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Controllable Hydrogen Sulfide Donors and Their Activity against Myocardial Ischemia-Reperfusion Injury

Yu Zhao,[†] Shashi Bhushan,[‡] Chuntao Yang,[§] Hiroyuki Otsuka,[‡] Jason D. Stein,[†] Armando Pacheco,[†] Bo Peng,[†] Nelmi O. Devarie-Baez,[†] Hector C. Aguilar,[∥] David J. Lefer,[‡] and Ming Xian*[,†]

[†]Department of Chemistry and [∥]Paul G. Allen School for Global Animal Health, Washington State University, Pullman, Washington 99164, United States

[‡]Department of Surgery, Division of Cardiothoracic Surgery, Emory University School of Medicine, Carlyle Fraser Heart Center, Atlanta, Georgia 30308, United States

[§]Department of Physiology, Guangzhou Medical University, Guangzhou 510182, China

Supporting Information

ABSTRACT: Hydrogen sulfide (H_2S), known as an important cellular signaling molecule, plays critical roles in many physiological and/or pathological processes. Modulation of H_2S levels could have tremendous therapeutic value. However, the study on H_2S has been hindered due to the lack of controllable H_2S releasing agents that could mimic the slow and moderate H_2S release *in vivo*. In this work we report the design, synthesis, and biological evaluation of a new class of controllable H_2S donors. Twenty-five donors were prepared and tested. Their structures were based on a perthiol template, which was suggested to be involved in H_2S biosynthesis. H_2S release mechanism from these donors was studied and proved to be thiol-dependent. We also developed a series of cell-based assays to access their H_2S-related activities. H9c2 cardiac myocytes were used in these experiments. We tested lead donors' cytotoxicity and confirmed their H_2S production in cells. Finally we demonstrated that selected donors showed potent protective effects in an *in vivo* murine model of myocardial ischemia-reperfusion injury, through a H_2S-related mechanism.

Hydrogen sulfide (H_2S) has been recognized as an important cellular signaling molecule, much like nitric oxide (NO).[1−6] The endogenous formation of H_2S is attributed to enzymes including cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE), and 3-mercaptopyruvate sulfurtransferase (MPST).[7−10] These enzymes convert cysteine or cysteine derivatives to H_2S in different tissues and organs. Recent studies have suggested that the production of endogenous H_2S and the exogenous administration of H_2S can exert protective effects in many pathologies.[1,2] For example, H_2S has been shown to relax vascular smooth muscle, induce vasodilation of isolated blood vessels, and reduce blood pressure. H_2S can also inhibit leukocyte adherence in mesenteric microcirculation during vascular inflammation in rats, suggesting H_2S is a potent anti-inflammatory molecule. In addition, H_2S may intereact with *S*-nitrosothiols to form thionitrous acid (HSNO), the smallest *S*-nitrosothiol, whose metabolites, such as NO^+, NO, and NO^-, have distinct but important physiological consequences.[11] These results strongly suggest that modulation of H_2S levels could have potential therapeutic values.

To explore the biological functions of H_2S, researchers started to use H_2S releasing compounds (also known as H_2S donors) to mimic endogenous H_2S generation.[12−14] The idea is similar to the well-studied nitric oxide (NO) donors. Currently there are many options for NO donors, including organic nitrates, nitrites, diazeniumdiolates, *N*-nitrosoamines, *N*-nitrosimines, *S*-nitrosothiols, hydroxylamines, *N*-hydroxyguanidines, etc. Moreover, many strategies, such as light, pH, enzymes, etc., can be used to trigger NO generation from these donors. In contrast, currently available H_2S donors are still very limited. These donors include the following: (1) sulfide salts, such as Na_2S, NaHS, and CaS, have been widely used in the field. These inorganic donors have the advantage of rapidly enhancing H_2S concentration. The maximum concentration of H_2S released from these salts can be reached within seconds. However such a fast generation may cause acute changes in blood pressure. In addition, since H_2S is highly volatile in solutions, the effective residence time of these donors in tissues may be very short.[15,16] (2) Naturally occurring polysulfide compounds such as diallyl trisulfide (DATS) are also employed as H_2S donors in some studies. DATS can vasodilate rat aortas[17] and protect rat ischemic myocardium[18] via a H_2S-related manner, but the simplicity of the structure limits its application as H_2S donors. (3) Synthetic H_2S donors have recently emerged as useful tools. GYY4137,[19] which is a Lawesson's reagent derivative, releases H_2S via hydrolysis both *in vitro* and *in vivo* and exhibits some interesting biological activities[20,21] such as anti-inflammation.[22−24] H_2S release from

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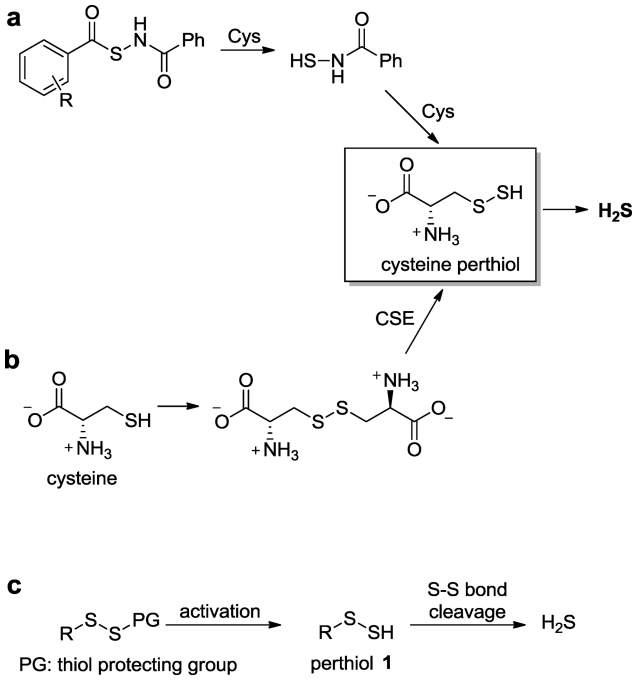
GYY4137 is relatively low (<10% of H_2S was released from this molecule after 7 days).[25] Dithiolthione is another structure that releases H_2S in aqueous solution.[13] However the detailed mechanism is still unclear. A major limitation of current donors is that H_2S release is largely uncontrollable. 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. In our opinion, ideal H_2S donors should be stable by themselves in aqueous solutions. The release of H_2S (both the time and the rate) should be controllable (upon activation by certain factors). Such donors will not only be useful research tools for H_2S researchers but also have unique therapeutic benefits themselves.

The research in our laboratory focuses on the development of controllable H_2S donors. In 2011 we discovered a series of *N*-(benzoylthio)benzamide derivatives as thiol-activated H_2S donors.[26] These compounds are stable in aqueous solutions and in the presence of some cellular nucleophiles. Upon activation by cysteine or reduced glutathione (GSH), the compounds could produce H_2S (Scheme 1a).[26] In this process,

Scheme 1. (a) Hydrogen Sulfide (H_2S) Release from *N*-(Benzoylthio)benzamide Derivatives; (b) H_2S Biosynthesis Catalyzed by Cystathionine γ-lyase (CSE); (c) Concept of Perthiol-Based H_2S Donors



cysteine perthiol (also known as thiocysteine) is believed to be a key intermediate. It should be noticed that cysteine perthiol is also involved in H_2S biosynthesis catalyzed by CSE (Scheme 1b).[13,27] These findings suggest that the perthiol (Scheme 1c) can be a useful template for the design of controllable H_2S donors. Herein we report the development of perthiol-based donors and their activities in myocardial ischemia-reperfusion (MI/R) injury.

RESULTS AND DISCUSSION

Primary Perthiol-Based H_2S Donors. Perthiols are known to be unstable species.[28−30] We expected a protecting group on -SH could enhance the stability. In addition, the protecting group could allow us to develop different strategies to retrieve perthiol, therefore achieving the regulation of H_2S release. With this idea in mind, we decided to test cysteine-based perthiol derivatives **2** (Scheme 2). Acyl groups were used as the protecting group on perthiol moieties.

The synthesis of four cysteine-based donors (**2a**−**d**) is described in Supplementary Scheme S1. *N*-Benzoyl cysteine methyl ester was first treated with 2-mercapto pyridine disulfide to provide a cysteine-pyridine disulfide intermediate, which was then treated with corresponding thioacids to give the desired donor compounds. Using the procedure reported previously,[26] we analyzed H_2S release capabilities of these donors in the presence of cysteine and GSH. As shown in Scheme 2, these donors indeed could generate H_2S in the presence of thiols. Compared to *N*-(benzoylthio)-benzamide type donors, however, these donors showed much decreased ability of H_2S generation (read from their peaking concentrations). The initial concentration of the donors was 150 μM, while the maximum H_2S concentration formed was less than 15% (by cysteine) or 8% (by GSH).

From the reaction mechanism point of view, we expected the free SH of cysteine or GSH would undergo a thioester exchange with the acyl group to produce perthiol **4** (pathway A, Scheme 2b), which in turn should lead to H_2S formation. However, it was also possible that SH reacted with the acyl-disulfide linkage to form a new disulfide **5** and thioacid **6** (pathway B). We found that thioacids could not release H_2S even in the presence of cysteine or GSH under the conditions used in our experiments (see Supplementary Figure S1).[31] Therefore, H_2S release from donors **2** was diminished due to the involvement of pathway B.

Tertiary Perthiol-Based H_2S Donors. We envisioned that the steric hindrance on the α-carbon of the disulfide bridge should prevent the reactions through pathway B and therefore enhance H_2S formation. As such we decided to prepare a series of penicillamine-based perthiol derivatives **8** (Scheme 3a). The general synthesis of this series of donors is described in Supplementary Scheme S2. Briefly, *C*- and *N*-protected penicillamine was first treated with 2,2′-dibenzothioazolyl disulfide to provide a penicillamine-benzothioazolyl disulfide intermediate and then treated with corresponding thioacids to furnish the desired penicillamine-based donors. Both steps gave good yields for all of the substrates. These donor compounds proved to be stable in aqueous solutions. In the presence of cysteine or GSH, we observed a time-dependent H_2S generation. The representative H_2S release curves of **8a** are shown in Scheme 3. With an initial concentration at 100 μM, the maximum H_2S concentration formed was ∼80 μM (with cysteine), which demonstrated the efficiency of this compound as H_2S donor. After reaching the maximum value, H_2S concentration started to drop due to volatilization.[15]

We expected that the change of acyl substitutions could affect the rate of thioester exchange and regulate H_2S generation. Therefore a series of acyl substitution modified donors (19 compounds in total) were prepared and tested. The H_2S generation data of 9 representative compounds are summerized in Scheme 3b. Data of the rest of the compounds are shown in Supplementary Table S1. Generally, electron-withdrawing groups on the phenyl group led to faster H_2S generation, while electron-donating groups led to slower H_2S release. We also observed significant steric effects on H_2S formation. More sterically hindered substrates (compounds **8o**, **8r**, **8s**) resulted in slower H_2S release (with decreased H_2S amounts) or even

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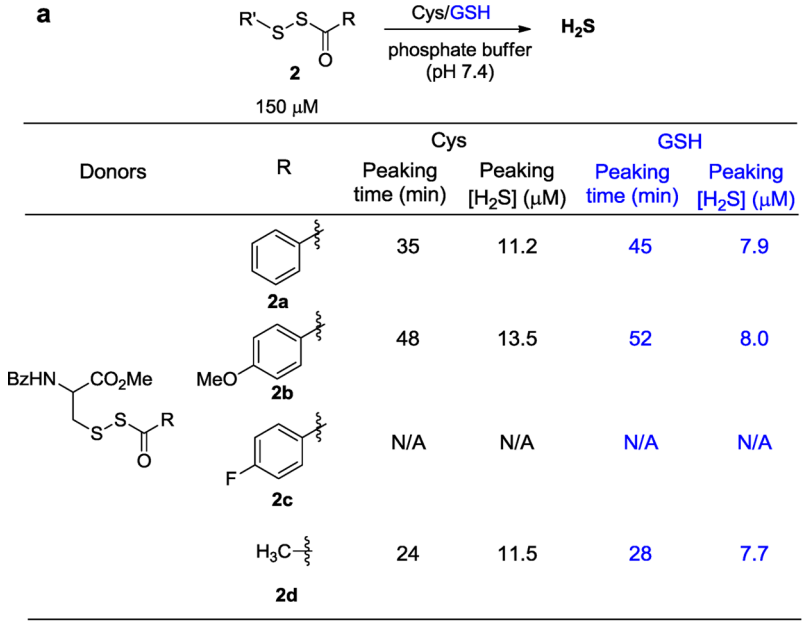
Scheme 2. (a) H_2S Generation Data of Cysteine-Based Donors 2; (b) Proposed Reactions of 2



no release at all. In addition, we found that cysteine always caused higher/faster H_2S release compared to GSH. This is likely due to the fact that cysteine, compared with GSH, is a smaller molecule and can react more quickly with the thioester group. These results demonstrated that H_2S release from these perthiol-based donors could be regulated via structural modifications.

H_2S Release Mechanism Study. To understand the mechanism of H_2S release from these donors, we studied the reaction between **8a** and a cysteine derivative **9** (3 equiv). As shown in Scheme 4, we confirmed the formation of a thioester **10**, an asymmetric disulfide **12**, a free thiol **13**, as well as cysteine disulfide **15**. On the basis of these reaction products, we proposed the mechanism as follows: the reaction is initiated by a thioester exchange between **8a** and **9** to form a new thioester **10** and perthiol **11**. Both S atoms of **11** can be attacked by cysteine.[32] Therefore two possible pathways exist: (a) the cysteine attacks the internal S to yield disulfide **12** and liberate H_2S, or (b) the external S is attacked to form thiol **13** and cysteine perthiol **14**. Then another molecule of cysteine **9** reacts with **14** to form disulfide **15** and release H_2S. In this process it is also possible that **13** reacts with **14** to form disulfide **12** and release H_2S.

Biological Evaluation of Perthiol-Based H_2S Donors. With these donors in hand, we next explored their therapeutic benefits. Recent studies with animal models suggested that H_2S can protect cardiovascular system against myocardial ischemia-reperfusion (MI/R) injury.[8,17,33−35] Several groups have shown that H_2S, when applied both at the time of reperfusion and as a preconditioning reagent, exhibits the cardioprotection by different mechanisms, such as preserving mitochondrial function,[36] reducing oxidative stress,[34] decreasing myocardial inflammation,[37] and improving angiogenesis.[38] We envisioned that our perthiol-based donors might exhibit similar myocardial protective effects in an *in vivo* model of murine MI/R injury.

Before conducting animal experiments, we tested cytotoxicity of two representative donors (**8a**/**8l**) in H9c2 cardiac myocytes. The cell viability was detected using cell counter kit (CCK)-8 assay (Figure 1). After 24-h exposure of H9c2 cells to **8a** and **8l** at varied concentrations (0 to 100 μM), cell viability did not decrease. Interestingly the exposure of cells to **8a** and to **8l** at concentrations of 12, 25, 50, and 100 μM, increased cell viability percentage (at the level compared to 400 μM NaHS). The results of these studies indicate that these perthiol-based donors do not promote cytotoxicity in cardiac cells at the doses we have tested. We also determined cell viability through the evaluation of reactive oxygen species (ROS) concentration. The results are shown in Supplementary Figure S3. Donors **8a** and **8l** did not lead to ROS increase, which confirms the safety of the donors.

We wondered if our donors indeed could release H_2S when interacting with myocytes and then conducted experiments to

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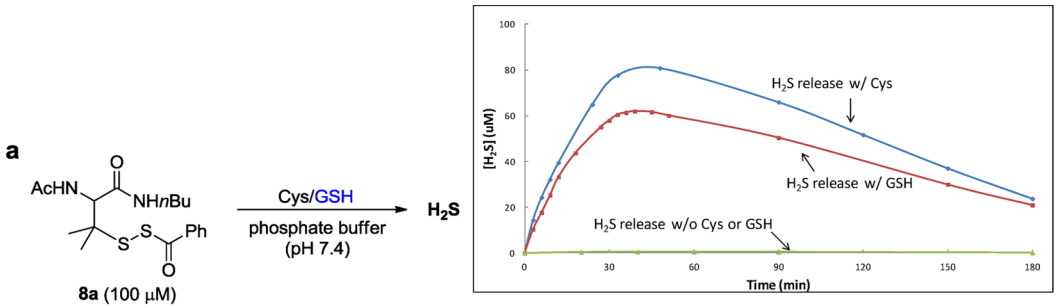
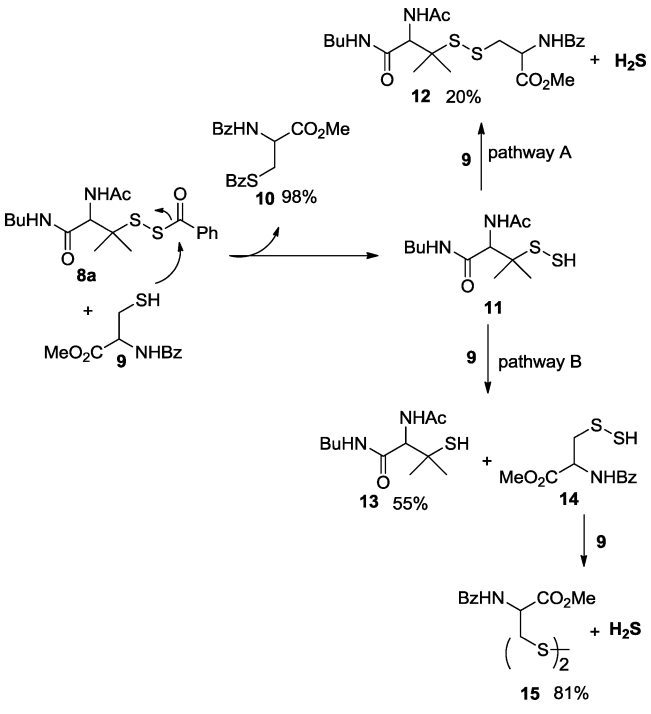
Scheme 3. (a) H_2S Release Curve of Donor 8a; (b) H_2S Generation Data of Selected Penicillamine-Based Donors



b

R	Peaking time (min)	Peaking [H_2S] (μM)	R	Peaking time (min)	Peaking [H_2S] (μM)	R	Peaking time (min)	Peaking [H_2S] (μM)
8a	39[a] (39)[b]	80.3 (61.9)	**8j**	50 (45)	63.0 (44.9)	**8o**	60 (70)	58.4 (14.4)
8b	34 (36)	48.3 (37.1)	**8l**	39 (42)	70.6 (61.3)	**8r**	60 (75)	52.7 (14.3)
8g	36 (48)	68.0 (53.9)	**8n**	48 (51)	50.8 (26.1)	**8s**	N/A	N/A

Scheme 4. Proposed Mechanism of H_2S Generation



address this question. As shown in Figure 2, H9c2 cells were incubated with the donors (**8a** and **8l**) for 30 min, respectively. After that a selective H_2S fluorescent probe, WSP-1,[39] was applied into the cells to monitor the production of H_2S. As expected, donor-treated cells (Figure 2b and c) showed much enhanced fluorescent signals compared to vehicle-treated cells (Figure 2a). The image of positive control (with H_2S) is shown

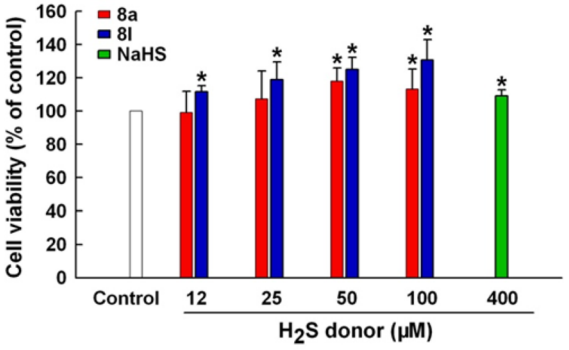


Figure 1. Effects of **8a** and **8l** on cell viability. H9c2 cells were treated with different concentrations of **8a** or **8l** (12−100 μM) for 24 h. The cell counter kit (CCK)-8 assay was performed to measure cell viability. Data were shown as the mean ± SD (n = 8). $^{**}P < 0.01$ versus control group.

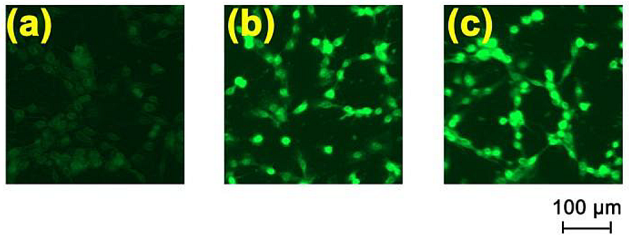


Figure 2. H_2S production from **8a** and **8l** in H9c2 cells. Cells were incubated with vehicle (A), 100 μM **8a** (B), and 100 μM of **8l** (C) for 30 min. After removal of excess donors, 250 μM concentration of a H_2S fluorescent probe (WSP-1) was added. Images were taken after 30 min.

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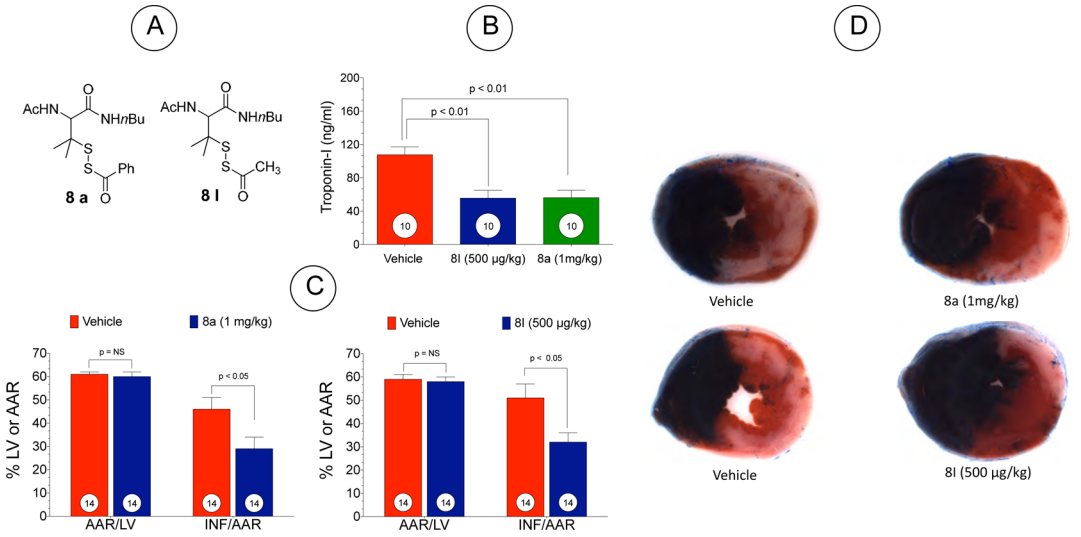

Figure 3. Cardioprotective effects of compounds **8a** and **8l** in myocardial ischemia-reperfusion injury. Compound **8a**, **8l**, or vehicle were injected *in vivo* after 22.5 min of ischemia. (A) Structures of donors. (B) Circulating cardiac troponin I levels at following 45 min of MI and 2 h of reperfusion. Troponin-I was significantly ($p < 0.01$) reduced with either **8a** or **8l**. (C) Myocardial area-at-risk (AAR) per left ventricle (AAR/LV) and infarct size per area-at-risk (INF/AAR) were assessed in vehicle- ($n = 14$) and donor-treated animals ($n = 14$) at 24 h following MI/R. AAR/LV was similar among all groups. INF/AAR was significantly ($p < 0.05$) smaller in animals treated with either **8a** or **8l** as compared to vehicle. (D) Representative photomicrographs of a midventricular slice after MI/R stained with Evan's blue and 2,3,5-triphenyltetrazolium chloride for both vehicle- and donor-treated hearts.

in Supplementary Figure S2. In addition, we did not observe shape changes of the cells after the treatment. These results demonstrated that perthiol-based donors can release H_2S when interacting with H9c2 cells and H_2S generation can be evaluated by fluorescent image.

Finally we tested myocardial protective effects of donors **8a** and **8l** 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. Compounds **8a**, **8l**, or vehicle were administered into the left ventricular lumen at the 22.5 min of myocardial ischemia. All animal groups displayed similar area-at-risk per left ventricle (AAR/LV), which means surgery caused similar risk. However, compared to vehicle-treated mice, mice receiving **8a** or **8l** displayed a significant reduction in circulating levels of cardiac troponin I and myocardial infract size per area-at-risk (Figure 3B and C). For example, a 500 μg/kg bolus of **8l** maximally reduced INF/AAR by ~50%, which was a significant protection. These results demonstrated that perthiol-based compounds can exhibit H_2S-mediated cardiac protection in MI/R injury and these compounds may have potential therapeutic benefits.

We also tested *in vivo* H_2S production from donors **8a** and **8l**. As such, donors (1 mg/kg for **8a** and 500 μg/kg for **8l**) were injected intravenously via tail vein injection. Blood and hearts were obtained at 15 min following injection. H_2S levels were determined using previously described gas chromatography and chemiluminesence methods.[40] As shown in Figure 4, blood and myocardial levels of H_2S were significantly ($p < 0.01$) increased following injection of the donors as compared to controls.

Conclusion. In summary, we have developed a series of new H_2S donors based on the perthiol template. Their H_2S generation is regulated by thiols such as cysteine or GSH. We also demonstrated that H_2S release capability from these donors can be manipulated by structural modifications. Moreover, these donors are nontoxic to cardiac cells and their H_2S production upon interacting with myocytes can be

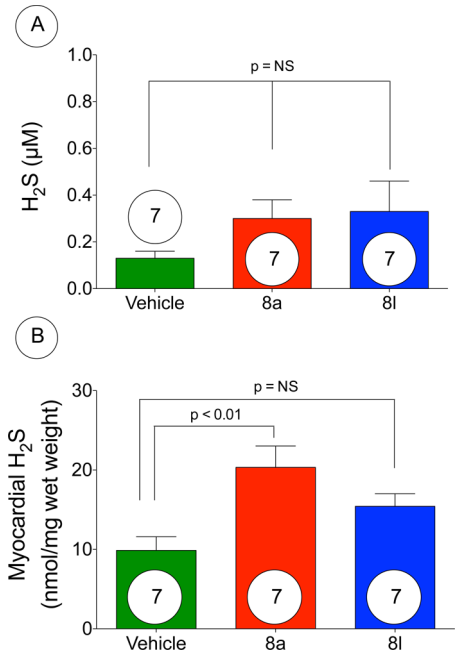

Figure 4. *In vivo* H_2S levels (μM) in blood (A) and hearts (B) obtained from mice treated with **8a** and **8l**.

detected. Some donors exhibited potent myocardial protective effects in MI/R injury, presumably due to H_2S generation. It should be noted that H_2S generation from these donors is not dependent on specific enzymes such as CBS and CSE. Recently our group tested the effects of some H_2S donors on CBS/CSE activity and we did not notice any changes even after weeks.[41] Taken together these donors may be potential therapeutic agents. In our *in vivo* experiments, **8l** exhibited better cardioprotective effects compared to those of **8a** (Figure 3). Interestingly, **8l** also exhibited better activity in cell viability test and H_2S generation test (Figures 1 and 2). These data suggest

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that *in vitro* evaluation of donors may allow us to predicate donors' *in vivo* behaviors. Further development of this type of donors and evaluation of their other H_2S related biological activities are currently ongoing in our laboratory.

METHODS

Synthesis of Compounds 2a−2d. 2-Mercapto pyridine disulfide (2.2 g, 10 mmol) was dissolved in 50 mL of $CHCl_3$. To this solution was added *N*-benzoyl cysteine methyl ester (1.2 g, 5 mmol). The reaction was stirred at RT for 1 h and then concentrated under vacuum, and 1.48 g of compound **a** was obtained as a white solid by flash chromatography (hexane/ethyl acetate = 2:1). Please see Supporting Information for characterization data of **a**. Synthetic intermediate **a**, 83 mg, 0.24 mmol, was dissolved in 5 mL of $CHCl_3$. To this solution was added thiobenzoic acid (42 mg, 0.3 mmol). The mixture was stirred at RT for 1 h. The excess thiobenzoic acid was removed by washing with aqueous $NaHCO_3$ solution. The organic layer was separated, dried, and concentrated under vacuum. The final product **2a** was purified as white solid by flash chromatography (hexane/ethyl acetate = 10: 4). Mp 94−96 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.97 (d, J = 6.9 Hz, 2H), 7.90 (d, J = 7.8 Hz, 2H), 7.80 (d, J = 7.5 Hz, 1H), 7.61 (t, J = 7.5 Hz, 1H), 7.47 (m, 5H), 5.06 (m, 1H), 3.70 (s, 3H), 3.57 (dd, J = 14.4, 4.8 Hz, 1H), 3.30 (dd, J = 14.4, 4.8 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 191.3, 170.8, 167.3, 135.3, 134.7, 133.7, 132.1, 129.2, 128.8, 128.0, 127.6, 53.0, 51.8, 40.9; IR (thin film) cm^{-1} 3326, 3056, 2955, 2927, 1748, 1683, 1638, 1520, 1489, 1319, 1203, 883; mass spectrum (ESI/MS) m/z 398.1 [M + Na]$^+$; HRMS m/z 398.0500 [M + Na]$^+$; calcd for $C_{18}H_{17}NNaO_4S_2$ 398.0497; yield 81%.

Compounds **2b−2d** were prepared from the corresponding thiolacids using the same procedure as **2a**. Their data are reported in the Supporting Information.

Synthesis of Compounds 8a−8s. 2,2′-Dibenzothiazolyl disulfide (4.32g, 13 mmol) was dissolved into 500 mL of $CHCl_3$. To this solution was added D,L-penicillamine derivative **13** (2.36 g, 9.6 mmol). The reaction mixture was stirred at RT for 48 h. Solvent was then removed, and the crude mixture was then purified by flash column chromatography (3% v/v MeOH in DCM) to provide the intermediate **d** as white solid. To a 15 mL $CHCl_3$ solution containing **d** (822 mg, 2 mmol) was added thiobenzoic acid (1.10 g, 8 mmol). The reaction was stirred at RT for 10 min. Excess thiobenzoic acid was removed by washing with $NaHCO_3$. The organic layer was separated, dried, and concentrated. The final product **8a** was purified by flash column chromatography (1% v/v MeOH in DCM) as white solid. Mp 132−134 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.16 (m, 1H), 8.03 (d, J = 7.5 Hz, 2H), 7.64 (t, J = 7.5 Hz, 1H), 7.49 (t, J = 7.5 Hz, 2H), 7.04 (d, J = 8.1 Hz, 1H), 4.46 (d, J = 8.4 Hz, 1H), 3.36 (m, 2H), 2.01 (s, 3H), 1.62 (m, 2H), 1.44 (m, 5H), 1.25 (s, 3H), 0.95 (t, J = 7.2 Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 194.0, 170.4, 168.8, 135.5, 134.8, 129.2, 128.3, 58.7, 53.8, 39.8, 31.6, 27.0, 24.0, 23.5, 20.5, 14.0; IR (thin film) cm^{-1} 3285, 3085, 2962, 2929, 2868, 1684, 1636, 1561, 1527, 1445, 1379, 1202, 1174, 1118, 890, 676; mass spectrum (ESI/MS) m/z 405.1 [M + Na]$^+$; HRMS m/z 383.1411 [M + H]$^+$; calcd for $C_{18}H_{27}N_2O_3S_2$ 383.1463; yield 94%.

Compounds **8b−8s** were prepared from corresponding thiolacids using the same procedure as **8a.**

H_2S Measurement. The reaction was initiated by adding 75 μL of stock solution of the donor (40 mM, in THF) into pH 7.4 phosphate buffer (30 mL) containing cysteine (1.0 mM). Then 1.0 mL of reaction aliquots were periodically taken and transferred to 4.0-mL vials containing zinc acetate (1% w/v, 100 μL) and *N*,*N*-dimethyl-1,4-phenylenediamine sulfate (20 mM, 200 μL) in 7.2 M HCl and ferric chloride (30 mM, 200 μL) in 1.2 M HCl. The absorbance (670 nm) of the resulted solution (1.5 mL) was determined 15 min thereafter using a UV−vis spectrometer (Thermo Evolution 300). The H_2S concentration of each sample was calculated against a calibration curve of Na_2S. The H_2S releasing curve was obtained by plotting H_2S concentration versus time.

Product Analysis. A 100 mg portion of **8a** (0.26 mmol) was dissolved in 10.0 mL of THF/phosphate buffer (pH 7.4) (1:1, v/v). Then cysteine derivative **9** (187 mg, 0.78 mmol) was added into the solution. The mixture was stirred at RT for 1 h. The reaction mixture was extracted with DCM 3 times. The organic layers were combined, dried with $MgSO_4$, and concentrated. Products **10**, **12**, **13**, and **15** were isolated by flash column chromatography (1% v/v MeOH in DCM).

10 and **15** are known compounds. Their data are given in Supporting Information.

12 (1:1 mixture of diastereoisomers): mp 73−75 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.84 (d, J = 6.6 Hz, 2H), 7.82 (d, J = 6.9 Hz, 2H), 7.46 (m, 6H), 7.29 (m, 2H), 6.96 (br, 1H), 6.78 (br, 1H), 6.69 (d, J = 9.3 Hz, 2H), 5.09 (m, 2H), 4.68 (d, J = 9..9 Hz, 1H), 4.65 (d, J = 9.9 Hz, 1H), 3.78 (s, 6H), 3.29 (m, 6H), 3.07 (m, 2H), 1.97 (s, 6H), 1.35 (m, 20H), 0.86 (t, J = 7.2 Hz, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 171.4, 171.2, 170.4 (2C), 169.3, 169.2, 167.4, 167.3, 133.8, 133.7, 132.2, 132.1, 128.8 (2C), 127.5, 127.4, 58.6, 58.4, 53.3, 53.1, 53.0, 52.8, 42.3, 42.2, 39.6, 34.9, 31.8 (2C), 31.5 (2C), 29.3, 25.5, 25.4, 25.3, 24.2, 23.5, 22.9, 20.3, 14.4, 14.0; IR (thin film) cm^{-1} 3300, 3072, 2962, 2934, 2871, 1739, 1645, 1535, 1366, 1228; mass spectrum (ESI/MS) m/z 506.1 [M + Na]$^+$; HRMS m/z 506.1752 [M + Na]$^+$; calcd for $C_{22}H_{33}N_3NaO_5S_2$ 506.1759; yield 20%.

13: mp 176−177 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.92 (br, 1H), 6.77 (d, J = 9.3 Hz, 1H), 4.51 (d, J = 9.3 Hz, 1H), 3.21 (m, 2H), 2.65 (s, 1H), 2.04 (s, 3H), 1.48 (m, 5H), 1.32 (m, 5H), 0.90 (t, J = 7.2 Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.4, 169.9, 60.4, 46.3, 39.4, 31.5, 31.2, 28.7, 23.6, 20.3, 14.0; IR (thin film) cm^{-1} 3267, 3084, 2967, 2935, 2874, 2558, 1667, 1638, 1537, 1456, 1371, 1241, 1136; mass spectrum (ESI/MS) m/z 269.1 [M + Na]$^+$; HRMS m/z 247.1473 [M + H]$^+$; calcd for $C_{11}H_{23}N_2O_2S$ 247.1480; yield 55%.

Cell Viability Assay. H9c2 (2-1) cardiomyocytes (H9c2 cells) were cultured in DMEM high glucose medium supplemented with 10% fetal bovine serum (FBS) at 37 °C under an atmosphere of 5% CO_2 and 95% air. H9c2 cells at a concentration of 1×10^5/mL were inoculated in 96-well plates and cultured overnight. H_2S donor (**8a** or **8l**) in FBS-free medium was administered and cultured for 24 h. The cell viability was measured by cell counter kit (CCK)-8. The absorbance at 450 nm was measured with a microplate reader (Molecular Devices, Sunnyvale, CA, USA). Optical density (OD) of the 8 wells in the indicated groups was used to calculate percentage of cell viability according to the formula below:

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

H_2S Release in H9c2 Cells. H9c2 cells were inoculated in 6-well plates and cultured overnight. The cells were co-incubated with 100 μM H_2S donor, **8a**, or **8l** dissolved in phosphate buffered solution (PBS) at 37 °C for 30 min, and then the solution in the wells was removed. The cells were then co-incubated with a H_2S probe (WSP-1) solution (250 μM in PBS) and surfactant CTAB (500 μM) in PBS at 37 °C for 30 min. After the PBS was removed, the fluorescence signal was observed by AMG fluorescent microscope (Advanced Microscopy Group, USA).

Cardioprotective Effects in MI/R. *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 (Publication 85-23, Revised 1996). All animal procedures were approved by the Emory University Institutional Animal Care and Use Committee.

Drug Preparation. On the day of experimentation, test compounds (**8a** or **8l**) were diluted in 0.5 mL of 100% THF solution. For *in vivo* experiments, the test compounds were further diluted in sterile saline to obtain the correct dosage to be delivered in a volume of 50 μL. The

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resulting concentration of THF in this dosage was 0.5% v/v. Vehicle consisted of a solution of 0.5% v/v THF in sterile saline.

Myocardial Ischemia/Reperfusion (MI/R) Protocol and Assessment of Myocardial Infarct Size.[42] Mice were fully anesthetized via intraperitoneal injection of ketamine (50 mg/kg) and pentobarbital sodium (60 mg/kg), intubated, and connected to a rodent ventilator. A median sternotomy was performed to gain access to and identify the left coronary artery (LCA). The LCA was surgically ligated with a 7-0 silk suture mated to a BV-1 needle to ensnare the LCA. A short segment of PE-10 tubing was placed between the LCA and the 7-0 suture to cushion the artery against trauma. Mice were subjected to 45 min of LCA ischemia, followed by reperfusion for 24 h. At 22.5 min of ischemia, a single dose of intracardiac injection (50 μL total volume administered with a 31-gauge needle directly into the left ventricular lumen via injection at the apex of the heart) of compound **8a**, compound **8l**, or vehicle (0.5% THF mixed with saline) was administered. After 24 h of reperfusion, mice were anesthetized and connected to a rodent ventilator. The LCA was religated at the same place as the previous day, and a catheter was placed inside the carotid artery to inject 7.0% Evans blue (1.2 mL) to delineate between ischemic and nonischemic zones. The heart was rapidly excised and cross-sectioned into 1-mm-thick sections, which were then incubated in 1.0% m/v 2,3,5-triphenyl tetrazolium chloride for 4 min to demarcate the viable and nonviable myocardium within the risk zone. Digital images of each side of heart section were taken and weighed, and the myocardial area-at-risk and infarct per left ventricle were determined by a blinded observer.

Cardiac Troponin-I Assay.[42] 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 high-sensitivity mouse cardiac troponin-I ELISA kit (Mouse Cardiac Tn-I ELISA Kit; Life Diagnostics, West Chester, PA) as previously described.

***In Vivo* Determination of H_2S Levels.** H_2S levels were measured according to previously described gas chromatography and chemiluminescence methods.[40] Myocardial tissue or blood were homogenized in 5 vol of PBS (pH 7.4), and 0.2 mL of the sample homogenate was placed in a small glass vial along with 0.4 mL of 1 M sodium citrate buffer, pH 6.0, and sealed. The mixture was incubated at 37 °C for 10 min with shaking at 125 rpm on a rotary shaker to facilitate the release of H_2S gas from the aqueous phase. After shaking, 0.1 mL of headspace gas was applied to a gas chromatograph (7890A GC, Agilent) equipped with a dual plasma controller and chemiluminescence sulfur detector (355, Agilent). H_2S concentrations were calculated using a standard curve of Na_2S as a source of H_2S. Chromatographs were captured and analyzed with Agilent ChemStation software (B.04.03).

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: mxian@wsu.edu.

Notes

The authors declare no competing financial interest.

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