Structure-function studies of peptide fragments derived from a defensin of the tick *Ornithodoros savignyi* Audouin (1827)

By

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Magister Scientiae Biochemistry

In the Faculty of Natural and Agricultural Sciences

University of Pretoria

Pretoria

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Acknowledgements

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To our Father in Heaven for without Him I would not be where I am today, Philippians 4:13:
“I can do all things through Him who strengthens me”.

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Overuse of conventional antibiotics has led to increased multidrug resistant micro-organisms. Therefore, development of alternative drugs with new mechanisms of action in the control of resistant micro-organisms is urgently needed. Defensins, one of the larger groups of naturally occurring antimicrobial peptides (AMPs), found in a variety of species, may serve as templates for the development of novel therapeutic agents. The work completed in this study is based on an antimicrobial peptide (AMP), Os, derived from the C-terminus of a tick *Ornithodoros savignyi* defensin isoform 2 (OsDef2). OsDef2 was found to be active against Gram-positive bacteria only, whereas Os, showed bactericidal activity towards both Gram-positive and Gram-negative bacteria. In this study a series of synthetic shorter peptides, based on the sequence of Os, was utilised in order to determine whether shorter peptides would retain their antibacterial activity and selectivity. Initial screening indicated that only two fragments, Os(3-12) and Os(11-22), were active towards the tested Gram-negative and Gram-positive bacteria. The minimum bactericidal concentrations (MBCs) of the two fragments were determined and ranged from 30 µg/ml to 120 µg/ml. The MBCs of the parent peptide, Os (1.88 to 15 µg/ml), was considerably lower than that of Os(3-12) and Os(11-22). As previously observed for Os, neither of the peptides showed cytotoxic effects towards eukaryotic cells. The amidated analogue of one of the active peptides, Os(11-22)NH₂, was further evaluated in terms of its secondary structure, antibacterial and antioxidant activities as well as cytotoxicity. Amidation increased the activity of Os(11-22) 16 fold towards *B. subtilis* (MBC of 1.88 µg/ml) and 32 fold towards both *Escherichia coli* and *Pseudomonas aeruginosa* (MBC of 3.75 µg/ml), whereas a 2 fold decrease in activity was observed against *Staphylococcus aureus* (MBC of 60 µg/ml). Circular dichroism data showed that amidation altered the secondary structure of Os(11-22) from α-helical to mostly random coiled. In the presence of 30% serum the activity of Os(11-22)NH₂ unexpectedly increased 8 fold against *S. aureus* (MBC of 7.5 µg/ml), but decreased 32 fold against *E. coli* (MBC of 120 µg/ml). The activity of Os(11-22)NH₂ in 100 mM NaCl decreased 4 fold against *E. coli* (MBC of 15 µg/ml), but was completely lost (MBC >120 µg/ml) against *S. aureus*. The kinetics of bactericidal activity indicated that Os(11-22)NH₂ killed *B. subtilis* and *E. coli* within 30 min and 120 min, respectively, whereas Os killed both bacteria within 5 min. Even at high concentrations Os(11-22)NH₂ was non-toxic towards human erythrocytes and SC-1 cells.
moreover an increase in SC-1 cell number was observed at 120 μg/ml. The peptide showed strong antioxidant activity and was found to be 4 fold more active than glutathione (GSH), however Os was 3.4 fold more antioxidative than Os(11-22)NH₂. Os(11-22)NH₂ can be considered a dual functional peptide, since it possesses both antibacterial and antioxidant activity. The amidated peptide has the potential for use against the damaging effects of oxidative stress associated with infectious diseases and recovery of chronic wounds. Further investigation into structure-function properties of Os(11-22)NH₂ is necessary.
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<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;H&gt;</td>
<td>Hydrophobicity</td>
</tr>
<tr>
<td>&lt;μH&gt;</td>
<td>Hydrophobic moment</td>
</tr>
</tbody>
</table>
| A            | AAPH  
2, 2’-azobis (2-amidino-propane) dihydrochloride  
AMP  
Antimicrobial peptide  
AMPs  
Antimicrobial peptides  
AUC  
Area under curve |
| C            | Caco-2  
Epithelial colonic tumor cells  
CD  
Circular dichroism  
CFU  
Colony forming units  
C-terminal  
Carboxy terminal  
CV  
Crystal Violet |
| D            | Da  
Dalton  
DCHF-DA  
2,7-dichlorodihydrofluorescein diacetate  
DPPH  
2,2-diphenyl-L-picrylhydrazyl  
DTT  
Dithiothreitol |
| F            | FL  
Fluorescein 3’,6’-dihydroxyspiro[isobenzofuran-1[3H], 9’[9H]-xanthen]-3-one |
| G            | GSH  
Glutathione |
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBD</td>
<td>Human beta-defensin</td>
</tr>
<tr>
<td>HNP</td>
<td>Human neutrophil peptide</td>
</tr>
<tr>
<td>HNP-1</td>
<td>Human neutrophil peptide-1</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>Iso-PBS</td>
<td>Isotonic phosphate buffered saline</td>
</tr>
<tr>
<td>kDa</td>
<td>kiloDaltons</td>
</tr>
<tr>
<td>LAL</td>
<td>Limulus ambocyte assay</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Bertani</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MBC</td>
<td>Minimum bactericidal concentration</td>
</tr>
<tr>
<td>MBCs</td>
<td>Minimum bactericidal concentrations</td>
</tr>
<tr>
<td>MIC</td>
<td>Minimum inhibitory concentration</td>
</tr>
<tr>
<td>MRE</td>
<td>Mean residue ellipticity</td>
</tr>
<tr>
<td>MRSA</td>
<td>Methicillin resistant <em>Staphylococcus aureus</em></td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>N-terminal</td>
<td>Amino-terminal</td>
</tr>
<tr>
<td>OD&lt;sub&gt;600&lt;/sub&gt;</td>
<td>Optical density measured at 600 nm</td>
</tr>
<tr>
<td>OMΔC</td>
<td><em>Ornithodoros moubata</em> defensin carboxy-terminal synthetic analogue</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>ORAC</td>
<td>Oxygen radical absorbance capacity</td>
</tr>
<tr>
<td>Os(11-22)</td>
<td>Synthetic analogue of Os, containing the last twelve amino acids of the carboxy-terminal of Os</td>
</tr>
<tr>
<td>Os(11-22)NH₂</td>
<td>Os(11-22) amidated at the carboxy-terminal</td>
</tr>
<tr>
<td>Os(3-12)</td>
<td>Synthetic analogue of Os, containing amino acids three to twelve of the amino-terminal of Os</td>
</tr>
<tr>
<td>Os</td>
<td>Last 22 C-terminal amino acids of <em>Ornitodoros savygnyi</em> defensin isoform 2 containing cysteine residues</td>
</tr>
<tr>
<td>Os-C</td>
<td>Analogue of Os lacking cysteine residues</td>
</tr>
<tr>
<td>OsDef1</td>
<td><em>Ornitodoros savygnyi</em> defensin isoform 1</td>
</tr>
<tr>
<td>OsDef2</td>
<td><em>Ornitodoros savygnyi</em> defensin isoform 2</td>
</tr>
<tr>
<td>P</td>
<td>Synthetic analogue of longicin</td>
</tr>
<tr>
<td>P4</td>
<td>Synthetic analogue of longicin</td>
</tr>
<tr>
<td>PAΔC</td>
<td><em>Pyrrhocoris apterus</em> defensin C-terminal synthetic analogue</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>RP-HPLC</td>
<td>Reverse-phase high-performance liquid chromatography</td>
</tr>
<tr>
<td>S</td>
<td>Embryonic mouse fibroblasts cells</td>
</tr>
<tr>
<td>SC-1</td>
<td>Embryonic mouse fibroblasts cells</td>
</tr>
<tr>
<td>SCΔC</td>
<td><em>Stomoxys calcitrans</em> defensin C-terminal synthetic analogue</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of mean</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SPB</td>
<td>Sodium phosphate buffer</td>
</tr>
<tr>
<td>T</td>
<td>Trolox equivalents</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic acid</td>
</tr>
</tbody>
</table>
1.1 Problem statement
Infectious diseases are one of the leading causes of death worldwide (Lozano et al., 2012). According to World Health Organization data, approximately 13 million deaths related to infectious diseases are reported annually. Infectious diseases cause approximately 45% of all deaths in developing countries (Allahverdiyev et al., 2013). The extensive use of conventional antibiotics has caused a growing number of multidrug resistant micro-organisms to emerge and spread rapidly in the environment (Levy, 2002; Chrudimska et al., 2011). This has led to strains of important human pathogens such as Mycobacterium tuberculosis, Staphylococcus aureus, Pseudomonas aeruginosa, Acinetobacter baumannii and Enterobacteriaceae to become resistant against the most important classes of antimicrobials used to treat infectious diseases (Giuliani et al., 2007; Aoki & Ueda, 2013).

If pathogens have the opportunity to contaminate wounds, wound healing can be significantly impaired leading to increased exposure to blood-borne pathogens and serious consequences for human health (Beltrami et al., 2000; Bowler et al., 2001). Approximately 1 to 2% of the population in developed countries will experience a chronic wound, related to antibacterial infections in their lifetime (Kirketerp-Møller et al., 2008). The most common bacterial isolates from wounds include S. aureus, Streptococcus spp., Enterococcus spp., Escherichia coli, P. aeruginosa, and Enterobacter spp. (Halbert et al., 1992; Pathare et al., 1998; Revathi et al., 1998).

As a result, investigation into the development of new antimicrobial drugs is urgently needed. Naturally occurring antimicrobial peptides (AMPs) are promising candidates in the search for novel therapeutic agents. They possess attractive properties in comparison to conventional antibiotics, including limited ability to induce resistance in pathogens, broad spectrum of antimicrobial activity and rapid killing of pathogens. Despite these attributes, some drawbacks associated with AMPs are cytotoxicity to eukaryotic cells, limited efficacy under physiological conditions and high cost of synthesis. Understanding the structure-activity relationship of AMPs and mechanisms of bacterial killing is necessary for the design of peptides with improved pharmaceutical efficacy (Hancock & Lehrer, 1998).
1.2 Antimicrobial peptides as novel therapeutic agents

AMPs are a diverse group of molecules produced by all types of living organisms, ranging from prokaryotes, insects, plants, amphibians, birds, fish, mammals and humans that form part of the host innate immune defence of these organisms (Giuliani et al., 2007). These peptides demonstrate powerful antimicrobial activity against a broad range of microbes, including viruses, Gram-positive and Gram-negative bacteria, protozoa, and fungi, whereas antibiotics and antifungal drugs are pathogen type specific and are only selective for bacteria and fungi, respectively (Hancock & Lehrer, 1998; Hancock, 2001). In addition, these molecules have been recorded to have activity against highly resistant bacteria such as Gram-negative P. aeruginosa and Stenotrophomonas maltophilia and Gram-positive methicillin resistant S. aureus (MRSA) (Wu et al., 1999; Zhang et al., 2000; Hancock, 2001). A notable difference in the modes of action between AMPs and antibiotics is that AMPs are not easily affected by antibiotic-resistance mechanisms, which often limits the use of conventional antibiotics (Jenssen et al., 2006). As a result, cationic AMPs have been identified as promising therapeutic agents as these peptides are specifically attracted to the negative charge of bacterial membranes. The main focus of antimicrobial peptide (AMP) research has been on cationic peptides (Hancock & Lehrer, 1998; Oren & Shai, 1998; Wu et al., 1999; Hancock, 2001; Giuliani et al., 2007; Jiang et al., 2008).

Although anionic AMPs exist, the majority is cationic with an overall positive charge ranging from +2 to +9, provided by excess arginine and lysine residues. Cationic AMPs are generally defined as small (less than 50 amino acids), amphipathic (both hydrophobic and hydrophilic properties) molecules containing a substantial portion of hydrophobic residues (≥30% or more) (Hancock, 2001; Giuliani et al., 2007). AMPs are diverse in their sequence and structure and have the ability to fold into amphipathic conformations. This is induced by interactions with membranes or under membrane-mimicking conditions, allowing positively charged hydrophilic domains to be separated from the hydrophobic domains (Hancock, 2001). The amphipathic structure of AMPs as well as the overall positive charge, therefore allow them to interact with bacterial cell membranes (Auvynet & Rosenstein, 2009).

It is difficult to classify cationic AMPs due to their large diversity. According to one such classification, they can be categorized into four classes according to their secondary structure. These classes include, mixed structures containing both β-sheets and α-helices, stabilized by disulfide bonds (Fig. 1.1 A), loops stabilized by disulfide bonds (Fig. 1.1 B), amphipathic α-helices (Fig. 1.1 C) and extended peptides (Fig. 1.1 D) (Hancock, 2001). The structural
conformation of these AMPs usually form upon phospholipid membrane interaction or in the presence of structure promoting solvents (Hancock & Lehrer, 1998). The β-sheet and α-helical cationic peptides are the most commonly found AMPs in nature (Hancock, 2001).

Based on their amino acid composition, cationic AMPs can also be divided into three subgroups (Brogden, 2005). Subgroup one contains a large group of short (less than 40 amino acids) α-helical peptides lacking cysteine residues (Fig. 1.1 C) and often with a hinge region in the middle. The second subgroup is comprised of peptides enriched with specific amino acids, such as proline or arginine. The third subgroup includes β-sheet peptides stabilised by disulfide bonds (Fig. 1.1 A and B). Table 1.1 lists the three subgroups, together with examples of each AMP and their origins.
Table 1.1: Characteristics and origins of various cationic AMPs based on amino acid composition (Brogden, 2005; Lazarev & Govoron, 2010)

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Name</th>
<th>Origin</th>
</tr>
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<tbody>
<tr>
<td>α-Helices lacking cysteine residues</td>
<td>Cecropins</td>
<td>Silk moth</td>
</tr>
<tr>
<td></td>
<td>Moricin</td>
<td>Silk moth</td>
</tr>
<tr>
<td></td>
<td>Andropin</td>
<td>Fruit fly</td>
</tr>
<tr>
<td></td>
<td>Ceratotoxin</td>
<td>Fruit fly</td>
</tr>
<tr>
<td></td>
<td>Melittin</td>
<td>Bee</td>
</tr>
<tr>
<td></td>
<td>Magainin</td>
<td>Frog</td>
</tr>
<tr>
<td></td>
<td>Dermaseptin</td>
<td>Frog</td>
</tr>
<tr>
<td></td>
<td>Bombinin</td>
<td>Toad</td>
</tr>
<tr>
<td></td>
<td>Brevinin-1</td>
<td>Frog</td>
</tr>
<tr>
<td></td>
<td>Buforin II</td>
<td>Toad</td>
</tr>
<tr>
<td></td>
<td>Esculentins</td>
<td>Frog</td>
</tr>
<tr>
<td></td>
<td>Pleurocidin</td>
<td>Winter flounder</td>
</tr>
<tr>
<td></td>
<td>Seminalplasmin</td>
<td>Bovine</td>
</tr>
<tr>
<td></td>
<td>CAP18</td>
<td>Rabbits</td>
</tr>
<tr>
<td></td>
<td>LL37</td>
<td>Humans</td>
</tr>
<tr>
<td>Enriched with specific amino acids but excluding cysteines</td>
<td>Pro</td>
<td>Abaecin</td>
</tr>
<tr>
<td></td>
<td>Pro and Arg</td>
<td>Apidaecin</td>
</tr>
<tr>
<td></td>
<td>Gly and Pro</td>
<td>Pyrrhocoricin</td>
</tr>
<tr>
<td></td>
<td>Pro and Phe</td>
<td>Coleoptericin</td>
</tr>
<tr>
<td></td>
<td>Trp</td>
<td>Prophenin</td>
</tr>
<tr>
<td></td>
<td>His</td>
<td>Indolicidin</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Histatins</td>
</tr>
</tbody>
</table>

**1 disulfide bond:**
- Bactenecin: Bovine
- Ranalexin: Rana frog

**2 disulfide bonds:**
- Protegrin: Pig
- Tachyplesin: Horseshoe crab

**3 disulfide bonds:**
- α-defensins: Humans
- β-defensins: Bovine
- Sapecin A: Insect

**More than 3 disulfide bonds:**
- Drosomycin: Fruit flies
- Defensins: Plants (Radish)
According to The Antimicrobial Peptide Database (http://aps.unmc.edu/AP/main.php, accessed on 15 October 2013), established and maintained by the researchers at the University of Nebraska Medical Center in Omaha, a total of 2295 AMPs with numerous different sequences have been identified from a wide range of organisms (Wang et al., 2009).

In addition to broad spectrum antimicrobial activity a few cationic AMPs have been found to have other beneficial properties. These properties include immunomodulatory and antioxidant activity, as well as stimulation and proliferation of epithelial cells potentially aiding in wound healing (Niyonsaba et al., 2007; Lu et al., 2010b; Winter & Wenghoefer, 2012). AMPs from ranid frogs have been reported to possess antioxidant activity in addition to antimicrobial activity (Lu et al., 2010b; Zhang et al., 2012). One study described the investigation of antioxidant activity of three AMPs isolated from the skin secretion of the Xizang plateau frog, Nanorana parkeri, captured in Lhasa, Tibet, China (Lu et al., 2010b). The latter evaluated the antioxidative capacities of the three frog AMPs in terms of free radical (2,2-diphenyl-L-picrylhydrazyl; DPPH) scavenging activity (von Gadow et al., 1997). All three peptides showed DPPH scavenging activity, suggesting that these multi-functional AMPs could play an essential role in defending N. parkeri against environmental oxidative stress and pathogenic micro-organisms. A study performed by Huang et al. (2012) showed that sweet potato defensin (SPD-1) possessed antioxidant activity in both in vitro and ex vivo experiments. Human neutrophil peptides (HNP) as well as human beta-defensins (hBD) have been shown to be associated with the process of wound healing by promoting cell proliferation and migration (Baroni et al., 2009; Han et al., 2009), however the antioxidant activity of these peptides has not been investigated.

Antioxidants are essential in reducing oxidative stress caused by free radicals, such as reactive oxygen species (ROS; superoxide and hydrogen peroxide). Although normally formed as by-products of metabolism, overproduction of free radicals is one of the main causes of damage to cell membranes, DNA and proteins (Birnboim, 1986; Cross et al., 1987; Uttara et al., 2009). ROS also play a role in degenerative and pathological processes such as aging, inflammation, cancer, Alzheimer’s disease, atherosclerosis and heart disease (Rosenfeld, 1998; Ashok & Ali, 1999; Hecht, 1999; Wettasinghe et al., 2000). ROS cause damage by scavenging the body to actively accept or donate electrons. Antioxidants are capable of neutralizing free radicals by donating one of their own electrons, thereby ending the electron scavenging reaction and ultimately promote cellular recovery and generation.
Therefore, antioxidants are thought to be valuable in the wound healing process by controlling wound oxidative stress. The typical process of wound healing depends on low levels of ROS and oxidative stress, whereas an overexposure can lead to impeded wound healing (Sen, 2003; Fitzmaurice et al., 2011). In addition, antioxidants are also beneficial in the prevention of human diseases such as cancer, cardiovascular disease and neurodegenerative diseases (Young & Woodside, 2001). AMPs are also known to improve and promote wound healing by controlling pathogens that cause infection, as well as stimulate the production and migration of epithelial cells (Jacobsen et al., 2005; Winter & Wenghoefer, 2012).

1.3 Mechanism of AMP action
The mode of action of AMPs has mostly focused on the interaction of cationic amphipathic helical peptides with model bacterial membrane systems, such as liposomes. Microscopic analyses revealed that different AMPs have different cellular targets and mechanisms of action (Brogden, 2005). Initially all AMPs interact with the microbial cytoplasmic membrane by electrostatic interaction (Hancock & Rozek, 2002). Generally AMPs act by permeabilizing the cell membrane resulting in pore formation and leakage of cellular contents causing cell death (Oren & Shai, 1998; Giuliani et al., 2007). Peptides can also act by targeting intracellular components after transportation across the membrane without the formation of stable pores or they can act by forming pores together with targeting intracellular components (Jenssen et al., 2006; Lazarev & Govoron, 2010). The mechanism of membrane permeabilization depends on various factors such as the amino acid composition, amphipathicity and concentration of the AMP as well as microbial lipid membrane composition.

Important differences exist between prokaryotic and eukaryotic cells that represent targets for AMPs (Zasloff, 1987). Bacteria are composed of a peptidoglycan cell wall and cytoplasmic membrane, whereas eukaryotic cells have a plasma membrane. Bacterial membranes are negatively charged, due to the presence of anionic teichoic acids and lipopolysaccharide (LPS), as well as negatively charged membrane phospholipids such as, phosphatidylglycerol, phosphatidyserine and cardiolipin. In contrast, mammalian membranes are mostly composed of zwitterionic phospholipids, including phosphatidylcholine, sphingomyelin and phosphatidylethanolamine (Giuliani et al., 2007). The different composition of cell membranes determines whether AMPs target microbial or host membranes.

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1.3.1 Gram-positive and Gram-negative membrane interaction with AMPs

Differences between Gram-positive and Gram-negative bacterial membranes also exist, causing different bactericidal concentrations and killing rates of AMPs. In the case of Gram-positive bacteria (Fig. 1.2 A), which lack an outer membrane, AMPs target the negatively charged lipoteichoic acids and amino acid carboxyl groups present on the outer surface of the peptidoglycan layer that is thicker compared to Gram-negative bacteria (Dennison et al., 2005). AMPs then pass through the peptidoglycan layer after which they aggregate and insert into the cytoplasmic membrane causing an overall disruptive effect (Tossi et al., 2000). It is suggested that AMPs are unstructured in aqueous environments but take on functional conformations upon interaction with the outer surface of the cytoplasmic membrane of bacteria (Tossi et al., 2000; Devine, 2003).

In the case of Gram-negative bacteria (Fig. 1.2 B), peptides must first traverse through the negatively charged outer membrane driven by hydrophobic interactions. The initial interaction of peptides with the Gram-negative bacterial outer membrane occurs through electrostatic interactions between the cationic peptide and the negatively charged lipopolysaccharides, which is stabilized by divalent cations such as Mg$^{2+}$ and Ca$^{2+}$. Cationic AMPs, being bulkier than cations, displace these ions and translocate across the outer membrane (Hancock & Patrzykat, 2002). The amphipathic characteristics of AMPs allow them to interact with negatively charged phospholipid head groups and hydrophobic fatty acid chains of the cytoplasmic membrane, resulting in pore formation and leakage of cytosol components (Fig. 1.3) (Pathak et al., 1995; Hancock & Lehrer, 1998; Kondejewski et al., 1999; Giuliani et al., 2007).
Several models explaining membrane targeting as mode of action of AMPs currently exist. The barrel stave, carpet, toroidal and aggregate models (Fig. 1.3 A-D) are the most popular models described for pore formation, leading to membrane permeabilization (Jenssen et al., 2006). Alternative mechanisms of AMP action have been suggested that act by inhibiting or disturbing multiple intracellular targets such as, DNA, RNA and protein synthesis (Fig. 1.3 E-G) (Oren & Shai, 1998; Wu et al., 1999; Giuliani et al., 2007). Buforin II, pleurocidin, and dermaseptin have all been shown to inhibit DNA and RNA synthesis at their minimal inhibitory concentrations (MIC) without destabilizing the membrane (Park et al., 1998; Subbalakshmi & Sitaram, 1998; Patrzykat et al., 2002).
Figure 1.3: Proposed mechanisms of action for AMPs. Models to explain mechanisms of membrane permeabilization are indicated (A to D). The toroidal pore model (A), carpet model (B), barrel-stave model (C), and aggregate model (D). The mechanisms of action of peptides which do not act by permeabilizing the bacterial membrane are suggested by E to G. The mechanisms of action are further discussed in detail in the text. Peptides are shown in red and the lipid membrane is shown in green and grey (Jenssen et al., 2006; Giuliani et al., 2007; Li et al., 2012).

1.3.2 Membrane permeabilization by AMPs

The mode of action of individual peptides may differ due to concentration, charge and amphipathic nature, target cell and the physical properties of the particular bacterial membrane. AMPs can possibly use more than one mechanism of action, for instance destabilization of the cell membrane combined with inhibition of one or more intracellular targets (Jenssen et al., 2006). The amphipathicity of AMPs is a main feature for membrane interaction, as hydrophobic regions are necessary to interact with the lipid components of the membrane and hydrophilic regions are important for interaction with the phospholipid head groups and formation of the aqueous face of pores (Brogden, 2005). The pore forming ability of \( \alpha \)-helical AMPs can generally be explained by the models mentioned in Figure 1.3 A to D. The killing mechanisms used by \( \beta \)-sheet containing peptides, such as defensins have not been
studied in much detail. Although amphipathic folds can be adopted by β-sheeted AMPs, there is still little experimental evidence to indicate which of the following models is valid for defensins (Jenssen et al., 2006).

1.3.2.1 The toroidal pore model
In the toroidal pore model, peptides can induce defects in the bilayer and form a toroidal pore as shown in Figure 1.3 A. Aggregates of peptides insert perpendicularly to the membrane to form a pore, causing the membrane to curve inwards forming a pore with the phospholipid head groups facing towards the centre of the pore (Jenssen et al., 2006). The polar faces of the peptides associate with the polar head groups of the phospholipids, together lining the aqueous core. The pores created cause membrane depolarization and leakage of ions and metabolites, ultimately leading to cell death (Brogden, 2005). The activity of peptide magainin-2 is mediated by the formation of toroidal pores (Kim et al., 2009).

1.3.2.2 The carpet model
The carpet model suggests that aggregates of the peptide orient parallel to the membrane surface to form high density clusters (Pouny et al., 1992). Unlike the barrel-stave model, the peptide does not insert into the hydrophobic membrane core, but is electrostatically attracted to the anionic phospholipid head groups. Acting as detergent forming micelles, AMPs cover the surface in a carpet like manner, causing considerable strain on membrane integrity at high concentrations, resulting in collapse of structural integrity and loss of cytosol components (Fig. 1.3 B) (Jenssen et al., 2006; Giuliani et al., 2007). Some peptides, such as the human cathelicidin derived LL37 act by this mechanism (Dean et al., 2010).

1.3.2.3 The barrel stave model
A common transmembrane pore is represented by the barrel-stave model as seen in Figure 1.3 C (Ehrenstein & Lecar, 1977). This model describes the perpendicular insertion of transmembrane amphipathic α-helical or β-sheet peptides leading to the formation of cylindrical pores (Giuliani et al., 2007). Peptides become the “staves” in a “barrel”-shaped cluster. The hydrophobic surfaces of the peptides interact with the lipid core of the membrane, while the hydrophilic surfaces form the interior region of the pore (Jenssen et al., 2006; Giuliani et al., 2007). Cell death will then be as a result of leakage of cellular contents, membrane depolarization or disturbance of membrane function from lipid redistribution. Melittin, a peptide isolated from the venom of a European honey bee, *Apis mellifera*, acts by
forming pores in the bacterial membrane by means of the barrel stave method (Oren & Shai, 1998; van den Bogaart et al., 2008).

1.3.2.4 The aggregate model
The models mentioned above predict that the bactericidal activity of AMPs occurs due to membrane permeabilization alone. Additional mechanisms have been thought to play a role in bacterial cell death as membrane permeability alone may not always be sufficient (Ganz & Lehrer, 1995; Li et al., 2012). An additional model, the aggregate model, has been suggested (Fig 1.3 D) (Hancock & Chapple, 1999; Wu et al., 1999). This model describes both membrane permeabilization caused by membrane depolarization, and translocation of peptides across the bilayer to interact with internal targets. Membrane depolarization is caused by peptides inserting into the membrane, forming unstructured membrane spanning aggregates with micelle-like complexes composed of lipids and peptides (Jenssen et al., 2006). As peptide aggregates translocate and dissociate from the membrane, intracellular targets can be accessed to exert killing activities (Hancock & Chapple, 1999). This leads to formation of random sized pores as well as additional translocation of peptides across the membrane to act upon internal targets (Wu et al., 2010; Li et al., 2012). A membrane-active AMP, named maculatin-1.1, from an Australian tree frog acts by this mechanism (Bond et al., 2008).

1.3.3. Intracellular targets of AMPs
It has been shown that some AMPs also act on intracellular targets (Fig. 1.3 E to G) when translocated across the lipid bilayer and accumulated intracellularly, without causing membrane permeabilization (Park et al., 1998; Subbalakshmi & Sitaram, 1998; Patrzykat et al., 2002; Tew et al., 2002; Jenssen et al., 2006). Once inside these peptides can bind to DNA, RNA and proteins, causing inhibition of nucleic acid synthesis, protein synthesis, interference with metabolic processes and respiration as well as inhibiting important enzymes and cell wall synthesis (Brogden, 2005). A frog AMP, buforin II, translocates across the membrane, binding both DNA and RNA of E. coli (Park et al., 1998). Alpha-helical peptides derived from fish and isolated from frog skin have been shown to cause inhibition of DNA and RNA synthesis in E. coli (Subbalakshmi & Sitaram, 1998; Patrzykat et al., 2002). Inhibition of nucleic acid synthesis has also been shown for the β-sheet human defensin, human neutrophil peptide-1 (HNP-1) (Tew et al., 2002).
1.4 Therapeutic applications of AMPs

AMPs have several advantages over conventional antibiotics and these are listed in Table 1.2.

Table 1.2: Advantages of AMPs

<table>
<thead>
<tr>
<th>Advantage</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Probability of developing resistance against AMPs is limited due to different mechanism of antimicrobial action compared to conventional antibiotics</td>
<td>(Zasloff, 2002; Hancock &amp; Sahl, 2006)</td>
</tr>
<tr>
<td>Local application of the drug</td>
<td>(Lazarev &amp; Govoron, 2010)</td>
</tr>
<tr>
<td>Activity in the nanomolar concentration range</td>
<td>(Breukink et al., 1999)</td>
</tr>
<tr>
<td>Unlimited possibilities of chemical synthesis of AMP analogues with modified biological properties such as decreased toxicity for eukaryotic cells</td>
<td>(Lazarev &amp; Govoron, 2010)</td>
</tr>
<tr>
<td>Wider spectrum of antimicrobial action including bacteria, fungi, viruses and parasites</td>
<td>(Hancock &amp; Lehrer, 1998)</td>
</tr>
<tr>
<td>Can act synergistically with conventional antibiotics</td>
<td>(Gordon et al., 2005)</td>
</tr>
<tr>
<td>As a result of their positive charge, cationic AMPs are initially attracted to the cytoplasmic membrane of bacteria by electrostatic interactions, thereafter following their respective modes of action</td>
<td>(Jenssen et al., 2006)</td>
</tr>
<tr>
<td>Rapid bactericidal action, some within 5 min</td>
<td>(Hancock &amp; Rozek, 2002)</td>
</tr>
<tr>
<td>May possess additional advantageous properties such as immunomodulatory, anti-inflammatory and antioxidant activity</td>
<td>(Yeung et al., 2011)</td>
</tr>
<tr>
<td></td>
<td>(Huang et al., 2012)</td>
</tr>
</tbody>
</table>

AMPs have been developed for topical application, such as the magainin 2 analogue, Pexiganan (Lamb & Wiseman, 1998). This was the first AMP to undergo commercial development as an antibiotic cream for the topical treatment of diabetic foot ulcers. The main developer, Magainin Pharmaceuticals, had merged with SmithKline Beecham (now GlaxoSmithKline) to take the product through clinical trials. Unfortunately, after phase III clinical trials, FDA approval was denied (Zasloff, 2001) due to early development difficulties, late requirement to change clinical trial design, shifting commercial priorities of the pharmaceutical partner and declining resources (Fox, 2013). However, Pexiganan is now being reconsidered as Locilex, under the sponsorship of Dipexium Pharmaceuticals of White Plains, New York. This company is negotiating with the FDA to bring Pexiganan through clinical trials for the treatment of antibiotic resistant bacterial infections associated with diabetic foot ulcers (Fox, 2013).
The indolicidin-based peptide variants, MBI-266 and MBI-594AN, developed by Migenix (Vancouver, British Columbia, Canada) for the treatment of catheter related infections and acne, respectively, showed some potential (Gordon *et al.*, 2005). Preclinical studies involving MBI-594AN confirmed the *in vitro* efficacy against sensitive and resistant *Propionibacterium acnes* strains and topical application of MBI-594AN was found to be non-toxic in animal models. MBI-594AN significantly reduced inflammation and acne lesions compared with vehicle controls in a phase IIb trial in 2003 and were licensed to Cutanea Life Sciences in late 2005 (www.mbiotix.com, Press Release November 17, 2003).

Currently on the market, Cubicin (Daptomycin for injection), a lipopeptide antibacterial agent approved by the FDA in September 2003, was developed by Cubist Pharmaceuticals in Lexington, Massachusetts, USA (http://www.cubicin.com/, accessed 15 October 2013). Daptomycin is limited to Gram-positive bacteria including highly resistant MRSA, for the treatment of complicated skin and soft tissue infections.

Surotomycin an antibacterial lipopeptide, also discovered by Cubist Pharmaceuticals, reached phase III clinical trials in late 2012 as treatment for *Clostridium difficile* infections. Surotomycin compared favourably to the conventional antibiotic vancomycin. This drug candidate was granted qualified infectious disease product status by the FDA in December 2012 as well as fast-track status early in 2013 to increase the rate of development and review of this drug (Cubist Pharmaceutical Inc. Lexington, MA, USA. Surotomyxin (CB-315), http://www.cubist.com/products/cdad, accessed 15 October 2013). Additional examples of AMPs involved in pharmaceutical development and clinical trials are shown in Table 1.3.
Systemic applications have previously been considered for AMPs, but there are some limitations associated with this, including cost-effectiveness, AMP susceptibility to proteases and toxicity towards mammalian cells. In addition to direct bacterial killing, AMPs possessing antioxidant activity may promote wound healing by means of advancing optimal cellular recovery and functioning by reducing the amount of free radicals (Ramos et al., 2011; Galli et al., 2012). When AMPs target cancer cells they show promising potential as anti-cancer agents (Yeung et al., 2011). Alternatively, AMPs can coat medical devices as a biodisinfectant to prevent adherence and biofilm formation of micro-organisms (Batoni et al., 2011; Li et al., 2012). Defensins and defensin-derived peptides are potential novel anti-infective agents and will be discussed in section 1.5.

<table>
<thead>
<tr>
<th>Name</th>
<th>Description</th>
<th>Application</th>
<th>Trial phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iseganan (IB-367)</td>
<td>Protegrin; β-hairpin, two disulfide bonds. Isolated from pig leucocytes</td>
<td>Mouthwash</td>
<td>III</td>
</tr>
<tr>
<td>Pexiganan (MSI-78)</td>
<td>α-Helical magainin-2. Isolated from frog skin</td>
<td>Cream</td>
<td>III</td>
</tr>
<tr>
<td>Omiganan (MBI-226)</td>
<td>Synthetic analogue of indolicidin</td>
<td>Cream</td>
<td>III and II</td>
</tr>
<tr>
<td>MBI-594AN</td>
<td>α-Helical indolicidin. Isolated from bovine erythrocytes</td>
<td>Cream</td>
<td>IIb</td>
</tr>
<tr>
<td>P113P113D</td>
<td>α-Helical human histatin</td>
<td>Mouthwash</td>
<td>II</td>
</tr>
<tr>
<td>Neuprex (rBP121)</td>
<td>Human cationic BPI* 450 amino acid residues</td>
<td>Injection</td>
<td>III</td>
</tr>
<tr>
<td>AP-214</td>
<td>Synthetic derivative of HDP** from α-MSH***</td>
<td>Injection</td>
<td>IIb</td>
</tr>
<tr>
<td>HB-1345</td>
<td>Human lipohexapeptide</td>
<td>Topical</td>
<td>Pre-I</td>
</tr>
<tr>
<td>Heptapeptide-7</td>
<td>7-mer from synthetic prototype HB-107. Derived from cecropin B</td>
<td>Cream</td>
<td>I</td>
</tr>
<tr>
<td>Lytixar</td>
<td>Synthetic, membrane-degrading peptide</td>
<td>Topical</td>
<td>IIA</td>
</tr>
<tr>
<td>CUBICIN</td>
<td>Daptomycin, lipopeptide</td>
<td>Injection</td>
<td>On market</td>
</tr>
</tbody>
</table>

*Bactericidal permeability increasing protein, histatin (human)

**Host defence peptides (α-melanocyte-stimulating hormone fused to hexalysine at C-terminus)

***α-melanocyte-stimulating hormone
1.5 Defensins

Defensins are small 3 to 5 kDa (34 to 51 amino acids), cysteine rich cationic peptides containing three to four disulfide bonds (Ganz, 2003; Chrudimska et al., 2011). These AMPs play an important role in the innate immunity of a wide variety of organisms including, vertebrates, invertebrates and plants. In mammals, defensins are found in epithelial cells, mucosal surfaces and phagocytes and are often found in millimolar concentrations (Ganz, 2003; Brandenburg et al., 2012). Mammalian defensins mainly adopt a three stranded antiparallel β-sheet structure, whereas the group of arthropod and plant defensins are composed of an α-helix which is linked to an antiparallel double stranded β-sheet by disulfide bridges (Schmitt et al., 2010).

Invertebrates do not develop acquired immunity, usually present in vertebrates, therefore invertebrates defend themselves from infection through innate immunity (Yamaguchi & Ouchi, 2012). AMPs such as defensins form a major part of the innate immune defence of invertebrates. Expression of invertebrate defensins have been found in tissues such as the hemolymph, hemocytes, fat bodies and the respiratory and digestive tracts (Bulet et al., 1999; Bulet et al., 2004; Varkey et al., 2006). Defensins have been identified from a variety of invertebrates such as scorpions, insects, molluscs and ticks (Cociancich et al., 1993; Charlet et al., 1996; Schmitt et al., 2010). All insect defensins have the same cysteine pairing: Cys1-Cys4, Cys2-Cys5 and Cys3-Cys6 as shown in Figure 1.4 A (Bulet et al., 2004). The common structural conformation observed is an amino-terminal loop and an α-helix linked to double stranded antiparallel β-sheets by disulfide bridges (Fig. 1.4 B) (Bulet & Stocklin, 2005; Gueguen et al., 2006). The Cys2-Cys5 and Cys3-Cys6 disulfide bonds connect the α-helix to the carboxy-terminal β-strand (Varkey et al., 2006). This study specifically, will focus on defensin-derived AMPs from ticks and for this reason tick defensins will be discussed in more detail.
1.5.1 Tick defensins

Compared to insects, little is known about innate immunity in ticks, but a number of AMPs have been identified in various tick species (Johns et al., 2001b; Fogaça et al., 2004; Lai et al., 2004a; Hynes et al., 2005; Sonenshine & Hynes, 2008; Rahman et al., 2010). Ticks are blood feeding external parasites that have many opportunities to encounter microbes due to their feeding behaviour and have been reported to control infections when challenged with bacteria (Johns et al., 1998; 2000; Johns et al., 2001a). The midgut of ticks has developed multiple antimicrobial factors that act as the innate immune system, with defensins being mainly expressed in the midgut after blood feeding or pathogen invasion (Nakajima et al., 2002; Saito et al., 2009). Defensins are the most studied AMPs in ticks (Taylor, 2006) and are mostly effective against Gram-positive bacteria, but some isoforms are also active against Gram-negative bacteria, yeast and parasites (Tsuji et al., 2007; Isogai et al., 2009; Saito et al., 2009). Most tick defensins are cationic peptides that consist primarily of six cysteine residues with the formation of three disulfide bridges (Bulet et al., 2004).

Disulfide bonds stabilize the molecule and maintain its tertiary structure, which is known as the defensin fold (Ganz, 2003). Isogai et al. (2011) showed that the folded form of the defensin, persulcatusin, was more active than the linear form against targeted bacteria. Thus, the antimicrobial activity of defensins is dependent on their structure and structural characterization of defensin molecules is important in understanding the activity of the molecule (Ganz, 2003; Isogai et al., 2011). Structural factors such as peptide conformation, high net positive charge, amphipathicity, hydrophobic moment and hydrophobicity are
considered to be important for antimicrobial activity (Yeaman & Yount, 2003; Rahman et al., 2010). The formation of intra-molecular disulfide bonds in the defensin molecule has been shown to be important for function (Table 1.4) (Ganz, 2003; Chrudimska et al., 2011; Isogai et al., 2011).

Table 1.4: Summary of physical properties and antimicrobial activity of defensins from ticks (Isogai et al., 2011)

<table>
<thead>
<tr>
<th>Origin</th>
<th>Disulfide bonds</th>
<th>Molecular mass (Da)</th>
<th>Net charge</th>
<th>Antimicrobial activity against S. aureus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ixodes persulcatus</td>
<td>yes</td>
<td>4193.8</td>
<td>+6</td>
<td>yes</td>
</tr>
<tr>
<td>Ixodes persulcatus</td>
<td>no</td>
<td>4199.8</td>
<td>+6</td>
<td>weak</td>
</tr>
<tr>
<td>Haemaphysalis longicornis</td>
<td>yes</td>
<td>5820.0</td>
<td>+6</td>
<td>yes</td>
</tr>
<tr>
<td>Ixodes ricinus</td>
<td>yes</td>
<td>4231.9</td>
<td>+5</td>
<td>yes</td>
</tr>
<tr>
<td>Ornithodoros moubata</td>
<td>yes</td>
<td>4062.6</td>
<td>+5</td>
<td>yes</td>
</tr>
<tr>
<td>Ixodes scapularis a</td>
<td>yes</td>
<td>8130.3 b</td>
<td>+6</td>
<td>yes</td>
</tr>
</tbody>
</table>

a(Wang & Zhu, 2011) and http://www.uniprot.org/uniprot/Q5ISE3 accessed 9 September 2013
bObtained from Expacy, Protparam [http://web.expasy.org/cgi-bin/protparam/protparam]
Accessed 9 September 2013

The first report of defensins in ticks was a partial amino acid sequence with high homology to a scorpion 4 kDa defensin purified from the hemolymph of the tick, *Ornithodoros moubata* (van der Goes van Naters-Yasui et al., 2000). Later, four defensin isoforms (A to D) from *O. moubata* were identified from the midgut and showed to possess antibacterial activity (Nakajima et al., 2001; 2002). The synthetic, oxidized form of a tick defensin, *Ornithodoros* defensin A, was shown to be mostly bactericidal against a range of Gram-positive bacteria including *Enterococcus faecalis* and MRSA, which are highly resistant to antibiotics (Nakajima et al., 2003).

Shortly after Nakajima’s (2001) report of the first two defensin isoforms identified in *O. moubata*, Johns et al. (2001b) reported the identification of a defensin from the hemolymph of the American dog tick *Dermacentor variabilis*. This defensin showed 83% similarity to a defensin isolated from scorpions. Defensins have also been identified and analyzed from other tick species, including *Boophilus microplus* in which, Fogaça et al. (2004), isolated a 4285 Da defensin from hemocytes and showed 65% similarity to the *O. moubata* defensins. Rudenko et al. (2005; 2007) showed that expression of a defensin gene is induced in *Ixodes ricinus* after a blood meal and revealed the presence of two defensin isoforms, Def1 (8231
Da) and Def2 (8228 Da). Chrudimska et al. (2011) showed that the reduced form of Def1 and Def2 were active against Gram-positive bacteria. Lu et al. (2010a), isolated a defensin like peptide, named longicornsin, from the tick *Haemophysalis longicornis*. Longicornsin is composed of 47 amino acids, containing six cysteine residues as in other defensin-like proteins. Longicornsin was found to have strong antimicrobial activity against a variety of drug resistant strains, such as *P. aeruginosa* and *S. aureus* (MIC between 0.8 and 1.6 µg/ml). The specificity of AMPs isolated form ticks against bacteria and fungi are listed in Table 1.5.

Table 1.5: AMPs and defensins isolated from ticks (Cheng, 2010)

<table>
<thead>
<tr>
<th>Tick</th>
<th>AMP</th>
<th>Source</th>
<th>Antimicrobial activity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>O. moubata</em></td>
<td>Defensin A</td>
<td>MG</td>
<td>Y</td>
<td>N N (Nakajima et al., 2001; 2002)</td>
</tr>
<tr>
<td></td>
<td>Defensin B</td>
<td>MG</td>
<td>Y</td>
<td>N N (Nakajima et al., 2001; 2002)</td>
</tr>
<tr>
<td></td>
<td>Defensin C</td>
<td>MG</td>
<td>Y</td>
<td>N N (Nakajima et al., 2001; 2002)</td>
</tr>
<tr>
<td></td>
<td>Defensin D</td>
<td>FB</td>
<td>Y</td>
<td>N N (Nakajima et al., 2001; 2002)</td>
</tr>
<tr>
<td>Rabbit α-HS</td>
<td></td>
<td>MG</td>
<td>Y</td>
<td>N N (Nakajima et al., 2003)</td>
</tr>
<tr>
<td><em>A. hebraeum</em></td>
<td>Peptide 1</td>
<td>HL</td>
<td>Y</td>
<td>Y N (Lai et al., 2004a)</td>
</tr>
<tr>
<td></td>
<td>Peptide 2</td>
<td>HL</td>
<td>Y</td>
<td>Y N (Lai et al., 2004b)</td>
</tr>
<tr>
<td></td>
<td>Hebraein</td>
<td>HL</td>
<td>Y</td>
<td>Y Y (Lai et al., 2004b)</td>
</tr>
<tr>
<td><em>B. microplus</em></td>
<td>Bovine α-HS</td>
<td>MG</td>
<td>Y</td>
<td>N Y (Fogaça et al., 1999)</td>
</tr>
<tr>
<td></td>
<td>Defensin</td>
<td>H,</td>
<td>Y</td>
<td>N N (Fogaça et al., 2004; Esteves et al., 2008)</td>
</tr>
<tr>
<td></td>
<td>Microplusin</td>
<td>H,</td>
<td>Y</td>
<td>N Y (Fogaça et al., 2004; Esteves et al., 2008; Silva et al., 2009)</td>
</tr>
<tr>
<td></td>
<td>Ixodidin</td>
<td>H</td>
<td>Y</td>
<td>Y N (Fogaça et al., 2006)</td>
</tr>
<tr>
<td><em>D. variaiblis</em></td>
<td>Varisin</td>
<td>HL</td>
<td>Y</td>
<td>Y N (Sonenshine et al., 2002)</td>
</tr>
<tr>
<td><em>H. longicornis</em></td>
<td>Gut-defensin</td>
<td>MG</td>
<td>Y</td>
<td>Y N (Zhou et al., 2007)</td>
</tr>
<tr>
<td></td>
<td>Hisal-defensin</td>
<td>SG</td>
<td>Y</td>
<td>Y N (Zhou et al., 2007)</td>
</tr>
<tr>
<td></td>
<td>Longicorn</td>
<td>MG</td>
<td>Y</td>
<td>Y Y (Tsuji et al., 2007)</td>
</tr>
<tr>
<td></td>
<td>Longicornsin</td>
<td>SG</td>
<td>Y</td>
<td>Y Y (Lu et al., 2010a)</td>
</tr>
<tr>
<td><em>I. ricinus</em></td>
<td>Defensin1 &amp; 2</td>
<td>WT</td>
<td>Y</td>
<td>N N (Rudenko et al., 2005; Rudenco et al., 2007; Chrudimska et al., 2011)</td>
</tr>
<tr>
<td><em>I. scapularis</em></td>
<td>ISAMP</td>
<td>H, SG</td>
<td>Y</td>
<td>Y N (Pichu et al., 2009)</td>
</tr>
<tr>
<td><em>I. sinensis</em></td>
<td>Ixosin</td>
<td>SG</td>
<td>Y</td>
<td>Y Y (Yu et al., 2006)</td>
</tr>
<tr>
<td></td>
<td>Ixosin</td>
<td>SG</td>
<td>Y</td>
<td>Y Y (Liu et al., 2008)</td>
</tr>
</tbody>
</table>

1.5.2 Tick defensins as templates for anti-infective agents

Ticks are becoming important sources of novel proteins and peptides that can serve as templates for the development of antimicrobial agents (Nakajima et al., 2003; Chrudimska et al., 2011). Defensins and defensin-derived peptides hold potential as promising pharmaceutical agents (Winter & Wenghoefer, 2012), especially if they have a broad range of activity against bacteria, fungi and parasites with low toxicity towards mammalian cells (Hancock & Sahl, 2006; Tsuji et al., 2007; Wang & Zhu, 2011).

Longicin was previously identified by Tsuji et al. (2007) in the hard tick *H. longicornis* and shown to have antibacterial, antifungal and antiparasitic activity. The authors found that the active region of this tick defensin-like molecule is the C-terminus 20 amino acid containing region and that this region is essential for antimicrobial activity. The synthetic peptide, P4, corresponding to the active region is shown in Table 1.6. The analysis of structural differences among longicin and its analogue, P4, was done in relation to their antimicrobial potential. Longicin is composed of α-helixes and β-sheet motifs, while P4 was predicted to have a β-sheet conformation but can adopt a helical conformation after interaction with membranes or membrane-like environments. The antimicrobial active sites were believed to be located in the C-terminus amino acid sequence that consists of a β-sheet instead of an α-helix as in insects (Osaki et al., 1999). Neither longicin nor P4 caused erythrocyte haemolysis, which is promising for pharmaceutical applications. P4 differed from the peptides used by Varkey et al. (2006) in that in P4 the cysteines were included.

Varkey et al. (2006) studied the antibacterial activities of synthetic peptides spanning the C-terminus regions (β-strands) of the defensins from the firebug, *Pyrrhocoris apterus* (PAΔC; 17-mer with a net charge of +6), the stable fly, *Stomoxys calcitrans* (SCΔC; 18-mer with a net charge of +3), and the tick *O. moubata* (OMΔC; 20-mer with a net charge of +5; isoform C) (Table 1.6). These peptides were synthesized lacking cysteine residues (ΔC), whereas the parent peptides naturally contain cysteine residues. It has been shown that shorter peptides derived from the C-terminus domain, without a specific sequence or presence of disulfide bonds, still exerted antimicrobial activity (Varkey et al., 2006). Interactions of the peptides with lipid vesicles were investigated by monitoring the fluorescence of tryptophan, added to the N-terminus of each peptide. It was observed that the OMΔC analogue had the ability to form pores in the lipid vesicles. Varying antibacterial activities were observed for each of the
peptides even though all three peptides had a positive net charge. Regardless of a charge
difference of only +1, PAΔC showed comparable activities against Gram-negative and Gram-
positive bacteria (Lethal concentration (LC) = 5.0 µM) whereas OMΔC was more active
against Gram-negative bacteria (LC = 5.0 µM versus 20 µM for Gram-positive bacteria). The
peptide SCΔC was not active against most of the bacteria tested except for Bacillus subtilis
(LC = 40 µM) in spite of having a net charge of +3. The native defensin from O. moubata
was only active against Gram-positive bacteria.

Wang and Zhu (2011) found that an even shorter peptide derived from the C-terminus
domain of the defensin, scapularisin-20, from Ixodes scapularis revealed a wide spectrum of
antibacterial activity at micromolar concentrations. Overall the shorter amidated peptide,
amino acids 26 to 39 of scapularisin-20 (14 amino acids, charge +4) (Table 1.6) showed
slightly more potency to Gram-positive bacteria (LC = 10 to 20 µM) than Gram-negative
bacteria (LC = 15 to 33 µM). No haemolytic activity on mouse erythrocytes was reported in
this study, even at very high peptide concentrations (400 µM).

Table 1.6: Summary of tick defensins as templates for anti-infective agents

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Peptide sequence</th>
<th>Charge</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAΔC</td>
<td>W-VIKGYKGGKQITVHRR-amide</td>
<td>+6</td>
</tr>
<tr>
<td>OMΔC</td>
<td>W-SIGRYKGYKYKGLFKQTNY-amide</td>
<td>+5</td>
</tr>
<tr>
<td>P4</td>
<td>SIGRRGGYCAIIKQTCTCYR</td>
<td>+4</td>
</tr>
<tr>
<td>Scapularisin-20(26-39)</td>
<td>CGGFLKKTICVMK-amide</td>
<td>+4</td>
</tr>
</tbody>
</table>

1.6 Background to this study

The tick used in this study, Ornithodoros savignyi, is a livestock parasite endemic to arid and
semi-arid regions of Africa. This tick is a rich source of bioactive molecules and by using it
as a model for tick investigations, we have described several anti-haemostatic components
(Maritz-Olivier et al., 2007). Previously, a Gram-positive AMP was purified from the
hemolymph of this tick (Olivier, 2002). The N-terminal sequence of this peptide was
obtained and degenerate primers designed using the latter sequence led to the molecular
characterization of two midgut defensin isoforms (M. Botha, Honours project), O. savignyi
defensin isoform one (OsDef1) and two (OsDef2) (Table 1.7). The two full-length defensin
isoforms are 42 amino acid residues in length, with the mature peptide represented by 37 of these residues. Both isoforms display cysteine residues characteristic of defensin molecules. The amino acid sequences for the mature forms of OsDef1 and OsDef2 differ only at positions 16 and 22. In position 16, serine is present in OsDef1 and lysine in case of OsDef2, whereas in position 22, OsDef1 has an arginine instead of a lysine residue (manuscript in preparation). The native mature peptide, OsDef2, has a net charge of +6 (five Lys and one Arg residues) and showed greater activity against Gram-positive bacteria compared to OsDef1 (Prinsloo et al., 2013). Additional properties of OsDef1 and OsDef2 are shown in Table 1.7. A recent study derived Os (Table 1.7) from the last 22 amino acids of the C-terminus of OsDef2 and evaluated the structural and functional properties of this peptide.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>Charge</th>
<th>MWa</th>
<th>No of amino acids</th>
</tr>
</thead>
<tbody>
<tr>
<td>OsDef1</td>
<td>GYGCPFNYQCHSHCGIRGYRGGYCKGAFKQTCKCY</td>
<td>+5</td>
<td>4172.7</td>
<td>37</td>
</tr>
<tr>
<td>OsDef2</td>
<td>GYGCPFNYQCHSHCKGIRGYKGGYCKGAFKQTCKCY</td>
<td>+6</td>
<td>4185.8</td>
<td>37</td>
</tr>
<tr>
<td>Os</td>
<td>KGIRGYKGGYCKGAFKQTCKCY</td>
<td>+6</td>
<td>2459.9</td>
<td>22</td>
</tr>
</tbody>
</table>

*Theoretical molecular weight, data obtained from Expacy, ProtParam [http://web.expasy.org/cgi-bin/protparam/protparam] accessed 1 October 2013. Differences in amino acid sequence of OsDef1 and 2 are highlighted in green. Cysteine residues and charged residues are highlighted in red and blue, respectively.

Prinsloo et al. (2013) showed that Os exhibits strong antibacterial activities against both Gram-positive and Gram-negative bacteria (Table 1.8) without causing any damage or toxicity towards mammalian cells, even at high concentrations (100 μM). In addition to Os possessing antibacterial activity, high antioxidant activity was observed and this was the first report of tick defensin-derived peptides showing antioxidant activity. The antibacterial activity was also investigated in the presence of 100 mM NaCl and revealed that Os retained its activity towards *E. coli*, whereas its MBC against *S. aureus* increased from 3.75 μg/ml to 120 μg/ml. However, in the presence of 30% human serum, Os retained all of its activity towards *S. aureus*, but was 32 fold less active against *E. coli.*
Table 1.8: Bactericidal activity of synthetic peptide Os derived from OsDef2 (Prinsloo et al., 2013)

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>MBC (µg/ml) Os</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gram-negative</td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>1.88 (0.77 μM)</td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td>0.94 (0.38 μM)</td>
</tr>
<tr>
<td>Gram-positive</td>
<td></td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>15.0 (6.10 µM)</td>
</tr>
<tr>
<td><em>B. subtilis</em></td>
<td>1.88 (0.77 µM)</td>
</tr>
</tbody>
</table>

1.7 Aims of study

As mentioned earlier, problems associated with the development of AMPs include high cost of manufacturing and cytotoxicity towards mammalian cells. Shorter AMPs, which retain their bactericidal activity with low toxicity, are particularly attractive since their synthesis cost will be lower. Thus, investigating the cytotoxicity of shorter peptides to eukaryotic cells is important before they can be considered for future development as novel antibiotics. Sensitivity of AMPs to increased salt concentrations poses a major obstacle in the development of AMPs, whereas interaction of AMPs with serum proteins may affect their bioavailability. It is therefore essential to investigate whether shorter peptides remain active under increased salt concentrations and physiological conditions.

The purpose of this study was to investigate whether shorter sequences derived from Os would retain their activity and selectivity towards bacteria. To this end, seven overlapping synthetic peptides, composed of ten amino acid residues each, spanning the entire sequence of Os, as well as one peptide comprising the last twelve C-terminus amino acid residues were evaluated for antibacterial activity. The amidated form of one of the two identified active peptides, Os(11-22)NH₂ was further evaluated in terms of its secondary structure, antibacterial, antioxidant and cytotoxic activities.

More specifically the aims were to:

1. Screen all shorter peptides derived from Os for antibacterial activity.
2. Determine the minimum bactericidal concentration (MBC) for the active peptides.
3. Establish the cytotoxicity of the active peptides using eukaryotic cells.
4. Investigate whether amidation of one of the shorter active peptides would increase its antibacterial activity.

5. Establish whether amidation alters the secondary structure of the unamidated peptide.

6. Determine the effects of increased salt concentration and serum on the activity of the amidated peptide.

7. Determine the kinetics of bactericidal activity for the amidated peptide.

8. Investigate the cytotoxicity of the amidated peptide to erythrocytes and eukaryotic cells.

9. Establish whether Os(11-22)NH$_2$ possesses antioxidant activity.
CHAPTER 2: Materials and Methods

2.1 Materials

All reagents used were of analytical grade and for all experiments double-distilled deionized sterile water (ddH2O) was used (Millipore system Q, Millipore, USA). Concentrations reported are those used in the final reaction volumes.

*Escherichia coli* (ATCC 700928), *Pseudomonas aeruginosa* (ATCC 10145), *Bacillus subtilis* (ATCC 13933) and *Staphylococcus aureus* (U3300) were used for the antibacterial assays.

A series of overlapping peptides based on the structure of Os were synthesized, together with the C-terminus of Os containing all three cysteine residues (Table 2.1). The overlapping 10-mer peptides as well as the 12-mer cysteine containing C-terminus of Os were obtained from GenScript (New Jersey, USA) that synthesized the peptides using their FlexPeptide™ technology. The amidated peptide was obtained from LifeTein (New Jersey, USA) synthesized by their PeptideSyn™ technology. Peptide synthesis by both companies used solid phase protein synthesis. The purity of the peptides determined by GenScript used reverse-phase high-performance liquid chromatography (RP-HPLC), with an AlltimaTM C18 column with mobile phase one as 0.065% trifluoroacetic acid (TFA) in 100% water (v/v) and mobile phase two as 0.05% TFA in 100% acetonitrile (v/v). The peptides obtained from LifeTein were purified by RP-HPLC using a SinoChrom ODS-BP C18 column with 0.1% TFA in 100% water (v/v) and 0.1% TFA in 100% acetonitrile (v/v) as mobile phases one and two, respectively. The molecular weights of the peptides were determined by mass spectrometry. The reducing agent, dithiothreitol (DTT, 10 nmol), was added by the supplier to the peptides prior to lyophilisation to maintain reducing conditions by excluding oxygen. No additional DTT was added to peptide stock solutions, Os(11-20) and Os(11-22). Table 2.1 lists these peptides and some of their properties.
Table 2.1: Properties of the series of synthetic overlapping peptide based on the structure of Os as well as the C-terminus of Os containing all three cysteine residues

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>Number of amino acids</th>
<th>Net charge</th>
</tr>
</thead>
<tbody>
<tr>
<td>Os</td>
<td>KGIRGYKGYCKGAFKQTCKCY</td>
<td>22</td>
<td>+6</td>
</tr>
<tr>
<td>Os (1-10)</td>
<td>KGIRGYKGGY</td>
<td>10</td>
<td>+3</td>
</tr>
<tr>
<td>Os (3-12)</td>
<td>IRGYKGYCK</td>
<td>10</td>
<td>+3</td>
</tr>
<tr>
<td>Os (5-14)</td>
<td>GYKGGYCKGA</td>
<td>10</td>
<td>+2</td>
</tr>
<tr>
<td>Os (7-16)</td>
<td>KGGYCKGAFK</td>
<td>10</td>
<td>+3</td>
</tr>
<tr>
<td>Os (9-18)</td>
<td>GYCKGAFKQT</td>
<td>10</td>
<td>+2</td>
</tr>
<tr>
<td>Os (11-20)</td>
<td>CKGAFKQTCK</td>
<td>10</td>
<td>+3</td>
</tr>
<tr>
<td>Os (13-22)</td>
<td>GAFKQTCKCY</td>
<td>10</td>
<td>+2</td>
</tr>
<tr>
<td>Os (11-22)</td>
<td>CKGAFKQTCKCY</td>
<td>12</td>
<td>+3</td>
</tr>
</tbody>
</table>

Melittin (Sigma Aldrich, RSA) was used as positive control in haemolytic and cytotoxicity studies. This peptide is an α-helical, cationic AMP composed of 26 amino acid residues (GIGAVLKVLTTLPALISWIKRKRQQ-NH₂, +6), found in the venom of the European honey bee, *Apis mellifera*.

Stock peptide solutions of 1.2 mg/ml were prepared in dddH₂O. Peptides were sterilized by filtration through a 0.45 μM filter, following concentration determination of each peptide using a Beer-Lambert Law based equation as follows:

\[ c = \frac{A \times df \times MW}{n(ε_{Tyr}) + n(ε_{Trp})} \]

where \( c \) is the peptide concentration (mg/ml); \( A \) is the absorbance at 280 nm; \( df \) the dilution factor; \( MW \) the molecular weight of each particular peptide; \( n \) the number of tyrosine or tryptophan residues of each respective peptide and \( ε \) the extinction coefficient of tyrosine (1200 Au/mmol/ml) or tryptophan (5560 Au/mmol/ml) residues.

2.2 Antibacterial assays

2.2.1 Determination of the bacterial cell count

The determination of the bacterial cell count was previously performed in our laboratory. Briefly, a standard curve of OD₆₀₀ against colony forming units per ml (CFU/ml) was constructed for each bacterial strain (Giuliani & Rinaldi, 2010). A series of eight serial dilutions (1:2, 1:4, up to 1:256) of a dense bacterial suspension (2.5 ml; OD₆₀₀ ≥ 1.5) in
sterile sodium phosphate buffer (0.1 M Na₂HPO₄·2H₂O and 1 M NaH₂PO₄, made up to 10 mM, pH 7.4; SPB) was prepared. The OD₆₀₀ was measured for each dilution. A series of eight 10 fold dilutions of the same initial suspension (1:10, 1:10² up to 1:10⁸) was prepared. Aliquots of 200 μl of the 10⁵, 10⁶, 10⁷, and 10⁸ dilutions were plated onto Luria Bertani (LB) agar plates (1% tryptone, 0.5% yeast extract, 1% sodium chloride (NaCl), pH 7.5, 2% (w/v) bacteriological agar) with incubation at 37°C overnight. The colonies on each plate containing ≥ 50 colonies were counted. The cell count in the original bacterial suspension was calculated. For example, if 82 colonies were counted on the plates prepared from the 10⁶ dilution, the original cell count in suspension would be 82 x 5 x 10⁶ = 4.1 x 10⁸ CFU/ml. A standard curve was constructed that represents cell count (CFU/ml) on the X - axis and OD₆₀₀ values on the Y - axis. The value obtained from the slope was used to dilute bacteria to 10⁶ CFU/ml. The slopes obtained for E. coli, B. subtilis, P. aeruginosa and S. aureus were 2 x10⁻⁹, 3 x 10⁻⁹, 3.8 x 10⁻⁹ and 3.67 x 10⁻⁹, respectively.

\[
\text{Dilution} = \frac{\text{OD}_600}{\text{Slope obtained for each bacteria}} \times 1 \times 10^{-6} \text{ CFU/ml}
\]

2.2.2 The turbidity method

A high-throughput liquid culture assay for measuring defensin antibacterial activity by turbidity measurements, described by Wanniarachchi et al. (2011), was used in this study. Initial screening for antibacterial activity and determination of the MBC of the shorter peptides were performed with E. coli and B. subtilis. Bacteria from stock solutions were streaked on LB agar plates and grown for 14 to 16 h at 37°C. Three to five colonies were picked and dispersed in 25 ml LB broth (1% tryptone, 0.5% yeast extract, 1% NaCl, pH 7.4) and grown for 14 to 16 h at 37°C with shaking at 150 rpm. The overnight culture was diluted 100 times in LB broth and sub-cultured for 2 to 3 h at 37°C with shaking at 150 rpm, until the optical density at 600 nm (OD₆₀₀) reached 0.6 (E. coli) or 0.4 (B. subtilis) to obtain bacteria in their exponential growth phase. The OD₆₀₀ was measured with a Shimadzu UV-160A, UV-visible recording spectrometer (Kyoto, Japan) using a 1 cm quartz cuvette. Bacteria (1 ml) were centrifuged at 14000 g for 90 sec. The supernatant was discarded and bacterial cells washed twice with 0.5 ml SPB for Gram-negative bacteria or 0.5 ml SPB supplemented with 1% LB for Gram-positive bacteria. The washed cell pellets for Gram-negative and Gram-positive bacteria were dispersed in their respective sodium phosphate buffers. Bacterial dilutions were made in the appropriate buffer to give approximately 10⁶ CFU/ml.
Using a sterile 96-well polypropylene micro-titre plate, a twelve step serial dilution series of the peptides were prepared (0.06 µg/ml to 120 µg/ml) and made up to a final volume of 10 µl. A volume of 90 µl bacteria was incubated with 10 µl of each of the peptide concentrations, in triplicate, for 2 h at 37°C with shaking at 150 rpm. A sterile control containing 90 µl SPB and 10 µl dddH₂O and a growth control containing 90 µl bacteria and 10 µl dddH₂O were included. After incubation, 100 µl, 2 x LB was added to each well and incubated for 5 h at 37°C with shaking. The absorbance at 595 nm was measured, by using a Multiscan Ascent V1.24 96-well micro-titre plate reader. The lowest concentration of peptide that killed 99% of bacteria was taken as the MBC. The peptides with MBCs ≤ 120 µg/ml obtained using this method were further evaluated using the CFU method against *E. coli*, *B. subtilis*, *P. aeruginosa* and *S. aureus*.

### 2.2.3 The colony forming unit method

The MBCs for the active peptides were determined by means of the CFU method as described by Varkey *et al.* (2006). One of the active peptides was synthetically amidated and the MBC was also determined. The respective bacteria were prepared as described previously for the turbidity method (section 2.2.2), except that *P. aeruginosa* and *S. aureus* were also included. Exponential phase *P. aeruginosa* and *S. aureus* were obtained after the OD₆₀₀ reached 0.5 and 0.4, respectively. The peptides were prepared as for the turbidity method and incubated with bacteria for 2 h at 37°C with shaking. After incubation, 10 µl of the incubation samples were diluted 500 times with SPB and LB for Gram-negative and Gram-positive bacteria, respectively. A volume of 100 µl was plated out on LB agar plates and incubated for 14 to 16 h at 37°C. The surviving colonies were counted to determine the number of CFUs for each peptide and compared to the growth control. The MBC was taken as the lowest concentration of peptide that killed 99% of bacteria.

### 2.3 Secondary structure prediction using PSIPRED

The secondary structure of the shorter active peptide was predicted using the PSIPRED secondary structure prediction program (Jones, 1999).
2.4 Secondary structure determination using circular dichroism (CD) spectroscopy

CD spectroscopy is used to study chiral molecules of all types and sizes to determine their secondary structure. CD is defined as the differential absorption of left-handed and right-handed circularly polarized light (Kelly et al., 2005; Greenfield, 2006). Secondary structure can be determined in the far-UV spectral region (190-250 nm). At these wavelengths the chromophores are the chiral peptide bonds (amide bond electrons absorb in this energy range), where the intensity and energy of these conversions depend on the angles the peptide bonds assume and therefore on the secondary structure of the protein. The absorbance measured between left and right circularly polarized light is in terms of ellipticity, $\theta$, in degrees (Kelly et al., 2005).

CD spectroscopy was performed with the help of Prof. Y. Sayed from the Protein Structure-Function Research Unit, School of Molecular and Cell Biology, University of Witwatersrand. Far-UV CD spectroscopy was used for the determination of the secondary structures of Os(11-22), Os(11-22)NH$_2$ and melittin, using a J-810 spectropolarimeter (Jasco, Tokyo, Japan) with cell path length of 0.2 cm over the 180 to 250 nm range. CD spectra were obtained at 20°C using a data pitch of 0.1 nm at a bandwidth of 0.5 nm. Ten scans with a scan speed of 200 nm/min were averaged for each peptide. A volume of 200 μl of each peptide (50 μM) was scanned in water, SPB and sodium dodecyl sulphate (SDS, 25 mM). Data acquired for the CD spectra above 800 volts were omitted due to the unreliability of data beyond that point. Graphs were generated by converting millidegree readings into the mean residue ellipticity, and plotting it against wavelength. The equation for this calculation incorporates the concentration and number of amino acid residues (Čeřovský et al., 2009; Rahman et al., 2010) and is as follows:

$$\theta_{\text{MRE}} = \frac{100 \times \theta}{CnI}$$

where $[\theta]_{\text{MRE}}$ is the mean residue ellipticity (deg.cm$^2$.dmol$^{-1}$); $\theta$ represents ellipticity, the unit for CD raw data reading in millidegree (mdeg); $C$ is the concentration of peptide (mM); $n$ is the number of amino acid residues and $I$ is the path length of the cell (cm).

Alpha-helical, $\beta$-sheet and random coiled structures each give rise to a characteristic profile and magnitude on the CD spectrum (Fig 2.1). For a random coiled structure the mean residue
ellipticity should be positive at 212 nm and negative at 195 nm. The mean residue ellipticity of an α-helical structure should be positive at 192 nm and negative at 209 nm and 222 nm (Kelly et al., 2005). For β-sheet structures, the mean residue ellipticity should be positive at 196 nm and negative at 218 nm (Greenfield, 2006). A characteristic CD spectrum of pure secondary structures can be observed in Figure 2.1 A. When none of these criteria are met, the secondary structure is thought to be unstructured.

**Figure 2.1: Typical CD spectra for α-helices, β-sheets and random coils.** (A) Characteristic shape of α-helical (green), β-sheet (blue) and random coiled (red) structures on the CD spectra (http://www.fbs.leeds.ac.uk/facilities/cd/) and (B) summary of characteristic CD spectrum magnitudes of α-helix, β-sheet and random coil structures taken from Kelly et al. (2005).

### 2.5 Effects of NaCl and serum on the MBC of Os(11-22)NH$_2$

The effect of 100 mM NaCl on the antibacterial activity of Os(11-22)NH$_2$ was determined. Bacteria were prepared (washed with SPB) as for the CFU method, following dilution ($10^6$ CFU/ml) in NaCl/SPB to obtain a final concentration of 100 mM NaCl. A volume of 90 μl bacteria were incubated with 10 μl peptide at concentrations ranging from 0.06 to 120 μg/ml for 2 h at 37°C, with shaking at 150 rpm. After 2 h, 10 μl of the incubation samples was diluted 500 times and plated out on LB agar plates. Plates were incubated at 37°C for 14 to 16 h. The MBC was defined as the concentration of peptide that killed 99% of bacteria. Experiments were repeated at least twice in triplicate.

To determine the effect of human serum on the antibacterial activity of Os(11-22)NH$_2$, human serum (5 mg lyophilized powder pooled, non-heat inactivated, Sigma-Aldrich, RSA)
was dissolved in 5 ml dddH2O (1 mg/ml human serum), thereafter a volume of 30 µl serum was incubated with 10 µl peptide (0.06 to 120 µg/ml) and 60 µl bacteria (1.5 x 10^6 CFU/ml) to obtain 30% serum (v/v) in the final reaction mixture. After 2 h, 10 µl of the incubation samples were diluted 500 times (E. coli) or 1000 times (S. aureus) and plated out on LB agar plates. Plates were incubated at 37⁰C for 16 h. The MBC was defined as the concentration of peptide that killed 99% of bacteria. Experiments were repeated three times in triplicate.

2.6 Kinetics of bactericidal activity
E. coli and B. subtilis, were prepared and diluted as for the turbidity method and incubated at specific time intervals (0, 5, 10, 30, 60, and 120 min), at 37⁰C with Os(11-22)NH₂, at the respective MBCs for each bacteria, while shaking at 150 rpm. At the specific time intervals, 10 µl of the incubation samples was diluted 500 times and plated on LB agar plates. Plates were incubated at 37⁰C for 14 to 16 h and the number of colonies counted. Successful killing time was defined as the time (min) after which no colonies were formed. Experiments were repeated at least twice in triplicate.

2.7 Cytotoxicity assays
2.7.1 Haemolytic assay
The haemolytic activity of Os(11-22)NH₂ and melittin (positive control) was studied, following a modified method of Stark et al. (2002). Blood was collected from healthy consenting donors (Ethical clearance obtained from the Research Ethics Committee (Protocol no. 61/201), Faculty of Health Sciences, University of Pretoria). A volume of 5 ml of freshly collected blood was centrifuged at 2500 x g to remove the plasma and buffy coat and to collect the erythrocytes. Erythrocytes obtained were washed with isotonic phosphate buffered saline (0.137 M NaCl, 3 mM KCl, 1.9 mM NaH₂PO₄·2H₂O, 8.1 mM Na₂HPO₄, pH 7.4; Iso-PBS) and resuspended in Iso-PBS to obtain 5% (v/v) erythrocytes. The peptides Os(11-22)NH₂ and melittin were diluted in Iso-PBS, to obtain a dilution range from 100 to 0.78 µM. A volume of 10 µl of the peptides was added to 90 µl of the erythrocyte suspension in triplicate and incubated at 37⁰C for 30 min. Following centrifugation at 1250 x g for 2 min, 75 µl of the supernatants were transferred to a 96-well micro-titre plate and the absorbance (A) measured with a BioTek plate reader at 570 nm for released hemoglobin (Stark et al., 2002; Rahman et al., 2010). The haemolysis assay measures hemoglobin release, as an indicator of red blood cell lysis, as a result of peptide exposure (Lubran, 1989). Cells were
incubated with either 2% SDS as positive control, representing 100% haemolysis or Iso-PBS as negative control represented 0% haemolysis. The results were expressed as % haemolysis, relative to the SDS control and determined using the following equation:

\[
\text{% Haemolysis} = \left( \frac{\text{Abs}_{\text{peptide}} - \text{Abs}_{\text{negative control}}}{\text{Abs}_{\text{100% control}} - \text{Abs}_{\text{negative control}}} \right) \times 100
\]

### 2.7.2 Crystal violet assay

The crystal violet (CV) assay was used to determine the cytotoxic effects of the peptides. The CV assay provides easy, inexpensive and quantitative data analysis by using 96-well plates. CV, a positively charged molecule (Fig. 2.2), will bind to negatively charged molecules such as nucleic acids and proteins (amino acid residues, glutamic acid and aspartic acid). Since CV binds and stains all these components of live and dead cells, extraction of the dye from stained cells is an indication of cell number. Although both alive and dead cells are stained, dead cells can not adhere to the surface of the micro-titre plate, due to loss of cell integrity by apoptosis. Therefore, after CV staining the plates are washed and non-adherent cells are washed away (Negri et al., 2010).

![Figure 2.2: The structure of crystal violet](http://www.chemicalbook.com/ChemicalProductProperty_EN_CB2161846.htm) accessed on 22 August 2013

The cytotoxic effects of the peptides were tested using two cell lines; SC-1 (embryo *Mus musculus*, fibroblasts, doubling time 48 h) and Caco-2 (adult, *Homo sapiens*, epithelial,
colorectal adenocarcinoma, doubling time 62 h) cells. The cytotoxicity of Os(3-12), Os(11-22) and melittin (positive control) was tested on Caco-2 cells, before Os(11-22)NH₂ was available. After Os(11-22)NH₂ was synthesized the cytotoxicity of this peptide and Os(11-22) was tested on SC-1 cells. The cells were cultured by the Department of Anatomy, Faculty of Health Sciences, University of Pretoria. Briefly, cells were maintained in Dulbecco’s Modified Eagle’s Medium supplemented with 10 % foetal calf serum (FCS) and 1 % antibiotic solution. Cells were maintained until confluent at 37°C and 5% CO₂. Cells were collected and resuspended in Eagle’s Minimal Essential Medium/10% FCS and used by the author in the CV assay as described below.

Peptides were serially diluted to obtain a peptide concentration range of 0.06 to 120 µg/ml after which 10 µl of peptide was added to 90 µl Caco-2 or SC-1 cells. Following 24 h exposure of Caco-2 or SC-1 cells to peptides, a volume of 10 µl 2% formaldehyde fixative solution was added to each well and removed after 20 minutes together with the cell culture medium. The plate was air dried and cells attached at the bottom of the plate stained by adding 500 µl of 0.1% (w/v) CV solution prepared in 200 mM formic acid (pH 3.5) to each well. After 30 minutes the plate was washed with distilled water and dried. Bound dye was dissolved in 200 µl 10% acetic acid prepared in distilled water. Absorbance was read at 630 nm using a BioTek plate reader. Results were expressed as % cell number relative to the control, with no addition of peptide.

2.8 Oxygen radical absorbance capacity (ORAC) antioxidant assay

The antioxidant activity of Os(11-22)NH₂, was investigated by means of the ORAC assay. The ORAC assay, initially developed by Cao et al. (1993), is a standardized assay that allows data comparison across laboratories and integrates both the time and extent of the antioxidant reaction by using biologically relevant free radicals in the assay (Cao et al., 1993; Zulueta et al., 2009). This assay consists of measuring the decrease in fluorescence of a fluorescent probe as a result of the change in its conformation when it undergoes oxidative damage caused by a source of peroxyl radicals (ROO•) (Fig. 2.3) (Ou et al., 2001; Zulueta et al., 2009). The decrease in fluorescence of fluorescein by the sample is compared to the reference antioxidant standard, Trolox®. The ORAC assay measures the ability of the antioxidants (peptides) in the sample to protect fluorescein from oxidative damage (Zulueta et al., 2009). The inhibition of free radical damage by the antioxidant (peptide) is a measure of its
antioxidant capacity with respect to a free radical. The uniqueness of the ORAC assay is that the reaction is driven to completion and the quantization is achieved using the area under the curve (AUC) (Ou et al., 2001; Huang et al., 2005).

Figure 2.3: Schematic presentation of free radical (∙*R*) neutralization. The free radical is neutralized by a phenolic side chain of tyrosine as a potential antioxidant amino acid. Images generated using ACD/ChemBasic in ACD/ChemSketch, Version 12.0.

The method used was based on a modified method of Ou et al. (2001). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), a water-soluble analogue of vitamin E, was serially diluted (0 to 800 μM) and used as a control standard, with SPB as a blank. A volume of 10 μl of the peptide, Os(11-22)NH₂, was used at a concentration range from 1.2 to 4.1 μM, with the tripeptide glutathione (GSH, 4.8 μM) as a positive antioxidant control. Fluorescein (0.139 nM; 30,60-dihydroxyiso[isobenzofuran-1][3H], 90[9H]- xanthen]-3-one) was used as the fluorescent probe, whereas AAPH (0.11 μM, 2, 2’-azobis (2-amidino-propane) dihydrochloride) was used as a free radical generator. Fluorescence was measured every 5 min for 4 h on a FLUOstar OPTIMA plate reader (BMG lab technologies, Offenburg, Germany). The excitation wavelength was 485 nm and emission wavelength was 520 nm. Calculations were done by exporting the data to an Excel spread sheet and determining the area under curve (Fig. 2.4) (netAUC = AUCₐntenioxidant – AUCₐnblank). The results were expressed as micromole trolox equivalents (μmol TE) (Ou et al., 2001).
Figure 2.4: The graphs represent the results obtained from the ORAC assay (A) Fluorescein control, (B) AAPH and Fluorescein and (C) to (F) increasing concentrations of Trolox. AUC for each peptide was determined and expressed as μmol TE.
CHAPTER 3: Results

3.1 Physicochemical properties of the synthetic peptide fragments

To identify the active region of Os, a series of 10-mer overlapping synthetic peptides based on the structure of Os, as well as the 12 amino acids corresponding to the cysteine containing C-terminus were obtained. The physicochemical properties of Os and the synthetic overlapping peptide fragments used in this study are listed in Table 3.1. The parameters computed by ProtParam for this study included the theoretical molecular weight and pI. The hydrophobic moment \(<\mu_H>\) and hydrophobicity \(<H>\) were computed by HeliQuest. The physicochemical properties such as \(<\mu_H>\) and \(<H>\) are an indication of the activity of peptides. Peptides with higher \(<\mu_H>\) and \(<H>\) are associated with higher antimicrobial activity (Wieprecht et al., 1997).

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>No A.A</th>
<th>Net charge</th>
<th>MW&lt;sup&gt;b&lt;/sup&gt;</th>
<th>pI&lt;sup&gt;a&lt;/sup&gt;</th>
<th>(&lt;\mu_H&gt;^c)</th>
<th>(&lt;H&gt;^d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Os</td>
<td>KGIRGYKGGYCKGAFKQTCKCY</td>
<td>22</td>
<td>6</td>
<td>2459.9</td>
<td>9.69</td>
<td>0.277</td>
<td>0.249</td>
</tr>
<tr>
<td>Os(1-10)</td>
<td>KGIRGYKGGY</td>
<td>10</td>
<td>3</td>
<td>1098.3</td>
<td>10</td>
<td>0.472</td>
<td>0.073</td>
</tr>
<tr>
<td>Os(3-12)</td>
<td>IRGYKGGYCK</td>
<td>10</td>
<td>3</td>
<td>1144.4</td>
<td>9.63</td>
<td>0.406</td>
<td>0.227</td>
</tr>
<tr>
<td>Os(5-14)</td>
<td>GYKGGYCKGA</td>
<td>10</td>
<td>2</td>
<td>1003.1</td>
<td>9.11</td>
<td>0.218</td>
<td>0.179</td>
</tr>
<tr>
<td>Os(7-16)</td>
<td>KGGYCKGAFK</td>
<td>10</td>
<td>3</td>
<td>1058.3</td>
<td>9.63</td>
<td>0.324</td>
<td>0.163</td>
</tr>
<tr>
<td>Os(9-18)</td>
<td>GYCKGAFKQT</td>
<td>10</td>
<td>2</td>
<td>1102.3</td>
<td>9.2</td>
<td>0.447</td>
<td>0.266</td>
</tr>
<tr>
<td>Os(11-20)</td>
<td>CKGAFKQTCK</td>
<td>10</td>
<td>3</td>
<td>1113.4</td>
<td>9.39</td>
<td>0.568</td>
<td>0.225</td>
</tr>
<tr>
<td>Os(13-22)</td>
<td>GAFKQTCKCY</td>
<td>10</td>
<td>2</td>
<td>1148.4</td>
<td>8.86</td>
<td>0.511</td>
<td>0.420 (50%)</td>
</tr>
<tr>
<td>Os(11-22)</td>
<td>CKGAFKQTCKCY</td>
<td>12</td>
<td>3</td>
<td>1379.7</td>
<td>9.06</td>
<td>0.565</td>
<td>0.396 (50%)</td>
</tr>
</tbody>
</table>

Note: Nr A.A: Number of amino acids, NC: Net charge, MW: Molecular weight.

<sup>a</sup>Data obtained from Expacy, ProtParam [http://web.expasy.org/cgi-bin/protparam/protparam].

<sup>b</sup>Data obtained from Genscript (MS/C18 RP-HPLC).

<sup>c</sup>Hydrophobic moment, obtained from HeliQuest [heliquest.ipmc.cnrs.fr/].

<sup>d</sup>Hydrophobicity, obtained from HeliQuest [heliquest.ipmc.cnrs.fr/].
3.2 Screening of peptides using the turbidity method

All the peptides were tested for activity against *B. subtilis* and *E. coli* by means of the turbidity method. The turbidity method provides convenient, simultaneous screening of peptides by using a 96-well plate to identify active peptides using a range of peptide concentrations. The MBCs for the respective peptides are listed in Table 3.2.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>MBC (μg/ml)</th>
<th>MBC (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><em>B. subtilis</em></td>
<td><em>E. coli</em></td>
</tr>
<tr>
<td>Os</td>
<td>KGIRGYKGGYCKGAFKQTCKCY</td>
<td>3.75</td>
<td>1.88</td>
</tr>
<tr>
<td>Os(1-10)</td>
<td>KGIRGYKGGY</td>
<td>&gt;120</td>
<td>&gt;120</td>
</tr>
<tr>
<td>Os(3-12)</td>
<td>IRGYKGGYCK</td>
<td>30.0</td>
<td>30.0</td>
</tr>
<tr>
<td>Os(5-14)</td>
<td>GYKGGYCKGA</td>
<td>&gt;120</td>
<td>&gt;120</td>
</tr>
<tr>
<td>Os(7-16)</td>
<td>KGGYCKGAFK</td>
<td>&gt;120</td>
<td>&gt;120</td>
</tr>
<tr>
<td>Os(9-18)</td>
<td>GYCKGAFKQT</td>
<td>&gt;120</td>
<td>&gt;120</td>
</tr>
<tr>
<td>Os(11-20)</td>
<td>CKGAFKQTCK</td>
<td>&gt;120</td>
<td>&gt;120</td>
</tr>
<tr>
<td>Os(13-22)</td>
<td>GAFKQTCKCY</td>
<td>&gt;120</td>
<td>&gt;120</td>
</tr>
<tr>
<td>Os(11-22)</td>
<td>CKGAFKQTCKCY</td>
<td>30.0</td>
<td>60.0</td>
</tr>
</tbody>
</table>

Note: G +: Gram-positive bacteria; G -: Gram-negative bacteria

The MBC of the parent peptide, Os, used as a control, was found to be 3.75 μg/ml against *B. subtilis* and 1.88 μg/ml against *E. coli*, respectively. The MBC values obtained for Os, by the turbidity method correlated with the MBC values obtained by the CFU method (Prinsloo *et al.*, 2013), thus validating the turbidity method as an alternative method for antibacterial screening. Of the peptide fragments tested only Os(3-12) and Os(11-22) were bactericidal. The remaining peptide fragments were inactive even when tested at 120 μg/ml. The MBC of Os(3-12) was found to be 30 μg/ml against both *B. subtilis* and *E. coli*. Os was observed to be 8 and 16 fold more active than Os(3-12) against *B. subtilis* and *E. coli*, respectively. The C-terminus peptide, Os(11-22), containing all three cysteine residues was found to have a MBC of 30 and 60 μg/ml against *B. subtilis* and *E. coli*, respectively. Also it was found that the parent molecule, Os, was shown be 8 and 32 fold more active than Os(11-22) against *B. subtilis* and *E. coli*, respectively.
3.3 Determination of MBC for Os(3-12) and Os(11-22) using the CFU method

The turbidity method may under or overestimate the activity of peptides to some degree, therefore the MBCs of the active peptides were confirmed by the CFU method (Wanniarachchi et al., 2011). The bactericidal activity of the two active peptides was determined and confirmed against both Gram-positive and Gram-negative bacteria. Table 3.3 lists the MBCs of the two active peptides against *E. coli*, *P. aeruginosa*, *S. aureus* and *B. subtilis*.

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>MBC (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Os*</td>
</tr>
<tr>
<td><strong>Gram-negative</strong></td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>1.88 (0.77 µM)</td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td>0.94 (0.38 µM)</td>
</tr>
<tr>
<td><strong>Gram-positive</strong></td>
<td></td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>15.0 (6.10 µM)</td>
</tr>
<tr>
<td><em>B. subtilis</em></td>
<td>1.88 (0.77 µM)</td>
</tr>
</tbody>
</table>

*Included in table for comparison purposes, obtained from Prinsloo et al. (2013)*

The peptides, Os(3-12) and Os(11-22), were active against the bacteria strains tested. Peptide Os(3-12) showed higher activity towards Gram-negative bacteria compared to Os(11-22), whereas Os(11-22) was more active against Gram-positive bacteria compared to Os(3-12). Although both peptides were active against both Gram-positive and Gram-negative bacteria, the MBC values for both peptides ranged from 30 to 120 µg/ml, whereas the MBC values for Os ranged from 1.88 to 15.0 µg/ml.

3.4 Cytotoxic activity of Os(3-12) and Os(11-22) against Caco-2 cells

It is important for AMPs to have low cytotoxicity to eukaryotic cells as peptide selectivity towards bacterial cells is important in pharmaceutical development (Nakajima et al., 2003). The cytotoxicity of Os(3-12) and Os(11-22) against Caco-2 cells was investigated. Melittin, known for its cytotoxicity, was used as positive control (Blondelle & Houghten, 1991) (Fig. 3.1). Neither of the peptides caused a change in cell number using a concentration range of 15 to 120 µg/ml after 24 h exposure (Fig. 3.1 A to E), compared to the untreated cells. Even at 120 µg/ml Os(3-12) and Os(11-22) showed no cytotoxic effects toward Caco-2 cells, while melittin reduced the cell number of Caco-2 cells by approximately 20%.
Figure 3.1: (A) Cytotoxicity of Os(3-12) (purple), Os(11-22) (green) and melittin (control – yellow) against Caco-2 cells. Untreated cells, without peptide, are shown in blue. Cell number determined using the CV assay. Data are expressed as means ± SEM of two independent experiments. Statistical analysis was performed using one-way ANOVA and showed a statistically significant difference between cytotoxic effects of the peptides, Os(3-12) and Os(11-22), and melittin at 120 μg/ml with *p ≤ 0.05. Images B to E show light microscopy images of CV stained Caco-2 cells in the presence of the highest cytotoxic concentrations of (B) Os(3-12), (C) Os(11-22), (D) melittin (positive control) and (E) untreated cells (negative control). The concentration range in μM corresponding to the concentration range in μg/ml are indicated as follows for Os(3-12) (13 to 105 μM), Os(11-22) (11 to 87 μM) and melittin (5.2 to 42 μM).
3.5 Determination of the MBC of the amidated peptide

Amidation increases the overall positive charge and stability of peptides, leading to increased antimicrobial activity (Arispe et al., 2008). It was therefore investigated whether amidation would increase the activity of Os(11-22). Since Os(11-22) showed higher activity towards Gram-positive bacteria compared to Os(3-12) and given that S. aureus is well known for its antibiotic resistance and cause of devastating diseases as well as one of the main causes of infection and reduced healing in wounds, Os(11-22) was synthetically amidated (Halbert et al., 1992; Bowler et al., 2001). The bactericidal activity of Os(11-22)NH$_2$ was compared to that of Os and Os(11-22) against representative bacteria and is shown in Table 3.4.

Table 3.4: Determination of the MBC of Os, Os(11-22) and Os(11-22)NH$_2$

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>MBC (µg/ml)</th>
<th>Os*</th>
<th>Os(11-22)</th>
<th>Os(11-22)NH$_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gram-negative</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. coli</td>
<td>1.88 (0.77 µM)</td>
<td>120 (87.0 µM)</td>
<td>3.75 (2.73 µM)</td>
<td></td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td>0.94 (0.38 µM)</td>
<td>120 (87.0 µM)</td>
<td>3.75 (2.73 µM)</td>
<td></td>
</tr>
<tr>
<td><strong>Gram-positive</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. aureus</td>
<td>15.0 (6.10 µM)</td>
<td>30.0 (21.7 µM)</td>
<td>60.0 (43.5 µM)</td>
<td></td>
</tr>
<tr>
<td>B. subtilis</td>
<td>1.88 (0.77 µM)</td>
<td>30.0 (21.7 µM)</td>
<td>1.88 (1.37 µM)</td>
<td></td>
</tr>
</tbody>
</table>

*Included for comparison purposes, obtained from Prinsloo et al. (2013)

Os(11-22)NH$_2$ (12 amino acids, net charge of +4) was more active against the tested bacterial strains compared to Os(11-22), except for S. aureus, where Os(11-22) was 2 fold more active (Table 3.4). The parent peptide, Os, was 2 and 4 fold more active than Os(11-22)NH$_2$ against E. coli and P. aeruginosa, respectively, 4 fold more active than Os(11-22)NH$_2$ against S. aureus and equally active as Os(11-22)NH$_2$ against B. subtilis. Overall, Os still possessed higher antibactericidal activity compared to the peptides tested.

3.6 Secondary structure prediction and helical wheel projections

AMPs are known to be mostly unstructured in aqueous solutions, but to adopt secondary structures when in contact with a membrane or membrane-mimicking environment (Carotenuto et al., 2008). In order to understand the function of proteins or peptides, it is necessary to predict and determine their secondary structure. The secondary structures of Os,
Os(11-22) and melittin were predicted using PSIPRED, a computer-based secondary structure prediction program, as shown in Figure 3.2 (Jones, 1999). Melittin is well known to be unstructured in water and assembles into an α-helical structure in membrane environments (Bello et al., 1982; Raghuraman & Chattopadhyay, 2005; 2007) and was therefore used as a control in both secondary structure prediction and determination.

Figure 3.2: PSIPRED predicted images of (A) Os, (B), Os(11-22) and (C) melittin (Jones, 1999).
The secondary structure of melittin was predicted to be almost entirely \( \alpha \)-helical. Os was predicted to contain some \( \alpha \)-helical content, with Os(11-22) being mostly \( \alpha \)-helical. A summary of the predicted secondary structures of the peptides is shown in Table 3.5. Due to limitations associated with the prediction program, an amidation group cannot be entered into the program and therefore the secondary structure of Os(11-22)NH\(_2\) could not be predicted.

Table 3.5: Summary of the predicted secondary structures of Os, Os(11-22) and melittin

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Secondary structure constituent</th>
<th>% Helix</th>
<th>% ( \beta )-Strand</th>
<th>% Coil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Os</td>
<td></td>
<td>32.0</td>
<td>0</td>
<td>68.0</td>
</tr>
<tr>
<td>Os(11-22)</td>
<td></td>
<td>66.7</td>
<td>0</td>
<td>33.3</td>
</tr>
<tr>
<td>Melittin</td>
<td></td>
<td>88.5</td>
<td>0</td>
<td>11.5</td>
</tr>
</tbody>
</table>

Helical wheels are used as an indication of the helical properties of peptides or proteins if the peptide indeed possess an \( \alpha \)-helical structure (Schiffer & Edmundson, 1967). These wheels represent projections of the amino acid side-chains of peptides or proteins onto a plane perpendicular to the axis of the helix, easily visualizing possible side-chain interactions and general characteristics of helices. Helical wheel projections of Os, melittin and Os(11-22) are shown in Fig. 3.3. The helical wheels show the arrangement and segregation of hydrophobic and hydrophilic amino acids of the peptides, rendering them amphipathic, providing the probability of possessing antibacterial activity. For melittin and Os(11-22) the segregation of hydrophobic and hydrophilic amino acids to opposite sides of the molecules is more visible (Fig. 3.3 B and C).

Figure 3.3: Helical wheel projections of (A) Os, (B) melittin and (C) Os(11-22) generated by HeliQuest (Gautier et al., 2008). Hydrophobic residues are in yellow, including alanine shown in gray circles, cationic residues are shaded blue, and polar residues are in purple and pink circles, as well as glycine shown in gray and argenine and lysine shown in blue.
3.7 Secondary structure determination using CD spectroscopy

CD spectroscopy is used for determining whether a protein is folded, and if so characterizing its secondary structure, which can provide information about the activity of the peptide. Many peptides are reported to have relatively unstructured or extended conformations in solution and to fold into their correct secondary structure when in contact with a membrane (Tossi et al., 2000). SDS was therefore used as a membrane-mimicking agent (Nguyen et al., 2010). SDS forms micelles (critical micelle concentration = 6 to 8 mM) and acts as a bacterial membrane-mimicking agent that possesses an anionic outer surface and a hydrophobic inner core (Carotenuto et al., 2008).

The secondary structures of melittin, Os(11-22) and Os(11-22)NH$_2$ (Fig. 3.4 A to C), were determined using CD spectroscopy in 25 mM SDS, water and 10 mM SPB. The CD spectra in water and buffer were determined because the peptides were diluted in water and the majority of the assays were performed in buffer.

![Figure 3.4: The effects of solvents on the CD spectra of melittin, Os(11-22) and Os(11-22)NH$_2$. The far UV CD spectra shown are those of (A) melittin, (B) Os(11-22) and (C) Os(11-22)NH$_2$ in water, 25 mM SDS and SPB recorded on a Jasco J-810 spectropolarimeter using 200 nm/min scan speed, 0.5 s time constant, 0.5 nm bandwidth and accumulation of 10 scans in each case.](image-url)
Alpha-helix, β-sheet and random coiled structures each bring about a characteristic profile on the CD spectrum (Fig. 2.1 A). Evaluation of the CD spectra of melittin (Fig. 3.4 A), a clear characteristic α-helical profile in SDS was observed with an unstructured conformation in water and buffer. No clear characteristic profile on the CD spectrum was observed for Os(11-22) (Fig. 3.4 B) in the respective solvents. The CD spectrum for Os(11-22)NH₂ (Fig. 3.4 C) showed a characteristic random coiled structure in SDS and no clear structure was observed in the remaining solvents.

Alpha-helix, β-sheet and random coiled structures also give rise to a characteristic magnitude of CD spectra (Fig. 2.1 B) (Kelly et al., 2005). According to the features shown in Figure 2.1, the CD spectra showed that in SDS, Os(11-22)NH₂ exhibited a random coiled structure, whereas Os(11-22) and melittin exhibited α-helical structures with two minima around 209 and 222 nm. In water the CD spectra showed that Os(11-22) and Os(11-22)NH₂ exhibited random coiled structures, whereas Os and melittin were unstructured. The CD spectra revealed that in buffer all peptides had unstructured conformations. These results are summarised in Table 3.6.

Table 3.6: Summary of the secondary structures of Os(11-22), Os(11-22)NH₂ and melittin according to CD spectra magnitude

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Os(11-22)</th>
<th>Os(11-22)NH₂</th>
<th>Melittin</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDS</td>
<td>α-helical</td>
<td>Random coiled</td>
<td>α-helical</td>
</tr>
<tr>
<td>Water</td>
<td>Random coiled</td>
<td>Random coiled</td>
<td>Unstructured</td>
</tr>
<tr>
<td>Buffer</td>
<td>Unstructured</td>
<td>Unstructured</td>
<td>Unstructured</td>
</tr>
</tbody>
</table>

3.8 Effects of NaCl and serum on the activity of Os(11-22)NH₂

Increased concentrations of NaCl and serum have been reported to influence the activity of AMPs. For this reason, the effect of 100 mM NaCl and 30% human serum on the MBC of Os(11-22)NH₂ was determined against *E. coli* and *S. aureus*, as representative Gram-negative and Gram-positive strains, respectively (Table 3.7).
Table 3.7: MBC of Os(11-22)NH\(_2\) in the presence of SPB, 100 mM NaCl and 30% human serum

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>MBC of Os(11-22)NH(_2) (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>In SPB</td>
</tr>
<tr>
<td><em>E. coli</em> (Gram -)</td>
<td>3.75 (2.73 (\mu)M)</td>
</tr>
<tr>
<td><em>S. aureus</em> (Gram +)</td>
<td>60.0 (43.5 (\mu)M)</td>
</tr>
</tbody>
</table>

\(^a\)NaCl dissolved in 10 mM SPB to obtain final reaction concentration of 100 mM

\(^b\)human serum diluted in water (30% v/v final reaction concentration)

From Table 3.7 it can be seen that the MBC of Os(11-22)NH\(_2\) increased from 3.75 \(\mu\)g/ml to 15 \(\mu\)g/ml against *E. coli* and lost activity against *S. aureus* in the presence of NaCl. In the presence of serum the MBC of Os(11-22)NH\(_2\) increased from 3.75 \(\mu\)g/ml to 120 \(\mu\)g/ml against *E. coli* and unexpectedly decreased from 60 \(\mu\)g/ml to 7.5 \(\mu\)g/ml against *S. aureus*.

### 3.9 Kinetics of bactericidal activity for Os(11-22)NH\(_2\)

The kinetics of bactericidal activity of Os(11-22)NH\(_2\) was determined using *E. coli* and *B. subtilis*, as representative Gram-negative and Gram-positive strains, respectively (Fig 3.5).

![Figure 3.5: Kinetics of bactericidal killing of Os(11-22)NH\(_2\) (red) against (A) *E. coli* and (B) *B. subtilis*. The log CFU/ml of Os(11-22)NH\(_2\) is given in comparison with the growth control (blue) wherein no peptide was added. Data are expressed as means ± SEM of at least two independent experiments in triplicate.](image-url)

The study showed that Os(11-22)NH\(_2\) eliminated 100% of Gram-negative (Fig. 3.5 A) and Gram-positive bacteria (Fig. 3.5 B) within 120 min and 30 min, respectively. The killing rate
was slower compared to Os, which killed both bacterial strains within 5 min (Prinsloo et al., 2013).

### 3.10 Haemolytic activity of Os(11-22)NH$_2$

Besides having significant antibacterial activity, for the further development as a pharmaceutical agent, Os(11-22)NH$_2$ must be shown to be non-toxic to eukaryotic cells. Os(11-22)NH$_2$ was tested against human erythrocytes for haemolytic activity (Fig. 3.6). Melittin was used as a positive control as it possesses both antimicrobial and haemolytic activity (Blondelle & Houghten, 1991). The peptides were tested at a concentration range of 0.78 to 100 μM (Os(11-22)NH$_2$; 1.07 to 138 μg/ml and melittin 2.22 to 284.6 μg/ml).

![Figure 3.6: Haemolytic activity of Os(11-22)NH$_2$ (blue) and melittin (green) against human erythrocytes. Cells incubated with 2% SDS (control-red) represent 100% haemolysis. % Haemolysis caused by Os(11-22)NH$_2$ is represented relative to haemolysis caused by SDS. Values are represented as means ± SEM of three independent experiments in triplicate. Linear regression analysis showed an equation of y = 24.20x + 25.80 with correlation coefficient ($R^2$) value of 0.997.](image)

Even the highest concentration (100 μM) of Os(11-22)NH$_2$ showed no erythrocyte haemolysis (Fig. 3.6). In comparison, melittin caused a dose-dependent increase in haemolysis ($R^2 = 0.997$).
3.11 Cytotoxic effects of Os(11-22) and Os(11-22)NH₂ against SC-1 cells

Prinsloo et al. (2013) showed that Os had no cytotoxic effect towards Caco-2 and SC-1 cells. Based on these findings, SC-1 cells were more susceptible towards cytotoxicity caused by melittin and since Os(11-22) was amidated and as a result, showed stronger antibacterial activity, the cytotoxicity of Os(11-22) and Os(11-22)NH₂ on SC-1 cells was investigated (Fig. 3.7).

![Graph showing cytotoxicity of Os(11-22)NH₂, Os(11-22), and melittin against SC-1 cells](image)

**Figure 3.7:** (A) Cytotoxicity of Os(11-22)NH₂ (red), Os(11-22) (blue) and melittin (positive control – green) against SC-1 cells. Untreated cells, without peptide, are shown in purple. Cell number determined using the CV assay. Values are expressed as means ± SEM of two independent experiments. Statistical analysis was performed using one-way ANOVA and showed statistically significant difference between cytotoxic effects of Os(11-22)NH₂, Os(11-22) and melittin at 120 μg/ml, *p ≤ 0.05. Images B to E show light microscopy images of CV stained SC-1 cells at the highest cytotoxic concentrations of (B) Os(11-22)NH₂, (C) Os(11-22), (D) Melittin (positive control) and (E) Untreated cells (negative control). The concentration range in μM corresponding to the concentration range in μg/ml are indicated as follows for Os(11-22)NH₂ (1.4 to 87 μM), Os(11-22) (11 to 87 μM) and melittin (0.7 to 42 μM).
Os(11-22) did not cause significant changes in cell number compared to the untreated cells, using a concentration range of 15 to 120 μg/ml after 24 h exposure (Fig. 3.7 A and C). No significant change in cell number was observed for Os(11-22)NH₂ at concentrations 1.88 to 30 μg/ml, however a significant increase in cell number was observed for Os(11-22)NH₂ at 120 μg/ml (Fig. 3.7 A and B), compared to untreated cells. At both 30 and 120 μg/ml, neither Os(11-22) nor Os(11-22)NH₂ showed cytotoxic effects toward SC-1 cells, whereas melittin showed a significant decrease, of approximately 23%, in SC-1 cell number.

3.12 Antioxidant activity of Os(11-22)NH₂

Antimicrobial peptides containing antioxidant activity have increased beneficial effects related to clinical applications. The parent peptide, Os, was shown to possess antioxidant activity (Prinsloo et al., 2013). It was investigated whether truncation of Os by 10 amino acids to yield Os(11-22)NH₂, retained its antioxidant activity. Glutathione (GSH), a known naturally occurring antioxidative peptide, was used as positive control and compared to Os(11-22)NH₂ for antioxidant activity (Fig. 3.8). The values obtained from this method were normalized to μM Trolox equivalents (μM TE).

Figure 3.8: Antioxidant activity of Os(11-22)NH₂ and GSH. ORAC antioxidant capacity for Os(11-22)NH₂ (green) and GSH (blue) expressed as μM TE. Data are expressed as means ± SEM of three independent experiments in triplicate. Linear regression analysis showed an equation of \( y = 6.309x + 13.72 \) with correlation coefficient \( R^2 \) value of 0.998.
A dose dependant linear increase ($R^2 = 0.998$) in antioxidant activity was observed for Os(11-22)NH$_2$ (Fig. 3.8). From this data, a molar ratio of 6.30 µM TE/µM peptide was determined for Os(11-22)NH$_2$. The antioxidant activity of Os was reported to be 21.6 µM TE/µM peptide, which is 3.4 fold higher than the molar ratio for Os(11-22)NH$_2$. The result for GSH is in agreement with previous reported results (Prinsloo et al., 2013). Even at four times lower concentration (1.2 µM), Os(11-22)NH$_2$ exhibited significantly higher antioxidant activity than the naturally occurring peptide, GSH.
Ticks rely solely on an innate immune defence of which AMPs are key components providing protection against microbes, such as bacteria, fungi and viruses. Due to their unique feeding behaviour, ticks have many opportunities to encounter microbes and therefore produce a variety of antimicrobial peptides, like defensins (Lu et al., 2010a). Bacterial resistance to antibiotics has increased considerably with their widespread use, therefore the need for new antibiotics. Synthetic AMPs are promising candidates for the development of novel antibacterial agents (Li et al., 2012). Some limitations associated with development of AMPs as novel antibiotics are high synthesis cost, cytotoxicity to eukaryotic cells and reduced activity in biological fluids (Loose, 2007; Prinsloo et al., 2013). The overall aim of this study was to determine whether shorter peptides derived from Os would retain their antibacterial activity, antioxidant activity as well as low cytotoxicity.

Of the eight peptide fragments screened for antibacterial activity by the turbidity method, only two peptides were found to be active, namely Os(3-12) and Os(11-22) (Table 3.2). Os(3-12) was bactericidal to both E. coli and B. subtilis (MBC of 30 μg/ml), whereas Os(11-22) was more active against B. subtilis (MBC of 30 μg/ml) than E. coli (MBC of 60 μg/ml). The fact that Os(13-22) and Os(11-20), which lack the two N-terminal amino acids (cysteine and lysine) and two C-terminus amino acids (cysteine and tyrosine), respectively, were devoid of activity, suggests that these residues are essential for the activity of Os(11-22). Comparing Os(13-22) to Os(11-22), loss of activity could possibly be as a result of the reduced net charge of Os(13-22) which is +2, whereas for Os(11-22) it is +3. In the case of Os(11-20), which also has a net charge of +3, the activity of this peptide compared to Os(11-22) could be due to its reduced hydrophobicity (<H> = 0.225 for Os(11-20) versus <H> = 0.396 for Os(11-22)).

Previous studies have shown that the functional region of insect defensins is located in a γ-core motif of the C-terminus domain (Yount & Yeaman, 2004). The γ-core comprises of a conserved GXCXₙC or the inverted CXGXₙC motif (X being any amino acid and n, any number of amino acids). Conformation alignments prepared by Yount et al., (2004), revealed the γ-core motif to be conserved across all classes of disulfide-stabilized antimicrobial
peptides. This motif has been conserved regardless of amino acid site-specific or orientation-specific variations in primary sequence. In some cases, the γ-core alone is sufficient for antimicrobial activity. Additional features that characterize the γ-core include, net positive charge, hydrophobicity to yield peptide amphipathicity and formation of one to four disulfide bonds.

Wang and Zhu (2011) reported similar results when they synthesized a peptide fragment corresponding to the γ-core of scapularisin-20, (scapularisin-20 amino acids (26-39), amidated, net charge +4) isolated from *I. scapularis*. The short 14 amino acid peptide showed slightly more potency to Gram-positive bacteria (LC ranged from 10 to 20 μM) than Gram-negative bacteria (LC ranged from 15 to 33 μM), consistent with the functional feature of insect defensins (Yeaman & Yount, 2003). Peptide, Os(11-22), could correspond to the γ-core of Os, as sequence similarity was present between scapularisin-20(26-39) and Os(11-22) (Fig. 4.1). The conserved γ-core motif, CXGX₄C, is present in Os(11-22) and may contribute to increased antibacterial activity against Gram-positive bacteria.

Figure 4.1: Sequence similarities of scapularisin-20(26-39) and Os(11-22). Different colours highlight the same amino acids present in both peptides. The γ-core motif, CXGX₄C, is underlined.

Another interesting observation was that the N-terminal region of Os, Os(1-10), was inactive but the fragment lacking the first two N-terminal amino acids and extended with cysteine and lysine, Os(3-12), was active against both Gram-positive and Gram-negative bacteria (Table 3.2). Compared to Os(1-10), Os(3-12) has the same charge, but their hydrophobicities differ, in that Os(3-12) has a higher <H> (0.227) than Os(1-10) (<H> = 0.072) (Table 3.1), possibly contributing to higher antimicrobial activity.

Following initial screening the MBCs of Os(3-12) and Os(11-22) were confirmed and determined by the CFU method for representative Gram-positive and Gram-negative bacteria. Both peptides showed antibacterial activity against both Gram-positive and Gram-negative bacteria (Table 3.3). Similar to the turbidity method, the CFU method indicated that Os(3-12) and Os(11-22) was bactericidal against *E. coli* and *B. subtilis* in the micromolar range (Table
Os(3-12) was equally active against *E. coli* and *B. subtilis* (60 μg/ml; 52 μM) and approximately 32 fold less active than Os (1.88 μg/ml; 0.77 μM). The peptide fragment, Os(11-22), was overall more active against Gram-positive bacteria (30 μg/ml; 21.7 μM), whereas it was less effective against Gram-negative bacteria (120 μg/ml; 87 μM) (Table 3.3). When the activity of Os(11-22) was compared to Os, it was 63 fold less active against *E. coli* and 16 fold less active against *B. subtilis*.

Wang and Zhu (2011) determined the MBC of the amidated scapularisin 20(26-39) peptide as previously mentioned. This peptide showed activity against Gram-positive and Gram-negative bacteria in the low micromolar range. More than 50% of peptide hormones and neurotransmitters require amidation through post-translational modifications at their C-terminus to reveal their full biological activities (Eipper *et al.*, 1992; Mirabeau *et al.*, 2007). Amidation is advantageous since the C-terminus of the peptide becomes uncharged, increasing the overall positive charge as well as stability towards carboxypeptidases, allowing it to closely mimic the peptide’s natural structure and to ultimately lead to increased biological activity of a peptide. In addition, an increase in positive charge can lead to increased antibacterial activity (Arispe *et al.*, 2008). Since Os(11-22) correspond to the γ-core of Os and because Gram-positive bacteria (mainly gram-positive cocci) like *S. aureus*, are especially important pathogens in the hospital environment, this peptide was synthetically amidated. It was then investigated whether amidation would increase the antibacterial activity of Os(11-22).

The bactericidal activity of the amidated peptide, Os(11-22)NH$_2$, was determined using the CFU method and compared to the activity of Os and unamidated Os(11-22) (Table 3.4). The amidated peptide showed increased bactericidal activity, against Gram-negative and Gram-positive bacteria, but was most potent against Gram-positive *B. subtilis*. The MBC against Gram-negative bacteria reduced from 120 μg/ml (87 μM) to 3.75 μg/ml (2.73 μM) with an enhancement in activity of 32 fold. The MBC against Gram-positive bacteria (*B. subtilis*) reduced from 30 μg/ml (21.7 μM) to 1.88 μg/ml (1.37 μM), with an enhancement in activity of 16 fold, however amidation decreased the activity of Os(11-22) against *S. aureus* by 2 fold. The parent peptide, Os, was equally active as Os(11-22)NH$_2$ against *B. subtilis*, but 2 fold more active against *E. coli* (Table 3.4). Taking in consideration that ten N-terminal amino acids of Os were removed to form Os(11-22)NH$_2$, it still possesses strong antibacterial activity in the low micromolar range (Table 3.4). Similar results were observed by Wang and
Zhu (2011), but Os(11-22)NH₂ was 11 fold more active against *B. subtilis* and 4 fold more active against *P. aeruginosa*, when compared to scapularisin 20(26-39).

Since an interaction between AMPs and the bacterial cytoplasmic and outer membrane is necessary to ensure antibacterial activity, it is important for peptides to be able to form amphipathic structures composed of both hydrophobic and hydrophilic amino acid residues to provide membrane interaction (Gifford et al., 2005). The charged and hydrophobic residues allow peptides to interact with negatively charged phospholipid head groups and hydrophobic fatty acid chains of microbial membranes, respectively (Gifford et al., 2005; Giuliani et al., 2007). All the peptides tested, showed amphipathic properties, projected by their respective helical wheels (Fig. 3.3), containing both hydrophilic (Cys, Arg, Lys, Gln and Thr) and hydrophobic (Ile, Ala, Phe and Tyr) amino acid residues. The amphipathicity of the peptides contribute to their antibacterial activity.

Although both Os(3-12) and Os(11-22) have the same net positive charge, they exhibit different antibacterial activities. Differences in the activities of Os(3-12) and Os(11-22) can be ascribed to different $\langle H \rangle$ and $<\mu H>$ of each peptide. Described by Eisenberg et al. (1984), $<\mu H>$ is the measure of the separation of the hydrophobic and hydrophilic segments of a peptide and aids in recognizing amphipathic structures by identifying when the residues on one side of the peptide are more hydrophobic than on the other (Eisenberg et al., 1984). Hydrophobic interactions play a major role in the activity of AMPs with phospholipid bilayers. Higher $\langle H \rangle$ and $<\mu H>$ values are an indication of higher antimicrobial activity (Carotenuto et al., 2008). Since Os(11-22) ($\langle H \rangle = 0.396; <\mu H> = 0.565$) has a higher $\langle H \rangle$ and $<\mu H>$ values than Os(3-12) ($\langle H \rangle = 0.227; <\mu H> = 0.406$) it can be predicted to possess better antibacterial activity. This is true for the peptides against Gram-positive bacteria. Even though the $\langle H \rangle$ (0.277) and $<\mu H>$ (0.249) values of Os are less than that for Os(11-22), Os revealed higher bactericidal activity since Os has a net charge of +6. When the C-terminus of Os(11-22) was amidated the charge increased from +3 to +4, which led to increased antibacterial activity, corresponding to the advantages described for peptide amidation.

Another reason for the difference in activity between the peptides could be due to different structures adopted by each peptide in the presence of a membrane environment. SDS micelles were used as a membrane-mimicking environment and are generally considered to be good models for bacterial membranes because it possesses an anionic (hydrophilic) outer surface.
and a hydrophobic inner core (Carotenuto et al., 2008). CD aids in identifying the secondary structure of a peptide, if at all present (Fábio et al., 2006). Alpha-helix, β-sheet, and random coil structures each produce a distinguishable shape and magnitude on the CD spectrum (Fig. 2.1). Generally, CD spectroscopy provides the most accurate results for helical structures, because helices tend to have a more regular conformation compared to β-sheet structures. Beta-sheets tend to be more variable in conformation, containing both parallel and antiparallel orientations of adjacent strands and different twists of each strand, leading to varying CD spectra (Wallace et al., 2004; Whitmore & Wallace, 2008). CD spectroscopy is quite sensitive to determine changes in the secondary structure of peptides or proteins with changes in solvent composition or interactions with biomolecules. However, deduction of the secondary structure of peptides from CD data has caused some difficulty (Manavalan & Johnson, 1985).

Previous studies showed that AMPs possessing α-helical and β-sheet conformations have increased antimicrobial activity (Cornet et al., 1995; Tsuji et al., 2007). In this study, melittin was used as a control and found to have a characteristic α-helical structure in a membrane-mimicking environment, whereas an unfolded structure was observed in water and buffer (Fig. 3.4 A) (Bello et al., 1982; Raghuraman & Chattopadhyay, 2005). It is evident that different solvents induce a change in the secondary structure of peptides. The CD spectroscopy data clearly showed that melittin, like most peptides, requires an environment with some hydrophobicity to form its distinct secondary structure (Nguyen et al., 2010). The conformational changes of melittin induced upon its interaction with SDS were more pronounced than the conformational changes of the other peptides induced upon their interaction with SDS, indicating that the α-helical content of melittin is higher than those of the other peptides in a membrane environment (Unneberg et al., 2001; Carotenuto et al., 2008).

Far-UV CD data showed that in membrane-mimicking environment Os(11-22) has α-helical properties, whereas Os(11-22)NH₂ has a random coiled conformation (Fig. 3.4). CD data for Os(11-22) (Fig. 3.4 B) indicated a random coiled conformation in water, whereas no defined structure was observed in buffer, thus rendering it unfolded. According to the CD data Os(11-22)NH₂ has a random coiled structure in water with no defined structure in buffer (Fig. 3.4 C). According to Prinsloo et al. (2013), Os was found to be an α-helix in SDS, but lacked a definite structure in water and buffer and therefore, was unfolded. Truncation of 10 amino
acids of Os, at the N-terminus to yield Os(11-22), retained its α-helical structure, but to a lesser degree. Os and Os(11-22) had α-helical conformations in SDS, but Os possessed higher antibacterial activity than Os(11-22). This can be as a result of Os having a stronger α-helical tendency than Os(11-22), as the CD minima at 208 and 222 nm are much larger (approximately -40000 and -20000 [θ] for Os and -5000 and -1500 [θ] for Os(11-22)) possibly contributing to the increased antimicrobial activity and α-helical structure of Os (Prinsloo et al., 2013). It was evident that truncation of 10 N-terminal amino acids of Os, following C-terminus amidation, led to a change in secondary structure, from mostly an α-helical to a random coiled structure and could be a reason, besides the difference in their charge, for the difference in antibacterial activities observed between Os and Os(11-22)NH₂.

The CD data also showed that the secondary structures for Os(11-22) and Os(11-22)NH₂ were similar in water and buffer, but differed in the presence of a membrane-mimicking environment. Os(11-22)NH₂ tends to have a more defined structure compared to Os(11-22) in a membrane-mimicking environment. The characteristic α-helical shape observed for Os(11-22) was not as obvious as the characteristic random coiled shape observed for Os(11-22)NH₂ on the CD spectrum in SDS (Fig. 3.4 B and C). This can be an indication that Os(11-22) has a weak α-helical structure and can in turn affect its antibacterial activity (Schibli et al., 1999). Since both peptides, Os(11-22) and Os(11-22)NH₂, were diluted without the addition of reducing agent, the peptides could have undergone oxidation, thereby inducing the formation of intra-disulfide bonds. Intra-disulfide bond formation can increase the stability of the peptide and possibly lead to increased activity, expecting similar activities for both peptides. However, Os(11-22)NH₂ was more active than Os(11-22). The increase in antibacterial activity of Os(11-22)NH₂ can be as a result of Os(11-22)NH₂ having a more defined conformation, due to increased structure stability caused by C-terminus amidation.

It can be concluded that the stability of the peptides as well as their secondary structures are important for antibacterial activity. Overall, the CD spectra for Os(11-22) showed that the structures obtained in the different solvents, did not appear to contain any regular backbone secondary structure and that the peptides probably adopt a conformation to yield the best separation of the hydrophobic and basic amino acid residues (Schibli et al., 1999).

All the peptides discussed have antibacterial activity, but their secondary structures differ, which indicates that these peptides could have different modes of action. The mode of action
of AMPs has been mostly focused on the interaction of cationic amphipathic α-helical peptides with model bacterial membrane systems (Brogden, 2005). Peptides that do not take on an α-helical conformation, would not be expected to orient themselves parallel to the surface of a bacterial membrane, observed with many α-helical AMPs that are associated with membrane disruption (Oren & Shai, 1998; Brogden, 2005; Respondek et al., 2007; Nguyen et al., 2010). It seems that an α-helical structure is not necessary for activity, since this has also been described previously (Oren et al., 1997; Oren & Shai, 1997; Shai, 1999). The mode of action of the shorter AMPs can target intracellular components, following peptide translocation across the membrane (Tossi et al., 2000). This has been found previously for short peptides as small as six residues (Hunter et al., 2005; Rezansoff et al., 2005; Nguyen et al., 2010).

The majority of AMPs are active in buffers, like SPB or PBS, but they show a reduction in their antibacterial activity in the presence of salts and complex biofluids such as serum (Marr et al., 2006). Biological fluids can inhibit the antimicrobial activity of peptides in several ways. Increased salt concentrations found in serum may compete with the peptides for specific binding sites on the surface of bacterial cells (Bellamy et al., 1992; Maisetta et al., 2008). Anionic proteins, like albumin, may bind to AMPs, lower their effective concentrations or reduce their accessibility to bacterial membranes by blocking the peptide binding sites (Bowdish et al., 2005; Svenson et al., 2007). Serum proteases may digest AMPs, thus rendering them inactive (Bowdish et al., 2005; Maisetta et al., 2008). The effect of increased NaCl concentration and serum was investigated on the MBC of Os(11-22)NH₂ and compared to Os. Serum is similar to plasma, but in serum fibrinogen and prothrombin are absent. Serum is a mixture of water, polysaccharides, lipids, salts and proteins. Albumin and globulin are major protein components found in serum. It is therefore important to test the activity of the peptides in conditions that more closely resemble those found in vivo (Maisetta et al., 2008).

The MBC of Os(11-22)NH₂ against E. coli was low (3.75 μg/ml) in SPB and increased 4 fold (15 μg/ml) and 32 fold (120 μg/ml) in the presence of 100 mM NaCl and 30% non-heat inactivated serum, respectively (Table 3.7). In other words, a decrease in peptide antibacterial activity was observed. The MBC of the parent peptide, Os, was not affected in NaCl against E. coli (1.88 μg/ml), but increased 32 fold in the presence of serum (unpublished data). Os(11-22)NH₂ did not have strong bactericidal activity against S. aureus in SPB (60 μg/ml).
and completely lost activity in 100 mM NaCl. In contrast, the activity of Os(11-22)NH$_2$ in 30% serum, increased 8 fold (from 60 in SPB to 7.5 μg/ml) against *S. aureus* (Table 3.7). This result was unexpected since the serum used was not heat inactivated, thus basal levels of serum proteases may still be present. In comparison, the MBC of Os, in 100 mM NaCl increased 32 fold (from 3.75 to 120 μg/ml), against *S. aureus*, whereas the MBC was unchanged in 30% serum (unpublished data). Maisetta and colleagues (2008) showed that hBD-3 was completely inhibited against *S. aureus* (MBC > 256 μg/ml) in the presence of NaCl and serum, whereas the opposite was found in the author’s study.

Binding of anionic albumin proteins to peptides has been recorded to reduce activity by decreasing the effective concentration of the peptide at the bacterial surface (Svenson *et al.*, 2007). In a previous study it was shown that HNP-1 completely lost its activity in the presence of NaCl against *E. coli* and *S. aureus* (Nagaoka *et al.*, 2000). Varkey *et al.* (2006) showed that the activities of PAΔC and OMΔC against *E. coli* were not affected in the presence of NaCl, but that the bactericidal activity is reduced by approximately 50% against *S. aureus*.

Salt inhibition of Os(11-22)NH$_2$ against Gram-positive bacteria strain was more noticeable compared to Gram-negative bacteria. A possible reason for the selectivity is the presence of molecules such as teichoic acids, on the cell walls of Gram-positive bacteria, with high affinity to mono and divalent cations, which could interfere with the electrostatic interactions between the peptide and bacterium (Brogden, 2005). As serum was not heat inactivated and proteolytic enzymes still present, the peptide does not seem to be affected by the proteolytic enzymes when tested against *S. aureus*, but against *E. coli* the activity decreased, but was still retained, in serum. The different effects observed in serum for Os(11-22)NH$_2$ against the two different bacteria strains could more likely be related to the bacterial strains rather than the effects of serum on the peptide. If serum affected the peptide, the results observed against the bacteria should be similar but this is not the case. Also, if albumin indeed binds to the peptide, rendering it inactive, then the MBC against *S. aureus* should also have increased in serum. This again indicates that the effects observed for Os(11-22)NH$_2$ in serum can be related to the bacterial strains tested and that serum not only affects the peptide, if at all, but also the bacterial strain. The morphology of the bacterial membrane could be affected by serum, causing the observed results.
The kinetics of bactericidal activity was performed with Os(11-22)NH₂. The rate of killing for Os(11-22)NH₂, at its MBC, was 4 fold more rapid against Gram-positive bacteria (killed after 30 min) compared to Gram-negative bacteria (killed after 120 min) (Fig. 3.5). The killing rate of Os was determined previously by Prinsloo et al. (2013) and found that Os killed both Gram-positive and Gram-negative bacteria within 5 min. Previous studies also showed faster killing of Gram-positive bacteria compared to Gram-negative bacteria by AMPs (Bellamy et al., 1992). However, Varkey et al. (2006), showed that the synthetic defensin peptides, PAΔC and OMΔC, killed Gram-negative bacteria more rapidly as compared to Gram-positive bacteria (S. aureus).

The difference in MBC and killing kinetics for Os(11-22)NH₂ against Gram-positive and Gram-negative bacteria could be an indication of different modes of action caused by the differences in the cell wall compositions of Gram-positive and Gram-negative bacteria (Fig. 1.2). Briefly, the cell envelope of Gram-negative bacteria is composed of an outer membrane, single peptidoglycan layer and an inner cytoplasmic membrane. The outer membrane of Gram-negative bacteria contains a negatively charged component, LPS. The delayed killing effect of Os(11-22)NH₂ against E. coli can be due to Os(11-22)NH₂ first having to cross the outer membrane, as the outer membrane can act as a barrier blocking direct contact of the peptides to the cytoplasmic membrane (Hartmann et al., 2010). LPS structure variations in the outer membrane have also been shown to affect the susceptibility of Gram-negative bacteria to peptides (Bellamy et al., 1992). Gram-positive bacteria on the other hand, consist of several layers of peptidoglycan and is absent from an outer membrane. A group of negatively charged molecules, teichoic acids, are found perpendicularly to the peptidoglycan layers, which are unique to the Gram-positive cell wall (Todar, 2008). Several AMPs do not cause membrane permeabilization, instead they translocate across the cytoplasmic membrane and accumulate intracellularly. Once inside the cell these peptides can target multiple cellular processes, including inhibition of nucleic acid synthesis, protein synthesis and cell wall synthesis.

The more rapid bactericidal effect of Os(11-22)NH₂ against Gram-positive bacteria is most likely due to the absence of a protective outer membrane. Initially, the cationic peptides are electrostatically attracted to the negatively charged membranes of bacteria. Bacterial membranes are rich in acidic phospholipids providing an overall negative charge (Yeaman & Yount, 2003). An additional negative charge to the surfaces of bacteria is provided by LPS.
and teichoic acids of Gram-negative and Gram-positive bacteria, respectively. The rapid lethal effect of Os is believed to occur due to interruption of membrane integrity (Prinsloo et al., 2013), whereas Os(11-22)NH₂ can act on intracellular targets when translocated across the cytoplasmic membrane. This is supported by the lag time observed in the bactericidal effects of Os(11-22)NH₂. Membrane permeabilization cannot yet be ruled out for Os(11-22)NH₂, since the exact bactericidal mechanism is unknown.

An essential requirement for any antimicrobial therapeutic agent is that it has selective toxicity for prokaryotic cells relative to eukaryotic cells (Yeaman & Yount, 2003). Several peptides have been shown to be toxic to eukaryotic cells at high concentrations. An ideal model system for the determination of the direct effect of AMPs on mammalian cell membranes is the erythrocyte. Erythrocytes are examples of typical cellular bilayers and contain no nucleus or subcellular metabolic structures. Therefore, if haemolysis occurs it is as a result of damage directly to the cellular membrane, resulting in leakage of hemoglobin that can be directly quantified (Lubran, 1989; Prinsloo et al., 2013). Melittin is known to possess both antibacterial and haemolytic activity and was therefore used as a positive control (DeGrado et al., 1982; Blondelle & Houghten, 1991; Raghuraman & Chattopadhyay, 2005; 2007). In the author’s study melittin caused a dose related increase in haemolysis, whereas no haemolysis was observed for Os(11-22)NH₂ (Fig. 3.6).

The bacterial membrane is rich in acidic phospholipids, therefore, the net positive charge of Os(11-22)NH₂ allows the peptide to have affinity for bacterial membranes. On the contrary, the membrane of erythrocytes is composed mostly of zwitterionic phosphatidylcholine, cholesterol and sphingomyelin phospholipids (Verkleij et al., 1973; Shai, 1999; Unger et al., 2001), possibly attributing to the low affinity of Os(11-22)NH₂ and Os towards erythrocytes. Melittin lacks selectivity between zwitterionic mammalian and negatively charged bacterial cell membranes, as it interacts with both membranes causing haemolysis (Batenburg & Kruijff, 1988; Matsuzaki et al., 1995; Unger et al., 2001). The buffer used to resuspend the erythrocytes, PBS, contains 137 mM NaCl and 3 mM KCl, yielding a total of 140 mM monovalent ions. As described, the activity of Os(11-22)NH₂ in 100 mM NaCl was retained (Table 3.7), therefore the salts are not likely to affect the activity of the peptide against erythrocytes, since haemolytic activity was observed for melittin despite the presence of salts.
The haemolytic activity of peptides is also influenced by the peptide’s $<H>$, where a higher hydrophobic content can lead to greater haemolytic activity (Gifford et al., 2005). The $<H>$ of melittin (0.511) is larger compared to those of Os (0.249) and Os(11-22)NH$_2$ (0.396), indicating that $<H>$ indeed affects haemolytic activity (Gifford et al., 2005; Gautier et al., 2008). From the helical wheel projections (Fig. 3.3) it can also be seen that melittin possesses a distinct hydrophobic face, contributing to its haemolytic effect. Helical wheels project the arrangement of amino acids of peptides, assuming that they take on an $\alpha$-helical structure. Os showed $\alpha$-helical properties in membrane environments, but did not possess a distinct hydrophobic face, according to the helical wheels (Fig. 3.3 A). On the other hand, Os(11-22)NH$_2$ showed fewer hydrophobic residues contributing to the hydrophobic face, but Os(11-22)NH$_2$ showed no $\alpha$-helical properties in a membrane environment, thus the arrangement of amino acids projected by the helical wheels may not be the true arrangement. Melittin is thought to bind and insert into membranes by interfacial binding of the unfolded peptide, mainly due to hydrophobic interactions, inserting the non-polar side chains into the hydrophobic core of the lipid membrane causing conversion from a random coil to an $\alpha$-helical structure allowing insertion of the peptide into the phospholipid bilayer (Wolfe et al., 1998; Ladokhin & White, 1999; Wieprecht et al., 1999; Unger et al., 2001).

A previous study showed that when tryptophan from the peptide, N-1, was substituted with phenylalanine, the haemolytic activity was significantly reduced without a significant change in antibacterial activity (Shin et al., 2001). Similar results were observed for indolicidin, derived from bovine neutrophils, when tryptophan was substituted by phenylalanine, haemolytic activity was reduced considerably (Subbalakshmi et al., 2000). These results indicated that tryptophan residues may be motif that possibly favours haemolytic activity. Melittin also contains a tryptophan residue at position 19 that has previously been reported to contribute to haemolytic activity (Blondelle & Houghten, 1991; Blondelle et al., 1993). Neither Os(11-22)NH$_2$ nor Os contains tryptophan residues and showed no haemolytic activity (Fig. 3.6). A leucine zipper motif was previously identified in melittin that seems to contribute to haemolytic activity and cytotoxicity (Asthana et al., 2004). No leucine residues were observed in the sequences of both Os and Os(11-22)NH$_2$ and also no haemolytic activity. Since tryptophan and leucine are both hydrophobic amino acids they contribute to the $<H>$ of melittin and as mentioned an increased hydrophobicity leads to increased haemolytic activity.
Mammalian cells are metabolically active entities, containing a nucleus. Effects on the membrane of cells may activate or inhibit cellular pathways that may cause cellular death. In the Caco-2 cell line, the CV assay revealed that Os(3-12) and Os(11-22) had no effect on cell number even when tested at high concentrations, whereas melittin significantly decreased the cell number at high concentrations (Fig. 3.1). Results found were in good agreement with Prinsloo et al. (2013), where Os, the parent molecule, showed no cytotoxicity. By means of the CV assay, similar results were observed for Os(11-22) and Os(11-22)NH₂, which caused no decrease in SC-1 cell number. In contrast melittin decreased the cell number (Fig. 3.7). Os(11-22)NH₂ at the highest concentration tested, caused a significant increase in cell number. Since cell culture media contain both salt and serum, these components could influence the results, but as described previously, NaCl would not necessarily affect the activity of the peptides against SC-1 cells. The proteins in serum, such as negatively charged albumin may bind peptides. However, cytotoxicity was observed for melittin (net charge +6) and if albumin bound to melittin it would affect its cytotoxicity. Therefore albumin most likely does not influence the activity of the peptides in the cytotoxicity assay.

The difference in selectivity of melittin, Os, Os(3-12), Os(11-22) and Os(11-22)NH₂ between microbial and mammalian cells can be attributed to the difference in bacterial and mammalian membrane composition. The outer surfaces of bacteria are negatively charged, allowing electrostatic binding of cationic peptides to the outside of the bacterial cell envelope (Gifford et al., 2005). Bacterial cell membranes are also composed predominantly of negatively charged phospholipids, such as phosphatidylycerol, cardiolipin, or phosphatidylinerine, increasing the affinity of cationic peptides towards these molecules. In contrast, mammalian cytoplasmic membranes are enriched with zwitterionic phospholipids, such as, phosphatidylethanolamine, phosphatidyldcholine and sphingomyelin (Yeaman & Yount, 2003), contributing to the low affinity for mammalian membranes. Such considerable selectivity for micro-organisms over mammalian cells as well as good bacteria killing kinetics and MBCs, provide these AMPs with the possibility to be suitable candidates for designing more effective antibiotics.

The increase in cell number observed for SC-1 cells exposed to Os(11-22)NH₂ can be as a result of stimulation of cell proliferation caused by Os(11-22)NH₂. Since SC-1 cells are fibroblast cells and since fibroblasts contribute to the wound healing process (Tettamanti et al., 2004; Deveci et al., 2005) and because Os(11-22)NH₂ caused a significant increase in
SC-1 cell number, this can be an indication that the peptide may possess wound healing properties. In addition, AMPs improve wound healing by controlling bacteria such as *S. aureus* and *P. aeruginosa* (Halbert *et al.*, 1992; Jacobsen *et al.*, 2005). Defensins, such as hBDs, have been shown to be involved in different cellular processes, including cell migration or proliferation, thereby positively affecting wound healing (Baroni *et al.*, 2009; Lai & Gallo, 2009). Niyonsaba *et al.* (2007) showed that hBD-2 to 4 participated in wound healing by enhancing the migration and production of keratinocytes, which is the most abundant cell type of the epidermis (Fitzpatrick *et al.*, 1978; Eckert & Rorke, 1989; Niyonsaba *et al.*, 2007). HBD-2 has also been shown to stimulate migration, proliferation and tube formation of endothelial cells (Baroni *et al.*, 2009). Endothelial cells form the internal surface of blood and lymphatic vessels and are important in the formation of these vessels (Michiels, 2003).

Multifunctional peptides have been receiving increased research attention (Yang *et al.*, 2009). It is known that AMPs play an important role in the innate immune system of vertebrates and invertebrates, providing the first form of defence against infections (Bulet *et al.*, 2004). In mammals, defensins are mainly found in epithelial (skin) and non-epithelial (mucosa) surfaces, where they maintain barrier function and prevent microbial invasions (Schroder, 2010; Brandenburg *et al.*, 2012). When these barriers are broken, additional protection is needed from antimicrobial topical or systemic applications. Bioactive peptides exhibiting both antimicrobial and antioxidant activity, will not only kill pathogens, but will also promote cellular recovery and generation by scavenging free radicals that cause oxidative damage, leading to promotion of wound healing (Yang *et al.*, 2009). It has been suggested that cells under oxidative stress do not function properly and that responses to mitogenic signals involved in wound healing occur adequately when cells are under low or no oxidative stress (Shukla *et al.*, 1997). GSH was shown to reverse the impaired proliferative activity of cultured fibroblast cells from diabetic patients with nephropathy (Morocutti *et al.*, 1998).

The ORAC assay revealed that Os(11-22)NH$_2$ exhibited antioxidant activity (6.3 μM TE/μM peptide) and was found to be significantly more active than GSH (Fig. 3.8). The parent peptide, Os, was shown to be a stronger antioxidative peptide (21.6 μM TE/μM peptide) than Os(11-22)NH$_2$ (Prinsloo *et al.*, 2013). It has been shown that the peptides tested have the ability to scavenge reactive oxygen species (ROS). Various amino acids, such as, methionine, and cysteine (containing sulphur), tyrosine and phenylalanine (aromatic side chains),
tryptophan, proline and histidine contribute to antioxidant activity (Yang et al., 2009; Shen et al., 2010). Yang et al. (2009) showed that replacing the mentioned amino acids of AMPs, possessing antioxidant properties, with glycine, the antioxidant activity decreased or was completely eliminated, but these peptides still retained their antimicrobial activity. Tryptophan has been shown to have the highest antioxidant activity amongst the amino acids, followed by tyrosine and methionine and then cysteine, histidine and phenylalanine (Shen et al., 2010). It has also been suggested that antioxidant peptides are sequence specific and that proline might be important for antioxidant activity (Yang et al., 2009). Neither Os nor Os(11-22)NH$_2$ contain proline residues in their sequence and still possess antioxidant activity.

From the antioxidant amino acids mentioned, only tyrosine, cysteine and phenylalanine are present in the sequences of Os and Os(11-22)NH$_2$. The phenolic side chain of tyrosine and the thiol group of cysteine allows for these amino acids to donate an electron to ROS and thereby stabilizing them. Os possesses more antioxidative amino acids (3 Tyr, 3 Cys and 1 Phe) than Os(11-22)NH$_2$ (1 Tyr, 3 Cys and 1 Phe), allowing Os to possess higher antioxidant activity. The result of Os having higher antioxidant activity can also be due to the presence of the dipeptide sequence, Ile-Arg according to BIOPEP [http://www.uwm.edu.pl/biochemia/index.php/pl/biopep/29-about-biopep]. This sequence may contribute to the observed antioxidant activity and is absent from the amino acid sequence of Os(11-22)NH$_2$ (Prinsloo et al., 2013).

According to BIOPEP analysis, Os(11-22)NH$_2$, exhibited no antioxidative contributing amino acid sequences. Studies done by Prinsloo et al. (2013) found that Os-C, an analogue of Os lacking cysteine residues, displayed similar antioxidant activity to Os. This finding confirms that the cysteine residues were not responsible for antioxidant activity; therefore dipeptide sequences such as Ile-Arg may be important. Once Os was diluted, in the absence of a reducing agent, the peptide could have been oxidized, rendering free thiol groups of cysteine residues unavailable. Os(11-22)NH$_2$ could have undergone similar reactions, since Os(11-22)NH$_2$ was also diluted in the absence of a reducing agent (DTT).
CHAPTER 5: Conclusion and Future Perspectives

Defensins and defensin-derived peptides are promising candidates for the development of novel anti-infective agents especially for wound healing. During this study, two short bioactive peptides based on the structure of Os, Os(3-12) and Os(11-22) were identified. Overall, Os(3-12) was more active against Gram-negative bacteria, but Os(11-22) more active against Gram-positive bacteria. Amidation of Os(11-22), increasing net charge (+3 to +4) and structure stability, resulted in higher bactericidal activity than Os(11-22) against the tested bacteria; however, Os(11-22) was 2 fold more active than Os(11-22)NH₂ against S. aureus. CD data revealed that the parent peptide, Os, possesses mostly α-helical properties, whereas Os(11-22) exhibits some α-helical content. Os(11-22)NH₂ was shown to be mostly random coiled. It appears that secondary structure is not essential for the activity of the amidated peptide.

For application in a physiological environment the effects of 100 mM NaCl and 30% serum on the activity of Os(11-22)NH₂ were investigated. The activity of Os(11-22)NH₂ in NaCl decreased 4 fold against E. coli and was completely lost against S. aureus. In the presence of serum the activity of Os(11-22)NH₂ towards E. coli reduced 32 fold and unexpectedly increased 8 fold against S. aureus. Further research into the effect of serum on the activity of Os(11-22)NH₂ against S. aureus is required. Different and more resistant S. aureus strains should be considered, because the effect may be strain specific. If the same effect is observed for additional S. aureus strains further investigation, into which components of serum are responsible for the observed effect, is necessary.

Bactericidal killing by Os(11-22)NH₂ occurred within 120 min against E. coli and 30 min against B. subtilis, whereas Os killed both bacteria within 5 min, indicating different modes of action for the peptides. AMPs kill by targeting the cell membrane and/or intracellular targets such as DNA and processes such as protein synthesis. Cell membrane effects can be demonstrated by scanning electron microscopy and the functional consequences to this damage can be determined using a triple-staining method described by Mangoni et al. (2004). In this method three different fluorochromes can be used to visualize the total viable and non-viable bacteria cells and to detect if the peptide causes death via membrane permeabilization.
This effect can then be quantified with the SYTOX green assay. A DNA binding assay can be performed to determine if Os(11-22)NH$_2$ binds to DNA and may indicate intracellular DNA binding and possibly inhibition of DNA replication. A detailed proteomic analysis of a bacterium with a well-defined proteome, such as *E. coli*, in the presence and absence of Os(11-22)NH$_2$ can be evaluated to determine which proteins are up- or down-regulated and may give further insight into its mode of action.

Os(11-22)NH$_2$ and Os showed no haemolytic activity towards human erythrocytes and none of the peptides tested, including Os, showed cytotoxic effects towards Caco-2 and SC-1 cells. Os(11-22)NH$_2$ caused a significant increase in cell number at the highest concentration tested, possibly stimulating cell proliferation, which may be beneficial especially related to wound healing. Future research can include an *in vitro* wound model to determine if Os(11-22)NH$_2$ does indeed promote wound healing. Factors contributing to wound healing such as the ability of Os(11-22)NH$_2$ to induce migration and proliferation of keratinocytes and endothelial cells can be investigated.

Both Os(11-22)NH$_2$ and Os exhibited antioxidant activity and can be regarded as dual functional peptides, since antioxidant activity was identified in addition to antibacterial activity. *In vitro* antioxidant activity, such as mammalian cell protection by the peptide against free radicals, should be investigated in the future. The use of the cellular DCHF-DA (2,7-dichlorodihydrofluorescein diacetate) assay should be considered (Valkonen & Kuusi, 1997) as this assay can be used to measure the ability of peptides to protect cells against oxidative damage.

This study revealed that Os(11-22)NH$_2$, a defensin-derived peptide, has pharmaceutical potential as a possible wound healing agent based on the antibacterial and antioxidant properties of this peptide. It can be concluded that two shorter peptides derived from Os, as well as the amidated peptide, retained antibacterial activity and selectivity towards bacterial cells. Further investigation regarding structure-function properties is needed as well as the identification of additional bioactivities for applications in the clinical field. Other activities associated with infection such as the effects of Os(11-22)NH$_2$ on inflammation and biofilms should be investigated. The anti-inflammatory activity of Os(11-22)NH$_2$ can be determined in terms of the ability of the peptide to neutralize LPS, measured by the limulus amebocyte lysate (LAL) assay, since LPS is considered one of the most powerful bacterial virulence
factors related to pro-inflammatory properties (Blais et al., 2005). The nitric oxide (NO) scavenging capacity of Os(11-22)NH$_2$ can also be determined using the Griess reaction assay (Griess, 1879). NO is recognized as a signalling molecule that plays an important role in initiation of inflammation (Sharma et al., 2007). Biofilms, which are microbial communities that cause serious chronic infections and dental plaque, form environments that significantly enhance antimicrobial resistance due to the production of biofilm exopolysaccharide structures (Otto, 2006). Biofilm formation of pathogenic bacteria poses a serious threat to the community and new anti-biofilm agents are needed. Therefore the anti-biofilm activity of Os(11-22)NH$_2$ should be investigated.


Birnboim, H.C. (1986) DNA strand breaks in human leukocytes induced by superoxide anion, hydrogen peroxide and tumor promoters are repaired slowly compared to breaks induced by ionizing radiation. Carcinogenesis 7, 1511-1517.


