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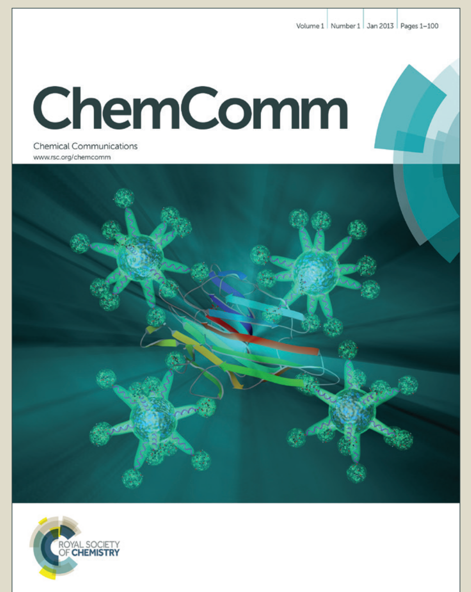

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Hydrogen Sulfide (H_2S) Releasing Agents: Chemistry and Biological Applications

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Hydrogen sulfide (H_2S) is a newly recognized signaling molecule with very potent cytoprotective actions. The fields of H_2S physiology and pharmacology have been rapidly growing in recent years, but a number of fundamental issues must be addressed to advance our understanding of the biology and clinical potential of H_2S in the future. Hydrogen sulfide releasing agents (also known as H_2S donors) have been widely used in the field. These compounds are not only useful research tools, but also potential therapeutic agents. It is therefore important to study the chemistry and pharmacology of exogenous H_2S and to be aware of the limitations associated with the choice of donors used to generate H_2S *in vitro* and *in vivo*. In this review we summarized the developments and limitations of current available donors including H_2S gas, sulfide salts, garlic-derived sulfur compounds, Lawesson's reagent/analogs, 1,2-dithiole-3-thiones, thiol-activated donors, photo-caged donors, and thioamino acids. Some biological applications of these donors were also discussed.

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1. INTRODUCTION

Hydrogen sulfide (H_2S), first discovered in 1777 by Carl Wilhelm Scheele, has been traditionally known as a toxic air pollutant with the characteristic odor of rotten eggs. However, this gaseous molecule has been recently recognized as a member of the gasotransmitter family along with its congeners nitric oxide (NO) and carbon monoxide (CO).[1-8] The production of H_2S in mammalian systems has been attributed to at least three enzymes: cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE), and 3-mercaptopyruvate sulfur-transferase (MPST) (Scheme 1).[9-13] CBS is found predominantly in the brain, nervous system and liver. It converts cysteine and homocysteine to cystathionine and releases H_2S. In comparison, CSE activity is higher than CBS in aorta, portal vein and other vascular tissue. CSE is responsible for H_2S production in the vasculature and heart through a reaction involving the generation of L-cysteine, pyruvate, and ammonia from L-cystathionine and cysteine. MPST is mainly localized in mitochondria.[14] Kimura and coworkers demonstrated that MPST, together with cysteine aminotransferase (CAT), produces H_2S from cysteine in the presence of α–ketoglutarate.[15] It has also been reported that MPST can convert D-cysteine to H_2S in the presence of D-amino acid oxidase.[16] Although the expression of these enzymes is tissue-specific, they all convert cysteine or cysteine derivatives to H_2S. These enzymes work collectively and precisely regulate H_2S levels in tissues, and therefore are crucial for H_2S homeostasis.

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Scheme 1. Enzymatic synthesis of H_2S.

In 1996 Kimura demonstrated that endogenous H_2S acts as a neuromodulator in the brain.[17] Following his work, a number of studies have revealed various biological effects of H_2S, which include the relaxation of blood vessels,[18-20] protection against myocardial ischemia injury,[21-23] and cytoprotection against oxidative stress.[24-26] In addition, some chemical and biochemical catabolic reactions of H_2S have also been disclosed, and these reactions may be responsible for the biological functions of H_2S. For example, H_2S is a powerful reducing agent and is likely to be consumed by endogenous oxidants such as peroxynitrite ($ONOO^-$), superoxide ($O_2^{\cdot -}$) and hydrogen peroxide (H_2O_2) (Scheme 2a).[27-29] H_2S reacts readily with methemoglobin to form sulfhemoglobin, which might act as a metabolic sink for H_2S (Scheme 2b).[30] It is reported that H_2S can cause protein S-sulfhydration (i.e. to form -*S*-SH) (Scheme 2c),[31-33] but the detailed mechanism is still unclear. Nevertheless this process is potentially significant as it provides a possible route

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by which H_2S can alter the functions of a wide range of cellular proteins and enzymes.[34-40] H_2S can also interact with *S*-nitrosothiols to form thionitrous acid (HSNO), the smallest *S*-nitrosothiol, whose metabolites, such as NO, NO^-, and NO^+, have significant physiological functions (Scheme 2d).[41] It is likely that many more important reactions of H_2S remain to be discovered.

a.

$ONOO^- \xrightarrow{H_2S} HS{-}N^{+}(=O){-}O^- \rightleftharpoons$ O=N–O–SH / H–S(=O)–N=O $\rightarrow HSO^\bullet + NO^\bullet$

$O_2^{\bullet -} \xrightarrow{H_2S} H_2O_2 + S^{\bullet -}$

$H_2O_2 \xrightarrow{H_2S} H_2O + HSOH$

b.

methemoglobin—Fe^{3+} $\xrightarrow{H_2S}$ methemoglobin—Fe^{3+}—SH

sulfhemoglobin

c.

Protein—S–X $\xrightarrow{H_2S}$ Protein—S–SH

X may be H, SR, OH, etc.

d.

$RSNO \xrightarrow{H_2S} HSNO + RSH$

Scheme 2. Some important biological reactions of H_2S.

Although the endogenous formation of H_2S and exogenous administration of H_2S have been proved beneficial in some pathophysiological conditions, the molecular mechanisms of H_2S action are still under investigation. It is therefore important to understand the chemistry and properties of H_2S and to be aware of the problems

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associated with the choice of resources used to generate H_2S in *in vitro* and *in vivo* experiments. H_2S is a colorless gas under ambient temperature and pressure. The toxicity of H_2S has been known for hundreds of years and is comparable to that of CO or hydrogen cyanide (HCN).[42-44] Exposure to 300 ppm of H_2S leads to pulmonary edema and 1000 ppm of H_2S causes immediate death. Caution should therefore be taken when working with H_2S. As a weak acid, H_2S is very water soluble. Its solubility was reported to be ~80 mM at 37 ^{o}C as an equilibrium between molecular and ionic forms ($H_2S_{aq} \rightleftharpoons HS^- \rightleftharpoons S^{2-}$). The p$K_a$ values for the first and second dissociation steps are 7.0 and >12.0, respectively.[45-47] Therefore, in aqueous state under the physiological pH of 7.4, the major form of hydrogen sulfide exists as HS^- with a minor form of free H_2S (the ratio of HS^-/H_2S is ~3:1). Very small amounts of sulfide anion (S^{2-}) are also present. Since it has not been possible to determine which form of H_2S (H_2S, HS^-, or S^{2-}) is the active species in biological systems, the term of H_2S is used to refer to the total sulfide present in the solution (i.e. $H_2S + HS^- + S^{2-}$).

So far, one of the major challenges in the H_2S field is precise measurement of H_2S concentrations. Traditional methods such as methylene blue (MB) assay, ion selective electrodes (ISE), and gas chromatography, require complicated post-mortem processing and/or destruction of samples.[48-50] Given the high reactivity of H_2S under biological environments, these methods may yield inconsistent results.[46, 51] Fluorescence based assays can be very useful due to high sensitivity and easy operation. Fluorescence methods are suitable for nondestructive detection of bio-targets in live cells or tissues with readily available instruments. In 2011 several groups reported the first reaction-based fluorescent probes for H_2S detection in cell and blood samples.[52-56] These works

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inspired researchers to develop new H_2S fluorescent probes and a number of papers have been published in the past two years.[57-62] All of these probes are based on reaction-based fluorescence turn-on strategies, i.e. using certain H_2S specific reactions to convert non-fluorescent substrates to materials with strong fluorescence. So far three types of reactions have been employed for the probe design (Scheme 3): 1) H_2S-mediated reductions, often using azide (N_3) substrates; 2) H_2S-mediated nucleophilic reactions, and 3) H_2S-mediated metal-sulfide precipitations. However, although a number of probes have been reported, few can be applied to real biological detections due to slow reaction rate and/or low sensitivity of many probes. In addition, the selectivity of these probes for H_2S vs other reactive sulfur species, especially the newly recognized persulfide species, is largely unaddressed. Due to these problems, further development of chemeospecific fluorescent probes for H_2S remains critical.

Scheme 3. Representative fluorescent probes for H_2S detection

In the study of H_2S's mechanisms and functions H_2S releasing agents (i.e. donors) are important research tools. In the past several years the development of novel H_2S donors has become a rapidly growing field, with several series of donors reported. [63, 64] These donors release H_2S through different mechanisms. It should be noted that although H_2S is biologically active, its functions sometimes appear inconsistent. For example, in addition

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to its anti-inflammatory effects, H_2S has also been reported as a pro-inflammatory molecule.[65, 66] These disparate results might be due to the use of different H_2S donors in the research. H_2S releasing capabilities of each donor category are quite different, which may lead to different results. Additionally, byproducts could form along with H_2S generation and it is unclear whether these byproducts have biological effects. Therefore, the selection of suitable H_2S donors is crucial. In this review we summarize the information about current available H_2S donors with a focus on the chemistry of their development. Some biological applications are also discussed.

2. H_2S DONORS

2.1. H_2S gas

As the authentic resource, H_2S gas has been directly used in this field. It has been reported that H_2S gas promotes glucose uptake and provides amelioration in type II diabetes.[67] In 2005 Roth found that H_2S (g) could induce a suspended animation-like state in mice.[68] The exposure of mice to 80 ppm of H_2S (g) caused a significant drop in their oxygen (O_2) consumption (by ~ 50%) and carbon dioxide (CO_2) output (by ~ 60%) within first 5 minutes. A 6-hour H_2S (g) exposure diminished mice's metabolic rate (MR) by ~ 90%. The decrease in MR was followed by a drop in core body temperature (CBT) to as low as 15 ^{o}C. When mice were returned to normal air and room temperature after this H_2S exposure, their MR and CBT returned to normal levels. It has been suggested that these effects were induced through reversible and competitive inhibition of the mitochondrial enzyme cytochrome c oxidase by H_2S, which slowed respiration.[69]

The reversible H_2S-induced hibernation state was later shown to protect mice from lethal hypoxia.[70] C57BL/6J mice cannot survive in 5% O_2 for more than 15 minutes.

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However, after inducing the suspended animation-like state by pretreating the animals with 150 ppm H_2S (g) for 20 minutes, mice survived the duration of 1-h experiment under normally lethal hypoxia conditions. The longest exposure to 5% O_2 was 6.5 hours and H_2S-pretreated mice survived without notable damage.

Although these interesting experiments revealed promising effects of H_2S gas in reducing mice metabolic rate, similar studies failed in large animals, such as sheep and piglets.[71-74] From experimental operation perspective, it is hard to consider H_2S gas as an ideal resource due to difficulties in obtaining precisely controlled concentrations and possible toxic impact of H_2S excess. Because of this reason, H_2S equivalents or releasing agents are often used in the field.

2.2. Inorganic sulfide salts

Inorganic sulfide salts such as sodium sulfide (Na_2S) and sodium hydrogen sulfide (NaHS) have been used as H_2S equivalents by many researchers. The treatments of cells, tissues, or animals with sulfide salts have shown protective effects against a number of disease states.[75-78] By using Na_2S as an exogenous H_2S donor, Lefer et al. confirmed that long-term H_2S therapy attenuates ischemia-induced heart failure.[79] In this study heart failure was induced by subjecting C57BL6/J mice to 60 minutes of left coronary artery occlusion followed by reperfusion for up to 4 weeks. 100 μg/kg of Na_2S was administered once at the time of reperfusion and then daily for the first 7 days of reperfusion. The results suggested that long-term H_2S therapy leads to a decrease in left ventricular (LV) dilation, decrease in cardiac hypertrophy, and improvement in cardiac function. In addition, treatment with Na_2S also reduced oxidative stress associated with heart failure. Although previous studies have provided solid evidence for the

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cardioprotective effects of short-term H_2S therapy,[80, 81] these results by Lefer showed for the first time that H_2S therapy can provide long-term protection against myocardial injury and this effect is believed to be induced by the reduction of oxidative stress.

In addition to cardioprotection sulfide salts also showed protective effects against other diseases such as inflammation.[82] Osteoarthritis (OA), a form of arthritis, is characterized by degenerative and inflammatory processes driven by the presence of enhanced levels of pro-inflammatory proteins such as IL-6 and IL-8. Kloesch et al demonstrated that short-term (15-30 minutes) treatment of human cells with NaHS is sufficient to down-regulate IL-6 and IL-8 expression, which may account for H_2S anti-inflammatory effects against OA.[82b] However, care should be taken when NaHS is applied because it was also found that the anti-inflammatory effects of NaHS were altered to be pro-inflammatory when the NaHS incubation time was extended from 15 minutes to 1 hour.

It is obvious that sulfide salts, as H_2S donors, have the advantage of boosting H_2S concentration rapidly. However, these compounds release H_2S spontaneously at the time the solution is prepared, making it hard to precisely control H_2S concentration. Modifications made between the time that a solution is prepared and the time that the biological effect is measured can dramatically affect results. This uncontrolled and rapid H_2S release can cause severe damages *in vivo*. In addition, H_2S can be quickly lost from solution due to volatilization under laboratory conditions. The effective residence time of sulfide salts in tissues is relatively short. Olson and co-workers conducted an experiment to test H_2S loss from aqueous solutions.[83] They found that H_2S were lost from solutions with $t_{1/2}$ values of about 5 min. After 12 hours, undetectable H_2S was left from an original

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10 μM of H_2S Hepes solution. It should also be noted that commercial sulfide salts, especially NaHS, always contains significant amount of impurities. Recent study revealed that polysulfides rapidly form in NaHS solution.[84] All of these problems should be kept in mind when using sulfide salts as H_2S donors.

2.3. Garlic and related sulfur compounds

For hundreds of years garlic has been considered as a magic medicine. Recent studies suggest that at least some of the beneficial effects of garlic are due to H_2S production. So far the best characterized compound from garlic is allicin (diallyl thiosulfinate). This compound is unstable in aqueous solutions and quickly decomposes to several compounds including diallyl sulfide (DAS), diallyl disulfide (DADS) and diallyl trisulfide (DATS) (Scheme 4).[85] Kraus and coworkers demonstrated that human blood cells (RBCs) can convert garlic-derived organic polysulfides into H_2S, which the vasoactivity of garlic is attributed to.[86] Among all of the sulfur compounds, i.e. DAS, DADS, DATS, allyl methyl sulfide (AMS), and dipropyl disulfide (DPDS) (Scheme 4), DATS produced the highest amount of H_2S in the presence of glutathione (GSH), followed by DADS. Apparently H_2S production from these sulfur compounds is facilitated by allyl substituents and by increasing the numbers of tethering sulfur atoms.

Diallyl Thiosulfinate (Allicin)
Diallyl Sulfide (DAS)
Diallyl Disulfide (DADS)
Diallyl Trisulfide (DATS)
Dipropyl Disulfide (DPDS)
Allyl Methyl Sulfide (AMS)

Scheme 4. Garlic-related sulfur compounds.

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From a reaction mechanism point of view, the regular thiol/disulfide exchange between DADS and GSH should not produce H_2S. Instead, H_2S generation is initiated by the nucleophilic substitution of GSH at the α-carbon of the allyl substituent, forming an allyl perthiol, which undergoes a thiol/disulfide exchange to release H_2S. Trisulfides (R-SSS-R') also undergo similar nucleophilic substitutions at the sulfur atom, yielding RSSH, and then H_2S (Scheme 5).

DADS → (GSH; GS-allyl) → Allyl perthiol (HS-S-allyl) → (GSH; GS-S-allyl) → H_2S

R'SSSR → (GSH; R'SSG) → RSSH → (GSH; GSSR) → H_2S

Scheme 5. H_2S generation from garlic-derived sulfur compounds.

The vasodilation effects caused by garlic and garlic-derived sulfur compounds were tested.[86] In these experiments, phenylephrine-precontracted aorta rings were suspended in a 37 ^{o}C organ bath containing 1 mM GSH under physiological O_2 conditions and treated with different doses of garlic (50, 200, and 500 μg/ml) or garlic-derived sulfur compounds. In the garlic-treated group, the aorta rings showed a concentration-dependent relaxation accompanied by H_2S production. In the polysulfide-treated group, DATS and DADS exhibited the maximum relaxation of aorta rings. However, DPDS and AMS showed minimum effects. These results are paralleled with the compounds' H_2S yields, suggesting a link between bioactivity and H_2S production.

It should be noted that DADS and DATS are reactive sulfane sulfur species. Sulfane sulfur refers to a sulfur atom with six valence electrons but no charge (represented as S^0).[42] Sulfane sulfur compounds have unique reactivity and exhibit regulatory effects in

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diverse biological systems.[87, 88] Biologically important sulfane sulfur compounds include perthiol (R–S–SH), polysulfides (R–S–S_n–S–R), and protein-bound elemental sulfur (S_8).[89] Sulfane sulfur and H_2S usually coexist, and recent work even suggests that sulfane sulfur species, derived from H_2S, may be the active signaling molecules and exhibit protection in mammals.[90-93] Therefore, garlic derived and related sulfur compounds that have shown protective effects in biological systems must be further investigated as to whether the effects are derived from sulfane sulfurs, or hydrogen sulfide.

2.4. Lawesson's reagent and analogs

2,4-Bis(4-methoxyphenyl)-1,3,2,4-dithiadiphosphetane-2,4-disulfide (Lawesson's reagent) is a widely used sulfurization reagent in organic synthesis [94] that can also be used as an H_2S donor. It can be easily synthesized by heating a mixture of anisole with phosphorus pentasulfide (P_4S_{10}) (Scheme 6a).[95, 96]

Scheme 6. Chemical synthesis of Lawesson's reagent (a) and GYY4137 (b).

As an H_2S donor, Lawesson's reagent showed some H_2S-related bioactivities, such as ion channel regulation and anti-inflammation. [97, 98] In 2009 Wallace et al investigated the effects of H_2S on inflammation and ulceration of the colon in a rat model of colitis.[98] Treatment with Lawesson's reagent dramatically reduced the severity of colitis.

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Additionally, Lawesson's reagent also significantly attenuated the increase in colonic thickness that occurs in rats with colitis. The results are comparable with those obtained in NaHS-treated group, confirming the potency of Lawesson's reagent as an H_2S donor. However, this donor releases H_2S upon spontaneous hydrolysis in aqueous solution. This uncontrollable release of H_2S makes it difficult to mimic endogenous H_2S formation. In addition, the poor solubility of Lawesson's reagent in aqueous solutions limits its applications.

Morpholin-4-ium 4 methoxyphenyl(morpholino) phosphinodithioate (GYY4137), a derivative of Lawesson's reagent, is water soluble. It can be synthesized by reacting Lawesson's reagent with morpholine in methylene chloride at room temperature (Scheme 6b).[99]

Similar to Lawesson's reagent, GYY4137 releases H_2S upon hydrolysis. The *in vitro* H_2S release was confirmed by colorimetric and amperometry assays.[99] Compared to sulfide salts, H_2S release from GYY4137 was much slower and the H_2S concentration reached the maximum value within 6-10 minutes but at a very low level. 1 mM of GYY4137 released 40 μM of H_2S within the first 10 minutes and another 50 μM of H_2S in the following 90 minutes in aqueous solution (pH 3.0). H_2S release from GYY4137 was pH- and temperature-dependent, with more release at acidic pH and less release at low temperatures. Under physiological conditions, H_2S production from GYY4137 maintained at low level (less than 10 %) even after 7 days.[106] For *in vivo* H_2S production, GYY4137 (133 μmol/kg) was administrated (intravenous or intraperitoneal injections) to anesthetized male Sprague-Dawley rats. The plasma H_2S concentration, measured by MB method, was increased at 30 minutes and remained elevated over 180 minutes.

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In a report by Moore et al, no detectable cytotoxicity, cell cycle distribution change, or p53 expression induction was observed after treating rat vascular smooth muscle cells with GYY4137 (up to 100 μM) for up to 72 hours.[99] Previous studies have shown that NaHS (at similar concentrations and time courses) promoted the apoptotic cell death of cultured fibroblasts and smooth muscle cells.[100, 101] The very slow H_2S release from GYY4137 may explain why GYY4137 did not cause apoptosis. In contrast to the rapid and reversible relaxation of precontracted aortic rings (~ 20 to 30 seconds) caused by NaHS, GYY4137 showed a slower onset (~ 10 minutes) and longer sustained effect (~ 40 minutes). NaHS (2.5 – 20 μmol/kg) caused fast (10 – 30 seconds) and dose-related decrease in blood pressure, but GYY4137 (26.6 to 133 μmol/kg) caused a slowly (apparent at 30 minutes) drop in blood pressure.

H_2S plays disparate roles on inflammation, with both pro- and anti-inflammatory effects illustrated.[65, 66, 102-104] In 2010, Moore and co-workers compared the effects of NaHS and GYY4137 on the release of pro- and anti-inflammatory mediators in lipopolysaccharide (LPS)-treated murine RAW 264.7 macrophages.[105] The purpose of this study was to test whether the effects of H_2S on inflammation were dependent on H_2S generation rate. In this study GYY4137 significantly inhibited the LPS-induced release of pro-inflammatory mediators such as IL-1β, IL-6, TNF-α, nitric oxide, and PGE_2, but increased the synthesis of the anti-inflammatory chemokine IL-10. In contrast the effects of NaHS were much less consistent. The results indicated that the effects of H_2S on inflammation are complex and may depend not only on H_2S concentration but also on the rate of H_2S generation.

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In addition to its roles in vasorelaxation and inflammation, anticancer effects of GYY4137 were recently reported.[106] Proliferation of cancer cells, such as breast adenocarcinoma (MCF-7), acute promyelocytic leukemia (MV4-11), and myelomonocytic leukemia (HL-60), were significantly reduced by a 5-day treatment with GYY4137 (400 μM). NaHS and ZYJ1122 (a structural analog of GYY4137 lacking sulfur) remained inactive at the same concentration. GYY4137 (800 μM) killed 75-95% of these cells, but it did not affect the survival of human non-cancer diploid fibroblasts (WI-38 and IMR90). Mechanistic investigation revealed that the treatment of MCF-7 cells with GYY4137 led to cell cycle arrest in G_2/M phase and promotion of apoptosis. The fact that the non-H_2S-releasing compound ZYJ1122 did not show inhibitory effects on any cell lines may suggest the anticancer effects of GYY4137 are due to H_2S release.

Although GYY4137 has been widely used, its fixed H_2S release capability may not fulfill the requirements of different biological applications. In addition, the exact mechanism of H_2S release from GYY4137 is still unclear as are the byproducts produced. As mentioned above, GYY4137 is proposed to exhibit anti-cancer effects due to H_2S release since its non-H_2S releasing analog, ZYJ1122, failed to show similar effects. This conclusion needs to be further clarified as it is unclear if ZYJ1122 is truly the byproduct of GYY4137. Therefore, control experiments should be conducted appropriately when using GYY4137 as an H_2S donor. Otherwise, GYY4137-induced biological effects may not be concluded to be H_2S-dependent.

Recently the Xian group developed a series of phosphorodithioate-based H_2S donors by replacing the phosphorus-carbon bond in GYY4137 with phosphorus-oxygen bonds.[107] It was expected that the structure modifications on phosphorodithioate core may

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result in H_2S release capability change and in turn lead to biological activity changes. The synthesis of such phosphorodithioate-based H_2S donors was achieved in 4 steps (Scheme 7): the starting material trichlorophosphine reacted with 1,2-dithioethane to yield 2-chloro-1,3,2-dithiaphospholane, which was then treated with aniline and followed by sulfurization with elemental sulfur to give the key intermediate 2-amine-1,3,2-dithiaphospholan-2-sulfide. Finally, this intermediate was condensed with different phenols and alcohols in the presence of 1,8-diazobicyclo[5.4.0]undec-7-ene (DBU) to afford the desired products.

PCl_3 → (HS–CH₂CH₂–SH) → 2-chloro-1,3,2-dithiaphospholane → 1) $PhNH_2$, DIEA; 2) S_8, CS_2 → 2-amine-1,3,2-dithiaphospholan-2-sulfide → ROH, DBU → PhHN–P(=S)(OR)–S^- (Phosphorodithioate-based donors)

ROH = HO–C₆H₄–OCH_3; HO–C₆H₄–Br; HO–C₆H₄–NO_2

Scheme 7. Chemical synthesis of phosphorodithioate-based H_2S donors.

H_2S release capabilities of these compounds were tested by spectroscopic methods and compared with GYY4137. In previous studies H_2S release from GYY4137 was mainly measured by MB assay, a standard H_2S detection method involving strong acidic conditions. It is known that the hydrolysis of phosphorodithioates is pH dependent and hydrolysis is much faster under acidic conditions than under neutral pH. Therefore, MB may not be a viable way to evaluate phosphorodithioate-based donors like GYY4137. Dansyl azide (DNS-Az), a fluorescent probe for H_2S,[55] was used to measure H_2S generation from phosphorodithioate-based H_2S donors under neutral pH. Similar to GYY4137, *O*-aryl substituted phosphorodithioate-based donors showed slow and low H_2S release. Up to 1 μM of H_2S was detected from 100 μM of donor in a mixed

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acetonitrile/phosphate buffer solution (1:1 v/v, pH 7.4) within 3 hours of experiment period. However *O*-alkyl substituted donors showed almost un-detectable H_2S release. Presumably the *O*-alkyl substitutions led to increased stability of phosphorodithioates and therefore decreased the rate of hydrolysis to generate H_2S. *O*-Aryl substituted phosphorodithioate-based donors were also shown to release H_2S in cells. The cell imaging experiments were conducted by incubating H9c2 cardiac myocytes with the donor at various concentrations (0, 100, and 200 μM) for 24 hours. Then WSP-1, an H_2S-specific fluorescent probe,[53] was applied to the cells to monitor H_2S production. Compared to vehicle-treated group, enhanced fluorescence in donor-treated group demonstrated a sustain H_2S generation.

Since H_2S has been known to exhibit cellular protection against oxidative injury,[108] it was hypothesized that phosphorodithioate-based H_2S donors may have similar effects due to H_2S release. Two *O*-aryl substituted donors were selected to evaluate their protective effects against H_2O_2-induced oxidative damage in H9c2 cells. In these experiments, H9c2 cells were incubated with each donor (50, 100 and 200 μM) for 24 hours, followed by 5-hour incubation with H_2O_2 (150 μM). Cell viability results showed that 35% of cells were killed if H_2S donors were absent. In comparison, cell viability increased significantly in the presence of donors, suggesting that H_2S donors may have some protective effects against oxidative injury.

It should be noted that although phosphorodithioate-based donors have shown H_2S-like biological activities, it is still premature to attribute those activities to H_2S production. H_2S production from those phosphorodithioate-based donors is very slow and at very low levels. The major species in media is still the donor molecule itself. It is known that

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phosphorodithioate core structures are biologically active. For example, phosphorodithioate DNA is resistant to nuclease degradation and has been reported as a potential therapeutic drug.[109, 110] In addition, phosphorodithioate oligodeoxycytidine has also shown inhibitive activities against human immunodeficiency virus.[111] Therefore whether phosphorodithioate-induced effects are due to H_2S release needs further confirmation.

2.5. 1, 2-Dithiole-3-thiones and H_2S-hybrid nonsteroidal anti-inflammatory drugs

1, 2-Dithiole-3-thiones (DTTs) are known to release H_2S in aqueous solutions. Although the detailed mechanism is still unclear, it has been demonstrated that DTTs decompose to the corresponding 1, 2-dithiole-3-one upon heating to 120 °C in a DMSO-aqueous phosphate buffer system. This observation implies that hydrolysis might be the mechanism of H_2S generation from DTTs (Scheme 8).[112-114]

1,2-Dithiole-3-thiones (DTTs)

R = Aryl substituents, such as

Scheme 8. Proposed mechanism for H_2S release from DTTs.

Several different methods have been applied to synthesize DTTs. In most cases, elemental sulfur or phosphorus pentasulfide is used to dehydrogenate and sulfurize an allylic methyl group to afford the desired products (Scheme 9a).[115-117]In an alternative

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method dithioic aicds, obtained from a reaction between ketones and carbon disulfide (CS_2), react with hexamethyldisilathiane (HMDT, a sulfur resource) and *N*-chlorosuccinimide (NCS, an oxidizing agent) to give substituted DTTs (Scheme 9b).[118, 119] In addition, β-ketoesters were also reported to react with Lawesson's reagent to form desired DTTs (Scheme 9c).[120]

Scheme 9. Chemical synthesis of DTTs.

DTTs have been widely used in studying H_2S-related biological effects in the alimentary system. The use of nonsteroidal anti-inflammatory drugs (NSAIDs) is associated with an unacceptable risk for gastrointestinal ulceration and bleeding.[121-123] DTTs have been coupled with NSAIDs and the resultant HS-hybrid NSAIDs (HS-NSAIDs) showed significant reduction of gastrointestinal damage compared to the parent NSAIDs.[114, 124-126] HS-NSAIDs are usually synthesized by coupling NSAID counterparts with 5-(4-hydroxyphenyl)-3H-1,2-dithiole-3-thione (ADT-OH), an H_2S releasing molecule, in the presence of dicyclohexylcarbodiimide (DCC) and 4-dimethylaminopyridine (DMAP) (Scheme 10a).[127] Some representative HS-NSAIDs and their parent NSAIDs are listed in Scheme 10b.

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Scheme 10. Chemical synthesis of HS-NSAIDs (a) and structures of representative HS-NSAIDs and corresponding NSAIDs (b).

Wallace and co-workers evaluated the anti-inflammatory effects of ATB-337 in rats.[103] H_2S release from ATB-337 and ADT-OH was measured by an *in vitro* system. Briefly, ATB-337 or ADT-OH (10 μM in polyethylene glycol) were incubated in a potassium phosphate buffer (100 mM, pH 7.4) alone or in the presence of rat liver homogenate (10% wt/vol) and pyridoxal 5'-phosphate (2 mM) for 30 minutes. The generation of H_2S was detected by a sulfide-sensitive electrode. Results suggested that when incubated in buffer, ADT-OH released negligible amounts of H_2S, while ~ 12 nmol/min of H_2S was released from ATB-337. On the other hand, incubation of both

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compounds in liver homogenate caused 3-fold greater levels of H_2S generated from ATB-337 (~ 43 nmol/min) than those from ADT-OH (~ 14 nmol/min). In order to measure plasma H_2S concentrations, male Wistar rats were fasted overnight and then orally treated with diclofenac, ATB-337 (both drugs at 50 μmol/kg), or vehicle. Results showed that plasma H_2S levels were significantly increased (by ~ 40%) after the administration of ATB-337 but unchanged in rats treated with diclofenac. These findings suggest that ATB-337 indeed releases H_2S both *in vitro* and *in vivo*.

Further investigations of the gastrointestinal damages caused by diclofenac and ATB-337 showed that oral administration of diclofenac led to hemorrhagic erosions in rat stomach. In comparison, the same dose of ATB-337 did not produce this damage. In order to determine whether the separate but concomitant administration of the NSAID moiety (diclofenac) and H_2S donor moiety (ADT-OH) of ATB-337 would induce the same degree of gastric damages as the intact compound, rats were treated with diclofenac alone or together with ADT-OH. Results showed that gastric damages caused by the co-administration of these two moieties were similar to that observed in diclofenac-treated group, indicating ADT-OH alone did not protect the stomach against the damages caused by NSAIDs. Considering ATB-337 released 3 times amount of H_2S more than ADT-OH in liver homogenate, it is possible that the gastric safety of ATB-337 was caused by its enhanced H_2S releasing ability. However, it is still unclear why ATB-337 released more H_2S than ADT-OH. It is possible that after the conjugation of diclofenac with ADT-OH, the latter's induction effect changes, making it hydrolyze more easily. Further studies are necessary regarding this question.

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In addition to the protective effects in gastric system, other biological effects of HS-NSAIDs have also been observed.[128-130] Kashfi and co-workers found that HS-ASA, HS-IBU, HS-SUL and HS-NAP can inhibit the growth of various human cancer cells including breast, prostate, lung, leukemia, pancreas, and colon cancer cells.[129] Studies of ATB-429 in a model of postinflammatory hypersensitivity showed that administration of ATB-429 reduces visceral sensitivity and pain perception in conscious healthy and postcolitic hypersensitive rats.[131]

Although HS-NSAIDs have shown promising H_2S-related effects in different tissues and organs, it is still unclear how these molecules release H_2S in living systems. Previous studies suggested that the hydrolysis of DTTs releases H_2S in aqueous buffer.[112-114] However, considering the complexity of biological systems, H_2S release from HS-NSAIDs is expected to be more complicated. The hydrolysis of HS-NSAIDs may initiate H_2S release. However, due to the abundant amount of cysteine and GSH in biological systems, the resultant thiolactone-like species may continue to react with these thiols to release more H_2S. More importantly, it should be noted that perthiols, which belong to reactive sulfane sulfur species, are formed in this process. Therefore, whether the biological effects of HS-NSAIDs are H_2S-related or sulfane sulfur-related needs to be further investigated.

2.6. Thiol-activated H_2S donors

N-mercapto-based H_2S donors. In 2011 Xian and coworkers introduced the concept of controllable H_2S donors and reported the first thiol-activated donors.[132] Their goal was to develop donors which are stable in aqueous solutions and during sample preparation. Ideally H_2S release from these donors should be controlled by factors like biomolecules,

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pH, light, etc. The first series of thiol-activated donors were based on an *N*-mercapto (*N*-SH) template. Since *N*-SH species are unstable, acyl groups were introduced as SH protecting groups to enhance the stability (Scheme 11a). A series of *N*-(benzoylthio)benzamide derivatives were prepared in a two-step synthesis: thiocarboxylic acids were treated with hydroxylamine-O-sulfonic acid in basic conditions to yield *S*-acylthiohydroxylamines, which were further reacted with benzoic anhydride to form the final products (Scheme 11b).

a

PG–S–N(R_1)(R_2) —Deprotection→ HS–N(R_1)(R_2) → H_2S

PG = Protecting group   *N*-SH species

b

RC(O)SH —NH_2SO_3H, KOH→ RC(O)S–NH_2 —Bz_2O→ RC(O)S–NH–C(O)Ph   R = Alkyl, Aryl, etc.

S-acylthiohydroxylamines   *N*-SH-based H_2S donors

Scheme 11. Design and synthesis of *N*-SH-based H_2S donors.

The protected *N*-SH compounds proved to be stable in aqueous solutions. They did not react with potential cellular nucleophiles, such as -OH and -NH_2 groups. However, in the presence of cysteine or GSH, a time-dependent decomposition of the donors accompanied by H_2S release was observed. The structure activity relationship studies showed that electron withdrawing groups (EWGs) led to faster H_2S release and electron donating groups (EDGs) resulted in slower H_2S release, demonstrating the achievement of H_2S release regulation by structural modifications. In addition, H_2S release from *N*-SH-based donors was also detected in plasma, suggesting *N*-SH compounds are active H_2S donors in complex systems.

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Reaction mechanism studies showed that H_2S generation in these donors is initiated by the thiol exchange between cysteine and donors to generate *S*-acylated cysteine and *N*-mercaptobenzamide. *S*-Acylated cysteine then undergoes a native chemical ligation (NCL) to form a stable *N*-acylated cysteine. The *N*-mercaptobenzamide intermediate interacts with cysteine to produce cysteine perthiol, which finally reacts with cysteine to form cystine and release H_2S (Scheme 12).

Scheme 12. Proposed mechanism of H_2S release from *N*-SH-based donors.

Perthiol-based H_2S donors. In the study of *N*-SH-based donors cysteine perthiol was found to be a key intermediate. It should be noted that cysteine perthiol is also involved in H_2S biosynthesis catalyzed by CSE (Scheme 1). This suggests that perthiol (*S*-SH) could be a useful template for H_2S donor design. With this idea in mind Xian and coworkers developed a series of perthiol-based donors.[133] They first employed cysteine perthiol (i.e. primary perthiol) as the structure backbone. Since *S*-SH compounds are very unstable, acyl groups were used again as protecting groups to enhance the stability (Scheme 13).

Scheme 13. Design of perthiol-based H_2S donors.

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The synthesis of Cys-*S*-SH-based donors was achieved in two steps: *N*-benzoyl cysteine methyl ester was treated with 2-mercapto pyridine disulfide to provide a reactive cysteine-pyridine disulfide intermediate, which then reacted with thioacids to give the desired donor compounds (Scheme 14a). H_2S release capabilities of these donors were evaluated. The results indicated that these donors indeed released H_2S in the presence of thiols (cysteine or GSH). However, compared to *N*-SH-based donors, primary perthiol-based donors showed much decreased ability of H_2S generation. Only less than 20 μM of H_2S was detected from 150 μM of donors. One possible explanation is that thiols can attack the acyldisulfide linkage to form a new disulfide and a thioacid (Scheme 14b, pathway a). This reaction prevents the formation of the key perthiol intermediate (Scheme 14b, pathway b), therefore, diminishing H_2S release from these donors.

Scheme 14. a) Chemical synthesis of cysteine perthiol-based H_2S donors; b) explanation of low H_2S release from primary perthiol-based donors.

In order to enhance H_2S generation capability tertiary perthiol-based donors were synthesized. The acyldisulfide linkage was blocked by two methyl groups on the α-

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carbon in order to prevent the unwanted disulfide formation. To synthesize these donors, C- and N-protected penicillamine was treated with 2, 2'-dibenzothioazolyl disulfide. The resultant penicillamine-benzothioazolyl disulfide intermediate reacted with different thioacids to furnish the desired donors (Scheme 15).

Scheme 15. Chemical synthesis of penicillamine perthiol-based H_2S donors.

These tertiary perthiol-based compounds were proved to be potent H_2S donors. Up to 80 μM of H_2S were generated from 100 μM of donors. The regulation of H_2S release from these donors could be achieved by structural modifications. Similar to *N*-SH-based donors, EWGs caused faster H_2S release and EDGs led to slower H_2S generation. In addition, steric effects were also observed as more hindered substrates resulted in slower H_2S release or even no release at all.

H_2S release mechanism of these donors was studied and proved to be similar as that of *N*-SH-based donors. Briefly, the thiol exchange initiated the reaction and resultant penicillamine perthiol released H_2S in two possible pathways: thiol attacked the acyldisulfide linkage to produce a disulfide and generate H_2S (Scheme 16, pathway a); the reaction between penicillamine perthiol and thiols would form a new perthiol species (cysteine perthiol or GSH perthiol) and this newly formed perthiol could interact with excess thiol to release H_2S (Scheme 16, pathway b).

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Scheme 16. Proposed mechanism of H_2S release from *S*-SH-based donors.

Since H_2S exhibited protective effects against myocardial ischemia/reperfusion (MI/R) injury,[134-136] perthiol based donors were expected to exhibit similar effects due to H_2S release. Myocardial protective effects of selected *S*-SH-based donors were tested in a murine model of MI/R. In these experiments MI/R injury was induced by subjecting mice to 45-min left ventricular ischemia followed by 24-h reperfusion. Donors or vehicle were administered into left ventricular lumen at 22.5 min of myocardial ischemia. Compared to vehicle-treated mice, mice receiving donors displayed a significant reduction in circulating levels of cardiac troponin I and myocardial infarct size per area-at-risk, suggesting that *S*-SH-based compounds can exhibit H_2S-mediated cardiac protection in MI/R injury and these compounds may have potential therapeutic benefits.

Although *S*-SH-based donors showed promising biological effects, the exact mechanisms *in vivo* are to be investigated. It should be noted that the reaction between donors and cysteine yields perthiols, which also belong to reactive sulfane sulfur species. Therefore, further studies on H_2S-related and sulfane sulfur-related mechanisms are needed.

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Dithioperoxyanhydrides. Similar to *N*-SH-based and *S*-SH-based donors, dithioperoxyanhydrides were recently reported as another class of thiol-activated H_2S donors.[137] These donors were synthesized by either iodine oxidation of the thiocarboxylates[138] or reactions between thiocarboxylic acids and methoxycarbonylsulfenyl chloride[139] (Scheme 17). H_2S release was confirmed in both buffers and cellular lysates. Additionally, one of the donors, $CH_3C(O)SSC(O)CH_3$ in Scheme 17, was also shown to induce concentration-dependent vasorelaxation on pre-contracted rat aortic rings.

Scheme 17. Chemical synthesis of dithioperoxyanhydride-based H_2S donors

Acylpersulfides were proposed to be the key intermediates for H_2S release from these donors. It is possible that acylpersulfides directly react with thiols (RSH) to give H_2S and RSSAc (Scheme 18, pathway a). Alternatively the reaction between acylpersulfides and thiols would produce a new perthiol species (RS-SH), which then reacts with excess thiols to yield H_2S (Scheme 18, pathway b).

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Scheme 18 (reaction scheme labels): RSH, RSAc, RSH Pathway a, H_2S + RSSAc, RSH Pathway b, AcSH, RS−SH, RSH, H_2S + RSSR; RSH = Cysteine or GSH

Scheme 18. Proposed mechanism for H_2S release from dithioperoxyanhydrides.

Arylthioamides. Arylthioamides were classified as the fourth class of thiol-activated H_2S donors (Scheme 19a).[140] The *p*-hydroxybenzothioamide was selected as the lead compound and a library of such compounds were synthesized by structural modifications as follows: 1) introduction of EWGs or EDGs at the 2- or 5-position of the phenyl ring; 2) replacement of the 4-hydroxy group with an amino group; and 3) replacement of the phenyl ring with heterocycles. Briefly, the non-heterocyclic compounds were prepared by mixing the corresponding benzonitrile with P_4S_{10} in ethanol at 70 °C for 10 hours (Scheme 19b); the heterocyclic compounds were obtained by the treatment of the amides with Lawesson's reagent for 12 hours (Scheme 19c).

a: *p*-hydroxybenzothioamide —Thiol→ H_2S

b: P_4S_{10}, EtOH, 70 °C; R_1 = OH or NH_2, R_2 = H, Cl, F, CF_3

c: Lawesson's reagent; Ar = 4-piridyl, 2-furyl, 2-thienyl, etc.

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Scheme 19. a) H_2S release from *p*-hydroxybenzothioamide; b) chemical synthesis of non-heterocyclic donors; and c) chemical synthesis of heterocyclic donors.

These compounds showed very weak H_2S generation in buffers. With 1 mM initial concentration, negligible amounts of H_2S were observed from lead donors in the absence of cysteine. When cysteine or GSH (4 mM) was presented, H_2S formation was detectable, but at a low level (~1%).

After confirming H_2S release ability, Calderone and coworkers tested the effects of the lead donor, *p*-hydroxybenzothioamide, on vasoconstriction induced by noradrenaline in isolated rat aortic rings. The results suggested that the pretreatment of aortic rings with 1 mM of *p*-hydroxybenzothioamide almost completely inhibited vasoconstriction. This observation is similar with that obtained in NaHS-treated group, indicating the anti-vasoconstriction effects of *p*-hydroxybenzothioamide are H_2S-related. In addition, the effects of *p*-hydroxybenzothioamide on blood pressure were tested. As expected, after oral administration of *p*-hydroxybenzothioamide (0.1 mg/kg) to rats, a decrease of blood pressure (89 ± 1%) was observed.

Although these data suggest arylthioamides can release H_2S and their biological effects are H_2S-related, some puzzling questions remain: 1) H_2S release from arylthioamides was only shown for 15 minutes in the paper. It is unclear if H_2S concentrations maintained elevated or dropped afterwards. 2) H_2S release mechanism (with and without cysteine) is not reported. It is unclear what the active intermediate(s) and final product(s) are. 3) Since the donors release only very limited H_2S (~ 1%), the major form of the donors *in vivo* should still be the donor molecules. Caution should be

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used in attributing their biological activities to H_2S unless careful control experiments using both the active intermediates and final products after H_2S release are conducted.

2.7. Photo-induced H_2S donors

Gem-dithiol-based-H_2S donors. In an effort to develop non-thiol dependent donors, geminal-dithiols (*gem*-dithiols) were recently identified as a useful structure template.[141] Gem-dithiol compounds are unstable in aqueous solutions. H_2S can be formed as a decomposition byproduct.[142-144] To make stable *gem*-dithiol-based donors, a photo cleavable 2-nitrobenzyl group[145-147] was introduced as the protecting group on SH. Upon light irradiation, the free *gem*-dithiol intermediates should be formed and subsequent hydrolysis of these intermediates would liberate H_2S (Scheme 20).

Scheme 20. Design of *gem*-dithiol-based H_2S donors.

A three-step synthesis of this type of donors was described as follows: commercially available 2-nitrobenzyl bromide was treated with thiourea in THF to produce the thiouroniumbromide salt. Hydrolysis of this salt in the presence of sodiummetabisulfite provided 2-nitrobenzenemethanethiol in high yield. Finally, 2-nitrobenzenemethanethiol was coupled with ketones in the presence of catalytic amount of titanium tetrachloride to yield the desired donor products (Scheme 21).

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Scheme 21. Chemical synthesis of *gem*-dithiol-based H_2S donors.

MB assay indicated that up to 36 μM of H_2S was generated after the irradiation of *gem*-dithiol-based donors (200 μM) at 365 nm. In comparison, no H_2S was detected without the exposure of donors under UV light. H_2S release of a selected donor was also detected in HeLa cells (under UV irradiation) by using an H_2S fluorescent probe.

It should be noted that H_2S generation from these donors depended on the hydrolysis of *gem*-dithiol in aqueous solution, which resulted in the difficulties to control the rate of H_2S release. Additionally, 2-nitrosobenzaldehyde, the byproduct of donor deprotection, may react with free *gem*-dithiol, therefore diminishing H_2S generation. Considering these drawbacks, 2-nitrobenzyl group may not be suitable in developing photo-induced H_2S donors for biological applications. The desired protecting groups should be photoremovable, and meanwhile not form any reactive byproducts.

Ketoprofenate-caged H_2S donors. Recently, Nakagawa's group employed ketoprofenate[148] as a photocage to develop photolabile H_2S donors.[149] The synthesis of this donor was illustrated in Scheme 22a. Briefly, bromination of the nitrile starting material yielded a bromide intermediate in the presence of lithium diisopropylamide (LDA) and dibromomethane. This bromide species was then converted to sulfide intermediate by Na_2S and the hydrolysis of this sulfide gave the desired donor. This

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ketoprofenate-caged donor can release H_2S by eliminating 2 equivalents of 2-propenylbenzophenone and CO_2 upon the irradiation at 300-350 nm (Scheme 22b). To evaluate H_2S releasing capability in a complex biological system, this ketoprofenate-caged donor was applied to fetal bovine serum. No H_2S was detected without irradiation. However, approximately 30 μM of H_2S was detected from 500 μM of the donor in serum after irradiating for 10 minutes.

Scheme 22. a) Synthesis of ketoprofenate-caged donor and b) H_2S release mechanism.

So far two classes of photo-inducible H_2S donors have been developed and evaluated. It should be noted that UV photolysis on biological samples such as cells or tissues may have cytotoxicity concerns, therefore limiting the applications of such H_2S donors. Nevertheless these works proved the concept that both *gem*-dithiols and ketoprofenate-caged thioether are viable H_2S donor precusors and that non-thiol dependent activation strategies can be used in triggering H_2S generation. Based on these results, we expect to see more donors with new H_2S release mechanisms in the near future. For example, since near-infrared (NIR) light can penetrate tissues and minimize the damage to biological samples, NIR-activated H_2S donors may be developed and be useful.

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2.8. Thioamino acids

In 2012 Giannis et al. reported that thioamino acids, such as thioglycine and thiovaline, could be H_2S donors.[150] They found that in the presence of bicarbonate both compounds were converted to the corresponding amino acid *N*-carboxyanhydrides with the generation of H_2S (Scheme 23).

Thioglycine, R = H
Thiovaline, R = *iso*-propyl

Amino acid
N-carboxyanhydrides

Scheme 23. Proposed mechanism for H_2S release from thioamino acids.

Considering the high bicarbonate concentration (~ 27 mM) in blood at physiological pH, Giannis et al. envisioned that thioamino acids could be good H_2S donors. By using a H_2S electrode, H_2S releasing capabilities of thioglycine and thiovaline were evaluated and compared with other H_2S donors. Results showed that H_2S release from these two thioamino acids plateau after 1 hour. In comparison, H_2S release from NaHS and Na_2S was much faster and completed within 20 minutes. Up to ~50 µM of H_2S could be detected from 100 µM of thioglycine, while GYY4137 released much less H_2S.

To test the pharmacological benefits of these donors, their effects on intracellular cyclic guanosine monophosphate (cGMP) levels were determined. The results indicated that thioglycine and thiovaline led to a concentration-dependent increase in cGMP levels (~ 10-fold increase). In comparison, NaHS only resulted in a 2-fold increase of cGMP and glycine/valine did not cause any increase of cGMP. In addition, both thioglycine and thiovaline were found to induce significant relaxation of precontracted mouse aortic rings.

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Although both thioglycine and thiovaline were proved to release H_2S, thioamino acids are highly reactive molecules. Under aerobic conditions, they can rapidly carry out amidation reactions[151-156] and can be easily oxidized to dithioperoxyanhydrides, which were also reported as H_2S donors.[137] All of these may lead to unwanted side-effects. Therefore, caution should be taken when using thioamino acids to study H_2S-related effects.

3. CONLUSIONS AND FUTURE DIRECTIONS

In this review, we summarized the information on currently available H_2S donors, with a focus on fundamental chemistry and some biological applications (Table 1). Given the importance of H_2S in biomedical research, H_2S donors are not only useful research tools, but also potential therapeutic agents. Although a number of donors have been developed and shown to release H_2S both *in vitro* and *in vivo*, it is hard to define a universal "best" donor. All the donors have their own advantages, as well as problems. A major problem is that H_2S release from many donors (i.e. sulfide salts, GYY4137, and DTTs) is not controllable and cannot mimic biological/endogenous H_2S generation. This fast and uncontrollable H_2S release can cause severe problems and sometimes can even be lethal. In addition, the byproducts associated with H_2S release from some widely used donors such as GYY4137 and DTTs are still unclear. It is necessary to identify these products and study their biological activities. As for controllable donors, current efforts have been mostly put on thiol-activated donors and four types of such donors (*N*-SH-based donors, *S*-SH-based donors, dithioperoxyanhydrides, and arylthioamides) have been developed. These donors require the consumption of biological thiols to promote H_2S generation. Considering biological levels of H_2S, it is anticipated that the active dose

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of these donors should be at low micromolar level. Therefore the consumption of free thiols by donors may not cause significant changes in thiol redox balance. However, it is possible that in some cases, free thiols' level might be low due to disulfide formation or binding to proteins. Caution should be taken when using these donors in such conditions or careful control experiments are needed. Last but not least, since reactive sulfane sulfur species, such as perthiols, are possibly involved in some donors (i.e. garlic-derived sulfur compounds, DTTs, dithioperoxyanhydrides, *N*-SH-based and *S*-SH-based donors), whether the biological activities of these donors are H_2S-dependent or sulfane sulfur-dependent needs to be further investigated. In conclusion, H_2S is a potential therapeutic molecule. The development of useful donors is critical. In our opinion future efforts should be focused on developing controllable H_2S donors and different H_2S releasing mechanisms should be explored. We expect to see more work coming out in this exciting field.

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Table 1. Summary of H_2S donors.

H_2S donors	Structures	H_2S release mechanism	Representative bioactivities	Key ref.
H_2S (gas)	H_2S	Authentic H_2S resource	Hibernation induction Type II diabetes amelioration	67-70
Sulfide salts	NaHS & Na_2S	Hydrolysis	Heart failure reduction Anti-inflammation	75-83
Garlic-derived sulfur compounds	$R-S-S_n-S-R$	Thiol activation	Vasodilation	86
Lawesson's reagent	MeO, OMe, P, S	Hydrolysis	Anti-inflammation Ion channel regulation	97, 98
GYY4137	MeO, P, S, S^-, N, O, $H_2\overset{+}{N}$	Hydrolysis	Vasodilation Anti-inflammation Anti-cancer	99, 105, 106
Phosphorodithioates	PhHN-P(=S)(OR)-S^-	Hydrolysis	Prevention against oxidative damages	107
DTTs	S, R, S, S	Hydrolysis	Anti-inflammation Anti-cancer	114, 124-126, 129
N-(acylthio)-benzamides	O, R, S, H, N, Ph, O	Thiol activation	No biological effects were reported to date.	132
S-SH compounds	AcHN, O, NHBu, S, S, R, O	Thiol activation	MI/R protection	133
Dithioperoxyanhydrides	O, R, S, S, R, O	Thiol activation	Vasodilation	137
Arylthioamides	S, Ar, NH_2	Thiol activation	Vasodilation	140
Gem-dithiol compounds	NO_2, O_2N, S, S, R_1, R_2	Light activation	No biological effects were reported to date.	141
Ketoprofenate-caged compound	O, CO_2H, S, CO_2H, O	Light activation	No biological effects were reported to date.	149
Thioamino acids	O, R, SH, NH_2	Bicarbonate activation	Vasodilation	150

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Abbreviations:

H_2S	Hydrogen sulfide
NO	Nitric oxide
CO	Carbon monoxide
CBS	Cystathionine β-synthase
CSE	Cystathionine γ-lyase
MPST	3-Mercaptopyruvate sulfur transferase
CAT	Cysteine amino transferase
$ONOO^-$	Peroxynitrite
$O_2^{\cdot -}$	Superoxide
H_2O_2	Hydrogen peroxide
HSNO	Thionitrous acid
HCN	Hydrogen cyanide
MB	Methylene blue
ISE	Ion selective electrodes
N_3	Azide
O_2	Oxygen
CO_2	Carbon dioxide
MR	Metabolic rate
CBT	Core body temperature
Na_2S	Sodium sulfide
NaHS	Sodium hydrogen sulfide
LV	Left ventricular
OA	Osteoarthritis
DAS	Diallyl sulfide
DADS	Diallyl disulfide
DATS	Diallyl trisulfide
RBCs	Red blood cells
PBS	Phosphate buffered saline
GSH	Glutathione
AMS	Allyl methyl sulfide
DPDS	Dipropyl disulfide
S_8	Elemental sulfur
P_4S_{10}	Phosphorus pentasulfide
TNBS	Trinitrobenzene sulfonic acid
DTNB	5,5’-Dithiobis-(2-nitrobenzoic acid)
DBU	1,8-Diazobicyclo[5.4.0]undec-7-ene
DNS-Az	Dansyl azide
DTTs	1,2-Dithiole-3-thiones
CS_2	Carbon disulfide
HMDT	Hexamethyldisilathiane
NCS	N-chlorosuccinimide
NSAIDs	Nonsteroidal anti-inflammatory drugs
HS-NSAIDs	HS-hybrid NSAIDs

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ADT-OH	5-(4-Hydroxyphenyl)-3H-1,2-dithiole-3-thione
DCC	Dicyclohexylcarbodiimide
DMAP	4-Dimethylaminopyridine
EWGs	Electron withdrawing groups
EDGs	Electron donating groups
NCL	Native chemical ligation
MI/R	Myocardial ischemia/reperfusion
gem-dithiol	Geminal-dithiols
LDA	Lithium diisopropylamide
NIR	Near infrared
cGMP	Cyclic guanosine monophosphate

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