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Cysteine-Activated Hydrogen Sulfide (H_2S) Donors

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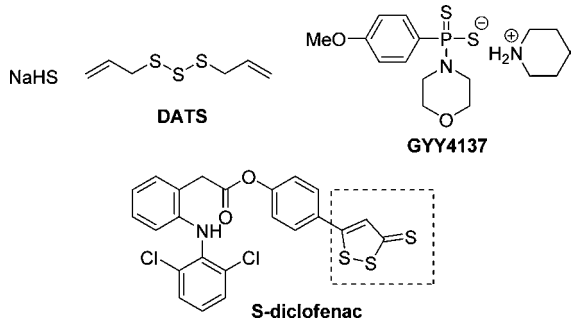
Abstract: H_2S, the newly discovered gasotransmitter, plays important roles in biological systems. However, the research on H_2S has been hindered by the lack of controllable H_2S donors that could mimic the slow and continuous H_2S generation process in vivo. Herein we report a series of cysteine-activated H_2S donors. Structural modifications of these molecules can regulate the rates of H_2S generation. These compounds can be useful tools in H_2S research.

Hydrogen sulfide (H_2S) is a noxious gas with the characteristic smell of rotten eggs. Recent studies have recognized H_2S as the third gaseous transmitter, in addition to nitric oxide (NO) and carbon monoxide (CO), that influences various physiological processes.[1] H_2S has been shown to relax vascular smooth muscles, mediate neurotransmission, elicit hibernation, inhibit insulin signaling, and regulate inflammation and blood vessel caliber.[1] Endogenous formation of H_2S is achieved by enzymes such as cystathionine-β-synthase (CBS) in the brain and cystathionine γ-lyase (CSE) in the liver and vascular and nonvascular smooth muscle. Although its exact chemical and biochemical modes of action are still not fully understood, levels of H_2S in the brain and vasculature have unambiguously been associated with human health and disease.[1]

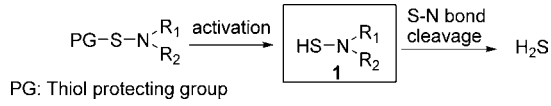
To study the physiological and pathophysiological properties of H_2S, the direct use of H_2S gas or NaHS in aqueous solutions is typical. However, the therapeutic potential of H_2S gas seems to be limited because of difficulties in obtaining precisely controlled concentrations and the possible toxic impact of excess H_2S. NaHS, although widely used as a research tool, is a short-lived donor that does not mimic the slow and continuous process of H_2S generation in vivo. In addition, NaHS in aqueous solution can be rapidly oxidized by O_2. Modifications that are made between the time that a solution is prepared and the time that the biological effect is measured can dramatically affect the results. Because of these limitations, H_2S-releasing agents (i.e., H_2S donors) are considered useful tools in the study of H_2S.[1,2] However, only a very limited number of H_2S donors are currently available.[1,2] Besides NaHS, only three types of H_2S donors have been reported (Scheme 1): (1) Garlic-derived polysulfide compounds, such as diallyl trisulfide (DATS). H_2S release from DATS has been suggested to mediate the vasoactivity of garlic.[3] (2) GYY4137, a derivative of Lawesson's reagent, is a synthetic H_2S donor.[4] This molecule decomposes spontaneously in aqueous buffers to release H_2S. (3) A dithiolthione moiety as a H_2S donor has been used to prepare H_2S−nonsteroidal anti-inflammatory drug hybrids such as S-diclofenac.[5] In addition, biological thiols such as cysteine and glutathione can be H_2S donors upon enzymatic or thermal treatment.[6] A limitation of these known donors is that the H_2S release is too fast to mimic biological H_2S generation. In view of the structural characters of these compounds, little can be done to modify their structures to control the release of H_2S. Therefore, the development of new H_2S donors with controllable H_2S generation capability is critical for this field. From the therapeutic point of view and for applications in H_2S-related biological research, ideal H_2S donors should release H_2S slowly and in moderate amounts.[2] The donors should also be stable compounds that can be easily handled by researchers.

Scheme 1. Current H_2S Donors



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 H_2S donors based on S−N bonds. We envisioned that *N*-mercapto compounds such as **1** could be potential H_2S donors (Scheme 2). As *N*-SH derivatives are unstable species, we expected that 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**, thereby achieving controllable H_2S release.

Scheme 2. *N*-Mercapto Compounds as H_2S Donors

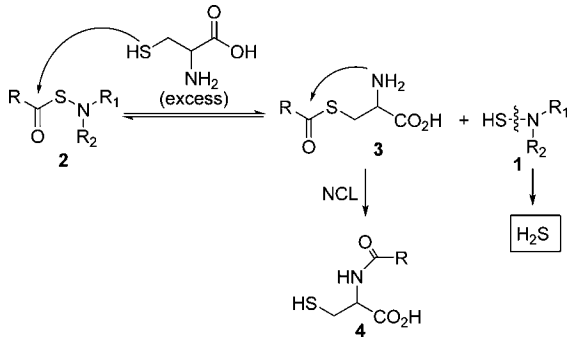


In our first-generation design of *N*-mercapto-based H_2S donors, we decided to use an acyl group as the protecting group. As shown in Scheme 3, we expected compounds such as **2** to react with cellular cysteine via native chemical ligation (NCL) to produce *N*-SH derivative **1**, and subsequent cleavage of the S−N bond in **1** should produce H_2S.

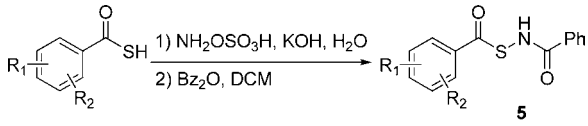
To test this idea, a series of *N*-(benzoylthio)benzamide derivatives **5a**−**l** were prepared from the corresponding thiobenzoic acids (Scheme 4; see the Supporting Information for details). We expected that different substituents would affect the reaction rate of compounds **5** with cysteine, thereby allowing the rate of H_2S generation to be regulated.

Compounds **5a**−**l** proved to be stable in aqueous buffers. As shown in Scheme 5, they do not react with potential cellular nucleophiles such as −OH and $-NH_2$ groups. However, in the presence of cysteine, we observed time-dependent decomposition of the donors accompanied by H_2S release. The formation of H_2S

Scheme 3. Proposed Cysteine-Activated H_2S Donors

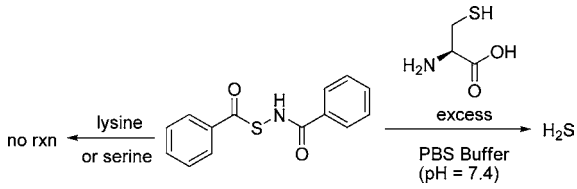


Scheme 4. Synthesis of *N*-(Benzoylthio)benzamides



5a: R_1 = H, R_2 = H, **5b**: R_1 = H, R_2 = *p*-F, **5c**: R_1 = H, R_2 = *p*-CF_3,
5d: R_1 = H, R_2 = *m*-Cl, **5e**: R_1 = H, R_2 = *o*-Me, **5f**: R_1 = H, R_2 = *m*-Me,
5g: R_1 = H, R_2 = *p*-Me, **5h**: R_1 = H, R_2 = *o*-OMe,
5i: R_1 = H, R_2 = *m*-OMe, **5j**: R_1 = H, R_2 = *p*-OMe,
5k: R_1 = *o*-OMe, R_2 = *p*-OMe, **5l**: R_1 = H, R_2 = *p*-$N(CH_3)_2$

Scheme 5. H_2S Generation from *N*-(Benzoylthio)benzamides



was monitored by a 2 mm H_2S-selective microelectrode (ISO-H_2S-2; WPI) attached to an Apollo 1100 free-radical analyzer (WPI). A typical H_2S generation curve in pH 7.4 buffer is shown in Figure 1. In the presence of excess of cysteine, the concentration of H_2S released from **5a** reached a maximum value at 18 min (the "peaking time"), and then started to decrease, presumably as a result of oxidation by air. We also measured H_2S generation at other pH, including pH 5.5 and 9.0. Similar release curves were observed (see the Supporting Information).

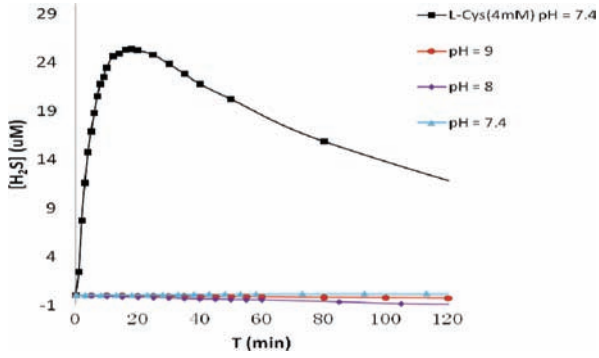
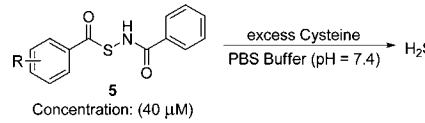
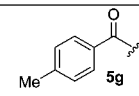
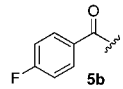
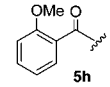
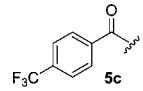
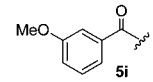
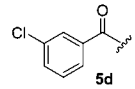
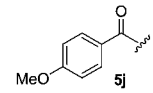
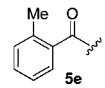
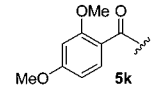
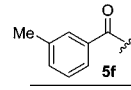
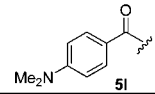


Figure 1. H_2S generation curves from **5a**.

We believe that the peaking time and the H_2S concentration at the peaking time are useful parameters to assess the rate of H_2S generation from donors. Therefore, the peaking times and corresponding H_2S concentrations of **5a**−**l** in a pH 7.4 phosphate-buffered saline buffer were measured, and the results are summarized in Table 1. In general, electron-donating groups led to slower generation of H_2S while electron-withdrawing groups led to faster generation. These results proved that controllable H_2S release can be achieved by structural modifications of the donors.

Table 1. H_2S Generation Peaking Times



donors	peaking time (min)	peaking time [H_2S] (μM)	donors	peaking time (min)	peaking time [H_2S] (μM)
 **5a**	18	25.4	 **5g**	22	31.0
 **5b**	16	35.2	 **5h**	25	20.8
 **5c**	13	35.6	 **5i**	30	24.2
 **5d**	14	30.7	 **5j**	25	21.5
 **5e**	25	26.1	 **5k**	50	23.0
 **5f**	18	31.4	 **5l**	22	17.5

It is known that plasma can contain significant amount of free cysteine.[8] We therefore measured H_2S generation of **5** in plasma containing ~500 μM cysteine using a colorimetry method.[9] We observed a time-dependent H_2S release similar to the one shown in Figure 1 (Figure 2 shows the results obtained using **5a**). However, when the plasma was first treated with *N*-methylmaleimide (NMM) to block free cysteine, no H_2S generation was observed. These results demonstrate the capability of *N*-(benzoylthio)benzamide-based donors to release H_2S in complex biological systems and also show that cysteine is the regulator of this type of donor.

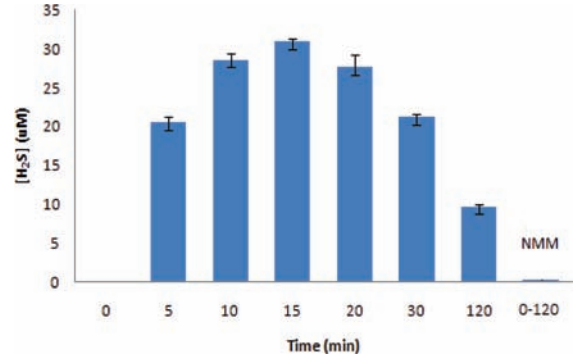
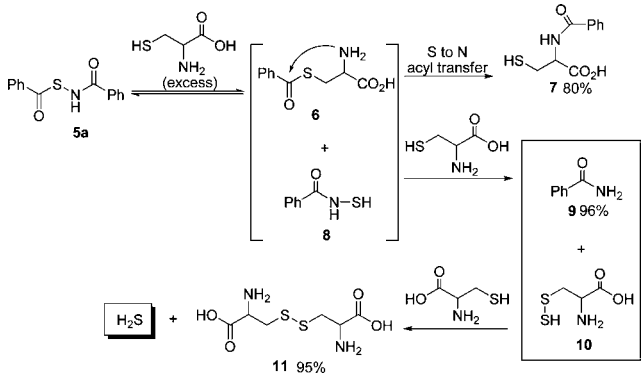


Figure 2. H_2S generation from **5a** in plasma.

Finally, to understand the mechanism of H_2S generation from *N*-(benzoylthio)benzamides, we analyzed the reaction between **5a**

and cysteine (10 equiv). As shown in Scheme 6, we confirmed the formation of *N*-acylcysteine **7**, benzamide (**9**), and cystine (**11**) in high yields. On the basis of the products observed, we propose the following mechanism: The reaction is initiated by reversible thiol exchange between **5a** and cysteine to first generate the new thioester **6** and *N*-mercaptobenzamide (**8**). Compound **6** then undergoes fast S-to-N acyl transfer to form amide **7**. This process is similar to the well-known NCL reaction. Meanwhile, the reaction between **8** and excess cysteine should lead to **9** and cysteine perthiol (**10**). Finally, the reaction between **10** and cysteine should complete the generation of H_2S and provide **11**.

Scheme 6. Proposed Mechanism of H_2S Generation



In summary, we have developed a series of new H_2S donors based on the *N*-(benzoylthio)benzamide template. These compounds are stable in aqueous buffers. H_2S generation from these compounds is regulated by cysteine. We have proved that H_2S release rates from these compounds are controllable through structural modifications. It should be noted that the H_2S release rates shown in Table 1 can serve only as a reference for predicting the H_2S release capabilities of these donors. In complex biological systems, the perthiol intermediate (i.e., compound **10** in Scheme 6) may react with other redox-active biomolecules. Therefore, the actual H_2S release rates in such systems might be quite different. In addition, in some biological systems, free cysteine might be lacking because of disulfide formation or binding to proteins. When one of these donors is applied in such a system, extra cysteine must be added together with the donor in order to produce H_2S, which may compromise the redox balance of the system under study. Therefore, careful control experiments are needed in order to clarify the potential problem. Nevertheless, *N*-(benzoylthio)benzamides provide researchers with new H_2S donor options, and we expect them to be useful tools in H_2S studies. Further development of *N*-mercapto-based H_2S donors and evaluation of their biological activities are currently ongoing in our laboratory.

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Supporting Information Available: Spectroscopic and analytical data and selected experimental procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

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