THE DEVELOPMENT AND SYNTHESES OF NITROREDUCTASE TRIGGERED RELEASABLE LINKERS FOR USE IN SIDEROPHORE DRUG CONJUGATES

A Thesis

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Bacterial resistance to antibiotics is becoming increasingly prevalent and dictates a desperate need for new therapies. A means of fighting this resistance is through the exploitation of microbial iron uptake. In order to obtain this necessary nutrient, bacteria synthesize a plethora of low molecular weight iron chelators known as siderophores. While drugs can be attached to these molecules and actively transported into cells in a “Trojan Horse” approach, activity is often lost when the target is present in the cytoplasm. As these drugs no longer reach their target or do not bind correctly, the incorporation of a releasable linker between the siderophore and antibiotic may restore activity and circumvent resistance due to decreased permeability of the bacterial outer membrane.

In this thesis, work towards the production of siderophore-drug-conjugates containing nitroreductase-triggered linkers is discussed. In chapter 2, the design and syntheses of linkers hypothesized to be reduced and cyclized by bacterial nitroreductase
enzymes are described. Initially, test molecules were synthesized and reduced to demonstrate the feasibility of triggered drug release. After this proof of principle, a first generation linker was prepared, however a nitroreductase assay suggested that linkers with adjusted reduction potential might be necessary for drug release. Therefore, additional molecules were synthesized and the three prepared linkers were coupled to ciprofloxacin to form linker-drug conjugates. Biological testing was completed for several synthesized molecules and dinitro containing compounds showed moderate to strong activity against *M. vaccae* and *M. tuberculosis*.

In chapter 3 the production of a mixed-ligand siderophore and attempts at preparing siderophore-drug conjugates are described. Many conditions were employed unsuccessfully to couple the siderophore and the linker-drug conjugates. Currently, a new pathway is underway with the hope that it will be successful in the formation of siderophore-drug conjugates. Further studies are required to see if nitroreductase-triggered linkers in siderophore-drug conjugates will create effective antibacterial agents.
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ABBREVIATIONS

AcOH…………………………acetic acid
ACN…………………………..acetonitrile
Bn……………………………..benzyl
Boc…………………………....tert-butoxycarbonyl
br, s…………………………....broad singlet
˚C……………………………..degrees Celsius
CDI…………………………..carbonyldiimidazole
CDMT………………………...2-chloro-4,6-dimethoxy-1,3,5-triazine
δ………………………………chemical shift in parts per million downfield from
tetramethylsilane
DCM………………………….dichloromethane
DFO…………………………..desferrioxamine
DMAP………………………….4-dimethylaminopyridine
DMF……………………….N,N-dimethylformamide
DMFDMA………………….N,N-dimethylformamide dimethyl acetal
DMSO………………………...dimethylsulfoxide
d……………………………..doublet
dd..............................doublet of doublets

dt..............................doublet of triplets

E..............................Escherichia

EDC............................1-ethyl-3-(3-dimethylaminopropyl)carbodiimide

EEDQ...........................N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline

Et₂O..............................diethyl ether

EtOAc..........................ethyl acetate

FMN..............................flavin mononucleotide

g...............................gram(s)

h...............................hour(s)

¹H...............................proton

HCl..............................hydrochloric acid

Hex..............................hexanes

Hz...............................hertz

HOBt............................hydroxybenztriazole

HPLC............................high performance liquid chromatography

LC-MS............................liquid chromatography mass spectrometry

μ...............................micro

m...............................multiplet

M...............................molarity (moles per liter)

M......................Mycobacterium

mg............................milligram

MHz.............................megahertz
PNB.......................... $p$-nitrobenzyl
q..............................quartet (spectral)
rt...........................room temperature
s............................singlet
$S$...........................$Staphylococcus$
SA...........................succinic anhydride
t..........................triplet
td...........................triplet of doublets
TFA..........................trifluoroacetic acid
THF..........................tetrahydrofuran
TLC...........................thin layer chromatography
troc..........................trichloroethyl chloroformate
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CHAPTER 1:
THE NEVER ENDING BACTERIAL WAR

1.1 Introduction to Antibiotics

Antibacterial agents selectively interfere with critical processes in the bacterial cell and can either be natural products, semisynthetic derivatives of these compounds, or completely synthetic materials. Most of the classes of antibiotics in use today are based on natural products while only three scaffolds (sulfa drugs, quinolones, and oxazolidinones) are synthetic.\textsuperscript{1} The discovery and subsequent development of these molecules has been paramount in the last century. Since its discovery, penicillin and related compounds have been hailed as miracle drugs due to their ability to kill bacteria responsible for diseases such as pneumonia and syphilis and are credited with half of the greater than 20-year increase in the average lifespan since the 1920s.\textsuperscript{2}

It was once believed that the battle against infectious diseases was won, causing many pharmaceutical companies to move away from antibiotic research.\textsuperscript{2} Unfortunately, this also coincided with the rise of resistance mechanisms to various antibacterial agents. Overuse and misuse of antibiotics by health professionals as well as overuse in food animals have exacerbated the problem of resistance.\textsuperscript{3} Cases of resistance in clinical settings have been reported for all currently approved antibiotics and this resistance is rapidly spreading to the outside community.\textsuperscript{4} Several strains of particularly resistant bacteria have emerged including methicillin-resistant \textit{Staphylococcus aureus} (MRSA),
which is estimated to cause around 19,000 deaths per year in the United States, multidrug-resistant and pandrug-resistant Gram-negative bacteria, and multidrug-resistant and extensively drug-resistant *Mycobacterium tuberculosis*, which is especially prevalent in developing countries.\(^5\) In combination with this rise in resistance, the decrease in antibiotic development could trigger a return to pre-antibiotic times if new drugs are not discovered.

![Resistant strains spread rapidly](image)

**Figure 1.1:** The rise of resistance (left) and the decrease of antibiotic development (right) could bring about a return to pre-antibiotic times. Figure taken from Taubes.\(^4\)

1.2 **Bacterial resistance mechanisms**

Within a few years of penicillin’s introduction to the drug market, the first strains of resistant *Staphylococcus aureus* were detected.\(^4\) Since then, a proliferation of antibiotic-resistant strains have emerged. Bacteria have developed several modes of resistance against antibiotics, the most well known of which are inactivating enzymes such as β-lactamases. This resistance mechanism occurs when a bacterium is exposed to an antibiotic agent and in turn produces an enzyme capable of degrading or inactivating the drug.\(^2\) In the case of β-lactamases, the β-lactam ring is hydrolyzed and therefore no longer effective. Many generations of this drug class have been produced in order to
restore activity; however resistance inevitably reemerges due to mutation or acquisition from another microbe. This issue has also been addressed through the use of specific β-lactamase inhibitors which are co-administered with β-lactam antibiotics to extend the utility of the drugs.\textsuperscript{2,6} Additionally, resistance to antibiotics through target modification has also been noted. When exposed to an antibiotic, some bacteria are capable of producing an alternative enzymatic target to which the drug binds less effectively. This has been seen in microbes such as MRSA, which are resistant due to the presence of an additional penicillin-binding protein that does not bind penicillins such as methicillin or oxacillin. Other strains are also able to adjust biosynthetic pathways so that the enzymatic target is no longer utilized and the drug will therefore be ineffective.\textsuperscript{6}

The use of inactivating enzymes as well as target modifications occurs commonly in Gram-positive bacteria. However, Gram-negative bacteria have additional resistance mechanisms due to the presence of an additional outer membrane.\textsuperscript{4} This membrane contains porins, proteins through which small molecules can diffuse. In order to reduce the concentration of drug in the bacterial cell, these porins can be adjusted or deleted. This reduced outer membrane permeability to antibiotics is especially harmful, as it potentiates all other modes of resistance due to reduced concentration of the drug at its target. Additionally, Gram-negative bacteria have the ability to synthesize efflux pumps which can actively remove specific antibiotics from the cell and prevent them from reaching their targets.\textsuperscript{2,6} These types of resistance are especially prevalent in strains such as \textit{Pseudomonas aeruginosa}, \textit{Acinetobactor baumannii}, and \textit{Klebsiella pneumonia} which can cause deadly infections due to their ability to become resistant to virtually all antibiotics.\textsuperscript{4}
1.3 Bacterial Iron Uptake Pathways

Although it is possible to restore antibiotic activity through the modification of drugs already on the market, new pathways must also be pursued in order to bypass the resistance pathways outlined above. One means of bypassing resistance due to reduced outer membrane permeability in Gram-negative bacteria involves the exploitation of iron uptake pathways. Iron is an essential element due to its important role in many enzymes as well as in biological functions such as the production of DNA, oxygen binding and transport with hemoglobin and myoglobin, and energy generation in the cytochrome and cytochrome oxidase systems. However, this element is difficult for microbes to obtain due to its low solubility in the environment (the $K_{sp}$ of Fe(OH)$_3$ is $10^{-39}$) as well as in the infected host. At the onset of infection, the first line of defense for the host is the sequestration of iron by the proteins lactoferrin, transferrin, and ferritin. The resulting competition for iron determines the course of the infection.

Since bacteria require micromolar levels of iron to grow, they synthesize and excrete low molecular weight iron chelators (500-1500 Daltons) known as siderophores.
of which there are over five hundred known structures from terrestrial microbes alone. Iron(III) is bound tightly and selectively by these molecules, often in an octahedral fashion, through the presence of ligands, such as catechols, hydroxamates, or polycarboxylates.\textsuperscript{8} While some species of bacteria only produce and secrete one siderophore, others produce multiple molecules in order to potentially increase virulence.\textsuperscript{9}

![Diagram of siderophores and functional groups](image)

**Figure 1.3**: Representative Siderophores (left) and Functional Groups Commonly Utilized for Tight Iron Binding (right).

For Gram-negative bacteria, siderophore complexes are recognized by outer membrane receptors which then interact with TonB, a protein bound to the inner membrane. A conformational change driven by a protein gradient across the inner membrane transports the siderophore into the periplasm where it is bound by a protein such as FhuD, which then transports the complex to the inner membrane. Finally, the siderophore is transported across the cytoplasmic membrane via an ATP binding cassette (ABC) transporter complex. In Gram-positive bacteria, siderophores are recognized by receptors in the plasma membrane and transported into the cytoplasm as discussed above. In both cases, outer membrane receptor proteins are often specifically designed for the functional groups the siderophore contains. Once the siderophore complex reaches the
cytoplasm iron is most often released through reduction from iron(III) to iron(II), for which the siderophore has a much lower affinity for, or through an esterase cleavage of the siderophore backbone. The siderophore is commonly excreted back into the extracellular space after iron has been released. Some strains of bacteria also express additional outer membrane receptors which sequester iron from heme, transferrin, or lactoferrin present in a host body. Once the iron(II) concentration in the cell is sufficient, uptake is halted by the Fur repressor protein, which blocks the biosynthesis of the components of the transport system. During times of iron starvation, such as infection, this pathway is upregulated, making it a suitable target for antimicrobial therapies.

**Figure 1.4:** Methods for iron uptake in Gram-negative bacteria. Figure taken from Sandy and Butler.°
1.4 Siderophores as Antibiotics

One method of utilizing bacterial iron uptake pathways as an antibiotic target is the use of siderophore-mediated drug delivery. In this “Trojan Horse” approach, an antibiotic can be actively transported into the bacterial cell when attached to a siderophore. Like many classes of antimicrobials, these drug conjugates have been produced in nature. While bacterial receptor proteins are specific for the iron complexes of native siderophores, the ability to acquire iron is absolutely essential and some strains have evolved the means of stealing iron from competing microbes though the uptake of xenosiderophores. In order to protect themselves from iron thievery and ensure survival through elimination of competing microbes, some bacteria synthesize natural examples of siderophore-drug conjugates, known as sideromycins.\textsuperscript{10,11} Examples of these molecules include the albomycins, produced by \emph{Actinomyces}, and the salmycins, produced by \emph{Streptomycyes}.\textsuperscript{12} The albomycins contain a toxic thionucleoside moiety which inhibits seryl tRNA synthesis and has activity against both Gram-positive and Gram-negative strains. The salmycins contain an aminoglycoside antibiotic which inhibits an unidentified step of protein synthesis in Gram-positive bacteria. Without attachment to the siderophore, the thionucleoside of the albomycins is 30,000-fold less effective against certain strains of bacteria, demonstrating the need for active transport into the cell as the antibiotic cannot diffuse into the cell.\textsuperscript{10,11} While the antibiotic moiety makes up a substantial part of these molecules, bacterial outer membrane receptors are still able to recognize and transport the complexes, suggesting that this is promising antibiotic target.\textsuperscript{11}
While siderophore drug conjugates have been produced in nature, there are also many synthetic examples which attempt to mimic this activity. The first synthetic drug conjugates, produced in 1977, utilized sulfonamides but showed only minimal activity when a spacer was present between the iron binder and the drug. Since then, hundreds of synthetic sideromycins have been produced, many of which have successfully utilized β-lactam antibiotics. In one case, the ampicillin conjugate of a rationally designed siderophore 1.6 was a thousand times more active than the free drug alone against \textit{P. aeruginosa} and \textit{S. maltophilia}, both of which are intrinsically resistant due to reduced outer membrane permeability. It was also determined that these molecules were not substrates for the efflux pumps in \textit{P. aeruginosa}. In a separate case, conjugates of ampicillin (1.7) and amoxicillin (1.8) and a tris-catecholate siderophore, which mimics the backbone of enterobactin, were prepared. These molecules showed strong activity against \textit{P. aeruginosa} (MICs ranging from 0.05 to 0.39 µM) and moderate activity against \textit{E. coli}. The success of β-lactam conjugates is likely because of the location of the penicillin binding proteins (PBPs) in the periplasm, as only the outer membrane must be crossed to reach the target, or because β-lactams tolerate significant side chain...
modification. Additionally, binding studies have shown that the presence of the siderophore does not prevent binding to the PBP.\textsuperscript{10,12} While in many cases β-lactam-siderophore conjugates showed very potent activity, resistance to these molecules does eventually develop. The isolated mutants have damaged iron uptake systems, with the deletion of the outer membrane receptors necessary for transport of the drug conjugate. However, these strains are non-pathogenic and do not survive well under iron-deficient conditions, suggesting that this type of antibiotic therapy is very promising.\textsuperscript{13}

1.5 Drug Release in Siderophore-Drug Conjugates

While β-lactam-siderophore conjugates have been shown to have strong activity, reduced activity relative to the free drug has been observed in conjugates with targets present in the cytoplasm, such as the quinolones. Linkage to the siderophore either prevents these drugs from reaching their target or significantly lowers the binding affinity of the drug. It is therefore necessary to modify these molecules so that drug release can
occur and the antibiotic activity of the drug can be restored. This problem has already been addressed in nature in regards to the aforementioned albomycins. The toxic thionucleoside pyrimidine moiety of this conjugate is released by a serine protease in the bacterial cell. Similarly, it is hypothesized that the salmycins are subject to a drug release process through a cyclization process upon iron reduction as seen in **Figure 1.7**.\(^\text{10}\)

![Figure 1.7: Hypothesized drug release process for the salmycins.](image)

While many siderophore drug conjugates have been produced, there are limited examples of synthetic molecules containing a linker allowing drug release. Previous studies have involved the use of fluoroquinolones which are an ideal choice as their targets are present in the cytoplasm. These antibiotics inhibit both DNA gyrase and topoisomerase II, preventing DNA replication.\(^\text{15}\) Early studies utilized conjugates of the primary siderophore of *Pseudomonas*, pyoverdin, and contained either a stable linker or an unspecifically hydrolyzable carbamate linker that was hypothesized to be
enzymatically cleaved. The nonreleasable conjugates had reduced activity relative to antibiotic alone while a releasable linker-norfloxacin conjugate showed enhanced activity against *P. aeruginosa*, suggesting that a drug release process was occurring.\textsuperscript{16} In later studies, the secondary siderophore of *Pseudomonas*, pyochelin, was utilized instead due to the strain specific nature of the pyoverdines. Again the stable conjugates showed reduced antibiotic activity relative to the free drug alone. However, conjugates containing the labile linker showed some activity, although less than that of the free drug. Further testing demonstrated that these conjugates displayed activity against a strain of *P. aeruginosa* that is unable to utilize pyochelin as a siderophore, suggesting that these linkers were hydrolyzed extracellularly.\textsuperscript{17, 18}

Figure 1.8: Example Pyochelin-norfloxacin conjugates with a stable linker (1.9) and a hydrolyzable linker (1.10)\textsuperscript{17}

Work towards the utilization of enzymatically-triggered linkers has also been pursued. Conjugates were designed that contained functionality allowing for esterase- or
phosphatase-mediated drug release. These enzymes are widely distributed in bacteria and would trigger lactonization, the rate of which is enhanced by $10^5$ due to the presence of the ‘trimethyl lock’ as seen in Figure 1.8. The phosphatase-triggered conjugate did not show activity, possibly due to the lack of active uptake through iron transport pathways. The esterase-triggered conjugate, although less active than a parent drug, showed moderate to good antibiotic activity against several bacterial strains. However, it is possible that these linkers could be hydrolyzed extracellularly or that reduced activity could be due to poor enzyme recognition.19

Figure 1.9: Esterase/Phosphatase triggered siderophore-drug conjugates utilizing a trimethyl lock.19
In order to prevent extracellular hydrolysis, the trimethyl lock-containing linker was adjusted to be triggered under reduction conditions. In this case, the linker is composed of a quinone that can be reduced to a hydroquinone by hydride sources such as flavin or NADPH present in the bacterial cell used to reduce iron (III) to iron (II). This hydroquinone would quickly lactonize and release the attached drug selectively in the cell. This reduction and release process was tested in vitro through the treatment of a ciprofloxacin-releasable linker-desferrioxamine conjugate 1.14 with sodium dithionate. Monitoring with LC-MS showed that the quinone was reduced to the hydroquinone and ciprofloxacin was released from the conjugate, suggesting that drug release in the bacterial cell was also feasible. 20

![Figure 1.10: Reduction, lactonization, and drug release of a DFO-releasable linker-ciprofloxacin conjugate](image)

While this drug release process was demonstrated to be successful in vitro, this was not necessarily the case in vivo. When tested against bacteria, the siderophore-drug-conjugates with this linker showed moderate to strong activity (0.5-32 µM) relative to the counterparts containing stable linkers. However, these conjugates still showed reduced activity relative to the free drug alone suggesting there was either difficulty in siderophore recognition or in reduction of the linker in the bacterial cell. 20 This work suggests that triggered-linkers are a promising means of restoring activity to siderophore-
drug conjugates with non β-lactam drugs. Since the use of iron uptake is a means of overcoming resistance due to decreased outer membrane permeability, more research into drug release processes should be pursued in the search of new antibiotics. One such possibility is the utilization of different enzymatic processes in bacteria to trigger drug release in the cell. As discussed below, nitroreductases are a promising target due to their promiscuity and prevalence in bacterial systems as well as absence in mammalian systems.
2.1 Introduction to Nitroreductase-Triggered Cyclization and Drug Release

As there has been some success with reduction-triggered linkers in siderophore-drug conjugates, it is likely that other enzymes can be utilized in a drug release process. It is hypothesized that through the use of a 2-nitrophenylacetate based linker in siderophore drug conjugates, bacterial nitroreductase enzymes can reduce the nitro group to produce a hydroxylamine or an amine, either of which can perform a nucleophilic attack at the carbonyl carbon and cause cyclization and release of an attached group as seen in Figure 2.1.

Figure 2.1: Proposed reduction and drug release in vivo of a siderophore drug conjugate with a 2-nitrophenylacetate based linker

Bacterial nitroreductases are a class of enzymes which are NADPH-dependent (nicotinamide adenine dinucleotide phosphate) and contain FMN (flavin mononucleotide) as a cofactor. These enzymes are capable of reducing a broad range of nitro-containing
substrates and are present in a large variety of bacterial strains.\textsuperscript{21,22} Human cells do not produce nitroreductase enzymes, although iodothyrosine deiodinase, located in thyroid cells, contains a nitroreductase like domain which is only responsible iodine scavenging.\textsuperscript{22} Nitroreductases are divided into two classes based on the reduction process utilized. Type I is the most common and utilizes a two electron transfer from NADPH to the nitro group to first form a nitroso moiety, which is quickly reduced again due its reactive nature to produce the hydroxylamine. The hydroxylamine can then be further reduced to an amine. In type II nitroreductases, a one-electron transfer process occurs producing a nitro radical which subsequently reacts with oxygen to produce a superoxide radical and regenerates the nitro group. These two processes can be seen in Figure 2.2.\textsuperscript{21,22}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig2.png}
\caption{Reaction pathways of type I and II nitroreductases.}
\end{figure}

There are several antibiotics which require nitroreductases for activity. Metronidazole is a nitro-containing antibiotic, which is used primarily against \textit{Helicobacter pylori} and several strains of anaerobic bacteria.\textsuperscript{22} The antibiotics nitrofurazone and nitrofurantoin, which are effective against both Gram-positive and Gram-negative bacteria, also require nitroreduction for activity. Nitrofurazone and
nitrofurantoin are used for the treatment of genitourinary infections and topically for the
treatment of burns respectively. Studies have shown that activation of these two drugs
occurs through the use of type I nitroreductases to produce the hydroxylamine, which is
believed to react with protein or DNA to cause cell damage.\textsuperscript{23} Resistance to nitro
containing drugs has been linked to mutations of the genes associated with type I
nitroreductase production.\textsuperscript{22,23} The utilization of these enzymes in antibiotic activation
suggests that the use of nitroreductase-triggered linkers in siderophore-drug conjugates is
a feasible approach.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure2.3.png}
\caption{Example of antibiotics requiring nitroreductases for activity}
\end{figure}

2.2 Synthesis of Test Molecules

In order to test the feasibility of nitroreduction-triggered drug release, a series of
cocules was synthesized and subjected to reduction conditions to determine the result.
As 2-nitrophenylacetic acid (2-NPAA) is the central component of the proposed
siderophore drug conjugate, potential leaving groups were attached to see if they would
be released once the nitro group was reduced. Initially three test molecules were
synthesized. 2-NPAA was treated with benzyl bromide and potassium carbonate to form
molecule 2.6. A Fischer esterification with methanol was also performed on the starting
material to produce compound 2.7. The acid was also coupled to benzylamine through

\textbf{2.3 Metronidazole} \quad \textbf{2.4 Nitrofurazone} \quad \textbf{2.5 Nitrofurantoin}
the use of EDC to generate 2.8. These three compounds were subsequently treated with iron powder and acetic acid to reduce the nitro group and facilitate cyclization. In each case oxindole 2.10 was isolated, suggesting that cyclization and release of the leaving group did occur. To further test this drug release process, a ciprofloxacin conjugate was prepared through the use of EDC in the presence of DMAP and HOBt to afford 2.9. While the yield was low as this was a coupling to a secondary amine, the obtained material was also reduced to afford 2.10 as with the previously synthesized molecules, suggesting that cyclization and drug release had occurred.

Scheme 2.1: Production and reduction of test molecules to determine the feasibility of nitroreduction triggered drug release

2.3 Preparation and Elaboration of the First Releasable Linker

As the test molecules previously demonstrated cyclization and release of a leaving group, work towards producing an adjusted 2-NPAA-based linker with a second handle for conjugation to a siderophore was pursued. Initially, aromatic halogenation of 2-
NPAA was attempted so that further elaboration through processes such as nucleophilic aromatic substitution or the use of an organometallic cross-coupling reaction could be utilized. However, since the presence of the nitro group deactivates the ring, harsh conditions were required and the only successful reaction produced multiple isomers which were difficult to separate. As halogenation proved to be a difficult route, a different synthetic pathway was attempted. Protected 4-methyl-3-nitrobenzoic acid would be further elaborated to produce a molecule with an acetic acid moiety, which could then be coupled to a drug. Initially, $p$-toluoyl chloride was reacted with methanol to produce methyl 4-methyl benzoate (2.11). This molecule was then nitrated to produce methyl 4-methyl 3-nitrobenzoate (2.12). Several different reactions were then attempted to install the acetic acid handle.

Molecule 2.12 contains fairly acidic protons on the methyl group ortho to the nitro group. Initially, treatment of this molecule with sodium methoxide and diethyl oxalate followed by sodium hydroxide and hydrogen peroxide was attempted but with no success. While this reaction was adjusted several times, only starting material was ever isolated. Instead, Brederick’s reagents, tert-butoxy bis(dimethylamino) methane, tris(dimethylamino) methane, or dimethylformamide dimethyl acetal (DMFDMA), were employed to add on an enamine moiety and produce 2.13. This molecule was hydrolyzed to produce aldehyde 2.14 which was then oxidized with chromium trioxide and sulfuric acid to produce the desired molecule 2.15.
Following the production of the first linker, work to install a spacer that would be present between the linker and the siderophore was pursued. In order to retain the ability of the nitro group to be a substrate for bacterial nitroreductases, this spacer may be necessary to leave sufficient distance between the linker and the siderophore. The free acid of linker 2.15 was first protected with a tert-butyl ester through an EDC coupling with tert-butyl alcohol to produce 2.16, which was treated with lithium hydroxide to cleave the methyl ester and afford 2.17. This molecule was then treated with N-hydroxysuccinimide and EDC to form an activated ester, which was treated with excess ethylenediamine to produce 2.18. This molecule could in turn be conjugated to a siderophore through the amine and to a drug after removal of the tert-butyl ester.
Scheme 2.3: Addition of an ethylenediamine spacer to the first linker.

Four of the previously synthesized molecules, \textbf{2.6}, \textbf{2.8}, \textbf{2.15}, and \textbf{2.17}, were incubated with wild-type \textit{E. coli} for 24 hours to see if the bacterial nitroreductase enzymes were able to reduce the nitro group and cause cyclization. After incubation, the bacteria were filtered out and the compounds were extracted from the broth and analyzed with HPLC. In all cases the isolated material showed a large peak for the starting material as well as smaller peaks which did not correspond to the cyclized product. Relative to the starting material and luria broth peaks, the other peaks were much smaller, making it difficult to identify other compounds. It is possible that the bacteria need longer incubation times or abnormally permeable outer membranes to be reduced by the bacteria. However, it may possible that the reduction potential of these molecules is not within the range of the bacteria. This suggested that this reduction potential might have to be adjusted in order to optimize the efficacy of this drug release system.

2.4 Adjustment of the Reduction Potential of the Releasable Linker

From the results of the nitroreductase assay discussed above, it was hypothesized that the addition of electron withdrawing groups to the ring of the linker would adjust the reduction potential of the nitro group such that it would be a substrate for these enzymes. Initially, the addition of iodine to the ring was attempted through the treatment of \textbf{2.12}
with \( I_2 \) in sulfuric acid. This reaction was not successful and instead a procedure utilizing \( N \)-bromosuccinimide (NBS) was attempted and produced the bromine-containing molecule \( 2.19 \). This molecule was reacted with DMFDMA to produce the enamine \( 2.20 \), which was hydrolyzed with 1N HCl in THF to afford the aldehyde \( 2.21 \). This aldehyde was then oxidized with chromium trioxide and sulfuric acid to afford the brominated linker \( 2.22 \). A similar process was pursued in order to produce a dinitro-containing linker which would theoretically be reduced more easily than the first linker and the bromine-containing linker. Initially, 3,5-dinitro \( p \)-toluic acid was treated with thionyl chloride in methanol to produce the methyl ester \( 2.23 \). This molecule was reacted with DMFDMA to produce the enamine \( 2.24 \). It is interesting to note that the additional nitro molecule lowered the reaction time from 8 hours for the mononitrated compound to 1 hour. The enamine was hydrolyzed under acidic conditions to afford the aldehyde \( 2.25 \) which was then oxidized to afford the dinitro linker \( 2.26 \).

**Scheme 2.4:** Synthesis of additional linkers containing an electron withdrawing group to adjust the reduction potential of the nitro group.

Once these new linkers were produced, their reduction potentials relative to the previously produced linker were qualitatively tested. Fisher esterifications of \( 2.15, 2.22 \),
and 2.26 were performed in order to produce the methyl esters of these molecules (2.27, 2.28, and 2.29 respectively). The mononitrated molecule 2.27 took 2.5 hours to reduce and cyclize while the bromine-containing molecule 2.28 only required 1.25 hours for complete conversion. The dinitro-containing linker 2.29 took only 25 minutes to fully react. This suggests that the new linkers may be better substrates for bacterial nitroreductase enzymes as they are easier to reduce than the previously synthesized mononitrated linker.

Scheme 2.5: Qualitative comparison of reduction potentials of the three synthesized linkers

Once these new linkers were produced, installing ethylene diamine spacers was pursued. While this had been a relatively straightforward process with the mononitrated
linker, this process was surprisingly difficult with the two new linkers. Coupling with EDC and tert-butyl alcohol proved completely unsuccessful, and instead a new procedure was attempted, using di-tert-butyl dicarbonate (Boc₂O) and 4-dimethylaminopyridine (DMAP) in tert-butanol and afforded the desired compounds 2.33 and 2.34. Following the installation of the tert-butyl protecting group, removal of the methyl protecting group was attempted. This would be followed by the formation of an NHS ester and subsequent reaction with ethylenediamine. However, saponification conditions caused decomposition suggesting that this pathway would have to be altered.

Since the methyl protecting group could not be removed without causing decomposition, allyl esters were installed in this system since they can be removed under milder conditions. 4-methyl 3-benzoic acid was treated with either NBS or nitric acid to produce the brominated compound 2.39 or the dinitro compound 2.40. These acids were allyl protected through reaction with allyl bromide and the resulting compounds 2.41 and 2.42 were treated with DMFDMA. It was expected that this would produce the desired enamines 2.45 and 2.46. However methoxide is produced during this reaction and the product isolated was actually the transesterified material 2.43 and 2.44. While some time was spent making different Brederick reagents such as DMF diisopropyl amine,²⁵ which

Scheme 2.6: Pathway attempted to install the ethylenediamine spacer to the new linkers.
would be less likely to cause transesterification due to the bulkier nature of isopropoxide, this pathway was not pursued further due to the development of a more efficient synthesis for the linkers as is discussed below.

**Scheme 2.7**: Attempts at producing linkers utilizing allyl protection.

2.5: A More Efficient Synthesis of Releasable Linkers

As an alternative plan for synthesis, it was decided to install the ethylenediamine spacer prior to further elaboration. Previously, the synthetic scheme involved the synthesis of the linker followed by the installation of the spacer and then the coupling of the spacer to the carboxylic acid of a siderophore. Following this, the protecting group present on the linker could be removed and a drug attached. However, not only were there issues with this pathway as discussed above, but this would require larger amounts of siderophore, which is a precious molecule due to the many steps required to produce it. Installing the spacer first would allow for installation of a drug prior to conjugation to a siderophore, preventing loss of this molecule in additional steps and allowing for easier purification. 4-Methyl-3-benzoic acid, the brominated acid 2.39, and the dinitro acid 2.40 were coupled to mono-Boc ethylenediamine with EDC in to afford 2.47, 2.48, and 2.49.
These molecules were then treated with DMFDMA to form the enamines 2.50, 2.51, 2.52. For the mononitrated molecule the reaction was very sluggish and reaction conditions had to be adjusted in order to obtain complete conversion. The use of two extra equivalents of DMFDMA allowed for success in this reaction. It is also notable that no tranesterification was observed in these reactions. These enamines were then hydrolyzed carefully with 1M HCl and THF to produce aldehydes 2.53, 2.54, and 2.55 with no loss of the Boc group. These aldehydes were oxidized using a Pinnick oxidation,26 as the previously utilized Jones oxidation involves the use of strong acid, which had the potential to remove the protecting group. These reactions were successful in moderate yields to produce the desired acids 2.56, 2.57, and 2.58. Once the three acids were produced, they were converted to the activated NHS esters and reacted with ciprofloxacin to produce 2.59, 2.60, and 2.61. Ciprofloxacin was chosen since siderophore-drug conjugates containing cytoplasmic drugs typically have reduced antibacterial activity relative to the free drug alone.10 The reaction with ciprofloxacin resulted in low yields for the dinitro containing molecule and the bromine-containing molecule. This is possibly due to steric hindrance of the additional groups on the ring. The Boc group can then be removed with HCl and the free amine can be conjugated to a siderophore as is discussed in the following chapter.
**Scheme 2.8:** Production of linker-ciprofloxacin conjugates through a more efficient synthesis

2.6 Biological Activity

Nine of the synthesized compounds from section 2.5 were subjected to testing against several strains of bacteria using the agar well diffusion test. The enamines 2.50-2.52 were not initially tested due to the possibility that they would hydrolyze in the media. Additionally, the ciprofloxacin conjugates 2.59-2.61 were not tested, as they were difficult to purify and likely contaminated by free ciprofloxacin, which would skew the results. While aldehydes 2.54 and 2.55 showed some activity against *B. subtilis*, the dinitro compounds 2.49, 2.55, and 2.58 showed exciting activity against *M. vaccae*. The bromo containing acid 2.57 also showed moderate activity against this strain of bacteria. Due to the strong activity of the dinitro compounds against *M. vaccae*, the enamine 2.52 was later submitted for testing as well. This molecule showed strong activity against *M. vaccae* as well as some activity against other Gram-positive strains. This data can be seen in **Table 2.1**. The agar plate containing *M. vaccae* and compounds 2.47-2.49 and 2.53-2.58 can be seen in **Figure 2.4**.
### TABLE 2.1

SPECTRUM OF ANTIMICROBIAL ACTIVITY IN THE AGAR DIFFUSION ASSAY

<table>
<thead>
<tr>
<th>Compound</th>
<th>Conc.</th>
<th>Solvent</th>
<th>Zone of Growth Inhibition (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>B. subtilis</td>
</tr>
<tr>
<td>ND-010954</td>
<td>2 mM</td>
<td>DMSO/MEOH</td>
<td>0</td>
</tr>
<tr>
<td>(2.47)</td>
<td></td>
<td></td>
<td>ATCC 6633</td>
</tr>
<tr>
<td>ND-010958</td>
<td>2 mM</td>
<td>DMSO/MEOH</td>
<td>0</td>
</tr>
<tr>
<td>(2.48)</td>
<td></td>
<td></td>
<td>ATCC 6633</td>
</tr>
<tr>
<td>ND-010959</td>
<td>2 mM</td>
<td>DMSO/MEOH</td>
<td>0</td>
</tr>
<tr>
<td>(2.49)</td>
<td></td>
<td></td>
<td>ATCC 6633</td>
</tr>
<tr>
<td>ND-010961</td>
<td>2 mM</td>
<td>DMSO/MEOH</td>
<td>0</td>
</tr>
<tr>
<td>(2.52)</td>
<td></td>
<td></td>
<td>ATCC 6633</td>
</tr>
<tr>
<td>ND-011094</td>
<td>2 mM</td>
<td>DMSO/MEOH</td>
<td>15P</td>
</tr>
<tr>
<td>(2.53)</td>
<td></td>
<td></td>
<td>ATCC 6633</td>
</tr>
<tr>
<td>ND-010962</td>
<td>2 mM</td>
<td>DMSO/MEOH</td>
<td>0</td>
</tr>
<tr>
<td>(2.54)</td>
<td></td>
<td></td>
<td>ATCC 6633</td>
</tr>
<tr>
<td>ND-010963</td>
<td>2 mM</td>
<td>DMSO/MEOH</td>
<td>15V</td>
</tr>
<tr>
<td>(2.55)</td>
<td></td>
<td></td>
<td>ATCC 6633</td>
</tr>
<tr>
<td>ND-010966</td>
<td>2 mM</td>
<td>DMSO/MEOH</td>
<td>0</td>
</tr>
<tr>
<td>(2.56)</td>
<td></td>
<td></td>
<td>ATCC 6633</td>
</tr>
<tr>
<td>ND-010964</td>
<td>2 mM</td>
<td>DMSO/MEOH</td>
<td>0</td>
</tr>
<tr>
<td>(2.57)</td>
<td></td>
<td></td>
<td>ATCC 6633</td>
</tr>
<tr>
<td>ND-010965</td>
<td>2 mM</td>
<td>DMSO/MEOH</td>
<td>0</td>
</tr>
<tr>
<td>(2.58)</td>
<td></td>
<td></td>
<td>ATCC 6633</td>
</tr>
<tr>
<td>DMSO/MEOH</td>
<td>1:10</td>
<td>DMSO/MEOH</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ATCC 6633</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>5 µg/mL</td>
<td>H₂O</td>
<td>1.66µg/ml</td>
</tr>
</tbody>
</table>

Notes: **Normal MHII Conditions**

All solutions were prepared by first making a 20 mM solution in DMSO and diluting 10-fold with MEOH to give 2 mM solution. Plates were incubated at 37°C for 22 hrs. M. succinogenes was incubated 37°C for 44 hrs.

- h: Indicates only a hint of growth inhibition detectable.
- p: Indicates unclear inhibition zone.
- V: Indicates a very unclear inhibition zone.
- *: Indicates a slightly missmaphen zone.
Figure 2.4: Agar plate containing *M. vaccae* with large zones of inhibition for dinitro containing compounds.

Additionally, several compounds were submitted for testing against *Mycobacterium tuberculosis*. These were tested by microplate alamar blue assay\textsuperscript{27} (MABA), in glycerol-alanine-salts media\textsuperscript{28} (GAS), and by low-oxygen recovery assay\textsuperscript{29} (LORA), which determines activity against non-replicating TB. All the assays were performed as per published protocols.\textsuperscript{27-29} The half maximal inhibitory concentration (IC\textsubscript{50}) was also determined for these compounds. Interestingly, the four dinitro compounds tested showed moderate to excellent activity against TB as seen in Table 2.2. All other compounds showed no activity.
TABLE 2.2
EVALUATION OF COMPOUNDS AGAINST M. TUBERCULOSIS

<table>
<thead>
<tr>
<th>Compounds</th>
<th>H$_{37}$R$_V$-TB</th>
<th></th>
<th></th>
<th>IC$_{50}$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MABA (µM)</td>
<td>GAS (µM)</td>
<td>LORA (µM)</td>
<td></td>
</tr>
<tr>
<td>ND-010931 (2.18)</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>nt$^a$</td>
<td>nt</td>
</tr>
<tr>
<td>ND-010948 (2.22)</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>nt</td>
<td>nt</td>
</tr>
<tr>
<td>ND-010947 (2.26)</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>nt</td>
<td>nt</td>
</tr>
<tr>
<td>ND-010958 (2.48)</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>nt</td>
<td>nt</td>
</tr>
<tr>
<td>ND-010959 (2.49)</td>
<td>2.36</td>
<td>2.81</td>
<td>&gt;50</td>
<td>47.59</td>
</tr>
<tr>
<td>ND-010960 (2.51)</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>nt</td>
<td>nt</td>
</tr>
<tr>
<td>ND-010961 (2.52)</td>
<td>4.18</td>
<td>24.37</td>
<td>&gt;50</td>
<td>nt</td>
</tr>
<tr>
<td>ND-010963 (2.54)</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>nt</td>
<td>nt</td>
</tr>
<tr>
<td>ND-010962 (2.55)</td>
<td>5.89</td>
<td>42.51</td>
<td>&gt;50</td>
<td>46.80</td>
</tr>
<tr>
<td>ND-010966 (2.56)</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>nt</td>
<td>nt</td>
</tr>
<tr>
<td>ND-010964 (2.57)</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>nt</td>
<td>nt</td>
</tr>
<tr>
<td>ND-010965 (2.58)</td>
<td>1.81</td>
<td>1.33</td>
<td>&gt;50</td>
<td>43.39</td>
</tr>
</tbody>
</table>

$^a$nt = not tested
3.1 Introduction to the Mixed Ligand Siderophore

Once the syntheses of nitroreductase-triggered releasable linkers were begun, it was necessary to select a siderophore to utilize in drug conjugates. As there have been many examples of siderophore-drug conjugates, there is a multitude from which to select. One factor to consider was the development of resistance to the previously synthesized molecules. In one case, separate catechol-based and hydroxamic acid-based siderophore-carbacephalosporin conjugates were synthesized and tested. While these molecules showed strong antibacterial activity, mutants to each compound were isolated. It was determined that the selected bacteria had a defect in siderophore receptors. When treated with the catechol-containing compound, mutants showed a decrease in expression of Cir, whereas bacteria resistant to the hydroxamate-containing compound showed less expression of FhuA. These proteins specifically recognize the functional groups present in the siderophore of the compound they were exposed to, and the mutants remained susceptible to the other conjugate. It was also discovered that co-administration of the conjugates resulted in better activity than either compound alone. This suggests that the use of a siderophore containing multiple functional groups would not only avoid resistance due to decrease in outer membrane protein expression, but may produce a conjugate with stronger overall activity. Therefore, a mixed catechol- and
hydroxamate-containing siderophore was selected for use in siderophore-nitroreductase triggered linker-drug conjugates.

Figure 3.1: Hydroxamate-based and catechol-based siderophore-carbacephalosporin conjugates

To date, several examples of siderophore-drug conjugates containing the mixed ligand siderophore have been produced. In one case, a loracarbef conjugate (3.3) was produced and showed potent activity against *Acinetobacter baumanii* (MIC = 0.03 µg/mL whereas loracarbef has a MIC = 64 µg/mL). Additionally, a mixed-ligand siderophore-vancomycin conjugate was prepared. However, this molecule showed reduced activity against Gram-positive bacteria and was inactive against the Gram-negative strains tested. Similarly, a ciprofloxacin conjugate (3.4) was also synthesized but showed no activity whatsoever. These results suggest that conjugates utilizing this siderophore and non-β-lactam drugs may benefit from the use of a drug release process. In order to test this hypothesis, a mixed-ligand siderophore-ciprofloxacin conjugate was prepared with the use of a reduction-triggered linker as discussed in Chapter 1. This molecule (3.5) showed stronger activity than its stable linker counterpart, but was less activity than the free drug alone. This suggests that drug release is occurring inside of the cell although
the release process is not optimized. Therefore, the mixed-ligand siderophore is ideal for continued study with releasable linkers.

![Siderophore Structure](image)

**Figure 3.2:** Previously studied mixed-ligand siderophore drug conjugates.²⁰, ³², ³⁴

3.2 Total Synthesis of the Siderophore

In order to utilize the mixed-ligand siderophore in drug conjugates, it was first necessary to prepare this molecule. While previous syntheses have been published, there were issues encountered throughout this process. First, 2,3-dihydroxybenzoic acid was converted to methyl 2,3-dihydroxybenzoate (3.6) through treatment with thionyl chloride in methanol. This molecule was then further protected with benzyl groups to produce methyl 2,3-bis(benzyloxy)benzoate (3.7) which was saponified to produce 2,3-bis(benzyloxy)benzoic acid (3.8). This reaction did not follow the literature procedure exactly and adjustments were necessary to improve the yields in this step. This acid was
in turn conjugated to a spermidine backbone through the use of carbonyldiimidazole (CDI). There were a few difficulties with this step as well. The published procedure used called for a reaction time of one day, which did not provide complete conversion, and a mobile phase for chromatography, which did not move the product from the baseline. Instead an adjusted procedure was utilized, which provided the product 3.9 in moderate to high yields. Interestingly, the yields diminished when the scale of the reaction was increased. After this reaction was optimized, a succinate spacer was installed. Initially, a procedure was attempted which reacted 3.9 with the NHS ester of \( p \)-nitrobenzyl (PNB) protected succinate in the presence of 4-dimethylaminopyridine (DMAP). However, as this reaction was never successful, direct treatment with succinic anhydride (SA) and DMAP was utilized instead to produce 3.10. While it would have been possible to determine the means to successfully react 3.9 with the NHS ester of PNB-protected succinate, this would also introduce an additional step to remove the PNB group, introducing an additional opportunity for loss of product.

**Scheme 3.1**: Initial steps in the synthesis of the mixed-ligand siderophore
Following production of 3.10, it was necessary to synthesize the hydroxamic acid-containing portion of the siderophore (3.13). This was completed through the Mitsonobu reaction of Boc-protected aminopentanol (3.11) and Troc-protected O-benzylhydroxylamine (3.12). Compound 3.13 was then treated with trifluoroacetic acid (TFA) and coupled to 3.10 through the use of N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) to produce 3.14. This reaction was completed many times in order to produce more siderophore for conjugation attempts. While initial yields were high, further attempts were inconsistent and varied from 16-75%. The Troc group of this molecule was then removed through the use of zinc and acetic acid to afford the fully protected siderophore 3.15. The benzyl protecting groups were removed through the use of 10% palladium on carbon (10% Pd/C) to produce the mixed ligand siderophore.

![Scheme 3.2: Completion of mixed-ligand siderophore synthesis](image-url)
3.3 Initial attempts at Siderophore-Drug-Conjugate Formation

Following the production of both linker-drug conjugates and the mixed-ligand siderophore, it was necessary to attempt coupling reactions to produce the desired siderophore-drug conjugates. The initial strategy to generate these molecules was to deprotect the siderophore prior to coupling, as hydrogenolysis conditions would likely also reduce the nitro group present in the linker. This dictated the need for reaction conditions that would selectively couple a carboxylic acid and an amine without competition from catechols or hydroxamates. Due to previous success in our group, 2-chloro-4,6-dimethoxy-1,3,5-triazine (CDMT) was selected for use. This coupling agent and a non-nucleophilic base (such as \(N\)-methylmorpholine) was first added to the carboxylic acid to produce an activated ester to which the amine was added.\(^{38,39}\) In this case, the amine 3.16 was produced through removal of the Boc group with concentrated HCl and used immediately, as this material decomposed over time. This amine was detected by LCMS, however peaks in the NMR spectra were so broad that this was a difficult means of characterization. All deprotected amines utilized in this chapter showed similar results. The reaction with CDMT was attempted multiple times and in each case, careful monitoring with LCMS revealed only the presence of the starting materials and the activated ester. Even after several days of stirring, no coupling was ever seen, and the addition of heat resulted only in the hydrolysis of the activated ester. Additionally, coupling of the iron-bound siderophore to the linker was also attempted. This was anticipated to prevent any competition from catechols and hydroxamates during coupling reactions. The bright purple iron complex was formed through the use of Fe(acac)\(_3\).\(^{34}\) As bacteria recognize the iron complex of siderophores for active uptake, it was
hypothesized that utilizing the iron-bound molecule would not affect activity. Coupling through the use of EDC and HOBt was attempted; however solubility of the iron complex was poor in most solvents making characterization and reaction monitoring difficult. Therefore, this reaction pathway was not extensively pursued.

Scheme 3.3: Initial coupling attempts utilizing CDMT.

Since there was no success in reacting the deprotected siderophore, work on coupling the protected siderophore to the linker-ciprofloxacin conjugates was then pursued. As deprotection of the final compound with Pd/C was not possible since it would reduce the nitro group, other procedures would be necessary once the protected conjugates were produced. Initially, a procedure used to make the aforementioned mixed-ligand siderophore-carbacephalosporin conjugates was attempted. This reaction which involved the production of an HOBt activated ester was attempted twice, once in THF, which resulted in low solubility of the deprotected linker-ciprofloxacin conjugate, and once in DMF where the solubility was improved. No desired product was seen in
either reaction. Since it was possible that there was coupling occurring between the carboxylic acid of ciprofloxacin and the amine of the linker to form dimers, it was decided that preformation of an activated ester would be a preferred coupling method. Therefore, the fully protected siderophore (3.15) was treated with N-hydroxysuccinimide and EDC to produce the NHS activated ester (3.19). A solution of the deprotected linker-ciprofloxacin conjugate and triethylamine would then be added to the NHS ester of the siderophore. Initially, LCMS indicated that there was a small amount of product (3.19) present when the deprotected mononitrated linker-ciprofloxacin conjugate (3.16) was used. However, it was not the main peak, and upon repeating the reaction, only the activated ester (3.19) and the hydrolyzed material (3.14) were seen, even after allowing the material to stir for several days. Since the deprotected bromine (3.20) or dinitro (3.21) linker-ciprofloxacin conjugates were different electronically, perhaps coupling these molecules might afford the desired compounds. When monitoring these reactions by TLC, color was an important indicator. Ciprofloxacin shines a bright blue color under UV light which could be indicative of the desired product. In the case of the bromine containing linker, a new blue spot appeared with a slightly lower R_f value than that of the NHS ester. However, upon analysis with LCMS, the desired product was not present. In regards to the dinitro-containing molecule, a new blue spot was also seen with a much lower R_f value than the NHS ester. Although this observation was promising, this spot again did not correspond to the desired product. Although these two reactions were checked with both LCMS and HRMS, only the NHS ester (3.20) and hydrolyzed material (3.15) were detected. In addition, this reaction was attempted in a mixture of methanol and water upon suggestion that this would better couple the zwitterionic linker-
ciprofloxacin conjugate. As before, no product was detected. It was believed that perhaps these couplings were unsuccessful due to the bulky nature of the two components, therefore the reaction of a deprotected linker that was not conjugated to ciprofloxacin was also attempted. However, no product was seen, suggesting that different procedures would need to be utilized in further coupling attempts.

**Scheme 3.4:** Attempted couplings of the protected mixed ligand siderophore

Instead of isolating the activated ester prior to reacting with the amine, conditions were utilized that formed activated molecules *in situ*. As CDI was previously used successfully, this reagent was utilized. This reaction again resulted in no desired product and LCMS showed only the activated ester and hydrolyzed material (3.14). Based on a
procedure utilized to make siderophore-β-lactam conjugates, preforming the mixed anhydride was also attempted through reaction of the siderophore with methyl chloroformate followed by addition of the deprotected linker-ciprofloxacin conjugate.\textsuperscript{41} Unfortunately, this reaction did not produce the desired material either. Additional work with methyl chloroformate was also pursued. Since it is possible to make the reactive mixed anhydride with a chloroformate, it was believed that treatment of the fully deprotected mixed ligand siderophore with several equivalents of this reagent would result in both a fully carbonate-protected molecule as well as the activated species. These carbonate groups would not affect activity as they are lost upon iron binding. This reaction was attempted twice under slightly different conditions; however, each attempt resulted in a mixture of starting material, di-reacted, tri-reacted, and tetra-reacted molecules, causing this pathway to be abandoned.

\textbf{Scheme 3.5:} Additional coupling reactions attempted to produce benzyl protected siderophore drug conjugates
From these results, a few possible conclusions were made. Since couplings with linker-ciprofloxacin conjugates or a deprotected linker with no drug attached were unsuccessful, it was thought that perhaps the ionizable groups present were preventing success. Additionally, it is possible that coupling large molecules is difficult due to steric hindrance. Based on these observations, adjustments were made in order to facilitate successful couplings.

3.4 Further Attempts at Producing Siderophore-Drug-Conjugates

As it was hypothesized that the ionizable groups were affecting coupling, a linker-benzyl protected ciprofloxacin conjugate 3.25 was produced. As the siderophore was also benzyl protected, the addition of one more benzyl group should not substantially affect deprotection. In each case, the Boc protecting group was removed from 3.25 first and used immediately to prevent decomposition of the molecule. Initially, this amine was reacted with the NHS ester of the siderophore (3.20), but the reaction was again unsuccessful. As there was now no carboxylic acid group present on ciprofloxacin, different reactions, such as EDC couplings, could also be attempted without concern for dimerization. In the first attempt, EDC and DMAP were used but no product was detected. Additionally, the production of an HOBt activated ester in situ was also employed but with no success.

As the utilization of protected ciprofloxacin did not seem to affect coupling success, it was decided that the use of a longer spacer between the siderophore and the linker might bypass coupling issues due to steric hindrance. Compound 3.26 was
prepared using the same synthetic pathway outlined in Chapter 2, but beginning with Boc-butanediamine. In case ionizable groups were also an issue, protected ciprofloxacin was used as well. As with previous reaction attempts, the Boc group of 3.26 was removed with HCl and added to the NHS ester of the siderophore 3.20. Again, coupling was unsuccessful. These results suggest that it may simply be impossible to make the bond between the amine of the linker and the carboxylic acid of the siderophore.

Scheme 3.6: Coupling reactions attempted with a linker-benzyl protected ciprofloxacin conjugate and a linker containing a longer amine spacer.

Due to the previous failures in coupling attempts, a new scheme was proposed involving a different order of steps. This was adapted from the preparation of the carbacephalosporin-mixed-ligand siderophore-conjugate. In this case, the hydroxamate-containing portion of the siderophore 3.13 was first attached to the linker-drug moiety and then the resulting molecule would be reacted with the backbone of the
siderophore 3.10. To accomplish this, 3.13 was first stirred with zinc and \( N \)-methylimidazole in the presence of succinic anhydride to both remove the Troc group and add on a succinate spacer and produce 3.28.\(^2\) In turn, this molecule was coupled with the deprotected longer linker (3.29). Initial attempts involving the use of EDC and DMAP were unsuccessful. However, when the acid was reacted with CDI followed by the addition of the amine, initial results showed the desired product (3.30) as the main peak by LCMS. Further studies of this reaction are necessary to determine if the 3.30 can be produced. If so, this molecule can be treated with TFA to to remove the Boc group and coupled to 3.10 to produce the protected siderophore-drug-conjugate as seen in Scheme 3.7.

**Scheme 3.7:** New pathway in progress to produce siderophore-drug-conjugates
4.1 Conclusions

Although the total synthesis of the desired siderophore-drug conjugates containing nitroreductase-triggered linkers has not yet been achieved, the work described here provides promising leads which may be applied to this project’s completion. In this thesis, the syntheses of linkers hypothesized to be reduced and cyclized by bacterial nitroreductase enzymes were described. Initial work with test molecules demonstrated the feasibility of nitroreduction triggered drug release. From there, the first generation linker (2.15) was produced, but a nitroreductase assay suggested that linkers with adjusted reduction potential might be needed for activity. With this in mind, two additional linkers (2.22 and 2.26) were produced, but the previously utilized pathway for installing a spacer was unsuccessful, demonstrating a need for an alternative scheme to produce the desired molecules. This new scheme proved successful and allowed for the syntheses of ciprofloxacin-releasable linker conjugates (2.59, 2.60, and 2.61). Biological testing was completed for several of these compounds. The dinitro-containing compounds 2.52, 2.55, and 2.58 showed moderate to strong activity against M. vaccae and M. tuberculosis.

Additionally, a mixed-ligand siderophore was selected for use in drug-conjugates as it is hypothesized that a siderophore containing multiple functional groups would
prevent resistance due to decreases in outer membrane protein expression. Therefore, the total synthesis of this molecule was completed for use in siderophore-drug conjugates. From there, a multitude of conditions were employed to couple the siderophore to the deprotected linker-drug conjugates. Thus far, successful conditions for this coupling remain elusive. A new pathway is currently underway with the hope that it will be successful in the formation of siderophore-drug conjugates. Further studies will be required to see if the use of nitroreductase-triggered linkers in siderophore drug-conjugates will create effective antibacterial agents.

4.2 Short-Term Future Directions

The next steps of this project involve the completion of the first siderophore drug conjugate as outlined in Scheme 3.7. If this is successful, a means of removing the benzyl protecting groups must be developed that will not also reduce the nitro group present in the linker. Different conditions, such as the use of Me₃SiI₄³ or Lewis acids such as BF₃•Et₂O₄⁴, SnCl₄, or FeCl₃₄⁵, can be utilized to see if the deprotection of the conjugate will occur without affecting the functional groups present. Additionally, it will be interesting to see if the length of the spacer present in the linker-drug conjugate has an effect upon coupling success in the new pathway as thus far only the longer linker has been used.

If the deprotected siderophore-drug conjugate can be produced, it will be interesting to see if there is antibacterial activity. If not, it may be possible to produce conjugates that contain the bromo- or dinitro-linker, as these may be more readily reduced by bacteria due to the adjusted reduction potential. Additional nitroreductase
assays might also be helpful in determining if reduction and cyclization are occurring in biological systems. Adjustments to the linker may be necessary if nitroreduction does not lead to lactamization. One possibility would be to introduce bulky substituents, such as two methyl groups, to the alpha carbon of the acetate moiety in order to make cyclization more favorable due to steric hindrance. This has been utilized in the aforementioned trimethyl lock system, which was utilized in a releasable linker in previously made siderophore drug conjugates.

Success in drug release in siderophore-drug conjugates would allow different antibiotics to be tested to see if activity is in fact restored. A few representative examples include cycloserine, the pantothenamides, and the sulfadrugs. Cycloserine inhibits D-alanine racemase and D-alanine D-alanine ligase, both of which are important enzymes in peptidoglycan synthesis. This drug is currently used in treatment of multidrug-resistant M. tuberculosis. Since the amino group is likely necessary for enzymatic recognition and therefore antibiotic activity, this drug would likely lose activity when incorporated into a non-releasable siderophore drug conjugate. Another example is N-pentylpantothenamide, which is an antimetabolite of pantothenic acid, a precursor of coenzyme A. This molecule does not inhibit CoA biosynthesis, but instead is an alternative substrate for the pathway, resulting in the production of an inactive CoA analog. N-Pentylpantothenamide has been shown to have an MIC of 2 µM against E. coli, but does not diffuse through cell membranes. Previously N-propylpantothenamide was conjugated to the siderophore danoxamine and was shown to have weak activity against certain bacterial strains. An additional class of drugs that would be interesting to utilize are the sulfonamides. These molecules interfere with folate biosynthesis in
bacteria specifically in the step catalyzed by dihydropteroate synthase. This class of antibiotics was utilized in the first example of artificial siderophore-drug-conjugates but did not retain activity. Therefore, cycloserine, the pantothenaamides, and the sulfonamides would be ideal candidates to test drug release in siderophore-drug-conjugates.

![Figure 4.1](image-url)

**Figure 4.1**: Example structures of drugs for potential use in testing drug release process in siderophore-drug-conjugates

### 4.3 Long term future directions

While there have been many studies involving siderophores and drug conjugates, there are still many questions to be answered regarding these molecules. For example, it has not yet been determined how large of a molecule can be actively transported into bacterial cells through iron uptake pathways. One example of a conjugate with a large drug attached is microcin E492m. This molecule consists of an 84-residue peptide which is posttranslationally modified to contain a catechol-type siderophore. MccE492m has been shown to have potent activity (40-80 nm) against certain Gram-positive strains and is 4-8 times more active than the unmodified microcin due to active uptake through iron acquisition pathways. The strong activity of this molecule suggests that very large molecules can be connected to siderophores and actively transported into bacterial cells.
Figure 4.2: Structure of microcin E492m

With this in mind, it is possible to design siderophore-drug conjugates containing very large molecules. One possibility would be the use of antisense oligonucleotides. These molecules bind to mRNA, preventing protein translation, or to DNA, preventing gene transcription. Antisense oligonucleotides are promising as antibiotics due to their ability to block essential functions. Additionally, it may be possible to target resistance genes, such as for β-lactamase or efflux pump biosynthesis, which could restore susceptibility to multiple classes of bacteria. As nucleic acids can be easily degraded by enzymes, the use of synthetic molecules such as phosphorodiamidate morpholino oligomers (PMOs) or peptide nucleic acids (PNAs) is necessary. PMOs are composed of nucleotides which are attached to morpholine rings through phosphorodiamidate linkages and have been utilized as antibiotics in several studies.
Antisense antibiotics have been used successfully against strains such as *Escherichia coli*, *Salmonella*, and *Burkholderia*. In one study, an 11 base PMO was prepared which targeted the gene for an acyl carrier protein necessary for survival. Mice infected with *E. coli* were treated with the targeted PMO and a scrambled PMO. Antibiotic activity was seen for the targeted PMO against strains with abnormally permeable outer membranes while the scrambled PMO was inactive. Additionally, a PMO which again targeted the acyl carrier protein was utilized against the *Burkholderia cepacia* complex. These strains are Gram-negative bacteria, which are deadly to immunocompromised patients, such as those with cystic fibrosis. This antisense PMO was shown to have MICs of 2.5 – 10 µM against a multitude of strains. From the studies of antisense antibiotics, it has been determined that an oligomer of at least 10 units is needed for activity. As these molecules are roughly 2-3 kDa in size, they are not able to passively diffuse into the bacterial cell. This suggests a need for an active transport process. One such example involves the use of a cell-permeabilizing peptide, which allows uptake through peptide permease enzymes present in the cytoplasmic membrane. Conjugation to these peptides is necessary for strains which do not have abnormally permeable outer membranes and was shown to increase efficacy in mice by 50 – 100 times. However, a recent study determined that resistance to these antibiotics was occurring. While determining the MICs of peptide-PMOs against *E. coli*, mutants were isolated which was missing the gene *sbmA*, which encodes an active transporter for peptides. Therefore it may be possible to utilize siderophores in the active transport of antisense PMOs. Not only could it be possible to increase the activity of PMOs by conjugation to siderophores, but this may also bypass resistance, as bacteria
missing siderophore receptors are non pathogenic. It is not known whether a drug-release process is necessary for success with antisense conjugates, however these molecules represent an exciting future direction for research in siderophore-drug-conjugates.
5.1 General

Commercially available reagents were used without further purification except as indicated. Dichloromethane (CH$_2$Cl$_2$) and acetonitrile (CH$_3$CN) were distilled from CaH$_2$. Tetrahydrofuran (THF) was distilled from a mixture of sodium metal and benzophenone. Unless otherwise noted, reactions were carried out in oven-dried glassware under an atmosphere of dry argon. All reactions were magnetically stirred and monitored by analytical thin-layer chromatography using aluminum-backed 0.2 mm silica gel 60 F-254 plates. Visualization was accomplished by UV light (254 nm), or by staining with ninhydrin or vanillin. Flash chromatography was performed with silica gel 60 (230–400 mesh). $^1$H NMR and $^{13}$C NMR spectra were obtained on a 300 or 600 MHz Varian DirectDrive spectrometer or a 400 or 500 MHz Bruker spectrometer and FIDs were processed using ACD/SpecManager version 11, with the residual solvent peaks as internal standards. The line positions of multiplets are given in ppm (δ) and the coupling constants (J) are given as absolute values in hertz (Hz).

Melting points were determined in capillary tubes using a Thomas Hoover melting point apparatus and are uncorrected. High resolution, accurate mass measurements were obtained with a Bruker micrOTOF II electrospray ionization time-of-flight mass spectrometer in positive ion mode. LCMS was performed on a Waters ZQ
Yields refer to chromatographically and spectrographically pure compounds, unless otherwise noted.

5.2 Experimental Procedures

**Benzyl 2-(2-nitrophenyl) acetate (2.6)**

2-Nitrophenylacetic acid (6.03g, 33.3 mmol) and potassium carbonate (4.62, 33.5 mmol) were dissolved in 90 mL of acetone to which benzyl bromide, (6.33 grams 37.0 mmol) was added. The reaction was allowed to reflux for three hours and monitored with TLC (2:1 ethyl acetate: hexanes). Upon completion, the reaction was filtered and the solvent removed under reduced pressure. The residue was dissolved in ether, washer with water and brine, dried with magnesium sulfate, filtered, and concentrated in vacuo. The crude product was recrystallized in a minimum amount of hot methanol. To afford a light yellow solid (6.03 g, 67%) mp 77-80 °C.  

$\text{H NMR (300MHz , CDCl}_3\text{)} \delta = 8.14 (d, J = 8.3 \text{ Hz, 1 H}), 7.61 (tt, J = 1.1, 5.0 \text{ Hz, 1 H}), 7.54 - 7.45 (m, 1 H), 7.40 - 7.37 (m, 1 H), 7.36 (s, 5 H), 5.18 (s, 2 H), 4.09 (s, 2 H).$  

$^{13}\text{C NMR (125MHz ,CDCl}_3\text{)} \delta = 169.2, 149.1, 138.3, 133.8, 133.7, 133.6, 130.5, 128.9, 128.6, 127.9, 127.7, 125.3, 44.0, 41.1.;$  

HRMS (ESI) calcd, for C$_{15}$H$_{14}$NO$_4$ (M+H): 272.0917, found 272.0944.

**Methyl 2-(2-nitrophenyl)acetate (2.7)**

2-Nitrophenylacetic acid (1.05 g, 5.80 mmol) was dissolved in 6 mL of methanol to which 2 drops of sulfuric acid were added. The reaction was allowed to reflux for three hours and monitored with TLC (2:1 ethyl acetate: hexanes). Reaction mixture was then diluted with 15 of mL ether, washed with saturated aqueous sodium bicarbonate and
brine, dried with magnesium sulfate, filtered, and concentrated under vacuum to afford a viscous yellow oil (0.605 g, 54%). $^1$H NMR (300MHz, CDCl$_3$) δ= 8.13 (dd, $J=8.29$, 1.38 Hz, 1H), 7.62 (td, $J=7.46$, 1.66 Hz, 1H), 7.49 (td, $J=8.01$, 1.38 Hz, 1H), 7.37 (dd, $J=7.46$, 1.66 Hz, 1H), 4.04 (s, 2H) 3.72 (s, 3H). Data were equivalent to the known compound.$^{57}$

\textbf{N-Benzyl-2-(2-nitrophenyl)acetamide (2.8)}

2-Nitrophenylacetic acid, (4.04 g, 22.3 mmol) was dissolved in 80 mL of acetonitrile. EDC (5.6 g, 36.1 mmol) was then added to the flask followed by 3.152 g (29.41 mmol) of benzylamine. Precipitate was noted after all additions were complete. Reaction was allowed to stir for three hours and monitored with TLC (2:1 ethyl acetate: hexanes). After the reaction was complete, saturated aqueous sodium bicarbonate was added, which caused full precipitation of the product. Isolated solid was isolated through vacuum filtration and recrystallized from methanol and water to afford an off white solid (3.79 g, 63% yield). mp 140-142 °C $^1$H NMR: (300 MHz, CDCl$_3$) δ 8.05 (d, $J = 8.0$ Hz, 1H), 7.62 (t, $J = 7.5$ Hz, 1H), 7.54 - 7.43 (m, 2H), 7.39 - 7.20 (m, 5H), 6.16 (br.s., 1H), 4.46 (d, $J = 5.8$ Hz, 2H), 3.89 (s, 2H); $^{13}$C NMR (125MHz, CDCl$_3$) δ =170.1, 149.0, 135.9, 133.9, 133.6, 129.9, 128.9, 128.8, 128.7, 128.6, 128.5, 125.5, 67.3, 39.5; HRMS (ESI) calcd, for C$_{15}$H$_{14}$N$_2$O$_3$ (M+H): 271.1078, found 271.1096.

\textbf{2-Nitrophenyl acetate-ciprofloxacin conjugate (2.9)}

2-Nitrophenylacetic acid (0.182g, 1.005 mmol) was dissolved in 6 mL of anhydrous DCM. This solution was cooled to 0°C and EDC (0.231g, 1.2 mmol) was
added. The solution was kept at this temperature for ten min prior to the addition of
ciprofloxacin (0.4g, 1.2 mmol), triethylamine (0.34 mL, 2.4 mmol), DMAP (2 mg), and
HOBt (0.163 g, 1.2 mmol). This reaction was allowed to stir for 24 h at room temperature
and monitored with TLC (10% methanol in ethyl acetate). After the reaction was
complete, the reaction mixture was washed with sat NaHCO₃, 5% aqueous HCl, and
brine, dried with magnesium sulfate, and filtered. The solvent was removed under
reduced pressure to afford a light yellow solid (0.170 g, 38% yield): mp 258-265 °C. ¹H
NMR (300MHz, CDCl₃) δ= 8.81 (s, 1 H), 8.08 (dd, J = 8.0, 13.3 Hz, 2 H), 7.75 - 7.58 (m,
1 H), 7.56 - 7.35 (m, 3 H), 4.15 (s, 2 H), 3.91 (br. s., 4 H), 3.72 - 3.42 (m, 3 H), 3.42 -
3.22 (m, 2 H), 1.44 (br. s., 2 H), 1.36 - 1.05 (m, 2 H). HRMS (ESI) calcd, for
C₂₅H₂₄FN₄O₆ (M+H): 495.1674, found 495.1701.

**General procedure for reduction with Iron Powder to produce oxindole (2.10)**

Starting material (2.56 mmol) was dissolved in 12.5 mL of glacial acetic acid to
which iron powder (2.56 g) was added. The reaction was allowed brought to 100 °C for 3
hours and monitored with TLC (1:1 ethyl acetate: hexanes). Upon completion, reaction
mixture was added slowly to saturated aqueous sodium bicarbonate to neutralize the acid.
The reaction mixture was then filtered to remove iron, extracted with ethyl acetate,
washed with aqueous sodium bicarbonate followed by saturated aqueous ascorbic acid,
and concentrated under vacuum. The obtained orange solid was washed with water to
remove impurities. 225 mg of an orange solid were obtained (1.69 mmol, 66% yield):
mp 119-121°C (lit 122-125 °C). ¹H NMR (300 MHz, CDCl₃) δ=8.37 (br. s., 1 H), 7.23
(t, J = 7.5 Hz, 1 H), 7.04 (t, J = 7.2 Hz, 1 H), 6.89 (d, J = 7.7 Hz, 1 H), 3.56 (s, 2 H).

Data were equivalent to the known compound.57

Methyl 4-methyl 3-nitrobenzoate (2.12)

To a solution of 3.6 mL of concentrated sulfuric acid cooled in an ice bath was added methyl 4-methyl benzoate (2g, 13.32 mmol). To this solution was added slowly a mixture of 1.1 mL of concentrated sulfuric acid and 1.1 mL of nitric acid so that the temperature of the reaction mixture did not exceed 15 degrees Celsius. After addition was complete the reaction was allowed to stir for an additional 15 minutes. The reaction mixture was then poured onto a small quantity of crushed ice and the isolated precipitate was washed with solid and a small amount of cold methanol. Solid was recrystallized from methanol and water to afford 2.53 g of a light yellow solid (97% yield): mp 44-49 °C. 1H NMR: (300 MHZ, CDCl3) δ= 8.76 - 8.38 (m, 1 H), 8.14 (dd, J = 1.8, 7.9 Hz, 9 H), 7.44 (d, J = 7.7 Hz, 10 H), 3.96 (s, 3 H), 2.66 (s, 3 H).

4-(2-(Dimethylamino)vinyl)-3-nitrobenzoate (2.13)

Method 1: To a solution of 2.12 (36 mg, 0.18445 mmon) in 0.18 mL of DMF was added 0.114 mL of tertbutoxy bis(dimethylamino)methane. The reaction mixture was allowed to stir overnight at room temperature and monitored with TLC (2:1 ethyl acetate: hexanes). The DMF was removed in vacuo and the resulting precipitate was washed with water to afford 45 mg (97% yield) of product as a bright red solid.: mp 125-128 °C. 1H NMR: (300MHz, CDCl3) δ= 8.50 (s, 1 H), 7.90 (d, J = 8.6 Hz, 2 H), 7.48 (d, J = 8.8 Hz, 16 H), 7.17 (d, J = 13.3 Hz, 1 H), 5.93 (d, J = 13.3 Hz, 4 H), 3.91 (s, 3 H), 2.99 (s, 6 H)
Method 2: 2.12 (840 mg, 3.85 mmol) was dissolved in 7.45 mL of DMF. Dimethylamino methane (0.84 g, 5.78 mmol) was added and the reaction was heated to 60 °C for 5 hours and monitored with TLC (2:1 ethyl acetate: hexanes). The solvent was removed in vacuo to give 0.675 g (70% yield) of product as a red solid. Properties were identical to those of 2.13 from method 1.

Method 3: To a solution of 2.12 (10g, 51.23 mmol) in 42.8 mL of DMF was added 10.72 mL of DMF dimethyl acetal. The reaction mixture was allowed to stir for 8 h at 110 °C and monitored with TLC (2:1 ethyl acetate: hexanes). Once cooled, the reaction mixture was added to 400 mL of ether and extracted with water and dried. The solvent was removed in vacuo to afford 12.543g as a red solid (98% yield). Properties were identical to those of 2.13 from method 1.

Methyl 3-nitro-4-(2-oxoethyl)benzoate (2.14)

Compound 2.13 (12.54 g, 50.11 mmol) was added to 210 mL of 7% HCl and allowed to stir for 3 hours at room temperature. Reaction mixture was extracted with chloroform, dried, and concentrated under reduced pressure to afford 10.5 g (94% yield) of product as a brown-red oil: $^1$H NMR (300MHz CDCl$_3$) δ= 9.86 (s, 1 H), 8.77 (d, J = 1.9 Hz, 1 H), 8.26 (dd, J = 1.7, 8.0 Hz, 1 H), 7.43 (d, J = 8.0 Hz, 1 H), 4.22 (s, 2 H), 3.99 (s, 3 H).

2-(4-(Methoxycarbonyl)-2-nitrophenyl)acetic acid (2.15).

Compound 2.14 (240 mg, 1.0754 mmol) was added to 12.3 mL of acetone. Solution was cooled in an ice bath and concentrated sulfuric acid (0.06 mL) was added
followed by chromium trioxide (100 mg, 1 mmol). Reaction was allowed to stir for 30 min at 0 °C and monitored with TLC (2:1 ethyl acetate: hexanes). Upon completion of the reaction, an equal portion of DCM was added and the solution was washed with 10% sulfuric acid followed by 10% ascorbic acid and concentrated in vacuo to afford 170 mg (66% yield) of product as a tan solid: mp 125-128 °C. \(\text{H NMR: } (400MHz, CD_3OD) \delta=8.63 \text{ (d, } J=1.57 \text{ Hz, 1 H), 8.23 (dd, } J=7.87, 1.57 \text{ Hz, 1 H), 7.60 (d, } J=7.87 \text{ Hz, 1 H), 4.11 (s, 2 H), 3.96 (s, 3 H). \text{C NMR (101MHz, CD}_3\text{OD) } \delta = 173.6, 166.4, 150.9, 136.5, 135.4, 134.9, 132.2, 126.9, 53.3, 40.3.} \) HRMS (ESI) calcd, for C\(_{10}\)H\(_9\)N\(_2\)O\(_6\) (M+Na): 262.0322, found 262.0346.

**Methyl 4-(2-(tert-butoxy)-2-oxoethyl)-3-nitrobenzoate (2.16)**

Compound **2.15** (526 mg, 1.867 mmol), DMAP (200 mg, 1.637 mmol), and tert-butyl alcohol (0.48 mL, 5.05 mmol) were dissolved in 8 mL of dichloromethane and cooled in an ice bath. EDC (745 mg, 3.89 mmol) was added and the reaction was stirred for 2 h at 0 °C and room temperature overnight and monitored with TLC (1:1 ethyl acetate: hexanes). The reaction mixture was concentrated under vacuum and the residue was taken up in ethyl acetate and water. The organic layer was washed with saturated sodium bicarbonate and water, dried with magnesium sulfate, and concentrated in vacuo to afford a brown oil. The product was purified with column chromatography (75:25 hexanes:ethyl acetate) to afford 384 mg as a brown oil (69%). \(\text{H NMR (300MHz CDCl}_3\text{) } \delta=8.75 \text{ (s, 1 H), 8.24 (d, } J = 7.2 \text{ Hz, 1 H), 7.46 (d, } J = 7.9 \text{ Hz, 1 H), 4.01 (s, 2 H), 3.98 (s, 3 H), 1.44 (s, 9 H). \delta = 173.6, 166.4, 150.9, 136.5, 135.4, 134.9, 132.2, 126.9, 53.3, 40.3.} \)
4-(2-\textit{tert}-Butoxy)-2-oxoethyl)-3-nitrobenzoic acid (2.17)

Compound \textbf{2.16} (200 mg, 0.677 mmol) was dissolved in 4 mL of 3:1 THF: water. Lithium hydroxide (52 mg, 2.16 mmol) was added and the reaction mixture was stirred for 2 hours at room temperature and monitored with TLC (1:1 ethyl acetate: hexanes). Upon completion, the reaction mixture was diluted with 20 mL of ether and washed with saturated aqueous citric acid and brine, dried with magnesium sulfate, and concentrated in vacuo to afford 128 mg as a tan solid (67%): mp 210-215 °C. $^1$H NMR: (300MHz, CDCl$_3$) $\delta$=8.82 (d, $J$ = 1.4 Hz, 1 H), 8.34 (dd, $J$ = 1.7, 6.4 Hz, 1 H), 7.52 (d, $J$ = 8.0 Hz, 1 H), 4.06 (s, 2 H), 1.47 (s, 9 H).

\textit{tert}-Butyl 2-((2-aminoethyl)carbamoyl)-2-nitrophenyl)acetate (2.18)

Step 1: Compound \textbf{2.17} (128 mg, 0.455 mmol) and $N$-hydroxysuccinimide (157.13 mg, 1.36 mmol) were dissolved in 10 mL of anhydrous DCM. After 15 minutes of stirring, EDC (208.4 mg, 0.92 mmol) was added and the reaction was allowed to stir for 2 hours. Upon completion, the reaction mixture was washed with, 5% citric acid, water, 10% sodium bicarbonate, and brine, dried with magnesium sulfate, and concentrated in vacuo to afford 114.4 mg as a tan solid (66%).

Step 2: The NHS ester of \textbf{2.17} (114.4 mg, 0.302 mmol) and triethylamine (0.08 mL, 0.6 mmol) were dissolved in 94 mL of methanol and cooled to 0 °C. To this solution was added ethylene diamine (1.08 mL, 16.2 mmol) and the reaction was allowed to stir for 10 minutes and monitored with TLC (3:1 ethyl acetate: hexanes). Upon completion, the solvent was removed under reduced pressure and the residue was partitioned between
ethyl acetate and water. The organic layer was washed with water and brine, dried with magnesium sulfate, and concentrated in vacuo to afford 2.18 mg as a yellow oil (85%). 

$^1$H NMR: (300 MHz, CDCl₃) $\delta$=8.38 (s, 1 H), 7.85 (d, $J$ = 7.2 Hz, 1 H), 7.24 (d, $J$ = 7.9 Hz, 0 H), 4.25 (br. s., 2 H), 3.86 (s, 2 H), 3.57 (s, 2 H), 3.08 (s, 2 H), 1.38 (s, 9 H). $^{13}$C NMR (100MHz ,CD$_3$OD) $\delta$ = 169.0, 165.6, 148.9, 134.7, 133.8, 133.5, 132.1, 123.9, 82.2, 40.8, 29.9, 28.1. HRMS (ESI) calcd, for C$_{15}$H$_{22}$N$_3$O$_5$ (M+H): 324.1554, found 324.1566.

**Methyl 3-bromo-4-methyl-5-nitrobenzoate (2.19)**

Compound 2.12 (1.5g, 7.69 mmol) was dissolved in 7 mL of concentrated sulfuric acid which was heated to 60 °C. N-bromosuccinimide (1.64g, 9.2 mmol) was then added in three portions over 15 min. After stirring for 4 h, TLC (3:1 hexanes: ethyl acetate) showed complete conversion and the reaction mixture was partitioned between ethyl acetate and water. The organic layer was washed with water, saturated sodium bicarbonate, and brine, dried with magnesium sulfate, filtered, and concentrated under vacuum. The resulting solid was recrystallized in ethyl acetate and hexanes to afford 1.2g (57%) as a tan solid; mp= 70-75 °C; $^1$H NMR: (300MHz, CDCl₃) $\delta$= 8.43 (s, 1 H), 8.36 (s, 1 H), 3.97 (d, $J$ = 1.0 Hz, 3 H), 2.62 (s, 3 H).

**Methyl 3-bromo-4-(2-(dimethylamino)vinyl)-5-nitrobenzoate (2.20)**

Compound 2.19 (0.452g, 1.65 mmol) was dissolved in 1.38 mL of anhydrous DMF. The solution was then heated to 110 °C and 0.48 mL of DMFDMA was added. The reaction was monitored with TLC (2:1 hexanes: ethyl acetate) and allowed to stir for
3 hours. Upon completion, the reaction was cooled and partitioned between ethyl acetate and 0.5 N HCl. The water layer was washed with ethyl acetate and the combined organic layers were washed with brine, dried with magnesium sulfate, filtered, and concentrated under reduced pressure to afford 0.486g (89%) as a red solid which required no further purification; mp= 69-72 °C. 1H NMR: (300MHz, CDCl₃) δ=8.26 (d, J = 1.7 Hz, 1 H), 8.05 (d, J = 1.7 Hz, 1 H), 6.80 (d, J = 13.5 Hz, 1 H), 5.18 (d, J = 13.5 Hz, 1 H), 3.91 (s, 2 H), 2.93 (s, 6 H).

Methyl 3-bromo-5-nitro-4-(2-oxoethyl)benzoate (2.21)

Compound 2.20 (0.486 g, 1.48 mmol) was dissolved in 7 mL of 7% HCl with a small amount of THF added to improve solubility. This reaction was allowed to stir overnight and was monitored by TLC (2:1 hexanes: ethyl acetate). Upon completion, the reaction was extracted with ethyl acetate which was washed with saturated sodium bicarbonate and brine, dried with magnesium sulfate, filtered, and concentrated under vacuum to afford 0.393g (88%) as a stick brown solid which required no further purification; mp= 68-70 °C. 1H NMR: (300MHz, CDCl₃) δ = 9.83 (s, 1 H), 8.59 (d, J = 1.7 Hz, 1 H), 8.54 (d, J = 1.7 Hz, 1 H), 4.40 (s, 2 H), 4.00 (s, 3 H).

2-(2-Bromo-4-(methoxycarbonyl)-6-nitrophenyl)acetic acid (2.22)

Compound 2.21 (424 mg, 1.5 mmol) was dissolved in 19 mL of acetone. This solution was cooled in an ice bath and sulfuric acid (0.1 mL) and chromium trioxide (153 mg, 1.53 mmol) were added. The reaction was allowed to stir for 30 min at 0 °C. The reaction was monitored with TLC (2:1 hexanes: ethyl acetate) and upon completion, the
reaction was partitioned between ethyl acetate and water and the organic layer was washed with water and brine. The organic layer was then washed with 1 M NaOH several times. The aqueous layer was the added to an equal portion of ethyl acetate and acidified slowly with stirring until a pH of 2 was reached. The organic layer was then washed with brine, dried with magnesium sulfate, filtered, and concentrated under vacuum to afford 418 mg (88%) as a tan solid; mp= 218-220 °C; \(^1\)H NMR: (300 MHz, CD\(_3\)OD) \(\delta\) 8.66 - 8.42 (m, 2 H), 4.99 (s, 2 H), 4.22 (s, 3 H); \(^{13}\)C NMR (125MHz, CD\(_3\)OD) \(\delta\) = 170.7, 164.7, 151.0, 137.6, 137.5, 134.0, 132.3, 127.8, 124.7, 40.3, 38.3. HRMS (ESI) calcd, for C\(_{10}\)H\(_8\)BrNNaO\(_6\) (M+Na): 339.9427, found 339.9426.

Methyl 4-methyl-3,5-dinitrobenzoate (2.23)

3,5-Dinitro-\(p\)-toluic acid (1g, 4.42 mmol) was dissolved in methanol (8.8 mL), which was cooled to 0 °C. Over 30 minutes thionyl chloride (0.5 mL) was added dropwise. The reaction was then warmed to room temperature and refluxed for 5 h. The reaction was monitored with TLC (4:1 hexanes: ethyl acetate). Upon completion, the reaction was concentrated under reduced pressure and the residue was taken up in ethyl acetate. The organic layer was washed with saturated sodium bicarbonate and brine, dried with magnesium sulfate, filtered, and concentrated in vacuo to afford the product which was recrystallized from ethyl acetate/hexanes to obtain 0.924 g (87%) as a tan solid; mp= 77-82 °C \(^1\)H NMR: (300 MHz, CDCl\(_3\)) \(\delta\)=8.60 (s, 3 H), 4.21 - 3.98 (m, 3 H), 2.64 (s, 2 H).
Methyl 4-(2-(dimethylamino)vinyl)-3,5-dinitrobenzoate (2.24)

Compound **2.24** (0.924 g, 3.89 mmol) was dissolved in anhydrous DMF (3.2 mL). Dimethylformamide dimethyl amine (1.126 mL) was added and the reaction was heated to 110 °C. The reaction was monitored with TLC (2:1 hexanes: ethyl acetate) and allowed to stir for 1 h. Upon completion, the reaction was cooled and partitioned between ethyl acetate and 0.5 N HCl. The water layer was washed with ethyl acetate and the combined organic layers were washed with brine, dried with magnesium sulfate, filtered, and concentrated under reduced pressure to afford 1.1 g (96%) as a red solid; mp= 191-121 °C; ¹H NMR: (300MHz, CDCl₃) δ= 8.32 (s, 2 H), 6.71 (d, J = 13.4 Hz, 1 H), 5.50 (d, J = 13.4 Hz, 1 H), 3.94 (s, 3 H), 2.96 (s, 6 H).

Methyl 3,5-dinitro-4-(2-oxoethyl)benzoate (2.25)

Compound **2.24** (1.1 g, 3.73 mmol) was dissolved in 16 mL of 7% HCl with a small amount of THF added to improve solubility. This reaction was allowed to stir overnight and was monitored by TLC (2:1 hexanes: ethyl acetate). Upon completion, the reaction was extracted with ethyl acetate which was washed with saturated sodium bicarbonate and brine, dried with magnesium sulfate, filtered, and concentrated under vacuum to afford 0.968 g (97%) as a brown sticky solid; mp= 85-90 °C ¹H NMR: (300MHz, CDCl₃) δ 9.82 (s, 1 H), 8.81 (s, 2 H), 4.41 (s, 2 H), 4.05 (s, 3 H).

2-(4-(Methoxycarbonyl)-2,6-dinitrophenyl)acetic acid (2.26)

Compound **2.25** (1.75 g, 6.53 mmol) was dissolved in 80 mL of acetone. This solution was cooled in an ice bath and sulfuric acid (0.4 mL) and chromium trioxide (664
mg, 6.64 mmol) were added. The reaction was allowed to stir for 30 min at 0 °C. The reaction was monitored with TLC (2:1 hexanes: ethyl acetate) and upon completion, the reaction was partitioned between ethyl acetate and water and the organic layer was washed with water and brine. The organic layer was then washed with 1 M NaOH several times. The aqueous layer was the added to an equal portion of ethyl acetate and acidified slowly with stirring until a pH of 2 was reached. The organic layer was then washed with brine, dried with magnesium sulfate, filtered, and concentrated under vacuum to afford 1.6g (86%) as a tan solid mp= 192-196 °C; $^1$H NMR: (300MHz, CDCl$_3$) $\delta$=9.82 (s, 1 H), 8.81 (s, 2 H), 4.41 (s, 2 H), 4.05 (s, 3 H); $^{13}$C NMR (125MHz, CD$_3$OD) $\delta$ = 170.3, 164.3, 164.1, 151.8, 151.4, 132.7, 131.0, 128.3, 33.6, 14.1. HRMS (ESI) calcd, for C$_{10}$H$_8$N$_2$O$_8$ (M+H): 285.0354, found 285.053.

**Methyl 4-(2-methoxy-2-oxoethyl)-3-nitrobenzoate (2.27)**

Compound 2.15 (0.5g, 2.09 mmol) was added to a 25 mL flask to which 10 mL of methanol and 2 drops of sulfuric acid were added. The reaction was allowed to reflux for 3 h and monitored with TLC (1:1 ethyl acetate: hexanes). Upon completion, the reaction mixture was partitioned between ether and water. The organic layer was washed with saturated aqueous sodium bicarbonate followed by brine, dried with magnesium sulfate, filtered, and concentrated under vacuum to afford 0.440g of a tan solid (83%); mp= 73-75 °C; $^1$H NMR: (300MHz, CDCl$_3$) $\delta$ 8.76 (d, $J = 1.7$ Hz, 1 H), 8.25 (dd, $J = 1.7$, 7.9 Hz, 1 H), 7.47 (d, $J = 7.9$ Hz, 1 H), 4.10 (s, 2 H), 3.99 (s, 3 H), 3.73 (s, 3 H)
Methyl 3-bromo-4-(2-methoxy-2-oxoethyl)-5-nitrobenzoate (2.28)

Compound 2.22 (0.2g, 0.628 mmol) was dissolved in methanol (5 mL) to which 2 drops of sulfuric acid were added. The reaction was allowed to reflux for 3 h and monitored with TLC (1:1 ethyl acetate: hexanes). Upon completion, the reaction mixture was partitioned between ether and water. The organic layer was washed with saturated aqueous sodium bicarbonate followed by brine, dried with magnesium sulfate, filtered, and concentrated under vacuum to afford 0.17g of an oil (80%); $^1$H NMR: (300MHz, CDCl$_3$) δ=8.58 (d, $J = 1.7$ Hz, 1 H), 8.52 (d, $J = 1.7$ Hz, 1 H), 4.26 (s, 2 H), 4.02 - 3.97 (m, 3 H), 3.75 (s, 3 H)

Methyl 4-(2-methoxy-2-oxoethyl)-3,5-dinitrobenzoate (2.29)

Compound 2.26 (0.2g, 0.7 mmol) was dissolved in methanol (5 mL) to which 2 drops of sulfuric acid were added. The reaction was allowed to reflux for 3 h and monitored with TLC (1:1 ethyl acetate: hexanes). Upon completion, the reaction mixture was partitioned between ether and water. The organic layer was washed with saturated aqueous sodium bicarbonate followed by brine, dried with magnesium sulfate, filtered, and concentrated under vacuum to afford 0.157g (75%) as an tan solid; mp= 100-105 °C $^1$H NMR: (CDCl$_3$) δ = 8.79 (s, 2 H), 4.25 (s, 2 H), 4.04 (s, 3 H), 3.77 (s, 3 H)

Methyl 2-oxoindoline-6-carboxylate (2.30)

Compound 2.27 (0.440g, 1.74 mmol) was added to a 50mL round bottom flask to which 1.71 grams of iron powder and 8.4 mL of glacial acetic acid were added. The reaction was allowed to reflux for 3 h (1:2 ethyl acetate: hexanes). Upon completion,
reaction mixture was added slowly to saturated aqueous sodium bicarbonate to neutralize the acid. The reaction mixture was then filtered to remove iron, extracted with ethyl acetate, washed with aqueous sodium bicarbonate followed by saturated aqueous ascorbic acid, and concentrated under vacuum. The obtained solid was washed with water to remove impurities to afford 216 mg of an orange solid (65%); mp= 192-197 °C ¹H NMR: (CDCl₃) δ= 8.50 (br. s., 1 H), 7.76 (dd, J = 1.4, 7.7 Hz, 1 H), 7.55 (s, 1 H), 7.31 (d, J = 7.9 Hz, 1 H), 3.93 (s, 3 H), 3.60 (s, 2 H). HRMS (ESI) calcd, for C₁₀H₁₀NO₃ (M+H): 192.0655, found 195.0632. Data were equivalent to the known compound.⁵⁹

**Methyl 4-bromo-2-oxoindoline-6-carboxylate (2.31)**

Compound 2.30 (50mg, 0.15 mmol) was added to a 10 mL round bottom flask to which 150 mg of iron powder and 0.73 mL of glacial acetic acid were added. The reaction was allowed to stir at 95 °C for 1.25 h (1:2 ethyl acetate: hexanes). Upon completion, the reaction mixture was filtered to remove iron, and partitioned between ethyl acetate and water. The organic layer was washed with water, saturated sodium bicarbonate, and brine, dried with magnesium sulfate, filtered, and concentrated under vacuum to afford a brown solid (30 mg, 74%); mp= 120-125 °C; ¹H NMR: (300MHz, CDCl₃) δ= 7.90 (s, 1 H), 7.48 (d, J = 1.0 Hz, 1 H), 3.94 (s, 3 H), 3.55 (s, 2 H); ¹³C NMR (125 MHz, CDCl₃) δ=175.8, 165.7, 143.5, 132.1, 131.3, 127.2, 119.1, 109.5, 52.8, 37.8; HRMS (ESI) calcd, for C₁₀H₉BrNO₃ (M+H): 269.9760, found 269.9775.
Methyl 4-nitro-2-oxoindoline-6-carboxylate (2.32)

Compound 2.31 (50mg, 0.17 mmol) was added to a 10mL round bottom flask to which 175 mg of iron powder and 0.875 mL of glacial acetic acid were added. The reaction was allowed to stir for 25 min at 95 °C (1:2 ethyl acetate: hexanes). Upon completion, the reaction mixture was filtered to remove iron, and partitioned between ethyl acetate and water. The organic layer was washed with water, saturated sodium bicarbonate, and brine, dried with magnesium sulfate, filtered, and concentrated under vacuum to afford a brown solid (25 mg, 64%); mp= 181-190 °C; $^1$H NMR: (300MHz, CD$_3$OD) δ= 9.13 (s, 1 H), 8.89 (s, 2 H), 5.87 (s, 2 H), 5.33 (br. s., 3 H); $^1$H NMR (101MHz, CD$_3$OD) δ=167.7, 143.8, 130.7, 117.5, 114.4, 111.3, 100.0, 51.3, 48.5, 33.8.

Methyl 3-bromo-4-(2-(tert-butoxy)-2-oxoethyl)-5-nitrobenzoate (2.33)

Compound 2.22 (300 mg, 0.94 mmol) was dissolved in 9 mL of tert-butanol and 2 mL of dimethylformamide. To this solution was added Boc anhydride (430 mg, 1.97 mmol) followed by DMAP (34.5 mg, 0.28 mmol) and the reaction flask was heated to 60 °C for 3 h. The reaction was monitored with TLC (4:1 hexanes:ethyl acetate). Upon completion, the tert-butanol was removed under reduced pressure and the residual DMF was partitioned between ethyl acetate and water. The organic layer was washed with saturated sodium bicarbonate and brine, dried with magnesium sulfate, filtered, and concentrated under reduced pressure. The residue was purified with column chromatography (9:1 hexanes:ethylacetate) to afford 300 mg (85%) as a yellow oil. $^1$H NMR: (300MHz, CDCl$_3$) δ=8.43 (s, 1H), 8.35 (s, 1H), 3.96 (s, 2H), 2.6 (s, 3H), 1.6 (s, 9H).
**Methyl 4-(2-(tert-butoxy)-2-oxoethyl)-3,5-dinitrobenzoate (2.34)**

Compound 2.26 (300 mg, 1.056 mmol) was dissolved in 10 mL of tert-butanol and 2 mL of dimethylformamide. To this solution was added Boc anhydride (489 mg, 2.24 mmol) followed by DMAP (39 mg, 0.32 mmol) and the reaction flask was heated to 60 °C for 3 h. The reaction was monitored with TLC (4:1 hexanes:ethyl acetate). Upon completion, the tert-butanol was removed under reduced pressure and the residual DMF was partitioned between ethyl acetate and water. The organic layer was washed with saturated sodium bicarbonate and brine, dried with magnesium sulfate, filtered, and concentrated under reduced pressure. The residue was purified with column chromatography (5:1 hexanes:ethylacetate) to afford 240 mg (66%) as a tan oil. \(^1\)H NMR: (300MHz, CDCl₃) δ=8.55 (s, 2H), 2.66 (s, 3H), 1.6 (s, 9H), 1.58 (s, 2H).

**3-Bromo-4-methyl-5-nitrobenzoic acid (2.39)**

4-Methyl-3-nitrobenzoic acid (5g, 27.6 mmol) was dissolved in 25 mL of concentrated sulfuric acid. The reaction mixture was heated to 60 °C and \(N\)-bromosuccinimide (5.91g, 33.2 mmol) was added in three portions. The reaction was allowed to stir for three h. Upon completion the solution was poured onto ice and the resulting precipitant was collected with vacuum filtration. The solid was washed with water and recrystallized from ethyl acetate/ hexanes to afford 6.2 g (86%) as an off white solid. mp= 170-175 °C (lit 184.5-185.5 °C) \(^6\) \(^0\) \(^1\)H NMR: (300MHz, CDCl₃) δ=8.47 (s, 1H), 8.39 (s, 1H), 2.64 (s, 3H).
4-Methyl-3,5-dinitrobenzoic acid (2.40)

4-methyl-3-nitrobenzoic acid (5g, 27.6 mmol) was dissolved in 40 mL of concentrated sulfuric acid. Reaction mixture was heated to 100 °C and 1.09 mL of concentrated nitric acid was added dropwise. The reaction was allowed to stir for 2 h and was poured onto ice to precipitate the product. The solid was collected with vacuum filtration, washed with water and recrystallized from ethyl acetate/ hexanes to afford 5.2 g (83%) as an off white solid; mp= 153-157 °C (lit 161 °C)61 1H NMR: (CDCl₃) δ 8.66 (s, 2H), 2.67 (s, 3H).

Allyl 3-bromo-4-methyl-5-nitrobenzoate (2.41)

Compound 2.39 (2g, 7.69 mmol) was dissolved in 10 mL of DMF to which sodium bicarbonate (1.288g, 15.3 mmol) was added portionwise. The reaction mixture was allowed to stir for 1 h prior to the addition of allyl bromide (1.14 mL, 11.5 mmol). The reaction was allowed to stir overnight (1:2 ethyl acetate: hexanes). Upon completion, the solvent was removed with the high vacuum rotary evaporator and the residue was partitioned between water and dichloromethane. The organic layer was washed with saturated sodium bicarbonate and brine, dried with magnesium sulfate, filtered, and concentrated under reduced pressure to afford 2.01g (87%) as an amber oil. 1H NMR: (300 MHz, CDCl₃) δ== 8.43 (d, J = 1.7 Hz, 1 H), 8.35 (d, J = 1.7 Hz, 1 H), 6.16 - 5.91 (m, 1 H), 5.38 (dd, J = 10.5, 17.2 Hz, 2 H), 4.85 (d, J = 6.0 Hz, 21 H), 2.61 (s, 3 H)
Allyl 4-methyl-3,5-dinitrobenzoate (2.42)

Compound 2.40 (2g, 8.8 mmol) was dissolved in 10 mL of DMF to which sodium bicarbonate (1.486g, 17.6 mmol) was added portionwise. The reaction mixture was allowed to stir for 1 h prior to the addition of allyl bromide (1.14 mL, 13.2 mmol). The reaction was allowed to stir overnight was monitored with TLC (1:2 ethyl acetate: hexanes). Upon completion, the solvent was removed with the high vacuum rotary evaporator and the residue was partitioned between water and dichloromethane. The organic layer was washed with saturated sodium bicarbonate and brine, dried with magnesium sulfate, filtered, and concentrated under reduced pressure to afford 1.43g (60%) as a tan solid; mp= 65-67 °C; $^1$H NMR: (CDCl$_3$) $\delta$= 8.62 (s, 2 H), 6.19 - 5.91 (m, 1 H), 5.41 (dd, $J$ = 9.3, 15.8 Hz, 2 H), 4.90 (dt, $J$ = 1.3, 6.0 Hz, 2 H), 2.65 (s, 3 H).

tert-Butyl (2-(4-methyl-3-nitrobenzamido)ethyl)carbamate (2.47)

4-Methyl-3-nitrobenzoic acid (1.5 g, 8.3 mmol) and N-Boc ethylenediamine (1.45 g, 9.05 mmol) were dissolved in 45 mL of THF. EDC (2.88 g, 15 mmol) was added and the reaction was allowed to stir at room temperature for 3 h was monitored with TLC (1:1 ethyl acetate: hexanes). Upon completion, the solvent was removed under vacuum and the residue was partitioned between ethyl acetate and water. The organic layer was washed with 10% citric acid, water, saturated sodium bicarbonate, and brine, dried with magnesium sulfate, filtered, and concentrated in vacuo to afford an off white solid (1.77 g, 66%) as which was recrystallized from ethyl acetate/ hexanes; mp= 204-206 °C; $^1$H
NMR:  (300MHz, CDCl₃) δ= 8.49 (s, 1 H), 7.99 (d, J = 6.9 Hz, 1 H), 7.69 (br. s., 1 H), 7.41 (d, J = 7.9 Hz, 3 H), 5.02 (br. s., 1 H), 3.66 - 3.51 (m, 2 H), 3.51 - 3.33 (m, 2 H), 2.66 (s, 3 H), 1.45 (s, 9 H).

tert-Butyl (2-(3-bromo-4-methyl-5-nitrobenzamido)ethyl)carbamate (2.48)

Compound 2.39 (2 g, 7.6 mmol) and N-Boc ethylenediamine (1.5 g, 9.36 mmol) were dissolved in 25 mL of THF. EDC (2.4 g, 12.5 mmol) was added and the reaction was allowed to stir at room temperature for 3 h was monitored with TLC (1:1 ethyl acetate: hexanes). Upon completion, the solvent was removed under vacuum and the residue was partitioned between ethyl acetate and water. The organic layer was washed with 10% citric acid, water, saturated sodium bicarbonate, and brine, dried with magnesium sulfate, filtered, and concentrated in vacuo to afford an off white solid (1.76 g, 58%) as which was recrystallized from ethyl acetate/ hexanes; mp= 208-211 °C; ¹H NMR:  (300MHz, CDCl₃) δ= 8.29 (s, 1 H), 8.25 - 8.17 (m, 1 H), 7.88 (br. s., 1 H), 5.07 (br. s., 1 H), 3.67 - 3.50 (m, 2 H), 3.50 - 3.32 (m, 2 H), 2.60 (s, 3 H), 1.47 (s, 9 H).

tert-Butyl (2-(4-methyl-3,5-dinitrobenzamido)ethyl)carbamate (2.49)

Compound 2.40 (1.5 g, 6.6 mmol) and N-Boc ethylenediamine (1.25 g, 7.8 mmol) were dissolved in 25 mL of THF. EDC (2.25 g, 11.7 mmol) was added and the reaction was allowed to stir at room temperature for 3 h and was monitored with TLC (1:1 ethyl acetate: hexanes). Upon completion, the solvent was removed under vacuum and the residue was partitioned between ethyl acetate and water. The organic layer was washed with 10% citric acid, water, saturated sodium bicarbonate, and brine, dried with
magnesium sulfate, filtered, and concentrated in vacuo to afford an off white solid (1.25 g, 51%) as which was recrystallized from ethyl acetate/ hexanes; mp= 205-207 °C; $^1$H NMR: (300MHz, CDCl$_3$) δ=8.51 (s, 2 H), 8.31 (br. s., 1 H), 5.10 (br. s., 1 H), 3.66 - 3.51 (m, 2 H), 3.51 - 3.37 (m, 2 H), 2.62 (s, 3 H), 1.46 (s, 9 H).

**tert-Butyl (2-(4-(2-(dimethylamino)vinyl)-3-nitrobenzamido)ethyl)carbamate (2.50)**

Compound 2.47 (1.1g, 1.95 mmol) was dissolved in 3 mL of DMF to which 2 mL of DMFDMA was added. The reaction was heated to 110 °C for 5 h and 80 °C overnight and was monitored with TLC (2:1 ethyl acetate: hexanes). Upon completion, the reaction mixture was taken up in 50 mL ethyl acetate and washed with water (3x 100 mL) and brine (1x 100 mL). The organic layer was dried with magnesium sulfate, filtered, and evaporated to afford 920 mg (75%) as a sticky red oil; $^1$H NMR: (300MHz, CDCl$_3$) δ= 8.34 (s, 1 H), 7.82 (d, $J$ = 6.7 Hz, 1 H), 7.53 (d, $J$ = 8.8 Hz, 1 H), 7.11 (d, $J$ = 13.4 Hz, 1 H), 5.93 (d, $J$ = 13.2 Hz, 1 H), 4.98 (br. s., 1 H), 3.71 - 3.51 (m, 2 H), 3.51 - 3.26 (m, 2 H), 2.97 (s, 6 H), 1.45 (s, 9 H).

**tert-Butyl (2-(3-bromo-4-(2-(dimethylamino)vinyl)-5-nitrobenzamido)ethyl)carbamate (2.51)**

Compound 2.48 (720 mg, 1.95 mmol) was dissolved in 2 mL of DMF to which 0.65 mL of DMFDMA was added. The reaction was heated to 110 °C for 5 h and was monitored with TLC (2:1 ethyl acetate: hexanes). Upon completion, the reaction mixture was taken up and washed with water and brine. The organic layer was dried with magnesium sulfate, filtered, and concentrated to afford 934 mg (94%) as a sticky red oil.
\(^1\)H NMR: (300 MHz, CDCl\(_3\)) \(\delta= 8.12\) (s, 1 H), 7.90 (s, 1 H), 7.55 (br. s., 1 H), 6.69 (d, \(J = 13.5\) Hz, 1 H), 5.13 (d, \(J = 13.5\) Hz, 1 H), 5.02 (br. s., 1 H), 3.61 - 3.47 (m, 2 H), 3.40 (br. s., 2 H), 2.90 (s, 6 H), 1.45 (s, 9 H).

**tert-Butyl (2-(4-(2-(dimethylamino)vinyl)-3,5-dinitrobenzamido)ethyl)carbamate**

(2.52)

Compound 2.49 (720 mg, 1.95 mmol) was dissolved in 2 mL of DMF to which 1 mL of DMFDMA was added. The reaction was heated to 110 °C for 3 h and was monitored with TLC (2:1 ethyl acetate: hexanes). Upon completion, the reaction mixture was taken up in 50 mL ethyl acetate and washed with water (3x 100 mL) and brine (1x 100 mL). The organic layer was dried with magnesium sulfate, filtered, and evaporated to afford 671 mg (81%) as a sticky red oil. \(^1\)H NMR: (300 MHz, CDCl\(_3\)) \(\delta= 8.22\) (s, 2 H), 7.86 (br. s., 1 H), 6.62 (d, \(J = 13.5\) Hz, 1 H), 5.46 (d, \(J = 13.5\) Hz, 1 H), 5.03 (br. s., 1 H), 3.59 - 3.50 (m, 2 H), 3.47 - 3.34 (m, 2 H), 2.92 (s, 6 H), 1.45 (s, 9 H).

**tert-Butyl (2-(3-nitro-4-(2-oxoethyl)benzamido)ethyl)carbamate**

(2.53)

Compound 2.50 (1.38 g, 3.63 mmol) was dissolved in 10 mL each of THF and 1 M HCl. The reaction was allowed to stir for 10 minutes and a color change from dark red to brown was observed. The reaction was partitioned between ethyl acetate and water. The organic layer was washed with 10% citric acid, water, saturated sodium bicarbonate, brine, dried with magnesium sulfate, and concentrated under reduced pressure to afford 1.139 g (89%) as a sticky brown oil. \(^1\)H NMR: (300MHz, CDCl\(_3\)) \(\delta=9.86\) (s, 1 H), 8.60
(s, 1 H), 8.12 (dd, J = 1.7, 6.2 Hz, 1 H), 7.85 (br. s., 1 H), 7.41 (d, J = 8.1 Hz, 1 H), 5.04 (br. s., 1 H), 4.19 (s, 2 H), 3.66 - 3.52 (m, 2 H), 3.51 - 3.29 (m, 2 H), 1.46 (s, 9 H).

**tert-Butyl (2-(3-bromo-5-nitro-4-(2-oxoethyl)benzamido)ethyl)carbamate (2.54)**

Compound 2.51 (930 mg, 2.03 mmol) was dissolved in 4 mL each of THF and 1 M HCl. The reaction was allowed to stir for 10 minutes and a color change from dark red to brown was observed. The reaction was partitioned between ethyl acetate and water. The organic layer was washed with 10% citric acid, water, saturated sodium bicarbonate, brine, dried with magnesium sulfate, filtered, and concentrated under reduced pressure to afford 714 mg (82%) as a light brown sticky oil. \(^1\)H NMR: (300MHZ, CDCl\(_3\)) \(\delta= 9.82\) (s, 1 H), 8.47 (s, 1 H), 8.41 (s, 1 H), 8.05 (br. s., 1 H), 5.08 (br. s., 1 H), 4.36 (s, 2 H), 3.69 - 3.51 (m, 2 H), 3.48 - 3.36 (m, 2 H), 1.51 - 1.33 (m, 9 H).

**tert-Butyl (2-(3,5-dinitro-4-(2-oxoethyl)benzamido)ethyl)carbamate (2.55)**

Compound 2.52 (671 mg, 1.58 mmol) was dissolved in 4 mL each of THF and 1 M HCl. The reaction was allowed to stir for 10 minutes and a color change from dark red to brown was observed. The reaction was partitioned between ethyl acetate and water. The organic layer was washed with 10% citric acid, water, saturated sodium bicarbonate, brine, dried with magnesium sulfate, and concentrated under reduced pressure to afford 608 mg (97%) as a dark brown sticky oil. \(^1\)H NMR: (CDCl\(_3\)) \(\delta= 9.80\) (s, 1 H), 8.70 (s, 2 H), 8.47 (br. s., 1 H), 5.16 (br. s., 1 H), 4.36 (s, 2 H), 3.68 - 3.51 (m, 2 H), 3.51 - 3.36 (m, 2 H), 1.45 (s, 9 H).
2-(4-((tert-Butoxycarbonyl)amino)ethyl)carbamoyl)-2-nitrophenyl)acetic acid (2.56)

Compound 2.53 (325 mg, 0.88 mmol) was dissolved in 18 mL of tert-butanol and 4.6 mL of 2-methyl-2-butene. Sodium chlorite (743 mg, 8.22 mmol) and monosodium phosphate (892 mg, 6.46 mmol) were dissolved in 7.4 mL of water and added dropwise to the reaction mixture and the reaction was monitored with TLC (100% ethyl acetate). After 30 min of stirring, the volatiles were removed in vacuo and 30 mL of water was added. The aqueous layer was extracted with hexanes (2 x 25 mL) and then acidified with 10% citric acid. The aqueous layer was then extracted with ethyl acetate (3 x 25 mL). The organic layer was washed with brine, dried with magnesium sulfate, filtered, and concentrated under reduced pressure to afford a sticky yellow solid which was recrystallized from ethyl acetate/ hexanes to afford 53% (180 mg) as an off white solid; mp = 146-148 °C; $^1$H NMR: (300MHz, CDCl$_3$) $\delta$ = 8.78 (s, 1 H), 8.50 (s, 1 H), 8.25 (br. s., 1 H), 5.08 (br. s., 1 H), 3.70 - 3.51 (m, 2 H), 3.51 - 3.25 (m, 2 H), 2.62 (s, 2 H), 1.46 (s, 9 H); $^{13}$C NMR (125MHz, CD$_3$OD) $\delta$ = 170.07, 166.5, 157.6, 149.1, 135.0, 133.8, 133.6, 131.7, 123.7, 79.0, 40.4, 39.5, 38.9, 27.5; HRMS (ESI) calcd, for C$_{16}$H$_{21}$N$_3$NaO$_7$ (M+Na): 390.1272, found 390.1248.
2-(2-Bromo-4-((2-((\text{\textit{tert}}-butoxycarbonyl)amino)ethyl)carbamoyl)-6-nitrophenyl)acetic acid (2.57)

Compound 2.54 (710 mg, 1.65 mmol) was dissolved in 32 mL of \textit{tert}-butanol and 8 mL of 2-methyl-2-butene. Sodium chlorite (1.31g, 14.5 mmol) and monosodium phosphate (1.57g, 11.4 mmol) were dissolved in 13 mL of water and added dropwise to the reaction mixture and the reaction was monitored with TLC (100% ethyl acetate). After 30 min of stirring, the volatiles were removed in vacuo and 30 mL of water was added. The aqueous layer was extracted with hexanes (2 x 25 mL) and then acidified with 10% citric acid. The aqueous layer was then extracted with ethyl acetate (3 x 25 mL). The organic layer was washed with brine, dried with magnesium sulfate, filtered, and concentrated under reduced pressure to afford a sticky yellow solid which was recrystallized from ethyl acetate/ hexanes to afford 52% (380 mg); mp 129-132 °C; $^1$H NMR: (300MHz, CDCl$_3$) $\delta$ = 8.71 - 8.28 (m, 2 H), 4.21 (s, 3 H), 3.61 - 3.39 (m, 2 H), 3.39 - 3.13 (m, 2 H), 1.42 (s, 9 H); $^{13}$C NMR (125MHz ,CD$_3$OD) $\delta$ = 170.8, 165.0, 157.6, 150.9, 132.7, 127.8, 122.8, 79.1, 40.6, 39.4, 38.2, 37.7, 27.6; HRMS (ESI) calcd, for C$_{16}$H$_{20}$BrN$_3$NaO$_7$ (M+Na): 468.0377, found 468.0366.

2-(4-((2-(\text{\textit{tert}}-Butoxycarbonyl)amino)ethyl)carbamoyl)-2,6-dinitrophenyl)acetic acid (2.58)

Compound 2.55 (608 mg, 1.53 mmol) was dissolved in 30 mL of \textit{tert}-butanol and 8.5 mL of 2-methyl-2-butene. Sodium chlorite (1.367g, 15.11 mmol) and monosodium phosphate (1.641g, 11.89 mmol) were dissolved in 14 mL of water and added dropwise to the reaction mixture and the reaction was monitored with TLC (100% ethyl acetate).
After 30 min of stirring, the volatiles were removed in vacuo and 30 mL of water was added. The aqueous layer was extracted with hexanes (2 x 25 mL) and then acidified with 10% citric acid. The aqueous layer was then extracted with ethyl acetate (3 x 25 mL). The organic layer was washed with brine, dried with magnesium sulfate, filtered, and concentrated under reduced pressure to afford a sticky yellow solid which was recrystallized from ethyl acetate/ hexanes to afford 380 mg (52%) as a yellow solid; mp 1H NMR: mp 200-205 °C; (300 MHz, CDCl3) δ= 8.35 (s, 2 H), 4.27 (s, 2 H), 3.85 - 3.69 (m, 2 H), 3.63 - 3.38 (m, 2 H), 1.48 (s, 9 H); 13C NMR (125MHz ,CD3OD) δ= 170.4, 164.2, 157.6, 151.4, 135.8, 127.7, 127.0, 79.1, 40.7, 39.4, 33.7, 27.6; HRMS (ESI) calcd, for C16H20N4NaO9 (M+Na): 435.1122, found 435.1137.

Ciprofloxacin – protected mononitro containing linker conjugate (2.59)

Step 1: Compound 2.56 (100mg, 0.27 mmol) and N-hydroxysuccinimide (86 mg, 0.75 mmol) were dissolved in 10 mL of DCM to which EDC (96 mg, 0.5 mmol) was added and the mixture was allowed to stir for 3 h. Upon completion, the THF was evaporated and the residue was partitioned between ethyl acetate and 10% citric acid. The organic layer was then washed with water, saturated sodium bicarbonate, and brine, dried with magnesium sulfate, filtered, and concentrated in vacuo to afford the NHS ester (138 mg, 0.279 mmol) as a tan solid.

Step 2: Ciprofloxacin (278 mg, 0.84 mmol) and triethylamine, (0.5 mL, 6.82 mmol) were added to 18 mL of DMF and heated on an steam bath until a cloudy yellow solution was observed. This solution was cooled to 50 °C and the NHS ester dissolved in 3 mL of DMF was added. This reaction was allowed to stir for 1 day and was monitored
with TLC (3% MeOH in CHCl₃). Upon completion, DMF was removed with high vacuum rotary evaporator and the residue was partitioned between chloroform and 10% citric acid. The organic layer was then washed with 10% citric acid, dried with magnesium sulfate, filtered, and concentrated in vacuo. The residue was recrystallized from DCM/hexanes to afford an off white solid (46%, 184 mg); mp 205-208 °C; ¹H NMR: (300 MHz, CDCl₃) δ = 8.82 (s, 1 H), 8.67 - 8.56 (m, 2 H), 8.09 (d, J = 12.7 Hz, 2 H), 7.82 (br. s., 1 H), 7.44 (s, 2 H), 5.22 - 4.88 (m, 1 H), 4.19 (s, 2 H), 3.99 - 3.78 (m, 4 H), 3.57 (m, 3 H), 3.54 - 3.40 (m, 4 H), 3.40 - 3.25 (m, 2 H), 1.79 - 1.54 (m, 3 H), 1.46 (s, 9 H), 1.32 - 1.12 (m, 2 H); HRMS (ESI) calcd, for C₃₃H₃₇FN₆NaO₉ (M+Na): 703.2498, found 703.2504.

Ciprofloxacin – protected bromine containing linker conjugate (2.60)

Step 1: Compound **2.57** (300 mg, 0.668 mmol) and N-hydroxysuccinimide (258 mg, 2.25 mmol) were dissolved in 20 mL of THF to which EDC (288 mg, 1.5 mmol) was added and the mixture was allowed to stir for 3 h. Upon completion, the THF was evaporated and the residue was partitioned between ethyl acetate and 10% citric acid. The organic layer was then washed with water, saturated sodium bicarbonate, and brine, dried with magnesium sulfate, filtered, and concentrated in vacuo to afford the NHS ester (360 mg, 0.66 mmol) as a tan solid.

Step 2: Ciprofloxacin (400 mg, 1.21 mmol) and triethylamine, (0.6 mL, 8.18 mmol) were added to 20 mL of DMF and heated on an steam bath until a cloudy yellow solution was observed. This solution was cooled to 50 °C and the NHS ester dissolved in 3 mL of DMF was added. This reaction was allowed to stir for 3 days and was monitored
with TLC (3% MeOH in CHCl₃). Upon completion, DMF was removed with high vacuum rotary evaporator and the residue was partitioned between chloroform and 10% citric acid. The organic layer was then washed with 10% citric acid, dried with magnesium sulfate, filtered, and concentrated in vacuo. The residue was recrystallized from DCM/hexanes to afford a light yellow solid (137 mg, 27%). mp 182-184 °C ¹H NMR: (300 MHz, CDCl₃) δ = 8.83 (s, 1 H), 8.47 (d, J = 23.0 Hz, 2 H), 8.12 (d, J = 12.7 Hz, 2 H), 8.06 - 7.95 (m, 1 H), 7.50 - 7.35 (m, 1 H), 5.03 (br. s., 1 H), 4.38 (s, 2 H), 3.98 - 3.75 (m, 4 H), 3.65 - 3.21 (m, 7 H), 1.73 - 1.54 (m, 3 H), 1.48 (s, 9 H), 1.34 - 1.18 (m, 2 H); HRMS (ESI) calcd, for C₃₃H₃₆BrFN₆NaO₉ (M+Na): 781.1603, found 781.1587.

Ciprofloxacin – protected dinitro containing linker conjugate (2.61)

Step 1: 41 (100 mg, 0.24 mmol) and N-hydroxysuccinimide (86 mg, 0.75 mmol) were dissolved in 10 mL of DCM to which EDC (96 mg, 0.5 mmol) was added and the mixture was allowed to stir for 3 h. Upon completion, the THF was evaporated and the residue was partitioned between ethyl acetate and 10% citric acid. The organic layer was then washed with water, saturated sodium bicarbonate, and brine, dried with magnesium sulfate, filtered, and concentrated in vacuo to afford the NHS ester as a tan solid.

Step 2: Ciprofloxacin (239 mg, 0.72 mmol) and triethylamine, (0.3 mL, 4.09 mmol) were added to 16 mL of DMF and heated on an steam bath until cloudy yellow solution was observed. This solution was cooled to 50 °C and the NHS ester dissolved in 3 mL of DMF was added. This reaction was allowed to stir for 7 days and was monitored with TLC (3% MeOH in CHCl₃). Upon completion, DMF was removed with high vacuum rotary evaporator and the residue was partitioned between chloroform and 10%
citric acid. The organic layer was then washed with 10% citric acid, dried with magnesium sulfate, filtered, and concentrated in vacuo. The residue was recrystallized from DCM/hexanes to afford as a light brown solid (25 mg, 14%); mp >280 °C ¹H NMR: (500 MHz, CDCl₃) δ = 8.82 (s, 1 H), 8.76 (s, 1 H), 8.71 (s, 1 H), 8.44 (br. s., 1 H), 8.15 - 7.97 (m, 2 H), 7.49 - 7.33 (m, 2 H), 5.67 (br. s., 1 H), 5.21 - 5.06 (m, 1 H), 4.39 (s, 2 H), 3.95 - 3.82 (m, 2 H), 3.81 - 3.69 (m, 1 H), 3.69 - 3.43 (m, 5 H), 3.43 - 3.30 (m, 4 H), 3.19 - 3.05 (m, 1 H), 2.74 - 2.64 (m, 1 H), 1.49 (s, 9 H), 1.46 - 1.35 (m, 3 H), 1.30 - 1.15 (m, 2 H)HRMS (ESI) calcd, for C₃₃H₃₆FN₇O₁₁ (M+H): 726.2530, found 726.2530.

Methyl 2,3-Dihydroxybenzoate (3.6)

2,3 Dihydroxybenzoic acid (4.5 g, 29.2 mmol) was dissolved in 75 mL of methanol and cooled to -78 °C. Thionyl chloride (6.41 mL, 88.25 mmol) was added dropwise and the solution was warmed to 40 °C for 72 h and was monitored with TLC (2:1 ethyl acetate: hexanes). The solvent was removed under reduced pressure and the residue was dissolved in ethyl acetate. The organic layer was washed with saturated sodium bicarbonate and brine, dried with magnesium sulfate, filtered, and concentrated in vacuo to afford the product which was recrystallized from ethyl acetate/hexanes to obtain 4.075 g as a tan solid (83%); mp = 75-78 °C (lit 72-74 °C);³⁶ ¹H NMR: (300 MHz, CDCl₃) δ = 10.91 (s, 1 H), 7.37 (dd, J = 1.5, 8.1 Hz, 1 H), 7.11 (s, J = 7.7 Hz, 1 H), 6.81 (t, J = 8.0 Hz, 1 H), 5.75 (s, 1 H), 3.96 (s, 3 H). Data were equivalent to the known compound.³⁶
Methyl 2,3- Bis(benzyloxy)benzoate (3.7)

Methyl 2,3-Dihydroxybenzoate (5.0 g, 29.74 mmol), anhydrous potassium carbonate (16.43 g, 118.9 mmol), and sodium iodide (0.14 g, 0.934 mmol) were dissolved in 71.67 mL of anhydrous DMF. To this solution was added benzyl bromide (7.75 mL, 65.51 mmol). The reaction was allowed to stir for 16 h, diluted with ether, and washed with 2% NaOH and water. The organic layer was dried with magnesium sulfate, filtered, and concentrated in vacuo to afford a light yellow solid. The product was recrystallized from ethyl acetate/hexanes to afford 8.48 g as a white solid (82%); mp 59-61 °C (lit 55-57 °C); \(^1\)H NMR: (300 MHz, CDCl\(_3\)) \(\delta= 7.56 - 7.29\) (m, 10 H), \(7.23 - 6.93\) (m, 3 H), \(5.16\) (s, 3 H), \(5.12\) (s, 2 H), \(3.86\) (s, 3 H). Data were equivalent to the known compound.

2,3- Bis(benzyloxy)benzoic acid (3.8)

Methyl 2,3- Bis(benzyloxy)benzoate (950 mg, 2.727 mmol) was dissolved in 75 mL of 1:1 THF: water. To this was added 6 mL of 1N KOH and the reaction was allowed to stir for 3 h at room temperature and was monitored with TLC (2:1 ethyl acetate: hexanes). The reaction mixture was diluted with ethyl acetate and acidified with 10% citric acid. The organic layer was washed with water and brine, dried with magnesium sulfate and evaporated under reduced pressure. The resulting white solid was recrystallized from methanol/water to afford 775 mg as a white fluffy solid (82%). mp 116-119 °C (lit 120 °C); \(^1\)H NMR: \((\text{CDCl}_3)\) \(\delta= 7.4-7.0\) (m, 13H), \(5.15\) (s, 2H), \(5.11\) (s, 2H). Data were equivalent to the known compound.
\(N^{1},N^{8}\)-Bis(2,3-bis(benzyloxy)benzoyl)spermidine (3.9)

2,3- Bis(benzyloxy)benzoic acid (3 g, 8.97 mmol) was dissolved in 40 mL of dry dichloromethane. To this was added carbonyldiimidazole (2.18g, 13.44 mmol). Reaction was allowed to stir until starting material was no longer seen via TLC (3:2 hexanes: ethyl acetate). Spermidine (0.64 g, 4.4 mmol) in 5 mL of dichloromethane was then added and the reaction was allowed to stir for 4 d at room temperature. Reaction was monitored with 16:3:1 ethyl acetate: methanol: triethylamine. Upon completion, the reaction was washed with aqueous sodium bicarbonate and brine, dried with magnesium sulfate, filtered, and concentrated under vacuum to afford a yellow liquid which was purified by column chromatography (16:3:1 ethyl acetate: methanol: triethylamine) to obtain 2.08 g (1.93 mmol, 60%) as a yellow oil; \(^1\)H NMR: (300 MHz, CDCl\(_3\)) \(\delta=8.13 \text{ (d, } J = 5.5 \text{ Hz, 1 H}), 8.00 \text{ (d, } J = 5.0 \text{ Hz, 1 H}), 7.74 - 7.65 \text{ (m, 2 H), 7.63 - 7.58 \text{ (m, 1 H), 7.51 - 7.28 \text{ (m, 19 H), 7.14 \text{ (t, } J = 4.1 \text{ Hz, 4 H), 7.05 \text{ (s, 1 H), 5.15 \text{ (s, 4 H), 5.07 \text{ (s, 4 H), 3.41 - 3.18 \text{ (m, 4 H), 2.55 - 2.36 \text{ (m, 4 H), 1.60 - 1.45 \text{ (m, 2 H), 1.42 - 1.25 \text{ (m, 5 H). Data were equivalent to the known compound.}^{36}}

\(N^{4}\)-Succinoyl- \(N^{1},N^{8}\)-Bis(2,3-bis(benzyloxy)benzoyl)spermidine (3.10)

\(N^{1},N^{8}\)-Bis(2,3-bis(benzyloxy)benzoyl)spermidine (1 g, 1.28 mmol), succinic anhydride, (193 mg, 1.929 mmol), and a catalytic amount of DMAP were dissolved in 30 mL of acetonitrile. This reaction was allowed to stir overnight at room temperature and was monitored with TLC (10% methanol in ethyl acetate). The solvent was then evaporated and the residue was dissolved in ethyl acetate, washed with 10% citric acid and brine, dried with magnesium sulfate, filtered, and concentrated under reduced
pressure to afford 0.94 g (83%) as a sticky yellow oil.  $^1$H NMR: (300 MHz, CDCl$_3$) δ=8.14 - 7.96 (m, 2 H), 7.79 - 7.60 (m, 2 H), 7.54 - 7.28 (m, 20 H), 7.22 - 7.05 (m, 4 H), 5.24 - 5.12 (m, 4 H), 5.12 - 5.00 (m, 4 H), 3.34 - 3.02 (m, 8 H), 2.70 - 2.45 (m, 4 H), 1.64 - 1.19 (m, 6 H). Data were equivalent to the known compound.$^{36}$

5-[(tertButoxycarbonyl)amino]-1-pentanol (3.11)

Di-(tert-butyl) dicarbonate (4.0 g, 18.32 mmol) was dissolved in 40mL of dioxane to which 5-amino-1-pentanol (2 g, 9.16 mmol) was added. The solution was stirred at room temperature for 3 hrs. The product was added to ether (100 mL) and the organic solutions were washed with water (50 mL X 3), dried with magnesium sulfate, filtered and concentrated in vacuo to afford 1.73 g (44%) as a clear oil. $^1$H NMR: (300 MHz, CDCl$_3$) δ= 4.63 (br. s., 1 H), 3.65 (t, $J = 6.3$ Hz, 2 H), 3.22 - 3.06 (m, 2 H), 1.81 (br. s., 1 H), 1.67 - 1.11 (m, 15 H).

N-[(Trichlorethoxy)carbonyl]-O-benzylhydroxylamine (3.12)

To a solution of O-benzyl hydroxylamine (1.6 g, 10 mmol) in 50 mL of 1:1 THF: water was added sodium bicarbonate (1.6g, 19 mmol) followed by 2,2,2 trichloroethoxycarbonyl chloride (1.65 mL, 12 mmol) was added dropwise and the reaction was allowed to stir overnight at room temperature and was monitored with TLC (1:2 ethyl acetate: hexanes). THF was removed under reduced pressure and the water layer was extracted with ether. The organic layer was then washed with 10% citric acid and water, dried with magnesium sulfate, filtered, and concentrated in vacuo to afford
2.923 g (98%) as a clear oil. \( ^1H \) NMR: (300 MHz, CDCl\(_3\)) \( \delta = 7.58 \) (br. s., 1 H), 7.48 - 7.31 (m, 5 H), 4.94 (s, 2 H), 4.81 (s, 2 H).

**1-[(tert-Butoxycarbonyl)amino]-5-[(trichloroethoxycarbonyl)(benzyloxy)amino]pentane (3.13)**

5-[(tert-Butoxycarbonyl)amino]-1-pentanol (3.11) (338 mg, 1.66 mmol), \( N\)-[(Trichlorethoxy)carbonyl]-O-benzylhydroxylamine (3.12) (500 mg, 1.67 mmol), and triphenylphosphine (577 mg, 2.2 mmol) were dissolved in 12 mL of anhydrous THF. DEAD in 3 mL of anhydrous THF was added dropwise over 20 minutes. The reaction was allowed to stir for 16 hours at room temperature and was monitored with TLC (1:1 ethyl acetate: hexanes). Upon completion, the reaction mixture was concentrated under reduced pressure and the residue was purified using column chromatography (20:80 ethyl acetate: hexanes) to afford 0.77g (96%) as a clear oil that solidified over time. \( ^1H \) NMR: (300MHz, CDCl\(_3\)) \( \delta = 7.70 - 7.30 \) (m, 5 H), 4.93 (s, 2 H), 4.84 (s, 2 H), 4.52 (br. s., 1 H), 3.51 (t, \( J = 7.2 \) Hz, 2 H), 3.24 - 2.86 (m, 2 H), 1.87 - 1.57 (m, 2 H), 1.57 - 1.04 (m, 14 H). Data were equivalent to the known compound.\(^{33}\)

**2,2,2-trichloroethyl benzyloxy(5-(4-((4-(2,3-bis(benzyloxy)benzamido)butyl)(3-(2,3-bis(benzyloxy)benzamido)propyl)amino)-4-oxobutanamido)pentyl)carbamate (3.16)**

Compound 3.13 (0.974 g, 2.014 mmol), was dissolved in 14 mL of trifluoroacetic acid and stirred for 5 minutes at room temperature. The TFA was removed by azeotropic evaporation with toluene and the resulting TFA salt was dissolved in 15 mL of dichloromethane to which 3.10 (1.77g g, 2.011 mmol), \( N\)-Ethoxycarbonyl-2-ethoxy-1,2-
dihydroquinoline (0.987g, 3.98 mmol), and triethylamine (0.43 mL, 5.78 mmol) were added. The reaction was allowed to stir overnight at room temperature and was monitored with TLC (10% methanol in ethyl acetate). Upon completion, the solvent was evaporated and the residue was taken up in ethyl acetate, washed with 0.5 M HCl, water, and brine, dried with magnesium sulfate, filtered, and concentrated under reduced pressure. The resulting oil was purified with column chromatography (10% methanol in chloroform) to afford 1.87 g (75%) as a yellow oil. \(^1\)H NMR: (300 MHz, CDCl\(_3\)) \(\delta = 8.18 - 7.83\) (m, 2 H), 7.83 - 7.53 (m, 2 H), 7.52 - 7.24 (m, 25 H), 7.20 - 7.09 (m, 4 H), 6.23 - 6.02 (m, 1 H), 5.22 - 5.03 (m, 8 H), 4.97 - 4.87 (m, 2 H), 4.87 - 4.72 (m, 2 H), 3.59 - 3.42 (m, 2 H), 3.34 - 2.98 (m, 10 H), 2.66 - 2.35 (m, 4 H), 1.84 - 1.19 (m, 12 H). Data were equivalent to the known compound.\(^3\)

**Protected Mixed Ligand Siderophore (3.15)**

Compound **3.16** (1.45 g, 1.16 mmol) was dissolved in 24 mL of 1:1 THF: AcOH. To this solution was added freshly activated zinc (0.76 g, 11.67 mmol) and succinic anhydride (1.16 g, 11.67 mmol) were added and the reaction was stirred at room temperature overnight and was monitored with TLC (10% methanol in ethyl acetate). The reaction was filtered, concentrated under vacuum, and the residue was taken up in ethyl acetate. The organic layer was washed with water, and brine, dried with magnesium sulfate, filtered, and concentrated in vacuo. The resulting compound was purified by column chromatography eluting with chloroform: isopropanol: acetic acid (90:10:1) to afford 1.11g (82%) as a yellow sticky oil; \(^1\)H NMR: (600MHz, CDCl\(_3\)) \(\delta = 8.09 - 7.87\) (m, 2 H), 7.76 - 7.60 (m, 2 H), 7.51 - 7.43 (m, 2 H), 7.43 - 7.28 (m, 12 H),
7.20 - 7.08 (m, 3 H), 6.95 (t, J = 5.0 Hz, 1 H), 6.88 (t, J = 5.0 Hz, 1 H), 5.21 - 5.12 (m, 4 H), 5.09 (dd, J = 6.7, 11.7 Hz, 3 H), 4.81 (d, J = 3.8 Hz, 1 H), 3.77 - 3.70 (m, 2 H), 3.32 - 3.05 (m, 8 H), 2.72 - 2.46 (m, 8 H), 1.69 - 1.59 (m, 2 H), 1.59 - 1.48 (m, 2 H), 1.48 - 1.30 (m, 6 H), 1.30 - 1.18 (m, 4 H). Data were equivalent to the known compound.33

**Mixed Ligand Siderophore (3.16)**

Compound 3.15 (290 mg, 0.246 mmol) was dissolved in 12 mL of methanol. The resulting solution was bubbled through with argon and 10% Pd-C (58 mg, 20% w/w) was added. This suspension was purged with argon which was then removed under vacuum and placed under a hydrogen atmosphere for 6 H and was monitored with TLC (10% methanol in ethyl acetate). Upon completion the reaction mixture was filtered through glass filter paper and concentrated under vacuum to afford 160 mg (90%) as a gummy pink oil. Data were equivalent to the known compound.33 1H NMR (300MHz, CD3OD) δ =7.34 - 7.13 (m, 2 H), 7.08 - 6.84 (m, 2 H), 6.84 - 6.58 (m, 2 H), 3.71 - 3.23 (m, 11 H), 3.23 - 2.99 (m, 3 H), 2.97 - 2.39 (m, 6 H), 2.03 - 1.17 (m, 12 H). Data were equivalent to the known compound.33

**OBn Ciprofloxacin – protected mononitro linker conjugate (3.25)**

Step 1: Compound 2.56 (100mg, 0.27 mmol) and N-hydroxysuccinimide (86 mg, 0.75 mmol) were dissolved in 10 mL of DCM to which EDC (96 mg, 0.5 mmol) was added and the mixture was allowed to stir for 3 h. Upon completion, the THF was evaporated and the residue was partitioned between ethyl acetate and 10% citric acid. The organic layer was then washed with water, saturated sodium bicarbonate, and brine,
dried with magnesium sulfate, filtered, and concentrated in vacuo to afford the NHS ester (100 mg, 0.215 mmol) as a tan solid.

Step 2: OBn-Ciprofloxacin (120 mg, 0.262 mmol) and triethylamine, (0.5 mL, 6.82 mmol) were added to 5 mL of DMF to which the NHS ester was added. This reaction was allowed to stir for 1 day and was monitored with TLC (10% MeOH in CHCl₃). Upon completion, DMF was removed with high vacuum rotary evaporator and the residue was partitioned between chloroform and 10% citric acid. The organic layer was then washed with 10% citric acid, dried with magnesium sulfate, filtered, and concentrated in vacuo. The residue was recrystallized from ethyl acetate /hexanes to afford an off white solid (40%, 83 mg); mp 140-145 °C; ¹H NMR: (300 MHz, CDCl₃) δ=8.66 - 8.52 (m, 2 H), 8.09 (d, J = 12.0 Hz, 2 H), 7.86 (br. s., 1 H), 7.57 - 7.29 (m, 7 H), 5.40 (s, 2 H), 5.21 - 5.06 (m, 1 H), 4.18 (s, 2 H), 3.97 - 3.77 (m, 4 H), 3.65 - 3.53 (m, 3 H), 3.53 - 3.35 (m, 4 H), 3.35 - 3.20 (m, 2 H), 1.45 (s, 9 H), 1.40 - 1.07 (m, 5 H); HRMS (ESI) calcd, for C₄₀H₄₄FN₆O₉ (M+H): 771.3148 found 771.3125.

**OBn Ciprofloxacin – protected mononitro linker conjugate containing longer spacer (3.28)**

Step 1: Starting acid (94 mg, 0.24 mmol) and N-hydroxysuccinimide (82 mg, 0.71 mmol) were dissolved in 5 mL each of THF and DCM to which EDC (91 mg, 0.47 mmol) was added and the mixture was allowed to stir for 3 h. Upon completion, the solvent was evaporated and the residue was partitioned between ethyl acetate and 10% citric acid. The organic layer was then washed with water, saturated sodium bicarbonate,
and brine, dried with magnesium sulfate, filtered, and concentrated in vacuo to afford the
NHS ester (80 mg, 0.16 mmol) as a tan solid.

Step 2: OBTN-Ciprofloxacin (89 mg, 0.20 mmol) and triethylamine, (0.25 mL,
2.27 mmol) were added to 5 mL of DMF to which the NHS ester was added. This
reaction was allowed to stir for 1 day and was monitored with TLC (10% MeOH in
CHCl₃). Upon completion, DMF was removed with high vacuum rotary evaporator and
the residue was partitioned between chloroform and 10% citric acid. The organic layer
was then washed with 10% citric acid, dried with magnesium sulfate, filtered, and
concentrated in vacuo. The residue was recrystallized from ethyl acetate /hexanes to
afford an off white solid (36%, 69 mg); mp 128-131 °C; ¹H NMR: (300 MHz, CDCl₃) δ=
8.58 (s, 2 H), 8.28 - 8.00 (m, 2 H), 7.62 - 7.47 (m, 2 H), 7.47 - 7.29 (m, 5 H), 7.09 (br. s.,
1 H), 5.40 (s, 2 H), 4.66 (br. s., 1 H), 4.18 (s, 2 H), 3.86 (br. s., 4 H), 3.52 (br. s., 3 H),
3.46 - 3.35 (m, 2 H), 3.35 - 3.23 (m, 2 H), 3.23 - 3.09 (m, 2 H), 1.97 - 1.53 (m, 5 H), 1.45
(s, 9 H), 1.39 - 1.26 (m, 2 H), 1.26 - 1.01 (m, 2 H); HRMS (ESI) calcd, for
C₄₂H₄₇F₇N₆O₉ (M+Na): 821.3281 found 821.3306.

4-((Benzyloxy)(5-((tert-butoxycarbonyl)amino)pentyl)amino)-4-oxobutanoic acid
(2.58)

Compound 3.13 (242 mg, 0.5 mmol) was dissolved in 5 mL of ethyl acetate to
which freshly activated zinc dust (327 mg, 5 mmol) and N-methylimidazole (123 mg, 1.5
mmol) were added. The reaction was heated to 70 °C and allowed to stir overnight and
was monitored with TLC (1:1 ethyl acetate: hexanes). Upon completion, ethyl acetate
was added (20 mL) and the reaction mixture was filtered, washed with 10% citric acid,
water, and brine, dried with magnesium dulfate, filtered, and concentrated under reduced pressure to afford a clear oil (150 mg, 74%); $^1$H NMR: (300 MHz, CDCl$_3$) $\delta$ = 7.39 (s, 5 H), 6.10 (br. s., 1 H), 4.83 (s, 2 H), 4.67 (br. s., 1 H), 3.81 - 3.54 (m, 2 H), 3.23 - 2.92 (m, 2 H), 2.64 (s, 4 H), 1.63 (d, $J$ = 7.2 Hz, 2 H), 1.44 (s, 11 H), 1.39 - 1.20 (m, 2 H); $^{13}$C NMR (125MHz ,CD$_3$OD) $\delta$ = 170.1, 174.0, 156.3, 134.7, 132.4, 129.5, 129.3, 129.0, 128.8, 76.6, 45.4, 44.3, 41.9, 40.6, 29.6, 29.1, 28.7, 26.8, 23.9; HRMS (ESI) calcd, for C$_{20}$H$_{33}$NO$_9$ (M+H): 431.2150 found 431.2171.
CHAPTER 5

SELECTED SPECTRAL DATA
$^{1}H$ NMR of 2.6 (300MHz, CDCl$_3$)
$^{13}$C NMR of 2.6 (126MHz, CDCl$_3$)


\(^1\)H NMR of 2.8 (300MHz, CDCl\(_3\))
\(^{13}\)C NMR of 2.8 (126MHz, CDCl\(_3\))
$^{1}$H NMR of 2.9 (300MHz, CDCl$_3$)
$^1$H NMR of 2.12 (300MHz, CDCl$_3$)
$^1$H NMR of 2.13 (300MHz, CDCl$_3$)
$^1$H NMR of 2.14 (300MHz, CDCl$_3$)
$^1$H NMR of 2.15 (400MHz, CD$_3$OD)
$^{13}$C NMR of 2.15 (100MHz, CD$_3$OD)
$^{1}H$ NMR of 2.16 (300MHz, CDCl$_3$)
$^1$H NMR of 2.17 (300MHz, CDCl$_3$)
$^{1}$H NMR of 2.18 (300MHz, CDCl$_3$)
$^{13}$C NMR of 2.18 (100MHz, CDCl$_3$)
$^1$H NMR of **2.19** (300MHz, CDCl$_3$)
$^1$H NMR of 2.20 (300MHz, CDCl$_3$)
$^1$H NMR of 2.21 (300MHz, CDCl$_3$)
¹H NMR of 2.22 (300MHz, CD₃OD)
$^{13}$C NMR of 2.22 (125 MHz, CD$_3$OD)
$^1$H NMR of 2.23 (300MHz, CDCl$_3$)
\(^1\)H NMR of 2.24 (300MHz, CDCl\(_3\))
$^1$H NMR of 2.25 (300MHz, CDCl$_3$)
$^1$H NMR of 2.26 (300MHz, CD$_3$OD)
$^{13}$C NMR of 2.26 (125MHz, CD$_3$OD)
$^1$H NMR of 2.27 (300MHz, CDCl$_3$)
$^{1}$H NMR of 2.28 (300MHz, CDCl₃)
\[ \text{\(^1\)H NMR of 2.29 (300MHz, CDCl}_3) \]
$^1$H NMR of 2.30 (300MHz, CDCl$_3$)
$^{1}$H NMR of 2.31 (300MHz, CDCl$_3$)
$^{13}$C NMR of 2.31 (125 MHz, CDCl$_3$)
$^1$H NMR of 2.32 (300MHz, CD$_3$OD)
$^{13}$C NMR of 2.32 (100MHz, CD$_3$OD)
$^1$H NMR of 2.41 (300MHz, CDCl$_3$)
$^1$H NMR of 2.42 (300MHz, CDCl$_3$)
$^1$H NMR of 2.47 (300 MHz, CDCl$_3$)
$^1$H NMR of 2.48 (300MHz, CDCl$_3$)
$^1$H NMR of 2.49 (300MHz, CDCl$_3$)
\[ \text{H NMR of 2.50 (300MHz, CDCl}_3\text{)} \]
$^1$H NMR of 2.51 (300MHz, CDCl$_3$)
$^1$H NMR of 2.52 (300MHz, CDCl$_3$)
$^1$H NMR of 2.53 (300MHz, CDCl$_3$)
$^1$H NMR of 2.54 (300MHz, CDCl$_3$)
$^1$H NMR of 2.55 (300MHz, CDCl$_3$)
$^1$H NMR of 2.56 (300MHz, CDCl$_3$)
$^{13}$C NMR of 2.56 (125MHz, CD$_3$OD)
$^{1}H$ NMR of 2.57 (300MHz, CD$_3$OD)
$^{13}$C NMR of 2.57 (125MHz, CD$_3$OD)
$^1$H NMR of 2.58 (300MHz, CDCl$_3$)
$^{13}$C NMR of 2.58 (125MHz, CD$_3$OD)
$^1$H NMR of 2.59 (300MHz, CDCl$_3$)
$^1$H NMR of 2.60 (300MHz, CDCl$_3$)
$^1$H NMR of 2.60 (300MHz, CDCl$_3$)
$^1$H NMR of 3.25 (300MHz, CDCl$_3$)
$^1$H NMR of **3.26** (300MHz, CDCl$_3$)
$^1$H NMR of 3.28 (300MHz, CDCl$_3$)
$^{13}$C NMR of 3.28 (125MHz, CDCl$_3$)
REFERENCES


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