11-9-2018


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Structure-based Design, Synthesis and Applications of a New Class of Peptidomimetics:

\( \gamma \)-AA Peptides and Their Derivatives

by

Ma Su

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
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Keywords: \( \gamma \)-AA peptide, antimicrobial agents, OBTC library, drug resistance

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DEDICATION

To dear parents and friends.
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I would like to thank my research advisor, Dr. Jianfeng Cai, for the support and guidance he has given during my academic study at USF. I would like to thank him for his guidance, encouragement and patience.

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ABSTRACT

Peptidomimetics can mimic hierarchical structures of peptides and proteins. Thus, they are extensively studied for therapeutic applications. To break the limitation of backbones and frameworks and expand the peptidomimetics family, a new class of peptidomimetics - “γ-AApeptides” was developed. Design of γ-AApeptides is based on the chiral peptide nucleic acids (PNAs) backbone.

The World Health Organization estimates that one-third of all deaths in the world are on account of infectious diseases. AMPs are important because of their high activity against broad spectrum microbes, less susceptible to grow resistance and selectivity in binding to bacterial cells over human cells. γ-AApeptides as a new class of peptidomimetics have increased stability and enhanced chemical diversity. We have developed polymyxin mimic cyclic peptides, small linear molecules and hydantoin derivatives as potent antibiotic agents with γ-AApeptides. They have good bioactivity and selectivity.

Combinatorial library is key technology for accelerating the discovery of novel therapeutic agents. One-bead-two-compound γ-AApeptides-based library was developed and screened against SMYD2 protein which is essential for tumor growing.
CHAPTER 1: INTRODUCTION

1.1 Antibiotic resistance overview

As a major cause of morbidity and mortality, infectious diseases have threatened human health throughout the mankind history. The World Health Organization estimates that one-third of all deaths in the world are on account of infectious diseases\(^1\). Pneumonia and influenza are the fourth-leading causes of death in older adults, and bacteremia is the ninth-leading cause of death\(^2\). Discovery of penicillin in 1928 represented an start of “antibiotic” era\(^2\). The major death cause change from infectious diseases to the chronic diseases.

However, bigger problem has been resulted due to misuse and overuse antibiotics. Drug-resistant strains initially showed up in places where most antibiotics were being used very soon after the introduction of penicillin\(^3\). For example, sulfonamide-resistant Streptococcus pyogenes appeared in military hospitals in the 1930s\(^4\), and penicillin-resistant Staphylococcus aureus emerged in civilian hospitals in the 1940s\(^5\). The frequency of resistance promoted in many different bacteria due to the increasing antimicrobial use. In the late 1950s to early 1960s, multiple drugs resistance (MDR) was first detected among Escherichia coli, Shigella and Salmonella\(^6\)\(^-\)\(^7\). Treatment of MDR infections are more precarious, costly and sometimes unsuccessful because all available drugs have failed. For example, E. faecium have plagued immunocompromised individuals in hospitals in the United States and other places for more than a decade as a MDR strain\(^8\). Methicillin-resistant Staphylococcus aureus (MRSA) has been the most frequently isolated resistant pathogens in the older population and was one of the earlier resistant pathogens described as a major clinical problem in long-term care facilities\(^9\).
Unfortunately, resistance to methicillin is not the only issue; MRSA is generally resistant to all quinolones and cephalosporins as well.\textsuperscript{10} Although vancomycin has become the main therapy for MRSA, recent studies point out the worrying evidence that Staphylococcus aureus with resistance to vancomycin already emerged. Among the Gram-negative bacteria, hospital infections caused by P. aeruginosa and A. baumanii can result in death because of their resistance to most antibiotics\textsuperscript{11}. The frequency of drug resistance emergence has extended the problem beyond the hospital and can be traced to the community.

What causes drug resistance? Microbes have been existing since primitive times. Their evolution to adapt themselves to innumerable ecological microcosms already took over a period of 3.5 billion years. Due to their rapid rate of multiplication and genetic makeup ability, microbes can survive from and adapt to environment changes by incorporating all the beneficial mutations to evolve. The antibiotic selects the resistant organisms by inhibiting susceptible ones, and the determinant resistance genes are selected\textsuperscript{12}. Under continued antimicrobial selection, resistance genes can be spread and propagated. Furthermore, the genes for resistance traits can be transferred among different bacteria groups by means of mobile genetic elements such as plasmids, transposons, naked DNA or bacteriophages\textsuperscript{13}. Resistance mechanisms are varied.

Resistance mechanisms vary. One type of mechanisms is directed at the antibiotic itself. For example, it was discovered that an enzyme produced by bacteria can destroy the $\beta$-lactam ring of antibiotics\textsuperscript{14}. Targeting drug transportation and altering intracellular target of the drug are other two types of mechanisms\textsuperscript{15}. It is also indicated that losing drug resistance is slow, even in the absence of the selecting antibiotic.

One of the approaches to manage and prevent drug resistance is continuing, steady development
of new antibiotics. From what we have learned, shorter-course therapies with highly active antibiotics will reduce multidrug resistance. The development of new antibiotics with different mechanisms—either attack new targets or circumvent resistance mechanisms—is essential.

### 1.2 Antimicrobial peptides (AMPs) overview

In 1922, the first bacteriolytic enzyme was found from nasal mucous by Alexander Fleming. Later in 1928, Fleming discovered penicillin extracted from *Penicillium notatum*.\(^{14}\) Subsequently, Stephens and Marshall isolated antimicrobial peptides (AMPs) from hemolymph of wax moth larvae in 1962.\(^{16}\) AMPs are defined as peptides produced by immune system to protect the body from bacteria, fungi and viruses and can be found in all forms of life in variable sequences. AMPs can be classified into different classes due to the secondary structure in solution: \(\alpha\)-helical peptides, \(\beta\)-sheet peptides, extended peptides and loop peptides.\(^{17}\) The most common characteristics of AMPs are positive charge and hydrophobic residues. Based on their different action modes, AMPs have been subdivided into two classes: membrane permeabilization and intracellular targeting.\(^{18}\) Membrane permeabilization is the most common mode of action. The cationic groups electrostatically attract and bind to bacteria membrane with negative charge and hydrophobic groups interact with lipid bilayers to burrow and penetrate into the bacterial cell.\(^{19-20}\) Different types of model mechanisms have been proposed: barrel stave model, carpet model and toroidal model.\(^{21}\) Intracellular targeting includes altering the cellular pathways of the bacteria and inhibiting the DNA, RNA and folic acid synthesis.\(^{22}\)

AMPs are important because of their high activity against broad spectrum microbes, less susceptible to grow resistance and selectivity in binding to bacterial cells over human cells.\(^{23}\) However, AMPs have to overcome limitations such as proteolytic degradation, rapid metabolism, poor pharmacodynamic and pharmacokinetic properties.\(^{24}\) To increase their potential as drug, synthesis of peptidomimetics is needed to
mimic the action mode and decrease the limitations.\textsuperscript{25}

1.3 $\gamma$-AA peptide overview

In the past decade, peptidomimetics offer important applications and exciting approaches in chemical biology\textsuperscript{26}. Due to their unnatural backbones, peptidomimetics could overcome obstacles of conventional peptides, including susceptibility to enzymatic hydrolysis, low bioavailability, and limited chemodiversity\textsuperscript{27}. Peptidomimetics can mimic hierarchical structures of peptides and proteins. Thus, they are extensively studied for therapeutic applications, such as peptoids\textsuperscript{28}, $\beta$-peptides\textsuperscript{29}, $\alpha/\beta$-peptides\textsuperscript{30}, oligoureas\textsuperscript{31}, azapeptides\textsuperscript{32}, and so forth.

To break the limitation of backbones and frameworks and expand the peptidomimetics family, a new class of peptidomimetics - “$\gamma$-AApeptides” was developed. Design of $\gamma$-AApeptides is based on the chiral peptide nucleic acids (PNAs) backbone\textsuperscript{33}. They are termed $\gamma$-AApeptides, as they are oligomers of $\gamma$-substituted-N-acylated-N-aminoethyl amino acids\textsuperscript{34}. As peptidomimetics, $\gamma$-AApeptides also possess resistance to proteolytic degradation and enhanced chemodiversity. In addition, one of the most attractive features of $\gamma$-AApeptides is that the secondary amines on the backbone can react with innumerable agents besides carboxylic acids to introduce half of their side chains.

We have developed several intriguing structures and applications of $\gamma$-AApeptides. For example, they could permeate cell membranes and specifically bind to HIV RNA by mimicking the Tat peptide\textsuperscript{35}. We also rationally designed some $\gamma$-AApeptides for a range of biological applications. By mimicking host-defense peptides (HDPs), $\gamma$-AApeptides displayed potent and broad-spectrum activity against a panel of drug resistant bacterial pathogens\textsuperscript{36}. Meanwhile, $\gamma$-AApeptides are also capable for combinatorial library screening because of their stability against proteolysis and their chemodiversity\textsuperscript{37}. Through combinatorial selection, certain $\gamma$-AApeptides can disrupt A$\beta$ peptide aggregation, and few $\gamma$-
AApeptides can bind to the DNA-binding domain of STAT3 and inhibit STAT3/DNA interactions. 

Our studies suggest that γ-AApeptides can mimic primary and secondary structures of bioactive peptides. In addition, they are promising candidates for combinatorial development for the identification of molecular probes and potential drug leads. With further development of both structures and functions, γ-AApeptides may play an important role in biomedical sciences as a new class of peptidomimetics.

1.4 Combinatorial library overview

In the 1990s, combinatorial chemistry has drawn substantial attention from the pharmaceutical industry due to its ability of greatly facilitating drug discovery process. Combinatorial chemistry is considered as one of the most important advances in medicinal chemistry. Moreover, it is also an enormously powerful tool in basic research.

Combinatorial library methods have various applications in many fields, such as peptides, oligonucleotides, proteins, synthetic oligomers, small molecules, and oligosaccharides. Three main steps are involved in all combinatorial library methods: (i) prepare the library, (ii) screen the library, and (iii) determine the chemical structures of active compounds. In general, combinatorial libraries are prepared on a solid phase. Manageably large collection of amino acids and well development of solid-phase coupling make solid-phase peptide synthesis become the combinatorial library approach. A beaded polymer using as solid phase needs to meet certain criteria according to the synthetic and screening approaches: size uniformity, substitution homogeneity, resistance to clusters formation and ability to swell in both organic and aqueous solvents. TentaGel was discovered as the choice of solid support for combinatorial library synthesis due to its uniformity in size and nonstickiness. In addition, functionalizable groups of TentaGel are located at the end of polyoxyethylene chains, which is essential for compound display on the resin bead surface for binding.
The one-bead-one-compound concept was first brought forward by Lam et al.\textsuperscript{51}. This library was prepared using a “split synthesis” approach (Figure 2), which was described recognized by Furka et al. in 1988. The synthesis of this library begins with \( x \) portions of the resin beads coupling with \( x \) different building blocks. Each portion is then divided into \( y \) pieces and distributed into \( y \) reaction vessels to react with \( y \) different reagents. The process can be repeated as many times as required and will produce a library of \( x \times y \times \ldots = N \) compounds on \( N \) resin beads.

Cleavable linkers fulfilling several main requirements are needed for screening and structures determining: (i) ability to stay stable during all the reactions of library synthesis, (ii) end products are readily cleaved from the resin beads, (iii) library compounds will not degrade in cleavage process, (iv) user friendly and provide the released compounds ready for screening.\textsuperscript{39} There are two screening approaches for on-bead screening: target binding to the ligand or other functional properties of the ligand. The binding of target to bead-bound ligand can be detected either by direct visualization such as color target, or indirectly by using a reporter group such as an enzyme or a fluorescent probe attached to a target.\textsuperscript{39} The most expedient method for compound detachment is exposing resin beads to gaseous reagents, such as ammonia, cyanogen bromide and hydrogen fluride.
Mass spectrometry is a very sensitive analytical tool for library characterization. Mass spectrometry can identify complement amino acid sequencing, unnatural amino acids sequences and all types of impurities from the synthesis or cleavage process. In addition, mass spectrometric analysis of several hundred samples can be performed automatically.

The molecular biology revolution enables researchers to clone and express biological receptors, enzymes, and proteins routinely. Many new drug targets for various diseases have now been identified. Since the initial publication of the one-bead-one-compound combinatorial library method, there have been numerous applications to various biological targets such as monoclonal antibodies, streptavidin/avidin, protein kinase, protease, and anticancer agent. The development of combinatorial chemistry is timely and undoubtedly will contribute to discovering new drugs that can benefit the whole mankind. Since peptide-protein, protein-nucleic acid, and protein-protein interactions represent the major molecular interactions and signal in living cells and organisms, peptide library methods will keep playing an important role in identifying drug leads and developing drug candidates.

Figure 1.1 Scheme of the “split synthesis” method. (Adapted with permission from Nature 1991, 354, 82. Copyright 1997 Springer Nature.)
CHAPTER 2: MEMBRANE-ACTIVE HYDANTOIN DERIVATIVES AS ANTIBIOTIC AGENTS

Note to Reader

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2.1 Introduction

Infection caused by drug-resistant bacteria has become one of the greatest threats to public health in the 21st century. Exploration for alternative therapeutic strategies is in a huge demand. One promising approach is to reinvestigate known antibiotics and design their derivatives, in the hope of identifying novel antibiotic agents that combat antibiotic resistance. Hydantoins, the derivatives of 2,4-imidazolidinedione, have been developed for antibacterial applications for long time. The mechanism of action for hydantoin derivatives is complex and not well understood, possibly due to their damage to bacterial DNA, as well as binding to bacterial ribosomes to inhibit synthesis of critical bacterial enzymes. To date, one hydantoin derivative, nitrofurantoin, was approved to treat urinary tract infections. As an old antibiotic, it recently attracted considerable interest due to their low probability of bacterial resistance compared to other conventional antibiotics such as fluoroquinolones, possibly owing to their mixed mechanisms of action. However, hydantoin derivatives including nitrofurantoin generally exhibit only moderate antibacterial activity, which may limit their further application in combating emergent antibiotic resistance. For instance, nitrofurantoin (Figure 2.1) show a MIC of 12.5 µg/mL for MRSA, and it is even not active towards P. aeruginosa up to 100 µg/mL.
Another alternative strategy to combat antibiotic resistance is to develop cationic host-defense peptides (HDPs) as potential antibiotic agents. Containing hydrophobic and cationic groups, HDPs and related peptidomimetics such as β-peptides, oligoureas, peptoids, AApeptides, etc., are able to selectively interaction with negatively charged bacterial membranes, leading to membrane damage and cell death. Cationic charges are critical for association of these molecules with bacterial membranes, while hydrophobic groups are of importance for membrane penetration and disruption. HDPs and their derivatives are believed to minimize the potential of bacterial resistance development, as the membrane interaction and disruption is rather biophysical and lack specific membrane targets. It should be noted that many HDPs could also permeate bacterial membranes and act on bacteria intracellular targets. Indeed, the mixed antibacterial mechanisms are expected to further strengthen their ability to combat bacterial resistance. Despite considerable enthusiasm, HDPs and oligomeric peptidomimetics encounter obstacles for antibiotic development, including moderate activity and systematic toxicity. In addition, their large molecular weights (normally >1000 Da) and structural complexity lead to tedious synthetic process and costly production, hampering the therapeutic development of HDPs.

Inspired by the structures of hydantoin derivatives such as nitrofurantoin and the mechanism of action of HDPs, herein we propose to design a new class of hydantoin based small molecules, so as to enhance and revitalize the antibacterial activity of old antibiotics by conferring their ability for bacterial membrane association. It is well known that the existing lipo-antibiotics including marketed drugs
daptomycin\textsuperscript{107-108} and polymyxin\textsuperscript{109-110}, the two “last-resort” antibiotics, all associate and interact with bacterial membranes using their lipid tails. Our previous findings also indicate that cationic peptidomimetics with lipidation could kill bacteria with greater potency by disrupting bacterial membranes.\textsuperscript{111-115} Thus, we hypothesized that hydantoin compounds bearing cationic groups and lipid tails would be membrane active, similar to the mechanism of action of HDPs (Figure 2.2, D2). As such, they could specifically interact with bacterial membranes and kill bacterial pathogens through bacterial membrane disruption. In addition, as the compounds still contain the hydantoin pharmacophore (Figure 2.2, D1), they could also pass bacterial membranes and directly act on the potential targets such as DNAs and ribosomes,\textsuperscript{74-75} analogous to nitrofurantoin. The synergistic effect on bacterial killing could lead to a new generation of antibiotics with high potency and novel mechanisms, as well as less probability for resistance development. Herein, we report the design, synthesis, and investigation of hydantoin derivatives containing hydrophobic tails and cationic charged groups. Our studies show that these compounds exhibit much enhanced antimicrobial activity against both Gram-positive and Gram-negative bacteria compared to nitrofurantoin (>50 fold for certain strains), including clinically relevant multidrug resistant bacterial strains. The lead compound also shows excellent in vivo activity towards MRSA-induced pneumonia on a rat model by eradicating MRSA and suppressing lung inflammation caused by pneumonia.

![Figure 2.2](image_url)

**Figure 2.2** Design of hydantoin compounds with membrane-acting capability. D1, the hydantoin core, in which R1 represents hydrophobic groups; D2, membrane interacting domain, in which R2 is the cationic group, and Cx represents lipid tails.
2.2 Results and Discussion

Our attempted to design membrane-active hydantoins in a very straightforward manner, making it possible for convenient optimization and production in the future. As shown in Figure 2, R2 was designated to be cationic NH2 group, R1 were hydrophobic groups, and Cx were lipid tails. The synthesis was also very straightforward (Figure 2.3), which allowed a series of compounds to be prepared rapidly on the solid phase. Briefly, the alloc protected building block\textsuperscript{[116-117]} bearing the R2 side chain was attached to Rink-amide resin. After the alloc group was removed, the R1NCO was added to react with the secondary amine to introduce urea functionality. Next, the alloc protecting group was removed, followed by reaction with the CxCOCl to introduce the lipid tail. The molecule was then cleaved from the solid support in the presence of 1:1 TFA/DCM, which cyclized spontaneously in situ to yield the desired hydantoin product with good yield.

![Diagram](image)

\begin{tabular}{|c|c|c|}
\hline
R₁ & R₂ & Cx \\
\hline
1 & \text{ } & \text{ } \\
2 & \text{ } & \text{ } \\
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\end{tabular}

\textit{Figure 2.3} The general approach to synthesize cationic lipidated hydantoins.
Table 2.1 Activity of cationic lipidated hydantoins.

<table>
<thead>
<tr>
<th>Compound</th>
<th>MIC (µg/mL)</th>
<th>Gram Positive</th>
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<tbody>
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<td></td>
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<td>MRSA</td>
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<tr>
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Table 2.1 Continued Activity of cationic lipidated hydantoins.

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<td>12.5</td>
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Bacteria included in the test were Methicillin-resistant *S. aureus* (MRSA) (ATCC 33591), Methicillin-resistant *S. epidermidis* (MRSE) (RP62A), vancomycin-resistant *E. faecalis* (ATCC 700802), *E. coli* (ATCC 25922), *P. aeruginosa* (ATCC 27853), *K. pneumoniae* (ATCC 13383) \(^{113, 118}\). Minimum concentration (MIC) was measured after incubating hydantoins with bacteria for 16 h. Nitrofurantoin (compound 26) was included in the test as the positive control.

Subsequently, these cationic hydantoin derivatives were tested for their antimicrobial activity against a panel of Gram-positive and Gram-negative bacteria, including multidrug-resistant clinically relevant strains. The compound 26, nitrofurantoin, exhibits antimicrobial activity against most of bacteria with MICs ranging from 6.25 to 25 \(\mu\)g/mL, which are highly consistent to previously reported antibacterial activity.\(^{79, 119}\) Although nitrofurantoin is the preferred antibiotic to treat bladder and urinary tract infections, as shown above, its antibacterial activity is moderate. It should also be noted that under the tested condition, nitrofurantoin failed to show any activity toward *Pseudomonas aeruginosa*, a notorious Gram-negative strain which could cause severe or even lethal infection.

As the proof of concept, we fixed the cationic group R2 shown in Figure 2, and explored the activity of the compounds with respect to the variation of the hydrophobic group R1 and the lipid tail Cx. When R1 is the ethyl group, with a C10 decanoic tail, the compound 1 doesn’t display any activity against all tested bacterial strains. It could be due to insufficient hydrophobicity of the ethyl group and short length of the lipid tail, rendering the compound 1 ineffective to interact with bacteria. It is therefore reasonable to observe that the antimicrobial activity of compounds 2 and 3 increase as their lipid tails
become longer, which makes them more membrane active. Indeed, compound 3 already has comparable activity to nitrofurantoin against most strains; in addition, it shows an encouraging MIC of 12.5 µg/mL toward *P. aeruginosa*, suggesting that hydantoin compounds could be developed to identify more potent antibiotic agents. However, it is intriguing that the longer lipid tail doesn’t necessary lead to more potent antimicrobial agents. As seen for 4, which contains the same ethyl group for R1 but a C16 palmitic lipid tail, albeit exhibiting enhanced activity against Gram-positive bacteria, abolishes its activity toward *P. aeruginosa*. It clearly implies that both hydantoin core (D1) and membrane interacting domain (D2) are required for good antimicrobial activity, and a balance of hydrophobicity for R1 and lipid tail length for Cx is needed. With the preliminary studies on this series of ethyl-containing hydantoins, we set out to test the activity of a few more compounds containing R1 of increased hydrophobicity. With the enhanced rigidity and hydrophobicity, the compounds are more active, even though they still contain the C10 lipid tails. For instance, compound 5, containing the butyl group, doesn’t have any antimicrobial activity. However, bearing the cyclohexyl group for R1, compound 9 starts to show activity toward a few bacterial strains. While with the adamantyl group, compound 13 already exhibits decent activity across the panel of bacteria. Again, in each series of compounds bearing the same R1 group, increasing the length of the lipid tail lead to enhancement and then detriment in antimicrobial activity. Consistent to the findings in the initial investigation, it seems that the compounds containing C12 or C14 tail possess the optimal antimicrobial activity. As aromatic groups are frequently identified in vast majority of antibiotic agents, we next tested the activity of hydantoins containing phenyl groups as the R1 group. Although para-methoxybenzyl group-containing compounds 17-20 do not yield better antibiotic agents, hydantoin possessing the meta-chlorobenzyl groups lead to new compounds with potent and broad-spectrum activity. The most potent compound 22 exhibits MICs less than 1 µg/mL against all tested
Gram-positive and Gram-negative strains. Compared with nitrofurantoin, it is 25-fold as effective toward MRSA, and at least 50-fold more effective toward *P. aeruginosa*. We lastly examined the impact of the cationic charge R2 on the antimicrobial activity. Surprisingly, replacement of the cationic aminobutyryl group in 22 with the phenyl group gives the highly hydrophobic compound 25, which fails to show any antimicrobial activity. It strongly supports that the cationic charge is of vital importance for antimicrobial activity, possible due to its ability for electrostatic interaction with negatively charged bacterial membranes.

**Table 2.2** Selectivity of hydantoin compounds.

<table>
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<tr>
<th>compound</th>
<th>MIC of MRSA (µg/mL)</th>
<th>HC&lt;sub&gt;50&lt;/sub&gt; (µg/mL)</th>
<th>SI (MIC/HC&lt;sub&gt;50&lt;/sub&gt;)</th>
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</tr>
<tr>
<td>26</td>
<td>12.5</td>
<td>&gt;200</td>
<td>&gt;16</td>
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HC<sub>50</sub> is the hemolytic activity of the compounds. SI is selectivity index, which is the ratio of MIC for MRSA to hemolytic activity. Nitrofurantoin (compound 26).

In order to evaluate the therapeutic potential of these hydantoin compounds, we next investigated the selectivity of the lead compounds which exhibited broad-spectrum antimicrobial activity against all tested strains. As shown in Table 2.2, these compounds all have better antimicrobial activity than nitrofurantoin 26, while exhibiting limited hemolytic activity. Most noticeably, the most potent compound 22, with much enhanced activity compared with nitrofurantoin, show excellent selectivity toward MRSA bacteria.
As aforementioned, the antimicrobial mechanisms of hydantoins are complex and elusive, however, since the compounds developed here were expected to at least possess the mechanism of action analogous to HDPs, their impact on bacterial membranes could be investigated by fluorescent microscopic studies.\textsuperscript{113,120} The most potent compound 22 was thus studied for its ability to compromise bacterial membranes of Gram-positive bacteria MRSA and Gram-negative bacteria \textit{E. coli}.\textsuperscript{66,121} Two dyes, 4',6-diamidino-2-phenylindole (DAPI), and propidium iodide (PI) (Figure 2.4), were used to differentiate cells with either intact or damaged membranes. DAPI could permeate intact cell membrane and as such it shows blue fluorescence regardless of cell viability. In contrast, PI is a DNA intercalator but lack of cell permeability. It only fluoresces in red color when cell membranes are disrupted. As shown in Figure 4, under the DAPI channel, both MRSA and \textit{E. coli} exhibit blue fluorescence (Figure 2.4a1 and 2.4a3) in the absence of the compound 22, whereas none of them display fluorescence under the PI channel (Figure 2.4b1 and 2.4b3), indicating the membranes of these bacteria were intact. However, after bacteria including both MRSA and \textit{E. coli} were incubated with 22 for 2 h, they are able to be stained by both DAPI and PI channels (Figure 2.4a2, 2.4b2, 4a4, 4b4), suggesting the membranes of both MRSA and \textit{E. coli} were damaged.
HDPs are well known for their ability to eradicate bacteria rapidly, due to their membrane disruptive bactericidal mechanism.\textsuperscript{122} It is compelling to know if our newly synthesized hydantoin compounds exhibit similar bacterial killing kinetics. As such, we subsequently carried out time-kill studies for 22 at different concentrations toward MRSA and \textit{E. coli}, respectively. As shown in Figure 2.5, at 25 and 50 \(\mu\)g/mL, 22 could completely eradicate MRSA in just 10 min (Figure 2.5a). Even at 12.5 \(\mu\)g/mL, MRSA were thoroughly removed in 30 min. Killing \textit{E. coli} is relatively slower, however, all bacteria were still eradicated in 30 min at all concentrations. It demonstrates that this class of
hydantoin compounds could rapidly kill both Gram-positive and Gram-negative pathogens, analogous to HDPs.

**Figure 2.5** Time-kill plots of 22 against MRSA (a) and E. coli (b).

One of the most appealing features of HDPs is that they do not readily elicit bacteria resistance, as they disrupt bacterial membranes rather than acting on specific targets.\(^{123}\) Since compound 22 was designed to be membrane active, in addition to the mechanism of action due to the hydantoin core, we hypothesized that 22 could also prevent the resistance development in bacteria. As such, we conducted the drug resistance studies for 22 against MRSA. 22 were incubated with bacteria at the concentration of half MIC overnight, and the new MIC was measured subsequently. It is intriguing that after 14 passages, MICs of 22 virtually remain unchanged (Figure 2.6), which strongly suggests that this class of hydantoin compounds do not readily induce resistance in bacteria, thereby augmenting their potential therapeutic applications.
Figure 2.6 Drug resistance study for compound 22.

One of the major causes of pneumonia is bacterial infection. Hospital-acquired and community-acquired MRSA pneumonia has become more prevalent in recent years and presented significant therapeutic challenges due to its increasing motility. As our hydantoin compounds exhibited potent in vitro antibacterial activity against both Gram-positive and Gram-negative bacteria including MRSA, it was intriguing to investigate their in vivo activity so as to assess their therapeutic potential. As such, we tested their efficacy on a rat model bearing MRSA pneumonia induced by intratracheal instillation. As shown in Figure 2.7, the control group, which was treated with PBS only, exhibited high level of MRSA after bacterial inoculation. The slightly decreased quantity of bacteria on day 5 compared with day 3 suggested that rats may fight MRSA through their host immune response. However, the impact on the bacterial clearance was very minimal. Vancomycin, which has long been considered as the “last-resort” antibiotic to treat infections caused by multidrug-resistant Gram-positive pathogens, was included as a positive control. As shown in Figure 2.7, vancomycin did exhibit efficacy in the inhibition of MRSA proliferation. On the day 3 after its administration, it
exhibited ~30% reduction of bacteria compared with the control. On the day 5, the reduction increased ~45%. It indicates that vancomycin could help to clear MRSA from lungs of rats. However, the compound 22 displayed a much superior in vivo efficacy in the eradication of MRSA bacteria. On the day 3 after the treatment of 22, a ~70% reduction in bacteria was observed. On the day 5, compared to the control, MRSA was reduced by 96%. The remarkable potency of 22 relative to the control and vancomycin demonstrates its future therapeutic potential.

![Figure 2.7](image)

**Figure 2.7.** In vivo efficacy of the compound 22 on a rat model bearing MRSA pneumonia bearing. Rats (n = 6 per group) were inoculated with 100 µL of 10^6 CFU/mL MRSA by intratracheal instillation, followed by i. v. injection of 22 (10 mg/kg) in the tail vein after 24h.

Given the findings that 22 could virtually completely eradicate the MRSA bacteria in lungs, we subsequently conducted the pathological analysis to find out if 22 could suppress lung inflammation induced by MRSA. As shown in Figure 8, all three groups (PBS control, vancomycin and 22) all demonstrate normal conditions right after MRSA inoculation (0 day), which suggests that the inflammation has not developed in lungs. However, the inflammation in the control group elevated rapidly due to the lack of treatment, as seen for the presence of heavily populated inflammatory cells including monocytes, macrophages, neutrophils, eosinophils, etc (blue spots in H&E staining) on the
day 5, which is the typical indication of severe lung pneumonia. Rats treated with vancomycin showed alleviated condition due to its ability to inhibit the proliferation of MRSA, which lowered down the inflammation. Remarkably, treatment with the compound 22 exhibited much more significant impact on the suppression of lung inflammation. On the day 3, only mild inflammation was observed, whilst on the day 5, the inflammation was further mitigated to almost the normal condition. The findings, consistent to the abovementioned MRSA proliferation studies, strongly suggest that the compound 22 could be superior to vancomycin as a novel therapeutic strategy to treat MRSA pneumonia.

![Pathological assay based on hematoxylin and eosin (H&E) staining.](image)

**Figure 2.8** Pathological assay based on hematoxylin and eosin (H&E) staining.

### 2.3 Conclusions

In summary, we have reported a new class of hydantoin derivatives as the potential antibiotic agents. These molecules, bearing cationic charge and hydrophobic lipid tail, were designed to be membrane active in bacterial killing. They exhibited significantly more potent antimicrobial activity against a panel of multidrug-resistant Gram-positive and Gram-negative bacteria compared with the
marketed antibiotic nitrofurantoin, a hydantoin derivative. Although mechanisms of action for hydantoin compounds are known to be complex, our investigation demonstrated that the hydantoin compounds reported here could compromise bacterial membranes and kill bacteria rapidly and do not induce resistance in MRSA even after 14 passages, which is similar to host-defense peptides (HDPs). Moreover, these molecules have also exhibited remarkable in vivo efficacy on a rat model bearing MRSA-induced pneumonia, by effectively eradicating MRSA bacteria and suppressing lung inflammation, which is superior to vancomycin. Together with the facile synthesis, these compounds could be an appealing class of antibiotic agents to combat emergent drug-resistance. Further optimization of lead compounds and efficacy studies on other in vivo models are currently underway.

2.4 Experimental

2.4.1 General information

Rink amide MBHA resins (0.7 mmol/g, 200–400 mesh) were purchased from Chem-Impex Int’l Inc. Solvents and other chemicals were ordered from either Fisher Scientific or Sigma-Aldrich, and were used without further purification. The $^1$HNMR spectra were obtained on a Varian Inova 400 instrument. The solid phase syntheses of all compounds were carried out in a peptide reaction vessel on a Burrell Wrist-Action shaker. All compounds were analyzed and purified using the Waters Breeze 2 HPLC system under 215 nm of UV detector equipped with both analytical and preparative modules. The desired fractions were lyophilized on a Labconco lyophilizer. Molar masses of compounds were identified by Agilent Technologies 6540 UHD Accurate-Mass Q-TOF LC/MS spectrometer.

2.4.2 Synthesis of γ-AAppeptide building blocks
Both γ-AA peptide building blocks shown above were used in the synthesis of hydantoin compounds; their synthetic procedure was reported previously.  

### 2.4.3 Synthesis of hydantoin compounds

![Figure 2.10 General synthetic procedure for hydantoin compounds.]

Synthetic procedure of the compound 22: 200 mg Rink-amide (MBHA) resin (0.14 mmol) was treated with 3 mL 20% piperidine/DMF (v/v) solution for 15 min (× 2) to remove the Fmoc protection group, followed by DMF (2 mL × 3) and DCM (2 mL × 3) wash. The attachment of the γ-AA peptide building block to the resin was achieved by adding γ-Lys-BB (238 mg, 0.4 mmol), DIC (101 mg, 114 μL, 0.8 mmol), and HOBt (122 mg, 0.8 mmol) in 3 mL DMF to the reaction vessel, and the reaction was allowed to shake at room temperature for 3 h. The solution was drained, and the beads were washed with DCM (3 mL × 3) and DMF (3 mL × 3). After that, beads were treated with Pd(PPh3)4 (24 mg, 0.02 mmol) and Me3NH.BH3 (70 mg, 1.2 mmol) in 3 mL DCM for 10 min (×2) to remove the alloc protein group, then washed with DCM (3 mL x3) and DMF (3 mL × 3). Next, 3-chlorophenyl isocyanate (77 mg, 61 μL, 0.5 mmol) and DIPEA (65 mg, 87 μL, 0.5 mmol) in 3 mL DCM were added to the resin and
allowed to react for 30 min at room temperature, and then the solution was drained. After DMF (2 mL ×3) and DCM (2 mL ×3) wash, beads were treated with 3 mL 20% piperidine/DMF (v/v) solution for 15 min (× 2) to remove the Fmoc protection group, followed by wash with DMF (2 mL ×3) and DCM (2 mL ×3). Subsequently, lauric acid (80 mg, 0.4 mmol), DIC (101 mg, 114 μL, 0.8 mmol), and HOBt (122 mg, 0.8 mmol) in 3 mL DMF were added to the reaction vessel and reacted for 3 h. After the solution was drained, the beads were washed with DMF (2 mL ×3) and DCM (2 mL ×3), followed by the incubation with 4 mL cocktail of 1:1 TFA: DCM 1:1 (v/v) for 2 h to achieve cleavage and global deprotection of the compound. After the solvent was removed in vacuo, the residue was analyzed and purified on the Waters HPLC system, and the desired fraction was lyophilized to give the pure product 22 which was subsequently characterized by NMR and MS.

Synthesis of other compounds: The other compounds were synthesized following the similar procedure of compound 22.

**Compound 1**

\[ {^1}H NMR (400 MHz, d6-DMSO) \delta 7.73 (brd, 3H), 7.61 (d, J=8.0 Hz, 1H), 4.01 (d, J =6.8 Hz, 1H), 3.81-3.89 (m, 2H), 3.33-3.36 (m, 3H), 3.09 (dd, J = 14.0, 4.4 Hz, 1H), 2.65-2.74 (m, 2H), 1.96 (t, J = 16.0 Hz, 2H), 1.31-1.49 (m, 6H), 1.10-1.30 (m, 14H), 1.01 (t, J = 14.4 Hz, 3H), 0.81 (t, J = 16.0 Hz, 3H). \]

\[ {^{13}C} NMR (125 MHz, d6-DMSO) \delta 173.0, 170.6, 156.8, 50.3, 46.8, 46.7, 39.1, 35.9, 33.4, 31.6, 31.0, 29.2, 29.1, 29.1, 28.9, 27.0, 25.7, 22.8, 22.5, 14.3, 13.6. \] HRMS (ESI) C_{21}H_{40}N_{4}O_{3} [M+H]^+ calc’d = 397.3175; found = 397.3176.

**Compound 2**

\[ {^1}H NMR (400 MHz, d6-DMSO) \delta 7.70 (brd, 3H), 7.62 (d, J=8.8 Hz, 1H), 3.80-4.05 (m, 3H), 3.31-3.36 (m, 3H), 3.03 (dd, J = 14.0, 4.0 Hz, 1H), 2.67-2.75 (m, 2H), 1.97 (t, J = 7.2 Hz, 2H), 1.36-1.49 (m, 6H), 24
1.10-1.25 (m, 18H), 1.02 (t, $J = 7.2$Hz, 3H), 0.82 (t, $J = 6.4$Hz, 3H). \textsuperscript{13}CNMR (125 MHz, d$_6$-DMSO) \\
$\delta$ 173.0, 170.7, 156.8, 50.3, 46.9, 46.7, 36.0, 33.4, 31.7, 31.0, 29.4, 29.4, 29.3, 29.2, 29.1, 29.0, 27.7, 25.7, 22.8, 22.5, 14.3, 13.7. HRMS (ESI) C$_{23}$H$_{44}$N$_4$O$_3$ [M+H]$^+$ calc’d = 425.3485; found = 425.3486.

**Compound 3**

$^1$HNMR (400 MHz, d$_6$-DMSO) $\delta$ 7.75 (brd, 3H), 7.63 (d, $J = 8$Hz, 1H), 4.02 (d, $J = 17.2$Hz, 1H), 3.85 (d, $J = 7.2$Hz, 1H), 3.31-3.33 (m, 3H), 3.03 (dd, $J = 14.0$, 4.0 Hz, 1H), 2.70-2.73 (m, 2H), 1.97 (t, $J = 7.2$Hz, 2H), 1.29-1.48 (m, 8H), 1.02 (t, $J = 7.2$Hz, 3H), 0.82 (t, $J = 6.4$Hz, 3H).

$^{13}$CNMR (125 MHz, d$_6$-DMSO) $\delta$ 172.8, 170.7, 156.8, 50.3, 46.9, 46.7, 39.3, 39.1, 36.0, 33.4, 31.7, 31.1, 29.4, 29.3, 29.2, 29.1, 29.0, 27.1, 25.7, 22.9, 22.5, 14.4, 13.7. HRMS (ESI) C$_{25}$H$_{48}$N$_4$O$_3$ [M+H]$^+$ calc’d = 453.3799; found = 453.3800.

**Compound 4**

$^1$HNMR (400 MHz, d$_6$-DMSO) $\delta$ 7.77 (brd, 3H), 7.62 (d, $J = 9.2$Hz, 1H), 3.79-4.09 (m, 3H), 3.30-3.37 (m, 3H), 3.08 (dd, $J = 14.0$, 4.0 Hz, 1H), 2.65-2.75 (m, 2H), 1.96 (t, $J = 7.6$Hz, 2H), 1.27-1.53 (m, 6H), 1.03-1.24 (m, 26H), 1.01 (t, $J = 7.2$Hz, 3H), 0.81 (t, $J = 6.4$Hz, 3H). $^{13}$CNMR (125 MHz, d$_6$-DMSO) $\delta$ 172.8, 170.6, 156.8, 50.3, 46.9, 46.7, 39.1, 36.0, 33.4, 31.7, 31.1, 29.5, 29.3, 29.2, 29.1, 29.1, 27.1, 25.7, 22.9, 22.5, 14.4, 13.7. HRMS (ESI) C$_{27}$H$_{52}$N$_4$O$_3$ [M+H]$^+$ calc’d = 481.4112; found = 481.4110.

**Compound 5**

$^1$HNMR (400 MHz, d$_6$-DMSO) $\delta$ 7.72 (brd, 3H), 7.62 (d, $J = 8.8$Hz, 1H), 3.85 (d, $J = 17.2$Hz, 2H), 3.26-3.45 (m, 3H), 3.00-3.15 (m, 1H), 2.68-2.73 (m, 2H), 1.96 (t, $J = 14.8$Hz, 2H), 1.32-1.52 (m, 8H), 1.10-1.25 (m, 16H), 0.79-0.83 (m, 6H). $^{13}$CNMR (125 MHz, d$_6$-DMSO) $\delta$ 173.0, 170.9, 157.0, 50.2, 46.9, 46.7, 39.2, 38.1, 36.0, 31.7, 31.0, 30.1, 29.3, 29.2, 29.1, 29.0, 27.1, 25.7, 22.8, 22.5, 19.7, 14.3 13.8. HRMS (ESI) C$_{25}$H$_{44}$N$_4$O$_3$ [M+H]$^+$ calc’d = 425.3486; found = 425.3487.
**Compound 6**

$^1$HNMR (400 MHz, $d_6$-DMSO) $\delta$ 7.75 (brd, 3H), 7.62 (d, $J$=9.2Hz, 1H), 4.02 (d, $J$=17.2Hz 1H), 3.82-3.94 (m, 2H), 3.25-3.39 (m, 3H), 3.07 (dd, $J$ = 14.0, 4.0 Hz, 1H), 2.68-2.74 (m, 2H), 1.96 (t, $J$ = 7.6Hz, 2H), 1.29-1.51 (m, 8H), 1.10-1.22 (m, 20H), 0.79-0.84 (m, 6H). $^{13}$CNMR (125 MHz, $d_6$-DMSO) $\delta$ 172.8, 170.8, 156.9, 50.2, 46.9, 46.7, 39.1, 38.1, 36.0, 31.7, 31.1, 30.1, 29.5, 29.4, 29.3, 29.2, 29.1, 27.1, 25.7, 22.9, 22.5, 19.7, 14.4, 13.9. HRMS (ESI) C$_{25}$H$_{48}$N$_4$O$_3$ [M+H]$^+$ calc’d = 453.3802; found = 453.3803.

**Compound 7**

$^1$HNMR (400 MHz, $d_6$-DMSO) $\delta$ 7.73 (brd, 3H), 7.62 (d, $J$=8.8Hz, 1H), 4.02 (d, $J$=17.6Hz 1H), 3.82-3.90 (m, 2H), 3.26-3.38 (m, 3H), 3.07 (dd, $J$ = 14.0, 4.0 Hz, 1H), 2.68-2.74 (m, 2H), 1.95 (t, $J$ = 7.2Hz, 2H), 1.30-1.54 (m, 8H), 1.12-1.25 (m, 24H), 0.78-0.84 (m, 6H). $^{13}$CNMR (125 MHz, $d_6$-DMSO) $\delta$ 172.8, 170.8, 156.9, 50.2, 46.9, 46.7, 39.1, 38.1, 36.0, 31.7, 31.1, 30.1, 29.5, 29.3, 29.2, 29.1, 27.1, 25.7, 22.9, 22.5, 19.7, 14.3, 13.9. HRMS (ESI) C$_{27}$H$_{52}$N$_4$O$_3$ [M+H]$^+$ calc’d = 481.4112; found = 481.4111.

**Compound 8**

$^1$HNMR (400 MHz, $d_6$-DMSO) $\delta$ 7.77 (brd, 3H), 7.62 (d, $J$=9.2Hz, 1H), 4.02 (d, $J$=17.2Hz 1H), 3.82-3.89 (m, 2H), 3.25-3.38(m, 3H), 3.07 (dd, $J$ = 13.6, 4.0 Hz, 1H), 2.68-2.73 (m, 2H), 1.95 (t, $J$ = 7.2Hz, 2H), 1.27-1.50 (m, 8H), 1.08-1.22 (m, 28H), 0.79-0.84 (m, 6H). $^{13}$CNMR (125 MHz, $d_6$-DMSO) $\delta$ 173.0, 170.8, 157.0, 50.1, 46.9, 46.7, 38.1, 36.0, 31.7, 31.1, 30.1, 29.4, 29.3, 29.2, 29.1, 27.1, 25.7, 22.8, 22.5, 19.7, 14.3, 13.8. HRMS (ESI) C$_{29}$H$_{56}$N$_4$O$_3$ [M+H]$^+$ calc’d = 509.4422; found = 509.4424.

**Compound 9**

$^1$HNMR (400 MHz, $d_6$-DMSO) $\delta$ 7.66 (brd, 3H), 7.59 (d, $J$=8.8Hz, 1H), 3.95-3.98 (m, 1H), 3.73-3.85 (m, 2H), 3.61-3.72(m, 1H), 3.29-3.35 (m, 1H), 3.03-3.08 (m, 1H), 2.65-2.72 (m, 2H), 1.93-1.98 (m,
4H), 1.72 (d, J=12.4Hz, 2H), 1.28-1.57 (m, 8H), 1.02-1.24 (m, 18H), 0.82 (t, J=6.8Hz, 3H).  $^{13}$CNMR (125 MHz, d$_6$-DMSO) δ 172.9, 170.8, 156.7, 50.9, 49.8, 46.9, 46.6, 36.0, 31.7, 31.0, 29.4, 29.4, 29.2, 29.2, 29.1, 27.1, 25.7, 25.2, 22.8, 22.5, 14.3. HRMS (ESI) C$_{25}$H$_{46}$N$_4$O$_3$ [M+H]$^+$ calc’d = 451.3643; found = 451.3644.

**Compound 10**

$^1$HNMR (400 MHz, d$_6$-DMSO) δ 7.64 (brd, 3H), 7.60 (d, J=9.2Hz, 1H), 3.95-4.05 (m, 3H), 3.34-3.36 (m, 1H), 3.25 (s, 1H), 3.00-3.09 (m, 1H), 2.71 (s, 2H), 1.90-2.00 (m, 4H), 1.72 (d, J=12.4Hz, 2H), 1.31-1.59 (m, 8H), 1.00-1.27 (m, 22H), 0.82 (t, J=6.0Hz, 3H). $^{13}$CNMR (125 MHz, d$_6$-DMSO) δ 173.2, 170.8, 156.7, 50.9, 49.8, 46.9, 46.6, 36.0, 31.7, 31.1, 29.4, 29.4, 29.3, 29.1, 27.1, 25.8, 25.3, 22.5. HRMS (ESI) C$_{27}$H$_{50}$N$_4$O$_3$ [M+H]$^+$ calc’d = 479.3955; found = 479.3956.

**Compound 11**

$^1$HNMR (400 MHz, d$_6$-DMSO) δ 7.72 (brd, 3H), 7.60 (d, J=9.2Hz, 1H), 3.92-4.00 (m, 1H), 3.61-3.85 (m, 2H), 3.25-3.36 (m, 2H), 3.01-3.10 (m, 1H), 2.68-2.71 (m, 2H), 1.91-2.01 (m, 4H), 1.72 (d, J=12.4Hz, 2H), 1.30-1.58 (m, 8H), 0.98-1.25 (m, 26H), 0.82 (t, J=6.4Hz, 3H). $^{13}$CNMR (125 MHz, d$_6$-DMSO) δ 172.7, 170.6, 156.7, 50.9, 49.8, 46.9, 46.6, 39.1, 36.1, 31.7, 31.1, 29.5, 29.4, 29.3, 29.2, 29.1, 27.1, 25.8, 25.2, 22.9, 22.5, 14.3. HRMS (ESI) C$_{29}$H$_{54}$N$_4$O$_3$ [M+H]$^+$ calc’d = 507.4269; found = 507.4272.

**Compound 12**

$^1$HNMR (400 MHz, d$_6$-DMSO) δ 7.77 (brd, 3H), 7.61 (d, J=9.2Hz, 1H), 3.96 (d, J=12.4Hz, 1H), 3.63-3.81 (m, 2H), 3.28-3.33 (m, 2H), 3.00-3.10 (m, 1H), 2.62-2.75 (m, 2H), 1.91-1.99 (m, 4H), 1.70 (d, J=12.0Hz, 2H), 1.31-1.59 (m, 8H), 0.99-1.24 (m, 30H), 0.81 (t, J=6.4Hz, 3H). $^{13}$CNMR (125 MHz, d$_6$-DMSO) δ 173.1, 170.7, 156.7, 51.0, 49.8, 46.9, 46.6, 36.1, 31.7, 31.0, 29.4, 29.3, 29.2, 29.1, 27.0, 25.8, 25.2, 22.8, 22.5, 14.3. HRMS (ESI) C$_{31}$H$_{58}$N$_4$O$_3$ [M+H]$^+$ calc’d = 535.4582; found = 535.4583.
Compound 13

$^1$HNMR (400 MHz, d$_6$-DMSO) $\delta$ 7.76 (brd, 3H), 7.58 (d, $J$=9.2Hz, 1H), 3.65-3.88 (m, 3H), 3.26-3.33 (m, 1H), 2.99 (dd, $J$ = 14.0, 4.0 Hz, 1H), 2.68-2.74 (m, 2H), 2.27 (d, $J$ = 2.4 Hz, 6H), 1.93-2.03 (m, 5H), 1.59 (s, 6H), 1.26-1.51 (m, 6H), 1.15-1.23 (m, 14H), 0.81 (t, $J$=7.2Hz, 3H). $^{13}$CNMR (125 MHz, d$_6$-DMSO) $\delta$ 172.7, 171.5, 157.3, 59.1, 49.7, 46.7, 46.5, 39.1, 36.2, 36.1, 31.7, 31.2, 29.5, 29.3, 29.2, 27.1, 25.8, 22.9, 22.5, 14.3. HRMS (ESI) C$_{29}$H$_{50}$N$_4$O$_3$ [M+H]$^+$ calc’d = 503.3955; found = 503.3955.

Compound 14

$^1$HNMR (400 MHz, d$_6$-DMSO) $\delta$ 7.70 (brd, 3H), 7.57 (d, $J$=9.2Hz, 1H), 3.85 (d, $J$ = 17.2 Hz, 2H), 3.67 (d, $J$ = 17.2Hz, 1H), 3.26-3.33 (m, 1H), 2.99 (dd, $J$ = 14.0, 4.0 Hz, 1H), 2.66-2.73 (m, 2H), 2.27 (d, $J$ = 2.4 Hz, 6H), 1.95-2.01 (m, 5H), 1.59 (s, 6H), 1.26-1.51 (m, 6H), 1.12-1.23 (m, 18H), 0.81 (t, $J$=6.4Hz, 3H). $^{13}$CNMR (125 MHz, d$_6$-DMSO) $\delta$ 172.9, 171.5, 157.3,59.1, 49.6, 46.6, 46.5, 36.2, 36.1, 31.7, 31.2, 29.5, 29.4, 29.3, 29.3, 29.2, 29.1, 27.1, 25.8, 22.9, 22.5 14.3. HRMS (ESI) C$_{31}$H$_{54}$N$_4$O$_3$ [M+H]$^+$ calc’d = 531.4269; found = 531.4269.

Compound 15

$^1$HNMR (400 MHz, d$_6$-DMSO) $\delta$ 7.72 (brd, 3H), 7.57 (d, $J$=9.2Hz, 1H), 3.85 (d, $J$ = 17.2 Hz, 2H), 3.68 (d, $J$ = 17.2Hz, 1H), 3.27-3.34 (m, 1H), 2.99 (dd, $J$ = 14.0, 4.0 Hz, 1H), 2.66-2.74 (m, 2H), 2.27 (d, $J$ = 2.4 Hz, 6H), 1.94-2.01 (m, 5H), 1.59 (s, 6H), 1.26-1.50 (m, 6H), 1.13-1.22 (m, 22H), 0.81 (t, $J$=6.4Hz, 3H). $^{13}$CNMR (125 MHz, d$_6$-DMSO) $\delta$ 172.9, 171.5, 157.3,59.1, 49.6, 46.6, 46.5, 39.1, 36.1, 36.1, 31.7, 31.1, 29.5, 29.4, 29.4, 29.3, 29.3, 29.2, 29.1, 27.1, 25.8, 22.8, 22.5, 14.3. HRMS (ESI) C$_{33}$H$_{58}$N$_4$O$_3$ [M+H]$^+$ calc’d = 559.4578; found = 559.4579.

Compound 16
\[^{1}\text{HNMR (400 MHz, d}_6\text{-DMSO)} \delta 7.63 \text{ (brd, 3H), 7.57 (d, J=8.8 Hz, 1H), 3.85 (d, J = 17.2 Hz, 2H), 3.68 (d, J = 17.2 Hz, 1H), 3.25-3.33 (m, 1H), 2.99 (dd, J = 14.0, 4.0 Hz, 1H), 2.68-2.73 (m, 2H), 2.27 (d, J = 2.4 Hz, 6H), 1.92-2.02 (m, 5H), 1.60 (s, 6H), 1.24-1.50 (m, 6H), 1.10-1.20 (m, 26H), 0.81 (t, J = 7.2 Hz, 3H).} \]

\[^{13}\text{C NMR (125 MHz, d}_6\text{-DMSO)} \delta 172.9, 171.5, 157.3, 59.1, 49.6, 46.6, 46.4, 39.1, 36.1, 36.1, 31.7, 31.1, 29.5, 29.4, 29.3, 29.2, 29.1, 27.1, 25.8, 22.8, 22.5, 14.3. \]

HRMS (ESI) C\(_{35}\)H\(_{62}\)N\(_4\)O\(_3\) \[M+H\]^+ calc’d = 587.4895; found = 587.4897.

**Compound 17**

\[^{1}\text{HNMR (400 MHz, d}_6\text{-DMSO)} \delta 7.74 \text{ (brd, 3H), 7.68 (d, J=8.8 Hz, 1H), 7.16 (d, J=9.2 Hz, 2H), 6.97 (d, J=8.8 Hz, 2H), 4.18 (d, J=17.2 Hz, 1H), 3.92-4.03 (m, 2H), 3.74 (s, 3H), 3.36-3.42 (m, 1H), 3.17 (dd, J = 14.0, 4.0 Hz, 1H), 2.68-2.76 (m, 2H), 1.99 (t, J = 7.2 Hz, 2H), 1.29-1.54 (m, 6H), 1.06-1.25 (m, 14H), 0.80 (t, J = 6.0 Hz, 3H).} \]

\[^{13}\text{C NMR (125 MHz, d}_6\text{-DMSO)} \delta 173.1, 170.1, 159.0, 156.2, 128.2, 125.2, 114.4, 55.8, 50.4, 47.2, 46.8, 36.1, 31.7, 31.0, 29.3, 29.1, 27.1, 25.8, 22.9, 22.5, 14.4. \]

HRMS (ESI) C\(_{26}\)H\(_{42}\)N\(_4\)O\(_3\) \[M+H\]^+ calc’d = 475.3279; found = 475.3279.

**Compound 18**

\[^{1}\text{HNMR (400 MHz, d}_6\text{-DMSO)} \delta 7.78 \text{ (brd, 3H), 7.68 (d, J=8.8 Hz, 1H), 7.16(d, J=9.2 Hz, 2H), 6.96(d, J=9.2 Hz, 2H), 4.18 (d, J=17.2 Hz, 1H), 3.90-4.03 (m, 2H), 3.74 (s, 3H), 3.37-3.43 (m, 1H), 3.13-3.20 (m, 1H), 2.65-2.80 (m, 2H), 1.99 (t, J = 7.2 Hz, 2H), 1.29-1.53 (m, 6H), 1.07-1.24 (m, 18H), 0.80 (t, J=6.0 Hz, 3H).} \]

\[^{13}\text{C NMR (125 MHz, d}_6\text{-DMSO)} \delta 173.1, 170.0, 158.9, 156.2, 128.2, 125.2, 114.5, 55.7, 50.4, 47.2, 46.8, 39.1, 36.0, 31.7, 31.0, 29.5, 29.4, 29.3, 29.2, 29.1, 27.1, 25.8, 22.8, 22.5, 14.3. \]

HRMS (ESI) C\(_{28}\)H\(_{46}\)N\(_4\)O\(_3\) \[M+H\]^+ calc’d = 503.3592; found = 503.3594

**Compound 19**
HNMR (400 MHz, d$_6$-DMSO) δ 7.77 (brd, 3H), 7.68 (d, J=9.2Hz, 1H), 7.16 (d, J=9.2Hz, 2H), 6.97 (d, J=9.2Hz, 2H), 4.19 (d, J=17.2Hz, 1H), 3.91-4.03 (m, 2H), 3.74 (s, 3H), 3.37-3.43 (m, 1H), 3.15-3.21 (m, 1H), 2.70-2.76 (m, 2H), 2.00 (t, J = 7.2 Hz, 2H), 1.30-1.57 (m, 6H), 1.10-1.24 (m, 22H), 0.82 (t, J=6.4Hz, 3H).

13CNMR (125 MHz, d$_6$-DMSO) δ 172.9, 170.0, 159.0, 156.2, 128.2, 125.3, 114.4, 55.8, 50.4, 47.2, 46.7, 39.1, 36.1, 31.7, 31.1, 29.5, 29.4, 29.3, 29.1, 27.1, 25.8, 22.9, 22.5, 14.4.

HRMS (ESI) C$_{23}$H$_{44}$N$_4$O$_3$ [M+H]$^+$ calc’d = 531.3896; found = 531.3894.

**Compound 20**

HNMR (400 MHz, d$_6$-DMSO) δ 7.76 (brd, 3H), 7.68 (d, J=9.2Hz, 1H), 7.15 (d, J=9.2Hz, 2H), 6.96 (d, J=9.2Hz, 2H), 4.18 (d, J=17.2Hz, 1H), 3.88-4.04 (m, 2H), 3.73 (s, 3H), 3.39-3.43 (m, 1H), 3.14-3.20 (m, 1H), 2.65-2.78 (m, 2H), 1.99 (t, J = 7.2 Hz, 2H), 1.29-1.53 (m, 6H), 1.10-1.24 (m, 26H), 0.81 (t, J=6.4Hz, 3H).

13CNMR (125 MHz, d$_6$-DMSO) δ 173.1, 170.0, 158.9, 156.2, 128.2, 125.2, 114.3, 55.7, 50.4, 47.2, 46.7, 39.1, 36.0, 31.7, 31.0, 29.4, 29.4, 29.3, 29.1, 27.1, 25.8, 22.8, 22.5, 14.3.

HRMS (ESI) C$_{32}$H$_{54}$N$_4$O$_3$ [M+H]$^+$ calc’d = 559.4218; found = 559.4217.

**Compound 21**

HNMR (400 MHz, d$_6$-DMSO) δ 7.74 (brd, 3H), 7.69 (d, J=9.2Hz, 1H), 7.19 (d, J=9.2Hz, 2H), 6.96 (d, J=9.2Hz, 2H), 3.32-3.47 (m, 2H), 3.13-3.21 (m, 1H), 2.73 (s, 2H), 1.99 (t, J = 7.2 Hz, 2H), 1.26-1.51 (m, 8H), 1.05-1.21 (m, 12H), 0.78 (t, J=6.4Hz, 3H).

13CNMR (125 MHz, d$_6$-DMSO) δ 173.0, 169.6, 155.4, 134.0, 133.2, 130.8, 128.0, 126.3, 125.2, 50.4, 47.3, 46.6, 36.0, 31.7, 31.0, 29.3, 29.1, 27.1, 25.8, 22.9, 22.5, 14.4.

HRMS (ESI) C$_{25}$H$_{39}$N$_4$O$_3$ [M+H]$^+$ calc’d = 479.2779; found = 479.2782.

**Compound 22**

HNMR (400 MHz, d$_6$-DMSO) δ 7.72 (brd, 3H), 7.68-7.72 (m, 1H), 6.78-7.50 (m, 4H), 4.01-4.25 (m, 2H), 3.32-3.47 (m, 2H), 3.13-3.21 (m, 1H), 2.73 (s, 2H), 1.99 (t, J = 7.2 Hz, 2H), 1.26-1.51 (m, 8H), 1.05-1.21 (m, 12H), 0.78 (t, J=6.4Hz, 3H).
\[ \delta_{13}^{\text{CNMR}} (125 \text{ MHz, d}_{6}\text{-DMSO}) \delta 173.0, 169.8, 155.6, 134.0, 133.2, 130.8, 128.0, 126.3, 125.2, 50.4, 47.3, 46.6, 39.1, 36.0, 31.7, 31.0, 29.5, 29.4, 29.3, 29.1, 27.1, 25.8, 22.9, 22.5, 14.4. \]

HRMS (ESI) \( \text{C}_{27}\text{H}_{43}\text{N}_{4}\text{O}_{3}[\text{M+H}]^+ \) calc’d = 507.3092; found = 507.3095

**Compound 23**

\[ ^{1}\text{HNMR} (400 \text{ MHz, d}_{6}\text{-DMSO}) \delta 7.69-7.73 (m, 1H), 7.67 (\text{brd}, 3H), 7.15-7.50 (m, 4H), 3.87-4.28 (m, 3H), 3.34-3.45 (m, 1H), 2.72 (s, 2H), 1.91-2.15 (m, 2H), 1.27-1.49 (m, 6H), 1.09-1.20 (m, 22H), 0.81 (t, \text{J}=6.4\text{Hz}, 3H). \]

\[ ^{13}\text{CNMR} (125 \text{ MHz, d}_{6}\text{-DMSO}) \delta 173.2, 169.6, 155.4, 133.9, 133.2, 130.8, 128.0, 126.3, 125.2, 50.3, 47.2, 46.6, 39.1, 36.0, 31.7, 31.0, 29.4, 29.2, 29.1, 27.0, 25.8, 22.8, 22.5, 14.3. \]

HRMS (ESI) \( \text{C}_{29}\text{H}_{47}\text{N}_{4}\text{O}_{3}[\text{M+H}]^+ \) calc’d = 535.3408; found = 535.3410.

**Compound 24**

\[ ^{1}\text{HNMR} (400 \text{ MHz, d}_{6}\text{-DMSO}) \delta 7.67 (d, \text{J}=8.8\text{Hz}, 1H), 7.63 (\text{brd}, 3H), 7.28-7.49 (m, 4H), 3.90-4.24 (m, 2H), 3.33-3.44 (m, 2H), 3.15-3.21 (m, 1H), 2.72 (s, 2H), 1.99 (m, \text{J}=7.6\text{Hz}, 2H), 1.30-1.52 (m, 6H), 1.10-1.22 (m, 26H), 0.81 (t, \text{J}=6.4\text{Hz}, 3H). \]

\[ ^{13}\text{CNMR} (125 \text{ MHz, d}_{6}\text{-DMSO}) \delta 173.1, 169.6, 155.4, 133.9, 133.2, 130.8, 128.0, 126.3, 125.2, 50.4, 47.2, 46.6, 39.2, 36.0, 31.7, 31.0, 29.4, 29.2, 29.1, 27.1, 25.8, 22.9, 22.5, 14.4. \]

HRMS (ESI) \( \text{C}_{31}\text{H}_{51}\text{N}_{4}\text{O}_{3}[\text{M+H}]^+ \) calc’d = 563.3722; found = 563.3723.

**Compound 25**

\[ ^{1}\text{HNMR} (400 \text{ MHz, d}_{6}\text{-DMSO}) \delta 7.79 (d, \text{J}=9.2\text{Hz}, 1H), 7.36-7.50 (m, 3H), 7.30 (d, \text{J}=8.0\text{Hz}, 1H), 7.22 (d, \text{J}=4.4\text{Hz}, 4H), 7.10-7.17 (m, 1H), 4.18-4.27 (m, 2H), 4.04 (d, \text{J}=17.6\text{Hz}, 1H), 3.47-3.54 (m, 1H), 3.31-3.27 (m, 1H), 2.76-2.82 (m, 1H), 2.60-2.68 (m, 1H), 1.82-1.95 (m, 2H), 1.02-1.32 (m, 18H), 0.81 (t, \text{J}=6.8\text{Hz}, 3H). \]

\[ ^{13}\text{CNMR} (125 \text{ MHz, d}_{6}\text{-DMSO}) \delta 172.8, 169.6, 155.5, 138.9, 134.0, 133.2, 133.2, 130.8, 128.0, 126.3, 125.2, 50.4, 47.2, 46.6, 39.2, 36.0, 31.7, 31.0, 29.4, 29.2, 29.1, 27.1, 25.8, 22.9, 22.5, 14.4. \]
130.8, 129.4, 128.5, 128.0, 126.5, 126.4, 125.3, 50.4, 48.5, 47.2, 40.8, 37.7, 36.0, 31.7, 29.4, 29.4, 29.2, 29.1, 28.9, 25.7, 22.5, 14.4. HRMS (ESI) C$_{31}$H$_{51}$N$_{4}$O$_{3}$ [M+H]$^+$ calc’d = 526.2826; found = 526.2849.

2.4.4 Minimum inhibitory concentrations (MICs) assay.

All compounds were tested against six different bacteria strains: methicillin-resistant *S. aureus* (MRSA, ATCC 33591), *E. coli* (ATCC 25922), methicillin-resistant *S. epidermidis* (MRSE, RP62A), *K. pneumoniae* (ATCC 13383), vancomycin-resistant *Enterococcus faecalis* (VREF, ATCC 700802), *P. aeruginosa* (ATCC 27853). One colony of each bacteria was inoculated in 4 mL TSB buffer at 37 °C overnight, which was then diluted 100 times, and the bacteria were allowed to grow to the mid-logarithmic phase. 50 µL hydantoin compounds in 2-fold serial dilution of TSB were added in the 96-well plate, then 50 µL diluted bacterial in TSB medium (1 × 10$^6$ CFU/mL) was added to each well. After 16 h of incubation at 37 °C, the absorption at 600 nm wavelength on a Biotek Synergy HT microtiter plate reader was recorded. Minimum inhibitory concentrations were determined as the lowest concentrations that inhibited bacteria growth completely.

2.4.5 Time kill assay.

Bacteria MRSA (Gram-positive) and *E. coli* (Gram-negative) suspensions were allowed to grow at 37 °C to the mid-logarithmic phase, and diluted to 1 × 10$^6$ CFU/mL, then incubated with the compound 22 at the concentration of 50, 25, 12.5 µg/mL at 10 min, 30 min, 1 h and 2 h, respectively. The resulted mixture was then diluted by 10$^2$ to 10$^4$ fold, from which 100 µL was spread on the TSB agar plate. The Number of bacteria colonies was counted after 20 h of incubation at 37 °C.

2.4.6 Drug resistance assay.
The lead compound 22 was chosen for drug resistance studies. Briefly, after its MIC against MRSA was determined, the bacteria solution from the well of the 1/2 MIC was withdrawn and diluted to $1 \times 10^6$ CFU/mL for the next MIC measurement. The measurement was repeated for 14 passages.

2.4.7 Hemolytic assay.\textsuperscript{127}

Fresh red blood cells (RBCs) of mice were washed with 1× PBS buffer and centrifuged 10 min at 3500 rpm for 3 times until the supernatant was clear, then RBCs were diluted into 5% v/v suspension in 1× PBS. 50 µL compounds in PBS were 2-fold serially diluted in a 96-well plate, and incubated with 50 µL RBCs suspension for 1 h at 37 °C. The mixture was then centrifuged for 10 min at 3500 rpm. Subsequently, 30 µL of the supernatant was added to 100 µL PBS, then the absorbance of mixture was read on a Biotek Synergy HT plate reader at 540 nm. The hemolytic activity was calculated by the formula

\[
\% \text{ hemolysis} = \frac{(\text{Abs}_{\text{sample}} - \text{Abs}_{\text{PBS}})}{(\text{Abs}_{\text{Triton}} - \text{Abs}_{\text{PBS}})} \times 100%.
\]

1% Triton X-100 were used as the positive control and 1× PBS buffer was used as the negative control.

2.4.8 Fluorescence microscopy.\textsuperscript{127}

Both propidium iodide (PI) and 4’, 6’-diamidino-2-phenylindole dihydrochloride (DAPI) fluorescent dyes were used in the studies to determine the ability of the compound 22 to compromise the membranes of MRSA and E. coli, respectively. In brief, bacterial suspensions were incubated at 37 °C to the mid-logarithmic phase and then diluted by 100 fold, followed by incubation with compound 22 for 2 h at 37 °C. After centrifugation for 15 min at 5000 rpm, cell pellets were washed with 1× PBS buffer, and incubated with PI (5 µg/mL) for 15 min on ice in the dark, then washed 2 times with PBS. Then the cell pellets were incubated with DAPI (10 µg/mL) in the similar way. The pellets were then diluted in 100 µL PBS, and 10–20 µL of the suspension was applied on chamber slides and observed under Zeiss Axio Image Zloptical microscope using 100× oil-immersion objective.
2.4.9 In vivo study

2.4.9.1 The rat model of MRSA-induced pneumonia\textsuperscript{125, 129}

The protocol of animal studies was approved by the institutional committee for animal care of Nanjing University, and the experiments were conducted following the regulation of the National Ministry of Health of China. In Brief, male Wistar rats (6-8 weeks, ~200 g in average weight) were subjected to fast for 12 h. Next, rats were anesthetized by intraperitoneal administration of 0.35 g/kg of chloral hydrate. To induce MRSA pneumonia, the trachea of rats were exposed, to which 100 μL PBS containing $2 \times 10^6$ CFU/mL MRSA in PBS was slowly injected. The rats were retained upright for 1 min, and the cut was sealed. The MRSA infection was allowed to develop for 24 h.

2.4.9.2 Bronchoalveolar lavage (BAL) assay.

The assay was carried out to quantify the bacteria in the lungs of rats. The thorax of rats was opened, and the lungs were collected in each group at different time points. Then the lungs were malleated and homogenized by a glass homogenizer, and diluted with PBS to the determined volume. After that, 10 μL solution was picked up, diluted with PBS by 100-fold, and daubed on the isolation medium. Bacteria were counted in each sample after incubation for 24 h at 37 °C, from which the number of bacteria (colonies/g tissue) was calculated.

2.4.9.3 Pathological analysis.

The hydantoin compound\textsuperscript{22} or vancomycin were used as antibiotics in the study. In brief, a dose of 10 mg/kg of the drug was injected to the tested rats intravenously. On the day of 3 and 5, rats were sacrificed, and lung sections were stained with hematoxylin and eosin (H&E), and their morphology was investigated under a light microscope at 100 × magnification. The existence (indicated by the
infiltration of neutrophils) and the extent of possible inflammatory response (indicated by the integrity of the alveolar structure and endothelium cillum) were recorded from at least five randomly picked sections by an experienced physician.
CHAPTER 3: POLYMYXIN MIMIC CYCLIC PEPTIDES AS BROAD-SPECTRUM ANTIBIOTIC AGENTS

3.1 Introduction

Polymyxins were secondary metabolite nonribosomal peptides produced by Paenibacillus polymyxa and first recognized as antibiotic agents in the 1940s. Among five polymyxins (polymyxins A to E), two of them have been used in clinic: polymyxin B and E (also known as colistin). Both polymyxin B and colistin exhibit antibacterial activities against a narrow spectrum of gram-negative pathogens such as Pseudomonas aeruginosa, Acinetobacter baumannii and Klebsiella pneumoniae. Colistin is a complex, multicomponent antibiotic mixture. Two major constituents are colistin A and colistin B, with identical head groups but fatty acyl tails of different lengths: colistin A fatty acid is 6-methyloctanoic acid, colistin B fatty acid is 6-methylheptanoic acid. In the clinical setting, colistin is administered in the form of colistin methane sulfonate (CMS), a less toxic and nonactive prodrug. Because the early clinical experience, before the 1970s, with parenteral administration of PMB and colistin (or its nonactive prodrug colistin methanesulfonate) led to concern over the potential for nephrotoxicity and neurotoxicity, their clinical use waned.

The outer membrane of Gram-negative bacteria constitutes a permeable barrier. Polymyxin can directly interact with the lipid A component of the lipopolysaccharide (LPS). The current understanding of structure-activity relationship (SAR) is that amphipathic nature of polymyxin is crucial: cationic residues and hydrophobic groups. There are several key domains crucial for interaction with lipid A: Dab side chain with positive charge, the heptapeptide backbone, hydrophobic
fatty acyl tail at N-terminal and hydrophobic motif at position 6 and 7. (figure 3.1)

Figure 3.1 (a) Structure of colistin (polymyxin E); (b) Structure of polymyxin. (Adapted with permission from Future Medicine. Copyright 2013.)

The WHO has identified antibiotic resistance as one of the three greatest threats to human health. The world is now facing an enormous threat from the emergence of bacteria that are resistant to almost all available antibiotics. In recent years, virtually no novel drugs targeting multidrug-resistant (MDR) Gram-negative bacteria (especially P. aeruginosa, A. baumannii and K. pneumoniae) have been developed. Meanwhile, the polymyxins are increasingly being used as last-line therapy to treat otherwise untreatable serious infections caused by gram-negative bacteria that are resistant to essentially all other currently available antibiotics. However, emergence of polymyxin resistance in Gram-negative bacteria has been reported. The most common way that gram-negative
bacteria survives from polymyxin by remodeling LPS.\textsuperscript{149}

Increasing drug resistance bring emergent demand of new antibiotic agents, no new antibiotics will be available for these ‘superbugs’ in the near future due to the dry antibiotic discovery pipeline.\textsuperscript{150} Over the last 30 years only two novel antibiotic classes have been introduced into the clinic (linezolid and daptomycin), illustrating that the post-antibiotic era is fast approaching.\textsuperscript{151} In the ‘Bad Bugs, No Drugs’ era, we must pursue structure–activity relationship-based approaches to develop novel polymyxin-like lipopeptides targeting polymyxin-resistant Gram-negative ‘superbugs’.\textsuperscript{152}

According to our previous antimicrobial peptides study, γ-AApeptides and long lipid tail can increase the stability and activity. Therefore, we set out to design a new class of polymyxin mimic cyclic peptides with antimicrobial activity against both Gram-positive and Gram-negative bacteria.

\textbf{3.2 Results and discussion}

According to our previous antibiotic agent study, we found out that long lipid tails and γ-AApeptides can increase antimicrobial activity and stability of antibiotic drugs. Therefore, we set out a class of polymyxin mimic cyclic peptides to obtain better potent antibiotic. As Table 3.1 showed, we made a few changes of colistin domains. First, we changed the length of fatty acyl group was changed from 8 carbons to 16 carbons to get compound P1. Secondly, to design compound P2, L-Dab residues on position 8 and 9 were changed to γ-AA Lys-BB-1, which contains two positive charged groups acting the same as L-Dab residues. For compound P3, we changed L-Dab and L-Thr residues on position 1 and 2 to γ-AA Lys-BB-2, which contains one positive charged and one negative charged group. At last, we designed compound P4, with a change from D-Leu and L-Leu residues to γ-AA Leu-BB. The length of fatty acyl groups of compound P2, P3 and P4 are all changed to 16 carbons. According to the SAR of polymyxin, positive charged group will contact by electrostatic effect with negative charged groups.
on bacteria outer membrane. Hydrophobic groups then help the molecule get through, penetrate and destruct the membrane.

**Table 3.1.** Structures of polymyxin mimic cyclic peptides.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Change Position</th>
<th>Change Structure</th>
<th>Fatty acyl group</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>None</td>
<td>None</td>
<td>C_{16}H_{31}O</td>
</tr>
<tr>
<td>P2</td>
<td>8,9</td>
<td><img src="image" alt="Structure" /></td>
<td>C_{16}H_{31}O</td>
</tr>
<tr>
<td>P3</td>
<td>1,2</td>
<td><img src="image" alt="Structure" /></td>
<td>C_{16}H_{31}O</td>
</tr>
<tr>
<td>P4</td>
<td>6,7</td>
<td><img src="image" alt="Structure" /></td>
<td>C_{16}H_{31}O</td>
</tr>
</tbody>
</table>

The antimicrobial activity was tested by MIC using 6 different bacterial strains including both Gram-positive and Gram-negative, results shown in Table 3.1 As mentioned in introduction, traditional polymyxin is only active against Gram-negative bacterial strains and does not have activity against Gram-positive bacteria strains. According to MIC results, all 4 polymyxin mimic cyclic peptides are active against both MRSA and E. coli, which indicates that increasing the length of lipid tail rationally will lead compounds to a broad-spectrum antibiotics. Since all 4 compounds have longer lipid tails compared with polymyxin. Compound **P1** has good antibiotic activity against MRSA, MRSE, VREF, E. coli and P.A. bacteria strains. Compound **P2-P4** also have broader antibacterial activity than polymyxin. Considering compound **P1** has the best activity among 4 compounds and not contain
changes of γ-AApeptide building blocks, the unique backbone of γ-AApeptide doesn’t improve the antibiotic activity in this case. However, γ-AApeptide can still increase the chemodiversity of compounds. Hemolytic activity of all 4 compounds was also investigated. As shown in Table X, all 4 compounds exhibit limited hemolytic activity and compound P1 has good selectivity toward MRSA.

Table 3.2 Activity and selectivity of polymyxin mimic peptides.

<table>
<thead>
<tr>
<th>Compound</th>
<th>MIC (µg/mL)</th>
<th>Hemolytic Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Gram Positive</td>
<td>Gram Negative</td>
</tr>
<tr>
<td></td>
<td>MRSA</td>
<td>MRSE</td>
</tr>
<tr>
<td>P1</td>
<td>6.25</td>
<td>3.12</td>
</tr>
<tr>
<td>P2</td>
<td>12.5</td>
<td>&gt;50</td>
</tr>
<tr>
<td>P3</td>
<td>25</td>
<td>&gt;50</td>
</tr>
<tr>
<td>P4</td>
<td>12.5</td>
<td>&gt;50</td>
</tr>
</tbody>
</table>

Compound P1 was investigated of its ability to disrupt bacteria membranes of MRSA and E. coli. Two dyes, 4′,6-diamidino-2-phenylindole (DAPI) and propidium iodide (PI) (Figure 3.2), were used to differentiate between cells with either an intact or a damaged membrane. DAPI can permeate the membrane of intact cells therefore shows blue fluorescence regardless of cell viability. In contrast, PI is a DNA intercalator but lacks cell permeability. It fluoresces in red only when cell membranes are disrupted. As shown in Figure 3.2, in the DAPI channel, both MRSA and E. coli exhibited blue fluorescence in the absence of compound P1. In the PI channel, neither strain showed red fluorescence indicating the membranes of these bacteria were intact. However, after treated with compound P1 for
2 h at 2 x MIC, both MRSA and E. coli exhibited red fluorescence, suggesting that the membranes of both MRSA and E. coli were disrupted.

<table>
<thead>
<tr>
<th></th>
<th>DAPI</th>
<th>PI</th>
<th>DAPI</th>
<th>PI</th>
</tr>
</thead>
<tbody>
<tr>
<td>MRSA</td>
<td><img src="image1.png" alt="Fluorescence image of MRSA" /></td>
<td><img src="image2.png" alt="Fluorescence image of MRSA" /></td>
<td><img src="image3.png" alt="Fluorescence image of E. coli" /></td>
<td><img src="image4.png" alt="Fluorescence image of E. coli" /></td>
</tr>
<tr>
<td>MRSA+1</td>
<td><img src="image5.png" alt="Fluorescence image of MRSA+1" /></td>
<td><img src="image6.png" alt="Fluorescence image of MRSA+1" /></td>
<td><img src="image7.png" alt="Fluorescence image of E. coli+1" /></td>
<td><img src="image8.png" alt="Fluorescence image of E. coli+1" /></td>
</tr>
</tbody>
</table>

**Figure 3.2** Fluorescence micrographs of MRSA and E. coli treated or not treated with 2 x MIC of compound **P1** for 2 h.

To understand the bacteria membrane-disruptive kinetics, time kill assay of compound **P1** was also carried out. MRSA and E. coli was treated with different concentrations of compound **P1**: 2× MIC, 4× MIC and 8× MIC. In Figure 3.3, MRSA can be eliminated within 60 min at all three concentrations. Growth of E. coli was effectively controlled and decreased at all three concentrations. This indicates that compound **P1** can rapidly kill both MRSA and E. coli bacteria strains.

**Figure 3.3** Time-kill plot of compound **P1** against MRSA and E. coli.

Since compound **P1** was designed to be membrane active and disrupt bacterial membranes rather
than acting on specific targets, we hypothesized that P1 could also prevent the development of resistance in bacteria. Therefore, we carried out drug resistance studies for P1 against E. coli. To do so, P1 was incubated with E. coli at half of its MIC overnight, and the new MIC was measured subsequently. After 14 passages, the MICs of P1 remained relatively stable (Figure 3.4), which strongly suggests that this class of polymyxin mimic cyclic compounds does not readily induce resistance in bacteria, thereby suggesting their potential therapeutic potent.

![Figure 3.4 Drug resistance study for compound P1.](image)

The development of membrane-active antibacterial peptides has been hindered by difficulties with systematic toxicity and tissue distribution, thus only a few compounds have been reported with *in vivo* activity and advanced into clinical trials. We envisioned that our polymyxin mimic cyclic peptides may possess better therapeutic potential. We employed the thigh burden model to evaluate the in vivo anti-infective activity of compounds P1, which is a widely used animal model for evaluating preclinical antimicrobial activity of compounds. Thigh muscle of neutropenic mice was inoculated with MRSA, followed by intravenous (i.v.) injections of the compound P1. As shown in Figure 3.5, significant activity was observed at the dose of 5 mg/kg when administered twice with a 6 h and 12 h interval
between each injection. A 1-log10 decrease in colony-forming unit (CFU) was observed when injections interval is 6 h, while a more significant decrease (4-log10 CFU) was observed when injections interval is 12 h. *In vivo* results suggested that compound P1 provided significant antibiotic activity against infection with MRSA.

![Graph showing bacterial load over time](image)

**Figure 3.5** *In vivo* efficacy of the compounds P1 in the thigh-infection mouse model.

### 3.3 Conclusions

In summary, we have developed a new class of polymyxin mimic cyclic peptides which possess broad-spectrum antimicrobial activity. These compounds exhibit remarkable potency against a panel of multidrug-resistant Gram-positive and Gram-negative bacteria. These compounds were designed to be membrane active with both cationic charged and hydrophobic groups. Our studies suggest that the lead compound could kill bacteria rapidly and the susceptibility of MRSA remained stable even after 14 passages. Furthermore, results of MRSA-infected thigh burden mouse model confirmed great antibiotic therapeutic potential of the lead compound. Therefore, this class of compounds can be potential broad-spectrum antibiotic agents to combat drug resistance. Further studies on the optimization of activity and selectivity are currently underway.
3.4 Experimental

3.4.1 General information

2-Chlorotrityl chloride (CTC) resins (0.972 mmol/g, 100–200 mesh) were purchased from Chem-Impex Int’l Inc. Solvents and other chemicals were ordered from either Fisher Scientific or Sigma-Aldrich, and were used without further purification. The solid phase syntheses of all compounds were carried out in a peptide reaction vessel on a Burrell Wrist-Action shaker. All compounds were analyzed and purified using the Waters Breeze 2 HPLC system under 215 nm of UV detector equipped with both analytical and preparative modules. The desired fractions were lyophilized on a Labconco lyophilizer.

3.4.2 Synthesis of building block

\[
\begin{align*}
\text{γ-Leu-BB} & \quad \text{γ-Lys-BB-1} & \quad \text{γ-Lys-BB-2}
\end{align*}
\]

Figure 3.6 Structures of γ-AApeptide building blocks

Synthesis of γ-AApeptide building blocks is similar as Chapter 2.

3.4.3 Synthesis of polymyxin mimic peptides
Figure 3.7 Synthetic procedure of the compound P1.

200 mg CTC resin (0.196 mmol) was reacted with Fmoc-Thr(tBu)-OH (0.23 g, 0.588 mmol) and DIPEA (50 μL, 0.287 mmol) in DCM solution for 3 h, followed by DMF (2 mL × 3) and DCM (2 mL × 3) wash. Then resin beads were capped in methanol for 30 min. The attachment of the Fmoc-Dab(Boc)-OH to the resin was achieved by adding Fmoc-Dab(Boc)-OH (176 mg, 0.4 mmol), DIC (100 μL, 0.7 mmol), and HOBt (0.1 g, 0.8 mmol) in 3 mL DMF to the reaction vessel, and the reaction was allowed to shake at room temperature for 3 h. Then the solution was drained, and the beads were washed with DCM (3 mL × 3) and DMF (3 mL × 3). After that, beads were treated with 3 mL 20% piperidine/DMF (v/v) solution for 15 min (× 2) to remove the Fmoc protection group, followed by DMF (2 mL × 3) and DCM (2 mL × 3) wash. This step was repeated once, Fmoc-Dab(Boc)-OH was added to the resin again. Next, Fmoc-L-leucine (141 mg, 0.4 mmol), DIC (100 μL, 0.7 mmol), and HOBt (0.1 g, 0.8 mmol) in 3 mL DMF were added to the resin and allowed to react for 3 h at room temperature after Fmoc protecting group was removed first. This step was repeated once, Fmoc-L-leucine was added to the resin again. Next, Fmoc-Dab(Boc)-OH (176 mg, 0.4 mmol), DIC (100 μL, 0.7 mmol), and HOBt (0.1 g, 0.8 mmol) in 3 mL DMF was added to the reaction vessel after Fmoc protecting group was removed. After beads were washed by DMF and DCM and Fmoc protecting group was removed, Fmoc-
Dab(Alloc)-OH (170mg, 0.4 mmol), DIC (100 μL, 0.7 mmol), and HOBT (0.1 g, 0.8 mmol) in 3 mL DMF was added to the reaction vessel. The solution was drained and beads were washed with DMF and DCM. Next, Fmoc-Dab(Boc)-OH (176 mg, 0.4 mmol), DIC (100 μL, 0.7 mmol), and HOBT (0.1 g, 0.8 mmol) in 3 mL DMF was added to the reaction vessel after Fmoc protecting group was removed. After that, Fmoc-Thr(tBu)-OH (0.23 g, 0.588 mmol), DIC (100 μL, 0.7 mmol), and HOBT (0.1 g, 0.8 mmol) in 3 mL DMF was added to the reaction vessel after Fmoc protecting group was removed. Next, Fmoc-Dab(Boc)-OH (176 mg, 0.4 mmol), DIC (100 μL, 0.7 mmol), and HOBT (0.1 g, 0.8 mmol) in 3 mL DMF was added to the reaction vessel after Fmoc protecting group was removed. Then, palmitic acid (0.1g, 0.4 mmol) DIC (100 μL, 0.7 mmol), and HOBT (0.1 g, 0.8 mmol) in 3 mL DMF was added to the reaction vessel after Fmoc protecting group was removed. Then beads were with Pd(PPh₃)₄ (24 mg, 0.02 mmol) and Me₂NH.BH₃ (70 mg, 1.2 mmol) in 3 mL DCM for 10 min (×2) to remove the alloc protein group, then washed with DCM (3 mL ×3) and DMF (3 mL ×3). Then, all remained protecting groups were removed by incubated with 20 mL cocktail of 1:1 TFA: DCM 1:1 (v/v/v) for 2 h, after the solution was extracted and solvent was evaporated. Then the compound was analyzed and purified on the Waters HPLC system, and the desired fraction was lyophilized to give the pure product compound P₁.

Compound P₂, P₃ and P₄ were synthesized with similar methods.
Figure 3.8 Structures of compound P1-P4.
3.4.4 In vivo study of mouse thigh burden infection model.

All protocols and methods associated with animal experiments were approved by University of South Florida (USF) Institutional Animal Care and Use Committee. The CD-1 female mice which were 6 to 8 weeks old and around 25 g in weights were used for the study. Neutropenic Mice were induced by injecting cyclophosphamide (150 mg/kg) intraperitoneally twice at 4 and 1 days before bacterial inoculation. One MRSA colony from TSA cultures was allowed to grow in TSB medium overnight at 37 °C, then 100 μL culture was withdrawn and diluted with TSB to a total volume of 4 mL, which was subsequently incubated at 37 °C for another 6 h. The bacterial culture was then diluted in sterile 1x PBS buffer. The thigh burden infection model was established by injecting both posterior thighs of mice with 100 μL of inoculums. Two doses of the compounds P1 were given at 1 h/7 h and 1 h/13 h by i.v. bolus injection in the tail vein at 5 mg/kg per dose of drugs after bacterial infection. Thighs were harvested at 25 h for both groups after bacterial inoculation. Thigh muscles were collected in a sterile tared tube, to which 5 mL sterile PBS buffer was added. The mixture was then homogenized with a tissue homogenizer (BioSpec product tissue tearor 985-370) for approximately 30 sec. 100 mL of serial diluted aliquots were plated on TSA plates, which were incubated for 24 h at 37 °C. Then bacteria colonies were counted to calculate CFU per thigh.

MICs assay, time kill assay, hemolytic assay, drug resistance assay and fluorescence microscopy were the same as Chapter 2.
CHAPTER 4: SMALL LINEAR MOLECULES AS ANTIBIOTIC AGENTS

4.1 Introduction

Due to the omnipresent threat of bacterial infection is still a serious public health concern, AMPs have gained considerable interest.\textsuperscript{155} AMPs perform their function by electrostatic and hydrophobic interaction, kill bacteria by damaging their cell membranes.\textsuperscript{93} However, development of AMPs is impeded by their intrinsic drawbacks, such as poor selectivity, susceptibility to proteolytic degradation, and low-to-moderate activity. Furthermore, most antimicrobial peptides have large molecular weights (>1000 Da), and synthesis is too complex for production. As such, antimicrobial peptidomimetics started to draw attentions, which are smaller in size but still retain potential broad-spectrum activity.\textsuperscript{99}

The new class of peptidomimetics “γ-AApeptide” developed by our group have been shown resistance to proteolytic degradation, and various side chain acylating agents can create almost limitless chemical diversity.\textsuperscript{156} These advantages make γ-AApeptide a promising candidate for paralleling function and structure of AMPs.\textsuperscript{157} Indeed, a variety of γ-AApeptides have been developed and displayed potent and broad-spectrum antimicrobial activity. However, most lead compounds are long sequences which require multi steps synthesis. If small molecules can be developed based on γ-AApeptides, it would enhance potential application significantly. Moreover, a few research groups have reported the study of peptidomimetic a-hybrid peptidic oligomers for antimicrobial activity, which had unexpected antimicrobial activity and low hemolytic activity.\textsuperscript{158-159} Previously, our group has developed a class of lipo-linear α/γ-AApeptides that utilize a hybrid backbone of α-peptide with γ-AApeptides.\textsuperscript{160} This class of peptides display broad-spectrum antimicrobial activity. Therefore, the heterogeneous backbone could further enhance the chemodiversity for future optimization and development.
4.2 Results and discussion

From previous studies in Chapter 2, we know that amphipathic property is essential for HDPs. Therefore, we designed a series of small linear molecules based on structures. Different hydrophobic groups and cationic charged groups were rationally introduced and distributed to find out the best potent antimicrobial agent. All small linear molecules were synthesized on solid phase. The synthesis is very straightforward (Scheme 4.1), which allowed compounds to be synthesized efficiently.

Figure 4.1 General approach of small linear molecules synthesis on solid phase.
Figure 4.2 Structure-based design of compound L1-L23.
<table>
<thead>
<tr>
<th>L11</th>
<th><img src="image1.png" alt="Structure" /></th>
<th><img src="image2.png" alt="Structure" /></th>
<th>NH₂</th>
<th>C₁₃H₂₇</th>
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<td><img src="image4.png" alt="Structure" /></td>
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<td><img src="image6.png" alt="Structure" /></td>
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<td><img src="image8.png" alt="Structure" /></td>
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<td><img src="image26.png" alt="Structure" /></td>
<td>NH₂</td>
<td>C₁₁H₂₃</td>
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Figure 4.2 Continued Structure-based design of compound L1-L23.
Table 4.1 Structure-based design of compounds L24-L28.

<table>
<thead>
<tr>
<th>Compound</th>
<th>R1</th>
<th>R2</th>
<th>R3</th>
<th>R4</th>
<th>Cx</th>
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</thead>
<tbody>
<tr>
<td>L24</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>C11H23</td>
</tr>
<tr>
<td>L25</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>C11H23</td>
</tr>
<tr>
<td>L26</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>C15H23</td>
</tr>
<tr>
<td>L27</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>C11H23</td>
</tr>
<tr>
<td>L28</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>C11H23</td>
</tr>
</tbody>
</table>

Figure 4.3 Structure-based design of compound L24-L28.

Table 4.2 Structure-based design of compounds L29-L30.

<table>
<thead>
<tr>
<th>Compound</th>
<th>R1</th>
<th>R2</th>
<th>R3</th>
<th>R4</th>
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<td></td>
<td></td>
<td>C15H31</td>
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</tbody>
</table>

Figure 4.4 Structure-based design of compound L29-L30.
All small linear molecules were tested against a panel of Gram-positive bacteria for their antimicrobial activity (Table 4.1). As shown in Figure 4.2, compounds L1-L23 are composed by 1
amino acid, 1 γ-AApeptide building block with urea side chain and Cx lipid tail. Compound L1-L5 have the same R1, R2 and R3 groups, but the length of Cx group is varied. When compared MICs results in Table 4.2, L3 and L4 have better antimicrobial activity than other compounds which indicated that C12 and C14 lipid tails lead to the activity increase. Compound L10 has the best overall antimicrobial activity among L3, L6, L7, L8, L9 and L10, which have the same R2, R3 and Cx groups and varied amino acid at R1 position. Compared to Lys, Arg, Leu, Try and Gly residue, Glu residue has the best activity that all MICs are lower than 4 µg/mL. When changing the length of C12 lipid tail on L10 to C14 and C16, L11 and L12 have no activity improvement. When compared MICs of compound L3 with L13-L20, it is obvious that L3 has the overall best antimicrobial activity. They have the same R1, R3 and Cx groups and different R2 groups. It’s indicated that 1-chloro-3-isocyanatobenzene provided the best antimicrobial activity among these urea functional groups. MICs of compound L23 indicates that there is no activity. Therefore, Lys residue on R3 position is essential. As shown in Figure 4.3, compounds L24-L28 have an extra amino acid compared to compounds in Figure 4.2. When compared MICs of L3, L24 and L25, L3 has better activity which indicates that one extra Lys amino acid and Phe amino acid could not increase antimicrobial activity of compound. Same results appeared when compared L10, L27 and L28. Furthermore, increasing length of C12 lipid tail to C16 decreased the activity when comparing MICs of L25 and L26. As shown in Figure 4.4 and 4.5, compound L29-L31 have one more amino acid compared with compounds in Figure 4.3. When compared MICs of L24 and L29, the increased Lys amino acid didn’t make obvious improvement. The same situation was happened when comparing L27, L30 and L31. Furthermore, L32 was designed by adding one Lys amino acid to compound L23, still no antimicrobial activity. As shown in Figure 4.7, compound L33 was designed by switching order of Lys amino acid and γ-Lys-BB of compound L3,
which did not increase the antimicrobial activity.

To summary all MICs results, it is indicated that the number and property of amino acids, length of Cx lipid tail and urea side chains are essential to antimicrobial activity and L10 is the lead compound.

Table 4.1 Antibiotic activity of linear small molecule compounds.

<table>
<thead>
<tr>
<th>Compound</th>
<th>MIC (µg/mL)</th>
<th>Gram Positive</th>
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<td>MRSE</td>
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<td>L11</td>
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</table>
Table 4.1 Continued Antibiotic activity of linear small molecule compounds.

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<td>L33</td>
<td>3.12</td>
<td>12.5</td>
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</tbody>
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To study the selectivity of small linear molecules, hemolytic assay was carried out. As shown in Table 4.2, compound L10 not only has the best antimicrobial activity but also has the best selectivity.
over MRSA among this series of small linear compounds.

**Table 4.2** Selectivity of linear small molecule compounds.

<table>
<thead>
<tr>
<th>Compound</th>
<th>MIC of MRSA (µg/mL)</th>
<th>HC50 (µg/mL)</th>
<th>SI (MIC/HC50)</th>
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<td>10</td>
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Table 4.2 Continued Selectivity of linear small molecule compounds.

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<thead>
<tr>
<th>Compound</th>
<th>Selectivity</th>
<th>MIC</th>
<th>CMC</th>
</tr>
</thead>
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<tr>
<td>L33</td>
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<td>80</td>
<td>25.6</td>
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We next conducted time-kill studies of compound L10 to study its bacteria killing kinetics. As shown in Figure 4.8, MRSA was treated with compound L10 at three different concentrations: 2× MIC, 4× MIC, and 8× MIC. Treatment L10 at 2× MIC, 4× MIC can control and slow down MRSA growing. Furthermore, at 8× MIC, compound L10 can eradicate MRSA completely within 30 min. This indicates that L10 can kill MRSA rapidly.

![Figure 4.8](image)

**Figure 4.8** Time-kill plots of L10 against MRSA.

Next, their impact on bacterial membranes was tested by fluorescent microscopic studies. Compound L10 was tested against MRSA at 2× MIC for 2 h. As shown in Figure 4.9, in the DAPI
channel, MRSA cells emitted blue fluorescence with and without L10 treatment. However, MRSA cells only emitted red fluorescence with L10 treatment, indicating that their membranes were disrupted therefore stained by PI dye.

<table>
<thead>
<tr>
<th></th>
<th>DAPI</th>
<th>PI</th>
</tr>
</thead>
<tbody>
<tr>
<td>MRSA</td>
<td><img src="image1" alt="a1" /></td>
<td><img src="image2" alt="b1" /></td>
</tr>
<tr>
<td>MRSA + P10</td>
<td><img src="image3" alt="a2" /></td>
<td><img src="image4" alt="b2" /></td>
</tr>
</tbody>
</table>

**Figure 4.9** Fluorescence micrographs of MRSA treated and not treated with 2XMIC. (a1) Control, no treatment, DAPI stained; (a2) control, no treatment, PI stained; (b1) MRSA treated with L10, DAPI stained; (b2) MRSA treated with L10, PI stained.

### 4.3 Conclusions

In summary, we investigated a new class of small linear molecules as potential antibiotic agents against Gram-positive bacteria. They were structure-based designed with both cationic charged groups and hydrophobic groups. Our studies suggest that these compounds can disrupt bacteria membranes and kill bacteria rapidly. Due to their small molecular weight and facile synthesis approach, they could be potential antibiotic agents. Further characterization of lead compound are currently underway in our lab.

### 4.4 Experimental

#### 4.4.1 General information
Rink amide MBHA resins (0.7 mmol/g, 200–400 mesh) were purchased from Chem-Impex Int’l Inc. The solid phase syntheses of all compounds were carried out in a peptide reaction vessel on a Burrell Wrist-Action shaker. Solvents and other chemicals were ordered from either Fisher Scientific or Sigma-Aldrich, and were used without further purification. All compounds were analyzed and purified using the Waters Breeze 2 HPLC system under 215 nm of UV detector equipped with both analytical and preparative modules. The desired fractions were lyophilized on a Labcono lyophilizer.

4.4.2 Synthesis of γ-AApeptide building blocks

\[ \text{γ-Lys-BB} \quad \text{γ-Phe-BB} \]

Figure 4.10 γ-AApeptide building blocks.

Method to synthesize γ-AApeptides building blocks is the same as Chapter 2.

4.4.3 Synthesis of small linear compounds on solid phase

Synthetic procedure of the compound L10: 200 mg Rink-amide (MBHA) resin (0.14 mmol) was treated with 3 mL 20% piperidine/DMF (v/v) solution for 15 min (× 2) to remove the Fmoc protection
group, followed by DMF (2 mL × 3) and DCM (2 mL × 3) wash. The attachment of Fmoc-L-Phenylalanine to the resin was achieved by adding Fmoc-L-Phenylalanine (155 mg, 0.4 mmol), DIC (101 mg, 114 μL, 0.8 mmol), and HOBt (122 mg, 0.8 mmol) in 3 mL DMF to the reaction vessel, and the reaction was allowed to shake at room temperature for 3 h. The solution was drained, and the beads were washed with DCM (3 mL × 3) and DMF (3 mL × 3). After that, beads were treated with 3 mL 20% piperidine/DMF (v/v) solution for 15 min (× 2) to remove the Fmoc protection group, followed by DMF (2 mL × 3) and DCM (2 mL × 3) wash. Then add γ-Lys-BB (238 mg, 0.4 mmol), DIC (101 mg, 114 μL, 0.8 mmol), and HOBt (122 mg, 0.8 mmol) in 3 mL DMF to the reaction vessel, and the reaction was allowed to shake at room temperature for 3 h. The solution was drained, and the beads were washed with DCM (3 mL × 3) and DMF (3 mL × 3). After that, the resin was treated with Pd(PPh₃)₄ (24 mg, 0.02 mmol) and Me₂NH.BH₃ (70 mg, 1.2 mmol) in 3 mL DCM for 10 min (× 2) to remove the alloc protein group, then washed with DCM (3 mL × 3) and DMF (3 mL × 3). Next, 3-chlorophenyl isocyanate (77 mg, 61 μL, 0.5 mmol) and DIPEA (65 mg, 87 μL, 0.5 mmol) in 3 mL DCM were added to the resin and allowed to react for 30 min at room temperature, and then the solution was drained. After DMF (2 mL × 3) and DCM (2 mL × 3) wash, beads were treated with 3 mL 20% piperidine/DMF (v/v) solution for 15 min (× 2) to remove the Fmoc protection group, followed by wash with DMF (2 mL × 3) and DCM (2 mL × 3). Subsequently, lauric acid (80 mg, 0.4 mmol), DIC (101 mg, 114 μL, 0.8 mmol), and HOBt (122 mg, 0.8 mmol) in 3 mL DMF were added to the reaction vessel and reacted for 3 h. After the solution was drained, the beads were washed with DMF (2 mL × 3) and DCM (2 mL × 3), followed by the incubation with 4 mL cocktail of 1:1 TFA: DCM 1:1 (v/v) for 2 h to achieve cleavage and global deprotection of the compound. After the solvent was removed in vacuo, the residue was analyzed and
purified on the Waters HPLC system, and the desired fraction was lyophilized to give the pure product L10.

Synthesis of other compounds: The other compounds were synthesized following the similar procedure of compound L10.

Figure 4.12 Structures of small linear compounds L1-L33.
Figure 4.12 Continued  Structures of small linear compounds L1-L33.
Figure 4.12 Continued Structures of small linear compounds L1-L33.
Figure 4.12 Continued Structures of small linear compounds L1-L33.

MICs assay, time kill assay, hemolytic assay and fluorescence microscopy were the same as Chapter 2.
CHAPTER 5: ONE-BEAD–TWO-COMPOUND THIOETHER BRIDGED MACROCYCLIC
γ-AA PEPTIDES SCREENING LIBRARY AGAINST SMYD2

5.1 Introduction

During this new era of development and discovery of numerous new disease targets, combinatorial chemistry is a powerful tool to identify ligands that recognize protein targets with high specificity and affinity, therefore could help to understand and diagnose function of proteins and lead to potential therapeutic treatment. Combinatorial library can synthesize large number of possible compounds and provide unbiased opportunity for ligand identification. In 1990s, combinatorial library methods became key technology for accelerating the discovery of novel therapeutic agents and had various applications in many fields, such as peptides, oligonucleotides, proteins, synthetic oligomers, small molecules, and oligosaccharides. However, only a few of peptidomimetic combinatorial libraries were investigated for protein ligand identification.

According to our previous study, γ-AApeptides have high resistance to proteolytic degradation and enhanced chemodiversity, which make them ideal candidates for molecular probes and therapeutic agents. These advantages of γ-AApeptides have been demonstrated by our previously developed one-bead-one-compound (OBOC) linear combinatorial libraries, which capable of finding hits to inhibit Aβ aggregation and disrupt STAT3/DNA interaction. Compared to linear γ-Aapeptides, cyclic γ-AApeptides are more stable against proteolytic degradation and possess cellular translocation capability. Thus, we developed a one-bead-two-compound (OBTC) cyclic γ-AApeptides-based (γ-substituted-N-acylated-N-aminoethyl amino acids) combinatorial library against SMYD2.

SET and MYND domain-containing protein 2 (SMYD2) is a lysine methyltransferase that is
highly expressed in pediatric acute lymphoblastic leukemia and esophageal squamous cell carcinoma.\textsuperscript{163} Its function as a methyltransferase has been reported to various substrates including retinoblastoma tumor suppressor (Rb) (K860),\textsuperscript{164} histone H3 (K36 and K4),\textsuperscript{165-166} and tumor suppressor p53 (K370).\textsuperscript{167} SMYD2 is believed to play an important role in the network of post-translational modifications that regulates tumor growth. Therefore, finding hits binding to SMYD2 protein is essential for potential cancer treatment.

5.2 Results and discussion

One-bead-two compound cyclic $\gamma$-AApeptides-based library was synthesized on Tenta-Gel due to it is non-sticky and uniform in size. Cyclic peptides were achieved by thioether-bridge mediated cyclization, which has proven to be highly efficient in cyclization. Compared with linear peptides, cyclic peptides have rigidified conformational freedom and enhanced metabolic stability. Heinis et al also adopted the thioether linkage to develop phage-display mediated bicyclic peptide libraries.\textsuperscript{169-171} As shown in Figure 5.1 we introduced a Dmt (4,4′-dimethoxytrityl) protected mercaptoethyl carbonyl group to the secondary amine in the first $\gamma$-AApeptide building block on the solid phase. The 4-(bromomethyl)benzoyl group was attached to the N-terminal amino group of the sequence after all $\gamma$-AApeptide building blocks were added. After that, Dmt protecting group was removed and cyclization was achieved with high efficiency of sulfur-mediated SN2 reaction (Figure 5.2).

![Figure 5.1 Structure design of one-bead-two compound cyclic $\gamma$-AApeptides-based library.](image-url)
As shown in Figure 5.1, five different N-Alloc protected γ-AApeptide building blocks were added to position X₁-X₄ and six diverse carboxylic acids or acyl chlorides side chains were added to position Y₂-Y₄ after deprotection of the alloc protecting group. Therefore, the theoretical diversity of the library was expected to be 5x5x6x5x6x5x6=135000, and 405000 beads were used in library synthesis with each compound has three copies.

Dde ((1-(4,4-dimethyl-2,6-dioxacyclohexylidene) ethyl) protected α-amino acids were used to encode and decode structures of compound because that deprotection of Dde was very mild using NH₂OH•HCl and imidazole and had no effect on other chemical reactions engaged in the synthesis of thioether-bridged cyclic γ-AApeptides. In Figure 5.2, corresponding Dde protected amino acid were added to position R₁-R₇.

Library screening was carried out after library synthesis was complete. To conduct screening, Tenta-Gel beads were incubated with SMYD2 protein first and then primary and secondary SMYD2 antibody. Beads emitting fluorescence were picked as hits (Figure 5.3).
Subsequently, hits were washed, denatured carefully and cleaved with CNBr. After MS/MS analysis, structures of hits were decoded and hits were resynthesized on rink amide resin. Among 10 hits, three hits were decoded and resynthesized (Figure 5.4).

Figure 5.4 Structures of three hits.

Further bioactivity tests are underway.
5.3 Experimental

5.3.1 General information

Rink amide MBHA resins (0.7 mmol/g, 200‒400 mesh) were purchased from Chem-Impex Int’l Inc. Tentagel resin (0.23 mmol/g) was purchased from RAPP Polymere. Solvents and other chemicals were ordered from either Fisher Scientific or Sigma-Aldrich. Fmoc-protected amino acids were purchased from Chem-impex. The solid phase syntheses of all compounds were carried out in a peptide reaction vessel on a Burrell Wrist-Action shaker. All compounds were analyzed and purified using the Waters Breeze 2 HPLC system under 215 nm of UV detector equipped with both analytical and preparative modules. The desired fractions were lyophilized on a Labcono lyophilizer. Masses of γ-AApeptides and the MS/MS analysis were obtained on an Applied Biosystems 4700 Proteomics Analyzer.

5.3.2 Synthesis of γ-AApeptide building blocks

\[ \text{γ-Ala-BB} \quad \text{γ-Leu-BB} \quad \text{γ-Phe-BB} \]
\[ \text{γ-Glu-BB} \quad \text{γ-Lys-BB} \]

Figure 5.5. γ-AApeptide building blocks.

All 5 γ-AApeptide building blocks shown above were used in the synthesis of One-bead Two-compound library; their synthetic procedure was reported previously.\(^\text{126}\)
5.3.3 Synthesis of Dde protected amino acids

\[
\begin{align*}
\text{5,5-dimethylcyclohexane-1,3-dione} & \quad + \quad \text{AcCl} \\
& \quad \xrightarrow{\text{DIPCA, DMAP, DCM}} \\
\end{align*}
\]

\[
\begin{align*}
\text{H}_{2}\text{N-CH}_{2}\text{COOH} & \quad + \quad \text{H}_{2}\text{O} \\
& \quad \xrightarrow{\text{TEA, Ethanol reflux}} \\
\end{align*}
\]

**Figure 5.6** Synthesis of Dde protected amino acids

![Structures of all Dde protected amino acids](image)

**Figure 5.7** Structures of all Dde protected amino acids.

To a 100 mL round bottom flask was added 5,5-dimethylcyclohexane-1,3-dione (10 g, 71.34 mmol), N,N-Diisopropylethylamine (14.91 mL, 85.6 mmol), 4-Dimethyaminopyridine (435.76 mg, 3.57 mmol) and 50 mL DCM. The mixture was stirred in an ice bath to which acetyl chloride (6.08 mL, 72
85.6 mmol) was added. The reaction was warmed up to room temperature and allowed to stir for 8 h. After solvent was removed and the residue was washed with 1M HCl and extracted with ethyl acetate for 3 times. The organic layer was dried with anhydrous Na$_2$SO$_4$ then was purified by flash column chromatography (Hexane/Ethyl acetate 1:1). The product 2-acetyl-5,5-dimethylcyclohexane-1,3-dione is a yellowish solid

The L-Amino acid (1 equiv), 2-acetyl-5,5-dimethylcyclohexane-1,3-dione (1.3 equiv) and triethylamine (1.5 equiv) was refluxed in 50mL ethanol for 18 h. The reacted yellow solution then was cooled and concentrated. The residue was dissolved in 50 mL DCM, and washed with 50 mL 1 M HCl twice. The organic layer was dried by Na$_2$SO$_4$ and filtered. After solvent DCM was removed, add 40mL Et$_2$O to the residue resulted in immediate white precipitate, which was filtered and washed with cold Et$_2$O to obtain off-white crystalline solid as product.

5.3.4 Synthesis of side chains and linkers

Figure 5.8 Structures and synthesis of 6 side chains.
Side chain 3 cyclopropylacetic acid was purchased from AK Scientific. Side chain 6 3,4-(methylenedioxy) phenylacetic acid was purchased from TCI.

Synthesis of side chain 1: the 3-Cyclohexanepropionic acid was refluxed in 10 mL thionyl chloride for 5 h. The excess thionyl chloride was removed under vacuum. The desired product is a white solid and can be used without purification.

Synthesis of side chain 2: same as side chain 1.

Synthesis of side chain 4: 4-Aminobutyric acid (5g, 40.5 mmol) was dissolved in 150 mL THF at 0 degree Celsius. Di-tert-butyl decarbonate (14 mL, 60.9 mmol) and 1M NaOH (49 mL) were then added to solution. The mixed was stirred at room temperature for 3 h. Remove the solvent and add water (30 mL) and ethyl acetate (20 mL). Adjust PH of solution to 2 by adding 1M HCl. The solution was exact with ethyl acetate three times, and organic layer was dried with anhydrous sodium sulfate. After flash column purification, the desired product is a white solid.

Synthesis of side chain 5: to a mixture of succinic anhydride (30g, 0.3 mol), N-hydroxysuccinimide (10g, 0.09 mol) and DMAP (3.5g, 0.03 mol) in toluene (150 mL), add tert-butyl alcohol (35mL) and Et3N (12.5mL, 0.09 mol). The mixture was refluxed for 24 h. Then cool the solution and add 150 ethyl acetate (150mL). Wash solution with 10% citric acid and dried with anhydrous sodium sulfate. The solvent was removed and residue was recrystallize by ether and petroleum ether at -20 degree Celsius. The desired product is a white solid.
Synthesis of linker 1: the 4-(bromomethyl) benzoic acid was refluxed in 10 mL thionyl chloride for 5 h. The excess thionyl chloride was removed under vacuum. The desired product is a white solid and can be used without purification.

Synthesis of linker 2: 4,4'-dimethoxytrityl chloride (6.38g, 18.82 mmol), 3-mercaptopropionic acid (1.64ml, 18.82 mmol) and triethylamine (3.93, 22.58 mmol) were dissolved in 40 mL DCM. The solution was stirred at room temperature for 4 h~ 6 h. After evaporated solvent, the residue was washed with saturated citric acid and extracted with ethyl acetate 3 times. Organic layer was dried with anhydrous Na₂SO₄ and the solvent was removed. After purified by flash column chromatography (Hexane/Ethyl acetate 1:1), desired product is a light yellow solid.

5.3.5 Synthesis of One-bead Two-compound library

The One-bead Two-compound cyclic γ-AApeptide library was prepared on solid phase using TentaGel NH₂ resin (2.64g, 0.61mmol, 405000 beads). Fmoc protecting group was removed by 20% (v/v) piperidine in DMF (10 min × 2). Alloc protecting group was removed by Pd(PPh₃)₄ (8 mg, 0.007
mmol) and Me₂NH·BH₃ (25 mg, 0.42 mmol) in 3 mL DCM (10 min × 2). All γ-AApeptide building blocks (2 equiv) and carboxylic acids (2 equiv) were coupled twice to desired amino groups using HOBt (4 equiv.) and DIC (4 equiv.) in DMF, each time is 6 h. All the acyl chlorides (2 equiv.) side chains were coupled to desired amino groups using DIPEA (4 equiv.) in DCM twice, each time is 30 min. All Dde protected amino acids (5.5 equiv.) were coupled to the desired amino groups with PyBop (5 equiv.) and NEM (11 equiv.) in DMF for 3 h. Dde protecting groups were removed by shaking in 5 mL Dde deprotection solution and 1 mL DCM twice, each time is 3 h. Dde deprotection solution was prepared by NH₂OH·HCl (1.25 g, 0.180 mmol) and imidazole (0.918 g, 0.135 mmol) dissolved in 5 mL NMP.

Briefly, TentaGel resin beads was soaked in water overnight to allow two layers separated from each other. Inner layer contains a coding peptide and outer layer composes of the cyclic γ-AApeptide ligand. Transfer beads into reaction vessel, then rinse the resin three times with 1:1 (v/v) DCM/diethyl ether. Add (Boc)₂O (0.5 equiv.) and 1:1 (v/v) DCM/diethyl ether to the vessel, then the mixture was shaken for 3 h. After reaction is complete, wash beads three times with DCM and DMF. Next, Fmoc-Met-OH (0.5 equiv.), HOBt (2 equiv.) and DIC (2 equiv.) were added to react with the inner layer of the resin. Remove Fmoc protecting group and split beads into 5 equal portions. To 5 reacting vessels, add Dde-Ala-OH, Dde-Phe-OH, Dde-Leu-OH, Dde-Val-OH, and Dde-Glu(OBn)-OH, respectively. Boc protecting group on the outer layer was removed by using mixture of 94% TFA, 2% TIS (triisopropylsilane), 2% H₂O and 2% Thioanisole for 1 h, and corresponding γ-AApeptide building blocks were coupled on the beads. Next, Alloc protecting group of the first γ-AApeptide building block was removed, and Dmt protected mercaptopropionic acid was added. After washed with DMF and DCM, all beads were pooled together and shaken to be mixed thoroughly. Next, split beads into 5 equal
portions again. Dde protecting group of the coding peptides on the inner layer was removed and 5 different Dde protected amino acids were coupled separately in each vessel. Then Fmoc protecting group of the first γ-AApeptides on the outer layer was removed and desired γ-AApeptide building blocks were added. The beads were pooled and split again, and the synthetic cycle was repeated three more times. Then Fmoc group of the outer layer was removed, then beads were reacted with the 4-(bromomethyl)benzoyl chloride. Next, Dmt protecting group was removed by mixture of 2% TFA, 2% triisopropylsilane and 96% DCM for several times until the deprotecting solution became colorless, each time is 2 min. The cyclization of γ-AApeptide was achieved with shaking beads in solution of (NH₄)₂CO₃ (10 equiv) in 1:1 (v/v) DMF/H₂O for 8 h, repeat twice. Wash beads with DMF and DCM. Finally, protecting groups on the sidechains were removed with 94% TFA, 2% triisopropylsilane, 2% H₂O and 2% Thioanisole for 1 h.

**Figure 5.10** Preparation of the thioether bridged cyclic library.¹⁷³ (Adapted with permission from ACS. Copyright 2017)
The SMYD2 protein was used as a target for the combinatorial library screening. The beads were screened and picked up under a Zeiss inverted fluorescence microscope installed with a 10x43HE filter. To avoid any possible nonspecific binding, SMYD2 and antibodies solution were all made in 1% BSA/TBST blocking buffer.

The TentaGel beads (2.64 g, 405000 beads) were soaked in DMF for 1 h. After being washed with 1xTris buffer for five times, the beads were equilibrated in Tris buffer overnight at room temperature.
temperature. Then beads were incubated with blocking buffer (1% BSA in 1×Tris buffer with a 1000× excess of cleared E. coli lysate) for 1 h.

E. coli lysate preparation: take two colony of E. coli and add to 25 ml TSB media, incubate for 16 h on the shaker, 250RPM. Then spin down E. coli solution in centrifuge machine (4 °C, 3000RPM, 15 min), and discard the supernatant liquid (wipe the wall with paper towel to fully remove the liquid). Put the solution on EtOH/Dry ice bath. Add 8 ml lysate buffer, re-suspend, combine solution in one centrifuge tube rest on ice for 30min. The solution was sonicated while cooling (10 times, each time 10 sec, rest on ice for 5 sec in between). Next, solution was centrifuged twice (6000rpm, 4 °C), each time is 15 min.

Prescreening: Beads were incubated with primary SMYD2 antibody (1:1000 dilution) for 2h at room temperature. After a thorough wash with 1× Tris buffer, the beads were then incubated with the secondary SMYD2 antibody(1:1000 dilution) for 2 h at room temperature. The beads were washed with 1× Tris buffer (3×), and then transferred into a 6-well plate to be observed under Zeiss inverted fluorescence microscope installed with the 10×43HE filter. Beads emitting red fluorescence were picked up and excluded from formal screening.

The rest of the beads were pooled together into the peptide vessel, washed with 1× Tris buffer, and then treated with 8 M guandine-HCl at room temperature for 1h to remove any bound proteins. The guandine-HCl was then washed away with both water and Tris buffer. The beads were then shaken in DMF for 1 h, followed by washing and equilibration in Tris buffer overnight.

Screening: The beads were incubated in 1% BSA/Tris buffer and 1000× excess of E. coli lysate for 1 h at room temperature. After wash with Tris buffer for 5 times, the beads were incubated with SMYD2 protein at a concentration of 50 nM for 4 h at room temperature with E. coli lysate. Next, the
library beads were incubated with 10 μL primary SMYD2 antibody in 10 mL solution for 2 h at room temperature. The beads were washed by Tris buffer and incubated with 10 μL secondary SMYD2 antibody for 2 h at room temperature. Beads were washed thoroughly with Tris buffer and then transferred into the 6-well plate. Under fluorescence microscope, beads emitting read fluorescence were picked up as hits. Each hit bead was transferred to an Eppendorf microtube, and denatured in 100 μL 8M guanidine•HCl for 1h at room temperature, respectively. Each bead was rinsed with Tris buffer, water, DMF, and ACN, each time is 10 min. At last, beads were soaked in ACN overnight in each Eppendorf microtube and then ACN was evaporated. The bead was incubated in mixture of 5:4:1 (v/v/v) ACN: glacial acetic acid: H₂O and cyanogen bromide (50 mg/mL) overnight at room temperature. The solution was then evaporated, and cleaved peptide was dissolved in ACN : H₂O (4:1) and run MALDI MS/MS analysis.

5.3.7 Synthesis of Hits

Synthesis of **Hit 1**: 200 mg Rink-amide (MBHA) resin (0.14 mmol) was treated with 3 mL 20% piperidine/DMF (v/v) solution for 15 min (× 2) to remove the Fmoc protection group, followed by DMF (2 mL × 3) and DCM (2 mL × 3) wash. Then the first γ-AApeptide building block γ-Ala-BB (176 mg, 0.4 mmol), DIC (101 mg, 114 μL, 0.8 mmol), and HOBt (122 mg, 0.8 mmol) in 3 mL DMF to the reaction vessel, and the reaction was allowed to shake at room temperature for 3 h. The solution was drained, and the beads were washed with DCM (3 mL × 3) and DMF (3 mL × 3). After that, beads were treated with Pd(PPh₃)₄ (24 mg, 0.02 mmol) and Me₂NH.BH₃ (70 mg, 1.2 mmol) in 3 mL DCM for 10 min (×2) to remove the alloc protein group, then washed with DCM (3 mL x3) and DMF (3 mL x3). Next, Dmt protected mercaptopropionic acid (166 mg, 0.5 mmol) and DIPEA (87 μL, 0.5 mmol) in 3 mL DCM were added to the resin and allowed to react for 30 min at room temperature, and then the
solution was drained. After DMF (2 mL × 3) and DCM (2 mL × 3) wash, beads were treated with 3 mL 20% piperidine/DMF (v/v) solution for 15 min (× 2) to remove the Fmoc protection group, followed by wash with DMF (2 mL × 3) and DCM (2 mL × 3). Then the second γ-AApeptide building block γ-Ala-BB (176 mg, 0.4 mmol), DIC (101 mg, 114 μL, 0.8 mmol), and HOBt (122 mg, 0.8 mmol) in 3 mL DMF to the reaction vessel, and the reaction was allowed to shake at room temperature for 3 h. The solution was drained, and the beads were washed with DCM (3 mL × 3) and DMF (3 mL × 3). After that, beads were treated with Pd(PPh₃)₄ (24 mg, 0.02 mmol) and Me₂NH.BH₃ (70 mg, 1.2 mmol) in 3 mL DCM for 10 min (× 2) to remove the alloc protein group, then washed with DCM and DMF. Subsequently, cyclopropylacetic acid (40 mg, 0.4 mmol), DIC (101 mg, 114 μL, 0.8 mmol), and HOBt (122 mg, 0.8 mmol) in 3 mL DMF were added to the reaction vessel and reacted for 3 h. After the solution was drained, the beads were washed with DMF and DCM, Fmoc protecting group was removed and the third γ-AApeptide building block Ala-BB was added. Next, Alloc protecting group was removed and 3-phenylpropanoyl chloride (84 mg, 0.5 mmol) and DIPEA (65 mg, 87 μL, 0.5 mmol) in 3 mL DCM were added to the resin and allowed to react for 30 min at room temperature. After that, the fourth γ-AApeptide building block Phe-BB was added after Fmoc protecting group was removed. Next, remove Alloc protecting group and add 3-cyclohexylpropanoyl chloride (87 mg, 0.5 mmol) and DIPEA (65 mg, 87 μL, 0.5 mmol) in 3 mL DCM to react for 30 min. Then remove Fmoc protecting group and add 4-(bromomethyl)benzoyl chloride (117 mg, 0.5 mmol) and DIPEA (65 mg, 87 μL, 0.5 mmol) in 3 mL DCM to react for 30 min. Next, Dmt protecting group was removed by treated resin beads with mixture of 2% TFA, 2% triisopropylsilane and 96% DCM for several times until the deprotecting solution became colorless, each time is 2 min.
Then cyclization was achieved with shaking beads in solution of (NH₄)₂CO₃ (10 equiv) in 1:1 (v/v) DMF/H₂O for 8 h, repeat twice. Wash beads with DMF and DCM. Finally, resin beads was incubated with 4 mL cocktail of 1:1 TFA: DCM 1:1 (v/v) for 2 h to achieve cleavage and global deprotection of the compound. After the solvent was removed in vacuo, the residue was analyzed and purified on the Waters HPLC system, and the desired fraction was lyophilized to give the pure compound Hit 1.

Synthesis of other 2 Hits is similar as Hit 1.
REFERENCES


52. Lam, K. S.; Salmon, S. E.; Hersh, E. M.; Hruby, V. J.; Kazmierski, W. M.; Knapp, R. J., A new


76. Tu, Y.; McCalla, D. R., Effect of activated nitrofurans on DNA. *Biochimica et Biophysica Acta (BBA) - Nucleic Acids and Protein Synthesis* 1975, 402 (2), 142-149.


85. McKinnell, J. A.; Stollenwerk, N. S.; Jung, C. W.; Miller, L. G., Nitrofurantoin compares favorably to recommended agents as empirical treatment of uncomplicated urinary tract infections in a decision


101. Shi, Y.; Teng, P.; Sang, P.; She, F.; Wei, L.; Cai, J., γ-AApeptides: Design, Structure, and
Applications. Accounts of chemical research 2016, 49 (3), 428-441.


109. Marr, A. K.; Gooderham, W. J.; Hancock, R. E. W., Antibacterial peptides for therapeutic use:
91


APPENDIX A: $^1$H AND $^{13}$C NMR SPECTRA

Compound 1
Compound 2
Compound 3
Compound 4
Compound 5
Compound 6
Compound 7
Compound 8
Compound 9
Compound 10
Compound 11
Compound 12
Compound 13
Compound 14
Compound 15
Compound 16
Compound 17
Compound 18
Compound 19
Compound 20
Compound 21
Compound 22
Compound 23
Compound 24
Compound 25
APPENDIX B: HPLC SPECTRA

Compound 1

Compound 2

Compound 3

Compound 4
Compound 5

Compound 6

Compound 7

Compound 8

Compound 9
Compound P4

Compound L1

Compound L2

Compound L3
Compound L4

Compound L5

Compound L6

Compound L7

Compound L8
Compound L12

Compound L13

Compound L14

Compound L15

Compound L16
Compound L25

Compound L26

Compound L27

Compound L28
Compound L33
APPENDIX C: PUBLISHING RIGHT

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Publication: Nature
Publisher: Springer Nature
Date: Nov 7, 1991

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APPENDIX D: IACUC APPROVAL

MEMORANDUM

TO: Chuanhai Cao,

FROM: Farah Moulvi, MSPH, IACUC Coordinator
Institutional Animal Care & Use Committee
Research Integrity & Compliance

DATE: 4/15/2015

PROJECT TITLE: Development of antimicrobial agents 1. Alpha-AApeptides as a novel class of antimicrobial biomaterials

FUNDING SOURCE: National Institutes of Health; USF department, institute, center, etc.

IACUC PROTOCOL #: R IS00001173

PROTOCOL STATUS: APPROVED

The Institutional Animal Care and Use Committee (IACUC) reviewed your application requesting the use of animals in research for the above-entitled study. The IACUC APPROVED your request to use the following animals in your protocol for a one-year period beginning 4/15/2015:

Mouse: CD-1 (6-8 weeks of age; range 132 of 20-26g in weight; equal number of males and females)

Please take note of the following:

• IACUC approval is granted for a one-year period at the end of which, an annual renewal form must be submitted for years two (2) and three (3) of the protocol through the eIACUC system. After three years all continuing studies must be completely re-described in a new electronic application and submitted to IACUC for review.

• All modifications to the IACUC-Approved Protocol must be approved by the IACUC prior to initiating the modification. Modifications can be submitted to the

4/15/2015
IACUC for review and approval as an Amendment or Procedural Change through the eIACUC system. These changes must be within the scope of the original research hypothesis, involve the original species and justified in writing. Any change in the IACUC-approved protocol that does not meet the latter definition is considered a major protocol change and requires the submission of a new application.

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