Design, Synthesis, and Cardioprotective Effects of N-Mercapto-Based Hydrogen Sulfide Donors

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Ⓢ Supporting Information

ABSTRACT: Hydrogen sulfide (H_2S) is a signaling molecule which plays regulatory roles in many physiological and/or pathological processes. Therefore, regulation of H_2S levels could have great potential therapeutic value. In this work, we report the design, synthesis, and evaluation of a class of N-mercapto (N-SH)-based H_2S donors. Thirty-three donors were synthesized and tested. Our results indicated that controllable H_2S release from these donors could be achieved upon structural modifications. Selected donors (**NSHD-1**, **NSHD-2**, and **NSHD-6**) were tested in cellular models of oxidative damage and showed significant cytoprotective effects. Moreover, **NSHD-1** and **NSHD-2** were also found to exhibit potent protective effects in a murine model of myocardial ischemia reperfusion (MI/R) injury.
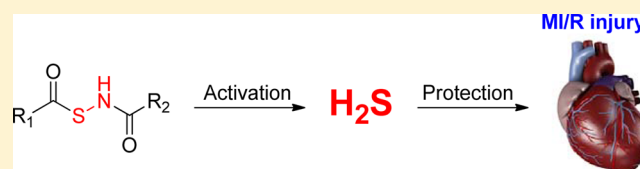
INTRODUCTION

Hydrogen sulfide (H_2S), which is known for its characteristic odor of rotten eggs, has been considered as a highly toxic air pollutant for centuries. However, this gaseous molecule has been recently recognized as a member of a gasotransmitter family along with nitric oxide (NO) and carbon monoxide (CO).[1−7] Endogenous H_2S formation has been attributed to at least three enzymes: cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE), and 3-mercaptopyruvate sulfur-transferase (MPST).[8−12] These enzymes convert cysteine or cysteine derivatives to H_2S in different tissues and organs. A number of studies have revealed a variety of biological effects of H_2S, which include vaso-dilation,[13−15] anti-inflammation,[16,17] anticancer,[18,19] and cardio-protection.[20−24] While the exact mechanisms of these actions are still under investigation, some interesting biochemical/catabolic reactions of H_2S have been disclosed. For example, H_2S reacts readily with methemoglobin to yield sulfhemoglobin.[25] H_2S inhibits cytochrome c oxidase, which accounts for its toxic action.[26] H_2S is an effective reducing agent and can react with reactive oxygen species such as hydrogen peroxide, superoxide, and peroxynitrite.[27−29] It has been reported that H_2S can cause protein S-sulfhydration (i.e., to form -S-SH),[30−32] and this process is significant as it provides a possible route by which H_2S can alter functions of a wide range of cellular proteins and enzymes.[33−35] H_2S can also interact with S-nitrosothiols to yield thionitrous acid (HSNO), the smallest S-nitrosothiol, which can freely diffuse through membranes to facilitate transnitrosation of proteins.[36] These reactions may be responsible for the biological

functions of H_2S. It is also possible that additional important biological reactions of H_2S will be discovered. All of these findings suggest that regulation of H_2S levels may have great therapeutic value.

In the H_2S field, H_2S releasing agents (also known as H_2S donors) are important research tools.[37,38] Moreover, H_2S donors could potentially be unique therapeutic agents, given the well-documented protective effects of H_2S in many disease states.[37,38] Among these donors, sulfide salts, including sodium sulfide (Na_2S) and sodium hydrogen sulfide (NaHS), are most frequently used. These salts have the advantage of boosting H_2S concentrations rapidly. However, they release H_2S spontaneously in aqueous solution, making it hard to control the precise H_2S concentrations. In addition, H_2S concentrations in aqueous solution can rapidly decrease due to volatilization, thus significantly limiting the use of these two H_2S precursors.[39]

Considering these drawbacks, synthetic H_2S donors have received considerable attention, and a number of donors have been reported.[40,41] For example, a widely used donor is GYY4137, a derivative of Lawesson's reagent.[42] GYY4137 has been demonstrated to release H_2S both in vitro and in vivo. GYY4137 can induce H_2S-related biological effects such as vasodilation and anti-inflammation.[42−45] However, the mechanism of H_2S release from GYY4137 is still unclear. The extremely slow release of H_2S from GYY4137 could also be problematic for
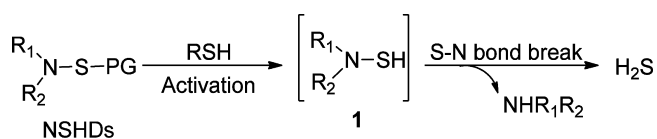
its applications.[46] Dithiolthiones (DTTs) are another class of synthetic H$_2$S donors.[38] DTTs have been widely used to prepare nonsteroidal anti-inflammatory drug (NSAIDs)-H$_2$S hybrids. The resultant HS-NSAIDs, compared to the parent NSAIDs, showed similar anti-inflammatory effects but significantly decreased gastric damage.[47−49] Although DTTs have shown promising H$_2$S-related biological activities, their H$_2$S release is also uncontrollable, and their H$_2$S release profile has not been explored yet.

The problem with the majority of currently available donors is that the H$_2$S generation from these compounds is uncontrollable. Commonly used donors such as sulfide salts and GYY4137 release H$_2$S spontaneously upon hydrolysis, making it very difficult to obtain the precise physiological or pharmacological H$_2$S levels needed. Modifications made between the time that a solution is prepared and the time that the biological effect is measured can dramatically affect research outcomes and sometimes can even get disparate results. From a therapeutic perspective and for applications in H$_2$S-related biological research, ideal H$_2$S donors should release H$_2$S slowly in moderate amounts and/or in a highly controllable manner. To this end, our group introduced thiol-activated H$_2$S donors in 2009.[50] The idea is described in Scheme 1. It is known that S−N bonds are weak

Scheme 1. Design of NSHDs

and can break under certain conditions.[51−53] This property makes N-mercapto (N-SH) species, such as **1**, useful templates for the design of H$_2$S donors. As N-SH species are unstable and highly reactive, we expected that the protecting groups on −SH should enhance their stability. Moreover, the removal of protecting groups can be achieved by different strategies, making controllable H$_2$S release possible. To prove this hypothesis, 12 N-(benzoylthio)benzamides were prepared as the first thiol-activated H$_2$S donors. Some preliminary results regarding their H$_2$S release profiles were reported.[50] Recently, we expanded the scope of this type of donors and studied 33 substrates. Herein, we report the comprehensive results about these N-SH-based donors (NSHDs), including their synthesis, H$_2$S release evaluation/mechanism, and structure−activity relationship (SAR). In addition, protective effects of selected donors on cellular oxidative damages and myocardial ischemia reperfusion (MI/R) injury are also reported.
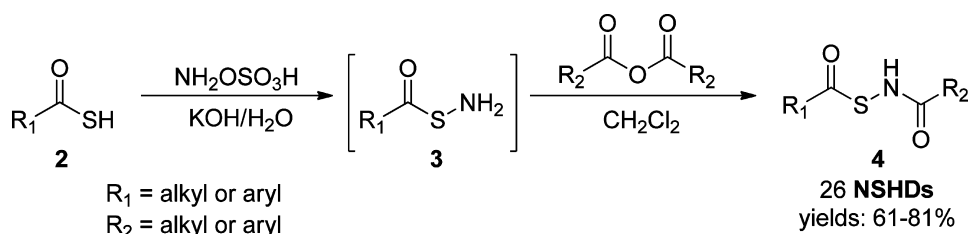
RESULTS AND DISCUSSION

Synthesis of N-(Acylthio)amides as NSHDs. In the design of thiol-activated NSHDs, acyl groups were selected as the protecting groups on −SH. Both alkyl- and aryl-based acyl groups were used to prepare the donors. The synthesis is illustrated in Scheme 2. Readily available thiocarboxylic acids **2** were the starting materials. The treatment of **2** with hydroxyl-amine-O-sulfonic acid gave S-acylthiohydroxylamines **3** as the reactive intermediates, which were further reacted (without separation) with acid anhydrides to provide NSHDs **4**. In total, 26 NSHDs were prepared using this route, and overall yields were 61−81%.

H$_2$S Release of N-(Acylthio)amides. With these compounds in hand, we first tested their H$_2$S release ability in aqueous buffers. N-(Acylthio)amides were found to be stable in buffers. Unlike hydrolysis-based donors, N-(acylthio)amides did not release H$_2$S spontaneously. In addition, no H$_2$S generation was observed in the presence of some potential cellular nucleophiles, such as serine and lysine. However, a time-dependent H$_2$S release was detected when cysteine was added into the solutions, indicating that thiols are essential to trigger H$_2$S release from these molecules.

In order to systematically compare the H$_2$S generation capabilities of these donors, we studied the effects of donor concentrations, cysteine concentrations, and solvent systems, as well as reaction time/temperatures. Eventually, the optimized conditions were found to use 100 μM donors and 1000 μM cysteine in phosphate buffered saline (PBS) (pH 7.4) containing 10% tetrahydrofuran (THF). H$_2$S release was monitored at room temperature for 2 h. The well-known methylene blue (MB) method was used to measure H$_2$S generation. Using **NSHD-1** as the example (Figure 1), a maximum of 56 μM of H$_2$S (peak H$_2$S concentration) at 50 min (peak time) was detected. As shown in Figure 1, higher concentrations of cysteine (i.e., 2 mM) did not significantly improve H$_2$S release, while lower concentrations of cysteine (i.e 0.5 mM) dramatically decreased H$_2$S production. In addition to cysteine, GSH's ability to promote H$_2$S release from NSHDs was also evaluated. Results showed that GSH (1 mM) can also successfully trigger H$_2$S release at levels comparable to those of 1 mM cysteine.

All NSHD substrates were then tested under the optimized conditions. Briefly, a solution of each donor (0.1 mM) and cysteine (or GSH, 1 mM) was prepared in a mixed solvent system PBS/THF (9:1, v/v). At different reaction times, 1.0 mL of reaction aliquots were taken and mixed with MB cocktails (0.5 mL). The MB reactions were carried out for 15 min, and absorbance at 670 nm was then measured. H$_2$S concentrations were calculated based on a Na$_2$S standard curve (please see the Experimental Procedures section for detailed procedures). Each donor was tested three times, and their average results are summarized in Table 1. The results showed that up to 68% of
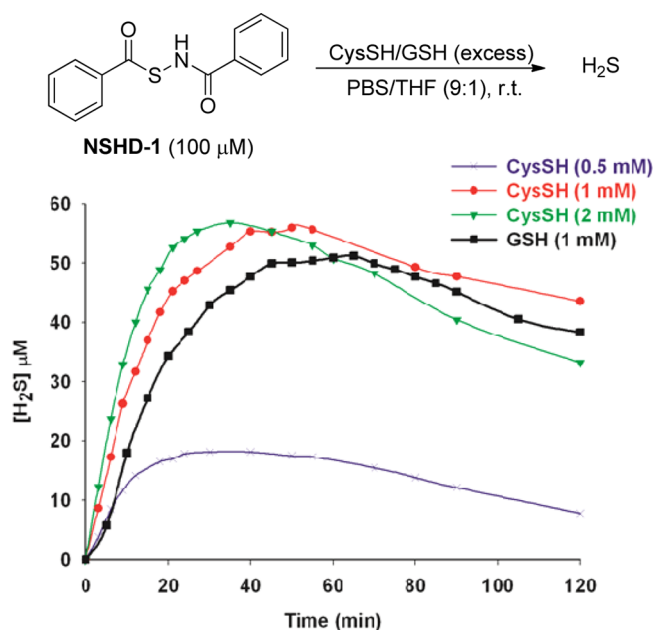
Scheme 2. Synthesis of NSHDs

Figure 1. Cysteine and GSH-mediated H$_2$S release from **NSHD-1**.

H$_2$S can be detected within a 2-h experimental period, indicating that *N*-(acylthio)amides are potent H$_2$S donors. As for SAR, electronic effects on H$_2$S release were observed. Compared to the parent donor **NSHD-1**, donors with electron withdrawing groups (**NSHD-2−NSHD-5**) showed faster H$_2$S release; donors with electron donating groups (**NSHD-6−NSHD-12**) exhibited slower H$_2$S release. Steric effects were also observed as more sterically hindered substrates showed slower H$_2$S release (**NSHD-13−NSHD-15**). In addition to the thioester moiety, SAR studies were also conducted on amide terminals (**NSHD-20−NSHD-26**). Results showed that all of these amide-modified donors exhibited similar H$_2$S releasing abilities, suggesting that the amide end had little effects on the reactivity. In all GSH-treated groups, peak times were constantly 5−20 min slower than those in cysteine-treated groups. This is presumably due to the increased steric hindrance of GSH, therefore causing a slower reaction to release H$_2$S. These findings demonstrated that structural modifications can regulate H$_2$S release from NSHDs, therefore, achieving controllable H$_2$S generation.

Synthesis and H$_2$S Release of 1-(Benzoylthio)-3-phenylureas. To expand the scope of the donors, we next replaced the amide moiety with urea templates. A series of 1-mercapto-3-phenylurea-based H$_2$S donors (**6**) were synthesized (Scheme 3). Briefly, readily available thiocarboxylic acids were treated with hydroxylamine-*O*-sulfonic acid to form *S*-acylthiohydroxylamines **3**, which were then conjugated with phenyl isocyanate derivatives (**5**) to provide 1-(benzoylthio)-3-phenylureas (**6**) as the desired products.

H$_2$S release from these donors was tested using the same protocol described above, and the results are shown in Table 2. Interestingly, these 1-(benzoylthio)-3-phenylurea based donors showed lower and slower H$_2$S release as compared to *N*-(acylthio)amides.

H$_2$S Release Mechanism. To understand the mechanism of H$_2$S release from these *N*-mercapto-based donors, a model reaction using **NSHD-1** and cysteine (10 equiv) was studied. Three products, *N*-benzoyl cysteine **8**, benzamide **10**, and cystine **12**, were obtained. On the basis of these products, the H$_2$S release mechanism was proposed as follows (Scheme 4): a reversible

thiol exchange between **NSHD-1** and cysteine initiates the reaction. The resultant *S*-benzoyl cysteine **7** should then undergo a native chemical ligation (NCL)-type acyl transfer to form a more stable *N*-benzoyl cysteine **8**. Meanwhile, the free *N*-SH species, *N*-mercaptobenzamide **9**, should react with cysteine to yield benzamide **10** and cysteine perthiol **11**. The latter product can further react with cysteine to liberate H$_2$S and form cystine **12**.

Cytotoxicity of NSHDs. With these NSHDs in hand, we next explored their H$_2$S-induced biological activities. Recent studies have suggested that H$_2$S can protect the cardiovascular system against myocardial ischemia reperfusion (MI/R) injury. It has been demonstrated that H$_2$S (using Na$_2$S or NaHS), when applied at the time of reperfusion or as a preconditioning reagent, exhibits significant cardioprotection by different mechanisms, such as reducing oxidative stress, preserving mitochondrial function, decreasing myocardial inflammation, and improving angiogenesis.[20−24] Among these, H$_2$S's effects as an antioxidant may play a prominent role. We envisioned that NSHDs should exhibit similar cardioprotective activities due to H$_2$S release.

Before conducting experiments to investigate NSHD's cardioprotective actions, we tested the cytotoxicity of three representative donors (**NSHD-1**, **NSHD-2**, and **NSHD-6**) in H9c2 cardiomyoblasts. These three donors were selected because of their varied H$_2$S release abilities. The cells, originating from rat heart ventricular tissue, have been widely used as an *in vitro* model for cardiac muscle in virtue of their morphological features and biochemical properties.[54] In our experiment, H9c2 cells were incubated with each donor at different concentrations (20−160 μM) for 1 h. Cells were then washed with PBS twice to remove extracellular donors, and cell viability was measured by the standard CCK-8 assay. As shown in Figure 2, these donors did not exhibit significant cytotoxicity at low concentrations (<40 μM). However, cytotoxicity was observed when higher concentrations (>80 μM) were applied. Considering that cysteine and GSH are rich in living systems, which are expected to react with NSHDs and trigger H$_2$S release, we envisioned NSHD-induced cytotoxicity could be diminished or avoided when cysteine or GSH is presented. To prove this hypothesis, H9c2 cells were then incubated with each donor (160 μM) in the presence of cysteine (480 μM). As expected, NSHD's cytotoxicity was completely suppressed, suggesting that NSHDs should be safe in cysteine-rich environments.

Establishment of an *in Vitro* Model of MI/R Injury. Under the condition of MI/R injury, oxidative stress (OS) plays a central role.[55] OS-induced cell injury can be triggered by reactive oxygen species (ROS), including superoxide (O$_2$$^{•−}$), hydroxyl radical ($^{•}$OH), singlet oxygen ($^{•}$O), and hydrogen peroxide (H$_2$O$_2$).[56,57] Among these, H$_2$O$_2$ is the dominant form due to its stability.[58] Therefore, H$_2$O$_2$-treated H9c2 cells are usually used as an *in vitro* model of MI/R injury.[59,60] To establish the cellular model of MI/R injury, H9c2 cells were treated with increasing concentrations of H$_2$O$_2$ (100−800 μM) for 5 h. As shown in Figure 3, exposure of H9c2 cells to H$_2$O$_2$ led to a dose-dependent inhibition in cell viability ($P < 0.01$), indicating that H$_2$O$_2$ was able to induce the oxidative injury in cardiomyocytes. This can partly imitate *in vivo* I/R-triggered effects. Since the median lethal concentration of H$_2$O$_2$ in H9c2 cells was approximately 400 μM, this concentration was used for the following studies.

Effects of NSHDs on H$_2$O$_2$-Induced Damage in H9c2 Cells. We next tested the protective capabilities of NSHDs against H$_2$O$_2$-induced cellular damage. In these experiments, H9c2 cells were pretreated with **NSHD-1**, **NSHD-2**, or **NSHD-6**
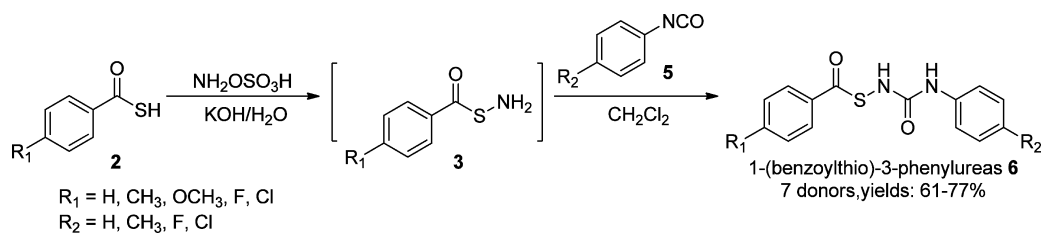
Table 1. H₂S Release Profiles of *N*-(Acylthio)amides

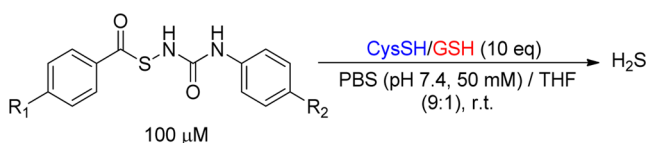| Donors	R₁	R₂	T_{peak} (min)	$[H_2S]_{peak}$ (μM)	Donors	R₁	R₂	T_{peak} (min)	$[H_2S]_{peak}$ (μM)
NSHD-1	H—⬡—	⬡—	55 / 65	55.8[a] / 51.2[b]	NSHD-14	(isopropyl)	⬡	65 / 70	40.7 / 58.4
NSHD-2	F—⬡—	⬡—	50 / 55	39.5 / 40.6	NSHD-15	(tert-butyl)	⬡	80 / 90	37.5 / 48.1
NSHD-3	CF₃—⬡—	⬡—	45 / 60	48.3 / 28.1	NSHD-16	(cyclohexyl)	⬡	65 / 80	48.1 / 68.2
NSHD-4	(3-Cl-phenyl)	⬡—	50 / 60	49.4 / 31.3	NSHD-17	(cyclopentyl)	⬡	60 / 65	40.9 / 49.9
NSHD-5	(4-methoxycarbonyl-phenyl)	⬡—	50 / 55	38.8 / 36.0	NSHD-18	(benzyl)	⬡	50 / 60	33.4 / 47.5
NSHD-6	(4-methoxy-phenyl)	⬡—	60 / 65	41.8 / 46.8	NSHD-19	(isopropylphenyl)	⬡	60 / 70	48.2 / 55.6
NSHD-7	(2-methoxy-phenyl)	⬡—	60 / 80	29.0 / 34.0	NSHD-20	⬡	(2-CH₃-phenyl)	55 / 65	51.4 / 57.6
NSHD-8	(3-methoxy-phenyl)	⬡—	55 / 80	47.0 / 39.5	NSHD-21	⬡	(2-OAc-phenyl)	60 / 60	35.1 / 44.5
NSHD-9	(4-methyl-phenyl)	⬡—	60 / 60	45.4 / 39.8	NSHD-22	⬡	(3-Cl-phenyl)	60 / 70	25.2 / 29.9
NSHD-10	(2-methyl-phenyl)	⬡—	60 / 70	29.4 / 19.8	NSHD-23	⬡	(4-F-phenyl)	55 / 65	38.5 / 43.3
NSHD-11	(3-methyl-phenyl)	⬡—	55 / 65	49.3 / 39.3	NSHD-24	⬡	H₃C—	45 / 50	46.4 / 58.3
NSHD-12	(4-dimethylamino-phenyl)	⬡—	65 / 80	19.5 / 7.6	NSHD-25	⬡	(ethyl/propyl)	55 / 60	44.1 / 54.1
NSHD-13	H₃C—⬡—	⬡—	60 / 70	48.4 / 63.1	NSHD-26	⬡	(butyl)	60 / 60	45.9 / 58.3

[a]Data were obtained with cysteine. [b]Data were obtained with GSH.

Scheme 3. Chemical Synthesis of 1-Mercapto-3-phenylurea-Based Donors

R₁ = H, CH₃, OCH₃, F, Cl
R₂ = H, CH₃, F, Cl

1-(benzoylthio)-3-phenylureas **6**
7 donors, yields: 61-77%

at varied concentrations (40, 80, and 160 μM) in the presence of cysteine (480 μM) for 1 h. Cells were then washed with PBS to remove extracellular NSHDs and cysteine. The donor-treated cells were challenged by H₂O₂ (400 μM) for another 5 h, after which cell viability was analyzed. Results showed that NSHDs exhibited significant attenuation of H₂O₂-induced damage

Table 2. H₂S Release Profiles of 1-(Benzoylthio)-3-phenylurea Based Donors[a]

donors	R_1	R_2	CysSH		GSH	
			T_{peak} (min)	$[H_2S]_{peak}$ (μM)	T_{peak} (min)	$[H_2S]_{peak}$ (μM)
NSHD-27	H	H	95	21.5	110	29.9
NSHD-28	OCH₃	H	105	14.1	120	26.4
NSHD-29	CH₃	H	105	16.5	130	20.0
NSHD-30	F	H	75	18.8	90	18.5
NSHD-31	Cl	H	95	18.2	120	24.9
NSHD-32	H	CH₃	90	21.8	130	27.4
NSHD-33	H	Cl	110	10.7	120	20.9

[a]Data were reported as the average value of three measurements.

(Figure 4A, B, and C). In order to compare these new donors with known donors, we also tested the effects of GYY4137 and NaHS, two well-studied H₂S donors, under the same conditions. Similar protective effects were observed with GYY4137 and NaHS (40, 80, and 160 μM) (Figure 4D and E). To further prove that the protective effects of NSHDs were due to H₂S release, we studied the effects of cysteine only (480 μM) and byproducts of NSHDs (i.e., N-benzoyl cysteine (NBC) and benzamide (BZM), at 160 μM). These species did not exhibit protection against H₂O₂-induced damage (Figure 4F). Taken together, these results suggested that NSHDs can protect cardiomyocytes from oxidative injury and that their protective activities are likely due to H₂S release.

Measurement of LDH and MMP Levels. In addition to the cell viability assay, several other methods were also used to validate NSHD's protective effects in cells. Lactate dehydrogenase (LDH) is a stable cytoplasmic enzyme located in the cytoplasm under normal conditions. When the heart suffers from
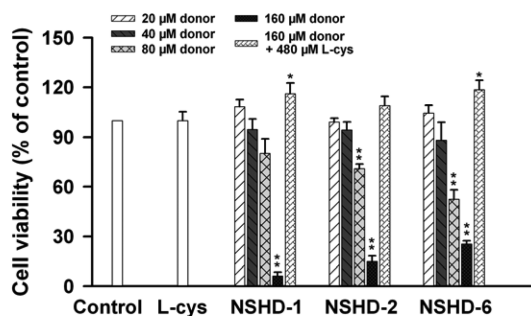

Figure 2. Cytotoxicity of NSHDs in the absence or presence of cysteine. H9c2 cells were incubated with **NSHD-1**, **NSHD-2**, and **NSHD-6** at varied concentrations (20−160 μM) for 1 h in the absence or presence of cysteine (480 μM). Excess NSHDs were then washed away with PBS. The CCK-8 assay was applied to measure cell viability. Data were expressed as the mean ± SEM. *$P < 0.05$, **$P < 0.01$ vs the control group.
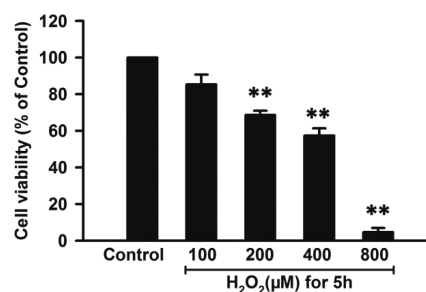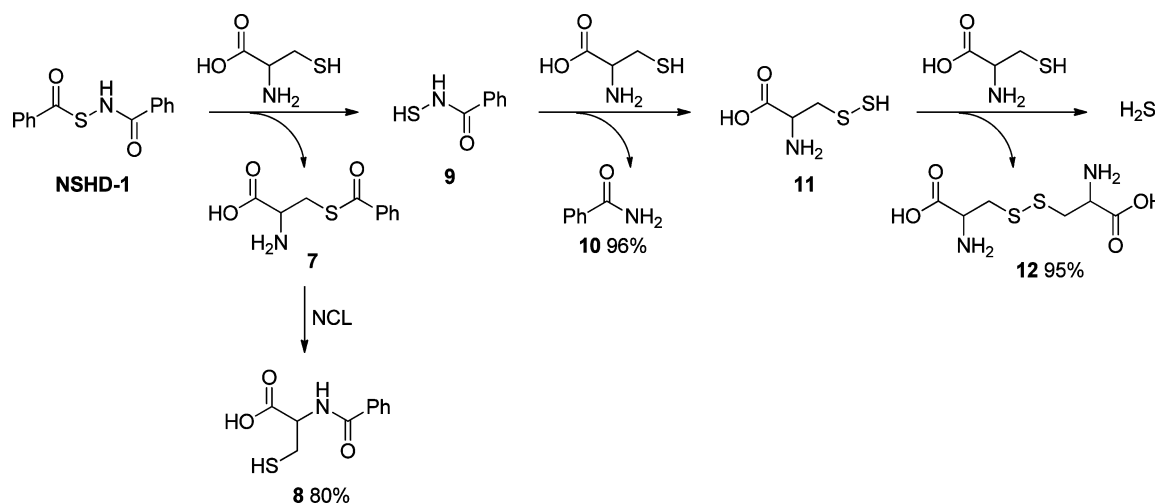

Figure 3. Effects of H₂O₂ on cell viability. H9c2 cells were treated with varied concentrations of H₂O₂ for 5 h. The CCK-8 assay was performed to detect cell viability. Data were expressed as the mean ± SEM. **$P < 0.01$ vs the control group.

MI/R injury, the cell membrane of cardiomyocytes is damaged, leading to the increase of cell membrane permeability and LDH leakage from the cells into the blood.[61] In the present study, by comparing the contents of LDH in cytoplasm and medium, the LDH release rate can be used to indicate the extent of cellular injury. As shown in Figure 5, preconditioning of H9c2 cells with cysteine (480 μM, 1 h) followed by the exposure to H₂O₂ (400 μM, 5 h) remarkably enhanced LDH release, indicating that H₂O₂ induced severe damage to cells and that cysteine (480 μM)

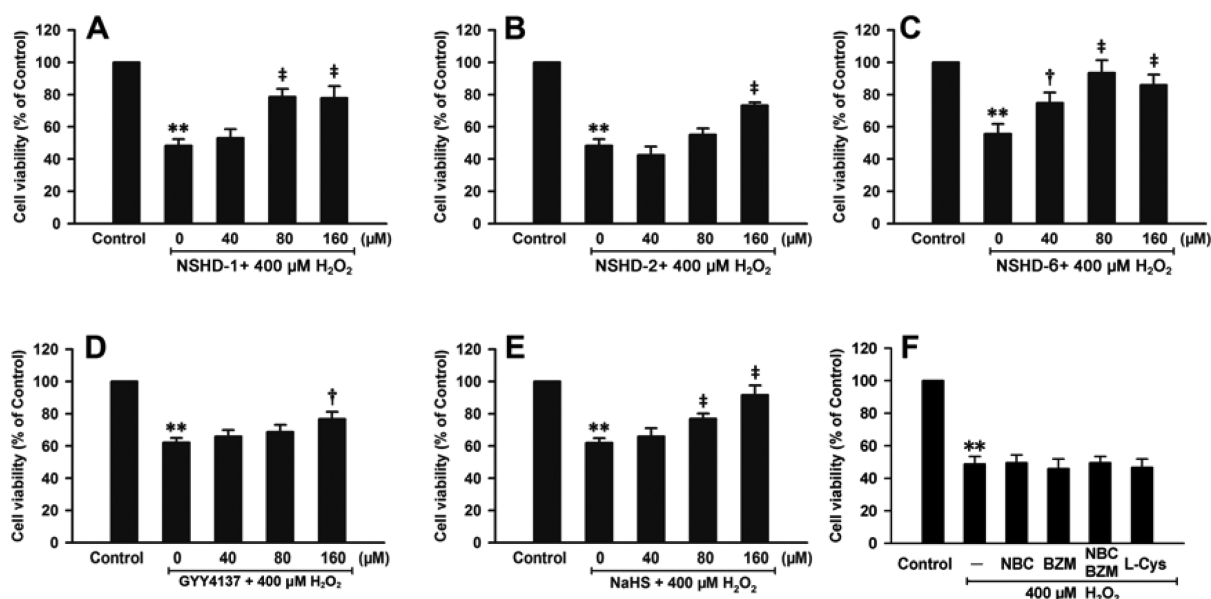## Scheme 4. Proposed Mechanism of H₂S Release from NSHDs

Figure 4. Effects of H$_2$S donors (A−E) and NSHD byproducts (F) on H$_2$O$_2$-induced cellular damage. Prior to the 5-h H$_2$O$_2$ (400 μM) treatment, H9c2 cells were preconditioned with **NSHD-1** (A), **NSHD-2** (B), and **NSHD-6** (C) in the presence of cysteine (480 μM) at the indicated concentrations for 1 h. GYY4137 (D) and NaHS (E) were used as positive controls under similar conditions. Effects of H$_2$S releasing byproducts, such as NBC and BZM (160 μM), and cysteine alone (480 μM) were also investigated (F). The CCK-8 assay was performed to detect cell viability. Data were expressed as the mean ± SEM. **$P < 0.01$ vs the control group. $^{\dagger}P < 0.05$, $^{\ddagger}P < 0.01$ vs the H$_2$O$_2$ treatment group.
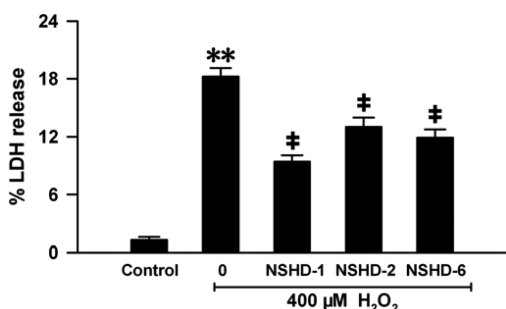

Figure 5. Effects of NSHDs on H$_2$O$_2$-induced LDH release. H9c2 cells were preconditioned with **NSHD-1**, **NSHD-2**, and **NSHD-6** (160 μM) in the presence of cysteine (480 μM) for 1 h followed by treatment with H$_2$O$_2$ (400 μM) for 5 h. LDH in cells and medium was measured with a commercial kit. Data were expressed as the mean ± SEM. **$P < 0.01$ vs the control group. $^{\ddagger}P < 0.01$ vs the H$_2$O$_2$ treatment group.

did not provide protection. However, LDH release was significantly reduced when cells were pretreated with 160 μM NSHD donors in the presence of cysteine (480 μM), demonstrating that NSHDs can protect cells from oxidative damage.

Mitochondria are the energy factory of cells and sensitive to noxious stimuli. Mitochondrial membrane potential (MMP) usually reflects whether mitochondria are healthy, which further indicates whether cells are suffering from noxious damage. Rh-123 staining is a useful tool to observe MMP. Figure 6A shows that under normal conditions H9c2 cells had bright green fluorescence. When cells were preconditioned with cysteine (480 μM) for 1 h and then treated with H$_2$O$_2$ (400 μM) for 5 h, a dramatic MMP loss was observed, evidenced by weak green fluorescence, suggesting that H$_2$O$_2$ induced severe damage to cells and that cysteine (480 μM) did not provide significant protection (Figure 6B). However, preconditioning with 160 μM of NSHDs greatly impeded this MMP loss (Figures 6C−F) by preserving mitochondrial functions. These results further

confirmed that NSHDs exhibited potent cellular protection against oxidative injury.

Cardioprotective Effects of NSHD-1 and NSHD-2 in MI/R Injury. Finally, we tested the protective effects of two representative donors, i.e., **NSHD-1** and **NSHD-2**, against myocardial ischemia/reperfusion (MI/R) injury in a murine model system. In these experiments, mice were subjected to 45 min of left ventricular ischemia followed by 24 h reperfusion. **NSHD-1**, **NSHD-2**, or vehicle was administered by direct intracardiac (i.c.) injection at the time of reperfusion at different doses. All animal groups displayed similar area-at-risk per left ventricle (AAR/LV), which means that surgery caused a similar risk. However, compared to vehicle treated mice, mice receiving **NSHD-1** and **NSHD-2** displayed a dose-dependent reduction in infarct size per area-at-risk (INF/AAR), assessed by 2,3,5-triphenyltetrazolium chloride (TTC) staining (Figure 7). A 100 μg/kg bolus of **NSHD-1** maximally reduced INF/AAR by ∼35%. Similarly, 50 μg/kg and 100 μg/kg of **NSHD-2** reduced INF/AAR by ∼50% ($p < 0.01$, $p < 0.001$, respectively). Moreover, circulating cardiac troponin I (TnI) levels, the marker for acute myocardial infarction, were significantly lowered with **NSHD-2** treated animals ($p < 0.05$ for 50 ug/kg and $p < 0.01$ for 100 ug/kg) (Figure 8). These results strongly suggest that N-mercapto compounds are potent H$_2$S donors and may have some pharmacological benefits in biological systems. In the present study, we injected these H$_2$S donors directly into the left ventricular cavity at the time of reperfusion. In the clinical setting, we believe that these agents could be administered via a number of routes to patients suffering from an acute myocardial infarction. These compounds could be administered via intravenous bolus injection or infusion or via intracoronary injection during coronary catheterization to open the occluded coronary artery. In previous studies, we have administered H$_2$S donors into the systemic circulation with excellent results.[62]
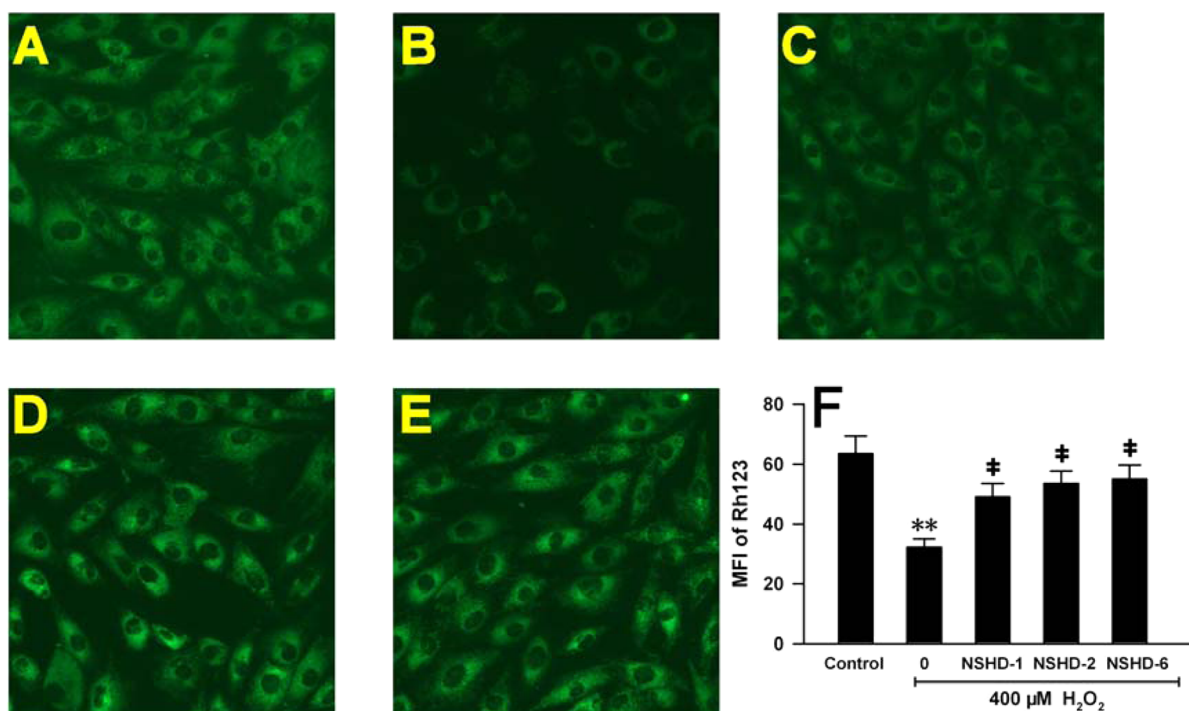
Figure 6. Effects of H_2S donors on H_2O_2-induced MMP loss in H9c2 cells. (A−E) Rh123 staining followed by photofluorography to observe MMP in H9c2 cells. (A) Control group. (B) Cells were pretreated with cysteine (480 μM) for 1 h, then exposed to H_2O_2 (400 μM) for 5 h. Cells were preconditioned with 160 μM **NSHD-1** (C), **NSHD-2** (D), and **NSHD-6** (E) in the presence of cysteine (480 μM) for 1 h prior to H_2O_2 treatment. (F) Quantitative analysis for Rh123 in panels A−E. Mean fluorescence intensities (MFI) were measured using IMAGEJ software. Data were expressed as the mean ± SEM. **$P < 0.01$ vs the control group. ‡$P < 0.01$ vs the H_2O_2 treated group.
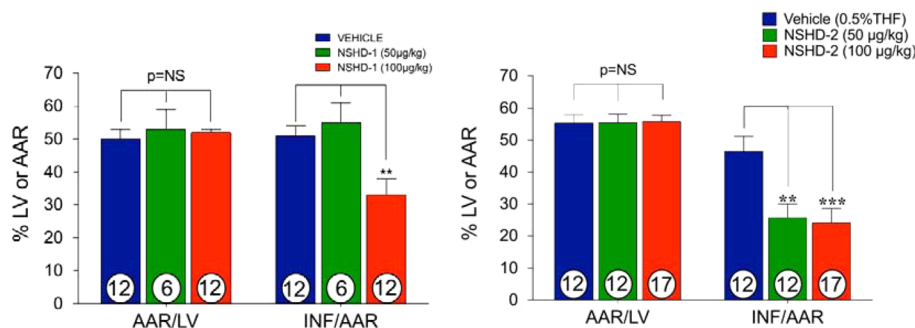

Figure 7. Cardioprotective effects of **NSHD-1** and **NSHD-2** in myocardial ischemia-reperfusion injury. Doses of **NSHD-1**, **NSHD-2**, or vehicle were injected at the time of reperfusion. (Left) Myocardial area-at-risk (AAR) per left ventricle (AAR/LV) and infarct per area-at-risk (INF/AAR) were assessed in vehicle ($n = 12$), **NSHD-1** (50 μg/kg) treated animals ($n = 6$), and **NSHD-1** (100 μg/kg) treated animals ($n = 12$). AAR/LV was similar among all groups. INF/AAR was significantly smaller in the 100 μg/kg dose treated animals. (Right) Myocardial area-at-risk (AAR) per left ventricle (AAR/LV) and infarct per area-at-risk (INF/AAR) were assessed in the vehicle ($n = 12$), **NSHD-2** (50 μg/kg) treated ($n = 12$), and **NSHD-2** (100 μg/kg) treated animals ($n = 17$). AAR/LV was similar among all groups. INF/AAR was significantly smaller in animals treated with either dose.

■ CONCLUSIONS

In summary, we have developed a series of H_2S donors based on a *N*-mercapto template. Their H_2S release is triggered by cellular thiols such as cysteine or GSH. H_2S release from these donors can be regulated by structural modifications. Moreover, we demonstrated that these donors are safe in cysteine-rich environments. Their protective effects on cellular oxidative injury and myocardial ischemia reperfusion injury were also observed. Taken together, our studies demonstrate that *N*-mercapto species are potent H_2S donors and that they possess potential therapeutic benefits.

■ EXPERIMENTAL PROCEDURES

Chemical Synthesis. All solvents were of reagent grade. THF was freshly distilled from sodium/benzophenone under argon. Reactions were magnetically stirred and monitored by thin layer chromatography (TLC) with 0.25 mm precoated silica gel plates. Flash chromatography was performed with silica gel 60 (particle size 0.040−0.062 mm). Yields refer to chromatographically and spectroscopically pure compounds, unless otherwise stated. Proton and carbon-13 NMR spectra were recorded on a 300 MHz spectrometer. Chemical shifts are reported relative to chloroform (δ7.26) for ^{1}H NMR and chloroform (δ77.0) for ^{13}C NMR. The purity of all of the compounds was at least 95%, determined by LC/MS. Compounds were also characterized by TLC, melting points, and NMR spectroscopy.

Synthesis of (*N*-Acylthio)amides (NSHD-1−NSHD-26). General procedure: thiocarboxylic acids (1 mmol) were mixed with KOH (5
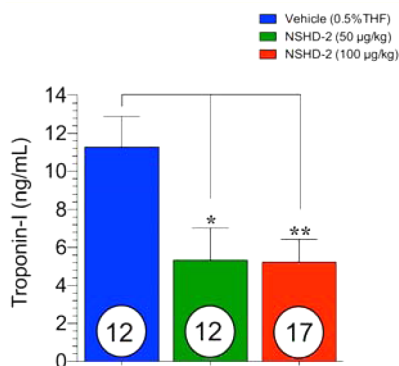
Figure 8. Blood was collected at 4 h of reperfusion, and circulating cardiac troponin I levels were measured. Troponin I level was significantly reduced with either 50 μg/kg or 100 μg/kg of NSHD-2 treatment.

mmol) in water (10 mL). To this mixture was added hydroxylamine-O-sulfonic acid (3 mmol). The reaction mixture was stirred at room temperature for 5 min and then extracted with 15 mL of CH_2Cl_2 three times. Organic layers were then combined, dried (by $MgSO_4$), and concentrated to give the crude product S-acylthiohydroxylamines **3**. Without further purification, **3** was dissolved in CH_2Cl_2 (5 mL) containing acid anhydride (2 mmol). The reaction solution was stirred at room temperature for 12 h. Crude products were precipitated out and collected. Purification by column chromatography or recrystallization gave the final products. The characterization data of each donor is provided in the Supporting Information.

Synthesis of 1-(Benzoylthio)-3-phenylureas (NSHD-27–NSHD-33). Each freshly prepared S-acylthiohydroxylamine **3** (1 mmol, see procedure described above) was mixed with the corresponding phenyl isocyanate derivative **5** (2 mmol) in CH_2Cl_2 (2 mL). The mixture was stirred at room temperature for 12 h. The crude products were precipitated out and collected. Recrystallization in CH_2Cl_2/hexanes gave the final products. The characterization data of each donor is provided in the Supporting Information.

H$_2$S Measurement. H_2S generation was initiated by adding 100 μL of a donor's stock solution (30 mM in THF) into a 30 mL PBS (pH 7.4, 50 mM)/THF (9:1) solution containing cysteine or GSH (1.0 mM). Then, 1.0 mL of reaction aliquots were periodically taken and transferred to UV cuvettes containing MB cocktail (100 μL of zinc acetate (1% w/v), 200 μL of N,N-dimethyl-1,4-phenylenediamine sulfate (20 mM in 7.2 M HCl), and 200 μL of ferric chloride (30 mM in 1.2 M HCl)). The MB reaction was carried out for 15 min, and the absorbance (670 nm) of the resultant solution was determined using an UV–vis spectrometer (Thermo Evolution 300). The H_2S concentration of each sample was calculated against a calibration curve, which was obtained with a series of Na_2S solutions.

Product Analysis. NSHD-1 (129 mg 0.5 mmol) in 2 mL of MeOH was added to a stirred solution of L-cysteine (605 mg, 5 mmol) in 50 mL of PBS buffer (pH 7.4, 20 mM). After 1 h, 0.1 mL of solution was taken via syringe equipped with a 45 μM syringe filter, diluted with 0.4 mL of MeOH, and analyzed by HPLC (Thermo Scientific Surveyor, Hypersil gold column, 4.6 × 100 mm, water/CH_3CN 80/20 2 min; 60/40 4 min; 20/80 2 min; 80/20 2 min, flow rate 1 mL/min). Compounds **8**, **10**, and **12** were identified and quantified as compared to standard compounds. These compounds were also isolated from the reaction mixture and characterized. The yields were 80%, 96%, and 95%, respectively.

Cell Culture. H9c2 cells were purchased from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) high glucose medium supplemented with 10% fetal bovine serum (FBS) at 37 °C under 5% CO_2.

Cell Viability Assay. The cell counting kit (CCK)-8 (Dojindo Laboratory, Kumamoto, Japan) was applied to measure the cell viability of H9c2 cells cultured in 96-well plates. After the indicated cell treatment, 100 μL of CCK-8 solution at 1:10 dilution was added to each

well, and cells were incubated for a further 3 h at 37 °C. Absorbance was measured at 450 nm with a microplate reader (Tecan Infinite F200, Switzerland). The mean optical density (OD) of 4 wells in each group was used to calculate cell viability as follows (experiments were performed in triplicate):

$$\%\text{cell viability} = \frac{\text{OD treatment group}}{\text{OD control group}} \times 100$$

Protective Activities of NSHDs on H$_2$O$_2$-Induced Cellular Damage. H9c2 cells were treated with **NSHD-1**, **NSHD-2**, and **NSHD-6** at varied concentrations (40−160 μM) for 1 h in the presence of cysteine (480 μM). Excess donors and cysteine were washed away with PBS (pH 7.4). Cells were then incubated with H_2O_2 (400 μM) for an additional 5 h. The control group was set up by incubating H9c2 cells with H_2O_2 (400 μM) only. Cell viability was measured using the CCK-8 assay. GYY4137 and NaHS were used as positive controls, and experiments were set up in a similar manner in the absence of cysteine. Cysteine (480 μM), NBC (160 μM), and BZM (160 μM) were incubated with cells as well to rule out their effects on cell viability.

LDH Measurement. The rate of LDH release was determined with a commercial LDH kit supplied by Thermo Fisher Scientific Inc. (Pittsburgh PA, US). Briefly, H9c2 cells were inoculated in a 96-well plate and grew to about 70% confluence. After the indicated treatments, 50 μL of supernatant per well was carefully removed and transferred into corresponding wells for the determination of extracellular LDH levels. Then, 100 μL of DMEM with 2% Triton X-100 was added to the adherent cells to lyse the cells. Fifty microliters of cell lysate was transferred to a 96-well plate to determine intracellular LDH levels. The same volume of prepared reaction mixture was added to the supernatant or homogenate, separately, and reacted for 10 min at room temperature by gentle shaking. Stopping solution was added to stop the reaction. Absorbance was measured at 490 and 600 nm with a microplate reader (Tecan Infinite F200, Switzerland). LDH release rate was calculated as follows (experiments were performed 4 times):

$$\%\text{LDH release} = \text{extracellular LDH}/(\text{extracellular LDH} + \text{intracellular LDH}) \times 100$$

MMP Measurement. Mitochondrial membrane potential (MMP) was observed using a fluorescent dye, rhodamine 123 (Rh123), a cell-permeable cationic dye that preferentially enters the mitochondria based on the highly negative MMP. Depolarization of MMP usually leads to a decreased intake of Rh123 and an intracellular weak fluorescence. After the indicated treatments, 10 mg/L Rh123 was added to cell cultures for 15 min at 37 °C, and fluorescence was measured over the entire field of vision using a fluorescent microscope (Advanced Microscopy Group, Seattle, US). The mean fluorescent intensity (MFI) of Rh123 from four random fields was analyzed using IMAGEJ 1.41o software.

Cardioprotective Effects in MI/R Injury. *Animals.* Male C57BL/6J mice, 10−12 weeks of age (Jackson Laboratories, Bar Harbor, ME) were used in the present study. All animals were housed in a temperature-controlled animal facility with a 12-h light/dark cycle, with water and rodent chow provided *ad libitum*. All animals received humane care in compliance with the Principles of Laboratory Animal Care formulated by the National Society of Medical Research and the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health. All animal procedures were approved by the Louisiana State University Health Science Center Institutional Animal Care and Use Committee.

Myocardial Ischemia/Reperfusion (MI/R) Protocol. Surgical occlusion of the left coronary artery (LCA), infarct size determination, and troponin I (TnI) measurements were performed as previously described.[63] C57BL6/J male mice (10−12 weeks of age) were anesthetized using pentobarbital (50 mg/kg) and ketamine (60 mg/kg) and maintained under anesthesia throughout the procedure with additional administration of pentobarbital (50 mg/kg). Mice were intubated and mechanically ventilated using a Hugo Sachs type 845 minivent. A hair removal agent was used to remove the hair on the chest of mice. The area was then cleaned using betadine and alcohol wipes alternating 3 times. An incision was made proximally left of the midline

of the chest, exposing the second and third ribs. The chest was opened by cauterizing through the second and third rib. The LCA was visualized, and using a 7−0 silk suture, a 3−5 mm piece of PE-10 tubing was secured over the LCA, occluding the artery. Mice were subjected to 45 min of myocardial injury, and at the time of reperfusion, an injection of vehicle (0.5% THF), **NSHD-1** (50 μg/kg and 100 μg/kg), or **NSHD-2** (50 μg/kg) was given i.c. The chest was closed in layers, closing the muscle layer first, then the skin. Mice were removed from ventilation once pedal reflex returned and it was determined that respiration was spontaneous. Mice were placed in a recovery cage and supplied with 100% O_2. Reperfusion was allowed for 24 h, and blood samples for TnI measurements were taken at 4 h after time of reperfusion. At 24 h of reperfusion, the LV area at risk (AAR) and infarct size were determined by Evans blue and 2,3,5-tetrazolium chloride stainin, as previously described.[63]

Drug Preparation. On the day of experimentation, test compounds (**NSHD-1** or **NSHD-2**) were diluted in 0.2 mL of 100% THF solution. For *in vivo* experiments, the test compounds were further diluted in sterile saline to final concentrations of 0.05 or 0.1 mg/mL. Then, various volumes were administered based on the animals' weights to obtain the correct dosage. The resulting concentration of THF in each dosage was 0.5% v/v. The vehicle consisted of a solution of 0.5% v/v THF in sterile saline.

Cardiac Troponin-I Assay. Blood samples were collected via a tail vein at 4 h of reperfusion. Cardiac troponin I level was measured in serum using the Life Diagnostic highsensitivity mouse cardiac troponin I ELISA kit (Mouse Cardiac Tn-I ELISA Kit; Life Diagnostics, West Chester, PA) following the manufacturer's instructions.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jmedchem.5b01033.

> Detailed synthetic procedures, characteristic data, and experimental procedures (PDF)

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Notes
The authors declare no competing financial interest.

ABBREVIATIONS USED

H_2S, hydrogen sulfide; NO, nitric oxide; CO, carbon monoxide; CBS, cystathionine β-synthase; CSE, cystathionine γ-lyase; MPST, 3-mercaptopyruvate sulfur-transferase; S-SH, *S*-sulfhydration; HSNO, thionitrous acid; Na_2S, sodium sulfide; NaHS, sodium hydrogen sulfide; DTTs, dithiolthiones; NSAIDs, nonsteroidal anti-inflammatory drugs; *N*-SH, *N*-mercapto; NSHDs, *N*-SH-based donors; SAR, structure−activity relationship; MI/R, myocardial ischemia reperfusion; MB, methylene blue; GSH, reduced glutathione; PBS, phosphate buffered saline; NCL, native chemical ligation; CCK-8, cell counting kit-8; OS, oxidative stress; ROS, reactive oxygen species; $O_2^{\bullet-}$, superoxide; $^{\bullet}OH$, hydroxyl radical; $^{\bullet}O$, singlet oxygen; H_2O_2, hydrogen peroxide; NBC, *N*-benzoyl cysteine; BZM, benzamide; LDH, lactate dehydrogenase; MMP, mitochondrial membrane potential; i.c., intracardiac; AAR/LV, area-at-risk per left ventricle; INF/AAR, infarct size per area-at-risk; TTC, 2,3,5-triphenyltetrazolium chloride; TnI, troponin I; AAR, area-at-risk; THF, tetrahydrofuran; TLC, thin layer chromatography; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; OD, optical density; MFI, mean fluorescent intensity; LCA, left coronary artery

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