NEWLY DESIGNED HNO TRIGGERED PRODRUGS

BY

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Abstract

Nitric oxide (NO) is an important species in many biological processes. The one-electron reduced and protonated derivative of NO, HNO has also been determined to play important biological roles. Because of HNO’s high reactivity, its detection has been difficult and many methods including fluorescent copper compounds and electrochemical methods have been devised. Our group has studied HNO for many years and recently developed a detection method based on HNO’s reaction with organophosphorus compounds.

Inflammation is associated with many pathological disorders, such as cancer and infection and production of reactive oxygen species (ROS), including H$_2$O$_2$, is central to many inflammatory diseases. ROS can react with NO to generate reactive nitrogen species (RNS) including HNO and HNO-triggered prodrugs might be used for targeting inflammatory sites.

In this thesis, we describe new HNO-triggered prodrugs based on our previously described HNO detection methods. Specifically, three esters of the drugs wintergreen, acetaminophen and metronidazole with an organo-phosphine were prepared and characterized. These compounds were treated with HNO, as generated by Angeli’s salt, and evaluated for their release of drug and other predicted byproducts. In all cases, the expected drug was recovered but the reactions of the wintergreen and acetaminophen derivatives were relatively complicated by numerous side products. These side products could result from various hydrolytic pathways or direct reactions of HNO with the prodrugs at sites besides the phosphorus atom. The kinetics of the process were determined using the wintergreen prodrug and shown to follow the kinetics of Angeli’s
salt decomposition indicating the prodrugs rapidly react with HNO. The prodrugs do not
react with biological reducing agents but oxidize to the corresponding phosphine oxide
upon treatment with NO or nitrite. Such reactivity may limit the usefulness of these
HNO-triggered prodrugs.

The results are discussed in context of future plans regarding cellular experiments and
the need to further define the mechanisms of the HNO-based reactions with these
compounds.
Chapter 1. Background

1.1 NO

NO, a diatomic free radical, is one of several oxides of nitrogen. Under standard conditions, it is a colorless gas. It is an important intermediate in the chemical industry and is unavoidably produced during combustion of fossil fuels. In early times, the biggest concern about NO was its role in air pollution. Since the 1980s, much research has been done on NO as NO also plays an important biological role in vivo. ¹ NO has a wide range of functions which include the regulation of neurotransmission, blood clotting, blood pressure and the ability to destroy tumor cells. Diverse fields such as neurobiology, immunology and cardiovascular pharmacology have focused on the study of NO. ¹

1.1.1 Biological Properties

NO can be synthesized in vivo via nitric oxide synthases (NOSs). There are 3 isoforms of the NOS enzyme: endothelial (eNOS), neuronal (nNOS), and inducible (iNOS) with different functions. ² Several factors including shear stress, acetylcholine, and cytokines stimulation can induce NO by endothelial nitric oxide synthase (eNOS). NOS produce NO from the terminal guanidine-nitrogen of L-arginine and oxygen. After NO synthesis in vivo, NO diffuses into smooth muscle cells of the blood vessel and activates soluble guanylate cyclase (sGC) that catalyzes the production of the second messenger cyclic guanosine monophosphate (cGMP) from guanosine triphosphate (GTP). After that, cGMP activates cyclic nucleotide-dependent protein kinase G (cGKI) which is a kinase
that phosphorylates a number of proteins. The phosphorylation finally results in
smooth muscle relaxation which can cause vasodilation. The brief scheme of
how NO works for vasodilation is shown in Figure 1. [1-3, 14]

![Figure 1. Physiology of NO in vasodilation](image)

Through muscle relaxation, NO plays important roles in blood pressure
control, penile erection and the immune system. The vasodilation can also help
the renal control of extracellular fluid homeostasis and is essential for the
regulation of blood flow and blood pressure. [4-6] Nitric oxide also serves as a
neurotransmitter between nerve cells. Unlike other transmitters, NO can diffuse
widely and readily into cells due to its small, uncharged, and fat-soluble
properties.[7, 8]

In addition to NO biosynthesis via NOS, dietary nitrite is swallowed and reacts
with acid and reducing substances in the stomach to produce high
concentrations of nitric oxide which is thought to be involved in the sterilization of
swallowed food, preventing food poisoning, and maintaining gastric mucosal blood flow. [7, 8]

1.1.2 Chemical Properties

Nitric oxide has N in the formal +2 oxidation state. Since the oxidation state of nitrogen ranges from -3 to +5, nitric oxide can both be oxidized and reduced. Several ways exist to prepare nitric oxide.

In a commercial setting, NO is produced by oxidation of ammonia with platinum as catalyst.[9]

$$4\text{NH}_3 + 5\text{O}_2 \xrightarrow{\text{Pt}} 4\text{NO} + 6\text{H}_2\text{O}$$

The uncatalyzed endothermic reaction of O$_2$ and N$_2$ can generate NO at a very high temperature

$$\text{N}_2 + \text{O}_2 \rightarrow 2\text{NO}$$

In the laboratory, nitric oxide can be generated by the reduction of nitric acid.

$$8 \text{HNO}_3 + 3 \text{Cu} \rightarrow 3 \text{Cu(NO}_3\text{)}_2 + 4 \text{H}_2\text{O} + 2 \text{NO}$$

In aerobic conditions, nitric oxide can be oxidized to NO$_2$ which is a brown toxic gas and considered as a major air pollutant.

$$2\text{NO} + \text{O}_2 \rightarrow 2\text{NO}_2$$

Moreover, NOx reacts with volatile organic compounds in the presence of sunlight to form ozone which can cause adverse effects such as damage to lung
tissue and reduction in lung function. \textsuperscript{[10]} Based on this toxicity, NO has historically been considered as a major concern of air pollution which also can be produced by the use of fuel.

1.2 HNO

HNO (Hydrogen oxonitrate, also called nitroxy, nitrosyl hydride, nitroso hydrogen, monomeric hyponitrous acid) has been found as an intermediate in a variety of thermal and photochemical reactions since the early 1900s.\textsuperscript{[11]} Since then, much research has been done focusing on the intermediacy of HNO in combustion of nitrogen-containing fuels, in the atmosphere in interstellar chemistry and in bacterial denitrification.\textsuperscript{[11]} Recently, the study of HNO has been more concerned with the pharmacological effects and potential physiological functions of HNO.\textsuperscript{[14]}

1.2.1 Chemical Properties

HNO is the one-electron reduced and protonated derivative of NO.\textsuperscript{[14]} It is a very reactive species that undergoes a rapid dimerization.\textsuperscript{[12, 13]} The dimerization rate constant is about $8 \times 10^6 \text{M}^{-1} \text{s}^{-1}$.\textsuperscript{[49]}

\[
2\text{HNO} \rightarrow [\text{HONNOH}] \rightarrow \text{N}_2\text{O} + \text{H}_2\text{O}
\]

Also, HNO can be treated as the conjugate acid of NO\textsuperscript{−} which, in turn, can be seen as the conjugate base of HNO. But, these species are not simply related through an acid-base relationship. Their different spins make the acid-base relationship of HNO and NO\textsuperscript{−} complicated.\textsuperscript{[11]}
Experiments show that $^1$HNO is about 18-19 kcal/mol more stable than $^3$HNO. \[11\] Also, the protonation of $^3$NO$^-$ always produces $^3$NOH, the isomer of $^3$HNO. The energy gap between the $^3$NOH and $^1$HNO is about 20-23 kcal/mol and $^1$HNO is more stable. \[15, 16\] The energy gap between $^1$NO$^-$ and $^3$NO$^-$ is about 16-21 kcal/mol. The energy relationship between different spin states are shown in Figure 2. \[11\]

HNO to NOH is thermally inaccessible under biological condition. However, discrete deprotonation of HNO and NOH could lead to acid-base equilibria as convoluted as depicted in Figure 2. \[17\]

![Figure 1. Different spins states of HNO](image)

The pKa of HNO was first determined to be 4.7 by Gratzel, and then Shafirovich and Lymar updated the pKa to 11.4 in 2002. \[27, 50\]
1.2.2 Biological Properties

Until the mid 1980s, attention to nitrogen oxide was generally limited to environmental concerns. In 1980, vasodilation was determined to be actively mediated by an unidentified species, which was labeled the endothelium-derived relaxing factor (EDRF).\cite{18} After chemical and biological research, the results led to the conclusion that NO was EDRF.\cite{19,20} However, some certain dissimilarities between the effects exerted by NO and EDRF were observed that led to the speculation that this species may be HNO.\cite{21} Angeli’s Salt (Na$_2$N$_2$O$_3$) and Piloty’s acid (benzenesulphonydraoxamic acid) are both bioactive HNO donors. When administered intraperitoneally or intraarterially to mice or rats, these donors result in vasorelaxation.\cite{11}
The vasoactivity is accompanied by increased cyclic guanosine monophosphate (cGMP) production. However, the vasoactivity is generally less effective than that elicited by NO donors, which indicates that HNO is converted to NO in vivo and that HNO is an intermediate form of EDRF. Fukuto et al. demonstrated that HNO can be easily oxidized to NO in the presence of SOD (superoxide dismutase). Interestingly, coinfusion of Angeli’s salt and the electron paramagnetic resonance (EPR) trap diethyldithiocarbonate (DETC) suggested that HNO was only minimally oxidized to NO in vivo (<5%) which also raises the speculation that HNO only serves as an intermediate when converted to NO in vivo.

In vitro, scientists noticed that Angeli’s Salt, an HNO donor, enhanced oxidative stress by peroxides while NO was protective under the same conditions. The cytotoxicity of Angeli’s salt was dependent on an aerobic environment and enhanced by the absence of glutathione (GSH). This research provides the evidence that HNO could affect cellular function by changing the redox status of the cell. Also, HNO can either associate with GSH or scavenge GSH, which may affect the activity of enzymes containing critical thiols.

The biological function of HNO in vivo is not fully understood. More research still needs to be done in the future to give a better understanding of HNO.
1.2.3 Biosynthesis of HNO

No unequivocal evidence for the endogenous generation of HNO in mammalian systems currently exists. However, some chemical and biochemical processes have been shown to be possible ways of endogenous HNO formation. For example, the reaction of S-nitrosothiols with other thiols can generate HNO and difsulfide.  

\[
\text{RS-NO+R'SH} \rightarrow \text{RSSR'+HNO}
\]

N-Hydroxy-L-arginine (NOHA), a NO biosynthetic intermediate, can be oxidized to HNO. HNO can also be generated from L-arginine or NOHA by the presence of NOS especially when it is deplete of one of its prosthetic groups. Another possible endogenous pathway to HNO is the reaction of NO and H\(_2\)S. NO and H\(_2\)S can enter a redox reaction with each other that lead to the formation of HNO.

1.3 HNO Detection

While both physiological and pathological roles of NO in vivo have been deeply studied, HNO has been much less thoroughly investigated. Much evidence shows that HNO plays important biological roles in potential pharmacological applications distinct from those of NO and developing efficient detection methods for HNO in vivo is very important.  

As stated before, HNO reacts rapidly with itself to form a dimer. Also, HNO can convert to NO in vivo. So an efficient probe must have a high rate constant for reacting with HNO and a high selectivity of reaction. For HNO, the most
efficient methods include Cu-based fluorescent probes and HNO-specific electrodes. This section will discuss some detection methods which have been developed in recent years.\cite{29, 30}

Rosenthal and Lippard developed a copper based fluorescent probe in 2010. The [Cu$^{II}$(BOT1)Cl] structure is shown in Figure 3.\cite{28}

![Figure 3. Structure of [Cu$^{II}$(BOT1)Cl]](image)

Just as SOD(Cu$^{II}$) reacts with HNO which generates NO and reduced SODCu$, [Cu$^{II}$(BOT1)Cl] also reacts with HNO and forms [Cu$^{I}$(BOT1)Cl]. While [Cu$^{II}$(BOT1)Cl] has no emission, [Cu$^{I}$(BOT1)Cl] fluoresces at 500-650nm. The probe shows increased fluorescence when treated with Angeli’s Salt. Meanwhile, treating the probe with other reactive nitrogen species (RNS) or reactive oxygen species (ROS) including NO$, NO_2^-$, NO_3^-, ONOO$, H_2O_2$, OCl$^-$ and NO do not cause fluorescence showing a high selectivity of this probe.\cite{28}

Another important detection method is a HNO-specific electrode whose structure is shown in Figure 4 developed by Martí and Doctorovich that detects HNO in a time resolved fashion at low nanomolar concentration.\cite{29}
Figure 5 shows the mechanism of detection. The electrode molecule is based on a Co$^{III}$ porphyrin, Co$^{III}$(P). The Co$^{II}$(P) can be oxidized to Co$^{III}$(P). Since the Co$^{III}$(P) is sensitive to HNO, it reacts with HNO and generates Co$^{III}$(P)NO$^-$ which is oxidized to Co$^{III}$(P)NO rapidly, yielding an electron. The resulting Co$^{III}$(P)NO complex releases the NO ligand rapidly and gives Co$^{III}$(P) allowing the catalytic cycle to start again. [29]
In the mechanism described above, the current can be detected in the presence of HNO due to the production of an electron. Also, the sensitivity and selectivity of this electrode are both good. The Co(P) electrode exhibits a linear response in transient HNO concentrations from 1 to 1000nM. The presence of oxygen and other reactive nitrogen and oxygen species (RNOS) don't affect electrode performance.\textsuperscript{[29]}

Both methods noted above are based on metal complexes which might raise concerns about water solubility and cytotoxicity. Our group has also developed a fluorescent probe based on an organic phosphorus-containing molecule,\textsuperscript{[30]} shown in Figure 6.

![Figure 6. Structure of an organic phosphine HNO probe](image)

This probe does not demonstrate fluorescence but upon reaction with HNO, the following reaction (Figure 7) occurs to give a fluorescent molecule which can be easily detected by fluorescence at 520 nm.\textsuperscript{[30]}
The selectivity of this probe has been evaluated. The probe was treated with NO⁻, NO₂⁻, NO₃⁻, H₂O₂, H₂S, GSH, S-nitrosoglutathione and S-nitrosocysteine. The probe does not react with most other redox active compounds and shows the strongest fluorescence with HNO.⁹² As the basis of my research relies on the same reaction, the mechanism is shown in detail in Figure 8.⁹³
The first phosphine (13) adds to HNO to form an adduct that can be drawn as a 3 membered ring (14). The second phosphine (13) opens the 3-membered ring resulting in an aza-ylide (16) and a phosphine oxide (15). The aza-ylide undergoes an intramolecular nucleophilic attack with release of the alcohol group and subsequent hydrolysis yields the phosphine oxide-based amide (8).

Research regarding the important biological role of HNO in the human body is based on the development of the reliable detection methods. To improve the
methods, sensitivity, selectivity and cytotoxicity must be taken into concern. The organic probe developed by our group is a very good detection method which operates through a very specific chemical pathway to release a fluorophore. However, this chemistry allows other molecules besides detection compounds to be released making new potential prodrugs.

1.4 HNO Donors

1.4.1 General Ways to Form HNO

HNO is highly reactive and its dimerization makes it impossible to store. [12, 13, 26] To study HNO, efficient HNO donors must be used. Several HNO donors exist that can be divided into organic and inorganic classes.

For the inorganic pathways, the simplest route to HNO is the reduction of NO. [32] The aerobic photolysis of ammonia also generates HNO. [33] A chain reaction may happen in the presence of O₂. Both of these pathways have atmospheric importance but are not relevant in biological research.

Organic compounds form another category of HNO donors. The Nef reaction produces HNO as shown in Figure 9. [34] The nitro group is hydrolyzed to give HNO with the by-product ketone.

![Figure 9. Scheme of Nef reaction](image.png)
Another pathway to HNO is the nitrosative cleavage of tertiary amines (Figure 10). Nitrosation of tertiary amines generate HNO by elimination of the adjacent H to give HNO.

![Figure 10. HNO formation from nitrosative cleavage](image)

Retro Diels-Alder reactions can also lead to HNO formation as shown in Figure 11. The retro Diels Alder reaction of appropriate cycloadducts will produce an acyl nitroso compound that is easily hydrolyzed to give nitroxyl.

![Figure 11. HNO formation from retro-Diels Alder reaction](image)

Simple decomposition of N-phosphinoylhydroxylamines also yield HNO (Figure 12).
The hydroxylamine can be oxidized to a nitroso group which is easily hydrolyzed to give HNO.

1.4.2 Biological HNO Donors

While these pathways give HNO, these compounds cannot always be used as efficient donors due to various drawbacks. Some of these compounds are hard to store and some cannot react in biological conditions. More efficient ways to yield HNO are required for biological research. The most commonly used HNO donors are Piloty’s acid and Angeli’s Salt.[11]

Piloty’s acid (N-hydroxybenzenesulfonamide or benzosulfohydroxamic acid) is an organic compound which hydrolyzes in basic conditions to yield HNO and a sulfinic acid. Piloty’s acid decomposes through a base-catalyzed deprotonation mechanism followed by S-N bond heterolysis. [11, 38] The mechanism is shown in Figure 13.

Piloty’s acid provides a convinient way to investigate HNO in both high and neutral pH. However, Piloty’s acid can be oxidized to the corresbonding nitroxide which then releases NO rather than HNO. [11, 39] Consequently, Piloty’s acid must be utilized in anaerobic and reducing environments. Indeed, the oxidation of Piloty’s acid to give NO is suggested to be the primary pathway in physiological conditions.
The most widely used donor is Angeli’s Salt whose decomposition mechanism is shown in Figure 14. $^{[11, 40, 41]}$

\[
\begin{align*}
\text{O} & \quad \text{N} = \text{N}^{+} + \text{O}^{-} \\
\text{H}^{+} & \quad \rightarrow \\
\text{H} & \quad \text{N} = \text{N}^{+} + \text{O}^{-} \\
\text{S} & \quad \text{H} \quad \text{N} & \quad \text{N}^{+} + \text{O}^{-} \\
\text{HNO} & \quad \text{NO}_{2}^{-}
\end{align*}
\]

Figure 14. Decomposition of Angeli’s Salt

The decomposition of Angeli’s Salt is essentially pH-independent from pH 4 to 8 and will accelerate in low pH and will diminish in high pH. $^{[11, 40]}$ Since Angeli’s Salt is more stable and it decomposes faster in neutral conditions, Angeli’s Salt is the most widely used biological HNO donor and will be used as the HNO donor in all of our experiments.

1.5 Prodrugs

A prodrug is a medication or compound that, after administration, is metabolized into a pharmacologically active drug. $^{[42]}$ Before metabolism, a prodrug is pharmacologically inactive. Prodrugs are designed for many purposes, for example, improving the absorption, bioavailability, or targeting and reducing side effects of drugs.

Prodrugs can be divided into 2 main types. Type 1 are bioactivated inside the cell (intracellularly). Type 2 are bioactivated outside the cell (extracellularly). $^{[43]}$ Prodrugs require some common properties. First, the prodrug does not have or has less bioactivity compared to the drug. $^{[44]}$ Second, the drug molecule is
connected to the carrier by a covalent bond that can be easily broken in vivo. \[^{[42]}\]

Third, the rate of decomposition of the prodrug in vivo must be fast enough so that the concentration of drug molecule can reach a certain level. Based on these principles, one of the most common strategies to design a prodrug are through an ester linkage.

HNO may play important roles in the human body and based on our current HNO detection model that releases a fluorophore, we considered whether an HNO triggered prodrug could be developed. Inflammation is associated with many pathological disorders such as cancers and infections and production of reactive oxygen species (ROS) including \(\text{H}_2\text{O}_2\) is central to many inflammatory diseases. \[^{[47]}\] ROS can act as both a signaling molecule and a mediator of inflammation. The ROS can also rapidly combine with NO to generate reactive nitrogen species (RNS) including S-nitrosothiols peroxynitrite, and possibly nitroxyl anion. \[^{[48]}\] Based on these property, HNO might be a good signaling marker for inflammation and an HNO-triggered prodrug can be efficient in targeting the drug, especially on anti-inflammatory agent to an inflammation site.

In the presence of HNO, the prodrug would decompose and release a drug molecule (Figure 15). The prodrug may improve the targeting properties of the drug molecule but also would only be released in the presence of HNO which could be of importance in redox biology. To determine whether the idea will work, drugs with a hydroxyl group will be linked to 2-(diphenylphosphino) benzoic acid (DPPBA) and our initial candidates include methyl salicylate, acetaminophen and metronidazole.
Methyl salicylate (wintergreen oil, Figure 16) is an organic ester naturally produced by many species of plants, particularly wintergreens. External application of methyl salicylate can reduce pain and relieve muscle pain and are also used as anti-herbivore defense by many plants.

Acetaminophen (Figure 17) is a widely used drug and is the most commonly used medication for pain and fever in the world. However, acute overdoes of acetaminophen can cause potentially fatal liver damage.

Metronidazole (Figure 18) is an antibiotic and antiprotozoal medication and is used either alone or with other antibiotics to treat pelvic inflammatory disease,
endocarditis. Common side effects include nausea, a metallic taste, loss of appetite, and headache.

![Metronidazole](image)

Figure 18. Metronidazole

In this thesis, the research goal will be to connect these 3 drugs with DPPBA to construct the prodrug molecules. The reaction of the prodrugs with HNO to release the drug will then be evaluated.

Using metronidazole as an example for the research plan. A metronidazole ester prodrug will be synthesized that decomposes in the presence of HNO to produce metronidazole. Once the reactivity is demonstrated, the proper product isolated, the rate of decomposition and selectivity of the prodrugs will also be studied.
Figure 19. Metronidazole prodrug decomposition in the presence of HNO
Chapter 2. Results and Discussion

2.1 Prodrug Synthesis

Based on the proposed idea from the background, the synthesis of new prodrug systems and the study of their reaction with HNO was pursued. Since the carrier is an acid, it requires the drug molecule to have a hydroxyl group which can form an ester with the carrier. We considered methyl salicylate (1, wintergreen oil) as a possible drug molecule. Although wintergreen oil is not an oral medication, it is still a good first choice based on its simple structure and widely studied properties.

Treatment of 1 with 2-(diphenylphosphino) benzoic acid (2) using DCC (N,N'-dicyclohexylcarbodiimide) as a coupling agent[30, 31] gives 3 in 83% yield and the purity was determined by $^1$H NMR and mass spectrometry (MS), which can be found in the appendix (Figure 20).

![Figure 20. Synthesis of 3](image)

Based on this success, other prodrugs were prepared. The requirement of a hydroxyl group makes acetaminophen a good choice as it is a widely used medication with a simple structure. The synthesis is similar to 3 in that DCC
coupling of 2 and 4 gave 5 in 81% yield (Figure 21). The purity is confirmed by $^1$H NMR and mass spectrometry which can be found in the appendix.

Figure 21. Synthesis of 5

An antibiotic prodrug would also be useful and for this reason, metronidazole was chosen. Metronidazole contains a nitro group that may be reduced to an amine that may interfere with the coupling reaction. However, coupling with DCC give 7 in 76% yield (Figure 22). The purity was determined by $^1$H NMR and MS which can be found in the appendix.

Figure 22. Synthesis of 7
Table 1 summarizes the synthetic results of the prodrugs (3, 5 and 7).

<table>
<thead>
<tr>
<th>Drug molecule</th>
<th>Percentage yield</th>
<th>Prodrug</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methyl Salicylate</td>
<td>83%</td>
<td><img src="image" alt="Methyl Salicylate Prodrug" /></td>
</tr>
<tr>
<td>Acetaminophen</td>
<td>81%</td>
<td><img src="image" alt="Acetaminophen Prodrug" /></td>
</tr>
<tr>
<td>Metronidazole</td>
<td>76%</td>
<td><img src="image" alt="Metronidazole Prodrug" /></td>
</tr>
</tbody>
</table>

Table 1. Synthesis of prodrugs (3, 5 and 7).
2.2 Reaction with HNO

Upon synthesis of 3, 5 and 7, the reaction of these prodrugs with HNO was examined. These compounds should behave by the mechanism previously proposed by our group for fluorophore generation which is shown in Figure 23.

![Reaction mechanism diagram]

Figure 23. Reaction mechanism

Addition of the phosphine to the N-O double bond of HNO will form an adduct that may exist in a 3-membered ring resonance structure. Another molecule of
the phosphine (13) attacks the 3-member ring (14) to form an aza-ylide (16) and phosphine oxide (15). An intramolecular nucleophilic attack of the aza-ylide (16) on the adjacent ester gives a tetrahedral intermediate (17) that decomposes to an alcohol and a phosphonium ion (18) that hydrolyzes to give an amide (8).

Angeli’s Salt was used as the HNO donor in the experiments. Angeli’s Salt can release HNO in neutral and slightly acid environments. If the pH of solution is too low, Angeli’s Salt will release NO instead of HNO.\cite{45} For this concern, the pH should be modified by buffer and both Tris buffer (tris (hydroxymethyl) aminomethane and its conjugate acid) and PBS (phosphate-buffered saline, a water-based salt solution containing sodium hydrogen phosphate, sodium chloride and, in some formulations, potassium chloride and potassium dihydrogen phosphate) buffer were used in the experiments. Since PBS contains phosphate that interferes with $^{31}$P NMR, Tris buffer was generally used in the experiments.

As previously determined,\cite{30} this reaction occurs in acetonitrile. However, these prodrugs do not dissolve well in the mixture of acetonitrile and Tris buffer. To yield a solution, THF was added to modify the solubility of the starting materials. After several trials, the best ratio of solvent was determined to be 3:1:2 acetonitrile:THF:Tris buffer.

The main goal of these reactions is to determine if the alcohol (i.e. the drug molecule) will be released through the proposed mechanism along with the corresponding phosphine oxide and the amide-phosphine oxide (8).
2.2.1 Wintergreen Ester (3)

The wintergreen ester (3) was synthesized as noted. This white solid was added to the solvent mixture, and 5 equivalents of Angeli’s Salt were added at room temperature. After 24-hours, methyl salicylate was recovered in 76% yield after chromatography as predicted by the proposed mechanism shown in Figure 24. The purity of methyl salicylate was determined by NMR and MS.

Further, experiments to show that the phosphine oxide and the amide were also produced as the proposed mechanism were performed. The isolation of 8 and 9 would support the proposed mechanism. Purification of the crude reaction mixture by column chromatography, similar to the isolation of methyl salicylate, unfortunately, did not yield 8 or 9.
To determine what was generated in the reaction, a small scale reaction of 3 with Angeli’s Salt in deuterated acetonitrile was performed and the crude $^{31}$P NMR was taken (Figure 25). For comparison, a standard of the phosphine oxide (9) was prepared by adding hydrogen peroxide to the ester (3). In addition, a standard of the amide (8) was prepared by the reaction of methyl 2-(diphenylphosphanyl) benzoate (12) with HNO.

Figure 25. Crude $^{31}$P-NMR and comparison. Panel A is the crude $^{31}$P-NMR of the reaction, Panel B is the $^{31}$P-NMR of 8 and Panel C is the $^{31}$P-NMR of 9. All the NMR spectra are prepared in deuterated acetonitrile.
In Figure 25, the peak in panel A ($\delta$ 33.47 ppm) aligns with C ($\delta$ 33.52 ppm) to confirming that 9 forms in the reaction.

Moreover, a peak in Panel A ($\delta$ 32.37 ppm) aligns with a peak in Panel B ($\delta$ 32.59 ppm) confirming the formation of 8 in the reaction. Other peaks appear in A that mean other byproducts exist other than the products the mechanism predicts.

To further investigate this reaction, LC-MS (liquid chromatography-mass spectrometry) of the mixture was used without any further purification (Figure 26).

![Figure 26. LC-MS of the mixture](image)

From the LC-MS results, the phosphine oxide (9) forms. However, other impurities also form and it is difficult to determine the other compounds from LC-MS. From the results, we can conclude that reaction of 3 with HNO forms 8 and 9
and methyl salicylate, but also yields other products compared to the mechanism we proposed previously.

Though impurities formed in the reaction of 3 with HNO, these results show that this kind of prodrug system works as an HNO triggered prodrug. From the analysis, the reason for multiple products might be that 2 ester groups exist in 3 which could be hydrolyzed or undergo other reactions. Based on this, we considered an acetaminophen ester (5) as a better substrate as acetaminophen does not have any other esters.

### 2.2.2 Acetaminophen Ester (5)

Treatment of the acetaminophen ester (5) with 5 equivalents of Angeli’s Salt in the 3:1:2 acetonitrile:THF:Tris buffer at room temperature (Figure 27) gave acetaminophen (4) in 90% yield and phosphine oxide (10) in 78% yield. However, no evidence of 8 could be found.

Figure 27. Acetaminophen ester reacts with HNO
Figure 28. $^{31}$P-NMR comparison. Panel A is the $^{31}$P-NMR of 8 and Panel B is the crude $^{31}$P-NMR for the reaction of 5 with Angeli’s Salt.

Upon comparison of $^{31}$P-NMR spectra, a peak exists in Panel B ($\delta$ 32.15 ppm) that aligns with Panel A ($\delta$ 32.10 ppm). From this comparison, we can conclude that some 8 is produced in the reaction along with many other phosphorus-containing compounds similar to the reaction of 3 with Angeli’s Salt. These results suggest that this reaction does not occur cleanly and may undergo other mechanisms than the originally proposed.

Considering the prodrugs work well in drug releasing, experiments with cells may be useful. For such a goal, a drug with antibiotic properties would provide
further evidence by killing bacterial cells after drug release. The final prodrug is based on the antibiotic, metronidazole.

### 2.2.3 Metronidazole Ester

Metronidazole is a good choice for a new prodrug molecule since it has a hydroxyl group and is a widely studied antibiotic. Furthermore, the structure of metronidazole is not complicated, which means we can analyze the reaction easily.

Just as the previous experiments, the same conditions were applied to the metronidazole ester (7). Treatment of 7 with 5 equivalents of Angeli’s Salt in 3:1:2 acetonitrile: THF: Tris buffer gave metronidazole in 75% yield after purification by chromatography (Figure 29).

![Figure 29. Reaction of metronidazole ester (7) with HNO](image-url)
Unfortunately, no other compounds could be separated by chromatography. To establish the presence of the amide (8) and the phosphine oxide (11), LC-MS and $^{31}$P-NMR were used.

Figure 30. $^{31}$P-NMR comparison. Panel A is the $^{31}$P-NMR of 11, Panel B is the $^{31}$P-NMR of the reaction of metronidazole ester with HNO and Panel C is the $^{31}$P-NMR of 8.

As Figure 30 shows, the crude reaction in panel B shows only 2 peaks which means only 2 compounds with phosphorous are in the mixture after reaction. In addition, the starting material (7) has a negative chemical shift which is absent in
the $^{31}$P-NMR indicating that starting materials react with HNO completely as the 2 phosphorous compounds are generated. In addition, the peak in Panel A ($\delta$ 33.35 ppm) aligns well with the peak in Panel B ($\delta$ 33.12 ppm) and the peak in Panel C ($\delta$ 32.10 ppm) also aligns well with the peak in Panel B ($\delta$ 32.17 ppm). This comparison indicates that the 2 compounds found in the reaction in B are likely 8 and 11 as predicted.

Unlike the previous results, this reaction appears clean and supports the proposed reaction mechanism. To further verify that the 2 phosphorous compounds are indeed 8 and 11, LC-MS was done.

![LC-MS of the mixture of the treatment of metronidazole ester (7) with HNO](image_url)

Figure 31. LC-MS of the mixture of the treatment of metronidazole ester (7) with HNO
From the LC-MS, two peaks are found that correspond to 8 and 11. The combination of these results (^31P-NMR and LC-MS) support the formation of 8 and 11 during the reaction of 7 with HNO.

2.2.4 Mechanism Discussion

As shown, except for 7, both 3 and 5 react with HNO to yield many products which complicates our mechanistic interpretation. For both 3 and 5 evidence exists that the amide (8) and the corresponding phosphine oxide from. In all of the reactions, isolation of all products proved difficult suggesting other reactions are occurring. As shown in Figure 23, the phosphine probe will first react with HNO to form a phosphine oxide and an aza-ylide. Competition between the hydrolysis of ylide and the nucleophilic attack can occur. If hydrolysis occurs, the phosphine oxide forms without the corresponding amide (8), in addition, direct oxidation of the probe would also lead to the phosphine oxide without 8. If the phosphine oxide hydrolyzes, carboxylic acid (19) will be produced as shown in Figure 32, and such a compound might be one of the impurities shown in the ^31P-NMR.
Moreover, the prodrug of wintergreen has a second ester group and acetaminophen has an amide group, so both 3 and 5 can in theory hydrolyze. While the amide is difficult to hydrolyze under normal conditions, the presence of HNO might aid the decomposition of the amide. Hydrolysis of these groups could also explain the many impurities in the reaction of 3 and 5 with HNO. Finally, HNO is a good electrophile and may undergo some reaction with the aromatic portion of these molecules, giving a new set of products.

2.3 Kinetic Analysis

As stated in the background, prodrugs require the important property that they need to be metabolized in vivo at a high reaction rate in order to achieve a certain concentration in the human body. The results show that the prodrugs can be activated by the presence of HNO. Another important part of proving the efficiency of the prodrug is to analyze the kinetics of the reaction.

$^{31}$P-NMR was used to study the kinetics of this reaction with HNO. The basic idea was to take a $^{31}$P-NMR spectrum at regular intervals and to judge its
concentration by integrating the peak of starting materials and determining the amount the concentration changes by comparing the change of the integral. Moreover, in order to compare the integral and make the results reliable, phosphoric acid, which has a chemical shift of δ 0 ppm in $^{31}$P-NMR was added as a standard.

Initially, 3 was dissolved in 3:1:2 acetonitrile: THF: Tris buffer and Angeli's Salt was added and the mixture was quickly transferred to an NMR tube along with a tiny amount of phosphoric acid. $^{31}$P-NMR spectra were taken every 15 minutes until all of 3 disappeared. Controls included both pure 3 and 9, produced by adding hydrogen peroxide to 3, and Figure 33 shows the results of these experiments.
Figure 33. Kinetic study. Panel A is the $^{31}$P-NMR of 9, Panel B is the $^{31}$P-NMR of 3. The other 9 are the $^{31}$P-NMR of the reaction mixture at the time labeled.

Figure 33 shows that over time, the peak of 3 (δ -4.95 ppm) decreases and the peak of 9 (δ 33.90 ppm) increases which shows the formation of phosphine oxide. In about 45 minutes, 3 is nearly gone, which means 3 reacts with HNO completely within 1 hour.

Figure 34 shows this data graphically by setting the integral of phosphoric acid as a standard and following the integral of 3 over time to give a relative amount.
Figure 34. Concentration of 3 vs. Time after Angeli’s Salt addition

From the graph, 3 decomposes quickly in the first 20 minutes, with almost 80% of 3 gone in the first 20 minutes. After 100 minutes, almost all of 3 is gone.

Actually, two reactions are occurring in the system, one is the decomposition of Angeli’s Salt to release HNO and the other is the reaction of 3 with HNO. To determine which reaction Figure 34 depicts, the observed rate constant was calculated to be $5.6 \times 10^{-4}$ s$^{-1}$. This value closely matches the reported rate constant for Angeli’s Salt decomposition at 25 °C of $6.8 \times 10^{-4}$ s$^{-1}$.[49] This rate constant is also much slower than the reported rate constants for the reaction of HNO with phosphines of $9 \times 10^{-5}$ s$^{-1}$ and $8.4 \times 10^6$ M$^{-1}$ s$^{-1}$.[49] These results indicate Figure 34 shows the slow decomposition of Angeli’s Salt that rapidly reacts with
the phosphine prodrug and reveals that the prodrugs have a rapid reaction rate with HNO as desired.

Based on these results, we can conclude that the newly designed prodrugs rapidly react with HNO to release drug within 1 hour. As required for prodrugs, the fast conversion meets the requirement of designing a prodrug.

2.4 Selectivity

With the basic reactivity and kinetic analysis established, a further need is to examine the selectivity of these compounds for reacting with HNO. We have shown that these prodrugs are activated by HNO but must show whether these prodrugs react with other compounds such as glutathione (GSH), cysteine (cys) or NO. To demonstrate the selectivity of the prodrug, experiments were done with 7 using GSH, cysteine, NaNO₂ and NO (DEANO as donor, Figure 35) whose structures are shown in Figure 35.

![Figure 35. Structure of biological reducing and oxidizing agents.](image)

Compound 7 was treated with 5 equivalents of each compound and a control was also included. After 24 hours, ³¹P-NMR spectra were taken to determine if a reaction occurred. Figure 36 shows the results of experiments with GSH, cys and a control.
Figure 36. $^{31}$P-NMR of selectivity experiments. Panel A is a control, Panel B mixture with cys and Panel C a mixture with GSH.

As shown, the peak around $\delta$=-5 ppm is the peak for 7 which means that most of 7 remains. The small peak at $\delta$=33 ppm corresponds to 11 that likely forms by air oxidation. From this comparison, we can conclude that the prodrug does not react with other reducing molecules.

However, upon mixing 7 with NO and NaNO$_2$, the peak for 7 disappears indicating that 7 reacts with these 2 reactive nitrogen species (RNS). The results are shown in Figure 37 and 38.
Figure 37. $^{31}$P-NMR of the reaction of 7 with NaNO$_2$. Panel A shows addition of 11 to the reaction, Panel B is the reaction of 7 with NaNO$_2$.

The difference between this reaction and the blank is that the peak for 7 totally disappears. As only one peak forms, we conclude that NaNO$_2$ can oxidize 7 to produce 11. To prove this, 11 was added to the mixture (Figure 37, Panel A), and we see that there is still only one peak in the $^{31}$P-NMR. Based on that result, we can conclude that 7 can react with NaNO$_2$ and produce 11. This result stands in contrast to previous experiments that show other triphenylphosphine derivatives do not react with NaNO$_2$. [49, 50]
Figure 38. $^{31}$P-NMR of the reaction of 7 with DEANO. Panel A shows the addition of 11 to the reaction, Panel B is the reaction of 7 reacting with DEANO.

Similar to NaNO$_2$, the $^{31}$P-NMR of the reaction of 7 with NO is shown in Figure 38. Panel A shows the addition of 11 to the reaction mixture and Panel B is the spectrum of the reaction of 7 with DEANO. These results are similar to previous results that show NO oxidizes phosphine to phosphine oxide. [49]

Based on these selectivity experiments, the prodrug does not react with reducing agents such as GSH and cysteine but does react with oxidants including NaNO$_2$, NO, and hydrogen peroxide. However, the reaction with the
oxidants, only forms phosphine oxide (11) and no ylide forms and does not lead to drug release.

Further, as stated, the decomposition of Angeli’s Salt will generate HNO and sodium nitrite. Though, sodium nitrite can react with 7 and ruin its activity, 7 can still be activated by Angeli’s Salt, which means the prodrug 7 has a higher reaction rate with HNO than sodium nitrite and shows a high selectivity with HNO.

2.5 Conclusion

Based on previous reactions described by our group (Figure 22), a type of prodrug has been designed. Three prodrugs (3, 5 and 7) have been synthesized in reasonable yield indicating that this type of prodrug can be easily produced.

The reaction of 3, 5 and 7 with HNO were studied. We recovered the drugs from all three reactions in good yields meaning all 3 prodrugs worked well for drug release. Though we assumed the drugs will be released by the proposed mechanism, some impurities formed during the drug release process. Focusing on those impurities, possible side reactions have also been proposed. The summary is shown in Figure 39.
The kinetics of drug release were studied and showed that the prodrugs quickly react with HNO, which is good for a prodrug. Selectivity experiments were also done and show that the prodrug can’t be activated by reducing reagents demonstrating a high selectivity. Prodrug 7 reacts with oxidants including NaNO₂, NO, and hydrogen peroxide, but will not release drug, as only HNO can activate drug release.

These initial results show the good efficiency of the newly designed compounds that can act as HNO mediated prodrugs. Further investigation will be needed to better define these compounds as prodrugs. Further work on the mechanism and selectivity is important for example isolation of the impurities and studying other prodrugs without hydrolytic sites

To examine if the prodrug (7) works in killing bacteria, cell experiments, as described in Figure 40, could also be done to further prove the efficiency of this prodrug.
Chapter 3. Experiment

3.1 General Chemistry

Analytical thin-layer chromatography (TLC) was performed on Sorbtech Silica G TLC plates with UV254, 200/pk. Proton NMR, Carbon-13 NMR and Phosphor-31 NMR spectra were taken on a Bruker (300 MHz) multinuclear spectrometer. LC-MS was performed on Agilent Technologies HPLC column. MS was done by an Agilent ion trap (ESI) with HPLC. Organic solvents were distilled from drying agents before use. Commercially available reagents were used without further purification.

3.2 Synthetic Procedure

2-(methoxycarbonyl)phenyl 2-(diphenylphosphanyl)benzoate (3). 2-(Diphenylphosphino) benzoic acid (0.92 g, 3.0 mmol) was added to a solution of methyl salicylate (1, 0.46 g, 3.0 mmol) in dichloromethane (20 mL) to give a yellow turbid solution. Dicyclohexylcarbodiimide (DCC, 0.93 g, 4.5 mmol) and 4-dimethylaminopyridine (DMAP, 0.074 g, 0.61 mmol) were added to this solution and stirred at room temperature. After 24 hours, the white mixture was filtered,
the filtrate was concentrated and purified by column chromatography (1:1 ethyl acetates: hexane) to give 3 as white solid (1.10 g, 2.4 mmol, 83%). $^1$H NMR(CDCl$_3$): δ 8.29 (m, 1H); 7.87 (d, 1H); 7.33 (m, 3H); 7.15 (m, 11H); 6.90 (m, 1H); 6.81 (d, 1H); 3.59 (s, 3H). $^{13}$C NMR (CDCl$_3$): δ 164.97, 150.52, 141.44, 137.90, 134.36, 134.22, 133.5, 132.53, 131.81, 131.55, 128.57, 126.01, 124.09, 123.47, 77.63, 77.21, 76.79, 60.42, 52.19, 21.10, 14.28. $^{31}$P NMR (CDCl$_3$) δ -5.20.

4-Acetamidophenyl 2-(diphenylphosphanyl)benzoate (5). 2-(Diphenylphosphino) benzoic acid (0.92 g, 3.0 mmol) was added to a solution of N-(4-hydroxyphenyl) ethanamide (acetaminophen, 0.45 g, 3.0 mmol) in dichloromethane (20 mL) to give a yellow turbid solution. Dicyclohexylcarbodiimide (DCC, 0.93 g, 4.5 mmol) and 4-dimethylaminopyridine (DMAP, 0.074 g, 0.61 mmol) were added to this solution and stirred at room temperature. After 24 hours, the white mixture was filtered, the filtrate was concentrated and purified by column chromatography (1:1 ethyl acetates: hexane) to give 5 as white solid (1.07 g, 2.4 mmol, 81%). $^1$H NMR(CDCl$_3$): δ 8.20 (m, 1H); 7.31 (m, 4H); 7.20 (m, 15H); 7.10 (m, 1H); 6.90 (m, 1H); 6.80 (d, 1H); 2.10 (s, 3H). $^{31}$P NMR (CDCl$_3$) δ -4.90. MS (LC-MSD-Trap-SL, ESI): 441.1

2-(2-Methyl-5-nitro-1H-imidazol-1-yl)ethyl 2-(diphenylphosphanyl)benzoate (7). 2-(Diphenylphosphino) benzoic acid (0.92 g, 3.0 mmol) was added to a solution of 2-(2-methyl-5-nitro-1H-imidazol-1-yl) ethanol (metronidazole, 0.51 g, 3.0 mmol) in dichloromethane (20 mL) to give a yellow turbid solution. Dicyclohexylcarbodiimide (DCC, 0.93 g, 4.5 mmol) and 4-
dimethylaminopyridine (DMAP, 0.074 g, 0.61 mmol) were added to this solution and stirred at room temperature. After 24 hours, the white mixture was filtered, the filtrate was concentrated and purified by column chromatography (1:1 ethyl acetates: hexane) to give 7 as yellow solid (1.05 g, 2.3 mmol, 83%). $^1$H NMR (CDCl$_3$): $\delta$7.25 (m, 15H); 4.45 (m, 2H); 4.39 (m, 2H); 2.25 (s, 3H). $^{31}$P NMR (CDCl$_3$) $\delta$ -4.80. MS (LC-MSD-Trap-SL, ESI): 460.2

**Methyl 2-(diphenylphosphanyl)benzoate (12).** 2-(Diphenylphosphino) benzoic acid (0.92 g, 3.0 mmol) was added to a solution of methanol (0.10 g, 3.0 mmol) in dichloromethane (20 mL) to give a yellow turbid solution. Dicyclohexylcarbodiimide (DCC, 0.93 g, 4.5 mmol) and 4-dimethylaminopyridine (DMAP, 0.074 g, 0.61 mmol) were added to this solution and stirred at room temperature. After 24 hours, the white mixture was filtered, the filtrate was concentrated and purified by column chromatography (1:1 ethyl acetates: hexane) to give 12 as white solid (0.77 g, 2.3 mmol, 80%). $^1$H NMR (CDCl$_3$): $\delta$8.05 (m, 1H); 7.28 (m, 12H); 6.93 (m, 1H); 3.73 (s, 3H). $^{31}$P NMR (CDCl$_3$) $\delta$ -4.34.

**2-(Diphenylphosphanyl)benzamide (8).** Angeli’s Salt (390 mg, 3.2 mmol) was added to a solution of 12 (0.20 g, 0.63 mmol) in CH$_3$CN: THF: Tris Buffer 3:1:2 (10 mL). After 12 hours stirring at room temperature, the solution became 2 layers and was extracted with dichloromethane (3 $\times$ 20 mL). The product was purified by column chromatography (5% methanol in chloroform) to give 8 as yellow solid (73 mg, 0.23 mmol, 72%). $^1$H-NMR (CD$_3$CN): $\delta$7.80 (m, 1H); 7.60 (m, 16H). $^{31}$P NMR (CD$_3$CN), $\delta$ 37.20.
**Phosphine Oxide 9.** Hydrogen peroxide (30%, 3 mL) was added to a solution of 3 (0.02 g, 0.045 mmol) in a mixture of CD₃CN: THF: Tris Buffer 3:1:2 (2mL). After 12 hours stirring at room temperature, ³¹P NMR (CD₃CN), δ 33.47.

**Phosphine Oxide 10.** Hydrogen peroxide (30%, 3 mL) was added to a solution of 5 (0.02 g, 0.046 mmol) in a mixture of CD₃CN: THF: Tris Buffer 3:1:2 (2mL). After 12 hours stirring at room temperature, ³¹P-NMR were taken. ¹P NMR (DMSO), δ 28.75.

**Phosphine Oxide 11.** Hydrogen peroxide (30%, 3 mL) was added to a solution of 7 (0.02 g, 0.044 mmol) in a mixture of CD₃CN: THF: Tris Buffer 3:1:2 (2mL). After 12 hours stirring at room temperature, ³¹P-NMR were taken. ¹P NMR (CD₃CN), δ 33.34.

**3.3 Treatment of Prodrugs with HNO**

**Treatment of 3 with HNO.** Angeli’s Salt (600 mg, 4.9 mmol) was added to a solution of prodrug 3 (0.440 g, 1.00 mmol) in a mixture of CH₃CN: THF: Tris Buffer 3:1:2 (20 mL). After 12 hours stirring at room temperature, the solution became 2 layers and was extracted with dichloromethane (3 x 20 mL). The product was purified by column chromatography (5% methanol in chloroform) to give 1 as white solid (116 mg, 0.76 mmol, 76%). ¹H-NMR (CDCl₃): δ 10.67 (s, 1H); 7.72 (m, 1H); 7.32 (m, 1H); 6.85 (m, 1H); 6.75 (m, 1H); 3.80 (s, 1H). ¹³C NMR (75 MHz, CDCl₃) δ 170.57, 161.60, 135.69, 129.90, 119.15, 117.57, 112.39, 77.47, 77.05, 76.63, 52.26.
**Treatment of 5 with HNO.** Angeli’s Salt (600 mg, 4.9 mmol) was added to a solution of 5 (0.439 g, 1.00 mmol) in a mixture of CH₃CN: THF: Tris Buffer 3:1:2 (20 mL). After 12 hours stirring at room temperature, the solution became 2 layers and was extracted with dichloromethane (3 × 20 mL). The product was purified by column chromatography (5% methanol in chloroform) to give 4 as white solid (136 mg, 0.90 mmol, 90%). $^1$H-NMR (DMSO-d): δ9.70 (s, 1H); 9.14 (s, 1H); 7.33 (d, 2H); 6.67 (d, 2H); 1.97 (s, 3H). $^{13}$C NMR (75 MHz, DMSO) δ 153.05, 130.98, 120.72, 114.92, 40.31, 40.03, 39.75, 39.48, 39.20, 38.92, 38.64, 23.69 and phosphine oxide (10) as yellow solid (354 mg, 0.78 mmol, 78%). $^1$H-NMR (CD$_3$Cl): δ10.00 (s, 1H); 7.90 (m, 1H); 7.75 (m, 1H); 7.40 (m, 1H); 6.55 (m, 17H); 2.00 (s, 3H). $^{31}$P NMR (DMSO-d): 38.00. MS (LC-MSD-Trap-SL, ESI): 456.1.

**Treatment of 7 with HNO.** Angeli’s Salt (300 mg, 2.5 mmol) was added to a solution of 7 (0.230 g, 1.00 mmol) in a mixture of CH₃CN: THF: Tris Buffer 3:1:2 (20 mL). After 12 hours stirring at room temperature, the solution became 2 layers and was extracted with dichloromethane (3×20 mL). The product was purified by column chromatography (5% methanol in chloroform) to give 6 as white solid (65 mg, 0.38 mmol, 75%). $^1$H-NMR (DMSO): δ12.79 (s, 1H); 9.77 (t, 1H); 9.12 (t, 2H); 8.45 (m, 2H); 7.23 (s, 3H). $^{13}$C-NMR (DMSO) δ 208.67, 192.24, 138.19, 64.96, 53.48, 19.48.

**3.4 Kinetic Analysis of the Reaction of 3 and HNO.**

Angeli’s Salt (12.6 mg, 0.103 mmol) was added to a solution of 3 (0.0104 g, 0.0236 mmol) in a mixture of CD$_3$CN: THF: Tris Buffer 3:1:2 (600 μL) in an NMR
tube. $^{31}$P-NMR spectra were taken every 15 minutes and overall 9 spectra were taken. A phosphoric acid standard was also added for the $^{31}$P-NMR. By setting the integral of the phosphoric acid as the standard, integrals were calculated from starting material 3 and compared. The time resolved graph was drawn and an observed rate constant was calculated.

### 3.5 Selectivity

Compound 7 (138 mg, 0.30 mmol) was dissolved in a mixture of CD$_3$CN: THF: Tris Buffer 3:1:2 (12 mL). The mixture was separated into 5 vials and each vial contained 2 mL of the mixture. GSH (0.47 mmol, 144 mg), cys (0.47 mmol, 57 mg), NaNO$_2$ (0.47 mmol, 32 mg), DEANO (0.16 mmol, 20 mg) and a blank were added. After 24 hours, $^{31}$P-NMR spectra were taken.
References


Appendix

Figure A1. $^1$H-NMR of 3
Figure A2. $^{13}$C-NMR of 3
Figure A3. $^{31}$P-NMR of 3
Figure A4. $^1$H-NMR for 1
Figure A5. $^{13}$C-NMR of 1
Figure A6. $^1$H NMR of 5
Figure A7. $^{31}$P NMR of 5
Figure A8. $^1$H NMR of 4
Figure A9. $^{13}$C NMR of 4
Figure A10. $^1$H NMR of 10
Figure A11. $^{31}$P NMR of 10
Figure A12. MS of 10
Figure A13. $^1$H NMR of 8
Figure A14. $^{31}$P NMR of 8
Figure A15. $^1$H NMR of 7
Figure A16. $^{31}$P NMR of 7
Figure A18. $^1$H NMR of 6
Figure A19. $^1$H NMR of 12
Figure A20. $^{31}$P NMR of 12
Wen-Rui Su       Curriculum Vitae

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Education

2010-2014  B. S.  Chemistry Department, Peking University, Beijing, China
            Major in Physical Chemistry (Advisor: Professor Kai Wu)
            Minor in Mathematics and Applied Mathematics

2010-2014  M. S.  Chemistry Department, Wake Forest University, U. S.
            Major in Organic Chemistry (Advisor: Professor S. B. King)

Courses related

Linear Algebra, Real Analysis, Complex Analysis, Statistics, Probability Theory,
Quantitative Analysis, C language, Data Structure and Algorithm.

Working Experience

2014-2016  Teaching Assistant in Wake Forest University
            I was T. A. for General Chemistry and Organic Chemistry and
            gained strong ability of communication in English.

Jul.-Aug. 2015  Internship in Bank of China
I was in the international settlement department and mainly responsible for the issue of Letter of Credit.

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**Achievements**

July, 2014  **Ph. D. Program in Wake Forest University**
I was admitted by Ph. D. program in Wake Forest University for full scholarship. Based on the interest, I talked with my advisor to change to M. S. program.

June, 2015  **CFA level 1 exam**
I passed the CFA level 1 exam at the first attempt. I got >70% in Cooprerate Finance, Equity Investment and Financial Reporting Analysis and 50-70% in most other subjects.

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**Interests**

- **Mathematics**
  Mathematics is my most favorite subject especially analysis part. I did well in Probability Theory and Statistic. I also audited the Applied Stochastic Process which made me interest in modeling analysis.

- **Quantitative Analysis**
  I took Quantitative Analysis about chemistry and audited the Applied Mathematical Software (about the using of SAS) which made me interest in applying mathematical and computational method in practical analysis.

- **Communication**
  I’m outgoing and want to communicate with others. 2-year T. A. work helps me a lot in learning how to communicate with others. Also, I enjoy that job and get lots of positive comments from undergraduate students in Wake Forest University.

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**Additional Skills**: 

**Technical**:
- STM, AFM, UV-Vis spectrum, NMR, Mass Spectrum, X-Ray Diffraction.

**Computing**:
- C, MS Windows, MS Office, Origin 8.0, SAS, SPSS, Matlab, MS Mathematics

Languages:
- Fluent in Chinese (Native) and English.