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pH-Controlled Hydrogen Sulfide Release for Myocardial Ischemia-Reperfusion Injury

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Supporting Information

ABSTRACT: Hydrogen sulfide (H_2S) is a critical signaling molecule that regulates many physiological and/or pathological processes. Modulation of H_2S levels could have potential therapeutic value. In this work, we report the rational design, synthesis, and biological evaluation of a class of phosphonamidothioate-based H_2S-releasing agents (i.e., H_2S donors). A novel pH-dependent intramolecular cyclization was employed to promote H_2S release from the donors. These water-soluble compounds showed slow, controllable, and pH-sensitive production of H_2S in aqueous solutions. The donors also showed significant cytoprotective effects in cellular models of oxidative damage. Most importantly, the donors were found to exhibit potent cardioprotective effects in an in vivo murine model of myocardial ischemia-reperfusion (MI/R) injury through a H_2S-related mechanism.

Hydrogen sulfide (H_2S) has been recently recognized as a member of the gasotransmitter family, along with nitric oxide (NO) and carbon monoxide (CO). Studies have demonstrated that the production of endogenous H_2S and the exogenous administration of H_2S can exert protective effects in many pathologies.[1] 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, suggesting that H_2S is a potent anti-inflammatory molecule.[2] In addition, H_2S may interact 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.[3] These results strongly suggest that modulation of H_2S levels could have therapeutic value.

In this field, chemical tools that allow precise detection and delivery of H_2S are critical.[4] In particular, H_2S-releasing agents (also known as H_2S donors) are not only important research tools but also potential therapeutic agents. 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 concentration. In addition, H_2S concentrations in aqueous solution can rapidly decrease as a result of volatilization, thus significantly limiting the use of these two H_2S sources.

In view of these drawbacks, synthetic H_2S donors have received considerable attention.[5] To date, several types of H_2S donors have been reported, and their H_2S releases are controlled by different mechanisms such as hydrolysis, cellular thiol activation, and photolysis.[5] Among currently available donors, GYY4137 is the most widely used and has shown some H_2S-like bioactivities.[6,7] This water-soluble derivative of Lawesson's reagent is believed to slowly release H_2S upon hydrolysis in water.[6] A recent work by Whiteman, Wood, and co-workers carefully studied the hydrolysis kinetics and byproducts of GYY4137.[8] The hydrolysis of GYY4137 in pure aqueous solutions was found to be too slow to be monitored by ^{31}P NMR spectroscopy. "Wet" organic solvents such as acetone or chloroform led to faster hydrolysis, but complete hydrolysis required 71 days. On the other hand, incubation of GYY4137 in cell lysates does lead to detectable H_2S production, but the mechanism is still unclear. Structural modifications of GYY4137 have been studied with the goal of improving the H_2S production ability.[7] However, the resulting analogues (e.g., phosphorodithioates; Scheme 1) did not show significantly improved H_2S release. We envisioned the core structure of GYY4137 or the phosphorothioate to be a valuable template for the design of useful H_2S donors, but new activation strategies should be invented. Moreover, understanding the mechanism of H_2S release and the identity of byproducts should be investigated for applications of the donors. With these considerations in mind, we expected that phosphorothioates might be activated by an intramolecular cyclization (as described in Scheme 1). The rates of cyclization are likely to be regulated by pH. Such a design should lead to more productive and pH-controlled H_2S release.

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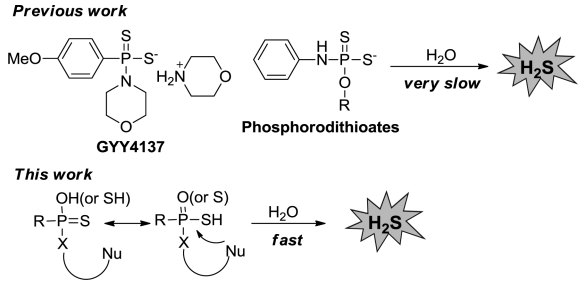
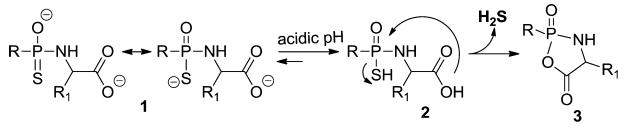
Scheme 1. Enhanced H_2S Production from Phosphorothioates



Herein we report the development of intramolecular-cyclization-based donors and their biological activities.

As shown in Scheme 2, we expected that protonation of phosphonamidothioates **1** (at neutral or slightly acidic pH)

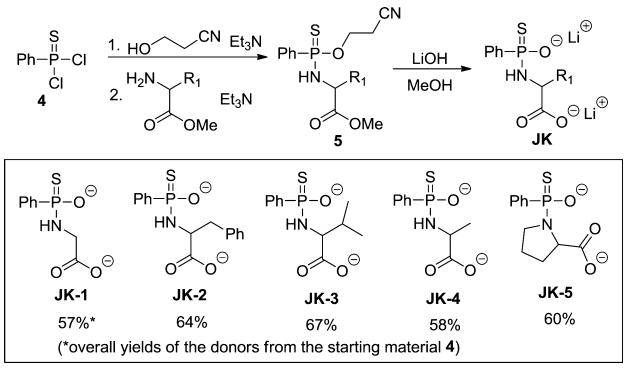
Scheme 2. Design of Intramolecular-Cyclization-Based Donors



should form the corresponding phosphorothiols **2**. This process should facilitate the release of H_2S if a nucleophilic carboxylate is presented at a suitable position. The formation of the five-membered-ring product **3** could be the driving force for H_2S release.[9] To the best of our knowledge, pH-controlled slow-release H_2S donors have not been reported. Such donors could have unique biological applications. For example, it has been proved that H_2S-based therapy is promising for myocardial ischemia-reperfusion (MI/R) injury.[10] Ischemic injury can lead to reduced local pH levels.[11] Thus, acid-promoted H_2S release would be very attractive for the treatment of ischemic injury. In our design, readily available amino acids were used to construct the donors. We also expected different side-chain residues (R_1) on the amino acids would affect the rates of intramolecular cyclization, thereby regulating the H_2S release profiles.

With this idea in mind, a series of donors (**JK-1**–**JK-5**) were synthesized from phenylphosphonothioic dichloride (**4**) (Scheme 3). Briefly, **4** was sequentially treated with 3-hydroxypropionitrile and a C-protected amino acid to provide the precursor **5**. LiOH-mediated hydrolysis of **5** then provided the donor product in good overall yield. Five amino acids

Scheme 3. Synthesis and Structures of New Donors



(glycine, phenylalanine, valine, alanine, and proline) were employed to prepare these donors. All of these products were fully characterized (see the Supporting Information (SI)).

With these new donors in hand, we first tested their H_2S-releasing ability in aqueous buffers. For comparison, GYY4137 was also tested. It should be noted that previous measurements of H_2S production from GYY4137 and similar donors were mainly done using the standard methylene blue (MB) method.[6,12] However, strongly acidic conditions are involved in this assay. It is known that acids can dramatically facilitate hydrolysis of phosphorothioates.[6,13] Therefore, the standard MB method is not appropriate for evaluating phosphorothioate-based donors. In this study, a modified zinc sulfide-precipitation-based MB method[14] was used (see the SI). This method avoids the false signals caused by acid-promoted hydrolysis. Using this method, we found that H_2S releases from the **JK** donors were significantly affected by pH. In general, weakly acidic pH (5 and 6) caused faster and more H_2S release, while neutral and weakly basic pH (7.4 and 8) caused slower and less H_2S release. As examples, the time-dependent H_2S release curves for **JK-1** and **JK-2** at different pH were compared with that for GYY4137 (Figure 1). Clearly,

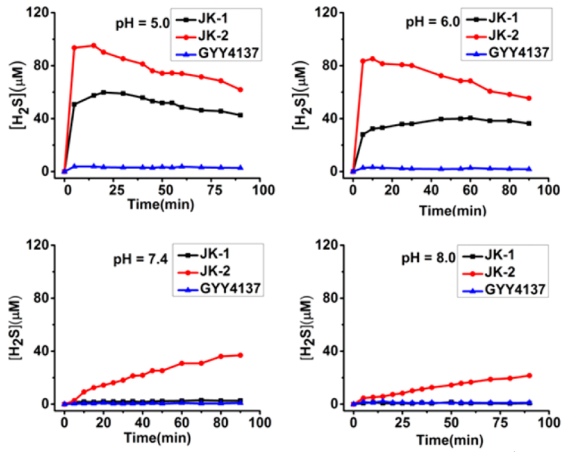


Figure 1. H_2S release profiles of **JK-1**, **JK-2**, and GYY4137 (100 μM) at different pH.

GYY4137 released very little H_2S under these conditions. In contrast **JK-1** and **JK-2** showed much-enhanced H_2S-releasing ability. Most interestingly, **JK-1** was found to be a donor that releases H_2S only at weakly acidic pH (5 and 6). It released barely detectable amounts of H_2S at pH 7.4 and 8. **JK-2**, however, showed slow and sustained H_2S release at pH 7.4 and 8 but much faster release at pH 5 and 6. The enhanced H_2S production from **JK-2** is likely due to the introduction of the benzyl group at the α-position, which facilitates the intramolecular cyclization. Two other donors, **JK-3** and **JK-4**, showed activities similar to that of **JK-2** (data for **JK-3** and **JK-4** are shown in Figure S1 in the SI). **JK-5**, on the other hand, was found not to release H_2S at all. Perhaps the rigid proline ring makes the cyclization very difficult. Quantum-chemical calculations on the cyclization step for **JK-1**, **JK-2**, and **JK-5** were carried out. The results (shown in Figures S2 and S3) confirmed their different behaviors in experiments. Moreover, the mechanism of H_2S release from the donors was also confirmed by analysis of the products (Scheme S1 in the SI). Overall, these results demonstrated that (1) the intramolecular cyclization was effective in promoting H_2S release from phosphorothioate-based donors, (2) the resultant donors showed unique pH-controlled H_2S release profiles, and (3)

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stuctural modifications could further tune the H_2S-releasing ability.

Having demonstrated H_2S release by the donors in buffers, we wondered whether they could be used to deliver H_2S to biological systems such as live cells. To this end, we first studied the donors' cytotoxicity. Under our conditions, the donors (up to 400 μM) showed no cytotoxicity toward H9c2 and HeLa cells. Next, we validated the donors' H_2S production in cells. As shown in Figure 2, HeLa cells were first incubated with a H_2S-selective fluorescent

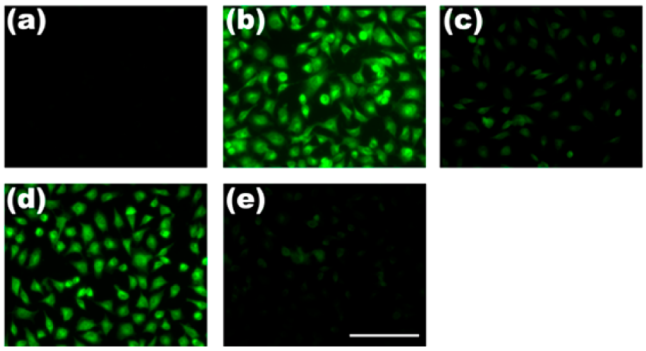

Figure 2. H_2S production from **JK-1**, **JK-2**, and GYY4137 in HeLa cells. Cells were incubated with **WSP-5** for 30 min. After removal of excess **WSP-5**, cells were treated with (a) vehicle, (b) Na_2S (200 μM), (c) **JK-1** (200 μM), (d) **JK-2** (200 μM), and (e) GYY4137 (200 μM). Images were taken after 1 h (scale bar = 50 μm).

probe, **WSP-5**, for 30 min. After removal of the excess probe, cells were treated with the donors (**JK-1**, **JK-2**, GYY4137, and Na_2S) for 1 h. As expected, **JK-2**-treated cells exhibited strong fluorescence comparable to that from Na_2S-treated cells. **JK-1**- and GYY4137-treated cells showed much weaker fluorescence, consistent with their decreased H_2S-releasing ability at neutral pH.

It is known that H_2S has cardioprotective effects against MI/R injury.[10] The mechanisms include reducing oxidative stress, preserving mitochondrial function, decreasing myocardial inflammation, and improving angiogenesis.[9] We envisioned that **JK** donors would exhibit similar cardioprotective activities due to H_2S release. To establish the cellular model of MI/R injury, H9c2 cardiomyoblasts were exposed to anoxia/reoxygenation (A/R) treatment together with increasing concentrations of H_2O_2 (100−400 μM). As shown in Figure S5, this treatment led to a dose-dependent inhibition in cell viability, indicating that it could imitate in vivo I/R-triggered effects. Since the median lethal concentration of H_2O_2 in H9c2 cells was approximately 400 μM, this concentration was used for our studies.

Next, we tested the protective effects of **JK** donors against A/R-induced cellular damage. In these experiments, H9c2 cells were pretreated with **JK-1** or **JK-2** at various concentrations (12, 25, and 50 μM) for 1 h during anoxia. Cells then underwent a reoxygenation process, after which cell viability was analyzed. The results showed that **JK-1** and **JK-2** exhibited significant attenuation of A/R-induced damage (Figure 3).

In addition to the cell viability assay, other methods were also used to validate the **JK** donors' protective effects in cells. Lactate dehydrogenase (LDH) is a cytoplasmic protein whose leakage from the cells indicates cell damage. As shown in Figure S6, preconditioning of H9c2 cells with A/R treatment remarkably enhanced LDH release, indicating that A/R treatment induced severe damage to the cells. However, LDH release was significantly reduced when cells were pretreated with 25 μM

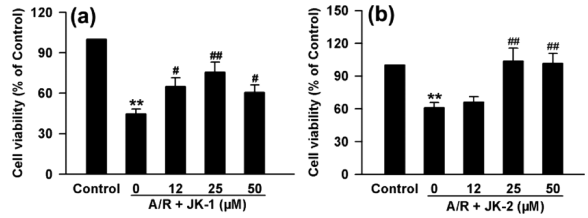

Figure 3. Effects of **JK-1** and **JK-2** on A/R-induced cellular injury. During anoxia various concentrations of **JK-1** and **JK-2** were added into medium and incubated for 1 h. Then the cells underwent a reoxygenation process. After the treatments, the CCK-8 assay was performed to detect cell viability. Data are expressed as mean ± SEM. $^{**}P < 0.01$ vs the control group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs the A/R-alone group.

JK. In another experiment, mitochondrial membrane potential (MMP) was measured by Rh123 staining to test cellular damage. Figure S7 shows that under normal conditions H9c2 cells had bright green fluorescence. When the cells were exposed to A/R treatment, a dramatic MMP loss was observed, as evidenced by weak green fluorescence. However, incubation with 25 μM **JK** greatly impeded this MMP loss by preserving mitochondrial function. These results further confirmed that the **JK** donors exhibited potent cellular protection against oxidative injury.

Finally, considering the reduced local pH level caused by ischemic injury, we tested the protective effects of the representative donors **JK-1** and **JK-2** against MI/R injury in a murine model. In these experiments, mice were subjected to 45 min of left ventricular ischemia followed by 24 h of reperfusion. The donors or vehicle were administered by intracardiac injection at the time of reperfusion at different doses. All of the animal groups displayed similar area-at-risk per left ventricle (AAR/LV), suggesting that the surgical procedure produced the same degree of ischemic damage. However, compared with vehicle-treated mice, those receiving the donors displayed significant reductions in infarct size per area-at-risk (INF/AAR), as assessed by 2,3,5-triphenyltetrazolium chloride (TTC) staining (Figure 4). A 50 or 100 μg/kg bolus of **JK-1** maximally

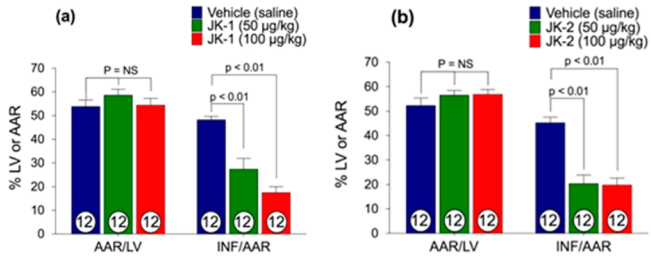

Figure 4. Cardioprotective effects of **JK-1** and **JK-2** in MI/R injury. Myocardial infarct size was significantly reduced in mice treated with (a) 50 or 100 μg/kg **JK-1** ($p < 0.01$) or (b) 50 or 100 μg/kg **JK-2** ($p < 0.01$) compared with vehicle-treated mice. Results are presented as mean ± SEM for $n = 12$ in each group.

reduced INF/AAR by 43% and 64%, respectively. Similarly, a 50 or 100 μg/kg bolus of **JK-2** reduced INF/AAR by 55% and 56%, respectively. These activities are significantly better than the activities measured with other known donors such as GYY4137 and DTT-2 (Figure S8).

Moreover, circulating cardiac troponin I levels, the marker for acute myocardial infarction, were significantly lowered with **JK-1**- or **JK-2**-treated animals ($p < 0.05$ for 50 μg/kg and $p < 0.01$ for 100 μg/kg in both groups) (Figure 5). We also validated H_2S

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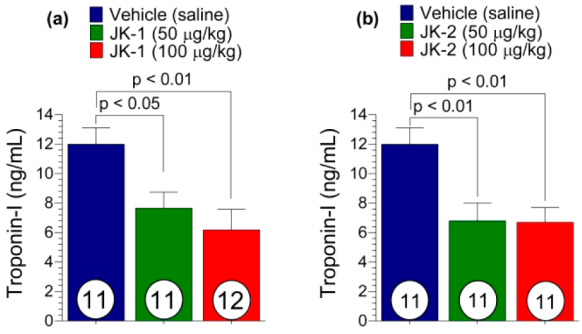

Figure 5. Circulating troponin I levels. Blood was collected at 4 h of reperfusion, and circulating cardiac troponin I levels were measured. The troponin I level was significantly reduced by treatment with either (a) 50 or 100 μg/kg **JK-1** ($p < 0.05$ and $p < 0.01$, respectively) or (b) 50 or 100 μg/kg **JK-2** ($p < 0.05$ and $p < 0.01$, respectively).

production from the donors. As shown in Figure S9, administration of **JK-2** led to a significant increase of free H_2S in blood. These results strongly suggest that the **JK** donors are potent H_2S donors and have valuable pharmacological benefits.

In summary, we have developed a series of novel H_2S donors based on the phosphonamidothioate template. Compared with other known H_2S donors, these compounds have the following advantages: (1) excellent solubility in aqueous solutions, (2) slow but significant production of H_2S, and (3) pH-controlled H_2S release profiles. It should also be noted that high solubility could result in decreased bioavailability and permeability. The fast release at low pH presumably might limit these compounds as orally administered drugs. However, if needed, these limitations could be solved by developing prodrugs of these cyclization-based donors. Nevertheless, this work provides a new and effective strategy for the design of H_2S donors. The protective effects of the new donors (**JK-1** and **JK-2**) on cellular and murine models of myocardial ischemia reperfusion injury have been demonstrated. Other H_2S-related activities of these donors are currently under investigation.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.6b01373.

Procedures and additional data (PDF)

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Notes

The authors declare no competing financial interest.

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