DEVELOPMENT OF NOVEL CONTROLLABLE HYDROGEN SULFIDE DONORS

By

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A dissertation submitted in partial fulfillment of
The requirement for the degree of

DOCTOR OF PHILOSOPHY

WASHINGTON STATE UNIVERSITY
Department of Chemistry

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The members of the Committee appointed to examine the dissertation of YU ZHAO find it satisfactory and recommend that it be accepted.

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Thank you again for all your support and help during these years. I would never forget my life at WSU.
DEVELOPMENT OF NOVEL CONTROLLABLE HYDROGEN SULFIDE DONORS

Abstract

by Yu Zhao, Ph.D.
Washington State University
May 2014

Chair: Ming Xian

Hydrogen sulfide (H₂S) is as an important cell signaling molecule. It has been recognized as a mediator of many physiological and/or pathological processes. The production of H₂S in mammalian systems has been attributed to three enzymes: cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE), and 3-mercaptopyruvate sulfur-transferase (MPST). Although H₂S’s exact chemical and biochemical modes of action are still not fully understood, the production of endogenous H₂S and exogenously administration of H₂S have been demonstrated to exert protective effects in many pathologies. In this field, H₂S-releasing agents (or donors) are important research tools for the study of H₂S biological functions. However, currently available H₂S donors are very limited and H₂S generations from these compounds are too fast and uncontrollable. Rapid release of H₂S may cause acute changes in blood pressure. Ideal H₂S donors, from
therapeutic point of view and for the applications in H$_2$S-related biological research, should release H$_2$S slowly in moderate amounts.

To this end, we have developed new controllable H$_2$S donors, such as $N$-mercapto-based donors and perthiol-based donors. These molecules were stable in aqueous solutions. However, in the presence of cellular thiols (i.e. cysteine and glutathione), time-dependent H$_2$S release was observed. In addition, protective effects of these donors in living systems were also observed. These findings suggest that both $N$-mercapto-based and perthiol-based molecules are potent H$_2$S donors and they have potential therapeutic benefits due to H$_2$S release.
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<tr>
<td>AAR</td>
<td>Area at risk</td>
</tr>
<tr>
<td>AAR/LV</td>
<td>Area at risk per left ventricle</td>
</tr>
<tr>
<td>ADT-OH</td>
<td>5-(4-Hydroxyphenyl)-3H-1,2-dithiole-3-thione</td>
</tr>
<tr>
<td>AMS</td>
<td>Allyl methyl sulfide</td>
</tr>
<tr>
<td>br</td>
<td>Broad</td>
</tr>
<tr>
<td>C</td>
<td>Carbon</td>
</tr>
<tr>
<td>Calcd</td>
<td>Calculated</td>
</tr>
<tr>
<td>CaS</td>
<td>Calcium sulfide</td>
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<tr>
<td>CAT</td>
<td>Cysteine amino transferase</td>
</tr>
<tr>
<td>CBS</td>
<td>Cystathionine β-synthase</td>
</tr>
<tr>
<td>CBT</td>
<td>Core body temperature</td>
</tr>
<tr>
<td>CCK</td>
<td>Cell counter kit</td>
</tr>
<tr>
<td>CDCl₃</td>
<td>Deuterated chloroform</td>
</tr>
<tr>
<td>CD₃OD</td>
<td>Deuterated methanol</td>
</tr>
<tr>
<td>cGMP</td>
<td>Cyclic guanosine monophosphate</td>
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<td>CH₃CN</td>
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<td>CO</td>
<td>Carbon monoxide</td>
</tr>
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<td>CO₂</td>
<td>Carbon dioxide</td>
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<tr>
<td>CS₂</td>
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<td>CSE</td>
<td>Cystathionine γ-lyase</td>
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<td>Description</td>
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</tr>
<tr>
<td>CTAB</td>
<td>Cetyltrimethylammonium bromide</td>
</tr>
<tr>
<td>d</td>
<td>Doublet</td>
</tr>
<tr>
<td>DAS</td>
<td>Diallyl sulfide</td>
</tr>
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<td>DADS</td>
<td>Diallyl disulfide</td>
</tr>
<tr>
<td>DATS</td>
<td>Diallyl trisulfide</td>
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<td>DBU</td>
<td>1,8-Diazobicyclo[5.4.0]undec-7-ene</td>
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<td>Dichloromethane</td>
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<tr>
<td>DI</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle medium</td>
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<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<td>DNS-Az</td>
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<td>DMAP</td>
<td>4-Dimethylaminopyridine</td>
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<td>D$_2$O</td>
<td>Deuterium oxide</td>
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<td>DPDS</td>
<td>Dipropyl disulfide</td>
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<td>DTNB</td>
<td>5,5’-Dithiobis-(2-nitrobenzoic acid)</td>
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<td>DTTs</td>
<td>1,2-Dithiole-3-thiones</td>
</tr>
<tr>
<td>EDGs</td>
<td>Electron donating groups</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diamine tetraacetic acid</td>
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<tr>
<td>ESI</td>
<td>Electron spray ionization</td>
</tr>
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<td>Et$_2$O</td>
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<td>EWGs</td>
<td>Electron withdrawing groups</td>
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<td>FBS</td>
<td>Fetal bovine serum</td>
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</tr>
<tr>
<td>FeCl₃</td>
<td>Ferric chloride</td>
</tr>
<tr>
<td>GC</td>
<td>Gas chromatography</td>
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<tr>
<td><em>gem</em>-dithiol</td>
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</tr>
<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>H</td>
<td>Hydrogen</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>HCN</td>
<td>Hydrogen cyanide</td>
</tr>
<tr>
<td>HMDT</td>
<td>Hexamethyldisilathiane</td>
</tr>
<tr>
<td>H₂O</td>
<td>Water</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>HPLC</td>
<td>High resolution liquid chromatography</td>
</tr>
<tr>
<td>HRMS</td>
<td>High resolution mass spectrometry</td>
</tr>
<tr>
<td>H₂S</td>
<td>Hydrogen sulfide</td>
</tr>
<tr>
<td>HSNO</td>
<td>Thionitrous acid</td>
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<tr>
<td>HS-NSAIDs</td>
<td>HS-hybrid NSAIDs</td>
</tr>
<tr>
<td>Hz</td>
<td>Hertz</td>
</tr>
<tr>
<td>INF/AAR</td>
<td>Infarct size per area at risk</td>
</tr>
<tr>
<td>ISE</td>
<td>Ion selective electrodes</td>
</tr>
<tr>
<td>IR</td>
<td>Infrared</td>
</tr>
<tr>
<td>J</td>
<td>NMR coupling constant</td>
</tr>
<tr>
<td>KBr</td>
<td>Potassium bromide</td>
</tr>
<tr>
<td>kg</td>
<td>Kilogram</td>
</tr>
<tr>
<td>KOH</td>
<td>Potassium hydroxide</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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</tr>
<tr>
<td>LCA</td>
<td>Left coronary artery</td>
</tr>
<tr>
<td>LDA</td>
<td>Lithium diisopropylamide</td>
</tr>
<tr>
<td>LV</td>
<td>Left ventricle</td>
</tr>
<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>m</td>
<td>Multiplet</td>
</tr>
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<td>MB</td>
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<tr>
<td>min</td>
<td>Minute</td>
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</tr>
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<td>mmol</td>
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</tr>
<tr>
<td>m.p.</td>
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</tr>
<tr>
<td>MPST</td>
<td>3-Mercaptopyruvate sulfur transferase</td>
</tr>
<tr>
<td>MI/R</td>
<td>Myocardial ischemia/reperfusion</td>
</tr>
<tr>
<td>MR</td>
<td>Metabolic rate</td>
</tr>
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<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>N₃</td>
<td>Azide</td>
</tr>
<tr>
<td>nA</td>
<td>Nanoamp</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>Sodium bicarbonate</td>
</tr>
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<td>NaHS</td>
<td>Sodium hydrogen sulfide</td>
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<td>Na₂S</td>
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</tr>
<tr>
<td>NCS</td>
<td>N-chlorosuccinimide</td>
</tr>
<tr>
<td>NCL</td>
<td>Native chemical ligation</td>
</tr>
<tr>
<td>NIR</td>
<td>Near infrared</td>
</tr>
<tr>
<td>nM</td>
<td>Nanomolar</td>
</tr>
<tr>
<td>nm</td>
<td>Nanometer</td>
</tr>
<tr>
<td>NMM</td>
<td>N-Methylmaleimide</td>
</tr>
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<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
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<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NSAIDs</td>
<td>Nonsteroidal anti-inflammatory drugs</td>
</tr>
<tr>
<td>O$_2$</td>
<td>Oxygen</td>
</tr>
<tr>
<td>O$_2^-$</td>
<td>Superoxide</td>
</tr>
<tr>
<td>OA</td>
<td>Osteoarthritis</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>ONOO$^-$</td>
<td>Peroxynitrite</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>P$<em>4$S$</em>{10}$</td>
<td>Phosphorus pentasulfide</td>
</tr>
<tr>
<td>PySSPy</td>
<td>2-Mercapto pyridine disulfide</td>
</tr>
<tr>
<td>RBCs</td>
<td>Red blood cells</td>
</tr>
<tr>
<td>r.t.</td>
<td>Room temperature</td>
</tr>
<tr>
<td>S$_8$</td>
<td>Elemental sulfur</td>
</tr>
<tr>
<td>t</td>
<td>Triplet</td>
</tr>
<tr>
<td>THF</td>
<td>Tetrahydrofuran</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin layer chromatography</td>
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<td>Description</td>
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<tr>
<td>--------------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td>TNBS</td>
<td>Trinitrobenzene sulfonic acid</td>
</tr>
<tr>
<td>µL</td>
<td>Microliter</td>
</tr>
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<td>µM</td>
<td>Micromolar</td>
</tr>
<tr>
<td>µmol</td>
<td>Micromole</td>
</tr>
<tr>
<td>UV-Vis</td>
<td>Ultraviolet-visible</td>
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Dedication

This dissertation is dedicated to my parents Mr. Xinming Zhao and Mrs. Wenhua Zhao, my lovely wife Ang Gong and her parents Mr. Pinren Gong and Mrs Yuhong Wang for their endless love and emotional support.
CHAPTER ONE

HYDROGEN SULFIDE (H₂S) RELEASING AGENTS:
CHEMISTRY AND BIOLOGICAL APPLICATIONS

1.1 ABSTRACT

Hydrogen sulfide (H₂S) is a newly recognized signaling molecule with very potent cytoprotective actions. The fields of H₂S 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 H₂S in the future. Hydrogen sulfide releasing agents (also known as H₂S 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 H₂S and to be aware of the limitations associated with the choice of donors used to generate H₂S in vitro and in vivo. Herein I summarized the developments and limitations of current available donors including H₂S 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.

1.2 INTRODUCTION

Hydrogen sulfide (H₂S), 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
$\text{H}_2\text{S}$ in mammalian systems has been attributed to at least three enzymes: cystathionine $\beta$-synthase (CBS), cystathionine $\gamma$-lyase (CSE), and 3-mercaptopropanolate sulfur-transferase (MPST) (Scheme 1.1). CBS is found predominantly in the brain, nervous system and liver. It converts cysteine and homocysteine to cystathionine and releases $\text{H}_2\text{S}$. In comparison, CSE activity is higher than CBS in aorta, portal vein and other vascular tissue. CSE is responsible for $\text{H}_2\text{S}$ 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. Kimura and coworkers demonstrated that MPST, together with cysteine aminotransferase (CAT), produces $\text{H}_2\text{S}$ from cysteine in the presence of $\alpha$-ketoglutarate. It has also been reported that MPST can convert D-cysteine to $\text{H}_2\text{S}$ in the presence of D-amino acid oxidase. Although the expression of these enzymes is tissue-specific, they all convert cysteine or cysteine derivatives to $\text{H}_2\text{S}$. These enzymes work collectively and precisely regulate $\text{H}_2\text{S}$ levels in tissues, and therefore are crucial for $\text{H}_2\text{S}$ homeostasis.

In 1996 Kimura demonstrated that endogenous $\text{H}_2\text{S}$ acts as a neuromodulator in the brain. Following his work, a number of studies have revealed various biological effects of $\text{H}_2\text{S}$, which include the relaxation of blood vessels, protection against myocardial ischemia injury, and cytoprotection against oxidative stress. In addition, some chemical and biochemical catabolic reactions of $\text{H}_2\text{S}$ have also been disclosed, and these reactions may be responsible for the biological functions of $\text{H}_2\text{S}$. For example, $\text{H}_2\text{S}$ is a powerful reducing agent and is likely to be consumed by endogenous oxidants such as peroxynitrite ($\text{ONOO}^-$), superoxide ($\text{O}_2^-$) and hydrogen peroxide ($\text{H}_2\text{O}_2$) (Scheme 1.2a). $\text{H}_2\text{S}$ reacts readily with methemoglobin to form
Sulfhemoglobin, which might act as a metabolic sink for H$_2$S (Scheme 1.2b).[30] It is reported that H$_2$S can cause protein $S$-sulfhydration (i.e. to form -$S$-SH) (Scheme 1.2c).[31-33] but the detailed mechanism is still unclear. Nevertheless this process is potentially significant as it provides a possible route by which H$_2$S can alter the functions of a wide range of cellular proteins and enzymes.[34-40] H$_2$S 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 1.2d).[41] It is likely that many more important reactions of H$_2$S remain to be discovered.
Although the endogenous formation of H\textsubscript{2}S and exogenous administration of H\textsubscript{2}S have been proved beneficial in some pathophysiological conditions, the molecular mechanisms of H\textsubscript{2}S action are still under investigation. It is therefore important to understand the chemistry and properties of H\textsubscript{2}S and to be aware of the problems associated with the choice of resources used to generate H\textsubscript{2}S in \textit{in vitro} and \textit{in vivo} experiments. H\textsubscript{2}S is a colorless gas under ambient temperature and pressure. The toxicity of H\textsubscript{2}S has been known for hundreds of years and is comparable to that of CO or hydrogen cyanide (HCN).\cite{42-44} Exposure to 300 ppm of H\textsubscript{2}S leads to pulmonary edema and 1000 ppm of H\textsubscript{2}S causes immediate death. Caution should therefore be taken when working with H\textsubscript{2}S. As a weak acid, H\textsubscript{2}S is very water soluble. Its solubility was

\textbf{Scheme 1.2.} Some important biological reactions of H\textsubscript{2}S.
reported to be ~80 mM at 37 °C as an equilibrium between molecular and ionic forms (H$_2$S$_{aq}$ ↔ HS$^-$ ↔ S$^2$). The pK$_a$ 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$_2$S (the ratio of HS$^-$/H$_2$S 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$_2$S (H$_2$S, HS$^-$, or S$^2$-) is the active species in biological systems, the term of H$_2$S is used to refer to the total sulfide present in the solution (i.e. H$_2$S + HS$^-$ + S$^2$-).

So far, one of the major challenges in the H$_2$S field is precise measurement of H$_2$S 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$_2$S 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$_2$S detection in cell and blood samples.[52-56] These works inspired researchers to develop new H$_2$S 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$_2$S 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 1.3): 1) H$_2$S-mediated reductions, often using azide (N$_3$) substrates; 2) H$_2$S-mediated nucleophilic reactions, and 3) H$_2$S-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\textsubscript{2}S vs other reactive sulfur species, especially the newly recognized persulfide species, is largely unaddressed. Due to these problems, further development of chemoospecific fluorescent probes for H\textsubscript{2}S remains critical.

**Scheme 1.3.** Representative fluorescent probes for H\textsubscript{2}S detection.

In the study of H\textsubscript{2}S’s mechanisms and functions H\textsubscript{2}S releasing agents (i.e. donors) are important research tools. In the past several years the development of novel H\textsubscript{2}S donors has become a rapidly growing field, with several series of donors reported.\[63, 64\] These donors release H\textsubscript{2}S through different mechanisms. It should be noted that although H\textsubscript{2}S is biologically active, its functions sometimes appear inconsistent. For example, in addition to its anti-inflammatory effects, H\textsubscript{2}S has also been reported as a pro-inflammatory molecule.\[65, 66\] These disparate results might be due to the use of different H\textsubscript{2}S donors in the research. H\textsubscript{2}S releasing capabilities of each donor category are quite different, which may lead to different results. Additionally, byproducts could form along with H\textsubscript{2}S generation and it is unclear whether these byproducts have biological effects. Therefore, the selection of suitable H\textsubscript{2}S donors is crucial. Here I summarize the information about current available H\textsubscript{2}S donors with a focus on the chemistry of their development. Some biological applications are also discussed.

**1.3 H\textsubscript{2}S DONORS**

**1.3.1 H\textsubscript{2}S Gas**
As the authentic resource, H₂S gas has been directly used in this field. It has been reported that H₂S gas promotes glucose uptake and provides amelioration in type II diabetes.[67] In 2005 Roth found that H₂S (g) could induce a suspended animation-like state in mice.[68] The exposure of mice to 80 ppm of H₂S (g) caused a significant drop in their oxygen (O₂) consumption (by ~ 50%) and carbon dioxide (CO₂) output (by ~ 60%) within first 5 minutes. A 6-hour H₂S (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 °C. When mice were returned to normal air and room temperature after this H₂S 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 H₂S, which slowed respiration.[69]

The reversible H₂S-induced hibernation state was later shown to protect mice from lethal hypoxia.[70] C57BL/6J mice cannot survive in 5% O₂ for more than 15 minutes. However, after inducing the suspended animation-like state by pretreating the animals with 150 ppm H₂S (g) for 20 minutes, mice survived the duration of 1-h experiment under normally lethal hypoxia conditions. The longest exposure to 5% O₂ was 6.5 hours and H₂S-pretreated mice survived without notable damage.

Although these interesting experiments revealed promising effects of H₂S 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 H₂S gas as an ideal resource due to difficulties in obtaining precisely controlled concentrations and possible toxic impact of H₂S excess. Because of this reason, H₂S equivalents or releasing agents are often used in the field.

1.3.2 Inorganic Sulfide Salts
Inorganic sulfide salts such as sodium sulfide (Na$_2$S) and sodium hydrogen sulfide (NaHS) have been used as H$_2$S 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$_2$S as an exogenous H$_2$S donor, Lefer et al. confirmed that long-term H$_2$S 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$_2$S 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$_2$S therapy leads to a decrease in left ventricular (LV) dilation, decrease in cardiac hypertrophy, and improvement in cardiac function. In addition, treatment with Na$_2$S also reduced oxidative stress associated with heart failure. Although previous studies have provided solid evidence for the cardioprotective effects of short-term H$_2$S therapy,[80, 81] these results by Lefer showed for the first time that H$_2$S 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$_2$S 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\textsubscript{2}S donors, have the advantage of boosting H\textsubscript{2}S concentration rapidly. However, these compounds release H\textsubscript{2}S spontaneously at the time the solution is prepared, making it hard to precisely control H\textsubscript{2}S 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\textsubscript{2}S release can cause severe damages \textit{in vivo}. In addition, H\textsubscript{2}S 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\textsubscript{2}S loss from aqueous solutions.\[83\] They found that H\textsubscript{2}S were lost in solutions with t_{1/2} values of about 5 min. After 12 hours, only 3.4 \times 10^{-43} \mu \text{M} of H\textsubscript{2}S was left from an original 10 \mu \text{M} of H\textsubscript{2}S Hepes solution. It should also be noted that commercial sulfide salts, especially NaHS, always contain 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\textsubscript{2}S donors.

\subsection*{1.3.3 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\textsubscript{2}S 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 1.4).\[85\] Kraus and coworkers demonstrated that human blood cells (RBCs) can convert garlic-derived organic polysulfides into H\textsubscript{2}S, 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 1.4), DATS produced the highest amount of H\textsubscript{2}S in the presence of glutathione.
(GSH), followed by DADS. Apparently H₂S production from these sulfur compounds is facilitated by allyl substituents and by increasing the numbers of tethering sulfur atoms.

![Scheme 1.4. Garlic-related sulfur compounds.](image)

From a reaction mechanism point of view, the regular thiol/disulfide exchange between DADS and GSH should not produce H₂S. Instead, H₂S 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 H₂S. Trisulfides (R-SSS-R’) also undergo similar nucleophilic substitutions at the sulfur atom, yielding RSSH, and then H₂S (Scheme 1.5).

![Scheme 1.5. H₂S generation from garlic-derived sulfur compounds.](image)

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 °C organ bath containing 1 mM GSH under physiological O₂ 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$_2$S 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$_2$S yields, suggesting a link between bioactivity and H$_2$S 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 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$_2$S usually coexist, and recent work even suggests that sulfane sulfur species, derived from H$_2$S, 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.

1.3.4 Lawesson’s Reagent and Analogs

2,4-Bis(4-methoxyphenyl)-1,3,2,4-dithiadiphosphetane-2,4-disulfide (also known as Lawesson’s reagent) is a widely used sulfurization reagent in organic synthesis [94] that can also be used as a H$_2$S donor. It can be easily synthesized by heating a mixture of anisole with phosphorus pentasulfide (P$_4$S$_{10}$) (Scheme 1.6a).[95, 96]
Scheme 1.6. Chemical synthesis of Lawesson’s reagent (a) and GYY4137 (b).

As a H$_2$S donor, Lawesson’s reagent showed some H$_2$S-related bioactivities, such as ion channel regulation and anti-inflammation.[97, 98] In 2009 Wallace et al investigated the effects of H$_2$S 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. 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 a H$_2$S donor. However, this donor releases H$_2$S upon spontaneous hydrolysis in aqueous solution. This uncontrollable release of H$_2$S makes it difficult to mimic endogenous H$_2$S 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 1.6b).[99]

Similar to Lawesson’s reagent, GYY4137 releases H$_2$S upon hydrolysis. The in vitro H$_2$S release was confirmed by colorimetric and amperometry assays.[99] Compared to sulfide salts, H$_2$S release from GYY4137 was much slower and the H$_2$S concentration reached the maximum
value within 6-10 minutes but at a very low level. 1 mM of GYY4137 released 40 µM of H₂S within the first 10 minutes and another 50 µM of H₂S in the following 90 minutes in aqueous solution (pH 3.0). H₂S release from GYY4137 was pH- and temperature-dependent, with more release at acidic pH and less release at low temperatures. Under physiological conditions, H₂S production from GYY4137 maintained at low level (less than 10 %) even after 7 days.[106] For \textit{in vivo} H₂S production, GYY4137 (133 µmol/kg) was administrated (intravenous or intraperitoneal injections) to anesthetized male Sprague-Dawley rats. The plasma H₂S concentration, measured by MB method, was increased at 30 minutes and remained elevated over 180 minutes.

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 H₂S 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.

H₂S 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\textsubscript{2}S on inflammation were dependent on H\textsubscript{2}S generation rate. In this study GYY4137 significantly inhibited the LPS-induced release of pro-inflammatory mediators such as IL-1\textbeta, IL-6, TNF-\alpha, nitric oxide, and PGE\textsubscript{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\textsubscript{2}S on inflammation are complex and may depend not only on H\textsubscript{2}S concentration but also on the rate of H\textsubscript{2}S generation.

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 \textmu M). NaHS and ZYJ1122 (a structural analog of GYY4137 lacking sulfur) remained inactive at the same concentration. GYY4137 (800 \textmu M) 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\textsubscript{2}/M phase and promotion of apoptosis. The fact that the non-H\textsubscript{2}S-releasing compound ZYJ1122 did not show inhibitory effects on any cell lines may suggest the anticancer effects of GYY4137 are due to H\textsubscript{2}S release.

Although GYY4137 has been widely used, its fixed H\textsubscript{2}S release capability may not fulfill the requirements of different biological applications. In addition, the exact mechanism of H\textsubscript{2}S 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\textsubscript{2}S release since its non-H\textsubscript{2}S 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 a H₂S donor. Otherwise, GYY4137-induced biological effects may not be concluded to be H₂S-dependent.

Recently the Xian group developed a series of phosphorodithioate-based H₂S 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 result in H₂S release capability change and in turn lead to biological activity changes. The synthesis of such phosphorodithioate-based H₂S donors was achieved in 4 steps (Scheme 1.7): 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.

\[
\begin{align*}
\text{PCl}_3 & \xrightarrow{\text{HS-SH}} \text{S-P-S} \xrightarrow{\text{1) PhNH}_2, \text{DIEA}} \text{S-P-S} \xrightarrow{\text{2) S}_8, \text{CS}_2} \text{PhHN-P-S} \xrightarrow{\text{ROH, DBU}} \text{PhHN-P-S} \\
\text{ROH} &= \text{OCH}_3, \text{Br}, \text{NO}_2
\end{align*}
\]

**Scheme 1.7.** Chemical synthesis of phosphorodithioate-based H₂S donors.

H₂S release capabilities of these compounds were tested by spectroscopic methods and compared with GYY4137. In previous studies H₂S release from GYY4137 was mainly measured by MB assay, a standard H₂S 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$_2$S,[55] was used to measure H$_2$S generation from phosphorodithioate-based H$_2$S donors under neutral pH. Similar to GYY4137, O-aryl substituted phosphorodithioate-based donors showed slow and low H$_2$S release. Up to 1 µM of H$_2$S was detected from 100 µM of donor in a mixed 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$_2$S release. Presumably the O-alkyl substitutions led to increased stability of phosphorodithioates and therefore decreased the rate of hydrolysis to generate H$_2$S. O-Aryl substituted phosphorodithioate-based donors were also shown to release H$_2$S 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, a H$_2$S-specific fluorescent probe,[53] was applied to the cells to monitor H$_2$S production. Compared to vehicle-treated group, enhanced fluorescence in donor-treated group demonstrated a sustain H$_2$S generation.

Since H$_2$S has been known to exhibit cellular protection against oxidative injury,[108] it was hypothesized that phosphorodithioate-based H$_2$S donors may have similar effects due to H$_2$S release. Two O-aryl substituted donors were selected to evaluate their protective effects against H$_2$O$_2$-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$_2$O$_2$ (150 µM). Cell viability results showed that 35% of cells were killed if H$_2$S donors were absent. In comparison, cell viability increased significantly in the presence of donors, suggesting that H$_2$S donors may have some protective effects against oxidative injury.

It should be noted that although phosphorodithioate-based donors have showed H$_2$S-like biological activities, it is still premature to attribute those activities to H$_2$S production. H$_2$S 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 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 oligodeoxyctydine has also showed inhibitive activities against human immunodeficiency virus.[111] Therefore whether phosphorodithioate-induced effects are due to H$_2$S release needs further confirmation.

1.3.5 1,2-Dithiole-3-Thiones and H$_2$S-Hybrid Nonsteroidal Anti-Inflammatory Drugs

1, 2-Dithiole-3-thiones (DTTs) are known to release H$_2$S 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$_2$S generation from DTTs (Scheme 1.8).[112-114]

![Scheme 1.8. Proposed mechanism for H$_2$S release from DTTs.](image)

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 1.9a).[115-117] In an alternative method
Dithiocic acids, obtained from a reaction between ketones and carbon disulfide (CS₂), react with hexamethyldisilathiane (HMDT, a sulfur resource) and N-chlorosuccinimide (NCS, an oxidizing agent) to give substituted DTTs (Scheme 1.9b).[118, 119] In addition, β-ketoesters were also reported to react with Lawesson’s reagent to form desired DTTs (Scheme 1.9c).[120]

**Scheme 1.9.** Chemical synthesis of DTTs.

DTTs have been widely used in studying H₂S-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 H₂S releasing molecule, in the presence of dicyclohexylcarbodiimide (DCC) and 4-dimethylaminopyridine (DMAP) (Scheme 1.10a).[127] Some representative HS-NSAIDs and their parent NSAIDs are listed in Scheme 1.10b.
Scheme 1.10. 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] H₂S 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 H₂S was detected by a sulfide-sensitive electrode. Results suggested that when incubated in buffer, ADT-OH released negligible amounts of H₂S, while ~ 12 nmol/min of H₂S was released from ATB-337. On the other hand, incubation of both compounds in liver homogenate caused 3-fold greater levels of H₂S generated from ATB-337 (~ 43 nmol/min) than those from ADT-OH (~ 14 nmol/min). In order to measure
plasma H$_2$S 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$_2$S 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$_2$S 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$_2$S 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$_2$S more than ADT-OH in liver homogenate, it is possible that the gastric safety of ATB-337 was caused by its enhanced H$_2$S releasing ability. However, it is still unclear why ATB-337 released more H$_2$S 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.

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 H₂S-related effects in different tissues and organs, it is still unclear how these molecules release H₂S in living systems. Previous studies suggested that the hydrolysis of DTTs releases H₂S in aqueous buffer.[112-114] However, considering the complexity of biological systems, H₂S release from HS-NSAIDs is expected to be more complicated. The hydrolysis of HS-NSAIDs may initiate H₂S 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 H₂S. 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 H₂S-related or sulfane sulfur-related needs to be further investigated.

1.3.6 Thiol-Activated H₂S Donors

1.3.6.1 N-mercapto-based H₂S donors

In 2011 Xian and coworkers introduced the concept of controllable H₂S 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 H₂S release from these donors should be controlled by factors like biomolecules, 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 1.11a). 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 1.11b).

**Scheme 1.11.** Design and synthesis of N-SH-based H\textsubscript{2}S 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\textsubscript{2} groups. However, in the presence of cysteine or GSH, a time-dependent decomposition of the donors accompanied by H\textsubscript{2}S release was observed. The structure activity relationship studies showed that electron withdrawing groups (EWGs) led to faster H\textsubscript{2}S release and electron donating groups (EDGs) resulted in slower H\textsubscript{2}S release, demonstrating the achievement of H\textsubscript{2}S release regulation by structural modifications. In addition, H\textsubscript{2}S release from N-SH-based donors was also detected in plasma, suggesting N-SH compounds are active H\textsubscript{2}S donors in complex systems.

Reaction mechanism studies showed that H\textsubscript{2}S generation in these donors is initiated by the thiol exchange between cysteine and donors to generate S-acylated cysteine and N-mercaptop benzamide. S-Acylated cysteine then undergoes a native chemical ligation (NCL) to form a stable N-acylated cysteine. The N-mercaptop benzamide intermediate interacts with cysteine to produce cysteine perthiol, which finally reacts with cysteine to form cystine and release H\textsubscript{2}S (Scheme 1.12).

1.3.6.2 Perthiol-based H$_2$S 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$_2$S biosynthesis catalyzed by CSE (Scheme 1.1). These suggest that perthiol (S-SH) could be a useful template for H$_2$S 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 1.13).

Scheme 1.13. Design of perthiol-based H$_2$S donors.

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 1.14a). H$_2$S release capabilities of these donors were evaluated. The results indicated that these donors indeed released H$_2$S in the presence of thiols (cysteine or GSH). However, compared to N-SH-based donors, primary perthiol-based donors showed much
decreased ability of H₂S generation. Only less than 20 µM of H₂S 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 1.14b, pathway a). This reaction prevents the formation of the key perthiol intermediate (Scheme 1.14b, pathway b), therefore, diminishing H₂S release from these donors.

**Scheme 1.14.** a) Chemical synthesis of cysteine perthiol-based H₂S donors; b) explanation of low H₂S release from primary perthiol-based donors.

In order to enhance H₂S generation capability tertiary perthiol-based donors were synthesized. The acyldisulfide linkage was blocked by two methyl groups on the α-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 1.15).
These tertiary perthiol-based compounds were proved to be potent \( \text{H}_2\text{S} \) donors. Up to 80 \( \mu \text{M} \) of \( \text{H}_2\text{S} \) were generated from 100 \( \mu \text{M} \) of donors. The regulation of \( \text{H}_2\text{S} \) release from these donors could be achieved by structural modifications. Similar to \( N\)-SH-based donors, EWGs caused faster \( \text{H}_2\text{S} \) release and EDGs led to slower \( \text{H}_2\text{S} \) generation. In addition, steric effects were also observed as more hindered substrates resulted in slower \( \text{H}_2\text{S} \) release or even no release at all.

\( \text{H}_2\text{S} \) 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 \( \text{H}_2\text{S} \) in two possible pathways: thiol attacked the acyldisulfide linkage to produce a disulfide and generate \( \text{H}_2\text{S} \) (Scheme 1.16, 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 \( \text{H}_2\text{S} \) (Scheme 1.16, pathway b).

Since H₂S exhibited protective effects against myocardial ischemia/reperfusion (MI/R) injury,[134-136] perthiol based donors were expected to exhibit similar effects due to H₂S 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 infract size per area-at-risk, suggesting that S-SH-based compounds can exhibit H₂S-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 H₂S-related and sulfane sulfur-related mechanisms are needed.

1.3.6.3 Dithioperoxyanhydrides

Similar to N-SH-based and S-SH-based donors, dithioperoxyanhydrides were recently reported as another class of thiol-activated H₂S donors.[137] These donors were synthesized by either iodine oxidation of the thiocarboxylates [138] or reactions between thiocarboxylic acids and methoxycarbonylsulfenyl chloride [139] (Scheme 1.17). H₂S release was confirmed in both buffers and cellular lysates. Additionally, one of the donors, CH₃C(O)SSC(O)CH₃ in Scheme 1.17, was also shown to induce concentration-dependent vasorelaxation on pre-contracted rat aortic rings.
Scheme 1.17. Chemical synthesis of dithioperoxyanhydride-based H₂S donors

Acylpersulfides were proposed to be the key intermediates for H₂S release from these donors. It is possible that acylpersulfides directly react with thiols (RSH) to give H₂S and RSSAc (Scheme 1.18, 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 H₂S (Scheme 1.18, pathway b).

Scheme 1.18. Proposed mechanism for H₂S release from dithioperoxyanhydrides.

1.3.6.4 Arylthioamides

Arylthioamides were classified as the fourth class of thiol-activated H₂S donors (Scheme 1.19a).[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 1.19b); the heterocyclic compounds were obtained by the treatment of the amides with Lawesson’s reagent for 12 hours (Scheme 1.19c).

![Chemical structure of $p$-hydroxybenzothioamide](image)

\[ p\text{-hydroxybenzothioamide} \]

\[ R_1 = \text{OH or NH}_2, \quad R_2 = \text{H, Cl, F, CF}_3 \]

**Scheme 1.19.** 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
\textit{p}-hydroxybenzothioamide are H\textsubscript{2}S-related. In addition, the effects of \textit{p}-hydroxybenzothioamide on blood pressure were tested. As expected, after oral administration of \textit{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\textsubscript{2}S and their biological effects are H\textsubscript{2}S-related, some puzzling questions remain: 1) H\textsubscript{2}S release from arylthioamides was only shown for 15 minutes in the paper. It is unclear if H\textsubscript{2}S concentrations maintained elevated or dropped afterwards. 2) H\textsubscript{2}S 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\textsubscript{2}S (~ 1\%), the major form of the donors \textit{in vivo} should still be the donor molecules. Caution should be used in attributing their biological activities to H\textsubscript{2}S unless careful control experiments using both the active intermediates and final products after H\textsubscript{2}S release are conducted.

1.3.7 Photo-Induced H\textsubscript{2}S Donors

1.3.7.1 Gem-dithiol-based-H\textsubscript{2}S donors

In an effort to develop non-thiol dependent donors, geminal-dithiols (\textit{gem}-dithiols) were recently identified as a useful structure template.[141] Gem-dithiol compounds are unstable in aqueous solutions. H\textsubscript{2}S can be formed as a decomposition byproduct.[142-144] To make stable \textit{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 \textit{gem}-dithiol intermediates should be formed and subsequent hydrolysis of these intermediates would liberate H\textsubscript{2}S (Scheme 1.20).
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 thioniumbromide salt. Hydrolysis of this salt in the presence of sodium metabisulfite 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 1.21).

MB assay indicated that up to 36 µM of H₂S was generated after the irradiation of gem-dithiol-based donors (200 µM) at 365 nm. In comparison, no H₂S was detected without the exposure of donors under UV light. H₂S release of a selected donor was also detected in HeLa cells (under UV irradiation) by using a H₂S fluorescent probe.

It should be noted that H₂S generation from these donors depended on the hydrolysis of gem-dithiol in aqueous solution, which resulted in the difficulties to control the rate of H₂S release. Additionally, 2-nitrosobenzaldehyde, the byproduct of donor deprotection, may react
with free gem-dithiol, therefore diminishing H$_2$S generation. Considering these drawbacks, 2-nitrobenzyl group may not be suitable in developing photo-induced H$_2$S donors for biological applications. The desired protecting groups should be photoremoveable, and meanwhile not form any reactive byproducts.

### 1.3.7.2 Ketoprofenate-caged H$_2$S donors

Recently, Nakagawa’s group employed ketoprofenate [148] as a photocage to develop photolabile H$_2$S donors.[149] The synthesis of this donor was illustrated in Scheme 1.22a. 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$_2$S and the hydrolysis of this sulfide gave the desired donor. This ketoprofenate-caged donor can release H$_2$S by eliminating 2 equivalents of 2-propenylbenzophenone and CO$_2$ upon the irradiation at 300-350 nm (Scheme 1.22b). To evaluate H$_2$S releasing capability in a complex biological system, this ketoprofenate-caged donor was applied to fetal bovine serum. No H$_2$S was detected without irradiation. However, approximately 30 µM of H$_2$S was detected from 500 µM of the donor in serum after irradiating for 10 minutes.
So far two classes of photo-inducible H₂S 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 H₂S donors. Nevertheless these works proved the concept that both gem-dithiols and ketoprofenate-caged thioether are viable H₂S donor precursors and that non-thiol dependent activation strategies can be used in triggering H₂S generation. Based on these results, we expect to see more donors with new H₂S 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 H₂S donors may be developed and be useful.

### 1.3.8 Thioamino Acids

In 2012 Giannis et al. reported that thioamino acids, such as thioglycine and thiovaline, could be H₂S donors. They found that in the presence of bicarbonate both compounds were converted to the corresponding amino acid N-carboxyanhydrides with the generation of H₂S.

![Scheme 1.23](image)

**Scheme 1.23.** Proposed mechanism for H₂S 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 H₂S donors. By using a H₂S
electrode, H$_2$S releasing capabilities of thioglycine and thiovaline were evaluated and compared with other H$_2$S donors. Results showed that H$_2$S release from these two thioamino acids plateau after 1 hour. In comparison, H$_2$S release from NaHS and Na$_2$S was much faster and completed within 20 minutes. Up to ~ 50 µM of H$_2$S could be detected from 100 µM of thioglycine, while GYY4137 released much less H$_2$S.

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.

Although both thioglycine and thiovaline were proved to release H$_2$S, 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$_2$S donors.[137] All of these may lead to unwanted side-effects. Therefore, caution should be taken when using thioamino acids to study H$_2$S-related effects.

**1.4 CONCLUSION AND FUTURE DIRECTIONS**

In this chapter, we summarized the information on currently available H$_2$S donors, with a focus on fundamental chemistry and some biological applications (Table 1.1). Given the importance of H$_2$S in biomedical research, H$_2$S 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$_2$S 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$_2$S release from many
donors (i.e. sulfide salts, GYY4137, and DTTs) is not controllable and cannot mimic biological/endogenous H\textsubscript{2}S generation. This fast and uncontrollable H\textsubscript{2}S release can cause severe problems and sometimes even lethal. In addition, the byproducts associated with H\textsubscript{2}S 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 (\textit{N-SH-based} donors, \textit{S-SH-based} donors, dithioperoxyanhydrides, and arylthioamides) have been developed. These donors require the consumption of biological thiols to promote H\textsubscript{2}S generation. Considering biological levels of H\textsubscript{2}S, it is anticipated that the active dose 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 bound 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, \textit{N-SH-based} and \textit{S-SH-based} donors), whether the biological activities of these donors are H\textsubscript{2}S-dependent or sulfane sulfur-dependent needs to be further investigated. In conclusion, H\textsubscript{2}S is a potential therapeutic molecule. The development of useful donors is critical. In our opinion future efforts should be focused on developing controllable H\textsubscript{2}S donors and different H\textsubscript{2}S releasing mechanisms should be explored. We expect to see more work coming out in this exciting field.
Table 1.1. Summary of H₂S donors.

<table>
<thead>
<tr>
<th>H₂S donors</th>
<th>Structures</th>
<th>H₂S release mechanism</th>
<th>Representative bioactivities</th>
<th>Key ref.</th>
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<td>Hydrolysis</td>
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<td>Thiol activation</td>
<td>Vasodilation</td>
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<tr>
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<td>Anti-inflammation Ion channel regulation</td>
<td>97, 98</td>
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<td>GYY4137</td>
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<td>Vasodilation Anti-inflammation Anti-cancer</td>
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<td>Bicarbonate activation</td>
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CHAPTER TWO

CYSTEINE ACTIVATED HYDROGEN SULFIDE (H$_2$S) DONORS


2.1 INTRODUCTION

Hydrogen sulfide (H$_2$S) is a noxious gas with the characteristic smell of rotten eggs. Recent studies have recognized H$_2$S as the third gaseous transmitter, in addition to nitric oxide (NO) and carbon monoxide (CO), that influences various physiological processes.[1] H$_2$S has been shown to relax vascular smooth muscles, mediate neurotransmission, elicit hibernation, inhibit insulin signaling, and regulate inflammation and blood vessel caliber.[1] Endogenous formation of H$_2$S is achieved by enzymes such as cystathionine-β-synthase (CBS) in the brain and cystathionine-γ-lyase (CSE) in the liver and vascular and nonvascular smooth muscle. Although its exact chemical and biochemical modes of action are still not fully understood, levels of H$_2$S in the brain and vasculature have unambiguously been associated with human health and disease.[1]

To study the physiological and pathophysiological properties of H$_2$S, the direct use of H$_2$S gas or NaHS in aqueous solutions is typical. However, the therapeutic potential of H$_2$S gas seems to be limited because of difficulties in obtaining precisely controlled concentrations and the possible toxic impact of excess H$_2$S. NaHS, although widely used as a research tool, is a short-lived donor that does not mimic the slow and continuous process of H$_2$S generation in vivo. In addition, NaHS in aqueous solution can be rapidly oxidized by O$_2$. Modifications that are made between the time that a solution is prepared and the time that the biological effect is measured can dramatically affect the results. Because of these limitations, H$_2$S-releasing agents
(i.e., H₂S donors) are considered useful tools in the study of H₂S.[1,2] However, only a very limited number of H₂S donors are currently available.[1,2] Besides NaHS, only three types of H₂S donors have been reported (Scheme 2.1): (1) Garlic-derived polysulfide compounds, such as diallyl trisulfide (DATS). H₂S release from DATS has been suggested to mediate the vasoactivity of garlic.[3] (2) GYY4137, a derivative of Lawesson’s reagent, is a synthetic H₂S donor.[4] This molecule decomposes spontaneously in aqueous buffers to release H₂S. (3) A dithiolthione moiety as a H₂S donor has been used to prepare H₂S-nonsteroidal anti-inflammatory drug hybrids such as S-diclofenac.[5] In addition, biological thiols such as cysteine and glutathione can be H₂S donors upon enzymatic or thermal treatment.[6] A limitation of these known donors is that the H₂S release is too fast to mimic biological H₂S generation. In view of the structural characters of these compounds, little can be done to modify their structures to control the release of H₂S. Therefore, the development of new H₂S donors with controllable H₂S generation capability is critical for this field. From the therapeutic point of view and for applications in H₂S-related biological research, ideal H₂S donors should release H₂S slowly and in moderate amounts.[2] The donors should also be stable compounds that can be easily handled by researchers.

Scheme 2.1. Current H₂S donors.

In our group’s recent studies of S-nitrosothiols,[7] we noticed that S-N bonds are unstable and easy to break under certain conditions. Such a property triggered our idea to develop controllable H₂S donors based on S-N bonds. We envisioned that N-mercapto compounds such
as 1 could be potential H₂S donors (Scheme 2.2). As N-SH derivatives are unstable species, we expected that a protecting group on SH should enhance the stability. In addition, the protecting group could allow us to design different activation strategies to generate 1, thereby achieving controllable H₂S release.

![Scheme 2.2. N-Mercapto compounds as H₂S donors.](image)

**2.2 RESULTS AND DISCUSSION**

In our first-generation design of N-mercapto-based H₂S donors, we decided to use an acyl group as the protecting group. As shown in Scheme 2.3, we expected compounds such as 2 to react with cellular cysteine via native chemical ligation (NCL) to produce N-SH derivative 1, and subsequent cleavage of the S-N bond in 1 should produce H₂S.

![Scheme 2.3. Proposed cysteine activated H₂S donors.](image)

To test this idea, a series of N-(benzoylthio)benzamide derivatives 5a-1 were prepared from the corresponding thiobenzoic acids (Scheme 2.4). We expected that different substituents
would affect the reaction rate of compounds 5 with cysteine, thereby allowing the rate of \( \text{H}_2\text{S} \) generation to be regulated.

\[
\begin{array}{c}
\text{R}_1 \quad \text{O} \quad \text{SH} \\
1) \quad \text{NH}_2\text{OSO}_3\text{H}, \text{KOH}, \text{H}_2\text{O} \\
2) \quad \text{Bz}_2\text{O}, \text{DCM} \\
\text{R}_1 \quad \text{O} \quad \text{S} \quad \text{N} \quad \text{R}_2
\end{array}
\]

5a: \( \text{R}_1 = \text{H}, \text{R}_2 = \text{H} \), 5b: \( \text{R}_1 = \text{H}, \text{R}_2 = p-\text{F} \), 5c: \( \text{R}_1 = \text{H}, \text{R}_2 = p-\text{CF}_3 \),
5d: \( \text{R}_1 = \text{H}, \text{R}_2 = m-\text{Cl} \), 5e: \( \text{R}_1 = \text{H}, \text{R}_2 = p-\text{Cl} \), 5f: \( \text{R}_1 = \text{H}, \text{R}_2 = o-\text{Me} \),
5g: \( \text{R}_1 = \text{H}, \text{R}_2 = m-\text{Me} \), 5h: \( \text{R}_1 = \text{H}, \text{R}_2 = p-\text{Me} \), 5i: \( \text{R}_1 = \text{H}, \text{R}_2 = o-\text{OMe} \),
5j: \( \text{R}_1 = \text{H}, \text{R}_2 = m-\text{OMe} \), 5k: \( \text{R}_1 = \text{H}, \text{R}_2 = p-\text{OMe} \), 5l: \( \text{R}_1 = o-\text{OMe}, \text{R}_2 = p-\text{OMe} \),
5m: \( \text{R}_1 = \text{H}, \text{R}_2 = p-\text{N(CH}_3\text{)}_2 \)

**Scheme 2.4.** Synthesis of \( N \)-(benzoylthio)benzamides.

Compounds 5a-l proved to be stable in aqueous buffers. As shown in Figure 2.1, they do not react with potential cellular nucleophiles such as -OH and -NH\(_2\) groups. However, in the presence of cysteine, we observed time-dependent decomposition of the donors accompanied by \( \text{H}_2\text{S} \) release. The formation of \( \text{H}_2\text{S} \) was monitored by a 2mm \( \text{H}_2\text{S} \)-selective microelectrode (ISO-\( \text{H}_2\text{S}-2\); WPI) attached to an Apollo 1100 free-radical analyzer (WPI). A typical \( \text{H}_2\text{S} \) generation curve in pH 7.4 buffer is shown in Figure 2.1. In the presence of excess of cysteine, the concentration of \( \text{H}_2\text{S} \) released from 5areached a maximum value at 18 min (the “peaking time”), and then started to decrease, presumably as a result of oxidation by air. \( \text{H}_2\text{S} \) generation at other pH, including pH 5.5 and 9.0, was also measured. Similar release curves were observed.
Figure 2.1. H\textsubscript{2}S generation from 5a.

It is believed that the peaking time and the H\textsubscript{2}S concentration at the peaking time are useful parameters to assess the rate of H\textsubscript{2}S generation from donors. Therefore, the peaking times and corresponding H\textsubscript{2}S concentrations of 5a-l in a pH 7.4 phosphate buffered saline buffer were measured, and the results are summarized in Table 2.1. In general, electron-donating groups led to slower generation of H\textsubscript{2}S while electron-withdrawing groups led to faster generation. These results proved that controllable H\textsubscript{2}S release can be achieved by structural modifications of the donors.

Table 2.1. H\textsubscript{2}S generations from 5a-5l.

<table>
<thead>
<tr>
<th>Donors</th>
<th>Peaking t (min)</th>
<th>Peaking [H\textsubscript{2}S] (µM)</th>
<th>Donors</th>
<th>Peaking t (min)</th>
<th>Peaking [H\textsubscript{2}S] (µM)</th>
<th>Donors</th>
<th>Peaking t (min)</th>
<th>Peaking [H\textsubscript{2}S] (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5a</td>
<td>18</td>
<td>25.4</td>
<td>5e</td>
<td>25</td>
<td>26.1</td>
<td>5i</td>
<td>30</td>
<td>24.2</td>
</tr>
<tr>
<td>5b</td>
<td>16</td>
<td>35.2</td>
<td>5f</td>
<td>18</td>
<td>31.4</td>
<td>5j</td>
<td>25</td>
<td>21.5</td>
</tr>
<tr>
<td>5c</td>
<td>13</td>
<td>35.6</td>
<td>5g</td>
<td>22</td>
<td>31.0</td>
<td>5k</td>
<td>50</td>
<td>23.0</td>
</tr>
<tr>
<td>5d</td>
<td>14</td>
<td>30.7</td>
<td>5h</td>
<td>25</td>
<td>20.8</td>
<td>5l</td>
<td>22</td>
<td>17.5</td>
</tr>
</tbody>
</table>

It is known that plasma can contain significant amount of free cysteine.[8] Therefore H\textsubscript{2}S generation of 5 in plasma containing \(~\)500 µM cysteine was measured using a colorimetry method.[9] We observed a time-dependent H\textsubscript{2}S release similar to the one shown in Figure 2.1
Figure 2.2 shows the results obtained using 5a. However, when the plasma was first treated with N-methylmaleimide (NMM) to block free cysteine, no H\textsubscript{2}S generation was observed. These results demonstrate the capability of N-(benzoylthio)benzamide based donors to release H\textsubscript{2}S in complex biological systems and also show that cysteine is the regulator of this type of donor.

**Figure 2.2.** H\textsubscript{2}S generation from 5a in plasma.

Finally, to understand the mechanism of H\textsubscript{2}S generation from N-(benzoylthio)benzamides, the reaction between 5a and cysteine (10 equiv) was analyzed. As shown in Scheme 2.5, we confirmed the formation of N-acylcysteine (7), benzamide (9), and cystine (11) in high yields. On the basis of the products observed, the following mechanism was proposed: The reaction is initiated by reversible thiol exchange between 5a and cysteine to first generate the new thioester 6 and N-mercaptopbenzamide (8). Compound 6 then undergoes fast S-to-N acyl transfer to form amide 7. This process is similar to the well-known NCL reaction. Meanwhile, the reaction between 8 and excess cysteine should lead to 9 and cysteine perthiol (10). Finally, the reaction between 10 and cysteine should complete the generation of H\textsubscript{2}S and provide 11.
2.3 CONCLUSION

In summary, a series of new H$_2$S donors have been developed based on the N-(benzoylthio)benzamide template. These compounds are stable in aqueous buffers. H$_2$S generation from these compounds is regulated by cysteine. It has been proved that H$_2$S release rates from these compounds are controllable through structural modifications. It should be noted that the H$_2$S release rates shown in Table 2.1 can serve only as a reference for predicting the H$_2$S release capabilities of these donors. In complex biological systems, the perthiol intermediate (i.e., compound 10 in Scheme 2.5) may react with other redox-active biomolecules. Therefore, the actual H$_2$S release rates in such systems might be quite different. In addition, in some biological systems, free cysteine might be lacking because of disulfide formation or binding to proteins. When one of these donors is applied in such a system, extra cysteine must be added together with the donor in order to produce H$_2$S, which may compromise the redox balance of the system under study. Therefore, careful control experiments are needed in order to clarify the potential
problem. Nevertheless, N-(benzoylthio)benzamides provide researchers with new H₂S donor options, and they are expected to be useful tools in H₂S studies.

2.4 METHODS

All solvents were reagent grade. Tetrahydrofuran (THF) was freshly distilled from sodium/benzophenone under argon. Reactions were magnetically stirred and monitored by thin layer chromatography (TLC) with 0.25 mm pre-coated silica gel plates. Flash chromatography was performed with silica gel 60 (particle size 0.040-0.062 mm). Yields refer to chromatographically and spectroscopically pure compounds, unless otherwise stated. Proton and carbon-13 NMR spectra were recorded on a 300 MHz spectrometer. Chemical shifts are reported relative to chloroform (δ 7.26) for ¹H NMR and chloroform (δ 77.0) for ¹³C NMR.

2.4.1 Chemical Synthesis of N-(Benzoylthio)benzamides

![Scheme 2.6. Synthesis of donor 5a.]

To a stirred solution of KOH (560 mg, 10 mmol) in water (15 mL) was added thiobenzoic acid (690 mg, 5 mmol) and hydroxylamine-O-sulfonic acid (565 mg, 5 mmol). The solution was stirred for 20 min at rt. The white solid (S-benzoylthiohydroxylamine) was collected by filtration and then dissolved in CH₂Cl₂ (10 mL). To this mixture was added benzoic anhydride (2.26 g, 10 mmol). The resulting solution was allowed to stir overnight at rt. The crude product was purified by recrystallization (CH₂Cl₂/hexane) to give 5a as white solid (Scheme 2.6). m.p. 138-140 °C; ¹H NMR (300 MHz, CDCl₃) δ 7.92 (m, 4H), 7.60 (m, 1H), 7.53 (m, 1H), 7.39
(m, 4H), 7.17 (s, 1H); $^{13}$C NMR (75 MHz, CDCl$_3$) $\delta$ 190.0, 159.1, 134.6, 134.4, 133.3, 132.8, 129.3, 129.0, 128.0, 127.3; IR (thin film) cm$^{-1}$ 3265, 3062, 1696, 1659, 1451, 1419, 1257, 1207; HRMS m/z 258.0598 [M+H]$^+$; calcd for C$_{14}$H$_{12}$NO$_2$S 258.0589; overall yield: 64% (2 steps).

![5b](image)

**5b** was prepared from p-fluorothiobenzoic acid using the same procedure as **5a**. m.p. 143-145 °C; $^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 7.88 (m, 4H), 7.61 (s, 1H), 7.50 (tt, $J$ = 7.5 Hz, $J$ = 1.5 Hz 1H), 7.39 (t, $J$ = 7.8 Hz, 2H), 7.12 (t, $J$ = 8.7 Hz, 2H); $^{13}$C NMR (75 MHz, CDCl$_3$) $\delta$ 189.7, 168.9, 133.0, 132.8, 130.7, 130.0, 129.9, 128.9, 128.1, 116.7; IR (thin film) cm$^{-1}$ 3241, 1693, 1652, 1595, 1453, 1428,1208, 1100, 908, 851; HRMS m/z 276.0504 [M+H]$^+$; calcd for C$_{14}$H$_{11}$FNO$_2$S 276.0495; overall yield: 36 % (2 steps).

![5c](image)

**5c** was prepared from p-trifluoromethylthiobenzoic acid using the same procedure as **5a**. m.p. 163-164 °C; $^1$H NMR (300 MHz, CD$_3$OD) $\delta$ 8.02 (d, $J$ = 7.8 Hz, 2H), 7.92 (d, $J$ = 7.5 Hz, 2H), 7.76 (d, $J$ = 8.1 Hz, 2H), 7.58 (t, $J$ = 7.5 Hz, 1H), 7.48 (t, $J$ = 7.5 Hz, 2H), 7.20 (s, 1H); $^{13}$C NMR (75 MHz, CD$_3$OD) $\delta$ 190.1, 170.3, 137.7, 133.2, 132.6, 128.6, 128.0, 127.5, 126.2, 126.1; IR (thin film) cm$^{-1}$ 3199, 1705, 1649, 1456, 1407, 1326, 1170, 1125, 1067, 905, 846; HRMS m/z 326.0471 [M+H]$^+$; calcd for C$_{15}$H$_{11}$F$_3$NO$_2$S 326.0463; overall yield: 27 % (2 steps).

![5d](image)
5d was prepared from m-chlorothiobenzoic acid using the same procedure as 5a. m.p. 130-132 °C; $^1$H NMR (300 MHz, CDCl$_3$) δ 7.91 (m, 2H), 7.86 (t, $J = 2.0$ Hz, 1H), 7.77 (dt, $J = 7.8$ Hz, $J = 1.2$ Hz 1H), 7.57 (m, 2H), 7.45 (m, 3H), 7.33 (s, 1H); $^{13}$C NMR (75 MHz, CDCl$_3$) δ 190.0, 168.8, 135.8, 135.6, 134.5, 133.0, 132.9, 130.6, 129.0, 128.0, 127.3, 125.4; IR (thin film) cm$^{-1}$ 3278, 1702, 1660, 1452, 1421, 1260, 1198; HRMS m/z 292.0207 [M+H]$^+$; calcd for C$_{14}$H$_{11}$ClNO$_2$S 292.0199; overall yield: 42 % (2 steps).

5e was prepared from o-methylthiobenzoic acid using the same procedure as 5a. m.p. 101-103 °C; $^1$H NMR (300 MHz, CDCl$_3$) δ 7.93 (d, $J = 8.7$ Hz, 2H), 7.76 (d, $J = 8.1$ Hz, 1H), 7.69 (s, 1H), 7.49 (m, 1H), 7.40 (m, 3H), 7.25 (t, $J = 7.5$ Hz, 2H), 2.47 (s, 3H); $^{13}$C NMR (75 MHz, CDCl$_3$) δ 193.1, 168.8, 138.1, 133.8, 133.2, 133.0, 132.7, 132.2, 128.9, 128.4, 128.1, 126.3, 20.8; IR (thin film) cm$^{-1}$3252, 3071, 1687, 1453, 1424, 1326, 1292, 896, 708; HRMS m/z 272.0759 [M+H]$^+$; calcd for C$_{15}$H$_{14}$NO$_2$S 272.0745; overall yield: 68 % (2 steps).

5f was prepared from m-methylthiobenzoic acid using the same procedure as 5a. m.p. 79-81 °C; $^1$H NMR (300 MHz, CDCl$_3$) δ 8.11 (d, $J = 7.5$ Hz, 1H), 7.93 (d, $J = 7.5$ Hz, 2H), 7.73 (s, 2H), 7.50 (m, 4H), 7.06 (s, 1H), 2.43 (s, 3H); $^{13}$C NMR (75 MHz, CDCl$_3$) δ 191.5, 169.2, 139.1, 135.3, 134.4, 133.0, 132.6, 129.0, 128.8, 128.2, 127.7, 124.5, 21.5; IR (thin film) cm$^{-1}$ 3258, 2911, 1693, 1661, 1450, 1418, 1245; HRMS m/z 272.0741 [M+H]$^+$; calcd for C$_{15}$H$_{14}$NO$_2$S 272.0745; overall yield: 71 % (2 steps).
5g was prepared from p-methylthiobenzoic acid using the same procedure as 5a. m.p. 135-137 °C; $^1$H NMR (300 MHz, CDCl$_3$) δ 7.93 (m, 2H), 7.82 (d, $J = 8.4$ Hz, 2H), 7.57 (m, 1H), 7.48 (m, 2H), 7.29 (d, $J = 8.1$ Hz, 2H), 7.10 (s, 1H), 2.43 (s, 3H); $^{13}$C NMR (75 MHz, CDCl$_3$) δ 190.8, 168.9, 145.7, 133.2, 132.7, 131.8, 129.9, 128.9, 128.0, 127.4, 22.1; IR (thin film) cm$^{-1}$ 3264, 2923, 1695, 1659, 1601, 1420, 1209, 1177, 903; HRMS m/z 272.0736 [M+H]$^+$; calcd for C$_{15}$H$_{14}$NO$_2$S 272.0745; overall yield: 66 % (2 steps).

5h was prepared from o-methoxythiobenzoic acid using the same procedure as 5a. m.p. 123-124 °C; $^1$H NMR (300 MHz, CDCl$_3$) δ 7.5 (m, 3H), 7.53 (m, 2H), 7.44 (m, 3H), 7.04 (m, 2H), 3.98 (s, 3H); $^{13}$C NMR (75 MHz, CDCl$_3$) δ 189.5, 160.1, 135.5, 133.7, 133.1, 132.4, 130.2, 128.9, 127.9, 123.1, 121.4, 112.0, 56.1; IR (thin film) cm$^{-1}$ 3279, 1654, 1597, 1485, 1452, 1434, 1288, 1247, 1111, 1016, 905; HRMS m/z 288.0535 [M+H]$^+$; calcd for C$_{15}$H$_{14}$NO$_3$S 288.0694; overall yield: 63 % (2 steps).

5i was prepared from m-methoxythiobenzoic acid using the same procedure as 5a. m.p. 78-80 °C; $^1$H NMR (300 MHz, CDCl$_3$) δ 7.90 (m, 2H), 7.79 (s, 1H), 7.44 (m, 2H), 7.32 (m, 4H), 7.11 (m, 1H), 3.79 (s, 3H); $^{13}$C NMR (75 MHz, CDCl$_3$) δ 191.2, 169.0, 160.1, 135.6, 133.1, 132.4, 130.2, 128.9, 127.9, 123.1, 121.4, 112.0, 56.1; IR (thin film) cm$^{-1}$ 3279, 1654, 1597, 1485, 1452, 1434, 1288, 1247, 1111, 1016, 905; HRMS m/z 288.0535 [M+H]$^+$; calcd for C$_{15}$H$_{14}$NO$_3$S 288.0694; overall yield: 63 % (2 steps).
132.7, 130.3, 128.9, 128.1, 121.0, 119.7, 111.4, 55.7; IR (thin film) cm$^{-1}$ 3260, 1696, 1663, 1597, 1582, 1452, 1426, 1260, 786, 693; HRMS m/z 288.0512 [M+H]$^+$; calcd for C$_{15}$H$_{14}$NO$_3$S 288.0694; overall yield: 60 % (2 steps).

![Structure 5j]

$5j$ was prepared from p-methoxythiobenzoic acid using the same procedure as $5a$. m.p. 131-132 °C; $^1$H NMR (300 MHz, CDCl$_3$) δ 7.90 (m, 4H), 7.53 (m, 1H), 7.45 (m, 2H), 7.22 (s, 1H), 6.95 (m, 2H), 3.78 (s, 3H); $^{13}$C NMR (75 MHz, CDCl$_3$) δ 189.3, 164.7, 140.9, 133.4, 132.7, 129.6, 129.0, 128.0, 127.1, 114.5, 55.8; IR (thin film) cm$^{-1}$ 3264, 1657, 1601, 1508, 1452, 1419, 1262, 1169, 903; HRMS m/z 288.0710 [M+H]$^+$; calcd for C$_{15}$H$_{14}$NO$_3$S 288.0694; overall yield: 66 % (2 steps).

![Structure 5k]

$5k$ was prepared from 2,4-dimethoxythiobenzoic acid using the same procedure as $5a$. m.p. 138-139 °C; $^1$H NMR (300 MHz, CDCl$_3$) δ 7.92 (m, 3H), 7.47 (m, 4H), 6.57 (d, J = 2.4 Hz, 1H), 6.46 (d, J = 2.4 Hz, 1H), 3.99 (s, 3H), 3.86 (s, 3H); $^{13}$C NMR (75 MHz, CDCl$_3$) δ 188.0, 169.1, 165.9, 161.9, 133.9, 132.3, 132.2, 128.9, 127.9, 116.6, 106.7, 98.1, 56.0, 55.9; IR (thin film) cm$^{-1}$ 3241, 2942, 1648, 1601, 1453, 1421, 1252, 1217, 1126, 1021; HRMS m/z 318.0751 [M+H]$^+$; calcd for C$_{16}$H$_{16}$NO$_4$S 318.0800; overall yield: 56 % (2 steps).

![Structure 5l]
5l was prepared from p-N,N-dimethylthiobenzoic acid using the same procedure as 5a. m.p. 192-194 °C; NMR (300 MHz, DMSO-d$_6$) δ 9.87 (s, 1H), 7.96 (d, $J = 8.4$ Hz, 2H), 7.73 (d, $J = 9.0$ Hz, 2H), 7.61 (t, $J = 6.9$ Hz, 1H), 7.52 (t, $J = 7.8$ Hz, 2H), 6.77 (d, $J = 9.3$ Hz, 2H), 3.03 (s, 6H); $^{13}$C NMR (75 MHz, DMSO-d$_6$) δ 187.8, 168.8, 154.7, 134.0, 132.9, 129.2, 128.7, 121.2, 111.8, 40.3; IR (thin film) cm$^{-1}$ 3267, 1679, 1648, 1449, 1414, 1240, 889, 815; HRMS m/z 301.1009 [M+H]$^+$; calcd for C$_{16}$H$_{17}$N$_2$O$_2$S 301.1011; overall yield: 69 % (2 steps).

2.4.2 Amperometry Experiment for H$_2$S Release in PBS Buffer

2.4.2.1 Na$_2$S standard curve for H$_2$S release

5 mg EDTA was dissolved in 100 mL DI water in a 100-mL volumetric flask. The solution was purged vigorously with argon gas for 15 minutes. Then 48.0 mg sodium sulfide (Na$_2$S$\cdot$9H$_2$O) was dissolved in the solution under argon atmospheres. The solution was 2.0 mM Na$_2$S.

A small stirring bar was placed in a 50-mL round bottom flask containing 20 mL PBS buffer (pH = 7.4, 20 mM). The flask was placed on a magnetic stirring plate. The H$_2$S sensor was immersed into this solution and the background current (usually less than 60 nA) was allowed to stabilize for few minutes.

Six aliquots of Na$_2$S solution at 10 µL, 20 µL, 40 µL, 80 µL, 90 µL and 160 µL were injected into the flask. The current increased rapidly upon the addition of the first aliquot and reached a plateau within a few seconds. The second aliquot of 20 µL was injected as soon as the first signal reached plateau. In the same way, other aliquots were injected. This corresponded to 1 µM, 3 µM, 7 µM, 15 µM, 24 µM and 40 µM, respectively.

The recorded data were used to construct a linear calibration curve of concentration vs. current.
2.4.2.2 H₂S release from 5a in the presence of cysteine in buffers

Cysteine stock solution in PBS buffer (400 mM, 200 µL) and N-(benzoylthio)benzamide 5a stock solution in THF (80 mM, 10 µL) were added into PBS buffer solution (20 mL, pH = 7.4), respectively. The current was recorded in every 2 minutes. The concentration of H₂S generated from 5a was then calculated by the Na₂S calibration curve. The H₂S releasing curve was obtained by plotting H₂S concentration versus time.

H₂S releasing curves of 5a were also measured in pH 5.5 and pH 9.0. Results were shown below (Figure 2.3).

The H₂S generation from 5b-5l was measured in pH 7.4 buffer using the same procedure for 5a.

![Figure 2.3. H₂S release from 5a at different pHs.](image)

2.4.2.3 Cysteine effects on H₂S release

H₂S releasing curves of 5a in the presence of different concentrations of cysteine (3 eq, 5 eq, 10 eq, 50 eq, and 100 eq) were measured using the procedure described above. The plots were
summarized in Figure 2.4. In all cases, H$_2$S generation was observed. 10 eq of cysteine can lead to effective H$_2$S generation (i.e. high H$_2$S concentration at peaking time).

![Figure 2.4. Cysteine effects on H$_2$S release.](image)

### 2.4.3 H$_2$S Release in Plasma

To 2.0 mL of bovine/calf plasma (with the addition of free cysteine ~2 µmol) was added 2 mL water solution containing 5a (25 µL of 80 mM in DMSO). The solution was stirred at room temperature. Reaction aliquots (1 mL) were collected into 2-mL vials containing zinc acetate (1% w/v, 100 µL) and trichloroacetic acid (10% w/v, 600 µL) in every 5 minutes. The resulting mixture was centrifuged for 10 minutes (5000 g) and the precipitate was filtered. The clear solution (1.2 mL) was transferred to another 2-mL vial. Subsequently, N, N-dimethyl-p-phenylenediamine sulfate (20 mM, 150 µL) in 7.2 M HCl was added followed by FeCl$_3$ (30 mM, 150 µL) in 1.2 M HCl, and absorbance (670 nm) of aliquots of the resulting solution (1.5 mL) was determined 20 minutes thereafter using a UV-Vis spectrometer. The absorbance was converted to H$_2$S concentration of each sample by a calibration curve.
To study the effect of \( N \)-methylmaleimide (NMM) in this reaction, the plasma sample was pretreated with NMM stock solution (200 mM in DMSO, 20 \( \mu \)L) for 10 hours. Then the same procedure described above was used to test \( \text{H}_2\text{S} \) generation.

### 2.4.4 Product Analysis

Compound 5a (123 mg 0.5 mmol) in 2 mL of MeOH was added to a stirred solution of cysteine (605 mg, 5 mmol) in 50 mL of PBS buffer (20 mM). After 1 hour, 0.1 mL of solution was taken via syringe equipped with a 45 \( \mu \)M syringe filter, diluted with 0.4 mL of MeOH and analyzed by HPLC (Thermo Scientific Surveyor, Hypersil gold column, 4.6 \( \times \) 100 mm, water/CH\(_3\)CN 80/20 2 min; 60/40 4 min; 20/80 2 min; 80/20 2 min, flow rate 1 mL/min). Compounds 7, 9 and 11 were identified and quantified in comparison to standard compounds. These compounds were also isolated from the reaction mixture and characterized.

\[
\text{HS} \quad \overset{\text{CO}_{2}\text{H}}{\text{NHBz}}
\]

\( ^1\text{H} \) NMR (300 MHz, CDCl\(_3\)) \( \delta \) 11.53 (s, 1H), 7.83-7.79 (m, 2H), 7.54-7.47 (m, 1H), 7.45-7.37 (m, 3H), 5.03 (dt, \( J = 7.4, 4.2 \) Hz, 1H), 3.20-3.04 (m, 2H), 1.49 (t, \( J = 9.4 \)Hz, 1H); \( ^{13}\text{C} \) NMR (75 MHz, CDCl\(_3\)) \( \delta \) 173.0, 168.8, 133.1, 132.7, 129.1, 127.6, 54.4, 26.7; IR (thin film) cm\(^{-1}\) 3650-2350 (br), 1731, 1633, 1538, 1487, 1337, 1214; HRMS m/z 248.0363 [M+Na]\(^+\); calcd for C\(_{10}\)H\(_{11}\)NO\(_3\)SNa: 248.0357.

\[
\text{O} \quad \overset{\text{NH}_2}{\text{C}}
\]
$^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 7.85-7.78 (m, 2H), 7.57-7.50 (m, 1H), 7.48-7.40 (m, 2H), 6.25 (br, 2H); $^{13}$C NMR (75 MHz, CDCl$_3$) $\delta$ 170.0, 133.6, 132.2, 128.8, 127.6; IR (thin film) cm$^{-1}$ 3366, 3173, 1649, 1624, 1577, 1405; HRMS m/z 144.0434 [M+Na]$^+$; calcd for C$_7$H$_7$NONa: 144.0425.

\[
\begin{align*}
\text{HO} & \quad \text{NH}_2 \\
\text{S} & \quad \text{S} \\
\text{O} & \quad \text{O} \\
\text{HO} & \quad \text{NH}_2
\end{align*}
\]

11

Compound 11 was isolated as HCl salt. $^1$H NMR (300 MHz, D$_2$O) $\delta$ 4.29 (dd, $J = 7.9$, 4.4 Hz, 2H), 3.25 (dd, $J = 15.2$, 4.3 Hz, 2H) 3.25 (dd, $J = 15.2$, 7.9 Hz, 2H); $^{13}$C NMR (75 MHz, CDCl$_3$) $\delta$ 170.4, 51.6, 36.1; IR (KBr) cm$^{-1}$ 3306-2198 (br), 1618, 1586, 1484, 1407, 1380, 1339, 1298, 1192, 1124, 1037, 959, 844, 776, 675, 616, 538; MS 241.1 [M+H].
2.5 REFERENCES


CHAPTER THREE

CONTROLLABLE HYDROGEN SULFIDE DONORS AND THEIR ACTIVITY AGAINST MYOCARDIAL ISCHEMIA-REPERFUSION INJURY


3.1 INTRODUCTION

Hydrogen sulfide (H$_2$S) has been recognized as an important cellular signaling molecule, much like nitric oxide (NO).[1–6] The endogenous formation of H$_2$S is attributed to enzymes including cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE), and 3-mercaptoppyruvate sulfurtransferase (MPST).[7–10] These enzymes convert cysteine or cysteine derivatives to H$_2$S in different tissues and organs. Recent studies have suggested that the production of endogenous H$_2$S and the exogenous administration of H$_2$S can exert protective effects in many pathologies.[1,2] For example, H$_2$S has been shown to relax vascular smooth muscle, induce vasodilation of isolated blood vessels, and reduce blood pressure. H$_2$S can also inhibit leukocyte adherence in mesenteric microcirculation during vascular inflammation in rats, suggesting H$_2$S is a potent anti-inflammatory molecule. In addition, H$_2$S may interreact 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$_2$S levels could have potential therapeutic values.

To explore the biological functions of H$_2$S, researchers started to use H$_2$S releasing compounds (also known as H$_2$S donors) to mimic endogenous H$_2$S 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 H₂S donors are still very limited. These donors include the following: (1) sulfide salts, such as Na₂S, NaHS, and CaS, have been widely used in the field. These inorganic donors have the advantage of rapidly enhancing H₂S concentration. The maximum concentration of H₂S released from these salts can be reached within seconds. However such a fast generation may cause acute changes in blood pressure. In addition, since H₂S 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 H₂S donors in some studies. DATS can vasodilate rat aortas [17] and protect rat ischemic myocardium [18] via a H₂S related manner, but the simplicity of the structure limits its application as H₂S donors. (3) Synthetic H₂S donors have recently emerged as useful tools. GYY4137,[19] which is a Lawesson’s reagent derivative, releases H₂S via hydrolysis both in vitro and in vivo and exhibits some interesting biological activities [20, 21] such as anti-inflammation.[22–24] H₂S release from GYY4137 is relatively low (< 10% of H₂S was released from this molecule after 7 days).[25] Dithiolthione is another structure that releases H₂S in aqueous solution.[13] However the detailed mechanism is still unclear. A major limitation of current donors is that H₂S 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 H₂S donors should be stable by themselves in aqueous solutions. The release of H₂S (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$_2$S researchers but also have unique therapeutic benefits themselves.

The research in our laboratory focuses on the development of controllable H$_2$S donors. In 2011 we discovered a series of N-(benzoylthio)benzamide derivatives as thiol-activated H$_2$S 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$_2$S (Scheme 3.1a).[26] In this process, 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$_2$S biosynthesis catalyzed by CSE (Scheme 3.1b). [13,27] These findings suggest that the perthiol (Scheme 3.1c) can be a useful template for the design of controllable H$_2$S donors. Herein we report the development of perthiol-based donors and their activities in myocardial ischemia-reperfusion (MI/R) injury.

**Scheme 3.1.** (a) H$_2$S release from N-(benzoylthio)benzamide derivatives; (b) H$_2$S biosynthesis catalyzed by cystathionineγ-lyase (CSE); (c) Concept of perthiol-based H$_2$S donors.
3.2 RESULTS AND DISCUSSION

3.2.1 Primary Perthiol-Based H$_2$S Donors

Perthiols are known to be unstable species.\cite{28-30} It is 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$_2$S release. With this idea in mind, cysteine-based perthiol derivatives were tested. Acyl groups were used as the protecting group on perthiol moieties.

Table 3.1 H$_2$S generation from cysteine-based H$_2$S donors.

<table>
<thead>
<tr>
<th>Donors</th>
<th>R</th>
<th>Cys Peaking time (min)</th>
<th>Cys Peaking $[H_2S]$ (μM)</th>
<th>GSH Peaking time (min)</th>
<th>GSH $[H_2S]$ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2a</td>
<td></td>
<td>35</td>
<td>11.2</td>
<td>45</td>
<td>7.9</td>
</tr>
<tr>
<td>2b</td>
<td></td>
<td>48</td>
<td>13.5</td>
<td>52</td>
<td>8.0</td>
</tr>
<tr>
<td>2c</td>
<td></td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>2d</td>
<td></td>
<td>24</td>
<td>11.5</td>
<td>28</td>
<td>7.7</td>
</tr>
</tbody>
</table>

Using the procedure reported previously,\cite{26} we analyzed H$_2$S release capabilities of these donors in the presence of cysteine and GSH. As shown in Table 3.1, these donors indeed could generate H$_2$S in the presence of thiols. Compared to $N$-(benzoylthio)benzamide type
donors, however, these donors showed much decreased ability of \( \text{H}_2\text{S} \) generation (read from their peaking concentrations). The initial concentration of the donors was 150 \( \mu \text{M} \), while the maximum \( \text{H}_2\text{S} \) 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 3.2), which in turn should lead to \( \text{H}_2\text{S} \) formation. However, it was also possible that SH reacted with the acyldisulfide linkage to form a new disulfide 5 and thioacid 6 (pathway B, Scheme 3.2). We found that thioacids could not release \( \text{H}_2\text{S} \) even in the presence of cysteine or GSH under the conditions used in our experiments.[31] Therefore, \( \text{H}_2\text{S} \) release from donors 2 was diminished due to the involvement of pathway B.

\[ \text{R}_2\text{O} - \text{S}^\text{S} - \text{R} \quad \text{NHR}_1 \quad \text{O} \quad \text{NH}_3^+ \quad \text{HS} \quad \text{O} \quad \text{R}_2\text{O} - \text{NHR}_1 \quad \text{O} - \text{NH}_3^+ \quad \text{HS} \quad \text{O} \quad \text{R} \]

\[ \text{pathway A} \quad \text{pathway B} \]

\[ \text{R}_2\text{O} - \text{S}^\text{S} - \text{R} \quad \text{NHR}_1 \quad \text{O} \quad \text{NH}_3^+ \quad \text{HS} \quad \text{O} \quad \text{R}_2\text{O} - \text{NHR}_1 \quad \text{O} - \text{NH}_3^+ \quad \text{HS} \quad \text{O} \quad \text{R} \]

\[ \text{pathway A} \quad \text{pathway B} \]

\[ \text{4} \quad \text{H}_2\text{S} \]

\[ \text{5} \quad \text{6} \]

**Scheme 3.2.** Proposed reactions of 2.

### 3.2.2 Tertiary Perthiol-Based \( \text{H}_2\text{S} \) Donors

We envisioned that the steric hindrance on the \( \alpha \)-carbon of the disulfide bridge should prevent the reactions through pathway B and therefore enhance \( \text{H}_2\text{S} \) formation. As such a series of penicillamine-based perthiol derivatives 8 were prepared (Figure 3.1). These donor
compounds proved to be stable in aqueous solutions. In the presence of cysteine or GSH, a time-dependent \( \text{H}_2\text{S} \) generation was observed. The representative \( \text{H}_2\text{S} \) release curves of 8a are shown in Figure 3.1. With an initial concentration at 100 \( \mu \text{M} \), the maximum \( \text{H}_2\text{S} \) concentration formed was \(~80 \mu \text{M} \) (with cysteine), which demonstrated the efficiency of this compound as \( \text{H}_2\text{S} \) donor. After reaching the maximum value, \( \text{H}_2\text{S} \) concentration started to drop probably due to volatilization.[15]

![Diagram of the reaction](image)

**Figure 3.1.** \( \text{H}_2\text{S} \) release curve of 8a.

It is expected that the change of acyl substitutions could affect the rate of thioester exchange and regulate \( \text{H}_2\text{S} \) generation. Therefore a series of acyl substitution modified donors (19 compounds in total) were prepared and tested (Table 3.2). Generally, electron-withdrawing
groups on the phenyl group led to faster H\textsubscript{2}S generation, while electron-donating groups led to slower H\textsubscript{2}S release. Significant steric effects on H\textsubscript{2}S formation was also observed. More sterically hindered substrates (compounds 8o, 8r, 8s) resulted in slower H\textsubscript{2}S release (with decreased H\textsubscript{2}S amounts) or even no release at all. In addition, it is found that cysteine always caused higher/faster H\textsubscript{2}S 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\textsubscript{2}S release from these perthiol-based donors could be regulated via structural modifications.

Table 3.2. H\textsubscript{2}S generation from penicillamine-based H\textsubscript{2}S donors.

<table>
<thead>
<tr>
<th>R</th>
<th>Peaking time (min)</th>
<th>Peaking time [H\textsubscript{2}S] (\mu M)</th>
<th>R</th>
<th>Peaking time (min)</th>
<th>Peaking time [H\textsubscript{2}S] (\mu M)</th>
<th>R</th>
<th>Peaking time (min)</th>
<th>Peaking time [H\textsubscript{2}S] (\mu M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8a</td>
<td>39 (38)</td>
<td>80.3 (51.9)</td>
<td>8g</td>
<td>36 (48)</td>
<td>68.0 (53.8)</td>
<td>8m</td>
<td>45 (42)</td>
<td>58.2 (30.5)</td>
</tr>
<tr>
<td>8b</td>
<td>34 (36)</td>
<td>48.3 (37.1)</td>
<td>8h</td>
<td>45 (55)</td>
<td>63.6 (49.6)</td>
<td>8n</td>
<td>48 (51)</td>
<td>50.8 (26.1)</td>
</tr>
<tr>
<td>8c</td>
<td>33 (38)</td>
<td>63.1 (44.2)</td>
<td>8i</td>
<td>40 (49)</td>
<td>61.6 (54.3)</td>
<td>8o</td>
<td>60 (70)</td>
<td>58.4 (14.4)</td>
</tr>
<tr>
<td>8d</td>
<td>33 (38)</td>
<td>52.9 (35.5)</td>
<td>8j</td>
<td>50 (45)</td>
<td>63.0 (44.9)</td>
<td>8p</td>
<td>42 (42)</td>
<td>70.0 (56.8)</td>
</tr>
<tr>
<td>8e</td>
<td>48 (52)</td>
<td>59.7 (54.7)</td>
<td>8k</td>
<td>55 (50)</td>
<td>55.7 (35.4)</td>
<td>8q</td>
<td>48 (60)</td>
<td>58.4 (47.1)</td>
</tr>
<tr>
<td>8f</td>
<td>48 (48)</td>
<td>64.3 (49.0)</td>
<td>8l</td>
<td>39 (42)</td>
<td>70.6 (61.3)</td>
<td>8r</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

3.2.3 H\textsubscript{2}S Release Mechanism Study
To understand the mechanism of H$_2$S release from these donors, the reaction between 8a and a cysteine derivative 9 (3 equiv) was studied. As shown in Scheme 3.3, 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$_2$S, 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$_2$S. In this process it is also possible that 13 reacts with 14 to form disulfide 12 and release H$_2$S.

Scheme 3.3. Proposed mechanism for H$_2$S release from penicillamine-based donors.

3.2.4 Biological Evaluation of Perthiol-Based H$_2$S Donors

With these donors in hand, their therapeutic benefits were explored. Recent studies with animal models suggested that H$_2$S can protect cardiovascular system against myocardial...
ischemiareperfusion (MI/R) injury.[8,17,33–35] Several groups have shown that H2S, 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] It is hypothesized 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, cytotoxicity of two representative donors (8a/8l) was tested in H9c2 cardiac myocytes. The cell viability was detected using cell counter kit (CCK)-8 assay (Figure 3.2). 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 tested.

**Figure 3.2.** 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.**
To prove perthiol-based donors indeed could release H$_2$S when interacting with myocytes, experiments were conducted by incubating H9c2 cells with donors (8a and 8l), respectively. As shown in Figure 3.3, after incubating cells with donors for 30 min, a selective H$_2$S fluorescent probe, WSP-1,[39] was applied into the cells to monitor the production of H$_2$S. As expected, donor-treated cells (Figure 3.3b and 3.3c) showed much enhanced fluorescent signals compared to vehicle-treated cells (Figure 3.3a). In addition, we did not observe shape changes of the cells after the treatment. These results demonstrated that perthiol-based donors can release H$_2$S when interacting with H9c2 cells and H$_2$S generation can be evaluated by fluorescent image.

**Figure 3.3.** H$_2$S 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$_2$S fluorescent probe (WSP-1) was added. Images were taken after 30 min.

Finally myocardial protective effects of donors 8a and 8l against myocardial ischemia/reperfusion (MI/R) injury were tested 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 3.4B and 3.4C). 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 H₂S-mediated cardiac protection in MI/R injury and these compounds may have potential therapeutic benefits.

**Figure 3.4.** Cardioprotective effects of compounds 8a and 8l in myocardial ischemia-reperfusion injury. Compound 8a, 8l, or vehicle was 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.

We also tested in vivo H₂S 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$_2$S levels were determined using previously described gas chromatography and chemiluminescence methods.[40] As shown in Figure 3.5, blood and myocardial levels of H$_2$S were significantly (p < 0.01) increased following injection of the donors as compared to controls.

![Figure 3.5](image)

**Figure 3.5.** In vivo H$_2$S levels (µM) in blood (A) and hearts (B) obtained from mice treated with 8a and 8l.

### 3.3 CONCLUSION

In summary, a series of new H$_2$S donors have been developed based on the perthiol template. Their H$_2$S generation is regulated by thiols such as cysteine or GSH. It is demonstrated that H$_2$S release capability from these donors can be manipulated by structural modifications. Moreover, these donors are nontoxic to cardiac cells and their H$_2$S production upon interacting
with myocytes can be detected. Some donors exhibited potent myocardial protective effects in MI/R injury, presumably due to H$_2$S generation. It should be noted that H$_2$S generation from these donors is not dependent on specific enzymes such as CBS and CSE. Recently the effects of some H$_2$S donors on CBS/CSE activity were tested in Dr. Lefer’s laboratory and no changes were observed even after weeks.[41] Taken together these donors may be potential therapeutic agents. In the in vivo experiments, 8l exhibited better cardioprotective effects compared to those of 8a (Figure 3.3). Interestingly, 8l also exhibited better activity in cell viability test and H$_2$S generation test (Figures 3.1 and 3.2). These data suggest that in vitro evaluation of donors may allow us to predicate donors’ in vivo behaviors.

3.4 METHODS

3.4.1 Chemical Synthesis

3.4.1.1 Synthesis of compounds 2a-2d (Scheme 3.4)

![Scheme 3.4. General synthesis of cysteine-based H$_2$S donors.]

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). m.p. 103-105 °C; $^1$H NMR (300 MHz, CDCl$_3$) δ 8.33 (d, J = 5.1 Hz, 1H), 8.15 (d, J = 7.5 Hz, 1H), 7.84 (d, J = 7.2 Hz, 2H), 7.49 (m, 5H), 7.06 (m, 1H), 5.06 (m, 1H), 3.69 (s, 3H), 3.53 (dd, J = 14.4, 5.4 Hz, 1H),
3.39 (dd, J = 14.4, 4.5 Hz, 1H); $^{13}$C NMR (75 MHz, CDCl$_3$) δ 170.9, 167.6, 159.0, 150.0, 137.3, 134.2, 132.0, 128.8, 127.5, 121.5, 121.1, 52.9, 52.2, 41.1; IR (thin film) cm$^{-1}$ 3334, 3054, 2974, 2921, 1746, 1641, 1573, 1519, 1451, 1216 1123; mass spectrum (ESI/MS) m/z 371.0 [M+Na]$^+$; calcd for C$_{16}$H$_{16}$N$_2$NaO$_3$S$_2$ 371.1; yield: 85%

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, 4.8Hz, 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$_4$S$_2$ 398.0497; yield 81%.

![2b](attachment:image)

2b was prepared from p-methoxythiobenzoic acid using the same procedure as 2a. m.p. 124-125 °C; $^1$H NMR (300 MHz, CDCl$_3$) δ 7.98 (d, J = 7.2 Hz, 2H), 7.90 (d, J = 8.7 Hz, 3H), 7.47 (m, 3H), 6.92 (d, J = 9.0 Hz, 2H), 5.05 (m, 1H), 3.86 (s, 3H), 3.69 (s, 3H), 3.58 (dd, J = 14.4, 4.8 Hz, 1H), 3.26 (dd, J = 14.4, 4.8 Hz, 1H); $^{13}$C NMR (75 MHz, CDCl$_3$) δ 189.5, 170.9, 167.3, 164.9, 133.8, 132.0, 130.4, 128.8, 128.0, 127.7, 114.4, 55.9, 53.0, 51.8, 41.1; IR (thin film)
cm⁻¹ 3346, 2950, 2836, 1741, 1693, 1636, 1523, 1487, 1321, 1262, 1215, 1165, 1026, 890; mass spectrum (ESI/MS) m/z 428.1 [M+Na]⁺; HRMS m/z 406.0777 [M+H]⁺; calcd for C₁₉H₂₀NO₅S 2406.0783; yield: 70%.

![2c](image)

2c was prepared from p-fluorothiobenzoic acid using the same procedure as 2a. m.p. 133-134 °C; ¹H NMR (300 MHz, CDCl₃) δ 7.93 (m, 4H), 7.74 (d, J = 7.5 Hz, 1H), 7.51 (t, J = 7.5 Hz, 1H), 7.43 (t, J = 7.8 Hz, 2H), 7.13 (t, J = 8.7 Hz, 2H), 5.06 (m, 1H), 3.72 (s, 3H), 3.56 (dd, J = 14.4, 4.8 Hz, 1H), 3.31 (dd, J = 14.4, 4.8 Hz, 1H); ¹³C NMR (75 MHz, CDCl₃) δ 189.8, 170.8, 167.3, 133.7, 132.1, 130.8, 130.6, 128.8, 127.6, 116.6, 116.3, 53.1, 51.9, 41.0; IR (thin film) cm⁻¹ 3342, 3066, 2953, 1747, 1671, 1641, 1596, 1523, 1500, 1486, 1319, 1201, 1162, 894; mass spectrum (ESI/MS) m/z 416.1 [M+Na]⁺; HRMS m/z 416.0386 [M+Na]⁺; calcd for C₁₈H₁₆FNNaO₄S₂ 416.0402; yield: 65%.

![2d](image)

2d was prepared from thioacetic acid using the same procedure as 2a. m.p. 68-70 °C; ¹H NMR (300 MHz, CDCl₃) δ 7.95 (d, J = 8.4 Hz, 2H), 7.51 (m, 4H), 5.03 (m, 1H), 3.79 (s, 3H), 3.48 (dd, J = 14.4, 4.5 Hz, 1H), 3.25 (dd, J = 14.4, 4.8 Hz, 1H), 2.36 (s, 3H); ¹³C NMR (75 MHz, CDCl₃) δ 195.5, 170.8, 167.2, 133.6, 132.2, 128.9, 127.6, 53.0, 51.7, 40.7, 29.1; IR (thin film) cm⁻¹ 3301, 3068, 2958, 2929, 1740, 1642, 1528, 1489, 1285, 1249, 1110, 1010, 889; mass spectrum (ESI/MS) m/z 336.1 [M+Na]⁺; HRMS m/z 314.0501 [M+H]⁺; calcd for C₁₃H₁₆NO₄S₂ 314.0521; yield: 60%.
3.4.1.2 Synthesis of compounds 8a-8s (Scheme 3.5)

Scheme 3.5. General synthesis of penicillamine-based H₂S donors.

2, 2’-Dibenzothiazolyl disulfide (4.32g, 13 mmol) was dissolved into 500 mL of CHCl₃. 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. m.p. 167-168 °C; ¹H NMR (300 MHz, CDCl₃) δ 8.04 (br, 1H), 7.78 (t, J = 9.0 Hz, 2H), 7.37 (m, 2H), 6.90 (d, J = 9.0 Hz, 1H), 4.87 (d, J = 9.0 Hz, 1H), 3.31 (m, 2H), 2.02 (s, 3H), 1.52 (m, 5H), 1.36 (m, 5H), 0.89 (t, J = 7.2 Hz, 3H); ¹³C NMR (75 MHz, CDCl₃) δ 170.4, 168.9, 153.9, 136.1, 126.6, 125.1, 121.9, 121.5, 58.7, 55.5, 39.7, 31.6, 31.2, 25.2, 23.6, 20.5, 14.0; IR (thin film) cm⁻¹ 3273, 3080, 2951, 2864, 1683, 1634, 1564, 1455, 1424, 1380, 1363, 1002, 749, 721; mass spectrum (ESI/MS) m/z 434.0 [M+Na]⁺; calcd for C₁₈H₂₅N₃NaO₂S₄ 434.1; yield: 95%.

To a 15 mL CHCl₃ 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 NaHCO₃. 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; ¹H NMR (300 MHz, CDCl₃) 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); ¹³C NMR (75 MHz, CDCl₃) 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\(_2\)O\(_3\)S\(_2\) 383.1463; yield 94%.

8b was prepared from p-(trifluoromethy)thiobenzoic acid using the same procedure as 8a. m.p. 90-92 °C; \(^{1}\)H NMR (300 MHz, CDCl\(_3\)) \(\delta\) 8.13 (d, \(J = 8.1\) Hz, 2H), 8.03 (s, 1H), 7.76 (t, \(J = 7.8\) Hz, 2H), 7.05 (d, \(J = 7.8\) Hz, 1H), 4.49 (d, \(J = 8.1\) Hz, 1H), 3.35 (m, 2H), 2.00 (s, 3H), 1.59 (m, 2H), 1.43 (m, 5H), 1.27 (s, 3H), 0.94 (t, \(J = 6.9\) Hz, 3H); \(^{13}\)C NMR (75 MHz, CDCl\(_3\)) \(\delta\) 193.5, 170.5, 168.7, 138.3, 136.1, 128.6, 126.3, 121.7, 58.6, 54.3, 39.8, 31.6, 26.7, 24.1, 23.5, 20.4, 14.0; IR (thin film) cm\(^{-1}\) 3281, 3085, 2958, 2933, 2868, 1695, 1638, 1541, 1408, 1323, 1172, 1130, 1065, 891, 848, 773; mass spectrum (ESI/MS) m/z 473.1 [M+Na]\(^+\); HRMS m/z 473.1138 [M+Na]\(^+\); calcd for C\(_{19}\)H\(_{25}\)F\(_3\)N\(_2\)NaO\(_3\)S\(_2\) 473.1156; yield: 89%.

8c was prepared from p-fluorothiobenzoic acid using the same procedure as 8a. m.p. 76-78 °C; \(^{1}\)H NMR (300 MHz, CDCl\(_3\)) \(\delta\) 8.16 (br, 1H), 8.05 (m, 2H), 7.14 (m, 3H), 4.50 (d, \(J = 8.7\) Hz, 1H), 3.36 (m, 2H), 1.98 (s, 3H), 1.58 (m, 2H), 1.40 (m, 5H), 1.25 (s, 3H), 0.92 (t, \(J = 7.5\) Hz,
$^{13}$C NMR (75 MHz, CDCl$_3$) $\delta$ 192.3, 170.4, 168.8, 165.1, 131.8, 131.0, 116.6, 58.5, 54.0, 39.7, 31.6, 26.6, 24.1, 23.4, 20.4, 14.0; IR (thin film) cm$^{-1}$ 3285, 3081, 2962, 2925, 2864, 1683, 1642, 1597, 1505, 1369, 1236, 1197, 1154, 892, 844; mass spectrum (ESI/MS) m/z 423.1 [M+Na]$^+$; HRMS m/z 423.1189 [M+Na]$^+$; calcd for C$_{18}$H$_{25}$FN$_2$NaO$_3$S$_2$ 423.1188; yield: 92%.

8d was prepared from m-chlorothiobenzoic acid using the same procedure as 8a. m.p. 155-157 °C; $^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 8.04 (br, 1H), 7.99 (m, 1H), 7.94 (d, $J = 7.8$ Hz, 1H), 7.62 (d, $J = 6.9$ Hz, 1H), 7.45 (t, $J = 7.8$ Hz, 1H), 7.00 (d, $J = 4.2$ Hz, 1H), 4.43 (d, $J = 8.4$ Hz, 1H), 3.36 (m, 2H), 2.02 (s, 3H), 1.61 (m, 2H), 1.45 (m, 5H), 1.26 (s, 3H), 0.95 (t, $J = 7.5$ Hz, 3H); $^{13}$C NMR (75 MHz, CDCl$_3$) $\delta$ 193.2, 170.5, 168.7, 136.9, 135.6, 134.7, 130.5, 128.1, 126.4, 58.7, 54.1, 39.8, 31.6, 27.0, 24.0, 23.5, 20.4, 14.0; IR (thin film) cm$^{-1}$ 3289, 3089, 2958, 2929, 2872, 1697, 1639, 1532, 1369, 1188, 942, 722, 688; mass spectrum (ESI/MS) m/z 439.0 [M+Na]$^+$; HRMS m/z 439.0896 [M+Na]$^+$; calcd for C$_{18}$H$_{25}$ClN$_2$NaO$_3$S$_2$ 439.0893; yield: 89%.

8e was prepared from o-methylthiobenzoic acid using the same procedure as 8a. m.p. 142-144 °C; $^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 8.21 (br, 1H), 7.87 (d, $J = 7.8$ Hz, 1H), 7.42 (t, $J =$
7.5 Hz, 1H), 7.29 (m, 2H), 7.13 (d, J = 8.4 Hz, 1H), 4.53 (d, J = 8.4 Hz, 1H), 3.33 (m, 2H), 2.48 (s, 3H), 2.01 (s, 3H), 1.59 (m, 2H), 1.43 (m, 5H), 1.26 (s, 3H), 0.93 (t, J = 7.5 Hz, 3H); $^{13}$C NMR (75 MHz, CDCl$_3$) δ 196.2, 170.4, 168.8, 137.5, 135.7, 133.0, 132.0, 129.2, 126.3, 58.5, 53.9, 39.7, 31.5, 26.8, 24.0, 23.5, 20.7, 20.4, 14.0; IR (thin film) cm$^{-1}$ 3269, 3077, 2958, 2925, 2872, 1699, 1638, 1543, 1456, 1368, 1189, 1125, 885, 765; mass spectrum (ESI/MS) m/z 397.0 [M+H]$^+$; HRMS m/z 397.1619 [M+H]$^+$; calcd for C$_{19}$H$_{29}$N$_2$O$_3$S$_2$ 397.1620; yield: 88 %.

![8f](image)

8f was prepared from m-methylthiobenzoic acid using the same procedure as 8a. m.p. 162-164 °C; $^1$H NMR (300 MHz, CDCl$_3$) δ 8.20 (br, 1H), 7.82 (m, 2H), 7.38 (m, 2H), 7.12 (d, J = 8.1 Hz, 1H), 4.49 (d, J = 8.4 Hz, 1H), 3.34 (m, 2H), 2.41 (s, 3H), 1.99 (s, 3H), 1.59 (m, 2H), 1.42 (m, 5H), 1.25 (s, 3H), 0.93 (t, J = 7.2 Hz, 3H); $^{13}$C NMR (75 MHz, CDCl$_3$) δ 193.9, 170.4, 168.8, 139.2, 135.6, 135.5, 129.1, 128.6, 125.5, 58.6, 53.8, 39.7, 31.6, 26.8, 24.1, 23.5, 21.5, 20.4, 14.0; IR (thin film) cm$^{-1}$ 3293, 3224, 3060, 2962, 2921, 2864, 1683, 1670, 1638, 1548, 1369, 1243, 1155, 1119, 928, 817; mass spectrum (ESI/MS) m/z 419.1 [M+Na]$^+$; HRMS m/z 397.1619 [M+H]$^+$; calcd for C$_{19}$H$_{29}$N$_2$O$_3$S$_2$ 397.1620; yield: 85 %.

![8g](image)
\textbf{8g} was prepared from p-methylthiobenzoic acid using the same procedure as \textbf{8a}. m.p. 131-133 °C; $^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 8.22 (br, 1H), 7.92 (d, $J =$ 8.4 Hz, 2H), 7.27 (d, $J =$ 7.2 Hz, 2H), 7.10 (d, $J =$ 8.1 Hz, 1H), 4.48 (d, $J =$ 8.4 Hz, 1H), 3.33 (m, 2H), 2.41 (s, 3H), 1.99 (s, 3H), 1.60 (m, 2H), 1.44 (m, 5H), 1.24 (s, 3H), 0.93 (t, $J =$ 7.5 Hz, 3H); $^{13}$C NMR (75 MHz, CDCl$_3$) $\delta$ 193.3, 170.4, 168.9, 146.0, 132.9, 129.8, 128.4, 58.6, 53.7, 39.7, 31.6, 26.8, 24.1, 23.5, 22.1, 20.4, 14.0; IR (thin film) cm$^{-1}$ 3281, 3089, 2958, 2934, 2868, 1691, 1636, 1541, 1367, 1201, 1171, 1118, 889, 788; mass spectrum (ESI/MS) m/z 419.2 [M+Na]$^+$; HRMS m/z 397.1625 [M+H]$^+$; calcd for C$_{19}$H$_{29}$N$_2$O$_3$S$_2$ 397.1620; yield: 99 %.

![8h](image)

\textbf{8h} was prepared from o-methoxythiobenzoic acid using the same procedure as \textbf{8a}. m.p. 103-105 °C; $^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 8.25 (br, 1H), 7.84 (dd, $J =$ 7.8, 1.8 Hz, 1H), 7.51 (td, $J =$ 7.5, 1.8 Hz, 1H), 7.18 (d, $J =$ 8.4 Hz, 2H), 7.01 (m, 2H), 4.48 (d, $J =$ 8.7 Hz, 1H), 3.93 (s, 3H), 3.33 (m, 2H), 2.00 (s, 3H), 1.59 (m, 2H), 1.41 (m, 5H), 1.23 (s, 3H), 0.92 (t, $J =$ 7.5 Hz, 3H); $^{13}$C NMR (75 MHz, CDCl$_3$) $\delta$ 193.5, 170.4, 168.9, 158.9, 135.3, 130.5, 124.9, 121.0, 112.3, 58.8, 56.1, 53.6, 39.7, 31.6, 27.0, 24.2, 23.5, 20.4, 14.0; IR (thin film) cm$^{-1}$ 3302, 2968, 2872, 1649, 1638, 1524, 1371, 1243, 1109, 1008, 889, 764; mass spectrum (ESI/MS) m/z 413.0 [M+H]$^+$; HRMS m/z 451.1133 [M+K]$^+$; calcd for C$_{19}$H$_{28}$KN$_2$O$_4$S$_2$ 451.1128; yield: 91 %.
8i was prepared from m-methoxythiobenzoic acid using the same procedure as 8a. m.p. 130-132 °C; $^1$H NMR (300 MHz, CDCl$_3$) δ 8.16 (br, 1H), 7.64 (d, $J = 7.8$, Hz, 1H), 7.47 (m, 1H), 7.38 (t, $J = 7.8$ Hz, 1H), 7.16 (dd, $J = 8.1$, 2.1 Hz, 1H), 7.08 (d, $J = 8.4$ Hz, 1H), 4.48 (d, $J = 8.7$ Hz, 1H), 3.85 (s, 3H), 3.34 (m, 2H), 2.00 (s, 3H), 1.60 (m, 2H), 1.44 (m, 5H), 1.25 (s, 3H), 0.94 (t, $J = 7.2$ Hz, 3H); $^{13}$C NMR (75 MHz, CDCl$_3$) δ 193.8, 170.4, 168.8, 160.1, 136.7, 130.2, 121.2, 120.9, 112.3, 58.7, 55.8, 53.9, 39.7, 31.6, 26.9, 24.1, 23.5, 20.5, 14.0; IR (thin film) cm$^{-1}$ 3314, 3240, 2958, 2934, 2864, 1688, 1666, 1636, 1548, 1369, 1256, 1152, 1050, 925; mass spectrum (ESI/MS) m/z 435.1 [M+Na]$^+$; HRMS m/z 451.1136 [M+K]$^+$; calcd for C$_{19}$H$_{28}$KN$_2$O$_4$S$_2$ 451.1128; yield: 99%.

8j was prepared from p-methoxythiobenzoic acid using the same procedure as 8a. m.p. 173-175 °C; $^1$H NMR (300 MHz, CDCl$_3$) δ 8.28 (br, 1H), 8.00 (d, $J = 8.7$ Hz, 2H), 7.13 (d, $J = 8.4$ Hz, 1H), 6.93 (d, $J = 9.0$ Hz, 2H), 4.48 (d, $J = 8.4$ Hz, 1H), 3.86 (s, 3H), 3.32 (m, 2H), 1.98 (s, 3H), 1.59 (m, 2H), 1.43 (m, 5H), 1.23 (s, 3H), 0.92 (t, $J = 7.5$ Hz, 3H); $^{13}$C NMR (75 MHz, CDCl$_3$) δ 191.8, 170.4, 168.9, 165.0, 130.7, 128.1, 114.4, 58.6, 55.9, 53.7, 39.7, 31.6, 26.8, 24.0,
23.5, 20.4, 14.0; IR (thin film) cm⁻¹ 3277, 3224, 3060, 2958, 2925, 2868, 1670, 1636, 1593, 1547, 1458, 1369, 1306, 1262, 1214, 1166, 1024; mass spectrum (ESI/MS) m/z 435.1 [M+Na]⁺; HRMS m/z 451.1129 [M+K]⁺; calcd for C₁₉H₂₈KN₂O₄S₂ 451.1128; yield: 95 %.

\[
\begin{align*}
8k
\end{align*}
\]

\[\text{8k was prepared from 2, 4-dimethoxythiobenzoic acid using the same procedure as 8a. m.p. 76-78 ^\circ C; } \]
\[\text{\textsuperscript{1}H NMR (300 MHz, CDCl}_3\text{) } \delta \text{ 8.40 (br, 1H), 7.90 (d, } J = 8.7 \text{ Hz, 1H), 7.14 (d, } J = 8.1 \text{ Hz, 1H), 6.53 (d, } J = 8.7 \text{ Hz, 1H), 6.46 (s, 1H), 4.44 (d, } J = 8.4 \text{ Hz, 1H), 3.93 (s, 3H), 3.85 (s, 3H), 3.33 (m, 2H), 1.99 (s, 3H), 1.57 (m, 2H), 1.43 (m, 5H), 1.22 (s, 3H), 0.93 (t, } J = 7.2 \text{ Hz, 3H); } \]
\[\text{\textsuperscript{13}C NMR (75 MHz, CDCl}_3\text{) } \delta \text{ 191.5, 170.4, 168.9, 165.9, 161.4, 132.9, 117.8, 106.1, 98.7, 58.8, 56.1, 55.9, 53.3, 39.7, 31.6, 27.1, 24.1, 23.5, 20.5, 14.0; IR (thin film) cm⁻¹ 3305, 3068, 2962, 2921, 1643, 1598, 1543, 1369, 1294, 1251, 1218, 1122, 1024, 873, 833; mass spectrum (ESI/MS) m/z 465.1 [M+Na]⁺; HRMS m/z 465.1459 [M+Na]⁺; calcd for C_{20}H_{30}N_{2}NaO_{5}S_{2} 465.1494; yield: 81 %.
\]

\[
\begin{align*}
8l
\end{align*}
\]

\[\text{8l was prepared from thioacetic acid using the same procedure as 8a. m.p. 168-171 ^\circ C; } \]
\[\text{\textsuperscript{1}H NMR (300 MHz, CDCl}_3\text{) } \delta \text{ 8.03 (br, 1H), 6.91 (br, 1H), 4.35 (d, } J = 8.1 \text{ Hz, 1H), 3.32 (m, } \]

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2H), 2.50 (s, 3H), 2.00 (s, 3H), 1.56 (m, 2H), 1.42 (m, 5H), 1.19 (s, 3H), 0.93 (t, \( J = 6.9 \) Hz, 3H);

\(^{13}\)C NMR (75 MHz, CDCl\(_3\)) \( \delta \) 198.6, 170.5, 168.7, 58.4, 53.6, 39.7, 31.5, 29.3, 26.7, 23.8, 23.5, 20.4, 14.0; IR (thin film) cm\(^{-1}\) 3289, 3089, 2958, 2925, 2868, 1732, 1704, 1643, 1536, 1370, 1115, 944; mass spectrum (ESI/MS) m/z 343.1 [M+Na]\(^+\); HRMS m/z 343.1124 [M+Na]\(^+\); calcd for C\(_{13}\)H\(_{24}\)N\(_2\)NaO\(_3\)S\(_2\) 343.1126; yield: 86 %.

\begin{center}
\includegraphics[width=0.2\textwidth]{8m.png}
\end{center}

**8m** was prepared from thiopropionic acid using the same procedure as **8a**, m.p. 143-144 \( ^\circ \)C; \(^1\)H NMR (300 MHz, CDCl\(_3\)) \( \delta \) 8.08 (br, 1H), 6.88 (d, \( J = 8.1 \) Hz, 1H), 4.34 (d, \( J = 8.4 \) Hz, 1H), 3.33 (m, 2H), 2.77 (m, 2H), 2.01 (s, 3H), 1.59 (m, 2H), 1.43 (m, 5H), 1.25 (t, \( J = 7.5 \) Hz, 3H), 1.19 (s, 3H), 0.93 (t, \( J = 7.2 \) Hz, 3H); \(^{13}\)C NMR (75 MHz, CDCl\(_3\)) \( \delta \) 202.8, 170.4, 168.6, 58.2, 53.5, 39.7, 36.4, 31.5, 26.7, 23.7, 23.5, 20.4, 14.0, 10.0; IR (thin film) cm\(^{-1}\) 3281, 3085, 2962, 2929, 2868, 1715, 1642, 1534, 1457, 1369, 1242, 1124, 1006, 915; mass spectrum (ESI/MS) m/z 357.1 [M+Na]\(^+\); HRMS m/z 335.1460 [M+H]\(^+\); calcd for C\(_{14}\)H\(_{27}\)N\(_2\)O\(_2\)S\(_2\) 335.1463; yield: 71 %.

\begin{center}
\includegraphics[width=0.2\textwidth]{8n.png}
\end{center}

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**8n** was prepared from thioisobutyric acid using the same procedure as 8a. m.p. 131-132°C; $^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 8.13 (br, 1H), 6.83 (d, $J = 8.1$ Hz, 1H), 4.31 (d, $J = 8.4$ Hz, 1H), 3.34 (m, 2H), 2.99 (quin, $J = 6.6$ Hz, 1H), 2.00 (s, 3H), 1.59 (m, 2H), 1.45 (m, 2H), 1.39 (s, 3H), 1.31 (d, $J = 6.9$ Hz, 3H), 1.26 (d, $J = 6.9$ Hz, 3H), 1.19 (s, 3H), 0.94 (t, $J = 7.5$ Hz, 3H); $^{13}$C NMR (75 MHz, CDCl$_3$) $\delta$ 206.5, 170.4, 168.6, 58.1, 53.5, 42.6, 39.8, 31.6, 26.7, 23.7, 23.5, 20.4, 19.9, 19.5, 14.0; IR (thin film) cm$^{-1}$ 3297, 3085, 2966, 2925, 2864, 1722, 1636, 1561, 1453, 1379, 948, 849, 694; mass spectrum (ESI/MS) m/z 371.2 [M+Na]$^+$; HRMS m/z 371.1418 [M+Na]$^+$; calcd for C$_{15}$H$_{28}$N$_2$NaO$_3$S$_2$ 371.1439; yield: 82%.

![Structure of 8n](image)

**8o** was prepared from thiopivalic acid using the same procedure as 8a. m.p. 152-154°C; $^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 8.18 (br, 1H), 6.85 (d, $J = 8.1$ Hz, 1H), 4.29 (d, $J = 8.1$ Hz, 1H), 3.34 (m, 2H), 2.01 (s, 3H), 1.59 (m, 2H), 1.46 (m, 2H), 1.39 (s, 3H), 1.35 (s, 9H), 1.18 (s, 3H), 0.94 (t, $J = 7.2$ Hz, 3H); $^{13}$C NMR (75 MHz, CDCl$_3$) $\delta$ 208.9, 170.4, 168.7, 58.1, 53.4, 47.5, 39.7, 31.6, 27.7, 26.7, 23.7, 23.5, 20.4, 14.0; IR (thin film) cm$^{-1}$ 3293, 3097, 2962, 2925, 2872, 1728, 1708, 1636, 1564, 1536, 1366, 1117, 922, 801; mass spectrum (ESI/MS) m/z 385.1 [M+Na]$^+$; HRMS m/z 401.1336 [M+K]$^+$; calcd for C$_{16}$H$_{30}$KN$_2$O$_3$S$_2$ 401.1335; yield: 80%.
**8p** was prepared from thiophenylacetic acid using the same procedure as **8a**. m.p. 145-146 °C; $^1$H NMR (300 MHz, CDCl$_3$) δ 7.89 (br, 1H), 7.34 (m, 5H), 6.88 (d, $J = 8.1$ Hz, 1H), 4.35 (d, $J = 8.4$ Hz, 1H), 4.01 (s, 2H), 3.30 (m, 2H), 2.01 (s, 3H), 1.56 (m, 2H), 1.40 (m, 5H), 1.17 (s, 3H), 0.92 (t, $J = 7.2$ Hz, 3H); $^{13}$C NMR (75 MHz, CDCl$_3$) δ 200.2, 170.4, 168.6, 132.6, 129.9, 129.1, 128.1, 58.5, 53.7, 49.3, 39.7, 31.5, 26.9, 23.9, 23.5, 20.4, 14.0; IR (thin film) cm$^{-1}$ 3285, 3087, 2962, 2872, 1715, 1635, 1540, 1453, 1369, 1120, 994, 702; mass spectrum (ESI/MS) m/z 419.2 [M+Na]$^+$; HRMS m/z 435.1167 [M+K]$^+$; calcd for C$_{19}$H$_{28}$KN$_2$O$_3$S$_2$ 435.1178; yield: 84 %.

**8q** (mixture of diastereoisomers dr 1:1) was prepared from 2-phenylpropanethioic S-acid using the same procedure as **8a**. m.p. 116-118 °C; $^1$H NMR (300 MHz, CDCl$_3$) δ 8.00 (br, 1H), 7.85 (br, 1H), 7.35 (m, 10H), 6.93 (d, $J = 8.4$ Hz, 2H), 4.36 (d, $J = 8.4$ Hz, 1H), 4.34 (d, $J = 8.4$ Hz, 1H), 4.13 (q, $J = 6.9$ Hz, 1H), 4.06 (q, $J = 6.9$ Hz, 1H), 3.37 (m, 2H), 3.25 (m, 2H), 2.01 (s, 3H), 2.00 (s, 3H), 1.61 (d, $J = 7.2$ Hz, 6H), 1.49 (m, 8H), 1.37 (s, 3H), 1.31 (s, 3H), 1.15 (s, 3H), 1.14 (s, 3H), 0.93 (t, $J = 7.2$ Hz, 6H); $^{13}$C NMR (75 MHz, CDCl$_3$) δ 203.7, 203.6, 170.4 (2C), 200.4, 170.4 (2C), 168.6, 132.6, 129.9, 129.1, 128.1, 58.5, 53.7, 49.3, 39.7, 31.5, 26.9, 23.9, 23.5, 20.4, 14.0; IR (thin film) cm$^{-1}$ 3285, 3087, 2962, 2872, 1715, 1635, 1540, 1453, 1369, 1120, 994, 702; mass spectrum (ESI/MS) m/z 419.2 [M+Na]$^+$; HRMS m/z 435.1167 [M+K]$^+$; calcd for C$_{19}$H$_{28}$KN$_2$O$_3$S$_2$ 435.1178; yield: 84 %.
168.8 (2C), 138.3 (2C), 129.2 (2C), 128.5 (2C), 128.2 (2C), 58.6, 58.4, 53.7 (2C), 39.7 (2C), 31.5 (4C), 26.6 (2C), 24.0 (2C), 23.5 (2C), 20.4 (2C), 18.9 (2C), 14.0 (2C); IR (thin film) cm$^{-1}$ 3288, 3090, 2955, 2925, 2866, 1729, 1635, 1541, 1366, 924; mass spectrum (ESI/MS) m/z 433.3 [M+Na]$^+$; HRMS m/z 449.1350 [M+K]$^+$; calcd for C$_{20}$H$_{30}$KN$_2$O$_3$S$_2$ 449.1335; yield: 85%.

8r was prepared from 2, 2-diphenylethanethioic S-acid using the same procedure as 8a. m.p. 113-116 °C; $^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 7.79 (br, 1H), 7.34 (m, 10H), 6.94 (d, $J$ = 8.4 Hz, 1H), 5.42 (s, 1H), 4.39 (d, $J$ = 8.4 Hz, 1H), 3.29 (m, 2H), 2.01 (s, 3H), 1.54 (m, 2H), 1.38 (m, 5H), 1.17 (s, 3H), 0.90 (t, $J$ = 7.2 Hz, 3H); 13C NMR (75 MHz, CDCl$_3$) $\delta$ 202.2, 170.4, 168.8, 137.3, 129.3, 129.1, 128.1, 64.3, 58.9, 53.9, 39.7, 31.5, 27.0, 24.1, 23.5, 20.4, 14.0; IR (thin film) cm$^{-1}$ 3277, 3088, 2956, 2925, 2872, 1721, 1674, 1632, 1557, 1540, 1495, 1378, 1128, 976; mass spectrum (ESI/MS) m/z 495.3 [M+Na]$^+$; HRMS m/z 495.1722 [M+Na]$^+$; calcd for C$_{25}$H$_{32}$N$_2$NaO$_3$S$_2$ 495.1752; yield: 91%.

$^8$s was prepared from triphenylethanethioic S-acid using the same procedure as 8a. m.p. 197-199 °C; $^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 7.99 (br, 1H), 7.25 (m, 16H), 4.37 (m, 1H), 3.23 (m, 2H), 2.01 (s, 3H), 1.54 (m, 2H), 1.38 (m, 5H), 1.17 (s, 3H), 0.90 (t, $J$ = 7.2 Hz, 3H); 13C NMR (75 MHz, CDCl$_3$) $\delta$ 202.2, 170.4, 168.8, 137.3, 129.3, 129.1, 128.1, 64.3, 58.9, 53.9, 39.7, 31.5, 27.0, 24.1, 23.5, 20.4, 14.0; IR (thin film) cm$^{-1}$ 3277, 3088, 2956, 2925, 2872, 1721, 1674, 1632, 1557, 1540, 1495, 1378, 1128, 976; mass spectrum (ESI/MS) m/z 495.3 [M+Na]$^+$; HRMS m/z 495.1722 [M+Na]$^+$; calcd for C$_{25}$H$_{32}$N$_2$NaO$_3$S$_2$ 495.1752; yield: 91%.
2H), 2.00 (s, 3H), 1.46 (m, 2H), 1.27 (m, 5H), 1.12 (s, 3H), 0.88 (t, J = 7.2 Hz, 3H); \(^{13}\)C NMR (75 MHz, CDCl\(_3\)) \(\delta\) 206.9, 170.3, 168.8, 141.7, 131.2, 128.3, 127.9, 73.3, 59.1, 54.0, 39.7, 31.5, 27.3, 24.1, 23.5, 20.4, 14.0; IR (thin film) cm\(^{-1}\) 3310, 2956, 2929, 1774, 1721, 1646, 1535, 1491, 1447, 1367, 1190, 1070, 1035, 738, 698; mass spectrum (ESI/MS) m/z 571.2 [M+Na]\(^+\); HRMS m/z 571.2074 [M+Na]\(^+\); calcd for C\(_{31}\)H\(_{36}\)N\(_2\)O\(_3\)S\(_2\) 571.2065; yield: 64 %.

### 3.4.2 H\(_2\)S Measurement

The reaction was initiated by adding 75 \(\mu\)L 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\(\mu\)L) and \(\text{N, N-dimethyl-1,4-phenylenediamine sulfate (20 mM, 200}\(\mu\)L) in 7.2 M HCl and ferric chloride (30 mM, 200\(\mu\)L) in 1.2 M HCl. The absorbance (670 nm) of the resultant solution (1.5 mL) was determined 15 min thereafter using a UV–vis spectrometer (Thermo Evolution 300). The H\(_2\)S concentration of each sample was calculated against a calibration curve of Na\(_2\)S. The H\(_2\)S releasing curve was obtained by plotting H\(_2\)S concentration versus time.

### 3.4.3 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: this is a known compound. m.p. 135-136 °C; \(^1\)H NMR (300 MHz, CDCl\(_3\)) \(\delta\) 7.95 (d, \(J = 8.7\) Hz, 2H), 7.80 (m, 2H), 7.59 (m, 1H), 7.46 (m, 5H), 7.16 (d, \(J = 6.9\) Hz, 1H), 5.07 (m, 1H), 3.81 (s, 3H), 3.69 (q, \(J = 2.4\) Hz, 2H); \(^{13}\)C NMR (75 MHz, CDCl\(_3\)) \(\delta\) 191.7, 171.0, 167.3, 136.5,
134.2, 133.6, 132.1, 129.0, 128.8, 127.7, 127.4, 53.3, 53.2, 30.8; IR (thin film) cm\(^{-1}\) 3314, 1741, 1650, 1531, 1325, 1209, 1095, 1035, 1000, 913; mass spectrum (ESI/MS) m/z 366.1 [M+Na]\(^+\); calcd for C\(_{18}\)H\(_{17}\)NNaO\(_{4}\)S 366.1; yield: 98%.

**15:** this is a known compound. m.p. 152-153 °C; \(^1\)H NMR (300 MHz, CDCl\(_3\)) \(\delta\) 7.81 (d, \(J = 8.7\) Hz, 4H), 7.50 (t, \(J = 6.0, 1.2\) Hz, 2H), 7.40 (m, 4H), 7.15 (d, \(J = 7.2\) Hz, 2H), 5.07 (m, 2H), 3.77 (s, 6H), 3.34 (d, \(J = 5.1\) Hz, 4H); \(^{13}\)C NMR (75 MHz, CDCl\(_3\)) \(\delta\) 171.2, 167.3, 133.6, 132.2, 128.9, 127.4, 53.1, 52.5, 41.1; IR (thin film) cm\(^{-1}\) 3309, 2954, 1737, 1638, 1580, 1525, 1488, 1433, 1217, 1163, 691; mass spectrum (ESI/MS) m/z 499.1 [M+Na]\(^+\); calcd for C\(_{22}\)H\(_{24}\)N\(_2\)NaO\(_6\)S\(_2\) 499.1; yield: 81%.

**12** (1:1 mixture of diastereoisomers): mp 73−75°C; \(^1\)H NMR (300 MHz, CDCl\(_3\)) \(\delta\) 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\(_3\)) \(\delta\) 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\(_3\)NaO\(_5\)S\(_2\) 506.1759; yield 20%.

**13:** mp 176−177°C; \(^1\)H NMR (300 MHz, CDCl\(_3\)) \(\delta\) 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\(_3\)) \(\delta\) 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_{2}O_{2}S 247.1480; yield 55%.

3.4.4 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% CO₂ and 95% air. H9c2 cells at a concentration of 1×10^5/mL were inoculated in 96-well plates and cultured overnight. H₂S 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:

\[
\% \text{ Cell Viability} = \frac{OD \text{ treatment group}}{OD \text{ control group}} \times 100\%
\]

3.4.5 H₂S Release in H9c2 Cells

H9c2 cells were inoculated in 6-well plates and cultured overnight. The cells were co-incubated with 100 µM H₂S 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 H₂S 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).

3.4.6 Cardioprotective effects in MI/R (This work has been done by our collaborator, Dr. David J Lefer)

3.4.6.1 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 Animal 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.

3.4.6.2 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 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.

3.4.6.3 Myocardial ischemia/reperfusion (MI/R) protocol and assessment of myocardial infarct size

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.

3.4.6.4 Cardiac troponin-I assay

Blood samples were collected via a tail vein at 4 h of reperfusion. Cardiac troponin-I level was measured in serum using the Life Diagnostic high-sensitivity mouse cardiac troponin-I ELISA kit (Mouse Cardiac Tn-I ELISA Kit; Life Diagnostics, West Chester, PA) as previously described.

3.4.7 In vivo determination of H$_2$S levels

H$_2$S 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$_2$S 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$_2$S concentrations were calculated using a standard curve of Na$_2$S as a source of H$_2$S. Chromatographs were captured and analyzed with Agilent Chem-Station software (B.04.03).
3.5 REFERENCES


APPENDIX
CHAPTER TWO
$^1$H NMR (300 MHz, CDCl$_3$) spectrum of 5a
$^1$H NMR (300 MHz, CDCl$_3$) spectrum of 5b
$^1$H NMR (300 MHz, CD$_3$OD) spectrum of 5c
$^{13}$C NMR (75 MHz, CDCl$_3$) spectrum of 5d

![Chemical Structure of 5d](image)
$^1$H NMR (200 MHz, CDCl$_3$) spectrum of 5e
$^{1}H$ NMR (300 MHz, CDCl$_3$) spectrum of 5f
$^1$H NMR (300 MHz, CDCl$_3$) spectrum of 5g
$^{13}$C NMR (75 MHz, CDCl$_3$) spectrum of 5g
$^1$H NMR (300 MHz, CDCl$_3$) spectrum of 5h
$^1$H NMR (300 MHz, CDCl$_3$) spectrum of 5i
$^1$H NMR (300 MHz, CDCl$_3$) spectrum of 5j
$^{13}$C NMR (75 MHz, CDCl$_3$) spectrum of 5j
$^{13}$C NMR (75 MHz, CDCl$_3$) spectrum of 5k
$^{13}$C NMR (75 MHz, DMSO-$d_6$) spectrum of 5l
$^1$H NMR (300 MHz, CDCl$_3$) spectrum of 7
$^{13}$C NMR (75 MHz, CDCl$_3$) spectrum of 7

![Molecular Structure]

7
$^{1}$H NMR (300 MHz, CDCl$_3$) spectrum of 9
$^{13}$C NMR (75 MHz, CDCl$_3$) spectrum of 9
$^1$H NMR (300 MHz, D$_2$O) spectrum of 11
$^{13}$C NMR (75 MHz, CDCl$_3$) spectrum of 11

![Chemical Structure](image-url)
CHAPTER THREE
$^1$H NMR (300 MHz, CDCl$_3$) spectrum of a
$^1$H NMR (300 MHz, CDCl$_3$) spectrum of d
$^1$H NMR (300 MHz, CDCl$_3$) spectrum of 2a
$^1$H NMR (75 MHz, CDCl$_3$) spectrum of 2a
$^1$H NMR (300 MHz, CDCl$_3$) spectrum of 2b
$^1$H NMR (300 MHz, CDCl$_3$) spectrum of 2c
$^{13}$C NMR (75 MHz, CDCl$_3$) spectrum of 2c
$^1$H NMR (300 MHz, CDCl$_3$) spectrum of 2d
$^1$H NMR (300 MHz, CDCl$_3$) spectrum of 8a
$^{13}$C NMR (75 MHz, CDCl$_3$) spectrum of 8a
$^1$H NMR (300 MHz, CDCl$_3$) spectrum of 8b
$^{13}$C NMR (75 MHz, CDCl$_3$) spectrum of 8b
$^1$H NMR (300 MHz, CDCl$_3$) spectrum of 8c
$^{13}$C NMR (75 MHz, CDCl$_3$) spectrum of 8e
$^{13}$C NMR (75 MHz, CDCl$_3$) spectrum of 8f
$^1$H NMR (300 MHz, CDCl$_3$) spectrum of 8g
$\text{H NMR (300 MHz, CDCl}_3\text{) spectrum of 8h}$

![Chemical structure of 8h](image)
$^{13}$C NMR (75 MHz, CDCl$_3$) spectrum of 8h

AcHN

$\text{NH}Bu$

8h

OCH$_3$

f$_2$ (ppm)
$^{13}$C NMR (75 MHz, CDCl$_3$) spectrum of 8i
$^1$H NMR (300 MHz, CDCl$_3$) spectrum of 8j
$^1$H NMR (300 MHz, CDCl$_3$) spectrum of 8I
\[^{13}\text{C} \text{NMR (75 MHz, CDCl}_3\text{)} \text{ spectrum of 8l}\]
$^1$H NMR (300 MHz, CDCl$_3$) spectrum of 8m
\[ ^{13}C \text{ NMR (75 MHz, CDCl}_3 \text{) spectrum of 8m} \]
$^{13}$C NMR (75 MHz, CDCl$_3$) spectrum of $8n$
^1H NMR (300 MHz, CDCl₃) spectrum of 8o
$^1$H NMR (300 MHz, CDCl$_3$) spectrum of 8p
$^1$H NMR (300 MHz, CDCl$_3$) spectrum of 8q
$^1$H NMR (300 MHz, CDCl$_3$) spectrum of 8r
$^{13}$C NMR (75 MHz, CDCl$_3$) spectrum of $8s$
$^1$H NMR (300 MHz, CDCl$_3$) spectrum of 12
$^{1} \text{H NMR (300 MHz, CDCl}_3\text{)}$ spectrum of 13
$^{13}$C NMR (75 MHz, CDCl$_3$) spectrum of 13
$^{13}$C NMR (75 MHz, CDCl$_3$) spectrum of 15