Synthesis and Analysis of Short Antimicrobial Peptides: RR, RIKA IGR, and HQ

Kathleen Pierce

Follow this and additional works at: https://digitalcommons.latech.edu/theses
SYNTHESIS AND ANALYSIS OF SHORT ANTIMICROBIAL PEPTIDES:

RR, RIKA, IGR, AND HQ

by

Kathleen Pierce B.S.

A Thesis Presented in Partial Fulfillment of the Requirements of the Degree Master of Science

COLLEGE OF APPLIED AND NATURAL SCIENCES
LOUISIANA TECH UNIVERSITY

August 2022
LOUISIANA TECH UNIVERSITY

GRADUATE SCHOOL

June 20, 2022
Date of thesis defense

We hereby recommend that the thesis prepared by

Kathleen Pierce

entitled Synthesis and Analysis of Short Antimicrobial Peptides: RR, RIKA
IGR, and HQ

be accepted in partial fulfillment of the requirements for the degree of

Master of Science in Biology

Rebecca Giorno-McConnell
Supervisor of Thesis Research

William Campbell
Head of Biological Sciences

Thesis Committee Members:
Rebecca Giorno-McConnell
Scott Poh
Jamie Newman
Kyle Kemege

Approved:
Gary A. Kennedy
Dean of Applied & Natural Sciences

Approved:
Ramu Ramachandran
Dean of the Graduate School
ABSTRACT

A significant impending threat to the public health system is the rise of multidrug resistant bacteria. An attractive potential option to combat this is antimicrobial peptides (AMPs). AMPs are peptide chains with lengths that range from 10 to 70 amino acid residues that show antimicrobial activity. AMPs tend to have less toxicity than other antibiotic therapies, have a broader range of activity, and have a decreased resistance development by the target cells. Short AMPs are especially attractive due to their stability as well as their ability to penetrate cell membranes. With a wide range of possible AMPs available, further characterization of these molecules is needed before using AMPs in a clinical setting. For this study, 4 previously identified AMPs (WLRRIKAWLRR [RR], WLRRIKAWLRIKA [RIKA], IIGGR [IGR], and HPQYNQR [HQ]) were synthesized using solid phase peptide synthesis and evaluated for antimicrobial activity. Previous studies regarding MIC assay methods indicate that special considerations, such as test vessel material and growth media, are required for AMPs to obtain accurate MIC data. Therefore, a resazurin colorimetric assay was conducted in a 96-well polypropylene microtiter plate with iso-sensitest broth. Eight species were challenged with these 4 peptides, many for the first time. Peptides RR and RIKA demonstrated antimicrobial activity in 6 and 7 species, respectively. RIKA consistently demonstrated more antimicrobial activity than RR. IGR and HQ showed no antibiotic activity in this study.
This novel study involved synthesizing four AMPs, designing a novel experimental method for antibiotic activity of AMPs, and expanding antimicrobial activity testing to eight bacterial species to allow for a comprehensive comparison of these potential novel antibiotic therapies. This comparison could renew interest in these AMPs and possible combination therapies.
APPROVAL FOR SCHOLARLY DISSEMINATION

The author grants to the Prescott Memorial Library of Louisiana Tech University the right to reproduce, by appropriate methods, upon request, any or all portions of this Thesis. It is understood that “proper request” consists of the agreement, on the part of the requesting party, that said reproduction is for his personal use and that subsequent reproduction will not occur without written approval of the author of this Thesis. Further, any portions of the Thesis used in books, papers, and other works must be appropriately referenced to this Thesis.

Finally, the author of this Thesis reserves the right to publish freely, in the literature, at any time, any or all portions of this Thesis.

Author  
Kathleen Pierce

Date 7/1/2022
DEDICATION

To my family, friends, and dog for providing support needed to finish this project.
TABLE OF CONTENTS

ABSTRACT ................................................................................................................................. iii
APPROVAL FOR SCHOLARLY DISSEMINATION ................................................................. v
DEDICATION ............................................................................................................................. vi
LIST OF FIGURES .................................................................................................................... ix
LIST OF TABLES ...................................................................................................................... xi
ACKNOWLEDGMENTS ............................................................................................................ xii
CHAPTER 1 INTRODUCTION ................................................................................................. 1
  1.1 Discovery and Development of Antibiotics ................................................................. 1
  1.2 Rise of Antibiotic Resistance ..................................................................................... 5
  1.3 Peptides with Antimicrobial Activity ......................................................................... 8
CHAPTER 2 Materials and methods .................................................................................... 12
  2.1 Materials ....................................................................................................................... 12
  2.1.1 Peptide Synthesis ................................................................................................. 12
  2.1.2 Analysis of Antimicrobial Activity .................................................................... 13
  2.2 Peptide Synthesis ......................................................................................................... 13
  2.2.1 Column Preparation and Deprotection Step ....................................................... 14
  2.2.2 SPPS Washing Cycle ............................................................................................ 15
  2.2.3 Coupling Reaction: Amino Acid Addition ......................................................... 16
  2.2.4 Cleavage from Resin ............................................................................................ 18
  2.3 Analysis of Antimicrobial Activity ............................................................................. 20
CHAPTER 3 Results ................................................................................................................ 23
3.1 Peptide Synthesis ........................................................................................................... 23

3.2 Antimicrobial Activity Analysis ...................................................................................... 23

CHAPTER 4 Discussion ........................................................................................................... 35

4.1 Peptide Synthesis ........................................................................................................... 35

4.2 Novel Experimental Design for determining AMP MICs ................................................ 35

4.3 Antimicrobial Activity of RR and RIKA ......................................................................... 38

4.4 Antimicrobial Activity of IGR and HQ ........................................................................... 40

CHAPTER 5 Conclusions and Future Directions ................................................................. 42

5.1 Conclusions ..................................................................................................................... 42

5.2 Future Directions ............................................................................................................ 43

CHAPTER 6 Appendix ........................................................................................................... 45

6.1 Amino Acids Used in this Study ...................................................................................... 45

6.2 MIC Assay General Plate Loading Scheme ..................................................................... 46

6.3 MALDI Results ............................................................................................................... 47

6.3.1 MALDI-IGR ............................................................................................................... 47

6.3.2 MALDI-HQ ............................................................................................................... 47

6.3.3 MALDI-RR ............................................................................................................... 48

6.3.4 MALDI-RIKA ........................................................................................................... 48

BIBLIOGRAPHY ..................................................................................................................... 49
LIST OF FIGURES

Figure 1-1: Timeline indicating the years antibiotics were approved as well as relevant developments ................................................................. 2

Figure 1-2: Five classes of antibiotic targets in Gram-positive and Gram-negative cells .................................................................................. 3

Figure 1-3: Antibiotic therapy pipeline ................................................................................................................................. 4

Figure 1-4: Mechanisms of Antibiotic Resistance .......................................................... 6

Figure 1-5: Common mechanisms of AMPs ........................................................................... 9

Figure 2-1: Fmoc-Rink Amide Resin ......................................................................................... 14

Figure 2-2: Mechanism for the Deprotection Step of the Fmoc-Rink Amide Resin to initiate Solid Phase Peptide Synthesis with 20% Piperidine ......................................................... 15

Figure 2-3: Coupling Reaction to Form a Peptide Chain ....................................................... 17

Figure 2-4: Cleavage of Newly formed AMP with. TFA, TIPS, and EDT in H₂O........ 19

Figure 3-1: Reduction of Resazurin to Resorufin to Dihydroresorufin .......................... 24

Figure 3-2: MIC assay for Bacillus anthracis ........................................................................... 26

Figure 3-3: MIC assay for Bacillus cereus ................................................................................. 27

Figure 3-4: MIC assay for Escherichia coli .............................................................................. 28

Figure 3-5: MIC assay for Klebsiella pneumoniae ................................................................... 29

Figure 3-6: MIC assay for Proteus mirabilis ........................................................................... 30

Figure 3-7: MIC assay for Pseudomonas aeruginosa .............................................................. 31

Figure 3-8: MIC assay for Staphylococcus aureus ................................................................. 32

Figure 3-9: MIC assay for Staphylococcus epidermidis .......................................................... 33
Figure 6-1: MIC Assay Plate Loading Scheme .................................................................46

Figure 6-2: MALDI Results for IGR ...................................................................................47

Figure 6-3: MALDI Results for HQ ...................................................................................47

Figure 6-4: MALDI Results for RR ...................................................................................48

Figure 6-5: MALDI Results for IGR ...................................................................................48
LIST OF TABLES

Table 1-1: AMPs Synthesized in this Project ................................................................. 10

Table 2-1: Bacterial Strains Used in this Project ............................................................ 21

Table 3-1: Minimum Inhibitory Concentrations of Synthesized AMPs. ...................... 24

Table 4-1: AMPs and Previously Tested Bacterial Strains ............................................. 38

Table 6-1: Amino Acids Used in this Study ................................................................. 45
ACKNOWLEDGMENTS

I am extremely grateful for Dr. Rebecca Giorno for all of the guidance, time, and patience she gave me throughout my time at Louisiana Tech University.

Thanks to my Committee Members for their guidance
Dr. Rebecca Giorno, Ph.D.
Dr. Scott Poh, Ph.D.
Dr. Jamie Newman, Ph.D.
Dr. Kyle Kemege, Ph.D.

Thanks to Those Who Contributed to this Project
Elizabeth Ruff
Dale Major

Thanks to Giorno Lab Members
Andrew Roser
Morgan Nall
Jacob Saucier
CHAPTER 1

INTRODUCTION

1.1 Discovery and Development of Antibiotics

Since their discovery, antibiotics have become the widespread clinical standard therapy for illnesses caused by bacteria. In 2015, the number of antibiotics consumed worldwide was estimated to be 42 billion defined daily doses and that number is projected to increase 200% by 2030.\(^1\) Arsphenamine was first synthesized in 1907 by Paul Ehrlich and released as the first antibiotic in 1910 as a new treatment for syphilis. This discovery led Ehrlich to develop the concept of a “magic bullet”, a drug that kills specific microbes without harming the body. The magic bullet concept led Ehrlich and other scientists to search for compounds with specificity for individual microbes. A few years later, the discovery of penicillin from *Penicillium rubens* in 1928 sparked the golden age of discovery of natural bacterial products that demonstrated antibacterial activity that continued through the 1940s and 1960s (**Figure 1-1**).\(^2\) The antibiotics developed during this era of discovery served as precursors for the antibiotics commonly used today.\(^3\)
During the golden age of discovery, novel antibiotics were sorted into 23 different classes of antibiotics. These classes were defined by the structure of the antibiotic as well as its target in bacterial cells and are still used today to define new drugs. Antibiotic targets include inhibition of cell wall synthesis, protein synthesis, DNA/RNA synthesis, folate synthesis, and membrane disruption. The first commercially available antibiotic class was sulfonamide, which disrupts folate synthesis. One of the most known and used antibiotic classes are beta lactams, which contain a four-membered beta lactam ring. Beta lactams target cell wall synthesis and are effective against both Gram-positive and Gram-negative bacteria, also referred to as having a broad spectrum of activity. Tetracyclines and macrolides are also considered broad spectrum antibiotics, as they target protein synthesis in both Gram-positive and Gram-negative bacteria. Quinolones and fluoroquinolones disrupt DNA synthesis and primarily target Gram-positive
bacteria. Polymyxins consist of five polypeptides that target Gram-negative bacteria by disrupting the cell membrane. The last novel classes of antibiotics in clinical use were discovered in the 1980s. Any developments since the 1980s are modifications of previously known antibiotics, known as the golden age of medicinal chemistry. This span of decades consists of modifications of previously discovered antibiotic compounds and the optimization of antibiotic therapies, including the introduction of combinational therapeutic approaches. Though the golden age of medicinal chemistry prolonged the effective usage of current antibiotics, there is a definitive need for the discovery of novel antibiotic agents.

**Figure 1-2. Classes of antibiotic targets in Gram-positive and Gram-negative cells.**

When considering antibiotic advancement and use, one must also consider the antibiotic pipeline. The antibiotic pipeline refers to all the potential antibiotic therapies in various stages of development before going into clinical use. During the golden age of discovery, the antibiotic pipeline was numerous in every stage of development.
Following the development of the antibiotic oxazolidinones in 1987, a drop-off in discovery occurred. The cause of this slowdown was the large number of antibiotics available for treatment as well as the significant costs of putting a new therapy through the required safety testing. This lull in the discovery of new antibiotics drastically decreased the number of antibiotics in the pipeline (Figure 1-3). Generally, a multitude of therapies are in the initial discovery and preclinical stages of the development pipeline, which involves testing for antimicrobial activity as well as toxicity to eukaryotic cells. After extensive testing in this stage, the therapies that continue to show promise will move to animal testing followed by clinical testing. The late stages of preclinical studies and the initial clinical studies cause many of the therapies to be abandoned due to inactivity, toxicity, or side effects. Today, the number of antibiotics in the pipeline is a fraction of that in the early years of discovery. This is cause for concern due to the recent rise in antibiotic resistant bacterial infections.

Figure 1-3. Antibiotic Therapy pipeline. Adapted from “Drugs for bad bugs: confronting the challenges of antibacterial discovery” using Biorender.com
1.2 Rise of Antibiotic Resistance

The concept of the magic bullet gives rise to the question whether or not one magic bullet drug could be used for multiple microorganisms. Each antibiotic targets different cell types as well as different parts of the cell. When evaluating antibiotics, one must consider the spectrum of activity and the mechanism of action. An antibiotic’s spectrum of activity is referring to the diversity of its targeted bacteria, which is directly related to the antibiotic’s specific target within the bacteria. There are five classes of antibiotic targets: inhibition of cell wall synthesis, membrane disruption, inhibition of protein synthesis, inhibition of folate synthesis, and inhibitions of DNA or RNA synthesis (Figure 1-2). Disruption of cell wall synthesis can cause changes in osmotic pressure, initiating cell lysis. Cell membrane disruption includes the leakage of ions and small molecules, depriving cells of nutrients and disrupting homeostasis. Inhibition of protein synthesis can cause disruption in cell growth, division, and metabolic processes. The disruption of DNA and RNA synthesis interferes with protein production as well as cell replication. The disruption of folate synthesis prevents the conversion of uracil to thiamine, which prevents DNA biosynthesis. Different bacteria types can have predisposed, or intrinsic, resistance to antibiotics due to the inability of the antibiotic to reach its target (Figure 1-4). For example, Gram-negative bacteria have a thinner peptidoglycan cell wall, but also an outer cell membrane. For many antibiotics that target inside the cell or the cell wall, Gram-negative bacteria are intrinsically resistant due to the impermeability of the outer cell membrane. Because of intrinsic resistance, developing a large library of antibiotics with varying targets is essential and so a more appropriate allocation of resources than trying to develop one antibiotic that can target all bacteria.
With the use of antibiotics, came the rise of acquired antibiotic resistance. Acquired antibiotic resistance involves bacteria that were once susceptible to a drug acquiring the ability to continue to grow and reproduce despite the presence of antibiotics. This occurs through genetic changes such as mutation, transformation, transduction, or conjugation. The mechanisms of resistance include modification or destruction of the antibiotic, the development of efflux pumps that decrease drug uptake, and modification of the antimicrobial target (Figure 1-4). Acquired antibiotic resistance can vary greatly in complexity and occur through various biochemical pathways, which makes accounting for this type of resistance difficult. Rather than developing entirely new antibiotic classes, medicinal chemistry focused on developing combinational therapies to account for acquired resistance, such as beta-lactamase inhibitors. In response to the introduction of beta-lactams, bacteria were able to show resistance to this antibiotic class by releasing beta-lactamase, which broke open the active site of beta-lactam drugs. The antibiotic activity of beta-lactams was revived when beta-lactamase inhibitors were taken in addition to the beta-lactam antibiotic. Though this

**Figure 1-4. Mechanisms of Antibiotic Resistance.**

With the use of antibiotics, came the rise of acquired antibiotic resistance. Acquired antibiotic resistance involves bacteria that were once susceptible to a drug acquiring the ability to continue to grow and reproduce despite the presence of antibiotics. This occurs through genetic changes such as mutation, transformation, transduction, or conjugation. The mechanisms of resistance include modification or destruction of the antibiotic, the development of efflux pumps that decrease drug uptake, and modification of the antimicrobial target (Figure 1-4). Acquired antibiotic resistance can vary greatly in complexity and occur through various biochemical pathways, which makes accounting for this type of resistance difficult. Rather than developing entirely new antibiotic classes, medicinal chemistry focused on developing combinational therapies to account for acquired resistance, such as beta-lactamase inhibitors. In response to the introduction of beta-lactams, bacteria were able to show resistance to this antibiotic class by releasing beta-lactamase, which broke open the active site of beta-lactam drugs. The antibiotic activity of beta-lactams was revived when beta-lactamase inhibitors were taken in addition to the beta-lactam antibiotic. Though this

**Figure 1-4. Mechanisms of Antibiotic Resistance.**

With the use of antibiotics, came the rise of acquired antibiotic resistance. Acquired antibiotic resistance involves bacteria that were once susceptible to a drug acquiring the ability to continue to grow and reproduce despite the presence of antibiotics. This occurs through genetic changes such as mutation, transformation, transduction, or conjugation. The mechanisms of resistance include modification or destruction of the antibiotic, the development of efflux pumps that decrease drug uptake, and modification of the antimicrobial target (Figure 1-4). Acquired antibiotic resistance can vary greatly in complexity and occur through various biochemical pathways, which makes accounting for this type of resistance difficult. Rather than developing entirely new antibiotic classes, medicinal chemistry focused on developing combinational therapies to account for acquired resistance, such as beta-lactamase inhibitors. In response to the introduction of beta-lactams, bacteria were able to show resistance to this antibiotic class by releasing beta-lactamase, which broke open the active site of beta-lactam drugs. The antibiotic activity of beta-lactams was revived when beta-lactamase inhibitors were taken in addition to the beta-lactam antibiotic. Though this
approach worked for beta-lactam antibiotics, it does not work for all antibiotics. Acquired resistance mechanisms, such as the overexpression of efflux pumps, still pose a significant threat to antibiotic therapies.

Though antibiotic resistance occurs naturally, certain behaviors and practices can promote the rapid growth of the antibiotic resistance crisis. Social actions that are unfavorable for bacteria can increase the rate at which resistance develops, as this adds selective pressure for mutation. Using antibacterial soaps and sanitizers too frequently selects for resistance by killing off the bacteria that cannot withstand the chemicals but the bacteria that happen to have a mutation, such as the presence of membrane efflux pumps, survive and are able to reproduce.\textsuperscript{3,5} Increased use of antibiotics also provides repeated exposure, which selects for the development of resistance. Recent studies have also suggested that the use of antibiotics that are commonly used in winter months, such as penicillin and macrolides, can cause increases in resistance in a wide range of antibiotic classes.\textsuperscript{10} This indicates that the overuse of one antibiotic can have lasting effects on the overall antibiotic resistance crisis. After the golden age of discovery for antibiotics, the antibiotic use in healthcare as well as agriculture soared with limited regulation.\textsuperscript{11} Here, antibiotics are often used in sub-therapeutic levels in animals to promote growth and prevent disease. These practices have shown to increase the resistome, which refers to the collection of all antibiotic resistance genes among bacteria.\textsuperscript{12} Though there is a critical need for novel antibiotics, there is also a great need to decrease behaviors that favor the rapid growth of the antibiotic resistome.

The use of antibiotics brought about the rise of acquired antibiotic resistance in pathogenic bacteria. The first recorded instance of antibiotic resistance was a bacterial
infection that was resistant to penicillin in 1942. Antibiotic resistance among clinically relevant bacterial strains is an impending threat to public health. A group of clinically concerning pathogens with high virulence and incidence of antibiotic resistance are known as the ESKAPE pathogens, which include Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, Enterobacter aerogenes, and Escherichia coli. Bacterial infections that were once easily treatable are now unresponsive to some of the strongest antibiotics on the market, like S. aureus and Mycobacterium tuberculosis. The introduction of antibiotics to the clinical setting provided life-saving care to millions of people for many years, though widespread use led to the novel challenge of antibiotic resistance. The number of multidrug resident bacteria has continued to increase and the World Health Organization (WHO) declared the condition of the antibiotic resistance crisis as “dire”. Multidrug resistant pathogens, such as MRSA as well as multidrug-resistant tuberculosis, are now a large concern in healthcare settings and are especially difficult to treat.

### 1.3 Peptides with Antimicrobial Activity

Antimicrobial peptides (AMPs) first became of interest in the 1980s, after several AMPs were isolated from insects and mammalian neutrophil granules. Though the characteristics of AMPs can vary, many AMPs with significant antimicrobial activity tend to have similarities. Having a cationic charge is one of the most common characteristics of AMPs, as the positive charge attracts to the negatively charged cell membrane of bacteria. Though residues lengths tend to range from 10 to 70 residues, shorter AMPs have added stability as well as increased ability to enter the cell
Concerns for all potentially therapeutic AMPs include having high cytotoxicity for human cells and maintaining stability in physiological conditions.

The mechanism of action for most AMPs are poorly understood, which makes predicting the mechanism of action for novel AMPs difficult. AMPs with strong cationic properties tend to be involved in cell membrane disruption in one of three proposed models: barrel-stave, toroidal pore, and carpet bomb (Figure 1-5). Barrel-stave and toroidal pore models involved AMPs with cationic and anionic sections that allow for the attraction and integration into the cell membrane, forming pores. The model for cell membrane disruption is named the “carpet bomb” model and involves the extensive coverage of the cell membrane by the AMPs, causing the membrane to destabilize. It is important to continue research on the mechanisms of actions of AMPs in order to gain a more comprehensive understanding of the potentially novel antibiotic therapies.

Figure 1-5. Common mechanisms of AMPs. A) Toroidal pore model. B) Barrel-stave model. C) Carpet Bomb model.

The focus of this research project was on the viability and activity of four previously identified AMPs (Table 1). Peptides WLRIKAWLRR (RR) and
WLRRIKAWLRRIKA (RIKA) were suspected to destabilize the cell membrane as their primary mechanism of action. Other antibiotic mechanisms focus on inhibiting critical cellular processes. Replication is prevented by inhibitions of nucleic acid biosynthesis as well as inhibition of cell division. Cellular maintenance pathways, such as protein biosynthesis and protease activity, can also be targeted by AMPs. The mechanisms of action for peptides IIGGR (IGR) and HPQYNQR (HQ) are still unknown, though both AMPs are derived from human leukocyte cathepsin G protein. Previous research has shown antimicrobial activity in a number of oral bacteria, such as Acintomyces viscous and Streptococcus sanguis. This suggests that they likely have similar activity, which is inhibition of protease activity. Further study is needed in order to pinpoint the exact mechanism of action of IGR and HQ.

Table 1-1. AMPs Synthesized in this Project

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Ref.</th>
<th>Strains Previously Tested</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGR</td>
<td>IIGGR</td>
<td>17</td>
<td><em>Nissicca gonorrhoeae</em>, <em>Acintobacter acintomycomitans</em>, <em>Escherichia coli</em>, <em>Staphylococcus aureus</em></td>
<td>500 μg/mL</td>
</tr>
<tr>
<td>HQ</td>
<td>HPQYNQR</td>
<td>17</td>
<td><em>Nissicca gonorrhoeae</em>, <em>Acintobacter acintomycomitans</em>, <em>Escherichia coli</em>, <em>Staphylococcus aureus</em></td>
<td>500 μg/mL</td>
</tr>
<tr>
<td>RR</td>
<td>WLRRIKAWLRR</td>
<td>18</td>
<td><em>Staphylococcus aureus</em>, <em>Staphylococcus epidermidis</em>, <em>Pseudomonas aeruginosa</em></td>
<td>424.96 μg/mL</td>
</tr>
<tr>
<td>RIKA</td>
<td>WLRRIKAWLRRIKA</td>
<td>18</td>
<td><em>Staphylococcus aureus</em>, <em>Staphylococcus epidermidis</em>, <em>Pseudomonas aeruginosa</em></td>
<td>498.2 μg/mL</td>
</tr>
</tbody>
</table>

Previous research has established that peptides RR and RIKA have antimicrobial activity against *S. aureus* and *Staphylococcus epidermidis*. However, there have not been extensive studies on the antimicrobial activity of a wide range of bacteria, especially
Gram-negative bacteria. In comparison, the antimicrobial activity of the peptides IGR and HQ were primarily focused on Gram-negative bacteria, while lacking data regarding Gram-positive bacteria. This research expands our knowledge in that it tested the spectrum of activity of these four AMPs to each other by achieving two objectives. The first objective involved synthesis and confirmation of IGR, HQ, RR, and RIKA by Solid Phase Peptide Synthesis. The second objective involved measuring the antibiotic activity of each peptide with a range of bacterial strains as well as comparing the four AMPs to each other. The bacteria included in this project are known to be clinically relevant, liable to antibiotic resistance, and provide Gram reaction variability. This project provides more insight on the spectrum of activity of these AMPs.
CHAPTER 2
MATERIALS AND METHODS

2.1 Materials

2.1.1 Peptide Synthesis

Solid phase peptide synthesis was conducted in a 50 mL Glass Solid Phase Peptide Synthesis Vessel (Catalog Number: CG-1866-12, Chemglass) using a Rink Amide resin (Fmoc-Rink Amide AM Resin, 0.61 mmol/g, Catalog Number: RRZ001, AAPPTec). Regents used for synthesis include Dimethylformamide (N,N-Dimethylformamide, Sequencing Grade, Catalog Number: BP1160-4, Fisher BioReagents), Piperidine (Hexahydropyridine, ≥99.5%, Catalog Number: 411027-1L, Sigma-Aldrich), Dichloromethane (Dichloromethane, ≥99.5%, Catalog Number: BDH1113-4LG, VWR), Isopropanol (2-Propanol, Certified ACS, Catalog Number: A416P-4, Fisher Scientific), 1-hydroxybenzotriazole monohydrate (HOBt, Catalog Number: CXZ010, PPTTec), O-Benzotriazole-N,N,N’,N’-tetramethyluronium-hexafluoro-phosphate (HBTU, Catalog Number: CXZ020, PPTTec), N,N-Diisopropylethylamine (DIPEA, ≥99%, Catalog Number: D125806-500ML, Sigma Aldrich), Triisopropylsilane (TIPS, 98%, Catalog Number: 233781, Sigma, Aldrich), 1,2-Ethanediithiol (EDT, 98+%, Catalog Number: L12865, Alfa Aesar), Trifluoroacetic acid (TFA, 99%, Catalog Number: T6508, Sigma Aldrich), and Ethyl Ether anhydrous

Laboratory equipment used for peptide synthesis included a Rotary evaporator (Digital Rotary Evaporator, Catalog Number: RE301, Yamato Scientific America), a Centrifuge (Benchtop General Purpose Centrifuge, Catalog Number: 10830-746, VWR), and a Freeze Dryer (FreeZone 2.5 Liter -50C Benchtop Freeze Dryer, Catalog Number: 700202000, Labconco). Amino acids were purchased from PPTTec (Appendix 6-1).

2.1.2 Analysis of Antimicrobial Activity

Mueller Hinton II agar was used for overnight cultures (Catalog Number: 211438, Fisher Scientific). Iso-sensitest broth was inoculated from overnight plate cultures and 3.3x iso-sensitest broth was used for 96-well MIC assay (Catalog Number: CM0473B, ThermoFisher Scientific). Normal saline (0.9%) was made with sodium chloride (ACS, Catalog Number: MSX0420-1, Fisher Scientific) and water. Samples were compared with a 0.5 McFarland Latex Standard (Catalog Number ML05, Hardy Diagnostics) visually and with a spectrophotometer (Thermo Spectronic 4001, ThermoFisher Scientific). AlamarBlue was used as the colorimetric indicator (BUF012A, BioRad). MIC assays were conducted in polypropylene 96-well plates (Catalog Number 3879, Corning Inc. through VWR).

2.2 Peptide Synthesis

The first objective of this project is synthesizing the four AMPs. This was completed with a Rink Amide resin and Fluorenylmethyloxycarbonyl (Fmoc) protecting group (Figure 2-1) via stepwise Solid Phase Peptide Synthesis (SPPS). This method involves attaching an initial Fmoc-protected amino acid to the Rink Amide resin, removing the Fmoc protecting group, and adding additional Fmoc-protected amino acids
until the desired peptide is complete. SPPS was chosen because synthesis performed on a solid support allows for the isolation of the product after each amino acid addition without using extraction, recrystallization, or chromatography and increases the synthesis yield.

![Figure 2-1. Fmoc-Rink Amide Resin. Fmoc protecting group (Red). Rink Amide Linker (Orange). Resin (Purple). Created using ChemDraw.](image)

2.2.1 **Column Preparation and Deprotection Step**

Preparation for SPPS required adding 300 mg of Rink Amide Resin to into a glass 50 mL SPPS vessel with 4-5 mL dimethylformamide (DMF) and N₂ gas for 30 minutes. The DMF was then drained. Next, 5 mL of 20% Piperidine in DMF was added with N₂ for 30 minutes, this is the initial reaction involving the removal of Fmoc from the Rink Amide linker by piperidine to give a reactive amine, also known as a deprotection step (Figure 2-2). Deprotection is the initial step of SPPS and is repeated before the addition of each amino acid.

2.2.2 SPPS Washing Cycle

A series of four steps is used following the deprotection to wash the resin prior to the addition of an amino acid. Each step is a specified number of washes with 5-6 mL of solution with N₂. The first step uses DMF and includes a 5-minute incubation with the resin prior to draining (repeated for a total of 3 rinses). The second step uses DCM and includes a 3-minute incubation with the resin prior to draining (repeated for a total of 2 rinses). The third step uses isopropyl and includes 3-minute incubation with the resin prior to draining. The final step uses DMF and includes a 5-minute incubation with the resin prior to draining (repeated for a total of 3 rinses).
2.2.3 Coupling Reaction: Amino Acid Addition

Next, a Fmoc-protected amino acid is added to the Rink Amide linker by a carbonyl substitution reaction, also known as a coupling reaction (Figure 2-3). The coupling reaction requires a mixture of coupling reagents including 0.07 g of 1-hydroxybenzotriazole monohydrate (HOBt), 0.1735 g of hexafluorophosphate benzotriazole tetramethyl uranium (HBTU), and the amino acid in enough dimethylformamide (DMF) to dissolve the mixture (Table 6-1 for amount of each amino acid added). This mixture was then added to the resin in the column with N₂ and finally, 160 μL of N,N-Diisopropylethylamine (DIPEA) was added. DIPEA and DMF function to maximize solvation and minimize hydrogen bonding while HOBt and HBTU function to activate the amino acid being added to the growing peptide chain. The amino acid addition order is the reverse compared to amino acid addition in physiological conditions, meaning peptides are synthesized from the C-terminus to N-terminus. A cycle consisting of deprotection, washing, coupling, and washing was repeated until all amino acids were chain-linked to the Rink Amide resin.
Figure 2-3. Coupling Reaction to Form a Peptide Chain. (A) Deprotonation of protected amino acid by DIPEA with DMF solvent. (B) Activation of protected amino acid with HBTU followed by HOBT. (C) Substitution reaction with deprotected peptide chain attached to Rink Amid resin. Fmoc (Red), resin (Purple sphere), R group of protected amino acid being added (Pink), Side chain protecting groups (Green circles). Created using ChemDraw.
2.2.4 **Cleavage from Resin**

The synthesized peptide was cleaved from the resin using a mixture containing 0.5 mL TIPS, 0.5 mL water, and 18.5 mL TFA (cleavage cocktail). TFA is a strong organic acid that separates the Rink Amide linker from the peptide. EDT and TIPS act as scavengers by quenching the cations formed during deprotection and coupling steps, preventing extraneous reactions such as the acid-catalyzed oxidation of tryptophan residues.

Once the last amino acid for the AMP was added (including a deprotection and wash cycle), 10 ml of the cleavage cocktail was added to the column with N\textsubscript{2} for 30 minutes. (Figure 2-4). N\textsubscript{2} was then turned off and the column removed from the flask to a sterile, pre-weighed 50 mL conical tube. Using a rubber suction ball to apply pressure to the column and filter the cleaved peptides into the 50 mL conical tube. The column was returned to the flask. The remaining 10 mL cleavage cocktail was added with N\textsubscript{2} for 10 minutes. N\textsubscript{2} was then turned off and the column removed from the flask to the same 50 mL conical tube. Using a rubber suction ball to apply pressure to the column and filter the cleaved peptides into the 50 mL conical tube. At this point the peptide is in a dilute suspension but removed from the resin.
Next, 6 mL of the peptide mixture was transferred to a sterile, pre-weighed 15 mL conical tube and TFA was evaporated off using rotary evaporation (72°C). TFA was removed by rotary evaporation and cold diethyl ether was used for the precipitation of the AMPs. Once evaporation has stopped, the remaining mixture was transferred to a new sterile, pre-weighed 50 mL conical tube and placed on ice for 10 minutes. Next, diethyl ether was added to the conical tube to 25 mL and mixed by vortexing. The crude peptide precipitates were isolated by centrifugation (4500 rpm, 4°C, 5 minutes), with the supernatant poured off and additional peptide/diethyl ether mixture was added and centrifuged until only crude peptide was left. The peptide was then suspended in 25 mL water and frozen overnight in -40°C. The peptides were then freeze-dried for 2-3 days and then stored at -20°C for long term storage.

To confirm the sequence and purity of the AMPs, the samples were sent to LSU mass spectrometry facility and confirmed by MALDI. Once confirmed, stock solutions were made at a final concentration of 1024 μg/mL. For each peptide, 0.01024 g suspended in 10 mL water, and distributed into 1 mL aliquots creating a stock solution of
1024 μg/mL. Aliquots were frozen in water and the remaining dry AMPs were stored at -20°C.

2.3 Analysis of Antimicrobial Activity

The second objective of this project is accessing the antimicrobial activity of the synthesized peptides, which will be determined by observing the minimum inhibitory concentrations (MICs) of each peptide using 8 bacteria (Table 2-1). MICs were determined using the colorimetric Resazurin microdilution assay in a 96-well format. AlamarBlue is a cell viability assay reagent that contains the indicator dye, resazurin (BioRad BUF012A). Isolated colonies were collected from fresh cultures on Mueller-Hinton agar and then transferred to 100 mL iso-sensitest broth (Thermo Scientific CM0471B). After overnight incubation at 35°C with 6% carbon dioxide, the culture was spun down (25 mL at a time, 4000 rpm, 4°C, 5 minutes each), resuspended in a total of 20 mL normal saline (0.9% NaCl), adjusted turbidity to a 0.5 McFarland standard using normal saline, and then further adjusted the suspension to an Optical Density at 550nm (OD\textsubscript{550}) of 0.09 to achieve a colony forming unit concentration near the 0.5 McFarland standard equivalent of 1.5 x 10\textsuperscript{8} cfu/mL of \textit{E. coli}. The suspension was then diluted 1:20 in normal saline, which roughly equates to 5 x10\textsuperscript{6} cfu/mL \textit{E. coli}.
Table 2-1. Bacterial Strains Used in this Project

<table>
<thead>
<tr>
<th>Strain</th>
<th>Strain Characteristics</th>
<th>Gram&lt;sup&gt;a&lt;/sup&gt;</th>
<th>BSL&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacillus anthracis</td>
<td>Sterne</td>
<td>+</td>
<td>2</td>
<td>Lab collection</td>
</tr>
<tr>
<td>Bacillus cereus</td>
<td>569</td>
<td>+</td>
<td>2</td>
<td>Lab collection</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>ATCC 25992, Serotype O6</td>
<td>-</td>
<td>1</td>
<td>VWR</td>
</tr>
<tr>
<td>Klebsiella pneumoniae</td>
<td>ATCC 13882, Serotype 3</td>
<td>-</td>
<td>2</td>
<td>VWR</td>
</tr>
<tr>
<td>Proteus mirabilis</td>
<td>NCIMB 13283, Wildtype</td>
<td>-</td>
<td>2</td>
<td>VWR</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>ATCC 10145, Schroeter</td>
<td>-</td>
<td>2</td>
<td>VWR</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>ATCC 6538, Rosenbach</td>
<td>+</td>
<td>2</td>
<td>Lab collection</td>
</tr>
<tr>
<td>Staphylococcus epidermidis</td>
<td>NCIMB 8853, Wildtype</td>
<td>+</td>
<td>1</td>
<td>VWR</td>
</tr>
</tbody>
</table>

<sup>a</sup>Gram: Gram reaction  
<sup>b</sup>BSL: Biosafety Level

To begin the preparation of 75 μL reaction in a 96-well polypropylene plate, 37.5 μL water was added to columns 2 through 9, then 75 μL of 1024 μg/mL peptide in water was added to column 1 (Figure 6-2). Using a multichannel pipettor, 37.5 μL was taken from column to column 2, mixed, and then 37.5 μL from column 2 was transferred to column 3. This continued through column 9, where the final 37.5 μL of peptide and water was discarded. Next, 22.5 μL 3.3x iso-sensitest broth, 7.5 μL AlamarBlue, and 7.5 μL bacterial suspension were added to each well. The final bacterial concentration per well was approximately 5x10⁵ cfu/mL. The peptide concentrations ranged from 512 μg/mL in column 1 to 2 μg/mL in column 9 in a two-fold serial dilution. Column 10 served as a positive control, containing the same components as test wells, only with water and no peptide. Column 11 served as a negative control, containing peptides, but no bacteria. Column 12, rows A-F served as antibiotic controls. Antibiotic concentrations were chosen based on preliminary MIC assays (data not shown). Well 12G contained bacteria, but no drug or peptide. Well 12H contained no drug, peptide, or bacteria. Resazurin microdilution assays were conducted in triplicate and the MIC will be interpreted as the
lowest peptide concentration that inhibits bacterial growth after 24-hour incubation.

Growth inhibition is indicated by the well remaining blue and growth is indicated by the well turning pink. The MICs were observed and demonstrated the differences in antimicrobial activity.
CHAPTER 3

RESULTS

3.1 Peptide Synthesis

The first objective of this study was to synthesize the four AMPs. Solid phase peptide synthesis (SPSS) was used to synthesize four previously identified, but not well characterized, AMPs with suspected antimicrobial properties: IGR, HQ, RR, and RIKA. SPPS was chosen because synthesis performed on a solid support allows for the isolation of the product after each amino acid addition without using extraction, recrystallization, or chromatography and increases the synthesis yield. The cycle of deprotection, washing coupling, and washing again allowed for the addition of each amino acid with limited extraneous products. MALDI analysis by Louisiana State University confirmed the successful synthesis of all four peptides (Appendix 6-3).

3.2 Antimicrobial Activity Analysis

One classic methodology to characterize and compare antimicrobial activities of antibiotics is to perform minimum inhibitory concentrations (MICs) studies. I chose to conduct MIC analysis of the 4 AMPs ranging in concentrations from 512 μg/ml to 2 μg/ml on eight bacterial strains. To determine the MICs for the AMPs, AlamarBlue was used as a qualitative indicator of bacterial cell viability. Resazurin is an oxidation-reduction indicator that undergoes a colorimetric change in response to cellular metabolic reduction.
(Figure 3-1). Bacterial cells that survive will reduce resazurin, which colors the solution pink. The MICs for the Resazurin assays were determined by observation of break from pink to blue in adjacent wells, with the AMP concentration of the blue wells listed in the table below as the MIC (Table 3-1). For each bacteria strain, assays were conducted in triplicate from the same overnight culture and were consistent across all three tests with the exception for one MIC for RIKA against E. coli.

![Reduction of Resazurin (Blue) to Resorufin (Pink) to Dihydroresorufin (Colorless).](image)

**Figure 3-1. Reduction of Resazurin (Blue) to Resorufin (Pink) to Dihydroresorufin (Colorless).**

**Table 3-1. Minimum Inhibitory Concentration of Synthesized AMPs**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Gram</th>
<th>IGR</th>
<th>HQ</th>
<th>RR</th>
<th>RIKA</th>
<th>MIC (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. anthracis</td>
<td>+</td>
<td>&gt;512</td>
<td>&gt;512</td>
<td>16</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>B. cereus</td>
<td>+</td>
<td>&gt;512</td>
<td>&gt;512</td>
<td>32</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>E. coli</td>
<td>-</td>
<td>&gt;512</td>
<td>&gt;512</td>
<td>64</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>K. pneumoniae</td>
<td>-</td>
<td>&gt;512</td>
<td>&gt;512</td>
<td>&gt;512</td>
<td>512</td>
<td></td>
</tr>
<tr>
<td>P. mirabilis</td>
<td>-</td>
<td>&gt;512</td>
<td>&gt;512</td>
<td>&gt;512</td>
<td>&gt;512</td>
<td></td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td>-</td>
<td>&gt;512</td>
<td>&gt;512</td>
<td>128</td>
<td>128</td>
<td></td>
</tr>
<tr>
<td>S. aureus</td>
<td>+</td>
<td>&gt;512</td>
<td>&gt;512</td>
<td>128</td>
<td>64</td>
<td></td>
</tr>
<tr>
<td>S. epidermidis</td>
<td>+</td>
<td>&gt;512&lt;sup&gt;b&lt;/sup&gt;</td>
<td>&gt;512&lt;sup&gt;b&lt;/sup&gt;</td>
<td>16&lt;sup&gt;c&lt;/sup&gt;</td>
<td>8&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Two out of three tests showed the MIC for RIKA against E. coli as 32 μg/mL. One showed 64 μg/mL.

<sup>b</sup>MIC tests for IGR and HQ against S. epidermidis showed purple wells, indicating nearing the MIC at 512 μg/mL.

<sup>c</sup>Two out of three tests showed the MIC for RR and RIKA at 16 μg/mL and 8 μg/mL, respectively. One test showed the MICs one two-fold dilution higher.

RR and RIKA consistently showed antimicrobial activity for both Gram-positive and Gram-negative bacteria, including *B. anthracis* (Figure 3-2), *B. cereus* (Figure 3-3), *E. coli* (Figure 3-4), *K. pneumoniae* (Figure 3-5, RIKA only), *P. aeruginosa* (Figure 3-
For B. anthracis as well as S. epidermidis, RIKA showed the lowest MIC with 8 μg/mL, followed by RR at 16 μg/mL. For B. cereus, RIKA had the lowest MIC with 16 μg/mL and RR with 16 μg/mL. The lowest MIC for E. coli was RIKA at 32 μg/mL, followed by RR with 64 μg/mL. For K. pneumoniae, RIKA had lowest MIC at 512 μg/mL and not inhibition for any other peptide. For P. mirabilis, the MICs for RR and RIKA were above 512 μg/mL (Figure 3-6). For S. aureus, the MIC with RIKA was 64 μg/mL and RR was 128 μg/mL. One MIC test for RIKA against S. aureus at 4 μg/mL showed no growth and this is likely due to pipetting error resulting in no bacteria in that well. For P. aeruginosa, both RIKA and RR showed 128 μg/mL for the MIC. RIKA showed more antibiotic activity over RR. In all but one bacterial species, P. aeruginosa, the MIC was one two-fold dilution higher for RR.
Figure 3-2. MIC assay for *Bacillus anthracis*.

**Rows A and E:** IGR starting at 512 μg/mL in column 1 descending in a two-fold dilution to 2 μg/mL in column 9. Wells A10 and E10 contained bacteria and no peptide. Wells A11 and E11 contained IGR at 512 μg/mL and no bacteria. **Rows B and F:** HQ starting at 512 μg/mL in column 1 descending in a two-fold dilution to 2 μg/mL in column 9. B10 and F10 contained bacteria and no peptide. B11 and F11 contained 512 μg/mL HQ and no bacteria. **Rows C and G:** RR starting at 512 μg/mL in column 1 descending in a two-fold dilution to 2 μg/mL in column 9. Wells C10 and G10 contained bacteria and no peptide. C11 and G11 contained 512 μg/mL RR and no bacteria. **Rows D and H:** RIKA starting at 512 μg/mL in column 1 descending in a two-fold dilution to 2 μg/mL in column 9. Wells D10 and H10 contained bacteria and no peptide. Wells D11 and H11 contained 512 μg/mL RIKA and no bacteria. **Column 12:** A12 contained 4 μg/mL erythromycin descending in a two-fold dilution to 0.125 μg/mL in well F12. G12 contained bacteria and no drug. H12 contained no drug or bacteria.
Figure 3-3. MIC assay for Bacillus cereus.

**Rows A and E**: IGR starting at 512 μg/mL in column 1 descending in a two-fold dilution to 2 μg/mL in column 9. Wells A10 and E10 contained bacteria and no peptide. Wells A11 and E11 contained IGR at 512 μg/mL and no bacteria. **Rows B and F**: HQ starting at 512 μg/mL in column 1 descending in a two-fold dilution to 2 μg/mL in column 9. B10 and F10 contained bacteria and no peptide. B11 and F11 contained 512 μg/mL HQ and no bacteria. **Rows C and G**: RR starting at 512 μg/mL in column 1 descending in a two-fold dilution to 2 μg/mL in column 9. Wells C10 and G10 contained bacteria and no peptide. C11 and G11 contained 512 μg/mL RR and no bacteria. **Rows D and H**: RIKA starting at 512 μg/mL in column 1 descending in a two-fold dilution to 2 μg/mL in column 9. Wells D10 and H10 contained bacteria and no peptide. Wells D11 and H11 contained 512 μg/mL RIKA and no bacteria. **Column 12**: A12 contained 2 μg/mL erythromycin descending in a two-fold dilution to 0.0625 μg/mL in well F12. G12 contained bacteria and no drug. H12 contained no drug or bacteria.
Figure 3-4. MIC assay for Escherichia coli.

**Rows A and E:** IGR starting at 512 μg/mL in column 1 descending in a two-fold dilution to 2 μg/mL in column 9. Wells A10 and E10 contained bacteria and no peptide. Wells A11 and E11 contained IGR at 512 μg/mL and no bacteria. **Rows B and F:** HQ starting at 512 μg/mL in column 1 descending in a two-fold dilution to 2 μg/mL in column 9. B10 and F10 contained bacteria and no peptide. B11 and F11 contained 512 μg/mL HQ and no bacteria. **Rows C and G:** RR starting at 512 μg/mL in column 1 descending in a two-fold dilution to 2 μg/mL in column 9. Wells C10 and G10 contained bacteria and no peptide. C11 and G11 contained 512 μg/mL RR and no bacteria. **Rows D and H:** Rika starting at 512 μg/mL in column 1 descending in a two-fold dilution to 2 μg/mL in column 9. Wells D10 and H10 contained bacteria and no peptide. Wells D11 and H11 contained 512 μg/mL Rika and no bacteria. **Column 12:** A12 contained 64 μg/mL carbenicillin descending in a two-fold dilution to 2 μg/mL in well F12. G12 contained bacteria and no drug. H12 contained no drug or bacteria.
Figure 3-5. MIC assay for *Klebsiella pneumoniae*.

**Rows A and E:** IGR starting at 512 μg/mL in column 1 descending in a two-fold dilution to 2 μg/mL in column 9. Wells A10 and E10 contained bacteria and no peptide. Wells A11 and E11 contained IGR at 512 μg/mL and no bacteria. **Rows B and F:** HQ starting at 512 μg/mL in column 1 descending in a two-fold dilution to 2 μg/mL in column 9. B10 and F10 contained bacteria and no peptide. B11 and F11 contained 512 μg/mL HQ and no bacteria. **Rows C and G:** RR starting at 512 μg/mL in column 1 descending in a two-fold dilution to 2 μg/mL in column 9. Wells C10 and G10 contained bacteria and no peptide. C11 and G11 contained 512 μg/mL RR and no bacteria. **Rows D and H:** RIKA starting at 512 μg/mL in column 1 descending in a two-fold dilution to 2 μg/mL in column 9. Wells D10 and H10 contained bacteria and no peptide. Wells D11 and H11 contained 512 μg/mL RIKA and no bacteria. **Column 12:** A12 contained 8 μg/mL chloramphenicol descending in a two-fold dilution to 0.25 μg/mL in well F12. G12 contained bacteria and no drug. H12 contained no drug or bacteria.
Figure 3-6. MIC assay for *Proteus mirabilis*.

**Rows A and E:** IGR starting at 512 μg/mL in column 1 descending in a two-fold dilution to 2 μg/mL in column 9. Wells A10 and E10 contained bacteria and no peptide. Wells A11 and E11 contained IGR at 512 μg/mL and no bacteria. **Rows B and F:** HQ starting at 512 μg/mL in column 1 descending in a two-fold dilution to 2 μg/mL in column 9. B10 and F10 contained bacteria and no peptide. B11 and F11 contained 512 μg/mL HQ and no bacteria. **Rows C and G:** RR starting at 512 μg/mL in column 1 descending in a two-fold dilution to 2 μg/mL in column 9. Wells C10 and G10 contained bacteria and no peptide. C11 and G11 contained 512 μg/mL RR and no bacteria. **Rows D and H:** RIKA starting at 512 μg/mL in column 1 descending in a two-fold dilution to 2 μg/mL in column 9. Wells D10 and H10 contained bacteria and no peptide. Wells D11 and H11 contained 512 μg/mL RIKA and no bacteria. **Column 12:** A12 contained 256 μg/mL chloramphenicol descending in a two-fold dilution to 8 μg/mL in well F12. G12 contained bacteria and no drug. H12 contained no drug or bacteria.
Figure 3-7. MIC assay for *Pseudomonas aeruginosa*.

**Rows A and E:** IGR starting at 512 μg/mL in column 1 descending in a two-fold dilution to 2 μg/mL in column 9. Wells A10 and E10 contained bacteria and no peptide. Wells A11 and E11 contained IGR at 512 μg/mL and no bacteria. **Rows B and F:** HQ starting at 512 μg/mL in column 1 descending in a two-fold dilution to 2 μg/mL in column 9. B10 and F10 contained bacteria and no peptide. B11 and F11 contained 512 μg/mL HQ and no bacteria. **Rows C and G:** RR starting at 512 μg/mL in column 1 descending in a two-fold dilution to 2 μg/mL in column 9. Wells C10 and G10 contained bacteria and no peptide. C11 and G11 contained 512 μg/mL RR and no bacteria. **Rows D and H:** RIKA starting at 512 μg/mL in column 1 descending in a two-fold dilution to 2 μg/mL in column 9. Wells D10 and H10 contained bacteria and no peptide. Wells D11 and H11 contained 512 μg/mL RIKA and no bacteria. **Column 12:** A12 contained 256 μg/mL carbenicillin descending in a two-fold dilution to 8 μg/mL in well F12. G12 contained bacteria and no drug. H12 contained no drug or bacteria.
Figure 3-8. MIC assay for Staphylococcus aureus.

Rows A and E: IGR starting at 512 μg/mL in column 1 descending in a two-fold dilution to 2 μg/mL in column 9. Wells A10 and E10 contained bacteria and no peptide. Wells A11 and E11 contained IGR at 512 μg/mL and no bacteria. Rows B and F: HQ starting at 512 μg/mL in column 1 descending in a two-fold dilution to 2 μg/mL in column 9. B10 and F10 contained bacteria and no peptide. B11 and F11 contained 512 μg/mL HQ and no bacteria. Rows C and G: RR starting at 512 μg/mL in column 1 descending in a two-fold dilution to 2 μg/mL in column 9. Wells C10 and G10 contained bacteria and no peptide. C11 and G11 contained 512 μg/mL RR and no bacteria. Rows D and H: RIKA starting at 512 μg/mL in column 1 descending in a two-fold dilution to 2 μg/mL in column 9. Wells D10 and H10 contained bacteria and no peptide. Wells D11 and H11 contained 512 μg/mL RIKA and no bacteria. D8 showed no growth and is likely a pipetting error (Top). Column 12: A12 contained 1 μg/mL erythromycin descending in a two-fold dilution to 0.03125 μg/mL in well F12. G12 contained bacteria and no drug. H12 contained no drug or bacteria.
Figure 3-9. MIC assay for *Staphylococcus epidermidis*.

**Row A and E**: IGR starting at 512 μg/mL in column 1 descending in a two-fold dilution to 2 μg/mL in column 9. Wells A10 and E10 contained bacteria and no peptide. Wells A11 and E11 contained IGR at 512 μg/mL and no bacteria. **Row B and F**: HQ starting at 512 μg/mL in column 1 descending in a two-fold dilution to 2 μg/mL in column 9. B10 and F10 contained bacteria and no peptide. B11 and F11 contained 512 μg/mL HQ and no bacteria. **Row C and G**: RR starting at 512 μg/mL in column 1 descending in a two-fold dilution to 2 μg/mL in column 9. Wells C10 and G10 contained bacteria and no peptide. C11 and G11 contained 512 μg/mL RR and no bacteria. **Row D and H**: RIKA starting at 512 μg/mL in column 1 descending in a two-fold dilution to 2 μg/mL in column 9. Wells D10 and H10 contained bacteria and no peptide. Wells D11 and H11 contained 512 μg/mL RIKA and no bacteria. **Column 12**: A12 contained 0.25 μg/mL erythromycin descending in a two-fold dilution to 0.008 μg/mL in well F12. G12 contained bacteria and no drug. H12 contained no drug or bacteria.
IGR and HQ showed no antibiotic activity with the bacteria tested in this study. The MICs for IGR and HQ were not determined and are above 512 μg/mL. The only notable result is a purple color showing for *S. epidermidis* at 256 μg/mL IGR and 512 μg/mL IGR (Figure 3-9, Top plate, Wells A1 and E1). The purple color might indicate some residual AlamarBlue is not being converted and therefore some antimicrobial activity (see Discussion). This suggests that the MIC for *S. epidermidis* with IGR and HQ are possibly between 512 and 1024 μg/mL.
CHAPTER 4
DISCUSSION

4.1 Peptide Synthesis

The MALDI confirmed that my synthesis produced AMPs of the correct mass, indicating correct sequence and high purity.

4.2 Novel Experimental Design for determining AMP MICs

Upon reviewing the literature on MIC protocols, I learned there is no one “gold standard” for determining MICs. This stems from the various growth requirements of pathogens (some fastidious), the absorption of AMPs (positively charged) by the test vessel (negatively charged), and the definitive identification of the MIC breakpoint. Keeping these issues in mind, I looked at several standard protocols and came up with my own experimental design.

A traditional MIC uses Mueller-Hinton broth, a two-fold dilution series of an antibiotic, a polystyrene 96-well microtiter plate, and a bacterial inoculum equivalent to $5 \times 10^5$ cfu/mL as the final concentration per well. Two possible issues with this test method are the media and the test vessel. Mueller-Hinton can serve as a suitable growth medium for fastidious pathogens, if properly supplemented. If not supplemented, the
MIC would be inaccurate due to insufficient growth and results would be unreliable. An alternative growth media used in antibiotic testing is iso-sensitest broth.\(^{20}\) The use of the iso-sensitest broth enables \(P. \text{aeruginosa}\) and \(S. \text{aureus}\) to grow without requiring supplementation, as would be the case with Mueller-Hinton broth. Considering eight organisms were tested in this study, using a universal growth medium simplified testing. Iso-sensitest broth acts as a more universal growth medium while maintaining comparable MIC results.\(^{20}\) Therefore, iso-sensitest broth was selected for this project.

Most MICs call for a polystyrene 96-well plate, which does not account for the characteristics of AMPs used in this study. In many cases, this would be suitable. However, previous studies of MIC assays involving AMPs found that the standard polystyrene 96-well plates decreased the observed antimicrobial activity.\(^{22}\) The strong negative charge of polystyrene causes the positively charged AMPs to stick to the sides of the wells rather than remain in solution. Because of the positive charges on the AMPs, adjustments must be made to standard MIC assay protocols to obtain more accurate antimicrobial activity data. The recommendation is to use a polypropylene 96-well plate, which does not carry a negative charge to eliminate this potential inconsistency with polystyrene.\(^{22}\) Therefore, I chose to use polypropylene 96-well plates.

Though polypropylene plates took away the concern of the plate charge interacting with the AMPs, the opaque appearance of polypropylene plates increases the difficulty of interpreting growth in individual wells. The standard method to determine growth in a MIC assay in a 96-well plate includes looking for turbidity and sediments at the bottom of the wells between 16 and 24 hours, something that increases in difficulty with an opaque test vessel.\(^{22}\) One work around is to use a resazurin colorimetric indicator,
like AlamarBlue. AlamarBlue is a cell viability indicator that turns pink from blue via a
reduction-oxidation reaction when living cells are present. This allows for a clear color
distinction between wells that contain living bacteria and wells that do not after 24
hours.\textsuperscript{20} Therefore, I chose to use AlamarBlue as a colorimetric indicator in the MIC
assays and 24-hour incubation periods.

There are extraneous colors, such as purple, light pink, and orange.\textsuperscript{19} Purple
reactions suggest some reduction of resazurin to resorufin and some unreacted resazurin.
This can be explained by the peptide concentration nearing the MIC or that the peptide
can have bacteriostatic activity, with the peptide limiting metabolic activity. Determining
bacteriostatic activity would require conducting minimum bactericidal concentrations
assays after conducting MIC assays. For light pink wells suggest that some of the
resorufin has been further reduced to dihydroresorufin, which is colorless, but the pH is
still above 4.\textsuperscript{20,24} Bacteria cultures with extensive growth will appear light pink to clear
because of the additional reduction reactions. Orange wells are indicative of a pH below
3.8. Organisms in orange wells likely either have surface properties or extracellular
secretions that cause a pH drop in the well.

The goal of this experimental design is to provide MIC data that uses standard
methods, while accounting for the special considerations of AMPs. Concentrations, size
of inoculum, and antibiotic controls were used in similar fashions of accepted MIC
standards. The uses of a colorimetric indicator, polypropylene 96-well plates, and iso-
sensitest broth were included to account for the charges of AMPs and to minimize growth
interpretation errors.
4.3 Antimicrobial Activity of RR and RIKA

Previous studies on RR and RIKA demonstrated antimicrobial activity in 3 pathogens, both Gram-positive and Gram-negative bacteria. This study expands the number of bacterial organisms tested to 8 to better define the spectrum of activity of these two AMPs (Table 4-1). The inclusion of novel organisms: *B. anthracis*, *B. cereus*, *E. coli*, *K. pneumoniae*, and *P. mirabilis* suggest a broader spectrum of activity for RR and RIKA than previously thought. RR and RIKA demonstrated MICs of 64 μg/mL and lower against *B. anthracis*, *B. cereus*, and *E. coli*, suggesting these AMPs might be developed into novel therapeutics. Though the MICs of *P. mirabilis* were not determined in this study, a foundation for further study was established and possible modifications of the AMPs can be explored to target this organism.

**Table 4-1. AMPs and Previously Tested Bacterial Strains**

<table>
<thead>
<tr>
<th>Strain</th>
<th>RR</th>
<th>RIKA</th>
<th>IGR</th>
<th>HQ</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. anthracis</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>B. cereus</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>K. pneumoniae</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. mirabilis</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. epidermidis</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Previously published data regarding RIKA demonstrated antimicrobial activity against *S. aureus*, *S. epidermidis*, and *P. aeruginosa*. For *S. aureus*, published MICs for RIKA ranged from 3.7 μg/ml (2 μM) to 7.5 μg/ml (4 μM). In contrast, this study determined the MIC was 64 μg/ml. For *S. epidermidis*, the previously published MIC for
RIKA was 3.7 μg/mL (2 μM). In this study, the MIC was 8 μg/mL. For *P. aeruginosa*, the previously published MIC for RIKA was 15.1 μg/mL (8 μM) and the MIC in this study was 128 μg/mL. Data published regarding RR showed antimicrobial activity typically one concentration higher in the two-fold serial dilution series among these bacteria. For *S. aureus*, the previously published MIC for RR was 24.8 μg/mL (16 μM) and 128 μg/mL in this study. For *S. epidermidis*, the previous MIC for RR was 12.4 μg/mL (8 μM) and 16 μg/mL in this study. For *P. aeruginosa*, the previous MIC for RR was 198.4 μg/mL (128 μM) and was 128 μg/mL in this study. Overall, the MICs measured in this study were markedly higher than previously published data except for *S. epidermidis* with RR and *P. aeruginosa* with RIKA.

RIKA consistently demonstrates a lower MIC than RR. The only difference between these two AMPs is the addition of isoleucine, lysine, and alanine to the RR sequence suggesting that the combination of isoleucine, lysine, and alanine provide additional antibiotic activity. The combination of cationic amino acids (lysine) and hydrophobic (isoleucine) amino acids follow the characteristics of AMPs that show a barrel-stave or toroidal pore mechanism of action.23 This disruption of the cell membrane causes leakage and eventual cell death. Further study is needed to confirm the mechanism of action for these AMPs.

The discrepancies in MICs between the two studies are notable but could be attributed to differences in methods. Previous publications used 16 over 24-hour incubation periods and it is unclear if the Mueller-Hinton was supplemented, both of which could cause inaccurate MICs due to reduced overall growth. They also depended on turbidity to detect growth instead of color. One of the benefits to using resazurin as an
indicator of growth is its high Kreft’s dichromaticity index, meaning the color change breakpoint is easy to interpret thereby minimizing human error. Given the limited amount of peptide available, I could not compare the two methods experimentally to see if these variables are the cause.

4.4 Antimicrobial Activity of IGR and HQ

Previous publications on IGR and HQ determined bactericidal activity (MBC) at 500 μg/mL for both AMPs against E. coli and S. aureus. MBCs are typically higher than MICs, but given the unique methodology used in the previous study and that they did not report a MIC, no comparison can be made. This study showed no antibiotic activity (MIC) at 512 μg/ml with one exception. S. epidermidis with IGR at 512 μg/mL consistently showed a purple color, which suggests some delay in growth possibly due to it approaching the MIC.

One explanation for the lack of antibiotic activity of these two AMPs with the 8 strains tested could be attributed to the distribution of charged amino acids in the AMPs. The mechanisms of action of AMPs are still poorly understood, but a slightly positive charge is thought to be important for the attraction to the negatively charged cell membrane of bacteria. IGR (IIIGGR) is made up of isoleucine, glycine, and arginine. Isoleucine has a hydrophobic side chain and is uncharged. The side chain for glycine is only a hydrogen atom, which likely does not add to any antibiotic activity. Arginine has a positive charge and would likely aid in the attraction to the cell membrane, though only a weak contribution due to the large number of isoleucine and glycine amino acids. Previously published data for IGR suggested the terminal arginine is significant to its antimicrobial activity. With HQ (HPQYNQR), there are more positively charged amino
acids within the peptide (histidine and arginine). Although when one considers the remaining amino acids, which are hydrophobic, this peptide has a weak positive charge.

AMPs are typically defined as peptides with antimicrobial activity that range from 10 to 70 amino acids.\textsuperscript{4,15} Considering IGR is only 5 amino acids in length and HQ is 7 amino acids in length, perhaps higher concentrations are needed for significant antibiotic activity. Both IGR and HQ are domains isolated from cathepsin G, a protein in humans and mammals that eliminates intracellular pathogens. IGR has previously been shown to increase antimicrobial activity when used in combination with other longer AMPs. Future combination studies with both AMPs could provide some novel therapeutics.
CHAPTER 5
CONCLUSIONS AND FUTURE DIRECTIONS

5.1 Conclusions

In this study, four AMPs were successfully synthesized with SPPS and MICs were determined by a refined protocol of my design. The use of iso-sensitest broth eliminated the need for supplementation for specific bacteria and acted as a universal growth medium for all 8 bacteria tested in this study. Polypropylene 96-well plates were used as a test vessel instead of polystyrene, ensuring the surface properties of the test vessel do not interfere with the MIC assay. Finally, the use of resazurin as a colorimetric indicator after 24-hour incubation diminished possible human error when interpreting the results. There were discrepancies between the results recorded here and previously published MIC data, though this is likely attributed to differences in methods used. Additional studies comparing the two MIC assays with the peptides could help to confirm or eliminate these discrepancies.

Identifying RIKA’s activity against K. pneumoniae and E. coli, along with confirming activity against S. aureus and P. aeruginosa is significant since many serovars of these organisms are multidrug-resistant ESKAPE pathogens. The antibiotic resistance crisis continues to be an impending threat to healthcare and steps to develop novel therapies is essential. Options for treating ESKAPE pathogens are especially

42
critical due to their increasing prevalence and pan-resistance. With the lull in the novel discovery of antibiotic drugs, alternative antibiotic therapies have become more important than ever before. The consistent outperformance of Rika over RR suggests significant antimicrobial activity stemming from the combination of isoleucine, lysine, and alanine.

IGR and HQ showed no activity in this study. This is not surprising since these AMPs are related to cathepsin G, a protein that is known to have antimicrobial properties against oral pathogens and the bacteria tested in this study were not associated with oral infections. Previous publications suggest that IGR and HQ can increase antimicrobial activity when added to other AMPs. Overall, this project demonstrates my ability to synthesize peptides using SPPS and to design an assay to overcome 3 major concerns for conducting MICs with AMPs, providing a more comprehensive evaluation of antimicrobial activity.

5.2 Future Directions

Given the results reported here there are several directions this project could develop in the future. Some are related to using these four AMPs and some to modifying the AMP sequences. Initially, additional MIC assays will help further define the exact MIC for each peptide followed by MBC assays and Time-kill kinetics assays. This study used a two-fold dilution series to report MICs for these four AMPs. Using a smaller dilution factor (i.e. 0.5-fold) would define a more precise MIC for these AMPs. These precise values will provide a benchmark for research focused on improving the antimicrobial activity by modifying the AMPs used in this study. Modifications to amino acid sequences, specifically adding residues, will provide insight on the residues or domains that provide the most antimicrobial activity.
Modifications of interest include adding more lysine residues, isoleucine residues, IGR domains, and HQ domains to RR and RIKA. Previous publications suggested that isoleucine and lysine are important for antimicrobial activity and found that adding IGR and HQ to the ends of peptides can improve antimicrobial activity.\textsuperscript{17,18} Next, combination therapy studies, which involve testing two or more peptides concurrently, will provide insight regarding potential synergy. Finally, to demonstrate safety of these AMPs by evaluating their cytotoxicity with various human cell types. This is one of the necessary and important steps for any antibiotic moving through the antibiotic pipeline prior to clinical studies.

Another direction is to continue defining the spectrum of activity of these AMPs. However, this direction also involves refining the MIC assay protocol by further modifying methods to account for fastidious bacteria. Oral bacteria \textit{Aggregatibacter actinomycetemcomitans} and \textit{Streptococcus mutans} are two bacteria of interest, as IGR and HQ previously showed antibiotic activity against them.\textsuperscript{17} A consideration for these strains is the required 48-72 incubation periods, while the MIC assay protocol used in this study is limited to 24-hour incubation.\textsuperscript{17} Testing additional ESKAPE pathogen species and clinical isolates of previously tested bacteria will also provide information regarding the clinically relevant antimicrobial activity of these four AMPs. Testing more ESKAPE pathogen strains such as \textit{Enterococcus faecium}, \textit{Acinetobacter baumannii}, and \textit{Enterobacter} strains will provide further insight into the activity of these AMPs on the pathogens posing significant threats to our healthcare system.

Once these studies are completed, applications such as inserting the AMPs into hydrogels as a wound healing application can be pursued.
## CHAPTER 6

### APPENDIX

### 6.1 Amino Acids Used in this Study

**Table 6-1. Amino Acids Used in this Study**

<table>
<thead>
<tr>
<th>Amino Acids (Acid)</th>
<th>Fmoc/Protecting Groups with Amino Acids</th>
<th>Amount used in this study (g)</th>
<th>Catalog Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>A Alanine</td>
<td>Fmoc-Ala-OH</td>
<td>0.0957</td>
<td>AFA101</td>
</tr>
<tr>
<td>G Glycine</td>
<td>Fmoc-Gly-OH</td>
<td>0.0914</td>
<td>AFG101</td>
</tr>
<tr>
<td>H Histidine</td>
<td>Fmoc-His(Trt)-OH</td>
<td>0.1906</td>
<td>AFH105</td>
</tr>
<tr>
<td>I Isoleucine</td>
<td>Fmoc-Ile-OH</td>
<td>0.1087</td>
<td>AFI101</td>
</tr>
<tr>
<td>K Lysine</td>
<td>Fmoc-Lys(Boc)-OH</td>
<td>0.1441</td>
<td>AFK105</td>
</tr>
<tr>
<td>L Leucine</td>
<td>Fmoc-Leu-OH</td>
<td>0.1087</td>
<td>AFL101</td>
</tr>
<tr>
<td>N Asparagine</td>
<td>Fmoc-Asn(trt)-OH</td>
<td>0.1835</td>
<td>AFN105</td>
</tr>
<tr>
<td>P Proline</td>
<td>Fmoc-Pro-OH</td>
<td>0.1038</td>
<td>AFP101</td>
</tr>
<tr>
<td>Q Glutamine</td>
<td>Fmoc-Gln(Trt)-OH</td>
<td>0.1878</td>
<td>AFQ105</td>
</tr>
<tr>
<td>R Arginine</td>
<td>Fmoc-Arg(Pbf)-OH</td>
<td>0.1995</td>
<td>AFR105</td>
</tr>
<tr>
<td>W Tryptophan</td>
<td>Fmoc-Trp(Boc)-OH</td>
<td>0.1619</td>
<td>AFW105</td>
</tr>
<tr>
<td>Y Tyrosine</td>
<td>Fmoc-Tyr(tBu)-OH</td>
<td>0.1413</td>
<td>AFY105</td>
</tr>
</tbody>
</table>
6.2 MIC Assay General Plate Loading Scheme

Water is added first and followed by adding the peptides. Serial dilution with only peptides and water. Serial dilution for antibiotic control. Next, 3.3x iso-sensitest broth is added to all wells and then AlamarBlue is added to all wells. Finally, bacteria is added to appropriate wells and all wells are mixed. Pipette tips must be changed for each well when adding bacteria and mixing the wells.
6.3 MALDI Results

6.3.1 MALDI-IGR

Figure 6-2. MALDI Results for IGR. IGR sample was acquired in the 500-2000 Da range with 20% attenuation and 35% energy. 514.42 is IGR. There is also a strong peak at 736.42, this is likely Fmoc attached to IGR.

6.3.2 MALDI-HQ

Figure 6-3. MALDI Results for HQ. HQ sample acquired in the 500-3000 Da range with 20% attenuation and 20% energy. 941.47 is HQ. This is because the C-terminus is CONH$_2$ instead of COOH.
6.3.3 MALDI-RR

Figure 6-4. MALDI Results for RR. RR sample acquired in 600-4000 Da range with 20% attenuation and 35% energy. 1553.01 is RR. This is because the C terminus is CONH$_2$ instead of COOH.

6.3.4 MALDI-RIKA

Figure 6-5. MALDI Results for RIKA. RIKA sample acquired in 500-3000 Da range with 20% attenuation and 35% energy. 1865.16 is RIKA.


21. Vinicius Waldow, CC BY-SA 4.0 <https://creativecommons.org/licenses/by-sa/4.0>, via Wikimedia Commons