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## Cysteine-Activated Hydrogen Sulfide (H<sub>2</sub>S) Delivery through Caged Carbonyl Sulfide (COS) Donor Motifs

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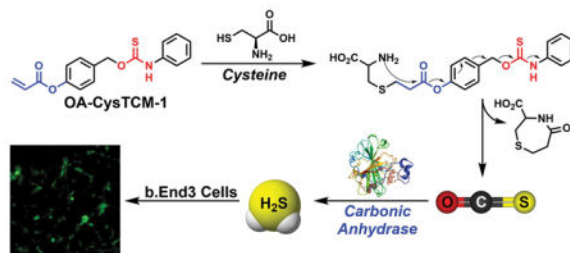
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### Abstract

Hydrogen sulfide (H<sub>2</sub>S) is an important biomolecule, and controllable H<sub>2</sub>S donors are needed to investigate H<sub>2</sub>S biological functions. Here we utilize cysteine-mediated addition/cyclization chemistry to unmask an acrylate-functionalized caged thiocarbamate and release carbonyl sulfide (COS), which is quickly converted to H<sub>2</sub>S by carbonic anhydrase (CA).

### Graphical Abstract

Cysteine-activated acrylate-functionalized caged thiocarbamate provides access to triggered COS/H<sub>2</sub>S donors



Hydrogen sulfide (H<sub>2</sub>S) has joined the gasotransmitter family since its first recognition as an endogenous neuromodulator in 1996.<sup>1</sup> Four main enzymes, including cystathionine  $\beta$ -synthase (CBS) and cystathionine  $\gamma$ -lyase (CSE), are responsible for endogenous H<sub>2</sub>S production, converting cysteine (Cys) and homocysteine (Hcy) to H<sub>2</sub>S.<sup>2</sup> Significant efforts have been contributed to develop H<sub>2</sub>S releasing agents (H<sub>2</sub>S donors) because the regulation of H<sub>2</sub>S levels has been found to mediate a wide variety of physiological processes, including anti-inflammation, oxidative stress reduction, and vasorelaxation.<sup>3</sup> Although sulfide salts, such as sodium hydrosulfide (NaHS) and sodium sulfide (Na<sub>2</sub>S), have been widely used in the field, they are far from ideal donors because they release H<sub>2</sub>S spontaneously, resulting in a concentrated bolus of sulfide that oxidizes rapidly and does not mimic well-regulated

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Electronic Supplementary Information (ESI) available: experimental details, H<sub>2</sub>S release measurements, NMR spectra. See DOI: 10.1039/x0xx00000x

### Conflicts of interest

There are no conflicts to declare

endogenous H<sub>2</sub>S production. The use of these inorganic sulfide sources has even led to contradictory results,<sup>3c</sup> demonstrating the need for improved sources of H<sub>2</sub>S. These limitations suggest that controllable H<sub>2</sub>S donors, which are stable, only release H<sub>2</sub>S upon activation by certain stimuli, and have slower and controllable kinetics of sulfide release, are key research tools for H<sub>2</sub>S investigations.

Aligned with this need, our group recently reported the use of caged-carbonyl sulfide (COS) molecules as new H<sub>2</sub>S donors.<sup>4</sup> Unlike other known H<sub>2</sub>S donors, which directly release H<sub>2</sub>S as the activation product, COS-based donors are activated to release COS, which is quickly converted to H<sub>2</sub>S by the ubiquitous enzyme carbonic anhydrase (CA). We have demonstrated that caged-thiocarbamates and thiocarbonates can serve as promising COS donors and can be activated to release COS through a self-immolative cascade reaction.<sup>5</sup> One important advantage of this strategy is that COS-releasing scaffolds can be designed to deliver H<sub>2</sub>S under well-defined conditions. For example, H<sub>2</sub>S delivery from these caged-COS donors can be modulated by judicious trigger selection, and the rate of release can be manipulated through modification of the donor structure.<sup>5</sup> Following our initial report, we, as well as others, have expended this strategy to include donors activated by different triggers, such as reactive oxygen species (ROS),<sup>5-6</sup> esterases,<sup>7</sup> nucleophiles,<sup>8</sup> click chemistry,<sup>9</sup> and light<sup>10</sup> (Figure 1).

Cellular nucleophiles play crucial roles in biological systems. Among these, thiol species, such as Cys and reduced glutathione (GSH), attract the most attention due to their cellular abundance and potent reactivity. Cys and GSH have been widely used to trigger biologically active molecules and prodrugs to release caged compounds, including sulfur dioxide (SO<sub>2</sub>),<sup>11</sup> nitroxyl (HNO),<sup>12</sup> and anti-cancer drugs.<sup>13</sup> Importantly, thiol activation strategies have been adopted in H<sub>2</sub>S donor development and several thiol labile H<sub>2</sub>S donors exhibit promising protections in animal models with some of them currently in clinical trials.<sup>3b, 3c, 3e</sup> Motivated by these findings, we report here the first example of a Cys-activated COS/H<sub>2</sub>S donor through functionalization of a thiocarbamate with a Cys-reactive acrylate moiety. We envision that such thiocarbamate compounds will expand the current COS-based H<sub>2</sub>S donor family and serve as promising research tools for H<sub>2</sub>S studies.

The reactions of Cys with acrylates in the preparation of substituted 1,4-thiazepines have been known for decades.<sup>14</sup> The initial attack by Cys on the acrylate generates a thioether, which then undergoes an intramolecular cyclization to yield 1,4-thiazepines. This cyclization strategy has been leveraged by Strongin,<sup>15</sup> as well as others,<sup>16</sup> to design a series of acrylate-based fluorescent probes for Cys detection. Similarly, the Berreau group has recently used a similar approach to develop a class of Cys-responsive CO donors.<sup>17</sup> Building from these approaches, we adopt the Cys-acrylate reaction as a triggering mechanism to access new COS/H<sub>2</sub>S donors in which an aryl acrylate-functionalized thiocarbamate is activated through a Cys-mediated addition/cyclization sequence. The resultant phenolic intermediate then undergoes a 1,6-elimination to release COS, which is quickly converted to H<sub>2</sub>S by CA (Scheme 1).

To test our hypothesis that acrylate-functionalized thiocarbamates could serve as Cys-triggered COS/H<sub>2</sub>S donors, we prepared *O*-alkyl cysteine-sensitive thiocarbamate (OA-

**CysTCM-1**) with an aryl acrylate trigger and an aniline payload by reacting 4-(hydroxymethyl)phenyl acrylate and phenyl isothiocyanate. Upon activation, **OA-CysTCM-1** releases COS, which is quickly hydrolyzed to H<sub>2</sub>S by CA. In addition to **OA-CysTCM-1**, we also prepared the corresponding carbamate (**OA-CysCM-1**) and triggerless thiocarbamate (**OA-TCM-1**)<sup>18</sup> control compounds. **OA-CysCM-1**, obtained from the reaction between 4-(hydroxymethyl)phenyl acrylate and phenyl isocyanate, is expected to undergo the same Cys activation but would release CO<sub>2</sub> instead of COS. **OA-TCM-1**, on the other hand, maintains the thiocarbamate scaffold but lacks the acrylate trigger, and thus is not expected to react with Cys or decompose to otherwise release COS (Scheme 2).

To evaluate Cys-activated H<sub>2</sub>S release from the donor motif, we used the methylene blue (MB) assay to monitor H<sub>2</sub>S release from **OA-CysTCM-1** (50 μM) in the presence of Cys (0 – 500 μM) in PBS buffer (pH 7.4, 10 mM) containing cellularly-relevant concentrations of CA (25 μg/mL). The MB assay was chosen to measure H<sub>2</sub>S production since it has been widely used to detect H<sub>2</sub>S from previously developed Cys-activated H<sub>2</sub>S donors. In the absence of Cys, **OA-CysTCM-1** was stable in aqueous buffer and did not release COS/H<sub>2</sub>S spontaneously. By contrast, the addition of Cys led to a dose-dependent COS/H<sub>2</sub>S release from **OA-CysTCM-1** (Figure 2). These results demonstrate that **OA-CysTCM-1** can be activated by Cys and the resultant COS is quickly converted to H<sub>2</sub>S in the presence of CA.

To further demonstrate that the observed H<sub>2</sub>S release is due to Cys activation via the proposed mechanism, we pretreated Cys with *N*-ethylmaleimide (NEM), a Cys scavenger, for 20 min, followed by the addition of **OA-CysTCM-1**. When compared to the regular activation conditions (Figure 3, bar 1), NEM pretreatment significantly diminished H<sub>2</sub>S release from **OA-CysTCM-1**, confirming that Cys was required for donor activation (Figure 3, bar 2). No H<sub>2</sub>S release was observed in the absence of CA, and, similarly, pretreatment of CA with acetazolamide (AAA), a CA inhibitor, also failed to provide H<sub>2</sub>S, confirming that H<sub>2</sub>S release from **OA-CysTCM-1** proceeds through a COS-dependent pathway (Figure 3, bars 3 and 4).

In addition to Cys, other biologically relevant nucleophiles, such as GSH, oxidized glutathione (GSSG), Hcy, *N*-acetylcysteine (NAC), serine (Ser), and lysine (Lys), were evaluated towards donor activation. As expected, none of these species triggered **OA-CysTCM-1**, and no COS/H<sub>2</sub>S release was observed due to the lack of the addition/cyclization activation sequence (Figure 3, bars 5–10). Considering the high abundance of GSH in cellular environment, we also evaluated Cys-triggered COS/H<sub>2</sub>S release from **OA-CysTCM-1** in the presence of GSH. In these experiments, **OA-CysTCM-1** (50 μM) was co-incubated with Cys (500 μM) and GSH (0 – 1000 μM) and COS/H<sub>2</sub>S release was monitored by MB assay (Figure S2). A decrease of H<sub>2</sub>S release was observed as the GSH concentration increased, indicating a potential GSH-induced donor consumption. Although the effects of GSH were not significant in aqueous buffer, it should be taken into consideration when applying donors in biological systems. Since the acrylate trigger may be prone to esterase-catalyzed hydrolysis, we also incubated **OA-CysTCM-1** with porcine liver esterase (PLE) to determine whether common esterases could generate COS/H<sub>2</sub>S release. Although we did observe H<sub>2</sub>S release, it was significantly less efficient than Cys activation (Figure 3, bar 11). As expected, treatment of control compounds **OA-CysCM-1** and **OA-TCM-1** with Cys in

the presence of CA failed to generate H<sub>2</sub>S, demonstrating that both the aryl-acrylate trigger and the caged COS-containing thiocarbamate scaffold are crucial for COS release from this scaffold (Figure 3, bars 12 and 13). Taken together, these selectivity studies demonstrate that **OA-CysTCM-1** is highly sensitive towards Cys activation to release COS/H<sub>2</sub>S and inert to activation by other biomolecules, such as GSH, GSSG, Hcy, NAC, Ser, and Lys.

We next sought to confirm that **OA-CysTCM-1** would release COS/H<sub>2</sub>S upon reaction with Cys in a cellular environment. We incubated bEnd.3 cells with **OA-CysTCM-1** in the presence of Cys and visualized H<sub>2</sub>S-release using SF7-AM, a cell-trappable H<sub>2</sub>S-responsive fluorescent probe.<sup>19</sup> In the absence of **OA-CysTCM-1**, negligible SF7-AM fluorescence was observed, suggesting a minimum amount of endogenous H<sub>2</sub>S present in bEnd.3 cells. By contrast, addition of **OA-CysTCM-1** resulted in a significant increase in SF7-AM fluorescence, confirming that **OA-CysTCM-1** can be activated by Cys to release H<sub>2</sub>S in a cellular environment (Figure 4). These results demonstrate that **OA-CysTCM-1** is a potent COS/H<sub>2</sub>S donor and Cys-triggered H<sub>2</sub>S delivery can be visualized in complex biological systems, indicating applications of **OA-CysTCM-1** as a potential H<sub>2</sub>S-related therapeutic or research tool.

In summary, we prepared and evaluated **OA-CysTCM-1** as a Cys-triggered COS/H<sub>2</sub>S donor. Our studies demonstrate that **OA-CysTCM-1** is stable in aqueous media and does not release COS/H<sub>2</sub>S until being activated by Cys. Importantly, H<sub>2</sub>S delivery from **OA-CysTCM-1** is observed in a cellular environment, indicating **OA-CysTCM-1** can be used as a new efficacious Cys labile COS/H<sub>2</sub>S donor in complex biological systems. Taken together, our investigations demonstrate that H<sub>2</sub>S delivery from **OA-CysTCM-1** can be controlled and regulated through a COS-dependent pathway, making **OA-CysTCM-1** a new member of COS-based H<sub>2</sub>S donor family with potential applications in the study of both H<sub>2</sub>S and COS chemical biology, especially when used in combination with available Cys-activated H<sub>2</sub>S donors. Further applications of this as well as other COS-releasing constructs are currently ongoing in our laboratory.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

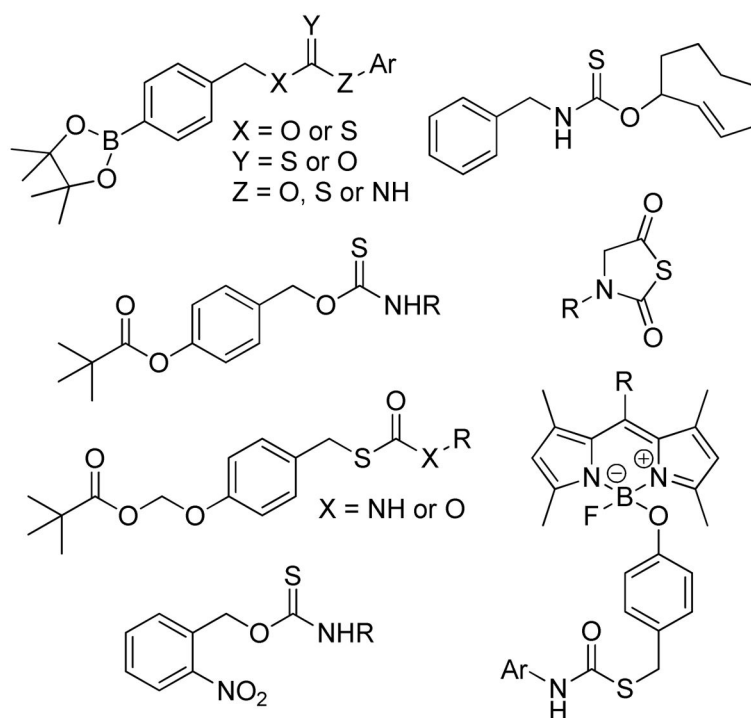
## Acknowledgments

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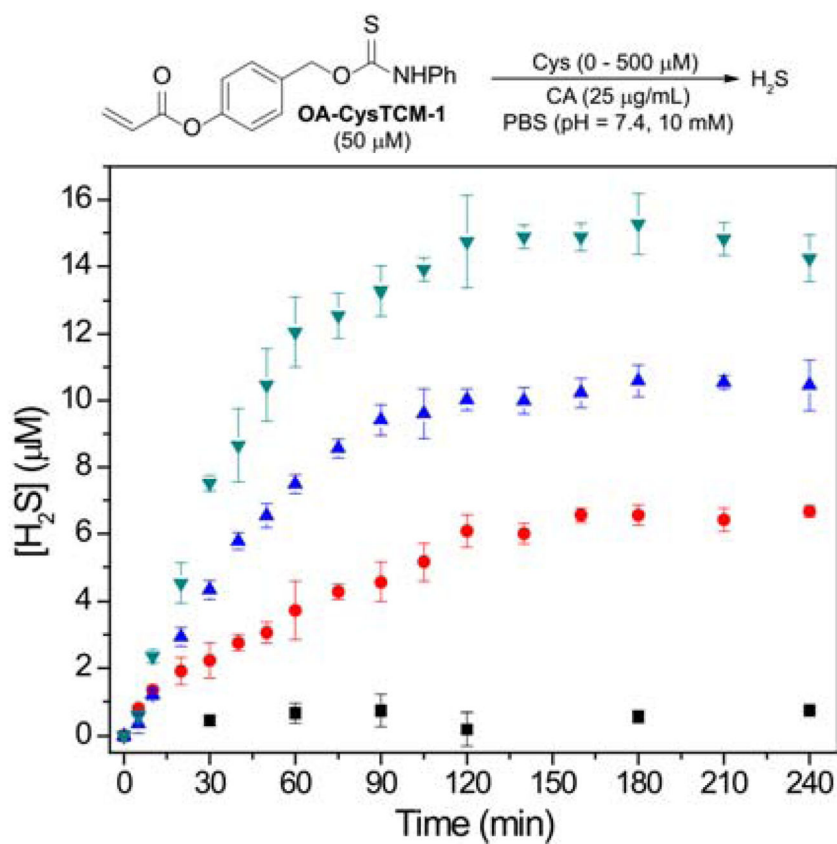
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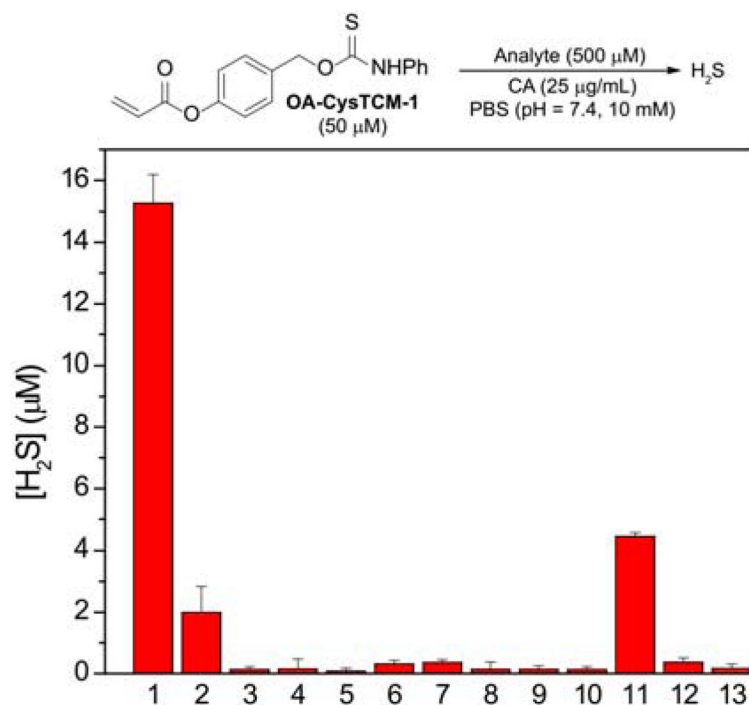
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**Figure 1.** Examples of currently-available COS-based H<sub>2</sub>S donors that are activated by different triggering stimuli.

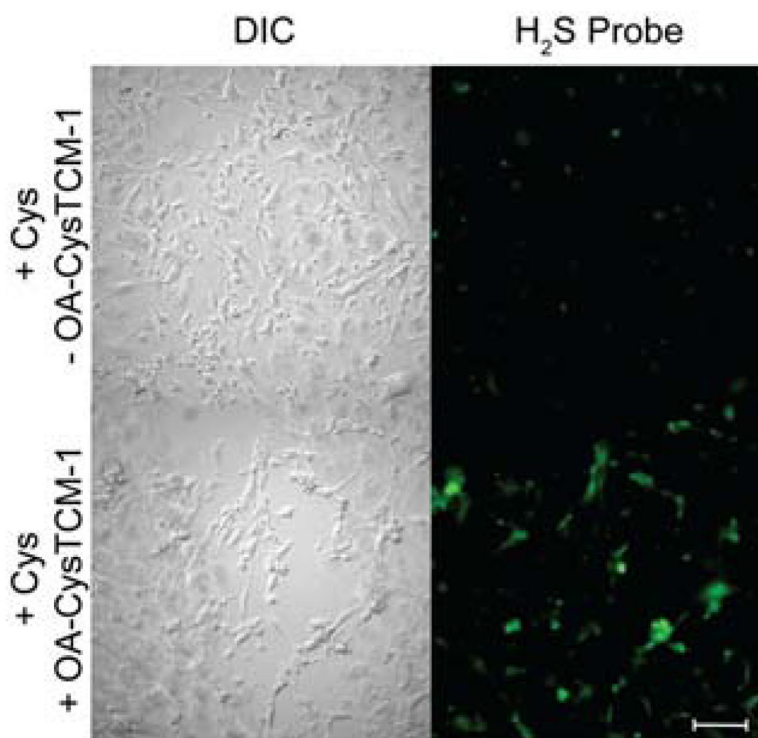


**Figure 2.** COS/ $\text{H}_2\text{S}$  Release from **OA-CysTCM-1** (50  $\mu\text{M}$ ) in the presence of 0  $\mu\text{M}$  (black), 50  $\mu\text{M}$  (red), 250  $\mu\text{M}$  (blue), and 500  $\mu\text{M}$  (green) Cys. The experiments were performed in triplicate and results are expressed as mean  $\pm$  SD ( $n = 3$ ).

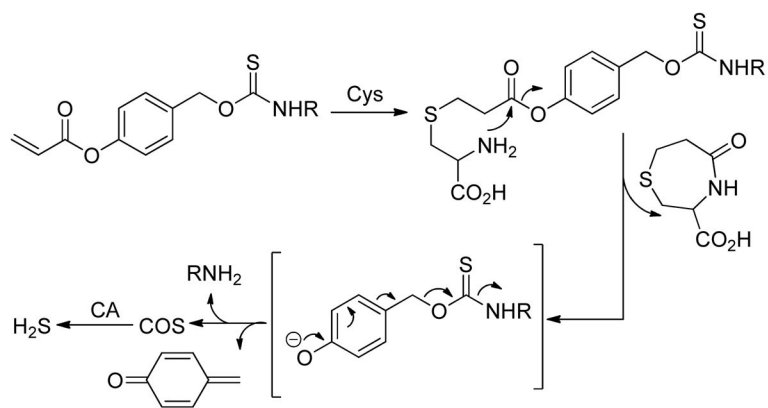


**Figure 3.** COS/H<sub>2</sub>S Release from **OA-CysTCM-1** (50 μM) in the presence of cellular nucleophiles (500 μM): (1) Cys, (2) Cys + NEM (10 mM), (3) Cys - CA, (4) Cys + AAA (10 μM), (5) Hcy, (6) NAC, (7) GSH (5.0 mM), (8) Ser, (9) Lys, (10) GSSG, and (11) PLE (1 U/mL). Cys (500 μM) effects on **OA-CysTCM-1** (12), and **OA-TCM-1** (13) toward COS/H<sub>2</sub>S release. H<sub>2</sub>S concentration was measured after 3-h incubation. The experiments were performed in triplicate and the results were expressed as mean ± SD (n = 3).

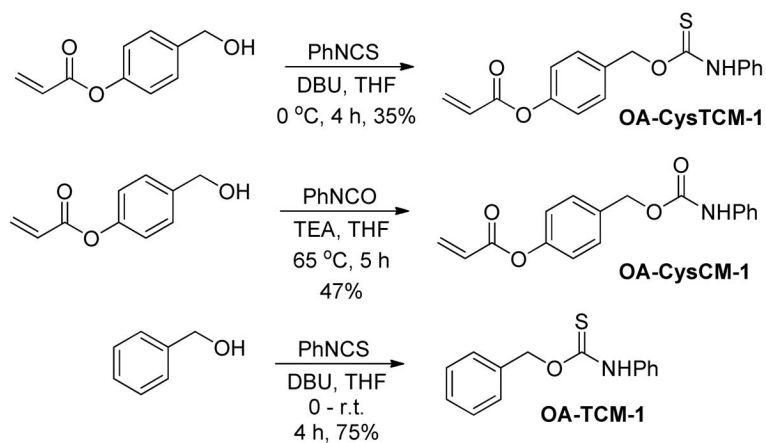




**Figure 4.** H<sub>2</sub>S Release from **OA-CysTCM-1** in bEnd.3 cells. Top: DIC (left) and GFP (right) channels with cysteine (250 μM) and SF7-AM (5 μM). Bottom: DIC (left) and GFP (right) channels with cysteine (250 μM), **OA-CysTCM-1** (100 μM), and SF7-AM (5 μM). Scale bar represents 100 μM.



**Scheme 1.**  
General design of Cys-triggered COS/H<sub>2</sub>S release from caged-thiocarbamate donors.



**Scheme 2.**  
Synthesis of **OA-CysTCM-1**, **OA-CysCM-1**, and **OA-TCM-1**.