ABSTRACT


The rise of multi-drug resistant bacteria is now considered one of the greatest threats to mankind. Traditional antibiotics work through either a bactericidal or bacteriostatic mechanism, which places an evolutionary pressure on the bacterium to develop resistance. An alternative strategy for the development of antibiotics, that in theory would not summon bacterial resistance, is through the identification of compounds that target antibiotic resistance mechanisms in a non-microbicidal manner. These adjuvants could then be used in combination therapies to restore the efficacy of previously identified antibiotics. Herein the synthesis and biological activity of novel 1,5-substituted 2-aminoimidazoles is described. These initial screens resulted in the identification of a 2-aminoimidazole/triazole conjugate that displayed the unique ability to suppress resistance of methicillin-resistant *Staphylococcus aureus* to oxacillin upwards of 512-fold when co-dosed at sub-MIC levels. Initial mechanism of action studies indicate that this compound interferes with VraSR two-component system signaling. Next, a 2-aminoimidazole adjuvant was shown to suppress colistin resistance in multi-drug resistant *Acinetobacter baumannii* and *Klebsiella pneumoniae*. Colistin resistance in *A. baumannii* is known to be regulated by the PmrAB two-component system, which controls a modification of the lipid A portion of the bacterial membrane. This modification reduces the affinity of colistin towards the Gram-negative bacterial cell membrane. The identified adjuvant was shown to down-regulate the *pmrCAB* operon, and reverse the membrane modification. Additionally, evolution experiments
indicate that the bacteria are unable to evolve resistance to the effects of the 2-aminoimidazole adjuvant.
Small Molecule Suppression of Antibiotic Resistance Mechanisms

by
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DEDICATION

I would like to dedicate this work to my parents, Charles and Donna Harris. Thank you for your love and support over all of the years.
BIOGRAPHY

Tyler Lee Harris was born in Greenville, North Carolina on July 25th 1984. He graduated high school from D.H. Conley in the spring of 2002. As a college freshman he attended East Carolina University but ultimately decided to transfer to The University of North Carolina at Wilmington (UNCW) in 2003. In 2006, he graduated from UNCW receiving a Bachelor of Science in Chemistry. During his time at UNCW he had the opportunity to perform undergraduate research in organic chemistry, and upon graduating he decided to continue his education under the guidance of Professor Pam Seaton. In 2009, he successfully defended his thesis titled "Photo-Induced Isomerization and Dimerization of Various Styryl Quinolines" and received a Master of Science in Chemistry.

After completing his Master of Science, Tyler continued his graduate education at North Carolina State University (NCSU) under the direction of Professor Christian Melander. During his time at NCSU, he studied the various mechanisms that bacteria employ to evade the effects of antibiotics. His work focused on the development of novel methods to disable these resistance mechanisms and thereby restore susceptibility to antibiotic therapy. Upon the completion of his Ph.D. in December 2013, he joined the laboratory of Professor Kim Janda as a postdoctoral research associate at The Scripps Research Institute in San Diego, California.
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CHAPTER 1
INTRODUCTION TO BACTERIAL RESISTANCE MECHANISMS

1.1: Classifying Relevant Bacteria

Bacteria are prokaryotic organisms that are generally divided into two broad groups based on cell wall structure. The differences in cell wall structure correlate with the reaction to the Gram staining procedure that was developed in 1884 by Christian Gram. Gram-negative bacteria are colored pink or red by the Gram staining technique, and Gram-positive bacteria stain purple. The reason for this difference is that the Gram-positive cell wall contains a layer just outside of the plasma membrane known as the peptidoglycan layer which is 20 to 80 nm thick. The Gram-negative cell wall contains the peptidoglycan layer which is only 2 to 7 nm thick in addition to an outer membrane (7 to 8 nm thick) (Figure 1.1). The difference in the peptidoglycan layer equates to the Gram-positive cell wall being more resistant to osmotic pressure, and thus retains the stain more efficiently.¹
Two common Gram-positive bacteria that are medically relevant today include *Staphylococcus aureus* and *Staphylococcus epidermidis*. *S. aureus* appears as grape-like clusters and is common in humans, particularly on external skin surfaces and in the nasal passage. *S. aureus* is common in hospitals, and can cause serious problems when infecting damaged tissue. As the bacteria fester in the wound they produce many toxins, including exotoxin TSST-1. This toxin can cause toxic shock syndrome, which is characterized by fever, hypertension, scarlatiniform rash, and the involvement of the major organ systems. If left untreated the disease can be fatal. Other infections related to *S. aureus* include osteomyelitis, endocarditis, and pneumonia. β-Lactams are a common class of antibiotics used to treat *S. aureus* infections, however resistant strains are now a major problem. It is
now estimated that approximately 70% of all *S. aureus* strains isolated in the clinic are classified as methicillin-resistant.³

*S. epidermidis* is common in mammalian mucous membranes and it is generally not pathogenic. However, patients with compromised immune systems are at a much higher risk of infection. This is particularly true for patients with indwelling medical devices such as catheters, as the bacterium can form a biofilm, thus making treatment very difficult.

*Acinetobacter baumannii* and *Klebsiella pneumoniae* are two common Gram-negative bacteria that are particularly relevant today. *A. baumannii* is responsible for a variety of infections, including septicemia, urinary tract infections, pneumonia, and wound infections. *A. baumannii* infections have become increasingly common, especially for soldiers of the Afghanistan and Iraq wars.⁴ The clinical significance of *A. baumannii* has grown rapidly over the past decade due to its ability to acquire resistance determinants. Resistant strains to all known antibiotics are now being reported.⁵

*K. pneumoniae* is typically found on the skin, as well as the mouth and intestines. *K. pneumoniae* infections are some of the most frequently observed Gram-negative infections in the world, and include urinary tract infections and pneumonia.⁶ This bacterium has received much attention recently because of its ability to rapidly evolve resistance to the carbapenem class of β-lactam antibiotics. In 2008, a strain of *K. pneumoniae* was isolated that was resistant to all β-lactam antibiotics except monobactams.⁷

1.2: Common Antibiotics and Bacterial Resistance

Bacterial resistance to commonly prescribed antibiotics is a major challenge facing the world today. Bacterial strains resistant to many, or even all, currently available
antibiotics are increasingly common. To compound the problem, scientists have been ineffective at discovering new classes of antibiotics in recent years. Almost all classes of antibiotics were discovered during the “Golden Age” of antibiotics (1945 – 1965). Tyrothricin, penicillin, streptomycin, chloramphenicol, chlortetracycline, neomycin, erythromycin, and more drugs were discovered during this era. These drugs were isolated from soil microbes and proved to be efficient when first introduced, however bacterial resistance has since rendered some completely useless.

Resistant strains of bacteria not only lead to increased mortality rates but also increased economic costs. It is estimated that antibiotic-resistant infections cost the United States approximately 20 billion dollars per year. A study involving New York City hospitals showed that methicillin-resistant \textit{Staphylococcus aureus} (MRSA) led to a three-fold increase in the number of deaths and a twenty-two percent increase in cost compared to methicillin-susceptible \textit{S. aureus} (MSSA).

To understand how bacteria have evolved resistance to every known antibiotic, one must understand the mechanism of action of the respective antibiotic. Almost all antibiotics work by targeting bacterial protein synthesis or bacterial cell-wall biosynthesis. Some antibiotics do operate under different mechanisms of action, such as inhibiting DNA replication/repair, however these will not be discussed. A brief introduction to inhibitors of protein synthesis is described next, followed by a more detailed description of cell-wall biosynthesis inhibitors.
Protein Synthesis Inhibitors

As bacteria are prokaryotic organisms, the ribosome within the cell is quite different from the eukaryotic equivalent. The various classes of protein synthesis inhibitors target different steps in ribosome action, which comes as no surprise given the large number of molecular steps involved in initiation, elongation, and termination of protein synthesis. Antibiotics of this class included aminoglycosides, macrolides, and tetracyclines; these bind to various sites on the protein or on the ribosomal DNA of the bacterial 70S ribosome (Figure 1.2). For example, it has been shown that aminoglycosides bind the 30S ribosomal subunit, but macrolides bind the 50S subunit.\textsuperscript{10,11}

![Macrolide](image1.png)

![Aminoglycoside](image2.png)

![Tetracycline](image3.png)

Figure 1.2: Structures of common protein synthesis inhibitors.
Tetracyclines inhibit protein synthesis by preventing the binding of aminoacyltransfer-RNA to the ribosome acceptor (A) site. This is accomplished by the molecule binding to the 30S ribosomal subunit.\textsuperscript{12} Resistance to tetracyclines occurs by the proteins TET(M), TET(O), and TET(Q). It is thought that these proteins interact with the ribosome, which allows protein biosynthesis to continue, even in the presence of bound tetracycline.\textsuperscript{13} Efflux pumps also play an important role in removing magnesium-chelated tetracyclines from the cell in exchange for protons, this is particularly true for Gram-negative bacteria.\textsuperscript{14}

\textit{Cell-wall Biosynthesis Inhibitors}

The bacterial cell wall contains a layer known as the peptidoglycan, and this barrier protects bacteria from lysis related to high internal osmotic pressures and helps maintain the shape of the bacterial cell.\textsuperscript{15} The peptidoglycan layer is comprised of a meshwork of peptide and glycan chains. The glycan chains are composed of a layer of alternating sugars, N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM) linked (1,4)-\(\beta\) in a long chain. To the carboxyl moieties of NAM, a pentapeptide chain is attached via an amide linkage. This peptide, consisting of L-Ala-D-Glu-L-Lys-D-Ala-D-Ala, is commonly referred to as the stem peptide. The stem peptide has a pentaglycine side-chain attached to the amino group of the lysine. Cross-linking (transpeptidation) between the lysine of the stem peptide and the displacement of the terminal D-Ala of the stem peptide from another glycan chain via a pentaglycine bridge completes the linkage between two separate glycan chains, thus strengthening the cell wall (Figure 1.3).\textsuperscript{16} Transpeptidation is catalyzed by cell wall transamidases, known as the penicillin-binding proteins or PBPs. The bacterial cell wall is
quite different compared to the outer layers of mammalian cells, thus providing an excellent target for selective chemotherapy agents.\textsuperscript{17}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{peptidoglycan.png}
\caption{Structure of the peptidoglycan layer of the bacterial cell wall.}
\end{figure}

PBP\textsubscript{s} target the D-Ala-D-Ala peptide sequence of the stem peptide chain attached to NAM. The active site on the enzyme employs a serine hydroxyl group to attack the peptide chain. The terminal D-Ala is removed and a temporary covalent bond is formed between the PBP and the remaining D-Ala unit. The amino group of the pentaglycyl unit of another
glycan chain attacks the newly formed acyl-enzyme complex. This regenerates the enzyme’s activity and completes the linkage between two separate glycan chains (Figure 1.4).\textsuperscript{18}

![Diagram of transpeptidation](image)

**Figure 1.4: Transpeptidation by PBP, R = L-Lys-D-Glu-L-Ala-NAM, R' = pentaglycyl unit of a different glycan chain.**

Most inhibitors of cell-wall synthesis are members of the $\beta$-lactam class of antibiotics, this includes the penicillins, cehalosporins, carbapenems, and monobactams (Figure 1.5). $\beta$-Lactams share a similar geometry to the acylated D-Ala-D-Ala. The PBP mistakes the $\beta$-lactam for its normal target and attacks the lactam ring. Upon hydrolysis of the 4-membered ring, there is a significant thermodynamic energy drop due to strain within the bicyclic system being relieved. This makes the reverse reaction highly unfavored, thus the substrate is irreversibly bound to the enzyme’s active site (Figure 1.6). The heterocyclic ring that is covalently bound to the PBP is also believed to provide sufficient steric hindrance to make nucleophilic attack unfavorable at the ester. Thus, the PBP is rendered inactive and can no longer continue transpeptidation. This results in a very weak bacterial cell wall that ultimately succumbs to osmotic stress.\textsuperscript{19}
Resistance to β-lactams generally occurs by one of two mechanisms, β-lactamases and an altered form of PBP. β-Lactamases are generally classified as either serine β-lactamases (SBL) or metallo-β-lactamases (MBL). Mechanistically, SBL inactivate the antibiotic in a similar fashion as the PBPs, however in this case, water is a sufficient nucleophile to yield the carboxylic acid and the free enzyme. This difference in reactivity is thought to be due to subtle modifications within the active site, which activate the ester towards hydrolysis. This is accomplished through hydrogen-bonding from the protein to the β-lactam carbonyl by the amide nitrogens of serine and alanine. MBLs are Zn(II)-dependent enzymes that are capable of hydrolyzing almost all known β-lactam antibiotics. This class of β-lactamase has become quite relevant in recent years due to Gram-negative
bacteria possessing plasmid encoded MBLs. Upon acquisition of these plasmids, numerous resistance genes can be expressed thus making some bacteria resistant to the majority of β-lactam antibiotics.\textsuperscript{20}

\textbf{Figure 1.6:} β-lactams bind irreversibly to PBP, but are inactivated by β-lactamases.

Methicillin-susceptible \textit{S. aureus} (MSSA) possesses four PBPs. The entire function of each PBP is not completely understood, but it is thought that the high molecular weight
PBP1, PBP2, and PBP3) are involved in transpeptidation and transglycosylation.\textsuperscript{21} PBP4 is thought to be involved in a secondary cross-linking reaction related to the synthesis of the peptidoglycan layer.\textsuperscript{22} PBP2a is known to be present in methicillin-resistant \textit{S. aureus} (MRSA), and it confers a 500-fold increase in minimum inhibitory concentration (MIC) for penicillins.\textsuperscript{23} PBP2a has a higher rate of release of bound drugs and a lower binding affinity compared to other PBPs. This allows it to take over the role of cross-linking the glycan chains, while other PBPs are inactive.\textsuperscript{20}

The glycopeptide antibiotic vancomycin is another important cell-wall biosynthesis inhibitor (Figure 1.7). The spectrum of activity of vancomycin is limited to Gram-positive pathogens, and it has become the antibiotic of choice for the treatment of MRSA infections. Vancomycin acts on the peptidoglycan by binding to the terminal D-Ala-D-Ala residues of the stem peptide, via five hydrogen bonds. The antibiotic effectively "cups" the uncross-linked pentapeptide, and prevents transglycosylation/transpeptidation. This makes the bacteria more susceptible to lysis from osmotic pressure.\textsuperscript{24}
Resistance to vancomycin occurs by the alteration of the D-Ala-D-Ala units on the peptidoglycan cell wall precursors to D-Ala-D-lactate. This change reduces vancomycin's affinity for the new depsipeptide linkage, and thus allows normal transglycosylation and transpeptidation activity to occur within the bacterial cell wall.\textsuperscript{25} Transcription of the genes responsible for this modification are controlled by a two-component system (TCS), and will be discussed in detail later.

1.3: Biofilms Impact on Society

Bacteria can exist as planktonic (free floating bacteria) or in microbial communities known as biofilms. Planktonic bacterial infections are much easier to treat, compared to bacterial biofilm infections, for reasons that are described below. Discovering novel ways to
eradicate biofilms, and ultimately provide new solutions to the growing problem of antibiotic resistance has been the focus of much work within the Melander Lab over the past decade.\textsuperscript{26-32}

In the 1970s, Costerton \textit{et al.} reported on the significance of biofilms. They stated that the majority of bacteria grow in matrix-enclosed biofilms that are capable of attaching to surfaces.\textsuperscript{33} Today, a biofilm is typically defined as a sessile community of microorganisms characterized by cells that are embedded in a matrix of extracellular polymeric substances (EPS) that are irreversibly attached to a surface, and exhibit an altered phenotype with respect to growth rate and gene transcription.\textsuperscript{34} Bacteria within biofilms are inherently insensitive to antiseptics and host immune responses, and residing within the biofilm state confers resistance to conventional antibiotics upwards of 1000 times that of planktonic bacteria. This is an alarming fact considering biofilms have been attributed to many ailments, such as lung infection in cystic fibrosis patients, otitis media, periodontitis, infections caused by surgical implants, urinary tract infections, and many others.\textsuperscript{35}

The large increase in antibiotic resistance that is observed for bacterial biofilms can be attributed its complex three dimensional structure and the EPS matrix that surrounds the cells (Figure 1.8). The EPS matrix is a complex structure of polysaccharides, small peptides, and nucleic acids. This mixture of highly polar compounds is believed to act as an adsorbent for charged compounds. It has been shown that some aminoglycosides are adsorbed by the EPS matrix that is produced from \textit{Pseudomonas aeruginosa}.\textsuperscript{36} The inability of antibiotics to penetrate all areas of the biofilm has been shown in various studies.\textsuperscript{37-39}
Another reason for the increase in antibiotic resistance within a biofilm, is that there are numerous microenvironments that have pH variations and anaerobic pockets. It is thought that this could aid in the degradation of various antibiotics.\textsuperscript{40} Additionally, biofilms have a heterogeneous population of cells that exist in different metabolic states. Cells that are deep within the biofilm might grow at a slower rate due to lack of nutrients, and this could also reduce the effectiveness of an antibiotic.\textsuperscript{41}

Biofilms can also lead to accumulation of slime and invertebrates on the bottom of ships. This is known as biofouling and can lead to a loss of drag beneath ships. This leads to increased fuel costs, which is a major burden on shipping industries and the military. In years past, agents such as bis(tributyltin) oxide were utilized in paints on the bottom of ships.
to counter biofouling. However, these chemicals are toxic by nature and can have dramatic effects on various aquatic ecosystems, as such they are rarely used today.

The summation of these two problems, antibiotic resistance and biofouling, have had a dramatic impact on society. It is estimated that the United States Navy spends between $300 - 500 million per year on fuel and cleaning costs related to biofouling. Additionally, it is estimated that the United States alone spends approximately $20 billion per year on antibiotic-resistant infections. The National Institutes of Health (NIH) has stated that over 80% of microbial infections that occur in the human body are mediated by biofilms. Indeed, bacterial biofilm infections are a very serious problem due to the ineffectiveness of antibiotics that would otherwise be more effective towards planktonic bacteria.

1.4: The Biofilm Development Cycle

The formation of *P. aeruginosa*, *Escherichia coli*, and *Vibrio cholera* biofilms has been studied extensively. This has led to the development of a stepwise model to explain the different stages of the biofilm lifecycle (Figure 1.9). Once the community has reached a certain population, the biofilm undergoes a dispersion stage whereby the bacteria can spread to infect nearby areas.
Figure 1.9: Biofilm development model.

The first step in biofilm formation involves the reversible attachment of planktonic bacteria to a surface. In the second step bacterial cells begin to release exopolymeric substances and attachment to the surface becomes irreversible. Three-dimensional architecture forms in the third step, and is denoted as early maturity. The morphology and topography of the biofilm become very distinct during the fourth step. Pillar shapes protrude from the biomass, so waste disposal and nutrient adsorption can be maximized. Cavities also form throughout the biofilm and these are used as a transportation system through the biofilm matrix. The movement of water and planktonic bacteria through the matrix signify the maturity of the biofilm. The last step in the development model involves detachment or dispersion of bacteria. This occurs as the hollow cavities within the biofilm fill with hypermotile cells, these cells are released when these channels are opened. Cells released in this stage are phenotypically similar to the planktonic cells involved in the initial steps of
biofilm development. This hypothesis is supported with reports that these cells are antibiotic-susceptible when treated with antimicrobial agents. After dispersion occurs the free-floating bacterial cells colonize new areas and the process is repeated. One way in which dispersion is initiated is by a process known as quorum sensing (QS).

1.5: Modulating Bacterial Biofilms

QS (cell to cell communication) pathways are known to influence virulence factors, bioluminescence, antibiotic production, sporulation, competence for DNA uptake, and biofilm formation. QS involves the release of small-molecules that relay information about population density and alter gene expression within the biofilm community. Most QS studies have involved Gram-negative bacteria such as *Vibrio fischeri*, *P. aeruginosa*, *Burkholderiacepacia*, *V. cholera*, and *Yersinia pestis*. It has been shown that many intraspecies signaling molecules are comprised of N-acyl homoserine lactone (AHL) derivatives, varying only at the acyl functionality (Figure 1.10). Once the molecule is released, it binds to a cognate cytoplasmic receptor protein reserved specifically for AHLs of a given acyl side-chain. This binding is thought to induce a conformational change and multimerization of the receptor protein, this is followed by DNA binding which activates or represses the transcription of targeted genes.
QS in Gram-positive systems is less well understood. There are many similarities between Gram-negative and Gram-positive QS, but there are important differences also. QS molecules in Gram-positive bacteria are post-translationally modified peptides called autoinducing peptides (AIPs) (Figure 1.10). AIPs do not diffuse across the cell membrane, thus transmembrane proteins are the receptors instead of cytoplasmic proteins. Once the AIP signal is bound by the receptor kinase, activation of complex phosphorylation events within the cell induces or represses gene transcription.\(^5\)

A third class of QS molecule is called autoinducer-2 (AI-2). AI-2 is produced by a variety of bacteria and is thought to enable interspecies communication.\(^5\) The AI-2 synthase, LuxS, produces a molecule called 4,5-dihydroxy-2,3-pentanedione (DPD). From this compound various species of bacteria produce unique AI-2s through a series of spontaneous rearrangements (Scheme 1.1). \textit{R}-THMF and \textit{S}-THMF-borate are the active AI-
2 signals in *V. harveyi* and *Salmonella enterica* serovar Typhimurium, respectively. The bacteria are then able to differentiate between their particular AI-2s. It should be noted that some bacteria are able to produce AI-2s and consume other AI-2s produced from other species. *E. coli* does this efficiently, which leads to neighboring species underestimating their own population density. The result is that other species fail to initiate or incorrectly terminate quorum sensing.

![Scheme 1.1: The DPD precursor is used to form different AI-2s depending on the bacterial species.](image)

Targeting QS mechanisms to modulate bacterial biofilms and reduce virulence has received much attention over the past decade. Most of these efforts have focused on the AHL mediated signaling cascade, as it is the best studied QS circuit. This has led to the use of various AHL analogs, most of these compounds are structurally similar to the naturally occurring signals. Modifications have included extending/shortening the acyl...
chain, the addition of aromatic substituents, and variations of the oxidation pattern (Figure 1.11). \(^{41,54}\)

![Chemical structures](image)

**Figure 1.11: Modified AHLs used to study quorum sensing pathways.**

Another approach to identify novel compounds that modulate biofilm activity, is to draw inspiration from nature in the form of natural products. Many marine organisms have evolved the ability to produce extremely toxic compounds as a means of chemical defense. These compounds are released into water and then rapidly diluted, thus it is required that they possess very high potency. It is hoped that the diverse populations within the oceans will yield promising biologically active compounds.\(^{55}\) The marine red algae *Delisea pulchra* produces a class of natural products known as halogenated furanones (Figure 1.12). These compounds have been shown to not only disrupt biofilms but they also sensitize *P. aeruginosa* biofilms to the effects of the antibiotic tobramycin.\(^{41}\)
Other marine natural products that incorporate a 2-aminoimidazole moiety have been shown to inhibit the formation of bacterial biofilms. Many marine sponges are soft bodied and live sedentary lifestyles. This leaves only a few options for self-defense against predators and/or parasites. Bromoageliferin 1.1 and oroidin 1.2 were isolated from the sponge Agelas conifera, and these compounds were effective at inhibiting the formation of biofilms by *Rhodospirillum salexigens* (Figure 1.13). It is thought that the sponge exploits these compounds as anti-biofouling agents.
1.6: Bacterial Two-Component Systems

To survive in the various harsh environments that bacteria routinely inhabit, they must be able to respond to particular stimuli such as pH, nutrient level, osmotic pressure, redox state, quorum signals, and antibiotics. To accomplish this, bacteria have evolved two-component signal transduction systems (TCS) that control gene expression related to various processes (Table 1.1). TCS are generally comprised of two proteins: a membrane-bound sensor histidine kinase (HK) and a cytoplasmic DNA-binding response regulator protein (RR).\textsuperscript{57} The cytoplasmic C-terminal domain of the HK contains a conserved histidine which is autophosphorylated in response to an external signal. Additionally, the cytoplasmic region of the HK contains a catalytic domain with an ATP-binding site that is quite different from the serine/threonine/tyrosine kinase found in eukaryotic organisms.\textsuperscript{58} Phosphotransfer from the histidine residue of the HK to an aspartic acid residue on the RR results in a
conformational rearrangement of the RR. This rearrangement generally leads to dimerization of two RRs, followed by DNA binding and thus changes in gene expression (Figure 1.14). 59

Table 1.1: Various cellular processes controlled by a TCS.

<table>
<thead>
<tr>
<th>Proteins Involved</th>
<th>Process Regulated</th>
</tr>
</thead>
<tbody>
<tr>
<td>CheA, CheY, CheB</td>
<td>bacterial chemotaxis</td>
</tr>
<tr>
<td>EnvZ, OmpR</td>
<td>osmoregulation</td>
</tr>
<tr>
<td>KinA, SpoOF</td>
<td>sporulation</td>
</tr>
<tr>
<td>ArcB, ArcA</td>
<td>regulation of genes involved in anaerobic respiration</td>
</tr>
<tr>
<td>NtrB, NtrC</td>
<td>nitrogen regulation</td>
</tr>
<tr>
<td>PhoR, PhoP, CreC</td>
<td>phosphate regulation</td>
</tr>
<tr>
<td>NarX, NarQ</td>
<td>nitrate regulation</td>
</tr>
<tr>
<td>VirA, BvgS</td>
<td>virulence</td>
</tr>
<tr>
<td>LemA</td>
<td>pathogenicity</td>
</tr>
<tr>
<td>Rpf C</td>
<td>synthesis of extracellular enzymes and polysaccharides</td>
</tr>
<tr>
<td>NifR1</td>
<td>nitrogen fixation</td>
</tr>
<tr>
<td>DegS</td>
<td>extracellular protease synthesis</td>
</tr>
<tr>
<td>RteA, RteB</td>
<td>regulation of tetracycline resistance elements</td>
</tr>
<tr>
<td>VanR, VanS</td>
<td>vancomycin resistance</td>
</tr>
</tbody>
</table>

TCSs are found in many prokaryotes, fungi, yeast, and some plants; however none have been identified in organisms belonging to the animal kingdom. 60,61 Importantly, these systems have been reported to exist within many pathogenic bacteria including *P. aeruginosa, E. coli, S. enterica, A. baumannii, K. pneumoniae*, and *S. aureus*. 58,62,63 As such, bacterial TCS have been targeted for the development of novel antibacterial compounds. 57,64,65
Many TCSs are not required for bacterial survival but aid significantly when the bacterium placed under an external stress such as an antibiotic. Thus TCSs represent an excellent target for the development of an adjuvant that could be used in combination with known antibiotics. This strategy would have the following advantages: 1) Selectively target bacterial HKs in the presence of mammalian HKs. 2) Restore the efficacy of an antibiotic by disrupting proper gene expression. 3) If the inhibitor operates under a non-toxic mechanism, the acquisition of resistance to the TCS inhibitor would be diminished.

Figure 1.14: Overview of a TCS signal transduction pathway.
1.7: The Role of a TCS in Antibacterial Resistance

Various TCSs are known to play important roles in coordinating a response to antibiotics in pathogenic bacteria, and thus are an attractive target for the identification of a small molecule inhibitor (Table 1.2). Exposure to the particular antibiotic generally results in up-regulation of genes encoding a phenotypic modification. For example, the PmrAB TCS is responsible for the addition of phosphoethanolamine on the lipid A component of the lipopolysaccharide portion of the bacterial membrane in *A. baumannii*. This modification reduces the net negative charge of the membrane, reducing the affinity of cationic antibiotics, resulting in a less susceptible strain of bacteria. Studies involving *A. baumannii* genetic knock-outs of the PmrAB TCS resulted in the reversal of this phenotype and thus restored susceptibility to the cationic antibiotic.\textsuperscript{62,66}

Table 1.2: Various TCSs with a role in antimicrobial resistance.

<table>
<thead>
<tr>
<th>Organism</th>
<th>TCS</th>
<th>Observed Antibiotic Resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. baumannii, P. aeruginosa</em></td>
<td>PmrAB</td>
<td>Polymyxins, antimicrobial peptides</td>
</tr>
<tr>
<td><em>Enterococcus faecium</em></td>
<td>VanRS</td>
<td>Vancomycin</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>VraRS</td>
<td>β-lactams, glycopeptides, daptomycin</td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td>PhoPQ</td>
<td>Polymyxins, antimicrobial peptides</td>
</tr>
<tr>
<td><em>K. pneumoniae</em></td>
<td>PhoBR</td>
<td>Carbapenems</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>BraRS</td>
<td>Antimicrobial peptides</td>
</tr>
</tbody>
</table>

The VanRS TCS confers resistance to the glycopeptide antibiotic vancomycin in enterococcal bacteria.\textsuperscript{67} Vancomycin normally binds to the N-acyl-D-Ala-D-Ala termini of
an uncrossed linked pentapeptide via five hydrogen bonds, and disrupts cell wall biosynthesis. The vanRSHAX operon is comprised of five genes and upon activation induces the biosynthesis of altered cell-wall intermediates (Table 1.3). At the present, it is unclear whether vancomycin is acting directly or indirectly as the external stimuli to activate this TCS. Transcription of the operon results in an altered cell wall, which differs from a normal bacterial cell wall by exchanging an amide for an ester. This results in a N-acyl-D-Ala-D-lactate termini which has a 1000-fold decrease in binding constant for vancomycin compared to the normal N-acyl-D-Ala-D-Ala.

Table 1.3: The role of each protein encoded by the vanRSHAX TCS.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Activity</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>VanS</td>
<td>Transmembrane histidine kinase</td>
<td>Sensor protein</td>
</tr>
<tr>
<td>VanR</td>
<td>Response regulator</td>
<td>Accepts PO₃²⁻ from VanS, activates vanH,A,X transcription</td>
</tr>
<tr>
<td>VanH</td>
<td>D-Specific α-keto acid reductase</td>
<td>Generates D-lactate for VanA</td>
</tr>
<tr>
<td>VanA</td>
<td>Depsipeptide ligase for D-Ala-D-lactate</td>
<td>Generates an ester in competition with the normal amide</td>
</tr>
<tr>
<td>VanX</td>
<td>Zn²⁺ dependent D-Ala-D-Ala dipeptidase</td>
<td>Removes D-Ala-D-Ala, allowing accumulation of D-Ala-D-lactate</td>
</tr>
</tbody>
</table>

The genome of S. aureus encodes 16 pairs of TCS, of these, VraRS TCS has been shown to control the expression of a variety of genes that are related to resistance to cell wall acting antibiotics. This system is known to be induced by exposing S. aureus to inhibitors of cell wall synthesis, and it has been shown that disruption of vraRS through the
use of a ΔvraS strain resulted in MIC reductions for both oxacillin and vancomycin.\textsuperscript{71,72}

Presently it is unclear what the exact stimuli is, but it is hypothesized that the sensor kinase responds to the damage of cell-wall structure or inhibition of cell-wall biosynthesis. Utilizing DNA microarrays, Kuroda \textit{et al}. successfully established that 139 genes were up-regulated by incubation with vancomycin, these included genes associated with cell-wall peptidoglycan synthesis such as \textit{pbp2, sgtB, murZ and fmtA}.\textsuperscript{70}

Transcripts for \textit{sgtB} encode a putative glycotransferase,\textsuperscript{72} \textit{murZ} encodes UDP-N-acetylglucosamine 1-carboxylvinyl transferase 2,\textsuperscript{73} while \textit{fmtA} encodes an accessory PBP with low affinity to β-lactams.\textsuperscript{74} All of these are important for the strength associated with the bacterial cell wall (Figure 1.15). UDP-N-acetylglucosamine and UDP-N-acetylmuramic acid-pentapeptide are synthesized by MurZ in the cytoplasm. A membrane carrier molecule is then attached to the peptidoglycan precursor via a di-phosphate bridge, which allows transportation of the molecule across the cytoplasmic membrane. Lastly, the transglycosylases (PBP2 and SgtB) and transpeptidases (FmtA, among other enzymes not transcriptionally controlled by VraSR) complete the sugar-peptide chains thus completing the synthesis of the peptidoglycan layer in \textit{S. aureus}.\textsuperscript{75}
Figure 1.15: The VraSR TCS is known to regulate gene expression of various proteins involved in cell-wall biosynthesis.

1.8: Known Inhibitors of TCS

Most small molecules that have been identified as an inhibitor of TCS signaling, operate by inhibiting autophosphorylation of the HK. Compound 1.3, imidazolium derivative, was shown to inhibit autophosphorylation of multiple HKs such as YycG, PhoQ, EnvZ, and posses antibacterial properties against Gram-positive organisms when present at the low μg/mL range (Figure 1.16). Compound 1.4 was shown to inhibit several HKs,
including VanS from *Enterococcus faecium* and EnvZ from *E. coli*, when present at a non-toxic concentration.\(^{77}\)

![Figure 1.16: Structure of small molecules known to inhibit HK function.](image)

Alternatively, there are reports of compounds that target the RR protein involved in TCS signaling (Figure 1.17). Compound 1.5 was identified via high-through-put screening and shown to down-regulate the expression of genes controlled by Alg-R1 in *P. aeruginosa*.\(^{78}\) In a later study, the Weisblum lab successfully demonstrated that compound 1.5 was able to prevent phosphotransfer of a phosphate group from the HK to the RR in the VanRS TCS of *E. faecium*.\(^{67}\) The Melander lab has shown a small molecule based on the 2-aminoimidazole scaffold, compound 1.6 is capable of binding to the RR BfmR in *A. baumannii*.\(^{32}\) BfmR is known to be a master controller of biofilm formation in *A. baumannii*, and Ballard *et al* demonstrated that 1.6 was able to inhibit and disperse biofilms via a non-toxic mechanism.\(^{79}\) Taken together, these results indicate the potential of targeting a TCS with small molecule, and thereby modulating bacterial behavior.
Our lab has made significant efforts to target TCSs responsible for antibiotic resistance. We successfully demonstrated that small molecules based on the 2-aminoimidazole motif can be used to inhibit these TCSs, and in the process restore antibiotic susceptibility to an otherwise resistant strain of bacteria (Figure 1.18). Compound 1.7 was shown to suppress oxacillin resistance in MRSA by targeting the VraSR TCS (discussed in Chapter 3), and compound 1.8 was shown to suppress colistin resistance in multi-drug resistant *A. baumannii* by targeting the PmrAB TCS (discussed in Chapter 4)\(^{80,81}\).
1.9: Conclusions

Bacterial biofilms represent a significant challenge to the scientific community. This is mainly due to the increased antimicrobial resistance observed when bacteria encase themselves within an extracellular matrix. Biofilm formation is controlled by a process known as quorum sensing. Controlling this process could lead to many novel therapies for treating biofilm-mediated infections. Additionally, nature has provided a variety of compounds that modulate biofilms, drawing inspiration from these compounds will ultimately lead to more active molecules.

Identifying compounds that target TCSs has been shown to be a viable means for the development of novel antibiotics. While the development of new antibiotics will benefit mankind in the short-term, it has been shown that bacteria inevitably evolve resistance to any external stimuli that places selective pressure on the organism. Alternatively, the development of adjuvants that target non-essential TCSs represents a novel approach to counter bacterial resistance mechanisms, as some of these non-essential TCSs have been shown to regulate genes associated with antibiotic resistance. As such, using combination therapy could extend the lifetime of current antibiotics and possibly restore the effectiveness of older antibiotics against an otherwise resistant organism.
References


(33) Costerton, J. W.; Geesey, G. G.; Cheng, K. J. *Scientific American* 1978, 238, 86


(49) Camilli, A.; Bassler, B. L. *Science* 2006, 311, 1113.


(58) Worthington, R. J.; Blackledge, M. S.; Melander, C. *Future Medicinal Chemistry* 2013, 5, 1265.


(60) Perry, J.; Koteva, K.; Wright, G. *Molecular Biosystems* 2011, 7, 1388.


(64) Matsushita, M.; Janda, K. D. Bioorganic and Medicinal Chemistry 2002, 10, 855.


CHAPTER 2
DEVELOPING A SYNTHETIC ROUTE TO ACCESS A DIVERSE LIBRARY OF 1,5-SUBSTITUED 2-AMINOIMIDAZOLES

2.1: Introduction

The Melander group has drawn inspiration from marine natural products in the search for anti-biofilm compounds. Marine coral and sponges are known to produce compounds that will inhibit the attachment of microorganisms which can lead to biofouling. Bromoageliferin 2.1 and oroidin 2.2 are two such compounds. Bromoageliferin 2.1 and oroidin 2.2 were shown to inhibit *Rhodospirillum salexigens* growth on a glass surface with IC$_{50}$ values of 2.43 nM and 169 μM, respectively.$^1$ The IC$_{50}$ value is defined as the concentration of a compound that inhibits 50% of biofilm formation relative to a control.

Huigens *et al.* postulated that the bicyclic core of bromoageliferin 2.1 was the key pharmacophore for the biological activity of this compound. As such, they synthesized two compounds TAGE (*trans*-bromoageliferin) 2.3 and CAGE (*cis*-bromoageliferin) 2.4 and tested them for biofilm inhibition of *P. aeruginosa* PAO1 (Figure 2.1).$^2$ TAGE 2.3 and CAGE 2.4 were able to inhibit the formation of a biofilm (IC$_{50}$ = 100 μM). Following this initial discovery, Richards *et al.* published a series of papers focused on the oroidin scaffold, thus providing the foundation for much of the work done within the Melander Lab.$^3$-$^5$
Over the years the Melander lab has developed several diverse libraries of compounds with anti-biofilm activity against a variety of bacterial strains, both Gram-positive and Gram-negative, in addition to compounds with fungal anti-biofilm activity.\textsuperscript{2-16} These studies have shown that the 2-aminoimidazole is a key functionality for the bioactivity of many of these compounds. Additionally, many of these anti-biofilm compounds inhibit the formation of a bacterial biofilm without exhibiting microbicidal activity towards planktonic bacteria. There is significant potential for molecules that possess the ability to inhibit and/or disperse bacterial biofilms via a non-toxic mechanism in infectious disease therapy, as if a molecule
does not directly kill bacteria there is a reduced likelihood of the bacteria acquiring resistance to the molecule.

Due to the robust biological activity of mono-substituted-2-aminoimidazoles, our lab has explored other substitution patterns. In 2010, Su and co-workers developed a synthesis to access 4,5-disubstituted-2-aminoimidazoles via a nitroenolate approach (Scheme 2.1). Many of these compounds displayed promising activity, initiating work on other substitution patterns.

Scheme 2.1: Synthetic route to 4,5-substituted-2-aminoimidazoles. m = 2-4, n = 0 - 4.

A route that would allow the placement of a substituent at one of the endocyclic nitrogen atoms would provide access to a 1,5-substituted-2-aminoimidazole library. Previously, reported methods for the preparation of 1,5-disubstituted-2-aminoimidazoles however, do not allow for the introduction of a diverse array of C-5 substituents. Kaila et al reported a method to prepare 2-aminoimidazoles with an acyl group at the 5-position. This was achieved by monocondensation of thiourea with dimethylformamide dimethyl acetal, followed by protection with trityl chloride to give the corresponding 1-amidino-3-trityl-thiourea. Reaction of the amidinothiourea with various anilines in the presence of mercury
(II) chloride yielded the respective guanidines. Cyclization with a variety of α-bromo ketones, followed by deprotection afforded the 1,5-disubstituted-2-aminoimidazole (Scheme 2.2).\textsuperscript{18}

Scheme 2.2: Preparation of 1,5-disubstituted-2-aminoimidazole. $R^1 = \text{Various acyl groups.}$

A method to prepare 5-aryl 1,5-disubstituted-2-aminoimidazoles has been reported via the formation of imidazo(1,2-α)pyrimidinium salts. Starting with a substituted 2-aminopyrimidine, the imidazo(1,2-α)pyrimidinium intermediate can be formed by heating with various α-bromocarbonyl compounds followed by acid-catalyzed elimination with HBr or PPA. Treatment with hydrazine in acetonitrile yielded various 5-aryl 2-aminoimidazole derivatives.\textsuperscript{19} A revised method utilizing microwave irradiation revealed a two-step
procedure to the desired product (Scheme 2.3). These compounds were then shown to inhibit biofilm formation against *Salmonella typhimurium* and *P. aeruginosa*.\(^{20}\)

**Scheme 2.3:** Synthesis of 5-aryl 2-aminoimidazoles from imidazo(1,2-α)pyrimidinium salts. \(R^3\) = various aryl groups.

The aforementioned routes are limited to acyl or aryl substitution patterns at the 5-position, we desired a method that utilized readily available building blocks and allowed for the introduction of diversity at both the 1-position and the 5-position of the 2-aminoimidazole ring. Ultimately the ability to make analogues possessing our previously determined active C-5 substituents would lead to better structure activity relationship studies between various libraries. To this end, we envisioned that the desired 1,5-disubstituted-2-aminoimidazole derivatives could be readily prepared from the condensation of N-substituted α-aminoaldehydes with cyanamide. In turn, these N-substituted α-aminoaldehydes could be
prepared from a reductive amination reaction between commercially available aldehydes and readily available amino acids, followed by reduction of the carboxylic acid to the aldehyde (Figure 2.2).

![Figure 2.2: Retrosynthetic analysis of 1,5-disubstituted-2-aminoimidazoles.](image)

2.2: Results and Discussion

The first step of our route to 1,5-substituted 2-aminoimidazoles involved the formation of N-substituted α-amino acids. We tested several conditions for the reductive amination of L-phenylalanine with benzaldehyde and determined the best method to be a two-step procedure using sodium borohydride as the reducing agent, which, after Boc-protection, afforded the desired N-substituted phenylalanine 2.5 in 70% yield. We then turned our attention to the conversion of the α-amino acid to its corresponding α-amino aldehyde. Several methods were investigated including thiol ester formation followed by reduction with triethylsilane and sodium amalgam (Akabori) reduction of the methyl ester of compound 2.5 (Scheme 2.4). It was decided that the most efficient route, and that which allowed for the greatest introduction of diversity, was via the formation of the N-methoxy-N-methylamide (Weinreb amide) and subsequent reduction (Scheme 2.5). Conversion of the Boc protected amino acid 2.5 to the Weinreb amide was achieved using (benzotriazol-1-
yloxy)tris(dimethylamino)phosphonium hexafluorophosphate (BOP) as the coupling reagent. Reduction of the Weinreb amide to the aldehyde was carried out using diisobutylaluminum hydride (DIBAL-H) followed by in situ Boc-deprotection with HCl, and cyclization with cyanamide to afford the 1,5-disubstituted-2-aminoimidazole.

![Chemical reaction](image)

**Scheme 2.4: Initially explored routes to α-amino aldehyde.**

Following these test reactions, an initial 20-member library was generated, possessing a variety of substituents including, straight chain and branched alkyl groups, phenyl and alkyl phenyl groups, and a more polar carbamate group. In summary, imine formation between equimolar amounts of commercially available amino acids and aldehydes in methanol in the presence of lithium hydroxide was followed by reduction with two equivalents of sodium borohydride. The crude secondary amines were Boc protected, using Boc anhydride in
acetonitrile with TMAH as base to furnish the N-substituted Boc amino acids. Amide coupling of these protected amino acids with \(N,O\)-dimethylhydroxylamine, using BOP as the coupling reagent in DCM in the presence of triethylamine, afforded the desired Weinreb amides. The Weinreb amides were then reduced with DIBAL-H in THF and after quenching the reaction, the crude aldehydes were extracted with diethyl ether then treated with diethyl ether/aqueous HCl or TFA/DCM to remove the Boc group. Following solvent removal the crude amino aldehydes were dissolved in a 1:1 mixture of ethanol/water, the pH adjusted to 4.3, and allowed to react with cyanamide at 95 °C. Following purification the respective 1,5-disubstituted-2-aminoimidazoles were converted to their HCl salts for biological testing.
Initially we investigated the antibiotic activity of this 1,5-disubstituted-2-aminoimidazole library. This was carried out by measurement of the minimum inhibitory concentration (MIC) of each derivative against a variety of representative pathogenic bacterial strains, both Gram-negative and Gram-positive, using a standard broth microdilution protocol. We tested activity against: *Escherichia coli, Acinetobacter*
baumannii, multi-drug resistant Acinetobacter baumannii (MDRAB), Staphylococcus epidermidis, methicillin resistant Staphylococcus epidermidis (MRSE), methicillin susceptible Staphylococcus aureus (MSSA), methicillin resistant Staphylococcus aureus (MRSA) and a carbapenem resistant strain of Klebsiella pneumoniae which produces the recently reported New Delhi metallo-ß-lactamase (NDM-1). It was found that compound 2.64 was the most active antibiotic, with MIC values of 2, 8, 8, 32, 0.125, 2, 2, and 2 μg/mL against E. coli, A. baumannii, MDRAB, K. pneumoniae S. epidermidis, MRSE, MSSA and MRSA respectively (complete data in Appendix). This compound demonstrated greater antibiotic activity against the Gram-positive strains tested than against the Gram-negative strains, and has particularly notable activity against the opportunistic bacterium S. epidermidis.

Comparing compound 2.64 with 2.63, it can be seen that increasing the chain length of the N-1 substituent leads to an increase in activity for Gram-negative strains and Gram-positive strains (Figure 2.2). Therefore it was decided to synthesize two more compounds with the same C-5 substituent and increasing alkyl chain lengths of the N-1 substituent to see whether this trend would be continued. These compounds (2.65 and 2.66) were synthesized in the same manner as the initial library and were tested for antibiotic activity against the same eight bacterial strains (Figure 2.3). Unfortunately, a significant increase in antibiotic activity from compound 2.64 was not observed, though these compounds still possess considerable antibiotic activity against S. epidermidis, MRSE, MSSA, MRSA and E. coli.
Next, the 1,5-disubstituted 2-aminoimidazole library was tested for the ability to inhibit bacterial biofilm formation. For this investigation five biofilm forming bacterial strains were selected, *E. coli*, *A. baumannii*, MDRAB, MSSA and MRSA. Compounds were initially screened at 200 μM using a crystal violet reporter assay.\textsuperscript{24} Several compounds, including 2.63 and 2.65, exhibited biofilm inhibition activity (complete data in Appendix). Compounds exhibiting greater than 90% inhibition were subject to a dose response assay in hopes of determining the IC\textsubscript{50} value for biofilm inhibition activity. However, upon performing the dose response assay, we noticed a rapid drop in activity over a narrow
concentration range, indicative of activity via a microbicidal mechanism. IC\textsubscript{50} values for biofilm inhibition activity for this library of compounds were therefore not determined.

2.3: Conclusions

In conclusion, we have developed a facile route to 1,5-disubstituted 2-aminoimidazoles that allows rapid assembly of a diverse array of 2-aminoimidazole derivatives from readily available starting materials. The lead compounds identified in this study show significant antibiotic activity against a wide variety of bacterial strains. Several of the simple analogues developed in this study demonstrated the ability to inhibit biofilm formation, albeit through a microbicidal mechanism.\textsuperscript{25} The synthetic route developed in this project can be used to access 1,5-substituted 2-aminoimidazoles based on previously identified highly active mono-substituted 2-aminoimidazoles.

2.4: Experimental Section

Synthesis Experimental

All reagents used for chemical synthesis were purchased from commercially available sources and used without further purification. Chromatography was performed using 60Å\textsuperscript{°} mesh standard grade silica gel from Sorbtech (Atlanta, GA, USA). NMR solvents were obtained from Cambridge Isotope Labs and used as is. \textsuperscript{1}H NMR (300 MHz or 400 MHz) and \textsuperscript{13}C NMR (75 MHz or 100 MHz) spectra were recorded at 25°C on Varian Mercury spectrometers. Chemical shifts (\(d\)) are given in ppm relative to tetramethylsilane or the respective NMR solvent; coupling constants (\(J\)) are in Hertz (Hz). Abbreviations used are s = singlet, br s = broad singlet, d = doublet, dd = doublet of doublets, t = triplet, dt = doublet of triplets, q = quartet and m = multiplet. Mass spectra were obtained at the NCSU Department
of Chemistry Mass Spectrometry Facility. Infrared spectra were obtained on a FT/IR-4100 spectrophotometer ($v_{\text{max}}$ in cm$^{-1}$). UV absorbance was recorded on a Genesys 10 scanning UV/visible spectrophotometer ($\lambda_{\text{max}}$ in nm).

**General synthetic procedure for reductive amination and Boc protection**

The amino acid specified below was dissolved in methanol. LiOH•H$_2$O was added, and the solution stirred at room temperature for 20 minutes. The corresponding aldehyde was added dropwise, and stirred for 1 hour. Sodium borohydride was then added portionwise. After 30 minutes, the solvent was removed under reduced pressure and the resulting solid was dissolved in water. Using 1M HCl, the pH was adjusted to 6.5 and then filtered. The crude white solid was carried on without further purification. The crude product and tetramethylammonium hydroxide (TMAH) were dissolved in CH$_3$CN, after stirring for 30 minutes, Di-tert-butyl dicarbonate (Boc$_2$O) was then added. The reaction was allowed to stir for 18 hours. The solvent was then removed under reduced pressure and the crude Boc-protected amino acid was dissolved in water. Using 1M HCl the pH was adjusted to 2, and then extracted with ethyl acetate. The organic layer was washed with water (3x), brine (1x), then dried over sodium sulfate. The solvent was removed under reduced pressure, and the crude product was purified via flash column chromatography (5% → 50% acetone/hexane).

**General synthetic procedure for Weinreb amide formation**

The Boc protected amino acid was stirred in dry dichloromethane (DCM) (20 mL) under nitrogen gas. To this solution, triethylamine was added followed by (benzotriazol-1-yl oxy)tris(dimethylamino)phosphonium hexafluorophosphate (BOP) and stirred at room temperature. After 15 minutes, $O,N$-dimethylhydroxylamine hydrochloride and
triethylamine were added. The reaction was stirred for 18 hours, diluted with DCM (60 mL) and then washed with 2M HCl (3 x), aqueous saturated sodium bicarbonate (3 x), and brine. The organic layer was dried over sodium sulfate and the solvent was removed under reduced pressure. The crude product was then purified via flash column chromatography (5% → 50% ethyl acetate/hexane).

**General synthetic procedure for cyanamide cyclization**

The α-amino Weinreb amide was added to dry tetrahydrofuran (10 mL) at -78 °C, under nitrogen gas. While stirring Diisobutylaluminium hydride (DIBAL-H) was added and the solution was stirred for 1 hour at -78 °C → room temperature. The reaction was quenched with aqueous 0.35 M sodium bisulfate, then extracted with diethyl ether (3 x). The organic layer was washed with 1M HCl (2 x), aqueous saturated sodium bicarbonate (2 x), brine (2 x), and then dried over sodium sulfate. The solvent was removed under reduced pressure, and the crude product was carried on without further purification. The crude product was dissolved in either 1:1 diethyl ether:4M HCl (15 mL) or 10:1 dichloromethane:trifluoroacetic acid (15 mL). The reaction was stirred for 2 hours at room temperature, and then the solvent was removed under reduced pressure. Without purification the crude product was dissolved in 1:1 ethanol:water (15 mL), and the pH was adjusted to 4.3 using 2M aqueous NaOH. While stirring, cyanamide was added to the solution which was then refluxed (95 °C) for 3 hours. The solvent was removed under reduced pressure and the crude product was purified via flash column chromatography (3% methanol(sat. NH₃)/CH₂Cl₂ → 10% methanol(sat. NH₃)/CH₂Cl₂). The pure compound was then dissolved in methanol (5 mL) and 1M HCl (0.2 mL) was added to afford the hydrochloride salt upon removal of solvent.
2-(Benzyl(tert-butoxycarbonyl)amino)-3-phenylpropanoic acid (2.5): 2.5 was synthesized as described in the general procedure using L-phenylalanine (12.11 mmol, 2 g), benzaldehyde (12.11 mmol, 1.22 mL), sodium borohydride (24.22 mmol, 916 mg). To the crude product (7.84 mmol, 2 g) TMAH (15.68 mmol, 2.84 g) was added, followed by Boc$_2$O (15.68 mmol, 3.42 g). After purification via flash column chromatography, 2.5 was obtained as a yellow oil (1.96 g, 71%).

$^1$H NMR (400 MHz, CDCl$_3$) δ 10.45 – 10.19 (br s, 1H), 7.33 – 7.12 (m, 10H), 4.71 and 4.67 and 3.90 and 2.58 (4 x br s, 2H, rotamers), 4.27 and 3.95 (2 x br s, 1H, rotamers), 3.36 and 3.15 (2 x br s, 2H, rotamers), 1.59 and 1.49 (s, 9H, rotamers); $^{13}$C NMR (100 MHz, CDCl$_3$) δ 176.9 and 176.1 (rotamers), 155.7 and 155.0 (rotamers), 137.8 and 137.7 (rotamers), 137.4 and 136.7 (rotamers), 129.2, 128.6 and 128.5 (rotamers), 128.4 and 128.2 (rotamers), 127.6, 127.3 and 127.0 (rotamers), 126.6 and 126.5 (rotamers), 81.4 and 81.0 (rotamers), 61.9 and 60.8 (rotamers), 52.5 and 52.0 (rotamers), 36.3 and 35.2 (rotamers), 28.3 and 28.2 (rotamers). IR (KBr) ν(cm$^{-1}$) 2928, 1703, 1460, 1250, 1164. $\lambda_{\text{max}}$ = 219 nm, 252 nm. HRMS (ESI): m/z: Calcd for C$_{21}$H$_{25}$NO$_4$: 378.1676 [M+Na]$^+$; found: 378.1677.
2-(tert-Butoxycarbonyl(3-phenylpropyl)amino)-3-phenylpropanoic acid (2.6): 2.6 was synthesized as described in the general procedure using L-phenylalanine (12.11 mmol, 2 g), hydrocinnamaldehyde (12.11 mmol, 1.60 mL), sodium borohydride (24.22 mmol, 916 mg). To the crude product (7.06 mmol, 2 g) TMAH (14.13 mmol, 2.56 g) was added, followed by Boc₂O (14.13 mmol, 3.09 g). After purification via flash column chromatography, 2.6 was obtained as a colorless oil (2.13 g, 79%).

\[ \text{^1H NMR (400 MHz, CDCl}_3\text{)} \delta 11.01 \text{ (br s, 1H), 7.33 – 7.09 (m, 10H), 4.16 and 3.97 (2 x br s, 2H, rotamers), 3.34 (s, 2H), 3.14 and 2.64 (2 x m, 2H, rotamers), 2.47 (m, 2H), 1.62 – 1.47 (m, 11H); } \]

\[ \text{^13C NMR (100 MHz, CDCl}_3\text{)} \delta 177.3 \text{ and 176.5 (rotamers), 155.9 and 154.7 (rotamers), 142.0 and 141.7 (rotamers), 138.1, 129.5 and 129.4 (rotamers), 128.9 and 128.8 (rotamers), 128.6, 128.4, 126.8 and 126.7 (rotamers), 126.0 and 125.8 (rotamers), 81.2 and 80.8 (rotamers), 63.3 and 62.9 (rotamers), 49.2, 36.3 and 35.2 (rotamers), 33.1, 30.1 and 29.8 (rotamers), 28.5. } \]

IR (KBr) \( \nu (\text{cm}^{-1}) \): 2931, 1692, 1403, 1159. \( \lambda_{\text{max}} = 217 \text{ nm, 258 nm. } \)


\[ \text{2-(tert-Butoxycarbonyl(pentyl)amino)-3-phenylpropanoic acid (2.7): 2.7 was synthesized as described in the general procedure using L-phenylalanine (12.11 mmol, 2 g), valeraldehyde (12.11 mmol, 1.29 mL), sodium borohydride (24.22 mmol, 916 mg). To the} \]

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crude product (8.50 mmol, 2 g) TMAH (17.00 mmol, 3.08 g) was added, followed by Boc₂O (17.00 mmol, 3.71 g). After purification via flash column chromatography, 2.7 was obtained as a colorless oil (1.93 g, 68%).

\(^1\)H NMR (300 MHz, CDCl₃) \(\delta 7.32 – 7.20 (m, 5H), 4.10\) and 3.96 (2 x br s, 1H, rotamers), 3.36 - 3.23 (m, 2H), 3.19 and 2.55 (2 x m, 2H, rotamers), 1.48 (s, 9H), 1.28 – 1.08 (m, 6H), 0.82 (t, 3H, \(J = 6.2\) Hz); \(^1\)3C NMR (75 MHz, CDCl₃) \(\delta 177.3\) and 176.3 (rotamers), 156.0 and 154.5 (rotamers), 137.9 and 137.8 (rotamers), 129.3 and 129.2 (rotamers), 128.5 and 128.4 (rotamers), 126.6, 80.8 and 80.7 (rotamers), 63.4 and 62.7 (rotamers), 49.6 and 49.5 (rotamers), 36.1 and 35.0 (rotamers), 28.8, 28.3, 28.0 and 27.6 (rotamers), 22.3 and 22.2 (rotamers), 13.9. IR (KBr) \(\nu (\text{cm}^{-1})\) 2961, 1691, 1409, 1171. \(\lambda_{\text{max}} = 219\) nm, 258 nm.


2-(tert-Butoxycarbonyl(octyl)amino)-3-phenylpropanoic acid (2.8): 2.8 was synthesized as described in the general procedure using L-phenylalanine (12.11 mmol, 2 g), octyl aldehyde (12.11 mmol, 1.89 mL), sodium borohydride (24.22 mmol, 916 mg). To the crude product (7.22 mmol, 2 g) TMAH (14.44 mmol, 2.62 g) was added, followed by Boc₂O (14.44 mmol, 3.15 g). After purification via flash column chromatography, 2.8 was obtained as a colorless oil (1.87 g, 69%).

\(^1\)H NMR (300 MHz, CDCl₃) \(\delta 7.33 – 7.19 (m, 5H), 4.08 - 3.98 (m, 1H), 3.37 - 3.27 (m, 2H), 3.08\) and 2.60 (2 x m, 2H, rotamers), 1.46 (s, 9H), 1.35 – 1.20 (m, 12H), 0.88 (t, 3H, \(J = 6.6\) Hz).
Hz; $^{13}$C NMR (75 MHz, CDCl$_3$) $\delta$ 177.3 and 175.5 (rotamers), 156.4 and 154.5 (rotamers), 138.0 and 137.7 (rotamers), 129.3 and 129.2 (rotamers), 128.5 and 128.4 (rotamers), 126.7, 81.0 and 80.8 (rotamers), 64.0 and 62.7 (rotamers), 49.9 and 49.5 (rotamers), 36.2 and 35.0 (rotamers), 31.7, 29.9 and 29.2 (rotamers), 29.1, 28.3 and 27.9 (rotamers), 26.7 and 26.6 (rotamers), 22.6, 14.1. IR (KBr) $\nu$ (cm$^{-1}$) 2926, 1699, 1254, 1169. $\lambda_{\text{max}}$ = 218 nm, 257 nm.

HRMS (ESI): $m/z$: Calcd for C$_{22}$H$_{35}$NO$_4$: 400.2458 [M+Na]$^+$; found: 400.2440.

2-(Benzyl(tert-butoxycarbonyl)amino)-2-phenylacetic acid (2.9): 2.9 was synthesized as described in the general procedure using L-\(\alpha\)-phenylglycine (13.23 mmol, 2 g), benzaldehyde (13.23 mmol, 1.34 mL), sodium borohydride (26.46 mmol, 1 g). To the crude product (8.30 mmol, 2 g) TMAH (16.60 mmol, 3.01 g) was added, followed by Boc$_2$O (16.60 mmol, 3.62 g). After purification via flash column chromatography, 2.9 was obtained as a colorless oil (2.23 g, 79%).

$^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 11.20 (br s, 1H), 7.31 – 7.02 (m, 10H), 5.85 and 5.25 (2 x br s, 1H, rotamers), 4.80 and 4.18 (2 x m, 2H, rotamers), 1.46 (br s, 9H); $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 175.9, 156.3, 138.6, 137.9, 133.8, 129.3, 128.4, 127.8, 127.3, 126.5, 81.5, 63.2, 49.2, 28.1. IR (KBr) $\nu$ (cm$^{-1}$) 2976, 1693, 1398, 1160. $\lambda_{\text{max}}$ = 212 nm, 258 nm. HRMS (ESI): $m/z$: Calcd for C$_{20}$H$_{23}$NO$_4$: 364.1519 [M+Na]$^+$; found: 364.152.
**2-(tert-Butoxycarbonyl(3-phenylpropyl)amino)-2-phenylacetic acid (2.10):**

2.10 was synthesized as described in the general procedure using L-α-phenylglycine (13.23 mmol, 2 g), hydrocinnamaldehyde (13.23 mmol, 1.75 mL), sodium borohydride (26.46 mmol, 1 g). To the crude product (7.43 mmol, 2 g) TMAH (14.86 mmol, 2.69 g) was added, followed by Boc₂O (14.86 mmol, 3.24 g). After purification via flash column chromatography, 2.10 was obtained as a pale yellow oil (1.66 g, 61%).

^1^H NMR (400 MHz, CDCl₃) δ 11.1 (br s, 1H), 7.42 – 7.07 (m, 10H), 5.96 and 5.51 (2 x br s, 1H, rotamers), 3.42 and 3.13 (2 x br s, 2H, rotamers), 2.76 and 2.45 (2 x br s, 2H, rotamers), 1.83 and 1.46 (2 x br s, 2H, rotamers), 1.56 (s, 9H); ^1^C NMR (100 MHz, CDCl₃) δ 175.8, 156.2 and 155.0 (rotamers), 141.4 and 139.9 (rotamers), 134.3 and 134.0 (rotamers), 129.1, 128.5 and 128.3 (rotamers), 128.2 and 128.1 (rotamers), 128.0, 125.8 and 125.7 (rotamers), 125.5, 80.9 and 80.6 (rotamers), 62.7 and 62.4 (rotamers), 45.6 and 45.3 (rotamers), 32.9, 30.5 and 30.1 (rotamers), 28.1 and 28.0 (rotamers). IR (KBr) ν (cm⁻¹) 2977, 1692, 1404, 1159. λ_max = 221 nm, 257 nm. HRMS (ESI): m/z: Calcd for C₂₂H₂₇NO₄: 392.1832 [M+Na]^+; found: 392.1833.

**2-(tert-Butoxycarbonyl(pentyl)amino)-2-phenylacetic acid (2.11):**

2.11 was synthesized as described in the general procedure using L-α-phenylglycine (13.23 mmol, 2 g),
valeraldehyde (13.23 mmol, 1.41 mL), sodium borohydride (26.46 mmol, 1 g). To the crude product (9.04 mmol, 2 g) TMAH (18.08 mmol, 3.28 g) was added, followed by Boc₂O (18.08 mmol, 3.95 g). After purification via flash column chromatography, 2.11 was obtained as a colorless oil (2.36 g, 81%).

\(^1\)H NMR (400 MHz, CDCl₃) δ 10.99 (br s, 1H), 7.34 – 7.26 (m, 5H), 5.79 and 5.41 (2 x br s, 1H, rotamers), 3.25 and 2.95 (2 x br s, 2H), 1.47 (s, 9H), 1.10 – 0.99 (m, 6H), 0.74 (t, 3H, \(J = 7.2\) Hz); \(^1\)³C NMR (100 MHz, CDCl₃) δ 175.9, 156.4 and 154.9 (rotamers), 134.4, 129.1, 128.4, 128.3, 80.6, 62.9 and 62.8 (rotamers), 45.8, 28.7, 28.4, 28.2, 21.9, 13.8. IR (KBr) \(\nu (\text{cm}^{-1})\) 2961, 1691, 1409, 1172. \(\lambda_{\text{max}}\) = 219 nm, 256 nm. HRMS (ESI): \(m/z\): Calcd for C₁₈H₂₇NO₄: 344.1832 [M+Na]⁺; found: 344.1841.

![Chemical structure of 2.11](image)

2-(tert-Butoxycarbonyl(octyl)amino)-2-phenylacetic acid (2.12): 2.12 was synthesized as described in the general procedure using L-\(\alpha\)-phenylglycine (13.23 mmol, 2 g), octyl aldehyde (13.23 mmol, 2.06 mL), sodium borohydride (26.46 mmol, 1 g). To the crude product (7.60 mmol, 2 g) TMAH (15.2 mmol, 2.75 g) was added, followed by Boc₂O (15.2 mmol, 3.32 g). After purification via flash column chromatography, 2.12 was obtained as a colorless oil (1.59 g, 58%).

\(^1\)H NMR (400 MHz, CDCl₃) δ 11.22 (br s, 1H), 7.41 – 7.32 (m, 5H), 5.80 and 5.40 (2 x br s, 1H, rotamers), 3.28 and 2.97 (2 x br s, 2H, rotamers), 1.49 – 1.04 (m, 21H), 0.86 (t, 3H, \(J = 7.2\) Hz); \(^1\)³C NMR (100 MHz, CDCl₃) δ 175.9, 156.6, 134.5, 129.1, 128.5, 128.4, 80.8, 63.3, 46.2, 31.7, 29.0, 28.9, 28.8, 28.3, 26.6, 22.5, 14.0. IR (KBr) \(\nu (\text{cm}^{-1})\) 2926, 1695, 1407,
$\lambda_{\text{max}} = 218$ nm, 250 nm. HRMS (ESI): $m/z$: Calcd for C$_{21}$H$_{33}$NO$_4$: 386.2302 [M+Na]$^+$; found: 386.2299.

2-(Benzyl(tert-butoxycarbonyl)amino)-4-methylpentanoic acid (2.13): 2.13 was synthesized as described in the general procedure using L-leucine (15.25 mmol, 2 g), benzaldehyde (15.25 mmol, 1.54 mL), sodium borohydride (30.5 mmol, 1.15 g). To the crude product (9.04 mmol, 2 g) TMAH (18.08 mmol, 3.28 g) was added, followed by Boc$_2$O (18.08 mmol, 3.95 g). After purification via flash column chromatography, 2.13 was obtained as a colorless oil (2.14 g, 74%).

$^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 10.98 – 10.75 (br s, 1H), 7.30 – 7.25 (m, 5H), 4.75 and 4.28 (2 x m, 2H, rotamers), 4.33 and 4.10 (2 x m, 1H, rotamers), 1.83 – 1.57 (m, 1H), 1.62 – 1.42 (m, 11H), 0.86 – 0.69 (m, 6H, rotamers); $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 178.2 and 177.4 (rotamers), 156.5 and 155.8 (rotamers), 138.6 and 138.0 (rotamers), 128.3, 127.3, 127.1, 81.1, 58.2 and 57.6 (rotamers), 50.8 and 50.6 (rotamers), 39.2 and 38.3 (rotamers), 28.2, 24.9 and 24.8 (rotamers), 22.5 and 21.8 (rotamers). IR (KBr) $\nu$(cm$^{-1}$) 2972, 1700, 1398, 1169. $\lambda_{\text{max}} = 218$ nm, 257 nm. HRMS (ESI): $m/z$: Calcd for C$_{18}$H$_{27}$NO$_4$: 344.1832 [M+Na]$^+$; found: 344.1834.
2-(tert-Butoxycarbonyl(3-phenylpropyl)amino)-4-methylpentanoic acid (2.14): 2.14 was synthesized as described in the general procedure using L-leucine (15.25 mmol, 2 g), hydrocinnamaldehyde (15.25 mmol, 2.02 mL), sodium borohydride (30.5 mmol, 1.15 g). To the crude product (8.03 mmol, 2 g) TMAH (16.06 mmol, 2.91 g) was added, followed by Boc₂O (16.06 mmol, 3.51 g). After purification via flash column chromatography, 2.14 was obtained as a colorless oil (1.41 g, 50%).

¹H NMR (400 MHz, CDCl₃) δ 10.98 – 10.75 (br s, 1H), 7.34 - 7.17 (m, 5H), 4.53 and 4.15 (2 x m, 1H, rotamers), 3.49 and 3.02 (2 x m, 2H, rotamers), 2.62 (t, 2H, J = 7.0 Hz), 2.02 – 1.43 (m, 14H), 0.94 (d, 6H, J = 5.4 Hz); ¹³C NMR (100 MHz, CDCl₃) δ 178.0 and 177.6 (rotamers), 156.1 and 154.9 (rotamers), 141.7 and 141.4 (rotamers), 128.2, 125.8, 125.7, 80.6 and 80.4 (rotamers), 58.2 and 57.8 (rotamers), 47.2 and 46.3 (rotamers), 38.9 and 38.0 (rotamers), 33.3 and 33.2 (rotamers), 30.9 and 30.2 (rotamers), 28.2, 24.8 and 24.6 (rotamers), 23.0 and 22.9 (rotamers). IR (KBr) ν(cm⁻¹) 2951, 1693, 1331, 1172. λₘₐₓ = 216 nm, 258 nm. HRMS (ESI): m/z: Calcd for C₂₀H₃₁NO₄: 372.2145 [M+Na]⁺; found: 372.2134.
2-(tert-Butoxycarbonyl(pentyl)amino)-4-methylpentanoic acid (2.15): 2.15 was synthesized as described in the general procedure using L-leucine (15.25 mmol, 2 g), valeraldehyde (15.25 mmol, 1.62 mL), sodium borohydride (30.5 mmol, 1.15 g). To the crude product (9.94 mmol, 2 g) TMAH (19.88 mmol, 3.60 g) was added, followed by Boc$_2$O (19.88 mmol, 4.34 g). After purification via flash column chromatography, 2.15 was obtained as a colorless oil (1.33 g, 45%).

$^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 4.35 and 4.19 (2 x br s, 1H, rotamers), 3.32 and 2.95 (2 x br s, 2H, rotamers), 1.82 – 1.25 (m, 18H), 0.96 (m, 9H); $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 178.1 and 176.3 (rotamers), 156.9 and 155.0 (rotamers), 80.9 and 80.4 (rotamers), 59.2 and 58.1 (rotamers), 47.7, 39.1 and 38.1 (rotamers), 29.1 and 28.9 (rotamers), 28.3, 24.8, 23.1 and 22.8 (rotamers), 22.3, 22.0 and 21.8 (rotamers), 14.0. IR (KBr) $\nu$(cm$^{-1}$) 2956, 1701, 1356, 1163. HRMS (ESI): $m$/z: Calcd for C$_{16}$H$_{31}$NO$_4$: 324.2145 [M+Na]$^+$; found: 324.2146.

2-(tert-Butoxycarbonyl(octyl)amino)-4-methylpentanoic acid (2.16): 2.16 was synthesized as described in the general procedure using L-leucine (15.25 mmol, 2 g), octyl aldehyde (15.25 mmol, 2.38 mL), sodium borohydride (30.5 mmol, 1.15 g). To the crude product (8.22 mmol, 2 g) TMAH (16.44 mmol, 2.98 g) was added, followed by Boc$_2$O (16.44
mmol, 3.59 g). After purification via flash column chromatography, 2.16 was obtained as a colorless oil (1.70 g, 60%).

$^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 4.36 and 4.17 (2 x br s, 1H, rotamers), 3.31 and 2.94 (2 x br s, 2H, rotamers), 1.81 – 1.70 (m, 2H, rotamers), 1.47 (br s, 9H), 1.27 (m, 13H), 0.96 – 0.75 (m, 9H); $^{13}$C NMR (75 MHz, CDCl$_3$) $\delta$ 178.4 and 176.7 (rotamers), 156.9 and 155.1 (rotamers), 80.9 and 80.5 (rotamers), 59.0 and 58.2 (rotamers), 47.7, 39.1 and 38.1 (rotamers), 31.8, 29.2, 28.7, 28.3, 26.9, 24.8, 23.1 and 22.9 (rotamers), 22.6, 21.9, 14.1. IR (KBr) $\nu$(cm$^{-1}$) 2927, 1705, 1301, 1162. HRMS (ESI): $m/z$: Calcd for C$_{19}$H$_{37}$NO$_4$: 366.2615 [M+Na]$^+$; found: 366.2618.

$^{1}$H NMR (400 MHz, CDCl$_3$) $\delta$ 7.30 – 7.23 (m, 5H), 4.68 and 4.43 (2 x m, 2H rotamers), 4.3 and 3.90 (2 x m, 1H, rotamers), 1.93 and 1.73 (2 x br s, 2H, rotamers), 1.48 and 1.41 (2 x br s, 9H, rotamers), 1.23 – 1.14 (m, 8H), 0.85 (t, 3H, $J = 7.0$ Hz); $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 177.6 and 177.3 (rotamers), 156.1 and 155.6 (rotamers), 138.6 and 137.7 (rotamers), 128.3,

2-(Benzyl(tert-butoxycarbonyl)amino)octanoic acid (2.17): 2.17 was synthesized as described in the general procedure using DL-$\alpha$-aminocaprylic acid (12.56 mmol, 2 g), benzaldehyde (12.56 mmol, 1.27 mL), sodium borohydride (25.12 mmol, 950 mg). To the crude product (8.03 mmol, 2 g) TMAH (16.06 mmol, 2.91 g) was added, followed by Boc$_2$O (16.06 mmol, 3.51 g). After purification via flash column chromatography, 2.17 was obtained as a colorless oil (1.45 g, 52%).

$^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 7.30 – 7.23 (m, 5H), 4.68 and 4.43 (2 x m, 2H rotamers), 4.3 and 3.90 (2 x m, 1H, rotamers), 1.93 and 1.73 (2 x br s, 2H, rotamers), 1.48 and 1.41 (2 x br s, 9H, rotamers), 1.23 – 1.14 (m, 8H), 0.85 (t, 3H, $J = 7.0$ Hz); $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 177.6 and 177.3 (rotamers), 156.1 and 155.6 (rotamers), 138.6 and 137.7 (rotamers), 128.3,
128.1, 127.2 and 126.9 (rotamers), 80.9, 59.6 and 59.5 (rotamers), 51.3 and 50.4 (rotamers), 31.4, 30.1 and 29.3 (rotamers), 28.7, 28.1, 26.3, 22.3, 13.9. IR (KBr) ν(cm⁻¹) 2958, 1703, 1352, 1164. λ_max = 218 nm, 257 nm. HRMS (ESI): m/z: Calcd for C₂₀H₃₁NO₄: 372.2135 [M+Na]⁺; found: 372.2142.

2-(tert-Butoxycarbonyl(3-phenylpropyl)amino)octanoic acid (2.18): 2.18 was synthesized as described in the general procedure using DL-α-aminocaprylic acid (12.56 mmol, 2 g), hydrocinnamaldehyde (12.56 mmol, 1.66 mL), sodium borohydride (25.12 mmol, 950 mg). To the crude product (7.22 mmol, 2 g) TMAH (14.44 mmol, 2.62 g) was added, followed by Boc₂O (14.44 mmol, 3.15 g). After purification via flash column chromatography, 2.18 was obtained as a colorless oil (1.89 g, 69%).

¹H NMR (400 MHz, CDCl₃) δ 7.28 – 7.17 (m, 5H), 4.18 and 3.90 (2 x br s, 1H), 3.54 and 3.08 (2 x m, 2H, rotamers), 2.60 (br s, 2H), 2.05 – 1.70 (m, 4H, rotamers), 1.44 – 1.28 (m, 17H), 0.89 (t, 3H, J = 6.4 Hz); ¹³C NMR (100 MHz, CDCl₃) δ 178.1 and 177.0 (rotamers), 156.8, 141.9 and 141.3 (rotamers), 128.4, 128.3, 125.9, 81.1, 61.2 and 60.3 (rotamers), 48.0 and 47.6 (rotamers), 33.3, 31.6, 30.8 and 30.3 (rotamers), 30.0 and 29.7 (rotamers), 28.9, 28.3, 26.3, 22.5, 14.0. IR (KBr) ν(cm⁻¹) 2960, 1699, 1302, 1162. λ_max = 217 nm, 258 nm. HRMS (ESI): m/z: Calcd for C₂₂H₃₅NO₄: 400.2458 [M+Na]⁺; found: 400.2451.
2-(tert-Butoxycarbonyl(pentyl)amino)octanoic acid (2.19): 2.19 was synthesized as described in the general procedure using DL-α-aminocaprylic acid (12.56 mmol, 2 g), valeraldehyde (12.56 mmol, 1.34 mL), sodium borohydride (25.12 mmol, 950 mg). To the crude product (8.73 mmol, 2 g) TMAH (17.46 mmol, 3.16 g) was added, followed by Boc$_2$O (17.46 mmol, 3.81 g). After purification via flash column chromatography, 2.19 was obtained as a colorless oil (1.49 g, 52%).

$^1$H NMR (400 MHz, CDCl$_3$) δ 9.35 (br s, 1H), 4.24 and 3.95 (2 x m, 1H, rotamers), 3.35 and 2.97 (2 x m, 2H, rotamers), 2.10 – 1.16 (m, 25H), 0.91 – 0.84 (m, 6H); $^{13}$C NMR (100 MHz, CDCl$_3$) δ 177.9 and 176.4 (rotamers), 156.7 and 154.8 (rotamers), 80.7, 60.7 and 60.3 (rotamers), 48.3 and 47.7 (rotamers), 31.6, 30.1, 28.9, 28.7, 28.3, 28.0, 26.3, 22.5, 22.4, 22.3, 14.0. IR (KBr) ν (cm$^{-1}$) 2929, 1701, 1298, 1167. HRMS (ESI): $m/z$: Calcd for C$_{18}$H$_{35}$NO$_4$: 352.2458 [M+Na]$^+; $ found: 352.2454.

2-(tert-Butoxycarbonyl(octyl)amino)octanoic acid (2.20): 2.20 was synthesized as described in the general procedure using DL-α-aminocaprylic acid (12.56 mmol, 2 g), octylaldehyde (12.56 mmol, 1.96 mL), sodium borohydride (25.12 mmol, 950 mg). To the crude product (7.37 mmol, 2 g) TMAH (14.74 mmol, 2.67 g) was added, followed by Boc$_2$O (14.74
mmol, 3.22 g). After purification via flash column chromatography, 2.20 was obtained as a colorless oil (1.47 g, 54%).

$^1$H NMR (400 MHz, CDCl$_3$) δ 4.26 and 3.93 (2 x br s, 1H, rotamers), 3.37 and 2.95 (2 x br s, 2H, rotamers), 1.95 and 1.77 (2 x br s, 2H, rotamers), 1.45 – 1.26 (m, 29H), 0.86 – 0.80 (m, 6H); $^{13}$C NMR (100 MHz, CDCl$_3$) δ 178.1 and 177.0 (rotamers), 156.5 and 155.0 (rotamers), 80.5, 60.3, 48.4, 47.5, 31.8, 31.6, 30.1, 29.2, 28.9, 28.5, 28.3, 27.0, 26.3, 22.6, 22.5, 14.0, 14.0. IR (KBr) ν(cm$^{-1}$) 2925, 2857, 1701, 1169. HRMS (ESI): m/z: Calcd for C$_{21}$H$_{41}$NO$_4$: 394.2928 [M+Na]$^+$; found: 394.2926.

2-(tert-Butoxycarbonyl(decyl)amino)octanoic acid (2.21): 2.21 was synthesized as described in the general procedure using DL-$\alpha$-aminocaprylic acid (12.56 mmol, 2 g), n-decanal (12.56 mmol, 2.37 mL), sodium borohydride (25.12 mmol, 950 mg). To the crude product (6.68 mmol, 2 g) TMAH (13.36 mmol, 2.42 g) was added, followed by Boc$_2$O (13.36 mmol, 2.92 g). After purification via flash column chromatography, 2.21 was obtained as a colorless oil (1.26 g, 47%).

$^1$H NMR (400 MHz, CDCl$_3$) δ 4.25 and 3.93 (2 x br s, 1H, rotamers), 3.33 and 2.96 (2 x br s, 2H, rotamers), 1.97 and 1.79 (2 x br s, 2H, rotamers), 1.58 – 1.27 (m, 33H), 0.89 – 0.85 (m, 6H); $^{13}$C NMR (100 MHz, CDCl$_3$) δ 178.2 and 176.9 (rotamers), 156.6 and 154.9 (rotamers), 80.6 and 80.5 (rotamers), 60.6 and 60.3 (rotamers), 48.4 and 47.7 (rotamers), 31.9, 31.6, 30.1, 29.6, 29.5, 29.3, 29.2, 29.0, 28.6, 28.3, 27.0, 26.3, 22.6, 22.5, 14.1, 14.0. IR
(KBr) $\nu$ (cm$^{-1}$) 2925, 2856, 1702, 1164. HRMS (ESI): $m/\ell$: Calcd for C$_{23}$H$_{45}$NO$_4$: 422.3241 [M+Na]$^+$; found: 422.3242.

2-(tert-Butoxycarbonyl(dodecyl)amino)octanoic acid (2.22): 2.22 was synthesized as described in the general procedure using DL-$\alpha$-aminocaprylic acid (12.56 mmol, 2 g), dodecyl aldehyde (12.56 mmol, 2.81 mL), sodium borohydride (25.12 mmol, 950 mg). To the crude product (6.11 mmol, 2 g) TMAH (12.22 mmol, 2.21 g) was added, followed by Boc$_2$O (12.22 mmol, 2.67 g). After purification via flash column chromatography, 2.22 was obtained as a colorless oil (1.18 g, 45%).

$^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 4.15 and 3.90 (2 x br s, 1H, rotamers), 3.38 and 3.0 (2 x br s, 2H, rotamers), 1.99 – 1.26 (m, 39H), 0.90 – 0.87 (m, 6H); $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 175.7, 157.0, 81.2 and 81.1 (rotamers), 61.4 and 60.3 (rotamers), 48.2, 31.9, 31.6, 30.2, 29.7, 29.6, 29.6, 29.3, 29.1, 28.9, 28.6, 28.3, 26.9, 26.3, 22.7, 22.5, 14.1, 14.0. IR (KBr) $\nu$ (cm$^{-1}$) 2923, 2855, 1702, 1164. HRMS (ESI): $m/\ell$: Calcd for C$_{25}$H$_{49}$NO$_4$: 450.3554 [M+Na]$^+$; found: 450.3552.
2-(benzyl(tert-Butoxycarbonyl)amino)-6-(benzyloxycarbonylamino)hexanoic acid (2.23): 2.23 was synthesized as described in the general procedure using Cbz-lysine (7.13 mmol, 2 g), benzaldehyde (7.13 mmol, 0.72 mL), sodium borohydride (14.26 mmol, 540 mg). To the crude product (5.40 mmol, 2 g) TMAH (10.8 mmol, 1.96 g) was added, followed by Boc$_2$O (10.8 mmol, 2.36 g). After purification via flash column chromatography, 2.23 was obtained as a colorless oil (1.18 g, 47%).

$^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 8.75 (br s, 1H), 7.35 – 7.28 (m, 10H), 5.93 (br s, 1H), 5.08 (s, 2H), 4.82 – 4.38 (m, 2H), 4.27 and 3.86 (2 x br s, 1H, rotamers), 3.02 (br s, 2H), 1.95 and 1.69 (2 x br s, 2H, rotamers), 1.46 – 1.10 (m, 13H); $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 176.5 and 175.9 (rotamers), 157.9, 156.4 and 155.7 (rotamers), 138.6 and 138.0 (rotamers), 136.5, 128.4, 128.3, 128.0, 127.8, 127.3, 127.1, 81.1, 67.0 and 66.5 (rotamers), 59.6, 51.4 and 50.7 (rotamers), 41.1 and 40.6 (rotamers), 29.9, 29.3 and 29.0 (rotamers), 28.2, 23.6. IR (KBr) $\nu$(cm$^{-1}$) 2956, 1694, 1254, 1163. $\lambda_{\text{max}}$ = 218 nm, 257 nm. HRMS (ESI): $m/z$: Calcd for C$_{26}$H$_{34}$N$_2$O$_6$: 493.2309 [M+Na]$^+$; found: 493.2306.
6-(Benzyloxycarbonylamino)-2-(tert-butoxycarbonyl(3-phenylpropyl)amino)hexanoic acid (2.24): 2.24 was synthesized as described in the general procedure using Cbz-lysine (7.13 mmol, 2 g), hydrocinnamaldehyde (7.13 mmol, 0.94 mL), sodium borohydride (14.26 mmol, 540 mg). To the crude product (5.02 mmol, 2 g) TMAH (10.04 mmol, 1.82 g) was added, followed by Boc₂O (10.04 mmol, 2.19 g). After purification via flash column chromatography, 2.24 was obtained as a colorless oil (1.30 g, 52%).

¹H NMR (400 MHz, CDCl₃) δ 10.30 (br s, 1H), 6.10 (br s, 1H), 5.16 and 4.94 (2 x br s, 1H), 5.10 (s, 2H), 4.28 and 3.85 (2 x br s, 2H, rotamers), 3.55 and 3.35 (2 x br s, 2H, rotamers), 3.17 - 2.65 (m, 2H), 2.63 – 2.59 (m, 2H), 1.90 and 1.82 (2 x br s, 2H, rotamers), 1.52 – 1.27 (m, 13H); ¹³C NMR (100 MHz, CDCl₃) δ 176.8 and 176.1 (rotamers), 156.4 and 156.2 (rotamers), 154.8, 141.8 and 141.4 (rotamers), 136.5, 128.4, 128.3, 128.1, 127.9, 125.9, 125.7, 80.7, 67.1 and 66.6 (rotamers), 60.2 and 59.9 (rotamers), 48.1 and 47.0 (rotamers), 41.2 and 40.7 (rotamers), 33.2, 30.9 and 30.2 (rotamers), 29.8 and 29.7 (rotamers), 29.4 and 28.8 (rotamers), 28.2 and 28.1 (rotamers), 23.7 and 23.4 (rotamers). IR (KBr) ν(cm⁻¹) 2924, 1692, 1421, 1162. λ_max = 220 nm, 257 nm. HRMS (ESI): m/z: Calcd for C₂₈H₃₈N₂O₆: 521.2622 [M+Na]⁺; found: 521.2621.
6-(Benzyloxy carbonylamino)-2-(tert-butoxycarbonyl(pentyl)amino)hexanoic acid (2.25): 2.25 was synthesized as described in the general procedure using Cbz-lysine (7.13 mmol, 2 g), valeraldehyde (7.13 mmol, 0.76 mL), sodium borohydride (14.26 mmol, 540 mg). To the crude product (5.71 mmol, 2 g) TMAH (11.42 mmol, 2.07 g) was added, followed by Boc₂O (11.42 mmol, 2.49 g). After purification via flash column chromatography, 2.25 was obtained as a colorless oil (2.24 g, 87%).

¹H NMR (400 MHz, CDCl₃) δ 10.25 (br s, 1H), 7.30 – 7.16 (m, 5H), 6.23 (br s, 1H), 5.20 – 4.65 (m, 2H, rotamers), 4.33 and 3.85 (2 x br s, 1H, rotamers), 3.38 – 2.92 (m, 4H, rotamers), 1.97 and 1.74 (2 x br s, 2H, rotamers), 1.58 – 1.27 (m, 19H), 0.86 (t, 3H, J = 6.6 Hz); ¹³C NMR (100 MHz, CDCl₃) δ 176.8 and 176.1 (rotamers), 157.2 and 156.4 (rotamers), 154.9, 136.5, 128.4, 128.0, 127.9, 80.5, 67.0 and 66.5 (rotamers), 60.2, 48.3 and 47.4 (rotamers), 41.2 and 40.7 (rotamers), 40.2, 29.7 and 29.4 (rotamers), 29.1 and 28.9 (rotamers), 28.3, 23.8 and 23.4 (rotamers), 22.3 and 22.2 (rotamers), 14.0. IR (KBr) ν(cm⁻¹) 2933, 1700, 1455, 1253. λₘₐₓ = 213 nm, 256 nm. HRMS (ESI): m/z: Calcd for C₂₄H₃₈N₂O₆: 473.2622 [M+Na]⁺; found: 473.2616.
6-(Benzyloxy carbonylamino)-2-(tert-butoxycarbonyl(octyl)amino)hexanoic acid (2.26): 2.26 was synthesized as described in the general procedure using Cbz-lysine (7.13 mmol, 2 g), octyl aldehyde (7.13 mmol, 1.11 mL), sodium borohydride (14.26 mmol, 540 mg). To the crude product (5.10 mmol, 2 g) TMAH (10.2 mmol, 1.85 g) was added, followed by Boc₂O (10.2 mmol, 2.23 g). After purification via flash column chromatography, 2.26 was obtained as a colorless oil (1.96 g, 78%).

¹H NMR (400 MHz, CDCl₃) δ 7.52 – 7.24 (m, 5H), 5.89 (br s, 1H), 5.13 and 4.93 (2 x br s, 1H, rotamers), 5.07 (s, 2H), 4.23 and 3.86 (2 x br s, 2H, rotamers), 3.39 – 2.93 (m, 4H), 1.98 and 1.77 (2 x br s, 2H, rotamers), 1.51 – 1.24 (m, 23H), 0.86 (s, 3H); ¹³C NMR (100 MHz, CDCl₃) δ 176.4 and 175.7 (rotamers), 161.5, 156.4 and 155.6 (rotamers), 136.5, 128.4, 128.0, 128.0, 80.7, 67.1 and 66.6 (rotamers), 60.1, 48.5 and 47.6 (rotamers), 41.2 and 40.7 (rotamers), 31.7, 29.7, 29.2, 28.8, 28.6, 28.3, 26.9, 23.8, 23.4, 22.6, 14.1. IR (KBr) ν(cm⁻¹) 2929, 1699, 1418, 1163. λmax = 214 nm, 256 nm. HRMS (ESI): m/z: Calcd for C₂₇H₄₄N₂O₄: 515.3092 [M+Na]+; found: 515.3076.

tert-Butyl benzyl(1-(methoxy(methyl)amino)-1-oxo-3-phenylpropan-2-yl)carbamate (2.27): 2.27 was synthesized as described in the general procedure using 2.5 (2 mmol, 710 g)
mg), TEA (2 x 2 mmol, 0.28 mL), BOP (2 mmol, 885 mg), and N,O-dimethylhydroxylamine hydrochloride (2.2 mmol, 204 mg). After purification via flash column chromatography, 2.27 was obtained as a yellow oil (449 mg, 56%).

\[ ^1H \text{NMR (400 MHz, CDCl}_3\delta 7.30 – 7.14 \text{ (m, 10H), 5.65 and 5.23 (2 x br s, 1H, rotamers), 4.82 – 4.32 \text{ (m, 2H, rotamers), 3.52 and 3.44 (2 x br s, 3H, rotamers), 3.19 – 2.96 \text{ (m, 2H, rotamers), 2.85 and 2.72 (2 x br s, 3H, rotamers), 1.32 and 1.30 \text{ (s, 9H, rotamers); ^13C NMR (100 MHz, CDCl}_3\delta 170.9 and 170.2 \text{ (rotamers), 155.4 and 155.2 \text{ (rotamers), 139.3 and 138.5 \text{ (rotamers), 137.9 and 137.3 \text{ (rotamers), 129.5, 128.2, 128.1, 127.9, 126.9 and 126.7 \text{ (rotamers), 126.4 and 126.3 \text{ (rotamers), 80.1 and 79.9 \text{ (rotamers), 61.5 and 61.2 \text{ (rotamers), 56.4 and 54.7 \text{ (rotamers), 47.0 and 46.3 \text{ (rotamers), 35.8 and 35.6 \text{ (rotamers), 31.7, 28.0. IR (KBr) \nu (cm}^{-1} \text{) 2976, 1689, 1454, 1170.} \lambda_{\text{max}} = 218 \text{ nm. HRMS (ESI): m/z: Calcd for C}_{23}\text{H}_{30}\text{N}_2\text{O}_4: 421.2098 \text{ [M+Na}^+\text{]; found: 421.2107.}}

\[
\begin{align*}
\text{\textbf{tert-Butyl 1-(methoxy(methyl)amino)-1-oxo-3-phenylpropan-2-yl(3-phenylpropyl)carbamate (2.28):}} \\
\text{2.28 was synthesized as described in the general procedure using 2.6 (2 mmol, 766 mg), TEA (2 x 2 mmol, 0.28 mL), BOP (2 mmol, 885 mg), and N,O-dimethylhydroxylamine hydrochloride (2.2 mmol, 204 mg). After purification via flash column chromatography, 2.28 was obtained as a colorless oil (402 mg, 47%).}
\end{align*}
\]

\[ ^1H \text{NMR (400 MHz, CDCl}_3\delta 7.32 – 7.20 \text{ (10H, m), 5.55 and 5.25 (2 x br s, 1H), 3.60 \text{ (s, 3H), 3.31 – 2.89 \text{ (m, 7H), 2.60 \text{ (br s, 2H), 1.82 \text{ (br s, 2H), 1.37 and 1.28 \text{ (2 x s, 9H, rotamers);}}}}
\]
\( ^{13}\)C NMR (100 MHz, CDCl\(_3\)) \(\delta\) 171.5 and 171.3 (rotamers), 155.2 and 154.4 (rotamers), 141.8, 137.9, 137.4, 129.3, 128.2, 128.2, 126.3, 125.7, 79.6, 61.4, 56.4 and 54.5 (rotamers), 43.6 and 43.5 (rotamers), 35.7, 33.5, 32.2, 31.3 and 30.5 (rotamers), 28.2 and 28.0 (rotamers). IR (KBr) \(\nu\) (cm\(^{-1}\)) 2978, 1692, 1405, 1159. \(\lambda_{\text{max}} = 228\) nm. HRMS (ESI): \(m/z\): Calcd for C\(_{25}\)H\(_{34}\)N\(_2\)O\(_4\): 449.2411 [M+Na]\(^+\); found: 449.2416.

**tert-Butyl 1-(methoxy(methyl)amino)-1-oxo-3-phenylpropan-2-yl(pentyl)carbamate (2.29):** 2.29 was synthesized as described in the general procedure using 2.7 (2 mmol, 670 mg), TEA (2 x 2 mmol, 0.28 mL), BOP (2 mmol, 885 mg), and \(N,O\)-dimethylhydroxylamine hydrochloride (2.2 mmol, 204 mg). After purification via flash column chromatography, 2.29 was obtained as a colorless oil (396 mg, 52%).

\(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 7.28 – 7.19 (m, 5H), 5.55 and 5.19 (2 x br s, 1H, rotamers), 3.59 (s, 3H), 3.32 – 2.88 (m, 7H), 1.48 – 1.25 (m, 15H), 0.88 (t, 3H, \(J = 7.2\) Hz); \(^{13}\)C NMR (100 MHz, CDCl\(_3\)) \(\delta\) 171.3, 155.3 and 154.5 (rotamers), 138.1 and 137.6 (rotamers), 129.5 and 129.4 (rotamers), 128.3 and 128.1 (rotamers), 126.3, 79.5, 61.4, 56.4 and 54.6 (rotamers), 43.7, 35.8, 32.3, 29.3, 28.4 and 28.2 (rotamers), 28.0, 22.4 and 22.3 (rotamers), 14.0. IR (KBr) \(\nu\) (cm\(^{-1}\)) 2964, 1681, 1399, 1175. \(\lambda_{\text{max}} = 222\) nm. HRMS (ESI): \(m/z\): Calcd for C\(_{21}\)H\(_{34}\)N\(_2\)O\(_4\): 401.2411 [M+Na]\(^+\); found: 401.2398.
tert-Butyl 1-(methoxy(methyl)amino)-1-oxo-3-phenylpropan-2-yl(octyl)carbamate (2.30): 2.30 was synthesized as described in the general procedure using 2.8 (2 mmol, 755 mg), TEA (2 x 2 mmol, 0.28 mL), BOP (2 mmol, 885 mg), and N,O-dimethylhydroxylamine hydrochloride (2.2 mmol, 204 mg). After purification via flash column chromatography, 2.30 was obtained as a colorless oil (504 mg, 59%).

$^{1}$H NMR (300 MHz, CDCl$_3$) δ 7.28 – 7.16 (m, 5H), 5.50 and 5.18 (2 x br s, 1H), 3.58 (s, 3H), 3.18 – 2.93 (m, 7H), 1.36 – 1.25 (m, 21H), 0.87 (t, 3H, J = 6.45 Hz); $^{13}$C NMR (100 MHz, CDCl$_3$) δ 171.5, 155.3 and 154.4 (rotamers), 138.1 and 137.5 (rotamers), 129.4 and 129.3 (rotamers), 128.3 and 128.1 (rotamers), 126.2, 79.5, 61.4, 56.4 and 54.6 (rotamers), 43.8, 35.7, 31.7, 29.6, 29.2, 28.8, 28.2, 28.0, 27.1, 22.6, 14.0. IR (KBr) ν(cm$^{-1}$) 2929, 1694, 1406, 1171. $\lambda_{\text{max}}$ = 218 nm. HRMS (ESI): m/z: Calcd for C$_{24}$H$_{40}$N$_{2}$O$_{4}$: 443.288 [M+Na]$^{+}$; found: 443.2877.

tert-Butyl benzyl(2-(methoxy(methyl)amino)-2-oxo-1-phenylethyl)carbamate (2.31): 2.31 was synthesized as described in the general procedure using 2.9 (2 mmol, 682 mg), TEA (2 x 2 mmol, 0.28 mL), BOP (2 mmol, 885 mg), and N,O-dimethylhydroxylamine hydrochloride (2.2 mmol, 204 mg). After purification via flash column chromatography, 2.31 was obtained as a colorless oil (462 mg, 60%).
1H NMR (300 MHz, CDCl3) δ 7.31 – 6.87 (m, 10H), 6.52 and 6.18 (2 x br s, 1H, rotamers), 4.70 and 4.22 (2 x d, 2H, J = 16.5 Hz, rotamers), 3.48 (s, 3H), 3.21 (s, 3H), 1.34 (s, 9H); 13C NMR (100 MHz, CDCl3) δ 172.2, 156.5, 140.2, 134.9, 129.6, 128.4, 128.2, 127.4, 126.5, 125.8, 80.3, 61.0, 59.8, 48.7, 32.1, 28.2. IR (KBr) ν(cm⁻¹) 2977, 1685, 1393, 1167. λmax = 226 nm. Calcd for C22H28N2O4: 407.1955 [M+Na]+; found: 407.1959.

tert-Butyl 2-(methoxy(methyl)amino)-2-oxo-1-phenylethyl(3-phenylpropyl)carbamate (2.32): 2.32 was synthesized as described in the general procedure using 2.10 (2 mmol, 738 mg), TEA (2 x 2 mmol, 0.28 mL), BOP (2 mmol, 885 mg), and N,O-dimethylhydroxylamine hydrochloride (2.2 mmol, 204 mg). After purification via flash column chromatography, 2.32 was obtained as a pale yellow oil (382 mg, 46%).

1H NMR (400 MHz, CDCl3) δ 7.32 – 6.91 (m, 10H), 6.40 and 6.16 (2 x br s, 1H), 3.42 – 3.10 (m, 10H), 2.29 (br s, 2H), 1.47 (s, 9H); 13C NMR (100 MHz, CDCl3) δ 171.8, 156.0 and 154.9 (rotamers), 141.5 and 139.0 (rotamers), 135.3 and 133.5 (rotamers), 129.1, 128.4, 128.0 and 127.9 (rotamers), 127.9, 127.8, 125.4 and 125.2 (rotamers), 79.5, 60.7 and 59.1 (rotamers), 44.7, 35.6 and 34.3 (rotamers), 33.0, 31.8 and 30.8 (rotamers), 30.3 and 29.5 (rotamers), 28.1 and 28.0 (rotamers). IR (KBr) ν(cm⁻¹) 2978, 1692, 1405, 1159. λmax = 224 nm. HRMS (ESI): m/z: Calcd for C24H32N2O4: 435.2254 [M+Na]+; found: 435.2259.
tert-Butyl 2-(methoxy(methyl)amino)-2-oxo-1-phenylethyl(pentyl)carbamate (2.33): 2.33 was synthesized as described in the general procedure using 2.11 (2 mmol, 738 mg), TEA (2 x 2 mmol, 0.28 mL), BOP (2 mmol, 885 mg), and N,O-dimethylhydroxylamine hydrochloride (2.2 mmol, 204 mg). After purification via flash column chromatography, 2.33 was obtained as a colorless oil (352 mg, 42%).

$^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 7.31 – 7.27 (m, 5H), 6.33 and 6.09 (2 x br s, 1H), 3.43 – 2.01 (m, 8H), 1.54 – 1.29 (m, 11H), 1.05 – 0.85 (m, 4H), 0.68 (t, 3H, $J$ = 7.4 Hz); $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 172.1, 156.3, 135.6, 129.3, 128.5, 128.1, 79.6, 61.0, 59.3, 45.2, 32.0, 29.0, 28.7, 28.4, 21.9, 13.8. IR (KBr) $\nu$(cm$^{-1}$) 2964, 1681, 1399, 1175. $\lambda_{max}$ = 227 nm. HRMS (ESI): $m/z$: Calcd for C$_{20}$H$_{32}$N$_2$O$_4$: 387.2254 [M+Na]$^+$; found: 387.2265.

tert-Butyl 2-(methoxy(methyl)amino)-2-oxo-1-phenylethyl(octyl)carbamate (2.34): 2.34 was synthesized as described in the general procedure using 2.12 (2 mmol, 727 mg), TEA (2 x 2 mmol, 0.28 mL), BOP (2 mmol, 885 mg), and N,O-dimethylhydroxylamine hydrochloride (2.2 mmol, 204 mg). After purification via flash column chromatography, 2.34 was obtained as a colorless oil (356 mg, 44%).

$^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 7.34 – 7.28 (m, 5H), 6.36 and 6.13 (2 x br s, 1H), 3.47 – 2.97 (m, 8H), 1.74 – 0.91 (m, 21H), 0.84 (t, 3H, $J$ = 7.2 Hz); $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$
172.2, 156.5, 135.7, 129.3, 128.6, 128.2, 79.7, 61.1 and 59.4 (rotamers), 45.3, 32.1, 31.7, 29.3, 29.1, 28.9, 28.5, 26.8, 22.6, 14.1. IR (KBr) \( \nu (\text{cm}^{-1}) \) 2928, 1683, 1399, 1165. \( \lambda_{\text{max}} = 221 \text{ nm} \). HRMS (ESI): \( m/z \): Calcd for C\(_{23}\)H\(_{38}\)N\(_2\)O\(_4\): 429.2724 [M+Na]\(^+\); found: 429.2731.

**tert-Butyl benzyl(1-(methoxy(methyl)amino)-4-methyl-1-oxopentan-2-yl)carbamate (2.35):** 2.35 was synthesized as described in the general procedure using 2.13 (2 mmol, 642 mg), TEA (2 x 2 mmol, 0.28 mL), BOP (2 mmol, 885 mg), and N,O-dimethylhydroxylamine hydrochloride (2.2 mmol, 204 mg). After purification via flash column chromatography, 2.35 was obtained as a colorless oil (346 mg, 48%).

\(^1\)H NMR (400 MHz, CDCl\(_3\)) \( \delta \) 7.27 – 7.18 (m, 5H), 5.39 and 5.09 (2 x br s, 1H, rotamers), 4.49 (m, 2H), 3.73 and 3.61 (2 x s, 3H, rotamers), 3.02 and 2.86 (2 x s, 3H, rotamers), 1.57 – 1.35 (m, 12H), 0.93 and 0.77 (2 x t, 6H, \( J = 6.4 \text{ Hz} \), rotamers); \(^{13}\)C NMR (100 MHz, CDCl\(_3\)) \( \delta \) 172.3, 155.9, 139.9 and 139.1 (rotamers),127.9, 126.9, 126.6 and 126.4 (rotamers), 80.1, 61.6 and 61.3 (rotamers), 53.1 and 52.2 (rotamers), 47.3 and 46.6 (rotamers), 38.4, 32.0, 28.4 and 28.2 (rotamers), 24.8 and 24.3 (rotamers), 22.7 and 22.3 (rotamers). IR (KBr) \( \nu (\text{cm}^{-1}) \) 2960, 1694, 1396, 1166. \( \lambda_{\text{max}} = 225 \text{ nm} \). HRMS (ESI): \( m/z \): Calcd for C\(_{20}\)H\(_{32}\)N\(_2\)O\(_4\): 387.2254 [M+Na]\(^+\); found: 387.2244.
tert-Butyl 1-(methoxy(methyl)amino)-4-methyl-1-oxopentan-2-yl(3-phenylpropyl)carbamate (2.36): 2.36 was synthesized as described in the general procedure using 2.14 (2 mmol, 699 mg), TEA (2 x 2 mmol, 0.28 mL), BOP (2 mmol, 885 mg), and N,O-dimethylhydroxylamine hydrochloride (2.2 mmol, 204 mg). After purification via flash column chromatography, 2.36 was obtained as a colorless oil (402 mg, 51%).

\[ \text{IR (KBr)} \nu (\text{cm}^{-1}) 2960, 1687, 1164. \lambda_{\text{max}} = 222 \text{ nm. HRMS (ESI): } m/z: \text{ Calcd for } C_{22}H_{36}N_2O_4: 415.2567 \ [M+Na]^+; \text{ found: 415.2561.} \]

tert-Butyl 1-(methoxy(methyl)amino)-4-methyl-1-oxopentan-2-yl(pentyl)carbamate (2.37): 2.37 was synthesized as described in the general procedure using 2.15 (2 mmol, 603 mg), TEA (2 x 2 mmol, 0.28 mL), BOP (2 mmol, 885 mg), and N,O-dimethylhydroxylamine
hydrochloride (2.2 mmol, 204 mg). After purification via flash column chromatography, 2.37 was obtained as a colorless oil (376 mg, 55%).

\(^{1}\)H NMR (300 MHz, CDCl\(_3\)) \(\delta\) 5.18 and 4.94 (2 x br s, 1H, rotamers), 3.61 (s, 3H), 3.07 - 2.99 (m, 5H), 1.47 - 1.35 (m, 14H), 1.24 - 1.07 (m, 4H), 0.86 - 0.74 (m, 9H); \(^{13}\)C NMR (75 MHz, CDCl\(_3\)) \(\delta\) 172.7, 155.5 and 154.7 (rotamers), 79.5 and 79.1 (rotamers), 61.2, 52.7, 51.6, 43.5, 38.5 and 38.2 (rotamers), 31.9, 29.3 and 29.2 (rotamers), 28.4 and 28.1 (rotamers), 24.4 and 24.1 (rotamers), 22.7, 22.1, 13.8. IR (KBr) \(\nu\) (cm\(^{-1}\)) 2945, 1681, 1406, 1170. HRMS (ESI): \(m/z:\) Calcd for C\(_{18}\)H\(_{36}\)N\(_2\)O\(_4\): 367.2567 [M+Na]\(^+\); found: 367.2566.

**tert-Butyl 1-(methoxy(methyl)amino)-4-methyl-1-oxopentan-2-yl(octyl)carbamate (2.38):** 2.38 was synthesized as described in the general procedure using 2.16 (2 mmol, 687 mg), TEA (2 x 2 mmol, 0.28 mL), BOP (2 mmol, 885 mg), and N,O-dimethylhydroxylamine hydrochloride (2.2 mmol, 204 mg). After purification via flash column chromatography, 2.38 was obtained as a colorless oil (399 mg, 52%).

\(^{1}\)H NMR (300 MHz, CDCl\(_3\)) \(\delta\) 5.24 and 4.99 (2 x br s, 1H, rotamers), 3.66 (s, 3H), 3.14 (s, 3H), 3.08 (t, 2H, \(J = 7.5\) Hz), 1.53 – 1.42 (m, 14H), 1.21 (br s, 10H), 0.90 (t, 3H, \(J = 6.3\) Hz), 0.83 (br s, 6H); \(^{13}\)C NMR (75 MHz, CDCl\(_3\)) 172.9, 155.7 and 154.8 (rotamers), 79.6 and 79.3 (rotamers), 61.3, 53.8, 51.8, 43.7, 38.6 and 38.3 (rotamers), 32.1, 31.7, 29.8, 29.2 and 28.9 (rotamers), 28.3, 27.1, 24.6 and 24.3 (rotamers), 22.8 and 22.5 (rotamers), 22.2, 14.0.
IR (KBr) $\nu$(cm$^{-1}$) 2928, 1688, 1377, 1169. HRMS (ESI): $m/z$: Calcd for C$_{21}$H$_{42}$N$_2$O$_4$: 409.3037 [M+Na]$^+${; found: 409.3023.

tert-Butyl benzyl(1-(methoxy(methyl)amino)-1-oxooctan-2-yl)carbamate (2.39): 2.39 was synthesized as described in the general procedure using 2.17 (2 mmol, 699 mg), TEA (2 x 2 mmol, 0.28 mL), BOP (2 mmol, 885 mg), and N,O-dimethylhydroxylamine hydrochloride (2.2 mmol, 204 mg). After purification via flash column chromatography, 2.39 was obtained as a colorless oil (306 mg, 52%).

$^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 7.32 – 7.24 (m, 5H), 5.32 and 5.04 (2 x br s, 1H, rotamers), 4.63 – 4.37 (m, 2H, rotamers), 3.77 and 3.64 (2 x br s, 3H, rotamers), 3.06 and 2.91 (2 x br s, 3H, rotamers), 1.82 – 1.26 (m, 19H), 0.90 (t, 3H, $J = 6.2$ Hz); $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 171.2, 155.9, 139.9 and 139.8 (rotamers), 127.9, 126.9, 126.6 and 126.4 (rotamers), 80.1, 61.7 and 61.3 (rotamers), 54.9 and 53.8 (rotamers), 47.2 and 46.5 (rotamers), 31.9 and 31.6 (rotamers), 29.6 and 29.3 (rotamers), 29.0, 28.4, 28.2, 26.0, 22.5, 14.0. IR (KBr) $\nu$(cm$^{-1}$) 2930, 1695, 1413, 1168. $\lambda_{max} = 221$ nm. HRMS (ESI): $m/z$: Calcd for C$_{22}$H$_{36}$N$_2$O$_4$: 415.2567 [M+Na]$^+${; found: 415.2564.}
tert-Butyl 1-(methoxy(methyl)amino)-1-oxooctan-2-yl(3-phenylpropyl)carbamate (2.40): 2.40 was synthesized as described in the general procedure using 2.18 (2 mmol, 755 mg), TEA (2 x 2 mmol, 0.28 mM), BOP (2 mmol, 885 mg), and N,O-dimethylhydroxylamine hydrochloride (2.2 mmol, 204 mg). After purification via flash column chromatography, 2.40 was obtained as a colorless oil (582 mg, 69%).

1H NMR (300 MHz, CDCl3) δ 7.29 – 7.16 (m, 5H), 5.18 and 4.93 (2 x br s, 1H, rotamers), 3.70 (s, 3H), 3.17 (br s, 5H), 2.56 (t, 2H, J = 7.8 Hz), 2.05 – 1.62 (m, 4H), 1.45 and 1.43 (2 x s, 9H, rotamers), 1.26 (br s, 8H), 0.87 (t, 3H, J = 6.3 Hz); C NMR (100 MHz, CDCl3) δ 172.6, 155.6 and 155.0 (rotamers), 141.8, 128.2, 125.7, 125.5, 79.5, 61.5 and 61.4 (rotamers), 54.7 and 53.4 (rotamers), 43.5 and 43.3 (rotamers), 33.6, 32.1, 31.6 and 31.5 (rotamers), 30.7, 29.5, 28.9, 28.3, 25.7, 22.5, 14.0. IR (KBr) ν(cm⁻¹) 2931, 1692, 1411, 1162. λ_max = 227 nm. HRMS (ESI): m/z: Calcd for C24H40N2O4: 443.2880 [M+Na]⁺; found: 443.2879.

tert-Butyl 1-(methoxy(methyl)amino)-1-oxooctan-2-yl(pentyl)carbamate (2.41): 2.41 was synthesized as described in the general procedure using 2.19 (2 mmol, 659 mg), TEA (2 x 2 mmol, 0.28 mL), BOP (2 mmol, 885 mg), and N,O-dimethylhydroxylamine hydrochloride (2.2 mmol, 204 mg). After purification via flash column chromatography, 2.41 was obtained as a colorless oil (638 mg, 72%).

1H NMR (300 MHz, CDCl3) δ 7.29 – 7.16 (m, 5H), 5.18 and 4.93 (2 x br s, 1H, rotamers), 3.70 (s, 3H), 3.17 (br s, 5H), 2.56 (t, 2H, J = 7.8 Hz), 2.05 – 1.62 (m, 4H), 1.45 and 1.43 (2 x s, 9H, rotamers), 1.26 (br s, 8H), 0.87 (t, 3H, J = 6.3 Hz); 13C NMR (100 MHz, CDCl3) δ 172.6, 155.6 and 155.0 (rotamers), 141.8, 128.2, 125.7, 125.5, 79.5, 61.5 and 61.4 (rotamers), 54.7 and 53.4 (rotamers), 43.5 and 43.3 (rotamers), 33.6, 32.1, 31.6 and 31.5 (rotamers), 30.7, 29.5, 28.9, 28.3, 25.7, 22.5, 14.0. IR (KBr) ν(cm⁻¹) 2931, 1692, 1411, 1162. λ_max = 227 nm. HRMS (ESI): m/z: Calcd for C24H40N2O4: 443.2880 [M+Na]⁺; found: 443.2879.
hydrochloride (2.2 mmol, 204 mg). After purification via flash column chromatography, 2.41 was obtained as a colorless oil (390 mg, 52%).

$^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 5.15 and 4.91 (2 x br s, 1H, rotamers), 3.69 (s, 3H), 3.17 – 3.07 (m, 5H), 1.78 – 1.14 (m, 25H), 0.87 (br s, 6H); $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 172.8, 155.8, 79.6 and 79.4 (rotamers), 61.6 and 61.4 (rotamers), 54.7 and 53.5 (rotamers), 43.6, 32.1, 31.8, 29.5, 29.3, 29.0, 28.6, 28.3, 25.8, 22.5, 22.4, 22.3, 14.0. IR (KBr) $\nu$(cm$^{-1}$) 2931, 1693, 1421, 1176. HRMS (ESI): $m/z$: Calcd for C$_{20}$H$_{40}$N$_2$O$_4$: 395.288 [M+Na]$^+${; found: 395.2877.

![Image](image_url)

tert-Butyl 1-(methoxy(methyl)amino)-1-oxooctan-2-yl(octyl)carbamate (2.42): 2.42 was synthesized as described in the general procedure using 2.20 (2 mmol, 743 mg), TEA (2 x 2 mmol, 0.28 mL), BOP (2 mmol, 885 mg), and N,O-dimethylhydroxylamine hydrochloride (2.2 mmol, 204 mg). After purification via flash column chromatography, 2.42 was obtained as a colorless oil (437 mg, 53%).

$^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 5.17 and 4.92 (2 x br s, 1H, rotamers), 3.71 (s, 3H), 3.18 (s, 3H), 3.11 (br s, 2H), 1.79 – 1.44 (m, 31H), 0.91 – 0.84 (m, 6H); $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 172.8 and 172.3 (rotamers), 155.8 and 155.5 (rotamers), 79.6 and 79 .4 (rotamers), 61.6 and 61.4 (rotamers), 54.7, 53.5, 43.8 and 43.7 (rotamers), 31.8 and 31.7 (rotamers), 29.9, 29.6, 29.3, 29.1, 28.9, 28.4, 27.2, 25.8, 22.6 and 22.5 (rotamers), 14.1, 14.0. IR (KBr)
ν(cm\(^{-1}\)) 2927, 1696, 1397, 1172. HRMS (ESI): \(m/z\): Calcd for C\(_{23}\)H\(_{46}\)N\(_2\)O\(_4\): 437.3350 [M+Na]\(^+\); found: 437.3351.

tert-Butyl decyl(1-(methoxy(methyl)amino)-1-oxooctan-2-yl)carbamate (2.43): 2.43 was synthesized as described in the general procedure using 2.21 (2 mmol, 799 mg), TEA (2 x 2 mmol, 0.28 mL), BOP (2 mmol, 885 mg), and \(N,O\)-dimethylhydroxylamine hydrochloride (2.2 mmol, 204 mg). After purification via flash column chromatography, 2.43 was obtained as a colorless oil (365 mg, 41%).

\(^1\)H NMR (400 MHz, CDCl\(_3\)) δ 5.17 and 4.88 (2 x br s, 1H, rotamers), 3.65 (s, 3H), 3.15 – 3.00 (m, 5H), 1.79 – 1.10 (m, 39H), 0.85 – 0.79 (m, 6H); \(^13\)C NMR (100 MHz, CDCl\(_3\)) δ 172.7, 155.7 and 154.9 (rotamers), 79.5 and 79.3 (rotamers), 61.5 and 61.3 (rotamers), 54.7 and 53.4 (rotamers), 43.7 and 43.6 (rotamers), 31.8, 31.6, 29.8, 29.5, 29.5, 29.4, 29.2, 29.0, 28.9, 28.3, 27.1, 25.7, 22.6, 22.5, 14.0, 13.9. IR (KBr) ν(cm\(^{-1}\)) 2926, 2854, 1695, 1171.

HRMS (ESI): \(m/z\): Calcd for C\(_{25}\)H\(_{50}\)N\(_2\)O\(_4\): 465.3655 [M+Na]\(^+\); found: 465.3661.

tert-Butyl dodecyl(1-(methoxy(methyl)amino)-1-oxooctan-2-yl)carbamate (2.44): 2.44 was synthesized as described in the general procedure using 2.22 (2 mmol, 855 mg), TEA (2 x 2 mmol, 0.28 mL), BOP (2 mmol, 885 mg), and \(N,O\)-dimethylhydroxylamine
hydrochloride (2.2 mmol, 204 mg). After purification via flash column chromatography, 2.44 was obtained as a colorless oil (340 mg, 40%).

$^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 5.12 and 4.87 (2 x br s, 1H, rotamers), 3.67 (s, 3H), 3.14 – 2.98 (m, 5H), 1.69 – 1.06 (m, 39H), 0.85 – 0.82 (m, 6H); $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 172.7 and 172.0 (rotamers), 155.7 and 154.9 (rotamers), 79.5 and 79.3 (rotamers), 61.3, 54.7, 53.5, 43.7, 43.6, 32.1, 31.8, 31.6, 29.8, 29.6, 29.5, 29.4, 29.2, 29.0, 28.3, 27.1, 25.7, 22.6, 22.5, 14.0, 13.9. IR (KBr) $\nu$(cm$^{-1}$) 2925, 2856, 1695, 1172. HRMS (ESI): $m/z$: Calcd for C$_{27}$H$_{54}$N$_2$O$_4$: 493.3976 [M+Na]$^+$; found: 493.3972.

2-(Benzyl(tert-butoxycarbonyl)amino)-6-(benzyloxy carbonylamino)-6-(methoxy(methyl)amino)-6-oxohexane (2.45): 2.45 was synthesized as described in the general procedure using 2.23 (2 mmol, 941 mg), TEA (2 x 2 mmol, 0.28 mL), BOP (2 mmol, 885 mg), and N,O-dimethylhydroxylamine hydrochloride (2.2 mmol, 204 mg). After purification via flash column chromatography, 2.45 was obtained as a colorless oil (526 mg, 51%).

$^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 7.92 – 7.14 (m, 10H), 5.19 – 4.86 (m, 5H), 4.56 – 4.31 (m, 2H), 3.64 and 3.53 (2 x s, 3H, rotamers), 3.06 – 2.81 (m, 5H), 1.72 – 1.10 (m, 15H); $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 171.5 and 170.9 (rotamers), 156.1, 155.6 and 155.4 (rotamers), 139.3 and 138.7 (rotamers), 136.4, 128.1, 127.9, 127.6, 126.7, 126.4, 126.2, 79.9, 66.0, 61.2
and 60.9 (rotamers), 54.7 and 53.3 (rotamers), 46.8 and 46.4 (rotamers), 41.0 and 40.4 (rotamers), 31.5, 29.2 and 29.0 (rotamers), 28.7, 28.0 and 27.8 (rotamers), 22.7. IR (KBr) ν(cm⁻¹) 2938, 1689, 1457, 1252, 1163. Λₘₐₓ = 224 nm, 251 nm. HRMS (ESI): m/z: Calcd for C₂₈H₃₉N₃O₆: 536.2731 [M+Na]⁺; found: 536.2728.

6-(Benzyloxy carbonylamino)-2-(tert-butoxycarbonyl(3-phenylpropyl)amino)6-(methoxy(methyl)amino)-6-oxohexane (2.46): 2.46 was synthesized as described in the general procedure using 2.24 (2 mmol, 997 mg), TEA (2 x 2 mmol, 0.28 mL), BOP (2 mmol, 885 mg), and N,O-dimethylhydroxylamine hydrochloride (2.2 mmol, 204 mg). After purification via flash column chromatography, 2.46 was obtained as a colorless oil (500 mg, 46%).

¹H NMR (400 MHz, CDCl₃) δ 7.35 – 7.16 (m, 10H), 5.12 (br s, 1H), 5.08 (s, 2H), 4.87 and 4.80 (2 x br s, 1H, rotamers), 3.68 (s, 3H), 3.17 (br s, 5H), 2.55 (br s, 2H), 1.82 – 1.26 (m, 19H); ¹³C NMR (100 MHz, CDCl₃) δ 176.2, 166.5, 156.4, 142.1, 136.6, 128.4, 128.3, 128.1, 128.0, 125.9, 125.7, 66.5, 61.5, 57.5, 47.8, 40.8, 33.5, 33.0, 32.2, 31.8, 29.7, 28.4, 23.1, 23.0. IR (KBr) ν(cm⁻¹) 2933, 1695, 1457, 1249, 1156. Λₘₐₓ = 225 nm. HRMS (ESI): m/z: Calcd for C₃₀H₄₃N₃O₆: 564.3044 [M+Na]⁺; found: 564.3043.
6-(Benzyloxy carbonylamino)-2-(tert-butoxycarbonyl(pentyl)amino)-6-(methoxy(methyl)amino)-6-oxohexane (2.47): 2.47 was synthesized as described in the general procedure using 2.25 (2 mmol, 901 mg), TEA (2 x 2 mmol, 0.28 mL), BOP (2 mmol, 885 mg), and N,O-dimethylhydroxylamine hydrochloride (2.2 mmol, 204 mg). After purification via flash column chromatography, 2.47 was obtained as a colorless oil (578 mg, 59%).

$^1$H NMR (400 MHz, CDCl$_3$) δ 7.28 – 7.23 (m, 5H), 5.08 (br s, 1H), 5.02 (s, 2H), 5.02 and 4.85 (2 x br s, 1H, rotamers), 3.62 (s, 3H), 3.11 – 3.01 (m, 7H), 1.49 – 1.12 (m, 21H), 0.83 (t, 3H, $J = 6.6$ Hz); $^{13}$C NMR (100 MHz, CDCl$_3$) δ 172.1 and 171.5 (rotamers), 156.2, 155.6 and 154.6 (rotamers), 136.5, 128.2, 127.8, 127.7, 79.6, 61.4, 54.6 and 53.0 (rotamers), 43.6 and 43.4 (rotamers), 41.2 and 40.6 (rotamers), 32.0, 29.5, 29.2, 29.1, 28.8, 28.4 and 28.1 (rotamers), 22.9, 22.6, 22.1, 13.9. IR (KBr) ν(cm$^{-1}$) 2937, 1694, 1455, 1250, 1171. $\lambda_{\text{max}} = 226$ nm. HRMS (ESI): $m/z$: Calcd for C$_{26}$H$_{43}$N$_3$O$_6$: 516.3044 [M+Na]$^+$; found: 516.3035.
6-(Benzyloxycarbonylamino)-2-(tert-butoxycarbonyl(Octyl)amino)-6-(methoxy(methyl)amino)-6-oxohexane (2.48): 2.48 was synthesized as described in the general procedure using 2.26 (2 mmol, 985 mg), TEA (2 x 2 mmol, 0.28 mL), BOP (2 mmol, 885 mg), and N,O-dimethylhydroxylamine hydrochloride (2.2 mmol, 204 mg). After purification via flash column chromatography, 2.48 was obtained as a colorless oil (470 mg, 44%).

\(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta \) 7.31 – 7.25 (m, 5H), 5.09 and 4.85 (2 x br s, 1H, rotamers), 5.04 (s, 2H), 3.65 (s, 3H), 3.13 – 3.02 (m, 7H), 1.73 – 1.17 (m, 27H), 0.84 (t, 3H, \(J = 6.4 \) Hz); \(^13\)C NMR (100 MHz, CDCl\(_3\)) \(\delta \) 172.2, 156.3, 155.7, 136.6, 128.3, 127.9, 127.8, 79.5, 66.3, 61.5 and 61.3 (rotamers), 54.7, 53.1, 43.8 and 43.6 (rotamers), 40.7, 31.6, 29.7, 29.2, 29.1, 28.8, 28.2, 27.0, 23.0, 22.7, 22.5, 14.0. IR (KBr) \(\nu (cm^{-1})\) 2929, 1671, 1406, 1168. \(\lambda_{\text{max}} \) = 227 nm. HRMS (ESI): \(m/z\): Calcd for C\(_{29}\)H\(_{49}\)N\(_3\)O\(_6\): 558.3514 [M+Na]\(^+\); found: 558.3512.

1,5-Dibenzyl-1H-imidazol-2-amine hydrochloride (2.49): 2.49 was synthesized as described in the general procedure using 2.27 (0.7 mmol, 279 mg) and DIBAL-H (1.05
mmol, 1.05 mL). Following work-up, the crude product was stirred in a 10:1 DCM/TFA solution. After completion of the reaction, removal of solvent, and pH adjustment, cyanamide (3.5 mmol, 147 mg) was added to the crude product in a 1:1 water:ethanol solution. After purification, 2.49 was converted to its hydrochloride salt as a yellow oil (79 mg, 43%).

\[ ^1H\text{ NMR (400 MHz, CD}_3\text{OD)} \delta 7.32 - 6.99 (m, 10H), 6.31 (s, 1H), 4.85 (s, 2H), 3.70 (s, 2H); \]
\[ ^{13}\text{C NMR (100 MHz, CD}_3\text{OD)} \delta 151.4, 139.9, 138.3, 129.9, 129.7, 128.7, 128.6, 127.7, 127.4, 122.3, 46.6, 31.8. \] IR (KBr) \( \nu(\text{cm}^{-1}) \) 3026, 1558, 1488. \( \lambda_{\text{max}} = 225 \text{ nm.} \) HRMS (ESI): \( m/z \): Calcd for C\(_{17}\)H\(_{17}\)N\(_3\): 264.1495 [M+H]\(^+\); found: 264.1598.

**5-Benzyl-1-(3-phenylpropyl)-1H-imidazol-2-amine hydrochloride (2.50):** 2.50 was synthesized as described in the general procedure using 2.28 (0.7 mmol, 298 mg) and DIBAL-H (1.05 mmol, 1.05 mL). Following work-up, the crude product was stirred in a 10:1 DCM/TFA solution. After completion of the reaction, removal of solvent, and pH adjustment, cyanamide (3.5 mmol, 147 mg) was added to the crude product in a 1:1 water:ethanol solution. After purification, 2.50 was converted to its hydrochloride salt as a yellow oil (79 mg, 39%).

\[ ^1H\text{ NMR (400 MHz, CD}_3\text{OD)} \delta 7.32 - 7.09 (m, 10H), 6.62 (s, 1H), 3.81 (s, 2H), 3.72 (t, 2H, J = 8 \text{ Hz}), 2.55 (t, 2H, J = 7.6 \text{ Hz}), 1.70 (\text{tt}, 2H, J = 6 \text{ Hz}); \]
\[ ^{13}\text{C NMR (100 MHz, CD}_3\text{OD)} \delta \]
5-Benzyl-1-pentyl-1H-imidazol-2-amine hydrochloride (2.51): 2.51 was synthesized as described in the general procedure using 2.29 (0.7 mmol, 271 mg) and DIBAL-H (1.05 mmol, 1.05 mL). Following work-up, the crude product was stirred in a 10:1 DCM/TFA solution. After completion of the reaction, removal of solvent, and pH adjustment, cyanamide (3.5 mmol, 147 mg) was added to the crude product in a 1:1 water:ethanol solution. After purification, 2.51 was converted to its hydrochloride salt as a yellow oil (85 mg, 49%).

$^1$H NMR (400 MHz, CD$_3$OD) δ 7.32 – 7.20 (m, 5H), 6.28 (s, 1H), 3.85 (s, 2H), 3.55 (t, 2H, $J = 8$ Hz), 1.36 – 1.35 (m, 2H), 1.24 – 1.18 (m, 4H), 0.86 (t, 3H, $J = 6.4$ Hz); $^{13}$C NMR (100 MHz, CD$_3$OD) δ 149.6, 139.2, 128.53, 128.51, 126.5, 125.9, 120.9, 42.5, 30.7, 28.9, 28.8, 22.4, 13.4. IR (KBr) ν(cm$^{-1}$) 2930, 1646, 1492. $\lambda_{\text{max}} = 227$ nm. HRMS (ESI): $m/z$: Calcd for C$_{19}$H$_{21}$N$_3$: 292.1808 [M+H]$^+$; found: 292.1807.

HRMS (ESI): $m/z$: Calcd for C$_{19}$H$_{21}$N$_3$: 292.1808 [M+H]$^+$; found: 292.1807.

HRMS (ESI): $m/z$: Calcd for C$_{19}$H$_{21}$N$_3$: 292.1808 [M+H]$^+$; found: 292.1807.

146.9, 140.7, 136.6, 128.8, 128.5, 128.4, 128.3, 127.6, 127.1, 126.1, 110.3, 42.4, 32.2, 29.6, 29.6. IR (KBr) ν(cm$^{-1}$) 3084, 1654, 1456. $\lambda_{\text{max}} = 229$ nm. HRMS (ESI): $m/z$: Calcd for C$_{19}$H$_{21}$N$_3$: 292.1808 [M+H]$^+$; found: 292.1807.

IR (KBr) ν(cm$^{-1}$) 3084, 1654, 1456. $\lambda_{\text{max}} = 229$ nm. HRMS (ESI): $m/z$: Calcd for C$_{19}$H$_{21}$N$_3$: 292.1808 [M+H]$^+$; found: 292.1807.
5-Benzyl-1-octyl-1H-imidazol-2-amine hydrochloride (2.52): 2.52 was synthesized as described in the general procedure using 2.30 (0.7 mmol, 294 mg) and DIBAL-H (1.05 mmol, 1.05 mL). Following work-up, the crude product was stirred in a 10:1 DCM/TFA solution. After completion of the reaction, removal of solvent, and pH adjustment, cyanamide (3.5 mmol, 147 mg) was added to the crude product in a 1:1 water:ethanol solution. After purification, 2.52 was converted to its hydrochloride salt as a yellow oil (95 mg, 48%).

$^1$H NMR (300 MHz, CD$_3$OD) δ 7.33 – 7.21 (m, 5H), 6.30 (s, 1H), 3.86 (s, 2H), 3.53 (t, 2H, $J$ = 7.8 Hz), 1.36 – 1.19 (m, 12H), 0.92 (t, 3H, $J$ = 6.9 Hz); $^{13}$C NMR (100 MHz, CD$_3$OD) δ 164.8, 150.5, 140.2, 129.5, 127.5, 121.4, 119.9, 43.6, 32.9, 31.7, 30.3, 30.1, 27.7, 23.7, 14.5. IR (KBr) $\nu$ (cm$^{-1}$) 3142, 1630, 1498. $\lambda_{max}$ = 223 nm. HRMS (ESI): m/z: Calcd for C$_{18}$H$_{27}$N$_3$: 286.2278 [M+H]$^+$; found: 286.2276.
1-Benzyl-5-phenyl-1H-imidazol-2-amine hydrochloride (2.53): 2.53 was synthesized as described in the general procedure using 2.31 (0.7 mmol, 269 mg) and DIBAL-H (1.05 mmol, 1.05 mL). Following work-up, the crude product was stirred in a 10:1 DCM/TFA solution. After completion of the reaction, removal of solvent, and pH adjustment, cyanamide (3.5 mmol, 147 mg) was added to the crude product in a 1:1 water:ethanol solution. After purification, 2.53 was converted to its hydrochloride salt as a yellow oil (101 mg, 59%).

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10:1 DCM/TFA solution. After completion of the reaction, removal of solvent, and pH adjustment, cyanamide (3.5 mmol, 147 mg) was added to the crude product in a 1:1 water:ethanol solution. After purification, **2.54** was converted to its hydrochloride salt as a yellow oil (99 mg, 51%).

\[ ^1\text{H NMR (400 MHz, CD}_3\text{OD) } 7.37 \text{–} 7.00 (m, 10H), 6.54 \text{ (s, 1H), 3.85 (t, 2H, } J = 7.6 \text{ Hz), 2.46 (t, 2H, } J = 7.6 \text{ Hz), 1.85 (q, 2H, } J = 7.6 \text{ Hz);} \]
\[ ^{13}\text{C NMR (100 MHz, CD}_3\text{OD) } \delta 151.8, 142.2, 132.2, 130.1, 129.8, 129.4, 129.2, 129.2, 128.3, 127.0, 122.8, 43.3, 33.5, 31.8. \]
\[ \text{IR (KBr) } \nu(\text{cm}^{-1}) \text{ 3135, 1658, 1239. } \]
\[ \Lambda_{\text{max}} = 229 \text{ nm, 241 nm, 258 nm. } \]
\[ \text{HRMS (ESI): } m/z: \]
Calcd for C\(_{18}\)H\(_{19}\)N\(_3\): 278.1652 [M+H]; found: 278.1653.

![Chemical Structure](image)

**1-Pentyl-5-phenyl-1H-imidazol-2-amine hydrochloride (2.55):** **2.55** was synthesized as described in the general procedure using **2.33** (0.7 mmol, 255 mg) and DIBAL-H (1.05 mmol, 1.05 mL). Following work-up, the crude product was stirred in a 10:1 DCM/TFA solution. After completion of the reaction, removal of solvent, and pH adjustment, cyanamide (3.5 mmol, 147 mg) was added to the crude product in a 1:1 water:ethanol solution. After purification, **2.55** was converted to its hydrochloride salt as a yellow oil (92 mg, 58%).

\[ ^1\text{H NMR (300 MHz, CD}_3\text{OD) } \delta 7.45 \text{–} 7.33 (m, 5H), 6.53 (s, 1H), 3.85 (t, 2H, } J = 7.65 \text{ Hz), 1.53 (p, } J = 7.2 \text{ Hz), 1.22 \text{–} 1.09 (m, 4H), 0.80 (t, 3H, } J = 7.05 \text{ Hz);} \]
\[ ^{13}\text{C NMR (100 MHz, CD}_3\text{OD) } \delta \]

\[ \]
(KBr) ν(cm⁻¹) 2959, 1632, 1488. \( \lambda_{\text{max}} = 206 \text{ nm}, 275 \text{ nm} \). HRMS (ESI): \( m/z \): Calcd for C_{14}H_{19}N_{3}: 230.1652 [M+H]^+; found: 230.1649.

1-Octyl-5-phenyl-1H-imidazol-2-amine hydrochloride (2.56): 2.56 was synthesized as described in the general procedure using 2.34 (0.7 mmol, 284 mg) and DIBAL-H (1.05 mmol, 1.05 mL). Following work-up, the crude product was stirred in a 10:1 DCM/TFA solution. After completion of the reaction, removal of solvent, and pH adjustment, cyanamide (3.5 mmol, 147 mg) was added to the crude product in a 1:1 water:ethanol solution. After purification, 2.56 was converted to its hydrochloride salt as a yellow oil (103 mg, 54%).

\(^1\)H NMR (400 MHz, CD\textsubscript{3}OD) δ 7.45 – 7.34 (m, 5H), 6.53 (s, 1H), 3.86 (t, 2H, \( J = 7.2 \text{ Hz} \)), 1.52 (t, 2H, \( J = 7.2 \text{ Hz} \)), 1.17 – 1.14 (m, 10H), 0.88 (t, 3H, \( J = 7.2 \text{ Hz} \)); \(^{13}\)C NMR (100 MHz, CD\textsubscript{3}OD) δ 151.8, 132.4, 130.2, 129.8, 129.4, 128.4, 122.7, 43.6, 32.8, 30.2, 30.0, 29.9, 27.2, 23.7, 14.4. IR (KBr) ν(cm⁻¹) 2923, 1602, 1544. \( \lambda_{\text{max}} = 215 \text{ nm}, 277 \text{ nm} \). HRMS (ESI): \( m/z \): Calcd for C_{17}H_{25}N_{3}: 272.2121 [M+H]^+; found: 272.2118.
1-Benzyl-5-isobutyl-1H-imidazol-2-amine hydrochloride (2.57): 2.57 was synthesized as described in the general procedure using 2.35 (0.7 mmol, 255 mg) and DIBAL-H (1.05 mmol, 1.05 mL). Following work-up, the crude product was stirred in a 1:1 diethyl ether:4M HCl. After completion of the reaction, removal of solvent, and pH adjustment, cyanamide (3.5 mmol, 147 mg) was added to the crude product in a 1:1 water:ethanol solution. After purification, 2.57 was converted to its hydrochloride salt as a yellow oil (75 mg, 47%).

$^1$H NMR (400 MHz, CD$_3$OD) $\delta$ 7.34-7.24 (m, 3H), 7.07-7.05 (m, 2H), 6.32 (s, 1H), 4.9 (s, 2H) 2.22 (d, 2H, $J = 7.2$ Hz), 1.69-1.62 (m, 1H) 0.87 (d, 6H, $J = 6.8$ Hz); $^{13}$C NMR (100 MHz, CD$_3$OD) $\delta$ 148.3, 136.0, 130.1, 129.2, 129.1, 127.2, 111.0, 46.7, 33.6, 27.9, 22.5. IR (KBr) $\nu$(cm$^{-1}$) 2958, 1646, 1454. $\lambda_{\text{max}}$ = 207 nm. HRMS (ESI): $m/z$: Calcd for C$_{14}$H$_{19}$N$_3$: 230.1652 [M+H]$^+$; found: 230.1654.

5-Isobutyl-1-(3-phenylpropyl)-1H-imidazol-2-amine hydrochloride (2.58): 2.58 was synthesized as described in the general procedure using 2.36 (0.7 mmol, 275 mg) and DIBAL-H (1.05 mmol, 1.05 mL). Following work-up, the crude product was stirred in a 1:1
diethyl ether:4M HCl. After completion of the reaction, removal of solvent, and pH adjustment, cyanamide (3.5 mmol, 147 mg) was added to the crude product in a 1:1 water:ethanol solution. After purification, 2.58 was converted to its hydrochloride salt as a yellow oil (90 mg, 50%).

$^1$H NMR (400 MHz, CD$_3$OD) δ 7.31-7.17 (m, 5H), 6.21 (s, 1H), 3.67 (t, 2H, $J = 7.8$ Hz), 2.66 (t, 2H, $J = 6$ Hz), 2.17 (d, 2H, 8.8 Hz), 1.95 (m, 2H) 1.63 (m, 1H), 0.86 (d, 6H, $J = 6.2$ Hz); $^{13}$C NMR (100 MHz, CD$_3$OD) δ 149.9, 142.4, 129.5, 129.4, 127.2, 121.0, 42.5, 34.4, 33.7, 32.1, 29.0, 22.7. IR (KBr) ν(cm$^{-1}$) 3098, 1652, 1457. $\lambda_{max} = 210$ nm. HRMS (ESI): m/z: Calcd for C$_{16}$H$_{23}$N$_3$: 258.1965 [M+H]$^+$; found: 258.1968.

5-Isobutyl-1-pentyl-1H-imidazol-2-amine hydrochloride (2.59): 2.59 was synthesized as described in the general procedure using 2.37 (0.7 mmol, 241 mg) and DIBAL-H (1.05 mmol, 1.05 mL). Following work-up, the crude product was stirred in a 1:1 diethyl ether:4M HCl. After completion of the reaction, removal of solvent, and pH adjustment, cyanamide (3.5 mmol, 147 mg) was added to the crude product in a 1:1 water:ethanol solution. After purification, 2.59 was converted to its hydrochloride salt as a yellow oil (60 mg, 41%).

$^1$H NMR (400 MHz, CD$_3$OD) δ 6.23 (s, 1H), 3.68 (t, 2H, $J = 6$ Hz), 2.33 (d, 2H, $J = 8$ Hz), 1.83-1.69 (m, 1H), 1.68-1.62 (m, 2H), 1.41-1.29 (m, 4H), 0.98-0.92 (m, 9H). $^{13}$C NMR (100 MHz, CD$_3$OD) δ 149.8, 127.3, 120.5, 43.2, 34.6, 30.3, 29.9, 29.0, 23.4, 22.8, 14.3. IR (KBr)
\(v(\text{cm}^{-1})\) 2934, 1647, 1527. \(\lambda_{\text{max}} = 230\) nm. HRMS (ESI): \(m/z\): Calcd for \(\text{C}_{12}\text{H}_{23}\text{N}_3\): 210.1965 \([\text{M}+\text{H}]^+\); found: 210.1964.

![structure](image)

5-Isobutyl-1-octyl-1H-imidazol-2-amine hydrochloride (2.60): 2.60 was synthesized as described in the general procedure using 2.38 (0.7 mmol, 270 mg) and DIBAL-H (1.05 mmol, 1.05 mL). Following work-up, the crude product was stirred in a 1:1 diethyl ether:4M HCl. After completion of the reaction, removal of solvent, and pH adjustment, cyanamide (3.5 mmol, 147 mg) was added to the crude product in a 1:1 water:ethanol solution. After purification, 2.60 was converted to its hydrochloride salt as a yellow oil (91 mg, 52%).

\(^1\text{H}\) NMR (400 MHz, \(\text{CD}_3\text{OD}\)) \(\delta\) 6.23 (s, 1H), 3.69 (t, \(J = 6.8\) Hz), 2.33 (d, \(J = 7.2\) Hz), 1.86-1.76 (1H, m), 1.68-1.60 (m, 2H), 1.38-1.28 (m, 10H), 0.97 (d, 3H, \(J = 6.4\)Hz), 0.92 (t, 6H, \(J = 6.0\) Hz); \(^{13}\text{C}\) NMR (100 MHz, \(\text{CD}_3\text{OD}\)) \(\delta\) 149.9, 127.3, 120.6, 43.2, 34.6, 32.9, 30.6, 30.4, 30.3, 29.1, 27.7, 23.7, 22.8, 14.4. IR (KBr) \(v(\text{cm}^{-1})\) 3109, 1644, 1526. \(\lambda_{\text{max}} = 220\) nm. HRMS (ESI): \(m/z\): Calcd for \(\text{C}_{15}\text{H}_{29}\text{N}_3\): 252.2434 \([\text{M}+\text{H}]^+\); found: 252.2432.
1-Benzyl-5-hexyl-1H-imidazol-2-amine hydrochloride (2.61): 2.61 was synthesized as described in the general procedure using 2.39 (0.7 mmol, 275 mg) and DIBAL-H (1.05 mmol, 1.05 mL). Following work-up, the crude product was stirred in a 1:1 diethyl ether:4M HCl. After completion of the reaction, removal of solvent, and pH adjustment, cyanamide (3.5 mmol, 147 mg) was added to the crude product in a 1:1 water:ethanol solution. After purification, 2.61 was converted to its hydrochloride salt as a yellow oil (82 mg, 46%).

$^1$H NMR (400 MHz, CD$_3$OD) $\delta$ 7.43-7.35 (m, 3H), 7.16-7.14 (m, 2H), 6.69 (s, 1H), 5.19 (s, 2H), 2.42 (t, 2H, 8.0 Hz), 1.49 (tt, 2H, $J = 7.4$, 6.9 Hz), 1.31-1.22 (m, 6H), 0.87 (t, 3H, $J = 6.9$ Hz); $^{13}$C NMR (100 MHz, CD$_3$OD) $\delta$ 148.5, 136.1, 130.6, 130.2, 129.2, 127.2, 110.0, 46.6, 32.5, 29.7, 28.1, 24.6, 23.5, 14.3. IR (KBr) $\nu$(cm$^{-1}$) 3105, 1655, 1456. $\lambda_{\text{max}} = 220$ nm.

HRMS (ESI): $m/z$: Calcd for C$_{16}$H$_{24}$N$_3$: 258.1965 [M+H]$^+$; found: 258.1964.

5-Hexyl-1-(3-phenylpropyl)-1H-imidazol-2-amine hydrochloride (2.62): 2.62 was synthesized as described in the general procedure using 2.40 (0.7 mmol, 294 mg) and DIBAL-H (1.05 mmol, 1.05 mL). Following work-up, the crude product was stirred in a 1:1
diethyl ether:4M HCl. After completion of the reaction, removal of solvent, and pH adjustment, cyanamide (3.5 mmol, 147 mg) was added to the crude product in a 1:1 water:ethanol solution. After purification, 2.62 was converted to its hydrochloride salt as a yellow oil (102 mg, 51%).

\[ \text{H NMR (400 MHz, CD}_3\text{OD)} \delta 7.31-7.18 \text{ (m, 5H), 6.20 (s, 1H), 3.71 (t, 2H, } J = 8 \text{ Hz), 2.67 (t, 2H, } J = 7.6 \text{ Hz), 2.29 (t, 2H, } J = 7.2 \text{ Hz), 1.95 (tt, 2H, } J = 7.6 \text{ Hz), 1.51-1.46 (m, 2H), 1.36-1.27 (m, 6H), 0.92 (t, 3H, } J = 7.2 \text{ Hz); } ^{13}\text{C NMR (100 MHz CD}_3\text{OD) } \delta 149.8, 142.4, 129.5, 128.6, 127.1, 119.9, 119.3, 42.5, 33.7, 32.7, 32.1, 30.0, 29.5, 25.2, 23.7, 14.4. \text{ IR (KBr) } \nu(\text{cm}^{-1}) \text{ 2925, 1637, 1552, 1336. } \lambda_{\text{max}} = 220 \text{ nm, 284 nm. HRMS (ESI): } m/z\text{: Calcd for C}_{18}\text{H}_{27}\text{N}_3: 286.2278 [M+H]^+; \text{ found: 286.2274.} \]

5-Hexyl-1-pentyl-1H-imidazol-2-amine hydrochloride (2.63): 2.63 was synthesized as described in the general procedure using 2.41 (0.7 mmol, 261 mg) and DIBAL-H (1.05 mmol, 1.05 mL). Following work-up, the crude product was stirred in a 1:1 diethyl ether:4M HCl. After completion of the reaction, removal of solvent, and pH adjustment, cyanamide (3.5 mmol, 147 mg) was added to the crude product in a 1:1 water:ethanol solution. After purification, 2.63 was converted to its hydrochloride salt as a yellow oil (38 mg, 23%).

\[ \text{H NMR (400 MHz, CD}_3\text{OD) } \delta 6.59 \text{ (s, 1H), 3.84 (t, 2H, } J = 7.65 \text{ Hz), 2.54 (t, 2H, } J = 7.65 \text{ Hz), 1.71 – 1.31 (m, 14H), 0.98 – 0.94 (m, 6H); } ^{13}\text{C NMR (100 MHz, CD}_3\text{OD) } \delta 158.1, \]

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130.4, 109.7, 43.8, 32.8, 30.0, 29.7, 29.5, 28.4, 24.7, 23.8, 23.5, 14.5, 14.4. IR (KBr) ν (cm⁻¹) 2924, 1648, 1460, 1267. λ_max = 210 nm. HRMS (ESI): m/z: Calcd for C₁₄H₂₇N₃: 238.2278 [M+H]⁺; found: 238.2280.

5-Hexyl-1-octyl-1H-imidazol-2-amine hydrochloride (2.64): 2.64 was synthesized as described in the general procedure using 2.42 (0.7 mmol, 290 mg) and DIBAL-H (1.05 mmol, 1.05 mL). Following work-up, the crude product was stirred in a 1:1 diethyl ether:4M HCl. After completion of the reaction, removal of solvent, and pH adjustment, cyanamide (3.5 mmol, 147 mg) was added to the crude product in a 1:1 water:ethanol solution. After purification, 2.64 was converted to its hydrochloride salt as a yellow oil (47 mg, 24%).

¹H NMR (300 MHz, CD₃OD) δ 6.59 (s, 1H), 3.84 (t, 2H, J = 7.5 Hz), 2.54 (t, 2H, J = 7.65 Hz), 1.70 – 1.63 (m, 4H), 1.46 – 1.33 (m, 16H), 0.97 – 0.90 (m, 6H); ¹³C NMR (100 MHz CD₃OD) δ 147.7, 130.3, 109.6, 43.7, 32.9, 32.8, 30.3, 29.9, 29.6, 28.3, 27.4, 24.6, 24.2, 23.7, 23.6, 14.4, 14.3. IR (KBr) ν (cm⁻¹) 2927, 1658, 1463. λ_max = 214 nm. HRMS (ESI): m/z: Calcd for C₁₇H₃₃N₅: 280.2747 [M+H]⁺; found: 280.2748.
1-Decyl-5-hexyl-1H-imidazol-2-amine hydrochloride (2.65): 2.65 was synthesized as described in the general procedure using 2.43 (0.7 mmol, 310 mg) and DIBAL-H (1.05 mmol, 1.05 mL). Following work-up, the crude product was stirred in a 10:1 DCM/TFA solution. After completion of the reaction, removal of solvent, and pH adjustment, cyanamide (3.5 mmol, 147 mg) was added to the crude product in a 1:1 water:ethanol solution. After purification, 2.65 was converted to its hydrochloride salt as a yellow oil (107 mg, 50%).

$^1$H NMR (400 MHz, CD$_3$OD) $\delta$ 6.22 (s, 1H), 3.70 (t, 2H, $J = 7.6$ Hz), 2.45 (t, 2H, $J = 7.6$ Hz), 1.67 – 1.59 (m, 4H), 1.44 – 1.32 (m, 29H), 0.95 – 0.90 (m, 6H); $^{13}$C NMR (100 MHz, CD$_3$OD) $\delta$ 149.8, 128.4, 119.3, 43.2, 33.1, 32.8, 30.7, 30.7, 30.5, 30.5, 30.4, 30.1, 29.4, 27.7, 25.4, 23.8, 23.7, 14.5, 14.5. IR (KBr) $\nu$(cm$^{-1}$) 2922, 2855, 1656, 1461. $\lambda_{\text{max}}$ = 226 nm. HRMS (ESI): m/z: Calcd for C$_{19}$H$_{37}$N$_3$: 308.3060 [M+H]$^+$; found: 308.3062.

1-Dodecyl-5-hexyl-1H-imidazol-2-amine hydrochloride (2.66): 2.66 was synthesized as described in the general procedure using 2.44 (0.7 mmol, 329 mg) and DIBAL-H (1.05 mmol, 1.05 mL). Following work-up, the crude product was stirred in a 10:1 DCM/TFA solution. After completion of the reaction, removal of solvent, and pH adjustment,
cyanamide (3.5 mmol, 147 mg) was added to the crude product in a 1:1 water:ethanol solution. After purification, **2.66** was converted to its hydrochloride salt as a yellow oil (136 mg, 58%).

$^1$H NMR (400 MHz, CD$_3$OD) $\delta$ 6.21 (s, 1H), 3.70 (t, 2H, $J = 7.8$ Hz), 2.46 (t, 2H, $J = 7.4$ Hz), 1.64 – 1.57 (m, 4H), 1.42 – 1.32 (m, 24H), 0.94 – 0.89 (m, 6H); $^{13}$C NMR (100 MHz, CD$_3$OD) $\delta$ 149.8, 128.4, 119.4, 43.2, 33.1, 32.9, 30.8, 30.8, 30.7, 30.7, 30.6, 30.5, 30.4, 30.2, 29.4, 27.7, 25.4, 23.8, 23.7, 14.5, 14.5. IR (KBr) $\nu$(cm$^{-1}$): 3093, 2917, 1654, 1455. $\lambda_{\text{max}} = 222$ nm. HRMS (ESI): $m/z$: Calcd for C$_{21}$H$_{41}$N$_3$: 336.3373 [M+H]$^+$; found: 336.3376.

**Benzyl 4-(2-amino-1-benzyl-1H-imidazol-5-yl)butylcarbamate hydrochloride (2.67):**

**2.67** was synthesized as described in the general procedure using **2.45** (0.7 mmol, 359 mg) and DIBAL-H (1.05 mmol, 1.05 mL). Following work-up, the crude product was stirred in a 10:1 DCM/TFA solution. After completion of the reaction, removal of solvent, and pH adjustment, cyanamide (3.5 mmol, 147 mg) was added to the crude product in a 1:1 water:ethanol solution. After purification, **2.67** was converted to its hydrochloride salt as a yellow oil (110 mg, 41%).

$^1$H NMR (400 MHz, CD$_3$OD) $\delta$ 7.36 – 7.06 (m, 10H), 6.33 (s, 1H), 5.06 (s, 2H), 5.09 (s, 2H), 3.07 (br s, 2H), 2.38 (br s, 2H), 1.49 (br s, 4H); $^{13}$C NMR (100 MHz, CD$_3$OD) $\delta$ 159.0, 150.7, 138.6, 138.5, 130.0, 130.0, 129.6, 129.1, 128.9, 128.7, 127.4, 120.3, 67.4, 46.4, 41.5,
Benzyl 4-(2-amino-1-(3-phenylpropyl)-1H-imidazol-5-yl)butylcarbamate hydrochloride (2.68): 2.68 was synthesized as described in the general procedure using 2.46 (0.7 mmol, 379 mg) and DIBAL-H (1.05 mmol, 1.05 mL). Following work-up, the crude product was stirred in a 10:1 DCM/TFA solution. After completion of the reaction, removal of solvent, and pH adjustment, cyanamide (3.5 mmol, 147 mg) was added to the crude product in a 1:1 water:ethanol solution. After purification, 2.68 was converted to its hydrochloride salt as a yellow oil (96 mg, 34%).

$^1$H NMR (300 MHz, CD$_3$OD) $\delta$ 7.37 – 7.18 (m, 10H), 6.54 (s, 1H), 5.08 (s, 2H), 3.84 (t, 2H, $J = 7.8$ Hz), 3.15 (t, 2H, $J = 6.3$ Hz), 2.71 (t, 2H, $J = 7.5$ Hz), 2.38 (br s, 2H), 1.99 (quintet, 2H, $J = 7.5$ Hz), 1.54 (s, 4H); $^{13}$C NMR (100 MHz, CD$_3$OD) $\delta$ 158.9, 150.0, 142.5, 138.5, 129.7, 129.5, 129.4, 128.9, 128.7, 128.1, 127.1, 119.8, 67.3, 42.6, 41.4, 33.7, 32.1, 30.5, 26.6, 24.8. IR (KBr) $\nu$(cm$^{-1}$) 2923, 1661, 1530, 1254. $\lambda_{\text{max}} = 215$ nm. HRMS (ESI): $m/z$: Calcd for C$_{24}$H$_{30}$N$_4$O$_2$: 407.2442 [M+H]$^+$; found: 407.2438.
Benzyl 4-(2-amino-1-pentyl-1H-imidazol-5-yl)butylcarbamate hydrochloride (2.69): 2.69 was synthesized as described in the general procedure using 2.47 (0.7 mmol, 345 mg) and DIBAL-H (1.05 mmol, 1.05 mL). Following work-up, the crude product was stirred in a 10:1 DCM/TFA solution. After completion of the reaction, removal of solvent, and pH adjustment, cyanamide (3.5 mmol, 147 mg) was added to the crude product in a 1:1 water:ethanol solution. After purification, 2.69 was converted to its hydrochloride salt as a yellow oil (79 mg, 32%).

$^1$H NMR (400 MHz, CD$_3$OD), δ 7.36 - 7.29 (m, 5H), 6.25 (s, 1H), 5.08 (s, 2H), 3.69 (t, 2H, $J$ = 7.2 Hz), 3.17 (t, 2H, $J$ = 6 Hz), 2.48 (t, 2H, $J$ = 6.4 Hz), 1.67-1.59 (m, 6H), 1.40-1.32 (m, 4H), 0.93 (t, 3H, $J$ = 7.2 Hz); $^{13}$C NMR (100 MHz, CD$_3$OD) δ 159.1, 150.0, 138.6, 129.6, 129.1, 128.9, 128.4, 119.3, 67.4, 43.4, 41.6, 30.7, 30.4, 30.1, 26.6, 25.0, 23.6, 14.5. IR (KBr) ν(cm$^{-1}$) 2944, 1660, 1455, 1257, 1135. $\lambda_{\text{max}}$ = 228 nm. HRMS (ESI): $m/z$: Calcd for C$_{20}$H$_{30}$N$_4$O$_2$: 359.2442 [M+H]$^+$; found: 359.2442.
Benzyl 4-(2-amino-1-octyl-1H-imidazol-5-yl)butylcarbamate hydrochloride (2.70): 2.70 was synthesized as described in the general procedure using 2.48 (0.7 mmol, 375 mg) and DIBAL-H (1.05 mmol, 1.05 mL). Following work-up, the crude product was stirred in a 10:1 DCM/TFA solution. After completion of the reaction, removal of solvent, and pH adjustment, cyanamide (3.5 mmol, 147 mg) was added to the crude product in a 1:1 water:ethanol solution. After purification, 2.70 was converted to its hydrochloride salt as a yellow oil (95 mg, 34%).

$^1$H NMR (400 MHz, CD$_3$OD) δ 7.37 – 7.30 (m, 5H), 6.26 (s, 1H), 5.08 (s, 2H), 3.70 (t, 2H, $J$ = 7.6 Hz), 3.17 (t, 2H, $J$ = 6.4 Hz), 2.48 (t, 2H, $J$ = 6.4 Hz), 1.62 – 1.60 (m, 6H), 1.34 – 1.31 (m, 10H), 0.91 (t, 3H, $J$ = 6.4 Hz); $^{13}$C NMR (100 MHz, CD$_3$OD) δ 164.8, 149.7, 138.4, 129.4, 128.9, 128.7, 128.4, 119.9, 118.6, 67.3, 43.3, 41.4, 32.9, 30.5, 30.4, 30.3, 27.7, 26.3, 24.8, 23.7, 14.4. IR (KBr) ν(cm$^{-1}$) 2926, 1692, 1458. $\lambda_{\text{max}}$ = 213 nm. HRMS (ESI): $m/z$: Calcd for C$_{23}$H$_{36}$N$_4$O$_2$: 401.2911 [M+H]$^+$; found: 401.2912.

**Bacterial Strains.** *S. aureus* (29213), *S. epidermidis* (29886), MRSE (51625) MRSA (BAA-44), MDRAB (BAA-1605), *A. baumannii* (19606), *K. pneumoniae* (BAA-2146), and *E. coli* (35695) were obtained from the ATCC.
**Broth Microdilution Method for MIC Determination.** Overnight bacterial cultures were subcultured to $5 \times 10^5$ CFU/mL in Mueller Hinton Broth (MHB). The resulting bacterial suspension was aliquoted (1.0 mL) into culture tubes and compound added from 100 mM stock samples in molecular biology grade DMSO to a final concentration of 128 µg/mL, samples containing no compound served as a control. Samples were then aliquoted (200 µL) into the first row of wells of a 96-well microtiter plate in which subsequent wells had been prefilled with 100 µL of the same bacterial subculture. Row one wells were mixed 8–10 times then 100 µL was withdrawn and transferred to row two. Row two wells were mixed 8–10 times followed by a 100 µL transfer to row three. This procedure was used to serially dilute the rest of the rows of the microtiter plate. The microtiter plate was then covered with a microtiter plate lid, placed in a covered plastic container and incubated under stationary conditions at 37°C. After 16 h, the lid was removed and MIC values were recorded.

**Biofilm Inhibition Assay Protocol.** Overnight cultures of *S. aureus*, *A. baumannii*, or *E. coli* in tryptic soy broth with 0.5% glucose (*S. aureus*) or Luria-Bertani (LB) (*A. baumannii*) media respectively, were subcultured to an OD$_{600}$ of 0.01 into the same media. Test compound was added to the inoculated media and 100 µL was aliquoted into the wells of a 96-well plate, inoculum without compound served as the control. Plates were covered, and incubated in a humidified container under stationary condition at 37 °C for 24 h. Media and planktonic bacteria were discarded and the plates were washed with water. Each well was stained with 110 µL 0.1% solution of crystal violet (CV) at room temperature for 30 min. After washed with water again, the remaining stain was dissolved in 200 µL 95% ethanol and
125 µL was transferred to polystyrene microtiter dish. Biofilm inhibition was quantified by measuring the OD$_{540}$ of each well. Blank wells were employed as background controls.
2.19
135
2.22
Pulse Sequence: z-grad
Solvent: CDCl3
Resonant frequency
H NMR (400 MHz, CDCl3)
Relaxation delay 1.00 sec
Pulse width 1.5 µsec
Power 250 W
Spectral width 10000 Hz
Dedicated 400.1363 MHz
Power 50 W

Compound: N
2.49
2.58
2.67
References


Appendix
Appendix A - MIC values (µg/mL) of all compounds against several strains

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Appendix B - Percentage biofilm inhibition at 200 µM

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CHAPTER 3
SUPPRESSING RESISTANCE TO β-LACTAM ANTIBIOTICS IN MRSA

3.1: Introduction

β-lactams represent one of the largest classes of antibiotics, as of 2004 more than 65% of antibiotics sold around the world are members of this class. This class of antibiotics can be further divided into sub-classes that include penicillins, carbapenems, cephalosporins, and monobactams (Figure 1.5). As previously mentioned, β-lactam antibiotics selectively inhibit bacterial cell wall biosynthesis by inhibiting the penicillin-binding proteins (PBPs). The outcome is a weakened cell wall that ultimately results in cell lysis due to osmotic pressure. Unfortunately, bacterial resistance has rendered many of these drugs, once considered miracle drugs, completely useless.

In the past, methicillin-resistant S. aureus (MRSA) was only encountered in the hospital setting but it is now becoming increasingly common in the community setting. In 2005, almost 95,000 people acquired MRSA infections in the United States, of which nearly 19,000 people died - more than die annually from HIV/AIDS, emphysema, Parkinson’s disease, and homicide combined. One strategy used to combat the aforementioned resistance mechanisms is to develop new β-lactam antibiotics. In 1940, penicillin was the first β-lactam antibiotic introduced to treat S. aureus infections. Four years later, reports of resistance began to emerge mostly due to S. aureus attaining the enzyme known as β-lactamase. To counter β-lactamase resistance, a structure modification strategy was employed that resulted in the antibiotic methicillin. The introduction of two methoxy-groups on the aromatic ring and the deletion of the methylene spacer provided sufficient steric
hindrance to protect the β-lactam ring (Figure 3.1).\textsuperscript{5} Methicillin was found to be effective against penicillin-resistant \textit{S. aureus} but within two years of its introduction to the clinic MRSA was isolated. Resistance to methicillin is the result of an additional PBP (PBP2a) (discussed in chapter 1).\textsuperscript{6} Over the next 50 years, this cycle of developing a new antibiotic followed by the inevitable development of bacterial resistance would be repeated multiple times. As of 2013, there have not been any new β-lactam class antibiotics discovered in the past 30 years, and derivatives based on previously discovered scaffolds represent a small portion of new antibiotics in clinical development.\textsuperscript{6}

![Penicillin G and Methicillin](image)

\textbf{Figure 3.1: Structure of penicillin G and methicillin.}

An alternative to developing new antibiotics is to use small molecule adjuvants in combination with previously discovered antibiotics. The ideal adjuvant is non-microbicidal when used alone, and instead targets a mechanism of resistance that the respective bacterium exploits to lower the effectiveness of an antibiotic. This allows an otherwise ineffective antibiotic to be useful again. This approach shows promise because it may also delay the
development of resistance to the adjuvant, due to the adjuvant acting on a non-essential pathway. One of the most successful examples of antibiotic/adjuvant combination therapy is Augmentin, which combines the β-lactam antibiotic amoxicillin with the β-lactamase inhibitor clavulanic acid (Figure 3.2). Clavulanic acid is able to form an acyl enzyme intermediate with β-lactamase, and ultimately inactivates the enzyme. Cell wall biosynthesis is then inhibited by amoxicillin as previously described.

![Clavulanic acid and Amoxicillin](image)

**Figure 3.2: Structure of clavulanic acid (β-lactamase inhibitor) and amoxicillin (β-lactam antibiotic).**

The Melander lab has shown that 2-aminoimidazoles can act as potential adjuvants for the suppression of antibiotic resistance mechanisms. In 2010, Rogers *et al.* successfully identified a 2-aminoimidazole/triazole conjugate that was able to resensitize multidrug-resistant strains of bacteria to the effects of conventional antibiotics. When compound 3.1 was present at 45 μM, it suppressed the MIC of penicillin G against MRSA 8-fold (from 32 μg/mL to 4 μg/mL). This was an exciting result, as compound 3.1 was determined to be nontoxic to planktonic bacteria at this concentration.
Figure 3.3: Structure of compound 3.1 that was shown to suppress antibiotic resistance.

Following the initial discovery of compound 3.1, we began screening other compounds from our in-house library for the ability to suppress β-lactam resistance in MRSA (BAA-44). Over the past decade the Melander lab has accumulated a library of over 500 compounds, and some of the most active compounds from various sets were initially tested. First the MIC of the respective compound was determined, and then the compound was tested in combination with penicillin G. To minimize toxicity effects all compounds were tested at sub-MIC levels. These compounds included 2-aminoimidazole/triazole amides, derivatives of compound 3.1, 2-aminoimidazole reverse amides, alkyl mono-substituted 2-aminoimidazoles, and 2-aminobenzimidazoles.\textsuperscript{9-12} It was determined that members of the 2-aminoimidazole/triazole (2-AIT) library contained the most active compounds (Figure 3.4). Compound 3.3 was approximately twice as active as 3.1, and it was able to suppress the MIC of penicillin G 8-fold when present at 25 μM.
Figure 3.4: A class of 2-aminoimidazole/triazole amide conjugates that suppresses resistance to β-lactam antibiotics.

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3.2: Results and Discussion

Upon identifying compound 3.3, we opted to test it against a more relevant USA300 MRSA strain (ATCC BAA-1556) and a common clinically administered β-lactam antibiotic (oxacillin). Using the CLSI broth microdilution protocol, we determined the oxacillin MIC in the absence and presence of 40% of the MIC of compound 3.3 (20 μM). Compound 3.3 lowered the oxacillin MIC by eight-fold at this concentration, from 32 μg/mL to 4 μg/mL. With the aim of developing a compound with increased activity, a series of analogues of compound 3.3, in which substituents were placed at the N1-position of the 2-aminoimidazole, were synthesized using the approach developed in Chapter 2.
Commercially available 2-bromoethylamine 3.8 was treated with sodium azide to yield the azido amine 3.9, which was subsequently amidated with 4-pentylbenzoyl chloride to deliver azido amide 3.10. Isomerization of 3.11 with NaH in ethylene diamine gave the terminal alkyne 3.12. Mesylation of the alcohol followed by displacement with sodium iodide afforded the primary iodide 3.13. The commercially available N-protected glycinate ethyl ester was converted to the corresponding enolate with KO{\textsuperscript{t}Bu}, followed by addition of 3.13. After stirring at room temperature for 48 hours, the alkylated glycinate ethyl ester 3.14 was obtained. This was treated with the azido amide 3.10 under copper-catalyzed Huisgen cycloaddition conditions followed by deprotection to deliver key intermediate 3.15. This common intermediate was synthesized in gram quantities and was used to introduce diversity into the library. Reductive amination of 3.15 with a variety of commercially available benzaldehydes, followed by Boc-protection gave 3.16. The ethyl ester was then converted into the Weinreb amide 3.17, which was then reduced to the corresponding α-amino aldehyde with DIBAL-H. Following Boc-deprotection and cyclization with cyanamide, the desired 1,5-substituted 2-aminoimidazole/triazole conjugates 3.18 - 3.31 were obtained (Scheme 3.1).
Scheme 3.1: Synthesis of 1,5-substituted 2-aminoimidazole/triazole conjugates.

As with compound 3.3, the MIC of each compound against MRSA (ATCC BAA-1556) was first established. The MIC of oxacillin in the presence of 40% of the MIC of each compound was then determined (Table 3.1). Most compounds exhibited a significantly reduced or abrogated ability to lower the oxacillin MIC. Three compounds however (3.21, 3.28, and 3.29), displayed a marked increase in activity, lowering the oxacillin MIC by 128-fold, 64-fold, and 64-fold, respectively. The ability of the pilot library to suppress oxacillin resistance in another CA-MRSA USA300 strain (JE2) was next investigated (Table 3.1) and activity for each compound against this strain was virtually identical to that of BAA-1556.
Table 3.1: MIC values and oxacillin resistance suppression activity against MRSA USA300 strains.

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</tbody>
</table>

Compound 3.21, which possess a 4-butylbenzyl substituent was selected as the lead compound for further study owing to the considerably lower concentration at which it displayed activity compared to 3.28 and 3.29 (5 µM compared to 40 µM and 20 µM respectively). A control compound, 3.32, which possesses only the N-1 substituent of compound 3.21 was synthesized in the same manner as the pilot library and shown to be devoid of both bactericidal activity (MIC >200 µM) and resistance suppression activity at concentrations as high as 50 µM.
To measure toxicity as a function of time, time-kill curves were constructed for strain JE2 cultured in the presence of combinations of oxacillin and compound 3.21 (Figure 3.5). Compound 3.21, when dosed alone at 5 µM, is bactericidal at early time points; however bacterial growth is similar to that of the control by the 24 h time point. When bacteria are cultured in the presence of combinations of oxacillin and compound 3.21, a large reduction in the number of colony forming units (CFU) compared to treatment with oxacillin alone is observed. Additionally a considerable synergistic effect can be observed at the 24 h time point. Compound 3.21 alone, at 5 µM, effected a 1.08 log reduction in CFU after 24 h, and oxacillin effected less than 0.4 log reduction at concentrations of 16 µg/mL and below. Combining 3.21 (5 µM) and oxacillin resulted in log CFU reductions of 6.41, 5.54 and 4.38 for oxacillin concentrations of 16, 4, and 1 µg/mL respectively.
Compound 3.21 was also tested at 5 μM for the ability to suppress oxacillin resistance in seven additional MRSA isolates from the ATCC (Table 3.2). Observed reduction in MIC values ranged from 4-fold to 512-fold. This data shows that compound 3.21 has broad applications against various MRSA isolates.
Table 3.2: Activity of compound 3.21 against various MRSA strains.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Oxacillin MIC (µg/mL)</th>
<th>Oxacillin MIC (µg/mL) in the presence of 3.21 (5 µM)</th>
<th>Fold reduction in oxacillin MIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>43300</td>
<td>32</td>
<td>1</td>
<td>32</td>
</tr>
<tr>
<td>33591</td>
<td>256</td>
<td>≤0.5</td>
<td>512</td>
</tr>
<tr>
<td>700789</td>
<td>64</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>BAA-1753</td>
<td>256</td>
<td>64</td>
<td>4</td>
</tr>
<tr>
<td>BAA-811</td>
<td>64</td>
<td>1</td>
<td>64</td>
</tr>
<tr>
<td>BAA-1770</td>
<td>32</td>
<td>≤0.5</td>
<td>64</td>
</tr>
<tr>
<td>BAA-1685</td>
<td>256</td>
<td>64</td>
<td>4</td>
</tr>
</tbody>
</table>

As the synthesized molecules are amphipathic, we next investigated the effect of the compounds on cell membrane integrity. The ability of compound 3.21 to permeabilize the bacterial cell membrane was quantified using the BacLight assay. This is a fluorescence based assay that measures the permeability of the bacterial membrane towards propidium iodide (a red fluorescent nucleic acid stain). Propidium iodide is excluded by healthy cells, and only stains cells with damaged bacterial membranes. This is compared to a green fluorescent, SYTO 9, that stains all cells. After exposure of strain JE2 to compound 3.21 for one hour, the ratio of intact/damaged cells was measured and compared to control (DMSO only treated) bacteria. At 4x the MIC, 96% of cells were damaged, while at 1x, 0.4x, and 0.25x the MIC, only 33%, 21%, and 9% of cells were damaged respectively. An inactive compound (3.22) was found to be comparable, with 83%, 24%, 23%, and 16% of cells damaged at 4x, 1x, 0.4x, and 0.25x the MIC respectively, suggesting that cell membrane
permeabilization is not the mechanism by which compound \(3.21\) suppresses resistance to oxacillin. Importantly for a potential antibiotic adjuvant, \(3.21\) exhibited little effect on eukaryotic cell membranes, as determined by measuring the hemolytic activity against mechanically defibrinated sheep blood.\(^{16}\) At its active resistance suppression concentration (5 \(\mu M\)), less than 1\% lysis was observed compared to triton x positive control, while only 5.6\% lysis was observed as high as 50 \(\mu M\).

To further probe the mechanism of action, we obtained a library of transposon mutants from the Network on Antimicrobial Resistance in \(S.\) \(aureus\) (NARSA) that had various non-essential two-component system (TCS) genes knocked-out. These mutants are all derived from JE2, allowing us to probe non-essential pathways that may be involved in suppression of oxacillin resistance. For this screen, we focused largely on mutants of non-essential TCS. Bacterial TCS, consisting of a membrane-bound histidine kinase and a response regulator, regulate adaptation to environmental changes and have been shown to play a role in resistance to certain antibiotics (discussed in Chapter 1).\(^{17,18}\) TCS have also been shown to regulate biofilm formation.\(^{19}\) Recently, Thompson et al. were able to show that biotinylated analogues of related 2-aminoimidazole anti-biofilm compounds employed in pull down assays bind to response regulators involved in biofilm formation.\(^{20}\) This lead to the hypothesis that similar 2-aminoimidazoles might also target other response regulators involved in antibiotic resistance.

First, the MICs of oxacillin and \(3.21\) were determined for each mutant strain (Table 3.3). A gene description for each mutant strain can be found in the Appendix. The MIC of \(3.21\) was fairly consistent against all mutant strains (either 6.25 or 12.5 \(\mu M\)), while a majority
of the strains examined, including several response regulator mutants (strains NE958, NE481, NE262, and NE49), histidine kinase mutants (strains NE218, NE147, NE618, NE873, NE820, NE116, and NE423), and a MecR1 regulatory protein mutant (strain NE839), did not exhibit a greater than two-fold difference in oxacillin MIC compared to the parent strain. However, three of the strains tested exhibited considerably lower oxacillin MIC values: NE481, an unidentified DNA-binding response regulator mutant, NE554 (vraR mutant), and NE823 (vraS mutant) exhibited oxacillin MICs that were reduced 16-fold, eight-fold, and eight-fold respectively. These results are in line with previous studies that show expression of VraSR contributes to oxacillin resistance.21
Table 3.3: MIC values and oxacillin resistance suppression activity of compound 3.21 against strains from the Nebraska Transposon Mutant Library.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Concentration of 3.21 (μM)</th>
<th>Oxacillin MIC (μg/mL)</th>
<th>Oxacillin MIC with 3.21 (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>JE2</td>
<td>5</td>
<td>32</td>
<td>0.5</td>
</tr>
<tr>
<td>NE218</td>
<td>2.5</td>
<td>32</td>
<td>8</td>
</tr>
<tr>
<td>NE147</td>
<td>2.5</td>
<td>32</td>
<td>0.25</td>
</tr>
<tr>
<td>NE958</td>
<td>5</td>
<td>32</td>
<td>0.25</td>
</tr>
<tr>
<td>NE481</td>
<td>5</td>
<td>2</td>
<td>0.25</td>
</tr>
<tr>
<td>NE262</td>
<td>5</td>
<td>32</td>
<td>0.5</td>
</tr>
<tr>
<td>NE618</td>
<td>5</td>
<td>32</td>
<td>1</td>
</tr>
<tr>
<td>NE554</td>
<td>2.5</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>NE823</td>
<td>2.5</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>NE873</td>
<td>5</td>
<td>32</td>
<td>0.25</td>
</tr>
<tr>
<td>NE210</td>
<td>5</td>
<td>32</td>
<td>0.5</td>
</tr>
<tr>
<td>NE820</td>
<td>5</td>
<td>32</td>
<td>0.25</td>
</tr>
<tr>
<td>NE839</td>
<td>5</td>
<td>32</td>
<td>0.5</td>
</tr>
<tr>
<td>NE49</td>
<td>2.5</td>
<td>32</td>
<td>16</td>
</tr>
<tr>
<td>NE116</td>
<td>2.5</td>
<td>32</td>
<td>32</td>
</tr>
<tr>
<td>NE95</td>
<td>5</td>
<td>16</td>
<td>0.5</td>
</tr>
<tr>
<td>NE423</td>
<td>2.5</td>
<td>16</td>
<td>4</td>
</tr>
</tbody>
</table>

The ability of 3.21 to lower the oxacillin MIC against the mutant strains was then examined in an identical manner to that used for the parent strain (at 40% of the MIC). Of the mutants that exhibited altered oxacillin MIC values compared to the parent, compound 3.21 failed to lower the MIC of both the VraSR two-component system mutant strains NE554 and NE823, suggesting the mode of action of oxacillin resistance suppression activity of compound 3.21 involves VraSR. Interestingly, compound 3.21 also failed to lower the oxacillin MIC by more than two-fold against strains NE116 and NE49. However as these two mutant strains did not exhibit oxacillin MICs that differed from the parent strain, these
pathways most likely do not relate to the mechanism of oxacillin resistance suppression by compound 3.21.

Compound 3.21 exhibited a lower MIC against a number of mutant strains than the parent strain and was therefore screened for resistance suppression at a lower concentration, 2.5 µM. To ensure that the lack of resistance suppression activity was not simply a result of lower bactericidal activity of the compound, time kill curves were constructed for NE554 in the presence of 2.5 µM 3.21 (40% MIC) and compared to the time kill curve of JE2 in the presence of 5 µM 3.21 (Figure 3.6). The bactericidal activity of compound 3.21, was slightly higher against strain NE554 than JE2 at the concentrations used in the resistance suppression assay. This result suggests that the lack of activity against NE554 is due to the absence of VraR, rather than altered bactericidal activity.
The expression of the VraSR TCS is induced upon exposure to cell-wall acting antibiotics, and it has been shown that VraSR mutants are treatable with an oxacillin regimen in vivo.\textsuperscript{22} Once induced, VraSR coordinates a response that involves the expression of a number of genes involved in antibiotic resistance.\textsuperscript{21} To further establish disruption of VraSR signaling in the mechanism of \textbf{3.21}, the ability of \textbf{3.21} to lower the MIC of vancomycin was tested (Table 3.4). The MIC of vancomycin against JE2 was established as 1 µg/mL, while in the presence of 5 µM \textbf{3.21} this is lowered to 0.25 µg/mL. The MIC of vancomycin is 0.5 µg/mL against both NE554 and NE823, and this remained unchanged in the presence of 2.5
Furthermore, compound 3.21 had little or no effect on the MICs of streptomycin or chloramphenicol (Table 3.4), non-cell wall acting antibiotics that do not activate the VraSR TCS. The fact that compound 3.21 did not lower the MIC of these latter antibiotics against the parent strain further suggests that the reduction in oxacillin MIC brought about by this compound is not simply due to a combined microbicidal effect, but due to disruption of the VraSR TCS pathway.

### Table 3.4: MIC values of various antibiotics without/with compound 3.21. All values reported in μg/mL.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Antibiotic MIC/MIC with 3.21</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>JE-2</td>
</tr>
<tr>
<td>Oxacillin</td>
<td>32.0/0.5</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>1.0/0.25</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>8.0/4.0</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>8.0/4.0</td>
</tr>
</tbody>
</table>

### 3.3: Conclusions

In conclusion, a library of fourteen 1,5-substituted 2-aminoimidazole/triazole conjugates were synthesized and tested for the ability to suppress oxacillin resistance. Compound 3.21 was shown to suppress resistance to oxacillin, upwards of 512-fold in diverse MRSA strains. Utilizing a BacLight assay, it was shown that this activity is not dependent upon membrane disruption. Preliminary screening of USA300 mutants indicated that the TCS VraSR plays an important role in the activity of this compound.
3.4: Experimental Section

Synthesis Experimental

All reagents used for chemical synthesis were purchased from commercially available sources and used without further purification. Chromatography was performed using 60A° mesh standard grade silica gel from Sorbtech (Atlanta, GA, USA). NMR solvents were obtained from Cambridge Isotope Labs and used as is. $^1$H NMR (300 MHz or 400 MHz) and $^{13}$C NMR (75 MHz or 100 MHz) spectra were recorded at 25°C on Varian Mercury spectrometers. Chemical shifts ($d$) are given in ppm relative to tetramethylsilane or the respective NMR solvent; coupling constants ($J$) are in Hertz (Hz). Abbreviations used are s = singlet, br s = broad singlet, d = doublet, dd = doublet of doublets, t = triplet, dt = doublet of triplets, q = quartet and m = multiplet. Mass spectra were obtained at the NCSU Department of Chemistry Mass Spectrometry Facility. Infrared spectra were obtained on a FT/IR-4100 spectrophotometer ($\nu_{\text{max}}$ in cm$^{-1}$). UV absorbance was recorded on a Genesys 10 scanning UV/visible spectrophotometer ($\lambda_{\text{max}}$ in nm).

General synthetic procedure for reductive amination

The $\alpha$-amino ethyl ester 3.33 was dissolved in methanol (30 mL). LiOH.H$_2$O was added to the solution and stirred at room temperature for 30 minutes. The respective aldehyde was added dropwise, and stirred for 2 hours. Sodium borohydride was then added portionwise, and the solution was allowed to stir for 1 hour. The solvent was removed under reduced pressure and the resulting solid was dissolved in ethyl acetate (75 mL), and washed with saturated sodium bicarbonate (3 x). The organic layer was then dried over sodium sulfate.
The solvent was removed under reduced pressure, and the crude product was purified via flash column chromatography (20% → 40% acetone/hexanes).

**General synthetic procedure for Boc-protection**

The appropriate secondary amine was dissolved in 1:1 dioxane/water (20 mL) and triethylamine was added. Di-tert-butyl dicarbonate was added and the solution was stirred at room temperature overnight (16 hours). The solvent was removed under reduced pressure, and the crude product was then dissolved in water and extracted with diethyl ether (3 x). The combined organic layers were washed with brine and then dried over sodium sulfate. Following the removal of the solvent under reduced pressure, the crude product was purified via flash column chromatography (40% → 75% ethyl acetate/hexanes).

**General synthetic procedure for Weinreb amide formation**

The appropriate Boc-protected secondary amine was stirred in dry tetrahydrofuran (15 mL) under nitrogen gas. To this solution, N,O-dimethylhydroxylamine hydrochloride was added and then cooled to -20 °C. 2.0M isopropylmagnesium chloride solution in tetrahydrofuran was added dropwise and the reaction was allowed to warm to room temperature. After 18 hours, the solution was cooled to 0 °C and saturated ammonium chloride (15 mL) was added dropwise. The aqueous layer was then extracted with ethyl acetate (3 x) and the combined organic layers were dried over sodium sulfate. The solvent was removed under reduced pressure, and the crude product was purified via flash column chromatography (20% → 40% acetone/hexanes).
General synthetic procedure for cyanamide cyclization

A solution of the appropriate Weinreb amide was stirred in dry tetrahydrofuran (8 mL) and then cooled to -78 °C under nitrogen gas. To this solution, 1.0M diisobutylaluminium hydride (DIBAL-H) in hexanes was added and the reaction was stirred until completion by TLC analysis (approximately 2 hours). The reaction was quenched with aqueous 0.35 M sodium bisulfate (15 mL), then extracted with diethyl ether (3 x). The combined organic fractions were then washed with aqueous 1M HCl (2 x), followed by aqueous saturated sodium bicarbonate (2 x), and then with brine (2 x). The organic layer was then dried over sodium sulfate, and the solvent was removed under reduced pressure.

The crude product was dissolved in 9:1 dichloromethane:trifluoroacetic acid (15 mL). This was stirred for 15 minutes and upon completion by TLC analysis the solvent was removed under reduced pressure. Following the removal of the solvent, the crude product was dissolved in 1:1 ethanol/water (8 mL) and the pH of the solution was adjusted to 4.3 with aqueous 2M sodium hydroxide. To this, cyanamide was added and the reaction was stirred at reflux (95 °C) for 3 hours. The solvent was removed under reduced pressure and the crude product was purified via flash column chromatography (4% → 10% methanol(saturated ammonia)/dichloromethane). The pure product was dissolved in methanol (3 mL) and concentrated HCl (0.3 mL) was added. The HCl salt was obtained upon the removal of solvent under reduced pressure.
Ethyl 2-amino-7-(1-(2-(4-pentylbenzamido)ethyl)-1H-1,2,3-triazol-4-yl)heptanoate hydrochloride (3.33): Ethyl 2-(diphenylmethyleneamino)non-8-ynoate was synthesized as described by Su et al.\textsuperscript{23} N-(2-azidoethyl)-4-pentylbenzamide was synthesized as described by Rogers et al.\textsuperscript{10} Ethyl 2-(diphenylmethyleneamino)non-8-ynoate (10 mmol) and N-(2-azidoethyl)-4-pentylbenzamide (10 mmol) were stirred in 1:1:1 DCM/H\textsubscript{2}O/EtOH (100 mL). To this solution, copper(II)sulfate (1.5 mmol) and sodium ascorbate (4.5 mmol) were added and the reaction stirred at room temperature for 3 hours. Upon completion of the reaction, H\textsubscript{2}O (200 mL) was added. The aqueous layer was extracted with DCM (3 \times 200 mL). The combined organic fractions were dried over sodium sulfate, and the solvent was removed under reduced pressure. The crude product was dissolved in 1:1 diethyl ether/2M aqueous HCl (150 mL). The reaction stirred at room temperature for 2 hours. Upon completion, H\textsubscript{2}O (75 mL) was added to the solution and the aqueous layer was washed with diethyl ether (3 x). H\textsubscript{2}O was then removed under reduced pressure and without further purification 5 was isolated as the HCl salt.

\textsuperscript{1}H NMR (300 MHz, CDCl\textsubscript{3}) δ 8.67 (s, 1H), 7.72 (d, 2H, \textit{J} = 8.1 Hz), 7.30 (d, 2H, \textit{J} = 8.1 Hz), 4.88 (t, 2H, \textit{J} = 5.3 Hz), 4.31 (q, 2H, \textit{J} = 7.2 Hz), 4.06 - 3.95 (m, 3H), 2.93 (t, 2H, \textit{J} = 7.4 Hz), 2.68 (t, 2H, \textit{J} = 7.7 Hz), 1.95 - 1.17 (m, 17H), 0.92 (t, 3H, \textit{J} = 6.8 Hz); \textsuperscript{13}C NMR (100 MHz, CDCl\textsubscript{3}) δ 170.6, 170.5, 148.8, 145.4, 132.2, 129.8, 128.9, 128.6, 63.7, 54.5, 54.0,
40.5, 36.8, 32.6, 32.2, 31.3, 29.2, 28.8, 25.5, 24.0, 23.7, 14.6, 14.5; IR (KBr) ν(cm⁻¹) 3448, 1645, 1239; λmax = 205 nm, 232 nm; HRMS (ESI): m/z: Calcd for C_{25}H_{39}N_{5}O_{3}: 458.3126 [M+H]^+, found: 458.3129.

**Ethyl 2-(benzylamino)-7-(1-(2-(4-pentylbenzamido)ethyl)-1H-1,2,3-triazol-4-yl)heptanoate (amine 3.18):** was synthesized as described in the general procedure using the α-amino ethyl ester HCl salt (1 mmol), LiOH.H_{2}O (1.1 mmol), benzaldehyde (1 mmol), sodium borohydride (2 mmol). Following purification via flash column chromatography, amine 3.18 was obtained as a clear oil (33% yield).

^1H NMR (400 MHz, CDCl₃) δ 7.68 (d, 2H, J = 8.4 Hz), 7.34 - 7.20 (m, 8H), 7.00 (br s, 1H), 4.55 (t, 2H, J = 5.6 Hz), 4.18 (q, 2H, J = 7.2 Hz), 3.95 (t, 2H, J = 5.6 Hz), 3.82 (d, 1H, J = 12.8 Hz), 3.68 (d, 1H, J = 22.0 Hz), 3.24 (t, 1H, J = 6.6 Hz), 2.68 - 2.60 (m, 4H), 2.16 (br s, 1H), 1.67 - 1.26 (m, 17H), 0.88 (t, 3H, J = 7.0 Hz) ; ^13C NMR (100 MHz, CDCl₃) δ 175.2, 167.8, 148.2, 147.2, 139.4, 131.1, 128.6, 128.4, 128.3, 127.1, 127.0, 121.8, 60.6, 60.5, 52.0, 49.3, 39.8, 35.7, 33.2, 31.3, 30.8, 29.1, 28.8, 25.4, 25.3, 22.5, 14.3, 14.0; IR (KBr) ν(cm⁻¹) 3431, 2989, 1733, 1270; λmax = 230 nm; HRMS (ESI): m/z: Calcd for C_{32}H_{45}N_{5}O_{3}: 548.3595 [M+H]^+, found: 548.3597.
Ethyl 2-(naphthalen-2-ylmethylamino)-7-(1-(2-(4-pentylbenzamido)ethyl)-1H-1,2,3-triazol-4-yl)heptanoate (amine 3.19): was synthesized as described in the general procedure using the α-amino ethyl ester HCl salt (1 mmol), LiOH.H₂O (1.1 mmol), 2-naphthaldehyde (1 mmol), sodium borohydride (2 mmol). Following purification via flash column chromatography, amine 3.19 was obtained as a clear oil (76% yield).

¹H NMR (400 MHz, CDCl₃) δ 7.81 - 7.67 (m, 6H), 7.47 - 7.44 (m, 3H), 7.25 (s, 1H), 7.20 (d, 2H, J = 8.4 Hz), 7.09 (br s, 1H), 4.52 (t, 2H, J = 5.6 Hz), 4.18 (q, 2H, J = 7.1 Hz), 4.00 - 3.71 (m, 4H), 3.29 (m, 1H), 2.66 - 2.59 (m, 4H), 2.37 (br s, 1H), 1.66 - 1.25 (m, 17H), 0.88 (t, 3H, J = 7.0 Hz) ; ¹³C NMR (100 MHz, CDCl₃) δ 175.2, 167.8, 148.1, 147.2, 136.8, 133.3, 132.7, 131.0, 128.6, 128.0, 127.7, 127.0, 126.7, 126.0, 125.6, 121.8, 60.7, 60.4, 52.1, 51.7, 49.3, 39.8, 35.7, 33.2, 31.3, 30.8, 29.1, 28.8, 25.3, 22.4, 14.3, 14.0; IR (KBr) ν(cm⁻¹) 3328, 2937, 1733, 1540, 1236; λₘₐₓ = 227 nm; HRMS (ESI): m/z: Calcd for C₃₆H₄₇N₅O₃: 598.3752 [M+H]⁺, found: 598.3759.
**Ethyl 2-(4-isopropylbenzylamino)-7-(1-(2-(4-pentylbenzamido)ethyl)-1H-1,2,3-triazol-4-yl)heptanoate (amine 3.20):** was synthesized as described in the general procedure using the α-amino ethyl ester HCl salt (1 mmol), LiOH·H₂O (1.1 mmol), 4-isopropylbenzaldehyde (1 mmol), sodium borohydride (2 mmol). Following purification via flash column chromatography, amine 3.20 was obtained as a clear oil (32% yield).

^1H NMR (400 MHz, CDCl₃) δ 7.71 (d, 2H, J = 8.8 Hz), 7.65 (t, 1H, J = 5.8 Hz), 7.26 - 7.13 (m, 7H), 4.51 (t, 2H, J = 5.8 Hz), 4.13 (q, 2H, J = 7.2 Hz), 3.88 (m, 2H), 3.72 (d, 1H, J = 12.4 Hz), 3.56 (d, 1H, J = 12.8 Hz), 3.20 (t, 1H, J = 6.6 Hz), 2.85 (sp, 1H, J = 7.0 Hz), 2.60 - 2.57 (m, 4H), 1.98 (br s, 1H), 1.56 - 1.19 (m, 23H), 0.85 (t, 3H, J = 6.8 Hz); ^13C NMR (100 MHz, CDCl₃) δ 175.3, 167.8, 147.7, 147.4, 146.8, 137.0, 131.0, 128.3, 128.0, 127.0, 126.2, 121.7, 60.5, 60.3, 51.7, 49.0, 39.8, 35.6, 33.6, 33.2, 31.2, 30.7, 29.0, 28.7, 25.2, 23.8, 22.3, 14.2, 13.8; IR (KBr) ν(cm⁻¹) 3349, 2946, 1722, 1644, 1164; λ_max = 203 nm; HRMS (ESI): m/z: Calcd for C_{35}H_{51}N_{5}O_{3}: 590.4065 [M+H]^+, found: 590.4065.
Ethyl 2-(4-butylbenzylamino)-7-(1-(2-(4-pentylbenzamido)ethyl)-1H-1,2,3-triazol-4-yl)heptanoate (amine 3.21): was synthesized as described in the general procedure using the α-amino ethyl ester HCl salt (1 mmol), LiOH.H₂O (1.1 mmol), 4-butylbenzaldehyde (1 mmol), sodium borohydride (2 mmol). Following purification via flash column chromatography, amine 3.21 was obtained as a clear oil (29% yield).

1H NMR (300 MHz, CDCl₃) δ 7.67 (d, 2H, J = 8.1 Hz), 7.29 - 7.11 (m, 7H), 6.92 (t, 1H, J = 5.6 Hz), 4.55 (t, 2H, J = 7.4 Hz), 4.17 (q, 2H, J = 7.2 Hz), 3.95 (m, 2H), 3.76 (d, 1H, J = 12.6 Hz), 3.59 (d, 1H, J = 12.6 Hz), 3.23 (t, 1H, J = 6.8 Hz), 2.70 - 2.56 (m, 6H), 1.87 (br s, 1H), 1.66 - 1.25 (m, 21H), 0.94 - 0.86 (m, 6H); 13C NMR (100 MHz, CDCl₃) δ 175.5, 167.8, 148.1, 147.1, 141.6, 136.9, 131.1, 128.5, 128.3, 128.1, 127.0, 121.7, 60.6, 60.5, 51.8, 49.2, 39.8, 35.7, 35.2, 33.6, 33.3, 31.3, 30.8, 29.1, 28.9, 25.4, 22.4, 22.3, 14.3, 13.9, 13.8; IR (KBr) ν(cm⁻¹) 3313, 2932, 1728, 1667, 1187; λmax = 221 nm; HRMS (ESI): m/z: Calcd for C₃₆H₅₃N₅O₃: 604.4221 [M+H]⁺, found: 604.4242. 
Ethyl 2-(4-chlorobenzylamino)-7-(1-(2-(4-pentylbenzamido)ethyl)-1H-1,2,3-triazol-4-yl)heptanoate (3.22): was synthesized as described in the general procedure using the α-amino ethyl ester HCl salt (1 mmol), LiOH.H$_2$O (1.1 mmol), 4-chlorobenzaldehyde (1 mmol), sodium borohydride (2 mmol). Following purification via flash column chromatography, amine 3.22 was obtained as a clear oil (73% yield).

$^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 7.69 (d, 2H, $J = 8.4$ Hz), 7.28 - 7.17 (m, 8H), 4.53 (t, 2H, $J = 5.8$ Hz), 4.16 (q, 2H, $J = 7.2$ Hz), 3.92 (m, 2H), 3.75 (d, 1H, $J = 13.2$ Hz), 3.55 (d, 1H, $J = 13.2$ Hz), 3.12 (t, 1H, $J = 6.6$ Hz), 2.64 - 2.58 (m, 4H), 1.95 (br s, 1H), 1.63 - 1.24 (m, 17H), 0.86 (t, 3H, $J = 7.0$ Hz); $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 175.3, 167.8, 148.0, 147.1, 138.3, 132.6, 131.1, 129.5, 128.5, 128.3, 127.0, 121.7, 60.5, 60.4, 51.3, 49.2, 39.8, 35.7, 33.3, 31.3, 30.8, 29.1, 28.8, 25.4, 25.3, 22.4, 14.3, 13.9; IR (KBr) $\nu$(cm$^{-1}$) 3301, 2926, 1733, 1644, 754; $\lambda_{max} = 224$ nm; HRMS (ESI): $m/z$: Calcd for C$_{32}$H$_{44}$ClN$_5$O$_3$: 582.3205 [M+H]$^+$, found: 582.3209.
Ethyl 2-(4-bromobenzylamino)-7-(1-(2-(4-pentylbenzamido)ethyl)-1H-1,2,3-triazol-4-yl)heptanoate (amine 3.23): was synthesized as described in the general procedure using the α-amino ethyl ester HCl salt (1 mmol), LiOH·H₂O (1.1 mmol), 4-bromobenzaldehyde (1 mmol), sodium borohydride (2 mmol). Following purification via flash column chromatography, amine 3.23 was obtained as a clear oil (52% yield).

¹H NMR (400 MHz, CDCl₃) δ 7.69 (d, 2H, J = 8.0 Hz), 7.41 - 7.17 (m, 8H), 4.53 (t, 2H, J = 5.8 Hz), 4.15 (q, 2H, J = 7.0 Hz), 3.92 (m, 2H), 3.74 (d, 1H, J = 13.2 Hz), 3.54 (d, 1H, J = 13.2 Hz), 3.15 (t, 1H, J = 6.6 Hz), 2.64 - 2.58 (m, 4H), 1.97 (br s, 1H), 1.63 - 1.25 (m, 17H), 0.86 (t, 3H, J = 7.0 Hz); ¹³C NMR (100 MHz, CDCl₃) δ 175.3, 167.8, 148.0, 147.1, 138.8, 131.3, 131.1, 129.9, 128.5, 127.0, 121.7, 120.7, 60.5, 60.4, 51.6, 51.3, 49.2, 39.8, 35.7, 33.3, 31.3, 30.7, 29.1, 28.8, 25.4, 22.4, 14.3, 13.9; IR (KBr) ν(cm⁻¹) 3319, 2932, 1726, 1653, 702; λₘₐₓ = 225 nm; HRMS (ESI): m/z: Calcd for C₃₂H₄₄BrN₅O₃: 626.2700 [M+H]⁺, found: 626.2691.
Ethyl 7-(1-(2-(4-pentylbenzamido)ethyl)-1H-1,2,3-triazol-4-yl)-2-(4-propoxybenzylamino)heptanoate (amine 3.24): was synthesized as described in the general procedure using the α-amino ethyl ester HCl salt (1 mmol), LiOH.H2O (1.1 mmol), 4-propoxybenzaldehyde (1 mmol), sodium borohydride (2 mmol). Following purification via flash column chromatography, amine 3.24 was obtained as a clear oil (41% yield).

1H NMR (400 MHz, CDCl3) δ 7.69 (d, 2H, J = 8.0 Hz), 7.31 - 7.17 (m, 6H), 6.82 (d, 2H, J = 8.4 Hz), 4.52 (t, 2H, J = 5.4 Hz), 4.16 (q, 2H, J = 7.2 Hz), 3.93 - 3.86 (m, 4H), 3.70 (m, 1H), 3.53 (m, 1H), 3.20 (m, 1H), 2.63 - 2.58 (m, 4H), 2.07 (br s, 1H), 1.82 - 1.24 (m, 19H), 1.00 (t, 3H, J = 7.4 Hz), 0.86 (t, 3H, J = 7.0 Hz); 13C NMR (100 MHz, CDCl3) δ 175.4, 167.8, 158.2, 148.0, 147.0, 131.5, 131.1, 129.3, 128.5, 127.0, 121.7, 114.2, 69.4, 60.4, 60.3, 51.5, 51.4, 49.2, 39.8, 35.7, 33.2, 31.3, 30.7, 29.1, 28.8, 25.3, 22.5, 22.4, 14.3, 13.9, 10.4; IR (KBr) ν(cm⁻¹) 3402, 2937, 1726, 1504, 1239; λmax = 201 nm, 226 nm; HRMS (ESI): m/z: Calcd for C35H51N5O4: 606.4014 [M+H]+, found: 606.4013.
Ethyl 2-(4-tert-butylbenzylamino)-7-(1-(2-(4-pentylbenzamido)ethyl)-1H-1,2,3-triazol-4-yl)heptanoate (amine 3.25): was synthesized as described in the general procedure using the α-amino ethyl ester HCl salt (1 mmol), LiOH.H₂O (1.1 mmol), 4-tert-butylbenzaldehyde (1 mmol), sodium borohydride (2 mmol). Following purification via flash column chromatography, amine 3.25 was obtained as a clear oil (71% yield).

¹H NMR (400 MHz, CDCl₃) δ 7.70 (d, 2H, J = 8.4 Hz), 7.34 - 7.18 (m, 8H), 4.53 (t, 2H, J = 5.6 Hz), 4.16 (q, 2H, J = 7.1 Hz), 3.92 (m, 2H), 3.76 (d, 1H, J = 12.8 Hz), 3.61 (d, 1H, J = 12.8 Hz), 3.24 (t, 1H, J = 6.6 Hz), 2.65 - 2.59 (m, 4H), 2.41 (br s, 1H), 1.64 - 1.24 (m, 26H), 0.88 (t, 3H, J = 7.0 Hz); ¹³C NMR (100 MHz, CDCl₃) δ 175.2, 167.8, 149.9, 148.0, 147.1, 136.3, 131.1, 128.5, 128.0, 127.0, 125.2, 121.7, 60.6, 60.5, 51.6, 49.2, 39.8, 35.7, 34.3, 33.2, 31.3, 31.2, 30.8, 29.1, 28.8, 25.4, 25.3, 22.4, 14.3, 13.9; IR (KBr) ν(cm⁻¹) 3368, 2926, 1733, 1641, 1186; λ_max = 202 nm, 220 nm; HRMS (ESI): m/z: Calcd for C₃₆H₅₃N₅O₃: 604.4221 [M+H]⁺, found: 604.4203.
Ethyl 2-(4-ethylbenzylamino)-7-(1-(2-(4-pentylbenzamido)ethyl)-1H-1,2,3-triazol-4-yl)heptanoate (amine 3.26): was synthesized as described in the general procedure using the α-amino ethyl ester HCl salt (1 mmol), LiOH.H₂O (1.1 mmol), 4-ethylbenzaldehyde (1 mmol), sodium borohydride (2 mmol). Following purification via flash column chromatography, amine 3.26 was obtained as a clear oil (27% yield).

¹H NMR (400 MHz, CDCl₃) δ 7.67 (d, 2H, J = 8.1 Hz), 7.46 - 7.20 (m, 7H), 6.92 (br s, 1H), 4.55 (t, 2H, J = 5.6 Hz), 4.17 (q, 2H, J = 7.0 Hz), 3.95 (m, 2H), 3.83 (d, 1H, J = 13.2 Hz), 3.62 (d, 1H, J = 13.2 Hz), 3.20 (t, 1H, J = 6.6 Hz), 3.06 (s, 1H), 2.70 - 2.60 (m, 4H), 2.17 (br s, 1H), 1.66 - 1.21 (m, 17H), 0.88 (t, 3H, J = 6.8 Hz); ¹³C NMR (100 MHz, CDCl₃) δ 176.0, 168.5, 148.7, 147.8, 141.3, 132.7, 131.8, 129.2, 128.8, 127.7, 122.4, 121.3, 84.2, 77.6, 61.3, 61.2, 52.3, 49.9, 40.5, 36.4, 34.0, 32.0, 31.5, 29.8, 29.5, 29.4, 26.1, 23.1, 15.0, 14.6; IR (KBr) ν(cm⁻¹) 3301, 2937, 2264, 1728, 1191; λ_max = 206 nm, 238 nm; HRMS (ESI): m/z: Calcd for C₃₄H₄₅N₅O₃: 572.3595 [M+H]⁺, found: 572.3584.
Ethyl 2-(biphenyl-4-ylmethylamino)-7-(1-(2-(4-pentylbenzamido)ethyl)-1H-1,2,3-triazol-4-yl)heptanoate (amine 3.27): was synthesized as described in the general procedure using the α-amino ethyl ester HCl salt (1 mmol), LiOH.H₂O (1.1 mmol), biphenyl-4-carbaldehyde (1 mmol), sodium borohydride (2 mmol). Following purification via flash column chromatography, amine 3.27 was obtained as a clear oil (40% yield).

$^1$H NMR (400 MHz, CDCl₃) δ 7.70 (d, 2H, $J = 8.0$ Hz), 7.59 - 7.27 (m, 10H), 7.20 (d, 2H, $J = 8.4$ Hz), 4.53 (t, 2H, $J = 5.6$ Hz), 4.19 (q, 2H, $J = 7.2$ Hz), 3.95 - 3.90 (m, 2H), 3.87 (d, 1H, $J = 13.2$ Hz), 3.69 (d, 1H, $J = 13.2$ Hz), 3.27 (t, 1H, $J = 6.6$ Hz), 2.67 - 2.59 (m, 4H), 1.68 - 1.24 (m, 18H), 0.88 (t, 3H, $J = 7.0$ Hz); $^{13}$C NMR (100 MHz, CDCl₃) δ 175.2, 167.8, 148.1, 147.1, 140.8, 140.0, 138.5, 131.1, 128.7, 128.6, 128.5, 127.2, 127.0, 126.9, 121.7, 60.6, 60.5, 51.6, 49.2, 39.8, 35.7, 33.2, 31.3, 30.8, 29.1, 28.8, 25.4, 25.3, 22.4, 14.3, 13.9; IR (KBr) ν (cm$^{-1}$) 3372, 2927, 1672, 1541, 1168; $\lambda_{max} = 210$ nm, 244 nm; HRMS (ESI): $m/z$: Calcd for C₃₈H₄₉N₅O₃: 624.3908 [M+H]$^+$, found: 624.3888.
Ethyl 2-(4-cyanobenzylamino)-7-(1-(2-(4-pentylbenzamido)ethyl)-1H-1,2,3-triazol-4-yl)heptanoate (amine 3.28): was synthesized as described in the general procedure using the α-amino ethyl ester HCl salt (1 mmol), LiOH·H₂O (1.1 mmol), 4-formylbenzonitrile (1 mmol), sodium borohydride (2 mmol). Following purification via flash column chromatography, amine 3.28 was obtained as a clear oil (40% yield).

¹H NMR (400 MHz, CDCl₃) δ 7.68 (d, 2H, J = 8.4 Hz), 7.57 (d, 2H, J = 8.4 Hz), 7.43 (d, 2H, J = 8.4 Hz), 7.29 (s, 1H), 7.23 (br s, 1H), 7.18 (d, 2H, J = 8.8 Hz), 4.53 (t, 2H, J = 5.8 Hz), 4.16 (q, 2H, J = 7.1 Hz), 3.90 (m, 2H, 3.71 - 3.62 (m, 2H), 3.15 (t, 1H, J = 6.6 Hz), 2.66 - 2.57 (m, 4H), 2.55 (br s, 1H), 1.64 - 1.21 (m, 17H), 0.85 (t, 3H, J = 7.0 Hz); ¹³C NMR (100 MHz, CDCl₃) δ 175.0, 167.8, 148.0, 147.1, 145.3, 132.3, 131.0, 128.7, 128.4, 127.0, 121.7, 118.8, 110.7, 60.6, 60.5, 51.4, 49.2, 39.7, 35.6, 33.2, 31.2, 30.7, 29.0, 28.8, 25.4, 25.3, 22.3, 14.2, 13.9; IR (KBr) ν cm⁻¹) 3415, 2929, 2231, 1634, 1150; λ max = 202 nm, 230 nm; HRMS (ESI): m/z: Calcd for C₃₃H₄₄N₆O₃: 573.3548 [M+H]⁺, found: 573.3548.
Ethyl 2-(3,4-diethoxybenzylamino)-7-(1-(2-(4-pentylbenzamido)ethyl)-1H-1,2,3-triazol-4-yl)heptanoate (amine 3.29): was synthesized as described in the general procedure using the α-amino ethyl ester HCl salt (1 mmol), LiOH·H₂O (1.1 mmol), 3,4-diethoxybenzaldehyde (1 mmol), sodium borohydride (2 mmol). Following purification via flash column chromatography, amine 3.29 was obtained as a clear oil (35% yield).

¹H NMR (400 MHz, CDCl₃) δ 7.70 (d, 2H, J = 8.0 Hz), 7.42 - 7.15 (m, 4H), 6.85 - 6.77 (m, 3H), 4.50 (t, 2H, J = 5.2 Hz), 4.17 - 3.49 (m, 10H), 3.17 (t, 1H, J = 6.6 Hz), 2.69 - 2.57 (m, 4H), 1.96 (br s, 1H), 1.58 - 1.22 (m, 23H), 0.85 (t, 3H, J = 6.4 Hz); ¹³C NMR (100 MHz, CDCl₃) δ 175.3, 167.8, 148.5, 147.9, 147.6, 147.0, 132.4, 131.1, 128.4, 127.0, 121.6, 120.4, 113.5, 113.2, 64.4, 64.2, 60.3, 51.7, 49.1, 39.7, 35.6, 33.2, 31.2, 31.0, 30.7, 30.6, 29.0, 28.8, 25.3, 25.2, 22.3, 14.7, 14.2, 13.8; IR (KBr) ν(cm⁻¹) 3405, 2936, 1634, 1262; λ_max = 204 nm; 230 nm; HRMS (ESI): m/z: Calcd for C₃₆H₅₃N₅O₅: 636.4119 [M+H]⁺, found: 636.4123.
**Ethyl 2-(4-(hexyloxy)benzylamino)-7-(1-(2-(4-pentylbenzamido)ethyl)-1H-1,2,3-triazol-4-yl)heptanoate (amine 3.30):** was synthesized as described in the general procedure using the α-amino ethyl ester HCl salt (1 mmol), LiOH.H$_2$O (1.1 mmol), 4-(hexyloxy)benzaldehyde (1 mmol), sodium borohydride (2 mmol). Following purification via flash column chromatography, amine 3.30 was obtained as a clear oil (63% yield).

$^1$H NMR (400 MHz, CDCl$_3$) δ 7.66 (d, 2H, $J$ = 8.4 Hz), 7.30 - 7.19 (m, 5H), 7.00 (t, 1H, $J$ = 5.4 Hz), 6.82 (d, 2H, $J$ = 8.7 Hz), 4.54 (t, 2H, $J$ = 5.6 Hz), 4.17 (q, 2H, $J$ = 7.2 Hz), 3.97 - 3.90 (m, 4H), 3.73 (d, 1H, $J$ = 12.6 Hz), 3.56 (d, 1H, $J$ = 12.6 Hz), 3.21 (t, 1H, $J$ = 6.6 Hz), 2.68 - 2.59 (m, 4H), 2.07 (br s, 1H), 1.80 - 1.24 (m, 25H), 0.91 - 0.85 (m, 6H); $^{13}$C NMR (100 MHz, CDCl$_3$) δ 175.2, 167.8, 158.1, 147.7, 146.8, 131.3, 131.0, 129.2, 128.3, 127.0, 121.6, 114.1, 67.7, 60.3, 60.2, 51.3, 49.0, 39.8, 35.2, 33.1, 31.3, 31.2, 30.6, 29.0, 28.9, 28.7, 25.5, 25.2, 25.1, 22.4, 22.2, 14.1, 13.8, 13.7; IR (KBr) $\nu$(cm$^{-1}$) 3411, 2934, 1702, 1219; $\lambda_{\text{max}}$ = 202 nm, 226 nm; HRMS (ESI): $m$/z: Calcd for C$_{38}$H$_{57}$N$_5$O$_4$: 648.4483 [M+H]$^+$, found: 648.4483.
Ethyl 2-(3,4-dichlorobenzylamino)-7-(1-(2-(4-pentylbenzamido)ethyl)-1H-1,2,3-triazol-4-yl)heptanoate (amine 3.31): was synthesized as described in the general procedure using the α-amino ethyl ester HCl salt (1 mmol), LiOH.H₂O (1.1 mmol), 3,4-dichlorobenzaldehyde (1 mmol), sodium borohydride (2 mmol). Following purification via flash column chromatography, amine 3.31 was obtained as a clear oil (43% yield).

¹H NMR (400 MHz, CDCl₃) δ 7.67 (d, 2H, J = 8.0 Hz), 7.46 - 7.17 (m, 6H), 6.96 (br s, 1H), 4.55 (t, 2H, J = 5.6 Hz), 4.18 (q, 2H, J = 7.1 Hz), 3.98 - 3.93 (m, 2H), 3.80 (d, 1H, J = 13.6 Hz), 3.59 (d, 1H, J = 13.6 Hz), 3.19 (t, 1H, J = 6.6 Hz), 2.69 - 2.60 (m, 4H), 2.17 (br s, 1H), 1.66 - 1.26 (m, 17H), 0.88 (t, 3H, J = 6.8 Hz); ¹³C NMR (100 MHz, CDCl₃) δ 174.7, 167.8, 148.0, 147.1, 139.4, 132.3, 131.1, 130.2, 130.1, 128.5, 127.7, 127.6, 127.0, 121.8, 60.8, 60.3, 50.6, 49.3, 39.8, 35.7, 33.0, 31.3, 30.8, 29.6, 29.0, 28.7, 25.3, 22.4, 14.3, 13.9; IR (KBr) ν (cm⁻¹) 3326, 2924, 1644, 751; λ max = 202 nm, 226 nm; HRMS (ESI): m/z:  
Calcd for C₃₂H₄₅Cl₂N₅O₃: 616.2816 [M+H]⁺, found: 616.2818.

Methyl 2-(4-butylbenzylamino)acetate (amine 3.32): was synthesized as described in the general procedure using glycine methyl ester hydrochloride (1 mmol), LiOH.H₂O (1.1
mmol), 4-butylbenzaldehyde (1 mmol), sodium borohydride (2 mmol). Following purification via flash column chromatography, 3.32 was obtained as a clear oil (49% yield).

$^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 7.24 (d, 2H, $J = 8.0$ Hz), 7.15 (d, 2H, $J = 8.0$ Hz), 3.78 (s, 2H), 3.73 (s, 3H), 3.43 (s, 2H), 2.60 (t, 2H, $J = 7.8$ Hz), 2.17 (br s, 1H), 1.60 (qn, 2H, $J = 7.6$ Hz), 1.36 (sx, 2H, $J = 7.6$ Hz), 0.93 (t, 3H, $J = 7.2$ Hz); $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 172.9, 141.8, 136.5, 128.5, 128.2, 53.0, 51.7, 49.9, 35.3, 33.6, 22.3, 13.9; IR (KBr) $\nu$(cm$^{-1}$) 3459, 2942, 1721, 1189; $\lambda_{\text{max}} = 219$ nm, 260 nm; HRMS (ESI): $m/z$: Calcd for C$_{14}$H$_{21}$NO$_2$: 236.1645 [M+H]$^+$, found: 236.1637.

Ethyl 2-(benzyl(tert-butoxycarbonyl)amino)-7-(1-(2-(4-pentylbenzamido)ethyl)-1H-1,2,3-triazol-4-yl)heptanoate (Boc 3.18): was synthesized as described in the general procedure using amine 3.18 (0.25 mmol), triethylamine (0.33 mmol), di-tert-butyl dicarbonate (0.75 mmol). Following completion of the reaction via TLC, the crude product was purified via flash column chromatography. Boc 3.18 was obtained as a clear oil (75% yield).

$^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 7.68 (d, 2H, $J = 8.4$ Hz), 7.30 - 7.21 (m, 8H), 6.93 (br s, 1H), 4.63 - 3.92 (m, 9H), 2.65 - 2.60 (m, 4H), 1.90 - 1.17 (m, 26H), 0.89 (t, 3H, $J = 7.0$ Hz); $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 171.7 and 171.5 (rotamers), 167.8, 155.8 and 155.6 (rotamers),
147.9, 147.0, 138.8 and 138.0 (rotamers), 131.0, 128.4, 128.1, 127.2, 127.1, 127.0, 121.7, 80.4, 60.8, 59.5 and 58.9 (rotamers), 51.0 and 49.7 (rotamers), 49.2, 39.8, 35.6, 31.2, 30.8, 30.1, 29.3, 28.7 and 28.5 (rotamers), 28.2, 26.2 and 26.0 (rotamers), 25.3, 22.3, 13.9, 13.8; IR (KBr) ν(cm⁻¹) 3317, 2926, 1697, 1536, 1166; λ_max = 202 nm, 232 nm; HRMS (ESI): m/z: Calcd for C_{37}H_{53}N_{5}O_{5}: 648.4119 [M+H]^+, found: 648.4134.

Ethyl 2-(tert-butoxycarbonyl(naphthalen-2-ylmethyl)amino)-7-(1-(2-(4-pentylbenzamido)ethyl)-1H-1,2,3-triazol-4-yl)heptanoate (Boc 3.19): was synthesized as described in the general procedure using amine 3.19 (0.25 mmol), triethylamine (0.33 mmol), di-tert-butyl dicarbonate (0.75 mmol). Following completion of the reaction via TLC, the crude product was purified via flash column chromatography. Boc 3.19 was obtained as a clear oil (73% yield).

¹H NMR (400 MHz, CDCl₃) δ 7.75 - 7.39 (m, 10H), 7.15 (d, 2H, J = 8.0 Hz), 7.11 (s, 1H), 4.78 - 4.42 (m, 5H), 4.00 - 3.85 (m, 4H), 2.57 (t, 2H, J = 7.8 Hz), 2.40 (br s, 2H), 1.87 - 1.11 (m, 26H), 0.84 (t, 3H, J = 6.8 Hz); ¹³C NMR (100 MHz, CDCl₃) δ 171.6 and 171.4 (rotamers), 167.7, 155.6 and 155.5 (rotamers), 147.6, 146.8, 136.1 and 135.4 (rotamers), 132.9, 132.5 and 132.3 (rotamers), 131.0, 128.3, 127.8, 127.6, 127.4, 127.0, 126.8, 126.6, 125.8, 125.5, 121.5, 80.4, 60.7, 59.4 and 58.9 (rotamers), 51.6 and 51.2 (rotamers), 49.9 and
49.5 (rotamers), 49.0, 39.7, 35.5, 31.1, 30.6, 29.9 and 29.2 (rotamers), 28.7 and 28.4 (rotamers), 28.1, 25.9, 25.0, 22.2, 13.8, 13.7; IR (KBr) ν(cm⁻¹) 3270, 2926, 1733, 1686, 1155; λ_max = 226 nm; HRMS (ESI): m/z: Calcd for C_{41}H_{44}N_{5}O_{5}: 698.4276 [M+H]^+, found: 698.4274.

**Ethyl 2-(tert-butoxycarbonyl(4-isopropylbenzyl)amino)-7-(1-(2-(4-pentylbenzamido)ethyl)-1H-1,2,3-triazol-4-yl)heptanoate (Boc 3.20):** was synthesized as described in the general procedure using amine 3.20 (0.25 mmol), triethylamine (0.33 mmol), di-tert-butyl dicarbonate (0.75 mmol). Following completion of the reaction via TLC, the crude product was purified via flash column chromatography. Boc 3.20 was obtained as a clear oil (70% yield).

^1H NMR (300 MHz, CDCl₃) δ 7.67 (d, 2H, J = 8.1 Hz), 7.28 - 7.13 (m, 7H), 6.95 (br s, 1H), 4.57 - 4.42 (m, 4H), 3.98 - 3.92 (m, 4H), 3.71 (s, 1H), 2.86 (sp, 1H, J = 6.9 Hz), 2.65 - 2.59 (m, 4H), 1.98 - 1.13 (m, 32H), 0.88 (t, 3H, J = 6.8 Hz); ^13C NMR (75 MHz, CDCl₃) δ 171.6, 167.8, 155.7 and 155.5 (rotamers), 147.9, 147.7 and 147.5 (rotamers), 147.0, 136.0 and 135.2 (rotamers), 131.0, 128.4, 127.5, 127.0, 126.0, 121.6, 80.3, 66.9, 60.7, 59.4 and 58.7 (rotamers), 50.7 and 49.5 (rotamers), 49.2, 39.8, 35.6, 33.6, 31.3, 30.8, 29.0, 28.8 and 28.6 (rotamers), 28.2, 26.1 and 26.0 (rotamers), 25.3, 23.9, 22.3, 13.9, 13.8; IR (KBr) ν(cm⁻¹)
3419, 2921, 1713, 1686, 1161; \( \lambda_{\text{max}} = 203 \) nm, 220; HRMS (ESI): \( m/z \): Calcd for C_{40}H_{59}N_{5}O_{5}: 690.4589 [M+H]^+, found: 690.4605.

![Chemical structure](image)

**Ethyl 2-(tert-butoxycarbonyl(4-butylbenzyl)amino)-7-(1-(2-(4-pentylbenzamido)ethyl)-1H-1,2,3-triazol-4-yl)heptanoate (Boc 3.21):** was synthesized as described in the general procedure using amine 3.21 (0.25 mmol), triethylamine (0.33 mmol), di-tert-butyl dicarbonate (0.75 mmol). Following completion of the reaction via TLC, the crude product was purified via flash column chromatography. **Boc 3.21** was obtained as a clear oil (81% yield).

\(^1\)H NMR (400 MHz, CDCl\(_3\)) \( \delta \) 7.70 (d, 2H, \( J = 7.6 \) Hz) 7.42 (br s, 1H), 7.19 - 7.14 (m, 5H), 7.07 (d, 2H, \( J = 7.6 \) Hz) 4.53 - 4.19 (m, 5H), 4.05 - 3.86 (m, 4H), 2.62 - 2.54 (m, 6H), 1.91 - 1.13 (m, 30H), 0.89 - 0.84 (m, 6H); \(^{13}\)C NMR (100 MHz, CDCl\(_3\)) \( \delta \) 171.7 and 171.6 (rotamers), 167.8, 155.7 and 155.5 (rotamers), 147.9, 147.0, 141.7 and 141.5 (rotamers), 135.8 and 135.0 (rotamers), 131.0, 128.4, 128.1, 127.3, 127.0, 121.6, 80.3, 60.7, 59.3 and 58.8 (rotamers), 50.7 and 49.5 (rotamers), 49.2, 39.8, 35.6, 35.1, 33.5, 31.2, 30.7, 30.0, 29.2 and 29.0 (rotamers), 28.7 and 28.5 (rotamers), 28.1, 26.1 and 25.9 (rotamers), 25.2, 22.3,
22.1, 13.9, 13.8, 13.7; IR (KBr) ν(cm⁻¹) 3422, 2932, 1686, 1634, 1165; λ_max = 206 nm, 221 nm; HRMS (ESI): m/z: Calcd for C_{41}H_{61}N_{5}O_{5}: 704.4745 [M+H]^+, found: 704.4758.

![Chemical structure](image)

**Ethyl 2-(tert-butoxycarbonyl(4-chlorobenzyl)amo)-7-(1-(2-(4-pentylbenzamido)ethyl)-1H-1,2,3-triazol-4-yl)heptanoate (Boc 3.22):** was synthesized as described in the general procedure using amine 3.22 (0.25 mmol), triethylamine (0.33 mmol), di-tert-butyl dicarbonate (0.75 mmol). Following completion of the reaction via TLC, the crude product was purified via flash column chromatography. **Boc 3.22** was obtained as a clear oil (74% yield).

^1^H NMR (400 MHz, CDCl₃) δ 7.69 (d, 2H, J = 8.0 Hz), 7.42 - 7.06 (m, 8H), 4.60 (t, 2H, J = 5.6 Hz), 4.50 - 3.95 (m, 7H), 2.68 - 2.61 (m, 4H), 1.72 - 1.18 (m, 26H), 0.89 (t, 3H, J = 7.0 Hz); ^1^C NMR (100 MHz, CDCl₃) δ 171.4 and 171.2 (rotamers), 167.7, 155.4, 147.6, 146.8, 137.4 and 136.6 (rotamers), 132.6 and 132.3 (rotamers), 131.0, 129.5, 128.2, 128.0, 127.0, 121.6, 80.5, 60.7, 59.6 and 58.8 (rotamers), 50.1, 49.0, 39.7, 35.5, 31.1, 30.6, 29.9, 29.2, 28.9 and 28.5 (rotamers), 28.0, 26.0, 25.1, 22.2, 13.8, 13.7; IR (KBr) ν(cm⁻¹) 3308, 2918, 1728, 1634, 732; λ_max = 227 nm; HRMS (ESI): m/z: Calcd for C_{37}H_{52}ClN_{5}O_{5}: 682.373 [M+H]^+, found: 682.3719.
Ethyl 2-((4-bromobenzyl)(tert-butoxy carbonyl) amino)-7-(1-(2-(4-pentylbenzamido)ethyl)-1H-1,2,3-triazol-4-yl)heptanoate (Boc 3.23): was synthesized as described in the general procedure using amine 3.23 (0.25 mmol), triethylamine (0.33 mmol), di-tert-butyl dicarbonate (0.75 mmol). Following completion of the reaction via TLC, the crude product was purified via flash column chromatography. Boc 3.23 was obtained as a clear oil (79% yield).

$^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 7.68 (d, 2H, $J = 8.0$ Hz), 7.51 (br s, 1H), 7.36 - 7.33 (m, 4H), 7.15 - 7.09 (m, 3H), 4.51 - 3.86 (m, 9H), 2.58 - 2.51 (m, 4H), 1.87 - 1.12 (m, 26H), 0.83 (t, 3H, $J = 6.6$ Hz); $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 171.5 and 171.2 (rotamers), 167.8, 155.4, 147.7, 146.9, 137.9 and 137.1 (rotamers), 131.0, 129.9, 128.8, 128.3, 127.0, 121.6, 120.8 and 120.4 (rotamers), 80.5, 60.8, 59.7 and 58.9 (rotamers), 50.2, 49.1, 39.7, 35.5, 31.2, 30.6, 29.9, 29.4, 28.9 and 28.6 (rotamers), 28.0, 26.0 and 25.9 (rotamers), 25.2, 22.2, 13.9, 13.8; IR (KBr) $\nu$(cm$^{-1}$) 3318, 2937, 1686, 1161, 492; $\lambda_{max}$ = 202 nm, 226 nm; HRMS (ESI): $m/z$: Calcd for C$_{37}$H$_{52}$BrN$_5$O$_5$: 726.3225 [M+H]$^+$, found: 726.3215.
Ethyl 2-(tert-butoxycarbonyl(4-propoxybenzyl)amino)-7-(1-(2-(4-pentylbenzamido)ethyl)-1H-1,2,3-triazol-4-yl)heptanoate (Boc 3.24): was synthesized as described in the general procedure using amine 3.24 (0.25 mmol), triethylamine (0.33 mmol), di-tert-butyl dicarbonate (0.75 mmol). Following completion of the reaction via TLC, the crude product was purified via flash column chromatography. Boc 3.24 was obtained as a clear oil (66% yield).

$^1$H NMR (400 MHz, CDCl$_3$) δ 7.68 (d, 2H, $J = 8.4$ Hz), 7.28 (d, 2H, $J = 6.8$ Hz), 7.22 - 7.05 (m, 4H), 6.81 (d, 2H, $J = 8.4$ Hz), 4.56 - 3.88 (m, 11H), 2.64 - 2.60 (m, 4H), 1.79 - 1.16 (m, 28H), 1.00 (t, 3H, $J = 7.6$ Hz), 0.88 (t, 3H, $J = 7.0$ Hz); $^{13}$C NMR (100 MHz, CDCl$_3$) δ 171.6, 167.8, 158.3, 155.5, 147.9, 147.0, 131.0, 129.7, 128.6, 128.4, 127.0, 121.7, 114.1, 80.4 and 80.3 (rotamers), 69.4, 60.7, 59.2 and 58.8 (rotamers), 51.7, 50.5, 49.2, 39.7, 35.6, 31.2, 30.7, 29.0 and 28.7 (rotamers), 28.5, 28.2, 26.1, 25.2, 22.4, 22.3, 13.9, 13.8, 10.3; IR (KBr) $\nu$(cm$^{-1}$) 3459, 2942, 1697, 1514, 1249; $\lambda_{\text{max}}$ = 205 nm, 227 nm; HRMS (ESI): $m/z$: Calcd for C$_{40}$H$_{59}$N$_5$O$_6$: 706.4538 [M+H]$^+$, found: 706.4541.
Ethyl 2-((tert-butoxycarbonyl)(4-tert-butylbenzyl)amino)-7-((1-(2-((4-pentylbenzamido)ethyl)-1H-1,2,3-triazol-4-yl)heptan-1yl)heptanoate (Boc 3.25): was synthesized as described in the general procedure using amine 3.25 (0.25 mmol), triethylamine (0.33 mmol), di-tert-butyl dicarbonate (0.75 mmol). Following completion of the reaction via TLC, the crude product was purified via flash column chromatography. Boc 3.25 was obtained as a clear oil (62% yield).

$^1$H NMR (400 MHz, CDCl$_3$) δ 7.68 (d, 2H, $J = 8.4$ Hz), 7.30 - 7.18 (m, 8H), 4.53 - 4.18 (m, 5H), 4.08 - 3.86 (m, 4H), 2.62 - 2.56 (m, 4H), 1.92 - 1.11 (m, 26H), 0.86 (t, 3H, $J = 6.8$ Hz);

$^{13}$C NMR (100 MHz, CDCl$_3$) δ 171.6, 167.8, 155.9 and 155.7 (rotamers), 149.9, 148.0, 147.1, 135.6 and 134.9 (rotamers), 131.1, 128.5, 127.3, 127.0, 125.0, 121.7, 80.4, 60.7, 59.4 and 58.8 (rotamers), 51.7 and 50.6 (rotamers), 49.2, 39.8, 35.7, 34.3, 31.3, 31.2, 30.8, 30.0, 29.1, 28.8 and 28.6 (rotamers), 28.2, 26.2 and 26.0 (rotamers), 25.3, 22.4, 13.9, 13.8; IR (KBr) ν(cm$^{-1}$) 3374, 2926, 1692, 1161; $\lambda_{max} = 204$ nm, 219 nm; HRMS (ESI): $m/z$: Calcd for C$_{41}$H$_{61}$N$_5$O$_5$: 704.4745 [M+H]$^+$, found: 704.4739.
Ethyl 2-(tert-butoxycarbonyl(4-ethynylbenzyl)amino)-7-(1-(2-(4-pentylbenzamido)ethyl)-1H-1,2,3-triazol-4-yl)heptanoate (Boc 3.26): was synthesized as described in the general procedure using amine 3.26 (0.25 mmol), triethylamine (0.33 mmol), di-tert-butyl dicarbonate (0.75 mmol). Following completion of the reaction via TLC, the crude product was purified via flash column chromatography. Boc 3.26 was obtained as a clear oil (75% yield).

$^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 7.68 (d, 2H, $J = 8.0$ Hz), 7.40 (d, 2H, $J = 8.0$ Hz), 7.29 - 7.13 (m, 6H), 4.56 - 3.91 (m, 9H), 3.07 (s, 1H), 2.63 - 2.57 (m, 4H), 1.91 - 1.16 (m, 26H), 0.87 (t, 3H, $J = 6.8$ Hz); $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 171.7, 167.8, 155.8, 148.0, 147.1, 140.0 and 139.2 (rotamers), 131.9, 131.1, 128.5, 128.2, 127.0, 121.8, 120.7, 83.5, 80.7, 60.9, 59.8 and 59.0 (rotamers), 51.8 and 51.0 (rotamers), 49.3, 39.8, 35.7, 31.3, 30.8, 30.1, 29.4, 29.0, 28.6, 28.2, 26.0, 25.3, 22.4, 14.0, 13.9; IR (KBr) $\nu$(cm$^{-1}$) 3296, 2926, 2358, 1697, 1165; $\lambda_{\text{max}}$ = 203 nm, 231 nm; HRMS (ESI): $m/z$: Calcd for C$_{39}$H$_{53}$N$_5$O$_5$: 694.3939 [M+Na]$^+$, found: 694.3933.
**Ethyl 2-((biphenyl-4-ylmethyl)(tert-butoxycarbonyl)amino)-7-(1-(2-(4-pentylbenzamido)ethyl)-1H-1,2,3-triazol-4-yl)heptanoate (Boc 3.27):** was synthesized as described in the general procedure using amine 3.27 (0.25 mmol), triethylamine (0.33 mmol), di-tert-butyl dicarbonate (0.75 mmol). Following completion of the reaction via TLC, the crude product was purified via flash column chromatography. **Boc 3.27** was obtained as a clear oil (63% yield).

$^1$H NMR (400 MHz, CDCl$_3$) δ 7.67 (d, 2H, $J = 8.0$ Hz), 7.57 - 7.12 (m, 11H), 6.98 (br s, 1H), 4.65 - 4.28 (m, 5H), 4.08 - 3.90 (m, 4H), 2.64 - 2.56 (m, 4H), 1.95 - 1.18 (m, 26H), 0.88 (t, 3H, $J = 7.0$ Hz); $^{13}$C NMR (100 MHz, CDCl$_3$) δ 171.9, 167.8, 155.8, 148.1, 147.2, 140.7 and 139.7 (rotamers), 137.7 and 137.3 (rotamers), 131.1, 128.9, 128.7, 128.6, 127.8, 127.2, 127.0, 126.9, 126.7, 121.6, 80.6, 60.9, 59.8 and 59.1 (rotamers), 50.8 and 49.9 (rotamers), 49.3, 39.7, 35.7, 31.3, 30.8, 30.1, 29.1, 28.8, 28.3, 26.2, 25.4, 22.4, 14.0, 13.9; IR (KBr) $\nu$(cm$^{-1}$) 3380, 2931, 1541, 1159; $\lambda_{\text{max}}$ = 204 nm, 244 nm; HRMS (ESI): $m/z$: Calcd for C$_{43}$H$_{57}$N$_5$O$_5$: 724.4432 [M+H]$^+$, found: 724.4417.
Ethyl 2-(tert-butoxycarbonyl(4-cyanobenzyl)amino)-7-(1-(2-(4-pentylbenzamido)ethyl)-1H-1,2,3-triazol-4-yl)heptanoate (Boc 3.28): was synthesized as described in the general procedure using amine 3.28 (0.25 mmol), triethylamine (0.33 mmol), di-tert-butyl dicarbonate (0.75 mmol). Following completion of the reaction via TLC, the crude product was purified via flash column chromatography. Boc 3.28 was obtained as a clear oil (90% yield).

$^1$H NMR (300 MHz, CDCl$_3$) δ 7.68 (d, 2H, $J = 8.4$ Hz), 7.59 (d, 2H, $J = 8.1$ Hz), 7.42 (br s, 2H), 7.31 (s, 1H), 7.22 (d, 2H, $J = 8.4$ Hz), 6.98 (br s, 1H), 4.62 - 3.95 (m, 9H), 2.65 - 2.60 (m, 4H), 1.93 - 1.18 (m, 26H), 0.88 (t, 3H, $J = 6.8$ Hz) ; $^{13}$C NMR (100 MHz, CDCl$_3$) δ 171.5 and 171.1 (rotamers), 167.7, 155.4, 147.7, 146.9, 144.9 and 144.0 (rotamers), 132.3, 131.0, 128.3, 127.4, 126.9, 121.6, 118.6, 110.6 and 110.3 (rotamers), 80.9, 60.9, 60.1 and 58.9 (rotamers), 51.8 and 50.3 (rotamers), 49.1, 39.7, 35.5, 31.2, 30.6, 29.9 and 29.4 (rotamers), 28.9, 28.5, 28.0, 26.1 and 25.9 (rotamers), 25.2, 22.2, 13.9, 13.8; IR (KBr) ν(cm$^{-1}$) 3422, 2932, 2234, 1640, 1161; $\lambda_{\text{max}}$ = 202 nm, 232 nm; HRMS (ESI): $m/z$: Calcd for C$_{38}$H$_{52}$N$_6$O$_5$: 673.4072 [M+H]$^+$, found: 673.0471.
Ethyl 2-(tert-butoxycarbonyl(3,4-diethoxybenzyl)amino)-7-(1-(2-(4-pentylenzamido)ethyl)-1H-1,2,3-triazol-4-yl)heptanoate (Boc 3.29): was synthesized as described in the general procedure using amine 3.29 (0.25 mmol), triethylamine (0.33 mmol), di-tert-butyl dicarbonate (0.75 mmol). Following completion of the reaction via TLC, the crude product was purified via flash column chromatography. Boc 3.29 was obtained as a clear oil (68% yield).

$^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 7.67 (d, 2H, $J = 8.0$ Hz), 7.42 (br s, 1H), 7.28 (s, 1H), 7.14 (d, 2H, $J = 8.0$ Hz), 6.90 - 6.74 (m, 3H), 4.50 - 3.82 (m, 13H), 2.58 - 2.53 (m, 4H), 1.91 - 1.11 (m, 32H), 0.83 (t, 3H, $J = 6.8$ Hz); $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 171.5, 167.7, 155.5, 148.4 and 147.6 (rotamers), 147.8, 146.9, 131.3 and 130.6 (rotamers), 131.0, 128.3, 127.0, 121.7, 120.8, 119.8, 113.8, 112.9, 80.2, 64.4, 64.2, 60.7, 59.2 and 58.9 (rotamers), 50.7 and 49.6 (rotamers), 49.1, 39.7, 35.6, 31.2, 30.7, 30.0, 28.9, 28.6 and 28.5 (rotamers), 28.1, 26.1, 25.2, 22.3, 14.6, 14.6, 13.9, 13.8; IR (KBr) $\nu$(cm$^{-1}$) 3396, 2925, 1686, 1554; $\lambda_{max}$ = 210 nm, 230 nm; HRMS (ESI): $m/z$: Calcd for C$_{41}$H$_{61}$N$_5$O$_7$: 736.4644 [M+H]$^+$, found: 736.4641.
Ethyl 2-(tert-butoxycarbonyl(4-(hexyloxy)benzyl)amino)-7-(1-(2-(4-pentylbenzamido)ethyl)-1H-1,2,3-triazol-4-yl)heptanoate (Boc 3.30): was synthesized as described in the general procedure using amine 3.30 (0.25 mmol), triethylamine (0.33 mmol), di-tert-butyl dicarbonate (0.75 mmol). Following completion of the reaction via TLC, the crude product was purified via flash column chromatography. Boc 3.30 was obtained as a clear oil (88% yield).

$^1$H NMR (300 MHz, CDCl$_3$) δ 7.69 (d, 2H, $J = 8.4$ Hz), 7.32 - 7.08 (m, 6H), 6.81 (d, 2H, $J = 8.4$ Hz), 4.59 - 3.89 (m, 11H), 2.66 - 2.60 (m, 4H), 1.97 - 1.16 (m, 34H), 0.92 - 0.86 (m, 6H); $^{13}$C NMR (100 MHz, CDCl$_3$) δ 171.5, 167.8, 158.2 and 158.0 (rotamers), 155.4, 147.6, 146.9, 131.0, 130.3 and 129.6 (rotamers), 128.5, 128.3, 127.0, 121.8, 114.0, 80.2, 67.8, 60.6, 59.1 and 58.8 (rotamers), 50.4 and 49.4 (rotamers), 49.2, 39.7, 35.6, 31.4, 31.2, 30.7, 30.0, 29.0, 28.9, 28.6 and 28.5 (rotamers), 28.1, 26.0, 25.5, 25.1, 22.4, 22.3, 13.9, 13.8, 13.7; IR (KBr) ν(cm$^{-1}$) 3389, 2922, 1718, 1499, 1218; $\lambda_{max} = 228$ nm; HRMS (ESI): m/z: Calcd for C$_{43}$H$_{65}$N$_3$O$_6$: 747.5008 [M+H]$^+$, found: 747.5006.
Ethyl 2-(tert-butoxycarbonyl(3,4-dichlorobenzyl)amino)-7-(1-(2-(4-pentylbenzamido)ethyl)-1H-1,2,3-triazol-4-yl)heptanoate (Boc 3.31): was synthesized as described in the general procedure using amine 3.31 (0.25 mmol), triethylamine (0.33 mmol), di-tert-butyl dicarbonate (0.75 mmol). Following completion of the reaction via TLC, the crude product was purified via flash column chromatography. Boc 3.31 was obtained as a clear oil (76% yield).

$^1$H NMR (400 MHz, CDCl$_3$) δ 7.70 (d, 2H, $J$ = 8.0 Hz), 7.45 - 7.07 (m, 7H), 4.58 - 3.88 (m, 9H), 2.62 - 2.57 (m, 4H), 1.60 - 1.16 (m, 26H), 0.85 (t, 3H, $J$ = 6.8 Hz); $^{13}$C NMR (100 MHz, CDCl$_3$) δ 171.4 and 171.1 (rotamers), 167.8, 155.4, 147.4, 147.0, 139.4 and 138.6 (rotamers), 132.0, 130.9, 129.9, 129.0, 128.4, 127.5, 127.1, 126.4, 122.2, 80.9, 61.0, 59.9 and 59.0 (rotamers), 49.8 and 48.6 (rotamers), 49.6, 39.7, 35.6, 31.2, 30.7, 29.9 and 29.4 (rotamers), 28.9, 28.7 and 28.5 (rotamers), 28.1, 26.0, 25.0, 22.3, 13.9, 13.8; IR (KBr) ν(cm$^{-1}$) 3334, 2922, 1672, 761; $\lambda_{\text{max}}$ = 204 nm, 222 nm; HRMS (ESI): m/z: Calcd for C$_{37}$H$_{51}$Cl$_2$N$_5$O$_5$: 716.3340 [M+H]$^+$, found: 716.3347.

Methyl 2-(tert-butoxycarbonyl(4-butylbenzyl)amino)acetate (Boc 3.32): was synthesized as described in the general procedure using methyl 2-(4-butylbenzylamino)acetate (0.25
mmol), triethylamine (0.33 mmol), di-tert-butyl dicarbonate (0.75 mmol). Following completion of the reaction via TLC, the crude product was purified via flash column chromatography. **3.32** was obtained as a clear oil (91% yield).

$^1$H NMR (400 MHz, CDCl$_3$) δ 7.16 - 7.14 (m, 4H), 4.51 and 4.48 (2 x s, 2H, rotamers), 3.92 and 3.78 (2 x s, 2H, rotamers), 3.71 (s, 3H), 2.59 (t, 2H, $J$ = 7.6 Hz), 1.60 - 1.33 (m, 13H), 0.93 (t, 3H, $J$ = 7.4 Hz); $^{13}$C NMR (100 MHz, CDCl$_3$) δ 170.5 and 170.4 (rotamers), 155.8 and 155.5 (rotamers), 142.1, 134.6 and 134.4 (rotamers), 128.6, 128.1 and 127.5 (rotamers), 80.5 and 80.3 (rotamers), 51.9 and 51.8 (rotamers), 51.1 and 50.6 (rotamers), 47.7 and 47.2 (rotamers), 35.2, 33.6, 28.3 and 28.2 (rotamers), 22.3, 13.9; IR (KBr) ν(cm$^{-1}$) 3458, 2932, 1759, 1187; $\lambda_{\text{max}}$ = 220 nm, 262 nm; HRMS (ESI): $m/z$: Calcd for C$_{19}$H$_{29}$NNaO$_4$: 358.1989 [M+Na]$^+$, found: 358.1987.

![Chemical Structure](image)

tert-Butyl benzyl(1-(methoxy(methyl)amino)-1-oxo-7-(1-(2-(4-pentylbenzamido)ethyl)-1H-1,2,3-triazol-4-yl)heptan-2-yl)carbamate (Amide 3.18): was synthesized as described in the general procedure using Boc 3.18 (0.20 mmol), O,N-dimethylhydroxylamine hydrochloride (1.00 mmol), and 2.0$M$ isopropylmagnesium chloride solution in tetrahydrofuran (1.6 mmol). Upon completion of the reaction via TLC analysis, the crude product was purified via flash column chromatography. **Amide 3.18** was obtained as a clear oil (71% yield).
**1H NMR (400 MHz, CDCl$_3$) $\delta$ 7.68 (d, 2H, $J$ = 8.0 Hz), 7.53 (br s, 1H), 7.26 - 7.11 (m, 8H), 5.18 and 4.87 (2 x br s, 1H, rotamers), 4.54 - 4.27 (m, 4H), 3.87 - 3.83 (m, 2H), 3.63 and 3.54 (2 x br s, 3H, rotamers), 2.92 and 2.78 (2 x br s, 3H, rotamers), 2.59 - 2.52 (m, 4H), 1.77 - 1.18 (m, 23H), 0.83 (t, 3H, $J$ = 6.8 Hz); $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 171.9 and 170.9 (rotamers), 167.8, 155.7, 147.8, 146.9, 139.5 and 138.7 (rotamers), 131.0, 128.3, 127.8, 127.0, 126.7, 126.5 and 126.3 (rotamers), 121.6, 80.0, 61.5, 54.5 and 53.5 (rotamers), 49.0, 47.0 and 46.4 (rotamers), 39.8, 35.6, 31.7, 31.2, 30.7, 29.2 and 28.9 (rotamers), 28.6, 28.2, 28.0, 25.5, 25.2, 22.3, 13.8; IR (KBr) $\nu$(cm$^{-1}$) 3437, 2932, 1660, 1160; $\lambda_{\text{max}}$ = 235 nm; HRMS (ESI): $m/z$: Calcd for C$_{37}$H$_{54}$N$_6$O$_5$: 663.4228 [M+H]$^+$, found: 663.423.

**tert-Butyl 1-(methoxy(methyl)amino)-1-oxo-7-(1-(2-(4-pentylbenzamido)ethyl)-1H-1,2,3-triazol-4-yl)heptan-2-yl(naphthalen-2-ylmethyl)carbamate (Amide 3.19):** was synthesized as described in the general procedure using Boc 3.19 (0.20 mmol), O,N-dimethylhydroxylamine hydrochloride (1.00 mmol), and 2.0M isopropylmagnesium chloride solution in tetrahydrofuran (1.6 mmol). Upon completion of the reaction via TLC analysis, the crude product was purified via flash column chromatography. **Amide 3.19** was obtained as a clear oil (63% yield).
\(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 7.73 - 7.32 (m, 10H), 7.17 - 7.14 (m, 3H), 5.28 and 4.89 (2 x br s, 1H, rotamers), 4.72 - 4.47 (m, 4H), 3.89 - 3.84 (m, 2H), 3.68 and 3.56 (2 x br s, 3H, rotamers), 2.91 and 2.75 (2 x br s, 3H, rotamers), 2.58 (t, 2H, \(J = 7.8\) Hz), 2.49 (t, 2H, \(J = 7.4\) Hz), 1.80 - 1.16 (m, 23H), 0.85 (t, 3H, \(J = 6.8\) Hz); \(^{13}\)C NMR (100 MHz, CDCl\(_3\)) \(\delta\) 171.9 and 171.0 (rotamers), 167.7, 155.8, 147.8, 146.9, 137.0 and 136.4 (rotamers), 133.0, 132.2, 131.0, 128.3, 127.4, 127.0, 126.1, 126.0, 125.7, 125.6, 125.2, 125.1, 121.6, 80.1, 61.5, 54.9 and 53.6 (rotamers), 49.0, 47.1 and 46.6 (rotamers), 39.7, 35.6, 31.8, 31.2, 30.7, 29.3, 28.9 and 28.6 (rotamers), 28.3, 28.0, 25.5, 25.1, 22.3, 13.8; IR (KBr) \(\nu (\text{cm}^{-1})\) 3395, 2937, 1689, 1537, 1169; \(\lambda_{\text{max}} = 224\) nm; HRMS (ESI): \(m/z\): Calcd for C\(_{41}\)H\(_{56}\)N\(_6\)O\(_5\): 713.4385 [M+H]\(^+\), found: 713.4381.

\(\text{ tert-Butyl 4-isopropylbenzyl(1-(methoxy(methyl)amino)-1-oxo-7-(1-(2-(4-pentylbenzamido)ethyl)-1H-1,2,3-triazol-4-yl)heptan-2-yl)carbamate (Amide 3.20): }\) was synthesized as described in the general procedure using Boc 3.20 (0.20 mmol), O,N-dimethylhydroxylamine hydrochloride (1.00 mmol), and 2.0\(M\) isopropylmagnesium chloride solution in tetrahydrofuran (1.6 mmol). Upon completion of the reaction via TLC analysis, the crude product was purified via flash column chromatography. Amide 3.20 was obtained as a clear oil (87% yield).
$^1$H NMR (400 MHz, CDCl$_3$)  $\delta$ 7.74 (br s, 1H), 7.70 (d, 2H, $J = 8.4$ Hz), 7.15 - 7.08 (m, 7H), 5.15 and 4.85 (2 x br s, 1H, rotamers), 4.51 - 4.23 (m, 4H), 3.86 - 3.82 (m, 2H), 3.61 and 3.53 (2 x br s, 3H, rotamers), 2.87 - 2.77 (m, 4H), 2.59 - 2.51 (m, 4H), 1.71 - 1.15 (m, 29H), 0.83 (t, 3H, $J = 7.0$ Hz); $^{13}$C NMR (100 MHz, CDCl$_3$)  $\delta$ 171.7 and 170.8 (rotamers), 167.7, 155.6, 147.6, 146.9, 146.7, 136.6 and 135.9 (rotamers), 131.0, 128.2, 127.6, 126.9, 125.6, 121.5, 79.8, 61.3, 54.4 and 53.4 (rotamers), 48.9, 46.6 and 46.0 (rotamers), 39.7, 35.5, 33.4, 31.5, 31.1, 30.6, 29.0, 28.9, 28.5, 28.1, 27.9, 25.4 and 25.1 (rotamers), 23.7, 22.2, 13.7; IR (KBr) $\nu$(cm$^{-1}$) 3416, 2941, 1678, 1541, 1152; $\lambda_{\text{max}}$ = 219 nm; HRMS (ESI): $m/z$: Calcd for C$_{40}$H$_{60}$N$_6$O$_5$: 705.4698 [M+H]$^+$, found: 705.4699.

![Chemical structure](image)

tert-Butyl 4-butylbenzyl(1-(methoxy(methyl)amino)-1-oxo-7-(1-(2-(4-pentylbenzamido)ethyl)-1H-1,2,3-triazol-4-yl)heptan-2-yl)carbamate (Amide 3.21): was synthesized as described in the general procedure using Boc 3.21 (0.20 mmol), O,N-dimethylhydroxylamine hydrochloride (1.00 mmol), and 2.0M isopropylmagnesium chloride solution in tetrahydrofuran (1.6 mmol). Upon completion of the reaction via TLC analysis, the crude product was purified via flash column chromatography. Amide 3.21 was obtained as a clear oil (66% yield).
\(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 7.68 (d, 2H, \(J = 8.0\) Hz), 7.34 - 7.03 (m, 8H), 5.18 and 4.85 (2 x br s, 1H, rotamers), 4.52 (t, 2H, \(J = 5.6\) Hz), 4.45 - 4.20 (m, 2H), 3.92 - 3.88 (m, 2H), 3.63 and 3.55 (2 x br s, 3H, rotamers), 2.92 and 2.82 (2 x br s, 3H, rotamers), 2.62 - 2.51 (m, 6H), 1.62 - 1.22 (m, 27H), 0.89 - 0.84 (m, 6H); \(^1^3\)C NMR (100 MHz, CDCl\(_3\)) \(\delta\) 171.9, 167.8, 155.8, 148.0, 147.0, 141.0, 136.6 and 135.9 (rotamers), 131.1, 128.4, 127.9, 127.0, 126.9, 121.7, 80.0, 61.5, 54.5 and 53.6 (rotamers), 49.2, 46.8 and 46.2 (rotamers), 39.8, 35.7, 35.1, 33.6, 31.8, 31.3, 30.8, 29.2 and 29.0 (rotamers), 28.7, 28.3, 28.1, 25.5, 25.3, 22.4, 22.1, 13.9, 13.8; IR (KBr) \(\nu\) (cm\(^{-1}\)) 3297, 2947, 1692, 1540, 1165; \(\lambda_{\text{max}}\) = 230 nm; HRMS (ESI): \(m/z\): Calcd for C\(_{41}\)H\(_{62}\)N\(_6\)O\(_5\): 719.4854 \([\text{M+H}]^+\), found: 719.4833.

**tert-Butyl 4-chlorobenzyl(1-(methoxy(methyl)amino)-1-oxo-7-(1-(2-(4-pentylbenzamido)ethyl)-1H-1,2,3-triazol-4-yl)heptan-2-yl)carbamate** (Amide 3.22): was synthesized as described in the general procedure using Boc 3.22 (0.20 mmol), O,N-dimethyldihydroxylamine hydrochloride (1.00 mmol), and 2.0\(M\) isopropylmagnesium chloride solution in tetrahydrofuran (1.6 mmol). Upon completion of the reaction via TLC analysis, the crude product was purified via flash column chromatography. **Amide 3.22** was obtained as a clear oil (84% yield).
$^1$H NMR (400 MHz, CDCl$_3$) δ 7.68 (d, 2H, $J = 8.4$ Hz), 7.29 - 7.10 (m, 8H), 5.23 and 4.92 (2 x br s, 1H, rotamers), 4.55 (t, 2H, $J = 5.6$ Hz), 4.50 - 4.29 (m, 2H), 3.96 - 3.92 (m, 2H), 3.69 and 3.60 (2 x br s, 3H, rotamers), 2.98 and 2.86 (2 x br s, 3H, rotamers), 2.65 - 2.60 (m, 4H), 1.75 - 1.25 (m, 23H), 0.87 (t, 3H, $J = 7.0$ Hz); $^{13}$C NMR (100 MHz, CDCl$_3$) δ 171.8 and 170.9 (rotamers), 167.7, 155.5, 147.8, 146.9, 137.4 and 136.9 (rotamers), 132.1 and 131.9 (rotamers), 131.1, 129.1, 128.3, 127.8, 127.0, 121.6, 80.2, 61.5 and 61.2 (rotamers), 54.6 and 53.4 (rotamers), 49.1, 46.3 and 45.7 (rotamers), 39.8, 35.6, 31.7, 31.2, 30.7, 29.5 and 29.2 (rotamers), 28.6, 28.2, 28.0, 25.5, 25.2, 22.3, 13.8; IR (KBr) $\nu$(cm$^{-1}$) 3322, 2932, 1697, 1165, 733; $\lambda_{\text{max}}$ = 223 nm; HRMS (ESI): $m/z$: Calcd for C$_{37}$H$_{53}$ClN$_6$NaO$_5$: 719.3658 [M+Na]$^+$, found: 719.3659.

**tert-Butyl 4-bromobenzyl(1-(methoxy(methyl)amino)-1-oxo-7-(1-(2-(4-pentylbenzamido)ethyl)-1H-1,2,3-triazol-4-yl)heptan-2-yl)carbamate (Amide 3.23):** was synthesized as described in the general procedure using Boc 3.23 (0.20 mmol), O,N-dimethylhydroxylamine hydrochloride (1.00 mmol), and 2.0M isopropylmagnesium chloride solution in tetrahydrofuran (1.6 mmol). Upon completion of the reaction via TLC analysis, the crude product was purified via flash column chromatography. **Amide 3.23** was obtained as a clear oil (63% yield).
1H NMR (300 MHz, CDCl₃) δ 7.68 (d, 2H, J = 8.1 Hz), 7.39 - 6.99 (m, 8H), 5.25 and 4.94 (2 x br s, 1H, rotamers), 4.56 (t, 2H, J = 5.4 Hz), 4.49 - 4.23 (m, 2H), 3.98 - 3.93 (m, 2H), 3.69 and 3.61 (2 x br s, 3H, rotamers), 2.99 and 2.88 (2 x br s, 3H, rotamers), 2.67 - 2.60 (m, 4H), 1.82 - 1.25 (m, 23H), 0.88 (t, 3H, J = 6.8 Hz); 13C NMR (100 MHz, CDCl₃) δ 171.9 and 170.9 (rotamers), 167.8, 155.6, 148.0, 147.0, 138.8 and 138.0 (rotamers), 131.1, 130.9, 129.5, 128.5, 127.0, 121.7, 120.4 and 120.0 (rotamers), 80.3, 61.6 and 61.3 (rotamers), 54.3 and 53.4 (rotamers), 49.2, 46.4 and 45.8 (rotamers), 39.8, 35.6, 31.8, 31.3, 30.7, 29.3 and 29.0 (rotamers), 28.7, 28.3, 28.1, 25.5, 25.3, 22.3, 13.9; IR (KBr) ν(cm⁻¹) 3354, 2932, 1655, 1171, 749; λ_max = 226 nm; HRMS (ESI): m/z: Calcd for C₃₇H₅₃BrN₆O₅: 741.3334 [M+H]^+, found: 741.3332.

![Chemical Structure](image)

**tert-Butyl 1-(methoxy(methyl)amino)-1-oxo-7-(1-(2-(4-pentylbenzamido)ethyl)-1H-1,2,3-triazol-4-yl)heptan-2-yl(4-propoxybenzyl)carbamate (Amide 3.24):** was synthesized as described in the general procedure using Boc 3.24 (0.20 mmol), O,N-dimethylhydroxylamine hydrochloride (1.00 mmol), and 2.0M isopropylmagnesium chloride solution in tetrahydrofuran (1.6 mmol). Upon completion of the reaction via TLC analysis,
the crude product was purified via flash column chromatography. **Amide 3.24** was obtained as a clear oil (59% yield).

$^1$H NMR (300 MHz, CDCl$_3$) δ 7.69 (d, 2H, $J = 8.1$ Hz), 7.29 - 7.11 (m, 6H), 6.79 (d, 2H, $J = 8.4$ Hz), 5.20 and 4.89 (2 x br s, 1H, rotamers), 4.55 (t, 2H, $J = 5.6$ Hz), 4.48 - 4.39 (m, 2H), 4.0 - 3.85 (m, 4H), 3.64 and 3.55 (2 x br s, 3H, rotamers), 3.00 and 2.88 (2 x br s, 3H, rotamers), 2.65 - 2.60 (m, 4H), 1.81 - 1.25 (m, 25H), 1.00 (t, 3H, $J = 7.4$ Hz), 0.88 (t, 3H, $J = 6.9$ Hz) ; $^{13}$C NMR (100 MHz, CDCl$_3$) δ 172.2 and 171.8 (rotamers), 168.1, 158.1, 156.1, 148.3, 147.3, 131.7 and 131.4 (rotamers), 129.6, 128.8, 128.6, 127.4, 122.0, 114.2, 80.3, 69.7, 61.8 and 61.5 (rotamers), 55.0 and 53.9 (rotamers), 49.5, 46.7 and 46.3 (rotamers), 40.1, 36.0, 32.2, 31.6, 31.1, 29.9, 29.5 and 29.4 (rotamers), 29.0, 28.5, 25.9, 25.6, 22.8, 22.7, 14.2, 10.7; IR (KBr) ν (cm$^{-1}$) 3318, 2932, 1676, 1241; $\lambda_{\text{max}} = 226$ nm; HRMS (ESI): $m/z$: Calcd for C$_{40}$H$_{60}$N$_6$O$_6$: 721.4647 [M+H]$^+$, found: 721.4639.

![Chemical Structure](image)

tert-Butyl 4-tert-butylbenzyl(1-(methoxy(methyl)amino)-1-oxo-7-(1-(2-(4-pentylbenzamido)ethyl)-1H-1,2,3-triazol-4-yl)heptan-2-yl)carbamate (Amide 3.25): was synthesized as described in the general procedure using **Boc 3.25** (0.20 mmol), O,N-dimethylhydroxylamine hydrochloride (1.00 mmol), and 2.0$M$ isopropylmagnesium chloride solution in tetrahydrofuran (1.6 mmol). Upon completion of the reaction via TLC analysis,
the crude product was purified via flash column chromatography. **Amide 3.25** was obtained as a clear oil (88% yield).

**H NMR (400 MHz, CDCl$_3$) δ 7.69 (d, 2H, $J = 8.0$ Hz), 7.32 - 7.17 (m, 8H), 5.18 and 4.76 (2 x br s, 1H, rotamers), 4.57 (t, 2H, $J = 5.6$ Hz), 4.49 - 4.27 (m, 2H), 3.98 - 3.93 (m, 2H), 3.65 and 3.57 (2 x br s, 3H, rotamers), 2.91 and 2.82 (2 x br s, 3H, rotamers), 2.67 - 2.61 (m, 2H), 1.76 - 1.28 (m, 32H), 0.89 (t, 3H, $J = 7.0$ Hz); $^{13}$C NMR (100 MHz, CDCl$_3$) δ 171.9 and 171.0 (rotamers), 167.8, 155.8, 149.3, 148.0, 147.0, 136.4 and 135.7 (rotamers), 131.1, 128.4, 127.5, 127.0, 124.7, 121.7, 80.0, 61.5, 54.7 and 53.6 (rotamers), 49.2, 46.7 and 46.1 (rotamers), 39.8, 35.7, 34.2, 31.8, 31.3, 31.2, 29.6, 29.2 and 29.0 (rotamers), 28.7, 28.3, 28.1, 25.5, 25.3, 22.4, 13.9; IR (KBr) $\nu$(cm$^{-1}$) 3429, 2921, 1686, 1452; $\lambda_{\text{max}} = 228$ nm; HRMS (ESI): $m/z$: Calcd for C$_{41}$H$_{62}$N$_6$O$_5$: 719.4854 [M+H]$^+$, found: 719.4837.

![Structure of Amide 3.25](image)

tert-Butyl 4-ethynylbenzyl(1-(methoxy(methyl)amino)-1-oxo-7-(1-(2-(4-pentylbenzamido)ethyl)-1H-1,2,3-triazol-4-yl)heptan-2-yl)carbamate (Amide 3.26): was synthesized as described in the general procedure using **Boc 3.26** (0.20 mmol), $O,N$-dimethylhydroxylamine hydrochloride (1.00 mmol), and 2.0M isopropylmagnesium chloride solution in tetrahydrofuran (1.6 mmol). Upon completion of the reaction via TLC analysis,
the crude product was purified via flash column chromatography. **Amide 3.26** was obtained as a clear oil (52% yield).

$^1$H NMR (300 MHz, CDCl$_3$) δ 7.70 (d, 2H, $J = 7.8$ Hz), 7.39 - 7.15 (m, 8H), 5.23 and 4.88 (2 x br s, 1H, rotamers), 4.62 (t, 2H, $J = 5.1$ Hz), 4.46 (s, 2H), 3.97 - 3.94 (m, 2H), 3.70 and 3.61 (2 x br s, 3H, rotamers), 3.05 (s, 1H), 3.01 and 2.88 (2 x br s, 3H, rotamers), 2.72 - 2.61 (m, 4H), 1.76 - 1.26 (m, 23H), 0.89 (t, 3H, $J = 6.5$ Hz); $^{13}$C NMR (100 MHz, CDCl$_3$) δ 172.0, 167.8, 155.7, 148.0, 147.1, 140.8 and 140.0 (rotamers), 131.7 and 131.1 (rotamers), 128.5, 127.7, 127.0, 126.7, 121.8, 120.0, 83.6, 80.3, 61.7, 54.8 and 53.5 (rotamers), 49.4, 46.9 and 46.2 (rotamers), 39.8, 35.7, 31.9, 31.3, 30.8, 29.6, 29.4, 29.0 and 28.7 (rotamers), 28.3, 28.1, 25.6, 25.3, 22.4, 13.9; IR (KBr) ν(cm$^{-1}$) 3281, 2937, 2322, 1686, 1165; $\lambda_{\text{max}}$ = 238 nm; HRMS (ESI): $m/z$: Calcd for C$_{39}$H$_{54}$N$_6$O$_5$: 687.4228 [M+H]$^+$, found: 687.424.

**tert-Butyl biphenyl-4-ylmethyl(1-(methoxy(methyl)amino)-1-oxo-7-(1-(2-(4-pentylbenzamido)ethyl)-1H-1,2,3-triazol-4-yl)heptan-2-yl)carbamate (Amide 3.27):** was synthesized as described in the general procedure using **Boc 3.27** (0.20 mmol), O,N-dimethylhydroxylamine hydrochloride (1.00 mmol), and 2.0M isopropylmagnesium chloride solution in tetrahydrofuran (1.6 mmol). Upon completion of the reaction via TLC analysis,
the crude product was purified via flash column chromatography. Amide 3.27 was obtained as a clear oil (56% yield).

$^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 7.69 (d, 2H, $J$ = 8.0 Hz), 7.56 - 7.18 (m, 13H), 5.32 an 4.88 (2 x br s, 1H, rotamers), 4.63 - 4.46 (m, 4H), 3.91 - 3.87 (m, 2H), 3.67 and 3.60 (2 x br s, 3H, rotamers), 2.98 and 2.87 (2 x br s, 3H, rotamers), 2.63 - 2.58 (m, 4H), 1.82 - 1.24 (m, 23H), 0.87 (t, 3H, $J$ = 7.0 Hz); $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 172.0, 167.8, 155.8, 147.9, 147.1, 140.7, 139.2, 138.8 and 138.1 (rotamers), 131.1, 128.7, 128.5, 128.2, 127.4, 127.0, 126.8, 126.5, 121.8, 80.2, 61.6 and 61.3 (rotamers), 54.7 and 53.7 (rotamers), 49.3, 46.8 and 46.3 (rotamers), 39.7, 35.7, 31.8, 31.3, 30.8, 29.3, 29.0 and 28.8 (rotamers), 28.4, 28.2, 25.6, 25.3, 22.4, 13.9; IR (KBr) $\nu$(cm$^{-1}$) 3427, 2932, 2120, 1644, 1171; $\lambda_{max}$ = 244 nm; HRMS (ESI): $m/z$: Calcd for C$_{43}$H$_{58}$N$_6$O$_5$: 739.4541 [M+H$^+$], found: 739.4542.

![Structure](image)

tert-Butyl 4-cyanobenzyl(1-(methoxy(methyl)amino)-1-oxo-7-(1-(2-(4-pentylbenzamido)ethyl)-1H-1,2,3-triazol-4-yl)heptan-2-yl)carbamate (Amide 3.28): was synthesized as described in the general procedure using Boc 3.28 (0.20 mmol), O,N-dimethylhydroxylamine hydrochloride (1.00 mmol), and 2.0$M$ isopropylmagnesium chloride solution in tetrahydrofuran (1.6 mmol). Upon completion of the reaction via TLC analysis,
the crude product was purified via flash column chromatography. **Amide 3.28** was obtained as a clear oil (88% yield).

\[^1\]H NMR (300 MHz, CDCl\(_3\))  δ 7.68 (d, 2H, \(J = 8.1\) Hz), 7.57 (d, 2H, \(J = 7.5\) Hz), 7.33 - 7.21 (m, 5H), 7.00 (br s, 1H), 5.28 and 4.97 (2 x br s, 1H, rotamers), 4.60 - 4.37 (m, 4H), 3.99 - 3.94 (m, 2H), 3.74 and 3.66 (2 x br s, 3H, rotamers), 3.01 and 2.86 (2 x br s, 3H, rotamers), 2.69 - 2.61 (m, 4H), 1.81 - 1.26 (m, 23H), 0.89 (t, 3H, \(J = 6.9\) Hz); \[^13\]C NMR (100 MHz, CDCl\(_3\))  δ 171.9 and 170.9 (rotamers), 167.8, 155.5, 147.9, 147.1, 145.7 and 144.7 (rotamers), 131.8, 131.1, 128.5 and 128.0 (rotamers), 127.2, 127.0, 121.7, 118.9, 110.1, 80.6, 61.7 and 61.4 (rotamers), 54.5 and 53.3 (rotamers), 49.2, 46.9 and 46.1 (rotamers), 39.8, 35.6, 31.8, 31.2, 30.7, 29.4, 29.0 and 28.7 (rotamers), 28.3, 28.0, 25.5, 25.3, 22.3, 13.9; IR (KBr) \(\nu(\text{cm}^{-1})\) 3426, 2932, 2223, 1655, 1166; \(\lambda_{\text{max}} = 234\) nm; HRMS (ESI): \(m/z\): Calcd for C\(_{38}\)H\(_{53}\)N\(_7\)O\(_5\): 688.4181 [M+H]\(^+\), found: 688.4175.

tert-Butyl 3,4-diethoxybenzyl(1-(methoxy(methyl)amino)-1-oxo-7-(1-(2-(4-pentylbenzamido)ethyl)-1H-1,2,3-triazol-4-yl)heptan-2-yl)carbamate (Amide 3.29): was synthesized as described in the general procedure using Boc 3.29 (0.20 mmol), O,N-dimethylhydroxylamine hydrochloride (1.00 mmol), and 2.0\(M\) isopropylmagnesium chloride solution in tetrahydrofuran (1.6 mmol). Upon completion of the reaction via TLC analysis,
the crude product was purified via flash column chromatography. **Amide 3.29** was obtained as a clear oil (95% yield).

$^1$H NMR (400 MHz, CDCl$_3$) δ 7.67 (d, 2H, $J = 8.0$ Hz), 7.41 (br s, 1H), 7.15 (d, 2H, $J = 8.0$ Hz), 6.91 - 6.70 (m, 4H), 5.18 and 4.86 (2 x br s, 1H, rotamers), 4.51 (t, 2H, $J = 5.6$ Hz), 4.40 - 3.85 (m, 8H), 3.60 and 3.51 (2 x br s, 3H, rotamers), 2.90 and 2.82 (2 x br s, 3H, rotamers), 2.59 - 2.54 (m, 4H), 1.59 - 1.18 (m, 29H), 0.83 (t, 3H, $J = 6.8$ Hz); $^{13}$C NMR (100 MHz, CDCl$_3$) δ 171.9, 167.7, 155.6, 148.1, 147.8, 147.1, 146.9, 132.2 and 131.7 (rotamers), 131.0, 128.4, 127.0, 121.7, 120.3 and 119.5 (rotamers), 113.3 and 113.0 (rotamers), 112.6, 79.9, 64.4, 64.1 and 64.0 (rotamers), 64.1, 54.5 and 53.6 (rotamers), 49.2, 46.6 and 46.1 (rotamers), 39.7, 35.6, 31.8, 31.2, 30.7, 29.2, 28.9, 28.7, 28.2, 25.5, 25.2, 22.3, 14.7, 14.6, 13.8; IR (KBr) v(cm$^{-1}$) 3378, 2922, 1672, 1233, 1138; $\lambda_{\text{max}}$ = 230 nm; HRMS (ESI): $m$/z: Calcd for C$_{41}$H$_{62}$N$_6$O$_7$: 751.1753 [M+H]$^+$, found: 751.4757.

tert-Butyl 4-(hexyloxy)benzyl(1-(methoxy(methyl)amino)-1-oxo-7-(1-(2-(4-pentylbenzamido)ethyl)-1H-1,2,3-triazol-4-yl)heptan-2-yl)carbamate (Amide 3.30): was synthesized as described in the general procedure using **Boc 3.30** (0.20 mmol), O,N-dimethylhydroxylamine hydrochloride (1.00 mmol), and 2.0M isopropylmagnesium chloride solution in tetrahydrofuran (1.6 mmol). Upon completion of the reaction via TLC analysis,
the crude product was purified via flash column chromatography. **Amide 3.30** was obtained as a clear oil (55% yield).

$^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 7.32 (d, 2H, $J = 7.8$ Hz), 7.42 - 7.11 (m, 6H), 6.79 (d, 2H, $J = 7.8$ Hz), 5.19 and 4.88 (2 x br s, 1H, rotamers), 4.61 (t, 2H, $J = 5.3$ Hz), 4.56 - 4.17 (m, 4H), 3.97 - 3.89 (m, 4H), 3.64 and 3.56 (2 x br s, 3H, rotamers), 2.97 and 2.89 (2 x br s, 3H, rotamers), 2.71 - 2.60 (m, 4H), 1.77 - 1.26 (m, 31H), 0.92 - 0.86 (m, 6H); $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 172.0, 167.8, 157.7, 155.8, 147.9, 147.0, 131.1, 129.2, 128.4, 128.2, 127.0, 121.8, 113.9, 80.0, 67.9, 61.5 and 61.2 (rotamers), 54.3 and 53.6 (rotamers), 49.3, 46.4 and 45.9 (rotamers), 39.8, 35.6, 31.8, 31.5, 31.3, 30.7, 29.2 and 29.0 (rotamers), 29.1, 28.7, 28.2, 28.1, 25.6, 25.5, 25.2, 22.5, 22.3, 13.9, 13.8; IR (KBr) $\nu$(cm$^{-1}$) 3401, 2932, 1644, 1236, 1151; $\lambda_{\text{max}}$ = 229 nm; HRMS (ESI): $m/z$: Calcd for C$_{43}$H$_{66}$N$_6$O$_6$: 763.5117 [M+H]$^+$, found: 763.5105.

**tert-Butyl 3,4-dichlorobenzyl(1-(methoxy(methyl)amino)-1-oxo-7-(1-(2-(4-pentylbenzamido)ethyl)-1H-1,2,3-triazol-4-yl)heptan-2-yl)carbamate (Amide 3.31):** was synthesized as described in the general procedure using Boc 3.31 (0.20 mmol), O,N-dimethylhydroxylamine hydrochloride (1.00 mmol), and 2.0$M$ isopropylmagnesium chloride solution in tetrahydrofuran (1.6 mmol). Upon completion of the reaction via TLC analysis,
the crude product was purified via flash column chromatography. **Amide 3.31** was obtained as a clear oil (79\% yield).

$^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 7.69 (d, 2H, $J = 8.4$ Hz), 7.37 - 7.03 (m, 7H), 5.26 and 4.97 (2 x br s, 1H, rotamers), 4.60 (t, 2H, $J = 5.4$ Hz), 4.41 (s, 2H), 4.05 - 3.97 (m, 2H), 3.72 and 3.65 (2 x br s, 3H, rotamers), 3.04 and 2.95 (2 x br s, 3H, rotamers), 2.72 - 2.61 (m, 4H), 1.82 - 1.26 (m, 23H), 0.89 (t, 3H, $J = 6.8$ Hz); $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 171.8, 167.8, 155.5, 147.7, 147.1, 140.2, 131.9, 131.0, 129.8, 129.4, 128.7, 128.5, 127.0, 126.2, 122.0, 80.6, 61.6, 54.2 and 53.3 (rotamers), 49.5, 46.0 and 45.4 (rotamers), 39.7, 35.7, 31.8, 31.3, 30.8, 29.3, 29.0 and 28.7 (rotamers), 28.3, 28.1, 25.5, 25.1, 22.4, 13.9; IR (KBr) $\nu$ (cm$^{-1}$) 3321, 2926, 1668, 755; $\lambda_{\text{max}}$ = 221 nm; HRMS (ESI): $m/z$: Calcd for $C_{37}H_{52}Cl_2N_7O_5$: 731.3449 [M+H]$^+$, found: 731.3442.

**tert-Butyl 4-butylbenzyl(2-(methoxy(methyl)amino)-2-oxoethyl)carbamate (Amide 3.32):** was synthesized as described in the general procedure using **Boc 3.32** (0.20 mmol), $O,N$-dimethylhydroxylamine hydrochloride (1.00 mmol), and 2.0M isopropylmagnesium chloride solution in tetrahydrofuran (1.6 mmol). Upon completion of the reaction via TLC analysis, the crude product was purified via flash column chromatography. **Amide 3.32** was obtained as a clear oil (77\% yield).

$^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 7.16 - 7.12 (m, 4H), 4.53 and 4.51 (2 x s, 2H, rotamers), 4.08 and 3.95 (2 x s, 2H, rotamers), 3.63 and 3.58 (2 x s, 3H, rotamers), 3.17 (s, 3H), 2.59 (t, 2H, $J = 7.8$ Hz), 1.60 - 1.34 (m, 13H), 0.92 (t, 3H, $J = 7.4$ Hz); $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$
170.4 and 170.1 (rotamers), 156.1 and 155.9 (rotamers), 141.9, 135.1 and 134.9 (rotamers), 128.4, 128.0 and 127.4 (rotamers), 80.1 and 79.9 (rotamers), 61.1 and 61.0 (rotamers), 51.1 and 50.5 (rotamers), 46.7 and 46.5 (rotamers), 35.2, 33.6, 32.3 and 32.2 (rotamers), 28.3 and 28.2 (rotamers), 22.2, 13.8; IR (KBr) ν(cm⁻¹) 3464, 2958, 1671, 1171; λmax = 223 nm, 261 nm; HRMS (ESI): m/z: Calcd for C20H32N2NaO4: 387.2254 [M+Na]⁺, found: 387.2247.

N-(2-(4-(5-(2-amino-1-benzyl-1H-imidazol-5-yl)pentyl)-1H-1,2,3-triazol-1-yl)ethyl)-4-pentylbenzamide hydrochloride (3.18): was synthesized as described in the general procedure using Amide 3.18 (0.1 mmol), DIBAL-H (0.15 mmol), and cyanamide (0.5 mmol). Upon completion of the reaction, the crude product was purified via flash column chromatography. Methanol and aqueous concentrated HCl were added, following the removal of the solvent the HCl salt 3.18 was obtained as a pale yellow solid (50% yield).

¹H NMR (400 MHz, CD3OD) δ 7.69 - 7.65 (m, 3H), 7.33 - 7.22 (m, 5H), 7.06 (d, 2H, J = 7.2 Hz), 6.29 (s, 1H), 4.99 (s, 2H), 4.60 (t, 2H, J = 6 Hz), 3.82 (t, 2H, J = 5.9 Hz), 2.65 - 2.58 (m, 4H), 2.30 (t, 2H, J = 7.6 Hz), 1.63 - 1.26 (m, 12H), 0.90 (t, 3H, J = 6.8 Hz); ¹³C NMR (100 MHz, CD3OD) δ 170.5, 150.6, 149.1, 148.6, 138.5, 132.8, 129.9, 129.7, 128.9, 128.6, 128.5, 127.4, 123.8, 120.1, 50.4, 46.4, 41.2, 36.8, 32.7, 32.2, 30.2, 29.6, 29.0, 26.1, 25.2, 23.7, 14.5; IR (KBr) ν(cm⁻¹) 3423, 2921, 1687; λmax = 232 nm; HRMS (ESI): m/z: Calcd for C31H41N7O: 528.3445 [M+H]⁺, found: 528.3447.
N-(2-(4-(5-(2-amino-1-(naphthalen-2-ylmethyl)-1H-imidazol-5-yl)pentyl)-1H-1,2,3-triazol-1-yl)ethyl)-4-pentylbenzamide hydrochloride (3.19): was synthesized as described in the general procedure using Amide 3.19 (0.1 mmol), DIBAL-H (0.15 mmol), and cyanamide (0.5 mmol). Upon completion of the reaction, the crude product was purified via flash column chromatography. Methanol and aqueous concentrated HCl were added, following the removal of the solvent the HCl salt 3.19 was obtained as a pale yellow solid (38% yield).

$^1$H NMR (400 MHz, CD$_3$OD) δ 7.81 - 7.65 (m, 5H), 7.48 (s, 1H), 7.43 - 7.40 (m, 3H), 7.25 (dd, 1H, $J = 5.9$ Hz, $J = 8.6$ Hz), 7.19 (d, 2H, $J = 8.4$ Hz), 6.32 (s, 1H), 5.13 (s, 2H), 4.55 (t, 2H, $J = 5.8$ Hz), 3.79 (t, 2H, $J = 5.8$ Hz), 2.58 (t, 2H, $J = 7.6$ Hz), 2.48 (t, 2H, $J = 7.2$ Hz), 2.30 (t, 2H, $J = 7.6$ Hz), 1.56 - 1.21 (m, 12H), 0.88 (t, 3H, $J = 7.0$ Hz); $^{13}$C NMR (100 MHz, CD$_3$OD) δ 170.4, 150.7, 149.1, 148.5, 136.1, 134.9, 134.3, 132.7, 129.8, 129.7, 128.9, 128.9, 128.5, 128.5, 127.6, 127.2, 125.8, 125.6, 123.7, 120.3, 50.3, 41.1, 36.8, 32.6, 32.2, 30.1, 29.5, 29.0, 26.1, 25.2, 23.7, 14.5; IR (KBr) ν (cm$^{-1}$) 3256, 2931, 1748; $\lambda_{\text{max}}$ = 229 nm; HRMS (ESI): m/z: Calcd for C$_{35}$H$_{43}$N$_7$O: 578.3602 [M+H]$^+$, found: 578.3592.
N-(2-(4-(5-(2-amino-1-(4-isopropylbenzyl)-1H-imidazol-5-yl)pentyl)-1H-1,2,3-triazol-1-yl)ethyl)-4-pentylbenzamide hydrochloride (3.20): was synthesized as described in the general procedure using Amide 3.20 (0.1 mmol), DIBAL-H (0.15 mmol), and cyanamide (0.5 mmol). Upon completion of the reaction, the crude product was purified via flash column chromatography. Methanol and aqueous concentrated HCl were added, following the removal of the solvent the HCl salt 3.20 was obtained as a pale yellow solid (38% yield).

$^1$H NMR (400 MHz, CD$_3$OD) $\delta$ 7.68 (s, 1H), 7.65 (d, 2H, $J = 4.4$ Hz), 7.23 (d, 2H, $J = 8.4$ Hz), 7.18 (d, 2H, $J = 8$ Hz), 6.99 (d, 2H, $J = 8$ Hz), 6.27 (s, 1H), 4.49 (s, 2H), 2.65 - 2.57 (m, 4H), 3.82 (t, 2H, $J = 6.2$ Hz), 2.86 (sp, 1H, $J = 7$ Hz), 1.65 - 1.19 (m, 18H), 0.90 (t, 3H, $J = 6.8$ Hz);

$^{13}$C NMR (100 MHz, CD$_3$OD) $\delta$ 170.5, 150.6, 149.6, 149.2, 148.6, 135.9, 132.8, 129.7, 128.9, 128.5, 127.9, 127.4, 123.8, 120.2, 50.4, 46.2, 41.2, 36.8, 35.2, 32.7, 32.3, 30.2, 29.6, 29.1, 26.1, 25.3, 24.6, 23.7, 14.5;

IR (KBr) $\nu$(cm$^{-1}$) 3321, 2915, 1701; $\lambda_{max} = 221$ nm; HRMS (ESI): $m/z$: Calcd for C$_{34}$H$_{47}$N$_7$O: 570.3915 [M+H]$^+$, found: 570.3898.
N-(2-(4-(5-(2-amino-1-(4-butylbenzyl)-1H-imidazol-5-yl)pentyl)-1H-1,2,3-triazol-1-yl)ethyl)-4-pentylbenzamide hydrochloride (3.21): was synthesized as described in the general procedure using Amide 3.21 (0.1 mmol), DIBAL-H (0.15 mmol), and cyanamide (0.5 mmol). Upon completion of the reaction, the crude product was purified via flash column chromatography. Methanol and aqueous concentrated HCl were added, following the removal of the solvent the HCl salt 3.21 was obtained as a pale yellow solid (62% yield).

$^1$H NMR (400 MHz, CD$_3$OD) $\delta$ 7.67 (d, 2H, $J = 6.8$ Hz), 7.65 (s, 1H), 7.23 (d, 2H, $J = 8.4$ Hz), 7.13 (d, 2H, $J = 8$ Hz), 6.97 (d, 2H, $J = 8$ Hz), 6.27 (s, 1H), 4.94 (s, 2H), 4.60 (t, 2H, $J = 5.8$ Hz), 3.82 (t, 2H, $J = 6.2$ Hz), 2.65 (m, 6H), 2.30 (t, 2H, $J = 7.4$ Hz), 1.63 - 1.26 (m, 16H), 0.91 (m, 6H); $^{13}$C NMR (100 MHz, CD$_3$OD) $\delta$ 170.5, 150.6, 149.2, 148.6, 143.5, 135.7, 132.7, 130.0, 129.7, 128.9, 128.5, 127.4, 123.8, 120.2, 50.4, 46.3, 41.1, 36.8, 36.3, 35.0, 32.7, 32.3, 30.2, 29.6, 29.0, 26.1, 25.3, 23.7, 23.4, 14.6, 14.4; IR (KBr) $\nu$(cm$^{-1}$) 3305, 2934, 1687; $\lambda_{\text{max}}$ = 225 nm; HRMS (ESI): $m/z$: Calcd for C$_{35}$H$_{49}$N$_7$O: 584.4071 [M+H]$^+$, found: 584.4070.
N-(2-(4-((5-((2-amino-1-(4-chlorobenzyl)-1H-imidazol-5-yl)pentyl)-1H-1,2,3-triazol-1-yl)ethyl)-4-pentylbenzamide hydrochloride (3.22): was synthesized as described in the general procedure using Amide 3.22 (0.1 mmol), DIBAL-H (0.15 mmol), and cyanamide (0.5 mmol). Upon completion of the reaction, the crude product was purified via flash column chromatography. Methanol and aqueous concentrated HCl were added, following the removal of the solvent the HCl salt 3.22 was obtained as a yellow solid (48% yield).

$^1$H NMR (400 MHz, CD$_3$OD) δ 7.67 - 7.65 (m, 3H), 7.33 (d, 2H, $J = 8.8$ Hz), 7.25 (d, 2H, $J = 8.4$ Hz), 7.05 (d, 2H, $J = 8.8$ Hz), 6.31 (s, 1H), 4.99 (s, 2H), 4.61 (t, 2H, $J = 6$ Hz), 3.83 (t, 2H, $J = 5.8$ Hz), 2.67 - 2.61 (m, 4H), 2.31 (t, 2H, $J = 7.6$ Hz), 1.64 - 1.28 (m, 12H), 0.91 (t, 3H, $J = 7.2$ Hz); $^{13}$C NMR (100 MHz, CD$_3$OD) δ 170.6, 150.6, 149.2, 148.6, 137.4, 134.4, 132.8, 130.0, 129.7, 129.1, 128.8, 128.5, 123.8, 119.9, 50.4, 45.8, 41.2, 36.8, 32.7, 32.3, 30.2, 29.6, 29.0, 26.1, 25.2, 23.7, 14.5; IR (KBr) ν (cm$^{-1}$) 3411, 2924, 1678, 764; $\lambda_{\text{max}}$ = 220 nm; HRMS (ESI): $m/z$: Calcd for C$_{31}$H$_{40}$ClN$_7$O: 562.3056 [M+H]$^+$, found: 562.3062.
N-(2-(4-(5-(2-amino-1-(4-bromobenzyl)-1H-imidazol-5-yl)pentyl)-1H-1,2,3-triazol-1-yl)ethyl)-4-pentylbenzamide hydrochloride (3.23): was synthesized as described in the general procedure using Amide 3.23 (0.1 mmol), DIBAL-H (0.15 mmol), and cyanamide (0.5 mmol). Upon completion of the reaction, the crude product was purified via flash column chromatography. Methanol and aqueous concentrated HCl were added, following the removal of the solvent the HCl salt 3.23 was obtained as a yellow solid (42% yield).

$^1$H NMR (400 MHz, CD$_3$OD) $\delta$ 7.68 (s, 1H), 7.67 (d, 2H, $J = 8.4$ Hz), 7.48 (d, 2H, $J = 8.4$ Hz), 7.24 (d, 2H, $J = 8.8$ Hz), 7.0 (d, 2H, $J = 8.4$ Hz), 6.30 (s, 1H), 4.97 (s, 2H), 4.62 (t, 2H, $J = 5.8$), 3.83 (t, 2H, $J = 6$ Hz), 2.67 - 2.61 (m, 4H), 2.30 (t, 2H, $J = 7.6$ Hz), 1.65 - 1.28 (m, 12H), 0.91 (t, 2H, $J = 7$ Hz); $^{13}$C NMR (100 MHz, CD$_3$OD) $\delta$ 170.6, 150.5, 149.2, 148.6, 137.9, 133.0, 132.8, 129.7, 129.4, 128.8, 128.5, 123.8, 122.3, 119.8, 50.4, 45.9, 41.2, 36.8, 32.7, 32.3, 30.2, 29.6, 29.0, 26.1, 25.2, 23.7, 14.5; IR (KBr) $\nu$(cm$^{-1}$) 3378, 2936, 1703, 622; $\lambda_{max}$ = 228 nm; HRMS (ESI): $m/z$: Calcd for C$_{31}$H$_{40}$BrN$_7$O: 606.255 [M+H]$^+$, found: 606.2548.
N-(2-(4-(5-(2-amino-1-(4-propoxybenzyl)-1H-imidazol-5-yl)pentyl)-1H-1,2,3-triazol-1-yl)ethyl)-4-pentylbenzamide hydrochloride (3.24): was synthesized as described in the general procedure using Amide 3.24 (0.1 mmol), DIBAL-H (0.15 mmol), and cyanamide (0.5 mmol). Upon completion of the reaction, the crude product was purified via flash column chromatography. Methanol and aqueous concentrated HCl were added, following the removal of the solvent the HCl salt 3.24 was obtained as a yellow oil (50% yield).

\(^1\)H NMR (400 MHz, CD\(_3\)OD) δ 7.67 (d, 2H, \(J = 8.2\) Hz), 7.66 (s, 1H), 7.24 (d, 2H, \(J = 8.4\) Hz), 7.00 (d, 2H, \(J = 8.8\) Hz), 6.86 (d, 2H, \(J = 8.8\) Hz), 6.30 (s, 1H), 4.61 (t, 2H, \(J = 5.8\) Hz), 3.89 (t, 2H, \(J = 6.4\) Hz), 3.83 (t, 2H, \(J = 5.8\) Hz), 2.65 - 2.60 (m, 4H), 2.32 (t, 2H, \(J = 7.6\) Hz), 1.78 - 1.30 (m, 14H), 1.02 (t, 3H, \(J = 7.2\) Hz), 0.91 (t, 3H, \(J = 6.8\) Hz); \(^{13}\)C NMR (100 MHz, CD\(_3\)OD) δ 170.6, 160.2, 150.3, 149.2, 148.6, 132.8, 130.0, 129.7, 129.1, 128.7, 128.5, 123.8, 119.0, 115.9, 70.7, 50.4, 46.1, 41.2, 36.8, 32.7, 32.3, 30.2, 29.6, 28.9, 26.1, 25.2, 23.8, 23.7, 14.5, 11.0; IR (KBr) ν (cm\(^{-1}\)) 3462, 2926, 1733, 1243; \(\lambda_{\text{max}}\) = 229 nm; HRMS (ESI): \(m/z\): Calcd for C\(_{34}\)H\(_{47}\)N\(_7\)O\(_2\): 586.3864 [M+H]\(^{+}\), found: 586.3861.
N-(2-(4-(5-(2-amino-1-(4-tert-butylbenzyl)-1H-imidazol-5-yl)penty1)-1H-1,2,3-triazol-1-yl)ethyl)-4-pentylbenzamide hydrochloride (3.25): was synthesized as described in the general procedure using Amide 3.25 (0.1 mmol), DIBAL-H (0.15 mmol), and cyanamide (0.5 mmol). Upon completion of the reaction, the crude product was purified via flash column chromatography. Methanol and aqueous concentrated HCl were added, following the removal of the solvent the HCl salt 3.25 was obtained as a yellow solid (40% yield).

$^1$H NMR (400 MHz, CD$_3$OD) δ 7.66 (d, 2H, $J = 8$ Hz), 7.65 (s, 1H), 7.39 (d, 2H, $J = 8.4$ Hz), 7.24 (d, 2H, $J = 8$ Hz), 7.00 (d, 2H, $J = 8.4$ Hz), 6.27 (s, 1H), 4.96 (s, 2H), 4.61 (t, 2H, $J = 5.8$ Hz), 3.83 (t, 2H, $J = 5.8$ Hz), 2.66 - 2.60 (m, 4H), 2.32 (t, 2H, $J = 7.6$ Hz), 1.64 - 1.26 (m, 21H), 0.91 (t, 3H, $J = 7.2$ Hz); $^{13}$C NMR (100 MHz, CD$_3$OD) δ 170.6, 152.6, 148.8, 148.6, 145.8, 133.2, 132.4, 130.5, 129.8, 128.5, 128.5, 128.5, 128.4, 127.2, 110.2, 54.3, 46.5, 40.5, 36.8, 35.6, 32.7, 32.3, 31.8, 29.2, 29.0, 27.8, 24.6, 24.1, 23.7, 14.5; IR (KBr) $\nu$(cm$^{-1}$) 3405, 2942, 1644; $\lambda_{\text{max}}$ = 218 nm; HRMS (ESI): $m/z$: Calcd for C$_{35}$H$_{49}$N$_7$O: 584.4071 [M+H]$^+$, found: 584.4089.
N-(2-(4-(5-(2-amino-1-(4-ethynylbenzyl)-1H-imidazol-5-yl)pentyl)-1H-1,2,3-triazol-1-yl)ethyl)-4-pentylbenzamide hydrochloride (3.26): was synthesized as described in the general procedure using Amide 3.26 (0.1 mmol), DIBAL-H (0.15 mmol), and cyanamide (0.5 mmol). Upon completion of the reaction, the crude product was purified via flash column chromatography. Methanol and aqueous concentrated HCl were added, following the removal of the solvent the HCl salt 3.26 was obtained as a pale yellow solid (28% yield).

$^1$H NMR (400 MHz, CD$_3$OD) $\delta$ 7.66 (s, 1H), 7.65 (d, 2H, $J = 8.4$ Hz), 7.42 (d, 2H, $J = 8$ Hz), 7.24 (d, 2H, $J = 8.4$ Hz), 7.04 (d, 2H, $J = 8.4$ Hz), 6.29 (s, 1H), 5.00 (s, 2H), 4.61 (t, 2H, $J = 5.8$ Hz), 3.83 (t, 2H, $J = 5.8$ Hz), 3.49 (s, 1H), 2.66 - 2.60 (m, 4H), 2.30 (t, 2H, $J = 7.6$ Hz), 1.62 - 1.29 (m, 12H), 0.91 (t, 3H, $J = 7.2$ Hz); $^{13}$C NMR (100 MHz, CD$_3$OD) $\delta$ 169.3, 149.1, 147.8, 147.3, 137.8, 132.3, 131.5, 128.4, 127.7, 127.2, 126.2, 122.5, 121.9, 117.5, 82.8, 77.9, 49.1, 44.9, 39.8, 35.5, 31.4, 31.0, 28.9, 28.2, 27.6, 24.8, 23.8, 22.4, 13.2; IR (KBr) $\nu$(cm$^{-1}$) 3296, 2929, 2318, 1699; $\lambda_{\text{max}} = 234$ nm; HRMS (ESI): $m/z$: Calcd for C$_{22}$H$_{41}$N$_7$O: 552.3445 [M+H]$^+$, found: 552.3442.
N-(2-(4-(5-(2-amino-1-(biphenyl-4-ylmethyl)-1H-imidazol-5-yl)pentyl)-1H-1,2,3-triazol-1-yl)ethyl)-4-pentylbenzamide hydrochloride (3.27): was synthesized as described in the general procedure using Amide 3.27 (0.1 mmol), DIBAL-H (0.15 mmol), and cyanamide (0.5 mmol). Upon completion of the reaction, the crude product was purified via flash column chromatography. Methanol and aqueous concentrated HCl were added, following the removal of the solvent the HCl salt 3.27 was obtained as a pale yellow solid (72% yield).

$^1$H NMR (400 MHz, CD$_3$OD) δ 7.67 - 7.14 (m, 14H), 6.30 (s, 1H), 5.04 (s, 2H), 4.57 (t, 2H, $J = 8$ Hz), 3.80 (t, 2H, $J = 8$ Hz), 2.66 - 2.58 (m, 4H), 2.35 (t, 2H, $J = 10$ Hz), 1.64 - 1.26 (m, 12H), 0.90 (t, 3H, $J = 9.2$ Hz); $^{13}$C NMR (100 MHz, CD$_3$OD) δ 170.5, 150.7, 149.2, 148.6, 141.9, 141.8, 137.7, 132.8, 130.1, 129.7, 128.9, 128.6, 128.5, 128.0, 127.9, 123.8, 120.4, 50.4, 46.2, 41.1, 36.8, 32.7, 32.2, 30.2, 29.6, 29.1, 26.1, 25.3, 23.7, 14.5; IR (KBr) ν(cm$^{-1}$) 3386, 2918, 1675; $\lambda_{max} = 242$ nm; HRMS (ESI): $m/z$: Calcd for C$_{37}$H$_{45}$N$_7$O: 604.3758 [M+H]$^+$, found: 604.3756.
N-(2-(4-(5-(2-amino-1-(4-cyanobenzyl)-1H-imidazol-5-yl)pentyl)-1H-1,2,3-triazol-1-yl)ethyl)-4-pentylbenzamide hydrochloride (3.28): was synthesized as described in the general procedure using Amide 3.28 (0.1 mmol), DIBAL-H (0.15 mmol), and cyanamide (0.5 mmol). Upon completion of the reaction, the crude product was purified via flash column chromatography. Methanol and aqueous concentrated HCl were added, following the removal of the solvent the HCl salt 3.28 was obtained as a yellow oil (44% yield).

$^1$H NMR (400 MHz, CD$_3$OD) δ 7.70 - 7.65 (m, 4H), 7.24 (d, 2H, $J = 8.4$ Hz), 7.21 (d, 2H, $J = 8.4$ Hz), 6.30 (s, 1H), 5.09 (s, 2H), 4.61 (t, 2H, $J = 6$ Hz), 3.83 (t, 2H, $J = 5.8$ Hz), 2.66 - 2.60 (m, 4H), 2.29 (t, 2H, $J = 7.4$ Hz), 1.64 - 1.30 (m, 12H), 0.91 (t, 3H, $J = 7.0$ Hz); $^{13}$C NMR (100 MHz, CD$_3$OD) δ 170.6, 150.7, 149.1, 148.6, 144.5, 133.8, 132.8, 129.7, 128.7, 128.5, 128.4, 123.8, 120.4, 119.6, 112.4, 61.7, 46.1, 41.2, 36.8, 32.7, 32.3, 30.9, 29.6, 26.1, 25.1, 23.7, 21.0, 14.5; IR (KBr) ν(cm$^{-1}$) 3406, 2922, 2214, 1643; $\lambda_{\text{max}}$ = 228 nm; HRMS (ESI): m/z: Calcd for C$_{32}$H$_{40}$N$_8$O: 553.3398 [M+H]$^+$, found: 553.3397.
N-(2-(4-(5-(2-amino-1-(3,4-diethoxybenzyl)-1H-imidazol-5-yl)pentyl)-1H-1,2,3-triazol-1-yl)ethyl)-4-pentylbenzamide hydrochloride (3.29): was synthesized as described in the general procedure using Amide 3.29 (0.1 mmol), DIBAL-H (0.15 mmol), and cyanamide (0.5 mmol). Upon completion of the reaction, the crude product was purified via flash column chromatography. Methanol and aqueous concentrated HCl were added, following the removal of the solvent the HCl salt 3.29 was obtained as a yellow oil (29% yield).

$^1$H NMR (400 MHz, CD$_3$OD) $\delta$ 7.68 (s, 1H), 7.66 (d, 2H, $J = 8.4$ Hz), 7.25 (d, 2H, $J = 8.6$ Hz), 6.89 (d, 1H, $J = 8.1$ Hz), 6.69 (d, 1H, $J = 2.8$ Hz), 6.58 (dd, 1H, $J = 8.3$ Hz, $J = 2.8$ Hz), 6.27 (s, 1H), 4.88 (s, 2H), 4.61 (t, 2H, $J = 8.0$ Hz), 4.06 - 3.95 (m, 4H), 3.82 (t, 2H, $J = 8.0$ Hz), 2.66 - 2.59 (m, 4H), 2.33 (t, 2H, $J = 7.4$ Hz), 1.65 - 1.28 (m, 18H), 0.91 (t, 3H, $J = 6.9$ Hz); $^{13}$C NMR (75 MHz, CD$_3$OD) $\delta$ 170.5, 150.4, 149.5, 149.1, 148.6, 132.7, 131.3, 129.7, 128.9, 128.5, 123.8, 120.2, 119.9, 118.0, 115.2, 113.2, 65.9, 65.8, 50.4, 46.1, 41.1, 36.8, 32.7, 32.2, 30.2, 29.6, 29.1, 26.2, 25.2, 23.7, 15.3, 15.2, 14.5; IR (KBr) $\nu$(cm$^{-1}$) 3386, 2938, 1691, 1124; $\lambda_{\text{max}}$ = 228 nm; HRMS (ESI): $m/z$: Calcd for C$_{35}$H$_{49}$N$_7$O$_3$: 616.3970 [M+H]$^+$, found: 616.3967.
N-(2-(4-(5-(2-amino-1-(4-(hexyloxy)benzyl)-1H-imidazol-5-yl)pentyl)-1H-1,2,3-triazol-1-yl)ethyl)-4-pentylbenzamide hydrochloride (3.30): was synthesized as described in the general procedure using Amide 3.30 (0.1 mmol), DIBAL-H (0.15 mmol), and cyanamide (0.5 mmol). Upon completion of the reaction, the crude product was purified via flash column chromatography. Methanol and aqueous concentrated HCl were added, following the removal of the solvent the HCl salt 3.30 was obtained as a yellow oil (77% yield).

$^1$H NMR (400 MHz, CD$_3$OD) δ 7.66 (d, 2H, $J = 7.2$ Hz), 7.65 (s, 1H), 7.24 (d, 2H, $J = 8.4$ Hz), 6.99 (d, 2H, $J = 8.8$ Hz), 6.86 (d, 2H, $J = 8.4$ Hz), 6.27 (s, 1H), 4.91 (s, 2H), 4.61 (t, 2H, $J = 6.0$ Hz), 3.93 (t, 2H, $J = 6.4$ Hz), 3.83 (t, 2H, $J = 5.8$ Hz), 2.66 - 2.60 (m, 4H), 2.32 (t, 2H, $J = 7.6$ Hz), 1.76 - 1.30 (m, 20H), 0.94 - 0.89 (m, 6H); $^{13}$C NMR (100 MHz, CD$_3$OD) δ 170.5, 164.9, 160.0, 149.2, 148.6, 132.7, 130.1, 129.7, 128.7, 128.5, 123.8, 119.9, 118.0, 115.9, 69.2, 50.4, 46.0, 41.1, 36.8, 32.9, 32.6, 32.2, 30.4, 30.2, 29.6, 29.0, 26.9, 26.1, 25.2, 23.8, 23.6, 14.5, 14.5; IR (KBr) ν(cm$^{-1}$) 3332, 2906, 1614, 1108; $\lambda_{\text{max}}$ = 229 nm; HRMS (ESI): m/z: Calcd for C$_{37}$H$_{53}$N$_7$O$_2$: 628.4334 [M+H]$^+$, found: 628.4330.
N-(2-(4-(5-(2-amino-1-(3,4-dichlorobenzyl)-1H-imidazol-5-yl)pentyl)-1H-1,2,3-triazol-1-yl)ethyl)-4-pentylbenzamide hydrochloride (3.31): was synthesized as described in the general procedure using Amide 3.31 (0.1 mmol), DIBAL-H (0.15 mmol), and cyanamide (0.5 mmol). Upon completion of the reaction, the crude product was purified via flash column chromatography. Methanol and aqueous concentrated HCl were added, following the removal of the solvent the HCl salt 3.31 was obtained as a pale yellow solid (46% yield).

$^1$H NMR (400 MHz, CD$_3$OD) δ 7.66 (s, 1H), 7.65 (d, 2H, $J = 8.8$ Hz), 7.48 (d, 1H, $J = 8.4$ Hz), 7.24 (d, 2H, $J = 8.4$ Hz), 7.19 (d, 1H, $J = 2.0$ Hz), 6.99 (dd, 1H, $J = 8.4$ Hz, $J = 2.0$ Hz), 6.31 (s, 1H), 4.99 (s, 2H), 4.61 (t, 2H, $J = 5.8$ Hz), 3.83 (t, 2H, $J = 5.8$ Hz), 2.66 - 2.61 (m, 4H), 2.31 (t, 2H, $J = 7.6$ Hz), 1.64 - 1.29 (m, 12H), 0.91 (t, 3H, $J = 7.0$ Hz); $^{13}$C NMR (100 MHz, CD$_3$OD) δ 170.6, 164.9, 150.5, 149.2, 148.7, 139.4, 133.8, 132.5, 132.0, 129.7, 129.4, 128.4, 127.3, 123.8, 120.0, 117.9, 50.4, 45.4, 41.1, 36.8, 32.6, 32.2, 30.2, 29.5, 28.9, 26.1, 25.1, 23.6, 14.5; IR (KBr) ν (cm$^{-1}$) 3304, 2941, 1636, 724; $\lambda_{\text{max}}$ = 226 nm; HRMS (ESI): $m/z$: Calcd for C$_{31}$H$_{39}$Cl$_2$N$_7$O: 596.2666 [M+H]$^+$, found: 596.2665.
1-(4-Butylbenzyl)-1H-imidazol-2-amine (3.32): was synthesized as described in the general procedure using Amide 3.32 (0.1 mmol), DIBAL-H (0.15 mmol), and cyanamide (0.5 mmol). Upon completion of the reaction, the crude product was purified via flash column chromatography. Methanol and aqueous concentrated HCl were added, following the removal of the solvent the HCl salt 3.32 was obtained as a pale yellow solid (39% yield).

$^1$H NMR (400 MHz, CD$_3$OD) δ 7.18 (d, 2H, $J = 8.0$ Hz), 7.12 (d, 2H, $J = 8.0$ Hz), 6.55 (d, 1H, $J = 2.0$ Hz), 6.51 (d, 1H, $J = 2.0$ Hz), 4.93 (s, 2H), 2.61 (t, 2H, $J = 7.8$ Hz), 1.60 (qn, 2H, $J = 7.6$ Hz), 1.36 (sx, 2H, $J = 7.6$ Hz), 0.95 (t, 3H, $J = 7.4$ Hz); $^{13}$C NMR (100 MHz, CD$_3$OD) δ 165.0, 143.8, 130.0, 128.4, 124.0 120.1, 48.9, 36.4, 35.0, 23.4, 14.4; IR (KBr) $\nu$(cm$^{-1}$) 3421, 2946; $\lambda_{\text{max}}$ = 218 nm; HRMS (ESI): $m/z$: Calcd for C$_{14}$H$_{19}$N$_3$: 230.1652 [M+H]$^+$, found: 230.1649.

Bacterial Strains, Media, and Antibiotics. Staphylococcus aureus strains were obtained from the ATCC (43300, 33591, 700789, BAA-1753, BAA-811, BAA-1770, BAA-1685, BAA-44, BAA-1556) or the Network on Antimicrobial Resistance in Staphylococcus aureus (NARSA) (JE-2, NE-218, NE-147, NE-958, NE-481, NE-262, NE-618, NE-554, NE-823, NE-873, NE-210, NE-820, NE-839, NE-49, NE-116, NE-95, NE-423) and colonies grown on solid media as instructed. Mueller-Hinton broth (MHB) (cat # 275710) was purchased from
BD Diagnostics. Vancomycin (cat # 861987) and streptomycin (cat # S1567) were purchased from Sigma Aldrich. Oxacillin (cat # O0353) was purchased from TCI. Chloramphenicol (cat # B20841) was purchased from Alfa Aesar. All assays were run in duplicate and repeated at least two separate times. All compounds were dissolved as their HCl salts in molecular biology grade DMSO as 100 mM or 10 mM stock solutions.

**Broth Microdilution Method for Determination of Minimum Inhibitory Concentrations.** *S. aureus* was grown in MHB for 6-8 h and this culture was used to inoculate fresh MHB (5 x 10^5 CFU/mL). The resulting bacterial suspension was aliquoted (5 mL) into culture tubes and compound, from a 100 mM or 10 mM DMSO stock, was added. Inoculated media not treated with compound served as the control. From each sample, 1 mL was transferred to a new culture tube and antibiotic, from water stock, was added. Rows 2-12 of a 96-well microtiter plate were filled at 100 µL/well from the remaining inoculated media, allowing the concentration of compound to be kept uniform throughout the antibiotic dilution procedure. The samples containing antibiotic were then aliquoted (200 µL) into the corresponding first row wells of the microtiter plate. Row 1 wells were mixed 6 to 8 times then 100 µL was transferred to row 2. Row 2 wells were mixed 6 to 8 times, followed by a 100 µL transfer from row 2 to row 3. This procedure was repeated to serially dilute the rest of the rows of the microtiter plate, with the exception of the final row, to which no antibiotic was added (to check for growth of bacteria in the presence of compound alone). The plate was then covered with a lid and incubated under stationary conditions at 37 °C. After 16 h, minimum inhibitory concentration (MIC) values were recorded as the lowest concentration of antibiotic at which no visible growth of bacteria was observed.
**Time Kill Curves.** *S. aureus* was grown in MHB overnight and this culture was used to inoculate fresh MHB (5 x 10^5 CFU/mL). Inoculated media was aliquoted (3 mL) into culture tubes and compound and/or oxacillin were added, untreated inoculated media served as the control. Tubes were incubated at 37 °C with shaking. Samples were taken at 2, 4, 6, 8, and 24 h time points, serially diluted in fresh MHB and plated on tryptic soy agar. Plates were incubated at 37 °C overnight and the number of colonies enumerated.

**Hemolysis Assay.** Hemolysis assays were performed on mechanically difibrinated sheep blood (Hemostat Labs: DSB100). Difibrinated blood (1.5 mL) was placed into a microcentrifuge tube and centrifuged for 10 min at 10,000 rpm. The supernatant was then removed and then the cells were resuspended in 1 mL of phosphate-buffered saline (PBS). The suspension was centrifuged, the supernatant was removed and cells were resuspended two additional times. The final cell suspension was then diluted 10-fold. Test compound solutions were made in PBS in small culture tubes and then added to aliquots of the 10-fold suspension dilution of blood. PBS was used as a negative control and a zero hemolysis marker. Triton X (a 1% sample) was used as a positive control serving as the 100% lysis marker. Samples were then placed in an incubator at 37 °C while being shaken at 200 rpm for one hour. After one hour, the samples were transferred to microcentrifuge tubes and centrifuged for 10 min at 10,000 rpm. The resulting supernatant was diluted by a factor of 40 in distilled water. The absorbance of the supernatant was then measured with a UV spectrometer at a 540 nm wavelength.

**Bacterial Membrane Permeabilization Assay.** The BacLight assay (Invitrogen) was used to assess membrane permeability. *S. aureus* was grown overnight in MHB at 37 °C with
shaking. The culture was diluted 1:40 in MHB and grown to an optical density at 600nm (OD$_{600}$) of ~1.0 (~4 h growth). The cultures were centrifuged at 10,000g for 15 min, and the cell pellet was washed once with sterile water, resuspended to 1/10 of the original volume and diluted 1:20 into either water or water containing test compounds. Suspensions were incubated at 37 °C with shaking for 1 h then centrifuged at 10,000g for 10 min, washed once with sterile water, and resuspended in water. A 1:1 mixture of SYTO-9 and propidium iodide were added to the suspension (3 μL/mL) and mixed well. 100 μL of the suspension was added to each well of a 96-well plate. And the plates were incubated in the dark for 15 min at room temperature. Green fluorescence (SYTO-9) was read at 530 nm, and red fluorescence (propidium iodide) was read at 645 nm (excitation wavelength, 485nm). The ratio of green to red fluorescence was expressed as a percentage of the control.
Amine 3.18
Amine 3.19
Amine 3.19
Amine 3.23
Amine 3.23
Amine 3.24
Amine 3.25
Amine 3.26
Amine 3.27
Amine 3.27
Amine 3.28
Amine 3.28
Amine 3.29
Amine 3.30
Amine 3.30
Amine 3.31
Amine 3.31
Amine 3.32
Amine 3.32
Boc 3.18
359
Boc 3.23
Boc 3.29
Boc 3.32
Boc 3.32
Amide 3.19
Amide 3.21
Amide 3.21
Amide 3.23
Amide 3.26
Amide 3.26
Amide 3.28
Amide 3.28
Amide 3.29
Amide 5.30
Amide 3.31
References

(1) Poole, K. *Cellular and Molecular Life Sciences* **2004**, *61*, 2200.


(3) IDSA Policy Paper d CID 2011:52 (Suppl 5) d S397.


(14) Harris, T. L.; Worthington, R. J.; Melander, C. Organic and Biomolecular Chemistry Letters 2011, 21, 4516.


(16) Worthington, R. J.; Bunders, C. A.; Reed, C. S.; Melander, C. ACS Medicinal Chemistry Letters, 3, 357.


Appendix
Appendix A- Gene description for NARSA mutant strains

<table>
<thead>
<tr>
<th>Strain Name</th>
<th>Gene Name</th>
<th>Description</th>
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<tr>
<td>JE2</td>
<td>USA300</td>
<td>Parent Strain</td>
</tr>
<tr>
<td>NE218</td>
<td>Sensor histidine kinase family protein</td>
<td></td>
</tr>
<tr>
<td>NE147</td>
<td>Sensor histidine kinase</td>
<td></td>
</tr>
<tr>
<td>NE958</td>
<td>Two-component response regulator</td>
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</tr>
<tr>
<td>NE481</td>
<td>DNA-binding response regulator</td>
<td></td>
</tr>
<tr>
<td>NE262</td>
<td>DNA-binding response regulator. LuxR family</td>
<td></td>
</tr>
<tr>
<td>NE618</td>
<td>phoR</td>
<td>Sensory box histidine kinase PhoR</td>
</tr>
<tr>
<td>NE554</td>
<td>vraR</td>
<td>DNA-binding response regulator</td>
</tr>
<tr>
<td>NE823</td>
<td>vraS</td>
<td>Two-component system sensor histidine kinase</td>
</tr>
<tr>
<td>NE873</td>
<td>agrC</td>
<td>Accessory gene regulator protein C</td>
</tr>
<tr>
<td>NE210</td>
<td>Staphylococcal accessory regulator</td>
<td></td>
</tr>
<tr>
<td>NE820</td>
<td>Sensor histidine kinase</td>
<td></td>
</tr>
<tr>
<td>NE839</td>
<td>Methicillin-resistance MecR1 regulatory protein</td>
<td></td>
</tr>
<tr>
<td>NE49</td>
<td>DNA-binding response regulator, AraC family</td>
<td></td>
</tr>
<tr>
<td>NE116</td>
<td>Putative sensor histidine kinase</td>
<td></td>
</tr>
<tr>
<td>NE95</td>
<td>agrB</td>
<td>Accessory gene regulator protein B</td>
</tr>
<tr>
<td>NE423</td>
<td>kdpD</td>
<td>Sensor histidine kinase, KdpD</td>
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</tbody>
</table>
CHAPTER 4

SUPPRESSING COLISTIN RESISTANCE IN GRAM-NEGATIVE BACTERIA BY INHIBITING TWO-COMPONENT SYSTEM SIGNALING

4.1: Introduction

Among the greatest current global health problems are bacterial infections that stem from multi-drug resistant (MDR) organisms. Infections that arise from the so-called ESKAPE pathogens (E. faecium, S. aureus, K. pneumoniae, A. baumannii, P. aeruginosa, and Enterobacter species) are considered the greatest threat to human health due to their prevalence and ability to escape the effects of antibiotics. To compound the problem, only two new classes of antibiotics have been introduced in the clinic in the last two decades, daptomycin and linezolid, both of which are only active against Gram-positive bacteria. The dearth of new antibiotics to treat infections caused by MDR Gram-negative bacteria is a considerable problem and the ever increasing isolation of carbapenem resistant strains means that colistin is now essentially the last line of defense against MDR Gram-negative infections, especially in wounded soldiers returning from combat operations in Iraq/Afghanistan.

Colistin is a polymyxin antibiotic that has shown to be effective at eradicating infections associated with Gram-negative bacteria (Figure 4.1). It became available for therapeutic use in the 1960s, but was later removed from use due to concerns about its neurotoxicity and nephrotoxicity However, due to the lack of novel antibiotic scaffolds and the emergence of multi-drug resistant Gram-negative bacteria, in recent years colistin has
become the only viable option for clinical use. Additionally, recent studies suggest that there are fewer issues with polymyxin toxicity than previously thought.\textsuperscript{8,9}

![Chemical structure of colistin](image)

**Figure 4.1: Chemical structure of colistin.**

Colistin has been shown to be bactericidal against Gram-negative bacteria by disrupting the outer membrane.\textsuperscript{10} The bactericidal activity is a consequence of the cationic antibiotic binding to anionic lipopolysaccharides and phospholipids in the outer cell membrane. Upon binding, it has been shown to competitively displace divalent cations (Ca\textsuperscript{2+} and Mg\textsuperscript{2+}) that normally link adjacent lipopolysaccharide molecules.\textsuperscript{11,12} This ultimately leads to leakage of intracellular contents and results in cellular death. Koike \textit{et al.} demonstrated this by examining the bacterial cytoplasmic membrane after exposure to colistin (via electron microscopy) and they were able to show that it was damaged enough to result in the release of cytoplasmic material.\textsuperscript{13}
As with all antibiotics, the rise of colistin therapy has lead to a rise in colistin-resistant strains of Gram-negative bacteria, particularly *A. baumannii* and *K. pneumoniae*. Polymyxin resistance in *A. baumannii* has been attributed to the modification of the lipid A component of the bacterial membrane. This modification results in the addition of phosphoethanolamine, which reduces the net anionic charge of the bacterial membrane, which results in reduced affinity for the cationic colistin (Figure 4.2). The PmrAB TCS is comprised of a response regulator protein, PmrA, a histidine kinase, PmrB, and this TCS is known to regulate this addition. Previous studies have utilized *pmrB* knockout strains, and observed an 8- to 16-fold decrease in polymyxin MICs.\textsuperscript{14,15}

![Modification of Lipid A](image)

**Figure 4.2:** Phosphoethanolamine modification of lipid A conveys resistance to polymyxin antibiotics.

As previously shown (Chapter 3), we have been successful in the development of small molecule adjuvants that interfere with TCS signaling.\textsuperscript{16} With this initial success in
Gram-positive bacteria, we sought to apply a similar approach to multi-drug resistant Gram-negative bacteria. Previously, Worthington et al. identified compound 4.1, a 2-aminoimidazole containing small molecule that suppresses carbapenem resistance of a New Delhi Metallo-β-lactamase producing strain of *K. pneumoniae* via an unknown mechanism (Figure 4.3). Using 4.1 as our lead, we began to investigate its ability to suppress colistin resistance in *A. baumannii*.

![Chemical structure of 2-aminoimidazole adjuvant](image)

**Figure 4.3: Chemical structure of a 2-aminoimidazole adjuvant.**

### 4.2: Results and Discussion

To initially validate the ability of compound 4.1 to break colistin resistance, we obtained five colistin resistant *A. baumannii* clinical isolates (MICs of 512 - 1024 µg/mL). These values represent extremely high levels of colistin resistance, as the Clinical and Laboratory Standards Institute defines colistin resistance as ≥8 µg/mL. We then determined the colistin MIC of each strain in the presence of 10, 20, and 30 µM compound 4.1 (3, 6, and 9 µg/mL respectively) (Table 4.1). We observed a dose responsive effect, with colistin MICs reduced to 0.5 – 4 µg/mL at 30 µM compound 4.1.
Table 4.1: Effect of compound 4.1 on colistin MIC (Values reported in µg/mL).

<table>
<thead>
<tr>
<th>4. beaumarii Strain</th>
<th>Colistin MIC</th>
<th>Colistin MIC + 10 µM 4.1</th>
<th>Colistin MIC + 20 µM 4.1</th>
<th>Colistin MIC + 30 µM 4.1</th>
</tr>
</thead>
<tbody>
<tr>
<td>3941</td>
<td>512</td>
<td>&gt;128</td>
<td>32</td>
<td>4</td>
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<tr>
<td>3942</td>
<td>512</td>
<td>&gt;128</td>
<td>16</td>
<td>2</td>
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<td>4106</td>
<td>&gt;512</td>
<td>&gt;128</td>
<td>16</td>
<td>2</td>
</tr>
<tr>
<td>4112</td>
<td>&gt;512</td>
<td>&gt;128</td>
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<td>4</td>
</tr>
<tr>
<td>4119</td>
<td>512</td>
<td>128</td>
<td>4</td>
<td>0.5</td>
</tr>
</tbody>
</table>

To quantify this effect, time kill curves were constructed for one representative isolate (4106) for colistin alone, compound 4.1 alone, and the combination of colistin and compound 4.1 (Figure 4.4). Compound 4.1 alone had a bacteriostatic effect on early bacterial growth at 30 µM; however, bacterial growth was identical to untreated bacteria by 8 h (<0.1 log difference in CFU/mL). After 8 h, the combination of 30 µM compound 4.1 and 2 µg/mL colistin effected a 2.4 log reduction in CFU/mL as compared to untreated bacteria. To achieve this level of reduction, colistin alone must be present at 512 µg/mL (reduction of 2.6 log units).
Figure 4.4: (A) Toxicity of compound 4.1 as a function of time. (B) Toxicity of colistin as a function of time. (C) Toxicity of colistin + 10 µM compound 4.1 as a function of time. (D) Toxicity of colistin + 20 µM compound 4.1 as a function of time. (E) Toxicity of colistin + 30 µM compound 4.1 as a function of time.

As colistin acts on the bacterial membrane, we wanted to first establish whether compound 4.1 had an effect on A. baumannii membrane permeability and, if so, if the observed MIC drop was due to membrane effects or, as we posited, interference with TCS signaling. Using the BacLight assay,\(^1\) which measures the permeability of the bacterial membrane toward propidium iodide, we determined that compound 4.1 potentiated the permeability of the membrane of strain 4106. In the presence of 30 µM compound 4.1, the
membrane permeability of 4106 increased 8.1 fold in comparison to an untreated control. As a control to separate membrane permeability from suppression of colistin resistance, we studied the effects that compound 4.2 had on membrane permeability (Figure 4.5).

Figure 4.5: Chemical structure of an inactive 2-aminoimidazole.

Compound 4.2 was chosen as a control compound as it is an analogue of compound 4.1 that does not suppress resistance to colistin in strain 4106 (lowering the MIC by only 2-fold to 256 µg/mL at a concentration of 50 µM), and would thereby allow us to deconvolute membrane disruption from resistance suppression. At 50 µM, compound 4.2 also significantly potentiated the permeability of the membrane of strain 4106, such that permeability was increased by 8.7 fold compared to untreated bacteria, indicating that increased membrane permeability was not the cause of the increased colistin susceptibility effected by compound 4.1.

We further delineated the mechanism of compound 4.1 by studying its effect on pmrCAB transcript levels in 4106 using real time RT-PCR analysis. The pmrCAB operon, encodes for the PmrAB TCS, in which PmrA is the response regulator and PmrB is the histidine kinase, in addition to a lipid A phosphoethanolamine transferase encoded by
We noted potent, dose-responsive suppression of *pmrCAB* expression in the presence of compound 4.1 such that at 30 μM, we observed upwards of 10-fold downregulation of the *pmrCAB* system (Figure 4.6). Control compound 4.2 upregulated *pmrCAB* expression (Figure 4.6) and did not suppress lipid A modification, most likely due to its ability to increase membrane permeability but lack of colistin-resistance suppression activity. Given the high level of colistin resistance associated with this strain, any upregulation of *pmrCAB* operon is not expected to affect the magnitude of colistin resistance.
Next, our collaborators at the University of Maryland, Baltimore verified that compound 4.1 suppressed the degree of lipid A modification by analyzing the mass spectrum of the lipid A fraction of 4106 grown in the presence and absence of compound 4.1 (Figure 4.7). This revealed that the presence of compound 4.1 significantly reduced the extent of
phosphoethanolamine addition to lipid A. Additionally, compound 4.2 was shown to not affect the degree of lipid A modification in strain 4106.

![Graph showing suppression of lipid A phosphoethanolamine modification](image)

**Figure 4.7:** Suppression of lipid A phosphoethanolamine modification, \( m/z \) 2033, via compound 4.1.

With any potential antimicrobial therapy, there exists the threat of resistance evolution. To assess the ability of bacteria to evolve resistance to the action of compound 4.1, we ran a number of experiments to establish the rate at which resistance was acquired.
We first grew 4106 in the presence of 1X the MIC of compound 4.1 and plated surviving colonies after 4 h of growth. Surviving colonies were picked and the MIC of compound 4.1, colistin, and colistin in the presence of 30 µM compound 4.1 was determined. Activity was identical to that determined previously (Table 4.1). We then continuously passaged 4106 in the presence of 30 µM compound 4.1 and colistin at 0.5X, 1X, and 2X its MIC for seven days. Growth at the highest concentration was carried forward with MICs being determined every one-two days against the passaged culture (Figure 4.8). Not only did the MIC of colistin not increase, but we observed that the colistin-only MIC of the resulting bacterial population after passaging in the presence of compound 4.1/colistin decreased over time compared to passaging the bacteria in media alone. Further experiments to delineate this effect are necessary.

Figure 4.8: Seven-day evolution experiment. Colistin MICs of A. baumannii (4106) cultured in the presence of 30 µM 4.1 + colistin.
We next repeated this experiment with a colistin susceptible laboratory strain of *A. baumannii* (ATCC 19606) (Figure 4.9) to evaluate the ability of compound 4.1 to suppress acquisition of resistance. During the timeframe of the experiment (seven days) the colistin MIC for bacteria exposed to only colistin increased from 1 µg/mL to >1024 µg/mL, while repetition of the experiment in the presence of 10 µM compound 4.1 completely inhibited resistance evolution with the MIC remaining at 1 µg/mL. Serially passaging the bacteria in the presence of compound 4.1 at 5 µM (1.5 µg/mL) slowed the evolution such that after seven days the colistin MIC had only risen to 16 µg/mL. The strain evolved against colistin only was also susceptible to the action of compound 4.1, as we observed suppression of colistin MICs to 128 µg/mL and 16 µg/mL in the presence of compound 4.1 at concentrations of 2.5 µM (0.75 µg/mL) and 5 µM respectively.
After establishing that compound 4.1 reduced colistin MICs 128-1024-fold against the initial clinical isolates, we sought to further establish the activity of compound 4.1 by screening against 24 additional A. baumannii isolates with varying colistin MICs (Table 4.2). We determined the MIC of colistin against each strain in the presence of 10, 20, or 30 µM compound 4.1. MIC$_{90}$ values for the collection (including the five original strains) are summarized in Table 4.3. Compound 4.1 substantially reduced the colistin MIC against each strain.
Table 4.2: Colistin MICs of 24 A. baumannii isolates and 14 K. pneumoniae isolates. MIC values reported in μg/mL.

<table>
<thead>
<tr>
<th>A. baumannii</th>
<th>Isolate</th>
<th>Colistin MIC</th>
</tr>
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<tbody>
<tr>
<td>A2</td>
<td>1</td>
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</tr>
<tr>
<td>A3</td>
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It has also been reported that colistin resistance in K. pneumoniae is driven by TCS signaling. To further establish the effect of compound 4.1 upon colistin resistance, we studied the ability of compound 4.1 to lower colistin MICs against 14 K. pneumoniae clinical isolates with varying degrees of colistin resistance (Table 4.2). MIC\textsubscript{90} values for the
collection are summarized in Table 4.3, and compound 4.1 significantly suppressed the colistin MIC against each strain.

### Table 4.3: Colistin MIC\textsubscript{90} values for 29 \textit{A. baumannii} isolates and 14 \textit{K. pneumoniae} isolates when dosed with varying concentrations of compound 4.1. (Values reported in \(\mu\text{g/mL}\)).

<table>
<thead>
<tr>
<th>Bacterial Strain</th>
<th>Strains Tested</th>
<th>No compd 4.1</th>
<th>10 \text{nM} compd 4.1</th>
<th>20 \text{nM} compd 4.1</th>
<th>30 \text{nM} compd 4.1</th>
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<td>\textit{K. pneumoniae}</td>
<td>14</td>
<td>512</td>
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### 4.3: Conclusions

In conclusion, there is no doubt that the rise of multi-drug resistant bacteria is placing a large burden on our healthcare system and infections caused by these bacteria lead to significant patient morbidity and mortality. Novel approaches to treat these infectious are sorely needed, regardless of whether they come from the development of novel antibiotics or antibiotic adjuvants. In this vein, we have demonstrated that a 2-aminoimidazole adjuvant (compound 4.1) has the ability to downregulate \textit{pmrCAB} expression, suppress lipid A modification, and in the process restore colistin sensitivity to colistin resistant \textit{A. baumannii} and \textit{K. pneumoniae}. To the best of our knowledge, this is the first example of a small molecule that directly modulates TCS signaling, reverses an antibiotic-resistance phenotype, and in the process breaks antibiotic resistance in Gram-negative bacteria. Serially passaging of either colistin-susceptible or colistin-resistant bacteria in the presence of compound 4.1 and colistin failed to deliver a resistant bacterial population, indicating that selection pressure to evolve resistance to this combination is severely reduced in comparison to conventional
antibiotics. Finally, compound 4.1 establishes that small molecule suppression of TCS activity is a viable approach to reversing antibiotic resistance and scaffolds based upon compound 4.1 or other small molecules that suppress TCS function can potentially be employed as adjuvants to restore antibiotic activity.

4.4: Experimental Section

Bacterial Strains, Media, and Antibiotics. Acinetobacter baumannii strains were obtained from the Walter Reed Army Institute of Research (3941, 3942, 4106, 4112, 4119) or The University of Maryland at Baltimore (A2, A3, A6, A7, B2, B5, D4, D5, D9, E1, E3, E4, E5, E6, E8, E9, F2, F3, F8, F9, G2, G3, G9, H1) or ATCC (19606) and colonies grown on nutrient agar. Klebsiella pneumoniae strains were obtained from The University of Maryland at Baltimore (A2, A5, B2, B3, B5, B6, B8, B9, C2, C3, C4, C5, D4, D7) and grown on nutrient agar. Mueller-Hinton II broth cation adjusted (CAMHB) (cat. # 212322) and nutrient agar (cat. # 213000) were purchased from BD Diagnostics. Colistin sulfate salt (cat. # C4461) was purchased from Sigma Aldrich. All assays were run in duplicate and repeated at least two separate times. All compounds were dissolved as their HCl salts in molecular biology grade DMSO as 100 mM or 10 mM stock solutions.

Broth Microdilution Method for Determination of Minimum Inhibitory Concentrations. A. baumannii or K. pneumoniae was grown in CAMHB for 6-8 h and this culture was used to inoculate fresh CAMHB (5 x 10^5 CFU/mL). The resulting bacterial suspension was aliquoted (5 mL) into culture tubes and compound, from a 100 mM or 10 mM DMSO stock, was added. Inoculated media not treated with compound served as the control. From each sample, 1 mL was transferred to a new culture tube and antibiotic, from
water stock, was added. Rows 2-12 of a 96-well microtiter plate were filled at 100 µL/well from the remaining inoculated media, allowing the concentration of compound to be kept uniform throughout the antibiotic dilution procedure. The samples containing antibiotic were then aliquoted (200 µL) into the corresponding first row wells of the microtiter plate. Row 1 wells were mixed 6 to 8 times then 100 µL was transferred to row 2. Row 2 wells were mixed 6 to 8 times, followed by a 100 µL transfer from row 2 to row 3. This procedure was repeated to serially dilute the rest of the rows of the microtiter plate, with the exception of the final row, to which no antibiotic was added (to check for growth of bacteria in the presence of compound alone). The plate was then covered with a lid and incubated under stationary conditions at 37 °C. After 16 h, minimum inhibitory concentration (MIC) values were recorded as the lowest concentration of antibiotic at which no visible growth of bacteria was observed.

**Evolution of Colistin Resistance A. baumannii 4106.** Colistin-resistant A. baumannii was grown in CAMHB overnight and this culture was used to inoculate fresh CAMHB (5 x 10^5 CFU/mL). Inoculated media was aliquoted (5 mL) into culture tubes and compound (30 μM) and colistin at 0.5X, 1.0X, and 2.0X its MIC were added. Growth at the highest concentration (compound/colistin combo) was carried forward and MICs (colistin only, colistin + 10 μM compound, colistin + 20 μM compound, colistin + 30 μM compound) were determined every one-two days. This was repeated for a period of seven days.

**Evolution of Colistin Resistance A. baumannii 19606.** Colistin-susceptible A. baumannii was grown in CAMHB overnight and this culture was used to inoculate fresh CAMHB (5 x 10^5 CFU/mL). Inoculated media was aliquoted (5 mL) into culture tubes and compound (5
μM or 10 μM) and colistin at 0.5X, 1.0X, and 2.0X its MIC were added. Growth at the highest concentration (compound/colistin combo) was carried forward and the colistin MIC was determined every one-two days. This was repeated for a period of seven days.

**Time Kill Curves.** *A. baumannii* was grown in CAMHB overnight and this culture was used to inoculate fresh CAMHB (5 x 10^5 CFU/mL). Inoculated media was aliquoted (3 mL) into culture tubes and compound and/or colistin were added, untreated inoculated media served as the control. Tubes were incubated at 37 ºC with shaking. Samples were taken at 2, 4, 8, and 16 h time points, serially diluted in fresh CAMHB and plated on nutrient agar. Plates were incubated at 37 ºC overnight and the number of colonies enumerated. Experiment was repeated four times for each data point.

**Bacterial Membrane Permeabilization Assay.** The BacLight assay (Invitrogen) was used to assess membrane permeability. *A. baumannii* was grown overnight in CAMHB at 37 ºC with shaking. The culture was diluted 1:40 in CAMHB and grown to an optical density at 600nm (OD_{600}) of ~1.0. The cultures were centrifuged at 10,000g for 15 min, and the cell pellet was washed once with sterile water, resuspended to 1/10 of the original volume and diluted 1:20 into either water or water containing test compounds. Suspensions were incubated at 37 ºC with shaking for 1 h then centrifuged at 10,000g for 10 min, washed once with sterile water, and resuspended in water. A 1:1 mixture of SYTO-9 and propidium iodide were added to the suspension (3 μL/mL) and mixed well. 100 μL of the suspension was added to each well of a 96-well plate. And the plates were incubated in the dark for 15 min at room temperature. Green fluorescence (SYTO-9) was read at 530 nm, and red fluorescence
(propidium iodide) was read at 645 nm (excitation wavelength, 485 nm). The ratio of green to red fluorescence was expressed as a percentage of the control.

**RNA Extraction Protocol and rtPCR.** *A. baumannii* was grown overnight in CAMHB, and sub-cultured 1:100 in the morning. Cells were grown to an OD₆₀₀ = ~0.6, then treated with compound for 1 hour. The cultures were mixed with bacterial RNA protect solution (Qiagen, cat. # 76506). Samples were centrifuged at 3,000 g for 20 mins and then cell pellets were collected and suspended in 1 mL TRIzol reagent (Invitrogen, cat. # 15596-026). Cell lysis was accomplished using a Branson sonifier 450. RNA was extracted and purified using a RNeasy mini kit, following the manufacturer's instructions (Qiagen, cat. # 74104). RNA was quantified using a NanoDrop ND-1000 spectrophotometer. Reverse transcriptase polymerase chain reaction (rtPCR) was accomplished using SuperScript III Platinum SYBR Green One-Step qRT-PCR Kit (Invitrogen, cat. # 11736-059) according to manufacturer's instructions. 16S rRNA gene was used as a reference gene in all experiments and relative levels of gene expression were calculated by Bio-Rad CFX Manager Software. All reactions were performed in triplicate using a C1000 Touch Thermal Cycler, CFX96 Real-Time System (Bio-Rad) on three independent samples. Forward and reverse primers for *pmrA, pmrB, pmrC,* and 16S rRNA are listed in Appendix as described previously. ²⁰
References


(17) Worthington, R. J.; Bunders, C. A.; Reed, C. S.; Melander, C. *ACS Medicinal Chemistry Letters*, 3, 357.


Appendix
## Appendix A - Forward and reverse primers

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<tr>
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</tr>
<tr>
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<td>GAACAGCTGAGCACCCTTTAA</td>
</tr>
<tr>
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<tr>
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<td>GTAAAAAGTAAAAACACCGACCA</td>
</tr>
<tr>
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