide Deactivates Common Nitrobenzofurazan-Based Fluorescent Thiol Labeling Reagents

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## S Supporting Information



**ABSTRACT:** Sulphydryl-containing compounds, including thiols and hydrogen sulfide (H<sub>2</sub>S), play important but differential roles in biological structure and function. One major challenge in separating the biological roles of thiols and H<sub>2</sub>S is developing tools to effectively separate the reactivity of these sulphydryl-containing compounds. To address this challenge, we report the differential responses of common electrophilic fluorescent thiol labeling reagents, including nitrobenzofurazan-based scaffolds, maleimides, alkylating agents, and electrophilic aldehydes, toward cysteine and H<sub>2</sub>S. Although H<sub>2</sub>S reacted with all of the investigated scaffolds, the photophysical response to each scaffold was significantly different. Maleimide-based, alkylating, and aldehydic thiol labeling reagents provided a diminished fluorescence response when treated with H<sub>2</sub>S. By contrast, nitrobenzofurazan-based labeling reagents were deactivated by H<sub>2</sub>S addition. Furthermore, the addition of H<sub>2</sub>S to thiol-activated nitrobenzofurazan-based reagents reduced the fluorescence signal, thus establishing the incompatibility of nitrobenzofurazan-based thiol labeling reagents in the presence of H<sub>2</sub>S. Taken together, these studies highlight the differential reactivity of thiols and H<sub>2</sub>S toward common thiol-labeling reagents and suggest that sufficient care must be taken when labeling or measuring thiols in cellular environments that produce H<sub>2</sub>S due to the potential for both false-positive and eroded responses.

Sulphydryl-containing compounds, such as cysteine (Cys), homocysteine (Hcy), glutathione (GSH), and hydrogen sulfide (H<sub>2</sub>S) are potent nucleophiles and play diverse and important roles in biological structure and function. As the only sulphydryl-containing canonical  $\alpha$ -amino acid, Cys imparts significant effects on protein structure through its reversible oxidation to form disulfide bonds with other cysteine residues.<sup>1</sup> Similarly, Cys can function as a powerful nucleophile in the active sites of enzymes operating under nucleophilic catalysis.<sup>2</sup> Homocysteine, the methylene homologue of Cys, also plays important biological roles and is a key sulphydryl-containing intermediate generated during the enzymatic generation of Cys from methionine.<sup>3</sup> Misregulation of Hcy is implicated in various cardiovascular diseases and neuropsychiatric conditions, and the elevated Hcy levels found in hyperhomocysteinemia have been implicated in stroke, pre-eclampsia, and Alzheimer's disease.<sup>4–8</sup> By comparison to the low micromolar concentrations of free Cys and Hcy, GSH is present in much higher cellular concentrations (1–10 mM) and is the most abundant source of nonprotein sulfur.<sup>9–12</sup> Glutathione plays key roles in

maintaining cellular redox homeostasis and provides protection against oxidative stress through its reversible oxidation to glutathione disulfide (GSSG).

Although much less understood than other sulphydryl-containing compounds, H<sub>2</sub>S has emerged as an important biological mediator and is implicated to play important roles in the cardiovascular, neuronal, endocrine, and immune systems.<sup>13–16</sup> As the smallest sulphydryl-containing molecule, H<sub>2</sub>S is now accepted as a gaseous signaling molecule, joining nitric oxide and carbon monoxide as a cellular gasotransmitter.<sup>17,18</sup> The majority of enzymatic H<sub>2</sub>S biosynthesis derives from metabolism of sulfur-containing substrates, such as Cys and Hcy, by cystathionine- $\beta$ -synthase (CBS) and cystathionine- $\gamma$ -lyase (CSE), as well as cysteine aminotransferase (CAT) working in concert with 3-mercaptopyruvate sulfurtransferase

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(3-MST).<sup>13</sup> In aqueous solution, H<sub>2</sub>S is significantly more acidic (pK<sub>a1</sub> 6.9) than Cys (8.10), Hcy (8.25), or GSH (8.72), which is manifested in that ~80% of H<sub>2</sub>S exists as HS<sup>−</sup> under physiological conditions. Additionally, the small size and variable protonation state of H<sub>2</sub>S, HS<sup>−</sup>, and S<sup>2−</sup> allows for modulation of lipid and water solubility. Taken together, these physiochemical differences make H<sub>2</sub>S a significantly more potent nucleophile than other biologically relevant sulfhydryl-containing compounds. Additionally, owing to its diprotic nature, H<sub>2</sub>S can participate in two sequential nucleophilic attacks, by comparison to the single nucleophilic addition possible for thiols.

Concomitant with the emerging and multifaceted biological roles of sulfhydryl-containing species, researchers have developed chemical tools for selectively labeling and detecting thiols and H<sub>2</sub>S. Most chemical methods for thiol labeling rely on the high nucleophilicity of thiols to covalently attach electrophilic labeling reagents. Using this nucleophilic attack strategy, researchers have developed a diverse palette of tools for thiol detection, quantification, and labeling protein Cys residues, thus greatly enabling investigation into processes associated with thiol biochemistry and redox homeostasis.<sup>19–22</sup> By contrast, development of chemical tools for H<sub>2</sub>S detection remains in early stages of development, with chemical tools emerging only in the past few years.<sup>19,23–25</sup> Such chemical tools have included fluorescent,<sup>26–40</sup> visible,<sup>41</sup> and chemiluminescent<sup>42</sup> methods for H<sub>2</sub>S visualization and quantification. Of such chemical tools, three primary strategies have emerged, including H<sub>2</sub>S-mediated reduction,<sup>26–34</sup> nucleophilic attack,<sup>35–39,43,44</sup> and precipitation of transition metals,<sup>40</sup> although the comparative efficacy of each approach under different physiological conditions remains to be determined.

Although most recently developed chemical tools for H<sub>2</sub>S detection have been tested with thiols and afford moderate to good selectivity for H<sub>2</sub>S over thiols, commonly used chemical tools for thiol detection have not been evaluated with H<sub>2</sub>S. Because thiols and H<sub>2</sub>S share similar reactivity profiles, electrophilic agents used to label thiols are expected to react with H<sub>2</sub>S, although the resultant responses remain unknown. Although biological thiol concentrations are typically higher than cellular H<sub>2</sub>S levels (high nM to low μM), the constant enzymatic production of H<sub>2</sub>S provides a continuous source of highly nucleophilic sulfide.<sup>45,46</sup> Evaluation of the reactivity profiles, as well as the photophysical properties of the resultant products of commonly used thiol labeling agents with thiols and H<sub>2</sub>S, is an important step toward separating the similar reactivities of these important sulfhydryl-containing biomolecules. Furthermore, these studies will provide insight into the potential cross-reactivity of different labeling reagents and potential false-positive response leading to experimental ambiguity. To investigate and address this potential cross reactivity, we report here the differences between thiol and H<sub>2</sub>S reactivity with common electrophilic tools for fluorescent detection and labeling of thiols and highlight the differential responses of different classes of electrophilic reagents toward thiols and H<sub>2</sub>S.

## ■ EXPERIMENTAL SECTION

**Materials and Methods.** *General.* NMR spectra were acquired on either a Varian INOVA 500 or Bruker 600 MHz spectrometer at 25 °C. Deuterated solvents were purchased from Cambridge Isotope Laboratories. Fluorescence spectra were obtained on a Photon Technology International (PTI)

Quanta Master 40 spectrofluorimeter equipped with a Quantum Northwest TLC-50 temperature controller. All cuvette-based spectroscopic measurements were made under anaerobic conditions, with solutions prepared under an inert atmosphere in 1.0 cm path length septum-sealed cuvettes obtained from Starna Scientific. Differential interference contrast (DIC) and fluorescent images were obtained using a Nikon Eclipse Ti Inverted Microscope equipped with a 20× APO ELWD objective using the NIS-Elements acquisition software. Cells used in live imaging experiments were incubated at 37 °C under 5% CO<sub>2</sub> during imaging. All fluorescent images were corrected by applying identical intensity cutoffs to exclude background noise. Fluorescent data were analyzed using ImageJ software.<sup>47</sup> All statistical comparisons were performed using Prism.<sup>48</sup>

**Spectroscopic Materials and Methods.** Piperazine-*N,N'*-bis(2-ethanesulfonic acid) (PIPES, Aldrich) and KCl (99.999%, Aldrich) were used to prepare buffered solutions (50 mM PIPES, 100 mM KCl, pH 7.0) with Millipore water. Buffered solutions were deoxygenated by vigorous sparging with nitrogen for at least 2 h. Samples for all spectroscopic measurements were prepared in an Innovative Technology N<sub>2</sub>-filled glovebox with O<sub>2</sub> levels less than 1.0 ppm. Anhydrous sodium hydrogen sulfide (NaSH) was purchased from Strem Chemicals and handled under nitrogen. Thiol labeling reagents **1** and **2** were obtained from TCI, **4** from Sigma-Aldrich, **5** from Echelon Biosciences, and **3** and **6** were prepared as described in the literature.<sup>41,49–51</sup> GYY4137 was prepared according to the published procedure.<sup>52</sup> Stock solutions of the different thiol probes were prepared in deoxygenated DMSO and stored in aliquots at −25 °C under nitrogen until immediately prior to use.

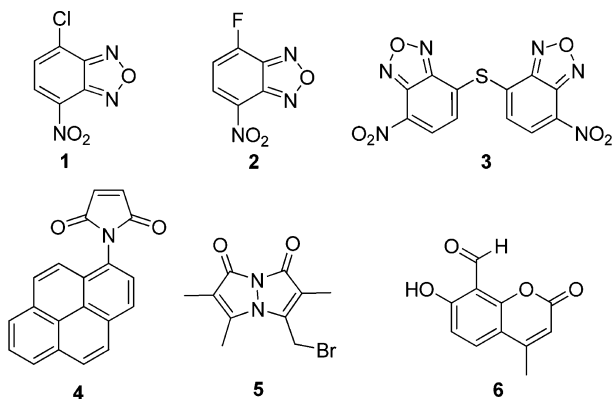
**General Procedure for Fluorescent Studies.** Stock solutions of each probe (10 mM) in DMSO were prepared in a glovebox. A 13 mL solution of each probe (5 μM) in pH 7.0 PIPES buffer was prepared, and 3.0 mL of the solution was distributed to individual cuvettes containing a stir bar and a septum cap. After removal from the glovebox, initial fluorescent readings were recorded, after which each probe was treated with 50 μM Cys or NaSH by syringe and monitored for 60 min. To investigate the effects of added NaSH after Cys addition, each reagent was incubated with 50 μM Cys for 60 min, after which 500 μM NaSH was added, and the cuvette was monitored for an additional 60 min. For each fluorescence experiment, reagents **1–3** were excited at 465 nm, **4** at 340 nm, **5** at 392 nm, and **6** at 365 nm. Fluorescence measurements are reported as integrated emissions over an emission window sufficient to capture the complete emission profile of each reagent.

**Cell Culture and Imaging Materials and Methods.** HeLa cells were obtained from ATCC and cultured in Dulbecco's Modified Eagle Medium (DMEM, GIBCO, MediaTek, Inc.) supplemented with 10% fetal bovine serum (FBS, HyClone) and 1% penicillin/streptomycin. Cells were passed and plated into six-well dishes (MatTek) containing 3.0 mL of DMEM and were incubated at 37 °C with 5% CO<sub>2</sub>. Prior to imaging, cells were washed with 1.5 mL of Dulbecco's Phosphate Buffered Saline (DPBS) and then bathed in 3.0 mL of DMEM without phenol red indicator supplemented with 1% penicillin/streptomycin during imaging. For cell imaging experiments, cells were treated with **1** or **4** (10 μM) and Hoechst 33258 nuclear dye (2.5 μM) in DMEM without phenol red indicator supplemented with 1% penicillin/streptomycin and monitored over a period of 30 min. The cells were then treated with NaSH

(200  $\mu\text{M}$ ) or GYY4137 (400  $\mu\text{M}$ ) and monitored for an additional 60 and 90 min, respectively.

## RESULTS

Because common fluorescent reagents for thiol labeling are typically activated by nucleophilic attack of the thiol on an



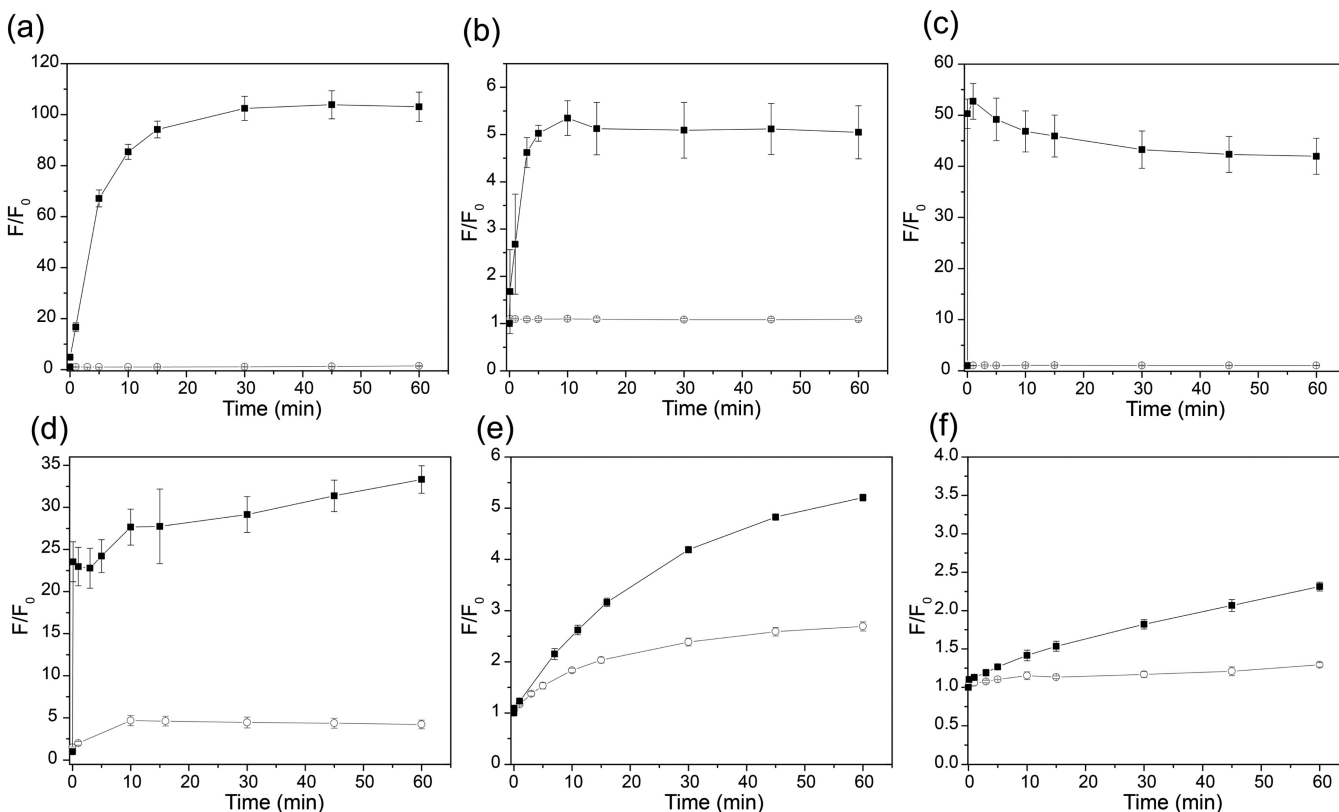
**Figure 1.** Commonly used reagents for labeling and detection of cellular thiols that were used to investigate the potential cross-reactivity with  $\text{H}_2\text{S}$ .

electrophilic moiety of the probe reagent, we reasoned that  $\text{H}_2\text{S}$ , which is a more potent nucleophile, could potentially react with and activate common thiol detection reagents. Additionally, we wanted to determine whether the thiol reaction products of such probes are stable in the presence of  $\text{H}_2\text{S}$ . To

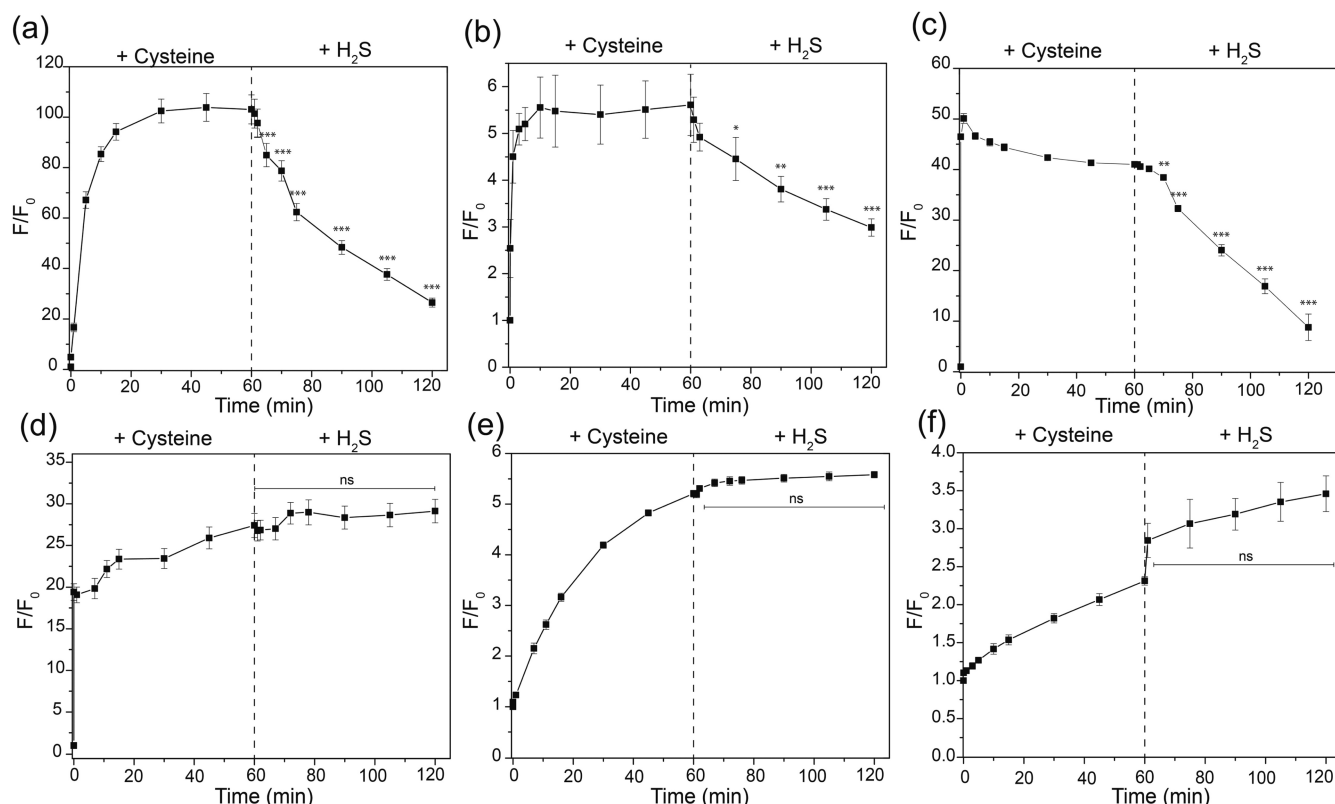
investigate such potential reactivity differences we chose electrophilic thiol labeling reagents including nitrobenzofurazan electrophiles (NBD-Cl, **1**; NBD-F, **2**; (NBD) $_2\text{S}$ , **3**), fluorophore-bound maleimides (*N*-(1-pyrene)maleimide, **4**), fluorescent alkylating agents (monobromobimane, **5**), and electrophilic aldehydes (coumarin carbaldehyde, **6**) due to their broad application in thiol labeling, detection, and quantification, as well as their different electrophilic moieties (Figure 1). To probe the differential reactivity of each class of characteristic probe toward  $\text{H}_2\text{S}$  and thiols, we measured the fluorescence response upon treatment with  $\text{H}_2\text{S}$  and Cys individually.

To determine the reaction profiles of each reagent with thiols, we incubated 5  $\mu\text{M}$  solutions of compounds **1–6** with 10 equiv of Cys at 37  $^\circ\text{C}$  and monitored the subsequent fluorescence response (Figure 2). As expected, each scaffold produced a fluorescence response upon the addition of Cys. After verifying the Cys-derived fluorescence response, we repeated each experiment with an equivalent amount of  $\text{H}_2\text{S}$ . Although **1–6** produced a fluorescence response to Cys, only **4–6** produced a fluorescent response to  $\text{H}_2\text{S}$ . This observed response, however, was significantly lower than that observed for Cys. Although treatment of **1–3** with  $\text{H}_2\text{S}$  did not generate a fluorescence response, a significant color change was observed (*vide infra*), which is consistent with the formation of NBD-SH.<sup>41</sup>

On the basis of the observed reactivity of **1–6** toward  $\text{H}_2\text{S}$ , we next investigated whether the fluorescent signal generated from the thiol reaction products of **1–6** could be affected by treatment with  $\text{H}_2\text{S}$ . To test this potential reactivity, 5  $\mu\text{M}$  solutions of **1–6** were treated with 10 equiv of Cys for 60 min,



**Figure 2.** Fluorescent data for (a) **1**, (b) **2**, (c) **3**, (d) **4**, (e) **5**, and (f) **6** reacting with Cys (—■—) or  $\text{H}_2\text{S}$  (—○—). Conditions: pH 7.0, 50 mM PIPES buffer, 100 mM KCl, 37  $^\circ\text{C}$ , 5  $\mu\text{M}$  thiol probe, 10 equiv of Cys or  $\text{H}_2\text{S}$ . Data were acquired at  $t = 0, 1, 5, 10, 15, 30, 45$ , and 60 min after addition. Error bars represent  $\pm$  SE,  $n = 4$ .



**Figure 3.** Impact of added  $\text{H}_2\text{S}$  on the fluorescence response from Cys for (a) 1, (b) 2, (c) 3, (d) 4, (e) 5, and (f) 6. Conditions: pH 7.0, 50 mM PIPES buffer, 100 mM KCl, 37 °C, 5  $\mu\text{M}$  thiol probe, 10 equiv of Cys followed by 100 equiv of  $\text{H}_2\text{S}$ . Data were acquired at  $t = 0, 1, 5, 10, 15, 30, 45$ , and 60 min after Cys addition and at  $t = 0, 1, 5, 10, 15, 30, 45$ , and 60 min after  $\text{H}_2\text{S}$  addition. Error bars represent  $\pm$  SE,  $n = 4$ , ns = not significant, \* =  $p < 0.05$ , \*\* =  $p < 0.01$ , \*\*\* =  $p < 0.001$ .

followed by 100 equiv of  $\text{H}_2\text{S}$  for an additional 60 min. For nitrobenzofurazan compounds 1–3, the addition of  $\text{H}_2\text{S}$  resulted in a significant reduction of the fluorescence response, signifying that the thiol-reaction products are not stable toward  $\text{H}_2\text{S}$  (Figure 3a–c). By contrast, reagents 4–6 showed negligible change after  $\text{H}_2\text{S}$  addition (Figure 3d–f), highlighting the different reactivity profiles of commonly used thiol labeling reagents.

To further demonstrate the prevalence of the observed reactivity, we used fluorescent microscopy to determine whether the same fluorescence response was observed in live cells. We chose 1 as the representative example from nitrobenzofurazan-based probes due to its significant reduction in fluorescence after treatment of the thiol ligated product with  $\text{H}_2\text{S}$ . Similarly, 4 was chosen as a control reactant due to its lack of reactivity toward  $\text{H}_2\text{S}$ . For live-cell imaging studies, HeLa cells were incubated with 1 or 4 (10  $\mu\text{M}$ ) for 30 min, after which either  $\text{H}_2\text{S}$  or GYY4137, a common slow-releasing  $\text{H}_2\text{S}$  donor,<sup>52</sup> was added. Treatment of HeLa cells with 1 (Figure 4a) or 4 (Figure 4d) for 30 min resulted in a fluorescence response consistent with intracellular thiol labeling. Upon the addition of  $\text{H}_2\text{S}$  or GYY4137, however, a significant decrease in fluorescence was observed for compound 1 (Figure 4b,c), but no fluorescence change was observed for compound 4 (Figure 4e,f). Furthermore, differences in both the magnitude and rate of fluorescence decrease from 1 were observed upon the addition of  $\text{H}_2\text{S}$  and GYY4137 (Figure 4g), which is consistent with the  $\text{H}_2\text{S}$  release profiles of these two sulfide sources. The cell based studies of 1 and 4 match the cuvette-based experiment and highlight the incompatibility of nitrobenzofur-

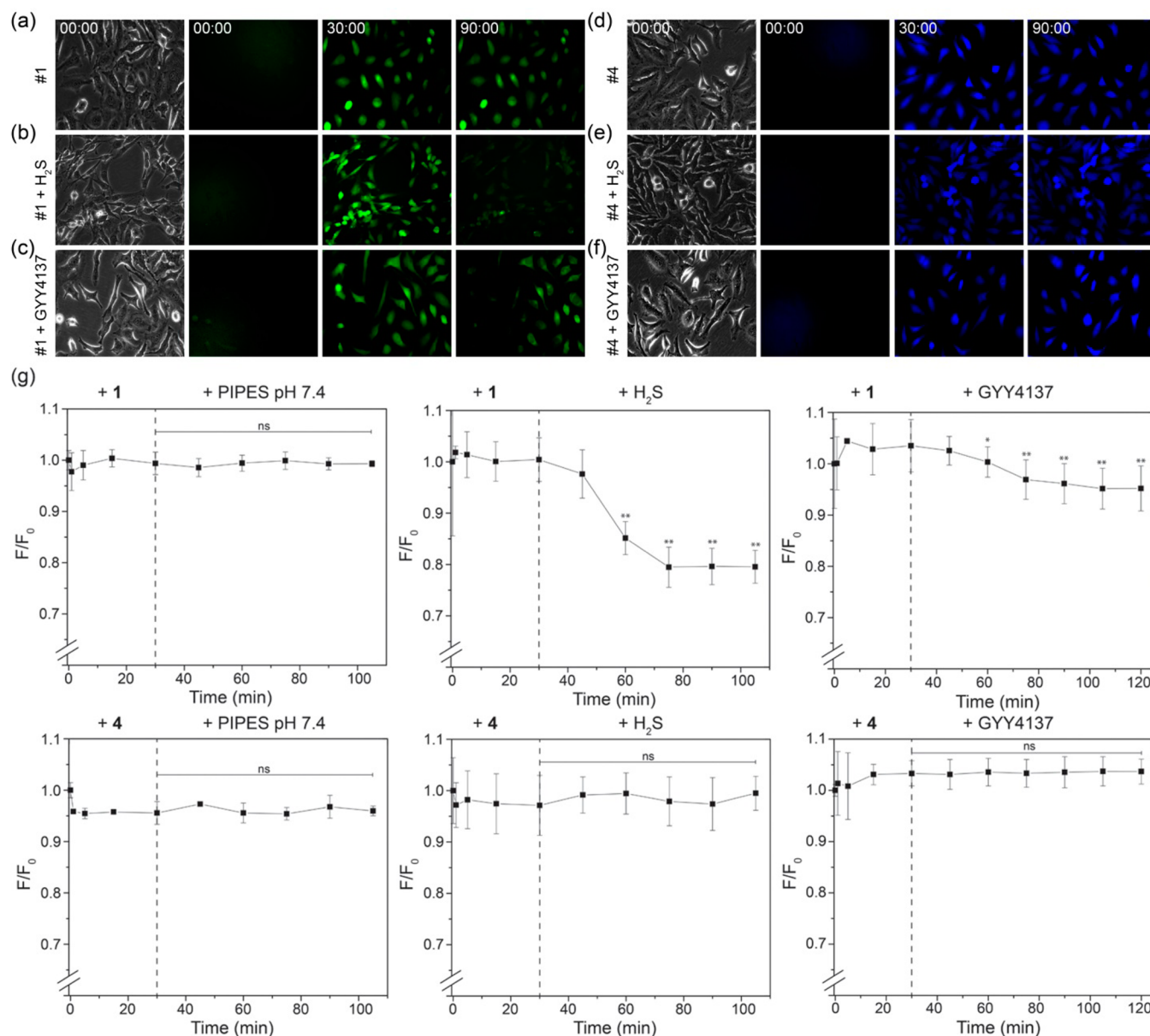
azan-based thiol labeling reagents with  $\text{H}_2\text{S}$ , even after thiol ligation.

## DISCUSSION

The observed differential reactivity of  $\text{H}_2\text{S}$  and thiols toward 1–6 highlights the need for a judicious choice of thiol labeling reagents for experiments in which elevated endogenous  $\text{H}_2\text{S}$  levels are present. Furthermore, because both thiols and  $\text{H}_2\text{S}$  play important roles in cellular redox chemistry and homeostasis, the possible cross-reactivity of  $\text{H}_2\text{S}$  with thiol labeling reagents requires significant consideration during experimental design. For example, because nitrobenzofurazan-based reagents are deactivated by  $\text{H}_2\text{S}$ , both before and after reaction with thiols, such reagents have poor utility for experiments in which  $\text{H}_2\text{S}$  is present. Alternatively, although electrophilic reagents 4–6 can react quickly with  $\text{H}_2\text{S}$  to generate a small false-positive fluorescence response, the thiol reaction products are stable in the presence of  $\text{H}_2\text{S}$ , thus highlighting their greater utility of these reagents for experiments in which  $\text{H}_2\text{S}$  is present.

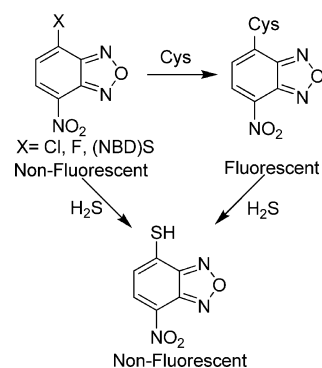
Although the exogenous levels of  $\text{H}_2\text{S}$  used in the experiments (200  $\mu\text{M}$ ) are higher than basal cellular  $\text{H}_2\text{S}$  levels, these concentrations were used to balance the rate of reaction with NBD-electrophiles and cell viability. Additionally, previous studies have demonstrated that much of administered exogenous  $\text{H}_2\text{S}$  is quickly metabolized, resulting in lower actual levels of accessible sulfide.<sup>53</sup> Based on cuvette-based studies, we expect that the same deactivation of nitrobenzofurazan-based reagents and erosion of nitrobenzofurazan-labeled thiols will still occur in a cellular environment at lower  $\text{H}_2\text{S}$  concentrations, albeit at a diminished rate. By contrast to  $\text{NaSH}$ ,





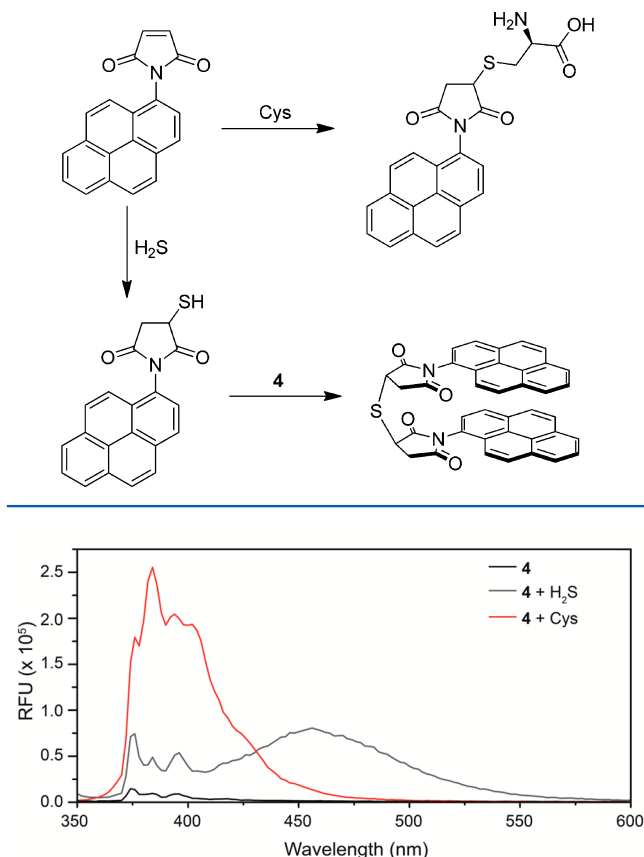
**Figure 4.** Fluorescence and brightfield images of live HeLa cells pretreated with **1** and **4** (10  $\mu$ M) for 30 min and then incubated with added PIPES buffer at pH 7.4 (a, d), H<sub>2</sub>S (200  $\mu$ M) (b, e), or GYY4137 (400  $\mu$ M) for 60 min (c, f). Integrated cellular fluorescence from individual HeLa cells (g) incubated with **1** and **4** after the addition of pH 7.4 PIPES buffer, H<sub>2</sub>S, or GYY4137. Conditions: 10  $\mu$ M thiol probe, 200  $\mu$ M or 400  $\mu$ M analyte, 37  $^{\circ}$ C, 5% CO<sub>2</sub>. Data were acquired at  $t = 0, 1, 10, 15$ , and 30 min after thiol probe addition and at  $t = 0, 15, 30, 45$ , and 60 min after analyte addition. Error bars represent  $\pm$  SE,  $n = 30$ , ns = not significant, \* =  $p < 0.05$ , \*\* =  $p < 0.01$ .

#### Scheme 1. Reaction Pathways of Nitrobenzofurazan-Based Thiol Labeling Reagents (**1**–**3**) with Cys and H<sub>2</sub>S

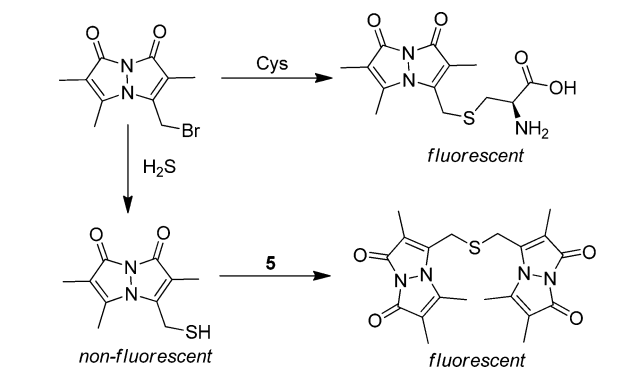


administration of a slow-release H<sub>2</sub>S donor, such as GYY4137, provides a continuous low level of H<sub>2</sub>S and thus is typically administered at higher dosages to generate physiological effects. The GYY4137 concentrations used here (400  $\mu$ M) are equivalent to those used in previous biological experiments and assays.<sup>54,55</sup> The above considerations suggest that nitrobenzofurazan-based thiol labeling reagents should not be used in experiments in which exogenous H<sub>2</sub>S, either from NaSH or sulfide donors, is administered. Additionally, exposure of nitrobenzofurazan-labeled thiols to endogenous H<sub>2</sub>S over prolonged time periods may provide deleterious effects.

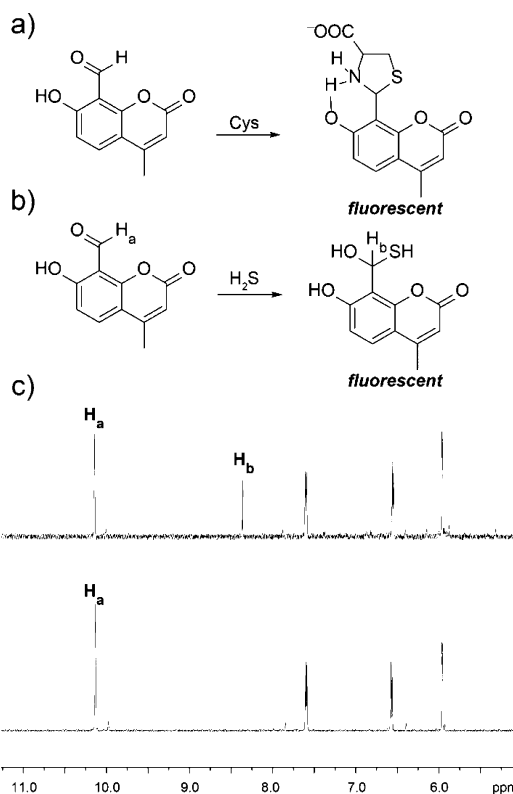
To further determine the different pathways by which Cys and H<sub>2</sub>S react with thiol labeling reagents **1**–**6** and subsequently increase the generality of the observed differential reactivity, we investigated the mechanisms by which probe

Scheme 2. Reaction Pathways of 4 with Cys and H<sub>2</sub>S

**Figure 5.** Emission spectra of 4, 4 + Cys, and 4 + H<sub>2</sub>S. Reaction with H<sub>2</sub>S generates a new band at 455 nm that is consistent with pyrene excimer formation. Conditions: pH 7.0, 50 mM PIPES buffer, 100 mM KCl, 37 °C, 5  $\mu$ M 4, and 10 equiv of Cys or NaSH.

Scheme 3. Reaction Pathway of 5 with Cys and H<sub>2</sub>S

activation or deactivation occurred. Although 1–3 react with both Cys and H<sub>2</sub>S, only the Cys reaction product is fluorescent. Upon reaction of 1–3 with Cys, however, the Cys-bound adduct remains sufficiently electrophilic to further react with H<sub>2</sub>S to generate NBD-SH (Scheme 1). This subsequent H<sub>2</sub>S reaction is irreversible, which we demonstrated previously in the study of the reactivity of NBD-based electrophiles toward H<sub>2</sub>S.<sup>41</sup> Taken together, this reactivity is consistent with the observed abolishment of the NBD-Cys fluorescence upon H<sub>2</sub>S addition and suggests that NBD-based thiol labeling reagents are incompatible with systems in which H<sub>2</sub>S is generated.



**Figure 6.** Reaction of 5 with (a) Cys and (b) H<sub>2</sub>S. (c) <sup>1</sup>H NMR spectrum of 5 before (bottom) and after (top) the addition of H<sub>2</sub>S. Conditions: 10 mM thiol probe, 10 equiv of H<sub>2</sub>S in D<sub>2</sub>O and HEPES buffer pH 7.4.

By contrast to the NBD scaffolds, the thiol-ligated product formed upon reaction of 4 with Cys is not sufficiently electrophilic to react further with H<sub>2</sub>S. This difference in reactivity is consistent with the stable fluorescence response observed upon treatment of the Cys-ligated 4 with H<sub>2</sub>S. Although H<sub>2</sub>S does not reduce the response from Cys-ligated 4, H<sub>2</sub>S can still react with 4 to generate a fluorescent product, although the magnitude of this response is lower than that observed for Cys alone. When H<sub>2</sub>S reacts with 4, the initial thiomaleimide product could further react with a second equiv of 4 to generate a dipyrenyl thioether (Scheme 2) leading to a mixture of mono- and dipyrenyl adduct formation. The dipyrenyl adduct would be expected to exhibit distinct photophysical properties from the Cys reaction product due to the close proximity of two pyrene moieties. Upon measuring the emission spectra of the reaction products from 4 with Cys and 4 with H<sub>2</sub>S we observed the appearance of a new fluorescence band at 455 nm upon treatment with H<sub>2</sub>S. This bathochromic and broad emission band is consistent with pyrene excimer formation,<sup>56–59</sup> which would be expected from formation of dipyrenyl thioether formation upon reaction with H<sub>2</sub>S (Figure 5). Supporting this hypothesis, mass spectrometric analysis of the reaction products revealed products with *m/z* values of 629.1510 and 651.1335 which match those expected for the dipyrenyl thioether product (*m/z* calculated for M + H<sup>+</sup>, 629.1535; M + Na<sup>+</sup>, 651.1354). In addition to highlighting the differential reactivity of 4 toward H<sub>2</sub>S and Cys, the different photophysical properties of the H<sub>2</sub>S reaction product provides a potential platform on which thiol and H<sub>2</sub>S reactivity can be separated.

Following similar reactivity to **4**, monobromobimane (**5**) reacts quickly with thiols to form fluorescent bimane-thiol adducts and is a common reagent for thiol labeling and quantification by HPLC.<sup>60–62</sup> Much like **4**, the bimane-Cys conjugate is unreactive toward H<sub>2</sub>S due to the thermodynamic stability of the thioether product. Upon reaction of **5** with H<sub>2</sub>S, however, the bimanethiol generated during the first nucleophilic attack remains sufficiently nucleophilic to react with a second equiv of **5** to form fluorescent sulfide dibimane (SdB), thus paralleling the observed reactivity of **4**. The addition of 2 equiv of **5** to trap H<sub>2</sub>S as stable SdB is well established and has been identified and used as a robust method for quantifying endogenous H<sub>2</sub>S concentrations using fluorescence HPLC.<sup>43,44,63</sup> Taken together, the reactivity of **5** toward H<sub>2</sub>S and Cys matches that observed for **4**, in that reaction with either H<sub>2</sub>S or Cys produces a fluorescent product, although the reaction stoichiometries of these two pathways are different (Scheme 3).

Use of aldehydic electrophiles is also an effective strategy for thiol detection. Such constructs, especially with ortho-hydroxy groups, have been used as selective labeling reagents for Cys and Hcy due to formation of a hydrogen-bond stabilized fluorescent thiazolidine product.<sup>64–67</sup> Unlike electrophilic **4** and **5**, in which H<sub>2</sub>S addition results in the formation of a nucleophilic thiol intermediate, the addition of H<sub>2</sub>S to aldehyde-based electrophiles, such as **6**, should produce a significantly lesser nucleophilic thiol product. For example, when **6** is treated with H<sub>2</sub>S, addition of H<sub>2</sub>S to the aldehyde generates the (mercapto)benzylalcohol adduct, which exhibits a diminished fluorescence response by comparison to Cys. To confirm that such nucleophilic addition was occurring, we treated **6** with H<sub>2</sub>S and monitored the subsequent reaction by <sup>1</sup>H NMR spectroscopy. Upon the addition of H<sub>2</sub>S, the aldehydic <sup>1</sup>H NMR signal at 10.1 ppm shifted upfield to 8.4 ppm, consistent with nucleophilic attack on the aldehyde by H<sub>2</sub>S (Figure 6). Similarly, the <sup>13</sup>C{<sup>1</sup>H} NMR resonance corresponding to the aldehyde carbon shifts from 192 to 62 ppm, which is also consistent with nucleophilic addition of sulfide to the aldehyde (Figure S3). Unlike **4** and **5**, this resultant thiol does not further react with a second electrophile but rather forms a stable final product.

## CONCLUSION

Investigation of the reaction of H<sub>2</sub>S with commonly used electrophilic thiol labeling reagents revealed highly differential responses between different classes of thiol-detection scaffolds. Both cuvette- and cell-based studies revealed that thiol probes based on nitrobenzofurazan scaffolds are deactivated by H<sub>2</sub>S. Furthermore, the initial response generated upon reaction with thiols is decreased by the addition of H<sub>2</sub>S, suggesting that nitrobenzofurazan-based thiol detection platforms are not compatible with cellular experiments in which H<sub>2</sub>S generation is prevalent. By contrast, the fluorescence response of other electrophilic thiol probes tested is not reduced upon the addition of H<sub>2</sub>S. In the absence of thiols, however, these probes also react with H<sub>2</sub>S to generate products with lower fluorescence signals. Taken together, these studies highlight the differential reactivity of sulfhydryl-containing compounds toward common thiol-labeling reagents and suggest that sufficient care must be taken when labeling or measuring thiols in cellular environments that produce H<sub>2</sub>S due to the potential for false-positive as well as erroneously reduced responses.

## ASSOCIATED CONTENT

### Supporting Information

Synthetic procedures, NMR spectra, and UV–vis spectra. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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### Notes

The authors declare no competing financial interest.

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