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Controllable Thioester-Based Hydrogen Sulfide Slow-releasing Donors as Cardioprotective Agents

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Hydrogen sulfide (H_2S) is an important signaling molecule with promising protective effects in many physiological and pathological processes. However, the study on H_2S has been impeded by the lack of appropriate H_2S donors that could mimic its slow-releasing process in vivo. Herein, we report the rational design, synthesis, and biological evaluation of a series of thioester-based H_2S donors. These cysteine-activated H_2S donors release H_2S in a slow and controllable manner. Most of the donors comprising allyl moiety showed significant cytoprotective effects in H9c2 cellular models of oxidative damage. The most potent donor 5e decreased the mitochondrial membrane potential (MMP) loss and lactate dehydrogenase (LDH) release in H_2O_2-stimulated H9c2 cells. More importantly, donor 5e exhibited potent cardioprotective effect in an in vivo myocardial infarction (MI) mice model by reducing myocardial infarct size and cardiomyocytes apoptosis. Taken together, our studies demonstrated that these new allyl thioesters are potential cardioprotective agents by releasing H_2S.

Introduction

H_2S has been recognized as an important signaling molecule owing to its significant functions in many aspects of human health and disease.[1] Many studies have shown various therapeutic effects of H_2S, including vasodilation, anti-inflammation, anti-oxidation, and cardiovascular protection.[2] In order to explore the physiological and pathological properties of H_2S, researchers used inorganic sulfide salts such as Na_2S and NaHS to mimic endogenous H_2S generation.[3] However, the uncontrollable and rapid release of H_2S from sulfide salts does not mimic the slow and controllable H_2S production in the living system, which greatly limits their usage.[4] Considering these disadvantages, small H_2S-releasing organic molecules (H_2S donors) have been developed and used as the primary tools, mainly including diallyl trisulfide (DATS), derivative of Lawesson's reagent (GYY4137), dithiolethione (ADT-OH), geminal-dithiol species (*gem*-dithiol) and so on (Figure 1).[5] More recently, donors based on esterase-activation, pH-modulation, ROS-activation and cysteine-activation as well as their H_2S-related biological effects have been reported.[6]

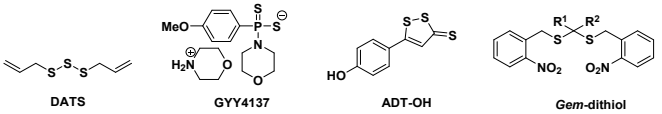

Figure 1. Representative H_2S donors.

It should be noted that although a number of H_2S donors have been reported, ideal donors with controllable and slow H_2S-releasing properties are still lacking.[7] Recently, Liang and co-workers clarified the misunderstanding of diallyl disulfide (DADS) as a rapid H_2S-releasing donor, which was attributed to its DATS impurity.[8] The results showed that H_2S was released in fact *via* a sluggish reaction of DADS with GSH through an α-carbon nucleophilic substitution pathway within hours. Thus, we envisioned the core structure of DADS (diallyl disulfide) to be valuable template for the design of H_2S slow-releasing donors (Scheme 1, Step c).

In addition, due to the ubiquitous targets of H_2S, localized generation of H_2S is highly desirable but remains a great challenge. Site-directed delivery of H_2S by donors ideally shall be triggered by specific cellular species or events.[9] H_2S has been found to improve cardiac functions and serve as a cardioprotectant for the treatment of heart failure and ischemic heart diseases.[10] Under the condition of cardiovascular injury, oxidative stress triggered by reactive oxygen species (ROS) including superoxide, hydroxyl radical and hydrogen peroxide plays a vital role.[11] As it is well known that the sulfide can be easily converted into disulfide under oxidative conditions, the oxidative stress in the injured cells could be slickly used to trigger the localized generation of H_2S within injured cells (Scheme 1, Step b). [12]

Based on the above researches, we envision that allyl mercaptan is a valuable template for the design of H_2S slow-releasing donors with cardioprotective effect. However, the simplicity and instability of allyl mercaptan limit its direct application. Since transthioesterification is a common strategy to transfer thioester to thiol in the presence of Cys or GSH (Scheme 1, Step a).[13] We

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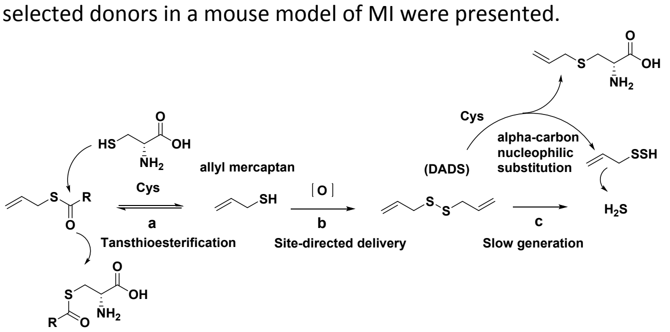
designed a series of thioester-based hydrogen sulfide donors, which could release H_2S in a controllable and slow manner.

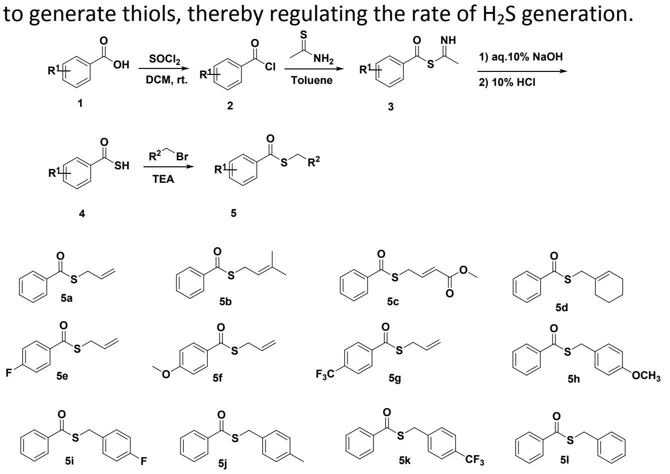
Herein, we reported a class of thioester-based H_2S donors with novel H_2S-releasing mechanism and *in vitro* H_2S-releasing kinetics. Also their protective effects on H9c2 cells subjected with H_2O_2 challenge were evaluated, and the cardioprotective effects of selected donors in a mouse model of MI were presented.



Scheme 1. Proposed controllable thioester-based hydrogen sulfide slow release donors.

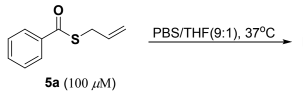
Result and discussion

To test this idea, a series of thioester derivatives **5a-l** were prepared and the synthesis route is illustrated in Scheme 2. Various readily available benzoic acids were used as the starting materials. The treatment of acids with thionyl chloride afforded acyl chlorides **2** as the reactive intermediates, which were further reacted with thioacetamide in toluene to give compounds **3** in almost quantitive yields. After hydrolysis, various thiobenzoic acids **4** were obtained, which were treated with alkyl bromides or benzyl bromides directly in the presence of triethylamine (TEA) to provide the desired compounds **5a-l** in 72-88% total yields. We expected that different substituents would affect the reaction rates of compounds **5** with Cys to generate thiols, thereby regulating the rate of H_2S generation.



Scheme 2. Synthesis of thioester-based hydrogen sulfide donors **5a-l**.

With these compounds in hand, we tested their H_2S-releasing kinetics in aqueous buffers firstly. These thioesters were stable in PBS buffers, but a time- and dose-dependent H_2S release was detected when Cys was added into the solutions using the standard methylene blue (MB) method.[14] Finally, the optimized condition was determined to be 100 μM donors in PBS buffer (pH 7.4, 50 mM) containing 10% THF in the presence of 10 equiv of Cys. As shown in Figure 2, taking compound **5a** as the example, a maximum of 41.5 μM of H_2S at 8 h was detected from 100 μM donor in PBS buffer containing 1 mM Cys. To our surprise, other amino acid, like Gly or Lys, couldn't trigger the H_2S release of **5a**, we supposed that it might be due to the weaker nucleophilicity of those amino acids. We then systematically compared the H_2S-releasing capabilities of these donors, and the data are summarized in Table 1. In contrast to DATS (48.8%, 0.35 h), the results of our H_2S donors showed that up to 52.2 % of the H_2S can be detected (compound **5f**) and the release time lasts up to 8.5 h (compound **5k**). All those thioester-based H_2S donors could release H_2S in a much slower manner than DATS.



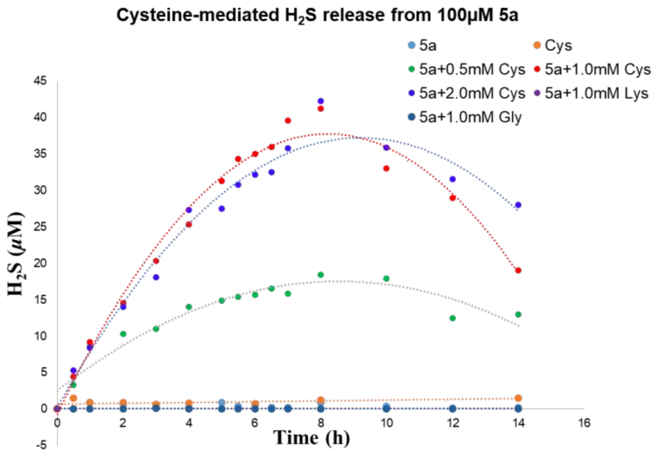
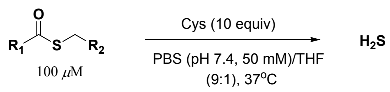


Figure 2. Cysteine mediated H_2S release from compound **5a**.

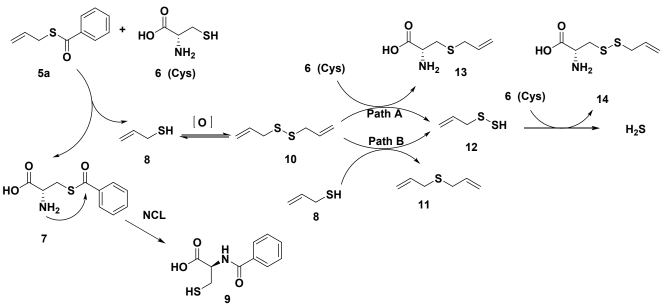
Table 1. H_2S-releasing profiles of thioester-based hydrogen sulfide donors.



Donors	T_{peak} (h)	$[H_2S]_{peak}$ (μM)	Donors	T_{peak} (h)	$[H_2S]_{peak}$ (μM)
5a	8.0	41.5	**5h**	3.5	19.4
5b	7.0	17.5	**5i**	5.0	15.6
5c	6.5	38.3	**5j**	3.0	24.3
5d	5.0	34.5	**5k**	8.5	15.8
5e	8.0	50.6	**5l**	7.5	15.3
5f	6.0	52.2	**DATS**	0.27	48.8
5g	7.0	42.4			

To better understand the mechanism of H_2S generation from these thioester-based hydrogen sulfide donors, we analyzed the reaction between **5a** and Cys (10 equiv). As shown in Scheme 3, the decomposition of **5a** to **8** was monitored by the formation of **9** using mass spectrometric analysis (Supporting Information, Fig. S1). The formation of disulfide **10** from allyl mercaptan (**8**) was also confirmed by mass spectra and NMR (Fig. S2). Besides, a great decline in the release of H_2S was observed under anaerobic conditions. On the basis of the products observed and the previous knowledge,[8] we proposed the following mechanism: The reaction is initiated by reversible thiol exchange between **5a** and Cys to generate the allyl mercaptan (**8**), which suffers oxidation to form DADS (**10**). Meanwhile, the resultant S-benzoyl cysteine **7** should undergo a native chemical ligation (NCL)-type acyl transfer to form a more stable *N*-benzoyl cystein **9**. Compound **10** then undergoes α-carbon nucleophilic substitution with Cys (Path A) or allyl mercaptan (Path

B) to generate perthiol intermediate (**12**), which releases H_2S in the presence of Cys. However, it is worth mentioning that allyl sulfides are substantially active because of the presence of allylic system, the precise and comprehensive H_2S-releaseing mechanism of these donors was not clear yet, additional pathways to generate the H_2S might exist in this complex system.[15]



Scheme 3. Proposed Mechanism of H_2S generation.

H_2S has been widely proposed to protect the cardiovascular system through its antioxidative role.[16] In order to test the protective effects of our newly synthesized H_2S donors in cells, the H_2S releasing abilities of these donors in cells were firstly verified in H9c2 cells by a selective H_2S fluorescent probe WSP-1 (Fig. S3).[17] The results showed that the typical donor **5a** released H_2S well in H_2O_2-stimulated H9c2 cells. Thus, *in vitro* model of cardiomyoblasts were established in H_2O_2-stimulated H9c2 cells. The cell viability was analyzed by MTT colorimetry and the results were exhibited in Table 2. Compounds **5a**, **5c-g** significantly attenuated H_2O_2-induced cell damage at concentrations of both 20 and 50 μM. The weaker protective effects of these donors at higher concentrations (> 100 μM) might be attributed to their poor solubility (data not shown). Overall, the protective effects of these donors were similar to that of NaHS, DADS and DATS, and were consistent with their H_2S release abilities, suggesting that their protective effects are likely due to H_2S release.

Table 2. Protective effect of compounds on H_2O_2-stimulated H9c2 cells.

NO	Comp.	Cell viability (%)			
		1 μM	10 μM	20 μM	50 μM
1	**5a**	58.1	61.1	74.5	68.3
2	**5b**	59.6	64.2	66.3	66.9
3	**5c**	64.6	69.3	77.9	65.5
4	**5d**	62.8	65.8	68.9	71.1
5	**5e**	65.0	71.9	71.3	77.7
6	**5f**	66.0	65.7	64.4	70.5
7	**5g**	63.1	66.2	72.1	63.3
8	**5h**	62.8	66.0	69.9	61.7
9	**5i**	63.3	59.5	59.0	54.8
10	**5j**	63.7	55.4	57.1	55.2
11	**5k**	63.3	62.9	64.7	57.1
12	**5l**	62.6	59.6	57.1	53.8
13	NaHS	58.6	67.0	68.5	69.6
14	DATS	60.3	67.5	71.7	70.8
15	DADS	59.6	64.8	71.2	69.6
16	Model	51.9			

[a]Values are expressed as means from three independent experiments, the bar graph expressed as mean ± SD was included in Supporting Information Fig. S4.

Cell viability is most often defined based on the integrity of the cell membrane. A common method for detecting cell viability is based on measuring the activity of cytoplasmic enzymes released by damaged cells, such as lactate dehydrogenase (LDH), which is a stable cytoplasmic enzyme found in all cells.[18] Thus, in this study, the contents of LDH in medium were measured to indicate the extent of cellular injury. As shown in Figure 3, the exposure of H9c2 cells to H_2O_2 (400 μM, 1 h) caused the damage of cell membrane, leading to the release of LDH to the medium. The LDH release was significantly reduced when cells were pretreated with different concentrations (10, 20, and 50 μM) of donors for 5 h in the presence of Cys (10 equiv), indicating the protective effects of these compounds. Among these donors, **5e** exhibited the most potent cardioprotective effect. Changes in the mitochondrial membrane potential (Δψm, MMP) have been postulated to be early events in H_2O_2-induced apoptosis signaling pathway.[19] Thus, fluorescent probe JC-1 was used to measure the changes of Δψm of cells treated with **5e**. As shown in Figure S5, normal H9c2 cells had bright red fluorescence, and cells treated with H_2O_2 appeared green fluorescence, indicating that H_2O_2 caused severe damage to cells and reduced Δψm. The pre-incubation of cells with 20 or 50 μM of **5e** greatly impeded this Δψm loss, as indicated by a decrease in green emission and an increase in red emission. These findings further confirmed that these H_2S donors exhibited potent cellular protection against oxidative injury.

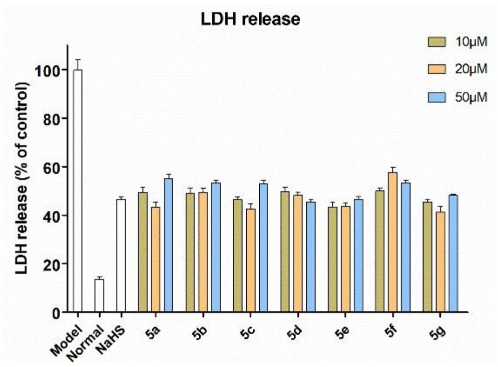


Figure 3. LDH assay of compounds **5a-g** on H9c2 cells. Cell viability was analyzed by LDH assay from culture media of indicated cells which were pretreated with different concentrations (10, 20, and 50 μM) of compounds for 5 h or NaHS (100 μM, 1 h). The data are expressed as percentages of H_2O_2-stimulated cells (model group).

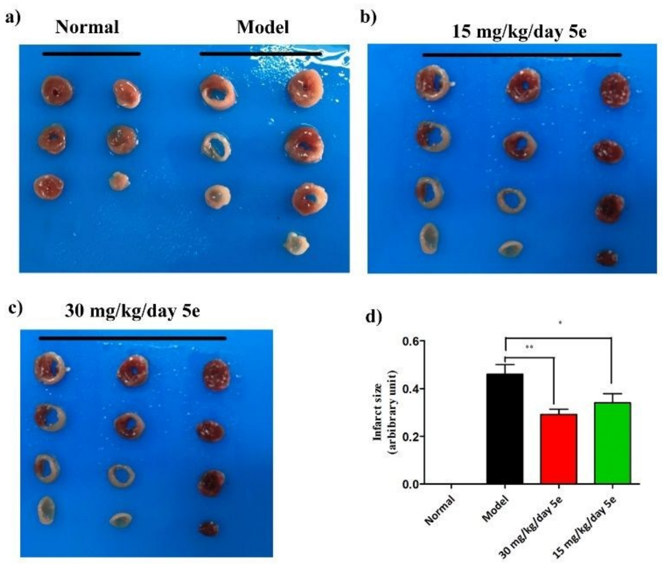


Figure 4. Isolated hearts from mice treated with different concentrations of **5e** (15 mg/kg/day, 30 mg/kg/day) were sliced into 2 mm sections and stained with TTC to determine the infarction size. (n=6 for each group). *p<0.05 versus model group, **p<0.01 versus model group.

The cardioprotective effects of **5e** were further verified *in vivo* in the MI mice model.[20] Mice treated with **5e** for 7 days were subjected to coronary occlusion and the hearts were harvested 72 h post MI. Infarct size was assessed with TTC staining (2,3,5-triphenyltetrazolium chloride), this white compound was enzymatically reduced to red TPF (1,3,5-triphenylformazan) in living tissues, while it remains as white TTC in areas of infarction since these enzymes have been degraded due to apoptosis. For this reason, healthy viable heart muscle will be stained in deep red, while areas of potential infarctions will be more pale. Infarction size was calculated as the ratio of scar average circumferences to left ventricular average inner circumferences. The results showed that infarct size was significantly larger in post MI mice (Model group) than that in sham-operated mice (Normal group) (Figure 4a). Remarkably, donor **5e** treatment relived myocardial infarction in a dose-dependent manner. Low (15 mg/kg/day) and high (30 mg/kg/day) dose of **5e** reduce infarction size to 74.75% and 63.97% of the model group, respectively (Figures 4b-c).

H&E staining was then applied to evaluate histological feature of infarcted hearts. As shown in Figure 5b, in peri-infarct heart, myofilaments condensed, leaving damaged gap junction with infiltrated nucleus from necrotic and apoptotic cells. However, donor **5e** significantly restrained the damage within epimyocardium and alleviated the morphological damage of cardiomyocytes (Figure 5c). Furthermore, TUNEL staining was used to determine apoptosis of cardiomyocytes in peri-infarction area. As shown in Figure S6, TUNEL positive staining cells were hardly found in Sham operated mice, while massive red fluorescent dots indicating fragments of DNA were found in heart section of model group. Donor **5e** displayed an obvious benefit in reducing the number of TUNEL-positive cells.

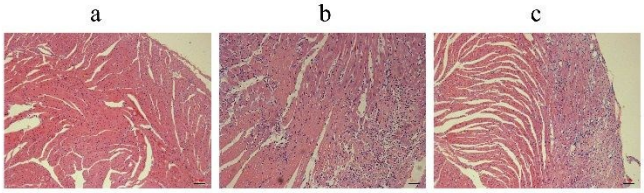


Figure 5. H&E staining was performed using heart sections to determine the morphological changes. (a) Normal group; (b) Model group; (c) Treated with **5e**, 30 mg/kg/day. Scale bar: 10 μm.

In summary, a series of thioester-based H_2S donors were developed. These donors are stable in aqueous solution, and release significant amounts of H_2S in a slow and controllable manner in the presence of Cys. The most potent donor **5e** exhibited promising cardioprotective activity against H_2O_2-induced oxidative stress by decreasing the mitochondrial membrane potential loss and lactate dehydrogenase release in H9c2 cells. More importantly, donor **5e** also exhibited potent cardioprotective effects in an *in vivo* myocardial infarction mice model by reducing myocardial infarct size and cardiomyoblasts apoptosis. Taken together, our study demonstrated that the new allyl thioesters may have potential as cardioprotective agents by releasing H_2S.

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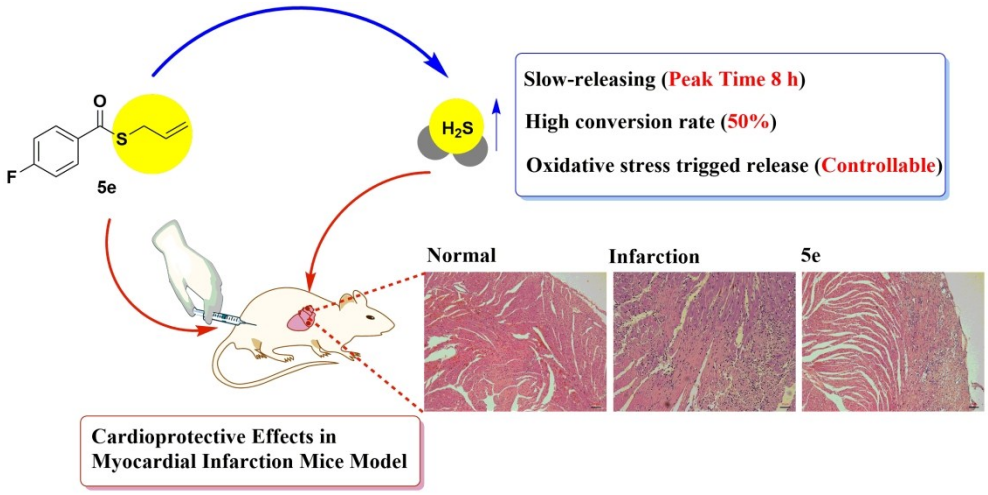

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