MECHANISM OF ACTION OF TWO ACTIVE ANTIMICROBIAL PEPTIDES

DERIVED FROM AUREIN 2.2

by

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The following individuals certify that they have read, and recommend to the Faculty of Graduate and Postdoctoral Studies for acceptance, the dissertation entitled:

Mechanism of Action of Two Active Antimicrobial Peptides Derived from Aurein 2.2

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Abstract

With the increasing problem of antibiotic resistance, coupled with the limited number of newly developed antibiotics, therapeutic alternatives are urgently required. Host defense peptides (HDPs), also known as antimicrobial peptides (AMPs), are promising candidates as they have multifaceted anti-infective properties as well as a lower likelihood of causing antibiotic resistance. HDPs are ubiquitous in nature and constitute an important part of the innate immune system of almost all life forms. However, only a few peptides are in clinical trials due to issues such as systemic toxicity, proteolytic degradation and short half-life from renal/hepatic clearance. Therefore, it is important to minimize the negative impacts while improving the antimicrobial activity at the same time. Part of this thesis describes the various functions of HDPs including antibacterial, antibiofilm, immunomodulatory activities, along with the associated mechanisms of action (MOAs). The determination of antibacterial and antibiofilm MOAs of active HDPs forms the basis of this thesis, with a description of commonly used methods.

From an array of aurein 2.2 analogue peptides, peptide 73 and peptide 77 were chosen for further study as they showed the highest antibacterial activity. Furthermore, both analogue peptides demonstrated better antibiofilm activity than aurein 2.2. Different biophysical techniques were used to investigate the structure – function relationship as well as the antibiofilm MOA of these analogue peptides. Specifically, the binding interaction with the alarmone nucleotide ppGpp was investigated, to determine whether the analogues function by inhibiting the bacterial stringent response. In addition, biological techniques were also employed to characterize their in vitro activity against bacteria.
Finally, the antibacterial and antibiofilm activities of several HDPs are compared to attempt to assess whether these anti-infective properties are linked or independent and future experiments are proposed.

Overall, the analogue peptides demonstrated higher antibacterial and antibiofilm activities compared to aurein 2.2. In addition, both have a slightly different antibiofilm mechanism of action compared to the antibiofilm peptide, IDR-1018. The results from this thesis will help to establish a foundation for future HDP design so that they can be used as therapeutics to combat antimicrobial resistance (AMR).
Lay Summary

Antimicrobial resistance (AMR) is a serious threat to global public health. Given that AMR is compounded by a decrease in antibiotic development, therapeutic alternatives are urgently needed. Host defense peptides (HDPs) are promising alternatives due to their multifaceted functions such as antibacterial, antibiofilm, immunomodulatory properties, in addition to the lower probability for bacteria to develop resistance mechanisms. This thesis describes the antibacterial and antibiofilm mechanism of action of two analogues derived from the natural HDP aurein 2.2. Understanding how peptides 73 and 77 function will lead to better antimicrobial designing principles for the development of potential HDPs as therapeutic alternatives against AMR.
Preface

The research described herein was conducted under the supervision of Dr. Suzana K. Straus (Department of Chemistry, UBC, Vancouver).

A version of Chapters 1 and 2 has been published: N. Raheem, S.K. Straus, "Mechanisms of Action for Antimicrobial Peptides With Antibacterial and Antibiofilm Functions", *Front. Microbiol.* 2019 Dec 12;10:2866 (10.3389/fmicb.2019.02866). I was responsible for doing a literature review and wrote a first draft of the paper, which was edited by my supervisor.

A version of Chapter 3 has been published: N. Raheem, P. Kumar, E. Lee, J.T.J. Cheng, R.E.W. Hancock and S.K. Straus, "Insights into the mechanism of action of two analogues of aurein 2.2", *BBA Biomembranes* Special Issue in honour of Michele Auger, 2020, 10.1016/j.bbamem.2020.183262. This study describes the investigation of the antibacterial mechanism of action of two aurein 2.2 analogues, namely peptide 73 and peptide 77. I designed the experiments with my supervisor, synthesized and purified the peptides and conducted most of the experiments except for the following: Dr. Prashant Kumar determined the minimum inhibitory concentrations for the peptides; Dr. John T. J. Cheng ran the DiSC₃5 assay in the Hancock laboratory (UBC). I completed the minimum bactericidal assay, the pyranine assay and the co-precipitation assay in the Hancock laboratory. The NMR experiments were completed with help from my supervisor. I was responsible for writing the first draft paper, which was edited by my supervisor.
Chapter 4 describes the study of antibiofilm mechanism of action determination of the two analogue peptides. Specifically, I examined whether the peptides target the stringent response in bacteria by binding to the alarmone nucleotide ppGpp. I synthesized the peptides, designed and ran all experiments. The co-precipitation experiments were conducted in the Hancock laboratory, IDR-1018 was provided by the Hancock lab. All the NMR experiments were completed with help from my supervisor. $^1$H NMR experiment was completed with help from Dr. Mark Okon (UBC).

This thesis may appear short because in addition to the work presented here, there is ongoing work. Preliminary data from our collaboration with Dr. Tanja Schneider (University of Bonn, Germany) showed cell wall biosynthesis inhibition activity of peptides 73 and 77. Specifically, both peptides were shown to interfere with peptidoglycan biosynthesis, by inducing an accumulation of the soluble cell wall precursor UDP-MurNAc-pentapeptide and inhibiting the MraY reaction at a concentration of 400 µM. Results from this experiment will lead to a publication. I have opted not to include a separate chapter in this thesis on this work, due to the collaborative nature and the delays incurred due to the Covid-19 pandemic.

**Publications**


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List of Symbols

°C  Degree Celsius

g  Gram

mg  Milligram (10^3 gram)

M  Molarity (moles per litre)

µg/mL  Microgram per millilitre

µM  Micromolar (10^-6 mole per litre)

µmole  Micromole (10^-3 mole)

Å  Angstrom (10^-10 meter)

s  Second

h  Hour

MHz  Megahertz (10^6 hertz)

mL  Millilitre (10^-3 litre)

µL  Microlitre (10^-6 litre)

µH  Mean hydrophobic moment

rpm  Rotations per minute
**List of Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AcN</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>AMP</td>
<td>Antimicrobial peptides</td>
</tr>
<tr>
<td>AMR</td>
<td>Antimicrobial resistance</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>ATR-FTIR</td>
<td>Attenuated total reflectance – Fourier transform infrared spectroscopy</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>CA</td>
<td>Community acquired</td>
</tr>
<tr>
<td>CD</td>
<td>Circular dichroism</td>
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<tr>
<td>CFU</td>
<td>Colony forming units</td>
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<tr>
<td>CL</td>
<td>Cardiolipin</td>
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<tr>
<td>CLSM</td>
<td>Confocal laser scanning microscopy</td>
</tr>
<tr>
<td>CPP</td>
<td>Cell penetrating peptide</td>
</tr>
<tr>
<td>ddH$_2$O</td>
<td>MilliQ filtered water</td>
</tr>
<tr>
<td>D$_2$O</td>
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<td>DCM</td>
<td>Dichloromethane</td>
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<td>Diisopropylethylamine</td>
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<td>DiSC$_{35}$</td>
<td>3,3-dipropylthiacarbocyanine</td>
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<td>DMF</td>
<td>N,N-dimethylformamide</td>
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<td>DMPC</td>
<td>1,2-dimyristoyl-sn-glycero-3-phosphocholine</td>
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<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>-----------</td>
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<tr>
<td>DSC</td>
<td>Differential scanning calorimetry</td>
</tr>
<tr>
<td>EDT</td>
<td>1,2-Ethanedithiol</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetra-acetic acid</td>
</tr>
<tr>
<td>EPS</td>
<td>Extracellular polymeric substance</td>
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<tr>
<td>GDP</td>
<td>Guanosine diphosphate</td>
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<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
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<td>HBTU</td>
<td>Tetramethyluronium hexafluorophosphate</td>
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<tr>
<td>HDP</td>
<td>Host defense peptide</td>
</tr>
<tr>
<td>HPG</td>
<td>Hyperbranched polyglycerol</td>
</tr>
<tr>
<td>HPLC</td>
<td>High-performance liquid chromatography</td>
</tr>
<tr>
<td>IDR</td>
<td>Innate defense regulator peptide</td>
</tr>
<tr>
<td>LB</td>
<td>Lysogeny broth</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
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<tr>
<td>LTA</td>
<td>Lipoteichoic acid</td>
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<tr>
<td>LUV</td>
<td>Large unilamellar vesicles</td>
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<tr>
<td>MALDI-TOF</td>
<td>Matrix-assisted laser desorption ionization - Time of flight mass spectrometry</td>
</tr>
<tr>
<td>MBEC</td>
<td>Minimum biofilm eradication concentration</td>
</tr>
<tr>
<td>MBIC</td>
<td>Minimum biofilm inhibitory concentration</td>
</tr>
<tr>
<td>MIC</td>
<td>Minimum inhibitory concentration</td>
</tr>
<tr>
<td>MOA</td>
<td>Mechanism of action</td>
</tr>
<tr>
<td>MRSA</td>
<td>Methicillin resistant <em>Staphylococcus aureus</em></td>
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<td>MTT</td>
<td>3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
</tr>
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<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>NOESY</td>
<td>Nuclear overhauser effect spectroscopy</td>
</tr>
<tr>
<td>OCD</td>
<td>Oriented circular dichroism</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>OM</td>
<td>Outer membrane</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>PG</td>
<td>Phosphatidylglycerol</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cells</td>
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<tr>
<td>POPC</td>
<td>1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylcholine</td>
</tr>
<tr>
<td>POPE</td>
<td>1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylethanolamine</td>
</tr>
<tr>
<td>POPG</td>
<td>1-palmitoyl-2-oleoyl-sn-glycero-3-[phosphatidyl-(1’-rac-glycerol)]</td>
</tr>
<tr>
<td>ppGpp</td>
<td>Guanosine 5’-diphosphate 3’-diphosphate</td>
</tr>
<tr>
<td>PS</td>
<td>Phosphatidylserine</td>
</tr>
<tr>
<td>RBC</td>
<td>Red blood cell</td>
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<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
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<tr>
<td>RP-HPLC</td>
<td>Reversed phase - High performance liquid chromatography</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
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<tr>
<td>SPR</td>
<td>Surface plasmon resonance</td>
</tr>
<tr>
<td>SUV</td>
<td>Small unilamellar vesicle</td>
</tr>
<tr>
<td>TES</td>
<td>Triethylsilane</td>
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<tr>
<td>TFA</td>
<td>Trifluoroacetic acid</td>
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<td>TFE</td>
<td>2,2,2-Trifluoroethanol</td>
</tr>
<tr>
<td>TFE-d3</td>
<td>Deuterated 2,2,2-trifluoroethanol</td>
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<td>-----------</td>
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<tr>
<td>TI</td>
<td>Therapeutic index</td>
</tr>
<tr>
<td>TOCSY</td>
<td>Total correlation spectroscopy</td>
</tr>
<tr>
<td>TSB</td>
<td>Tryptic soy broth</td>
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<tr>
<td>UV</td>
<td>Ultraviolet</td>
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</table>
Acknowledgements

First and foremost, I would like to express my deepest gratitude to my research supervisor, Dr. Suzana Straus, for letting me be a part of her research group and carry out research in the field of biophysical chemistry for the past five years. Dr. Straus’ expertise and enthusiasm towards research and teaching have motivated throughout my study. I am very grateful for her constant support, guidance and encouragement.

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Last but not least, I am forever grateful to my parents for their unconditional love and endless support throughout my life. I wouldn’t be who I am today without the guidance, inspiration and encouragement from my parents.
Dedication

To my parents.
Chapter 1: Introduction

1.1 Synopsis

The increasing prevalence of antibiotic resistance has led researchers to explore alternatives. Host defense peptides offer many advantages, as outlined in this Chapter. The aurein peptides, HDPs from frogs, are interesting candidates to explore further. This Chapter will provide the framework and background for this thesis.

1.2 Antibiotics

1.2.1 Antibiotic Discovery

An antibiotic is a subset of compounds that are antimicrobial, i.e. kill or inhibit the growth of microorganisms, such as bacteria, fungi, viruses, and parasites. Antibiotics specifically target bacteria. Although antibiotics were developed in the early 20th century, humanity has used a number of compounds to fend off infections throughout history. For instance, investigations of the ancient human skeletal remains from the Sudanese Nubia and the Dakhleh Oasis of Egypt in the late Roman period revealed the presence of tetracycline, indicating its intake via the diet of these civilizations. In addition, mouldy bread, herbs and honey have been used in treating infections since ancient times. The therapeutic applications of antibiotics in the modern era began with the discovery of Salvarsan in 1909 by Paul Ehrlich and his team. Ehrlich’s search for a “magic bullet” that selectively kills the microbe without targeting the host, led to the

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systematic screening of chemicals and eventually to the application of Salvarsan in the treatment of syphilis \(^1,^4\), which arises from an infection by the bacterium *Treponema pallidum*.

In 1928, Sir Alexander Fleming noticed that *Penicillium* mold contaminated culture plates inhibited the growth of Staphylococcus colonies \(^5\). The active compound from the mold colony was termed penicillin and exhibited antibacterial activity towards pathogens other than staphylococci, such as streptococci and pneumococci \(^5,^6\). The serendipitous discovery of penicillin was followed by its purification, with a technique developed by Ernst Chain and Howard Florey from Oxford in 1940, which eventually led to its mass production and distribution by 1945 \(^1,^2,^6\).

During the golden era of antibiotic discovery from the 1950s to 1970s, most of the antibiotic classes were discovered, including sulfonamides, \(\beta\)-lactams, chloramphenicols, quinolones and others \(^7\). Although modified versions of antibiotics belonging to these classes were introduced in subsequent years, no new classes of antibiotics have been discovered since the end of the golden era \(^1,^7\), with the exception of lipopeptides, such as daptomycin \(^8\).

### 1.2.2 Antibiotic Resistance

The ability of bacteria to become resistant to antibiotics was first proposed by Fleming himself and was documented as early as 1940 by Abraham and Chain. Despite this, up until recently most antibiotics were still effective against most pathogenic bacteria \(^9,^{10}\). Bacterial resistance to antibiotics is either intrinsic or acquired by genetic mutations and horizontal gene transfer \(^11\). Intrinsic resistance is due to the presence of structural and functional components that are
inherently present in a bacterial species. In contrast, acquired resistance can occur via different mechanisms that lead to a lowered intracellular antibiotic concentration, the inability of the antibiotic to reach its target, as well as antibiotic inactivation.

Because of the misuse of antibiotics in the 20th century, many bacteria are now resistant to drugs of last resort. These bacteria are commonly referred to as "superbugs". For example, *Staphylococcus aureus* is a Gram positive bacteria contributing to a high proportion of hospital acquired infections. Persistent *S. aureus* colonization occurs in 20% of the population and can cause severe pathogenic infections including sepsis, pneumonia, meningitis and even death. Methicillin resistant strains of *S. aureus* (MRSA) are treated with vancomycin administration, however, vancomycin resistant strains have emerged as well. In summary, the emergence and prevalence of multidrug resistant bacterial strains are becoming commonplace.

With the problem of increasing antibiotic resistance and shortage of new antibiotics, the need for novel and effective alternatives is essential. Alternative strategies, which make use of small molecules or peptides to block pathways specific to bacteria (e.g. rhamnose biosynthesis), small multidrug resistant proteins have been proposed. Another important strategy is to use antimicrobial peptides (AMPs), which target bacterial cells in non-traditional ways and often act by more than one mechanism.

### 1.3 Antimicrobial Peptides (AMPs)

Antimicrobial peptides (AMPs) are considered to be viable alternatives to antibiotics because they demonstrate selectivity towards bacterial cells and they are touted as causing little to no
bacterial resistance. AMPs are ubiquitous in nature. They are naturally occurring polypeptide sequences comprised of cationic and hydrophobic amino acids (~12-50 residues) with direct antibacterial activity. AMP discovery and development generally starts with identifying novel peptides from natural sources (e.g. organisms and tissue extracts, excised predicted antimicrobial sequences from larger proteins) and then optimizing antibacterial activity by making synthetic variants. Since their discovery in the 1980s, the number of known AMPs has increased to over tens of thousands of sequences, as reported in the many databases available. Examples of databases include: the Antimicrobial Peptide Database with over 3000 entries, DRAMP with over 17000 entries, and finally DBAASP. AMPs are often introduced in the literature as “promising alternative to antibiotics”, having the “potential to address the growing problem of antibiotic resistance” and holding "promise to be developed as novel antibiotics". Some studies suggest that AMPs are unlikely to give rise to bacterial resistance due to the fact that AMPs have low-affinity and target bacteria in multiple ways. Moreover, their presence in nature for millions of years and their effectiveness in combating infections further suggest that they have evolved in line with bacteria to maintain a strong defense. However, AMPs are not commonly used as therapeutic agents, due to issues such as proteolytic degradation, hepatic/renal clearance as well as systemic cytotoxicity. Indeed, AMP delivery is most commonly achieved by local administration via creams or nasal sprays to the site of infection. Moreover, other than polymyxin B and gramicidin S being used as topical agents, only a few peptides have entered clinical trials. Recent research has aimed to minimize the negative effects of AMPs by exploring a variety of drug delivery methods. For
instance, advancements have been made such as the use of surfactants to improve peptide transport across membranes, co-administration of protease inhibitors in order to prevent AMP degradation, as well as the attachment of polymers or encapsulation with nanocarriers for improved bioavailability.

In recent years, it is becoming increasingly recognized that AMPs act more than just by killing or inhibiting the growth of bacteria: many AMPs have been shown to possess immunomodulatory, anti-cancer and antibiofilm functions in addition to their antimicrobial properties. Indeed, this ability to act on multiple fronts is what makes AMPs attractive and has led to them being referred to as host defense peptides (HDPs).

1.4 Host Defense Peptide (HDP) Functions

HDPs are involved in a breadth of biological processes due to their versatility; they are ubiquitous in nature and are part of the innate immune defense system of almost all life forms. They can modulate the immune response, demonstrate anti-cancer activity and inhibit or eradicate biofilms. They can kill bacteria directly, by either targeting a broad spectrum of bacteria, or by being selective for Gram-positive or Gram-negative bacteria. Finally, HDPs are also active against pathogenic species such as viruses, fungi and parasites. Figure 1.1 summarizes all currently known functions of these diverse biomolecules, as recently reviewed in.

Although an increasing number of studies examine more than just the AMP function (i.e. direct killing of bacteria, Figure 1.1), much remains to be understood about how HDPs work. In
particular, it remains to be determined whether the multiple functions of HDPs are independent from one another or whether some functions have commonalities. In the following sections, some of the functions will be described in more detail.

![Diagram showing the known multiple functions of host defense peptides.](image)

**Figure 1.1** The known multiple functions of host defense peptides. Reproduced with permission from 62.

### 1.4.1 HDP Antibacterial Function

HDP antimicrobial activity can be broad-spectrum, or selective for Gram-positive or Gram-negative bacteria 19,43,61. HDPs are typically unstructured in solution and adopt amphiphilic structures in the presence of lipid membranes or membrane mimicking environments 24,55. These amphiphilic structures are formed via intramolecular or intermolecular hydrogen bond formation, and facilitate peptide binding in the interfacial region of the phospholipid bilayer 29,63. Major
classes of folded peptide structures include α-helix, β-sheet (with two to four disulfide bridges), loop (with one disulfide bridge) and extended peptides that are rich in specific amino acid residues.\(^{19,42,64,65}\)

The manners in which HDPs exhibit their antibacterial function, termed mechanism of action or MOA, can be diverse. HDP MOAs include direct membrane disruption, translocation across the membrane bilayer to reach intracellular targets, or a combination of both.\(^{19,29,66}\) Lytic peptides kill bacterial cells by membrane disruption, while others cause cell killing by inhibition of essential processes including cell wall biosynthesis, cell division, DNA/RNA synthesis, protein synthesis and others. The antibacterial MOA for HDPs are listed in Figure 1.2.

![Antibacterial mechanisms of action (MOA) of HDPs. Reproduced with permission from 62.](image)

Regardless of the mechanism of action, HDPs first have to interact with the outer layer of bacteria. Since most HDPs are cationic, this interaction is primarily electrostatic in nature given that the bacterial membrane is negatively charged.\(^{27,64,67}\) The negative charge of the bacterial membrane arises from the presence of anionic phospholipids such as phosphatidylglycerol (PG), phosphatidylserine (PS), and cardiolipin (CL).\(^{68}\) In addition, there are specific components in
Gram-positive and Gram-negative bacteria that contribute to the negative charge. Specifically, the negative charge of the Gram-negative bacterial membrane is from the lipopolysaccharide (LPS) phosphate groups, while for Gram-positive bacteria lipoteichoic acid (LTA) phosphate groups contribute to the negative charge. This initial electrostatic attraction gives the target cell selectivity of HDPs, since eukaryotic cell membranes are not negatively charged. Once bound, HDPs adopt a membrane bound structure and then either permeabilize or disrupt the membrane or simply translocate through the membrane, to interact with internal targets via the membrane non-disruptive mechanisms (Figure 1.2).

1.4.1.1 Membrane Disruptive HDPs

Many HDPs have a membrane disruptive mechanism of action, which is mainly driven by the stress induced in the bilayer organization by the peptide. In addition to the electrostatic attraction presented in the previous section, hydrophobic interactions also play a role in the adsorption of HDPs onto membranes. The membrane disruptive mode of action includes barrel-stave, toroidal pore, carpet and micelle aggregate formation, according to the Shai-Matsuzaki-Huang model, as well as a few others. An illustration of these various MOAs is given in Figure 1.3 and discussed in more detail below.

In the barrel-stave model (Figure 1.3), HDPs orient themselves perpendicular to the lipid membrane, with the hydrophobic side chains pointing outwards into the lipid environment and the hydrophilic surface facing inwards forming transmembrane pores. The transmembrane pores facilitate the leakage of cellular content and eventually disrupt membrane integrity. Prior to peptide insertion, HDP monomers first bind to the membrane and adopt structure. In this
model, the peptide is able to span the hydrophobic core of the lipid bilayer, and the peptide insertion process is driven predominantly by hydrophobic interactions \(^{72-74}\).

The toroidal pore model is similar to the barrel-stave model as HDPs also induce pore formation in this case. However, the difference is that the lipid bilayer bends back on itself and connects the top and bottom monolayers (Figure 1.3), forming a structure similar to the inside of a torus \(^{63,76}\). This mechanism is common for peptides that are too short to span the membrane bilayer.

In the carpet model (Figure 1.3), the HDPs aggregate on the surface of the membrane bilayer and are aligned parallel to the membrane surface, after the initial electrostatic interaction with phospholipid head groups \(^{73}\). After reaching a certain threshold concentration of the peptide, the bilayer curvature is disrupted leading to membrane disintegration \(^{73,74}\), i.e. the carpet model leads to the toroidal pore or aggregate model (see below). Transient pores may form prior to complete

**Figure 1.3** A comparison of the membrane-disruptive versus membrane non-disruptive MOAs. Reproduced with permission from \(^{62}\).
membrane lysis.73,74

Another membrane-lytic mode of action is explained by the aggregate model, in which the HDPs cluster to form water-containing micellar aggregates (Figure 1.3) 55,77. Specifically, these HDPs are shorter peptides unable to span the hydrophobic core of the lipid bilayer, eventually leading to membrane micellization 72. These aggregates span the lipid membrane and can collapse to either the inner or outer region, facilitating peptide translocation 67.

Although traditionally most HDPs were thought to function by perturbing membranes, more and more studies suggest that HDPs function using non-membrane disruptive MOAs or by using more than one mechanism 78–81. For instance, several groups have shown that membrane perturbation and cell killing are not necessarily related events by demonstrating that permeabilization by HDPs does not result in bacterial death 68,82–84. In fact, Zhang et al. have shown that even though polymyxins B and E1 did not cause cytoplasmic membrane depolarization, rapid cell death was observed 68,84.

### 1.4.1.2 Membrane non-Disruptive HDPs

Mechanisms that fall under the non-disruptive category generally involve peptides that permeate through the membrane (Figure 1.2). These peptides are generally known as cell penetrating peptides (CPPs). When they translocate into cells and display inhibition of bacterial cell wall-, nucleic acid-, protein-synthesis or other enzyme activities 25,55,64,72 then they are HDPs. In addition to being an antibiotic alternative, cell penetrating HDPs can also act as drug transporters in clinical applications 85. In addition, some HDPs are reported to target lipid II, the cell wall
precursor, thereby blocking cell wall synthesis\textsuperscript{80}. Proteins are also the target of non-lytic peptides. Such protein targets can be identified using the proteome microarray\textsuperscript{80}.

In order to reach intracellular targets, HDPs can enter into the bacterial cytoplasm via either the collapse of peptide-formed pores/channels or through a process known as lipid flip-flop\textsuperscript{25,63}. Lipid flip-flop is the trans-bilayer motion of lipids across the membrane bilayer\textsuperscript{86}. An example of membrane non-disruptive HDP is buforin 2, which has higher antimicrobial activity and a similar amphipathic $\alpha$-helical structure as the membrane lytic peptide, magainin 2\textsuperscript{83,87}. Buforin 2 was shown to translocate across the membrane bilayer upon the disintegration of formed pores, and then bind to DNA and RNA to inhibit cellular functions\textsuperscript{83,87}. These unstable toroidal pores with a shorter lifetime lead to increased peptide translocation and a decreased membrane permeabilization\textsuperscript{85}.

1.4.2 HDP Antibiofilm Function

Typically, bacteria are found in biofilms rather than in the free-swimming or planktonic state. This is because bacteria are readily associated with surfaces or microbial mats as a response to environmental stress\textsuperscript{54}. Biofilm infections make up more than 65\% of human infections, as they can form on medical implants and tissues\textsuperscript{88-91}. They are more difficult to treat because they are comparatively more resistant to antibiotics than planktonic bacteria (10- to 1000-fold higher\textsuperscript{92}). Indeed, in patients, the best way to remove a biofilm-related infection is through surgical removal\textsuperscript{54,92,93}. Furthermore, there has not been any antibiotic developed specifically for biofilm infection treatment\textsuperscript{54}. As a consequence, novel therapeutic agents such as antibiofilm HDPs are sought after.
Biofilms are microorganism aggregates that are surrounded by an extracellular polymeric substance (EPS) consisting of polysaccharides, extracellular DNA, proteins, lipids and water (Figures 1.4 and 1.5) 88,93,94. The EPS provides enhanced antimicrobial resistance to biofilms compared to planktonic bacteria 88 because antibiotics have to maneuver through this layer to get to the bacteria. Biofilm formation is an adaptation of planktonic bacteria to environmental stress factors such as antibiotics, host immune system, starvation and others 54,94. Planktonic bacteria respond to stress factors by triggering the stringent response (Figure 1.5), a conserved mechanism in both Gram-positive and Gram-negative bacteria mediating the synthesis of signaling nucleotide (p)ppGpp 54,93. The nucleotide then binds to RNA polymerase and changes its specificity, consequently diverting cellular energy from cell division to a stress-coping mechanism 54. The correlation between ppGpp production and biofilm formation was confirmed by de la Fuente-Núñez et al., as the bacterial cells unable to synthesize (p)ppGpp nucleotide showed a decreased ability in surface adhesion and an inability to form biofilms 93,94.

Antibiofilm HDPs typically demonstrate antibiofilm activity (Figure 1.4) at concentrations below their minimum inhibitory concentration (MIC), with a distinct structure activity relationship compared to the direct killing antimicrobial activity 20,94–96. They are able to inhibit biofilm formation as well as eradicate preformed biofilms 94,97. Some HDPs have synergistic effects with other antibiotics and improve their effectivity 20,94,97. In addition, biomedical devices incorporated with HDPs can also inhibit biofilm development 94.
In order to prevent biofilm formation, HDPs can target a number of different processes including the quorum sensing pathway, adhesion organelles, matrix components and others \(^54\) (Figure 1.4). In particular, antibiofilm peptides can prevent biofilm formation by inhibiting the stringent response \(^93\) (Figure 1.5). For example, antibiofilm peptide IDR-1018 was found to bind directly to ppGpp nucleotide, leading to its degradation and subsequent prevention of biofilm formation and eradication of mature biofilms \(^92,93\).

Figure 1.4 Antibiofilm mechanisms of action (MOA) for HDPs. EPS: Extracellular polymeric substance.

1.4.3 Other HDP Functions

1.4.3.1 Immunomodulatory HDPs

Many HDPs have immunomodulatory activity (Figure 1.1), a feature which is an integral part of that innate host defense system and in inflammatory response \(^65\). This function enables HDPs to selectively upregulate innate immunity, while also suppressing proinflammatory cytokine response \(^19\). For instance, a recent study of innate defense regulator peptide (IDR-1) showed that
even though it has no antimicrobial activity *in vitro*, it shows anti-infective ability against bothGram-positive and Gram-negative bacteria *in vivo*.

Figure 1.5 Details relating to the stringent response mechanism of action of HDPs. When bacteria are stressed or starved, proteins such as RelA and SpoT are up-regulated. This leads to the conversion of the nucleotides guanosine triphosphate (GTP) or guanosine diphosphate (GDP) to the alarmone ppGpp, whose structure is shown on the left. The increased production of ppGpp leads to biofilm formation, illustrated in the lower half of the box (EPS = extracellular polymeric substance, as discussed in Section 1.4.2). Reproduced with permission from 62.

Depending on the cell type and peptide sequence, immunomodulatory HDPs target one or more receptors and processes in cells. Some of their functions include modulation of gene expression in monocytes, epithelial cells and others, as well as the promotion of the wound healing response and induction of chemokines. However, compared to direct antimicrobial activity, little is known about the structure and sequence requirement of immunomodulatory activity. As the work presented in this thesis is not focused on this activity, the reader is invited to read excellent reviews on the subject.
1.4.3.2 Anticancer HDPs

Some HDPs demonstrate anticancer activity towards tumor cells in addition to their antimicrobial activity (Figure 1.1). Anticancer HDPs target malignant cells due to the fact that cancer cells have membranes that are more negatively charged compared to normal mammalian cells. Specifically, tumor cells are negatively charged due to the presence of anionic molecules such as the phospholipid phosphatidylserine (PS), O-glycosylated mucins, sialylated gangliosides and heparin sulfate\textsuperscript{101}. In addition, cancer cell membranes are more fluid than healthy cells due to a decreased amount of cholesterol, allowing greater penetration of anticancer HDPs. Furthermore, the increased presence of microvilli on cancer cells leads to higher cell-surface area. This factor also helps the HDPs have greater access to the surface of cancerous cells\textsuperscript{101}. As the work presented in this thesis is not focused on this activity, the reader is invited to read excellent research papers on the subject\textsuperscript{101, 102} for further details.

1.4.3.3 Other Functions

Besides direct antimicrobial activity against bacteria, immunomodulatory and anticancer abilities, HDPs also have other anti-infective functions as shown in Figure 1.1. For example, some HDPs with antiviral activity can participate in viral pathogenesis, including the entry and replication stages\textsuperscript{103}. These antiviral HDPs interact with the virus envelope and lead to its permeation and consequently to lysis\textsuperscript{104}. Other mechanisms of action of antiviral peptides include inhibition of the virus' ability to induce cell fusion, as well as the inhibition of virus binding and entry to human cells\textsuperscript{104}. In addition, some HDPs have wound healing ability as demonstrated from \textit{in vitro} and \textit{in vivo} experiments\textsuperscript{98}. The results indicate that it is unrelated to antimicrobial activity, as certain HDPs are able to promote wound healing despite their weak or
lack of antimicrobial activity \textsuperscript{98}. Furthermore, certain HDPs are able to promote angiogenesis and also maintain homeostasis \textsuperscript{98,105}. A detailed summary of these functions and relation to disease can be found in \textsuperscript{20}.

1.5 Natural HDPs From Amphibians: Aurein Peptides

As mentioned in Section 1.3, HDPs are found in every life form. Amphibians secrete a large number of HDPs on their skin to protect them from environmental factors such as microorganisms and parasites \textsuperscript{61,106,107}. To date, over 300 amphibian HDPs have been identified and explored for antibiotic activity \textsuperscript{28}. Amphibian HDPs include some of the very early AMPs discovered, such as magainin, but also numerous other families, including temporins, brevenins and aureins \textsuperscript{101,106,108,109}.

The aurein peptides are cationic antimicrobial peptides secreted from the Australian southern bell frogs \textit{Litoria aurea} and \textit{Litoria raniformis} \textsuperscript{109}. There are five families of aurein peptides that include short and active aurein peptides (designated aurein 1.x, 2.x, and 3.x; where x = 1-6 and indicates the subfamily of peptides of the same length and > 68\% sequence identity; Table 1.1), as well as longer and typically inactive aurein 4.x and 5.x peptides \textsuperscript{108,109}. Truncated versions of these peptides are designated with an additional number, e.g. aurein 3.1.2 is missing the first two residues of aurein 3.1. Families 1-3 exhibit moderate broad spectrum activity against pathogens, with higher activity against Gram-positive bacteria versus Gram-negative bacteria \textsuperscript{109}. In addition, some aurein 1-3 peptides have anticancer activity \textsuperscript{108}.
Table 1.1 Aurein Peptides from the Australian southern bell frogs *Litoria aurea* and *Litoria raniformis*. NH₂: amidated C-terminus. OH: Carboxy C-terminus.

<table>
<thead>
<tr>
<th>Aurein</th>
<th>Sequence</th>
<th>MW</th>
<th>L.a.</th>
<th>L.r.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>GLFDIIKKIAESI (NH₂)</td>
<td>1444</td>
<td></td>
<td>*</td>
</tr>
<tr>
<td>1.2</td>
<td>GLFDIIKKIAESF (NH₂)</td>
<td>1478</td>
<td></td>
<td>*</td>
</tr>
<tr>
<td>2.1</td>
<td>GLLDIVKVVGAFGLS (NH₂)</td>
<td>1613</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>2.1.1</td>
<td>LDIVKVVGAFGLS (NH₂)</td>
<td>1443</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>2.2</td>
<td>GLFDIVKVVGALGS (NH₂)</td>
<td>1613</td>
<td></td>
<td>*</td>
</tr>
<tr>
<td>2.3</td>
<td>GLFDIVKVGAIGSL (NH₂)</td>
<td>1613</td>
<td></td>
<td>*</td>
</tr>
<tr>
<td>2.4</td>
<td>GLFDIVKVVGTLAG (NH₂)</td>
<td>1627</td>
<td>*</td>
<td></td>
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<tr>
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<td>1457</td>
<td>*</td>
<td></td>
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<tr>
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<td>GLFDIVKVGVGLSL (NH₂)</td>
<td>1647</td>
<td>*</td>
<td>*</td>
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<tr>
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<tr>
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<td>GLFDIVKKIAGHISI (NH₂)</td>
<td>1736</td>
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<td>*</td>
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<td>1766</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>3.3</td>
<td>GLFDIVKKIAGHIVSSI (NH₂)</td>
<td>1794</td>
<td>*</td>
<td></td>
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<td>*</td>
<td></td>
</tr>
<tr>
<td>4.1</td>
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<td>*</td>
<td></td>
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<tr>
<td>4.2</td>
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<td>*</td>
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<tr>
<td>4.3</td>
<td>GLLQTIKEKLKEFAGGLVTVGQS (OH)</td>
<td>2414</td>
<td>*</td>
<td></td>
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<tr>
<td>4.4</td>
<td>GLLQTIKEKLKELATGGLVIGVQS (OH)</td>
<td>2424</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>5.1</td>
<td>GLLDIVTGLGLNLIVDVLKPKTPAS (OH)</td>
<td>2544</td>
<td>*</td>
<td>*</td>
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<tr>
<td>5.2</td>
<td>GLMSISIGKALKGLIVDVLKPKTPAS (OH)</td>
<td>2450</td>
<td>*</td>
<td>*</td>
</tr>
</tbody>
</table>
Two members of the aurein peptide family, namely aurein 2.2 and 2.3, have been studied extensively in the Straus group. These two peptides each consist of 16 amino acids, have a net charge of +2 and an amidated C-terminus. They adopt a highly α-helical structure. Although initial reports suggested that aurein 2.2 and 2.3 have vastly different MICs, both of these peptides are equally active against Gram positive bacteria such as Staphylococcus aureus and Staphylococcus epidermidis, with MICs of 16-32 µg/mL. Cheng et al. found that the N-terminus is required for its activity, whereas truncation of three residues from the C-terminus has no effect on its antimicrobial function. Moreover, the mechanism of action of aurein 2.2 and 2.3 was found to be pore formation, using model membranes as well as in the Gram-positive bacteria Bacillus subtilis (B. subtilis). Specifically, aurein 2.2 and 2.3 were found to form toroidal pores (Figure 1.3): the mismatch between the hydrophobic length of aurein 2.2 of ~24 Å and the POPC/POPG bilayer thickness of ~39 Å favours such a mechanism. In addition, the aurein peptides cause selective leakage of potassium, magnesium and iron, thereby disturbing cellular ion homeostasis. Wenzel et al. also confirmed that truncation of aurein 2.2 by 3 amino acids at the C-terminus (denoted henceforth as aurein 2.2-Δ3) had no impact on the mechanism of action relative to the full-length natural peptide.

1.6 Developing More Active Analogues of Aurein2.2-Δ3: Peptides 73 and 77

In the search for more active versions of aurein 2.2-Δ3, a peptide array was designed based on the peptide sequence of the parent peptide. Note that the shorter version of the peptide was chosen for further study due to cost considerations (US $75-100 per gram per residue). Previous work had shown that cationic residues such as arginine (R) and lysine (K) mediate the
initial electrostatic interaction between HDPs and the bacterial cytoplasmic membrane $^{29,51}$. In addition, tryptophan (W) residues prefer to bind in the interfacial region of lipid membranes to facilitate peptide-lipid interactions $^{29}$. Furthermore, the use of both R and W amino acids allows for the formation of cation-π interactions which have also been found to improve peptide-membrane interactions $^{51}$. Consequently, the analogues were designed such that the hydrophobic and basic amino acid residues of aurein 2.2-Δ3 were substituted with W and R, respectively, in order to increase favorable interactions.

The peptide array was generated using the SPOT-synthesis technique by Kinexus Inc. (Vancouver, BC, Canada) on cellulose membranes $^{39,116–118}$. To test the impact of the amino acid substitutions on activity $^{29}$, the MIC value of each peptide was determined against $S. aureus$ $^{39}$. Of the list of 91 peptides produced (Appendix A.1), two of them, namely peptides 73 and 77, were retained for further study as they had MIC values of 4 µg/mL against $S. aureus$, which was 8-fold smaller than the parent peptide (MIC: 32 µg/mL) (Table 1.2). Furthermore, both peptides show antibiofilm activities based on minimum biofilm inhibitory concentration (MBIC) values (see Chapter 2 for detailed description), with MBIC$_{85}$ of 2 µg/mL against $S. aureus$ (Table 1.2).

Peptides 73 and 77 have similar primary sequences as the parent peptide, aurein 2.2-Δ3. Although some residue changes are more significant, the overall sequence of these peptides are not changed drastically as hydrophobic residues were replaced with another hydrophobic residue (tryptophan), and basic residues were substituted by another basic residue (arginine). In fact, sequence alignment of aurein 2.2-Δ3, peptide 73 and peptide 77 was performed using EXPASY local similarity (SIM) program, the percent identity between aurein 2.2-Δ3 and analogue peptides
was found to be 58.3% respectively (not including the N-terminus residue) \(^{119}\). Clustal Omega (1.2.4) program was also used to compare sequence similarity, as shown in Figure 1.6, the primary sequence of these peptides have some features in common \(^{120}\).

<table>
<thead>
<tr>
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<th>Sequence</th>
<th>Length</th>
</tr>
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<tbody>
<tr>
<td>aurein2.2-Δ3</td>
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<td>13</td>
</tr>
<tr>
<td>peptide73</td>
<td>RLWDIVRWWVGWL</td>
<td>13</td>
</tr>
<tr>
<td>peptide77</td>
<td>RLWDIVRRWVGWL</td>
<td>13</td>
</tr>
</tbody>
</table>

*;**;***; **

Figure 1.6 Sequence alignment of aurein 2.2-Δ3 and analogue peptides 73 and 77 using the Clustal O (1.2.4) multiple sequence alignment program \(^{120}\). Due to the sequence similarity, aurein 2.2-Δ3 is also referred to as the parent peptide (see section 3.4.1 for more details).

Interestingly, peptides 73 and 77 have very similar sequences, with the residues at positions 9 and 10 being either W followed by valine (V) or V-W, respectively. Inspection of the helical wheel projections for all 13 residue peptides listed in Table 1.2 shows that the new analogues have an increase in charge of +1 and slightly higher mean hydrophobic moments (Figure 1.7). According to Yount et al., ideally \(\alpha\)-helical HDPs have a signature mean hydrophobic moment of 0.5 \(^{121}\). Furthermore, in both peptide 73 and peptide 77, W3 would be in close proximity to R7 in an \(\alpha\)-helical peptide, making a cation-\(\pi\) interaction possible (distance ~ 4.5-6.0 Å). An additional interaction between R8 and W12 may also be present in both peptides, if a continuous \(\alpha\) – helical structure is present (Figure 1.7 and 1.8). How these changes in hydrophobicity and the presence of cation - \(\pi\) interactions impact the mechanism of action of peptides 73 and 77 will be explored in this thesis.
Table 1.2 Sequences and activities of aurein 2.2-Δ3 and the analogue peptides 73 and 77. All have amidated C-termini. The Minimum Inhibitory Concentrations (MIC), Minimum Biofilm Inhibition Concentration (MBIC) and Minimum Biofilm Eradication Concentration (MBEC) for the various peptides against the strains indicated (in μg/mL): *S. aureus* C622 is a standard strain; CA-MRSA USA 300 is a community-acquired methicillin-resistant *S. aureus* strain; *P. aeruginosa* is the Gram-negative bacterium *Pseudomonas aeruginosa*.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>MIC</th>
<th>MIC</th>
<th>MIC</th>
<th>MBIC&lt;sub&gt;85&lt;/sub&gt;</th>
<th>MBIC&lt;sub&gt;85&lt;/sub&gt;</th>
<th>MBEC&lt;sub&gt;80&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>S. aureus</em></td>
<td>CA-MRSA USA300</td>
<td><em>P. aeruginosa</em> PA01</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Aurein 2.2</strong> (GLFDIVKKVVGALGSL)</td>
<td>16</td>
<td>32</td>
<td>&gt;64</td>
<td>16</td>
<td>&gt;64</td>
<td>&gt;64</td>
</tr>
<tr>
<td><strong>Aurein 2.2-Δ3</strong> (GLFDIVKKVVGAL)</td>
<td>32</td>
<td>64</td>
<td>&gt;64</td>
<td>32</td>
<td>&gt;64</td>
<td>&gt;64</td>
</tr>
<tr>
<td><strong>peptide 73</strong> (RLWDIVRRWVGWL)</td>
<td>4</td>
<td>4</td>
<td>64</td>
<td>2</td>
<td>&gt;64</td>
<td>16</td>
</tr>
<tr>
<td><strong>peptide 77</strong> (RLWDIVRRVWGWL)</td>
<td>4</td>
<td>4</td>
<td>64</td>
<td>2</td>
<td>&gt;64</td>
<td>-</td>
</tr>
</tbody>
</table>

Figure 1.7 Helical wheel projections for aurein 2.2-Δ3 and the analogue peptides 73 and 77. Images generated using http://heliquest.ipmc.cnrs.fr/cgi-bin/CompuParams.py. The mean hydrophobic moment (μH) is also given, as well as the net charge. Positively charged residues are shown in blue; negatively charged residues are in red; hydrophobic residues are in yellow. In both peptides 73 and 77, W3/R7 and R8/W12 are highlighted by a shadow to indicate that these two pairs are close enough to form cation-π interactions. The N and C terminal amino acids are indicated by the small red letters.
As one of the limitations of host defense peptides is their toxicity, studies were completed to determine the toxicity profiles of peptides 73 and 77. Specifically, it was found that the analogue peptides 73 and 77 demonstrate a higher human red blood cells (RBCs) lysis activity as well as an increased cytotoxicity against human peripheral blood mononuclear cells (PBMCs) \cite{56,125}. Table 1.3 gives a summary of the RBC lysis behaviour and the therapeutic indices of aurein 2.2, aurein 2.2-Δ3 and the analogue peptides \cite{125}. The therapeutic index (TI) is defined as the ratio of peptide concentration that causes 50% RBC lysis and the MIC value of the peptide. The higher the TI value, the higher the therapeutic potential of the select peptide. As shown in Table 1.3, peptide 73 has the highest in vitro TI value, followed by aurein 2.2.
Table 1.3 Toxicity profile of aurein 2.2, aurein 2.2-∆3 and the analogue peptides 73 and 77. MIC values were obtained against *S. aureus* C622 strain.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>50% RBC Lysis (µg/mL)</th>
<th>Therapeutic Index [50% RBC lysis] / MIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aurein 2.2</td>
<td>1350</td>
<td>42</td>
</tr>
<tr>
<td>Aurein 2.2 -∆3</td>
<td>1577</td>
<td>25</td>
</tr>
<tr>
<td>Peptide 73</td>
<td>221</td>
<td>55</td>
</tr>
<tr>
<td>Peptide 77</td>
<td>107</td>
<td>27</td>
</tr>
</tbody>
</table>

Table 1.3 clearly shows that both analogue peptides 73 and 77 demonstrate increased toxicity towards human RBC and PMBC cells and, consequently, would probably never go into clinical trials. The study of their mechanism of action is still useful for developing formulation methods, including peptide conjugation to hyperbranched polyglycerol (HPG), or micellar encapsulation of peptides with polyethylene glycol (PEG)-modified phospholipids and others. These formulation methods aim to alleviate peptide cytotoxicity while maintaining/enhancing their effectiveness in treating infections. Therefore, it is important to study how these peptides function against pathogens to complement the formulation study at the same time.

### 1.7 Thesis Aims

With the emergence of antimicrobial resistance, it is increasingly important to develop novel HDPs that are highly active, that preferably act by using more than one mechanism of action and that have more than one function. Human redesigned peptides are also important because with the currently known technologies, we are able to design and develop libraries of peptides, and test for peptide variations very quickly. When looking for more active derivatives of aurein 2.2,
peptides 73 and 77 were discovered. As presented in Section 1.5, these HDPs have many features in common with the parent aurein 2.2-Δ3 peptide (and some minor differences), yet these peptides are much more active. In addition, the new analogues not only display antibacterial activity, but are also good antibiofilm peptides. Despite our ability to find new and more active sequences, a deep understanding of how amino acid sequence modulates activities remains to be elucidated. Moreover, our understanding of whether the diverse functions listed in Figure 1.1 are independent of each other or not is currently unknown.

Hence, the aims of my thesis work are: 1) to determine the detailed mechanism of antibacterial action (Figure 1.3) of peptides 73 and 77; 2) to determine the detailed mechanism of antibiofilm action of peptides 73 and 77; and 3) to determine, based on the findings of the first two aims, whether antibacterial and antibiofilm functions should be viewed as independent or not. These goals will be described briefly below and in subsequent Chapters. Prior to presenting the data relating to these objectives, I will describe in Chapter 2 many of the current methods used to determine antibacterial (MIC) and antibiofilm activity (MBIC, MBEC), as well as mechanisms of action for HDPs with antibacterial and/or antibiofilm properties. Many of these methods in this Chapter will then be used in Chapters 3 and 4.

1.7.1 Determining the Mechanism of Antibacterial Action of Peptides 73 and 77.

Based on the helical wheels shown in Figure 1.7 and the fact that the mean hydrophobic moments and net charges are only slightly different between aurein 2.2-Δ3 and peptides 73 and 77, I would hypothesize that the mechanism of action all three peptides involves membrane perturbation and pore formation, as was found for aurein 2.2-Δ3. The slight increase in charge
and the higher activity of peptides 73 and 77 suggest that these peptides may interact even better with membrane than aurein 2.2-Δ3 does. In addition, since both peptides contain two cation-π interactions, I hypothesize that the enhanced activity of the peptides relative to aurein 2.2 may be related to their improved peptide-membrane interactions. To test these hypotheses, the specific aims are to:

1. Determine the structure of peptides 73 and 77 to verify whether the helical structure is preserved.
2. Determine whether peptides 73 and 77 insert into the membrane bilayer.
3. Determine whether peptides 73 and 77 can cause leakage and whether it is selective for specific ions (e.g. potassium versus sodium), as was previously found for aurein 2.2.
4. If the mechanism of action does not involve membrane perturbation, to explore alternative MOAs.

Details pertaining to these specific aims will be reported in Chapter 3.

1.7.2 Determining the Mechanism of Antibiofilm Action of Peptides 73 and 77.

Unlike aurein 2.2-Δ3, peptides 73 and 77 can also be considered as effective antibiofilm HDPs. In recent years, a number of other peptides, such as IDR-1018 (VRLIVAVRIWRR-CONH₂) and DJK-5 (vqwrairvrvir-CONH₂), have also been shown to inhibit and eradicate biofilms. Moreover, studies have demonstrated that the stringent response (Figure 1.5) plays a central role in the antibiofilm MOA for these peptides. Since peptides 73 and 77 are as rich in R and W as
IDR-1018 and DJK-5, I hypothesize that the peptides might interact with the alarmone ppGpp. Hence, in Chapter 4, I will specifically:

1. Determine whether peptides 73 and 77 co-precipitate preferentially with ppGpp versus other nucleotides.
2. If this interaction is confirmed, then I will explore whether this interaction is driven by electrostatic interactions.
3. If this interaction is not confirmed, then I will explore alternative MOAs, e.g. membrane disruption (Figure 1.4). Since in Section 1.7.1. I hypothesize that the peptides might interact with membranes effectively, then this could be a plausible alternative MOA.

1.7.3 Addressing the Question of whether Antibacterial and Antibiofilm Functions are Separate.

As discussed by Haney et al. \(^ {20} \) and in Section 1.3, much remains to be understood about the HDP landscape, i.e. how the physicochemical characteristics associated with peptide sequence translate into specific HDP functions. By comparing the findings presented in Chapters 3 and 4 to other HDP studies reported in the literature, I will examine what insights can be obtained from such studies and outline future directions. This will be presented in Chapter 5.

1.8 Summary

With the increasing prevalence of antimicrobial resistance (AMR), it is crucial to find molecules that display varied mechanisms of action and functions. HDPs are promising in this regard, because they have been found to act using a variety of ways (Figure 1.1). If one considers a 13
residue peptide, as in this thesis, the number of possible sequences that one would have to explore to find the ultimate antibacterial, antibiofilm, antiviral, etc. or HDPs with combinations of functions would be $10^{13}$ or $20^{13}$ depending on whether we consider all possible amino acid substitutions (resulting in $20^{13}$) or a subset $^{20}$. Clearly, the number of possibilities is too large to explore completely.

Mechanistic studies can allow us to narrow this search space significantly. The ultimate goal of my research is to better understand the HDP landscape so that ultimately select HDPs are identified and used in the fight against AMR in a timely manner.
Chapter 2: Methods to Determine Activity and Mechanisms of Action for HDPs with Multiple Biological Functions

2.1 Synopsis

The antibiotic crisis has led to a pressing need for alternatives such as host defense peptides (HDPs). Recent work has shown that these molecules have great potential not only as antimicrobials, but also as antibiofilm agents, immune modulators, anti-cancer agents and anti-inflammatories as described in Chapter 1. A better understanding of the mechanism of action (MOA) of HDPs is an important part of the discovery of more potent and less toxic HDPs. Many models and techniques have been utilized to describe the MOA. This Chapter will review how biological assays and biophysical methods can be utilized in the context of the multiple biological functions of HDPs, with a particular bias on the antibacterial and antibiofilm functions (Figure 1.1).

2.2 Introduction

As described in Chapter 1, host defense peptides hold promise as alternatives to currently used antibiotics. HDPs are part of the innate immune defense system of almost all life forms. They display a variety of functions, summarized in Figure 1.1. Ever since AMPs were first discovered four decades ago, scientists have tried to relate amino acid sequence to antibacterial activity, i.e. to derive design rules to yield “better” peptides. The typical approach is to substitute amino acids

in the sequence in order to manipulate cationic charge and hydrophobicity. This generally results in a small library of \( \sim 5\text{–}10 \) peptides, which are tested for antimicrobial activity. In most published examples, some derivatives exhibit moderately enhanced antimicrobial potency relative to the parent sequence \( ^{29,40,41,53,127} \), or perhaps lower toxicity (i.e. more selectivity towards bacterial cells versus mammalian cells), and perhaps \( \sim 1\text{–}4 \) of these peptides are studied further to determine their mechanism of action (MOA). So although databases with large numbers of sequences exist, e.g. the Antimicrobial Peptide Database with over 3000 entries (http://aps.unmc.edu/AP/main.php) \(^{44} \), DRAMP with over 17000 entries \(^{47} \), DBAASP (http://dbaasp.org) \(^{48} \) and others, the MOA for only a small proportion of these peptides is known. The primary reason for this, of course, is that determining MOA can be labour intensive, as it requires multiple experiments. In addition, the MOA is usually characterized for the antibacterial function of the peptide and not necessarily for its other functions.

However, in order to truly understand the HDP activity landscape \(^{20} \), more information is required: i.e. we need to generate a large number of sequences (e.g. using SPOT synthesis \(^{89,117,118} \)), to collect activity data for each of these peptides, and to determine MOAs for representative members. In the following, we examine some of the methods available to us to determine activity and MOA and discuss how future studies (and databases) may contain sufficient information to strengthen HDP design rules. Because HDPs have many functions and because there are many methods to determine similar parameters, the sections below do not represent an exhaustive list of assays available, but rather some of the more common ones. Moreover, we will limit ourselves to the antibacterial and antibiofilm MOAs of HDPs (Figures
Table 2.1 Assays and techniques used to characterize the antibacterial and antibiofilm MOAs of representative HDPs. Adapted from 62.

<table>
<thead>
<tr>
<th>Mechanism of action</th>
<th>Assays/Techniques</th>
<th>Representative HDPs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antibacterial</td>
<td>Membrane target: MIC; MBC – bactericidal*/Membrane depolarization (DiSC3 assay; pyranine leakage); Membrane damage (Sytox Green, PI, calcine leakage, ion leakage, DNA/RNA release, OCD, DSC, NMR, SEM); Cell wall targets (e.g., LPS, lipid II) – NMR, ITC, SPR</td>
<td>Aurein 2.2, 110,111, Magainin 128, HNP-1 129, Cg-Deh1 130, Thanatin 131, Esculentin-1a 132, LL-37 133</td>
</tr>
<tr>
<td>DNA target</td>
<td>MIC; MBC – bactericidal*/Gel electrophoresis</td>
<td>Buforin II 83,87,134,135, Indolicidin 79,136–138</td>
</tr>
<tr>
<td>RNA target</td>
<td>MIC; MBC – generally bacteriostatic*/Gel electrophoresis</td>
<td>Attacin 139</td>
</tr>
<tr>
<td>Protein target</td>
<td>MIC; MBC – generally bacteriostatic*/Co-precipitation; fluorescence</td>
<td>Bac71-35 140, Api137 141, Tur1A 142</td>
</tr>
<tr>
<td>Other target</td>
<td>MIC; MBC e.g. autolysin release</td>
<td>Mel4 143</td>
</tr>
<tr>
<td>Antibiofilm</td>
<td>Membrane disruption: MBIC; MBEC/Membrane depolarization (DiBAC4(3) assay); Membrane damage (Sytox Green, PI, Syto-9, ATP release)</td>
<td>Esculentin-1a 132, Nisin A, Lacticin Q, Nukacin ISK-1 144</td>
</tr>
<tr>
<td>Cell signaling</td>
<td>MBIC; MBEC</td>
<td>LL-37 and Indolicidin 90, 1037 96</td>
</tr>
<tr>
<td>EPS degradation</td>
<td>MBIC; MBEC</td>
<td>Hepcidin-20 145</td>
</tr>
<tr>
<td>Stringent response inhibition</td>
<td>MBIC; MBEC/Co-precipitation; 31P NMR</td>
<td>1018 95</td>
</tr>
<tr>
<td>Other target</td>
<td>MBIC; MBEC e.g., gene down-regulation/targeting</td>
<td>Nal-P-113 146, human β-defensin 3 147</td>
</tr>
</tbody>
</table>

* Based on 148

2.3 Biological Assays

In this Chapter, we will use the term “biological” to refer to methods which make use of bacteria (as opposed to individual components, i.e. model lipid membranes, DNA, etc.), in either planktonic (free swimming) or biofilm form.
In addition, we will limit ourselves to *in vitro* assays. For excellent articles on the use of *in vivo* models to characterize HDP antibacterial and antibiofilm activity, the reader is invited to consult references 24,149–151 and references therein.

### 2.3.1 Biological Assays to Determine Activity

**2.3.1.1 Minimum Inhibitory Concentration**

Minimum inhibitory concentration (MIC) is the lowest concentration (typically reported in μg/mL or μM) required of an antimicrobial agent to prevent the growth of planktonic bacteria. It is not only a useful parameter for assessing the therapeutic potential of new antibiotic candidates, but also permits the classification of bacteria as being clinically susceptible or resistant to the tested antibiotic. *In vitro* techniques such as agar dilution or broth dilution are commonly used for determining the MIC of antimicrobials.

In the agar dilution method, solutions containing a certain number of bacterial cells are spotted onto agar plates that contain different antibiotic concentrations. The bacterial colonies observed on these plates after incubation indicates bacterial growth. The broth dilution method involves the use of a liquid growth medium containing a certain number of bacterial cells for inoculation and to which an increasing concentration of the antibiotic is added. The growth of bacteria is indicated by the presence of turbidity or sedimentation after a period of incubation. For both methods, the lowest concentration of antimicrobial agent needed to prevent the visible growth of bacteria is defined as MIC.
Other methods to determine MIC include the agar diffusion method and the antimicrobial gradient method (Etest) \(^{152-154}\). In the agar diffusion method, an agar plate is inoculated with the test bacterial strain. And then filter paper discs, wells, strips or cups containing known concentrations of the test antimicrobial agent are placed on or punched into the agar plate \(^{153}\). The diffusion of an antibiotic into the agar medium inhibits bacterial growth. The MIC value can be determined from the relationship between the size of growth inhibition zones and the antibiotic concentration \(^{153}\). A disadvantage of this technique is that the obtained MIC value is often not accurate as the test antibiotic may not diffuse freely in the solid medium and could lead to partial inactivation \(^{153}\). The Etest method uses a strip containing an increasing concentration of the test antimicrobial agent, which is placed onto the agar plate already inoculated with the test microorganism. After incubation, the MIC value is obtained from the intersection of the growth inhibition zone with the test strip \(^{154}\). Studies have shown good correlation of MIC results obtained from Etest and broth/agar dilution method \(^{154}\). However, the disadvantages of this method include the cost for testing numerous antimicrobial agents, as well as the limited antibiotic concentration range set by the manufacturer \(^{152}\).

The methods described above can be readily adapted to screen a number of peptides, with the broth dilution method being commonly used to screen large peptide libraries \(^{24,39,155}\) obtained from SPOT-synthesis \(^{117,118}\). In this case, 96-well plates are typically used and the turbidity is measured by recording the optical density (OD) using a plate reader.
2.3.1.2 Minimum Biofilm Inhibitory Concentration

The techniques presented in the previous section enable the assessment of the antimicrobial activity against planktonic bacteria. Since bacteria can also exist as biofilms, assays specific to bacteria in this state are required. The minimum biofilm inhibitory concentration (MBIC) is the lowest concentration of antimicrobial agent required to inhibit biofilm formation. An HDP is considered to be antibiofilm if the MBIC is below the minimum inhibitory concentration (MIC), with a distinct structure activity relationship compared to the direct killing antimicrobial activity.

Different susceptibility tests can be done to determine the MBIC, including the use of an abiotic solid surface as well as the flow cell technique.

For instance, 96-well microtitre plates with sterile growth media are first inoculated with bacteria to allow growth, and then different concentrations of antimicrobial agents are added to observe biofilm prevention. After a period of incubation, the spent culture fluid and planktonic bacteria are removed prior to cell staining with crystal violet dye, which is then dissolved in ethanol or glacial acetic acid. Pipetting helps detach the biofilm in each well and the optical density at 570 nm can be measured using a microplate reader for biomass assessment. Since crystal violet stains both dead and living cells, alternative dyes have been used. For example, the metabolic reducing dye MTT (3-(4,5-dimethyl-2-thiazoly)-2,5-diphenyl-2H-tetrazolium bromide) can be utilized. Viable cells reduce the MTT dye and lead to colour formation: the optical density at 570 nm can hence be correlated to the number of living cells per well. The lack of visible colour from an MTT-assay indicates the MBIC value of the antimicrobial agent.

Alternatively, biofilms can be incubated with the blue phenoxyazine dye resazurin, which is converted to resorufin in the presence of viable cells. Measuring resorufin fluorescence using a
plate spectrophotometer indicates the metabolic activity of biofilm cells. Biofilm cells can be quantified afterwards by suspending the biofilm in each well, followed by the agar plate count method.

In addition to being able to distinguish between dead and living cells, assessment of biofilm inhibition requires that a clear distinction be made between planktonic and biofilm bacteria. To test whether the antimicrobial agent acts on a uniform biofilm, a so-called Calgary biofilm device can be used. This device consists of an upper lid containing 96 polystyrene pegs, which can be fitted in the channels of the bottom component and the wells of a 96-well microtitre plate. The bottom component allows consistent medium flow to all pegs to ensure even biofilm deposition. Afterwards, the peg lid is rinsed and transferred to a 96-well plate with different concentrations of antimicrobial agent in each well and then incubated overnight. The peg lid is rinsed again and placed into a second 96-well plate to transfer the biofilms from each peg into the wells containing growth medium, which is achieved by sonication or light centrifugation. Viability of biofilm cells can be assessed by measuring turbidity at 650 nm. For MBIC determination, OD$_{650}$ is measured both before and after plate incubation at 37°C for 6 hours and then compared to the positive control wells. The concentration of antimicrobial agent that leads to an OD difference which is $\leq$ 10% of the average of two positive control wells is defined as the MBIC value.

The above-mentioned techniques are simple and reproducible, but do not fully capture in vivo conditions. The flow cell system is a more sophisticated method for evaluating antibiofilm activity. In this technique, biofilms are grown in flow cell chambers in the presence of HDPs.
at specific concentrations and imaged non-destructively using confocal laser scanning microscopy (CLSM) or Attenuated Total Reflectance–Fourier Transform InfraRed (ATR–FTIR) spectroscopy. Bacteria are fluorescently tagged for CLSM analysis by using green fluorescent protein, cyan fluorescent protein, or yellow fluorescent protein. In addition, propidium iodide (PI) can be used to stain dead cells for studying the bactericidal ability of an antibiofilm agent. Dispersed bacteria cell counts can also be measured by plating the output flow on LB agar plates. For the ATR-FTIR experiment, no labelling is required: instead the characteristic IR signals arising from the proteins, nucleic acids and polysaccharides in the biofilm are tracked as a function of time. From the flow cell data, both biofilm growth inhibition and eradication of existing biofilms can be studied. Finally, instead of flow cell chambers, a suspended substratum reactor, also known as the CDC biofilm reactor, can be utilized. This reactor contains coupons suspended from the coupon holder lid into the growth medium-containing reactor. Bacterial incubation allows biofilm growth on the coupons. The presence of antimicrobial agents in the fluid phase allows complete exposure of all coupons. The coupons can be removed at different time intervals during the experiment and quantification can be achieved afterwards by plate counting. The biofilm structure can be examined by CLSM, similar to the flow cell method.

2.3.1.3 Minimum Biofilm Eradication Concentration

Minimum biofilm eradication concentration (MBEC) is the minimum concentration of an antimicrobial agent required to eliminate pre-formed biofilms. Similar to MBIC determination, the methods outlined above can be used. Generally, MBECs are larger than MBICs, as eradication of biofilms is more difficult than inhibition. Bacterial biofilms need to be formed...
before the addition of antimicrobial agents to test eradication. Recently, an improved method which relies on the use of a dissolvable bead has helped to improve the reliability and robustness of MBEC determinations. The alginate beads result in a more homogeneous biofilm and a more homogeneous exposure of the antimicrobial, resulting in a more responsive assay than surface-based methods.

2.3.2 Biological Assays to Determine MOA

Once the concentration required to kill planktonic bacteria, to inhibit or eradicate biofilms has been determined, it is useful to assess how these processes occur. For instance, the MIC does not allow a differentiation between the bacteriostatic and bactericidal activities of an HDP. Furthermore, an HDP may be bactericidal and eradicate biofilms. Bacteriostatic antibiotics act by fully or partially inhibiting bacterial growth; however, growth will resume after removal of the antibiotic. In contrast, bactericidal antibiotics cause cell death. Several studies have suggested that in general terms, bacteriostatic and bactericidal activities can be linked to MOA, though exceptions exist. In the following, we present methods to determine the bactericidal activity of HDPs, as well as methods to look at membrane perturbation. As before, these methods rely on the use of bacteria.

2.3.2.1 Minimum Bactericidal Concentration

The minimum bactericidal concentration (MBC) is defined as the minimum concentration for an antimicrobial agent to kill 99.9% of a bacterial inoculum. The assay is generally performed by broth macrodilution or microdilution of the bacterial sub-culture and plating an appropriate amount of antimicrobial agent on non-selective agar plates for 24 hours incubation.
remaining colony forming units (CFU) are counted to determine the MBC. In contrast to MIC which provides inhibitory activity information, MBC gives information on the bactericidal activity of the test antibacterial or antibiofilm agent at a specific time point.

Alternatively, the MBC value can be determined at different time points. This is known as a time-kill assay and allows the determination of the bacterial killing rate, as well as the concentration dependence of the antimicrobial agent. Using this approach, bacterial growth is studied in the absence (growth control) and presence of different concentrations of the antimicrobial agent, typically expressed as fractions or multiples of MIC (e.g. 0.25-2 x MIC). Serial dilutions are performed on the aliquot of culture removed at certain time intervals, followed by the agar plate count method to determine viable cells after a period of incubation. The result is usually plotted with the number of surviving cells (typically expressed as \( \log_{10}(\text{CFU/mL}) \)) on the ordinate and time (in hours or minutes) on the abscissa. A greater than 3 \( \log_{10} \)-fold decrease in surviving cells corresponds to a 99.9% killing of the initial inoculum, which is indicative of bactericidal activity at the test antimicrobial concentration. Time-kill assays can also be used to test the synergistic behavior of different antimicrobial agents.

### 2.3.2.2 Cytoplasmic Membrane Disruption

The membrane disruptive activity of an HDP can be examined by using a number of dyes. The first dye, 3,3-dipropylthiacyrbocyanine (DiSC\(_3\)) \(^{170} \), can be used to examine membrane depolarization. Alteration of the trans-membrane electric potential is correlated with a change in membrane permeability as well as cell death once past a critical point \(^{171} \). The fluorescent cyanine dye can be taken up into the bacterial cytoplasm depending on the trans-membrane
potential; the accumulation of the dye leads to a quenching of its fluorescence. Upon addition of the antimicrobial agent, the cytoplasmic membrane is permeabilized and depolarized, leading to dye release and fluorescence increase. The increase in fluorescence indicates the extent of membrane potential depolarization, as well as the integrity of the bacterial cells. The viability of cells upon antimicrobial action can be assessed at different time intervals by plate counting to determine the CFU.

In contrast to Gram-positive bacteria, the presence of outer membrane (OM) in Gram-negative bacteria acts as a barrier to prevent DiSC₃(5) dye from reaching the cytoplasmic membrane. In order to allow dye accumulation within the cytoplasm, several agents can be used to permeabilize the OM while not causing cell death. For instance, the metal ion chelator ethylene diamine tetra-acetic acid (EDTA) can be used to bind divalent ions such as Ca²⁺ and Mg²⁺ that are present in the OM, which then leads to dye uptake into the cytoplasm. Polymyxin derivatives such as polymyxin B nonapeptide can perturb the OM of Gram-negative bacteria by binding to lipopolysaccharide (LPS), which leads to increased OM permeability towards hydrophobic antimicrobial agents. In addition, the antimicrobial peptide ceragenin can also increase permeability of the OM, hence increasing the sensitization of Gram-negative bacteria towards hydrophobic antibiotics.

Other dyes such as Sytox Green or PI can be used to establish whether HDPs damage cytoplasmic membranes and interact with intracellular nucleic acids. These dyes penetrate through membranes in which pores are present and can be used to probe differences in pore size and structure. The protocol typically involves suspending bacterial cells and
dispensing them into wells of 96-well plates along with the dye. After a period of incubation, HDP is added and a change in fluorescence is observed. For example, once PI enters cells with damaged membranes (i.e. dead cells), it binds to nucleic acids and leads to an increase in red fluorescence. Triton X-100 is often used as the control for complete membrane damage. Alternatively, flow cytometry techniques can be used in a high-throughput fashion and dyes that stain viable cells, e.g. Syto-9, can be used in conjunction with membrane impermeable dyes to obtain a complete picture of the extent of cytoplasmic membrane disruption by an HDP.

2.3.2.3 Leakage of Intracellular Components

Membrane disruption can be further characterized by measuring which components leak out of bacterial cells, i.e. ions, ATP, or DNA/RNA. For example, cellular ions, phosphorus and sulfur can be detected by atomic emission spectroscopy after exposing bacterial cells to HDPs. For select ions, such as potassium, leakage can also be determined using a selective electrode. Likewise leakage of ATP can be assessed by using an ATP bioluminescence kit and comparing total vs. released amounts. This can also be done in the context of biofilms. Finally, DNA and RNA can be detected by using the fact that these molecules contain chromophores which can be detected using UV spectrometry (i.e. by measuring at OD$_{250nm}$). These methods are generally not used in a high-throughput fashion, but are useful for pinpointing details of the mechanism of action.

2.3.2.4 Other Methods

Most of the methods outlined above are used to separate out HDPs which function by membrane disruption from those that have a different MOA (Figure 1.2). In other words, since traditionally...
AMPs have been studied in the context of their ability to kill bacteria by disrupting bacterial membranes 19,53,189,190, most methods used to determine MOA focus first on determining whether the HDP is membrane perturbing. If it is not, very often, then and only then are other MOAs explored.

Rather than using a process of elimination, it is conceivable that future studies will rely on other parameters. As outlined in 20, an increasing number of studies show that HDPs exert their antibacterial functions at least in part by targeting other cell components or through a variety of MOAs 191. Instead of focusing solely on membrane damage, proteomic or transcriptomic profiling could be used since several HDPs have been observed to cause substantial changes in gene expression responses 95,113,146,147,183,192,193. For example, Wenzel et al. used 2D-PAGE to investigate which proteins were up- or down-regulated in *Bacillus subtilis* upon exposure to a variety of lantibiotics 185. Select protein markers were identified to distinguish the impact of the tested lantibiotics on the cell envelope. Alternatively, Wuerth et al. examined the host response to *Pseudomonas aeruginosa* in the absence and presence of the HDP IDR-1002 194 using RNA-Seq to provide insight into the mechanism of action of 1002.

### 2.4 Biophysical Techniques to Determine MOA

In addition to probing MOA in the presence of bacteria, a number of biophysical methods can be used to pinpoint HDP function. These typically rely on bacterial components (e.g. DNA from *Staphylococcus aureus* to test whether an HDP targets DNA) or model systems (e.g. synthetic lipids or lipid extracts, nucleotides, etc.). Although these approaches are usually quite a bit more labor-intensive than the biological assays outlined above, they provide crucial information on the
mechanism of action at a molecular level. In particular, biophysical *in vitro* experiments provide important insights when the MOA does not involve membrane damage. In the following sections, we will briefly summarize some of the typical methods used.

2.4.1 Membrane Disruption

There are many biophysical techniques to determine whether an HDP disrupts membrane integrity. Again, this is likely due to the fact that the membrane perturbation MOA has been the focus of many studies over the years [19,53,189,190].

2.4.1.1 Pyranine Leakage Assay

Pyranine (3-hydroxy-1,3,6-pyrenetrisulfonate) is a pH sensitive fluorescent probe that can be used to detect proton concentration within lipid vesicles. It is a hydrophilic polyanionic molecule with an ionizable 8-hydroxyl group (pKₐ = 7.2) dependent on the pH of the surrounding medium. The anionic character ensures no significant binding between pyranine and the negatively charged phospholipid vesicles, typically used as a model for the bacterial cytoplasmic membrane. Also, the fluorescence intensity is dependent on the extent of ionization in a pH 6-10 range [195]. These characteristics enable the use of pyranine to detect proton and counterion transport across vesicle membranes in the presence of HDPs that function by causing membrane damage [196,197]. This assay can be viewed as a counterpart to the DiSC₃5 assay described above. The additional information that can be probed using this assay is how the leakage depends on membrane composition or the presence of ions important for activity, e.g. Ca²⁺ [198].
2.4.1.2 Calcein Leakage Assay

Damage induced to model membranes by HDPs, such as large unilamellar vesicles (LUVs) or small unilamellar vesicles (SUVs) can be studied using the calcein leakage assay. Calcein or carboxyfluorescein are fluorescent probes that are membrane impermeable and self-quench at high concentrations \(^{199,200}\). Similar to the pyranine leakage assay, the dye is mixed with liposomes (e.g. using a number of freeze-thaw cycles) and the non-entrapped calcein is removed by using gel filtration. HDP-induced membrane damage leads to calcein leakage, and results in an increase in fluorescence \(^{199,201}\). This assay can also be adapted for use in bacterial cells to test whether an HDP has an effect on bacterial cytoplasmic membrane integrity \(^{202}\). Finally, smaller fluorophores, such as the Ca\(^{2+}\)-dependent fluorophore Quin-2 \(^{200}\), can be used to probe pores of a smaller diameter (< 1 nm).

2.4.2 Membrane Interaction

Since many HDPs function by translocating through the membrane \(^{20,203,204}\), methods to determine how HDPs interact with model membranes are important. One approach is to use oriented circular dichroism (OCD). In OCD, oriented lipid bilayers are used to provide information about the membrane alignment of peptides \(^{205}\). The observed signal allows a clear distinction to be made between a parallel versus perpendicular orientation of a peptide relative to the membrane bilayer \(^{205,206}\). Although this method can be applied to HDPs that adopt β-sheet conformations \(^{207}\), most examples in the literature are for α-helical peptides \(^{26,110,208}\). Peptides that form clearly defined pores will display a change in the CD signal as a function of increasing peptide concentration. HDPs that translocate through the membrane do not display this change in CD signal (Chapter 3).
Alternatively, methods such as differential scanning calorimetry (DSC)\textsuperscript{188,209} and \textsuperscript{2}H or \textsuperscript{31}P solid-state NMR\textsuperscript{26,110,201} can be used to determine whether HDPs affect lipid packing. HDPs that perturb the arrangement of fatty acyl chains result in changes in thermotropic-phase behavior as compared to lipid alone, as observed in the DSC thermogram. This can be used to indicate peptide integration, as demonstrated in a number of studies in the literature\textsuperscript{183,210–212}. Changes in \textsuperscript{31}P chemical shift depend on the change in orientation of the phosphorus nuclei in lipid membranes and provide additional information on HDP mode of action\textsuperscript{213}. By using phospholipids with deuterated acyl chains, \textsuperscript{2}H NMR can examine the effect of the HDP on acyl chain order\textsuperscript{214}. HDPs that translocate through the membrane do not show any changes in the DSC thermogram\textsuperscript{215}. Recently, DSC has been used to probe the interaction of the AMP MSI-78 in whole bacteria\textsuperscript{216}. Likewise, \textsuperscript{31}P NMR studies on whole bacterial cells have also been reported\textsuperscript{217}.

### 2.4.3 DNA or RNA Interaction

The interaction of HDPs with DNA or RNA can be monitored using gel electrophoresis. The electrophoretic mobility of the DNA (or RNA) bands is typically examined as a function of HDP concentration. Binding between the HDP and DNA is defined as decrease in the band migration rate or a complete inhibition of band migration\textsuperscript{218}. The bands are stained with ethidium bromide, a fluorophore that can intercalate between base pairs of the DNA double helix and leads to a fluorescence increase, visualized under UV illumination\textsuperscript{219}. For example, the DNA and RNA binding activity of burforin II, a potent HDP that causes rapid cell death of \textit{Escherichia coli} without cell lysis, was determined using agarose gel electrophoresis\textsuperscript{83}. Similarly, the DNA and
RNA binding activity of 13-amino acid long indolicidin was studied. From the gel retardation experiments, it was found that indolicin, an HDP that also causes *E. coli* membrane permeabilization without cell lysis, binds DNA but not RNA \(^{136}\).

### 2.4.4 Nucleotide Interaction

In biofilms, one important mechanism of action is for HDPs to interact with the signaling nucleotide (p)ppGpp. This interaction can be examined by the co-precipitation assay, in which samples are mixed in microtitre plates and the increase in turbidity is quantified by an absorbance measurement at 620 nm. Using this assay, IDR-1018, a broad spectrum antibiofilm peptide, was found to preferentially bind ppGpp \(^{93}\). The binding interaction leads to ppGpp degradation in bacterial cells and blocks the stress response, which consequently leads to biofilm prevention or eradication/dispersal of preformed biofilms. Alternatively, HDP/nucleotide interactions can be monitored using \(^{31}\)P NMR, by either using the signal of individual nucleotides in solution or whole bacteria \(^{93}\).

### 2.4.5 Other Methods

Alternate approaches involve examining other types of binding interactions, such as for example, the ability of HDPs to bind proteins or to coat surfaces to prevent biofilm formation \(^{160}\). In the first case, methods such as co-precipitation can be used. For instance, the ribosomal protein binding activity of Bac71-35, an HDP within the cathelicidin family, was determined by checking its co-sedimentation activity with purified ribosomes. Initially, *E. coli* 70S ribosomes were incubated with different concentrations of the peptide, and then the ribosome bound peptide was separated by ultracentrifugation. The ribosome pellets were analyzed by immunoblotting in
order to confirm the presence of a Ba71-35/ribosomal protein interaction. Alternatively, the presence of peptide on the surface of or within bacteria or on a solid support can be detected by labelling the peptide with rhodamine or other fluorescent dyes, or by adding a His-tag. In this case, it is important to verify that the label does not affect the structure and activity of the peptide.

2.5 Conclusions

A number of studies and the large number of HDP database entries clearly indicate that the host defense peptide landscape is large and diverse. Not only are there many sequences, but HDPs can function using one or more MOAs (Figure 1.2). To add to this complexity, some HDPs can have more than one function: antibacterial and/or antibiofilm, as considered in this Chapter; or any combination of functions listed in Figure 1.1. Currently, HDP sequences are modified "by hand" or using more sophisticated library-based approaches (e.g. using SPOT synthesis) in order to optimize one function. However, the manner in which peptide sequence relates to specific functions or combinations of functions remains elusive. One way to improve our understanding of HDPs is to elucidate the mechanistic details underpinning the multiple functions of HDPs.

In this Chapter, we examined the various tools at our disposal to probe the mechanism of action of HDPs that function as antibacterial and/or antibiofilm agents, techniques which will be used in Chapters 3 and 4. In a first step, it is crucial to have methods to determine the activity of a large set of HDPs. Methods to determine MICs, MBICs, and MBECs in a rapid and reliable manner are widely available. Next, assays and techniques to determine MOAs are applied. Depending on
the mechanism of action (Figure 1.2), the choice of method can vary. For HDPs that function by perturbing the membrane for example, there are many biological and biophysical methods that provide complementary information. On the other hand, there are only a few select biophysical techniques to probe select MOAs. In this Chapter, a number of examples that make use of biophysical methods to determine MOAs that involve DNA/RNA targets \(^{183,136,218}\), protein targets \(^{140}\), stringent response inhibition \(^{93,151}\), and degradation of biofilms \(^{160,220}\) were presented. A number of examples of how these biophysical approaches can be applied to bacteria (i.e. termed "biological" assays here) \(^{93,202,216,217}\) serve to illustrate not only how the lines between these methods may become increasingly blurred in the future, but also how all the methods presented in this Chapter will continue to be relevant.

As it is clear from the examples given in \(^{222}\), it is only in the last decade of the AMPs forty year history \(^{46}\) that researchers have examined in detail how HDPs function as antibacterial and/or antibiofilm agents. As more studies will report on the multifunctional, multi-MOA characteristics of HDPs, our understanding of the HDP landscape will improve. The details provided by the combination of sequence and MOA data will allow us to rapidly zoom in to select areas of the HDP landscape to find effective drugs against diseases, including antimicrobial resistant ones. Many methods at our disposal are already high-throughput. A judicious combination of these methods and techniques that provide mechanistic details will enable us to find concrete solutions to AMR in a timely manner.
Chapter 3: Insights into the Mechanism of Action of Two Analogues of Aurein 2.2

3.1 Synopsis

The naturally occurring host defense peptide (HDP), aurein 2.2, secreted by the amphibian *Litoria aurea*, acts as a moderate antibacterial, affecting Gram positive bacteria such as *Staphylococcus aureus* by forming ion selective pores. In a quest to find more active analogues of aurein 2.2, peptides 73 and 77 were discovered. These peptides were rich in arginine and tryptophan and found to have MICs of 4 µg/mL. In this chapter, I report the impact of increased charge from +2 to +3 and a slight increase in hydrophobic moment relative to aurein 2.2 on the mechanism of action of these two analogues. Using a time-kill assay, both peptides 73 and 77 were found to kill bacteria more effectively than the parent peptide. Using solution CD and NMR, the peptides were found to adopt a partial α-helical structure, i.e. the analogues were not a continuous helix like the parent peptide. Results obtained from oriented CD (OCD), DiSC₃,5 and pyranine assays and a gel retardation experiment showed that the peptides did not function by membrane perturbation and further showed that peptides 73 and 77 did not interact with DNA. Overall, the data were consistent with these peptides acting as cell penetrating peptides with intracellular targets, which did not appear to be DNA.

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3.2 Introduction

As discussed in Chapter 1, host defense peptides (HDPs) are ubiquitous in nature and represent an integral part of the innate immune defense system of many organisms. HDPs function in a variety of ways, demonstrating immunomodulatory, anti-cancer as well as biofilm inhibition and eradication activities. In addition, they can directly kill a broad spectrum of bacteria, or selectively target Gram-positive or Gram-negative bacteria. HDPs are also active against other pathogenic species such as viruses, fungi and parasites.

Amphibian HDPs represent an important group of peptide antibiotics. To date, more than 300 amphibian HDPs have been identified and explored for antibiotic activity. As described in Chapter 1, aurein peptides are natural HDPs secreted from the Australian southern bell frogs. The short and active aurein peptides from families 1-3 demonstrate a higher activity against Gram-positive bacteria compared to Gram-negative bacteria.

In the last decade, the Straus group has extensively studied two members of the aurein peptide family, namely aurein 2.2 and 2.3. Both peptides are 16 amino acid residues in length and highly \( \alpha \)-helical, with a net charge of +2 and an amidated C-terminus. The mechanism of action of both peptides was found to be toroidal pore formation. Aurein 2.2-\( \Delta 3 \), a 3 amino acids truncated version of aurein 2.2, also has the same mechanism of action as the full-length natural peptide. As discussed in Chapter 1, in order to find more active versions of aurein 2.2-\( \Delta 3 \), a peptide array was designed and generated using the SPOT-synthesis technique on cellulose membranes. Two of the peptides from the peptide array (Appendix A.1), namely peptides...
73 and 77, were selected for further study since they had MIC values of 4 µg/mL against *S. aureus*, which was 8-fold smaller than the parent peptide (MIC: 32 µg/mL).

As evident from Figure 1.7, Peptides 73 (RLWDIVRRWVGWL-CONH₂) and 77 (RLWDIVRRVWGWL-CONH₂) have very similar sequences, with the underlined residues indicating sequence changes relative to aurein 2.2-Δ3 (GLFDIVKKVVGAL-CONH₂). The analogue peptides are rich in arginine and tryptophan residues, have an increased charge of +1 and a slightly higher mean hydrophobic moment (Figure 1.7). Furthermore, in both peptides 73 and 77, residues W3 and R7, as well as R8 and W12, would be in close proximity in an α-helical peptide, hence making two cation-π interactions possible (distance ~ 4.5-6.0 Å).

In this chapter, I will present the results from functional and biophysical studies to demonstrate how these relatively subtle changes in charge, hydrophobic moment and introduction of cation-π interactions impacted the mechanism of action of peptides 73 and 77, relative to the parent peptide.

### 3.3 Materials and Methods

#### 3.3.1 Materials

Fmoc-protected amino acids, Rink resin, and 2-(1 H-benzotriazol-1-yl)-1,1,3,3-tetramethyluroniumhexafluorophosphate (HBTU) were purchased from Advanced ChemTech (Louisville, KY). N,N-dimethylformamide (DMF), dichloromethane (DCM), acetonitrile (AcN) and potassium nitrate were purchased from Fisher Chemicals (Nepean, ON). N,N-
diisopropylethylamine (DIEA), trifluoroacetic acid (TFA), ethane dithiol (EDT), triethylsilane (TES) were obtained from Sigma-Aldrich (St. Louis, MO). 1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-sn-glycero-3-[phospho-(1'-rac-glycerol)] (POPG) were purchased from Avanti Polar Lipids (Alabaster, AL) and obtained dissolved in chloroform. Tryptic Soy Broth (TSB) and Lysogeny Broth (LB) agar were purchased from Bacto and Fisher. MilliQ filtered water (ddH2O) was used. The plasmid pRMC2 was a gift from Tim Foster (Addgene plasmid # 68940; http://n2t.net/addgene:68940; RRID: Addgene_68940).

3.3.2 Peptide Synthesis and Purification

Aurein 2.2-Δ3, peptide 73 and peptide 77 were synthesized using a solid phase peptide synthesizer (CS Bio Co., Menlo Park, CA, USA) using Fmoc chemistry, as described previously. Briefly, in all cases, the first amino acid from the C-terminus end, Fmoc-Leu, was double coupled to the Rink resin for C-terminal amidated peptide synthesis. The six amino acids from the N-terminus end were also double coupled in order to improve yield. The crude product was purified using reversed-phase high performance liquid chromatography (RP-HPLC), using a Phenomenex (Torrance, CA, USA) C4 preparative column (20.0 µm, 2.1 cm × 25.0 cm) on a Waters 600 system (Missisauga, Ontario, Canada). UV-detection was set to λmax of 229 nm. Gradient elution at a flow rate of 10 mL/min was used with buffer A (90% ddH2O, 10% AcN and 0.1% TFA) and buffer B (90% AcN, 10% ddH2O and 0.1% TFA). Peptide samples were purified twice to ensure a purity of greater than 95%. Matrix assisted laser desorption ionization - time of flight (MALDI-TOF) mass spectrometry was used to confirm the product. The experimental
masses for peptides 73 and 77 were 1754.1 g/mol (theoretical: 1754.1 g/mol) and for aurein 2.2-Δ3 the experimental mass was 1357.9 g/mol (theoretical: 1357.7 g/mol).

3.3.3 Time-kill Assay

_S. aureus_ strain C622 (ATCC 25923) was used. Tryptic Soy broth (TSB) and Lysogeny broth (LB) agar were used for bacterial growth. _S. aureus_ C622 was inoculated and grown overnight in TSB broth at 37 °C at 200 rpm. The bacteria were transferred to a fresh TSB tube the next day and grown at 37 °C to mid-logarithmic phase (OD₆₀₀ = 0.5). The sub-culture was washed once with TSB and diluted to ca. 2.5x10⁶ CFU/mL. Subsequently, 0.5x, 1x, 2x and 4x MIC concentrations of peptide ³⁹,⁵⁶,¹¹¹ were added to the culture and incubated at 37 °C under shaking conditions (~169 rpm) for up to 4 hours. The peptide/bacteria mixture was serially diluted and plated on LB-agar plates in 10 µL portions at every 1-hour interval. Finally, colonies were counted after 14.5-17 hours of incubation at 37 °C.

3.3.4 Circular Dichroism (CD)

Samples for solution CD were prepared by adding peptides to 2,2,2-Trifluoroethanol (TFE) or 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1′-rac-glycerol) (sodium salt) (POPG). POPC and POPC/POPG (1:1 molar) liposomes were used to mimic mammalian or bacterial membranes specifically ²⁶,¹¹⁰–¹¹². Lipid stock solutions were first prepared by transferring appropriate volumes of lipids in chloroform into a round bottom flask. The lipids were dried in a stream of air and then vacuum dried overnight. MillQ filtered water (ddH₂O) was added afterwards to reach the required concentration. Finally, the lipid suspension was sonicated for 30 minutes to yield small unilamellar vesicles (SUV).
Solution CD experiments using TFE/ddH₂O and POPC/POPG (1:1) were carried out with a JASCO J-815 spectrophotometer at room temperature in the Shared Instrument Facility (SIF, UBC), as previously described \(^{26,110-112}\). The POPC experiment was performed using a JASCO J-810 spectrophotometer at 25 °C in the Life Sciences Centre (LSC, UBC). The signal was measured over a wavelength range of 190-250 nm using continuous scanning mode, with a digital integration time of 1 second. The scanning speed was set to 20 nm/min with a data pitch of 0.5 nm and a bandwidth of 1.50 nm. The peptide to lipid ratios tested were 1:15, 1:50 and 1:100. All samples were background subtracted for signal correction. One scan was used for the TFE/ddH₂O experiments, whereas three scans were used for the POPC and POPC/POPG experiments in order to increase the signal/noise (S/N) ratio. Since phospholipids contribute to noise in UV-CD signals, particularly at less than 190 nm wavelength, CD signals of greater than 600 Volts were discarded due to excess noise.

### 3.3.5 Nuclear Magnetic Resonance Spectroscopy (NMR)

Samples of peptides 73 and 77 were prepared to give a final peptide concentration of 1 mM, dissolved in 25% deuterated TFE, 10% D₂O and 65% ddH₂O, and a total volume of 600 µL. A Bruker 500-MHz instrument (Milton, Ontario, Canada) was used for data acquisition. The \(^1\)H operating frequency was 499.4 MHz. Total Correlation Spectroscopy (TOCSY) and Nuclear Overhauser Effect Spectroscopy (NOESY) 2D NMR measurements were conducted in phase sensitive mode \(^{225}\). All spectra were referenced to the residual methylene protons present in d₃-TFE (3.918 ppm). TOCSY experiments used the MLEV17 sequence with 70 ms mixing time \(^{226}\). NOESY experiments had a 150 ms mixing time \(^{226,227}\). The 2D NMR spectra were acquired with
4096 data points in F2 and 256 data points in F1 and were transformed to a final 1k x 1k matrix. For peptide 73 measurements, the TOCSY signal was averaged over 32 scans, while the NOESY signal was averaged over 128 scans. For peptide 77 measurements, the TOCSY signal was averaged over 64 scans, while the NOESY signal was averaged over 256 scans.

### 3.3.6 Oriented CD

Samples were prepared at peptide to lipid ratios of 1:15, 1:80 and 1:120. The lipid was composed of a 1:1 molar ratio of POPC and POPG. The peptide concentration was 40 µM, with a volume of 250 µL. Liposome preparation was the same as described in the solution CD sample preparation section. The samples were deposited onto clean quartz slides (3 cm × 1 cm, d = 1 mm) in 125 µL portions. The sample was first left to dry before the next deposition. Afterwards, the quartz slide containing the dried sample was half covered with a top slide with a spacer in between 7 layers of parafilm. The slides were placed in a Petri dish and then enclosed inside a 93% relative humidity chamber (saturated potassium nitrate containing desiccator). Samples were incubated at 37 °C over 3-6 days for rehydration. The oven temperature fluctuation was within 7 °C. Corresponding background samples were also prepared.

Oriented CD experiments were performed in LSC using the JASCO J-810 spectrophotometer at 25 °C. The sample containing quartz plates were placed directly into the sample compartment for measurement. All instrument parameters were the same as for the solution CD measurements. Three scans were used for better signal averaging. Linear dichroism effects were tested for and found not to contribute significantly to the signal.
3.3.7 **DiSC$_3$5 Assay**

Membrane depolarization of *S. aureus* C622 was determined using the membrane potential-sensitive dye 3,3'-dipropylthiadicarbocyanine iodide (DiSC$_3$5), as previously described. Briefly, bacteria were grown to mid-logarithmic phase in LB media, centrifuged, washed in 5 mM HEPES and 20 mM glucose. They were then resuspended in the same buffer to a final OD$_{600}$ of 0.05. A final concentration of 200 mM KCl was added to the cells and left for 30 min at room temperature to equilibrate cytoplasmic and external K$^+$ concentration before the dye was added at a final concentration of 0.8 µM for 30 min. The changes in fluorescence were measured using a Perkin-Elmer 640-10S spectrofluorimeter (Waltham, MA) using an excitation wavelength of 622 nm and a emission wavelength of 670 nm. Peptide concentrations of 0.03125x MIC were used for aurein and 0.5x MIC for peptides 73 and 77. The experiments were repeated 3 times.

3.3.8 **Pyranine Assay**

Translocation experiments were performed for aurein 2.2 (positive control), peptide 73, peptide 77 and daptomycin (negative control), using the pH-sensitive dye pyranine. Previously reported experimental methods were followed, using POPC/POPG (1:1 molar). The experiment was conducted using a PerkinElmer LS50B luminescence spectrometer at room temperature. The excitation and emission wavelengths were set to 454 nm and 511 nm, respectively. An aqueous solution of 10% Triton X-100 was used to set the maximum fluorescence intensity to around 800 arbitrary units (a.u.).
Liposomes were prepared in a manner similar to CD sample preparation. In brief, the vacuum dried POPC/POPG lipid was suspended in an internal buffer containing pyranine and sonicated for 45 minutes. The suspension composed of liposomes, pyranine and Na$_2$HPO$_4$ at a pH of 6.2 was filtered through a 0.22 µm filter and then purified with a Sephadex G-50 column (1.8 × 15 cm) to remove the pyranine which was not trapped within vesicles. Elution buffer was composed of Na$_2$HPO$_4$ and MCl (i.e. either NaCl or KCl), at a pH of 6.2. An external buffer solution with the same composition as the elution buffer at pH 7.2 was also prepared.

For peptides 73, 77 and aurein 2.2 data measurements, 100 µL of pyranine loaded vesicle solution was added to 3 mL external buffer (pH 7.2) in the 1 cm pathlength cuvette cell. Ca$^{2+}$ stock solution was added additionally for the daptomycin experiments, since calcium ion is required for its activity. The solution mixture was stirred at a low speed for homogeneous vesicle distribution. After signal stabilization for around 100 s, 20 µL of peptide solution was added to reach final peptide concentration of 2.5 µM. Lastly, Triton solution was added at ca. 1000 seconds to completely lyse the vesicles. The fluorescence intensity data were converted to K$^+$ or Na$^+$ ion transport efficiency by using the formula: % transport efficiency = (F - F$_0$) / (F$_{max}$ - F$_0$); where F$_0$ is the fluorescence intensity prior to peptide addition, F is the measured fluorescence intensity and F$_{max}$ is the maximum intensity upon Triton addition.

### 3.3.9 Gel Electrophoresis

Agarose gel electrophoresis was employed to test the DNA binding activity of peptides 73 and 77 following the protocols outlined in 188,218 and references therein. Peptides at concentrations
ranging from 0.15625x MIC to 10x MIC were mixed with 60 ng plasmid DNA (pRMC2) from *S. aureus* in 20 µL of binding buffer (5 % glycerol, 10 mM Tris/HCl (pH 8), 1 mM EDTA, 1 mM dithiothreitol, 20 mM KCl and 50 µg/mL BSA) and incubated for 1 h at room temperature before being loaded on the gel. The rate of migration of DNA bands through the gels was observed under UV illumination, using ethidium bromide staining (stock solution concentration of 1 mg/mL). The peptide IDR-1018, which is rich in R and W, and is known to bind to nucleotides, was used as a control.

### 3.4 Results
#### 3.4.1 Peptides 73 and 77 were more bactericidal than the parent peptide

In the quest to find more active peptides, an array of sequences derived from aurein 2.2-Δ3 was designed. The shorter version of aurein 2.2 was chosen based on cost considerations, and the observation that truncation of 3 residues at the C-terminus of aurein 2.2 has no impact on activity. A peptide array was constructed consisting of sequences in which hydrophobic residues were generally replaced by W and K was replaced with R. Single to multiple (max. 5 individual W; 3 individual R; or a mixture of 8 W and R residues) substitutions were made, as described in. Peptides 73 and 77 were also synthesized for comparative studies because they had MICs (against *S. aureus*) that were 8 times lower than that of aurein 2.2-Δ3.

To determine whether the introduction of W and R residues enhanced the killing ability of peptides 73 and 77, time-kill assays were conducted. As illustrated in Figure 3.1, both peptides 73 and 77 had more rapid killing kinetics than the parent peptide. Indeed, peptide 77, where the
position of the R and W residues has the potential of forming two cation-π interactions (W3/R7, R8/W12), was most effective with killing being observed already at 1 hour, with 0.5xMIC = 2 μg/mL (Figure 3.1b). The same behaviour was observed for peptide 73 at 1x MIC or higher concentrations (Figure 3.1a). Some error bars seen in Figure 3.1 are larger than the others, however they are typically similar or smaller than what is reported in other MBC determination studies. 

Figure 3.1 Time-kill kinetics of a) peptide 73, b) peptide 77, and c) aurein 2.2 against *S. aureus* C622 as determined from time-kill assays. Data points represent the means ± standard deviations from 3 independent experiments. The control represents no addition of peptide. The starting culture contained ca. 2.5x10⁶ CFU/mL colonies. When colony count was zero, a value of “1” was added to calculate logCFU, as is typical for this experiment.
3.4.2 Peptides 73 and 77 were \(\alpha\)-helical but less so than aurein 2.2-\(\Delta\)3

To compare the mean hydrophobic moment shown in Figure 1.7 for peptides 73 and 77 relative to the parent peptide, it was assumed that all peptides were \(\alpha\)-helical. If indeed these peptides are completely \(\alpha\)-helical, then W3 and R7 and R8 and W12 should be sufficiently close to form a cation-\(\pi\) interaction. To verify whether this is the case, solution CD experiments were conducted in trifluoroethanol (Figure 3.2), as well as in the mammalian and bacterial mimetic membranes, i.e. POPC (Figure 3.3) and POPC/POPG (1:1)\(^{110-112}\), respectively. As shown in Figure 3.2a and 3.2b, both peptides 73 and 77 were unstructured in water alone and adopted an \(\alpha\)-helical structure in the presence of TFE. To assess the extent of helical content, all CD spectra were fitted using the CDPro package, i.e. CDSSTR\(^{233}\), CONTINLL\(^{234}\) and SELCON3\(^{235}\). The results are listed in Table 3.1 for all conditions tested. Since TFE is known to favour \(\alpha\)-helical structures, it was important to also perform the experiments in liposomes.

Interestingly, the results showed that the helical content (i.e. 45-60 \%) was similar in all membrane mimetic environments and that there was no significant preference for the model bacterial vs. mammalian vesicles. This was in sharp contrast to previous work: aurein 2.2 was more helical in DMPC/PG than DMPC alone\(^{26}\); and aurein 2.2-\(\Delta\)3 had an \(\alpha\)-helical content of 96-98\% in both POPC/POPG and POPE/POPG\(^{111}\). In addition, the positive CD signal observed in the 220-230 nm range in the spectrum of peptide 77 in 100\% water was most likely due to the Trp residues\(^{236}\). Normally, there are two minima in the CD spectra of \(\alpha\)-helical peptides, with the mean residue ellipticity at 222 nm being of the same magnitude as that observed at 208 nm. In our case, the smaller ellipticity observed at 222 nm was reminiscent of the CD spectra for
model α-helical peptides with Arg and Trp residues positioned at residues $i$ and $i+4$ respectively, where the cation-π interaction is not strong $^{237}$. Indeed, the NMR NOESY spectra for peptides 73 (Figure 3.2c) and 77 did not show many $i$ to $i+4$ contacts, as expected for a continuous α-helix, and as also observed for aurein 2.2 $^{26}$. Finally, the number of NOE connectivities that were observed (Figure 3.2c for peptide 73, with the similar pattern for peptide 77, as shown in Figure 3.4) suggested that the segment W3-R7 was helical and hence this cation-π interaction would be likely to be present.

Figure 3.2 Structural data for peptides 73 and 77. a) Solution CD of peptide 73 in 0%, 25%, and 50% TFE (remaining fraction is water); b) Solution CD of peptide 77 in 0%, 25%, and 50% TFE (remaining fraction is water); c) Fingerprint region of the NOESY spectrum for peptide 73 in 25% TFE-d$_3$, with the $i$ to $i+4$ and $i$ to $i+3$ connectivities shown. The ambiguous connectivities, which arise due to spectral overlap, are shown using a dotted-line arrow.
Table 3.1 Average α-helical content obtained for peptides 73 and 77 in various amount of TFE and various peptide to lipid ratios in POPC and POPC/POPG (1:1). The average was calculated by taking the resulting values from the CDPro programs CDSSTR, CONTINLL and SELCON3.

<table>
<thead>
<tr>
<th>Condition</th>
<th>α-helical content (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Peptide 73</td>
</tr>
<tr>
<td>0% TFE; 100% H$_2$O</td>
<td>6 ± 5</td>
</tr>
<tr>
<td>25% TFE; 75% H$_2$O</td>
<td>50 ± 6</td>
</tr>
<tr>
<td>50% TFE; 50% H$_2$O</td>
<td>49 ± 7</td>
</tr>
<tr>
<td>1:15 peptide: POPC$^b$</td>
<td>44 ± 3</td>
</tr>
<tr>
<td>1:50 peptide: POPC</td>
<td>56 ± 2</td>
</tr>
<tr>
<td>1:100 peptide: POPC</td>
<td>49 ± 3</td>
</tr>
<tr>
<td>1:15 peptide: POPC/POPG</td>
<td>57 ± 3</td>
</tr>
<tr>
<td>1:50 peptide: POPC/POPG</td>
<td>55 ± 6</td>
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<tr>
<td>1:100 peptide: POPC/POPG</td>
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</tbody>
</table>

$^a$ Since the error associated with % secondary structure is generally ± 5, this error is not realistic. We report here, as with all numbers, the standard deviation obtained from the averaging of three program outputs. Generally, the error is well represented by the standard deviation.

$^b$ CD spectra of POPC shown in Figure 3.3.

Figure 3.3 Solution CD spectra of analogue peptides: a) peptide 73 in POPC with peptide to lipid ratios of 1:15, 1:50 and 1:100; b) peptide 77 in POPC with peptide to lipid ratios of 1:15, 1:50 and 1:100.
3.4.3 Peptides 73 and 77 did not readily insert in the membrane

As indicated in Table 2.1, the mechanism of action of bactericidal peptides generally involves DNA or membrane targets. Consequently, the fact that peptides 73 and 77 were bactericidal might have suggested that these peptides functioned by membrane perturbation, i.e. pore formation. To test this, we examined the insertion profile of peptides 73 and 77 as a function of increasing concentrations in POPC/POPG (1:1) bacterial mimetic membranes using oriented circular dichroism (OCD). As the data in Figure 3.5 show, these peptides do not readily insert into a bilayer, since even at high concentrations (i.e. P/L = 1:15) the peptides were still in a surface adsorbed state (i.e. two clear minima observed at 208 and 222 nm). Experiments using aurein 2.2-Δ3 under the same conditions were also performed and yielded the same results as previously reported. These results may suggest that peptides 73 and 77 did not form clearly defined pores like aurein.
Figure 3.5 OCD spectra of a) peptide 73 and b) peptide 77 in POPC/POPG (1:1) as a function of P/L ratio. The spectra clearly indicate that the peptide remain in the surface adsorbed state, i.e. do not insert in the membrane like aurein 2.2-Δ3 does. The spectra were normalized at 222 nm and the experiments were repeated 3 times.

3.4.4 Peptides 73 and 77 did not cause membrane permeabilization

To verify that peptides 73 and 77 indeed do not insert into the membrane and damage it, DiSC₃(5) and pyranine assays were conducted to assess impacts on the transmembrane electrical potential and pH gradient respectively. These assays were chosen in particular, because previous work showed that aurein causes membrane depolarization in *S. aureus* and leads to the selective leakage of K⁺ over Na⁺. As seen in Figure 3.6, peptide 73 and peptide 77 caused some membrane depolarization, as well as modest leakage of ions. Experiments using aurein 2.2-Δ3 under the same conditions were also conducted and yielded the same results as previously reported, i.e. significant membrane depolarization, ≈ 74% leakage of K⁺ and ≈ 45% leakage of Na⁺.
3.4.5 Alternate modes of action

Since both peptides 73 and 77 are bactericidal and since neither function by disrupting membranes, alternative mechanisms were tested. A number of studies have shown that some bactericidal peptides inhibit intracellular functions by interfering with DNA/RNA synthesis \(^{83,203}\). Consequently, the ability of peptides 73 and 77 to interact with DNA was examined using gel retardation. Figure 3.7 shows that neither peptides interacted with DNA strongly since the positions of bands were not perturbed regardless of the peptide concentration. The experiment was also conducted using the peptide IDR-1018, which is also rich in Arg and Trp residues. Figure 3.7c shows that there were some retardation effects at high IDR-1018 concentrations (≥ 5x MIC). Although IDR-1018 has some antibacterial activity \(^{215}\), this peptide primarily functions as an antibiofilm peptide \(^{93}\). It has also been shown to interact strongly with nucleotides, such as ppGpp \(^{93}\).
Figure 3.7 Agarose gel electrophoresis of a) peptide 73, b) peptide 77 and c) IDR-1018, incubated with 60 ng plasmid DNA (pRMC2). The control consists of DNA alone in binding buffer. The peptide concentrations (in µg/ml) are multiples of the individual MIC values, as indicated above each lane. It is common to observe two bands in this plasmid DNA system, with the intense band below the weaker ones, as seen in the published paper by Gottschalk et al.

3.5 Discussion

The increase in levels of bacterial antibiotic resistance has led researchers to look for alternatives to current antibiotics. HDPs or synthetic analogues are considered to be interesting substitutes because they can function in a variety of ways. Recently, a number of studies have shown that incorporation of amino acids such as Arg and Trp results in very active analogues. The inclusion of these residues allows for strong electrostatic interactions between the positively charged residues (Arg, Lys) and negatively charged bacterial membranes, and favorable membrane association since Trp preferentially interacts with the interfacial region of membranes.
Addition of both Arg and Trp also allows for the potential formation of cation-π interactions which also serve to stabilize structures and enhance peptide-membrane interactions. Starting from a truncated version of the naturally occurring HDP aurein 2.2, a library of 13 amino acid residue peptides with varying amounts of Arg and Trp was generated to form 91 new analogues (Appendix A.1). For most peptides, residues D4 and I5 were kept unchanged and generally, aromatic (e.g. F3) and/or hydrophobic residues (e.g. V9) were replaced by Trp and lysines (i.e. K7 and K8) were replaced by Arg. Residue D4 was preserved in most analogues as it has been previously suggested that it may play an important role in the mechanism of action of aurein 2.2, specifically the ion selectivity of the HDP. Peptides in this library were synthesized by SPOT synthesis and tested for activity against S. aureus. Interestingly, peptides with D4 substituted by N, K, E, Q or A were all found to be 2- to 4-fold less active than aurein 2.2-D3. Furthermore, peptides that either contained a Trp at position i and an Arg at position i +4 or the converse (i.e. Argi - Trpi+4), which is typical of model peptides that contain a cation-π interaction influencing an α-helical configuration, did not show significantly improved activity (1-2x MIC of parent aurein 2.2-D3), with the exception of two peptides, peptide 73 and peptide 77, which had MICs that were 8x better. This subset of cation-π containing peptides represented 46% of the R/W containing peptides in the library (Appendix A.1). This suggests that the presence of a cation-π interaction is not the sole determining factor for improved function and activity.

Many studies over the years have shown that the balance between charge and hydrophobicity plays an important role in activity of HDPs. The R/W analogues (Appendix A.1) generated here varied from +2 to +3 charge, with 2 exceptions at +4. The mean hydrophobic
moment, as calculated using HeliQuest and assuming an α-helical structure, varied from 0.578 to 0.899. Examination of the data in Appendix A.1 did not reveal any specific trends relating physicochemical properties to activity. Indeed, this is not surprising as it is well known that a large number of peptide features, termed molecular descriptors, are required in order to predict activity with any reliability 24,89,100. Nevertheless, the generation of this list of sequences did yield two candidates, peptides 73 and 77 that were more active than the parent peptide.

3.6 Summary

In this Chapter, the question of how the amino acid substitutions leading to modest increases in charge and mean hydrophobic moment (Figure 1.7), impact the structure and membrane interaction of an HDP was addressed. Given that the MIC was better and both peptides 73 and 77 are more bactericidal than aurein, it might have been anticipated that these peptides would interact more readily with membranes and cause more damage. Instead, the data presented here clearly show that the new analogues behave like cell-penetrating peptides, i.e. more like indolicidin 51,95,136,137 than aurein 2.2. Nevertheless, unlike indolicidin, peptides 73 and 77 do not appear to interact with DNA 136,137. This is perhaps not surprising given the relatively low charge of peptides 73 and 77.

Overall, the data presented here suggest that peptides 73 and 77 transit through membranes and most likely target something other than the membrane (e.g. cell wall biosynthesis, such as other R/W rich peptides 183; and/or other known targets such as antagonism of heat shock proteins/chaperones 247). Given that both these peptides have minimum biofilm inhibitory concentrations (MBIC) that are less than the MIC 56, it is however also possible that these
peptides should be solely considered as antibiofilm peptides and not as having any antibacterial activity. Work to explore and characterize the antibiofilm mechanism of action of these peptides is described in Chapter 4.
Chapter 4: Antibiofilm Function of Peptides 73 and 77

4.1 Synopsis

As described in Chapter 3, peptide 73 and peptide 77 both have antibacterial and antibiofilm activity, with the minimum biofilm inhibitory concentration (MBIC) being slightly lower than the minimum inhibitory concentration (MIC) which is measured for planktonic bacteria. This suggests that these peptides should perhaps be considered as having a primary antibiofilm function. In this chapter, I will examine the mechanism of antibiofilm action. Since these peptides are rich in R and W, as IDR-1018 is, and since IDR-1018 has been found to preferentially bind the alarmone ppGpp, I will examine here how peptides 73 and 77 interact with a variety of nucleotides (ATP, ADP, GTP, GDP, and ppGpp, Figure 4.1). Co-precipitation assays, ITC and NMR experiments will be used. The data will be discussed in light of the complex equilibria that exists between the peptides and nucleotides.

![Figure 4.1 Various nucleotides used in this study. Adenosine triphosphate (ATP), adenosine diphosphate (ADP), guanosine triphosphate (GTP), guanosine diphosphate (GDP) and guanosine 5′-diphosphate 3′-diphosphate (ppGpp).](image-url)
4.2 Introduction

As described by Kumar et al. and discussed in Chapter 3, peptides 73 and 77 have antibiofilm activity in addition to their antibacterial activity. Table 4.1 lists the MIC and MBIC values of the analogue peptides and the parent peptide and shows that the MBIC values for peptides 73 and 77 are lower than their MIC values. This is analogous to what was reported for IDR-1018, a recently discovered broad spectrum antibiofilm peptide. IDR-1018 functions by binding to and degrading bacterial signaling nucleotides that arise due to the stringent response, namely guanosine 5’-diphosphate 3’-diphosphate (ppGpp) and guanosine 5’-triphosphate 3’-diphosphate (pppGpp), collectively termed (p)ppGpp. The interaction of 1018 with (p)ppGpp leads to biofilm inhibition or cell death in biofilms.

Table 4.1 MIC and MBIC values of aurein 2.2, aurein 2.2-Δ3, peptide 73, peptide 77 and IDR-1018 against S. aureus. MIC values were determined by Dr. Prashant Kumar (Straus group). MBIC values were determined by Dr. John T.J. Cheng (Hancock group). *For IDR-1018, MIC and MBIC values are from 93,215. The 50% RBC lysis results are from 125.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>MIC S. aureus (µg/mL)</th>
<th>MBIC S. aureus (µg/mL)</th>
<th>50% RBC lysis (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aurein 2.2</td>
<td>16</td>
<td>16</td>
<td>1350</td>
</tr>
<tr>
<td>Aurein 2.2-Δ3</td>
<td>32</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>peptide 73</td>
<td>4</td>
<td>2</td>
<td>221</td>
</tr>
<tr>
<td>peptide 77</td>
<td>4</td>
<td>2</td>
<td>107</td>
</tr>
<tr>
<td>IDR-1018*</td>
<td>5</td>
<td>2.5</td>
<td></td>
</tr>
</tbody>
</table>

Although it is possible that peptides 73 and 77 function as antibiofilm peptides by interacting with (p)ppGpp as 1018 does, there are other mechanisms of action possible, as detailed in
Chapter 1. In fact, it has been suggested that antibiofilm peptides can function in the following ways (Figure 1.5, Table 2.1): membrane disruption, cell signaling, EPS degradation, stringent response inhibition, or others targets, such as gene downregulation. As described in the literature and in Chapter 2, few methods exist, however, to easily probe these various mechanisms of action in detail. Moreover, it is also important to note that antibiofilm activity is often distinct from anti-planktonic antimicrobial activity. Hence, the studies presented in Chapter 3 cannot be used here to directly infer the antibiofilm mechanism of action of peptides 73 and 77.

Nevertheless, from the data reported in Chapter 3, it seems likely that peptides that do not interact with and perturb membranes of bacteria in the planktonic form will not perturb membranes when these bacteria form biofilms. In other words, of the possible mechanisms of action that can be readily investigated, I will focus on stringent response inhibition and determine whether peptides 73 and 77 behave like IDR-1018 and other inhibitors, e.g. DJK-5. As described by de la Fuente-Núñez et al., this interaction will be probed using a co-precipitation assay. For 1018, the results of this assay showed a preferential binding interaction between ppGpp and 1018 compared to other nucleotides (i.e. ATP, ADP, GTP, GDP). Finally, a different study showed that an analog of 1018, with its amino acid sequence reversed, was equally able to co-precipitate ppGpp in a test tube.

In general, interactions between two biomolecules is driven by a number of weak intermolecular forces, such as electrostatics, hydrophobic interactions, hydrogen bonding, etc. In order to characterize binding, experiments typically probe changes in enthalpy upon binding (e.g. using
isothermal titration calorimetry) or by determining binding kinetics (e.g. using NMR, fluorescence, SPR, etc.). Interestingly, de la Fuente-Núñez et al. chose to determine which nucleotide preferentially bound to the host defense peptides by using a "salting-out" method. When the positively charged peptides interact with the negatively charged nucleotides, they precipitate. Given that it has been shown that anion-driven peptide aggregation has an impact on activity \(^{250}\), it is unclear whether this assay is a true reflection on how ppGpp inhibition occurs in bacterial biofilms.

Peptide aggregation is a complex phenomenon influenced by different factors such as peptide secondary structure, hydrophobicity, charge, as well as solute characteristics in the environment \(^{250}\). Aggregation occurs when a biomolecule exceeds a critical concentration, which is dependent on a variety of conditions including temperature, pH, ionic strength and the structure of the biomolecule \(^{251}\). Aggregates can vary in size. When they are small, they can be invisible to the eye and remain suspended in the solution. When the aggregate concentration is high and the size of the aggregate is large, visible precipitation from the solution is typically observed. In addition, depending on the techniques used to probe the interaction, we may see one or the other phenomenon.

In this chapter, I will present the results from experiments performed to probe the binding interaction between peptide and nucleotides. Specifically, results from co-precipitation assay, \(^{31}\)P-NMR as well as ITC experiments will be presented, in order to determine the antibiofilm mechanism of action of aurein analogue peptides 73 and 77.
4.3 Materials and Methods

Peptides 73 and 77 were synthesized using a solid phase peptide synthesizer (Fmoc), the peptides were purified using RP-HPLC and characterized with MALDI-TOF mass spectrometry as described in Chapter 3. IDR-1018 was kindly provided by the Hancock lab, UBC. The nucleotide ppGpp was purchased from TriLink Biotechnologies. NaH$_2$PO$_4$ was purchased from EMD Chemicals. All other nucleotides were from Sigma-Aldrich.

4.3.1 Co-precipitation Assay

The ability of peptides 73 and 77 to bind with different nucleotides was tested with the co-precipitation assay as described in $^{93,138}$. Peptide 73 or peptide 77 was mixed individually with increasing concentrations of ATP, ADP, GTP, GDP, ppGpp and NaH$_2$PO$_4$ in 25 mM Tris buffer at pH 7.4 using a 96-well flat bottom plate. The peptide concentration was kept fixed at 0.8 mM, with the nucleotide concentration changing from 0.025 – 0.8 mM to obtain N/P ratios in the range of 0.03125 to 1. Peptide only, Tris only and nucleotide only controls were prepared to confirm each reagent did not precipitate on its own. Co-precipitation was examined/quantified using Biotek Instruments microplate reader by measuring absorbance at 620 nm. The experiment was performed at room temperature. Four repeats were completed for each peptide.

4.3.2 Co-precipitation Assay as a Function of Time and Temperature

Peptide 73 or IDR-1018 was mixed with the nucleotide ATP in 25 mM Tris buffer at pH 7.4 using a 96-well flat bottom plate to observe the effect of time and temperature on peptide – nucleotide precipitation. Sample preparation was the same as described in Section 4.3.1, the difference being that an additional incubation step was added to the procedure. The plate was
parafilmed during incubation (at 0.5 hour intervals) at either room temperature or 37°C, to prevent the solution from drying out. Turbidity measurements were performed in the time range of 0 to 18.5 hours, with a gap between time points representing 3.5 and 16.5 hours. For each peptide, the experiment was repeated three times.

4.3.3 **Isothermal Titration Calorimetry (ITC)**

ITC experiments were performed using a MicroCal iTC200 (Malvern Instruments) in the Michael Smith Laboratories (UBC, Vancouver). ITC titrations were conducted in order to investigate the binding affinity between peptide 73 and the specific nucleotides GTP and ADP. Nucleotide solution in Tris buffer was loaded into the syringe and added into the peptide solution in the same Tris buffer in the sample cell. All samples were degassed in order to prevent spikes during titration. The samples were also stirred at a constant speed to ensure thorough mixing of components. The Tris buffer concentration was 25 mM at pH 7.4 and the peptide concentration was 25 µM. After a preliminary injection of 0.2 µL of 250 µM nucleotide solution (GTP or ADP) into the sample cell (200 µL cell volume), 18 consecutive injections of 2 µL nucleotide solution was added with a time interval of 180 seconds. Blank experiments were performed by titrating the nucleotide into peptide free buffer in the sample cell for both nucleotides. Experiments were performed at 25°C and 37°C. The nucleotide to peptide ratios ranged from 0 to 1.81.

4.3.4 **31P-NMR spectroscopy**

In order to examine the binding interaction of the peptide with nucleotides, 31P-NMR experiments were conducted on a Bruker Avance 500MHz spectrometer. Samples containing 0.1
mM ATP or ppGpp were prepared in 25 mM Tris buffer at pH 7.4. Peptide 73 or IDR-1018 was titrated in, so as to reach nucleotide to peptide (N/P) ratios in the range of 0.5:1 – 16:1. At each N/P ratio, $^{31}$P signals were averaged over 14k scans. All samples contained 10% D$_2$O and 0.5 µL trimethylphosphate as the internal calibration. $^{31}$P NMR spectra were recorded at room temperature.

A second titration was performed in which both ATP and ppGpp were present in order to determine whether the peptides bound preferentially to one nucleotide versus the other. Samples containing 0.1 mM of ATP and 0.1 mM ppGpp were prepared in 25 mM Tris at pH 7.4. Peptide 73 or IDR-1018 was then added separately in order to sample a N/P range of 0.5:1 – 4:1. As previously, 10% D$_2$O and 0.5 µL trimethylphosphate were added to each sample.

### 4.3.5 $^1$H NMR spectroscopy

In order to determine whether precipitation involved peptide 73, $^1$H NMR spectra were obtained for two samples: one of the peptide dissolved in 25 mM Tris at pH 7.4, 10% D$_2$O; and one of peptide 73 with ATP and ppGpp at a N/P ratio of 0.5:0.5:1 in 25 mM Tris at pH 7.4, 10% D$_2$O, 0.5 µL trimethylphosphate. The peptide concentration in both samples was 0.2 mM, each nucleotide had a concentration of 0.1 mM. Spectra were recorded at 25°C on a BRUKER 500MHz spectrometer with the help of Dr. Mark Okon (UBC). The number of scans was 128 for both samples.
4.4 Results

4.4.1 Co-precipitation assay

In order to test whether peptides 73 and peptide 77 target the stringent response by binding to alarmone nucleotide ppGpp, a co-precipitation assay was used, as previously described by de la Fuente-Núñez et al. 93. Nucleotides with differing number of phosphate groups and different nitrogenous bases, i.e. ATP, ADP, GTP, GDP, ppGpp, were tested. As compared to IDR-1018 93, both peptide 73 and peptide 77 did not show a higher OD reading at a N/P ratio of 1:1 for ppGpp over the other nucleotides that were examined (Figure 4.2). This would hence suggest that peptides 73 and 77 do not show preferential binding to ppGpp.

Figure 4.2 Binding of peptide 73 (left) and peptide 77 (right) with various nucleotides based on co-precipitation, over 4 trials. Each peptide was mixed with increasing concentrations of nucleotides (ATP, ADP, GTP, GDP, ppGpp) in Tris buffer (25 mM, pH 7.4). The peptide concentration was 0.8 mM and nucleotide concentrations ranged from 0.025 – 0.8 mM. The extent of co-precipitation was quantified by measuring absorbance at 620 nm. NaH₂PO₄ was used as a control to confirm that phosphate ions did not induce peptide precipitation in the tested N/P range.
Looking at the results at N/P of 0.25:1 or lower, the OD readings follow the trend that the higher the negative charge of the nucleotide (Figure 4.2), the stronger the interaction. At these low ratios, the data suggest that the electrostatic interaction is most likely the driving factor. The leveling off behavior observed for ppGpp after reaching N/P ratio of 0.25 seems to suggest some saturation behaviour. Interestingly, the data at N/P ratio of 1:1 for peptide 73 suggest a preference for adenine nucleotide compared to guanine nucleotides. Finally, it is important to note that this assay is conducted in such a way that the absorbance readings are taken quickly after all components are mixed in the wells of the 96-well plate. Hence, it represents an instantaneous, non-equilibrium condition for the peptide-nucleotide co-precipitation. In addition, because of the nature of the experiment and the large error bars, it is not typical to fit the curves to extract binding constants in this experiment.

4.4.2 Isothermal Titration Calorimetry

In order to further characterize the interaction between the peptides and nucleotides, ITC experiments were conducted. The advantage of the ITC experiment over the co-precipitation experiment described above is that the concentrations required for ITC experiments are approximately ten times lower, i.e. 10-100 µM versus 800 µM. Given that the concentration of (p)ppGpp nucleotide is ca. 50 µM in bacterial cells, this experiment should probe the interaction under potentially more biologically relevant concentration. An additional advantage of working at these low concentrations is the potential to minimize peptide precipitation, as peptides 73 and 77 as well as IDR-1018 have been shown to precipitate readily.
The raw ITC data from the experiment are shown in Figure 4.3 and Figure 4.4 for peptide 73 and various nucleotides. The ITC profiles obtained show that the heats evolved with each injection is small and that the overall profile for the peptide – nucleotide binding interaction indicated a weak non-specific interaction. As evidenced from some of the irregularities in the traces, the experiment was complicated by precipitation of the sample, even at these concentrations. Consequently, the data were not interpreted further.

Figure 4.3 Raw ITC data of peptide 73 and GTP binding interaction at 25°C (left image) and 37°C (right image). Blue curves: buffer runs. Black curves: sample runs.
Figure 4.4 Raw ITC data of peptide 73 and ADP binding interaction at 25°C (left image) and 37°C (right image). Blue curves: buffer runs. Black curves: sample runs.

4.4.3 $^{31}$P NMR

In light of the ITC data obtained above, $^{31}$P NMR experiments were performed instead to study the co-precipitation behaviour, as this technique is well suited to weak interactions and reasonable spectra could be obtained for concentrations in the range of 100 µM. In order to determine whether the data obtained in Figure 4.2 truly reflect a preferential interaction of peptide 73 with ATP over ppGpp, the peptide was titrated in an ATP solution. The $^{31}$P-NMR chemical shift assignment of ATP is as follows: the α-phosphate group has a resonance at approximately -10 ppm, the β-phosphate group has a resonance at around -20 ppm and the γ-phosphate group has a chemical shift near -5 ppm \cite{253}. Based on the chemical shift assignment from Wilkinson et al., the authors indicate that some signals are overlapped for ppGpp, such as the 5’-α and 3’-α phosphate group signals near -6 ppm. They also seem to suggest that the 5’-β phosphate group has a resonance around -11 ppm, while the 3’-β phosphate group has a chemical shift close to -6 ppm \cite{254}. 

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As the spectra in the left column of Figure 4.5 show, high peptide concentrations (i.e. at N/P ratio of 0.5:1) are required in order to see a decrease in the signal, which arises due to precipitation. This is in stark contrast to the concentration required to observe a similar decrease in signal for IDR-1018 and ATP. This decrease, as seen in the second column of Figure 4.5, occurs at N/P ratios of 1:1. Given that IDR-1018 has a +5 charge and peptide 73 has a +3 charge, this behavior is consistent with an electrostatic interaction. Interestingly, when the titration is performed using peptide 73 and ppGpp (Figure 4.5, 3rd column), the $^{31}$P nucleotide signal persists even at N/P ratio of 0.5:1, suggesting a weaker interaction than that seen between peptide 73 and ATP. The mismatch between the +3 charge of the peptide and the -6 charge of ppGpp (versus -4 for ATP) may account for these observations, i.e. even higher concentrations of peptide 73 than those probed here are required to neutralize the charge (e.g. N/P=3/6=0.5:1). A similar trend is observed for IDR-1018 and ppGpp (Figure 4.5, last column), with $^{31}$P NMR signal still being present at N/P=1:1.
Figure 4.5 $^{31}$P NMR spectra of ATP (left) and ppGpp (right) as a function of decreasing N/P ratios (top to bottom). All samples were in Tris buffer (25 mM, pH 7.4), nucleotide concentration was 0.1 mM and the peptide concentrations varied from 0.00625 – 0.2 mM. All samples contained 10% D$_2$O and 0.5 µL trimethylphosphate for internal calibration.

Overall, the $^{31}$P NMR data shown in Figure 4.5 could be interpreted as suggesting that both peptide 73 and IDR-1018 interact preferentially with ATP given that less peptide is required for complete precipitation. This is in stark contrast to what was previously reported for IDR-1018 and ppGpp: IDR-1018 was found to preferentially bind to ppGpp as demonstrated by $^{31}$P-NMR results, specifically, upon peptide exposure to a sample mixture of equal concentrations of GTP and ppGpp, the signals corresponding to ppGpp almost completely disappeared, while the GTP signals still remained although with a lower peak intensity. Furthermore, a quicker peak intensity decrease of ppGpp phosphorus signals was observed, when increasing concentrations of IDR-1018 were added to an equimolar mixture of GTP and ppGpp, confirming the higher
affinity between IDR-1018 and ppGpp. Therefore, before confirming the observation that both peptide 73 and IDR-1018 show preferential interaction with ATP, I first verified that the precipitate formed contained both nucleotide and peptide. The decrease in $^{31}\text{P}$ NMR signal clearly indicated nucleotide precipitation. To test peptide precipitation, $^1\text{H}$ NMR spectra were recorded.

4.4.4 $^1\text{H}$ NMR

$^1\text{H}$ NMR experiment was performed to confirm that the precipitation behavior seen from the $^{31}\text{P}$ NMR experiment was due to the binding interaction of peptide and nucleotide and not from nucleotide simply precipitating out of solution. Figure 4.6 clearly shows the reduction in the amide backbone and other peaks corresponding to the peptide in the peptide 73/ATP:ppGpp sample. This clearly shows that the precipitate formed is composed of both nucleotides and the peptide.
4.4.5 $^{31}$P NMR: Competition between ATP and ppGpp

In order to further characterize the binding interaction between peptide 73 and the nucleotide ppGpp, $^{31}$P NMR experiments were performed by titrating either peptide 73 or IDR-1018 into a solution containing an equal amount of the nucleotides ATP and ppGpp. All the signals between -4.5 ppm and -20 ppm belong to both nucleotides respectively, the signals unique to ppGpp and ATP are clearly identified in Figure 4.7, as could be seen by overlaying the spectra shown in Figure 4.5. Figure 4.7 shows that for both peptide 73 and IDR-1018, the peak corresponding to ppGpp disappears first. This is consistent with previous reports of a preferential interaction between IDR-1018 and ppGpp. Interestingly, the peak is absent for N/P ratios of 1:1:1 for
peptide 73 and at 0.5:0.5:1 for 1018. This would suggest that peptide 73 interacts more strongly with ppGpp than 1018 does, as less peptide is required. In addition, the disappearance of the signals corresponding to ATP in the spectrum obtained at N/P ratio of 0.5:0.5:1 for peptide 73 suggests that this peptide also interacts more strongly with this nucleotide.

Figure 4.7 Competitive binding behavior of peptides to nucleotides as shown by $^{31}$P NMR. Samples containing equal concentrations of ATP (0.1 mM) and ppGpp (0.1 mM) were prepared in Tris buffer (25 mM, pH 7.4). Peptide 73 (left) or IDR-1018 (right) was titrated in to reach concentrations of 0.025 – 0.2 mM as a function of decreasing N/P ratios (top to bottom). All samples contained 10% D$_2$O and 0.5 µL trimethylphosphosphate for internal calibration.
4.4.6 Co-precipitation Assay as a Function of Time and Temperature

Given the data presented this far and the complex equilibria that are most likely present in the samples (i.e. peptide-peptide and peptide-nucleotide interactions), the co-precipitation experiments described in section 4.4.1 were repeated as a function of time and temperature. The motivation for extending the measurements as function of time stem from the fact that the $^{31}$P NMR experiments occurred over a period of ca. 19 hours. The motivation to perform the assay at a different temperature was to see whether the phenomena observed are temperature dependent.

Using peptide 73 and IDR-1018 and the nucleotide ATP, co-precipitation experiments were performed at room temperature and 37°C over a period of 17.5 – 18.5 hours as a function of increasing N/P ratio. As seen in Figures 4.8 and 4.9, the turbidity of the peptide/nucleotide solutions consistently decreases over time, independent of temperature. Moreover, the reduction in co-precipitation occurs more rapidly at higher temperature for peptide 73 (Figure 4.10) than for IDR-1018 (Figure 4.11), specifically at N/P=1:1.
Figure 4.8 Co-precipitation between peptide 73 and ATP at room temperature (left) and 37°C (right) over 17.5 – 18.5 hours as a function of increasing N/P ratios, over 3 trials. Peptide 73 (0.8 mM) was mixed with increasing concentrations of ATP (0.025 – 0.8 mM) in Tris buffer (25 mM, pH 7.4). The extent of co-precipitation was determined by measuring absorbance at 620 nm.

Figure 4.9 Co-precipitation between IDR-1018 and ATP at room temperature (left) and 37°C (right) over 17.5 – 18.5 hours as a function of increasing N/P ratios, over 3 trials. IDR-1018 (0.8 mM) was mixed with increasing concentrations of ATP (0.025 – 0.8 mM) in Tris buffer (25 mM, pH 7.4). The extent of co-precipitation was determined by measuring absorbance at 620 nm.
Figure 4.10 Change in co-precipitation between peptide 73 and ATP at room temperature (left) and 37°C (right) over 17.5 – 18.5 hours, with a gap between time points 3.5 and 16.5 hours. Turbidity measurements for N/P ratios of 1 and 0.25 are shown, over 3 trials. Peptide 73 (0.8 mM) was mixed with increasing concentrations of ATP (0.2 mM and 0.8 mM) in Tris buffer (25 mM, pH 7.4). The extent of co-precipitation was determined by measuring absorbance at 620 nm.

Figure 4.11 Change in co-precipitation between IDR-1018 and ATP at room temperature (left) and 37°C (right) over 17.5 – 18.5 hours, with a gap between time points 3.5 and 16.5 hours. Turbidity measurements for N/P ratios of 1 and 0.25 are shown, over 3 trials. IDR-1018 (0.8 mM) was mixed with increasing concentrations of ATP (0.2 mM and 0.8 mM) in Tris buffer (25 mM, pH 7.4). The extent of co-precipitation was determined by measuring absorbance at 620 nm.
4.5 Discussion

As presented in Chapter 2, there are relatively few biophysical methods to determine HDP antibiofilm mechanisms of action (Table 2.1). The co-precipitation assay, which measures the turbidity that arises as a result of an interaction between an antibiofilm peptide (e.g. IDR-1018 and DJK-5) and the alarmone ppGpp, has been used in the past to suggest that these HDPs function by perturbing ppGpp levels and hence breaking the cycle shown in Figure 1.5. However, the assay relies on higher than biologically-relevant concentrations and relies on the formation of a precipitate. Numerous protein-ligand interaction studies show strong binding interactions, but the resulting complex remains soluble. The interpretation of the results obtained from this assay can be further complicated by the fact that many HDPs (e.g. IDR-1018) are known to aggregate and "salt out" under certain conditions.

In this Chapter, the interaction of peptide 73 and peptide 77 with a series of nucleotides was examined. A number of techniques were used to characterize this interaction. The fact that the results are method dependent suggests that the binding interaction between these HDPs and nucleotides is complex and path dependent. For example, maximum precipitation was observed at N/P ratios of 1:1 in the co-precipitation assay (Figure 4.2), whereas in the $^{31}$P NMR experiments, the peptide/nucleotide mixtures were completely soluble at N/P ratios of 16:1 (Figure 4.5). This would correspond to a data point to the right of the graph in Figure 4.2. In other words, concentrations of the components, the order of addition, time and temperature (Figures 4.8-4.11) all play a role in the observed amount of precipitation, as seen in the results presented here. It should be noted that in the NMR experiments, the precipitate remained at the bottom of the tube, i.e. was not filtered out. Furthermore, measuring turbidity in the co-
precipitation assay requires extensive mixing of the solution (which is done with a pipettor) just prior to when the OD reading is made (with potential differences in extent of mixing being accounted for by performing multiple repeats). Both of these methods should yield the same result if the interaction is a straightforward rapid interaction between component A and component B: NMR measures what is left in solution, whereas the assay measures the resulting precipitate. Due to conservation of mass, the resulting curves obtained in this case as a function of N/P ratio should be mirror images. The results presented here showed that the interaction between peptide 73 or IDR-1018 and nucleotides cannot be described in such simple terms. Such complex behavior has been seen before for systems where multiple binding sites or non-specific binding occurs. Indeed, the ITC data obtained here lend support to this hypothesis. In particular, since the shape of the obtained ITC titration plots was almost linear, it is an indication of low binding affinity and non-specific interactions between the peptide and nucleotides.

Of all the methods presented here to probe HDP/nucleotide interactions, the one that appears to provide the most reliable information is the \(^{31}\text{P}\) NMR experiment where peptide is added to a mixture of two nucleotides present in equal molar ratio. Indeed, as seen in Figure 4.7, this experiment clearly demonstrates that IDR-1018 preferentially interacts with ppGpp. Co-precipitation results aside, the study by de la Fuente-Núñez et al. showed through a variety of experiments that this preference appears to play an important role in IDR-1018's antibiofilm function. Indeed, de la Fuente-Núñez et al. showed that 1018 interferes directly with the stringent response cycle shown in Figure 1.5. RelA and SpoT deletion mutants replicated the effects seen
by IDR-1018, both *in vitro* biofilm experiments $^{93}$ and in animal models $^{126}$. These highly conserved enzymes synthesize and breakdown ppGpp.

If the same holds true for peptide 73, the results of the $^{31}$P experiments presented in Figure 4.7 would indicate that this peptide functions somewhat differently from IDR-1018, since peptide 73 interacts readily with both ppGpp and ATP, as seen by the decrease in intensity of both signals in Figure 4.7. Given that ATP and GTP are also crucial to the stringent response (Figure 1.5), it is possible that peptide 73 functions by perturbing the cycle by removing the energy sources required for ppGpp production. In other words, the effect would be an indirect regulation of ppGpp alarmone levels. Of course, ATP and GTP are implicated in many processes required to keep bacteria alive, so perturbation of other types of responses also need to be considered. These hypotheses need to be substantiated with additional experiments which probe the effect of peptide 73 on ATP levels in bacteria in both planktonic and biofilms forms. Based on the results presented in this Chapter, IDR-1018 would be expected to have a limited impact on ATP levels and could be a useful control.

### 4.6 Conclusion

The results presented in this Chapter suggest that the antibiofilm function of peptide 73 may involve the regulation of the stringent response, as was found for IDR-1018, but that the exact nature of this regulation is different. Unlike IDR-1018 which preferentially interacts with ppGpp, peptide 73 appears to bind both ppGpp and ATP. Overall, the results also show that the co-precipitation assay should most likely not be used on its own to indicate preferential binding, in particular in systems where complex HDP-HDP and HDP-nucleotide interactions are present.
Alternative techniques, such as $^{31}$P NMR competitive binding titrations offer some insight but should be supported with *in vitro* biofilm data (e.g. such as the flow cell system experiments described in Chapter 2) and data obtained from animal models. For instance, the data obtained from Figure 4.5 and Figure 4.7 are contradictory because we are not just looking at one to one binding interactions, i.e. other interactions such as peptide-nucleotide and nucleotide-nucleotide interactions are likely also present. Therefore, we will need additional assays (e.g. measurement of ATP levels in biofilms) to determine this interaction, which will be described in further detail in section 5.3.
Chapter 5: Conclusions and Future Studies

5.1 General Conclusions

With the increasing number of bacteria that are resistant to antibiotics and the low number of new compounds being developed and approved for use, it is becoming increasingly important to look for alternatives. Host defense peptides (HDPs) offer a viable solution because they function through a variety of ways. Specifically, they have antibacterial, antibiofilm, immunomodulatory, anticancer and many other activities, as described in detail in Chapter 1. Since their discovery forty years ago, most HDPs have been optimized and studied in light of their antibacterial mechanisms of action. It is only in the last decade that more researchers have explored the other functions of HDPs and tried to determine detailed mechanisms of action associated with these other functions. In the context of antibacterial versus antibiofilm function, this time difference manifests itself in the number of methods available to study MOAs, as discussed in Chapter 2 and highlighted in particular in Table 2.1. Some researchers have suggested that the antibiofilm MOA of HDPs is distinct from their antimicrobial activity against planktonic bacteria. Others on the other hand have proposed that some aspects of the MOAs may be linked. For example, given that membrane perturbation is possible for both antibacterial and antibiofilm HDPs, it is conceivable that this occurs through similar mechanisms.

In order to try to expand our knowledge on the relationships between the various HDP functions, my thesis focused on characterizing two peptides that have both antibacterial and antibiofilm function. These peptides were derived from the naturally occurring HDP aurein 2.2. The strategy to generate these peptides was twofold: 1) to start from a peptide whose MOA was well
characterized and clearly defined; and 2) to introduce residues, such as Arg and Trp, at varied locations in the peptide sequence to generate more active synthetic HDP analogues. As outlined in Chapter 3, previous studies had shown that incorporation of these amino acids resulted in very active analogues $^{29,51,183}$. To improve the sampling of the HDP functional landscape, the approach developed in the Hancock lab was used: a library of peptides was generated using SPOT synthesis $^{117,118}$. From this, two peptides, peptide 73 and peptide 77, were identified as warranting further study, given that they displayed improved MICs over aurein 2.2 and have antibiofilm activity. Although the analogue peptides demonstrate toxicity effects towards human cells such as red blood cells (RBCs) and human peripheral blood mononuclear cells (PBMCs) as described in Chapter 1, it is still useful to study and understand their MOA individually, a key missing element to aid future HDP design.

We anticipated little change in the analogue peptides’ MOA compared to aurein 2.2, yet it appeared different for both antibacterial and antibiofilm functions based on experimental results. The data presented in Chapter 3 showed that peptides 73 and 77 were both more bactericidal than aurein 2.2, but that they did not form well defined pores $^{266}$. Instead, they appear to act more like cell penetrating peptides, most likely targeting other cell components. The specific aims of Chapter 3 are individually addressed:

1. Determine the structure of peptides 73 and 77 to verify whether the helical structure is preserved.
Solution CD and NMR spectroscopy results demonstrate that the analogue peptides adopt α-helical structures in the presence of TFE or liposomes. However, both peptides are not continuous α-helices and have a lower α-helicity compared to the parent peptide.

2. Determine whether peptides 73 and 77 insert into the membrane bilayer.

3. Determine whether peptides 73 and 77 can cause leakage and whether it is selective for specific ions (e.g. potassium versus sodium), as was previously found for aurein 2.2.

Oriented CD spectroscopy, pyranine assay and DiSC35 assay results indicate that peptides 73 and 77 do not show membrane insertion behavior or the ability to form well-defined pores like aurein 2.2. In addition, both analogue peptides demonstrate less membrane perturbation and cause no selective ion leakage in contrast to aurein 2.2.

4. If the mechanism of action does not involve membrane perturbation, to explore alternative MOAs.

As both analogue peptides have higher antimicrobial activity and are more bactericidal compared to aurein 2.2, as well as the fact that they do not cause membrane damage, alternative MOAs such as whether the peptides function via a DNA binding interaction. As the agarose gel electrophoresis results show, it would appear that peptides 73 and 77 do not interact strongly with DNA. However, this does not rule out the fact that they may still interfere with the DNA synthesis process, therefore further experiments are required. In addition, other non-membrane targets should also be investigated to determine their antibacterial activity MOA.
In terms of the antibiofilm function of the peptide analogues, the data presented in Chapter 4 suggested that peptide 73 functions by interacting with the alarmone ppGpp and the nucleotide ATP. Whether this interaction translates into a direct interference in the stringent response remains to be shown. The specific aims of Chapter 4 are individually addressed:

1. Determine whether peptides 73 and 77 co-precipitate preferentially with ppGpp versus other nucleotides.

Unlike IDR-1018, no preferential binding activity to ppGpp was observed for both analogue peptides based on the results from co-precipitation assay. Interestingly, the results suggest a preferential interaction with the adenine nucleotide ATP instead.

2. If this interaction is confirmed, then I will explore whether this interaction is driven by electrostatic interactions.

3. If this interaction is not confirmed, then I will explore alternative MOAs, e.g. membrane disruption (Figure 1.4). Since in Section 1.6.1. I hypothesize that the peptides might interact with membranes effectively, then this could be a plausible alternative MOA.

The membrane disruptive antibiofilm MOA was not investigated, as the peptide analogues did not show membrane disruptive activity in Chapter 3. Instead, the peptide–nucleotide binding interaction was examined. The data indicate a weak and non-specific binding interaction between peptide 73 and the tested nucleotides. Peptide 73 was also found to bind with nucleotides ppGpp and ATP readily. In addition, the results show that the co-precipitation assay should not be used on its own to determine preferential binding between peptide and nucleotides. Other techniques such as $^{31}$P-NMR, in vitro biofilm data and animal model studies should be used in conjunction.
Overall, peptides 73 and 77 represent two new synthetic HDPs with both antibacterial and antibiofilm function. In order to compare the antimicrobial function of the analogue peptides to other similar HDPs, various HDPs containing more than one antimicrobial function was reviewed. Four other similar HDPs have been studied in equal detail as peptides 73 and 77. They are described in the following section.

5.2 HDPs with More than One Function

Cathelicidin LL-37 was one of the first peptides found to have antibiofilm function. Since then, three other HDPs, namely IDR–1018, DJK-5 and DJK-6\textsuperscript{265}, were developed. Some of their MOA are described below.

5.2.1 LL-37

Cathelicidin LL-37 is a 37 residue long cationic α-helical peptide, it is part of the human innate immune defence system\textsuperscript{95}. LL-37 has antibiofilm activity, immunomodulatory activity as well as weak antimicrobial activity\textsuperscript{95}. At concentrations much lower than required for its antimicrobial effect, the peptide shows inhibitory activity against biofilm formation and disperses preformed biofilms of \textit{Pseudomonas aeruginosa} (\textit{P. aeruginosa}). The LL-37 mechanism of action includes: 1) reduction in the initial attachment of \textit{P. aeruginosa} cells to surfaces, 2) increases in cell twitching motility and 3) downregulation of genes that are involved in bacterial quorum-sensing. Additional studies demonstrated that LL-37 has antibiofilm activity against \textit{S. aureus}, \textit{Escherichia coli} (\textit{E. coli}) as well as methicillin-resistant \textit{S. aureus} (MRSA) biofilms\textsuperscript{54,267}. 
5.2.2 IDR-1018

IDR-1018 (VRLIVAVRIWRR-NH2) is a synthetic peptide with antimicrobial, immunomodulatory and broad spectrum antibiofilm activity. Its sequence is derived from the peptide Bac2a (RLARIVVIRVAR-NH2), which is a modified version of the natural bovine peptide bactenecin. It has antimicrobial activity against Gram-positive and Gram-negative bacteria, with minimum inhibitory concentrations (MICs) of 5 µg/mL against S. aureus and 19 µg/mL against P. aeruginosa. IDR-1018 is unstructured in water but adopts an α-helical structure in DPC micelles (mammalian model membrane). However, IDR-1018 adopts a β-turn conformation in SDS and 1:1 POPC/POPG (bacterial model membrane).

Differential scanning calorimetry (DSC) experiments using DMPC, DMPC/DMPG and DiPoPE lipid showed no significant membrane perturbation caused by IDR-1018. Hence it was concluded that IDR-1018 does not function via membrane perturbation, but rather mechanisms such as membrane translocation to reach intracellular targets, interference with cell wall biosynthesis, cellular division, energy transduction, or active transport.

IDR-1018 has broad spectrum antibiofilm activity against P. aeruginosa, E. coli, Acinetobacter baumannii, Klebsiella pneumoniae, MRSA, Salmonella Typhimurium and Burkholderia cenocepania at concentrations lower than its MIC value. Its antibiofilm activity includes biofilm inhibition, eradication of preformed biofilms as well as biofilm dispersal promotion. The minimum concentration of IDR-1018 required to inhibit 50% of biofilm biomass (MBIC) and
100% of biomass (MBIC\textsubscript{100}) compared to the growth control are given in Table 5.1. As discussed elsewhere in the thesis, its MOA involves the ability of 1018 to bind to (p)ppGpp\textsuperscript{93}.

Table 5.1 IDR-1018 minimum inhibitory concentrations against planktonic and biofilm state bacteria. Minimum biofilm eradication concentration (MBEC) against MRSA is also provided, however the exact percentage of eradication extent is unknown\textsuperscript{93,269}.

<table>
<thead>
<tr>
<th>Bacterial strains</th>
<th>MIC (µg/mL)</th>
<th>MBIC\textsubscript{50} (µg/mL)</th>
<th>MBIC\textsubscript{100} (µg/mL)</th>
<th>MBEC (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. aeruginosa</em> PA01</td>
<td>64</td>
<td>5</td>
<td>10</td>
<td>/</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> PA14</td>
<td>64</td>
<td>5</td>
<td>10</td>
<td>/</td>
</tr>
<tr>
<td><em>S. aureus</em> MRSA #SAP0017</td>
<td>64</td>
<td>2</td>
<td>2.5</td>
<td>2.5</td>
</tr>
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</table>

In addition, IDR-1018 has potent immunomodulatory activity: it reduces pro-inflammatory response and promotes wound healing\textsuperscript{92}. In addition, it shows synergistic effects with conventional antibiotics to prevent and eradicate existing biofilms\textsuperscript{248}. Furthermore, the peptide shows low toxicity from *in vitro* and *in vivo* experimental results, as well as rapid distribution in blood, liver, brain and spleen, reaching steady state concentrations\textsuperscript{268}.

### 5.2.3 DJK-5

DJK-5 (VQWRAIRRVIR) is a broad-spectrum antibiofilm peptide composed of D-amino acids. It is active against Gram positive MRSA and all Gram-negative bacteria of the ESKAPE pathogens\textsuperscript{126,270}. The antibiofilm activity of DJK-5 includes biofilm inhibition, biofilm eradication as well as biofilm dispersal\textsuperscript{270}.
Table 5.2 Antimicrobial and antibiofilm activities of DJK-5\textsuperscript{84,270}.
*Results from\textsuperscript{126}, MBEC value against MRSA#SAP0017 represents 90% eradication of biofilm\textsuperscript{126}, however the exact percentage of MBEC against\textit{P. aeruginosa} is unknown.

<table>
<thead>
<tr>
<th>Bacterial strains</th>
<th>MIC (µg/mL)</th>
<th>MBIC\textsubscript{50} (µg/mL)</th>
<th>MBIC\textsubscript{100} (µg/mL)</th>
<th>MBEC (µg/mL)</th>
</tr>
</thead>
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<tr>
<td>\textit{P. aeruginosa} PA14</td>
<td>16</td>
<td>1</td>
<td>10 **</td>
<td>2.5</td>
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<tr>
<td>\textit{E. coli 0157}</td>
<td>1.6</td>
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<td>/</td>
<td>/</td>
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<td>\textit{A. baumannii}</td>
<td>8</td>
<td>4</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td>\textit{K. Pneumoniae}</td>
<td>3.2</td>
<td>1.6</td>
<td>10 ***</td>
<td>/</td>
</tr>
<tr>
<td>\textit{S. enterica}</td>
<td>3.2</td>
<td>0.8</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td>S. aureus MRSA #SAP0017 *</td>
<td>/</td>
<td>5</td>
<td>40</td>
<td>2.5</td>
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<tr>
<td>MRSA USA300 *</td>
<td>32</td>
<td>/</td>
<td>/</td>
<td>/</td>
</tr>
</tbody>
</table>

** 10 µg/mL for MBIC\textsubscript{90.7} ***10 µg/mL for MBIC\textsubscript{99.8}

At high concentration (128 µg/mL), DJK-5 causes significant propidium iodide uptake by MRSA, indicating bacterial membrane perturbation at this concentration\textsuperscript{126}. In comparison to its antibiofilm activity, the antibacterial activity of DJK-5 is weaker (Table 5.2)\textsuperscript{126}. In addition, its antibiofilm mechanism of action is similar to IDR-1018, involving binding to intracellular nucleotides (p)ppGpp and causing their degradation, which consequently prevents biofilm formation and eradicates mature biofilms in Gram-negative bacteria such as \textit{P. aeruginosa}, as well as Gram positive bacteria such as \textit{S. aureus}\textsuperscript{126,270}.

DJK-5 is protease-resistant and demonstrates activity both \textit{in vitro} and \textit{in vivo}. The peptide protects invertebrate organisms from \textit{P. aeruginosa} infections and was shown to be non-toxic\textsuperscript{270}. In addition, DJK-5 was able to decrease the cytotoxicity of MRSA(USA300) towards eukaryotic cells\textsuperscript{126}. 

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5.3 Distinct MOAs or not? Future directions

As seen from the data available in the literature and the results presented in this thesis, the MOAs of the HDPs studied with more than one function are very diverse. In fact, one could argue that the only common feature that can be seen from the findings summarized in Section 5.2 is that all of these HDPs have MBICs that are lower than MICs. In other words, more research is required to fully flesh out the relationship between HDP function and mechanism of action.

For peptides 73 and 77, additional work is also required to better understand their mechanism of action. In terms of their antibacterial function, it would be important to determine what the intracellular targets are. Given that these peptides are bactericidal, it is highly likely that they interfere with proteins involved in cell wall biosynthesis. Assays to test accumulation of cell wall precursors or inhibition of enzymes could be used \(^{271,272}\). Whole-cell NMR experiments could also help with quantifying the change in cell wall composition in bacterial cells, thereby allowing to determine a detailed MOA of these analogues \(^{273}\). In fact, a collaboration has begun with the laboratory of Dr. Tanja Schneider, University of Bonn, Germany, to determine whether the peptides impact cell wall biogenesis. Preliminary data suggests peptides 73 and 77 demonstrate inhibitory activity in cell wall biosynthesis, particularly by interfering with the peptidoglycan biosynthesis process. Specifically, both peptides induced an accumulation of the soluble cell wall precursor UDP-MurNAc-pentapeptide, suggesting an inhibition of a later membrane associated step in cell wall biosynthesis. Additionally, both peptides were found to inhibit the MraY reaction at a concentration of 400µM. Both processes are essential in the formation of Lipid I and then Lipid II, which is eventually polymerized into the growing peptidoglycan network \(^{274,275}\). Although I anticipated more preliminary results by the time of thesis submission, due to
the collaborative nature of this experiment and world events, I have opted not to include it in this thesis. Nevertheless, results from this experiment should lead to a publication.

In terms of their antibiofilm function, further studies to determine the impact of ATP and GTP sequestration in bacteria and whether peptide 73 and peptide 77 play a direct role in the stringent response are required. One potential way to do this is to adapt the method described by Kinniment and Wimpenny to *S. aureus* biofilms, in order to determine the adenine nucleotide pool and the stored potential energy, which indicates the energy status of living biofilm cells\(^{276}\). Specifically, the biofilm could be generated with a constant-depth film fermentor, and then frozen and sectioned to determine the adenylate pool variation as a function of biofilm depth\(^{276}\). Experiments will be designed and tested in collaboration with Dr. Haney in the Hancock lab at UBC. Furthermore, antibiofilm targets other than the stringent response could also be investigated, such as the quorum sensing pathway. Quorum sensing regulates the expression of various genes in bacteria including those that encode biofilm formation and virulence factors\(^{277}\). One way to determine the interference of analogue peptides in quorum sensing mechanism is to measure the reduction of phenol soluble modulins (PSMs) production, which are biosurfactants critical in the biofilm structuring and channel formation process\(^{126}\).

Finally, the ultimate goal of my research is to better understand the HDP landscape so that ultimately select HDPs are identified and used in the fight against AMR in a timely manner. The work presented in this thesis demonstrates the multifaceted nature of HDPs and the importance of considering the various functions in HDP research. The research findings could provide
guidelines towards future HDP design as well as their development into therapeutic alternatives to combat AMR.
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Appendices

Appendix A

A.1 List of peptides generated by Dr. Prashant Kumar and my research supervisor, as well as some of their properties.

<table>
<thead>
<tr>
<th>Peptide # and sequence&lt;sup&gt;a&lt;/sup&gt;</th>
<th>MIC&lt;sup&gt;b&lt;/sup&gt;</th>
<th>net charge</th>
<th>mean hydrophobic moment&lt;sup&gt;b&lt;/sup&gt;</th>
<th>cation-π interaction&lt;sup&gt;c&lt;/sup&gt;</th>
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a All peptides have amidated C-termini  
b Calculated using http://heliquest.ipmc.cnrs.fr/cgi-bin/ComputParams.py  
c Assuming the peptides are α-helical; Number of WxxxR or RxxW motifs