DISSERTATION

SYNTHESIS, CHARACTERIZATION, AND INVESTIGATION OF NITRIC OXIDE DONORS FOR BACTERIAL DETECTION AND ANTIBACTERIAL ACTIVITY

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ABSTRACT

SYNTHESIS, CHARACTERIZATION, AND INVESTIGATION OF NITRIC OXIDE DONORS FOR BACTERIAL DETECTION AND ANTIBACTERIAL ACTIVITY

Antibiotic resistance is a critical problem, especially with the emergence of "superbugs," which are bacteria species that have become immune to most common antibiotics. To address this alarming issue, it is necessary to both prevent the overuse of current antimicrobial therapeutics, and to develop new, effective antibacterial treatments. By detecting when the source of a patient's infection is bacterial, and which species is causing the infection, antibiotics can be prescribed only when they are needed, and the most effective antibiotic can be chosen. The development of new antibiotics with new mechanisms of action are also a productive path to help solve the problem of antibiotic resistance. Bacteria that encounter antibiotics with novel mechanisms of action have not yet evolved pathways to resistance against the therapeutic, so these innovative compounds will be highly successful at eradicating infections.

Nitric oxide is a small molecule with potent antibacterial activity due to its reactivity and ability to form reactive nitrogen species, which induces nitrosative stress in bacteria cells and cell death. The development of a new antibiotic incorporating nitric oxide could lead to a very powerful antibacterial agent. Combining the ideas of detecting bacterial infections with the antibiotic potential of nitric oxide into a multifunctional molecule would give the benefits of both detection of a bacterial infection, and potent antimicrobial action. The focus of this work is to develop a small molecule to both sense and kill bacteria. This goal is accomplished through the development of a dual-function small molecule to detect and kill bacteria through a colorimetric

change and release of nitric oxide to kill bacteria. Another approach to achieve this goal is the synthesis of a molecule that bacteria can sense, an inactive drug until bacterial enzymes cause the release of nitric oxide to kill the bacteria. Nitric oxide release is localized to bacteria causing an infection, which can help prevent bacteria from developing resistance by avoiding unnecessary exposure.

Toward the goal of addressing the problem of antibiotic resistance, a nitric oxide donor attached to a fluorescent compound is synthesized, creating a compound that can both detect and kill the deadly multi-drug resistant bacteria strain, *Pseudomonas aeruginosa*. Detection occurs through a bacterial enzyme-activated color change, showing a visible color change from blue to yellow under UV light. The synthesized compound spontaneously releases 853 μmol of nitric oxide/g at a 10 mM initial concentration of the compound. Antibacterial efficacy studies after exposing *Pseudomonas aeruginosa* to a 10 mM dose of the synthesized compound show a 65% reduction in bacteria after 24 hours. This work is the first instance of a small molecule dual-function material that can both detect and kill bacteria.

To address the goal of developing a bacteria-specific nitric oxide releasing compound, novel nitroaromatic-protected piperazine diazeniumdiolate (nitric oxide donor) prodrugs are synthesized to release nitric oxide upon enzyme activation to kill bacteria. These prodrugs are activated by an enzyme in the nitroreductase family, which are found almost exclusively in bacteria, and reduces the nitroaromatic-protecting group of the synthesized compounds, catalyzing the release of nitric oxide. Experiments show that nitric oxide release from the synthesized compounds only occurs in the presence of a bacteria-derived nitroreductase enzyme, demonstrating the possibility of site-specific delivery of an antibacterial therapeutic. The amount of nitric oxide release is measured at concentrations of 0.01, 0.1, and 1 mM, and is well within

known antibacterial levels at concentrations of 0.1 and 1 mM, reaching nitric oxide concentrations of up to 4.8 μM. The antibacterial activity of the compounds is demonstrated after exposure of the compounds to *Escherichia coli*, a nitroreductase-producing bacterial species and common infection forming species, leading to up to a 94% reduction in the number of viable bacteria after 24 hours at 1 mM concentrations of the prodrug. This study is the first example of an antibacterial diazeniumdiolate prodrug activated by a nitroreductase enzyme, and further demonstrates the possibilities of antibacterial prodrugs.

Medical devices are a site where bacterial infections can develop, and these infections are often incredibly difficult to treat, sometimes requiring the removal of the device. Medical devices could be coated with an antibacterial material that releases antibiotics to prevent infections. To investigate the application of the nitroreductase enzyme-activated nitric oxide releasing prodrugs for antibacterial medical device coatings, the prodrugs are incorporated into polyvinyl chloride and polyurethane films to create antibacterial prodrug polymer composite materials. Characterization of nitric oxide release from the surface of the composite films is observed only after metabolism by a bacterial nitroreductase enzyme, demonstrating the prodrug nature of the polymer composite. Excitingly, antibacterial efficacy experiments resulted in a 66% reduction in *Escherichia coli* after exposure to the diazeniumdiolate-composite films. This work details the first example of an antibacterial enzyme-activated NO-releasing polymer.

The development of these novel compounds and materials represents significant advances in research to develop new ways to detect and treat bacterial infections.

ACKNOWLEDGMENTS

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CHAPTER 1

INTRODUCTION

1.1 Addressing the Problem of Antibiotic Resistance

Bacteria are microscopic single-cell organisms ubiquitous in our environment. They can play beneficial roles in our environment and in our bodies, but if a certain species grows out of control, there can be severe detrimental effects. Since Paul Erlich discovered an antibiotic to treat syphilis in 1911 and Alexander Fleming discovered penicillin in 1928, leading to the mass production of antibiotics in the 1940s, health care providers have had a "miracle cure" for bacterial infections.[1] However, with the discovery of antibiotics, antibiotic resistance has followed not far behind.[2] Antibiotic resistance has high costs to hospitals and society at a whole.[3] Resistance to antibiotics is due the development and spread of resistance genes in bacteria and the overuse of antibiotics.[4] If bacteria develop resistance genes to antibiotics, which allow them to produce proteins that neutralize the antibiotic's effects, these genes can spread among the community of bacteria and sometimes even between bacterial species. This developed resistance then renders a specific antibiotic or class of antibiotics inert for that type of bacterial infection. Resistance is a natural development for bacteria in their fight for survival, so it cannot be completely prevented, but there are ways to slow its spread.[5,6]

One way to limit the development of resistance development in bacteria is to limit use of antibiotics except in times where it is strictly necessary. If antibiotics are used unnecessarily or incorrectly, bacteria with resistance genes can avoid eradication, then multiply and spread, creating "superbugs," bacterial strains that are resistant to many or all of current common antibitotics.[7] To prevent the overuse of antibiotics, it is important to only use antibiotics when it

is certain that an infection or illness is caused by bacteria, and which species is responsible. To do this, it is important to have reliable methods to detect bacterial infections and identify which species is causing the infection. Many methods have been developed to detect bacteria, including colorimetric, electrochemical, and biological detection. By detecting that an infection is in fact caused by bacteria, and which bacterial species is the cause, a patient can be treated effectively while limiting the spread of resistance.

Another way to address resistance development is to circumvent bacterial resistance mechanisms by developing new antibiotics with new mechanisms of action. While the development of resistance is inevitable, new antibiotics can buy more time. As more bacterial species become resistant to all known antibiotics, it becomes more critical than ever to create new antibiotics.[7] Often, pharmaceutical companies will make derivatives of known antibiotics, such as β-lactam antibiotics like penicillin and amoxycillin. However, these antibiotics usually have the same or similar mechanisms of action, such as targeting the same protein in bacteria to kill or disable them. This means that bacteria often quickly develop resistance to these derivatives, as their resistance genes quickly develop mutations that render the new antibiotic ineffective. For this reason, it is important to develop new antibiotics with new mechanisms of action, although is a difficult task.

1.2 Physiological and Therapeutic Role of Nitric Oxide

For many decades, nitric oxide was known only as one of the nitrogen oxide (NO_x) air pollutants. In the past few decades, however, researchers have discovered that nitric oxide also has beneficial effects, first as an important cardiovascular cell signaling molecule in the body.[8] In 1998, Robert Furchgott, Louis Ignarro, and Ferid Murad were awarded the Nobel Prize for their work in the 1980s demonstrating the role of NO as a cardiovascular signaling molecule.[9] In

1992, *Science* named NO the "Molecule of the Year," demonstrating the impact that the discovery of the role of NO has had on the field.[10]

In addition to cell signaling, NO also plays many other roles in the body. NO is synthesized in the body from the amino acid L-arginine, converted to citrulline and NO via various NO synthase (NOS) enzymes.[11,12] NO has been shown to improve wound healing by increasing the rate of cell proliferation.[13] NO can act as a vasodilator to increase blood flow, a role exploited by the administration of nitroglycerin to treat various conditions such as heart attacks and acute hypertension.[14–16] In addition to these functions, NO is produced as part of the immune response to infectious agents invading the body.[17,18] When applied exogenously, NO has been used to treat certain types of cancers, promote wound healing, and kill bacterial infections and disperse biofilms.[18–20]

The antimicrobial activity of NO is particularly important in stemming the spread of antibiotic-resistant bacteria due to its multiple mechanisms of action. NO can react under physiological conditions, forming nitrites (NO_2^-) and nitrates (NO_3^-), as well as reactive nitrogen species (RNS) like peroxynitrite (ONO_2^-) that have additional toxic effects.[21,22] The effects of NO and RNS produced from NO are referred to as nitrosative stress, which can cause a myriad of negative effects against bacteria.[23,24] NO or the RNS it forms kill or harm bacteria by mutating its DNA structure, inhibiting DNA repair and synthesis, or increasing alkylating agents and hydrogen peroxide that can react with DNA.[25,26] It can also inhibit protein synthesis, alter proteins by *S*-nitrosylation, inactivate enzymes, and peroxidate cell membranes. For bacteria to develop resistance to all of the mechanisms of action of NO, multiple mutations must happen simultaneously, which is unlikely. Studies by the Schoenfisch group showed that bacteria are unable to develop resistance to exogenously applied NO, even after 20 days.[27] In addition, NO is

able to virtually eradicate NO, achieving up to a log-8 reduction (99.999999%) in a number of different bacterial species,[28] as well as successfully dispersing biofilms using NO as a therapeutic.[29,30] Neufeld showed that the concentration of NO needed to achieve a 90% reduction in *Pseudomonas aeruginosa* biofilm viability was 2.73 mM of NO from *S*-nitrosoglutathione.[29]

The inherent properties of NO give it the potential to be a very effective therapeutic. NO is nonpolar, uncharged, and hydrophobic.[31,32] NO has a very small radius compared to other gaseous molecules, even oxygen or carbon dioxide.[33] It can easily cross cell membranes due to it small radius, and its hydrophobic nature increases its ability to enter cells.[34] These properties make NO a leading target for development into a therapeutic, but its inherent nature as a gas makes its delivery as a drug difficult.

1.3 Categories of Nitric Oxide Donors

As NO has been shown to have many therapeutic benefits, it is of high interest to develop NO-releasing drugs. NO is naturally in a gaseous state, and gaseous NO is an approved treatment for pulmonary hypertension.[35] However, it is difficult to deliver NO otherwise due to its gaseous nature, short half-life, and near-instantaneous reaction with oxygen to form nitrogen dioxide, a toxic gas. For these reasons, many NO donors have been developed to store and deliver NO in a stable, controlled way, with a multitude of release rates and mechanisms.[36] Broadly, the most common NO donors fall into the categories of organic nitrites/nitrates, metal nitrosyls, *S*-nitrosothiols, and diazeniumdiolates.[36–40]

Organic nitrites and nitrates are compounds with structures made up of organic R groups attached to nitrite (NO_2^-) or nitrate (NO_3^-) groups (Figure 1.1a-b). These compounds are typically known to release NO via specific NO-generating enzymes.[41] Examples of organic nitrites and

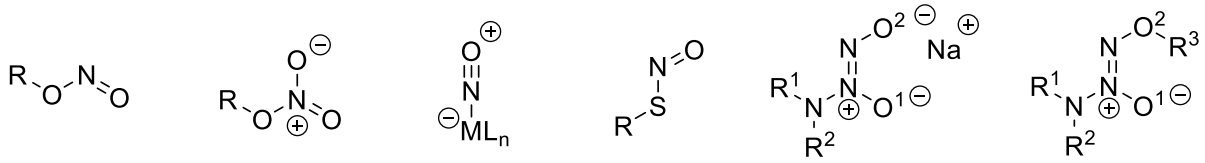

Fig. 1.1 Examples of NO donor structures **a)** organic nitrite **b)** organic nitrate **c)** metal nitrosyl **d)** S-nitrosothiol **e)** unprotected diazeniumdiolate **f)** O^2-protected diazeniumdiolate

nitrates used as therapeutics include amyl nitrite and nitroglycerin, which can treat hypertension and reduce pain in heart attacks.[42]

Metal nitrosyls are complexes involving a NO ligand attached to a transition metal (Figure 1.1c). These compounds can release NO under physiological conditions or with a light-trigger.[43,44] One common metal nitrosyl, sodium nitroprusside, releases NO by binding to oxyhemoglobin, and releasing cyanide, methemoglobin, and NO.[45]

S-nitrosothiols (RSNOs) are structures involving organic R groups attached to a nitrosated thiol group (Figure 1.1d). These compounds are produced endogenously and are found in the bloodstream in low concentrations, as well as bound to proteins, typically on nitrosated cysteine residues.[46,47] RSNOs release NO through thermal, photocatalytic, or transition-metal catalyzed methods, releasing NO and a corresponding disulfide formed from two thiol groups.[48]

Diazeniumdiolates (also called NONOates) are made up of secondary amines with the same or different R groups, attached to two NO groups (Figure 1.1e-f). "Diazen" refers to the nitrogen-nitrogen double bond, "ium" to the positive charge on nitrogen, and "diolate" to the two negatively charged oxygen atoms.[49] It is standard in the field to refer to the two oxygen atoms as 1 and 2 (Figure 1.1e),[49] and they are usually named with an abbreviation of the secondary amine and forward slash NO in uppercase letters, such as PROLI/NO, a diazeniumdiolate formed from proline.[50] These types of donors will be discussed more in depth in the next section.

1.4 Synthesis, Characterization, and Properties of Diazeniumdiolates

Diazeniumdiolates are of special interest as NO donors due to their ability to be protected and prevent spontaneous NO release prior to enzyme activation.[51] Instead of the typical spontaneous NO release characteristics of most other NO donors or release by specific native enzymes, diazeniumdiolates are unique in that they can be protected by a variety of different organic groups to allow activation by a chosen enzyme to meet the requirements for the intended application.

Diazeniumdiolates are typically synthesized by reacting secondary amines with NO gas under high pressure, commonly under basic conditions.[38,49] Solvents used in these reactions can range from polar protic (methanol), polar aprotic (acetonitrile), to non-polar (diethyl ether).[50,52,53] There are two main proposed mechanisms for diazeniumdiolate formation (Figure 1.2). At low pressures (0-30 psi), there is first order dependence in NO.[54] In a stepwise reaction, the amine performs a nucleophilic attack on one molecule of NO, forming a nitrosamine radical anion.[54–56] Next, the nitrogen of the nitrosamine anion forms a radical bond with a second molecule of NO.

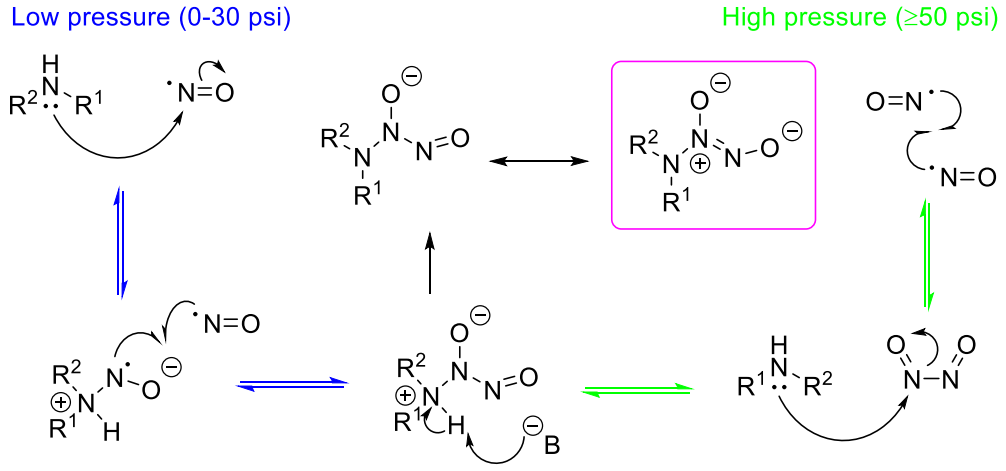

Fig. 1.2 Two proposed mechanisms for the formation of diazeniumdiolates. Mechanism 1 (left, blue arrows) is proposed for reactions at 0-30 psi, and has a first order dependence on NO. Mechanism 2 (right, green arrows) is proposed for reactions at ≥50 psi, and has a second order dependence on NO.

The base (usually sodium methoxide, NaOMe, or a second amine molecule) deprotonates the amine, and the molecule tautomerizes to the diazeniumdiolate structure. At high pressures (50 psi and higher), Bohle and coworkers proposes a second order dependence in NO, where NO forms an electrophilic dimer (N_2O_2), then the lone pair of the amine attacks one nitrogen of the NO dimer (Figure 1.2).[54] The mechanism from that point is the same as at low pressures. Secondary amines form diazeniumdiolates much more readily as they are better nucleophiles than primary amines.[55] X-ray crystal structures of diazeniumdiolates have shown that the cis form of diazeniumdiolates with both oxygens on the same side (Figure 1.1e) is the most stable structure.[49]

As NO gas is a strongly oxidizing chemical, safety precautions must be undertaken when using it as a reagent. A reactor system to run these types of reactions should be built completely with stainless steel parts, which are inert to NO gas. These reactions are run at high pressure, so the reactor system should be equipped to handle the necessary pressure and not be pressurized past the maximum rating of the parts of the reactor system. NO forms nitrogen dioxide (NO_2) upon contact with oxygen, which is a toxic environmental pollutant gas, so when NO is released from the reaction vessel, it must be bubbled through an appropriate solution to neutralize the NO gas waste. To address some of the hazards associated with these types of reactions, to scale up these reactions for their potential use in pharmaceuticals, Zhong and coworkers as part of the process chemistry team at Merck have developed an alternative synthesis.[57] They found that during the course of diazeniumdiolate-forming reactions, sodium methoxide can react with NO to form dangerously high levels of nitrous oxide (N_2O) in a side reaction, which was previously noted by DeRosa, et. al.[58] Zhong and coworkers even noted “two unexpected explosions were reported in a 500 mL autoclave reactor (during venting), because of the rapid depressurization of

high-pressure nitric oxide (NO) from the headspace through Teflon-lined steel-braided tubing to atmospheric pressure."[57] To avoid potential explosion risk upon scale-up, the Merck team developed an alternative synthetic procedure involving $Ca(OH)_2$ as the base and water as the solvent in the presence of NO at pressures ranging from 15-350 psi. They were able to show that the reaction conditions were successful across a broad range of amine starting materials in high yield. This novel procedure could offer greater risk mitigation for future syntheses of diazeniumdiolates.

The success of diazeniumdiolate-forming reactions can usually be monitored visually by the formation of a precipitate in the reaction, as the product is highly ionic and does not dissolve well in the organic solvents typically used to run the reaction. The precipitate is filtered and rinsed once the reaction is complete, isolating a free flowing white solid. Diazeniumdiolates have an overall negative charge, balanced by the base counter ion (usually sodium, Figure 1.1e).

The most direct way to analyze the product of diazeniumdiolate reactions is by UV-vis. Diazeniumdiolates typically have an absorbance peak at 250 nm, with a molar extinction coefficient between 7.2-9.4 $M^{-1}cm^{-1}$.[49] This peak can be shifted to ~235 nm by protecting groups on the diazeniumdiolate (Figure 1.1f).[49] Analysis of the reactions can also be based on NMR spectra, which can be taken in a mixture of deuterated sodium hydroxide and deuterated water to stabilize the compound. Diazeniumdiolates are typically stable as solids stored at -20 °C, or in basic solutions (pH 12). They dissolve easily in water and aqueous solutions due to their charged nature and display low solubility in most organic solvents. Diazeniumdiolates release two moles of NO per mole of diazeniumdiolate spontaneously in neutral or acidic solutions or when heated. The half-life of NO release from diazeniumdiolates can range from seconds to hours depending on the identity of the amine the diazeniumdiolate functional group is formed on.[38,51] They

display first order kinetics in NO release.[49] The release properties of diazeniumdiolates can be tuned by the identity of the amine and the surrounding conditions.

Diazeniumdiolates are proposed to release NO via a hydrolysis mechanism (Figure 1.3).[59,60] The amine is protonated, giving it a positive charge. The negative charge on O^2 pushes onto the adjacent nitrogen, forming a double bond, and the double bond between the two nitrogens breaks, pushing the electrons to form a double bond between the other nitrogen and O^1 of the diazeniumdiolate functional group. The secondary amine is released and homolytic cleavage of the bond between the two nitrogens in the remaining NO dimer releases two molecules of NO. This proposed mechanism explains why a protecting group on O^2 prevents NO release, as the negative charge is not there to push onto the adjacent nitrogen, causing the compound to break apart.[51] The amine can be protonated, but no further reaction occurs.

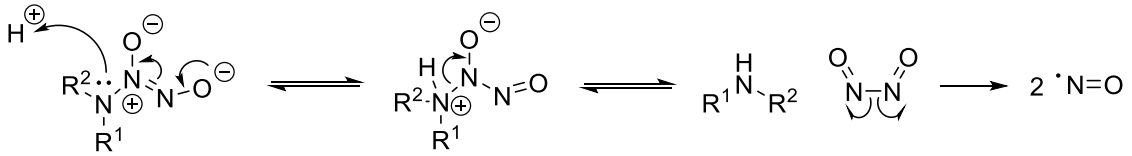

Fig. 1.3 Proposed mechanism for NO release from a diazeniumdiolate. Two moles of NO are released per one mole of diazeniumdiolate.

One challenge with diazeniumdiolates is that they can form *N*-nitrosamines as a side product or decompose to form *N*-nitrosamines (Figure 1.4a).[38,61] Most nitrosamines have been shown to be activated to form carcinogenic species.[62,63] However, PROLI/NO is a common diazeniumdiolate which can decompose to form *N*-nitrosoproline, but nitrosoproline has been shown to be non-carcinogenic (Figure 1.4b-c).[50,64] Nitrosamines are typically yellow in color and display UV-vis peaks at 230-240 and 330-350 nm, which can help distinguish this decomposition product from diazeniumdiolate product formation.[36] When working with diazeniumdiolates, it is important to make sure they are under stable conditions to avoid decomposition to a carcinogenic

species, and to test these decomposition products to determine their toxicity. In the case of using these compounds as therapeutics, it is especially important to investigate decomposition products that could potentially form under physiological conditions.

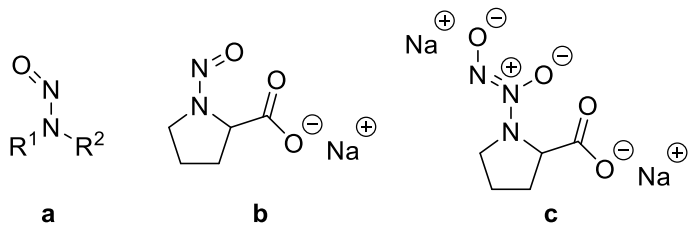

Fig. 1.4 Structures of (a) general nitrosamine, which can form after decomposition of diazeniumdiolates and are typically carcinogenic (b) *N*-nitrosamine, a non-carcinogenic nitrosamine (c) PROLI/NO, a common diazeniumdiolate formed from the amino acid proline

1.5 Enzyme-Activated Nitric Oxide Prodrugs

Because NO plays many roles in the body and is highly reactive, it is important to deliver it site specifically to avoid side effects. NO is cell permeable, and has a very short half-life in blood, making it an excellent candidate for site-specific delivery. A wide variety of O^2-protected diazeniumdiolates have been synthesized, the general structure of which are shown in Figure 1.1f, with a variety of triggers for NO release.[51] These protected diazeniumdiolates are often prodrugs, inactive until they encounter a trigger such as an enzyme to release NO as the active therapeutic.

Ionic (unprotected, Figure 1.1e) diazeniumdiolates release NO spontaneously and can be unstable, which can be problematic for their application as therapeutics. To address these problems, diazeniumdiolates can be protected by covalently binding an R group at the O^2 position (Figure 1.1f). As O^2 is nucleophilic in nature, these protection reactions are typically selective and relatively simple.[65] Protected diazeniumdiolates are synthesized by first synthesizing the ionic diazeniumdiolate, then functionalizing with a protecting group, usually from an electrophilic source such as an alkyl halide, sulfate ester, or epoxide.[49] Protected

diazeniumdiolates release NO more slowly or only in the presence of a specific trigger, such as an enzyme.[51]

MOM-PIPERAZI/NO is an example of a protected diazeniumdiolate that is not enzyme-activated. It is a piperazine-based diazeniumdiolate protected by a methoxymethyl ether (MOM) group at the O^2 position (Figure 1.5). The MOM protecting group slows NO release considerably, from a half-life of 5 minutes for unprotected PIPERAZI/NO to 17 days with a MOM protecting group.[66] For MOM-PIPERAZI/NO, NO release is still spontaneous as the MOM protecting group is naturally hydrolyzed over time, but it is significantly slowed. However, for most protected diazeniumdiolates, the protecting group is not hydrolyzable, and NO release is only activated with a specific enzyme or other compound.

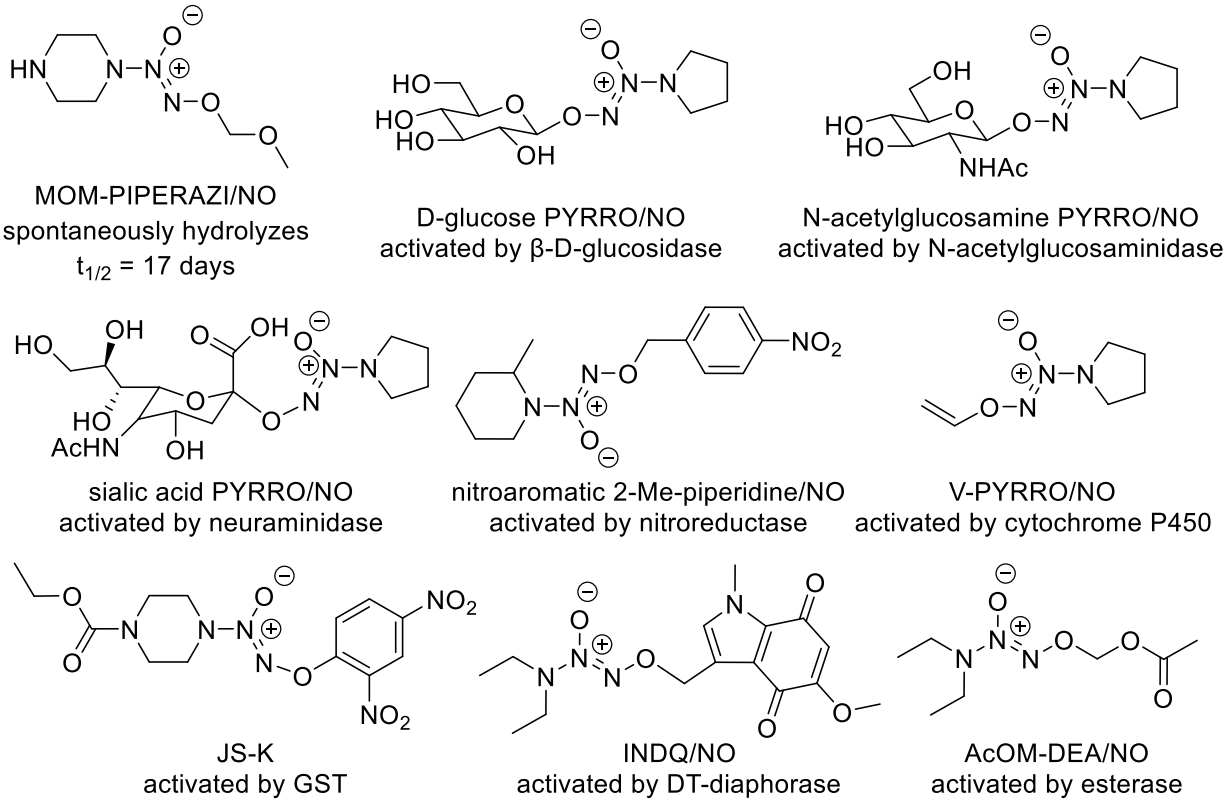

Fig. 1.5 Selected examples of enzyme-activated diazeniumdiolates and trigger, typically an enzyme, that leads to the release of NO

There are many other examples of diazeniumdiolates that are enzyme-activated by a variety of enzymes. Diazeniumdiolates can be protected by carbohydrates and NO release is activated by a carbohydrate-cleaving enzyme. A diazeniumdiolate is reacted with a halogenated glycoside, forming a glycosidic bond with the diazeniumdiolate. NO release is then caused by the corresponding glycosidase. For example, the Wang group developed a series of glycoside-protected diazeniumdiolates, including D-galactose cleaved by a β-D-galactosidase, D-glucose cleaved by β-D-glucosidase (Figure 1.5), and D-mannose cleaved by α-D-mannosidase, for potential application as sugar transporters into cells.[67] Valdez and coworkers demonstrated that these glycosylated diazeniumdiolates are pH sensitive, and synthesized a *N*-acetylglucosamine protected structure activated by a *N*-acetylglucosaminidase enzyme for antileishmanial applications (Figure 1.5).[68] Nandurdikar, et. al. synthesized *N*-acetylglucosamine protected PROLI/NO with potentially biocompatible byproducts.[69] Showalter and coworkers investigated glycosylated diazeniumdiolates as a protection-deprotection system.[70] The Wang group also synthesized a novel sialated diazeniumdiolate, activated by a neuraminidase enzyme to target influenza viruses (Figure 1.5).[71]

Another class of enzyme-activated diazeniumdiolates are protected by esters or related functional groups and are cleaved by esterases. Several examples from Saavedra, et. al. are acetoxymethylated diazeniumdiolates formed by reacting bromomethyl acetate with an ionic diazeniumdiolate, such as AcOM-DEA/NO (Figure 1.5). The resulting ester-protected diazeniumdiolates are activated to release NO by a porcine liver esterase, and are shown to induce apoptosis in leukemia cells.[72]

Cytochrome P450 enzymes have also been used to trigger liver-specific NO release from enzyme-activated diazeniumdiolates. Saavedra and coworkers synthesized V-PYRRO/NO

(Figure 1.5) by attaching a vinyl group to a pyrrolidine-based diazeniumdiolate, and demonstrated that this compound can protect liver cells from apoptosis, which could potentially prevent liver toxicity and failure.[53] Chakrapani et. al. synthesized an alternative to V-PYRRO/NO, V-PROLI/NO, a proline-based diazeniumdiolate, to increase water solubility, and showed that NO release is still activated by cytochrome P450 enzymes from the liver.[73]

Glutathione-*S*-transferases (GSTs) are a family of enzymes that work to detoxify and remove foreign compounds from the body.[74] These enzymes are often over-expressed in cancer cells, so they have been a target for enzyme-activated diazeniumdiolates for anticancer applications. Several of these diazeniumdiolates have been synthesized, including JS-K, a 2,4-dinitroaryl GST-activated diazeniumdiolate (Figure 1.5).[74,75] This compound displays potent anti-proliferative effects and other mechanisms of anticancer activity against a variety of cancer cell lines.[76,77] Another GST-activated diazeniumdiolate, PABA/NO (Figure 1.5), was synthesized by Findlay and others, and also displays anticancer activity in a human ovarian cancer model.[78] Chakrapani and coworkers synthesized PABA/NO analogues to further investigate the anticancer therapeutic potential.[79] Saavedra and coworkers also investigated these 2,4-dinitroaryl-protected compounds for their use as HIV antiviral therapeutics.[80]

Other enzyme-activated diazeniumdiolates have been synthesized for specific applications. One example is a DT-diaphorase enzyme-activated diazeniumdiolate, INDQ/NO, protected by an indolequinone (Figure 1.5).[74] DT-diaphorase is an over-expressed enzyme in cancer cells, and Sharma and coworkers demonstrated that the INDQ/NO therapeutic inhibits cancer cell proliferation.[81] Tang and coworkers coupled diazeniumdiolates to small peptide chains to make α-chymotrypsin-activated NO-releasing therapeutics to treat prostate cancer.[82] Nitroreductase enzymes are found in bacteria, and have been used in gene-directed enzyme

prodrug therapy to treat cancer. Sharma et. al. synthesized several nitroreductase activated diazeniumdiolates and demonstrated their anti-proliferative effects in cancer cells (Figure 1.5).[83] Hibbard, et. al also synthesized novel nitroreductase-activated diazeniumdiolates for applications to treat bacterial infections, as nitroreductase enzymes are found almost exclusively in bacteria.[84]

1.6 Antibacterial Diazeniumdiolates

NO is known to be a potent antimicrobial agent, and several groups have used diazeniumdiolates to make antibacterial materials due to their stability and controlled, predictable release.[85] Clinical studies with DETA/NO (Figure 1.6), a diethylenetriamine based diazeniumdiolate, showed that it had potent activity against both *Candida* and other fungal species.[86] Chen, et. al. showed that *Escherichia coli* transformed with a *lacZ* gene were able to hydrolyze a *β*-galactosidase-activated diazeniumdiolate, *β*-Gal-NONOate (Figure 1.6), and kill the galactosidase-producing *E. coli* cells up to 87.6%.[87] The Chakrapani group synthesized novel boronic acid-protected diazeniumdiolates (BORO/NO, Figure 1.6) that are activated by hydrogen peroxide (H_2O_2).[88] They showed that cotreatment of various bacteria species such as *E. coli*, MRSA, and *Pseudomonas aeruginosa*, with BORO/NO and H_2O_2 increased the amount of

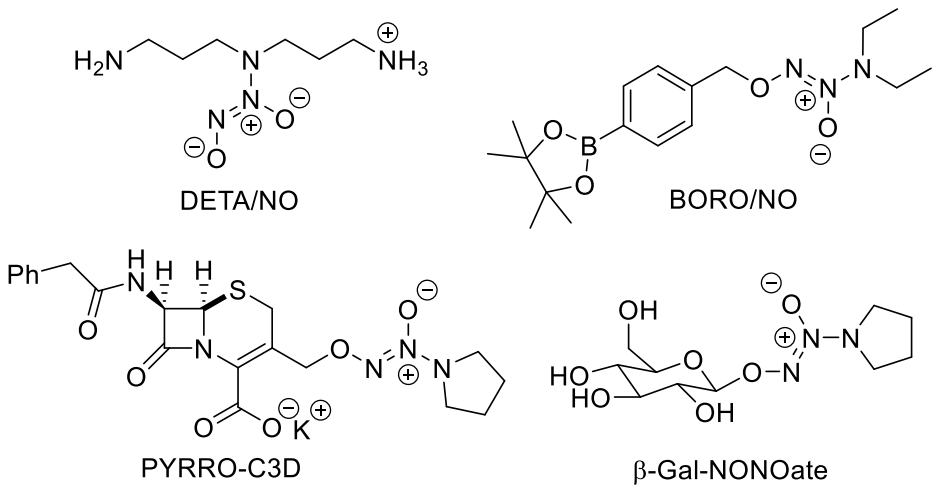

Fig. 1.6 Selected examples of antibacterial diazeniumdiolates

intercellular NO, which would likely correlate to increased antibacterial activity. Collins, Allan, and coworkers developed PYRRO-C3D (Figure 1.6), a cephalosporin-3-diazeniumdiolate prodrug, activated to release NO with transpeptidases or β-lactamases.[89,90] They showed that PYRRO-C3D has anti-biofilm activity against *Haemophilus influenzae* biofilms with coadministration of the antibiotic azithromycin, and against *Streptococcus pneumoniae* bacteria in both planktonic and biofilm form.[89,90]

While some examples of small molecule diazeniumdiolates have been used to induce antibacterial activity, often they are incorporated into polymers or macromolecules to make antibacterial materials. A variety of polymeric and macromolecular platforms have been investigated, from nanoparticles, synthetic polymers like polyurethane (PU) or polyamidoamines (PAMAMs), to natural polymers like chitosan.[91] These materials have been investigated to kill planktonic bacteria,[92,93] biofilms,[94] or both.[93,95] Xu and Siedlecki wrote a review detailing antibacterial polyurethanes, including NO-releasing PUs with antibacterial properties.[96] The Schoenfisch group developed composite PU electrospun fibers doped with diazeniumdiolate groups and PAMAM dendrimers and demonstrated their broad spectrum antibacterial activity.[97] Yang and coworkers synthesized hyperbranched PAMAM polymer chains, modified the polymers with propylene oxide, and functionalized the polymers with diazeniumdiolate groups, then showed that the material is able to eradicate oral bacteria.[98] Backlund, et. al. derivatized PAMAMs functionalized with diazeniumdiolates to create scaffolds with bactericidal activity against *Streptococcus mutans* biofilms, a bacteria species causing dental cavities.[99] Shim, et. al. showed that a branched, diazeniumdiolate functionalized-polyethylenimine (PEI) displayed antibacterial activity against several periodontal pathogens.[100] Nurhasni and coworkers synthesized nanoparticles made of poly(lactic-*co*-glycolic acid) (PLGA) and diazeniumdiolated

PEI, and investigated the nanoparticles antibacterial activity against MRSA and *P. aeruginosa*.[101] Park and coworkers made hollow polydopamine nanoparticles functionalized with diazeniumdiolate groups, and demonstrated the nanoparticles potent antibacterial activity against *E. coli* and *P. aeruginosa*.[102] Other groups have functionalized the natural oligosaccharide chitosan with diazeniumdiolate groups for antibacterial effects. Liu, et. al. derivatized chitosan and functionalized the polymer with diazeniumdiolate groups, then tested its antibacterial efficacy against *E. coli*.[103] The Schoenfisch group also derivatized chitosan with secondary amine groups, then functionalized with diazeniumdiolate groups on the chitosan oligosaccharides and demonstrated the 5-log reduction in *P. aeruginosa* biofilms after exposure to the NO-releasing polymer.[104] These examples demonstrate the wide variety of NO-releasing diazeniumdiolate macromolecules designed and investigated for their antibacterial activity.

Many antibacterial diazeniumdiolates have been synthesized and analyzed, both small molecules and polymeric or macromolecular materials. Many enzyme-activated diazeniumdiolates have also been synthesized, and some tested for their antibacterial efficacy. However, there is a severe lack of work developing diazeniumdiolates activated by wild type bacterial enzymes and investigation into their antibacterial activity.

1.7 Dissertation Overview

The overarching goal of this dissertation is to synthesize a new antibiotic incorporating nitric oxide that can both detect and kill bacteria. To do this, I aim to create a small molecule that can sense bacteria and kill bacteria, ideally an enzyme-activated prodrug. The research question I aimed to answer in my research was: can a small molecule nitric oxide-releasing prodrug be synthesized that functions to both sense and kill bacteria?

In the first project I worked on, I synthesized a fluorescent small molecule that changed color in the presence of bacteria. The goal of this project was to functionalize the fluorescent amide with a diazeniumdiolate. Bacterial enzymes would then metabolize the fluorescent molecule, changing the color of the molecule and indicating the presence of bacteria. This would also destabilize the molecule and trigger the release of NO from the diazeniumdiolate. Unfortunately, I was unable to successfully functionalize the fluorescent amide moiety with a diazeniumdiolate group. As a new approach, I synthesized an analogue of the fluorescent molecule which had a secondary amine functionality, and I was able to functionalize the compound with a diazeniumdiolate group. In this case, however, NO is released spontaneously, killing *P. aeruginosa*, then the bacterial enzymes cause a color change to indicate bacterial presence. With this first project, I was able to accomplish the goal of creating a molecule that could both sense and kill bacteria, although only the color change is enzyme-activated.

With my second project, I wanted to address the problem of creating an enzyme-activated NO-releasing antibacterial prodrug. This molecule would ideally sense bacteria, be metabolized by a bacterial enzyme, then release NO as the active therapeutic to kill bacteria. To accomplish this, I synthesized several nitroaromatic-protected diazeniumdiolates. Many bacteria species have nitroreductase enzymes that can reduce nitroaromatics. These enzymes can metabolize the protecting group, leading to the removal of the nitroaromatic group and causing the release of NO. In this way, I was able to achieve the goal of creating a bacterial enzyme-activated NO-releasing small molecule. Exposure of the therapeutic to bacteria (*Escherichia coli*) and subsequent NO release then led to significant eradication of the bacteria. While there is not a visible color change like the compound in my first project, bacteria could potentially be detected through NO release.

After synthesizing these small molecules, I wanted to make a material that would have applications in medical device development. Antibacterial coatings for medical devices could help prevent infections from forming on medical devices, which are often very difficult to treat. Incorporation of an antibiotic, such as the nitroaromatic-protected diazeniumdiolates I synthesized, into a device coating could release NO to kill bacteria, preventing infections from forming and increasing the lifetime of medical devices. I incorporated the nitroreductase-activated diazeniumdiolate compound into a polyvinyl chloride/polyurethane composite and measured NO release from the polymer composite. NO release continued to be enzyme-activated, unchanged from the powder small molecule form. I also showed that the diazeniumdiolate polymer composites display antibacterial activity. My research demonstrates some of the first examples of dual-function molecules that sense and kill bacteria.

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CHAPTER 2

L-ALANYL AMINOPEPTIDASE-ACTIVATED BACTERIAL INDICATOR

2.1 Introduction

2.1.1 Overview

The CDC estimates that there were 700,000 cases of hospital-acquired infections, or nosocomial infections, in 2011, resulting in about 75,000 deaths.[1] The most common of these infections are caused by antibiotic-resistant bacteria, such as *Pseudomonas aeruginosa* and *Acinetobacter baumannii*.[2] Bacterial resistance to antibiotics is spreading at an alarming rate and is one of the most significant current health threats according to the World Health Organization.[3] Without the development of new antibiotics or new ways to treat bacterial infections soon, there will be no way to treat infections that were once easy to cure. My approach to addressing the antibiotic resistance problem is to design and synthesize a nitric oxide releasing compound to detect and kill bacteria. Nitric oxide is a small, gaseous molecule known to have potent antimicrobial activity.[4] The project detailed in this chapter outlines progress toward the synthesis of a new antibiotic incorporating nitric oxide.

2.1.2 Nitric oxide and its antimicrobial activity

Within the past few decades, nitric oxide (NO) has become increasingly recognized as a biologically important molecule. The many roles NO plays in the body and its importance as a potential drug candidate have been discovered and studied extensively.[5] NO acts as a vasodilator to expand blood vessels and lower blood pressure or as a cell signaling molecule in the cardiovascular system.[6] NO is produced as part of the immune response to infectious agents

invading the body.[7] When applied exogenously, NO has been used to treat certain types of cancers, promote wound healing, and as an antimicrobial.[8–10]

The antimicrobial activity of NO is particularly important in stemming the spread of antibiotic-resistant bacteria due to its multiple mechanisms of action. NO is a highly reactive radical molecule that forms reactive species in the body that can kill or harm bacteria by reacting directly with DNA structure, inhibiting DNA repair, or increasing alkylating agents and hydrogen peroxide that can react with DNA.[4] In order for bacteria to develop resistance to NO, multiple mutations must happen simultaneously, which is unlikely. Studies by the Schoenfisch group showed that several common species of bacteria, including methicillin-resistant *Staphylococcus aureus* (MRSA), are unable to develop resistance to exogenously applied NO, even after 20 days.[11]

2.1.3 Prodrugs

Because NO is involved in varied functions throughout the body, it is critical to deliver NO site-specifically, so as not to interfere with other functions. While NO plays several beneficial roles in the body, in high doses, it can also have negative effects on the body, killing certain cells.[12] NO is also an unstable gaseous molecule, reacting quickly with oxygen and forming toxic derivatives.[13,14] When considering NO as a therapeutic, these problems must be addressed.

Targeted drug delivery can be extremely beneficial to avoid adverse side effects from drugs. Site-specific delivery can be achieved with prodrugs, which are inactive drug compounds that release the active form of a drug after being metabolized, either through an enzymatic or chemical transformation.[15] Prodrugs can help avoid adverse or unintended side effects by preventing the active drug compound from being released in areas where it is not needed.

Prodrugs also increase chemical stability, stabilizing the active compound until it is metabolized and preventing unwanted side reactions. Due to all of these properties, NO-releasing prodrugs are ideal for addressing the issues of using NO as a therapeutic, such as instability, reactivity, and side effects.

NO-releasing prodrugs have been synthesized previously, usually dependent on enzyme activation to release NO.[16,17] In the form of a prodrug, NO is no longer free to react and form toxic derivatives or to interfere with other normal processes in the body. The prodrug circulates in the body until it encounters a specific enzyme that metabolizes the prodrug and releases NO as the active drug.[16,18–20] If the enzyme that activates the drug is only found in a specific location, such as the liver, then NO will only be released when the prodrug reaches the liver.[20] Enzyme-activated prodrugs allow for a very specific, targeted release of NO as a drug, while avoiding adverse side effects caused by NO.

2.1.4 N-Diazeniumdiolates

As NO is naturally a gaseous compound, several types of NO donor compounds have been developed to trap NO in a stable form for delivery. *N*-diazeniumdiolates are the most commonly used NO donor functional group for enzyme-activated NO prodrugs. Their general structure is shown in Figure 2.1, and it is standard in the field to refer to the two oxygen atoms as 1 and 2 as shown in Figure 2.1a.[21] Diazeniumdiolates release two moles of NO per mole of diazeniumdiolate, with half-lives ranging from seconds to days.[16] NO is released when the nitrogen atom with R^1 and R^2 substituents is protonated (Figure 2.1), so the basicity of that nitrogen determines the half-life, and changes depending on the nature of the R^1 and R^2 groups.[21] Diazeniumdiolates are typically formed by reacting a secondary amine under a high pressure NO atmosphere, usually 40-100 psi, until a precipitate forms.[21]

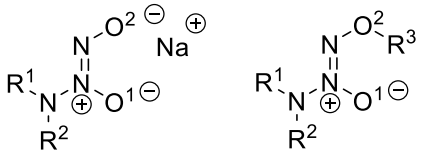


Fig. 2.1 General structure of an **a)** unprotected diazeniumdiolate **b)** O^2-protected diazeniumdiolate

To increase the stability and half-lives of diazeniumdiolates, various groups have been attached to O^2 to protect the diazeniumdiolate (Figure 2.1b).[16] These groups can simply increase the half-life, or they can prevent release of NO entirely until the protecting group is removed. Often, enzymes are used to cleave the O^2-protecting group, allowing the specific release of NO.[16] Diazeniumdiolates are a logical choice to use as an NO donor functional group when developing an enzyme-activated NO prodrug.

2.1.5 Project Idea

The goal of my research project is to synthesize a small molecule prodrug that detects the presence of bacteria and kills bacteria. When the prodrug is in the presence of bacteria, a bacterial enzyme will metabolize the prodrug, releasing the active drug to kill the bacteria. By detecting a bacterial infection, and localizing release of the active therapeutic, this compound will help address the problem of antibiotic resistance. To accomplish this idea, I envisioned synthesizing a small molecule sensor that is functionalized with a NO donor that detects bacteria, and upon activation by a bacterial enzyme, releases NO as an antimicrobial agent. A publication by Marie Cellier and coworkers sets a precedent for enzyme-activated bacterial detection.[22] They synthesized several small molecules that changed their fluorescent color in the presence of several strains of gram-negative bacteria, including *P. aeruginosa* and *A. baumannii*.[22] The molecules consisted of a fluorescent tag attached to the amino acid L-alanine. The bacterial strains they investigated release a protease enzyme called L-alanyl aminopeptidase that cleaves

the alanine group from the fluorescent tag. A protease is a protein-specific enzyme that cleaves peptide bonds, removing amino acids. In the molecules synthesized by Cellier, the amino acid/fluorescent compound fluoresces blue. When the compound was in the presence of bacteria, the protease L-alanyl aminopeptidase cleaved the amino acid and released the free fluorescent tag which appears yellow. This fluorescent color change was used to visualize the presence of bacteria. Colorimetric detection would be ideal for the intended application of determining infections on bandages. For health care workers, it is important to change bandages when necessary, but avoid disturbance of a patient's wound when unnecessary. The ability to visually determine if a wound is becoming infected by the color of the bandage would be extremely helpful for indicating when bandages should be changed, and if antibiotics should be applied.

Based on colorimetric bacterial sensing molecules, I envisioned synthesizing the bacterial sensing portion of the molecule and then functionalizing it with a diazeniumdiolate group that would only be activated once the amino acid was cleaved, represented in Figure 2.2. This figure shows a representation of ideal function of the final synthesized dual ability compound. The initial molecule would not be fluorescent or would fluoresce one color, but once the bacterial protease cleaves the amino acid, the remaining compound would be unstable. The diazeniumdiolate group and fluorescent sensor portions of the molecule would spontaneously break apart and release NO and the fluorescent molecule, changing color (yellow star, Figure 2.2). The bacteria I am focused on inhibiting are two antibiotic-resistant strains that cause many

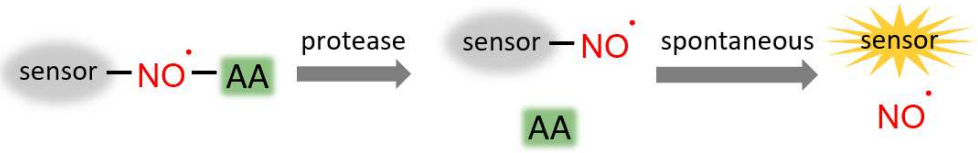


Fig. 2.2 Representation of protease-activated NO release. An amino acid (AA) protects a NO donor, then is removed by a protease enzyme. NO is released spontaneously and the sensor bond breaks and the sensor changes color.

nosocomial infections, *P. aeruginosa* and *A. baumannii*.[2,23] The fluorescent function of the molecule could be used to visualize *P. aeruginosa* and *A. baumannii*, which could be useful in the case of an external wound, or as a laboratory technique to determine if the bacteria are present. If the fluorescent portion proved unnecessary, such as if the prodrug was used internally where fluorescence cannot be seen, I also envisioned a simpler molecule without the fluorescent moiety. In this case, the sensing function would come only from detecting NO release.

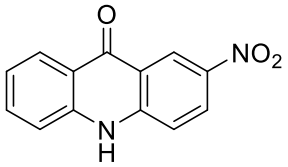
2.1.6 Project Objectives

The rapid spread of antibiotic resistance calls for new antibiotics to be developed. The goal of my project is to synthesize an enzyme-activated NO-releasing antimicrobial prodrug. The research question the work in this chapter attempts to answer is: can a dual function small molecule be synthesized with a fluorescent tag that changes color in the presence of bacteria and leads to NO release to kill bacteria? This will be accomplished by:

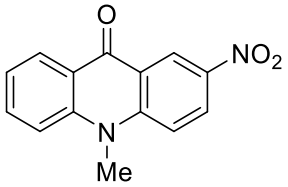
I. Synthesizing the fluorescent molecule
II. Labeling an amino acid with the fluorescent tag
III. Functionalizing the fluorescent tagged amino acid with a diazeniumdiolate group
IV. Adding an O^2-protecting group to prevent spontaneous release of NO
V. Measuring NO release in the presence of and without L-alanyl aminopeptidase
VI. Testing the compound for antimicrobial activity against *P. aeruginosa* and *A. baumannii*

The completion of all six of these steps would complete the development of an antibacterial prodrug that indicates the presence of bacteria and releases NO to kill bacteria only when bacteria are present. This would fully address the goal of this research project, although unfortunately, after experimentation, this goal was not fully addressed, and I changed the direction of the project to better address this goal.

2.2 Experimental Methods

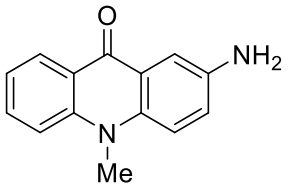


*2.2.1 2-nitroacridin-9(10H)-one (**2**).* 9(10H)-Acridone (0.976 g, 5 mmol) was stirred in 36% acetic acid (65 mL, 0.077 M) for 5 minutes. A mixture of concentrated nitric acid (68%, 4.7 mL, 1 M) and glacial acetic acid (10.43 mL, 0.5 M) was slowly added, and the reaction was stirred for 5 hours at 55 °C. The hot reaction mixture was poured over 50 g of ice, and the precipitate was filtered. The precipitate was heated to boiling in absolute ethanol and filtered while hot to remove acridone. The solid was heated in boiling glacial acetic acid, filtered while hot, and rinsed with water to remove the 4-isomer. A pure yellow powder was obtained (821 mg, 67%). ^{1}H NMR (400 MHz, d_6-DMSO) δ 12.34 (s, 1H), 8.97 (d, *J* = 2.7 Hz, 1H), 8.45 (dd, *J* = 9.2, 2.7 Hz, 1H), 8.23 (d, *J* = 7.9 Hz, 1H), 7.81 (t, *J* = 7.0 Hz, 1H), 7.66 (d, *J* = 9.2 Hz, 1H), 7.58 (d, *J* = 8.3 Hz, 1H), 7.36 (t, *J* = 7.5 Hz, 1H). Spectra matched the previously reported values.[24]

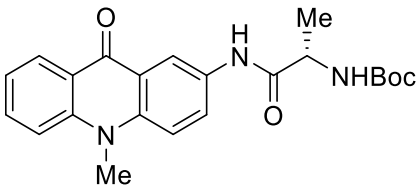


*2.2.2 10-methyl-2-nitroacridin-9(10H)-one (**3**).* A previously reported synthetic procedure was modified as follows.[25] *Safety note: DMF and NaH can pose a potential explosion risk, so it is important to use a blast shield when running the reaction, avoid scaling up the reaction, do not decrease the ratio of solvent to NaH, and ensure the reaction is cooled to 0 °C before adding NaH and during the reaction.* To a solution of **2** (200 mg, 0.833 mmol) in DMF (6.7 mL, 0.125 M) at 0 °C was added NaH (83.2 mg, 2.08 mmol), and the reaction was stirred 30 minutes at 0

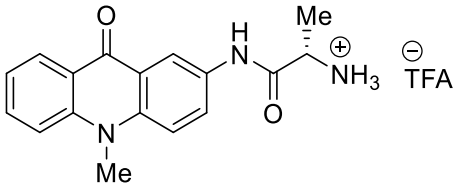
°C. Iodomethane (129 µL, 2.08 mmol) was added and the reaction was heated at 60 °C for 18 hours. The reaction was cooled to rt, quenched with 15 mL of deionized water, and then the precipitate was filtered. The crude precipitate was recrystallized from 25:1 DMF:H_2O and rinsed with diethyl ether, affording the title compound as yellow crystals (145.2 mg, 69%). ^{1}H NMR (400 MHz, d_6-DMSO) δ 9.03 (d, *J* = 2.9 Hz, 1H), 8.51 (dd, *J* = 9.6, 2.8 Hz, 1H), 8.34 (d, *J* = 8.2 Hz, 1H), 8.04 (d, *J* = 9.6 Hz, 1H), 7.98-7.87 (m, 2H), 7.45 (t, *J* = 7.3 Hz, 1H), 4.00 (s, 3H). Spectra matched the previously reported values.[25]



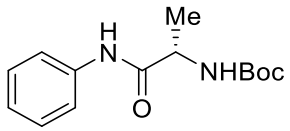
*2.2.3 2-amino-10-methylacridin-9(10H)-one (**4**).* A previously reported synthetic procedure was modified as follows.[26] To a solution of **3** (254.3 mg, 1 mmol) in 8 N HCl (19.2 mL, 0.052 M), a mixture of $SnCl_2 \cdot H_2O$ (67.7 mg, 3 mmol) in 8 N HCl (28.6 mL, 0.035 M) was added. The solution was heated under reflux at 120 °C for 1 hour. After cooling to rt, 4 M NaOH was added until the mixture reached pH 11, then the precipitate was filtered and rinsed with water. The crude product was purified by flash column chromatography (SiO_2, 70% ethyl acetate in hexanes, 100% ethyl acetate gradient), affording the title compound as a bright orange solid (122.8 mg, 55%). ^{1}H NMR (400 MHz, d_6-DMSO) δ 8.27 (d, *J* = 8.1 Hz, 1H), 7.76-7.67 (m, 2H), 7.61 (d, *J* = 9.2 Hz, 1H), 7.46 (d, *J* = 2.8 Hz, 1H), 7.25 – 7.12 (m, 2H), 5.23 (s, 2H), 3.85 (s, 3H). Spectra matched the previously reported values.[26]



*2.2.4 tert-butyl (S)-(1-((10-methyl-9-oxo-9,10-dihydroacridin-2-yl)amino)-1-oxopropan-2-yl)carbamate (**5**).* A previously reported synthetic procedure was modified as follows.[22] N-Boc-L-alanine (248.3 mg, 1.05 equiv) was dissolved in dry THF (12.5 mL, 0.1 M) under Argon and cooled to -10 ºC in salt ice bath. Added N-methyl morpholine (137.4 µL, 1 equiv) and isobutyl chloroformate (163.4 µL, 1 equiv), then added **4** (280.3 mg, 1.25 mmol) after 90 seconds. Stirred 20 hours, added 10 mL of DCM, washed with 0.1 M citric acid (15 mL), sat. $NaHCO_3$ (15 mL), deionized water (15 mL), brine (15 mL). Washed combined aqueous with 3×15 mL of DCM. Dried over $MgSO_4$, filtered, concentrated. Recrystallized crude product from isopropanol and diethyl ether (1:1). Product is a yellow powder (312.1 mg, 63% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.53 (d, *J* = 7.9 Hz, 1H), 8.33 (d, *J* = 2.3 Hz, 1H), 7.65 (t, *J* = 7.5 Hz, 1H), 7.44 (dd, *J* = 23.3, 9.0 Hz, 2H), 7.31 – 7.22 (m, 2H), 3.83 (s, 3H), 1.76 (s, 1H), 1.48 (s, 9H), 1.46 (s, 3H). Spectra matched the previously reported values.[22]



*2.2.5 (S)-1-((10-methyl-9-oxo-9,10-dihydroacridin-2-yl)amino)-1-oxopropan-2-aminium, trifluoroacetic acid salt (**6**).* Added trifluoroacetic acid (5 mL, 0.025 M) to **5**, stirred 2 hours, concentrated *in vacuo*. Precipitated out with diethyl ether (7 mL), dissolved crude product in water (2 mL), washed with ethyl acetate (2 mL), concentrated. Collected pure product as a greenish-yellow powder (51.6 mg, 97% yield). ^{1}H NMR (400 MHz, DMSO) δ 10.63 (s, 1H), 8.59 (d, *J* = 2.6 Hz, 1H), 8.31 (d, *J* = 8.1 Hz, 1H), 8.21 (s, 3H), 8.01 (dd, *J* = 9.3, 2.6 Hz, 1H), 7.90 (d, *J* = 9.4 Hz, 1H), 7.86 – 7.77 (m, 2H), 7.31 (t, *J* = 7.1 Hz, 1H), 4.02 (q, *J* = 7.1 Hz, 1H), 3.91 (s, 3H), 1.48 (d, *J* = 7.0 Hz, 3H). Spectra matched the previously reported values.[22]



*2.2.6 Tert-butyl (S)-(1-oxo-1-(phenylamino)propan-2-yl)carbamate (**7**).* Followed literature procedure.[27] In brief, N-Boc-L-alanine (1.45 mmol, 1 eq) and triethylamine (2.28 mmol, 1.6 eq) were dissolved in anhydrous THF (7.3 mL, 0.2 M) and cooled to -15 °C under Ar. Ethyl chloroformate (1.74 mmol, 1.2 eq) was added dropwise over 5 minutes, then the reaction stirred for 20 minutes, then aniline (1.5 mmol, 1.03 eq) was added. The reaction was allowed to stir at -15 °C, then warmed to rt and stirred overnight (18-20 h). 5% $NaHCO_3$ solution (2.5 mL) was added to the reaction and stirred for 30 minutes. Added 5 mL of water to reaction, then extracted with ethyl acetate (10 mL) three times, washed with 10% citric acid (15 mL), brine (15 mL), and dried over Na_2SO_4. Pure product was collected as a white powder (300 mg, 78%) after purification by flash column chromatography (SiO_2, 100% hexanes, 50% hexanes in diethyl ether, 20% hexanes in diethyl ether gradient). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.36 (s, 1H), 7.50 (d, *J* = 8.1 Hz, 2H), 7.28 (dd, *J* = 16.9, 8.9 Hz, 2H), 7.08 (t, *J* = 7.3 Hz, 1H), 4.30 (s, 1H), 1.46 (s, 9H), 1.43 (d, *J* = 7.0 Hz, 3H). Spectra matched the previously reported values.[27]

2.3 Synthetic Routes to L-alanyl Aminopeptidase Indicator

I conceived of three compounds to synthesize (Figure 2.3) in the development process of the dual function antibacterial indicator. The first was the compound published by Cellier, with the fluorescent attachment, **1** in Figure 2.3.[22] Compound **1** fluoresces blue with the amino acid attachment, but when the amino acid is cleaved, it fluoresces yellow. The alanine moiety would remain Boc-protected during the diazeniumdiolate reaction to prevent potential reaction with the alanine amine. Since this compound requires multiple steps to synthesize, I also wanted to synthesize a less complex model compound (**2**) that could be used to optimize the

diazeniumdiolate-forming reaction conditions. This model compound is structurally similar to **1** but would require fewer steps to synthesize. The model compound **2** in Figure 2.3 requires only one step to synthesize from commercially available starting materials, versus four steps to synthesize the fluorescent compound **1**. Compound **3** in Figure 2.3 does not include any aromatic or fluorescent functionality, which could be useful if visualizing the presence of bacteria through fluorescence proves unnecessary. The bacteria-sensing function of compound **3** could come from sensing NO directly.

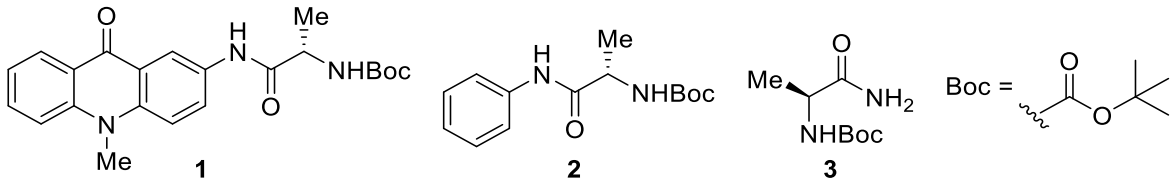


Fig. 2.3 Structures of fluorescent (**1**), model (**2**), and non-fluorescent (**3**) L-alanyl aminopeptidase indicators

2.3.1 Development of Synthetic Procedure for Fluorescent Indicator

I began the synthesis of **1** with nitrating acridone **4** to form **5** (Figure 2.4), then methylating the amine to form **6**. The next step is reducing the nitro group to an amine, then coupling the Boc-protected amino acid to **7** to form the product **1**. I attempted to follow the synthetic procedure published by Cellier et. al. to synthesize **1**, but their experimental procedures often lacked necessary details and several of the reactions did not work as they reported, potentially due to ambiguity in their procedures.[22] To overcome this challenge, I modified Cellier's reported procedures or found new procedures to achieve improved yield (Figure 2.4.)

First, I nitrated acridone (**4**) with nitric acid in the presence of acetic acid to form **5**, 2-nitroacridone, based on previously reported procedure by Gao and coworkers.[24] Based on the ^{1}H-NMR spectrum of the product, this reaction forms a 4:1 ratio of the desired product with the nitro

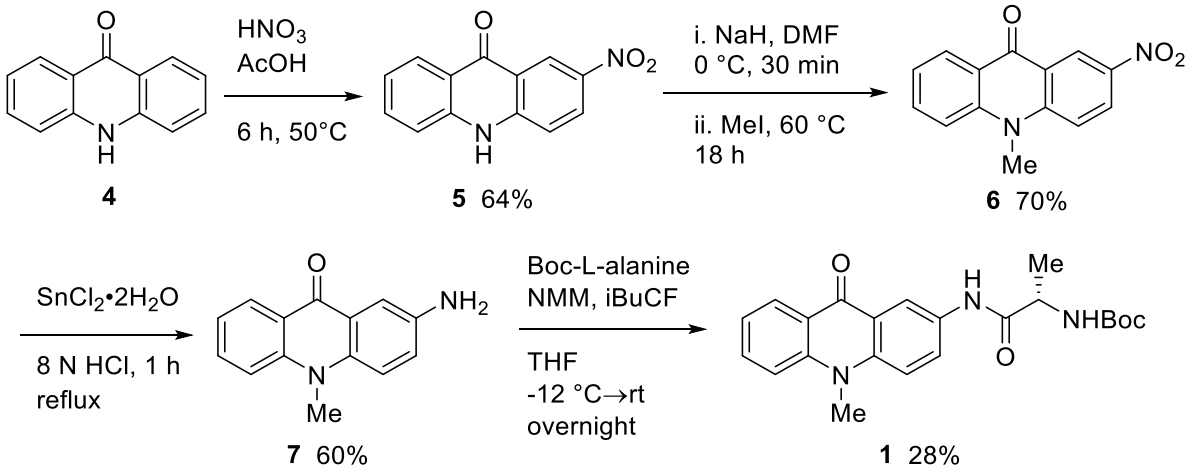


Fig. 2.4 Overall reaction scheme for synthesis of Boc-protected fluorescent L-alanylaminopeptidase indicator **1**

group in the 2-position to an undesired side product with the nitro group in the 4-position (Figure 2.5). Gao's experimental procedure did not mention formation of this side product, and the original method of purification, recrystallization from absolute ethanol, did not remove the undesired side product. To attempt to avoid forming the 4-isomer, I followed another synthetic procedure of **5** reported by Chen, but encountered the same problem of forming the undesired isomer, as well as being left with a small amount of unreacted starting material.[28] Increasing the reaction time did not decrease the amount of unreacted starting material. Cellier did mention forming the 4-isomer as a side product and their solution was to dissolve the mixture of isomers in boiling acetic acid. I found that this method worked, dissolving the 4-isomer and leaving the 2-isomer as a solid. After several attempts at this synthesis, I optimized the method to purify

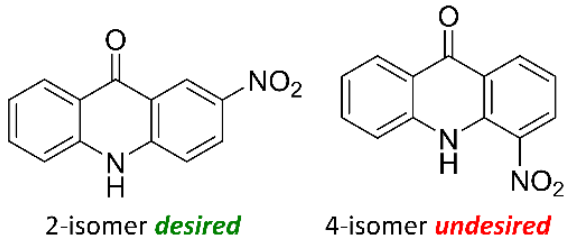


Fig. 2.5 The 2-isomer and 4-isomer products resulting from the reaction of acridone **4** to nitroacridone. The 2-isomer is the desired product, which was separated from the undesired 4-isomer in a later step.

product **5** by combining the procedures of Cellier and Gao and coworkers.[22,24] I used the amounts and procedure reported by Gao and worked up the reaction following Cellier's procedure. The starting material acridone (**4**) dissolves in hot ethanol while the product does not, so this provides a way to remove any leftover starting material. Boiling the crude product in glacial acetic acid dissolves the 4-isomer while the desired 2-isomer remains solid and filtering immediately after removes any 4-isomer. Recrystallization from 25:1 DMF:water further purifies the product. This procedure gives an average yield of 64% of a mixture of isomers.

The next step of the synthesis, methylation of **5** to **6** in Figure 2.4, was straightforward. I followed a synthesis reported by Stopka and colleagues to methylate **5**.[25] I deprotonated the amine with sodium hydride (NaH) in *N,N*-dimethylformamide (DMF), then added methyl iodide (MeI) as the methyl group donor. As a safety note, although DMF is a common solvent to use in reactions with NaH, it can pose an explosion risk.[29] To account for these risks, it is important for the scientist to be aware of the risk and place a blast shield in front of the reaction in case of an explosion. In addition, the risk can be minimized by increasing the solvent to NaH ratio (8.7% NaH, 3.7% mineral oil, and 87.6% DMF in the experimental methods described in this chapter), running the reaction at lower temperatures (0 °C in the experimental methods described in this chapter), running the reaction on small scale, or using a different solvent that does not pose these risks, such as THF.[29] After completing the work-up procedure reported by Stopka, my product still contained impurities. I used Cellier's purification technique of recrystallization from DMF and water to further purify my product. This technique gave the desired results, and NMR analysis showed that the product was pure. If I used starting material containing both the 2- and 4-isomers of nitroacridone (Figure 2.5), the NMR of **6** after the methylation reaction showed no sign of the 4-isomer. This was an excellent observation, as it meant I did not need to purify **5**

before carrying the product on to the next step. My procedure for this step produced an average yield of 70%.

To reduce the nitro group, **6** to **7** in Figure 2.4, I tried different procedures before finding success. I first followed Cellier's reported experimental procedure using tin(II) chloride dihydrate ($SnCl_2 \cdot 2H_2O$) in ethanol, but the reaction did not produce any product. I found a different synthesis reported by Manfroni and coworkers, using tin(II) chloride dihydrate and hydrochloric acid (HCl) that was succesful.[26] The crude product showed evidence of the undesirable 4-isomer as well as the desired 2-isomer, similar to Figure 2.5. Fortunately, I was able to develop column chromatography conditions using a gradient solvent system, 70% ethyl acetate:30% hexanes, then 100% ethyl acetate on silica gel to separate the isomers, giving an isolated yield of **7** of 60%. When dissolved in ethanol, I observe that compound **7** appears yellow under a 365 nm UV light.

For the reaction of **7** to form **1** in Figure 2.4, I followed the procedure reported by Cellier.[22] I added *N*-methylmorpholine (NMM) and isobutyl chloroformate (*i*BuCF) to *N*-Boc-L-alanine, then added **7**. It is necessary to couple Boc-protected L-alanine to **7** because NO reacts readily with amines to form diazeniumdiolates. To push the diazeniumdiolate to form at the amide position as desired, the amine group on the amino acid needs to be protected. Cellier's procedure for this reaction did not report amounts of solvents or a specific reaction time, so I chose reasonable values for these variables. The reaction worked, although their reported purification technique of recrystallizing from isopropanol and isopropyl ether did not successfully purify my product. Instead, I found that by pouring cold ether over the oily crude product, my product precipitates out. My yield for this reaction is 28% on average.

2.3.2 Synthesis of Model Compound

To avoid depleting my supply of synthesized compound **1** while optimizing conditions to form the diazeniumdiolate, I decided to synthesize a structurally similar model compound **2** that could be used for optimization instead. Compound **2** is synthesized through a one-step coupling reaction between aniline **9**, and *N*-Boc-L-alanine **8** (Figure 2.6), using ethylchloroformate and triethylamine (Et_3N) in tetrahydrofuran (THF). The phenyl group on **2** is similar enough to the acridone group on **1** for the diazeniumdiolate reaction conditions to be applicable. To synthesize compound **2**, I reacted aniline with Boc-L-alanine to form the amide, following a procedure published by Chen and colleagues.[27] The reaction was successful (Figure 2.6), and after purification by column chromatography, I obtained compound **2** with an average yield of 85%.

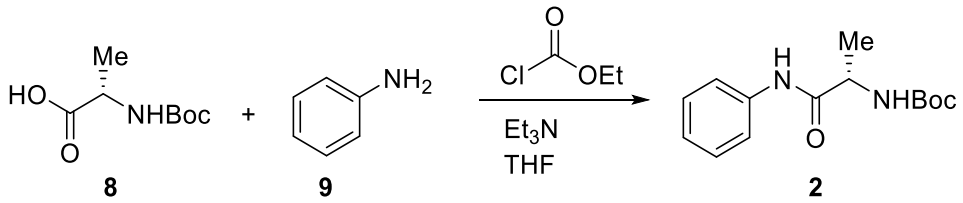


Fig. 2.6 Coupling reaction between Boc-L-alanine **8** and aniline **9** to synthesize model compound **2**

2.3.3 Synthesis of Non-Fluorescent Indicator

As the visible fluorescent color change is not useful in all applications, I also decided to synthesize a version of the fluorescent indicator without a fluorescent attachment, **3** in Figure 2.7. In this case, there will be no way to visualize the presence of bacteria with UV light, so by detecting the release of NO after functionalization with a diazeniumdiolate group, the presence of bacteria can be detected. To synthesize compound **3**, I followed a procedure by Islam, et. al, starting with *N*-Boc-L-alanine **8** and esterifying it with trimethylsilyldiazomethane (TMSD) in methanol (MeOH) and benzene, forming **10** (Figure 2.7).[30] From **10**, I performed an amidation reaction with ammonium hydroxide (NH_4OH) in MeOH to give **4**, following a procedure by

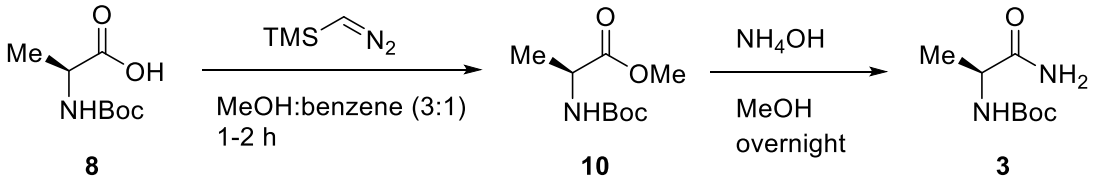


Fig. 2.7 Reaction scheme for synthesis of non-fluorescent indicator **3**

Singh and coworkers.[31] According to literature, these reaction steps should give quantitative yields.[30,31] However, based on the NMR data for my final product, there were major impurities present, which I removed with a basic work-up step. After this work-up step, the highest yield I achieved was 16% over both steps, which is not close to literature reports of quantitative yield. I hypothesized that water might be reacting with the reagents and decreasing the yield, so I tried running the reaction under an inert atmosphere with distilled solvents, but this did not improve the yield. My distilled solvents most likely still contained small amounts of water even after storage over activated molecular sieves, so I purchased anhydrous solvents under inert gas. However, using anhydrous solvents did not improve my yields either. It is possible that the TMS group was not removed, forming product **11** (Figure 2.8) instead of the methyl ester, proposed as a potential issue in a paper by Kühnel et. al.[32] It proved to be less expensive and less time consuming to purchase compound **4** instead of continuing in my attempts to improve my yield.

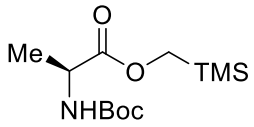
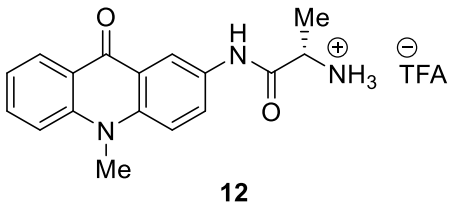


Fig. 2.8 Potential side product of non-fluorescent indicator synthesis without TMS group removed

2.3.4 Experiments Investigating Bacterial Indicator Properties of Synthesized Compounds

With compound **1** synthesized, I wanted to replicate the bacterial studies published by Cellier *et. al.* as a proof of concept.[22] These cell studies were performed by Bella Neufeld of the Reynolds group. Because the bacteria produce the protease L-alanyl aminopeptidase, which



12

Fig. 2.9 Deprotected fluorescent indicator **12**

recognizes only the unprotected form of L-alanine, I removed the Boc group in **1** by stirring it with trifluoroacetic acid to give **12** in Figure 2.9. Using Cellier's procedure as a guide, Bella dissolved the compounds I synthesized, **1** and **12**, in dimethyl sulfoxide (DMSO) and added them to Colombia agar. She then inoculated the plates of agar with *P. aeruginosa* at 37˚C for 18 hours. After incubation, the plates were illuminated under 365 nm UV light to determine fluorescence, shown in Figure 2.10. Yellow fluorescence indicates that enzymatic cleavage occurred, releasing the free amino acid **8** and aminoacridone **7** to fluoresce yellow. Blue fluorescence indicates that no enzymatic cleavage occurred; it is the color of the background fluorescence. All the columns of cell culture plates in Figure 2.10 are identical replicates; they differ across the rows. On the left is the positive control, which was inoculated with *P.*

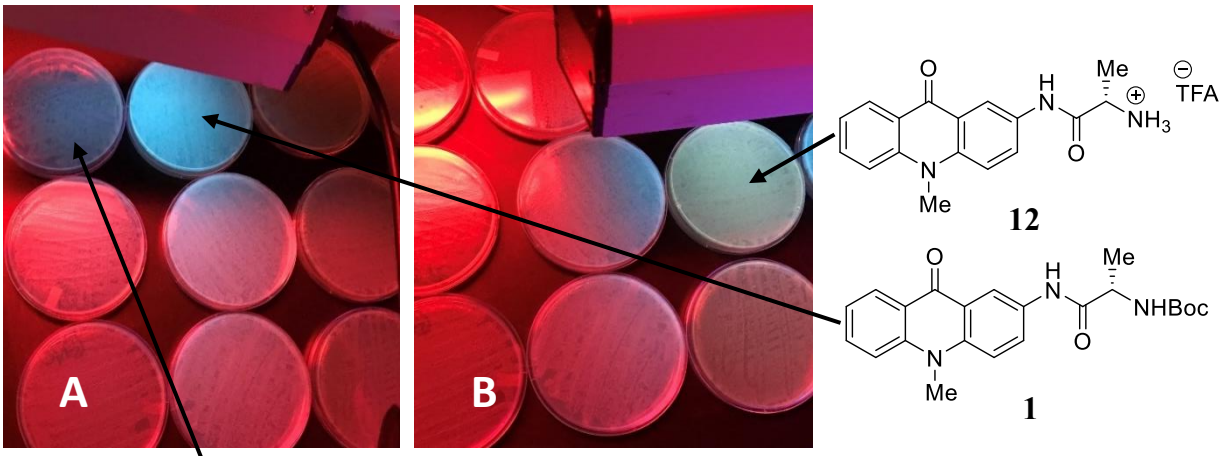


Fig. 2.10 Cell culture plates of bacterial studies under UV light. Left column: positive control (PC, *P. aeruginosa* only); middle column: compound **1**, Boc-protected fluorescent compound; right column: compound **12**, Boc deprotected fluorescent compound in both images A and B.

aeruginosa, but did not contain any of the compounds I synthesized. The positive control in image A (Figure 2.10) has weak blue fluorescence, which is due to the inherent ability of *P. aeruginosa* to fluoresce on its own.[33] In the middle column of plates is compound **1** in the presence of *P. aeruginosa*, a control to show that the enzyme cleaves only the unprotected amino acid. In image A in Figure 2.10, strong blue fluorescence is observed for the middle plates, which matches my expectations, as the compound should not be metabolized and change color. The bacterial enzyme should not recognize the Boc-protected amino acid, so no cleavage occurs and **1** fluoresces blue. On the right plates is compound **12**, and image B in Figure 2.10 clearly shows yellow fluorescence. This indicates that the L-alanyl aminopeptidase in *P. aeruginosa* cleaved the compound as expected, and that the indicator produces a visible color change in the presence of bacteria. These bacterial studies with *P. aeruginosa* showed that blue fluorescence occurred with the Boc-protected compound, as expected for the background color. Yellow fluorescence was observed only with the deprotected compound, providing a proof of concept that my compounds act as a bacterial indicator as Cellier's paper showed they should.[22]

2.3.5 Boc Group Deprotection

Removing the Boc group from my compound once the diazeniumdiolate reaction is complete will be one of the last steps before the synthesis is finished, so I started testing reaction conditions to remove the Boc group on the model compound **2**. Boc groups are typically removed under acidic conditions, but diazeniumdiolates are acid sensitive, so I searched the literature for basic conditions to remove the Boc group. Tom, et. al. used sodium tert-butoxide (NaO*t*Bu), sodium ethoxide (NaOEt), or sodium methoxide (NaOMe) in refluxing THF to remove primary Boc groups.[34] El Kazzouli and coworkers removed the Boc group with sodium carbonate in refluxing dimethoxyethane (DME) and water.[35] Yin and Zhang tested several

conditions based on the paper published by El Kazzouli *et. al.*, and found that for their system, sodium hydroxide (NaOH) in THF and water at room temperature removed the Boc group.[36] I tested all of these reported conditions and many variations on these variables with the model compound **2** to attempt to form the deprotected product **13** (Equation 1). I investigated variables such as the identity and amount of the base, solvent, additives, temperature, and reaction time. Using stronger bases, such as NaOMe, versus a weaker base, like Na_2CO_3, could lead to the desired product or to side products. Different solvents change the solubility of the reagents, leading to a more homogeneous or heterogeneous solution, which affects how the reagents interact. Additives like water could also change the solubility of the base. Lower temperatures and shorter reaction times could lead to the formation of lower amounts of side products. Higher temperatures or longer reaction times could allow reactions that did not previously work to happen or lead to higher yields. The conditions tested are shown in Table 2.1, but unfortunately, I found limited success.

Entries 1-8 show variations of the reaction described by Tom.[34] In entries 1 and 2, I tried the reaction conditions as described in Tom's paper, but I found that NaOMe afforded only a small amount of deprotected product. NaOEt, on the other hand, cleaved the starting material at the wrong location, producing aniline. To avoid this unwanted pathway, I decreased the reaction time, as shown in entries 3 and 4. This also produced aniline, along with unreacted starting material. I also tried lower temperatures (Table 2.1, entries 5-6), and fewer equivalents of NaOEt (Entries 7-8). While this lead to a small increase in the yield of the desired product, none of the conditions afforded a large amount of deprotected product, so I decided to test other conditions.

Entry 9 shows the optimized conditions published by Yin,[36] and entry 10 shows the reaction conditions from El Kazzouli.[35] Both afforded very small amounts of deprotected

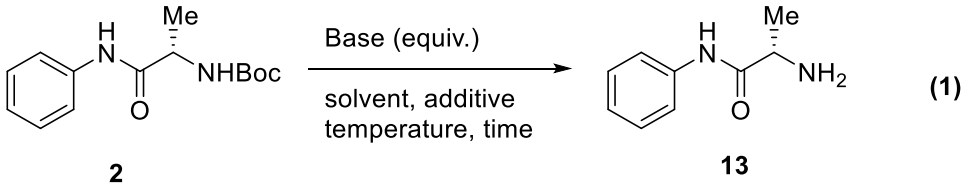


Table 2.1 Optimizing Basic Boc Deprotection Conditions

Entry	Base	Equiv. base	Solvent	Additives	Temperature (˚C)	Time (hours)	% Boc group removed
1	NaOMe	4	THF	H_2O (10 eq)	70	18	8
2	NaOEt	4	THF	H_2O (10 eq)	70	18	Aniline
3	NaOEt	4	THF	H_2O (10 eq)	70	2	Aniline
4	NaOEt	4	THF	H_2O (10 eq)	70	6	Aniline
5	NaOEt	4	THF	H_2O (10 eq)	rt	19	13
6	NaOEt	4	THF	H_2O (10 eq)	40	19	Aniline
7	NaOEt	1	THF	H_2O (10 eq)	40	19	13
8	NaOEt	2	THF	H_2O (10 eq)	40	19	19
9	NaOH	2.4	THF:H_2O (1:1)	-	rt	19	13
10	Na_2CO_3	1.2	DME:H_2O (1:1)	-	40	19	12
11	Na_2CO_3	1.2	THF:H_2O (1:1)	-	40	19	16
12	Na_2CO_3	1.2	Et_2O:H_2O (1:1)	-	40	19	14
13	Na_2CO_3	1.2	dioxane:H_2O (1:1)	-	40	19	16
14	K_2CO_3	2.4	DME:H_2O (1:1)	-	80	17	13
15	NaOH	2.4	DME:H_2O (1:1)	-	80	17	12
16	Na_2CO_3	1.2	DME:H_2O (1:1)	-	80	23	13
17	Na_2CO_3	1.2	DMF:H_2O (1:1)	-	85	20	35
18	Na_2CO_3	1.2	dioxane:H_2O (1:1)	-	80	23	13
19	Na_2CO_3	1.2	DMF:H_2O (1:1)	-	110	18	12
20	Na_2CO_3	1.2	DMF:H_2O (1:1)	-	140	18	52
21	K_2CO_3	1.2	DMF:H_2O (1:1)	-	85	18	9
22	NaOH	1.2	DMF:H_2O (1:1)	-	85	18	13
23	NaOMe	1.2	DMF:H_2O (1:1)	-	85	18	10
24	NaOEt	1.2	DMF:H_2O (1:1)	-	85	18	8
25	Na_2CO_3	1.2	DMF:H_2O (1:1)	-	85	18	0
26	Na_2CO_3	2.4	DMF:H_2O (1:1)	-	85	18	3
27	Na_2CO_3	5	DMF:H_2O (1:1)	-	85	18	5
28	Na_2CO_3	1.2	DMF:H_2O (1:1)	-	80	20	13
29	Na_2CO_3	1.2	DMF:H_2O (1:1)	-	85	18	16
30	Na_2CO_3	1.2	DMF:H_2O (1:1)	-	85	18	18

product, so I varied the conditions to try to increase the yield. Entries 14 and 15 show different bases, potassium carbonate (K_2CO_3) and NaOH, that Yin tried in their optimization, neither of which provided any improvement. Entries 11-13 and 16-18 show various polar aprotic solvents, THF, diethyl ether (Et_2O), 1,4-dioxane, DME, and DMF, some at higher temperatures. The only solvent that provided any significant improvement in yield was DMF.

Based on these results, I optimized the reaction using DMF as the solvent. I increased the temperature, entries 19 and 20, and found that at 140 ˚C, higher amounts of deprotection were possible, but the reaction also produced several side products and decomposed some of the starting material. Furthermore, temperatures in that range will most likely decompose the diazeniumdiolate group. Entries 21-24 show several different bases in DMF, but none of these increased the amount of product. In entries 25-27, I varied the equivalents of base, but found no improvement. Strangely, entry 25 was run in almost identical conditions as entry 17 which previously showed an increased yield, but in this case, no deprotection occurred at all. I ran the reaction again to see if perhaps entry 17 was anomaly, and based on entries 28-30, it seems it must have been. None of the three identical reactions gave yields even close to what I had observed previously. So far, none of the basic Boc deprotection strategies that I have employed have been very successful, so a new strategy will be necessary to afford the final unprotected, product with a diazeniumdiolate group attached.

2.4 Chemical Approach to Functionalization of Indicator with Nitric Oxide Donor

2.4.1 Development of High-Pressure NO Reactor Set Up

In order to synthesize diazeniumdiolates, a high-pressure gas reactor set-up is necessary, so I set up our NO reactor system in order to run these reactions. The NO reactor is made up completely of stainless-steel parts, as NO is a strongly oxidizing gas and would react with other

materials. The metal tubing (Figure 2.11) is connected by Swagelok fittings for a leak-proof seal. The reactor on the left is connected to an argon tank to purge the system (Figure 2.11a-b), and there are positions for five possible reactions at one time (Figure 2.11c). On the right, the system is connected to a high purity (>99%) NO tank (Figure 2.11c). The Swagelok connections must be tightened properly, but not overtightened, in order to prevent leaks in the system. Leaks can allow NO to be released into the atmosphere, which is dangerous, and the reaction will not hold pressure over the course of the reaction, which can prevent the reaction from being successful. To test for leaks while setting up the reaction system, I filled the system with argon gas, closed the valves, and checked each gauge at particular time points to determine if the system was leaking (indicated by decreasing gauge readings) and where the leak was occurring. Once I determined that there were leaks in the system, I used Snoop, a soapy solution, to help find the leak. The solution is sprayed on joints, and if bubbles form, that is a sign that gas is leaking at that point. Once the leak is found, the joint should be checked for damage and replaced if so or

Fig. 2.11 NO reactor system set-up a) argon tank to purge the reactor system of oxygen b) tubing and gauge connecting argon tank to NO reactor system c) reactor set-up with high purity NO tank to the right of the system d) reaction vessel attached to reactor system

tightened slightly if there is no apparent damage. Teflon tape can also be used to prevent leaks for certain types of joints. The process of filling the system with argon and checking the gauges should be repeated until pressure is maintained at each gauge for at least 24 hours, or the longest potential reaction time. Once the reactor system is determined to be leak free, a reaction vessel and valve set up (Figure 2.11d) should be attached to the reactor system and pressurized with argon gas, then left for at least 24 hours to ensure that the reaction set up and valve also do not have leaks. If the set-up leaks, the rubber gaskets on the quick release valve should be replaced and retested.

To set up a reaction, the reaction mixture is placed in a reaction vessel with a stir bar. If the reaction volume is small (<10 mL), a glass vial can be placed in the coated reaction vessel to hold the reaction solution. The vessel is sealed with a valve, and the valve is connected to the reactor system and stirred below on a stir plate (Figure 2.11d). The system is purged with argon, then filled with NO to the pressure needed for the reaction. The reactor system is only rated to be pressurized up to 100 psi, and reactions requiring pressures higher than that should not be run on our system. The reaction is then sealed off separately from the rest of the system, and the reaction is allowed to run until complete. The rest of the reactor system is purged with argon to remove any leftover NO. Once complete, the reaction is opened to the rest of the system, and the system is again purged with argon several times. The reaction vessel can then be removed from the reactor system and opened to workup the reaction and purify the product.

There are several safety guards to ensure that the risks of using the NO reactor are mitigated. NO is a P-listed chemical and can be hazardous to work with. In our applications, it is used in a high-pressure gas reactor system, which also poses its own risks. The reaction mixture is placed in a polymer-coated reaction vessel (Figure 2.12a) which is sealed with a quick release

valve (Figure 2.12c, 2.12e). The polymer coating means that if the reaction explodes, the vessel and reaction will be contained within the coating. The quick release valve means that the reaction vessel can be immediately removed from the reactor at any time without purging the vessel, as it is a fully contained unit (Figure 2.12e). As NO reacts readily with oxygen, the system must be purged with argon gas several times before adding NO to the system, after NO is added to the system, and when the reaction is complete. Any waste NO that needs to be released from the reactor system is absorbed through the waste solution, a solution of potassium permanganate ($KMnO_4$) and potassium hydroxide (KOH), which reacts with the NO to form solid manganese oxide (MnO), neutralizing the NO gas (Figure 2.12f).[37] The solution appears bright purple in color when freshly prepared due to the $KMnO_4$, but when it is no longer active and needs to be replaced, it is clear with a brown solid, which is the MnO. To prevent any water in the system

Fig. 2.12 Safety features of the NO reactor set-up a) polymer coated reaction vessel to contain vessel in case of explosion b) tubing on reactor connecting to quick release valve c) quick release valve d) $NaOH/CaSO_4$ capsule e) coated vessel attached to quick release valve f) $KMnO_4$/KOH waste solution to absorb waste NO gas

and to help neutralize any NO leaks in the system, a capsule containing sodium hydroxide (NaOH) pellets and calcium sulfate ($CaSO_4$, Drierite) is part of the system (Figure 2.12d). Cotton wool is placed at each end of the capsule to prevent the pellets from falling out into the tubing. All of these safety precautions and proper training of the reactor user helps to mitigate the risks of working with a high-pressure NO reactor.

2.4.2 Analyzing Reaction Products of UNC Chapel Hill Samples

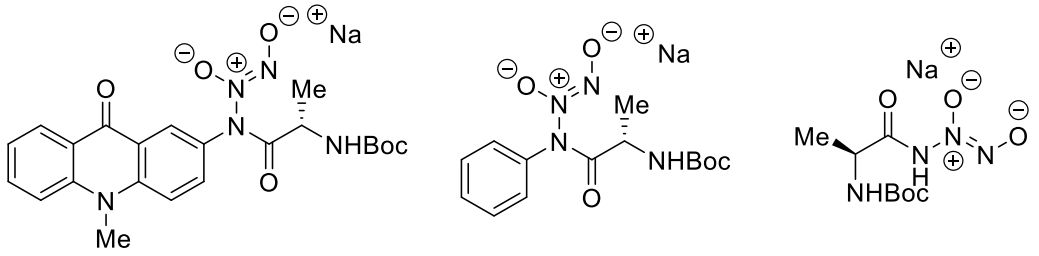


Fig. 2.13 Potential diazeniumdiolate products with fluorescent, model, and non-fluorescent indicator starting materials

While I was in the process of setting up our in-house NO reactor system, I sent samples of my starting materials to UNC Chapel Hill, where Lei Yang of Mark Schoenfisch's group performed initial reactions with NO to attempt to form the proposed diazeniumdiolate structures (Figure 2.13). When the reacted samples were sent back, I used UV-Vis and NMR spectroscopy to confirm whether the reaction was successful or not. Diazeniumdiolates have a very distinctive peak around 250 nm in their UV-Vis spectrum, so this feature is an easy way to determine if the reaction was successful.[21] Diazeniumdiolates are unstable in non-basic solutions, so I took UV-Vis measurements for **14**-**16** in 0.01 M NaOH, shown in Figure 2.14. The UV-Vis spectrum of **14** showed peaks at 263 and 413 nm, which is not indicative of a diazeniumdiolate. To determine what these peaks were, I took a UV-Vis spectrum of compound **2** in EtOH, as it is too non-polar to dissolve in aqueous solutions. This spectrum showed peaks at 261 and 414 nm, very similar to **14** in Figure 2.14, so these peaks are most likely due to the fluorescent compound alone. The

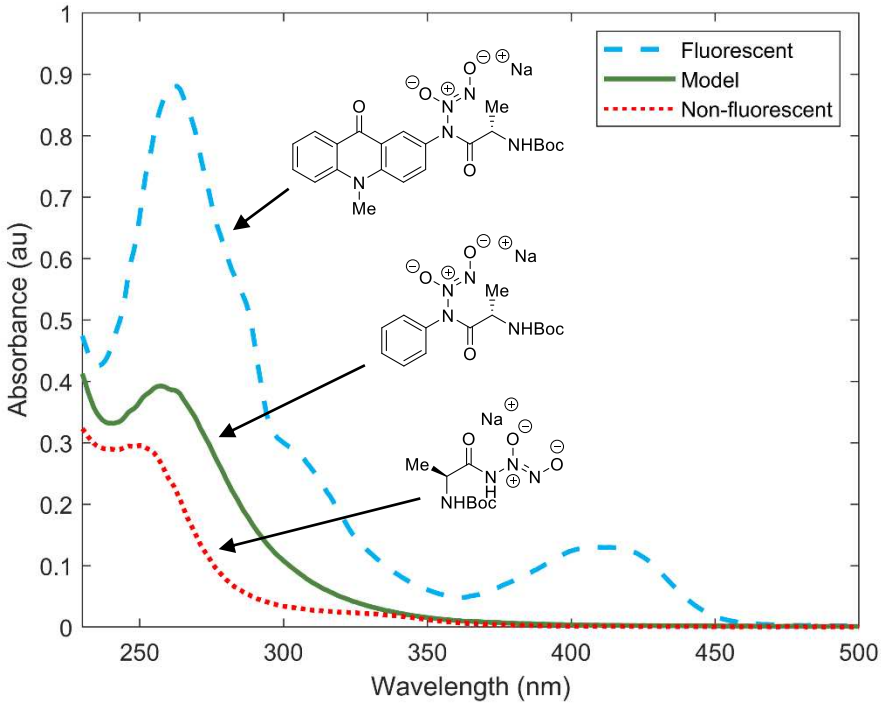


Fig. 2.14 UV-vis spectra for potential diazeniumdiolate compounds **14-16** in 0.01 M NaOH, after reaction of NO with fluorescent, model, and non-fluorescent starting materials.

UV-Vis spectrum of **15** showed a peak at 258 nm, which is a higher wavelength than a normal diazeniumdiolate feature but could still possibly be indicative of diazeniumdiolate formation. The UV-Vis spectrum of **16** showed a peak at 250 nm, which is a good indication that the diazeniumdiolate was formed. This data indicated to me that further testing would be needed to determine if **15** or **16** were formed in the reaction.

While **14** was almost certainly not formed successfully based on the UV-Vis data, further studies would confirm if **15** and **16** were formed or not. Because diazeniumdiolates are unstable in water at pH 7, their decomposition can be tracked by UV-Vis spectroscopy by observing the disappearance of the diazeniumdiolate peak at 250 nm when the compounds are dissolved in water. I dissolved compounds **15** and **16** in water and immediately collected UV-Vis spectra, finding a peak at 260 nm for **15** and 250 nm for **16**. The absorbance was 0.347 for **15** at 0 minutes and 0.820 for **16**. As can be seen in Figure 2.15, absorbance did not decrease at all for

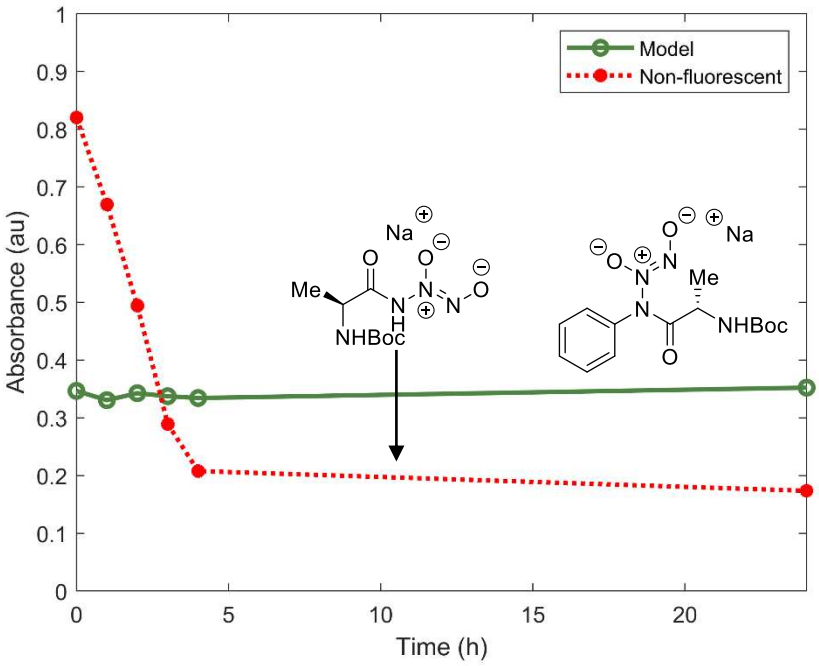


Fig. 2.15 Tracking decomposition of potential diazeniumdiolate compounds **15** and **16** in water over 24 hours by measuring peak absorbance at 260 or 250 nm

15 over 24 hours. For compound **16**, however, absorbance steadily decreased over the first four hours of the experiment and decreased further after 24 hours. This water decomposition study indicates that the diazeniumdiolate reaction was successful for the non-fluorescent compound and **16** was formed, but **15** was not successfully formed.

I also attempted to use NMR studies to confirm whether the reaction with NO was successful in forming compounds **14**-**16**. Because diazeniumdiolates are unstable in non-basic solutions, an NMR spectrum of a diazeniumdiolate is typically collected in a solution of deuterated sodium hydroxide (NaOD) and deuterated water (D_2O). I first collected NMR data with the samples dissolved in 10% NaOD in D_2O (Figure 2.16). The NMR spectra of all three compounds showed none of the expected peaks. All of the spectra displayed solvent peaks and some unknown impurities with three singlets between 1.5-3 ppm. The NMR of compound **15** also had some small peaks in the aromatic region, but the spectrum was noisy and the peaks were difficult to interpret.

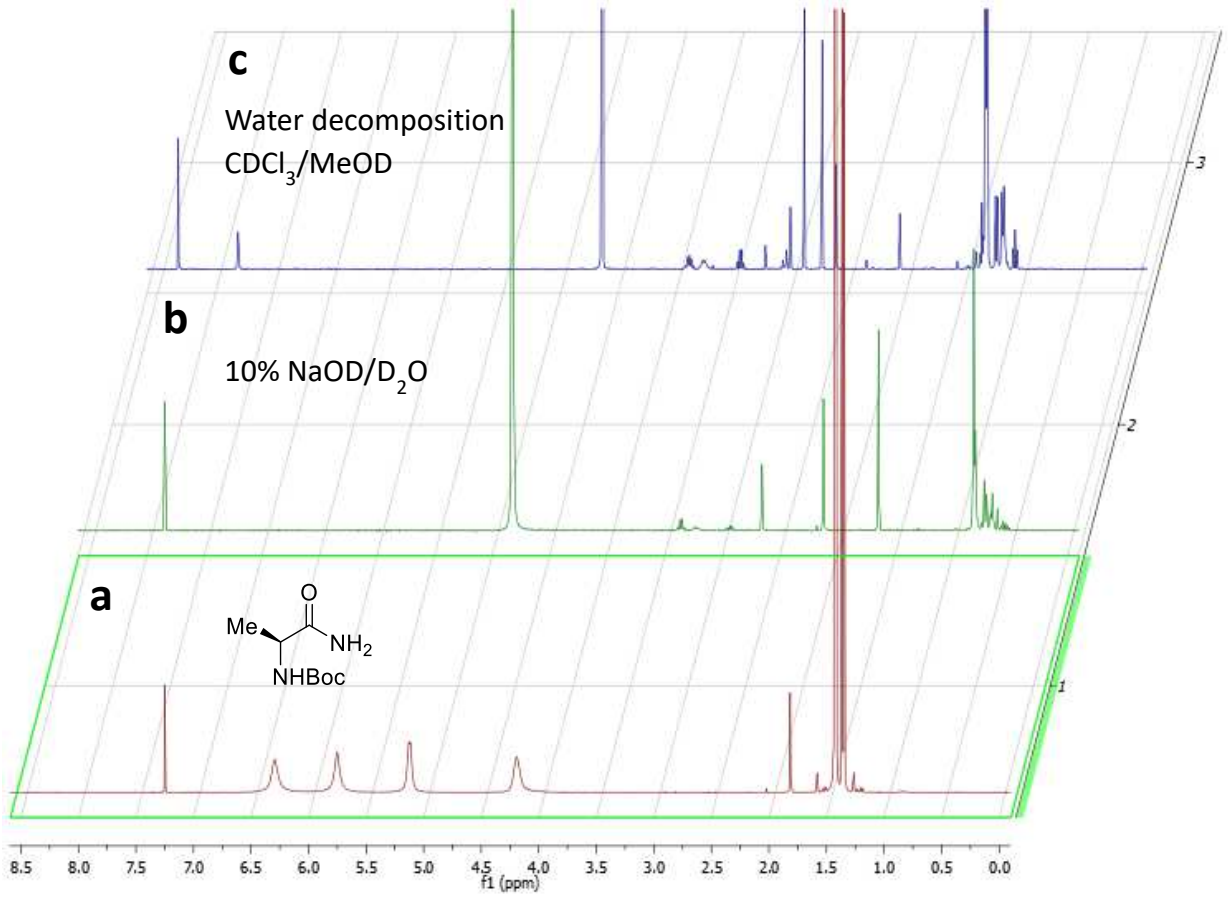


Fig. 2.16 a) NMR spectrum of pure model compound **2**. b) NMR spectrum of reaction product of potential diazeniumdiolate **15** formation in 10% NaOD/D_2O solvent. c) NMR spectrum of reaction product of model compound potential diazeniumdiolate formation **15** after decomposition in water to attempt to return to starting material in $CDCl_3$/MeOD solvent.

Because the NMR spectra in NaOD were inconclusive, I attempted a similar water decomposition experiment to corroborate the UV-Vis data. I dissolved the three compounds in water and let them sit for at least one hour to decompose the diazeniumdiolate group if present. I then removed the water under vacuum and dissolved the remaining solids in deuterated chloroform and deuterated methanol. However, the NMR spectra for all three compounds showed the same unknown impurity as in NaOD, and none of the peaks for the starting materials were observed (Figure 2.16). After collecting this NMR data, the results are inconclusive. I believe that my compounds are unstable in the NMR solvents I have used and are decomposing before I can get a clear spectrum. More work in the future is needed to collect conclusive NMR

data. Solely based on UV-Vis data, compounds **14** and **15** were not successfully synthesized, but compound **16** was synthesized.

2.4.3 In-House Process to Synthesize Diazeniumdiolates

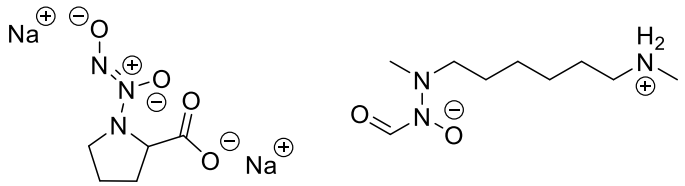


Fig. 2.17 Structures of common diazeniumdiolates PROLI/NO and MAHMA/NO synthesized to test function of NO reactor set-up

Once our in-house NO reactor system was set up and ready to use, to ensure that the reactor was functioning properly, I synthesized the common diazeniumdiolates PROLI/NO[19] and MAHMA/NO[38] (Figure 2.17) based on previously reported procedures. These reactions were successful based on UV-vis peaks at 251 nm for PROLI/NO and 249 nm for MAHMA/NO. After establishing the proper reactor function, I began testing reaction conditions to form the diazeniumdiolates **15** and **16** with the model compound **2** and the non-fluorescent compound **3**. Diazeniumdiolates are usually formed by reacting amines with NO, but I will be adding NO at the amide position to form the diazeniumdiolate group on my compounds. I tried conditions based on a report from Holland where they successfully formed a diazeniumdiolate on nicotinamide.[39] They used a mixture of methanol and ether as solvents, NaOMe in methanol as the base, and pressurized NO to 50 psi, allowing the reaction to run for 72 hours (Figure 2.18). I tried these reaction conditions, shown in Table 2.2, entries 1 and 8. Unfortunately, these reactions seemed to form the nitrosamine product (Figure 2.18) based on the UV-vis analysis (Figure 2.19). Nitrosamines typically have UV-vis peaks around 230 nm and 340 nm,[40] which can be observed in the UV-vis spectra of the products of these conditions in Figure 2.19. The product of the model compound reaction had UV-vis peaks at 239 and 351 nm, and the product

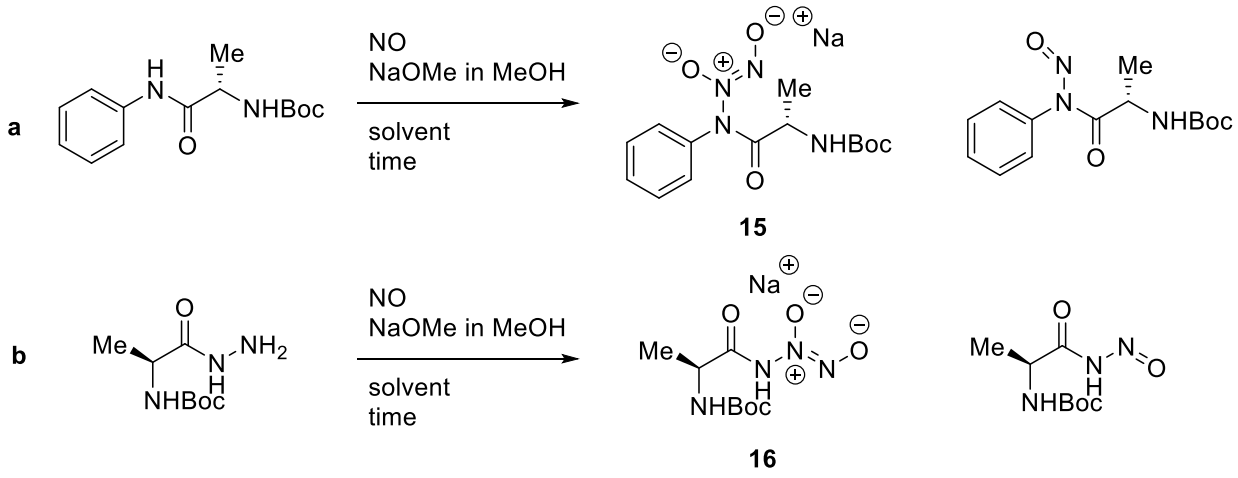


Fig. 2.18 Attempted diazeniumdiolate reactions and conditions and potential nitrosamine products on right a) model compound reaction b) non-fluorescent compound reaction

of the non-fluorescent compound reaction had peaks at 231 and 342 nm, which are indicative of nitrosamines. I tried changing reaction variables such as solvent ratio (Table 2.2, entries 2-3, 9-10), which could improve solubility of the starting materials. I tried changing NO pressure (Table 2.2, entries 3, 10), as higher NO pressures can promote diazeniumdiolate formation, but the desired product still was not formed. Changing reaction time (Table 2.2, entries 3, 5, 7, 12) also did not change product formation. One hypothesis was that the diazeniumdiolate was

Table 2.2 Reaction conditions attempted to functionalize model and non-fluorescent compounds with diazeniumdiolate

Entry	Compound	Temperature (°C)	NO pressure (psi)	Solvent ratio	Time (h)
1	Model	21	50	MeOH:ether (3:2)	72
2	Model	21	50	MeOH:ether (2:3)	72
3	Model	21	80	MeOH:ether (1:9)	72
4	Model	-20	80	MeOH:ether (1:9)	48
5	Model	21	80	MeOH:ether (1:9)	24
6	Model	-15	80	MeOH:ether (1:9)	24
7	Model	-15	80	MeOH:ether (1:9)	5.5
8	Non-fluorescent	21	50	MeOH:ether (3:2)	72
9	Non-fluorescent	21	50	MeOH:ether (1:4)	72
10	Non-fluorescent	21	80	MeOH:ether (1:9)	72
11	Non-fluorescent	-20	80	MeOH:ether (1:9)	48
12	Non-fluorescent	21	80	MeOH:ether (1:9)	24

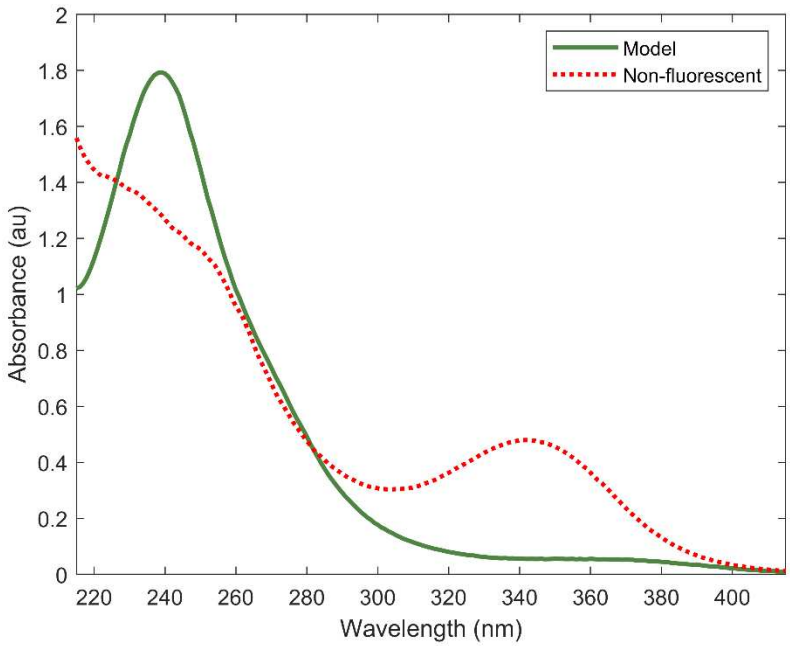


Fig. 2.19 UV-vis spectra of products of reactions to attempt to form diazeniumdiolate on model and non-fluorescent compounds showing potential nitrosamine formation.

unstable at room temperature and was decomposing to the nitrosamine before the reaction could be analyzed, so I ran the reactions at lower temperatures (Table 2.2, entries 4, 6-7, 11). However, these reactions also showed formation of the nitrosamine. Based on this unsuccessful reaction optimization and the fact that diazeniumdiolates do not typically form on amides, this strategy was abandoned in favor of a more successful approach.

2.5 Conclusion

The goal of this project was to synthesize a small molecule antibiotic prodrug that could detect and kill bacteria. To accomplish this, I envisioned a compound with a fluorescent sensor, a NO donor group, and an amino acid that would be cleaved by a bacterial protease to release NO. My approach was to synthesize 2-aminoacridone-tagged L-alanine, which would fluoresce yellow after the protease L-alanyl aminopeptidase in bacteria cleaved L-alanine from my compound, releasing NO from a diazeniumdiolate group. I successfully synthesized a fluorescent

sensor with a fluorescent molecule and an amino acid attachment that fluoresces in the presence of *P. aeruginosa,* based on our bacterial studies.

In addition to the fluorescent sensor, I synthesized a model compound similar to the fluorescent sensor to use to perfect the conditions of the NO addition reaction. I also synthesized a non-fluorescent version of the sensor that would be able to detect bacteria by sensing NO release. I tried many variations on a basic Boc deprotection reaction, investigating different bases, additives, solvents, and temperatures, but found limited success, likely due to the difficulty of removing a Boc group under basic conditions. Future experiments could investigate further basic deprotection conditions, but my project moved in a different direction, making the need for basic deprotection conditions unnecessary.

I also analyzed samples of my compounds that the Schoenfisch group at UNC Chapel Hill reacted with NO. Using UV-Vis spectroscopy, I found that the diazeniumdiolate group likely was successfully formed on the non-fluorescent compound. Unfortunately, despite several attempts and varied conditions, such as solvent, temperature, and NO pressure, I was unable to functionalize the model compound or non-fluorescent compound with a diazeniumdiolate group using my NO reactor set-up. This challenge is likely due to the rarity of forming diazeniumdiolates on amides rather than secondary amines. There is only one literature report of forming a diazeniumdiolate on an amide, in which Holland and coworkers formed a diazeniumdiolate on nicotinamide, highlighting the difficulty of this reaction. Basing my approach on functionalizing an amide with a diazeniumdiolate turned out to be infeasible.

In answer to the research question posed at the beginning of this chapter, can a dual function small molecule be synthesized with a fluorescent tag that changes color in the presence of bacteria and leads to NO release to kill bacteria? At this point in the research project, I have

not achieved this goal. I ran into challenges in completing the third step in the goals laid out in the beginning of this chapter, functionalizing the fluorescently tagged amino acid with a diazeniumdiolate group. This chapter details several failures encountered while attempting to reach this goal, indicating that this is not a feasible approach. Despite these setbacks, based on this work, I was able to develop a new, more feasible approach to continue this project, which will be discussed in Chapter 3 of this dissertation.

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CHAPTER 3

SYNTHESIS, CHARACTERIZATION, AND APPLICATION OF PRO/FLUORO/NO

3.1 Background

To continue to progress toward the goal of addressing the problem of antibiotic resistance by synthesizing a small molecule to detect and kill bacteria, I searched for a new approach. The challenges with forming the diazeniumdiolate on the L-alanylaminopeptidase indicator discussed in chapter 2 lead me to decide to redesign the structure. The difficulties arose with forming a diazeniumdiolate on an amide functionality, which has only one report of being successful.[1] However, I wanted to keep the color-changing bacterial indicator function. To keep similar functionality with an aminopeptidase enzyme, I considered all of the other amino acids and chose proline, which has a secondary amine functionality that could be functionalized with a diazeniumdiolate group. This approach has precedence for being feasible, as proline is a starting material to make a common diazeniumdiolate, PROLI/NO. In addition, if PROLI/NO decomposes to form a nitrosamine, which are typically carcinogenic compounds, *N*-nitrosoproline has been shown to be non-carcinogenic.[2,3] Certain bacterial species also contain prolylaminopeptidases, which cleave proline from peptide bonds, so the compound could still act as a fluorescent enzyme-activated bacterial indicator. Based on these reasons, I decided to add proline to the aminoacridone fluorescent compound instead of alanine, then functionalize the secondary amine of proline with a diazeniumdiolate, a more viable pathway.

I would like to thank Jesus B. Tapia for performing MS experiments for this project. I would also like to thank Dr. Joe Saavedra (National Institutes of Health) for helpful discussions and advice and Dr. Brad Borlee (Colorado State University) for providing the bacteria strain,

Pseudomonas aeruginosa PAO1. This work was supported by a grant from the Monfort Foundation. This project was originally published in *Journal of Materials Chemistry B* (Hibbard, H. A. J., Reynolds, M. M. Fluorescent nitric oxide donor for the detection and killing of *Pseudomonas aeruginosa*. *J. Mater. Chem. B.* **2019**, *7*, 2009-2018).

3.2 Introduction

The Review on Antimicrobial Resistance predicts that by 2050, one person will die every three seconds from a bacterial infection,[4] surpassing both heart disease and cancer as the most common cause of death.[5] This epidemic of antibiotic-resistant bacteria creates a critical need for researchers to develop better methods for detection and treatment of bacterial infections. A multifunctional material that could both detect and eradicate bacterial infections would be ideal.

Multifunctional materials are a promising new area of research, offering improved efficiency and versatility, while reducing costs. Unfortunately, few examples exist of materials that can both detect and kill bacteria, and these examples are exclusively larger molecules, including polymers and nanoparticles.[6–8] Small molecules, on the other hand, tend to release active drug compounds much faster.[9] Additionally, the small molecule could be incorporated into a polymer support to make biomedical devices.

In this work, a dual-function small molecule is specifically designed, synthesized, and tested to detect the presence of and kill bacteria, shown in Figure 3.1. Briefly, a derivative of a

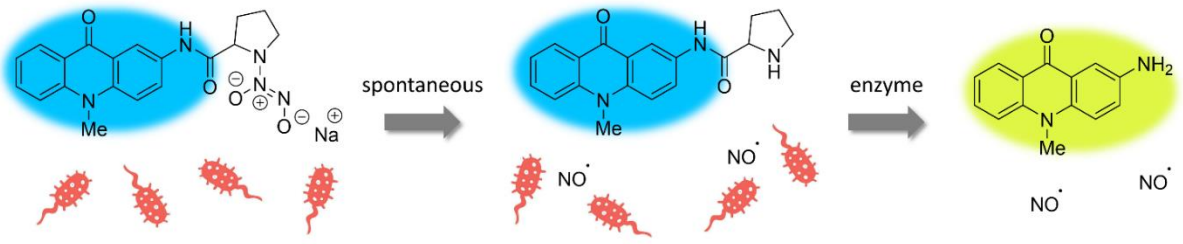


Fig. 3.1 Schematic showing nitric oxide release and color change of sensor. Nitric oxide is released from the diazeniumdiolate functional group spontaneously, killing *Pseudomonas aeruginosa* bacteria cells. A bacterial enzyme then cleaves proline from the fluorescent aminoacridone/proline compound, changing the fluorescent color from blue to yellow.

well-studied class of fluorescent molecules, aminoacridones, is attached to the amino acid proline to form an indicator for the bacterial enzyme prolyl aminopeptidase, which is known to cleave N-terminal proline from peptides.[10] Next, a nitric oxide (NO) donor, a diazeniumdiolate similar to PROLI/NO, shown in Figure 3.2, is added to impart antibacterial activity.

The fluorescent compound 2-aminoacridone is a commercially available, well-characterized fluorescent reporter.[11] It is commonly used to derivatize oligosaccharides for fluorescent analysis.[12] Previous reports have also shown that attaching amino acids to derivatives of 2-aminoacridones allows for enzymatic detection of bacteria.[13] For these reasons, a 2-aminoacridone derivative is chosen as the fluorescent reporter to detect bacteria in this work.

By attaching an amino acid to the fluorescent aminoacridone compound, the wavelength the emitted light changes, and a color change can be relied upon for bacterial detection. In this work, proline is attached to an aminoacridone, offering several advantages. Proline is cleaved by prolyl aminopeptidase, an enzyme found in Pseudomonas aeruginosa, a deadly bacteria species that is growing increasingly resistant to antibiotics and causes a significant amount of hospital-acquired infections.[10,14] In addition, a known NO donor, PROLI/NO (Figure 3.2), is formed from the secondary amine on proline, conveying antibacterial activity. There are a few recent reports of NO donors with fluorescent reporters, however, fluorescent changes in these compounds allow quantification of the amount of NO released, not detection of an external stimulus.[15–18] The compound in this work uses fluorescence to detect bacteria and NO release to kill bacteria.

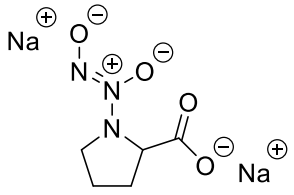


Fig. 3.2 Structure of proline-based diazeniumdiolate, PROLI/NO, a previously synthesized diazeniumdiolate formed by reacting nitric oxide with proline

Nitric oxide (NO) is an excellent small molecule candidate for incorporation into a new antibiotic. It plays many roles in the body, in addition to its broad-spectrum antimicrobial activity, killing both gram-positive and gram-negative strains of bacteria.[19–22] Researchers have shown that NO uses multiple mechanisms of action to kill bacteria, meaning that bacterial resistance is much more difficult to develop, which is critical in stemming the spread of antibiotic-resistant bacterial infections.[23,24] Studies by the Schoenfisch group show that bacteria are unable to develop resistance to exogenously applied NO, even after 20 days.[23]

Nitric oxide is a potent antibacterial agent, but due to its high reactivity and gaseous nature, NO is difficult to deliver and dose as a stable drug compound.[25] As a result, NO is almost always delivered as a prodrug, in the form of an NO donor that is metabolized into free radical NO via a chemical or enzymatic transformation. Some common NO donors include S-nitrosothiols, organic nitrites and nitrates, metal nitrosyls, and diazeniumdiolates.[26–30] Diazeniumdiolates have the advantage of being very stable as solids and in basic media, releasing two equivalents of NO per equivalent of diazeniumdiolate, and having predictable half-lives for spontaneous NO release.[31] One disadvantage of diazeniumdiolates is that they are known to decompose into nitrosamines, which are typically carcinogenic.[32] However, the nitrosamine formed from proline, *N*-nitrosoproline, is one of the few non-carcinogenic nitrosamines.[2,3] This could mean that if the nitrosamine forms from our synthesized proline aminoacridone compound, it would also be non-carcinogenic, although future studies would be necessary to confirm its non-toxicity. Because of these advantages, a derivative of the diazeniumdiolate PROLI/NO (Figure 3.2) is chosen as the NO donor in this work.

This report details the design and synthesis of a compound for its application as a dual-function bacterial indicator and antibacterial agent. The research question posed in this chapter

is: can a dual function small molecule be synthesized with a fluorescent tag that changes color in the presence of bacteria and leads to NO release to kill bacteria? The proposed process is shown in Figure 3.1, where the proline-based diazeniumdiolate releases NO spontaneously to kill bacteria. The bacterial enzyme prolyl aminopeptidase found in *P. aeruginosa* then cleaves proline from the fluorescent aminoacridone compound, causing a fluorescent color change from blue to yellow that indicates the presence of bacteria. *P. aeruginosa* commonly causes infections in burn wounds[33] and our compound could be useful in this case, releasing NO as site-specific broad-spectrum antibacterial agent to kill bacteria in the wound, and determining if the infection is caused by *P. aeruginosa*. To our knowledge, this is the first example of a multifunctional small molecule that can both detect and kill *P. aeruginosa*.

3.3 Experimental Methods

3.3.1 Materials and Methods. Acridone (98%) was purchased from Oxchem Corp (Wood Dale, IL, USA). Nitric acid (68-70%), iodomethane (98%), PBS tablets, and triethylamine (99.5%) were purchased from EMD Chemicals (Gibbstown, NJ, USA). Acetic acid (glacial) was purchased from BDH Chemicals (Radnor, PA, USA). Sodium hydride (57-63% oil dispersion), tin(II) chloride dihydrate, isobutyl chloroformate (98%), and anhydrous methanol (99.9%) were purchased from Alfa Aesar (Ward Hill, MA, USA). *N,N*-dimethylformamide (99.9%), hydrochloric acid (1 N), sodium hydroxide (98.8%), tetrahydrofuran, Oxoid nutrient broth (OXCM0001B), and Oxoid nutrient agar (OXCM0003B) were purchased from Fisher Scientific (Hampton, NH, USA). Boc anhydride (99.5%) was purchased from Chem Impex (Bensenville, IL, USA). Dichloromethane (99.5%) and trifluoroacetic acid (99%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Nitric oxide (99%) was purchased from Matheson Tri-gas (Montgomeryville, PA). Sodium methoxide (30% in methanol) was purchased from Acros

Organics (Geel, Belgium). CellTiter Blue was purchased from Promega (Madison, WI, USA). 24-well and 96-well tissue culture nontreated plates were obtained from Corning (Corning, NY, USA). Argon (ultrahigh purity), nitrogen (ultrahigh purity), and oxygen were purchased from Airgas (Denver, CO, USA). Deionized water (18.2 MΩ·cm) was obtained from a Millipore Direct-Q water purification system (EMD Millipore, Billerica, MA, USA). *Pseudomonas aeruginosa* (PAO1) was provided by Dr. Brad Borlee at Colorado State University. Commercially available reagents were purchased and used without further purification unless otherwise indicated.

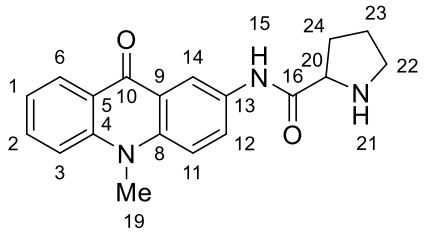
All nuclear magnetic resonance (NMR) spectra were obtained on Varian Inova 400 (400 MHz for ^{1}H; 101 MHz for ^{13}C) and/or Bruker US400 (400 MHz for ^{1}H; 101 MHz for ^{13}C) at room temperature unless noted otherwise. Chemicals shifts were reported in parts per million (δ scale), and referenced according the following standards: chloroform residual signal (δ 7.16), water residual signal (δ 4.79), or dimethyl sulfoxide residual signal (δ 2.50) for ^{1}H signals; deuterated chloroform or deuterium dimethyl sulfoxide carbon resonances (middle peak is δ 77.1, or δ 39.5, respectively) or tetramethylsilane for samples in deuterated water for ^{13}C signals. Coupling constants were reported in Hertz (Hz) and multiplicities were reported as follows: singlet (s), doublet (d), doublet of doublets (dd), triplet (t), quartet (q), and multiplet (m). IR spectra were obtained on a Thermo Scientific Nicolet 6700 FT-IR spectrometer. Electrospray ionization mass spectrometry data were obtained on an Agilent 6224 TOF mass spectrometer equipped with a dual electrospray ion source operated in positive and negative mode. Excitation and emission spectra were obtained on a Horiba Jobin-Yvon FluoroLog-3 Spectrofluorometer. Melting points were obtained with an Electrothermal Mel-Temp apparatus. UV-Vis measurements were obtained with a Thermo Scientific Nicolet Evolution 300 spectrometer. Well

plate absorbance measurements were obtained with a Biotek Synergy 2 Multi-Mode Reader. Reactions were analyzed by thin layer chromatography (TLC) on aluminum sheets that were pre-coated with silica gel 60 F_{254}, and the reactions were purified by column chromatography using Alfa Aesar silica gel 60 (0.06-0.2 mm).

3.3.2 Abbreviations. HNO_3=nitric acid, AcOH= acetic acid, NaH= sodium hydride, DMF= N,N-dimethylformamide, MeI= methyl iodide, $SnCl_2 \cdot 2H_2O$= tin (II) chloride dihydrate, HCl= hydrochloric acid, *i*BuCF= isobutyl chloroformate, Et_3N= triethylamine, TFA= trifluoroacetic acid, DCM= dichloromethane, Boc= tert-Butyloxycarbonyl), TFA= trifluoroacetic acid, DCM= dichloromethane, NO= nitric oxide, NaOMe= sodium methoxide, MeOH= methanol

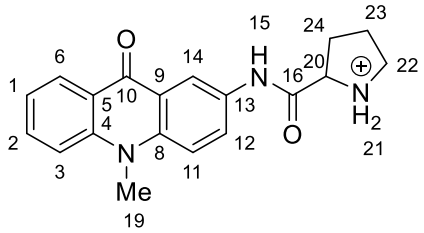
3.3.3 Synthetic Procedures and Characterization

Compounds **2**-**5** were synthesized according to previously reported procedures; see Chapter 2, Section 2 for details.



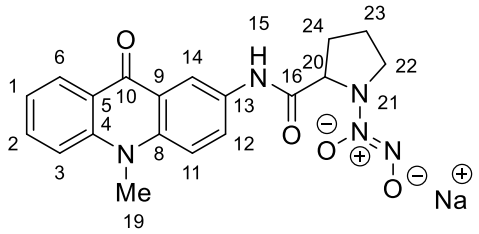
*3.3.3.1 N-(10-methyl-9-oxo-9,10-dihydroacridin-2-yl)pyrrolidine-2-carboxamide (**6**, Pro/Fluoro).* To a solution of **5** (430.5 mg, 2 mmol) in distilled DCM dried over 3 Å molecular sieves (20 mL, 0.1 M) at 0 °C, isobutyl chloroformate (285 µL, 2.2 mmol) and triethylamine (307 µL, 2.2 mmol) were added. After stirring for 30 min at 0 °C, **4** (493.4 mg, 2.2 mmol) was added and the reaction was warmed to rt and stirred for 18 h. The reaction was washed sequentially with aq. HCl (1 M, 20 mL), sat. aq. $NaHCO_3$ (20 mL) and brine (20 mL). The organic layer was dried over Na_2SO_4 and concentrated in vacuo to afford an oily orange crude

solid. The crude product was dissolved in DCM (6.7 mL, 0.3 M) and trifluoroacetic acid (3.1 mL, 40 mmol) was added. The reaction was stirred at rt for 3 h, and the solvent was then removed in vacuo. The concentrate was dissolved in 15 mL of DCM, then washed with sat. aq. $NaHCO_3$ (10 mL) and extracted with DCM (3 × 10 mL). The organic layer was dried over Na_2SO_4 and concentrated in vacuo to afford an orange oily solid. The crude product was purified by flash column chromatography (SiO_2, 100% EtOAc, 5% MeOH/DCM, 10% MeOH/DCM gradient), affording the title compound as an orange solid (188 mg, 29%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.02 (s, 1H, H15), 8.64 (dd, J = 9.3, 2.7 Hz, 1H, H6), 8.56 (dd, J = 8.0, 1.5 Hz, 1H, H14), 8.20 (d, J = 2.6 Hz, 1H, H2), 7.73 (t, J = 8.7 Hz, 1H, H1), 7.54 (dd, J = 8.9, 6.8 Hz, 2H, H3, H11), 7.29 (t, J = 7.5 Hz, 1H, H12), 3.92 (s, 3H, H19), 3.91 – 3.81 (m, 1H, H20), 3.08 (dq, J = 16.4, 6.5 Hz, 2H, H22), 2.23 (m, 1H, H24), 2.08 (m, 1H, H24), 1.84 – 1.73 (m, 2H, H23).[34] ^{13}C NMR (101 MHz, $CDCl_3$) δ 177.68 (C10), 173.60 (C16), 142.34 (C4), 139.11 (C13), 133.76 (C8), 132.35 (C2), 127.72 (C6), 126.30 (C9), 122.47 (C5), 121.88 (C1), 121.06 (C12), 116.31 (C11), 115.66 (C14), 114.70 (C3), 60.95 (C20), 47.35 (C22), 33.68 (C19), 30.80 (C24), 26.29 (C23). HRMS (+ESI) Found 322.1556 (Calcd. 322.1556 for $C_{19}H_{20}N_3O_2$; $[M+H]^+$). m.p. 54-56 ^oC. ν_{max}/cm^{-1} 3676br, 3255br, 2989w, 2972w, 2901w, 1675w, 1589s, 1504s, 1423s, 1275s, 1179s, 1066s, 879w, 806s, 749s, 684s. λ_{EX} = 419 nm, λ_{EM} = 499 nm (EtOH).



*3.3.3.2 2-((10-methyl-9-oxo-9,10-dihydroacridin-2-yl)carbamoyl)pyrrolidin-1-ium (**7**, Pro/Fluoro/H$^+$).* Crude **6** (200 mg, 0.47 mmol, post work-up, prior to column chromatography) was dissolved in DCM (2 mL, 0.235 M) and trifluoroacetic acid (1 mL, 13.1 mmol) was added to

the reaction. The reaction stirred at rt for 2 h, and the solvent was then removed in vacuo. The product was precipitated out in diethyl ether and filtered, affording the title compound as a yellow powder (145 mg, 96%). ^{1}H NMR (400 MHz, d_6-DMSO) δ 10.76 (s, 1H, H15), 9.33 (s, 1H, H21), 8.73 (s, 1H, H21), 8.63 (d, J = 2.6 Hz, 1H, H6), 8.34 (d, J = 8.3 Hz, 1H, H14), 8.04 (dd, J = 9.3, 2.6 Hz, 1H, H2), 7.96 – 7.77 (m, 3H, H1, H3, H11), 7.34 (t, J = 7.8 Hz, 1H, H12), 4.37 (d, J = 7.9 Hz, 1H, H20), 3.96 (s, 3H, H19), 3.31 (m, 2H, H22), 2.40 (m, 1H, H24), 2.02 – 1.93 (m, 2H, H23).^{13}C NMR (101 MHz, d_6-DMSO) δ 176.62 (C10), 167.22 (C16), 142.50 (C4), 139.48 (C13), 134.43 (C8), 132.41 (C2), 126.92 (C6), 126.78 (C9), 122.08 (C5), 121.56 (C1), 117.56 (C12), 116.53 (C11), 116.41 (C14), 116.39 (C5), 60.20 (C20), 46.28 (C22), 34.12 (C19), 29.99 (C24), 24.05 (C23). HRMS (+ESI) Found 322.1563 (Calcd. 322.1556 for $C_{19}H_{20}N_3O_2$; $[M]^+$). m.p. 210.5-211.5 °C. ν_{max}/cm^{-1} 3084br, 1665s, 1637w, 1617w, 1586s, 1550w, 1507s, 1466w, 1430w, 1351w, 1292w, 1280w, 1200s, 1181s, 1128s, 1051w, 1039w, 1002w. λ_{EX} = 421 nm, λ_{EM} = 489 nm (H_2O).



*3.3.3.3 Sodium 1-(N-(10-methyl-9-oxo-9,10-dihydroacridin-2-yl)pyrrolidine-2-carboxamide)diazen-1-ium-1,2-diolate (**8**, Pro/Fluoro/NO).* A solution of **7** (100 mg, 0.31 mmol) in anhydrous MeOH (3.1 mL, 0.1 M) was mixed with 30% sodium methoxide in methanol (133 µL, 0.62 mmol) that had previously been dried over activated 3 Å molecular sieves for at least 24 h. The resulting solution was flushed with argon, then charged with 5 atm of NO and stirred at rt. A thick yellow precipitate began to form within 3 h of exposure to NO. After 24 h, the pressure was released, and the product was collected by filtration, washed with ether, and dried under

vacuum to give the title compound as a yellow powder (110 mg, 88%). ^{1}H NMR (400 MHz, 0.5 mM NaOD in D_2O) δ 7.64 (d, J = 7.7 Hz, 1H, H6), 7.57 (d, J = 2.3 Hz, 1H, H14), 7.32 (dd, J = 9.2, 2.4 Hz, 1H, H2), 7.28 – 7.13 (m, 2H, H1, H3), 6.98 (d, J = 8.9 Hz, 1H, H11), 6.78 (t, J = 7.5 Hz, 1H, H12), 3.71 (t, J = 7.2 Hz, 2H, H22), 3.22 (s, 3H, H19), 3.21 – 3.09 (m, 2H, H24), 2.81 – 2.66 (m, 1H, H20), 2.07 (m, 1H, H23), 1.93 (m, 1H, H23). ^{13}C NMR (101 MHz, 0.5 mM NaOD in D_2O) δ 177.60 (C10), 165.99 (C16), 143.53 (C4), 140.28 (C13), 137.49 (C8), 133.44 (C2), 132.13 (C6), 125.00 (C9), 120.78 (C5), 120.55 (C1), 119.07 (C12) , 117.63 (C11), 115.89 (C14), 114.67 (C3), 93.98 (C20), 47.46 (C22), 34.36 (C24), 32.84(C19), 18.89 (C23). HRMS (+ESI) Found 381.1427 (Calcd. 381.1437 for $C_{19}H_{19}N_5O_4$; $[M+H]^+$). m.p. 174 ^{o}C decomp. ν_{max}/cm^{-1} 3678br, 2989s, 2901w, 1668s, 1581s, 1506s, 1466s, 1406s, 1279w, 1250w, 1241w, 1177s, 1125s, 1066s. λ_{EX} = 397 nm, λ_{EM} = 462 nm (0.01 M NaOH).

3.3.4 Nitric Oxide Release Measurements. Nitric oxide generation was recorded in real time using a chemiluminescence-based GE Nitric Oxide Analyzer (NOA). Before each use, the NOA was calibrated using a 39.6 ppm NO calibration gas cylinder. Pro/Fluoro/NO **8** powder was added into the NOA sample cell in the dark at rt. The powder was purged continuously with ultrapure nitrogen gas for 5-10 min to collect a baseline release. An aliquot of deoxygenated nutrient broth (2.6 g in 200 mL of Millipore water, pH 7.4) was added through the side injection port of the NOA vessel to give the specified initial concentration. The lights were turned on and the vessel was placed in a 37 °C water bath. The nutrient broth solution was purged continuously with ultrapure nitrogen gas for the duration of the experiment. NO release was recorded in 1 s intervals as parts per billion with a gas sampling rate of 200 mL/min. The solution was maintained in a nitrogen atmosphere with bubbling rate of 48-54 mL/min N_2 directly into the solution and flow gas introduced into the remaining headspace.

3.3.5 Bacteria Studies

3.3.5.1 Pseudomonas aeruginosa *Bacteria Culture.* Initial stock culture of *Pseudomonas aeruginosa* (PAO1) was made by streaking with a sterile inoculating loop onto agar plates and incubating overnight at 37 °C. A colony was inoculated in sterilized nutrient broth media (NBM, 13 g nutrient broth/1 L Millipore water) and grown overnight at 37 °C until an optical density at 600 nm (OD_{600nm}) ≈0.7 was reached. This bacterial solution was combined with glycerol (30% v/v) in a 1:1 fashion to obtain a final glycerol concentration of 15% (v/v). These solutions were stored at −80 °C until use. Prior to each bacterial experiment, a frozen culture was thawed at rt and then centrifuged at 4700 rpm for 10 min. The supernatant was discarded, and the pellet was resuspended using 5 mL NBM. This was transferred to an additional NBM (15 mL) and allowed to grow overnight with shaking at 120 rpm until the OD_{600nm} ≈1.0. The culture was diluted to an OD_{600nm} ≈0.35 using warmed NBM prior to beginning experiments.

3.3.5.2 Bacterial Detection Study. Pro/Fluoro/H^+ **7** (5 mg) was dissolved in dimethyl sulfoxide (DSMO, 200 μL) and added to sterilized nutrient agar [powder base (2.8 g) in Millipore water (100 mL)] at 50 °C to a final concentration of 50 mg/L. The mixture was divided among five sterile Petri dishes and allowed to set overnight before inoculating each plate with 20 μL of a *Pseudomonas aeruginosa* suspension (OD_{600}=0.033-0.038) using a sterile plastic spreader. The positive control was made using the same procedure by mixing DMSO (200 μL) with sterile nutrient agar [powder base (2.8 g) in Millipore water (100 mL)] before inoculation. The plates were incubated at 37 °C for 24 h before analysis. The plates were illuminated by an Aldrich Spectroline F-series hand-held UV lamp at a wavelength of 365 nm.

3.3.5.3 Cell Viability Assay Solutions. Solutions of Pro/Fluoro/NO (0.1 mM, 40 mg/L; 1 mM, 400 mg/L; and 10 mM, 4,000 mg/L) were made in *Pseudomonas aeruginosa* culture solution in

NBM. To a 24 well plate was added the Pro/Fluoro/NO solutions (1 mL; 0.1 mM, 1 mM, or 10 mM) in triplicate. The positive control, Pseudomonas aeruginosa culture only, and the negative control, Pro/Fluoro/NO in sterile nutrient broth, were plated in the same manner. The plates were incubated at 37 °C for 24 h before analysis. The plates were illuminated by an Aldrich Spectroline F-series hand-held UV lamp at a wavelength of 365 nm. A standard Cell Titer Blue cell viability assay was performed to determine antibacterial efficacy of Pro/Fluoro/NO. Three aliquots (100 µL) of each solution in the 24 well plate were transferred to a 96 well plate. To each well containing solution, 20 µL of Cell Titer Blue stock solution (1:5 Cell Titer Blue reagent:nutrient broth), warmed to 37 °C, was added. The plate was incubated at 37 °C for 2 h, then the absorbance was measured at 570 and 600 nm using a plate reader.

3.3.5.4 Statistical Analysis. All biological assays were performed using at least three samples and assayed in replicate form. Viability assays are reported as the mean and 95% confidence interval. All data were evaluated for potential outliers using the Grubbs Test. The statistical differences in data were evaluated using the Student's *t* test ($p < 0.05$) at the 95% confidence level.

3.4 Development of Synthetic Route to Pro/Fluoro/NO

The steps I developed to synthesize the enzyme-activated bacterial indicator Pro/Fluoro (**6**) are shown in Figure 3.3. I used the steps developed and discussed in depth in Chapter 2, Section 2.3 to make the fluorescent aminoacridone, then attached proline instead of alanine to form the indicator Pro/Fluoro. In the first step of the synthesis, I nitrated acridone (**1**) with nitric acid in the presence of acetic acid to form **2**, 2-nitroacridone. This procedure gives a yield of 64% of a mixture of the 2-nitro and 4-nitro isomers, which are separated in a later step. In next step of the synthesis, I methylated **2** to form **3** in Figure 3.3. I modified a previously reported synthesis using sodium hydride and methyl iodide to methylate the amine, giving a 70% isolated

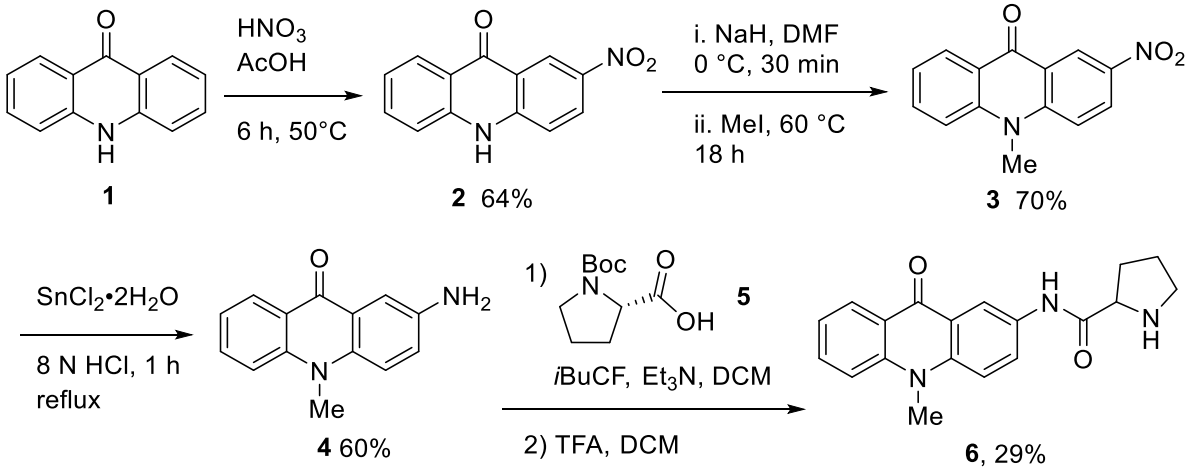


Fig. 3.3 Overall reaction scheme developed for the synthesis of fluorescent indicator Pro/Fluoro **6**, for an overall yield of 8% over 5 steps.

yield.[35] I reduced the nitro group, **3** to **4** in Figure 3.3, using tin(II) chloride dihydrate and hydrochloric acid, following a previously published procedure.[36] The crude product showed evidence of the undesirable 4-isomer as well as the desired 2-isomer. Fortunately, I developed column chromatography conditions on silica gel to separate the isomers, giving an isolated yield of **4** of 60%. When dissolved in ethanol, I observe that compound **4** appears yellow under a UV light (365 nm).

In the next step to make the indicator, I coupled Boc-protected proline (**5**) to the aminoacridone, **4**, using isobutyl chloroformate and triethylamine. After an aqueous work-up, I removed the Boc protecting group using trifluoroacetic acid to give compound **6**, Pro/Fluoro. I purified the deprotected product by column chromatography, giving a 29% yield. We used mass spectrometry and ^{1}H- and ^{13}C-NMR spectra to confirm the synthesis of Pro/Fluoro **6** (Figures 3.17-3.18). I observe that Pro/Fluoro **6** appears blue under a UV light (365 nm) when dissolved in ethanol. The synthetic route to Pro/Fluoro **6** I developed gives an overall yield of 8% over five steps. This is the first synthesis of an indicator designed to detect prolyl aminopeptidase in bacteria.

3.5 Pro/Fluoro/NO as a Bacterial Indicator

With the synthesis of Pro/Fluoro **6** complete, I wanted to test the bacterial detection ability of Pro/Fluoro **6** as a proof of concept, to ensure that a color change would be possible once NO is released (Figure 3.1). I selected *P. aeruginosa* for the bacterial studies because it produces a prolyl aminopeptidase enzyme, which I hypothesized would cleave N-terminal proline from Pro/Fluoro **6**, causing a color change.[37] *P. aeruginosa* is also a deadly gram-negative bacteria strain, resistant to many common antibiotics and is the cause of death in many immunocompromised hospital patients.[14]

I attempted bacterial detection experiments with Pro/Fluoro **6**, but it is not soluble enough in aqueous-based agar and precipitated out before the agar set, preventing sensing. To overcome this problem, Pro/Fluoro **6** was protonated using trifluoroacetic acid to form **7** (Pro/Fluoro/H^+), shown in Figure 3.4. Protonation solubilized Pro/Fluoro/H^+ **7** in the agar, forming a homogenous solution.

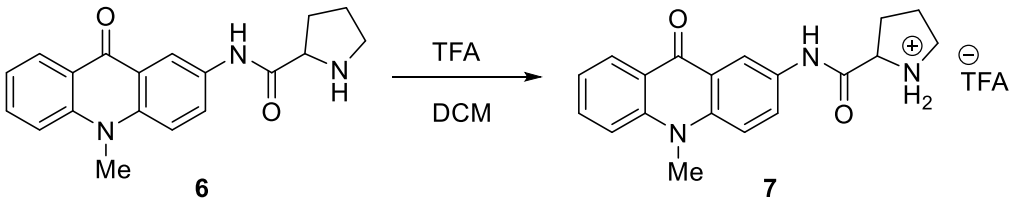


Fig. 3.4 Synthesis of fluorescent indicator Pro/Fluoro/H^+ **7**, used for *Pseudomonas aeruginosa* detection experiments.

To perform the bacterial sensing experiments, I dissolved Pro/Fluoro/H^+ **7** in dimethyl sulfoxide (DMSO) and mix it with cooled sterilized nutrient agar. I inoculated the agar plates with *P. aeruginosa* and incubated them at 37°C for 18 hours. The positive control plates consist of agar mixed with DMSO but without Pro/Fluoro/H^+ **7**, which I inoculated with *P. aeruginosa*. For the negative control plates, I mixed agar with Pro/Fluoro/H^+ **7** in DMSO but did not inoculate the plates with *P. aeruginosa*. After incubation, I illuminated the plates with a 365 nm

UV light to determine if a color change occurred. Yellow fluorescence indicates that enzymatic cleavage occurred, releasing proline and compound **4** to fluoresce yellow (Figure 3.1). Blue fluorescence indicates that no enzymatic cleavage occurred; it is the color of fluorescence of intact Pro/Fluoro/H^+ **7**. Each experiment had five samples and was repeated three times.

The results of these experiments can be seen in Figure 3.5, showing that there is a clear color change due to the presence of *P. aeruginosa*. The positive control shows no fluorescence under UV light (Figure 3.6), indicating that fluorescence is due entirely to the synthesized Pro/Fluoro/H^+ **7** compound. The negative control, left in Figure 3.5, clearly fluoresces blue, indicating that no cleavage of Pro/Fluoro/H^+ **7** occurs when *P. aeruginosa* is not present. The experimental plate, right in Figure 3.5, fluoresces yellow under UV light, indicating that proline is cleaved from Pro/Fluoro/H^+ **7** by *P. aeruginosa*, leaving compound **4** to fluoresce yellow.

Based on the above experimental observations, I conclude that Pro/Fluoro/H^+ **7** can be used as an indicator to detect if *P. aeruginosa* is present using UV light. I observe a clear and obvious color change from blue to yellow indicating the presence of *P. aeruginosa*. My

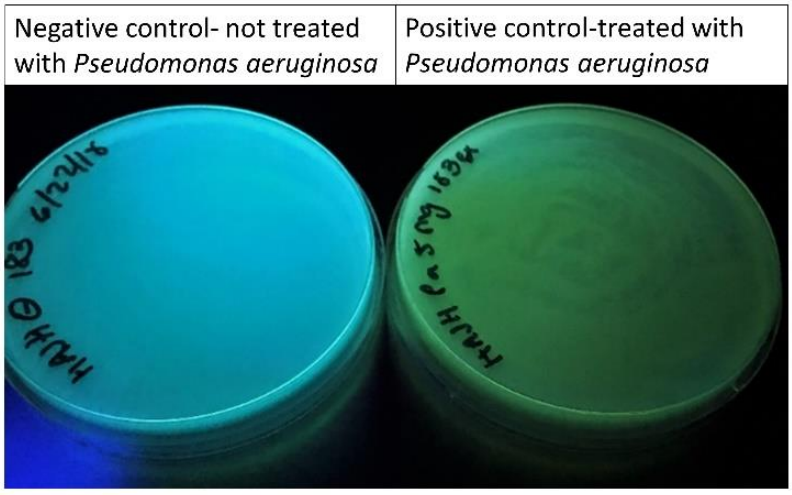


Fig. 3.5 Representative agar plates showing color change from blue to yellow under a 365 nm UV lamp in the presence of *Pseudomonas aeruginosa*, as expected. Left: negative control- not inoculated with *Pseudomonas aeruginosa*, Pro/Fluoro/H^+ **7**, appears blue; right- inoculated with *Pseudomonas aeruginosa*, Pro/Fluoro/H^+ **7**, appears yellow

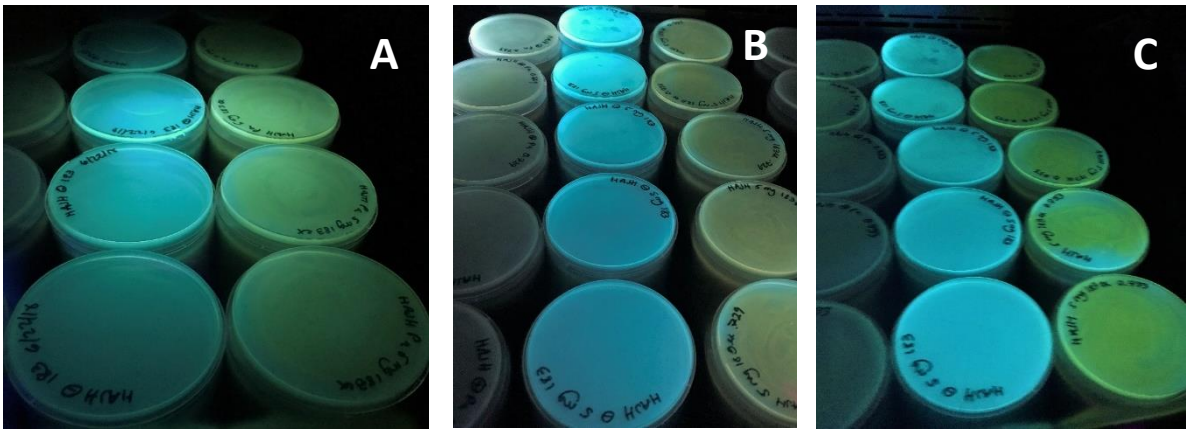


Fig. 3.6 A-C are photos of repeated experiments for bacterial detection, colonies of *Pseudomonas aeruginosa* after incubation in the presence of Pro/Fluoro/H^+ **7** illuminated under a 365 nm UV light. The positive control (left column of plates in images A-C) is inoculated with *P. aeruginosa* with no Pro/Fluoro/H^+ **7** present and shows no fluorescence or color. The negative control (middle column of plates) is not inoculated with *P. aeruginosa* but contains Pro/Fluoro/H^+ **7** and shows a blue color, indicating that Pro/Fluoro/H^+ **7** is not cleaved. The experiment plates are in the right column and are inoculated with *P. aeruginosa* and contain Pro/Fluoro/H^+ **7**, and show a yellow color, indicating that Pro/Fluoro/H^+ **7** is cleaved apart and changes color.

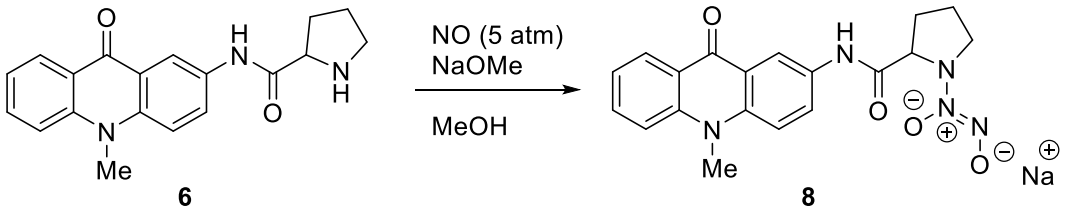
hypothesis is that the enzyme prolyl aminopeptidase, produced by *P. aeruginosa*,[10] cleaves proline, causing the color change. If my hypothesis is correct, this is the first example of a prolyl aminopeptidase-specific indicator. Based on my hypothesis, any bacteria species containing this enzyme would be detectable via this sensing mechanism, such as *Clostridium difficile*, which can cause life-threatening gastrointestinal infections.[38]

3.6 Synthesis and Characterization of Properties of Pro/Fluoro/NO

3.6.1 Synthesis of Pro/Fluoro/NO

Once I demonstrated the functionality of the bacterial indicator, the last step in the synthesis is to attach a diazeniumdiolate group to Pro/Fluoro **6**. Adding the diazeniumdiolate group to the compound imparts the ability to release NO, giving it antibacterial activity and creating a dual-function material. Diazeniumdiolates are typically formed by reacting secondary amines with gaseous NO at elevated pressures,[32] which in my case is at the proline amine

location, seen in Figure 3.7. The diazeniumdiolate reaction is based on the previously reported procedure to synthesize the proline-based diazeniumdiolate, PROLI/NO (Figure 3.2).[39] As can be seen in Figure 3.7, I reacted Pro/Fluoro **6** with NO at high pressure, 5 atm, in the presence of sodium methoxide in methanol. I discovered that it is important to use anhydrous methanol and to dry the methanolic sodium methoxide over activated 3 Å molecular sieves before running the reaction, as water decomposes diazeniumdiolates.[32] During the reaction, I observed the formation of a yellow precipitate product **8**, Pro/Fluoro/NO, and upon filtration and drying, a yield of 88% is achieved. Diazeniumdiolates are typically white powders, but in my case, the yellow color of the product is due to the fluorescent acridone compound, and is not indicative of diazeniumdiolate decomposition.[31]



6 **8**

Fig. 3.7 Reaction of Pro/Fluoro **6** under high pressure NO in the presence of NaOMe in methanol developed to form the fluorescent diazeniumdiolate Pro/Fluoro/NO **8**.

The formation of diazeniumdiolates is typically confirmed by UV-Vis spectroscopy due to their distinctive peak at 250 nm.[31] However, an absorbance peak due to the fluorescent acridone component occurs at 260 nm (Figure 3.8), interfering with the diazeniumdiolate absorbance peak. To confirm the formation of the diazeniumdiolate Pro/Fluoro/NO **8**, we used several techniques. I observe the formation of a precipitate in the course of the reaction shown in Figure 3.7, which is typical of diazeniumdiolate-forming reactions.[39] The physical properties of the product of the reaction are different than the starting material; the product is no longer soluble in organic solvents as Pro/Fluoro **6** is. It is, however, soluble in water and basic solutions,

which is indicative of the formation of a diazeniumdiolate salt.[40] The ^{1}H-NMR spectrum of Pro/Fluoro/NO **8** (Figure 3.9) shows the disappearance of the proline N-H peak and a shift in the other peaks compared to the ^{1}H-NMR spectrum of Pro/Fluoro **6** (Figure 3.9), which provides indirect evidence that the desired reaction took place. Electrospray ionization time-of-flight mass spectrometry (ESI-TOF MS) was performed in a basic solvent mixture, and the accurate mass of the diazeniumdiolate was found at 381 *m/z* (Figure 3.10), further leading us to conclude that the reaction was successful.

Taken together, this evidence shows that we successfully synthesized the diazeniumdiolate, Pro/Fluoro/NO **8**. The overall yield of the synthesis of Pro/Fluoro/NO **8** over six steps is 7%. Compared to total syntheses of other common antibiotics and their analogs, our synthetic procedure requires significantly fewer steps, as well as giving a much higher overall yield.[41] This is the first time that this diazeniumdiolate is synthesized in the literature. It also one of very few diazeniumdiolates with a fluorescent component, which use fluorescence to quantify

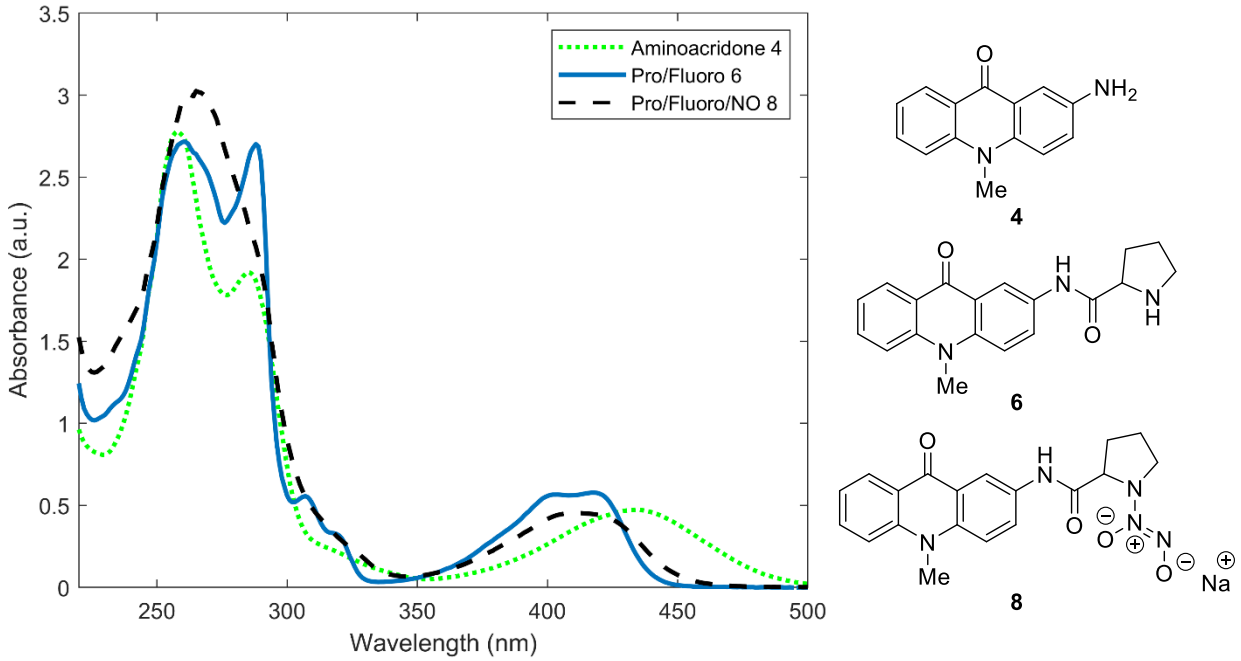


Fig. 3.8 UV-Vis spectra of aminoacridone compound **4** (1 mM in EtOH), Pro/Fluoro **6** (1 mM in EtOH) Pro/Fluoro/NO **8** (1 mM in 0.01 M NaOH)

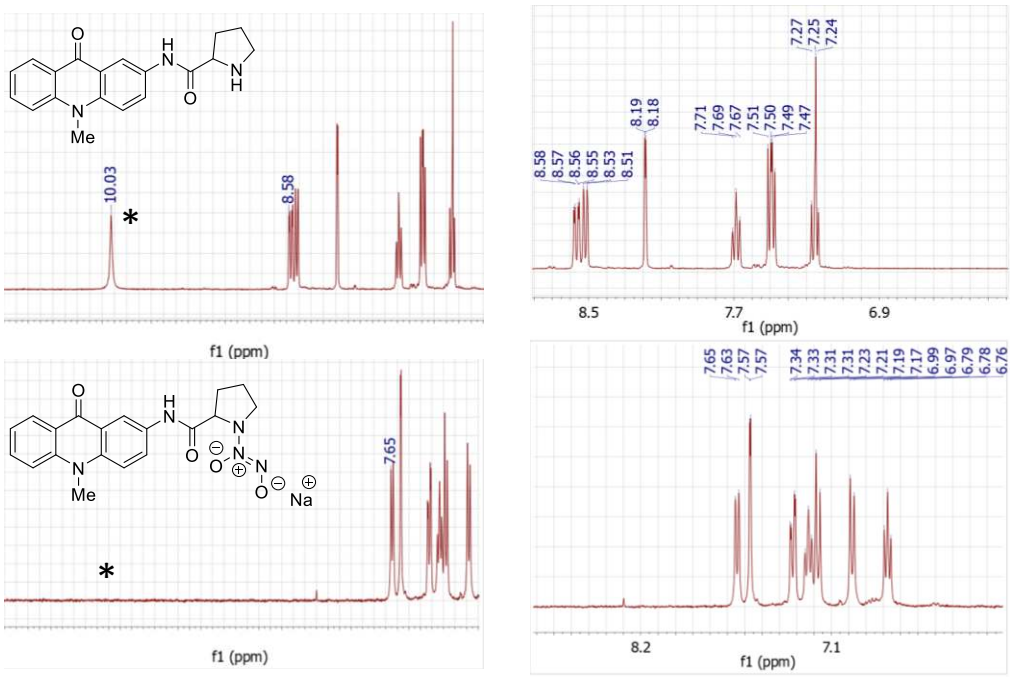


Fig. 3.9 Left: showing disappearance of peak at 10.03 ppm of proline H on Pro/Fluoro **6** (top) after formation of Pro/Fluoro/NO **8** (bottom). Right: showing peak shifts from Pro/Fluoro **6** (top) to formation of Pro/Fluoro/NO **8** (bottom).

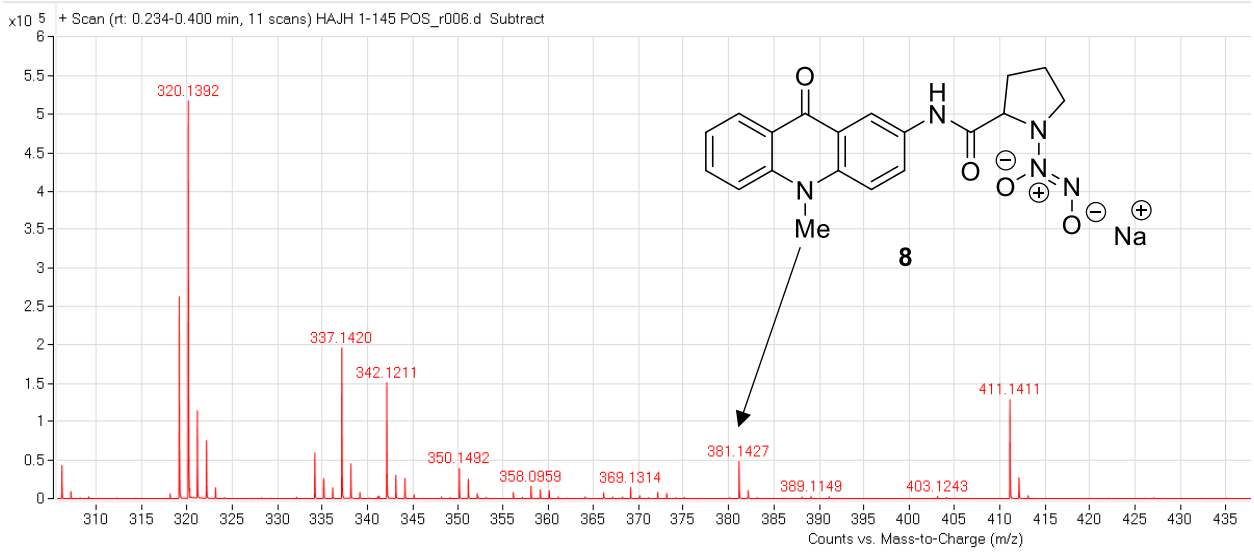


Fig. 3.10 Electrospray ionization time-of-flight mass spectrometry (ESI-TOF MS) spectrum showing correct mass of **8**. The experiment was run in a solvent system of 80 v/v% methanol, 19.5 v/v% water, and 0.5 v/v% ammonia, with a pH ≈ 12 to stabilize the diazeniumdiolate.

the amount of NO release.[15,18] Our synthesized compound Pro/Fluoro/NO **8** is the first example of a diazeniumdiolate with a fluorescent marker to detect bacteria.

3.6.2 NO Release Measurements

Once we synthesized Pro/Fluoro/NO **8**, I measured the amount of NO released at different concentrations using a nitric oxide analyzer (NOA). An NOA uses chemiluminescence to directly quantify the amount of NO released by oxidizing NO with ozone, forming nitrogen dioxide in an excited state. Upon relaxation, it emits a certain amount of light, which is measured and related to the moles of NO released via a previously determined calibration constant. I performed NOA experiments to determine the NO release profile of Pro/Fluoro/NO **8** and to quantify the amount of NO release. During the NOA experiments, I observed that NO release from Pro/Fluoro/NO **8** is light sensitive. I saw a visible decrease in NO release when the overhead lights were turned off, and a return to previous NO release levels when the lights were turned on again, although there is still above-baseline NO release when the lights are off. This light sensitivity could be due to the photosensitivity of acridone compounds, which has been previously reported.[42] Light could be exciting the acridone piece of Pro/Fluoro/NO **8**, leading to an increase in the release of NO.

First, I determined the total amount of possible NO release from Pro/Fluoro/NO **8**. Previous reports show that NO release from diazeniumdiolates displays first order kinetics, and occurs when the amine on which the diazeniumdiolate is protonated, causing up to two molecules of NO to be released from the compound.[32] I chose experimental conditions to release all the NO in Pro/Fluoro/NO **8**. Most diazeniumdiolates are stable as solids when stored at -20 ˚C or below, or in solutions at pH 12 or above.[43] Diazeniumdiolates are unstable in acidic and neutral conditions, so exposure to these conditions forces the release of NO. To determine the total amount of NO released, I forced NO off the diazeniumdiolate by injecting a basic solution containing the diazeniumdiolate into an acidic buffer (pH = 3), as well as shining a UV light on

the solution to address the light sensitivity of the diazeniumdiolate. By comparing the observed total moles of NO release from Pro/Fluoro/NO **8** in these experiments to the theoretical amount of NO release possible, the amount of NO released from the diazeniumdiolate can be calculated. In these calculations, we assume two moles of NO per mole of Pro/Fluoro/NO **8**, as is typical in a diazeniumdiolate.[32] Based on our calculations, Pro/Fluoro/NO **8** releases 75 ± 15% of the theoretical 200% of NO possible. Previous literature reports have shown that based on their structure, some diazeniumdiolates do not release the entirety of the theoretical 200% of NO, due to decomposition or other factors.[44–48] For example, Bushan, et. al. reported that the stability of the diazeniumdiolate protecting group is important, as photochemical instability can lead to decomposition into the nitrosamine, decreasing diazeniumdiolate yield.[44,45] Maragos and coworkers found that some diazeniumdiolates decompose in solution, resulting in less than 200% of the theoretical release of NO, and that increasing the pH of the solution decreases NO release from diazeniumdiolates.[46,47]

Next, I measured the amount of NO release at initial concentrations of 0.1, 1, and 10 mM Pro/Fluoro/NO **8**. I chose the conditions of the NO release measurements to most closely mimic the future cell viability assay conditions. The experiments are run in nutrient broth media (NBM, pH 7.4) at 37 °C for 24 hours with overhead lights on, and all experiments were run at least three times. A representative NO release profile can be seen in Figure 3.11, showing an initial spike of high NO release, then dropping off to a steady release after about one hour, as can be seen in the inset. This release profile matches the release profile of other diazeniumdiolates.[49] The triplicate experimental plots for each concentration can be seen in Figures 3.12-3.14. The results of these experiments can be found in Table 3.1. In column 2 of Table 3.1, entries 1-3, the amount of NO

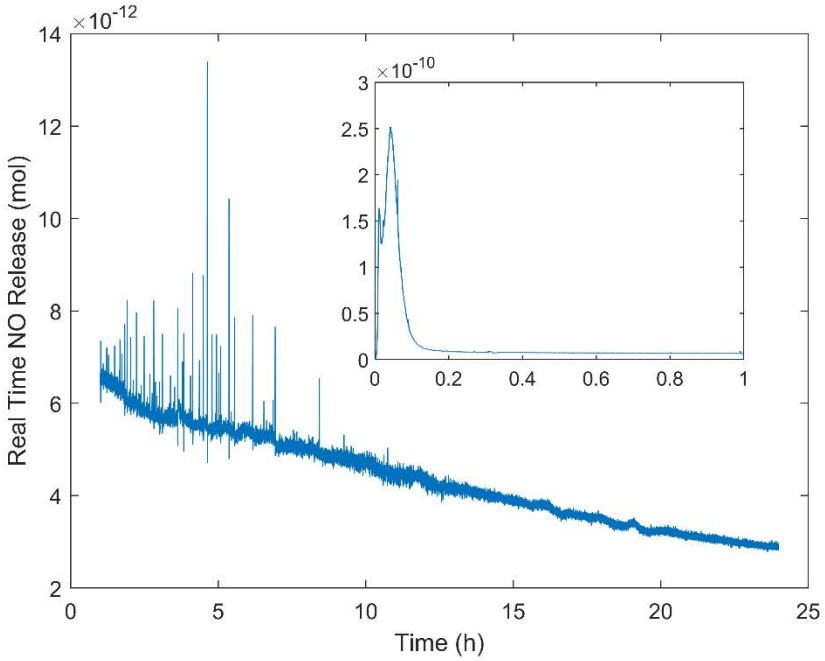


Fig. 3.11 Representative real-time NO release profile of Pro/Fluoro/NO **8** at an initial concentration of 1 mM in nutrient broth at 37 °C, showing steady release of NO from 1–24 hours. Inset shows an initial spike of NO release in the first hour.

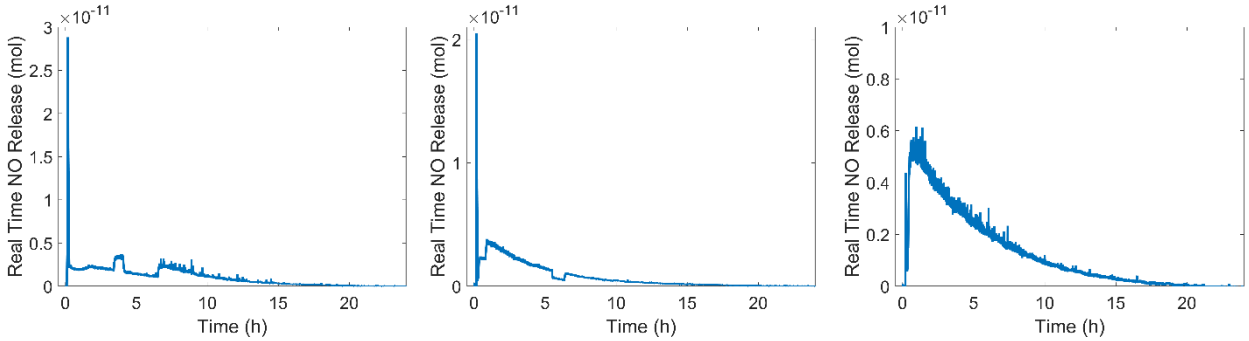


Fig. 3.12 Three trials of real-time NO release plots of 0.1 mM Pro/Fluoro/NO **8** in NBM

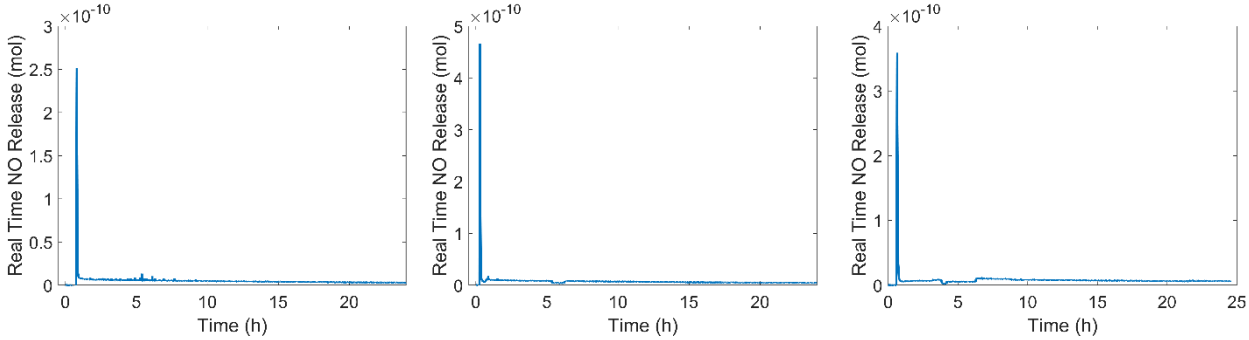


Fig. 3.13 Three trials of real-time NO release plots of 1 mM Pro/Fluoro/NO **8** in NBM

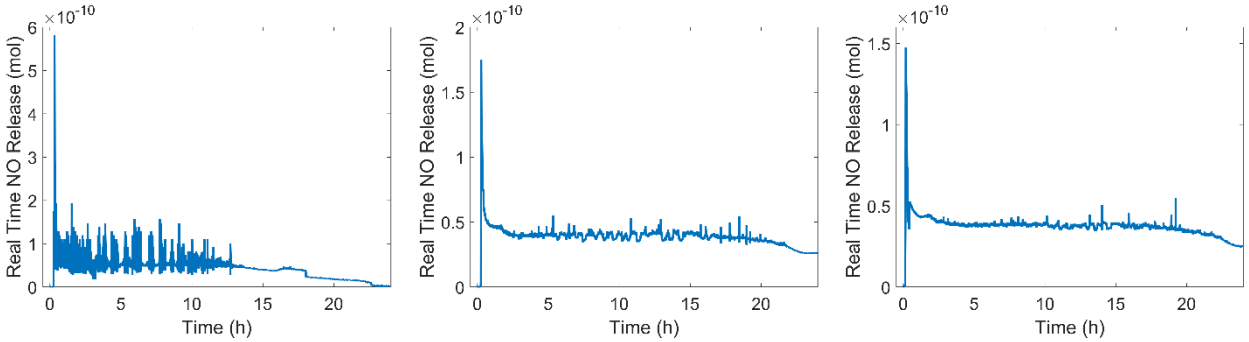


Fig. 3.14 Three trials of real-time NO release plots of 10 mM Pro/Fluoro/NO **8** in NBM

release is normalized to the mass of Pro/Fluoro/NO **8** used in each experiment. At 10 mM, 853 µmol of NO/g of Pro/Fluoro/NO **8** is released (Table 3.1, Entry 3), compared to only 322 µmol of NO/g of Pro/Fluoro/NO **8** at a concentration of 0.1 mM (Table 3.1, Entry 1). As can be seen in column 3 of Table 3.1, this corresponds to 46% of the total possible NO released at 10 mM (Table 3.1, Entry 3) and 17% at 0.1 mM (Table 3.1, Entry 1), assuming 75% of the theoretical NO is released based on previous experiments. The data in Table 3.1 show that less NO is released at the 0.1 mM concentration. This could be due to less availability of NO to be released at lower concentrations or because the level of NO release approaches the limit of detection of the NOA instrument. An increased amount of NO release observed at concentrations of 10 mM should lead to increased antibacterial activity, which I observe in the cell viability studies.

3.7 Antibacterial Activity of Pro/Fluoro/NO with *Pseudomonas aeruginosa*

With the amount of NO release measured, I determined the antibacterial efficacy of Pro/Fluoro/NO **8**. To study this, I exposed *P. aeruginosa* to Pro/Fluoro/NO **8**, and then I performed a spectroscopic assay, CellTiter Blue (CTB), to determine the percent of viable

Table 3.1 Normalized amount of NO released and total NO released at different concentrations

Entry	Initial concentration (mM)	µmol NO/g Pro/Fluoro/NO **8**[a]	Percentage of total possible NO released (%)[a]
1	0.1	322 ± 45	17 ± 2
2	1	731 ± 87	40 ± 5
3	10	853 ± 35	46 ± 2

[a]Data is presented as mean ± standard deviation, n=3

bacteria cells after exposure to the therapeutic. The CTB assay relies on the transformation of blue resazurin (λ_{max} = 600 nm) to pink resorufin (λ_{max} = 570 nm) accomplished by healthy bacteria, where the absorbance is measured to determine an increase or decrease in the metabolic activity of viable bacteria cells compared to a positive control (PC).[50] The positive control in these experiments is *P. aeruginosa* culture with no therapeutic. For these experiments, I mixed *P. aeruginosa* culture in NBM with Pro/Fluoro/NO **8** to initial concentrations of 0.1, 1, and 10 mM, and then incubated for 24 hours at 37 °C.

The results of the CTB assay of *P. aeruginosa* after exposure to Pro/Fluoro/NO **8** (Figure 3.15) indicate that it has antibacterial activity at certain doses. At concentrations of 0.1 mM Pro/Fluoro/NO **8**, I observed a 93 ± 7% viability, a 7% reduction in bacteria which is not significant compared to the positive control (100 ± 2% viability). The 1 mM dose causes a decrease in bacteria viability, as I observed 77 ± 9% cell viability, corresponding to a 23% reduction, a statistically significant reduction in bacteria compared to the positive control. The 10 mM dose is the most effective, with 35 ± 10% cell viability after exposure, a 65% reduction after exposure to the therapeutic. This reduction is statistically significant compared to the positive control, as well as compared to the reduction from the 1 mM dose. While typically a more significant reduction in the number of bacteria remaining is necessary for antibacterial materials,[51] I demonstrate that this compound has antibacterial effects as a proof of concept. I show that Pro/Fluoro/NO **8** has antibacterial effects statistically significant compared to the positive control at concentrations of 1 and 10 mM.

Wink and Mitchell, as well as Schairer and co-workers, show that concentrations of NO in the low micromolar range have antibacterial effects.[24,52] At an initial concentration of 0.1 mM Pro/Fluoro/NO **8**, at the peak of the instantaneous NO release measurements represented by the

spike of NO release in Figure 3.11, the concentration of NO in the solution ranges from 0.6-4.1 nM. Because the concentration of NO does not reach micromolar ranges, this could explain why at an initial concentration of 0.1 mM Pro/Fluoro/NO **8**, there is not a significant reduction in the number of bacteria cells. The NO concentration does not reach levels high enough to have antibacterial activity, based on literature values (>1 μM).[24,52] The experiments at an initial concentration of 1 mM Pro/Fluoro/NO **8** reach concentrations of NO ranging from 167-196 nM. The experiments at an initial concentration of 10 mM Pro/Fluoro/NO **8** reach concentrations of NO ranging from 149-576 nM. At concentrations of 1 and 10 mM, the concentration of NO is one to two orders of magnitude higher than at 0.1 mM. This could explain why bacteria cell viability is significantly lower at 1 and 10 mM concentrations, as NO concentrations are closer to the micromolar range.

After exposure, I observed a color change from blue to yellow in the presence of *P. aeruginosa* (Figure 3.16), similar to the previous detection experiments. The solutions of *P.*

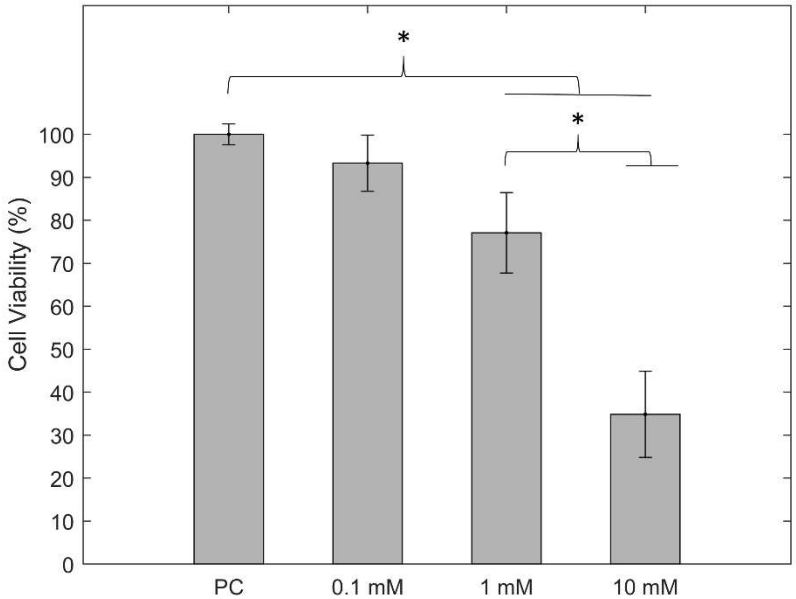


Fig. 3.15 Cell viability after 24-hour exposure of *Pseudomonas aeruginosa* to Pro/Fluoro/NO **8** at initial concentrations of 0.1, 1, and 10 mM as determined by CellTiter Blue assay. Average and 95% confidence interval displayed, n = 9. Statistically significant differences between cellular viabilities are indicated (*) as determined by a student's t test. PC=positive control

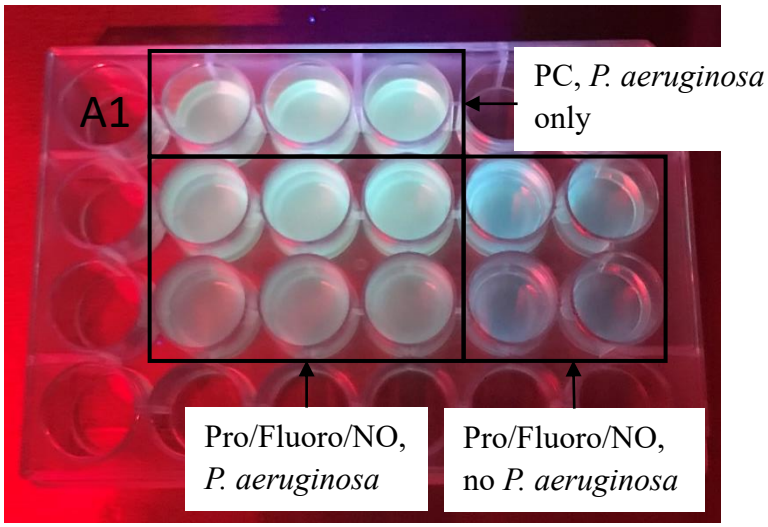


Fig. 3.16 24 well plate under UV light after 24-hour incubation. A2-A4: *P. aeruginosa*, positive control (PC); B2-B4: 1 mM Pro/Fluoro/NO, *P. aeruginosa*, yellow fluorescent color; B5-B6: 1 mM Pro/Fluoro/NO, blue fluorescent color; C2-C4: 100 µM Pro/Fluoro/NO, *P. aeruginosa*, yellow fluorescent color; C5-C6: 100 µM Pro/Fluoro/NO, blue fluorescent color

aeruginosa in NBM that are exposed to Pro/Fluoro/NO **8** at all concentrations appear yellow under a UV light at 365 nm. Solutions of Pro/Fluoro/NO **8** at varying concentrations in nutrient broth media without *P. aeruginosa* are blue under UV light as expected. This demonstrates that the detection function also works post-NO release. Together, the results of these experiments demonstrate the dual-function of Pro/Fluoro/NO **8** to both detect and kill *Pseudomonas aeruginosa*.

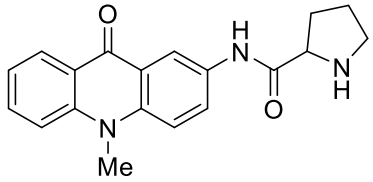
3.8 Conclusion

The research question posed at the beginning of this chapter was: can a dual function small molecule be synthesized with a fluorescent tag that changes color in the presence of bacteria and leads to NO release to kill bacteria? This work shows that yes, it is possible. To the best of our knowledge, work described in this chapter is the first example of a small molecule multifunctional material that can both detect and kill bacteria. A synthetic procedure is developed to attach proline to a fluorescent aminoacridone compound, Pro/Fluoro **6**. Upon

protonation, bacterial studies show a clear and distinct color change from blue to yellow under UV light in the presence of *Pseudomonas aeruginosa*, the first example of a prolyl aminopeptidase-specific indicator. A reaction is developed to react Pro/Fluoro **6** with NO to form the novel diazeniumdiolate Pro/Fluoro/NO **8**. NO release measurements show that Pro/Fluoro/NO **8** releases 853 μmol of NO/g at an initial concentration of 10 mM. *P. aeruginosa* is exposed to Pro/Fluoro/NO **8**, and bacteria cell viability is reduced by 65 ± 10% at a concentration of 10 mM Pro/Fluoro/NO **8** after 24 hours of exposure. After exposure, a color change from blue to yellow is observed under UV light in the presence of *P. aeruginosa*. These results demonstrate the dual function of Pro/Fluoro/NO **8** to detect and kill *P. aeruginosa*, creating a very versatile material.

In the future, the amount of NO release from Pro/Fluoro/NO **8** could be increased by increasing the amount of nitrosation, potentially improving antibacterial efficacy. This could allow a lower dose of the diazeniumdiolate to be applied with the same or better results. More controlled release of NO could be achieved with a protecting group on O^2 of the diazeniumdiolate.[32] Incorporation into a polymer could also help to control NO release. Pro/Fluoro/NO **8** could also be tested with other bacteria species that produce the prolyl aminopeptidase enzyme, such as *Clostridium difficile*, a gram-positive bacteria strain that causes severe gastrointestinal infections. In the long term, Pro/Fluoro/NO **8** could be incorporated into a polymer support,[53] which could be made into a bandage that would have antibacterial properties, as well as visualizing when a bacterial infection is present. While there are complications to overcome in the practical application of the synthesized compound, this is an exciting proof of concept of a dual function compound to detect and kill bacteria.

3.9 Nuclear Magnetic Resonance Spectra



6

$CDCl_3$, 400 MHz

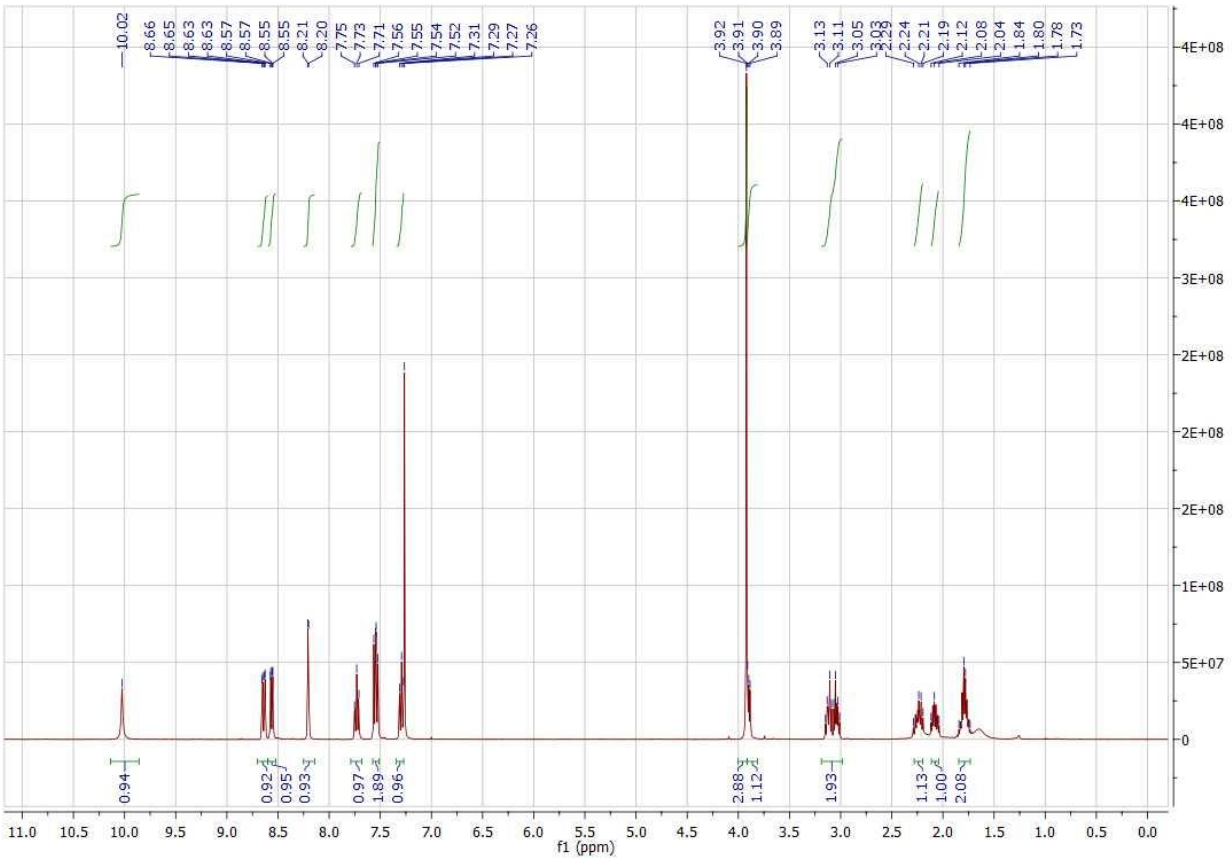
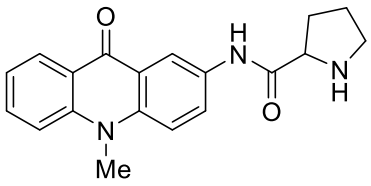


Fig. 3.17 ^1H NMR of **6** in $CDCl_3$



6

$CDCl_3$, 101 MHz

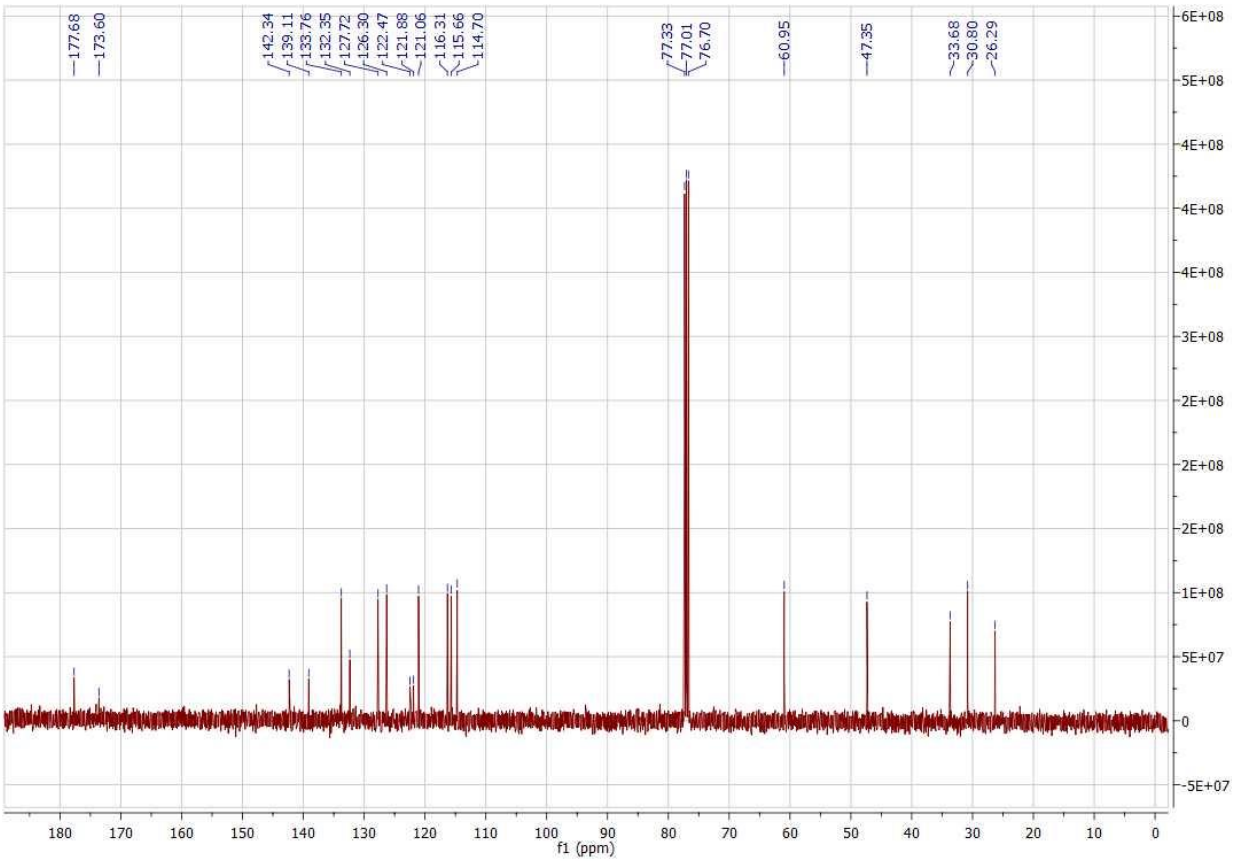
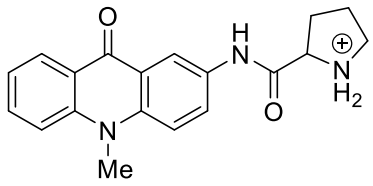


Fig. 3.18 ^{13}C NMR of **6** in $CDCl_3$



7

d_6-DMSO, 400 MHz

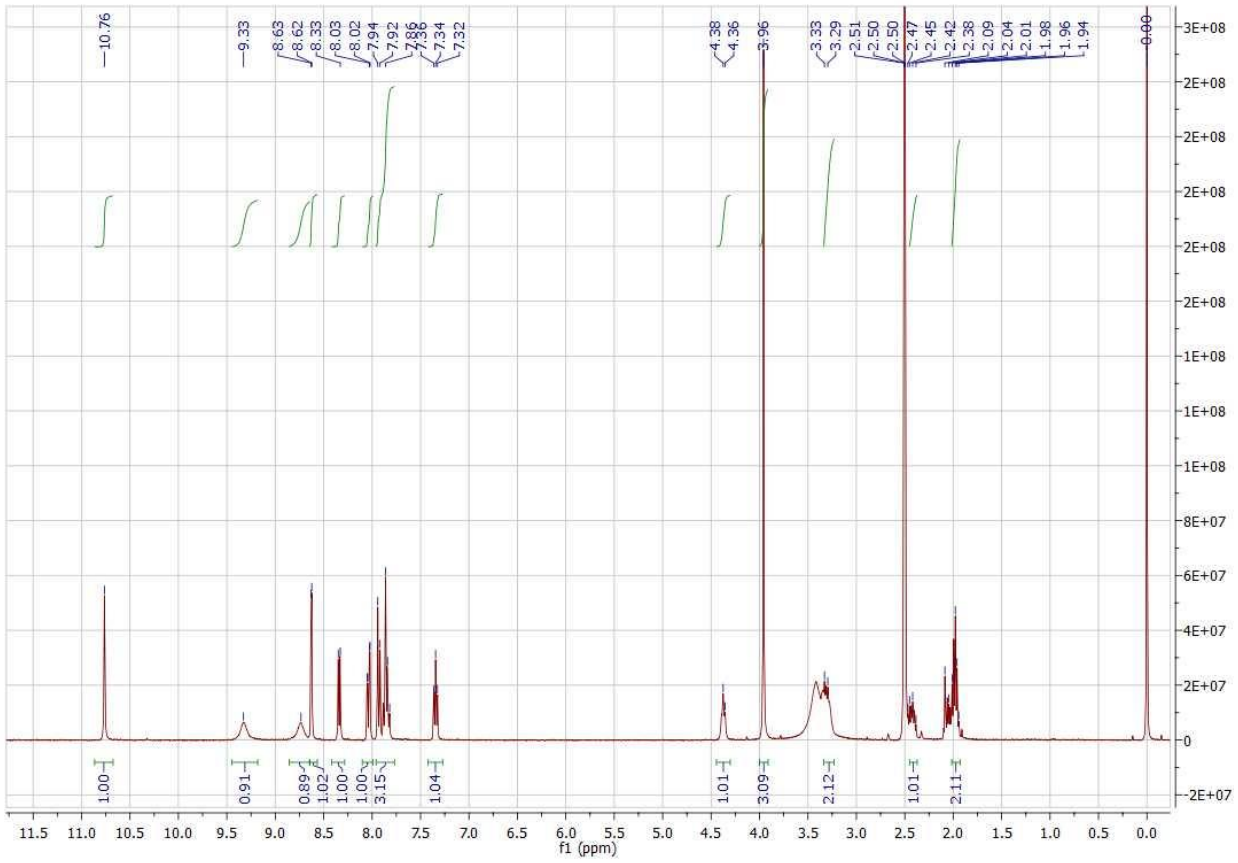
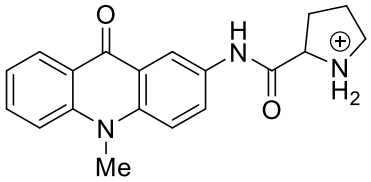


Fig. 3.19 ^{1}H NMR of **7** in d_6-DMSO



7

d_6-DMSO, 101 MHz

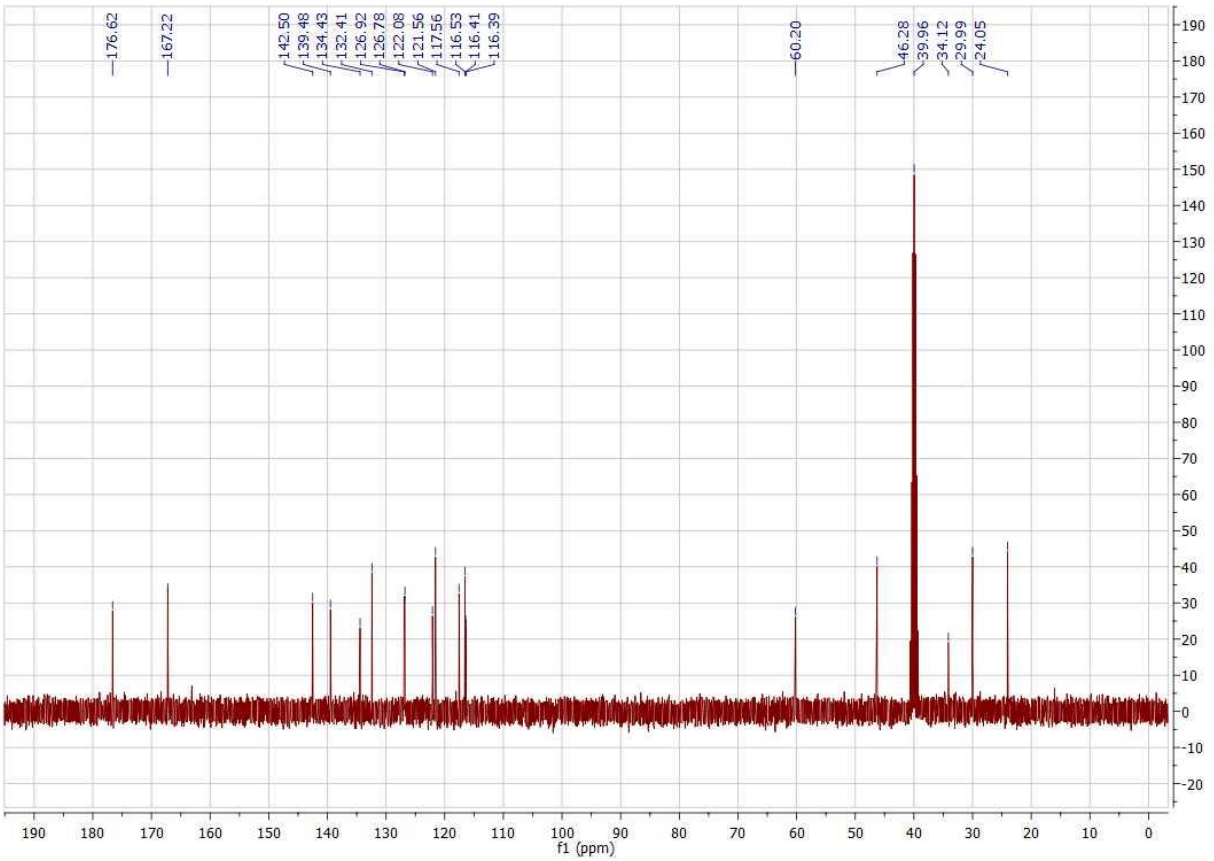
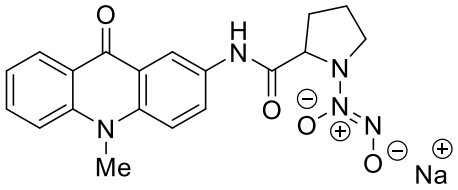


Fig. 3.20 ^{13}C NMR of **7** in d_6-DMSO



8

0.5 mM NaOD in D_2O, 400 MHz

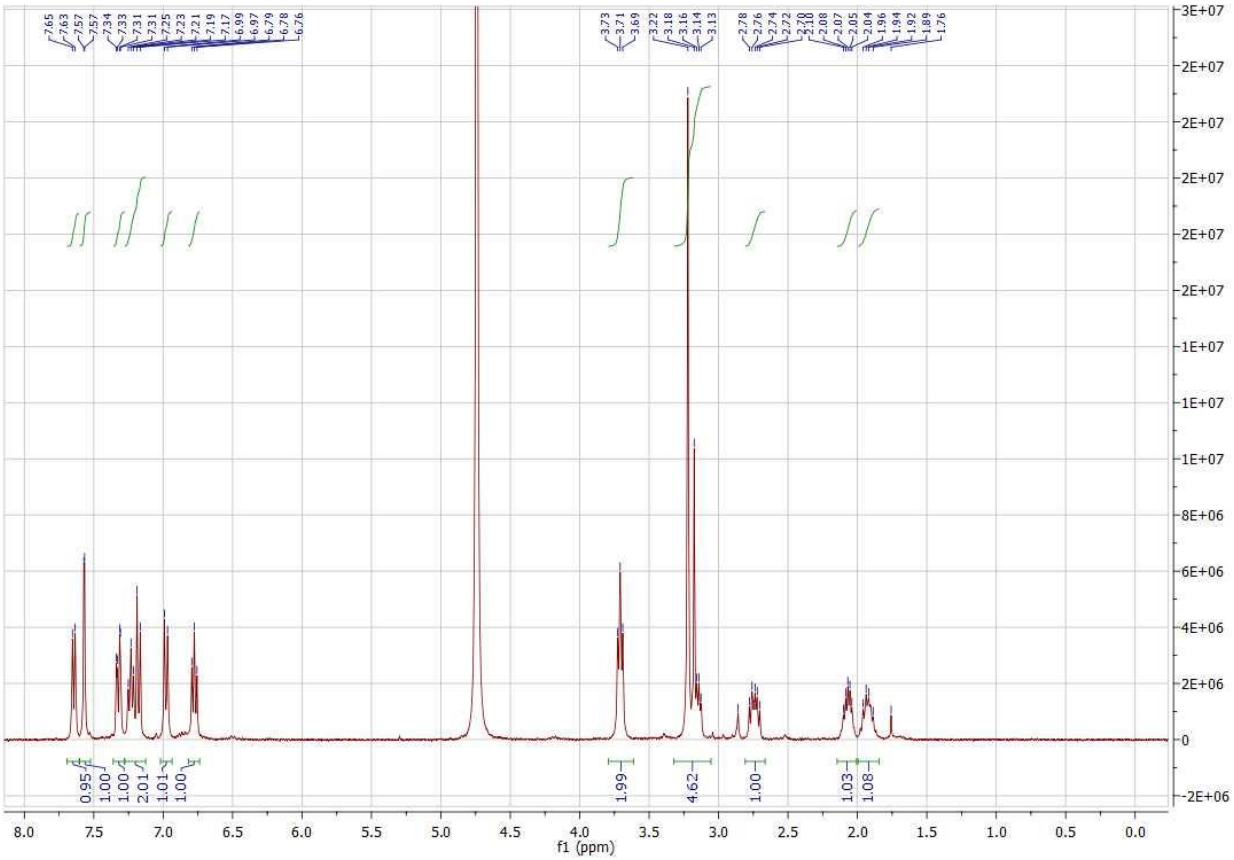
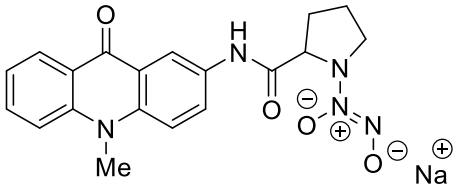


Fig. 3.21 ^{1}H NMR of **8** 0.5 mM NaOD in D_2O



8

0.5 mM NaOD in D_2O, 101 MHz

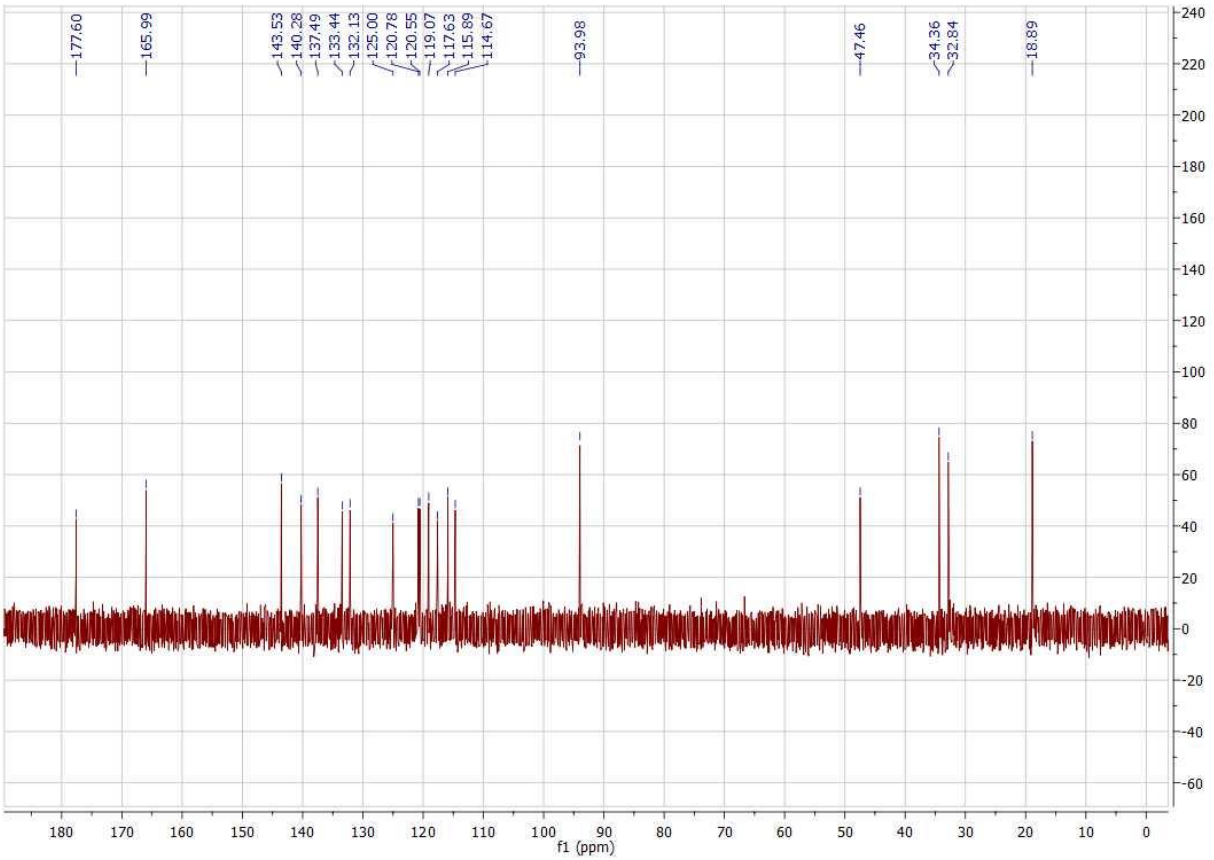


Fig. 3.22 ^{13}C NMR of **8** 0.5 mM NaOD in D_2O

3.10 References

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CHAPTER 4

NITROREDUCTASE-ACTIVATED PIPERAZINE DIAZENIUMDIOLATES

4.1 Background

This work was supported by grants from the Monfort Foundation and the National Institutes of Health (5R21EB016838-02). This project was originally published in *Bioorganic Chemistry* (Hibbard, H. A. J., Reynolds, M. M. Synthesis of Novel Nitroreductase Enzyme-Activated Nitric Oxide Prodrugs to Site-Specifically Kill Bacteria. *Bioorg. Chem.* **2019**, *93*, 103318.)

4.2 Introduction

The alarming trend of increasing antibiotic resistance in bacteria illustrates the dire need for new antibiotics. One major cause of the rise in antibiotic-resistant strains of bacteria is the overuse of antibiotics.[1] Antibiotic prodrugs that only release the active antibiotic when bacteria are present, such as enzyme-activated antibacterial compounds, can help overcome this problem.[2,3] In this work, we show that the bacterial enzyme nitroreductase activates the release of nitric oxide, a known antimicrobial agent, from a novel prodrug to kill bacteria.

Nitroreductases are a class of enzymes that reduce nitro groups to hydroxylamine or amine moieties.[4,5] These enzymes have been used in biocatalysis applications to reduce nitroaromatic compounds.[6–8] Nitroreductase enzymes have also been studied in prodrug applications to trigger the release of anti-cancer therapeutics at the specific location of a tumor in directed enzyme prodrug therapy (DEPT) to treat cancer.[9–14] Nitroreductases are found in bacteria and very few eukaryotes, but are not found in humans.[15] For this reason, nitroreductases could be used to catalyze the release of an antibiotic site-specifically in humans, concentrating

the active drug where an infection is located. The compound would only release the therapeutic in large amounts if an infection develops, preventing the overuse of antibiotics. This would help address the issue of bacteria developing antibiotic resistance, and potentially reduce or eliminate side effects from antibiotics. Antibacterial prodrugs could also prevent recurring, chronic infections by accumulating in dormant persister cells, and be metabolized by an enzyme inside the cell, releasing the active drug and killing the cell.[2] Nitroreductase enzymes have previously been used to reduce nitro-containing antibiotics, such as nitrofurazone and nitrofurantoin,[16] demonstrating the viability of this study. In this paper, we use the *Escherichia coli*-derived nitroreductase NfsB, an oxygen-insensitive enzyme, to reduce the nitro group of a prodrug to an amine, initiating the release of an antibacterial therapeutic, nitric oxide (NO).

Nitric oxide has been shown to have potent antibacterial effects. It kills bacteria via multiple mechanisms by reacting with oxygen and superoxide species in the body, forming reactive nitrogen and oxygen species, which can alkylate DNA and inhibit enzyme function in bacteria, among other detrimental effects.[17,18] Studies have shown that bacteria are unable to develop resistance to exogenously-applied NO, even after 21 days.[19] In addition, NO has a very short half-life, which could minimize side effects from the antibiotic.[20] For these reasons, NO is of interest to be used in new antibiotics.

In addition to its antibacterial activity, NO plays many roles in the body as a cell-signaling molecule, as part of the immune response, and as a vasodilator to lower blood pressure.[21–23] Because of the multitude of effects it can have on the body, it is critical to deliver NO site-specifically as a therapeutic to avoid major side effects. Enzyme-activated nitric oxide-releasing prodrugs have previously been synthesized, typically employing diazeniumdiolates as the NO donor.[24] A diazeniumdiolate is a type of nitric oxide-releasing functional group (Figure

4.1), releasing two equivalents of NO per one equivalent of diazeniumdiolate spontaneously in biological medium with a predictable half-life.[25] A few antimicrobial diazeniumdiolates have been published which release NO spontaneously, but these compounds could lead to serious side effects due to non-specific release of NO. By attaching a group to the O^2 position of a diazeniumdiolate (Figure 4.1), spontaneous NO release can be slowed or even prevented entirely.[24] Examples include protecting groups cleaved by cytochrome p450 enzymes, glycosidases, or esterases to activate the release of NO from O^2-protected diazeniumdiolates.[26–28] To the best of my knowledge, there are no examples of small molecule antibiotic diazeniumdiolate prodrugs. There are a limited number of related examples of antimicrobial diazeniumdiolate prodrugs, in which Collins, Allan, and coworkers use transpeptidases or β-lactamases to activate NO release for anti-biofilm applications.[29,30]

Precedence for nitroreductase-activated diazeniumdiolates is demonstrated by the Chakrapani group, where they used nitroaromatic-protected diazeniumdiolates for applications in cancer therapeutics.[31] Based on this precedence, I applied the idea of nitroaromatic-protected diazeniumdiolate to antibacterial applications. The hypothesis posed at the beginning of this work is: can a small molecule antibacterial prodrug be developed to be activated to release NO only in the presence of bacterial enzymes? In this work, I investigated a variety of diazeniumdiolates to protect with a nitroaromatic group. Eventually, I optimized the synthetic conditions with nitroaromatic-protected piperazine-based diazeniumdiolates. I demonstrated in this work that a nitroreductase enzyme derived from *Escherichia coli* catalyzes the release of

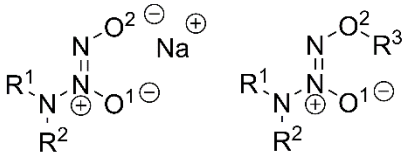


Fig. 4.1 General structure of unprotected (left) and protected (right) diazeniumdiolate functional group.

NO. In the absence of the enzyme, NO is not released, demonstrating that NO release is specific to the presence of the nitroreductase enzyme. Excitingly, when *E. coli* is exposed to my synthesized diazeniumdiolates, significant reductions in bacteria are observed. This is the first report of antibacterial nitroreductase-activated diazeniumdiolates, demonstrating the potential use of antibiotic prodrugs for site-specific delivery of therapeutics to treat bacterial infections.

4.3 Experimental Methods

4.3.1 Chemicals: Ethyl 1-piperazinecarboxylate (99%), 4-nitrobenzyl bromide (99%), 15-crown-5 (98%), nitroreductase from *Escherichia coli* (90%), and β-Nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt hydrate (NADPH, 97%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). 1-(tert-Butoxycarbonyl)piperazine (98%) and 1-Carbobenzoxypiperazine (95%) were purchased from TCI (Cambridge, MA, USA). Nitric oxide (99%) was purchased from Matheson Tri-gas (Montgomeryville, PA, USA). Sodium methoxide (30% in methanol) was purchased from Acros Organics (Geel, Belgium). Oxoid nutrient broth (OXCM0001B) and Oxoid nutrient agar (OXCM0003B) were purchased from Fisher Scientific (Hampton, NH, USA). *Escherichia coli* (ATCC 25922) was obtained from American Type Culture Collection (ATCC, USA). Argon (ultrahigh purity), nitrogen (ultrahigh purity), and oxygen were purchased from Airgas (Denver, CO, USA). Deionized water (18.2 MΩ·cm) was obtained from a Millipore Direct-Q water purification system (EMD Millipore, Billerica, MA, USA).

4.3.2 Abbreviations: The following abbreviations are used throughout: rt= room temperature, h= hour, min= minute, THF= tetrahydrofuran, 15-crown-5= 1,4,7,10,13-Pentaoxacyclopentadecane, NaOH= sodium hydroxide, DCM= dichloromethane, Na_2SO_4= sodium sulfate, EtOAc= ethyl

acetate, SiO_2= silica, PBS= phosphate-buffered saline, DMSO= dimethyl sulfoxide, Boc= tert-Butyloxycarbonyl, Cbz= carboxybenzyl.

4.3.3 Instruments: All nuclear magnetic resonance (NMR) spectra were obtained on Varian Inova 400 (400 MHz for ^1H; 101 MHz for ^{13}C) and/or Bruker US400 (400 MHz for ^1H; 101 MHz for ^{13}C) at room temperature unless noted otherwise. Chemicals shifts were reported in parts per million (δ scale) and referenced according the following standards: chloroform residual signal (δ 7.16) for ^1H signals; deuterated chloroform carbon resonances (middle peak is δ 77.1) for ^{13}C signals. Coupling constants were reported in Hertz (Hz) and multiplicities were reported as follows: singlet (s), doublet (d), doublet of doublets (dd), triplet (t), quartet (q), and multiplet (m). IR spectra were obtained on a Thermo Scientific Nicolet 6700 FT-IR spectrometer. Electrospray ionization mass spectrometry data were obtained on an Agilent 6224 TOF mass spectrometer equipped with a dual electrospray ion source operated in positive and negative mode. Melting points were obtained with an Electrothermal Mel-Temp apparatus. UV-Vis measurements were obtained with a Thermo Scientific Nicolet Evolution 300 spectrometer. Reactions were analyzed by thin layer chromatography (TLC) on aluminum sheets that were pre-coated with silica gel 60 F_{254}, and the reactions were purified by column chromatography using Alfa Aesar silica gel 60 (0.06-0.2 mm).

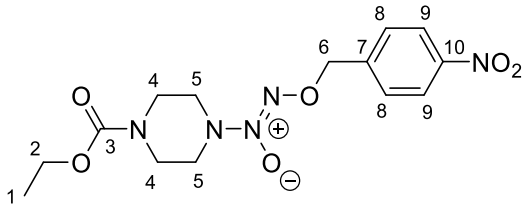
4.3.4 Experimental Procedures

Diazeniumdiolates were synthesized according to previously reported procedures.[32] In brief, the piperazine starting material (1 equiv.) was stirred to dissolve in anhydrous methanol (2.1 M) in a high pressure reaction vessel. Sodium methoxide (30% in methanol, 1 equiv.) was added. The reaction vessel was attached to a high-pressure stainless-steel nitric oxide reactor system, purged with high purity argon, then charged with nitric oxide gas (80 psi, 99%). After stirring under

pressure for 24 h, the white precipitate that formed was washed with methanol, then diethyl ether and dried under vacuum for 1 h. The products were stored at -20 ˚C until further use. Spectra matched the previously reported values.

4.3.4.1 General procedure for synthesis of O^2-(4-nitrobenzyl) diazeniumdiolates

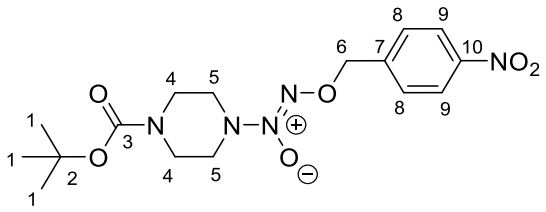
To a 0 ˚C solution of diazeniumdiolate (0.75 mmol, 1.5 eq.) in anhydrous THF (0.1 M), 15-crown-5 (1 mmol, 2 eq.) was added. After 30 minutes stirring at 0 ˚C, 4-nitrobenzyl bromide (0.5 mmol, 1 eq.) was added and the reaction was allowed to warm to rt. After stirring for 2-4 h, the reaction mixture was concentrated *in vacuo* to remove THF. The crude product was purified by flash column chromatography, affording the title compound.



4.3.4.2 O^2-(4-Nitrobenzyl) 1-(4-ethoxycarbonylpiperazin-1-yl)diazen-1-ium-1,2-diolate, ***3a.***

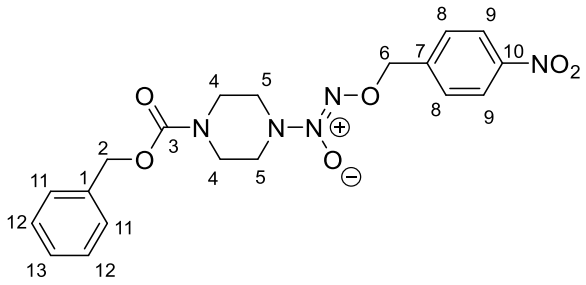
Following the general procedure, stirring for 4 h, prior to column chromatography purification, the crude mixture was dissolved in DCM (10 mL) and NaOH (1 M, 10 mL) was added. The aqueous layer was extracted with DCM (3 × 10 mL), then the combined organic layers were washed sequentially with deionized water (10 mL) and brine (15 mL), then dried over Na_2SO_4 and concentrated *in vacuo* to afford a yellow oil. After purification by column chromatography (SiO_2, 10%, 30% EtOAc in hexanes gradient), **3a** is collected as a pure pale yellow crystalline solid (128-154 mg, 79 ± 7%, n=4). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.15 (d, *J* = 8.5 Hz, 2H, H9), 7.46 (d, *J* = 9.5 Hz, 2H, H8), 5.24 (s, 2H, H6), 4.07 (q, *J* = 7.1 Hz, 2H, H2), 3.56 (t, *J* = 5.2 Hz, 4H, H4), 3.30 (t, *J* = 5.1 Hz, 4H, H5), 1.19 (t, *J* = 7.1 Hz, 3H, H1). ^{13}C NMR (101 MHz, $CDCl_3$) δ 155.04 (C3), 147.98 (C10), 142.81 (C7), 128.66 (C8), 123.87 (C9), 73.94 (C6), 61.87 (C2),

51.01 (C5), 42.33 (C4), 14.59 (C1). HRMS (+ESI) Found 354.1408 (Calcd. 354.1414 for $C_{14}H_{19}N_5O_6$; $[M+H]^+$). UV (DCM) λ_{max} (ε) 241 nm (30.4 $mM^{-1}cm^{-1}$); 263 nm (15.8 $mM^{-1}cm^{-1}$). m.p. 105.5-108 °C. ν_{max}/cm^{-1} 3110 w, 3081 w, 2876 br, 1683 s, 1602 m, 1519 s, 1423 m, 1343 s, 1221 s, 1121 s, 1031 s, 956 s, 848 s, 739 s.



4.3.4.3 O^2-(4-Nitrobenzyl) 1-(4-tert-butyoxycarbonylpiperazin-1-yl)diazen-1-ium-1,2-diolate, ***3b.***

Following the general procedure stirring for 2 h, after purification by column chromatography (SiO_2, 5%, 15%, 40% EtOAc in hexanes gradient), **3b** is collected as a pure pale yellow crystalline solid (46-123 mg, 42 ± 21%, n=3). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.24 (d, *J* = 8.7 Hz, 2H, H9), 7.55 (d, *J* = 8.7 Hz, 2H, H8), 5.31 (s, 2H, H6), 3.58 (t, *J* = 6 Hz, 4H, H4), 3.35 (t, *J* = 6 Hz, 4H, H5), 1.46 (s, 9H, H1). ^{13}C NMR (101 MHz, $CDCl_3$) δ 154.17 (C3), 147.98 (C10), 142.85 (C7), 128.65 (C8), 123.85 (C9), 80.57 (C2), 73.91 (C6), 51.07 (C5), 42.32 (C4), 28.31 (C1). HRMS (+ESI) Found 382.1721 (Calcd. 382.1727 for $C_{16}H_{23}N_5O_6$; $[M+H]^+$). UV (DCM) λ_{max} (ε) 240 nm (32.8 $mM^{-1}cm^{-1}$); 264 nm (12.6 $mM^{-1}cm^{-1}$). m.p. 132-135 °C. ν_{max}/cm^{-1} 2980 br, 2932 br, 1677 s, 1604 w, 1521 s, 1406 s, 1343 s, 1224 m, 1124 s, 1033 s, 845 s, 738 s.



4.3.4.4 O^2-(4-Nitrobenzyl) 1-(4-benzyloxycarbonylpiperazin-1-yl)diazen-1-ium-1,2-diolate, ***3c.***

Following the general procedure stirring for 2 h, after purification by column chromatography

(SiO_2, 15%, 40% EtOAc in hexanes gradient), **3c** is collected as a pure pale yellow crystalline solid (159-184 mg, 81 ± 7%, n=3). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.16 (d, *J* = 8.7 Hz, 2H, H9), 7.47 (d, *J* = 8.8 Hz, 2H, H8), 7.28 (m, 5H, H11, H12, H13), 5.24 (s, 2H, H6), 5.06 (s, 2H, H2), 3.60 (m, 4H, H4), 3.30 (m, 4H, H5). ^{13}C NMR (101 MHz, $CDCl_3$) δ 154.80 (C3), 147.98 (C10), 142.80 (C7), 136.17 (C1), 128.67 (C8), 128.58 (C12), 128.29 (C13), 128.07 (C11), 123.86 (C9), 73.95 (C6), 67.62 (C2), 50.97 (C5), 42.45 (C4). HRMS (+ESI) Found 416.1564 (Calcd. 416.1570 for $C_{19}H_{21}N_5O_6$; $[M+H]^+$). UV (DCM) λ_{max} (ε) 240 nm (32.8 $mM^{-1}cm^{-1}$); 264 nm (13.0 $mM^{-1}cm^{-1}$). m.p. 74-77 °C. ν_{max}/cm^{-1} 2935 w, 2850 br, 1704 s, 1605 w, 1513 s, 1439 s, 1348 s, 1215 s, 1126 s, 1013 s, 848 m, 744 s, 698 s.

4.3.5 Nitric Oxide Release Measurements: Nitric oxide generation was recorded in real time using a chemiluminescence-based GE Nitric Oxide Analyzer (NOA). Before each use, the NOA was calibrated using a 43.83 ppm NO calibration gas cylinder (Airgas). For the experiments to determine NO release at different concentrations, solutions of **3a-c** in DMSO were made (1, 10 and 100 mM). PBS (1480 µL,10 mM in Millipore water), NADPH (400 µL, 10 mM in PBS), and the corresponding **3a-c** solution (20 µL, 1, 10, or 100 mM in DMSO) to a final concentration of 0.01, 0.1, and 1 mM are added to NOA cell in a 37 °C water bath. The solution is purged continuously with ultrapure nitrogen gas for 5-10 min to collect a baseline release. Nitroreductase enzyme (50 µL, 1 mg/mL in PBS) is then added through the side injection port of the NOA vessel. The solution is purged continuously with ultrapure nitrogen gas with bubbling rate of 55 mL/min directly into the solution and flow gas introduced into the remaining headspace for the duration of the experiment. NO release is recorded in 15 s intervals as parts per billion. NO release data is presented as the average of at least 3 trials ± standard deviation.

4.3.6 NADPH Consumption Studies: A baseline spectrum is collected of a solution of 100 µM **3a** (10 µL, 10 mM solution in DMSO) in PBS (970 µL, 10 mM in Millipore water). NADPH (10 µL, 20 mM in PBS) is added to a concentration of 200 µM, and a time zero spectrum from 200-400 nm was collected, as well as a spectrum after 5 minutes as a control to ensure that NADPH absorbance does not decrease over time without enzyme present. Nitroreductase enzyme (10 µL, 1 mg/mL in PBS) is added and data is collected in cycles in intervals (approximately every 5 seconds for 60 cycles) from 335-345 nm at rt. Absorbances are collected at 340 nm and plotted over time. The data is presented as the average of 3 trials ± standard deviation.

4.3.7 Bacteria Studies

4.3.7.1 Escherichia coli *Bacteria Culture:* Initial stock culture of *Escherichia coli* was made by reconstituting lyophilized bacteria in warm nutrient broth media (NBM, 13 g nutrient broth/1 L Millipore water) and grown overnight at 37 °C and 150 rpm until an optical density at 600 nm (OD_{600nm}) ≈1.0 was reached. This bacterial solution was combined with glycerol (30% v/v) in a 1:1 fashion to obtain a final glycerol concentration of 15% (v/v). These solutions were stored at −80 °C until use. Prior to each bacterial experiment, a frozen culture was thawed at rt and then centrifuged at 4700 rpm for 10 min. The supernatant was discarded, and the pellet was resuspended in NBM. This was transferred to additional NBM and allowed to grow overnight with shaking at 120 rpm until the OD_{600nm} ≈1.0. The culture was diluted to an OD_{600nm} ≈0.3-0.35 using warmed NBM prior to beginning experiments.

4.3.7.2 Bacterial Viability The previously made **3a-c** solutions in DMSO (10 µL; 1, 10 or 100 mM) were added to *E. coli* culture (10^7 CFU/mL) in NBM (990 µL, OD_{600nm} ≈0.3-0.35) in 1.5 mL Eppendorf microcentrifuge tubes to final concentrations of 0.01, 0.1, and 1 mM. The positive control was *E. coli* culture in NBM (1 mL, OD_{600nm} ≈0.3-0.35). The solutions were placed in a

static 37 ˚C incubator for 24 h. Agar plates were made by adding 15 mL of sterilized nutrient agar [powder base (14 g) in Millipore water (500 mL)] to sterile Petri dishes and allowed to set for at least 2 h. After 24 h exposure, the *E. coli* solutions underwent serial tenfold dilution with sterile NBM to reach 10^4 or 10^5 dilution factors, and the agar plates were inoculated with 20 µL of the diluted *E. coli* solutions using a sterile plastic or glass spreader. The agar plates were placed in a static 37 ˚C incubator overnight and colony-forming units (CFUs) were counted after 24 h (n = 9). The number of CFUs were calculated using the equation:

$$\frac{CFU}{mL} = \frac{(\#\ CFU\ counted)(Dilution\ factor)}{Volume\ plated\ (mL)}$$

The percent reduction in the number of viable bacteria was calculated as follows:

$$\%\ reduction = \frac{CFU\ after\ exposure\ to\ therapeutic/mL}{CFU\ positive\ control/mL}$$

4.3.7.3 Statistical Analysis All biological experiments were performed using at least nine samples. Viability assays are reported as the mean and 95% confidence interval. All data were evaluated for potential outliers using the Grubbs Test. The statistical differences in data were evaluated using the Student's t test ($p < 0.05$) at the 95% confidence level.

4.4 Synthesis of Nitroaromatic-Protected Diazeniumdiolates

I designed the antibacterial diazeniumdiolate prodrugs with specific function and requirements in mind, beginning with known diazeniumdiolates that would be stable under the nitroaromatic protection conditions. These molecules would display nitroreductase-activated NO release. I was first interested in looking at a variety of diazeniumdiolates with different half-lives, as this could potentially affect NO release and the therapeutic effects of the compounds. Diazeniumdiolate half-lives can range from seconds to hours, so I investigated diazeniumdiolates representing this range of half-lives, from PROLI/NO and PYRRO/NO with half-lives of a few

seconds, to piperazine-based diazeniumdiolates with half-lives of a few minutes, to DPTA/NO with a half-life of 30 minutes to a few hours (Figure 4.2).[32,33] Unfortunately, I found that the protection conditions led to formation of large amounts of side products for PROLI/NO, PYRRO/NO, and DPTA/NO. A basic workup step to try to remove starting material and other side products did not help with purification, and attempting different column chromatography conditions also did not afford the pure product. Ultimately, I decided to move ahead with the piperazine-based diazeniumdiolates, as the purification of these compounds was more straight forward.

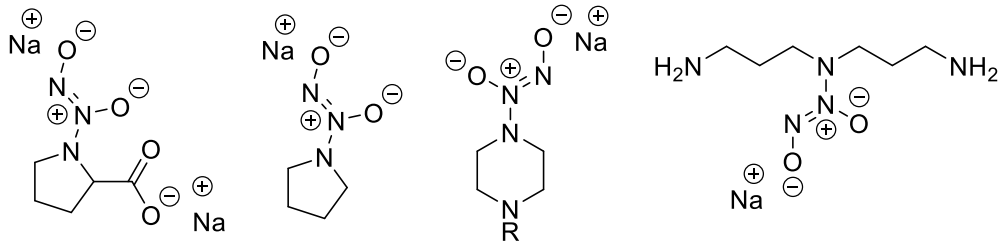


Fig. 4.2 Diazeniumdiolates with varied half-lives and attempted to protect with nitroaromatic group

Piperazine diazeniumdiolates are known to have half-lives ranging from two to five minutes, a mid-range half-life for diazeniumdiolates, and this range was deemed to be ideal for initial testing for the intended antibacterial applications.[20,32] Some piperazines have antibacterial activity, depending on substituents, which could further enhance the antibacterial activity of our compounds.[34,35] Piperazines are similar to the piperidine derivative previously published, providing precedence that protection conditions would not decompose the diazeniumdiolate.[31]

To synthesize these novel nitroaromatic-protected diazeniumdiolates, several piperazine diazeniumdiolates were synthesized. Based on a known synthetic procedure,[32] the piperazine

starting material was reacted under high pressure NO gas in the presence of sodium methoxide, forming **2a-c** (Figure 4.3).

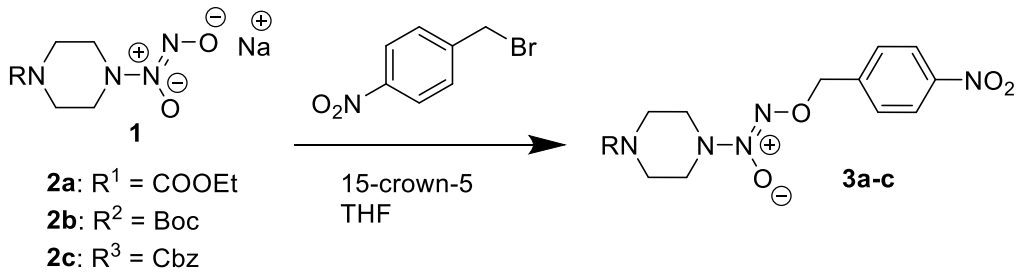


Fig. 4.3 Reaction scheme showing protection of various piperazine diazeniumdiolates with a nitroaromatic group.

After the successful synthesis of the piperazine diazeniumdiolates, I developed a procedure to O^2-protect the diazeniumdiolates with a nitroaromatic group, based on a previous literature report.[31] Figure 4.3 shows these protecting conditions, in which the diazeniumdiolate was mixed with 15-crown-5 to trap the sodium counterion, then 4-nitrobenzyl bromide was added to form the nitroaromatic-protected diazeniumdiolates **3a-c** in an S_N2-style reaction. I developed a basic work-up procedure to purify the ethyl carboxylate derivative **3a**, followed by column chromatography, significantly improving the yield of **3a** to 79 ± 7% (mean ± standard deviation, $n \geq 3$). Boc- (**3b**, 42 ± 21%) and Cbz- (**3c**, 81 ± 7%) protected piperazine derivatives were also synthesized to evaluate if the group on the piperazine leads to significant differences in the functionality of the compound.

The different piperazine protecting groups also allow for varied deprotecting conditions if the piperazine functional handle is desired. The nitroaromatic protecting group on the diazeniumdiolate should improve the stability to various deprotection conditions of the piperazine. With the piperazine functional handle, the compound could be incorporated into a

polymer, such as PVC or polyurethane, and used to make antibacterial medical devices or coatings.[36–38]

4.5 Nitric Oxide Release Properties of Nitroreductase-Activated Diazeniumdiolates

Once the nitroaromatic-protected diazeniumdiolates were synthesized, I evaluated them for their ability to release NO in the presence of a nitroreductase (NTR) enzyme using a nitric oxide analyzer (NOA). An NOA uses chemiluminescence to directly quantify the amount of NO released by oxidizing NO with ozone, forming nitrogen dioxide in an excited state. Upon relaxation, excited state NO_2 emits a photon, which is measured and related to the moles of NO released via a previously determined calibration constant. This instrument provides direct measurement of NO release as a function of time.

For the NO release quantification experiments, the diazeniumdiolate compounds were added to a solution of PBS and warmed to 37 °C to mimic physiological conditions and future bacterial viability studies. NADPH was added to the solution, as it necessary as a reducing agent for the NTR enzyme to function. A baseline release was collected to determine if the protected diazeniumdiolates release NO spontaneously under these conditions. The NTR enzyme was then added to the solution to induce NO release, which was quantified until the baseline returned to 0 ppb. The NTR enzyme used in these experiments was the commercially available nitroreductase NfsB, isolated from *Escherichia coli,* a well-studied enzyme that has previously been shown to reduce various nitroaromatic compounds.[16] The NO release experiments were repeated three times for each compound at each concentration to demonstrate reproducible results.

The diazeniumdiolate compounds **3a-c** do not release NO when the NTR enzyme is not present (Figure 4.4). When the diazeniumdiolate compounds **3a-c** are in solution, the baseline release of NO is below the limit of detection, indicating that there is no observable spontaneous

release of NO. Excitingly, upon injection of a solution of the NTR enzyme in PBS, there was an immediate response shown by a steep release of NO (Figure 4.4). NO release quickly reaches a maximum, then more slowly releases NO until all of the diazeniumdiolate groups decompose. One reported hypothesis for how NO release occurs in the presence of nitroreductase is that the enzyme reduces the nitro group first to a hydroxylamine, then to a primary amine.[31] The amine then tautomerizes to the imine, resulting in the loss of the group from the diazeniumdiolate. The amine component of the diazeniumdiolate is then protonated, causing two molecules of NO to be released from the compound, which displays first order kinetics.[39] Alternatively, the NTR enzyme could reduce the nitroaromatic group to the hydroxylamine, which then tautomerizes to an oxime species, then the protecting group is released from the diazeniumdiolate. The amine of the diazeniumdiolate is protonated, causing the release of NO. Either of these mechanisms are plausible, but as nitroreductase enzymes reduce nitro groups to amines via a hydroxylamine

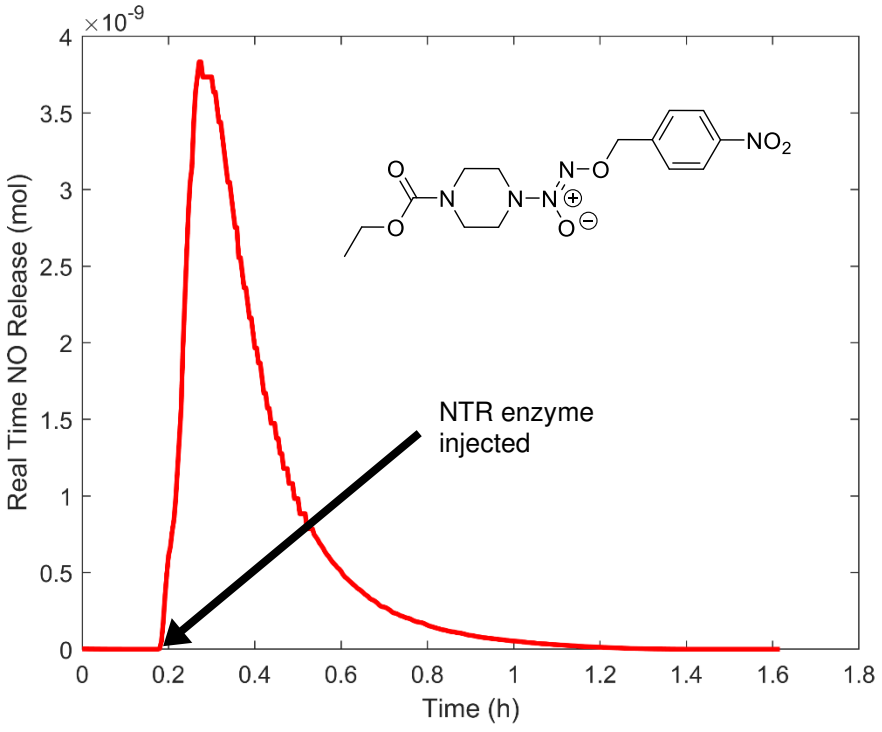


Fig. 4.4 A representative NO release plot of COOEt **3a** showing that NO release is dependent upon the injection of *E. coli*-derived NfsB nitroreductase (NTR) enzyme (0.1 mM in PBS).

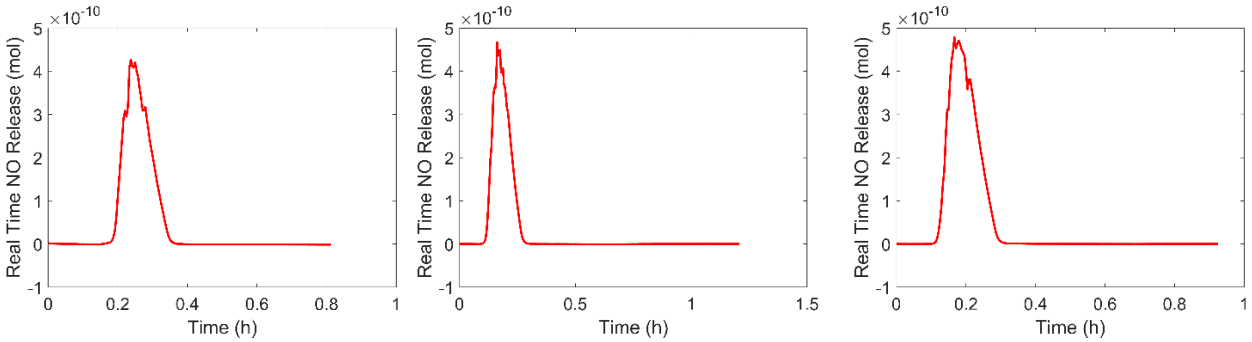


Fig. 4.5 NO release plots for 3 trials of COOEt **3a** (0.01 mM in PBS).

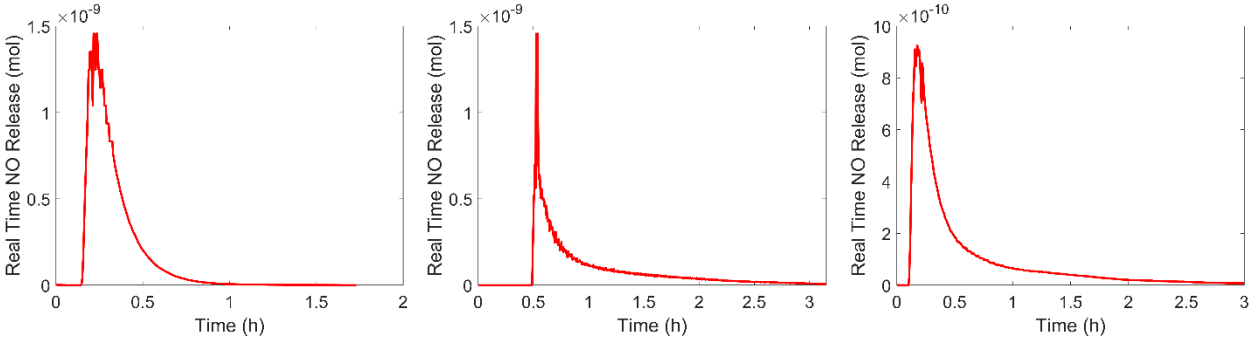


Fig. 4.6 NO release plots for 3 trials of COOEt **3a** (0.1 mM in PBS).

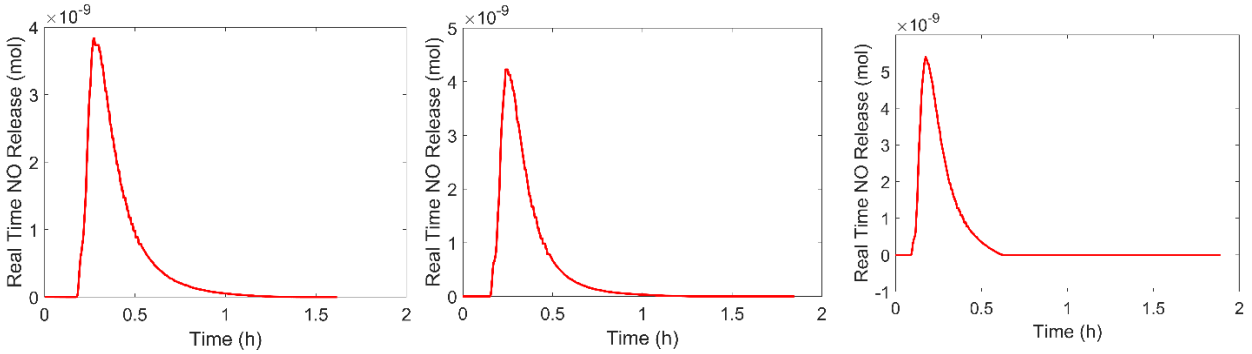


Fig. 4.7 NO release plots for 3 trials of COOEt **3a** (1 mM in PBS).

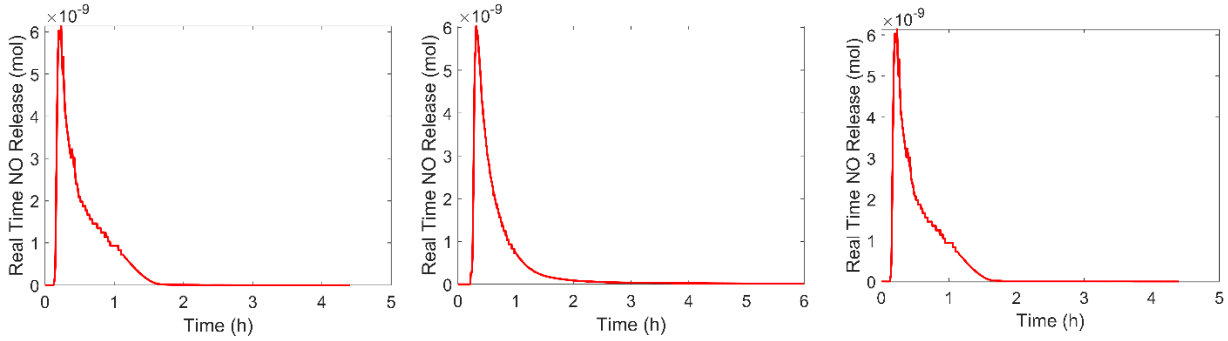


Fig. 4.8 NO release plots for 3 trials of Boc **3b** (0.1 mM in PBS).

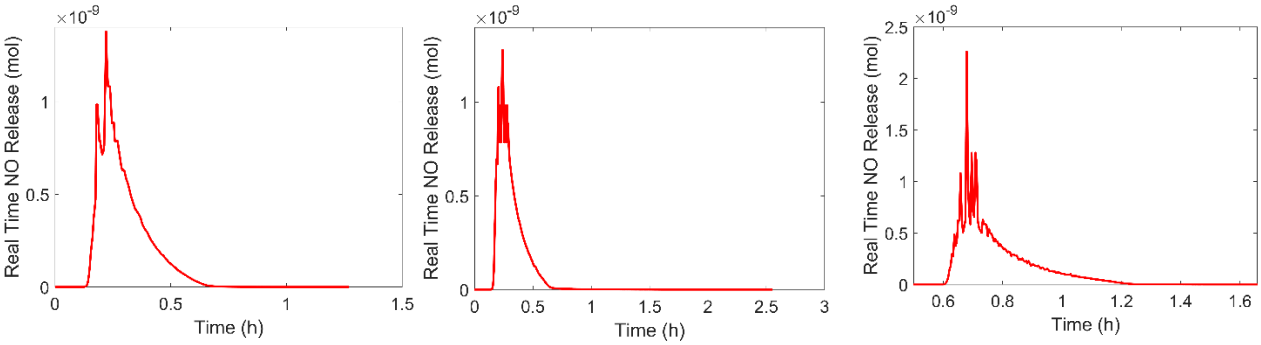


Fig. 4.9 NO release plots for 3 trials of Cbz **3c** (0.1 mM in PBS).

intermediate, the latter mechanism is potentially more likely. The NO release data shown in Figure 4.4 provides strong support that the prodrug is activated by the NTR enzyme. Experiments were performed with at least 3 trials at varying concentrations and the three different piperazine substituents (Figures 4.5-4.9).

To provide further evidence that the prodrug is enzyme activated, the consumption of NADPH by the nitroreductase enzyme is measured, as NADPH is a necessary cofactor for enzyme function. In this experiment, UV-vis absorbance is measured at 340 nm, where NADPH is known to have a peak, but disappears as it is converted to $NADP^+$ by the enzyme.[40] By

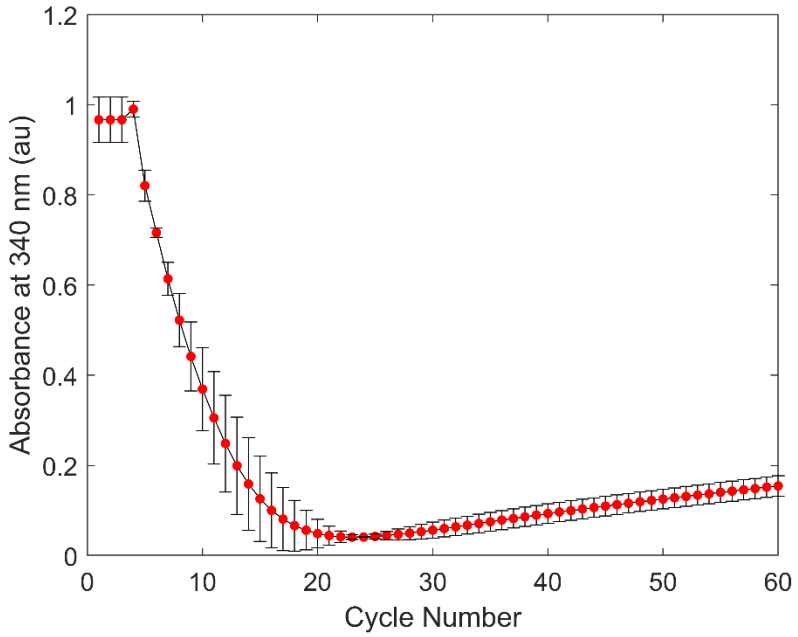


Fig. 4.10 Absorbance at 340 nm of NADPH and COOEt **3a** in the presence of NTR enzyme showing decreasing NADPH concentration over time as it is consumed by NTR enzyme.

tracking the absorbance at 340 nm, it is assumed that NADPH is being consumed by the enzyme, indicating that the enzyme is activated. The NTR enzyme is added to a solution of **3a** and NADPH in PBS, then absorbance is measured over time. The data (Figure 4.10) shows that NADPH concentration decreases in the presence of the substrate **3a** after the NTR enzyme is added, as expected based on literature reports.[41] Previous literature data shows that NADPH should be consumed over time (absorbance at 340 nm decreases) as enzymes consume the cofactor as they function, which is exactly what I observe in this experiment. The structure of the cofactor NADPH is changed in the presence of the NTR enzyme, showing that the enzyme is converting the structure of the cofactor in solution. This experiment demonstrates that the enzyme is activated during the experimental conditions and corroborates the results observed in the NO release experiments.

Total NO release is measured for **3a** at concentrations of 0.01, 0.1, and 1 mM (Table 4.1, entries 1-3), and for **3b-c** at a concentration of 0.1 mM (Table 4.1, entries 4-5). Table 4.1 shows that the total amount of NO release does not match the theoretical yield, but several literature reports indicate that diazeniumdiolates do not always release 100% of the theoretical NO available, based on their structure or due to decomposition.[42–44] Reports have demonstrated that diazeniumdiolates can decompose in solutions of low pH,[43] or that photochemically activated

Table 4.1 NO release data showing the normalized amount and the percent of total NO released at different concentrations for **3a-c**

Entry	R group	Conc. (mM)	Total moles of NO released[a]	% of theoretical NO[a]	Highest instantaneous NO release (μM)
1	COOEt (**3a**)	0.01	$9.7 \pm 1.2 \times 10^{-9}$	24 ± 3	0.2
2	COOEt (**3a**)	0.1	$2.1 \pm 0.1 \times 10^{-7}$	52 ± 3	2.7
3	COOEt (**3a**)	1	$5.3 \pm 0.5 \times 10^{-7}$	13 ± 1	4.8
4	Boc (**3b**)	0.1	$4.4 \pm 0.4 \times 10^{-8}$	11 ± 1	1.1
5	Cbz (**3c**)	0.1	$6.7 \pm 0.6 \times 10^{-8}$	17 ± 2	0.7

[a]Data is presented as mean ± standard deviation, n=3

diazeniumdiolates can decompose to the nitrosamine.[42,45] Our hypothesis for the observed less than 100% recovery of NO is based on the aqueous experimental conditions. Based on our observations, the synthesized compounds **3a-c** have limited solubility in aqueous solutions at higher concentrations. The NTR enzyme is soluble in water and is unable to access the prodrugs **3a-c** when they are not dissolved in solution, resulting in lower NO recovery. These solubility observations are supported in Table 4.1, as the COOEt derivative **3a** releases the highest percentage of NO (entry 2) and is the least hydrophobic protecting group. At the highest concentration of the COOEt derivative **3a** (entry 3), less of the theoretical NO is released, as less of it dissolves in the aqueous solution. The Boc- **3b** and Cbz-protected derivatives **3c** are even more hydrophobic, and less of the theoretical NO is released at the same concentration as the ethyl carboxylate derivative **3a** (entries 2, 4-5), further demonstrating that solubility plays a role in NO release.

4.6 Antibacterial Activity of Piperazine Diazeniumdiolates

To investigate the antibacterial activity of the synthesized prodrugs, *E. coli* was chosen as the initial species of interest. *E. coli* is well-known to contain a nitroreductase enzyme, which is commercially available and was used for the NO release experiments (section 3.2). *E. coli* is a very common infection-forming species and worryingly, is growing increasingly resistant to common antibiotics, indicating a pressing need for new antibacterial agents to treat *E. coli* infections.[46,47] To determine the antibacterial activity of the synthesized nitroreductase-activated diazeniumdiolates **3a-c**, *E. coli* was exposed to varying concentrations of the compounds. The positive control in these experiments was *E. coli* with no synthesized compound present. The initial hypothesis was that *E. coli* would metabolize the synthesized compounds *via* the nitroreductase enzyme it produces, inducing NO release and killing the bacteria.

The results of the cell viability experiments with *E. coli* are shown in Figure 4.11. Figure 4.11a shows a decrease in viability of *E. coli* cells when exposed to higher concentrations of the COOEt derivative **3a**, indicating that the compound effectively kills *E. coli.* There is no statistically significant difference between the positive control and the COOEt derivative at a 0.01 mM concentration (Figure 4.11a). Excitingly, however, there is a 56% reduction in the number of viable bacteria at concentrations of 0.1 mM **3a**, from $4.6 \pm 0.7 \times 10^8$ CFU/mL to $2.0 \pm 0.7 \times 10^8$ CFU/mL. At a dose of 1 mM **3a**, a 94% reduction in bacterial viability was observed, from $4.6 \pm 0.7 \times 10^8$ CFU/mL to $3.1 \pm 0.6 \times 10^7$ CFU/mL. Likely, the correlation between improved antibacterial activity and **3a** concentration is due to the observed higher amount of NO release (Table 4.1, entries 1-3). This observation is supported by Wink and Mitchell, as well as Schairer and co-workers, who show that concentrations of NO in the low micromolar range (>1 μM) have antibacterial effects.[17,48] At the time of the highest peak of NO release, instantaneous concentrations of NO reach micromolar levels for COOEt **3a**, 2.7 μM (0.1 mM, Table 4.1, entry 2) and 4.8 μM (1 mM, Table 4.1, entry 3), which could explain the significant antibacterial

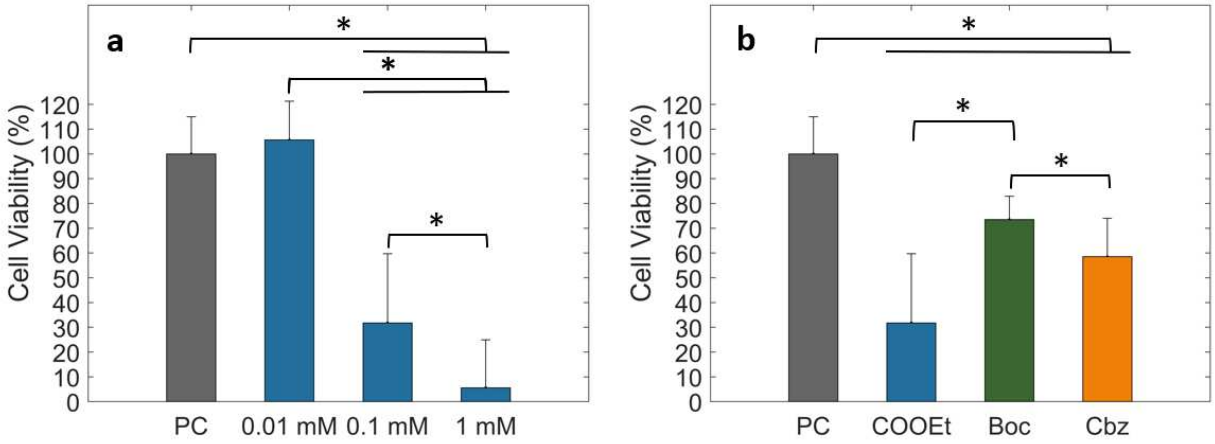


Fig. 4.11 a) Percent viability of *E. coli* after exposure to **3a** at concentrations of 0.01 mM, 0.1 mM, and 1 mM. b) Percent viability of *E. coli* after exposure to **3a, 3b,** or **3c** at a concentration of 0.1 mM. Average and 95% confidence interval displayed, $n \geq 9$. Statistically significant differences between cellular viabilities are indicated (*) as determined by a student's t test. PC = positive control

activity at these concentrations. Figure 4.11b shows that the Boc and Cbz-protected derivatives, **3b** and **3c**, also provoke a reduction in the amount of viable *E. coli*. While this result is exciting, the amount of reduction does not match the antibacterial activity of the COOEt derivative **3a**. Instead at a concentration of 0.1 mM, **3b** induces a 26% decrease in the number of viable bacteria, from $3.7 \pm 0.8 \times 10^8$ CFU/mL to $2.8 \pm 0.3 \times 10^8$ CFU/mL. The Cbz derivative **3c** induces a more significant decrease in the amount of viable *E. coli* by 41%, to $2.2 \pm 0.3 \times 10^8$ CFU/mL. At the same concentration (0.1 mM), the COOEt derivative **3a** causes a higher reduction in the number of bacteria (56%). These experimental results are consistent with Wink and Mitchell's report that greater than micromolar concentrations of NO have antibacterial effects;[48] the Boc-protected derivative **3b** peaks at an instantaneous concentration of 1.1 μM of NO, and the Cbz derivative **3c** peaks at an instantaneous concentration 0.7 μM, which is close to the antibacterial range of ≥1 μM (Table 4.1, entries 4-5). Because these values are less than the instantaneous release concentration (2.7 μM) for 0.1 mM COOEt **3a**, however, I observe a lower reduction in the number of bacteria. In contrast to the experiments performed with **3a**, preliminary experiments exposing *E. coli* to increased concentrations of the Boc **3b** and Cbz derivatives **3c** does not lead to a further reduction in the number of bacteria. This could be due to the solubility issues observed the NO release experiments discussed in section 3.2. Due to the more hydrophobic nature of the Boc and Cbz protecting groups compared to the COOEt group, **3b** and **3c** do not dissolve as well in aqueous solutions at higher concentrations, and therefore do not kill more bacteria at higher concentrations.

As piperazines have been shown to have antibacterial activity in some cases, it is important to determine the extent to which the observed antibacterial effects are due to NO release. To support the claim that NO is responsible for the observed antibacterial effects, *E. coli*

are exposed to the non-NO releasing structural parts of the compound in a control experiment. These structures are shown in Figure 4.12, 4-nitrotoluene and the corresponding piperazine derivative to the diazeniumdiolate (**1a-c**). For these control experiments, *E. coli* was exposed to both 4-nitrotoluene and the corresponding piperazine, either COOEt **1a**, Boc **1b**, or Cbz piperazine **1c** at the relevant concentrations (0.01, 0.1, or 1 mM, Figure 4.13). Based on these experiments, the COOEt piperazine **1a** and 4-nitrotoluene combination shows no significant reduction compared to the positive control at concentrations of 0.01, 0.1 and 1 mM. At 0.01 mM COOEt **1a** and 4-nitrotoluene, there is an increase in bacterial growth, 120% of the positive control. At a concentration of 0.1 mM, the Boc **1b** and Cbz **1c** piperazine and 4-nitrotoluene combination does not cause a significant reduction in *E. coli*. This experiment also showed a small increase in bacterial viability, to 124% (**3b**) and 126% (**3c**). These control experiments demonstrate that the antibacterial activity of compounds **3a-c** is due to the NO- releasing diazeniumdiolate functional group in the compound, not due to the possible antibacterial activity of the parent structure.

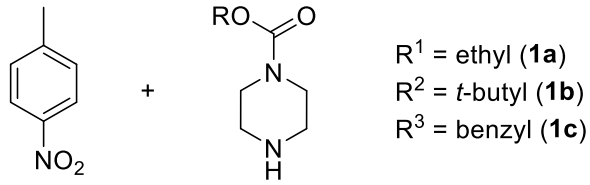


Fig. 4.12 Control compounds exposed to bacteria, 4-nitrotoluene on the left and piperazine starting materials **1a-c** on the right.

After testing the antibacterial activity of our compounds against *E. coli*, I was interested in exploring possible antibacterial activity with other nitroreductase-producing bacteria species. *Pseudomonas aeruginosa* contains a nitroreductase enzyme of the same nitroreductase subgroup as *E. coli* NfsB, indicating that similar antibacterial activity would be observed with *P.*

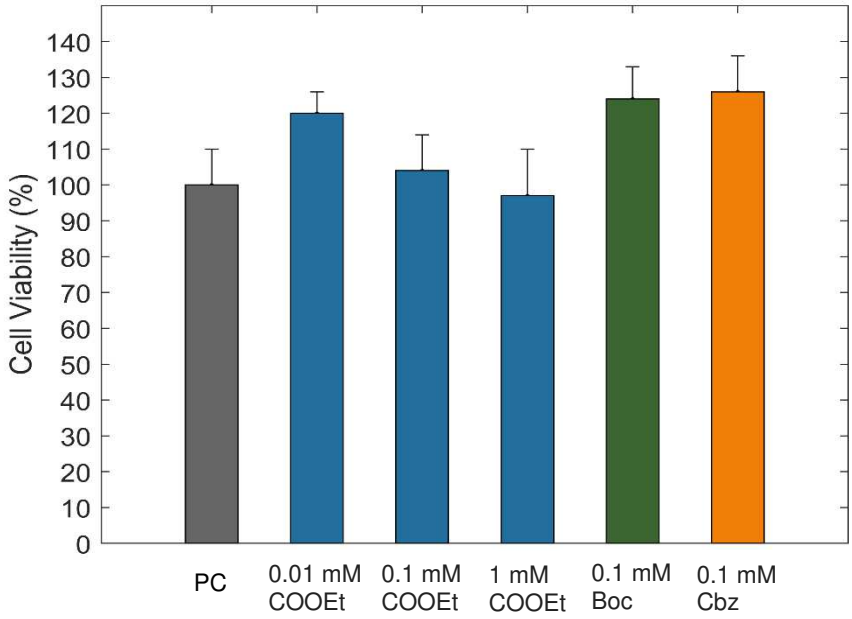


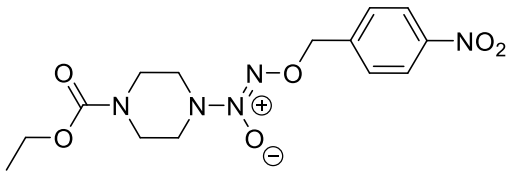
Fig. 4.13 Control experiments with *E. coli* exposed to COOEt **3a** at concentrations of 0.01, 0.1, and 1 mM and Boc **3b** and Cbz **3c** at a concentration of 0.1 mM. Data is reported as mean and standard deviation bars with $n \geq 9$

aeruginosa upon exposure to our compounds.[49] *P. aeruginosa* is a deadly bacterial species, growing increasingly resistant to most common antibiotics, illustrating a critical need to develop new antibacterial agents to kill this particular bacterial strain.[50] Using the previously stated plating and CFU counting method, during preliminary testing we observed a decrease in the number of viable bacteria after exposure to **3a**. However, the same controls previously discussed in which the bacteria are exposed to the compounds in Figure 4.13 showed the same or greater reduction in the number of viable bacteria. Unfortunately, I am not able to conclude that NO release from **3a-3c** induces the same antibacterial activity against *P. aeruginosa* as with *E. coli*. This could be due to possible lower catalytic activity of the *P. aeruginosa* nitroreductase enzyme, or lack of access to the enzyme in *P. aeruginosa*. In the future, this issue could be addressed by simultaneous application of the compounds and isolated NfsB nitroreductase enzyme to kill *P. aeruginosa* infections.

4.7 Conclusion

The research question posed at the beginning of this chapter is: can a small molecule antibacterial prodrug be developed to be activated to release NO only in the presence of bacterial enzymes? This work shows that yes, antibacterial enzyme-activated diazeniumdiolate prodrugs are synthesized and activated in the presence of a bacterial nitroreductase enzyme. Novel nitroaromatic-protected piperazine diazeniumdiolates were synthesized, COOEt, Boc, and Cbz piperazine derivatives. Analysis of NO release from the compounds demonstrates that NO release occurs only in the presence of a nitroreductase enzyme, releasing up to $5.3 \pm 0.5 \times 10^{-7}$ moles of NO. The antibacterial activity of the compounds against *E. coli* was analyzed, causing up to a 94% reduction in the number of bacteria. This study demonstrates the possibility of antibiotic nitric oxide prodrugs, which could allow targeted delivery of NO to infections. In the future, the compounds could be derivatized and tested against other bacterial species containing nitroreductase enzymes to achieve broad spectrum activity.

4.8 Nuclear Magnetic Resonance Spectra



3a
$CDCl_3$, 400 MHz

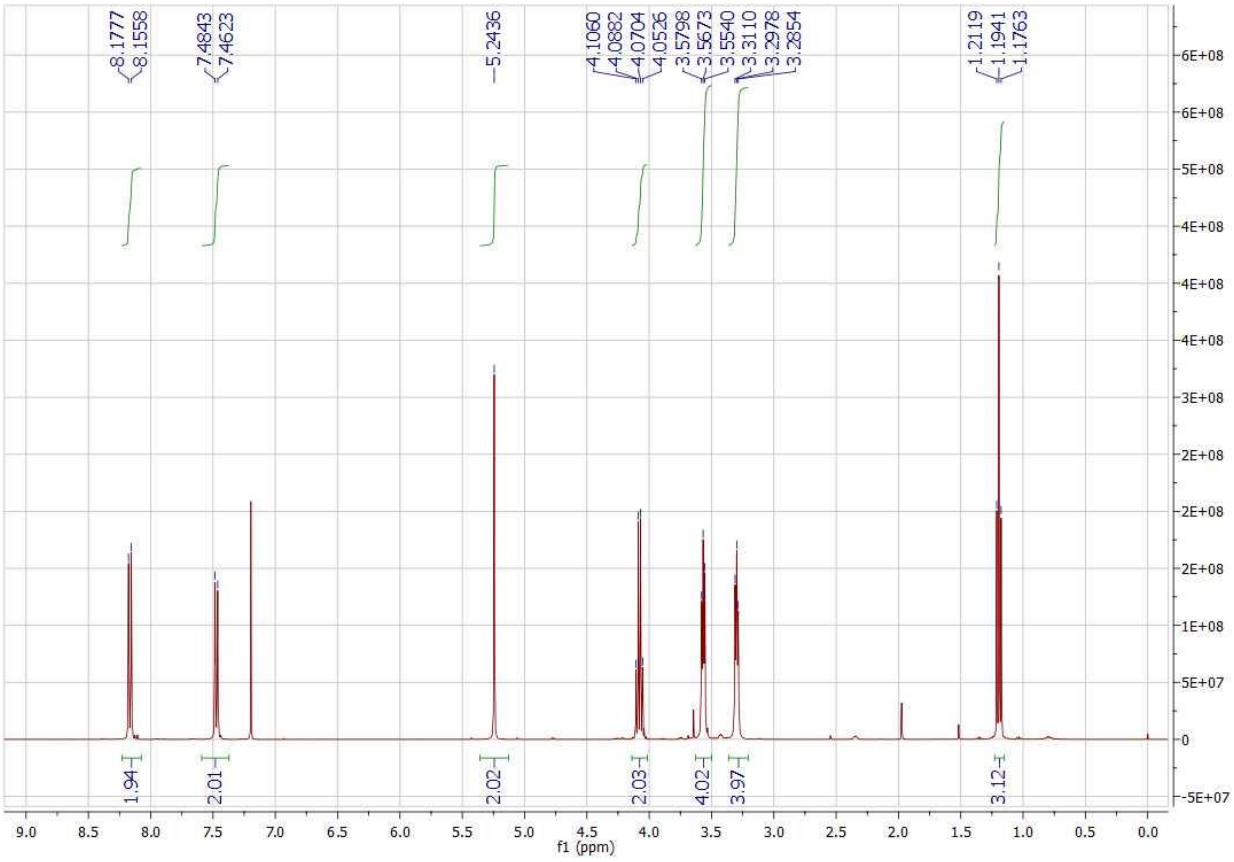
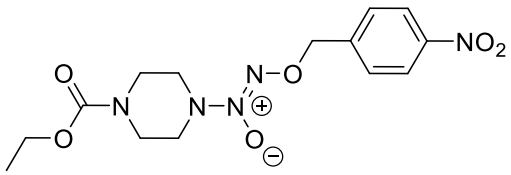


Fig. 4.14 ^{1}H NMR of **3a** in $CDCl_3$



3a
$CDCl_3$, 101 MHz

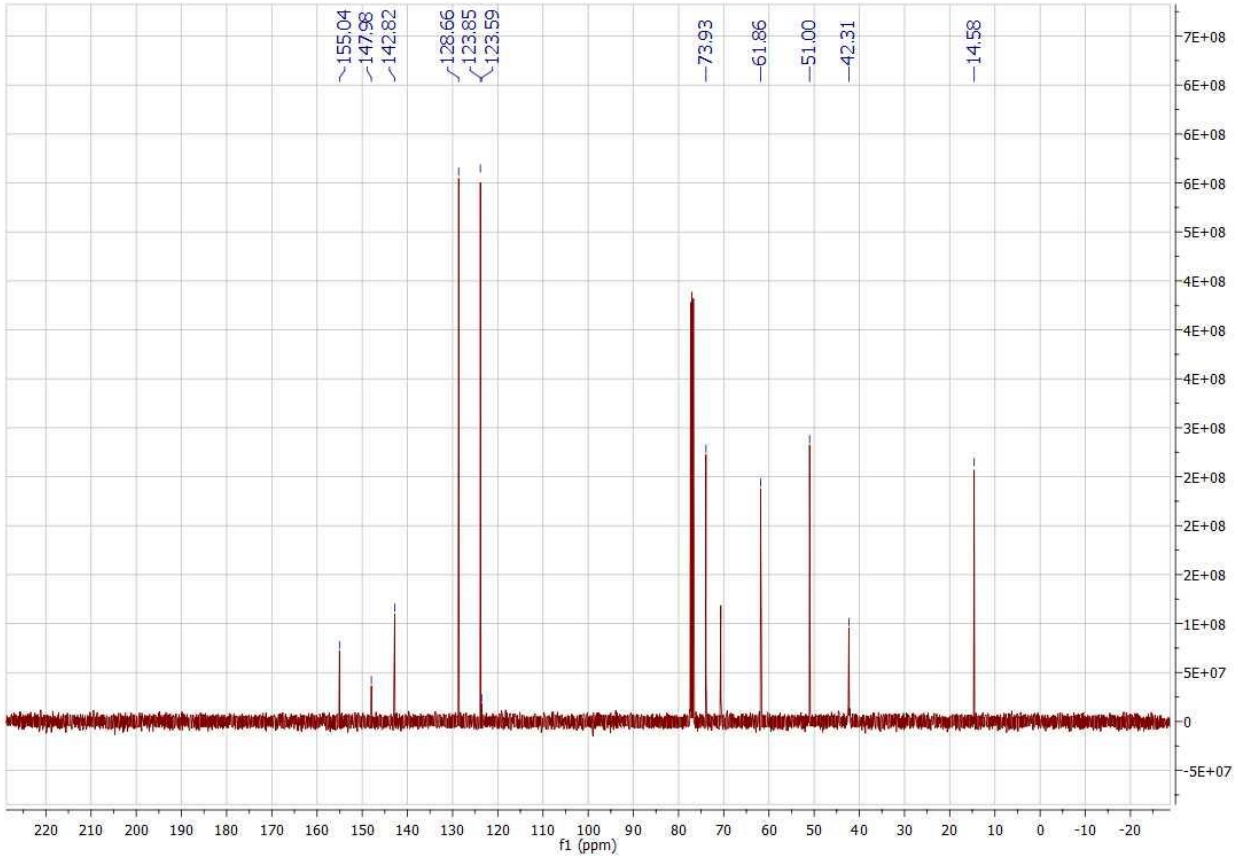
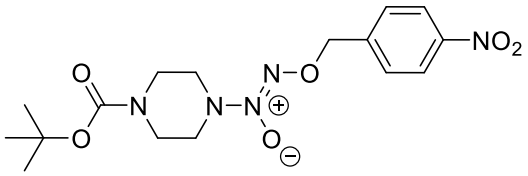


Fig. 4.15 ^{13}C NMR of **3a** in $CDCl_3$



3b
$CDCl_3$, 400 MHz

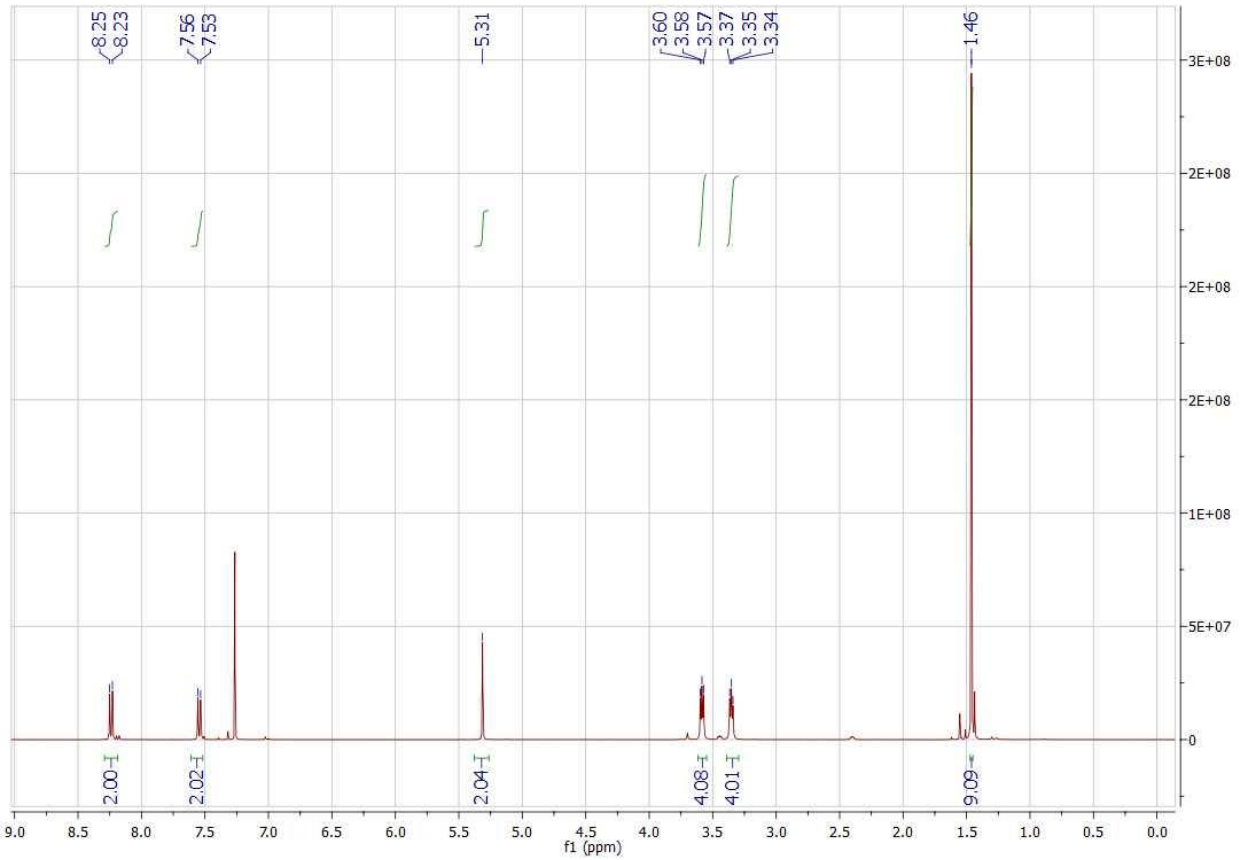
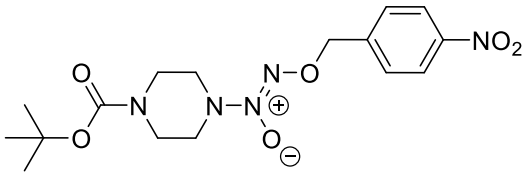


Fig. 4.16 ^1H NMR of **3b** in $CDCl_3$



3b
$CDCl_3$, 101 MHz

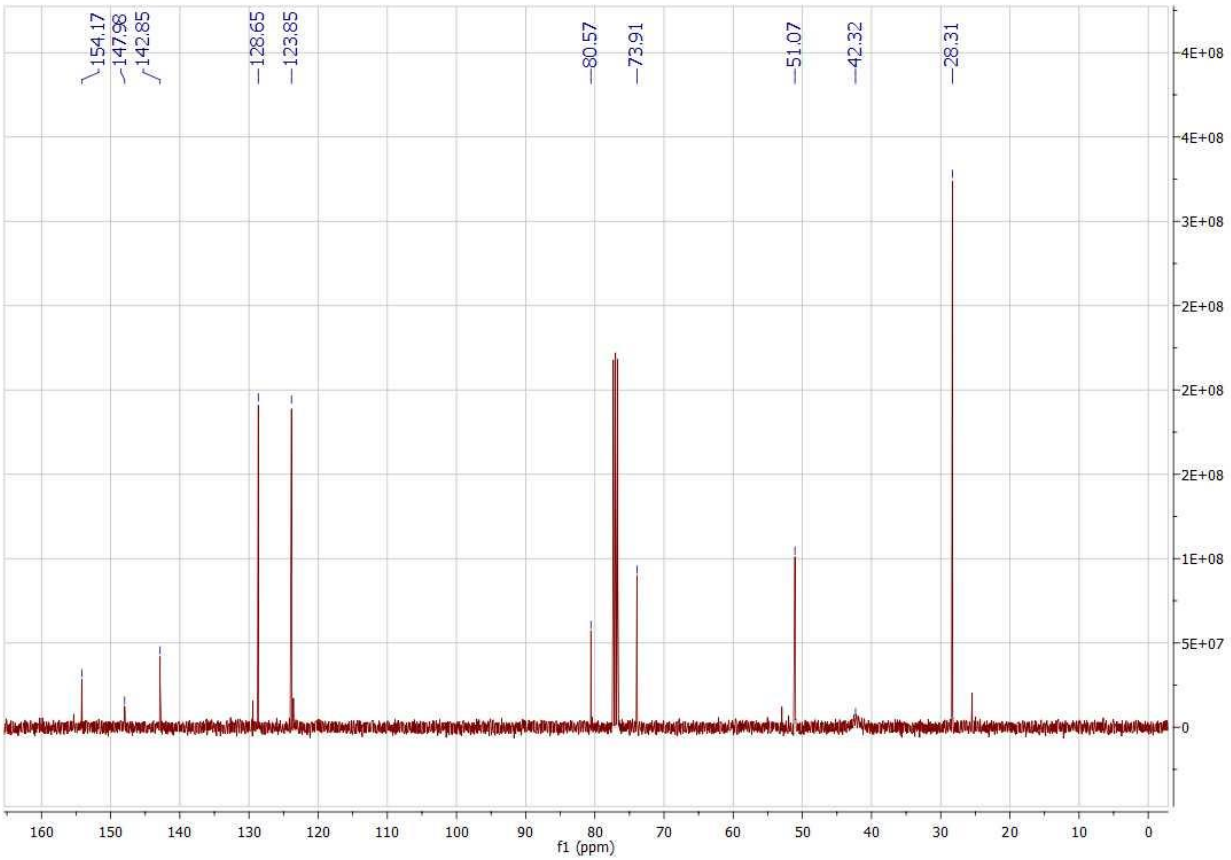
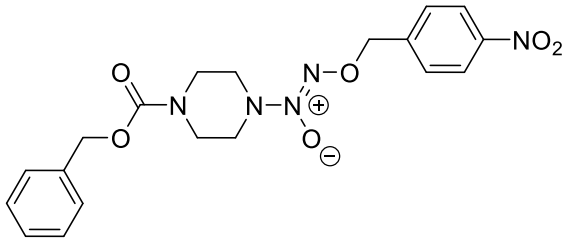


Fig. 4.17 ^{13}C NMR of **3b** in $CDCl_3$



3c
$CDCl_3$, 400 MHz

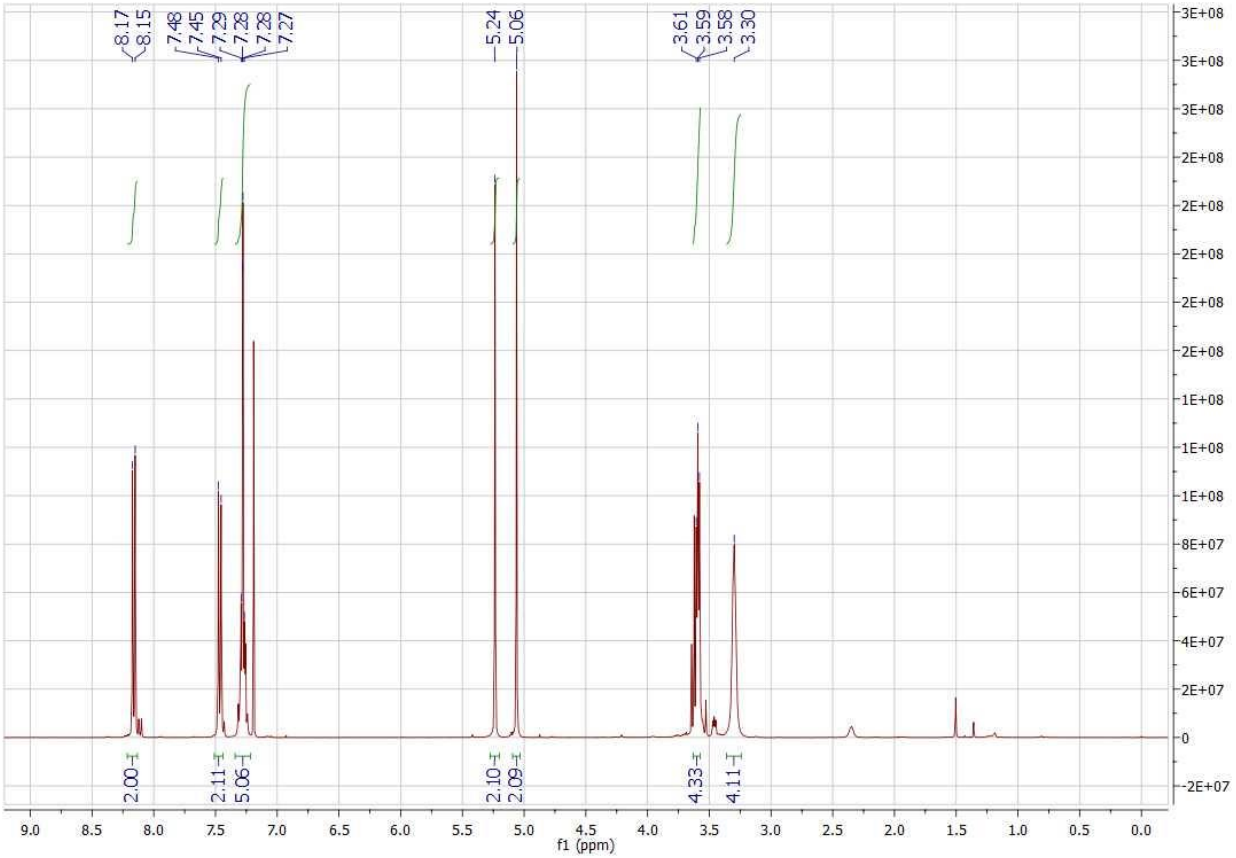
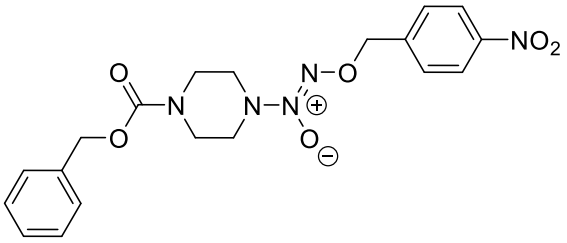


Fig. 4.18 ^1H NMR of **3c** in $CDCl_3$



3c
$CDCl_3$, 101 MHz

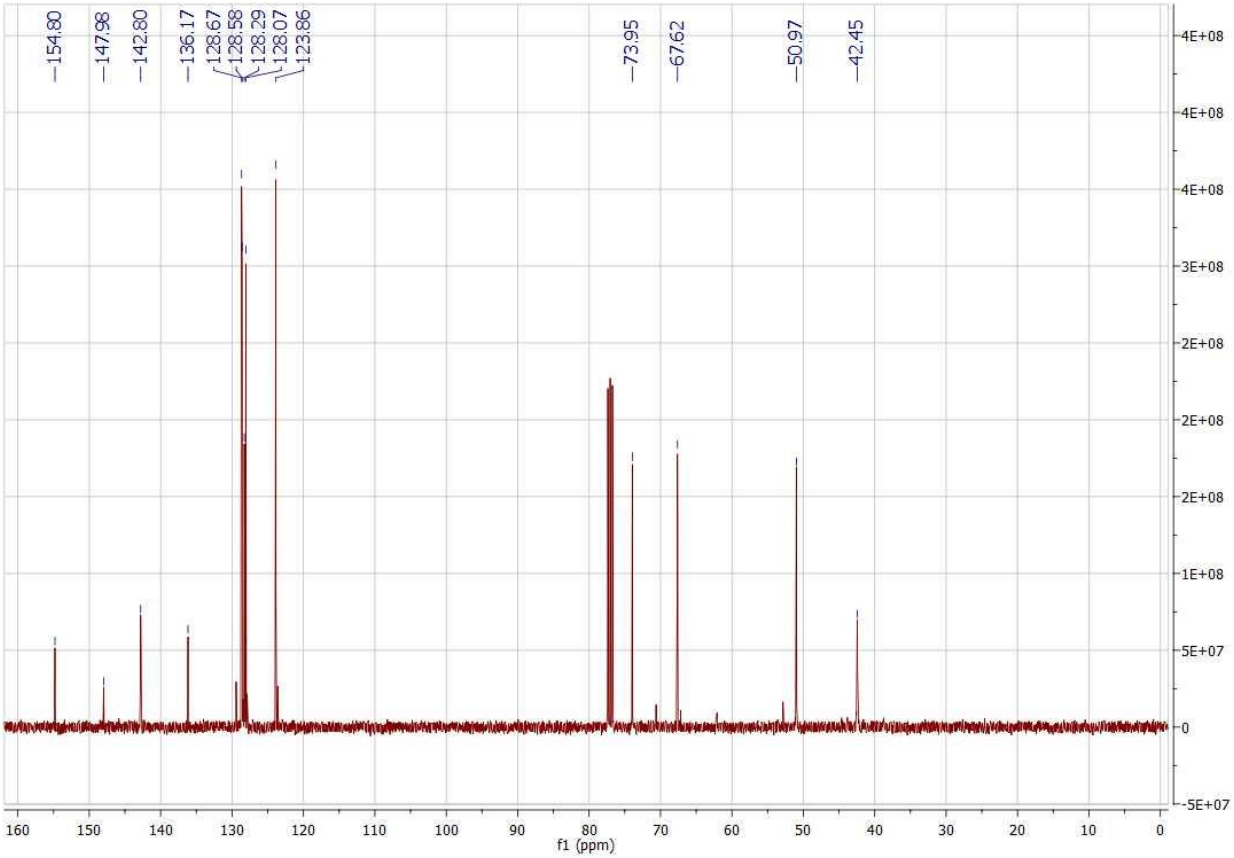


Fig. 4.19 ^{13}C NMR of **3c** in $CDCl_3$

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CHAPTER 5

POLYMER BLENDS OF NITROREDUCTASE-ACTIVATED PIPERAZINE DIAZENIUMDIOLATES

5.1 Background

After the success of synthesizing an enzyme-activated diazeniumdiolate specific to bacterial enzymes (discussed in Chapter 4), I wanted to incorporate the compound into a polymeric matrix. Through this process, new antibacterial materials could be developed. These materials could be used to coat or make new medical devices that have long-term antibacterial activity, preventing infection complications with medical implants. This work was supported by grants from the Monfort Foundation and the National Institutes of Health (5R21EB016838-02). The research described in this chapter will be submitted to the journal *ACS Applied Biomaterials*.

5.2 Introduction

The growing trend of antibiotic resistant bacterial infections calls for new ways to treat bacterial infections. Antibiotic prodrugs, which only release the active therapeutic in the presence of bacteria, can help reduce the opportunity for bacteria to develop resistance.[1] Medical devices are a site where bacterial infections can develop, and these infections are often incredibly difficult to treat and can require removal of the device to treat the infection. The Centers for Disease Control and Prevention (CDC) estimates that 1 out of every 31 hospital patients acquire a healthcare-associated infection (HAI), 25.6% of which are device-associated infections, costing billions of dollars per year.[2,3] To help address this problem, antibacterial coatings can be added to devices and implants to prevent infections.[4] Bacteria can attach to the surface of a medical device and multiply to form an infection. A coating on the device that releases an antibiotic

could prevent bacterial attachment and eventual infection. An antibacterial prodrug coating that only releases the active drug in the presence of bacteria could prevent medical device infections, battle resistance development, and have long-term antibacterial activity.

Nitric oxide (NO) is a gaseous small molecule that plays several roles in the body, such as cell signaling, immune response, and vasodilation, as well as being a potent therapeutic when applied exogenously, promoting would healing, killing cancer cells, and killing bacteria. Its antibacterial activity is of particular interest, as it is potent, and a report from Privett, et. al., showed that bacterial resistance to NO does not develop even after 21 days of exposure.[5] NO donors are found in many forms, including S-nitrosothiols (RSNOs), metal nitrosyls, organic nitrates and nitrites, and diazeniumdiolates.[6–11]

To increase the usability of NO donors for medical device applications, they must be incorporated into a polymer, either non-covalently or covalently. There are many examples of NO releasing polymers[12–15], used for many applications.[16,17] Many types of NO donors incorporated into polymeric matrices have been used for varied applications, most commonly RSNOs[18–20] and diazeniumdiolates[21–23].

Diazeniumdiolates are a functional group made up of two NO groups attached to a secondary amine that release two moles of NO per mole of substrate. They typically release NO spontaneously with a well-defined half-life ranging from seconds to hours. NO release can be prevented by protecting the O^2 position of the diazeniumdiolate functional group, stopping NO release until the removal of the protecting group. If this group is metabolized by an enzyme, then NO release from the compound is enzyme-activated. Diazeniumdiolate-containing polymers can increase the stability of the functional group and lengthen the time-release profile of the compound, allowing for more controllable NO release.[24] To the best of our knowledge, there is

only one report of an enzyme-activated diazeniumdiolate polymer, in which Zhao and coworkers synthesize a chitosan polymer containing a glycosylated diazeniumdiolate activated by a glycosidase enzyme and show its application in increasing cell proliferation for improved wound healing.[25] A similar idea is employed in this work, applying the concept of enzyme-activated diazeniumdiolate polymers to antibacterial treatments.

In a previous report from our lab, we described the synthesis, characterization, and analysis of some of the first bacterial enzyme-activated diazeniumdiolates.[26] Piperazine-based diazeniumdiolates were synthesized, then protected with a nitroaromatic group (Figure 5.1). Upon exposure to a nitroreductase (NTR) enzyme, a family of enzymes found almost exclusively in bacteria and known to reduce nitro groups, the protecting group is removed, leading to NO release. These novel compounds release NO only in the presence of the enzyme, indicating that these compounds could be used as bacterial prodrugs. The research question posed in this chapter is: will incorporating a nitroaromatic-protected diazeniumdiolate into a polymer composite maintain the NO release properties of the compound and create a material with enzyme-activated antibacterial properties? To investigate the possible use of these compounds as enzyme-activated antibacterial medical device coatings, I describe the preparation of polymer films containing Et-PIPER/NO-NO_2 (**1a**, Figure 5.1), the compound with the highest antibacterial performance based on previous experimental results.[26] I fully characterized the

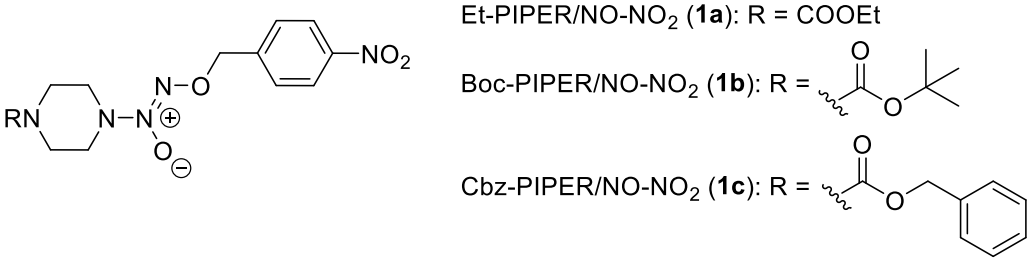

Fig. 5.1 Structure of enzyme activated nitric oxide releasing piperazine-based nitroaromatic-protected diazeniumdiolates

properties of the films using scanning electron microscopy (SEM), energy-dispersive x-ray analysis (EDX), and nitric oxide analyzers to quantify NO release, and I analyzed the antibacterial activity of the films by counting colony-forming units (CFUs) after exposure to the films. This is the first report of an antibacterial enzyme-activated NO releasing polymer material.

5.3 Experimental Methods

5.3.1 Materials. Ethyl 1-piperazinecarboxylate (99%), 4-nitrobenzyl bromide (99%), 15-crown-5 (98%), high molecular weight poly(vinyl chloride), nitroreductase from *Escherichia coli* (90%), and β-Nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt hydrate (NADPH, 97%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Anhydrous (Dri-Solv, 99.8%) tetrahydrofuran was purchased from EMD Millipore (Billerica, MA, USA). Nitric oxide (99%) was purchased from Matheson Tri-gas (Montgomeryville, PA, USA). Sodium methoxide (30% in methanol) and dioctyl sebacate (97%) were purchased from Acros Organics (Geel, Belgium). Medical grade polyurethane (Tecophilic SP 80A-150) was obtained from Lubrizol (Wickcliffe, OH, USA). Oxoid nutrient broth (OXCM0001B), Oxoid nutrient agar (OXCM0003B), and tetrahydrofuran were purchased from Fisher Scientific (Hampton, NH, USA). *Escherichia coli* (ATCC 25922) was obtained from American Type Culture Collection (ATCC, USA). Argon (ultrahigh purity), nitrogen (ultrahigh purity), and oxygen were purchased from Airgas (Denver, CO, USA). Deionized water (18.2 MΩ·cm) was obtained from a Millipore Direct-Q water purification system (EMD Millipore, Billerica, MA, USA).

5.3.2 Abbreviations. The following abbreviations are used throughout: rt= room temperature, h= hour, min= minute, THF= tetrahydrofuran, 15-crown-5= 1,4,7,10,13-Pentaoxacyclopentadecane, NaOH= sodium hydroxide, DCM= dichloromethane, Na_2SO_4= sodium sulfate, EtOAc= ethyl acetate, SiO_2= silica, NADPH= β-Nicotinamide adenine dinucleotide 2′-phosphate reduced

tetrasodium salt hydrate, NBM= nutrient broth media, PBS= phosphate-buffered saline, PVC= poly(vinyl chloride), DOS= dioctyl sebacate, PU= polyurethane, NO= nitric oxide, SEM= scanning electron microscope, EDX= energy dispersive X-ray spectrometer

5.3.3 Instruments. NO release measurements were obtained using a Sievers 280i Nitric Oxide Analyzer (NOA, GE Analytical Instruments, Boulder, CO, USA). Reactions were analyzed by thin layer chromatography (TLC) on aluminum sheets that were pre-coated with silica gel 60 F_{254}, and the reactions were purified by column chromatography using Alfa Aesar silica gel 60 (0.06-0.2 mm). SEM imaging was performed using a JEOL JSM-6500F scanning electron microscope. Gold coating was performed using a Denton Vacuum Desk II Gold Sputter Coater. SEM/EDX analysis was performed using the SEM equipped with a Thermo Electron energy dispersive X-ray spectrometer (EDX) for qualitative and quantitative elemental analysis.

5.3.4 Experimental Procedures

5.3.4.1 Synthesis of sodium 1-(4-Ethoxycarbonylpiperazin-1-yl)diazen-1-ium-1,2-diolate. The diazeniumdiolate was synthesized according to a previously reported procedure.[26] In brief, ethyl 1-piperazinecarboxylate (1 equiv.) was stirred to dissolve in anhydrous methanol (2.1 M) in a high pressure reaction vessel. Sodium methoxide (30% in methanol, 1 equiv.) was added. The reaction vessel was attached to a high-pressure stainless-steel nitric oxide reactor system, purged with high purity argon, then charged with nitric oxide gas (80 psi, 99%). After stirring under pressure for 24 h, the white precipitate that formed was washed with methanol, then diethyl ether and dried under vacuum for 1 h. The products were stored at -20 ˚C until further use. Spectra matched the previously reported values.[26]

5.3.4.2 Synthesis of O^2-(4-Nitrobenzyl) 1-(4-ethoxycarbonylpiperazin-1-yl)diazen-1-ium-1,2-diolate. The diazeniumdiolate was protected according to a previously reported procedure.[25] In

brief, to a 0 ˚C solution of diazeniumdiolate (0.75 mmol, 1.5 eq.) in anhydrous THF (0.1 M), 15-crown-5 (1 mmol, 2 eq.) was added. After 30 minutes stirring at 0 ˚C, 4-nitrobenzyl bromide (0.5 mmol, 1 eq.) was added and the reaction was allowed to warm to rt. After stirring for 4 h, the reaction mixture was concentrated *in vacuo*. The crude mixture was dissolved in DCM (10 mL) and NaOH (1 M, 10 mL) was added. The aqueous layer was extracted with DCM (3 × 10 mL), then the organic layers were washed sequentially with deionized water (10 mL) and brine (15 mL), dried over Na_2SO_4 and concentrated *in vacuo* to afford a yellow oil. After purification by column chromatography (SiO_2, 10%, 30% EtOAc in hexanes gradient), the pure product is collected as a pale yellow crystalline solid. Spectra matched the previously reported values.[25]

5.3.4.3 Preparation of polyvinyl chloride/polyurethane films. High molecular weight polyvinyl chloride (PVC, 2 g) was mixed with plasticizer dioctyl sebacate (DOS, 1.1 mL) in a 2:1 PVC:DOS ratio in THF (20 mL, 10% w/v), stirring at 1200 rpm for at least 1 h to dissolve. 500 µL aliquots of PVC were cast in 10 mL Pyrex beakers, three times, allowing for layers to dry 1 h in between additions, for a total of three layers of PVC per film. Medical grade SG 80A-150 polyurethane (PU, 100 mg) was dissolved in THF (2 mL, 5 w/v%), stirring at 1200 rpm for at least 3 h to dissolve. The NO_2-protected diazeniumdiolate was then added in the appropriate w/v% (control= 0%, 0.25%= 5 mg/2 mL PU/THF mixture, 1%= 20 mg/2 mL PU/THF mixture, 2.5%= 50 mg/2 mL PU/THF mixture), stirring to dissolve in the PU mixture. One 400 µL aliquot of the appropriate PU mixture was cast on top of the PVC layered films. The films were allowed to cure in a fume hood overnight, then removed from the beaker and cut to 15 x 15 mm squares for NO release experiments, and 7.5 x 15 mm rectangles for bacteria experiments.

5.3.4.4 SEM and SEM/EDX Analysis. SEM images were obtained using an accelerating voltage of 10.0 kV and a working distance of 10.0 mm. For SEM images, all films were sputtered with a

20 nm layer of gold. SEM/EDX analysis was performed with uncoated films with an accelerating voltage of 2 kV. Quantitative elemental analysis data was obtained for carbon, oxygen, and nitrogen.

5.3.4.5 Nitric oxide release measurements under physiological conditions. The amount of NO release from each w/v% film (0%, 0.25%, 1%, and 2.5%) was assessed over a 24 h period using a nitric oxide analyzer (NOA, Sievers 280i, GE Analytical Instruments, Boulder, CO, USA). Before each experiment, the NOA was calibrated using ultra-high purity nitrogen as zero gas and a 45 ppm NO calibration gas cylinder (Airgas), according to the manufacturer's standard operating procedures. The NOA sample holder was under nitrogen, contained 1500 µL degassed PBS, 400 µL NADPH (10 mM in PBS), and 1 film (15 x 15 mm), and was maintained at 37 °C, to mimic both experimental and physiological conditions. Sufficient baseline of the PBS, NADPH, and film solution was obtained before injection of the nitroreductase enzyme. Nitroreductase enzyme (50 µL, 1 mg/mL in PBS) was then added through the side injection port of the NOA vessel. The solution was purged continuously with ultra-high purity nitrogen gas with bubbling rate of 55 mL/min directly into the solution and flow gas introduced into the remaining headspace for the duration of the experiment. NO release was recorded in 5 s intervals as parts per billion. Each experiment was allowed to run for 24 h before analyzing total moles released over that time period. NO release data is presented as the average of at least 3 trials ± standard deviation. NO flux from the polymer films were calculated using the equation below.

$$NO\ flux = \frac{total\ moles\ NO\ released}{(surface\ area\ of\ film)(time)}$$

5.3.4.6 Nitric oxide release during thermal decomposition of films. A similar procedure was used as the experiments measuring NO release under physiological conditions. A used 2.5% film from

the physiological conditions experiment was placed in an empty NOA cell and a baseline release was established. The cell was heated to 120 °C in a sand bath and NO release was measured for four hours until the film began to turn black and melt.

5.3.4.7 Preparation of and nitric oxide release from KTpClPB-containing films. The potassium tetrakis(4-chlorophenylborate (KTpClPB) films were made in a similar manner to the previously described procedure. The plasticized PVC layers were made and cast in the same way. The PU layer was made by dissolving medical grade SG 80A-150 polyurethane (PU, 50 mg) in THF (1 mL, 5 w/v%), stirring at 1200 rpm for at least 3 h to dissolve. The NO_2-protected diazeniumdiolate (2.5 mg) and KTpClPB (0.125% w/v, 1.125 mg/1 mL PU/THF mixture) were then added, stirring to dissolve in the PU mixture. One 400 µL aliquot of the appropriate PU mixture was cast on top of the PVC layered films. The films were allowed to cure in a fume hood overnight, then removed from the beaker and cut to 15 x 15 mm squares for NO release experiments. The NO release experiments were run exactly as previously described under physiological conditions with the KTpClPB-containing 0.25% films.

5.3.5 Bacterial Studies

5.3.5.1 Escherichia coli Bacteria Culture. Initial stock culture of *Escherichia coli* was made by reconstituting lyophilized bacteria in warm nutrient broth media (NBM, 13 g nutrient broth/1 L Millipore water) and grown overnight at 37 °C and 150 rpm until an optical density at 600 nm (OD_{600nm}) ≈1.0 was reached. This bacterial solution was combined with glycerol (30% v/v) in a 1:1 fashion to obtain a final glycerol concentration of 15% (v/v). These solutions were stored at −80 °C until use. Prior to each bacterial experiment, a frozen culture was thawed at rt and then centrifuged at 4700 rpm for 10 min. The supernatant was discarded, and the pellet was resuspended in NBM. This was transferred to additional NBM and allowed to grow overnight in

a 37 °C incubator with shaking at 120 rpm until the OD_{600nm} ≈1.0. The culture was diluted to an OD_{600nm} ≈0.3-0.35 using warmed NBM prior to beginning experiments.

5.3.5.2 Bacterial Viability. The PVC/PU/diazeniumdiolate films (0.25%, 1%, and 2.5%; 7.5 x 15 mm) were added to *E. coli* culture (10^7 CFU/mL) in NBM (1 mL, OD_{600nm} ≈0.3-0.35) in 1.5 mL Eppendorf microcentrifuge tubes. The positive control was *E. coli* culture in NBM (1 mL, OD_{600nm} ≈0.3-0.35) with a PVC/PU control film (0% diazeniumdiolate). The negative control was *E. coli* culture in NBM (1 mL, OD_{600nm} ≈0.3-0.35). The solutions were placed in a static 37 ˚C incubator for 24 h. Agar plates were made by adding 15 mL of sterilized nutrient agar [powder base (14 g) in Millipore water (500 mL)] to sterile Petri dishes and allowed to set for at least 2 h. After 24 h exposure, the *E. coli* solutions underwent serial tenfold dilution with sterile NBM to reach a 10^5 dilution factor, and the agar plates were inoculated with 20 µL of the diluted *E. coli* solutions using a sterile plastic or glass spreader. The agar plates were placed in a static 37 °C incubator overnight and colony-forming units (CFUs) were counted after 24 h (n = 9). The number of CFUs were calculated using the equation:

$$\frac{CFU}{mL} = \frac{(\#\ CFU\ counted)(Dilution\ factor)}{Volume\ plated\ (mL)}$$

The percent reduction in the number of viable bacteria was calculated as follows:

$$\%\ reduction = \frac{CFU\ after\ exposure\ to\ film/mL}{CFU\ positive\ control/mL}$$

5.3.5.3 Statistical Analysis. All biological experiments were performed using at least three replicates with at least three samples per replicate. Viability assays are reported as the mean and 95% confidence interval. All data were evaluated for potential outliers using the Grubbs Test.

The statistical differences in data were evaluated using the Student's t test ($p < 0.05$) at the 95% confidence level.

5.4 Synthesis of PVC/PU Blends with Piperazine Diazeniumdiolates

Toward the goal of using enzyme-activated diazeniumdiolates in an antibacterial medical device coating, I prepared polymer composite films containing varying amounts of the protected diazeniumdiolate **1a** (Figure 5.1). To prepare the films, I cast plasticized PVC in three layers, allowing the layers to dry in between additions. The plasticizer dioctyl sebacate (DOS) was used to improve the flexibility and decrease the brittleness of the PVC. PU was mixed with 0.25, 1, or 2.5% w/v Et-PIPER/NO-NO_2 (**1a**), then one layer of the PU mixture was cast on top of the PVC layers, and the films were cured overnight. The resulting films can be seen in Figure 5.2. The films are referred to throughout this chapter in terms of the percent w/v of Et-PIPER/NO-NO_2 diazeniumdiolate **1a** in the film (0.25, 1, or 2.5% w/v).

The choice of materials used to make the films was intentional based on experience, experimentation, and intended application. Both PVC and PU are commonly used to make medical devices such as catheters and tubing, indicating the relative safety and applicability of these polymers.[27,28] I chose to layer plasticized polyvinyl chloride (PVC) and medical grade polyurethane (PU) to balance mechanical properties and usability. PVC adds sturdiness to the films, as hydrophilic PU alone in water can have a lower viscosity, making it impracticable for many applications. The layered PVC gave the films a visibly smoother surface texture, as well as adding durability to the films. Less than three layers of PVC caused the films to curl up in solution, potentially preventing complete aqueous saturation of the PU/diazeniumdiolate layer and limiting NO release. I chose a hydrophilic PU to mix with the diazeniumdiolate **1a**, hypothesizing that PU uptake of water in solution would better allow the NTR enzyme to diffuse

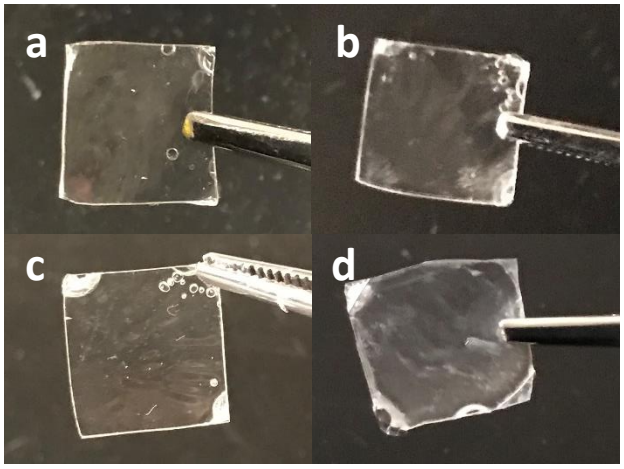

Fig. 5.2 Images of varying percent w/v Et-PIPER/NO-NO_2 polymer films a) control (0%) b) 0.25% c) 1% d) 2.5%

into the PU layer of the films, initiating enzyme-activated NO release. In addition, diazeniumdiolate-containing PU materials have previously been reported, demonstrating the feasibility of this approach.[29] Adding more than 2.5% w/v of diazeniumdiolate to the films led to significant opacity of the films, which could limit the applications of the material.

5.5 Characterization of PVC/PU Blends with Piperazine Diazeniumdiolates

5.5.1 SEM and SEM/EDX Analysis

To investigate the surface composition of the prepared diazeniumdiolate polymer films, the films were characterized by scanning electron microscopy (SEM) and energy-dispersive x-ray analysis (EDX). SEM images of films show that the higher the content of the diazeniumdiolate (higher percent w/v), the more diazeniumdiolate crystals are observed (Figure 5.3). The 2.5% w/v films appear to be entirely coated in diazeniumdiolate (Figure 5.4a). The SEM images of the control films (containing 0% diazeniumdiolate) appear different than the diazeniumdiolate-containing films. At low magnification (200x, Figure 5.3), the films appear uniform, and upon higher magnification, there appear to be polymer contaminants or undissolved pieces of PVC or PU (Figures 5.6-5.7). For the diazeniumdiolate containing films, the higher the

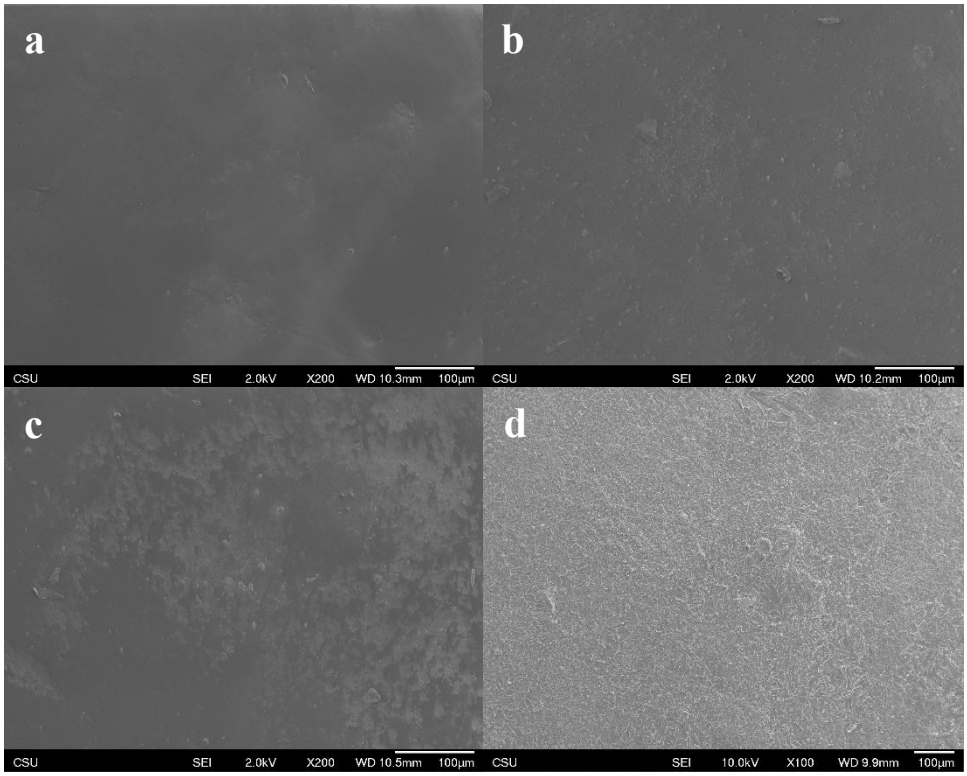

Fig. 5.3 Representative SEM images of a) control (0%) b) 0.25% c) 1% d) 2.5% films at 200x magnification showing increasing amounts of diazeniumdiolate on the surface with increasing % w/v

percent w/v, the higher the amount of diazeniumdiolate observed on the surface (Figure 5.3). For the 0.25% films, the distribution of the diazeniumdiolate crystals appears to be visibly uniform, although there are some larger aggregates of diazeniumdiolate (Figures 5.8-5.10). Images of the the 1% films show more diazeniumdiolate across the surface of the films, and they appear similar

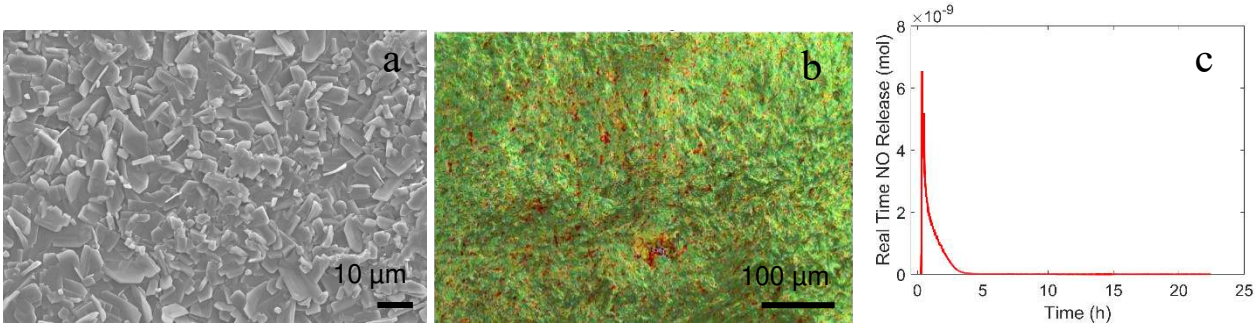

Fig. 5.4 Characterization data for polymer films. (a) Representative SEM image of 2.5% w/v film at 1000x magnification. (b) Representative EDX layered elemental surface map of 2.5% w/v film. Blue pixels represent nitrogen, green pixels represent oxygen, and red pixels represent carbon. (c) Representative NO release plot for 2.5% w/v film

to the 0.25% films, with diazeniumdiolate crystals spread across the polymer matrix (Figure 5.11-5.13). The 2.5% films appear to be entirely coated in diazeniumdiolate, with no visibly apparent polymer observable (Figures 5.4, 5.14-5.16). The 2.5% w/v films became charged very easily when acquiring SEM images, potentially due to the higher amount of charged diazeniumdiolate groups present on the surface. These SEM images show that the higher the percent w/v of diazeniumdiolate in the polymer films, the more diazeniumdiolate is observable on the surface of the films, as expected.

SEM/EDX analysis was performed to determine the elemental surface composition of the films. EDX analysis involves focusing a high energy beam of electrons on the surface of a sample and analyzing the characteristic X-ray pattern emitted by the excited atoms to determine the elemental composition of the sample. The SEM/EDX surface map analysis shows that the films are made up of mostly of carbon, followed by oxygen, and then nitrogen in the diazeniumdiolate-containing films (Table 5.1). The control films (0% w/v Et-PIPER/NO-NO_2, PU/PVC only) showed 0% nitrogen as no diazeniumdiolate is present and the surface is 91.7% carbon and 8.3% oxygen (Table 5.1, entry 1), likely from the composition of the polymers. The 0.25% films contain 0.2% nitrogen, demonstrating that the presence of diazeniumdiolate in the films increases the amount of nitrogen on the surface of the films (Table 5.1, entry 2). The 1% films contain more nitrogen, 2.4%, indicating a trend of increasing amounts of nitrogen on the surface with increasing amounts of diazeniumdiolate present in the film, as would be expected (Table 5.1, entry 3). This trend continues with the 2.5% films, made up of 13.2% nitrogen (Table 5.1, entry 4; Figure 5.3b). The SEM/EDX analysis provides quantitative evidence complementing the qualitative SEM observations that the higher percent w/v diazeniumdiolate-containing films contain more diazeniumdiolate on the surface.

Table 5.1 Surface composition of diazeniumdiolate polymer films determined by SEM/EDX

Entry	% w/v of Et-PIPER/NO-NO_2 in film	Carbon (wt.%)[a]	Oxygen (wt.%)[a]	Nitrogen (wt.%)[a]
1	Control (0%)	91.7 ± 0.1%	8.3 ± 0.1%	0.0 ± 0.1%
2	0.25%	76.4 ± 0.1%	23.5 ± 0.1%	0.2 ± 0.1%
3	1%	75.2 ± 0.1%	22.5 ± 0.1%	2.4 ± 0.1%
4	2.5%	72.3 ± 0.1%	14.5 ± 0.1%	13.2 ± 0.1%

[a]Data is reported as the mean ± standard deviation, n=3

5.5.2 Quantification of NO Release from Polymer Films

NO release properties of the films were investigated in the absence and presence of the NTR enzyme (NfsB isolated from *E. coli*). NO release is characterized using a nitric oxide analyzer (NOA), which uses chemiluminescence to directly quantify the amount of NO released. NO released from a substrate is oxidized by ozone generated *in situ* in the instrument, forming excited state nitrogen dioxide (NO_2). Excited state NO_2, upon relaxation, emits a photon which is measured and related to the moles of NO released with a previously determined calibration constant, providing direct measurement of NO release as a function of time.

To quantify the amount of NO released from the polymer films, the films were placed in a solution of phosphate-buffered saline (PBS) and β-Nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt hydrate (NADPH), an essential cofactor to the NTR enzyme acting as a reducing agent. A baseline measurement was collected without the NTR enzyme present. No NO release was observed from the films when the NTR enzyme is not present. This observation is expected based on previously reported results in which the diazeniumdiolate powder did not release appreciable NO when the enzyme was not present, providing strong evidence that the diazeniumdiolate is enzyme-activated.[25] The NTR enzyme is injected into the solution, and NO release is measured for 24 hours. Experiments with each percent w/v film were repeated three times to demonstrate reproducible results (Figures 5.17-5.19).

All the percent w/v films containing diazeniumdiolate (0.25, 1, and 2.5%) release similar amounts of NO (Figure 5.4c, Table 5.2, entries 1-3), ranging from 2.8-4.3 × 10^{-6} moles of NO. Control films containing 0% diazeniumdiolate were tested for NO release under identical conditions to the diazeniumdiolate-containing films and released a negligible amount of NO (data not shown). The concentration of NO release is in the micromolar range, which is the concentration level (>1 μM) known to have antibacterial effects.[30,31] The amount of NO release from each of the percent w/v films showed no discernible pattern correlating to the amount of NO donor loading (Table 5.2, entries 1-3). This could be attributed to a localized basic environment in the films, which can happen as NO is released and the amount of secondary amine byproducts increases, increasing the basicity of the local environment.[32] This would stabilize the diazeniumdiolate, as diazeniumdiolates are more stable in higher pH environments, and prevent further NO release. To test this, I made films with a PU layer containing 0.12% potassium tetrakis(4-chlorophenylborate (KTpClPB) and 0.25% diazeniumdiolate. KTpClPB is a salt with a large organic anion that can buffer the environment within the polymer film and prevent a high pH environment from forming as the concentration of secondary amines increases.[32] After making these KTpClPB-containing 0.25% diazeniumdiolate films, I ran NO release experiments to determine if additional NO would be released as compared to the amounts of NO released under physiological conditions. However, I found that these films released 4.0 × 10^{-6} moles of NO, which is very similar to the amount of NO (3.6 ± 0.7 × 10^{-6} moles) released

Table 5.2 NO release from Et-PIPER/NO-NO_2 polymer films

Entry	% w/v of Et-PIPER/NO-NO_2 in film	Total moles of NO (moles)[a]	NO flux (moles NO cm^{-2} min^{-1})[a]
1	0.25%	3.6 ± 0.7 × 10^{-6}	2.7 ± 0.5 × 10^{-9}
2	1%	4.3 ± 0.7 × 10^{-6}	2.7 ± 0.8 × 10^{-9}
3	2.5%	2.8 ± 0.1 × 10^{-6}	2.4 ± 0.8 × 10^{-9}

[a]Data is reported as the mean ± standard deviation, n=3

from the films containing no KTpClPB. This experiment shows that it is unlikely that a local basic environment forming in the films is preventing complete NO release.

Other hypotheses as to the similar amounts of release from each percent w/v film could be limited diffusion of the enzyme into the polymer to catalyze NO release or to limited solubility of the NO donor in aqueous conditions, which can affect NO release, as I previously observed.[25] This would lead to incomplete release of NO from the films. To determine if the films still contain NO after measuring NO release under physiological conditions, heat could be used to force any remaining NO in the films out. To determine this, I ran an experiment with a 2.5% w/v film that the amount of NO release had previously been determined by exposure to NADPH and NTR enzyme. I placed the used film in an NOA cell with no solution, heated the film to 120 °C in a sand bath, and measured the amount of NO release. I observed an additional amount of NO released from the film of up to 20% more NO (5.8×10^{-7} moles of NO) before the polymer started to melt and decompose and I ended the experiment. This shows that there is additional NO in the films, which could explain why the different percent w/v films show similar amounts of total NO release. A certain amount of NO is accessible in the films and is able to be released, while some additional NO is trapped in the films.

I also calculated the NO flux from the polymer films (Table 5.2). The flux from each of the percent w/v films is remarkably similar. The flux measurement is much higher than the natural endothelial flux of NO in the body, 1×10^{-10} mol cm^{-2} min^{-1}.[33] The amount of NO release observed and the NO flux levels indicate that the films will have antibacterial activity.[30]

5.6 Antibacterial Activity of Polymer Blends with *Escherichia coli*

E. coli is a common infection-forming species that is troublingly growing increasingly resistant to many antibiotics.[34,35] Because of this, *E. coli* was chosen as the bacterial species of

interest in the antibacterial efficacy studies. In addition, it is known to contain an NTR enzyme, one of which was the isolated NTR enzyme used in the NO release experiments, and activates NO release from Et-PIPER/NO-NO_2.[25]

To determine the antibacterial efficacy of the diazeniumdiolate films, *E. coli* was exposed to the films (0.25%, 1%, and 2.5%) for 24 hours. The bacterial solution was diluted and plated on agar, then colony forming units (CFUs) were counted. The negative control in these experiments was *E. coli* solution with no film present. The positive control was *E. coli* exposed to a PVC/PU film containing 0% diazeniumdiolate. Based on our previous work, *E. coli* should metabolize the diazeniumdiolate, catalyzing NO release with the NTR enzymes it produces.[25]

As can be seen in Figure 5.5, all percent w/v films containing the therapeutic cause a decrease in the number of viable bacteria, in similar amounts. The 0.25% film reduces cell viability by 64% and the 1% and 2.5% films both cause a 66% reduction. The negative control, on the other hand, does not significantly reduce the number of viable bacteria, with a 0% reduction. The positive control with *E. coli* exposed to the 0% polymer film causes an insignificant 3% reduction, showing that polymer films alone do not have antibacterial activity. This data shows that the diazeniumdiolate Et-PIPER/NO-NO_2 films have significant antibacterial effects. All of the percent w/v films cause similar amounts of reduction in bacteria, which fits with the NO release data. This could be due to potential difficulties of the bacteria with accessing the diazeniumdiolate to activate NO release within the films if bacteria are only able to access NO on the surface of the films. These results are an exciting proof of concept of the first example of a bacterial enzyme-activated diazeniumdiolate-polymer composite with antibacterial activity.

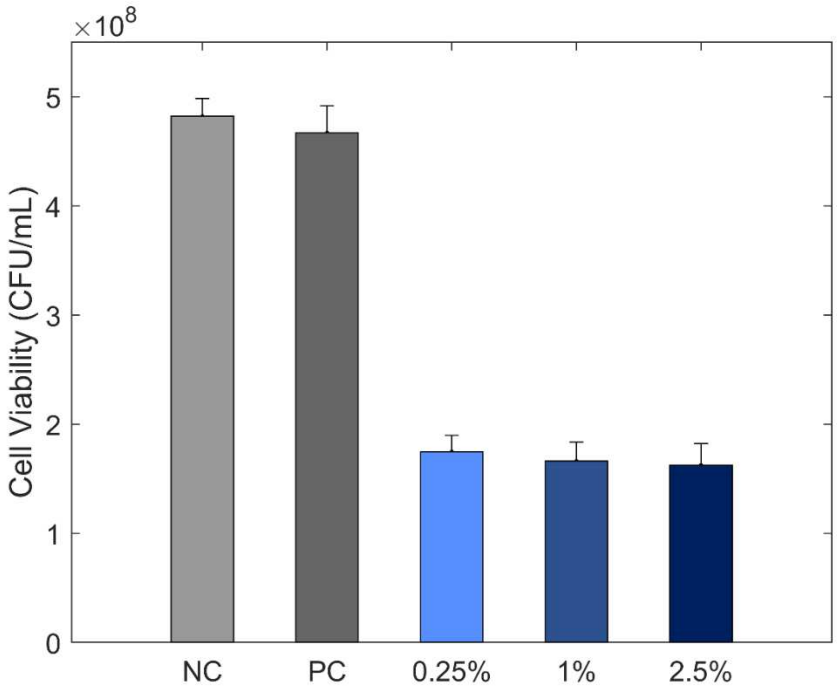

Fig. 5.5 Cell viability of *E. coli* after exposure to Et-PIPER/NO-NO_2 % w/v films. NC=negative control, *E. coli* with no polymer film; PC=positive control, *E. coli* with 0% w/v polymer film. Average and standard error bars displayed, $n \geq 9$. Statistically significant differences between cellular viabilities are indicated (*) as determined by a student's t test.

5.7 Conclusion

The research question posed at the beginning of this chapter is: will incorporating a nitroaromatic-protected diazeniumdiolate into a polymer composite maintain the NO release properties of the compound and create a material with enzyme-activated antibacterial properties? The research described in this chapter describes the first antibacterial enzyme-activated NO-releasing polymer composite. Through extensive characterization, NO release from the polymers is shown to be enzyme activated, and NO is released at concentrations known to have antibacterial effects. The NO release properties are similar to the powder form of the diazeniumdiolate. NO release from the polymer composite remains nitroreductase enzyme activated. Investigation of the antibacterial activity of the polymer composites against *E. coli* excitingly showed that the films have significant antibacterial effects, reducing bacterial cell

viability by up to 66%. The diazeniumdiolate composite films continue to have antibacterial effects as the powder form does, albeit with lower reductions, possibly due to lower solubility and aqueous saturation of the polymer composites.

Future work will focus on increasing the amount of NO release from the polymer films in the presence of bacteria, leading to more potent antibacterial activity. This could potentially be achieved by increasing the surface area of the therapeutic to allow greater access and increase antibacterial activity. These polymer composite films could be used to make or coat medical devices to add long-term antibacterial properties, helping to address the problem of implant infection and improving patient outcomes.

5.8 Representative SEM Images

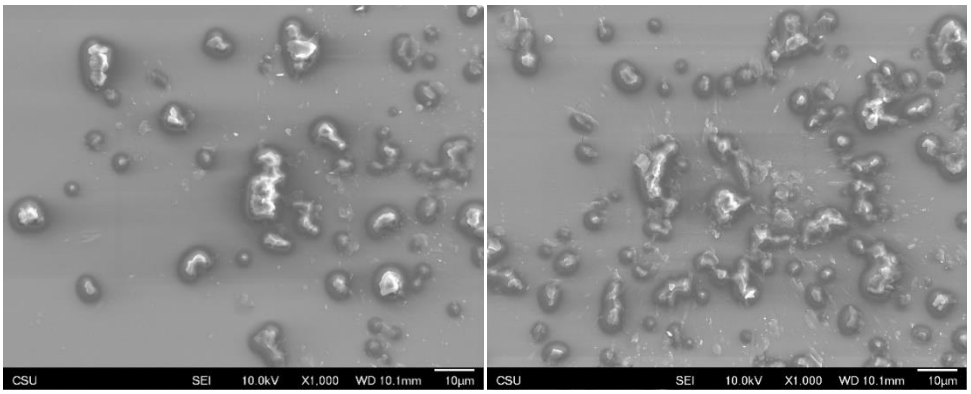

Fig. 5.6 Representative SEM images of control (0%) films at 1000x magnification.

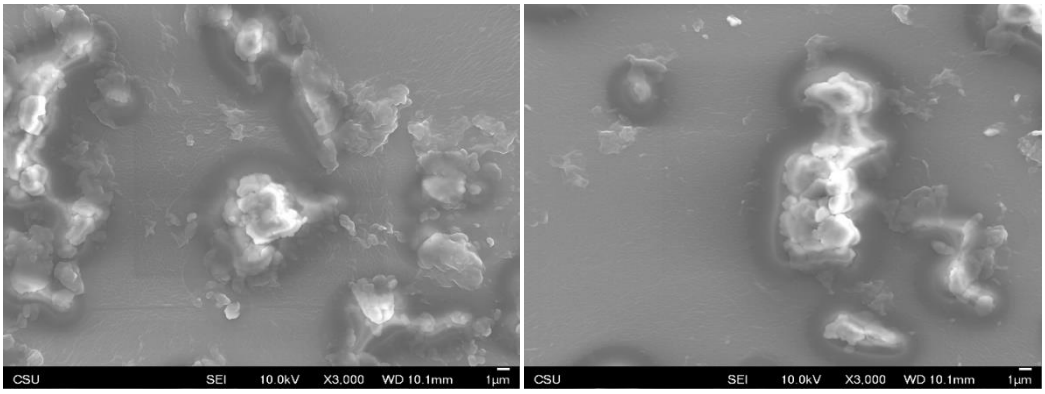

Fig. 5.7 Representative SEM images of control (0%) films at 3000x magnification.

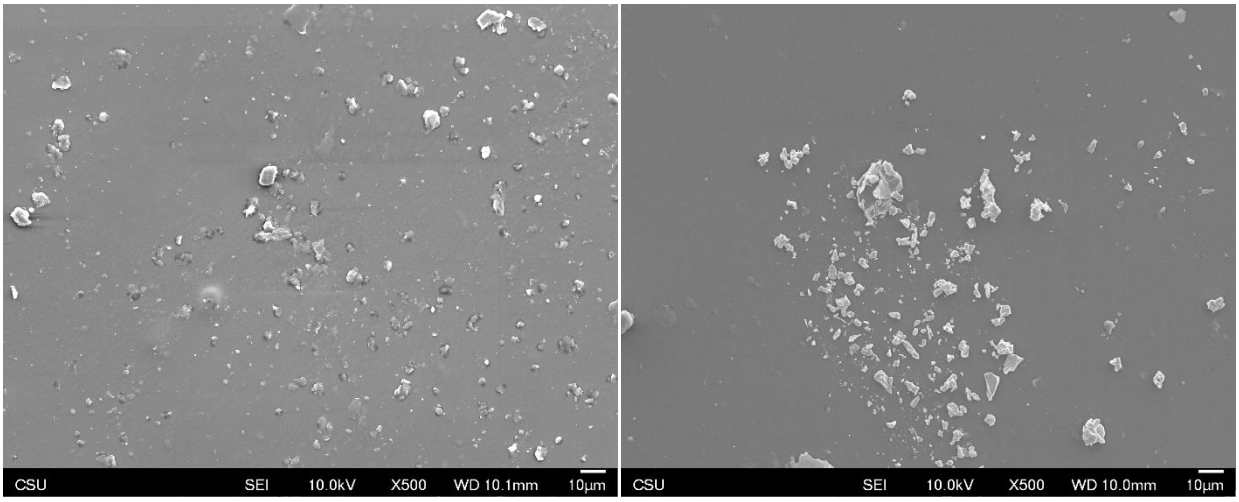

Fig. 5.8 Representative SEM images of 0.25% films at 500x magnification.

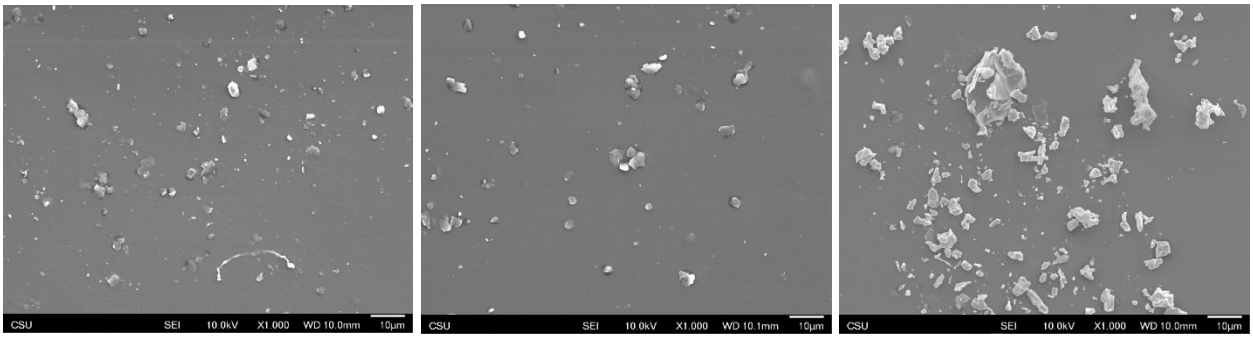

Fig. 5.9 Representative SEM images of 0.25% films at 1000x magnification.

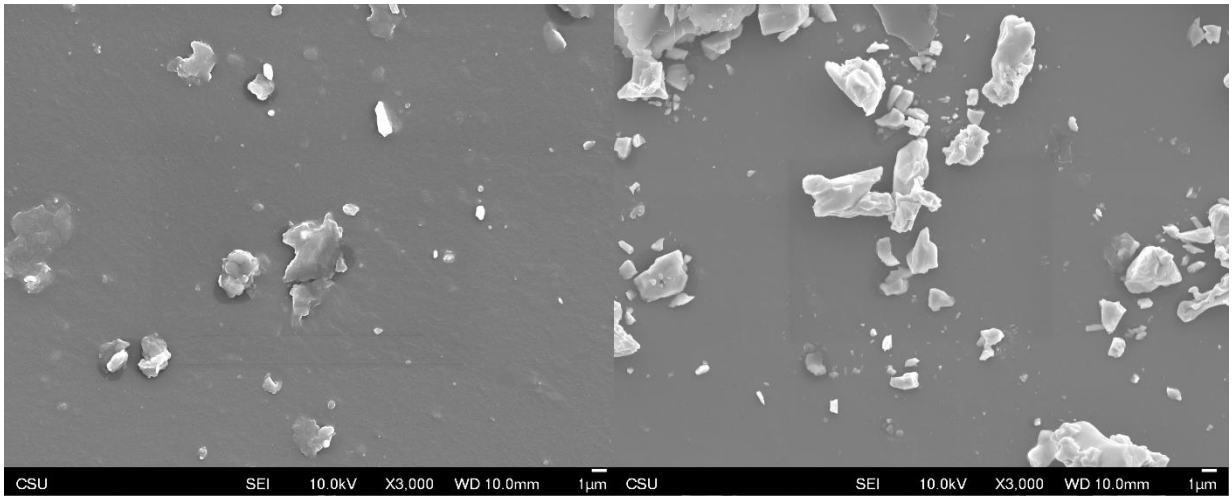

Fig. 5.10 Representative SEM images of 0.25% films at 3000x magnification.

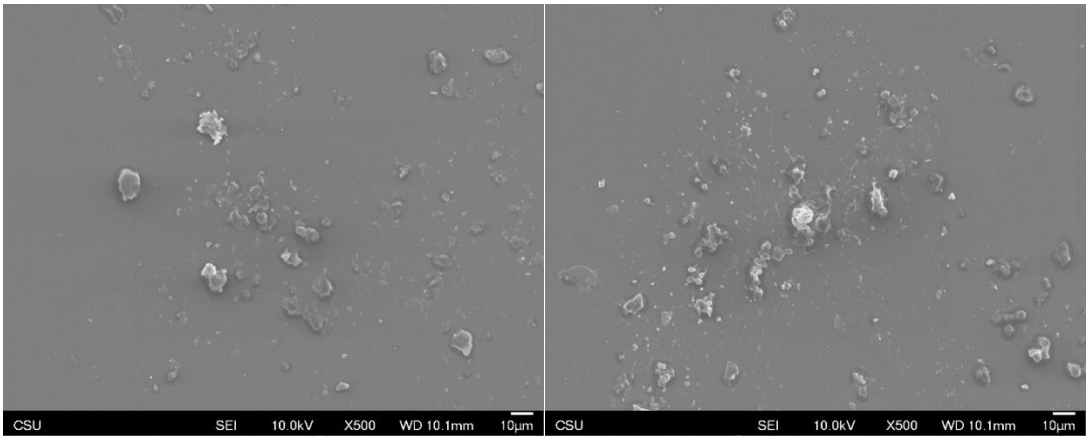

Fig. 5.11 Representative SEM images of 1% films at 500x magnification.

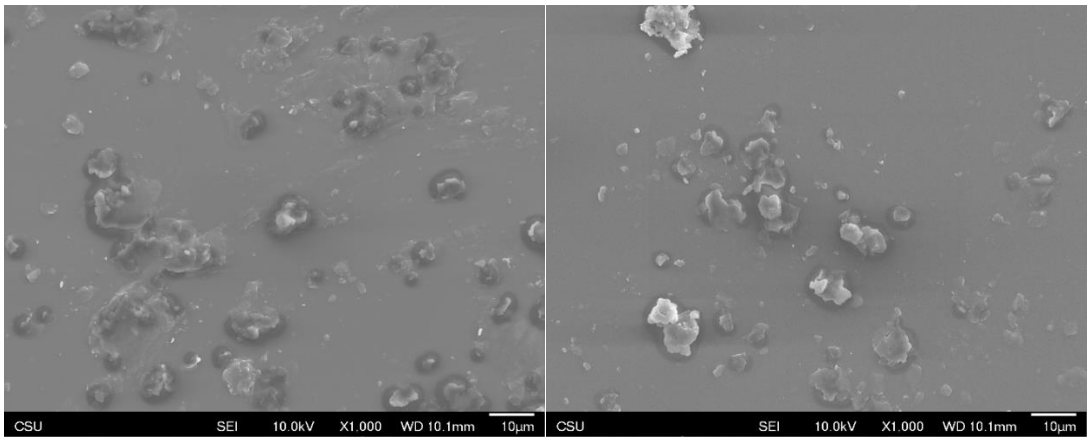

Fig. 5.12 Representative SEM images of 1% films at 1000x magnification.

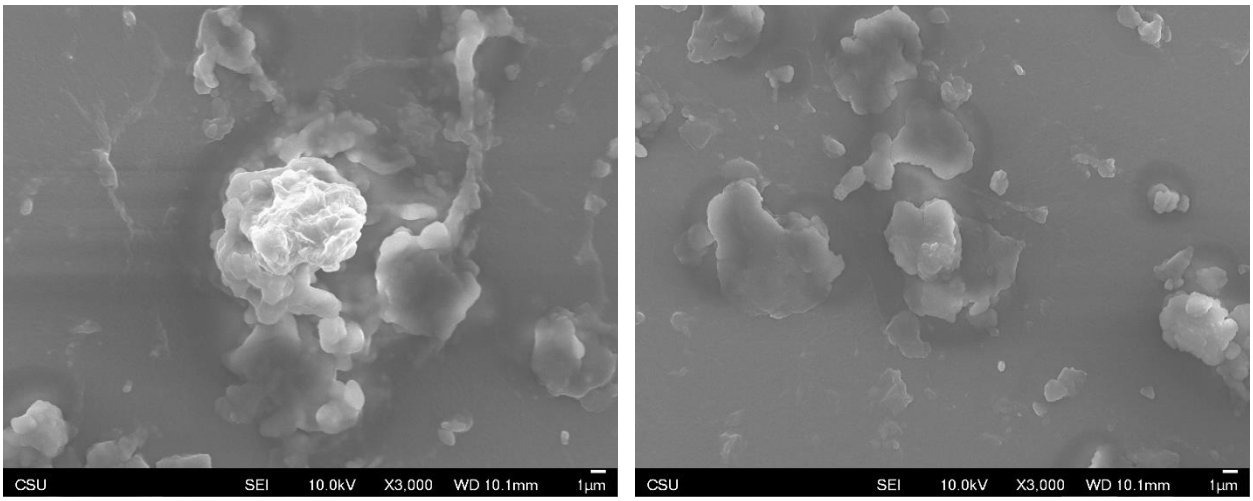

Fig. 5.13 Representative SEM images of 1% films at 3000x magnification.

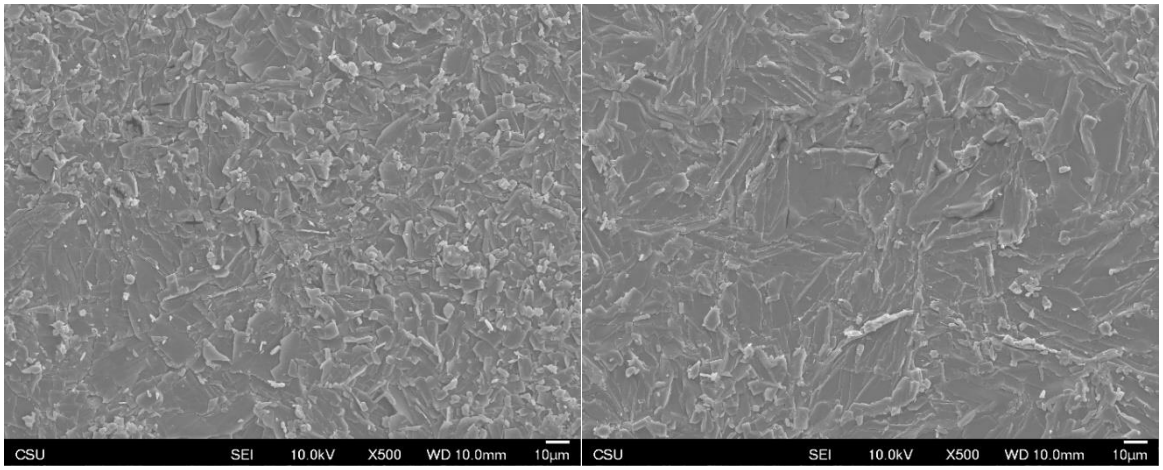

Fig. 5.14 Representative SEM images of 2.5% films at 500x magnification.

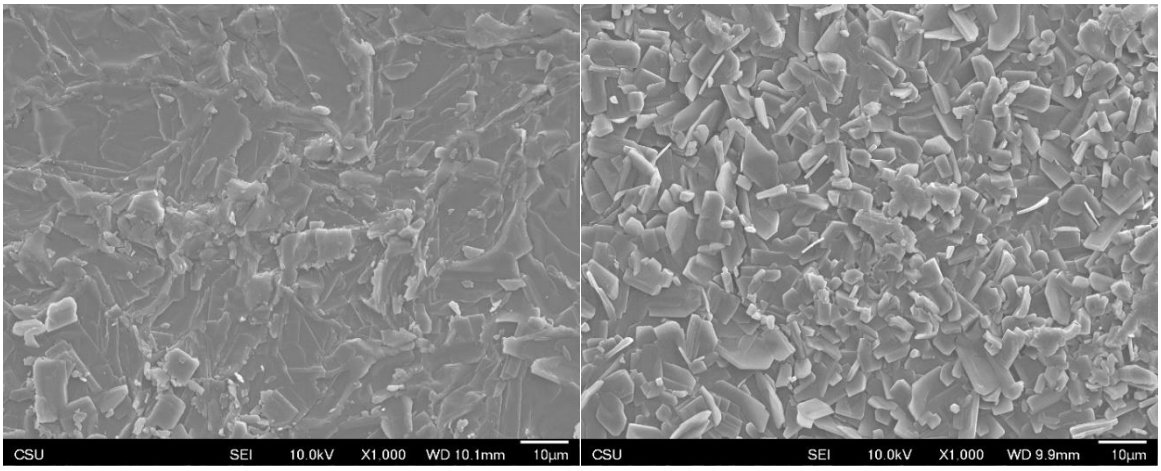

Fig. 5.15 Representative SEM images of 2.5% films at 1000x magnification.

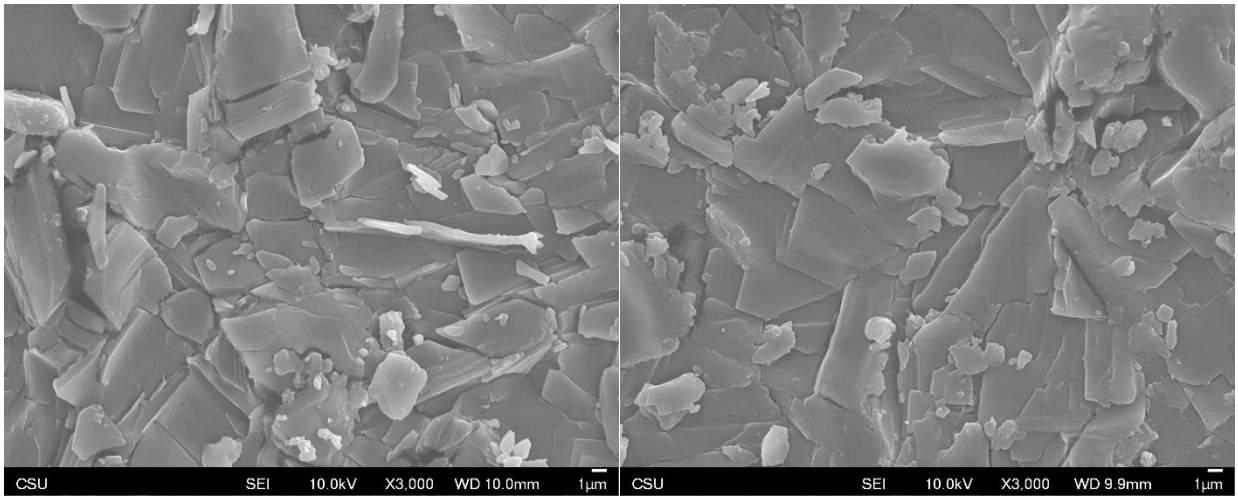

Fig. 5.16 Representative SEM images of 2.5% films at 3000x magnification.

5.9 Experimental NO Release Plots

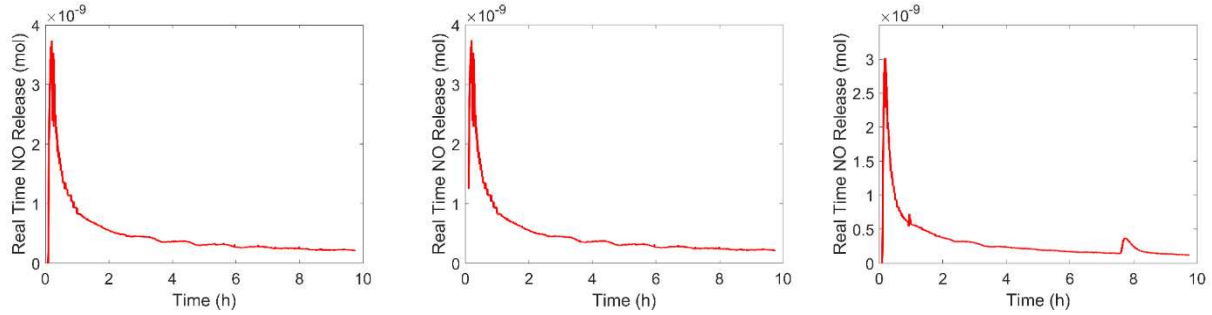

Fig. 5.17 Triplicate experiments of NO release for 0.25% w/v Et-PIPER/NO-NO_2 films

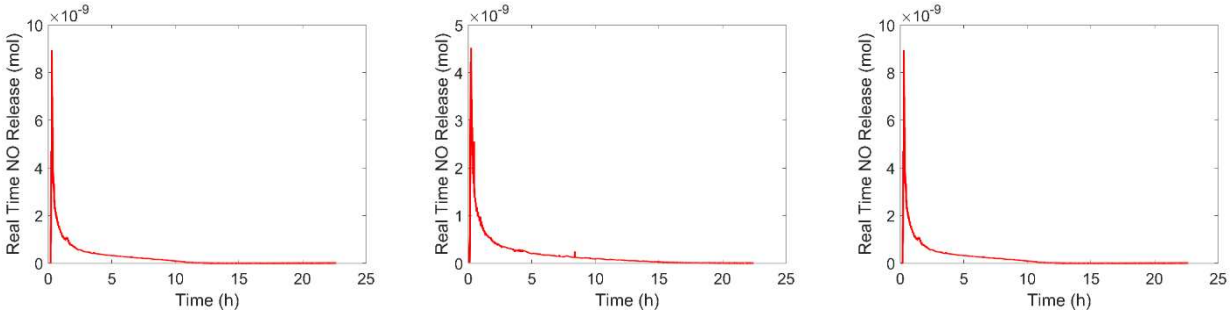

Fig. 5.18 Triplicate experiments of NO release for 1% w/v Et-PIPER/NO-NO_2 films

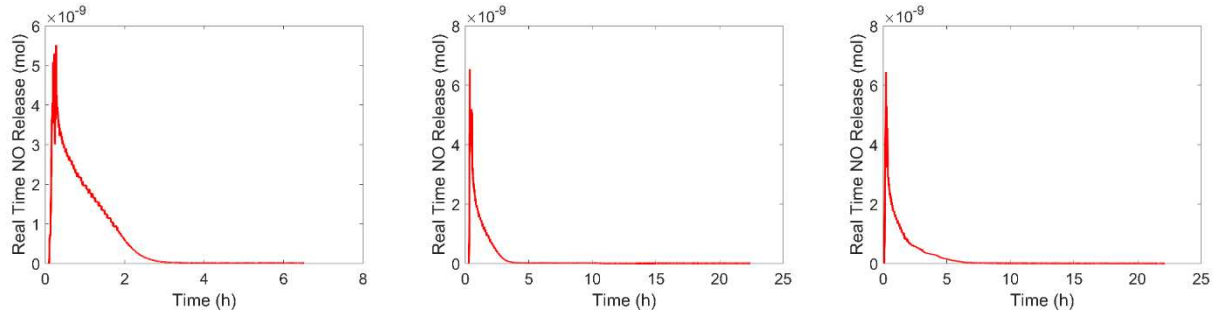

Fig. 5.19 Triplicate experiments of NO release for 2.5% w/v Et-PIPER/NO-NO_2 films

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CHAPTER 6

CONCLUSIONS AND FUTURE DIRECTIONS

6.1 Conclusions

The contributions made to the advancement of novel antibacterial materials research described in this dissertation are significant. The research began in this dissertation can be continued to develop practical NO-releasing antibiotics and antimicrobial materials. The project described in Chapters 2 and 3 represents the first example of a small molecule capable of both detecting and killing bacteria.[1] The synthesized molecule combines the idea of an enzyme-activated colorimetric bacterial indicator with a NO donor to impart antibacterial properties. Limited examples of dual function macromolecules have been previously described,[2–4] but a small molecule with multiple functions offers the advantages of being more versatile and can be incorporated into a polymeric matrix if desired. Through this work, I demonstrated the synthesis of a fluorescent indicator small molecule and its ability to change color in the presence of *P. aeruginosa*. I also showed that it releases NO in amounts known to have antibacterial activity, and that it causes up to a 65% reduction in *P. aeruginosa*. This research represents one of the first forays into developing multifunctional small molecule antibiotics, which is extremely important to address the complex problem of antibiotic resistance. As the current antibiotic resistance problem highlights, bacteria quickly evolve new pathways to develop resistance, and research aiming to combat this problem must focus on new areas of potential.

The work described in Chapters 4 and 5 represents some of the first examples of a bacterial enzyme-activated diazeniumdiolate prodrug materials.[5] NO has been known to be a potent antimicrobial agent for many years, but challenges have arisen in creating a stable

delivery system. Diazeniumdiolates hold great potential for controlled release of NO and incorporating them into a molecule activated by a bacterial enzyme could be a useful way to prevent bacterial resistance from developing. An enzyme-activated NO donor prodrug allows localized release of NO, avoiding indiscriminate exposure of bacteria to the therapeutic and the potential for resistance to develop. As discussed in Chapter 4, I successfully synthesized a nitroaromatic-protected diazeniumdiolate and demonstrated that NO release from the compound is nitroreductase enzyme activated. Antibacterial efficacy experiments demonstrated that the compounds lead to up to a 94% reduction in *E. coli*.

After synthesizing a novel nitroreductase-activated diazeniumdiolate, I wanted to incorporate the small molecule into a polymer material to demonstrate its potential biomedical application preventing infections on medical devices, as described in Chapter 5. Thorough characterization of the material by SEM, EDX, and NOA analysis ensured that the properties of the small molecule are maintained in the polymer composite. Incorporated into a polymer, the material demonstrated similar enzyme-activated NO-release and antibacterial activity against *E. coli*. This is one of the first examples of an enzyme-activated NO-releasing material with antibacterial activity, with the potential to prevent bacterial adhesion and biofilm formation by releasing NO when bacteria encounter the medical implant. This material could be used as a coating for medical devices to impart long-term antibacterial activity, preventing infections on in-dwelling devices and significantly improving patient outcomes.

6.2 Future Directions

6.2.1 Dual function fluorescent indicator

Based on the work described in this dissertation, there are several directions future work could take. For the dual function color changing small molecule project, it would be of interest to

determine the limit of detection (LOD) of the indicator. This would be useful to determine the smallest number of bacteria that need to be present to observe a visible color change and determine the limits of usability of the indicator.

Increasing the amount of nitrosation of the compound would potentially increase the antibacterial efficacy of the compound. Based on calculations from the NO release studies, the compound is only 37% nitrosated, releasing only 75% of the theoretical 200% NO release that is possible. Increasing the amount of NO release could potentially increase the antibacterial activity of the compound, as higher amounts of NO release should lead to higher antimicrobial activity. Increasing amounts of NO release could be accomplished by optimizing the diazeniumdiolate reaction conditions, through investigation of the effects of solvent, temperature, and reaction time, to favor higher amounts of product formation (diazeniumdiolate-functionalized material). This could also potentially be accomplished by first forming the PROLI/NO diazeniumdiolate and protecting it, then coupling the diazeniumdiolate to the fluorescent compound. This method would ensure that each molecule has a diazeniumdiolate functional group. A protecting group on the diazeniumdiolate would also prevent spontaneous release of NO, which could prevent detrimental side effects.

When considering diazeniumdiolates as therapeutics, it is important to consider that they can decompose into carcinogenic nitrosamines.[6] Cytotoxicity studies would be necessary before the synthesized fluorescent diazeniumdiolate compound is used in any *in vitro* study. The diazeniumdiolate formed is a derivative of PROLI/NO, which can form *N*-nitrosoproline, one of a few nitrosamines that has been shown to be non-carcinogenic.[7] This is a promising sign that the diazeniumdiolate would not decompose into a carcinogenic nitrosamine, but further toxicity

testing would be needed to ensure its safety. It would also be important to determine the cytotoxicity of the fluorescent aminoacridone, as this has not been previously investigated.

6.2.2 Nitroreductase-Activated Diazeniumdiolate and Polymer Composite

As previously stated, it is important to detect bacterial infections to help prevent the unnecessary prescription of antibiotics. Toward this goal, a method could be developed to determine if bacteria are present and to quantify the number of bacteria by relating the number of bacteria to the amount of nitroreductase with the nitroreductase activated diazeniumdiolates. This could be accomplished by quantifying the decrease in absorbance due to the nitro group of the nitroaromatic protecting group on the diazeniumdiolate, using UV-vis or another technique. As the nitro group is metabolized by the nitroreductase enzyme, its absorbance peak at around 375 nm will disappear in proportion.[8] The disappearance of the nitro group of the diazeniumdiolate protecting group is correlated to the presence of bacterial nitroreductase enzyme and the release of NO from the compound, so the disappearance of this peak can be used to indicate the presence of bacteria. Once this correlation is determined, the compound can be used to quantify the number of bacteria present, helping to diagnose a patient with a bacterial infection.

Another direction to improve the function and applicability of these compounds would be to try to increase the activity of the nitroreductase enzyme to the substrate. By using a different nitro-protecting group that bacteria have a higher affinity for, higher potency could potentially be achieved. There are several substrates that have been tested with nitroreductase enzymes and a few nitroreductase-activated antibiotics. For example, the cancer prodrugs CB1954 and PR-104A both are activated by nitroreductases found in *E. coli*.[9,10] Introducing substrates with similar substituents as these compounds could potentially increase the activity of the nitroreductase enzyme to the diazeniumdiolate, leading to higher amounts of NO release and higher

antibacterial efficacy. Antibiotics such as metronidazole and nitrofurantoin can be activated by nitroreductases and are other potential models for enzyme-activated diazeniumdiolate protecting groups that would allow for higher activity of the nitroreductases toward the substrate.[11,12]

Another direction would be to investigate bacteria species beyond *E. coli* and *P. aeruginosa* as I investigated in this dissertation. Most bacteria contain a nitroreductase enzyme, so other bacteria could potentially be killed by the nitroreductase-activated prodrugs I synthesized. For example, nitroreductases found in *Mycobacterium smegmatis, Bacillus amyloliquefaciens, Gluconobacter oxydans* have been characterized, among others.[13–15] These bacteria species could potentially activate the nitroaromatic-protected prodrugs similarly to the *E. coli*-derived nitroreductase NfsB.

In addition, as with the dual function compound, it will be important to determine the cytotoxicity of the compounds before their use in any *in vitro* experiments. It would be important to determine any degradation byproducts under physiological conditions, especially if the polymer composites were to be used for long term implants. The polymer composites would need to be tested to determine if there are any leachates from the material.

If cytotoxicity from byproducts or leachates from the polymer composite materials proved to be a challenge after testing, one direction to address this would be to covalently attach the NO donor molecule to the surface of the polyurethane substrate. Previous work has shown that this is a viable strategy, as polyurethanes can be functionalized with diazeniumdiolates[16] and piperazines can be used as linkers for diazeniumdiolate groups.[17] Polymers have also been functionalized with piperazine groups, which would be necessary for attachment of the Et-PIPER/NO-NO_2 diazeniumdiolate group.[18–20] By functionalizing polyurethane with piperazine, the piperazine could then be reacted with NO to form the diazeniumdiolate group, which would

then be covalently attached to the polymer, then the diazeniumdiolate could be protected with the nitroaromatic group to prevent spontaneous release. Covalent attachment of the diazeniumdiolate group to the polymer material would prevent release of byproducts from the material, which could help avoid potential cytotoxicity problems. As the application of this material is for medical device coatings, covalent attachment of the diazeniumdiolate to the surface of the polymer would allow optimal access of bacteria to the enzyme-activated therapeutic.

It would also be of interest to investigate amount of bacterial adhesion to the surface of the polymer films. In the experiments that I described in Chapter 5 of this dissertation, I counted the CFUs in the bacterial solution after removing the polymer composite materials. These experiments show that the polymer materials are able to reduce the number of bacteria in the surrounding solution, but to further probe how these materials would interact in a medical device coating, it would ideal to test bacterial adhesion to the surface of the materials. This would be interesting because the bacteria must interact with the polymer composite in order for NO to be released, but it would be interesting to see if the subsequent NO release is sufficient to prevent bacterial adhesion to the surface. This is especially important, as bacterial adhesion is the first step in biofilm formation, which NO is known to prevent.[21,22]

6.3 References

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