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Cysteine Activated Hydrogen Sulfide (H₂S) Donors

Yu Zhao, Hua Wang, and Ming Xian

Department of Chemistry, Washington State University, Pullman, WA 99164.

Ming Xian: mxian@wsu.edu

Abstract

H₂S, the newly discovered gasotransmitter, plays important roles in biological systems. However, the research on H₂S has been hindered by lacking controllable H₂S donors which could mimic the slow and continuous H₂S generation process in vivo. Herein we report a series of cysteine-activated H₂S donors. Structural modifications on these molecules can regulate the rates of H₂S generation. These compounds can be useful tools in H₂S research.

Hydrogen sulfide (H₂S) is a noxious gas with the characteristic smell of rotten eggs. Recent studies recognized H₂S as the third gaseous transmitter beside nitric oxide (NO) and carbon monoxide (CO) that influence various physiological processes.¹ H₂S has been shown to relax vascular smooth muscles, mediate neurotransmission, elicit hibernation, inhibit insulin signalling, regulate inflammation and blood vessel caliber.¹ Endogenous formation of H₂S is achieved by enzymes such as cystathionine-β-synthase (CBS) in the brain and cystathionine γ-lyase (CSE) in liver, vascular and non-vascular smooth muscle. Although its exact chemical and biochemical modes of action are still not fully understood, levels of H₂S in the brain and vasculature have unambiguously been associated with human health and disease.¹

To study the physiological and pathophysiological properties of H₂S, the direct use of H₂S gas or NaHS in aqueous solutions are typical. However, the therapeutic potential of H₂S gas seems to be limited due to difficulties in obtaining precisely controlled concentrations and possible toxic impact of H₂S excess. NaHS, although widely used as a research tool, is a short-lasting donor which does not mimic the slow and continuous process of H₂S generation in vivo. In addition, NaHS in aqueous solution can be rapidly oxidized by O₂. Modifications that are made between the time that a solution is prepared and the time that the biological effect is measured can dramatically affect results. Due to these limitations, H₂S-releasing agents (i.e. H₂S donors) are considered useful tools in the study of H₂S.^{1,2} However, currently available H₂S donors are very limited.^{1,2} Besides NaHS, only three types of H₂S donors have been reported (Scheme 1): 1) garlic-derived polysulfide compounds, such as diallyl trisulfide (DATS). H₂S release from DATS was suggested to mediate the vasoactivity of garlic.³ 2) GYY4137, a Lawesson's reagent derivative, is a synthetic H₂S donor.⁴ This molecule decomposes spontaneously in aqueous buffers to release H₂S. 3) A dithiolthione moiety as a H₂S donor has been used to prepare H₂S-nonsteroidal anti-inflammatory drug hybrids like S-diclofenac.⁵ In addition, biological thiols such as cysteine and glutathione can be H₂S donors upon enzymatic or thermal treatment.⁶ A limitation of these known donors is that H₂S release is too fast to mimic biological H₂S generation. Given the structural characters of these compounds, little can be done to modify their structures to control the release of H₂S. Therefore, developing new H₂S donors with

Correspondence to: Ming Xian, mxian@wsu.edu.

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controllable H₂S generation capability is critical for this field. Ideal H₂S donors, from therapeutic point of view and for the applications in H₂S-related biological research, should release H₂S slowly and in moderate amounts.² The donors should also be stable compounds that can be easily handled by researchers.

In our recent studies of *S*-nitrosothiols,⁷ we noticed that S-N bonds are unstable and easy to break under certain conditions. Such a property triggered our idea to develop controllable H₂S donors based on S-N bonds. We envisioned that *N*-mercapto compounds like **1** could be potential H₂S donors (Scheme 2). As *N*-SH derivatives are unstable species, we expected a protecting group on SH should enhance the stability. In addition, the protecting group could allow us to design different activation strategies to generate **1**, therefore achieving controllable H₂S release.

In our first generation design of *N*-mercapto based H₂S donors, we decided to use acyl groups as the protecting group. As shown in Scheme 3, we expected compounds like **2** should react with cellular cysteine via a native chemical ligation (NCL) to produce *N*-SH **1** and then the cleavage of S-N bond of **1** should produce H₂S.

To test this idea, a series of *N*-(benzoylthio)benzamide derivatives (**5a–5l**) were prepared from the corresponding thiobenzoic acids (Scheme 4, see supporting information for details). We expected different substituents could affect the reaction rates of compounds **5** with cysteine, therefore regulating the rate of H₂S generation.

Compounds **5a–5l** proved to be stable in aqueous buffers. As shown in Scheme 5, they do not react with potential cellular nucleophiles such as –OH and –NH₂ groups. However, in the presence of cysteine, we observed a time-dependent decomposition of the donors and H₂S release. The formation of H₂S was monitored by a 2-mm H₂S-selective microelectrode (ISO-H₂S-2; WPI) attached to an Apollo 1100 Free Radical Analyser (WPI). A typical H₂S generation curve in pH 7.4 buffer is shown in Figure 1. In the presence of excess of cysteine, the concentration of H₂S released from **5a** reached a maximum value at 18 min (peaking time), and then started to drop, presumably due to oxidation by air. We also measured H₂S generation under other pH including pH 5.5 and pH 9.0. Similar releasing curves were observed (see supporting information).

We believe peaking time and H₂S concentration at peaking time are useful parameters to assess the rate of H₂S generation from donors. Therefore, peaking time and H₂S concentration of **5a–5l** in pH 7.4 PBS buffer were measured and summarized in Table 1. In general, electron donating groups led to slow generation of H₂S while electron withdrawing groups led to fast generation. These results proved that controllable H₂S release can be achieved by structural modifications on donors.

It is known that plasma could contain significant amount of free cysteine.⁸ We therefore measured H₂S generation of **5** in plasma (containing ~500 μM cysteine) using a colorimetry method.⁹ We observed a similar time-dependent H₂S release (Figure 2 illustrated an example using **5a**) as the one shown in Figure 1. However, when plasma was first treated with *N*-methylmaleimide (NMM) to block free cysteine, no H₂S generation was observed. These results demonstrated the capability of H₂S release from *N*-(benzoylthio)benzamide-based donors in complex biological systems. It is also demonstrated that cysteine is the regulator of this type of donors.

Finally, to understand the mechanism of H₂S generation from *N*-(benzoylthio)benzamides, we analyzed the reaction between **5a** and cysteine (10 eq). As shown in Scheme 6, we confirmed the formation of *N*-acyl cysteine **7**, benzamide **9**, and cystine **11** in high yields. Based on the products observed, we proposed the following mechanism: this reaction is

initiated by a reversible thiol exchange between **5a** and cysteine to first generate a new thioester **6** and *N*-mercapto-benzamide **8**. Compound **6** then undergoes a fast *S* to *N* acyl transfer to form amide **7**. This process is similar to the well-known native chemical ligation. Meanwhile the reaction between **8** and excess cysteine should lead to benzamide **9** and cysteine perthiol **10**. Finally the reaction between **10** and cysteine should complete the generation of H₂S and provide cystine **11**.

In summary, a series of new H₂S donors have been developed based on the *N*-(benzoylthio)benzamide template. These compounds are stable in aqueous buffers. H₂S generation from these compounds is regulated by cysteine. We have proved that H₂S release rates from these compounds are controllable upon structural modifications. It should note that H₂S release rates shown in Table 1 can only serve as a reference to predict H₂S release capability of these donors. In complex biological systems the perthiol intermediate (i.e. compound **10**-Scheme 6) may react with other redox active biomolecules. Therefore, actual H₂S release rates in such systems might be quite different. In addition, in some biological systems, free cysteine might be lacking due to disulfide formation or bound to proteins. When these donors are applied in such systems, extra cysteine must be added together with the donors in order to produce H₂S, which may compromise the redox balance of the system under study. Therefore, careful control experiments are needed to clarify the potential problem. Nevertheless, *N*-(benzoylthio)benzamides provide a new H₂S donor option to the researchers and we expect them will be useful tools in H₂S studies. Further development of *N*-mercapto based H₂S donors and evaluation of their biological activities are currently undergoing in our laboratory.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.
H₂S generation curves from **5a**.

Figure 2.
H₂S generation from **5a** in plasma.

Scheme 1.
Current H₂S donors.

Scheme 2.

N-Mercapto compounds as H₂S donors.

Scheme 3.
Proposed cysteine-activated H₂S donors.

5a: $R_1 = \text{H}$, $R_2 = \text{H}$, **5b:** $R_1 = \text{H}$, $R_2 = p\text{-F}$, **5c:** $R_1 = \text{H}$, $R_2 = p\text{-CF}_3$,
5d: $R_1 = \text{H}$, $R_2 = m\text{-Cl}$, **5e:** $R_1 = \text{H}$, $R_2 = o\text{-Me}$, **5f:** $R_1 = \text{H}$, $R_2 = m\text{-Me}$,
5g: $R_1 = \text{H}$, $R_2 = p\text{-Me}$, **5h:** $R_1 = \text{H}$, $R_2 = o\text{-OMe}$,
5i: $R_1 = \text{H}$, $R_2 = m\text{-OMe}$, **5j:** $R_1 = \text{H}$, $R_2 = p\text{-OMe}$,
5k: $R_1 = o\text{-OMe}$, $R_2 = p\text{-OMe}$, **5l:** $R_1 = \text{H}$, $R_2 = p\text{-N}(\text{CH}_3)_2$

Scheme 4.
Synthesis of *N*-(benzoylthio)benzamides.

Scheme 5.
 H_2S generation from *N*-(benzoylthio)benzamides.

Scheme 6.
Proposed H₂S generation mechanism.

Table 1

H₂S generation peaking time.

donors	peaking time (min)	[H ₂ S] (μM)	donors	peaking time (min)	[H ₂ S] (μM)
18	25.4		22	31.0
16	35.2		25	20.8
13	35.6		30	24.2

donors	peaking time time (min)	peaking time [H ₂ S] (μM)	donors	peaking time time (min)	peaking time [H ₂ S] (μM)
 5d	14	30.7	 5j	25	21.5
 5e	25	26.1	 5k	50	23.0

donors	peaking time [H ₂ S] (μM)	peaking time (min)	donors	peaking time (min)	peaking time [H ₂ S] (μM)
	18	31.4		22	17.5