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Esterase Activated Carbonyl Sulfide/Hydrogen Sulfide (H_2S) Donors

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Supporting Information

ABSTRACT: Hydrogen sulfide (H_2S) is a mediator of a number of cellular processes, and modulating cellular levels of this gas has emerged as an important therapeutic area. Localized generation of H_2S is thus very useful but highly challenging. Here, we report pivaloyloxymethyl-based carbonothioates and carbamothioates that are activated by the enzyme, esterase, to generate carbonyl sulfide (COS), which is hydrolyzed to H_2S.

Gaseous entities such as nitric oxide (NO),[1] carbon monoxide (CO),[2,3] sulfur dioxide (SO_2)[4−6] and hydrogen sulfide (H_2S)[7] have emerged as major mediators of cellular processes and have therapeutic applications.[3] For example, H_2S is involved in regulating the homeostasis of various physiological systems which include−cardiovascular, neuronal, gastrointestinal, renal, liver and reproductive systems.[8−11] Modulating levels of H_2S within cells has tremendous impact on disease biology.[10,12,13] Thus, methodologies for controlled generation as well as dissipation assume importance.[14] Due to its gaseous nature, site-specific delivery of H_2S is challenging. A common strategy to deliver gaseous species is to mask them as organic or inorganic compounds that dissociate to generate the gas.[15] These compounds, known as H_2S donors, are widely used to generate this gas within cells and in animal models.[10,15,16] Due to the ubiquitous targets of H_2S, localized delivery of H_2S is highly desirable but remains a major hurdle. Site-directed delivery requires installation of a structural trigger in the H_2S donor which is cleaved by a metabolic stimulus to generate H_2S.[17−24]

Carbonyl sulfide (COS) is a naturally occurring gas that hydrolyzes to H_2S. This reaction is accelerated by the widely prevalent enzyme, carbonic anhydrase (CA).[25] Thus, generating COS within cells is a newly emerging and an attractive strategy to localize H_2S within cells. Recently, Pluth and coworkers reported a new class of H_2S generating small molecules that are triggered by H_2S.[26] These thiocarbamates generate COS, which undergoes hydrolysis to produce H_2S (Figure 1a).[27] Thiocarbamates were proposed as analytical tools for detection of H_2S and may aid in analyte homeostasis. Matson and co-workers, more recently, reported *N*-thiocarboxyanhydride (NTA) as a nucleophile activated COS/H_2S donor (Figure 1b).[25] Here, a nucleophile, such as an amine reacts with NTA to generate COS/H_2S. However, due to the ubiquitous nature of nucleophiles in various biological media, selectivity of this donor may be compromised. Our laboratory considered the possibility of using enzyme-activated donors of COS/H_2S. The use of enzymes to deliver drugs, latent fluorophores (for imaging), or biological species[28,29] has been widely used and presents distinct advantages.[30] We considered carbonothioates (Figure 1c, **1**; X = O) as well as carbamothioates (Figure 1c, **2**; X = NH) as enzyme activated COS/H_2S donors. The significantly lower bond dissociation energy of a typical C−S bond (shown in green, Figure 1c) when compared with a C−O bond (shown in blue, Figure 1a) might facilitate the process of self-immolation.[31] It is anticipated that, upon activation, a carbonothioate (X = O) will dissociate rapidly in pH 7.4 buffer to produce COS and an alcohol (Figure 1c).

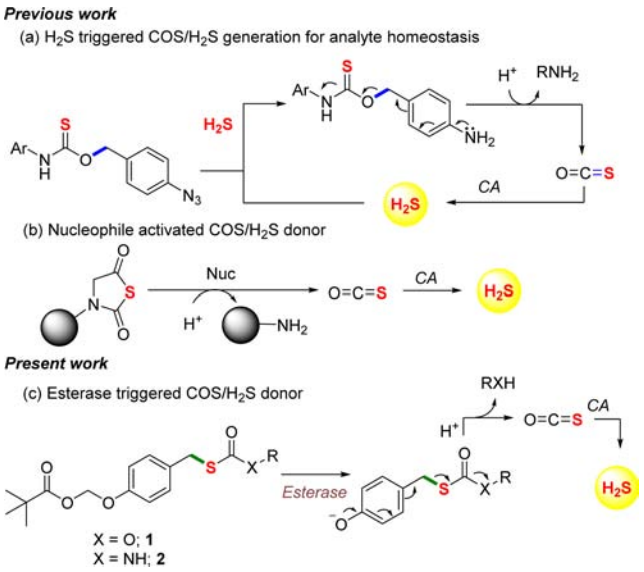

Figure 1. (a) Analyte homeostasis during H_2S detection is possible using azide-based thiocarbamates. (b) Nucleophile-activated COS/H_2S donors and their corresponding polymeric analogue. (c) Proposed enzyme-activated carbonothioate (**1**) and carbamothioate (**2**) COS/H_2S donors.

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In order to test if the nature of the leaving group X affects the rate of decomposition, carbamothioates (X = NH) were also considered (Figure 1c). Together, these compounds will offer an enzymatic trigger for H_2S generation and a better understanding of the nature of COS/H_2S donors. The enzyme that we chose was esterase (ES). Due to its wide occurrence in nearly all cells, this metabolic trigger will find broad use for the study of H_2S biology. The choice of a pivaloyloxymethyl group as the protective group will ensure rapid activation of the donor and help with localization of the bioactive molecule.[32]

In order to test this hypothesis, the thiol **4** was synthesized from **3** (Scheme 1). Reaction of **4** independently with **5a**, **5b**, and **5c** gave the desired compounds **1a**, **1b**, and **1c** (Table 1). Next, carbamothioates **2a**, **2b**, and **2c** were synthesized from the corresponding 4-nitrophenolates **6a**, **6b**, and **6c**, respectively by reaction with the thiol **4** (Table 1).[33]

Scheme 1. Synthesis of Thiol 4

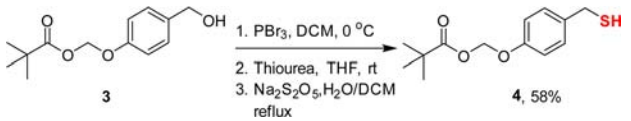
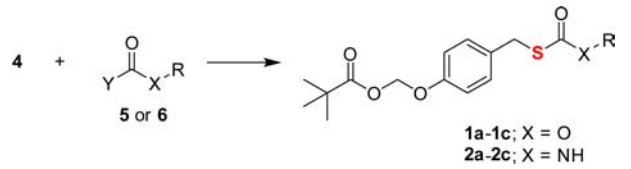


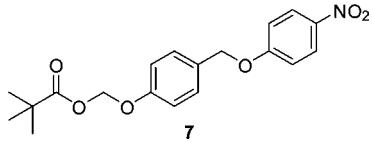
Table 1. Synthesis of Carbonothioates and Carbamothioates



X	Y	R	reactant	prod	yield, %[a]
O	Cl	4-NO_2Ph	**5a**	**1a**	77
O	Cl	Ph	**5b**	**1b**	64
O	Cl	CH_2Ph	**5c**	**1c**	16
NH	OAr[b]	Ph	**6a**	**2a**	11
NH	OAr[b]	4-OMePh	**6b**	**2b**	44
NH	OAr[b]	CH_2Ph	**6c**	**2c**	37

[a]Unoptimized isolated yields. [b]Ar = 4-NO_2Ph.

(4-((4-Nitrophenoxy)methyl)phenoxy)methyl pivalate **7** was prepared and used as a negative control. This compound should be cleaved by esterases but will not produce COS. The formation of 4-nitrophenol (as in the case of **1a**) will serve as a colorimetric indicator for mechanistic investigations.



Using Dansyl-Azide (Dn-N_3), a H_2S-sensitive fluorogenic dye, the formation of H_2S was assessed. This probe is known to react with HS^- to produce dansylamine (Dn-NH_2), which has a distinct fluorescence signal at 535 nm (excitation 340 nm). When authentic H_2S was treated with Dn-N_3, we find a fluorescence signal at 535 nm (excitation 340 nm) for Dn-NH_2 (Figure 2a).[34] Compound **1a** was incubated in the presence of ES and CA for 1 h, followed by addition of Dn-N_3. A fluorescence signal at 535 nm was indicative of the capability of this compound to generate H_2S under these conditions (Figure 2a). All other compounds **1**–**2** were similarly found to generate H_2S under these conditions (Figure 2a). Compound **7**, which is expected to dissociate in the presence of ES but does not generate H_2S, did not show a significant signal attributable to Dn-NH_2 in this assay (Figure 2a). The formation of the Dn-NH_2 fluorophore (see Supporting Information (SI), Figures S1, S2) was also independently confirmed by HPLC analysis of a reaction mixture containing **1c**, ES, CA, and Dn-N_3 (Figure 2b). The formation of H_2S during decomposition was also inferred by a lead acetate assay.[35] Here, incubation of **1c** with ES and CA followed by addition of an aliquot to lead acetate paper showed a distinct dark coloration that is indicative of the formation of lead sulfide (see SI, Figure S3).

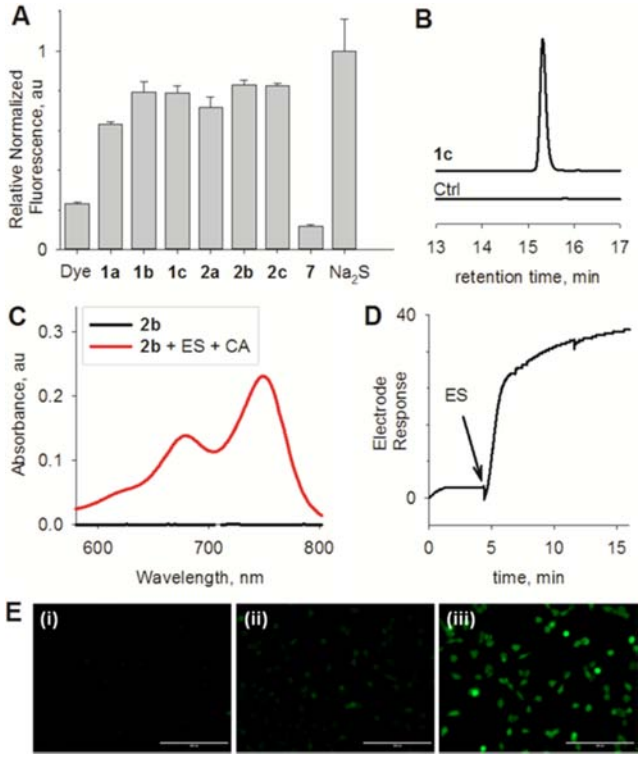


Figure 2. (a) H_2S yields were measured using a Dn-N_3-based assay. Compounds were incubated with ES and CA in pH 7.4 buffer for 60 min followed by addition of Dn-N_3. Fluorescence intensity at 535 nm (excitation 340 nm) was measured. (b) HPLC analysis of a reaction mixture consisting of **1c**, ES, CA, and Dn-N_3. Ctrl is Dn-N_3. Fluorescence detector was used: excitation 340 nm; emission 535 nm. Positive control: Na_2S (see SI, Figure S2). (c) Formation of methylene blue as determined by spectrophotometry during incubation of **2b** in the presence of ES and CA. Na_2S was used as the positive control in this experiment. (d) Representative trace for H_2S detection using a H_2S-sensitive electrode: **1a** was incubated in pH 7.4 buffer in the presence of CA. After 5 min, ES was added to the reaction mixture. (e) Representative images of MCF-7 cells. The H_2S-sensitive fluorogenic probe NBD-fluorescein (10 μM) was cotreated with (i) DMSO; (ii) **2b**, 50 μM; (iii) **2b**, 100 μM, for 40 min followed by imaging using a fluorescence microscope. Scale bar is 200 μm.

Independent verification of H_2S release from this class of compounds was carried out by a colorimetric assay where methylene blue formation is monitored (Figure 2c; see SI, Figure S5).[25] An absorbance profile that correlated well with authentic Na_2S was observed (see SI, Figure S4). When this experiment was carried out with acetazolamide, a known inhibitor of CA, we find nearly complete abrogation of the absorbance for methylene blue formation (see SI, Figure

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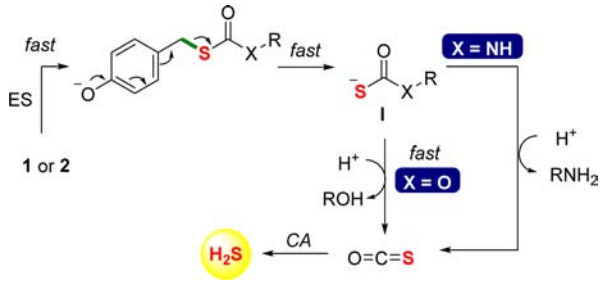
S6).[24,26] Lastly, carbonothioate **1a** was incubated with CA and a H_2S-selective electrode was used for detection of H_2S (Figure 2d). The formation of COS was verified by a reported mass spectrometry methodology, and a profile that is consistent with COS was recorded (see SI, Figure S7).[36]

The H_2S donors prepared herein were evaluated for their ability to inhibit proliferation of human breast cancer MCF-7 cells using a standard cell viability assay. We find no significant cytotoxicity at 25 μM (see SI, Figure S13). The H_2S generating capability of **2b** within cells was evaluated using a fluorogenic probe NBD-Fluorescein for H_2S (see SI, Figure S14).[37] Upon incubation of MCF-7 cells with this probe and subsequent treatment of cells with **2b**, we find a distinct fluorescence signal suggesting increased intracellular H_2S (Figure 2e). Together, these data support the formation of COS/H_2S under the assay conditions from donors prepared in this study and their compatibility for use in cellular studies.

Next, the kinetics of H_2S release from these compounds was studied. Using the Dn-N_3 method, the time course of Dn-NH_2 formation, which is a proxy for H_2S release, was monitored. A representative curve for H_2S release from **1a** is shown (see SI, Figure S1). The rate constant for H_2S release was found by fitting the initial rate data to first-order kinetics (see SI, Figure S1). The rate constants for H_2S release from **1a**, **1b**, and **1c** were found to be similar in magnitude suggesting no major dependence on the nature of the leaving group (see SI, Table S1, entries 1−3). In order to better understand the mechanism of decomposition (Scheme 2), the decomposition of **1a** was monitored by HPLC (see SI, Figure S8).

Scheme 2. Proposed Mechanism for Decomposition of Carbonothioates and Carbamothioates in the Presence of ES and CA



Upon addition of esterase, nearly complete disappearance of **1a** within 15 min was recorded suggesting that cleavage of the pivaloyloxymethyl group is fast.[32] During this study, concomitant formation of 4-nitrophenol was observed, which was confirmed by using an authentic sample of this compound (see SI, Figure S8). The time course of 4-nitrophenol formation during decomposition of **1a** was next independently recorded by spectrophotometry by monitoring the absorbance of 4-nitrophenol at 405 nm (see SI, Figure S9). The rate constant was found as 0.14 min^{-1}. When a similar experiment was conducted with **7** where 4-nitrophenol release does not involve the formation of COS, a comparable rate constant of 0.15 min^{-1} was recorded. Together, these data suggest the short-lived nature of intermediate I in the case of **1a**. Although the rate of 4-nitrophenol formation is faster than H_2S release as determined by Dn-NH_2 formation, these relatively small differences do not support significant accumulation of intermediate I (Scheme 2).[38] Next, H_2S release profiles of carbamothioates **2a**, **2b**, and **2c** in pH 7.4 buffer in the presence of ES and CA were recorded by using a similar Dn-N_3 assay. The relative rates for Dn-NH_2 formation during incubation of **2a** and **2b** were found to be comparable with those of **1a**−**1c** (see SI, Table S1, entries 4−5); it is thus likely that a similar mechanism is operational. The rate of H_2S release from **2b** was monitored by a methylene blue formation assay (see SI, Figure S5), and we found a similar rate when compared with the dansyl azide method.[39] HPLC analysis of the decomposition of **2b** (see SI, Figure S10) showed the formation of an intermediate (possibly I, Scheme 2) that decomposed to produce *p*-anisidine. Thus, rapid cleavage of the pivaloyloxymethyl group followed by self-immolation produces a short-lived intermediate I, which dissociates to produce COS. Hydrolysis of COS in the presence of CA produces H_2S.

When H_2S release from **2c** was monitored by the Dn-N_3 assay as well as methylene blue formation, we found a slower rate of release when compared with other compounds tested (see SI, Table S1 and Figure S5).[39] Decomposition of **2c** in the presence of ES was carried out: HPLC analysis revealed nearly complete disappearance of **2c** within 15 min suggesting the triggerable nature of this donor in the presence of the stimulus, esterase (see SI, Figure S11). An intermediate (possibly I, Scheme 2) was observed which gradually disappeared during the course of the reaction (see SI, Figure S12). We were unable to characterize this intermediate, but it appears that the rate of COS release may, in part, depend on the decomposition of this intermediate. Hence, the use of carbamothioates such as the ones developed in this work may offer distinct advantages in modulating H_2S release through stereoelectronic effects on the nitrogen.[40] Although these offer mechanistic insight, the differences among the release profiles are small, and in cellular experiments, the concentrations of these enzymes will, in part, determine the kinetics of H_2S release. Wang and co-workers reported an esterase triggered small molecule with tunable H_2S generating capability.[21] Here, the nature of the ester was modified to attain differences in rates of H_2S generation. The compounds prepared in our study can perhaps similarly be modified to achieve tunability. Furthermore, this scaffold is also amenable to incorporation of other triggers of interest that facilitate localized delivery of H_2S: Pluth and co-workers have reported a hydrogen peroxide inducible H_2S donor based on the thiocarbamate scaffold.[40] However, carbonothioates and carbamothioates prepared in this study, to our knowledge, have thus far not been reported as H_2S donors.[41]

In summary, we show that carbonothioates as well as carbamothioates can be suitably modified to be triggered to generate COS under physiologically relevant conditions. The importance of gaseous species in mediating cellular processes as well as close association with pathophysiological conditions has been widely studied. In order to better understand the precise roles of these gases, new and improved tools are necessary. While these tools are being developed, they are also being evaluated for their therapeutic potential. Numerous investigations have shown that H_2S has enormous potential but is limited by its toxicity. Therefore, being able to trigger H_2S generation intracellularly in a site of interest is of fundamental importance. Our data reveal that the cleavage of these compounds by esterase is rapid followed by generation of COS/H_2S. Although esterases are widely present in numerous cell types, this study lays the foundation for site-directed delivery of COS/H_2S.

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■ ASSOCIATED CONTENT

Supporting Information

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Preparative procedures, characterization data, and spectra (PDF)

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Notes

The authors declare no competing financial interest.

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(39) Morcos, E. F.; Kussrow, A.; Enders, C.; Bornhop, D. *Electrophoresis* **2010**, *31*, 3691. Although Dn-NH_2 is known to inhibit CA, we find that the rate of H_2S release from **2b** as independently determined by the Dn-N_3 and methylene blue methods are comparable (see SI, Figure S5). The origin of this effect is not clear, but as expected, a known CA inhibitor (acetazolamide) slows down H_2S release from **2b** (see SI, Figure S6).
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(41) The formation of a *p*-quinone-methide intermediate may potentially limit the utility of the compounds reported here as well as the ones reported in ref 40.

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