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Esterase-sensitive trithiane-based hydrogen sulfide donors†

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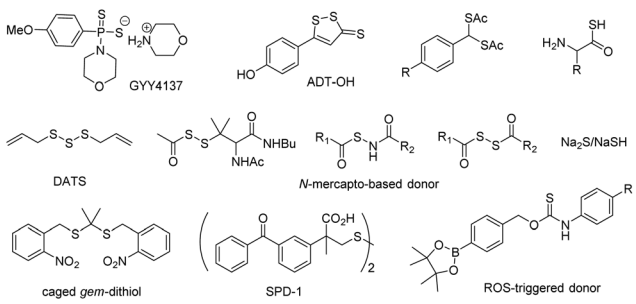
1,3,5-Trithiane functionalized with esterase-sensitive ester groups on the methylene linkers was developed as a class of enzymatic hydrolysis-based hydrogen sulfide (H_2S) donors. The amount of H_2S released from the donors was dependent on the number of ester bonds. The donors release H_2S in a controllable manner in the presence of an enzyme.

Hydrogen sulfide (H_2S) is a stinking, colorless, and toxic pollutant. Despite these demerits, it is an important biological molecule owing to its unique association with human health and disease.[3] Similar to carbon monoxide (CO) and nitric oxide (NO), it is a gasotransmitter and hence, an important gaseous signaling molecule in living systems.[1,2] In mammalian systems, biological H_2S is produced by endogenous enzymes such as cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE), 3-mercaptopyruvate sulfurtransferase (3-MST), and cysteine aminotransferase (CAT).[4–8] These enzymes primarily act on cysteine and homocysteine and convert them into H_2S either individually or cooperatively. This simple and general chemical process may release H_2S from the intracellular sulfane sulfur pool.[9] Chemical and biochemical catabolic reactions of both endogenous and exogenous H_2S are known to protect cells and organs against pathological damage or damage caused due to environmental exposure.[10–12] For example, H_2S can protect against myocardial ischemia reperfusion (MI/R) injury caused by endogenous reactive oxygen species (ROS) generated in the mitochondria.[13,14] Additionally, H_2S has been proven to be a potent anti-inflammatory biomolecule in animal models.[15] It relaxes vascular smooth muscles, reduces high blood pressure, and induces vasodilation of blood vessels.[16] These facts strongly establish the importance of H_2S in living systems. It is also evident that modulation of cellular H_2S levels can have potential therapeutic effects.

Researchers typically administer exogenous H_2S to modulate the H_2S concentration in cells. Easy access to relevant commercial reagents such as NaHS and Na_2S, which are H_2S sources, expedites the progress in research related to the use of this gas as a biomolecule. The sulfide salts are expected to be rapid H_2S-releasing species, based on the inherent pK_a values of H_2S under physiological conditions.[17,18] This phenomenon is substantially different from the endogenous production of H_2S in cells, and it may cause excess exposure to H_2S, resulting in unavoidable side effects.[19,20] Considering this, several types of H_2S-releasing synthetic molecules have been developed and categorized according to the manner in which they generate H_2S in a cell (Scheme 1), in order to explore the biological functions of H_2S.

The most common class of H_2S donors comprises the biothiol (Cys or GSH)-activated donors such as diallyl trisulfide (DATS), which is a natural organosulfur species[21] with a series of *N*-(benzoyl)-thiobenzamide derivatives.[22] Hydrolysis-based H_2S donors include GYY4137,[23] 1,2-dithiole-3-thiones (DTTs), and arylthioamide derivatives.[24,25] Photo-induced H_2S donors include caged *gem*-dithiols,[26] ketoprofenate-caged donors,[27] and *o*-nitrobenzyl-caged thiocarbamates.[28] Enzyme-triggered H_2S donors have also been reported.[29–32] Dithiolethione (ADT)



Scheme 1 Structures of H_2S donors.

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derivatives have been conjugated with a variety of therapeutics to enhance their biological activities.[33,34] H_2S production from lactonization of thioacids was found to exhibit anti-inflammatory effects.[22,35–37] Recently, ROS-triggered H_2S donors have also been developed; it was demonstrated that carbonyl sulfide (COS) generated from the donors is degraded by an enzyme and rapidly converted to H_2S.[38] pH-Dependent H_2S releasing donors have also been reported,[39,40] **3**, and are found to exhibit cardioprotective effects against myocardial ischemia-reperfusion (MI/R) injury.[39]

More recently, linear and cyclic polysulfides have been studied as H_2S donors and are classified as biothiol-activated H_2S donors (Fig. 1).[41] Among these cyclic polysulfides (Fig. 1), which are composed of methylene linkers and sulfur atoms, we found that 1,3,5-trithiane has a unique structure that is composed of a six-membered ring with alternating methylene linkers and thioether groups. The functionalization of three methylene linkers of this sulfur-enriched species may lead to a new class of H_2S donors. This encouraged us to design trithiane-based enzyme-triggered H_2S donors. Herein, we report the syntheses and evaluation of such trithiane-based donors that release H_2S in the presence of an esterase.

1,3,5-Trithiane, which has three sulfur atoms, could be considered as an ideal H_2S donor. If it can be hydrolyzed in aqueous media, the major products would be thioformic acids (O═CHSH), which could release H_2S by further hydrolysis. However, trithiane derivatives are very stable in air and water, although they can be decomposed at high temperatures.[42] To render the compound susceptible to hydrolysis, in order to trigger the release of H_2S from the hydrolyzed species under ambient conditions, we introduced acetate functionality to each methylene linker of 1,3,5-trithiane. The acetate functional groups may be sensitive to esterase, thereby producing an unstable intermediate that may undergo further hydrolysis to release H_2S (Scheme 2).

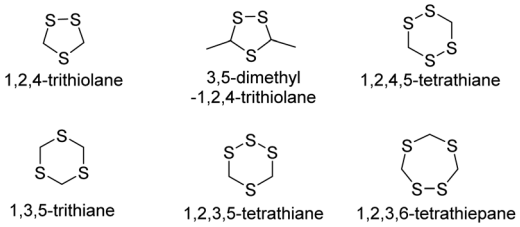
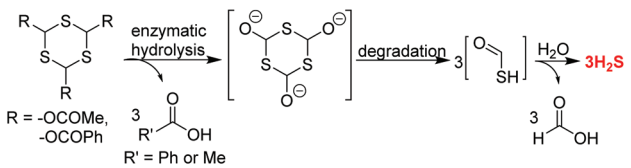
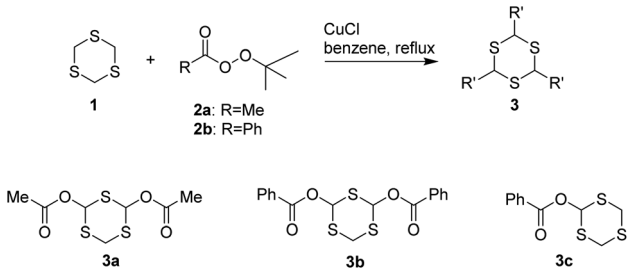


Fig. 1 Cyclic polysulfides established as H_2S donors.



Scheme 2 General concept of cyclic polysulfide-based hydrogen sulfide donors.



Scheme 3 Synthesis of trithiane-based H_2S donors.

To this end, acyloxylation of a cyclic thioether by peroxy esters in the presence of a catalytic amount of a cuprous salt (CuCl) was performed according to a modified method (Scheme 3).[43] 1,3,5-Trithiane was treated with excess *tert*-butyl peroxyacetate in dry benzene to introduce an acetate group into each methylene linker of the trithiane skeleton. However, the desired compound was not formed owing to steric hindrance. Instead, we obtained trithiane bisacetate **3a** as the major product. When **3a** was exposed to moist air, it quickly decomposed even at a low temperature and produced a distinctive rotten egg sulfur odor. To obtain trithiane monoacetate, we changed the molar ratio of **1** and **2a**. However, the compound could not be isolated due to its instability under the work-up conditions. To enhance the stability of the compound, we introduced a benzoate functionality on **1** using *tert*-butyl peroxybenzoate. A 3.5 : 1 mixture of peroxy ester **1** and **2b** was refluxed in the presence of 10 mol% CuCl in dry benzene. Trithiane bisbenzoate **3b** was obtained as the major product. A reversal in the ratio of the mixture of **1** and **2b** under the same reaction conditions afforded trithiane monobenzoate **3c**.

Although we could not obtain the functionalized trithiane donor depicted in Scheme 2, we speculated that **3b** and **3c** would be susceptible to enzymatic hydrolysis, affording fragments having thiols on them—these could undergo further hydrolysis to produce H_2S.

With these compounds in hand, we examined the feasibility of the concept of enzyme-responsive H_2S release from donors **3b** and **3c**. The amount of H_2S release was measured using an H_2S-sensitive fluorescent probe. Recently, a number of fluorescent probes have been developed for H_2S detection.[44,45] WSP-5, a fluorescent probe,[46] was selected to test the trithiane-based H_2S donors due to its fast reaction response. In these experiments, 200 μM of each donor was incubated in 5% DMSO in PBS buffer (pH 7.4) containing 50 μM of WSP-5. The changes in the fluorescence emission signals at 535 nm were detected for 4 h at the physiological temperature in the presence of porcine liver esterase (PLE) prepared in PBS. The fluorescence signals were finally converted into H_2S concentrations based on the standard calibration curve prepared from a series of NaHS standard solutions. First, we examined the capacity of esterase to catalyze H_2S release from the synthesized donors. Fig. 2 shows that **3b** and **3c** slightly decomposed in aqueous buffer and produced a negligible amount of H_2S. However, H_2S release from donors **3b** and **3c** in the presence of PLE led

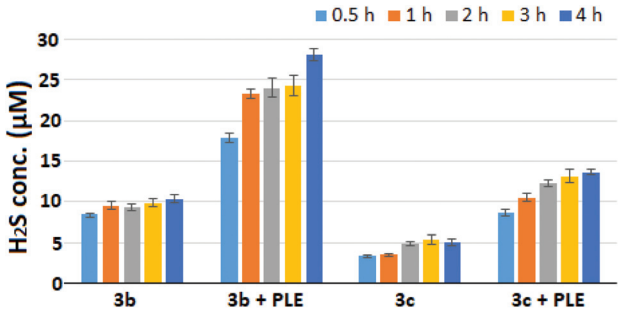


Fig. 2 Time-dependent H_2S-release profiles of donors **3b** and **3c** detected using a fluorescent probe, WSP-5. Each donor (200 μM) was incubated in PBS (5% DMSO) at 37 °C with 1 unit per mL of PLE. The concentration of WSP-5 was 50 μM, and fluorescence emission was recorded during the stated time intervals (p = 0.95, n = 3).

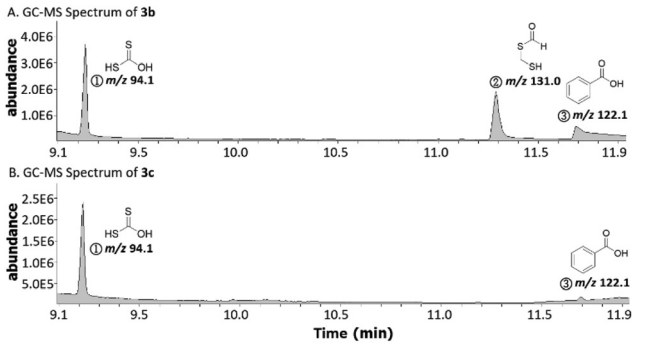
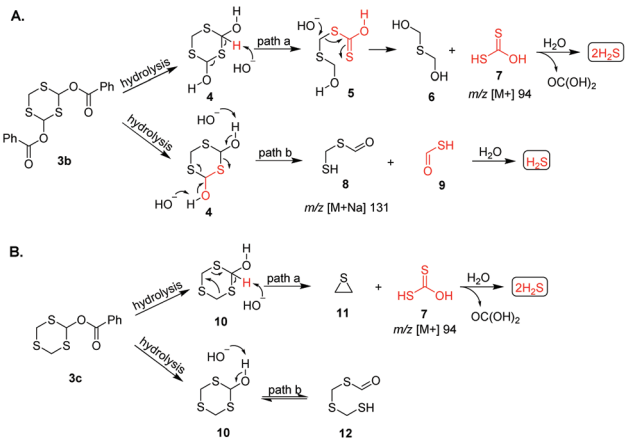


Fig. 3 GC-MS chromatograms of alkaline-hydrolyzed products of **3b** (A) and **3c** (B).



Scheme 4 Proposed mechanism of H_2S generation from the hydrolysis of **3b** (A) and **3c** (B) based on GC/MS analysis.

to more intense fluorescence than when **they** were incubated in aqueous buffer alone.

This indicates that esterase is responsive to the unusual ester bond, and the donors **3b** and **3c** indeed release H_2S by enzyme-catalyzed hydrolysis. Although the susceptibility of the donor to enzymatic hydrolysis was not high, they consistently released H_2S in a slow and sustainable manner. After 4 h, **3b** produced about 27.8 μM H_2S, while the concentration of H_2S released from **3c** was almost half that from **3b**. This observation led us to investigate the H_2S release mechanism, in which the esterase-catalyzed hydrolysis should be controlled by the number of ester bonds in the donors. The degradation of the bonds in **3b** and **3c** may generate the corresponding sulfur-containing intermediates, followed by the sustained release of H_2S in aqueous buffer.

To investigate the mechanism, we performed the alkaline hydrolysis of **3b** and **3c** using lithium hydroxide, in order to cleave the ester bonds. We expected that the hydrolysis of ester bonds accelerated the decomposition of donors, resulting in unstable fragments that release H_2S. To identify the important species, we tried to isolate the hydrolyzed products; however, we were not successful. Instead, we analyzed and identified the resulting hydrolyzed products by gas chromatography coupled with mass spectrometry (GC-MS). Interestingly, the GC-MS chromatograms showed only a few peaks upon the fast hydrolysis of compounds **3b** and **3c** (Fig. 3). Three major peaks at 9.2, 11.3, and 11.7 min were observed upon the hydrolysis of **3b** (Fig. 3A), while two major peaks at 9.2 and 11.7 min were observed upon the hydrolysis of **3c**; these were identical to the two peaks in the chromatogram of **3b** (Fig. 3B).

In the two chromatograms, we could clearly observe a peak corresponding to benzoic acid, which is the first hydrolyzed product. Peak 3 observed at a latter retention time (11.7 min) corresponded to the typical mass spectrum pattern of benzoic acid (m/z = 122.1) (ESI, Fig. S3†). Next, we analyzed peak 1 (m/z = 94.1), which was seen in the GC-MS chromatogram of both **3b** and **3c**. Based on the mass fragmentation pattern of this peak (m/z = 44.1, 61.0, 77.0, and 94.1, ESI, Fig. S1†), it was determined to arise from intermediate **4** and was attributed to carbonothionic acid **7** (Scheme 4). More specifically, under the hydrolysis conditions employed herein, species **4** underwent a rapid ring opening reaction by the abstraction of a labile acidic proton at the anomeric center, resulting in the formation of **7** (Scheme 4A, path a). This peak shows a typical M + 2 peak which is larger than usual due to the presence of a sulfur atom. Further hydrolysis of **7** should then afford H_2S through its tautomeric equilibrium leading to carbonic acid and 2 equiv. of H_2S like the hydrolysis pattern of thioacetic acid.[47] The ring opening of **4** also took place during enzymatic hydrolysis (Scheme 4A, path b), which resulted in the formation of Na-adduct species **8** (m/z = 131, see ESI, Fig. S2†). In this process, thioformic acid **9** is also formed, and should be hydrolyzed rapidly to H_2S due to its instability. **9** undergoes hydrolysis like thioacetic acid and gives formic acid and H_2S too.[47] In the case of **3c**, we can expect the ring opening of **10** to take place in a similar manner to that of **4** (Scheme 4B, path a), thereby yielding species **7**, as confirmed by the corresponding GC-MS chromatogram. Hydrolyzed species **10** can then undergo continued ring opening following enzymatic hydrolysis (Scheme 4B, path b). However, we were unable to detect **12** by GC/MS analysis, potentially due to the dominant

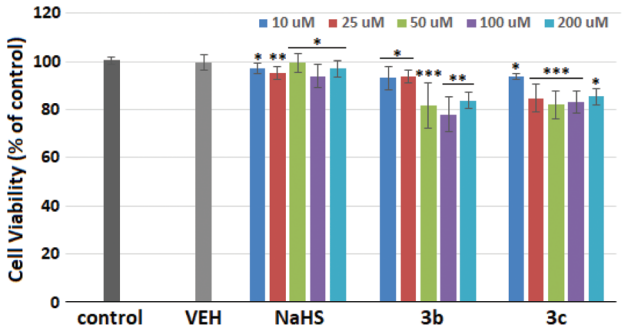


Fig. 4 Effect of the different H_2S donors on the cell viability of H9c2 cells. The cells were treated with various concentrations of **3b**, **3c**, and NaHS for 24 h. The cell counting kit-8 (CCK-8) assay was performed to measure the cell viability. The significant difference *versus* vehicle (***$p < 0.001$, **$P < 0.01$, *$P < 0.05$, $n = 4$).

path a. Based on this mechanistic study, we therefore assumed that **3b** produces about twice the quantity of H_2S compared to **3c** due to the presence of two benzoate groups in its structure. This is consistent with the H_2S measurements presented in Fig. 2.

Finally, we tested the H9c2 cell viability upon treatment with donors **3b** and **3c** to understand trithiane-based cellular cytotoxicity of the donors. For this purpose, sodium hydrogen sulfide (NaHS) was used as a control. Thus, the cells were treated with 10, 25, 50, 100, and 200 μM of each donor for 24 h, after which time the cell viability was determined using the cell counting kit-8 (CCK-8) assay. As shown in Fig. 4, neither **3b** or **3c**, nor NaHS exhibited any significant cellular toxicity to H9c2 cells at these doses.

Conclusions

In conclusion, we herein report the design and synthesis of trithiane-based H_2S donors that release H_2S following esterase-triggered hydrolysis. As a result of our evaluation of the H_2S releasing capabilities of these donors using fluorescence-based methods, we concluded that the structure containing two benzoate groups produced about twice the quantity of H_2S compared to an analogous structure containing only one benzoate group. Furthermore, following exploration of the H_2S release mechanism by GC-MS analysis, carbonothionic acid and thioformic acid were identified as the hydrolysis products for the donor containing two ester groups. These two compounds then undergo further hydrolysis to afford H_2S. In contrast, the donor containing a single ester group produced only carbonothionic acid. Studies are now ongoing in our group to determine the effects of these donors on various disease models.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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