PHOSPHINE BASED FLUORESCENT PROBES FOR NITROXYL DETECTION AND THEIR REACTIVITY TOWARD S-NITROSOTHIOLS

BY

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<td>protein kinase B</td>
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<td>ALDH</td>
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<td>AS</td>
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Nitrogen oxides are a unique class of molecules with profound physiology. Nitric oxide (NO), the most well-studied nitrogen oxide, plays a key role in modulating numerous physiological processes such as blood pressure control and the immune response. Nitroxy (HNO), the one-electron reduced/protonated relative of NO, possesses a distinct physiological profile from NO. HNO inhibits the activity of various thiol-containing enzymes and regulates cardiovascular signaling, making it an intriguing candidate for many physiological disorders such as alcoholism and congestive heart failure (CHF).

Despite its physiological significance, the lack of reliable detection methods still hampers our understanding of HNO. To help overcome this problem, we developed a novel fluorescein-derived fluorescent probe for HNO based on the reductive Staudinger ligation. This probe allows detection of HNO from various sources without the interference of other biological redox species. This probe also successfully detects HNO by fluorescence in HeLa cells following the designed mechanism.

We noticed similar reactivity between S-nitrosothiols (RSNO) with this type of probe. To investigate this potential cross-reactivity, we compared the phosphine-based detection strategies for HNO and RSNO. Phosphorus NMR studies show that azaylides derived from HNO or organic RSNO efficiently participate in the reductive ligation required for fluorescence generation while S-azaylides derived from biological RSNO containing free amine and carboxylic acid groups primarily yield phosphine oxides, allowing the use of phosphine probes for selective HNO detection. Flow cytometry experiments in HeLa cells reinforce the reactivity difference and offer a potential fast
screening approach for endogenous HNO sources.

We also proposed the existence of $S$-nitrosobacillithiol (BSNO), the corresponding RSNO for bacillithiol (BSH). We attempted the synthesis and characterization of BSNO. Though this work is still on-going, we believe the success of this project will boost the understanding of the roles that BSH and BSNO play in low-guanine-cytosine (G+C) content Gram-positive bacteria.
CHAPTER 1

INTRODUCTION

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Part of the writing contained in this chapter was published in *Nitric Oxide* in 2016.\(^1\) The manuscript, including figures and schemes, was drafted and edited by Zhengrui Miao and S. Bruce King. Since the publication, changes in both format and content were made to adapt this work for the dissertation format.
Dynamic physiological processes related to feedback mechanisms, metabolism, growth, proliferation, and carcinogenesis are well regulated via a sophisticated signaling network in response to endogenous and exogenous stimuli. A multitude of small molecules known as reactive oxygen and nitrogen species (ROS/RNS respectively) play key roles in cell signaling eliciting profound physiological effects. The biological signaling matrix associated with ROS and RNS has become a subject of increased research interest. These species are produced endogenously or introduced externally as pharmaceutical therapies. They are traditionally toxic species; however, growing data indicates their direct involvement in a diverse range of signaling events at lower concentrations. Nitrogen oxides represent a unique class of RNS, which have gained increasing attention over the past decades due to their significance in mammalian physiology. Established through dietary consumption or endogenous synthetic pathways, these molecules are special physiological messengers. The interconversion of these nitrogen oxides (Table 1) with the oxidation state of nitrogen ranging from -3 to +5 *in vivo* by mediated oxidation or reduction is responsible for the fine regulation of protein activities.

<table>
<thead>
<tr>
<th>Nitrogen Oxides</th>
<th>Oxidation State</th>
<th>Relevant Species</th>
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<tbody>
<tr>
<td>NO₃⁻</td>
<td>+5</td>
<td>Nitroglycerin</td>
</tr>
<tr>
<td>NO₂</td>
<td>+4</td>
<td></td>
</tr>
<tr>
<td>NO₂⁻</td>
<td>+3</td>
<td><em>S</em>-nitrosothiols</td>
</tr>
<tr>
<td>NO</td>
<td>+2</td>
<td>Diazeniumdiolates</td>
</tr>
<tr>
<td>HNO</td>
<td>+1</td>
<td>Nitrous Oxide</td>
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<tr>
<td>N₂</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>NH₂OH</td>
<td>-1</td>
<td><em>N</em>-hydroxy-<em>L</em>-arginine</td>
</tr>
<tr>
<td>NH₃</td>
<td>-3</td>
<td>Arginine</td>
</tr>
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</table>
Table 1. The physiologically relevant oxidation states of nitrogen.

1.1 NITRIC OXIDE

Nitric oxide (NO) is one of the most well-studied nitrogen oxides. The discovery of NO as a biological signaling agent helped establish the importance of endogenously produced gaseous small molecules as modulators of numerous physiological processes.\(^4\)-\(^6\) NO-mediated signaling plays widely-recognized roles in blood pressure and flow control, the immune response and neurotransmission, and NO’s role in blood pressure control explains the mechanism of action of clinically long-used drugs like nitroglycerin.\(^3\), \(^5\), \(^7\)-\(^8\) These initial findings triggered wide-ranging investigations to support and explore NO-based physiological signaling.\(^7\), \(^9\)-\(^10\) For example, metallo-heme proteins, particularly soluble guanylate cyclase (sGC) in terms of smooth muscle relaxation, were identified as valid biological targets of NO that ultimately allowed the development of new clinical phosphodiesterase inhibitors that modulate NO’s actions.\(^3\), \(^7\), \(^11\)-\(^14\) Protein thiols react with NO or its redox forms to yield S-nitrosothiols (RSNO), an NO-based post-translational modification that will be covered in Section 1.2.\(^15\)-\(^16\) Other early work clearly defined the nitric oxide synthase (NOS) catalyzed oxidation of L-arginine to NO and L-citrulline as a pathway of NO biosynthesis and demonstrated the existence of this system in endothelial and neural tissue and macrophages.\(^3\), \(^10\), \(^17\)-\(^22\) Numerous analytical methods either existed or were developed, including spectroscopic, electrochemical and chemiluminescence techniques, to measure NO levels allowing researchers to track and monitor this reactive signaling agent.\(^23\)-\(^26\) Commercially available nitric oxide gas provides direct access to solutions of NO but many other NO donors, especially the diazeniumdiolates (NONOates) achieve more controlled NO delivery.\(^27\) The contributions of these basic
studies in pharmacology, biochemistry, molecular biology, enzymology, chemistry and physics have driven the current understanding of NO signaling and its translation to physiology and medicine.

1.2 S-NITROSO ThiOLS

S-Nitrosothiol formation and protein S-nitrosation represents one of the two major NO-dependent post-translational modifications that requires temporal and spatial regulation.\(^{28}\) The precise mediation of S-nitrosation/de-nitrosation plays a key role in the NO dependent signaling paradigm that controls NO transport and preservation.\(^{29}\) Besides redox signaling, RSNO have also been found in cardiomyocytes and cardiomyocyte mitochondria and have a role in cardioprotection.\(^{30}\) Until now, the full chemical biological profile of RSNO has not been established. Typically, the reaction of NO with a thiol radical, \(\text{N}_2\text{O}_3\), transition metal assisted nitrosation and oxidation of the direct adduct of NO with a thiol are considered responsible for protein S-nitrosation.\(^{28, 31}\)

S-Nitrosation is the most important and frequent process that is involved in ischemia/reperfusion and cardioprotection.\(^{30}\) Moreover, several studies have shown that the formation of RSNO in biological systems is probably an important pathway to mediate many protein functions.\(^{30}\) Similar to other post-translational modifications, S-nitrosation can alter the structure, activity, localization, and/or stability of proteins, leading to mediation of protein function.\(^{30}\) Many physiological cellular functions including excitation-contraction coupling, G-protein coupled receptor signaling, homeostasis, proliferation and programmed cell death are relevant to direct protein S-nitrosation and/or biological processes that follow S-nitrosation.\(^{30}\) In addition, S-nitrosation represents a
mechanism for both cardioprotection and the protection of cysteine residues from irreversible oxidation. For instance, both protein kinase B (Akt, whose phosphorylated form plays an important role in signaling cardioprotection) and PTEN (phosphatase and tensin homologue deleted on Chromosome 10) are subject to S-nitrosation. While the S-nitrosation process protects Akt from the formation of a disulfide and reduces its activity, S-nitrosation of PTEN leads to its ubiquitination and degradation, thus limiting the dephosphorylation of Akt.

One of the challenges with the study of RSNO and protein nitrosation is accurate and sensitive detection. It has been a major issue in the study of post-translational modifications of NO. However, efforts have been devoted and several new methods have been developed for the determination of the S-nitrosothiol proteome. In chapter 3, we will examine a new fluorescent probe for S-nitrosothiols.

1.3 NITROXYL

Nitroxyl (or azanone, HNO) differs from nitric oxide only by the addition of a hydrogen atom to give the formal one-electron reduction and protonation product of NO. Given the close structural similarity of HNO to NO, HNO initially gained significant consideration as a possible component of NO’s biological response or intermediate in NO biosynthesis. Despite the structural similarity, the high chemical reactivity of HNO has hindered the overall development of our understanding of HNO’s basic chemical and biological properties as compared to NO. HNO represents the smallest and simplest nitroso compound (R-N=O, R = H for nitroxyl) and exhibits reactivity like other C-nitroso compounds as HNO reacts with itself to form a dimer (k = 8 x 10^6 M^-1 s^-1) that
dehydration to ultimately give nitrous oxide (N₂O, Scheme 1). This property alone requires nitroxyl to be introduced to systems by the use of donor molecules such as Angeli’s salt (AS, Na₂N₂O₃) and Piloty’s acid (PA, PhSO₂NHOH), which will be reviewed in Section 1.3.4. HNO’s self-reactivity limits HNO detection and the identification (Section 1.3.3) of any endogenous generating systems (Section 1.3.5). While the stability of C-nitroso compounds and nitrous oxide indicates the accessibility of the formal +1 nitrogen oxidation state of HNO and suggests possible biological roles for HNO, the self-reactivity and the lack of HNO specific metabolites or protein modifications limits our overall understanding of its biological chemistry.

\[
\text{H-NO} \quad \xrightarrow{\text{HNO}} \quad \text{O}^+ \quad \text{N}^- \quad \text{N}^+ \quad \quad \xrightarrow{\text{HON=NOH}} \quad \text{N}_2\text{O} \quad + \quad \text{H}_2\text{O}
\]

**Scheme 1.** Self-dimerization of HNO.

### 1.3.1 HNO Reactivity

Due to the fast self-dimerization, other HNO reactions must possess associated rate constants that are comparable to or greater than that of the self-consuming pathway to be considered chemically and biologically relevant. Miranda and co-workers have approximated the rate constant for some of the physiological relevant species: oxymyoglobin (1 × 10⁷ M⁻¹ s⁻¹) > horseradish peroxidase, glutathione (2 × 10⁶ M⁻¹ s⁻¹) > N-acetylcysteine, MnSOD, CuZnSOD, catalase, metmyoglobin (3-10 × 10⁵ M⁻¹ s⁻¹) > tempol, ferricytochrome c (4-8 × 10⁴ M⁻¹ s⁻¹) > O₂ (3×10³ M⁻¹ s⁻¹). The small rate constant for the reaction of HNO with oxygen suggests this chemistry is not likely to be biologically relevant. The most well-established and physiological relevant chemistry for
HNO includes its reaction with nucleophiles and metalloproteins. HNO reacts as a Lewis acid/base or redox partner with numerous metalloproteins (including both ferrous and ferric hemes).\textsuperscript{41, 44} Similarly, nitroxyl avidly reacts with soft nucleophiles, especially thiols, to yield either disulfides or sulfinamides, making both thiol and metal-containing proteins valid biological targets for HNO.\textsuperscript{45} These reactions are either responsible for many of the observed biological roles such as a vasodilator and enzyme regulator (Section 1.3.2) or used for HNO detection and quantification (Section 1.3.3).

Metalloprotein complexes directly coordinated by HNO are difficult to prepare and up to now only a few examples have been reported.\textsuperscript{41, 46} The association between HNO and Fe\textsuperscript{II} is generally considered to be weak despite the report that deoxygenated ferrous myoglobin reacts with HNO to form a stable complex (MbHNO) anaerobically, which is prone to oxidation.\textsuperscript{41, 44, 47} Direct reaction of HNO with metalloproteins results in reductive nitrosylation of oxidized metals especially Fe\textsuperscript{III} and remains one of the most important reactions in HNO physiology.\textsuperscript{41} Similar to NO-heme interactions, reactions of HNO with hemoglobin (Hb) and myoglobin (Mb) are the best studied. However, NO tends to react with ferrous proteins while HNO reacts preferentially with ferric (Met) proteins through reductive nitrosylation.\textsuperscript{9, 43} For instance, metMb reacts with HNO with a rate constant of 8 x 10\textsuperscript{5} M\textsuperscript{-1} s\textsuperscript{-1} (37 °C) forming ferrous nitrosyl complexes, which exist in resonance with Fe\textsuperscript{III}NO\textsuperscript{−}, a putative HNO source (Scheme 2).\textsuperscript{43}

\[
\text{MetMb} + \text{HNO} \rightarrow \text{H}^+ + \text{Mb[Fe\textsuperscript{II}]NO} \leftrightarrow \text{Mb[Fe\textsuperscript{III}]NO}^\text{−}
\]

\textbf{Scheme 2.} Reductive nitrosylation of ferric myoglobin by HNO.

Oxygenated ferrous hemes can rapidly react with HNO to provide NO and
peroxide.\textsuperscript{48} The nascent NO will then quickly be captured by the surrounding oxygenated heme to give the observed products ferric heme and nitrate.\textsuperscript{41, 48} For instance, oxymyoglobin (MbO\textsubscript{2}) is one of the best-known traps for HNO with a rate constant of 1 x 10\textsuperscript{7} M\textsuperscript{-1} s\textsuperscript{-1} (37 °C) (Scheme 3).\textsuperscript{43}

\begin{equation}
\text{MbO}_2 + \text{HNO} \rightarrow \text{MetMb} \quad \text{NO} \quad \text{MbO}_2 \rightarrow \text{MetMb} + \text{NO}_3^-
\end{equation}

**Scheme 3.** HNO-mediated oxidation of oxymyoglobin.

The reaction of nucleophiles with HNO is another important chemical approach toward its biological significance. Quantum mechanical calculations suggest that HNO will primarily react with soft nucleophiles, such as thiols, phosphines and amines.\textsuperscript{49} A hallmark of the physiologically relevant reactivity of HNO is its thiophilicity. HNO can rapidly react with cysteine thiols via nucleophilic attack at the nitrogen to give an \textit{N}-hydroxysulfenamide.\textsuperscript{50} This nascent species can either react with excessive thiol to provide disulfide, which may be reduced back to thiol species through an established process, or rearrange to sulfinamide followed by possible hydrolysis to the sulfinic acid as the final fate under low reduced thiol condition (Scheme 4).\textsuperscript{41} Physiologically, these reactions usually lead to an alteration of protein function and/or lifetime.
Scheme 4. Oxidative modification of thiols by HNO.

HNO also reacts with nucleophilic nitrogen oxides such as hydroxylamine to generate \( \text{N}_2 \) gas and water (Scheme 5).\(^{51}\) Angeli’s salt generates diazenes from secondary amines from the nucleophilic addition of amines to HNO, a highly thermodynamically favorable reaction in solution.\(^{52}\)

\[
\text{H-N=O} + \text{NH}_2\text{OH} \rightarrow \text{N}_2 + 2\text{H}_2\text{O}
\]

Scheme 5. Reaction of HNO with hydroxylamine.

1.3.2 The Physiological Role of HNO

Nitroxyln has been reported to regulate numerous significant processes in physiological systems. Reaction with cysteine thiols in proteins is the major mechanism of action for HNO to achieve physiological/pharmacological mediation. As discussed, the nucleophilic attack of an active site thiolate of a protein on HNO results in a covalent modification, altering protein activity. One of its first pharmacological uses was as an anti-alcoholism agent generated from the metabolism of cyanamide. The metabolic oxidation of cyanamide to HNO leads to the inhibition of aldehyde dehydrogenase (ALDH).\(^{53-54}\) Recently, HNO was reported to inhibit the activity of glyceraldehyde phosphate dehydrogenase (GAPDH) in yeast with a mechanism similar to that of ALDH at very low
levels of HNO. Moreover, the negligible change of glutathione (GSH) levels or GSH/GSSG (glutathione disulfide) ratios suggests the modifications occur without significantly altering the cellular redox environment. Although not defined, the inhibition of glycolysis due to the GAPDH deactivation may be responsible for fundamental metabolic changes such as inhibition of ischemia–reperfusion injury or elevations in calcitonin gene-related peptide (CGRP) levels. In addition, HNO covalently modifies the cysteine on a subunit of the N-methyl-D-aspartate (NMDA) receptor, thwarting Ca\textsuperscript{2+} influx and affording protection against excitotoxicity. Data collected from previous research also indicates that HNO interacts with these selected proteins without altering the cellular redox status.

The most well-known physiological significance of HNO lies in cardiovascular physiology. Various HNO sources were reported to elicit vasorelaxation and stimulate cyclic guanosine monophosphate (cGMP) production, presumably via the activation of sGC either by HNO directly or due to HNO conversion to NO via in situ one electron oxidation. The use of NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethyl-imidazole-1-oxy-3-oxide (carboxy-PTIO) does not affect the Angeli’s salt induced vasorelaxation, suggesting HNO directly activates sGC. However, studies with partially purified sGC suggests only NO is capable of activating sGC, despite the fact that these studies were performed in high levels of dithiothreitol, which could scavenge HNO before interaction with sGC. The possible interaction between HNO and sGC remains to be established, as recent work has led to more confusion by examining the biological effects of HNO on purified sGC due to the nature of HNO interaction with metalloproteins.
Another rationale for the HNO induced vasorelaxation involves an increase in CGRP.\textsuperscript{60} Originally shown in intact animals, the HNO induced vasorelaxant and inotropic (modifying the force or speed of cardiac contractility) properties are further confirmed in rat coronary tissue.\textsuperscript{60-61} Data suggests CGRP activates the calcitonin-receptor-like receptor and stimulates cyclic adenosine monophosphate (cAMP) production, a different mechanism distinct from the cGMP pathway associated with sGC activation.\textsuperscript{62-63} However, although the decrease of peripheral vascular resistance is undoubtedly associated with HNO-stimulated CGRP generation, recent studies suggest HNO-induced inotropy may be attributed to other effects.\textsuperscript{64}

The second key cardiovascular significance of HNO infusion involves an increase in systolic force and decrease in diastolic pressure in both normal and failing heart via specific and covalent thiol modification on the sensitive target ryanodine receptor (RyR).\textsuperscript{62-66} The cardiac contraction of striated muscle is largely determined by the release of Ca\textsuperscript{2+} from the sarcoplasmic reticulum (SR), a result of activation of the RyR.\textsuperscript{67} Insufficient Ca\textsuperscript{2+} release from the SR to the myocardium leads to inefficient contraction and a failing heart.\textsuperscript{68} The RyRs are modulated by redox reactions via modifications to critical cysteine residues.\textsuperscript{69} Studies with HNO donors indicate the ability of HNO to increase the RyR channel open probability, eliciting a Ca\textsuperscript{2+} release from both skeletal and cardiac SR vesicles, improving contractility and leading to a positive inotropic effect.\textsuperscript{70}

It is worth noting that HNO induces an increase in overall Ca\textsuperscript{2+} cycling.\textsuperscript{67} The subsequent uptake of cellular calcium facilitates myocardial relaxation and demonstrates a positive lusitropic (modifying the force or speed of cardiac relaxation) effect.\textsuperscript{66} This effect is proposed to be due to an effect on sarcoplasmic reticulum Ca\textsuperscript{2+}-ATPase (SERCA2a),
presumably via HNO-mediated covalent modification on thiol group of the SERCA2a-regulating protein phospholamban (PLN) or SERCA2a itself.66, 71-72

1.3.3 Improved HNO Detection

The development of new HNO donors and a complete establishment of HNO’s biological profile require fast and reliable HNO detection methods. Traditionally, the identification and quantification of the dimerization/dehydration product, nitrous oxide ($N_2O$), by headspace gas chromatography is the most frequently utilized method to detect HNO production in solution. However, this technique is less applied in bioassays since $N_2O$ might not specifically come from HNO and HNO consumption in vivo occurs via complicated competitive pathways.18 Physiologically abundant thiols can trap HNO faster than dimerization giving sulfinamides, which can be detected by high performance liquid-chromatography mass spectrometry (HPLC-MS).73 This technique requires the amount of thiols to be appropriate---high enough to compete with dimerization and low enough to minimize disulfide formation, though the product is specific for HNO. UV-visible spectroscopy (UV-vis) detection of MbNO generated from metMb serves as another HNO identification method, though NO also can give a false positive and the product is prone to air oxidation.13, 74-75 In addition, labeling with $^{15}$N and trapping by hemes to produce electron paramagnetic resonance spectroscopy (EPR) active species is also used for HNO detection, but all these traditional methods are indirect analytical techniques that suffer from either low sensitivity or selectivity.76-77 These limitations have led to the development of several new detection strategies, including a series of fluorescent probes based on Cu$^{II}$ complexes, triphenylphosphine derivatives or 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO) and both electrochemical or mass spectrometric
techniques.\textsuperscript{78-81}

In 2010, Lippard developed the first small-molecule fluorescent probe selective for HNO, a Cu\textsuperscript{II} complex of a boron-dipyrrromethene (BODIPY) reporter linked to a tripodal dipicolylamine-appended receptor via a triazole bridge (Cu\textsuperscript{II}[BOT1], Scheme 6).\textsuperscript{82} The BODIPY dye has relatively long-wavelength absorption and emission properties suitable for cellular imaging and the metal free organic moiety BOT1 displays typical BODIPY optical properties with a maximum emission at 526 nm.\textsuperscript{82} Introduction of Cu\textsuperscript{II} immediately quenches the fluorescence via photoinduced electron transfer (PET) from the BODIPY singlet excited state to the bound Cu\textsuperscript{II} ion.\textsuperscript{82} Addition of excess AS to Cu\textsuperscript{II}[BOT1] under physiological conditions restores emission inducing a 4.3-fold fluorescence increase revealing HNO detection with significant emission turn-on (Scheme 6).\textsuperscript{82} Further analysis suggests that this probe senses HNO in the 0.5 to 5 mM range and displays selectivity over other biological relevant species such as NO, NO\textsubscript{3}\textsuperscript{-}, NO\textsubscript{2}\textsuperscript{-}, ONOO\textsuperscript{-}, H\textsubscript{2}O\textsubscript{2} or OCl\textsuperscript{-}.\textsuperscript{82-83} The probe detects HNO in biological systems as HeLa cells incubated with Cu\textsuperscript{II}[BOT1] show only faint intracellular fluorescence but introduction of AS induces an increase of intracellular fluorescence.\textsuperscript{82} Similar experiments with an NO donor do not enhance fluorescence.\textsuperscript{82}
Scheme 6. Structure and mechanism of the HNO reaction for Cu\(\text{II}\)[BOT1].

After the initial report, various improved Cu\(\text{II}\) based fluorescent HNO probes including Cu\(\text{II}\)[COT1], Cu\(\text{II}\)[COET], a series of Cu\(\text{II}\)-benzoresorufin complexes, Cu\(\text{II}\)[DHX1] and a solid-phase prepared modular, lysine-based platform Cu\(\text{II}\) complexes have been prepared and investigated.\(^8\) However, as this type of probe relies on the reducing power of HNO, other biological reductants may reduce Cu\(\text{II}\) in these complexes yielding fluorescence and generating false positives. Treatment of Cu\(\text{II}\)[BOT1] with cysteine restores the emission of BOT1 and positive ion electrospray mass spectrometry shows the reduced species as the Cu\(\text{I}\) complex.\(^8\) The lack of a substantial fluorescence signal in HeLa cells incubated with Cu\(\text{II}\)[BOT1] suggests normal levels of intracellular cysteine and other thiols do not reduce this complex.\(^8\)

HNO reacts with 4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl (TEMPOL) through a hydrogen abstraction from HNO by the nitroxide radical (Scheme 7).\(^4\) Based on this reaction, Toscano designed a nitroxide based pre-fluorescent probe 4-((9-acridinecarbonyl)amino)-2,2,6,6-tetramethylpiperidin-1-oxyl (TEMPO-9-AC) that demonstrates weak fluorescence.\(^7\) Introduction of HNO from various sources transforms
TEMPO-9-AC to the highly fluorescent TEMPO-9-AC-H and this probe displays selectivity for HNO over NO in aqueous solution (Scheme 7).\textsuperscript{79} The use of TEMPO-9-AC may be complicated by intermolecular fluorescence quenching and competitive HNO trapping by the nascent NO (a potential drawback in the Cu\textsuperscript{II} probes as well) limiting its use in complicated biological environments.

![Scheme 7](image)

\textbf{Scheme 7}. The reaction of HNO with TEMPOL and TEMPO-9-AC.

Membrane inlet (or introduction) mass spectrometry (MIMS), a technique for gas detection since 1963, also detects HNO. In 2011, Toscano re-engineered and modified the membrane inlet following the original design of Silverman.\textsuperscript{80} This specific analytical technique has been successfully applied to monitor HNO generated from AS and 2-bromo-Piloty's acid (2-BPA) in aqueous solution by detecting NO\textsuperscript{+}, HNO\textsuperscript{+} and N\textsubscript{2}O\textsuperscript{+}, the respective ions of the ions of NO, HNO and N\textsubscript{2}O.\textsuperscript{80} Careful control experiments distinguish these species and allow for real-time HNO detection.\textsuperscript{80}
Electrochemical HNO detection represents another area of rapid and extensive research and progress in the identification of HNO. In 2010, Doctorovich developed a gold electrode with a cobalt porphyrin covalently attached through Au-S bonds. The CoII 5,10,15,20-tetrakis[3-(p-acetyltiopropoxy)phenyl]-porphyrin modified electrodes were thoroughly characterized and electrochemical methods demonstrate its ability to discriminate HNO from NO. This method allows time-resolved electrochemical quantification of HNO with a linear response in transient HNO concentration as low as 1 to 1000 nM. Control experiments indicate that several common biological species do not affect electrode performance, although the presence of O2 leads to a notable decrease in HNO concentration.

Organophosphines have long been known to react with electrophilic nitroso compounds to afford the corresponding ylides and phosphine oxides. In 2009, our group discovered HNO also reacts with phosphines in a similar manner. For instance, triphenylphosphine rapidly reacts with HNO to give an equal amount of triphenylphosphine oxide and an azaylide, which can serve as a reactive nucleophile allowing possible modified phosphine reagents as potential HNO markers (Scheme 8). New phosphine compounds have enabled HNO detection with the help of phosphorus NMR, UV-vis and colorimetric detection. In Chapter 2, we will detail the chemical basics of using phosphines to trap HNO and the design, synthesis and evaluation of a phosphine-based fluorescent probe for HNO detection.

Scheme 8. Reaction of HNO with triphenylphosphine

\[
\text{H-NO} \xrightarrow{2 \text{PPH}_3} \text{O=PPH}_3 + \text{HN=PPH}_3
\]
Similarly, S-nitrosothiols also react with organophosphines.\textsuperscript{91} In 1972, Haake reported the reaction of S-nitroso triphenylmethanethiol with triphenylphosphine produces triphenylphosphine oxide and the corresponding S-azaylide as an isolable solid (Scheme 9).\textsuperscript{91} In 2010, our group repeated this reaction and provided the first X-ray crystallographic and \textsuperscript{31}P NMR characterization of this S-azaylide.\textsuperscript{94} Xian’s work revealed that S-azaylides possess similar reactivity compared to the azaylde derived from HNO.\textsuperscript{95} This cross-reactivity renders the detection of HNO with phosphine based probes potentially unreliable in the presence of biologically abundant S-nitrosothiols. Chapter 3 investigates this problem and addresses this concern by direct comparing the reactivity of these two ylides.

![Scheme 9](image)

**Scheme 9.** Reaction of S-nitroso tritylthiol with triphenylphosphine

### 1.3.4 HNO Donors

Compounds that release HNO demonstrate unique biological properties especially in terms of cardiac muscle contractility and show promise as new therapeutics for congestive heart failure. A need exists to both develop improved HNO donors for basic studies and therapeutics and to better understand endogenous HNO production. Currently, two popular strategies exist for HNO release: disproportionation/oxidation of hydroxylamine derivatives with good leaving groups attached to the nitrogen atom (\textit{type a}, Scheme 10), while the other is the decomposition of nitroso compounds from nucleophilic attack (\textit{type b}, Scheme 10).\textsuperscript{96} For hydroxylamine derivatives (\textit{type a}), the nitrogen atom
possesses a formal -1 oxidation state and disproportionates to HNO (+1 oxidation state). Transformation of nitroso compounds (type b) to HNO usually occurs through hydrolysis or some type of displacement reaction. Traditional donors such as Angeli’s Salt, isopropylamine diazeniumdiolate (IPA/NO) and its analogs, Piloty’s acid and cyanamide are type a donors while acyl and acyloxy nitroso compounds release HNO via a type b mechanism. Inspired by the potential of endogenous HNO production and its promising pharmaceutical application, efforts have been made to generate physiologically useful donors/prodrugs that release HNO in a controlled, sustainable and tunable fashion.

Scheme 10. Chemical strategies for HNO generation.

Developed in 1896, sodium trioxodinitrate (Na₂N₂O₃, Angeli’s Salt or AS) remains the most widely used HNO donor. The production of HNO from AS follows a typical type a mechanism with nitrite as leaving group (Scheme 11). The decomposition occurs in a first-order and pH-dependent fashion. At pH 4–8, AS decomposes to generate HNO and nitrite with a rate constant of \( k = 4.6 \times 10^{-4} \text{ s}^{-1} \) at 37 °C. Under basic conditions (pH > 8), the decomposition rate is negligible due to the difficulty in \( \text{N}_2\text{O}_3^- \) protonation. When pH < 4, AS generates NO instead as the only nitrogen-containing product. AS can be easily synthesized and has a short half-life (~2.8 min) at physiological pH. Thus, AS only allows the short-term properties of HNO to be examined.
Scheme 11. Decomposition of AS under physiological pH.

IPA/NO represents an organic analog of AS. Different from its secondary amine analog diethylamine NONOate (DEA/NO), the primary amine derivative releases both HNO and NO at a rate constant comparable to AS at pH 7.4.\textsuperscript{96, 100} IPA/NO evolves only HNO at a pH above 8 and generates only NO below pH 3.\textsuperscript{101} In addition, IPA/NO only effectively acts as an HNO donor in vivo via a thiol sensitive reductive nitrosylation of ferric Mb.\textsuperscript{96, 101} Scheme 12 gives the proposed mechanism for the generation of HNO based on theoretical calculations and further chemical analysis.\textsuperscript{96, 102-103} Different from AS, which is an easily synthesized inorganic salt that has not been successfully structurally modified, the organic character of IPA/NO allows the preparation of derivatives with versatile HNO releasing properties. Over the years, a series of more stable, easily purified IPA/NO derivatives have been prepared with controllable HNO releasing properties and half-lives that range from minutes to hours.\textsuperscript{104-108} These compounds demonstrate promising physiological effects and may serve as leads for potential therapeutics for cardiovascular diseases and cancer.\textsuperscript{104-108}
Scheme 12. pH-Dependent decomposition of primary amine NONOates.

Piloty’s acid (PA) is another well-known HNO donor that finds less use in biological experiments due to its relatively low production of HNO at physiological pH. PA only generates HNO at comparable rates to AS under basic conditions (Scheme 13). Structural modification of PA can greatly improve HNO release and a library of PA derivatives with various substitutions at different positions or based on different aromatic heterocycles has been reported. Some of these derivatives release significant amounts of HNO at neutral pH such as 2-BPA and 2-nitro-PA. Besides aromatic substitutions, N-,O-diacylated or alkylated derivatives of PA have been prepared as HNO prodrugs that first generate acyl nitroso compounds that release HNO through hydrolysis. In addition, a library of N-,O-bis-acylated N-hydroxysulfonamide derivatives with tunable half-lives (minutes to hours) demonstrates efficient HNO release at physiological pH. Knaus prepared ethanesulfohydroxamic acid esters of indomethacin, (S)-naproxen, and ibuprofen as potential clinical prodrugs. Recently, Toscano reported a new series of highly tunable HNO donors based on N-substituted hydroxylamines with carbon-based leaving groups including barbituric acid and pyrazolone. These new structurally diverse HNO donors quantitatively produce HNO with extended half-lives holding promise as pharmacological tools and therapeutics.
Nitroso compounds are a relatively new type of HNO precursors including the acyl nitroso and the acyloxy nitroso compounds. Acyl nitroso compounds are highly reactive species and no stable acyl nitroso compounds have been successfully isolated.\textsuperscript{96} Traditional strategies for acyl nitroso compound formation include oxidation of hydroxamic acids or hydroxyureas by either chemical oxidants or enzymatic systems, such as catalase or horseradish peroxidase (HRP).\textsuperscript{116-117} Recently, a group of photo cleavable HNO donors based on acyl nitroso precursors have been reported to release HNO in a photo-controllable and tunable pattern.\textsuperscript{118-121} Acyloxy nitroso compounds represent another type of recently developed HNO donor. Acid or base catalyzed cleavage of the ester group leads to an unstable nitroso alcohol intermediate that quickly decomposes to HNO and ketone (Scheme 14).\textsuperscript{122} The original report on 1-nitrosocyclohexyl acetate (NCA) revealed the formation of HNO in a mixture of methanol and neutral phosphate buffer.\textsuperscript{122} NCA is relatively stable under buffered conditions but quickly hydrolyzes under basic conditions.\textsuperscript{122} Structural modification of NCA allows the improvement of its HNO releasing properties.\textsuperscript{122} Recently, our lab engineered several NCA derivatives with improved water solubility and various half-lives.\textsuperscript{122-125}
Metalloproteins play important roles in HNO chemistry and metal nitrosyl complexes (MNO) represent an alternative type of HNO-releasing agents. Harrop reported the first potentially useful MNO for HNO delivery.\textsuperscript{126} The \{CoNO\}\textsuperscript{8} complex in a pyrrole/imine ligand frame is relatively stable at physiological pH but addition of stoichiometric amounts of acid releases HNO.\textsuperscript{126} Later, a ruthenium complex, trans-[Ru(NO)(NH\textsubscript{3})P(O')(OEt)\textsubscript{2}](PF\textsubscript{6})\textsubscript{2}, has also been identified as a potential NO/HNO donor in aqueous media.\textsuperscript{127}

**1.3.5 Proposed Endogenous Sources of HNO**

Despite the recently described interest in HNO biochemistry, pharmacology and physiology, endogenous production of HNO remains questioned and poorly understood. Due to the previous limitations in HNO detection, only proposed pathways of endogenous HNO formation exist including NOS-catalyzed reactions, oxidation of hydroxylamine-derivatives, reductions of NO, and the direct reaction of S-nitrosothiols with nucleophiles.\textsuperscript{128-130} The development of new methods of HNO detection in the last few years greatly aids the identification of endogenous HNO formation. Recently, several pathways including the reaction of NO and hydrogen sulfide (H\textsubscript{2}S), reactions of RSNO with H\textsubscript{2}S and proton coupled NO reduction have been proposed for potential HNO

\textbf{Scheme 14.} Generation of HNO by acyloxy nitroso compounds.
Early work by Moore shows the addition of H$_2$S to different NO donors scavenges released NO or blocks NO release possibly forming HSNO. Bian similarly reports that administration of NO donors and H$_2$S (as NaSH) to cardiac myocytes elicits distinct responses compared to either the NO donor or NaSH alone. Specifically, NaSH does not affect myocyte contractility and three mechanistically distinct NO donors including sodium nitroprusside (SNP) decrease contractility (negative inotropic effect). Addition of both NaSH and the NO donors, however, yields an increase in myocyte contractility (positive inotropic effect). The SNP + NaSH system also increases the resting calcium level in the cell, suggesting increased calcium cycling within these cells. These results mimic the effects of HNO on cardiac myocytes leading to the suggestion that the reaction of NO and H$_2$S generates HNO presumably via direct displacement of thionitrous acid (HSNO) by H$_2$S.

By using a combination of various spectroscopic and chemical methods as well as pharmacological measures, Filipovic further defined HNO release from SNP upon reaction with H$_2$S. Using a copper-based fluorescence HNO probe, these experiments show HNO generation in human umbilical vein endothelial cells (HUVECs) treated with both SNP and H$_2$S. Additionally, only the combination of SNP and H$_2$S result in the release of CGRP, a potent vasodilator and known HNO marker in an isolated mouse heart model.

Recently, Filipovic showed the reaction of H$_2$S and NO directly produces HNO using the newly developed HNO electrode for detection as well as following the
disappearance of NO and H₂S electrochemically. Experiments indicate HNO formation in dorsal root ganglion (DRG) neurons using a copper-based HNO fluorescent probe from enzymatically generated NO and H₂S. Fluorescence decreases upon addition of inhibitors of either NOS or cystathione β-synthetase (CBS) inhibitors or by depleting the substrates L-arginine or L-cysteine revealing the requirement of both NO and H₂S for HNO formation. The combination of NO and H₂S also causes CGRP release in this system and these results provide some of the strongest evidence for endogenous HNO formation arising from the reaction of enzymatically generated NO and H₂S. The HNO generated in the DRG neurons reacts with the transient receptor potential channel A1 (TRPA1) resulting in an increase in calcium flux that leads to CGRP release and subsequent local and systemic vasodilation. This work provides some of the strongest evidence for endogenous HNO formation arising from the reaction of enzymatically generated NO and H₂S and clearly defines an HNO-TRPA1-CGRP pathway that describes HNO’s biological effects.

Similarly, the reactions of RSNO with H₂S have garnered increasing attention as a pathway to HNO formation. In 2012, Filipovic reported the generation of HSNO via pulse radiolysis, treatment of H₂S with acidified nitrite and the trans-nitrosation of S-nitrosoglutathione (GSNO) with H₂S. The product of the last reaction was characterized by high resolution MS, infrared spectroscopy (IR) and ¹⁵N nuclear magnetic resonance spectroscopy (NMR) spectroscopy to provide evidence of HSNO formation. Further experiments show that HSNO decomposes to yield NO, likely through S-N bond homolysis, and HNO as judged by methemoglobin trapping and identification of nitrous oxide and hydroxylamine. Treatment of HUVECs with GSNO and H₂S yields HNO
within the cells as judged by a copper-based fluorescent probe. Filipovic extended this work showing that nitrite reacts with H\textsubscript{2}S to form HSNO and HNO in the presence of water soluble ferric iron porphyrin complexes. The combination of nitrite and H\textsubscript{2}S applied to HUVECs generates more NO (than nitrite alone) and HNO localized to the mitochondria.

Feelisch has also examined the chemistry and biology of the reaction of NO or RSNO with H\textsubscript{2}S and found that similar to previous reports the mixture of NO and H\textsubscript{2}S elicits modified biological effects compared to either NO or H\textsubscript{2}S. However, Feelisch proposes a different mechanism including the involvement of nitrosopersulfide (\textsuperscript{−}SSNO), polysulfides (HS\textsubscript{n}) and dintrosulfite (or N-nitrosohydroxylamine N-sulfonate) (SULFI/NO). Despite the disagreement, this enforces HNO formation in these reactions. Clearly chemical and biological “crosstalk” between NO and H\textsubscript{2}S exists that likely includes HNO but questions remain regarding the chemistry.

New exciting work shows the ability of biologically relevant alcohols to reduce NO to HNO through a process termed proton coupled nucleophilic attack. Addition of various alcohols including ascorbic acid, tyrosine and hydroquinone to a solution of NO results in HNO formation as determined by electrochemical methods. Both N\textsubscript{2}O and nitrite form during these reactions presumably from HNO dimerization or the reaction of HNO with NO. Treatment of endothelial and macrophage cells that produce NO with excess ascorbic acid shows evidence of HNO formation by fluorescence and electrochemical detection, respectively. Extended work shows the ability of other biologically relevant phenols including vitamin E (tocopherol) and the drugs acetaminophen and salicylic acid facilitate the conversion of NO to HNO. Overall,
these results provide a unique potential endogenous pathway for the conversion of NO to HNO in the presence of these “reducing” alcohols and theoretically make any NO source a source of HNO.

1.4 CONCLUSION

The chemistry, biochemistry and physiology outlined in this chapter clearly demonstrate that HNO is a biologically unique species with significant physiological effects and largely undeveloped therapeutic potentials. As detection still impedes ongoing work in this field, we continue to work on the development of a reliable and robust detection and quantification approach for a better understanding of HNO. Earlier work done in our lab has discovered the methodology of triarylphosphine mediated Staudinger ligation to trap HNO.\textsuperscript{92-93} We now move on to the design and development of sensitive and selective fluorescent HNO probes that are suitable for biological application. The successful development of this probe will advance the development of new HNO sources, understanding of endogenous HNO formation and establishment of its full chemical and physiological profile.

Chapter 2 details the design, synthesis and chemical basis of the new fluorescent probe \textbf{1b} for HNO. These studies, published in 2015, demonstrate this probe successfully detects HNO from various sources leading to HNO-dependent fluorescence both in test tube and in HeLa cells (Scheme 15).\textsuperscript{141} Subsequent studies examine its selectivity for HNO over other biological relevant species. The fluorescence generation mechanism in cells is also confirmed by HPLC-MS analysis of cell lysates.
**Scheme 15.** Detection of HNO by a phosphine-based fluorescent probe.

The investigations described in Chapter 3 specifically compare the reductive ligation-based detection strategies for HNO and RSNO. These studies show that azaylides derived from HNO or organic RSNO efficiently undergo reductive ligation while S-azaylides derived from biological RSNO containing free amine and carboxylic acid groups primarily yield phosphine oxides, confirming phosphine based fluorescent probes are selective for HNO in biological systems (Scheme 16).
Lastly, as RSNO represents an important post-translational modification of protein thiols, we proposed the potential existence of $S$-nitrosobacillithiol (BSNO), the corresponding $S$-nitrosothiol of the major low-molecular-weight (LMW) thiols in Bacillus. Starting with $D$-glucosamine, $L$-malic acid and a protected version of cysteine, we attempted the synthesis and characterization of BSNO (Scheme 17).
Scheme 17. Preparation of BSNO.
CHAPTER 2

A SELECTIVE PHOSPHINE-BASED FLUORESCENT PROBE FOR
NITROXYL IN LIVING CELLS

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A portion of the work contained in this chapter was initially published in Bioorganic &
Medicinal Chemistry Letters in 2015. The manuscript, including figures, schemes and
supporting information, was drafted by Zhengrui Miao and edited by S. Bruce King and
Ming Xian. Since the publication, changes in both format and content were made to
adapt this work for the dissertation format. The research described herein was
performed by Zhengrui Miao with the exception of the cell, confocal microscopy and
following mass spectrometry work was done by Julie A. Reisz and Susan M. Mitroka.
The probe (1b) was first synthesized by Jia Pan.
As discussed in the Introduction of this dissertation, the discovery and establishment of NO as an important signaling agent involved in many physiological processes including blood pressure control, neurotransmission, and the immune response initiated the further study of the biological roles of many redox-related nitrogen-containing compounds. \textsuperscript{3-5, 20, 143} HNO, the one-electron reduced and protonated derivative of NO, possesses distinguishable physiological and pharmacological properties from NO. \textsuperscript{36, 38, 41} HNO-releasing prodrugs increase cardiac inotropy and lusitropy and elicit arterial and venous dilation without building tolerance, properties that make these compounds intriguing candidates for the treatment of congestive heart failure. \textsuperscript{60, 62, 67, 144-148} Such biological properties drive the search for new HNO donors and detection methods as well as the definition of an endogenous biochemical pathway of HNO formation. \textsuperscript{66, 70, 96, 104, 111, 113, 122, 149-150}

Given the fact that HNO rapidly dimerizes and dehydrates to yield N\textsubscript{2}O, a full chemical and biological understanding of HNO depends on fast, reliable and selective detection. Development of new HNO donors and understanding endogenous HNO production requires robust HNO detection methods. Early methods of HNO detection (e.g., identification of N\textsubscript{2}O, trapping with thiols or ferric heme proteins) either lack the sensitivity or the selectivity to unambiguously demonstrate endogenous HNO formation. \textsuperscript{35, 38} These limitations have led to the development of several new detection strategies, including a series of fluorescent probes based on Cu\textsuperscript{II} complexes or TEMPO and some electrochemical or mass spectrometry based techniques. \textsuperscript{78-81}
2.1 PHOSPHINE REACTIVITY WITH NITROSO COMPOUNDS

Nitroso compounds are electrophilic and known to react with various nucleophiles at the polar N=O bond. For example, triphenylphosphine reduces $\alpha$-nitroso-$\beta$-napthol to give phosphine oxide and an azaylide.\textsuperscript{90} In addition to C-nitroso compounds, triphenylphosphine also reacts with trityl S-nitrosothiol in a similar pattern, generating S-azaylide and triphenylphosphine oxide in equal amounts.\textsuperscript{91} By viewing HNO as the simplest nitroso compound and its known reactivity with soft nucleophiles, our group first reported the reaction of HNO with organic phosphines.\textsuperscript{92} Triphenylphosphine reacts with HNO generated from AS (2:1 ratio) to give equal amounts of triphenylphosphine oxide and azaylide, a Staudinger reduction intermediate, that quickly hydrolyzes to the corresponding phosphine oxide.\textsuperscript{92} Nitroso compounds are believed to react with phosphines via a nitroso compound addition product that may be represented as hetero three-membered ring intermediate. The direct reduction of the intermediate by another phosphine would produce the corresponding phosphine oxide and azaylide in a 1:1 ratio (Scheme 18)\textsuperscript{92, 95}

\[
R_1\text{-N}=O \quad : PR_3 \quad \xrightarrow{} \quad \left[ \begin{array}{c} R_1\text{-N}\text{-O} \\ P \\ R_3 \end{array} \right] \quad \xleftrightarrow{} \quad \left[ \begin{array}{c} ^{+}PR_3 \\ R_1\text{-N}\text{-O} \\ R_3 \end{array} \right] \quad : PR_3 \quad \xrightarrow{} \quad R_1\text{-N}=PR_3 \quad + \quad O=PR_3
\]

Scheme 18. Trapping nitroso compounds with phosphine.

Azaylides produced from these reactions are reactive nucleophilic intermediates that can be possibly captured by Staudinger ligation forming the basis of a new HNO detection strategy. An electrophilic ester adjacent to the phosphine allows the intramolecular nucleophilic attack from the azaylide, producing a stable and unique
HNO-derived amide that can be tracked for HNO identification and quantification. Scheme 19 depicts a triaryl phosphine trapping HNO through reductive Staudinger ligation with an appropriately positioned electrophilic group.\textsuperscript{92} Reaction of phosphine (1) with HNO first generates an equal amount of phosphine oxide (2) and azaylide (3), which will be trapped by the adjacent electrophilic ester group forming a tetrahedral intermediate.\textsuperscript{92} The decomposition of the species yields a free alcohol (5) and a phosphonium ion that hydrolyzes to benzamide phosphine oxide (4) as the final product (Scheme 19).\textsuperscript{92} The benzamide (4) is a unique product from the process and represents a stable and chemically distinct marker of HNO. In addition, the alcohol release process provides new perspective for the further design and development of colorimetric and fluorescent HNO probes.\textsuperscript{92-93} Based on this strategy, a series of 2-(diphenylphosphino)benzoic acid derived esters (1) including phenyl 2-(diphenylphosphino)benzoate (1a, Scheme 19) have been intensively evaluated for HNO detection and analyzed by $^{31}$P NMR, UV-Vis or HPLC.\textsuperscript{92-93}
2.2 RESULTS AND DISCUSSION

Organophosphines are suitable for HNO detection based on their documented ligation reaction with HNO, their rapid rate of HNO trapping and lack of cross-reactivity with other physiologically relevant nitrogen oxides, such as NO, nitrite, nitrate, and peroxynitrite. In light of this phosphine mediated ligation chemistry described for HNO, we envisioned the development of phosphine probes with fluorophore leaving groups. In the presence of an internal electrophilic ester, these azaylides undergo Staudinger ligation to yield the thermodynamically stable amide (4, Scheme 20) and the corresponding ester-derived alcohol. Based on this mechanism, we designed and synthesized 1b, which produces HNO-dependent fluorescence by generating the known

Scheme 19. Phosphorus-mediated Staudinger ligation.
fluorophore, fluorescein monomethyl ether (5b, Scheme 20).

Scheme 20. Proposed mechanism of 1b with HNO.

Compound 1b was prepared by using the straightforward DCC coupling strategy of 2-(diphenylphosphino)benzoic acid with fluorescein methyl ether, a previously reported fluorescein derivative, in 61% yield (Scheme 21).  

Scheme 21. Synthesis of 1b.

We first investigated the feasibility of 1b to detect HNO in buffered solutions. In the initial ligation experiment, solutions of 1b (40 μM) were incubated with increasing concentrations of AS (0-5 eq.) in 1:3 MeCN:phosphate buffered saline (PBS, containing 0.1 mM EDTA, pH 7.4) at ambient temperature and excitation at 465 nm led to a concentration-dependent increase in emission intensity at 520 nm with a 73.8-fold
maximum response observed at 5 eq. AS (Figure 1, Panel A). The solution immediately changed from clear to yellow with more intense hues at higher AS concentrations, and fluorescence intensity remained unchanged after 2 h (Figure 1, Panel B and C). Control experiments with increasing amounts of nitrite, the by-product of AS decomposition, and 1b (40 μM) do not generate a fluorescence response, illustrating the response depends on the HNO mediated release of the fluorophore.
**Figure 1.** Fluorescence response of 1b toward AS/NaNO₂.

Panel A: Fluorescence responses of 1b (40 μM) to 0.005, 0.05, 0.5, 5 eq. of AS or NaNO₂ in CH₃CN/PBS after 2 h incubation at room temperature. Panel B: The ligation induces a distinct color change. Panel C: Fluorescence response of 1b (40 μM) to AS (200 μM) in CH₃CN/PBS over 2 h incubation at room temperature.

The commercial availability, water solubility and rapid HNO release rate make AS the donor of choice for most chemical and biological studies, including recent studies regarding HNO detection with both phosphine and Cu²⁺-based fluorophores. Treatment of 1b with 4-bromo Piloty’s acid (4-BPA), a structurally distinct HNO donor, also results in fluorescence enhancement proportional to 4-BPA concentration (Figure 2). The slower continual release of HNO from this compound may better mimic its endogenous production and these results demonstrate HNO detection by 1b from a source besides AS.
Figure 2. Fluorescence response of 1b (40 μM) to 0.005, 0.05, 0.5, 5 eq. of 4-BPA in CH₃CN/PBS after 2 h incubation at room temperature.

Before attempting HNO detection in cells, 1b was assessed for the selectivity toward HNO compared to other biological redox species. Figure 3 shows the comparative fluorescence response of 1b to excess amounts (200 eq.) of NO, NO₂⁻, NO₃⁻, H₂O₂, H₂S, GSH, GSNO and S-nitrosocysteine (CysNO). NO and a series of oxidants (NO₂⁻, NO₃⁻, H₂O₂) gave no or little fluorescence response. Formation of the phosphine oxide (2b) by treatment of 1b with H₂O₂ does not yield a fluorescence response indicating that oxidation of these probes does not produce a false fluorescence response. Additionally, 1b does not react with the biological reductants (H₂S and GSH), which may reduce the Cu⁺⁺-based probes giving potential false positives.⁸²⁻⁸⁴ These results reinforce the general selectivity of these agents for HNO.
Given the ability of phosphines to react with $S$-nitrosothiols, 1b (40 μM) was treated with HNO, GSNO and CysNO (2 eq.) to determine the relative fluorescence response under identical conditions. Reaction of 1b with HNO yields a 58.5-fold increase in fluorescence while incubation with GSNO and CysNO only gives a 2.3 and 3.2-fold increase, respectively (Figure 4). These results clearly demonstrate the difference in fluorescence response and relative selectivity. A deep investigation regarding the reaction difference will be discussed in Chapter 3.

**Figure 3.** Fluorescence responses of 1b (40 μM) in CH$_3$CN/PBS at room temperature for 2 h after addition of 200 eq. of GSH, H$_2$O$_2$, NaNO$_2$, NaNO$_3$, Na$_2$S, DEA/NO, GSNO, CysNO, AS.
Figure 4. Fluorescence response of 1b (40 μM) to 2 eq. of GSNO/CysNO/AS in CH₃CN/PBS after 2 h incubation at room temperature.

After determining the ability of 1b to detect HNO in vitro, we applied this probe for the detection of HNO in cells. Treatment of HeLa cells with 1b (12.5 μM) for 10 min gives little or no background signal as judged by fluorescence microscopy (Figure 5a). Control experiments show a slow increase in background fluorescence during longer (30 min) incubation, presumably due to esterase-mediated hydrolysis. Addition of AS to the cells immediately initiated intracellular fluorescence (Figure 5b). These findings highlight the ability of 1b to detect HNO within a cellular environment.
**Figure 5.** HNO-induced fluorescence images of HeLa cells treated with 1b (12.5 μM) after 10 min (a), then treated with AS (500 μM) followed by 30 min incubation (b): (top) confocal image and (bottom) bright field. Scale bars represent 20 μm.

Following fluorescence microscopy, the control and AS-treated HeLa cells were lysed and the extracted contents analyzed by mass spectrometry to confirm the conversion of 1b to the expected products, particularly HNO-derived amide 4 (Figure 6). Orbitrap MS analyses of the AS-treated cells show the formation of the phosphine oxide amide (4, [M+H]+ 322.0991) and mono-methylated fluorescein (5b) in the cell lysates, confirmed by comparison to authentic standards. Control experiments using cells not treated with AS show the presence of 5b, possibly from background hydrolysis, but no evidence of 4 (Figure 6). As 4 results from the HNO-mediated ligation of 1b, this finding confirms the ability of 1b to trap HNO within cells and further validates 4 as an HNO marker. Identical experiments using $^{15}$N-AS, which generates H$^{15}$NO, followed by MS reveals that formation of $^{15}$N-4 and 5b further confirming HNO as the nitrogen source in
and the ability of 1 to trap HNO within cells via the proposed mechanism (Scheme 18).
Figure 6. LC-MS analysis of cell lysates.

A) phosphine oxide amide 4 synthetic standard, theoretical [M+H]$^+$ m/z 322.0991; B) lysate from cells treated with 1b (12.5 μM), theoretical [M+H]$^+$ m/z 322.0991 (4), 347.0914 (5b), 323.0822 (phosphine oxide carboxylic acid formed from the hydrolysis of 2b; C) Lysate from cells treated with 1b (12.5 μM) and AS (0.5 mM); D) Lysate from cells treated with 1b (12.5 μM) and NaNO$_2$ (0.5 mM); E) lysate from cells treated with 1b (12.5 μM) and $^{15}$N-AS (0.5 mM), theoretical [M+H]$^+$ m/z for $^{15}$N-4 323.0962; F) lysate from cells treated with 1b (12.5 μM), AS (0.5 mM) and Na$^{15}$NO$_2$ (0.5 mM).

Compound 1b represents the first generation phosphine based fluorescent probe directly derived from 2-(diphenylphosphino)benzoic acid. The above-mentioned work demonstrates its ability to detect HNO following the anticipated mechanism; however, a slow hydrolysis was observed in cell experiments presumably due to the esterase mediated cleavage of the ester structure that releases the fluorescence. We speculated a
carbamate backbone should circumvent the esterase catalyzed hydrolysis, providing a better fluorescent probe for HNO. Hence, we designed and synthesized a second generation probe 6 with a carbamate backbone (Scheme 22).

![Scheme 22. New fluorescent probe 6 with improved esterase resistance.](image)

As an analog of a previously reported 4-nitrophenyl (2-(diphenylphosphino)ethyl)carbamate, probe 6 can be synthesized in a similar approach.\textsuperscript{93} The fluorescein methyl ether chloroformate can be obtained by reaction of fluorescein methyl ether and triphosgene. A subsequent nucleophilic attack from 2-(diphenylphosphino)ethylamine affords the desired product in 15% yield as a stable pale yellow powder (Scheme 23).

We next examined the ability of 6 to detect HNO *in vitro*. Similar to earlier experiments, solutions of 6 (40 μM) were incubated at ambient temperature and the fluorescence of the solution was recorded every 5 minutes over 2 hours (Figure 7). As anticipated, addition of AS (5 eq.) under physiological conditions induces significant fluorescence increase (green, Figure 7). Surprisingly, the control experiment reveals that 6 is unstable in aqueous solution and spontaneously hydrolyzes to produce fluorescence in the absence of AS (purple, Figure 7). Interestingly, increased acidity of the buffered solution seems to slow down the self-hydrolysis (red, Figure 7) but also decelerates the HNO induced ligation process (blue, Figure 7). This spontaneous hydrolysis makes 6 an unsuitable HNO probe and further *in vivo* examination seems unnecessary.
**Figure 7.** Fluorescence change of 6 (40 μM) incubated in pH 7.4 buffer (blue), pH 6.2 buffer (red), or with 2 eq. AS in pH 7.4 buffer (purple), pH 6.2 buffer (green) over 2 h.

Besides 6, this hydrolysis process was also observed with its previously reported analogs phenyl (2-(diphenylphosphino)ethyl)carbamate and 4-nitrophenyl (2-(diphenylphosphino)ethyl)carbamate.\(^9\) To troubleshoot this problem, we rationalized that a phosphorus facilitated hydrolysis pathway is responsible for the instability of 6 (Scheme 24). Intramolecular cyclization has been widely used for prodrug activation where a nucleophilic atom nitrogen or oxygen plays a key role.\(^1\) Phosphorus, with a better nucleophilicity, should also facilitate this hydrolysis. In our proposed mechanism, the intramolecular nucleophilic attack on the carbamate leads to the formation of a five-membered ring intermediate that decomposes to give the leaving group and 7 as the final product. LC-MS analysis of the solution of 6 in pH 7.4 buffer detects a peak with a
mass/charge ratio of 274 ([M+H⁺]) that corresponds to 7 from the solution, providing support for the proposed mechanism.

![Reaction Scheme 24](image)

**Scheme 24.** Phosphorus facilitated hydrolysis of 6.

Noticing that a five-membered ring nucleophilic attack is key step for hydrolysis, an idea to overcome this issue is simply shortening the carbon chain. Scheme 25 outlines a series of different compounds designed under this idea. However, efforts to prepare them consistently failed presumably due to these compounds’ instability as the carbon between the phosphorus and nitrogen would be hydrolytically sensitive (at the acetal oxidation state).
2.3 CONCLUSION

In conclusion, the triarylphosphine based HNO probe (1b) was designed and synthesized to produce a fluorescent response based on the fast phosphine/HNO chemistry previously reported. HNO donors, including AS and 4-BPA, activate 1b with a concentration dependent increase in fluorescence intensity. Probe 1b is highly sensitive and selective for HNO detection under physiological conditions. Fluorescence microscopy experiments demonstrate that 1b detects release of HNO from AS in HeLa cells. Subsequent MS analyses of these cell lysates identify the amide phosphine oxide (4), a distinct HNO-derived product, whose detection confirms the suggested mechanism. The successful detection of HNO in HeLa cells with 1b offers an approach for the screening of endogenous HNO sources. Synthetic efforts to overcome the potential esterase mediated hydrolysis in cells were unsuccessful due to the inherent instability of

![Scheme 25. Structures of improved potential probes.](image-url)
designed compounds.

2.4 EXPERIMENTAL METHODS

General

Reagents were obtained from commercial sources and used without additional purification. AS was prepared as described and stored at -20 °C. Reaction solvents were anhydrous and purged with argon prior to use. Extraction, silica, and preparative reverse phase chromatography solvents were technical grade. LC-MS and electrospray ionisation (ESI)-MS solvents were Optima HPLC grade. Analytical thin layer chromatography (TLC) was performed on silica gel plates (normal phase) or C18 silica gel plates (reverse phase), and visualization was accomplished with UV light. £H NMR spectra were recorded on Bruker Avance DPX-300 and DRX-500 instruments at 300.13 and 500.13 MHz, respectively. £3C NMR spectra were recorded on the described instruments operating at 75.48 and 125.76 MHz, respectively. £3P NMR spectra were recorded on the described instruments operating at 121.49 MHz and 202.46 MHz, respectively. £3P chemical shifts are referenced to 85% H3PO4 (δ = 0 ppm) in a concentric internal capillary (Wilmad). NMR spectra were obtained using Bruker 5 mm BBO and QNP probes held at 25 °C. Low-resolution mass spectra were obtained using an Agilent Technologies 1100 LC/MSD ion trap mass spectrometer equipped with an atmospheric pressure electrospray ionization source and operating in positive ion mode. A high resolution mass spectrum for Ib was obtained using a Thermo Scientific LTQ Orbitrap XL mass spectrometer with electrospray ionization in positive ion mode. Fluorescence spectra were acquired at ambient temperature using a PerkinElmer LS50B luminescence
Synthesis of 1b

\( N,N'-\text{Dicyclohexylcarbodiimide} \) (DCC, 163 mg, 0.79 mmol) and 4-dimethylaminopyridine (DMAP, 5 mg, 0.039 mmol) were added to a solution of 2-((diphenylphosphino)benzoic acid (220.0 mg, 0.72 mmol) in anhydrous \( \text{CH}_2\text{Cl}_2 \) (30 mL) under argon with stirring at room temperature. After 30 min, fluorescein monomethyl ether (249 mg, 0.72 mmol) was added and the resulting mixture was stirred at room temperature overnight. The reaction mixture was concentrated under vacuum and cold acetone was added to dissolve the crude product. The insoluble urea by-product was removed by filtration. The filtrate was concentrated and the residue was purified by silica gel column chromatography (EtOAc /pet. Ether = 1:2) to provide 1b as a pale yellow solid (280 mg, 61%). \( R_f = 0.37 \) (EtOAc /pet. ether = 1:2). \( ^1\text{H} \) NMR (300 MHz, CDCl\(_3\)) \( \delta \) 8.17-8.06 (m, 1H), 7.87 (dd, \( J = 6.4, 1.4 \) Hz, 1H), 7.54-7.41 (m, 2H), 7.35-7.25 (m, 2H), 7.23-7.10 (m, 10H), 7.02-6.96 (m, 1H), 6.92-6.86 (m, 1H), 6.85 (d, \( J = 2.3 \) Hz, 1H), 6.65-6.59 (m, 2H), 6.56 (d, \( J = 8.8 \) Hz, 1H), 6.49 (ddd, \( J = 11.1, 8.7, 2.4 \) Hz, 2H), 3.64 (s, 3H). \( ^{13}\text{C} \) NMR (75 MHz, CDCl\(_3\)) \( \delta \) 169.3, 164.7 (d, \( ^3J_{\text{P-C}} = 2.5 \) Hz), 161.5, 153.1, 152.3, 151.8, 151.8, 141.6 (d, \( ^2J_{\text{P-C}} = 28.3 \) Hz), 137.5 (d, \( ^3J_{\text{P-C}} = 11.3 \) Hz), 135.2, 134.5, 134.0 (d, \( ^2J_{\text{P-C}} = 20.8 \) Hz), 133.0 (d, \( ^2J_{\text{P-C}} = 18.4 \) Hz), 132.8, 131.4 (d, \( ^3J_{\text{P-C}} = 1.8 \) Hz), 129.9, 128.98, 128.89, 128.6 (d, \( ^3J_{\text{P-C}} = 7.3 \) Hz), 128.4, 126.5, 125.0, 124.0, 117.6, 116.8, 112.0, 110.9, 110.5, 100.9, 82.5, 55.6. \( ^{31}\text{P} \) NMR (121 MHz, CDCl\(_3\)) \( \delta \) -4.01. ESI-MS positive ion mode (m/z) 635.2 [M+H]⁺
Fluorescein monomethyl ether (347 mg, 1 mmol) was dissolved in anhydrous CH$_2$Cl$_2$ (5 mL) under argon with stirring and cooled to 0 °C, to which a solution of triphosgene (107 mg, 0.36 mmol) in anhydrous CH$_2$Cl$_2$ (2 mL) was added. Pyridine (80 μL, 1 mmol) was then added dropwise and the resulting solution was stirred for 1 hour at 0 °C. The reaction mixture was allowed to warm to room temperature and stirred overnight. The reaction mixture was diluted with ethyl acetate and partitioned between ethyl acetate and 1 N aqueous HCl. The organic phase was washed with 1 N HCl (aq) and brine and dried over anhydrous Na$_2$SO$_4$. The resulting solution was concentrated under vacuum and the resulting oil was dissolved in anhydrous CH$_2$Cl$_2$ (10 mL) at 0 °C without further purification. A solution of 2-(diphenylphosphino)ethylamine (229 mg, 1 mmol) in anhydrous CH$_2$Cl$_2$ (2 mL) and pyridine (80 μL, 1 mmol) was added slowly at 0 °C. The reaction was stirred for 1 hour and then gradually warmed to room temperature for overnight. The final mixture was concentrated and purified by silica gel column chromatography to provide 6 as a pale yellow solid (90 mg, 15%). R$_f$ = 0.8 (methanol/CHCl$_3$ = 1:10). $^1$H NMR (300 MHz, CDCl$_3$) δ 8.11-7.91 (m, 1H), 7.71-7.56 (m, 2H), 7.56-7.27 (m, 10H), 7.20-7.05 (m, 2H), 6.81-6.73 (m, 3H), 6.72-6.58 (m, 2H), 5.28 (d, $J$ = 5.5 Hz, 1H), 3.83 (s, 3H), 3.42 (t, $J$ = 8.3 Hz, 2H), 2.43-2.34 (m, 2H). $^{13}$C NMR (75 MHz, CDCl$_3$) δ 169.27 (d, $^2J_{P-C}$ = 13.3 Hz), 161.44 (d, $^3J_{P-C}$ = 9.1 Hz), 153.66, 153.03, 152.88, 152.29 (d, $^3J_{P-C}$ = 3.8 Hz), 151.98, 151.68 (d, $^3J_{P-C}$ = 3.4 Hz), 137.52 (d, $^3J_{P-C}$ = 11.9 Hz), 135.94, 135.15 (d, $^3J_{P-C}$ = 9.5 Hz), 132.63 (d, $^2J_{P-C}$ = 19.0 Hz), 129.93, 129.78, 129.14, 128.92, 128.82, 128.71, 128.56 (d, $^3J_{P-C}$ = 6.9 Hz), 126.37 (d, $^3J_{P-C}$ = 8.4 Hz), 125.04 (d, $^3J_{P-C}$ = 11.8 Hz), 124.07 (d, $^3J_{P-C}$ = 10.7 Hz), 123.77, 117.82, 117.31 (d, $^2J_{P-C}$ = 12.3 Hz), 115.92, 111.94 (d, $^2J_{P-C}$ = 19.8 Hz), 110.88, 110.70,
110.07, 100.88 (d, $^3J_{P\text{-}C} = 3.9$ Hz), 82.62, 82.15, 55.54 (d, $^3J_{P\text{-}C} = 2.6$ Hz), 38.58 (d, $^2J_{P\text{-}C} = 22.0$ Hz), 28.66 (d, $^2J_{P\text{-}C} = 14.0$ Hz). $^{31}$P NMR (121 MHz, CDCl$_3$) $\delta$ -21.77. ESI-MS positive ion mode (m/z) 602.3 [M+H]$^+$. 

**Fluorescence Spectroscopy for HNO Detection**

*Fluorescence detection of AS (or 4-BPA) derived HNO by 1b:* A stock solution of 1b (10.1 mM) in anhydrous CH$_3$CN was prepared. Similarly, stock solutions of AS (0.5 mM and 5 mM in 15 mM NaOH) or 4-BPA (0.5 mM and 5 mM in CH$_3$CN) were also prepared. All solutions were used within 36 hours and stored at -20 $^\circ$C during that time. The solvent for each ligation reaction (3.5 mL) was composed of a 1:3 mixture of CH$_3$CN and PBS (containing 0.1 mM EDTA, pH 7.4). For each reaction, an aliquot of the phosphine probe solution (in CH$_3$CN) was diluted with the appropriate volumes of solvents. AS (or 4-BPA) was quickly added to initiate the ligation reaction, which was incubated for 2 h at ambient temperature. For the nitrite control experiments, stock solutions (0.5 mM and 5 mM) of NaNO$_2$ prepared in water were used in place of AS (or 4-BPA).

At 2 h of incubation time, a portion of the reaction solution (3.5 mL) was transferred to a quartz fluorometer cell (1 cm) for fluorescence spectroscopy. Solutions were excited at $\lambda_{ex} = 465$ nm and emission was monitored over the range of 485 - 700 nm ($\lambda_{em} = 520$ nm).

*Fluorescence response of 1b toward other biological species:* Probe 1b, AS and GSNO were stored dry under argon at -20 $^\circ$C until needed. CysNO was freshly prepared by adding t-tutyl nitrite (12 μL, 0.1 mmol) to a solution of the L-cysteine (0.1 mmol) in
PBS buffer at 0 °C. The reaction mixture was allowed to stir for 25 min and diluted with PBS buffer as detailed above to afford the stock solution. A solution of DEA/NO was prepared with degassed NaOH solution and the reaction of DEA/NO and 1b was performed anaerobically.

A stock solution of 1b (3.5 mM) in anhydrous acetonitrile was prepared. Similarly, other stock solutions of GSH, H₂O₂, NaNO₂, NaNO₃ (350 mM) in PBS; DEA/NO (350 mM), Na₂S (350 mM) in NaOH (15 mM); GSNO, CysNO (100 mM) in PBS; AS (350 mM) in NaOH (15 mM) were also prepared. The solvent for each reaction (3.5 mL) was composed of a 1:3 mixture of CH₃CN and PBS as detailed above. For each reaction, the stock solution of 1b (40 μL) was added to give a final concentration of 40 μM. An appropriate amount of AS or other biological species was added quickly to the solution to give a final concentration of 8 mM. Reaction mixtures were covered with aluminum foil immediately and incubated at room temperature in the dark for 2 h.

At 2 h of incubation time, a portion of the reaction solution (3.5 mL) was transferred to a quartz fluorometer cell (1 cm) for fluorescence spectroscopy. Ligation solutions were excited at λₑₓ = 465 nm and emission was monitored over the range of 485-700 nm (λₑₘ = 520 nm).

**Kinetic fluorescence study of probes 1b & 6:** A stock solution of 1b or 6 (10.1 mM) in anhydrous CH₃CN and the stock solution of AS (5 mM in 15 mM NaOH) were prepared. All solutions were used within 36 hours and stored at -20 °C before use. The solvent for each ligation reaction (3.5 mL) was composed of a 1:3 mixture of CH₃CN and PBS pH 7.4 or potassium phosphate buffer pH 6.2. For each reaction, an aliquot of the
phosphine probe solution (in CH$_3$CN) was diluted with the appropriate volumes of solvents. In a ligation experiment, AS was quickly added to initiate the reaction. The resulting solution was monitored by fluorimeter every 5 min over 2 hours.

**Cellular Experiments**

*Confocal Microscopy:* HeLa cells were seeded on 35 mm glass bottomed poly-D-lysine coated culture dishes (MatTek) at a density of 27,000 cells/dish. Cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM, 1x), supplemented with 10% FBS (Invitrogen) at 37 °C and 5% CO$_2$.

A solution of 1b (20 mM) in anhydrous dimethyl sulfoxide (DMSO) was freshly prepared and subsequently diluted into fresh media (15 mL) to a concentration of 12.5 μM. The media was removed from the cells and replaced with media containing 1b (2 mL, 12.5 μM) followed by incubation for 1 h at 37 °C, 5% CO$_2$. At this time, the media was removed and the cells washed three times with sterile PBS buffer (10 mM, pH 7.4). The cells were imaged in PBS, and either AS or NaNO$_2$ (10 mM in 15 mM NaOH) was added to the cells directly on the microscope stage, which was maintained at 37 °C for the duration of the imaging.

Cells were imaged on a Zeiss LSM 710 laser scanning confocal microscope and maintained at 37 °C with 5% CO$_2$ for the duration of imaging. Images were acquired using a 63x, 1.3NA water immersion objective with a zoom of 1.5, giving a pixel size of 0.09 μm. The 488 nm line of a 35 mW Argon laser, set at 80% power, was used for excitation, with a 488 nm beam splitter. The emission range was set to 500 - 600 nm. The pinhole was set to 1 AU (airy units). Master gain and offset were adjusted to ensure
maximal signal without exceeding the linear range of the detector.

**HR-LC/MS:** Following imaging, the cells were lysed with a solution of MeOH:CHCl₃:H₂O = 2.5:1:1. The solution above the lysed cells was removed, concentrated under vacuum, and then dissolved in 5% MeOH in H₂O (Optima HPLC grade, 0.2 mL) for analysis by LC-MS.

Organic extracts of HeLa cell lysates were analyzed on an Accela Open UPLC coupled to a Thermo Orbitrap LTQ XL high-resolution mass spectrometer. Separations were achieved using a Thermo Hypersil GOLD column (50 x 2.1 mm, 1.9 μm) with a flow rate of 250 μL/min. The column was conditioned using a linear gradient of 100% MeCN to 95% MeOH and 5% H₂O over 5 minutes, and held for 5 minutes. Separations were achieved using MeOH and water with a gradient of 5% MeOH to 95% MeOH over 10 minutes, followed by a decrease to 5% MeOH over 0.1 minutes, and holding at 5% MeOH for 4.9 minutes for a total run time of 15 minutes. Two blanks (5% MeOH in H₂O) were run using this method between analyses. Eluent was introduced to the mass spectrometer via positive ESI with the following settings: sheath gas 80, spray voltage of 4.0 kV, a capillary temperature of 350 °C, a capillary voltage of 25.0 V, and the tube lens held at 105.0 V. Mass spectra were acquired at a resolution of 60,000 over the range of 150-2000 m/z. The acquired raw data were analyzed using Xcalibur v 2.1 (Thermo).
CHAPTER 3

COMPARISON OF REDUCTIVE LIGATION BASED DETECTION STRATEGIES
FOR NITROXYL (HNO) AND S-NITROSOThIOLS

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Most of the work contained in this chapter was initially published in ChemistryOpen in 2016. The manuscript, including figures, schemes and supporting information, was drafted and edited by Zhengrui Miao and S. Bruce King. Since the publication, changes in both format and contents were made to adapt this work for the dissertation format. A small portion of the work contained in this chapter was initially published in Chemical Communications in 2014 in collaboration with Dr. Ming Xian, Washington State University, Pullman, Washington. The research described herein was performed by Zhengrui Miao unless otherwise stated.
3.1 BACKGROUND AND SIGNIFICANCE

As discussed in the Introduction, S-nitrosation is an important post-translational modification that not only modulates biological activity of nitric oxides but also regulates protein functions.\textsuperscript{157-160} The levels of S-nitrosation are finely regulated, and dysregulation is associated with the etiology of several pathologies. Currently, the detection of S-nitrosation in biological systems is still a challenge due to the lability of the products of S-nitrosation, i.e. S-nitrosothiols (RSNO).\textsuperscript{161-162} Since RSNO are unstable species, most current methods (such as chemiluminescence-based assays, colorimetry-based assays, and biotin switch-based assays) are indirect methods, which detect the decomposition products of SNO (either the S or the NO part).\textsuperscript{163-164} Careful control experiments are needed if these methods are used or false positive results could be generated. In this regard, direct methods that target the entire RSNO moiety would have distinct advantages.

3.2 RESULTS AND DISCUSSION

In the previous chapter, we discussed the reaction of phosphines with HNO and its further application in developing fluorescent HNO probes. As mentioned, 2-(diphenylphosphino)benzoic acid esters (1) also react with RSNO in a similar pattern to HNO, generating an S-azaylide and phosphine oxide in equal amounts. S-Azaylide, similar to the azaylide derived from HNO, can also be trapped through reductive ligation (Scheme 26).\textsuperscript{95}
Scheme 26. Design of reductive ligation based probes for RSNO.

Scheme 26 depicts the mechanism of the phosphorus mediated reductive ligation of an RSNO. The RSNO first reacts with triaryl phosphate (1) to form the S-azaylide intermediate (8), which in turn undergoes a rapid intramolecular acyl transfer and hydrolysis to give sulfenamide (9) and free alcohol. This reaction provides a unique and specific way to remove the acylated group on hydroxyl groups, which is widely used in the design of many reaction-based fluorescent probes. We envisioned the possibility for a fluorescent RSNO probe based on this chemistry (Scheme 26). In collaboration with Dr. Ming Xian’s group, we designed the first reductive ligation-based fluorescent probe for RSNO (SNOP1) derived from fluorescein (Scheme 27).
Scheme 27. RSNO induced fluorescence turn-on of SNOP1.

With the probe in hand, we first tested its fluorescence properties in aqueous buffers. The Tris-HCl buffer system gave the best results, so this system was used in all the experiments described. As expected, the probe itself showed low fluorescence intensity. One CysNO derivative, S-nitroso N-acetylcysteine methyl ester (N-Ac-CysNO-OMe, Scheme 28), was used as a model RSNO to test the feasibility of this probe. When the substrate was added to the solution of the probe (10 mM), a significant increase of fluorescence intensity (~90 fold) was observed. The fluorescence turn-on response was also found to be fast and the maximum intensity was reached in 25 min. This model reaction generates both free fluorescein and mono-acylated fluorescein as products, confirming the reaction mechanism proposed in Scheme 27.165

Scheme 28. Four commonly used S-nitroso cysteine derivatives
To further investigate whether **SNOP1** effectively detects biological \(S\)-nitrosothiols, we tested the response of **SNOP1** to GSNO. The reaction of **SNOP1** with GSNO also led to an obvious fluorescence increase, but at a smaller level (~18 fold) and a slower rate (reaching the maximum at ~40 min) compared to \(S\)-nitroso \(N\)-acetylcysteine methyl ester.

To demonstrate the efficiency of the probe in determining RSNO concentrations, a series of different concentrations of GSNO were treated with **SNOP1** (10 \(\mu\)M). For consistency, the fluorescence intensity was recorded after 45 min. We found that the intensities increased almost linearly in the range of 0-30 \(\mu\)M for GSNO (Figure 8). The detection limits were calculated to be 90 nM for GSNO, suggesting that the probe is reasonably sensitive.

![Fluorescence emission spectra of SNOP1 (10 \(\mu\)M) with GSNO.](image)

**Figure 8.** Fluorescence emission spectra of **SNOP1** (10 \(\mu\)M) with GSNO. GSNO concentration: 0, 0.3, 0.5, 1, 3, 5, 7, 10, 15, 20, 25, 40, 50 \(\mu\)M. \(\lambda_{ex} = 490\) nm. The
reactions were carried out for 45 min at 37 °C in Tris-HCl buffer (50 mM, pH = 7.4) with 1% DMSO ($\lambda_{ex/em} = 490/518$ nm).

To test the activity of SNOP1 toward HNO, we treated the probe (10 μM) with a solution of AS (10 μM). The fluorescence signals were recorded at 5 min in Tris-HCl buffer (50 mM, pH = 7.4, with 1% DMSO) at 37 °C. The probe showed a dramatic fluorescence increase (~ 235 fold). However, HNO is a highly reactive species, which quickly undergoes dimerization to hyponitrous acid followed by dehydration to give nitrous oxide (N$_2$O) and water. Thus, the time-dependent stability of HNO using SNOP1 was examined. A solution of Angeli’s salt (10 μM) was prepared and HNO was monitored by SNOP1 at different time intervals (0–60min). As shown in Figure 9, the fluorescence intensities decrease quickly. After about 10 min, there was almost no fluorescence detected, suggesting the complete decomposition of HNO. These results suggest that although SNOP1 gives a strong fluorescence response to HNO, its signal can be observed only when it is continuously formed and is present at the time of the measurement. Because of this property, SNOP1 may be useful for selective detection of RSNO, at least in some situations and might be able to distinguish RSNO from HNO based on its time-dependent response.
Figure 9. Time-dependent fluorescence changes of HNO in Tris-HCl buffer (50 mM, pH = 7.4) with 1% DMSO.

Note: AS (10 μM) was used as HNO equivalent. The solutions of AS (10 μM) were kept at 37 °C for 0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60 min, and then monitored by SNOPI (10 μM), respectively.

Besides the probes mentioned in the previous chapter, many other phosphine-based fluorescent probes for HNO have been developed with improved properties over the past five years (Scheme 29).\textsuperscript{152-154, 167-172} The Rhodamine derivative (P-Rhod, Scheme 29) is the first report fluorescent probe of this type.\textsuperscript{152} The coumarin-based fluorescent probe (P-CM, Scheme 29) is much easier to prepare and has an improved sensitivity and linear response toward low HNO concentrations.\textsuperscript{154} The 1,8-naphthalimide-derived probe (P-Nap, Scheme 29) shifts fluorescence from blue (418 nm) to green (546 nm) upon introduction of AS, allowing the ratiometric detection of HNO.\textsuperscript{153} A lysosomal directed near-infrared probe (Lyso-JN, Scheme 29) permits organelle
specific HNO detection. This strategy has been extended to prepare several other probes including a FRET (coumarin/fluorescein pair) based ratiometric fluorescent probe (CF), a two photon fluorescence turn-on probe (GCTPOC-1), a near-infrared fluorescent probe (Cyto-JN) and an excited state intramolecular proton transfer (ESIPT) based ratiometric fluorescent probe (HNOHBT, Scheme 29).

Scheme 29. New phosphine-based fluorescent probes for HNO.

While increasing efforts have been made in designing new phosphine-based
fluorescent probes for HNO and RSNO detection, little attention has been paid to the cross-reactivity of HNO and RSNO with the same phosphine based detection systems. Prior experiments consistently demonstrate that HNO induces a greater fluorescence response than GSNO or CysNO upon reaction with the same phosphine.\textsuperscript{141, 152, 154, 165, 172} Such differences suggest a relative specificity for HNO over RSNO in their reaction with organic phosphines. The overall similarity of these two described reaction pathways complicates phosphine based detection strategies of both species and opens the possibility of false positive results \textit{in vivo}. Concerns regarding the reliability of these probes exist and \textit{in vivo} screening of HNO with these probes remains risky without a rationale for the diminished fluorescence response from GSNO. We directly investigated the reactions of two previously mentioned phosphines (1a-b) with HNO and RSNO and revealed important differences vital for the fluorescence response. Unlike the HNO-derived azaylide or the S-azaylide described in earlier reports, the S-azaylide formed from the reaction of phosphines with GSNO or CysNO does not efficiently participate in the reductive ligation needed for fluorophore generation. This difference was further confirmed by using 1b to detect HNO (vs. RSNO) in HeLa cells using flow cytometry.\textsuperscript{141}
Fluorescence generation described in Scheme 30 relies on productive ylide formation and reductive ligation with the release of a competent fluorophore. For the reaction of 1 with HNO, a productive ligation sequence should yield an equivalent of phosphine oxide amide (4), phosphine oxide ester (2), and the alcohol (or fluorophore, 5). Given that 2 and 5 could arise from either oxidative or hydrolytic pathways, 4 represents a distinct indicator of a productive ligation process. For the RSNO reaction, the sulfenamide (9) represents an analogous ligation product, which can be reduced to 4 in the presence of excess phosphine (Scheme 30).
We monitored the reactions of phosphine probes with HNO and GSNO by phosphorus nuclear magnetic resonance (NMR) spectroscopy and HPLC-MS (Figures 10-17). Treatment of phenyl 2-(diphenylphosphino)benzoate (1a) with Angeli’s salt (AS), a common HNO donor, reveals the formation of 4 (δ = 34.8 ppm), the corresponding phosphine oxide (2a, δ = 33.7 ppm) as expected, and an additional peak (δ = 35.8 ppm), identified by HPLC-MS as 2-(diphenylphosphino)benzoic acid oxide, possibly from the hydrolysis of 2a (Figure 10A). The amide peak (4, δ = 34.8 ppm) indicates productive ligation. In contrast to reported efficient reductive ligation results, treatment of this model phosphine with GSNO only yields 2a (δ = 33.7 ppm) with trace amounts of 4 or sulfenamide (9, Figure 10B), revealing inefficient ligation. Incubation of fluorescent probe (1b) with AS gives a similar 31P NMR spectrum showing complete conversion of 1b to 4 (δ = 34.8 ppm), the corresponding phosphine oxide (2b, δ = 33.7 ppm, Figure 10C) and 2-(diphenylphosphino)benzoic acid oxide (δ = 35.8 ppm). The formation of 4 correlates with a strong and rapid fluorescence response. Similarly, incubation of 1b with GSNO yields mostly phosphine oxide (2b, δ = 33.7 ppm) and a trace amount of another phosphorus product (Figure 10D). A previously reported coumarin-derived fluorescent HNO probe (P-CM) demonstrates similar results (Figure 11, 16-17). These results clearly show different reactivity of phosphine probes with HNO and GSNO under these conditions. The HNO reaction reliably gives Staudinger ligation products (that yield fluorescence in properly designed compounds) but the GSNO reaction primarily yields phosphine oxide with little evidence of ligation, which corresponds to the low fluorescence response observed in previous reports.
Figure 10. $^{31}$P NMR spectra for the reaction of 1a-b (0.01 mmol) with HNO or GSNO (0.02 mmol) in CD$_3$CN/THF/Tris-HCl Buffer (100 mM, pH 7.4) = 3:1:2 after 60 min.

Panel A-B: resonances correspond to 4 (34.8 ppm) and 2a (33.7 ppm); Panel C-D: resonances correspond to 4 (34.8 ppm) and 2b (33.7 ppm)

Structure of the coumarin-based HNO probe P-CM.
Figure 11. $^{31}$P NMR spectra for reaction of P-CM (0.01 mmol) with AS or GSNO (0.02 mmol) in CD$_3$CN/THF/Tris-HCl Buffer (100 mM, pH 7.4) = 3:1:2 after 1 h.
Figure 12. HPLC-MS analysis for reaction of 1a (0.01 mmol) with AS (0.02 mmol) in CD₃CN/THF/Tris-HCl Buffer (100 mM, pH 7.4) = 300 μL/100 μL/200 μL at 30 min.
Figure 13. HPLC-MS analysis for reaction of 1a (0.01 mmol) with GSNO (0.02 mmol) in CD$_3$CN/THF/Tris-HCl Buffer (100 mM, pH 7.4) = 300 μL/100 μL/200 μL at 30 min.
Figure 14. HPLC-MS analysis for reaction of 1b (0.01 mmol) with AS (0.02 mmol) in CD$_3$CN/THF/Tris-HCl Buffer (100 mM, pH 7.4) = 300 μL/100 μL/200 μL at 30 min.
Figure 15. HPLC-MS analysis for reaction of 1b (0.01 mmol) with GSNO (0.02 mmol) in CD$_3$CN/THF/Tris-HCl Buffer (100 mM, pH 7.4) = 300 μL/100 μL/200 μL at 30 min.
Figure 16. HPLC-MS analysis for reaction of P-CM (0.01 mmol) with AS (0.02 mmol) in CD$_3$CN/THF/Tris-HCl Buffer (100 mM, pH 7.4) = 300 μL/100 μL/200 μL at 30 min.
Figure 17. HPLC-MS analysis for reaction of P-CM (0.01 mmol) with GSNO (0.02 mmol) in CD$_3$CN/THF/Tris-HCl buffer (100 mM, pH 7.4) = 300 μL/100 μL/200 μL at 30 min.

While reductive ligation phosphine-based fluorescent detection strategies for HNO are becoming well-established, the use of similar compounds for RSNO detection remains to be fully developed$^{1,165}$ Early studies show the ability of phosphines to react
with RSNO to form \(S\)-azaylides that undergo ligation reactions but these discoveries have not been translated to a robust RSNO detection system.\(^{95}\) Work previously described in this chapter with \textbf{SNOP1} shows this probe reacts with both RSNO and HNO to some extent.\(^{165}\) A mass spectrometric phosphine-based method for GSNO has been reported and this work also shows that probes with similar structures to \textbf{1a-b} do not undergo ligation with GSNO.\(^{173}\) The reasons for the observed differences in the ligation reactivity of phosphines and HNO (reliable) and RSNO (unreliable) remain to be defined. Kinetic studies on the reaction of phosphines with HNO or RSNO show that initial phosphine addition occurs rapidly (with the HNO reaction being slightly faster).\(^{93, 173-174}\) Differences in the reactivity of the azaylides intermediates (\textbf{3} or \textbf{8}) likely play a role in the observed reactivity as the rate determining step and rate of classical Staudinger ligations vary significantly with azaylide stability.\(^{175}\) Examination of previous work shows that in general only organic RSNOs or fully protected versions of biological RSNO derivatives (fully protected CysNO) yield successful and reliable reductive ligation.\(^{95, 141, 152, 154, 165, 173}\) We speculate that the \(S\)-azaylides derived from GSNO and CysNO that contain both free carboxylic acid and amine groups may be less stable than those from the organic RSNO. Both of these RSNO exist as a zwitterion at physiological pH and these functional groups may react with the \(S\)-azaylide facilitating other possible reaction pathways such as hydrolysis to yield the observed phosphine oxide.

To explore this idea, the reaction of various CysNO derivatives (Scheme 28) with \textbf{1a} followed by phosphorus NMR and HPLC-MS analysis (Figures 18-22) provides further information regarding RSNO structure in these reactions. Previous work shows \textit{N-}acetyl CysNO methyl ester, a fully protected CysNO derivative, reacts with \textbf{1a} via
ligation to give the sulpenamide in 84% yield.\textsuperscript{95} In our hands, treatment of \textbf{1a} with \textit{N-}
acetyl CysNO methyl ester yields three phosphorus-containing products, the expected
phosphine oxide (2\textit{a}, $\delta = 33.7$ ppm), sulpenamide (9, $\delta = 34.3$ ppm) and the amide (4, $\delta =$
34.8 ppm), a ligation product that forms from the reduction of 9 by \textbf{1a} (Figure 18A).\textsuperscript{95}
Both 9 and 4 result from a reductive ligation process and confirm previous work.\textsuperscript{95}
However, treatment of CysNO methyl ester, which contains a free amine, with \textbf{1a} only
provides small amounts of 9 and 4 with the phosphine oxide as the major species (Figure
18B). Incubation of \textit{N}-acetyl CysNO, which contains a free carboxylic acid, results in
primarily phosphine oxide (2\textit{a}) with a small amount of another downfield phosphorus-
containing product ($\delta = 53.5$ ppm, Figure 18C), presumed to be a phosphonium salt as
judged by HPLC-MS (Figure 21).\textsuperscript{94, 173} Reaction of CysNO with \textbf{1a} yields phosphine
oxide (2\textit{a}) as the only phosphorus-containing product (Figure 18D). These results
indicate that the structure of the RSNO influences the final product selectivity possibly
by influencing the stability of the S-azaylide. The reactions with RSNO substrates
containing free carboxylic acid and amine groups predominantly generate phosphine
oxide (Figure 18, B-D) suggesting the presence of these groups facilitate S-azaylide
hydrolysis as the most direct mechanism of phosphine oxide formation.
**Figure 18.** $^{31}$P NMR spectra for the reaction of 1a (0.02 mmol) with CysNO and its derivatives (0.01 mmol).

Panel A) $N$-acetyl-CysNO-methyl ester; Panel B) CysNO-methyl ester; Panel C) $N$-acetyl-CysNO; Panel D) CysNO in CD$_3$CN/THF/Tris-HCl Buffer (100 mM, pH 7.4) = 3:1:2 in dark after 30 min.
Figure 19. HPLC-MS analysis for reaction of 1a (0.02 mmol) with N-acetyl-CysNO-methyl ester (0.01 mmol) in CD$_3$CN/THF/Tris-HCl Buffer (100 mM, pH 7.4) = 300 μL/100 μL/200 μL in dark at 30 min.
Figure 20. HPLC-MS analysis for reaction of 1a (0.02 mmol) with CysNO-methyl ester (0.01 mmol) in CD$_3$CN/THF/Tris-HCl Buffer (100 mM, pH 7.4) = 300 µL/100 µL/200 µL in dark at 30 min.
Figure 21. HPLC-MS analysis for reaction of 1a (0.02 mmol) with N-acetyl-CysNO (0.01 mmol) in CD$_3$CN/THF/Tris-HCl Buffer (100 mM, pH 7.4) = 300 μL/100 μL/200 μL in dark at 30 min.
As described in Scheme 30 for phosphine based fluorescent probes, successful ligation processes occur accompanied by the generation of fluorescence. Hence, the fluorescence response of 1b toward these four CysNO derivatives should remain
consistent with the data presented in Figure 16. Indeed, addition of 2 eq. of \(N\)-acetyl CysNO methyl ester to 1b induces a significant (10.1-fold) fluorescence increase compared to CysNO methyl ester (2.9-fold), which is much greater than \(N\)-acetyl CysNO and CysNO (Figure 23). These fluorescence results correlate with the amount of ligation product observed by \(^{31}\)P NMR (more fluorescence with more ligation product) and support the idea that RSNO structure ultimately controls the stability of the derived \(S\)-azaylde.

![Figure 23](image)

**Figure 23.** Fluorescence responses of 1b (40 \(\mu\)M) in CH\(_3\)CN/PBS at room temperature for 60 min after addition of 2 eq. of CysNO derivatives.

Additional \(^{31}\)P NMR experiments followed by HPLC-MS of the reactions of these \(S\)-nitroso cysteine derivatives with triphenylphosphine reinforce these ideas (Figure 24-28). The reaction of \(N\)-acetyl CysNO methyl ester with PPh\(_3\) gives an expected 1:1 ratio of phosphine oxide and the proposed \(S\)-azaylde while CysNO derivatives containing either the free carboxylic acid or amine groups predominantly generate phosphine oxide.
Although we did not observe any S-azaylide signal in the following HPLC-MS analyses of these reaction mixtures at 60 min, the observed azaylide signal still supports this idea since it can only be produced stoichiometrically via reduction of the S-azaylides by phosphine (Figure 25-28).

**Figure 24.** $^{31}$P NMR spectra for the reaction of PPh$_3$ (0.024 mmol) with CysNO and its derivatives (0.012 mmol).

Panel A) $N$-acetyl-CysNO-methyl ester; Panel B) CysNO-methyl ester; Panel C) $N$-acetyl-CysNO; Panel D) CysNO in CD$_2$CN/THF/Tris-HCl Buffer (100 mM, pH 7.4) = 200 μL/180 μL/220 μL in dark after 1 h.
Figure 25. HPLC-MS analysis for reaction of PPh₃ (0.024 mmol) with N-acetyl-CysNO-methyl ester (0.012 mmol) in CD₃CN/THF/Tris-HCl Buffer (100 mM, pH 7.4) = 200 μL/180 μL/220 μL in dark at 30 min.

Note: No S-azaylide signal was observed. Only azaylide was observed. That is probably generated via reduction of S-azaylide by phosphine. The amount of azaylide should be consistent with S-azaylide formed.
Figure 26. HPLC-MS analysis for reaction of PPh₃ (0.024 mmol) with CysNO-methyl ester (0.012 mmol) in CD₃CN/THF/Tris-HCl Buffer (100 mM, pH 7.4) = 200 μL/180 μL/220 μL in dark at 30 min.

Note: No S-azaylide signal was observed. Only azaylide was observed. That is probably generated via reduction of S-azaylide by phosphine. The amount of azaylide should be consistent with S-azaylide formed.
Figure 27. HPLC-MS analysis for reaction of PPh₃ (0.024 mmol) with N-acetyl-CysNO (0.012 mmol) in CD₃CN/THF/Tris-HCl Buffer (100 mM, pH 7.4) = 200 μL/180 μL/220 μL in dark at 30 min.

Note: No S-azaylide signal was observed. Only azaylide was observed. That is probably generated via reduction of S-azaylide by phosphine. The amount of azaylide should be consistent with S-azaylide formed.
Figure 28. HPLC-MS analysis for reaction of PPh₃ (0.024 mmol) with CysNO (0.012 mmol) in CD₃CN/THF/Tris-HCl Buffer (100 mM, pH 7.4) = 200 μL/180 μL/220 μL in dark at 30 min.

Note: No S-azaylide signal was observed. Only azaylide was observed. That is probably generated via reduction of S-azaylide by phosphine. The amount of azaylide should be consistent with S-azaylide formed.
These experiments along with previously reported work indicate that HNO and RSNO distinctively react with triarylphosphines. Triarylphosphine probes react rapidly with both HNO and RSNO to generate a 1:1 mixture of the corresponding azaylidelide and the phosphine oxide (Scheme 30). In the case of HNO, these simple unsubstituted azaylidelides efficiently undergo Staudinger ligation with properly positioned esters to generate the fluorophore and the amide phosphine oxide (Scheme 30). While RSNO also quickly generate an S-azaylidelide, these species appear only to undergo efficient ligation reactions (required for fluorophore release) with simple organic RSNO or with fully-protected peptide or amino acid RSNO derivatives. Under predominantly aqueous conditions in the presence of free carboxylic acid and/or amine, the non-fluorescent phosphine oxide represents the major phosphorus-containing product indicating that other non-ligation reaction pathways of the S-azaylidelide dominate. Given the zwitterion state of the derived S-azaylidelides under physiological condition, the most likely pathway would be free carboxylic acid/amine assisted hydrolysis (Scheme 31). Since the free amine exists in a protonated state, it may act as a better electrophile to the azaylidelide, blocking the ligation pathway and forcing the S-azaylidelide to hydrolyse. The carboxylate could also direct attack the positively charged phosphorus catalyzing the hydrolysis (Scheme 31).

Considering the physiological environment and the presence of amine and carboxylic acid groups in many proteins or biological constituents, protein RSNO will most likely react with phosphine probes to yield phosphine oxides. Such reactivity suggests that phosphine based fluorescent probes demonstrate selectivity for HNO and will not be interfered by RSNO.
Scheme 31. Proposed free carboxylic acid/amine assisted hydrolysis of S-azaylides

To further confirm the reactivity difference of these probes with HNO vs RSNO, we measured the intracellular fluorescence increase in HeLa cells treated with 1b by adding an HNO donor and RSNO. Intracellular fluorescence was determined by flow cytometry that generates more statistically reliable data by simultaneous measurement of millions of cells and focuses on normal cells, avoiding faulty data from abnormal and/or dead cells. Figure 29 and Figure 30-31 show the mean fluorescence intensity value difference between 1b treated HeLa cells with different substrates. Direct addition of GSNO to cells does not yield an increase in fluorescence (Figure 29). Possible intracellular formation of protein RSNO by adding DEA/NO, a nitric oxide donor, also does not yield a fluorescence response. Addition of AS to cells containing 1b immediately results in an increase in fluorescence that grows over 30 min. The successful detection of HNO-induced fluorescence in numerous cells and the lack of response from GSNO or protein RSNO-derived from NO treatment confirm the difference in reactivity and reveal the possibility for HNO detection *in vivo* using these phosphine probes. The combination of such probes with flow cytometry may facilitate the search for endogenous HNO formation from different primary cells.
Figure 29. Mean fluorescence intensity (MFI) change ($\Delta$MFI = MFI - MFI_{control}) of HeLa cells treated with 1b (2 μM) toward DEA/NO, AS or GSNO (4 μM).
Figure 30. Fluorescence response of 1b (2 μM) treated HeLa cells to GSNO and AS derived HNO (4 μM).

X axis: Fluorescence intensity in logarithmic scale; y axis: cell counts. Cells were treated with: a) 1b, 10 min; b) 1b, 20 min; c) 1b, 30 min; d) 1b + GSNO, 10 min; e) 1b + GSNO, 20 min; f) 1b + GSNO, 30 min; g) 1b + AS, 10 min; h) 1b + AS, 20 min; i) 1b + AS, 30 min; j) none, 0 min; k) Overlay of all of the above. Mean fluorescence intensity values: a) 7.09; b) 6.88; c) 7.04; d) 6.92; e) 6.95; f) 7.03; g) 12.73; h) 16.77; i) 20.03; j) 3.28.
Figure 31. Fluorescence response of 1b (2 μM) treated HeLa cells to DEA/NO (4 μM).

X axis: fluorescence intensity in logarithmic scale; y axis: cell counts. Cells were treated with: a) 1b, 10 min; b) 1b, 20 min; c) 1b, 30 min; d) 1b + DEA/NONOate, 10 min; e) 1b + DEA/NONOate, 20 min; f) 1b + DEA/NONOate, 30 min; g) none, 0 min; h) Overlay of all of the above. Mean fluorescence intensity values: a) 4.65; b) 4.73; c) 4.69; d) 4.44; e) 4.58; f) 4.44; g) 2.87.

With this successful in vivo detection of HNO compared to RSNO and NO by organophosphine probes, we sought to establish a reliable and universal protocol for future identification of HNO to search for endogenous HNO sources. To further confirm the reliability of this system for endogenous HNO sources, we first tried to detect HNO from 2-bomo-Piloty’s acid (2-BPA) and 4-bomo-Piloty’s acid (4-BPA). As is shown in
Figure 32, addition of 2 equivalents of 2-BPA induces a fluorescence increase over 30 min. 4-BPA is known to generate HNO at a much slower rate than 2-BPA that may better mimic endogenous production of HNO.\textsuperscript{110} The fluorescence response of 1b toward 4-BPA was examined and 2 equivalents of 4-BPA does not yield an observable fluorescence response. Increasing the amount of 4-BPA to 20 equivalents promotes a weak fluorescence response (Figure 32). We next examined whether this method could be used to detect enzymatically produced HNO. Hydrogen peroxide is known to oxidize hydroxylamine to form HNO with the catalysis of horseradish peroxidase (HRP).\textsuperscript{116, 176} With this system, we also noticed that comparable concentration of substrates (2 eq.) to AS does not produce a noticeable amount of fluorescence increase but a greater concentration of substrate (100 eq.) yields a dramatic increase in fluorescence change (Figure 32). These experiments clearly indicate our general protocol can successfully identify HNO regardless of its source and generation rate, suggesting its feasibility for the screening of endogenous HNO sources.
Figure 32. Mean fluorescence intensity (MFI) change ($\Delta$MFI = MFI - MFI$_{\text{control}}$) of HeLa cells treated with 1b (2 μM) toward 2-BPA (4 μM), 4-BPA (40 μM) and HRP (0.4 μM) + NH$_2$OH (200 μM) + H$_2$O$_2$ (200 μM).

After the successful detection of HNO produced from these known HNO donors, we attempted the identification of HNO from potential endogenous reactions. As mentioned in Section 1.3.5, various groups believe the reaction of GSNO or NO with H$_2$S produces HNO either directly or via the intermediacy of HSNO.$^{131-137}$ With our established detection protocol, we tested whether these two potential HNO generation systems would yield fluorescence. Surprisingly, neither of these two reactions yields a fluorescence increase (Figure 33). Instead, a slight fluorescence decrease was observed for both. This result contradicts previous reports leading us to question the ability of these two systems to produce HNO.$^{131-137}$.
Figure 33. Mean fluorescence intensity (MFI) change ($\Delta$MFI = MFI - $\text{MFI}_{\text{control}}$) of HeLa cells treated with 1b (2 μM) toward GSNO (4 μM) + Na$_2$S (4 μM) and DEA/NONOate (4 μM) + Na$_2$S (4 μM).

3.3 CONCLUSION

In summary, we first designed a fluorescent probe SNP1 for RSNO based on phosphine mediated reductive ligation. SNP1 is capable of generating fluorescence with the introduction of RSNO. SNP1 displays excellent selectivity for RSNO over most biologically relevant species except HNO. However, certain techniques could be applied to avoid the interference from HNO. We then compared the phosphine detection strategies for HNO and RSNO. The reactions of phosphines with HNO, organic or biological RSNO demonstrate clear differences in the ability of the intermediate ylides to
undergo ligation leading to fluorophore release. Ylides derived from HNO or organic RSNO tend to participate in the ligation process while ylides from biological RSNO that contain free carboxylate and amino groups do not readily undergo ligation and preferentially react (perhaps through hydrolysis) to form the phosphine oxide. This reactivity difference was confirmed by monitoring the fluorescence response in HeLa cells. Successful detection of HNO in cells using 1b by flow cytometry without biological RSNO interference illustrates the reliability of phosphine probes for HNO. Additional experiments were also carried out in order to establish and confirm the general protocol for the identification of HNO by 1b incorporated with flow cytometric analysis, which may serve as a fast robust screening approach for endogenous HNO sources. Later this approach was also used to examine HNO from two potential endogenous HNO sources together with $^{31}$P NMR technique; however, no sign of HNO formation was observed.

3.4 EXPERIMENTAL METHODS

General

Reagents were obtained from commercial sources and used without additional purification. AS was prepared as described and stored at -20 °C. Reaction solvents were anhydrous and purged with argon prior to use. LC-MS and ESI-MS solvents were Optima HPLC grade. $^{31}$P NMR spectra were recorded on a Bruker Avance DPX-300 operating at 121.49 MHz. $^{31}$P chemical shifts are referenced to 85% H$_3$PO$_4$ (δ = 0 ppm) in a concentric internal capillary (Wilmad). NMR spectra were obtained using Bruker 5 mm BBO and QNP probes held at 25 °C. Low-resolution mass spectra were obtained using an
Agilent Technologies 1100 LC/MSD ion trap mass spectrometer equipped with an atmospheric pressure electrospray ionization source and operating in positive ion mode.

**Preparation of cysteine based S-nitrosothiols**

General procedure: $t$-Butyl nitrite (12 μL, 0.1 mmol) was added to a solution of the $L$-cysteine derivative (0.1 mmol) in Tris buffer (100 mM, pH 7.4, 1 mL) at 0 °C. The reaction mixture was allowed to stir in the dark at 0 °C for 25 min to give a final bright pink solution of the desired RSNO (0.1 mol/L) that was used in the following fluorescence study and $^3$P NMR experiments without further purification.

**Fluorescence Spectroscopy**

*Fluorescence studies for SNOP1*

The stock solution of SNOP1 (2 mM) was prepared in DMSO. The stock solution of GSNO (5 mM) was prepared in 50 mM Tris-HCl buffer (pH 7.4), Angeli’s salt (1 mM) was prepared in 10 mM NaOH solution. All solutions were freshly prepared before use.

An aliquot (20 μL) of SNOP1 stock solution was added to Tris-HCl buffer (3.96 mL, 50 mM, pH 7.4) and DMSO (20 μL) in a 4 mL glass vial and mixed well. Similarly, stock solution of GSNO (40 μL, 5 mM) and AS (40 μL, 1 mM) were added to 50 mM Tris-HCl buffer (pH 7.4, 3.96 mL), DMSO (20 μL) and SNOP1 stock (20 μL) in a 4 mL glass vial and mixed well.

These solutions were incubated at 37 °C in the dark. The incubation time for RSNO reaction was 45 min and 5 min for AS reaction. The solution was transferred to a
1-cm quartz cell to measure fluorescence with λ<sub>ex</sub> = 490 nm. All the measurements were repeated three times and data reported were averages.

**Fluorescence response of 1b toward CysNO derivatives**

A stock solution of 1b (3.5 mM) was prepared in anhydrous acetonitrile before use. Stock solutions of CysNO derivatives (28 mM) were freshly prepared following the procedure described above. The solvent for each ligation reaction (3.5 mL) was composed of a 3:1 mixture of CH<sub>3</sub>CN and phosphate-buffered saline (PBS, containing 0.1 mM EDTA, pH 7.4). For each reaction, stock solution of 1b (40 μL) was added to give a final concentration of 40 μM. An appropriate amount of the stock solution of CysNO derivative was quickly added to give a final concentration of 80 μM. Reaction mixtures were immediately covered with aluminum foil and incubated at room temperature in the dark for 1 h. After 1 h, a portion of the reaction solution (3.5 mL) was transferred to a quartz fluorometer cell (1 cm) for fluorescence spectroscopy. Ligation solutions were excited at λ<sub>ex</sub> = 465 nm and emission was monitored over the range of 485-700 nm (λ<sub>em</sub> = 520 nm). Data presented is the average ±SD of two independent experiments.

**Nuclear magnetic resonance experiments**

**31P NMR study of reactions of 1a-b with AS/GSNO**

AS/GSNO (0.02 mmol) was added to Tris-HCl buffer (100 mM, pH = 7.4, 200 μL) and the resulting solution was immediately added to an NMR tube containing a solution of 1a-b (0.01 mmol) in CD<sub>3</sub>CN/THF (300 μL/100 μL). The solution was
thoroughly mixed and monitored by $^{31}$P NMR over time in the dark.

$^{31}$P NMR study of 1a reactions with cysteine based RSNO

An aliquot of freshly prepared RSNO (0.1 mol/L, 100 μL) stock was added to an NMR tube containing a solution of 1a (7.6 mg, 0.02 mmol) in CD$_3$CN/THF/Tris buffer (300 μL/100 μL/100 μL). The solution was thoroughly mixed and monitored by $^{31}$P NMR over time in the dark.

$^{31}$P NMR study of PPh$_3$ reactions with cysteine based RSNO

An aliquot of freshly prepared RSNO (0.1 mol/L, 120 μL) stock was added to an NMR tube containing a solution of triphenylphosphine (6.2 mg, 0.024 mmol) in CD$_3$CN/THF/Tris buffer (200 μL/180 μL/100 μL). The solution was thoroughly mixed and monitored by $^{31}$P NMR over time in the dark.

Flow Cytometry experiments

Stock solutions

Stock solutions of 1b (0.1 mM) in anhydrous DMSO, GSNO (0.2 mM or 2 mM) in Dulbecco’s Phosphate-Buffered Saline (DPBS) buffer (Lonza), AS (0.2 mM) in NaOH (15 mM), DEA/NONOate (0.2 mM or 2 mM) in NaOH (15 mM), 2-BPA (0.2 mM or 2 mM) in DPBS buffer, 4-BPA (0.2 mM or 2 mM) in DPBS buffer, HRP (0.2 μM or 20 μM) in DPBS buffer, NH$_2$OH (0.1 mM or 10 mM) in DPBS buffer, H$_2$O$_2$ (0.1 mM or 10 mM) in DPBS buffer, Na$_2$S (2 mM) in NaOH (15 mM), were prepared 24 h before experiments and kept at 4 °C before use.
**GSNO and AS**

HeLa cells were detached, re-suspended and diluted with DPBS buffer to give a cell suspension (10 mL), which was evenly split into 10 tubes. Stock solution (20 μL) of 1b was added to nine of the tubes to give a final concentration of 2 μM while DMSO (20 μL) was added to the remaining tube. All tubes were then incubated for 10 min. For cells (tubes) treated with 1b, 3 tubes were treated with GSNO stock (20 μL), 3 tubes were treated with AS stock (20 μL) and 3 tubes were treated with DPBS (20 μL) as controls. The cells without 1b were treated with DPBS buffer (20 μL) and used to set up the flow cytometry experiment while the rest were incubated at 37 °C prior to analysis. At 10, 20, 30 min, the GSNO treated, the AS treated and control tubes were analyzed by flow cytometry.

**DEA/NO, 2-BPA, 4-BPA, HRP-system**

HeLa cells were detached, re-suspended and diluted with DPBS buffer to give a cell suspension (7 mL), which was evenly split into 7 tubes. Stock solution (20 μL) of 1b was added to six of the tubes to give a final concentration of 2 μM while DMSO (20 μL) was added to the remaining tube. All tubes were then incubated for 10 min. For cells (tubes) treated with 1b, 3 tubes were treated with DEA/NO stock (or corresponding 2-BPA, 4-BPA stock solution, 20 μL; for experiments with HRP-system, 20 μL NH₂OH, 20 μL H₂O₂, 20 μL HRP stock solution) and 3 tubes were treated with DPBS (20 μL) as controls. The cells without 1b were treated with DPBS buffer (20 μL) and used to set up the flow cytometry experiment while the other six tubes were incubated at 37 °C prior to analysis. At 10, 20, 30 min, the substrate treated and control tubes were analyzed by flow
GSNO + Na₂S, DEA/NO + Na₂S

HeLa cells were detached, re-suspended and diluted with DPBS buffer to give a cell suspension (7 mL), which was evenly split into 7 tubes. Stock solution (20 μL) of 1b was added to six of the tubes to give a final concentration of 2 μM while DMSO (20 μL) was added to the remaining tube. All tubes were then incubated for 10 min. For cells (tubes) treated with 1b, 3 tubes were treated with Na₂S stock (20 μL) followed by the addition of GSNO or DEA/NO stock (20 μL) and 3 tubes were treated with DPBS (20 μL) as controls. The cells without 1b were treated with DPBS buffer (20 μL) and used to set up the flow cytometry experiment while the other six tubes were incubated at 37 °C prior to analysis. At 10, 20, 30 min, the substrate treated and control tubes were analyzed by flow cytometry.
CHAPTER 4

THE SYNTHESIS AND CHARACTERIZATION OF S-
NITROSOBACILLITHIOL (BSNO)

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The work contained in this chapter is still on-going and had not been published at the
time this Dissertation was prepared.
4.1 BACKGROUND AND SIGNIFICANCE

Oxidative stress is involved in various physiological phenomena such as inflammation, aging, and complex diseases.\textsuperscript{177} The redox environment in living organism is sophisticatedly regulated to avoid excess oxidative stress.\textsuperscript{177} Most living organisms contain high levels of low-molecular-weight (LMW) thiols that play an important role in maintaining the redox environment of the cytosol and protect the cells against a variety of reactive species.\textsuperscript{178} Most LMW thiols co-exist with their coupled disulfides \textit{in vivo} to finely regulate various metabolic processes with the mediation of kinetically controlled thiol-disulfide ratios via S-thiolation and thiol-disulfide exchange (Figure 34).\textsuperscript{177}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Figure34.png}
\caption{LMW thiols in S-thiolation and thiol-disulfide exchange.}
\end{figure}

Glutathione (GSH, Scheme 32) is the best studied LMW thiol that exists at millimolar concentrations among eukaryotes and most Gram-negative bacteria.\textsuperscript{177} It is known to play a key role in a complex network including thioredoxin (Trx), glutaredoxin (Grx), and thiol-requiring enzymes.\textsuperscript{179} Its depletion increases antibiotic and metal toxicity, affects thiol related metabolism and impairs redox responses. In Archaea and
most Gram positive bacteria, maintenance of thiol redox homeostasis is instead achieved by other LMW cysteine derived thiols such as mycothiol (MSH) and bacillithiol (BSH) due to a lack of GSH (Scheme 32). Over the past 20 years, intensive studies have revealed MSH is involved in an equally complex system. BSH, the main LMW thiol among many low-guanine-cytosine (G+C) Gram positive bacteria (Firmicutes) discovered in the studies on the redox regulation of peroxiredoxin transcription regulator (OhrR), is also believed to be a dedicated thiol buffer.

![Scheme 32. Structure of some LMW thiols.](image)

Structurally, BSH is a combination of L-cysteine (L-Cys) linked to glucosamine (GlcN) and malic acid (L-Mal, Scheme 33). BSH shares the same GlcN-Cys scaffold as MSH with the free amino group on Cys and the myo-inositol replaced by L-malate. Inspired by the well-documented MSH biosynthetic pathway, the biosynthesis of BSH was proposed in three steps. The glycosyltransferase first couples GlcNAc and L-malate (BshA) to generate GlcNAc-Mal, which subsequently deacetylate (BshB) to GlcN-Mal. This intermediate is then coupled with a still uncharacterized cysteine
ligase (BshC) to produce the desired BSH (Scheme 33).\textsuperscript{179}

![Scheme 33. Proposed biosynthetic pathway for BSH.](image)

By analogy with other LMW thiols, BSH is also believed to be a key player in thiol-disulfide homeostasis.\textsuperscript{179} Figure 35 outlines the summary of known or proposed physiological processes with BSH participation.\textsuperscript{179} Similar to GSH and MSH, the intracellular ratio of BSH/BSSB is finely regulated ranging from 40:1 (in \textit{B. anthracis}) to 400:1 (in \textit{B. subtilis}).\textsuperscript{183} The ratio is expected to be maintained by a BSSB reductase, an enzyme that has not been fully identified.\textsuperscript{177} BSH is thought to be the preferred reducing agent among the LMW thiols given its dominant concentration and low pKa of the thiol group (7.97).\textsuperscript{184} In response to oxidative stress, BSH undergoes oxidation to form BSSB.\textsuperscript{179} Bacillithiolation is another type of response to stress.\textsuperscript{179} In \textit{B. subtilis}, BSH reacts with the redox sensitive OhrR to form a mixed disulfide during cumene hydroperoxide stress.\textsuperscript{177, 185}
Figure 35. Summary of processes known or proposed to involve BSH.

Including formation of BSSB, reversible chelation of intracellular metal ions, formation of mixed disulfides with OhrR in response, detoxification, the NADH-dependent reduction of SNO compounds, detoxification of methylglyoxal and formaldehyde.

The identification of BSH biosynthesis related genes boosted the understanding of the physiological role of BSH. Early work on BSH null cells showed more sensitivity to thiol oxidizing reagents, ROS, RNS, reactive electrophilic species, toxic metal ions and the antibiotic fosfomycin. A number of proteins in B. subtilis were noticed to undergo bacillithiolation when exposed to NaOCl. A study on the BSH structure suggested that the malate group may potentially act as an intracellular metal chelator. BSH chelates Zn\textsuperscript{II} and Co\textsuperscript{II} \textit{in vitro} and is also suggested as a major buffer of the labile zinc pool. It is also indicated that BSH is involved in the processing of other metals, such as Fe, Mn, As, Cd and Cu. However, no direct ligation to these metals was confirmed. Detoxification of fosfomycin via the nucleophilic attack on the epoxide ring has been noticed with several different enzymes including FosA, FosX in Gram (-) and FosB in Gram (+) bacteria (Scheme 34). BSH in purified FosB from \textit{S. aureus} and
B. subtilis was greatly involved in the detoxification pathway of fosfomycin via direct nucleophilic addition (Scheme 34). ^{190-191}

![Scheme 34. Detoxification pathways for fosfomycin.](image)

As is depicted in Figure 35, BSH may also undergo S-nitrosation to form S-nitrosobacillithiol (BSNO). ^{179} Previous studies in B. subtilis revealed the induction of complex stress responses upon the introduction of NO and the S-nitrosating agent S-nitroprusside. ^{192} NO has been known to react with Fe^{II} in metal dependent repressors to regulate enzyme activity. ^{179} For example, NO induces the release of active catalase via catalase mediated S-nitrosation of the heme-bound thiol. ^{179} LMW thiols play key roles in resistance to S-nitrosothiols via trans-nitrosylation. ^{179} Thereby, the resulting LMW S-nitrosothiols conveys the ubiquitous influence of NO on cellular signal transduction, acting as a key role in the redox-based physiological regulation matrix. ^{29} To date, both GSNO and S-nitrosomycophiol (MSNO) have been identified and prepared and the profile of GSNO has been very well established. ^{193-194} We believe the likely existence of BSNO and its key role as a signaling messenger in the mediation of various physiological processes. In this chapter, we will discuss our efforts to prepare and characterize BSNO.

4.2 RESULTS AND DISCUSSION

Preparation of S-nitrosothiols from thiols is straightforward. Reaction of free thiol
with NaNO₂ under acidic conditions or nitroso transfer from agents such as t-butyl nitrite provides the desired S-nitrosothiol. However at this time, BSH is not easily accessible. BSH is not commercially available and yields of BSH from bacteria remain low since BSH has only been isolated as BSmB (S-bimane derivative of BSH) with a yield of 50 mg/L from *D. radiodurans*. Hence, successful preparation of decent amounts of BSH via synthesis is a prerequisite for the study of BSNO.

Successful attempts to synthesize BSH have been reported by two different groups after the initial report of the BSH structure. The first synthesis was reported by Hamilton and coworkers in 2011, where they started with a *D*-glucosamine derivative and *L*-malic acid and obtained BSH in 9 steps with an overall yield of 13.2%. In 2012, Armstrong and coworkers started with *D*-glucosamine and *L*-malic acid and successfully prepared BSH with an overall yield 8-9% in 11 steps. In addition, this approach has proven successful in large scale reactions up to grams.

While both teams claimed the successful preparation of BSH, the reported NMR spectra (both proton and carbon) of BSH appear inconsistent. This data adds another level of complexity to the preparation of BSH. To resolve this complexity, we decided to move forward and start the synthesis of BSH following the Armstrong procedure with slight modifications.

Unlike Hamilton’s approach, this synthetic strategy targets the disulfide, BSSB, the oxidized form of BSH. As depicted in Scheme 32, BSH has three major components: *D*-glucosamine, *L*-malate and *L*-cysteine. Installation of these three parts followed by appropriate modifications should provide the desired product BSSB, which
can be reduced to BSH (Scheme 35).\textsuperscript{190}

Scheme 35. Overview of the preparation of BSH.

Prior to this attempt, a previous lab member completed a BSSB synthesis using this approach (Scheme 35). Mass spectrometric analysis of the obtained compound gave the parent peak of BSSB as the major peak along with other peaks. Both of the $^1$H and $^{13}$C NMR spectra in D$_2$O appear clean and can be interpreted to correspond to BSSB. However, the spectra are inconsistent with other reported spectra.\textsuperscript{190, 196} Despite this inconsistency, we tried the synthesis of BSNO by treating the reduced BSSB with NaNO$_2$ under acidic conditions but failed after several attempts as judged by UV-vis spectroscopy and mass spectrometry. These failures led us to examine the synthesis of BSH.

We initially tried the reaction of $L$-malic acid with $N,N$-dimethylformamide di-tert-butyl acetal for the synthesis of di-tert-butylmalate (10, Scheme 36).\textsuperscript{197} While this reaction worked nicely and gave the desired product in decent yield, the high price of the
acetal makes this route impractical for scaling up. Thus, we followed a known preparation through an isourea to esterify malic acid. The initial attempt was to esterify L-malic acid with the isourea, which is generated from addition of t-butanol to DCC with Cu\textsuperscript{1} catalysis (Scheme 36). This approach proved unsuccessful presumably due to the difficulty to isolate and purify the isourea. A switch from DCC to N,N-diisopropylcarbodiimide (DIC) overcame this problem and we were able to purify the N,N-diisopropyl-O-tert-butilisourea via vacuum distillation. The malate (10) then could be obtained by reacting malic acid with excess isourea (Scheme 36).

![Scheme 36. Synthesis of 10.](image)

The next component, trichloroacetimidate, is derived from D-glucosamine. As shown in Scheme 37, treatment of D-glucosamine with sodium methoxide, trifluoromethanesulfonyl azide followed by acetic anhyride provides the fully protected azide (15). Selective removal of the C1 acetate by hydrazine gives the free alcohol (16), which can add to trichloroacetonitrile with 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) catalysis to form the desired trichloroacetimidate (13, Scheme 37).
Scheme 37. Preparation of 13.

Scheme 38 depicts the rest of the synthesis for BSSB. Coupling of trichloroacetimidate (11) with di-tert-butylmalate (10) with the activation of trimethylsilyl trifluoromethanesulfonate (TMSOTf) affords a mixture of alpha (12a) and beta (12b) glycosides. These two isomers can be separated by column chromatography to give the desired azide (12a). Straightforward hydrogenation of the azide (12a) with the catalysis of palladium/carbon in methanol affords the amine (13). On-going work directly examines the reaction of amine (13) with the commercially available protected cysteine disulfide (cystine) to obtain the amide disulfide (14, Scheme 38). The removal of the remaining acetyl groups on 14 by sodium methoxide followed by the deprotection of the t-butyl ester with trifluoroacetic acid (TFA) will afford the desired disulfide BSSB. Purification of BSSB may be achieved by cation exchange column chromatography.
Scheme 38. Preparation of BSSB from 10 and 13.

The first coupling step is successful and the correct diastereoisomer 12a has been isolated and confirmed by comparing the reported MS and NMR data. Reduction of the azide to 13 also proved successful by mass spectrometry. However, coupling of 13 to \( N,N'\)-di-Boc-\( L \)-cystine is not successful after several attempts. LC-MS and NMR provides no sign of successful coupling.

The double coupling process may complicate this reaction since the second coupling may be sterically hindered after the first bulky amine coupled to the cystine. We followed Hamilton’s procedure in which they coupled the amine (13) to a tritylated cystine to make a protected BSH.\(^{196}\) We first prepared \( N \)-(tert-butoxycarbonyl)-\( S \)-trityl-\( L \)-cysteine pentafluorophenyl ester (17) from \( N \)-Boc- \( S \)-Trt-\( L \)-cystine (Scheme 39).\(^{196}\) Ongoing work attempts to build a protected version of BSH (18) by the reaction of the pentafluorophenyl ester (17) with the amine (13, Scheme 39). For use, BSH can be
readily prepared by de-protection of the protected BSH (18).

Future work will treat fresh BSH with NaNO₂ under acidic conditions or with t-butylnitrite for trans-nitrosation for the preparation of BSNO. The formation of BSNO should induce a significant color change due to the N=O bond, which could also be monitored by UV-vis spectroscopy. Isotopic labeling with \(^{15}\)N coupled with high resolution mass spectrometry will be used to confirm the existence of BSNO. If the stability of BSNO allows, BSNO will be further purified by HPLC followed by a full characterization by NMR. Further work on its biological significance can be carried out beyond that point.

4.3 EXPERIMENTAL METHODS

General
Reagents were obtained from commercial sources and used without additional purification. Reaction solvents were anhydrous and purged with argon prior to use. Extraction, silica, and preparative reverse phase chromatography solvents were technical grade. LC-MS and ESI-MS solvents were Optima HPLC grade. Analytical TLC was performed on silica gel plates (normal phase) or C18 silica gel plates (reverse phase), and visualization was accomplished with UV light. \(^1\)H NMR spectra were recorded on Bruker Avance DPX-300 and DRX-500 instruments at 300.13 and 500.13 MHz, respectively. \(^{13}\)C NMR spectra were recorded on the described instruments operating at 75.48 and 125.76 MHz, respectively. NMR spectra were obtained using Bruker 5 mm BBO and QNP probes held at 25 °C. Low-resolution mass spectra were obtained using an Agilent Technologies 1100 LC/MSD ion trap mass spectrometer equipped with an atmospheric pressure electrospray ionization source and operating in positive ion mode.

**Preparation of \(N,N'\)-diisopropyl-\(O\)-tert-butylisourea**

\(\text{CuCl} (120 \text{ mg, 1.21 mmol,})\) was added to a solution of \(\text{DCC} (18.9 \text{ mL, 121 mmol})\) in \(\text{t-BuOH} (13.2 \text{ mL, 139 mmol})\). The reaction mixture was stirred overnight at room temperature and subsequently distilled under reduced pressure to afford the desired product as a colorless oil (20.1 g, 83.1%). The product is immediately used for the esterification of L-malic acid. \(^1\)H NMR (300 MHz, CDCl\(_3\)) \(\delta\) 3.50-3.86 (m, 1H), 2.99-3.35 (m 1H), 1.47 (s, 9H), 1.38 (s, 1H), 0.97-1.19 (m, 12H). \(^{13}\)C NMR (75 MHz, CDCl3) \(\delta\) 149.54, 78.14, 46.22, 43.53, 43.45, 29.06, 28.42, 24.33, 23.87.

**Preparation of di-tert-butyl-(2R)-malate 10.**

A solution of \(N,N'\)-diisopropyl-\(O\)-tert-butylisourea (20.1 g, 100.0 mmol) and L-
malic acid (2.12 g, 15.8 mmol) in anhydrous CH₂Cl₂ (200 mL) in a dry 500 mL round bottom flask was stirred at room temperature for 20 h. The mixture was concentrated and the resulting white solid was dissolved in petroleum ether and filtered through a Celite pad. The solid and Celite pad were rinsed with petroleum ether (2 x 50 mL). The combined filtrates were concentrated to a crude product that was purified by flash chromatography (EtOAc /petroleum ether = 1:5) to provide **10** as a colorless oil (2.5 g, 64.3 %). Rf = 0.44 (EtOAc /petroleum ether = 1:5). ¹H NMR (300 MHz, CD₃Cl) δ 4.30-4.17 (m, 1H), 3.17 (d, J = 5.5 Hz, 1H), 2.72-2.51 (m, 2H), 1.42 (s, 9H), 1.39 (s, 9H). ¹³C NMR (75 MHz, CD₃Cl) δ 172.77, 169.76, 82.60, 81.30, 67.56, 39.91, 28.06, 27.96.

**Preparation of 10 by N,N-dimethylformamide di-tert-butyl acetal**

N,N-Dimethylformamide di-tert-butyl acetal (2.15 mL, 8.95 mmol) was added dropwise to a solution of L-malic acid (300 mg, 2.24 mmol) in dry toluene over 20 min at 80 °C. The reaction was stirred for 40 min at 80 °C and allowed to cool to room temperature. The reaction mixture was concentrated under vacuum and the crude product was purified by flash chromatography (EtOAc /petroleum ether = 1:5) to provide **10** as a colorless oil (280 mg, 50.8%).

**Preparation of trifluoromethanesulfonil azide (TfN₃)**

Trifluoromethanesulfonic anhydride (4.1 mL, 25 mmol) was added slowly to a vigorously stirred mixture of sodium azide (8.0 g, 123.0 mmol) initially dissolved at room temperature in water (20 mL) in a 100 mL round bottom flask followed by adding CH₂Cl₂ (25 mL) and cooled to 0 °C. After stirring for 2 hours at 0 °C, the organic layer was separated and the aqueous layer was extracted with CH₂Cl₂ (2 x 10 mL). The organic
layers were combined and washed with saturated NaHCO₃ (20 mL) and water (20 mL),
dried over MgSO₄ and filtered. The resulting TfN₃ solution (0.4 M) was used
immediately for the preparation of 11.

**Preparation of 15**

A solution of sodium methoxide (4.37 M) in methanol (2.6 mL) was added to a
suspension of D-glucosamine hydrochloride (2.0 g, 9.3 mmol) in methanol (18.5 mL) at
room temperature. The suspension becomes a colorless solution and a precipitate (NaCl)
forms. After 30 min of stirring, DMAP (1.20 g, 9.8 mmol) was added. Freshly prepared
TfN₃ (0.4 M) was added over 15 min and the resulting reaction mixture was stirred
overnight. The mixture was condensed under vacuum and pyridine (27 mL) was added.
This reaction was cooled to 0 °C and acetic anhydride (18.9 mL, 200 mmol) was added
dropwise. After stirring overnight, the reaction mixture was diluted with CH₂Cl₂ (50 mL)
and washed with 1 N HCl (3 x 100 mL), saturated NaHCO₃ and brine. The organic layer
was dried over MgSO₄, filtered and condensed under vacuum and the resulting crude
product was purified by flash chromatography (EtOAc /petroleum ether=1:2) to provide
15 as a colorless gummy oil (α:β = 3:7, 1.82 g, 52.6%). Rf = 0.38 (EtOAc /petroleum
ether = 1:2). ¹H NMR (300 MHz, CD₃Cl) δ 6.16 (dd, J = 3.8, 1.5 Hz, 0.3H, αH), 5.48
(dd, J = 8.5, 1.5 Hz, 0.7H, βH), 5.36-5.20 (m, 0.3H, αH), 5.07-4.94 (m, 1H, α+βH), 4.89
(td, J = 9.7, 1.6 Hz, 0.7H, βH), 4.23-4.06 (m, 1H), 4.03-3.83 (m, 1.3H), 3.75 (ddd, J =
9.9, 4.3, 2.1 Hz, 0.7H), 3.66-3.46 (m, 1H), 2.04 (d, J = 1.7 Hz, 3H), 1.98-1.76 (m, 9H).
¹³C NMR (75 MHz, CDCl₃) δ 169.73, 169.46, 169.36, 169.27, 168.32, 168.28, 92.30,
89.82, 72.42, 72.39, 70.55, 69.57, 67.78, 67.75, 62.44, 61.29, 20.58, 20.55, 20.35, 20.33,
20.31, 20.24. ESI-MS positive ion mode (m/z) 374.2 [M+H]⁺
Preparation of 16

Hydrazine acetate (0.54 g, 5.79 mmol) was added to a solution of azide 15 (1.8 g, 4.82 mmol) in DMF (14 mL) at 50 °C and the reaction was stirred until all salts were dissolved. The reaction solution was cooled to room temperature and stirred for another 30 min. The mixture was diluted with water (30 mL) and extracted with EtOAc (mL). The organic layers were combined, washed (water and brine), dried over MgSO₄, filtered and condensed under vacuum. The resulting crude product was purified by flash chromatography to give 16 as a yellow oil (0.8 g, 50.2%). Rf = 0.2 (EtOAc /pet. ether = 1:2). ¹H NMR (300 MHz, CD₃Cl) δ 5.49 (dd, J = 10.5, 9.3 Hz, 0.7H), 5.35 (d, J = 3.4 Hz, 0.7H), 5.09-4.95 (m, 1.3H), 4.82 (bs, 0.7H), 4.71 (d, J = 8, 0.3H), 4.31-4.15 (m, 1.3H), 4.15-4.00 (m, 0.7H), 3.70 (bs, 0.3H), 3.52-3.42 (m, 0.3H), 3.37 (dd, J = 10.5, 3.4 Hz, 0.7H), 2.06 (d, J = 2.4 Hz, 6H), 2.01 (s, 3H). ¹³C NMR (75 MHz, CDCl₃) δ 171.00, 170.30, 170.22, 169.96, 169.84, 96.11, 91.93, 72.64, 71.71, 70.48, 68.65, 68.42, 67.24, 64.78, 62.07, 61.41, 20.97, 20.63, 20.60, 20.53. ESI-MS positive ion mode (m/z) 332.2 [M+H]⁺

Preparation of trichloroimidate 11

DBU (0.09 mL, 0.7 mmol) was added to a solution of 16 (0.8 g, 2.416 mmol) and trichloroacetonitrile (0.8 mL, 8 mmol) in CH₂Cl₂ (12 mL) and allowed to stir overnight. The reaction mixture was concentrated and purified by flash chromatography to give 11 as a yellow oil (400 mg, 34.8 %). Rf = 0.42 (EtOAc /pet. ether = 1:2). ¹H NMR (300 MHz, CD₃Cl) δ 8.77 (s, 1H), 6.43 (d, J = 3.6 Hz, 1H), 5.46 (dd, J = 10.5, 9.3 Hz, 1H), 5.25-4.90 (m, 1H), 4.36-3.91 (m, 3H), 3.71 (dd, J = 10.5, 3.6 Hz, 1H), 2.05 (s, 3H), 1.99
(d, J = 1.7 Hz, 6H). $^{13}$C NMR (75 MHz, CDCl$_3$) δ 169.37, 168.75, 168.58, 159.42, 93.01, 89.52, 76.61, 76.19, 75.76, 69.64, 69.12, 66.94, 60.40, 59.62, 19.63, 19.60, 19.54.

**Preparation of 12a**

Molecular sieves (4 Å) was added to a solution of trichloroimidate (11, 400 mg, 0.84 mmol) and di-t-butylmalate (10, 248 mg, 1.01 mmol) in CH$_2$Cl$_2$ (4 mL) and stirred for 30 min. The solution was cooled to -40 °C and TMSOTf (0.08 mL, 0.42 mmol) added dropwise. The reaction mixture was allowed to warm to -20 °C, stirred for an additional 2 h and quenched with Et$_3$N (20 mL). The reaction mixture was filtered through a Celite pad and the filtrate concentrated. The residue was purified by column chromatography to afford 12a as white gummy solid (130 mg, 27.7%). Rf = 0.43 (EtOAc /pet. ether = 1:2). $^1$H NMR (300 MHz, CD$_3$Cl) δ 5.36 (t, J = 10.7 Hz, 1H), 5.06 (d, J = 3.8 Hz, 1H), 4.94 (t, J = 10.4, Hz, 1H), 4.47 (dt, J = 10.4, 2.8 Hz, 1H), 4.27 (t, J = 6.3 Hz, 1H), 4.18 (dd, J = 12.6, 3.3 Hz, 1H), 3.79 (dd, J = 12.5, 2.3 Hz, 1H), 3.13 (dd, J = 10.7, 3.8 Hz, 1H), 2.60 (d, J = 6.3 Hz, 2H), 1.92 (d, J = 2.5 Hz, 6H), 1.88 (s, 3H), 1.33 (s, 18H). $^{13}$C NMR (75 MHz, CDCl$_3$) δ 170.58, 169.84, 169.65, 169.61, 169.10, 100.07, 82.32, 81.64, 76.16, 70.23, 68.14, 68.09, 61.42, 60.80, 38.99, 27.90, 20.65, 20.62, 20.54. ESI-MS positive ion mode (m/z) 560.2 [M+H]$^+$

**Preparation of amine 13**

A solution of azide (12a, 130 mg, 0.23 mmol) in MeOH (5 mL) was slowly added to a slurry of 10% Pd/C in MeOH under an atmosphere of argon. The resulting suspension was purged and hydrogenated (3x) with a balloon full of hydrogen overnight. The reaction solution was passed through a Celite pad and the filtrate concentrated to
afford 13 as an oil (90 mg, 73%) of sufficient purity to be used without further purification. ESI-MS positive ion mode (m/z) 534.2 [M+H]^+  

**Preparation of 17**

Pentafluorophenyltrifluoroacetate (0.70 mL, 4.04 mmol) was added to a solution of N-Boc-Cys(S-Trt)-OH (1.04 g, 2.24 mmol) in anhydrous DMF (4 mL) and pyridine (0.35 mL). The reaction mixture was allowed to stir for 45 min at room temperature. After completion, the crude mixture was dissolved in CHCl₃ (50 mL) and washed with H₂O. The organic layer was then separated, dried (Na₂SO₄) and the solvent evaporated to give a yellow oil that was purified by flash chromatography to give 17 (1.30 g, 2.06 mmol, 92%) as a white gummy solid. ¹H NMR (300 MHz, CD₃Cl) δ 7.35 (m, 6H), 7.27 - 7.07 (m, 9H), 4.97 (d, J = 8.1 Hz, 1H), 4.36 - 4.08 (m, 1H), 2.82 - 2.67 (m, 1H), 2.59 (dd, J = 12.9, 4.7 Hz, 1H), 1.35 (s, 9H). ¹³C NMR (75 MHz, CDCl₃) 167.4, 155.0, 144.1, 129.5, 128.3, 128.05, 128.02, 127.4, 127.2, 81.0, 67.5, 52.7, 33.5, 28.3. ESI-MS positive ion mode (m/z) 652.2 [M+Na]^+
CONCLUSIONS AND FUTURE OUTLOOK

This dissertation focuses on the development of a new fluorescent detection method for HNO. Chapter 1 serves as a background and covers the basics of NO and RSNO and extensively reviews the chemistry, biochemistry and physiology of HNO. To overcome the limitations of the traditional detection methods, the improvement of the detection and quantification approaches for HNO has been improved, leading to the development of more diversified HNO donating agents and a better understanding of its physiological role as well as its endogenous sources.

Previous work in our lab developed a reductive Staudinger ligation based methodology for trapping HNO effectively. In Chapter 2 we extend this chemistry to a novel fluorescein-based fluorescent probe (1b) for HNO detection. This probe reacts with HNO derived from AS and 4-BPA leading to a concentration dependent fluorescence increase. Probe 1b also demonstrates high sensitivity and selectivity over other biological relevant species. Subsequent confocal microscopy experiments further display its ability to detect HNO by fluorescence in HeLa cells. Following mass spectrometric analyses of cell lysates successfully identify the amide phosphine oxide ligation product, confirming the proposed mechanism.

As earlier reports demonstrate the ability of RSNO to activate reductive ligation based probes, we carefully investigated and compared the phosphine-based detection strategies for both HNO and RSNO in Chapter 3. Phosphorus NMR and HPLC-MS studies reveal the difference that azaylides derived from HNO or organic RSNO efficiently participate in subsequent reductive ligation while the ligation process for
biologically relevant RSNO is not productive. This difference allows the use of phosphine probes for the detection of HNO in biological systems without interference from RSNO. Further successful detection of HNO in cells using \textbf{1b} by flow cytometry illustrates the reliability of these phosphine probes to serve as a fast robust screening approach for endogenous HNO sources. Application of our established HNO screening protocol to two potential endogenous HNO generation systems (H$_2$S + NO or RSNO) reveals these two systems do not produce notable amount of HNO spontaneously as judged by this detection.

In Chapter 4, we propose the potential existence of BSNO and the significant role it may play in signaling. We make efforts to prepare BSH from \textit{D}-glucosamine, \textit{L}-malic acid and the protected cysteine derivatives followed by its conversion to BSNO. Although this project is not complete, we believe the successful preparation and characterization of BSNO will help understand the biological roles that BSH/BSNO play in low-G+C content Gram-positive bacteria.

Currently, our knowledge on HNO is still somewhat limited. Upcoming work in the field of HNO should include application of the developed probes to better understand HNO related physiology and continue to explore endogenous HNO sources. Development of a standard and convenient protocol for fast screening may greatly assist this process. In the area of RSNO, detection of RSNO has always been a difficult bottleneck research endeavors and the development of RSNO specific detection approaches remains urgent.
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### APPENDIX

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PUBLICATIONS:


PRESENTATIONS AND ABSTRACTS:


2. Zhengrui Miao, Julie A. Reisz, Susan M. Mitroka, Jia Pan, Ming Xian and S.
Bruce King, “Phosphine-based Fluorescent Probes for Nitroxy (HNO)” **Oral. 66th Southeastern Regional Meeting of the American Chemical Society, Nashville, TN, October 16-19, 2014.**
